 Bull. Chem. Soc. Jpn. Vol. 79, No. 10, 1501–1508 (2006)

Kinetic Study on the Free Radical-Scavenging and Vitamin E-Regenerating Actions of Caffeic Acid and Its Related Compounds

Keishi Ohara,* Yoko Ichimura, Kumi Tsukamoto, Mayumi Ogata, Shin-ichi Nagaoka, and Kazuo Mukai

Department of Chemistry, Faculty of Science, Ehime University, Matsuyama 790-8577

Received March 14, 2006; E-mail: ohara@chem.sci.ehime-u.ac.jp

A kinetic study involving 4-hydroxycinnamic acid derivatives (HCAs) was performed in order to clarify the mechanism for free radical-scavenging and vitamin E-regenerating. The second-order rate constants of the radical-scavenging reaction (k_s) observed for caffeic acid derivatives are larger than those for ferulic acid derivatives. The result may be explained by i) the lower oxidation potential coming from the existence of the more electron-donating hydroxy group comparing with methoxy group, and ii) the stabilization effect of the corresponding radicals produced in the radical-scavenging reaction through intramolecular hydrogen bonding. The radical-scavenging activity of HCAs in ethanol mainly occurs via the hydrogen atom transfer from the phenolic OH-protons. The pH dependence of k_s for ferulic acid in the aqueous Triton X-100 micelle solutions suggests the importance of the phenolic OH proton on the radical-scavenging reaction. On the other hand, the pH dependence of k_s for caffeic acid and chlorogenic acid suggests the occurence of intramolecular hydrogen bonding and an electron-transfer process in the radical-scavenging reaction.

A group of 4-hydroxycinnamic acid derivatives (HCAs) including caffeic acid (CA), ferulic acid (FA), chlorogenic acid (CGA), and rosmarinic acid (RA) (Fig. 1) are a few of the polyphenols found in a variety of plants, such as beans, fruits, herbs, and vegetables.[1–5] For example, CGA is found in coffee beans, burdocks, apples, pears, berries, and tomatoes. These HCAs are produced from phenylalanine or L-tyrosine via an organic pathway in plants. The total content of CGA and CA in roasted coffee beans is about 2%, and the amount of RA in the herb rosemary is 10 mg g^{-1}.[2,3] Human being and animals usually take a certain amount of these HCAs from daily meals and drinks.

Because a considerable amount of HCAs is always taken and absorbed from a diet into human, the health effects of ingested HCAs are of great interest to many researchers. Recent pharmacological reports suggested that plant-origin HCAs have pharmacological activity, such as the inhibition of lipid-peroxidation[6–10] and DNA-damage,[11] reduction of non-heme iron in blood,[12] antiallergic activities,[13] and cancer-preventive effects.[14,15] Many of these effects are considered to result from antioxidant activity toward active-oxygen species (AOS), such as singlet oxygen and free radicals that are produced in the body. The antioxidant activity of natural materials is generally considered to be one of the most important factors in pharmacological activities, because it protects living bodies and tissues from diseases and injuries caused by AOS.[6–24] Kinetic studies on the antioxidant activities of these HCAs versus several free radicals, such as DPPH (2,2-diphenyl-1-picrylhydra-

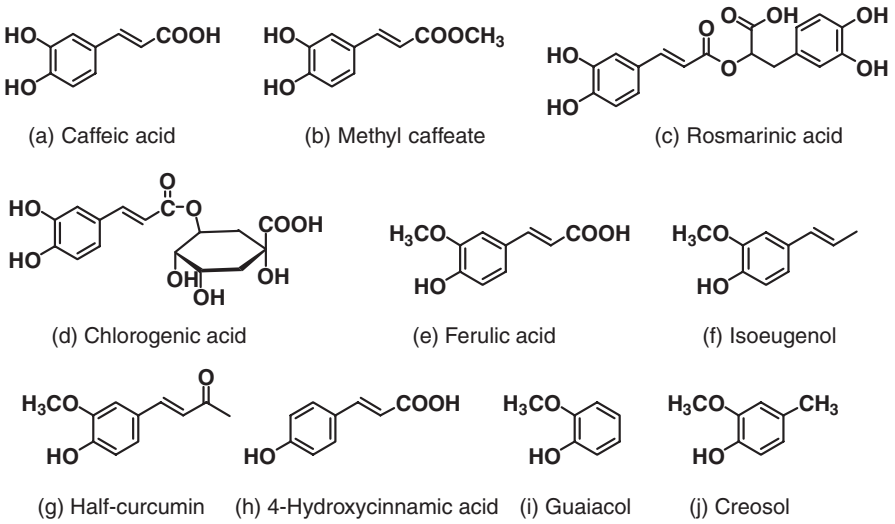

Fig. 1. Molecular structures of HCAs and the related phenols.

Published on the web October 10, 2006; doi:10.1246/bcsj.79.1501

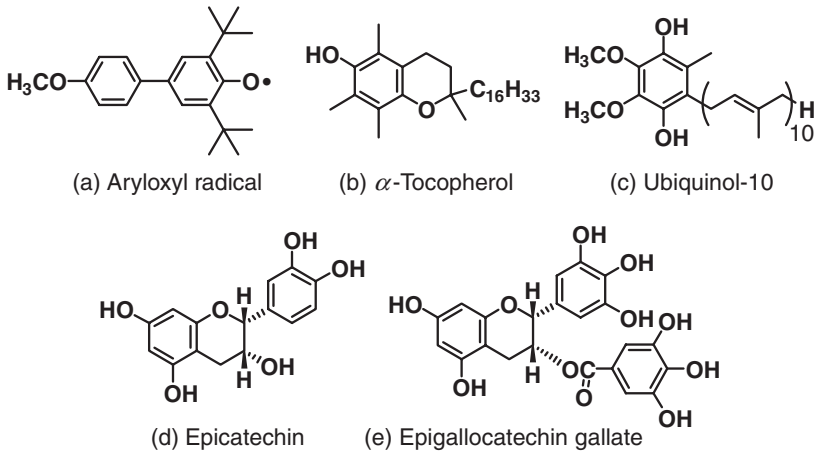

Fig. 2. Molecular structures of aryloxyl radical (ArO•) and some antioxidants.

$$\mathrm{ArO}\cdot + \mathrm{HCA} \xrightarrow{k_s} \mathrm{ArOH} + \mathrm{HCA}\cdot$$

Scheme 1.

$$\alpha\text{-Toc}\cdot + \mathrm{HCA} \xrightarrow{k_r} \alpha\text{-TocH} + \mathrm{HCA}\cdot$$

Scheme 2.

zyl), hydroxyl (•OH), superoxide ($\bullet O_2^-$), and azide radical ($\bullet N_3$), in solutions, have been reported.[16–24] It has been suggested that HCAs have a certain antioxidant activity as well as other naturally existing polyphenols.

