Kinetics and Mechanism for the Scavenging Reaction of the 2,2-Diphenyl-1-picrylhydrazyl Radical by Synthetic Artepillin C Analogues

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The kinetics for the reaction of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^\bullet$) with artepillin C, a prenylated phenylpropanoid found specifically in Brazilian propolis, and its analogues was examined in deaerated acetonitrile (MeCN) to shed light on the mechanism for the radical-scavenging reaction of phenolic antioxidants as well as on the structure–activity relationship. Among the examined analogues, a compound having a catechol moiety is found to have the largest second-order rate constant (k) for the DPPH$^\bullet$-scavenging reaction. The deuterium kinetic isotope effect of 1.6 was observed for the DPPH$^\bullet$-scavenging reaction of artepillin C in the presence of 0.13 M CD_3OD or CH_3OH in deaerated MeCN at 298 K. The $\log k$ values were found to be linearly correlated with calculated energy difference values (D_{HT}, HT: hydrogen transfer) between the artepillin C analogues and the corresponding phenoxyl radicals, while such a linear correlation cannot be observed between the $\log k$ values and calculated ionization potentials (IP), $D_{HT} - IP$, or experimental one-electron-oxidation potentials of the artepillin C analogues. These results together with a calculated structure of the transition state for the reaction between the artepillin C analogue and DPPH$^\bullet$ suggest that the DPPH$^\bullet$-scavenging reaction of the artepillin C analogues in deaerated MeCN proceeds via a one-step hydrogen-atom transfer from the phenolic OH group to DPPH$^\bullet$ rather than an electron transfer followed by proton transfer.

Artepillin C [(*E*)-3-{4-hydroxy-3,5-bis(3-methyl-2-butenyl)-phenyl}-2-propenoic acid] (**1H**) (Chart 1), a major component (>5%) of Brazilian propolis,[1] is a prenylated phenylpropanoid, which is found specifically in Brazilian propolis and exhibits important biological activities, such as antitumor,[2] apoptosis-inducing,[3] immunomodulating,[4] tumor anti-angiogenesis,[5] potential chemopreventive,[6] anti-leukemic,[7] and antioxidative activities.[8] It is known that there are two possible pathways for antioxidative radical-scavenging reactions of phenolic compounds.[9–12] One is a one-step hydrogen-atom transfer (Scheme 1a). The other is an electron transfer followed by proton transfer (Scheme 1b).[9–12] These mechanisms are also known to strongly depend on solvent.[10b,10d,13] However, very little is known about the kinetics, mechanism, and structure–activity relationship for the radical-scavenging reaction of **1H** and its analogues. Recently, we have reported that the scavenging reaction of the α,α-dimethylbenzylperoxyl (cumylperoxyl) radical ($PhCMe_2OO^\bullet$) by **1H** proceeds via the one-step hydrogen-atom transfer mechanism in propionitrile (EtCN) at a low temperature (203 K).[14] We have also reported the structure–activity relationship by comparing the rates of $PhCMe_2OO^\bullet$-scavenging reaction by structurally modified artepillin C derivatives.[15] The energy difference values (D_{HT}, HT: hydrogen transfer) between the artepillin C derivatives and the corresponding phenoxyl radicals are linearly correlated with the logarithm of the second-order rate constants (k) for $PhCMe_2OO^\bullet$-scavenging reaction.[15] However, for the kinetic investigation, stable radicals are advantageous, because their concentrations are readily directly measurable at room temperature. Among them, the 2,2-diphenyl-1-picrylhydrazyl radical

