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Electron-transfer mechanism in radical-scavenging reactions by a vitamin E model in a protic medium

Ikuo Nakanishi,^{a,*} Tomonori Kawashima,^{a,c} Kei Ohkubo,^b Hideko Kanazawa,^c Keiko Inami,^d Masataka Mochizuki,^d Kiyoshi Fukuhara,^c Haruhiro Okuda,^c Toshihiko Ozawa,^a Shinobu Itoh,^f Shunichi Fukuzumi^b and Nobuo Ikota^{a*}

^a Redox Regulation Research Group, Research Center for Radiation Safety, National Institute of Radiological Sciences, Inage-ku, Chiba, 263-8555, Japan. E-mail: nakanis@nirs.go.jp; Fax: +81-43-255-6819; Tel: +81-43-206-3131

^b Department of Material and Life Science, Graduate School of Engineering, Osaka University, SORST, Japan Science and Technology Agency (JST), Suita, Osaka, 565-0871, Japan

^c Department of Physical Pharmaceutical Chemistry, Kyoritsu University of Pharmacy, Minato-ku, Tokyo, 105-8512, Japan

^d Division of Organic and Bioorganic Chemistry, Kyoritsu University of Pharmacy, Minato-ku, Tokyo, 105-8512, Japan

^e Division of Organic Chemistry, National Institute of Health Sciences, Setagaya-ku, Tokyo, 158-8501, Japan

^f Department of Chemistry, Graduate School of Science, Osaka City University, Sumiyoshi-ku, Osaka, 558-8585, Japan

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The scavenging reaction of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]) or galvinoxyl radical (GO[•]) by a vitamin E model, 2,2,5,7,8-pentamethylchroman-6-ol (**III**), was significantly accelerated by the presence of Mg(ClO₄)₂ in de-aerated methanol (MeOH). Such an acceleration indicates that the radical-scavenging reaction of **III** in MeOH proceeds *via* an electron transfer from **III** to the radical, followed by a proton transfer, rather than the one-step hydrogen atom transfer which has been observed in acetonitrile (MeCN). A significant negative shift of the one-electron oxidation potential of **III** in MeOH (0.63 V vs. SCE), due to strong solvation as compared to that in MeCN (0.97 V vs. SCE), may result in change of the radical-scavenging mechanisms between protic and aprotic media.

Introduction

Recently, much attention has been paid to the mechanisms of radical-scavenging reactions of phenolic antioxidants, such as vitamin E (*α*-tocopherol) and flavonoids, with regard to the development of chemopreventive agents against oxidative stress and associated diseases. There are two mechanisms for the radical-scavenging reactions of phenolic antioxidants: a one-step hydrogen atom transfer from the phenolic OH group; and an electron transfer followed by a proton transfer.^{1–3} Metal ions are a powerful tool that can be used to distinguish between these two mechanisms, since electron-transfer reactions are known to be significantly accelerated by their presence.⁴ In fact, we have recently reported that scavenging reactions of the galvinoxyl radical (GO[•]) and the cumylperoxyl radical by (+)-catechin in aprotic media, such as acetonitrile (MeCN) and propionitrile, proceed *via* an electron transfer from (+)-catechin to the radicals (which is significantly accelerated by the presence of metal ions, such as Mg²⁺ and Se²⁺) followed by a proton transfer.^{5,6} On the other hand, no effect of Mg²⁺ on the hydrogen-transfer rate from a vitamin E model, 2,2,5,7,8-pentamethylchroman-6-ol (**III**), to 2,2-bis(4-*tert*-octylphenyl)-1-picrylhydrazyl radical (DOPPH[•]) or GO[•] in de-aerated MeCN has been observed, indicating that the radical-scavenging reactions of **III** in MeCN proceed *via* a one-step hydrogen atom transfer rather than *via* electron transfer.^{7,8} However, the effects of solvents on the mechanism of radical-scavenging reactions of phenolic antioxidants have yet to be clarified. Leopoldini *et al.* have reported that the bond dissociation enthalpies for O–H bonds and the adiabatic ionization potentials for phenolic antioxidants, calculated with use of density functional theory, do not follow the same trends in gas, water and benzene.⁹ Thus, it is of considerable importance

to investigate the effects of metal ions on radical-scavenging reactions in various solvents with different polarity.⁹

We report herein that the scavenging reactions of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]) or GO[•] by the vitamin E model **III** in de-aerated methanol (MeOH) proceed *via* an electron transfer mechanism rather than *via* a one-step hydrogen atom transfer, which has been observed in de-aerated MeCN. Effects of bases on the radical-scavenging rates were also examined, to clarify whether the actual electron donor is **III** or the corresponding phenolate anion **I** in MeOH. Different mechanisms in protic and aprotic solvents are discussed based on kinetic, electrochemical, and EPR data obtained in this study, providing valuable and fundamental information about the radical-scavenging mechanism of phenolic antioxidants.

Experimental

Materials

2,2,5,7,8-Pentamethylchroman-6-ol (**III**) was purchased from Wako Pure Chemical Ind. Ltd., Japan. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH[•]) and galvinoxyl radical (GO[•]) were commercially obtained from Aldrich. Tetra-*n*-butylammonium perchlorate (Bu₄NClO₄), used as a supporting electrolyte for the electrochemical measurements, was purchased from Tokyo Chemical Industry Co., Ltd., Japan, recrystallized from ethanol, and dried under vacuum at 313 K. Mg(ClO₄)₂ and methanol (MeOH; spectral grade) were purchased from Nacalai Tesque, Inc., Japan and used as received. Pyridine and 2,6-lutidine were commercially obtained from Wako Pure Chemical Ind. Ltd., Japan and purified by the standard procedure.¹⁰

Spectral and kinetic measurements

Since the phenoxyl radical of **III** (**I'**) generated in the reaction of **III** with radicals readily reacts with molecular oxygen (O_2), reactions were carried out under strictly de-aerated conditions. A continuous flow of Ar gas was bubbled through a MeOH solution (3.0 mL) containing DPPH $^{\cdot}$ ($4.8 \cdot 10^{-5}$ M) and $Mg(ClO_4)_2$ (0.3 M) in a square quartz cuvette (10 mm id) with a glass tube neck for 10 min. Air was prevented from leaking into neck of the cuvette with a rubber septum. Typically, an aliquot of **III** ($2.0 \cdot 10^{-7}$ M), which was also in de-aerated MeOH, was added to the cuvette with a microsyringe. This led to a reaction of **III** with DPPH $^{\cdot}$. UV-vis spectral changes associated with the reaction were monitored using an Agilent 8453 photodiode array spectrophotometer. The rates of the DPPH $^{\cdot}$ -scavenging reactions of **III** were determined by monitoring the absorbance change at 516 nm due to DPPH $^{\cdot}$ ($\epsilon = 1.13 \cdot 10^4$ M $^{-1}$ cm $^{-1}$) using a stopped-flow technique on a UNISOKU $^{\text{TM}}$ RSP-1000-02NM spectrophotometer. The pseudo-first-order rate constants (k_{app}) were determined by a least-squares curve fit using an Apple Macintosh personal computer. The first-order plots of $\ln(A/A_0)$ vs. time (A and A_0 are denoted as the absorbance at the reaction time and the final absorbance, respectively) were linear until three or more half-lives with the correlation coefficient $r > 0.999$. The reaction of **III** with GO $^{\cdot}$ was carried out in the same manner and the rates were determined from the absorbance change at 428 nm due to GO $^{\cdot}$ ($\epsilon = 1.32 \cdot 10^5$ M $^{-1}$ cm $^{-1}$). The rate constants of the reactions in the presence of base (pyridine or 2,6-lutidine) were determined in the same manner.

Electrochemical measurements

The cyclic voltammetry (CV) and second-harmonic alternating current voltammetry (SHACV) 11,16 measurements were performed on an ALS-630A electrochemical analyzer in de-aerated MeOH containing 0.1 M Bu_4NClO_4 as a supporting electrolyte. The Pt working electrode (BAS) was polished with BAS polishing alumina suspension and rinsed with acetone before use. The counter electrode was a platinum wire. The measured potentials were recorded with respect to an $Ag/AgNO_3$ (0.01 M) reference electrode. The E_1 values (vs. $Ag/AgNO_3$) were converted to those vs. SCE by adding 0.29 V. 17 All electrochemical measurements were carried out at 298 K under 1 atm Ar.

EPR measurements

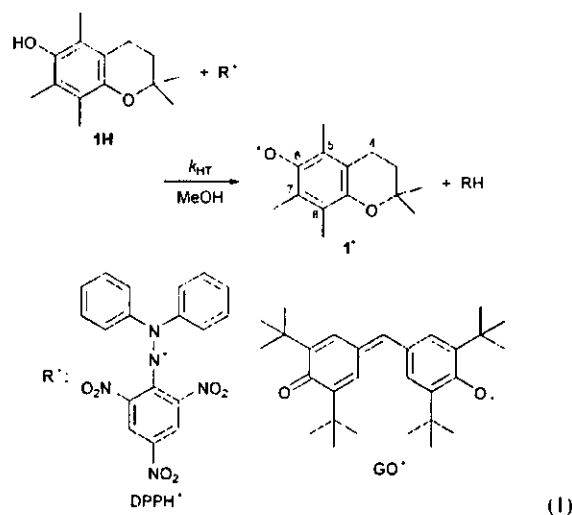
Typically, an aliquot of a stock solution of **III** ($2.0 \cdot 10^{-7}$ M) in de-aerated MeOH was added to the EPR sample tube (0.8 mm id) containing a de-aerated MeOH solution of DPPH $^{\cdot}$ ($2.0 \cdot 10^{-4}$ M) with a microsyringe under 1 atm Ar. EPR spectra of the phenoxyl radical **I'** produced in the reaction between **III** and DPPH $^{\cdot}$ were taken on a JEOL X-band spectrometer (JES-REIXE). The EPR spectra were recorded under non-saturating microwave power conditions. The magnitude of modulation was chosen to optimize the resolution and the signal-to-noise ratio of the observed spectra. The g values and the hyperfine splitting constants were calibrated with a Mn^{2+} marker. Computer simulation of the EPR spectra was carried out using Calleo ESR Version 1.2 program (Calleo Scientific Publisher) on an Apple Macintosh personal computer.

