Stability and Reactivity of Aryloxyl Radicals Derived from a Novel Antioxidant BO-653 and Related Compounds. Effects of Substituent and Side Chain in Solution and Membranes

Akira Watanabe,[†] Noriko Noguchi,[†] Akio Fujisawa,[†] Tatsuhiko Kodama,[†] Kunio Tamura,[§] Osamu Cynshi,[§] and Etsuo Niki*,[†]

Contribution from the Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Tokyo 153-8904, Japan, and Fujigotemba Research Laboratories, Chugai Pharmaceutical Co., Ltd., 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan

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Abstract: 2,3-Dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran (BO-653) is a novel antioxidant designed as a drug for inhibition of lipid peroxidation in vivo. To understand the dynamics of action of BO-653 as an antioxidant, the effects of substituents and side chains on the stability and reactivity of the BO-653 derived radical were studied and compared with the aryloxyl radicals derived from related compounds including α-tocopherol. The rate constants for the reactions of the aryloxyl radicals with themselves, lipid, hydroperoxide, and ascorbate were measured with a stopped-flow electron spin resonance (ESR) spectroscope equipped with a rapid mixing device. The ortho substituents exerted profound effects on the rate of bimolecular decay reactions and the reaction with ascorbic acid, while the effects on the reactions with methyl linoleate and *tert*-butyl hydroperoxide were much less significant. The side chain at the 2-position did not exert any effect in organic solution but the two pentyl side chains, when compared with two methyl side chains, diminished the apparent reactivities in the liposomal membranes.

Introduction

The free radical-mediated autoxidation of organic compounds by molecular oxygen is a double-edged sword and has both positive and negative aspects. This is one of the key processes in the present industrial chemistry and is applied for the production of chemicals such as terephthalic acid and cyclohexanol, important raw materials for synthetic polymers. On the other hand, such oxidation also induces oxidative degradation of plastics and rubbers and deterioration of foods and oils, and therefore various antioxidants have been developed to inhibit such oxidation. From the biological viewpoints, molecular oxygen is essential to us for energy production, synthesis of biologically active compounds, phagocytosis, and signal transduction in vivo. At the same time, there is now increasing experimental and clinical evidence which shows the involvement of free radical-induced oxidative damage of lipids, proteins, and DNA in a variety of pathological events, cancer, and aging.[1] As a result, the role of antioxidants has received renewed attention and both natural and synthetic antioxidants have been extensively explored.[2]

It is now generally accepted that oxidative modification of low density lipoprotein (LDL) is a key initial event in the progression of atherosclerosis which eventually causes coronary heart disease and cerebral hemorrhage.[3] The animal studies show that the antioxidant suppresses atherosclerosis[4,5] and the epidemiological studies suggest that high intake of vitamin E reduces the risk of coronary heart disease.[6] Probucol, 4,4′-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol), is a synthetic antiatherogenic drug used commercially, but it is known to have the side effect of decreasing high-density lipoprotein in plasma. BO-653, 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofran, is a novel antioxidant that has been designed, synthesized, and found to exert a potent antioxidant activity against lipid peroxidation[7] and LDL oxidative modification in vitro[8,9] and also antiatherogenic activity in three different animal models.[5] Special attention has been paid in designing BO-653 to choose appropriate substituents and side chains which determine the localization, retainment, and mobility within LDL particle. The potency as an antioxidant is determined by many factors as well as the chemical reactivity toward radicals.[10] For example, probucol is chemically much less reactive toward

[†] University of Tokyo.

[§] Chugai Pharmaceutical Co., Ltd.