The plant-origin HCAs are classified into caffeic acid type which has an *o*-hydroxyphenol (catechol) moiety and ferulic acid type which has an *o*-methoxyphenol (guaiacol) moiety. The former contains CA, CGA, and RA, and the latter contains FA and curcumin. The *o*-hydroxy- and *o*-methoxy-HCAs are superior to 4-hydroxycinnamic acid (*p*-coumaric acid), and CA derivatives usually have higher activity than FA derivatives.[7,19,23] Among the natural HCAs, the dietary contents, the adsorption in living bodies, the distribution in tissues, the solubility, and the antioxidant activities are different for each HCA. Further systematic studies involving HCAs on the relationship between their chemical structure and activity are needed for understanding their health and pharmacological effects and their antioxidant mechanisms.

In the present work, a kinetic investigation on the free radical-scavenging and vitamin E (VE)-regenerating reactions involving a group of HCAs and related phenols was carried out in order to clarify their structure–activity relationship. To investigate the difference in antioxidant activities between *o*-methoxyphenol (guaiacol) and *o*-hydroxyphenol (catechol) moieties, and the effect of the conjugated double bond in the side chain of HCAs, we used CA, methyl caffeate, CGA, RA, FA, isoeugenol, 4-hydroxycinnamic acid, guaiacol (2-methoxyphenol), and creosol (2-methoxy-4-methylphenol) (Fig. 1). The second-order rate constants of the HCAs and related phenols for the reactions with the model free-radical, aryloxyl (2,6-di-*tert*-butyl-4-(4-methoxyphenyl)phenoxyl, ArO•, Fig. 2a) (Scheme 1), and the regeneration reaction of VE (α-tocopherol, α-TocH) from α-tocopheroxyl (α-Toc•) (Scheme 2) have been measured using a stopped-flow spectrophotometer in ethanol. Furthermore, to investigate the contribution of the acid–base dissociation equilibrium to the antioxidant activity, the second-order rate constants of FA, CA, and CGA for the ArO• scavenging reaction have been measured in aqueous Triton X-100 (TX-100) micelle solutions at various pH values. TX-100 micelle systems are frequently used as a model for biomembranes in the kinetic studies for the reaction between water-soluble antioxidants and lipophilic free radicals, such as Toc• and ArO•. The mechanism and the structure–activity relationship for HCAs are discussed on the basis of results obtained from these rate constants.

Experimental

Caffeic acid, chlorogenic acid, rosmarinic acid, ferulic acid, isoeugenol, 4-hydroxycinnamic acid, guaiacol, and creosol were purchased from Nacalai Tesque and were used as received. Methyl caffeate was synthesized from CA and methanol. α-Tocopherol was obtained from Wako and was used as received. ArO• was prepared according to a published procedure.[25–29] Ethanol (Wako) and acetonitrile (Aldrich) were dried and purified by distillation. Triton X-100 is an extra-pure grade reagent commercially available from Nacalai Tesque and was used as received. All buffer solutions were prepared using deionized water purified with a Millipore-Q system. The pH of the solutions was adjusted using the following 0.1 M buffer solutions; pH 4.0–5.0, CH_3COONa–CH_3COOH; pH 6.0–8.0, Na_2HPO_4–KH_2PO_4; pH 9.0–11.0, Na_2CO_3–$NaHCO_3$.[25,26] The concentration of TX-100 in the micelle solutions was kept at 5.0 wt %. The sample solutions were deoxygenated by bubbling nitrogen gas before the experiments.

The kinetic data were obtained using a Unisoku RS-450 or RSP-1000 stopped-flow spectrophotometer by mixing equal volumes of a HCA solution and ArO• or α-Toc• solution at 25 °C.[27] The α-Toc• radical was prepared by mixing equal volumes of α-tocopherol and ArO• solutions 2 seconds prior to the mixing of HCA and α-Toc• solutions using the double-mixing unit of the RSP-1000.[27] The reactions were studied under pseudo-first-order conditions for HCA, and the absorption decay of ArO• (α-Toc•) was well-characterized as a single-exponential decay. Detailed experimental procedures have been reported elsewhere.[25–29]

Pseudo-first-order rate constants (k_{obsd}) for the scavenging reaction of ArO• or α-Toc• by HCAs were estimated by using a non-

linear least-squares fitting method to a single-exponential curve from the decrease in the absorbance at 385 or 580 nm of ArO• or the absorbance at 420 nm of α-Toc• (Fig. 3). The rate constant (k_{obsd}) is given by[25–27]

$$k_{obsd} = k_0 + k_s[HCA], \quad (1)$$

where k_0 is the first-order rate constant for the natural decay of ArO• or α-Toc• in the medium. The second-order rate constant (k_s or k_r) was obtained as a slope of plots of k_{obsd} versus the concentration of HCA ([HCA]).

Cyclic voltammetry measurements were performed in acetonitrile containing 0.10 M tetrabutylammonium perchlorate under a nitrogen atmosphere at 15 °C using platinum electrodes and an Ag/Ag^+ reference electrode with a BAS CV-50W cyclic voltammetric analyzer. Under these conditions, ferrocene, as a standard sample, had a half-peak potential ($E_{1/2}$) of +40 mV vs Ag/Ag^+.

