Photochemistry and photophysics of cholesta-5,7,9(11)-trien-3 β -ol: a fluorescent analogue of cholesterol

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Abstract

Cholesta-5,7,9(11)-trien-3 β -ol (9,11-dehydroprovitamin D₃, CTL) is used as a fluorescent probe to track the presence and migration of cholesterol in vivo. CTL is known to be photochemically active, but little consideration has been given to the formation efficiency and possible toxicity of its photoproducts. In degassed tetrahydrofuran (THF) solution, we isolated the photoproduct of CTL and of its 25-hydroxy derivative (HOCTL), and X-ray crystal structures were obtained for HOCTL and the photorearrangement product. The X-ray crystal structure and its ¹H NMR spectrum confirm the product structure as a pentacyclic HOCTL isomer. In the presence of air in THF, endoperoxide formation via [2+4] addition of ¹O₂* across the B ring of CTL or HOCTL becomes the dominant photoreaction. The UV spectrum and decay kinetics of the triplet state of HOCTL, the precursor of ¹O₂*, are determined by transient absorption spectroscopy. We confirm the proposed structure of the endoperoxide by X-ray crystallography. Kinetics analysis of quantum yields provides rate constants for photophysical and photochemical events.

Graphical abstract

Keywords Cholestatrienol rearrangement · Endoperoxide photoproducts · Fluorescence triplet yields

1 Introduction

The natural products cholesta-5,7,9(11)-trien-3 β -ol (CTL) and its close relative 9(11)-dehydroergosterol (DHE) differ from provitamin D₃ (Pro) and ergosterol (E), respectively, by the additional C₉–C₁₁ double bond [1–6]. The cholestane skeleton lends rigidity to their embedded conjugated triene moieties increasing S₁ lifetimes by diminishing torsional relaxation, thereby rendering them fluorescent [7]. Their structural similarity to cholesterol, Chart 1, accounts for their use as ideal fluorescence probes for studies of cholesterol trafficking and membrane organization in vivo [8–11]. Their

fluorescence intensity is diminished on prolonged excitation leading to photobleaching and fluorescence recovery studies [12]. The photoproducts obtained on direct excitation of CTL and DHE in vitro were investigated in pioneering studies by the Windaus and Barton groups [13–15]. We determine the correct diastereomer of the structure, Scheme 1, proposed by Barton and Kende for the photoproduct [15]. Windaus and Linsert also reported the eosin photosensitized oxidation of DHE in ethanol [13]. The accepted structure of the photooxidation product (DHEP, 5 α ,8 α -epidioxy-22-*E*-ergosta-6,9(11),22-trien-3 β -ol) was proposed by Fieser [16], before the role of ¹ Δ_g singlet oxygen (¹O₂*) in such reactions was known. ¹O₂* luminescence observed on irradiation of an air-saturated solution of CTL in CCl₄ established formation of CTL triplets that transfer energy to ³O₂ [15]. It was suggested that CTL, naturally present in skin, may contribute to skin photoaging and UV-induced skin cancer by sensitizing

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the formation of $^1\text{O}_2^*$ [17]. Furthermore, hydroperoxide formation via the $^1\text{O}_2^*$ oxidation of ergosterol may be a route to DHE [18]. The peroxides of CTL and DHE, CTLP, and DHEP, respectively, are also known natural products [5, 19]. Both ergosterol peroxide, EP, and DHEP have been shown to be cytotoxic [19].

In view of their wide use as fluorescence probes, there has been surprisingly little consideration of the formation efficiency and possible toxicity of CTL and DHE photoproducts. In this paper, we identify photoproducts formed following direct excitation of CTL and HOCTL in degassed and air-saturated THF solutions. A 1930 study that reported the UV evolution of the photochemical loss of DHE in the course of its direct excitation in ethanol [20] found the λ_{max} of the photoproduct mixture at 243 nm instead of at 261 nm, as expected for the major isolated photoproduct [13–15]. The participation of ethanol in the photoreactions of CTL and HOCTL leads to several additional photoproducts and will be addressed separately [21]. We show here that on irradiation in degassed THF, HOCTL quantitatively rearranges by 9,10 σ -bond cross-addition to the 5,6 π -bond to give the pentacyclic isomer (3*R*,3*aR*,5*bS*,7*S*,9*aS*,9*bS*)-3-((*R*)-6-hydroxy-6-methylheptan-2-yl)-3*a*,9*a*-dimethyl-1,2,3,3*a*,4,6,7,8,9,9*a*,9*b*,10*b*-dodecahydrobenzo[2,3]cyclopropa[1,2-*b*]-*as*-indacen-7-ol (HORP, $\lambda_{\text{max}} = 261$ nm). In the presence of air, at sufficiently high [CTL], peroxide formation dominates. The 25-OH substituent does not influence the photochemistry or the photophysics, Scheme 1.

