catalysts

MDPI

Article

Enzymatic Synthesis of *O*-Methylated Phenophospholipids by Lipase-Catalyzed Acidolysis of Egg-Yolk Phosphatidylcholine with Anisic and Veratric Acids

Marta Okulus * and Anna Gliszczyńska *

Department of Chemistry, Wrocław University of Environmental and Life Sciences, Norwida 25, 50-375 Wrocław, Poland

* Correspondence: marta.okulus@gmail.com (M.O.); anna.gliszczynska@wp.pl (A.G.); Tel.: +48-71-320-5183 (M.O. & A.G.)

Received: 18 April 2020; Accepted: 12 May 2020; Published: 13 May 2020

Abstract: Lipase-catalyzed acidolysis reactions of egg-yolk phosphatidylcholine (PC) with anisic (ANISA) and veratric (VERA) acids were investigated to develop a biotechnological method for the production of corresponding biologically active *O*-methylated phenophospholipids. Screening experiments with four commercially available immobilized lipases indicated that the most effective biocatalyst for the incorporation of ANISA into phospholipids was Novozym 435. None of the tested enzymes were able to catalyze the synthesis of PC structured with VERA. The effects of different solvents, substrate molar ratios, temperature, enzyme loading, and time of the reaction on the process of incorporation of ANISA into the phospholipids were evaluated in the next step of the study. The mixture of toluene/chloroform in the ratio 9:1 (*v*/*v*) significantly increased the incorporation of ANISA into PC. The acidolysis reaction was carried out using the selected binary solvent system, 1/15 substrate molar ratio PC/ANISA, 30% (*w*/*w*) enzyme load, and temperature of 50 °C afforded after 72 h anisoylated lysophosphatidylcholine (ANISA-LPC) and anisoylated phosphatidylcholine (ANISA-PC) in isolated yields of 28.5% and 2.5% (*w*/*w*), respectively. This is the first study reporting the production of ANISA-LPC and ANISA-PC via a one-step enzymatic method, which is an environmentally friendly alternative to the chemical synthesis of these biologically active compounds.

Keywords: anisic acid; methoxy derivatives of phenolic acids; immobilized lipases; enzymatic synthesis; structured phosphatidylcholine; acidolysis; egg-yolk phosphatidylcholine

1. Introduction

Phenolic acids are a class of plant secondary metabolites that are of particular interest because of their well-documented pro-health and nutritional properties. In this group, methoxy derivatives of benzoic acid are considered as important food constituents with potential preventive effects on several chronic diseases. *p*-Anisic acid (4-methoxybenzoic acid; ANISA), a constituent of Chinese star anise (*Illicium verum* Hook. f.) [1] and *Capparis spinosa* L. (*Capparidaceae*) [2], has been described as a compound that possesses the ability to protect the liver against the toxicity of carbon tetrachloride (CCl_4) and paracetamol (Pcl). Its activity was evaluated as comparable to the activity of commercially used silymarin [2]. Some studies have suggested that ANISA might also be useful in cancer prevention and treatment by affecting the activity of COX-2, which is a mediator in tumorigenesis [3]. Veratric acid (3,4-dimethoxybenzoic acid; VERA), occurring in vegetables, fruits, and medicinal mushrooms, also possesses biologically valuable properties. It has been evaluated that its oral administration at

the dosage of 40 mg/kg strongly decreases systolic and diastolic blood pressure, attenuates oxidative stress, and improves plasma nitric oxide level [4]. Recent studies have revealed that this natural dimethoxy derivative of benzoic acid could play a role as an effective therapeutic agent, which is able to eliminate atherosclerotic lesions [5] and protect against UVB-induced skin disorders [6] and bacterial infections [7].

Despite promising therapeutic effects, practical application of methoxy derivatives of benzoic acid as well as other phenolic compounds is difficult due to their low oral bioavailability. These compounds in food are mainly conjugated or bound to the dietary fiber matrix [8]. The majority of them reach the colon, but their bioavailability requires the activity of degradation enzymes of intestinal microbiota. Only around 5% of dietary phenolics occur in the form of free acids [9], but even their supplementation does not afford expected results, because they undergo rapid metabolism and their distribution in the body is limited to the organs responsible for the detoxification and excretion of xenobiotics [10].

A method that allows these limitations to be overcome is the lipophilization of phenolic acids and their conjugation with the lipid molecule, which allows their physico-chemical nature to be changed and increases their bioavailability in in vivo models, improving pro-health properties. Chemical synthesis of phenolipids is often difficult due to their heat sensitivity and susceptibility to oxidation in alkaline media. Therefore, enzymatic lipophilization of phenolic acids and their *O*-methylated derivatives with fatty alcohols (FA) and triacylglycerols (TAGs) has been widely studied during the last two decades [11–16].

Guyot examined the enzymatic esterification of *ortho*-, *meta*-, and *para*- hydroxybenzoic acid with octan-1-ol. Using thermostable immobilized lipase B from *C. antarctica* (Novozym 435) as a biocatalyst, he obtained corresponding esters in 4, 14, and 4% yields, respectively, after 30 days of reaction [11]. These findings were next continued and expanded by Buisman, who investigated the ability of lipase CALB to esterify various hydroxy (gallic and gentisic acids) and methoxy derivatives of benzoic acid (vanillic and syringic acids) with hexan-1-ol [12]. However, this time, the esterified products were also produced only in trace amounts (below 2%) after a one-week reaction. In later studies, it was found that the polarity of alcohols used as the substrates and solubility of phenolic acids in the reaction medium strongly affect the process of their lipophilization. Yu and co-workers obtained propyl gallate in a high (44.3%) yield after 12 h of reaction using benzene as the solvent and microencapsulated tannase isolated from *Aspergillus niger* as the biocatalyst [17]. For more hydrophobic alcohols such as hexanol, octanol, dodecanol, which have been studied previously by Guyot and Buisman, it was confirmed that they cannot solvate gallic acid efficiently and their long chains cannot establish favorable interactions with the active site of tannase [17].

Enzymatic incorporation of benzoic acid derivatives into TAGs was extensively investigated by Safari and Karboune [15,18], but did not bring satisfactory results because of the low bioconversion yields of studied phenolic compounds. In the face of this fact, the main goal of various research groups became the development of a new method of phenolipid production. For this purpose, phospholipids (PLs) have started being employed as the lipid substrate. Nowadays, the production of phospholipid conjugates with drugs [19,20] and other bioactive compounds [21–23] is an extensively employed approach. Soybean and egg-yolk phosphatidylcholine is widely used in these studies due to its health-promoting activity, biocompatibility with biological membranes, and the key role that it plays in the biochemistry and physiology of the human organism. Structural modifications of PLs with phenolic acids and their *O*-methylated derivatives are a new area of research. Up to now, only a few papers concerning this topic have appeared in the literature. Conjugates of syringic, vanillic, sinapic, and ferulic acids covalently bond to DPPC and egg-yolk PC with significantly higher antioxidant and antimicrobial activities in comparison to the free forms of these acids that have been described by Balakrishna and co-workers [24]. Guo et al. recently published the chemical synthesis of novel caffeoyl phosphatidylcholines with multifunctional properties indicating that obtained products could find high potential application in food, cosmetics, and pharmaceutical formulations [25]. The enzymatic

process of production of feruloylated phospholipids as pro-health food additives was also successfully developed by the two research groups of Yang and Gliszczyńska [26,27].

