Scavenging Effects of Metal Complexes of Water-Soluble Thiacalix[4]arenetetrasulfonate on Superoxide Anion Radicals

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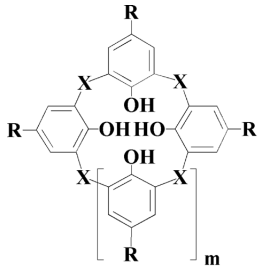
The scavenging effects of metal complexes of thiacalix[4]arenetetrasulfonate (Me-TCAS[4], Me=H_2, Fe^{3+}, Mn^{3+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}) on superoxide anion radicals (O_2^-) generated from the xanthine–xanthine oxidase system were investigated by the nitroblue tetrazolium (NBT) method and electron spin resonance (ESR) spin-trapping method using 5,5-dimethyl-1-pyrroline-*N*-oxide as a trapping reagent. As a reference, calix[4]arenetetrasulfonate (H_2-CAS[4]), calix[6]arenehexasulfonate (H_2-CAS[6]) and calix[8]areneoctasulfonate (H_2-CAS[8]) were also examined. The results by the NBT method indicated that Fe^{3+}- and Mn^{3+}-TCAS[4] exhibited the highest O_2^- scavenging activity among Me-TCAS[4] and H_2-CAS[*n*] (*n*=4, 6, 8) in this study. The IC_{50} values of Fe^{3+}- and Mn^{3+}-TCAS[4] for O_2^- scavenging activity were estimated to be 5.3 and 7.8 μM, respectively, and were almost the same as those of tannin acid, catechin and their derivatives, which are known as very effective scavengers of O_2^-. Scavenging activities were in the order of Fe^{3+}- and Mn^{3+}-TCAS[4]≫Mn^{2+}-, Cu^{2+}-, and Zn^{2+}-TCAS[4]≫H_2-TCAS[4] and H_2-CAS[*n*] (*n*=4, 6, 8). Each activity of Me-TCAS[4] (Me=Fe^{3+}, Mn^{3+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}) was higher than that of the corresponding metal ion, indicating that H_2-TCAS[4] has the ability to raise the activity of the metal ion itself by forming a complex. Also, the ESR spin-trapping method revealed that Fe^{3+}- and Mn^{3+}-TCAS[4] showed high O_2^- scavenging activities, similarly to the results by the NBT method.

Key words thiacalix[*n*]arene; calix[*n*]arene; superoxide anion radical; scavenger; nitroblue tetrazolium; electron spin resonance

Superoxide anion radicals (O_2^-) are well known to be continually produced in aerobic cells under normal conditions, and induce various injuries to the surrounding organism.[1,2] Consequentially, O_2^- has been shown to be implicated as a cause of some cancers, symptoms of aging, tissue inflammation, ischemia, arthritis, and so on[2,3]; therefore, scavenging O_2^- is one of the most effective defenses of a living body against oxidative stress. Recently, antioxidants against the potential toxicity of O_2^- have attracted a great deal of attention for their effect on preventing diseases due to oxidative stress, which leads to many pathological diseases.[4,5] Thus far, much interest has been focused on metal complexes with high O_2^- scavenging activities.[6—8]

On the other hand, calix[*n*]arenes as a class of macrocycles have been extensively investigated in various fields because of their high potential for forming host–guest complexes.[9,10] With marked progress, a large number of calix[*n*]arene derivatives have been developed by modifying either the upper or lower rims in order to apply them not only as useful tools for separation and sensors of various ions and molecules,[11—14] but also as useful catalysts for several reactions.[15,16] In these investigations, calix[*n*]arenes themselves has been utilized as a platform to add new functions by modifying the upper and/or lower rims with various functional groups. Only a few investigations have been performed on the activities of calix[*n*]arene derivatives without modifying their rims; however, thiacalix[*n*]arenes, recently developed by Kumagai *et al.*,[17] have a specific structure in that the methylene units of the parent calix[*n*]arenes are replaced by –S– atom linkages, as shown in Fig. 1. They possess several interesting features, the most remarkable being the ability to form very stable metal complexes without modifying their upper and/or lower rims.[18—20] With conventional calix[*n*]arenes, it is necessary to modify the upper and/or lower rims with suitable functional groups to prepare stable metal complexes.[10,21] Because



thiacalix[*n*]arenes (X: S)

H_2-TCAS[4] (m = 1, R: SO_3Na)

calix[*n*]arenes (X: CH_2)

H_2-CAS[4] (m = 1, R: SO_3Na)

H_2-CAS[6] (m = 3, R: SO_3Na)

H_2-CAS[8] (m = 5, R: SO_3Na)

