Coordination (Ag^+) Ion Spray−Mass Spectrometry of Peroxidation Products of Cholesterol Linoleate and Cholesterol Arachidonate: High-Performance Liquid Chromatography−Mass Spectrometry Analysis of Peroxide Products from Polyunsaturated Lipid Autoxidation

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Abstract: Lipid peroxidation of polyunsaturated fatty acids and esters leads to a complex mixture of hydroperoxides and cyclic peroxides, some of which possess potent biological activity. These product mixtures contain dozens of diastereomers and regioisomers. The technique of coordination ion spray−mass spectrometry (CIS−MS), recently reported by Bayer and collaborators, proves to be a powerful tool for the analysis of complex peroxide mixtures. Silver ion forms readily detected Ag^+ adducts of peroxides and hydroperoxides. These ions, observed at [M+107] and [M+109], undergo fragmentation typical of hydroperoxides, and cyclic peroxides. Thus, Hock fragmentation is observed from many of the silver ion adducts of hydroperoxides and cyclic peroxides undergo fragmentation to give aldehydes and epoxides. Silver ion coordination ion spray−mass spectrometry (Ag^+CIS−MS) can be coupled to normal-phase high-performance liquid chromatography (HPLC) by postcolumn addition of $AgBF_4$, allowing the use of powerful techniques such as selected ion monitoring and selected reaction monitoring. This coupling permits, for the first time, the combination of powerful normal-phase separation techniques with detection methods that provide unambiguous structural information of complex peroxide compounds.

The study of the free radical-mediated reaction of lipids with molecular oxygen has been of interest for decades. In the recent past, this process has received attention because of its probable role in destructive biological processes. In living systems, autoxidation of lipids has been implicated in DNA and protein modification, radiation damage, aging, modification of membrane structure, tumor initiation, and deposition of arterial plaque.[1]

Low-density lipoprotein (LDL) is the major carrier of cholesteryl esters in human blood plasma, and the free radical-mediated modification of LDL may play an important role in the development of atherosclerosis.[2] For this reason, much effort has been devoted to the study of lipoprotein oxidation and its prevention over the past decade.[3] Peroxidation of LDL neutral lipids affords peroxides mainly from cholesteryl linoleate (Ch18:2), and cholesteryl arachidonate (Ch 20:4), but the mixture of products formed from these compounds is complex.[4] Oxidation of cholesteryl linoleate, **1**, leads to several different hydroperoxide (R−OOH) products, and methods of analysis have been reported for the corresponding alcohols (R−OH) formed by reduction of the hydroperoxides.[5] The identification of peroxide products formed from lipids is tedious and time consuming, usually requiring several chromatography steps or conversions to known compounds.[6]

Analysis of free radical oxidation products from cholesteryl arachidonate poses an even more formidable problem. Cholesteryl arachidonate has sites for free radical oxidation on the arachidonate chain at carbons 7, 10, and 13 as well as on the

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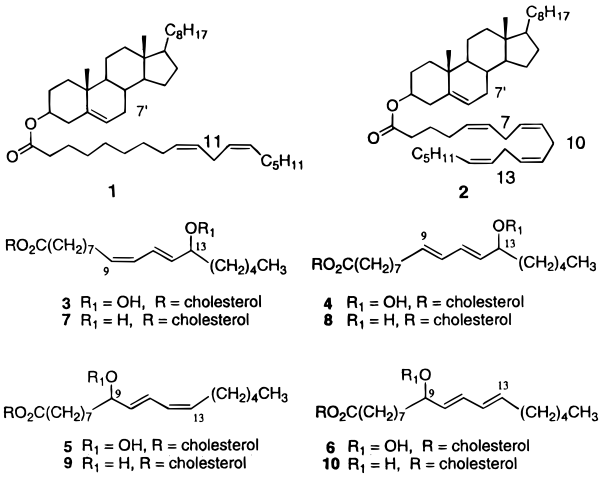


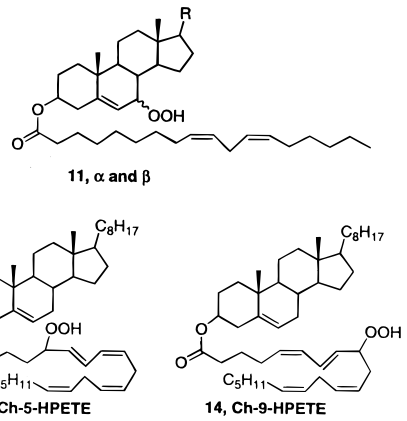
Figure 1. Free radical oxidation products formed from cholesteryl linoleate.

cholesterol backbone at, for example, carbon 7. These reactive centers are indicated in structure **2**.

One can quickly come to the conclusion that several dozen peroxide- or hydroperoxide-containing compounds could be formed in the oxidation of **2**. Conventional techniques (product isolation, spectroscopic identification, and conversion to and comparison with known compounds) are unrealistic for the analysis of oxidation products formed from complex biological lipid sources. For that reason, we sought new methods that are appropriate for peroxide and hydroperoxide analysis. Mass spectrometric methods that provide structural information and that are compatible with normal-phase high-performance liquid chromatography (HPLC) would prove particularly useful for examination of the oxidation products derived from cholesteryl esters such as **1** and **2**.

Results and Discussion

Cholesteryl Linoleate. We have previously developed a method for analysis of the oxidation products of cholesteryl linoleate[5] and we have reported the use of this protocol for the analysis of products formed in the azo-initiated peroxidation of LDL. The four hydroperoxide products, **3**−**6**, are shown in Figure 1. Direct analysis of the hydroperoxides or the corresponding alcohols, **7**−**10**, by mass spectrometry (MS) is complicated by the fact that most MS ionization methods result in loss of water from the parent ion.[7] Furthermore, the resultant mass spectrum is a composite of the sterol and fatty acid fragmentation pathways. Dehydration of secondary hydroperoxides such as **3**−**6** leads to ketones, which give fragment ions that can provide structural information. Most of the useful MS ionization techniques are not compatible with normal-phase HPLC however, which is the method of choice for the separation of complex mixtures of oxidation products from lipids such as cholesteryl esters. Indeed, normal-phase HPLC separation of the cholesteryl linoleate oxidation products **7**−**10** was possible, and structures were assigned, after separation, by conversion of the cholesteryl ester to the corresponding methyl esters and comparison with samples previously characterized.[5] The procedure was tedious and time consuming.



Several new ionization techniques were explored to provide a more direct solution to the peroxide and hydroperoxide analytical problem, and the method that we have found to be most useful is coordination ion-spray−mass spectrometry (CIS−MS), a technique reported recently by Bayer and collaborators who have used this method to analyze unsaturated organic compounds.[8] This ionization method, when combined with techniques available to modern tandem-mass spectrometers, provides valuable structural information for several peroxides and hydroperoxides. One particular advantage of CIS−MS is that it can be coupled to normal-phase HPLC, whereas most ionization methods are normally used in conjunction with reversed-phase chromatography, in aqueous solvents.

A typical CIS−MS spectrum is shown in Figure 2 for compound **3**, the trans,cis-conjugated diene with peroxide substitution on carbon 13 of the linoleate chain. The spectrum results from a direct liquid injection (DLI) experiment of **3** in 1:1 hexane:2-propanol with 2 equivalents of $AgBF_4$. The dominant ions observed are the $[M_3+Ag]^+$ adducts at m/z = 787 and 789, formed from ^{107}Ag and ^{109}Ag isotopes that are present in a ratio of ∼1:1. Collision Induced Dissociation (CID) experiments on the complex of **3** and $^{107}Ag^+$ at $m/z = 787$ gave fragment ions at $m/z = 769$ $[M_3+Ag^+-H_2O]^+$ and $m/z = 687$ (loss of hexanal). CID on the $^{109}Ag^+$ peak gave corresponding fragment ions at $m/z = 771$ $[M_3+Ag^+-H_2O]^+$ and $m/z = 689$. Analysis of **4**, the trans,trans-substituted geometric isomer of **3**, gave essentially the same spectra as those observed for **3**.

Analysis of the two hydroperoxides **5** and **6** having the peroxide substitution at the 9 position on the linoleate chain gave the $[M_5+Ag]^+$ ion doublet at m/z = 787 and 789. CID experiments on the Ag adducts of **5** (or **6**) with $^{107}Ag^+$ at m/z = 787 gave fragment ions at $m/z = 769$ $[M_5+Ag^+-H_2O]^+$ and m/z = 647. The spectra of the carbon-13-substituted hydroperoxides (**3** and **4**) and the carbon-9-substituted isomers (**5** and **6**) are essentially identical with the exception that **3** and **4** give CID fragments at 687/689 whereas **5** and **6** give fragments at 647/649.

We suggest that this fragmentation pattern observed is due to Hock fragmentation of the silver ion hydroperoxide complex. Hock fragmentation (or rearrangement), shown in Figure 3, is commonly observed in solution for lipid hydroperoxides such as **3**−**6**.[9] This fragmentation is promoted by protic or Lewis acids at moderate temperatures. The process catalyzed by silver

(7) (a) MacMillian, D. K.; Murphy, R. C. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 1190−1201. (b) See also, Nakamura, T.; Hall, L.; Murphy R. C. In *Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Radiation Injury*, *4*; Honn, K. V., Ed.; Advances in Experimental Medicine and Biology; Kluwer Academic/Plenum: New York, 1999; Vol. 469, pp 539−545.

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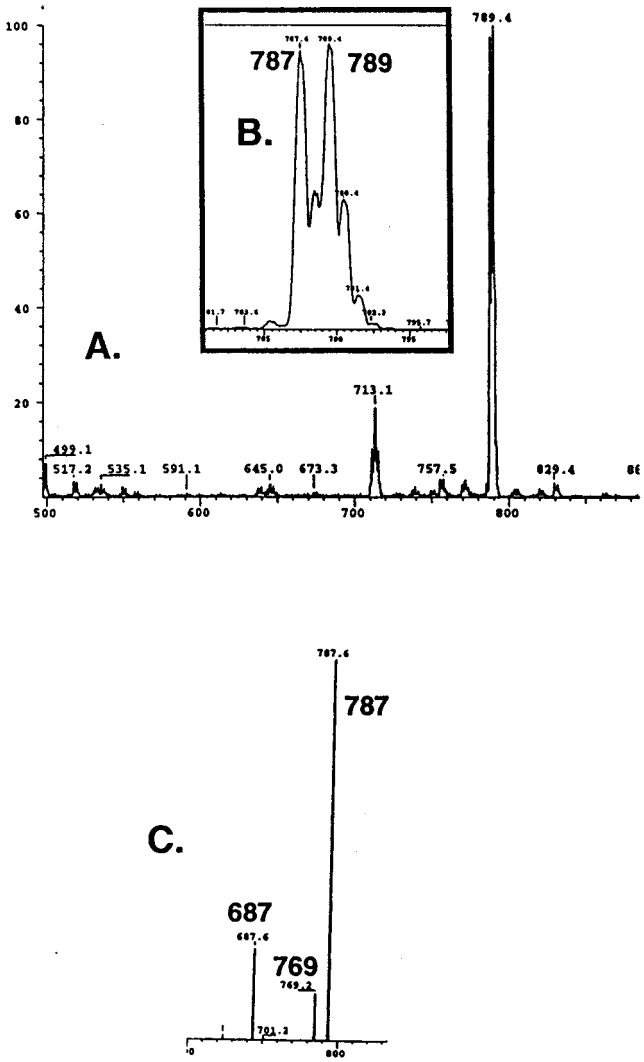


Figure 2. Direct injection silver ion coordination ion spray mass spectrum of **3**: (a) full scan; (b) blow up of $[M+Ag]^+$ region; (c) collision induced dissociation spectrum of $m/z = 787$.