Results and discussion

Radical-scavenging reactions of the vitamin E model in de-aerated MeOH

Upon addition of **III** to a de-aerated MeOH solution of DPPH $^{\cdot}$, the absorption band at 516 nm due to DPPH $^{\cdot}$ disappeared immediately, accompanied by an appearance of the absorption band at 427 nm. Since the absorption band at 427 nm is diagnostic of the phenoxyl radical derived from **III** (**I'**) in MeOH, 18 this spectral change indicates that hydrogen transfer

from the phenolic OH group of **III** to DPPH $^{\cdot}$ takes place to produce **I'** (eqn. (1)). The absorption band of **I'** was shifted from 423 nm in MeCN to 427 nm in MeOH. 18 Such a shift in the absorption band of **I'** may be due to a stronger solvation of **I'** in MeOH than in MeCN.



The rate of the DPPH $^{\cdot}$ -scavenging reaction of **III** was measured by monitoring the decrease in absorbance at 516 nm due to DPPH $^{\cdot}$ using a stopped-flow technique. The decay of the absorbance at 516 nm due to DPPH $^{\cdot}$ obeyed pseudo-first-order kinetics when the concentration of **III** (**[III]**) was maintained at more than a 10-fold excess of the DPPH $^{\cdot}$ concentration. The pseudo-first-order rate constants (k_{app}) increase with increasing **[III]**, exhibiting first-order dependence on **[III]**. From the slope of the linear plot of k_{app} vs. **[III]**, the second-order rate constant (k_{HI}) was determined for the radical-scavenging reaction as $1.07 \cdot 10^3$ M $^{-1}$ s $^{-1}$, in de-aerated MeOH at 298 K. The k_{HI} value thus obtained in de-aerated MeOH is significantly larger than that determined in de-aerated MeCN ($4.35 \cdot 10^2$ M $^{-1}$ s $^{-1}$). 7 A similar result has been reported by Litwinienko and Ingold. 19 Intermolecularly hydrogen-bonded phenolic OH groups of hydrogen-bond accepting solvents, such as alcohols, are known to be essentially unreactive against radicals. 19 Thus, the enhanced k_{HI} value in MeOH suggested that the reaction mechanism in MeOH may be different from that in MeCN. The GO $^{\cdot}$ -scavenging rate constant by **III** in de-aerated MeOH has also been determined in a same manner by monitoring the decrease in absorbance at 428 nm due to GO $^{\cdot}$ as $2.54 \cdot 10^3$ M $^{-1}$ s $^{-1}$, which is slightly smaller than that in de-aerated MeCN ($3.32 \cdot 10^3$ M $^{-1}$ s $^{-1}$).

Effect of magnesium ion on the rates of radical scavenging reactions

If the radical-scavenging reactions of **III** involve an electron-transfer process as the rate-determining step, the rates of radical scavenging would be accelerated by the presence of metal ions. 5,6 This was investigated by examining the effect of $Mg(ClO_4)_2$ on the radical-scavenging rates by **III** in de-aerated MeOH. When $Mg(ClO_4)_2$ is added to the **III** DPPH $^{\cdot}$ system in de-aerated MeOH, the rate of DPPH $^{\cdot}$ -scavenging reaction by **III** was significantly accelerated. Such an acceleration was not observed for the DPPH $^{\cdot}$ -scavenging reaction by **III** in MeCN. 7 The k_{HI} value increases linearly with increasing Mg^{2+} concentration ($[Mg^{2+}]$) as shown in Fig. 1a. A similar acceleration effect of Mg^{2+} has been observed for the GO $^{\cdot}$ -scavenging reaction by **III** in de-aerated MeOH (Fig. 1b). Thus, the radical-scavenging reactions in de-aerated MeOH may proceed *via* an electron transfer from **III** to DPPH $^{\cdot}$ or GO $^{\cdot}$, which is accelerated by the presence of Mg^{2+} , followed by proton transfer from **III'** to DPPH $^{\cdot}$ or GO $^{\cdot}$ as shown in Scheme 1. In such a case,

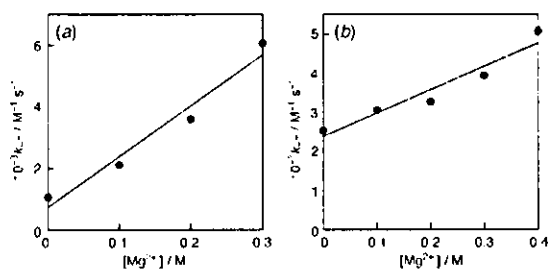
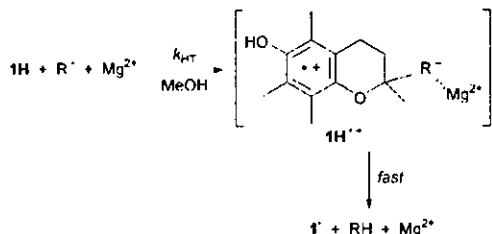


Fig. 1 Plots of k_{H} vs $[\text{Mg}^{2+}]$ in the reaction of **III** with (a) DPPH \cdot and (b) GO in de-aerated MeOH at 298 K.



Scheme 1 Radical-scavenging reaction by **III** via an electron transfer in MeOH

the coordination of Mg^{2+} to DPPH \cdot or GO may stabilize the product, resulting in the acceleration of the electron transfer.

Effect of base on the rates of radical scavenging reactions

In protic media, such as alcohols and water, **III** may be in equilibrium with the corresponding phenolate anion **1** $^-$, which is a much stronger electron donor as compared to the parent **III**.^{2b} In such a case, **1** $^-$ may act as an electron donor rather than the parent **III** in MeOH.

In order to clarify an actual electron donor in MeOH, the effect of base on the radical-scavenging rates of **III** was examined. The addition of pyridine to the **III**-DPPH \cdot system results in a significant increase in the rate of the DPPH \cdot -scavenging reaction by **III**. The k_{H} value increases with increasing pyridine concentration to reach a constant value as shown in Fig. 2. When pyridine is replaced by 2,6-lutidine, a stronger base than pyridine, as shown in Fig. 2. If the rate of acceleration is due to the deprotonation of the phenolic OH group of **III** in the presence of base, the limiting k_{H} value should be the same regardless of the basicity of pyridines. The different limiting k_{H} values between pyridine and 2,6-lutidine in Fig. 2 suggest that little deprotonation occurs to produce **1** $^-$ and that the actual electron donor is the parent **III** rather than **1** $^-$ in MeOH, as shown in Scheme 1. In such a case, the coordination of pyridines

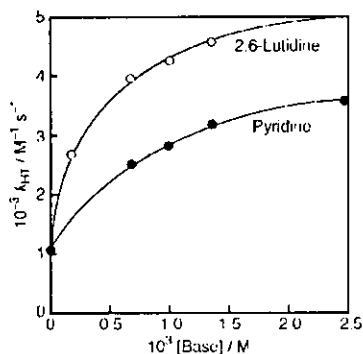


Fig. 2 Plot of k_{H} vs $[\text{base}]$ for the reaction of **III** with DPPH \cdot in the presence of pyridine (black circles) or 2,6-lutidine (white circles) in de-aerated MeOH at 298 K.

to **III** $^\cdot$ may stabilize the product, resulting in the acceleration of the initial electron-transfer process. In the presence of a large amount of a strong Lewis acid, such as $\text{Mg}(\text{ClO}_4)_2$, no deprotonation of **III** occurs in MeOH.

Solvent effect on the one-electron oxidation potential of the vitamin E model

The solvent effect on the one-electron oxidation potential (E_{ox}°) of **III** was examined by cyclic voltammetry (CV) and second-harmonic alternating current voltammetry (SHACV) measurements.^{11–16} Very recently, Williams and Webster have reported that the one-electron oxidation of α -tocopherol itself occurs at about 0.97 V vs. SCE in MeCN (0.25 M Bu_4NPF_6) based on the detailed electrochemical analyses.²¹ A similar cyclic voltammogram was observed for the electrochemical oxidation of **III** in MeCN (0.1 M Bu_4NClO_4) (data not shown), from which was determined the E_{ox}° value (vs. SCE) of **III** in MeCN as 0.97 V. On the other hand, the CV wave of **III** in MeOH (0.1 M Bu_4NClO_4) was irreversible. Thus, SHACV measurement was carried out to determine the E_{ox}° value of **III** in MeOH. The E_{ox}° value (vs. SCE) of **III** in MeOH (0.1 M Bu_4NClO_4), determined from the intersection of an SHACV wave (Fig. 3), is located at 0.63 V, which is significantly more negative than the value in MeCN (0.97 V). Such a negative shift of the E_{ox}° value in MeOH as compared to that in MeCN may be ascribed to a stronger solvation of **III** $^\cdot$ in MeOH than in MeCN. Thus, the ease of one-electron oxidation of **III** in MeOH as compared to in MeCN may result in the difference in the radical-scavenging mechanism.