Fig. 1. Structures of H_2-TCAS[4] and H_2-CAS[*n*]

of these features, some thiacalix[*n*]arene derivatives have been used as analytical reagents for the separation of various metal ions.[18,22] Recently, we have demonstrated that Fe^{3+} complexes of thiacalix[4]arenetetrasulfonate (TCAS[4]) modified on the anion-exchangers (Fe^{3+}-TCAS[4]$_{A\text{-}500}$) exhibited high peroxidase-like activity for catalyzing the oxidation of a substrate with H_2O_2 to scavenge H_2O_2.[23—25] Moreover, we have demonstrated that Fe^{3+}- and Mn^{3+}-TCAS[4]$_{A\text{-}500}$ exhibited high catalase-like activity for catalyzing the decomposition of H_2O_2.[26] In this way, we have demonstrated that Me-TCAS[4] exhibited very high activity for scavenging H_2O_2, which is an active oxygen species.

In this study, in an attempt to develop new calix[*n*]arene derivatives that scavenge O_2^-, a reactive oxygen species, the scavenging effects of Me-TCAS[4] (Me=H_2, Fe^{3+}, Mn^{3+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}) on O_2^- were investigated by the nitro-

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blue tetrazolium (NBT) method as an indirect monitoring method and by the electron spin resonance (ESR) spin-trapping method using 5,5-dimethyl-1-pyrroline-*N*-oxide as a direct monitoring method. To the best of our knowledge, no report has been published on the O_2^- scavenging activities of metal complexes of calix[*n*]arenes and thiacalix[*n*]arenes.

Experimental

Materials Sodium thiacalix[4]arenetetrasulfonate (Fig. 1, H_2-TCAS[4]), prepared as described in the literature,[17] was kindly provided by Cosmo Oil Co. Sodium calix[4]arenetetrasulfonate, sodium calix[6]arenehexasulfonate and sodium calix[8]areneoctasulfonate (Fig. 1, H_2-CAS[*n*]; n=4, 6, 8, respectively) were purchased from Sugai Kagaku Kogyo Co. (Wakayama, Japan). Nitroblue tetrazolium (NBT) and xanthine oxidase (XOD) were purchased from Sigma Chemical Co. (Tokyo, Japan) and Oriental Yeast Co. (Tokyo, Japan), respectively. The high purity spin-trapping reagent, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), was purchased from Labotec Co. (Tokyo, Japan), and the metal-chelating reagent, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DETAPAC), was from Nacalai Tesque Co. (Kyoto, Japan) (+)-Catechin, (−)-epicatechin, gallic acid, and tannic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of reagent grade and used without further purification.

Instruments The absorption spectra and absorbances were recorded on a Shimadzu UV-1600 PC double beam spectrophotometer with a 10 mm quartz cell. ESR spectra were observed on a JEOL JES-FA300 spectrometer using a flat quartz ESR cell (JEOL JESLC11, inner size 50 mm×4.2 mm×0.4 mm, effective volume 70 μl) at room temperature. The ESR spectroscopic conditions were as follows: magnetic field, 336.00±5.00 mT; microwave frequency, 9.43 GHz; modulation frequency, 100.0 kHz; modulation width, 0.10 mT; microwave power, 10 mW; sweep time, 2.0 min; time constant, 0.1 s. FAB-MS spectra were measured on a JMS-700 spectrometer (JEOL, Japan).

Preparation of Me-TCAS[4] Fe^{3+}-TCAS[4](Cl)·11H_2O was prepared as follows: $FeCl_3$ solution (0.13 mol/l, 1 ml) was added while stirring H_2-TCAS[4] solution (0.11 mol/l, 1 ml) adjusted to pH 7 with a 0.1 mol/l NaOH solution, and this mixture was stirred for an additional 2 h. Saturated NaCl solution (1 ml) was added to the mixture, which was stirred for an additional 1 h. EtOH (4 ml) was then added and the mixture was allowed to stand in a refrigerator overnight. The precipitate (Fe^{3+}-TCAS[4](Cl)·11H_2O) was washed with a mixture of EtOH and H_2O several times, and dried over P_2O_5 under reduced pressure.

Mn^{2+}-TCAS[4]·11H_2O, Cu^{2+}-TCAS[4]·8H_2O, and Zn^{2+}-TCAS[4]·11H_2O were prepared as were Fe^{3+}-TCAS[4](Cl)·11H_2O using $MnCl_2$·4H_2O, $CuCl_2$·2H_2O, and $Zn(CH_3COO)_2$·2H_2O instead of $FeCl_3$, respectively.