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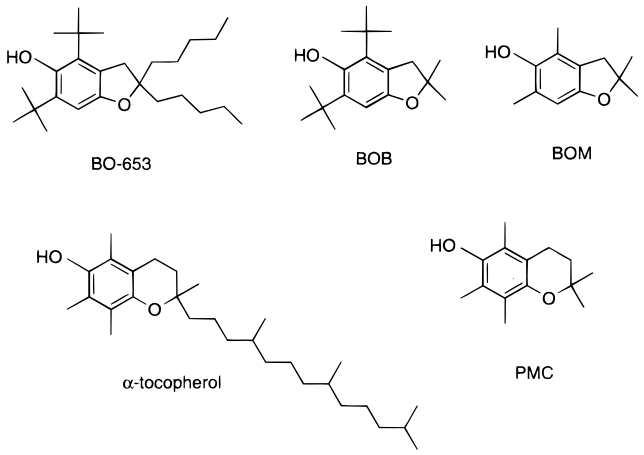

Figure 1. Antioxidants used in this study and their abbreviations.

radicals than α-tocopherol, the most abundant and active form of vitamin E in vivo, but probucol inhibits lipid peroxidation in LDL more efficiently than α-tocopherol in vitro.[11] When antioxidant scavenges radical, the antioxidant-derived radical is formed and the fate of this radical is also one of the important factors which determine antioxidant potency. To understand the basic chemistry of the mechanism and dynamics of the antioxidant action of BO-653, this study was carried out to investigate the stability and reactivity of the aryloxyl radicals derived from BO-653, its related compounds BOB and BOM, α-tocopherol, and its analogue PMC (Figure 1), aiming specifically at elucidating the effect of substituents and side chain, in solution and membranes.

Results

First, the reactions of the antioxidants shown in Figure 1 and stable phenoxyl radical galvinoxyl were studied with ESR spectroscopy. When excess antioxidant was reacted with galvinoxyl at 37 °C in ethanol under nitrogen, the ESR signal of galvinoxyl disappeared and a new ESR signal appeared. Typical examples are shown in Figure 2. As reported previously,[12] the time elapsed between the mixing of reactants and the beginning of recording operations (~1 s) corresponded to full consumption of galvinoxyl and the ESR signal intensity of the new aryloxyl radical was proportional to the initial concentration of galvinoxyl (data not shown). BO-653 and BOB gave an identical ESR signal, while α-tocopherol and PMC gave the same ESR signal (data not shown). The ESR signal intensities of the aryloxyl radicals decayed at different rates. The decay was followed with a rapid-mixing stopped-flow ESR at constant magnetic field corresponding to the main peak in the ESR spectrum and found to be second order with respect to the aryloxyl radical concentrations (Figure 3).

The decay rate of aryloxyl radicals, $I^{\bullet}$, is given by eq 1,

$$-d[I^{\bullet}]/dt = k_2[I^{\bullet}]^2 \quad (1)$$

and hence

$$1/[I^{\bullet}] = k_2 t + [I^{\bullet}]_0^{-1} \quad (2)$$

where k_2, t, $[I^{\bullet}]$, and $[I^{\bullet}]_0$ are the rate constant for bimolecular interactions of aryloxyl radicals, time, and the concentrations of aryloxyl radical at time t and start, respectively. The plot of $1/[I^{\bullet}]$ as a function of time gave a straight line and the rate constnts were obtained from the slope of the plots. They are summarized in Table 1. It shows that (i) the radicals derived from BO-653 and BOB with *tert*-butyl substituents at both ortho-positions were persistent and decayed much slower than BOM with two *o*-methyl substituents, (ii) the radical from BOM with the five-membered heterocyclic ring decayed slower than the PMC radical with the six-membered ring, and (iii) the side chain exerted little effect. When BOM was added to a solution containing BOB radical, the ESR spectrum did not change, but when BOB was reacted with BOM radical, the ESR signal of the BOM radical was rapidly replaced by that of the BOB radical (Figure 2). It was observed previously that when BO-653 was added to a solution of α-tocopheroxyl radical, the ESR signal of the α-tocopheroxyl radical disappeared rapidly and that of the BO-653 radical appeared and the addition of galvinoxyl to a mixture of BO-653 and α-tocopherol gave the ESR spectrum of only the BO-653 aryloxyl radical.[7]

To understand the fate of the antioxidant-derived radical, the rates of reaction of the aryloxyl radicals with lipid, hydroperoxide, and ascorbic acid were also measured in ethanol from the decay of the ESR signal of the aryloxyl radical. Methyl linoleate and *tert*-butyl hydroperoxide were chosen as a representative model of polyunsaturated lipids and lipid hydroperoxide, respectively. The interaction with ascorbic acid was also studied. The antioxidant was first reacted with galvinoxyl and then mixed with the substrate. The concentrations of the antioxidant and galvinoxyl were chosen so that galvinoxyl was depleted by the reaction with an excess of antioxidant before introducing a substrate.

