Chemistry and Physics of Lipids xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Short communication

# Peptidophospholipids: Synthesis, phospholipase $A_2$ catalyzed hydrolysis, and application to development of phospholipid prodrugs

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ARTICLE INFO

*Article history:*
Received 11 April 2014
Received in revised form 29 May 2014
Accepted 1 June 2014
Available online xxx

*Keywords:*
Phospholipid synthesis
Antiviral prodrugs
Phospholipase $A_2$ catalysis
Lipid-prodrug design

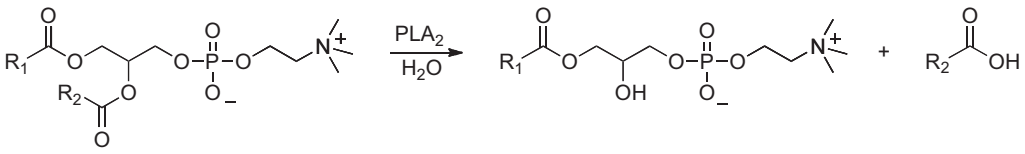
ABSTRACT

New phospholipid analogues incorporating *sn*-2-peptide substituents have been prepared to probe the fundamental structural requirements for phospholipase $A_2$ catalyzed hydrolysis of $PLA_2$-directed synthetic substrates. Two structurally different antiviral oligopeptides with C-terminal glycine were introduced separately at the *sn*-2-carboxylic ester position of phospholipids to assess the role of the α-methylene group adjacent to the ester carbonyl in allowing hydrolytic cleavage by the enzyme. The oligopeptide-carrying phospholipid derivatives were readily incorporated into mixed micelles consisting of natural phospholipid (dipalmitoyl phosphatidylcholine, DPPC) and Triton X-100 as surfactant. Hydrolytic cleavage of the synthetic peptidophospholipids by the phospholipase $A_2$ occurred slower, but within the same order of magnitude as the natural substrate alone. The results provide useful information toward better understanding the mechanism of action of the enzyme, and to improve the design and synthesis of phospholipid prodrugs targeted at secretory $PLA_2$ enzymes.



## 1. Introduction

Phospholipases $A_2$ ($PLA_2$s) comprise a superfamily of intracellular and secreted enzymes that catalyze the hydrolysis of the *sn*-2-ester bond of glycerophospholipids to yield fatty acids such as arachidonic acid and lysophospholipids (Dennis et al., 2011; Eq. (1)).




(1)

$R_1$, $R_2$ = naturally occurring fatty acids

The products are precursors of signaling molecules with a wide range of biological functions (Murakami and Lambeau, 2013). Along these lines arachidonic acid is converted to eicosanoids that have been shown to be involved in immune response, inflammation, pain perception and sleep regulation (Funk, 2001; Murakami et al., 2011a), while lysophospholipids are precursors of lipid mediators such as lysophosphatidic acid (LPA) and platelet activating factor (PAF). Specifically, LPA is involved in cell proliferation, survival and migration (Rivera and Chun, 2008; Zhao and Natarajan, 2009), while PAF is involved in inflammatory processes (Prescott et al., 2000).

Secretory phospholipases $A_2$ ($sPLA_2$s) occur widely in nature (Murakami et al., 2011b). The members of the $sPLA_2$ family were first isolated from insects and snake venoms, and subsequently they were found in plants, bacteria, fungi, viruses and mammals as well. To date more than 30 isozymes have been identified in mammals, and they have been classified based on their structures, catalytic mechanisms, localization, and evolutionary relationships (Schaloske and Dennis, 2006). The mammalian $PLA_2$ family

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http://dx.doi.org/10.1016/j.chemphyslip.2014.06.001
0009-3084/

includes 10 catalytically active isoforms (Lambeau and Gelb, 2008). Secretory $PLA_2$s isolated from a variety of sources share a series of common structural features. They are low molecular weight (14–18 kDa) secreted proteins, with a compact structure stabilized by six conserved disulfide bonds and two additional disulfides that are unique to each member (Dennis et al., 2011). Studies focusing on their mechanism of action have shown that an active site histidine and a highly conserved neighboring aspartate form a catalytic dyad involved in the reaction, requiring $Ca^{2+}$ for activation (Murakami et al., 2011b).

Mammalian $sPLA_2$s have been implicated in a variety of physiological and pathophysiological processes including lipid digestion, cell-proliferation, neurosecretion, antibacterial defense, cancer, tissue injury, and atherosclerosis (Murakami and Lambeau, 2013). Furthermore, it has become apparent that individual secretory phospholipase $A_2$ enzymes play important and diverse roles in biological events by acting through multiple mechanisms: (1) involving production of lipid mediators, and (2) executing their own unique action on their specific extracellular targets in lipid mediator-independent processes (Murakami et al., 2011b). In this context $sPLA_2$s can also act on non-cellular phospholipids, such as those in microvesicles, lipoproteins, microbial membranes and nutrient phospholipids.

Secretory $PLA_2$s are present extensively in a number of mammalian tissues including pancreas, kidney, and cancer (Arouri et al., 2013). In addition, it has been found that $sPLA_2$ enzymes, particularly subtype IIA, are overexpressed in several cancer types, specifically in prostate, pancreas, breast, and colon cancers (Yamashita et al., 1994), and that they may also be associated with tumorigenesis and tumor metastasis (Tribler et al., 2007; Scott et al., 2010). Thus, with the recognition that phospholipase $A_2$ activity has been demonstrated in a number of pathological conditions, the idea of designing $sPLA_2$-targeted prodrugs seemed a promising approach to improve the pharmacodynamic properties of tissue-directed drugs (Arouri et al., 2013). The concept was originally based upon replacement of the *sn*-2-ester group of the natural phospholipid **1**, (Fig. 1), by an ester group carrying the pharmacophore, directed at the tissue specific $sPLA_2$ isozyme, with the objective that hydrolysis by the enzyme will release the drug. Along these lines a number of $sPLA_2$-targeted prodrugs have been prepared, with the main emphasis on incorporation of pharmacophores with anticancer activities (Andresen et al., 2005; Arouri et al., 2013). One of the recently developed methods of delivery involved the use of phospholipid prodrugs capable of liposome formation, that were pre-mixed with natural phospholipids to provide enhanced formulation stability and performance (Arouri and Mouritsen, 2012), This strategy has been developed as an improved alternative to conventional liposome delivery that circumvents issues of limited efficiency of drug loading, and premature and uncontrolled drug release. However, a significant percentage of these $PLA_2$-targeted prodrugs turned out to be "$PLA_2$ resistant", (i.e., failed to undergo hydrolysis by the enzyme, Arouri et al., 2013)

