Chain-Breaking Fused Heterocyclic Antioxidants: Antioxidant Activities of 9*H*-Xanthene-2,7-diols and α-Tocopherol upon Liposomal Membranes

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ABSTRACT: We evaluated the antioxidant activities of 9*H*-xanthene-2,7-diols and α-tocopherol (α-Toc) upon the oxidation of soybean phosphatidylcholine liposomal membranes, induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile (AMVN). The stoichiometric factors of 9*H*-xanthene-2,7-diols, initiated with water-soluble AAPH and lipid-soluble AMVN, were 1.9–2.7 and 1.2–1.8-fold greater than those of α-Toc, respectively. The consumption profile of the antioxidant confirmed that 9*H*-xanthene-2,7-diol was completely consumed within the induction period (t_{inh}) and that the 9*H*-xanthene-2,7-diol oxidation product was formed. When all oxidation product was depleted, t_{inh} was terminated, and rapid oxidation occurred. These results suggested that the antioxidant activities of 9*H*-xanthene-2,7-diol depend not only on the initial hydrogen abstraction from 9*H*-xanthene-2,7-diol but also on a second hydrogen abstraction from the residual phenolic OH group of the oxidation product. Ascorbic acid (AsA) could not scavenge the radicals by itself in the lipid bilayer. However, when 9*H*-xanthene-2,7-diol was located in the lipid bilayer, the addition of AsA into the aqueous phase prolonged t_{inh} and reduced the rate of decay of 9*H*-xanthene-2,7-diol.
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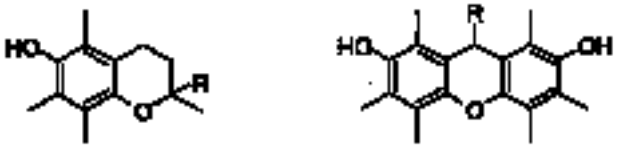
KEY WORDS: Antioxidant, ascorbic acid, soybean phosphatidylcholine liposome, synergistic effect, α-tocopherol, 9*H*-xanthene-2,7-diol.

A variety of polyunsaturated fatty acids and their esters play an important role in the various functions of cell membranes, for example, cellular communication, cellular contact processes, and regulation of the extracellular milieu (1–3). However, polyunsaturated fatty acids easily convert into lipid peroxides by reacting with free radicals or active oxygen species. The autoxidation of lipids in biological membranes has been implicated in a number of pathological events (4,5), including cancer (6), and in the aging process (7,8).

Natural antioxidants, such as tocopherols (Toc), are retained in the membrane and suppress the oxidation of phospholipid liposomal membranes, although the antioxidant activities of α-Toc are remarkably reduced in aqueous model membrane systems compared with those in homogeneous solution (9). For efficient use of these compounds, they are regenerated, most probably by ascorbic acid (AsA), at the lipid/water interface (10).



α-Toc, R = $C_{16}H_{33}$

1a, R = H **1d,** R = $CH(CH_3)_2$
1b, R = CH_3 **1e,** R = CH_6H_5
1c, R = CH_2H_5

SCHEME 1

We synthesized new antioxidants, 9*H*-xanthene-2,7-diols **1a–e** (Scheme 1), which are structurally related to α-Toc, and examined their antioxidant activities in the autoxidation of tetralin and linoleic acid in a homogeneous solution and in an aqueous micelle dispersion (11,12). Experimental results showed that 9*H*-xanthene-2,7-diols act as hydrogen donors and suppress the oxidation of tetralin and linoleic acid.

In this study, we investigated the antioxidant activity of 9*H*-xanthene-2,7-diols, as well as the synergistic effect of 9*H*-xanthene-2,7-diols and AsA, during the autoxidation of soybean phosphatidylcholine (PC) liposomes in an aqueous dispersion medium by using water-soluble as well as lipid-soluble initiators. We also describe the role of electronic effects of a fused heterocyclic 9*H*-xanthene-2,7-diol with an electron-donating group, bonded to the *para* heteroatom, which should further delocalize the unpaired electron in the phenoxyl radical and increase the antioxidant activity.

