Hydrolysis of Phospholipids in Presence of Phospholipase D: Thrmodynamic and Kinetic Studies of Hydrolysis in Water and Alcoholic Environments

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Article's Information	Abstract
	Phospholipase D (PLD) catalyzes the hydrolysis of phospholipids to produce phosphatidic acid and hydroxyl compounds. Phosphatidylcholine (PC) is the most abundant phospholipid in animals and plants, often constituting nearly 50% of total complex lipids in animals and plants. However, Phosphatidylserine (PS) makes up around 5–10% of all phospholipids. This work includes the monitoring thermodynamic and kinetic of the enzymatic hydrolysis of PC and PS in both water and ethanol. All experiments were performed using the Schlink line technique in N_2 present as an inert gas to prevent the oxidative stress. UV/Vis. spectrophotometer was used to observe the kinetic of all enzymatic reactions. In addition, the Origin 2019 software was used to analyze and find the kinetic parameters of the enzymatic hydrolysis reactions. The results show that the enzymatic hydrolysis reactions of PC in water and in ethanol occurred at the same conditions, enzymatic activity 1.752 U/mg, temperature 37 °C, and pH =7. However, the enzymatic hydrolysis reactions of PS in water occurred at different conditions than in ethanol. The enzymatic substitution reaction of PS in ethanol was thermodynamic favorable reaction due to the value of the $\Delta G = -164.868$ J, but in water was thermodynamically unfavorable $\Delta G = 65.048$ J. However, the enzymatic hydrolysis reaction of PC in water was thermodynamically unfavorable $\Delta G = 345.319$ J as well as in ethanol $\Delta G = 74.433$ J. The study shows that there is clear impact of present nitrogen bases of PC and the environment of the hydrolysis on the activity of the enzymatic catalyzing.

1. Introduction

A group of enzymes known as phospholipase D (PLD, EC 3.1.4.4), or phosphatidylcholine phospholipid hydrolase, works on the phosphodiester bond in phospholipid molecules. It catalyzes the hydrolysis of phospholipids to produce phosphatidic acid and hydroxyl compounds as well as, under specific conditions, the combining of various hydroxyl containing compounds with the bases of phospholipids to new phospholipids [1]. Phospholipids also known as glycerophospholipids, these lipids have a glycerol backbone that binds a hydrophobic tail (made up of two long-chain fatty acids bonded at the *sn*-1 and *sn*-2 positions) and *sn*-3 position bind to phosphate and nitrogen base as a hydrophilic head group [2-6]. Since PLs are biologically active substances, they are extensively researched and examined in addition to being the primary element of all biological membranes [7].

The three PLs were found in lecithin, which frequently are phosphate-dylcholine (PC),

phosphate-dylethanolamine (PE), and phosphate-dylinositol (PI) [8]. Although lecithin makes up between 40 and 50 percent of all phospholipids, phosphatidylcholine (PC) is mostly found in the cells' outer layer. Phosphatidylcholine or 1,2-diacyl-sn-glycero phosphocholine (formerly the common name "lecithin") is a neutral or zwitterionic phospholipid with a pH range from strongly acidic to strongly alkaline [9-11]. It is normally the most abundant phospholipid in animals and plants, often constituting nearly 50% of total complex lipids, and as such appears to be a key component of membrane bilayers [12]. Phosphatidylserine (PS), which makes up around 5–10% of all phospholipids, and phosphate-dylethanolamine (PE), which accounts for 20–30% of all phospholipids, are the two main types in the inner layer of cell membranes [13-15]. An analogy to other phospholipids, PS has a polar head group neutral serine group attached to the phosphate and thus has an overall negative charged linked to position sn-3 and two acyl chains at locations *sn*-1 and *sn*-2 of the glycerol moiety [16-18]. Saturated fatty acids with 16 or more carbons are often connected to the *sn*-1 position in PS, whereas unsaturated fatty acids are typically located at the *sn*-2 position, even though the acyl chains in PS differ among cell types and organelles [19,20]. PS makes up a small portion of most cellular membranes [21,22]. The PS has an important role in the removal of apoptotic cells. In addition, the PS plays a further role in the recruitment and activation of several enzymes and structural elements. [23-25]. This work focuses on the enzymatic hydrolysis reactions of the two PLs derivatives, which are phosphatidylcholine and phosphatidylserine, in water as well as in ethanol catalyzing by phospholipase D.

