

- effects as the bacteria are shifted from stationary phase in a rich medium to growth in a more minimal medium. The mechanism for synchronization is not understood but may involve secreted factors, such as signaling molecules, that are diluted out and need to be generated over time. We are currently investigating this phenomenon.
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Vitamin C-Induced Decomposition of Lipid Hydroperoxides to Endogenous Genotoxins

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Epidemiological data suggest that dietary antioxidants play a protective role against cancer. This has led to the proposal that dietary supplementation with antioxidants such as vitamin C (vit C) may be useful in disease prevention. However, vit C has proved to be ineffective in cancer chemoprevention studies. In addition, concerns have been raised over potentially deleterious transition metal ion-mediated pro-oxidant effects. We have now determined that vit C induces lipid hydroperoxide decomposition to the DNA-reactive bifunctional electrophiles 4-oxo-2-nonenal, 4,5-epoxy-2(E)-decenal, and 4-hydroxy-2-nonenal. The compound 4,5-Epoxy-2(E)-decenal is a precursor of etheno-2'-deoxyadenosine, a highly mutagenic lesion found in human DNA. Vitamin C-mediated formation of genotoxins from lipid hydroperoxides in the absence of transition metal ions could help explain its lack of efficacy as a cancer chemoprevention agent.

Molecular oxygen undergoes three successive one-electron reductions (1) to reactive oxygen species (ROS) that can damage cellular macromolecules such as DNA and proteins (2). DNA damage results directly from ROS (3) or from ROS-derived lipid hydroperoxides that break down to form endogenous genotoxins (4, 5). Covalent modifications to DNA by ROS (6) and lipid hydroperoxide-derived genotoxins (7, 8) have been unequivocally characterized in mammalian DNA. Lipid hydroperoxides are formed nonenzymatically by the action of ROS on polyunsaturated fatty acids (PUFAs) (4) or enzymatically from lipoxygenases (LOXs) (9) and cyclooxygenases (COXs) (10). Linoleic acid, the major ω -6 PUFA present in plasma lipids, is converted to 13(S)-hydroperoxy-(Z,E)-9,11-octadecadienoic acid (13-HPODE) by human 15-LOX (11). COX-1 and COX-2 produce mainly 9(R)-hydroperoxy-(E,Z)-10,12-octadec-

adienoic acid (9-HPODE) and 13-HPODE (10). The HPODEs are subsequently reduced to the corresponding 9(R)- and 13(S)-hydroxy-octadecadienoic acids (HODEs) through the peroxidase activity of the COXs (12, 13).

Lipid hydroperoxides undergo transition metal ion-dependent decomposition to the α,β -unsaturated aldehyde genotoxins 4-oxo-2-nonenal and 4-hydroxy-2-nonenal (14). 4-Oxo-2-nonenal is a particularly potent lipid hydroperoxide-derived genotoxin (15), which reacts with DNA bases to form heptanone-etheno-adducts (Fig. 1) (16). Failure to repair these DNA lesions can lead to mutations (4, 5) or apoptosis (17). The use of vitamin C (vit C) for antioxidant therapy has been advocated because of its ability to scavenge ROS, although its potential for pro-oxidant activity in the presence of transition metal ions has also been recognized (18). Transition metal ion-mediated decomposition of lipid hydroperoxides is thought to be initiated by a one-electron reduction to an alkoxy radical (14, 19). This raised the possibility that an alkoxy radical intermediate would also be formed by a one-electron re-

duction of the lipid hydroperoxide by vit C (Fig. 1). When a one-electron reduction of hydrogen peroxide occurs, the resulting hydroxy radical (1) is scavenged through a termination reaction either with the vit C radical anion (20) or with vit C itself. We reasoned that when an alkoxy radical is attached to a PUFA, intramolecular radical propagation could proceed more rapidly than intermolecular vit C-mediated termination reactions. Therefore, the same α,β -unsaturated aldehyde genotoxins observed with transition metal ions (14) may also be observed during vit C-induced decomposition of lipid hydroperoxides (Fig. 1). We have developed liquid chromatography (LC)/atmospheric pressure chemical ionization (APCI)/mass spectrometry (MS)/ultraviolet (UV) methodology to identify the α,β -unsaturated aldehydic bifunctional electrophiles 4,5-epoxy-2(E)-decenal (21), 4-oxo-2-nonenal (14), 4-hydroperoxy-2-nonenal (14, 22), and 4-hydroxy-2-nonenal (14) that could potentially be formed during homolytic lipid hydroperoxide decomposition (Fig. 2) (23–25).

