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Reactivities and Products of Free Radical Oxidation of Cholestadienols

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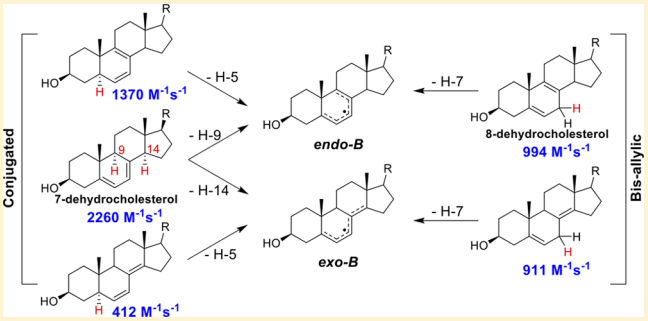
Supporting Information

ABSTRACT: 7-Dehydrocholesterol (7-DHC) is the most oxidizable lipid molecule reported to date, with a propagation rate constant for free radical peroxidation that is 200 times that of cholesterol. To better understand the high reactivity of 7-DHC and elucidate the reaction mechanism, we synthesized conjugated and skipped nonconjugated cholestadienols that would give one of the two putative pentadienyl-radical intermediates formed in 7-DHC peroxidation. The additional dienols include 6,8(9)-dienol, 5,8(14)-dienol, 6,8(14)-dienol, and the biologically important 8-dehydrocholesterol (8-DHC; 5,8(9)-dienol). We found that all of the dienols are significantly (at least 40 times) more reactive than cholesterol. Among them, dienols leading to the formation of the pentadienyl radical in ring B (termed ***endo-B***) of the sterol are more reactive than those leading to the pentadienyl radical spanning rings B and C (termed ***exo-B***). By comparing the oxysterol profile formed from 7-DHC and those formed from 8-DHC and 5,8(14)-dienol, products formed from abstraction of the hydrogen atoms at C-9 and C-14 (*H-9* or *H-14 mechanism*) were clearly differentiated. When the oxidation was carried out in the presence of the good hydrogen atom donor α-tocopherol, the oxysterol profile of 7-DHC peroxidation differed distinctly from the profile observed in the absence of the antioxidant and resembles more closely the profile observed in biological systems. This study suggests that oxidative stress and the accumulation of oxysterols should be considered as two key factors in cholesterol biosynthesis or metabolism disorders, where dienyl sterol intermediates are accumulated.



INTRODUCTION

Lipid peroxidation plays important roles in the pathophysiology of various human diseases, such as atherosclerosis,[1] asthma,[2] Alzheimer's disease,[3] and nonalcoholic fatty liver disease.[4] Increasing research effort has been devoted to the oxidation products of lipids as they not only serve as important biomarkers for disease[5] but also exert a variety of biological activities.[6−8] The rate-determining step of free radical-mediated lipid peroxidation is the hydrogen atom transfer from a lipid substrate to a propagating peroxyl radical.[9] Previously we determined the propagation rate constants (k_p) of free radical peroxidation of different lipids including polyunsaturated fatty acids, cholesterol, and 7-dehydrocholesterol (7-DHC) in solution and in liposomes.[10] The k_p for 7-DHC was exceptionally large at 2260 M^{-1} s^{-1} in solution, a value that is 200 times that for cholesterol,[10] and 20 times that for the simple 1,3-cyclohexadiene when normalized to the number of reactive hydrogen atoms.[11]

Peroxidation of 7-DHC leads to the formation of over a dozen oxysterols,[12] and although a detailed reaction mechanism has been proposed, the origin of some of the oxysterols is still ambiguous.[12] Briefly, the reaction of 7-DHC involves hydrogen atom transfer from C-9 or C-14 of the sterol to a propagating peroxyl radical (*H-9* or *H-14 mechanism*), leading to the formation of two different pentadienyl radical intermediates (C5−C6−C7−C8−C9 and C5−C6−C7−C8−C14) (Scheme 1). For convenience, we designate the C5−C6−C7−C8−C9 radical as ***endo-B*** since it is endocyclic in ring B of the sterol and the C5−C6−C7−C8−C14 radical as ***exo-B*** since one arm of this radical is exocyclic to ring B. The formation of both ***endo-B*** and ***exo-B*** is followed by precedented free radical transformations such as oxygen addition, 5-*exo* cyclization, and intramolecular radical substitution (S_Hi)[9] that lead to the oxysterol products isolated and identified.[12]

To further understand the high reactivity of 7-DHC and better define the reaction mechanisms, we synthesized conjugated and methylene-skipped nonconjugated cholesta-dien-3β-ols (abbreviated as "cholestadienols") that would readily give either the ***endo-B*** or ***exo-B*** pentadienyl radicals upon abstraction of the reactive hydrogen atom(s) (Scheme 2). Here we report the following: (i) the structure−reactivity studies on the free radical oxidation of different cholestadienols that include 7-DHC (5,7-dienol), 8-DHC (5,8(9)-dienol), 6,8(9)-dienol, 5,8(14)-dienol, and 6,8(14)-dienol; (ii) analysis of oxidation products of these dienols and elucidation of the