EXPERIMENTAL PROCEDURES

Materials. Commercial soybean PC, purchased from Nihon Seiyaku Co. (Tokyo, Japan), was purified by silica-gel chromatography. The composition of fatty acids in soybean PC

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was determined by gas–liquid chromatography after hydrolysis and esterification with boron trifluoride methanol complex (13). It consisted of palmitic acid 27.0, stearic acid 6.3, oleic acid 6.4, linoleic acid 50.5, and linolenic acid 9.8 mol%. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), and AsA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals and reagents purchased from commercial sources were of analytical grade. 9*H*-Xanthene-2,7-diols **1a**–**e** were prepared by the acid-catalyzed reaction of 2,3,5-trimethylhydroquinone with aldehydes (11).

Oxidation of liposomal membranes. The liposomal membranes were prepared as follows. Soybean PC and antioxidant were dissolved in chloroform, then the solvent was removed with a rotary evaporator to leave a thin film on the flask wall that was further evacuated under a high vacuum. NaCl aqueous solution (0.1 M, 10 mL) was then added, and the film was dispersed by sonication for 20 min to obtain a clear aqueous solution. The PC liposome solution was transferred to a reaction vessel with an attached oxygen electrode. The mixture was stirred at 37 or 50°C for about 10 min to saturate the solution with oxygen. Oxidation of the liposome system was initiated with the addition of water-soluble AAPH aqueous solution at 37°C or lipid-soluble AMVN methanol solution at 50°C. Oxygen concentration in the solution was monitored with a Biological Oxygen Monitor, Model YSI 5300 (Yellow Springs Instrument Co., Inc., Yellow Springs, OH), equipped with a Clark-type oxygen electrode. The induction period (t_{inh}) was determined graphically as the cross-point for the inhibited and uninhibited lines (14). Antioxidant activities were measured three times under the same conditions. Mean values and standard deviations are reported for each case. Student's *t*-test was applied for statistical evaluations.

High-performance liquid chromatography (HPLC). A 150 mm × 4.6 mm ODS column (Shiseido Co. Ltd., Tokyo, Japan) with a mobile phase of methanol/water (8:2, vol/vol) at a flow rate of 1.0 mL/min was used for 9*H*-xanthene-2,7-diol analysis. A Shimadzu LC-10A pump (Kyoto, Japan) was used. The components were detected with an ultraviolet-visible detector (Shimadzu SPD-6A), set at a wavelength of 254 nm, and a Shimadzu Chromatopac C-R4A recording data processor.

RESULTS AND DISCUSSION

The autoxidation of lipids under mild conditions, initiated by the azo initiator, such as AAPH or AMVN, and inhibited by antioxidant, can be represented by the following chain-reaction schemes (15):

$$\text{initiation: azo compound; } LH \xrightarrow{O_2} LO_2\cdot \quad \text{rate} = R_i \qquad [1]$$

$$\text{propagation: } LO_2\cdot + LH \xrightarrow{k_p} LOOH + L\cdot \qquad [2]$$

$$L\cdot + O_2 \longrightarrow LO_2\cdot \qquad [3]$$

$$\text{termination: } 2LO_2\cdot \xrightarrow{2k_t} \text{nonradical products} \qquad [4]$$

where LH denotes polyunsaturated fatty acid residues of soybean PC.

In the presence of the phenolic chain-breaking antioxidant, AH, the oxidation chains are shortened, termination by reaction 4 is suppressed, and 5 and 6 occur instead of reaction 4.

$$LO_2\cdot + AH \xrightarrow{k_{inh}} LOOH + A\cdot \qquad [5]$$

$$(n-1)LO_2\cdot + A\cdot \longrightarrow \text{nonradical products} \qquad [6]$$

In the presence of an antioxidant, the rate of oxidation, R_{inh}, during the induction period can be represented by Equation [7],

$$R_{inh} = k_p R_i[LH]/nk_{inh}[AH] \qquad [7]$$

where k_p is the propagation rate constant of the chain reaction, R_i is the rate of chain initiation, and k_{inh} is the rate constant of inhibition.

Figures 1 and 2 show typical oxygen uptake curves for the autoxidation of soybean PC liposomes initiated by water-soluble AAPH at 37°C and water-insoluble and lipid-soluble AMVN at 50°C, respectively. Scheme 1 gives the structures of the compounds studied. The rate of oxygen uptake was constant in the control solution in the absence of an antioxidant. When α-Toc or the 9*H*-xanthene-2,7-diols were added to the soybean PC liposomes, oxidation was suppressed, and there was a measurable induction period.

