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Kinetic Studies of Antioxidant Activity of New Tocopherol Model Compounds in Solution

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The second-order rate constants k_s for the reaction of 10 kinds of tocopherol (vitamin E) model compounds with stable phenoxyl radical in ethanol have been measured at 25.0 °C, using a stopped-flow spectrophotometer, as a model reaction of tocopherols with unstable free radicals (ROO·, RO·, and HO·) in biological systems. The absolute k_s values of α-, β-, γ-, and δ-tocopherol models are similar to or slightly smaller than those of the corresponding tocopherols having a long-phytyl-chain. The relative k_s values (α : β : γ : δ=100 : 53 : 50 : 24), that is, relative antioxidant activities, of α-, β-, γ-, and δ-tocopherol models are in good agreement with those (100 : 44 : 47 : 20) of α-, β-, γ-, and δ-tocopherols. The antioxidant activities of tocopherol models having two alkyl substituents, such as methyl, ethyl, isopropyl, and *t*-butyl groups, at ortho positions of OH group are similar to each other, suggesting that the effect of steric hindrance on the reaction rate is small. 5,7-Dimethyltocol model has quite similar rate constants with those of β- and γ-tocopherol models, whereas δ-tocopherol model is only ca. 24% as reactive as α-tocopherol model and tocol model is only ca. 10% as reactive as α-tocopherol model. The result indicates that the rate constants increase as the total electron donating capacity of the alkyl substituents at aromatic ring increases. For the tocopherol models log k_s was found to correlate with $\Sigma\sigma^+$ substituent constants with a ρ^+ value of −1.0.

Vitamin E (α-, β-, γ-, and δ-tocopherols) is known to scavenge peroxyl, alkoxyl, and hydroxyl radicals.[1,2] The above free radical scavenging actions by vitamin E have been ascribed to the initial reaction of the phenolic hydroxyl group with the production of a tocopheroxyl† radical.[3-7] Recently, Burton et al. have reported absolute second-order rate constants, k_1, for the reaction of α-, β-, γ-, and δ-tocopherols with poly(styrylperoxyl) radicals, using the inhibited autoxidation of styrene method (reaction 1).[8-10]

$$\mathrm{ROO\cdot} + \mathrm{Toc} \xrightarrow{k_1} \mathrm{ROOH} + \mathrm{Toc\cdot} \quad (1)$$

They suggested that the relative magnitude of the second-order rate constant, k_1, of α-, β-, γ-, and δ-tocopherols is in agreement with in vivo and in vitro tests of their relative biological activities which yield: α>β>γ>δ-tocopherol.[9]

In a previous work, we have determined spectrophotometrically the rates of reaction of α-, β-, γ-, and δ-tocopherols with stable phenoxyl radical (2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl (PhO·) (see Fig. 1)) in ethanol solution using stopped-flow technique, as a model reaction of tocopherols with unstable free radicals (ROO·, RO·, and HO·) in biological systems (reaction 2).[11]

$$\mathrm{PhO\cdot} + \mathrm{Toc} \xrightarrow{k_s} \mathrm{PhOH} + \mathrm{Toc\cdot} \quad (2)$$

The second-order rate constants, k_s, obtained are $(5.12\pm0.36)\times10^3$ (α-Toc), $(2.24\pm0.04)\times10^3$ (β-Toc), $(2.42\pm0.16)\times10^3$ (γ-Toc), and $(1.02\pm0.10)\times10^3$ (δ-Toc) $M^{-1}s^{-1}$ in ethanol at 25.0 °C. The relative k_s values (α : β : γ : δ=100 : 44 : 47 : 20) obtained by the stopped-flow technique are in good agreement with the values (100 : 41 : 44 : 14)[10] obtained by the inhibited autoxidation of styrene method, although the absolute k_s values are about 600 times smaller than those for the reaction of tocopherols with poly(styrylperoxyl) radical in chlorobenzene. The results suggest that the relative reactivities, that is, relative antioxidant activities of tocopherols in homogeneous solution do not change depending on the kinds of radicals (phenoxyl and poly(styrylperoxyl) radicals) used, while the absolute