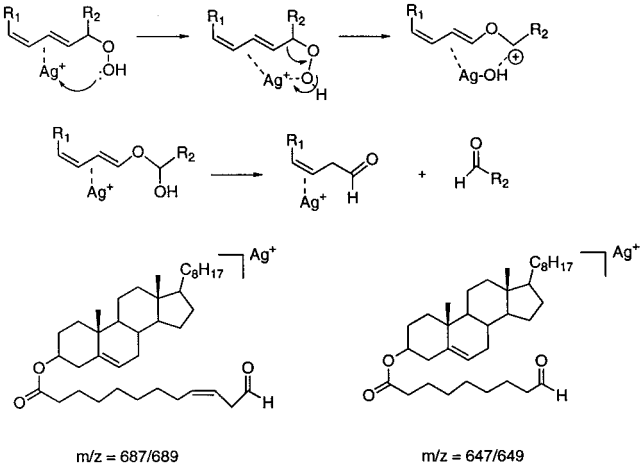


Figure 3. Hock fragmentation of a diene hydroperoxide and silver ion adducts observed for **3** and **5**.

ion Lewis acid is shown in Figure 3, but a proton could promote a similar rearrangment. Hock fragmentation in the Ag^+−CID experiment apparently occurs in the low-pressure collision cell and it is promoted by energy deposition in the collision process. It provides unambiguous information about the position of peroxide substitution on the linoleate chain. The structures assigned to the CID fragments $m/z = 687/689$ from hydroperoxides **3** and **4** and those assigned to the CID fragments $m/z = 647/649$ from hydroperoxides **5** and **6** are shown in Figure 3.

Coupling HPLC and Ag^+ coordination ion spray−mass spectrometry (HPLC−Ag^+CIS−MS) was possible by postcolumn mixing of a 0.26 mM $AgBF_4$ in 2-propanol with the effluent from a normal-phase separation. Chromatography was carried out with two Beckman Ultrasphere narrow bore silica columns at 150 μL/min of mobile phase (0.35% 2-propanol in hexane). The mixing of column effluent with the $AgBF_4$ coordinating ion was done with a Upchurch PEEK high-pressure mixing tee, with the silver solution added via syringe pump at a rate of 75 μL/min. The tetrafluoroborate salt of silver is soluble in most organic solvents making it ideal for use in this application.

A typical chromatogram for the mixture of hydroperoxides formed in the autoxidation of **1** is shown in Figure 4.[10] Panel A shows the chromatogram resulting from UV detection at 234 nm, and Panel B shows the total ion chromatogram of the Ag adducts. The chromatograms shown as Panels C and D in Figure 4 are the result of operation of the spectrometer in the selected reaction monitoring (SRM) mode. In SRM, a specific parent-to-daughter mass conversion produced at a characteristic energy in the collision cell is monitored. This mode clearly distinguishes the carbon-9-substituted hydroperoxides (Panel C), from the carbon-13-substituted compounds (Panel D). SRM essentially utilizes the differences in Hock fragmentation of the hydroperoxides to differentiate the eluting regioisomers.

Comment should also be made about differences observed between the chromatogram with UV detection (Figure 4, Panel A) and detection by CIS−MS (Figure 4, Panel B). Two peaks are observed between 24 and 25 min when CIS−MS detection is used that are not observed in the chromatograms obtained with UV detection at 234 nm. The first compound eluting in this region of the chromatogram gives $[M+Ag]^+ = 787$ adducts that undergo CID to give $m/z = 769$ $[M+Ag-H_2O]^+$ and a fragment at $m/z = 387$. The $m/z = 387$ fragment is assigned to $Ag^+ + C_{18}H_{32}O_2$, the silver ion complex of unoxidized linoleic acid. This fragment is observed in CID spectra of the parent cholesteryl linoleate, with the side-chain fragmentation from the cholesterol backbone apparently being an important dissociation mode. This compound is clearly a hydroperoxide of cholesteryl linoleate as evidenced by HPLC−CIS−MS, and it undergoes dehydration in CID mode but has an intact linoleate chain. Based on this evidence, we make the tentative structural assignment to this compound as the B-ring hydroperoxides of cholesteryl linoleate, **11**α or **11**β.[4] Some cholesterol backbone oxidation apparently competes with linoleate side-chain oxidation. The compound eluting at 25 min is also clearly a hydroperoxide of cholesteryl linoleate, but its CID spectrum is not instructive.

Cholesteryl Arachidonate. The free radical oxidation products from cholesteryl arachidonate have not been previously characterized. Based on the products of oxidation formed from other arachidonate esters, one anticipates formation of six hydroperoxides, **12**−**17**, analogous to the linoleate hydroperoxides. The acyclic hydroperoxides **12**−**17** are indeed formed if oxidation of the ester is carried out in the presence of good hydrogen atom donors, such as 1,4-cyclohexadiene. Structures for two of the hydroperoxides, **12** and **14** [cholesteryl-5-hydroperoxy eicosatetraenoate (Ch-5-HPETE) and cholesteryl-9-hydroperoxy eicosatetraenoate, (Ch-9-HPETE), respectively]

(10) Autoxidation was initiated by 10% di-*tert*-butyl hyponitrite (DTBN), see (a) Mendenhall, G. D. *Tetrahedron Lett.* **1983**, *24*, 451−454. (b) Traylor, T. G.; Kiefer, H. *Tetrahedron Lett.* **1966**, *49*, 6163−6168.

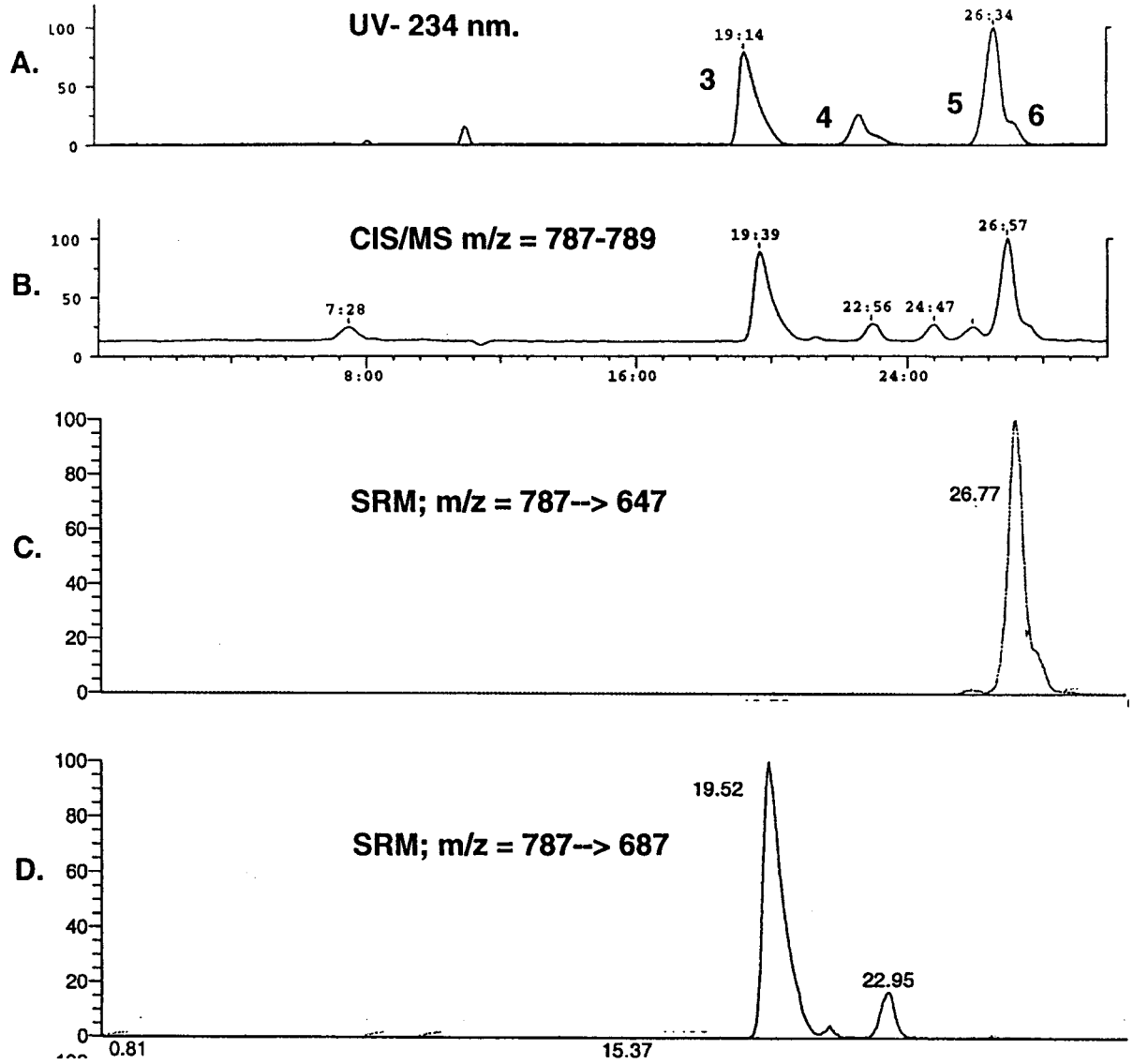


Figure 4. Chromatograms of cholesteryl linoleate oxidation mixture: (a) UV detection at 234 nm; (b) HPLC−CIS−MS in selected ion monitoring mode for $m/z = 787$ and 789; (c) HPLC−CIS−MS in selected reaction monitoring mode for $m/z = 787 \rightarrow 647$; (d) HPLC−CIS−MS in selected reaction monitoring mode for $m/z = 787 \rightarrow 687$.

are shown, and analogous compounds are also formed as follows: **13** (Ch-8-HPETE), **15** (Ch-11-HPETE), **16** (Ch-12-HPETE), and **17** (Ch-15-HPETE). HPLC analysis of the hydroperoxides **12**−**17** on silica separates all but **13** and **14**, which are only partially resolved. Reduction of the hydroperoxides to the corresponding alcohols and preparative HPLC provided pure compounds that were characterized by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and by conversion to the hydroxy eicosatetraenoate (HETE) methyl esters by reaction with sodium methoxide in methanol. The HETE methyl esters have been previously characterized, and comparison of the compounds derived from **12**−**17** with the known standards permitted assignments of structures to the hydroperoxides.

HPLC−Ag^+CIS−MS of the mixture of **12**−**17**, formed by autoxidation of **2**, provided support for the assigned structures. The chromatogram is presented in Figure 5, with UV chromatography detection shown in Panel A. SRM analysis of the parent $^{107}Ag^+$ adduct at $m/z = 811$ gives fragments that identify the position of substitution on the arachidonate chain. All of the SRM fragments result from cleavage of the cholesterol fatty acid link with characteristic fragments, shown in Figure 5, derived from Hock fragmentation of the arachidonate. Hock fragmentation of Ch-15-HPETE gives a fragment at $m/z = 343$ (Panel C). The 11- and 12-hydroperoxides of cholesteryl arachidonate give identical Hock fragmentation ions, and Panel D shows the sum of signals detected from the SRM for $m/z = 303$. Ch-8-HPETE and Ch-9-HPETE both give the same Hock fragmentation product and Panel C shows the SRM for this ion at $m/z = 283$, and the corresponding fragmentation of the Ch-5-HPETE gives $m/z = 327$.

