

## Chemoenzymatic Synthesis of Phospholipid Hydroperoxides

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**Chemoenzymatic synthesis of 1-stearoyl-2-hydroperoxyacyl-*sn*-glycerophospholipids including phosphatidylcholine (PC-OOH), phosphatidic acid (PA-OOH), phosphatidylethanolamine (PE-OOH), phosphatidylglycerol (PG-OOH) and phosphatidylserine (PS-OOH). The hydroperoxy acyl moieties were prepared *via* hydroperoxidation of linoleic, dihomo- $\gamma$ -linolenic and arachidonic acids by soybean, potato lipoxygenase or autoxidation. Their hydroperoxy group was protected as a dimethylperacetal before condensation with lysophosphatidylcholine. Optically active lysophosphatidylcholine was prepared via short pathway involving lipase-catalyzed direct enantioselective stearylation of 2-*O*-benzylglycerol and choline phosphate synthesis. Peroxy fatty acids and lysophosphatidylcholine thus obtained were condensed using dicyclohexylcarbodiimide in chloroform. Removing the peracetal group in the product and purification by reverse-phase chromatography afforded the desired PC-OOH's. PA-OOH, PG-OOH, PE-OOH and PS-OOH were obtained by phospholipase-D catalyzed transphosphatidylation from PC-OOH. As a reference compound for biological studies of hydroperoxy phospholipid, PC-OH's were also prepared in which hydroxy unsaturated fatty acyl group was linked to the *sn*-2 position of the glycerophospholipids.**

**Key words :** phospholipid, peroxide, hydroperoxide, phospholipase D, lipoxygenase

### Introduction

In recent years, the importance of polyunsaturated lipids like docosahexaenoic acid (DHA) and arachidonic acid (AA) have attracted wide interests due to their relevant diverse biological functions<sup>1-5)</sup>. The functions include chemical and physiological alterations of biological membranes<sup>6,7)</sup>, perturbation to arachidonic acid cascade<sup>8)</sup>, cell signal transductions<sup>9-11)</sup>, cancer cell growth regulations and many other physiological functions<sup>1)</sup>. Despite their outstanding roles in life, however, the non-conjugate *cis*-polyene structures in these lipids are extremely sensitive to oxidation by oxygen in air<sup>12)</sup> and by reactive oxygen species produced by leukocytes such as macrophages, monocytes and neutrophils in biological defense systems<sup>13)</sup>. These oxidative processes cause peroxidation of polyunsaturated lipids<sup>15)</sup>. Here, the lipid hydroperoxides may be fatty acids, triacylglycerols or phospholipids. Most of them are generally very unstable against light, heat, acylating agents, metal ions such as ferrous and ferric ions, and they decompose very easily to afford diverse oxidation prod-

ucts including unsaturated fatty acids and aldehydes<sup>16)</sup>. These degradation products are believed to cause food deterioration and affect on important biological functions through their interaction with DNA, proteins and other biological substances<sup>17)</sup>. Lipid peroxidation products and their degradation products have been extensively discussed in relation to their roles on physiological dysfunctions including arteriosclerosis<sup>19)</sup>, tumor promotion<sup>20,21)</sup> and activation of carcinogens<sup>22)</sup>. On the other hand, in our blood, even in those of younger and healthy persons, hydroperoxides of phospholipids such as phosphatidylcholine hydroperoxide (PC-OOH), phosphatidylethanolamine hydroperoxide (PE-OOH) and phosphatidylserine hydroperoxide (PS-OOH) are known to exist at very low concentration (5–20 nM), and the concentration increase in blood with diseases such as arteriosclerosis and aging as shown by the studies using high performance liquid chromatography with chemilu-

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minescence detector<sup>23</sup>). Their biological function of these hydroperoxides, however, remains uncovered yet, and it is even unknown that their formation *in vivo* is a cause of the diseases or simply results of the diseases or aging. Furthermore, it has been unclear whether those phospholipid hydroperoxides are toxic or deleterious against our health or not. Thus, unforeseen but important aspects of phospholipid hydroperoxide including their possible salutary effects must be revealed in biological systems.

A number of studies reported in recent years involves unexpected so far but rather salutary effects on human biological systems. Lipid peroxidation products externally administered to cultured human cancer cells or formed *in vivo* in mice bearing breast cancer were shown to negatively regulate the cancer cell proliferation or growth<sup>24</sup>. These salutary effects were confirmed by simultaneous addition of antioxidant such as  $\alpha$ -tocopherol. The similar observation was also reported in negative growth regulation of malaria parasite by lipid bearing polyunsaturated fatty acyl group and chemical substances that are responsible for the regulation were shown to be lipid peroxidation products formed *in vivo* although the detail remains uncovered<sup>25</sup>. Oxidized phospholipids were reported to inhibit expression of inflammatory adhesion molecules. It was shown very recently that they inhibited LPS-induced upregulation of inflammatory genes, by blocking the interaction of LPS with LPS-binding protein and CD14. By this inhibition, LPS-injected mice was protected from lethal endotoxin shock. The authors suggest possible application of oxidized phospholipids for the developments of new drugs for treatment of sepsis<sup>26</sup>. Phosphatidylserine (PS) molecules that move from inside to outside of cell membrane when the cells begin apoptosis and the PS on the surface become a marker or a messenger by which fagocytes are triggered to execute fagocytosis on the apoptotic cells. In this study, PS bearing parinaric acid was used as a model phospholipid<sup>27</sup>. Parinaric acid and its derivatives are very sensitive to oxidation giving fluorescence therefrom and has been used as a convenient marker for studies on biological lipid peroxidations. However, the real species of the lipid peroxide responsible for the externalization remains to be discovered. External application of ROS such as hydroxy radical is known to induce apoptosis and this pathway involves leak of cytochrom C from mitochondria. Very recently, it was shown that when such the oxidative stress was applied, cardiolipin molecules in the mitochondria are peroxidized and they enhance the leak of cytochrom C which trigger apoptosis cascade<sup>28</sup>. Thus, lipid peroxidations have been demonstrated to involve a

number of biological phenomena including cancer cell growth regulation, apoptosis, regulation of inflammatory in recent years as well as atherosclerosis, aging and many other physiological dysfunctions reported so far. One difficult aspect in biological studies related to the lipid peroxidation is that lipid peroxidation products, especially lipid hydroperoxides, that form by non-enzymatic oxidation of lipids having polyunsaturated fatty acyl groups with oxygen or ROS constitute complex mixtures of their degradation products, regio- and stereoisomers. Isolation by chromatographic separation to each fraction is usually very difficult. Moreover, formation of the lipid peroxidation products in living systems is transient and their amount is extremely small. Therefore, it is obvious that ample supply of chemically well-defined lipid peroxidation products in a pure state and in a large amount is essential. Thus, we have addressed chemical enzymatic synthesis of lipid peroxidation products, especially phospholipid hydroperoxides, since phospholipid is a grand component of cell membranes and hydroperoxides are prime peroxidation products from which a variety of degradation products are formed including phospholipids with functional groups such as aldehyde, ketone and carboxylic acid. This article describes details of the synthesis of phospholipid hydroperoxides and some of their degradation products. The phospholipids thus obtained may hopefully contribute on the foregoing quests concerning not only their physiological functions in life but also salutary applications to disease improvements.

## Results and Discussion

### Outline of the Synthesis

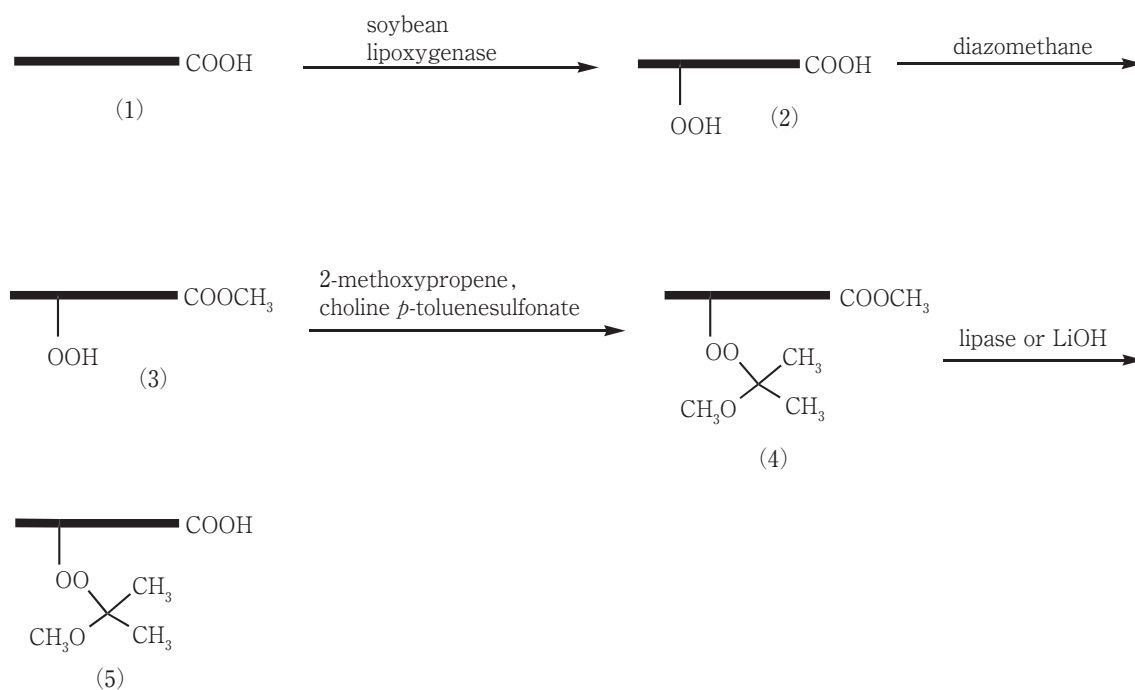
A general structure for glycerophospholipid hydroperoxides is shown below. Here, bold straight line simply express saturated or unsaturated hydrocarbon chain and X represents choline, ethanolamine, glycerol, serine or a proton. An asymmetric center in the glyceride backbone should be (*S*)-configuration according to the configuration of natural glycerophospholipids in living systems. It has been considered that coupling of hydroperoxy unsaturated fatty acid activated by dicyclohexylcarbodiimide (DCC) and optically active lysophosphatidylcholine via ester linkage was the best way according to the conventional synthetic concept for glycerophospholipids. Here, there is a problem that acylation of hydroperoxy group may also occur at the ester coupling stage and it is well known that, by the acylation, primary and secondary hydroperoxy groups change immediately to carbonyl oxygens<sup>29</sup>. In the study of enantiomer separation of racemic hydroperoxy fatty acid esters, Porter successfully employed chiral

