# *IN VITRO* AND *EX VIVO* FREE RADICAL SCAVENGING ACTIVITIES OF CARAZOSTATIN, CARBAZOMYCIN B AND THEIR DERIVATIVES

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Free radical scavenging activities of various carbazole compounds, carazostatin, carbazomycin B and their chemically modified derivatives were studied *in vitro* and *ex vivo*. Among these compounds, carazostatin, which was isolated as a free radical scavenger from the culture of *Streptomyces chromofuscus*, showed the most potent inhibitory activity against lipid peroxidation of rat brain homogenate *in vitro*. Carbazomycin B, a known antimicrobial antibiotic, also exhibited strong activity in this system. Although *O*-modified derivatives of carazostatin and carbazomycin B retained considerable activity, *N*,*O*-dimethyl derivatives did not suppress the peroxidation. On the other hand, the results from the *ex vivo* evaluation of these carbazoles in the lipid peroxidation system of mouse blood plasma showed that the original compounds as well as their *O*-modified derivatives had a strong inhibitory activity upon oral administration to mice. These findings suggest that these natural carbazoles and their effective derivatives can protect tissues from the peroxidative damage due to generation of free radicals.

Generation of oxygen free radicals has been recently suggested to play a crucial role in the pathological development of various diseases such as cerebral ischemia, renal failure and rheumatoid arthritis[1~3]. Especially subsequent peroxidative disintegration of cells and organellar membranes has been deeply implicated in various pathological processes[4]. Much effort has been made for therapeutic treatment of these peroxidative processes, in which some free radical scavengers and antioxidants such as vitamin E (α-tocopherol) have been recently used[5]. However, these attempts have not yet been satisfactory because of their insufficient potency.

In the course of our screening program for strong free radical scavengers from microorganisms, we have isolated several carbazole compounds such as carazostatin[6] and neocarazostatins[7], as reported previously. Further investigation has resulted in the finding of a new biological activity as a free radical scavenger for an antibacterial antibiotic, carbazomycin B[8~10], which also contains a carbazole nucleus in its structure. These carbazoles are therefore expected as a new class of therapeutic agents for peroxidative processes.

To examine the structure-activity relationship we semi-synthesized several derivatives of carazostatin (**1**) and carbazomycin B (**5**), and measured an inhibitory activity against lipid peroxidation in rat brain homogenate induced by free radicals and a radical scavenging activity against diphenyl-*p*-picrylhydrazyl (DPPH).

Recently, a new *ex vivo* system for the determination of inhibitory activities against lipid peroxidation in rat blood plasma has been reported by USHIYAMA *et al.*[11]. We developed it into a more convenient system in mouse, in which the required amount of drugs is much smaller than in the original system. This system provides us with a useful approach for evaluating bioavailability of free radical scavengers and antioxidants, and gives us information about their effective doses. We evaluated carbazoles in this *ex vivo* system, and examined whether these compounds could be absorbed and were effective in blood plasma by oral administration to mice.

## Results

### Chemistry

**1** was isolated from the culture of *Streptomyces chromofuscus* DC118, and purified as reported previously[6]. Reaction of **1** with triethylamine and acetic anhydride in $CH_2Cl_2$ afforded 3-*O*-acetylcarazostatin (**2**). *O*-Methylation of **1** with methyl iodide and $K_2CO_3$ in dry acetone under reflux gave 3-*O*-methylcarazostatin (**3**). *N,O*-Dimethylation of **1** with methyl iodide and NaH in DMF afforded *N*-3-*O*-dimethylcarazostatin (**4**).

**5** was purified by the method of SAKANO *et al.*[8] with some modification from the culture of *Streptoverticillium* species isolated from a soil sample in our screening. *O*-Acetylation of **5** with triethylamine and acetic anhydride in $CH_2Cl_2$ afforded 4-*O*-acetylcarbazomycin B (**6**). *O*-Methylation of **5** with methyl iodide and $K_2CO_3$ in dry acetone under reflux gave carbazomycin A (**7**). *N,O*-Dimethylation of **5** with methyl iodide and NaH in DMF afforded *N*-methylcarbazomycin A (**8**). **5**, **6** and **7** are known compounds reported by SAKANO *et al.*[9].

