A 13-Oxo-9,10-epoxytridecenoate Phospholipid Analogue of the Genotoxic 4,5-Epoxy-2*E*-decenal: Detection in Vivo, Chemical Synthesis, and Adduction with DNA

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Often guided by analogy with nonphospholipid products from oxidative cleavage of polyunsaturated fatty acids, we previously identified a variety of biologically active oxidatively truncated phospholipids. Previously, 4,5-epoxy-2(*E*)-decenal (4,5-EDE) was found to be produced by oxidative cleavage of 13-(*S*)-hydroperoxy-9,11-(*Z*,*E*)-octadeca-dienoic acid (13-HPODE). 4,5-EDE reacts with deoxy-adenosine (dAdo) and deoxy-guanosine (dGuo) to form mutagenic etheno derivatives. We hypothesized that a functionally similar and potentially mutagenic compound, that is, 13-oxo-9,10-epoxytridecenoic acid (OETA), would be generated from 9-HPODE through an analogous fragmentation. We expected that an ester of 2-lysophosphatidylcoline (PC), OETA-PC, would be produced by oxidative cleavage of 9-HPODE-PC in biological membranes. An efficient, unambiguous total synthesis of *trans*-OETA-PC was first executed to provide a standard that could facilitate the identification of this phospholipid epoxyalkenal that was shown to be produced during oxidation of the linoleic acid ester of 2-lysoPC. Finally, *trans*-OETA-PC was detected in a lipid extract from rat retina. The identity of the naturally occurring oxidatively truncated phospholipid was further confirmed by derivatization with methoxylamine that produced characteristic mono and bis adducts. The average amount of *trans*-OETA-PC in rat retina, 0.33 pmol, is relatively low as compared to other oxidatively truncated PCs, for example, the 4-hydroxy-7-oxohept-5-enoic acid PC ester (2.5 pmol) or the 4-keto-7-oxohept-5-enoic acid PC ester (1.7 pmol), derived from the docosahexaenoic acid ester of 2-lysoPC. This, most likely, is because docosahexaenoate PCs are particularly abundant in the retina as compared to the linoleate PC ester precursor of OETA-PC. As predicted by analogy with 4,5-EDE, OETA-PC reacts with dAdo and dGuo, as well as with DNA, to form mutagenic etheno adducts.

Introduction

The polyunsaturated fatty acyl (PUFA)[1] chains of membrane phospholipids (PLs) are susceptible to oxidative cleavages that generate oxidized phospholipids (oxPLs) with abbreviated acyl chains containing aldehydes, the corresponding acids, as well as hydroxyl, hydroperoxyl, and epoxy groups. OxPLs remain anchored in the membrane. However, because of their polar functionality, the oxidatively truncated acyl chains protrude from the membrane into the aqueous phase (*1*) where they can interact with proteins. They can act as receptor ligands, for example, for CD_{36} receptor-mediated endocytosis of oxidized low-density lipoprotein (oxLDL) by macrophage cells (*2*) that contributes to the pathogenesis of cardiovascular disease. Similar oxPL-induced CD_{36}-mediated endocytosis of photoreceptor cell outer segment tips by retinal pigmented endothelial cells contributes to rapid turnover of photoreceptor cells under conditions of oxidative stress (*3*). Binding of oxPL with CD_{36} receptors in platelet membranes promotes platelet activation and thrombosis (*4*). Binding of certain oxPL with the scavenger receptor (SR) B1 prevents binding to SR-B1 of its physiological ligand, high-density lipoprotein (*5*). Furthermore, oxPLs interfere with SR-B1-mediated selective uptake of cholesteryl esters in hepatocytes and thus may have an inhibitory effect on reverse cholesterol transport. Some oxPLs covalently modify proteins, for example, generating carboxyalkylpyrroles that incorporate the ε-amino group of lysine residues (*6*). For example, oxidative cleavage of PLs incorporating docosaheaxenoic acid (DHA), the most abundant PUFA in photoreceptor cell disk membranes, generates the 4-hydroxy-7-oxohept-5-enoic acid phosphatidylcoline ester (HOHA-PC). This bis electrophile reacts with proteins to produce carboxyethylpyrroles (*7*) that are angiogenic and immunogenic. These biological activities contribute, respectively, to the pathogenesis of choroidal neovascularization (*8*)

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[1] Abbreviations: BHT, butylated hydroxytoluene; dAdo, deoxy-adenosine; DCC, dicyclohexylcarbodiimide; dGuo, deoxy-guanosine; DHA, docosaheaxenoic acid; DHP, dihydropyran; DMAP, *N*,*N*-dimethylaminopyridine; DM-PE, 1,2-dimyristoyl-*sn*-glycero-phosphatidyl-3-ethanoalmine; DTPA, diethylenetriaminepentaacetic acid; 4,5-EDE, 4,5-epoxy-2(*E*)-decenal; HNE, 4-hydroxynon-2-enal; HOHA, 4-hydroxy-7-oxohept-5-enoic acid; 9-HPODE, 9-hydroperoxy-(*Z*,*E*)-9,11-octadecadienoic acid; 13-HPODE, 13-hydroperoxy-(*Z*,*E*)-9,11-octadecadienoic acid; KOHA, 4-keto-7-oxohept-5-enoic acid; MPO, myeloperoxidase; MRM, multiple reaction monitoring; NMO, 4-methylmorpholine *N*-oxide; OETA, 13-oxo-9,10-epoxytridecenoic acid; oxLDL, oxidized low-density lipoprotein; oxPLs, oxidized phospholipids; PA-PC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcoline; PPTS, pyridinium *p*-toluenesulfonate; PUFA, polyunsaturated fatty acyl; SPE, solid-phase extraction; SR, scavenger receptor; TLC, thin-layer chromatography; TPAP, tetrapropylammonium perruthenate.

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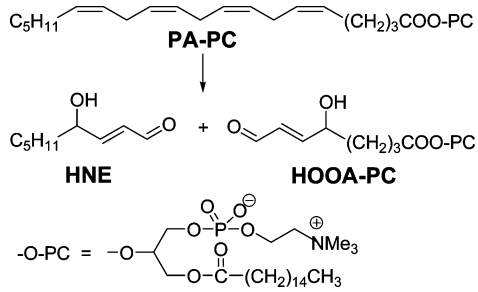
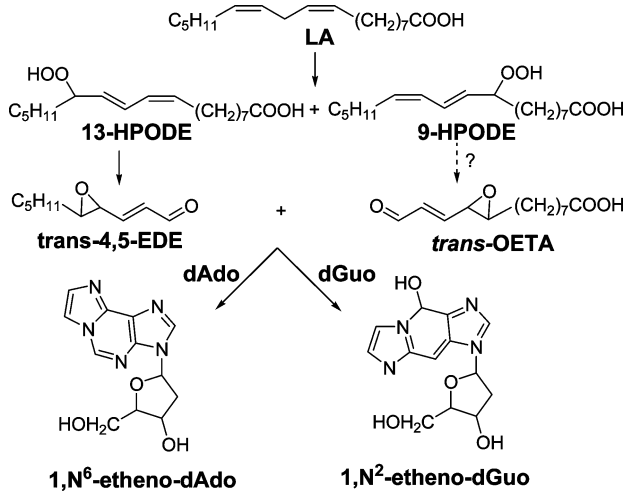
Scheme 1. Formation of HNE and HOOA-PC from PA-PC



Scheme 2. Formation of 4,5-EDE from 13-HPODE and Postulated Formation of OETA from 9-HPODE



and retinal degeneration (*9*), the "wet" and "dry" forms, respectively, of age-related macular degeneration.

Analogy with "mirror image" nonphospholipid products from oxidative cleavage of PUFAs has guided our efforts to identify naturally occurring oxPLs. Previously, we postulated the formation of a 5-hydroxy-8-oxooct-6-enoic acid PC ester HOOA-PC (Scheme 1) from 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PA-PC) by analogy with the well-known formation of 4-hydroxynon-2-enal (HNE). An authentic sample prepared by unambiguous total synthesis (*10*) confirmed that HOOA-PC is formed from PA-PC (*11*) and subsequently allowed the demonstration of its presence in oxLDL (*2*) and its CD_{36}-mediated biological activities (*12*). Total synthesis of more oxidized derivatives of HOOA-PC (*13*) similarly allowed their identification in oxLDL and the demonstration of their biological activities.

"Mirror image" hydroperoxides 9- and 13-hydroperoxy-(*Z*,*E*)-9,11-octadecadienoic acid (9- and 13-HPODE) are major primary oxidation products (Scheme 2) from linoleic acid (LA), the most abundant PUFA in the human body (*14*). Cell injury can initiate lipid peroxidation, leading to generation of these hydroperoxides through nonenzymatic reactions, induced by free radicals. Various lipoxygenases enantioselectively transform LA into specific enantiomers of 9- or 13-HPODE (*15*). 9(*S*)-HPODE is produced by the action of cyclooxygenases 1 and 2 on LA (*16*). Free radical-induced oxidation delivers a racemic mixture of these hydroperoxides. Previously, 4,5-epoxy-2(*E*)-decenal (4,5-EDE) was shown to be produced by oxidative cleavage of 13(*S*)-HPODE (Scheme 2), a fragmentation that is promoted by vitamin C in vitro under oxidative conditions (*17*). Furthermore, vitamin C levels comparable to the intracellular concentration reached with a dose of 200 mg per day—the recommended daily dose of vitamin C for healthy adults—was found to cause substantial amounts of DNA damage in vivo through the known reaction of 4,5-EDE with DNA (*18*). 4,5-EDE reacts with deoxy-adenosine (dAdo) and deoxy-guanosine (dGuo) to form etheno derivatives (*19*) (Scheme 2). This chemistry provides a significant correlation between a product of lipid peroxidation and a mutagenic DNA lesion. The etheno adducts of DNA, when formed in vivo, are normally repaired. However, in diseased individuals, these lesions are not repaired and subsequent DNA replication leads to mutations (*20*) or apoptosis (*21*). Mutations in protooncogenes and tumor suppressor genes have been directly implicated in human cancer (*20*). Mutagenic etheno adducts have been detected in human tissue samples (*22*).