From the oxygen uptake traces, the t_{inh} during which oxidation is reduced can be measured. The length of t_{inh} for liposomal membranes that contained 9*H*-xanthene-2,7-diols and α-Toc are listed in Table 1. The t_{inh} values of 9*H*-xanthene-2,7-diols **1a**–**e** were affected by the nature of the substituent

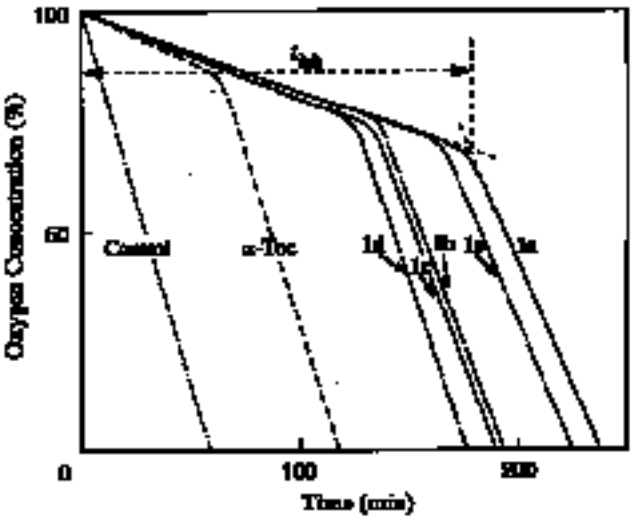

FIG. 1. Rate of oxygen uptake in the oxidation of 1.56 mM soybean phosphatidylcholine liposome initiated by 0.64 mM 2,2′-azobis(2-amidinopropane) dihydrochloride in the absence and presence of 0.012 mM antioxidant [9*H*-xanthene-2,7-diols **1a**–**e** and α-tocopherol (α-Toc)] at 37°C under oxygen; t_{inh}, induction period.

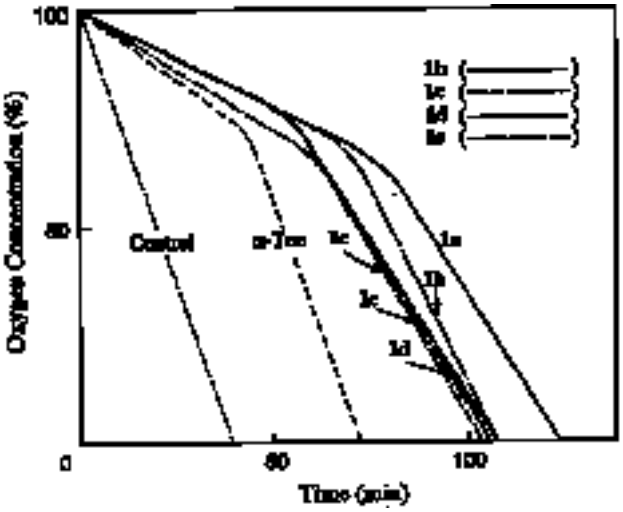

FIG. 2. Rate of oxygen uptake in the oxidation of 1.28 mM soybean phosphatidylcholine liposome initiated by 2.0 mM 2,2′-azobis(2,4-dimethylvaleronitrile) in the absence and presence of 0.02 mM antioxidant (9*H*-xanthene-2,7-diols **1a**–**e** and α-Toc) at 50°C under oxygen. See Figure 1 for other abbreviation.

group at the 9-position in the chemical structure. In particular, compound **1a** was the most ($P < 0.01$) active antioxidant under both conditions, being 2–3 times as active as α-Toc. The reported t_{inh} values (11) obtained under the same conditions for the autoxidation of tetralin in the presence of 9*H*-xanthene-2,7-diols and α-Toc are also summarized in Table 1 for comparison. The order of the t_{inh} values of 9*H*-xanthene-2,7-diols for soybean PC liposomes was similar to those found in the autoxidation of tetralin initiated with azobisisobutyronitrile (11).

