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# Action of the novel antioxidants 4GBE43 and 2BBE43 against lipid peroxidation

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**Abstract**

The action and the effect of the newly synthesized compounds 4GBE43 [*N*-(1,2-diethyltetrahydro-1*H*-pyrazol-4-yl)-4-[(2*E*)-3,7-diethyl-2,6-octadienyl]oxybenzamide] and 2BBE43 [2-(benzyloxy)-*N*-(1,2-diethyltetrahydro-1*H*-pyrazol-4-yl)benzamide] against lipid peroxidation were studied. 4GBE43 and 2BBE43 quenched the ESR signal of diphenylpicrylhydrazyl (DPPH), suggesting that 4GBE43 and 2BBE43 act as scavengers of free radicals and that each compound quenched 6 free radical molecules. These compounds suppressed the oxidation of methyl linoleate emulsions and soybean phosphatidylcholine liposomes by a free radical initiator, suggesting that these compounds quench the lipid peroxyl radical. 4GBE43 and 2BBE43 also suppressed the spontaneous oxidation of rat brain homogenates. The inhibitory effect of 2BBE43 was of the same order of magnitude ($IC_{50}$) as that of probucol. The $IC_{50}$ of 4GBE43 was on the same order of magnitude as that of $\alpha$-tocopherol. However, 4GBE43 at $10^{-4}$–$10^{-5}$ M completely inhibited peroxidation, showing it to be more effective than $\alpha$-tocopherol. These results suggest that 4GBE43 and 2BBE43 act as antioxidants by quenching the lipid peroxyl radical. 

*Keywords:* Antioxidant; Lipid peroxidation; Free radical; $\alpha$-Tocopherol; 4GBE43; 2BBE43

## 1. Introduction

It has been proposed that free radicals are involved in a variety of pathological events [1–6]. They are produced by neutrophiles and xanthine oxidase, induce lipid peroxidation by chain reaction, and cause cell injury. To prevent this oxidative injury, the following drug mechanisms are possible [7]: (i) preventing production of free radicals, (ii) quenching free radicals, and (iii) inhibiting lipid peroxidation by slowing down chain oxidation. However, the first mechanism is considered to be unlikely to prevent oxidative injury, because there are various production sites of radicals and they change with time. Regarding the second mechanism, superoxide ($O_2^-$) and hydroxyl (·OH) free radicals are the targets, but ·OH is so reactive that it is difficult to quench completely [8]. Furthermore, ·OH is produced not only by $O_2^-$ but also by prostaglandin metabolism. Thus, quenching $O_2^-$ is insufficient to prevent oxidative injury. In contrast, the third mechanism is considered to be effective, because it does not depend on the radical species or production sites.

Therefore, we synthesized various novel compounds and performed a wide range of screening tests for the purpose of developing an antioxidant that inhibits lipid peroxidation induced by chain oxidation, and found a pyrazolidine derivative to be effective. Based upon the pyrazolidine structure, we synthesized various other novel compounds and subjected them to screening. Consequently, two compounds, 4GBE43 [*N*-(1,2-diethyltetrahydro-1*H*-pyrazol-4-yl)-4-[(2*E*)-3,7-diethyl-2,6-octadienyl]oxybenzamide] and 2BBE43 [2-(benzyloxy)-*N*-(1,2-diethyltetrahydro-1*H*-pyrazol-4-yl)benzamide], were selected for their efficiency. The purpose of this study was to investigate the action and effect of these two newly synthesized compounds on lipid peroxidation.

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*Abbreviations*: DPPH, diphenylpicrylhydrazyl; TEP, 1,1,3,3-tetraethoxypropane; AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis (2,4-dimethylvaleronitrile); MLV, multilamellar vesicle; ULV, unilamellar vesicle; TBARs, thiobarbituric acid reactive substances; and PC-OOH, phosphatidylcholine hydroperoxides.

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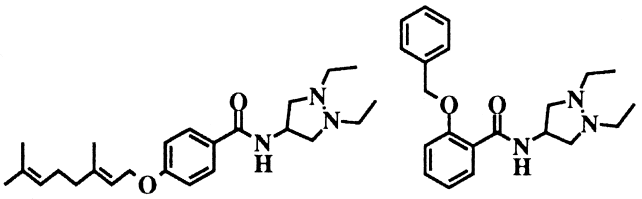

4GBE43 2BBE43

Fig. 1. Structures of 4GBE43 and 2BBE43.