We hypothesized that a functionally similar and potentially mutagenic compound, that is, 13-oxo-9,10-epoxytridecenoic acid (OETA), would be generated from 9-HPODE through an analogous fragmentation (Scheme 2). We expected that an ester OETA-PC of 2-lyso-PC would be produced by oxidative cleavage of 9-HPODE-PC in biological membranes. We now report an unambiguous total synthesis of *trans*-9,10-OETA-PC as well as its generation by oxidation of the linoleic acid ester LA-PC of 2-lysoPC. The natural occurrence of OETA-PC in vivo was established by detection in a lipid extract from rat retina. As predicted by analogy with 4,5-EDE, OETA-PC reacts with dAdo and dGuo, as well as with DNA, to form mutagenic etheno adducts (Scheme 2).

Materials and Methods

Materials. 1-Palmitoyl-2-linoleyl- *sn*-glycero-3-phosphatidylcholine (PL-PC) and 1,2-dimyristoyl-*sn*-glycero-phosphatidyl-3-ethanoalmine (DM-PE) were obtained from Avanti Polar Lipids (Alabaster, AL), and myeloperoxidase (MPO) was supplied by Calbiochem (La Jolla, CA). Dry pyridine and chloroform were supplied by ACROS (New Jersey). Ammonium acetate, dGuo, and dAdo were purchased from Sigma-Aldrich. (St. Louis, MO). Supelclean LC-18 solid-phase extraction (SPE) columns were from Supelco (Bellefonte, PA). Chelex-100 chelating ion-exchange resin (100−200 mesh size) was from Bio-Red Laboratories (Hercules, CA). HPLC grade water and acetonitrile were obtained from Fisher Scientific Co. (Fair Lawn, NJ). ACS grade ethanol was obtained from Pharmco (Brookfield, CT). Gases were supplied by BOC Gases (Lebanon, NJ).

General Methods. ^{1}H NMR spectra were recorded on Varian Gemini spectrometers operating at 200 or 300 MHz using $CHCl_3$ (δ 7.24 ppm) as an internal standard. All chemical shifts are reported in parts per million (ppm) on the δ scale relative to the solvent used. ^{1}H NMR spectra are presented as follows: Multiplicity is given as s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; qAB, quartet AB; and m, multiplet; coupling constants (Hz), number of protons. ^{13}C NMR spectra were recorded on Varian Gemini spectrometers operating at 50 and 75 MHz using $CDCl_3$ (δ 77.0) as an internal standard. Signal multiplicities were established by DEPT experiments. High-resolution mass spectra were recorded on a Kratos AEI MS25 RFA high-resolution mass spectrometer at 20 eV.

Thin-layer chromatography (TLC) was performed on glass plates precoated with silica gel (Kieselgel 60 F_{254}, E. Merck, Darmstadt, Germany); R_f values are quoted for plates of thickness 0.25 mm. The plates were visualized by viewing the plates under short-wavelength UV light or by exposure to iodine vapor. PL spots were visualized using a molybdenum-based detection spray (*23*). Flash chromathography was performed using Silica Gel 60A, 32−63 μM from Sorbent Technologies (Atlanta, GA) or ICN SiliTech 32−63 D 60A from ICN Biomedicals Gmbh Eschwege (Germany).

2-(8-Bromo-octyloxy)-tetrahydropyran. Pyridinium *p*-toluenesulfonate (PPTS, 217 mg, 0.86 mmol) was added to a solution of the alcohol 8-bromo-octanol (1942 mg, 8.67 mmol) and dihydropyran (DHP, 1.1 g, 13 mmol, freshly distilled) in dry methylene

chloride (40.0 mL) (*24*). The resulting solution was stirred overnight at room temperature and then diluted with water, and the resuting mixture was extracted with ethyl acetate, washed with brine, and dried on sodium sulfate. The solvent was removed with a rotary evaporator. The crude product was purified by flash chromatography on a silica gel column (10% ethyl acetate in hexanes) to afford 2-(8-bromo-octyloxy)-tetrahydropyran (2.5 g, 98%): TLC (8% ethyl acetate in hexanes) $R_f = 0.28$. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.55 (t, $J = 3$ Hz, 1H), 3.81−3.88 (m, 1H), 3.67−3.74 (tt, $J_1 = 6$ Hz, $J_2 = 6$ Hz, 1H), 3.51−3.32 (m, 2H), 1.30−1.87 (18H). ^{13}C NMR (75 MHz, $CDCl_3$): 98.92 (CH), 67.65 (CH_2), 62.43 (CH_2), 34.06 (CH_2), 32.84 (CH_2), 30.83 (CH_2), 29.74 (CH_2), 29.30 (CH_2), 28.74 (CH_2), 28.15 (CH_2), 26.18 (CH_2), 25.54 (CH_2), 19.76 (CH_2).

11-(Tetrahydropyran-2-yloxy)-undec-2-en-1-ol (2). Liquid ammonia (33 mL) and iron(III) nitrate (20 mg) were placed in a 100 mL three-necked flask equipped with a magnetic stirrer, dry ice/acetone-cooled condenser, and drying tube with potassium hydroxide for protection from moisture. To this mixture, lithium (200 mg, 28.8 mmol) was added in portions allowing the blue color to dissipate between additions. Propargyl alcohol (1 mL, 17 mmol) in tetrahydrofuran (8 mL, freshly distilled) was added over 25 min, and the reaction mixture was then allowed to boil under reflux for 100 min. 2-(8-Bromo-octyloxy)-tetrahydropyran (2.5 g, 8.5 mmol) in tetrahydrofuran (7 mL) was then added over 35 min, and the mixture was allowed to boil under reflux for 135 min. Lithium wire (50 mg, 7.2 mmol) was added resulting in a persistent blue color in the mixture (*25*). After 30 min, sufficient ammonium chloride was added to dispel the blue color. Most of the ammonia was the evaporated under a gentle stream of nitrogen, and the residue was poured onto ice (50 g). The resultant mixture was extracted with diethyl ether (3 × 30 mL) and then ethyl acetate (2 × 50 mL). The combined organic extract was dried and concentrated by rotary evaporation to give a residue that was purified by flash chromatography on a silica gel column with 25% ethyl acetate in hexanes to afford 11-(tetrahydropyran-2-yloxy)-undec-2-en-1-ol (2.1 g, 91%): TLC (ethyl acetate/hexanes 1:4) $R_f = 0.3$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.55−5.72 (m, 2H), 4.55 (t, $J = 3$ Hz, 1H), 3.81−3.88 (m, 1H), 3.67−3.74 (tt, $J_1 = 6$ Hz, $J_2 = 6$ Hz, 1H), 3.51−3.32 (m, 2H), 1.98−2.04 (m, 2H), 1.30−1.87 (18H). ^{13}C NMR (75 MHz, $CDCl_3$): 133.52 (CH), 128.9 (CH), 98.89 (CH), 67.71 (CH_2), 63.86(CH_2), 62.39 (CH_2), 32.22 (CH_2), 30.82 (CH_2), 29.77 (CH_2), 29.43 (CH_2), 29.12 (CH_2), 26.24 (CH_2), 25.54 (CH_2), 19.74 (CH_2).

Acetic Acid, 11-(Tetrahydro-pyran-2-yloxy)-undec-2-enyl Ester (4). A solution of acetic anhydride from a newly opened bottle (∼1.3 g, 12.4 mmol) in pyridine (5 mL, freshly distilled) was cooled in an ice bath. A solution of 11-(tetrahydropyran-2-yloxy)-undec-2-en-1-ol (2.2 g, 8 mmol) in pyridine (4 mL, freshly distilled) was added in one portion, and the mixture was stirred for 3 h at 0 °C and then for 3 more hours while it was warmed to room temperature (*26*). It was then poured onto a mixture of ice (30 g) and hexanes (20 mL) and shaken, and then, the layers were separated. The aqueous layer was extracted with hexanes (3 × 10 mL). The combined organic layers were washed with water and then brine and then dried over Na_2SO_4. The extract was concentrated by rotary evaporation to give a residue that was purified by flash chromatography on a silica gel column with 12% ethyl acetate in hexanes to afford the title compound (2.37 g, 95%): TLC (ethyl acetate/hexanes 1:9) $R_f = 0.32$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.48−5.57 (m, 1H), 5.69−5.79 (m, 1H), 4.55 (t, $J = 3$ Hz, 1H), 3.81−3.88 (m, 1H), 3.67−3.74 (tt, $J_1 = 6$ Hz, $J_2 = 6$ Hz, 1H), 3.51−3.32 (m, 2H), 2.00 (s, 3H), 1.98−2.04 (m, 2H), 1.30−1.87 (18H).

Acetic Acid, 11-Hydroxy-undec-2-enyl Ester (5). A solution of acetic acid, 11-(tetrahydro-pyran-2-yloxy)-undec-2-enyl ester (2.3 g, 7.5 mmol), and PPTS (237 mg, 0.75 mmol) in absolute ethanol (12.5 mL, freshly distilled over CaH_2) was heated for 6 h at 50 °C. The solution was then concentrated by rotary evaporation, and the residue was taken up in ethyl acetate (10 mL) to give a milky suspension that was transferred to a separatory funnel (*24*). The reaction flask was rinsed with water (5 mL) and ethyl acetate (5 mL), and then, the washes were added to the separatory funnel. The mixture was then shaken to give two clear phases that were separated. The organic layer was washed with water (3 mL), 1% $NaHCO_3$ (3 mL), and brine and then dried on Na_2SO_4. The solvent was removed with a rotary evaporator, and the residue was purified by flash chromatography on a silica gel column with 30% ethyl acetate in hexanes to afford the title compound (1.64 g, 96%): TLC (ethyl acetate/hexanes 3:7) $R_f = 0.37$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.48−5.58 (m, 1H), 5.69−5.79 (m, 1H), 4.48 (d, $J = 6$ Hz, 2H), 3.58−3.64 (m, 2H), 2.03 (s, 3H), 1.98−2.04 (m, 2H), 1.23−1.56 (14H).

