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Scavenging Effects of Metal Complexes of Tetrakis(*N*-methylpyridinium-4-yl)porphine and its β-Octabromonated Analogues on Superoxide Anion Radicals by Electron Spin Resonance Spectrometry

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The scavenging effects of metal complexes of tetrakis(*N*-methylpyridinium-4-yl)porphine (Me-TMPyP, Me = H_2, Mn^{3+}, Fe^{3+}, Co^{3+}, Cu^{2+}, Zn^{2+}, and Ni^{2+}) and β-octabromo-*meso*-tetrakis(*N*-methylpyridinium-4-yl)porphine (Me-TM(Br_8), Me = H_2, Mn^{2+}, Fe^{3+}, Co^{3+}, Cu^{2+}, Zn^{2+}, and Ni^{2+}) on superoxide anion radicals (O_2^-) generated from the hypoxanthine-xanthine oxidase system were investigated by ESR (electron spin resonance) spin trapping technique using 5,5-dimethyl-1-pyrroline-*N*-oxide as a trapping reagent. ESR spin trapping technique showed that Mn^{2+}-TM(Br_8) exhibited the highest O_2^- scavenging activity among metal porphyrins in this study. The O_2^- scavenging activities of Me-TM(Br_8) (Me = Mn^{2+}, Fe^{3+}, Co^{3+}, and Cu^{2+}) were higher than those of the corresponding Me-TMPyP (Me = Mn^{3+}, Fe^{3+}, Co^{3+}, and Cu^{2+}), respectively. Me-TMPyP and -TM(Br_8) (Me = H_2, Ni^{2+}, and Zn^{2+}) showed no activity. As a reference, the cytochrome c (Cyt. C) method was also applied for evaluating the O_2^- scavenging activities. In case of Me-TM(Br_8) (Me = Mn^{2+}, Fe^{3+}, Co^{3+}, and Cu^{2+}), a satisfactory linear relationship was shown between the IC_{50} values by ESR spin trapping technique and those by the Cyt. C method.

Keywords: metalloporphine; octabromoporphine; superoxide anion radical; scavenger; electron spin resonance; cytochrome c method.

Introduction

Superoxide anion radicals (O_2^-), one of reactive oxygen species, are well known to be continually produced in aerobic cells under normal conditions, and have been shown to be implicated as a cause of tissue inflammation, symptoms of aging, some cancers, ischemia, anemia, arthritis, and so on.[1,2] 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 in preventing diseases due to oxidative stress, which leads to many pathological diseases.[3,4] Thus far much interest has been focused on metal complexes with high O_2^- scavenging activities.[5-8] Especially, metal porphyrins have attracted much interest because not only they play an important role as an active center in hemeproteins, such as peroxidase and catalase that scavenge H_2O_2, but also some of them exhibit high O_2^- scavenging activities.[9,10] For example, Mn^{3+}- and Fe^{3+}-porphyrins have been shown to exhibit high O_2^- scavenging activities.[11-13] In these investigations, the cytochrome c (Cyt. C) method and the

nitro blue tetrazorium (NBT) method were frequently applied for evaluating the O_2^- scavenging activity. As point out by Weiss *et al.*,[14] these methods have possibility to give false positives for the O_2^- scavenging activity, because those are indirect assays for monitoring the scavenging of O_2^-. Therefore, a direct assay that can directly monitor the scavenging of O_2^-, such as ESR spectrometry, is needed to evaluate O_2^- scavenging activities. However, to the best of our knowledge, no report has been published on O_2^- scavenging activities of metal porphyrins by ESR spectrometry using spin trapping technique that can directly monitor the scavenging of O_2^-.

In this study, the scavenging effects of Me-TMPyP (Me = H_2, Mn^{3+}, Fe^{3+}, Co^{3+}, Cu^{2+}, Zn^{2+}, and Ni^{2+}) and Me-TM(Br_8) (Me = H_2, Mn^{2+}, Fe^{3+}, Co^{3+}, Cu^{2+}, Zn^{2+}, and Ni^{2+}) on O_2^-, shown in Fig. 1, were investigated by ESR spin trapping technique using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a trapping reagent. The Cyt. C method was also applied to the evaluation of the scavenging effects of Me-TMPyP and Me-TM(Br_8) on O_2^-, as an indirect monitoring method for evaluating the scavenging of O_2^-.