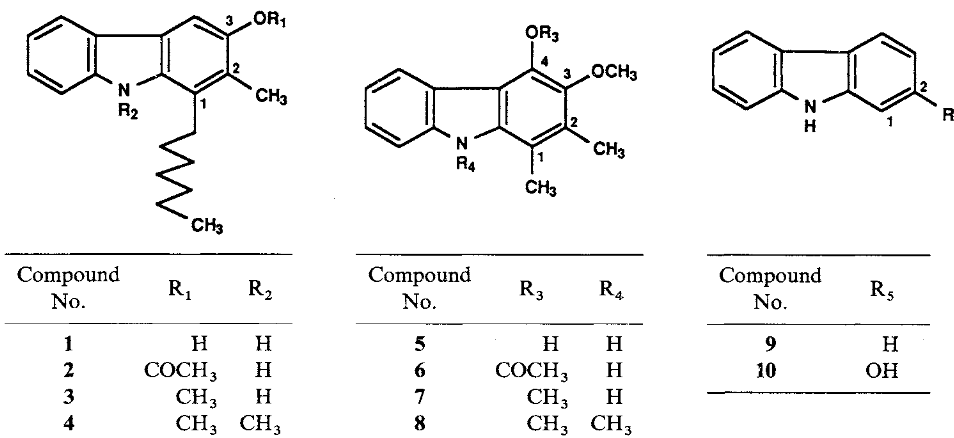
The structures of these semi-synthetic compounds were confirmed by mass, IR and $^1H$ NMR spectra. Physico-chemical properties of **5**, **6** and **7** coincided with the previously reported data[8,9]. The structures of the natural and semi-synthetic carbazoles are shown in Fig. 1.

### *In Vitro* Activity

*In vitro* inhibitory activity of the carbazoles against lipid peroxidation in rat brain homogenate was determined in the presence of $Fe^{2+}$ and ascorbic acid as initiators for generation of oxygen radicals. $IC_{50}$ values of these compounds and other related drugs are shown in the Table.

**1** showed the most potent activity among the carbazoles, and the activity was much stronger than that of known antioxidants such as α-tocopherol or butylhydroxytoluene (BHT). While 3-*O*-modified derivatives of **1** (**2** and **3**) were less effective than **1**, the activity of these derivatives was stronger than that of α-tocopherol or flunarizine, a calcium antagonist with free radical scavenging activity[12]. *N,O*-Dimethyl derivative of **1** (**4**) was inactive against lipid peroxidation at 100 μM. **5** had potent activity, but showed weaker activity than **1**. 4-*O*-Modified derivatives of **5** (**6** and **7**) were less effective than the original compound, but retained

Fig. 1. Structures of carazostatin (**1**), carbazomycin B (**5**), carbazomycin A (**7**), their semi-synthetic derivatives and related compounds.




| Compound No. | $R_1$ | $R_2$ |
|---|---|---|
| 1 | H | H |
| 2 | $COCH_3$ | H |
| 3 | $CH_3$ | H |
| 4 | $CH_3$ | $CH_3$ |

| Compound No. | $R_3$ | $R_4$ |
|---|---|---|
| 5 | H | H |
| 6 | $COCH_3$ | H |
| 7 | $CH_3$ | H |
| 8 | $CH_3$ | $CH_3$ |

| Compound No. | $R_5$ |
|---|---|
| 9 | H |
| 10 | OH |

Table 1. *In vitro* and *ex vivo* activities of various carbazoles.