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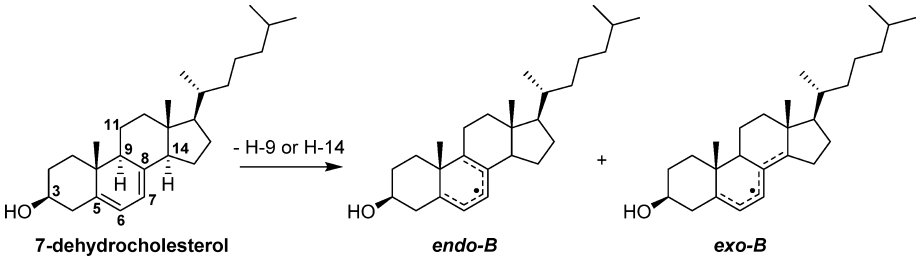
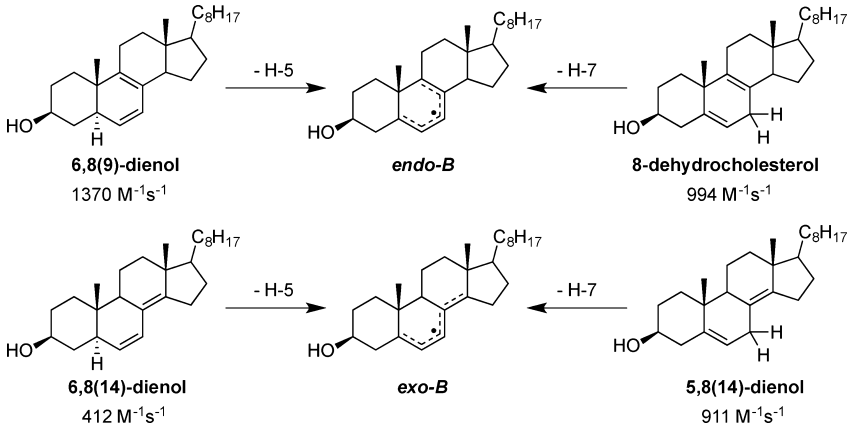
Scheme 1. Formation of Two Pentadienyl Radicals after Losing H-9 or H-14 in the Initial Step of Free Radical Oxidation of 7-Dehydrocholesterol



Scheme 2. Cholestadienols Synthesized in This Study That Serve As Precursors to *endo-B* or *exo-B* Pentadienyl Radicals[a]



[a]Rate constants for hydrogen atom transfer from the dienols to a linoleate peroxyl radical were shown (see Table 1).

reaction mechanism; (iii) the effect of an antioxidant, α-tocopherol, on the product distribution of the free radical oxidation of the dienols.

RESULTS

Structure–Reactivity Studies of Free Radical Oxidation of Cholestadienols. Hydrogen atoms on bis-allylic carbon centers are normally more reactive than monoallylic hydrogen atoms toward reaction with peroxyl radicals because of the lower bond dissociation enthalpy of the reactive C–H bond.[13,14] Thus linoleate (31 M^{-1} s^{-1} per H-atom) is much more prone to give up a hydrogen atom to a peroxyl radical than oleate (0.22 M^{-1} s^{-1} per H-atom).[11,15] To compare the reactivities of monoallylic and bis-allylic hydrogen atoms in the ring system of cholesterol, we prepared a series of cholestadienols with double bonds spanning ring B and/or C as shown in Scheme 2 and measured their propagation rate constants using the linoleate peroxyl radical clock.[16] 8-DHC, 5,8(14)-dienol, and 6,8(14)-dienol were synthesized as previously reported.[17−19] The 6,8(9)-dienol was synthesized from TBS-protected 7-DHC *via* selective hydroboration/oxidation at the C5 double bond,[20] mesylation/elimination of the resulting alcohol at C6, and deprotection (Scheme S1).

In the "slow" linoleate peroxyl radical clock, oxidation of methyl linoleate gave *trans,cis*-hydroxyoctadecadienoates (HODEs) and *trans,trans*-HODEs as the primary products and the ratio of *trans,cis*-/*trans,trans*-HODEs is proportional to the hydrogen atom transfer rate constant (k_H) for a given hydrogen atom donor (R–H) times the concentration of that donor ([R–H]).[16] Thus, under pseudo-first-order reaction conditions, by varying the concentrations of R–H while keeping the concentration of linoleate constant, k_p can be solved from the plot of *trans,cis*-/*trans,trans*-HODEs vs [R–H]. The propagation rate constants of the cholestadienols are summarized in Table 1 and Scheme 2, and the clocking plots are shown in Figure S1. As seen in the table, the reactivities of the cholestadienols decrease in the order of 7-DHC (5,7-dienol), 6,8(9)-dienol, 8-DHC (5,8-dienol), 5,8(14)-dienol, and 6,8(14)-dienol.

Table 1. Free Radical Oxidation Propagation Rate Constants of Different Unsaturated Sterols Determined by the Linoleate Peroxyl Radical Clock[a]

sterols (R–H)	k_p (M^{-1} s^{-1})
7-dehydrocholesterol (5,7-dienol)	2260[b]
6,8(9)-dienol	1370 ± 40
8-dehydrocholesterol (5,8-dienol)	994 ± 33
5,8(14)-dienol	911 ± 43
6,8(14)-dienol	412 ± 22
cholesterol	11[b]

[a]At 37 °C in benzene or chlorobenzene; errors are 2σ. [b]From ref 10.

