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Propagation Rate Constants for the Peroxidation of Sterols on the Biosynthetic Pathway to Cholesterol

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Abstract

The free radical chain autoxidation of cholesterol and the oxidation products formed, *i.e.* oxysterols, have been the focus of intensive study for decades. The peroxidation of sterol precursors to cholesterol such as 7-dehydrocholesterol (7-DHC) and desmosterol as well as their oxysterols has received less attention. The peroxidation of these sterol precursors can become important under circumstances in which genetic conditions or exposures to small molecules leads to an increase of these biosynthetic intermediates in tissues and fluids. 7-DHC, for example, has a propagation rate constant for peroxidation some 200 times that of cholesterol and this sterol is found at elevated levels in a devastating human genetic condition, Smith-Lemli-Opitz syndrome (SLOS). The propagation rate constants for peroxidation of sterol intermediates on the biosynthetic pathway to cholesterol were determined by a competition kinetic method, *i.e.* a peroxy radical clock. In this work, propagation rate constants for lathosterol, zymosterol, desmosterol, 7-dehydrodesmosterol and other sterols in the Bloch and Kandutsch-Russell pathways are assigned and these rate constants are related to sterol structural features. Furthermore, potential oxysterols products are proposed for sterols whose oxysterol products have not been determined.

Keywords

sterols; oxysterols; peroxidation; free radicals; rate constants; radical clock

1. INTRODUCTION

The free radical oxidation of cholesterol has been implicated in a number of human disorders including atherosclerosis, (Brown and Jessup, 1999) Alzheimer's disease, (Vaya and Schipper, 2007) cancer, retinal degeneration, (Rodriguez and Fliesler, 2009) and cataract. (Girao et al., 1998), (Vejux et al., 2011) Oxysterols formed from peroxidation of cholesterol have important biological activities and the study of these products has been the focus of extensive recent study. (Gill et al., 2008) The peroxidation of other sterol intermediates on the biosynthetic pathway from lanosterol to cholesterol has also seen recent

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research interest. For example, 7- and 8-dehydrocholesterol (7-DHC and 8-DHC), and the oxysterols formed from these sterols, have been the focus of several recent publications which showed that these compounds are much more prone to peroxidation than cholesterol. (Xu et al., 2009; Xu et al., 2010; Xu et al., 2011; Xu and Porter, 2014) 7-DHC, the immediate biosynthetic precursor of cholesterol on the Kandutsch-Russell pathway, is found at increased levels in tissues and fluids of individuals diagnosed with the developmental disorder Smith-Lemli-Opitz syndrome (SLOS). (Porter and Herman, 2011) SLOS is an autosomal recessive disorder that affects about 1 in 10,000 to 60,000 individuals. (Cross et al., 2015) It is characterized by elevated levels of 7-DHC and by decreased levels of cholesterol. Mutations in the gene that encodes 7-dehydrocholesterol reductase (DHCR7), the enzyme that catalyzes the reduction of the 7,8-double bond of 7-DHC to form cholesterol affect the conversion of 7-DHC to cholesterol (Figure 1). Oxysterols derived from 7-DHC are found in SLOS tissues and fluids and toxicities associated with these oxysterols have been reported. (Korade et al., 2013; Korade et al., 2010)

The determination of kinetic rate constants (k_p) associated with the rate determining step in the peroxidation of lipids provides a quantitative basis for assigning the susceptibility of individual lipids to oxidation, see Figure 2. Indeed, it was the report of the propagation rate constant for peroxidation of 7-DHC that provided a basis for suggesting that 7-DHC is some 200 times more reactive than cholesterol in this chain reaction. (Xu et al., 2009) And it was the unusually high propagation rate constant for 7-DHC, and the oxysterols formed during its autoxidation, that have been implicated in the patho physiology of SLOS. (Gaoua et al., 1999; Korade et al., 2010; Richards et al., 2006)