In comparing the structures of the "$PLA_2$-labile" vs. "$PLA_2$-resistant" variants of the prodrugs, it becomes apparent, that in the course of designing the compounds rather limited attention was directed towards one key substrate requirement for efficient $PLA_2$ hydrolysis, i.e., the need for the presence of an α-methylene group adjacent to the *sn*-2-ester carbonyl of the substrate (Bonsen et al., 1972). Specifically, among the $PLA_2$ resistant series, the anticancer prodrug derived from ATRA (all-*trans*-retinoic acid; Christensen et al., 2010) has a carbon–carbon double bond, and the one targeting RAR (retinoic acid receptor; Pedersen et al., 2010) carries an aromatic ester group at the *sn*-2-position. The phospholipid derivative of the NSAID ibuprofen (Kurz and Scriba, 2000) carries a methyl group in place of one of the α-methylene hydrogens, and the acyl chain of valproic acid (an anticonvulsant; Kurz and Scriba, 2000; Dahan et al., 2008) has a branching propyl group at the α-position adjacent to the *sn*-2-ester carbonyl. Indeed, due to the structural differences between these prodrugs and the requirements for $PLA_2$ catalysis none of these compounds was hydrolyzed by the enzyme.

In this communication we set out to test the working hypothesis that $sPLA_2$ enzymes can hydrolyze phospholipid-based prodrugs equipped with an intact α-methylene group at the *sn*-2-ester function. Based on the minimum structural requirements for $PLA_2$ catalysis **2** (Fig. 1).

We have designed two structurally modified phosphatidylcholine analogues **3** incorporating antiviral oligopeptides (Callebaut, et al., 1993; Epand et al., 1993; Epand, 2003) at the scissile *sn*-2-position of the substrate. Replacement of the naturally occurring fatty acyl chain with a membrane fusion inhibitory peptidyl group appeared to yield promising new prodrug candidates to test the feasibility of the release of their pharmacophores by a secretory $PLA_2$ enzyme.

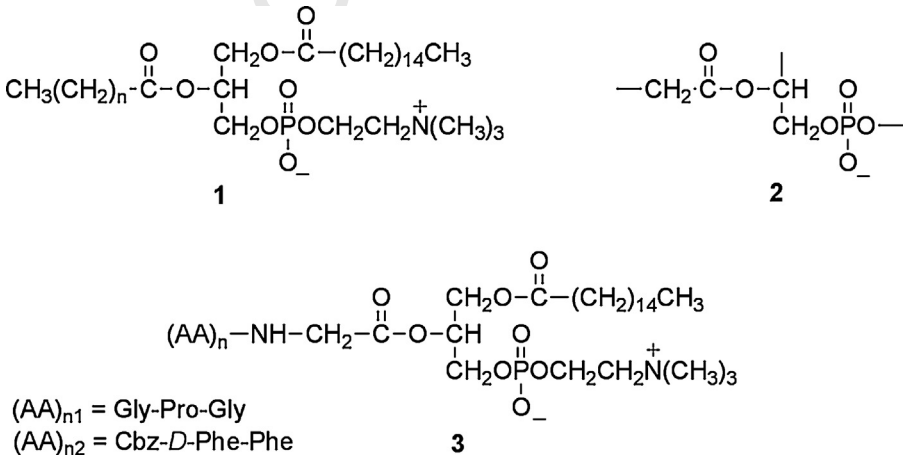


**Fig. 1.** Design of the phospholipase $A_2$-directed peptidophospholipids: the naturally occurring phosphatidylcholine **1**; the proposed minimum structural requirement for $PLA_2$ catalysis **2**, and the designed phosphatidylcholine conjugates carrying oligopeptides **3**.

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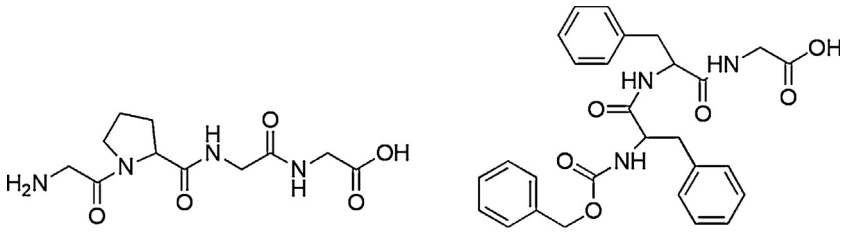