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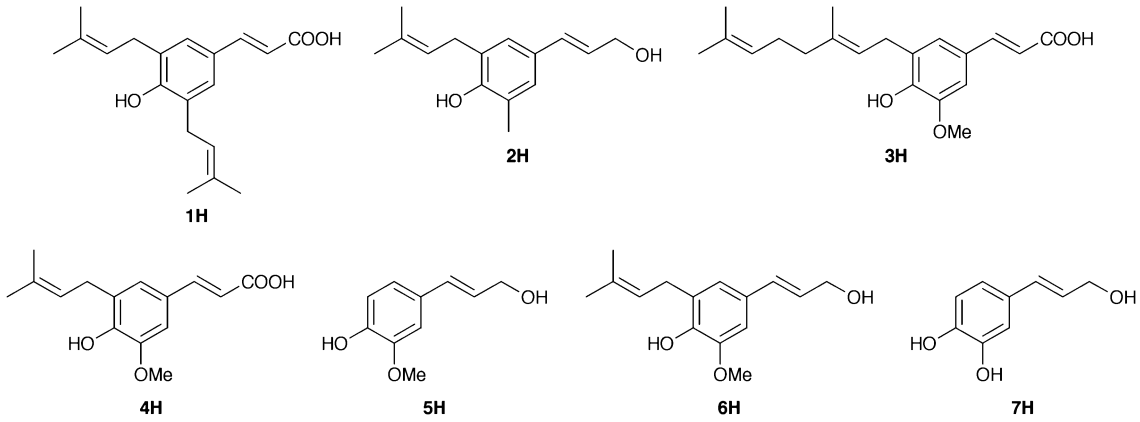
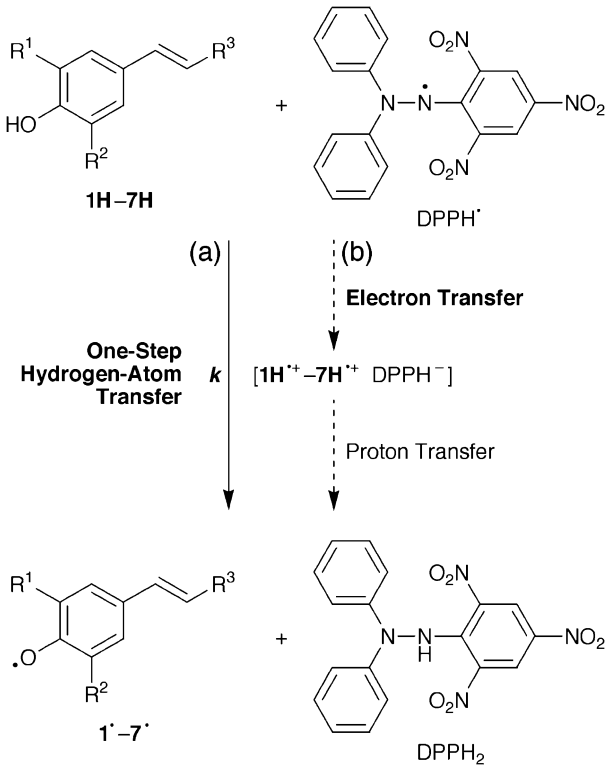


Chart 1.



Scheme 1. Two possible pathways for the DPPH•-scavenging reaction of **1H–7H** (R^1, R^2, R^3: See Chart 1).

(DPPH•) has been reported as a reactive hydrogen acceptor and found to be useful to examine antioxidative activity.[10a,13,16,17] In contrast to the case of $PhCMe_2OO^•$, DPPH• is also known to act as a good electron acceptor in hydrogen-transfer reactions with NADH (β-nicotinamide adenine dinucleotide reduced form) analogues.[18] Thus, it is interesting to investigate the mechanism of hydrogen-transfer reaction from artepillin C analogues to DPPH• in terms of the one-step hydrogen-atom transfer vs. electron transfer followed by proton transfer. We report herein the kinetics and mechanism for the reaction of **1H** and its analogues **2H–7H** (Chart 1) with DPPH• in deaerated acetonitrile (MeCN) at 298 K. The correlation between the $\log k$ values determined by the stopped-flow technique and calculated D_{HT} and ionization potential (IP) values as well as experimentally determined one-electron-oxidation potentials (E^0_{ox}) of **1H–7H** provides a valuable insight into the mechanism and structure–activity relationship of the radical-scavenging reactions of phenolic antioxidants.