2. Materials and Methods
Phosphatidylcholine (α-L-Lecithin, 98%) and phosphatidylserine (Ptd-L-Ser, 99%) were provided by Chemfish Tokyo Co., Ltd, Japan. Phospholipase D (PLD), hydrochloric acid (ACS reagent, 37%), and absolute ethanol were provided by Sigma-Aldrich, USA. This study was conducted at department of chemistry, collage of science, university of Kerbala, Iraq. All experiments were performed at an inert environment (in presence of N_2 as inert gas) using the Schlenk line technique. The data for enzymatic hydrolysis reaction of PC and PS were observed using Shimadzu UV-1800 spectrophotometer and Shimadzu (FTIR-8400S) spectrophotometer. The Origin 2019 software provided by Origin Lab cooperation, Northampton, Massachusetts, USA was used to analyze and find the kinetic parameters of the enzymatic hydrolysis reactions.

2.1. Concentration of the Substrates (PC and PS)
Experiments were performed to finding the appropriate concentration of both PC and PS for the enzymatic hydrolysis reactions by using the substrate concentrations at a physiological temperature (37 ± 2) and enzymatic activity (1.46) U/mg of the PLD enzyme. The reaction was monitored by withdrawing a 1 ml of the mixture (PLs, PLD, and dissociative nitrogen base) every 10 minutes and measuring its absorbance by spectrophotometer analyzer after fixing the wavelength at the maximum wavelength of the lipid derivative (λ_{max} =360 nm). After monitoring the results, the ideal substrate concentrations (PC = 0.8 mM, PS = 0.08 mM) were reached by taking the concentration that provided the most regular readings during work. Furthermore, the enzymatic hydrolysis and substitution experiments for derivatives (choline, serine, and phosphatidic acid) were carried out by taking different activities of PLD (0.292, 0.73, 1.168, 1.46, and 1.752) U/mg after fixing the concentration of the substrate at optimum concentration, the temperature at (37 ± 2) °C and the pH at 7.4, repeating the same steps in precious experiments and following the absorbance readings to obtain the suitable enzymatic activity in water and ethanol.

2.2. Finding the Michaelis-Menten Parameters for Enzymatic Hydrolysis of Phospholipids (PC and PS)
Both the Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad \ldots (1)$$

and Lineweaver-Burke equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad \ldots (2)$$

were applied to determine the values of maximum velocity of the enzymatic hydrolysis reaction of PLs (V_{max}) and the Michaelis-Menten constant K_m of the enzymatic reaction.
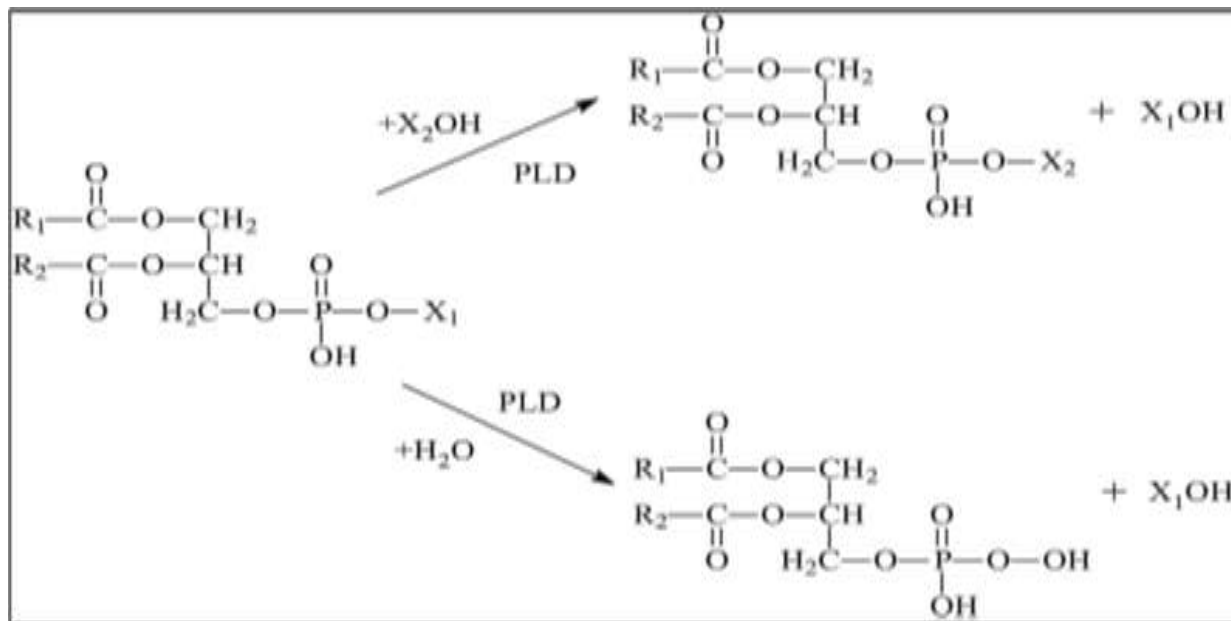