## 2. Materials and methods

### 2.1. Chemicals and biological materials

DPPH, TEP, AAPH, and AMVN were obtained from the Wako Pure Chemical Co. l-Ascorbic acid, $\alpha$-tocopherol, probucol, and linoleic acid were purchased from the Sigma Chemical Co. Thiobarbituric acid was obtained from the Merck Co. Soybean phosphatidylcholine was purified at the University of Tokyo by silica-gel column. Sprague–Dawley male rats (200–250 g) were purchased from the Japan SLC Co. They were given a diet of standard laboratory chow and water *ad lib.* and were maintained on 12-hr light periods. 4GBE43 and 2BBE43 were synthesized by the Shiseido Co. (Fig. 1). Other chemicals were of the highest grade commercially available.

### 2.2. Measurement of the reduction of the DPPH radical

The reactivities of 4GBE43 and 2BBE43 with DPPH were estimated by the slightly modified procedures of Blois [9] and Uchiyama *et al.* [10]. A 0.1 mM concentration of DPPH in ethanol was added to 20 mM acetate buffer (pH 5.5) containing 60% ethanol and various concentrations of 4GBE43 or 2BBE43. Incubation was performed at room temperature, and the absorbance at 517 nm was followed spectrophotometrically. 4GBE43 and 2BBE43 were dissolved in DMSO, and the final concentration of DMSO in the reaction mixtures was 10%. However, no effect of DMSO at this concentration was detected. The percent reduction of DPPH was calculated using the following equation:

$$\% \text{ Reduction} = \{1 - (A_t/A_i)\} \times 100$$

where $A_t$ represents absorbance at measuring time, and $A_i$ represents absorbance at initial time.

### 2.3. Measurement of radical quenching by ESR

The effect of radical quenching was measured with ESR according to Hiramatu and Packer [11] with some modifications. 4GBE43 and 2BBE43 at 100 $\mu$M were added to 100 $\mu$M DPPH in ethanol. After 10 sec of mixing, the spectra were measured with ESR at room temperature. The ESR spectra were recorded by an X-band JEOL JESR1X spectrometer under the following conditions: magnetic center field, 335 mT; scan width, 10 mT; sweep time, 0.5 min; microwave power, 8 mW; modulation frequency, 100 kHz; and modulation amplitude, 0.2 mT.

### 2.4. Measurement of the rate of oxidation of linoleic acid

The oxidation of methyl linoleate in aqueous dispersions was performed according to Pryor *et al.* [12] with some modifications. A 5 mM solution of AAPH was added to 50 mM phosphate buffer (pH 7.4) containing 50 mM methyl linoleate, 500 mM SDS, and various concentrations of 4GBE43 or 2BBE43, and incubated at 37°. The rate of oxidation was followed by measuring the oxygen concentration in solution with an oxygen electrode (MD-100; Iijima Denshi Kogyo Co.).

### 2.5. Measurement of the oxidation of PC liposomes

The oxidation of soybean PC liposomes was performed according to Niki [13]. The MLV solution contained 5.15 mM PC, 1 mM AMVN, and 10 $\mu$M 4GBE43 or 2BBE43. The oxidation was initiated by heating the solution at 37°. The ULV solution contained 2.8 mM PC and 10 $\mu$M 4GBE43 or 2BBE43. The oxidation was initiated by adding 1.0 mM AAPH and was carried out at 37°. The PC-OOH were followed by measuring the conjugated-diene absorption at 234 nm with HPLC. The HPLC conditions were as follows: the eluent was methanol:40 mM phosphate (90:10, v/v), the eluent speed was 1.0 mL/min, and an LC-Si column was used (particle size 5 $\mu$m, 4.6 $\times$ 25 cm; Supelco).

### 2.6. Inhibition of lipid peroxidation

The spontaneous oxidation of rat brain homogenates was performed according to Shimamoto *et al.* [14] with some modifications. Rat brain homogenate in 19 vol. of 20 mM phosphate buffer (pH 7.4) with various concentrations of the test compounds was incubated at 37° for 60 min under aerobic conditions. Lipid peroxide was measured as malondialdehyde by TBARs [15]. The test compounds were dissolved in DMSO, and the final concentration of DMSO in the reaction mixtures was 2%. No effect of DMSO was detected at this concentration. Percent inhibition of lipid peroxidation was calculated using the following equation:

$$\% \text{ Inhibition of lipid peroxidation} = \{1 - (m/M) + \times 100$$

where m represents the increase in the amount of lipid peroxide in the mixture upon the addition of test compounds, and M represents the increase in the amount of lipid peroxide in the mixture upon the addition of DMSO.

