Mechanistic Study of the Photooxidation of Squalene Sensitized with 2,2′:5′,2″-Terthiophene and 2,2′-Bithiophene

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To understand the photochemical basis of photodermatitis caused by 2,2′:5′,2″-terthiophene, photooxidation of squalene sensitized with 2,2′:5′,2″-terthiophene was studied with respect to mediation by singlet molecular oxygen, the activation energy, and the apparent quantum yield. Squalene was peroxidized in ethanol solution by irradiation with UV-A light in the presence of 2,2′:5′,2″-terthiophene and the related compound 2,2′-bithiophene. Involvement of singlet oxygen in the peroxidation was suggested on the basis of the suppressive effect of sodium azide and the enhancing effects of organic solvents which elongate the lifetime of singlet oxygen. The activation energy obtained, 11.2 kJ mol^{-1}, was a reasonable value for oxidation of olefins by singlet oxygen. The quantum yields of peroxidation were estimated to 12.6×10^{-2} and 8.3×10^{-2} for photosensitization with 2,2′:5′,2″-terthiophene and 2,2′-bithiophene, respectively.

2,2′:5′,2″-Terthiophene (α-terthienyl, αTT), a secondary metabolite in marigolds, induces a variety of photobiological effects at the molecular level, the cellular level, and the individual level.[1] It also exhibits phototoxic effects on human skin.[2] The pathological mechanism of photodermatitis caused by αTT is unknown. We recently found that αTT could be taken up into human buccal cells and it injured the lysosomal membranes on near-ultraviolet irradiation.[3]

In the present study, we dealt with the *in vitro* photooxidation of squalene, which is a major component of skin surface lipids, in the presence of αTT and a related compound, 2,2′-bithiophene (BT) to understand the photochemical basis of photodermatitis caused by αTT. First, we confirmed the potencies of the thiophenes to oxidize squalene on irradiation with near-ultraviolet light (300—400 nm). The suppressive effect of sodium azide on the peroxidation and the variation of the peroxidation rate in different organic solvents were compatible with oxidation by a Type II mechanism (oxidation mediated by singlet oxygen). Second, the rate constant of the photooxidation of squalene sensitized with αTT was measured at different temperatures to obtain the activation energy. Finally, the apparent quantum yields of the oxidation of squalene were measured for both photosensitizers.

Experimental

Materials. Commercial αTT (Tokyo Kasei Chemical Industries, Tokyo) was recrystallized from ethyl acetate:petroleum ether (8:2 v/v). Squalene and BT were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium azide was from Merck (Darmstadt, Germany), thiobarbituric acid (TBA) and ethanol were from Wako Pure Chemical Industries (Osaka), methanol, benzene, and chloroform were from Dojindo Laboratories (Kumamoto), *t*-butyl hydroperoxide was from Aldrich Chemical Co. (Milwaukee, WI, USA), and 1,1,3,3-tetraethoxypropane was from Tokyo Kasei Industries (Tokyo).

Spectroscopy. Absorption spectra of αTT, BT, and TBA reactive substances were measured with a Shimadzu spectrophotometer (Model MPS-2000).

UV-A Irradiation. One milliliter of an ethanol solution of squalene containing αTT or BT was irradiated in a polystyrene rectangular vessel (a Corning tissue culture flask, 25 cm^2) with near-ultraviolet light under a bank of 3 fluorescent BLB tubes (Nippo Electric Co., Hiratsuka). The thickness of the solution was 0.04 cm. The experimental conditions were selected so as to be optically thin ($2.3\times\varepsilon cl\ll1$) at the peak absorption wavelengths of both photosensitizers. The emission of the light source covered the range 300 to 400 nm, and the peak was at 355 nm (Fig. 1). (We designate the light as UV-A light for convenience' sake hereafter.) The fluence rate was measured to be 10 J m^{-2} s^{-1} with a Topcon UV-meter, Model UVR-1. To find the effect of a singlet oxygen quencher on the peroxidation, an ethanol solution of sodium azide was included in the sample solutions. (The stock solution of sodium azide contained a minimal amount of water to solubilize it.) To examine the solvent effects on the peroxidation rate, ethanol was replaced by methanol, benzene, or chloroform, and the sample solutions (0.8 ml) were irradiated in cylindrical quartz cells (4.7 cm in diameter; thickness of the solutions, 0.046 cm).

