γ-Hydroxyalkenals Are Oxidatively Cleaved through Michael Addition of Acylperoxy Radicals and Fragmentation of Intermediate β-Hydroxyperesters

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Abstract: Oxidative cleavage of arachidonate (C_{20}) and linoleate (C_{18}) phospholipids generates truncated C_8 or C_{12} γ-hydroxyalkenal phospholipids as well as C_5 or C_9 carboxyalkanoate phospholipids, which are abundant in atherosclerotic plaques. The γ-hydroxyalkenals promote foam cell formation by scavenger receptor CD36-mediated endocytosis. The carboxyalkanoates are potent regulators of endothelial cell functions that may promote atherogenesis. We now report an unexpected biosynthetic interconnection; the carboxyalkanoates can be generated through oxidative cleavage of the γ-hydroxyalkenals with the loss of three carbons. This unprecedented transformation is shown to involve Michael addition of an acylperoxy radical and fragmentation of the resulting β-hydroxyperester.

Lipid peroxidation is the subject of intense current interest. Mounting evidence indicates that lipid peroxidation is involved in the pathogenesis of many chronic diseases, e.g., atherosclerosis, Alzheimer's disease, Parkinson disease, stroke, as well as in aging.[1] The most abundant polyunsaturated lipids in human low-density lipoprotein (LDL), the so-called "bad cholesterol", are esters of arachidonic acid (AA) and linoleic acid (LA) with glycerol, cholesterol, or 2-lyso-phosphatidylcholine (PC).[2] Oxidative fragmentation of both AA-PC (**1a**) and LA-PC (**1b**) generates 4-hydroxy-2-nonenal (HNE, **2**) that is a key mediator of oxidative stress.[3] This γ-hydroxyalkenal has been widely studied because it is a cytotoxic electrophile that covalently modifies proteins resulting, inter alia, in enzyme inhibition.[4–6] Its proclivity toward forming Michael adducts with biological thiols underlies its ability to inactivate a large number of thiol-dependent enzymes, in particular cysteine proteases.[7–9] Recent studies revealed that free radical-induced oxidative fragmentation of polyunsaturated phospholipids **1** generates truncated ω-oxoalkanoate phospholipids **3** as well as γ-hydroxyalkenal phospholipids **5**, which are analogues of HNE, and γ-ketoalkenal phospholipids **7** (Scheme 1). Further oxidation produces the corresponding carboxylic acids **4**, **6**, and **8**, respectively.

Scheme 1
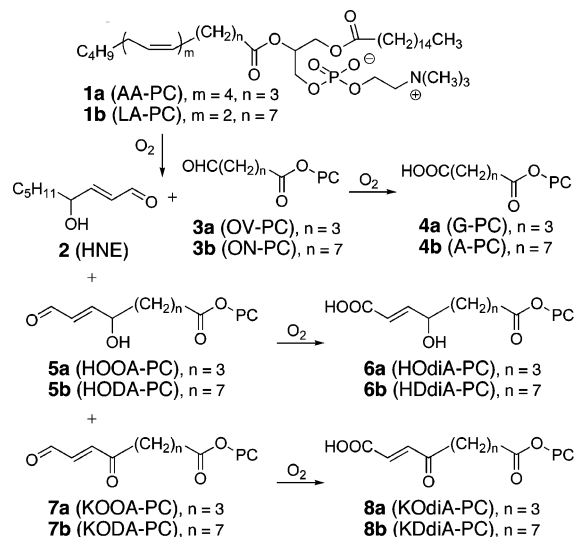
Our interest in oxidatively truncated phospholipids was aroused by a lecture at the Cleveland Clinic Foundation in which Dr. A. M. Fogelman reported that the 5-oxovaleric acid ester **3a** (OV-PC), which is generated upon free radical-induced oxidation of 2-arachidonyl phosphatidylcholine (**1a**, AA-PC), possesses important biological activities. During our effort to confirm the structure of **3a** with an unambiguous synthesis, we inadvertently generated traces of another product of AA-PC oxidation, the glutarate monoester **4a** (G-PC), which showed even greater biological activity than the aldehyde **3a**.[10] Both

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Scheme 2. Mechanistic Hypothesis for Oxidative Fragmentation of γ-Hydroxy-α,β-unsaturated Aldehydes
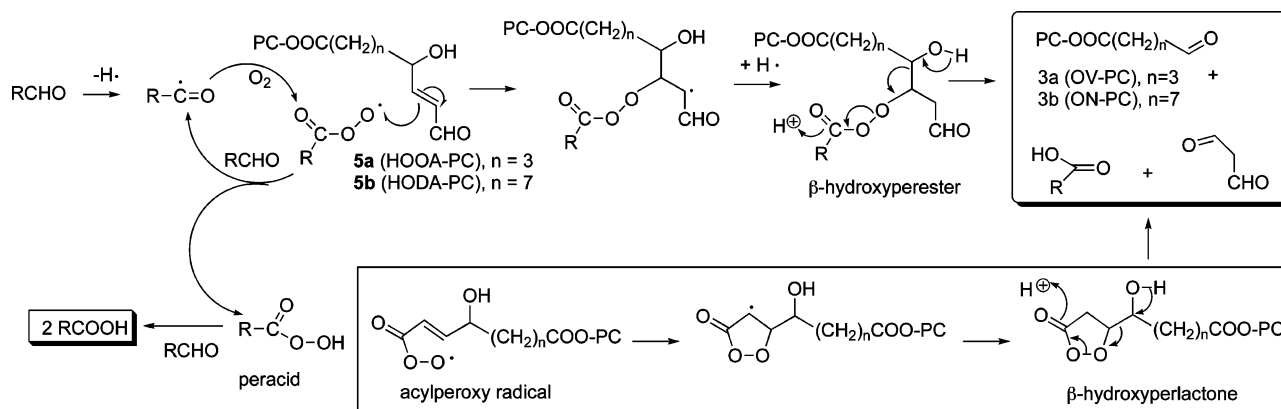
OV-PC and G-PC activate endothelial cells to bind monocytes and, consequently, may promote atherogenesis by fostering entry of monocytes into the vessel wall. Others have also reported the free radical-induced oxidative generation, natural occurrence and biological activities of the aldehydes OV-PC (**3a**) and ON-PC (**3b**),[11–14] as well as G-PC (**4a**) and the corresponding azelate monoester **4b** (A-PC).[14–17]

