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Formation of Isoprostane Bicyclic Endoperoxides from the Autoxidation of Cholesteryl Arachidonate

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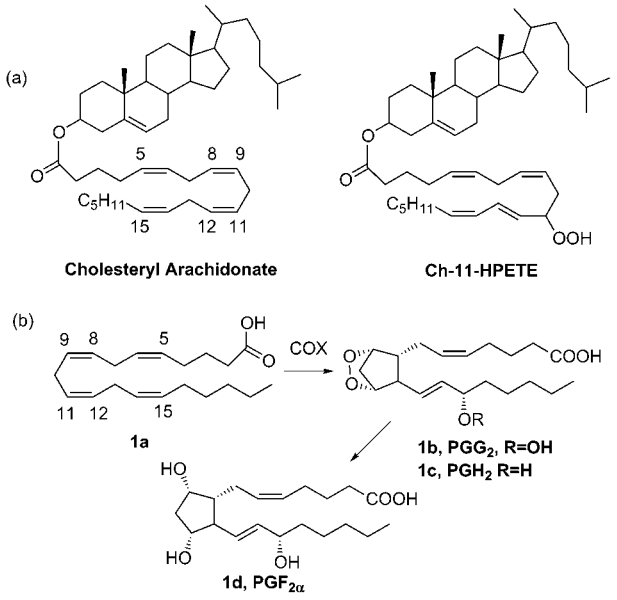
Abstract: Autoxidation of polyunsaturated fatty acids and esters leads to a complex mixture containing hydroperoxides and cyclic peroxides. Prostaglandin bicyclic endoperoxides have been detected from the autoxidation of cholesteryl arachidonate by LC-MS and GC-MS techniques. All four possible types (I−IV) of bicyclic endoperoxides have been found starting from different regioisomeric hydroperoxides of cholesteryl arachidonate. Furthermore, the stereochemistry of Type IV bicyclic endoperoxides has been determined by conversion to pentafluorobenzyl (PFB) ester, trimethylsilyl (TMS) derivatives, and comparison with synthetic standards by the use of GC-MS. All eight possible diastereomers of the derivatized isoprostanes were observed and were separated by gas chromatography. The bicyclic endoperoxides with the two alkyl chains syn on the cyclopentane ring were formed preferentially to those with anti configuration, a result anticipated from earlier work. Substantial amounts of the anti-substituted isoprostanes, including $PGF_{2\alpha}$, were, however, observed in the product mixture.

Introduction

Free radical-initiated lipid peroxidation has been linked to many human diseases such as atherosclerosis, cancer, and neurodegenerative disorders.[1] In particular, oxidative modification of the lipid components of low-density lipoprotein (LDL), the major carrier of cholesteryl esters in human blood, has been attributed to the pathogenesis and progression of atherosclerosis.[2] Deposition of the oxidized cholesteryl esters in cells and vascular tissues is a pathological hallmark of atherosclerotic lesions.[3] However, the relevant lipid oxidation products and the mechanism of the LDL oxidation process have not been well-defined. It has been shown that oxidized lipids extracted from the atherosclerotic lesions are rich in cholesterol and cholesterol esters.[4] Therefore, characterization of the oxidation products of cholesterol esters may have relevance to this disease. Cholesteryl linoleate (Ch 18:2) and cholesteryl arachidonate (Ch 20:4) comprise a substantial fraction of the neutral lipids present in LDL.

The oxidation of cholesteryl linoleate leads to several primary hydroperoxides. However, the mixture of oxidation products of cholesteryl arachidonate is extremely complicated because insertion of molecular oxygen may occur at C_5, C_8, C_9, C_{11}, C_{12}, or C_{15} of the 20 carbon fatty acid chain, Scheme 1.[5] Furthermore, the resultant peroxyl radicals can undergo reactions to give a complex mixture of primary hydroperoxides and cyclic peroxides with dozens of regioisomers and diastereomers possible. Conventional techniques of product identification

Scheme 1



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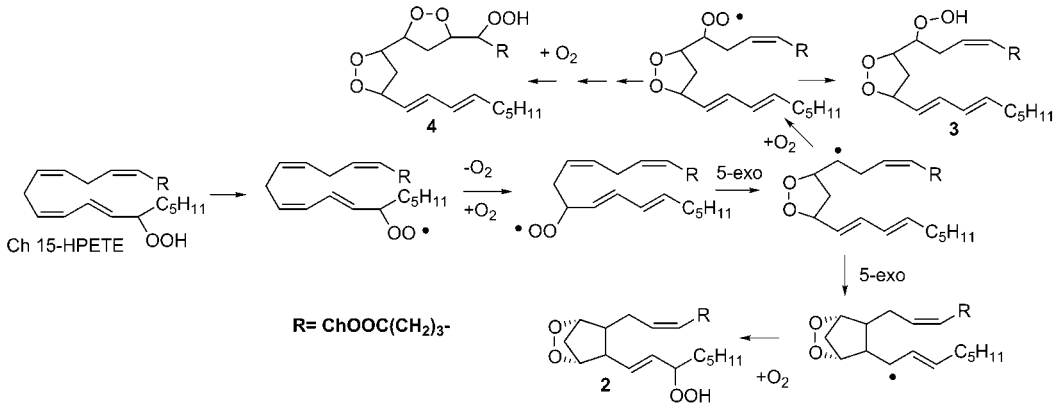
(1) (a) Chisolm, G. M.; Steinberg, D. *Free Radical Biol. Med.* **2000**, *28*, 1815−1826. (b) Nicholson, A. C.; Hajjar, D. P. *Am. Sci.* **1995**, *83*, 460−470.
(2) (a) Patel, R. P.; Moellering, D.; Murphy-Ullrich, J.; Jo, H.; Bechman, J. S.; Darley-Usmar, V. M. *Free Radical Biol. Med.* **2000**, *28*, 1780−1794. (b) Dhaliwal, B. S.; Steinbrecher, U. P. *J. Lipid Res.* **2000**, *41*, 1658−1665.
(3) (a) Heineke, J. W. *Atherosclerosis* **1998**, 141, 1−15. (b) Berliner, J.; Leitinger, N.; Watson, A.; Huber, J.; Fogelman, A.; Navab, M. *Thromb. Haemostasis* **1997**, *78*, 195−199.
(4) (a) Carpenter, K. L. H.; Tarlor, S. E.; van der Veen, C.; Williamson, B. K.; Balloantine, J. A.; Mitchinson, M. J. *Biochim. Biophys. Acta* **1995**, 1256, 141−150. (b) Suarna, C.; Dean, R. T.; May, J.; Stocker, R. *Arterioscler. Thromb. Vasc. Biol.* **1995**, *15*, 1616−1624.
(5) (a) Porter, N. A. *Free Radicals Biol.* **1980**, *4*, 261−294. (b) Porter, N. A.; Funk, M. O. *J. Org. Chem.* **1975**, *40*, 3614−3615. (c) Porter, N. A.; Funk, M. O.; Gilmore, D.; Isaac, R.; Nixon, J. *J. Am. Chem. Soc.* **1976**, *98*, 6000. (d) Marnett, L. J. *Curr. Opin. Chem. Biol.* **2000**, *4*, 545−552.

Scheme 2



(purification by chromatography, spectroscopic characterization, and conversion to or comparison with known compounds) are not possible for the analysis of the oxidation products of cholesteryl arachidonate.[6] The challenges of chromatography of such complex product mixtures alone makes analysis by classical methods unrealistic. In response to this analytical problem, we have developed methods for the analysis of complex mixtures of peroxide compounds based upon Ag^+ coordination ionspray mass spectrometry. This technique relies on coordination of the soft Lewis acid, Ag^+ to soft Lewis base sites such as carbon−carbon double bonds in unsaturated molecules and it provides characteristic fragmentation patterns for peroxidic compounds.

The bicyclic endoperoxide **1b** (prostaglandin G_2, PGG_2) is formed in the oxidation of arachidonic acid promoted by cyclooxygenases (COX) enzymes.[7] Formation of analogous endoperoxides has been implied in the nonenzymatic free radical oxidation of arachidonic acid or its esters and these endoperoxides are presumed intermediates in the formation of isoprostanes, compounds that have been used as a reliable index for oxidative injury in vivo.[5,7,8]

Given the fact that PGG_2 and its closely related analogue PGH_2, **1c**, are involved in important physiological events such as smooth muscle contraction and the inflammatory process, it seems likely that the many other regioisomeric and stereoisomeric endoperoxides formed in the free radical oxidation of arachidonates will have their own biological activities. Compounds derived from the endoperoxide mixtures that are analogous to compounds such as **1d** ($PGF_{2\alpha}$) and other PG's and thromboxanes may have interesting physiological properties as well. Furthermore, the mixture of regioisomers and stereoisomers having structures analogous to $PGF_{2\alpha}$ make up the class of compounds known as isoprostanes.