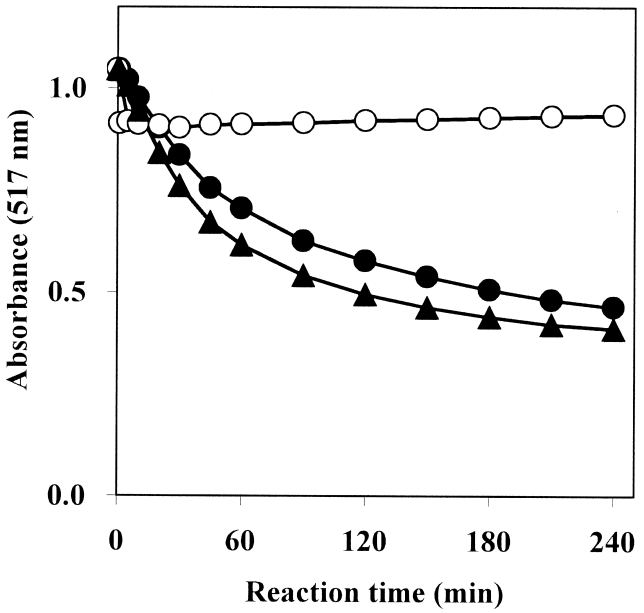


Fig. 2. Effects of 10 μM α-tocopherol, 4GBE43, and 2BBE43 on the DPPH radical (100 μM) in solution. Key: (○) α-tocopherol; (●) 4GBE43; and (▲) 2BBE43. The data represent three experiments.

## 3. Results

### *3.1. Activity of 4GBE43 and 2BBE43 as radical quenching antioxidants*

The radical scavenging activities of 4GBE43 and 2BBE43 were estimated by reactivity with DPPH. The absorbance of DPPH did not change for 240 min in the absence of the test compounds. When 10 μM α-tocopherol was added to the mixture as a positive control known to trap free radicals [9], the absorbance decreased quickly and reached a steady state after 1 min. The percent of DPPH reduced was 19.4 ± 0.4% after 240 min (Fig. 2). The addition of 10 μM 4GBE43 and 2BBE43 decreased the absorbance. After 240 min, the percent of DPPH reduced reached 59.5 ± 0.4 and 66.4 ± 1.0%, respectively. These reductions were higher than that produced by α-tocopherol. The percent reduction after 240 min increased with increasing concentrations of the test compounds (Table 1). To determine if the decrease in absorbance seen in Fig. 2 resulted from the quenching of the DPPH radical, the ESR signals of DPPH were recorded after 240 min, the time when the reactions reached steady state (Fig. 3). A 5-lined spectrum (pentad signals) of ESR was observed in the 100 μM DPPH ethanolic solution. A 100 μM concentration of either 4GBE43 or 2BBE43 completely quenched this signal, indicating their radical quenching activity.

### *3.2. Effects of 4GBE43 and 2BBE43 on the oxidation of methyl linoleate*

The antioxidant actions of 4GBE43 and 2BBE43 were estimated by the oxidation of methyl linoleate. The addition of AAPH induced the oxidation of methyl linoleate and oxygen consumption at a constant rate after 8 min. The rate of oxidation was 43.3 ± 2.2 nM/sec (Fig. 4). The addition of 100 μM α-tocopherol, a positive control known to inhibit the peroxidation of methyl linoleate [12], inhibited the rate of oxidation to 13.0 ± 1.0 nM/sec. The same concentration of 4GBE43 and 2BBE43 inhibited the rate of oxidation to 26.7 ± 1.2 and 32.7 ± 3.0 nM/sec, respectively, but these inhibitory activities were less than that of α-tocopherol.