Total syntheses[18,19] also facilitated our discoveries that another biologically active truncated phospholipid, 5-hydroxy-8-oxo-6-octenoic acid ester **5a** (HOOA-PC), is produced upon oxidation of AA-PC,[20] and that oxidation of the linoleic acid ester LA-PC generates the 9-hydroxy-12-oxo-10-dodecenoic acid ester **5b** (HODA-PC) as well as the keto analogues KOOA-PC (**7a**) and KODA-PC (**7b**) and the carboxylic acids **6** and **8**.[21,22] All of the γ-oxgenated α,β-unsaturated aldehydes and carboxylic acids **5–8** are strong ligands for the scavenger receptor CD-36 of macrophage cells. They promote recognition of oxidatively damaged low-density lipoprotein (oxLDL) leading to unregulated endocytosis. This avid uptake contributes to the accumulation of cholesterol esters and the production of foam cells and atherosclerotic plaques. HODA-PC also may impair processing of oxLDL in macrophage cells because it inhibits the cysteine protease cathepsin B and blocks essential posttranslational modification of the fusion protein Rab5a that is required for maturation of endosomes.[23]

In view of their diverse biological involvements, understanding the chemistry of γ-hydroxyalkenals, such as HNE, HOOA-

PC, and HODA-PC, is especially important. We now find that *γ-hydroxyalkenals are susceptible to further oxidative fragmentation with the loss of three carbons to generate shorter chain carboxylic acids*. Our investigation of this oxidative fragmentation indicates an unprecedented mechanism involving *Michael addition of a peracyl radical and fragmentation of the resulting β-hydroxyperester*.

While preparing oxidized phospholipids and studying their biological activities, we discovered that γ-hydroxyalkenal phospholipids readily fragment, e.g., ON-PC (**3b**) is produced upon autoxidation of HODA-PC (**5b**). The formation of ON-PC from HODA-PC is noteworthy not only because it involves novel chemistry but also because these two phospholipids exhibit divergent biological activities. Therefore, the mechanism of this process not only is chemically interesting but also may be biologically important. We postulated that the well-known reaction of aldehydes with oxygen to give carboxylic acids and the fragmentation of HODA-PC (**5b**) to give ON-PC (**3b**) are interrelated. The aerobic conversion of aldehydes to carboxylic acids involves peracid intermediates that undergo a Bayer–Villiger reaction with a second equivalent of aldehyde to produce 2 equiv of carboxylic acid (Scheme 2).

A plausible mechanism for the oxidative fragmentation involves diversion of an intermediate acylperoxy radical precursor of the peracid. Thus, Michael addition of the acylperoxy radical to HODA-PC followed by hydrogen transfer to the resulting carbonyl-stabilized radical would produce a β-hydroxyperester. Fragmentation of the β-hydroxyperester should occur readily because the reaction generates two carbonyl groups, one in a malondialdehyde byproduct and one in ON-PC (**3b**). An alternative intramolecular Michael addition to generate an intermediate β-hydroxyperlactone, is less likely. Thus, although 5-endo-trig radical cyclizations are not unknown,[24] they are generally disfavored stereoelectronically.[25] We now report mechanistic studies that indicate the feasibility of a β-hydroxyperester mechanism for the autoxidative fragmentation of γ-hydroxyalkenals.

Results and Discussion

Autoxidation of HODA-PC. A quantitative assessment was conducted on the autoxidation of HODA-PC (**5b**) at 37 °C. We

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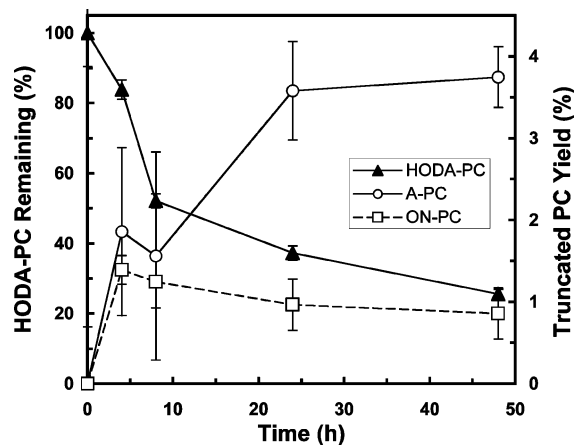
Figure 1. Oxidative fragmentation of HODA-PC.

confirmed the identities and determined the time course for generation of the fragmentation products ON-PC (**3b**) and A-PC (**4b**). After 8 h, half the HODA-PC had been consumed and ON-PC and A-PC were each produced in about 1% yield (Figure 1). Presumably fragmentation continues to generate ON-PC, but this aldehyde is consumed by further autoxidation to produce A-PC. Thus, while the yield of the truncated aldehyde ON-PC remained constant at about 1%, the yield of the truncated acid A-PC gradually rose within 1 day to about 4%.