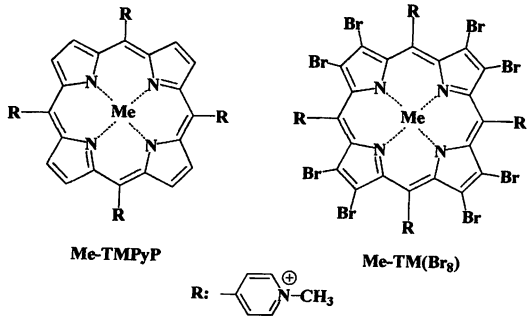

Fig. 1 Structures of Me-TMPyP and Me-TM(Br_8)

Experimental

Materials

Metal-free tetrakis(*N*-methylpyridinium-4-yl)porphine (H_2-TMPyP) was purchased from Tokyo Kasei Kogyo Co. (Japan, Tokyo) Cytochrome c (oxidized form) and hypoxanthine (HPX) were purchased from Sigma Chemical Co. Xanthine oxidase (XOD) was purchased from Oriental Yeast Co. The high purity DMPO was purchased from Labotec Co., and the metal-chelating reagent diethylenetriamine-*N,N,N',N'',N''*-pentaacetic acid (DETAPAC) from Nacalai Tesque Co. All other reagents were of reagent grade and used without further purification.

Preparation of Me-TMPyP and Me-TMPyP(Br_8)

Chloride salts of Me-TMPyP (Me = Mn^{3+}, Fe^{3+}, Co^{3+}, Cu^{2+}, Zn^{2+}, and Ni^{2+}) were prepared by metallation of metal-free H_2-TMPyP according to the method in the literature.[15] Me-TM(Br_8) was prepared by using the method in the literature.[16] First H_2-TM(Br_8) was prepared by demetallation of Cu^{2+}-TM(Br_8) that was synthesized *via* bromination of Cu^{2+}-TMPyP with Br_2, and next Me-TM(Br_8) (Me = Mn^{3+}, Fe^{3+}, Co^{3+}, Cu^{2+}, Zn^{2+}, and Ni^{2+}) was prepared by metallation of H_2-TM(Br_8). Chloride salts of Me-TM(Br_8) were analyzed by elemental analysis. *Anal.* Calcd for Cu^{2+}-TM(Br_8)Cl_4•$4H_2O$ ($CuC_{44}H_{36}N_8Cl_5Br_8$): C, 33.29; H, 2.29; N, 7.06. Found: C, 33.54; H, 2.75; N, 6.86. Calcd for Fe^{3+}-TM(Br_8)Cl_5 ($FeC_{44}H_{28}N_8O_4Cl_4Br_8$): C, 34.29; H, 1.83; N, 7.27. Found: C, 35.30; H, 3.26; N, 6.61. Calcd for Zn^{2+}-TM(Br_8)Cl_4•H_2O ($ZnC_{44}H_{30}N_8OCl_4Br_8$): C, 34.47; H, 1.97; N, 7.31. Found: C, 34.76; H, 2.73; N, 6.94. Calcd for Mn^{2+}-TM(Br_8)Cl_5•$7H_2O$ ($MnC_{44}H_{42}N_8O_7Cl_5Br_8$): C, 31.72; H, 2.54; N, 6.72. Found: C, 31.89; H, 2.74; N, 6.40. Calcd for Co^{3+}-TM(Br_8)Cl_5•H_2O ($CoC_{44}H_{30}N_8OCl_5Br_8$): C, 33.83; H, 1.94; N, 7.17. Found: C, 33.79; H, 2.65; N, 6.85. Calcd for Ni^{2+}-TM(Br_8)Cl_4•$5H_2O$ ($NiC_{44}H_{38}N_8O_5Cl_4Br_8$): C, 33.06; H, 2.40; N, 7.01. Found: C, 33.27; H, 2.84; N, 6.73.