11-Hydroxy-undec-9-enoic Acid (6). A 250 mL round-bottom flask was charged with Jones reagent (8 mL, 2.67 M $Na_2Cr_2O_7$ in 4 M H_2SO_4) and acetone (40 mL). A solution of acetic acid and 11-hydroxy-undec-2-enyl ester (1.2 g, 5.2 mmol) in acetone (40 mL) was added dropwise at 0 °C from an addition funnel over 2 h. The color changed from orange to dark brown. The mixture was stirred at room temperature for an additional 4 h, and then, the organic solution was decanted from an orange residue. The residue was dissolved in water (30 mL), and the color changed to green-blue. This was extracted with ethyl acetate (3 × 10 mL). The organic extracts were added to the decanted solution. Solvents were then removed by rotary evaporation to give a brown residue. This was dissolved in water (30 mL) and extracted with ethyl acetate (3 × 20 mL). The organic solution was washed with water (20 mL) to remove residual chromium species (*26*) and then extracted with 10% NaOH (20 mL). The aqueous phase was acidified with 6 M HCl to pH 1 and then extracted with ethyl acetate (4 × 10 mL). The combined organic extracts were washed with water (10 mL) and brine (10 mL) and then dried on Na_2SO_4. The yellow-green color of the organic phase gradually faded to become colorless. The solvent was removed with a rotary evaporator, and the white fluffy solid residue was purified by flash chromatography on a silica gel column with 70% ethyl acetate in hexanes to afford 11-hydroxy-undec-9-enoic acid (1.04 g, 86%): TLC (ethyl acetate/hexanes 1:1) $R_f = 0.28$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.55−5.72 (m, 2H), 4.06 (d, $J = 3$ Hz, 2H), 2.32 (t, $J = 6$ Hz, 2H), 2.0−2.03 (m, 2H), 1.58−1.63 (m, 2H), 1.21−1.35 (8H).

8-(3-Hydroxymethyl-oxiranyl)octanoic Acid (7). A solution of dioxirane in acetone (*27*) (8.8 mL, 60 mM) was added dropwise to a solution of 11-hydroxy-undec-9-enoic acid (120 mg, 0.6 mmol) in chloroform (4 mL) at room temperature. The resulting mixture was stirred for an additional 15 min at room temperature. The solvents were removed by rotary evaporation. The title compound was obtained in quantitative yield. ^{1}H NMR (300 MHz, $CDCl_3$,): δ 3.91−3.56 (2H), 2.96−2.89 (m, 2H), 2.32 (t, 2H, $J = 7.3$ Hz), 1.63−1.31 (12H). ^{13}C NMR (75 MHz, $CDCl_3$): 179.3 (C), 61.6 (CH_2), 58.98 (CH), 56.39 (CH), 34.00 (CH_2), 31.45 (CH_2), 29.11 (CH_2), 29.08 (CH_2), 28.90 (CH_2), 25.83 (CH_2), 24.63 (CH_2). HRMS (FAB): *m*/*z* calculated for $C_{11}H_{20}O_4$ [$(M - H)^+$], 217.1440; found, 217.1445.

8-(3-Formyl-oxiranyl)-octanoic Acid (8). 8-(3-Hydroxymethyl-oxiranyl)octanoic acid (130 mg, 0.6 mmol) was dissolved in dichloromethane (60 mL, freshly distilled) containing 4 Å molecular sieves and 4-methylmorpholine *N*-oxide (NMO, 105 mg, 0.9 mmol). Solid tetrapropylammonium perruthenate (TPAP, 32 mg, 0.091 mmol, 0.15 equiv) was then added under argon (*28*), and the resulting green mixture was stirred at room temperature for 2 h, until TLC analysis with 40% ethyl acetate in hexanes showed the complete disappearance of the starting material and the appearance of a new spot ($R_f = 0.26$). The molecular sieves were washed with four times the initial volume of the ethyl acetate to recover the entire product. The solution was filtered through a 1 cm layer of silica gel on a 60 mL separatory funnel. The solvent was removed by rotary evaporation to afford the title compound (123 mg, 96% yield) in high purity. ^{1}H NMR (75 MHz, $CDCl_3$,): δ 8.99 (d, 1H, $J = 6.2$), 3.20 (dt, 1H, $J = 6.9$, $J = 1.9$), 3.11 (dd, 1H, $J = 6.3$, $J = 1.9$), 2.33 (t, 2H, $J = 7.4$), 1.63−1.31 (12H). ^{13}C NMR (300 MHz, $CDCl_3$): 198.56 (CH), 179.83 (C), 59.17 (CH), 56.79 (CH), 33.97 (CH_2), 31.19 (CH_2), 29.04 (CH_2), 29.01 (CH_2), 28.87 (CH_2),

25.73 (CH_2), 24.59 (CH_2). HRMS (FAB): *m/z* calculated for $C_{11}H_{19}O_4$ [$(M - H)^+$], 215.1283; found, 215.1292.

8-[3-(3-Oxo-propenyl)-*trans*-oxiranyl]-octanoic Acid (*trans*-OETA, 1). To a stirred suspension of formylmethylene triphenylphosphorane (150 mg, 0.495 mmol) (*29*) in 4 mL of dry toluene at 0 °C, the above aldehyde (70 mg, 0.327 mmol) was added. The resulting mixture was stirred for 3 days at 4 °C under argon. The solvent was then removed by rotary evaporation. The residue was extracted with ethyl acetate (4 × 5 mL). The aqueous phase was acidified with 2 M HCl to pH 4 and then extracted with ethyl acetate (4 × 10 mL). The combined organic extracts were washed with water (10 mL) and brine (10 mL) and then dried on Na_2SO_4. The sample was concentrated by rotary evaporation to give a residue that was purified by flash chromatography on a silica gel column with 75% ethyl acetate in hexanes to afford OETA (70 mg, 90%): TLC (ethyl acetate/hexanes 1:1) $R_f = 0.3$. ^{1}H NMR (300 MHz, $CDCl_3$,): δ 9.53 (d, 1H, $J = 7.6$), 6.52 (dd, 1H, $J = 15.8$, $J = 6.8$) 6.35 (dd, 1H, $J = 15.8$, $J = 7.4$) 3.29 (dd, 1H, $J = 6.9$, $J = 1.9$), 2.92 (dt, 1H, $J = 5.6$, $J = 1.9$), 2.33 (t, 2H, $J = 7.4$), 1.63−1.31 (12H). ^{13}C NMR (75 MHz, $CDCl_3$): 192.64 (CH), 179.83 (C), 153.12 (CH), 133.57 (CH), 61.91 (CH), 56.21 (CH), 33.90 (CH_2), 31.87 (CH_2), 29.09 (CH_2), 28.90 (CH_2), 28.87 (CH_2), 25.73 (CH_2), 24.59 (CH_2). HRMS (FAB): *m/z* calculated for $C_{13}H_{21}O_4$ [$(M - H)^+$], 241.1440; found, 241.1447.

1-Palmitoyl-2-(13-oxo-9,10-*trans*-epoxy-octadeca-11-enoyl)-*sn*-glycero-3-phosphatidylcholine (*trans*-OETA-PC). A mixture of the acid (OETA, 9 mg, 0.037 mmol) and 2-lyso-phosphatidylcholine (lyso-PC, 9.2 mg, 0.018 mmol) was dried overnight on a vacuum pump (0.1 mmHg) equipped with a dry ice−acetone trap, at room temperature, and then was dissolved in dry $CHCl_3$ (1 mL, shaken with P_2O_5 for 0.5 h and distilled). Dicyclohexylcarbodiimide (DCC, 18.5 mg, 0.09 mmol) and *N*,*N*-dimethylaminopyridine (DMAP, 2.2 mg, 0.018 mmol) were added. The mixture was stirred for 10 days under argon. The mixture was then concentrated on a rotary evaporator, and the residue was purified by flash chromatography on a silica gel column with $CHCl_3$/MeOH/H_2O (16/9/1, TLC: $R_f = 0.27$) to produce the PL, OETA-PC (10 mg, 87%). ^{1}H NMR (300 MHz, $CDCl_3$,): δ 9.53 (d, $J = 7.6$, 1H), 6.52 (dd, $J = 15.8$, $J = 6.8$, 1H) 6.35 (dd, $J = 15.8$, $J = 7.4$, 1H), 5.17 (m, 1H), 4.29−4.40 (m, 2H), 3.80−4.12 (m, 4H), 3.35 (s, 9H), 3.29 (dd, $J = 6.9$, $J = 1.9$, 1H), 2.92 (dt, $J = 5.6$, $J = 1.9$, 1H), 2.33 (t, $J = 7.4$, 2H), 1.22−1.61 (42H), 0.85 (t, $J = 6$,3H). ^{13}C NMR (300 MHz, $CDCl_3$): 192.64 (CH), 179.83 (C), 153.12 (CH), 133.57 (CH), 61.91 (CH), 56.21 (CH), 33.90 (CH_2), 31.87 (CH_2), 29.09 (CH_2), 28.90 (CH_2), 28.87 (CH_2), 25.73 (CH_2), 24.59 (CH_2). HRMS (FAB): *m/z* calculated for $C_{37}H_{68}NO_{10}P$ [$(M - H)^+$], 718.4660; found, 718.4664.

Oxidative Generation of OETA-PC from PL-PC. Lipid Oxidation. The dry lipid, PL-PC (1 mg), was hydrated at 37 °C for 1 h in phosphate buffer (1 mL) supplemented with DTPA to minimize transition metal-induced autoxidation. Then, the hydrated PL-PC was made into unilamellar vesicles by extrusion using an Avanti Mini-Extruder (Avanti Polar Lipids, Inc.). For the Cu(II) oxidation, 10 μL of a solution of 500 μM $CuCl_2$ was added to 1.99 mL of vesicles. For the UV-induced oxidation, the vesicles (2 mL) were kept in a quartz tube placed in the center of a Rayonet photochemical reactor with three 80 W low pressure mercury UV (350 nm) Rayonet lamps (Southern New England Ultraviolet, Midtown, CT). The temperature was maintained at about 28 °C. The MPO oxidation was performed with the MPO-NO_2^--glucose/glucose oxidase system (*7*). Sampling (200 μL) was performed at various times, and the reaction was stopped by adding butylated hydroxytoluene (BHT, 2 μL, 40 μM) and catalase (7 μL, 300 μM) (*7*). For quantification purposes, the lipid extraction was performed immediately using a slightly modified Bligh and Dyer method (*30*), and the samples were dried in a stream of nitrogen at room temperature. The dried samples were stored in vials sealed under argon at −80 °C.

