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DOUBLY ALLYLIC HYDROPEROXIDE FORMED IN THE REACTION BETWEEN STEROL 5,7-DIENES AND SINGLET OXYGEN

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Abstract—Ergosterol and 7-dehydrocholesterol, common 5,7-conjugated diene sterols, react with photochemically produced singlet oxygen very efficiently to yield, in parallel pathways, the corresponding 5,8-endoperoxides and the 7β-hydroperoxy-5,8(9),22-trienol or -5,8(9)-dienol, respectively. The hydroperoxides decompose in an acid-catalyzed reaction to generate hydrogen peroxide and the 5,7,9(11),22-tetraenol or 5,7,9(11) trienol, respectively, with 1:1 stochiometry. The molar ratio of endoperoxide to hydroperoxide was constant (16:5) with two different reaction solvents, two different photosensitizers, and at all time points between 5 min and 3 h from the start of irradiation. Ergosterol did not react with either hydrogen peroxide or superoxide ion under our reaction conditions. Inhibition studies with nitrogen, 2,5-dimethylfuran, β-carotene, and *tert*-butanol confirmed the involvement of singlet oxygen in these reactions. The unstable hydroperoxide would be expected to have undesirable biological consequences if formed *in vivo*.

INTRODUCTION

Sterols containing a ring-bridging endoperoxy group (5,8-epidioxy sterols) are widespread in nature, being found in sponges,[1–3] tunicates,[3] coral,[4] the sea hare,[3] fungi[5,6] and lichens.[7] The most common representative of this structure is ergosta-5,8-epidioxy-6-ene-3β-ol (EEP).† The 5,8-epidioxy derivative may be produced in good yield when a 5,7-dienoic sterol such as ergosterol or 7-dehydrocholesterol is exposed to singlet oxygen.[8] These endoperoxides are relatively stable and are easily purified by column[5] or preparative thin-layer adsorption chromatography.[1] The side products have been reported to consist primarily of the highly fluorescent 9(11)-dehydro derivative of the parent sterol[9] or the 7-keto, 5,8-diene.[10]

5,8-Epidioxy sterols appear to be relatively nontoxic, but EEP is reported to be selectively toxic to some viruses[8] and tumor cells.[5,11] In the course of our efforts to synthesize a quantity of EEP through the reaction of ergosterol with photochemically produced singlet oxygen, we obtained evidence that the 7-keto and 9(11)-dehydro products described previously were not formed during the sterol–oxygen reaction. Instead, both types of products appeared to be handling artifacts derived from the 5,8-dienoic 7-hydroperoxide, which could be isolated and characterized under appropriate conditions.

It has been proposed that 5,7-dienoic sterols may interact with other compounds to serve as efficient singlet oxygen sensitizers *in vivo*, as well as reacting with the singlet oxygen so formed.[12] In such a case the cosynthesis of a product that is much more reactive than the endoperoxide might have toxicological significance.

MATERIALS AND METHODS

High-performance liquid chromatography (HPLC) was performed with an Eldex 9600 pumping system, a Rheodyne 7125 sample injector (20 μL loop), a 250 × 4.6 mm column packed with 5 μ Spherisorb C18 and a Varian 9060 diode array detector monitoring from 190 to 373 nm. The liquid phases were (a) methanol:water, 94:6 (vol/vol) and (b) acetonitrile:water, 95:5 (vol/vol), both at 1 cc/min. Samples were injected in methyl *t*-butyl ether (MTBE), which is miscible with both solvent systems. Peak areas were measured with a Hewlett-Packard model 3394A electronic integrator. All solvents were HPLC grade (from Burdick & Jackson, Muskeegan, MI).

