

## Oxysterols from Free Radical Chain Oxidation of 7-Dehydrocholesterol: Product and Mechanistic Studies

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**Abstract:** Free radical chain oxidation of highly oxidizable 7-dehydrocholesterol (7-DHC), initiated by 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile), was carried out at 37 °C in benzene for 24 h. Fifteen oxysterols derived from 7-DHC were isolated and characterized with 1D and 2D NMR spectroscopy and mass spectrometry. A mechanism that involves abstraction of hydrogen atoms at C-9 and/or C-14 is proposed to account for the formation of all of the oxysterols and the reaction progress profile. In either the *H-9* or *H-14 mechanism*, a pentadienyl radical intermediate is formed after abstraction of H-9 or H-14 by a peroxy radical. This step is followed by the well-precedented transformations observed in peroxidation reactions of polyunsaturated fatty acids such as oxygen addition, peroxy radical 5-exo cyclization, and S<sub>H</sub>i carbon radical attack on the peroxide bond. The mechanism for peroxidation of 7-DHC also accounts for the formation of numerous oxysterol natural products isolated from fungal species, marine sponges, and cactaceous species. In a cell viability test, the oxysterol mixture from 7-DHC peroxidation was found to be cytotoxic to Neuro2a neuroblastoma cells in the micromolar concentration range. We propose that the high reactivity of 7-DHC and the oxysterols generated from its peroxidation may play important roles in the pathogenesis of Smith–Lemli–Opitz syndrome, X-linked dominant chondrodysplasia punctata, and cerebrotendinous xanthomatosis, all of these being metabolic disorders characterized by an elevated level of 7-DHC.

### Introduction

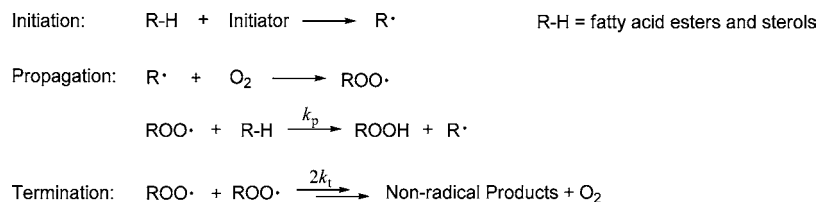
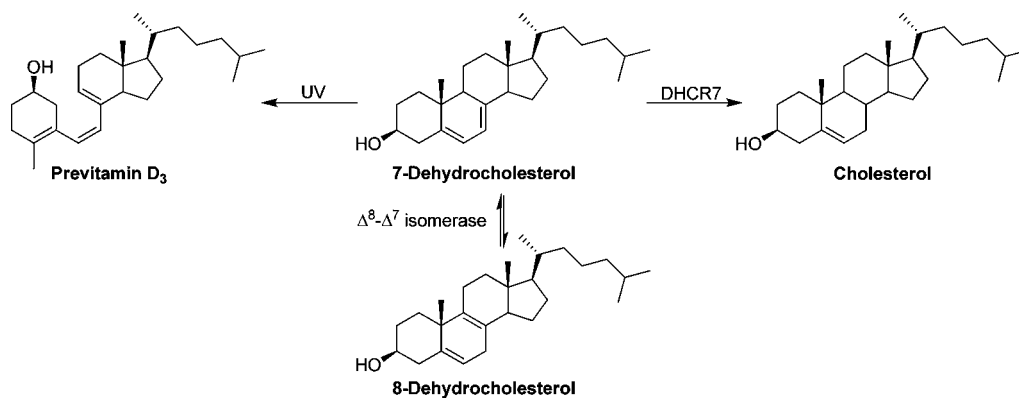
The free radical chain oxidation of polyunsaturated fatty acids (PUFAs)/esters and sterols, a process known as lipid peroxidation or lipid autoxidation (Chart 1),<sup>1–5</sup> has attracted increased research attention over the past few decades due to its involvement in the pathophysiology of common diseases including atherosclerosis,<sup>6–9</sup> asthma,<sup>10,11</sup> neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease,<sup>12–16</sup> and age-

related macular degeneration and cataracts.<sup>17–20</sup> Oxysterols, compounds largely derived from cholesterol chemical or enzymatic oxidation,<sup>21–24</sup> have been demonstrated to exert important biological activities such as cytotoxicity,<sup>24,25</sup> regulating cholesterol homeostasis,<sup>24,26–30</sup> suppressing the immune

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**Chart 1.** Typical Radical-Mediated Lipid Peroxidation Reaction**Scheme 1**

response,<sup>31–34</sup> and interacting with the hedgehog-signaling pathway.<sup>35–37</sup> Unusual oxysterols can be formed from cholesterol biosynthetic intermediates when elevated levels of these compounds are present,<sup>29</sup> such as in various cholesterol synthesis disorders.<sup>38</sup>

7-Dehydrocholesterol (7-DHC) is a biosynthetic precursor of cholesterol and vitamin D<sub>3</sub> (Scheme 1).<sup>37,39,40</sup> 7-DHC is present in relatively high concentrations in skin, where it is converted to pre-vitamin D<sub>3</sub> upon UV irradiation and where it is also exposed to exogenous radical sources and oxygen. 7-DHC accumulates in patients with Smith–Lemli–Opitz syndrome (SLOS), a metabolic disorder resulting from mutations in the gene encoding 7-dehydrocholesterol reductase (DHCR7), the enzyme that catalyzes the reduction of 7-DHC to cholesterol.<sup>41–46</sup>

In previous studies on radical-mediated peroxidation reactions, we reported that 7-DHC is extremely reactive to chain oxidation, with a propagation rate constant ( $k_p$  in Chart 1) in solution ( $2260 \text{ M}^{-1} \text{ s}^{-1}$ ) that is some 200 times that of cholesterol ( $11 \text{ M}^{-1} \text{ s}^{-1}$ ).<sup>47</sup> Indeed, 7-DHC has a propagation rate constant for oxidation that is more than 10 times that of arachidonic acid ( $197 \text{ M}^{-1} \text{ s}^{-1}$ ),<sup>47</sup> a PUFA that is considered to be highly susceptible to oxidation. Individuals with metabolic disorders in which 7-DHC levels are elevated in tissues and fluids will form significantly higher yields of oxidation products than normal individuals, given identical free radical chain initiation and termination in both. The unusually high oxidizability of 7-DHC will lead to increased formation of oxysterols, which may have important biological consequences for individuals having high levels of this sterol present in their tissues and fluids.

We report here a systematic study of the free radical chain oxidation of 7-DHC, aiming to elucidate the peroxidation reaction mechanism and the structures of oxysterols generated. A preliminary study on the effects of 7-DHC-derived oxysterols on cell viability is also reported.

## Results

**Isolation and Characterization of Oxysterols from 7-DHC Peroxidation.** We first sought to isolate and characterize the 7-DHC oxidation products. In a typical experiment, 0.1–0.2 M 7-DHC in benzene was oxidized at 37 °C under an O<sub>2</sub> atmosphere for 24–32 h in reactions initiated by 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeOAMVN, 1 mol %). The reaction mixture was reduced with PPh<sub>3</sub> before products were isolated in order to avoid decomposition of any unstable hydroperoxides present.