Mn^{3+}-TCAS[4](CH_3COO)·10H_2O was prepared in EtOH as a solvent as follows: $Mn^{3+}(CH_3COO)_3$·2H_2O (30.8 mg) and H_2-TCAS[4] (100 mg) was added to 50 ml EtOH, and refluxed while stirring for 2 h at 70—75 °C. After cooling, the precipitate (Mn^{3+}-TCAS[4](CH_3COO)·10H_2O) was washed with EtOH several times, and dried over P_2O_5 under reduced pressure.

The results of each elementary analysis of Me-TCAS[4] (Me: Fe^{3+}, Mn^{3+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}) were as follows, respectively. *Anal.* Calcd for $C_{24}H_{10}O_{16}ClNa_4S_8Fe$·11$H_2O$: C, 24.18; H, 2.71; N, 0.00. Found: C, 24.33; H, 2.83; N, 0.02. Calcd for $C_{26}H_{13}O_{18}Na_4S_8Mn$·10$H_2O$: C, 26.09; H, 2.78; N, 0.00. Found: C, 26.10; H, 2.93; N, 0.09. Calcd for $C_{24}H_{10}O_{16}Na_4S_8Mn$·11$H_2O$: C, 24.94; H, 2.79; N, 0.00. Found: C, 25.11; H, 3.05; N, 0.05. Calcd for $C_{24}H_{10}O_{16}Na_4S_8Cu$·8$H_2O$: C, 25.96; H, 2.36; N, 0.00. Found: C, 26.30; H, 2.85; N, 0.02. Calcd for $C_{24}H_{10}O_{16}Na_4S_8Zn$·11$H_2O$: C, 24.71; H, 2.77; N, 0.00. Found: C, 24.34; H, 2.36; N, 0.00. These results indicate that the molar ratio of each metal ion to TCAS[4] is 1 : 1 in Me-TCAS[4] complexes.

Evaluation of O_2^- Scavenging Activity by the NBT Method For the evaluation of O_2^- scavenging activity, the improved method by Imanari *et al.* was applied,[27] in which NBT reduction with O_2^- was stopped by the addition of $CuCl_2$. O_2^- was supplied to the evaluating system from the xanthine–XOD reaction, as described in the literature.[28] Sample solution (0.2 ml) was added to a mixture solution containing 0.05 M carbonate buffer (pH 10.2, 4.8 ml), 3 mM xanthine (0.2 ml), 3 mM ethylenediaminetetraacetic acid (EDTA)-2Na (0.2 ml), 0.15% bovine serum albumin (BSA) (0.2 ml), and 0.75 mM NBT (0.2 ml), and the mixture was incubated for 10 min at 25 °C. After 0.1 units/ml XOD (0.2 ml) was added, the reaction mixture was further incubated for 20 min at 25 °C. To stop the NBT reduction with O_2^- to form formazan dye, 6 mM $CuCl_2$ (0.2 ml) was added to the mixture. The absorbance at 560 nm of formazan dye formed in the mixture was observed against the reagent blank. The O_2^- scavenging activities of sample materials were expressed in terms of IC_{50}, which was the sample concentration required to inhibit NBT reduction with O_2^- by 50%. With a sample, the O_2^- scavenging activities of Fe^{3+}- and Mn^{3+}-TCAS[4] were evaluated by monitoring the formation of formazan dye, as shown in Fig. 4. IC_{50} was calculated by fitting the linear regression obtained from various concentrations of sample to a dose curve. Analyses were conducted three times for each test material concentration.

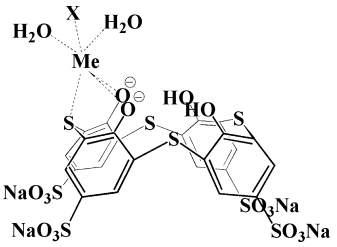

Fig. 2. Proposed Structure of Me-TCAS[4]

Evaluation of O_2^- Scavenging Activity by the ESR Spin-Trapping Method The ESR spin-trapping method was carried out according to the method of Mitsuta *et al.*[29] O_2^- was supplied to a evaluation system from the xanthine–XOD reaction, similarly to the NBT method. The O_2^- scavenging activity of Fe^{3+}-TCAS[4] was evaluated by monitoring the formation of DMPO-O_2^- adducts, as shown in Fig. 5. The intensities of DMPO-O_2^- signals were monitored, and their signal intensities were evaluated by comparing the peak height of the first DMPO-O_2^- signal relative to that of the Mn^{2+} signal as an internal standard. A solution of XOD (0.27 units/ml in 0.05 M sodium phosphate buffer (pH 7.4), 50 μl) was added to a mixture of 1.5 mM xanthine (800 μl), 10 mM DETAPAC (100 μl), and a solution of each of Me-TCAS[4] (100 μl), and DMPO (10 μl). After mixing for 2 s on a vortex mixer, the mixed solution was placed in a flat cell for the ESR measurement. Distilled water was used instead of test sample solution as the control. All experiments were performed at room temperature.