Experimental

Materials. Artepillin C (**1H**) and its analogues **2H**, **4H**, and **6H** were synthesized by regioselective C-prenylation of the corresponding *ortho*-substituted phenols according to our established procedure.[15,19] Coniferyl alcohol (**5H**) was purchased from Aldrich. Caffeyl alcohol (**7H**) was obtained according to a literature procedure.[20] The synthesis of **3H** was carried out with 4-iodo-2-methoxyphenol as a starting material according to a literature procedure,[21] although the detailed procedure will be reported elsewhere. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.60 (s, 3H, $C(CH_3)_2$), 1.68 (d, $J =$ 0.90 Hz, 3H, $C(CH_3)_2$), 1.72 (s, 3H, $ArCH_2CHC(CH_3)$), 1.98–2.12 (m, 4H, $2CH_2$), 3.36 (d, $J = 7.3$ Hz, 2H, $ArCH_2$), 3.92 (s, 3H, $ArOCH_3$), 5.11 (m, 1H, $C(CH_3)CH_2CH_2CH$), 5.32 (m, 1H, $ArCH_2CH$), 5.98 (s, 1H, OH), 6.28 (d, $J = 15.8$ Hz, 1H, CHCOOH), 6.93 (d, $J = 1.7$ Hz, 1H, H_3–Ar), 6.98 (d, $J =$ 1.2 Hz, 1H, H_5–Ar), 7.65 (d, $J = 15.9$ Hz, 1H, ArCH); EI-MS (m/z) 331 (M^+). Anal. Calcd for $C_{20}H_{26}O_4$: C, 72.79; H, 7.93%. Found: C, 72.97; H, 7.99%. The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) was commercially obtained from Wako Pure Chemical Ind. Ltd., Japan and used as received. MeCN used as a solvent in both spectrophotometric and electrochemical measurements was purchased from Nacalai Tesque, Inc. Japan, and used as received. CH_3OH and CD_3OD used for measurements of the deuterium kinetic isotope effect were obtained from Nacalai Tesque, Inc. Japan and Wako Pure Chemical Ind. Ltd., Japan, respectively, and used as received. Tetrabutylammonium perchlorate (Bu_4NClO_4) used as a supporting electrolyte in electrochemical measurements was purchased from Tokyo Chemical Industry Co., Ltd., Japan, recrystallized from CH_3CH_2OH, and dried under vacuum at 313 K prior to use.[22]

Spectral and Kinetic Measurements. To avoid the effect of molecular oxygen (O_2), the reactions were carried out under strictly deaerated conditions. A continuous flow of argon (Ar) gas was bubbled through an MeCN solution (3.0 mL) containing DPPH• (4.0×10^{-5} mol dm^{-3}) or an antioxidant (5.0×10^{-4}–2.0×10^{-3} mol dm^{-3}) in a reservoir of the spectropho-

tometer for 7 min. The rates of DPPH•-scavenging reactions by **1H–7H** in deaerated MeCN were determined by monitoring the absorbance change at 516 nm due to DPPH• by a stopped-flow technique on a UNISOKU RSP-1000-02NM spectrophotometer at 298 K. The pseudo-first-order rate constants (k_{obs}) were determined by a least-squares curve fit. 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 $\rho > 0.999$.

Electrochemical Measurements. Second-harmonic alternating current voltammetry (SHACV)[23] was carried out with three conventional electrodes on an ALS-630A electrochemical analyzer with glassy carbon disk working electrode, platinum wire counter electrode, and Ag/AgCl reference electrode. Solution concentrations for the SHACV analysis were 1.0×10^{-3} mol dm^{-3} for all artepillin C derivatives. The working electrode was polished with BAS polishing alumina suspension and a polishing cloth and rinsed with Millipore Milli-Q water followed by CH_3OH prior to each measurement. The electrode was cycled several times in MeCN containing 0.1 mol dm^{-3} Bu_4NClO_4 within the range of 0 to +1.9 V to reassure the absence of voltammetric signal due to the supporting electrolyte. All electrochemical measurements were performed at 298 K. To minimize the effect of O_2 on the electrochemical behavior of these antioxidants, the inert atmosphere in the electrochemical cell was maintained throughout the experiments by bubbling Ar gas in the solution before the experiments and a continuous Ar gas flow over the solution during the measurements. Addition of 45 mV to the E^0_{ox} values vs. Ag/AgCl were carried out, to report the E^0_{ox} values vs. the saturated calomel electrode (SCE).[24]