Evaluation of O_2^- scavenging activity by ESR spin trapping technique

ESR spin trapping technique was carried out according to the method by Mitsuta *et al.*[17,18] O_2^- was supplied to a evaluating system from the HPX-XOD reaction. As shown in Fig. 2, the O_2^- scavenging activities of Me-TMPyP and Me-TM(Br_8) were evaluated by monitoring the formation of DMPO-O_2^- adducts. Actually, 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 0.05M sodium phosphate buffer (pH7.4), 50 μl) was added to a mixture of 1.5 mM HPX (800 μl), 10 mM DETAPAC (100 μl), a solution of each metal porphyrin (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. The O_2^- scavenging activities were expressed in terms of IC_{50} value, that is, a concentration required to reduce the relative peak height of DMPO-O_2^- by 50 %.

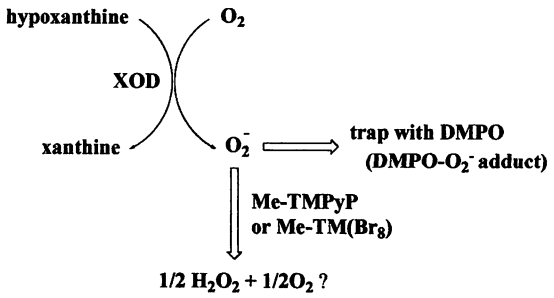

Fig. 2 ESR spin trapping technique using DMPO for evaluating the O_2^- scavenging activity

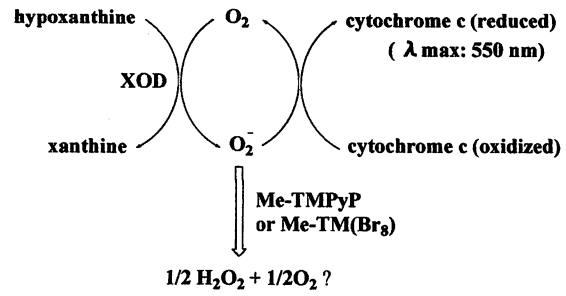

Fig. 3 Cyt. C method for evaluating the O_2^- scavenging activity

Evaluation of O_2^- scavenging activity by the Cyt. C method

The Cyt. C method, shown in Fig. 3, was performed according to the literatures.[7,19] This method has been developed to evaluate the O_2^- scavenging activity in which an amount of O_2^- in a system is estimated by reaction of O_2^- with cytochrome c (oxidized form). As shown in Fig. 3, the O_2^- scavenging activities of Me-TMPyP and Me-TM(Br_8) were evaluated by monitoring a decrease of an absorbance at 550 nm derived from inhibition of cytochrome c reduction by O_2^-. A XOD solution was prepared to contain an amount of XOD sufficient to give an initial rate of $\Delta A_{550\ nm}$/min = 0.012 ~ 0.013 in the reference. A sample solution (0.3 ml) was added to mixture solution containing 30 mM potassium phosphate buffer solution (pH7.4, 1.1 ml), 60 μM cytochrme c (0.5 ml), and 300 μM HPX (0.5 ml). After XOD solution was added to this mixture solution, the absorbance of 550 nm of the reaction solution was monitored to get an initial rate of $\Delta A_{550\,nm}$/min. As a reference, an experiment was performed in the absence of sample under the same condition. From these results, a concentration of sample that causes 50 % of the inhibition of the cytochrome c reduction by O_2^-, IC_{50} value, was determined. All experiments were performed at room temperature.

Apparatus

The absorption spectra and absorbances were measured on a Shimadzu UV-1600 PC double beam spectrophotometer with a 10 mm quartz cell. The ESR spectra were measured on a JEOL JES-PX2300 spectrometer with 100 kHz field modulation frequency and 1 G modulation amplitude at an output power of 10 mW unless otherwise indicated. Mn^{2+} in MnO was used as an internal standard.