Analytical thin-layer chromatography (TLC) was performed on Brinkmann Silplate 22 (250 μ) hard-layer silica gel GF (fluorescent) plates, developed in *n*-hexane:ethyl acetate 1:1 (vol/vol). Spots were visualized using iodine vapors, iodide/starch spray combination for peroxides,[13] 0.1% *N,N,N',N'*-tetramethylphenylene diamine (TMPD) in 50% aqueous methanol with 1% acetic acid at room temperature for hydroperoxides,[14] a saturated solution of 2,4-dinitrophenyl hydrazine (DNPH) in 2 *N* aqueous HCl for ketones and aldehydes (although some peroxides also react[13]), a saturated solution of phosphomolybdic acid in isopropanol for reducing compounds and a 5:5:3 (vol/vol/vol) mixture of sulfuric acid, nitric acid and water for charring at 130°C. Self fluorescence was sought under long-wave UV light and quenching of background fluorescence under short-wave UV (UltraViolet Products, Inc., San Gabriel, CA). Gas–liquid chromatography (GLC) of sterol trimethylsilyl (TMS) derivatives was as described previously.[15]

Proton NMR spectra were obtained in d_6-benzene using an NT-360 Fourier transform nuclear magnetic resonance (FT-NMR; Nicolet Magnetics Corp., 360 MHz) instrument. Benzene (7.16 ppm) was used as internal reference. Spectra were also obtained in $CDCl_3$ ($CHCl_3$, 7.26 ppm, internal reference) using a GT-500 FT-NMR (General Electric, 500 MHz). Attempts were made to obtain natural abundance ^{13}C spectra, but the sterol hydroperoxides were not sufficiently stable for the lengthy accumulation required.

Mass spectra were obtained at 70 eV using VG 12-250 or Kratos Concept 1SQ mass spectrometers with direct inlet probes. Ultraviolet-visible spectra were recorded on a Beckman DU-7 instrument

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†*Abbreviations:* CEP and EEP, 5α,8α-epidioxycholest-6-ene-3β-ol and 5α,8α-epidioxyergosta-6,22-diene-3β-ol, respectively; CHP and EHP, the compounds identified as the 5α-7-hydroperoxy-5,8-diene-3β-ols from 7-dehydrocholesterol and ergosterol, respectively; DHE, 9(11)-dehydroergosterol; DMPD, *N,N*-dimethylphenylenediamine; DMSO, dimethyl sulfoxide; DNPH, dinitrophenyl hydrazine; FT-NMR, Fourier transform nuclear magnetic resonance; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; MTBE, methyl *t*-butyl ether; TLC, thin-layer chromatography; TMCS, trimethylchlorosilane; TMPD, *N,N,N',N'*-tetramethylphenylene diamine; TMS, trimethylsilyl; TTMA-rose bengal, the di(tetradecyltrimethylammonium) salt of rose bengal; UTMA-eosin, the di(undecyltrimethylammonium) salt of eosin Y.

with 0.5 nm resolution, in addition to the low resolution spectra provided by the Varian diode array detector mentioned above. Fluorescence spectra were obtained using a Perkin-Elmer model MPF-3L fluorescence spectrometer and quartz cells. Infrared spectra were taken as dilute solutions in carbon tetrachloride, using a Perkin-Elmer model 1320 infrared spectrophotometer.

Sterols used as reference standards were primarily from the sources described previously.[15] 9(11)-Dehydroergosterol (DHE), 5,6-epoxy cholesterol, 7-keto cholesterol, 22-keto cholesterol and 6-keto cholestanol were from Sigma Chemical Co. (St. Louis, MO). Other sterols (5α-ergostan-3β-ol,[16] cholesterol-5-hydroperoxide,[17] the 7α-hydroperoxide[18] and EEP[3]) were synthesized by established methods. The di(undecyltrimethylammonium) salt of eosin Y (UTMA-eosin) and the di(tetradecyltrimethylammonium) salt of rose bengal (TTMA-rose bengal) were made as described by Bilski *et al.*[19] Halogenated solvents were passed through basic alumina (Fisher A540, activated at 130°C overnight before use) to remove any HCl that might be present. All reagent chemicals were of the highest purities available from Aldrich Chemical Co. (Milwaukee, WI) and Fisher Scientific (Raleigh, NC).