A complex product mixture was formed from 7-DHC free radical oxidation, as indicated by TLC, HPLC-UV, and HPLC-MS analyses. The PPh<sub>3</sub>-reduced reaction mixture was separated

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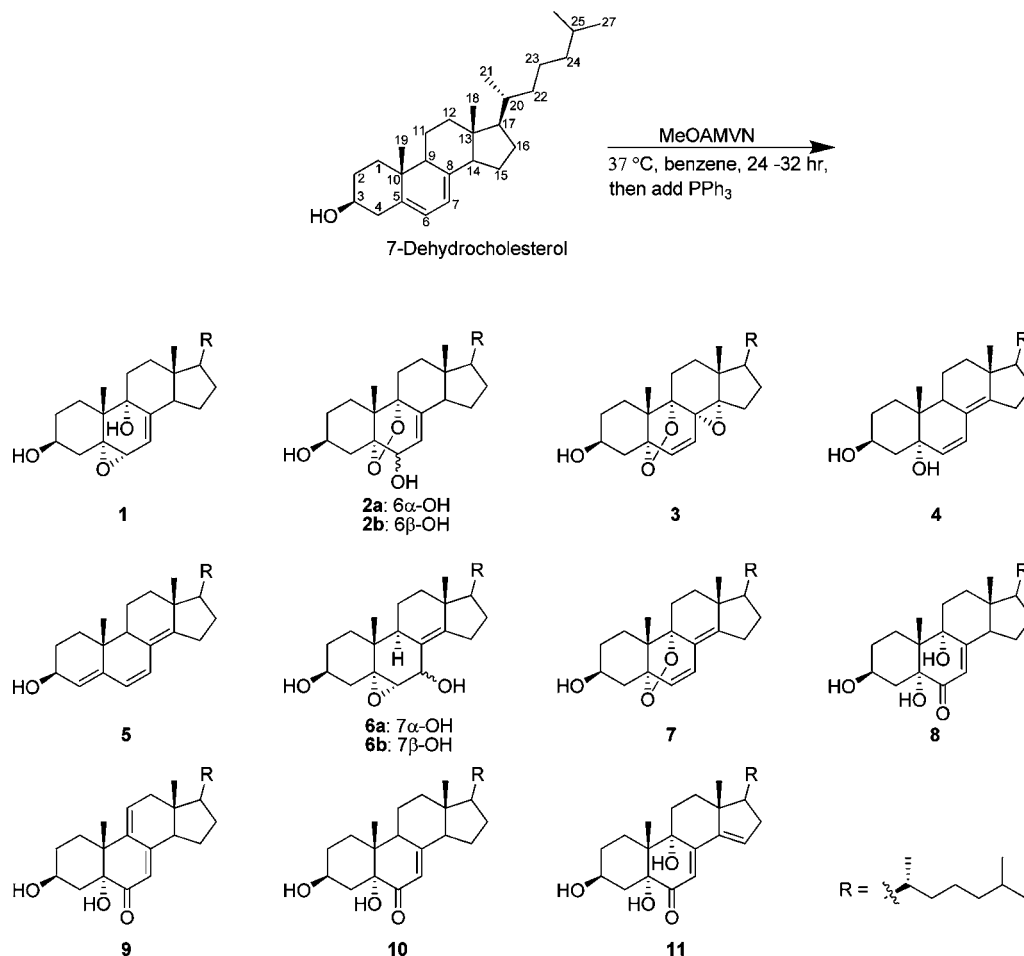
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**Figure 1.** Products isolated from free radical chain oxidation of 7-DHC after PPh<sub>3</sub> reduction.

into several fractions by flash column chromatography on silica gel, eluting with methanol in methylene chloride (1/20). The resulting fractions were again applied to flash column separation with different combinations of elution solvents, including hexane/ethyl acetate, methylene chloride/ethyl acetate, and methylene chloride/methanol, until pure product fractions were obtained. The remaining mixed fractions were examined by the use of normal-phase HPLC-UV, eluting with 10% 2-propanol in hexanes. Still-inseparable fractions were successfully separated by reverse-phase C-18 HPLC, eluting with a mixture of acetonitrile and methanol (see Experimental Section). Over a dozen oxysterols derived from 7-DHC were characterized by 1D and 2D NMR spectroscopy and mass spectrometry, and structures were assigned (Figure 1).

A combination of 2D NMR spectroscopy methods such as HSQC, HMBC, and NOESY is critical in solving the structure of the purified products. We illustrate the general approach by analysis of compound **1** as an example (Figure 2). From the HSQC spectrum, the correlation between each carbon and the directly connected protons can be obtained along with the number of attached protons. Protons on methyl groups such as C-18, C-19, and C-21 are the anchors of the structure elucidation, as they exhibit strong correlations to the carbons that are two or three bonds away in the HMBC spectrum. Typically, H-18 can be differentiated from H-19 by its coupling to C17, an atom that is also coupled to H-21, which is a distinct doublet. Thus, from the HMBC spectrum, C-1, C-5, C-9, and C-10 can be assigned on the basis of their correlation with H-19 and C-12,

C-13, C-14, and C-17 can be assigned on the basis of their H-18 correlations. The connectivity was further confirmed by the correlation between H-7 and C-5, C-6, C-9, and C-14. In the end, stereochemistry was assigned on the basis of NOESY experiments and consistency with the reaction mechanism (*vide infra*). 2D NMR correlations of compounds **2a**, **6a**, and **6b** are also illustrated in Figure 2. Tables of <sup>1</sup>H and <sup>13</sup>C NMR data are presented in the Supporting Information, as well as 1D and 2D NMR spectra.

Ring-opening of epoxide **1** under basic conditions and reduction of endoperoxide **2b** by LiAlH<sub>4</sub> affords the same tetraol **12b**, further confirming the stereochemical assignment (Scheme 2). Likewise, reduction of **2a** gives tetraol **12a** (Scheme 2). Both **12a** and **12b** were characterized in the same way as the other oxysterols shown in Figure 1.

In Figure 3, the HPLC-MS-MS chromatogram of the oxidation product mixture that includes compounds **1–11** is presented. Since sterols are known to be insensitive to electrospray ionization (ESI) in mass spectrometry, an atmospheric pressure chemical ionization (APCI) source was employed in this case. Typically, molecular ions of the sterols were not observed under APCI condition, and loss of one or more water molecules occurred easily.<sup>48,49</sup> There are exceptions to this rule, compounds **3**, **7**, **9**, and **10** giving readily observable molecular ions. No