Peroxyl Radical Cyclization. Hydroperoxide products **7**−**12** are major products of free radical oxidation of **2** only in the presence of good hydrogen atom donors, such as 1,4-cyclohexadiene. The product mixture becomes much more complex when cyclohexadiene is not used. Peroxyl radicals derived from **2** can undergo peroxyl radical cyclization in competition with hydrogen atom abstraction, and cyclization can lead to a host of different products.[11] Although cyclization products have not been previously reported from cholesteryl arachidonate, one expects them to form based on studies carried out and reported on the corresponding free acids and methyl esters.[12]

Scheme 1 outlines a mechanism that is useful in understanding the mixture of products that can form in the oxidation of

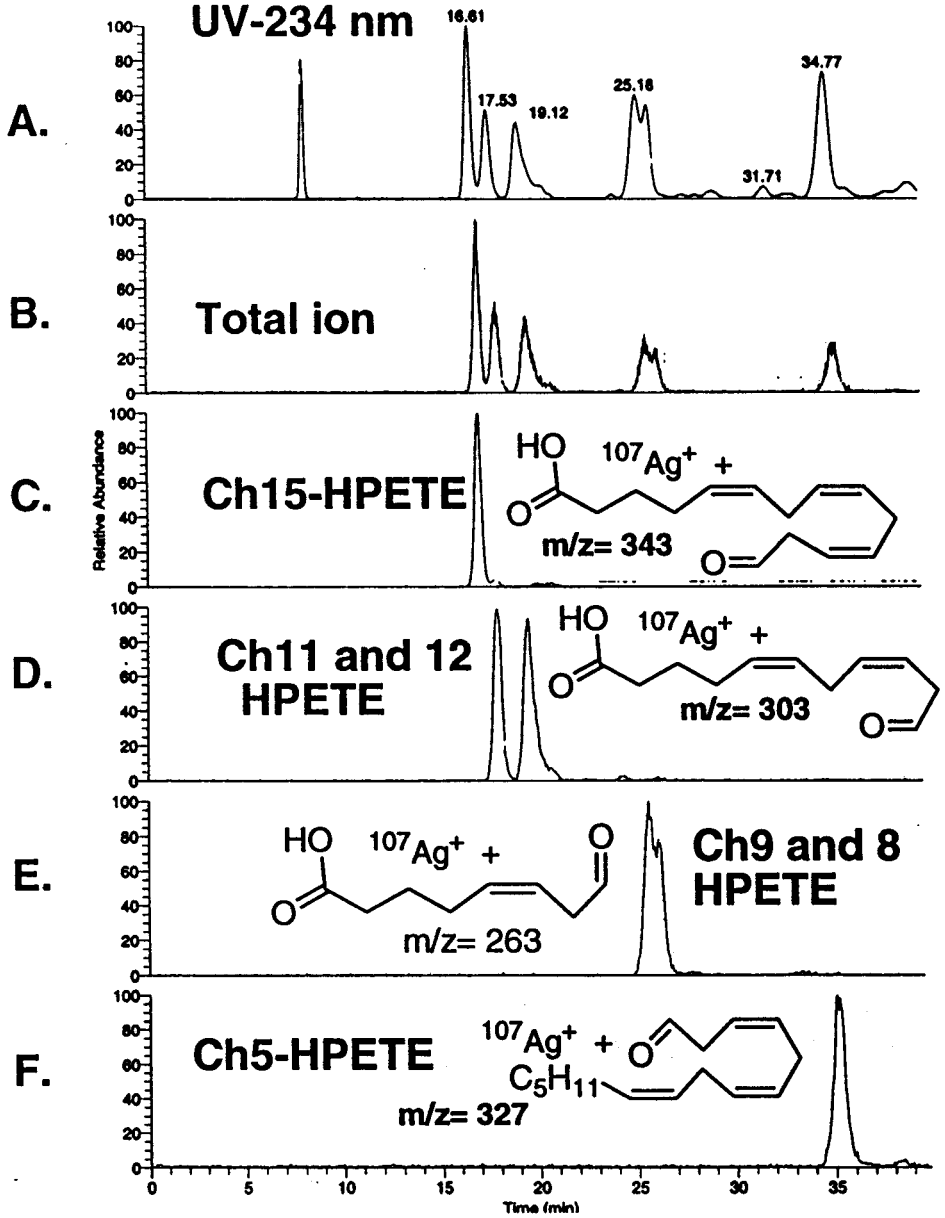
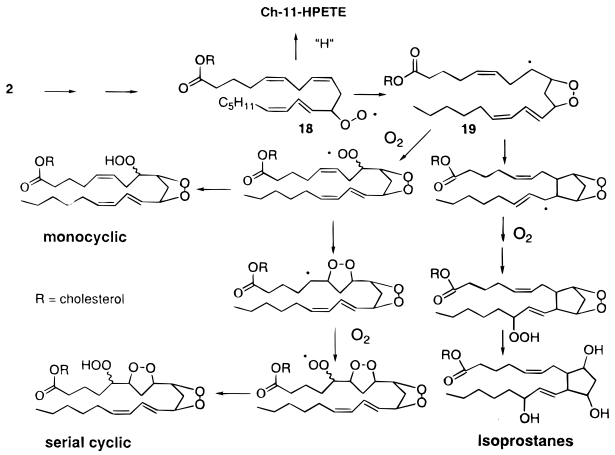


Figure 5. Chromatograms of cholesteryl arachidonate oxidation mixture formed by co−oxidation with cyclohexadiene: (a) UV detection at 234 nm; (b) HPLC−CIS−MS total ion current; (c) HPLC−CIS−MS in selected reaction monitoring mode for $m/z = 787 \rightarrow 343$; (d) HPLC−CIS−MS in selected reaction monitoring mode for $m/z = 787 \rightarrow 303$; (e) HPLC−CIS−MS in selected reaction monitoring mode for $m/z = 787 \rightarrow 327$.

Scheme 1

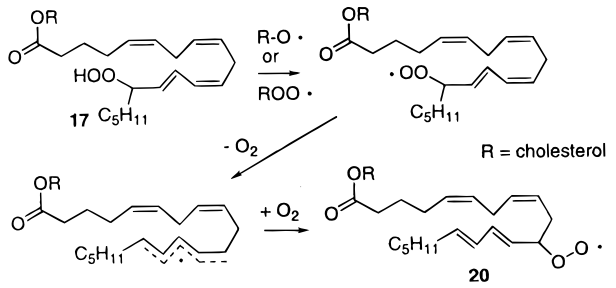


cholesteryl arachidonate. Six different peroxyl radicals are formed in oxidation of arachidonate, corresponding to the six different products **7**−**12**. Only one of these peroxyl radicals, **18**, is shown in the Scheme, but similar chemistry derives from the other radicals. Radical **18** can undergo hydrogen atom abstraction to give the corresponding hydroperoxide or 5-*exo* cyclization of the radical can occur giving **19**. Two competing pathways exist for reactions of the carbon radical **19**. Cyclization onto the conjugated diene leads ultimately to isoprostane structures, whereas addition of oxygen, which occurs presumably at the diffusion-controlled rate, gives a new peroxyl radical that can abstract hydrogen or cyclize again to form a second 1,2-dioxolane. The addition of oxygen to radical **19** would give

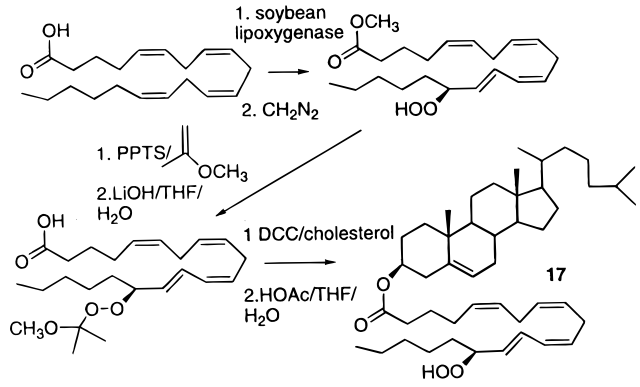
(11) (a) Porter, N. A., Caldwell, S. E., Mills, K. A. *Lipids* **1995**, *30*, 277−290. (b) Porter, N. A. *Acc. Chem. Res.* **1986**, *19*, 262−268. (c) O'Connor, D. E.; Mihelech, E. D.; and Coleman, M. C. *J. Am. Chem. Soc.* **1983**, *106*, 3577−3584. (d) Porter, N. A.; Funk, M. O.; Gilmore, D.; Isaac, R.; Nixon, J. *J. Am. Chem. Soc.* **1976**, *98*, 6000−6005. (e) Funk, M. O.; Isaac, R.; Porter, N. A. *J. Am. Chem. Soc.* **1975**, *97*, 1281−1282. (f) Porter, N. A.; Funk, M. O. *J. Org. Chem.* **1975**, *40*, 3614−3615. (g) Pryor, W. A.; Stanley, J. P. *J. Org. Chem.* **1975**, *40*, 3615−3617.

(12) Khan, J. A.; Porter, N. A., *Angew. Chem.* **1982**, *94*, 220−221.

Scheme 2



Scheme 3



members of the class of compounds identified in Scheme 1 as monocyclic and serial cyclic products. Note that the monocyclic compound contains three stereogenic centers, giving rise to eight possible stereoisomers (four pairs of enantiomers), whereas the five stereogenic centers in the serial cyclic structure leads to 16 pairs of enantiomers.

To simplify the mixture of higher oxidation products of cholesteryl arachidonate with the hope of isolating and characterizing pure compounds, we sought to generate one specific peroxyl radical capable of cyclization. The approach, which has been used in analogous systems,[9c−f] is outlined in Scheme 2. The hydroperoxide **17**, which can be prepared in large quantities by a chemo-enzymatic synthesis, may be used as a source of peroxyl radical **20**. Hydroperoxide hydrogens are readily abstracted by alkoxyl radicals, and hydrogen atom exchange between hydroperoxides is also a facile process. Thus, treatment of **17** with radical initiators that provide alkoxyl radicals starts a chain process in which a peroxyl radical is generated from **17**, and this peroxyl radical undergoes fragmentation and re-addition of oxygen, giving peroxyl radical **20**.[12] Note that radical **20** has a conjugated diene with trans,trans geometry, whereas peroxyl **18** shown in Scheme 1, has trans,cis diene geometry. This diene geometry is the only difference between the radical formed directly in the oxidation of cholesteryl arachidonate (**18**) and a radical generated indirectly (**20**) via a primary oxidation product **17**. In an oxidation mixture derived from cholesteryl arachidonate, one would likely see products derived from both **18** and **20**. Thus, products containing either trans,cis or trans,-trans diene geometry are expected.

The cholesteryl ester hydroperoxide **17** was prepared in quantity by the sequence outlined in Scheme 3. Dussault's peroxyketal group[13] was used to protect 15-hydroperoxyeicosatetraenoic acid (15-HPETE) and the coupling to cholesterol was achieved with DCC. Deprotection of the peroxyketal with acetic acid/THF/water gave **17**. Treatment of 0.15 M **17** in benzene with di-*tert*-butyl hyponitrite (DTBN; 0.10 eq), a room-temperature source of *t*-butoxy radicals, led to a complex product mixture of more polar peroxidic compounds. HPLC analysis of this product mixture gave a chromatogram that is displayed in Figure 6. The chromatogram shown is in SIM detection mode at $m/z = 875$ or 877, although essentially the same trace is obtained by UV detection (at 234 mm) or by SRM mode $m/z = 875 \rightarrow 591$. Several fractions were isolated by semipreparative chromatography of this product mixture. Material sufficient for analysis by NMR spectroscopy and direct-injection MS was obtained from five of the fractions identified as B, C, E, F/G, and I in Figure 6.

In Figure 7 is presented the Ag^+CIS−MS spectrum obtained by direct injection of fraction B of the chromatogram shown in Figure 6. The dominant ions observed are the $[M_{21}+Ag]^+$ ion at $m/z = 875$ and 877 (see insert B), which is consistent with the addition of four oxygen atoms (+64) to the starting hydroperoxide, **17**. The CID experiments on the complex of $^{107}Ag^+$ at $m/z = 875$ gave fragment ions at $m/z = 709$, 651, 637, 591, and 475, as shown in Figure 7C. Similar CID spectra were obtained from each of the fractions isolated. The data from the CID experiments suggest that the compounds eluting in fractions B, C, E, F/G, and I are diastereomers having the serial cyclic structure, **21** (Figure 8). The molecular ion of **21** + Ag^+ is 875/877, and the fragments observed in the CID spectra are consistent with structure **21**. Decomposition of peroxides initiated by heat, light, protic acid, Lewis acid, or reducing metal usually results in fragmentation of bonds β to peroxides and hydroperoxides.[14] The fragment ions observed in the CID spectra and shown in Figure 8 are all the result of such fragmentations in **21**. The fragments may provide unique markers for the serial cyclic structure, with β fragmentation leading to aldehyde and epoxide functional groups at multiple sites along the chain being the dominant CID pathway. In fact, chromatograms essentially identical to the one shown in Figure 6 can be obtained by operation in the SRM mode, with reaction of the m+$^{107}Ag^+$ complex ($m/z = 875$) to fragments at $m/z = 709$, 651, 637, 591, 475 being used for detection. Multiple-ion SRM confirms the structure because each of these ions is observed in the CID spectrum of **21**.

