ORIGINAL ARTICLE

Characterization of inclusion complex of vitamin E compound with 2,6-di-*O*-methylated *β*-cyclodextrin as the solubility enhancer and its kinetic determination for radical scavenging ability

Yoshimi Sueishi · Masashi Hori · Naoya Inazumi

Abstract The structure of the inclusion complex of α-tocopherol (vitamin E compound) with 2,6-di-*O*-methylated *β*-cyclodextrin (DM-*β*-CD) was characterized by 2D ROESY NMR measurements, suggesting that DM-*β*-CD includes the side-chain moiety of α-tocopherol. The inclusion complexation of DM-*β*-CD showed the usefulness of water solubilizer for the radical scavenging assay of vitamin E compounds in aqueous solution. Using the electron paramagnetic resonance (EPR) competitive spin trapping method, we determined the oxygen radical (RO$^\bullet$) scavenging abilities of seven vitamin E compounds (tocopherols and tocotrienols), which were solubilized by DM-*β*-CD in water. The order of the RO$^\bullet$ radical scavenging abilities for vitamin E compounds solubilized by DM-*β*-CD are α- > β- ≈ γ- > δ-, which is in agreement with the oxidation potential values of antioxidants. It is noted that the RO$^\bullet$ radical scavenging abilities of tocotrienols are comparable to those of tocopherols. Based on the results, the mechanism of the antioxidant reaction of vitamin E compounds with the RO$^\bullet$ radical is discussed.

Keywords Vitamin E · Inclusion complex · Radical scavenging ability · Spin trap · NMR · EPR

Introduction

Vitamin E compounds (tocopherols and tocotrienols) are recognized for their effective inhibition of lipid oxidation in foods and biological systems [1]. Tocopherol and tocotrienol are two forms of vitamin E constituents having the same aromatic chromanol head but different hydrocarbon tails. The differences in the biopotencies among them have been actively reported [1–3]. For instance, Suzuki et al. suggested that α-tocotrienol is more potent in scavenging the active radical than is α-tocopherol [3]. However, the credible determination of the free radical scavenging abilities of vitamin E compounds has not yet been established.

Recently, evaluations of antioxidant capacity of pure antioxidant compounds of food extracts have been performed by using the fluorescence-based indirect method (ORAC-FL) that was originated from Glazer's laboratory [4, 5]. To overcome a few experimental disadvantages such as the complexity of computer-aided analysis, we have proposed a new method of ORAC evaluation (ORAC-EPR) using an electron paramagnetic resonance (EPR) spin trapping technique [6]. The ORAC-EPR method, in which the free radicals are measured directly with EPR spin trapping, is useful for the radical scavenging assays of antioxidants. The ORAC evaluations are water-based methods, however lipophilic antioxidants such as tocopherols are compounds with poor water solubility. Cyclodextrins (CDs) have been used extensively as pharmaceutical excipients to increase the solubility of compounds with poor water solubility by forming inclusion complexes [7]. In fact, Huang et al. employed modified *β*-CD as the solubility enhancer for the ORAC-FL assay of lipophilic antioxidants [8].

CDs are cyclic α-(1,4)-linked glucopyranose oligomers that possess hydrophobic cavities capable of forming guest–host inclusion complexes with a variety of organic molecules in aqueous solution. The hydrophobic moieties of compounds with poor water solubility interact non-covalently

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with the CD cavity to form inclusion complexes, which are highly water soluble. Native β-CD is the most used CD because of its low cost and suitable cavity size. However, because native β-CD has poor water-solubility, several modified β-CDs such as heptakis(2,6-di-O-methyl)-β-CD (DM-β-CD) with high water solubility are used as water solubilizers. The resulting inclusion complexes can improve the solubility and bioavailability of antioxidant molecules.

In this study, the structure of the inclusion complex of typical antioxidant α-tocopherol with modified β-CD (DM-β-CD) has been characterized by 2D ROESY NMR measurements and the efficiency of water solubilizer for the radical scavenging assay has been examined. Further, by using the EPR spin trapping method, we conducted kinetic determinations of the radical scavenging abilities for tocopherols and tocotrienols solubilized with DM-β-CD in aqueous solution.