All laboratory operations except the actual irradiations were performed in the dark. Samples containing either UTMA-eosin at 40 μg/mL in MTBE or absolute ethanol, or TTMA-rose bengal at 17 μg/mL in ethanol, with sterol at 2 mg/mL (4.8 m*M*) were irradiated in open Pyrex glass flasks. The light source (15 cm from the sample solution) was a set of three evenly spaced incandescent lamps, color temperature 3200°C, providing a total output of 77.5 mW/cm^2. The geometry of the sample solutions was such that the surface area available for absorption of oxygen was at least 5 cm^2 with a depth not exceeding 0.7 cm. Oxygen was never rate limiting as evidenced by a linear rate of decrease in ergosterol concentration, culminating in its complete disappearance in 80–90 min. Potential inhibitors of the oxygenation reaction (nitrogen in place of air, 1 m*M* *t*-butanol, 1 m*M* 2,5-dimethylfuran and 0.01 m*M* *trans*-β-carotene) were tested by including them in the standard reaction mixture above, monitoring (by HPLC) the products at times ranging from 5 min to 3 h of irradiation.

Products for analysis by NMR, IR or mass spectrometry were purified from the reaction mixtures by preparative TLC on Anisil 1 mm thick plates (Analabs, Inc., North Haven, CT). The solvent system was *n*-hexane:ethyl acetate 1:1 (vol/vol) in unlined tanks. While the endoperoxide could be adequately purified in this way, the more polar reaction product, after elution from the TLC scrapings, was further purified on a column of CC-7 (pH 7) silicic acid (Mallinckrodt, St. Louis, MO), 1 g per 5 mg of sample, topped with anhydrous sodium sulfate. Fluorescent breakdown products that formed during TLC were eluted with *n*-hexane:MTBE, 80:20 (vol/vol) after which the desired product was eluted with *n*-hexane:MTBE 1:1 (vol/vol), 20 mL/g silicic acid.

Catalytic hydrogenation was accomplished in *n*-heptane over Adams catalyst with vigorous magnetic stirring under hydrogen at atmospheric pressure. Free hydrogen peroxide was measured using the scopoletin/peroxidase assay.[20] Total lipid peroxide was measured using DMPD as described by Dugan and O'Neill.[21] Enols were sought both by proton NMR (scanning the region from δ 10–18 ppm) and using the ferric chloride/chloroform assay.[13] The effect of trace acid was examined by bubbling HCl gas into chloroform, dissolving the sterol derivative and immediately removing the solvent in a rotary evaporator. All quantitative measurements are reported as mean ± SD (N = 3).

That cholesterol does not react with either superoxide or H_2O_2 is known,[22] but ergosterol had not been tested. A solution of ergosterol (2 mg/mL) in ether was shaken against an equal volume of 3% hydrogen peroxide for 1 h, centrifuged and the ether phase taken for analysis. A dimethyl sulfoxide (DMSO) solution of ergosterol (1 mg/mL) and potassium superoxide (tested for activity by its ability to form the blue formazan from nitro blue tetrazolium[23]), 0.2 mg/mL, was incubated at room temperature for 20 min in the dark. The products were extracted into MTBE and washed with water. Finally, ergosterol, 1.5 mg/mL in benzene, was treated with the benzene-soluble 18-Crown-6 complex of potassium superoxide[24] for 20 min in the dark. All products were examined by HPLC as above.

The EEP and DHE in reaction product mixtures were identified as ergosta-5,8-epidioxy-6-ene-3β-ol and 5,7,9(11),22-ergosta-tetraen-3β-ol, respectively, by comparison of their proton NMR spectra in $CDCl_3$ and in d_6-benzene, their 70 eV electron impact mass spectra, infrared and UV spectra, chromatographic properties on both HPLC and TLC and chemical reactivity on TLC plates to those of the corresponding reference compounds and to published reference data.[1,3,25,26] 9(11)-Dehydroergosterol was additional identified by its fluorescence spectrum and by co-GLC of its TMS derivative with that of the reference compound.