Autoxidation of HODA. Two autoxidation products (m/z 187 and m/z 243) were identified in the product mixture (Figure 2A) from autoxidation of the aldehydic acid HODA (**9**). The oxidation reaction mixture was injected into an LC-MS by infusion and analyzed by full scan in the negative ion mode. An anion with m/z 187 was shown to be the $[M - H]^-$ of azeleic acid (**11**), and an anion with m/z 243 was shown to be the $[M - H]^-$ of 4-hydroxydodec-2-enedioic acid (**10**). MS/MS
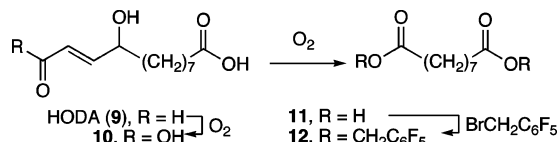
analysis of these ions produced the same fragmentation patterns (Figure 2B and D) as those from authentic **10** and **11** (Figure 2E and C). The identity of azeleic acid (**11**) was further confirmed by derivatization with pentaflurobenzyl bromide (PFBBr). After derivatization, the diPFB ester **12** of azeleic acid (**11**) appeared in the MRM chromatogram in the positive ion mode and exhibited the same retention time as authentic azeleic acid diPFB ester (Figure 3).

To monitor the disappearance of HODA (**9**) and the appearance of oxidation products, diacid **10** and azeleic acid (**11**) were quantified using LC/ESI/MS/MS (see Experimental Procedures for details). Only about 40% of HODA (**9**) remains after heating in air for 4 h. Diacid **10** accounted for about 4% of HODA (**9**) consumed after 18 h and azeleic acid **11** accounted for about 2% (Figure 4).

Comparison of the rates of autoxidation of HODA and HODA-PC suggests that the phosphatidylcholine headgroup has little or no effect on the rate of autoxidation. Thus, the 3-fold higher rate for HODA ($t_{1/2} \approx 3$ h) versus HODA-PC ($t_{1/2} \approx$ 8 h) is the expected consequence of the 3-fold higher concentration of neat HODA ($\sim$4.2 M) versus neat HODA-PC ($\sim$1.4 M).
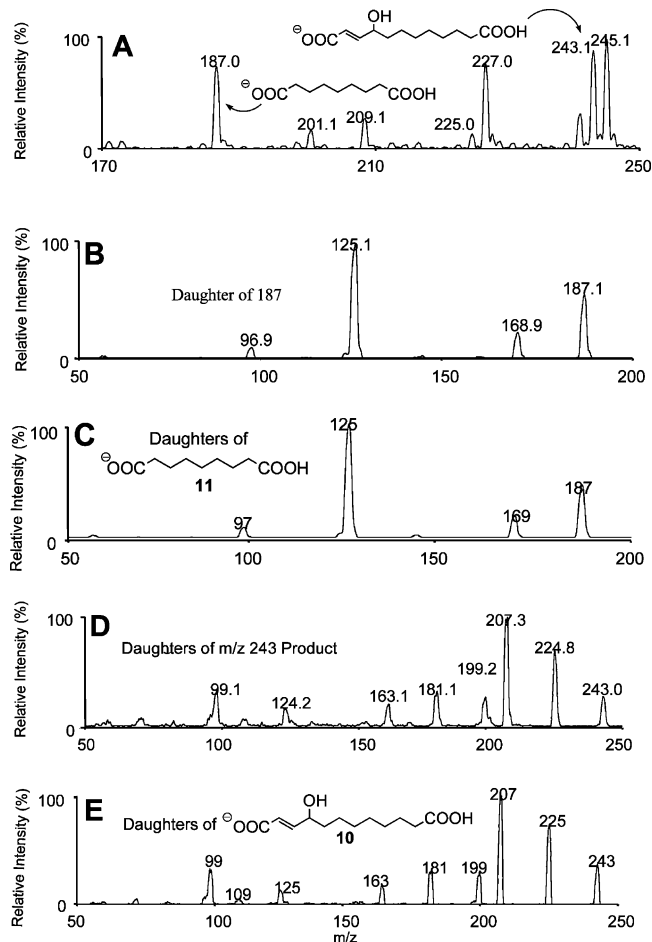
Figure 2. ESI-MS/MS analysis of oxidation product mixture from HODA (**9**). (A) Negative ion full scan spectrum of HODA (300 μg) after heating at 37 °C for 24 h. (B) MS/MS of m/z 187 product. (C) MS/MS of authentic azeleic acid (**11**). (D) MS/MS of m/z 243 product. (E) MS/MS of authentic dicarboxylic acid **10**.
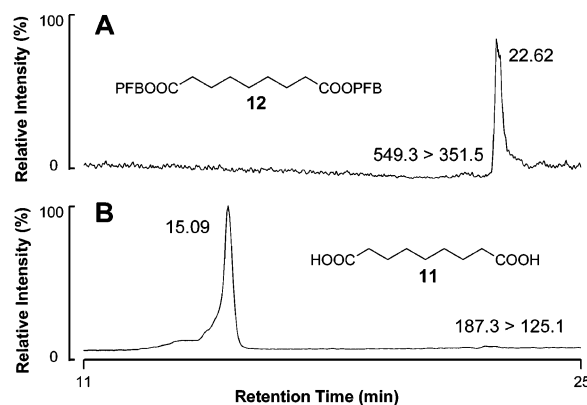

Figure 3. Chromatogram of azeleic acid from oxidized HODA before and after pentafluorobenzyl ester derivatization. (A) Diester **12** was detected by multiple reaction monitoring (MRM) in the positive ion mode after derivatization. (B) Diacid **11** detected by MRM in the negative ion mode before derivatization.