## Experimental

Materials and reagents

Antioxidant vitamin E compounds (tocopherols and tocotrienols, shown in Fig. 1) were purchased from Wako Pure Chemicals (Osaka, Japan) and Funakoshi (Tokyo, Japan), respectively, and used as received. Modified β-CD (heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD), Fig. 1) was employed to improve the water solubility of lipophilic vitamin E compounds because DM-β-CD has high water solubility and high encapsulation ability [9]. Spin-trap 5,5-dimethyl-pyrroline N-oxide (DMPO) was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). 2,2′-Azobis(2-amidino-propane) dihydrochloride (AAPH) was purchased from Wako Pure Chemicals and was used as a source of active oxygen radicals.

EPR and NMR measurements

A phosphate buffer (0.1 mol dm^{-3}, pH = 7.4) was used as a solvent. The active oxygen radical was generated with UV irradiation (1s irradiation, 200 W mercury arc RUF-203s, Radical Research Inc.). A competitive reaction method between spin trap and antioxidants (AO$_x$) was employed to determine the radical scavenging ability of AO$_x$ [6]. The EPR signals of radical adducts with DMPO were recorded in a JEOL FA200 X-band spectrometer (Akishima, Japan). Tocopherols and tocotrienols, which are hardly soluble in aqueous solution, were dissolved in acetonitrile having negligible antioxidant ability as a stock solution. The stock

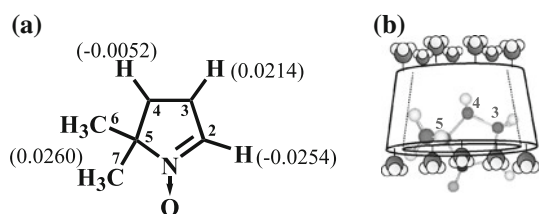Fig. 1 Structures of DMPO, DM-β-CD, and vitamin E compounds
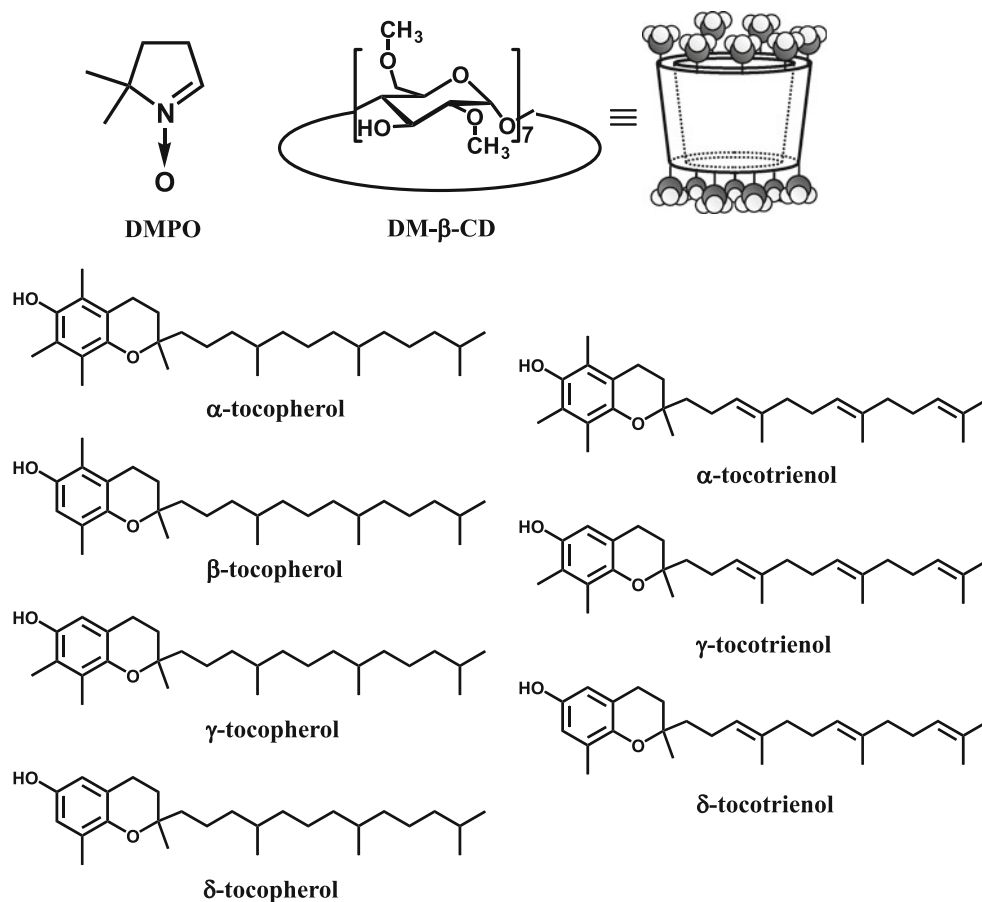
solution was diluted with phosphate buffer containing DM-β-CD; in the final solutions, the acetonitrile content was less than 3%. The solution of DMPO (5×10^{-3} mol dm^{-3}), AAPH (5×10^{-3} mol dm^{-3}) and DM-β-CD (6×10^{-2} mol dm^{-3}) was used as blank. The sample solution temperature was controlled at 298 ± 0.1 K.