**Fig. 2.** The structures of the selected antiviral peptides **4** and **5**.

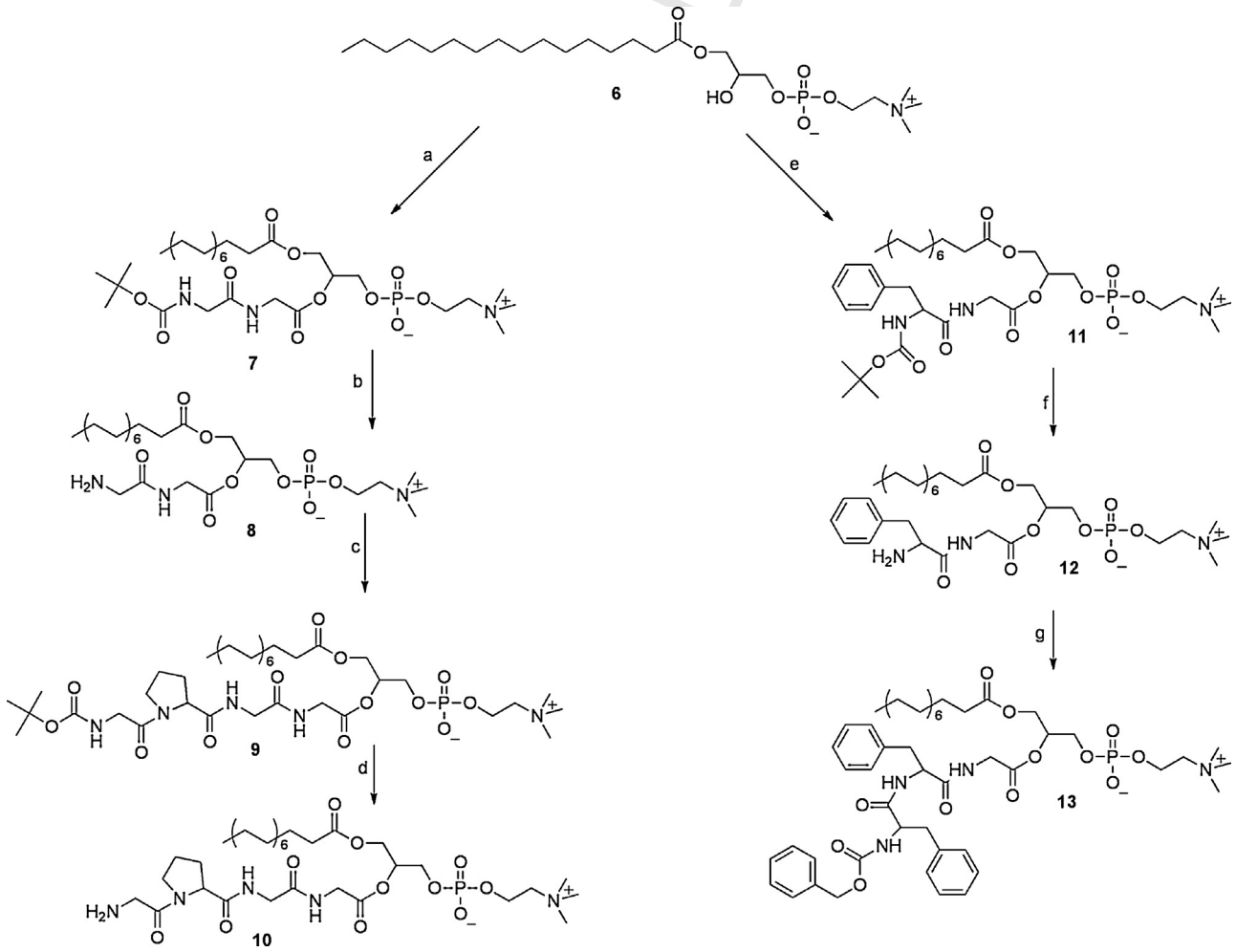
## 2. Results and discussion

### 2.1. Syntheses

For construction of the $PLA_2$-directed prodrugs we selected peptides **4** and **5**, shown in Fig. 2, as two antiviral peptides to prepare the target peptidophospholipids.

Specifically, compound **4** is an inhibitor of dipeptidyl peptidase IV, and it has been shown to inhibit entry of HIV-1 and HIV-2 into T limphoblastoid and monocytoid cell lines (Callebaut et al., 1993), while compound **5** is an antiviral peptide blocking viral infection by inhibiting membrane fusion, a required step in viral entry to the cell (Epand, 2003). While the structures of the two peptides are quite different, what they have in common is the α-methylene group adjacent to the C-terminal carboxyl group, that when incorporated into the phospholipid skeleton at the *sn*-2-position, makes them suitable to test the working hypothesis regarding the role of the methylene group in the hydrolytic cleavage of $PLA_2$-targeted prodrugs.

Our strategy for the synthesis of the target prodrugs relied on introducing the peptidyl ester group at the *sn*-2-position in a stepwise chain-extension sequence, shown in Scheme 1.




**Scheme 1.** Reagents and conditions: (a) BOC-gly-gly/DCC/DMAP, $CHCl_3$ 25 °C 4 h; (b) (i) 4.0 M HCl/dioxane, 40 min; (ii) $Et_3N$; (c) BOC-gly-pro-*p*-nitrophenyl ester/DMAP, $CHCl_3$, rt, 36 h; (d) 4.0 M HCl/dioxane, 30 min; (e) BOC-phe-gly/DCC/DMAP, $CHCl_3$, 25 °C, 3 h; (f) 4.0 M HCl/dioxane, 2.5 h; (g) *N*-Cbz-D-phe-*p*-nitrophenyl ester/DMAP, $CHCl_3$, rt 72 h.

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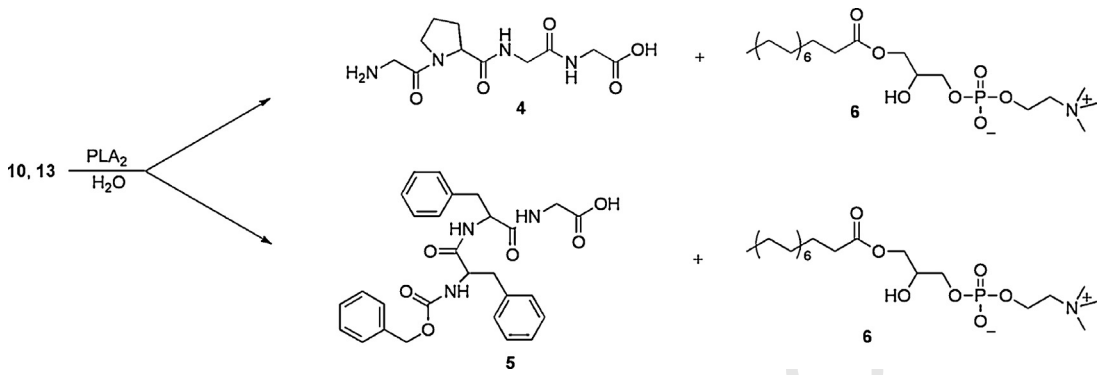
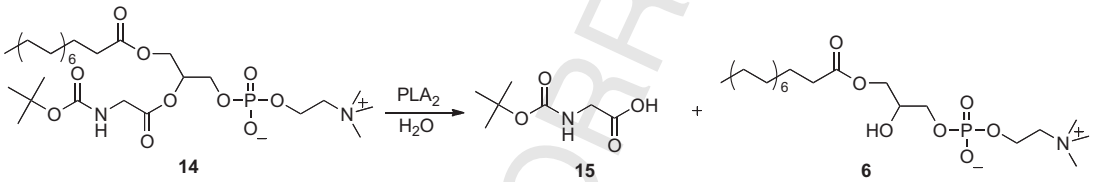