Results and Discussion

Coordination Structures of Me-TCAS[4] As previously described, thiacalix[*n*]arenes have ability to form very stable metal complexes without any modification of their upper and/or lower rims, because the –S– of the epithio groups of thiacalix[*n*]arenes is able to coordinate firmly to metal ions. According to X-ray analysis of the Zn^{2+} complex of *p-tert*-butylthiacalix[4]arene by Iki *et al.*,[19] the Zn^{2+} ion was coordinated to the –S– of the epithio groups and phenolic oxygen atoms. In fact, H_2-TCAS[4] reacted with metal ions to form stable Me-TCAS[4] complexes under the conditions of this study. Me-TCAS[4] (Me=Fe^{3+}, Mn^{3+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}) were water-soluble, and were black, dark brown, white, yellow, and white, respectively. In the case of H_2-TCAS[4], not only the –S– of the epithio groups and phenolic oxygen atoms but also the $-SO_3^-$ groups may coordinate to metal ions. As a reference, H_2-CAS[*n*] (n=4, 6, 8) without the –S– of the epithio groups were examined to react with metal ions; however, no matter how many metal ions of high concentration were allowed to react with H_2-CAS[*n*] (n=4, 6, 8), no metal complex was found under the conditions of this study, indicating that the $-SO_3^-$ groups of H_2-TCAS[4] and H_2-CAS[*n*] (n=4, 6, 8) did not coordinate to the metal ions under the conditions of this study. As previously described, elementary analyses indicate that the molar ratio of each metal ion to H_2-TCAS[4] is 1 : 1 in each Me-TCAS[4] complex. From these results, it is reasonable that each metal ion is coordinated to the –S– of the epithio groups and phenolic oxygen atoms of H_2-TCAS[4], as shown in Fig. 2. Also, the fact that Me-TCAS[4] (Me=Fe^{3+}, Mn^{3+}, and Cu^{2+}) was black, dark brown, and yellow, respectively, may

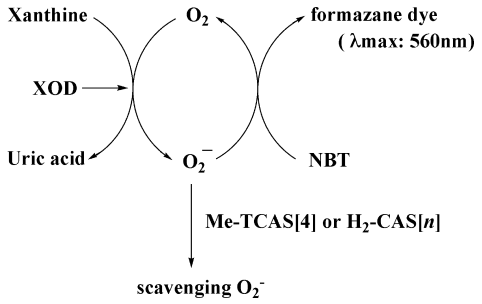

Fig. 3. Evaluation Mechanism of O_2^- Scavenging Activity by the NBT Method

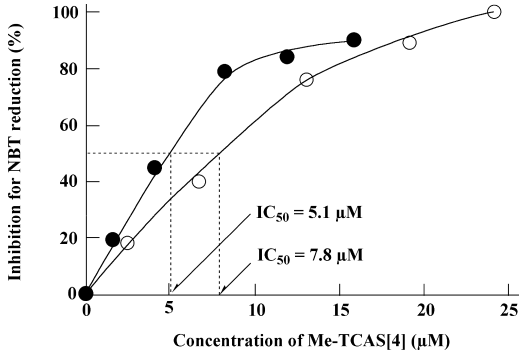

Fig. 4. Inhibition for NBT Reduction by Me-TCAS[4]
—●—, Fe^{3+}-TCAS[4]; —○—, Mn^{3+}-TCAS[4].

support that these metal ions are at least coordinated to the –S– of the epithio groups. From the proposed structure of Me-TCAS[4], shown in Fig. 2, the metal ion in Me-TCAS[4] may be favorably located to react with O_2^- efficiently.

The FAB-MS spectra of Mn^{3+}-TCAS[4] in a glycerol or thioglycerol matrix were investigated. Unfortunately, no peak related to Mn^{3+}-TCAS[4] was observed; however, the peaks corresponding to $[H_2\text{-TCAS[4]}+H]^+$, $[H_2\text{-TCAS[4]}+Na]^+$, and $[H_2\text{-TCAS[4]}+2Na]^+$ were observed at *m/z* 905, 927, and 949, respectively. These indicate that Mn^{3+}-TCAS[4] was dissociated under the ionization conditions in this study for measuring the FAB-MS spectra.