RESULTS

The HPLC chromatograms (methanol:water system monitoring at 215 nm) of the total products immediately after a 90 min irradiation of ergosterol with visible light in the presence of TTMA-rose bengal (17 μg/mL) and air with ethanol as solvent indicated that EEP and EHP represented 95% of the reaction products. Three minor products were seen; these are unidentified beyond the observation that their UV spectra show end absorption only. Ergosterol and DHE were absent. An essentially identical chromatogram resulted when 7-dehydrocholesterol was substituted for ergosterol except that the peaks, being based on end absorption, were proportionately larger for the ergosterol derivatives because of the additional side-chain double bond. Substituting UTMA-eosin (40 μg/mL) for TTMA-rose bengal made no detectable difference in the chromatographic profile. The only apparent effect from substituting MTBE for ethanol was a slight relative increase in the amounts of the minor peaks.

The Rf values for TLC of the ergosterol reaction products were: ergosterol = 0.86; DHE = 0.81; EEP = 0.56; EHP = 0.45; trace products = 0.34 and 0.25. Neither EEP nor EHP were fluorescent, and they showed little or no quenching of the silica gel GF fluorescent background.

The ratio of HPLC peak areas for EEP/EHP was constant for samples exposed to dye and light for 5, 15, 30, 60, 90 and 180 min. Ergosterol had disappeared by 90 min, indicating that the reaction products are themselves fairly unreactive to singlet oxygen. Integrated proton NMR spectra of the total reaction products from irradiation of either ergosterol or 7-dehydrocholesterol, while confirming the absence of DHE and CHE, indicated that the molar ratio of EEP to EHP was 2.85 ± 0.14, while the ratio of CEP to CHP was 2.88 ± 0.14 under these reaction conditions, regardless of the dye used as singlet oxygen sensitizer.

We looked carefully for the transient appearance of a second hydroperoxide at early time points and during irradiation at 0°C, analogous to the initial production of cholesterol-5-hydroperoxide isomerizing to the 7-hydroperoxide[18] but did not find it. Heating purified EEP at 60°C in MTBE or methanol did not cause any detectable decomposition.

Dimeric dehydrogenation products, commonly observed when either 7-dehydrocholesterol or ergosterol is irradiated at high concentration in ethanol, were not seen in these experiments (no poorly soluble products, no diols, no NMR spectral doublet of doublets for the C7 proton). The concentration of sterol, less than 5 μmol/mL, was well below that at which the condensation products are known to form.

Dimethylfuran (1 m*M*) reduced the initial rate of disappearance of ergosterol during irradiation in the presence of UTMA-eosin by 50%. β-Carotene at 0.01 m*M* concentration reduced the rate of ergosterol disappearance by 58.4 ± 2.1% measured at 30 min of irradiation. *Tert*-butanol at 1 m*M*

Table 1. Distinctive proton NMR peaks for EHP*

Functionality	In d-chloroform			In d_6-benzene		
	Shift, ppm	Type	J, Hz	Shift, ppm	Type	J, Hz
C18-methyl	0.661	s	—	0.677	s	—
C19-methyl	1.307	s	—	1.024	s	—
C21-methyl	1.036	d	6.48	1.112	d	6.60
C26-methyl†	0.819	d	7.21	0.913	d	6.67
C27-methyl†	0.834	d	7.47	0.918	d	6.71
C28-methyl	0.915	d	6.83	1.006	d	6.69
H-3	3.617	m		3.365	m	
H-6	5.698	d	1.44	5.693	d	2.15
H-7	4.675	bs‡		4.695	bs	
-OOH§	7.379	s		8.85	s	
H-22,23	~5.198	ddd	22.1, 10.3, <2	~5.264	ddd	obs
H-14	2.441	ddd	18.5, 10, 2.1	2.278	ddd	13.7, 8.3, 3.2

*For the concentration range 1–2 mg/mL. Spectra in $CDCl_3$ obtained using a 500 MHz instrument; spectra in d_6-benzene obtained with a 360 MHz instrument. s = Singlet; d = doublet; m = multiplet; bs = broad singlet or unresolved doublet.
†Could be reversed.
‡Width equal to combined H-6 doublet.
§Very concentration dependent. This is for 2 mg/mL.

concentration had no effect on the rate of ergosterol disappearance. There was no ergosterol disappearance when the irradiation was performed under nitrogen.

