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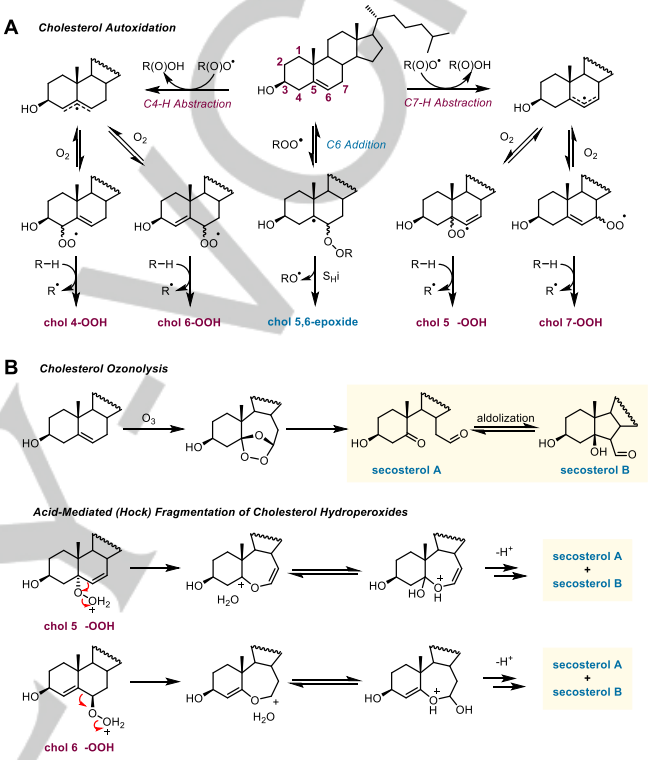
On the Products of Cholesterol Autoxidation in Phospholipid Bilayers and the Formation of Secosterols Derived Therefrom

Emily L. Schaefer, Nadia Zopyrus, Zosia A. M. Zielinski, Glenn A. Facey, and Derek A. Pratt*[a]

Dedicated to Dr. Keith U. Ingold, in recognition of his lifetime contributions to chemistry, on the occasion of his 90th birthday.

Abstract: In homogenous organic solution, cholesterol autoxidation leads to a mixture of epimers of 5 primary products, whose concentrations vary in the presence/absence of antioxidants, such as Vitamin E. Two of the products (5α-OOH and 6β-OOH) undergo Hock fragmentation to yield electrophilic secosterols implicated in disease. Herein, we show that the product distribution is similar in phospholipid bilayers, in that the 7-OOHs are the major products, but the presence of Vitamin E has no effect on the product distribution. Cholesterol 7α-OOH, but not 7β-OOH, undergoes Hock fragmentation to yield a mixture of unprecedented A-ring cleavage products and 6,7-epoxides. When subjected to typical derivatization conditions, 7α-OOH yields products with essentially indistinguishable chromatographic and spectroscopic features from the previously identified secosterols, casting further doubt on their controversial origin from endogenous O_3.

Cholesterol (chol) is among the most abundant of lipids in mammalian phospholipid bilayers[1] and elevated levels thereof, as part of circulating low-density lipoproteins, is a key risk factor for cardiovascular disease.[2,3] In organic solution, chol autoxidizes to a variety of products (**Scheme 1A**), of which the epimers of the 7-hydroperoxide (7-OOH) and 5,6-epoxide (5,6-epoxy) are predominant.[4,5] Cholesterol undergoes a variety of other oxidative transformations leading to so-called oxysterols,[6–8] several of which have been implicated in disease pathogenesis.[9–11] Among the more intriguing[12–14] oxidation products are the electrophilic secosterols A and B (secA and secB).[15,16] These compounds, which were allegedly identified in diseased heart and brain tissue,[17,18] were proposed to arise from the oxidation of chol by ozone formed from the antibody-catalyzed oxidation of water.[19,20] In previous work, we demonstrated alternative pathways for their formation (**Scheme 1B**). Specifically, we showed that chol 5α-OOH, (the major product of the reaction of $^1O_2$ with cholesterol and a minor product of chol autoxidation in the presence of Vitamin E), undergoes acid-catalyzed (Hock) fragmentation to yield secA and secB.[21,22] Similarly, chol 6β-OOH, a minor product of the reaction of $^1O_2$ with chol as well as chol autoxidation, yields secA and secB.[4] The acid-mediated reactions of these hydroperoxides are relevant given the presence of acidic microenvironments within the cell[23–25] and that the alleged identification of secA and secB was made following acid-catalyzed derivatization to their corresponding 2,4-dinitrophenylhydrazones. However, it was hitherto unknown if