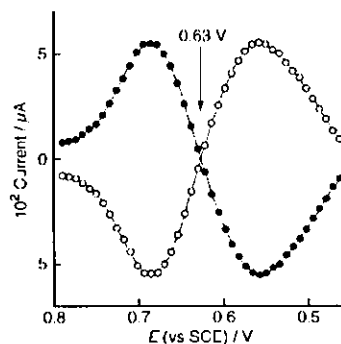


Fig. 3 SHACV of **III** recorded at the scan rate of 4 mV s^{-1} on Pt working electrode in de-aerated MeOH (0.1 M Bu_4NClO_4) at 298 K.

EPR spectrum of the phenoxy radical derived from the vitamin E model in de-aerated MeOH

The EPR detection of radical species derived from **III** would provide valuable information about the solvation of the radical species.^{22,23} The EPR spectrum of **1** $^\cdot$ in de-aerated MeOH at 298 K is shown in Fig. 4a. It should be noted that the g value of the EPR spectrum of **1** $^\cdot$ in MeOH (2.0040) is apparently smaller than that in MeCN (2.0047).⁷ The observed hyperfine structure in Fig. 4a is well reproduced by the computer simulation (Fig. 4b) with four hyperfine splitting constants (hfc) listed in Table 1. Table 1 also shows the hfc values of **1** $^\cdot$ in MeCN.⁷ All the hfc values in MeOH are also smaller than those in MeCN. The smaller g value of the EPR spectrum of **1** $^\cdot$ as well as the smaller hfc values in MeOH than those in MeCN indicates that the stronger solvation of **1** $^\cdot$ may occur in MeOH than in MeCN. Although the EPR spectrum of **III** $^\cdot$ could not be observed because of the fast deprotonation to produce **1** $^\cdot$ (Scheme 1), stronger solvation of **III** $^\cdot$ may also occur in MeOH than in MeCN, resulting in the ease of one-electron oxidation of **III** in MeOH than in MeCN.

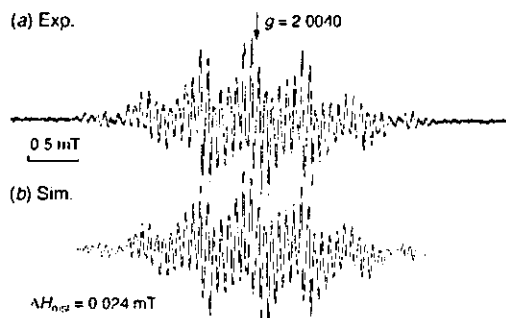


Fig. 4 (a) EPR spectrum of T^{\bullet} generated in the reaction of **III** (1.0×10^{-4} M) with DPPH $^{\bullet}$ (2.0×10^{-3} M) in de-aerated MeOH at 298 K. (b) The computer simulation spectrum. The hfc values used for the simulation are listed in Table 1.

Table 1 Hyperfine splitting constants (hfc ; in mT) and g values of T^{\bullet} in de-aerated solvents

Solvent	g	$a(3H^{\bullet})$	$a(3H^{\bullet})$	$a(3H^{\bullet})$	$a(2H^{\bullet})$
MeOH	2.0040	0.577	0.423	0.073	0.126
MeCN	2.0047 ^a	0.587 ^a	0.440 ^a	0.086 ^a	0.139 ^a

^a Taken from ref. 7.

In conclusion, the scavenging reaction of DPPH $^{\bullet}$ or GO $^{\bullet}$ by **III** in MeOH proceeds *via* the electron transfer from **III** to DPPH $^{\bullet}$ or GO $^{\bullet}$ followed by proton transfer rather than *via* the one-step hydrogen atom transfer, which has been observed in MeCN. Such a difference in the mechanism of radical-scavenging reactions by the vitamin E model depending on the solvents provides valuable information for the biological antioxidative reactions.

Acknowledgements

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EPR Study on Stable Magnesium Complexes of the Phenoxyl Radicals Derived from a Vitamin E Model and Its Deuterated Derivatives

Ikuo Nakanishi,^{*1,2} Shigenobu Matsumoto,³ Kei Ohkubo,² Kiyoshi Fukuhara,⁴ Haruhiro Okuda,⁴ Keiko Inami,⁵ Masataka Mochizuki,⁵ Toshihiko Ozawa,¹ Shinobu Itoh,⁶ Shunichi Fukuzumi,^{*2} and Nobuo Ikota^{*1}

¹Redox Regulation Research Group, Research Center for Radiation Safety, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555

²Department of Material and Life Science, Graduate School of Engineering, Osaka University, CREST, Japan Science and Technology Agency, 2-1 Yamada-oka, Suita, Osaka 565-0871

³Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015

⁴Division of Organic Chemistry, National Institute of Health Sciences, 1-18-1 Setagaya-ku, Tokyo 158-8501

⁵Division of Organic and Bioorganic Chemistry, Kyoritsu College of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512

⁶Department of Chemistry, Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585

Received April 8, 2004; E-mail: nakanis@nirs.go.jp

The phenoxyl radical **1**[•], generated by the reaction of a vitamin E model, 2,2,5,7,8-pentamethylchroman-6-ol (**1H**), with 2,2-bis(4-*tert*-octylphenyl)-1-picrylhydrazyl (DOPPH[•]), was significantly stabilized by complex formation with Mg²⁺ in deaerated acetonitrile at 298 K. The assignments of the hyperfine coupling constants (hfc) obtained by computer simulations of the observed EPR spectrum of the Mg²⁺ complex of **1**[•] (Mg²⁺-**1**[•]), were carried out using three deuterated isotopomers of **1**[•], i.e., 5-CD₃-**1**[•], 7-CD₃-**1**[•], and 8-CD₃-**1**[•], where a methyl group at the C5, C7, or C8 position is replaced by a CD₃ group, respectively. The decreased spin density of the benzene ring in the Mg²⁺-**1**[•] complex indicates that delocalization of the unpaired electron in **1**[•] into Mg²⁺ by complexation between Mg²⁺ and **1**[•] results in the enhanced stability of **1**[•] in the presence of Mg²⁺.

Most biological antioxidants, such as vitamin E (α -tocopherol) and flavonoids, have one or more phenolic hydroxy groups, and are converted into phenoxyl radical intermediates as the result of antioxidative radical-scavenging reactions with active oxygen radicals, such as hydroxyl radical ($\cdot\text{OH}$), superoxide anion ($\text{O}_2^{\cdot-}$), and lipid peroxy radical ($\text{LOO}\cdot$).¹⁻⁴ Thus, it is of great importance to detect and characterize the phenoxyl radicals of such antioxidants in order to shed light on the mechanism of the antioxidative radical-scavenging reactions in biological systems as well as to develop novel antioxidants with more effective antioxidative activities than the natural occurring ones. However, the phenoxyl radical of α -tocopherol is known to be unstable, because of disproportionation, even in the absence of molecular oxygen (O_2).⁵ Furthermore, in the presence of O_2 , a radical coupling between the phenoxyl radical derived from vitamin E and O_2 is known to produce a wide variety of oxidation products of the phenoxyl radical.⁶⁻²³ On the other hand, we have recently reported that the phenoxyl radical **1**[•] of a vitamin E model, 2,2,5,7,8-pentamethylchroman-6-ol (**1H**), generated by hydrogen transfer from **1H** to 2,2-bis(4-*tert*-octylphenyl)-1-picrylhydrazyl radical (DOPPH[•]) or galvinoxyl radical ($\text{G}\cdot$), is significantly stabilized by the presence of Mg²⁺ via the complexation of **1**[•] with

Mg²⁺.²⁴ The well-resolved 14 lines were observed in the EPR spectrum of the Mg²⁺ complex of **1**[•] (Mg²⁺-**1**[•]).²⁴ However, the hyperfine structure of the observed EPR spectrum of the Mg²⁺-**1**[•] complex has yet to be sufficiently characterized, since there are three methyl groups in the **1**[•] molecule, each of which gives a quartet hyperfine coupling structure. On the other hand, it is known that deuterium substitution at appropriate known sites in the molecule permits an experimental verification of the assignment of the hyperfine coupling constants (hfc) for the EPR spectrum of the observed radical species.²⁵⁻²⁹

Here, we report on an experimental assignment of the hfc values of the EPR spectrum of the Mg²⁺ complex of **1**[•] using three deuterated isotopomers of **1**[•], i.e., 5-CD₃-**1**[•], 7-CD₃-**1**[•], and 8-CD₃-**1**[•], where a methyl group at the C5, C7, or C8 position is replaced by a CD₃ group, respectively (Chart 1). A comparison of the hfc values of the Mg²⁺-**1**[•] complexes obtained in this study with those of the parent **1**[•] provides fundamental information about the spin distribution and stabilization of the phenoxyl radical species of phenolic antioxidants in the presence of metal ions, as well as mechanistic insight into the antioxidative radical-scavenging reactions of phenolic antioxidants.