LC/ESI-MS/MS Analysis. A standard curve was generated by incorporating a fixed amount of internal standard (1,2-myristoyl-*sn*-glycero-3-phosphatidylethanolamine, DM-PE), varying the levels of the analyte, and plotting peak area ratio versus analyte mol ratio for each sample. LC-MS analysis of oxidized lipids was performed on a Quattro Ultima mass spectrometer (Micromass, Wythenshawe, United Kingdom) equipped with an electrospray ionization (ESI) probe interfaced with a Waters 2790 (Waters, Milford, MA) HPLC system. OETA-PC (50 μg, determined by aliquoting a solution of a known concentration (determined by microphosphorous assay) was dissolved in 1 mL of chloroform/methanol (1:1). The solution (100 μL) was diluted with 400 μL of chloroform/methanol (1:1). To this solution (50 μL) the DM-PE standard (50 μL, 5−15 ng) was added, and the mixture was chromatographed on a Prodigy ODS C18 column (150 mm × 2 mm, 5 μm, Phenomenex, Torrance, CA.) with a binary solvent gradient, starting from 50% water in methanol with a linear gradient to 100% methanol over 10 min and then 100% methanol for 20 min at a flow rate of 0.2 mL/min. All of the mobile phase solvents contained 0.2% formic acid to enhance the MS signal. The total ion current was obtained in the mass range of *m/z* 200−1000 at 30 V of cone energy in the positive ion mode. Three kilovolts was applied to the electrospray capillary. All compounds were monitored in the positive ion mode for the parent ion and the major daughter ion, previously determined by direct injection using ESI. The optimal collision energy determined to obtain the most intense daughters was 20 eV. In Table 1 are given the calibration equation for OETA-PC and PL-PC, as well as the extraction efficiencies determined as follows. Liposomes containing 10 μg of OETA-PC and the corresponding precursors (PL-PC) and 130 μg (50% of the total lipid content of liposomes) of DP-PC were prepared as described previously (vide infra). After Bligh and Dyer extraction, the amounts recovered were determined by LC-MS relative to DM-PE added prior to analysis and compared to a mixture composed of 10 μg from the OETA-PC and precursors and the same amount of DM-PE. The extraction efficiency for OETA-PC was 37.6 ± 6.7%.

Table 1. Calibration Equations, Detection Limits, and Extraction Efficiency

compound	calibration equation	detection limit (fmol)	extraction efficiency
OETA-PC	$y = 2.1835 \times$ ($R^2 = 0.9993$)	9	37.6 ± 6.7
PL-PC	$y = 0.920 \times$ ($R^2 = 0.9913$)	2	58.8 ± 1.6

Derivatization of PLs. Methoxime derivatives of lipids were prepared by suspending the lipid (50 μg), previously dried in a stream of nitrogen in 200 μL of freshly dried pyridine with 10% methoxylamine hydrochloride. The reaction mixture was incubated for 2 h at room temperature. Solvents and volatile byproducts were removed under a nitrogen stream, and residues were resuspended in chloroform:methanol (1:1). Nonlipid components were removed by passing the solution through a C18 cartridge (Supelclean, 3 mL), as follows. The reaction mixture dissolved in 50% methanol (1 mL) was loaded onto an S-PE cartridge, which was previously equilibrated by passing 10 mL of water. The column then was eluted with a gradient of water and methanol: 50% methanol (10 mL), 60% methanol (5 mL), 75% methanol (5 mL), 90% methanol (10 mL), and then pure methanol. The derivatized lipids were eluted with 90−100% methanol. Lipids were dried again under a nitrogen stream. Derivatives of standard were characterized by ESI-MS/MS and chromatographed on the same Prodigy ODS C18 column (150 mm × 2 mm, 5 μm) using the same HPLC conditions described above for oxidized lipids. The LC/ESI-MS/MS was operated in the positive ion mode. The analytes were detected by multiple reaction monitoring (MRM). Mass transitions, *m/z* 747.9→184.5 and *m/z* 794.7→184.5 for OETA-PC methoxime derivatives were monitored.

Detection and Quantification of *trans*-OETA-PC in Rat Retina. Extraction of PLs from Retina. Rat retinas were harvested from five normal albino rats by Mary Rayborn and Dr. Xiaorong Gu. To prevent contamination by blood, or in vitro oxidation, the retinas were rinsed with saline antioxidant cocktail (saline PBS pH 7.4, containing 40 μM DTPA, and 4 nM BHT). The retinas were immediately homogenized manually in a plastic vial using a stainless steel pestle coated with Teflon, and lipid extraction was

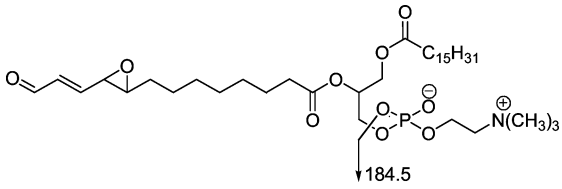


Figure 1. Mass spectrometric fragmentation of OETA-PC.

then performed immediately after homogenization using the method of Bligh and Dyer (*30*). The extract was dried under a stream of nitrogen, sealed in a vial under argon, and kept at −80 °C for 24 h before being analyzed by LC-MS.

Quantitative Analysis of PLs. The same LC-MS system used for qualitative analysis was used here. The MRM transitions used to detect the oxPL were the mass to charge ratio (*m*/*z*) for the molecular cation $[M + H]^+$ and their respective daughter ion. Mass transitions, *m*/*z* 718.3→ 184.5 for OETA-PC (Figure 1) and *m*/*z* 759.1→184 for PL-PC, were monitored simultaneously. Calibration curves for quantitative analysis of OETA-PC and PL-PC were constructed by adding a fixed amount of internal standard (DM-PE) into various amounts of authentic samples. The chromatograms are presented in Figure 2 (vide infra). Equations describing the calibration curves are presented in Table 1 (vide infra). Although we did not have the authentic standard, we also monitored theoretical ion pairs parent/daughter for the corresponding acid of OETA-PC, that is, in which the carboxaldehyde has been oxidized to a carboxyl group: *m*/*z* 734.5→184. When standard become available, these data can be used to provide identification and quantification of the acid. Calculation of *trans*-OETA-PC levels in rat retina is as follows: $n_A = y/(n_{st})(a)(EE)$, where n_A = mole analyte, y = peak area ratio, a = from calibration curves ($y = ax$), n_{st} = moles of internal standard used, and EE = extraction efficiency (from Table 1).

Reaction of OETA-PC with dAdo, dGuo, and Calf Thymus DNA. Liquid Chromatography. Chromatography for LC/MS/UV experiments was performed using a Waters Alliance 2690 HPLC system (Waters Corp.) and a Hitachi L-4200 UV detector at 231 nm. Gradient system 1 consisted of a Synergi Polar RP column (250 mm × 4.6 mm i.d., 4 μm; Phenomenex) at a flow rate of 1 mL/min. Solvent A was 5 mM ammonium acetate in water, and solvent B was 5 mM ammonium acetate in acetonitrile. The linear gradient (gradient 1) was as follows: 6% B at 0 min, 6% B at 2 min, 10% B at 15 min, 80% B at 25 min, 80% B at 35 min, 6% B at 37 min, and 6% B at 45 min. Separations were performed at room temperature.

Mass Spectrometry. Mass spectrometry was conducted using a Thermo Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an APCI source in the positive ion mode. The LCQ operating conditions were as follows: vaporizer temperature at 450 °C, heated capillary temperature at 150 °C, with a discharge current of 5 μA applied to the corona needle. Nitrogen was used as the sheath (80 psi) and auxiliary (5 arbitrary units) gas to assist with nebulization. Full scanning analyses were performed in the range of *m*/*z* 100 to *m*/*z* 800. Collision-induced dissociation (CID) experiments coupled with multiple tandem mass spectrometry (MS^n) employed helium as the collision gas. The relative collision energy was set at 20% of the maximum (1 V).

Reaction of OETA-PC with DNA Bases. A solution of OETA-PC (1.44 mg, 2 μmol) in ethanol (36 μL) was added to dGuo (2.67 mg, 10 μmol) or dAdo (2.52 mg, 10 μmol) in Chelex-treated 100 mM phosphate buffer (pH 7.4, 314 μL). After sonication for 15 min, the reaction mixture was incubated at 60 °C for 48 h. The samples were filtered through a 0.2 μm Costar cartridge prior to analysis of a portion of the sample (20 μL) by LC/MS using gradient system 1.

Preparation of OETA-PC-Modified Calf Thymus DNA. Calf thymus DNA (500 μg, 1.68 μmol) was dissolved in 100 mM phosphate buffer (pH 7.4, 314 μL) and treated with OETA-PC (1.44 mg, 2 μmol) in ethanol (36 μL). After sonication for 15 min, the reaction mixture was incubated at 60 °C for 48 h. Samples were placed on ice for 30 min, and the DNA was precipitated by adding ice-cold ethanol (1050 μL) followed by ice-cold 2.9 M sodium acetate (35 μL). The samples were centrifuged at 4000*g* for 15 min, and the supernatant was removed. The DNA pellet was washed with ethanol/water (1 mL, 7:3 v/v), and residual solvent was removed by evaporation under nitrogen.