Theoretical Calculations. Density functional theory (DFT) calculations were performed on an 8CPU workstation (PQS, Quantum Cube QS8-2400C-064). Geometry optimizations were carried out using the Becke3LYP and 6-31G(d) basis set for the phenoxyl radicals with the unrestricted Hartree–Fock (UHF) formalism as implemented in the Gaussian 09 program Revision A.02.[25] The D_{HT} values were determined by the single point energy calculations at the B3LYP/6-31G(d) basis set with the restricted open shell Hartree–Fock (ROHF) formalism. Transition state search on the geometry of DPPH•–**7H** was obtained from the highest energy point from scanning the bond length (1.2–2.5 Å) between the N atom of DPPH• and OH group of **7H**. The transition state was further optimized at the DFT using the option "opt = (ts, calcfc, noeigentest)" applying B3LYP/6-31G(d) basis set. To verify the character of the stationary points, they were subjected to frequency analyses. The optimized transition structure was characterized as possessing a single imaginary frequency corresponding to the transition vector (or reaction coordinate mode) for a particular chemical transformation, in contrast to energy minima with all-real vibrational frequencies.

Results and Discussion

DPPH•-Scavenging Rates of Artepillin C and Its Analogues. Upon addition of **1H–7H** to a deaerated MeCN solution of DPPH•, the absorbance at 516 nm due to DPPH• immediately decreased, as shown in Figure 1 (**7H** as a representative example). These spectral changes suggest that hydrogen transfer from the phenolic OH group in **1H–7H** to DPPH• may take place to scavenge DPPH•. The rate of the DPPH•-scavenging reaction of **1H–7H** was measured by monitoring the decrease in the absorbance at 516 nm due to DPPH• using a stopped-flow technique. The decay of the absorbance at 516 nm obeyed pseudo-first-order kinetics when the concentration of **1H–7H** ([**1H–7H**]) was maintained at more than a 10-fold excess of DPPH• concentration (inset of Figure 1). The k_{obs} values increase with increasing [**1H–7H**], exhibiting first-order dependence on [**1H–7H**], as shown in Figure 2. From the slopes of the linear plots of k_{obs} vs. [**1H–7H**], the second-order rate constants (k) were determined and are listed in Table 1. Structurally modified artepillin C analogues **2H–7H** could afford significantly larger k values than the parent artepillin C (**1H**). The k value for **7H** (3.1×10 mol^{-1} dm^3 s^{-1}) is the largest among the examined artepillin C analogues in this study. It is also found that the DPPH•-scavenging reactivity of the artepillin C derivatives in deaerated MeCN at 298 K

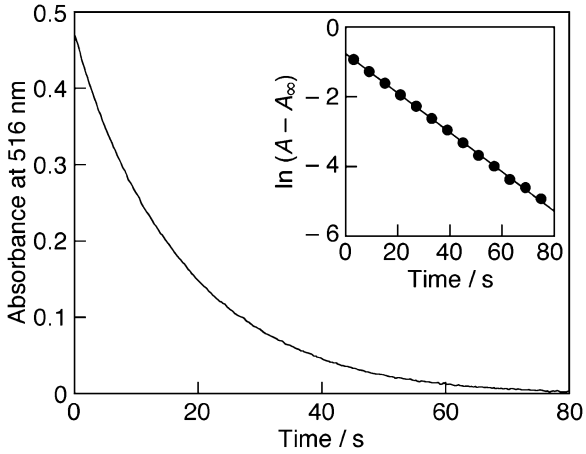


Figure 1. Time course of the absorbance change at 516 nm in the reaction of **7H** (2.0×10^{-3} mol dm^{-3}) with DPPH• (4.0×10^{-5} mol dm^{-3}) in deaerated MeCN at 298 K. Inset: The pseudo-first-order plot.