In order to elucidate the structure of the DM-β-CD inclusion complex, the ^{1}H-NMR spectra were measured in D$_2$O with a Varian Mercury 300 NMR spectrometer (300 MHz) at room temperature. Chemical shifts were reported as δ values relative to HOD (δ 4.79) as an internal standard [10]. A 2D ROESY NMR experiment was recorded at 600 MHz in D$_2$O on a Varian Inova AS600 NMR spectrometer at 303 K. The mixing time for the ROESY NMR experiments was set at 100 ms.

Results and discussion

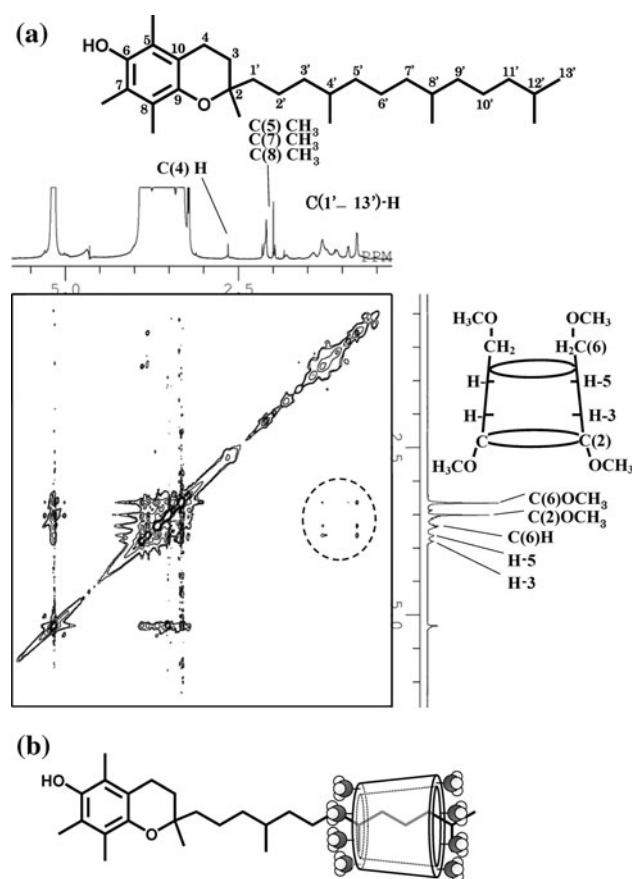### Inclusion of DMPO and α-tocopherol with DM-β-CD

