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Antioxidant Action of 2,2,4,6-Tetra-Substituted 2,3-Dihydro-5-hydroxybenzofuran Against Lipid Peroxidation: Effects of Substituents and Side Chain

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With increasing evidence suggesting the involvement of oxidative stress in various disorders and diseases, the role of antioxidants *in vivo* has received much attention. 2,3-Dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran (BO-653) was designed, synthesized and has been evaluated as a novel antiatherogenic drug. In order to further understand the action of BO-653 and also radical-scavenging antioxidants in general, the dynamics of inhibition of oxidation by BO-653 were compared with those of the related compounds, 2,3-dihydro-5-hydroxy-2,2-dimethyl-4,6-di-*tert*-butylbenzofuran (BOB), 2,3-dihydro-5-hydroxy-2,2,4,6-tetramethylbenzofuran (BOM), α-tocopherol and 2,2,5,7,8-pentamethyl-6-chromanol (PMC), aiming specifically at elucidating the effects of substituents and side chain length of the phenolic antioxidants. These five antioxidants exerted substantially the same reactivities toward radicals and antioxidant capacities against lipid peroxidation in organic solution. When compared with di-methyl side chains, the di-pentyl side chains of BO-653 reduced its inter-membrane mobility but exerted less significant effect than the phytyl side chain of α-tocopherol on the efficacy of radical scavenging within the membranes. Di-*tert*-butyl groups at both ortho-positions made BO-653 and BOB more lipophilic than di-methyl substituents and reduced markedly the reactivity toward Cu(II) and also the synergistic interaction with ascorbate. The results of the present study together with those of the previous work on the effect of substituents on the stabilities of aryloxyl radicals suggest that *tert*-butyl group is more favorable than methyl group as the substituent at the ortho-positions and that di-pentyl side chains may be superior to a phytyl side chain.

Keywords: Antioxidant; Free radical; Lipid peroxidation; Vitamin E; Hydroxybenzofuran

INTRODUCTION

There is now increasing experimental and clinical evidence which suggests the involvement of oxidative stress induced by active oxygen and nitrogen species in the pathogenesis of various diseases, cancer, and aging.[1] As a consequence, the role of antioxidants has received much attention.[2–4] One of the important functions of the antioxidants is to scavenge active radicals to inhibit lipid peroxidation, protein modification, and DNA damage. Numerous natural radical-scavenging antioxidants have been explored, while novel synthetic antioxidants have been also tested. The antioxidant activities have been measured often in homogeneous solution, where the activity is determined primarily by the chemical reactivity toward radicals. However, it is known that the potency of radical-scavenging antioxidants *in vivo*

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is determined not only by the chemical reactivity toward radicals but also by other factors such as localization, concentration and mobility at the microenvironment, fate of antioxidant-derived radical, and interaction with other antioxidants. On the basis of these considerations, we have designed and synthesized a novel radical-scavenging antioxidant, 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran (BO-653) and evaluated its antioxidant activity against lipid peroxidation,[5] oxidative modification of low density lipoprotein (LDL),[6] and atherogenesis in animal models.[7,8] BO-653 was also found to exert potent antioxidant effect in macrophage-mediated oxidation of LDL.[9] Additionally, it was found that BO-653 acted as a potent antioxidant in every system tested and is a promising candidate as a drug for atherosclerosis. It was recently found by DNA microarray analysis that BO-653 down-regulated gene expression of proteasome in endothelial cells.[10,11] In order to understand the dynamics of action of BO-653 and furthermore radical-scavenging compounds as an antioxidant, the effects of substituents and side chain on the inhibition of lipid peroxidation were studied in several model systems. For this purpose, two relevant compounds were synthesized, 2,3-dihydro-5-hydroxy-2,2-dimethyl-4,6-di-*tert*-butylbenzofuran (BOB) and 2,3-dihydro-5-hydroxy-2,2,4,6-tetramethylbenzofuran (BOM) (Fig. 1). These compounds together with α-tocopherol (α-TOC) and its model compound 2,2,5,7,8-pentamethyl-6-chromanol (PMC) enabled us to clarify the effects of substituents at phenolic ortho positions and side chain on the action of BO-653 and radical-scavenging antioxidants in organic homogeneous solution and in micelles and membranes in aqueous dispersions. It has been shown previously that the side chain at the 2-position does not affect the chemical reactivity of the chromanol but that a long side chain is required for incorporation and retainment in the membranes and lipoproteins, although the longer the side chain the less mobile the antioxidant becomes. This is the first report on the effects of side chain and also substituents on the action of BO-653 as a radical-scavenging antioxidant.