**Scheme 2.** Catalytic hydrolysis of the peptidophospholipids by bee-venom phospholipase $A_2$.

In the first step of the sequence palmitoyl lysophosphatidylcholine **6** was acylated at the *sn*-2-hydroxyl group with the respective BOC-protected dipeptides using dicyclohexyl carbodiimide (DCC) with 4-dimethylaminopyridine (DMAP) as catalyst. In order to achieve efficient and migration-free acylation, we employed the conditions that we have developed for acylation of lysophospholipids (Rosseto and Hajdu, 2005): (1) increasing the glass-surface of the reaction vessel, where the reaction is believed to take place, by addition of glass-beads, while using sonication rather than stirring the reaction mixture, and (2) keeping the temperature below 25 °C to prevent intramolecular acyl migration. Under these conditions the reactions reached completion in 3–4 h. The products **7**, and **11**, were readily isolated and purified on silica gel chromatography eluted with a stepwise gradient of $CHCl_3$-MeOH, followed by $CHCl_3$-MeOH-$H_2O$ (65:25:4). We found that using the acid-labile BOC protection of the amino group produced the *sn*-2-substituted phospholipids in substantially higher yield (90–96%) than the method employing the respective FMOC-derivatives (i.e., **11′** was obtained in 58% yield). Subsequent acid catalyzed cleavage of the tert.butoxycarbonyl group in anhydrous dioxane, followed by freeze-drying of the product solution was carried out in close to quantitative yield.




(2)

Chain-extension of the dipeptidyl phospholipid derivatives was carried out using the active ester method. Specifically, the *p*-nitrophenyl ester of BOC-glycylproline was allowed to react with phospholipid conjugate **8** in chloroform, in the presence of DMAP as catalyst, producing compound **9** in 96% yield. Next, acid catalyzed removal of the BOC protecting group yielded the amine hydrochloride of the peptidophospholipid prodrug **10** (96%). Similarly, the *sn*-2-phenylalanylglycyl chain of compound **12** was extended in a reaction with *p*-nitrophenyl Cbz-D-phenylalanine in chloroform, catalyzed by DMAP, to afford the corresponding target prodrug **13** in 81% isolated yield.

### 2.2. Enzymatic hydrolysis

Catalytic hydrolysis of the antiviral phospholipid prodrugs **10** and **13** was carried out with bee-venom phospholipase $A_2$, a widely used, readily available representative of secreted $PLA_2$ enzymes (Arouri and Mouritsen, 2012; Arouri et al., 2013; Valentin et al., 2000) in an assay system containing Triton X-100-phospholipid mixed micelles (Roodsari et al., 1999) in the presence of the catalytically essential $Ca^{2+}$ ions. Specifically, the phospholipid component of the micelles included a combination of the antiviral phospholipid prodrugs mixed with the natural phospholipid dipalmitoyl phosphatidylcholine (DPPC) in molar ratios of 1:4, and 1:3 respectively, using Triton X-100 as the surfactant. Both synthetic phospholipid analogues were completely hydrolyzed by the enzyme yielding lysophatidylcholine **6**, and the antiviral peptides **4** and **5**. The products were readily identified by thin layer chromatography. The disappearance of the synthetic substrates occurred slower, but within the same order of magnitude as the $PLA_2$ catalyzed hydrolysis of diplamitoyl phosphatidylcholine (DPPC) in the mixed micelles in the absence of the synthetic peptidophospholipids (Scheme 2).

Finally, we tested the prediction of the idea presented earlier as our working hypothesis, focusing on the need for the α-methylene group adjacent to the *sn*-2-ester function of the substrate to achieve $PLA_2$ catalyzed hydrolysis, by following the $PLA_2$ catalyzed hydrolysis of the aminoacyl analogue **14** carrying BOC-protected glycyl ester at the *sn*-2-position of the substrate **14**. (Eq. (2))

Specifically, we found that compound **14** was readily hydrolyzed by bee-venom $PLA_2$ to yield the BOC-protected glycine **15** and lysophosphatidylcholine **6**, under similar assay conditions as those used for the enzymatic hydrolysis of the antiviral peptidophospholipids. Preliminary studies, using mixed micellar substrates composed of compound **14** and palmitoyl phosphatidylcholine in 1:1 molar ratio with Triton X-100, indicate that hydrolysis of the synthetic analogue **14** occurred slower, by a factor of two, compared to the rate of $PLA_2$ catalyzed hydrolysis of mixed micelles containing dipalmitoyl phosphatidylcholine and Triton X-100, in absence of the synthetic compound **14**.