Results and Discussion

Aryloxyl Radical-Scavenging Rate Constant (k_s) in Ethanol. The second-order rate constants (k_s) for the ArO• scavenging reaction involving HCAs and related phenols (Fig. 1) are listed in Table 1, together with those reported for epicatechin and epigallocatechin gallate (EGCG) (Fig. 2).[27]

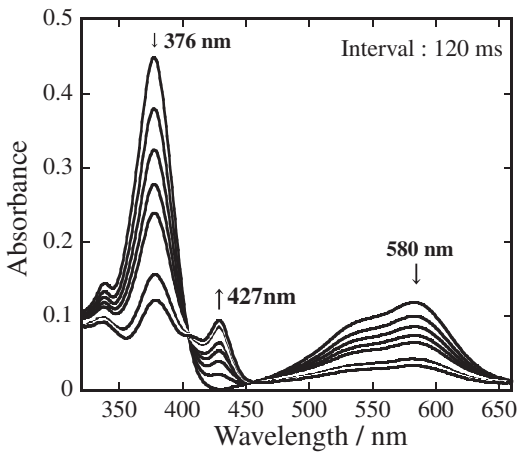

Fig. 3. Time evolution of the absorption spectrum of ArO• in the reaction with α-tocopherol in ethanol at 25 °C.

The k_s values were widely spread (0.1 to $5.21 \times 10^3\ M^{-1}\ s^{-1}$). The k_s value of CA ($5.54 \times 10\ M^{-1}\ s^{-1}$) was much smaller than those of α-tocopherol ($5.21 \times 10^3\ M^{-1}\ s^{-1}$) and EGCG ($k_s = 3.36 \times 10^2\ M^{-1}\ s^{-1}$), and on the same order as that for BHT (butylated hydroxytoluene, 2,6-di-*t*-butyl-4-methylphenol, $k_s = 3.50 \times 10\ M^{-1}\ s^{-1}$).[27] The k_s values for methyl caffeate and CGA were on the same order as that of CA, and RA ($k_s = 9.92 \times 10\ M^{-1}\ s^{-1}$) had the highest activity among the HCAs. The k_s values for FA ($1.44 \times 10\ M^{-1}\ s^{-1}$) was comparable to the value reported for half-curcumin (4-(4-hydroxy-3-methoxyphenyl)-3-buten-2-one, $k_s = 1.53 \times 10\ M^{-1}\ s^{-1}$),[26] and 1/4 of that for CA. The activity of isoeugenol which has no carboxyl group in its side-chain was higher than FA and half-curcumin. 4-Hydroxycinnamic acid which has no substituent at the position ortho to its phenol OH had almost no activity ($k_s = 1.0 \times 10^{-1}\ M^{-1}\ s^{-1}$). The k_s value of creosol (2-methoxy-4-methylphenol) was 9 times larger than that of guaiacol (2-methoxyphenol). The results suggest that the free radical-scavenging activities of these phenol compounds strongly depend on both ortho- and para-substituents on the phenol moiety. Existence of strong electron-donating substituents, such as hydroxy, methoxy, and methyl groups, causes an increase in the k_s values.

The free radical-scavenging activities of these phenolic compounds should be related to their oxidation potentials. Figure 4 shows the semi-logarithm plots for k_s versus the anodic peak oxidation potential (E_p) for HCAs and related phenols. A pseudo-linear relationship between $\log k_s$ and E_p was observed for the HCAs except for RA. This behavior is similar to one of the features of hydrogen atom transfer (HAT) reactions. Therefore, similar to half-curcumin,[26] the free radical-scavenging reactions involving HCAs and related phenols in ethanol progress as a typical HAT. As described above, higher radical-scavenging activity was observed for CA derivatives comparing with FA derivatives. It can be explained by the lower oxidation potential of CA derivatives due to the existence of the *o*-hydroxy group which is more electron-donating than the *o*-methoxy group. The large activity of isoeugenol, which is one of the *o*-methoxyphenols, also comes from its lower oxidation potential compared to those of FA and half-curcumin. Isoeugenol has an electron-donating methyl group in place

Table 1. Second-Order Rate Constants for the Scavenging Reactions of ArO• (k_s) and α-Tocopheroxyl (k_r) in Ethanol and Peak Oxidation Potentials (E_p)

	$k_s/M^{-1}\ s^{-1}$	$k_r/M^{-1}\ s^{-1}$	E_p/mV vs Ag/Ag^+
	in Ethanol		in Acetonitrile
Caffeic acid (CA)	5.54×10	2.31×10^2	900
Methyl caffeate	4.56×10	—	937
Chlorogenic acid (CGA)	6.83×10	2.63×10^2	881
Rosmarinic acid (RA)	9.92×10	9.05×10^2	939
Isoeugenol	9.76×10	9.28×10^2	844
Ferulic acid (FA)	1.44×10	3.68	966
4-Hydroxycinnamic acid	1.0×10^{-1}	—	1200
Half-curcumin	1.53×10	—	953
Guaiacol	4.8	$\approx 10^{-1}$	1050
Creosol	4.23×10	3.5×10^{-1}	986
Epicatechin (EC)	1.32×10^2	7.58×10^{2} a)	865
Epigallocatechin gallate (EGCG)	3.36×10^2	2.39×10^{4} a)	798

a) The values in 5:1 (v/v) ethanol–water mixture.[27]

of the electron-withdrawing carboxyl group in the side chain. Another factor for larger activity of CA derivatives is the stabilization effect of the corresponding radicals produced in the radical-scavenging reaction through intramolecular hydrogen bonding.[31,32] As shown in Fig. 5, intramolecular hydrogen bonding between two hydroxy groups should exist in the catechol moiety of CA derivatives. Through the intramolecular hydrogen migration, the corresponding phenoxyl radical generated by the radical-scavenging reaction is stabilized by the delocalization of the unpaired electron. This was also suggested in the theoretical studies for CA derivatives.[31,32] This type stabilization of the radical is not expected in the case of FA derivatives. This might be the reason why the activities of CA derivatives are larger than those of FA derivatives.