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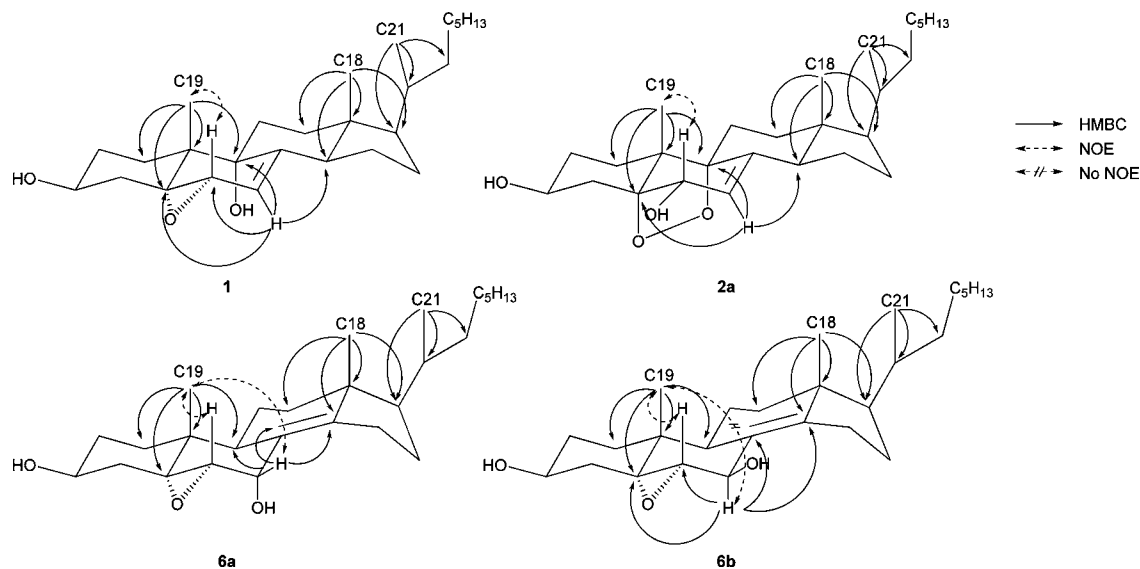
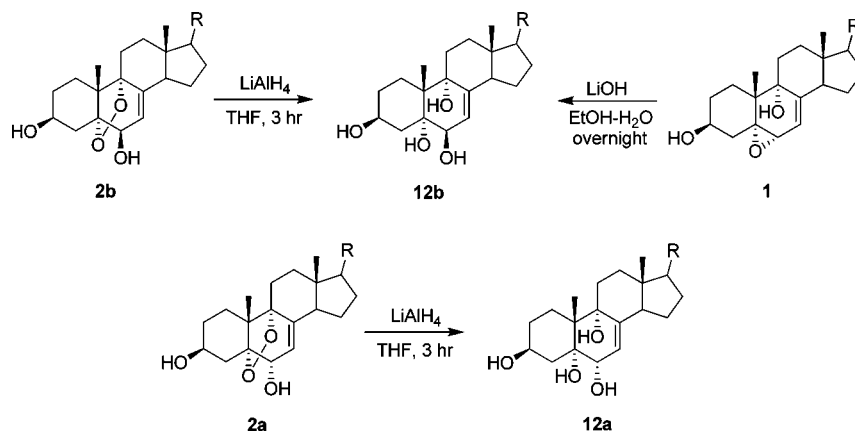


Figure 2. HMBC and NOE correlations of selected oxysterols derived from 7-DHC.

### Scheme 2



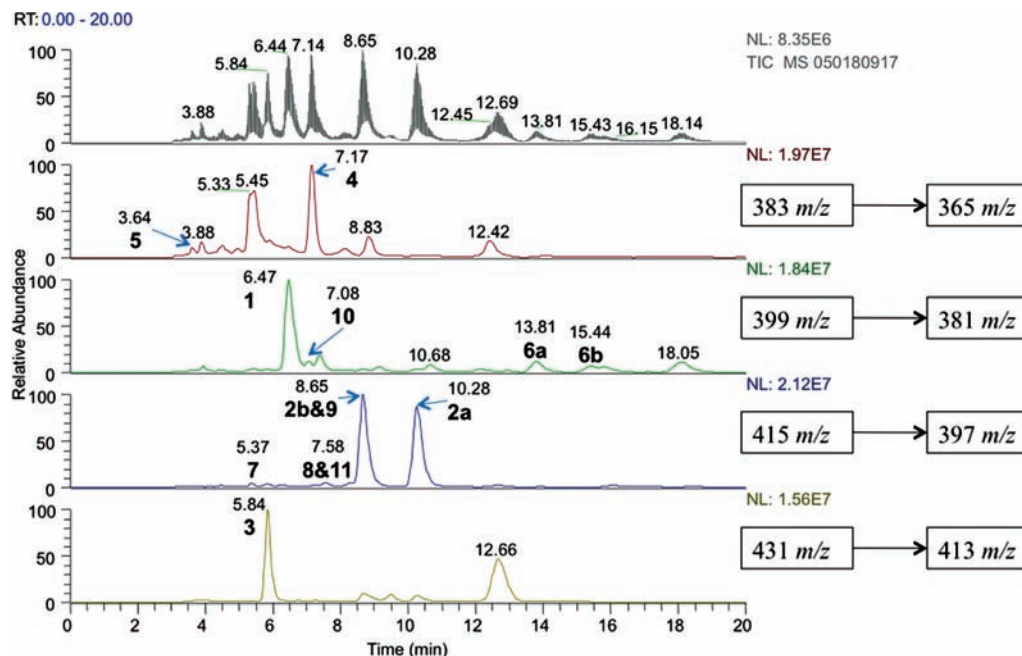
characteristic fragmentation was detected from any of the oxysterols other than dehydration ions (see Supporting Information). However, utilizing this dehydration property, selective reaction monitoring (SRM) was employed to monitor the reaction mixture, e.g., from  $[M + H - H_2O]^+$  to  $[M + H - 2H_2O]^+$ , and was achieved with enhanced sensitivity relative to total ion current (TIC). Thus,  $m/z$  corresponding to the dehydration ions of 7-DHC plus one, two, three, and four oxygen atoms could be monitored, as shown in the four panels of the chromatogram in Figure 3. No major peaks from 7-DHC plus five or more oxygen atoms were observed.

Oxysterols isolated by flash column chromatography and HPLC as described earlier were assigned to the corresponding peaks in the HPLC-MS-MS chromatogram on the basis of the mass spectrum and the corresponding retention time (Figure 3). Note that molecular ion  $[M + H]^+$  of compound **3** has the same  $m/z$  as the dehydration ion of 7-DHC plus four oxygen atoms,  $[7\text{-DHC} + 4O + H - H_2O]^+$ , due to an extra degree of unsaturation.

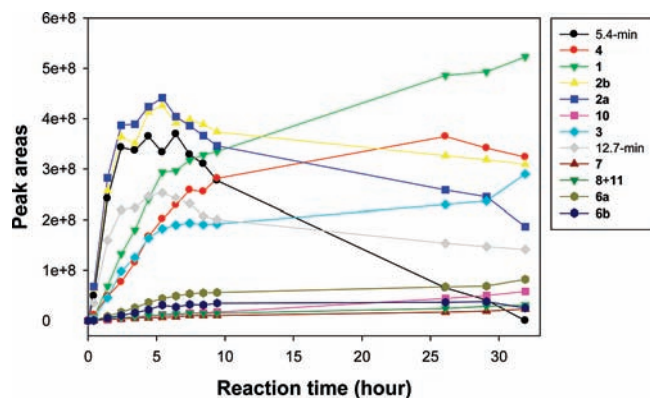
Among these oxysterols, compounds **1**, **2a**, **2b**, **3**, and **4** are the major products observed. The compound or compounds that elute in the peaks shown at 5.4 min in the second panel eventually disappeared over the course of the reaction (see Discussion). Neither the compounds eluting in the 5.4-min peaks

nor the one eluting at 12.7 min in the fifth panel of Figure 3 could be purified and identified due to their instability on the silica flash column (see Discussion). Note that the minor product **9** co-elutes with **2b**, and **11** co-elutes with **8** under the conditions of normal-phase chromatography. The separation of these compounds was achieved by reverse-phase HPLC (see Experimental Section).