peracetal group derived from (–)-menthol to a diastereomeric mixture of the peroxide which was separated to each other by HPLC and the peracetal group was found to be removed very easily under weak acidic condition<sup>30</sup>. In our present study, the peracetal group like this might be one of the best candidate for protection of the hydroperoxy group against the acylation. In this case, however, the group should also be stable against hydrolytic condition of ester and DCC activation of the carboxylic acid. Easy accessibility of the reagent for the protection is also very important. As a result of examination of several candidates, simple dimethylperacetal protective group was found to be most suitable for our plan since it could be prepared very easily using commercially available 2-methoxypropene, stable under ester hydrolysis and DCC activation of the carboxyl group, and the removal was also easy under the Porter's condition<sup>30</sup>. Thus, as shown in Scheme 1, a combination of lipoxygenase-catalyzed hydroperoxidation and protection of the hydroperoxy group afforded key intermediates, peroxy acids (1)–(5). The hydroperoxy fatty acid thus obtained was esterified with optically active lysophosphatidylcholine which was synthesized via short route utilizing lipase-catalyzed enantioselective stearylation of 2-*O*-benzylpropanediol<sup>31</sup>. Removal of the peracetal group for hydroperoxy group afforded phosphatidylcholine hydroperoxide (PC-OOH). Other phospholipid hydroperoxides were also obtained from PC-OOH via phospholipase D-catalyzed transphos-

phatidylation. According to this process, the phospholipid hydroperoxide (7)–(20) were synthesized in the present study.

#### Preparation of Peroxy Unsaturated Fatty Acids (1)–(5).

Unsaturated hydroperoxy fatty acid could be prepared via three ways, i.e., lipoxygenase-catalyzed hydroperoxidation, chemical synthesis<sup>32</sup> and autoxidation of unsaturated fatty acid<sup>33</sup>. Among them, most convenient and preparative scale method is lipoxygenase-catalyzed hydroperoxidation. The enzyme exists ubiquitously in living materials including vegetables like tomato, potato, soybean and tea leaves. In the present study, soybean lipoxygenase was employed for preparation of hydroperoxy unsaturated fatty acid affording gram scale quantity. The enzyme is commercially available and has high regio- and stereoselectivities. Thus, linoleic, dihomo- $\gamma$ -linolenic and arachidonic acid were hydroperoxidized with the enzyme in a borate buffer (pH 9) bubbled by oxygen gas at 0–5 °C with mechanical stirring for about 6 h in the presence of emulsifier. The product was extracted with ethyl acetate and esterified as such with diazomethane followed by silica gel column purification. The hydroperoxide was submitted to the reaction with methoxypropene in the presence of pyridinium *p*-toluenesulfonate in THF overnight. The peroxide esters, after silica gel column purification, was hydrolyzed by lipase or LiOH and the column purification afforded the key intermediates (1)–



Scheme 1. Preparation of Protected Form of Hydroperoxyfatty Acid (general)

(5) in gram scale. The preparation of a variety of unsaturated fatty acid hydroperoxides at will including regio- and stereoisomers in a pure state. Although we did not follow this approach in the present study, it could be an interesting way for synthesis of phospholipids and others bearing structurally unnatural hydroperoxy fatty acyl group in particular.

Most of hydroperoxidations in foods as well as in living systems are caused by radical-initiated autoxidation and this process is known to give very complicate mixture of oxidized lipids as well as their degradation products<sup>33</sup>. Therefore, synthesis of such phospholipids as those produced by autoxidation is also important, and autoxidation of unsaturated fatty acids were conducted. A complicate mixture of the autoxidation products was submitted to peracetalization after esterifying with diazomethane. This process allowed the hydroperoxides alone to convert to peracetals leading them to more non-polar and stable derivatives. The esters of the peroxy fatty acid mixture thus obtained including regio- and stereoisomers was successfully purified by silica gel column, and LiOH- or lipase-catalyzed hydrolysis of the esters afforded a key intermediates (4) and (5) from linoleic and arachidonic acids respectively.

#### **Short Route Synthesis of Optically Active Lysophosphatidylcholine.**

Optically active 1-stearoyllysophosphatidylcholine has been prepared from optically active 1, 2-isopropylidenglycerol or other building blocks via several steps. In the present experiment, the title compound could be much simply synthesized via short route based on our finding that lipase P from *Pseudomonas fluorescens* catalyzed monoacylation of 2-*O*-benzylpropanediol by use of vinyl stearate in diisopropyl ether and the process is highly enantioselective affording 1-stearoyl-2-*O*-benzylglycerol having ~100% optical purity<sup>31</sup>. This very simple process could afford more than 1 g of the glycerol in one batch. The optically active glyceride was linked to choline phosphate head group and the benzyl group was removed to give the desired lysophosphatidylcholine (6).

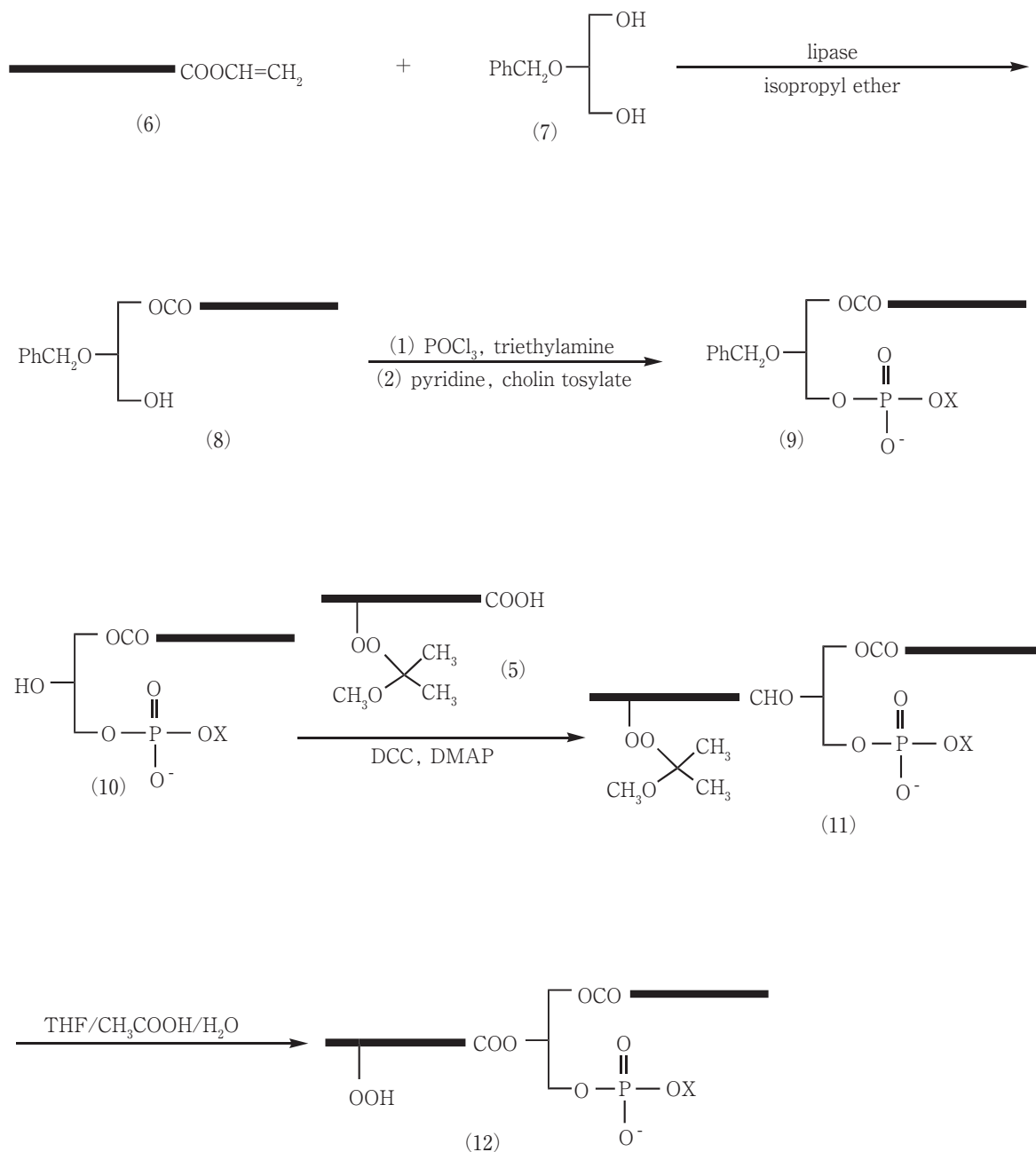
#### **Condensation of the Acids (1)-(5) with the Lysophosphatidylcholine (6).**