Table 1
Percent reduction of the DPPH radical by α -tocopherol, 4GBE43, and 2BBE43

| Concentration (μM) | % Reduction of DPPH radical | | |
|---|---|---|---|
| | 4GBE43 | 2BBE43 | α-Tocopherol |
| 5 | 31.8 ± 0.3 | 37.1 ± 1.2 | 8.6 ± 0.7 |
| 10 | 59.5 ± 0.4 | 66.4 ± 1.0 | 19.4 ± 0.4 |
| 20 | 80.2 ± 0.6 | 81.4 ± 0.2 | 39.5 ± 1.5 |
| 50 | 81.0 ± 0.3 | 81.1 ± 0.3 | 81.1 ± 0.5 |
| 100 | 81.3 ± 0.1 | 81.2 ± 0.2 | 79.4 ± 0.0 |

Percent reduction was calculated by the data of Fig. 2. The data are the means ± SEM of three experiments.

### *3.3. Effects of 4GBE43 and 2BBE43 on the oxidation of PC liposomes*

The antioxidant actions of 4GBE43 and 2BBE43 were also estimated by the oxidation of PC liposomes. In MLV, PC-OOH were formed by AMVN and reached a concentration of 269.3 μM after 240 min (Fig. 5). The rate of

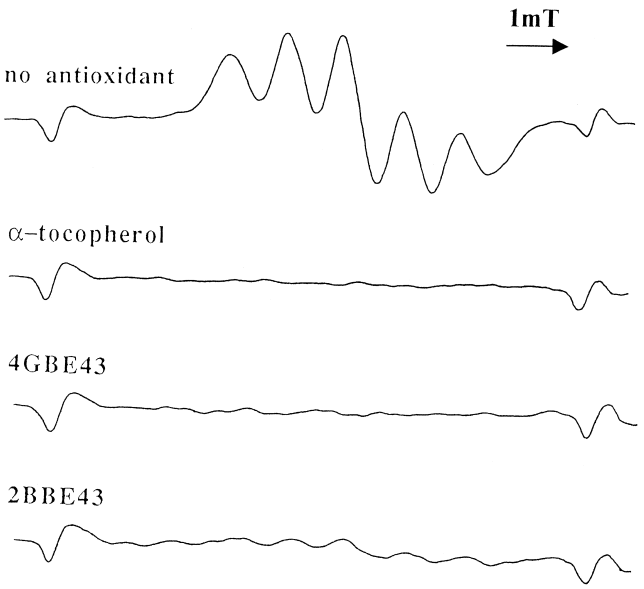


Fig. 3. Effects of 100 μM α-tocopherol, 4GBE43, and 2BBE43 on the ESR spectrum of the DPPH radical (100 μM) in ethanolic solution. The spectra were recorded under the following conditions: magnetic center field, 335 mT; scan width, 10 mT; sweep time, 0.5 min; microwave power, 8 mW; modulation frequency, 100 kHz; and modulation amplitude, 0.2 mT. Spectra represent three experiments.

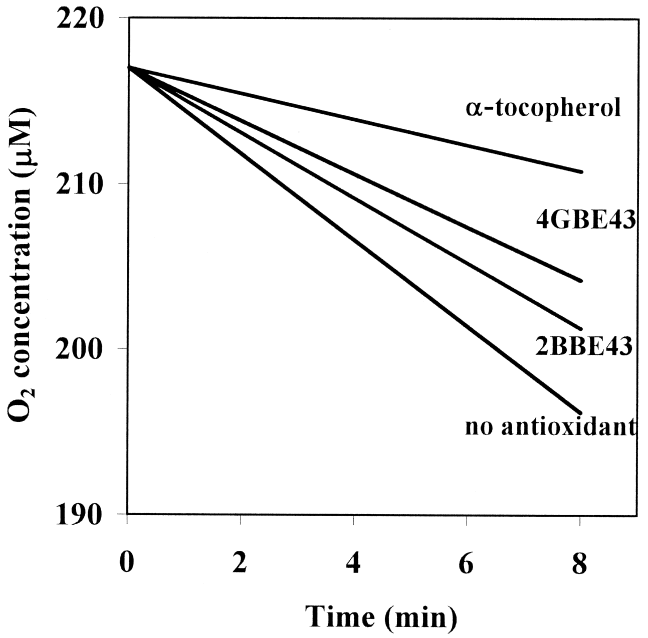


Fig. 4. Effects of 100 $\mu$M $\alpha$-tocopherol, 4GBE43, and 2BBE43 on linoleic acid (50 mM) oxidation induced by AAPH (5 mM) in phosphate buffer (50 mM, pH 7.4) containing SDS (500 mM) at 37°. $O_2$ concentration was measured with an oxgen electrode. Curves represent three experiments.

