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Tunneling in Tocopherol-Mediated Peroxidation of 7-Dehydrocholesterol

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The peroxidation of 7-dehydrocholesterol (7-DHC), a biosynthetic precursor to vitamin D_3 and cholesterol, has been linked to the pathophysiology of Smith-Lemli-Optiz syndrome (SLOS), a devastating human disorder. In SLOS, 7-DHC plasma and tissue levels are elevated because of defects in the enzyme that convert it to cholesterol. α-Tocopherol can mediate the peroxidation of 7-DHC under certain circumstances and this prompted us to investigate the kinetic isotope effect (KIE) during this process. Thus, 9,14-d_2-7-DHC was synthesized using a photochemical cyclization of deuterium-reinforced previtamin D_3 (retro to its biosynthesis). Subsequently, we carried out co-oxidation of 9,14-h_2-25,26,26,26,27,27,27-d_7- and 9,14-d_2-7-DHC in the presence of α-tocopherol under conditions that favor TMP. By monitoring the products formed from each precursor using mass spectrometry, the KIE for the hydrogen (deuterium) atom removal at C9 was found to be 21 ± 1. This large KIE value indicates that tunneling plays a role in the hydrogen atom transfer step in the tocopherol-mediated peroxidation of 7-DHC.

Introduction

Polyunsaturated fatty acids and esters undergo free radical chain oxidation readily and this process, lipid peroxidation, and the toxic products derived from it have been the subject of intense scrutiny in recent years.[1] Arachidonic acid is particularly prone to radical chain oxidation and the products of peroxidation of this fatty acid and its esters have been used extensively as biomarkers for oxidative stress *in vivo*.[2] There is a growing consensus that lipid peroxidation and oxidative stress play an important role in the pathophysiology of a number of human disorders.[3]

The rate-limiting step in radical chain oxidation is H-atom transfer to a propagating peroxyl radical, Eq. 1 in Figure 1, and polyunsaturated fatty acids are indeed highly reactive, the rate constant for propagation, k_p, of arachidonic acid being 197 $M^{-1}s^{-1}$. It is therefore of some interest that the sterol 7-dehydrocholesterol (**7-DHC**) has a k_p of 2260 $M^{-1}s^{-1}$, a value that is higher than the propagation rate constant measured for any other lipid, making it a likely target for chain-carrying peroxyl radicals.[4]

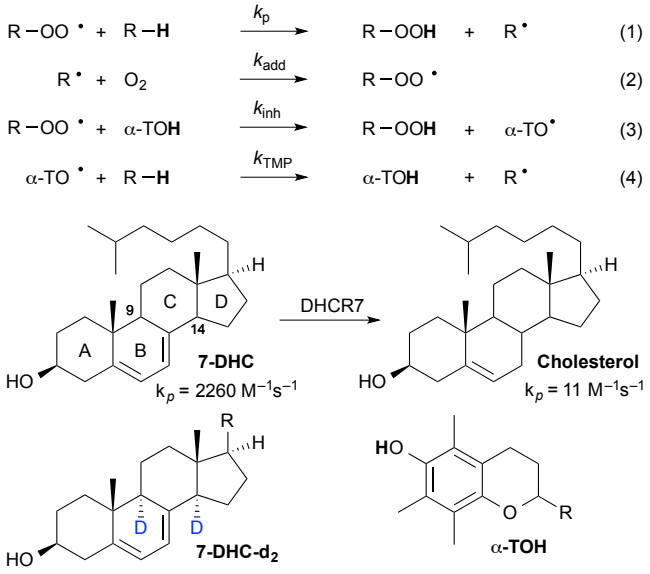


Fig. 1 Free radical peroxidation, inhibition and tocopherol-mediated chain propagation

7-DHC serves as the biosynthetic precursor to cholesterol (k_p = 11 $M^{-1}s^{-1}$) and it plays a central role in one of the most common autosomal recessive human disorders, Smith–Lemli–Opitz syndrome (SLOS).[5] SLOS is characterized by excessive plasma and tissue levels of 7-DHC and lower than normal levels of cholesterol, a consequence of mutations in the enzyme 7-dehydrocholesterol reductase (*DHCR7)* that converts 7-DHC to cholesterol.[5-6] A link between the pathology of SLOS and the