Results and discussion

Evaluation of O_2^- scavenging effects by ESR spin trapping technique

DMPO, one of radical trapping reagents, is well known to react with O_2^- to form its O_2^- radical adduct (DMPO-O_2^-), as shown in Fig. 2. The O_2^- scavenging activities of Me-TMPyP and Me-TM(Br_8) were investigated by monitoring characteristic ESR signals of DMPO-O_2^- produced in a reaction system. If metal porphyrins exhibited the O_2^- scavenging activities, the signal intensities of DMPO-O_2^- could be decreased due to a competition reaction between DMPO and metal porphyrins for O_2^-.

In Figs. 4 and 5, the ESR spectra after the addition of Mn^{3+} (or Mn^{2+}) and Cu^{2+} porphyrins at various concentrations to a HPX-POD reaction system were shown, respectively. As shown in Fig. 4(A), a typical ESR spectrum 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=1.42mT, one hydrogen of β-position, $a_{H\beta}$=1.15mT, and one hydrogen of γ-position, $a_{H\gamma}$=0.13mT. The addition of Mn^{3+}-TMPyP and Mn^{2+}-TM(Br_8) to the solution at various concentrations caused attenuation of the relative intensities of DMPO-O_2^- signals to those of Mn^{2+} signals as an internal standard. As shown in Fig. 4(A) to 4(C), the higher was the concentration of Mn^{3+}-TMPyP or Mn^{2+}-TM(Br_8), the lower was the relative intensities of DMPO-O_2^- signals to those of Mn^{2+} signals. These spectral changes indicate that Mn^{3+}-TMPyP and Mn^{2+}-TM(Br_8) inhibited the reaction between DMPO and O_2^-, and thus exhibited the O_2^- scavenging activity. In Figs. 4(D) and 4(E), a typical

ESR spectrum of DMPO-·OH (·OH adduct of DMPO) was observed. Hydroxyl radical (·OH) might be formed by a degradation reaction of H_2O_2 that was produced through the scavenging of O_2^- by these porphyrins.

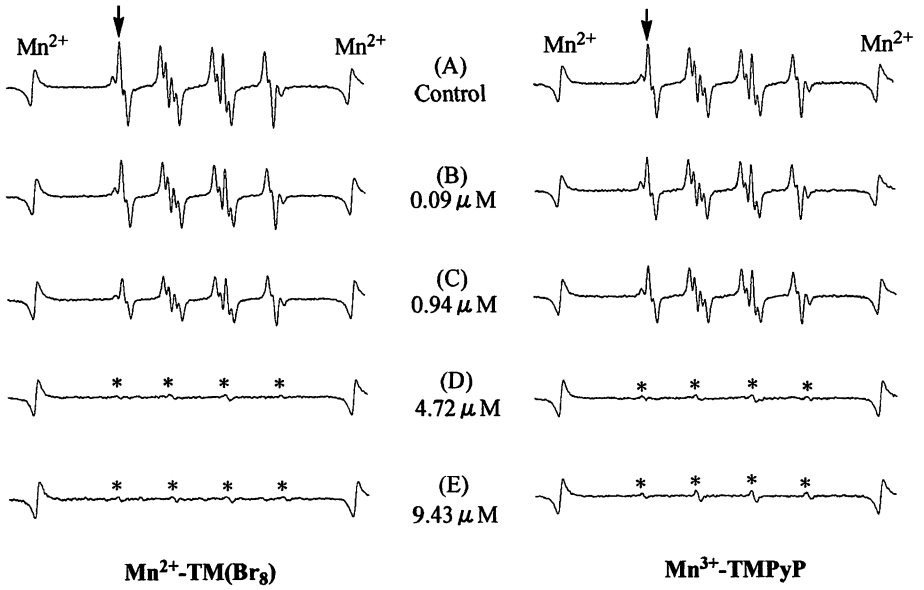

Fig. 4 ESR spectra observed at various concentrations (0 ~ 9.43 μM) of Mn^{3+}-TMPyP or Mn^{2+}-$TM(Br_8)$ in the reaction system. The arrow shows the first peak of the DMPO-O_2^- adduct. A spectrum of DMPO-·OH adduct (*) was obseved in the spectra.

