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Cholesterol Autoxidation Revisited: Debunking the Dogma Associated with the Most Vilified of Lipids

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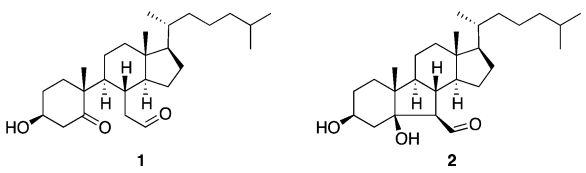
Supporting Information

ABSTRACT: The longstanding dogma that cholesterol (chol) autoxidation gives chol 7-hydroperoxide (7-OOH) as the sole primary product is shown to be invalid. In fact, the epimers of each of chol 4-OOH, 6-OOH, and 7-OOH are readily formed. Although the C4−H bond that must be cleaved to produce the chol 4-OOH and 6-OOH products is significantly stronger than the C7−H bond, H-atom abstraction from C4 is facilitated by H-bond formation between the attacking peroxyl radical and the 3β-OH. Chol 5α-OOH is also formed, but only in the presence of a good H-atom donor. Chol 5α-OOH *and* 6-OOH undergo Hock fragmentation to yield the secosterols implicated in cardiovascular and neurodegenerative diseases, suggesting that they are likely to arise simply from autoxidation and not from reactions with O_3 or $^1O_2$.

Cholesterol (chol) constitutes up to 50% of all lipids present in the plasma membrane (on a molar basis).[1] Its levels, as esters of polyunsaturated fatty acids in circulating low-density lipoprotein (LDL), are an established risk determinant of cardiovascular disease.[2] Furthermore, chol-derived oxidation products (and those of related sterols[3]) have been implicated in the pathogenesis of various degenerative diseases.[4] Of particular interest are the electrophilic secosterols **1** and **2**, which are

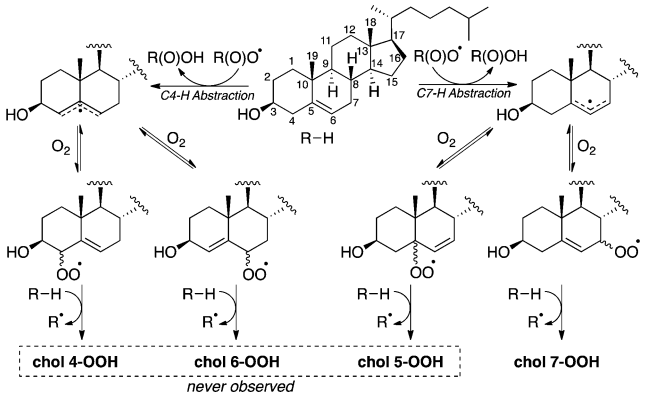


known products of chol ozonolysis and may contribute to the respiratory toxicity of O_3 present in polluted air.[5] More recently, **1** and **2** were identified following acid-catalyzed derivatization of extracts of atherosclerotic heart tissue[6] and brain tissue of Alzheimer's patients[7] with 2,4-dinitrophenylhydrazine. These results have prompted studies of the pathogenic potential of **1** and **2** in cardiovascular disease,[8] cancer,[9] and neurodegeneration (including Alzheimer's,[10] Parkinson's[11] and multiple sclerosis[12]) and the highly controversial[13] suggestion that O_3 is formed endogenously.[6]

A short time ago, we demonstrated that the formation of **1** and **2** is not unique to ozonolysis. In fact, these products also arise upon (acid-catalyzed) Hock fragmentation of chol 5α-hydroperoxide (chol 5α-OOH)[14]—the product of the reaction of chol with $^1O_2$. Of course, if chol 5α-OOH were formed simply from chol autoxidation, no highly energetic oxidants (i.e., O_3 or $^1O_2$) would need to be implicated in the formation of **1** and **2**. Thus, **1** and **2** could be considered among the nefarious products of lipid peroxidation, such as 4-hydroxynonenal or malondialdehyde, which are well-known to derive from oxidized linoleic acid (linoleate) and arachidonic acid (arachidonate), respectively.[15] However, a survey of the vast literature on chol autoxidation reveals that the primary products are limited to the epimers of chol 7-OOH,[16] the immediate precursors to the corresponding epimers of 7-hydroxycholesterol (chol 7-OH) and 7-ketocholesterol, each of which are used as biomarkers of lipid oxidation.[17]