| Compound No. | *In vitro* activities | | *Ex vivo* activity | |
|---|---|---|---|---|
| | Inhibition against lipid peroxidation in rat brain homogenate | Radical scavenging activity against DPPH | Inhibition against lipid peroxidation in mouse plasma (administered orally) | |
| | $IC_{50}$ (μM) | $IC_{0.2}$[a] (μM) | Inhibition at 100 mg/kg (%) | $ID_{50}$ (mg/kg) |
| **1** | 0.17 | 11.5 | 95.8 | 21.7 |
| **2** | 0.58 | >100 | 95.2 | 40.1 |
| **3** | 1.73 | >100 | 96.5 | 12.0 |
| **4** | >100 | >100 | 0 | >100 |
| **5** | 0.55 | 10.0 | 92.8 | 21.9 |
| **6** | 4.20 | >100 | 61.7 | 68.6 |
| **7** | 8.13 | >100 | 95.0 | 38.1 |
| **8** | >100 | >100 | 71.7 | 53.6 |
| **9** | >100 | n.d. | n.d. | n.d. |
| **10** | 10.9 | n.d. | n.d. | n.d. |
| α-Tocopherol | >100 | 10.0 | 74.5[b] | 76.9[b] |
| BHT | 4.89 | 8.71 | n.d. | n.d. |
| Flunarizine·2HCl | 55.0 | >100 | n.d. | n.d. |

[a] Concentration of each compound needed to decrease the absorbance at 517 nm by 0.2.
[b] The data of intravenously injected α-tocopherol acetate.
n.d.: Not determined.

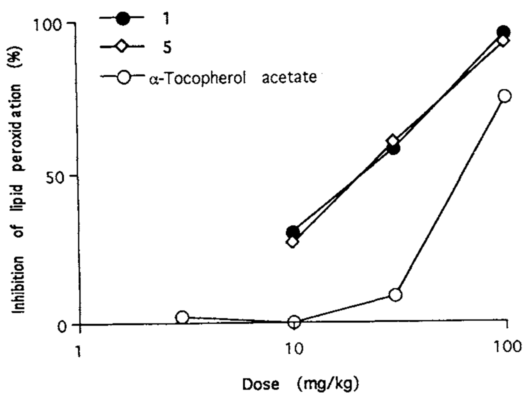
almost the same activity as BHT. *N,O*-Dimethylation of **5** (**8**) greatly reduced the activity. While 2-hydroxycarbazole (**10**) had a weak activity, carbazole (**9**) itself was inactive.

In summary, the two original carbazoles with a free phenolic hydroxy group had very strong activity, and *O*-modified carbazoles retained considerable activity. *N,O*-Dimethyl derivatives were not effective.

Radical scavenging activity of the carbazoles against diphenyl-*p*-picrylhydrazyl (DPPH), a stable free radical, was measured based on the decrease of the absorbance at 517 nm according to the method of MELLORS and TAPPEL[13]. A comparison of the radical scavenging activity of the carbazoles and other related drugs is shown in the Table.

Fig. 2. *Ex vivo* inhibitory effects of carazostatin (**1**), carbazomycin B (**5**) and α-tocopherol acetate on lipid peroxidation in mouse blood plasma[a].

● **1**, ◇ **5**, ○ α-Tocopherol acetate.




[a] Administration: **1** and **5**, po; α-tocopherol acetate, iv.

Both original compounds (**1** and **5**), as well as α-tocopherol and BHT, showed the potent radical scavenging activity against DPPH radical. Flunarizine did not show this activity at 100 μM. In contrast, all other derivatives (**2**, **3**, **4**, **6**, **7** and **8**) without a free phenolic hydroxy group showed no scavenging activity at 100 μM.