Analysis of fraction B by ^{1}H NMR showed the presence of five vinylic Hs, one being the cholesterol C-7 vinylic H observed at 5.38 δ, and the other four observed in a pattern consistent with a trans,trans-conjugated diene. In addition to conjugated diene, other protons were observed between 3.8 and 4.8δ, a region of the spectrum where protons α to peroxides and hydroperoxides are observed, and between 2.5 and 3.2δ, where protons substituted at C-4 of a 1,2-dioxolane are observed. Above 2δ, the complex spectrum typical of steroid derivatives made simple analysis impossible. The spectra of all other fractions (C, F/G, and I) showed evidence of trans,trans-conjugated diene structure and other features similar to those observed for fraction B. Most of the fractions appeared to contain one major component, but analysis of the ^{1}H NMR spectra obtained was not straightforward because most of the fractions were apparently not completely homogeneous. Attempts to obtain ^{13}C NMR spectra were abandoned because of the long acquisition times required. The NMR experiments defy simple analysis, but they are also supportive of the structures suggested on the basis of MS data.

(13) Dussault, P.; Sahli, A. *J. Org. Chem.* **1992**, *57*, 1009−1012.

(14) (a) Adam, W.; Duran, N. *J. Am. Chem. Soc.* **1977**, *99*, 2729−2734. (b) Adam, W.; Duran, N. *J. Org. Chem.* **1973**, *38*, 1434−1436. (c) Bloodworth, A. J.; Baker, D. S. *Chem. Commun.* **1981**, *11*, 547−549. (d) Bloodworth, A. J.; Eggelte, H. J. *Tetrahedron Lett.* **1984**, *25*, 1525−1528. (e) Balci, M.; Suetbeyaz, Y. *Tetrahedron Lett.* **1983**, *24*, 311−314. (f) Yoshida, M.; Miura, M.; Nojima, M.; Kusabayashi, S. *J. Am.Chem. Soc.* **1983**, *105*, 6279−6285.

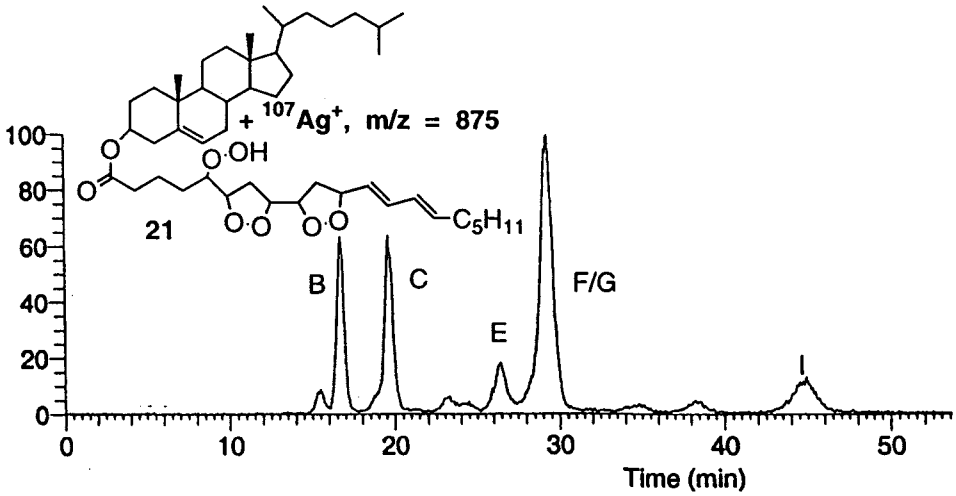
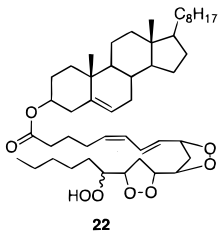


Figure 6. Chromatograms of product mixture formed from Ch-15-HPETE rearrangement selected ion monitoring of *m*/*z* = 875.

The serial cyclic compounds with the structure **21** result from *5-exo* cyclization of a peroxyl radical substituted at C-11 onto the Δ-8 double bond of the arachidonate (see Scheme 2). A different set of serial cyclic products is expected starting from a peroxyl radical substituted at C-9 that is formed by H atom abstraction at C-7 and oxygen addition at C-9. To access members of this serial cyclic class, we isolated milligram quantities of the 5-hydroperoxy cholesteryl arachidonate, **12**, from a product mixture of a cholesteryl arachidonate oxidation in the presence of cyclohexadiene.

Compound **12** was treated with the free radical initiator (DTBN) under conditions identical to those used to generate the serial cyclic structures **21** from **17**. This procedure led to a product mixture that was chromatographed on silica with detection by UV (234 nm) as well as by Ag^+CIS−MS in the SIM (*m*/*z* = 875−877) and SRM modes. The chromatograms obtained were essentially identical for the different detection methods, and an illustrative HPLC chromatogram is presented in Figure 9. In Figure 9, UV detection of the chromatogram is shown in Panel A, and Panel B shows the SRM of the reaction for ion *m*/*z* = 875 → 657. Indeed, the CID of this set of serial cyclic products, **22**, is much simpler than the CID obtained from serial cyclic products **21** shown in Figure 7. The fragment ion **23** shown in Panel B of Figure 9 is the dominant fragment. CID showed other ions consistent with the structure **22**, but they were formed to a much lesser extent than was **23**.



22

The studies just outlined provide a basis for analysis of an autoxidation mixture derived from cholesteryl arachidonate. The product mixture will likely include diastereomers with the general structures **21** and **22**. Another set of serial cyclic products is also expected, as shown in Scheme 1. Thus, products analogous to **21** and **22** with trans,cis-conjugated diene stereochemistry are expected in a direct autoxidation of **2**. The trans,-cis products are formed from the first-formed set of peroxyl radicals (see Scheme 1), whereas the trans,trans products form after β fragmentation−re-addition, as illustrated in Scheme 2.

Autoxidation of cholesteryl arachidonate in benzene with DTBN initiator gave a mixture that was chromatographed under conditions similar to those used to analyze the serial cyclic products **21** and **22**. An illustrative chromatogram obtained from the autoxidation mixture is shown in Figure 10. Panel A of Figure 10 shows UV detection of this mixture at 234 nm, Panel B is an SRM chromatogram that detects the formation of serial cyclic stereoisomers of **21** by detecting the CID reaction of *m*/*z* 875 → 591, and Panel C is an SRM that detects stereoisomers of structure **22** by detecting the CID reaction of *m*/*z* 875 → 657. It is noteworthy that most of the fractions observed by UV detection at 234 nm are also observed by the SRM method. The MS detection method apparently is detecting the principal compounds in the product mixture that have conjugated diene absorption at 234 nm. SRM chromatograms detecting fragments other than *m*/*z* 657 or 591 but that are observed in the CID of **21** or **22** were similar to those shown in Panels B and C. Thus, SRM of the conversion *m*/*z* 875 → 709 gave a chromatogram essentially identical to that shown in Panel B. This result can be understood based on the fact that *m*/*z* 709 and 591 are both observed in the CID spectrum of **21** and either fragment detects elution of a stereoisomer of **21**.

As can be seen in Figure 10, the mixture of products with fragmentation indicative of **21** is more complex than the chromatogram of **21** containing only the trans,trans diene functionality (compare Panel B of Figure 10 and Figure 6). Similarly, the mixture of products with fragmentation consistent with structure **22** is more complex than a mixture of **22** containing only trans,trans diene functionality (compare Panel C of Figure 10 and Figure 9). As noted, the mixture obtained from direct oxidation (Figure 10) contains both trans,cis and trans,trans dienes, and we have assigned trans,trans compounds in the Panels B and C of Figure 10 by comparison with the chromatograms shown in Figures 6 and 9. The other compounds eluting in the chromatograms that have essentially the same fragmentation patterns are assigned the structure of the corresponding trans,cis isomers of the serial cyclic products. Thus, in Panel B, the pair of peaks identified as "B" and "C" (trans,-trans-conjugated diene), based on comparison with the chromatogram shown in Figure 6, are immediately preceded by two peaks that we tentatively assign as the corresponding trans,cis isomers. We hesitate to provide more than this tentative identification at this time because to do so would require product isolation and a more classical structure proof.

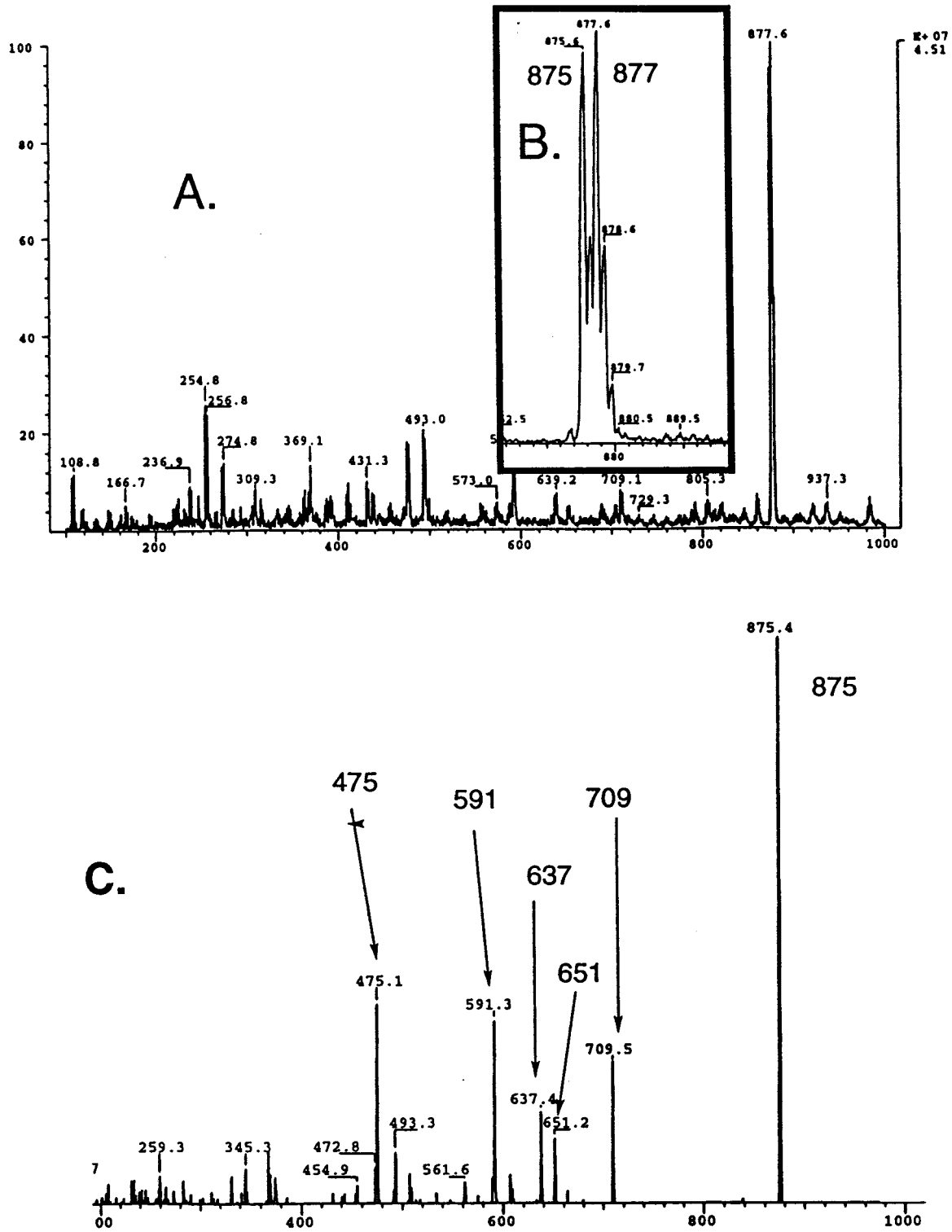


Figure 7. Direct injection silver ion coordination ion spray mass spectrum of fraction b from the chromatogram shown in Figure 6: (a) full scan; (b) blow up of $[M+Ag]^+$ region; (c) collision induced dissociation spectrum of $m/z = 875$.