Ester formation between the acid components and lysophosphatidylcholine (6) could be easily attained by activation of the carboxyl groups with dicyclohexylcarbodiimide (DCC) in chloroform in the presence of dimethylaminopyridine as an acylation catalyst according to Scheme 3. In the case where peroxyicosatrienoic acid (2) and peroxyicosatetraenoic acid (3) were submitted to the reaction, a trace of butylated hydroxytoluene (BHT) was added and the reaction period should be as

shorter as possible since the two acid components have one or two isolated double bonds respectively which are very susceptible to autoxidation. As a final procedure, the dimethylperacetal group was removed with a mixture of solvents (CH<sub>3</sub>COOH/THF/H<sub>2</sub>O, 4 : 2 : 1) in the presence of BHT and the product was purified by reverse phase HPLC. To assure the structural integrity of 13- or 15-hydroperoxy-*cis*, *trans*-olefinic moiety, the acyl group was removed from the glycerophosphocholine by porcine pancreatic phospholipase A<sub>2</sub>. The acid liberated was esterified with diazomethane and purified by silica gel column chromatography. <sup>1</sup>H NMR spectrum of this ester showed that no isomerization of the conjugate double bonds with hydroperoxy group at their allylic position and no autoxidation at the isolated double bonds occurred.

#### **Synthesis of Other Phospholipid Hydroperoxides Having Different Polar Head Group by Phospholipase D-Catalyzed Transphosphatidylation.**

Phospholipase D catalyzes transfer of phosphatidyl group from glycerophosphatidylcholine (PC) to other hydroxy compounds like glycerol, ethanolamine, serine and various primary alcohols and its substrate specificity is known to be very broad. Indeed, this enzyme has been widely employed for synthesis of phospholipids of biologically important compounds including nucleoside. In our present study, synthesis of PG-OOH, PE-OOH and PS-OOH from PC-OOH have been attained by use of this enzyme. In the first experiment, a substrate, phosphatidylcholine peroxide, in which the hydroperoxy group was protected as dimethylperacetal was submitted to the enzymatic reaction taking care of possible degradation of the hydroperoxy group as well as inactivation of the enzyme. This reaction was found to produce PG-OOH and PE-OOH in moderate yield. In the case of conversion to PS-OOH, however, the reaction gave a complicate mixture of unknown products. Accordingly, the reaction was examined using PC-OOH instead of its protected form. Contrary to our expectation, the phospholipase D showed full activity toward PC-OOH and, PS-OOH was obtained successfully in a pure state after column purification. This nature of the phospholipase D is of particular interest from the facts that porcine pancreatic phospholipase A<sub>2</sub> showed higher activity to PC-OOH than its normal unoxidized form as demonstrated recently by us<sup>34</sup> and, Snyder and Lamb<sup>35</sup> also reported catalytic activity of phospholipase C toward phospholipid hydroperoxide. These three enzymes are typical ones for conversion of glycerophospholipid. PG-OOH, PE-OOH and PS-OOH thus obtained were purified by cellulose column to



Scheme 2. Synthesis of Glycerophospholipid Hydroperoxide (general).

afford single desired products.

### Synthesis of Phospholipids Bearing Hydroxy Unsaturated Fatty Acyl Group.

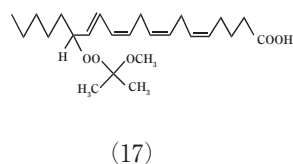
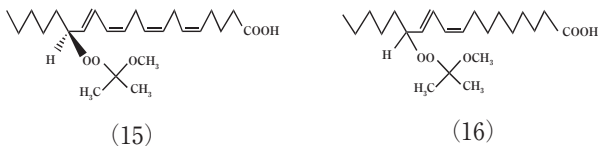
In general, biological study of phospholipid hydroperoxides requires reference compounds. They may be unoxidized forms in which intact unsaturated fatty acid like linoleic, linolenic or arachidonic acid is linked to the phospholipids. To know real biological effect of hydroperoxy group itself, however, its congener having hydroxy group instead of hydroperoxy group should be

examined. From this view, we prepared them simply by reduction of (7) and (9) with triphenylphosphine and the products were purified by silica gel chromatography affording PC-OH (12) and (13).

### Experimental Section

#### Materials and General Methods.

$^1\text{H}$  NMR spectra were recorded on a Varian VXR-500 Instrument, Mass spectra on a JEOL JMS-DX 300 mass spectrometer (Japan Electron Optical Laboratories, Oome, Japan). TLC was done on pre-



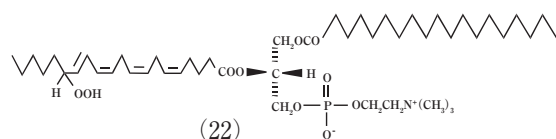
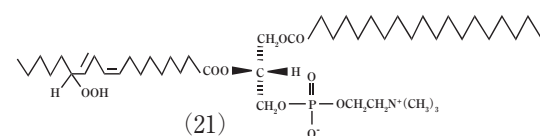
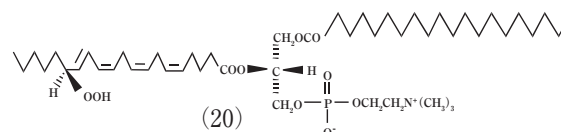
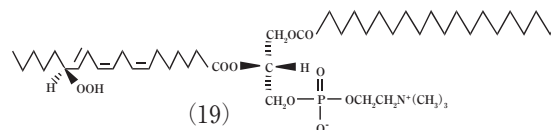
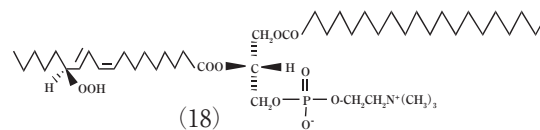
Scheme 3. Chemical Structure of Protected Peroxyfatty Acids as Key Intermediates

coated silica gel 60 F254 plates (E. Merck, Art 5554). Crystalline soybean lipoxygenase (EC 1.13.11.12, 104000 units/mg solid) purchased from Sigma Ltd. was used. Linoleic acid (90% purity) was purchased from Tokyo Kasei Ltd. Lipase (Amano P, 30 units/mg) was provided from Amano Seiyaku Ltd. Phospholipase D (1.5 units, from cabbage) was purchased from Sigma Ltd.

### Synthesis.

#### 13-(S)-(1'-Methoxy-1'-methylethylperoxy)-(9Z, 11E)-octadecadienoic acid (1).

Linoleic acid (8 g, 28.5 mmol) was converted into their ammonium salts by the addition of  $\text{NH}_4\text{OH}$  (0.019 M, 2 L). Soybean lipoxygenase (100 mg, 104000 units/mg solid) was dissolved in borate buffer (pH 9.0, 10 mM, 1.6 l) containing AF-emulsion (100 mg) and butylated hydroxy toluene (BHT) (10 mg, 0.45 mmol). The enzymatic reaction was started by mixing both the solutions at 0 °C with bubbling of oxygen gas. After 6 hours, the reaction mixture was acidified to pH 4-5 by 1.0 N-HCl, and the product was extracted with EtOAc three times. The solvent was removed, and the residual oil was esterified with diazomethane followed by evaporation of the solvent under reduced pressure. The residual oil was chromatographed on silica gel with hexane-EtOAc (9 : 1) as an eluent to afford hydroperoxy fatty acid methyl ester. A solution of the ester (6.7 g, 20.5 mmol), 2-methoxypropene (4.4 g, 61.1 mmol), pyridinium *p*-toluenesulfonate (150 mg, 0.60 mmol) and BHT (50 mg, 0.23 mmol) in THF (20 ml) was stirred for 4 hours at room temperature under nitrogen atmosphere. After removal of the solvent, residual oil was



Scheme 4. Chemical Structure of Phosphatidylcholine Hydroperoxides (18)-(22).

chromatographed on silica gel with hexane-EtOAc (95 : 5) as an eluant to give the protected hydroperoxy fatty acid methyl ester. The obtained methyl ester was hydrolyzed by lipase from *Pseudomonas fluorescense* (100 mg, 30 units/mg) in deionized water (100 ml). After 24 hr. the reaction solution was extracted with  $\text{Et}_2\text{O}$ , and obtained  $\text{Et}_2\text{O}$  phase was chromatographed on silica gel with hexane/EtOAc (7 : 3) as an eluent to give peracetal acid (1) (1.0 mmol, 35% yield). TLC (hexane : EtOAc = 75 : 25)  $R_f = 0.35$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  0.87 (3H, t,  $\text{CH}_3$ ), 1.29 (16H, m,  $\text{CH}_2 \times 8$ ), 1.38 (6H, s,  $\text{OO-C}(\text{CH}_3)_2$ ), 1.61 (2H, t,  $\text{OCOCH}_2\text{CH}_2$ ), 2.18 (2H, m,  $\text{C}_8\text{-H}_2$ ), 2.29 (2H, t,  $\text{OCOCH}_2\text{CH}_2$ ), 3.29 (3H, s,  $\text{OO-C-CCH}_3$ ), 4.40 (1H, m,  $\text{C}_{13}\text{-H}$ ), 5.44 (1H, m,  $\text{C}_9\text{-H}$ ), 5.60 (1H, m,  $\text{C}_{12}\text{-H}$ ), 5.98 (1H, m,  $\text{C}_{10}\text{-H}$ ), 6.47 (1H, m,  $\text{C}_{11}\text{-H}$ ).  $[\alpha]_{\text{D}}^{25} = -2.73$  ( $c = 2.20$ , MeOH).