We were admittedly puzzled by this dogma, since Porter has shown that the autoxidation of other monounsaturated lipids, such as oleic acid (oleate), yields hydroperoxides derived from O_2 addition to both ends of the allylic radical intermediate.[18] In principle, chol autoxidation should give eight products (cf. Scheme 1) resulting from H-atom abstraction from either C4

Scheme 1. Putative Mechanism of Cholesterol Autoxidation



or C7 followed by O_2 addition to either end of the respective allylic radical intermediates on either the α- or β-face of the sterol framework. Since it has been demonstrated that the reversibility of O_2 addition to intermediate radicals can be key to understanding product distributions arising in autoxidations of polyunsaturated lipids,[19] we elected to revisit the autoxidation of chol in the presence of H-atom donors that could serve to trap labile peroxyl radical intermediates.

Chol autoxidations were first carried out in aerated chlorobenzene (0.5 M) at 37 °C for 16 h, initiated by

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decomposition of the azo compound MeOAMVN (20 mM). The resultant mixtures were analyzed by normal-phase HPLC with APCI-MS/MS detection. In each instance, the samples were first treated with PPh_3 to convert the hydroperoxide products to their corresponding alcohols, which are more stable toward chromatography and MS analysis. Somewhat surprisingly, we observed six primary products (Figure 1A). The

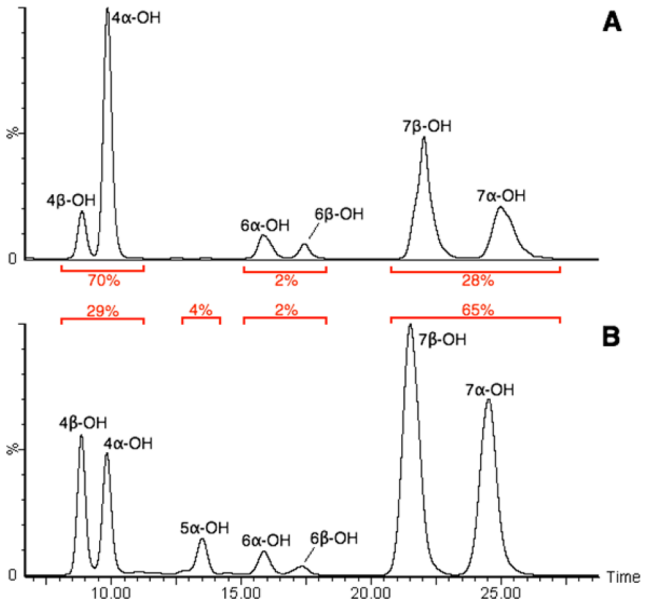


Figure 1. Product distributions from MeOAMVN-initiated autoxidations of chol in chlorobenzene at 37 °C with no added antioxidant (A) and with 250 mM 4-*tert*-butyl-2,6-dimethylphenol (B). Data were obtained by HPLC (95:5 hexane/*i*-PrOH, 1.5 mL/min) with $APCI^+$-MS/MS detection (m/z 385.35 → m/z 367.37). The alcohols are observed as a result of pretreatment with PPh_3.

compounds (chol 4α-OH, 4β-OH, 6α-OH, 6β-OH, 7α-OH, and 7β-OH) were identified by comparison with authentic standards, synthesized according to literature precedent, and determined relative to a newly synthesized d_6-chol 5α-OH standard (see the Supporting Information for details).