CD forms inclusion complexes with a large number of organic molecules, and the spin-trap DMPO used in this study for the radical scavenging assay might also be encapsulated in the hydrophobic CD cavity. ^{1}H-NMR measurements are useful for discussing the molecular position of inclusion complexes. The induced chemical shifts of the guest protons indicate an inclusion of the proton moiety into the CD cavity [11]. Figure 2a shows the induced changes in the chemical shifts of DMPO in the presence of DM-β-CD. We are able to make a few interesting observations. In DMPO, the induced chemical shift values of the –CH$_3$, C(2)–H, and C(3)–H moieties are large compared with the C(4)–H chemical shift. The upfield chemical shift indicates that the C(2)–H moiety is located close to the polar –OCH$_3$ groups of the CD portals and the downfield chemical shift indicates that the –CH$_3$ and C(3)–H moieties are encapsulated into the DM-β-CD cavity. Figure 2b depicts a plausible structure of the inclusion complex with DM-β-CD, which is in agreement with the

optimized structure of the inclusion complex of native β-CD with DMPO-OH adduct using Gaussian 03 software [12].

The 2D ROESY NMR experiments are informative for the disposition of the guest molecule in the CD cavity. Figure 3 shows the ROESY NMR spectrum of α-tocopherol/DM-β-CD in D$_2$O. The NMR protons of α-tocopherol were assigned with reference to the NMR spectrum reported by Baker and Myers [13]. The cross peaks of the H-5, C(6)–H, and C(6)OCH$_3$ protons of DM-β-CD were detected with the C($1'$–$13'$)–H protons in the side chain of α-tocopherol, as enclosed in the ellipse in Fig. 3a. However, the cross peaks between the C(4,5,7,8)–H and –CH$_3$ protons on the chromane ring and inner H-3,5 protons of DM-β-CD did not appear. This indicates that the side chain of α-tocopherol is encapsulated into the DM-β-CD cavity. Figure 3b depicts a plausible structure of the inclusion complex of α-tocopherol with DM-β-CD. In the α-tocopherol/DM-β-CD complex, the reactive OH group in the chromane ring is exposed to the bulk solution. Therefore, the inclusion effect of DM-β-CD solubilizer on the

Fig. 2 **a** Induced chemical shifts ($\Delta\delta$ (ppm) = δ(complex) − δ(free) in parentheses) of the ^{1}H NMR protons of spin trap DMPO upon inclusion complexation with DM-β-CD: [DM-β-CD]$_0$ = 0.1 mol dm^{-3} and [DMPO]$_0$ = 0.020 mol dm^{-3}. **b** A plausible structure of the inclusion complex of DMPO with DM-β-CD

Fig. 3 **a** 2D ROESY NMR spectra (100 ms mixing time) at 303 K in D$_2$O solution: [α-tocopherol]$_0$ = 10.0×10^{-3} mol dm^{-3} and [DM-β-CD]$_0$ = 10.3×10^{-3} mol dm^{-3}. **b** A plausible structure of the inclusion complex of α-tocopherol with DM-β-CD

radical scavenging assay is negligible for tocopherols and tocotrienols.