Evaluation of O_2^- Scavenging Activities by the NBT Method To evaluate the scavenging activities of Me-TCAS[4] on O_2^- generated from a xanthine–XOD system, the most convenient and commonly used NBT method was utilized in this study. As shown in Fig. 3, the scavenging activity of O_2^- was assayed by its ability to inhibit NBT reduction with O_2^- to form formazan dye. The course of the reaction was followed by a increase in absorbance at 560 nm as NBT was converted to formazan dye. In Fig. 4, the inhibition of NBT reduction by adding Fe^{3+}- and Mn^{3+}-TCAS[4] at various concentrations is shown. From these results, each IC_{50} value of Fe^{3+}- and Mn^{3+}-TCAS[4], the concentration of Me-TCAS[4] that causes 50% inhibition of NBT reduction with O_2^-, was determined. The IC_{50} values obtained for Me-TCAS[4] and H_2-CAS[*n*] (*n*=4, 6, 8) are summarized in Table 1. It was clear that Fe^{3+}- and Mn^{3+}-TCAS[4] exhibited the highest O_2^- scavenging activities among Me-TCAS[4] and H_2-CAS[*n*] (*n*=4, 6, 8). The activities of Fe^{3+}- and Mn^{3+}-TCAS[4] were almost the same to those observed for tannic aid, catechin and their derivatives, which are well known to be very effective for scavenging O_2^-.[30—32] The activities of Me-TCAS[4] were in the order of Fe^{3+}- and Mn^{3+}-TCAS[4]≫Mn^{2+}-, Cu^{2+}-, and Zn^{2+}-TCAS[4]≫H_2-TCAS[4]. The activities of metal ions were also determined. So far, some metal ions are known to exhibit O_2^- scavenging activities. In fact, Fe^{3+}, Mn^{3+}, and Cu^{2+} ions exhibited high activities, as shown in Table 1. Each activity of Me-TCAS[4] (Me=Fe^{3+}, Mn^{3+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}) was higher than that of the corresponding metal ion, indicating that the activity of each metal ion became stronger by forming a complex with H_2-TCAS[4]. Unexpectedly, H_2-CAS[8] exhibited relatively high activity (IC_{50}: 14.6 μM), while no activity was found for H_2-TCAS[4] and H_2-CAS[*n*] (*n*=4, 6). It is well known that calix[*n*]arenes are very effective for incorporating a small compound into the cavity between their upper and/or lower rims. As H_2-CAS[8] has the largest cavity among H_2-TCAS[4] and H_2-CAS[*n*] (*n*=4, 6, 8), H_2-CAS[8] may incorporate NBT into its large cavity. This incorporation of NBT into H_2-CAS[8] may lead to decreased formazan dye formation, and consequently result in seemingly high activity for H_2-CAS[8].

Table 1. IC_{50} Values by the NBT Method

Compounds	IC_{50} (μM)	Compounds	IC_{50} (μM)
Fe^{3+}-TCAS[4]	5.1±1.0	Fe^{3+}	13.3±1.3
Mn^{3+}-TCAS[4]	7.8±0.3	Mn^{3+}	19.8±1.0
Mn^{2+}-TCAS[4]	32.8±4.8	Mn^{2+}	>100
Cu^{2+}-TCAS[4]	54.2±6.2	Cu^{2+}	84.1±4.0
Zn^{2+}-TCAS[4]	51.8±2.6	Zn^{2+}	>100
H_2-TCAS[4]	>100	Catechin	7.4±0.4
H_2-CAS[4]	>100	Tannic acid	6.8±0.3
H_2-CAS[6]	>100	Epicatechin	5.6±0.2
H_2-CAS[8]	14.6±2.0	Gallic acid	1.2±0.1

Superoxide dismutase (SOD) is well known to scavenge O_2^- by catalyzing the reaction (1) to form O_2 and H_2O_2. If each Fe^{3+}- and Mn^{3+}-TCAS[4] catalyzed reaction (1) similarly to SOD, H_2O_2 would be increased in the reaction system

$$2O_2^- \xrightarrow{2H^+} O_2 + H_2O_2 \quad (1)$$

by scavenging O_2^-; however, almost no increase of H_2O_2 was observed in either Fe^{3+}- or Mn^{3+}-TCAS[4]. Fe^{3+}- and Mn^{3+}-TCAS[4] may decompose H_2O_2 produced in the reaction system because they have very high catalase-like activities for catalyzing the decomposition of H_2O_2, as we previously demonstrated.[26]

Evaluation of O_2^- Scavenging Activities by ESR Spin-Trapping Method As a direct method for evaluating the scavenging activities of Me-TCAS[4] on O_2^-, the ESR spin-trapping method using DMPO as a spin-trapping reagent was utilized. DMPO is well known to react with active oxygen radicals, such as O_2^- and hydroxyl radical ($^{\bullet}OH$), to form radical adducts (DMPO-O_2^- and DMPO-$^{\bullet}OH$, respectively),[33] as shown in Fig. 5. The O_2^- scavenging activities of Me-TCAS[4] were investigated by monitoring the characteristic ESR signals of DMPO-O_2^- produced in a reaction system. If Me-TCAS[4] exhibited O_2^- scavenging activities, the signal intensities of DMPO-O_2^- could be decreased due to a competition reaction between DMPO and Me-TCAS[4] for O_2^-.