Scheme 1 Photochemistry of HOCTL in THF

2 Experimental section

2.1 Materials

Unless otherwise stated, reagent grade solvents from commercial sources were used as received. We recrystallized 7-dehydrocholesterol (provitamin D₃, Pro, US Biochemical Corp.) from ethanol. 25-Hydroxyprovitamin D₃ (Isogen, 96.5%) was recrystallized twice from methanol and twice from a 3/1 ethyl acetate/hexane mixture. Alternatively, purification was by column chromatography on silica. For spectroscopic measurements, purification was by HPLC. Tetrahydrofuran (THF, Sigma-Aldrich, ACS reagent, 99%, containing 250 ppm BHT as inhibitor) was freshly distilled prior to use. Ethanol (Pharmco-AAPER) was 200 proof, absolute/anhydrous. CHCl_3 (Aldrich) and glacial acetic acid (Fisher) were used as received. $\text{Hg}(\text{OAc})_2$ (Acros) and benzophenone (Fisher Scientific, 99.69% GC purity) were used as received.

2.2 Analytical methods

Analysis of product compositions was done by GC, by ^1H NMR, by UV–Vis, by fluorescence, by MS, and by GC/MS. We used a Bruker/Varian CP-3800 gas chromatograph equipped with an electronic integrator for CTL and for stilbene actinometry. Semi-preparative HPLC was performed using a Beckman Coulter System Gold 125 solvent module with a System Gold 168 detector. UV–Vis absorption spectra were measured on a Varian Cary 300B Spectrometer. Fluorescence spectra were measured using a Horiba Fluoromax 4 fluorometer. Fluorescence lifetimes were determined using a different Horiba Fluoromax 4 instrument equipped with a time-correlated single-photon counting accessory and an R928 PMT detector (Hamamatsu). The light source was a 296 nm nanoLED (Horiba) having a pulse duration of < 0.75 ns and 1 MHz repetition rate. Photon count was set at 10,000 and the time-to-amplitude converter range was set at 50 ns. The instrument response function was obtained by collecting Rayleigh scatter at 296 nm. Fluorescence lifetimes were determined by reconvoluting the instrument response function with exponential decay using DAS6 (Horiba) fluorescence decay analysis software. The quality of the fits was judged by χ^2 values, standard deviations of derived lifetimes, and visual inspection of the residuals. Absolute fluorescence quantum yields were measured with the use of a Hamamatsu Quantaurus-QY Spectrometer equipped with a 150 W Xenon arc lamp. The instrument employs an integrated sphere sample chamber with a cooled back-thinned 1024 channel charge coupled device sensor as detector. It avoids the need for a fluorescence standard using the attenuation in the area

of the Rayleigh scattered light peak at $\lambda_{\text{exc}} = 325$ nm to measure photon absorption. HOCTL solutions were outgassed with oxygen or argon for 2 h prior to quantum yield and lifetime measurements. Transient absorption measurements and triplet decay kinetics were measured using an Edinburgh Instruments LP980-KS Laser Flash Photolysis Spectrometer. A continuum Nd:YAG laser provided the excitation pulse and transient absorption was monitored with a 150 W Xenon lamp. Bruker 500 MHz NMR spectrometer was used to measure NMR spectra in CDCl_3 . We used a Bruker Autoflex III MALDI TOF Mass Spectrometer for matrix-assisted laser desorption/ionization measurements.

2.3 Crystallography

HOCTL, HOPR, and HOCTLP crystals were mounted on a nylon loop and centered in the beam of 0.71073 Å Mo X-rays. After indexing, frame data were obtained on a Bruker SMART APEX 2 diffractometer at 103 K using a detector distance of 6 cm. Frame collection times of 40 and 60 s were used in an effort to obtain data at higher angles. The data-to-parameter ratio was always more than 10:1. The integration was performed using the program SAINT, which is part of the Bruker suite of programs. An absorption correction was done by SADABS and the program XPREP used to determine the space group. The structures were solved by direct methods and refined by SHELXTL. The non-hydrogen atoms were refined anisotropically. The hydrogen atoms were assigned except for those on the oxygen atoms of the hydroxyl substituents and of water solvent molecules.

2.4 Irradiation procedures

Sample preparation and degassing procedures were described previously [22]. We used Hanovia reactors for preparative experiments and a Moses merry-go-round [23] apparatus, immersed in a thermostated water bath, for quantum yield measurements. We employed medium pressure 200-W and 450-W Hg lamps. The 313 nm Hg line was isolated using a potassium perchromate/potassium carbonate filter solution [22]. The *trans* → *cis* photoisomerization of stilbene in pentane ($\lambda_{\text{exc}} = 313$ nm) was used for actinometry, $\phi_{\text{tc}} = 0.52$ [22]. Pyrex tubes, 13 mm o.d., fitted with standard taper joints and grease traps were loaded with 3.0 mL aliquots of solutions. They were degassed using 4–6 freeze–pump–thaw cycles to $< 10^{-4}$ Torr and flame sealed at a constriction. All operations, including analyses, were performed under nearly complete darkness (red light). Ar, N_2 , and O_2 outgassed solutions were used in some experiments.

