9*H*-Xanthene-2,7-Diols as Antioxidants for Autoxidation of Linoleic Acid

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ABSTRACT: The antioxidant activities of 9*H*-xanthene-2,7-diols and α-tocopherol were studied during the oxidation of linoleic acid in a homogeneous solution and in an aqueous micelle dispersion. The antioxidant activities of 9*H*-xanthene-2,7-diols for both systems were 1.0–2.4 times greater relative to α-tocopherol. In addition, the 1,3,4,5,6,8-hexamethylxanthene-2,7-diol showed less cytotoxicity toward human fibroblasts than did 2,6-di-*t*-butyl-4-methylphenol.
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KEY WORDS: Antioxidant, cytotoxicity, linoleic acid, α-tocopherol, 9*H*-xanthene-2,7-diol.

Saturated oils and fats are generally used as cosmetic ingredients because unsaturated oils and fats are easily changed to lipid peroxides by oxidation with the peroxyl radical. These lipid peroxides cause a change in color, rancidity and skin irritation. It is well known, however, that unsaturated fatty acids and their esters improve the texture and feeling of cosmetic products as a result of their physical properties. Further, essential fatty acids, especially linoleic acid, are known to be related to skin hydration (1–3). This fact suggests that dry skin is improved by the application of essential fatty acids. Therefore, inhibition of the autoxidation of lipids has important implications, not only for preserving the quality of cosmetics but also for developing novel products.

Several antioxidants, such as 2,6-di-*t*-butyl-4-methylphenol (BMP, often called BHT) and tocopherols (Toc), generally have been used to suppress quality deterioration of cosmetic products. α-Toc is one of the most effective natural antioxidants (4–6). The structural characteristics of α-Toc include a chroman ring bound to a phenolic hydroxyl group and a long phytyl side chain. The phytyl side chain has no effect on antioxidant activity, at least in a homogeneous solution (7). The phytyl side chain is only required for the incorporation and retention within biological membranes. Studies on many Toc derivatives have suggested that the phenol moiety and the ether-type oxygen at the position *para* to the hydroxyl groups may be important for its antioxidant activity (8,9).

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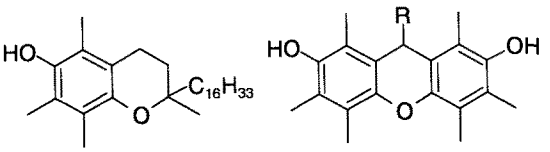
Recently, we synthesized 9*H*-xanthene-2,7-diols (**1a–e**) (Scheme 1) that are structurally related to α-Toc, and evaluated their antioxidant activity during the autoxidation of tetralin (10). The 9*H*-xanthene-2,7-diols, having two hydroxyl groups and an ether-type oxygen at the position *para* to the hydroxyl groups, exhibited strong potency relative to α-Toc for the autoxidation of tetralin initiated with azo-*bis*-isobutyronitrile (AIBN).

In this study, we investigated the antioxidant activity of 9*H*-xanthene-2,7-diols (**1a–e**) (Scheme 1) during the autoxidation of linoleic acid in a homogeneous solution and in an aqueous micelle solution. We also compared cytotoxicities of the 9*H*-xanthene-2,7-diols with commercially available antioxidants, BHT and α-Toc, in normal human fibroblasts.



α-Toc

1a, R = H **1d**, R = $CH(CH_3)_2$
1b, R = CH_3 **1e**, R = C_6H_5
1c, R = C_2H_5

SCHEME 1

EXPERIMENTAL PROCEDURES

Materials. Linoleic acid, AIBN, 2,2′-azo-*bis*-(2-amidinopropane) dihydrochloride (AAPH), 2,2′-azo-*bis*-(2,4-dimethylvaleronitrile) (AMVN), and crystal violet were purchased from Wako Pure Chemical Co. (Osaka, Japan). 2-(4,5-Dimethyl-2-thiazolyl)-3,5-diphenyl-2*H*-tetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents purchased from commercial sources were of analytical grade.