**Reaction Progress Monitored by Normal-Phase HPLC-MS-MS.** A 7-DHC oxidation reaction was carried out as described in the previous section, and aliquots of the reaction mixture were collected at different times. Each aliquot was reduced by  $PPh_3$  and analyzed by the HPLC-MS-MS protocols illustrated in Figure 3. The reaction progress product profile obtained in this way is shown in Figure 4. Compounds **2a**, **2b**, the unknown 5.4-min peak in the second HPLC panel, and the unknown 12.7-min peak in the fifth panel increased over the first 6 h of reaction and decayed slowly over the subsequent 26 h. The unknown eluting at 5.4 min ultimately disappeared from the product mixture. This product reaction progress profile suggests that these compounds are primary products that convert to other compounds over the course of the reaction. While compounds **1**, **3**, and **4** increased in the beginning 6-h period of the reaction, they also continued to form slowly throughout



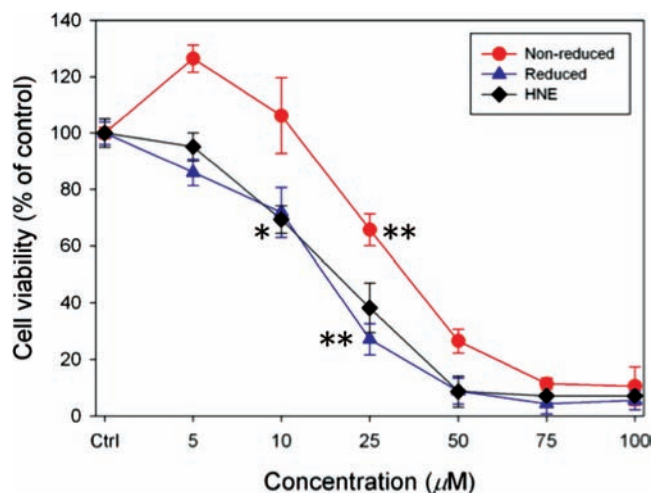
**Figure 3.** Normal-phase HPLC-APCI-MS-MS chromatogram (silica 4.6 mm  $\times$  25 cm column; 5  $\mu$ m; 1.0 mL/min; elution solvent, 10% 2-propanol in hexanes; source energy, 12 eV; collision pressure, 1.40 mTorr; collision energy, 10 eV) of oxidation mixture from the free radical oxidation of 7-DHC after 8.5 h of reaction followed by reduction with PPh<sub>3</sub>. Assignment of structural constituents of some of the chromatogram peaks is given by the compound number in bold close to the peak.



**Figure 4.** Reaction progress of 7-DHC free radical chain oxidation (0.13 M 7-DHC, 0.0013 M MeOAMVN, 37 °C in benzene).

the rest of the reaction progress. Formation of other minor products seems to follow the pattern observed for **1**, **3**, and **4**.

**Effects of Oxysterol Mixture Derived from 7-DHC Peroxidation on Cell Viability.** Oxysterols, derived from cholesterol by either enzymatic or non-enzymatic oxidation, are a large and diverse group of compounds with a multiplicity of biological activities. While some oxysterols have important physiological roles, others, especially those encountered under pathological conditions, may be harmful. Previous studies suggested that 7-DHC or its photo-oxidation-derived oxysterols have damaging biological activities. For example, 7-DHC-derived lipid hydroperoxides promote retinal degeneration in the rat model of SLOS.<sup>50,51</sup> Since we now know the composition and structure of non-enzymatically derived 7-DHC oxysterols, we decided to test their biological activity in our neuronal cell



**Figure 5.** 7-DHC-derived oxysterols reduce viability of Neuro2a cells. Neuro2a cells were grown in the presence of different concentrations of non-reduced or reduced 7-DHC oxysterol mixtures or 4-HNE for 48 h. The *x*-axis shows concentration of compounds, and the *y*-axis shows the percentage of live cells compared to untreated control cells. The bars are standard errors. \*  $p < 0.05$ , \*\*  $p < 0.001$

culture model. We used a mouse neuroblastoma, Neuro2a cells, which are widely used in neuroscience research and have been established as a cellular model of DHCR7 deficiency using the shRNA silencing method.<sup>52</sup> Cells were plated in 96-well plates at a density of 5000 cells/well and were treated with compounds with concentrations ranging from 0 to 100  $\mu$ M.

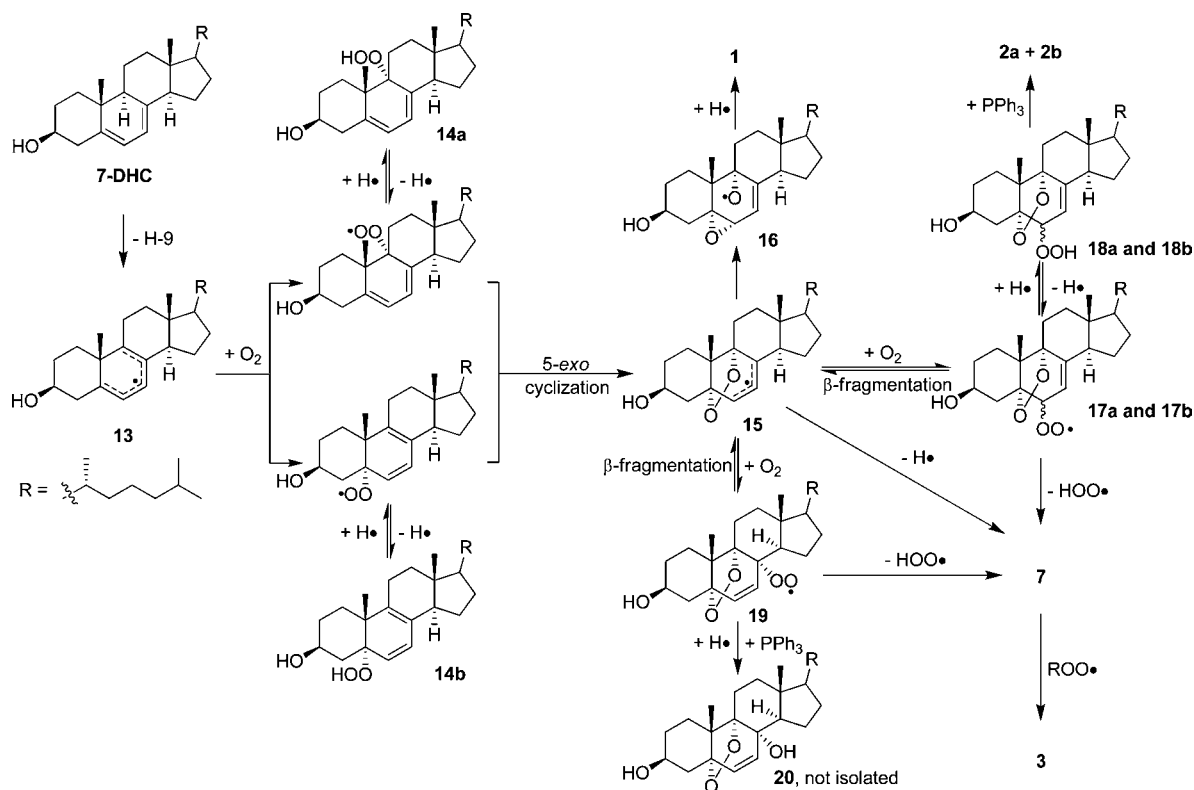
The Neuro2a cell viability after 48 h of incubation with oxysterol was determined by a standard method as described in the Experimental Section; the results are shown in Figure 5. We tested the activity of the mixture of 7-DHC oxysterols

