Pergamon

Bioorganic & Medicinal Chemistry, Vol. 3, No. 10, pp. 1397–1403, 1995

0968-0896/95 $9.50 + .00

0968-0896(95)00120-4

New Phospholipase A_2 Inhibitor: Synthesis and Inhibition Mechanism of Oxazolidinone Phospholipid Analog

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Abstract—(*R*)-3-Dodecanoyl-4-phosphatidylcholino-hydroxymethyl-2-oxazolidinone (**7**), which is a new glycerophospholipid analog, was synthesized starting from (*S*)-glycidol through a 4-alkylsilyloxymethyl derivative and *N*-acyl-4-hydroxymethyl derivative. The cyclic amide analog **7** showed strong inhibitory activity toward both Group I and II PLA_{2S}, but the inhibitory potency of **7** was slightly weaker than that of the linear amide analog (*R*)-**1**, which had been developed by de Haas *et al.* (*Biochem. Biophys. Acta* **1990**, *1043*, 67). The interactions of **7** with human secretory PLA_2 was investigated by computer modeling in comparison with those of the linear amide analog **1**. The results of the computer modeling were very compatible with those of the inhibitory activities toward PLA_{2S}, and the both results showed that the binding mode of the oxazolidinone analog **7** was very similar to that of the genuine substrate and was different from that of the linear amide analog **1**.

Introduction

Phospholipase A_2 (PLA_2) specifically catalyzes the hydrolysis of the ester linkage at the *sn*-2 position of glycerophospholipids, and the release of arachidonic acid from the *sn*-2 position of the phospholipids is the rate-limiting step in the production of eicosanoid mediators of inflammation.[1] Recently, various kinds of small molecules of PLA_2 inhibitor have been reported, and they are classified into phospholipid analogs and others.[2] Among them, the phospholipid analogs have been very extensively examined. The representative PLA_2 inhibitors of the phospholipid analogs are listed in Figure 1. Analogs **1** and **2**, which were developed by de Haas *et al.* in 1990,[3] contain an amide in place of the enzyme susceptible ester linkage at the *sn*-2 position, and they have received significant attention not only because of their very strong inhibitory activities toward PLA_2 but also being very useful compounds for the elucidation of the catalytic mechanism of PLA_2. The

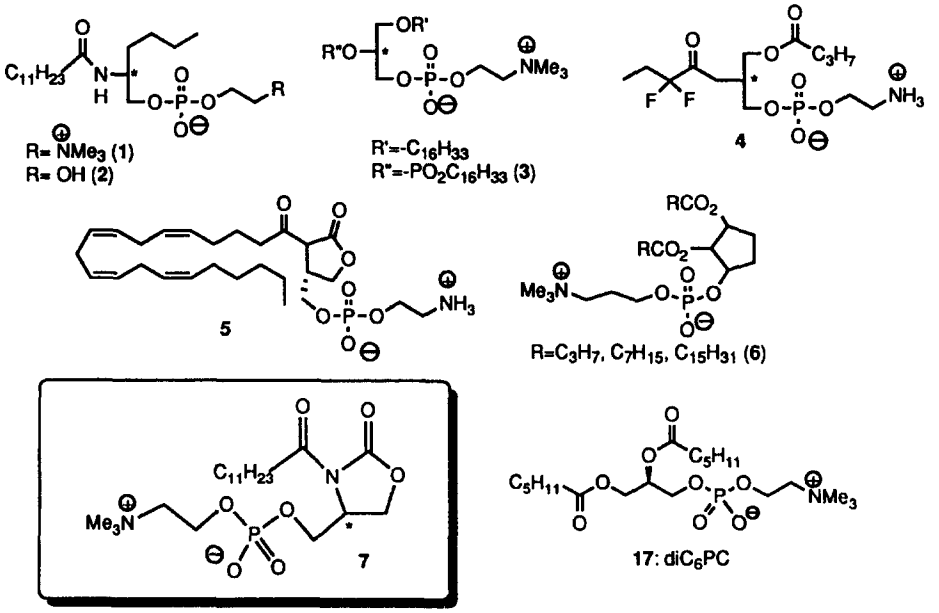


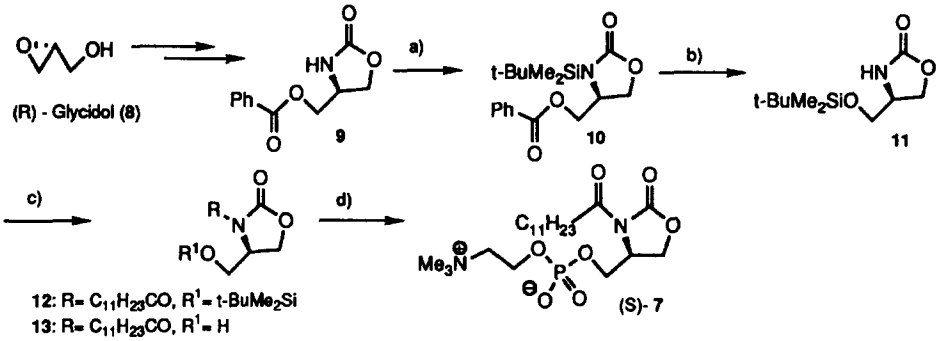
Figure 1.