A possible alternative mechanism for oxidative truncation of hydroxyalkenals **5** involves retroaldol cleavage of the hydration product **13** (Scheme 3). Reaction of the resulting α-hydroxyaldehyde **14** with a peracid, generated by autoxidation of an aldehyde, is expected to lead to a formic hemiacylal **16** through Criegee rearrangement of a hemiperacylal **15**. Similar rearrangements are known, and the postulated intermediacy of
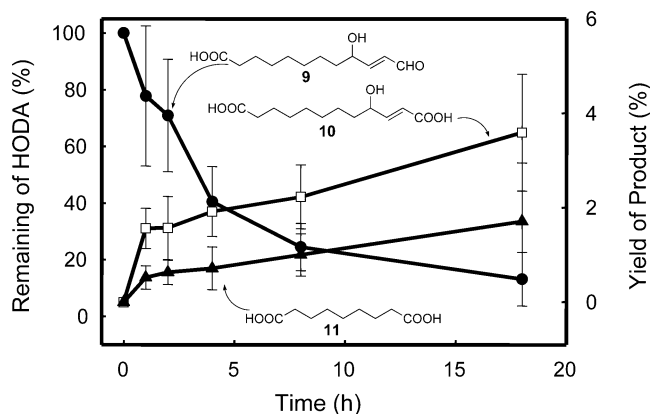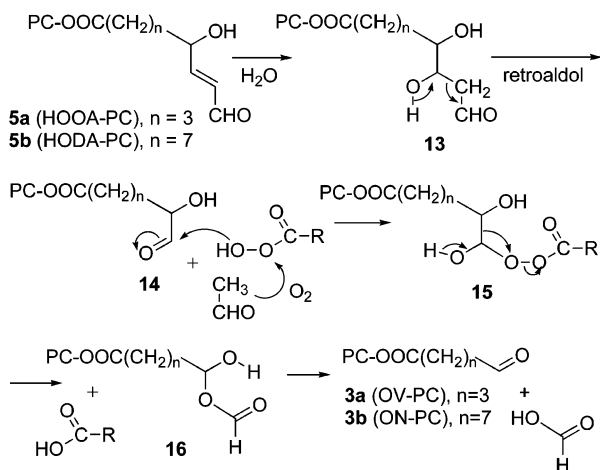
Figure 4. Evolution profiles of azeleic acid (**11**) and diacid **10** from HODA (**9**) heated at 37 °C in air.
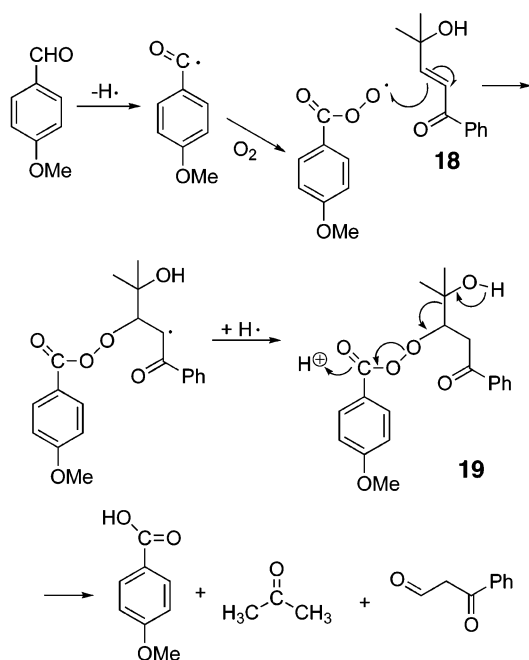
Scheme 3

formic hemiacylals is supported by the production of hexadecyl formate when the reaction is conducted in the presence of hexadecanol.[26] We favor the mechanism of Scheme 2 over this alternative because a similar fragmentation was observed in *anhydrous* benzene for a γ-hydroxyenone **18** (vide infra) in the presence of anisaldehyde while *no reaction* occurred in the absence of anisaldehyde. In this case, a hydration–retroaldol fragmentation sequence is precluded, and no evidence for it was found.

Coautoxidation of (*E*)-4-Hydroxy-4-methyl-1-phenyl-2-penten-1-one (18) with Anisaldehyde. Because the γ-hydroxyenone **18** lacks an aldehyde group (Scheme 4), it cannot undergo autoxidative fragmentation analogous to that proposed in Scheme 2. However, it should undergo Michael addition of an aldehyde-derived acylperoxy radical in analogy with the reaction of Scheme 2. Therefore, as outlined in Scheme 4, coautoxidation of γ-hydroxyenone **18** with anisaldehyde should generate the fragmentation products acetone and α-formylacetophenone.

To test this hypothesis we reacted a mixture of γ-hydroxyenone **18** and anisaldehyde in deuterated benzene solvent in a sealed glass tube under oxygen. The ^{1}H NMR signal for acetone in C6D6 appears as a singlet at δ 1.56 ppm, whereas in CDCl3 it appears as a singlet at 2.17 ppm. The peaks corresponding to benzene and chloroform were referenced at 7.157 and 7.27 ppm, respectively. After 72 h of heating at 55 °C, a resonance at δ

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Scheme 4

1.56 was readily apparent in the reaction product mixture that coincides with the chemical shift of acetone in C6D6. Similar results were observed upon coautoxidation of **18** and anisaldehyde in CDCl3 except that the resonance for acetone appeared at δ 2.17.

