Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

Fused Heterocyclic Antioxidants: Antioxidative Activities of Hydrocoumarins in a Homogeneous Solution

Tomihiro NISHIYAMA[a], Junichi OHNISHI[b] & Yasuhiro HASHIGUCHI[b]

[a] Department of Applied Chemistry, Faculty of Engineering and High Technology Research Center, Kansai University

[b] Department of Applied Chemistry, Faculty of Engineering, Kansai University

Published online: 22 May 2014.

To cite this article: Tomihiro NISHIYAMA, Junichi OHNISHI & Yasuhiro HASHIGUCHI (2001) Fused Heterocyclic Antioxidants: Antioxidative Activities of Hydrocoumarins in a Homogeneous Solution, Bioscience, Biotechnology, and Biochemistry, 65:5, 1127-1133, DOI: 10.1271/bbb.65.1127

To link to this article: http://dx.doi.org/10.1271/bbb.65.1127

Biosci. Biotechnol. Biochem., **65** (5), 1127–1133, 2001

Fused Heterocyclic Antioxidants: Antioxidative Activities of Hydrocoumarins in a Homogeneous Solution

Tomihiro NISHIYAMA,[1,†] Junichi OHNISHI,[2] and Yasuhiro HASHIGUCHI[2]

[1]*Department of Applied Chemistry, Faculty of Engineering and High Technology Research Center, Kansai University, Senriyama, Suita, Osaka 564-0073, Japan*
[2]*Department of Applied Chemistry, Faculty of Engineering, Kansai University, Senriyama, Suita, Osaka 564-0073, Japan*

Received November 7, 2000; Accepted January 13, 2001

We compared the antioxidative activities of seven hydrocoumarins with those of α-tocopherol for the oxidation of tetralin and linoleic acid in a homogeneous solution. Hydrocoumarins exhibited a higher induction period than that of α-Toc in both systems. However, the rate of oxygen absorption during the induction period for α-Toc was slower than that of the hydrocoumarins in both systems. In addition, 6,7-dihydroxy-4,4-dimethylhydrocoumarin showed less cytotoxicity toward human fibroblasts than did 2,6-di-*t*-butyl-4-methylphenol.

Key words: antioxidant; hydrocoumarin; cytotoxicity; bond dissociation energy

The peroxidation of unsaturated lipids has been implicated in a number of diseases, including cancer, and in the aging process.[1,2] Consequently, the mechanism for the action and activity of protective free radical chain-breaking antioxidants continues to be of outstanding interest. It is generally recognized that vitamin E (α-Toc) is a potent lipid-soluble chain-breaking antioxidant in biological systems. The high activities of α-Toc have been interpreted by Ingold and co-workers in terms of steric and electronic effects.[3,4] That is, the rate constant for H-atom abstraction caused by peroxyl radicals for Toc and related compounds depends on the degree of stabilization of the phenoxyl radical formed upon abstraction of the phenolic hydrogen. The p-type lone pair on the ether-type oxygen atom in a position *para* to the OH group can help to stabilize the phenoxyl radical, implying that the ether oxygen atom plays an important role in stabilizing the phenoxyl radical.

Coumarins which have a similar structure to that of Toc occur naturally in many plants such as Umbelliferae, Rutaceae, Leguminosae, and Compositae.[5] However, there have been few reports concerning the antioxidative activity of coumarins.[6] Liu[7] has recently reported the antioxidative activity of the three coumarin derivatives, 4-methylcoumarin (MC), 6-hydroxy-4-methylcoumarin (HMC) and 7,8-dihydroxy-4-methylcoumarin (DHMC) (Fig. 1), against the oxidation of linoleic acid in micellar systems. They found that MC and HMC showed no appreciable effect on oxygen absorption, while the addition of DHMC significantly decreased the rate of oxygen absorption. A comparison of the antioxidative activity between DHMC and α-Toc showed α-Toc to be more reactive than DHMC. The difference between the basic skeleton of Toc and hydroxycoumarins is the saturated and unsaturated hydrocarbons at the 3- and 4-positions. Moreover, hydroxycoumarins have a carbonyl oxygen instead of a phytyl side chain and methyl group. Both the carbonyl group and the $C_3=C_4$ double bond would suggest that hydroxycoumarins might be poor antioxidants compared with Toc. In other words, the p-type lone pair on the 1-oxygen of hydroxycoumarins would be able to interact with π electrons on the carbonyl carbon atom and the $C_3=C_4$ double bond.