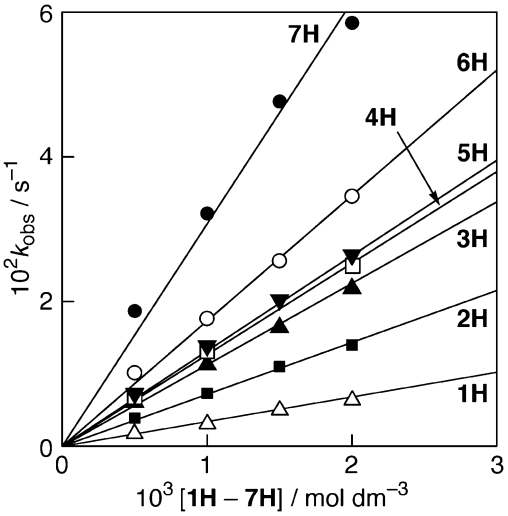


Figure 2. Plots of k_{obs} vs. [**1H–7H**] for the reaction of **1H–7H** with DPPH• in deaerated MeCN at 298 K.

Table 1. Second-Order Rate Constants (k) for DPPH$^{\bullet}$-Scavenging Reaction, Energy Difference Values (D_{HT}), Ionization Potentials (IP), D_{HT} − IP, and One-Electron-Oxidation Potentials (E^0_{ox}) of **1H–7H**

Compound	k[a] /mol^{-1} dm^3 s^{-1}	D_{HT}[b] /kcal mol^{-1}	IP[b] /kcal mol^{-1}	D_{HT} − IP[b] /kcal mol^{-1}	E^0_{ox} (vs. SCE)[c] /V
1H	3.4	398.3	162.4	235.9	+0.93
2H	7.2	396.2	154.3	241.9	+0.83
3H	1.1 × 10	394.2	162.6	231.6	+0.94
4H	1.3 × 10	394.1	162.3	231.8	+1.10
5H	1.3 × 10	393.8	158.2	235.6	+0.85
6H	1.7 × 10	392.3	153.2	239.1	+0.84
7H	3.1 × 10	390.2	161.0	229.2	+0.91

a) Determined in deaerated MeCN at 298 K. b) Calculated by DFT. c) Determined by SHACV with a glassy carbon working electrode in deaerated MeCN (0.1 mol dm^{-3} Bu_4NClO_4).

