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Published on Web 08/23/2008

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

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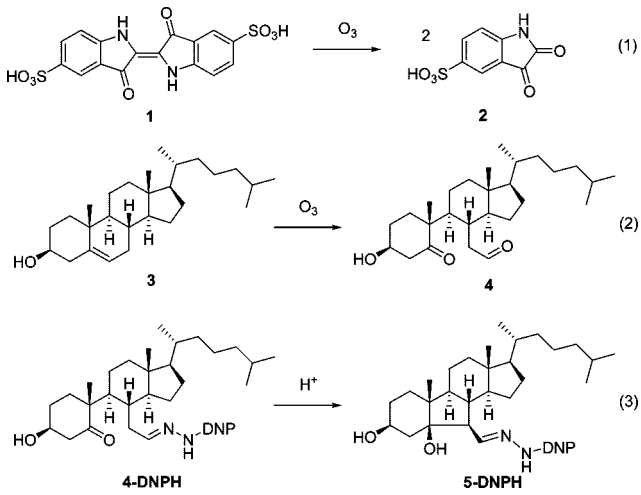
In a series of fascinating papers in the early 2000s, Wentworth and co-workers suggested that ozone is produced endogenously by antibody-catalyzed oxidation of water by singlet dioxygen.[1,2] Two substrates believed to undergo reactions unique to ozone were used as probes to obtain evidence for ozone 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 $H_2{}^{18}O$ 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's[6] and Parkinson's[7] 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 $H_2{}^{18}O$ 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

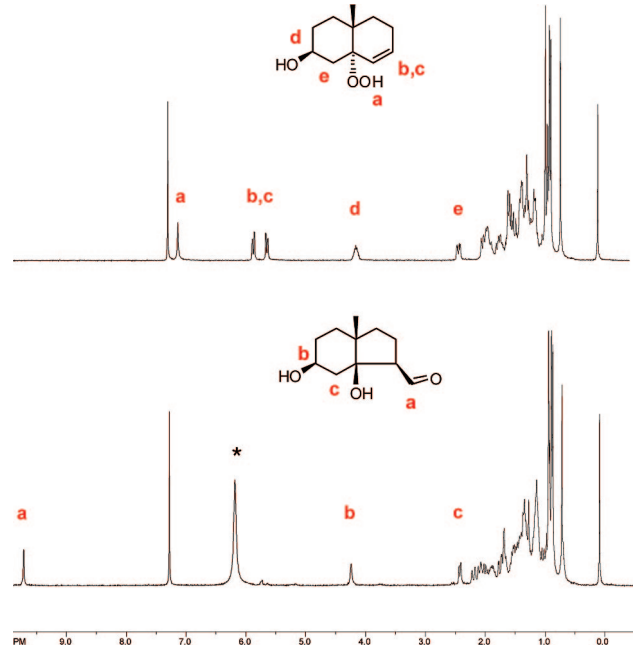
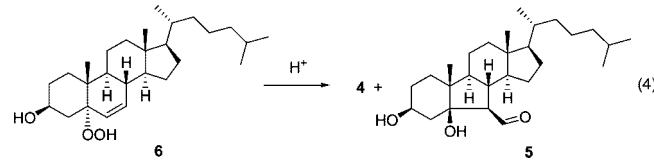


Figure 1. ^{1}H NMR spectra (400 MHz; $CDCl_3$) of **6** (top) and **5** (bottom) formed immediately following addition of 1 uL of trifluoroacetic acid (∗ = H_2O). Relevant protons are indicated in structures of **6** and **5** (substituents on A,B rings are omitted for clarity).

compounds are known to arise from decomposition of lipid hydroperoxides derived from $^3O_2$- and/or $^1O_2$-mediated lipid peroxidation,[11] and we wondered whether cholesterol hydroperoxides could serve as precursors to **4** (and **5**). Here, we show that cholesterol 5α-hydroperoxide (**6**), the major product of $^1O_2$-oxidation of cholesterol,[12] readily undergoes acid-catalyzed (Hock) cleavage[13] of the C5−C6 bond.

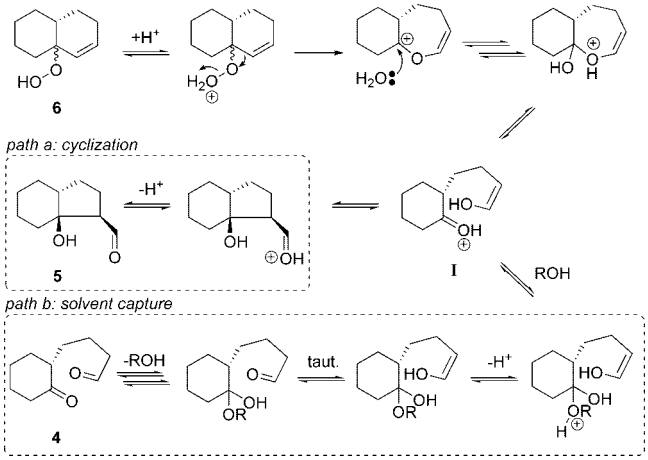


We prepared **6** by photosensitized (singlet) oxidation of cholesterol[14] and subjected it to a variety of protic acids (e.g., HCl, *p*-TsOH, TFA) in different solvents (e.g., EtOH, THF, $CHCl_3$), under all of which Hock cleavage was found to proceed quite readily as evidenced by ^{1}H NMR (e.g., Figure 1), ^{13}C NMR, and HRMS. In fact, conversion could be detected in NMR samples of **6**, presumably due to adventitious DCl in the $CDCl_3$.