On this basis, we expected that hydrocoumarins **1**–**7**, each lacking the π electrons at the C-3 and C-4 carbons, would be better antioxidants than hydroxycoumarins. However, their antioxidative activity has scarcely been studied.

In the course of our studies on the inhibition of autoxidation, we have reported the antioxidative activities of phenols having fused oxygen-containing heterocyclic compounds.[8–10] In this paper, we compare the

† To whom correspondence should be addressed. Phone: +81-6-6368-0901; Fax: +81-6-6339-4026; E-mail: organic@ipcku.kansai-u.ac.jp

Abbrevations: Toc, vitamin E; MC, 4-methylcoumarin; HMC, 6-hydroxy-4-methylcoumarin; DHMC, dihydroxy-4-methylcoumarin; HC, 7-hydroxycoumarin; BHT, 2,6-di-*t*-butyl-4-methylphenol; AIBN, α,α′-azobisisobutyronitrile; t_{inh}, induction period; k_{inh}, rate constant of inhibition; k_p, propagation rate constant; R_{inh}, rate of oxidation; n, stoichiometric factor; R_i, rate of chain initiation; D(O-H), dissociation energy of O-H bond

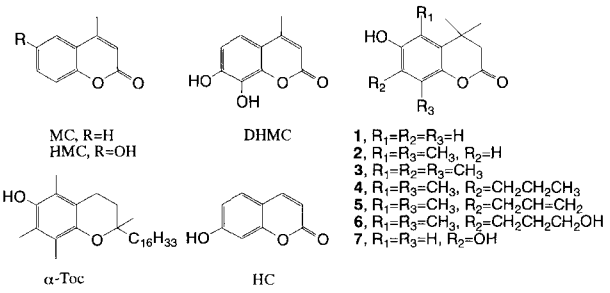

Fig. 1. Chemical Structures of Hydrocoumarins **1-7**, Coumarins, and α-Toc.

peroxyl radical-trapping ability of 6-hydroxy-3,4-dihydrocoumarins **1-6** and 6,7-dihydroxy-3,4-dihydrocoumarin **7**, which each have fused oxygen-containing heterocyclic compounds, with α-Toc and commercially available 7-hydroxycoumarin (HC). We also compare the cytoxicity of the hydrocoumarins with that of 2,6-di-*t*-butyl-4-methylphenol (BHT) and α-Toc in normal human fibroblasts.

Materials and Methods

General. Melting point (mp) data were measured with Yanaco MP-J3 micro-melting apparatus and are uncorrected. Nuclear magnetic resonance spectra were recorded with a Jeol GSX-400 spectrometer operated at 400 MHz for ^1H and at 100.6 MHz for ^{13}C in $CDCl_3$ unless otherwise noted, and chemical shift data are with reference to $(CH_3)_4Si$. Mass spectra were recorded with a Perkin-Elmer model 910 gas chromatograph-mass spectrometer at 70 eV.

Assay for antioxidative activity. The rate of oxygen absorption was measured as a function of time under 760 Torr (1 Torr = 133.322 Pa) of O_2 with 50.0 g of tetralin or 7.0 g of linoleic acid in 18.0 g of chlorobenzene containing an antioxidant (1 mmol dm^{-3}) and AIBN (10 mmol dm^{-3}) as the initiator. The oxidation temperature was maintained at 61 ± 0.1 °C. The t_{inh} value was graphically determined from the length of time between initiator injection and the point of intersection of the tangents to the oxidation curve corresponding to the initial inhibited and final uninhibited rates of oxidation.