Other disorders associated with deficiencies in cholesterol biosynthesis have been identified. These pathologies include desmosterolosis, CDPX2 (Kelley et al., 1999; Martanova et al., 2007; Porter and Herman, 2011) and lathosterolosis, (Herman, 2003; Krakowiak et al., 2003) and oxidative stress has been suggested to be a component of each. Indeed, genetic disorders are known that are associated with elevated levels of desmosterol, zymosterol, lathosterol and lanosterol, among others. (Porter and Herman, 2011) Due to the importance of the biosynthesis of cholesterol and the damage that can occur when sterol homeostasis is perturbed, the propagation rate constants for the peroxidation of several sterols on the cholesterol biosynthesis pathway are of potential interest. To provide a framework for understanding the susceptibility of cholesterol biosynthetic precursor to peroxidation, we have undertaken a study to determine the rate constants for free radical propagation by the use of a competition kinetic method (Roschek et al., 2006) and we report the results here.

2. MATERIALS AND METHODS

2.1 Materials

2-methyl-2-heptene (98%) and benzene (anhydrous, 99.8%) were obtained from Sigma-Aldrich (St. Louis, MO). Benzene was passed through a plug of neutral alumina and stored over 4 Å molecular sieves prior to use. Sterols were either purchased from Sigma-Aldrich or synthesized by established procedures. They were purified by HPLC before use. The synthetic approaches for the sterols prepared are described in Supporting Information.

2.2 Measurement of Propagation Rate Constants for Sterols

Propagation rate constants for various sterols and the unsaturated side chain analog, 2-methyl-2-heptene, were carried out using the methyl linoleate clock procedure that has been described in detail. (Roschek et al., 2006) Prior to all experiments, linoleate and sterols were purified by flash column chromatography (10% to 20% ethyl acetate in hexanes, depending on the compound) and dried overnight under vacuum. 2-methyl-2-heptene was purified by distillation at 122 °C. A 0.1 M stock solution of 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile (MeOAMVN) in benzene was used to initiate all reactions. Reagents were added in the order of: (1) benzene, (2) sterol or 2-methyl-2-heptene, (3) linoleate, (4) MeOAMVN. Reaction vials were vortexed for 5 s, followed by heating at 37 °C for 1 h. Each reaction was quenched by the addition of 25 µL of both 0.5 M BHT and 0.5 M PPh₃. Linoleate oxidation products, hydroxyoctadecadienoates (HODEs) were monitored by normal phase HPLC-UV (250 × 4.6 mm silica column; 5 µm; elution solvent, 0.5% 2-propanol in hexanes; monitoring wavelength 234 nm) as previously described. (Xu et al., 2009)

3. RESULTS

3.1 Peroxyl Radical Clocks

Propagation rate constants for various sterols and sterol analogs were measured using a competition kinetic system based upon the oxidation of methyl linoleate. (Roschek et al., 2006) This method relies on the fact that the distribution of conjugated diene hydroperoxide linoleate products formed in peroxidation depends on the concentration and propagation rate constant k_p of any H-atom donor present during the oxidation. The kinetic competition at the heart of this method, the so-called linoleate “radical clock”, is shown in Figure 3. (Roschek et al., 2006; Xu et al., 2009) The critical step in this peroxyl radical clock is the bimolecular transfer of a hydrogen atom from R₁-H to intermediate *trans,cis*-peroxyl radicals (Figure 3A) which is in competition with the unimolecular loss of oxygen from the *trans,cis*-radicals, *via* β-fragmentation following σ-bond rotation, a step that leads to *trans,trans*-diene products. If linoleate is the only H-atom donor present in an experiment, the *trans,cis/trans,trans* linoleate product ratio depends only on the concentration of linoleate as shown in Figure 3B.

To determine the propagation rate constant for any compound R₁-H, a low and constant concentration of methyl linoleate is co-oxidized in an organic solvent with various concentrations of the hydrogen atom donor and the products of linoleate oxidation are assayed by HPLC/UV or HPLC/MS. A plot of the *trans,cis/trans,trans* linoleate products vs. the concentration of R₁-H gives this ratio of products vs. [R₁-H] with a slope of $k_p/214$ and an intercept of 0.16, according to Equation 1. The concentration of linoleate chosen for these studies is low and constant so that the product ratio is dependent only on the concentration of R₁-H.