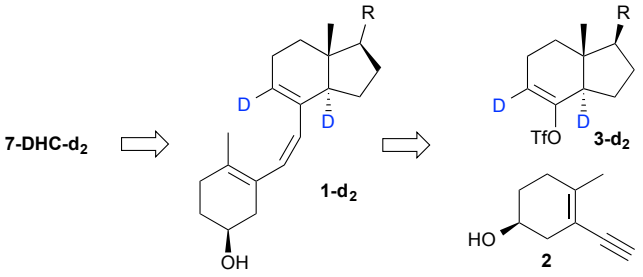
peroxidation of 7-DHC has been made with the discovery that oxysterols derived from 7-DHC are found in tissues and fluids of SLOS mouse models and in skin fibroblasts and plasma of SLOS patients.[7] The fact that many of the 7-DHC-derived oxysterols have potent biological activity provides further support for the notion that SLOS has a significant oxidative stress component.[8]

The free radical oxidation of 7-DHC is a complex process, with over a dozen products formed in the azo-initiated solution oxidation of the lipid.[9] Mechanistic studies suggest that the hydrogen atoms at C9 and C14 are the reactive atoms.[9] Co-oxidation of 7-DHC in the presence of Nature's major chain-breaking antioxidant, α-tocopherol (k_{inh} = 3.5×10^6 M^{-1} s^{-1}),[10] affects not only the rate of peroxidation but also the distribution of products formed during the process.[11] In fact, the product mixture is greatly simplified if peroxidation of 7-DHC is initiated in the presence of the antioxidant.[11]

Under certain circumstances, α-tocopherol can mediate the peroxidation of reactive lipids like 7-DHC with the tocopheryl radical abstracting a hydrogen atom from the reactive lipid, see Eq. 4 in Figure 1.[12] This process, *tocopherol mediated peroxidation* (TMP), likely becomes a major propagation pathway when radical intermediates are isolated in cellular organelles or lipid particles such as low-density lipoproteins.[12-13] Because of the biological relevance of TMP of 7-DHC and the importance of this sterol in a devastating human syndrome, we have focused our attention on the mechanism of this transformation. We report here that H-atom tunneling facilitates the propagation step in this process.

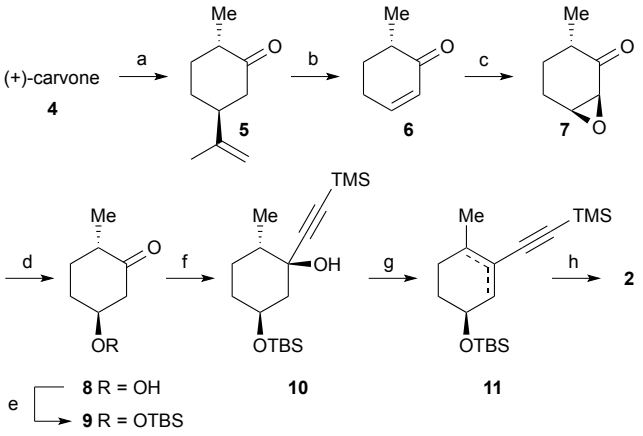
Results and discussion

To study the key atom-transfer step of TMP of 7-DHC, we designed and synthesized deuterium-reinforced 7-DHC at the reactive C9 and C14, **7-DHC-d$_2$**. Efforts to synthesize **7-DHC-d$_2$** starting from 7-DHC proved unsuccessful; a bromination–radical reduction strategy led to undesired diene products with rearranged double bonds. We successfully achieved the synthesis, however, using a photochemical reaction that is essentially a retro-synthesis of previtamin D$_3$ as shown in Scheme 1. Thus, the construction of ring B utilizes a 6π conrotatory photochemical cyclization of deutero-previtamin D$_3$ **1-d$_2$** (Scheme 3). It is the reverse of the reaction by which vitamin D$_3$ is produced biosynthetically and industrially.[14] Dauben and co-workers determined that the quantum yield for ring closure increases with wavelength applied (0.08 at 325 nm).[15] However, the photoequilibrium is heavily in favor of previtamin D$_3$ at all wavelengths and the amount of 7-DHC present is very small. For this reason we developed a gram scale synthesis of previtamin D$_3$ with deuterium at C-9 and C-14 (Scheme 1).