## 3. Conclusions

In addition to the synthesis of a series of $sPLA_2$ targeted antiviral prodrugs the significance of the work here presented is in its contribution to advance the design principles of secretory



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phospholipase $A_2$ directed substrates, including the preparation of phospholipid prodrugs. The principle that emerged from the work is the prediction that successful design of $PLA_2$ directed prodrugs should include an α-methylene group at the *sn*-2-ester carbonyl to achieve efficient catalytic hydrolysis by the enzyme. The results also explain why some of the previously prepared phospholipid prodrugs turned out to be "$PLA_2$-resistant", and opens the way to design new "$PLA_2$-labile" analogues. For example, oligopeptides with aspartic and glutamic acid side-chains that carry the required methylene groups are likely candidates to form $PLA_2$-cleavable *sn*-2-ester linkages as well, to incorporate new peptide-based pharmacophores built on a phospholipid scaffold.

The principle, however, does not limit the scope of the design and synthesis of successful $PLA_2$-directed prodrugs, by excluding drugs that lack the critical α-methylene group next to the carboxylic function, to attach the pharmacophore at the *sn*-2-position. Specifically, in that case, a suitable short-chain "spacer" equipped with a methylene bridged carboxylate might be used to link the drug molecule to the phospholipid skeleton (Pedersen et al., 2010; Rosseto and Hajdu, 2010; Arouri and Mouritsen, 2012). The use of such "linkers" can effectively target secretory $PLA_2$ enzymes, that will release the drug in the form of the respective conjugated prodrug.

## 4. Experimental procedures

### 4.1. 1-Palmitoyl-2-(BOC-gly-gly)-sn-glycero-3-phosphocholine (**7**)

To a suspension of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (0.5002 g, 1 mmol) in 25 mL of $CHCl_3$ was added BOC-gly-gly (0.7012 g, 3 mmol), followed by DCC (0.6189 g, 3 mmol), DMAP (0.3665 g, 3 mmol) and 1 g of glass beads. The reaction was sonicated for 4 h at 25 °C. The mixture was then filtered to remove DCC-urea and glass beads, the solvent collected was evaporated under reduced pressure to one third of its volume and loaded on a silica gel column, eluted with a stepwise gradient of $CHCl_3$/MeOH (5:1 and 5:2) to remove DMAP and the impurities, followed by $CHCl_3$/MeOH/$H_2O$ (65:25:4). The fractions corresponding to the product were combined, evaporated, re-dissolved in benzene and freeze-dried to give a white solid **7** (0.6702 g, 0.94 mmol, 94.4%). IR (Nujol): 3300 br m, 1744 vs, 1709 s, 1686 vs, 1248 m $cm^{-1}$. $^1H$ NMR ($CDCl_3$, 200 MHz) δ 0.87 (br t, 3H), 1.24 (br s, 24H), 1.41 (s, 9H), 1.56 (m, 2H), 2.29 (t, 2H, *J* = 7 Hz), 3.31 (br s, 9H), 3.37–3.83 (m, 4H), 4.01 (m, 4H), 4.22–4.26 (m, 4H), 4.57 (m, 1H), 5.22 (m, 1H), 5.73 (m, 1H). $^{13}C$ NMR ($CDCl_3$, 50 MHz) δ 14.09, 22.66, 24.78, 27.93, 28.38, 29.16, 29.33, 29.52, 29.68, 31.89, 33.93, 41.17, 43.65, 54.18, 59.47, 62.22, 63.93, 66.01, 71.84, 79.49, 156.23, 169.80, 170.51, 173.56. $R_f$ ($CHCl_3$/MeOH/$H_2O$ 65:25:4) 0.44. Anal. Cald for $C_{33}H_{64}N_3O_{11}P \cdot 2.5H_2O$: C, 52.50; H, 9.21; N, 5.57; found: C, 52.57; H, 8.88; N, 5.58. HRMS $MH^+$ $C_{33}H_{64}N_3O_{11}PH$ Cald: 710.4351, found: 710.4320. $[\alpha]_D^{25}$ +6.03 (c 0.96, $CHCl_3$/MeOH 4:1).

### 4.2. 1-Palmitoyl-2-(BOC-N-gly-pro-gly-gly)-sn-glycero-3-phosphocholine (**9**)

To a solution of **7** (0.3012 g, 0.42 mmol) in 20 mL of 1,4-dioxane was added 4 M HCl in dioxane solution dropwise at room temperature. After 40 min stirring the mixture became cloudy and a pale yellow precipitate formed, while **7** completely disappeared as observed by TLC ($CHCl_3$/MeOH/$H_2O$, 65:25:4/ $R_f$ > 0.44). The precipitate was separated from solution and was freeze-dried from a suspension of 30 mL of benzene. The pale yellow product obtained was washed with $CHCl_3$, yielding a white solid (**8**). $^1H$ NMR ($CD_3OD$, 200 MHz) δ 0.85 (br t, 3H), 1.25 (br s, 24H), 1.55 (m, 4H), 2.32 (br t, 2H), 3.36 (br s, 9H), 3.40–4.55 (br m), 5.35 (m, 1H). To this white precipitate dispersed in 15 mL $CHCl_3$ was added triethylamine until the pH of solution reached 8. When pH 8 was reached, the mixture became clear. To this solution was added BOC-gly-pro-*p*-nitrophenyl ester (0.2532 g, 0.64 mmol) followed by DMAP (97 mg, 0.8 mmol). After 36 h stirring at room temperature, to the mixture was added of Dowex-$H^+$ (10 mL) and it was stirred for an additional 15 min. The suspension was then filtered and the resin was washed with 30 mL $CHCl_3$/MeOH (1:1). The solvents were collected, evaporated under reduced pressure to one third of the volume and loaded on silica gel column, eluted first with
$CHCl_3$/MeOH (3:1) followed by $CHCl_3$/MeOH/$H_2O$ (65:25:4). The fractions corresponding to the product were combined, evaporated, re-dissolved in benzene and freeze-dried to give a white solid **9** (0.3472 g, 0.4 mmol, 95.7%). IR (Nujol): 3298 br m, 1743 br s, 1655 vs, 1534 w, 1245 m $cm^{-1}$. $^1H$ NMR ($CDCl_3$, 200 MHz) δ 0.85 (t, 3H, *J* = 6.7 Hz), 1.23 (br s, 29H), 1.40 (s, 9H), 1.51 (m, 2H), 2.09 (m, 2H), 2.28 (t, 2H, 6.7 Hz), 3.26 (br s, 9H), 3.65 (m, 3H), 3.82–4.10 (m, 6H), 4.25 (m, 3H), 4.40–4.60 (m, 3H), 5.20 (m, 1H), 5.80 (m, 1H), 8.25 (m, 1H). $^{13}C$ NMR ($CDCl_3$, 50 MHz) δ 14.05, 22.61, 24.74, 28.34, 29.14, 29.29, 29.50, 29.64, 31.84, 33.89, 42.70, 46.59, 54.10, 59.49, 60.78, 62.18, 63.96, 65.99, 71.75, 79.47, 156.05, 168.76, 169.64, 170.57, 172.60, 173.49. $R_f$ ($CHCl_3$/MeOH/$H_2O$ 65:25:4) 0.41. Anal. Cald for $C_{40}H_{74}N_5O_{13}P \cdot 2.5H_2O$ C, 52.85; H, 8.76; N, 7.70; found: C, 53.04; H, 8.41; N, 7.74. HRMS $MH^+$ $C_{40}H_{74}N_5O_{13}PH$ Calcd: 864.5094, found: 864.5094. $[\alpha]_D^{25}$ −12.06 (c 0.95, $CHCl_3$/MeOH 4:1).