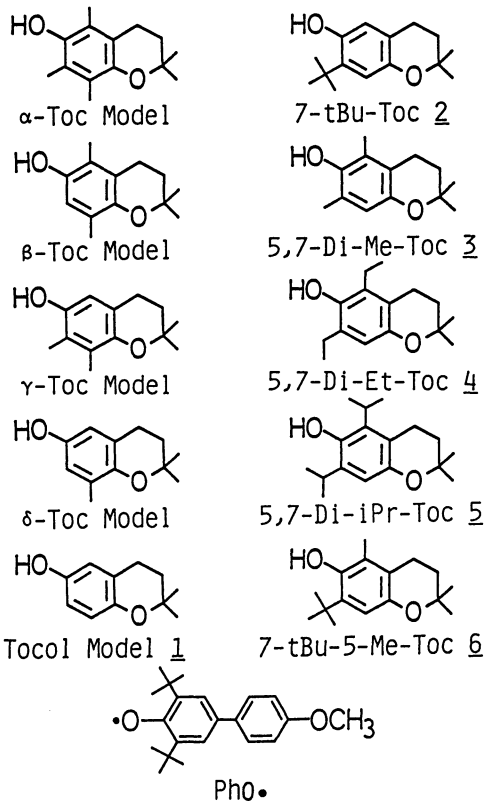

Fig. 1. Molecular structures of tocopherol model compounds and phenoxyl radical (PhO·).

†The phenoxyl radicals generated from tocopherols are named in this paper as widely used tocopheroxyl radicals for convenience rather than tocopheryloxyl radicals that conform to rules of nomenclature.

rates are considerably different from each other. Further, the above results indicate that α-tocopherol having two methyl groups at ortho positions of OH group is about two times as reactive as β- and γ-tocopherols having one methyl group at ortho positions, and, then, δ-tocopherol which has no methyl substituents at ortho positions is only ca. 20% as reactive as α-tocopherol. Antioxidant activities of tocopherols seem to increase in the order of the number of methyl substituents at ortho positions of OH group. These findings suggest that the presence of two methyl groups at ortho positions in tocopherols is of great importance to their antioxidant action.

Therefore, in the present work, in order to get the tocopherol compounds having higher antioxidant activity than α-tocopherol and in order to clear the structure-activity relationship in antioxidant action of tocopherols, we have prepared several tocopherol model compounds which have especially two alkyl substituents, such as methyl, ethyl, isopropyl, and *t*-butyl groups, at ortho positions of OH group (see Fig. 1), and measured the rates of reaction of tocopherol models with phenoxyl radical in ethanol solution, using stopped-flow spectrophotometer.[11]

Experimental

Materials. α-, β-, γ-, δ-Tocopherol models, tocol model (**1**), and 5,7-dimethyl-tocol model (**3**) were prepared according to the method of Nilsson et al.[12] Preparation of 7-*t*-butyl-tocol model (**2**), 5,7-diisopropyl-tocol model (**5**), and 7-*t*-butyl-5-methyl-tocol model (**6**) was reported in a previous paper.[13] 2,6-Diethylhydroquinone (mp 105.0—106.0 °C) were prepared from 2,6-diethylphenol by the standard procedure, as reported for the syntheses of the 2,3-, 2,5-, and 2,6-dimethylhydroquinone.[14]

The 2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl (PhO·) was prepared according to the method of Müller et al.[15] Radical concentration of phenoxyl radical was obtained from the results of the paramagnetic susceptibility measurements at 20 °C. The value was 100% for phenoxyl, assuming the Curie law.