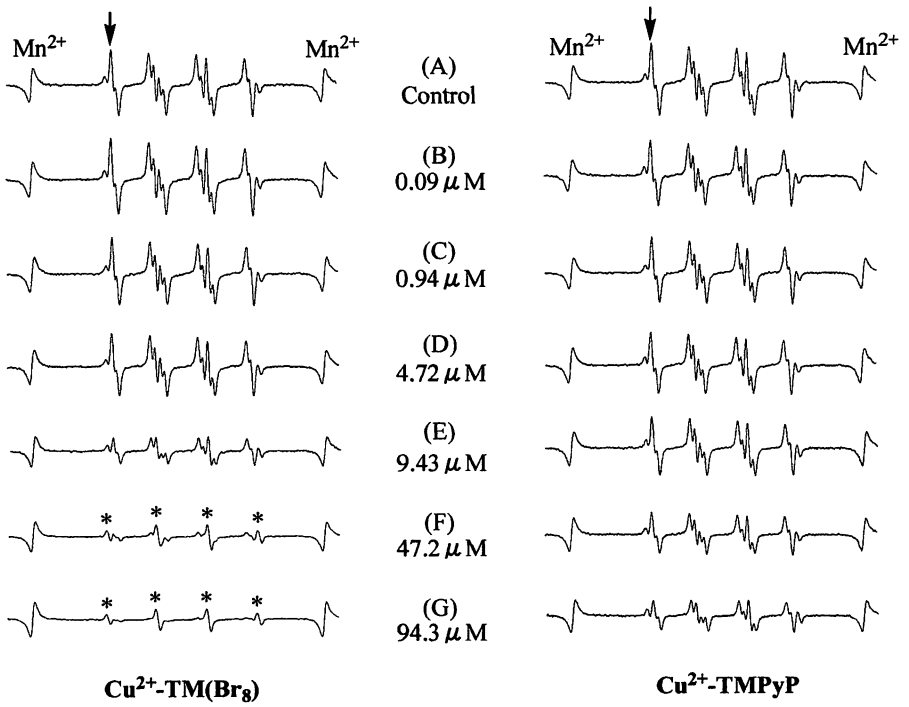

Fig. 5 ESR spectra observed at various concentrations (0 ~ 94.3 μM) of Cu^{2+}-TMPyP or Cu^{2+}-$TM(Br_8)$ in the reaction system. The arrow shows the first peak of the DMPO-O_2^- adduct. A spectrum of DMPO-·OH adduct (*) was obseved in the spectra.

Similarly, Fig. 5 shows the ESR spectra after the addition of Cu^{2+}-TMPyP or Cu^{2+}-TM(Br_8) to the HPX-POD reaction system. In Fig. 5(A) without addition of Cu^{2+} porphyrin, a typical ESR spectrum of DMPO-O_2^- was observed. When Cu^{2+} porphyrins at various concentrations were added to the reaction system, the relative intensities of DMPO-O_2^- signals to those of Mn^{2+} signals were decreased with increasing concentration of Cu^{2+} porphyrins. As shown in Figs. 5(F) and 5(G), a typical signal of DMPO-$^{\bullet}$OH was observed, in analogy with both cases of Mn^{3+}-TMPyP and Mn^{2+}-TM(Br_8) shown in Figs. 4(D) and 4(E).