Derivatization of Acetone with Phenylhydrazine. After coautoxidation of γ-hydroxyenone **18** with anisaldehyde in C6D6 at 55 °C for 3 days, excess phenylhydrazine was added to the reaction mixture. After half an hour, the acetone resonance at δ 1.56 disappeared and a peak at δ 1.79, characteristic of acetone phenylhydrazone in C6D6, was identified by comparison with an authentic sample. Karabatsos[27] observed that bubbling oxygen through a benzene solution of acetone phenylhydrazone generates a hydroperoxide (Me2C(OOH)—N=NC6H5) that exhibits a singlet at δ = 1.47. After 24 h, our acetone phenylhydrazone NMR spectrum showed a peak at 1.47 ppm. Confirmation of the formation of acetone phenylhydrazone was achieved by mass spectroscopic comparison of authentic acetone phenylhydrazone. Thus, MS/MS analysis was performed on the product generated after treatment of the autoxidation reaction mixture with phenylhydrazine. The full scan of the reaction mixture in the positive ion mode (Figure 5B) shows parent [M + H]+ ions at *m/z* 149 and 227 corresponding to the phenylhydrazones of acetone and anisaldehyde, respectively. The MS/MS spectrum of the reaction product *m/z* 149 parent ion produces daughter ions (Figure 5A) that are identical with those from an authentic sample of acetone phenylhydrazone (Figure 5C). Figure 5D shows the *m/z* 149 corresponding to the [M + H]+ parent ion peak of authentic acetone phenylhydrazone. This confirms that coautoxidation of γ-hydroxyenone **18** and anisaldehyde generates acetone as predicted by the β-hydroxyperester fragmentation hypothesis.

Yield of Acetone and Time Course of the Fragmentation Reaction. To monitor the time course for fragmentation of the γ-hydroxyenone **18** during coautoxidation with anisaldehyde,

(27) Karabatsos, G. J.; Taller, R. A. *J. Am. Chem. Soc.* **1963**, *85*, 3624–3629.
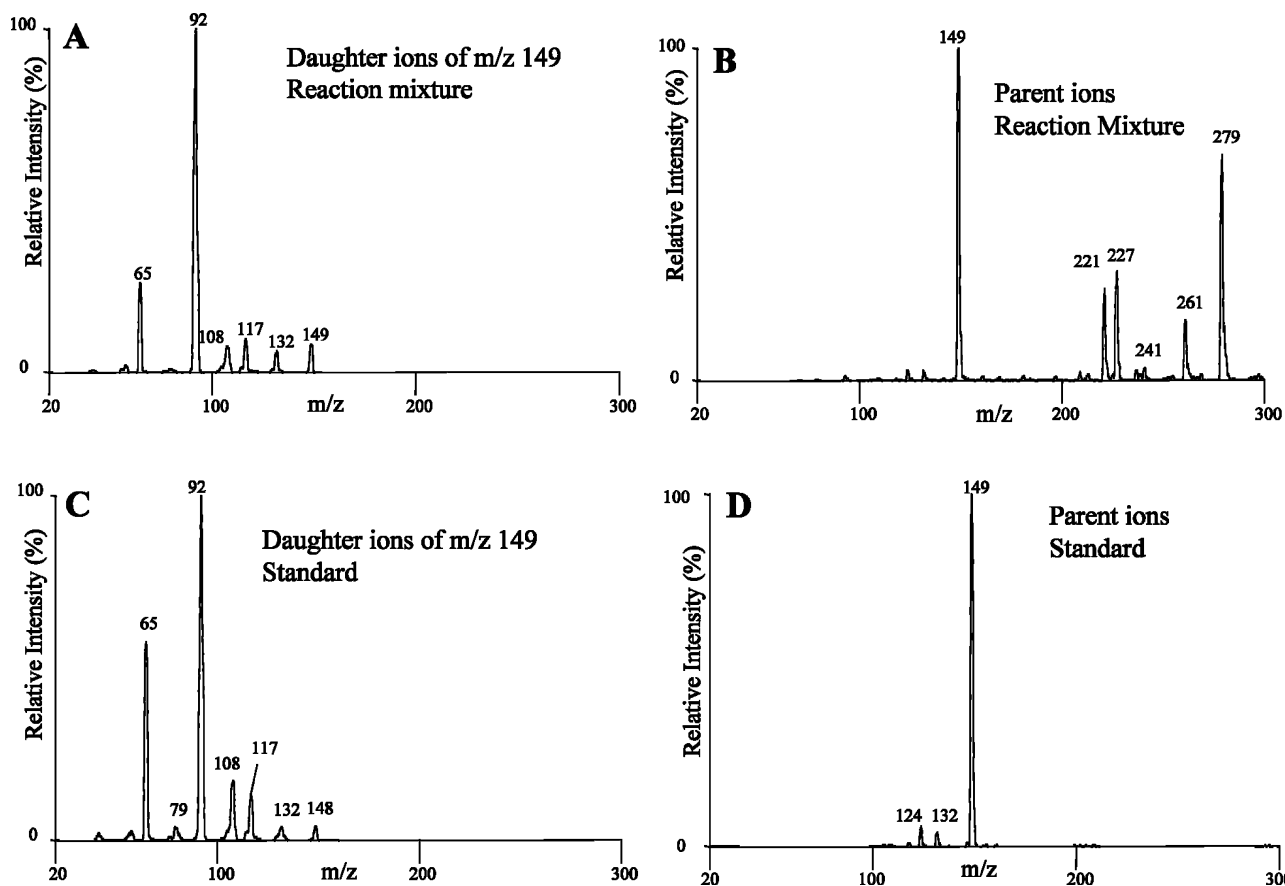
Figure 5. Mass spectra of acetone phenylhydrazone derivative. Parent ion (B) and daughter ions (A) corresponding to *m/z* 149 of the of acetone phenylhydrazone produced in the reaction product mixture after treatment with phenylhydrazine. Parent ion (D) and daughter ions (C) corresponding to *m/z* 149 of authentic acetone phenylhydrazone.
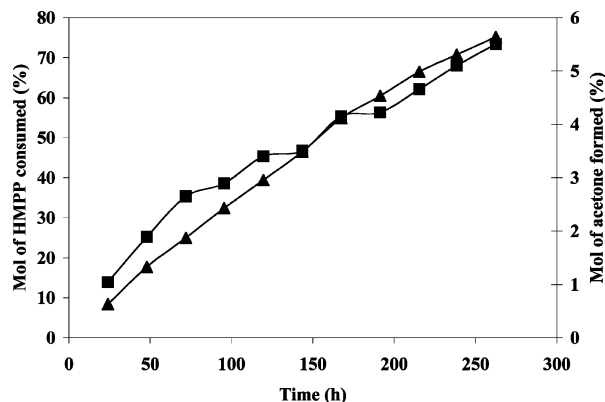