5,7-Diethyl-Tocol Model (4). 2,6-Diethylhydroquinone (3.7 g, 22 mmol) was dissolved in formic acid (99%) (50 ml) and THF (tetrahydrofuran) (5 ml), and the solution was heated to reflux. A THF (2 ml) solution of 2-methyl-3-buten-2-ol (1.9 g, 22 mmol) was added dropwise slowly during 1 h, and the refluxing was continued for an additional 3 h with stirring. The reaction mixture was poured onto crushed ice (150 g), and extracted with diethyl ether (5×25 ml). Petroleum ether (bp 40—50 °C) (25 ml) was added to the combined diethyl ether extracts and the mixture was washed with water (5×25 ml). After removal of the solvent, the residue was dissolved in methanol (75 ml), 1 ml of concn HCl was added, and the solution refluxed for 20 min to hydrolyze formate of **4** produced. The methanol was then evaporated, and the residue was taken up in diethyl ether (100 ml), washed with $NaHCO_3$ solution and with water, and the solution was dried over anhydrous sodium sulfate. The solvent was evaporated, and the residual oil was extracted three times with hot petroleum ether (bp 50—60 °C) (3×100 ml). After removal of the solvent, a viscous oil remained. The oily residue obtained was chromatographed on 50 g of silica gel with ether-petroleum ether (1 : 5) as the eluting agent. After removal of the solvent, the oily solids remained were recrystallized from petroleum ether (bp 60—70 °C), giving white crystals. Mp 80.0—80.4 °C. (Found: C, 76.63; H, 9.50%. Calcd for $C_{15}H_{22}O_2$: C, 76.88; H, 9.46%). UV spectrum (λ_{max}=294 nm, log ε=3.55 in ethanol). NMR spectrum (δ=1.13 (3H, t, 5- or 7-CH_2CH_3, *J*=7.3 Hz), 1.21 (3H, t, 5- or 7-CH_2CH_3, *J*=7.3 Hz), 1.28 (6H, s, 2-CH_3), 1.78 (2H, t, 3-CH_2, *J*=6.6 Hz), 2.56 (2H, q, 5- or 7-CH_2CH_3, *J*=7.3 Hz), 2.63 (2H, q, 5- or 7-CH_2CH_3, *J*=7.3 Hz), 2.67 (2H, t, 4-CH_2, *J*=6.6 Hz), 4.17 (1H, s, 6-OH), 6.46 (1H, s, 8-H); δ in $CDCl_3$ with TMS as an internal standard).

Measurement of k_s Values by the Stopped-Flow Spectrophotometer. The stopped-flow data were obtained on a UNISOKU stopped-flow spectrophotometer Model RS-450 by mixing equal volumes of ethanol solutions of phenoxyl and tocopherol model. The oxidation reactions were studied under pseudo-first-order conditions, and the observed rate constants, k_{obsd}, were calculated in the usual way using a standard least-squares analysis. All measurements were performed at 25.0±0.5 °C.

Results

The phenoxyl radical (PhO·) is stable in the absence of tocopherol models, and shows absorption peaks at λ_{max}=376 nm (ε=17600 $M^{-1}cm^{-1}$(1 M=1 mol dm^{-3})) and 580 nm (ε=4280 $M^{-1}cm^{-1}$) in ethanol. By adding an ethanol solution of α-tocopherol model (1.00 mM) to an ethanol solution of phenoxyl (0.10 mM) (1 : 1 in volume) at 25.0 °C, the absorption spectrum of the phenoxyl immediately changed to that of α-tocopheroxyl model. The absorption spectrum of α-tocopheroxyl model shows weak absorption peaks at 427 and 405 nm.[16] However, the α-tocopheroxyl model is unstable, and the absorption peaks rapidly disappeared. Similarly, the phenoxyl radical has been reacted with excess tocopherol models, showing rapid decrease of the absorption spectrum, respectively. Therefore, the oxidation rates of tocopherol model compounds by phenoxyl were studied spectrophotometrically using