We report here on the bicyclic endoperoxides formed in the oxidation of cholesterol arachidonate. Arachidonate present in LDL is esterified primarily to phospholipids and cholesterol esters and the formation of PGG_2-like bicyclic endoperoxides from cholesteryl esters (or phospholipids) has not been studied due to the complexity of the product mixture and the lack of appropriate analytical techniques.[9] Both Ag^+ coordination ionspray mass spectrometry and GC-MS of suitable isoprostane derivatives made from the isolated endoperoxides were used to provide information about the distribution of products formed in lipid peroxidation of cholesteryl arachidonate.

Results

Autoxidation of cholesteryl arachidonate in the presence of good hydrogen atom donors such as 1,4-cyclohexadiene or methyl Trolox leads to six hydroperoxides of cholesteryl arachidonate: Ch-5-HPETE, Ch-8-HPETE, Ch-9-HPETE, Ch-11-HPETE, Ch-12-HPETE, and Ch-15-HPETE.[6a] These regioisomeric hydroperoxides of cholesteryl arachidonate can be separated by semipreparative HPLC except for the 8 and 9 pair of regioisomers. The separated regioisomeric hydroperoxides can, themselves, serve as a source of specific peroxyl radicals when reacted under free radical autoxidation conditions. In this way, the complexity of the arachidonate autoxidation problem can be simplified, the product mixture that results from reaction of an individual arachidonyl peroxyl radical being simpler than the mixture that results from reaction of the six peroxyl radicals formed from arachidonate.

Characterization of Isoprostane Bicyclic Endoperoxides of Cholesteryl Esters by LC-MS Techniques (Ag^+ Coordination Ionspray Mass Spectrometry). (a) Isoprostane (Prostaglandin) Bicyclic Endoperoxides from Ch-15-HPETE (Type IV). The technique of Ag^+ coordination ionspray MS has proved to be a powerful tool for analyzing the oxidation mixture derived from Ch-15-HPETE. Coupled with normal phase HPLC, this CIS-MS technique provides information about different classes of peroxides that result from peroxidation.[6a,10] The possible oxidation products from Ch-15-HPETE are illustrated in Scheme 2. According to the mechanism shown in Scheme 2, a peroxyl radical forms by H-abstraction from the hydroperoxide of Ch-15-HPETE. This peroxyl radical can undergo β-fragmentation and O_2 addition to give a peroxyl radical at C_{11} that can undergo double cyclization to give the bicyclic endoperoxide, **2**. Other cyclic peroxide products can

(6) (a) Havrilla, C. M.; Hachey, D. L.; Porter, N. A. *J. Am. Chem. Soc*. **2000**, *122*, 8042−8055. (b) Khan J. A.; Porter, N. A. *Angew. Chem.* **1982**, *94*, 220−221.

(7) (a) Needleman, P.; Kulkarni, P. S.; Raz, A. *Science* **1977**, *195*, 409. (b) Kuehl, F. A.; Humes, J. L.; Egan, R. W.; Ham, E. A.; Beveridge, G. C.; Van Arman, C. G. *Nature* **1977**, *265*, 171. (c) Needleman, P.; Moncada, S.; Bunting, S.; Vane, J.; Hamberg, M.; Samuelsson, B. *Nature* **1976**, *261*, 558. (d) Funk, C. D. *Science* **2001**, *294*, 1871−1875.

(8) (a) Morrow, J. D.; Hill, K. E.; Burk, R. F.; Nammor, T.; Badr, K. F.; Roberts, L. J. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9383−9387. (b) Pratico, D.; Lawson, J. A.; Rokach, J.; FitzGerald, G. A. *TRENDS Endocrin Met*. **2001**, *12* (6), 243−247. (c) Lawson, J. A.; Rokach, J.; FitzGerald, G. A. *J. Biol. Chem*. **1999**, *274*, 24441−24444. (d) Rokach, J.; Khanapure, S. P.; Hwang, S. W.; Adiyaman, M.; Lawson, J. A.; FitzGerald, G. A. *Prostaglandins* **1997**, *54*, 823−851.

(9) Van Heek, M.; Schmitt, D.; Toren, P.; Cathcart, M. K.; DiCorleto, P. E. *J. Biol. Chem.* **1998**, *273*, 19405−19410.

(10) (a) Bayer, E.; Gfrorer, P.; Rentel, C. *Angew. Chem., Int Ed*. **1999**, *38*, 992−995. (b) Yin, H.; Hachey, D. L.; Porter, N. A. *J. Am. Soc. Mass. Spectrom.* **2001**, *12*, 449−455.

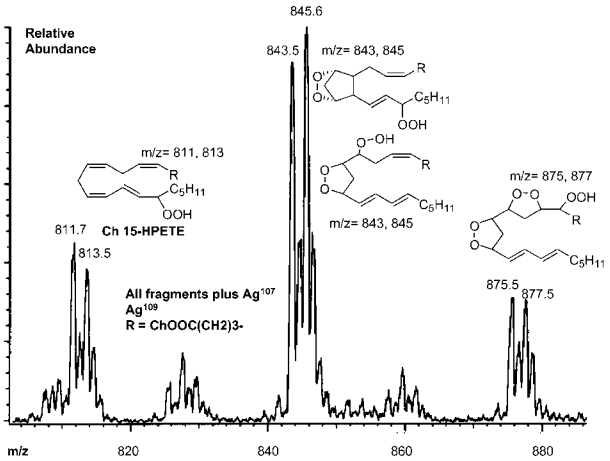


Figure 1. The oxidation products of Ch-15-HPETE detected by direct liquid infusion with Ag^+ CIS-MS.

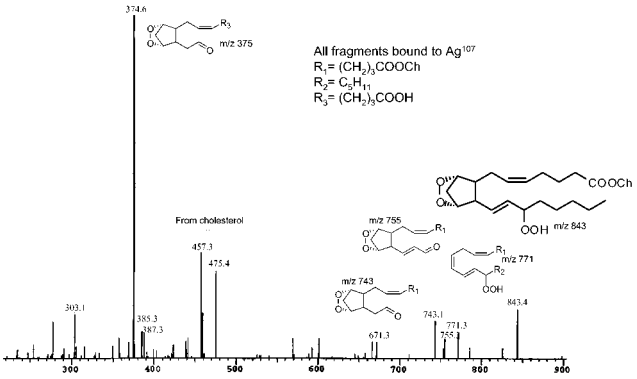


Figure 2. CID of Type IV bicyclic endoperoxides, **2**.

form.[11] Thus, addition of oxygen to the intermediate carbon-centered radical could give a peroxyl radical that would lead to the monocyclic peroxides, **3**, or the serial cyclic peroxides, **4**.

Figure 1 shows the results of a Ag^+ CIS-MS direct liquid infusion (DLI) experiment on the product mixture derived from exposure of Ch-15-HPETE to a free radical initiator in benzene under air at 37 °C. The doublet peaks observed are characteristic of the Ag^+ adducts of peroxidic compounds formed in the reaction. Silver ion adducts appear as doublets due to the fact that there are two isotopes of Ag 107 and 109, present in a 1:1 ratio. The *m*/*z* of 811.7/813.5 corresponds to the Ag^+ adduct of the starting hydroperoxide, Ch-15-HPETE. The *m*/*z* 843.5/845.6 corresponds to the Ag^+ adducts of either prostaglandin bicyclic endoperoxide **2** or monocyclic peroxide **3** since they have the same mass, resulting from addition of one molecule of oxygen to the starting material. The Ag^+ adduct of the serial cyclic stereoisomeric peroxides **4** appears at *m*/*z* 875.5/877.5.