crystal structure of a complex between porcine pancreatic PLA_2 and the analog **2** was determined by X-ray analysis.[4]

In analogs **3** and **4**, which are called transition state analogs, the ester linkage at the *sn*-2 position of the phospholipids has been replaced by phosphonate or an easily hydrated fluoroketone for mimicking a tetrahedral intermediate during the process of PLA_2 catalyzed hydrolysis.[5] The analog **3** is also interesting for understanding the catalytic mechanism of PLA_2, and the X-ray crystal structure of a complex between human nonpancreatic secretory PLA_2 and **3** was reported.[6] An alternative approach for the glycerophospholipid analog is to design cyclic analogs which are conformationally restricted. Compounds **5** or **6** possess a γ-lactone ring, or a cyclopentane ring, respectively,[7] and the analog **5**, which was designed using molecular graphics and molecular mechanics by Campbell *et al.* in 1988, showed significant inhibitory activity toward PLA_2. Now, our interest was the inhibitory activity of compound **7**, which is regarded as a cyclized form of the linear amide analog and is also regarded as an aza derivative of the γ-lactone analog. Herein, we report in detail the syntheses and inhibitory activities of a new cyclic analog **7**[8] and de Haas' linear amide analog **1**. Both compounds were synthesized from the same chiral building block **11** prepared from (+)- or (−)-glycidol. We also describe the analytical results of the interactions of these phospholipids amide analogs toward PLA_2 using computer modeling.

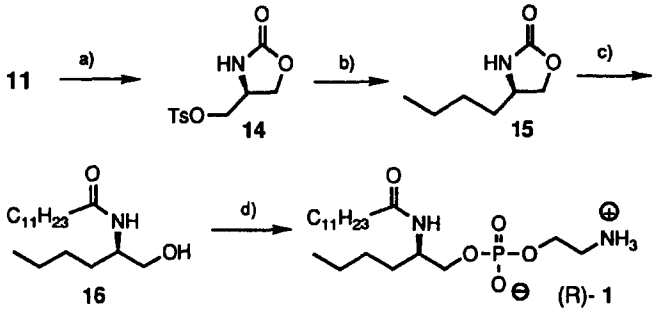
Syntheses of (S)- and (R)-3-dodecanoyl-4-phosphatidylcholinohydroxymethyl-2-oxazolidinone (7) and (R)-2-dodecanoyl-amino-1-hexanol-1-phosphatidylcholine (1)

Previously, we developed enantiomerically pure (*R*)-4-hydroxymethyl-2-oxazolidinone derivatives as chiral building blocks from (*R*)-glycidol,[9] and reported a new route for protected amino alcohols via the copper(I) mediated alkylation of a tosylate which was derived from the oxazolidinone chiral building block.[10] In that procedure, silyl ether **11** in Figure 2 was synthesized from (*R*)-glycidol (**8**) through benzoates **9** and **10**. The silyl group in compound **10** completely migrated from the nitrogen of the carbamate to the hydroxy group without any racemization due to recyclization of the oxazolidinone ring. The syntheses of both the (*S*)-oxazolidinone phospholipid (**7**) and linear (*R*)-2-amidophospholipid (**1**) were achieved from the silyl ether **11** as shown in Figures 2 and 3. Thus, treatment of **11** with dodecanoyl chloride in the presence of DMAP and triethylamine in THF afforded an acylated compound **12**, which was treated with 2 M aq. HCl in THF to give the desired alcohol **13** in 74% yield for two steps without any migration and racemization. The



a) t- $BuMe_2SiCl$, DMAP, Et_3N / DMF (95%); b) KOH / MeOH (93%); c) 1) $C_{11}H_{23}COCl$, Et_3N, DMAP / THF; 2) 2M HCl / THF (74%) ; d) 1) 2- chloro-2-oxo-1,3,2-dioxaphospholane, DMAP, Et_3N / benzene; 2) Me_3N / benzene (39%).

Figure 2.



a) 1) 2M HCl / THF 2) TsCl / pyridine (89%) ; b) Pr_2CuLi / THF (78%); c) 1) 6M NaOH / dioxane 2) $C_{11}H_{23}COCl$ (88%) ; d) 1) 2-chloro-2-oxo-1,3,2-dioxaphospholane, DMAP, Et_3N / benzene 2) Me_3N / benzene(45%).