Profiling the Oxysterols Formed from Free Radical Oxidation of the Cholestadienols. In order to unequivocally elucidate the *H-9* and *H-14-mechanisms* of 7-DHC peroxidation and to identify the reactive hydrogen atoms in the cholestadienols under study, peroxidation products of these dienols were analyzed by HPLC-MS. Free radical oxidation of the sterol dienols (0.01–0.08 M) in benzene at 37 °C was set up as described previously using (2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeOAMVN) as the radical initiator.[10,12] The product mixture was treated with PPh_3 to reduce hydroperoxides, and this product mixture was analyzed by

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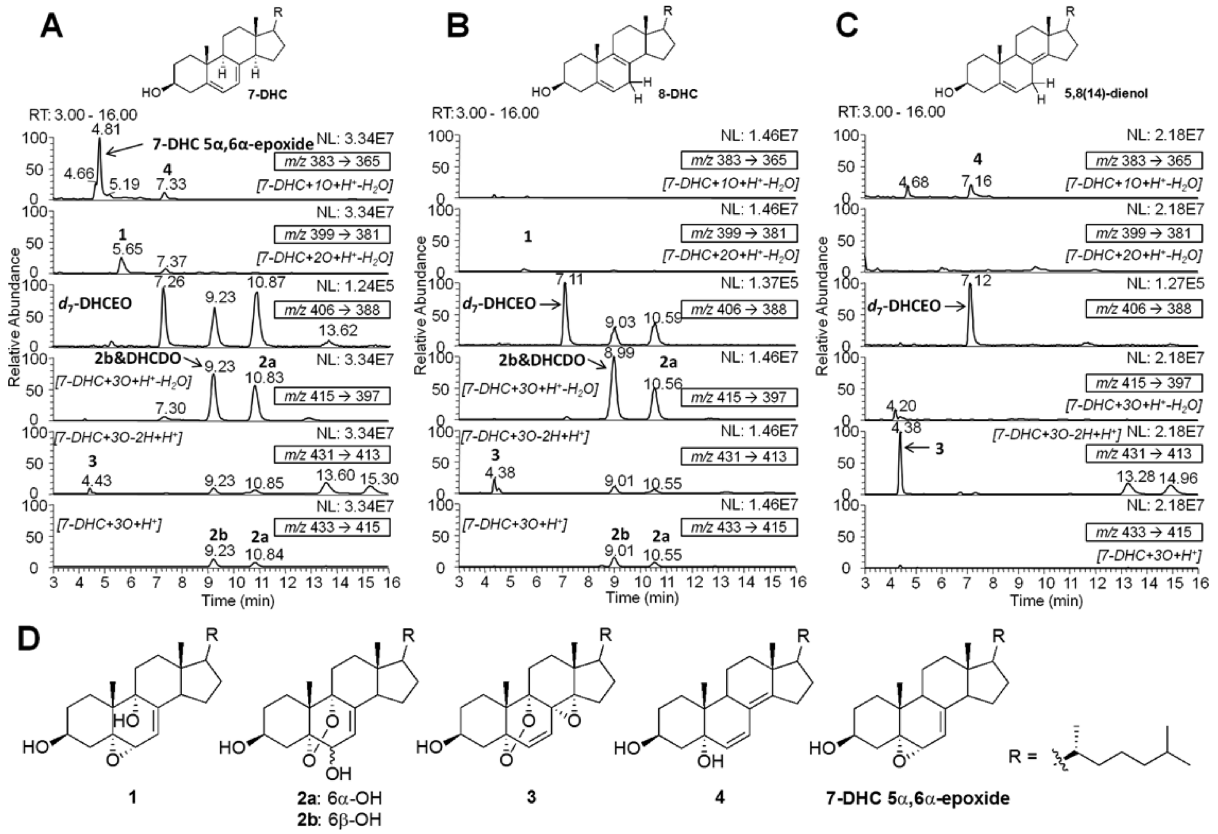

Figure 1. Normal phase HPLC-APCI-MS-MS analysis of oxysterols formed from free radical oxidation of 7-DHC (A), 8-DHC (B), and cholesta-5,8(14)-dienol (C). (D) Major oxysterols identified. D_7-3,5-dihydroxycholest-7-en-6-one (DHCEO) was added to each sample as a standard for proper comparison of the retention times between different chromatograms. The parent ion of each chromatogram and the MS/MS transition were marked in the corresponding MS panel.

normal phase HPLC-MS. Typical chromatograms of oxidation product mixtures of individual dienols are shown in Figures 1A–C and S2, and the structures of major oxysterols identified are shown in Figure 1D. Based on the proposed reaction mechanisms, oxidation of 8-DHC should exclusively give the products formed from the *H-9 mechanism* (***endo-B***) while oxidation of the 5,8(14)-dienol should only give products from the *H-14 mechanism* (***exo-B***) after initial hydrogen abstraction at the bis-allylic positions. Comparison of the oxysterol profile from the peroxidation of 7-DHC with the profiles from these two unconjugated dienols shows conclusively that compounds **1**, **2a**, and **2b** are formed from the *H-9 mechanism*, while compound **4** is formed from the *H-14 mechanism* and compound **3** can be formed from either mechanism with the *H-14 mechanism* being the primary route. Under the chromatographic conditions used here, compound **2b** coelutes with a minor product of 7-DHC peroxidation, 3*β*,5*α*-dihydroxycholesta-7,9(11)-dien-6-one (DHCDO; see Figure 2), as shown previously.[12] However, these two compounds can be differentiated by observing the SRM transition 433→415, which DHCDO lacks (see also Figure S4). Although 7-DHC-5*α*,6*α*-epoxide can only form from 7-DHC *via* peroxyl radical addition, we note that there is an oxysterol derived from the *H-14 mechanism* (4.68-min peak in Figure 1C) that might have a small contribution to the peak intensity of the epoxide (4.81 min) in Figure 1A.