Structural information was obtained by collision-induced dissociation (CID), using a triple quadrupole mass spectrometer. The CID experiment was carried out by selecting the parent ion (*m*/*z* 843.4) in the first quadrupole and fragmentation in the second quadrupole by collisional activation with argon. The third quadrupole was set to scan all of the resulting fragments. A CID spectrum of the parent ion *m*/*z* 843.4 is shown in Figure 2. The fragmentation patterns consistent with the CID experiment are summarized in Scheme 3. An apparent fragment with *m*/*z* 771 is obtained by the loss of malondialdehyde (MDA), a well-known process for the endoperoxides.[12] The Hock rearrangement has also been observed in Ag^+ coordination experiments and in this experiment, aldehydes with *m*/*z* 743 (R = Ch) or 375 (R = H) are obtained.[13] The α-cleavage of hydroperoxides can also occur to give aldehydes with *m*/*z* 755 (R = Ch) or 387 (R = H). The fragments with *m*/*z* 753 and 385 can be rationalized as resulting from dehydration of the fragment at *m*/*z* 771 and subsequent loss of cholesterol. The fragment with *m*/*z* 475.4 corresponds to the loss of cholesterol from the parent ion and its subsequent dehydration gives *m*/*z* 457.3. Overall, the CID experiment supports the notion that bicyclic endoperoxide compounds are present in the reaction mixture.

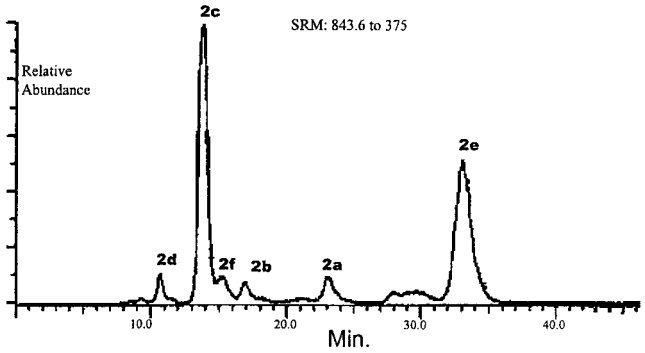


Figure 3. Selective reaction monitoring of **2** from *m*/*z* 843 to 375.

Selective reaction monitoring (SRM) was carried out by coupling HPLC to Ag^+CIS-MS, see Figure 3. The SRM chromatogram from the parent ion *m*/*z* 843 to the characteristic endoperoxide fragment, *m*/*z* 375, is shown in this chromatogram. SRM chromatograms monitoring fragmentation to other characteristic ions from Figure 2 are essentially identical with the one shown in Figure 3. We conclude that these chromatograms indicate the formation of several stereoisomeric bicyclic endoperoxides in the free radical reactions of Ch-15-HPETE.

Chromatography of the same product mixture with detection by UV at 234 or 207 nm gave the chromatograms shown in Figure 4. The endoperoxide structures do not have a conjugated diene and they are therefore not detected at 234 nm. Other products formed from the reaction of arachidonate hydroperoxides include serial cyclic and monocyclic peroxide compounds that contain a conjugated diene substructure. These compounds are detected at 234 nm and peaks in the HPLC-UV (234 nm) chromatogram shown in Figure 4a have previously been identified as having the serial cyclic structure.[6a]

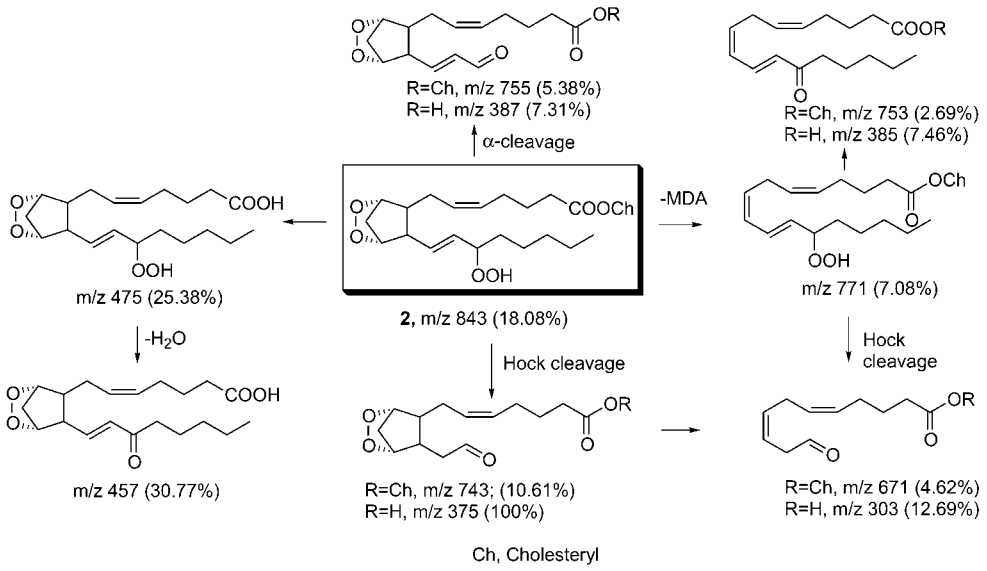
The HPLC-UV (207 nm) chromatogram shown in Figure 4b does give evidence of compounds having no conjugated diene. These compounds elute in regions of the HPLC-MS chromatogram that correspond to isoprostane endoperoxides, as identified by SRM. The isoprostane endoperoxides have no UV chromophore above 210 nm and they are therefore detected at 207 nm but not at 234 nm. Comparison of the chromatograms shown in Figure 4a,b and the SRM chromatogram shown in Figure 3

(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.

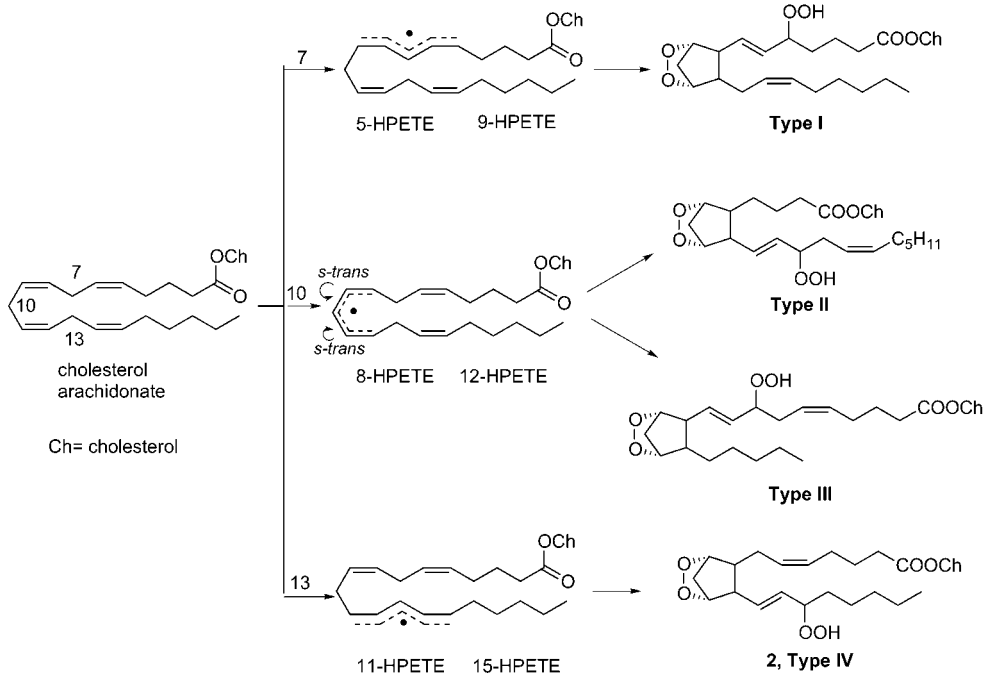
(12) Chaudhary, A. K.; Nokubo, M.; Reddy, G. R.; Yeola, S. N.; Morrow, J. D.; Blair, I. A.; Marnett, L. J. *Science* **1994**, *265*, 1580−1582.

(13) (a) Hock, H. *Angew. Chem.* **1936**, *49*, 595. (b) For a review see: Frimer, A. A. *Chem. Rev.* **1979**, *79*, 359−387. (c) Tanigawa, S.; Kajiware, T.; Hatanaka, A. *Phytochemistry* **1984**, *23*, 2349−2344. (d) Gardner, H. W.; Planter, R. D. *Lipids* **1984**, *19*, 294−298.

Scheme 3



Scheme 4



gives independent support for the formation of isoprostane endoperoxide structures in the reaction.

The mechanism for formation of the Type IV endoperoxides from Ch-15-HPETE requires fragmentation of the 15-peroxyl radical, which ultimately forms the 11-substituted peroxyl radical, see Scheme 2. This approach was dictated by the fact that it was relatively easy to obtain significant quantities of Ch-15-HPETE by a chemo-enzymatic pathway while the acquisition of the regioisomeric hydroperoxides required tedious preparative chromatography. Ch-11-HPETE should also give access to Type IV endoperoxides and indeed, when Ch-11-HPETE was reacted under conditions similar to those described above for the 15-isomer, an LC-MS chromatogram identical with the one presented in Figure 3 was obtained. This provides additional evidence in support of the mechanism for formation of the endoperoxides.

