

**REACTIONS OF 1-STEAROYL-2-(13'-OXO-9',11'-TRIDECADIENOYL)-SN-GLYCERO-3-PHOSPHOCHOLINE WITH AMINO ACIDS AND PEPTIDES AND ITS DIFFERENTIAL GENERATION FROM HYDROPEROXIDES OF 1-STEAROYL-2- $\alpha$ -LINOLENOYL-SN-GLYCERO-3-PHOSPHOCHOLINE AND 1-STEAROYL-2-LINOLEOYL-SN-GLYCERO-3-PHOSPHOCHOLINE**

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**ABSTRACT.** Phosphatidylcholines (PCs) bearing various kinds of aldehydic acyl chains at the *sn*-2 position have been detected in atherosclerotic tissues. However, 1-acyl-2-(13'-oxo-9',11'-tridecadienoyl)-*sn*-glycero-3-phosphocholine and other  $\alpha,\beta,\gamma,\delta$ -unsaturated aldehyde PCs have not. To determine whether this might be due to their high chemical reactivity with biomolecules, we investigated the reactions of 1-stearoyl-2-(13'-oxo-9',11'-tridecadienoyl)-*sn*-glycero-3-phosphocholine (OTDA-PC, where OTDA refers to the oxo-tridecadienoyl moiety) with nucleophilic amino acids and peptides by means of electrospray mass spectroscopy. OTDA-PC formed Michael adducts with lysine, arginine, histidine, hippuryl lysine and hippuryl arginine, but was surprisingly unreactive with cysteine or glutathione. When 1-stearoyl-2-(13'-hydroperoxy-9'Z,11'E,15'Z-octadecadienoyl)-*sn*-glycero-3-phosphocholine (PC-LNA-OOH, where LNA-OOH denotes the linolenic acid hydroperoxide moiety) was decomposed in the presence of the reactive lysine, OTDA-PC was still detected as a major product. However, OTDA-PC could not be detected when 1-stearoyl-2-(13'-hydroperoxy-9'Z,11'E-octadecadienoyl)-*sn*-glycero-3-phosphocholine (PC-LA-OOH, where LA-OOH refers to linoleic acid hydroperoxide) was decomposed in the presence or absence of lysine. Since linoleic acid is the major polyunsaturated fatty acid in atherosclerotic tissues, these results indicate that formation of OTDA-PC in only minor amounts in such tissues may explain its not having been detected in them. Surprisingly, 1-stearoyl-2-(9'-oxononanoyl)-*sn*-glycero-3-phosphocholine was the major aldehydic product of the decomposition of PC-LA-OOH under anaerobic conditions.

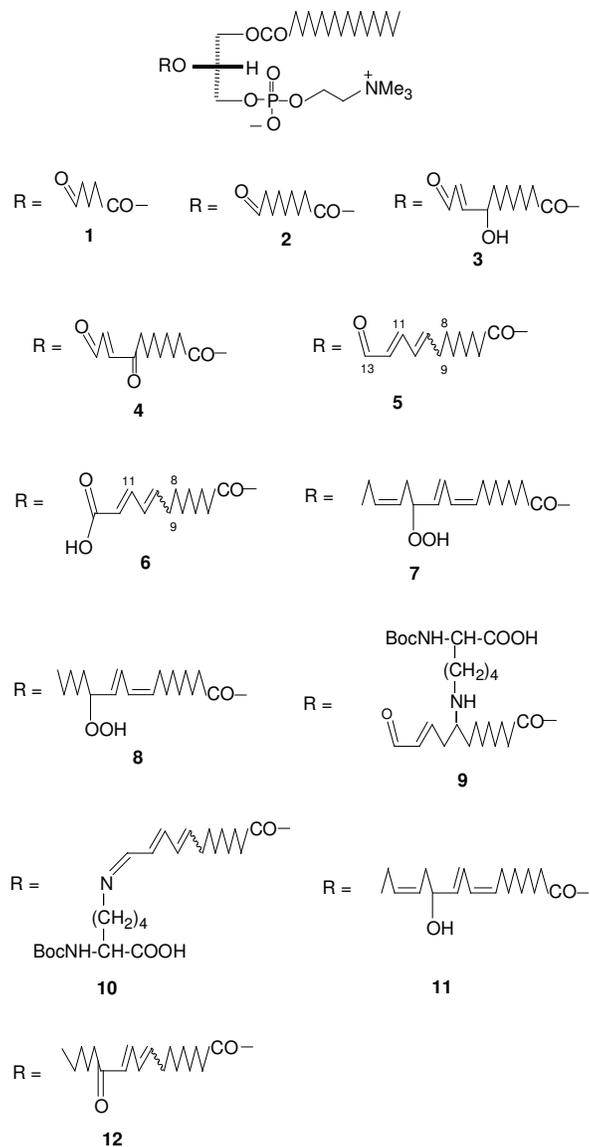
**KEY WORDS:** Lipid oxidation, Bioactive phospholipid aldehydes (core aldehydes), Michael addition