Monocyclic Peroxides and Isoprostanes.[15] Product mixtures derived from Ch-15-HPETE containing **21** and from Ch-5-HPETE containing **22** showed only small amounts of compounds with molecular weights $[(M + Ag^+) = 843/845]$ consistent with monocyclic peroxide or isoprostane structures (see Scheme 1). Consider the experiments in which Ch-15-HPETE, **17**, served as precursor for the serial cyclic compounds **21**, as shown in Scheme 4. The hydroperoxide **17** was converted to the peroxyl radical by H atom transfer and a monocyclic compound, **24**, would be formed from *5-exo* cyclization and oxygen addition. Compound **24** is, itself, also a hydroperoxide, which means that **24** can serve as a hydrogen-atom donor and generate a peroxyl radical that can cyclize again. Thus, even if **24** is formed in the reaction, it can be converted subsequently to **21** by further hydrogen-atom exchange, cyclization, oxygen addition, and hydrogen-atom transfer.

(15) Morrow, J. D.; Minton, T. A.; Mukundan, C. R.; Campbell, M. D.; Zacker, W. E.; Daniel, V. C.; Badr, K. R.; Blair, I. A.; Roberts, J. L., Jr. *J. Biol. Chem.* **1994**, *269*, 4317−4326 and references cited.

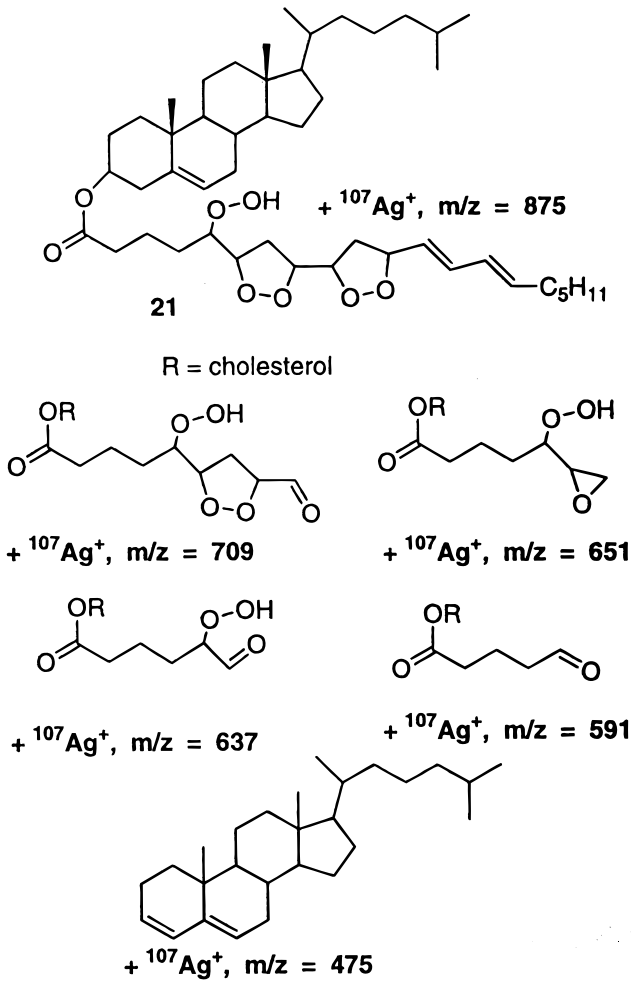


Figure 8. Structure of serial cyclic product **21** and fragment ions formed by collision induced dissociation of the $[M+Ag]^+$ adduct.

The experiments that lead to formation of the serial cyclic compounds from the isolated hydroperoxide precursors were biased toward the isolation of serial cyclic compounds. This fate is not, however, expected for monocyclic peroxides formed from precursor peroxyl radicals substituted at the 8 or 12 position of the arachidonate chain. Thus, monocyclic peroxide **25**, derived from Ch-12-HPETE, cannot form a serial cyclic product by subsequent reactions. There is some expectation, therefore, that monocyclic peroxides might be formed in the autoxidation of Ch-20:4 because compounds such as **25** are possible products.

In Figure 11 is presented the chromatogram obtained from analysis of the mixture formed by autoxidation of Ch-20:4. Detection of products formed was by UV at 234 nm (Panel A) and by SIM at m/z = 843/845 (Panel B). Serial cyclic compounds are detected in HPLC−Ag^+CIS−MS at m/z = 875 and 877, monocyclic compounds and isoprostanes at m/z = 843 and 845, and Ch-HPETEs at m/z = 811 and 813. A reconstructed ion current (RIC) chromatogram of ions at m/z = 875/877, 843/845, and 811/813 is presented in Panel C. It seems clear from the chromatogram shown in Panel B that monocyclic peroxides or isoprostane endoperoxides are present in the reaction mixture. Based on the fact that monocyclic compounds have a conjugated diene whereas isoprostanes do not (see Scheme 1) and by comparison of Panels A and B, the fractions identified with an "*m*" are suggested to be monocyclic peroxides. This assignment is based on the observation that these fractions have m/z ions at 843/845 and also have a conjugated diene, as is evidenced from UV detection. It should also be noted that there is a fraction observed in Panels B and C of Figure 11 at 15 min that does not have significant absorbance in the UV spectrum at 234. The compound eluting in this fraction may be an endoperoxide precursor to an isoprostane based on the MS and UV evidence. Assignment of structure to fractions based on the information obtained from only HPLC−MS and UV is risky, and further experiments are clearly in order to confirm or deny these structural assignments.

Conclusions. Ag^+CIS−MS provides a powerful new tool for the study of lipid peroxidation. The technique relies on the interaction of the soft Lewis acid, Ag^+, with soft Lewis base centers such as carbon−carbon double bonds. Highly oxidizable lipids generally have polyunsaturation and the products of lipid peroxidation are also highly unsaturated, making the technique appropriate to the problem. In addition, the silver ion complexes of hydroperoxides and polyperoxides provide useful structural information based on understandable fragmentation patterns.

The analysis of cholesteryl linoleate hydroperoxides, Ch-HPETEs and complex peroxide mixtures derived from rearrangements of Ch-5- and Ch-15-HPETEs, as well as products formed by direct oxidation of Ch-20:4 illustrate that complex mixtures of diverse peroxides can be studied. Coupling Ag^+-CIS−MS to HPLC makes possible the use of the powerful tools of SIM and SRM, which can dissect complex mixtures into constituent molecular classes. The SRM chromatograms shown in Figure 10, for example, provide detailed information about the serial cyclic structures that are present in the product mixture, whereas Figure 11 is an analysis of the same reaction mixture but with focus on monocyclic or isoprostane structures. The two analyses serve to deconvolute the chromatogram of the complex mixture into chromatograms of constituent classes of peroxide compounds.

This report is intended to demonstrate the potential utlility of this method and it is not intended to be exhaustive in its scope. Extensive studies of oxidation product mixtures of diverse lipid classes are ongoing and will be reported in due course.

Experimental Section

General Methods. Reactions involving hydroperoxides were monitored by TLC using a stain of 1.5 g of *N*,*N*′-dimethyl-*p*-phenylenediamine dihydrochloride/25 mL of H_2O/125 mL of MeOH/1 mL of acetic acid. Hydroperoxides yield an immediate pink color,[4] whereas protected hydroperoxides exhibit a pink color after mild charring. TLC was carried out using 0.2-mm layer thickness, Si-coated aluminum columns (EM Scientific) that were visualized by UV_{254}, phosphomolybdic acid char, or the peroxide stain. In general, hydroperoxides were stored as dilute solutions with 1 mol % butylated hydroxytoluene (BHT) in either hexanes or benzene at −78 °C, and they were never exposed to temperatures >40 °C. All HPLC solvents were filtered through Whatman Nylon membrane filters (0.45-μm pore size) prior to use.

Chemicals. All lipids were purchased from Nu Chek Prep (Elysian, MI) and were of the highest purity (>99+%). Soybean lipoxidase (Type I−B, EC. 1.13.11.12) was purchased from Sigma Chemical Company (St. Louis, MO). Sodium hyponitrite was purchased from Michigan Technological Institute (Houghton, MI). Chelex-100 Resin was purchased from Bio-Rad Laboratories (Hercules, CA) as the 50−100 mesh, sodium form. Organic solvents, such as 2-propanol, hexane, benzene, and ethyl acetate, were HPLC quality and purchased from either Mallinckrodt, Inc. (St. Louis, MO) or Fisher Chemical (Phillipsburg, NJ). Hexanes for LC−MS were purchased from Burdick & Jackson (Muskegon, MI). All other reagents were purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification. The free radical initiator di-*tert*-butyl hyponitrite (DTBN) was synthesized prior to use.[10] Borate buffer (pH 9.0, 0.2 M) was prepared and treated with Chelex-100 resin (10 g/L buffer) for at least 24 h to remove transition metal contaminants.

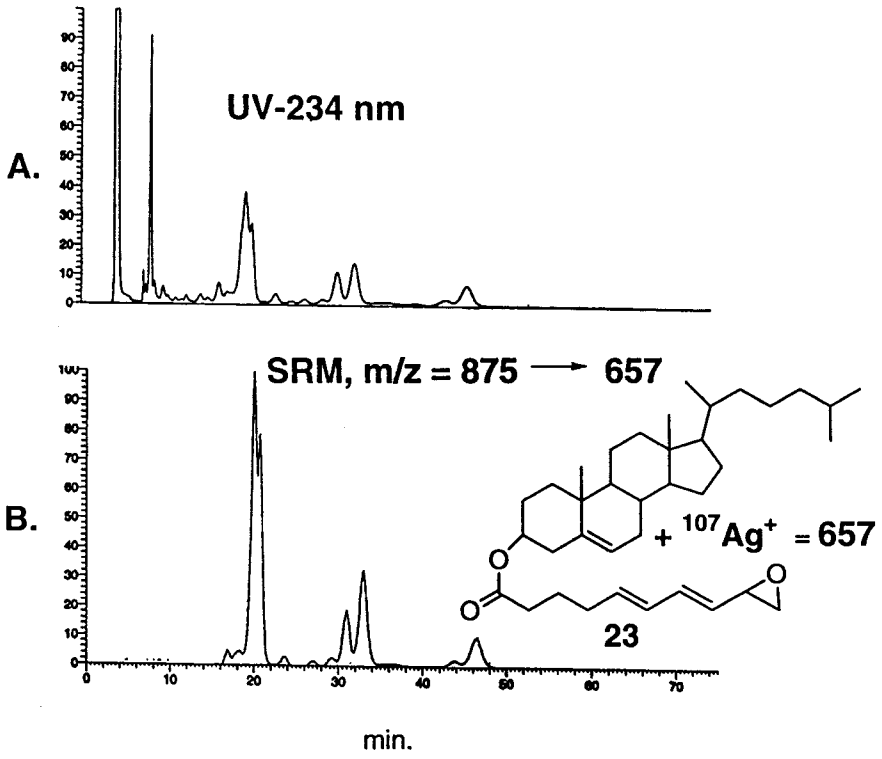


Figure 9. Chromatograms of product mixture formed from Ch-5-HPETE rearrangement: (a) UV detection at 234 nm; (b) selected reaction monitoring of $m/z = 875 \rightarrow 657$.