The k_s value of RA was about twice of those of CA and methyl caffeate, while their E_p values were similar. This might suggest that RA almost acts as a dimer of CA molecules in the free radical-scavenging reaction. In the literature, the high activity of EGCG in the VE-regeneration reaction was explained by intramolecular π–π stacking interaction between epigallocatechin and gallate moieties in EGCG,[25] and the condensed structures of polyphenols sometimes contribute to the free radical-scavenging.[26]

VE-Regeneration Rate Constant (k_r) in Ethanol. The second-order rate constants (k_r) for HCAs on the natural VE (α-tocopherol)-regeneration reaction are listed in Table 1. The k_r value for CA ($2.31 \times 10^2\ M^{-1}\ s^{-1}$) in ethanol is 3–4 orders smaller than those of ubiquinol-10 ($2.15 \times 10^5\ M^{-1}\ s^{-1}$) and sodium ascorbate ($2.68 \times 10^6\ M^{-1}\ s^{-1}$).[33] RA has the largest k_r ($9.05 \times 10^2\ M^{-1}\ s^{-1}$) in the HCAs, while the k_r value of FA is very small ($3.68\ M^{-1}\ s^{-1}$). *o*-Methoxyphenols (guaiacol and creosol) have almost no activity for the VE-regeneration reaction ($k_r \lessapprox 10^{-1}\ M^{-1}\ s^{-1}$). This behavior for the VE-regenerating activity of HCAs was slightly different from that for the previous free radical scavenging. For CA derivatives, the k_r values were 5–10 times larger than the k_s values. On the other hand, a notable decrease in k_r was observed for FA and *o*-methoxyphenols. In other words, for efficient VE-regeneration, the antioxidants are required to have lower oxidation potentials than the threshold value. From the k_r value, the oxidation potential of FA is nearly equal to the threshold.

The free radical-scavenging and VE-regenerating activities of these HCAs are not high compared to those of other naturally existing antioxidants. However, taking into account the large daily intakes by animals and human, HCAs may also act as good antioxidants for both the free radical and the VE radical which is produced by the antioxidant reaction involving VE towards AOS, such as lipid-peroxyl radicals in living bodies.

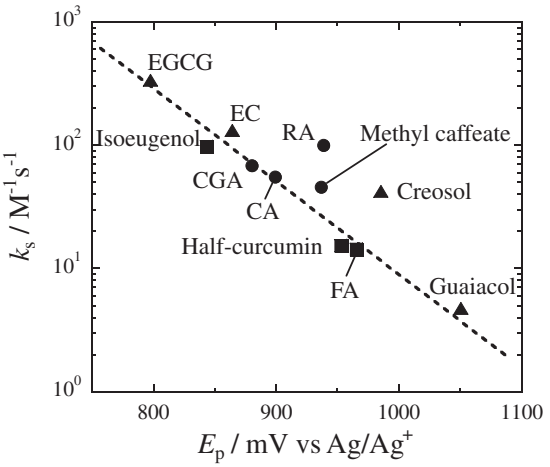

Fig. 4. Semi-logarithm plots of k_s versus E_p for CA derivatives (●), FA derivatives (■), and the related phenols (▲).

pH Dependence of the Aryloxyl Radical-Scavenging Rate (k_s) in TX-100 Micelle Solutions. The second-order rate constants (k_s) of the ArO• scavenging reaction by FA, CA, and CGA in the TX-100 micelle solutions were measured at various pH values. Previous kinetic studies showed that the second-order rate constants for the radical-scavenging reaction of vitamin C, flavonoids, and curcumin have notable pH dependence according to the variation of the mole fractions of some species produced by the acid–base dissociation equilibrium.[25–30] Figure 6a shows the plots of k_s observed for FA versus pH in the TX-100 micelle solutions. The k_s values in the micelle solution at pH 6–8 are one order of magnitude larger than that obtained in ethanol (see Table 2). With an increase in pH, the k_s values in the micelle solution decrease gradually between pH 4–6, are constant at pH 6–8, and decreased rapidly at pH 8–10. The measurements at pH < 4 were unsuccessful, because ArO• is unstable in these conditions. This pH-dependent behavior of k_s is similar to that observed for half-curcumin reported previously.[26]

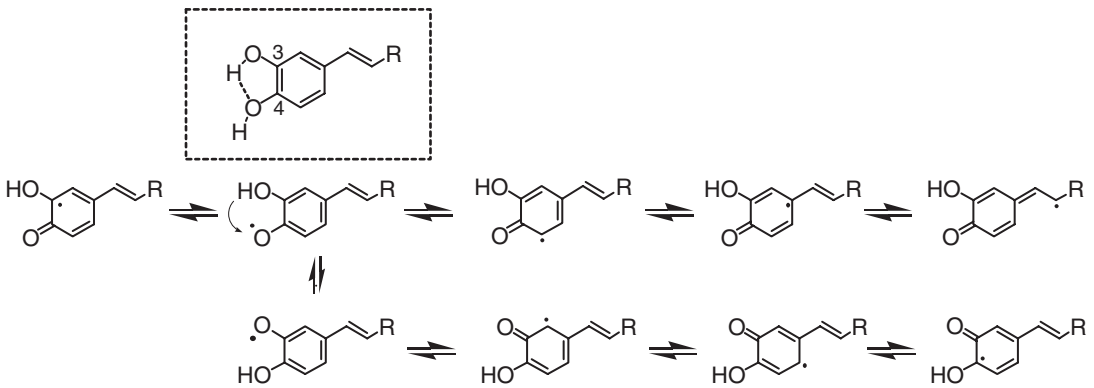

Fig. 5. Possible resonance structure of the radical produced from caffeic acid.