Figure 3.

enantiomerical purity of **13** was checked by high performance liquid chromatography using a chiral column. The synthesis of (*S*)-**7** was achieved by the reaction of **13** with 2-chloro-2-oxo-1,3,2-dioxaphospholane in the presence of DMAP and triethylamine in benzene, followed by treatment with trimethylamine in benzene at 75 °C in a pressure bottle for 3 days in 39% yield. The (*R*)-isomer of **7** was also synthesized using the same procedure starting from (*S*)-glycidol.

The synthesis of the linear (*R*)-2-amidophospholipid analog **1** from silyl ether **11** is as follows (Fig. 3). Treatment of **11** with aqueous hydrogen chloride in THF followed by the reaction with tosyl chloride in pyridine without any purification gave tosylate **14**, which was reacted with lithium dipropylcuprate to afford 4-butyl-2-oxazolidinone (**15**) in 78% yield. Upon treatment of **15** with 6 M NaOH followed by the successive reaction with dodecanoyl chloride, *N*-dodecanoyl-2-aminohexanol (**16**) was obtained in 88% yield for two steps. Introduction of the phosphatidylcholino part was performed by the same procedure as the case of oxazolidinone phospholipid **7**. Thus, the treatment of **16** with 2-chloro-2-oxo-1,3,2-dioxaphospholane followed by a reaction with trimethylamine afforded (*R*)-**1** in 45% yield for two steps. The enantiomeric (*S*)-**1** was also synthesized by the same procedure starting from (*S*)-glycidol.

As mentioned above, the syntheses of (*R*)-**1**, (*S*)-**1**, (*R*)-**7**, and (*S*)-**7** were achieved from the same enantiomerically pure oxazolidinone chiral building block, respectively, and we obtained a sufficient quan-tity of the both enantiomers of the cyclic and the linear 2-amidophospholipid analogs.

Inhibitory activities of the synthesized linear amide and oxazolidinone phospholipid analogs, 1 and 7

The inhibitory activities of the synthesized analogs, (*R*)-**1**, (*S*)-**1**, (*R*)-**7**, and (*S*)-**7** were measured toward phospholipase A_2s, which were isolated from cobra (*Naja naja atra*) venom as a Group I PLA_2,[11] and from Japanese mamushi (*Agkistrodon halys blomhoffii*) venom as a Group II PLA_2.[12] The enzymatic activities were measured toward a monodispersed substrate, (*R*)-1,2-dihexanoylglycero-3-phosphorylcholine (**17**: diC_6PC)[13] by the pH-stat method. The results are summarized in Figure 4. The analog (*R*)-**7** showed a 50% inhibition at a concentration of 36 μM (IC_{50} value) toward *N. naja atra* and 31 μM toward *A. halys blomhoffii* PLA_2s. The binding constants of (*R*)-**7** ($1/K_i$ value) were calculated to be 3.8×10^4 M^{-1} toward *N. naja atra* and 4.5×10^4 M^{-1} toward *A. halys blomhoffii* PLA_2s. In the case of (*R*)-**1**, the IC_{50} and $1/K_i$ values were 3.0 μM and 4.5×10^5 M^{-1} toward *N. naja atra*, and 5.3 μM and 2.6×10^5 M^{-1} toward *A. halys blomhoffii* PLA_2s, respectively. The diastereomer, (*S*)-**7**, showed no significant inhibitory activity. Thus, the inhibitory potencies of the oxazolidinone analog (*R*)-**7** toward two kinds of PLA_2s were slightly weaker than those of the amide analog (*R*)-**1**. Furthermore, from a biochemical viewpoint, the detailed inhibitory mechanism of the analog **7** towards both of Group I and II PLA_2s were studied by comparison with the analog **1**.[14] The obtained results are summarized as follows. (1) The oxazolidinone analog (*R*)-**7** was bound to the catalytic site of PLA_2, and competitively inhibited the hydrolyzing activity of PLA_2 toward monodispersed (*R*)-diC_6PC (**17**). (2) The binding of either the monodispersed oxazolidinone analog (*R*)-**7** or monodispersed diC_6PC (**17**) toward Group I PLA_2 was independent of the Ca^{2+} binding to the enzyme, whereas the binding toward Group II PLA_2 was facilitated by the Ca^{2+} binding. On the other hand, the binding of the monodispersed linear amide analog (*R*)-**1** was facilitated by the Ca^{2+} binding to the both Group I and II PLA_2s. (3) The binding constants of both the oxazolidinone analog (*R*)-**7** and (*R*)-diC_6PC (**17**) were pH independent, whereas the binding constant of the linear amide analog (*R*)-**1** significantly decreased at pH 6.8 in comparison with that at pH 8.2.