**Figure 6.** Formation of acetone (■) as γ-hydroxyenone **18** is consumed (▲).

we incubated a mixture of **18** and anisaldehyde in deuterated benzene in the presence of oxygen in a sealed NMR tube at 55 °C for 12 days. Periodically NMR spectra were recorded. Since the absorption arising from the methoxy singlet in anisaldehyde and in anisic acid have the same chemical shift at δ 3.11, this resonance was exploited as an internal reference standard for calculating the amount of acetone formed in the reaction. Figure 6 shows the liner plot of the percentage consumption of γ-hydroxyenone **18** (determined by integrating the signal at δ 1.05) on the left axis and on the right axis mole percent of acetone formed (determined by integrating the signal at δ 1.56). A 6% yield of acetone was generated over a period of 12 days, and 80% of **18** was consumed. This corresponds to

a 7.5% yield for the fragmentation reaction. In the absence of oxygen, a mixture of γ-hydroxyenone **18** and anisaldehyde in deuterated benzene was *unchanged* after heating at 55 °C for 4 days under argon. No trace of acetone was generated, and no retroaldol reaction was detected (vide supra). Thus, oxygen is essential for *any* reaction to occur. Furthermore, γ-hydroxyenone **18** is stable in deuterated benzene in the presence of oxygen at 55 °C but in the absence of anisaldehyde. No trace of acetone was detected, and there was no change in the spectrum of the mixture. Thus, coautoxidation of an aldehyde is essential for any reaction of **18** to occur.

The rate of anisaldehyde-promoted autoxidation of **18** ($t_{1/2} \approx 150$ h) at 55 °C is ∼19-fold slower than the rate of autoxidation of HODA-PC ($t_{1/2} \approx 8$ h) at 37 °C. In view of the 20-fold lower concentration of **18** and anisaldehyde (∼0.07 M) in benzene solution versus neat HODA-PC (≈1.4 M), this rate difference is not remarkable. Therefore, there is no reason to invoke a special mechanism, such as an intramolecular pathway (see Scheme 2) to explain the reactivity of HODA-PC compared with that of **18** in the presence of anisaldehyde.

Anisic Acid Does Not Promote Fragmentation of the γ-Hydroxyenone 18. Anisic acid is produced by autoxidation of anisaldehyde. To test whether anisic acid promotes fragmentation of γ-hydroxyenone **18**, we heated **18** in the presence of anisic acid and oxygen at 55 °C for 4 days. New resonances appeared at 1.2 to 1.4 ppm and but no trace of a resonance for acetone at 1.56 ppm was detected, indicating that no acetone is produced by fragmentation of **18** under these conditions. The

reaction of **18** that is catalyzed by anisic acid presumably generates dihydrofuran **20**. A pair of resonances at 1.34 and 1.53 ppm in the NMR spectrum of the reaction mixture are consistent with the two nonequivalent methyl groups of the putative dihydrofuran **20**.
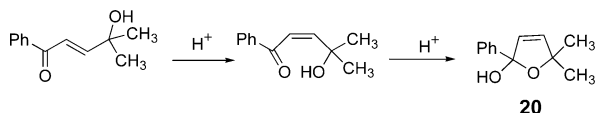
The key step in our proposed mechanism for the oxidative cleavage HODA-PC to give ON-PC is the fragmentation of a β-hydroxyperester. Precedent for the fragmentation of our putative intermediate **19** is provided by the closely analogous fragmentation of β-hydroxyperester **21**.[28] The same driving force, simultaneous generation of two carbonyl groups, promotes the analogous fragmentations of the β-hydroxydialkylperoxide **22**[29] and the β-hydroxyhydroperoxide **23**.[30] The oxidative cleavage of ribulose bisphosphate is an especially pertinent example that occurs in vivo during photorespiration. It involves fragmentation of the β-hydroxyhydroperoxide **24**.[31]
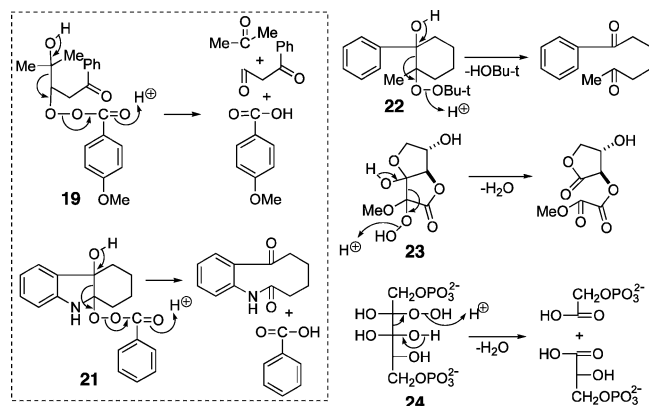
Experimental Procedures

General Methods. All proton magnetic resonance (^{1}H NMR) spectra were recorded on Varian Gemini spectrometers operating at 200 or 300 MHz. Proton chemical shifts are reported in parts per million on the δ scale relative to tetramethylsilane (δ 0.00) or CDCl$_3$ (δ 7.27) or CD$_3$OD (δ 3.30) or C$_6$D$_6$ (δ 7.157). ^{1}H NMR spectral data are tabulated in terms of multiplicity of proton absorption (s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet; br broad), coupling constants (Hz), number of protons. Carbon magnetic resonance (^{13}C NMR) spectra were recorded on a Varian Gemini spectrometer operating at 50 MHz. These spectra are reported in ppm on the δ scale relative to CDCl$_3$ (δ 77.23) or CD$_3$OD (δ 49.0). Proton and carbon NMR samples were analyzed as solutions in CDCl$_3$ or CD$_3$OD or C$_6$D$_6$.

