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], Mn3+, Mn2+, Cu2+, and Zn2+) on superoxide anion radicals (O2−) 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 (H2-CAS[4]), calix[6]arenehexasulfonate (H2-CAS[6]) and calix[8]areneoctasulfonate (H2-CAS[8]) were also examined. The results by the NBT method indicated that Fe3+ and Mn3+-TCAS[4] exhibited the highest scavenging activity among Me-TCAS[4] and H2-CAS[n] (n=4, 6, 8) in this study. The IC50 values of Fe3+ and Mn3+-TCAS[4] for O2− 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 O2−. Scavenging activities were in the order of Fe3+ and Mn3+-TCAS[4]>>Mn2+, Cu2+, and Zn2+-TCAS[4]>H2-TCAS[4] and H2-CAS[n] (n=4, 6, 8). Each activity of Me-TCAS[4] (Me=Fe3+, Mn3+, Mn2+, Cu2+, and Zn2+) was higher than that of the corresponding metal ion, indicating that H2-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 Fe3+ and Mn3+-TCAS[4] showed high O2− 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 (O2−) are well known to be continually produced in aerobic cells under normal conditions, and induce various injuries to the surrounding organism.1,2) Consequently, O2− 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 O2− is one of the most effective defenses of a living body against oxidative stress. Recently, antioxidants against the potential toxicity of O2− 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 O2− 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 have been utilized as a platform to add new functions by modifying the upper and/or lower rims with various functional groups.10,21) Because 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 Fe3+-complexes of thiacalix[4]arenetetrasulfonate (TCAS[4]) modified on the anion-exchangers (Fe3+-TCAS[4]AA) exhibited high peroxidase-like activity for catalyzing the oxidation of a substrate with H2O2 to scavenge H2O2.23–25) Moreover, we have demonstrated that Fe3+- and Mn3+-TCAS[4]AA exhibited high catalase-like activity for catalyzing the decomposition of H2O2.26) In this way, we have demonstrated that Me-TCAS[4] exhibited very high activity for scavenging H2O2, which is an active oxygen species.

In this study, in an attempt to develop new calix[n]arene derivatives that scavenge O2−, a reactive oxygen species, the scavenging effects of Me-TCAS[4] (Me=H2, Fe3+, Mn3+, Mn2+, Cu2+, and Zn2+) on O2− were investigated by the nitro-
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 \( \text{O}_2^- \) scavenging activities of metal complexes of calix[n]arenes and thiacalix[n]arenes.

### Experimental

**Materials** Sodium thiacalix[4]arenetetrasulfonate (Fig. 1, \( \text{H}_2\text{-CAS[4]} \)), prepared as described in the literature,\(^{17}\) was kindly provided by Cosmo Oil Co. Sodium calix[8]arenesulfonate, sodium calix[6]arenehexasulfonate and sodium calix[4]arenesulfonate (Fig. 1, \( \text{H}_2\text{-CAS[8]}; n = 4, 6, 8 \), 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 Labo tec Co. (Tokyo, Japan), and the metal-chelating reagent, diethylenetriamine-N,N',N''-tert-pentaacetic acid (DETPAC), 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 \( \mu \text{L} \)) at room temperature. The ESR spectroscopic conditions were as follows: magnetic field, 336.00 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]** \( \text{Fe}^{3+}\text{-TCAS[4](Cl)} \cdot 11\text{H}_2\text{O} \) was prepared as follows: \( \text{FeCl}_3 \) solution (0.13 mol/l, 1 ml) was added while stirring \( \text{H}_2\text{-TCAS[4]} \) solution (0.11 mol/l, 1 ml) adjusted to pH 7 with 0.1 mol/l NaOH solution, and this mixture was stirred for an additional 2 h. The precipitate (\( \text{Fe}_3\text{Cl}_4 \)) was washed with EtOH several times, and dried over P\(_2\)O\(_5\) under reduced pressure.

**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 \( \text{H}_2\text{-tert-butylthiacalix[4]} \)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, \( \text{H}_2\text{-TCAS[4]} \) reacted with metal ions to form stable Me-TCAS[4] complexes under the conditions of this study. Me-TCAS[4] (Me = \( \text{Fe}^{3+}, \text{Mn}^{2+}, \text{Mn}^{3+}, \text{Cu}^{2+}, \text{Zn}^{2+} \)) were water-soluble, and were black, dark brown, white, yellow, and white, respectively. In the case of \( \text{H}_2\text{-TCAS[4]} \), 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, \( \text{H}_2\text{-CAS[4]} \) \( (n = 4, 6, 8) \) did not coordinate to the metal ions under the conditions of this study. As previously described, elemental analyses indicate that the molar ratio of each metal ion to \( \text{H}_2\text{-TCAS[4]} \) is 1:1 in Me-TCAS[4] complexes.