Scheme 1 Retrosynthesis of **7-DHC-d$_2$**

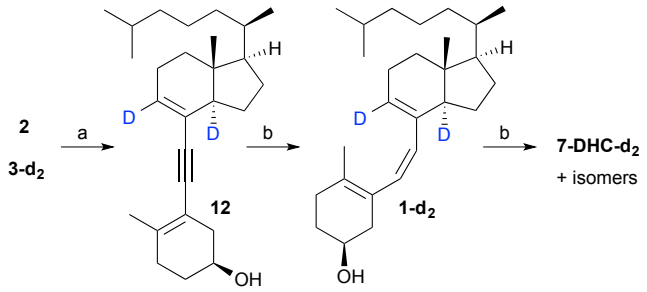
Synthesis of previtamin D$_3$ (**1**) is generally approached by coupling of a CD-ring fragment derived from Grundmann's ketone with an appropriate ring A fragment. Okamura and co-workers used this strategy to prepare several deuterated analogues of previtamin D to study the kinetic isotope effects in thermal [1,7]-sigmatropic shift.[16] The synthesis of alkynol **2** with a deuterated methyl group was part of that work but the introduction of CD$_3$ group and late stage resolution made the overall synthesis lengthy and not suitable for gram scale needs. We prepared alkynol **2** from (+)-carvone as shown in Scheme 2.[†]



Scheme 2 Synthesis of ring A fragment **2**: a) ref. 15; b) ; ref 16 c). H_2O_2, K_2CO_3, H_2O–MeOH (3:1), –20 °C, 1 h, 48% (d.r.≥19:1 +16% of the minor isomer); d) $(PhSe)_2$, $NaBH_4$, 2-propanol, AcOH, 0 °C, **7**, 15 min, 88%; e) TBSCl, imidazole, DMF, 0 °C to rt, 12 h, 96%; f) trimethylsilylacetylene, *n*-BuLi, THF, –78 °C, 30 min, **9**, –78 °C, 3 h, 99%; g) Martin's sulfurane dehydrating agent, CH_2Cl_2, rt, 2 h, 82% (d.r.=4.5:1); h) TBAF, THF, 0 °C to RT, 6 h, 56% (d.r.≥19:1).

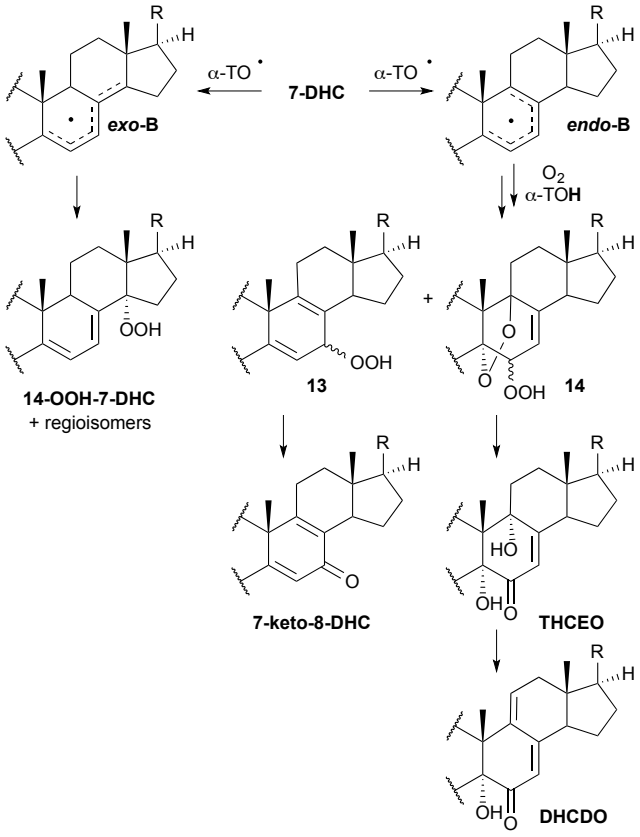
With both cross-coupling partners in hand, standard Sonogashira conditions afforded the conjugated alkyne **12** which was immediately reduced to a deutero-previtamin D$_3$ (**1-d$_2$**) using deactivated Pd/$CaCO_3$–quinoline semireduction protocol (Scheme 3). Once appreciable amounts of the deuterated previtamin D$_3$ were secured, the construction of ring B of 9,14-*d$_2$*-7-DHC was achieved *via* a 6π conrotatory photocyclization of **1-d$_2$**. For practical reasons we employed a