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Scheme 3. H-9 Mechanism



isolated before and after reduction with phosphine as well as 4-hydroxynon-2-enal (4-HNE), a well-known cytotoxin. While a 25  $\mu\text{M}$  concentration of all tested compounds reduces cell viability ( $p < 0.001$ ), the phosphine-reduced 7-DHC mix shows the effect at 10  $\mu\text{M}$  ( $p < 0.05$ ), indicating that it is more toxic than the non-reduced mixture. 4-HNE shows a similar toxicity in assays with a colon cancer cell line.<sup>53</sup>

## Discussion

**Mechanism of Oxidation.** 1,3-Cyclohexadienes normally undergo free radical chain oxidations by a mechanism that involves peroxy radical addition, the 1,4-isomers are usually aromatized in a process involving protonated superoxide as the chain-carrying species.<sup>54–56</sup> In contrast, 7-DHC follows a complex pathway that apparently involves initial hydrogen atom transfer to chain carrying peroxy radicals. Indeed, the array of products isolated suggests that the mechanism of free radical chain oxidation of 7-DHC involves abstraction of hydrogen atoms at the C-9 and/or C-14 position of the sterol. Molecular mechanics calculations indicate that the reactive allylic hydrogen atoms, H-9 and H-14, are well positioned for abstraction by peroxy radicals, with the dihedral angles of C(7)–C(8)–C(9)–H(9) and C(7)–C(8)–C(14)–H(14) being close to  $90^\circ$ .<sup>47</sup> Hydrogen atoms on C-4, however, are not well aligned for abstraction with one axial hydrogen atom in the  $\beta$ -face of the sterol and

the other being equatorial. The list of products formed, combined with the observations of reaction progress shown in Figure 4, suggests a reaction mechanism that accounts for all of the oxysterols characterized. To simplify the discussion, we consider separately the pathway starting with abstraction of H-9 (Schemes 3 and 4) and the one that follows abstraction of H-14 (Scheme 5).

We suggest that, in the *H-9 mechanism* (Scheme 3), abstraction of the C-9 hydrogen by a peroxy radical affords the pentadienyl radical **13**, which reacts with molecular oxygen at a diffusion-controlled rate.<sup>57</sup> The resulting dienyl peroxy radicals abstract a hydrogen atom and form hydroperoxides **14a** and **14b** that would be reduced to the corresponding alcohols by triphenylphosphine. We speculate that the unassigned 5.4-min peak in the chromatogram (Figure 3) is a mixture of these alcohols, the conjugated 6,8-diene-3,5-diol and the 5,7-diene-3,9-diol. Hydroperoxides **14a** and **14b** are primary products that subsequently are converted to the downstream products of the scheme. This accounts for the product time course presented in Figure 4. As the 5.4-min peak (reduced **14a** and **14b**) disappears over time, products **1** and **3** continue to form. Amounts of compounds **2a** and **2b** slowly decreased after 6 h of reaction because their hydroperoxide precursors, **18a** and **18b**, can be converted to the thermodynamically more stable compound **1** via H-atom abstraction and  $\beta$ -fragmentation.

The intermediate peroxy radicals can also undergo 5-*exo* cyclization to give the endoperoxyl allyl radical **15**.<sup>58</sup> Intramolecular homolytic substitution ( $S_{\text{H}1}$ ) on the endoperoxy group

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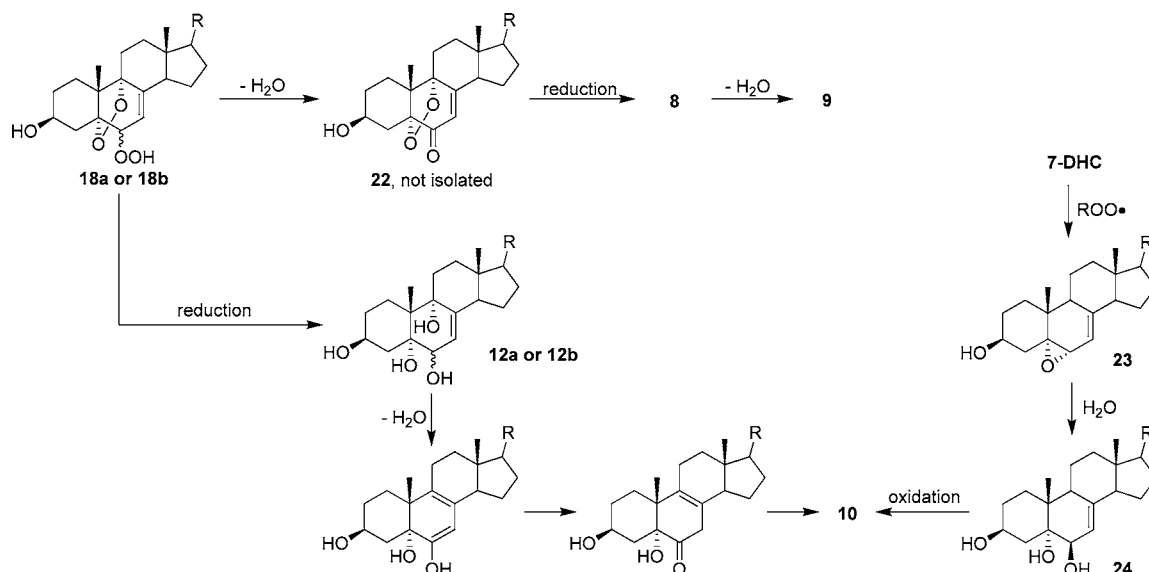
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Scheme 4. Extended H-9 Mechanism



of **15** leads to alkoxy radical **16**,<sup>59,60</sup> which gives compound **1** after abstracting a hydrogen atom from a donor. Allyl radical **15** can also be trapped by oxygen at C-6 to yield peroxy radicals **17a** and **17b**, which are precursors to **2a** and **2b** after H-atom transfer and PPh<sub>3</sub> reduction.

We did not find evidence for the formation of compound **20**, which could form from peroxy radical **19**. Elimination of HOO• from **19** or **17** and removal of H• from radical **15** would produce the diene endoperoxide **7**, a compound formed as a minor part of the product mixture. It seems likely that **7** is the precursor to **3**, a major constituent of the product mixture. Alkenes can be epoxidized by a peroxy radical mechanism,<sup>61</sup> and this seems to be a reasonable sequence for the formation of **3**, a compound whose formation is delayed relative to other major products formed (see Figure 4).