In our previous study, we synthesized a series of methoxy derivatives of benzoic and cinnamic acid after conjugation with phosphatidylcholine and lysophosphatidylcholine and showed that they could act as effective anticancer agents and were able to stimulate insulin secretion via G protein coupled receptors [28–30]. The active dosages of synthesized conjugates of anisic and veratric acids with phospholipids, which inhibited the proliferation of leukemia cells, were 12- to 38-fold lower than those determined for free forms of these acids. The huge potential of synthesized conjugates for multidisciplinary applications has encouraged us to develop a novel environmentally attractive enzymatic route for their synthesis via a one-step acidolysis reaction of egg-yolk phosphatidylcholine with ANISA and VERA using immobilized lipases as biocatalysts. The effects of the structure of methoxy derivatives of benzoic acid, reaction time, type of biocatalyst, substrate molar ratio, organic solvent, temperature, and enzyme content on the process of the enzymatic production of selected *O*-methylated phenophospholipids were investigated.

2. Results and Discussion

2.1. Analysis and Identification of Reaction Products

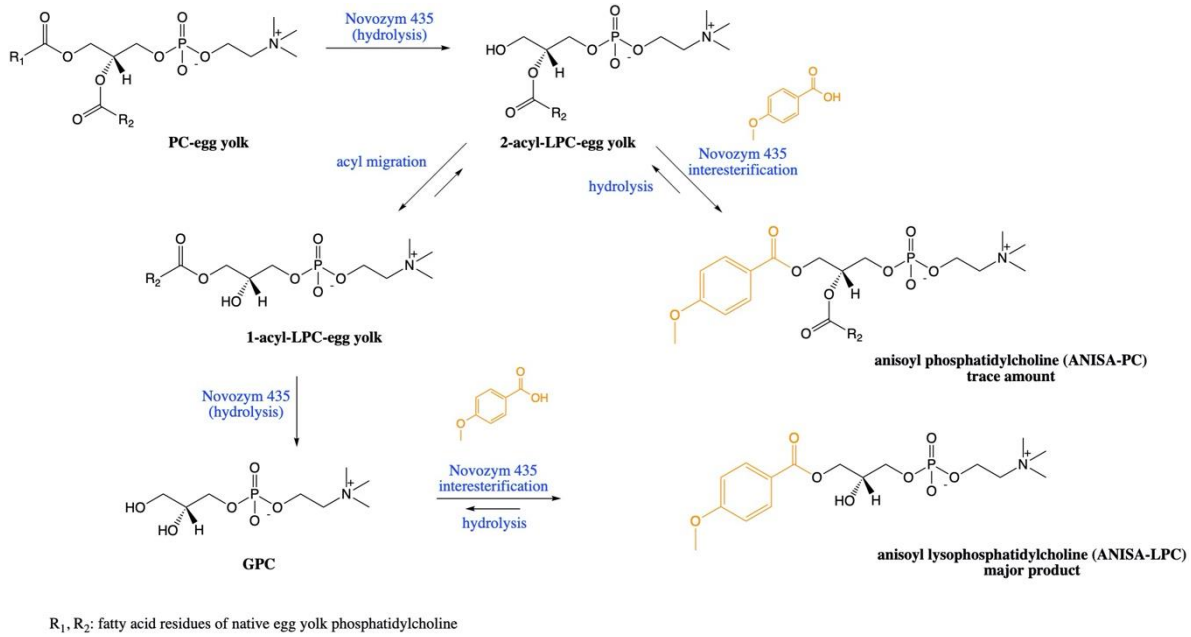
Biological pro-health activities of previously synthesized anisoylated and veratroylated phosphatidylcholines [28,30] encouraged us to develop a biotechnological process for their production. For this purpose, we focused our attention on a one-step acidolysis method in which hydrolysis and esterification reactions occur simultaneously, and fatty acids from the *sn*-1 position of the PC substrate are gradually exchanged for added free acids until equilibrium is reached. Our aim was to incorporate ANISA and VERA into the *sn*-1 position of phosphatidylcholine and retain most of the unsaturated fatty acids naturally occurring in the *sn*-2 position of PC. Therefore 1,3-regioselective lipases or non-specific enzymes, but known from the literature as highly selective toward the *sn*-1 position, were chosen. The selection of lipases was based on reports describing their ability to structurally modify TAGs and PLs [31,32]. As a lipid substrate, we used the main dietary glycerophospholipid egg-yolk phosphatidylcholine [33], which is characterized by high absorption from the digestive system (over 90%) and intense circulation in the bloodstream [34].

Reaction conditions were evaluated with respect to the selection of lipase, organic medium, substrate molar ratio, reaction temperature and enzyme dosage. Among the tested lipases, Novozym 435 was found to be the most effective biocatalyst for the production of anisoyl phospholipids. However, even this lipase was not able to catalyze the synthesis of PC structured with veratric acid. Both acids have electron-donating groups in the *para* position and in the case of veratric acid, also in the *meta* position on the ring. Lack of lipase affinity toward VERA can thus be explained by the electron effect, where electron donating groups on the ring cause deactivation of the electrophilic carbon center of the carboxylic group [11,13]. The steric effect is crucial in enzymatic reactions; therefore, most authors attribute low enzyme activity to the combined effect of these factors along with reaction conditions [11–13].

Under the selected parameters—reaction medium toluene/chloroform 9:1 (*v*/*v*), 1:15 PC:ANISA molar ratio, temperature 50 °C, 30% (*w*/*w*) enzyme load, and reaction time 72 h—we performed the reaction of acidolysis of PC with ANISA on a larger scale. Identification of products of Novozym 435-catalyzed acidolysis reaction of egg-yolk PC with ANISA was performed by TLC and RP-HPLC using PC-egg yolk, LPC-egg yolk, and previously synthesized 1-anisoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (1-ANISA-2-PA-PC) and 1-anisoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (1-ANISA-2-OH-LPC) as the reference standards [28]. In the mixture of products spotted in the TLC, we observed two new bands with higher polarity than PC-egg yolk and LPC-egg yolk, respectively. Based on the standards, we identified that phospholipid fraction contained not only PC-egg and LPC-egg, but also modified PC (ANISA-PC) and modified LPC (ANISA-LPC), whose retention factors corresponded to R_f of 1-ANISA-2-PA-PC and

1-ANISA-2-OH-LPC. The two new products were separated from fatty acids, unreacted anisic acid, PC-egg and LPC-egg, and fractioned by the chromatography column. The main product of the reaction anisoyl-LPC was obtained in a 28.5% *w/w* isolated yield, whereas anisoyl-PC was formed only in a trace amount in a 2.5% *w/w* isolated yield. Their enzymatic synthesis was confirmed by reversed phase HPLC with a UV/DAD detector. The analysis of modified phospholipids was possible because of the production of fluorescent absorption under UV light of 270 nm by the aromatic ring with a methoxy group attached to the glycerol backbone of PC and LPC (Supplementary Materials).

Novozym 435 is considered as a non-regiospecific lipase, but in the majority of lipid modifications, it showed high selectivity toward the *sn*-1 position of PC [27,32,35]. Hence, below, we present the possible pathway of the reaction of enzymatic acidolysis of PC with ANISA catalyzed by this enzyme (Scheme 1).



Scheme 1. Possible pathway of the enzymatic synthesis of anisoylated phospholipids during the acidolysis reaction of egg-yolk phosphatidylcholine (PC) with anisic acid (ANISA).