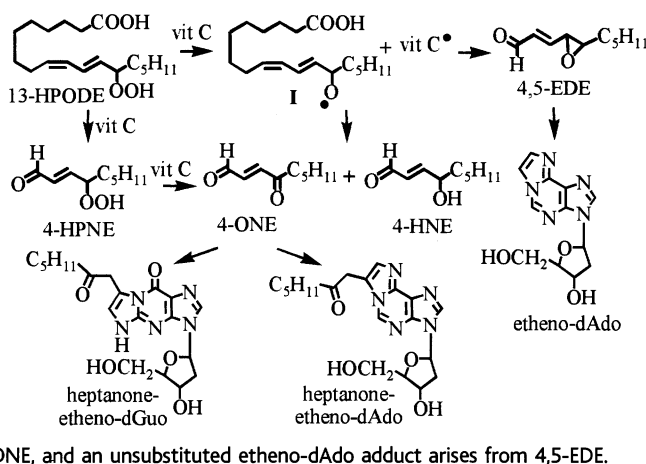
The prototypic ω -6 lipid hydroperoxide 13-HPODE was allowed to decompose in the presence of vit C in Chelex-treated Mops buffer at pH 7.0 and 37°C. Reaction products were then analyzed by LC/APCI/MS with concomitant UV monitoring (24). At early time points, 4-hydroperoxy-2-nonenal was the major product. After 30 min, the 4-hydroperoxy-2-nonenal level started to decline slowly with a concomitant increase in *trans*-4,5-epoxy-2(E)-decenal, 4-oxo-2-nonenal, and 4-hydroxy-2-nonenal. The reaction was complete at 2 hours, with 4-oxo-2-nonenal, 4-hydroperoxy-2-nonenal, and 4-hydroxy-2-nonenal as the major products (Fig. 3A). At higher vit C concentrations, *trans*-4,5-epoxy-2(E)-decenal, 4-oxo-2-nonenal, and 4-hydroxy-2-nonenal were produced in greater amounts, and *cis*-4,5-epoxy-2(E)-decenal could be readily detected (Fig. 3B). The level of 4-hydroperoxy-2-nonenal declined much more rapidly, so that after 2 hours it was undetectable. Unequivocal proof of structure for the aldehydes was obtained by normal-phase LC/tandem MS (MS/MS) analysis (23, 24).

In separate experiments, 13-HPODE was

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Fig. 1. Formation of alkoxy radicals by treatment of 13-HPODE with vit C with concomitant formation of vit C radical anion (vit C[•]). The initially formed alkoxy radical can then decompose to the α,β -unsaturated aldehydic bifunctional electrophiles, 4,5-epoxy-2(*E*)-decenal (4,5-EDE), 4-oxo-2-nonenal (4-ONE), and 4-hydroxy-2-nonenal (4-HNE). 4-Hydroperoxy-2-nonenal (4-HPNE) is formed by a quite different pathway. Heptanone-etheno-dGuo and -dAdo adducts are formed from 4-ONE, and an unsubstituted etheno-dAdo adduct arises from 4,5-EDE.



treated with increasing amounts of vit C, and the products were quantified by normal-phase LC/UV (24) (Fig. 4A). At low vit C concentrations, the major products were *trans*-4,5-epoxy-2(*E*)-decenal, 4-hydroperoxy-2-nonenal, and 4-oxo-2-nonenal. As the vit C concentration increased, 4-hydroperoxy-2-nonenal amounts decreased, with a concomitant increase in *trans*- and *cis*-4,5-epoxy-2(*E*)-decenal, 4-oxo-2-nonenal, and 4-hydroxy-2-nonenal. Maximal yields of 4-oxo-2-nonenal, 4,5-epoxy-2(*E*)-decenal, and 4-hydroxy-2-nonenal were obtained with an excess of vit C (Fig. 4A). When 1 mM vit C was used, these bifunctional electrophiles accounted for 27% of the starting 13-HPODE (Fig. 4A). A stoichiometric amount of vit C was consumed during the 13-HPODE decomposition. When 13-HPODE was treated with 1 mM Fe^{II} for 2 hours, the bifunctional electrophiles accounted for only 11% of the starting 13-HPODE. Thus, vit C was more efficient than transition metal ions at initiating the decomposition of 13-HPODE to bifunctional electrophiles.