In a typical experiment, sterols or sterol analogs and methyl linoleate were purified and dried under vacuum prior to oxidation. In oxidations, methyl linoleate was held constant at 0.3 M, and concentrations of the lipid co-oxidant were varied depending on their reactivity and solubility. All oxidations were initiated with the thermally labile azo initiator MeOAMVN (5 mol %). Reactions were initiated at 37 °C and quenched after 1 h with the

addition of BHT and PPh_3 . The linoleate hydroxy octadecadienoates (HODEs) were analyzed by HPLC-UV, monitoring at 234 nm. Under these conditions of oxidation, the consumption of oxidizable substrates is less than 2% and the starting concentration of reactants can be used in Equation 1.

3.2 Side Chain Analog of Bloch Pathway Sterols

One pathway for cholesterol biosynthesis is the Bloch pathway, (Bloch, 1992) in which the 20(*R*)-side chain bears a double bond between carbons C24-C25, see Figure 1. This unit of unsaturation adds eight allylic hydrogen atoms to the sterol structure that could potentially be abstracted by a peroxy radical. Side chain unsaturation could increase propagation rate constants for the Bloch pathway sterol intermediates compared to their Kandutsch–Russell pathway analogs, which have a saturated C24-C25 bond. DHCR24 reduces all Bloch intermediates to the Kandutsch–Russell analogs.

In order to understand the contribution of the side chain unsaturation to the overall propagation rate constant of Bloch pathway sterols, a side chain model of desmosterol was chosen for study. This simple olefin, 2-methyl-2-heptene provides a reactive substructure that models the Bloch sterol side chain. The propagation rate constant for this olefin was determined to be $5.6 \pm 0.2 \text{ M}^{-1} \text{ s}^{-1}$ by the linoleate radical clock, see Figure 4.

3.3 Desmosterol

Desmosterol is a Bloch pathway sterol intermediate that is converted to cholesterol by the reductase enzyme DHCR24, which acts on the C24-C25 double bond (Figure 1). The solubility of desmosterol is low in organic solvents and the highest concentration of this sterol studied was 0.625 M. Due to the small range of concentrations possible for this sterol and its relatively low reactivity, errors in the propagation rate constant were found to be particularly high. An estimate of the desmosterol k_p can be made however, based on the known rate constant for cholesterol ($11 \text{ M}^{-1} \text{ s}^{-1}$) and the k_p determined for 2-methyl-2-heptene, the substructure model for the desmosterol side chain. We thus suggest a desmosterol k_p of $17 \text{ M}^{-1} \text{ s}^{-1}$ based on the sum of rate constants for the reactive centers in the molecule.

3.4 Lathosterol and Zymostenol

Propagation rate constants for lathosterol and zymostenol were also measured. Both sterols are on the Kandutsch–Russell pathway and have very low levels of solubility. The concentration ranges tested for both sterols ranged from 0.05 to roughly 0.17 M and the rate constants determined were $57 \pm 3 \text{ M}^{-1} \text{ s}^{-1}$ and $77 \pm 5 \text{ M}^{-1} \text{ s}^{-1}$, respectively. Structures and plots of *trans,cis*-/*trans,trans*-HODEs versus the concentration of sterol are shown in Figure 5 for both compounds.

3.5 Dihydrolanosterol

Attempts to initiate peroxidation of dihydrolanosterol (see the structure in Figure 1) were unsuccessful under the conditions of oxidation used to determine propagation rate constants. This sterol used in co-oxidations with linoleate also had no effect on the *trans,cis*/*trans,trans*

product ratio. We conclude from these studies that dihydrolanosterol has a $k_p < 1 \text{ M}^{-1}\text{s}^{-1}$ and will not participate in peroxidation reactions in any significant way.

4. DISCUSSION

4.1 Estimated Rate Constants and Oxysterol Product Ratios

The experimental and estimated propagation rate constants for several sterols on the cholesterol biosynthetic pathway are shown in Figure 6. The estimated rate constants are for the sterols in the Bloch pathway and the estimates are made based on the rate constant for the corresponding Kandutsch–Russell sterol summed with $5.6 \text{ M}^{-1}\text{s}^{-1}$, the value determined for the sterol side chain substructure 2-methyl-2-heptene. Thus, the experimentally determined rate constant for 7-DHC is $2260 \pm 40 \text{ M}^{-1}\text{s}^{-1}$ and the estimated rate constant for 7-dehydrodesmosterol would be only $5.6 \text{ M}^{-1}\text{s}^{-1}$ higher, well within the uncertainty of the experimentally determined 7-DHC rate constant.