On the basis of the mechanism in Scheme 1, chol 4-OOH and 6-OOH presumably arise from initial H-atom abstraction from C4—previously assumed to be an uncompetitive pathway. Indeed, high-accuracy CBS-QB3[20] calculations on the A–B ring system of chol suggest that the C4–H bond is ca. 6 kcal/mol stronger than the C7–H bond (89.0 and 83.2 kcal/mol, respectively). However, since the H-atom abstraction step is likely to be irreversible, the product ratio should be determined by kinetics. We therefore calculated the barriers for each pathway with MeOO· as a model chain-carrying peroxyl radical using the density functional theory step of the CBS-QB3 calculation (B3LYP/CBSB7).[21] α-H abstraction from C7 by MeOO· is predicted to be favored over β-H abstraction due to steric hindrance imposed by the β-CH_3 at C10. More interestingly, H-atom abstraction from C4 by MeOO· is predicted to be competitive with abstraction from C7, as the barrier to β-H abstraction from C4 is lowered by a H-bonding interaction between the internal oxygen atom of the peroxyl radical and the β-OH group at C3 (cf. Figure 2). This interaction is relatively unaffected by sterics (i.e., the same trend exists with cyclohexylperoxyl; see the Supporting Information).

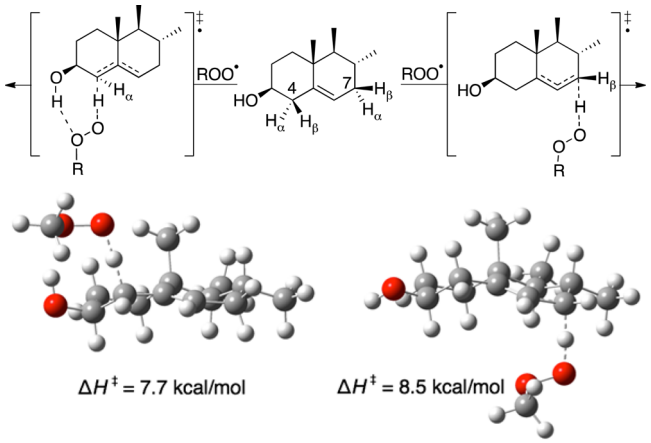


Figure 2. Lowest-energy transition state structures and associated enthalpic barriers for the H-atom abstractions from C4 and C7 (chol numbering) by a methylperoxyl radical.

The foregoing accounts for the observed products in Figure 1A, but it remains unclear why no chol 5-OOH products are observed. We wondered whether the peroxyl radicals resulting from O_2 addition to C5 are particularly unstable. In fact, calculations suggest that the 5α- and 5β-peroxyl radicals are ca. 3 and 6 kcal/mol less stable, respectively, than the peroxyl radicals formed from O_2 addition at the 7-position. It is predicted that the 5β-peroxyl radical is particularly unstable because of a conformational change in the steroid backbone that accompanies O_2 addition (cf. Figure 3).

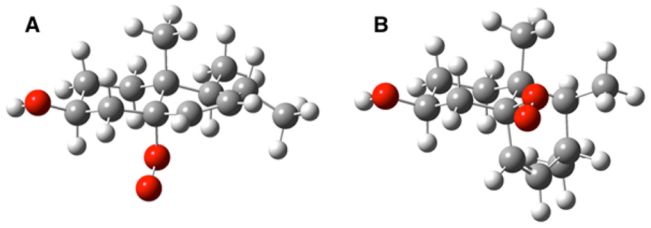
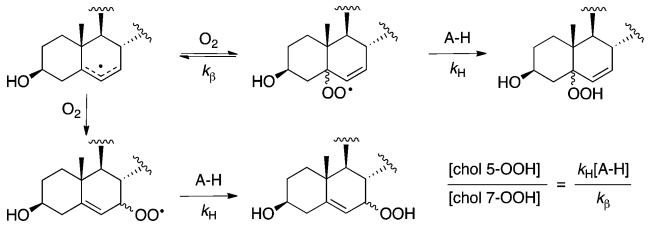


Figure 3. Calculated minimum-energy structures of models of chol 5α- (A) and 5β- (B) peroxyl radicals.