3 Results

3.1 Synthesis of CTL and HOCTL

The dehydrogenation of ergosterol to DHE using mercuric acetate was first described by Windaus and Linsert [13] and the method was applied to the conversion of several $\Delta^{5,7}$ -steroids, including Pro, to the corresponding $\Delta^{5,7,9}$ -steroids by Antonucci et al. [24] We based our synthesis on the somewhat modified version described by Fischer et al. [7] The following procedure for the synthesis of CTL is typical: Pro (2.0 g, 5.2 mmol) and $\text{Hg}(\text{OAc})_2$ (3.0 g, 9.4 mmol) were added to 40 mL of CHCl_3 in a three-necked 100 mL round-bottom flask equipped with a stirring bar and rubber septa at the three necks. Glacial acetic acid (17 mL) was added to the solution, and after the reaction mixture was outgassed with N_2 using entry and exit syringe needles, it was placed in a thermostated oil bath. The reaction mixture was stirred under N_2 at 40 °C for 18 h. The progress of the reaction was followed by TLC (10% ethyl acetate/hexanes). Precipitated mercury salts were removed by filtration and washed with CHCl_3 . The combined filtrate and washings were transferred to a 100 mL Erlenmeyer flask and neutralized using a saturated aqueous NaHCO_3 solution. The organic layer was separated and the aqueous layer was washed 3 × with CHCl_3 . After drying the combined organic solutions using anhydrous sodium sulfate, we removed the solvent under reduced pressure to obtain 1.54 g of a brown gummy material. This residue was purified by repeated column chromatographies on silica using progressively increasing ethyl acetate content in hexanes (up to 6%, initially and up to 3% in the third chromatography) eluents were monitored by UV–Vis. The final yield was 400 mg (20%) of CTL. HOCTL was similarly obtained with the exception that, due to its greater polarity, the ethyl acetate eluent was replaced by ethanol. In numerous runs, yields of CTL and HOCTL never exceeded 30%. The starting materials Pro and HOPro eluted very close to the CTL and HOCTL products and were exceedingly difficult to remove completely by column chromatography. This was accomplished for the final experiments by the use of semi-preparative HPLC. Residual HOPro in initial HOCTL samples did not interfere with fluorescence and most photochemical measurements, because its UV absorption is well to the blue of CTL (Fig. S1 in the Supporting Information, SI). For quantum yield and X-ray measurements, further purification of CTL and HOCTL was done by HPLC and by recrystallization. We obtained crystals of HOCTL suitable for X-ray diffraction analysis from CH_3CN and benzene in 3:1 ratio with a drop of deionized H_2O . Figure 1 shows the X-ray HOCTL structure.

Fig. 1 The X-ray structure of HOCTL

Table 1 O₂ Quenching of HOCTL fluorescence in THF, 20 °C

	10 ³ [O ₂]/M	ϕ_f	τ_f /ns	χ^2	10 ⁻⁸ ϕ_f/τ_f
Ar	0	0.033 ₅	0.22 ₅	1.24	1.49
Air	1.72	0.032 ₄	0.22 ₁	1.18	1.47
O ₂	8.27	0.029 ₂	0.20 ₈	1.08	1.40

Fig. 2 Irradiation of **a** [HOCTL]₀ = 5.23 × 10⁻⁵ M in THF; **b** the UV spectrum of pure HORP in THF

3.2 Fluorescence measurements

We determined the effects of O₂ on HOCTL fluorescence quantum yield and lifetime by measuring the fluorescence of Ar-bubbled, air-saturated and O₂-bubbled 1.05 × 10⁻⁵ M THF solutions at room *T* (20.3 °C), Table 1. The oxygen and the argon-saturated HOCTL solutions were outgassed with O₂ and Ar for 2 h prior to the quantum yield ($\lambda_{\text{exc}} = 325$ nm)) and lifetime measurements. A pre-bubbler was employed to minimize solvent loss. For the lifetime measurements, samples were excited with a 295 nm, < 0.75 ns diode pulse, and fluorescence decay was monitored at 367 nm. The contribution of a small (< 3%) longer lived component, $\tau = 11.9$ ns under Ar, diminishes and its lifetime decreases as the concentration of oxygen is increased. Lifetime and χ^2 values are given in Table 1.

3.3 Photochemical observations

The degassed THF solution (six freeze–pump–thaw cycles to < 10⁻⁴ Torr and flame sealed at a constriction) was irradiated in a 13 mm Pyrex test tube provided with a side-arm to which a 0.50 × 1.0 cm quartz cell was attached via a graded seal. Figure 2 shows the UV evolution of HOCTL

Fig. 3 X-ray crystal structures of HORP (upper) and HOCTLP

irradiated at $\lambda_{\text{exc}} = 313$ nm at 23.5 °C. The concentration given in the caption of Fig. 2 is based on our measured molar absorptivity coefficient of HOCTL at $\lambda_{\text{max}} = 324$ nm (1.1₀ × 10⁴ M⁻¹ cm⁻¹, Fig. S2 in SI). Figure S3 in SI gives the Beer–Lambert plot for HORP in THF.

The MALDI spectrum measured for CTL photoproducts in air-saturated THF shows *m/z* peaks at 404.72 and 436.78 for rearrangement product RP (382 for CTL+23 for Na⁺) and peroxide product CTLP (382 for CTL+23 for Na⁺ + 32 for 2xO), respectively, (Fig. S4 in SI). Together, the two spectra account for all product peaks in the ¹H NMR spectrum of the product mixture in degassed solutions. The ¹H NMR spectrum of RP (CDCl₃, 500 MHz): 5.42 (s, 1H); 5.35 (dd, 1H, *J* = 5.4 Hz); 3.68 (“q”, 1H, *J* = 9.5 Hz); 2.37–2.31 (m, 2H); 2.25 (“t”, 1H, *J* = 8.9 Hz); 2.10 (“d”, 1H, *J* = 17 Hz); 0.92 (d, 3H, *J* = 6.5 Hz); 0.87–0.85 (2 doublets, 10H); 0.54 (s, 3H). Due to the difficulty in separating CTL and HOCTL from their Pro and HOPro precursors, some experiments were performed with 90/10 CTL/Pro mixtures. The presence of ~ 10% Pro contamination is readily observed as structure at $\lambda < 310$ nm in UV spectra (Fig. S1 in SI) and in ¹H NMR spectra.