When the quantum yields of peroxidation were measured, 1.0-ml sample solutions containing 3 mM (M=mol dm^{-3}) squalene and 100 μM photosensitizer were irradiated in Corning tissue culture flasks at 316±3.5 nm with a JASCO spectroirradiator (Model CRM-FA). The fluence rate was measured as 2 J m^{-2} s^{-1} with a silicon photovoltaic detector (Model 550-2, EG & G Electro-Optics, Salem, MA, USA) in this case.

Measurements of Peroxidation Products. The concentrations of hydroperoxides and TBA reactive substances produced by the photosensitized oxidation were determined by iodometry and the TBA method, respectively, as reported previously.[4] *t*-Butyl hydroperoxide and 1,1,3,3-tetraethoxypropane were used as the standard substances to draw the calibration curves for quantification. The concentrations of hydroperoxide residues (–OOH) were expressed

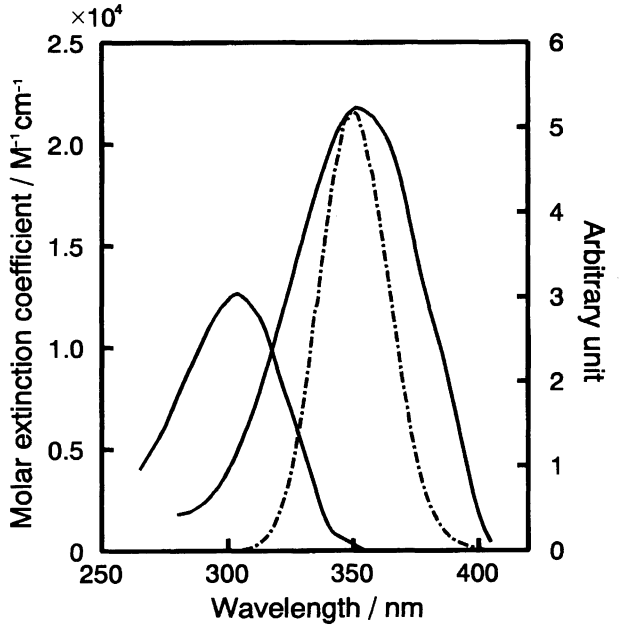

Fig. 1. Absorption spectra of αTT and BT, and the emission spectrum of the light source. —, absorption spectra (right peak, αTT; left peak, BT); –·–, emission spectrum.

in units of equivalents per liter, because multihydroperoxides can be produced by extensive peroxidation.

Results

Peroxidation of Squalene. To examine whether αTT is able to sensitize photooxidation of squalene as observed for membrane lipids,[5,6] iodometry and the TBA method were applied to follow the peroxidation of squalene by UV-A irradiation of an ethanol solution containing the lipid and αTT. Both the hydroperoxide residues and TBA-reactive substances increased linearly with increasing irradiated fluence (Figs. 2 and 3). The amounts of the TBA-reactive substances were always about 1/28 of the amounts of the hydroperoxide residues.

Since the absorption peak of BT was out of the region of the emission spectrum (Fig. 1), the photosensitized peroxidation efficiency of BT was much smaller than that of αTT when compared as a function of incident fluence. If the efficiencies were compared on a quantum yield basis, αTT was 1.5-times more efficient than BT in sensitizing the photooxidation of squalene (Fig. 2). The relative amounts of quanta absorbed were estimated from the overlapping areas of the emission spectrum and the absorption spectrum of each photosensitizer in the sample solutions.