The range of propagation rate constants for sterols is impressively large, but the side chain unsaturation at C24-C25 contributes little to the overall propagation rate constant for Bloch pathway sterols. Indeed, the propagation rate constant for peroxidation will be largely determined by the nature of unsaturation located in the B-ring of the sterol. As a consequence, oxysterol products that result from peroxidation will be mainly products that are the result of B-ring oxidation. One can estimate the ratio of products formed from B-ring oxidation vs. side chain oxidation based on these rate constants. For desmosterol, the ring to side-chain product ratio will be 11:5.6, the ratio of the rate constants for propagation for the substructures undergoing oxidation. The B-ring to side-chain oxidation product ratio for any Bloch sterol can be calculated in the same way, for zymosterol this ratio will be 77:5.6, for 7-dehydrodesmosterol the value will be 2260:5.6 and so forth. For lanosterol, B-ring oxidation is very slow, (Smith, 1981) if it occurs at all, and the major peroxidation products from lanosterol will be on the side-chain.

Values for propagation rate constants for peroxidation also permit estimates to be made of oxysterol product ratios in complex sterol mixtures. For example, under conditions that initiate peroxidation, a sterol mixture containing 3% of 7-DHC and 97% cholesterol would give rise to a product mixture of oxysterols in which ~ 85% of the oxysterols formed would derive from 7-DHC.

$$\frac{7\text{-DHC oxysterols}}{\text{Chol oxysterols}} = \frac{[7\text{-DHC}] \times k_{p7\text{-DHC}}}{[\text{Chol}] \times k_{p\text{Chol}}} = \frac{(0.03)(2260 \text{ M}^{-1}\text{s}^{-1})}{(0.97)(11 \text{ M}^{-1}\text{s}^{-1})} = \frac{68}{11}$$

This analysis provides particular insight when peroxidation of lipids from individuals having inborn errors in cholesterol biosynthesis is considered. Typically, sterols in the biosynthetic pathway are present at low levels relative to cholesterol. For instance, physiological concentrations of 7-DHC in healthy human plasma are very low (0.03% or less) compared to cholesterol, (Castelli et al., 1986) suggesting that the oxysterols formed from peroxidation of human plasma lipids would be less than 1% of 7-DHC-derived oxysterols compared to those formed from cholesterol. Patients with SLOS typically have much higher plasma levels of 7-

DHC and substantially reduced levels of cholesterol and the oxysterols derived from 7-DHC will dominate these product profiles. (Xu and Porter, 2015)

There have been recent reports of prescribed pharmaceuticals that have marked effects on plasma and tissue sterol profiles as well, even leading to elevated plasma levels for otherwise healthy individuals of 7-DHC on par with those seen in SLOS patients. (Korade et al., 2016) Some drugs linked to these increases include aripiprazole, an atypical antipsychotic, and trazodone, an antidepressant. (Canfran-Duque et al., 2013; Hall et al., 2013; Kim et al., 2016) It seems likely that individuals taking these prescribed medications will have elevated levels of 7-DHC derived oxysterols. In a similar way, several prescribed medications affect the biosynthetic conversion of zymostenol to lathosterol by inhibiting the isomerase enzyme that promotes that conversion. Oxysterols derived from zymostenol would likely be found at increased levels in individuals taking prescription medications such as tamoxifen, for example, since this selective estrogen receptor modulator (SERM) inhibits the isomerase enzyme. (de Medina et al., 2011; de Medina et al., 2010) It is of some interest that oxidative stress has been associated with SERM medications and zymostenol has been linked to this therapy.