We observed no differences in irradiations of similar solutions of HOCTL and CTL, indicating that the 25-OH substituent does not influence photochemical and photo-physical properties. HORP is the sole photoproduct in carefully N₂-outgassed or degassed THF and the endoperoxide, HOCTLP, is the major photoproduct in air-saturated THF, provided that a relatively high concentration of HOCTL is employed (~ 6 × 10⁻³ M). As in the case of HOCTL, the presence of the 25-OH substituent led to X-ray diffraction quality crystals and enabled definitive product identification. Crystallization of HORP from a 3:1 wet CH₃CN/benzene mixture afforded small transparent monohydrate crystals. The structure obtained from the X-ray diffraction of these crystals is shown in Fig. 3 and Scheme 1. It differs from the structure proposed by Barton et al. in the stereochemistry

Fig. 4 The UV spectrum of HOCTL in ethanol

of the bond-crossing reaction. Crystals of HOCTL were obtained from an ethanol/deionized water mixture X-ray analysis revealed the structure in Fig. 3 in agreement with Fieser's proposal [16]. The UV spectrum of HPLC-purified HOCTL in ethanol, Fig. 4, shows λ_{max} at 202 nm, a shoulder at 215 nm and very weak featureless absorption in the 250–350 nm region. Previously reported longer wavelength bands for CTLP, DHEP and the peroxides of their provitamin D precursors are not likely to be correct [19]. Ashour et al. gave λ_{max} at 204 nm, for ProP in methanol [25], but their 243 nm peak is not supported by our spectrum. The spectrum for DHEP by Albro et al. of end absorption with a shoulder at 222 nm [26] is closer to our own.

3.4 HOCTL triplet

A 3 mL aliquot of a THF solution containing 5.0×10^{-3} M HOCTL and 2.5×10^{-3} M benzophenone was transferred into a Pyrex tube equipped with a standard taper joint, provided with a grease trap and attached via a graded seal to a standard 1.0 cm² UV cell. The solution was degassed using five freeze–pump–thaw cycles to $< 10^{-4}$ Torr and flame sealed at a constriction. Pulse excitation at 370 nm led to transient spectra recorded periodically in the 0–100 μs time scale, Fig. S5 in SI. The bleach observed at 0-time is probably due to stimulated fluorescence from $^1\text{HOCTL}^*$ formed by direct excitation due to the high [HOCTL] employed. At longer times, we observed the known transient absorptions of the benzophenone triplet and the benzophenone ketyl radical [27, 28], and a new transient absorption, assigned to $^3\text{HOCTL}^*$, with $\lambda_{\text{max}} = 395$ nm and a shoulder at 416 nm, Fig. 5a. Decay monitored at 395 nm, Fig. 5b, is monoexponential and gives $\tau = 80$ μs . The small peak at 452 nm does not belong to this transient as it does not appear when HOCTL is excited directly or when benzophenone triplet energy transfer to HOCTL is in ethanol [21].

3.5 Photoproduct quantum yields

The quantum yield of RP formation was measured using HOCTL contaminated with 11% Pro based on ^1H NMR and UV analysis. Three mL aliquots of degassed HOCTL

Fig. 5 **a** The $^3\text{HOCTL}^*$ transient. **b** Triplet decay at 395 nm

solutions, 3.92×10^{-3} M in THF were irradiated in parallel in the Moses merry-go-round [23] together with a 3.0 mL 4.27×10^{-2} M degassed pentane solution of *trans*-stilbene (0.011% *cis*-stilbene by GC), used for actinometry. The samples were immersed in a water bath whose T was maintained at 25 °C. A 450-W Hanovia lamp was employed together with the 313 nm filter solution. Pro is transparent at $\lambda_{\text{exc}} = 313$ nm and does not interfere in these measurements. Following 12 h irradiation, HOCTL conversion to HOPR (8.3% – see the Supporting Information) was measured by ^1H NMR and *trans* → *cis*-stilbene conversion ($11.6 \pm 0.2\%$) was measured by GC. Correction for back reaction as previously described [22] gave $12.4 \pm 0.2\%$ *cis*-stilbene. With the use of $\phi_{\text{ic}} = 0.52$ in the actinometer [22], we obtain $\phi_{\text{RP}} = 0.025_2$. An independent experiment carried out as above but using pure [HOCTL] = 1.90×10^{-3} M, [*t*-St] = 4.01×10^{-2} M and the 550 W Hanovia lamp gave 9.3 ± 0.2 and $8.8_8\%$ conversions for RP and *c*-St (corrected for back reaction), respectively, yielding $\phi_{\text{RP}} = 0.025_7$.