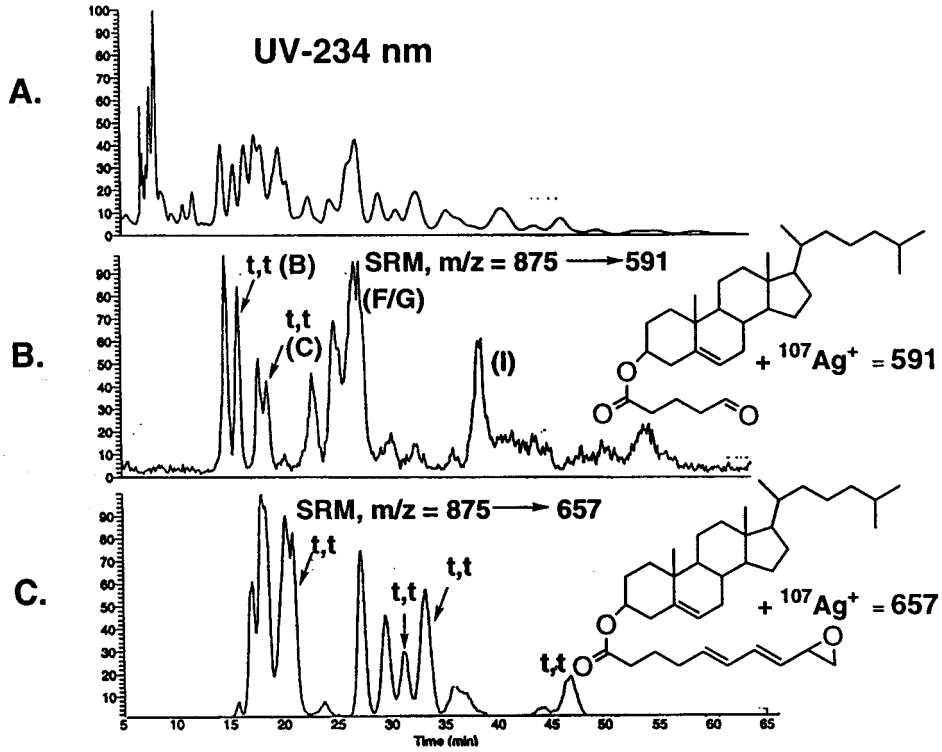
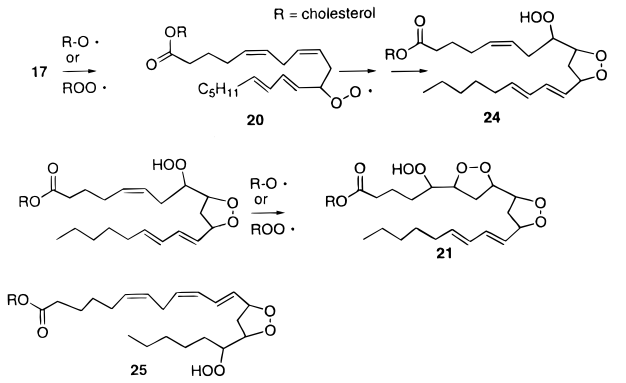


Figure 10. Chromatograms of cholesteryl arachidonate oxidation mixture: (a) UV detection at 234 nm; (b) HPLC−CIS−MS in selected ion monitoring mode for $m/z = 875 \rightarrow 591$; (c) HPLC−CIS−MS in selected reaction monitoring mode for $m/z = 875 \rightarrow 657$.

Instruments. ^{1}H NMR spectra were recorded on either a Varian INOVA 400 MHz spectrometer or a Bruker DRX-400 spectrometer in $CDCl_3$. Chemical shifts are reported in ppm (δ) with respect to the residual H signal in $CDCl_3$ ($\delta = 7.26$ ppm) and coupling constants (J values) are given in Hz. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), and m (multiplet). Immediately prior to NMR sample preparation, $CDCl_3$ was passed through a plug of basic alumina to remove adventitious HCl.

Analytical HPLC was conducted on a Waters model 600 HPLC instrument with a Hewlett-Packard 1050 Multiwavelength detector and a Hewlett-Packard 3396 Series III integrator. For cholesteryl linoleate and cholesteryl arachidonate hydroperoxide analysis, the HPLC was equipped with two tandem Beckman Ultrasphere 5-μm silica columns (4.6 mm × 25 cm), and 0.5% 2-propanol in hexane at a delivery rate of 1.0 mL/min was used. For analytical analysis of the more polar cyclic peroxides, HPLC was conducted on a Waters model 600E pump with

Scheme 4



a Waters 717plus Autosampler and a Waters 996 Photodiode array detector. The autosampler and PDA detector were controlled by Millenium chromatography software (Waters Corp., Milford, MA). A single Beckman Ultrasphere 5-μm silica column (4.6 mm × 25 cm) with a flow rate of 1.0 mL/min of 1.0% 2-propanol in hexanes was utilized for analytical cyclic lipid hydroperoxide analysis. Semipreparative HPLC was conducted on a Waters model 600E HPLC instrument, with a Waters model 481 variable wavelength detector operating at 234 nm and with output to a Fisher Record-All Series 5000 strip chart recorder. A Dynamax-60 Å silica 83−121-C column (21.4 mm × 25 cm × 8 μm particle) purchased from Rainin Instrument Company (Woburn, MA), 0.66% 2-propanol in hexane, and a solvent delivery rate of 10 mL/min were used for the preparative scale separations of cholesteryl arachidonate hydroperoxides (Ch-HPETEs). Semipreparative isolation of the more polar cyclic peroxides was accomplished using the same column with 1.1% 2-propanol in hexane at a flow of 10 mL/min.

Mass Spectrometry. CIS-MS was performed using a Finnigan TSQ-7000 (San Jose, CA) triple quadrupole mass spectrometer equipped with a standard API-1 electrospray ionization source outfitted with a 100-μm deactivated fused Si capillary. Data acquisition and spectral analysis were conducted with ICIS software, version 8.3.2, on a Digital Equipment Corp. Alpha Station 200 4/166. Data collected for selected reaction monitoring (SRM) experiments was also processed using Xcalibur, version 1 (Finnigan, San Jose, CA). Nitrogen gas served both as the sheath and auxiliary gas, and argon served as the collision gas. The electrospray needle was maintained at 4.6 kV, and the heated capillary temperature was 200 °C. The tube lens and capillary voltages were optimized to maximize ion current for electrospray, with the optimals determined to be 80 and 20 V, respectively, for cholesteryl ester analysis. For CID experiments, the collision gas pressure was typically 2.30−2.56 mTorr. To obtain fragmentation information of each compound, the dependence of offset-voltage and relative ion current (RIC) was studied. The collision energy offset was varied from 10 to 40 eV depending on the compound being analyzed. Positive ions were detected scanning from 100 to 1000 amu in both the parent and daughter ion scans, with a scan duration of 1 s. Profile data were recorded for 1 min (∼60 scans) and averaged for analysis.

Samples were introduced either by direct liquid infusion or HPLC. For direct liquid injection, stock solutions of the lipids (100 ng/μL in 1% 2-propanol in hexane) were prepared and mixed 1:1 with silver tetrafluoroborate (51.4 ng/μL in 2-propanol). Samples were introduced to the ESI source with a syringe pump at a rate of 10 μL/min. For HPLC sample introduction, a Hewlett-Packard 1090 HPLC system was used. The auxiliary gas flow rate was increased to between 5 and 10 units to assist in desolvation of the samples. For cholesteryl linoleate and cholesteryl arachidonate hydroperoxide analysis, normal-phase HPLC sample introduction was carried out using two tandem Beckman Ultrasphere narrowbore 5-μm silica columns (2.0 mm × 25 cm) operated in isocratic mode with 0.35% 2-propanol in hexanes. For analysis of cyclic peroxide mixtures, sample introduction was carried out using a single Beckman Ultrasphere narrowbore 5-μm silica column (2.0 mm × 25 cm) operated in isocratic mode with 1.0% 2-propanol in hexanes. The flow rate for both modes of chromatography was 150 μL/min. Column effluent was passed through an Applied Biosystems 785A programmable absorbance UV detector, with detection at 234 nm. An Upchurch PEEK high-pressure mixing tee was connected next in series for the postcolumn addition of the silver salts. The silver tertafluoroborate ($AgBF_4$) solution (0.25 mM in 2-propanol) was added via a Harvard Apparatus (Cambridge, MA) syringe pump at a flow rate of 75 μL/min. A section of PEEK tubing (1.04 m, 0.25 mm i.d.) allowed time for the complexation of the silver to the lipid while delivering effluent to the mass spectrometer. A Rheodyne 7725 injector was fitted with a 100-μL PEEK loop for 20−50 μL sample injections.

Autoxidation of Cholesteryl Esters for Lipid Hydroperoxide Analysis. Oxidation of Cholesteryl Linoleate. A round-bottomed flask was charged with cholesteryl linoleate (300 mg, 0.462 mmol) and was diluted to 0.20 M with 1,4-cyclohexadiene (0.876 mL, 9.26 mmol) and dry benzene (1.43 mL). DTBN (2 mg) was added to the solution and the sealed flask was heated to 37 °C under an oxygen atmosphere. After 24 h, TLC indicated the formation of peroxidic products. Butylated hydroxytoluene (BHT, ∼2 mg) was added to the reaction. Analytical HPLC (tandem Si columns, 0.5% 2-propanol in hexanes, λ = 234 nm) indicated the formation of four major components in the mixture: **3**, t_R = 15.0 min; **4**, t_R = 17.5 min; **5**, t_R = 20.1 min; and **6**, t_R = 20.5 min. Semipreparative HPLC (0.66% 2-propanol in hexane) was used to separate the components. Compounds **5** and **6** were isolated as a mixture.

Oxidation of Cholesteryl Arachidonate. In a round-bottomed flask, cholesteryl arachidonate (400 mg, 0.594 mmol) was dissolved in 1,4-cyclohexadiene (1.6 mL, 17.0 mmol) and dry benzene (2.7 mL) to give a solution 0.15 M in lipid and 4.0 M in 1,4-cyclohexadiene. DTBN (3 mg) was added and the reaction was stirred under oxygen at 37 °C for 24 h, after which time BHT (2 mg) was added. Analytical HPLC (tandem Si columns, 0.5% 2-propanol in hexanes, λ = 234 nm) indicated the formation of six major fractions: A, t_R = 11.44 min; B, t_R = 12.14 min; C, t_R = 13.12 min; D.1, t_R = 16.97 min; D.2, t_R = 17.29 min; and E, t_R = 22.57 min. Preparative HPLC (0.66% 2-propanol in hexane) was used to separate the components. Compounds D.1 and D.2 were isolated as a mixture.

Identification of Cholesteryl Arachidonate Oxidation Products. A mixture of cholesteryl arachidonate hydroperoxides (Ch-HPETEs) was prepared as already described. Preparative-scale HPLC isolation of the five major fractions was achieved with a Dynamax-60 Å Si 83−121-C column (21.4 mm × 25 cm × 8 μm particle) in isocratic mode, with 0.66% 2-propanol in hexane as solvent at a delivery rate of 10 mL/min. Each of the eluting hydroperoxides was collected into a round-bottomed flask containing BHT (3 mg) and an excess of triphenylphosphine (50 mg) for in situ reduction of the hydroperoxides to the corresponding alcohols. Each of the fractions was concentrated and rechromatographed under the same solvent conditions to give pure cholesteryl arachidonate alcohols, which were examined by ^{1}H NMR and by conversion to their corresponding methyl esters, which were previously fully characterized.[6]

Cholesteryl 15-hydroxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoate (fraction A). Analytical HPLC (0.5% IPA in hexanes, λ = 234 nm): t_R 11.44 min (−OOH), t_R 15.36 min (−OH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.50 (dd, J = 15.1, 11.2 Hz, 1H), 5.98 (t, J = 10.9, 10.9 Hz, 1H), 5.68 (dd, J = 15.1, 6.7 Hz, 1H), 5.38 (m, 6H), 4.59 (m, 1H), 4.15 (m, 1H), 3.46 (bs, 1H), 2.94 (m, 2H), 2.81 (m, 2H), 2.30−0.83 (m, 57H), 0.66 (s, 3H).