Enzymatic Hydrolysis of OETA-PC-Modified Calf Thymus DNA. The modified DNA pellet was dissolved in 1 mL of 10 mM MOPS containing 100 mM NaCl (pH 7.0) by sonication for 5 min. DNase I (556 units) dissolved in 200 μL of 10 mM MOPS containing 120 mM $MgCl_2$ (pH 7.0) was added, and the sample was incubated for 90 min at 37 °C. At the end of this incubation, 50 μL of 10 mM MOPS containing 100 mM NaCl (pH 7.0) and 15.5 units of nuclease P1 was added followed by 25 mM $ZnCl_2$ (50 μL). The incubation was then continued for 2 h at 37 °C. Finally, 30 units of alkaline phosphatase in 0.5 mL of 0.4 M MOPS (pH 7.8) was added, and this was followed by an additional 1-h incubation.

Isolation of DNA Adducts from Modified Calf Thymus DNA. The hydrolysate was applied directly to an SPE cartridge (1 g, 6 mL, Supelclean) that had been prewashed with acetonitrile (15 mL) followed by water (15 mL). The cartridge was washed with water (4 mL) and methanol/water (1 mL; 5:95, v/v). Etheno-dGuo and etheno-dAdo were eluted in acetonitrile/water (6 mL; 50:50, v/v). The elutes were evaporated to dryness under nitrogen and dissolved in water (100 μL). LC/MS analysis was conducted on a 20 μL aliquot of this solution using gradient system 1.

Results

Synthesis of *trans*-OETA-PC. To facilitate studies of the generation, chemistry, and biology of OETA-PC, we engaged in a synthetic program targeting this product. The route established is short and utilizes straightforward steps that are easy to execute. The synthetic target was envisioned to be available from the acid **1** via esterification of 2-lyso-phosphatidylcholine. Intermediate **1** could be generated from an allylic alcohol **2** by epoxidation of the *E* double bond and chain homologation (Scheme 3). In turn, intermediate **2** might be assembled through alkylation of an acetylide nucleophile, derived from propargyl alcohol, with a hydroxyl-protected derivative of the commercially available bromohydrin **3**.

Previously, Goerger and Hudson (*26*) reported a total synthesis of parinaric acid, starting from 9-decen-1-ol. In five rather elaborate steps, they obtained the intermediate **2**, starting from the bromo-alcohol **3**. We arrived at the same intermediate in only two steps in 92% overall yield. The very convenient, one-pot reaction developed by Patterson (*25*) for the conversion of terminal acetylenic alcohols into (*E*)-olefinic alkenols, succeeded when we used a 2:1 ratio of propargyl alcohol to the electrophile **3** to generate the allylic alcohol **2**. This protocol produced exclusively the requisite *E* isomer of **2** (Scheme 4) as expected.

The allylic hydroxyl of **2** was protected as an acetate and the primary alcohol was deprotected to **5** via established procedures (*24*). The alcohol **5** was then oxidized and deprotected during workup to provide the acid **6** in one step, as the only product (by NMR). The conversion of the intermediate acetoxy acid to the hydroxy acid **6** was the serendipitous result of acidifying the reaction mixture to pH 1 using 6 M HCl. This catalyzed solvolysis of the allylic acetate group. In anticipation of the Wittig homologation, we had envisioned a route involving protection of the acid with simultaneous hydrolysis of the allylic acetate (*13*). We decided, however, to carry on with the acid **6**, thus saving two steps. Protection of the acid functionality as an ester proved indeed to be unnecessary (*31*). Epoxidation of

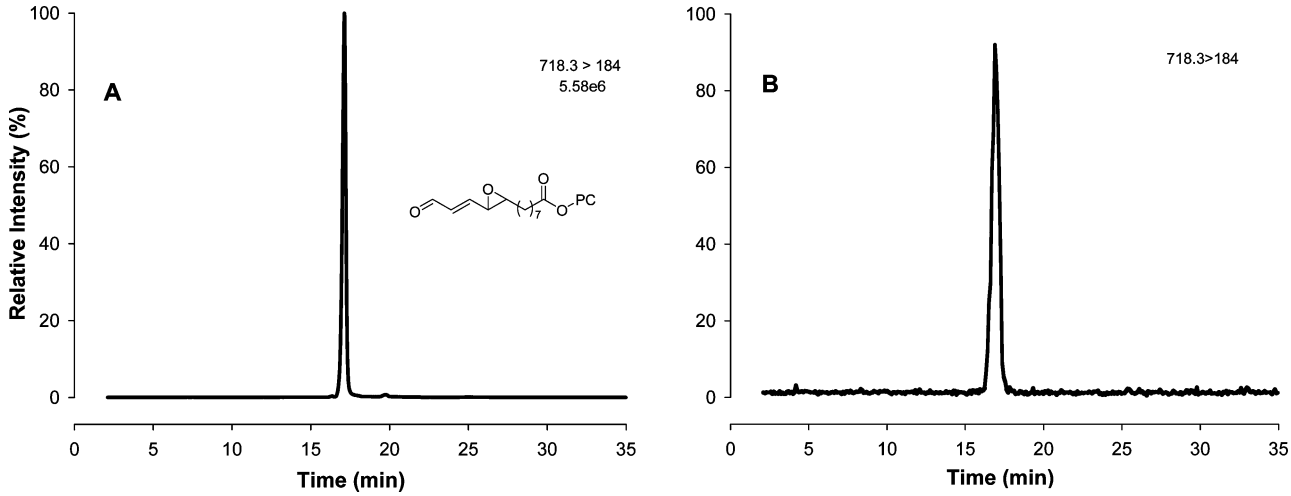
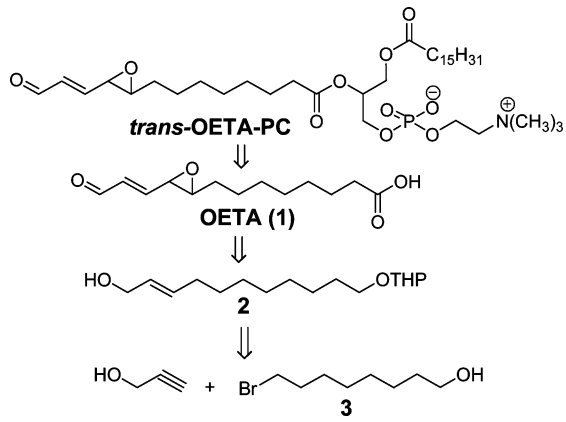


Figure 2. LC/ESI-MS/MS analysis of OETA-PC from oxidized PL-PC liposomes. (A) Synthetic OETA-PC standard (parent *m/z* 718.3), MRM chromatogram (daughter *m/z* 184.5). (B) OETA-PC detected in oxidized PL-PC liposomes, MRM chromatogram (718.3→184.5).

Scheme 3. Strategy for Synthesis of *trans*-OETA-PC



allylic alcohol **6** with a freshly prepared solution of dimethyldioxirane in acetone (*27*) was completed in 30 min at room temperature and gave pure *trans*-epoxide **7** quantitatively after removal of the solvents and volatile byproducts under reduced pressure. Oxidation of the free alcohol in **7** with TPAP/NMO (*28*) proceeded in excellent yield to afford the aldehyde **8**. Epoxyalkenal acid **1** was obtained in good yield from the aldehyde-epoxy-acid **8** by a Wittig reaction (*29*). The DMAP/DCC-promoted coupling of the acid **1** with lyso-PC gave *trans*-OETA-PC in 87% yield.

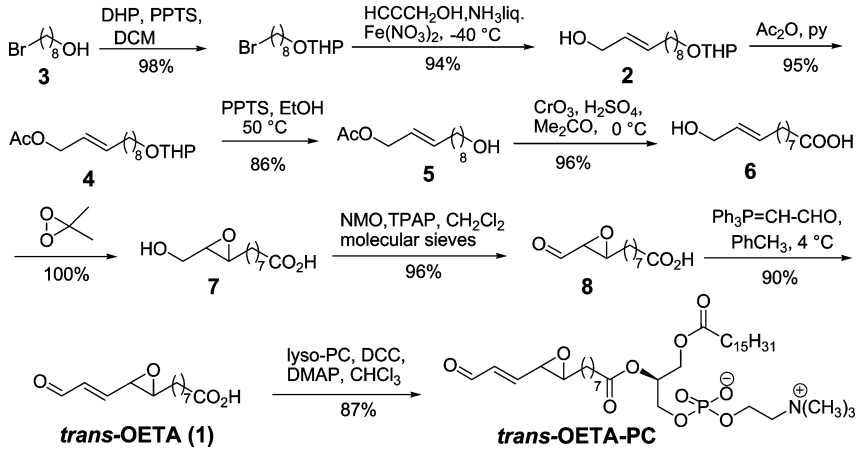
Production of OETA-PC in Vitro from PL-PC. To demonstrate that OETA-PC can be produced by oxidation of unsaturated PLs in vitro, PL-PC liposomes were oxidized under various reaction conditions. In one experiment, the liposomes were irradiated with 350 nm UV light in phosphate-buffered saline (PBS) at room temperature in the air. In parallel experiments, PL-PC liposomes were oxidized with copper(II) or peroxynitrate produced from a MPO-NO_2^--glucose/glucose oxidase system (*7*). To minimize transition metal-induced oxidation, the buffer was supplemented with a metal chelator, diethylenetriaminepentaacetic acid (DTPA). The oxidation reaction mixture was sampled at various times. Catalase was added to quench the reactions by removing hydroperoxides from the samples. To prevent further autoxidation of the samples during processing and storage, BHT was also added. Lipids were extracted using a slightly modified Bligh and Dyer method (*30*) as described in the experimental part, dried under a nitrogen stream at room temperature, stored under argon in the dark at −80 °C until they were analyzed (usually not more than 24 h). The extracts were analyzed by LC/ESI-MS/MS. Preliminary identification of product peaks was achieved by comparing retention times with authentic samples. The authentic sample of OETA-PC, synthesized as described above, was characterized by high-resolution mass spectrometry and multinuclear NMR. Positive ion ESI-MS/MS analysis of the authentic lipid yielded both parent and daughter ions that were used as the specific mass transition ion pairs in LC/ESI-MS/MS analysis. A common daughter ion *m/z* 184 was produced in all samples, as found for all other phosphatidylcholines detected previously in our lab. The retention time and the parent/daughter ion pair were collectively used to identify a peak in the oxidation reaction product mixture chromatograms.