Under these conditions, the reactions proceed as follows:

$$G^{\bullet} + IH \rightarrow GH + I^{\bullet} \quad (3)$$

$$I^{\bullet} + I^{\bullet} \xrightarrow{k_2} I{-}I \quad (4)$$

$$I^{\bullet} + SH \xrightarrow{k_s} IH + S^{\bullet} \quad (5)$$

where $G^{\bullet}$, IH, I−I, and SH are galvinoxyl, antioxidant, antioxidant-dimer, and substrate, respectively. The rate of decay of antioxidant radical $I^{\bullet}$ is given by eq 6, where k_2 and k_S are

$$-d[I^{\bullet}]/dt = k_2[I^{\bullet}]^2 + k_S[I^{\bullet}][SH] \quad (6)$$

the rate constant for reactions 4 and 5, respectively. The concentration of $I^{\bullet}$ was calculated from eq 7.[12]

$$[I^{\bullet}] = \frac{\dfrac{k_S[SH][I^{\bullet}]_0}{k_2[I^{\bullet}]_0 + k_s[SH]}\exp(-k_s[SH]t)}{1 - \dfrac{k_2[I^{\bullet}]_0}{k_2[I^{\bullet}]_0 + k_s[SH]}\exp(-k_s[SH]t)} \quad (7)$$

The rate constant k_S was obtained from the best fit to the experimental decay curve. The rate constants thus obtained for the reactions of aryloxyl radicals from the antioxidants and methyl linoleate, *tert*-butyl hydroperoxide, and ascorbic acid are included in Table 1. In ethanol solution, the effect of side chain was minimal. The rate constants of hydrogen atom abstraction from the three substrates by the antioxidant-derived radicals decreased in the order of α-tocopherol ~ PMC > BOM > BO-653 ~ BOB, but the difference in the reactivities varied

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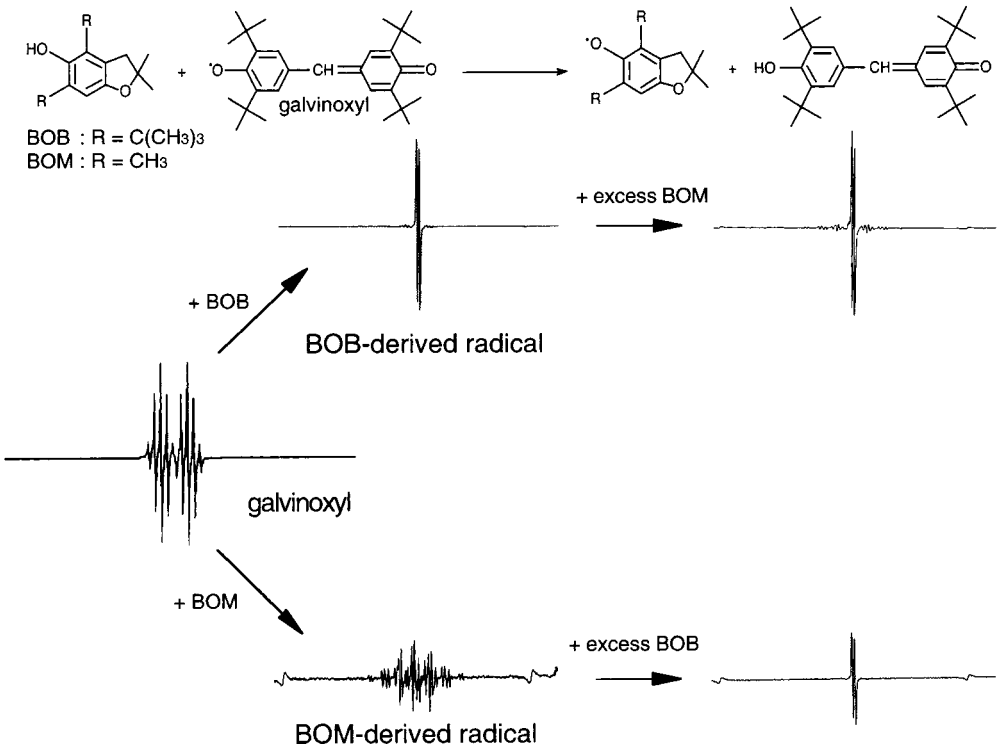