Suppressive Effect of Sodium Azide. Peroxidation of squalene sensitized with αTT did not occur under anaerobic conditions performed by bubbling argon gas. Addition of a low concentration (on the order of a millimole per liter) of sodium azide, a singlet oxygen quencher,[7] effectively suppressed the peroxidation under aerobic conditions. (Fig. 3). This result was consistent with the view that the rate-determining step of the peroxidation is mediated by singlet oxygen (Type II mechanism), because the contribution of sodium azide to quench radicals and triplet excited states of photosensitizers may not be prominent at such low concentrations.[8]

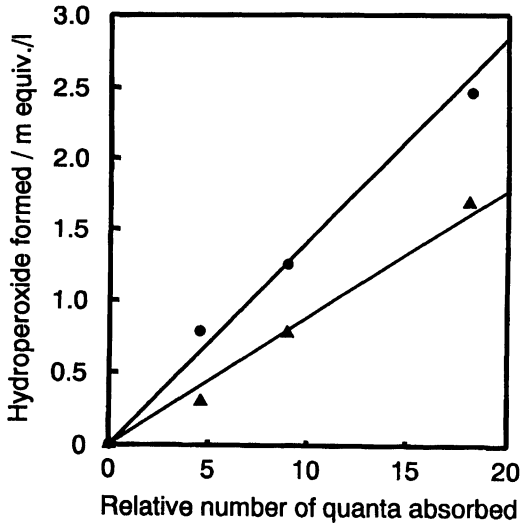

Fig. 2. Formation of hydroperoxide residues by irradiation of squalene in the presence of αTT and BT. Squalene, 3 mM; αTT (●) or BT (▲), 100 μM.

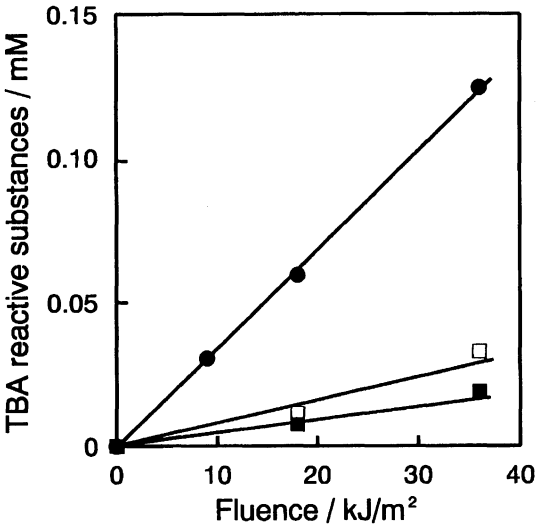

Fig. 3. Suppressing effect of sodium azide on the peroxidation of squalene by irradiation in the presence of αTT. Squalene, 3 mM; αTT, 48 μM. NaN_3, ●, 0 mM; □, 2 mM; ■, 4 mM.

Variation of the Peroxidation Rate in Different Organic Solvents. To obtain other supporting evidence for the participation of singlet oxygen in the photooxidation of squalene sensitized with αTT, UV-A irradiation was performed in other organic solvents such as benzene and chloroform which elongate the life-

time of singlet oxygen in that order.[9] More peroxides of squalene were produced by irradiation with a fixed fluence in these solvents (Fig. 4). This result was consistent with the expectation provided that production yields of singlet oxygen are not largely different in these solvents.[10]

Determination of the Activation Energy of Peroxidation. Peroxidation of squalene with singlet oxygen proceeded as a second-order reaction. Since there are no reports concerning the rate constant for the peroxidation of squalene, we attempted to measure the rate constants in the present system. Under steady-state conditions for the triplet excited state of αTT and singlet oxygen, the following equation can be derived:[11]

$$[\text{Sq–OOH}]^{-1} = I_{\text{abs}}{}^{-1} \Phi_{\text{isc}}{}^{-1} \left\{1 + (k_1/k_2)\,[\text{Sq}]^{-1}\right\}$$

where [Sq] and [Sq–OOH] are the concentrations of squalene and hydroperoxide residues of squalene, respectively, I_{abs} is the number of mole quanta per liter absorbed per irradiation time, Φ_{isc} is the effective quantum yield of intersystem crossing for αTT, and k_1 and k_2 are the rate constants of the decay of singlet oxygen and that of the reaction of singlet oxygen with squalene, respectively. We can obtain the value of k_2 because the value of k_1 is known to be 8.3×10^4 s^{-1} in ethanol.[12] To obtain the value of k_2, the photooxidation reaction was performed by irradiation of squalene at different concentrations (15—100 mM) in the presence of a fixed concentration of αTT (50 μM) with a fixed fluence (6 kJ m^{-2}). Figure 5 shows the reciprocal plots for the reactions at different temperatures. The value of k_2 was calculated to be 4.2×10^6 M^{-1} s^{-1} in ethanol solution at 25 °C. This value is one order of magnitude higher than that for the peroxidation of multiunsaturated fatty acid methyl esters.[13] The activation energy was estimated as 11.2 kJ mol^{-1} from the Arrhenius plot. This is a reasonable value for the oxidation of olefins by singlet oxygen.[14]