## INTRODUCTION

Recently, the role of phospholipids bearing aldehydic acyl chains (the so called core aldehydes) in the development of diseases has received much attention. Phosphatidylcholine (PC) core aldehydes that have been isolated from atherosclerotic lesions and shown to be proatherogenic include saturated ones such as 1-stearoyl-2-(5'-oxovaleroyl)-*sn*-glycero-3-phosphocholine **1** and 1-stearoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine **2** [1] as well as some PCs having  $\gamma$ -hydroxy (or oxo)- $\alpha,\beta$ -unsaturated aldehyde terminals, such as **3** and **4** [2]. However, those having  $\alpha,\beta,\gamma,\delta$ -unsaturation, like 1-stearoyl-2-(13'-oxo-9',11'-tridecadienoyl)-*sn*-glycero-3-phosphocholine (OTDA-PC **5**), have not been detected. We previously reported that OTDA-PC **5** was very prone to oxidation (for example to 1-stearoyl-2-(13'-carboxy-9',11'-tridecadienoyl)-*sn*-glycero-3-phosphocholine **6** if not isolated from other products of PC hydroperoxide decomposition [3]. It has also been postulated that OTDA-PC **5** may be highly reactive with biomolecules such as proteins [4]. If the latter type of reactions is important in hindering the detection of OTDA-PC **5** in biological tissues, this compound should be highly relevant in the development of diseases. Here we determined, by electrospray mass spectroscopy, (i) the relative reactivity of OTDA-PC **5** with various nucleophilic amino acids and peptides and (ii)

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the ability of such reactions and/or oxidation to prevent detection of OTDA-PC **5** generated from the decomposition of 1-stearoyl-2-(13'-hydroperoxy-9'Z,11'E,15' Z-octadecatrienoyl)-*sn*-glycero-3-phosphocholine (PC-LNA-OOH **7**) or 1-stearoyl-2-(13'-hydroperoxy-9'Z,11'E-octadecadienoyl)-*sn*-3-phosphocholine (PC-LA-OOH **8**) at physiological temperature. Here LNA and LA denote  $\alpha$ -linolenic and linoleic acids, respectively.



Scheme 1. Structures of oxidatively modified phosphatidylcholines mentioned in the text.

## EXPERIMENTAL

### *Materials*

OTDA-PC **5**, PC-LNA-OOH **7** and PC-LA-OOH **8** were synthesized according to our previously reported methods *via* soybean lipoxygenase-catalysed oxidation of  $\alpha$ -linolenic acid (for **5** and **7**) or linoleic acid [5, 6]. N-acetyl cysteine and glutathione (reduced form) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), N- $\alpha$ -(tert-butoxycarbonyl)-L-lysine ( $\alpha$ -Boc-lysine) and N- $\alpha$ -benzoyl-L-arginine ( $\alpha$ -benzoyl arginine) were obtained from Tokyo Kasei Co. (Tokyo, Japan), N- $\alpha$ -(tert-butoxycarbonyl)-L-histidine ( $\alpha$ -Boc-histidine) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, USA), and hippuryl lysine and hippuryl arginine were obtained from Peptides Institute Inc. (Kyoto, Japan).