## *Ex Vivo* Activity

Evaluation of the carbazoles for *ex vivo* antioxidant activity was made by testing the inhibition of

lipid peroxidation in mouse blood plasma. Each compound was administered orally to mice ($n=6$) 1 hour prior to blood collection. Since orally administered α-tocopherol showed no inhibition at 100 mg/kg, a solution of α-tocopherol acetate, a prodrug of α-tocopherol, was injected intravenously to mice. The *ex vivo* activity of **1**, **5** and α-tocopherol acetate at various doses is shown in Fig. 2. The mean inhibitory activity at 100 mg/kg and the $ID_{50}$ values of the drugs, a dose of each drug needed to inhibit the lipid peroxidation to 50% of the control, are listed in the Table.

**1** inhibited the *ex vivo* lipid peroxidation of blood plasma in a dose dependent manner (Fig. 2), and the activity based on $ID_{50}$ values was stronger than that of intravenously injected α-tocopherol acetate. **5** had nearly the same $ID_{50}$ value as **1**. *O*-Modified derivatives (**2**, **3**, **6** and **7**) retained the inhibitory activity, which was almost the same as or somewhat weaker than that of the original compounds. Of the two *N*,*O*-dimethyl derivatives (**4** and **8**), which did not suppress the *in vitro* lipid peroxidation, **8** showed a weak *ex vivo* activity in contrast to a no inhibitory effect of **4**.

All of the carbazoles studied had no toxicity to mice at 400 mg/kg po.

## Discussion

There has been much interest in free radical scavengers because of a crucial role of free radicals on the pathogenesis of various diseases. We have recently reported a strong free radical scavenger, carazostatin (**1**)[6]. In our extensive screening, we have found a radical scavenging activity for a known antibiotic, carbazomycin B (**5**)[8~10]. Both compounds with a carbazole nucleus in their structures had a potent inhibitory activity against *in vitro* lipid peroxidation in rat brain homogenate as described here. Because carbazole itself had no activity, a free phenolic hydroxy group in these structures may account for the activity as in α-tocopherol and BHT. This observation was also supported by the results that both **1** and **5** bearing a free phenolic hydroxy group scavenged DPPH radical, and agreed well with the findings that the inhibitory activity of carbazomycins and epocarbazolins bearing the free phenolic hydroxy group against 5-lipoxygenase, an enzyme which converts arachidonic acid to a lipid hydroperoxide, one of free radicals, was due to their radical scavenging activity[14,15].

Our *in vitro* experiments revealed that **1** had a stronger inhibitory activity against lipid peroxidation than **5**. Recently, we also showed similar results in the oxidation systems of methyl linoleate[16]. These results suggest that, in agreement with the previous result with 6-hydroxy-1,4-dimethylcarbazole reported by YAMAMOTO *et al.*[17], 3-hydroxycarbazole such as **1** inhibits lipid peroxidation more potently than 4-hydroxycarbazole such as **5**, and that the position of a free phenolic hydroxy group in the carbazole nucleus is important for the radical scavenging activity.

The presence of other substituents on the carbazole nucleus such as a *n*-heptyl group in **1** may also contribute to enhance the activity. This is supported by two observations that **10** had a weaker activity than **1** or **5**, and that neocarazostatins, derivatives of 6-(3-methyl-2-butenyl)-1-propylcarbazomycin B, had more potent activity ($IC_{50}$; 0.37~0.51 μM) than **5**[7].

In order to further investigate the relation of the structures to free radical scavenging activity, we synthesized some derivatives of these carbazoles and examined their activities. The *O*-acetyl derivatives had strong inhibitory activities against *in vitro* and *ex vivo* lipid peroxidation. On the contrary, they showed no DPPH radical scavenging activity. These results indicate that the acetyl group in these structures is readily removed in the brain homogenate or *in vivo* to give active compounds probably with a free phenolic hydroxy group. Interestingly, *O*-methyl derivatives exhibited almost the same activity as *O*-acetyl derivatives not only *in vitro* but also *ex vivo*. They had no DPPH radical scavenging activity similar to *O*-acetyl derivatives. These compounds may be metabolized by an unknown mechanism to give some biologically active compounds in these systems. *N*,*O*-Dimethyl derivatives did not suppress the *in vitro* lipid peroxidation and showed weak or no *ex vivo* activity. These results suggest that the modification of the imine group in the carbazole nucleus makes metabolism of these compounds into active form difficult. The metabolism of these carbazoles remains to be studied in detail.