Hanovia 450 W medium pressure mercury lamp equipped with a Vycor® filter to cut off high energy UV and minimize formation of undesired isomers.[17] A solution of **1-d_2** in ethanol–hexanes was irradiated at 0 °C for 50 min. Preparative HPLC separation of the product mixture afforded **7-DHC-d_2** in 4% yield.†



Scheme 2 Synthesis of **7-DHC-d_2**: a) $Pd(OAc)_2$, PPh_3, Et_2NH, CuI, DMF, rt, 30 min, degas (freeze–pump–thaw, 3 cycles) add **2** and **3-d_2**, DMF, 1 h in the dark, aqueous work-up; b) $Pd/CaCO_3$, quinoline, H_2, EtOAc–hexanes (3:7), 52%, 2 steps; c) Medium pressure Hg lamp with a Vycor filter, EtOH–hexanes (2:8 v/v, degassed), 50 min, 4% (+ unreacted **1-d_2** + isomers)

TMP of 7-DHC occurs by abstraction of hydrogen (Eq.4 in Figure 1) at C9 or C14 of the lipid. Reaction at C9 proceeds *via* the intermediate radical ***endo-B***, see Scheme 4, and gives 7-keto-8-DHC and the two ketones, 3β,5α,9α-trihydroxy-cholest-7-en-6-one (THCEO) and 3β,5α-dihydroxy-cholesta-7,9(11)-dien-6-one (DHCDO) as shown in the Scheme.[11] Likely intermediates in the formation of these products are a non-conjugated hydroperoxide **13** formed by reaction of oxygen at the center carbon of ***endo-B*** and the cyclic peroxide-hydroperoxide **14** that results from coupling with oxygen at either terminus of ***endo-B***. Dehydration and reduction of these intermediates leads to 7-keto-8-DHC and THCEO, which dehydrates to give DHCDO.[13b] It is of some interest that these oxysterols have been found in brain tissue of SLOS mouse models.[18] Products derived from H-atom removal at C14 of 7-DHC are formed *via* the intermediate radical ***exo-B***.[11] Conjugated and non-conjugated diene hydroperoxides are formed, one of which, 14-OOH-7-DHC, is shown in Scheme 4. This hydroperoxide is still a highly oxidizable substrate, with a reactive hydrogen atom remaining at C9 of the molecule.



Scheme 4 Mechanistic pathway for the formation of 7-keto-8-DHC, THCEO and DHCDO

To study the kinetic isotope effect (KIE) of TMP of 7-DHC, a mixture having a defined ratio of **7-DHC-d_7** and **7-DHC-d_2**,[19] was oxidized in benzene in the presence of 0.9 M α-tocopherol at 37 °C using 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile (MeOAMVN) as the radical initiator. The concentration of the **7-DHC-d_2** was kept in large excess due to its resistance to oxidation so that comparable levels of oxidation products from both 7-DHC substrates were formed. A time-course study suggested that 8 h of oxidation gave significant more products (> 10 times) than those at t = 0 while the consumption of the starting sterols was still low. These conditions were chosen for subsequent studies.†

