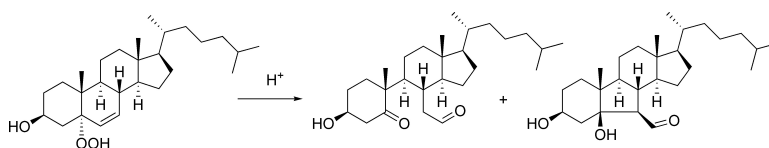


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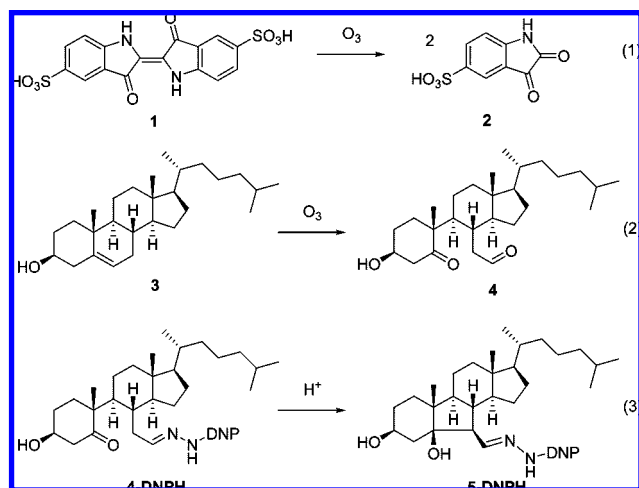
Hock Cleavage of Cholesterol 5 α -Hydroperoxide: An Ozone-Free Pathway to the Cholesterol Ozonolysis Products Identified in Arterial Plaque and Brain Tissue

Johan Brinkhorst, Susheel J. Nara, and Derek A. Pratt*

Department of Chemistry, Queen's University, 90 Bader Lane, Ontario, Canada K7L 3N6

Received June 2, 2008; E-mail: pratt@chem.queensu.ca

In a series of fascinating papers in the early 2000s, Wentworth and co-workers suggested that ozone is produced endogenously by antibody-catalyzed oxidation of water by singlet dioxygen.^{1,2} Two substrates believed to undergo reactions unique to ozone were used as probes to obtain evidence for ozone formation and its reactivity to biomolecules: the cleavage of the central bond in indigo carmine **1** to yield isatin sulfonic acid **2** with isotope incorporation from H₂¹⁸O into the lactam carbonyl of **2** (eq 1) and the cleavage of the $\Delta^{5,6}$ bond in cholesterol (**3**) to yield the 5,6-secosterol ketoaldehyde **4** (eq 2), respectively.



Since the identification of **4** (following derivatization to the 2,4-dinitrophenylhydrazones, **4-DNPH** and **5-DNPH**)³ in extracts of human arterial plaque,² the proatherogenic effects of **4** (and **5**) have been studied to assess their roles in the pathogenesis of cardiovascular disease.⁴ This work has been complemented by the identification of **4** (again, following derivatization to **4-DNPH** and **5-DNPH**) in human brain tissue,⁵ and associated investigations of the role of **4** (and **5**) in the pathogenesis of Alzheimer's⁶ and Parkinson's⁷ disease.

Many have expressed their reservations about the conclusions drawn in the foregoing works.⁸ Pessimism has been fueled further by Winterbourne and co-workers,⁹ who demonstrated that the conversion of **1** to **2** with isotope incorporation from H₂¹⁸O into the lactam carbonyl of **2** is not unique to ozone, but can also be carried out by superoxide (hydroperoxyl radical). While this is an important control experiment for evaluating the validity of the suggestion that ozone is produced endogenously, it does not provide an alternative explanation for the appearance of the 2,4-dinitrophenylhydrazones derived from **4**, the known cholesterol ozonolysis product,¹⁰ in derivatized extracts of arterial plaque and brain tissue.