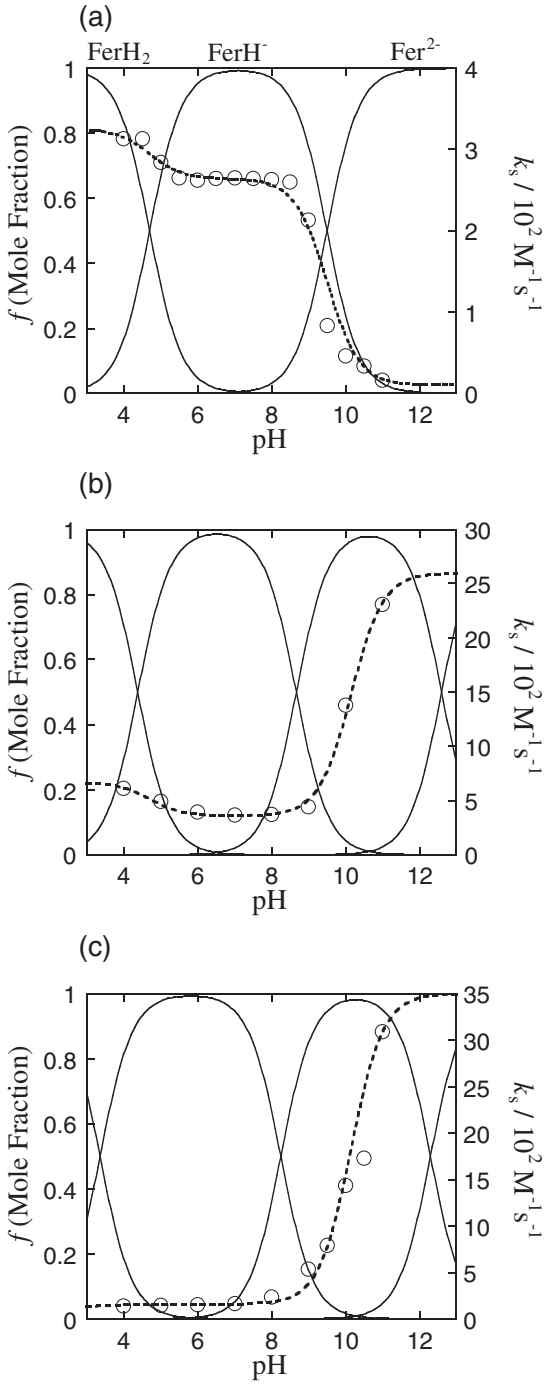

Fig. 6. Plots of k_s versus pH in TX-100 micelle solutions for (a) ferulic acid, (b) caffeic acid, and (c) chlorogenic acid. Solid lines show calculated mole-fraction of neutral and dissociated anion forms versus pH. Broken lines show the simulated k_s^{total} versus pH plots (see text).

The proton-dissociation equilibrium for FA is shown in Fig. 7a. Proton dissociation from the carboxyl group of FA due to acid–base equilibrium was reported to occur around pH 4.6 ($pK_{a1} = 4.58$), and the proton dissociation from its phenolic OH was to do around pH 9.4 ($pK_{a2} = 9.39$).[34] The neutral form ($FerH_2$), dissociated monoanion form ($FerH^-$), and dissociated dianion form (Fer^{2-}) of FA should have different rate constants for radical scavenging. The pH dependence

Table 2. pH Dependence of the Second-Order Rate Constants (k_s) for the ArO• Scavenging Reactions of Ferulic Acid, Caffeic Acid, and Chlorogenic Acid in TX-100 Micelle Solution (5.0 wt %) at 25.0 °C

	$k_s/M^{-1}\,s^{-1}$		
pH	Ferulic acid	Caffeic acid	Chlorogenic acid
4.0	3.13×10^2	6.15×10^2	1.40×10^2
4.5	3.13×10^2		
5.0	2.84×10^2	4.92×10^2	1.48×10^2
5.5	2.65×10^2		
6.0	2.62×10^2	3.94×10^2	1.57×10^2
6.5	2.64×10^2		
7.0	2.65×10^2	3.68×10^2	1.67×10^2
7.5	2.64×10^2		
8.0	2.63×10^2	3.75×10^2	2.37×10^2
8.5	2.60×10^2		
9.0	2.13×10^2	4.43×10^2	5.39×10^2
9.5	8.32×10		7.96×10^2
10.0	4.62×10	1.38×10^3	1.44×10^3
10.5	3.34×10		1.73×10^3
11.0	1.63×10	2.31×10^3	3.09×10^3

of the total reaction rate (k_s^{total}) may be represented as the sum of contributions for these species (Eq. 2),[26]

$$k_s^{total} = k_s^a f(FerH_2) + k_s^b f(FerH^-) + k_s^c f(Fer^{2-}), \qquad (2)$$

where k_s^a, k_s^b, and k_s^c are second-order rate constants (independent of pH) for $FerH_2$, $FerH^-$, and Fer^{2-}, respectively, and $f(FerH_2)$, $f(FerH^-)$, and $f(Fer^{2-})$ are pH dependent mole-fractions of $FerH_2$, $FerH^-$, and Fer^{2-}, respectively. Using a similar procedure as reported for curcuminoids,[26] the determination of these rate constants (k_s^a, k_s^b, and k_s^c) and the dissociation constants (pK_{a1} and pK_{a2}) for FA was performed. The k_s^b value was determined to be $2.64 \times 10^2\,M^{-1}\,s^{-1}$ by averaging the observed k_s values at pH 5.5–8. The k_s^a value was assumed to be slightly larger than the k_s value at pH 4.0 ($3.13 \times 10^2\,M^{-1}\,s^{-1}$), because the mole fraction of the neutral form ($FerH_2$) will be more than 0.8 at pH 4. The k_s^c value is considered to be slightly smaller than the k_s value at pH 11 ($1.63 \times 10\,M^{-1}\,s^{-1}$) because the di-anion form (Fer^{2-}) shows the smallest activity amongst the three species and the largest contribution at pH 11. Simulations of the pH dependence of k_s were performed by varying k_s^a, k_s^c, pK_{a1}, and pK_{a2} values. From the best fit, each value for FA was estimated to be $pK_{a1} = 4.70$, $pK_{a2} = 9.50$, $k_s^a = 3.25 \times 10^2\,M^{-1}\,s^{-1}$, and $k_s^c = 1.0 \times 10\,M^{-1}\,s^{-1}$. These pK_a values estimated in the present study agree well with those reported ($pK_{a1} = 4.58$, $pK_{a2} = 9.39$).[34] Solid lines in Fig. 6a show mole fractions of three FA species ($FerH_2$, $FerH^-$, and Fer^{2-}) versus pH, calculated using $pK_{a1} = 4.70$ and $pK_{a2} = 9.50$. As shown in Fig. 6a, good accordance between the measured rate constants and simulated curve (the broken line) was obtained. In the pH > 5.5 region, the pH-dependent behavior of FA was similar to that of half-curcumin reported.[26] As reported for half-curcumin, the phenolic OH proton of the FA derivatives is necessary to scavenge free radicals, indicating that the free radical scavenging is HAT reaction.[26] The free radical scavenging of Fer^{2-} should progress as an electron-transfer (ET) process because Fer^{2-} has no phenol-proton for the HAT activity. The radi-