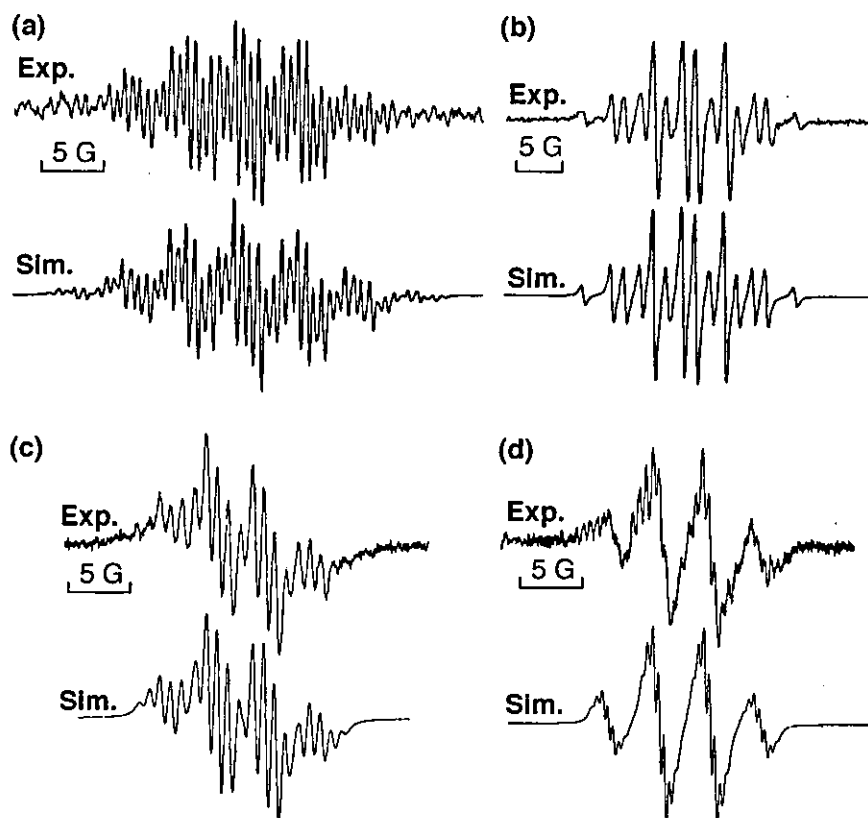


Fig. 1. EPR spectra of (a) 1^\bullet , (b) $\text{Mg}^{2+}-1^\bullet$, (c) $\text{Mg}^{2+}-5\text{-CD}_3-1^\bullet$, and (d) $\text{Mg}^{2+}-7\text{-CD}_3-1^\bullet$ in deaerated MeCN at 298 K with the corresponding computer simulation spectra. The hfc values used for the simulation are listed in Table 1.

Table 1. Hyperfine Splitting Constants (hfc) (in mT) of 1^\bullet and the Mg^{2+} Complexes of 1^\bullet and Its Deuterated Radicals in Deaerated MeCN

Radical	$a(3\text{H}^5)$	$a(3\text{H}^7)$	$a(3\text{H}^8)$	$a(2\text{H}^4)$
1^\bullet	0.587	0.440	0.086	0.139
$\text{Mg}^{2+}-1^\bullet$	0.486	0.335	— ^{a)}	— ^{a)}
$\text{Mg}^{2+}-5\text{-CD}_3-1^\bullet$	0.075 ^{b)}	0.335	— ^{a)}	— ^{a)}
$\text{Mg}^{2+}-7\text{-CD}_3-1^\bullet$	0.486	0.052 ^{b)}	— ^{a)}	— ^{a)}
$\text{Mg}^{2+}-8\text{-CD}_3-1^\bullet$	0.486	0.335	— ^{a)}	— ^{a)}

a) Too small to be determined. b) Deuterium splitting value.

trum in Fig. 1(b) to that in Fig. 1(c) for the $\text{Mg}^{2+}-5\text{-CD}_3-1^\bullet$ complex generated in the same way. The hfc value of 0.486 mT of the $\text{Mg}^{2+}-1^\bullet$ complex is decreased by the factor of the magnetogyric ratio of proton to deuterium (0.153) to 0.075 mT due to the CD_3 deuterons at the C-5 position of the $\text{Mg}^{2+}-5\text{-CD}_3-1^\bullet$ complex, while the other hfc value (0.335 mT) remains identical, as shown in Fig. 1(c) and Table 1. From such a decrease in the hfc value at the C-5 position by the deuterium substitution, was assigned the hfc value at the C-5 position of the $\text{Mg}^{2+}-1^\bullet$ complex as 0.486 mT.

A change in the splitting pattern was also observed upon deuterium substitution of the methyl group at the C-7 position of the $\text{Mg}^{2+}-1^\bullet$ complex, as shown in Fig. 1(d). The computer simulation spectrum using the same hfc value, except for the deuterium at the C-7 position, which are reduced by a factor of 0.153, agrees well with the observed EPR spectrum of the $\text{Mg}^{2+}-7\text{-CD}_3-1^\bullet$ complex [Fig. 1(d)]. On the other hand, a

deuterium substitution of the methyl protons at the C-8 position of $\text{Mg}^{2+}-1^\bullet$ resulted in no change in the splitting pattern in the EPR spectrum of the $\text{Mg}^{2+}-8\text{-CD}_3-1^\bullet$ complex, as compared to the $\text{Mg}^{2+}-1^\bullet$ complex (data not shown). From the above results, the hfc values of the $\text{Mg}^{2+}-1^\bullet$ complex were assigned as listed in Table 1. The hfc values due to the methyl protons at the C-5 (0.587 mT) and C-7 (0.440 mT) positions are significantly decreased by complexation with Mg^{2+} (0.486 and 0.335 mT, respectively). No hyperfine structure was observed due to the methylene protons at the C-4 position, as well as the methyl protons at the C-8 protons in the $\text{Mg}^{2+}-1^\bullet$ complex. It is clearly shown that the spin densities on the aromatic ring in the $\text{Mg}^{2+}-1^\bullet$ complex are significantly decreased by the coordination of Mg^{2+} . Thus, an unpaired electron in 1^\bullet is significantly delocalized into Mg^{2+} by complexation between 1^\bullet and Mg^{2+} via the phenolic O atom.

In conclusion, the deuterium substitutions of the methyl group in 1H enabled us to assign the hfc values of 1^\bullet in the presence of Mg^{2+} experimentally. The decreased spin densities on the benzene ring in 1^\bullet , the smaller g value of the EPR spectrum of 1^\bullet , and the red shift of the absorption bands of 1^\bullet in the presence of Mg^{2+} indicate that Mg^{2+} coordinates to the phenoxyl radical 1^\bullet via the phenolic O atom of 1^\bullet . Such complex formation between 1^\bullet and Mg^{2+} precludes the disproportionation of 1^\bullet , leading to the enhanced stability of 1^\bullet . We are in the process of further exploring the effect of metal ions on the stability of phenoxyl radicals derived from phenolic antioxidants with a catechol moiety, such as (+)-catechin and quercetin.

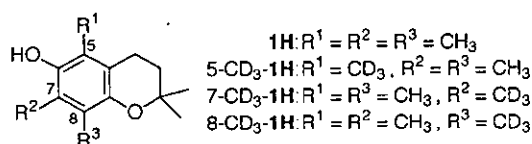


Chart 1. Vitamin E models.

Experimental

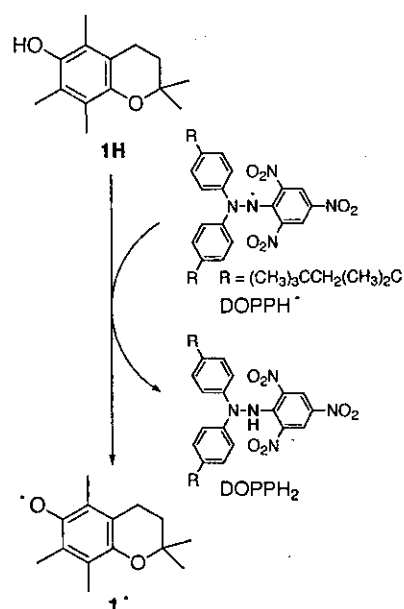
Materials. 2,2,5,7,8-Pentamethylchroman-6-ol (**1H**) was purchased from Wako Pure Chemical Ind. Ltd., Japan. 2,2-Bis(4-*tert*-octylphenyl)-1-picrylhydrazyl radical (DOPPH[•]) was obtained commercially from Aldrich. Mg(ClO₄)₂ and acetonitrile (MeCN; spectral grade) were purchased from Nacalai Tesque, Inc., Japan, and used as received. Three deuterated isotopomers (>98% isotopic purity) of **1H**, 5-CD₃-**1H**, 7-CD₃-**1H**, and 8-CD₃-**1H**, were synthesized according to the literature procedures.³⁰

Spectral Measurements. A continuous flow of Ar gas was bubbled through a MeCN solution (3.0 mL) containing DOPPH[•] (1.4×10^{-5} M) ($1 \text{ M} = 1 \text{ mol dm}^{-3}$) in a square quartz cuvette (10 mm i.d.) with a glass tube neck for 10 min. The neck of the cuvette was sealed to ensure that air would not leak into the cuvette by using a rubber septum. A microsyringe was used to inject **1H** (2.0×10^{-2} M), which was also deaerated, into the cuvette. This led to a hydrogen-transfer reaction from **1H** to DOPPH[•]. UV-vis spectral changes associated with this reaction were monitored using an Agilent 8453 photodiode array spectrophotometer.