Cholesteryl 12-hydroxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoate (fraction B). Analytical HPLC (0.5% IPA in hexanes, λ = 234 nm): t_R 12.14 min (−OOH), t_R 14.87 min (−OH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.54 (dd, J = 15.2, 11.3 Hz, 1H), 5.97 (t, J = 11.1, 11.1 Hz, 1H), 5.71 (dd, J = 15.2, 6.3 Hz, 1H), 5.55 (m, 1H), 5.37 (m, 5H), 4.59 (m, 1H), 4.20 (m, 1H), 3.49 (bs, 1H), 2.91 (m, 2H), 2.34−0.83 (59H), 0.65 (s, 3H).

Cholesteryl 11-hydroxy-5(*Z*),8(*Z*),12(*E*),14(*Z*)-eicosatetraenoate (fraction C). Analytical HPLC (0.5% IPA in hexanes, λ = 234 nm): t_R 13.12 min (−OOH); t_R 17.76 min (−OH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.51 (dd, J = 15.3, 11.0 Hz, 1H), 5.95 (t, J = 10.5, 10.5 Hz, 1H), 5.67 (dd, J = 15.1, 6.3 Hz, 1H), 5.45 (m, 6H), 4.60 (m, 1H), 4.21 (m, 1H), 3.56 (bs, 1H), 2.78 (m, 2H), 2.37−0.82 (59H), 0.66 (s, 3H).

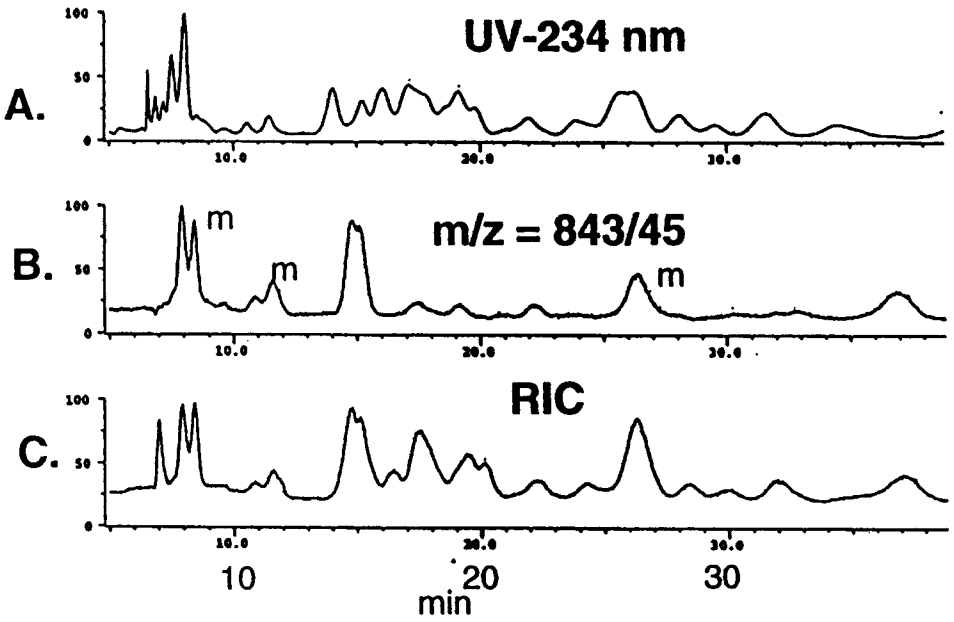


Figure 11. Chromatograms of cholesteryl arachidonate oxidation mixture: (a) UV detection at 234 nm; (b) HPLC−CIS−MS in selected ion monitoring mode for m/z = 843 and 845; (c) HPLC−CIS−MS, reconstructed ion current (RIC) for ions 811 and 813 (monohydroperoxides), 843 and 845 (monocyclic peroxides or isoprostanes), and 875 and 877 (serial cyclic peroxides).

Cholesteryl 9-hydroxy-5(*Z*),7(*E*),11(*Z*),14(*Z*)-eicosatetraenoate (fraction D.1). Analytical HPLC (0.5% IPA in hexanes, λ = 234 nm): t_R 16.97 min (−OOH); t_R 22.89 min (−OH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.48 (dd, J = 15.1, 11.2 Hz, 1H), 6.00 (t, J = 10.8, 10.8 Hz, 1H), 5.69 (dd, J = 15.5, 6.6 Hz, 1H), 5.53 (m, 1H), 5.36 (m, 5H), 4.60 (m, 1H), 4.20 (m, 1H), 3.53 (bs, 1H), 2.79 (m, 2H), 2.37−0.83 (59H), 0.65 (3H).

Cholesteryl 8-hydroxy-5(*Z*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoate (fraction D.2). Analytical HPLC (0.5% IPA in hexanes, λ = 234 nm): t_R 17.29 min (−OOH), t_R 25.80 min (−OH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.57 (dd, J = 15.1, 11.2 Hz, 1H), 5.97 (t, J = 10.9, 10.9 Hz, 1H), 5.69 (dd, J = 15.1, 6.6 Hz, 1H), 5.41 (m, 6H), 4.60 (m, 1H), 4.21 (m, 1H), 3.50 (bs, 1H), 2.91 (m, 2H), 2.34−0.83 (59H), 0.66 (s, 3H).

Cholesteryl 5-hydroxy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoate (fraction E). Analytical HPLC (0.5% IPA in hexanes, λ = 234 nm): t_R 22.57 min (−OOH), t_R 41.31 min (−OH);^{1}H NMR (400 MHz, $CDCl_3$): δ 6.51 (dd, J = 15.1, 11.2 Hz, 1H), 5.97 (t, J = 10.9, 10.9 Hz, 1H), 5.67 (dd, J = 15.4, 6.6 Hz, 1H), 5.36 (m, 6H), 4.60 (m, 1H), 4.17 (m, 1H), 3.52 (bs, 1H), 2.94 (m, 2H), 2.80 (m, 2H), 2.32−0.82 (57H), 0.66 (s, 3H).

Conversion of Cholesteryl Esters to Methyl Esters. Methyl arachidonate hydroperoxides were prepared as described[5,6] by autoxidation, analogous to that described for cholesteryl arachidonate. The mixture of methyl arachidonate hydroperoxides was treated with an excess of triphenylphosphine to generate the corresponding alcohols for comparison purposes. Each isolated cholesteryl arachidonate alcohol was dissolved in benzene (0.5 mL) and converted to the methyl ester by treatment with an excess of NaOMe in methanol for 2 h. The reactions were worked up by the addition of deionized water (1.0 mL) and glacial acetic acid (100 μL), followed by the extraction of the aqueous layer with hexane (2 × 5 mL). The combined organic layers were dried, concentrated, and analyzed by analytical HPLC equipped with two tandem Beckman Ultrasphere 5μm silica columns (4.6 mm × 25 cm) in isocratic mode with 0.6% 2-propanol in hexanes containing 0.1% acetic acid at a flow rate of 3.0 mL/min with UV detection at 234 nm. Co-injection of each transesterified fraction with the methyl arachidonate alcohol mixture confirmed assignment of the elution order. The elution order of the transesterified fractions was confirmed and correlated with the original cholesteryl arachidonate compounds: **16**, t_R 11.187 min; **17**, t_R 11.675 min; **15**, t_R 13.052 min; **14**, t_R 17.541 min; **13**, t_R 17.81 min; **12**, t_R 28.598 min.

CIS−MS Analysis of Cholesteryl Ester Hydroperoxides. Direct Liquid Injection Analysis (DLI). Individual cholesteryl linoleate and arachidonate hydroperoxide fractions were isolated by semipreparative HPLC (0.66% IPA in hexanes, 10.0 mL/min) and analyzed by direct liquid infusion of the sample. Briefly, each isolated lipid fraction was concentrated to dryness and a lipid solution (100 ng/μL) was prepared. This lipid solution was mixed 1:1 with $AgBF_4$ in 2-propanol (51.4 ng/μL) to give a solution of ∼50 ng/μL lipid and 25 ng/μL Ag^+. On a molar basis, the silver-to-lipid concentration is approximately 2:1. The lipid + Ag^+ solution is introduced to the ESI source via a syringe pump at a rate of 10 μL/min. Parent ion scans were collected over the mass range 100−1000 amu for a scan duration of 1 s. Mass spectra were typically collected in profile mode for 1 min (60 scans) and averaged. LC−MS−MS experiments were carried out with a argon gas pressure of 2.56 mTorr. Collision offset voltages were optimized with respect to each compound and ranged from 10 to 40 eV for cholesteryl linoleate and cholesteryl arachidonate hydroperoxides.

HPLC−MS Analysis (LC−CIS−MS). For on-line LC−MS separation and analysis of the cholesteryl ester hydroperoxides, the peroxidic oxidation products were isolated from the unoxidized lipid by semipreparative chromatography (0.5% 2-propanol in hexanes, 10 mL/min). The oxidized fractions for cholesteryl linoleate (10−25 min) and cholesteryl arachidonate (10−35 min) were collected, concentrated, and analyzed by LC−CIS-MS. A stock solution of the oxidized fraction (1 mg/mL in mobile phase) was prepared, and typically 2−20 μg of the mixture was injected per analysis. Offset voltages for SRM experiments were as determined by optimization in the DLI experiments.

Chemo-enzymatic Synthesis of Cholesteryl 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoate, 17. 15(*S*)-Hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic Acid. The following reaction was prepared on a 1.0 L scale. The reaction sequence was repeated six times for a total of 1 g of arachidonic acid substrate. An ethanolic solution of arachidonic acid (167 mg, 7 mL) was added in one aliquot to 1000 mL of boric acid buffer (pH 9.0, 0.2 M) that was saturated with oxygen. To the slightly cloudy solution was added a solution of soybean lipoxidase (3.1 mg/mL, 2.5 mL) in boric acid buffer. Oxygen was slowly bubbled into the solution for 10 min. The reaction mixture was acidified to pH 4 by the addition of HCl and extracted with EtO_2 (3 × 250 mL). The ether extracts were dried and concentrated in vacuo to a pale yellow oil. The resulting crude hydroperoxide was immediately carried forward without purification or characterization.

Methyl 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoate. The crude hydroperoxide was dissolved in EtO_2 (10 mL) and treated with an excess of an Etheral solution of freshly prepared diazomethane. The reaction was allowed to stir at 0 °C for 30 min. Excess diazomethane was removed by gently bubbling with argon until the

solution was colorless. BHT (~3 mg) was added and the ether removed in vacuo. The resulting bright yellow oil was purified by flash chromatography on silica gel (20% Et_2O in hexanes) yielding 0.9736 g of a pale yellow oil (80.5%): TLC: R_f 0.21 (25% Et_2O in hexanes); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.42 (s, 1H), 6.51 (dd, J = 15.2, 11.1 Hz, 1H), 5.94 (t, J = 10.9, 10.9 Hz, 1H), 5.55 (dd, J = 15.2, 8.0 Hz, 1H), 5.32 (m, 5H), 4.30 (m, 1H), 3.59 (s, 3H), 2.89 (m, 2H), 2.73 (m, 2H), 2.25 (m, 2H), 2.03 (m, 2H), 1.61 (m, 3H), 1.43−1.16 (8H), 0.81 (t, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.26, 133.52, 132.28, 130.62, 128.78, 128.47, 127.69, 127.21, 86.21, 60.41, 51.41, 33.20, 32.40, 31.56, 26.33, 25.95, 25.43, 24.77, 24.51, 22.31, 13.81; FTIR (neat, NaCl plates): 3417.7 (s), 1717.26 (s) cm^{-1}; CIS−MS (DLI, $AgBF_4$): m/z 457.0/459.1[M + Ag]$^+$.