The relative instability of the peroxyl radicals derived from addition at C5 suggests that they may undergo facile β-fragmentation under the autoxidation conditions to favor the C7 oxidation products at the expense of the C5 oxidation products—an accepted mechanism for the known rearrangement of chol 5α-OOH to 7α-OOH.[22] Thus, in the presence of a good H-atom donor, the C5 peroxyl radicals may be trapped to give chol 5-OOHs (cf. Scheme 2). It should be pointed out that chol autoxidations carried out in vitro have typically

Scheme 2. Kinetically Controlled Autoxidation of Chol Enables the Determination of k_β for Chol 5α-OO·



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depended on the H-atom donor being chol itself (k_H = 11 M^{-1} s^{-1});[23] however, chol autoxidation in vivo can occur in the presence of far better H-atom donors. For example, α-tocopherol (α-TOH), nature's premier lipid-soluble radical-trapping antioxidant, has k_H = 3.2 × 10^6 M^{-1} s^{-1},[24] and is present within cell membranes and chol-rich lipid rafts. Hence, product distributions were determined in autoxidations of chol in the presence of 2,2,5,7,8-pentamethyl-6-chromanol (PMC), a truncated α-TOH.

Chol autoxidations to which PMC (25 mM) were added (and analyzed as above) did indeed contain chol 5α-OH, in addition to 4α-OH, 4β-OH, 6α-OH, 6β-OH, 7α-OH, and 7β-OH (cf. Figure S4). Since in principle a determination of the product distribution as a function of PMC concentration would reveal the rate constant at which the C5 peroxyl radical undergoes β-fragmentation (as in Scheme 2), we carried out further autoxidations at higher PMC concentrations. Unfortunately, too little products were formed to reliably quantify. When PMC was exchanged for 4-*tert*-butyl-2,6-dimethylphenol (BDMP), a less reactive radical-trapping antioxidant (vide infra), chol 5α-OH was again observed (Figure S5), but in a lower amount than observed at the same concentration of PMC. However, upon an increase in [BDMP] (250 mM), the chol 5α-OH/7-OH ratio increased, as shown in Figure 1B. Furthermore, the ratio of products was clearly linearly dependent on the concentration of BDMP (Figure 4), and

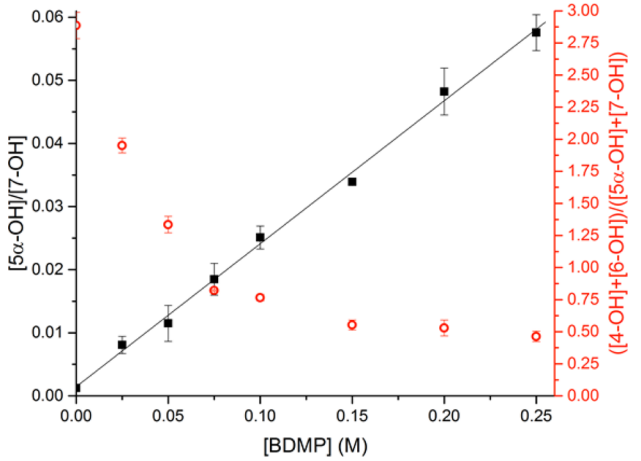


Figure 4. Dependence of the chol 5α-OH to 7-OH ratio (■) and the chol 4-OH and 6-OH to 5α-OH and 7-OH ratio (○) on the 4-*tert*-butyl-2,6-dimethylphenol (BDMP) concentration.

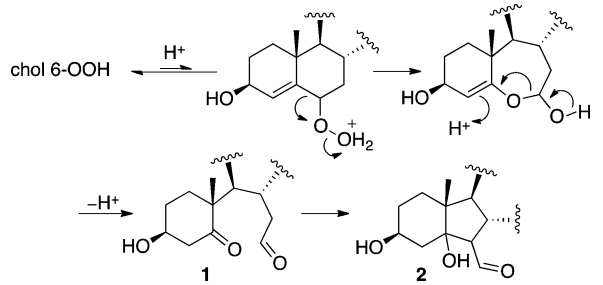
from the simple expression in Scheme 2 we obtained k_β = (5.6 ± 0.1) × 10^5 s^{-1} (using k_H = (1.3 ± 0.1) × 10^5 M^{-1} s^{-1} for BDMP, as determined by inhibited autoxidation of styrene;[25] see the Supporting Information). Similarly, k_β = (8.6 ± 0.4) × 10^3 s^{-1} for chol 4β-OO· was determined from the chol 4β-OH/4α-OH ratio with varying BDMP (cf. Figure S13).