EPR Measurements. Typically, an aliquot of a stock solution of **1H** (1.0×10^{-3} M) was added to LABOTEC LLC-04B EPR sample tube containing a deaerated MeCN solution of DOPPH[•] (1.0×10^{-3} M) in the presence or absence of 0.1 M Mg(ClO₄)₂ under an atmospheric pressure of Ar. The EPR spectra of the phenoxyl radical **1•** produced in the reaction between **1H** and DOPPH[•] were taken on a JEOL X-band spectrometer (JES-RE1-XE). The EPR spectra were recorded under nonsaturating microwave power conditions. The magnitude of modulation was chosen so as to optimize the resolution and the signal-to-noise (S/N) ratio of the observed spectra. The *g* values and the hyperfine splitting constants were calibrated with a Mn²⁺ marker. A computer simulation of the EPR spectra was carried out using the Calleo ESR Version 1.2 program (Calleo Scientific Publisher) on an Apple Macintosh personal computer.

Results and Discussion

Upon the addition of vitamin E model **1H** to an acetonitrile (MeCN) solution of DOPPH[•], the absorption band due to DOPPH[•] ($\lambda_{\text{max}} = 543 \text{ nm}$) decreased, accompanied by an increase in the absorption bands at 402 and 423 nm due to the phenoxyl radical **1•** with clear isosbestic points at 343, 374, and 437 nm. The absorption bands around 400 nm are typical for phenoxyl radical species of α -tocopherol.^{5,31} Thus, this spectral change is ascribed to a hydrogen transfer from **1H** to DOPPH[•] to produce **1•** and hydrogenated DOPPH[•] (DOPPH₂) (Scheme 1). In fact, the characteristic EPR spectrum due to **1•** having a *g* value of 2.0047 was observed in the reaction of **1H** with DOPPH[•] in deaerated MeCN at 298 K, as shown in Fig. 1(a), although the observed EPR signal gradually decreased because of the disproportionation of **1•**, even in the absence of O₂.⁵ The hyperfine coupling constants (hfc) of the observed EPR spectrum of **1•** were determined by a comparison of the observed spectrum with the comput-

Scheme 1. Hydrogen transfer from **1H** to DOPPH[•] to produce **1•**.

er-simulated spectrum, as shown in Fig. 1(a); the thus-obtained hfc values were assigned as listed in Table 1.^{24,32,33}

On the other hand, in the presence of Mg(ClO₄)₂ (0.1 M), the absorption bands due to **1•** were shifted from 402 and 423 nm to 412 and 437 nm, respectively.²⁴ Such a red shift of the absorption bands indicates a complex formation between Mg²⁺ and **1•**. The EPR spectrum of the Mg²⁺-**1•** complex was observed at *g* = 2.0040 [Fig. 1(b)], which is appreciably smaller than the *g* value of **1•** (2.0047), indicating that the spin density on oxygen nuclei in **1•** in the presence of Mg²⁺ is decreased by complexation with Mg²⁺.³⁴ It should be noted that no decay of the EPR signal was observed, significantly demonstrating the enhanced stability of the phenoxyl radical species in the presence of Mg(ClO₄)₂.²⁴ This behavior is similar to that found for the α -tocopheroxyl radical in the presence of a fluorinated alcohol, which acts as a hydrogen-bond donor, by Lucarini et al.³⁵ The hyperfine structure can be reproduced by a computer simulation with the hyperfine coupling constants (hfc) of only two sets of methyl protons (0.486 and 0.335 mT), as shown in Fig. 1(b). However, the hfc values of the methylene protons and the remaining methyl protons become undetectably small. Since there are three methyl groups in the **1•** molecule, the assignment of these two hfc values due to two sets of methyl protons is quite complex.

In order to assign these hfc values obtained for the Mg²⁺-**1•** complex, we synthesized three deuterated isotopomers of **1•**, i.e., 5-CD₃-**1•**, 7-CD₃-**1•**, and 8-CD₃-**1•**, where the methyl group at the C-5, C-7, or C-8 position is replaced by the CD₃ group, respectively (Chart 1),³⁰ since deuterium substitution at appropriate known sites in the molecule permits an experimental verification of the assignment of the observed radical species (*vide supra*). A single deuterium gives a triplet (instead of a doublet) hyperfine pattern and the deuterium splitting should decrease due to the magnetogyric ratio of a proton to a deuterium (0.153).²⁵⁻²⁹ In fact, deuterium substitution of the methyl group at the C-5 position of the Mg²⁺-**1•** complex resulted in a drastic change in the splitting pattern from the spec-

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Correlation of sister chromatid exchange formation through homologous recombination with ribonucleotide reductase inhibition

Atsuko Matsuoka^{a,*}, Cecilia Lundin^b, Fredrik Johansson^b, Margareta Sahlin^c, Kiyoshi Fukuhara^d, Britt-Marie Sjöberg^c, Dag Jensen^b, Agneta Önfelt^b

^a Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

^b Department of Genetic and Cellular Toxicology, Stockholm University, S-106 91 Stockholm, Sweden

^c Department of Molecular Biology and Functional Genomics, Stockholm University, S-106 91 Stockholm, Sweden

^d Division of Organic Chemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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Abstract

We conducted the recombination and sister chromatid exchange (SCE) assays with five chemicals (hydroxyurea (HU), resveratrol, 4-hydroxy-*trans*-stilbene, 3-hydroxy-*trans*-stilbene, and mitomycin C) in Chinese hamster cell line SPD8 V79 to confirm directly that SCE is a result of homologous recombination (HR). SPD8 has a partial duplication in exon 7 of the endogenous *hprt* gene and can revert to wild type by homologous recombination. All chemicals were positive in both assays except for 3-hydroxy-*trans*-stilbene, which was negative in both. HU, resveratrol, and 4-hydroxy-*trans*-stilbene were scavengers of the tyrosyl free radical of the R2 subunit of mammalian ribonucleotide reductase. Tyrosyl free radical scavengers disturb normal DNA replication, causing replication fork arrest. Mitomycin C is a DNA cross-linking agent that also causes replication fork arrest. The present study suggests that replication fork arrest, which is similar to the early phases of HR, leads to a high frequency of recombination, resulting in SCEs. The findings show that SCE may be mediated by HR.

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Keywords: Sister chromatid exchange; Homologous recombination; Hypoxanthine-guanine phosphoribosyltransferase; Ribonucleotide reductase

1. Introduction

Sister chromatid exchanges (SCEs) are induced during DNA replication [1,2], and evidence suggests that they are formed during homologous recombination (HR) [2–4]. Sonoda et al. [3] showed that spontaneous

and mitomycin C-induced SCE frequencies were reduced in chicken DT40 B cells lacking the key HR genes *RAD51* and *RAD54*, but not in *KU70*^{-/-} cells defective in non-homologous DNA end joining. When a human *RAD51* gene was inserted, HR activity was restored and SCE levels returned to normal [3]. Other experiments have also suggested that SCE formation is mediated by HR [5–8].

Resveratrol and some of its synthesized derivatives are potent inducers of SCEs in Chinese hamster lung

* Corresponding author. Tel.: +81-3-3700-9264.

fax: +81-3-3707-6950.

E-mail address: matsuoka@nihs.go.jp (A. Matsuoka).

fibroblast (CIII) cells [9,10]. The active moiety is the 4'-hydroxy group [10]. Resveratrol, like hydroxyurea (HU), inhibits ribonucleotide reductase (RNR), which is essential for formation of deoxyribonucleotides and, therefore, DNA synthesis [11,12]. It appears to act by replication fork arrest in SCE formation [2] and in the SP108 V79 cell line, HR [13], which carries a duplication in the *hprt* locus that renders it insensitive to thioguanine [14]. Following HR and loss of the duplication, the cells regain wild-type sensitivity to thioguanine [15,16].

In this work, we investigated resveratrol and its derivatives in an attempt to learn the mechanisms by which they induce cytogenetic effects. We applied resveratrol and derivatives that had or did not have a 4'-hydroxy group, and compared their effects on (1) tyrosyl radical decay in the purified R2 subunit of mammalian RNR and (2) SCE and HR in the SP108 cell line. The chemicals we used in the biological tests were HU, a selective inhibitor of RNR, and mitomycin C, an efficient inducer of SCEs. However, while HU stimulated HR, mitomycin C appeared to be much less efficient in this respect despite its pronounced effect on SCE. The results have clear implications for the links between RNR inhibition, SCE formation, and HR, and the role of the 4'-hydroxy group.

2. Materials and methods

2.1. Cells

The SP108 cell line was maintained in Dulbecco's modified eagle medium (GIBCO 11885-084) supplemented with 9% fetal bovine serum and penicillin-streptomycin (90 units/ml) (DMEM). 6-Thioguanine (5 µg/ml) was added to minimize the frequency of spontaneous reversion prior to treatment. The doubling time was around 12 h and the modal chromosome number was 22. SP108 cells carry a duplication of exon 7 of the *hprt* gene and can be reverted by an exchange of Rad51-supported HR [16].

2.2. Chemicals

Resveratrol (CAS no. 501-36-0) purchased from Sigma Chemical Co. (St. Louis, MO, USA) and 3-hy-

droxy-*trans*-stilbene (17861-18-6) and 4-hydroxy-*trans*-stilbene (6554-98-9) synthesized as previously reported [17] were suspended homogeneously in physiological saline for the assays. Mitomycin C (MMC, 50-07-7) purchased from Kyowa Hakko Kogyo Co. Ltd. (Tokyo) was dissolved in distilled water and the solution was diluted with physiological saline. Hydroxyurea (HU, 127-07-1) purchased from Sigma was dissolved in physiological saline just before use. 6-Thioguanine (Sigma), hypoxanthine (Wako Pure Chemical Industries, Ltd. Osaka, Japan), and thymidine (Sigma) were first dissolved in as little 5 M sodium hydroxide as possible, then diluted with physiological saline and stored at -20 °C until use. 1-Azaserine (Sigma) was dissolved in physiological saline and stored at -20 °C until use.