Initial observations revealed that, in the presence of air, the relative yield of peroxide formation increases at higher CTL or HOCTL concentrations. Accordingly, quantum yields of HOCTL formation, ϕ_{per} , were obtained at three [HOCTL] using HOCTL contaminated with 10% HOPR in air-saturated THF solutions. Three mL aliquots were irradiated at 313 nm in the merry-go-round in parallel with degassed *trans*-stilbene actinometry solutions, 0.026 M in pentane. The highest HOCTL concentration was irradiated twice as long. Stilbene conversions, corrected for back reaction, were 6.9 ± 0.3 and $12.9 \pm 0.7\%$ for 2 and 4 h irradiation, respectively. Conversions to peroxide were determined by ^1H NMR using the areas of vinyl protons (δ 5.7 for HOCTL and 6.31 and 6.62 for HOCTLP). The quantum yields are shown in Table 2. The [HOCTL] values in Table 2 are based on the absorbance of the solutions at 324 nm using $\epsilon_{324} = 1.1_0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The first row in Table 2 gives results from a separate analogous experiment in which air- and O₂-saturated THF solutions of pure HOCTL were irradiated in parallel with the *trans*-stilbene actinometer.

Table 2 Peroxide (HOCTL) quantum yields

[HOCTL]/mM	$\phi_{\text{per Air}}$	$\phi_{\text{per O}_2}$	$\tau_{\Delta}/\mu\text{s}$ (see text)
1.12	0.059 ₄	0.080 ₅	30
1.31 ^a	0.058		28.4
1.96 ^a	0.083		27.6
3.92 ^a	0.110		25.6

^aSolutions are contaminated by 11% HOPro—see text

4 Discussion

Windaus and Linsert isolated photodehydroergosterol (RP) in 40% yield from the UV irradiation of DHE [13]. Their UV spectrum agrees with our spectrum in Fig. 2b except that our minimum at 220 nm is less deep. Conjugation of the diene moiety with a cyclopropane ring was inferred from this UV spectrum by Barton and Kende who used chemical reactions and IR spectra to eliminate two other possible structures in assigning the structure to RP, a remarkable pre-¹H NMR achievement. Our X-ray structure in Fig. 3 confirms the proposed cross-addition of the C₉C₁₀ σ -bond to the C₅C₆ π -bond and provides correct orientational detail. The product evolution in the UV spectra in THF in Fig. 2a is significantly different from that reported by Morton et al. in ethanol [20]. The shift of the product λ_{max} from 243 nm in ethanol to 261 in THF reflects mainly the formation of addition and reduction photoproducts in ethanol that are absent in THF [21]. The presence of an isosbestic point in Fig. 2a shows that the photoproduct composition does not change in the course of the reaction. GC (used for CTL only) and ¹H NMR analyses were consistent with formation of RP as the sole product in degassed THF solution. In Fig. 2a, quantitative conversion of HOCTL to HORP predicts $\epsilon_{261} = 7.8_7 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for HORP. Our independently measured value for HORP in THF, $\epsilon_{\text{max}} = 7.8_5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (Beer–Lambert plots for HOCTL and HORP in THF are given in Figs. S2 and S3, respectively, in the Supporting Information) is in excellent agreement with this value. A much higher value of $\epsilon_{\text{max}} = 1.13 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ was reported by Barton and Kende for the acetate of RP from ergosterol at $\lambda_{\text{max}} = 260 \text{ nm}$ in ethanol [15].

4.1 Mechanism

Equations (1, 2, 3, 4, 5, 6) account for the photochemistry and photophysics of CTL and HOCTL in degassed THF.

In the presence of ³O₂, Eqs. (7, 8, 9, 10) augment this sequence.

In Eq. (7) quenching of the triplet state is shown, tentatively, with concomitant formation of ¹O₂^{*}. This is possible [29] when the S₁–T₁ energy gap exceeds 22.6 kcal/mol, the energy of ¹Δ_g ¹O₂^{*} [30], a condition that is satisfied in a conjugated triene moiety. Based on the overlap of absorption and fluorescence spectra of CTL, the energy of the S₁ state is about 82 kcal/mol. The energy of T₁ probably lies 40 kcal/mol lower, because triplet sensitized *cis*–*trans* photoisomerization of the 1,3,5-hexatrienes [31] and of previtamin and tachysterol D₃ [32] were achieved with sensitizers having E_TS as low as 42 kcal/mol.

4.2 Photophysics

On the basis of the above mechanism, the effect of O₂ on HOCTL fluorescence quantum yields is given by Eq. (11), the Stern–Volmer equation

$$\frac{\phi_f^0}{\phi_f} = 1 + k_{\text{ox}}^S \tau_f^0 [\text{O}_2], \quad (11)$$

where $\phi_f^0 = k_f \tau_f^0$ and $\tau_f^0 = (k_f + k_{\text{RP}} + k_{\text{is}} + k_{\text{d}})^{-1}$ are the fluorescence quantum yield and lifetime in the absence of O₂. The analogous expression for the effect of O₂ on the fluorescence lifetime is given in Eq. (12).

$$\frac{1}{\tau_f} = \frac{1}{\tau_f^0} + k_{\text{ox}}^S [\text{O}_2], \quad (12)$$

We calculated the [O₂] values in Table 1 for air- and O₂-saturated THF using O₂ mole fraction data in THF [33] and THF densities [34] and vapour pressures at 20 °C [34, 35]. Plots of the data in Table 1 according to Eqs. (11