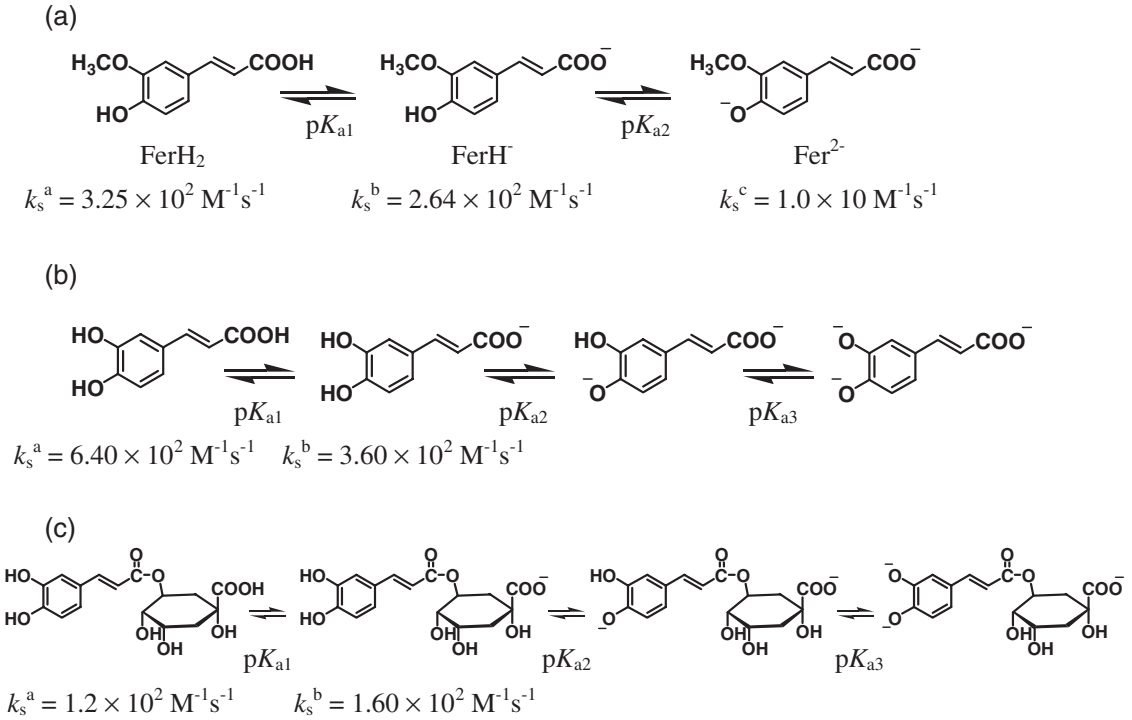

Fig. 7. Acid–base dissociation equilibriums for (a) ferulic acid, (b) caffeic acid, and (c) chlorogenic acid.

cal-scavenging activity of Fer^{2-} by ET was quite lower than that of $FerH^-$ by HAT.

On the other hand, a small decrease in k_s at low pH (pH < 5) is caused by the ArO• radical which exists inside the micelle because of its lipophilic property. It is difficult for ionic species, such as $FerH^-$ and Fer^{2-}, to access the inside of a micelle because of poor solubility in the hydrophobic phase.[35] The rate constant for the ArO• scavenging reaction in the micelle solution is controlled by both the amount of the antioxidant close to ArO• inside the micelle and the reactivity of the antioxidant in a molecular unit. Although the reactivity of the neutral form ($FerH_2$) is almost the same as or less than that of the monoanion form ($FerH^-$), the k_s^a value for the neutral form at the low pH range are apparently larger than the k_s^b value for the monoanion form because the neutral form has a higher solubility to the micelle.[27,35] Therefore, in the micelle system, the neutral form has the highest activity among three forms of FA arising from its acid–base dissociation equilibrium. The dissociation of the carboxyl-proton in FA has little effect on the antioxidant activity.

Figures 6b and 6c show the plots of k_s for CA and CGA versus pH in TX-100 micelle solutions, respectively. The pH-dependent behaviors of k_s observed for these *o*-dihydroxycinnamic acid derivatives are different from those of FA and half-curcumin.[26] The k_s values of CA (Fig. 6b) decrease gradually at pH 4–6, are constant at pH 6–8, and increase rapidly at pH 9–11 with an increase in pH. In the case of CGA (Fig. 6c), with an increase in pH, the k_s values increase slightly from 1.40×10^2 at pH 4.0 to 1.67×10^2 at pH 7.0, and then from $2.37 \times 10^2\ M^{-1}\ s^{-1}$ at pH 8.0 to $3.09 \times 10^3\ M^{-1}\ s^{-1}$ at pH 11.0.

The proton-dissociation equilibriums for CA and CGA are shown in Figs. 7b and 7c, respectively. The acid–base dissociation constants were reported as $pK_{a1} = 4.38$, $pK_{a2} = 8.67$, and $pK_{a3} = 12.6$ for CA, and $pK_{a1} = 3.36$, $pK_{a2} = 8.25$, and $pK_{a3} = 12.3$ for CGA.[34] The value of $pK_{a2} = 8.67$ for CA (8.25 for CGA) for the dissociation of the first phenolic OH proton is smaller than those (9.2–9.7) for FA, half-curcumin, dihydrocaffeic acid, and catechol.[26,34] This might be due to both the stabilization effect of the catechol anion structure through intramolecular hydrogen bonding with the neighboring OH proton (Fig. 5) and a resonance effect induced by the conjugated double bond in the side chain. The decrease in k_s at low pH (pH < 5) for CA is explained the same way as that in the case of FA, i.e., it is due to the difference in the molecular activity and the solubility into the micelle between the neutral and monoanion forms generated by the acid–base dissociation equilibrium of the carboxyl-proton of CA.