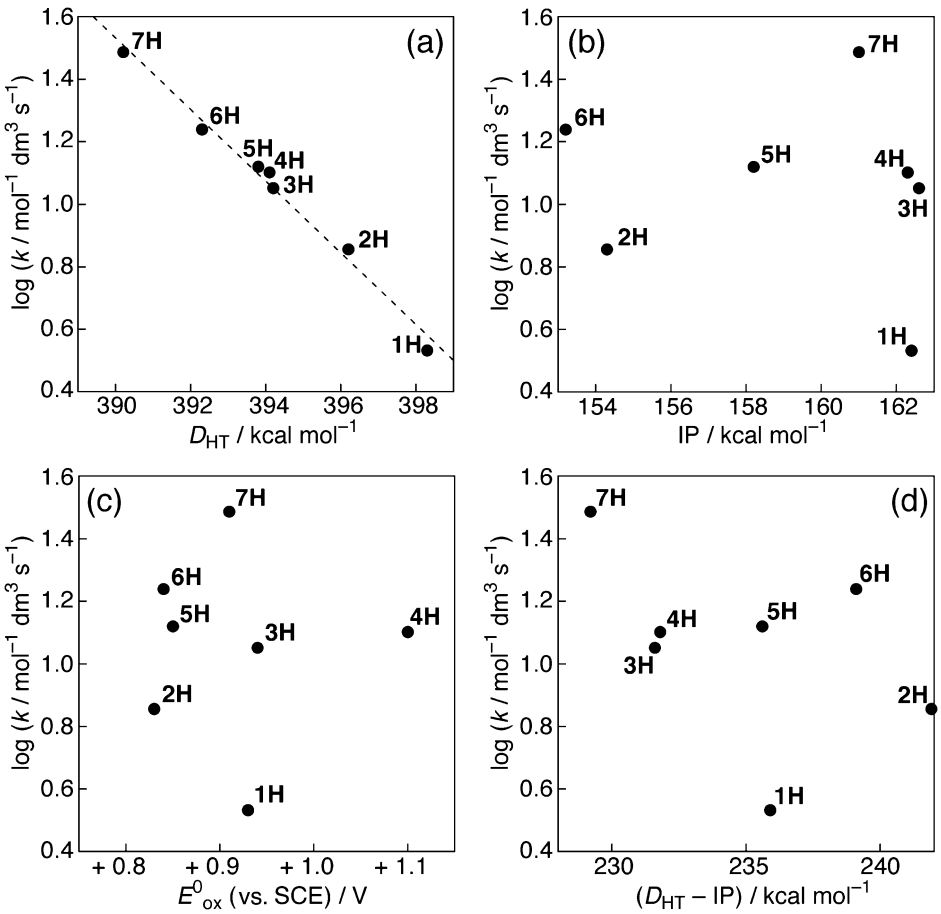


Figure 3. Plots of log k vs. (a) D_{HT}, (b) IP, (c) E^0_{ox}, and (d) D_{HT} − IP.

follows a similar trend to their $PhCMe_2OO^{\bullet}$-scavenging reactivity in EtCN at 203 K in our recent report.[15] Thus, DPPH$^{\bullet}$ is a useful tool to evaluated the scavenging reactivity of phenolic antioxidants against peroxyl radicals.

DPPH$^{\bullet}$-Scavenging Mechanism of Artepillin C and Its Analogues. If the radical-scavenging reaction of phenolic compounds proceeds via one-step hydrogen-atom transfer (Scheme 1a) as the rate-determining step, the O–H bond dissociation energy of phenolic OH group is an important parameter to control the reactivity of the radical-scavenging reaction.[9,10d,26] Thus, the O–H bond dissociation energies of phenolic OH group in **1H–7H**, which equal the D_{HT} values between **1H–7H** and the corresponding phenoxyl radicals **1**$^{\bullet}$**–7**$^{\bullet}$, were determined by DFT calculations at the B3LYP/6-31G(d) level (see the Experimental Section). The results are listed in Table 1. The lowest D_{HT} value of **7H** results from the electron-donating (ED) property of the *ortho* OH group and the intramolecular hydrogen bonding, which significantly stabilizes **7**$^{\bullet}$. Comparison of the D_{HT} values of the examined artepillin C analogues with their log k values revealed the linear correlation between these two parameters, as shown in Figure 3a. The smaller the D_{HT} value is, the larger the k value becomes. Thus, the DPPH$^{\bullet}$-scavenging reaction of **1H–7H** may proceed via the one-step hydrogen-atom transfer mechanism

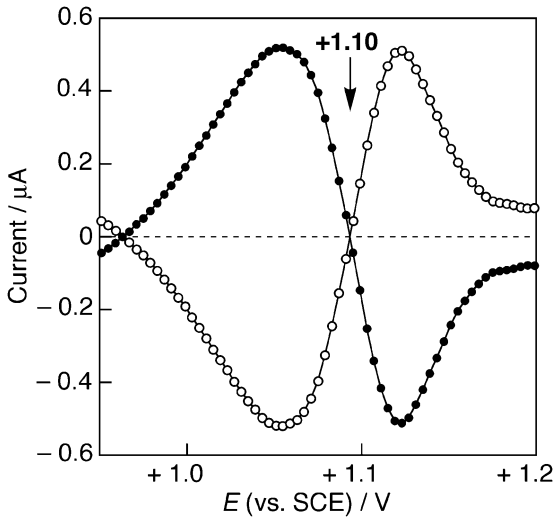


Figure 4. Second-harmonic alternating current voltammogram for the one-electron oxidation of **4H** (1.0×10^{-3} mol dm^{-3}) in deaerated MeCN (0.1 mol dm^{-3} Bu_4NClO_4) at 298 K. Scan rate: 4 mV s^{-1}; amplitude: 25 mV.