The treatment of **6** with acid in $CHCl_3$ and THF led to the quantitative formation of **5** (as shown in Figure 1) which can be understood by the precedented[13] mechanism in Scheme 1 (path a).

10.1021/ja804162d CCC: $40.75

Scheme 1. Proposed mechanism of Hock cleavage of cholesterol 5α-OOH in $CHCl_3$ and THF (path a from I) and EtOH (where path b from I competes). Substituents on A, B rings are omitted for clarity.

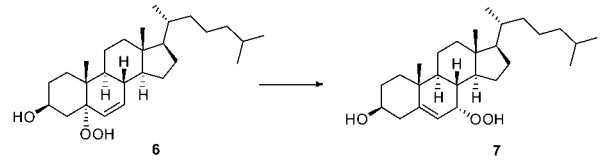


Reactions in EtOH, however, led to mixtures of **5** and **4** in a ratio of ~5:1. The appearance of **4** in EtOH can be explained on the basis of the competing capture of oxocarbenium ion I by EtOH to yield the hemiketal of **4** (path b, Scheme 1), which prevents cyclization of I to give **5** as in $CHCl_3$ 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 arterial[2] and brain[5] 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 the 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 conditions[2] (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 5α-OOH, which is known to rearrange readily to the more thermodynamically stable cholesterol 7α-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.



(5)

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 5α-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.

Acknowledgment. We are grateful for the support of the Natural Sciences and Engineering Research Council of Canada and the Ontario Ministry of Innovation. D.A.P. acknowledges the support of the Canada Research Chairs program.

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.

References

(1) (a) Wentworth, P., Jr; et al. *Science* **2002**, *298*, 2195–2199. (b) Wentworth, P., Jr.; Wentworth, A. D.; Zhu, X.; Wilson, I. A.; Janda, K. D.; Eschenmoser, A.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1490–1493. (c) Babior, B. M.; Takeuchi, C.; Ruedi, J.; Gutirrez, A.; Wentworth, P., Jr *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3031–3034.
(2) Wentworth, P., Jr.; et al. *Science* **2003**, *302*, 1053–1056.
(3) Wang, K.; Bermúdez, E.; Pryor, W. A. *Steroids* **1993**, *58*, 225–229.
(4) Takeuchi, C.; Galve, R.; Nieva, J.; Witter, D. P.; Wentworth, A. D.; Troseth, R. P.; Lerner, R. A.; Wentworth, P. *Biochemistry* **2006**, *45*, 7162–7170.
(5) Zhang, Q.; et al. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4752–4757.
(6) Bieschke, J.; Zhang, Q.; Powers, E. T.; Lerner, R. A.; Kelly, J. W. *Biochemistry* **2005**, *44*, 4977–4983.
(7) (a) Bosco, D. A.; Fowler, D. M.; Zhang, Q. H.; Nieva, J.; Powers, E. T.; Wentworth, P.; Lerner, R. A.; Kelly, J. W. *Nat. Chem. Biol.* **2006**, *2*, 249–253. (b) Stewart, C. R.; Wilson, L. M.; Zhang, Q.; Pham, C. L. L.; Waddington, L. J.; Staples, M. K.; Stapleton, D.; Kelly, J. W.; Howlett, G. J. *Biochemistry* **2007**, *46*, 5552–5561.
(8) Most notably: Smith, L. L. *Free Radical Biol. Med.* **2004**, *37*, 318–324. Sies, H. *Angew. Chem. Intl. Ed.* **2004**, *43*, 3514–3515.
(9) Kettle, A. J.; Clark, B. M.; Winterbourne, C. C. *J. Biol. Chem.* **2004**, *279*, 18521–18525.
(10) Smith, L. L. *Chem. Phys. Lipids* **1987**, *44*, 87–125.
(11) Esterbauer, H.; Schaur, R. J.; Zollner, H. *Free Radical Biol. Med.* **1991**, *11*, 81–128.
(12) Schenck, G. O.; Neumüller, O.-A.; Eisfeld, W. *Justus Liebigs Ann. Chem.* **1958**, *618*, 202.
(13) Frimer, A. A. *Chem. Rev.* **1979**, *79*, 359–387.
(14) Beckwith, A. L. J.; Davies, A. G.; Davison, I. G. E.; Maccoll, A.; Mruzek, M. H. *J. Chem. Soc., Perkin Trans. II* **1989**, 815–824.
(15) If the first and second oxocarbenium ion are trapped by EtOH this would lead to the ketal, which would also prevent cyclization. Regardless, it should be pointed out that **4** and **5** exist as their diethyl acetals under these conditions, which prevents cyclization of **4** to **5**.
(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.
(17) The change in product distribution in the presence of DNPH may be the result of hydrazone formation on the (hemi)ketal form of **4** generated directly from the Hock fragmentation; the enamine form would readily cyclize upon formation of any oxocarbenium intermediate upon interconversion of the (hemi)ketal and ketone.
(18) Yamazaki, S.; Ozawa, N.; Hiratsuka, A.; Watabe, T. *Free Radical Biol. Med.* **1999**, *27*, 301–308.

JA804162D