4.2 Peroxidation Reactivity of Sterols

We suggest that three factors contribute to the reactivity of sterols toward hydrogen atom abstraction by peroxy radicals: (Xu and Porter, 2015) *a) The bottom face of the sterol is relatively open for reaction with peroxy radicals, the top face is protected by two angular methyl groups.* The reactive hydrogens on the sterol tend to be α -C-H bonds. *b) The number of alkyl substituents on delocalized carbon radical intermediates in the peroxidation sequence.* Sterols normally have more alkyl substituents on double bond(s) than the double bonds in fatty acids. Substituents stabilize the transition state for H-atom abstraction and the resulting radical intermediate *via* hyperconjugation, thus lowering the activation energy of the atom transfer process. *c) The rigidity of the sterol ring and well-aligned allylic C-H bonds.* For example, as seen in 7-DHC (Figure 7), the planarity of the double bonds and the orthogonality of the C-H bonds allow maximum overlap between the π -orbitals and the reactive C-H bonds in the transition state. A minimum amount of molecular reorientation is required to reach the transition state for H-atom removal from either C9 or C14 of 7-DHC, and there is less entropy demand for the process. Thus the torsion angles between the conjugated diene in 7-DHC and the C-H bonds on C-9 and C-14 are close to the preferred torsion angle of 90° (92° and 99° respectively). Furthermore, the delocalized pentadienyl radical intermediate formed from abstraction of either hydrogen is highly substituted, accounting for the high reactivity of this sterol. The radicals derived from C14 and C9 abstraction are shown in Figure 7A. (Xu and Porter, 2015)

The reactive center on 8-DHC is at C-7 and the B-ring is puckered, resulting in a torsion angle of 129.8° for the α -H at that carbon and the C8-C9 double bond. (Xu and Porter, 2015) This is a less favored geometry than those found in 7-DHC and this accounts for the fact that 8-DHC has a propagation rate constant that is less than half that of 7-DHC. Even though the pentadienyl carbon radical formed from 8-DHC is identical to one that is intermediate in the reaction of 7-DHC, the stereoelectronics of the hydrogen abstraction is

more favorable for 7-DHC than it is for 8-DHC, resulting in a larger propagation rate constant for the 7-isomer.

Similar arguments can be made to account for the high reactivity of zymostenol and lathosterol relative to that of cholesterol. Substituted allyl radicals are intermediates in the peroxidation of these substrates and based on the radical structure and C-H stereoelectronics, the abstraction of the zymostenol H-14 should be particularly favorable. The torsion angle for this C-H bond relative to the C8-C9 double bond is 86.3°, close to the preferred 90° angle. The radical intermediate formed by removal of H-14, shown in Figure 8, would be embedded at the ring junction with carbons attached to each position of the intermediate radical.

Lathosterol has a less favorable stereoelectronic orientation of C-H bonds at C9 (102.3°) and C14 (108.6°) than zymostenol and the intermediate radicals formed after removal of H at either site is not fully substituted with carbon substituents (there is an H-substituent at C7). As a consequence, lathosterol is somewhat less reactive than zymostenol, but it is still more reactive than cholesterol which has its reactive α -hydrogen at C7 nearly equatorial relative to the C5-C6 double bond.

4.3 Oxysterol Products of Peroxidation

The free radical oxidation products of cholesterol, 7-DHC and 8-DHC have been isolated and characterized. Several recent publications describe the mechanism of peroxidation for these sterols in some detail and the effect of antioxidants on the product oxysterol profiles have also been discussed. (Muchalski et al., 2014; Xu et al., 2010; Xu and Porter, 2014; Zielinski and Pratt, 2016) The products and mechanism for formation of oxysterols formed in the peroxidation of lathosterol, zymostenol and lanosterol have not been reported in any detail (see however but one can suggest structures of likely products based upon the structure of anticipated intermediate radicals, see Figure 8. While the cholesterol side-chain oxysterols like those shown in Figure 8 have been reported, see page 88 of (Smith, 1981), determination of the product profile of oxysterols derived from lathosterol, zymostenol and the corresponding C24-C25 Bloch pathway oxysterols will require experimentation. The structures shown in Figure 8 represent likely products based on the mechanism of peroxidation and the reactivity guidelines proposed in Section 4.2. We suggest that the tertiary alcohols from zymostenol will be major products of oxidation, since they derive from abstraction of the reactive hydrogen at C14. Products that arise from C7 and C11 H-abstraction from zymostenol may also be formed but they will presumably be minor since reaction at these centers is not favored compared to abstraction at C14. In the same way, products formed from abstraction at C6 of lathosterol may be formed, but will likely be minor compared to the products shown in Figure 8 that derive from H-abstraction at C9 and C14 of this sterol.