On the other hand, the large increases in k_s at high pH (pH > 9) for CA and CGA cannot be explained via the HAT mechanism. The solid lines in Figs. 6b and 6c show the pH dependence of the mole fractions calculated for four kinds of species generated from CA and CGA by the acid–base equilibriums, using the pK_a values reported, respectively. The pH dependence curves of k_s for CA and CGA in the high pH region (pH > 9) are not consistent with the corresponding mole-fraction curves. Tentatively, assuming that the pH dependent behavior of k_s for CA and CGA occurs the same way as for FA, the determination of the rate constants (k_s^a, k_s^b, and k_s^c) and the dissociation constants (pK_{a1} and pK_{a2}) was done by fitting k_s^{total} calculated from Eq. 2 to k_s observed. The broken lines in Figs. 6b and 6c show plots of the simulated k_s^{total} versus pH using the estimated values (for CA: $pK_{a1} = 4.70$, $pK_{a2} = 10.15$, $k_s^a = 6.40 \times 10^2\ M^{-1}\ s^{-1}$, $k_s^b = 3.60 \times 10^2\ M^{-1}\ s^{-1}$, and $k_s^c = 2.6 \times 10^3\ M^{-1}\ s^{-1}$; for CGA: $pK_{a1} =$

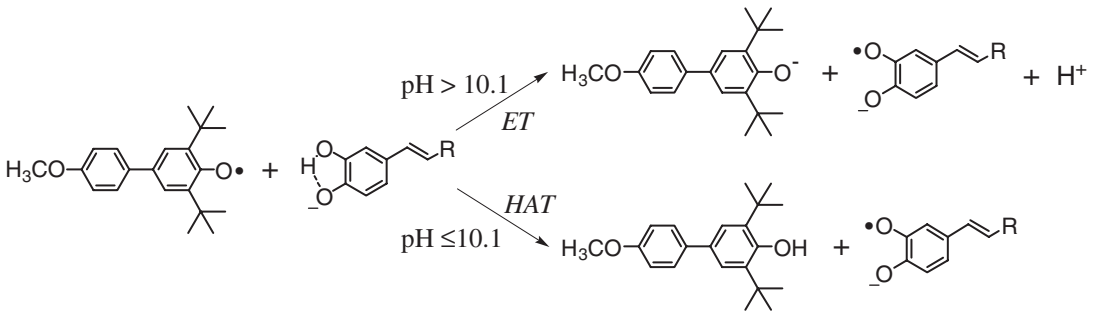

Fig. 8. Possible mechanism of pH-dependent antioxidant action of CA and CGA.

3.4, $pK_{a2} = 10.15$, $k_s^a = 1.2 \times 10^2\ M^{-1}\ s^{-1}$, $k_s^b = 1.60 \times 10^2\ M^{-1}\ s^{-1}$, and $k_s^c = 3.5 \times 10^3\ M^{-1}\ s^{-1}$). There was good accordance between the measured rate constants and simulated curves, however, the estimated pK_{a2} values are different from the reported values. Furthermore, the UV–visible absorption spectrum measured for CGA in the TX-100 (5.0 wt %) micelle solution red-shifted from 325 nm at pH 7.3 to 370 nm at pH 9.0, probably due to the phenolic proton dissociation. This result is consistent with $pK_{a2} = 8.25$ reported for the dissociation of the first phenolic OH proton of CGA.[34] In other words, it cannot be determined that the rapid increases of k_s in high pH region observed for CA and CGA are due to the dissociation of the phenolic OH-proton.

The pH dependent behavior is probably due to strong intramolecular hydrogen bonding in the catechol monoanion moiety of CA (CGA) and the contribution of the ET process in the antioxidant action. As previously described, several antioxidants that have multiple OH groups in one molecule, such as ascorbic acid, catechins, flavonoids, and curcumin, show an increase in the free radical-scavenging activity with an increase of pH.[25–30] This behavior was explained by the decrease in the oxidation potential of the antioxidants in the anion forms by the electron-donating effect of the minus charge. In the case of CA, while the first OH-proton of its catechol moiety dissociates around pH 8.7, an increase of k_s was not observed there. This might be explained as follows. When the first OH proton dissociation occurs, the second OH proton, which contributes to the HAT reaction, may be trapped via the intramolecular hydrogen bonding between 3- and 4-phenolate-oxygens. The hydrogen bonding is known to stabilize protons strongly. As a result, the pK_a value for the dissociation of the second OH proton is much larger ($pK_{a3} = 12.6$) than that for the first one ($pK_{a2} = 8.67$). Therefore, HAT activity for the second OH does not increase with the first OH dissociation.

In this situation, the increase in k_s starting around pH 9 can be explained by the ET process from CA (CGA) to ArO•. As reported before, the ArO• scavenging through the ET process occurs in the cases where the antioxidants have comparatively low oxidation potential.[26,27] For example, the anion forms of curcumin and edaravone, which have no reactive hydrogen for the HAT reaction, can scavenge ArO• by ET. Why does the increase in k_s start around pH 9, while the OH-proton of CA (CGA) starts to dissociate around pH 7? The mechanism of ArO• scavenging reaction changes. The dissociation constant of the phenol OH-proton of ArOH, which is the reduced form of ArO•, is estimated to be 10.0–10.5. Therefore, at pH > 10, the product from ArO• in the scavenging reaction should be ArO^- (the anion form of ArOH) (Fig. 8). Accordingly, it is reasonable to consider that the ET process to ArO• from CA (CGA) can occur at pH $\geq$ 10. The reaction rate of this ET process may be faster than that of the HAT process producing ArOH because the product is more stable.

On the other hand, for FA and half-curcumin, the contribution of ET in ArO• scavenging was very small. The results suggest that the threshold oxidation potential exists in the ArO• scavenging action by ET. In fact, the oxidation potential of CA and CGA are 50–90 mV smaller than those for FA and half-curcumin.

According to the present kinetic study, we concluded that the radical-scavenging activity of HCAs in ethanol mainly comes from the HAT reaction of the phenolic OH-protons. The higher radical-scavenging activity observed for CA derivatives than for FA derivatives is explained by the lower oxidation potential arising from the existence of more electron-donating hydroxy groups and the stabilization of the corresponding radicals induced by intramolecular hydrogen bonding. The pH dependence of k_s for FA in the aqueous micelle solutions suggests that phenolic OH proton is important in the radical-scavenging reaction. On the other hand, the pH dependence of k_s for CA and CGA suggests that other factors are involved in the radical-scavenging action; for example, the intramolecular hydrogen bonding and ET. The free radical-scavenging and VE-regenerating activities of these HCAs are not that remarkable compared to those of other naturally existing antioxidants, such as tocopherols and catechins. However, taking into account the large daily intake by animals and human and high solubility in both aqueous and organic media, HCAs may act as good antioxidants in living bodies both in water-rich regions, such as body fluids, and in hydrophobic regions, such as membranes inside of living bodies.