Figure 2. ESR spectra of aryloxyl radicals derived from BOB and BOM. BOB or BOM (20 μM) was reacted with galvinoxyl (10 μM) and then BOM or BOB (20 μM) was added to the solution. The ESR spectra were taken in ethanol at 25 °C under nitrogen under the conditions described in the Experimental Section.

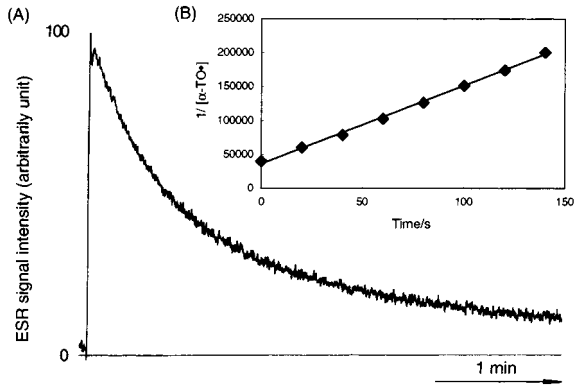

Figure 3. The decay of α-tocopheroxyl radical. α-Tocopherol (500 μM) and galvinoxyl (25 μM) were mixed in ethanol and the ESR signal intensity of α-tocopheroxyl radical was followed in ethanol at 37 °C under air. The insert is a second-order plot.

significantly with the substrates. The rate constant for the reaction of BOM radical with BOB was obtained as $(2.27 \pm 0.40) \times 10^3$ M^{-1} s^{-1} in ethanol at 37 °C under nitrogen.

The lipophilic antioxidants such as α-tocopherol and BO-653 are localized in vivo in the lipophilic domain of the membranes and lipoproteins, where the antioxidant efficacy is affected by physical factors such as fluidity and mobility of the microenvironment. Therefore, the action of antioxidant-derived aryloxyl radicals was also studied in liposomal membranes. The antioxidant was incorporated into the liposomal membranes simultaneously with dimyristoyl phosphatidylcholine (PC) and reacted with galvinoxyl to generate the aryloxyl radical. Since the local concentration of the aryloxyl radical within the lipophilic compartment was quite high,[13] the rates of spontaneous decay of α-tocopheroxyl and PMC radicals were too fast to measure the rate constant accurately. The rate constants for bimolecular interactions of the aryloxyl radicals derived from BOM, BOB, and BO-653 in PC liposomal membranes were obtained as 3.8×10^2, 0.52, and 0.28 M^{-1} s^{-1}, respectively. These rate constants in membranes were smaller than those in homogeneous solution by 54, 48, and 25%. The decay of BO-653 and BOB radicals was also measured in liposomal membranes composed of soybean phosphatidylcholine which contained about 70% linoleic acid moiety, but the decay rates were similar to those in dimyristoyl PC liposomal membranes, suggesting that the interaction of these aryloxyl radicals with polyunsaturated lipids was not rapid enough. The reduction of BOB radical and BO-653 radical in liposomal membranes by aqueous ascorbate was also measured (Figure 4). It shows that, as described above, BOB radical undergoes bimolecular decay faster than BO-653 radical in the absence of ascorbic acid and that ascorbic acid reduces BOB radical faster than BO-653 radical. The rate constants for the reaction of aryloxyl radicals from BO-653 and BOB with ascorbate were obtained as 2 and 14 M^{-1} s^{-1}, respectively, in liposomal membranes in the presence of 0.50 mM ascorbic acid. The rate constants obtained with 12.5 mM ascorbic acid concentration were smaller. This may be ascribed to the autoxidation of ascorbic acid at such high concentration.[14] Table 1 shows that the rate constants for interaction of BO-653 radical and BOB radical with ascorbic acid in liposomal membranes were 16 and 2 times smaller than those in ethanol solution, respectively. These results suggest that side chains with different lengths affect the apparent reactivity of aryloxyl radicals in the membranes.