Determination of the Apparent Quantum Yields. To obtain the apparent quantum yields of the peroxidation of squalene sensitized with αTT and BT, monochromatic irradiation of squalene (3 mM) containing one of the photosensitizers (100 μM) was performed at 316 nm where their molar extinction coefficients are the same value. The quantum yields for the production of hydroperoxide residues were 12.6×10^{-2} and 8.3×10^{-2} for αTT and BT, respectively. Photooperoxidation with αTT was again about 1.5-times more efficient than that with BT. This result suggests that the larger yield of the intersystem crossing of αTT compared to the yield of BT caused more efficient production of singlet oxygen on excitation of αTT.

Discussion

UV-A excitation of αTT is known to affect cell membranes[5,15] and liposomal membranes.[6] Although squalene is not a constituent of membrane lipids and it has an ethylene-interrupted structure different from

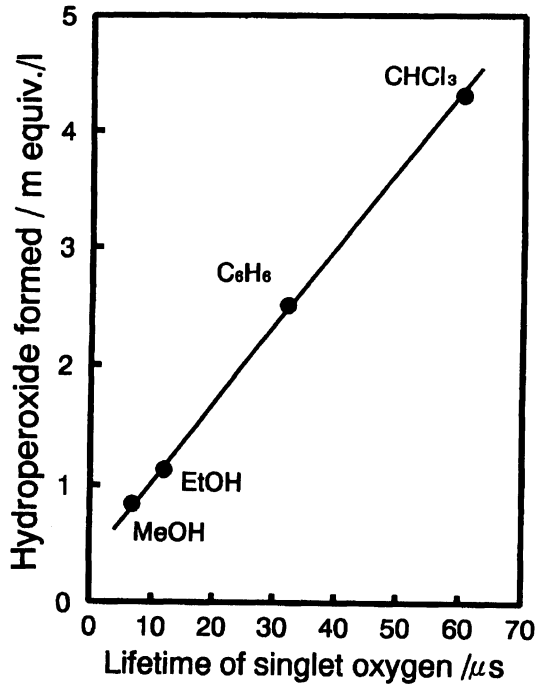

Fig. 4. Correlation between the production yield of hydroperoxides and the lifetime of singlet oxygen in different organic solvents. Squalene, 3 mM; αTT, 100 μM; irradiated fluence, 9 kJ m^{-2}.

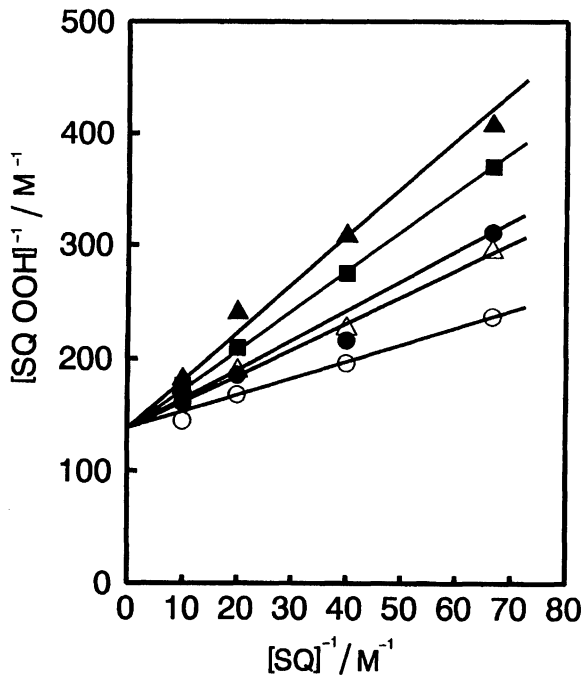

Fig. 5. Temperature dependence of the peroxidation at different concentrations of squalene. αTT, 50 μM; irradiated fluence, 6 kJ m^{-2}. ▲, −2 °C; ■, 5 °C; ●, 25 °C; △, 42 °C; ○, 53 °C.