(b) Isoprostane Bicyclic Endoperoxides from Regioisomeric Ch-HPETEs (Types I−III). As summarized in Scheme 4, H-abstraction from cholesteryl arachidonate can occur at C_7, C_{10}, or C_{13} (bisallylic positions) and this results in different regioisomeric hydroperoxides of cholesteryl arachidonate.[14] By analogy to the chemistry observed for Ch-15-HPETE, Type I isoprostane bicyclic endoperoxides could form from Ch-5-HPETE and Ch-9-HPETE. Type II and Type III endoperoxides could form either from Ch-8-HPETE or Ch-12-HPETE. To provide evidence for formation of these endoperoxides, the 5- and 12-Ch-HPETEs and the mixture of the 8- and 9-Ch-HPETEs were purified by semipreparative HPLC and reacted as described for the 15- and 11-Ch-HPETEs.

(14) (a) Morrow, J. D.; Harris, T. M.; Roberts, L. J., II *Anal. Biochem.* **1990**, *184*, 1−10. (b) Roberts, L. J., II; Morrow, J. D. *Adv. Prostaglandin, Thromboxane, Leukotriene Res.* **1995**, *23*, 219. For an alternate nomenclature for the isoprostanes, see refs 8c and 8d.

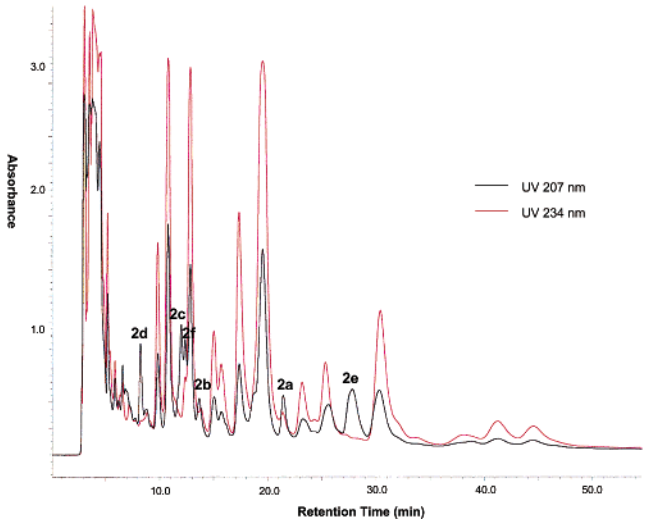


Figure 4. HPLC chromatogram of the oxidation mixture from Ch-15-HPETE monitored at UV 234 and 207 nm.

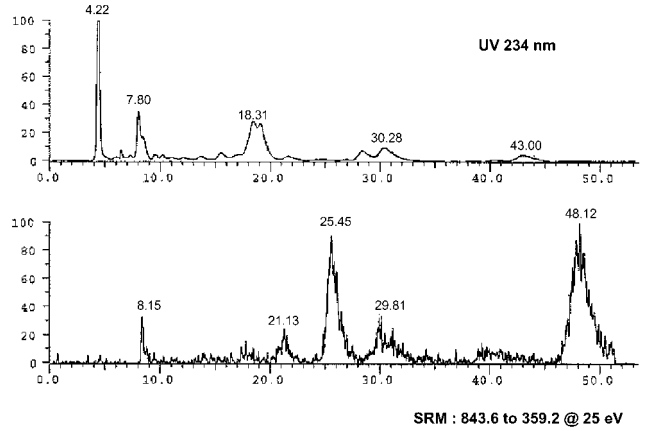


Figure 5. Selective reaction monitoring of Type I bicyclic endoperoxides from Ch-5-HPETE: *m*/*z* 843.6 to 359 (UV 234 nm).

Each of the purified hydroperoxides (and the mixture of the 8 and 9 isomers) was reacted with a free radical initiator in benzene under air at 37 °C. Mass spectral fragmentation patterns (DLI) and CID spectra obtained from the various Ch-HPETE's are presented in Supporting Information. In short, mass spectral fragmentation of endoperoxides of Type I−III occurs by the same pathways described for the Type IV compounds. Loss of malondialdehyde, Hock fragmentation of allylic hydroperoxides, and α-cleavage reactions of hydroperoxides were observed for all of these compounds.

SRM experiments were carried out in an attempt to analyze the isoprostane bicyclic endoperoxides of Type I, Type II, and Type III. Shown in Figure 5 are the SRM and UV (234 nm) chromatograms of the reaction mixture. The SRM chromatogram gives strong evidence for the formation of Type I endoperoxides, based upon fragmentation patterns typical of the structures. Experiments (SRM) that monitored several characteristic fragmentation pathways gave similar chromatograms, supporting the assignment of structure to compounds.

Attempts to find endoperoxides of Type II and Type III by SRM HPLC-MS were not successful. These isoprostanes derive from the 8 and 12 peroxyl radicals and isolation of the requisite Ch-HPETE precursors in substantial quantities in pure form is difficult. HPLC-MS SRM chromatograms that monitor characteristic fragmentation pathways gave poor signal-to-noise and conclusive identification of isoprostane endoperoxide fractions in these chromatograms was not possible. Evidence for the formation of Type II and Type III isoprostane endoperoxides was obtained, however, by GC-MS as described in the following section.

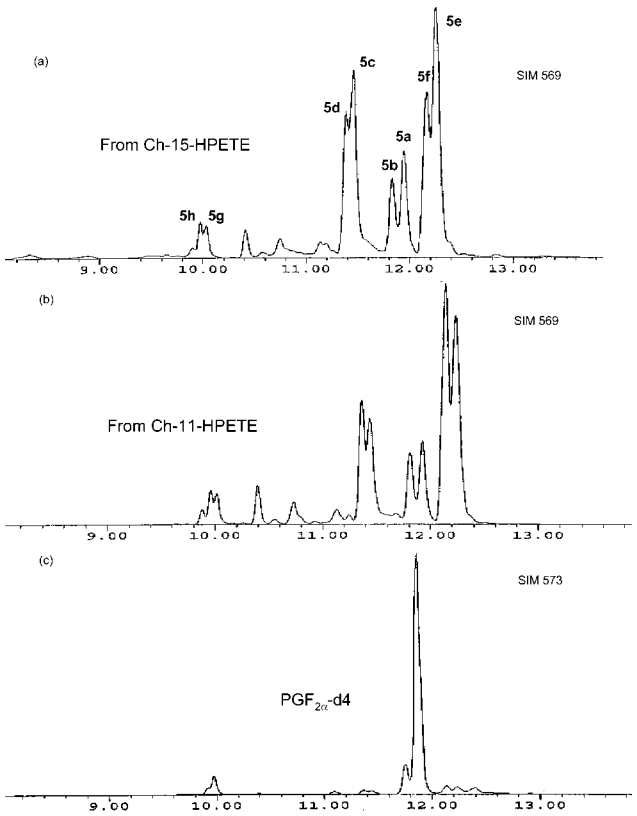
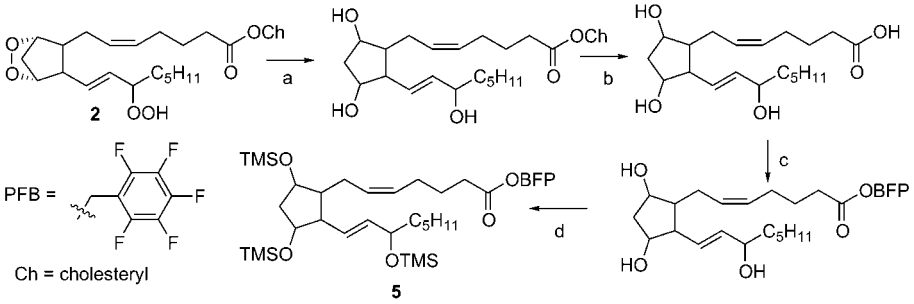


Figure 6. GC-NICI MS SIM (*m*/*z* 569) chromatograms of isoprostane derivatives from (a) Ch-15-HPETE and (b) Ch-11-HPETE. (c) Internal standard of PGF_{2a}-d_4 (*m*/*z* 573).