Hydroperoxides and endoperoxides are reasonably reactive functional groups, and it seems likely that some of the minor products isolated result from dehydration of hydroperoxides or reduction of both hydroperoxides and endoperoxides. We speculate that compounds **8**, **9**, and **10** are likely the result of further transformation of the endoperoxy hydroperoxides **18a** and **18b** (Scheme 4). Dehydration–reduction of **18a** and **18b** could afford compound **8**, which would yield **9** by elimination of water. Loss of water from the tetraols **12a** and **12b**, followed by a multistep isomerization, could lead to compound **10**. Direct epoxidation of 7-DHC followed by ring-opening and oxidation could also lead to the formation of **10**. We provide no direct evidence of either pathway for the formation of **10**, although we note that the cholesterol analogue of **10** has been identified as a peroxidation product formed via the epoxide intermediate.<sup>62,63</sup>

In the *H-14 mechanism* (Scheme 5), abstraction of the hydrogen atom at C-14 gives the extended pentadienyl radical **25** that can be trapped by O<sub>2</sub> to give peroxy radicals **26** and

**27**. H-atom transfer to **26** followed by reduction leads to compound **4**, a major constituent of the product mixture. Hydroperoxide **28** is the likely precursor to **6a** and **6b**, dienyl hydroperoxides derived from PUFAs and esters known to undergo analogous transformations.<sup>64,65</sup> Hydroperoxide **30** would still be a reactive molecule toward free radical chain oxidation, and abstraction of the hydrogen atom at C-9 from **30** could provide routes to **3** and **11**. Compound **5** can be formed from **4** by dehydration or from **25**, **26**, and **28** by elimination of H•, HOO•, and H<sub>2</sub>O<sub>2</sub>, respectively.

Independent confirmation of the H-9 mechanism was obtained by free radical oxidation of hydroperoxide **32** (Scheme 6), which is isolated as a minor product from the reaction of singlet oxygen with 7-DHC.<sup>66,67</sup> Abstraction of the hydrogen atom from the hydroperoxyl group, followed by β-fragmentation,<sup>68</sup> gives the key pentadienyl radical **13** of the H-9 mechanism (Scheme 3). The HPLC-MS chromatogram of the PPh<sub>3</sub>-reduced reaction mixture from oxidation of **32** shows the presence of **1**, **2a**, **2b**, and **3** in the product mixture, providing support for the proposed H-9 mechanism (see Supporting Information).

The key steps in the H-9 and H-14 mechanisms are well-known transformations in PUFA peroxidation reactions.<sup>1–4</sup> Thus, abstraction of a hydrogen atom from a diene to give a pentadienyl radical is the first step in 7-DHC oxidation and PUFA oxidation, and reversible oxygen addition to these intermediates is proposed in both systems. Radical cyclization is a critical step in the formation of isoprostanes from arachidonic acid, and a similar 5-*exo* peroxy radical cyclization is proposed in both the H-9 and H-14 mechanisms. Intramolecular homolytic substitution (S<sub>HI</sub>) of a carbon radical on the peroxide bond has good precedent in PUFA peroxidation chemistry, and an S<sub>HI</sub> transformation, **15** to **16**, is a key step in the formation of product **1**.

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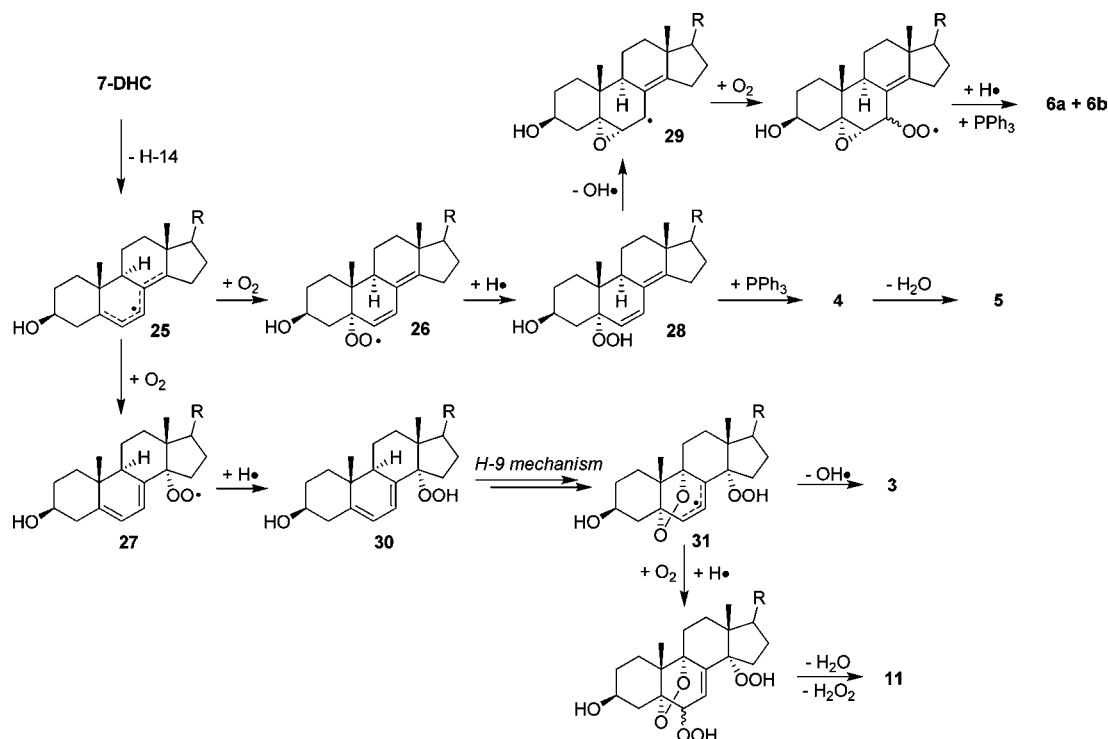
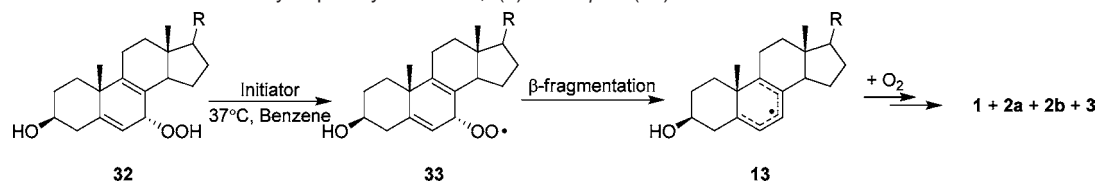
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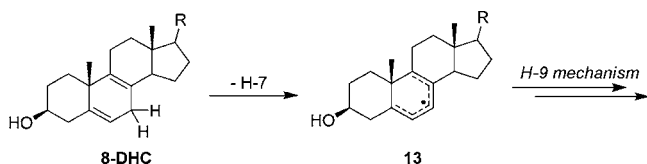
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Scheme 5. H-14 Mechanism

Scheme 6. Free Radical Oxidation of 7-Hydroperoxycholesta-5,8(9)-dien-3 $\beta$ -ol (**32**)

It is worth noting that 8-dehydrocholesterol (8-DHC) levels are also elevated in SLOS, and it is almost certain that 8-DHC will be reactive toward peroxidation because of its bis-allylic H-atoms on C-7.<sup>41</sup> The peroxidation mechanism for 8-DHC will follow essentially the H-9 mechanism since the same pentadienyl radical intermediate, **13**, will form after loss of a hydrogen atom at C-7 of 8-DHC as is formed from loss of H-9 in 7-DHC.