While we were also fascinated by the suggestion that ozone is produced endogenously, we wondered whether a more simple explanation for the formation of **4** (and **5**) could exist. Carbonyl

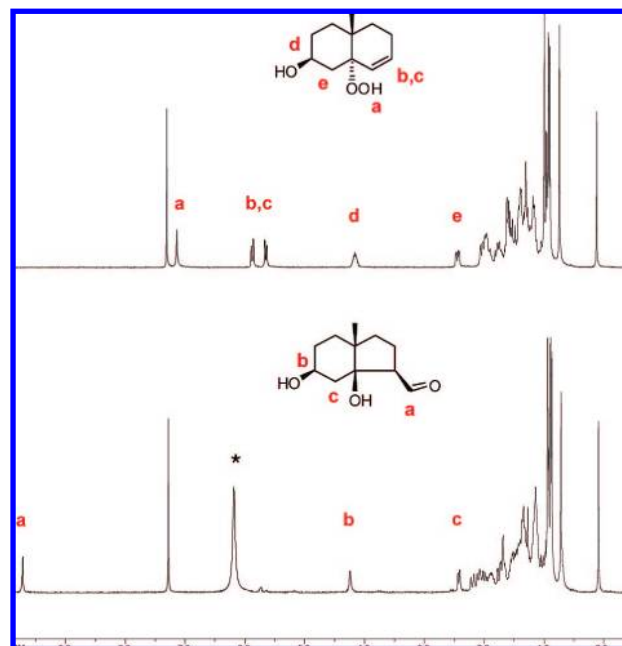
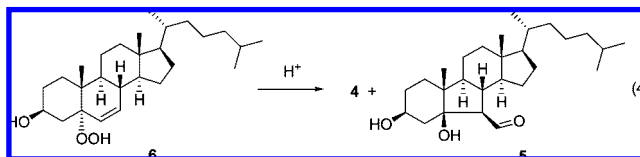


Figure 1. ¹H NMR spectra (400 MHz; CDCl₃) of **6** (top) and **5** (bottom) formed immediately following addition of 1 μ L of trifluoroacetic acid (* = H₂O). Relevant protons are indicated in structures of **6** and **5** (substituents on A,B rings are omitted for clarity).

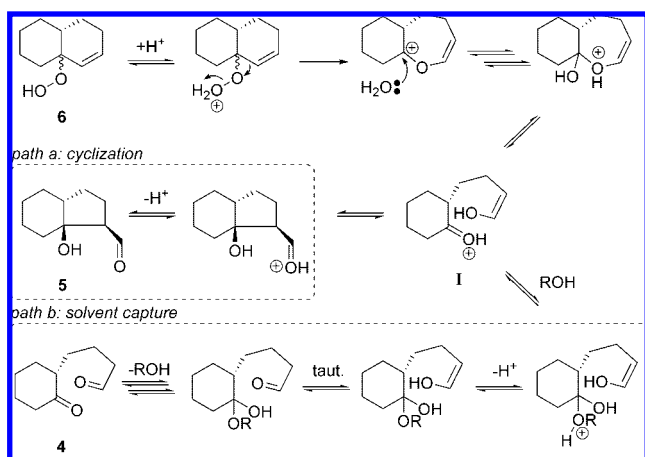
compounds are known to arise from decomposition of lipid hydroperoxides derived from ³O₂- and/or ¹O₂-mediated lipid peroxidation,¹¹ and we wondered whether cholesterol hydroperoxides could serve as precursors to **4** (and **5**). Here, we show that cholesterol 5 α -hydroperoxide (**6**), the major product of ¹O₂-oxidation of cholesterol,¹² readily undergoes acid-catalyzed (Hock) cleavage¹³ of the C5–C6 bond.



We prepared **6** by photosensitized (singlet) oxidation of cholesterol¹⁴ and subjected it to a variety of protic acids (e.g., HCl, *p*-TsOH, TFA) in different solvents (e.g., EtOH, THF, CHCl₃), under all of which Hock cleavage was found to proceed quite readily as evidenced by ¹H NMR (e.g., Figure 1), ¹³C NMR, and HRMS. In fact, conversion could be detected in NMR samples of **6**, presumably due to adventitious DCl in the CDCl₃.

The treatment of **6** with acid in CHCl₃ and THF led to the quantitative formation of **5** (as shown in Figure 1) which can be understood by the precedented¹³ mechanism in Scheme 1 (path a).

Scheme 1. Proposed mechanism of Hock cleavage of cholesterol 5 α -OOH in CHCl₃ and THF (path a from I) and EtOH (where path b from I competes). Substituents on A, B rings are omitted for clarity.