Derivatization of Isoprostane Endoperoxides Formed from Ch-HPETEs: Characterization by Gas Chromatography−Negative Ion Chemical Ionization Mass Spectrometry (NICI-MS). The GC-NICIMS technique has been extremely useful in the study of the isoprostanes due to its high sensitivity. Moreover, the isoprostanes have been employed as a reliable index for oxidative injury.[15] Oxidized lipid mixtures, either from in vitro oxidation of arachidonate or from biological sources, are typically converted to pentafluorobenzyl (PFB) esters and trimethylsilyl ether (TMS) derivatives in preparation for analysis by GC-NICIMS. The derivatization procedures of the bicyclic endoperoxides of cholesteryl arachidonate used in this study are illustrated in Scheme 5. The bicyclic endoperoxides were reduced by triphenyl phosphine to give the trihydroxyl cholesterol ester. We found that PPh_3 gave better results than other reductants such as $SnCl_2$, $NaBH_4$, and SmI_2.[16] After hydrolysis of the ester under aqueous basic condition, esterification to the pentafluorobenzyl ester, this product was converted to the TMS ether derivative **5** by reaction with BSTFA.[17]

GC-MS chromatograms of the isoprostane derivatives, **5**, from oxidation mixtures derived from Ch-15-HPETE and Ch-11-HPETE are presented in Figure 6. Panel a is the selective ion monitoring (SIM) chromatogram from reaction of the 15 isomer and panel b presents a similar chromatogram from the reaction

Scheme 5



Reagents: (a) PPh_3, (b) KOH, THF, (c) PFB-Br, diethylpropylamine (d) BSTFA, DMF

of Ch-11-HPETE. The chromatograms are remarkably similar with the exception that the relative intensities of some peaks are reversed. Thus **5c** and **5d** as well as **5e** and **5f** have reversed intensities depending upon the particular precursor. Panel c shows the internal standard $PGF_{2\alpha}$-d_4.

The isoprostanes from the other types of bicyclic endoperoxides have also been studied by this technique and the SIM chromatograms are presented in Figure 7. The Type II and Type III derivatives from Ch-12-HPETE are shown in panel a. The chromatograms of isoprostane derivatives from the mixture of 8-HPETE/9-HPETE (b) and Ch-5-HPETE (c) are also presented in Figure 7.

The similarity of the chromatograms shown in panels b and c of Figure 7 is noteworthy. Ch-5-HPETE can give only Type I isoprostanes and the chromatogram shown in Figure 7c must therefore represent a stereoisomeric mixture of Type I isoprostanes. On the other hand, the mixture of Ch-8- and -9-HPETE's that give rise to the chromatogram shown in Figure 7b can give rise, at least in theory, to Type I, Type II, and Type III isoprostanes. We conclude from these studies that the formation of Type II or Type III isoprostanes from 8-HPETE occurs to a lesser extent than the formation of Type I hydroperoxides from Ch-5 or 9-HPETE. This suggestion is consistent with the results from autoxidation of arachidonic acid and in vivo studies of the rat, i.e., Type I and Type IV isoprostanes are the major regioisomers.[18] The preference for formation of Type I and Type IV isoprostanes is not well understood and experiments are ongoing to provide further information on this point.

The GC-MS method has been widely used to study lipid oxidation products from biological sources. Compared to the LC-MS techniques reported in the previous section, the GC-MS method gives much better sensitivity. Furthermore, the LC-MS method provides excellent qualitative information about peroxide structures but its utility in quantitative analysis is limited. Silver ion complexes of stereosomeric endoperoxides give significantly different response factors in the mass spectrometer.

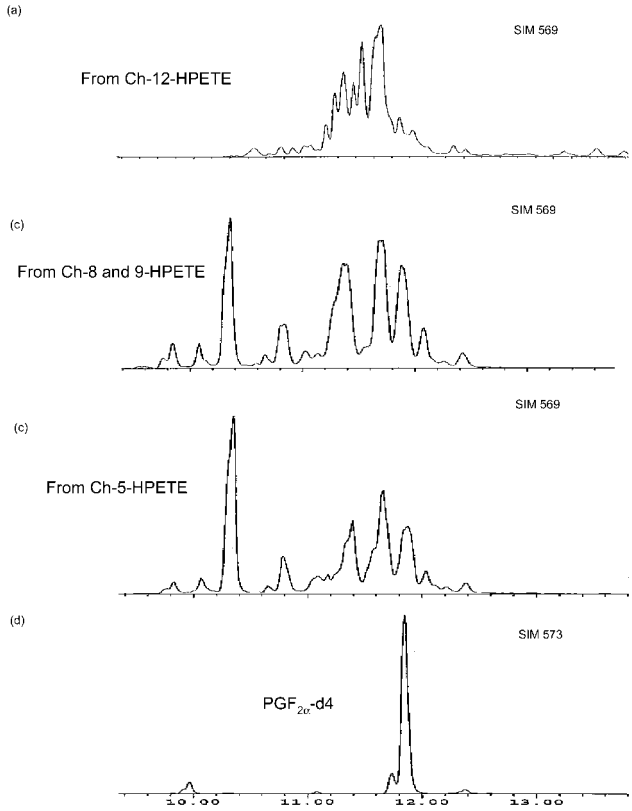


Figure 7. GC-NICI MS SIM (*m*/*z* 569) chromatograms of isoprostane derivatives from (a) Ch-12-HPETE, (b) Ch-8/9-HPETE, and (c) Ch-5-HPETE. (d) Internal standard of PGF_{2a}-d_4 (*m*/*z* 573).

Separation of the Stereoisomers of Type IV Isoprostanes. The bicyclic endoperoxide **2** has five stereogenic centers that can form 16 racemic diastereomers. There are, however, only eight possible racemic diastereomers observed due to the required cis substitution of the peroxide bond on the cyclopentane ring, which leads only to the *cis*-1,3-dihydroxycyclopentane substructures. The eight possible derivatives are shown in Scheme 6. Isoprostanes **5a**−**f** have been synthesized by Taber et al.,[19] and by comparing these known isoprostanes with the products formed from Ch-15 and 11-HPETE, assignment of structures to most of the products, both isoprostanes and cholesteryl ester endoperoxide precursors are possible. The six major peaks in the chromatogram shown in Figure 6a were

(15) (a) Morrow, J. D.; Zackert, W. E.; Yang, J. P.; Kurkhts, E. H.; Callewaert; Dworski, R.; Kanai, K.; Taber, D.; Moore, K.; Oates, J. A.; Roberts, J. L., II *Anal. Biochem*. **1999**, *269*, 326−331. (b) Mori, T. A.; Croft, K. D.; Puddey, I. B.; Beilin, L. J. *Anal. Biochem*. **1999**, *268*, 117−125.

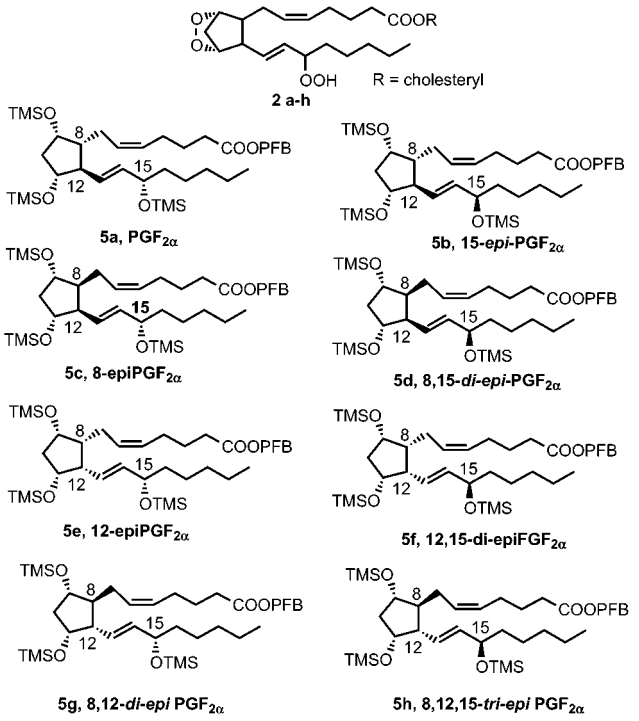
(16) (a) Hamberg, M.; Svensson, J.; Wakabayashi, T.; Samuelsson, B. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 345−349. (b) Suzuki, M.; Noyori, R.; Hamanaka, N. *J. Am. Chem. Soc*. **1981**, *103*, 5606−5607. (c) Feldman, K. S.; Simpson, R. E. *Tetrahedron Lett*. **1989**, *30*, 6985−6988.

(17) (a) Pouzar, V.; Slavikowa, T.; Cerny, I. *Collet. Czech. Chem. Commun*. **1998**, *63*, 1623−1634. (b) Linnet, K. *Clin. Biochem*. **1994**, *27*, 177−182.