Evaluation of radical scavenging ability

Recent spin-trapping studies revealed that the AAPH radical produced after thermal or photolytic decomposition of AAPH was not the peroxyl radical (ROO·), but the alkoxyl radical (RO$^\bullet$) [14, 15]. Figure 4a shows the EPR spectrum obtained from the UV-irradiated solution of AAPH in the presence of the spin-trap DMPO; its hyperfine splitting constant (hfsc) was obtained with a computer spectrum simulation: $A_N = 1.44$ mT and $A_H = 1.52$ mT. Figure 4b, c show the EPR spectra of DMPO-OR adducts in the presence of DM-β-CD. In the presence of an increasing amount of DM-β-CD, the EPR spectra changed from Fig. 4a–c, indicating the formation of the inclusion complex with DM-β-CD. The EPR spectrum of Fig. 4b was readily reproduced by computer simulation by superimposing two sets of four-line spectra. In the excess of DM-β-CD ([DM-β-CD]/[DMPO] = 12), the EPR spectrum for only the inclusion complex of the DMPO-OR adduct could be observed: $A_N = 1.38$ mT and $A_H = 1.33$ mT. The
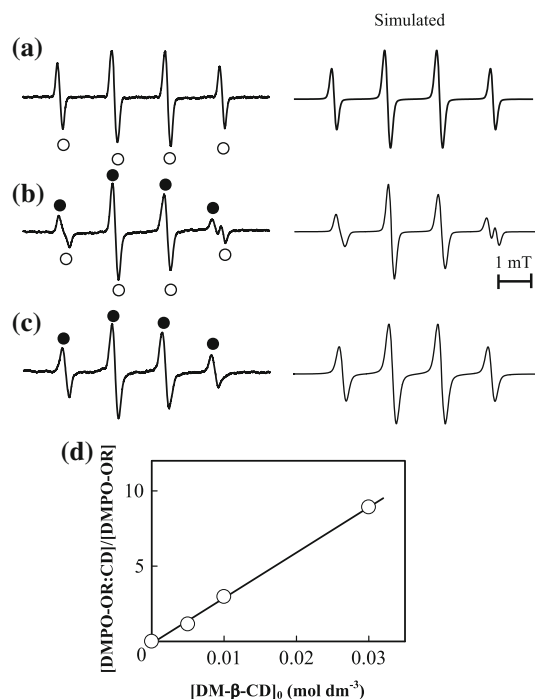
Fig. 4 EPR spectra obtained after photolysis of phosphate buffer (0.1 mol dm^{-3}, pH = 7.4) containing DMPO (5.0×10^{-3} mol dm^{-3}), AAPH (5.2×10^{-3} mol dm^{-3}), and DM-β-CD (**a** 0, **b** 10.2×10^{-3} mol dm^{-3} and **c** 60.7×10^{-3} mol dm^{-3}). The peaks marked with *open circles* and *closed circles* are assigned to DMPO-OR adduct and its inclusion complex, respectively. **d** Relative abundance of the inclusion complex and free DMPO-OR radical, plotted as a function of the initial concentration of DM-β-CD

decrease in A_N indicates that the NO group is surrounded by a less polar environment in the CD cavity. In the excess of DM-β-CD, all spin adducts exist as inclusion complexes, although DM-β-CD favors the inclusion of the neutral DMPO trap rather than the ionic spin adduct of the AAPH RO$^\bullet$ radical. Therefore, in the excess DM-β-CD ([DM-β-CD]/[DMPO] > 10), all molecules of spin trap DMPO and adduct are assumed to be included in the CD cavity.

An inclusion equilibrium constant was calculated based on the relative concentration of the inclusion complex and free species, which was also determined with spectral simulation. The equilibrium for the inclusion complex formation of DMPO-OR with DM-β-CD is expressed as:
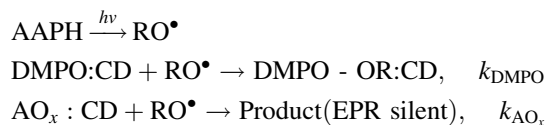
$$K = \frac{[\text{DMPO} - \text{OR} : \text{CD}]}{[\text{DMPO - OR}][\text{DM -} \beta \text{ - CD}]} \quad (1)$$

where DMPO-OR:CD denotes the inclusion complex. Under the condition of [DM-β-CD] $\gg$ [DMPO-OR], Eq. 1 can be rewritten as:

$$\frac{[\text{DMPO} - \text{OR} : \text{CD}]}{[\text{DMPO - OR}]} = K[\text{DM -} \beta \text{ - CD}]_0 \quad (2)$$

where [DM-β-CD]$_0$ denotes the initial concentration of DM-β-CD. Thus, the inclusion equilibrium constants (K) can be calculated from plots of the relative abundance of free DMPO-OR and the inclusion complex against the initial concentration of DM-β-CD. In Fig. 4d, a plot of [DMPO-OR:CD]/[DMPO-OR] vs. [DM-β-CD]$_0$ is a straight line with slope K and passes through the origin, indicating 1:1 inclusion complexation. Equilibrium constant (K) obtained for the inclusion complexes of DMPO-OR with DM-β-CD is estimated to be 296 ± 11 mol^{-1} dm^3.