Fig. 6 Plots of the data in Table 1: **a** Stern–Volmer plot and **b** decay rates

and 12) are shown in Fig. 6. The slope of the lifetime plot in Fig. 6b gives $k_{\text{ox}}^S = 4.5 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ and the plot in Fig. 6a gives $7.9 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$. The average of these two values, $k_{\text{ox}}^S = 6.2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$, is about twice as large as rate constants for O_2 quenching of excited singlet states of arenes in cyclohexane [29]. The higher value in THF is due almost entirely to its lower viscosity [36, 37] (the cyclohexane to THF viscosity ratio is 1.91 at 20 °C) [34]. We conclude that the quenching of $^1\text{HOCTL}^*$ by O_2 is at or close to the dif-

[41, 42]. These workers did not provide the experimental ϕ_f and τ_f values in ethanol, but their anomalously low $\varepsilon_{\text{max}} = 7.7 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for CTL in Fig. 1 should have led to a low rather than a high calculated k_f value.

4.3 Photochemistry

Application of the steady-state approximation on all excited species in the above mechanism leads to the quantum yield expression in Eq. 13 for RP formation. Our quantum yields

$$\phi_{\text{RP}} = \frac{k_{\text{RP}}\tau_f}{1 + k_{\text{ox}}^S\tau_f[\text{O}_2]}, \quad (13)$$

for RP formation in degassed THF, 0.025%, and $\tau_s = 0.22 \text{ ns}$ from Table 1 give $k_{\text{RP}} = 1.14 \times 10^8 \text{ s}^{-1}$ for the HOCTL rearrangement rate constant.

The quantum yield of peroxide formation is a function of the effective intersystem crossing quantum yield, the sum of the fractions of $^1\text{CTL}^*$ and $^3\text{CTL}^*$ that give $^1\text{O}_2^*$ by energy transfer, and the fraction of $^1\text{O}_2^*$ that reacts with ^1CTL to give CTLP, Eq. 14

$$\phi_{\text{CTLP}} = \left(\frac{k_{\text{per}}\tau_{\Delta}[\text{CTL}]}{1 + k_{\text{per}}\tau_{\Delta}[\text{CTL}]} \right) \left\{ \frac{k_{\text{ox}}^S\tau_s[\text{O}_2]}{1 + k_{\text{ox}}^S\tau_s[\text{O}_2]} + \frac{k_{\text{ox}}^T\tau_t[\text{O}_2]}{1 + k_{\text{ox}}^T\tau_t[\text{O}_2]} \left(\frac{k_{\text{is}}\tau_s + k_{\text{ox}}^S\tau_s[\text{O}_2]}{1 + k_{\text{ox}}^S\tau_s[\text{O}_2]} \right) \right\}, \quad (14)$$

fusion controlled limit [29]. A $4.5 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ k_{ox}^S value in THF at 25 °C was reported recently for O_2 quenching of cycloparaphenylene fluorescence [38]. Correcting the $[\text{O}_2]$ values in ref 38 by including the THF vapour pressure raises that value to $5.7 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ improving the agreement with our value.

The ϕ_f/τ_f ratios in Table 1 give $k_f = 1.45 \times 10^8 \text{ s}^{-1}$. Our results can be compared with fluorescence quantum yields and lifetimes measured for DHE in several air-saturated solvents [39]. Not surprisingly, in view of the identical triene chromophores in the two molecules, they were found to be the same as those for CTL in three solvents used for comparison [39]. Discounting anomalously low DHE ϕ_f values in acetone and chloroform, ϕ_f values in the remaining seven solvents fall in the 0.024–0.058 range and the range of corresponding τ_f values is 0.18–0.39 ns. The uncertainty in these values is evident when one considers that the range of k_f values calculated from the ϕ_f/τ_f ratios, $1.3\text{--}2.2 \times 10^8 \text{ s}^{-1}$, is much wider than expected based on the nearly solvent independent $\varepsilon_{\text{max}} = 1.09 \pm 0.06 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. [38] Our ϕ_f and τ_f values in THF are very close to the values for diethyl ether reported by Smutzer et al. [39]. Hyslop et al. reported $k_f = 1.79 \times 10^8 \text{ s}^{-1}$ CTL in ethanol at 37 °C, [40] a value that exceeds ours by ~23%. Almost the same value was calculated [40] using the Strickler–Berg relationship

where the τ 's, in order of appearance, are the lifetimes of $^1\text{O}_2^*$ and of the singlet and triplet excited states of CTL. The second term in Eq. (14), ϕ_{Δ} , is the quantum yield of $^1\Delta_g$ $^1\text{O}_2^*$ formation and is a constant at constant $[\text{O}_2]$. To evaluate the effect of HOCTL concentration on peroxide quantum yields, Eq. (14) can be rearranged to Eq. (15).