MATERIALS AND METHODS

Materials

BO-653, BOB, and BOM were prepared as described earlier.[12] Natural 2R,4R′,8R′-α-tocopherol and PMC were kindly supplied by Eisai Co. Ltd (Tokyo, Japan). Methyl linoleate and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (PLPC) used as oxidizable substrate were obtained from Tokyo Kasei Co. (Tokyo, Japan) and Sigma (St. Louis, MO). The azo compounds, 2,2′azobis-(2,4-dimethylvaleronitrile) (AMVN), 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN), and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) obtained from Wako Pure Chemical Ind. (Osaka, Japan) were used as received as a radical initiator. Other chemicals were those of the highest grade available commercially.

Interaction of Antioxidants with Galvinoxyl

The kinetics of the reaction of antioxidant and galvinoxyl were studied by following the decrease in

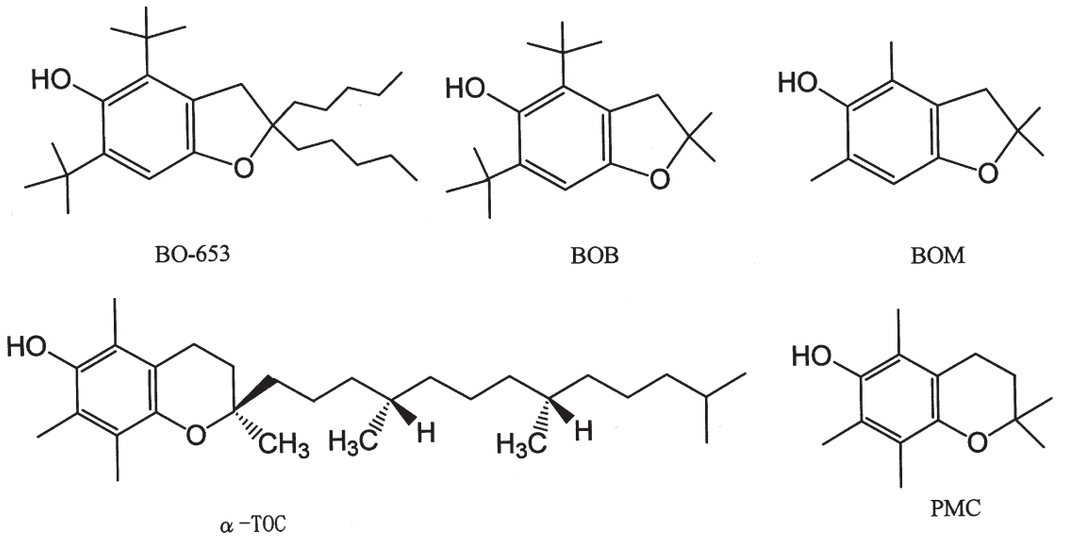

FIGURE 1 Antioxidants used in this study and their abbreviations. BO-653: 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran; BOB: 2,3-dihydro-5-hydroxy-2,2-dimethyl-4,6-di-*tert*-butylbenzofuran; BOM: 2,3-dihydro-5-hydroxy-2,2,4,6-tetramethylbenzofuran; PMC: 2,2,5,7,8-pentamethyl-6-chromanol; α-TOC: α-tocopherol.

absorption of galvinoxyl at 428 nm at 25°C with a stopped-flow spectrophotometer equipped with a processing system (RA-2000, Photal Otsuka Electronics, Osaka, Japan). Kinetic runs were carried out under pseudo-first order conditions, that is, the concentrations of galvinoxyl being kept several times lower than those of antioxidants. Each kinetic run was performed at least five times and the average first-order rate constant was plotted against antioxidant concentration to obtain the second-order rate constant.