2.2. Selectivity of Lipases

The enzymatic acidolysis of egg-yolk phosphatidylcholine with ANISA and VERA was investigated for the synthesis of corresponding *O*-methylated phenophospholipids. In order to determine the appropriate biocatalyst, four commercial preparations containing different lipases were evaluated. Lipozyme (lipase from *Rhizomucor miehei* RM IM immobilized on an anion exchange resin), Lipozyme TL IM (a silica granulated *Thermomyces lanuginosus* lipase preparation), and two other preparations containing the same lipase B from *Candida antarctica*, but immobilized on different carriers: Novozym 435 (immobilized on microporous acrylic resin) and CALB (immobilized on resin Immobead 150) were screened for their specificity with respect to the egg-yolk PC and selected aromatic acids. All studied biocatalysts were used in immobilized form, which is of great importance for industry from an economic point of view when scaling up the process. Moreover, usage of immobilized lipases increases the contact surface area of the enzyme with the substrate and ensures retention of more stable activity [36]. All lipases were applied at the same weight ratio, although they exhibited different activities according to the suppliers. Reactions were carried out at 50 °C for 72 h using a 1:5 PC/ANISA or VERA and 30% (w/w) enzyme dosage in 2 mL of heptane. The progress of the reaction of acidolysis was monitored by collecting samples of product mixtures in selected time intervals. The phospholipid fractions PC/LPC were separated from the fatty acids and unreacted aromatic

acids by solid phase extraction (SPE). The composition of acyls in the structured PL fractions was analyzed by gas chromatography (GC). In this stage, the PLs were not fractioned into individual PC and lysophosphatidylcholine (LPC), which was formed in this process due to partial hydrolysis of phosphatidylcholine during the acidolysis reaction. In Figure 1, only the results for the reaction of acidolysis with ANISA are presented because in the case of VERA, none of the tested biocatalysts were able to catalyze the process of its incorporation into the PC structure. This is in accordance with Karboune, who reported previously that when two methoxy groups are substituted in the aromatic ring of cinnamic acid, the bioconversion is very low [15]. Lack of satisfactory results from the process of lipophilization of VERA can be explained by a spatial barrier affecting the slowdown or inhibition of the reaction. In the acidolysis of PC with ANISA catalyzed by Novozym 435 as a biocatalyst, a significant increase in the incorporation degree of anisic acid (6 mol %) was obtained within the first 72 h of the reaction. The CALB-catalyzed acidolysis reaction showed a different trend, with a lower extent of incorporation at the level of 2 mol % in all tested time intervals. RM IM lipase was almost inactive in the acidolysis process, giving only 1 mol % of ANISA incorporation, whereas TL IM lipases did not show activity. Since the incorporation degree of ANISA was the highest in the reaction catalyzed by Novozym 435, this enzyme was chosen for further study.

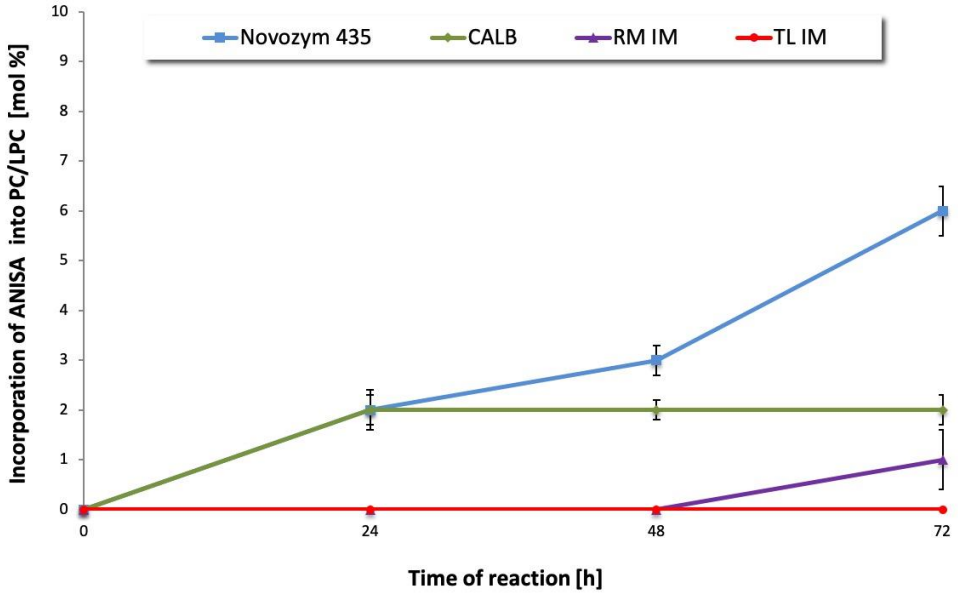


Figure 1. Time course of different lipase-catalyzed acidolysis between egg-yolk PC and ANISA (reaction conditions: 1:5 PC/ANISA molar ratio, solvent: heptane 2 mL, enzyme load 30% (*w*/*w*), temperature 50 °C). Vertical bars represent SD, n = 3.

2.3. Effect of Organic Media

Reaction media play an important role in maintaining enzyme catalytic activity and stability. It also influences the relative solubility of the substrates, which is a crucial point in the case of enzymatic reactions with phenolic acids and their derivatives. In order to investigate the most suitable organic media for the acidolysis of PC with ANISA and VERA, three different solvents with log*P* ranging from 2.5 to 4.5, mostly used in the enzymatic lipophilization of phenolic acids, were examined [26,37]. The results showed that acidolysis carried out in toluene (log*P* = 2.5) and heptane (log*P* = 4) gave the incorporation of ANISA into the PLs fraction at the level around 6 mol % in both organic solvents. Lower incorporation of ANISA, only 2 mol %, was obtained for the reaction performed in isooctane (log*P* = 4.5). The obtained results were somewhat inconsistent, with general reports indicating that solvents with log*P* values > 3 are more suitable for enzymatic modifications of lipids [38] because hydrophobic solvents have lower capability to remove water from the enzyme, thus providing higher

enzyme activity. However, it is worth noting that not the log*P* alone, but the cumulative effect of various other factors such as the solubility of the substrate, determines the enzyme activity in the organic solvent system. To overcome the limited solubility of the hydrophilic ANISA in the hydrophobic organic solvents, previously studied as effective media for the synthesis of phenoylated lipids, binary organic solvent mixtures of hexane/butan-2-one (85:15 *v*/*v*) [39] and toluene/chloroform (9:1, *v*/*v*) [26] were used. The results presented in Figure 2 show that the change of the reaction medium from toluene to a mixture of toluene with chloroform in the proportion 9:1 v/v resulted in higher ANISA incorporation from 6 to 10 mol % after 72 h. The second binary solvent system hexane/butan-2-one was reported not to be an appropriate reaction medium for the synthesis of phenolic lipids, leading to lower incorporation at the level of 1 mol %. The low enzymatic activities in the more polar solvent mixtures may be due to the poor solvation of PC in this reaction medium, which could hinder the interaction between Novozym 435 and ANISA. On the basis of the findings, the toluene/chloroform (9:1 *v*/*v*) mixture was used as a reaction medium throughout this study. At this stage of the study all five reaction media were also evaluated for the synthesis of PC enriched with VERA, but no significant results were obtained (incorporation less than 1 mol %, data not shown in Figure 2).