Profiling Oxysterols Formed from the Free Radical Oxidation of Cholestadienols in the Presence of *α*-Tocopherol. Human tissues and cells are rich in antioxidants, and *α*-tocopherol is the major hydrophobic chain-breaking phenolic antioxidant present *in vivo*.[21] Studies of lipid oxidation in the presence of *α*-tocopherol have biological relevance, and we therefore carried out the free radical oxidation of the series of cholestadienols, including 7-DHC, in the presence of *α*-tocopherol (1.0 M). Under these conditions (*i.e.*, high concentration of *α*-tocopherol but low rate of initiation), the tocopheryl radical becomes the main chain-propagating radical even though at a much slower rate ($0.10\ M^{-1}\ s^{-1}$),[22] *i.e.*, tocopherol-mediated peroxidation.[23,24] Typical HPLC-MS chromatograms of product mixtures and the oxysterol products identified in these oxidations are shown in Figures 2 and S3.

Oxidation of 7-DHC gave three partially overlapping peaks with the mass of the dehydration ion of [7-DHC + 1O + H^+] and these products are the only products formed from the oxidation of 5,8(14)-dienol in the presence of *α*-tocopherol, suggesting that these three products were derived from ***exo-B*** and the *H-14 mechanism*. Epoxidation of 7-DHC was not observed under these conditions, likely because of rapid trapping of peroxyl radicals by *α*-tocopherol before addition to a C═C bond can occur.

Three of the 7-DHC products are identical to the products of peroxidation of 8-DHC, suggesting that they are formed from ***endo-B*** and the *H-9 mechanism*. Two products observed in the SRM panel of 415 → 397 were identified to be 3*β*,5*α*,9*α*-trihydroxycholest-7-en-6-one (THCEO) and DHCDO (Figure 2), by comparing retention times and SRM transition characteristics with standards isolated from free radical oxidation of 7-DHC (Figures S4 and S5).[12] Note that

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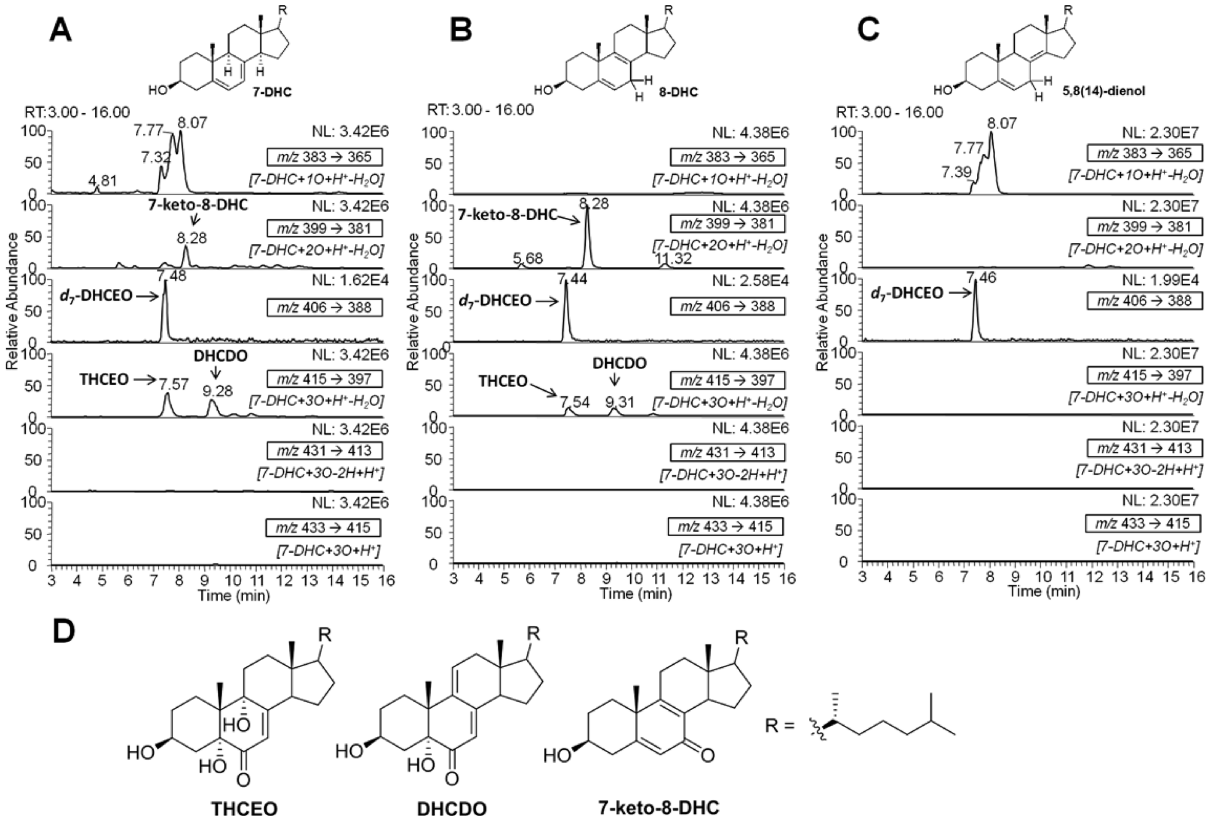
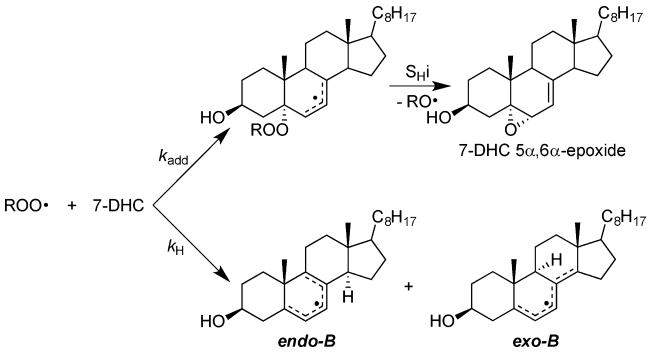