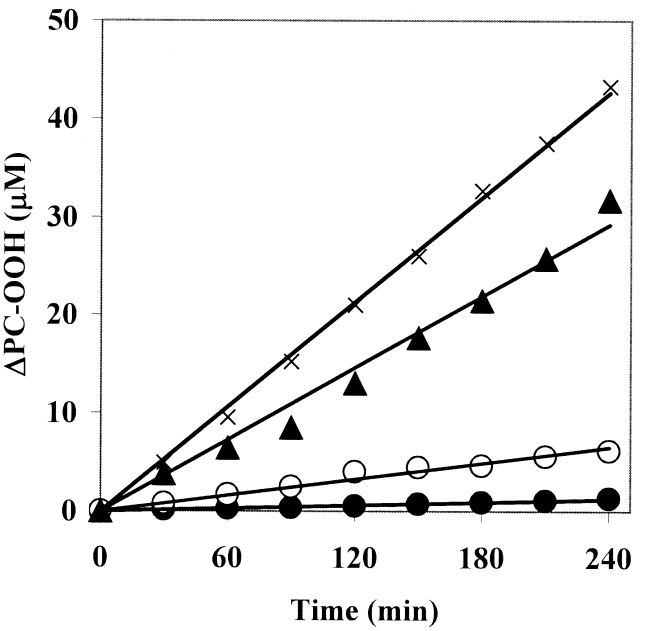


Fig. 6. Effects of 10 $\mu$M $\alpha$-tocopherol, 4GBE43, and 2BBE43 on the AAPH-induced oxidation of soybean PC liposomes. Key: (×) no antioxidant; (○) $\alpha$-tocopherol; (●) 4GBE43; and (▲) 2BBE43. Similar results were obtained in one additional experiment.

oxidation (Rp) was $1.87 \times 10^{-8}$ M/sec. The addition of 10 $\mu$M $\alpha$-tocopherol, a positive control known to inhibit the peroxidation of PC liposomes [13], inhibited the oxidation. The concentration of PC-OOH was 7.6 $\mu$M after 240 min, and the Rp was $5.28 \times 10^{-10}$ M/sec. 4GBE43 completely inhibited the oxidation similarly to $\alpha$-tocopherol; the concentration of PC-OOH was 14.8 $\mu$M after 240 min, and the Rp was $1.03 \times 10^{-9}$ M/sec. 2BBE43 also inhibited the oxidation; the concentration of PC-OOH was 184.3 $\mu$M after 240 min, and the Rp was $1.28 \times 10^{-8}$ M/sec. In L-ULV, PC-OOH were formed by AAPH and reached a concentration of 43.8 $\mu$M after 240 min of the reaction at an Rp of $3.04 \times 10^{-9}$ M/sec (Fig. 6). $\alpha$-Tocopherol inhibited the oxidation; the concentration of PC-OOH was 6.2 $\mu$M, and the Rp was $4.34 \times 10^{-10}$ M/sec. 4GBE43 completely inhibited the oxidation; the concentration of PC-OOH was 1.30 $\mu$M, and the Rp was $9.02 \times 10^{-11}$ M/sec. 2BBE43 also inhibited the oxidation; the concentration of PC-OOH after 240 min was 31.0 $\mu$M, and the Rp was $2.15 \times 10^{-9}$ M/sec.