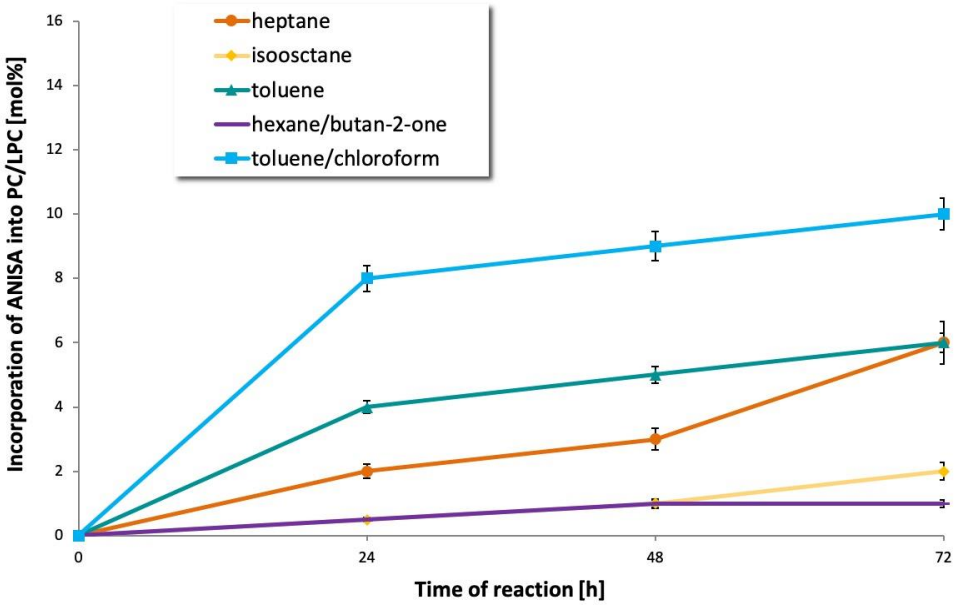


Figure 2. Effect of organic solvent on incorporation of ANISA into egg-yolk PC in the acidolysis reaction (reaction conditions: 1:5 PC/ANISA molar ratio, solvent 2 mL, enzyme load 30% (*w*/*w*) Novozym 435, temperature 50 °C). Vertical bars represent SD, n = 3.

2.4. Effect of Substrate Molar Ratio

In order to maximize the enzymatic synthesis of *O*-methylated phenophospholipids, the effect of substrate molar ratio on the time course of the acidolysis reaction was investigated by varying PC to anisic and veratric acids from 1:5 to 1:20 and using a toluene and chloroform mixture (9:1 *v*/*v*) as the reaction medium. Reactions were carried out at 50 °C and Novozyme 435 was used at a dose of 30% (*w*/*w*). Figure 3 only illustrates the profile of incorporation of ANISA into the PL fraction because despite the use of increasing concentrations of veratric acid in the reaction medium, we did not observe an increase in its incorporation into PC. In the case of anisic acid, the results demonstrate that its incorporation increases with increasing molar ratios of ANISA to PC. The highest incorporation (22 mol %) occurred at a 1:15 substrate molar ratio of PC to ANISA. This value was significantly higher than that reported at the 1:10 and 1:5 substrate molar ratios. Previous studies have shown that a higher acid to PC molar ratio directs the reaction equilibrium toward the synthesis of the product [26,27] and our results support this observation. However, here, no further increase in the incorporation of

ANISA was observed when the substrate molar ratio was beyond 1:15. At the substrate molar ratio 1:20, we observed that incorporation of ANISA into the phospholipid fraction drastically decreased. This phenomenon may be explained by the inhibition effect of high concentration of the substrate in the reaction medium on the enzyme activity. As highlighted above, solubility limitation of the substrate may also be a key factor. The increase in substrate amounts higher than the solubility limitation may not increase the concentration of substrate available for reaction, instead increasing viscosity and decreasing mass transfer, which might result in a decrease of incorporation.

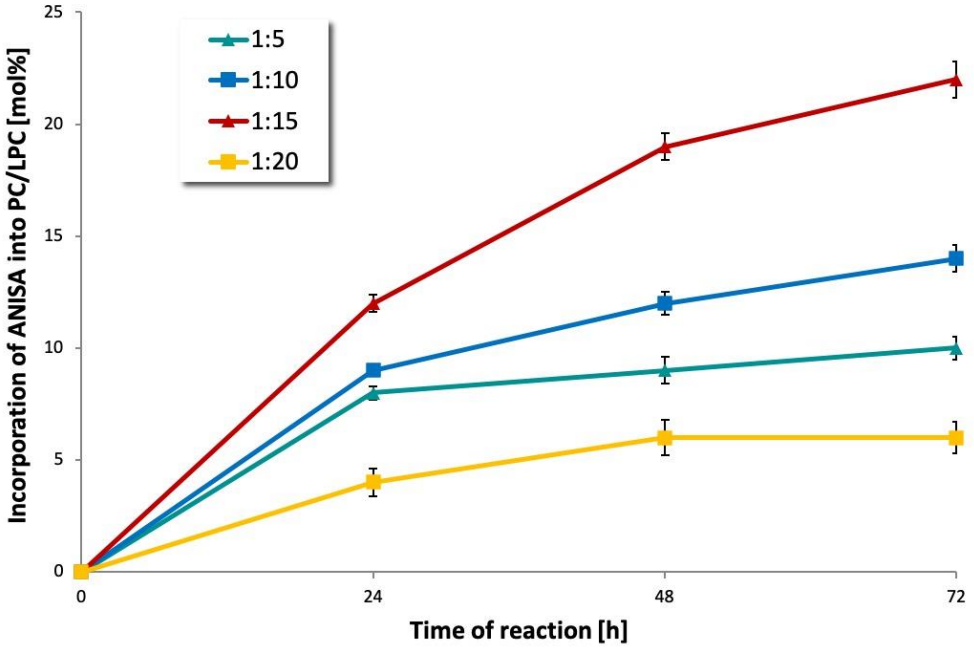


Figure 3. Effect of different substrate molar ratio on the incorporation of ANISA into egg-yolk PC in the acidolysis reaction (reaction conditions: solvent: toluene:chloroform (9:1 *v*/*v*) 2 mL, enzyme load 30% (*w*/*w*) Novozyme 435, temperature 50 °C). Vertical bars represent SD, n = 3.

2.5. Effect of Reaction Temperature

Reaction temperature is an important factor in lipase-catalyzed acidolysis, which influences the activity, selectivity, and stability of the enzyme. Generally, higher temperatures favor higher yields and rate of acidolysis, activating substrate molecules, increasing their solubility, and decreasing the viscosity of reaction solutions [40]. However, it must be pointed out that high temperature can also reduce the reaction rate due to irreversible denaturation of the enzyme and enhance side reactions such as acyl migration and hydrolysis [41,42]. The optimal temperature should be selected in terms of the overall performance of the reaction. The present results show that in the range of 30–50 °C, the incorporation rate increased significantly (Figure 4).