Oxidation of Methyl Linoleate and PLPC

Oxidation of methyl linoleate was carried out at 37°C under air either in organic homogeneous solution or in aqueous dispersions. The emulsions were prepared by vigorously mixing methyl linoleate and SDS in a phosphate-buffered saline (PBS, pH 7.4) with a vortex mixer for 2 min. The oxidation was induced by the addition of azo initiator or copper. The rate of oxidation of methyl linoleate was measured either from the increase in absorption at 234 nm due to conjugated diene or from the accumulation of methyl linoleate hydroperoxides measured with an HPLC.[6] ODS column was used and CH_3CN/H_2O (9/1 by volume) was delivered as eluent at 1 ml/min. The oxidation of PC was carried out in liposomal membranes as described before.[6] Silica-gel column was used and methanol/40 mM phosphate buffer (9/1 by volume) was delivered as eluent at 1 ml/min.

Analysis of Antioxidant

BO-653, BOB, BOM, α-tocopherol, and PMC were detected using an HPLC by an amperometric electrochemical detector (NANOSPACE SI-1, Shiseido, Tokyo, Japan) set at 800 mV, with an ODS column (LC-18, 5 μm, 250 × 4.6 mm, Supelco, Japan) and methanol/*tert*-butyl alcohol (90/10 by volume) as eluent at 1 ml/min.

Analysis of α-tocopheryl Quinone

α-Tocopheryl quinone was analyzed with both spectrophotometer by following the maximum absorption at 268 nm and an HPLC by a photodiode array detector (SPD-M10AVP, Shimadzu, Kyoto, Japan) with an ODS column (LC-18, 5 μm, 250 × 4.6 mm, Supelco, Japan) and methanol/*tert*-butyl alcohol (90/10 by volume) as eluent at 1 ml/min.

The experiments were carried out repeatedly and the result of the representative ones is shown. The reproducibility was within ±10%.

RESULTS

Reactivities Toward Oxygen Radicals and Inhibition of Oxidation in Homogeneous Solution

Obviously, one of the important factors that determine the antioxidant capacity is the reactivity toward radicals, that is, how rapidly does the antioxidant scavenge the radical. This can be measured by using a stable radical or by a competition method. In the present study, galvinoxyl was chosen as a stable radical and the reactivities of BO-653, BOB, BOM, α-tocopherol, and PMC toward galvinoxyl were measured with a stopped-flow spectrophotometer equipped with a rapid mixer by following a decrease in maximum absorption of galvinoxyl at 428 nm as reported previously.[13,14] It was found that all these antioxidants have similar reactivities toward galvinoxyl (data not shown).

The antioxidant activities of these compounds were measured first in the oxidation of methyl linoleate in homogeneous solution initiated by a lipophilic initiator AMVN. BO-653, BOB, BOM, α-tocopherol, and PMC exerted substantially the same antioxidant activities: they were consumed at the same rate, suppressed the rate of oxidation to the same extent, and produced the induction period of the similar length.

The above results suggest that these antioxidants exerted similar antioxidant capacities in homogeneous solution independent of substituent and side chain.

Relative Efficacy of Radical Scavenging in Membranes

The action of antioxidants was then studied in the liposomal membranes prepared from phosphatidylcholine (PC). The efficacy of antioxidant for radical scavenging can be evaluated from the consumption of antioxidant. BO-653, BOB, and BOM were consumed at the similar rates when one of them was incorporated into either PLPC or dimyristoyl PC (14:0 PC) liposomes and oxidized by AMVN (Fig. 2). The competition between the antioxidants for scavenging radicals was assessed by following the disappearance of the antioxidants. Equal concentrations of BO-653 and BOB were incorporated into PC multilamellar liposomal membranes and oxidized either by hydrophobic AMVN or by hydrophilic radical initiator AAPH. AMVN was mixed with PC beforehand and simultaneously incorporated together into the membranes, while AAPH was added as an aqueous solution after preparation of the membranes. When BOB and BO-653 were incorporated into either PLPC liposomes or 14:0 PC liposomes and oxidized with AMVN, both antioxidants were consumed at the similar rate