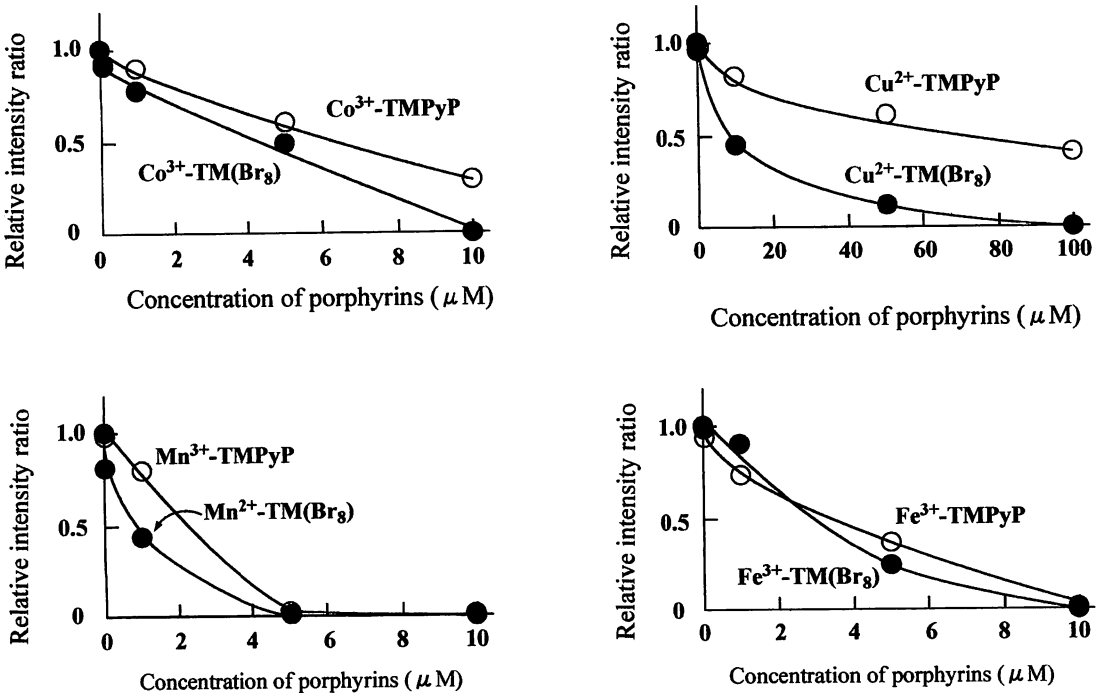

Fig. 6 Relationships between the relative intensities of the DMPO-O_2^- adduct and the concentration of Me-TMPyP and Me-TM(Br_8)

Additionally, the ESR spectra after the addition of other Me-TMPyP and Me-TM(Br_8) (Me = H_2, Zn^{2+}, Ni^{2+}, Fe^{3+}, and Co^{3+}) were investigated. Me-TMPyP and Me-TM(Br_8) (Me = Fe^{3+} and Co^{3+}) also exhibited the O_2^- scavenging activities, because the relative intensities of DMPO-O_2^- signals to those of Mn^{2+} signals were decreased with increasing concentration of these metal porphyrins (data not shown). However, in cases of Me-TMPyP and Me-TM(Br_8) (Me = H_2, Zn^{2+}, Ni^{2+}), no ESR spectral change was observed even if a large excess of these metal porphyrins was added in the reaction system.

In order to compare the O_2^- scavenging activities of Me-TMPyP or Me-TM(Br_8) with each other, their relative intensity ratios of DMPO-O_2^- signals to Mn^{2+} signals were determined at each concentration of metal porphyrins. The intensity of the first peak of DMPO-O_2^-, shown in Figs. 4(A) and 5(A), was selected for evaluating this relative intensity ratio. The relative intensity ratio without addition of meal porphyrins was estimated to be 1.0. In Fig. 6, the relationship between the concentration of each metal porphyrin and the corresponding relative intensity ratio was shown. Fig. 6 shows that the higher was the concentration of each metal porphyrin, the lower was the relative intensity ratio, indicating that all metal porphyrins in this study exhibited the O_2^- scavenging activities except for both Me-TMPyP and Me-TM(Br_8) (Me = H_2, Zn^{2+}, Ni^{2+}). From the results shown in Fig. 6, a concentration of Me-TMPyP or Me-TM(Br_8) required to reduce the relative peak height of DMPO-O_2^- by 50 %, the IC_{50} value, was determined, and was summarized in Table 1.

Table 1 IC_{50} values of Me-TMPyP and Me-TM(Br_8) by ESR spin trapping technique and the Cyt. C method

Me-TMPyP	IC_{50} (μM)		Me-TM(Br_8)	IC_{50} (μM)	
	ESR spin trapping technique	Cyt. C method		ESR spin trapping technique	Cyt. C method
Fe^{3+}-TMPyP	3.6	2.3	Fe^{3+}-TM(Br_8)	3.0	0.13
Mn^{3+}-TMPyP	2.3	12.8	Mn^{2+}-TM(Br_8)	0.9	0.09
Co^{3+}-TMPyP	6.5	0.50	Co^{3+}-TM(Br_8)	4.5	0.38
Cu^{2+}-TMPyP	72.6	28.3	Cu^{2+}-TM(Br_8)	9.0	1.09
Me-TMPyP (Me: H_2, Zn^{2+}, Ni^{2+})	>100	>100	Me-TM(Br_8) (Me: H_2, Zn^{2+}, Ni^{2+})	>100	>100