It is well known that biological buffers contain substantial amounts of transition metal ions, which can be removed by Chelex treatment (20). When 13-HPODE was heated for 2 hours at 37°C in Chelex-treated Mops buffer, no decomposition occurred, indicating that there were few or no transition metal ions in the buffer (14). The presence of transition metal ions in vit C solutions causes a relatively rapid loss in absorbance at 265 nm resulting from oxidation of vit C to its dehydro form. Cu^{II} is approximately 80 times more effective than Fe^{III} at oxidizing vit C (20). Mops-buffered vit C solutions containing 1 μ M Cu^{II} showed a decline in absorbance at 265 nm of 31% over 2 hours. Chelex-treated Mops-buffered vit C solutions showed no decline in absorbance at 265 nm over 2 hours unless they contained ≥ 50 nM of added Cu^{II}. This suggested that the maximum amount of Cu^{II} in the Mops buffer used in the present study was < 50 nM. Concentrations of Cu and Fe in the Mops buffer were subsequently determined by graphite furnace atomic

absorption spectrophotometry and inductively coupled plasma/MS, respectively. Chelex treatment of the Mops buffer reduced the Cu from 234 nM to <16 nM and Fe from 250 nM to <36 nM. No other transition metal ions were detected in the Chelex-treated buffer.

We have shown previously that trace amounts of transition metal ions can induce 13-HPODE decomposition to bifunctional electrophiles (14). Furthermore, it is well known that the Cu/ascorbate system exhibits a pro-oxidant effect with trace amounts of Cu (20). Therefore, we determined whether Cu or Fe had an additive effect on the vit C-mediated decomposition of 13-HPODE. In fact, there was no increased formation of bifunctional electrophiles from 13-HPODE when either 1 μ M Cu^{II} or 1 μ M Fe^{II} was added to the vit C. This confirmed that trace amounts of these transition metal ions did not substantially affect the reaction between vit C and 13-HPODE. In separate experiments, we established that synergistic effects between vit C and transition metal ions only occurred at concentrations that were two orders of magnitude greater than were present in the Chelex-treated Mops buffer (10 to 20 μ M).

The initial formation of 4-hydroperoxy-2-nonenal from 13-HPODE at low vit C concentrations and its subsequent decline at higher concentrations (Fig. 4A) suggested that it was a precursor to 4-oxo-2-nonenal and 4-hydroxy-2-nonenal. To test this possibility, we treated authentic 4-hydroperoxy-2-nonenal (22) with increasing concentrations of vit C. As the concentration of vit C increased, there was a decline in 4-hydroperoxy-2-nonenal with a concomitant increase in 4-oxo-2-nonenal and 4-hydroxy-2-nonenal (Fig. 4B). When the vit C was in excess, no residual 4-hydroperoxy-2-nonenal was observed (Fig. 4B). Stoichiometric amounts of 4-oxo-2-nonenal and 4-hydroxy-2-nonenal were formed in a ratio that was identical with that from the reaction of 13-HPODE with vit C. This confirmed that 4-hydroperoxy-2-non-