N. naja atra

	1/ K_m (M^{-1})	IC_{50} (M)
(R) - 1	450000	3.0×10^{-6}
(R) - 7	38000	3.6×10^{-5}
(S) - 1	15000	9.0×10^{-5}
(S) - 7	N. D.#	N. D.#

A. halys blomhoffii

	1/ K_m (M^{-1})	IC_{50} (M)
(R) - 1	259000	5.3×10^{-6}
(R) - 7	45000	3.1×10^{-5}
(S) - 1	18000	7.0×10^{-4}
(S) - 7	7200	1.6×10^{-4}

N. D. : not detected

Figure 4.

Thus, the binding mode of the oxazolidinone analog (*R*)-**7** was found to be very similar to that of the genuine substrate diC_6PC (**17**), and to be different from that of the linear amide analog (*R*)-**1**.

In 1981, a hypothetical catalytic mechanism of PLA_2 as shown in Figure 5 has been proposed by Verheij *et al.* on the basis of the X-ray crystallographic result for the Ca^{2+} complex of bovine pancreatic PLA_2.[15] In this mechanism, an intermediate complex was assumed to be stabilized by a coordination of the bound Ca^{2+} ion to the phosphoryl group and to the carbonyl group at the *sn*-2 position of the phospholipid molecule bound to the enzyme. Furthermore, one water molecule about 3 Å away from the Nδ1 atom of the catalytic group His-48 of the enzyme was assumed to perform a nucleophilic attack on the *sn*-2 ester linkage of the bound glycerophospholipid. Recently, Thunnissen *et al.* reported the X-ray crystallographic analysis of a complex of a porcine pancreatic PLA_2 with the linear amide glycol analog **2**.[4] The result showed the existence of direct interactions of the bound Ca^{2+} ion with the phosphoryl group, and with the carbonyl group of the *sn*-2 amide group of the linear amide glycol analog **2**. Furthermore, the Nδ1 atom of His-48 in the enzyme molecule was shown to form a hydrogen bond with the NH group of the *sn*-2 amide bond of **2**. In other words, in a complex of **2** with porcine pancreatic PLA_2, His-48, which is essential for the catalytic mechanism of PLA_2, has been masked by a hydrogen bond with the amide proton

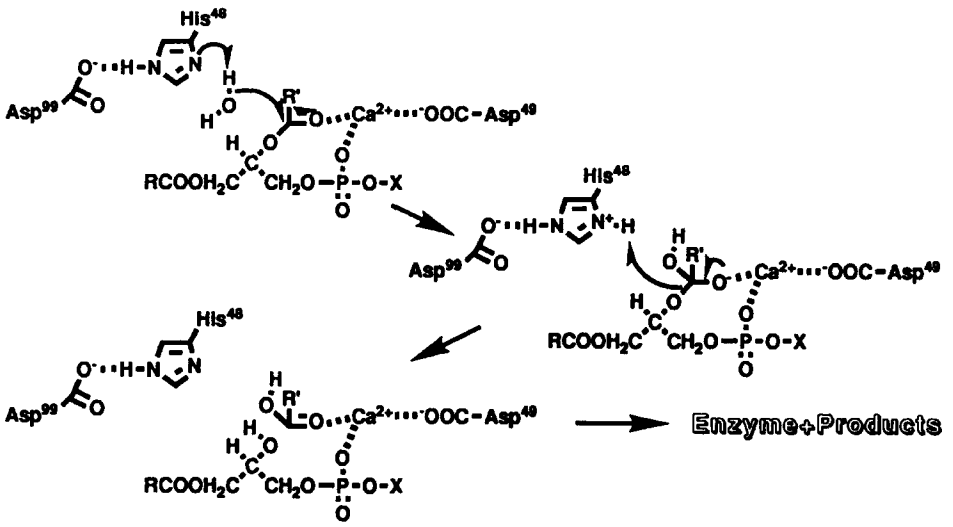


Figure 5. Verheij's hypothetical mechanism.

of **2**. The interactions between the Ca^{2+} ion and **2** are well compatible with the hypothesis of Verheij except for the mode of hydrogen bondings. On the other hand, the binding mode of the oxazolidinone analog **7** to Type I PLA_2 is totally well compatible with that of the genuine substrate, and therefore, with Verheij's hypothesis.

We next studied the binding mode of (*R*)-**7** toward a PLA_2 using a computer modeling analysis.