Discussion

BO-653 was designed to serve as a potent, lipophilic radical-scavenging antioxidant in the lipoproteins and cell membranes,

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Table 1. Rate Constants of Aryloxyl Radicals Derived from Antioxidants for the Spontaneous Decay Reaction and the Hydrogen Atom Abstraction Reaction from Methyl Linoleate, *tert*-Butyl Hydroperoxide, and Asorcic Acid in Ethanol and Phosphatidylcholine Liposomal Membranes at 37 °C under Nitrogen

	in ethanol[a]				in membranes[b]	
	hydrogen atom abstraction (M^{-1} s^{-1})					
	spontaneous decay (M^{-1} s^{-1})	methyl linoleate	*t*-BuOOH	ascorbic acid	spontaeous decay (M^{-1} $^{-1}$)	H- atom abstraction from ascorbic acid (500 μM) (M^{-1} s^{-1})
α-tocopherol	$1.03 (\pm 0.03) \times 10^3$	$2.7 (\pm 0.4) \times 10^{-2}$	$4.1 (\pm 0.1) \times 10^{-1}$	$1.1 (\pm 0.2) \times 10^5$	*c*	*c*
PMC	$1.10 (\pm 0.12) \times 10^3$	$2.3 (\pm 0.4) \times 10^{-2}$	$3.7 (\pm 0.2) \times 10^{-1}$	$9.5 (\pm 0.0) \times 10^4$	*c*	*c*
BOM	$7.03 (\pm 0.02) \times 10^2$	$2.4 (\pm 0.3) \times 10^{-1}$	$2.6 (\pm 0.5) \times 10^{-1}$	$8.3 (\pm 0.1) \times 10^4$	3.8×10^2	*c*
BOB	$1.08 (\pm 0.10)$	$2.5 (\pm 0.2) \times 10^{-3}$	$1.1 (\pm 0.4) \times 10^{-1}$	29 ± 8	0.52	14
BO-653	$1.13 (\pm 0.15)$	$2.8 (\pm 0.2) \times 10^{-3}$	$1.4 (\pm 0.2) \times 10^{-1}$	33 ± 5	0.28	2

[a] Data are mean ± standard deviation of four independent determinations. [b] The anitoxident was incorporated into dimyristoyl phosphatidylcholine liposomal membranes. [c] The aryloxyl radicals decayed too fast to be measured accurately.

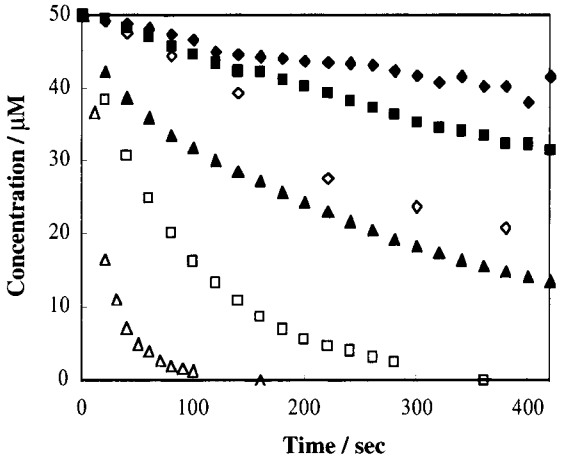

Figure 4. Decay of aryloxyl radicals derived from BO-653 (solid mark) and BOB (open mark) in dimyristoyl phosphatidylcholine liposomal membranes in the absence (diamond symbol) and presence of ascorbic acid. [Ascorbic acid] = 0.5 (square) and 12.5 mM (triangle).