Figure 1. Hydrolysis reaction of phospholipid in presence of phospholipase D

2.3. Thermodynamic Study for Enzymatic Hydrolysis of PC and PS

To finding the thermodynamic parameters that include enthalpy change (ΔH), Gibbs free energy (ΔG), and entropy change (ΔS) for the enzymatic hydrolysis reactions of PC and PS in presence of PLD as catalyst. The rates of enzymatic reactions were observed at different temperatures (10, 15, 20, 25, 30, 37, 40, and 45) °C. In addition, the values of ΔH and ΔS were found by applying the Van 't Hoff equation:

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad \ldots (3)$$

The ΔG value was calculated using the following equation:

$$\Delta G = \Delta H - T\Delta S \quad \ldots (4)$$

3. Results and Discussion
3.1. Enzymatic hydrolysis of PC and PS in Water

The values for V_{max} for the enzymatic hydrolysis of PC and PS in water were 0.2124 and 0.274 in addition to the values the K_m were 0.25 and 0.01 respectively, as shown in Figures 2 and 3.

The thermodynamics parameters for the enzymatic hydrolysis of PC in water were $\Delta H = 136.747$ J.mol^{-1}, $\Delta G = 65.048$ J and $\Delta S = 0.23$ J.K^{-1}, see Figure 4. The parameters for the enzymatic hydrolysis of PS in water were $\Delta H = 302.195$ J.mol^{-1}, $\Delta G = 345.319$ J, and $\Delta S = -0.193$ J.K^{-1}, see Figure 5.

3.2. Enzymatic hydrolysis of PC and PS in ethanol

The results of enzymatic substitution reaction of PC and PS in ethanol by using different concentrations, which include the values of the maximum velocity (V_{max}) of the enzymatic reaction were 0.253 and 0.234 in addition to the reaction rate constant (K_m) were 3.664 and 0.73 respectively as shown in Figures 6 and 7.

The thermodynamic values of the change in (ΔH), (ΔS), and (ΔG) for the substitution enzymatic reactions for PC and PS with ethanol are presented in Figures 8 and 9.

The kinetics of the enzymatic hydrolysis reaction of PC in the presence of the PLD revealed enzymatic activity 1.752 U/mg, temperature 37 °C,

and pH =7. In addition, the optimal circumstances of enzymatic substitution reaction of PC in ethanol pH = 7, enzyme activity 1.168 U/mg, and temperature 37 ᵒC. Consequently, it can be concluded that both reactions occurred under the same conditions.

However, it was appeared that the enzymatic hydrolysis reaction of PS occurred in different conditions for the enzymatic substitution reaction of PS with ethanol. The enzymatic hydrolysis reaction of PS occurred at pH = 9, enzymatic activity = 1.752 U/mg and 35 °C, while the enzymatic substitution reaction of PS with ethanol occurred at pH = 6, enzyme activity = 1.46 U/mg, and 37 ᵒC. ─The K_m value for the enzymatic hydrolysis reaction of PS was the lowest 0.01 in all reactions, indicating the least affinity between the PLD enzyme and the PS.

The enzymatic hydrolysis of PS in water was thermodynamically unfavorable ΔG = 345.319 J. However, the substitution reaction of PS with ethanol was thermodynamic favorable reaction due to the value of the ΔG = ₋164.868 J.