**Evaluation of O\(_2\) Scavenging Activity by the ESR Spin-Tracking Method** The ESR spin-tracking method was carried out according to the method of Mitsuta et al.\(^{29}\) \( \text{O}_2^- \) was supplied to a evaluation system from the xanthine–XOD reaction, similarly to the NBT method. The \( \text{O}_2^- \) scavenging activity of \( \text{Fe}^{3+}\text{-TCAS[4]} \) was 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.

**Evaluation of O\(_2\) Scavenging Activity by the NBT Method** For the evaluation of the \( \text{O}_2^- \) scavenging activity, the improved method by Imanari et al.\(^{28}\) was applied in which NBT reduction with \( \text{O}_2^- \) was stopped by the addition of CuCl\(_2\). CuCl\(_2\) was supplied to the evaluating system from the xanthine–XOD reaction, as described in the literature.\(^{29}\) Sample solution (0.2 ml) was added to a mixture solution containing 0.05 mol 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 \( \text{O}_2^- \) 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 \( \text{O}_2^- \) scavenging activities of sample materials were expressed in terms of IC\(_{50}\), which was the sample concentration required to inhibit NBT reduction with \( \text{O}_2^- \) by 50%. With a sample, the \( \text{O}_2^- \) scavenging activities of \( \text{Fe}^{3+}\text{-} \text{Mn}^{2+}\text{-} \text{Mn}^{3+}\text{-} \text{Cu}^{2+}\text{-} \text{Zn}^{2+} \) were black, dark brown, and yellow, respectively, may
and 949, respectively. These indicate that Mn$_3^+$
with O$_2$ value of Fe$_3^+$ was converted to formazan dye. In Fig. 4, the inhibition of the reaction may be favorably located to react with O$_2$. 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$_2$-TCAS[4]+H$^+$], [H$_2$-TCAS[4]+Na$^+$], and [H$_2$-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.

**Figure 3. Evaluation Mechanism of O$_2^-$ Scavenging Activity by the NBT Method**

**Figure 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$_2$-TCAS[4]+H$^+$], [H$_2$-TCAS[4]+Na$^+$], and [H$_2$-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 an increase in absorbance at 560 nm as NBT was converted to formazan dye. In Fig. 4, the inhibition of NBT reduction by adding Fe$_3^{3+}$- and Mn$_3^{3+}$-TCAS[4] at various concentrations is shown. From these results, each IC$_{50}$ value of Fe$_3^{3+}$- and Mn$_3^{3+}$-TCAS[4] at various concentrations is shown. From these results, each IC$_{50}$ value of Fe$_3^{3+}$- and Mn$_3^{3+}$-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$_3^{3+}$- and Mn$_3^{3+}$-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$_3^{3+}$- and Mn$_3^{3+}$-TCAS[4] were almost the same to those observed for tannic acid and their derivatives, which are well known to be very effective for scavenging O$_2^-$. The activities of Me-TCAS[4] were in the order of Fe$_3^{3+}$- and Mn$_3^{3+}$-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$_3^{3+}$-, Mn$^{2+}$-, 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**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (µM)</th>
<th>Compounds</th>
<th>IC$_{50}$ (µM)</th>
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</thead>
<tbody>
<tr>
<td>Fe$_3^{3+}$-TCAS[4]</td>
<td>5.1 ± 1.0</td>
<td>Fe$^{3+}$</td>
<td>13.3 ± 1.3</td>
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<tr>
<td>Mn$_3^{3+}$-TCAS[4]</td>
<td>7.8 ± 0.3</td>
<td>Mn$_3^{3+}$</td>
<td>19.8 ± 1.0</td>
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<tr>
<td>Mn$_3^{2+}$-TCAS[4]</td>
<td>32.8 ± 4.8</td>
<td>Mn$^{2+}$</td>
<td>&gt; 100</td>
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<tr>
<td>Cu$^{2+}$-TCAS[4]</td>
<td>54.2 ± 6.2</td>
<td>Cu$^{2+}$</td>
<td>84.1 ± 4.0</td>
</tr>
<tr>
<td>Zn$^{2+}$-TCAS[4]</td>
<td>51.8 ± 2.6</td>
<td>Zn$^{2+}$</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>H$_2$-TCAS[4]</td>
<td>&gt; 100</td>
<td>Catechin</td>
<td>7.4 ± 0.4</td>
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<tr>
<td>H$_2$-CAS[4]</td>
<td>&gt; 100</td>
<td>Tannic acid</td>
<td>6.8 ± 0.3</td>
</tr>
<tr>
<td>H$_2$-CAS[6]</td>
<td>&gt; 100</td>
<td>Epicatechin</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td>H$_2$-CAS[8]</td>
<td>14.6 ± 2.0</td>
<td>Gallic acid</td>
<td>1.2 ± 0.1</td>
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</table>