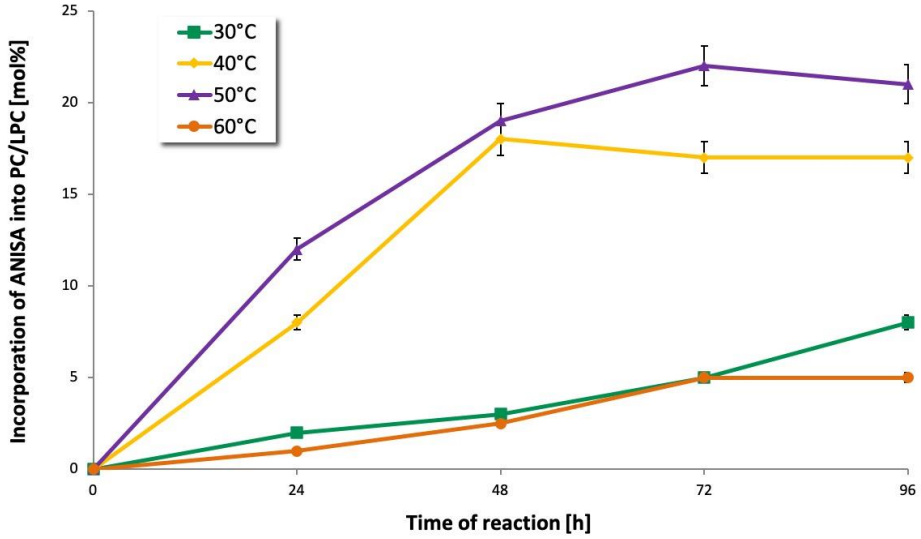


Figure 4. Effect of different temperatures on the incorporation of ANISA into egg-yolk PC in the acidolysis reaction (reaction conditions: 1:15 PC/ANISA molar ratio, solvent: toluene:chloroform (9:1 *v*/*v*) 2 mL, enzyme load 30% (*w*/*w*) Novozym 435). Vertical bars represent SD, n = 3.

Incorporation level through Novozym 435 increased from 5 to 22 mol % when the temperature increased from 30 to 50 °C. Further increase in the reaction temperature (to 60 °C) resulted in a decrease in ANISA incorporation for Novozym 435, which may be attributed to the partial deactivation of the enzyme [43]. The optimal temperature of 50 °C for lipase catalyzed acidolysis PC with ANISA falls within the range of 40–60 °C, which is reported in the literature as optimal for transesterification between selected phenolic acids with lipid molecules catalyzed by Novozym 435 [15,18,26].

2.6. Effect of Lipase Dosage

In biotechnological processes, it is always essential to determine the optimal enzyme loading to achieve the maximum efficiency and to reduce the costs. Therefore, in the next step of the study, the effect of different quantities of *C. antarctica* lipase B on the incorporation of ANISA was evaluated by varying the amount of enzyme load used in the reactions. The relation between the incorporation of ANISA into PL and lipase quantity was studied (Figure 5) using a range of enzyme concentration varying from 10% to 40% (based on the total weight of substrate) immobilized Novozym 435 in a 2 mL mixture of toluene:chloroform (9:1, *v*/*v*). Using 10% and 20% of Novozym 435, the incorporation of ANISA into the PL fraction was very low and achieved only 1 mol % and 2 mol %, respectively. Using 30% of lipase, the synthesis of anisoylated phenophospholipids increased rapidly and resulted in a higher level of ANISA incorporation into the PL fraction (22 mol %). The increase in the incorporation of acyl through the increase in the enzyme load has already been documented [43]. It has also been reported that a higher enzyme load can improve the incorporation of acyl in the acidolysis. However, Savaghebi et al. proved that beyond a certain value, an increase in the enzyme concentration does not increase productivity of the lipase and may lead to the hydrolysis of PC, which resulted in a low isolated yield of the product [44]. In our study, lowered incorporation of ANISA could also be the effect of increased viscosity. A similar observation has also been reported previously by Shaw [45]. A large difference observed for incorporation of ANISA into phospholipid fraction between enzyme loads of 20% and 30% (*w*/*w*) could be the result of the slow rate for acyl-enzyme complex formation. Jakovetić et al. reported a four times higher reaction rate constant for ethyl cinnamate than for ethyl-*p*-coumarate [46]. The higher incorporation at the presence of the higher amount of lipase (30% (*w*/*w*)) can then be the result of an increase in the probability of enzyme-substrate collisions and the subsequent reaction of esterification. Overall, the optimum enzyme load was estimated as 30%.

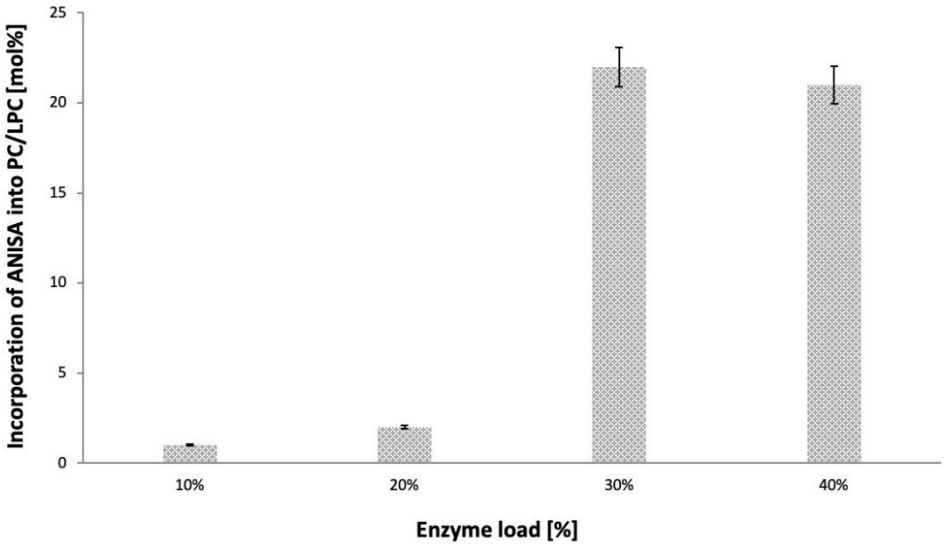


Figure 5. Effect of different lipase dosages on the incorporation of ANISA into egg-yolk PC in the acidolysis reaction (reaction conditions: 1:15 PC/ANISA molar ratio, solvent: toluene:chloroform (9:1 *v*/*v*) 2 mL, enzyme load 30% (*w*/*w*) Novozyme 435, temperature 50 °C, reaction time 72 h). Vertical bars represent SD, n = 3.

3. Materials and Methods

3.1. Substrates, Enzymes, and Chemicals

Egg-yolk phosphatidylcholine (PC) for enzymatic acidolysis was obtained according to the procedure described previously [32]. Immobilized lipase from *Thermomyces lanuginosus* (Lipozyme TL IM, 250 U/g) was provided by Novozymes A/S (Bagsvaeered, Denmark) while immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM, > 30 U/g) was supplied by Fluka (Buchs, Switzerland). Lipase B from *Candida antarctica* (CALB > 1800 U/g) immobilized on resin Immobead 150 and lipase B from *Candida antarctica* immobilized in a macroporous acrylic resin (Novozym 435 > 5000 U/g) were purchased from Sigma-Aldrich (St. Louis, MO, USA) as well as anisic acid (ANISA) and veratric acid (VERA).

1-Anisoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (1-ANISA-2-PA-PC) and 1-anisoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (1-ANISA-2-OH-LPC) were synthesized as described in an earlier paper [28] and used as reference standards.

All chemicals and organic solvents were of analytical grade and were supplied by Sigma-Aldrich (St. Louis, MO, USA). Silica gel (Kieselgel 60, 230–400 mesh), the thin layer pre-coated silica gel plates (Kieselgel 60 F254, 0.2 mm) used for column chromatography, and all solvents (Merck LiChrosolv Reag.) for high pressure liquid chromatography were purchased from Merck (Darmstadt, Germany).