Scheme 1. Products of cholesterol autoxidation via H-atom abstraction and addition pathways (**A**) and demonstrated routes to secosterols A and B (**B**).

5α-OOH and 6β-OOH are formed from chol autoxidation in phospholipid bilayers. Moreover, it was hitherto unknown if the epimers of chol 7-OOH undergo Hock fragmentation to give secosterol products. Herein we address these outstanding points.

The product distribution shown in **Scheme 1A** was established from analyses of samples of autoxidations of chol in chlorobenzene (PhCl) by normal phase HPLC-APCI$^+$-MS/MS.[5] The compounds were identified by direct comparison to authentic standards obtained by chemical synthesis and quantified against deuterated internal standards. The hydroperoxides were identified following reduction to their corresponding alcohols by the $[M+H^+–H_2O] \rightarrow [M+H^+–2H_2O]$ MS/MS transition (385.4 → 367.4). The 5,6-epoxides (the β-epimer of which unfortunately co-elutes with chol 4α-OH), were identified by the $[M+H^+] \rightarrow [M+H^+–H_2O]$ MS/MS transition (403.4 → 385.4).[26] Using this approach (see Supporting Information),[27] we investigated the product distribution in azo-initiated (MeOAMVN) autoxidations of cholesterol-embedded unilamellar liposomes prepared from either egg phosphatidylcholine (PC) or soy lecithin (43.5 mol% chol, ~130 nm diameter). The results are shown alongside those previously obtained in PhCl in **Figure 1**.

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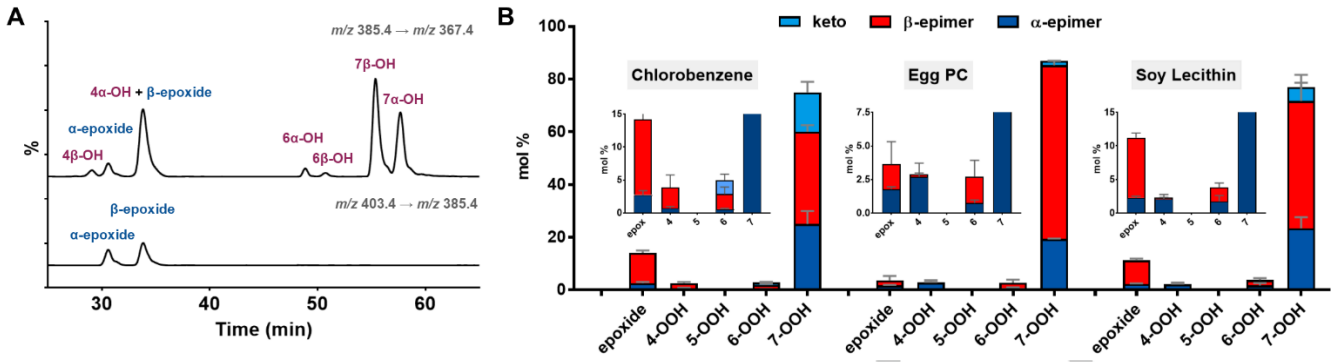

Figure 1. Representative chromatograms of chol autoxidation products in unilamellar liposomes of egg phosphatidylcholine. Hydroperoxide species were reduced to their corresponding alcohols for detection by APCI$^+$-MS/MS (**A**). Product distributions from MeOAMVN-initiated (20 mM) autoxidations of chol in PhCl at 37°C for 16 h (as previously reported)[5] compared to product distributions of MeOAMVN-initiated (1 mM) autoxidations of chol in liposomal egg phosphatidylcholine or soy lecithin at 37°C after 16 h; inset: distributions of minor products (**B**).