(18) (a) Waugh, R. J.; Morrow, J. D.; Roberts, L. J., II; Murphy, R. C. *Free Radical Biol. Med*. **1997**, *23*, 943−954. (b) Waugh R. J.; Murphy, R. C. *J. Am. Soc. Mass. Spectrom*. **1996**, *7*, 490−499.

(19) (a) Taber, D. F.; Herr, R. J.; Gleave, D.; Mark. *J. Org. Chem*. **1997**, *62*, 194−198. (b) Taber, D. F.; Kanai, K. *Tetrahedron* **1998**, *54*, 11767−11782. (c) Taber, D. F.; Jiang, Q. *J. Org. Chem*. **2001**, *66*, 1876−1884. (d) Taber, D. F.; Kanai, K.; Pina, R. *J. Am. Chem. Soc*. **1999**, *121*, 7773−7777. (e) Taber, D. F.; Hoerrner, R. S. *J. Org. Chem*. **1992**, *57*, 441−447.

Scheme 6



assigned structure by adding the known isoprostane derivatives to the oxidation mixtures followed by GC-MS. The results were unequivocal. The predominant stereoisomers are the C-15 epimeric pair, **5f** and **5e**, as well as the **5d** and **5c** pair. Both of these epimeric pairs have cis substitution of the alkyl chains on the cyclopentane ring. As to the relative geometry of the hydroxyl at C_{15}, the diastereomers with the hydroxyl group on the side chain at C_{15} having *R* configuration (based upon prostaglandin stereochemistry) elute first on the chromatogram for each of the known pairs. The first pair of peaks at retention times around 10 min is tentatively assigned to **5h** and **5g**, respectively, although no standards for comparison are available.[15b]

To provide evidence of stereochemical configuration for the isoprostane endoperoxides, the oxidation mixture of Ch-15-HPETE was separated into fractions based upon the SRM chromatograms shown in Figure 3. The separated fractions were reduced with triphenylphosphine, converted to PFB-TMS derivatives, and analyzed by SIM GC-MS. The stereochemistry of the known isoprostanes was used to prove the stereochemistry of the original endoperoxide, **2**. The six major diastereomers of the endoperoxides, **2**, were found and their stereochemical configuration was assigned. The assignment of endoperoxide stereochemistry is shown in Figure 3, in which the configuration of the endoperoxides, **2a**−**h**, is shown, **2a** having the same configuration as **5a** and so on.

Discussion

Although mass spectrometry plays an indispensable role in structural elucidation of organic and biological compounds, conventional MS techniques cause difficulties in the analysis of peroxidation product mixtures because most of the ionization techniques result in the fragmentation of peroxide molecular ions.[20] Electrospray MS is becoming more and more important in the analysis of biological compounds because it involves the soft ionization of analytes. Addition of a Lewis acid such as silver ion to the analyte in conjunction with electrospray, Coordination Ionspray MS, has proved to be extremely useful in providing information about the structure of organic peroxides. Silver ion is a soft Lewis acid that can coordinate to soft Lewis basic sites, such as carbon−carbon double bonds. Moreover, fragmentation and rearrangement reactions of the silver ion−molecule adduct usually give valuable structural insight. Information about the location of the peroxide functionality in an analyte molecule can be obtained in this way. The technique of Ag^+ CIS/MS has opened the possibility of analyzing intact peroxides formed from free radical chain oxidation of cholesteryl arachidonate.[6a,10b]

The bicyclic endoperoxides formed in the peroxidation of cholesteryl arachidonate have not been studied due to the lack of appropriate analytical techniques. Four types of bicyclic endoperoxides are expected based on the position of H-abstraction on the arachidonate chain. As shown in Schemes 2 and 4, the Type IV bicyclic endoperoxides can be formed from Ch-15-HPETE or 11-HPETE. In this mechanism, generation of the peroxyl radical from the hydroperoxide of Ch-15-HPETE is followed by β fragmentation and re-addition of oxygen at the 11-position. Subsequent bicyclization of the 11-peroxyl provides the Type IV compounds. In a similar way, Type I bicyclic endoperoxides may form from Ch-5-HPETE or 9-H-PETE.

The situation with Ch-8-HPETE and Ch-12-HPETE is distinctly different from that of the other regioisomers. For these compounds, both Type II and Type III bicyclic endoperoxides can be formed from a single hydroperoxide, either the 8- or 12-substituted precursor. The mechanism for forming both Type II and Type III bicyclic endoperoxides from Ch-12-HPETE is shown in Scheme 7. The peroxyl radical **12-t,c-OO•** can undergo either 5-exo cyclization or β-fragmentation. The cyclization leads to the Type III endoperoxides while fragmentation, followed by re-addition of O_2 at C_8 gives the radical **8-t,t-OO•**. (The nomenclature for these intermediate peroxyl radicals indicates the position of oxygen substitution on the C-20 chain and the configuration of the conjugated diene double bonds.) Subsequent bicyclization of **8-t,t-OO•** provides entry to the Type II endoperoxides.

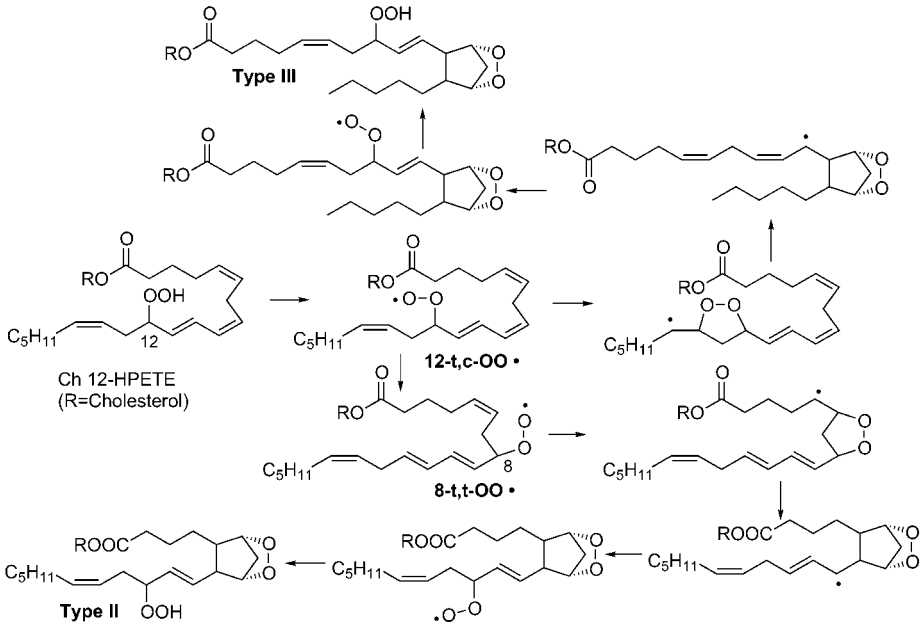
The rate constant for 5-exo cyclization of peroxyl radicals has been estimated to be 800 s^{-1}, while the rate constant for β-fragmentation is approximately 400 s^{-1}.[11] These two competitive reactions for **12-t,c-OO•** account for the final formation of both Type II and Type III bicyclic endoperoxides with the Type III compounds being the major products formed from Ch-12-HPETE. The same mechanism applied to Ch-8-HPETE would give both Type II and Type III, the former being the major endoperoxides in this instance.

The stereochemistry of the Type IV bicyclic endoperoxides was determined in this study by conversion of the oxidation mixtures to pentafluorobenzyl (PFB) ester−trimethylsilyl (TMS) derivatives and by comparison of the GC-MS chromatogram of the reaction mixture with those of the synthetic standards.[21]

(20) (a) Murphy, R. C. *Chem. Res. Toxicol.* **2001**, *14*, 1−10. (b) Murphy, R. C.; Fieldler, J.; Helvko, J. *Chem. Rev.* **2001**, *101*, 479−526. (c) Yin, H.; Hachey, D. L.; Porter, N. A. *Rapid Commun. Mass. Spectrom.* **2000**, *14*, 1248−1254.

(21) Thomas, M. J.; Chen, Q.; Sorci-Thomas, M. G.; Rudel, L. L. *Free. Radical Biol. Med.* **2001**, *30*, 1337−1346.