Oxidized PL-PC liposomes from all three experiments produced the expected oxidatively truncated OETA-PC (Figure 2) in various yields. A single peak with a retention time that is identical to the *trans*-OETA-PC standard (Figure 2A) is present in the MRM chromatogram of PL-PC oxidation product mixtures (Figure 2B, reconstructed MRM chromatograms), demonstrating the generation of OETA-PC in these oxidation experiments.

To further confirm the identity of the peak detected, the product mixtures of oxidation of PL-PC liposomes were treated with methoxylamine hydrochloride to derivatize the aldehyde functional group. Methoxime derivatization of an aldehyde carbonyl results in a net mass increase of 29 Da. Methoxime derivatives of pure OETA-PC were prepared to provide authentic samples for analyzing product mixtures from PL-PC oxidation. Each derivative was purified using a C18 mini-column and then analyzed by LC-MS in the positive ion mode. Methoxime derivatization of the synthetic standard of OETA-PC resulted in the expected *m/z* 747.9, which corresponds to a methoxime modification (29 Da) of the aldehyde group of OETA-PC (*m/z* 718.3) (Figure 3A). In addition, an unexpected bis-methoxylamine derivative (*m/z* 794.7) was produced, in which both the aldehyde and the epoxy group of OETA-PC are modified by methoxylamine (Figure 3C,D).

Both derivatives display a common daughter ion *m/z* 184.5 ($C_5H_{15}NO_4P$). The bis-methoxylamine derivative of OETA-PC is presumably generated through opening of the epoxy ring. Typical MRM chromatograms of oxidized PL-PC liposomes after derivatization are presented in Figure 2. Before derivatization, no peaks corresponding to derivatized standards were

Scheme 4. Synthesis of OETA-PC



observed (data not shown). After derivatization, new peaks corresponding to derivatized standards appeared with a parallel disappearance of the peak corresponding to OETA-PC.

Evolution Profiles of OETA-PC from Oxidation of PL-PC. Quantification of OETA-PC in reaction product mixtures from oxidation of PL-PC was achieved by LC/ESI-MS/MS in the positive ion mode using the MRM function. Appropriate $[M + H]^+$ and a common daughter ion *m/z* 184 were monitored. Calibration curves were produced using an authentic sample of OETA-PC prepared by the unambiguous total synthesis described above and commercially available PL-PC. Accurate determination of the amounts of these PLs was achieved by a microphosphorous assay. Standard curves were generated by incorporating a fixed amount of DM-PE as internal standard and various amounts of the analyte and plotting the peak area ratio versus analyte mole ratio. Trace amounts of OETA-PC were detected in the commercial PL-PC. The amounts of OETA-PC detected in the commercial PL-PC were subtracted from the initial values of the oxidation product. The consumption of PL-PC was greatest under UV light (Figure 4),

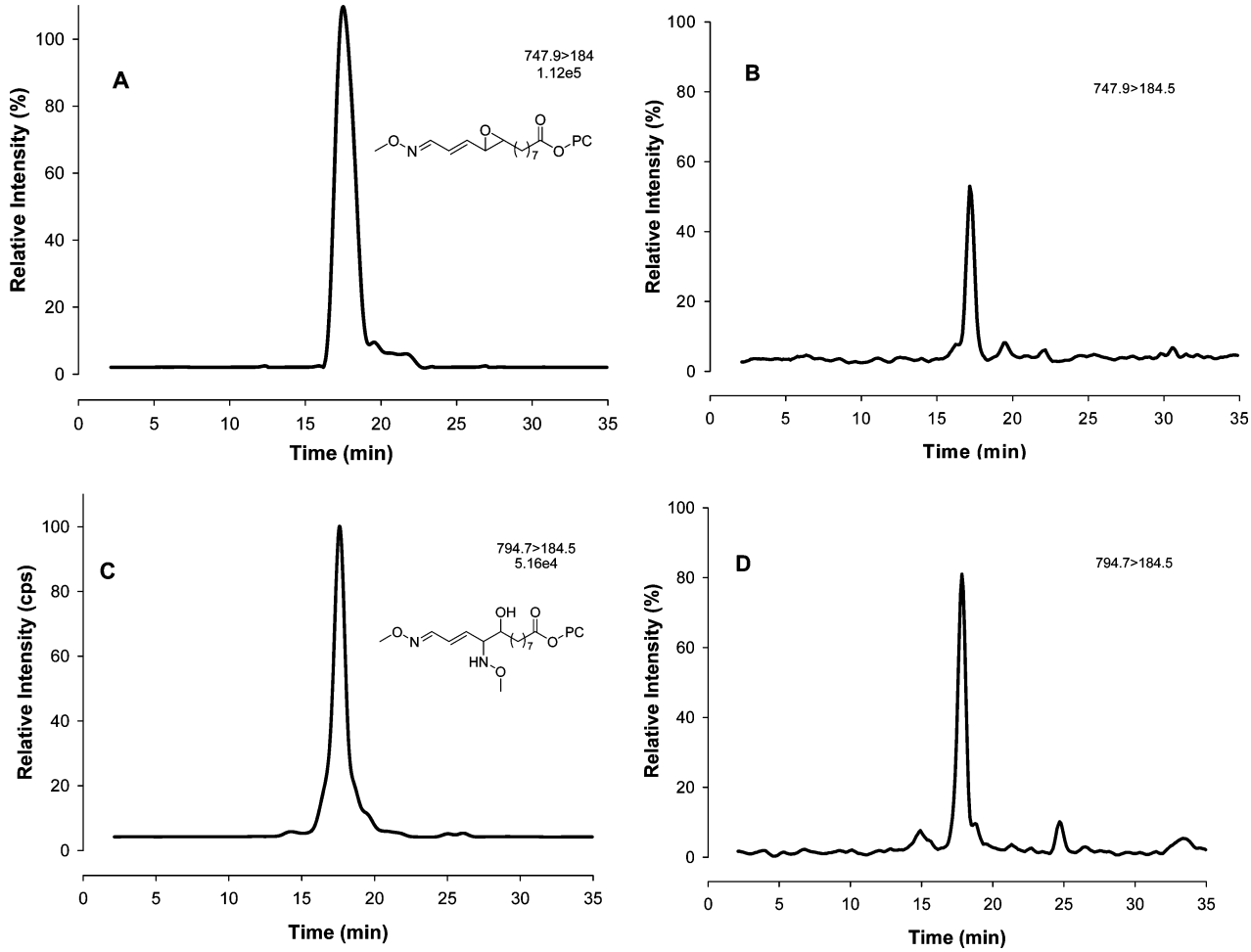


Figure 3. LC/ESI-MS/MS analysis of OETA-PC derivatives. (A) The methoxime of OETA-PC standard (parent *m/z* 747.9), MRM chromatogram (daughter *m/z* 184). (B) The methoxime of OETA-PC detected in derivatized oxidized PL-PC liposomes, MRM chromatogram (747.9→184.5). (C) The bis-methoxylamine adduct from OETA-PC standard (parent *m/z* 794.7), MRM chromatogram (daughter *m/z* 184). (D) The bis-methoxylamine adduct of OETA-PC detected in derivatized oxidized PL-PC liposomes, MRM chromatogram (794.7→184.5).

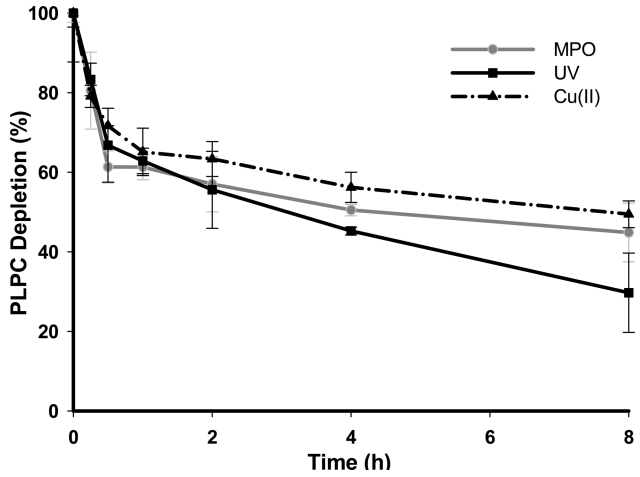


Figure 4. Consumption of PL-PC under various oxidation conditions. Data are the average of two sets of independent experiments.

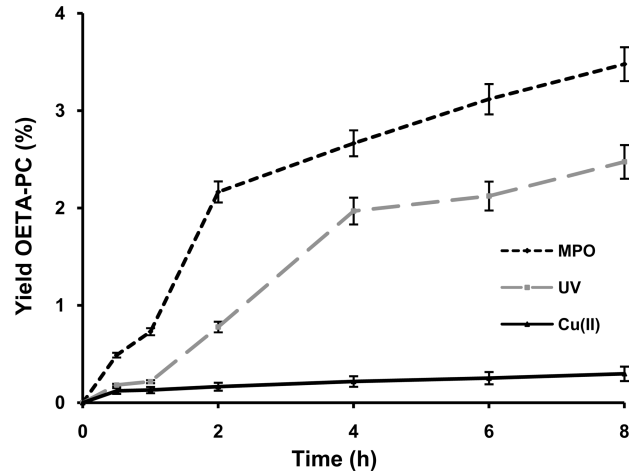


Figure 5. Evolution profile for production of OETA-PC from aerial oxidation of PL-PC liposomes promoted by Cu(II), UV, or MPO at 37 °C. Quantification was achieved with LC/ESI-MS/MS. The yields were calculated by dividing the amount of each analyte by the amount of starting PL-PC. Data are the average of two sets of independent experiments.

Table 2. Amounts of OETA-PC Per Rat Retina[a]

animal	OETA-PC (pmol) average*
1	0.27 ± 0.01
2	0.42 ± 0.32
3	0.47 ± 0.30
4	0.20 ± 0.30
5	0.30 ± 0.08

[a] Amounts are the average of four measurements from both eyes of same animal.

but the yield of OETA-PC was higher when the vesicles were oxidized with the MPO-NO_2^--glucose/glucose oxidase system (Figure 5).