Incorporation of chol into liposomes did not afford a substantial difference in the product distribution. The epimers of chol 7-OOH were still the predominant products, although their relative abundance in egg PC relative to PhCl was bolstered somewhat at the expense of the second most abundant 5,6-epoxy chol products. Interestingly, more epoxide was observed in the more highly unsaturated soy lecithin liposomes (ca. 64% polyunsaturated fatty acid content compared to 15% in egg PC), suggesting that a more fluid membrane facilitates peroxyl radical addition over H-atom abstraction. Interestingly, the minor 4-OOH and 6-OOH products were also observed in liposomes.[28] Although 4-OOH have been indirectly observed in tissue samples (as 4α/β-OH presumably derived from 4-OOH),[29,30] to the best of our knowledge, no mention of 6-OOH derived from autoxidation has been made. Instead, observation of 6-OOH has long been considered a biomarker for $^1O_2$.[31]

Fully consistent with the results in solution, no 5α-OOH was observed in phospholipids. This was expected based upon the rapid rate of β-fragmentation of the 5α-peroxyl radical (k_β = 3.8 × 10^5 s^{-1} in chlorobenzene).[4] In our previous studies, we showed that good H-atom donors (e.g. pentamethylchromanol, a truncated analog of α-tocopherol (α-TOH), the most potent form of Vitamin E), can effectively compete with β-fragmentation, trapping the 5α-peroxyl radical and leading to the observation of 5α-OOH (**Figure 2A**). In stark contrast, no 5α-OOH was observed in liposomal autoxidations loaded with α-TOH (**Figure 2B**). We surmised this may be due to the dramatically lower H-atom transfer reactivity exhibited by α-TOH in phospholipids (k_{inh} = 4.7 × 10^3 $M^{-1}s^{-1}$ in egg PC compared to 3.6 × 10^6 $M^{-1}s^{-1}$ in PhCl).[32] This difference has been attributed to the strong H-bonding interaction between α-TOH and the phospholipid phosphate diester moiety, which renders it unreactive to H-atom transfer (**Figure 2C**).[33] Consistent with this view, when autoxidations were carried out on chol-loaded liposomes supplemented with the more reactive, less strongly H-bonding antioxidant, phenoxazine (k_{inh} = 2.3 × 10^5 in egg PC),[32] 5α-OOH was observed and its concentration increased with increasing phenoxazine concentration (**Figure 2D**). Thus, it is clear that the exact same mechanistic scheme operates in phospholipid bilayers as in solution. However, these results also demonstrate that the medium impacts the product distribution directly (chol 7-OOH/epoxide ratio) and indirectly (whether chol 5-OOH is formed or not). Moreover, it suggests that under physiological conditions, where α-TOH is the most reactive lipid-soluble antioxidant, 5α-OOH is unlikely to form by chol autoxidation.

Given the overwhelming abundance of 7-OOH in chol autoxidations in solution and phospholipid bilayers, we sought to investigate its propensity to undergo the Hock fragmentation reactions that are so facile for 5α-OOH and 6β-OOH (**Figure 3A**). Initial treatment of chol autoxidation samples with acid revealed that while the 7α-OOH was readily consumed, 7β-OOH persisted (**Figure 3B**). As such, treatment of chol autoxidation mixtures or authentic chol 7α-OOH[34] with ethanolic HCl in the presence of 2,4-dinitrophenylhydrazine (DNPH) to trap the carbonyls formed upon Hock fragmentation yielded very similar HPLC/UV-Vis/MS profiles. Representative results are shown in **Figure 4A**, in which four prominent product peaks are evident in the chromatogram. Interestingly, the first two peaks co-eluted with the hydrazones of secA and secB, respectively, and the latest eluting peak corresponded to the hydrazine derivative of 7-ketocholesterol, the dehydration/oxidation product of 7-OOH.