On the other hand, the enzymatic hydrolysis reaction of PC in water was thermodynamically less favorable ΔG = 65.048 J, and the enzymatic substitution reaction of PC in ethanol was also thermodynamically less favorable ΔG = 74.433 J.The thermodynamic differences can be attributed to the impact of the alcoholic environment on the enzymatic reactions.
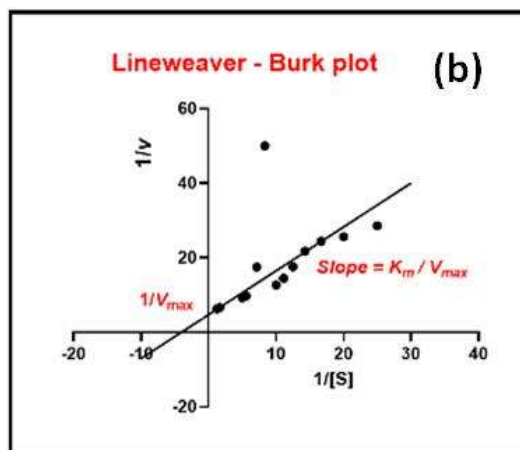

Figure 2. Michaelis-Menten and Lineweaver-Burke diagrams for the enzymatic hydrolysis reaction of PC in water.
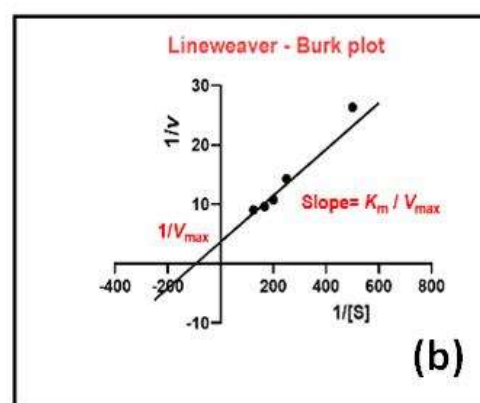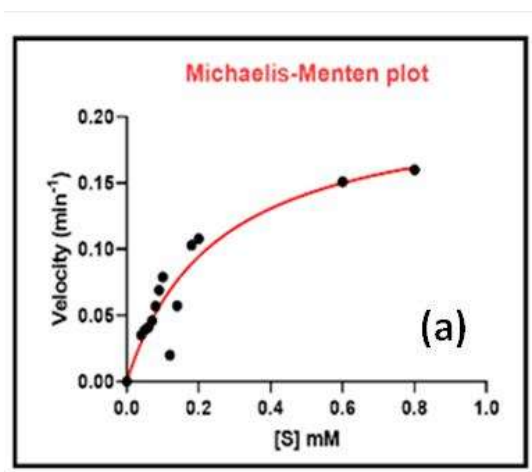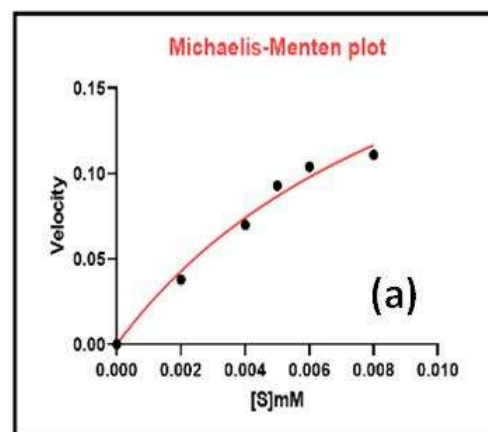