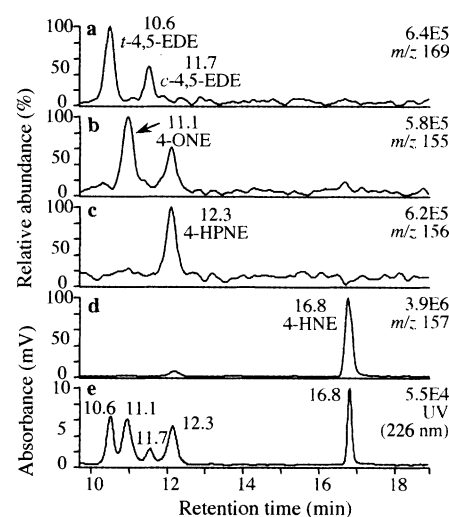


Fig. 2. LC/MS/UV analysis was done with the following amounts of authentic standards: *trans*-4,5-epoxy-2(*E*)-decenal (*t*-4,5-EDE; 100 ng, 0.6 nmol), 4-ONE (200 ng, 1.3 nmol), *cis*-4,5-epoxy-2(*E*)-decenal (*c*-4,5-EDE; 20 ng, 0.1 nmol), 4-HPNE (200 ng, 1.2 nmol), and 4-HNE (200 ng, 1.3 nmol). The samples were injected in ether (20 μ l) and analyzed by normal-phase LC system 1 (24). (a) Ion chromatograms (m/z 169) for the protonated molecular ion (MH^+) of *t*-4,5-EDE and *c*-4,5-EDE. (b) Ion chromatogram (m/z 155) for MH^+ of 4-ONE. (c) Ion chromatogram (m/z 156) for ($MH-OH$)⁺ of 4-HPNE. (d) Ion chromatogram (m/z 157) for MH^+ of 4-HNE. (e) UV chromatogram monitoring 226 nm.

enal was indeed a major precursor to 4-oxo-2-nonenal and 4-hydroxy-2-nonenal. Vitamin C-mediated formation of bifunctional electrophiles is most likely initiated in part by the formation of alkoxy radical I (Fig. 1), as suggested by Pryor and Porter for Fe^{II}-mediated decomposition (19), but our data indicate that another pathway exists, which involves the surprising intermediate formation of 4-hydroperoxy-2-nonenal (Fig. 1). The 4-hydroperoxy-2-nonenal was characterized as follows: (i) it had the same LC retention time and mass spectrum as authentic 4-hydroperoxy-2-nonenal (22); (ii) product ion spectra of MH^+ [mass-to-charge ratio (m/z) 173] and [$MH-OH$]⁺ (m/z 156) were identical with an authentic sample; (iii) its oxime derivative had syn- and anti-isomers with identical LC retention times, mass spectra, and isomer ratios as oxime isomers from an authentic standard.

13-HPODE and 9-HPODE (the initial products of linoleic acid oxidation by COX-1 and COX-2) are normally reduced to the 13- and 9-HODES (12, 13). In settings of oxidative stress, when reducing cofactors have been depleted, COX-mediated lipid hydroperoxide formation may become more important. The catalytic efficiency [enzyme turnover number (k_{cat})/Michaelis-Menten constant (K_m)] of linoleic acid oxidation by COX-2 (relative $k_{cat}/K_m = 2.8$) is somewhat

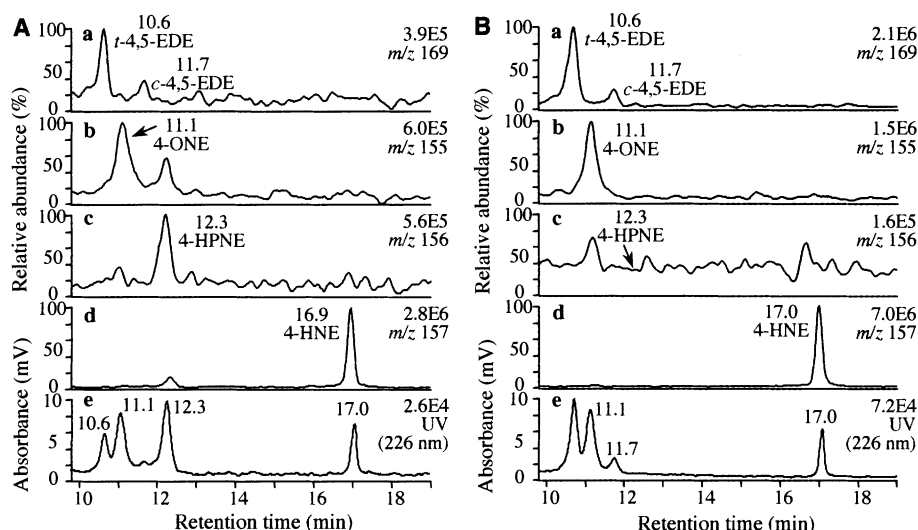


Fig. 3. LC/MS/UV analysis of reaction between 13-HPODE and vit C. Pure 13-HPODE (400 μ M) was treated with vit C in 2 ml of transition metal ion-free Mops buffer (100 mM) containing sodium chloride (150 mM) at pH 7.0 and 37°C for 2 hours. The buffer solution was then extracted with ether (0.5 ml). LC was conducted on the ether extract (20 μ l) with normal-phase system 1. Ion (a through d) and UV (e) chromatograms are the same as in Fig. 2. (A) Reaction conducted with an excess of 13-HPODE (400 μ M) compared with vit C (100 μ M). (B) Reaction conducted with an excess of vit C (1 mM) compared with 13-HPODE (400 μ M).