### *Reactions of OTDA-PC (5) with amino acids and peptides*

OTDA-PC (1 mg, 1.4  $\mu$ mol) was reacted with 3 molar equivalents of the amino acids or peptides in 1 mL of 0.1 M phosphate buffer (pH 7.0 or 7.4) at 37 °C under a nitrogen atmosphere. For N-acetyl-cysteine and glutathione, additional reactions were conducted at pH 8.0 and 9.0. Aliquots were taken from the reaction mixtures after 1, 6 or 24 hours and analysed by electrospray mass spectroscopy (ES MS).

### *Decomposition of PC-LNA-OOH (7) in the presence of lysine*

PC-LNA-OOH **7** (1 mg, 1.2  $\mu$ mol) was dissolved in 1 mL of 0.1 M phosphate buffer (pH 7.0 or 7.4) containing FeSO<sub>4</sub>·7H<sub>2</sub>O (0.5 mg, 1.8  $\mu$ mol) and Boc-lysine (3 mg, 12.2  $\mu$ mol). The reaction mixture was stirred at 37 °C under a nitrogen atmosphere for 1 hour, after which an aliquot was analysed by ES MS.

### *Decomposition of PC-LA-OOH (8)*

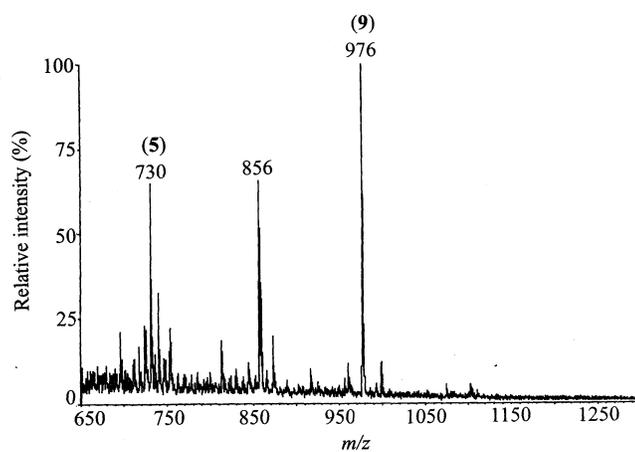
PC-LA-OOH **8** (6 mg, 7.3  $\mu$ mol) was dissolved in 12 mL of ethanol containing FeSO<sub>4</sub>·7H<sub>2</sub>O (0.5 mg, 1.8  $\mu$ mol). The reaction mixture was stirred in the dark under a nitrogen atmosphere for 1 hour. An aliquot of the reaction mixture was taken for ES MS analysis.

### *ES MS analysis*

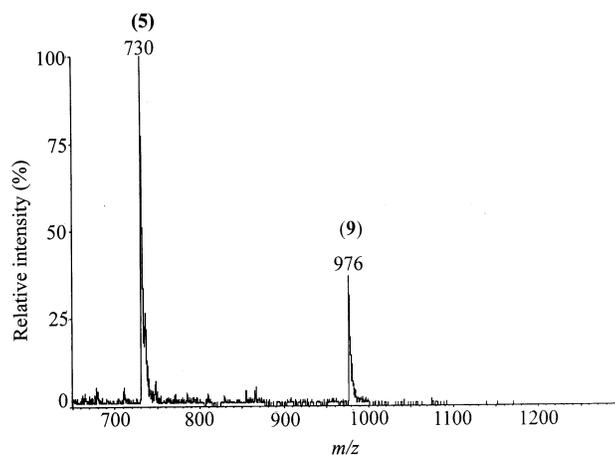
ES MS analysis was performed on an API III triple quadrupole mass spectrometer (PE-Sciex; Thorn Hill, ON, Canada) equipped with an electrospray interface. Aliquots taken from the decomposition experiments were diluted with THF-MeOH-H<sub>2</sub>O to less than 50 ppm and introduced directly into the spectrometer with a microsyringe (250  $\mu$ L) through an infusion pump and a fused silica capillary tubing (0.25 mm diameter) at a rate of 5  $\mu$ L/min. Spectra were acquired in the positive ion mode with the orifice at 70 V. Both normal scans for protonated molecular ion peaks and scans for the parents of  $m/z$  184 (corresponding to the phosphocholine ion characteristic to all PCs) were done.