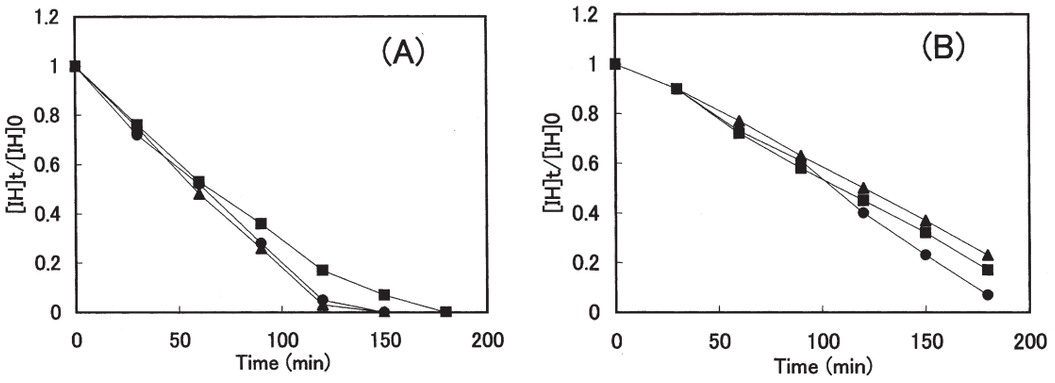

FIGURE 2 Consumption of either BOB (▲), BOM (●) or BO-653 (■) in 5 mM 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (PLPC) liposomes (A) and in 5 mM dimyristoyl PC (14:0 PC) liposomes (B) induced by 1 mM AMVN in aqueous dispersions (PBS, pH = 7.4) at 37°C. Initially, 3 μM BO-653, BOB, or BOM was incorporated into liposomal membranes.

concomitantly (Fig. 3A,B). In contrast, when they were oxidized by AAPH, BOB was consumed faster than BO-653 (Fig. 3C,D). These results suggest that BOB and BO-653 scavenge lipophilic radicals within the membranes by similar efficacy and that the effect of di-pentyl side chains is small. This is in contrast to the large effect of phytyl side chain of α-tocopherol, which reduces the mobility and activity in the membranes significantly.[15,16] In support of this, it was confirmed that only PMC was consumed preferentially and α-tocopherol was spared almost completely under the similar conditions (Fig. 4). The results that, when AAPH was used, BOB was consumed faster than BO-653 in both PLPC and 14:0 PC multilamellar vesicles suggest that BOB has higher mobility than BO-653 between the membranes.

The competition between BOB and BOM was also studied to examine the effect of ortho-substituents in four different systems: in PLPC and 14:0 PC