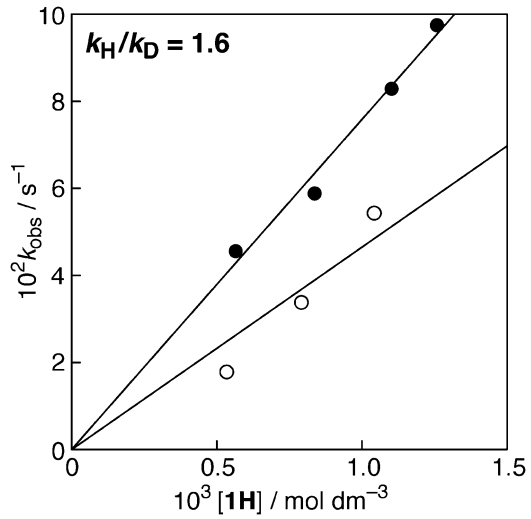


Figure 5. Plots of k_{obs} vs. [**1H**] in the presence of CH_3OH (3.5×10^{-1} mol dm^{-3}) (closed circles) and CD_3OD (3.5×10^{-1} mol dm^{-3}) (open circles) for the reaction between **1H** and DPPH$^{\bullet}$ (6.6×10^{-5} mol dm^{-3}) in deaerated MeCN at 298 K.

(Scheme 1a). On the other hand, the reactivity of the electron-transfer reaction (Scheme 1b) of antioxidants is related to their IP or E^0_{ox} values.[9,10d,26] Thus, the IP values for **1H**–**7H** were also calculated by DFT and the results are shown in Table 1. No linear correlation was observed in the plot of log k vs. IP, as shown in Figure 3b, suggesting that the electron-transfer process may not be involved as the rate-determining step in the DPPH$^{\bullet}$-scavenging reaction of **1H**–**7H**. In addition, the E^0_{ox} values of **1H**–**7H** were directly determined by the SHACV.[23] A representative SHACV wave for **4H** using a glassy carbon working electrode is shown in Figure 4. The E^0_{ox} value (vs. SCE) of **4H** in MeCN (0.1 mol dm^{-3} Bu_4NClO_4) was determined from the intersection of the SHACV wave (Figure 4) to be +1.10 V. The E^0_{ox} values of other artepillin C derivatives were also determined in a similar manner (Table 1). No linear correlation was also observed in the plot of log k vs. E^0_{ox}, as shown in Figure 3c, supporting the ruining of the possibility of the electron transfer as the rate-determining step in the DPPH$^{\bullet}$-scavenging reaction of **1H**–**7H**. Furthermore, the reactivity of the proton-transfer process following the initial electron transfer (Scheme 1b) can be evaluated by the calculated deprotonation energies of the radical cation intermediates **1**$^{\bullet+}$–**7**$^{\bullet+}$, which correspond to $D_{HT} - IP$ values in Table 1. As shown in Figure 3d, no linear correlation was observed between log k and $D_{HT} - IP$ values, suggesting that the proton-transfer process is also not the rate-determining step of the DPPH$^{\bullet}$-scavenging reaction of **1H**–**7H**. These results strongly suggest that the DPPH$^{\bullet}$-scavenging reaction of **1H**–**7H** proceeds via the one-step hydrogen-atom transfer as the case of the $PhCMe_2OO^{\bullet}$-scavenging reaction by artepillin C analogues (Scheme 1a).[15] In fact, the deuterium kinetic isotope effect (KIE, k_H/k_D) of 1.6 ($k_H = 7.6 \times 10$ mol^{-1} dm^3 s^{-1}, $k_D = 4.7 \times 10$ mol^{-1} dm^3 s^{-1}) was observed for the DPPH$^{\bullet}$-scavenging reaction of **1H** in the presence of 0.13 M CD_3OD or CH_3OH in deaerated MeCN at 298 K (Figure 5),[27] although the acceleration by the presence of CD_3OD or CH_3OH was observed as compared to its absence. Such a KIE value strongly supports