#### 15-(S)-(1'-Methoxy-1'-methylethylperoxy)-(8Z, 11Z, 13E)-icosatrienoic acid (2).

Ethyl dihomogamma-linolenate (2 g, 6.0 mmol, 90% purity) produced by *Mortierella alpina*. 1S-4 was stirred in a mixture of 2.0 N-lithium hydroxide (5 ml), THF (3 ml) and BHT (10 mg, 0.05 mmol) at room temperature. After slightly acidifying the solution, the acid liberated was extracted with ether. The fatty acid

(1.84 g, 6.0 mmol) thus obtained was dissolved as such in a solution of  $\text{NH}_4\text{OH}$  (0.019 M), AF-emulsion (50 mg) and BHT (50 mg, 0.25 mmol) in borate buffer (10 mM, pH 9.0, 400 ml). To the solution was added soy-bean lipoxygenase (200 mg, 104000 units/mg solid) at 0 °C and oxygen gas was bubbled for 4 h with magnetic stirring. After neutralizing the solution to ca. pH 7.0, with 1.0N-HCl the product hydroperoxide was extracted with  $\text{Et}_2\text{O}$  three times. After treating the ether solution with diazomethane the ester hydroperoxide as an oily residue was chromatographed on silica gel with hexane/EtOAc (9 : 1) as eluant to afford the hydroperoxy fatty acid methyl ester. The conversion of the ester to the title compound was the same with those in the case of compound (1). (39% yield) TLC hexane/EtOAc (75 : 25)  $R_f = 0.35$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  0.88 (3H, t,  $\text{CH}_3$ ), 1.38 (6H, s,  $\text{OO-C}(\text{CH}_3)_2$ ), 1.61 (2H, t,  $\text{OCOCH}_2\text{CH}_2$ ), 2.06 (2H, m, C7-H<sub>2</sub>), 2.30 (2H, t,  $\text{OCOCH}_2\text{CH}_2$ ), 2.93 (2H, m, C10-H), 3.29 (3H, s,  $\text{OO-C-CCH}_3$ ), 4.38 (1H, m, C15-H), 5.45 (1H, m, C11-H), 5.60 (1H, m, C14-H), 6.02 (1H, m, C12-H), 6.60 (1H, m, C13-H).

**15-(S)-(1'-Methoxy-1'-methylethylperoxy)-(5Z, 8Z, 11Z, 13E)-icosatetraenoic acid (3).**

The synthetic method to the end compound was the same that in the case of compound (2) except for employing ethyl arachidonate (96% purity) produced by *Mortierella alpina* as unsaturated fatty acid. (45% yield). TLC (hexane : EtOAc = 75 : 25)  $R_f = 0.35$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  0.88 (3H, t,  $\text{CH}_3$ ), 1.38 (6H, s,  $\text{OO-C}(\text{CH}_3)_2$ ), 1.71 (2H, t,  $\text{OCOCH}_2\text{CH}_2$ ), 2.14 (2H, m, C4-H<sub>2</sub>), 2.36 (2H, t,  $\text{OCOCH}_2\text{CH}_2$ ), 2.81 (2H, m, C7-H), 2.93 (2H, m, C10-H), 3.29 (3H, s,  $\text{OO-C-CCH}_3$ ), 4.42 (1H, m, C15-H), 5.64 (1H, m, C14-H), 6.02 (1H, m, C12-H), 6.50 (1H, m, C13-H).

**Protective form of autoxidized linoleic acid (4).**

Linoleic acid (2.34 g, 8.34 mmol) was stirring at room temp. for 7 days under oxygen atmosphere. Diazomethane in  $\text{Et}_2\text{O}$  was added to the autoxidized mixture converting the acid to the methyl ester. After evaporating off the organic solvent, the oily residue was chromatographed on silica gel with hexane/EtOAc (8 : 2) to afford the hydroperoxy fatty acid methyl ester. To protect hydroperoxy group as dimethylperacetal, the ester (520 mg, 1.48 mmol) in THF (10 mL) was stirred for 4 h with 2-methoxypropene (265 mg, 4.44 mmol), pyridinium *p*-toluene sulfonate (20 mg, 0.08 mmol), and BHT (20 mg, 0.09 mmol) under nitrogen atmosphere. After removal of organic solvent, the residue was chromatographed on silica gel with hexane/EtOAc (9 : 1) to give the peracetal methylester. The

ester (180 mg, 0.52 mmol) was hydrolyzed with lipase P (300 mg) to give the title compound (5, 170 mg, 6.2% yield). TLC hexane/EtOAc (75 : 25)  $R_f = 0.35$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz) The proton NMR spectrum of the olefin moiety was very complicated since the product constituted a mixture of regio- and stereoisomers produced by the autoxidation. The other proton signals, however, were coincided with those of 1.

**Protective form of autoxidized arachidonic acid (5).**

Arachidonic acid (500 mg, 1.6 mmol) obtained by hydrolysis of ethyl arachidonate with 2.0 N-lithium hydroxide was stirred at room temp for 10 hours under oxygen atmosphere. The procedure for preparation of the title compound from this autoxidized mixture was the same as for the compound (5) described above (9.4% yield). TLC hexane/EtOAc (75 : 25)  $R_f = 0.35$ . The proton NMR spectrum of the olefin moiety was very complicated. The other signals, however, were coincided with those of 3.

**1-Stearoyl-*sn*-glycero-3-lysophosphocholine (6).**

The direct acylation of 2-*O*-benzylglycerol (2.0 g, 11 mmol) with vinyl stearate (5.6 g, 18 mmol) was done in diisopropyl ether (30 mL) using lipase P (400 mg, 30 units/mg, from *Pseudomonas fluorescense*). The reaction was continued at room temperature until the yield of the monostearate amounted to 70% that was determined by quantitative TLC analysis. The enzyme was filtered off and the filtrate was concentrated under reduced pressure. The residue was chromatographed on silica gel column with hexane/EtOAc (75 : 25) to give 1-stearoyl-2-*O*-benzyl-*sn*-glycerol in 50% yield and 92% e. e. (the ratio of *S* to *R* was 96 : 4.). 1-Stearoyl-2-*O*-benzylglycerol (504 mg, 1.12 mmol) thus obtained was dissolved in EtOH-free  $\text{CHCl}_3$  (2 ml) containing triethylamine (142 mg, 1.40 mmol). Phosphorus oxychloride (215 mg, 1.40 mmol) was added dropwise to the solution for 10 min. and stirred at 0 °C for 30 min. and then at room temp. for 30 min. To the reaction mixture was added choline tosylate (463 mg, 1.68 mmol) and pyridine (0.5 mL), and the solution was stirred at room temperature for 12 hr. After the period, deionized water (1.0 mL) was added to the reaction mixture and stirred at room temperature for 30 min. The reaction mixture was extracted with  $\text{CHCl}_3$  and the organic phase was washed sequentially with water, 5% -HCl, and water. The extract was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and concentrated under reduced pressure. The residue was chromatographed on silica gel with  $\text{CHCl}_3/\text{MeOH}/28\% \text{NH}_4\text{OH}$  (60 : 30 : 0 to 60 : 35 : 4) to afford 1-stearoyl-2-*O*-benzyl-*sn*-glycero-3-phosphocholine. The obtained glycerophosphocholine was dissolved in organic solvent ( $\text{MeOH}/\text{H}_2\text{O} = 10 : 1$ , 10 ml)

and was hydrogenated in the presence of 20% Pd(OH)<sub>2</sub>/C (100mg) for 24 hr. to remove benzyl group. After the reaction, the solution was filtrated and evaporated *in vacuo* to afford the title compound (**6**, 496mg, 72.0% yield). TLC CHCl<sub>3</sub>/MeOH/28% NH<sub>4</sub>OH (60 : 30 : 4) *Rf* 0.14 <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 0.88 (3H, t, ω-CH<sub>3</sub>), 1.24 (28H, s, 1-CH<sub>2</sub> x 4), 1.58 (2H, m, OCOCH<sub>2</sub>CH<sub>2</sub>), 2.28 (2H, t, OCOCH<sub>2</sub>), 3.18 (9H, s, NMe<sub>3</sub>), 3.60, (2H, br, OCH<sub>2</sub>CH<sub>2</sub>N), 3.81 (1H, m, -OCH<sub>2</sub>-CH-CH<sub>2</sub>O-), 3.97 (2H, m, -OCH<sub>2</sub>-CH-CH<sub>2</sub>O-), 4.15-4.30 (4H, m, -OCH<sub>2</sub>-CH-CH<sub>2</sub>O-, -OCH<sub>2</sub>CH<sub>2</sub>N). **1-Stearoyl-2-[13'-(S)-hydroperoxy-(9'Z, 11'E)-octadecadienoyl]-sn-glycero-3-phosphocholine (7)**.