**Implications of the High Reactivity of 7-DHC and the Peroxidation-Derived Oxysterols in Human Diseases.** The oxysterol mixture from 7-DHC peroxidation is toxic to Neuro2a cells in the micromolar concentration range, as demonstrated in the preliminary cell viability study presented in Figure 5. It is known that cholesterol-derived oxysterols can induce apoptosis and necrosis,<sup>69–73</sup> and a similar cytotoxicity of cholesterol oxysterols has been reported in a different cell line.<sup>62</sup> We note that the mixture examined in the current study is composed of over a dozen oxysterols, and it seems likely that there will be a range of toxicities from compounds present in the mixture. Experiments to determine the most active cytotoxic constituents of the mixture are ongoing and will be reported in due course, but we note that the combined oxysterols are as cytotoxic as

4-hydroxynonenal (4-HNE), see Figure 5. After its discovery in biological tissues and fluids by Esterbauer and colleagues some 20 years ago, 4-HNE has achieved the status of one of the best recognized and most studied of the cytotoxic products of lipid peroxidation.<sup>53</sup> It is therefore of some interest that the peroxidation product mixture of the sterol 7-DHC shows similar toxicity to the fatty acid peroxidation product, 4-HNE.

We propose that the reactivity of 7-DHC and its peroxidation-generated oxysterols may play an important role in the pathogenesis of SLOS. Because of the unusually high oxidizability of 7-DHC, elevated levels of this sterol should serve notice for the possibility of high levels of 7-DHC-derived oxysterols in human tissues or fluids.<sup>74</sup> At the same concentration, environment, and conditions of radical initiation, the amount of oxysterols generated from 7-DHC will be 200 times

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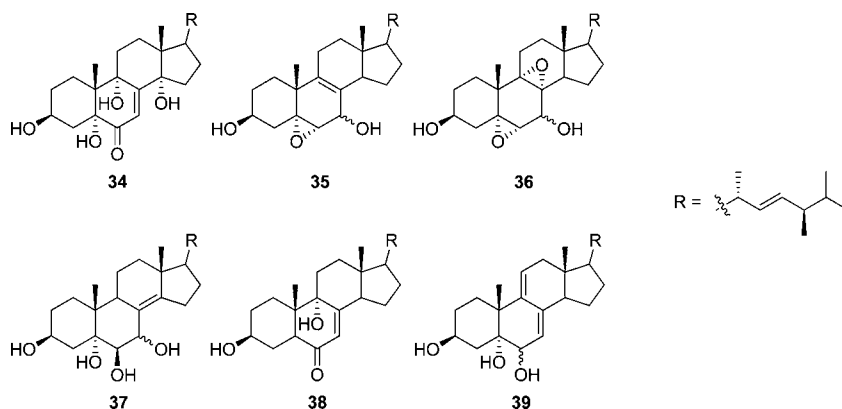
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Chart 2



that of oxysterols formed from cholesterol during the same period of time. SLOS patients are reported to show inhibited cholesterol synthesis caused by suppressed activity of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase,<sup>75</sup> a typical activity of oxysterols,<sup>76</sup> suggesting another possible role in SLOS for the 7-DHC-derived oxysterols.

Elevated levels of 7-DHC and/or 8-DHC have been observed in other metabolic diseases, such as X-linked dominant chondrodysplasia punctata (CDPX2)<sup>77,78</sup> and cerebrotendinous xanthomatosis (CTX).<sup>79,80</sup> CDPX2 is caused by mutations in the gene encoding 3 $\beta$ -hydroxysteroid- $\Delta^8, \Delta^7$ -isomerase,<sup>81</sup> and CTX is caused by mutations in the gene encoding the p450 enzyme sterol 27-hydroxylase (CYP27).<sup>82,83</sup> In CDPX2, decreased synthesis of cholesterol is observed, similar to SLOS, and the inhibition effect of oxysterols on cholesterol biosynthesis may account for this observation in both disorders. In CTX, up-regulation of cholesterol biosynthesis leads to the formation of increased sterol intermediates such as 7-DHC and 8-DHC. We should also note that steroid hormones and bile acids could be enzymatically derived from 7-DHC and 8-DHC.<sup>84–87</sup> It is conceivable that these enzymatically derived steroids would be as reactive as 7-DHC and 8-DHC toward peroxidation, since the core structure on ring B of the steroid is unchanged.

**Oxysterol Natural Products Derived from 7-DHC or Analogues.** Ergosterol is a 7-DHC analogue found in fungi, and numerous oxysterol natural products derived from ergosterol or analogous  $\Delta^{5,7}$ -sterols with different side chains have been isolated from fungal species,<sup>88–96</sup> marine sponges,<sup>97–105</sup> and cactaceae species.<sup>106,107</sup> We suggest that many of these natural oxysterols may be formed by a free radical chain oxidation of the corresponding  $\Delta^{5,7}$ -sterol by essentially the same mechanisms proposed in Schemes 3–5 for 7-DHC peroxidation. Specifically, ergosterol analogues of compounds **2a**, **2b**, **3**, **6a**, **6b**, **8**, **10**, **12a**, and **12b** have been isolated from fungal species,<sup>88–96</sup> and analogues of compounds **3**, **6a**, **8**, **10**, **12a**, and **12b** have been isolated from marine sponges.<sup>97–104</sup> Other reported natural products that can be generated from the free radical oxidation process are analogues of compounds **22–24**, **34–39** (Chart 2). For example, compound **34** can be formed from the precursor to **11** in the H-14 pathway, and compound **35** can derive from hydroperoxide **14b** via a reaction similar to the formation of **6a** and **6b**. Straightforward mechanisms can also be written for the formation of **36–39**.

Many of these natural oxysterols have shown diverse biological activities, such as being cytotoxic,<sup>95–100,104,108,109</sup> antima-

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larial,<sup>104</sup> antitubercular,<sup>110</sup> anti-inflammatory,<sup>111</sup> and an agonist of the liver X receptor (LXR).<sup>112</sup> LXRs are nuclear receptors that control cellular cholesterol efflux.<sup>113</sup> Certain oxysterols can up-regulate proteins involved in cholesterol export by activating LXRs, which is one of the means for oxysterols to affect cholesterol homeostasis.<sup>26,28</sup>

## Summary

Over a dozen oxysterols formed from the free radical chain oxidation of 7-DHC were isolated and characterized. A mechanism that involves abstraction of hydrogen atoms at C-9 and/or C-14 is proposed to account for the formation of all of the isolated oxysterols. The same peroxidation mechanism can be applied to account for the formation of numerous natural oxysterols, many of which exert important biological activities. The oxysterol mixture formed from 7-DHC peroxidation is toxic to Neuro2a neuroblastoma cells in the micromolar concentration range. These 7-DHC-derived oxysterols may play important roles in the pathogenesis of SLOS, CDPX2, and CTX, metabolic syndromes characterized by elevated levels of 7-DHC.

## Experimental Section

**General Methods and Materials.** The initiator, MeOAMVN, which has a half-life of 6 h at 37 °C, was purchased from Wako Chemicals, dried under vacuum, and then stored at -40 °C. 7-Dehydrocholesterol (>98%) and Rose Bengal (95%) were purchased from Sigma-Aldrich Co. and were used without further purification. Benzene (HPLC grade) was passed through a column of neutral alumina and stored over molecular sieves. Hexanes, ethyl acetate, methylene chloride, and methanol (all 99.9%) were purchased from Thermo Fisher Scientific Inc. The neuroblastoma cell line Neuro2a was purchased from the American Type Culture Collection (Rockville, MD). Fetal bovine serum (FBS) was purchased from Thermo Scientific HyClone (Logan, UT). DMEM (with glucose, and without sodium pyruvate and L-glutamine), L-glutamine, and penicillin/streptomycin were purchased from Mediatech, Inc. DMEM without phenol red was purchased from Invitrogen Corp. (SKU no. 21063-029).