Figure 2. Normal phase HPLC-APCI-MS-MS analysis of oxysterols formed from free radical oxidation of 7-DHC (A), 8-DHC (B), and cholesta-5,8(14)-dienol (C) in the presence of 1.0 M α-tocopherol. (D) Structures of identified major oxysterols. D_7-3,5-dihydroxycholest-7-en-6-one (DHCEO) was added to each sample as a standard for proper comparison of the retention times between different chromatograms. The parent ion of each chromatogram and the MS/MS transition were marked in the corresponding MS panel.

DHCDO does not display the SRM transition 433→415. These two compounds were minor products of the free radical oxidation of 7-DHC in the absence of α-tocopherol while THCEO was identified as one of the major 7-DHC-derived oxysterols observed in brain tissue of *Dhcr7*-KO mouse, a genetic animal model for Smith−Lemli−Opitz syndrome (SLOS).[25,26] The product eluting in the peak in the SRM panel of 399 → 381 was identified to be 7-oxo-5,8-dien-3β-ol (7-keto-8-DHC) by comparing its SRM transition characteristics and retention time with those of the known compound that was synthesized from dehydration of 7-hydroperoxy-5,8-dien-3β-ol,[25] which was prepared in turn by photooxidation of 7-DHC.[27] The mechanism of its formation is discussed in the following section.

Scheme 3. Competition between Peroxyl Radical Addition to 7-DHC (k_{add}) and Hydrogen Atom Transfer (k_H)



DISCUSSION

In the absence of α-tocopherol, product analysis by HPLC-MS suggests that 7-DHC undergoes free radical oxidation *via* three pathways designated *H-9* or *H-14 abstraction* and peroxyl radical addition. The formation of the 7-DHC-5α,6α-epoxide suggests that the addition of a propagating peroxyl radical to 7-DHC can compete with abstraction of a hydrogen atom by the same radical (Figure 1; Scheme 3). Peroxyl radical addition at C-5 of 7-DHC eventually leads *via* an S_Hi process to the formation of the epoxide and an alkoxyl radical that can propagate the radical chain reaction. In the presence of α-tocopherol (1.0 M), however, the addition pathway is completely suppressed due to the competition from hydrogen atom transfer from tocopherol to the peroxyl radical (k_H(α-tocopherol) = 3.5 × 10^6 M^{-1} s^{-1}) (Figure 2).[16,28,29]

For each pentadienyl radical, molecular oxygen can add to three positions that have high spin density, C-5, C-7, or C-9 for ***endo-B*** and C-5, C-7, or C-14 for ***exo-B*** (Figure 3). Thus, in the *H-9 mechanism*, three peroxyl radical regioisomers could form as shown in Scheme 4. In our previous studies of 7-DHC peroxidation, products derived from the bis-allylic peroxyl radical were not observed likely due to the rapid β-fragmentation of this radical to give back its precursor pentadienyl radical.[12,16] The rate constant of such a fragmentation in an acyclic system (linoleic acid) was found to be 2.6 × 10^6 s^{-1}.[16] In the presence of α-tocopherol, however, a product derived from the bis-allylic peroxyl radical, 7-keto-8-

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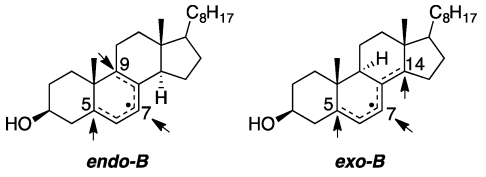


Figure 3. Potential sites in the pentadienyl radicals ***endo-B*** and ***exo-B*** for addition of molecular oxygen.

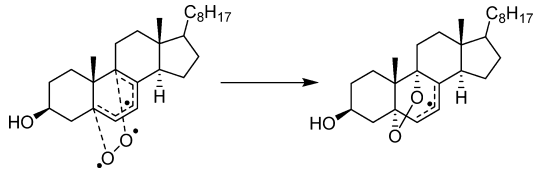


Figure 4. Proposed concerted mechanism for the addition of oxygen to ***endo-B***.