A solution of dicyclohexylcarbodiimide (0.416 g, 2.02 mmol), dimethylaminopyridine (25mg, 0.20 mmol), 1-stearoyl-*sn*-glycero-3-phosphocholine (**6**, 0.283 g, 0.54 mmol) and the peracid (**1**, 0.520 g, 1.35 mmol) in EtOH-free CHCl<sub>3</sub> (50 ml) was stirred at room temperature under nitrogen atmosphere for 48 hr. The reaction mixture was concentrated and separated dicyclohexylurea was filtered off. The filtrate was concentrated and the residue was chromatographed on silica gel column with CHCl<sub>3</sub>/MeOH/28% NH<sub>4</sub>OH (60 : 25 : 1.5) to afford peracetal phospholipid. A solution of the phospholipid and a trace amount of BHT in a mixture (5 ml), THF/CH<sub>3</sub>COOH/H<sub>2</sub>O (4 : 2 : 1) was stirred at room temperature overnight, and the reaction solution was concentrated under reduced pressure in the presence of carbon tetrachloride and isopropanol to remove moisture by azeotropic distillation. The residue was purified by reverse-phase column chromatography (RP-8 Rober column) using a mixture, CHCl<sub>3</sub>/MeOH/water (1 : 10 : 1) to afford the hydroperoxide (**8**) (60 mg, 67% yield). TLC *Rf* = 0.35 (CHCl<sub>3</sub> : MeOH : 28% NH<sub>4</sub>OH = 60 : 30 : 5) <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz) δ 0.87 (6H, m, ω-CH<sub>3</sub> x 2), 1.28 (44H, m, CH<sub>2</sub> x 22), 1.58 (4H, m, OCOCH<sub>2</sub>CH<sub>2</sub>), 2.15 (2H, m, C8'-H), 2.28 (4H, m, OCOCH<sub>2</sub>), 3.30 (9H, s, N(CH<sub>3</sub>)<sub>3</sub>), 3.75 (2H, m, -OCH<sub>2</sub>CH<sub>2</sub>N), 3.98 (2H, m, -OCH<sub>2</sub>-CH-CH<sub>2</sub>O-), 4.14 (1H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>O-), 4.34 (4H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>O-, -OCH<sub>2</sub>CH<sub>2</sub>N, C13'-H), 5.22 (1H, m, C10'-H), 6.52 (1H, m, C11'-H). FAB-MAS, *m/z* 818(M+H)<sup>+</sup>. [α]<sub>D</sub><sup>25</sup> = +2.00° [*c* = 1.90, CHCl<sub>3</sub>/MeOH (1 : 1)]: Anal. calcd for C<sub>44</sub>H<sub>84</sub>O<sub>10</sub>PN · H<sub>2</sub>O; C, 63.21; H, 10.37; N, 1.68, found C, 63.57; H, 10.34; N, 1.57.

**1-Stearoyl-2-[15'-(S)-hydroperoxy-(8'Z, 11'Z, 13'E)-icosatrienoyl]-sn-glycero-3-phosphocholine (8)**.

The same procedure as those described for **6** and **7** followed using the peroxy fatty acid (**2**) (3.0 g, 7.3

mmol) and lyso form (**7**) (1.5 g, 3.0 mmol) to afford the title compound (**8**) (1.26 g, 58.0% yield). TLC *Rf* = 0.5 (CHCl<sub>3</sub> : MeOH : 28% NH<sub>4</sub>OH = 60 : 30 : 1). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz) δ 0.88 (6H, t, ω-CH<sub>3</sub> x 2), 1.24 (28H, m, 1-CH<sub>2</sub> x 14), 1.58 (2H, m, 2-OCOCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.05 (2H, m, 2-OCOCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.28 (2H, t, 2-OCOCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.90 (2H, t, C10'-CH<sub>2</sub>), 3.36 (9H, s, N(CH<sub>3</sub>)<sub>3</sub>), 3.79 (2H, m, -OCH<sub>2</sub>CH<sub>2</sub>N), 3.94 (2H, m, CH<sub>2</sub>OP), 4.12 (1H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP), 4.32 (2H, m, OCH<sub>2</sub>CH<sub>2</sub>N), 4.38 (1H, m, C15'-H), 4.40 (1H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP), 5.20 (1H, m, -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP), 5.38 (2H, m, C8', C9'-H), 5.40 (1H, m, C11'-H), 5.64 (1H, m, C14'-H), 6.03 (1H, t, C12'-H), 6.51 (1H, m, C13'-H); FAB-MS: *m/z* 842(M-H)<sup>-</sup>.

**1-Stearoyl-2-[15'-(S)-hydroperoxy-(5'Z, 8'Z, 11'Z, 13'E)-icosatetraenoyl]-sn-glycero-3-phosphocholine (9)**.

The same procedure as those described for **7** and **8** followed using the peroxy fatty acid (**3**) (3.0 g, 7.3 mmol) and the lyso form (**6**) (1.5 g, 3.0 mmol) to afford the title compound (**9**) (1.41 g, 56.0% yield). TLC *Rf* = 0.5 (CHCl<sub>3</sub> : MeOH : 28% NH<sub>4</sub>OH = 60 : 30 : 1). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 0.88 (6H, t, ω-CH<sub>3</sub> x 2), 1.24 (28H, m, 1-CH<sub>2</sub> x 14), 1.29 (8H, m, 2-CH<sub>2</sub> x 4), 1.71 (2H, m, 2-OCOCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.13 (2H, m, 2-OCOCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.38 (2H, t, 2-OCOCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.80 (2H, t, C7'-CH<sub>2</sub>), 2.96 (2H, t, C10'-CH<sub>2</sub>), 3.35 (9H, s, N(CH<sub>3</sub>)<sub>3</sub>), 3.79 (2H, m, -OCH<sub>2</sub>CH<sub>2</sub>N), 3.94 (2H, m, CH<sub>2</sub>OP), 4.12 (1H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP), 4.32 (2H, m, OCH<sub>2</sub>CH<sub>2</sub>N), 4.38 (1H, m, C15'-H), 4.40 (1H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP), 5.20 (1H, m, -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP), 5.38 (4H, m, C5', C6', C8', C9'-H), 5.40 (1H, m, C11'-H), 5.64 (1H, m, C14'-H), 6.03 (1H, t, C12'-H), 6.51 (1H, m, C13'-H); FAB-MS: *m/z* 840(M-H)<sup>-</sup>.

**1-Stearoyl-2-hydroperoxyoctadecadienoyl-sn-glycero-3-phosphocholine (10)** (autoxidized glycerophosphocholine).

The same esterification and removal of the peracetal group as those described for **6** and **7** followed using the acid (**4**) (170mg, 0.52 mmol) and the lyso form (**7**) (225mg, 0.52 mmol) to afford the title compound (140 mg, 0.17 mmol, 32.7% yield). The proton NMR spectrum of the olefin moiety was very complicated. The other signals, however, were coincident with those of **8**.

**Autoxidized hydroperoxy form of 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (11)**.

The compound (**5**) (0.28 g, 0.68 mmol) was esteri-



fied with 1-stearoyl-*sn*-glycero-3-lysophosphocholine (**6**) (0.20 g, 0.46 mmol) by stirring with dicyclohexylcarbodiimide (0.43 g, 2 mmol), 4-dimethylaminopyridine (10 mg, 0.08 mmol) and a trace amount of BHT in EtOH-free dist. CHCl<sub>3</sub> (20 ml) for 24 hours at room temp. in nitrogen atmosphere. The procedure from here to end compound (**13**) was the same that as employed for the preparation of (**8**) (18.9% yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz) The proton NMR spectrum of the olefin moiety was very complicated. The other signals, however, were coincident with those of **10**.