Computer modeling analysis of the interactions of the linear amide analog (1) and the oxazolidinone analog (7) to human nonpancreatic secretory PLA_2

The analysis of the interactions of the oxazolidinone analog (*R*)-**7** and the linear amide analog (*R*)-**1** with a PLA_2 by computer modeling was investigated. Human secretory PLA_2, whose structure had been determined by X-ray crystallography,[6,16] was used as an enzyme molecule for computer modeling, since an X-ray analytical structure of *A. halys blomhoffii* had not been registered at the protein data bank. The model building and structural analysis were accomplished using the software packages Insight II.[17] In order to build models of the complexes, which are formed between human secretary PLA_2 and the genuine substrate diC_6PC (**17**), the X-ray structure of a complex of (*R*)-**2** with porcine PLA_2 has been applied as a reference. Human PLA_2 was superposed on the complex between (*R*)-**2** and porcine PLA_2 by sequences homology, and (*R*)-**2** was projected onto the catalytic site of human PLA_2 to produce an initial structure. The resulting complex was minimized by conjugate gradient method. In order to perform molecular mechanics (MM) and dynamics (MD) simulation,[18] the consistent valence force field (CVFF) of discover was used. After minimization, water molecules were generated in the 30 Å region from the Cδ1 atom of His-48 in the catalytic site. The MD simulations *in vacuo* were performed at the constant temperature (300 K) with dielectric constant of 1, and total simulation time was 100 ps. The (*R*)-**2** molecule in the catalytic site of human PLA_2 was then replaced by the (*R*)-**1**, (*R*)-**7**, and diC_6PC(**17**) molecules, respectively. These complexes were performed minimization and dynamic runs with a dielectric of 80 without the water molecules. The aspect of the continuously conformational changes accompanied by minimizations of the linear amide analog (*R*)-**1** is shown in Figure 6. Very little conformational change of (*R*)-**1** was admitted between the initial and the optimized structure. This result indicated that the present model was very reasonable for the analysis of the binding mode of the substrate analogs toward human PLA_2. In a model of a complex between the oxazolidinone analog (*R*)-**7** and human PLA_2 as shown in Figure 7, the optimized conformation of (*R*)-**7** to the catalytic site was different from the initial one. The oxazolidinone ring of the analog **7** moved from the inside to the entrance of the pocket of the catalytic site. In the minimized conformation of the oxazolidinone analog, however, the phosphoryl group coordinated with Ca^{2+}, and the C-2 acyl group of (*R*)-**7** was still placed in the hydrophobic channel which consisted of lipophilic amino acid residues, Leu-2, Phe-5, Ile-9, Ala-17, Ala-18, Leu-19, Phe-24, Cys-29, Val-31, and Cys-45 as pointed out by Scott *et al.*[6] Therefore, the oxazolidinone analog (*R*)-**7** is still able to bind to the catalytic site of human PLA_2. The distance between Nδ1 of His-48 and the C-2 carbonyl carbon in the graphics model of a complex between human PLA_2 and (*R*)-**7** was 4.8 Å, whereas that of the complex with (*R*)-**1** was 4.0 Å. Thus, the results obtained by the computer analysis corresponded well to the relative inhibitory activities of the oxazolidinone analog (*R*)-**7** and the linear amide analog (*R*)-**1**.

In conclusion, the new substrate analog (*R*)-**7** forms complexes with PLA_2s similar to those formed by the genuine substrate with PLA_2s, where one water molecule participates in the binding of His-48 with the *sn*-2 acyl group of (*R*)-**7** and the genuine substrate. Whereas, the binding mode of the linear amide analog (*R*)-**1** to PLA_2s is different from that of the genuine substrate.