above all against oxidative modification of LDL, and the substituents and side chains were carefully considered. Admittedly, the antioxidant should have high reactivity toward radicals, but it may be noteworthy that the chemical reactivity of the antioxidant toward radicals may be less important in LDL particles than in homogeneous solution. For example, α-tocopherol inhibits lipid peroxidation much more efficiently than 2,6-di-*tert*-butyl-4-methylphenol or probucol in organic solution, but the difference in antioxidant activities against lipid peroxidation is smaller in the membranes.[11] It appears considering the wealth of information from the extensive studies on antioxidants that the localization, mobility, and fate of antioxidant radical are more important factors which determine the overall antioxidant capacity in the membranes and lipoproteins. The ortho *tert*-butyl substituents hinder sterically the access of radical to the phenolic hydrogen atom but they also hinder the coupling reaction of aryloxyl radicals to make the radicals more persistent and also reduce their reactivities toward substrates. The data in Table 1 show that the rate constants for bimolecular decay of aryloxyl radicals derived from α-tocopherol and PMC are the same and the largest. About 30% smaller rate constant for BOM radical may be interpreted by a higher resonance stabilization due to the five-membered heterocylic ring than the six-membered ring.[15,16] The bimolecular decay rate constants for BO-653 and BOB radicals were found to be more than 600 times smaller than that for BOM, suggesting a significant effect of ortho substituents. The rate constants were measured in the absence of oxygen, but it has been reported that α-tocopheroxyl radical has low reactivity toward oxygen.[17]

The differences in the reactivities of aryloxyl radicals toward methyl linoleate and *tert*-butyl hydroperoxide were smaller than those in the bimolecular decay reaction. The radicals from BO-653 and BOB reacted with methyl linoleate 10 times slower than the BOM radical,[18] while they reacted with *tert*-butyl hydroperoxide at about half the rate of BOM radicals. Probably, the hydrogen atom of *tert*-butyl hydroperoxide is less sterically hindered than that of methyl linoleate. On the other hand, quite a striking steric effect was observed for the reaction with ascorbic acid. The rate constants for the reduction of aryloxyl radicals of BO-653 and BOB by ascorbic acid in ethanol were smaller than those from BOM, PMC, and α-tocopherol by more than 3 orders of magnitude. The reaction between antioxidant-derived aryloxyl radical and ascorbate is important since it regenerates and spares phenolic antioxidant and also inhibits the prooxidant action of the aryloxyl radicals. The most well-known example is the reduction of α-tocopheroxyl radical by ascorbate[19] and the synergistic inhibition of oxidation by a combination of vitamin E and vitamin C has been observed in many cases.[10,20] The present results show that ascorbate reduces the BO-653 radical quite slowly, about 3000 times slower than it reduces the α-tocopheroxyl radical. In agreement with this, it was found previously that BO-653 was not spared by ascorbic acid during the oxidation of phosphatidylcholine liposomal membranes[7] and LDL.[8] Furthermore, BO-653 reduces the α-tocopheroxyl radical to regenerate α-tocopherol,[7,8] the rate being obtained as 1.3×10^3 M^{-1} s^{-1} in ethanol under nitrogen at 37 °C.

Another interesting issue that can be seen from Table 1 is that the side chain has little effect in organic solution on the stability and reactivity of aryloxyl radicals: similar rate constants were obtained for α-tocopherol and PMC and also for BO-653 and BOB. A side chain of phenolic compound is another important factor which determines biological antioxidant capacity. α-Tocopherol and PMC exert similar antioxidant activity in homogeneous solution,[21] but PMC is more potent against lipid

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(18) We believe that the aryloxyl radicals react with methyl linoleate by hydrogen atom abstraction rather than addition reaction, because the reaction yields four isomeric conjugated hydroperoxides.