**1-Stearoyl-2-[13'-(S)-hydroxy-(9'Z, 11'E)-octadecadienyl]-*sn*-glycero-3-phosphocholine (**12**).**

The hydroperoxy phospholipid (**7**) (136 mg) was reduced by triphenylphosphine (135 mg) in CHCl<sub>3</sub> (10 ml) for 24 hours at room temp. in nitrogen atmosphere. After removal of CHCl<sub>3</sub>, the residue was purified on silica gel chromatography with CHCl<sub>3</sub>-MeOH-28% NH<sub>4</sub>OH (60 : 25 : 5) as an eluent to give the hydroxy phospholipid (**12**) (109 mg). (80% yield) <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz) δ 0.87 (6H, m, w-CH<sub>3</sub> x 2), 1.28 (44H, m, CH<sub>2</sub> x 22), 1.58 (4H, m, OCOCH<sub>2</sub>CH<sub>2</sub>), 2.15 (2H, m, C8'-H), 2.28 (4A, m, OCOCH<sub>2</sub>), 3.30 (9H, s, N(CH<sub>3</sub>)<sub>3</sub>), 3.75 (2H, m, -OCH<sub>2</sub>CH<sub>2</sub>N), 3.98 (2H, m, -OCH<sub>2</sub>-CH-CH<sub>2</sub>O-), 4.14 (1H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>O-), 4.34 (4H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>O-, -OCH<sub>2</sub>CH<sub>2</sub>N, C13'-H), 5.22 (1H, m, C10'-H), 6.52 (1H, m, C11'-H).

**1-Stearoyl-2-[15'-(S)-hydroxy-(5'Z, 8'Z, 11'Z, 13'E)-icosatetraenyl]-*sn*-glycero-3-phosphocholine (**13**).**

The compound (**9**) (38 mg, 0.05 mmol) was treated with triphenylphosphine by the same procedure as employed for the preparation of the compound (**12**) to give the compound (**13**) (24 mg, 60.0% yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz) δ 0.88 (6H, t, w-CH<sub>3</sub> x 2), 1.24 (28H, m, 1-CH<sub>2</sub> x 14), 1.29 (8H, m, 2-CH<sub>2</sub> x 4), 1.71 (2H, m, 2-OCOCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.13 (2H, m, 2-OCOCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.38 (2H, t, 2-OCOCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.80 (2H, t, C7'-CH<sub>2</sub>), 2.96 (2H, t, C10'-CH<sub>2</sub>), 3.35 (9H, s, N(CH<sub>3</sub>)<sub>3</sub>), 3.79 (2H, m, -OCH<sub>2</sub>CH<sub>2</sub>N), 3.94 (2H, m, CH<sub>2</sub>OP), 4.12 (1H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP), 4.32 (2H, m, OCH<sub>2</sub>CH<sub>2</sub>N), 4.38 (1H, m, C15'-H), 4.40 (1H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP), 5.20 (1H, m, -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP), 5.38 (4H, m, C5', C6', C8', C9'-H), 5.40 (1H, m, C11'-H), 5.64 (1H, m, C14'-H), 6.03 (1H, t, C12'-H), 6.51 (1H, m, C13'-H), FAB-MS; *m/z* 824 (M-H)<sup>-</sup>.

**1-Stearoyl-2-[13'-(S)-hydroperoxy-(9'Z, 11'E)-octadecadienyl]-*sn*-glycero-3-phosphoglycerol (**14**).**

The protective form of the compound (**7**), 1-

stearoyl-2-[13'-(S)-(1''-methoxy-1''-methylethylperoxy)-(9'Z, 11'E)-octadecadienyl]-*sn*-glycero-3-phosphocholine (50 mg, 0.056 mmol), in Et<sub>2</sub>O (3.5 ml), and 0.1 M acetate buffer [(pH 5.6, containing of 2-aminoethanol (1.65 g, 27.0 mmol)) was treated with phospholipase D (1 mg, 153 U/mg, from *Streptomyces sp.* AA 586) in the presence of a trace amount of BHT at 30 °C for 4 h under nitrogen atmosphere. The reaction solution was extracted with the mixture of CHCl<sub>3</sub> and MeOH (5 : 8). After removal of organic solvent from the CHCl<sub>3</sub> phase, the residue was chromatographed on silica gel with 60 : 30 : 1 CHCl<sub>3</sub>-MeOH-28% NH<sub>4</sub>OH as eluent to provide peracetal phosphatidylglycerol hydroperoxide. The peracetal hydroperoxide was deprotected by treating with a mixture of THF, AcOH, and water (4 : 2 : 1) in the presence of a trace amount of BHT. After removal of organic solvent, the residue was chromatographed on silica gel with 60 : 30 : 1 CHCl<sub>3</sub>-MeOH-28% NH<sub>4</sub>OH as eluent to provide hydroperoxy phosphatidylglycerol (**14**) (40 mg, 81% yield). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 0.88 (6H, m, w-CH<sub>3</sub> x 2), 1.28 (44H, m, CH<sub>2</sub> x 22), 1.57 (4H, m, OCOCH<sub>2</sub>CH<sub>2</sub> in each chain), 2.17 (2H, m, C8'-H), 2.28 (4H, m, OCOCH<sub>2</sub> in each chain), 3.62 (2H, m, PO-CH<sub>2</sub>-CHOH-CH<sub>2</sub>), 3.90 (5H, m, CH<sub>2</sub>OP x 2, CHOH), 4.15 (1H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>O-), 4.35 (2H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>O-, C13'-H), 5.22 (1H, br, CHOCO), 5.46 (1H, m, C9'-H), 5.58 (1H, m, C12'-H), 6.00 (1H, m, C10'-H), 6.54 (1H, m, C11'-H). FAB MASS : *m/z* 805 (M-H)<sup>-</sup>. [α]<sub>D</sub><sup>25</sup> = +3.76° (c = 1.07 in MeOH/CHCl<sub>3</sub> = 1 : 1 v/v).

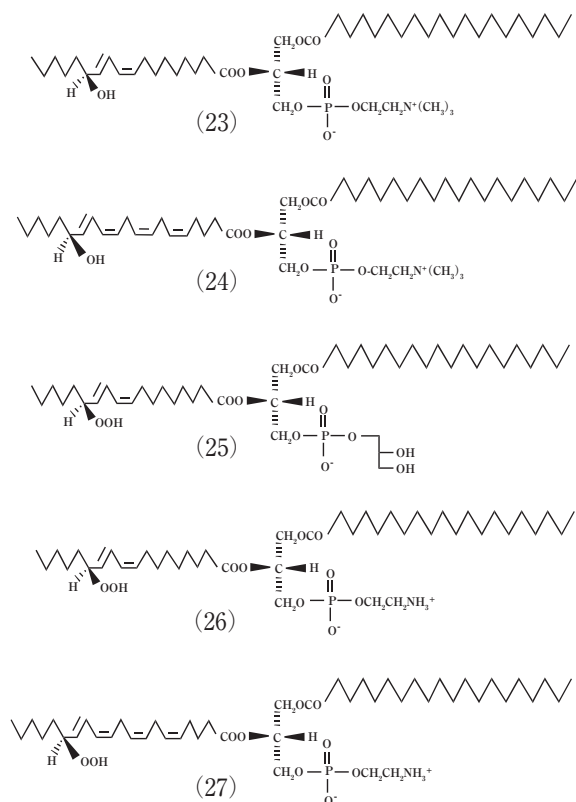
**1-Stearoyl-2-[13'-(S)-hydroperoxy-(9'Z, 11'E)-octadecadienyl]-*sn*-glycero-3-phosphoethanolamine (**15**).**

The protective form of the compound (**7**), 1-stearoyl-2-[13'-(S)-(1''-methoxy-1''-methylethylperoxy)-(9'Z, 11'E)-octadecadienyl]-*sn*-glycero-3-phosphocholine (50 mg, 0.056 mmol), in Et<sub>2</sub>O (2 ml), and 0.1 M acetate buffer (pH 5.6, containing of 10<sup>-3</sup> M 2-aminoethanol) was treated with phospholipase D (1 mg, 153 U/mg, from *Streptomyces sp.* AA 586) in the presence of a trace amount of BHT at room temp. for 6 hours under nitrogen atmosphere. The reaction solution was extracted with a mixture of CHCl<sub>3</sub> and MeOH (4 : 1). After removal of organic solvent from the CHCl<sub>3</sub> phase, the residue was chromatographed on silica gel with 60 : 30 : 1.5 CHCl<sub>3</sub>-MeOH-28% NH<sub>4</sub>OH as eluent to provide peracetal phosphoethanolamine hydroperoxide. The peracetal hydroperoxide was deprotected by treating with a mixture of THF, AcOH, and water (4 : 2 : 1) in the presence of a trace amount of BHT.

After removal of organic solvent, the residue was chromatographed on reverse-phase column chromatography (RP-8 Rober column) using a mixture of  $\text{CHCl}_3$ , MeOH, and water (1 : 10 : 1) as eluent to give the phosphatidylethanolamine hydroperoxide (**17**) (24.6 mg, 51.7% yield). TLC  $R_f = 0.53$  ( $\text{CHCl}_3$  : MeOH : 28%  $\text{NH}_4\text{OH} = 60 : 30 : 3$ ).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  0.88 (6H, m,  $\omega$ -Me x 2), 1.24 (44H, m,  $\text{CH}_2$  x 22), 1.58 (4H, m,  $\text{OCOCH}_2\text{CH}_2$  in each chain), 2.18 (2H, m,  $\text{C8}'\text{-H}$ ), 2.28 (4H, m,  $\text{OCOCH}_2$  in each chain), 3.13 (2H, m,  $\text{CH}_2\text{NH}_3$ ), 3.94 (2H, m,  $-\text{OCH}_2-\text{CH}-\text{CH}_2\text{O}-$ ), 4.07 (2H, m,  $\text{OCH}_2\text{CH}_2\text{NH}_3$ ), 4.14 (1H, m, one proton of  $-\text{OCH}_2-\text{CH}-\text{CH}_2\text{O}-$ ), 4.36 (2H, m, one proton of  $-\text{OCH}_2-\text{CH}-\text{CH}_2\text{O}-$ ,  $\text{C13}'\text{-H}$ ), 5.21 (1H, m,  $\text{CHOCO}$ ), 5.46 (1H, m,  $\text{C9}'\text{-H}$ ), 5.72 (1H, m,  $\text{C12}'\text{-H}$ ), 6.00 (1H, dd,  $\text{C10}'\text{-H}$ ), 6.53 (1H, m,  $\text{C11}'\text{-H}$ ). FAB MAS :  $m/z$  775 ( $\text{M}+\text{H}$ ) $^+$ .