The stoichiometric factor, *n*, for antioxidants was obtained from

$$t_{inh} = n[AH]/R_i \quad [8]$$

The t_{inh} values observed experimentally were proportional to the antioxidant concentration and the stoichiometric factor, as predicted from Equation 8. Because [AH] is known in Equation 8, the rate constant of chain initiation R_i can be calculated if *n* is known. The stoichiometric factor *n* is 2 for an efficient antioxidant, such as α-Toc (15). Therefore, the rate of initiation, R_i, was determined from the t_{inh} measured in the presence of α-Toc, for which $n = 2$ was assumed. The R_i values of PC liposomes initiated by AAPH and AMVN were 6.23×10^{-9} and 1.54×10^{-8} M s^{-1}, respectively. Because 9*H*-xanthene-2,7-diols have two active OH groups in the chemical structure, they should react with more peroxyl radicals than α-Toc. The stoichiometric factor obtained from Equation 8 for the series of xanthene-2,7-diols and α-Toc is summarized in Table 1. The stoichiometric factor for **1** was higher than that of α-Toc. Furthermore, the 9*H*-xanthene-2,7-diols initiated by AAPH (n = 3.3–5.5 peroxyls/molecule) exhibited higher stoichiometric factors in comparison with those of AMVN (2.5–3.6 peroxyls/molecule). This may in part reflect the generation of more reactive peroxyl radicals in the aqueous phase. In view of their chemical structure, 9*H*-xanthene-2,7-diols are lipid-soluble antioxidants and have a more polar group than α-Toc. This suggests that 9*H*-xanthene-2,7-diol would be located near the polar groups of the PC bilayers, because the polarity of 9*H*-xanthene-2,7-diols is higher than that of α-Toc. Therefore, 9*H*-xanthene-2,7-diols can react with radicals generated in the water phase at the polar region of the liposomal membranes.

Figure 3 shows the consumption of **1a** during the oxidation of the PC liposomes initiated by AMVN. The 9*H*-xanthene-2,7-diol **1a** was consumed linearly with time, and an oxidation product that was probably derived from **1a** ap-

TABLE 1
Antioxidant Activities of 9*H*-Xanthene-2,7-diols (1a–e) and α-Toc in the Oxidation of Soybean PC Liposomal Membranes and Tetralin

Compound number	Liposome (AAPH)[a]			Liposome (AMVN)[b]			Tetralin[c]
	t_{inh}[d] (min)	n[e]	R_{inh} ($\times 10^7$ M/min)	t_{inh}[d] (min)	n[e]	R_{inh}[f] ($\times 10^6$ M/min)	t_{inh} (min)
1a	174.8 ± 2.1[a]	5.5	9.30 ± 1.03	78.6 ± 1.8[a]	3.6	3.05 ± 0.15[f]	985
1b	136.7 ± 1.5[c]	4.3	9.58 ± 0.65	69.2 ± 3.1[b]	3.2	3.04 ± 0.28[f]	957
1c	134.6 ± 8.8[c]	4.1	10.13 ± 1.30	59.4 ± 2.6[c]	2.5	3.74 ± 0.16[g]	741
1d	123.6 ± 1.5[d]	3.8	10.00 ± 0.94	54.7 ± 2.2[c]	2.5	2.99 ± 0.26[f]	738
1e	163.3 ± 2.5[b]	5.1	9.53 ± 1.21	54.7 ± 3.1[c]	2.5	2.97 ± 0.25[f]	788
α-Toc	64.3 ± 1.7[e]	(2.0)	11.33 ± 1.21	43.4 ± 1.7[d]	(2.0)	4.25 ± 0.14[h]	345
Control	0.0 ± 0.0	—	—	0.0 ± 0.0	—	—	—

[a]Inhibition of oxidation of 1.56 mM soybean PC liposome by 0.012 mM antioxidant at 37°C, [AAPH] = 0.64 mM. Data are mean values ± SD of triplicate measurements.
[b]Inhibition of oxidation of 1.28 mM soybean PC liposome by 0.02 mM antioxidant at 50°C, [AMVN] = 2.0 mM. Data are mean values ± SD of triplicate measurements.
[c]Inhibition of tetralin oxidation by 1 mM antioxidant at 60°C, [AIBN] = 10 mM. Data for t_{inh} values were taken from Reference 11.
[d]Values within a column followed by different superscript letters (a–e) are significantly different ($P < 0.01$).
[e]Stoichiometric factor determined relative to standard α-Toc.
[f]Values within a column followed by different superscript letters (f–h) are significantly different ($P < 0.05$). Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); PC, phosphatidylcholine; AIBN, azobisisobutyronitrile.