A competitive spin-trapping method was applied to evaluate the radical scavenging abilities of tocopherols and tocotrienols (antioxidant, AO$_x$) [6]. Since DM-β-CD favors the inclusion of neutral spin trap DMPO than the ionic AAPH adduct, we assume that in excess of DM-β-CD all molecules of spin trap DMPO are included in the CD cavity. The competitive reaction in excess DM-β-CD takes place between the spin trap DMPO and AO$_x$ encapsulated in the CD cavity as follows:

$$\text{AAPH} \xrightarrow{h\nu} \text{RO}^\bullet$$
$$\text{DMPO:CD} + \text{RO}^\bullet \rightarrow \text{DMPO - OR:CD}, \quad k_{\text{DMPO}}$$
$$\text{AO}_x : \text{CD} + \text{RO}^\bullet \rightarrow \text{Product(EPR silent)}, \quad k_{\text{AO}_x}$$

where DMPO:CD and AO$_x$:CD denote the inclusion complexes. A simple formulation for the radical scavenging calculation can be derived from the above reaction scheme and has been reported elsewhere [6].

$$\frac{I_0 - I}{I} = \frac{k_{\text{AO}_x}}{k_{\text{DMPO}}} \frac{[\text{AO}_x : \text{CD}]}{[\text{DMPO:CD}]} = \frac{k_{\text{AO}_x}}{k_{\text{DMPO}}} \frac{[\text{AO}_x]_0}{[\text{DMPO}]_0} \quad (3)$$

where I and I_0 are EPR signal heights for the radical adduct in the presence and absence of AO_x and the $[\ ]_0$ symbol denotes the initial concentrations of DMPO and AO_x. Since AO_x is completely solubilized ($[DM\text{-}\beta\text{-}CD] \gg [AO_x]$), $[AO_x{:}CD]_0$ is equal to $[AO_x]_0$. A linear plot of $(I_0 - I)/I$ against $[AO_x]_0/[DMPO]_0$ gives the slope k_{AO_x}/k_{DMPO}.

Figure 5a shows the EPR spectra obtained from the UV-irradiated solution of AAPH in the presence of DMPO and the solubilizer DM-β-CD. The EPR active species is observed and its hfsc was in agreement with that of DMPO-OR encapsulated in the DM-β-CD cavity. When DMPO, AAPH, and AO_x (α-tocotrienol) were mixed, the EPR peak intensity was decreased, compared with that in the absence of AO_x (Fig. 5a), indicating that part of the RO$^\bullet$ radical was scavenged by the antioxidant. Using Eq. 3, the decrease in the EPR signal height is converted into the relative radical scavenging rate constants. Figure 5c shows a typical plot for the α-tocotrienol/DMPO system. As predicted by Eq. 3, the plot of the radical scavenging rates gives a straight line that passes through the origin, indicating that the reaction scheme and the calculation of the relative radical scavenging rate constants using Eq. 3 are justified. The RO$^\bullet$ scavenging
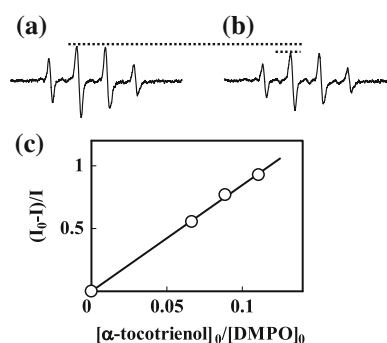
Fig. 5 EPR spectra of RO$^\bullet$ radical adduct of DMPO obtained after the UV-photolysis of phosphate buffer, containing AAPH (5.0×10^{-3} mol dm^{-3}), DMPO (5.0×10^{-3} mol dm^{-3}) and α-tocotrienol solubilized with DM-β-CD (60×10^{-3} mol dm^{-3}): [α-tocotrienol]$_0$ = 0 (a) and [α-tocotrienol]$_0$ = 1.48×10^{-4} mol dm^{-3} (b). *Horizontal broken lines* in the spectra demonstrate the change in EPR signal height of the selected peak by the addition of the antioxidant α-tocotrienol. c A plot of $(I_0 - I)/I$ vs. [α-tocotrienol]$_0$/[DMPO]$_0$ in the presence of DM-β-CD according to Eq. 3

rate constants of vitamin E compounds against DMPO are listed in Table 1. The radical scavenging rate constants (relative rate k_{AO_x}/k_{DMPO}) for vitamin E compounds were on the order of α-tocopherol > β-tocopherol $\approx$ γ-tocopherol > δ-tocopherol. It is noted that the scavenging rate constants for antioxidant tocotrienols are comparable to those of tocopherols.