**RESULTS AND DISCUSSION**

OTDA-PC **5** may be expected to react with nucleophilic compounds by formation of Michael adducts or Schiff's bases (such as compounds **9** and **10**, respectively). Figure 1A shows a typical ES MS spectrum obtained after reaction of OTDA-PC **5** with  $\alpha$ -Boc-lysine for 6 hours. The peak at  $m/z$  730 corresponds to unreacted OTDA-PC **5** while the prominent peak at  $m/z$  976 corresponds to the Michael adduct **9**. These assignments were confirmed by analysis in the parent ion scan mode for the parents of  $m/z$  184, which corresponds to the phosphocholine ion (Figure 1B).



1A



1B

Figure 1. ES MS spectra obtained after reaction of OTDA-PC **5** with 3 equivalents of  $\alpha$ -Boc-lysine in phosphate buffer (pH 7.4) at 37 °C for 6 hours. A, normal scan. B, parent ion scan mode (parents of  $m/z$  184). Numbers in bold refer to the assigned compounds

Table 1 shows a comparison of the relative intensities of peaks for OTDA-PC **5** with those for its adducts with the various compounds after reaction for 6 hours. These results indicate that OTDA-PC reacted at varying rates with amino acids or peptides having a free amino group in the R-chain, but not with the thiol-containing compounds, cysteine or glutathione. OTDA-PC's failure to react with cysteine and glutathione was contrary to the prediction that it should have similar adduct formation chemistry as the highly bioactive 2,4-decadienal, which rapidly reacts with cysteine and glutathione [4]. This finding also shows OTDA-PC **5** to be unlike 4-hydroxy-2-nonenal and 4-oxo-2-nonenal, two highly bioactive  $\alpha$ - $\beta$ -unsaturated aldehydic lipid peroxidation products which are more reactive with cysteine than with lysine, histidine and arginine [7].

Table 1. Comparison of relative intensities (RI) for ES-MS peaks of unreacted OTDA-PC **5** with those of OTDA-PC-amino acid/peptide adducts. OTDA-PC **5** was reacted with 3 molar equivalents of the amino acids or peptides at 37 °C in phosphate buffer (pH 7.4) for 6 hours.

Amino acid/peptide	RI (%) of OTDA-PC	RI (%) of adduct
$\alpha$ -Boc-lysine	68	100
$\alpha$ -Benzoyl arginine	65	100
$\alpha$ -Boc-histidine	100	62
Hippuryl lysine	100	14
Hippuryl arginine	100	15
N-acetyl cysteine*	100	Negligible
Glutathione*	100	Negligible

\* These compounds were not reactive even at pH 8 or 9 which would favour formation of the thiolate anion.