The 9*H*-xanthene-2,7-diols studied in this work were prepared by the acid-catalyzed reaction of 2,3,5-trimethylhydroquinone with aldehydes (10).

The micelle solution was prepared as follows: Linoleic acid (5 mmol) and antioxidant (0.002 mmol) were dissolved

in Tween 20 (3.6 g) and then dispersed in 100 mL distilled water. The micelle solutions were subjected to oxidation immediately after preparation.

Determination of antioxidant activities. The rate of oxidation was determined either by following the oxygen concentration in solution or by measuring the pressure decrease due to oxygen uptake. Oxygen concentration in solution was monitored with a Dissolved Oxygen Meter, Model DOL-40 (Denki Kagaku Keiki Co., Ltd., Tokyo, Japan) at 37°C. Oxygen pressure was measured as a function of time under 760 Torr (1 Torr = 133.3 Pa) of O_2 with linoleic acid in chlorobenzene containing an antioxidant and AIBN as the initiator. The oxidation temperature was maintained at 60 ± 0.1°C. The induction period (IP) was determined graphically (11,12) from the length of time between antioxidant injection and the point of intersection of tangents to the oxidation curve corresponding to the initial inhibited and final uninhibited rates of oxidation. All measurements of antioxidant activities were replicated three times. Mean values ± standard deviation are reported for each case. Student's *t*-test was applied for statistical evaluations.

Cell culture. Human dermal (foreskin) fibroblasts were obtained as primary cultures by explantation. The fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum.

Cytotoxicity test by MTT method. A secondary culture of the fibroblasts was harvested by trypsinization when the culture was about 80% confluent. The suspended cells were collected by centrifugation at 180 × *g* for 5 min and diluted in DMEM containing 10% fetal bovine serum. The cells (1×10^4 cells/0.1 mL of the medium, measured by Burker-Turle counting chamber (Erma Inc., Tokyo, Japan) were inoculated into each well of a 96-well tissue culture plate and cultured for 24 h at 37°C in a 5% CO_2 humidified atmosphere. The culture medium was replaced with MCDB153 medium (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) with 1.8 mM Ca^{2+} and cultured again for 24 h at 37°C in a 5% CO_2 humidified atmosphere. The cells were then treated with the antioxidants for 6 or 24 h. The antioxidants were dissolved in dimethyl sulfoxide and diluted with MCDB153 medium. The final concentration of the solvent was 0.5% (vol/vol) and showed no effects on cell growth in controlled growth experiments. After treatment with the antioxidant, the medium was replaced with 0.2 mL DMEM supplemented with 10% fetal bovine serum and MTT (0.5 mg/mL) in each well, and incubated at 37°C for 2 h. After being washed with phosphate-buffered saline (PBS), 2-propanol containing 0.04 N HCl was added to each well for the dissolution of formazan to give a homogeneous blue solution suitable for absorbance measurement. The resulting color intensity was measured a microplate reader at 590 nm. The value of formazan formation was determined as mean values of triplicate experiments in each concentration of antioxidants.

Cytotoxicity was represented as the concentration of antioxidant that caused a 50% reduction in formazan formation by a treated cell culture compared with the untreated control culture (LD_{50}). The value of LD_{50} was determined on the plot of formazan formation vs. antioxidant concentration.

Cytotoxicity test by the crystal violet staining method. For cell fixation, each well received 0.2 mL formalin (10%) and was left for at least 30 min. After being washed with PBS, the plates were stained with 0.1% crystal violet solution in methanol for 30 min. The resulting color intensity was measured by a microplate reader at 550 nm. The value of cell number was determined as means of triplicate experiments in each concentration of antioxidants.

Cytotoxicity was represented as the concentration of antioxidant that caused a 50% reduction in viable cells by a treated cell culture compared with the LD_{50}. The value of LD_{50} was determined on the plot of cell number vs. antioxidant concentration.