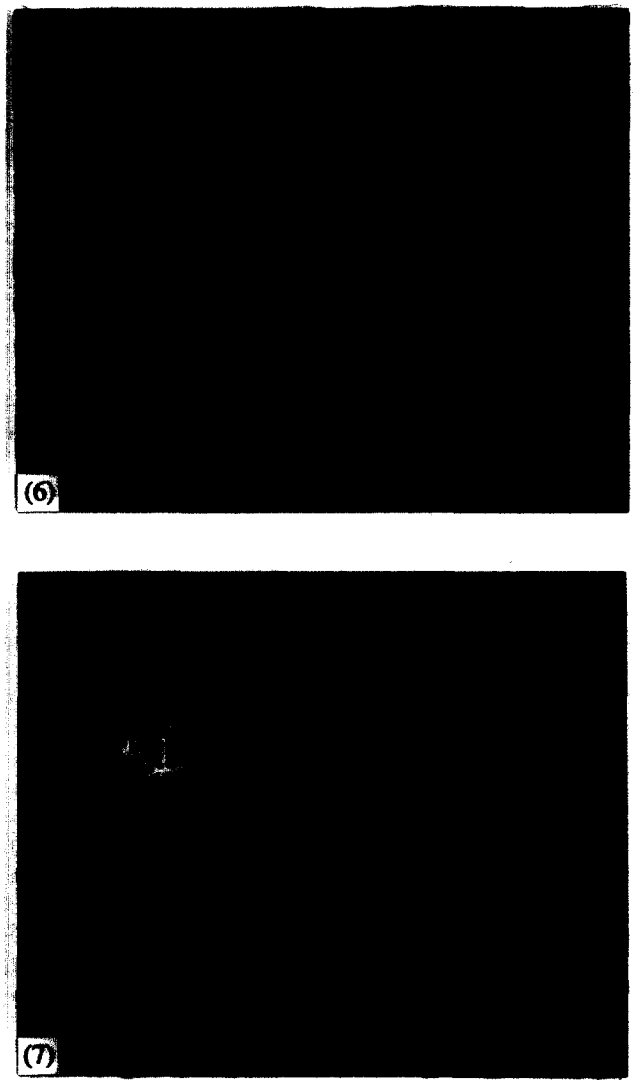


Figure 7.

The assumed binding modes of the PLA_2 molecule with the genuine substrate diC_6PC(**17**), the oxazolidinone analog (*R*)-**7**, and the linear amide analog (*R*)-**1** are shown in Figure 8.

Thus, the new type substrate analog, the oxazolidinone analog (*R*)-**7**, may be an instructive compound for elucidating the catalytic mechanism of PLA_2. The study to find more favorable substrate analogs having a oxazolidinone structure are now in progress.

Experimental

The melting points were measured with a Yanagimoto micro melting-point apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were taken on a JEOL A-400 spectrometer. Tetramethylsilane (δ 0.00) for ^1H and chloroform-*d* (δ 77.0) for ^{13}C were used as internal standards. IR spectra were recorded on a Hitachi 270-30 spectrometer. MS spectra were obtained on a JEOL SX-102 spectrometer. Elemental analyses were measured with a Perkin–Elmer Model 240.

*(S)-3-Dodecanoyl-4-*t*-butyldimethylsilyloxymethyl-2-oxazolidinone (12).* To a stirred solution of **11** (2.34 g, 10.1 mmol), triethylamine (1.69 mL, 12.1 mmol), and 4-dimethylaminopyridine (494 mg, 4.04 mmol) in THF (15.2 mL) were added dodecanoyl chloride (2.80 mL, 12.1 mmol). The stirring was continued for 18 h at room temperature, then the solution was treated with brine, and the mixture was extracted with EtOAc. The extracts were dried over $MgSO_4$ and evaporated *in vacuo*. The crude products were purified by column chromatography over silica gel to give **12** (4.03 g, 96%): $[\alpha]_D^{22}$ –55.7° (*c* 1.20, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 0.03 (6H, *d*, *J* = 10.0 Hz), 0.88 (9H, *s*), 1.25–1.30 (16H, *m*), 1.62 (2H,

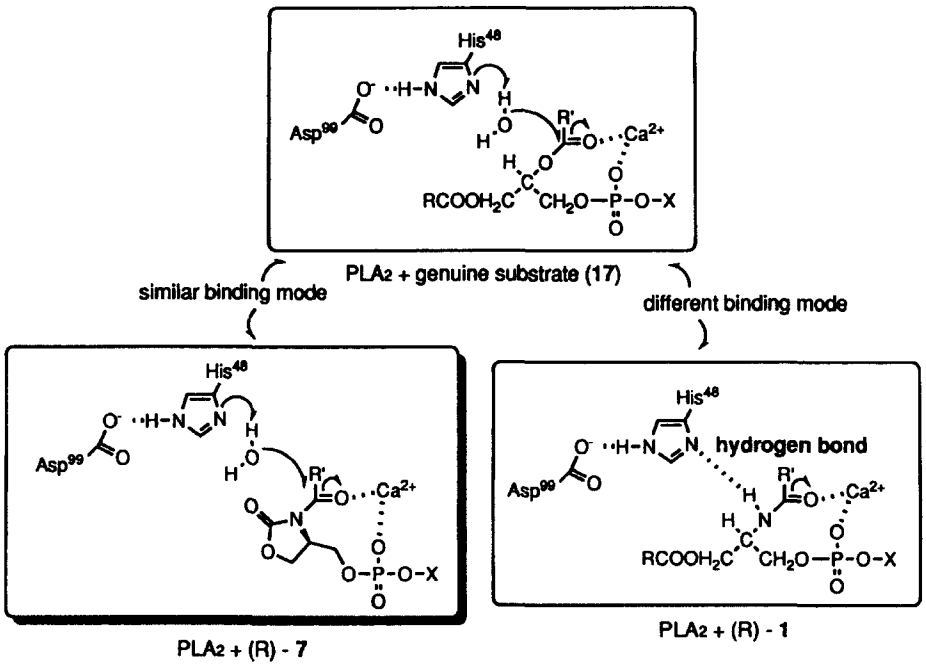


Figure 8. Assumed binding mode of PLA_2 molecule with the genuine substrate and its substrate analogs.