Figure 3. Michaelis-Menten and Lineweaver-Burke diagrams for the enzymatic hydrolysis reaction of PS in water.
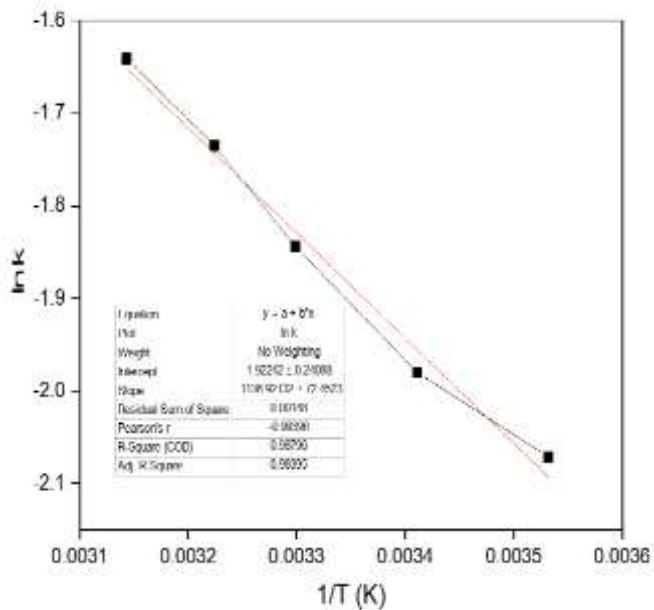
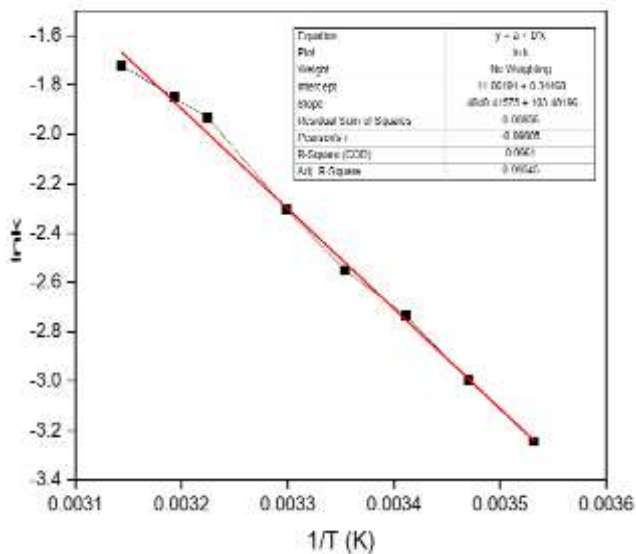**Figure 4.** Van't Hoff diagram for the enzymatic hydrolysis of PC in water.