Interestingly, the ratio of products derived from H-atom abstractions from C4 and C7 was also dependent on the BDMP concentration (also shown in Figure 4). Increasing the BDMP concentration led to a marked decrease in 4-OH and 6-OH products relative to 5-OH and 7-OH products. This result reflects the increasing contribution of the antioxidant-mediated peroxidation mechanism[26] in the autoxidation with increasing [BDMP]. That is, as the concentration of BDMP increases, the BDMP-derived phenoxyl radicals (BDMP·) become the prominent chain-carrying species. Since BDMP· lacks a good H-bond acceptor to interact with the β-OH group at C3, the barrier to abstraction of the C4 β-H is increased relative to that for abstraction at C7. Our calculations suggest that the difference is ca. 1.5 kcal/mol.

The foregoing observations directly address the longstanding speculation about the C4−H abstraction pathway in chol autoxidation.[4] Although both epimers of chol 4-OH have been detected in vitro[27] and in vivo,[28,29] their formation was only tenuously tied to autoxidation by Diczfalusy and co-workers, who showed that ^{18}O was incorporated into chol 4α-OH and 4β-OH in LDL oxidized in an $^{18}O_2$-enriched atmosphere.[29] Interestingly, the 4α-OH/4β-OH ratio observed in their experiments was ca. 1:1, consistent with the presence of a significant amount of antioxidant in the LDL (cf. Figure 1). However, it is likely that free chol and the cholesterol esters that predominate in LDL give rise to different product distributions (arising from initial C7−H vs C4−H abstraction). Indeed, the regiochemistry of H-atom abstraction is presumably influenced by both the identity of the abstracting radical and the possibility of H-bonding interactions with the medium, e.g., at the aqueous interface of a lipid bilayer or lipoprotein. This is currently under investigation.

It remains to reiterate that the observation of chol 5α-OOH in chol autoxidations offers a pathway to the secosterols **1** and **2** that does not require high-energy oxidants (i.e., O_3 or $^1O_2$). However, our elucidation of the complete set of products of chol autoxidations prompts the suggestion of an even more compelling pathway: the (acid-catalyzed) Hock fragmentation of chol 6-OOH (Scheme 3). Although it is a minor product,

Scheme 3. Proposed Mechanism of the Hock Fragmentation of Chol 6-OOH



chol 6-OOH arises from the autoxidation of chol regardless of the presence of antioxidants. As expected from the transformation shown in Scheme 3, when authentic chol 6β-OOH was subjected to the same derivatization conditions used for the identification of **1** and **2** in human heart and brain tissue (2,4-dinitrophenylhydrazine-HCl),[6,7] the (derivatized) secosterols **1** and **2** were the only products observed (see the Supporting Information for details). Thus, the formation of **1** and **2** can be linked directly to the ubiquitous process of lipid peroxidation by *two of the four possible autoxidation products*. However, since the derivatization processes used to identify **1** and **2** from tissue samples employ acid as a catalyst,[6,7,11] it remains to be determined unambiguously whether **1** and **2** are formed in vivo or ex vivo.

■ ASSOCIATED CONTENT

Supporting Information

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Computational and experimental details, compound characterization, standardization data, autoxidations carried out at various PMC and BDMP concentrations, and Cartesian coordinates and energies of calculated structures (PDF)

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Notes

The authors declare no competing financial interest.

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(3) Perhaps the best example is that of 7-dehydrocholesterol, a precursor to vitamin D_3 and chol, which builds up in patients with Smith−Lemli−Opitz syndrome and oxidizes readily to form the oxysterols believed to be important in the pathogenesis of the disease. See: (a) Xu, L.; Porter, N. A. *J. Am. Chem. Soc.* **2014**, *136*, 5443. (b) Xu, L.; Korade, Z.; Porter, N. A. *J. Am. Chem. Soc.* **2010**, *132*, 2222.

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