The EHP decomposed over a period of several hours at room temperature in neutral chloroform or methylene chloride to give only DHE. This conversion was extremely rapid (complete in less than 5 min) when either the total reaction products or purified EHP was placed in chloroform containing 5×10^{-4} *M* HCl. In contrast, the compound was stable for at least several days at −20°C in neutral, nonhalogenated solvents (MTBE, ethanol, benzene).

Known amounts of EHP were dissolved in 2 mL of chloroform containing 5×10^{-4} *M* HCl and allowed to decompose for 15 min at room temperature. The solutions were partitioned against 5 mL of HPLC-grade water, centrifuged and aliquots of the aqueous phases assayed for free H_2O_2.[20] Controls included equivalent amounts of similarly processed ergosterol, known aqueous solutions of H_2O_2 and the same solutions back extracted with chloroform. Recovery of H_2O_2 was 94.5 ± 8.7%, rather variable, possibly due to the extremely dilute solutions employed (~1–2 μmol/mL). The chloroform phases from the EHP samples were monitored for production of DHE (λ_{max} 326.5 nm in $CHCl_3$, ϵ = 12 100). We found 3.08 ± 0.23 μmol of H_2O_2 and 3.13 ± 0.06 μmol of DHE, indicating 1:1 stoichiometry. No hydrogen peroxide (less than 0.25 μmol, the lower limit of measurement under these conditions) or DHE were produced from the ergosterol control. Neither DHE nor EHP are extracted from chloroform into water under these conditions, so the scopoletin assay should be specific for H_2O_2.[20]

Identification of EHP

The *N,N*-dimethylphenylenediamine (DMPD) assay indicated that after 90 min of irradiation the reaction products from 24.0 μmol of ergosterol contained 23.0 ± 0.9 μeq of peroxyl group. Thus all of the major products appeared to be peroxides or hydroperoxides. Enols were not detected with ferric chloride, while neither enols nor epoxides were detected in the NMR spectra. There was no detectable reaction of ergosterol with either H_2O_2 or superoxide under the conditions described. None of the vitamin D-related intermediates could be detected by HPLC in the acetonitrile : water system in any of the experiments described here, confirming that light below 300 nm wavelength did not reach the samples.

The EHP reacted rapidly and strongly with both TMPDA and iodide/starch reagents on TLC but was initially negative to phosphomolybdate. It reacted slowly with 2,4-DNPH to give a brown rather than orange product. This behavior is typical of hydroperoxides. Treatment with acid, or with alcoholic $NaBH_4$, or attempted direct probe introduction into the mass spectrometer caused rapid breakdown to DHE, while attempted hydrogenation resulted in hydrogenolysis.

The proton NMR spectra of EHP are described in Table 1. The single proton associated with a ring double bond is assigned to C6 on the bases of the chemical shift for the C3 proton[1,3,11] and the splitting due to coupling to the proton at C7. The extreme shift of the C19 methyl group implies the presence of a double bond at C9, but it is greater than would be expected for a 9(11) double bond; therefore the double bond is assigned to C8(9). The hydroperoxyl group is assigned to C7 again because of the coupling to the C6 proton, the necessity for this compound to be able to decompose with loss of H_2O_2 to give DHE and the absence of a distinct UV peak above 200 nm, indicating the absence of conjugated double bonds. Attack at C5, C8, C9 or C11 would have left the double bonds conjugated; attack at C6 would have produced a C4(5) double bond that would have conspicuously affected the chemical shift and multiplicity for the C3 proton. The peaks associated with the side chain methyl groups and the C22 double bond were consistent with there having been no change in the side chain from the original ergosterol structure. The presence of a proton at C14 and the absence of a large downfield shift in the peak assigned to the C-18 methyl group ruled out all structures with a C8(14) double bond.