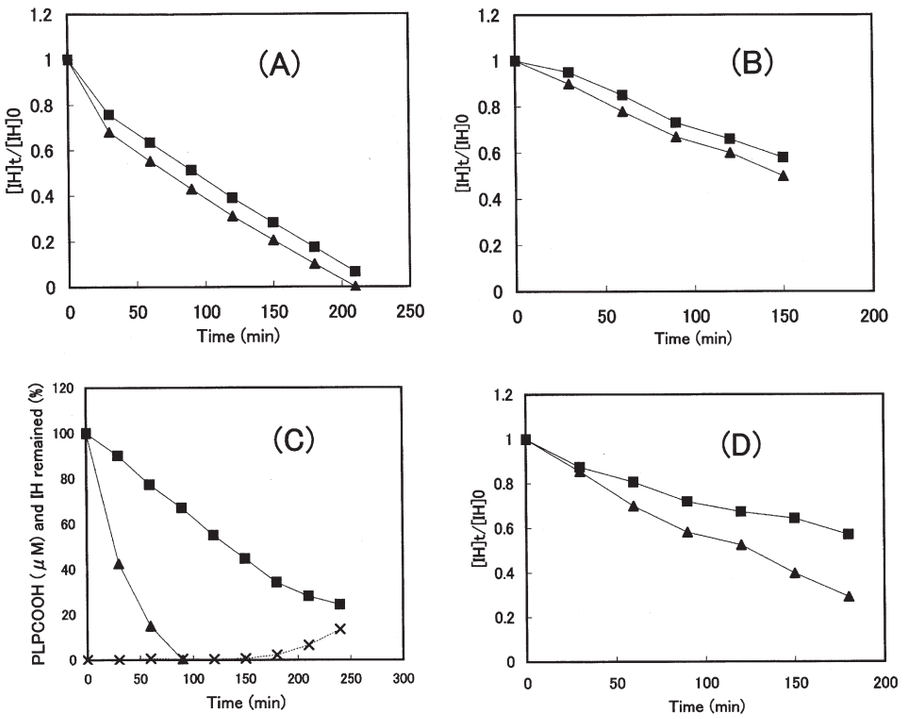

FIGURE 3 Consumption of BO-653 (■) and BOB (▲) in 5 mM 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (PLPC) liposomes (A, C) and in 5 mM dimyristoyl PC (14:0 PC) liposomes (B, D) induced by either 1 mM AMVN (A, B) or 3 mM AAPH (C, D) in aqueous dispersions (PBS, pH = 7.4) at 37°C. Equal concentrations of BO-653 and BOB (3 μM each) were incorporated into PC multilamellar liposomal membranes. The symbol × in Fig. 4C shows the formation of PLPC hydroperoxides (PLPCOOH).

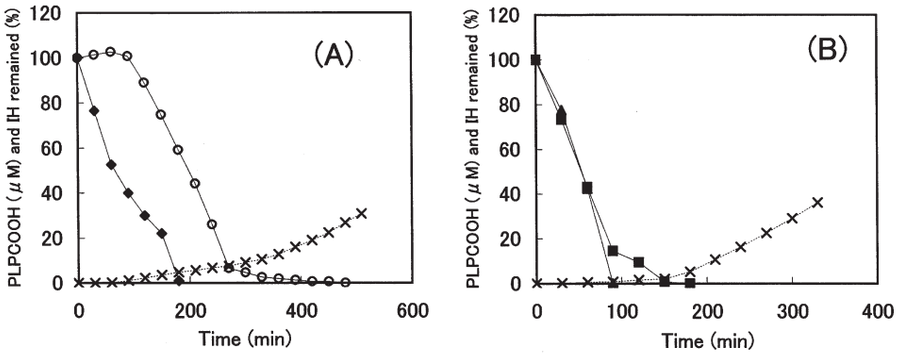

FIGURE 4 Consumption of PMC (◆) and α-tocopherol (○) (A) and that of BO-653 (■) and BOB (▲) (B) in 2.83 mM 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (PLPC) liposomes induced by 0.1 mM 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) in aqueous dispersions (PBS, pH = 7.4) at 37°C. Equal concentrations of two antioxidants (2 μM, each) were incorporated into PLPC multilamellar liposomal membranes. The symbol × shows the formation of PLPC hydroperoxides (PLPCOOH).

multilamellar vesicles induced by either AAPH or AMVN (Fig. 5). The relative rates of consumption of BOB and BOM are determined by the rate of scavenging of radicals by antioxidants and also by the reduction of BOM-derived radical by BOB.[12] It was found that BOB was consumed faster than BOM in all four systems implying that BOB reduces BOM-derived radical independent of the localization of radical formation.

Synergistic Inhibition of Lipid Peroxidation with Ascorbic Acid

It has been well-documented that the combination of α-tocopherol and ascorbic acid exerts synergistic effect against the lipid peroxidation in the membranes,[17,18] micelles,[19] and LDL,[20–22] since ascorbate reduces α-tocopheroxyl radical to regenerate α-tocopherol, although ascorbate is not capable of