**1-Stearoyl-2-[15'-(S)-hydroperoxy-(5'Z, 8'Z, 11'Z, 13'E)-icosatetraenoyl]-sn-glycero-3-phosphoethanolamine (16).**

To prepare the compound (**16**), the peracetal form of the compound (**9**), 1-stearoyl-2-[15'-(S)-(1''-methoxy-1''-methylethylperoxy)-(5'Z, 8'Z, 11'Z, 13'E)-icosatetraenoyl]-sn-glycero-3-phosphocholine, was

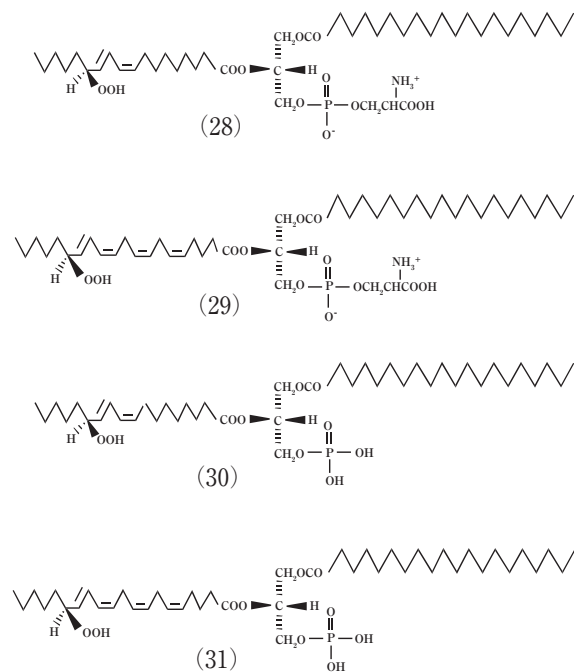


Scheme 5. Chemical Structure of Phospholipid Hydroperoxides (23) - (27) .

employed as a substrate for transphosphatidylation by the phospholipase D. The other reaction conditions and methods were the same that as employed for the synthesis of the phosphatidylethanolamine hydroperoxide (**15**) (68.0% yield). TLC  $R_f = 0.5$  ( $\text{CHCl}_2$  : MeOH : 28%  $\text{NH}_4\text{OH} = 60 : 30 : 1$ ).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  0.88 (6H, t,  $\omega$ - $\text{CH}_3$  x 2), 1.24 (28H, m,  $1-\text{CH}_2$  x 14), 1.29 (8H, m,  $2-\text{CH}_2$  x 4), 1.71 (2H, m,  $2-\text{OCOCH}_2\text{CH}_2\text{CH}_2$ ), 2.13 (2H, m,  $2-\text{OCOCH}_2\text{CH}_2\text{CH}_2$ ), 2.38 (2H, t,  $2-\text{OCOCH}_2\text{CH}_2\text{CH}_2$ ), 2.80 (2H, t,  $\text{C7}'\text{-CH}_2$ ), 2.96 (2H, t,  $\text{C10}'\text{-CH}_2$ ), 3.16 (2H, m,  $-\text{OCH}_2\text{CH}_2\text{N}$ ), 3.94 (2H, m,  $\text{CH}_2\text{OP}$ ), 4.06 (2H, m,  $\text{OCH}_2\text{CH}_2\text{N}$ ), 4.12 (1H, m, one proton of  $-\text{OCH}_2-\text{CH}-\text{CH}_2\text{OP}$ ), 4.35 (1H, m, one proton of  $-\text{OCH}_2-\text{CH}-\text{CH}_2\text{OP}$ ), 4.38 (1H, m,  $\text{C15}'\text{-H}$ ), 5.22 (1H, m,  $-\text{OCH}_2-\text{CH}-\text{CH}_2\text{OP}$ ), 5.38 (4H, m,  $\text{C5}'$ ,  $\text{C6}'$ ,  $\text{C8}'$ ,  $\text{C9}'\text{-H}$ ), 5.40 (1H, m,  $\text{C11}'\text{-H}$ ), 5.62 (1H, m,  $\text{C14}'\text{-H}$ ), 6.01 (1H, t,  $\text{C12}'\text{-H}$ ), 6.56 (1H, m,  $\text{C13}'\text{-H}$ ) ; 8.20 (3H, b,  $\text{NH}_3$ ). FAB-MS :  $m/z$  798 ( $\text{M}-\text{H}$ ) $^-$ .

**1-Stearoyl-2-[13'-(S)-hydroperoxy-(9'Z, 11'E)-octadecadienoyl]-sn-glycero-3-phosphoserine (17).**

The phospholipid hydroperoxide (**7**) (39mg, 0.048 mmol) in  $\text{Et}_2\text{O}$  (3.5 ml) and 0.1 M acetate buffer [pH 6.5, 3.5 ml containing of serine (783mg, 7.46 mmol)] was treated with phospholipase D (1 mg, 153 U/mg, from *Streptomyces sp.* AA 586) in the presence of a trace amount of BHT at 30 °C for 3 hours under nitrogen atmosphere. The reaction solution was extracted with the mixture of  $\text{CHCl}_3$  and MeOH (5 : 8). After



Scheme 6. Chemical Structure of Phospholipid Hydroperoxides (28) - (31) .

removal of organic solvent from the  $\text{CHCl}_3$  phase, the residue was chromatographed on CM-cellulose column with 85:15  $\text{CHCl}_3$ -MeOH as eluent to provide hydroperoxy-phosphatidylserine (**17**) (13mg, 15.9 mmol, 33% yield). TLC:  $R_f = 0.24$  ( $\text{CHCl}_3$ : MeOH: 28%  $\text{NH}_4\text{OH} = 65:25:4$ ).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  0.88 (6H, m,  $\omega$ -Me x 2), 1.28 (44H, m,  $\text{CH}_2$  x 22), 1.57 (4H, m,  $\text{OCOCH}_2\text{CH}_2$  in each chain), 2.18 (2H, m,  $\text{C8}'\text{-H}$ ), 2.30 (4H, m,  $\text{OCOCH}_2$  in each chain), 3.7-4.2 (6H, br,  $-\text{CH}_2\text{-CH-CH}_2-$ , one proton of  $-\text{OCH}_2\text{-CH-CH}_2\text{OP-}$ ,  $\text{POCH}_2\text{CHCOO}$ ), 4.39 (2H, m, one proton of  $-\text{OCH}_2\text{-CH-CH}_2\text{OP-}$ ,  $\text{C13}'\text{-H}$ ), 5.21 (1H, br,  $\text{CHOCO}$ ), 5.45 (1H, m,  $\text{C9}'\text{-H}$ ), 5.56 (1H, dd,  $\text{C12}'\text{-H}$ ), 6.00 (1H, t,  $\text{C10}'\text{-H}$ ), 6.53 (1H, dd,  $\text{C11}'\text{-H}$ ).  $[\alpha]_D^{25} = +5.34^\circ$  ( $c = 1.16$  in MeOH/ $\text{CHCl}_3 = 1:1$  v/v), FAB-MS;  $m/z$  818 ( $\text{M-H}$ ) $^-$ .