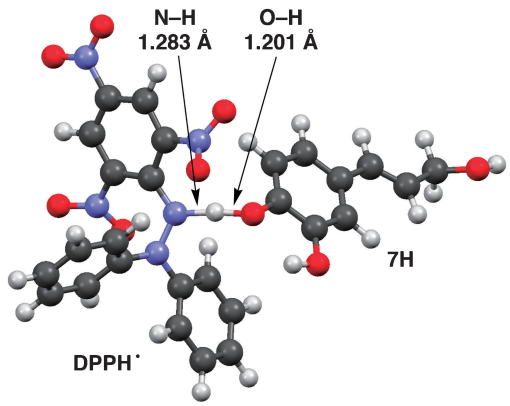


Figure 6. The optimized transition structure of **7H**–DPPH$^{\bullet}$ calculated by DFT (B3LYP/6-31G(d) level).

the one-step hydrogen atom transfer mechanism. We also evaluated the transition state structure of the reaction between **7H** and DPPH$^{\bullet}$ by DFT calculation (see the Experimental Section). The optimized transition structure of **7H**–DPPH$^{\bullet}$ is shown in Figure 6. The distance between the hydrogen atom of the phenolic OH group in **7H** and the nitrogen atom in DPPH$^{\bullet}$ is 1.283 Å, which is close enough to occur the hydrogen-atom transfer.

Structure–Activity Relationship. The enhancement in the radical-scavenging activity of **2H**–**7H** as compared to **1H** can be explained by the fact that the ED groups reduce the D_{HT} values and electron-withdrawing (EW) groups have a reverse effect on it.[15,28,29] The presence of ED groups and absence of EW groups in synthetic artepillin C analogues lowers their D_{HT} values and increases their k values. For example, the replacement of a methyl group in **2H** with a methoxy group to become **6H** resulted in more than 2-fold enhancement in the DPPH$^{\bullet}$-scavenging activity. A slight increase in the k values was observed by replacing a carboxy group in **4H** with a hydroxy-

ethyl group to become **6H** or by introducing a 3-methyl-2-butenyl (prenyl) group to **5H** to give **6H**. On the other hand, comparing **3H** with **4H**, the length of the prenyl side chain showed little effect on the k and D_{HT} values. By comparing the k values for **1H**–**7H**, it is clear that the electron richer environment the compound has, the lower is its D_{HT} value, and the higher is its radical-scavenging activity. On the other hand, **7H** having a catechol structure in the molecule has the highest k and the lowest D_{HT} values among the examined artepillin C analogues. The advantage of catechol in enhancing the radical-scavenging activity has been predicted by a series of previous studies, which showed that the phenoxyl radical **7**$^{\bullet}$ derived from catechol could be well stabilized by the *ortho*-hydroxy group and the intramolecular hydrogen bonding.[26,30] This also results in the low O–H bond dissociation energy of catechol. Hence, the reactivity of **7H** with DPPH$^{\bullet}$ ($k = 3.1 \times 10\,\mathrm{mol^{-1}\,dm^3\,s^{-1}}$) is increased 9 times higher than that of **1H** ($k = 3.4\,\mathrm{mol^{-1}\,dm^3\,s^{-1}}$), although **1H** has more ED groups than **7H**. A similar structural feature but the absence of catechol moiety in **5H** makes it less reactive than **7H**.

Conclusion

Artepillin C (**1H**) and its analogues **2H**–**7H** showed an efficient DPPH$^{\bullet}$-scavenging activity in deaerated MeCN at 298 K. Among them, **7H** having a catechol structure showed the highest DPPH$^{\bullet}$-scavenging activity due to the stabilization of the corresponding phenoxyl radical **7**$^{\bullet}$ via the intramolecular hydrogen bonding. The linear correlation between the $\log k$ and D_{HT} values as well as the deuterium kinetic isotope effect and the calculated transition state structure strongly suggests that artepillin C and its analogues may follow the one-step hydrogen-atom transfer rather than the electron transfer followed by proton transfer in the DPPH$^{\bullet}$-scavenging reaction in deaerated MeCN. Furthermore, the DPPH$^{\bullet}$-scavenging activity of artepillin C analogues can be predicted by the D_{HT} values calculated by DFT. It is clear that the electron richer environment the compound has, the lower its D_{HT} value is and the higher its radical-scavenging activity becomes. The structure–activity relationship obtained in this study provides valuable and fundamental information about the radical-scavenging mechanism of phenolic antioxidants in biological systems as well as about the development of novel antioxidants with higher activity than natural occurring ones.

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