RESULTS AND DISCUSSION

The antioxidant activities of 9*H*-xanthene-2,7-diols during the autoxidation of linoleic acid were evaluated in a homogeneous chlorobenzene solution and in an aqueous micelle dispersion containing Tween 20. Figure 1 shows the typical oxygen uptake curves for the oxidation of linoleic acid in a homogeneous solution initiated by AIBN at 60°C. After a brief initiation period, a constant rate of oxygen uptake was observed in the control solution in the absence of an antioxidant. When α-Toc or 9*H*-xanthene-2,7-diols (**1a–e**) were added to the linoleic acid solution, oxidation was suppressed, and there was a measurable IP. The IP values for solutions containing **1a–e** and α-Toc examined in this work are listed in Table 1,

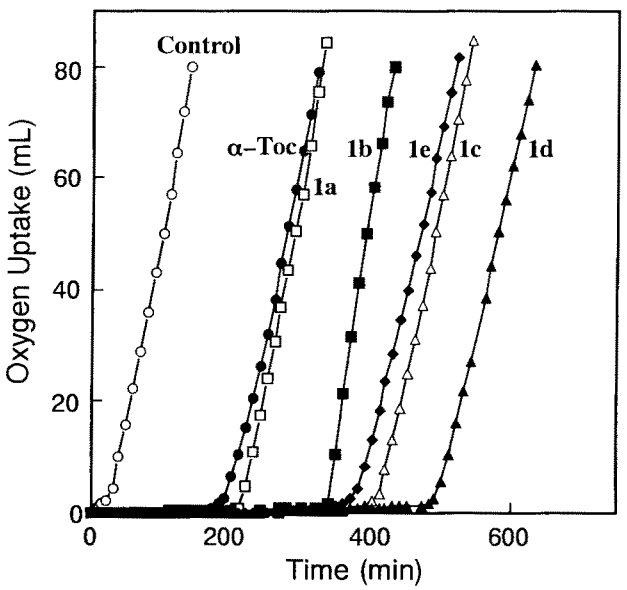


FIG. 1. Rate of oxygen uptake in the oxidation of 1 M linoleic acid in chlorobenzene initiated by 10 mM azo-*bis*-isobutyronitrile in the absence and presence of 1 mM 9*H*-xanthene-2,7-diols (**1a–e**) and α-tocopherol (α-Toc) at 60°C under oxygen.

TABLE 1
Antioxidant Activities of 9*H*-Xanthene-2,7-Diols (1a–e) and α-Tocopherol (α-Toc) in the Oxidation of Linoleic Acid in Homogeneous Solution, in Aqueous Micelle Dispersions, and in Tetralin

	IP (min)			
	Linoleic acid[a]			
Compound number	Homogeneous[b]	Micelle[c] (AAPH)	Micelle[d] (AMVN)	Tetralin[e]
1a	210.6 ± 2.1	111.6 ± 5.8	44.9 ± 1.7	985
1b	337.2 ± 5.4	132.1 ± 8.4	198.6 ± 6.6	957
1c	414.7 ± 4.6	135.6 ± 3.8	224.2 ± 8.4	741
1d	494.2 ± 6.2	164.9 ± 5.6	237.5 ± 6.9	738
1e	382.5 ± 3.6	163.0 ± 3.1	214.1 ± 4.5	788
α-Toc	201.9 ± 3.1	79.2 ± 3.7	149.5 ± 9.4	345
Control	34.7 ± 0.7	0.2 ± 0.0	0.6 ± 0.0	13

[a]Mean ± standard deviation of triplicate measurements.
[b]Oxidation of 1 M linoleic acid in chlorobenzene initiated by 10 mM AIBN at 60°C.
[c]Oxidation of 50 mM linoleic acid in Tween 20 aqueous micelle dispersions initiated by 5 mM 2,2′-azo-*bis*-(2-amidinopropane) dihydrochloride (AAPH) at 37°C.
[d]Oxidation of 50 mM linoleic acid in Tween 20 aqueous micelle dispersions initiated by 5 mM 2,2′-azo-*bis*-(2,4-dimethylvaleronitrile) (AMVN) at 37°C.
[e]Oxidation of tetralin initiated by 10 mM azo-*bis*-isobutyronitrile (AIBN) at 60°C. Data of induction period (IP) values were taken from Reference 10.