An HPLC-APCI-MS chromatogram of the product mixture formed from oxidation of a 10:1 mixture of **7-DHC-d_2** and **7-DHC-d_7** is shown in Figure 2B (See Figure S18 in SI for full chromatogram). By monitoring the ratio of the isotopically differentiated products 7-keto-8-DHC-d_7 and 7-keto-8-DHC-d_1 and knowing the ratio of their respective precursors, the kinetic isotope effect for the removal of H(D)-9 could be determined. The parent ion for 7-keto-8-DHC undergoes fragmentation with loss of water and for the d_7 compound this is observed at *m/z* 406→388 while for the d_1 product the transition is found at *m/z* 400→382. We conclude from these studies that k_H/k_D for removal of the hydrogen at C9 of 7-DHC by a tocopherol radical is 21±1 (Table S3 in SI†). Similar HPLC-MS analyses

were carried out on the d_1- and d_7-products of THCEO and DHCDO and the KIE values of 22±1 and 29±4 were determined, respectively. We note in passing that the KIE values obtained from 7-keto-8-DHC (21±1) and THCEO (22±1) are more reliable than that determined from DHCDO (29±4) since the latter compound is a minor constituent of the products formed from C9 H(D) abstraction. (see **Figure S18**).

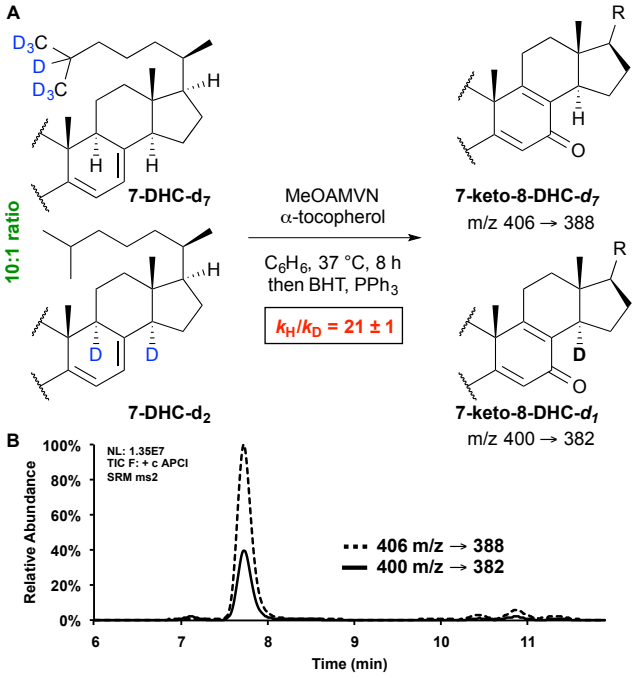


Figure 2 Co-oxidation of deuterium-substituted 7-DHC. A. Conditions of oxidation and structures of isotopically labeled 7-DHCs. B. Chromatogram of oxidation products obtained by normal phase HPLC-MS-MS using selected reaction monitoring (SRM) (see SI for details).

Another significant observation in this study was a large KIE observed even in the t=0 sample. The mixture of d_2- and d_7-7-DHC was collected directly into a flask containing α-tocopherol which was kept at -78 °C but oxidation products were still observed after thawing and quenching the solutons with triphenylphosphine. By analyzing the ratio of d_1/d_7-7-keto-8-DHC in the t=0 sample, a KIE of 20 was determined, similar to the value observed at 37 °C. This observation suggests that temperature does not have significant effect on the KIE of the H-atom transfer from 7-DHC to the tocopheryl radical.

The product distribution of 14-OOH-7-DHC and the regioisomers formed from removal of H(D) at C14 (formation of radical ***exo-B***) was affected by isotopic substitution. The hydroperoxide intermediate, 14-OOH-7-DHC, retains a reactive C9 hydrogen atom and is still a highly oxidizable compound. Subsequent free radical oxidation would be subject to a second KIE, preferentially removing 14-OOH-7-DHC-d_7 from the product mixture since it bears an H at C9 while 14-OOH-7-DHC-d_1 that has D at C9 would be resistant to further oxidation, thus accounting for the product-directing effect observed. This complication in reaction mechanism (*i.e.*, subsequent H-atom transfer at C9) prevented determination of the KIE of H/D transfer from C14. One can only speculate that a large KIE for H(D) atom removal from C14 would be found since the torsion angles between the reactive C-H bonds and the diene plane are similar for C9 and C14 (92.3° and 99.4°, respectively).