Methyl 15(*S*)-[(1-methoxy-1-methylethyl)dioxy]-5(*Z*),8(*Z*),11(*Z*),-13(*E*)-eicosatetraenoate. Methyl arachidonate hydroperoxide (0.8538 g, 2.44 mmol) was dissolved in dry CH_2Cl_2 (14 mL). To this solution, 2-methoxypropene (360 μL, 0.263 g, 3.65 mmol) and pyridinium-p-toluenesulfonate (~2 mg) were added. The reaction was stirred overnight at room temperature under argon. The reaction was added to a separatory funnel with CH_2Cl_2 (50 mL). The organic layer was washed with $NaHCO_3$ (1 × 20 mL) and saturated NaCl (1 × 20 mL), dried with $MgSO_4$, and concentrated to dryness to yield 1.22 g of a yellow oil (100%): TLC: R_f 0.35 (25% Et_2O in hexanes); ^{1}H NMR (400mHz, $CDCl_3$): δ 6.47 (dd, J = 15.2, 11.1 Hz, 1H), 5.98 (t, J = 11.2, 11.2 Hz, 1H), 5.61 (dd, J = 15.2, 7.9 Hz, 1H), 5.35 (m, 5H), 4.37 (m, 1H), 3.63 (s, 3H), 3.25 (s, 3H), 2.92 (m, 2H), 2.77 (m, 2H), 2.28 (t, 2H), 2.07 (q, 2H), 1.66 (m, 3H), 1.43−1.22 (13H), 0.84 (t, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.95, 133.60, 130.08, 128.98, 128.65, 128.53, 128.17, 127.56, 127.40, 104.51, 84.56, 51.41, 49.20, 33.35, 33.03, 31.73, 26.49, 26.06, 25.56, 25.05, 24.71, 22.98, 22.79, 22.47, 13.98; FTIR (neat, NaCl plate) 1738.12 cm^{-1}; CIS−MS (DLI, $AgBF_4$): m/z 529.1/531.1 [M + Ag]$^+$.

15(*S*)-[(1-Methoxy-1-methylethyl)dioxy]-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic Acid. The protected methyl arachidonate hydroperoxide (1.0358 g, 2.4510 mmol) was dissolved in tetrahydrofuran (THF, 10 mL) and LiOH (4.0 M, 10 mL). The biphasic solution was vigorously stirred at room temperature overnight. The reaction was poured into a separatory funnel containing 1 M HCl (60 mL) and EtOAc (50 mL). The acidified layer was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (1 × 25 mL), dried with anhydrous $MgSO_4$, and concentrated to 1.0228 g of a bright yellow oil (100% yield): TLC: R_f 0.09 (25% Et_2O in hexanes), R_f 0.27 (50% Et_2O in hexanes); ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.91 (bs, 1H), 6.48 (dd, J = 15.2, 11.1 Hz, 1H), 5.98 (t, J = 11.2, 11.2 Hz, 1H), 5.61 (dd, J = 15.2, 7.9 Hz, 1H), 5.36 (m, 5H), 4.38 (q, 1H), 3.26 (s, 3H), 2.93 (t, 2H), 2.78 (t, 2H), 2.33 (t, 2H), 2.10 (q, 2H), 1.67 (m, 3H), 1.44−1.20 (13H), 0.85 (t, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.49, 133.51, 130.08, 128.81, 128.46, 128.16, 127.57, 127.44, 104.57, 84.54, 49.18, 33.30, 33.02, 31.72, 26.39, 26.06, 25.57, 25.02, 24.43, 22.94, 22.78, 22.46, 13.98; FTIR (neat, NaCl plates): 2923.21 (s), 1693.61 (s); CIS−MS (DLI, $AgBF_4$): m/z 515.1/517.1 [M + Ag$^+$].

Cholesteryl 15(*S*)-[(1-methoxy-1-methylethyl)dioxy]-5(*Z*),8(*Z*),-11(*Z*),13(*E*)-eicosatetraenoate. The protected arachidonic acid hydroperoxide (0.6023 g, 1.47 mmol) was dissolved in dry CH_2Cl_2 (20 mL). dimethylaminopyridine (0.3978 g, 3.26 mmol) and cholesterol (0.6932 g, 1.792 mmol) were added and stirred for 5 min. dicyclohexylcarbodiimide (0.6744 g, 3.26 mmol) was added in one portion, and the reaction was stirred under argon for 24 h. TLC indicated the formation of a less polar fraction, which stained pink with the hydroperoxide stain after heating. After three flash columns (5% EtOAc in hexanes), the protected cholesteryl ester hydroperoxide was obtained as 0.7519 g of a thick, colorless oil (65.7%): TLC: R_f 0.33 (10% EtOAc in hexanes), R_f 0.51 (15% EtOAc in hexanes); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.48 (dd, J = 15.4, 11.1 Hz, 1H), 5.99 (t, J = 11.2, 11.2 Hz, 1H), 5.62 (dd, J = 15.5, 8.0 Hz, 1H), 5.36 (m, 6H), 4.59 (m, 1H), 4.39 (q, 14.0, 6.7 Hz, 1H), 3.27 (s, 3H), 2.94 (m, 2H), 2.79 (m, 2H), 2.27 (m, 4H), 2.11−0.83 (59H), 0.65 (s, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 172.95, 139.62, 133.62, 130.11, 129.14, 128.61,128.59, 128.21, 127.59, 127.44, 122.60, 104.54, 84.59, 73.77, 56.66, 56.10, 49.99, 49.24, 42.28, 39.70, 39.49, 38.13, 36.97, 36.57, 36.16, 35.77, 34.90, 34. 02, 33.06, 31.88, 31.83, 31.77, 28.21, 27.99, 27.79, 26.54, 26.10, 25.59, 25.43, 25.08, 24.93, 24.86, 24.67, 24.26, 23.80, 23.02, 22.83, 22.80, 22.54, 22.50, 21.00, 19.30, 18.69. 14.03, 11.83; FTIR (neat, NaCl plates): 1732.56 (s) cm^{-1}; CIS−MS (DLI, $AgBF_4$): m/z 883.6/885.6 [M + Ag]$^+$.

Cholesteryl 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoate, 17. The protected cholesteryl arachidonate hydroperoxide (0.7519 g, 0.967 mmol) was dissolved in THF (9 mL). BHT (~3 mg) was added to the solution to prevent hydroperoxide rearrangement. Glacial acetic acid (5 mL) and deionized water (3 mL) were added with stirring resulting in a cloudy white solution, which was stirred overnight under argon. The reaction was worked up by drying with anhydrous $NaSO_4$, followed by removal of the acetic acid by azeotrope with cyclohexane. The crude material was purified by flash column chromatography (5% EtOAc in hexanes), resulting in the recovery of 0.0321 g of the starting protected hydroperoxide and 0.5183 g of the desired hydroperoxide (76.0%): TLC: R_f 0.38 (15% EtOAc in hexanes); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.33 (s, 1H), 6.49 (dd, J = 15.2, 11.1 Hz, 1H), 5.91 (t, J = 10.9, 10.9 Hz, 1H), 5.51 (dd, J = 15.2, 8.0 Hz, 1H), 5.30 (m, 6H), 4.52 (m, 1H), 4.27 (q, 14.0, 6.7 Hz, 1H), 2.86 (m, 2H), 2.71 (m, 2H), 2.19 (m, 4H), 2.03−0.75 (53H), 0.57 (s, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 173.26, 139.50, 132.22, 130.90, 129.10, 129.03, 128.70, 128.55, 128.22, 127.73, 127.27, 122.58, 86.41, 73.89, 56.60, 56.06, 49.93, 42.23, 39.65, 39.45, 38.05, 36.90, 36.50, 36.12, 35.73, 33.97, 32.54, 31.82, 31.77, 31.71, 28.16, 27.94, 27.72, 26.50, 26.12, 25.58, 24.92, 24.78, 24.21, 23.77, 22.76, 22.50, 20.96, 19.24, 18.651, 13.97, 11.79; CIS−MS (DLI, $AgBF_4$): m/z 811.6/813.6 [M + Ag]$^+$.

Conversion of 15-OOH Cholesteryl Arachidonate (Ch-15-HPETE) to More Polar Compounds. A solution of Ch-15-HPETE (**17**, 0.2858 g, 0.405 mmol) was dissolved in dry benzene (2.7 mL) for a final lipid concentration of 0.15 M. DTBN (7.2 mg, 0.041 mmol, 10 mol %) was added, and the lipid mixture was stirred at 37 °C in an oil bath under an atmosphere of oxygen for 16−18 h. BHT (~2 mg) was added and the reaction was stored at −78 °C until HPLC and MS analysis. The reaction was repeated for a total of 0.5781 g of Ch-15-HPETE converted to more polar products. The analytical traces of each individual conversion were identical to one another, so the mixtures were pooled. The polar compounds were isolated by semipreparative HPLC (1.0% 2-propanol in hexanes, 10 mL/min). Typically, 15−20 mg of the oxidized material was separated per injection to prevent column overloading. Several major UV active (λ = 234 nm) fractions were collected (B, C, E, F/G, I). Minor fractions (A, D, H, J) were also collected, but were typically mixtures of 2−4 compounds. The major fractions were analyzed by ^{1}H NMR, two-dimensional correlation spectroscopy (2D COSY), and CIS−MS.

Direct Liquid Injection Analysis (DLI) and HPLC−MS Analysis of More Polar Cyclic Peroxides. Individual isolated fractions (B, C, E, F/G, I) were analyzed by direct liquid infusion of the sample as described for the cholesteryl linoleate hydroperoxides. For on-line LC−CIS-MS analysis of cyclic peroxide mixtures, sample introduction was carried out using a single Beckman Ultrasphere narrowbore 5-μm silica column (2.0 mm × 25 cm) operated in the isocratic mode with 1.0% 2-propanol in hexanes at a flow rate of 150 μL/min. A stock solution of the Ch-15-HPETE conversion mixture (1 mg/mL in mobile phase) was prepared in mobile phase and typically 20−40 μL (20−40 μg lipid) of the solution was injected per analysis. Offset voltages for SRM experiments, usually 20 eV were set as determined by optimization in the DLI experiments

Conversion of 5-OOH Cholesteryl Arachidonate (Ch-5-HPETE) to More Polar Compounds. Ch-5-HPETE (**12**) was isolated from a cholesteryl arachidonate autoxidation with 1,4-cyclohexadiene, as already described. The isolated hydroperoxide (6.8 mg, 9.64 × 10^{-3} mmol) was dissolved in benzene (195 μL). DTBN was added as a benzene solution (65 μL of 2.7 mg/mL, 0.176 mg, 1 × 10^{-3} mmol, 10 mol % DTBN) for a final lipid concentration of 0.0375 M. The reaction vial was flushed with oxygen, sealed, and stirred at 37 °C for 17 h. BHT (~2 mg) was added and the reaction was stored at −78 °C until analysis. LC/CIS-MS was performed as described for the above cyclic product mixtures.

Autoxidation of Cholesteryl Arachidonate without 1,4-Cyclohexadiene. Cholesteryl arachidonate (0.400 g, 0.594 mmol) was

dissolved in dry benzene (4.0 mL) for a final lipid concentration of 0.15 M. Oxidation was initiated by the addition of DTBN (~3 mg). The reaction was placed into a 37 °C oil bath under air for 48 h. Reaction was inhibited by the addition of BHT (~2 mg). A portion of the reaction mixture (1 mL) was removed and concentrated to dryness. The residue was diluted with $CHCl_3$ (100 μL) and applied to a Burdick & Jackson Inert SPE System (silica, 500 mg) prewashed with hexane (10 mL) and equilibrated with 1.5% MTBE in hexane (10 mL). The unoxidized cholesteryl arachidonate was eluted with 1.5% MTBE in hexanes (3 × 5 mL). The oxidized cholesteryl arachidonate material was eluted from the column with 5% methanol in MTBE (3 × 5 mL). The oxidized fractions were combined and concentrated to yield 61.2 mg oxidized material. A stock solution of the oxidized material was prepared in benzene (2 mL) for a concentration of 30.6 mg/mL. Subsequent dilutions of this stock solution were used for MS analysis. Analytical HPLC analysis (single Si column, 1.0% 2-propanol in hexane) indicated no difference in the product distribution of polar products after removal of the unoxidized lipid. The complex oxidation product mixture was analyzed by LC−CIS−MS as described for the cyclic peroxide analysis. Typically, 20−60 μg of the lipid oxidation mixture was injected for SIM and SRM analysis.

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