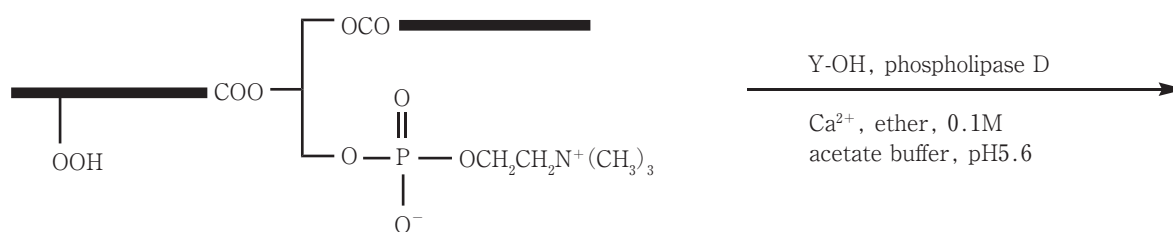
**1-Stearoyl-2-[15'-(S)-hydroperoxy-(5'Z, 8'Z, 11'Z, 13'E)-icosatetraenoyl]-sn-glycerol-3-phosphoserine (18):**

To prepare the compound (**18**), the hydroperoxy phosphatidylcholine (**9**) (76.1mg, 0.09 mmol) was employed as a substrate for transphosphatidylation by the phospholipase D. The other reaction conditions and methods were the same that as employed for the synthesis of the phosphatidylserine hydroperoxide (**17**) (37.2mg, 0.05 mmol, 55.6% yield). TLC:  $R_f = 0.24$  ( $\text{CHCl}_3$ : MeOH: 28%  $\text{NH}_4\text{OH} = 65:25:4$ ).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  0.88 (6H, m,  $\omega$ -Me x 2), 1.28 (44H, m,  $\text{CH}_2$  x 22), 1.57 (4H, m,  $\text{OCOCH}_2\text{CH}_2$  in

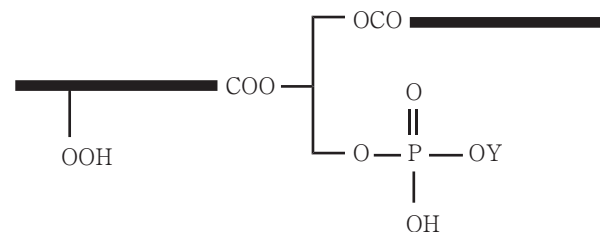
each chain), 2.18 (2H, m,  $\text{C8}'\text{-H}$ ), 2.30 (4H, m,  $\text{OCOCH}_2$  in each chain), 3.7-4.2 (6H, br,  $-\text{CH}_2\text{-CH-CH}_2-$ , one proton of  $-\text{OCH}_2\text{-CH-CH}_2\text{O-}$ ,  $-\text{POCH}_2\text{CHCOO}$ ), 4.39 (2H, m, one proton of  $-\text{OCH}_2\text{-CH-CH}_2\text{OP-}$ ,  $\text{C13}'\text{-H}$ ), 5.22 (1H, br,  $-\text{OCH}_2\text{-CH-CH}_2\text{O-}$ ), 5.38 (5H, m,  $\text{C5}'$ ,  $\text{C6}'$ ,  $\text{C8}'$ ,  $\text{C9}'$ ,  $\text{C11}'\text{-H}$ ), 5.61 (1H, dd,  $\text{C14}'\text{-H}$ ), 6.02 (1H, t,  $\text{C12}'\text{-H}$ ), 6.56 (1H, dd,  $\text{C13}'\text{-H}$ ) 8.10 (3H, br,  $\text{NH}_3$ ), FAB-MS;  $m/z$  842 ( $\text{M-H}$ ) $^-$ .

**1-Stearoyl-2-[13'-(S)-hydroperoxy-(9'Z, 11'E)-octadecadienoyl]-sn-glycerol-3-phosphatidic acid (19):**

A solution of the peroxide (**7**) (115mg, 0.13 mmol) in a mixture,  $\text{Et}_2\text{O}$  (5 mL)/acetate buffer (2 mL, 0.1 M, pH 5.6, containing 0.1 M  $\text{CaCl}_2$ ) was incubated with phospholipase D [60mg/ 5 ml (water), 1.5 units, from cabbage] at 37 °C. The reaction mixture was extracted with  $\text{Et}_2\text{O}$ , and solvent was removed under reduced pressure. The residue was chromatographed on silica gel with  $\text{CHCl}_3$ /MeOH/28%  $\text{NH}_4\text{OH}$  (65:25:5) to provide protective form of compound (**19**). The obtained phospholipid was treated with a mixture of THF-AcOH- $\text{H}_2\text{O}$  (4:2:1) in the presence of a trace amount of BHT at room temp. in nitrogen atmosphere. After removal of organic solvent, the residue was chromatographed on silica gel column with 65:25:7  $\text{CHCl}_3$ -MeOH-28%  $\text{NH}_4\text{OH}$  as eluent to give the hydroperoxy phosphatidic acid (**19**). (49.1% yield) TLC:  $R_f = 0.05$  ( $\text{CHCl}_3$ : MeOH: 28%  $\text{NH}_4\text{OH} = 65:25:5$ ).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  0.87 (6H, t,  $\text{CH}_3$  x 2),



Y-OH: ethanolamine, glycerol, serine



Scheme 7. Synthesis of Hydroperoxide of Phosphatidylethanolamide, Phosphatidylglycerol and Phosphatidylserine by Phospholipase D-Catalyzed Transphosphatidylation.

1.25 (44H, m, CH<sub>2</sub> x 22), 1.56 (4H, m, OCOCH<sub>2</sub>CH<sub>2</sub>), 2.16 (2H, m, C8'-H), 2.27 (4H, m, OCOCH<sub>2</sub>), 3.94 (2H, m, -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP-), 4.12 (1H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP-), 4.35 (2H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP-, C13'-H), 5.19 (1H, m, -OCH<sub>2</sub>-CH-CH<sub>2</sub>O-), 5.43 (1H, m, C9'-H), 5.60 (1H, m, C12'-H), 5.98 (1H, m, C10'-H), 6.47 (1H, m, C11'-H).

**1-Stearoyl-2-[15'-(S)-hydroperoxy-(5'Z, 8'Z, 11'Z, 13'E)-icosatetraenoyl]-sn-glycero-3-phosphatidic acid (20) :**

To prepare the compound (20), the peracetal form of the compound (9), 1-stearoyl-2-[15'-(S)-(1''-methoxy-1''-methylperoxy)-(5'Z, 8'Z, 11'Z, 13'E)-icosatetraenoyl]-sn-glycero-3-phosphocholine, was employed as a substrate for the hydrolysis by the phospholipase D. The other reaction conditions and methods were the same that as employed for the synthesis of the phosphatidylethanolamine hydroperoxide (19). (49% yield) TLC : R<sub>f</sub> = 0.05 (CHCl<sub>3</sub> : MeOH : 28% NH<sub>4</sub>OH = 65 : 25 : 5). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 0.87 (6H, t, CH<sub>3</sub> x 2), 1.25 (44H, m, CH<sub>2</sub> x 22), 1.56 (2H, m, 1-OCOCH<sub>2</sub>CH<sub>2</sub>), 1.66 (2-OCOCH<sub>2</sub>CH<sub>2</sub>), 2.27 (2H, t, 1-OCOCH<sub>2</sub>), 2.32 (2H, t, 2-OCOCH<sub>2</sub>), 3.94 (2H, m, -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP-), 4.12 (1H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP-), 4.35 (2H, m, one proton of -OCH<sub>2</sub>-CH-CH<sub>2</sub>OP-, C15'-H), 5.24 (1H, m, -OCH<sub>2</sub>-CH-CH<sub>2</sub>O-), 5.38 (5H, m, C5', C6', C8', C9', C11'-H), 5.60 (1H, dd, C14'-H), 6.00 (1H, t, C12'-H), 6.50 (1H, dd, C13'-H).

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## リン脂質過酸化物の生物有機化学的合成

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健康正常人の血液などの組織中にはリン脂質過酸化物が極微量で存在し、疾病や老化によってその濃度が顕著に上昇する事が知られている。その事が明らかにされた当初は、極めて複雑な混合物をなす生体脂質中に含まれる不安定な極微量の過酸化脂質を単離・構造決定する事は殆ど不可能と考えられていた。

現在もなお、そのような脂質過酸化物を生体組織から純粋に取り出し、構造決定したという報告は無い。従ってそのような分子種の化学的・生理学的性質は不明であったが、脂肪酸過酸化物が毒性を示す事から、リン脂質過酸化物もおそらく毒性を示すだろうと考えられてきた。このような漠然とした推定を科学的に明らかにするためには、化学合成によらざるを得ない。我々はこの未知の合成に取りかかった。しかし、従来の化学的手法のみでは不可能である事も明らかであった。その中で予想された困難の一つは極めて不安定なヒドロペルオキシ基を不飽和脂肪酸のある特定の位置にどのように導入するかという問題と、ヒドロペルオキシ基に影響を与える事なく合成中間体をどのように化学変換するかであった。第一の問題に対する解決策として、不飽和脂肪酸に大豆リポキシゲナーゼを作用させる事で解決する事ができた。植物に広く分布する酵素であるリポキシゲナーゼは植物中でリノール酸に作用して過酸化し、その生成物にヒドロペルオキシドリアーゼという酵素が作用して種々のアルデヒドが精製し、これは植物の青臭みを与える。第二の問題に対しては、リノール酸に導入された不安定なヒドロペルオキシ基をパーアセタールとしての保護する事により解決した。この保護基は、中間体から最終生成物に至るまでの反応条件、例えばDCCによるアシル化反応に対して安定である事が明らかとなった。この二つの問題を解決する事によって、リン脂質過酸化物の一つであるホスファチジルコリン過酸化物を世界に先駆けて成功した。さらにこのホスファチジルコリン過酸化物に微生物由来のホスホリパーゼDを作用させる事によってホスファチジルエタノールアミン過酸化物、ホスファチジルセリン過酸化物やホスファチジルグリセロール過酸化物の合成にも成功した。また、トリグリセリド過酸化物の合成も可能になった。これらの脂質過酸化物が化学的に実態のあるものとして認識されてから、その生理作用に関する研究が広範に行われている。

しかし、生体組織に存在するリン脂質過酸化物の生理学的役割は依然として明らかになっていない。ある種のリン脂質過酸化物が動物の免疫系を活性化するという報告もあり、必ずしも生体に対して悪い作用をするばかりではないようである。

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