Several derivatives of **1** and **5** as well as these original compounds showed more potent activity *in vitro* than that of known free radical scavengers and antioxidants such as flunarizine[12] or α-tocopherol. Furthermore, they were also effective in our *ex vivo* system. This indicates that they were well absorbed in mice by oral administration and can exist as an active form in the blood. These results suggest that these natural and semi-synthetic carbazoles may be useful as a new class of therapeutic agents for various diseases such as myocardial and cerebral ischemia, atheroscrelosis, renal failure, inflammation and rheumatoid arthritis, which might be caused by tissue damage due to generation of free radicals and subsequent peroxidative disintegration of cell membranes.

## Experimental

### General Methods

Melting points (MP) were determined with a Yanagimoto micro melting-point apparatus. IR and $^1$H NMR spectra were taken on a Jasco A-3 spectrophotometer and a JEOL JNM-GX500 (at 500 MHz) spectrometer, respectively. Mass spectra were obtained on a Hitachi M-80 spectrometer. TLC was carried out on Silica gel 60 $F_{254}$ TLC plates (E. Merck).

α-Tocopherol was purchased from Wako Pure Chemical Industries, Ltd.; BHT and carbazole were from Tokyo Chemical Industry Co., Ltd.; 2-hydroxycarbazole was from Aldrich Chemical Co., Inc.; and flunarizine·2HCl was from Sigma Chemical Co.

### Carazostatin (1)

Isolation and purification of **1** were carried out by the method described previously[6].

### 3-*O*-Acetylcarazostatin (2)

To a stirred solution of **1** (700 mg, 2.37 mmole) in $CH_2Cl_2$ (35 ml) were added triethylamine (3.31 ml, 10 equiv.) and acetic anhydride (2.23 ml, 10 equiv.) at 0°C. The mixture was stirred for 30 minutes at 0°C and for 18.5 hours at room temperature. The reaction was stopped by addition of MeOH (3 ml). The mixture was suspended in $CHCl_3$, and the organic solution was washed with saturated aqueous $NaHCO_3$ solution and water, dried and concentrated under reduced pressure. The residue was chromatographed on silica gel with hexane - EtOAc (20 : 1) as an eluent. The fractions containing **2** were combined and concentrated to give 552.6 mg (69% yield) of **2** as a pale yellowish powder. MP 92~95°C; TLC, Rf 0.58 ($CHCl_3$); IR (KBr) cm$^{-1}$ 3470, 2920, 2850, 1750, 1430, 1370, 1320, 1240, 1220, 1060, 740; $^1$H NMR (acetone-$d_6$) δ 7.61 (1H, s, 4-H), 8.01 (1H, d, $J$=7.9 Hz, 5-H), 7.12 (1H, dd, $J$=7.9 and 7.9 Hz, 6-H), 7.33 (1H, dd, $J$=7.9 and 7.9 Hz, 7-H), 7.45 (1H, d, $J$=7.9 Hz, 8-H), 3.00 (2H, t, $J$=7.9 Hz, 10-H), 1.66 (2H, m, 11-H), 1.51~1.29 (8H, m, 12-H~15-H), 0.87 (3H, t, $J$=6.7 Hz, 16-H), 2.33 (3H, s, 17-H), 2.26 (3H, s, 3-$OCOCH_3$), 10.21 (1H, br s, NH); FD-MS $m/z$ 337 ($M^+$).