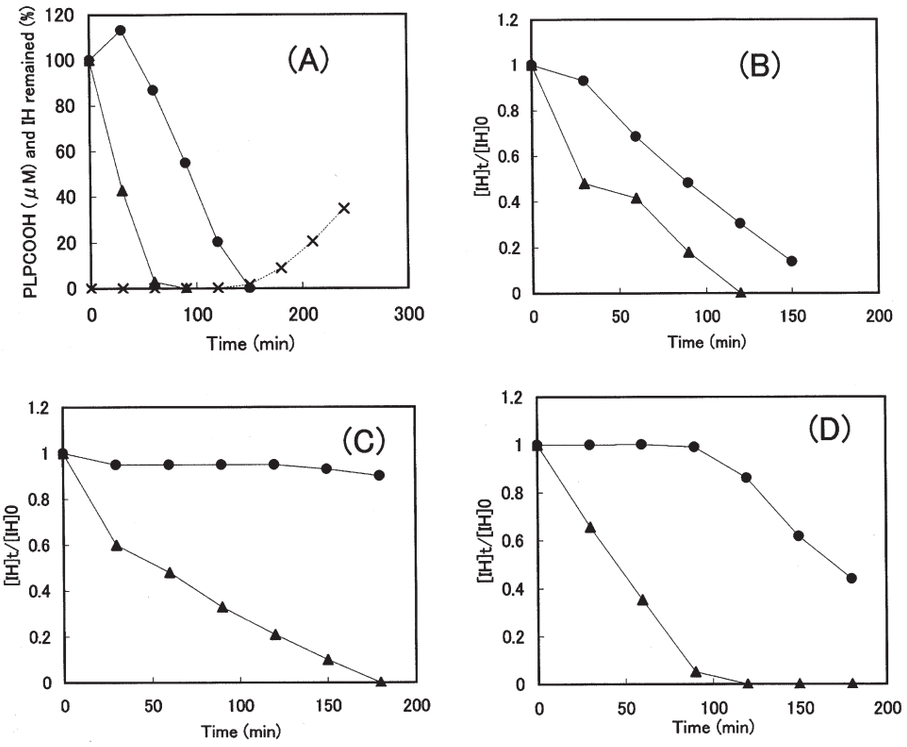

FIGURE 5 Consumption of BOM (●) and BOB (▲) in 5 mM 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (PLPC) liposomes (A, C) and in 5 mM dimyristoyl PC (14:0 PC) liposomes (B, D) induced by either 3 mM AAPH (A, B) or 1 mM AMVN (C, D) in aqueous dispersions (PBS, pH = 7.4) at 37°C. Equal concentrations of BOM and BOB (3 μM each) were incorporated into PC multilamellar liposomal membranes. The symbol × in Fig. 6A shows the formation of PLPC hydroperoxides (PLPCOOH).

scavenging lipophilic radicals within lipophilic compartment efficiently.[23,24] The effects of combination of ascorbic acid with BOB or BOM in the oxidation of methyl linoleate micelles are shown in Fig. 6. BOB was used to compare with BOM, since the efficacy of reducing aryloxyl radicals by ascorbate depends on the side chain. As shown in Fig. 6, ascorbic acid at higher concentration suppressed the oxidation by itself. Interestingly, the combination of ascorbic acid with BOM inhibited the oxidation completely and the inhibition period was prolonged markedly, while the combination with BOB resulted simply in an additive effect, suggesting that the reduction of BOB-derived radical by ascorbate is not efficient. These results are in accordance with the fact that the rate of reduction of aryloxyl radicals by ascorbate is strongly dependent on the ortho-substituents, the rate constant for reduction of BOB-derived radical by ascorbate in ethanol being about 3×10^3 times smaller than that of BOM radical.[12]

Pro-oxidant Effect of Antioxidant Induced by Copper

A potent radical-scavenging antioxidant acts as a reductant as well and may act as a pro-oxidant under certain circumstances. For example, ascorbate, the primary hydrophilic antioxidant *in vivo*, acts as an oxidant *in vitro* in combination with Fe(III). It is known that α-tocopherol can also act as a prooxidant *in vitro* in combination with Fe(III) or Cu(II).[25–31] The prooxidant action stems from the reduction by α-tocopherol of Fe(III) or Cu(II) to the corresponding lower valency state, Fe(II) or Cu(I), which decomposes hydroperoxides to give oxygen radicals much faster than Fe(III) or Cu(II), respectively. The effect of BO-653, BOB, and BOM as a prooxidant in combination with Cu(II) was studied in the oxidation of methyl linoleate micelles in aqueous dispersions containing 0.5 M SDS (PBS, pH 7.4). α-Tocopherol was oxidized by Cu(II) to give α-tocopheryl quinone, whose formation can be followed by an increase in absorption at 268 nm. It was found that α-tocopherol, PMC, and BOM were oxidized by Cu(II), but neither BO-653 nor BOB was oxidized (Fig. 7). Interestingly, BOM and PMC were consumed faster than α-tocopherol and the absorption at 268 nm due to quinone increased much faster from BOM and PMC than α-tocopherol. These results suggest that the oxidation by Cu(II), that is, reduction of Cu(II), depends on the ortho-substituents of phenols and also side chain in this micelle system.