The effects of the addition of Fe^{3+}-TCAS[4] to the xanthine–XOD reaction system on the ESR spectra were investi-

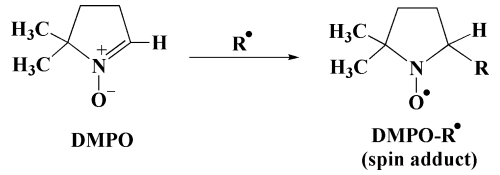

Fig. 5. DMPO and Its Spin Adduct

R˙ represents reactive oxygen species, such as O_2^- and ˙OH.

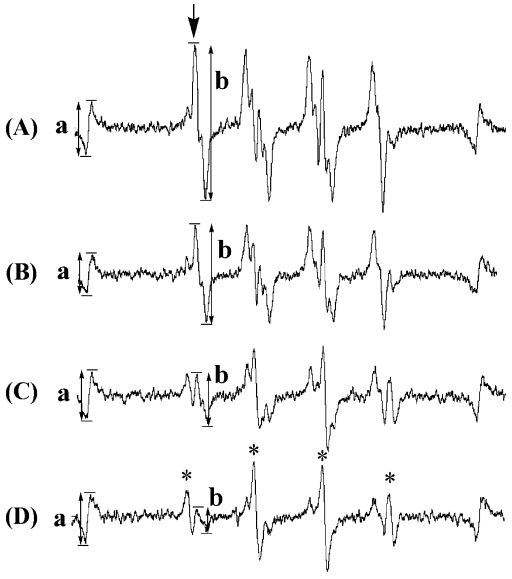

Fig. 6. ESR Spectra Observed at Various Concentrations of Fe^{3+}-TCAS[4] in the Reaction System

The arrow (↓) shows the first peak of the DMPO-O_2^- adduct used for the evaluation of O_2^- scavenging activity. The signals (*) were assigned to DMPO-˙OH adduct. (A) 0 μM, (B) 11.3 μM, (C) 56.6 μM, (D) 113.2 μM.

gated at various concentrations of Fe^{3+}-TCAS[4]. As shown in Fig. 6A, the typical ESR spectrum[33] of DMPO-O_2^- was immediately observed after DMPO was added to the reaction system. Hyperfine coupling constants of the signals were analyzed as one nitrogen, A_N=1.43 mT, one hydrogen at β-position, A_H^β=1.16 mT, and one hydrogen at γ-position, A_H^γ=0.13 mT. The addition of Fe^{3+}-TCAS[4] at various concentrations to the solution caused attenuation of the relative intensities of DMPO-O_2^- signals to Mn^{2+} signals as an internal standard. As shown in Figs. 6A to C, the higher the concentration of Fe^{3+}-TCAS[4], the lower the relative intensities of DMPO-O_2^- signals to Mn^{2+} signals. These spectral changes indicate that Fe^{3+}-TCAS[4] inhibited the reaction between DMPO and O_2^-, and thus exhibited O_2^- scavenging activity. In Figs. 6C and D, the typical ESR spectrum of DMPO-˙OH (A_N=A_H=1.48 mT)[33] was observed. As previously described, if Fe^{3+}-TCAS[4] exhibited the same activity as SOD, H_2O_2 might be formed according to reactions (2) and (3). As pointed out by Halliwell, hydroxyl radical (˙OH) might be formed by the degradation reaction of H_2O_2 by Fe^{2+}-TCAS[4] according to reaction (4).[34,35]

$$Fe^{3+}\text{-}TCAS[4] + O_2^- \longrightarrow Fe^{2+}\text{-}TCAS[4] + O_2 \quad (2)$$

$$Fe^{2+}\text{-}TCAS[4] + O_2^- \xrightarrow{2H^+} Fe^{3+}\text{-}TCAS[4] + H_2O_2 \quad (3)$$

$$Fe^{2+}\text{-}TCAS[4] + H_2O_2 \longrightarrow Fe^{3+}\text{-}TCAS[4] + {}^{\cdot}OH + OH^- \quad (4)$$