As shown in Table 1, Mn^{2+}-TM(Br_8), whose IC_{50} value was estimated to be 0.9 μM, exhibited the highest activity among metal porphyrins in this study. The activities were in the order of Mn^{2+}-TM(Br_8) > Fe^{3+}-TM(Br_8) > Co^{3+}-TM(Br_8) > Cu^{2+}-TM(Br_8) for Me-TM(Br_8), whereas Mn^{3+}-TMPyP > Fe^{3+}-TMPyP > Co^{3+}-TMPyP > Cu^{2+}-TMPyP for Me-TMPyP. The activities of Me-TM(Br_8) were higher than those of the corresponding Me-TMPyP, although the activities of Fe^{3+} porphyrins were almost same with each other. As pointed out by Carrier *et al.*, a halogenation of porphyrin-rings or side-chains of metal porphyrins leaded to raise their enzymatic activities.[20-22] Similarlly in this study, the bromonation of Me-TMPyP resulted in raising their O_2^- scavenging activities of the parent metal porphyrin, namely Me-TM(Br_8).

We tried to compare the O_2^- scavenging activities in this study with those of other metal porphyrins. However, no attempt to evaluate by ESR spectrometry has been reported for metal porphyrins including Me-TMPyP and Me-TM(Br_8). Therefore, this study is the first example to evaluate the O_2^- scavenging activities of Me-TMPyP and Me-TM(Br_8) by ESR spin trapping technique. On the other hand, Sakurai *et al.*[18] investigated the O_2^- scavenging activities of polyphenols, which have recently attracted much interest because of their high O_2^- scavenging activities, by ESR spin strapping technique. They evaluated their IC_{50} values of pyrogallol, gallic acid, and quercetin to be 1.45, 1.57, and 2.75 μM, respectively. Compared with these results, the activity of Mn^{2+}-TM(Br_8) may be slightly higher than those of these polyphenols.

Evaluation of O_2^- scavenging effects by the Cyt. C method

As described above, no study by ESR spectrometry has been reported on evaluating the O_2^- scavenging activities of metal porphyrins including Me-TMPyP and Me-TM(Br_8). However, some reports have been published on the O_2^- scavenging activities of metal porphyrins by using the Cyt. C method. For example, Batinic-Haberle *et al.*[11-13] investigated the O_2^- scavenging activities of Me-TMPyP and Me-TM(Br_8), and showed that especially Mn^{2+}-TM(Br_8) exhibited very high activity. Faulkner *et al.*[24] also investigated the O_2^- scavenging activities of Mn^{3+}-TMPyP. However, no attempt by the Cyt. C method has been made to investigate the O_2^- scavenging activities of Me-TMPyP (Me = Co^{3+}, Zn^{2+}, Ni^{2+}) and Me-TM(Br_8) (Me = Fe^{3+}, Co^{3+}, Zn^{2+}, Ni^{2+}).

According to the Cyt. C method in the literatures,[7,19] the IC_{50} value, a concentration of metal porphyrin that causes 50 % of the inhibition of the cytochrome c reduction by O_2^-, was determined. The IC_{50} values obtained for Me-TMPyP and Me-TM(Br_8) were summarized in Table 1. It is obvious that both Me-TMPyP and Me-TM(Br_8) (Me = Fe^{3+}, Mn^{3+}, Co^{3+}, Cu^{2+}) exhibited the O_2^- scavenging activities except for metal-free, Zn^{2+}, and Ni^{2+} porphyrins. Similar to the result by Batinic-Haberle *et al.*,[11-13] Mn^{2+}-TM(Br_8) exhibited the highest activity among all metal porphyrins in this study. The activities were in the order of Mn^{2+}-TM(Br_8) > Fe^{3+}-TM(Br_8) > Co^{3+}-TM(Br_8) > Cu^{2+}-TM(Br_8) for Me-TM(Br_8), whereas Co^{3+}-TMPyP > Fe^{3+}-TMPyP > Mn^{3+}-TMPyP > Cu^{2+}-TMPyP for

Me-TMPyP. Interestingly Co^{3+}-TMPyP exhibited the highest activity among Me-TMPyP in this study, and moreover Co^{3+}-TM(Br_8) also showed relatively high activity. The activities of Me-TM(Br_8) were higher than those of the corresponding Me-TMPyP, although the activities of Co^{3+} porphyrins were almost same with each other.