Chromatography was performed with ACS grade solvents (ethyl acetate, hexane, chloroform, and methanol). Thin-layer chromatography (TLC) was performed on glass plates precoated with silica gel (Kieselgel 60 F$_{254}$, E. Merck, Darmstadt, West Germany). R_f values are quoted for plates of thickness 0.25 mm. TLC plates were visualized by viewing the developed plates under short-wavelength UV light or with iodine. Aldehydes were visualized with Purpald reagent,[32] peroxides with thiocyanate reagent,[33,34] and phospholipids with molybdenum spray.[35]

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High-performance liquid chromatography (HPLC) was performed with HPLC grade solvents using a Waters M600A solvent delivery system and a Waters U6K injector. The eluents were monitored using a SEDEX 55 evaporative light scattering detector and an ISCO V4 UV−visable detector. Analytical RP-HPLC was performed on a Phenomenex LUNA C18 (2) column (4.6 mm i.d. × 25 cm). Semipreparative RP-HPLC was performed on a Phenomenex LUNA C18 (2) column (10 mm i.d. x 25 cm). Flash column chromatography was performed on 230−400 mesh silica gel supplied by E. Merck. All reactions in an inert atmosphere were in argon or nitrogen. All chemicals were obtained from Aldrich unless otherwise specified.

Liquid Chromatography/Mass Spectrometry. LC/ESI/MS/MS analysis of autoxidation reaction product mixtures was performed on a Quattro Ultima (Micromass, Wythenshawe, UK) connected to a Waters 2790 solvent delivery system with an auto-injector. The source temperature was maintained at 100 °C, and the desolvation temperature at 200 °C. The drying gas (N$_2$) was maintained at ca. 450 L/h, and the cone flow gas at ca. 50 L/h. The multiplier was set at an absolute value of 500. MS scans at m/z 20−400 were obtained for standard compounds. Argon was used as collision gas at a pressure of 5 psi for MS/MS analysis. For MS/MS analysis, the collision energy was optimized for each compound. For multiple reaction monitoring (MRM) experiments, the optimum collision energy (giving the strongest signal) was determined for each m/z ion pair. For acidic compounds, the mass spectrometer was operated in the negative ion mode. For PFB derivatives, it was operated in the positive ion mode. Online chromatographic separation was achieved using a 150 × 2.0 mm i.d. Prodigy ODS-2, 5 μ column (Phenomenex, UK), with a binary solvent (water and methanol) gradient. The solvents were supplemented with 0.2% formic acid, whenever the mass spectrometer was operated in positive mode. For acidic compounds, the gradient started with 100% water and rose to 100% methanol linearly in 15 min, and elution was continued for 5 min with 100% methanol. Then the gradient was reversed to 100% water in 0.5 min and then held for 9.5 min at 100% water. For dipentaflurobenzyl ester compounds, the gradient started with 85% methanol, rose to 88% methanol linearly in 12 min, and then rose to 100% methanol linearly over 3 min, which was held for 12 min. Then the gradient was reversed to 85% methanol in 4 min and held for 10 min. The solvents were delivered at 200 μL/min.

4-Hydroxydodec-2-enedioic acid (10) was synthesized by selective oxidation of HODA (**9**). A mixture of NaClO$_2$ (11 mg, 0.12 mmol), NaH$_2$PO$_4$ (8.3 mg, 0.06 mmol), 2-methyl-2-butene, and *t*-BuOH/H$_2$O (5/1 (v/v), 0.5 mL) was added to a flask containing HODA (9 mg, 0.04 mmol). The resulting mixture was stirred for 2 h. The product was extracted with ethyl acetate. Flash chromatography (hexane/ethyl acetate (30/70)) produced **10** (7 mg, 71%). ^{1}H NMR (CD$_3$OD, 200 MHz) δ 6.79 (dd, J_1 = 15.6 Hz, J_2 = 5.3 Hz, 1H), 5.97 (dd, J_1 = 15.6 Hz, J_2 = 1.5 Hz, 1H), 6.28 (dd, J_1 = 15.6 Hz, J_2 = 7.8 Hz, 1H), 4.1−4.2 (m, 1H), 2.26 (t, J = 7.2 Hz, 2 H), 1.4−1.7 (4H), 1.2−1.4 (8H).

Dipentaflurobenzyl azeleate (12) was prepared by stirring a mixture of azeleic acid (**11**, 100 μg) in dry acetonitrile (100 μL), containing 10 wt % pentafluorobenzyl bromide and 20 wt % *N,N*-diisopropylethylamine, for 2 h. After the solvent was evaporated with nitrogen, the residue was dissolved in 1 mL of water, and the products were extracted with 1 mL of ethyl acetate. ESI-MS/MS analysis in the positive ion mode produced [MH]$^+$ at 549 and characteristic fragmentations: at m/z 351.5, m/z 181.