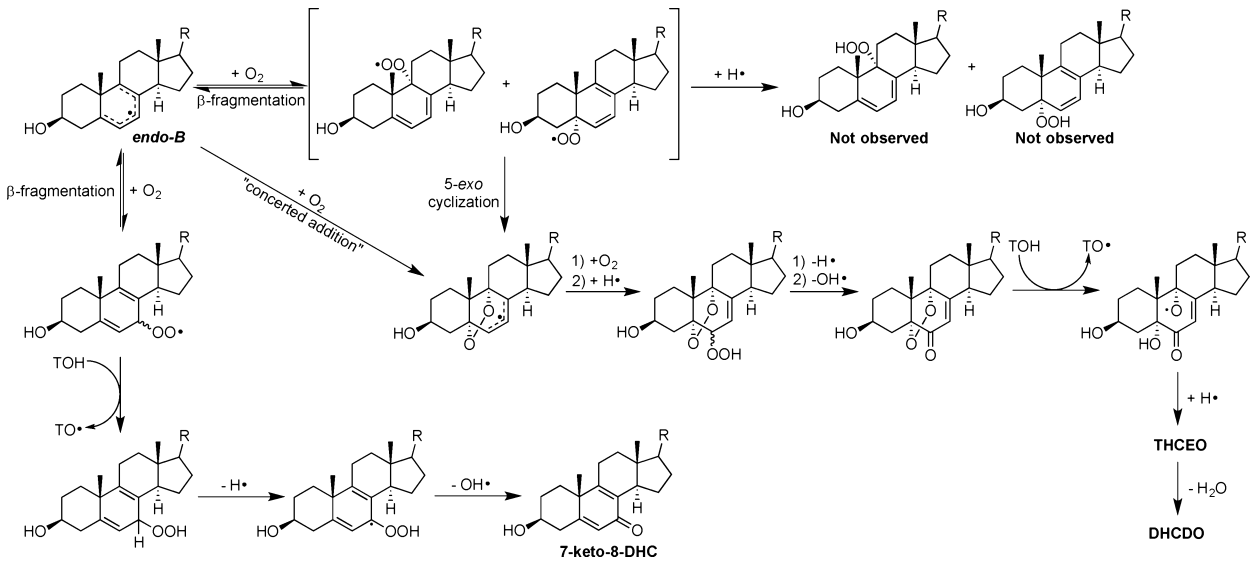
DHC, was observed as one of the major products formed. On the other hand, even in the presence of 1.0 M α-tocopherol, products formed from direct trapping of the 5- or 9-peroxyl radical were not observed but rather those formed from 5-*exo* cyclization were detected (see below) (Figure 2; Scheme 4), suggesting that the 5-*exo* cyclization of these peroxyl radicals is fast enough to compete with hydrogen atom transfer from α-tocopherol (pseudo-first-order rate constant = $k_H(\alpha\text{-tocopherol})\cdot[\alpha\text{-tocopherol}] = 3.5 \times 10^6\ s^{-1}$). This large peroxyl radical 5-*exo* cyclization rate constant is surprising since the rate constant for the same reaction in an acyclic system was estimated to be ca. 800 s^{-1} and the corresponding carbon radical cyclization is only $4.1 \times 10^5\ s^{-1}$ at 37 °C.[30,31] An alternative mechanism to be considered for this transformation is the concerted addition of oxygen to ***endo-B***, leading directly to the five-membered peroxide-allyl radical (Figure 4), although a concerted transformation of this type has, to our knowledge, no precedent. We note that oxygen-radical complex species have been suggested as intermediates in the oxidation of acyclic dienols such as linoleic acid, which proceeds *via* extended chain pentadienyl radical intermediates.[32] An oxygen-radical complex of the ring constrained ***endo-B*** pentadienyl radical would appear to be well on its way to the product allyl radical.

We suggest that the ketone products observed in the *H-9 mechanism* were formed *via* abstraction of the remaining bis-allylic or allylic hydrogen atom at the α-position of hydroperoxide, followed by β-fragmentation of the peroxide bond (Scheme 4). An alternative pathway could be that the hydroperoxide was reduced by α-tocopherol to give an alkoxyl radical, which is followed by elimination of the α-hydrogen atom. Ketones formation has been observed in free radical oxidation reactions, either from termination reactions of two peroxyl radicals *via* the "Russell mechanism" or from decomposition of hydroperoxides (via alkoxyl radical),[33−35] such as those observed in the oxidation of linoleate.[35−37] However, complete conversion of a peroxide to a ketone has not been previously reported.

The formation of THCEO requires the reduction of the cyclic peroxide bond, which may be achieved by proton-coupled electron transfer from the most reducing agent in the reaction, α-tocopherol, as shown in Scheme 4. We suggest that even though cyclic peroxides are normally considered stable to PPh_3 and α-tocopherol, the strain of the 5,9-cyclic peroxide and electron-withdrawing effect of the carbonyl group at C6 may lead to higher reactivity of this peroxide toward reduction. One such example is that isoprostanes bicyclic endoperoxides can be readily reduced by PPh_3 to give diols.[38] THCEO was found to be one of the major 7-DHC-derived oxysterols in the brain of *Dhcr7*-KO mouse.[25] We reported previously that THCEO is a metabolite of compounds **1** and **2b** in Neuro2a and human fibroblast cells,[25] and our results here suggest that THCEO could form directly from 7-DHC *via* the *H-9 mechanism* in the presence of α-tocopherol.

In the *H-14 mechanism*, oxygen can be added to three different positions of the ***exo-B*** pentadienyl radical (Scheme 5). However, after reduction by PPh_3, only three products corresponding to the molecular weight of [7-DHC + 1O] were observed, possibly because 5-*exo* cyclization is not favored for any of these peroxyl radical intermediates (Figure 2). We speculate that these three products are the hydroxydienols shown in Scheme 5. The peak eluting at 7.8 min in Figure 2

Scheme 4. *H-9 Mechanism* of the Free Radical Oxidation of 7-DHC in the Presence of α-Tocopherol (TOH)