The product evolution profiles for oxidation promoted by the MPO-NO_2^--glucose/glucose oxidase system and UV are similar, rising steadily in the first 2 h and more slowly afterward. The Cu(II) oxidation resulted in a much lower yield of OETA-PC. This may be because Cu(II) not only catalyzes the oxidation of PL-PC (**6**) but also promotes further oxidation of OETA-PC to other products, for example, the corresponding carboxylic acid. Table 2 shows the maximum yields for OETA-PC produced by different oxidation methods.

Quantification of OETA-PC in Rat Retina. Because many reports indicated that light damage induces the peroxidation of PUFAs in retina, we expected that the peroxidation of PL-PC in retina would produce OETA-PC as found in our in vitro experiments. Other oxidized PCs (oxPCs), derived from PL-PC, were identified previously in rat retina (*3*). To examine if OETA-PC is present in intact retinas, retinas harvested from albino rats were analyzed by LC-ESI/MS/MS. The retinas from five rats were harvested. To prevent contamination by blood, or in vitro oxidation, the retinas were rinsed with a saline antioxidant cocktail (PBS, DTPA, and BHT). The retinas were immediately homogenized manually in a plastic vial using a stainless steel pestle coated with Teflon. Lipid extraction using the procedure of Bligh and Dyer (*30*) was then performed immediately after homogenization. The extracted lipids were analyzed by LC-ESI/MS/MS in the positive ion mode. After the solvents were removed, the residue was redissolved in chloroform:methanol (1:1), one-fifth of the sample was injected into the LC-MS. OETA-PC, and the standard DM-PE was monitored simultaneously. Figure 6B presents the reconstructed MRM chromatogram showing the presence of OETA-PC in rat retina. The retention time (17.1 min), parent ion, and daughter ion were collectively used to identify LC-ESI/MS/MS peak by comparison with the authentic standard (Figure 6A) as described above for monitoring the generation of OETA-PC during in vitro oxidation of PL-PC.

Multiple peaks are present in the chromatogram of the rat retina sample (Figure 6B). A peak at 17.1 min has the same retention time and MRM as the authentic OETA-PC (Figure 6B). Because they produce a 184.5 Da fragment, the other peaks in Figure 6B apparently correspond to PCs that are isomeric with OETA-PC. To further verify the structure of the HPLC peak presumed to be OETA-PC, the sample from the rat retina was derivatized with methoxylamine hydrochloride (Figure S20 of the Supporting Information).

Before reaction with methoxylamine, no peaks corresponding to the mono and bis methoxylamine derivatives appeared (Figure S21 of the Supporting Information). After derivatization, new peaks appeared, corresponding to the mono and bis methoxylamine adducts with a concomitant disappearance of the peak corresponding to OETA-PC. The chromatogram of a methoxime derivative of pure OETA-PC, prepared as described in the previous paragraph, is presented in Figure S20A of the Supporting Information. A poorly resolved peak in the chromatogram of the derivatized rat retina extract (Figure S20B of the Supporting Information) exhibited the same net increase of 29 Da and retention time in the MRM as the derivatized standard. This corresponds to the addition of one molecule of methoxylamine and loss of water to generate a methoxime derivative of the aldehyde group. A well-resolved peak in the chromatogram of the derivatized rat retina extract (Figure 7D) exhibited the same additional increase of 47 Da and retention time MRM as the standard bis adduct (Figure S20C of the Supporting Information). This corresponds to the addition of a second molecule of methoxylamine by nucleophilic attack on the epoxide group.

The absolute amounts of OETA-PC detected in five rats are presented in Table 2. The values were calculated employing a calibration curve and extraction coefficient determined by LC/ESI-MS/MS as described in the experimental part.

The average amount of OETA-PC in rat retina, 0.33 pmol, is relatively low, as compared to oxPCs derived from DHA-PC, for example, HOHA-PC (2.5 pmol) or 4-keto-7-oxohept-5-enoic acid (KOHA)-PC (1.7 pmol). In part, this is because PL-PC is present in lower amounts (*3*) than DHA-PC in the rat retina (see Table 3).

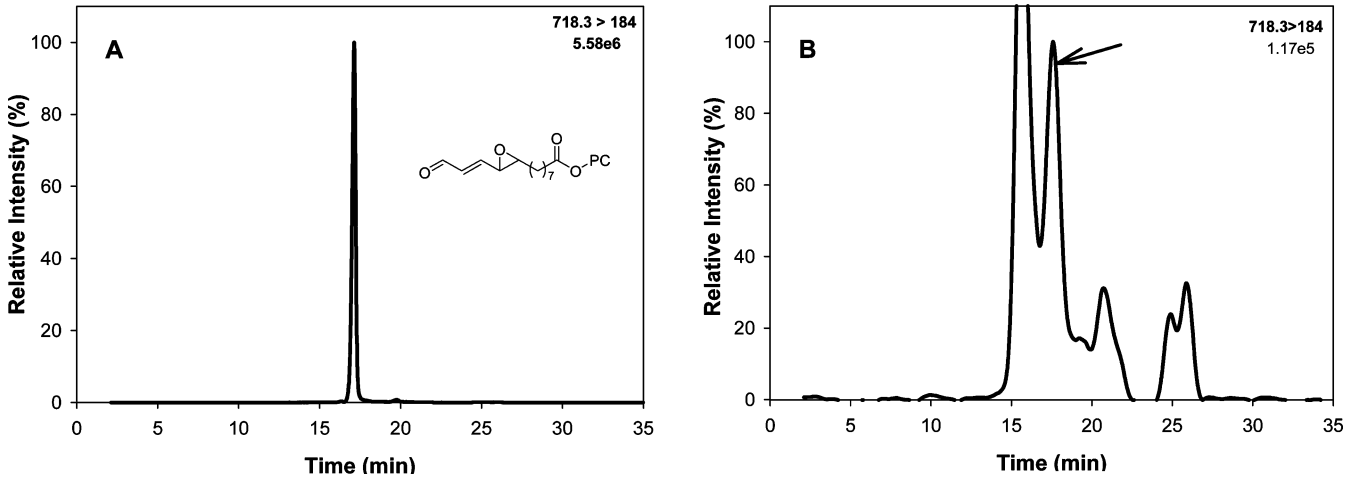


Figure 6. LC/ESI-MS/MS detection of OETA-PC (718.3→184.5) from rat retina. (A) Synthetic standard OETA-PC. (B) Rat retina extract.

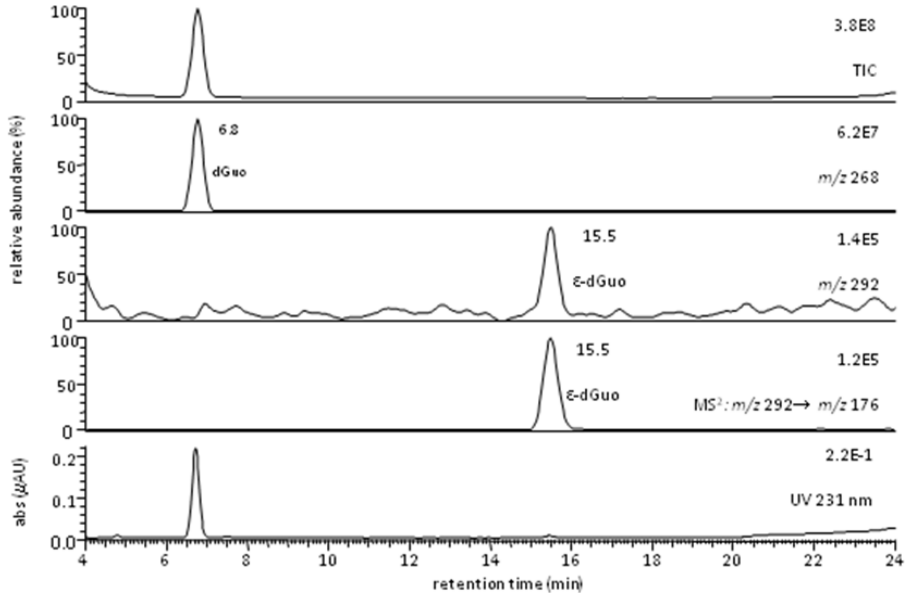


Figure 7. LC-APCI/MS analysis of reaction between OETA-PC and dGuo at 60 °C for 48 h.

Table 3. Amounts of Some PLs Detected Per Rat Retina (*3*)[a]

PL	absolute amount (nmol)
PA-PC	2.64 ± 0.32
PL-PC	2.06 ± 0.32
DHA-PC	7.0 ± 0.24
HOHA-PC	0.0025 ± 0.0023
KOHA-PC	0.0017 ± 0.0012

[a] Amounts are the average of four measurements from both eyes of the same animal.

Nucleotide Etheno Adduct Formation by OETA-PC. Reaction with dAdo. A solution of OETA-PC was added to dGuo or dAdo in Chelex-treated phosphate buffer, and the mixture was incubated for 48 h at 60 °C. LC/APCI/MS analysis of the reaction product mixture from dGuo after 48 h revealed the presence of one major adduct together with residual dGuo. The dGuo adduct eluted at a retention time of 15.5 min in system 1 (Figure 7). Its APCI mass spectrum revealed an intense MH^+ at *m/z* 292, together with a BH_2^+ ion at *m/z* 176. MS^2 analysis MH^+ (*m/z* 292) resulted in a BH_2^+ product ion at *m/z* 176. These LC/MS characteristics are identical to those for authentic1,N^2-etheno-dGuo (*19*).

Reaction with dGuo. LC/APCI/MS analysis of the reaction mixture from dAdo after 48 h using gradient system 1 revealed the presence of one dAdo adduct (retention time, 21.0 min) together with residual dAdo at a retention time of 12.8 min (Figure 8). The LC/MS characteristics of this adduct were identical to authentic 1,N^6-etheno-dAdo (*19*). Its mass spectrum exhibited an intense MH^+ at *m/z* 276, together with a BH_2^+ ion at *m/z* 160. MS^2 analysis of MH^+ *m/z* 276 gave rise to exclusive formation of the BH_2^+ product ion at *m/z* 160.