m), 2.89 (2H, *m*), 3.67 (1H, *d*, *J* = 2.5 Hz), 3.92 (1H, *d*, *J* = 2.5 Hz), 4.35 (2H, *m*), 4.49 (1H, *m*); ^{13}C NMR ($CDCl_3$): δ –5.6, 14.1, 18.0, 22.7, 24.3, 25.6, 29.1, 29.3, 29.4, 29.6, 31.9, 35.4, 55.1, 61.4, 65.0, 153.8, 173.6; IR (nujol) 1780, 1700 cm^{-1}; HRMS (FAB), Found: m/z 414.3039. Calcd for $C_{22}H_{44}O_4NSi$: M+H, 414.3028.

(S)-3-Dodecanoyl-4-hydroxymethyl-2-oxazolidinone (13). A solution of **12** (3.33 g, 8.04 mmol) in THF (40.2 mL) was treated with 2 M HCl (8.04 mL) for 17 h at room temperature. The mixture was diluted with EtOAc and H_2O, and extracted with EtOAc. The extracts were washed with brine, dried over $MgSO_4$ and evaporated *in vacuo*. Chromatography of the residue gave **14** (2.02 g, 84%) as colorless crystals: mp 68–69 °C; $[\alpha]_D^{21}$ –51.8° (*c* 1.06; $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 0.88 (3H, *t*, *J* = 6.7 Hz), 1.26 (16H, *m*), 1.65 (2H, *m*), 2.56 (1H, OH), 2.90 (2H, *m*), 3.84 (2H, *m*), 4.31 (1H, *q*), 4.43 (1H, *t*, *J* = 8.8 Hz), 4.55 (1H, *m*); ^{13}C NMR ($CDCl_3$): δ 14.1, 22.7, 24.3, 29.1, 29.4, 29.5, 29.6, 31.9, 35.5, 55.8, 62.3, 65.0, 153.7, 174.6; IR ($CHCl_3$) 3460, 1780, 1700 cm^{-1}; HRMS (FAB), Found: m/z 300.2173. Calcd for $C_{16}H_{30}O_4N$: M+H, 300.2167.

(S)-3-Dodecanoyl-4-phosphatidylcholinohydroxymethyl-2-oxazolidinone (7). To a solution of **13** (200 mg, 0.668 mmol) in benzene (10 mL) was added triethylamine (0.139 mL, 1.00 mmol), 4-dimethylaminopyridine (32 mg, 0.267 mmol), and 2-chloro-2-oxo-1,3,2-dioxaphospholane (142 mg, 1.00 mmol) at room temperature. The mixture was stirred for 18 h at room temperature. The precipitated Et_3N·HCl was filtered off, and the solvent removed *in vacuo* to give the phosphate ester which was used for the next step without purification. The obtained phosphate ester was stirred in a pressure bottle with trimethylamine (2 mL) in benzene (6 mL) for 3 days at 75 °C, and then the solvent was evaporated *in vacuo*. The crude products were purified by column chromatography over silica gel to give (*S*)-**7** (121 mg, 39% for two steps). The analytical samples were purified by reversed-phase HPLC on a Develosil Packed Column C8-5 (Nomura Chemical) with an acetonitrile concentration gradient from 30 to 100%: $[\alpha]_D^{23}$ –55.2° (*c* 1.06, $CHCl_3$). ^{1}H NMR (CD_3OD): δ 0.90 (3H, *t*, *J* = 6.9 Hz), 1.30 (16H, *s*), 1.63 (2H, *m*), 2.89 (2H, *m*), 3.22 (9H, *s*), 3.63 (2H, *t*, *J* = 4.6 Hz), 4.00 (1H, *m*), 4.23 (3H, *m*), 4.46 (2H, *d*, *J* = 2.5 Hz), 4.65 (1H, *m*); ^{13}C NMR ($CDCl_3$): δ 14.1, 22.7, 24.3, 29.2, 29.4, 29.5, 29.6, 29.7, 31.9, 35.4, 54.3, 59.4, 63.8, 65.6, 66.2, 154.1, 173.4; IR ($CHCl_3$) 1790, 1720 cm^{-1}; Found: C, 46.67, H, 8.98, N, 5.17%. Calcd. for $C_{21}H_{41}N_2O_7PNa·H_2O$: C, 46.57; H, 8.74; N, 5.17%.