Although it was entirely reasonable that secA and secB would appear in chol autoxidation samples that had undergone derivatization (since they contain 6β-OOH which we have shown to yield secA and secB under these conditions)[4], we were surprised to see these products derived from derivatization of authentic chol 7α-OOH. To confirm the identity of these products – and ascertain the identity of the compound in the peak eluting at 21 minutes – we carried out a preparative-scale experiment and isolated the compounds for characterization. Doing so revealed that the compounds eluting at 12.5 and 19 minutes were neither the DNPH derivatives of secA and secB, respectively, nor the expected Hock fragmentation products of chol 7α-OOH shown in **Figure 3A.** Although we were unable to confidently assign a structure to the compound co-eluting with the hydrazone of secA, the compound co-eluting with the hydrazone of secB at 19 minutes, as well as that contained in the following peak at 21 minutes, were confidently assigned to the unprecedented A-ring cleavage products shown in **Figure 4B**.

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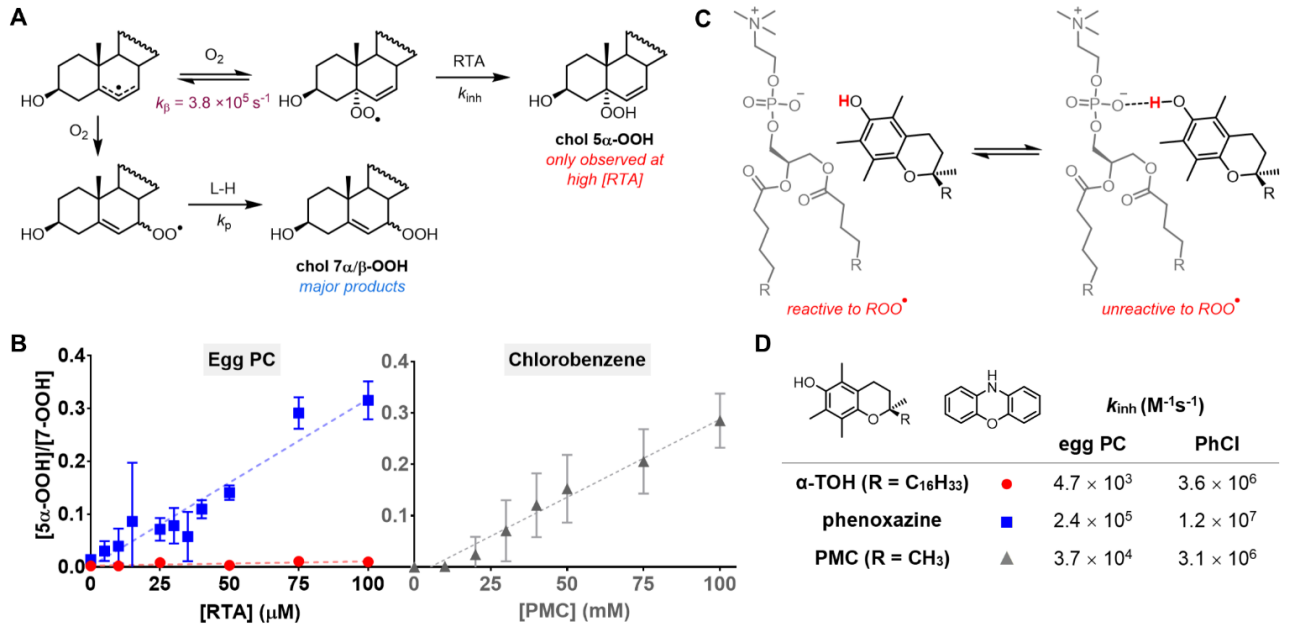