lower than that for arachidonic acid oxidation (relative $k_{cat}/K_m = 19$) (12). However, COX-2 (relative $k_{cat}/K_m = 2.8$) oxidizes linoleic acid more efficiently than COX-1 (relative $k_{cat}/K_m = 0.9$) (12). COX-2 is up-regulated in intestinal cancer (26), and so DNA-reactive α,β -unsaturated bifunctional electrophiles could be formed in increased amounts. This provides a genotoxic pathway, which is independent of eicosanoid biosynthesis. We note that 4-hydroxy-2-nonenal upregulates COX-2 expression (27). Thus, lipid hydroperoxide-derived bifunctional electrophiles may be capable of inducing their own formation through a COX-2-mediated pathway.

Unsubstituted etheno-2'-deoxyadenosine (dAdo) adducts are more mutagenic in mammalian cells than are adducts that arise from direct ROS-mediated damage such as 7,8-dihydro-8-hydroxy-2'-deoxyguanosine (8-oxo-dGuo) (28). Etheno-dAdo adducts identified in human tissue DNA (8) have been postulated to arise from the reaction of DNA bases with the epoxide of 4-hydroxy-2-nonenal (29). Although epoxidation of 4-hydroxy-2-nonenal can be performed chemically, it is questionable whether this reaction occurs in vivo, because 4-hydroxy-2-nonenal is such a good substrate for aldo-keto reductases and glutathione S-transferases (30). The identification of 4,5-epoxy-2(E)-decenal as a major product of lipid hydroperoxide decomposition provides an alternative route to etheno adducts. Our demonstration that it forms an unsubstituted etheno adduct with dAdo in vitro provides an important link between lipid peroxidation and the presence

of unsubstituted etheno-DNA adducts in human tissues (8).

Oral dosing with vit C results in plasma concentrations that reach a plateau of 80 μ M, and intracellular concentrations reach a plateau in the range 1.4 to 3.4 mM with a dose of 200 mg per day (31). This means that the concentrations of vit C used in our in vitro study are comparable to those found in vivo. The finding that vit C generates bifunctional electrophiles explains why hydroperoxide-dependent lipid peroxidation is enhanced by vit C in vitro (32) and could also help to explain why vit C has not demonstrated substantial efficacy in cancer chemoprevention trials (33). The efficiency of vit C in inducing the decomposition of lipid hydroperoxides suggests that this process could give rise to substantial amounts of DNA damage in vivo. Quantitation of etheno-dAdo adducts in human tissue samples may provide a dosimeter for populations at risk for lipid hydroperoxide-mediated DNA damage. It will be particularly important to monitor such lesions during cancer chemoprevention studies with vit C and COX-2 inhibitors.

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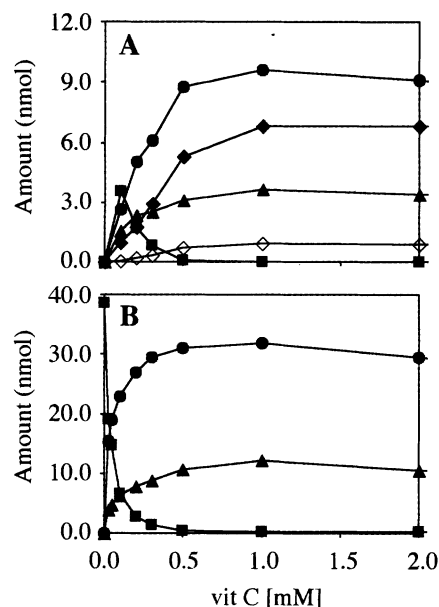