### 4.3. 1-Palmitoyl-2-(gly-pro-gly-gly)-sn-glycero-3-phosphocholine hydrochloride (**10**)

Compound **10** was obtained from the analytical pure **7** by acid catalyzed deprotection. To a solution of **9** (0.28 g, 0.28 mmol) in 15 mL 1,4-dioxane was added dropwise a solution of 4 M HCl in 1,4-dioxane (3 mL) at room temperature. After 30 min stirring the mixture became cloudy and an oily precipitate formed. To the precipitate was added 20 mL benzene followed by freeze-drying. The freeze-dried product was washed with chloroform, and then dried in vacuum to give **8** as a white solid (215 mg, 0.26 mmol, 96%). The $^1H$ NMR ($CD_3OD$, 200 MHz) was identical to the spectrum of **7**, except for the absence of the signal of the protons at δ 1.40 (s, 9H) assigned to the removed tBOC protecting group.

### 4.4. 1-Palmitoyl-2-(BOC-phe-gly)-sn-glycero-3-phosphocholine (**11**)

To a suspension of **6** (0.5002 g, 1 mmol) in 25 mL of $CHCl_3$ was added BOC-phe-gly-OH (1.0021 g, 3 mmol), followed by DCC (0.6408 g, 3 mmol), DMAP (0.3798 g, 3 mmol), and 1 g of glass beads. The reaction was sonicated for 3 h at 25 °C. After 3 h, the sonication was stopped and to the mixture was added 10 mL of Dowex-$H^+$ and the suspension was stirred for 10 min. The mixture was then filtered and the resin was washed with 40 mL of $CHCl_3$/MeOH (1:1). The solvents collected were evaporated under reduced pressure to one third of the volume and then directly loaded on a silica gel column for chromatography, using $CHCl_3$/MeOH (7:3) eluent, followed by $CHCl_3$/MeOH/$H_2O$ (65:25:4). The fractions corresponding to the product were combined, evaporated, re-dissolved in benzene and freeze-dried to give an off-white pale-yellow solid 11 (0.7194 g, 0.90 mmol, 90%). IR (Nujol): 3188 br w, 1742 vs, 1680 br vs, 1250 m $cm^{-1}$. $^1H$ NMR ($CDCl_3$, 200 MHz) δ 0.84 (br t, 3H), 1.25 (br s, 24H), 1.31 (br s, 9H), 1.54 (m, 2H), 2.25 (t, 2H, *J* = 6.7 Hz), 2.78 (m, 2H), 3.27 (br s, 9H), 3.76 (m, 2H), 4.14 (m, 3H), 4.21–4.71 (m, 4H), 4.76 (m, 2H), 5.25 (m, 1H), 5.47 (m, 1H), 7.22 (br s, 5H), 8.62 (m, 1H). $^{13}C$ NMR ($CDCl_3$, 50 MHz) δ 14.08, 22.64, 24.74, 28.25, 29.12, 29.28, 29.32, 29.50, 29.62, 29.67, 31.88, 33.89, 39.07, 41.21, 54.22, 55.06, 59.44, 62.32, 63.95, 66.17, 71.79, 79.43, 126.60, 128.29, 129.48, 136.95, 155.33, 169.65, 172.30, 173.52. $R_f$ ($CHCl_3$/MeOH/$H_2O$ 65:25:4) 0.40. Anal.



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Cald for $C_{40}H_{70}N_3O_{11}P{\cdot}0.5H_2O$ C, 59.39; H, 8.85; N, 5.19; found: C, 59.07; H, 8.80; N, 5.33. FAB-MS $MH^+$ $C_{40}H_{70}N_3O_{11}PH$ Calcd: 800.4821, found: 800.4827. $[\alpha]_D^{25}$ +1.44 (c 1.04, $CHCl_3$/MeOH 4:1)