2.3. Recombination assay

Cells were seeded at a density of 1.0×10^6 /20 ml DMEM/75 cm² flask, cultured for 4 h, and treated with a chemical for 20 h. Cells were rinsed, 20 ml DMEM was added, and the cells were allowed to recover for 30 h. We selected revertants by plating three dishes (3×10^5 cell/dish, 100 mm in diameter) in each treatment group and culturing them in the presence of HAT (50 µM hypoxanthine, 10 µM 1-azaserine, and 5 µM thymidine). Five hundred cells per dish were plated in duplicate for determination of the cloning efficiency. Seven days later, the colonies on the cloning plates were fixed with methanol and stained with Giemsa. The cells on the selection plates were also grown for 8 days, fixed, and stained. The reversion frequency was calculated as the total number of revertants on the selection plate divided by the total number of cells cloned at the same dose. The experiments were performed at least twice and representative data are shown.

2.4. Sister chromatid exchange assay

Cells were seeded at a density of 1.5×10^5 per plate (60 mm in diameter) and incubated for 17 h. The test chemical, then 5-bromodeoxyuridine (5 µM), was added, and the plate was incubated for 24 or 48 h. Chromosome preparations were made as follows: colcemid (final concentration 0.2 µg/ml) was added

to the culture; 2 h later, the cells were trypsinized, incubated in hypotonic solution for 20 min at room temperature, and fixed three times with ice-cold fixative (glacial acetic acid: methanol, 1:3). A drop of the fixed cell suspension was placed on a clean glass slide and air-dried. A fluorescence-plus-Giemsa technique [18] was used for sister chromatid differentiation staining as previously reported [19]. SCEs were scored with the aid of a microscope in 25 second metaphase (M2) cells having 22 chromosomes at 600 \times magnification. Centromeric SCEs were indistinguishable from centric twists and were not scored. Solvent-treated cells served as the negative control. We analyzed SCE data using the Mann-Whitney *U*-test (the normal, two-tailed version).

2.5. Expression and purification of protein R2 from mouse ribonucleotide reductase

Protein R2 from mouse RNR was prepared from overexpressing BL21(DE3)pLX's cells containing the native pETR2 plasmid [20]. The purified protein, which was essentially apoprotein, was reconstituted to form the iron-tyrosyl radical center.

2.6. Experimental setup for kinetics of tyrosyl decay with test chemicals

The R2 apoprotein, ca. 7 μ M in 50 mM aerobic Tris, pH 7.6, was reactivated by the addition of anaerobic 7 mM ferrous ammonium sulfate-5 mM ascorbic acid, in 50 mM Tris, pH 7.6, to the cuvette to give ca. 22 μ M iron. The recorded reconstituted spectrum constituted the start spectrum for incubation with the different chemicals. The iron radical center of R2 has absorption bands in the 300–430 nm region. The tyrosyl radical has an absorption maximum at 416 nm. We followed the decay of the tyrosyl radical upon addition of a test chemical by observing the decay at 416 nm. The spectra were recorded on a Perkin-Elmer lambda 2 spectrophotometer, either with consecutive scans or as kinetic traces at 416 nm. The protein R2 concentration was typically 7 μ M and we used equimolar concentrations of the chemicals. Because of the insolubility of the test chemicals in water, we dissolved them in acetone (final concentration 1%) for the spectroscopic measurements.

3. Results

3.1. Cytogenetic and recombination assays

The spontaneous reversion frequency in the recombination assay and baseline SCE frequency were $1.72 \pm 0.54 \cdot 10^{-5}$ cells ($n = 36$) and 6.4 ± 3.0 /cell ($n = 200$), respectively. Fig. 1 shows the SCE frequency at various treatment times with the higher ratio of M2 to total cells presented.

HU, the positive control for the recombination assay, induced revertants in a concentration-dependent manner up to 0.2 mM. HU induced a statistically significant increase in SCE frequency with a concentration-response relationship. SCEs were observed at 0–0.05 mM in 24-h treatment and at 0.1 mM in 48-h treatment.

Resveratrol and 4-hydroxy-*trans*-stilbene were positive in both assays with clear concentration-response relationships. 3-Hydroxy-*trans*-stilbene was negative in both assays and induced no delay in the cell cycle.

HU, resveratrol, and 4-hydroxy-*trans*-stilbene showed humped curves in the recombination assay and an increase in reversion frequency at concentrations that induced SCEs. MMC, the positive control for SCE induction, induced SCEs and revertants concentration-dependently, but at a higher concentration range for the latter (Fig. 1).

While MMC showed the peak reversion frequency at concentrations that induced severe toxicity, the other positive chemicals showed it at concentrations where the cloning efficiency was around 50% (Table 1). HU, resveratrol, and 4-hydroxy-*trans*-stilbene caused cell cycle delay, and peak SCE frequencies were seen at 48 h. At higher concentrations than those shown in Table 1, no metaphase cells were seen even at 48 h.

3.2. Tyrosyl radical decay in R2 of RNR

Decay of the tyrosyl radical of the *Escherichia coli* R2 unit of RNR was unaffected by resveratrol (data not shown). The decay of tyrosyl radical of R2 from mouse during incubation with 7.2 μ M with each of the test chemicals is shown in Fig. 2. Decay was fastest with 4-hydroxy-*trans*-stilbene (estimated half life ($t_{1/2}$), ca. 57 s). For resveratrol, a small increase in absorption at 416 nm was observed at the beginning of the incubation time, and after the

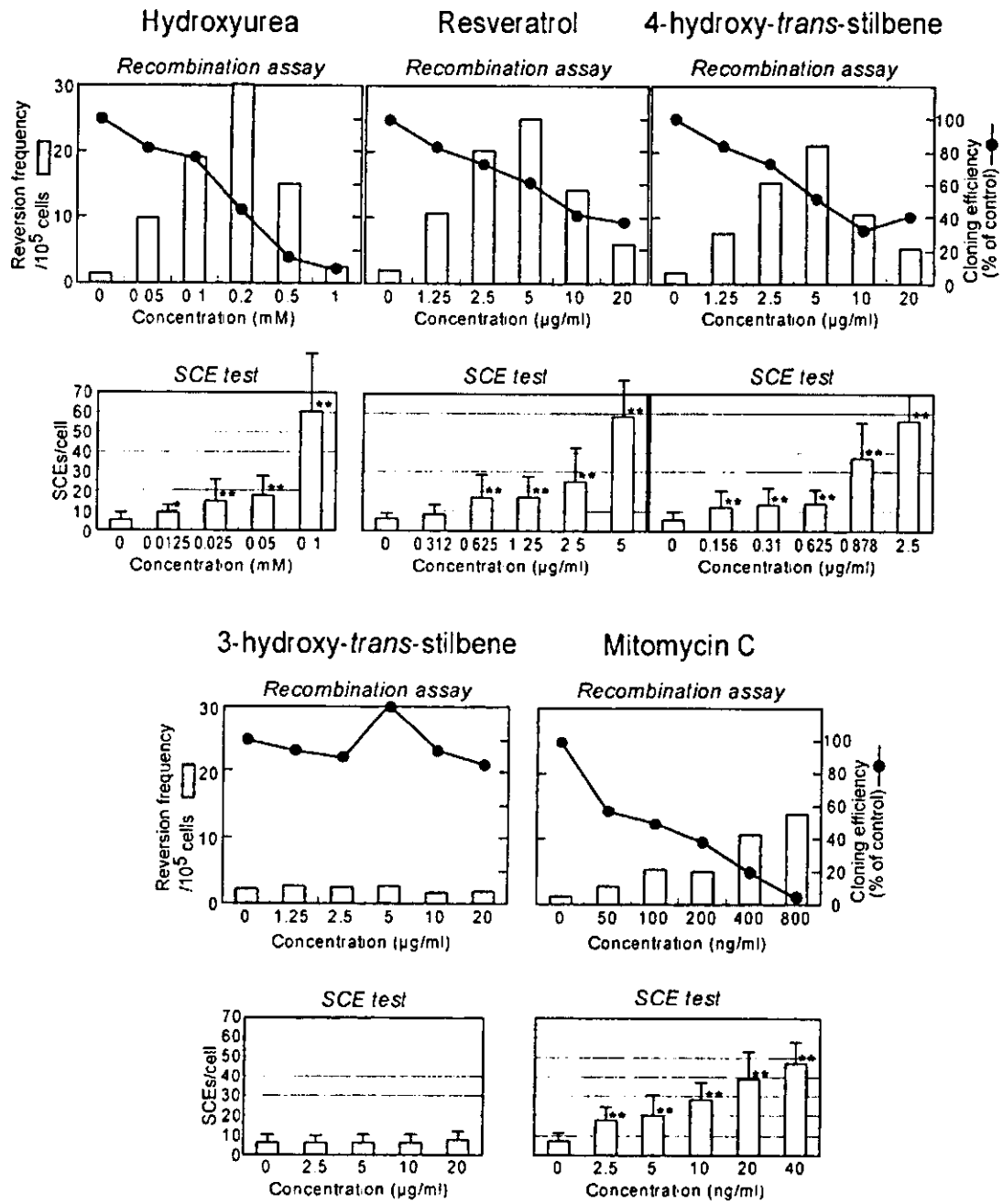


Fig. 1. Top: results of the recombination assay. The reversion frequency was calculated as the total number of revertants on the selection plate divided by the cloning efficiency at the same treatment concentration. Bottom: results of the SCE test in M2 cells ($n = 25$). Bars indicate S.D. * $P < 0.001$. ** $P < 0.0001$.