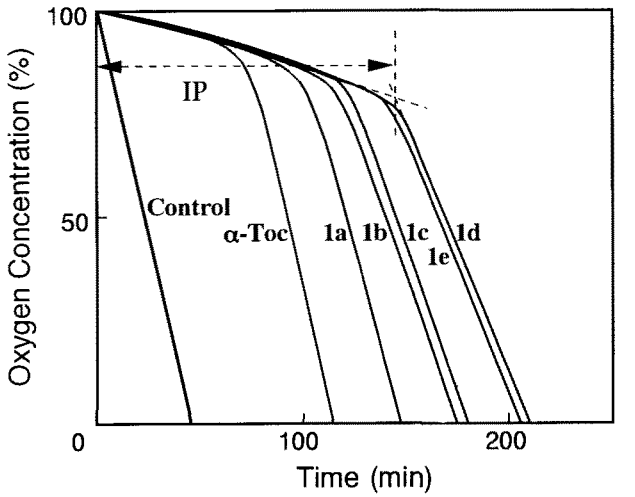


FIG. 2. Rate of oxygen uptake in the oxidation of 50 mM linoleic acid in Tween 20 aqueous micelle dispersions initiated by 5 mM 2,2′-azo-*bis*-(2-amidinopropane) dihydrochloride in the absence and presence of 0.02 mM 9*H*-xanthene-2,7-diols (**1a–e**) and α-tocopherol (α-Toc) at 37°C under oxygen. IP, induction period.

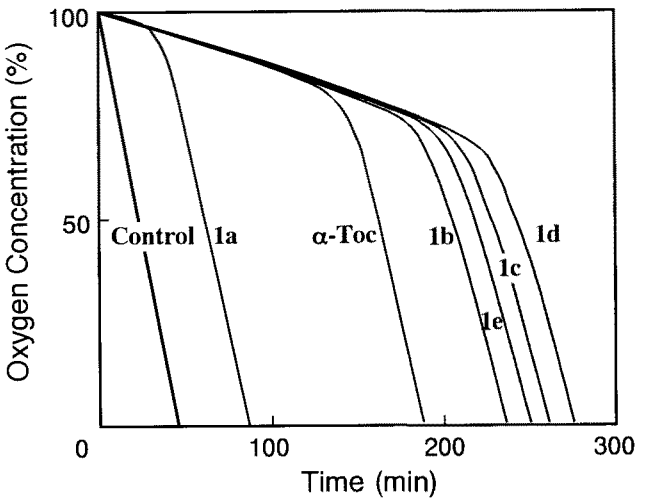


FIG. 3. Rate of oxygen uptake in the oxidation of 50 mM linoleic acid in Tween 20 aqueous micelle dispersions initiated by 5 mM 2,2′-azo-*bis*-(2,4-dimethylvaleronitrile) in the absence and presence of 0.02 mM 9*H*-xanthene-2,7-diols (**1a–e**) and α-tocopherol (α-Toc) at 37°C under oxygen.