5. CONCLUSIONS

Several of the sterols on the biosynthetic pathway from lanosterol to cholesterol are more reactive than either of these sterols toward free radical chain oxidation. The rate constants for the rate determining step for peroxidation of the Kandutsch–Russell sterols zymostenol,

7-DHC, 8-DHC, lathosterol and the corresponding Bloch sterols (zymosterol, 7-DHC etc.) are substantially higher than the rate constant found for cholesterol. Zymosterol and lathosterol have rate constants that suggest that these sterols are as oxidizable as linoleate, a fatty ester that is generally considered to be highly oxidizable. 7-DHC and 8-DHC are more reactive toward free radical chain peroxidation than any other lipid, making these sterols particularly vulnerable to conditions of oxidative stress. The relative reactivity of these sterol substrates can be understood based on steric, thermodynamic and stereoelectronic effects. Furthermore, the products of peroxidation can be predicted based upon the relative reactivity of abstractable hydrogens and the structures of intermediate carbon radicals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

7-DHC	7-dehydrocholesterol
8-DHC	8-dehydrocholesterol
SLOS	Smith- Lemli-Opitz syndrome
CDPX2	X-linked dominant chondrodysplasia punctata
DHCR7	7-dehydrocholesterol reductase
MeOAMVN	2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile)
HPLC	high-performance liquid chromatography
MS	mass spectrometry

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Figure 1.
Post-Lanosterol Biosynthesis of Cholesterol.

Figure 2.
Propagation Rate Constant for Peroxidation.

Figure 3.

Radical Clock for Determining k_p for Peroxidation. A. The mechanism of linoleate peroxidation, formation of *trans,cis* and *trans,trans* products (only one of the two possible *trans, cis* peroxy radicals is shown). Equation 1 describes the product ratio based on the concentration of H-atom donors present $[\text{R}_i - \text{H}]$ and the k_p of $\text{R}_i - \text{H}$. B. Product ratio as a function of linoleate concentration.

Figure 4.

The Propagation Rate Constant for 2-Methyl-2-heptene. $k_p = 5.6 \pm 0.2 \text{ M}^{-1}\text{s}^{-1}$ by the use of the Linoleate Clock. The structure of the substrate and plot of *trans,cis/trans,trans*-HODEs versus the concentration of 2-methyl-2-heptene are shown.

Figure 5. Results from measurement of propagation rate constants for A. Lathosterol and B. Zymostenol. Propagation rate constants were measured by plotting the ratio of *trans,cis-/trans,trans-HODEs* versus the concentration of the respective sterol. Propagation rate constants were calculated to be $57 \pm 3 \text{ M}^{-1} \text{ s}^{-1}$ for lathosterol and $77 \pm 5 \text{ M}^{-1} \text{ s}^{-1}$ for zymostenol.

Figure 6. Experimental and Estimated Propagation Rate Constants for the Peroxidation of Sterols on the Cholesterol Biosynthesis Pathway. Experimental rate constants are in **Bold**, estimated rate constants are in *[brackets]*. Values are in units of $\text{M}^{-1} \text{s}^{-1}$.

Figure 7.
3-D Structures of Oxidizable Sterols and Favored Radical Intermediates. Chemical and MM2-minimized structures of **A.** 7-dehydrocholesterol; **B.** 8-dehydrocholesterol; **C.** lathosterol; **D.** zymostenol. Favored radicals formed after peroxy radical H-atom abstraction are shown.

Figure 8.

Proposed Structures for Oxysterols Derived from Lathosterol, Zymostenol and Bloch sterol peroxidation at C24-C25. Primary products of free radical oxidation would be hydroperoxides, the corresponding alcohols are shown here.