Table 1
Toxicity of test chemicals at peak response in the recombination assay and the SCE test

	Recombination assay				SCE test			
	Concentration	Cloning efficiency (% of control)	Reversion frequency/ 10^5 cells	Ratio to ^a controls	Concentration	Treatment time (h)	SCEs/cell	Ratio to ^a controls
Hydroxyurea	0.2 mM	43	30.2	19.4	0.1 mM	48	60.9 ± 28.8	10.7
Resveratrol	5 μg/ml	60	25.1	11.8	5 μg/ml	48	58.7 ± 17.7	8.9
4-hydroxy- <i>trans</i> -stilbene	5 μg/ml	51	21.1	12.3	2.5 μg/ml	48	56.2 ± 13.1	9.6
Mitomycin C	400 ng/ml	19	10.7	8.3	40 ng/ml	24	47.5 ± 10.4	6.8
	800 ng/ml	3	13.9	10.8				

^a Ratio to controls indicates the ratio of the reversion frequency or SCEs/cell to that of concurrent controls.

delay, the $t_{1/2}$ was ca. 67 s. At the lower concentrations of 4-hydroxy-*trans*-stilbene, we observed a similar increase in absorption prior to the onset of decay (data not shown). The increases may have been caused by the formation of a phenoxy-like radical in resveratrol and 4-hydroxy-*trans*-stilbene. Strikingly, 3-hydroxy-*trans*-stilbene did not cause decay of the tyrosyl radical.

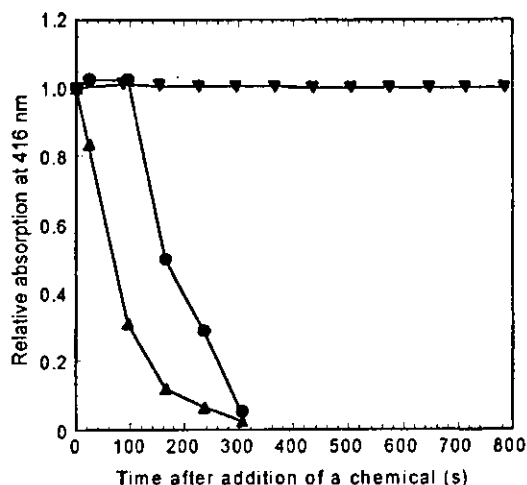


Fig. 2. Decay of tyrosyl radical of R2 protein of mouse ribonucleotide reductase. Solutions of R2 (7 μM) in 50 mM Tris buffer (pH 7.6) were incubated with 7.2 μM of resveratrol (●), 4-hydroxy-*trans*-stilbene (▲), or 3-hydroxy-*trans*-stilbene (▼). Spectra were recorded between 300 and 500 nm. Relative absorption at 416 nm (tyrosyl Abs_{max}) was calculated as relative absorption after subtraction of endpoint spectrum. Time 0 was set to 1.0 relative absorption. For 3-hydroxy-*trans*-stilbene no subtraction was performed since the tyrosyl radical did not decay.

4. Discussion

Micronucleus induction, SCE, mutation, and somatic recombination have been used to detect DNA damage and repair in genotoxic studies. These endpoints are usually applied independently, and they reflect different aspects of the repair processes. In the present study, we investigated the relationship between RNR-inhibition, SCE, and IIR after exposure to five chemicals with different mode of action.

One of these, HU, scavenges the tyrosyl free radical in the R2 subunit of RNR, causing depletion of nucleotides [21,22] resulting in replication fork arrest [23]. The molecular conformation at the replication fork is similar to that at the recombination initiation site. That is, two DNA double strands of similar sequence align and a nick is formed in one of the strands. We suggest that the conformation may favor recombination, resulting in a high frequency of SCEs and IIR.

Others have reported that resveratrol scavenges tyrosyl radicals of the R2 subunits from mice and *Arabidopsis thaliana* [12]. Here, we confirmed that observation in mice and showed that its analogue 4-hydroxy-*trans*-stilbene did the same while 3-hydroxy-*trans*-stilbene did not. HU, resveratrol, and 4-hydroxy-*trans*-stilbene caused IIR and SCEs, while 3-hydroxy-*trans*-stilbene had no effect on either process. Thus, all these compounds should similarly induce RNR inhibition, SCE induction, and IIR. Yet, the relationship between SCE and IIR was complex. MMC, for example, which efficiently

induced SCEs was a poor inducer of HR via loss of the partial duplication. The response to very high and strongly cytotoxic concentrations suggests either a random effect coupled to survival, or another process facilitating HR. In any case, it appears that DNA damage leads into a pathway that stimulates SCE formation, but in some way prohibits the HR that leads to loss of the partial duplication and reversion to wild type. It remains to be investigated if this is coupled to less flexible conformations elicited by repair processes, possibly impairing the type of recombination required for reversion to wild type.

Using cytostatic drugs with different mode of action, Arnaudeau et al. [13] reported that inhibition of DNA synthesis induces HR in SPD8 cells. They used recombination indices (RIs) as an indicator of recombination potency. RI represents the ratio between the reversion frequency observed at a concentration associated with 50% cloning efficiency and the control reversion frequency. RIs of HU, resveratrol, 4-hydroxy-*trans*-stilbene, and MMC in the present study were 18, 9, 12, and 4, respectively. The RI of HU and MMC reported by Arnaudeau et al. [13] were close to ours, at 20 and 4, respectively. The findings suggest that recombination potency is related to the mechanism of action, as Arnaudeau et al. [13] noted.

At a concentration range where no metaphases were observed in the SCE test, reversion frequency of HU, resveratrol, 4-hydroxy-*trans*-stilbene decreased, indicating saturation of either the recombination machinery or the initiating event as shown in Fig. 1. HU induced structural chromosome aberrations in 10–30% of the cells at 0.1 and 0.2 mM, and resveratrol in 9–50% of the cells at 5 and 10 µg/ml (SPD8 cells, data not shown). Thus, at higher concentrations, repair may not catch up with the increasing amount of DNA damage; many nicks may remain unrepaired, leading to chromosome aberrations in metaphase cells that escaped from S phase arrest.

In conclusion, all the chemicals induced both HR and SCE except for 3-hydroxy-*trans*-stilbene, which induced neither. We suggest that the observed correlation between tyrosyl radical decay in protein R2 of RNR and the frequency of HR and SCE support the view that SCE may be formed through HR during replication fork arrest.

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Water-Accelerated Radical-Scavenging Reaction of (+)-Catechin in an Aprotic Medium

Ikuo Nakanishi,^{1,2,*} Tomonori Kawashima,^{1,3} Kiyoshi Fukuhara,⁴ Hideko Kanazawa,³ Haruhiro Okuda,⁴ Shunichi Fukuzumi,^{2,*} Toshihiko Ozawa,¹ and Nobuo Ikota^{1,*}

¹Redox Regulation Research Group, Research Center for Radiation Safety, National Institute of Radiological Sciences, Inage-ku, Chiba 263-8555, Japan

²Department of Material and Life Science, Graduate School of Engineering, Osaka University, CREST, Japan Science and Technology Agency (JST), Suita, Osaka 565-0871, Japan

³Department of Physical Pharmaceutical Chemistry, Kyoritsu University of Pharmacy, Minato-ku, Tokyo 105-8512, Japan

⁴Division of Organic Chemistry, National Institute of Health Sciences, Setagaya-ku, Tokyo 158-8501, Japan

*Correspondence and requests for materials should be addressed to I.N. (E-mail: nakanis@nirs.go.jp; Fax: +81-43-255-6819).

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ABSTRACT

The scavenging reaction of galvinoxyl radical (GO^\cdot) by (+)-catechin (1H_2) in deaerated acetonitrile (MeCN), which is reported to proceed via an electron transfer from 1H_2 to GO^\cdot to produce $1\text{H}_2^{\cdot+}$ and GO^- followed by proton transfer, was significantly accelerated by the addition of H_2O . The strong solvation of H_2O to $1\text{H}_2^{\cdot+}$ may result in the largely negative shift of the one-electron oxidation potential of 1H_2 , resulting in the acceleration of the initial electron-transfer oxidation of 1H_2 by GO^\cdot .

KEYWORDS: Antioxidant; Oxidative stress; Catechin; Radical; Electron transfer; Water

INTRODUCTION

The mechanism of radical-scavenging reactions by phenolic antioxidants, such as polyphenols and tocopherols (vitamin E), has attracted considerable interest with regard to the development of novel chemopreventive agents against oxidative stress and associated diseases [1–3]. There are two mechanisms for the radical-scavenging reactions of phenolic antioxidants, i.e., a one-step hydrogen atom transfer from the phenolic OH group or an electron transfer followed by proton transfer [1–3]. Metal ions are a powerful tool to distinguish between these two mechanisms, since electron-transfer reactions are known to be significantly accelerated by the presence of metal ions [4]. In fact, we have recently reported that the scavenging reactions of the galvinoxyl radical (GO^\cdot) or cumylperoxyl radical ($\text{PhMe}_2\text{COO}^\cdot$) by (+)-catechin (1H_2) (Fig. 1) in acetonitrile (MeCN) or in

propionitrile, proceed via an electron transfer from 1H_2 to the radical, which was significantly accelerated by the presence of metal ions, such as Mg^{2+} and Sc^{3+} , followed by proton transfer [5,6]. In such a case, the coordination of metal ions to the one-electron reduced species of the radical may stabilize the product, resulting in the acceleration of the electron transfer [4]. These results suggest that polar solvents, such as water and alcohols, may stabilize the one-electron oxidized species of 1H_2 ($1\text{H}_2^{\cdot+}$) via strong solvation to accelerate the electron-transfer process.