Autoxidation of HODA-PC. Glass tubes (1 mL) containing a dry film of HODA-PC (100 ng) were heated in air at 37 °C. After incubation, the vials were stored at −78 °C until analysis. Before analysis, 1,2-ditridecanoyl-*sn*-glycero-3-phosphatidylcholine (DT-PC,

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4 ng) in methanol/water (85/15, 100 μL) was added to each vial. The content was then analyzed by LC/MS/MS, and the amount of each analyte was calculated according to standard curves using DT-PC as an internal standard as described previously.[22]

Autoxidation of HODA. Six 1-mL glass vials filled with 500 ng of HODA (**9**) in each were heated at 37 °C in an incubator. At 0, 1, 2, 4, 8, and 18 h, a vial was removed from the incubator and stored at −80 °C. Before analysis, 150 μL of a solution of standard (0.2 μg/mL) in methanol were added, and 50 μL were injected into the LC-MS/MS system. HPLC conditions and MS conditions are the same as those used in other experiments for other acidic compounds. For quantification of HDdiA, (**10**) m/z 243 and 207 were monitored with a collision energy of 25 V. For azelaic acid (**11**), m/z 187 and 125 were monitored with a collision energy of 15 V.

Derivatization of Oxidized HODA. After 100 μg of HODA (**9**) was heated at 37 °C for 4 h, 100 μL of acetonitrile, which contains 10 wt % pentafluorobenzyl bromide and 20 wt % *N,N*-diisopropylethylamine, were added and the mixture was stirred for 2 h. After the solvent was evaporated with nitrogen, the residue was dissolved in 1 mL of water, and the products were extracted with 1 mL of ethyl acetate.

Quantification of HDdiA and Azeleic Acid: HDdiA (**10**) and azeleic acid (**11**) were quantified by LC-ESI-MS/MS with an MRM function. For HDdiA, the mass transition m/z 242.7 to 207.5 was monitored. For azeleic acid, the mass transition m/z 187 to 125.1 was monitored. For internal standard, the mass transition m/z 382.9 to 100.6 was monitored. Calibration curves were built by injecting various amounts of 13-HPODE, 13-HODE, 9-HODE, and HODA and 10 ng of internal standard into the LC/MS/MS (Figure 7).

Coautoxidation of (*E***)-4-Hydroxy-4-methyl-1-phenyl-2-penten-1-one (18)[36] with Anisaldehyde.** An NMR tube containing the γ-hydroxyenone **15** (10 mg, 52 μmol) and anisaldehyde (11.2 mg, 82 μmol) in d^6-benzene or d^1-chloroform (700 μL) saturated with oxygen was cooled to −20 °C in a dry ice ethanol mixture while flushing the tube with oxygen. The tube was flame sealed and then incubated at 55 °C. The formation of acetone in the reaction product mixture was monitored by ^{1}H NMR.

Derivatization of Acetone with Phenylhydrazine. Phenylhydrazine (15 μL, 16.5 mg, 0.15 mmol) was added to the reaction mixture in C$_6$D$_6$ from coautoxidation of (*E*)-4-hydroxy-4-methyl-1-phenyl-2-penten-1-one (**18**) with anisaldehyde at 55 °C for 3 days. After half an hour, the acetone resonance at δ 1.56 disappeared and a peak at δ 1.79, characteristic of acetone phenylhydrazone in C$_6$D$_6$, was identified by comparison with an authentic sample prepared by adding phenylhydrazine (4 μL, 4.5 mg, 0.04 mmol) to acetone (3 μL, 2.4 mg, 0.04 mmol) in d^6-benzene (700 μL). Karabatsos[27] observed that bubbling oxygen through a benzene solution of acetone phenylhydrazone

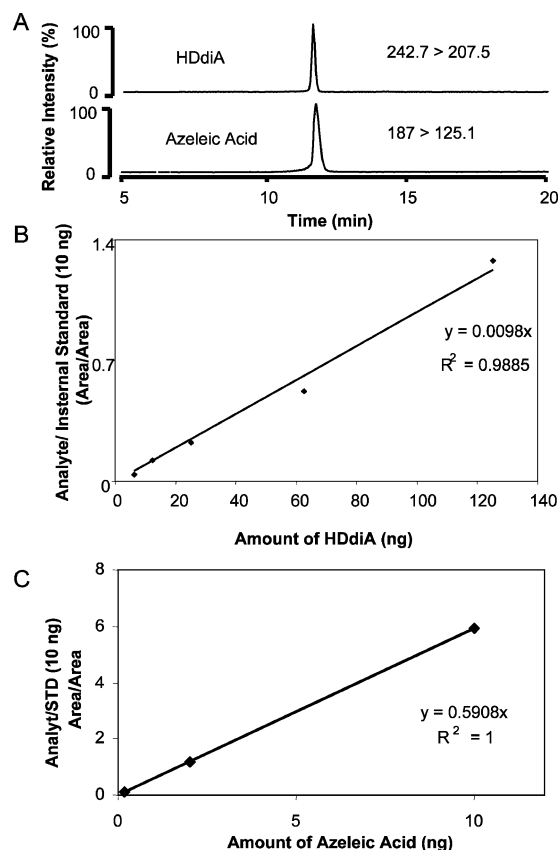(36) Sabol, M. R.; Wiglesworth, C.; Watt, D. S. *Synth. Commun.* **1988**, *18*, 1−12.

Figure 7. (A) LC-MS chromatogram of HDdiA (**10**) and azeleic acid (**11**); (B) calibration curve of HDdiA (**10**); (C) calibration curve of azeleic acid (**11**).

generates a hydroperoxide (Me$_2$C(OOH)−N=NC$_6$H$_5$) that exhibits a singlet at δ = 1.47. After 24 h, our acetone phenylhydrazone NMR spectrum showed a peak at 1.47 ppm.

Coautoxidation of (*E*)-4-hydroxy-4-methyl-1-phenyl-2-penten-1-one (**18**) with anisic acid. An NMR tube containing the γ-hydroxyenone **18** (10 mg, 52 μmol) and anisic acid (5 mg, 33 μmol) in d^6-benzene (700 μL) saturated with oxygen was incubated at 55 °C for 4 days.