Materials. 6-Hydroxy-4,4,5,8-tetramethylhydrocoumarin **(2)** and 7-allyl-6-hydroxy-4,4,5,8-tetramethylhydrocoumarin **(5)** were synthesized by following a published procedure.[11] These compounds showed spectral data in agreement with the published values. 6-Hydroxy-2,2-dimethylhydrocoumarin **(1)** and 6-hydroxy-4,4,5,7,8-pentamethylhydrocoumarin **(3)** were prepared according to a modification of the method reported in the literature:[11] 2,3,6-Trimethylhydroquinone was mixed with 3,3-dimethylacrylic acid and methanesulfonic acid, and the mixture was stirred at 85 °C under a nitrogen atmosphere for 3 h. After the usual work-up, the crude product was purified by SiO_2 column chromatography to afford compound **3** in an 85% yield. 6-Hydroxy-4,4,5,8-tetramethyl-7-propylhydrocoumarin **(4)** was prepared by reducing the double-bond of **5** with H_2/Pd-C at room temperature in an 37% yield. 7-(3-Hydroxy-1-propyl)-4,4,5,8-tetramethyl-3,4-dihydrocoumarin **(6)** was prepared by deprotecting the methoxymethoxy group of 7-(3-hydroxy-1-propyl)-6-(methoxymethoxy)-4,4,5,8-tetramethylhydrocoumarin[11] according to the same procedure as that used for the synthesis of compound **3**. 6,7-Dihydroxy-4,4-dimethylhydrocoumarin **(7)** was prepared from the reaction of 1,2,4-benzenetriol and 3,3-dimethylacrylic acid in an 86% yield.

6-Hydroxy-2,2-dimethylhydrocoumarin (1). Yield, 42%. Mp 93–94 °C. NMR δ_H: 1.32(s, 6H), 2.62(s, 2H), 6.23(s, 1H), 6.74–6.93(m, 3H). NMR δ_C: 27.5, 33.3, 43.5, 111.2, 114.7, 117.9, 132.9, 144.2, 152.9, 169.5. MS *m/z* (rel. intensity, %): 1924(M^+, 100), 177(44), 150(70), 149(17), 135(30).

6-Hydroxy-4,4,5,7,8-pentamethylhydrocoumarin (3). Yield, 85%. Mp 198–199 °C. NMR δ_H: 1.45(s, 6H), 2.19(s, 3H), 2.22(s, 3H), 2.36(s, 3H), 2.55(s, 2H), 4.72(s, 1H). NMR (acetone-d_6) δ_C: 15.2, 15.4, 17.8, 30.5, 38.8, 49.1, 123.9, 125.9, 126.7, 131.8, 147.1, 153.2, 171.3. MS *m/z* (rel. intensity, %): 234(M^+, 100), 219(23), 218(20), 192(70), 118(44), 177(44), 175(47), 160(18), 149(16).

6-Hydroxy-4,4,5,8-tetramethyl-7-propylhydrocoumarin (4). Yield, 37%. Mp 191–192°C. NMR δ_H: 1.02(t, J=7.3 Hz, 3H), 1.45(s, 6H), 1.51–1.58(m, 2H), 2.23(s, 3H), 2.35(s, 3H), 2.55(s, 2H), 2.55–2.62(m, 2H), 4.58(s, 1H). NMR δ_C: 15.1, 17.2, 17.3, 25.3, 30.6, 31.9, 38.4, 49.0, 122.0, 126.2, 129.5, 131.4, 146.7, 151.7, 171.9. MS *m/z* (rel. intensity, %): 262(M^+, 100), 247(29), 233(25), 232(15), 220(43), 219(36), 205(21), 203(33), 191(55), 175(13), 173(15).

7-(3-Hydroxy-1-propyl)-4,4,5,8-tetramethylhydrocoumarin (6). Yield, 70%. Mp 139–140 °C. NMR δ_H: 1.45(s, 6H), 1.86(m, 2H), 2.23(s, 1H), 2.37(s, 3H), 2.55(s, 2H), 2.84(t, J=6.6 Hz, 2H), 3.65(t, J=6.6 Hz, 3H). NMR δ_C: 12.1, 14.7, 21.9, 27.7, 29.9, 35.6, 46.1, 60.6, 120.8, 122.9, 124.9, 128.8, 143.5, 1550.2, 169.0. MS *m/z* (rel. intensity, %): 278(M^+, 96), 260(31), 245(20), 232(15), 218(100), 204(27), 203(38), 201(29), 190(31), 189(38), 175(38), 161(17), 147(14), 145(14).