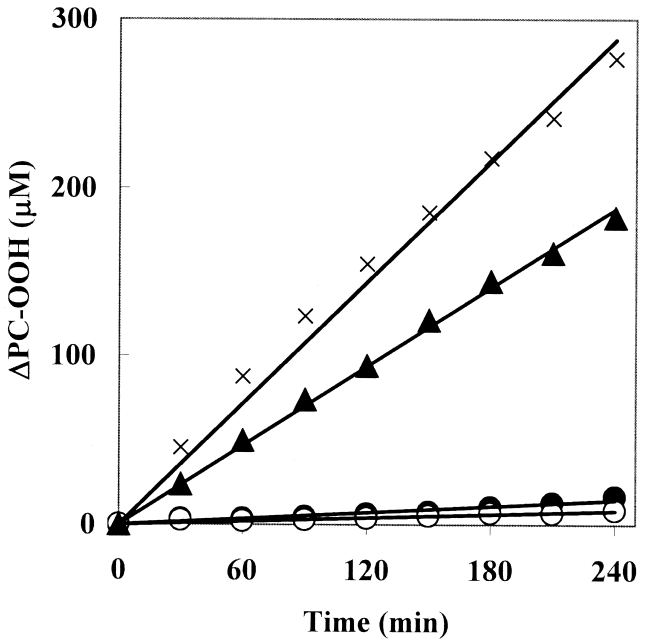


Fig. 5. Effects of 10 $\mu$M $\alpha$-tocopherol, 4GBE43, and 2BBE43 on the AMVN-induced oxidation of soybean PC liposomes. Key: (×) no antioxidant; (○) $\alpha$-tocopherol; (●) 4GBE43; and (▲) 2BBE43. Similar results were obtained in one additional experiment.

### *3.4. Effects of 4GBE43 and 2BBE43 on the spontaneous oxidation of rat brain homogenates*

The effects of the antioxidants 4GBE43 and 2BBE43 were compared with that of $\alpha$-tocopherol on the spontaneous oxidation of rat brain homogenates. The incubation of rat brain homogenates for 1 hr increased TBARs, or lipid hydroperoxide, from 13.6 ± 3.5 to 199.9 ± 14.9 nmol/g wet weight. $\alpha$-Tocopherol [16] and probucol [17], positive controls known to inhibit the peroxidation of rat brain homogenates, inhibited lipid peroxidation concentration dependently (Fig. 7). 4GBE43 and 2BBE43 also inhibited lipid peroxidation concentration dependently. The addition of $10^{-4}$–$10^{-5}$ M of 4GBE43 completely inhibited lipid peroxidation. The $IC_{50}$ values of the test compounds are shown

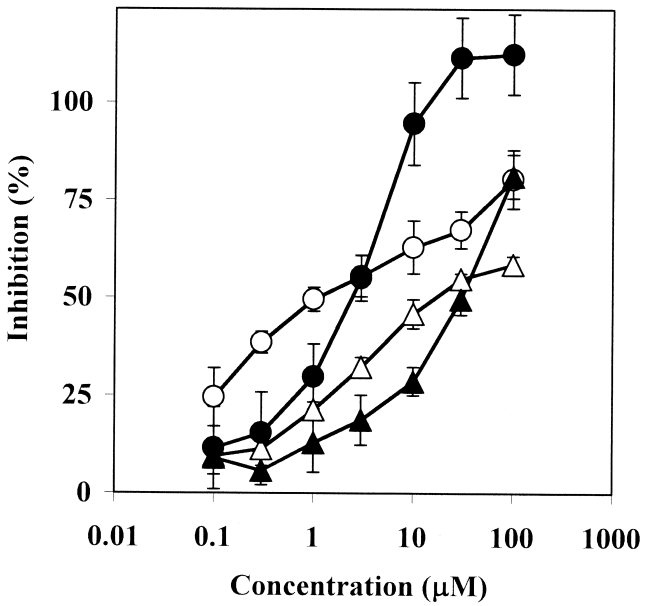


Fig. 7. Effects of α-tocopherol, 4GBE43, and 2BBE43 on the formation of TBARs in spontaneous oxidation of rat brain homogenate (5%, w/v) in phosphate buffer (20 mM, pH 7.4) for 1 hr at 37° in air. Key: (○) α-tocopherol; (Δ) probucol; (●) 4GBE43; and (▲) 2BBE43. Each point is the mean ± SEM of three experiments.

in Table 2. The $IC_{50}$ of 4GBE43 was 1.3 μM, on the same order of magnitude as that of α-tocopherol. The $ic_{50}$ of 2BBE43 was 21.9 μM, on the same order of magnitude as that of probucol.