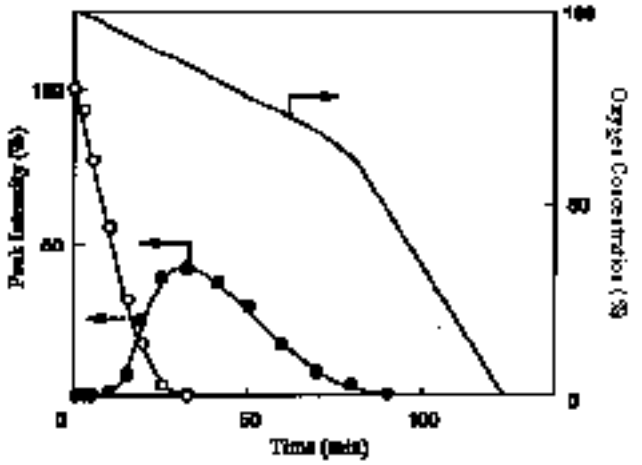

FIG. 3. Relationships between the consumption of 9*H*-xanthene-2,7-diol (**1a**) (○), profile of the oxidation product (●), and oxygen uptake during the oxidation of 1.28 mM soybean phosphatidylcholine liposome initiated by 2.0 mM 2,2′-azobis(2,4-dimethylvaleronitrile) at 50°C under oxygen.

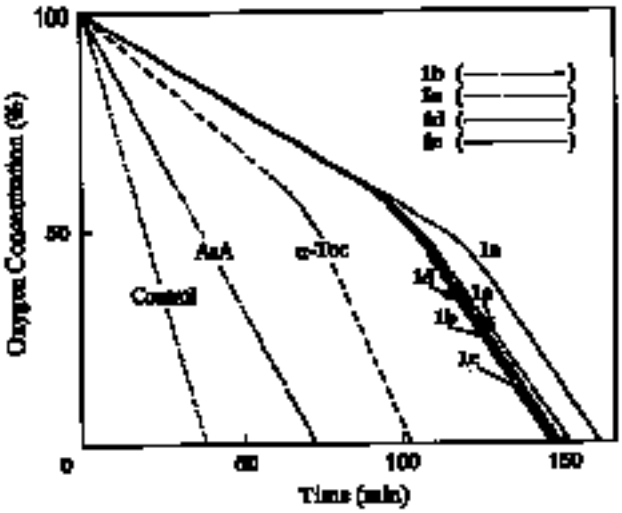

FIG. 4. Rate of oxygen uptake in the oxidation of 1.28 mM soybean phosphatidylcholine liposome containing 0.036 mM ascorbic acid initiated by 2.0 mM 2,2′-azobis(2,4-dimethylvaleronitrile) in the absence and presence of 0.02 mM antioxidant (9*H*-xanthene-2,7-diols **1a**–**e** and α-Toc) at 50°C under oxygen. See Figure 1 for abbreviation.

peared. The change in the concentration of **1a** oxidation product during the t_{inh} is also shown in Figure 3. The concentration of the oxidation product reached a maximum around 30 min. However, after all **1a** was consumed, the oxidation product of **1a** suppressed oxidation at a rate similar to that of **1a**. When all oxidation product was depleted, the t_{inh} was over, and oxidation became rapid. Although the structure of the 9*H*-xanthene-2,7-diol oxidation product remains unknown, these results indicated that suppression of oxidation depended not only on the 9*H*-xanthene-2,7-diol but also on the oxidation product.

AsA acts as a synergist for α-Toc on vegetable oil and common land animal fats (16,17). Niki *et al*. (10) have reported that a combination of α-Toc and AsA has a synergistic effect in the oxidation of PC liposomal membranes. AsA can directly scavenge free radicals that are generated in the aqueous phase and suppress oxidation of the liposomal membranes, but AsA cannot efficiently scavenge peroxyl radicals located in the liposomal lipid region. However, when α-Toc was located in the liposomal membranes, AsA extended the t_{inh} by regenerating α-Toc from the α-Toc radical.