along with the results of the oxidation of tetralin in a homogeneous system [reported from a previous paper (10) for comparison]. The effectiveness of 9*H*-xanthene-2,7-diols as antioxidants was affected by the kind of substituent group at the 9-position in the chemical structure. That is, 9*H*-xanthene-2,7-diols (**1b–e**) with substituent at the 9-position exhibited IP values that were 1.7–2.5 times greater than that of α-Toc ($P < 0.01$). In particular, **1d** exhibited a much longer IP compared with those of other 9*H*-xanthene-2,7-diols and α-Toc. On the other hand, unsubstituted 9*H*-xanthene-2,7-diol (**1a**) showed only a slightly higher antioxidant activity than did α-Toc ($P < 0.05$). The antioxidant activities of 9*H*-xanthene-2,7-diols (**1a–e**) in linoleic acid were in marked contrast to results for the oxidation of tetralin. That is, the antioxidant activities of **1a–e** depended on the reaction medium, and varied as follows: **1d** > **1c** > **1e** > **1b** > **1a** > α-Toc in a linoleic acid solution and **1a** > **1b** > **1e** > **1c**, **1d** > α-Toc in a tetralin solution.

Typical oxygen uptake curves for the autoxidation of linoleic acid micelle solutions initiated by AAPH and AMVN at 37°C are shown in Figures 2 and 3, respectively. In both cases, a constant rate of oxygen consumption without any noticeable IP was observed in the control solution. The antioxidant incorporated into linoleic acid micelles suppressed the oxidation markedly and produced a clear IP. After the IP, rapid oxidation took place at a rate similar to that in the absence of antioxidant. The IP values for the autoxidation of linoleic acid in these micelle dispersions are shown in Table 1. There was a similarity in the order of effectiveness of the antioxidants, as measured by IP, in both micelle dispersions and homogeneous solutions. That is, the IP values decreased in the order **1d** > **1c**, **1e** > **1b** > **1a** in both systems. In particular, compound **1d** was the most active antioxidant, being twice as active as α-Toc. On the other hand, there was a remarkable decrease in antioxidant activity of **1a** for the autoxidation of linoleic acid in a micelle solution initiated with AMVN as compared with that initiated by AAPH. These results suggest that the antioxidant activity of 9*H*-xanthene-2,7-diols is dependent on the solubility of linoleic acid as the substrate. Especially, it is speculated that **1a** does not homogeneously exist in the oil phase of linoleic acid but is localized in the interface of the micelle with Tween 20 because of the low solubility of **1a** in linoleic acid. Therefore, **1a** is capable of reacting with radicals generated in the water phase at the

TABLE 2
LD_{50} Values of 9*H*-Xanthene-2,7-Diols (1a–e), α-Tocopherol (α-Toc), and 2,6-Di-*t*-Butyl-4-Methylphenol (BHT) on Normal Human Fibroblasts Measured by MTT and Crystal Violet Staining Methods

Compound number	LD_{50} (μg/mL)[a]			
	MTT method[b]		Crystal violet staining[c]	
	6 h[d]	24 h[e]	6 h[d]	24 h[e]
1a	420.0	240.0	380.0	200.0
1b	36.0	29.0	38.0	34.0
1c	9.8	9.2	10.0	7.6
1d	19.0	9.2	15.0	11.0
1e	20.0	9.4	22.0	21.0
α-Toc	>500.0	>500.0	>500.0	>500.0
BHT	8.1	7.2	9.8	7.4

[a]Determined from means of triplicate experiments.

[b]Antioxidant concentration of 50% reduction in formazan formation compared with control culture. MTT,2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2*H*-tetrazolium bromide.

[c]Antioxidant concentration of 50% reduction in viable cells compared with control culture.

[d]Human fibroblasts were treated with antioxidant for 6 h.

[e]Human fibroblasts were treated with antioxidant for 24 h.

interface of the micelle but not with radicals generated in the oil phase, i.e., inside the micelle.

Burton *et al.* (9) reported that the rate constant for the H-atom abstraction by peroxyl radicals for α-Toc and related compounds depends on the degree of stabilization of the phenoxyl radical. Stabilization of the phenoxyl radical depends on two factors: (i) the extent of orbital overlap between the 2p-type lone pair of electrons on the *para* oxygen atom and the aromatic π-electron system, and (ii) the electron-donating ability of the group bonded to the *para* oxygen atom. On this basis, comparison of the electronic structure of 9*H*-xanthene-2,7-diols (**1a–e**) with α-Toc indicated that **1a–e** all have an electron-donating group bonded to the ether-type oxygen atom. On the other hand, α-Toc has a long phytyl side chain. Therefore, it is possible that the differences in antioxidant activity between **1a–e** and α-Toc can be attributed to the electron-donating ability of the substituent bonded to the ether-type oxygen atom.