Additionally, the ESR spectra were investigated after the addition of other Me-TCAS[4] (Me=H_2, Zn^{2+}, Cu^{2+}, Mn^{2+}, and Mn^{3+}), H_2-CAS[n] (n=4, 6, 8), and catechin. Mn^{3+}-TCAS[4] and catechin exhibited O_2^- scavenging activities because the relative intensities of DMPO-O_2^- signals to Mn^{2+} signals decreased with increasing concentrations (data not shown). However, with Me-TCAS[4] (Me=H_2, Zn^{2+}, Cu^{2+}, and Mn^{2+}) and H_2-CAS[n] (n=4, 6, 8), almost no ESR spectral change was observed even if an excess of these compounds was added to the reaction system. Although H_2-CAS[8] was evaluated as a relatively strong O_2^- scavenger by the NBT method, no activity was found by the ESR spin-trapping method. H_2-CAS[8] unambiguously incorporated NBT into its cavity under the conditions of the NBT method, indicating that the O_2^- scavenging activity of calix[n]arenes and thiacalix[n]arenes with high incorporation activity should be evaluated not only by the NBT method as an indirect method but also by the ESR spin-trapping method as a direct method.

In order to compare the O_2^- scavenging activities of Me-TCAS[4], the relative intensity ratios of DMPO-O_2^- signals to Mn^{2+} signals were determined at each concentration of Me-TCAS[4]. The intensity of the first peak of DMPO-O_2^-, shown in Fig. 6A, was selected to evaluate the relative intensity ratio, which was estimated to be 1.0 without the addition of Me-TCAS[4], H_2-CAS[n] (n=4, 6, 8), or catechin. In Figs. 7 and 8, the relationships among the concentrations of each Me-TCAS[4], H_2-CAS[n] (n=4, 6, 8), and catechins, and the corresponding relative intensity ratio are shown. Figure 7 shows that the higher the concentration of Fe^{3+}- and Mn^{3+}-TCAS[4], the lower the relative intensity ratio, indicating that Fe^{3+}- and Mn^{3+}-TCAS[4] exhibited O_2^- scavenging activities. Fe^{3+}-TCAS[4] exhibited almost the same high activity as catechins, shown in Fig. 8. Previously, we have demonstrated that Fe^{3+}- and Mn^{3+}-TCAS[4]$_{A\text{-}500}$ exhibited high catalase-like catalytic activity for scavenging H_2O_2.[26] Accordingly, Fe^{3+}- and Mn^{3+}-TCAS[4] were shown to be effective scavengers of both O_2^- and H_2O_2.

Effects of the Complexation for the Activity H_2-TCAS[4] and H_2-CAS[n] in this study are a cyclicphenololigomer with plural phenolic OH groups, and so are classified as a kind of polyphenols. So far, metal complexes with various ligands, such as aminoacids, peptides, macrocyclicpolyamines, porphyrins, polyphenols, shiff-bases, and salens, have been shown to exhibit the O_2^- scavenging activities.[6—8,36—40] Among these ligands, polyphenols have attracted much interest because that not only polyphenol compounds themselves exhibited high activities for scavenging O_2^- but also their activities were more elevated by forming a complex with metal ions.[32,41—46]

As shown in Tables 1 and 2, conventional polyphenol compound themselves exhibit high O_2^- scavenging activities. This is because that these polyphenol compounds have the phenolic OH groups which are said to be responsible for the activity for scavenging O_2^-.[40,47] As described before, H_2-TCAS[4] and H_2-CAS[n] in this study showed no activity in spite of possessing the phenolic OH groups similarly to polyphenol compounds. Iki *et al.* indicated the pK_a values for the most acidic phenolic hydrogen of H_2-TCAS[4] and H_2-CAS[4] were 2.18 and 3.26, respectively.[48,49] In contrast the corresponding pK_a values of polyphenol compounds with

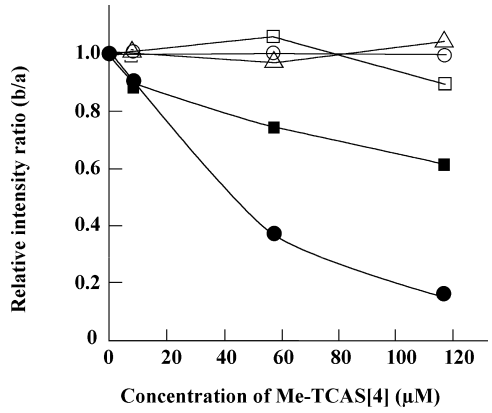

Fig. 7. Effects of the Concentration of Me-TCAS[4] on Relative Intensity Ratio of DMPO-O_2^- Signal (b) to Mn^{2+} Signal (a)

—●—, Fe^{3+}-TCAS[4]; —■—, Mn^{3+}-TCAS[4]; —□—, Cu^{2+}-TCAS[4]; —○—, Mn^{2+}-TCAS[4]; —△—, Zn^{2+}-TCAS[4].