(R)-N-Dodecanoyl-2-aminohexanol (16). To a solution of **15** (100 mg, 0.69 mmol) in dioxane (2.8 mL) was added 6 M NaOH (1.4 mL) at room temperature. The mixture was stirred for 1 day at 95 °C, and treated with dodecanoyl chloride (0.20 mL, 0.86 mmol). The resulting solution was stirred for 24 h at room temperature, neutralized with 2 M HCl, and then diluted with EtOAc. The mixture was extracted with EtOAc. The extracts were washed with brine, dried over $MgSO_4$, and evaporated *in vacuo*. Chromatography of the residue gave **17** (184 mg, 88% for two steps) as colorless crystals: mp 91–92 °C; $[\alpha]_D^{23}$ +20.7° (*c* 0.89; $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 0.88 (6H, *m*), 1.26–1.63 (24H, *m*), 2.20 (2H, *t*, *J* = 7.7 Hz), 2.99 (1H, *bt*, *J* = 5.3 Hz), 3.56 (1H, *m*), 3.66 (1H, *m*), 3.93 (1H, *m*), 5.66 (1H, *bd*, *J* = 7.6 Hz); ^{13}C NMR ($CDCl_3$): δ 13.9, 14.1, 22.5, 22.6, 25.8, 28.2, 29.2, 29.3, 29.5, 29.6, 30.9, 31.9, 36.9, 51.9, 66.0, 174.2; IR (Nujol) 3305, 1641 cm^{-1}; HRMS (FAB), Found: m/z 300.2910. Calcd for $C_{18}H_{38}O_2N$: M+H, 300.2893.

(R)-N-Dodecanoyl-O-phosphatidylcholino-2-aminohexanol (1). To a solution of **16** (800 mg, 2.67 mmol) in benzene (30 mL) were added triethylamine (0.41 mL, 2.93 mmol) and 2-chloro-2-oxo-1,3,2-dioxaphospholane (419 mg, 2.93 mmol) at room temperature. The mixture was stirred for 6 h at room temperature. The precipitated Et_3N·HCl was filtered off, and the solvent removed *in vacuo* to give the phosphate ester which was used for the next step without purification. The obtained phosphate ester was stirred in a pressure bottle with trimethylamine (*ca* 10 mL) in benzene (13.4 mL) for 2 days at 75 °C, and then the solvent was evaporated *in vacuo*. The crude products were purified by column chromatography over silica gel to give (*R*)-**1** (554 mg, 45% for two steps). The analytical samples were purified by reversed-phase HPLC on a Develosil Packed Column C8-5 (Nomura Chemical) with an acetonitrile concentration gradient from 30 to 100%: $[\alpha]_D^{24}$ +6.67° (*c* 0.90, $CHCl_3$); ^{1}H NMR (CD_3OD): δ 0.90 (6H, *m*), 1.17–1.62 (24H, *m*), 2.20 (2H, *m*), 3.22 (9H, *s*), 3.63 (2H, *m*), 3.81 (2H, *t*, *J* = 5.6 Hz), 3.99 (1H, *m*), 4.25 (1H, *d*, *J* = 10.5 Hz), 4.26 (2H, *m*); ^{13}C NMR ($CDCl_3$): δ 13.8, 14.0, 22.3, 22.6, 26.0, 27.8, 29.1, 29.2, 29.3, 29.5, 29.6, 30.5, 31.8, 36.3, 49.9, 54.5, 59.8, 66.2, 68.0, 175.4; IR ($CHCl_3$) 3400, 1660 cm^{-1}; HRMS (FAB), Found: m/z 465.3438. Calcd for $C_{23}H_{50}O_5N_2$: M+H, 465.3445.

Kinetics of hydrolysis of diC$_6$PC and inhibition studies

Enzymatic hydrolysis of the monodispersed genuine substrate (diC$_6$PC) was followed at 25 °C and an ionic strength of 0.1 by the pH-stat assay method using a system consisting of a Radiometere PHM 82 standard pH meter, a TTT 80 titrator, and an ABU 80 autoburette. The 2.8 mM diC$_6$PC solution (1.0 mL) containing 3.3 mM of $CaCl_2$ in the presence or absence of the monodispersed substrate analogs were transferred to the cells, and the pH values of the solutions were adjusted to 8.2 by the addition of small volumes of 30 mM NaOH solution. To these solutions, 5–50 μL of the enzyme stock solutions were added, and the released *n*-caproic acids were titrated with 30 mM NaOH under a nitrogen stream so as to keep the pHs at the initial values. The titration volumes recorded during the enzymatic hydrolysis of the substrate were corrected by subtracting the volumes due to spontaneous reactions without enzymes.

Acknowledgement

The authors are grateful to Daiso Co., Ltd for the supply of enantiomerically pure glycidol. The authors also thank the Ministry of Education, Science and Culture of Japan for their financial support. Part of this work was also supported by the Ciba-Geigy Foundation (Japan).

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(*Received in Japan* 29 *May* 1995; *accepted* 10 *June* 1995)