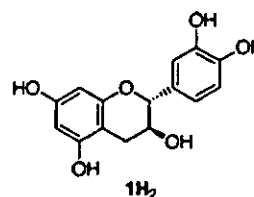


Fig. 1. Chemical structure of (+)-catechin (1H_2).

We herein report that the scavenging-reaction of GO^\bullet by (+)-catechin (1H_2) in deaerated MeCN is significantly accelerated by the addition of water. The results obtained in this study provide a valuable insight into the development of novel antioxidants with a more powerful radical-scavenging ability than natural-occurring ones.

MATERIALS AND METHODS

Materials

(+)-Catechin (1H_2) was purchased from Sigma and purified by column chromatography on silica gel using toluene/acetone/methanol as eluents with further recrystallization from *n*-hexane/ethyl acetate. The galvinoxyl radical (GO^\bullet) was commercially obtained from Aldrich. Acetoritrile (MeCN; spectral grade) was purchased from Nacalai Tesque, Inc., Japan, and used as received.

Kinetic measurements

Typically, an aliquot of (+)-catechin (1H_2 ; 2.0×10^{-2} M) in deaerated MeCN was added to a quartz cuvette (10 mm i.d.), which contained the galvinoxyl radical (GO^\bullet ; 8.4×10^{-2} M) in deaerated MeCN (3.0 mL). UV-vis spectral changes associated with the reaction were monitored using an Agilent 8453 photodiode array spectrophotometer. The rates of the GO^\bullet -scavenging reactions by 1H_2 were determined by monitoring the absorbance change at 428 nm due to GO^\bullet ($\epsilon = 1.43 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) using a stopped-flow technique on a UNISOKU RSP-1000-02NM spectrophotometer. The pseudo-first-order rate constants were determined by a least-squares curve fit using an Apple Macintosh personal computer. The first-order plots of $\ln(A - A_\infty)$ vs. time (A and A_∞ are denoted as the absorbance at the reaction time and the final absorbance, respectively) were linear until three or more half-lives with the correlation coefficient $\rho > 0.999$.

RESULTS AND DISCUSSION

Upon the addition of 1H_2 to a deaerated MeCN solution of GO^\bullet , the absorption band at 428 nm due to GO^\bullet immediately disappeared. This indicates that GO^\bullet was efficiently scavenged by 1H_2 via an electron

transfer from 1H_2 to GO^\bullet to produce $1\text{H}_2^{+\bullet}$ and GO^- , followed by proton transfer [5,6]. The rates of the GO^\bullet -scavenging reaction by 1H_2 were measured by monitoring the decrease in absorbance at 428 nm due to GO^\bullet using a stopped-flow technique on a UNISOKU RSP-1000-02NM spectrophotometer. The decay of the absorbance at 428 nm due to GO^\bullet obeyed pseudo-first-order kinetics when the concentration of 1H_2 ($[1\text{H}_2]$) was maintained at more than a 10-fold excess of the GO^\bullet concentration. The pseudo-first-order rate constants (k_{obs}) increase with the increasing $[1\text{H}_2]$ exhibited a first-order dependence on $[1\text{H}_2]$. From the slope of the linear plot of k_{obs} vs. $[1\text{H}_2]$, the second-order rate constant (k_{HT}) for the GO^\bullet -scavenging reaction by 1H_2 was determined as $2.64 \times 10 \text{ M}^{-1} \text{ s}^{-1}$ in deaerated MeCN at 298 K.

When H_2O is added to the 1H_2 - GO^\bullet system, the rate of the GO^\bullet -scavenging reaction was significantly accelerated. The k_{HT} value linearly increases with the increasing H_2O concentration as shown in Fig. 2. Such an acceleration of the GO^\bullet -scavenging rates by H_2O may be explained by the strong solvation of H_2O with (+)-catechin radical cation ($1\text{H}_2^{+\bullet}$) generated in the initial electron transfer from 1H_2 to GO^\bullet as shown in Fig. 3. The complex formation of 1H_2 and GO^\bullet due to the strong solvation should result in the negative shift of the one-electron oxidation potential (E_{ox}^0) of 1H_2 , leading to a decrease in the free energy change of the electron transfer. The deprotonation of 1H_2 may also be facilitated by H_2O . The E_{ox}^0 value of 1H_2 in MeCN containing H_2O could not be measured because of insolubility of the supporting electrolyte (Bu_4NClO_4) toward H_2O . However, the E_{ox}^0 value of 1H_2 in

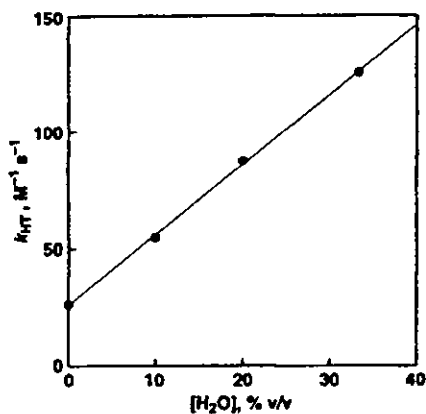


Fig. 2. Plot of k_{HT} vs. $[\text{H}_2\text{O}]$ for the reaction of 1H_2 with GO^\bullet in deaerated MeCN/ H_2O at 298 K.

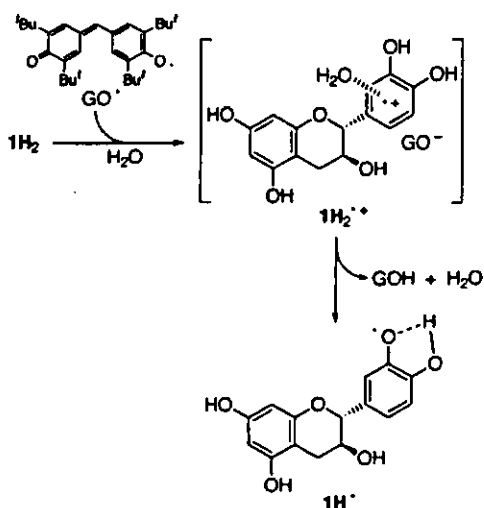


Fig. 3. Mechanism of water-accelerated GO^{•-}-scavenging by 1H₂.

methanol/phosphate buffer (0.1 M) (pH 7.5) (1:1 v/v) has been reported by flow-through electrolysis as 0.12V vs. SCE [7], which is significantly more negative than that in MeCN containing 0.1 M Bu₄NClO₄ (1.18 V vs. SCE [6]). A similar catalysis of H₂O has been observed for the multielectron oxidation of anthracene and its derivatives by one-electron oxidants, such as [Ru(bpy)₃]³⁺ (bpy = 2,2'-bipyridine), where the water-accelerated electron-transfer disproportionation of the radical cations of the anthracenes occurs [8].

In conclusion, the GO^{•-}-scavenging ability of 1H₂ in deaerated MeCN is significantly enhanced in the presence of H₂O. The strong solvation of water to 1H₂^{•+} generated by the initial electron transfer from 1H₂ to GO^{•-} and the enhanced deprotonation of 1H₂ may result in the significant negative shift of the one-electron oxidation potential of 1H₂, leading to the acceleration of the reaction. The results obtained in this study suggest that the introduction of substituents, which can stabilize 1H₂^{•+}, into 1H₂ may lead to the development of effective chemopreventive agents against oxidative stress and associated diseases.

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Ikuo Nakanishi, Researcher of Redox Regulation Research Group, National Institute of Radiological Sciences.
Areas: Physical chemistry for life science and electron-transfer chemistry.



Haruhiro Okuda, Director of Division of Organic Chemistry, National Institute of Health Sciences.
Areas: Bioorganic chemistry and regulatory science.



Tomonori Kawashima, Undergraduate student of Department of Physical Pharmaceutical Chemistry, Kyoritsu University of Pharmacy.
Areas: Physical pharmaceutical chemistry.



Shunichi Fukuzumi, Prof. of Department of Material and Life Science, Graduate School of Engineering, Osaka University.
Areas: Electron-transfer chemistry.



Kiyoshi Fukuhara, Section Chief of Division of Organic Chemistry, National Institute of Health Sciences.
Areas: Bioorganic chemistry and synthetic chemistry.



Toshihiko Ozawa, Executive Director of National Institute of Radiological Sciences.
Areas: Redox chemistry of reactive oxygens and free radicals; electron spin resonance application to biological systems.



Hideko Kanazawa, Prof. of Department of Physical Pharmaceutical Chemistry, Kyoritsu University of Pharmacy.
Areas: Physical pharmaceutical chemistry for analytical science and drug delivery system.



Nobuo Ikota, Director of Redox Regulation Research Group, National Institute of Radiological Sciences.
Areas: Synthetic organic chemistry and bioorganic chemistry.