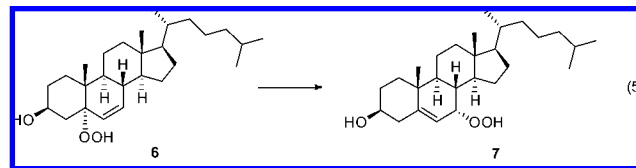
Reactions in EtOH, however, led to mixtures of **5** and **4** in a ratio of ~5:1. The appearance of **4** in EtOH can be explained on the basis of the competing capture of oxocarbenium ion I by EtOH to yield the hemiketal of **4** (path b, Scheme 1), which prevents cyclization of I to give **5** as in CHCl₃ and THF.¹⁵ Conversion of **6** to **5** and **4** was followed in ethanolic solutions containing HCl at concentrations that spanned 4 orders of magnitude (0.1 M – 0.0001 M) to confirm the dependence of the rate of conversion on acid concentration as well as to confirm that complete conversion was observed at substoichiometric amounts of acid, supporting acid-catalysis in the reaction (see Supporting Information).

Interestingly, upon DNPH derivatization of both arterial² and brain⁵ tissue extracts, Wentworth and co-workers found **5-DNPH** to be the major product under conditions that they indicated resulted in ~20% aldolization of **4-DNPH** to **5-DNPH** (eq 3). If ozonolysis was the route by which they had been formed, **4-DNPH** should have been the major product. It was suggested that amino acids or other entities containing 1° or 2° amino groups were responsible for catalyzing the aldolization of **4** to **5** in vivo, leading to the greater than expected amount of **5-DNPH** in the derivatized samples. In light of our results, the predominance of **5-DNPH** in the chromatograms of the derivatized tissue extracts tempts us to suggest that endogenous ozonolysis of cholesterol does not occur to yield **4** and **5**, but instead that Hock fragmentation of **6**—either in vivo or under the acidic conditions of the derivatization—is responsible for the predominance of **5**.¹⁶

When **6** was subjected to typical derivatization conditions² (0.2 mM DNPH, 0.1 M HCl in EtOH, stirred for 2 h at room temperature under argon), the same dinitrophenylhydrazones were formed as those obtained following derivatization of the cholesterol ozonolysis product **4**, but in ratios reflective of Hock fragmentations, where **5** predominates. However, under these conditions, the observed ratio of **5-DNPH**:**4-DNPH** is much larger than the ratio of ~5:1 observed in the absence of DNPH (see Supporting Information).¹⁷ This suggests that if Hock fragmentation of **6** is responsible for the appearance of **4** and **5** in the tissue extracts, it occurs in vivo and not upon derivatization.

The likelihood that Hock fragmentation occurs in vivo is reinforced by the expected transient nature of cholesterol 5 α -OOH, which is known to rearrange readily to the more thermodynamically stable cholesterol 7 α -OOH in solution (**7**, eq 4).¹⁴ While this rearrangement, which is believed to occur via an intermediate allylperoxyl radical, is slowed somewhat in H-bond accepting

solvents,¹⁴ it is difficult to envision **6** having a sufficient lifetime to survive surgical tissue removal, homogenization, extraction, and concentration all under an aerobic atmosphere.



In summary, we have shown that the cholesterol-derived keto-aldehyde **4** and its aldolization product **5** can arise from Hock cleavage of the cholesterol 5 α -OOH **6**, which has been shown to occur due to singlet oxygen oxidation of cholesterol (or some hitherto unknown mechanism) in vivo.¹⁸ While our findings do not unequivocally dismiss the possibility that ozone is produced endogenously, or that ozone is responsible for the cleavage of the $\Delta^{5,6}$ bond in cholesterol in vivo, they do clearly demonstrate that **4** and **5** are cholesterol oxidation products not unique to ozonolysis.

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Supporting Information Available: Complete refs 1a, 2, and 5; experimental procedures for Hock cleavage reactions, derivatizations and analyses. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (16) It should be pointed out that Arnold and Staples have shown that allylic hydroperoxides readily undergo Hock cleavage under standard DNPH derivatization conditions, see: Arnold, A. R.; Staples, R. *Polymer* **1992**, *33*, 1739–1741.
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