### 4.5. 1-Palmitoyl-2-(CBZ-D-phe-phe-gly)-sn-glycero-3-phosphocholine (**13**)

To a solution of **9** (0.3850 g, 0.48 mmol) in 20 mL 1,4-dioxane was added 4 M HCl in 1,4-dioxane (7 mL) dropwise at room temperature. The reaction mixture was stirred for 2.5 h, followed by the addition of 30 mL benzene and it was freeze-dried to give the deprotected amine 10 as a white solid. The $^1$H NMR ($CD_3OD$, 200 MHz) spectrum of the compound **12** showed the same pattern as the spectrum of compound **11**, except for the absence of the signal assigned to the protons at $\delta$ 1.40 (s, 9H) of the removed BOC protecting group. To the white precipitate of **12** dissolved in 20 mL of $CHCl_3$ was added DMAP (0.2987 g, 2.5 mmol) until pH of solution reached 8, followed by the active ester *p*-nitrophenyl *N*-Cbz-D-phenylalanine (0.2652 g, 0.63 mmol) at room temperature. After 24 h more active ester (0.1802 g, 0.43 mmol) was added. After 48 h stirring at room temperature, to the mixture was added 15 mL Dowex-$H^+$ and it was stirred for 10 min. The suspension was filtered and the resin was washed with 30 mL $CHCl_3$/MeOH (1:1). The solvents collected were evaporated under reduced pressure to one third of the volume and loaded on a silica gel column, eluted first with $CHCl_3$/MeOH (3:1), followed by $CHCl_3$/MeOH/$H_2O$ (65:25:4). The fractions corresponding to the product were combined, evaporated, re-dissolved in benzene and freeze-dried to give a white solid 13 (0.3815 g, 0.39 mmol, 81.3%). IR (Nujol): 3292 w, 1728 s, 1693 m, 1643 vs, 1540 m, 1301 w $cm^{-1}$. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.85 (br t, 3H), 1.25 (br s, 24H), 1.52 (m, 2H), 2.23 (t, 2H, $J$ = 6.7 Hz), 2.73 (m, 2H), 3.05 (m, 2H), 3.17 (br s, 9H), 3.71 (m, 2H), 4.05–4.20 (m, 4H), 4.35 (m, 2H), 4.62 (m, 2H), 4.81–5.02 (m, 4H), 5.25 (m, 1H), 6.03 (m, 1H), 6.90–7.26 (m, 15H), 8.01 (m, 1H), 8.32 (m, 1H). $^{13}$C NMR ($CDCl_3$, 50 MHz) $\delta$ 14.10, 22.65, 24.74, 29.12, 29.33, 29.51, 29.63, 29.68, 31.89, 33.86, 37.69, 38.43, 41.32, 54.21, 54.70, 55.89, 59.85, 62.08, 64.43, 65.93, 66.58, 71.52, 126.66, 126.83, 127.65, 128.02, 128.33, 128.48, 129.33, 129.46, 136.47, 137.05, 155.97, 169.54, 171.60, 172.27, 173.55. $R_f$ ($CHCl_3$/MeOH/$H_2O$ 65:25:4) 0.55. Anal. Cald for $C_{52}H_{77}N_4O_{12}P{\cdot}4H_2O$ C, 59.30; H, 8.13; N, 5.32; found: C, 59.75; H, 7.78; N, 5.51. FAB-MS $MH^+$ $C_{52}H_{77}N_4O_{12}PH$ Calcd: 981.5348, found: 981.5375. $[\alpha]_D^{25}$ −6.57 (c 0.97, $CHCl_3$/MeOH 4:1).

### 4.6. 1-Palmitoyl-2-(FMOC-phe-gly)-sn-glycero-3-phosphocholine (**11′**)

To a suspension of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine **6** (0.5002 g, 1 mmol) in 25 mL of $CHCl_3$ were added FMOC-phe-gly-OH (0.5393 g, 1.2 mmol), DCC (0.2498 g, 1.2 mmol), DMAP (0.1479 g, 1.2 mmol) and 1 g of glass beads. The reaction was sonicated for 48 h at 25 °C, the mixture was then filtered to remove DCC-urea and glass beads. The solvent was evaporated to one third of the volume and then loaded on a silica gel column for chromatography. A stepwise gradient of $CHCl_3$/MeOH (5:1 and 5:2) was applied to elute DMAP and some impurities, followed by $CHCl_3$/MeOH/$H_2O$ (65:25:4). The fractions corresponding to the product were combined, evaporated, re-dissolved in benzene and freeze-dried to give **11′** as a white solid (0.5352 g, 0.58 mmol, 58%). IR (Nujol): 3297 br m, 1728 vs, 1693 s, 1654 vs, 1536 m, 1252 w $cm^{-1}$. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.85 (br t, 3H), 1.25 (br s, 24H), 1.50 (m, 2H), 2.20 (t, 2H, $J$ = 6.7 Hz), 2.95 (m, 2H), 3.17 (br s, 9H), 3.67 (br s, 2H), 3.95–4.30 (br m, 10H), 4.44 (m, 2H), 5.36 (m, 1H), 6.15 (m, 1H), 7.21–7.47 (m, 11H), 7.72 (d, 2H, $J$ = 7.4 Hz), 8.66 (m, 1H). $^{13}$C NMR ($CDCl_3$, 50 MHz) $\delta$ 14.28, 22.85, 24.92, 29.31, 29.52, 29.71, 29.83, 29.87, 32.08, 34.04, 38.94, 41.47, 47.13, 54.34, 55.90, 59.63, 62.44, 64.18, 66.28, 67.07, 71.96, 120.08, 125.27, 125.47, 126.94, 127.26, 127.86, 128.57, 129.63, 137.05, 141.30, 143.90, 156.17, 169.83, 172.39, 173.72. $R_f$ ($CHCl_3$/MeOH/$H_2O$ 65:25:4) 0.48. Anal. Cald for $C_{50}H_{72}N_3O_{11}P{\cdot}2.5H_2O$ C, 62.09; H, 8.02; N, 4.34; found: C, 62.33; H, 8.03; N, 4.04. FAB-MS $MH^+$ $C_{50}H_{72}N_3O_{11}PH$ Calcd: 922.4977, found: 922.4981. $[\alpha]_D^{25\,°C}$ −6.73 (c 0.98, $CHCl_3$/MeOH 4:1).