3.2. Enzymatic Acidolysis of Egg-Yolk Phosphatidylcholine with Anisic/Veratric Acids

First, four different lipases (Lipozyme TL IM, Lipozyme RM IM, CALB and Novozym 435) were studied for the production of O-methylated phenophospholipids. The native egg-yolk PC (20 mg, 0.026 mmol) with anisic acid (ANISA) or veratric acid (VERA) at molar ratio 1:5 (PC/ANISA or PC/VERA) in 2 mL of heptane was mixed. Acidolysis was initiated by the addition of 30% of immobilized lipase (calculated in relation to substrate) to the substrate solution. The reactions were performed in screw-capped vials on a magnetic stirrer at a temperature of 50 °C at 300 rpm in an N_2 atmosphere. The different organic solvents (heptane, isooctane, toluene, toluene/chloroform (9:1 *v*/*v*), hexane:2-butanone (85:15 *v*/*v*), effect of molar ratio of PC to ANISA (1:5, 1:10, 1:15, 1:20), temperature

30–60 °C), time of reaction and enzyme load (10, 20, 30, 40%) were tested in another set of experiments using Novozym 435 as a biocatalyst. All reactions were performed in triplicate.

To evaluate the esterification rate, the reactions were stopped at specified time intervals for measurement of the incorporation of ANISA/VERA by enzyme filtration on a G4 Shott funnel with a Celite layer. Organic fractions were evaporated in vacuo and then modified phospholipid fractions were separated from the free fatty acids and unreacted methoxybenzoic acid using solid-phase extraction (SPE), according to the procedure described previously [27]. The phospholipid fraction was analyzed by thin-layer chromatography (TLC) and the profile of acids in the modified phospholipid fraction was analyzed by gas chromatography (GC) (Section 3.4.2).

3.3. Preparative Scale of Novozym 435-Catalyzed Acidolysis of Egg-Yolk Phosphatidylcholine with Anisic Acids

Enzymatic acidolysis was performed at 300 rpm in a N_2 atmosphere at 50 °C for 72 h. The reaction mixture contained 200 mg of egg-yolk phosphatidylcholine (PC) (0.026 mmol), anisic acid (at molar ratio of substrates 1/15, PC/ANISA), and 30% Novozym 435 (by weight of substrates) in 25 mL of binary solvent system toluene:chloroform (9:1 *v*/*v*). After 72 h, the crude reaction mixture was filtered and the solvent was evaporated in vacuo. Products were separated and purified by column chromatography. Initially, the column was eluted with chloroform/methanol/water (65:25:2 *v*/*v*/*v*) for the elution of the free fatty acids and unreacted anisic acid, followed by chloroform/methanol/water (65:25:4 *v*/*v*/*v*) to obtain the individual phospholipid fractions. The eluted fractions were collected in test tubes and identified by TLC on silica gel-coated aluminum plates according to the procedure described in Section 3.4.1. Separated and identified fractions of PC-egg, LPC-egg and modified PC (ANISA-PC), and LPC (ANISA-LPC) were combined and dried with magnesium sulfate ($MgSO_4$). After filtration, the solvent was evaporated in vacuo. The structures of ANISA-PC and ANISA-LPC were confirmed by HPLC.

3.4. Analysis of Substrates and Products

3.4.1. Thin-Layer Chromatography (TLC)

Qualitative analysis of the reaction mixtures of enzymatic acidolysis was performed by TLC on silica gel-coated aluminum plates using a mixture of chloroform/methanol/water (65:25:4 *v*/*v*/*v*) as a developing system. The reaction products were identified by spraying the TLC plates with 0.05% primuline solution (acetone:water, 8:2 *v*/*v*) and then exposing the plates to UV light (λ = 365 nm). R_f of different bands was identified as follows: 0.32 PC-egg, 0.13 LPC-egg, 0.19 ANISA-PC, and 0.06 ANISA-LPC.

3.4.2. Gas Chromatography (GC)

Fatty acid profiles of native PC and the modified phospholipid fraction (PC/LPC) were analyzed by gas chromatography after their conversion to the corresponding methyl esters (FAME). The same procedure was used for derivatization of anisic/veratric acid. A total of 10 mg of substrate was heated under reflux (3 min) with 3 mL of BF_3 × MeOH complex solution. After cooling, the mixtures were extracted with 2 mL of hexane and the organic layer was washed with a saturated NaCl solution. The hexane extract was dried over anhydrous $MgSO_4$. Samples performed in two replications were next analyzed by GC.

Methyl esters of fatty acids and anisic/veratric acids were analyzed on an Agilent 6890N chromatograph equipped with a flame ionization detector (FID) (Agilent, Santa Clara, CA, USA). The separation was performed using a DB-WAX capillary column (30 m × 0.32 mm × 0.25 µm film thickness) manufactured by Agilent Technologies (Santa Clara, CA, USA). The carrier gas was hydrogen with a constant flow of 1.5 mL/min. The oven temperature was first set at 90 °C and then raised to 200 °C (rate of 5 °C/min) and held there for 2 min. The total analysis time was 34 min. The injector temperature and the flame ionization detector temperatures were set at 260 °C. The FAME were identified by comparing their retention times with those of a standard FAME mixture (Supelco 37 FAME

Mix) purchased from Sigma Aldrich (St. Louis, MO, USA) and prepared methyl ester of ANISA and VERA. Quantitative analysis of incorporation of ANISA/VERA into the PL fraction (PC/LPC), expressed as ANISA/VERA mol %, was carried out based on their peak areas and were calculated using GC ChemStation Version A.10.02.

3.4.3. High Performance Liquid Chromatography (HPLC)

The native phosphatidylcholine and modified PC (ANISA-PC) were analyzed by HPLC on an DIONEX UltiMate 3000 chromatograph from Thermo Fisher Scientific (Olten, Switzerland) equipped with a Corona charged aerosol detector (CAD) from ESA Biosciences (Chelmsford, MA, USA) and a UV/CAD detector (at 270 nm). A BetaSil DIOL column (Thermo Scientific, 150 × 4.6 mm, 5 µm) was used for analysis. The injection volume was 15 µL for all analyzed samples. The temperature for the autosampler compartment was 20 °C and column temperature was maintained at 30 °C. The analysis was performed in a gradient mode with a constant flow of 1.5 mL/min. Solvent A (1% HCOOH, 0.1% TEA (triethylamine) in water), solvent B (hexane), and solvent C (2-propanol) were used. The elution program started with 3/40/57 (%A/%B/%C (*v*/*v*/*v*)), at 5 min = 10/40/50, at 9 min = 10/40/50, at 9.1 min = 3/40/57, and at 19 min = 43/40/57. Total time of analysis was 19 min. R_t for ANISA-PC was 11.408.

The ANISA-LPC was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) at 30 °C. The chromatographic separation was carried out with an Ascentis Express C18 column (250 × 4.6 mm, 5 µm) with UV/DAD detector (at 270 nm), according to the procedure described by Yang [26]. The mobile phase was a binary solvent of A (water containing 3% acetic acid) and B (acetonitrile) at a flow rate of 0.5 mL/min. The gradient was operated as follows: 0 min: 90% A and 10% B; 0–10 min: changed to 30% A and 70% B; 10–20 min: maintained 30% A and 70% B; and 20–20.5 min: changed to 90% A and 10% B. Total analysis time was 31 min. R_t for ANISA-LPC was 4.490. The sample was diluted in solvent A and the injection volume was 10 µL.