Since the proton NMR peak associated with the C6 hydrogen was a single sharp doublet, it appears that only a single epimer of the C7 hydroperoxyl was formed in these

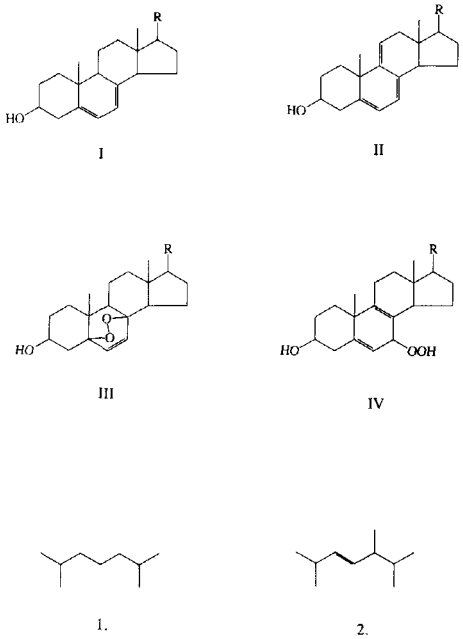

Figure 1. Structures of the compounds discussed in this paper. When R = side chain 1, the compounds are derivatives of 7-dehydrocholesterol; if R = 2, the compounds are derived from ergosterol. Ring structure I represents the parent compounds, II the dehydro forms (5,7,9[11]-cholestatrien-3β-ol, DHE). Ring structure III corresponds to the 5,8-epidioxy derivatives CEP and EEP, while ring structure IV represents the 7-hydroperoxy-5,8(9)-en-3β-ols CHP and EHP.

experiments. The very small J value for the C6–C7 proton coupling suggests that the C7 proton is quasiaxial,[27] so the hydroperoxy group should be quasiequatorial, *i.e.* 7β. Failure to detect multiple forms of hydroperoxide on HPLC, under conditions giving complete resolution of ergosterol and its 5β isomer lumisterol (retention times of 20.0 and 23.0 min, respectively, in the acetonitrile: water system) suggests that ring opening is probably not involved in the oxygenation reaction.

Infrared spectra of EHP showed small, sharp peaks at 3618 and 3520 cm^{-1}. More concentrated solutions showed a broad peak at 3350 cm^{-1} and a sharper peak at 3430 cm^{-1}. Thus there appear to be -OH and/or -OOH groupings in two environments, one of which may be less prone to hydrogen bonding than the other. The peak thought to be associated with C-O-O- was at 849 cm^{-1}, compared to the peak at 859 cm^{-1} for EEP or the pair of peaks at 835, 870 cm^{-1} for cholesterol 5,6-epoxide. The side chain double bond appeared to be *trans* (970 cm^{-1}) as in ergosterol itself.

We have discussed only the ergosterol-derived products in detail. Virtually identical results were seen with 7-dehydrocholesterol. The corresponding products comigrate on TLC and show the same colorimetric reactions. 5,7,9(11)-Cholestatrien-3β-ol is formed from CHP under the same conditions giving DHE from EHP.

The structures proposed for EHP and CHP are shown along with other relevant structures in Fig. 1. These structures are compatible with all of the spectral and chemical data but must be considered tentative in the absence of reference standards for direct comparison.

DISCUSSION

We consider the 7-hydroperoxide to be a primary product of the reaction of sterol 5,7-dienes with singlet oxygen for several reasons. First, it is formed in parallel with and at a constant ratio to the endoperoxide, a known $^1O_2$ reaction product.[1,3] Its formation requires molecular oxygen. The reaction is strongly inhibited by both 2,5-dimethylfuran, a chemical quencher, and β-carotene at low concentration, a physical quencher of singlet oxygen.[28] The reaction is not inhibited by *t*-butanol, an inhibitor of free radical reactions. The product ratio (endoperoxide/hydroperoxide) was the same whether eosin Y or rose bengal was the photosensitizing dye; these differ in the extent to which they produce superoxide as a side product under irradiation.[29] The sterols are apparently resistant to reaction with both H_2O_2 and superoxide. The hydroperoxide was not formed by decomposition of the endoperoxide.