**Free Radical Chain Oxidation of 7-DHC.** To a solution of 7-DHC (5.0 g, 0.013 mol) in benzene (100 mL) at 37 °C was added the radical initiator MeOAMVN (40 mg, 0.13 mmol). The reaction mixture was kept at 37 °C under an oxygen atmosphere for 26 h. Butylated hydroxytoluene (BHT) (286 mg, 1.3 mmol) and PPh<sub>3</sub> (1.7 g, 6.5 mmol) were added to quench the reaction. The resulting mixture was then evaporated and separated into several fractions by flash column chromatography on silica gel, eluting with methylene chloride/methanol from 20/1 to 5/1. The crude fractions were then applied to separation multiple times on a silica flash column, eluting with different solvent combinations and ratios,

including methylene chloride/methanol (40/1, 30/1, or 20/1), hexane/ethyl acetate (9/1, 1/1, 2/3, or 3/4), and methylene chloride/ethyl acetate (18/1, 3/1, or 1/1). The combinations of solvents were chosen on the basis of the TLC separation observed on silica gel. Pure compounds **1**, **2a**, **3**, **4**, **5**, **6a**, and **6b** were obtained by this flash column chromatography method. Inseparable fractions were applied to separation on normal-phase HPLC-UV (silica 4.6 mm × 25 cm column, 5 μm; 1.0 mL/min; elution solvent, 10% 2-propanol in hexanes), which gave pure minor products **7** and **10**, a mixture of **2b** and **9**, and a mixture of **8** and **11**. The separation of **9** from **2b** and **11** from **8** was achieved by reverse-phase HPLC-UV (Supelco Discovery C18 4.6 mm × 25 cm column, 5 μm; 1.0 mL/min; elution solvent, acetonitrile/methanol = 70/30).

**Reaction Progress of 7-DHC Oxidation.** The reaction was carried out in the same way as described above. An aliquot (10 μL) was taken at the time points shown in Figure 4. To each aliquot was added BHT in methylene chloride (0.1 M, 20 μL) and PPh<sub>3</sub> in methylene chloride (0.1 M, 20 μL). The mixture was vortexed and then diluted with hexane (100 μL) and methylene chloride (850 μL) for normal-phase HPLC-APCI-MS-MS analysis. HPLC conditions: silica 4.6 mm × 25 cm column, 5 μm; 1.0 mL/min; elution solvent, 10% 2-propanol in hexanes. Mass spectrometry was optimized for compound **1**, and the MS parameters chosen are as follow: discharge current, 2 μA; sheath gas pressure, 20 mTorr; ion sweep gas pressure, 4 mTorr; auxiliary gas pressure, 5 mTorr; tube lens, 80 eV; skimmer offset, 12 eV; collision pressure, 1.40 mTorr; collision energy, 10 eV.

**Singlet Oxygen Oxidation of 7-DHC.** The reaction was carried out following a reported procedure using Rose Bengal as the photosensitizer.<sup>66,67</sup> 7-Hydroperoxycholesta-5,8(9)-dien-3β-ol (**32**) was isolated in a yield of 10%.

**Free Radical Oxidation of 7-Hydroperoxycholesta-5,8(9)-dien-3β-ol (**32**).** Compound **32** (0.005 M) was incubated in benzene (200 μL) at 37 °C under air in the presence of radical initiator MeOAMVN (10 mol %) for 2 h. For HPLC analysis, an aliquot of the reaction mixture (50 μL) was quenched by adding BHT (0.1 M, 20 μL) and PPh<sub>3</sub> (0.1 M, 20 μL) and then diluted with hexanes (910 μL). The HPLC-MS-MS analysis was similar to the one described above.

**Cell Viability Test of Oxysterol Mixtures on Neuro2a Cells.** A non-reduced oxysterol mixture was obtained from a 32-h oxidation of 7-DHC as described above, with the exception that the PPh<sub>3</sub> step was omitted. The reduced mixture of oxysterols was obtained by reducing the reaction mixture with trimethyl phosphite (P(OMe)<sub>3</sub>). The excess trimethyl phosphite and its oxidation product, trimethyl phosphate, are volatile and were removed under vacuum over 24 h. Note that PPh<sub>3</sub>, instead of P(OMe)<sub>3</sub>, was used as the reductant in the isolation experiments described above because of the extreme smell of P(OMe)<sub>3</sub>. Both non-reduced and reduced mixtures were dissolved in DMSO (100% DMSO, 10 mM stock solution was used at 1000-fold dilutions to obtain working concentrations of 0–100 μM). The concentration for the oxysterol mixture was calculated on the assumption that the averaged molecular weight of the oxysterol mixture was 432 (7-DHC + 3O). Neuro2a cells were maintained in DMEM and supplemented with L-glutamine, 10% FBS, and penicillin/streptomycin at 37 °C and under 5% CO<sub>2</sub>. To evaluate the effect of 7-DHC oxysterol mixtures on cell viability, Neuro2a cells were cultured in DMEM without phenol red in the presence or absence of 7-DHC oxysterol mixtures in a 96-well plate. Briefly, the Neuro2a cells were plated at a density of 5000 cells/well. On the following day, the cell culture medium was replaced with fresh medium, with or without the 7-DHC oxysterol mixtures. The cell viability was evaluated after 48 h of oxysterol treatment using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corp., Madison WI), a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays (see below).

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**CellTiter 96 Aqueous One Solution Cell Proliferation Assay.** The CellTiter 96 Aqueous One Solution contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt, MTS] and an electron coupling reagent (phenazine ethosulfate, PES). The MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Assays are performed by adding a small amount of the CellTiter 96 Aqueous One Solution Reagent directly to culture wells, incubating for 1–4 h, and then recording the absorbance at 490 nm with a SPECTRA Fluor Plus plate reader (Tecan, Austria). The quantity of formazan product, as measured by the absorbance at 490 nm, is directly proportional to the number of living cells in culture.

**Abbreviations:** 7-DHC, 7-dehydrocholesterol; 8-DHC, 8-dehydrocholesterol; NMR, nuclear magnetic resonance; SLOS, Smith–Lemli–Opitz syndrome; CDPX2, X-linked dominant chondrodysplasia punctata; CTX, cerebrotendinous xanthomatosis; PUFA, polyunsaturated fatty acid; DHCR7, 7-dehydrocholesterol reductase; MeOAMVN, 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile); HPLC, high-performance liquid chromatography; MS, mass spectrometry; HSQC, heteronuclear single quantum coherence; HMBC,

heteronuclear multiple bond coherence; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; SRM, selective reaction monitoring; TIC, total ion current; *m/z*, mass-to-charge ratio; HNE, *trans*-4-hydroxy-2-nonenal; S<sub>Hi</sub>, intramolecular homolytic substitution; LXR, liver X receptor; FBS, fetal bovine serum; BHT, butylated hydroxytoluene; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's Modified Eagle Medium.

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**Supporting Information Available:** Complete refs 42 and 112; tables of <sup>1</sup>H and <sup>13</sup>C NMR chemical shifts; and 1D and 2D NMR, UV, and MS spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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