4. Conclusions

In the present paper, we describe the enzymatic synthesis approach for the preparation of anisoyl-structured phospholipids via a one-step acidolysis of anisic acid and egg-yolk phosphatidylcholine. Of the four lipases tested, Novozym 435 showed the highest catalytic activity for synthesis of anisoylated PC. The enzymatic synthesis is strongly dependent on the organic solvent, substrate molar ratio, and temperature. HPLC analysis confirmed that anisoyl-lysophosphatidylcholine (ANISA-LPC) was formed as the major product of the reaction, whereas modified phosphatidylcholine (ANI1SA-PC) was detected only in a trace amount. Under the optimized conditions, ANISA-LPC and ANISA-PC were obtained in 28.5% and 2.5% isolated yield (*w*/*w*), respectively. The developed method is an effective alternative to the previously described chemical synthesis of these compounds.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/10/5/538/s1, Figure S1: HPLC chromatogram of ANISA-PC, Figure S2: HPLC chromatogram of ANISA-LPC, Figure S3: GC chromatogram of fatty acid composition of modified phospholipid fraction PC/LPC.

Author Contributions: Conceptualization, A.G.; Investigation, M.O.; Methodology, M.O. and A.G.; Validation, A.G.; Writing—original draft preparation, M.O. and A.G.; Writing—Review and editing, A.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financed by the Wrocław University of Environmental and Life Sciences from the research project no. D220/0005/17 as part of the program "Innovative Doctorate". The Article Processing Charge (APC) was financed under the Leading Research Groups support project from the subsidy increased for the period 2020–2025 in the amount of 2% of the subsidy referred to Art. 387 (3) of the Law of 20 July 2018 on Higher Education and Science, obtained in 2019.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Ohira, H.; Torii, N.; Aida, T.M.; Watanabe, M.; Smith, R.L. Rapid separation of shikimic acid from Chinese star anise (*Illicium verum* Hook. f.) with hot water extraction. *Sep. Purif. Technol.* **2009**, *69*, 102–108. [CrossRef]
2. Gadgoli, C.; Mishra, S.H. Antihepatotoxic activity of p-methoxy benzoic acid from Capparis spinosa. *J. Ethnopharmacol.* **1999**, *66*, 187–192. [CrossRef]
3. Tao, L.; Wang, S.; Zhao, Y.; Sheng, X.; Wang, A.; Zheng, S.; Lu, Y. Phenolcarboxylic acids from medicinal herbs exert anticancer effects through disruption of COX-2 activity. *Phytomedicine* **2014**, *21*, 1473–1482. [CrossRef] [PubMed]
4. Saravanakumar, M.; Raja, B. Veratric acid, a phenolic acid attenuates blood pressure and oxidative stress in L-NAME induced hypertensive rats. *Eur. J. Pharmacol.* **2011**, *671*, 87–94. [CrossRef] [PubMed]
5. Raja, B.; Saravanakumar, M.; Sathya, G. Veratric acid ameliorates hyperlipidemia and oxidative stress in Wistar rats fed an atherogenic diet. *Mol. Cell. Biochem.* **2012**, *366*, 21–30. [CrossRef]
6. Shin, S.W.; Jung, E.; Kim, S.; Lee, K.E.; Youm, J.K.; Park, D. Antagonist effects of veratric acid against UVB-induced cell damages. *Molecules* **2013**, *18*, 5405–5419. [CrossRef]
7. Liu, L.J.; Wu, X.W.; Wang, R.F.; Peng, Y.S.; Yang, X.; Liu, J.X. Absorption properties and mechanism of trolline and veratric acid and their implication to an evaluation of the effective components of the flowers of Trollius chinensis. *Chin. J. Nat. Med.* **2014**, *12*, 700–704. [CrossRef]
8. Bento-Silva, A.; Koistinen, V.M.; Mena, P.; Bronze, M.R.; Hanhineva, K.; Sahlstrøm, S.; Kitryte, V.; Moco, S.; Aura, A.M. Factors affecting intake, metabolism and health benefits of phenolic acids: Do we understand individual variability? *Eur. J. Nutr.* **2019**. [CrossRef]
9. Clifford, M.N. Diet-derived phenols in plasma and tissues and their implications for health. *Planta Med.* **2004**, *70*, 1103–1114. [CrossRef]
10. Zhao, Z.; Moghadasian, M. Bioavailability of hydroxycinnamates: A brief review of in vivo and in vitro studies. *Phytochem. Rev.* **2010**, *9*, 133–145. [CrossRef]
11. Guyot, B.; Bosquette, B.; Pina, M.; Graille, J. Esterification of phenolic acids from green coffee with an immobilized lipase from Candida antarctica in solvent-free medium. *Biotechnol. Lett.* **1997**, *19*, 529–532. [CrossRef]
12. Buisman, G.J.H.; Van Helteren, C.T.W.; Kramer, G.F.H.; Veldsink, J.W.; Derksen, J.T.P.; Cuperus, F.P. Enzymatic esterifications of functionalized phenols for the synthesis of lipophilic antioxidants. *Biotechnol. Lett.* **1998**, *20*, 131–136. [CrossRef]
13. Stamatis, H.; Sereti, V.; Kolisis, F.N. Studies on the Enzymatic Synthesis of Lipophilic Derivatives of Natural Antioxidants. *J. Am. Oil Chem. Soc.* **1999**, *76*, 1505–1510. [CrossRef]
14. Compton, D.L.; Kenar, J.A.; Laszlo, J.A.; Felker, F.C. Starch-encapsulated, soy-based, ultraviolet-absorbing composites with feruoylated monoacyl- and diacylglycerol lipids. *Ind. Crops Prod.* **2007**, *25*, 17–23. [CrossRef]
15. Karboune, S.; St-louis, R.; Kermasha, S. Enzymatic synthesis of structured phenolic lipids by acidolysis of flaxseed oil with selected phenolic acids. *J. Mol. Catal. B Enzym.* **2008**, *53*, 96–105. [CrossRef]
16. Sun, S.; Zhu, S.; Bi, Y. Solvent-free enzymatic synthesis of feruloylated structured lipids by the transesterification of ethyl ferulate with castor oil. *Food Chem.* **2014**, *158*, 292–295. [CrossRef]
17. Yu, X.; Li, Y.; Wu, D. Enzymatic synthesis of gallic acid esters using microencapsulated tannase: Effect of organic solvents and enzyme specificity. *J. Mol. Catal. B Enzym.* **2004**, *30*, 69–73. [CrossRef]
18. Safari, M.; Karboune, S.; St-louis, R. Enzymatic synthesis of structured phenolic lipids by incorporation of selected phenolic acids into triolein. *Biocatal. Biotransform.* **2006**, *24*, 272–279. [CrossRef]
19. Dahan, A.; Markovic, M.; Epstein, S.; Cohen, N.; Zimmermann, E.M.; Aponick, A.; Ben-Shabat, S. Phospholipid-drug conjugates as a novel oral drug targeting approach for the treatment of inflammatory bowel disease. *Eur. J. Pharm. Sci.* **2017**, *108*, 78–85. [CrossRef]
20. Markovic, M.; Ben-Shabat, S.; Keinan, S.; Aponick, A.; Zimmermann, E.M.; Dahan, A. Prospects and challenges of phospholipid-based prodrugs. *Pharmaceutics* **2018**, *10*, 210. [CrossRef]
21. Gliszczyńska, A.; Niezgoda, N.; Gładkowski, W.; Czarnecka, M.; Świtalska, M.; Wietrzyk, J. Synthesis and biological evaluation of novel phosphatidylcholine analogues containing monoterpene acids as potent antiproliferative agents. *PLoS ONE* **2016**, *11*, e0157278. [CrossRef] [PubMed]