## 4. Discussion

The above results demonstrate that 4GBE43 and 2BBE43 inhibit lipid peroxidation. First, we discussed the inhibition mechanism of lipid peroxidation. 4GBE43 and 2BBE43 quenched the ESR signal of DPPH (Fig. 3), suggesting that these compounds could scavenge free radicals. Thus, we evaluated if these compounds could inhibit lipid peroxidation induced by the chain reaction of free radicals. Azo initiators (A-N = N-A), such as AAPH and AMVN used in our study, generate peroxyl radicals by thermal decomposition [18,19]. Thus, the potent radical scavengers are considered to inhibit lipid peroxidation [20]. 4GBE43 and 2BBE43 inhibited peroxidation of methyl linoleate (Fig. 4). Similarly, oxidation of PC liposomes induced by AMVN or AAPH was inhibited by 4GBE43 and 2BBE43 (Figs. 5 and 6). These results suggest that 4GBE43 and 2BBE43 can inhibit free radical chain reaction-induced lipid peroxidation. This inhibition is considered to be the result of lipid peroxyl radical quenching by these radical scavenging compounds. These compounds also inhibited lipid peroxidation of rat brain homogenates (Fig. 7). This lipid peroxidation was induced by the alkoxyl radical. The alkoxyl radical was formed by a Fenton-like reaction between lipid hydroperoxide and iron existing in the system, and this alkoxyl radical induced lipid peroxidation by chain oxidation [21]. Thus, the possible mechanisms considered to inhibit lipid peroxidation in this system were the chelating of iron [22], the scavenging of the radical [23], or the reducing of lipid hydroperoxides [24]. 4GBE43 and 2BBE43 cannot chelate iron or reduce lipid hydroperoxides in molecular form. Therefore, these compounds must have inhibited lipid peroxidation by quenching the lipid peroxyl radical.

Table 2
$IC_{50}$ of α-tocopherol, 4GBE43, and 2BBE43 on the formation of TBARs in spontaneous oxidation of rat brain homogenate

| Compounds | $IC_{50}$ (μM) |
|---|---|
| 4GBE43 | 1.3 |
| α-Tocopherol | 1.8 |
| Probucol | 16.2 |
| 2BBE43 | 21.9 |

The $IC_{50}$ values were calculated by the data of Fig. 7.

Next, we discussed the efficiency of 4GBE43 and 2BBE43 in inhibiting lipid peroxidation. The mole ratio of the test compounds to DPPH was 10:1, and the reaction was at steady state. Thus, the number of radical molecules that reacted with the test compound was estimated. Blois [9] reported that 1 molecule of ascorbate and α-tocopherol quenched 2 molecules of radical, estimated by the number of radical molecules that reacted with cysteine. A 10 μM solution of α-tocopherol reduced 20% of DPPH, suggesting that 10% of the reduction corresponded to 1 molecule of radical quenched by 1 molecule of test compound. 4GBE43 and 2BBE43 reduced 59.5 and 66.4% of DPPH, respectively. Thus, these compounds quenched an estimate of 6 molecules of radical. However, the reaction of α-tocopherol reached steady state in 1 min, but these compounds needed 4 hr. Hence, even though 4GBE43 and 2BBE43 reacted with more radicals than α-tocopherol, their reaction rate was slower than that of α-tocopherol. Thus, the efficiencies of the antioxidants were tested further in the lipid peroxidation system. AAPH generated free radicals outside the membrane, whereas AMVN generated free radicals inside the membrane. 4GBE43 and 2BBE43 inhibited lipid peroxidation initiated by both AAPH and AMVN, suggesting that these compounds were effective against radicals inside and outside the membrane. When methyl linoleate was used as the lipid and AAPH as the azo initiator, 4GBE43 was less effective than α-tocopherol. However, when PC was the lipid and AAPH was the azo initiator, 4GBE43 was more effective than α-tocopherol. Similarly, probucol, a known potent antioxidant [17], showed less antioxidant activity than α-tocopherol with methyl linoleate but was as efficient as α-tocopherol with low-density lipoproteins. The efficiency of the antioxidant was determined by various factors, such as the number of radical molecules quenched by 1 molecule, reaction rate, local concentration, and mobility in the microenvironment [18,25]. Since the antioxidant activ-

ity was different in various lipid assays, the efficiency of the antioxidant was finally determined in a lipid sample derived from tissue (Fig. 7). The $IC_{50}$ of 4GBE43 was as efficient as that of $\alpha$-tocopherol. However, it inhibited lipid peroxidation completely at $10^{-4}$–$10^{-5}$ M, which was more effective than $\alpha$-tocopherol. The $IC_{50}$ of 2BBE43 was as effective as the $IC_{50}$ of probucol.

In conclusion, 4GBE43 and 2BBE43 inhibited lipid peroxidation by quenching the lipid peroxyl radical; thus these compounds can be effective in treating various injuries derived from oxidative damage.

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