To verify the synergistic interaction between 9*H*-xanthene-2,7-diols and AsA, we studied the oxidation of PC liposomes initiated by AMVN. Figure 4 shows the effects of AsA in the oxidation of PC liposomes initiated with AMVN. AsA alone in the aqueous phase retarded the oxidation but did not produce a clear t_{inh}. On the other hand, when 9*H*-xanthene-2,7-diols **1** or α-Toc and AsA were present in the PC bilayers and in the aqueous phase, respectively, t_{inh} was lengthened as shown in Figure 4. The values of t_{inh} and R_{inh} of the 9*H*-xanthene-2,7-diols **1a**–**e** or α-Toc with AsA during oxidation of PC liposomal membranes are listed in Table 2. There were no significant differences in the R_{inh} values without and with AsA for **1** or α-Toc, except for **1c**. In the presence of AsA, however, the t_{inh} values for **1** and α-Toc were 1.4-2.0 times longer ($P < 0.01$) than those measured in the absence of AsA. These results support the concept that AsA acts in the aqueous phase, and the 9*H*-xanthene-2,7-diol radical acts in the lipid region. That is, AsA is more accessible to the 9*H*-xanthene-2,7-diol radical than to peroxyl radicals in the lipid region because 9*H*-xanthene-2,7-diol must be located near the membrane surface.

Figure 5 shows the consumption of **1a** in the presence of AsA for the oxidation of PC liposomes initiated by AMVN. The results indicate that the consumption of 9*H*-xanthene-2,7-diol **1a** and the yield of the xanthene-2,7-diol oxidation

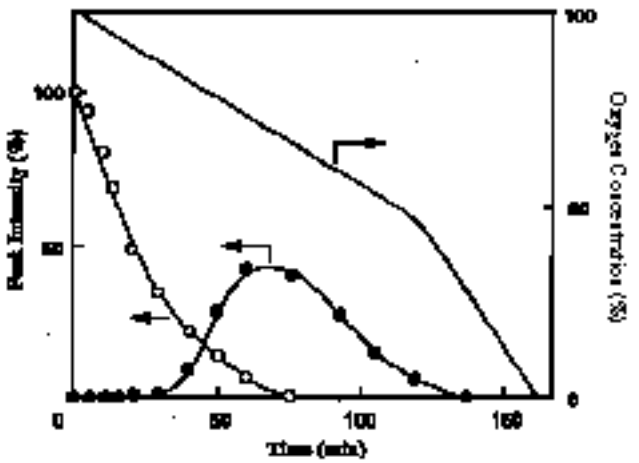

FIG. 5. Relationships between the consumption of 9*H*-xanthene-2,7-diol (**1a**) (○), profile of the oxidation product (●) and oxygen uptake during the oxidation of 1.28 mM soybean phosphatidylcholine liposome containing 0.036 mM ascorbic acid initiated by 2.0 mM 2,2′-azobis(2,4-dimethylvaleronitrile) at 50°C under oxygen.

TABLE 2
Antioxidant Activities of 9*H*-Xanthene-2,7-diols (1a–e) and α-Toc in the Oxidation of Soybean PC Liposomal Membranes without and with Ascorbic Acid

Compound number	Without ascorbic acid[a]		With ascorbic acid[b]	
	t_{inh}[c] (min)	R_{inh}[d] ($\times 10^6$ M/min)	t_{inh}[c] (min)	R_{inh}[d] ($\times 10^6$ M/min)
1a	78.6 ± 1.8[b]	3.05 ± 0.15	120.4 ± 3.9[a]	2.98 ± 0.12
1b	69.2 ± 3.1[b]	3.04 ± 0.28	98.5 ± 4.9[a]	3.06 ± 0.24
1c	59.4 ± 2.6[b]	3.74 ± 0.16[d]	103.6 ± 5.1[a]	3.09 ± 0.30[c]
1d	54.7 ± 2.2[b]	2.99 ± 0.26	98.7 ± 3.6[a]	3.10 ± 0.15
1e	54.7 ± 3.1[b]	2.97 ± 0.25	107.6 ± 5.0[a]	3.07 ± 0.11
α-Toc	43.4 ± 1.7[b]	4.25 ± 0.14	70.0 ± 2.2[a]	4.15 ± 0.17
Control	0.0 ± 0.0	—	0.0 ± 0.0	—