DISCUSSION

The reactivities of natural and synthetic radical-scavenging antioxidants have been studied extensively,[2–4] however, relatively less attention has been paid to the dynamics of antioxidant action in heterogeneous systems. It has been shown that efficacy of radical scavenging by antioxidants varies markedly depending on the environment.[32,33] For example, it is accepted that vitamin C (ascorbic acid) is the primary hydrophilic radical-scavenging antioxidant in human whole blood[34] and plasma.[35] However, the efficacy of scavenging radicals within lipophilic domain by vitamin C is low and it decreases as the radical goes deeper into the interior of membranes or lipoproteins.[23,24] α-Tocopherol, PMC, and its water-soluble analogue, 2-carboxy-2,5,7,8-tetramethyl-6-chromanol (Trolox) have similar reactivities toward peroxyl radicals and exert substantially the same antioxidant activity against lipid peroxidation in homogeneous solution, that is,

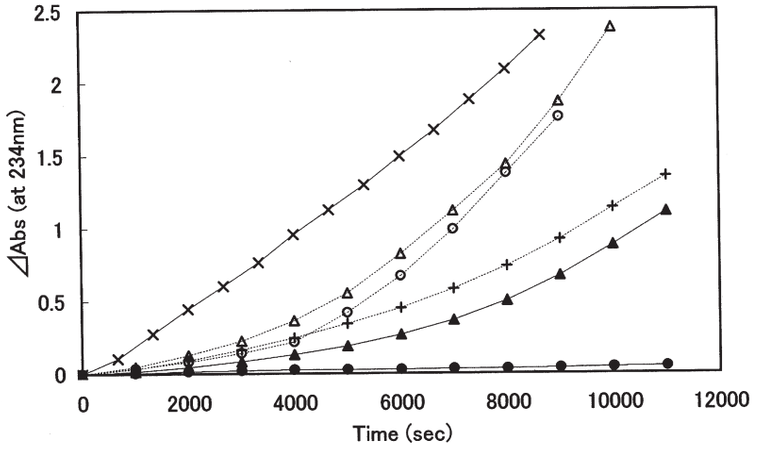

FIGURE 6 Increase in absorption at 234 nm in the oxidation of methyl linoleate (7.4 mM) SDS (0.5 M) micelles in the absence (×) and presence of antioxidants incorporated into micelle induced by 1 mM AMVN in aqueous dispersions (PBS, pH = 7.4) at 37°C. △: 2 μM BOB; ○; 2 μM BOM; ▲: 2 μM BOB with 50 μM ascorbate; ●: 2 μM BOM with 50 μM ascorbate; +: 50 μM ascorbate.