Figure 2. Influence of radical-trapping antioxidant (RTA) on the product distribution of chol autoxidation (**A**). The chol 5α-OOH to chol 7-OOH ratio in chol autoxidations as a function of both media and identity of RTA (**B**).[5] The potency of α-TOH is diminished in phospholipid bilayers relative to organic solution (e.g. PhCl) due to formation of strong H-bonds with the phosphate diester moiety (**C**), resulting in a significant decrease in reactivity to peroxyl radicals (k_{inh}) (**D**).[32,35,36]

These structural assignments were enabled by the results of parallel experiments carried out in the absence of DNPH (see Supporting Information). These revealed that the two primary reaction paths which are followed in the acid-mediated decomposition of chol 7α-OOH are distinct from that expected based on the reactivity of chol 5α-OOH and chol 6β-OOH (**Figure 3A**). Instead, the major product is not an aldehyde at all, but the α-hydroxyepoxides shown in **Scheme 2A** (or α-alkoxyepoxides when the reactions were carried out in alcohol). These products are, of course, 'invisible' in the above derivatizations because they lack carbonyls with which DNPH can react.[37] Unambiguous elucidation of the structures of the pair of epimers was made possible by single crystal x-ray crystallographic characterization of the β-epimer of both the α-hydroxy- and α-methoxyepoxides (see Supporting Information). The majority of the remaining mass balance consisted of the A-ring cleavage product also shown in **Scheme 2A**. To the best of our knowledge, this is the first occurrence of an A-ring cleavage product derived from chol oxidation. Thus, in addition to our exhaustive characterization efforts (see Supporting Information), we prepared 2,2,4,4-d_4-chol 7α-OOH and subjected it to the same reaction conditions to support the assignment of the exomethylene protons and the protons adjacent the aldehydic center as originating from C2 and C4, respectively (see Supporting Information). Furthermore, we subjected 7α-OOH in which the 3-OH was acetylated to the same conditions, to find that A-ring cleavage was significantly suppressed in favour of the α-hydroxyepoxides (see Supporting Information).

It should be noted that this is the most compelling evidence to date supporting the intermediacy of an epoxycarbenium ion intermediate in the mechanism of Hock fragmentation,[38] and is fully consistent with the fact that only chol 7α-OOH underwent fragmentation; the π orbital of the C5-C6 double bond and the σ_{O-}

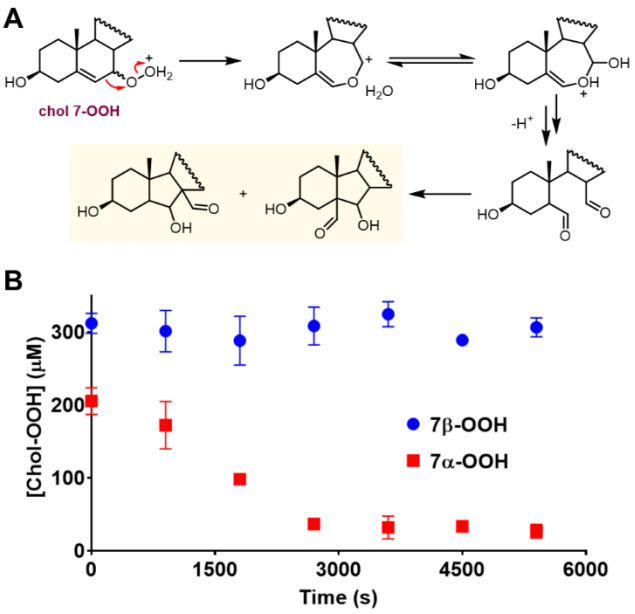

Figure 3. Expected products of acid-catalyzed (Hock) fragmentation of chol 7-OOH based on previous observations with chol 5α-OOH and chol 6β-OOH (**A**) and the comparative consumption of the 7α-OOH versus 7β-OOH isomer under acid-catalyzed (Hock) fragmentation conditions (**B**). A mixture of chol autoxidation products (obtained from MeOAMVN-initiated autoxidation of chol in chlorobenzene) was subjected to acidic conditions (0.01 M HCl in acetone). Consumption of chol 7α- and 7β-OOH was monitored by HPLC-APCI+-MS/MS.