This work was supported by Grant-in-Aids for the Scientific Research C (No. 16550016) and Scientific Research on Priority Areas "Application of Molecular Spins: Nanomagnets to Biological Spin Systems" (Area No. 769, 15087104) from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT).

References

1 K. Herrmann, *Crit. Rev. Food Sci. Nutr.* **1989**, *28*, 315.

2 H. Wang, G. J. Provan, K. Helliwell, *Food Chem.* **2004**, *87*, 307.

3 M. R. Olthof, P. C. H. Hollman, M. B. Katan, *J. Nutr.* **2001**, *131*, 66.

4 M. J. del Bano, J. Lorente, J. Castillo, O. Benavente-Garcia, J. A. del Rio, A. Ortuno, K.-W. Quirin, D. Gerard, *J. Agric. Food Chem.* **2003**, *51*, 4247.

5 M. Petersen, M. S. J. Simmonds, *Phytochemistry* **2003**, *62*, 121.

6 M. Nardini, M. D'Aquino, G. Tomassi, V. Gentili, M. Di Felice, C. Scaccini, *Free Radical Biol. Med.* **1995**, *19*, 541.

7 J. Laranjinha, O. Vieira, V. Madeira, L. Almeida, *Arch. Biochem. Biophys.* **1995**, *323*, 373.

8 J. Laranjinha, E. Cadenas, *Life* **1999**, *48*, 57.

9 V. Roginsky, *Arch. Biochem. Biophys.* **2003**, *414*, 261.

10 V. Raneva, H. Shimasaki, Y. Ishida, N. Ueta, E. Niki, *Lipids* **2001**, *36*, 1111.

11 W. Wenfeng, L. Jian, Y. Side, L. Zhirui, Z. Zhihua, Z. Jiashan, L. Nianyun, *Radiat. Phys. Chem.* **1995**, *46*, 41.

12 S. Samman, B. Sandström, M. B. Toft, K. Bukhave, M. Jensen, S. S. Sørensen, M. Hansen, *Am. J. Clin. Nutr.* **2001**, *73*, 607.

13 H. Ito, T. Miyazaki, M. Ono, H. Sakurai, *Bioorg. Med. Chem.* **1998**, *6*, 1051.

14 C. A. Gomes, T. G. Girão Da Cruz, J. L. Andrade, N. Milhazes, F. Borges, M. P. M. Marques, *J. Med. Chem.* **2003**, *46*, 5395.

15 M. Kampa, V. I. Alexaki, G. Notas, A. P. Nifli, A. Nistikaki, A. Hatzoglou, E. Bakogeorgou, E. Kouimtzoglou, G. Blekas, D. Boskou, A. Gravanis, E. Castanas, *Breast Cancer Res.* **2004**, *6*, R63.

16 S. Foley, S. Navaratnam, D. J. McGarvey, E. J. Land, T. G. Truscott, C. A. Rice-Evans, *Free Radical Biol. Med.* **1999**, *26*, 1202.

17 J. H. Chen, C.-T. Ho, *J. Agric. Food Chem.* **1997**, *45*, 2374.

18 R. H. Bisby, A. W. Parker, *Free Radical Res.* **2001**, *35*, 85.

19 H. Hotta, S. Nagano, M. Ueda, Y. Tsujino, J. Koyama, T. Osakai, *Biochim. Biophys. Acta* **2002**, *1572*, 123.

20 H. Kikuzaki, M. Hisamoto, K. Hirose, K. Akiyama, H. Taniguchi, *J. Agric. Food Chem.* **2002**, *50*, 2161.

21 W. Bors, C. Michel, K. Stettmaier, Y. Lu, L. Y. Foo, *Biol. Res.* **2004**, *37*, 301.

22 W. Bors, C. Michel, K. Stettmaier, Y. Lu, L. Y. Foo, *Biochim. Biophys. Acta* **2003**, *1620*, 97.

23 M. C. Foti, C. Daquino, C. Geraci, *J. Org. Chem.* **2004**, *69*, 2309.

24 F. A. M. Silva, F. Borges, C. Guimaraes, J. L. F. C. Lima, C. Matos, S. Reis, *J. Agric. Food Chem.* **2000**, *48*, 2122.

25 K. Mukai, S. Mitani, K. Ohara, S. Nagaoka, *Free Radical Biol. Med.* **2005**, *38*, 1243.

26 K. Ohara, W. Mizukami, A. Tokunaga, S. Nagaoka, H. Uno, K. Mukai, *Bull. Chem. Soc. Jpn.* **2005**, *78*, 615.

27 K. Ohara, A. Fujii, Y. Ichimura, K. Sato, K. Mukai, *Bull. Chem. Soc. Jpn.* **2006**, *79*, 421.

28 K. Mukai, W. Oka, K. Watanabe, Y. Egawa, S. Nagaoka, J. Terao, *J. Phys. Chem. A* **1997**, *101*, 3746.

29 K. Mukai, M. Nishimura, S. Kikuchi, *J. Biol. Chem.* **1991**, *266*, 274.

30 K. Mukai, Y. Kanesaki, Y. Egawa, S. Nagaoka, *Phytochemicals and Phytopharmaceuticals*, ed. by F. Shahidi, C.-T. Ho, AOCS Press, Champaign, Illinois, **2000**, Chap. 20.

31 L. Kong, Z.-L. Sun, L.-F. Wang, H.-Y. Zhang, S.-D. Yao, *Helv. Chim. Acta* **2004**, *87*, 511.

32 H. Cao, W.-X. Cheng, C. Li, X.-L. Pan, X.-G. Xie, T.-H. Li, *THEOCHEM* **2005**, *719*, 177.

33 K. Mukai, S. Itoh, H. Morimoto, *J. Biol. Chem.* **1992**, *267*, 22277.

34 M. L. Adams, B. O'Sullivan, A. J. Downard, K. J. Powell, *J. Chem. Eng. Data* **2002**, *47*, 289, and reference there in.

35 K. Ohara, R. Watanabe, Y. Mizuta, S. Nagaoka, K. Mukai, *J. Phys. Chem. B* **2003**, *107*, 11527.