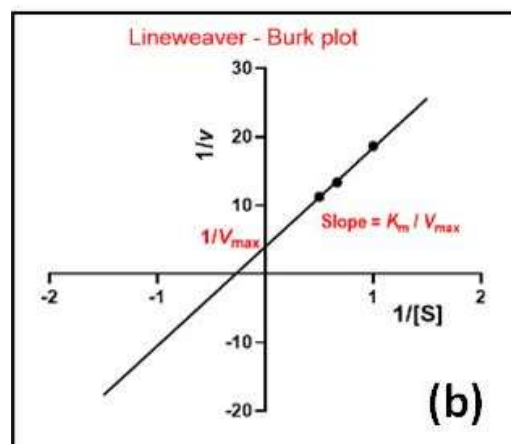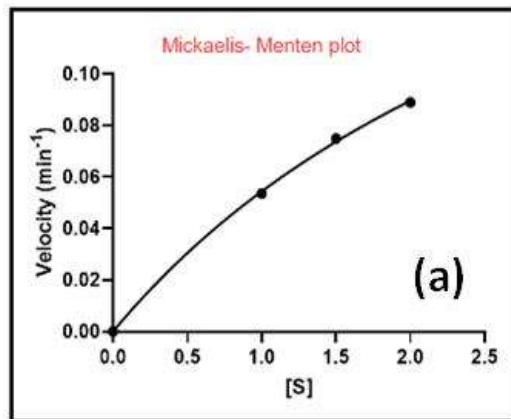**Figure 5.** Van't Hoff diagram for the enzymatic hydrolysis of PS in water.
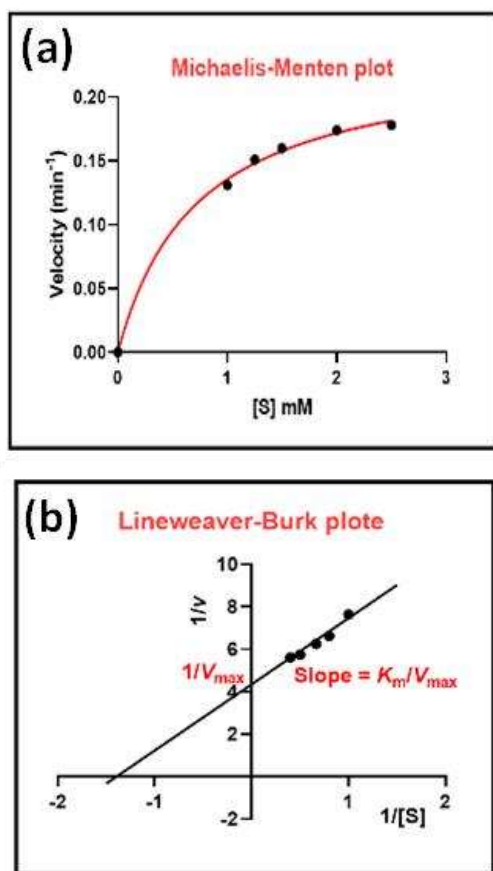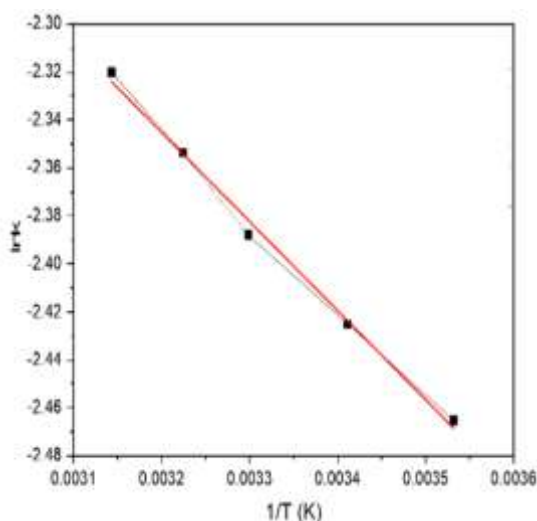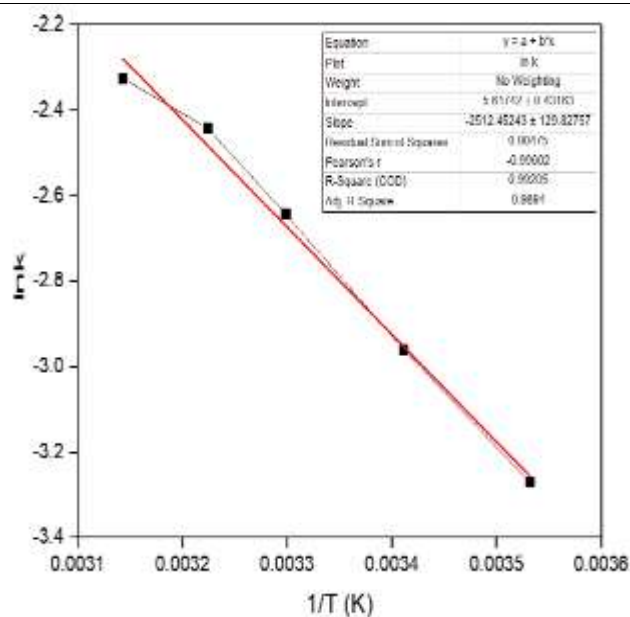

Figure 6. Michaelis-Menten and Lineweaver-Burke diagrams for the enzymatic substitution reaction of PC in ethanol.

Figure 7. Michaelis-Menten and Lineweaver-Burke diagrams for the enzymatic substitution reaction of PS in ethanol.

Figure 8. Van't Hoff diagram for the enzymatic substitution reaction of PC with ethanol. $\Delta H = 487.095$ J.mol^{-1}, $\Delta S = 1.33$ J.K^{-1} and $\Delta G = 74.433$ J.

Figure 9. Van't Hoff diagram for the enzymatic substitution reaction of PS with ethanol, $\Delta H = 44.671$ J.mol^{-1}, $\Delta S = 0.675$ J.K^{-1} and $\Delta G = -164.868$ J.

4. Conclusions

It is important to study the impact of ethanol on the hydrolysis of phospholipids due to the various applications of phosphatidylcholine and phosphatidylserine, so understanding of the effect of the alcoholic environment can help pharmaceutical, cosmetic, and food factories to design appropriate experiments to reduce the impact of these byproducts on the main product efficiency and yield.

Conflict of Interest:
All authors declare that there are no conflicts of interest.

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