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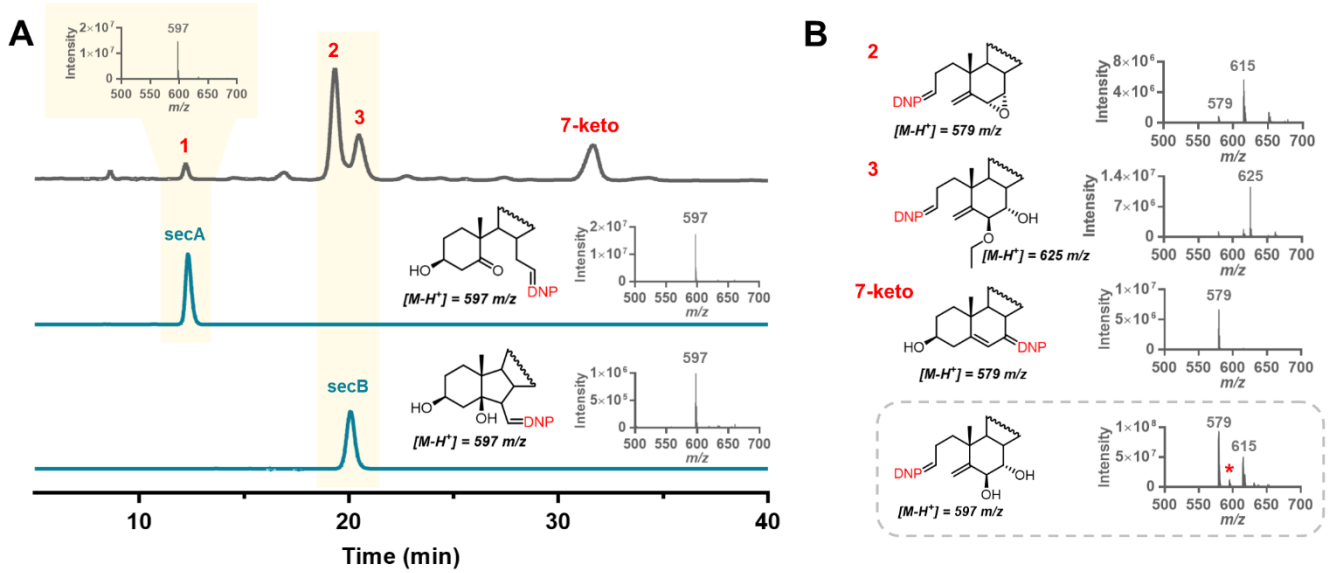
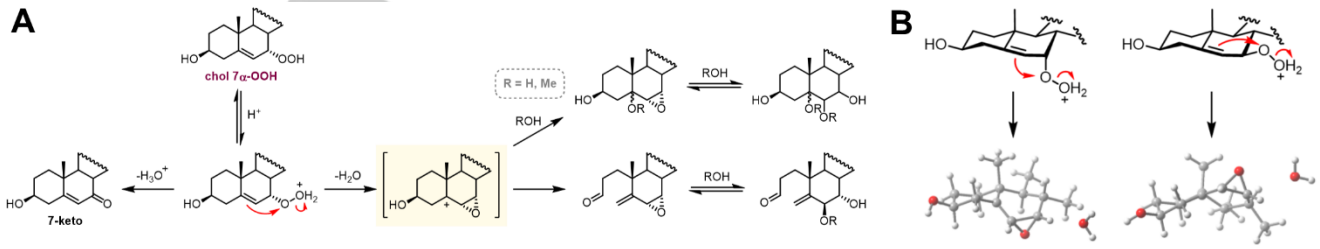


Figure 4. Chromatographic analysis of products observed when chol 7α-OOH is treated with DNPH (200 μM) in ethanolic HCl (0.1 M) for 1 hour (**A**, grey trace). Products were analysed by reverse phase HPLC-ESI$^-$-MS (4.6 × 150 mm Atlantis C18 HPLC column, flow rate = 1 mL/min, 75:20:5 ACN:MeOH:H_2O) with simultaneous UV detection (360 nm). Representative chromatograms for authentic DNPH hydrazone products derived from secA and B (**A**, teal traces) are shown along with the corresponding mass spectra. DNPH-derived hydrazones derived from chol 7α-OOH are indicated along with their corresponding mass spectra (**B**).