Radical scavenging ability and reaction mechanism

Tocopherols and tocotrienols are two vitamin E constituents having the same reactive chromanol moiety. The above results indicate that the RO$^\bullet$ radical scavenging abilities of tocotrienols in aqueous solution are comparable with those of tocopherols. Suzuki et al. suggested that α-tocotrienol exhibits significantly greater peroxyl radical scavenging ability than that of α-tocopherol in phosphatidylcholine liposomes [3]. Suarra et al. reported that the free radical scavenging effects of α-tocotrienol and α-tocopherol are similar in solution [2], which is in agreement with our observations. The spin trapping-based determination of the radical scavenging abilities for tocopherols and tocotrienols indicates that the large difference in biopotency among the tocopherols and tocotrienols are mostly due to differences in retention in tissues and membranes, as suggested by Kamal-Eldin and Appelqvist [1].

Antioxidants can deactivate radicals by two major mechanisms: hydrogen transfer and electron transfer. Investigation of the oxidation potentials (E_p) of antioxidants might be helpful in understanding the reaction processes of RO$^\bullet$ with antioxidants. The E_p values of tocopherols, reported by Nagaoka et al. [16], are listed on the right in Table 1. As can be seen in Table 1, the tocopherols that have smaller E_p values show higher RO$^\bullet$ radical scavenging abilities, which is in agreement with the results reported by Nagaoka et al. who show the relationship between the E_p values of tocopherols and PhO$^\bullet$ radical scavenging abilities. As suggested by Nagaoka et al. [16], we believe that both charge and proton transfer play an important role in the antioxidant reaction of tocopherols with AAPH RO$^\bullet$ radical and that the transition state has the property of the charge transfer species.

Table 1 Relative rate constants (k_{AO_x}/k_{DMPO} (AO_x/DMPO)) of tocopherols and tocotrienols solubilized with DM-β-CD	Antioxidants solubilized with DM-β-CD	k_{AO_x}/k_{DMPO}	$k/k_{\alpha\text{-tocopherol}}$	E_p (mV)[a]
	α-Tocopherol:CD	7.32 ± 0.30	1.0	860
	β-Tocopherol:CD	4.14 ± 0.25	0.57	920
	γ-Tocopherol:CD	4.96 ± 0.42	0.68	930
	δ-Tocopherol:CD	2.99 ± 0.11	0.41	990
	α-Tocotrienol:CD	8.46 ± 0.09	1.16	–
	γ-Tocotrienol:CD	3.49 ± 0.10	0.48	–
[a] Oxidation potential values reported by Nagaoka et al. [16]	δ-Tocotrienol:CD	2.96 ± 0.10	0.40	–

All seven antioxidants tested in this study showed the relative rate $k/k_{\alpha\text{-tocopherol}}$ values ranging from 0.40 to 1.16. Solubilized α-tocotrienol and α-tocopherol showed the highest antioxidant activity. The antioxidant potency of four tocopherols was in the order of α-tocopherol $>$ β-tocopherol $\approx$ γ-tocopherol $>$ δ-tocopherol, which is not only dependent on the stabilization of antioxidant phenoxyl radical by conjugative electron delocalization, but also on the facility of H-transfer [1, 17].

In summary, the inclusion complexation of vitamin E α-tocopherol with DM-β-CD has been characterized by 2D ROESY NMR measurements. Using the spin trapping method, we have determined the relative RO$^\bullet$ radical scavenging rate constants of vitamin E compounds (tocopherols and tocotrienols) solubilized with DM-β-CD. The RO$^\bullet$ radical scavenging abilities of tocotrienols in aqueous solution were compared with those of tocopherols. A good correlation between the radical scavenging abilities and oxidation potential values supports the belief that the AAPH RO$^\bullet$ radical scavenging reactions of vitamin E compounds proceed via a transition state such as the charge-transfer species.

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