In order to mimic the generation of OTDA-PC **5** in tissues and its possible depletion by adduct formation, we decomposed PC-LNA-OOH **7** in the presence of lysine under anaerobic conditions. Even when the decomposition was done in the presence of a large excess of lysine, OTDA-PC was still detected in greater relative intensity than OTDA-PC- $\alpha$ -Boc lysine adduct **9** (Figure 2). This indicated that adduct formation with nucleophiles may not be fast enough to prevent the detection of OTDA-PC **5** generated from PC-LNA-OOH **7**. We previously found that under anaerobic conditions for the decomposition of PC-LNA-OOH **7**, OTDA-PC **5** formed thereby was considerably oxidized to acid PC **6** (under aerobic conditions, various products are formed) [3]. However, lack of a significant peak for acid PC **6** ( $m/z$  746) in Figure 2 indicates that OTDA-PC **5** was resistant to oxidation in the presence of excess lysine. The reason for this is not clear.

In mammalian atherosclerotic tissues, the source of OTDA-PC **5** would be PC-LA-OOH **8** rather than PC-LNA-OOH **7** since in these tissues, linoleic acid is the major unsaturated fatty acid [8]. We previously explained that generation of OTDA-PC **5** from the former is expected to be less facile than from the latter due to differences in the kinds of carbon-centred radicals formed upon  $\beta$ -scission of their respective alkoxy radicals. We therefore felt that this difference might partly explain why OTDA-PC **5** has not been detected in atherosclerotic tissues although we have not previously determined how little is the amount of OTDA-PC **5** formed from PC-LA-OOH **8**. When we decomposed PC-LA-OOH **8** anaerobically for 1 hour, OTDA-PC **5** ( $m/z$  730) was not detected among the decomposition products (Figure 3). However, a small amount of its oxidation product, acid PC **6** was detected ( $m/z$  746). The complete oxidation of the little OTDA-PC **5** produced here might be explained as follows; since the first step in the oxidation of OTDA-PC **5** involves radical abstraction of the carbonyl hydrogen [3], rapid depletion is expected when the ratio of OTDA-PC **5** to radicals (such as alkoxy, peroxy and carbon-centered radicals) is low.

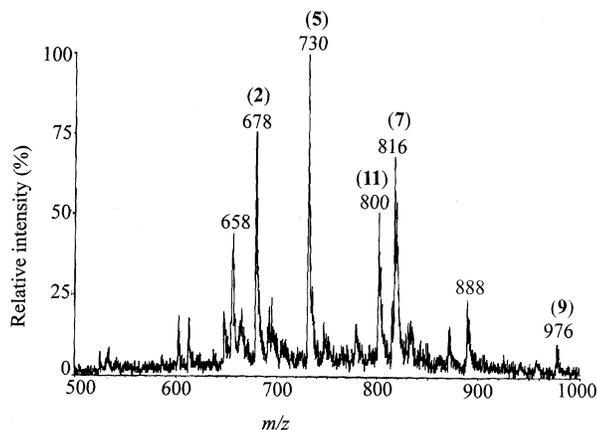


Figure 2. ES MS spectrum (parent ion scan mode) obtained after decomposition of PC-LNA-OOH **7** in the presence of 10 molar equivalents of  $\alpha$ -Boc-lysine in phosphate buffer (pH 7.4) at 37 °C for 1 hour. In the normal scan mode (not shown) OTDA-PC **5** and Michael adduct **9** had 100 % and 70 % RI's, respectively. Structural assignments for the compounds were based not only on their molecular ion peaks but also on chemical derivatization and GC-MS analysis as described previously [3].