Formation of Etheno-dGuo and Etheno-dAdo in Calf Thymus DNA. Calf-thymus DNA was treated with an approximately equimolar amount of OETA-PC. The DNA was then hydrolyzed with a mixture of DNase I, nuclease P1, and alkaline phosphatase in MOPS buffer. These hydrolysis conditions were shown to give essentially quantitative recovery of normal DNA bases. Modified DNA bases were separated from normal bases using the SPE procedure described in the Materials and Methods. Etheno-dGuo and etheno-dAdo were detected in the DNA hydrolysate (Figure 9). On the basis of a 71% recovery through the extraction and hydrolysis procedure, the signals for etheno-dGuo and etheno-dAdo corresponded to 3.5 and 5.5 adducts/10 (*7*) normal bases, respectively.

Discussion

An efficient, practical, total synthesis was accomplished for OETA and OETA-PC starting from a commercially available

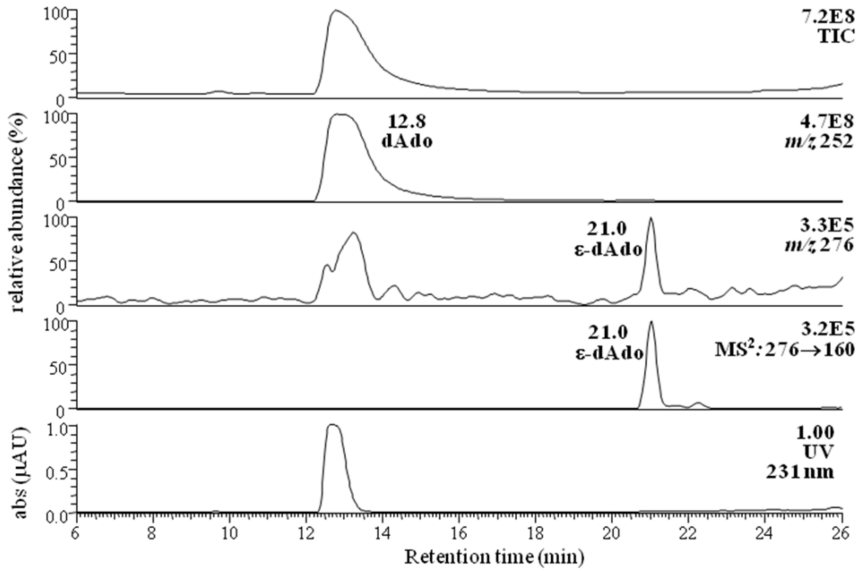


Figure 8. LC-APCI/MS analysis of reaction between OETA-PC and dAdo at 60 °C for 48 h.

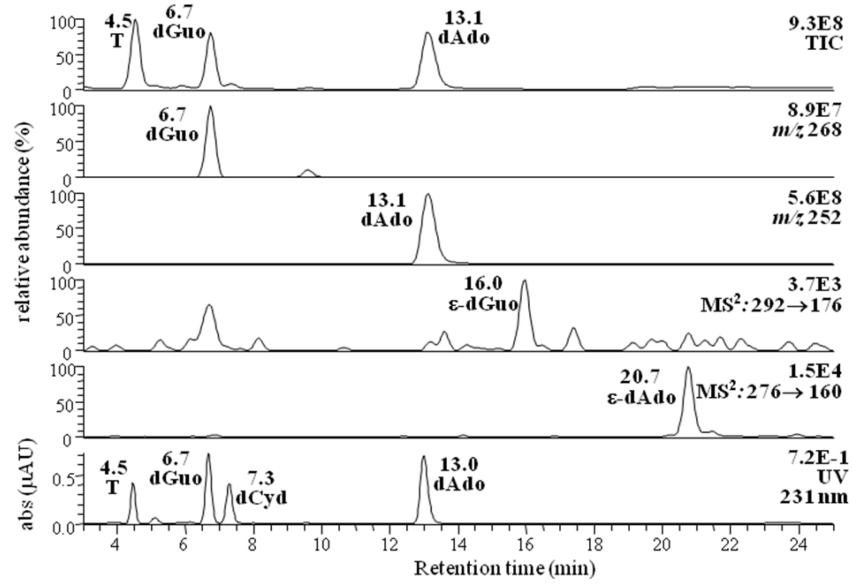
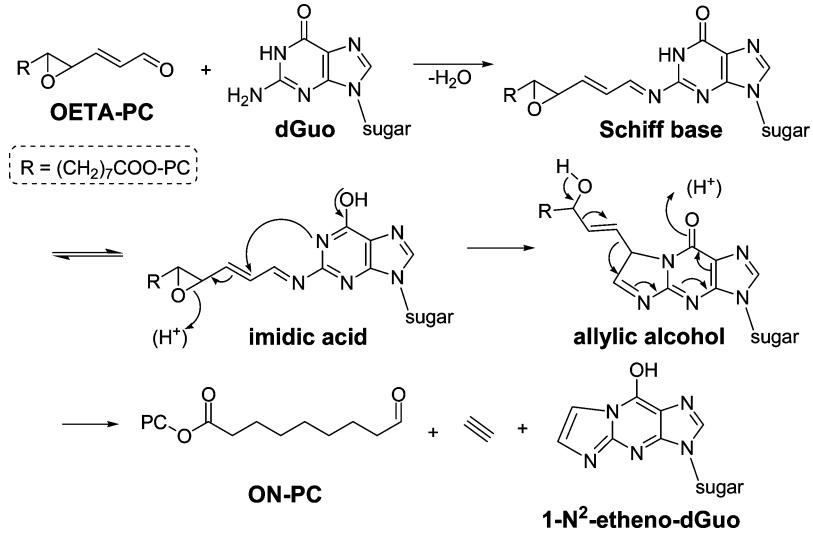


Figure 9. LC-APCI/MS analysis of reaction of OETA-PC with calf thymus DNA at 60 °C for 48 h.

Scheme 5. Plausible Mechanism for Formation of 1,N^2-Etheno-dGuo



bromo-alcohol. This synthesis provides ready access to adequate amounts of the pure lipids needed for biological studies. The pure OETA-PC served as a standard that enabled the identification of this epoxyaldehyde in the oxidation product mixture from

PL-PC initiated via three different methods: Cu(II), MPO, and UV light. The MPO treatment gave the highest yield (3.4%), while the Cu(II) oxidation produced OETA-PC in the lowest amount (0.2%). Because the yield obtained by UV induction was higher (2.4%) than the one by Cu(II), we considered that OETA-PC might be formed in vivo in UV-exposed organs, for example, the eye. OETA-PC was also identified in vivo in the lipid extract of the rat retina. The presence of numerous lipids in this extract that apparently react with 1 and 2 equiv of methoxylamine (see Figure S20B,D of the Supporting Information) is the reasonable consequence of the formation of a mixture of stereo- and regioisomers during the free radical-induced oxidation of LA-PC in vivo. Our synthetic standard is the pure *trans*-9,10-epoxide. Both *cis*- and *trans*-3,5-EDE are formed upon oxidative fragmentation of LA hydroperoxides (*32*). It is highly likely that *cis*-OETA-PC is also present among the oxidatively truncated PLs present in retina.

OETA-PC forms mutagenic etheno adducts upon reaction with dAdo and dGuo, as well as with the DNA. We did not look for the DNA adducts in the rat retina. The DNA adducts are not formed in high yields from OETA-PC and DNA (3.5 or 5.5 adducts/10^7 normal bases). OETA-PC is also not very abundant in the retina, so we did not expect to be able to detect these adducts. Future studies will look for the DNA adducts extracted from blood or epithelial cell lines, which endocytose lipoproteins, since PL-PC is one of the major constituents of LDL. Furthermore, the detection of the DNA adducts in vivo will most likely require additional enhancement in the sensitivity, possibly by immunoafinity purification. A plausible mechanism for the reaction of OETA-PC with dAdo or dGuo to give the *unsubstituted* etheno-dAdo adduct 1,N^6-etheno-dAdo or 1,N^2-etheno-dGuo (see Scheme 2) is presented in Scheme 5 for the case of dGuo.

Condensation of the aldehyde group of OETA-PC with the primary amino group of dGuo generates the corresponding Schiff base. Then, S_Ni attack of a nitrogen nucleophile in the corresponding imidic acid tautomer opens the allylic epoxide to generate an allylic alcohol. A Grob fragmentation (a vinylogous retroaldol reaction) of this intermediate delivers etheno-dGuo, 9-oxononanoyl-PC, and acetylene. The energy released because of aromatization of the tricyclic purine-derived base in 1,N^2-etheno-dGuo and the favorable entropy of this fragmentation reaction,that is, decomposition of the allylic alcohol intermediate into three molecules, facilitate the cleavage of two C−C bonds (*19*). The proposed pathway is in agreement with the finding that 4,5-EDE is stable under the reaction conditions *in the absence of dGuo or dAdo* (*19*).

As noted above, the relatively polar acyl chains of oxidatively truncated PLs protrude from membranes into the aqueous phase. This may contribute to their uniquely high susceptibility to lipolysis, for example, by platelet-activating factor-acetylhydrolase, which hydrolyzes the truncated acyl groups of oxPCs in addition to platelet-activating factor (*33, 34*). Therefore, it can be anticipated that the formation of OETA-PC in vivo will be accompanied by the release of the free acid OETA that is also expected to react with DNA to produce mutagenic etheno derivatives. Linoleic-containing PC is the major PL in lipoproteins, and thus, in cells that endocytose oxidized lipoproteins, OETA-PC may be an important DNA-damaging agent.

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Supporting Information Available: ^1H and ^{13}C NMR spectra of new compounds and a description of the statistical data analysis for rat retina MS quantification of OETA-PC. This material is available free of charge via the Internet at http://pubs.acs.org.

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