22. Gliszczyńska, A.; Niezgoda, N.; Gładkowski, W.; Świtalska, M.; Wietrzyk, J. Isoprenoid-phospholipid conjugates as potential therapeutic agents: Synthesis, characterization and antiproliferative studies. *PLoS ONE* **2017**, *12*, e0172238. [CrossRef] [PubMed]
23. Niezgoda, N.; Gliszczyńska, A. Lipase catalyzed acidolysis for efficient synthesis of phospholipids enriched with isomerically pure cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid. *Catalysts* **2019**, *9*, 1012. [CrossRef]
24. Balakrishna, M.; Kaki, S.S.; Karuna, M.S.L.; Sarada, S.; Kumar, C.G.; Prasad, R.B.N. Synthesis and in vitro antioxidant and antimicrobial studies of novel structured phosphatidylcholines with phenolic acids. *Food Chem.* **2017**, *221*, 664–672. [CrossRef]
25. Anankanbil, S.; Pérez, B.; Banerjee, C.; Guo, Z. New phenophospholipids equipped with multi-functionalities: Regiospecific synthesis and characterization. *J. Colloid Interface Sci.* **2018**, *523*, 169–178. [CrossRef]
26. Yang, H.; Mu, Y.; Chen, H.; Xiu, Z.; Yang, T. Enzymatic synthesis of feruloylated lysophospholipid in a selected organic solvent medium. *Food Chem.* **2013**, *141*, 3317–3322. [CrossRef]
27. Rychlicka, M.; Maciejewska, G.; Niezgoda, N.; Gliszczyńska, A. Production of feruloylated lysophosphatidylcholine via a one-step enzymatic interesterification. *Food Chem.* **2020**, *323*, 126802. [CrossRef]
28. Czarnecka, M.; Switalska, M.; Wietrzyk, J.; Gliszczynska, A. Growth Inhibition Evaluation of Novel Phosphatidylcholines with Anisic and Veratric Acids. *Molecules* **2018**, *23*, 2022. [CrossRef]
29. Czarnecka, M.; Switalska, M.; Wietrzyk, J.; Gliszczynska, A. Synthesis and biological evaluation of phosphatidylcholines with cinnamic and 3-methoxycinnamic acids with potent antiproliferative activity. *RSC Adv.* **2018**, *8*, 35744–35752. [CrossRef]
30. Drzazga, A.; Okulus, M.; Rychlicka, M.; Biegała, Ł.; Gliszczyńska, A.; Gendaszewska-Darmach, E. Lysophosphatidylcholine containing anisic acid is able to stimulate insuline secretion via G protein coupled receptors. *Nutrients* **2020**, *12*, 1173. [CrossRef]
31. Svensson, I.; Adlercreutz, P.; Mattiasson, B. Interesterification of phosphatidylcholine with lipases in organic media. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 255–258. [CrossRef] [PubMed]
32. Rychlicka, M.; Niezgoda, N.; Gliszczyńska, A. Lipase-catalyzed acidolysis of egg-yolk phosphatidylcholine with citronellic acid. New insight into synthesis of isoprenoid-phospholipids. *Molecules* **2018**, *23*, 314. [CrossRef]
33. Robert, C.; Couëdelo, L.; Vaysse, C.; Michalski, M.C. Vegetable lecithins: A review of their compositional diversity, impact on lipid metabolism and potential in cardiometabolic disease prevention. *Biochimie* **2020**, *169*, 121–132. [CrossRef] [PubMed]
34. Zierenberg, O.; Grundy, S.M. Intestinal absorption of polyenephosphatidylcholine in man. *J. Lipid Res.* **1982**, *23*, 1136–1142. [PubMed]
35. Chojnacka, A.; Gładkowski, W.; Grudniewska, A. Lipase-catalyzed transesterification of egg-yolk phosphatidylcholine with concentrate of n-3 polyunsaturated fatty acids from cod liver oil. *Molecules* **2017**, *22*, 1771. [CrossRef]
36. Liu, K.J.; Liu, K.M.; Chang, H.M. Biocatalytic synthesis of palmitoyl vanillylamide in supercritical carbon dioxide through amidation of vanillylamine hydrochloride and palmitic anhydride by lipase. *Food Chem.* **2007**, *102*, 1020–1026. [CrossRef]
37. Lue, B.M.; Karboune, S.; Yeboah, F.K.; Kermasha, S. Lipase-catalyzed esterification of cinnamic acid and oleyl alcohol in organic solvent media. *J. Chem. Technol. Biotechnol.* **2005**, *80*, 462–468. [CrossRef]
38. Kim, I.H.; Yoon, C.S.; Lee, K.W. Transesterification of conjugated linoleic acid and tricaprylin by lipases in organic media. *Food Res. Int.* **2001**, *34*, 301–306. [CrossRef]
39. Sabally, K.; Karboune, S.; St-Louis, R.; Kermasha, S. Lipase-catalyzed transesterification of dihydrocaffeic acid with flaxseed oil for the synthesis of phenolic lipids. *J. Biotechnol.* **2006**, *127*, 167–176. [CrossRef]
40. Peng, L.; Xu, X.; Mu, H.; Hoy, C.-E.; Adler-Nissen, J. Production of structured phospholipids by lipase-catalyzed acidolysis, optimization using response surface methodology. *Enzym. Microb. Technol.* **2002**, *31*, 523–532. [CrossRef]
41. Karabulut, I.; Durmaz, G.; Hayaloglu, A.A. Fatty acid selectivity of lipases during acidolysis reaction between triolein and saturated fatty acids varying from caproic to behenic acids. *J. Agric. Food Chem.* **2009**, *57*, 7584–7590. [CrossRef] [PubMed]

42. Vikbjerg, A.F.; Mu, H.; Xu, X. Parameters affecting incorporation and by-product formation during the production of structured phospholipids by lipase-catalyzed acidolysis in solvent-free system. *J. Mol. Catal. B Enzym.* **2005**, *36*, 14–21. [CrossRef]
43. Zhao, H.; Lu, Z.; Bie, X.; Lu, F.; Liu, Z. Lipase catalyzed acidolysis of loard with capric acid in organic solvent. *J. Food Eng.* **2007**, *78*, 41–46. [CrossRef]
44. Savaghebi, D.; Safari, M.; Rezaei, K.; Ashtari, P.; Farmani, J. Structured lipids produced through lipase-catalyzed acidolysis of canola oil. *J. Agric. Sci. Technol.* **2012**, *14*, 1297–1310.
45. Shaw, J.F.; Wu, H.Z.; Shieh, C.J. Optimized enzymatic synthesis of propylene glycol monolaurate by direct esterification. *Food Chem.* **2003**, *81*, 91–96. [CrossRef]
46. Jakovetić, S.M.; Jugović, B.Z.; Gvozdenović, M.M.; Bezbradica, D.I.; Antov, M.G.; Mijin, D.Ž.; Knežević-Jugović, Z.D. Synthesis of aliphatic esters of cinnamic acid as potential lipophilic antioxidant catalyzed by lipase B from *Candida antarctica*. *Appl. Biochem. Biotechnol.* **2013**, *170*, 1560–1573. [CrossRef]