Historically, KIE values reported for the autoxidation of hydrocarbons are less than 7, such as those reported for diphenylmethane and cumene by Ingold and Russell, respectively. The large KIEs observed in this study (> 20) suggest that tunneling is involved in the H-atom transfer from 7-DHC to tocopheryl radical. While tunneling is not uncommon in H-atom transfer processes catalyzed by enzymes, such as lipoxygenase (ref), the phenomenon has not been observed in solution-based inhibited lipid peroxidation reactions until our recent report on TMP of polyunsaturated fatty acids (ref, Connor JACS). Tunneling has also been suggested to play a role in regeneration of tocopherol form tocopheryl radical by ubiquinol (refs, Ouchi J Phys Chem B, 2009 and 2010). These earlier studies and our current report suggest that tunneling may be a common phenomenon for H-atom transfer to tocopheryl radical, irrespective of the H-atom donor being fatty acids, a sterol, or another phenolic antioxidant.

Experimental

In four screw cap vials containing solution of **7-DHC-d2**, **7-DHC-d7** (ca. 10:1 ratio, 0.03 M), and α-tocopherol (0.9 M) in benzene (150 mL) was added the MeOAMVN initiator (10 mL, 0.03 M). To the first vial was added PPh_3 (0.5 M in benzene, 20 mL) and BHT (0.5 M in benzene, 20 mL) and benzene (300 mL) and the vial was transferred to –80 °C freezer for storage. Three remaining vials were capped and incubated at 37 °C for 8 h and then was added PPh_3 (0.5 M in benzene, 20 mL) and BHT (0.5 M in benzene, 20 µL) and benzene (300 mL). A small fraction of each reaction was directly injected into MS via syringe pump at a flow rate of 20 µL/min (with a make up flow of 1.0 mL/min by the HPLC) to obtain the ratio of the **7-DHC-d_2** and **7-DHC-d_7** by comparing the intensity of *m/z* at 369 and 390, respectively. From each vial, 200 µL was taken for LCMS analysis. HPLC-APCI-MS-MS analysis was carried out similarly to the previously reported method for 7-DHC-derived oxysterols with modification of the masses being monitored.[7a, 9, 18] For example, for **7-DHC-d_7**-derived oxysterols, masses with 7 additional mass units relative to the non-deuterated oxysterols were monitored; for **7-DHC-d_2**-derived oxysterols (giving d_1-oxysterols after losing D-9 or D-14), masses with one additional mass unit were monitored. In general, selective reaction monitoring (SRM) was employed to monitor the dehydration process of the ion $[M+H]^+$ or $[M+H-H_2O]^+$ in the mass spectrometry. HPLC conditions (on Waters Alliance 2695): 3-µm 150 × 4.6 mm silica column (Phenomenex, Inc.); 10% 2-propanol in hexanes; 1.0 mL/min. MS conditions (on Thermo Finnigan TSQ Quantum Ultra): discharge current, 10 µA; sheath gas pressure, 20 mTorr; ion sweep gas pressure, 2

mTorr; auxiliary gas pressure, 15 mTorr; tube lens, 92 V; skimmer offset, 6 V; collision pressure, 1.50 mTorr; collision energy, 13 V; vaporizer temperature: 300 °C.

Conclusions

To summarize, this work is a first study of the KIE in TMP of a reactive sterol. The KIE obtained for the H-9 transfer is significantly larger than classical KIE values reported for hydrogen atom transfer in peroxidation (typically < 7).[20] This result suggests that tunneling is important in the H-transfer to a tocopheryl radical in the TMP reaction, a conclusion that is consistent with our earlier finding in polyunsaturated fatty acid peroxidations.[13b]. As TMP is thought to play an important role in the peroxidation of low-density lipoprotein (LDL) and sterol esters, including those from 7-DHC, are major constituents of LDL, our study suggests that tunneling contributes to the peroxidation of 7-DHC and hence to the pathophysiology associated with Smith–Lemli–Opitz syndrome in humans.

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Notes and references

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† Electronic Supplementary Information (SI) available: Full experimental details including synthetic and analytical procedures, characterization of all new compounds, and additional supplementary data can be found in the SI. See DOI: 10.1039/b000000x/

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