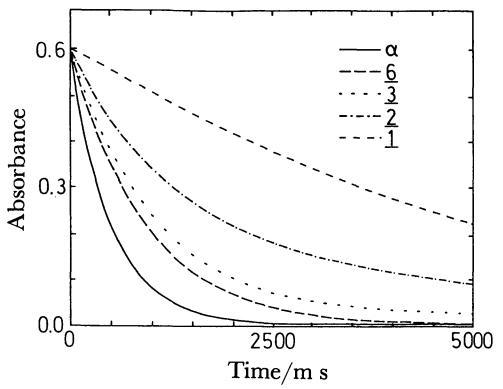

Fig. 2. The decay of phenoxyl radicals for the reaction of phenoxyls with tocopherol model compounds in ethanol at 25.0 °C. $[PhO\cdot]_{t=0}$ 0.05 mM and $[Toc]_{t=0}$ 0.50 mM. At 376 nm.

stopped-flow technique in the presence of excess tocopherol model in ethanol. The details of experiments are reported in a previous paper.[11] The rate was measured by following the decrease in absorbance at 376 and 580 nm of phenoxyl radical. The time dependence of the decrease in absorbance at 376 nm observed when 0.10 mM ethanol solution of phenoxyl is mixed with 1.00 mM ethanol solution of tocopherol models (1 : 1 in volume; final concentration of phenoxyl is 0.05 mM) is shown in Fig. 2. The stopped-flow reactions were performed at 25.0±0.5 °C.

The pseudo-first-order rate constants, k_{obsd}, observed at 376 nm and at 580 nm were linearly dependent on the concentration of tocopherol model, and, thus, the rate law is expressed as

$$-d[PhO\cdot]/dt = k_{obsd}[PhO\cdot] = k_s[Toc][PhO\cdot] \quad (3)$$

where k_s is the second-order rate constant for oxidation of tocopherol model by phenoxyl radical. The values of k_s calculated from k_{obsd} are listed in Table 1. For each tocopherol model, k_s was measured in at least 4 independent experiments. The experimental errors in k_s values was $<\pm 10\%$ in every case.

Discussion

The effect of phytyl side chain of vitamin E on its antioxidant activity has been studied by Fukuzawa et al.[17] and Niki et al.[18] They examined the inhibition effect of the oxidation of unsaturated lipids in homogeneous solution and in liposomes by α-tocopherol and α-tocopherol model. It was concluded that the antioxidative properties of α-tocopherol and α-tocopherol model without the phytyl side chain are quite similar within liposomes as well as in homogeneous solution but that the phytyl side chain enhances the retainment of α-tocopherol in liposomes and suppresses the transfer of α-tocopherol between liposomal membranes. Burton et al. have measured the rate constants k_1 for the reaction of α-tocopherol and α-tocopherol model with poly(styrylperoxyl) radical in homogeneous solution.[9,10,19] In an early report, they suggested that α-tocopherol and α-tocopherol model have similar in vitro antioxidant activity in solution.[9] However, both the absolute rate constants k_1 of α-tocopherol and α-tocopherol model and the relative rate constants ($k_1/k_1(\alpha\text{-Toc})\times 100$) reported by Burton et al. varied from early work to recent work. The values are as follows: i) α-tocopherol: $k_1 = (2.35\pm0.50)\times10^6\ M^{-1}s^{-1}$ (100) and α-tocopherol model: $(2.14\pm0.81)\times10^6$ (91) (1981);[9] ii) α-tocopherol: $(3.24\pm0.15)\times10^6$ (100) and α-tocopherol model: $(3.77\pm0.19)\times10^6$ (116) (1983);[19] iii) α-tocopherol: $(3.20\pm0.32)\times10^6$ (100) and α-tocopherol model: $(3.80\pm0.38)\times10^6$ (119) (1985).[10]

In the present work, we have measured the rate constants k_s for the reaction of α-, β-, γ-, and δ-tocopherol models with the stable phenoxyl radical in ethanol, using a stopped-flow spectrophotometer. The results listed in Table 1 indicate that α-, β-, γ-, and δ-tocopherol models have reactivities similar to or slightly less than those of corresponding α-, β-, γ-, and δ-tocopherols (see Table 2) in homogeneous solution. Further, the relative k_s values ($\alpha:\beta:\gamma:\delta = 100:53:50:24$), that is, relative antioxidant activities, obtained for α-, β-, γ-, and δ-tocopherol models are in good agreement with those (100 : 44 : 47 : 20) for α-, β-, γ-,