that of multiunsaturated fatty acids, photosensitized peroxidation of squalene was disclosed on irradiation with UV-A light in the presence of αTT and BT. Since αTT can generate singlet oxygen on UV-A excitation,[10,16—18] a Type II mechanism is likely for the photooxidation of the lipid sensitized with αTT. Singlet oxygen reacts with unsaturated lipids by a concerted 'ene' addition to form allylic hydroperoxides. Squalene, a sexiisoprene, can be readily oxidized by photosensitization[4] similar to other isoprene compounds.[11,19] The rate constant (k_2) for the photooxidation of squalene at 25 °C was much greater than those for unsaturated fatty acid esters.

We also applied the TBA method to detect the peroxidation of squalene. Although malonaldehyde (MA) is believed to be a major compound of TBA–reactive substances, only 5% of the hydroperoxides of linoleated and linolenate can produce MA.[20] The adduct ("red pigment") of TBA and MA shows an absorption peak at 532 nm.[21] TBA–reactive substances involve not only MA but also many other related compounds. For example, alka-2,4-dienals (R–CH=CH–CH=CH–CHO) can also produce the red pigment, although the potency to form the pigment is smaller than that of MA.[22] Peroxidation products of linoleate also give "yellow pigment" whose absorption peak is at 450 nm. Many other intermediary compounds are known to produce the yellow pigment. For example, saturated and unsaturated aldehydes give the yellow pigment.[23] The yellow pigment is converted to the red pigment by oxidation. Considerable amounts of the yellow pigment were involved in the peroxidation products of squalene.

Dennis and Shibamoto[24] determined the presence of free MA in squalene samples irradiated with a large fluence of UV-A light by detection of the reaction products with methylhydrazine by means of gas chromatography. Their results, however, indicated the production of much less amounts of free MA compared to the so-called TBA-values, i.e., the TBA-value was 50-times overestimated for UV-A irradiation products compared to the measured amounts of free MA. Since the conventional TBA method is weak in chemistry, the direct determination of hydroperoxide residues is favored to follow lipid peroxidation unless sample solutions involve other redox systems.

The quantum yield of singlet oxygen production, Φ_Δ, by UV-A excitation of αTT is known to be as high as 0.6—0.8 in polar and nonpolar solvents.[10] The quantum yield of the oxidation with BT was measured as 1/1.5 of that with αTT, implying a lower Φ_Δ value for BT. Although the quantum yield of intersystem crossing, Φ_{isc}, for αTT is 0.9—1.0,[10] no Φ_{isc} value for BT has appeared in the literature.

Since the skin is exposed to light, photosensitized reactions are possible in the cutaneous layers if any exogenous photosensitive compounds adsorb on cutaneous cells or are incorporated by the cells. Some of the photosensitive drugs applied systemically distribute to the cutaneous tissue by the circulation of blood. Unsaturated fatty acid residues of membrane lipids and cholesterol involved in cellular and subcellular membranes can be subjected to photosensitized oxidation (both of Type I and Type II). Photosensitized oxidation of lipids and proteins in cellular and subcellular membranes exerts deleterious effects on cellular functions. Squalene peroxides produced on the skin surface may lead cutaneous cells to secondary peroxidation of the membrane lipids by chain radical propagation reactions. Possible involvement of squalene peroxides in the induction of photodermatitis due to exogenous photosensitizers was proposed.[25] One of the prominent effects would be the release of physiologically-active chemical mediators such as histamine, leukotrienes, and prostaglandins from injured cells.

In summary, squalene was peroxidized by a Type II mechanism on irradiation of an ethanol solution with UV-A light in the presence of αTT, and the activation energy was a reasonable value for the oxidation of olefins with singlet oxygen. The quantum yields of peroxidation were 12.6×10^{-2} and 8.3×10^{-2} when αTT and BT, respectively, were used as photosensitizers.

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