Gorman *et al.*[12] have reported that the rate of quenching of $^1O_2$ by ergosterol in the millimolar concentration range is approximately 2.1×10^7 L mol^{-1} s^{-1} in benzene, as compared to a value of 1.1×10^7 in pyridine.[30] The reaction rate for 2,5-dimethylfuran is usually given as $1–2 \times 10^8$.[31] In our study 1 m*M* dimethylfuran reduced the initial rate of oxidation of 4.8 m*M* ergosterol in ether by 50%, which is quite compatible with the reported relative reactivities.

The doubly allylic hydroperoxide was made even less stable by our attempts to derivatize it. If the reaction mixture was treated with $NaBH_4$, then as soon as the resulting products were spotted on a thin-layer plate the presumptive diol decomposed to DHE. The hydroxyl derivative lost water more easily than the original hydroperoxide lost H_2O_2. It was not possible to hydrogenate the isolated double bonds of the hydroperoxide. This may be explained by the tendency of an 8(9) unsaturated steroid to isomerize in the presence of an activated metal catalyst to an 8(14) double bond, which is not ordinarily reducible. Any derivatization reaction involving an acid catalyst, for example trimethylsilylation in the presence of trimethylchlorosilane (TMCS), caused breakdown to multiple products including dehydrosterols.

If the 7-OOH had formed by allylic isomerization from a 5-OOH precursor, one would have expected a 7α configuration by analogy with that of cholesterol hydroperoxide.[18] But the equatorial center is more easily attacked directly for C7, and the equatorial (β) substitution product is also more stable than the axial epimer.[32]

It seems therefore that the 7-hydroperoxide is formed by a mechanism analogous or identical to an ene reaction in which singlet oxygen attacks from the α face of the sterol molecule, directed by steric hindrance from the C-19 methyl group, *i.e.* attack by singlet oxygen at C7 to cause a shift in the 7(8) double bond to the 8(9) position, with transfer of a proton to the resulting anion to yield the hydroperoxide.[33]

Dauben and Fonken[9] stated that formation of endoperoxide from compounds such as ergosterol is always accompanied by production of the 9(11)-dehydro compound. This appears to be incorrect, as long as acidic conditions or long standing at room temperature are avoided.

Suzuki and Tsuda[10] irradiated 3-acetoxy-7-dehydrocholesterol in the presence of eosin I in oxygen-saturated ethanol, with light from a 300 W tungsten lamp for 2.5 h at 40–60°C,

then separated the products by fractional crystallization. Their second most abundant product was identified as 3-acetoxycholest-5,8-dien-7-one (UV λ_{max} 246.5 nm). The analogous product was subsequently found when ergosterol was substituted for dehydrocholesterol[34]; in this case the alcohol was irradiated and the products treated with acetic anhydride before fractionation. These authors speculated that the 7-hydroperoxide might have been an intermediate in formation of the ketone but did not observe it directly. We did not observe any products having UV λ_{max} near 246 nm under our reaction conditions. However, passing the ergosterol irradiation products through activated A540 basic alumina resulted in essentially complete conversion of the 7-hydroperoxide to a slightly less polar compound having UV λ_{max} at approximately 247 nm, and infrared at 1661, 1611, 1595 cm^{-1}. This was probably the 7-keto-5,8,22-triene but was not fully characterized in this study. Treatment with acetic anhydride would also be expected to dehydrate the hydroperoxide to a ketone.[27]

Smith[22] speculated that the various oxidation products of 7-dehydrocholesterol were all derived from the 5α, 8α-endoperoxide, since no other primary reaction products of this compound with molecular oxygen were known. The 7-hydroperoxide now appears to be a more versatile precursor.

The instability of the 7-hydroperoxides would be expected to preclude their detection in extracts of biological material. Formation of the hydroperoxide would be expected to have biological consequences through its oxidizing ability, potential enzyme inhibition,[35] condensation reactions and through its decomposition to hydrogen peroxide.

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