[a]Inhibition of 1.28 mM soybean PC liposome oxidation by 0.02 mM antioxidant at 50°C, [AMVN] = 2.0 mM. Data are mean values ± SD of triplicate measurements.
[b]Inhibition of 1.28 mM soybean PC liposome oxidation by 0.02 mM antioxidant and 0.036 mM ascorbic acid at 50°C, [AMVN] = 2.0 mM. Data are mean values ± SD of triplicate measurements.
[c]Values for t_{inh} within the same row followed by different superscript letters (a,b) are significantly different ($P < 0.01$).
[d]Values for R_{inh} within the same row followed by different superscript letters (c,d) are significantly different ($P < 0.05$). See Table 1 for abbreviations.

product were suppressed when AsA existed in the aqueous phase. Generation of the oxidation products was completely inhibited during the initial stage of oxidation. It can be speculated that AsA interacts with phenoxyl radicals derived from 9*H*-xanthene-2,7-diol. However, there are no differences in the rates of consumption of the oxidation product during the t_{inh} in the absence (Fig. 3) and presence of AsA. The results suggest that 9*H*-xanthene-2,7-diol scavenges the peroxyl radical more quickly than AsA, and that the resultant 9*H*-xanthene-2,7-diol radical reacts with AsA to regenerate 9*H*-xanthene-2,7-diol. The difference in the extended t_{inh} values of 9*H*-xanthene-2,7-diols and α-Toc may be attributed to their location in the bilayers and the stability of the phenoxyl radicals. That is, 9*H*-xanthene-2,7-diols were localized near the polar group of PC bilayers.

The peroxyl radical trapping activities of phenolic antioxidants, such as 2,6-di-*tert*-butyl-4-methylphenol (BHT) and α-Toc, can be expressed by the values k_{inh}, t_{inh}, and n. However, the 9*H*-xanthene-2,7-diols underwent a two-step hydrogen abstraction. That is, the antioxidant mechanisms of 9*H*-xanthene-2,7-diols were governed not only on the initial hydrogen abstraction from 9*H*-xanthene-2,7-diol but also on the second, from the residual OH group in the oxidation product. The rate constant, k_{inh}, of reaction 5, which indicates the reactivity of the initial hydrogen abstraction, is difficult to obtain experimentally for 9*H*-xanthene-2,7-diols. In this study, therefore, the antioxidant activities were indicated by t_{inh}, n, and R_{inh}, which reflect the total antioxidant actions of the 9*H*-xanthene-2,7-diols.

As shown in Table 1, the rates of oxidation, R_{inh}, of 9*H*-xanthene-2,7-diols **1a**–**e** and α-Toc were 9.3–10.1 and 11.3×10^{-7} M/min (no significant differences), during the peroxidation of PC liposomes initiated by the water-soluble initiator AAPH. The R_{inh} values of **1a**–**e** and α-Toc were 3.0–3.7 and 4.3×10^{-6} M/min ($P < 0.01$ or $P < 0.05$) when initiated by lipid-soluble AMVN. Judging from the R_{inh} and n values, the antioxidant activities of **1a**–**e** in PC liposome bilayers were higher than those of α-Toc. Burton *et al*. (18) reported that the rate constant for H-atom abstraction by peroxyl radicals for α-Toc and related compounds depends on the degree of stabilization of the phenoxyl radical, which itself depends on two factors: (i) the extent of orbital overlap between the 2 *p*-type lone pair 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. Thus, the higher reactivities of the 9*H*-xanthene-2,7-diols are probably due to the electronic structure. That is, the electron-donating group bonded to the ether-type oxygen and *para* ether oxygen held in a fused ring system would stabilize the phenoxyl radical formed in reaction 5 by delocalizing the unpaired electron to the *p*-type orbital of the *para* ether oxygen. Therefore, it is possible that the differences in antioxidant activity between 9*H*-xanthene-2,7-diols and α-Toc may be attributed to the electron-donating ability of the substituent bonded to the ether-type oxygen atom.

In conclusion, 9*H*-xanthene-2,7-diols behave as good chain-breaking antioxidants for the oxidation of PC liposomes against the free radicals generated in the liposomal lipid region and the outer aqueous phase. In addition, t_{inh} was extended when 9*H*-xanthene-2,7-diols and AsA were present in the liposomal bilayers and in the aqueous phase, respectively. Furthermore, we reported that 9*H*-xanthene-2,7-diols are less cytotoxic toward human fibroblasts than BHT (12). Thus, 9*H*-xanthene-2,7-diols may be effective antioxidants for preventing lipid oxidation in biological membranes.

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