The quantum yields obtained in the presence of air in Table 2 are plotted according to Eq. (15) in Fig. 7a.

$$\frac{1}{\phi_{\text{HOCTLP}}} = \left(1 + \frac{1}{k_{\text{per}}\tau_{\Delta}[\text{HOCTL}]} \right) \frac{1}{\phi_{\Delta}}; \quad (15)$$

The intercept/slope ratio of the linear plot gives $k_{\text{per}}\tau_{\Delta} = 420 \text{ M}^{-1}$. Reported lifetimes of $^1\text{O}_2^*$ are 30 [43] and 20 [44] μs . The experimental rate constant for the

Fig. 7 **a** The effect of [HOCTL] on ϕ_{CTLP} , **b** as in **a** but accounting for variation in τ_{Δ}

stereospecific insertion of $^1\text{O}_2^*$ into a α -H of THF [45] is $3.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. That reaction gives the hydroperoxide and γ -butyrolactone, its rearrangement product, with a reported quantum yield of 0.93 [45]. If there were no other decay paths, in neat THF, this corresponds to $\tau_{\Delta} = 23 \text{ } \mu\text{s}$, indicating that the insertion reaction accounts for most, if not all, the lifetime of $^1\text{O}_2^*$. With the use of $\tau_{\Delta} = 30 \text{ } \mu\text{s}$, we obtain $k_{\text{per}} = 1.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. However, in contrast to the entries in the first row of Table 2 that were obtained with pure HOCTL, entries for the other three rows were obtained with HOCTL samples that were contaminated by 11% HOPro. While the presence of HOPro in the experiments for Fig. 7 does not interfere with incident light absorption, its reaction with $^1\text{O}_2^*$ alters the effective lifetime of $^1\text{O}_2^*$. Reported rate constants for the reactions of ergosterol with $^1\text{O}_2^*$ are $1.2_1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $2.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ in *tert*-butyl methyl ether [26] and benzene [46], respectively. Assuming that the reactivities of Pro and HOPro with $^1\text{O}_2^*$ are the same as the reactivity of ergosterol, the $^1\text{O}_2^*$ lifetime in Eq. (15) is not a constant, but varies with the HO-Pro concentration, Eq. (16).

$$\tau_{\Delta} = \frac{1}{k_{\text{dox}} + k_{\text{Pro}}[\text{HOPro}]} \quad (16)$$

With the use of $\tau_{\Delta} = 30 \text{ } \mu\text{s}$ in the absence of HOPro impurity [43], and the rate constant in *tert*-butyl methyl ether, lifetimes based on Eq. (16) are given in the last column of Table 2. The intercept/slope ratio of the plot of the inverse of the peroxide quantum yield against $1/(\tau_{\Delta}[\text{HOCTL}])$ in Fig. 7b gives $k_{\text{per}} = 1.0_7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. This is a bit larger than expected in view of an earlier finding by Albro et al. that in *tert*-butyl methyl ether, the reaction of DHE with $^1\text{O}_2^*$ is half as fast as the reaction with ergosterol [26]. Albro et al. also found that the conjugated trienols, present as minor impurities in commercial ergosterol and provitamin D₃ samples, function as photosensitizers for the $^1\text{O}_2^*$ oxidation of ergosterol and provitamin D₃ in vitro, and, because they are present in the skin, in vivo. [26] The conclusion that endoperoxide formation is faster with HOPro than with HOCTL is not surprising, because angle strain due to the exocyclic double bond in HOCTLP should raise its energy, thereby raising the energy of the transition state for its formation.

The first row of Table 2 shows a significant increase in ϕ_{HOCTLP} when the reaction is carried out under an O_2 atmosphere. Our $^3\text{HOCTL}^*$ lifetime from Fig. 5b and the expectation [29] that $k_{\text{ox}}^t = (1/9)k_{\text{ox}}^s$ lead to the conclusion that, in air-saturated THF solutions, all $^3\text{HOCTL}^*$ are quenched by oxygen. We further simplify Eq. (14) by noting that the first term, f_{CTL} , is constant at constant [HOCTL]. We then factor out $f_{\text{O}_2} = k_{\text{ox}}^s \tau_s [\text{O}_2] / (1 + k_{\text{ox}}^s \tau_s [\text{O}_2])$ and cross multiply by the f terms to obtain Eq. (17)

Fig. 8 The effect of oxygen concentration on ϕ_{CTLP}

$$\frac{\phi_{\text{HOCTLP}}}{f_{\text{HOCTL}}f_{\text{O}_2}} = \left(n + \frac{k_{\text{is}}}{k_{\text{ox}}^s [\text{O}_2]} \right), \quad (17)$$

where n is 2 if oxygen quenching of $^1\text{HOCTL}^*$ gives $^3\text{HOCTL}^*$, as shown in Eq. (7), but n is 1 if it gives ground-state HOCTL. Plotted according to Eq. (17) in Fig. 8, the quantum yields in Table 2 are well fit with an intercept of 1. We conclude that Eq. (7) is not correct. Quenching of $^1\text{HOCTL}^*$ by oxygen does not give $^3\text{HOCTL}^*$ as we had expected. The slope of the line in Fig. 8 gives $(k_{\text{is}}/k_{\text{ox}}^s) = 0.0139 \text{ M}$ from which we obtain $k_{\text{is}} = 8.9 \times 10^8 \text{ s}^{-1}$ and using the lifetime in Table 1, $\phi_{\text{is}} = 0.20$. This value is subject to $\pm 25\%$ error, because it is affected by the large uncertainty in our k_{ox}^s . It is in good agreement with 0.22, the quantum yield for $^1\text{O}_2^*$ formation that we obtain from the inverse of the intercept in Fig. 7b. A significantly smaller quantum yield of 0.09 for the formation of $^1\text{O}_2^*$ using dehydroergosterol as the photosensitizer in *tert*-butyl methyl ether was based on the relative phosphorescence intensity of $^1\text{O}_2^*$ [26].