$_O$* are not properly aligned for fragmentation to occur in the 7β-OOH, as illustrated in **Scheme 2B**. The conformational change required to achieve the epoxycarbenium ion from protonated 7β-OOH forces both the A- and B-rings into sub-optimal half-chair conformations, as opposed to the more favourable A-ring chair and B-ring twist-boat arising from protonation of chol 7α-OOH.

DNPH derivatization of the isolated A-ring cleavage products yielded the corresponding hydrazones. The epoxide and diol coincided with the mixture of compounds eluting at 19 minutes and the monoethylated product coincided with the compound eluting at 21 minutes. Interconversion of the epoxide and diol was noted in both the DNPH derivatized and underivatized samples, leading to differing ratios as a function of derivatization conditions and time. Related to this point, it should be noted that the ionization for the molecular ion of the derivatized diol (*m/z* = 597) was not particularly strong by ESI; instead, *m/z* = 579 and 615 were prominent peaks, implying facile dehydration to the epoxide (or a ketone) and hydration of the imine in the source. Nevertheless, carrying out selected ion monitoring of derivatized samples of either chol autoxidations or simply chol 7α-OOH at *m/z* = 597 yielded chromatograms where these products and secB were essentially indistinguishable.

In conclusion, consistent with earlier reports,[39,40] the autoxidation of cholesterol in phospholipid bilayers yields primarily chol 7-OOH and chol 5,6-epoxide. Chol 6-OOHs and 4-OOHs are also observed, but to a significantly lower extent, and importantly, we find that the ratio of the major products is influenced by the level of unsaturation in the bilayer. Chol 5α-OOH is not observed – even in the presence of Vitamin E, which is capable of trapping chol 5α-OO• in solution, but not in phospholipid bilayers where it is less effective due to H-bonding to the phosphate diester moiety. Thus, it appears likely that chol 6β-OOH is the source of electrophilic secA and secB in tissue samples subjected to acid-catalyzed derivatization. Alternatively, given the predominance of chol 7-OOH and the foregoing results that demonstrate fragmentation of the 7α-OOH epimer under derivatization conditions yields distinct products that have similar chromatographic and spectroscopic properties to secA and secB, it is possible that they were misidentified.[41]



Scheme 2. Chol 7α-OOH undergoes Hock fragmentation via an α-epoxycarbenium ion intermediate to generate α-hydroxy/alkoxyepoxide and A-ring cleavage products (**A**). Chol 7β-OOH does not undergo Hock fragmentation due to the poor overlap between the π-MO of the C5-C6 double bond and the $\sigma_{O\text{-}O}$* MO of the protonated hydroperoxide moiety. Driving the protonated hydroperoxide along this reaction coordinate forces the A- and B-rings into half-chair conformations when starting from the β-epimer *vs* an A-ring chair and B-ring twist-boat in the case of the α-epimer, the former being 4 kcal/mol lower in energy than the latter (**B**).

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Keywords: cholesterol • Vitamin E • lipid peroxidation • secosterols • Hock fragmentation

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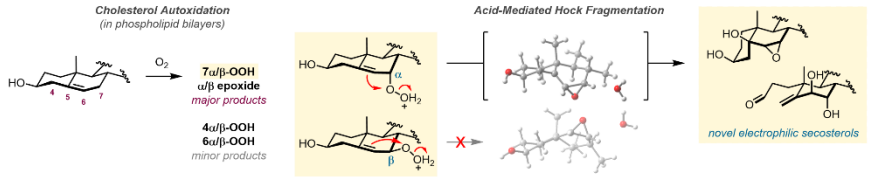

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