The cytotoxicities for 9*H*-xanthene-2,7-diols on normal human fibroblasts, measured by the MTT method (13) and crystal violet staining method (14), are summarized in Table 2. There were no significant differences in the LD_{50} values obtained by both methods. In addition, the LD_{50} values of each antioxidant slightly decreased with increasing treatment time. There is no clear criterion for the safety of antioxidants in the present method because interpretation of the cytotoxicity tests to human safety has not been done. Hence, the safety of 9*H*-xanthene-2,7-diols was estimated by comparing the LD_{50} values with those of α-Toc or BHT. With the present methods, toxicity for α-Toc could not be determined at <500 μg/mL. On the other hand, cytotoxicity of BHT, which is a popular synthetic antioxidant for polymers, rubber, lubricating oils, cosmetics, and some foods, showed values at relatively low concentrations (LD_{50} = 7.2–9.8 μg/mL). In contrast, the LD_{50} values of the 9*H*-xanthene-2,7-diols were greater than that of BHT. Especially, the LD_{50} values of **1a** and **1b** showed high concentrations that were 30–50 times and 4 times greater than that of BHT, respectively. These results suggest that the 9*H*-xanthene-2,7-diols are safer antioxidants than BHT, although the safety is inferior to that of α-Toc.

In summary, the antioxidant activity of 9*H*-xanthene-2,7-diols in linoleic acid was higher than that of α-Toc, and it was less cytotoxic toward human fibroblasts than was BHT. Therefore, 9*H*-xanthene-2,7-diols may be effective antioxidants for preventing the oxidation of oils and fats, not only in a homogeneous system but also in aqueous micelle dispersions such as cosmetics and foods.

REFERENCES

1. Wertz, P.W., E.S. Cho and D.T. Downing, *Biochim. Biophys. Acta 753*:350 (1983).
2. Hansen, H.S., and B. Jensen, *Ibid. 834*:357 (1985).
3. Nugterern, D.H., E. Christ-Hazelhof, A. van der Beek and U.M.T. Houtsmuller, *Ibid. 834*:429 (1985).
4. Niki, E., Y. Yamamoto, E. Komuro and K. Sato, *Am. J. Clin. Nutr. 53*:201S (1991).
5. Takahashi, M., J. Tsuchiya and E. Niki, *J. Am. Chem. Soc. 111*:6350 (1989).
6. Niki, E., *Chem. Phy. Lipids 44*:227 (1987).
7. L.R.C. Barclay, M.R. Vingvit, K. Makai, S. Itoh and H. Morimoto, *J. Org. Chem. 58*:7416 (1993).
8. Niki, E., A. Kawakami, M. Saito, Y. Yamamoto, J. Tsuchyia and Y. Kamiya, *J. Biol. Chem. 260*:2191 (1985).
9. Burton, G.W., T. Doba, E.J. Gabe, L. Hughes, F.L. Lee, L. Prasad and K.U. Ingold, *J. Am. Chem. Soc. 107*:7053 (1985).
10. Nishiyama, T., T. Ishida, S. Miyazaki, R. Tsuji and F. Yamada, *Bull. Chem. Soc. Jpn. 66*:2430 (1993).
11. Burton, G.W., and K.U. Ingold, *J. Am. Chem. Soc. 103*:6472 (1981).
12. Kharitonov, A.A., Z.G. Kozlova, V.F. Tsepalow and G.P. Gladyshev, *Kinet. Catal. 20*:593 (1979).
13. Mosmann, T., *J. Immunol. Methods 65*:55 (1983).
14. Saotome, K., H. Morita and M. Umeda, *Toxic. in Vitro 3*:317 (1989).

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