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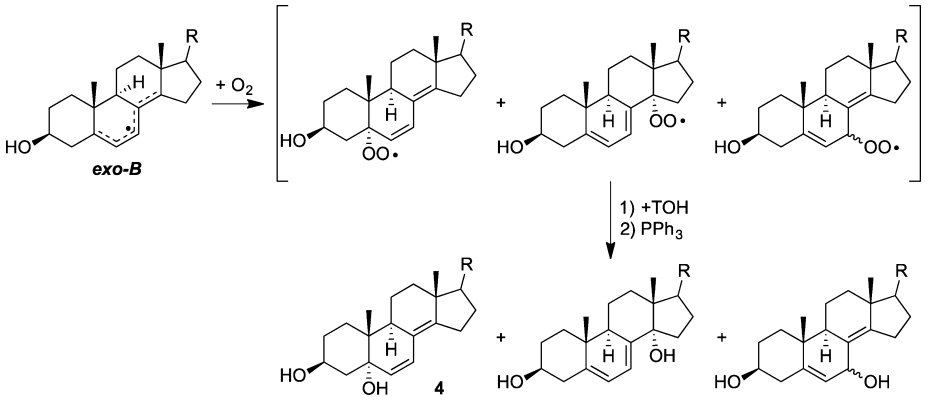
Scheme 5. ***H-14 Mechanism*** **of the Free Radical Oxidation of 7-DHC in the Presence of** α**-Tocopherol (TOH)**



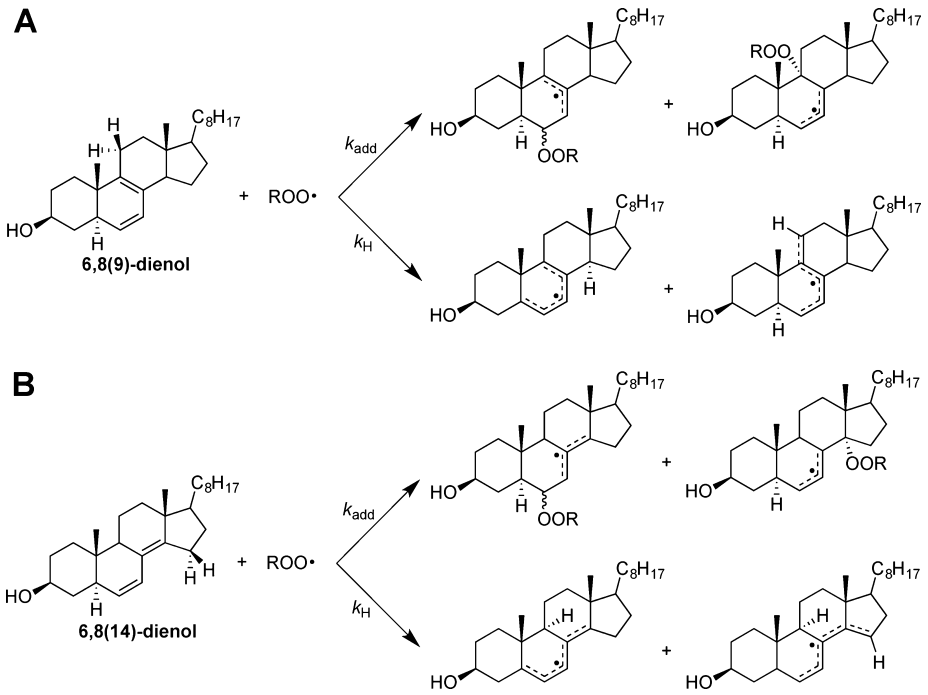


Figure 5. Proposed radical intermediates formed from cholesta-6,8(9)-dienol (A) or cholesta-6,8(14)-dienol (B) *via* peroxyl radical addition (k_{add}) or hydrogen atom transfer mechanism (k_H). Note that the peroxyl radical addition pathways are expected to be completely suppressed in the presence of 1.0 M α-tocopherol as observed in the reaction of 7-DHC.

was tentatively assigned to the known compound **4** based on its HPLC retention time.

In free radical oxidation of 6,8(9)-dienol or 6,8(14)-dienol with or without α-tocopherol, additional products that were not derived from the *H-9* or *H-14 mechanism* were observed (Figures S2 and S3). We speculate that these products were derived from peroxyl radical addition to the double bond (to give an epoxide; only in the absence of α-tocopherol) or from hydrogen atom abstraction at the allylic position C-11 (for 6,8(9)-dienol) or C-17 (for 6,8(14)-dienol) (Figure 5).

We found that cholestadienols displayed much higher reactivity than the acyclic dienes toward free radical oxidation even though some of them do not possess a bis-allylic methylene group. Even the least reactive dienol tested, the 6,8(14)-dienol, has a large propagation rate constant of 412 M^{-1} s^{-1}, a value that is ca. 40 times that of cholesterol (11 M^{-1} s^{-1}) and ca. 7 times that of the methylene-skipped acyclic diene, linoleate (62 M^{-1} s^{-1}). The overall reactivity trend for the cholestadienols suggests that the dienols that lead to the formation of the pentadienyl radical ***endo-B*** are more reactive than those leading to the pentadienyl radical ***exo-B***. It is known that *cisoid* 1,3-dienes have higher enthalpies than *transoid* 1,3-dienes,[39] which would imply a smaller activation energy of hydrogen abstraction from the allylic positions of the *cisoid* dienes, thus supporting our results on the reactivities of the conjugated dienes: 5,7-dienol (7-DHC) > 6,8(9)-dienol ≫ 6,8(14)-dienol.