Table 1. Second-Order Rate Constants (k_s) and Relative Rate Constants for Oxidation of Tocopherol Models by Phenoxyl Radical in Ethanol at 25.0 °C, and $\sum\sigma^+$ Values

Tocopherol Model	$10^{-3}\,k_s$ ($M^{-1}s^{-1}$)	$\frac{k_s\text{ (Toc Model)}\times 100}{k_s\text{ (α-Toc Model)}}$	$\sum\sigma^+$
α-Toc Model	4.20±0.04	100	−0.688
β-Toc Model	2.24±0.07	53	−0.377
γ-Toc Model	2.10±0.17	50	−0.377
δ-Toc Model	1.00±0.01	24	−0.066
Toc Model **1**	0.41±0.01	10	0.000
Toc Model **2**	1.36±0.13	32	−0.256
Toc Model **3**	2.00±0.01	48	−0.622
Toc Model **4**	1.91±0.01	45	−0.590
Toc Model **5**	2.82±0.02	67	−0.560
Toc Model **6**	2.34±0.15	56	−0.567

Table 2. Second-Order Rate Constants (k_s) and Relative Rate Constants for Oxidation of Tocopherols by Phenoxyl radical in Ethanol at 25.0 °C

Tocopherol	$10^{-3}\,k_s$ ($M^{-1}s^{-1}$)	$\frac{k_s\text{ (Toc)}\times 100}{k_s\text{ (α-Toc)}}$	$\frac{k_s\text{ (Toc Model)}}{k_s\text{ (Toc)}}$
α-Toc	5.12±0.36[a]	100	0.82
β-Toc	2.24±0.04	44	1.00
γ-Toc	2.42±0.16	47	0.87
δ-Toc	1.02±0.10	20	0.98

a) See Ref. 11.

and δ-tocopherols having a long-phytyl-chain, respectively.

As described in a previous section, α-tocopherol which has two methyl substituents at ortho positions of OH group showed the highest antioxidant activity among α-, β-, γ-, and δ-tocopherols. Therefore, we have prepared several tocopherol model compounds especially having two alkyl substituents, such as methyl, ethyl, isopropyl, and *t*-butyl groups, and measured the antioxidant activity of tocopherol models in ethanol solution. By comparing the second-order rate constants observed for 5,7-dimethyl-tocol model (**3**) having two methyl groups at ortho position with that of α-tocopherol model, the former is only ca. 48% as reactive as the latter, against to our expectation. On the other hand, as is clear from the results listed in Table 1, the antioxidant activities of tocopherol models (**3**), (**4**), (**5**), and (**6**) having two alkyl substituents at ortho position of OH group are similar to each other, although the 5,7-diisopropyl-tocol model (**5**) shows 12—15% higher antioxidant activity than the other tocopherol models. The result suggests that the effect of steric hindrance on the reaction rate is not remarkable, but small. Further, to our surprise, 5,7-dimethyl-tocol model (**3**) has quite similar rate constants with those of β- and γ-tocopherol models, whereas δ-tocopherol model is only ca. 24% as reactive as α-tocopherol model and tocol model (**1**) is only ca. 10% as reactive as α-tocopherol model. Therefore, the relative rate constants are plotted against to the number of alkyl substituents at aromatic ring in tocopherol models. The results shown in Fig. 3 clearly suggest that the antioxidant activity of tocopherol models depends on the number of alkyl substituents at aromatic ring rather than the position of substitution. This implies that the values of the rate constants for this reaction increase as the total electron donating capacity of the alkyl substituents increases.