### 4.7. 1-palmitoyl-2-(N-BOC-glycyl)-sn-glycero-3-phosphocholine (**14**)

To a suspension of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine 6 (0.3704 g, 0.7 mmol) in 25 mL of $CHCl_3$ was added *N*-BOC-gly (0.5305 g, 3 mmol), followed by DCC (0.6204 g, 3 mmol), DMAP (0.3704 g, 3 mmol) and 1 g of glass beads. The reaction was sonicated for 1 h at 25 °C. Next, to the mixture were added 8 mL of Dowex-$H^+$ and stirred for 10 min. The resin was filtered and washed with 30 mL of $CHCl_3$:MeOH (1:1). The combined solution was evaporated under reduced pressure to one third of volume and then was promoted the chromatographic purification on silica gel using as eluent. and then was loaded on a silica gel column, eluted first with $CHCl_3$/MeOH (7:3), folloed by $CHCl_3$/MeOH/$H_2O$ (65:25:4). The fractions corresponding to the product were combined, evaporated, re-dissolved in benzene and freeze-dried to give a white solid **14** (0.4325 g, 0.66 mmol, 94.5%). IR (Nujol): 3364 br m, 1746 vs, 1714 vs, 1253 m, 1168 m $cm^{-1}$. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.85 (br t, 3H), 1.23 (br s, 24H), 1.40 (s, 9H), 1.52 (m, 2H), 2.26 (t, 2H, $J$ = 6.7 Hz), 3.26 (br s, 9H), 3.75–4.01 (m, 6H), 4.10–4.18 (m, 2H), 4.25 (m, 2H), 5.22 (m, 1H), 6.21 (m, 1H). $^{13}$C NMR ($CDCl_3$, 50 MHz) $\delta$ 14.01, 22.58, 24.71, 28.33, 29.08, 29.22, 29.25, 29.44, 29.56, 29.60, 31.82, 33.89, 42.31, 54.16, 59.36, 62.43, 63.62, 65.98, 71.50, 79.39, 156.04, 170.36, 173.47. $R_f$ ($CHCl_3$/MeOH/$H_2O$ 65:25:4) 0.38. Anal. Cald for $C_{31}H_{61}N_2O_{10}P{\cdot}H_2O$ C, 55.50; H, 9.47; N, 4.18, found: C, 55.50; H, 9.49; N, 4.04. FAB-MS $MH^+$ $C_{31}H_{61}N_2O_{10}PH$ Calcd: 653.4137, found: 653.4165. $[\alpha]_D^{25\,°C}$ +8.80 (c 1.00, $CHCl_3$/MeOH 4:1).

### 4.8. Enzymatic hydrolysis of the phospholipids

In a typical experiment prodrug **10** (4.7 mg, 5.8 μmol) was added to a mixture containing dipalmitoyl phosphatidylcholine (DPPC, 17.9 mg, 23.4 μmol), in 4.1 mL Tris buffer (0.05 M, pH 8.50), with 0.1 mL Triton X-100 and $CaCl_2$ (7.2 mg, 0.049 mmol) The mixture was vortexed, for 5 min, followed by incubation of the resulting dispersion at 40 °C for 10 min in a constant-temperature water-bath. To the optically clear dispersion that resulted was added bee-venom phospholipase $A_2$ (40 μg in 200 μL buffer) to initiate the reaction. The reaction mixture was kept at 40 °C, and formation of the products was analyzed by thin layer chromatography ($CHCl_3$/MeOH/$H_2O$, 65:25:4). The compounds were visualized by iodine adsorption, molybdic acid spray and ninhydrin spray. TLC analysis showed complete hydrolysis of the phospholipids (DPPC and the synthetic phospholipid prodrug **10**) by $PLA_2$ within 90 min, leading to the formation of lysophosphatidylcholine **6**, and the oligopeptide **4**. $PLA_2$ catalyzed hydrolysis of DPPC under the same conditions in absence of compound **10** was completed in 10 min.

In a somewhat similar experimental setup, prodrug **13** (3.4 mg, 0.5 μmol) was added to a mixture containing DPPC (15 mg, 1.5 μmol), in 4.1 mL Tris buffer (0.05 M, pH 8.50), with 0.1 mL Triton X-100 and 50 mM $CaCl_2$. The mixture was vortexed, for 5 min, kept at 40 °C for 10 min in a constant-temperature water-bath. To the resulting dispersion was added bee-venom phospholipase $A_2$ (16 μg in 80 μL 0.05 M Tris buffer, pH 8.5) to initiate the reaction. The reaction mixture was kept at 40 °C, and formation of the products was analyzed by thin layer chromatography ($CHCl_3$/MeOH/$H_2O$, 65:25:4). The compounds were visualized by UV-absorption, iodine adsorption, and molybdic acid spray. TLC analysis showed complete hydrolysis of the DPPC



Please cite this article in press as: Rosseto, R., Hajdu, J., Peptidophospholipids: Synthesis, phospholipase $A_2$ catalyzed hydrolysis, and application to development of phospholipid prodrugs, Chem. Phys. Lipids (2014), http://dx.doi.org/10.1016/j.chemphyslip.2014.06.001

and the synthetic phospholipid prodrug **13** within 90 min, producing lysophosphatidylcholine **6**, and the oligopeptide **5**.

The synthetic phospholipid analogue with *sn*-2-*N*-BOC-gly **14**, was hydrolyzed by bee-venom $PLA_2$ under similar experimental conditions to those used for the catalytic hydrolysis of the peptide substituted analogues. TLC showed that the reaction was completed in 20 min, while the hydrolysis of DPPC in the same assay mixture without the aminoacyl phospholipid **14** was completed in 10 min.

The prodrugs did not change in the absence of the enzyme.

## Uncited references

Dong et al. (2006), Fujioka and Kugiyama (2009), and Skaug et al. (2011).

## Transparency document

The Transparency document associated with this article can be found in the online version.

## Acknowledgement

We are grateful to the National Institutes of Health, grant 1SC3GM096878 for financial support.

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Please cite this article in press as: Rosseto, R., Hajdu, J., Peptidophospholipids: Synthesis, phospholipase $A_2$ catalyzed hydrolysis, and application to development of phospholipid prodrugs, Chem. Phys. Lipids (2014), http://dx.doi.org/10.1016/j.chemphyslip.2014.06.001