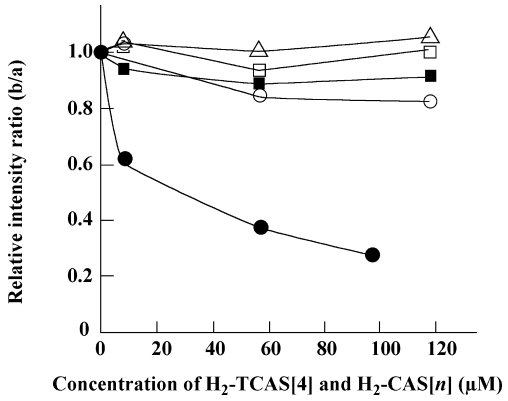

Fig. 8. Effects of the Concentration of H_2-TCA[n] and H_2-TCAS[4] on Relative Intensity Ratio of DMPO-O_2^- Signal (b) to Mn^{2+} Signal (a)

—●—, catechin; —○—, H_2-CAS[6]; —■—, H_2-CAS[8]; —□—, H_2-CAS[4]; —△—, H_2-TCAS[4].

high O_2^- scavenging activities, such as (−)-epicatechin-3-gallate, (−)-epicatechin, and protocatechuic acid, were determined to be 7.74, 8.76, and 8.90, respectively.[40,50—52] Clearly their pK_a values are very different from each other. As the phenolic OH groups in polyphenol compounds are said to be responsible for not only their complexation abilities with metal ions but also their activities for scavenging O_2^- , the nature of both phenolic OH groups will influence activities of free ligands.

As pointed out by Perron and Brumaghim, the stability constants of metal complexes of polyphenol compounds were shown to be responsible for the O_2^- scavenging activities.[40] For example, polyphenol compounds with catechol and gallol moieties have an ability to form a stable complex with metal ions, such as Fe^{2+} and Fe^{3+}, because of possessing the neighboring phenolic OH groups.[40] As shown in Tables 1 and 2, the metal complexes of such polyphenol compounds were actually shown to exhibit higher activity than the corresponding free ligands. On the other hand, Miyano and coworkers demonstrated that thiacalix[n]arenes have significantly larger ability to form a stable complex than calix[n]arenes, based on various experimental results.[18,53,54] In analogy with polyphenol compounds, also H_2-TCAS[4] exhibited higher activity for scavenging O_2^- by forming a complex with

Table 2. Effects of the Complexation for IC_{50} Values of Polyphenols

Ligands	IC_{50} (μM)		Methods	References
	Free ligands	Metal complexes		
H_2-TCAS[4]	>100	5.1±1.0 (Fe^{3+}), 7.8±0.3 (Mn^{3+}), 32.8±4.8 (Mn^{2+}), 54.2±6.2 (Cu^{2+})	NBT	This study
H_2-CAS[n] (n=4, 6, 8)	>100	—	NBT	This study
Rutin	35±0.3	22±2 (Fe^{3+}), 2.5±0.2 (Cu^{2+})	Cyt. $c^{a)}$	45)
(−)-Epicatechin	1.3	0.30 (Fe^{3+}), 0.32 (Cu^{2+})	NBT	46)
Rutin	9.0	2.5 (Fe^{3+}), 0.50 (Cu^{2+})	NBT	46)
Taxifolin	1.9	0.55 (Fe^{3+}), 0.80 (Cu^{2+})	NBT	46)
Luteolin	14.2	2.5 (Fe^{3+}), 0.80 (Cu^{2+})	NBT	46)

a) Cytochrome *c* method.

metal ions, as shown in Table 2. In this way, the –S– moiety in connection with the phenolic OH groups in H_2-TCAS[4] plays a characteristic role in the complexation with metal ions. Such a characteristic complexation ability will not be seen in other ligands including polyphenol compounds.

In conclusion, Fe^{3+}- and Mn^{3+}-TCAS[4] among Me-TCAS[4] in this study exhibited high activities for scavenging O_2^-. Their activities were almost the same as those of tannic acid, catechin, and their derivatives as very strong scavengers of O_2^-. As each O_2^- scavenging activity of Me-TCAS[4] (Me=Fe^{3+}, Mn^{3+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}) was higher than that of the corresponding metal ion, H_2-TCAS[4] showed the ability to raise the activity of each metal ion itself by forming a complex. This study is the first report on the O_2^- scavenging activity of calix[n]arenes and thiacalix[n]arenes without modifying their upper and lower rims.

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