### 3-*O*-Methylcarazostatin (3)

Methyl iodide (13.62 ml, 92 equiv.) and $K_2CO_3$ (927 mg, 2.83 equiv.) were added to a solution of **1** (700 mg, 2.37 mmole) in dry acetone (15 ml). The mixture was heated to reflux for 25.5 hours. The mixture was concentrated under reduced pressure. The residue was dissolved in EtOAc, and the organic solution was washed with water, dried and evaporated. The residue was chromatographed on silica gel with hexane - EtOAc (20 : 1) as an eluent. The fractions containing **3** were combined and concentrated to give 499.6 mg (68% yield) of **3** as a pale yellowish powder. MP 83~86°C; TLC, Rf 0.63 (hexane - EtOAc (5 : 1)); IR (KBr) cm$^{-1}$ 3430, 2940, 2870, 1590, 1500, 1460, 1430, 1310, 1210, 1120, 740; $^1$H NMR (acetone-$d_6$) δ 7.50 (1H, s, 4-H), 8.01 (1H, d, $J$=7.9 Hz, 5-H), 7.09 (1H, dd, $J$=7.9 and 7.9 Hz, 6-H), 7.28 (1H, dd, $J$=7.9 and 7.9 Hz, 7-H), 7.42 (1H, d, $J$=7.9 Hz, 8-H), 2.98 (2H, t, $J$=7.9 Hz, 10-H), 1.66 (2H, m, 11-H), 1.50~1.29 (8H, m, 12-H~15-H), 0.87 (3H, t, $J$=6.7 Hz, 16-H), 2.32 (3H, s, 17-H), 3.92 (3H, s, 3-$OCH_3$), 9.98 (1H, br s, NH); FD-MS $m/z$ 309 ($M^+$).

### *N*-3-*O*-Dimethylcarazostatin (4)

To a stirred solution of **1** (650 mg, 2.20 mmole) and methyl iodide (0.31 ml, 2.2 equiv.) in DMF (10 ml) was added NaH (60% oil dispersion; 200 mg, 2.2 equiv.) at 0°C. The mixture was stirred for 30 minutes

at 0°C and for 1 hour at room temperature. The mixture was concentrated under reduced pressure. The residue was dissolved in EtOAc, and the organic solution was washed with water, dried and concentrated. The residue was chromatographed on silica gel with hexane - EtOAc (20 : 1) as an eluent. The fractions containing **4** were combined and concentrated to give 647.4 mg (91% yield) of **4** as a pale yellowish powder. MP 47 ~ 50°C; TLC, Rf 0.82 (hexane - EtOAc (5 : 1)); IR (KBr) $cm^{-1}$ 2920, 2860, 1460, 1410, 1210, 1110, 750, 740; $^1H$ NMR (acetone-$d_6$) $\delta$ 7.56 (1H, s, 4-H), 8.03 (1H, d, $J$=7.9 Hz, 5-H), 7.12 (1H, dd, $J$=7.9 and 7.9 Hz, 6-H), 7.38 (1H, dd, $J$=7.9 and 7.9 Hz, 7-H), 7.46 (1H, d, $J$=7.9 Hz, 8-H), 3.18 (2H, t, $J$=7.9 Hz, 10-H), 1.70 (2H, m, 11-H), 1.60 ~ 1.29 (8H, m, 12-H ~ 15-H), 0.80 (3H, t, $J$=6.7 Hz, 16-H), 2.34 (3H, s, 17-H), 3.92 (3H, s, 3-$OCH_3$), 4.10 (3H, s, $NCH_3$); FD-MS $m/z$ 323 ($M^+$).

Carbazomycin B (5)

Purification of **5** from the culture of a soil isolate *Streptoverticillium* sp. was performed by the method of SAKANO *et al.*[8] with slight modification.