Howard and Ingold have measured the rate constants k_4 for the reaction of ortho-alkyl phenols with poly(styrylperoxyl) radicals.[20] They found that the logarithms of rate constants correlate with the sum of the Brown's σ^+ constants[21] for all the substituents on the phenol ($\Sigma\sigma^+$). They also reported that the steric effects due to ortho alkyl substituents are important. However, for the tocopherol models, it was observed that the steric effect on the reaction rate is not remarkable. Therefore, the values of log k_s for tocopherol models have been plotted against $\Sigma\sigma^+$. As shown in Fig. 4, the logarithms of rate constants (k_s) were found to roughly correlate with $\Sigma\sigma^+$ substituent constants. The slope of the best straight line, i.e., the ρ factor, was −1.0.

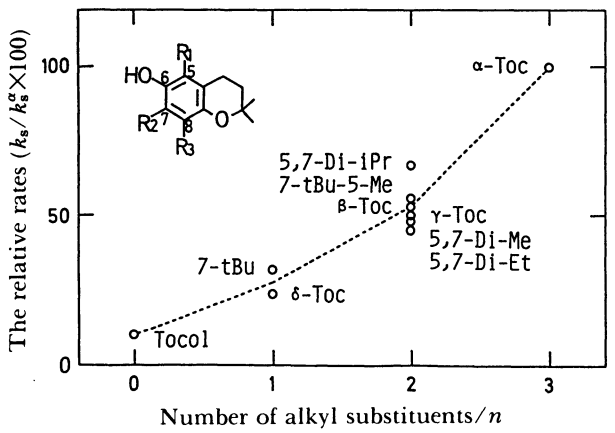

Fig. 3. Plot of the relative rate constant (k_s(Toc Model)/k_s(α-Toc Model)×100) vs. the number of alkyl substituents (n) at aromatic ring in tocopherol model compounds.

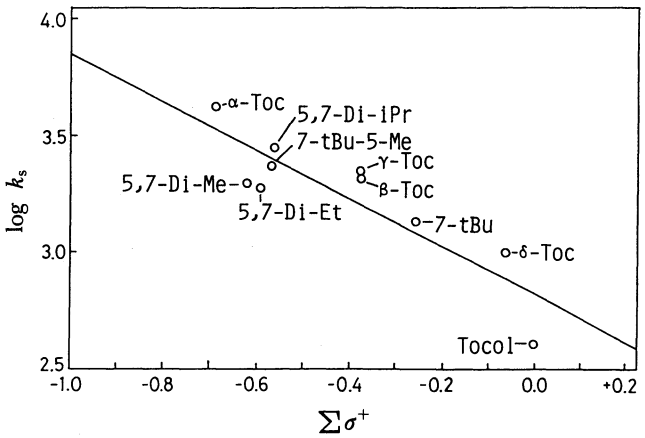

Fig. 4. Plot of log k_s for tocopherol models vs. $\Sigma\sigma^+$. The σ^+ values are from Ref. 21.

Recently, Burton et al. have reported absolute rate constants k_1 for the reaction of α-tocopherol and some related phenols (for example, 4-methoxy-2,3,5,6-tetramethylphenol) with peroxyl radicals (ROO·) (reaction 1).[9,10,19,22] By comparing the k_1 value for α-tocopherol with those found for structurally related phenols that lacked the 6-membered heterocyclic ring, they suggested that the structure of this ring was largely responsible for the high reactivity of α-tocopherol. The relative magnitudes of k_1 values for α-tocopherols and phenols can be correlated with the degree of stabilization of the radical produced by oxidation. Here, stabilization of radical depends on two factors: (i) the extent of orbital overlap between the 2p type lone pair on the para oxygen atom and the aromatic π electron system and (ii) the electron-donating or withdrawing character of the group bonded to the para oxygen atom. Therefore, Burton et al. have prepared new tocopherol model compounds having 5-membered heterocyclic ring, which is generally more planar than 6-membered ring. They found that two tocopherol models having 5-membered ring are 1.7—1.8 times as reactive as α-tocopherol. However, to our regret, all the tocopherol model compounds we have studied in the present work showed smaller reactivity than that of α-tocopherol.

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