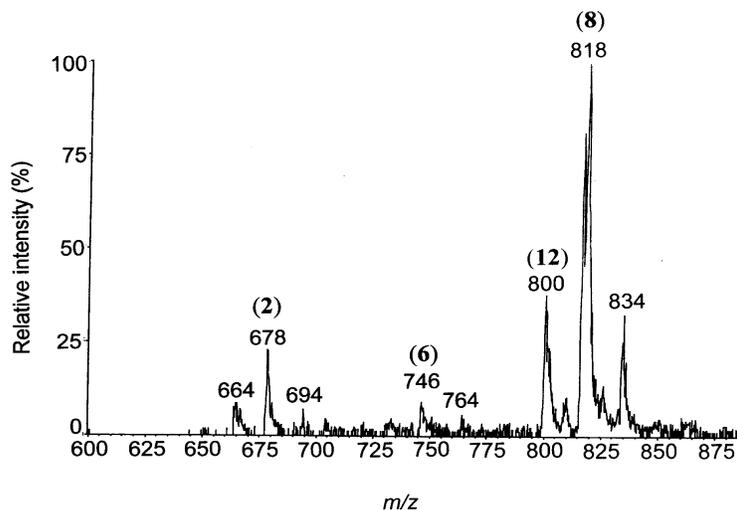
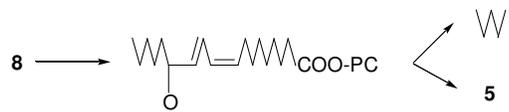


Figure 3. ES MS spectrum of the products of the decomposition of PC-LA-OOH **8** in the presence of  $\text{Fe}^{2+}$  at 37 °C for 1 hour in ethanol under anaerobic conditions.

Although  $\beta$ -scission of the alkoxy radical derived from PC-LA-OOH **7** is expected to primarily generate OTDA-PC **5** (Scheme 2), 9-oxononanoyl PC **2** was formed as the major aldehydic PC (Figure 3).



Scheme 2. Conversion of PC-LA-OOH **8** to OTDA-PC **5** via  $\beta$ -scission of an alkoxy radical

Spiteller and others previously found similar results that 9-oxononanoic acid rather than 13-oxo-9,11-tridecadienoic acid (OTDA) was the main aldehydic product of the decomposition of the 13-hydroperoxide of linoleic acid [8]. They explained that 13-oxo-9,11-tridecadienoic acid was formed first, but that it was converted to 9-oxononanoic acid by Michael addition of water to form 9-hydroxy-13-oxo-11-tridecenoic acid, followed by a retroaldol reaction. However, we have since found that pure OTDA-PC **5** was very stable against such Michael addition of water [3]. The involvement of Michael addition of water by OTDA-PC **5** in the formation of ONA-PC **2** from PC-LA-OOH **8** under anaerobic conditions in the present study is further doubtful because (i) decomposition of PC-LA-OOH **8** was done in ethanol and (ii) Michael addition of water did not prevent detection of OTDA-PC **5** generated from PC-LNA-OOH **7** (Figure 2). Our previously proposed oxidative shortening of the *sn*-2 chain of OTDA-PC **5** via  $\alpha$ -peroxylactone intermediates [3] may also not be relevant here because those degradative reactions require oxygen. Although isomerization of the hydroperoxy group of PC-LA-OOH **8** from C-13 to C-9 prior to decomposition might generate some ONA-PC **2**, the amount of ONA-PC **2** thus formed would be expected to be at most equal to the amount of OTDA-PC **5** if equilibration between 13-hydroperoxy-9,11-octadecadienoic acid (LA-OOH) and its 9-hydroperoxy isomer is slow, as has been previously found [8]. Moreover, theoretically,  $\beta$ -scission of alkoxy radicals derived from PC-LA-OOH **8** and its 9-hydroperoxy isomer should be equally facile (or unfavourable) due to similarities in the respective carbon-centered radicals thus generated (both are alkyl radicals). Thus the reason for the greater formation of ONA-PC **2** than OTDA-PC **5** under these conditions remains unclear and deserves further investigations.

In summary, we found that OTDA-PC **5** was reactive with amino acids and peptides having a free amino group but not with those having a thiol group. Whereas adduct formation with nucleophiles and oxidation cannot prevent detection of the large amounts of OTDA-PC **5** generated from PC-LNA-OOH **7**, the small amounts of OTDA-PC **5** generated from PC-LA-OOH **8** are easily depleted by oxidation. Thus we conclude that failure to detect OTDA-PC in atherosclerotic tissues should not be due to a high reactivity with biological nucleophiles, but rather its formation in minor quantities.

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