5 Conclusion

Photophysical and photochemical quantities determined in this work for HOCTL in THF are summarized in Table 3. We expect them to apply also to CTL and DHE. As conjugated trienes, these steroidal trienols are unusual in that they fluoresce and intersystem cross in solution. By comparison, their diene precursors, Pro and ergosterol, fluoresce only in low T glassy media [47–50] and undergo no intersystem crossing [46]. The unusual behavior is no doubt due to the structural rigidity that inhibits the torsional relaxation pathways that dominate the photochemistry and radiationless decay of the parent 1,3,5-hexatrienes [51]. It is reminiscent of the dramatic enhancement of fluorescence that structural rigidity induces on the *trans*-stilbene chromophore [52]. Structural rigidity also increases almost 1000-fold the lifetime of the

Table 3 Quantum yields and rate constants: HOCTL in THF

Quantum yield		Rate constant	
ϕ_f	0.033 ₅	k_f	$1.4_3 \times 10^8 \text{ s}^{-1}$
ϕ_{is}	0.20	k_{is}	$8.9 \times 10^8 \text{ s}^{-1}$
ϕ_{RP}	0.025 ₇	k_{RP}	$1.1_4 \times 10^8 \text{ s}^{-1}$
ϕ_{ic}	0.74	k_{ic}	$3.3 \times 10^9 \text{ s}^{-1}$
		k_{dt}	$1.25 \times 10^4 \text{ s}^{-1}$

trienol triplet state [53] rendering it a more effective $^1\text{O}_2^*$ sensitizer. As noted above, the strong structural similarity of the steroidal trienols to cholesterol has made them ideal fluorescent probes for cholesterol trafficking in vivo. Although weakly fluorescent in solution, they fluoresce more brightly in the confining environments of biological membranes and phospholipid bilayers, generally. Fluorescence lifetimes of 0.97 [39] and 0.9 [54] ns, measured at room temperature in such media, and the radiative rate constant in Table 3 allow estimation of $\phi_f = 0.13$ –0.14 at room temperature, substantially higher than the solution value in Table 3. Similarly estimated, but based on $k_f = 1.78 \times 10^8 \text{ s}^{-1}$, is $\phi_f = 0.11$ for CTL in liposomes at 37 °C [40]. That value drops to $\phi_f = 0.090$ with the use of our k_f . By analogy with ergosterol whose low T fluorescence competes with activated radiationless decay [48], the decrease in ϕ_f at the higher temperature is expected. Relevant to the use of the cholesteryl trienols as fluorescent probes for cholesterol trafficking is the report that the CTL fluorescence lifetime increases to 2.2 ns for CTL in cholesterol aggregates at 37 °C [40]. Use of our k_f value gives $\phi_f = 0.32$ under those conditions.

The lifetime increases must come at the expense of the sum of the three radiationless processes listed in Table 3. Of these, the major one, internal conversion to the ground state, must surely be diminished in more rigid media, but whether formation of the rearrangement product is similarly affected remains to be established. It is also possible that internal conversion and rearrangement are simultaneous processes that reflect the partitioning between product and reactant on decay through a single conical intersection.

The important role of intersystem crossing in solution was known before our work, and is further documented here in THF. Although the tenfold increase in the fluorescence lifetime in cholesterol aggregates [40] leads us to expect a higher ϕ_{is} under those conditions, the value of k_{is} must be considerably lower than in THF as ϕ_{is} cannot exceed unity. As observed by Albro et al. [26] the steroidal trienol triplets are efficient sensitizers of $^1\text{O}_2^*$ which, in turn, gives endoperoxides by 1,4-addition to the cyclohexadiene moiety of the B ring of the trienols and of the provitamin Ds. The presence of CTL as an impurity in commercial Pro probably accounts for the formation of Pro endoperoxide on 254 nm irradiation of a Pro solution in CDCl_3 [55]. DHE photobleaching

observed by Hao and coworkers when DHE was used as a fluorescent probe to monitor cholesterol trafficking in living cells [12] showed that it is photochemically active under physiological conditions. The relative role of rearrangement and peroxide formation in biological media is unknown.

Side photoproducts formed on prolonged irradiation of the vitamin D precursors have been collectively referred to as toxisterols [56–58], although we are aware of no definitive studies confirming their toxicity. Thus, the biological properties of the RP photoproducts are unknown. On the other hand, isolated as natural products, the peroxide photoproducts have been shown to be moderately cytotoxic against a variety of cancer cells [19, 25, 55, 59]. It has also been speculated that endoperoxide formation in exposed skin may account for the tumor promoting action of UVA ($\lambda > 315 \text{ nm}$) [26].

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Author contributions SBK performed most experimental measurements described in this paper. Initial experiments were performed by SG and EAH. RJC carried out the X-ray crystallographic measurements and determined the X-ray structures. JS directed the research and prepared the manuscript for publication.

Declarations

Conflict of interest The authors declare no competing financial interests.

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