4-*O*-Acetylcarbazomycin B (6)

**6** was prepared from **5** by the method reported by SAKANO *et al.*[9].

Carbazomycin A (4-*O*-methylcarbazomycin B) (7)

**7** was prepared from **5** by the procedure similar to that of **3**. **7** was reported by SAKANO *et al.*[9].

*N*-Methylcarbazomycin A (8)

Compound **8** was prepared from **5** (600 mg) by the procedure similar to that of **4**, and 648.6 mg (97% yield) of **8** was obtained as a white powder. MP 126 ~ 128°C; TLC, Rf 0.48 (hexane - EtOAc (5 : 1)); IR (KBr) $cm^{-1}$ 2930, 1460, 1390, 1300, 1090, 1050, 740; $^1H$ NMR (acetone-$d_6$) $\delta$ 8.21 (1H, d, $J$=7.9 Hz, 5-H), 7.14 (1H, dd, $J$=7.9 and 7.9 Hz, 6-H), 7.39 (1H, dd, $J$=7.9 and 7.9 Hz, 7-H), 7.43 (1H, d, $J$=7.9 Hz, 8-H), 2.71 (3H, s, 10-H), 2.35 (3H, s, 11-H), 3.83 (3H, s, 12-H), 4.04 (3H, s, 4-$OCH_3$), 4.10 (3H, s, $NCH_3$); FD-MS $m/z$ 269 ($M^+$).

*In Vitro* Inhibitory Activity against Lipid Peroxidation

Rat brain homogenate was prepared according to the method of KUBO *et al.*[12] with some modification as follows: A male Wistar rat weighing about 300 g was sacrificed by decapitation. The whole brain except cerebellum was immediately homogenized with a glass and teflon homogenizer for 30 seconds in 15 ml of an ice-cold 100 mM phosphate buffer (pH 7.4). The reaction mixture consisted of 0.5% (w/v) of the rat brain homogenate, 100 $\mu$M of sodium ascorbate, 10 $\mu$M of $FeSO_4 \cdot 7H_2O$ and a sample dissolved in MeOH. The mixture was incubated at 37°C for 1 hour with reciprocal agitation. Malondialdehyde (MDA) was formed in the reaction mixture in proportion to the amount of lipid peroxides. MDA was further allowed to react with thiobarbituric acid[18], and was determined by measuring the absorbance at 532 nm ($A_{532}$) of the reaction product. Percent of inhibition was calculated as follows; $[1-(T-B)/(C-B)] \times 100$ (%), where T, C and B are $A_{532}$ of the drug treatment, the control (spontaneous peroxidation) and the time 0 control (no peroxidation), respectively.

DPPH Radical Scavenging Activity

Radical scavenging activity against diphenyl-*p*-picrylhydrazyl (DPPH) was measured according to the method of MELLORS and TAPPEL[13]. DPPH, a stable free radical was dissolved in ethanol to give a 100 $\mu$M solution. To 3 ml of the DPPH solution were added 0.5 ml of ethanol solutions with or without test drug. The absorbance at 517 nm ($A_{517}$) was measured 30 minutes later. The actual decrease of $A_{517}$ induced by the drugs was calculated by subtracting $A_{517}$ of the treatment from that of the control. Concentration of each drug needed for 0.2 of the actual decrease at $A_{517}$ was determined as $IC_{0.2}$ ($\mu$M).

*Ex Vivo* Inhibitory Activity against Lipid Peroxidation

This assay system was referred to the method of USHIYAMA *et al.*[11]. Drugs suspended in aqueous 10% cremophor EL (Sigma Chemical Co.) solution were administered orally (10 ml/kg) to male ddY mice ($n$=6) weighing about 30 g. The same volume of the vehicle was administered to mice as a control. One

hour after administration, blood was collected and citrated plasma was obtained by centrifugation. The reaction mixture (1 ml), which consisted of 0.2 ml of mouse plasma, 10 mM of $FeSO_4$ and 30 mM of Tris HCl buffer (pH 7.4), was incubated at 37°C with vigorous agitation for 2 hours. Concentration of malondialdehyde (MDA) in the mixture was determined as described in the *in vitro* system. Percent of inhibition was calculated as follows; $(1-T/C)\times 100$ (%), where T and C were the mean values of $A_{532}$ of the drug treatment and the control, respectively.

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