As shown in Table 1, the IC_{50} values obtained by ESR spin trapping technique were very different from those obtained by the Cyt. C method for the corresponding metal porphyrins. For example, the IC_{50} values of Mn^{2+}-TM(Br_8) and Fe^{3+}-TM(Br_8) obtained by ESR spin trapping technique were almost 10 times and 30 times those by the Cyt. C method, respectively. Previously, Unno *et al.*[23] investigated the O_2^- scavenging activities of tea catechins by ESR spin trapping technique under different concentrations of DMPO. They pointed out that the IC_{50} values of tea catechins depended on a concentration of DMPO in a reaction system, that is, the IC_{50} values of tea catechins were decreased with decreasing concentration of DMPO. Therefore, it may be difficult to compare the IC_{50} values by ESR spin trapping technique with those by other methods, such as the Cyt. C method.

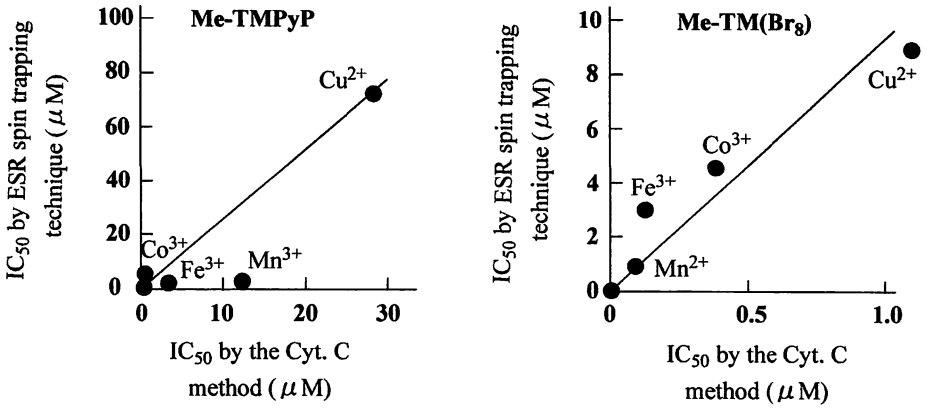

Fig. 7 Relationships between IC_{50} values by ESR spin trapping tecunique and those by the Cyt. C method for Me-TMPyP and Me-TM(Br_8)

The relationship was investigated between the O_2^- scavenging activities by ESR spin trapping technique and those by the Cyt. C method. Fig. 7 shows the correlation between the IC_{50} values obtained by both evaluation methods for Me-TMPyP and Me-TM(Br_8), respectively. Me-TMPyP showed no linear relationship between each IC_{50} value, whereas Me-TM(Br_8) showed relatively good a linear relationship between each IC_{50} value. In case of Me-TMPyP, other oxygen radical species except for O_2^- radical, such as $^{\bullet}OH$ radical, may influence the evaluation system in this study. Therefore, both direct and indirect monitoring may be necessary to obtain accurate and reliable O_2^- scavenging activity.

In this way, ESR spin trapping technique with DMPO showed that Mn^{2+}-TM(Br_8) exhibited the highest O_2^- scavenging activity among all metal porphyrins in this study, and the O_2^- scavenging activities of Me-TM(Br_8) were higher than those of the corresponding Me-TMPyP. The bromonation of Me-TMPyP was effective for raising their O_2^- scavenging activities of the parent metal porphyrins. Me-TM(Br_8) showed relatively a linear relationship between each IC_{50} value estimated by ESR spin trapping technique and by the Cyt. C method.

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