**Superoxide dismutase (SOD)** is well known to scavenge O$_2^-$ by catalyzing the reaction (1) to form O$_2$ and H$_2$O$_2$. If each Fe$_3^{3+}$- and Mn$_3^{3+}$-TCAS[4] catalyzed reaction (1) similarly to SOD, H$_2$O$_2$ would be increased in the reaction system.

$$2O_2^- + H_2O_2 \rightarrow O_2 + H_2O_2$$

(1)

**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 (·OH), to form radical adducts (DMPO-O$_2$ and DMPO·OH, respectively), 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 because they have very high catalase-like activities for catalyzing the decomposition of H$_2$O$_2$, as we previously demonstrated. The effects of the addition of Fe$_3^{3+}$-TCAS[4] to the xanthine–XOD reaction system on the ESR spectra were investi-
Additionally, the ESR spectra were investigated after the addition of other Me-TCAS[4] (Me=H2, Zn2+, Cu2+, Mn2+, and Mn3+), H2-CAS[n] (n=4, 6, 8), and catechin. Mn3+-TCAS[4] and catechin exhibited O2 scavenging activities because the relative intensities of DMPO-O2 signals to Mn2+ signals decreased with increasing concentrations (data not shown). However, with Me-TCAS[4] (Me=H2, Zn2+, Cu2+, and Mn3+) and H2-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 H2-CAS[8] was evaluated as a relatively strong O2 scavenger by the NBT method, no activity was found by the ESR spin-trapping method. H2-CAS[8] unambiguously incorporated NBT into its cavity under the conditions of the NBT method, indicating that the O2 scavenging activity of calix[n]arenes and thiacafox[n]arenes with high incorporation activity should be evaluated not only by the NBT method but also by the ESR spin-trapping method as a direct method.

In order to compare the O2 scavenging activities of Me-TCAS[4], the relative intensity ratios of DMPO-O2 signals to Mn2+ signals were determined at each concentration of Me-TCAS[4]. The intensity of the first peak of DMPO-O2, 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], H2-CAS[n] (n=4, 6, 8), or catechin. In Figs. 7 and 8, the relationships among the concentrations of each Me-TCAS[4], H2-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 Fe3+- and Mn3+-TCAS[4], the lower the relative intensity ratio, indicating that Fe3+- and Mn3+-TCAS[4] exhibited O2 scavenging activities. Fe3+-TCAS[4] exhibited almost the same high activity as catechins, shown in Fig. 8. Previously, we have demonstrated that Fe3+- and Mn3+-TCAS[4] exhibited high catalase-like catalytic activity for scavenging H2O2.26 Accordingly, Fe3+- and Mn3+-TCAS[4] were shown to be effective scavengers of both O2 and H2O2.

**Effects of the Complexation for the Activity H2 TCAS[4] and H2-CAS[n] in this study are a cyclopeanollogomer 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 O2 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 O2 but also their activities were more elevated by forming a complex with metal ions.2,24-46**

As shown in Tables 1 and 2, conventional polyphenol compound themselves exhibit high O2 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 O2.40,47 As described before, H2 TCAS[4] and H2-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 values for the most acidic phenolic hydrogen of H2 TCAS[4] and H2-CAS[4] were 2.18 and 3.26, respectively.38,40 In contrast the corresponding pK values of polyphenol compounds with
high $O_2^-$ scavenging activities, such as ($\neg$)-epicatechin-3-gallate, ($\neg$)-epicatechin, and protocatechuic acid, were determined to be 7.74, 8.76, and 8.90, respectively.\(^{30,50,52}\) 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.\(^{30}\) For example, polyphenol compounds with catechol and galloyl 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.\(^{30}\) 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 co-workers 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 metal ions, as shown in Table 2. In this way, the $\neg$-S-- moiety in connection with the phenolic OH groups in $H_2$-TCAS[4] plays a characteristic role in the complexion with metal ions. Such a characteristic complexion 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.

Acknowledgments The authors thank Cosmo Oil Co. for supplying sodium thiacalix[4]arenetetrasulfonate used in this study.

Table 2. Effects of the Complexation for IC$_{50}$ Values of Polyphenols

<table>
<thead>
<tr>
<th>Ligands</th>
<th>IC$_{50}$ (μM)</th>
<th>Methods</th>
<th>References</th>
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<tr>
<td>Free ligands</td>
<td>Metal complexes</td>
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<tr>
<td>$H_2$-TCAS[4]</td>
<td>&gt;100</td>
<td>NBT</td>
<td>This study</td>
</tr>
<tr>
<td>(n=4, 6, 8)</td>
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<tr>
<td>Rutin</td>
<td>35±0.3</td>
<td>Cyt. c(^{45})</td>
<td>46</td>
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<tr>
<td>Taxifolin</td>
<td>1.9</td>
<td>NBT</td>
<td>46</td>
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<tr>
<td>Luteolin</td>
<td>14.2</td>
<td>NBT</td>
<td>46</td>
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</table>

\(^{a}\) Cytochrome c method.

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