Scheme 7

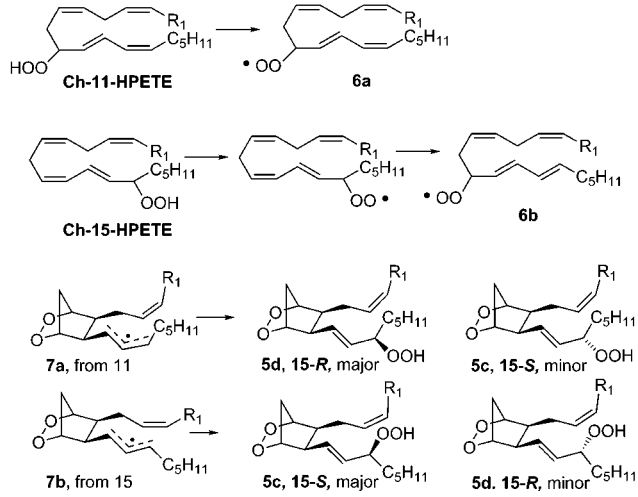


Eight possible racemic diastereomers were identified in the oxidation mixture of Ch-15-HPETE, a reaction that provides Type IV endoperoxides. Our results are consistent with those of O'Connor and Mihelich in which the endoperoxides with syn configuration of the cyclopentane side chains are favored compared to products having anti side chain arrangement.[22,23] Our results also suggest that the endo-endo diastereomers are more abundant than exo-exo products. The stereochemical course of the free radical process is in sharp contrast to that of the cyclooxygenase (COX) promoted conversion in which the anti product, **2a**, is formed exclusively.[7]

According to the proposed mechanism, the bicyclic endoperoxides formed from Ch-15-HPETE should be identical with those formed from Ch-11-HPETE. As shown in Figure 6, similar chromatograms are indeed obtained from reactions of both of these compounds. We note, however, that the relative intensity of the C-15 epimeric products corresponding to **5c** and **5d** is dependent upon the precursor hydroperoxide. Ch-15-HPETE gives more **5c** while the 11-regioisomer gives more **5d** product. Precursor-dependent product stereochemistry is also observed for the C-15 epimers **5e** and **5f** while this dependence is not observed for products **5a** and **5b**.

The only substantive difference between the mechanisms of reaction of Ch-11- and 15-HPETE is the geometry of the conjugated diene present in the first-formed peroxyl radicals, **6a** and **6b**. The conjugated diene of Ch-11-HPETE and the 11-peroxyl derived from this precursor, **6a**, have Δ_{12-13} trans, Δ_{14-15} cis geometry while the corresponding diene peroxyl **6b**, formed from Ch-15-HPETE, has Δ_{12-13} trans, Δ_{14-15} trans configuration. These intermediate peroxyls are stereoisomeric. We speculate that this stereochemical difference, translated through the reaction course, results in stereoisomeric allyl radicals **7a** and **7b**. Furthermore, we suggest that product

Scheme 8



stereochemistry translates directly from allyl radical geometry to the C-15 epimers **5c** and **5d**. The radical **7a**, having trans,cis substitution, adds oxygen preferentially to give **5d** while **7b**, having trans,trans allyl geometry, adds oxygen to give **5c** as the major product. Scheme 8 shows this reaction course for the oxygen addition. The geometry of **7a** and **7b** is chosen such that it is consistent with the products observed, if oxygen adds to the face of the radical opposite to the vicinal side chain on the endoperoxide.

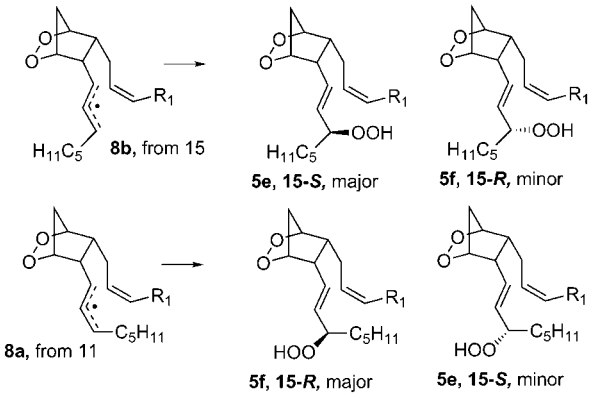
A similar analysis of the epimeric products **5e** and **5f**, formed from radicals **8a** and **8b**, suggests that the preferred geometry for these radicals is as shown in Scheme 9. Again, preferential addition of oxygen to the radical face opposite to the vicinal chain gives the preferred product. We note that the formation of 15-*R* isoprostane epimers from 11-HPETE (**5d** and **5f**) is consistent with the observations reported earlier for the simpler linolenate system.[22]

It is also noteworthy that product distribution is apparently independent of precursor diene geometry for the other isoprostane C-15 epimeric pairs, **5a/b** and **5h/g**, which have side chains substituted trans on the endoperoxide ring. A cis vicinal side

(22) (a) O'Connor, D. E.; Mihelich, E. D.; Coleman, M. C. *J. Am. Chem. Soc.* **1984**, *106*, 3577−3584. (b) Mihelich, E. D. *J. Am. Chem. Soc.* **1980**, *102*, 7141−7143. (c) O'Connor, D. E.; Mihelich, E. D.; Coleman, M. C. *J. Am. Chem. Soc.* **1981**, *103*, 223−224.

(23) (a) Corey, E. J.; Shih, C.; Shih, N.-Y.; Shimoji, K. *Tetrahedron Lett.* **1984**, *25*, 5013−5016. (b) Corey, E. J.; Wang, Z. *Tetrahedron Lett.* **1994**, *35*, 539−544.

Scheme 9



chain on the endoperoxide is an important factor for the modest stereoselection observed. Steric shielding arguments have been invoked to explain stereoselectivity in radical additions to carbon−carbon double bonds or atom transfer reactions. One expects that steric shielding would have a marginal effect in the addition of oxygen to carbon radicals, a reaction to proceed at or near diffusion control, and this is confirmed by our observations.

Our experiments are relevant to a novel alternate mechanism for isoprostane formation described in Scheme 10a.[23b,8c,d] In this mechanism, the Type IV isoprostanes are formed from 15-HPETE by a 4-*exo*-peroxyl radical cyclization followed by oxygen addition and double 5-exo cyclization. This mechanism does not account for the formation of monocyclic and serial cyclic peroxides, **3** and **4**, which are major products in the reaction and which are consistent with the mechanism described in Scheme 2. The mechanism described in Scheme 10 also does not account for the formation of Type IV isoprostanes from Ch-11-HPETE, see Scheme 10b. In fact, we see very similar product distributions starting from either the 15 or the 11 regioisomers, Figure 6, again consistent with the mechanism outlined in Schemes 2 and 4. Finally, our LC-MS analysis of reactions of Ch-15-HPETE shows evidence for the formation of Ch-11-HPETE during the reaction. This observation, which is consistent with reactions of other lipid hydroperoxides, supports the notion that fragmentation of the 15-peroxyl radical and re-addition of oxygen to give the 11-peroxyl radical accompanies the formation of isoprostane products. This observation is consistent with the mechanism shown in Scheme 2. The weight of the experimental evidence would appear to support the mechanism we propose.

Experimental Section

General Methods. Reactions involving hydroperoxides were monitored by TLC by 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. TLC was carried out by using a 0.2 mm layer thickness of Si coated on aluminum (EM Scientific) and was visualized by UV 254, phosphomolybdic acid char, or the peroxide stain. In general, peroxides were stored as dilute solutions in either hexanes or benzene at −78 °C and were never exposed to temperatures above 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+%). Pentafluorobenzyl bromide and diisopropylethylamine were purchased without further purification from Aldrich Chemical Co. (Milwaukee, WI). *N*,*O*-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Supelco Inc. (Bellefonte, PA). Organic solvents such as dichloromethane, hexane, benzene, and ethyl acetate were HPLC quality purchased from Allied Signal (Burdick and Jackson Brand, Muskegon, MI). Preparative TLC was performed on silica gel 60ALK6D plates (Whatman International Ltd., Maidstone, UK). The free radical initiator di-*tert*-butyl hyponitrite (DTBN) was synthesized according to the literature procedures.[24]

Instruments. Analytical HPLC was carried out with a Waters Model 600E pump and a Waters 996 Photodiode array detector. Millenium32 chromatography software (Waters Corp., Milford, MA) was used to control the 996 and to collect and process data. Cyclic peroxide analysis by analytical HPLC utilized a single Beckman Ultrasphere 5 μm (4.6 mm × 25 cm) silica column. A flow rate of 1 mL/min was used for analytical NP HPLC. Preparative NP HPLC was performed with a Dynamax-60 Å (83-121-C) silica column (21.4 mm × 25 cm × 8 μm particles) with a flow rate of 10 mL/min. Narrowbore HPLC for MS analysis used a single Beckman Ultrasphere 5 μm (2.0 mm × 25 cm) silica column for analysis of cyclic peroxides and two Beckman Ultrasphere 5 μm (2.0 mm × 25 cm) silica columns for acyclic hydroperoxide analysis. The flow rate for the LC-MS separations and analysis was 150 μL/min.