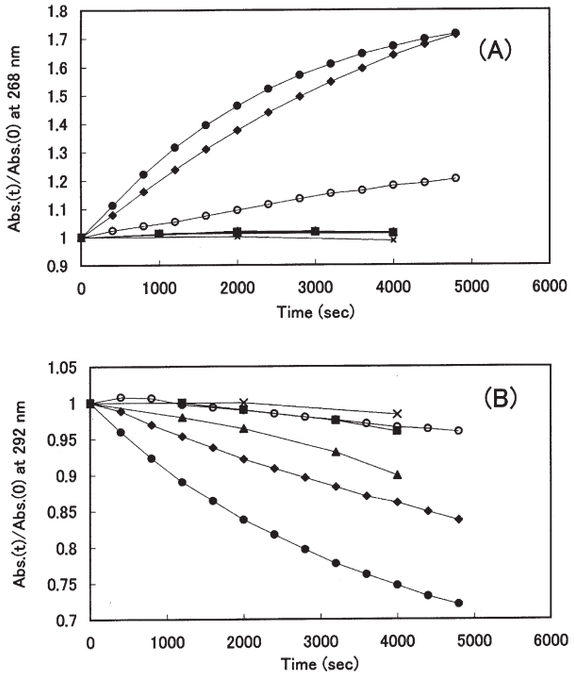

FIGURE 7 Interaction of either BOB (▲), BOM (●), BO-653 (■), PMC (◆), or α-tocopherol (○) (100 μM each) in methyl linoleate (7.4 mM) SDS (0.5 M) micelles with cupric sulfate (100 μM) in aqueous dispersions (PBS, pH = 7.4) at 37°C. (A) and (B) shows the change of absorption at 268 and 292 nm, respectively. The results of control run (without antioxidant) are shown by the symbol × in the figures.

they suppress the rate of oxidation and produce induction period to the same level. However, Trolox acts as the primary antioxidant against hydrophilic radicals, while PMC acts as the primary antioxidant in the membranes.[32,33] Furthermore, probucol an anti-atherogenic drug, has much smaller reactivity toward peroxyl radicals than α-tocopherol but it is as effective as α-tocopherol against the oxidation in low density lipoprotein particle.

Thus, the antioxidant capacities in heterogeneous systems are determined by several factors to which the side chain and substituents contribute significantly. These issues have not received as much attention as they should. The compounds listed in Fig. 1 are suitable for studying such effects. The results given above show that the five compounds tested have similar reactivities toward oxygen radicals and exert similar antioxidant activities against lipid peroxidation in homogeneous solution. However, several interesting effects of side chain and substituents were revealed in heterogeneous systems. It has been shown previously that the side chain of α-tocopherol plays an important role in the action and role as an antioxidant.[15,16,36,37] The phytyl side chain reduces the inter-membrane and intra-membrane mobility,[15,16,36,37] but it is essential for incorporation and retainment into the membranes and lipoproteins. PMC often exerts higher antioxidant activity than α-tocopherol in many kinds of oxidation *in vitro*,[38] but PMC has little biological activity.

The results in Figs. 3 and 4 show that PMC scavenges peroxyl radicals much faster than α-tocopherol in the liposomal membranes, but BOB and BO-653 have similar activities. The mobility of the antioxidant in the membranes has been shown to decrease with increasing length and number of side chain.[39] The above results suggest that the two pentyl side chains of BO-653 give less significant effect than phytyl side chain and BO-653 exerts substantially the same radical-scavenging efficacy as BOB in the membranes. However, the two pentyl side chains of BO-653 reduce the inter-membrane mobility.

This study demonstrated two significant effects of ortho-substituents. Firstly, the *tert*-butyl substituents reduced the reactivity of the aryloxyl radicals toward ascorbate and inhibited the synergistic inhibition with ascorbate. This is ascribed to a smaller rate constant for reduction of di-*tert*-butyl-substituted aryloxyl radicals by ascorbate than di-methyl-substituted aryloxyl radicals.[12] Another effect was observed in the reaction with Cu(II). It has been reported that under certain conditions, α-tocopherol reduces Cu(II) to Cu(I) and acts as a pro-oxidant.[25–30] The present study showed that α-tocopherol, PMC, and BOM reacted with Cu(II) to give corresponding quinone such as α-tocopheryl quinone, but that BOB and BO-653 did not, implying a significant ortho-substituent effect. Similarly, it was reported that di-*tert*-butylhydroxylated flavonoids neither reduced nor chelated copper.[40] It is not clear whether this is due to a steric effect or a difference in the redox potential. Although it is not clear whether the pro-oxidant effect of α-tocopherol and Cu(II) observed in the *in vitro* model systems is relevant to physiological conditions, the results of the present study show that BO-653 does not exert pro-oxidant effect in combination with copper.

The above results of the present study and previous ones[5–9,12] suggest that BO-653 has suitable structure in that it has high reactivity toward radicals, its aryloxyl radical is stable and unreactive, it is more lipophilic and more mobile in the membranes and probably in lipoproteins than α-tocopherol, and it does not reduce Cu(II), although its aryloxyl radical is reduced by ascorbate less efficiently than α-tocopheroxyl radical.

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