(19) The pK_{a1} and pK_{a2} values for ascorbic acid are 4.25 and 11.79, respectively, and hence it should be present as the monoanion form of ascorbate at pH 7.4.

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peroxidation in the membranes[21,22] and LDL[23,24] than α-tocopherol in vitro. However, PMC has little biological activity because it is readily excreted. The side chain is important for incorporation and retainment in the membranes and lipoproteins. At the same time, however, the side chain diminishes mobility of the antioxidant within and between the membranes, the effects being more significant with increasing side chain length.[21,22] As shown in Table 1, the rate constants for bimolecular decay reaction for BOM and BOB radicals in liposomal membranes were about half as large as those in solution, but that for the BO-653 radical with two pentyl side chains decreased to a quarter in the membranes. The rate constants for the reduction of aryloxyl radicals derived from BOB and BO-653 by ascorbate were smaller in the membranes than in solution. It has been shown that the reduction of radicals in the membranes and lipoproteins by ascorbate becomes slower as the radicals go deeper into the interior.[25,26] The more significant effect of the two pentyl side chains than two methyl groups within the liposomal membranes may be ascribed to lower mobility in the membranes, as observed for phytyl and methyl side chains of α-tocopherol and PMC.[22]

Experimental Section

Materials. BO-653 was synthesized from 4-acetoxy-3,5-di-*tert*-butylphenol and purified by column chromatography with 10% ethyl acetate in hexane (v/v) as eluent.[27] BOB and BOM were synthesized and purified similarly.[27] The synthesis of BOM (2,3-dihydro-2,2,4,6-tetramethyl-5-benzofuranol) was performed as follows. Claisen rearrangement of the allyl ether prepared by alkylation of 3,5-dimethylphenol with 3-chloro-2-methyl-1-propene gave 3,5-dimethyl-2-(2-methyl-2-propenyl)phenol. Then, the phenol derivative was converted into BOM through the following three-step reactions: oxidation to the benzoquinone derivative using Fremy's salt, reduction to the hydroquinone with sodium hydrosulfite, and cyclization of the resultant hydroquinone using boron trifluride diethyl etherate. Natural 2*R*,4′*R*,8′*R*-α-tocopherol and PMC were kindly supplied by Eisai Co. Ltd. (Tokyo, Japan). Methyl linoleate purchased from Sigma Chemical Co. (St. Louis, MO) was purified before use as reported previously.[11] Dimyristoyl phosphatidylcholine, *tert*-butyl hydroperoxide, and ascorbic acid were obtained from Sigma Chemical Co. and used as received. Other chemicals were those of the highest grade available commercially. Phosphatidylcholine liposomal membranes were prepared as reported previously.[11]

ESR Spectra of Galvinoxyl and Antioxidant-Derived Aryloxyl Radicals. The solution of galvinoxyl was placed into a quarts ESR tube and an appropriate solution of the antioxidant was taken into the sidearm. When required, the second antioxidant was taken into another sidearm. The solutions were frozen and evacuated and then the antioxidant was vacuum-transferred into the ESR tube. The solutions were thawed, galvinoxyl and the antioxidant were mixed, and the ESR spectra were recovered on an X-band JEOL JES-TE100 spectrometer at room temperature under vacuum under the following conditions: modulation frequency, 9.43 GHz; time constant, 0.30 s; scan time, 2 min; and microwave power, 1.00 mW. When required, the second antioxidant was introduced to the solution.

Stopped-Flow ESR Spectroscopy. Appropriate ethanol solutions or aqueous suspensions of liposomal membranes containing antioxidant and substrate were taken into the reservoir after removing air by bubbling nitrogen and they were introduced into a rapid mixer (type ES-SE2, JEOL, Tokyo) and then into an ESR flat cell by a pump.

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