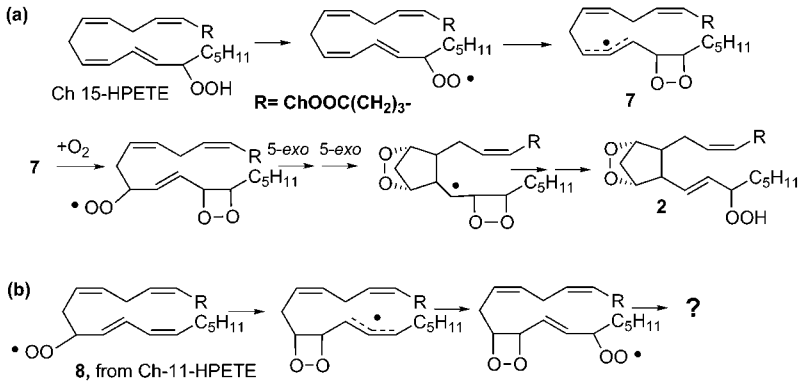
Mass Spectrometry. CIS-MS was accomplished with a Finnigan TSQ-7000 (San Jose, CA) triple quadrupole mass spectrometer operating in positive ion mode equipped with a standard API-1 electrospray ionization source. The source was outfitted with a 100 μm deactivated fused Si capillary. Data acquisition and evaluation were conducted on ICIS EXECUTIVE INST, version 8.3.2, and TSQ 7000 software INST, version 8.3. Data collected for Selected Reaction Monitoring (SRM) experiments was also processed with Xcalibur, version 1 (Finnigan, San Jose, CA).

Nitrogen gas served both as a sheath gas and an auxiliary gas; 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 potential and capillary voltage were optimized to maximize ion current for electrospray, with the optimal determined to be 80 and 20 V, respectively, for cholesteryl ester analysis. Positive ions were detected scanning from 100 to 1000 amu with a scan duration of 2 s. Profile data was recorded for 1 min and averaged for analysis. For CID experiments the collision gas pressure was set from 2.3 to 2.56 mT. To obtain fragmentation information of each compound, the dependence of offset-voltage and reconstructed ion current (RIC) was studied. The collision energy offset was varied from 10 to 40 eV depending on the compound being analyzed.

Samples were introduced either by direct liquid infusion or by HPLC. For direct liquid injection, stock solutions of the lipids (100 ng/μL in 1% IPA in hexane) were prepared and mixed 1:1 with silver tetrafluoroborate (51.4 ng/μL in IPA). Samples were introduced to the ESI source by 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 to the ESI interface was increased to between 5 and 10 units to assist in desolvation of the samples. For cholesteryl arachidonate hydroperoxide analysis, normal phase HPLC sample introduction was carried out by using two tandem Beckman Ultrasphere narrowbore 5μ silica columns (2.0 mm × 25 cm) operated in isocratic mode with 0.5% 2-propanol in hexanes. For analysis of cyclic peroxide mixtures, sample introduction was carried out with 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 high-pressure mixing tee was

(24) (a) Mendenhall, G. D. *Tetrahedron Lett.* **1983**, *24*, 451−454. (b) Traylor, T. G.; Kiefer, H. *Tetrahedron Lett.* **1966**, *49*, 6163−6168.

Scheme 10



connected next in series for the postcolumn addition of the silver salts. The silver tetrafluoroborate ($AgBF_4$) 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 long 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.

GC-NICI MS was performed with a Hewlett-Packard HP5989A GC/MS instrument interfaced with an IBM Pentium II computer system. GC was performed with a 30 m, 0.25 mm diameter, 0.25 μm film thickness, DB-Wax column (J & W Scientific, Folsom, CA). The column temperature was programmed from 190 to 260 °C at 10 deg/min. Methane was used as the carrier gas at a flow rate of 1 mL/min. The ion source temperature was 250 °C, the electron energy was 70 eV, and the filament current was 0.25 mA.

Oxidation of Cholesteryl Arachidonate to Primary Hydroperoxides. In a round-bottomed flask, cholesteryl arachidonate (400 mg, 0.584 mmol) was dissolved in benzene to make a 0.15 M solution. To the mixture were added 0.5 equiv of methyl trolox (methyl 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid ester)[25] and 0.1 equiv of DTBN. The reaction was stirred under oxygen at 37 °C for 24 h. The reaction was quenched by adding 2 mg of BHT. Analytical HPLC (tandem Si column, 0.5% 2-propanol in hexane, λ_{max} = 234 nm) indicated seven major fractions: A, t_R = 13.80 min, 15-HPETE; B, t_R = 14.54 min, 12-HPETE; C, t_R = 16.11 min, 11-HPETE; D, t_R = 20.91 min, 9-HPETE; E, t_R = 21.29 min, 8-HPETE; F, t_R = 26.11 min, 5-HPETE; G, t_R = 51.30 min, methyl trolox. The excess of hydrogen atom donor methyl trolox was separated by flash column chromatography on silica with use of a solvent mixture of hexane: ethyl acetate (9:1). All the peroxide stain positive fractions were combined and separated by semipreparative HPLC (0.5% 2-propanol in hexane, 234 nm). Components D and E were collected as a mixture.

Autoxidation of the Hydroperoxides of Cholesteryl Arachidonate. The primary hydroperoxides separated by HPLC were converted to more highly oxidized peroxides according to the literature.[6a]

Derivatization of the Oxidation Products of Cholesteryl Arachidonate. To 100 μL of the oxidation mixture was added 10 ng of $PGF_{2\alpha}$-d_4 as internal standard. An excess amount of PPh_3 was added to the mixture. After evaporation of the solvent, the residue was dissolved in 200 μL of ethanol and to the solution was added 200 μL of 8 M aqueous KOH solution. After being stirred at 40 °C for an hour, the mixture was cooled to room temperature and the pH of the solution was adjusted to 3 by adding dilute HCl. The aqueous solution was extracted by ethyl acetate three times. The combined organic phases were dried over Na_2SO_4. After evaporation of the solvent, the residue was dissolved in 20 μL of CH_3CN. To the resulting solution was added 20 μL of 10% pentafluorobenzyl bromide in acetonitrile and 10 μL of 10% *N*,*N*-diisopropylethylamine in acetonitrile and the mixture was kept at room temperature for 30 min. The reagent was dried under nitrogen and the residue was subjected to TLC separation by using the solvent ethyl acetate/methanol (98:2, v/v). Approximately 5 μg of the PFB ester of $PGF_{2\alpha}$ was applied on a separate lane and visualized by spraying with a 10% solution of phosphomolybdic acid in ethanol followed by heating. Compounds migrating in the region of the PFB ester of $PGF_{2\alpha}$ (R_f = 0.4) and the adjacent area 2 cm above and under were scrapped and extracted from the silica gel by ethyl acetate.

After evaporation of the ethyl acetate, 20 μL of BSTFA and 10 μL of dimethylformamide were added to the residue and the mixture was incubated at 40 °C for 20 min. The reagents were dried under nitrogen and the derivatives were dissolved in 10 μL of dry undecane for analysis of GC-MS.

Acknowledgment. We thank Lisa M. Manier and Dr. David L Hachey at the Vanderbilt University Mass Spectrometry Research Center for their assistance with MS measurements and Dr. Douglass F. Taber of the Department of Chemistry and Biochemistry at the University of Delaware for providing isoprostane samples. Financial support from NIH HL17921, GM15431, DK 48831, CA 77839, P30 ES00267, and NSF CHE 0107697 is gratefully acknowledged. H.Y. acknowledges the Warren Graduate Fellowship of the Chemistry Department at Vanderbilt University. J.D.M. is the recipient of a Burroughs Wellcome Fund Clinical Scientist Award in Translational Research.

Supporting Information Available: DLI and CID spectra obtained from the various Ch-HPETE (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0201092

(25) (a) Cohen, N.; Lopresti, R. J.; Neukom, C. *J. Org. Chem.* **1981**, *46*, 2445−2450. (b) Yoda, H.; Takabe, K. *Chem. Lett.* **1989**, 465−468. (c) Suarna, C.; Dean, R. T.; Southwellkeely, P. T. *Aust. J. Chem.* **1997**, *50*, 1129.