Fig. 4. Vit C-mediated conversion of hydroperoxides to α,β -aldheydic bifunctional electrophiles. (A) 13-HPODE-derived electrophiles. 13-HPODE (80 nmol, 400 μ M) was treated with increasing concentrations of vit C in the range from 20 nmol (100 μ M) to 400 nmol (2 mM). Reactions were performed in duplicate in transition metal ion-free Mops buffer (100 mM) containing sodium chloride (150 mM) at pH 7.0 and 37°C for 2 hours. 4-ONE, circles; t-4,5-EDE, solid diamonds; c-4,5-EDE, open diamonds; 4-HPNE, squares; 4-HNE, triangles. (B) 4-HPNE-derived bifunctional electrophiles. 4-HPNE (40 nmol, 200 μ M) was treated with increasing concentrations of vit C in the range from 5 nmol (25 μ M) to 400 nmol (2 mM). Reactions were performed in duplicate with transition metal ion-free Mops buffer (100 mM) containing sodium chloride (150 mM) at pH 7.0 and 37°C for 2 hours. 4-ONE, circles; 4-HPNE, squares; 4-HNE, triangles.

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23. Mass spectrometry was conducted with a Thermo

Finnigan LCQ ion trap mass spectrometer equipped with an APCI source. The mass spectrometer was operated in the positive ion mode with a discharge current of 5 μ A applied to the corona needle. Nitrogen was used as the sheath (80 psi) and auxiliary (10 units) gas. The vaporizer and heated capillary temperatures were set at 450°C and 150°C, respectively. Full scanning analyses were performed in the range of m/z 100 to m/z 800. Collision-induced dissociation experiments coupled with MS/MS analysis used helium as the collision gas. The relative collision energy was set at 20% of maximum (1 V). Ionization of the polar 4-hydroxy-2-nonenal was more efficient than the ionization of the other bifunctional electrophiles, so the mass spectrometer response was almost an order of magnitude greater.

24. For normal-phase LC system 1, we used two silica columns in series (250 mm by 4.6 mm internal diameter, 5 μ m) with a post-column split of 160 μ l/min to the UV detector (226 nm) and 640 μ l/min to the mass spectrometer. Solvent A was hexane/2-propanol (197:3, v/v), and solvent B was hexane/2-propanol (70:30, v/v). Isocratic elution with 21% B was conducted for 13 min at a flow rate of 800 μ l/min. A linear gradient was then run to 25% B for 18 min. For normal-phase LC system 2, we used only one of the silica columns and no split to the

UV detector (226 nm). Isocratic elution with 3% B was conducted for 15 min at 1.0 ml/min. Retention times were as follows: *trans*-4,5-epoxy-2(E)-decal (4.9 min), 4-oxo-2-nonenal (5.1 min), *cis*-4,5-epoxy-2(E)-decal (5.5 min), 4-hydroperoxy-2-nonenal (7.5 min), 4-hydroxy-2-nonenal (12.3 min). For LC/MS experiments, 13-HPODE (800 nmol, 400 μ M) was allowed to decompose in the presence of 200 nmol of vit C (100 μ M) or 2000 nmol of Vit C (1 mM) in 2 ml of pH 7.0 Chelex-treated Mops buffer (100 mM) containing NaCl (150 mM) (14) at pH 7.0 and 37°C. Aldehydes were extracted with diethyl ether (0.5 ml) and an aliquot (20 μ l) was analyzed by LC/APCI/MS with concomitant UV monitoring with system 1. For quantitation experiments, 13-HPODE (80 nmol, 400 μ M) in 200 μ l of Chelex-treated pH 7.0 Mops buffer (100 mM) containing NaCl (150 mM) was reacted with increasing amounts of vit C for 2 hours at 37°C. Aldehydes were extracted into diethyl ether (200 μ l), and an aliquot (25 μ l) was analyzed by LC/UV with system 2. Quantitation was performed from standard curves constructed by quantitation of known amounts of authentic standards extracted from Mops buffer with ether.

25. Authentic 4-oxo-2-nonenal was prepared as described in (15), authentic *trans*-4,5-epoxy-2(E)-de-

nal and *cis*-4,5-epoxy-2(E)-decal were prepared as in (21), authentic 4-hydroperoxy-2-nonenal was prepared enzymatically from 3(Z)-nonenal as described in (22), and 4-hydroxy-2-nonenal was obtained from Cayman (Ann Arbor, MI). All samples were >98.5% pure as determined by normal-phase LC with system 2.

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