It is unusual to observe that the Ring-B conjugated dienes, 7-DHC and 6,8(9)-dienol, display significantly higher reactivity than the dienols bearing a bis-allylic methylene group, 8-DHC and 5,8(14)-dienol, since the bis-allylic C−H bond normally is more reactive than the monoallylic C−H bond in acyclic structures (i.e., in polyunsaturated fatty acids).[11] We reported previously that, in 7-DHC, the torsion angles between the active C−H bond (at C9 or C14) and the diene plane are close to 90°, so that the two hydrogen atoms are well aligned for removal from the α-face of the steroid ring; *i.e.*, maximum delocalization stabilization is achieved in the hydrogen atom

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transfer transition state (Figure S6A).[10] Similar molecular mechanics calculation suggests that, in the 6,8(9)-dienol, the torsion angle for H5−C5−C6−C7 is 79.2°, also close to 90° (Figure S6B). In 8-DHC, the torsion angels for the C−H bonds at C7 are 111.5° (C5−C6−C7−$H7_\beta$) and 108.6° ($H7_\beta$−C7−C8−C9) for H_β and 133.5° (C5−C6−C7−$H7_\alpha$) and 129.8° ($H7_\alpha$−C7−C8−C9) for H_α, respectively (Figure S6C). Although the diene in 8-DHC appears to be in the same plane, the reactive C−H bonds are not positioned well for hydrogen atom transfer. In the 5,8(14)-dienol, the diene does not appear to be in the same plane, which would require extensive molecular reorientation in order to achieve maximum delocalization (Figure S6D). Further theoretical investigation would be worthwhile to elucidate the differences in the reactivities of these cholestadienols.

Implication in Cholesterol Biosynthesis and Metabolism Diseases. Elevated dienyl cholesterol precursors have been observed in a number of cholesterol biosynthesis and metabolism disorders, such as 7-DHC and 8-DHC in SLOS,[40,41] 7-DHC in cerebrotendinous xanthomatosis (CTX),[42] 8-DHC in X-linked dominant chondrodysplasia punctata (CDPX2),[43] 8(9),14-dienol in HEM dysplasia,[44] etc.[45] The high reactivity of cholestadienols suggests that oxidative stress should be considered as a factor in disorders where dienyl sterol intermediates are accumulated. Oxidation of the reactive sterols would lead to the formation of oxysterols with diverse structures and functions,[8,12,46−50] metabolites that may ultimately play significant roles in the pathophysiology of these diseases.

SUMMARY

In this study, we found that dienes in the rigid cyclic systems (cholestadienols) display much higher reactivities toward free radical oxidation than the known acyclic dienyl fatty acids. By comparing the oxysterol profiles from the oxidation of several custom-designed cholestadienols with those of 7-DHC, the free radical oxidation mechanism of 7-DHC was refined, with or without the presence of α-tocopherol. From the reactions carried out in the presence of α-tocopherol, evidence was presented that supports either an unusually fast 5-*exo* peroxyl radical cyclization or a concerted addition of molecular oxygen to a pentadienyl radical, both of which are unprecedented. Also notable was the complete conversion of a hydroperoxide to a ketone during the process of free radical oxidation. The knowledge of the reactivity and oxidation product profiles of these cholestadienols revealed in this study contributes to the overall understanding of the pathobiology of related cholesterol biosynthesis/metabolism disorders.

EXPERIMENTAL SECTION

General Methods and Materials. The initiator, MeOAMVN, was purchased from Wako Chemicals, dried under vacuum, and then stored at −40 °C. 7-Dehydrocholesterol (>98%), *tert*-butyldimethylsilyl chloride, borane·THF (1.0 M in THF), and methanesulfonyl chloride were purchased from Sigma-Aldrich Co. and were used without further purification. Methyl linoleate (Nu-Chek-Prep, Inc.) was purified on silica gel prior to use (10% ethyl acetate in hexane) and was stored under argon. Benzene (HPLC grade) was passed through a column of neutral alumina and stored over molecular sieves. HPLC grade hexanes and 2-propanol were purchased from Thermo Fisher Scientific Inc. 8-DHC, 5,8(14)-dienol, and 6,8(14)-dienol were synthesized following previously reported procedures.[17−19] The synthesis of 6,8(9)-dienol was described in the Supporting Information, and the NMR spectra matched those reported in the literature, in which the compound was synthesized by a different method.[18]

General Procedure for Clocking Experiments of Sterols Using Methyl Linoleate. The reaction was carried out as previously reported.[16] Briefly, for each series of reactions, the same amount of a stock solution of methyl linoleate in benzene was added to each reaction vial, followed by the addition of a stock solution of the specific sterol, an appropriate amount of benzene, and a stock solution of MeOAMVN in benzene in order to make the total reaction volume 100 μL. The final concentrations for methyl linoleate, the sterol under study, and MeOAMVN are 0.302 M, 0.01−0.08 M, and 0.001−0.002 M, respectively. The reaction was carried out at 37 °C for 1 h and was quenched by the addition of BHT (50 μL; 0.1 M in benzene) and PPh_3 (50 μL; 0.1 M in benzene). The resulting mixture was blown dry under nitrogen and redissolved in hexanes (500 μL) for the analysis of HODEs by HPLC-UV as previously described.[16] For oxysterol analysis, the reactions were carried out similarly as described above, but the resulting reaction mixtures were diluted to 1 mL with benzene before being analyzed by HPLC-APCI-MS/MS using the method that was described previously.[12]

General Procedure for Free Radical Oxidation of Sterols in the Presence of α-Tocopherol. The oxidation was carried out similarly as the clocking experiments, but with an additional reaction component (1.0 M α-tocopherol) and a longer reaction time (2 h). The reactions were quenched similarly and diluted to 1 mL with benzene for the analysis of oxysterols using the same HPLC-MS method.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures for the synthesis of 6,8(9)-dienol; plots of clocking experiments; mass spectra of **2b**, DHCDO, THCEO, and 7-keto-8-DHC; HPLC-MS chromatograms of oxysterols formed from oxidation of 6,8(9)- and 6,8(14)-dienols in the absence and presence of α-tocopherol; NMR and UV spectra of each cholestadienol. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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