6,7-Dihydroxy-4,4-dimethylhydrocoumarin (7). Yield, 86%. M.p 212–213°C. NMR δ_H: 1.39(s, 6H), 2.61(s, 2H), 4.87(s, 2H), 6.29(s, 1H), 7.12(s, 1H).

NMR δ_C: 14.9, 68.3, 92.8, 99.4, 101.5, 129.8, 144.4, 145.7, 181.9.

Cell culture. Human dermal (foreskin) fibroblasts were obtained as primary cultures by explantation. The fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM); (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 10% fetal bovine serum.

Cytotoxicity test by the MTT method. A secondary culture of the fibroblasts was harvested by trypsinization when the culture was about 80% confluent. The suspended cells were collected by centrifugation at $180 \times g$ for 5 min and diluted in DMEM containing 10% fetal bovine serum. The cells (1×10^4 cells/0.1 ml of the medium, measured by a Burker-Turle counting chamber) were inoculated into each well of a 96-well tissue culture plate and cultured for 24 h at 37°C in a 5% CO_2 humidified atmosphere. The culture medium was replaced with an MCDB153 medium (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan) with 1.8 mM Ca^{2+} and cultured again for 24 h at 37°C in a 5% CO_2 humidified atmosphere. The cells were then treated with each antioxidant for 24 h. Each antioxidant was dissolved in dimethyl sulfoxide and diluted with the MCDB153 medium. The final concentration of the solvent was 0.5% (v/v) and showed no effect on cell growth in a controlled growth experiment. After treating with an antioxidant, the medium was replaced with 0.2 ml of DMEM supplemented with 10% fetal bovine serum and MTT (0.5 mg/ml) in each well, and the culture incubated at 37°C for 2 h. After being washed with phosphate-buffered saline (PBS), 2-propanol containing 0.04 N HCl was added to each well for the dissolution of formazan to give a homogeneous blue solution suitable for absorbance measurement. The resulting color intensity was measured by using a micro-plate reader at 590 nm. The value for formazan formation was determined as the mean value of triplicate experiments at each concentration of an antioxidant.

Results

Scavenging effect of hydrocoumarins on the AIBN-induced peroxidation in a homogeneous solution

Figure 2 shows examples of oxygen-uptake curves for the oxidation of tetralin initiated by α,α'-azobisisobutyronitrile (AIBN) at 61°C. In the absence of an antioxidant (control), the oxidation initiated by AIBN proceeded with a very brief initiation period at a constant rate of oxygen uptake. The addition of 7-hydroxycoumarin (HC) did not delay the onset of oxidation, and a constant rate of oxygen uptake was observed without any appreciable induction

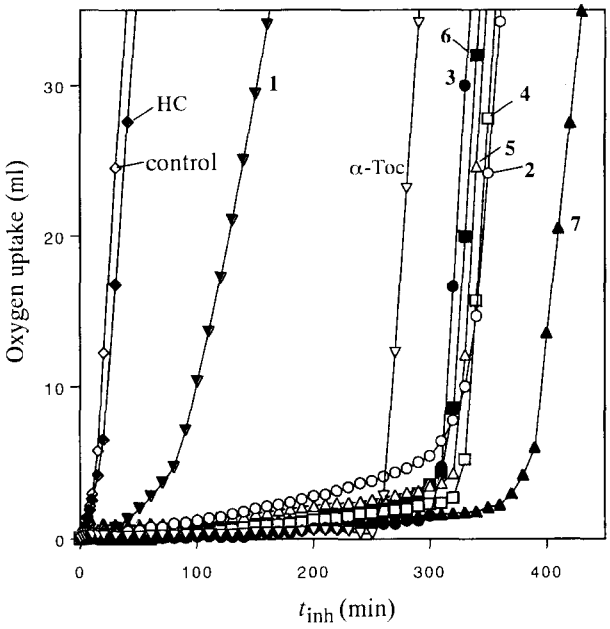

Fig. 2. Rate of Oxygen Uptake in the Oxidation of 50 g of Tetralin in the Absence (Control) and Presence of 1 mM of an Antioxidant Initiated by 10 mM AIBN at 61°C under Oxygen.

period (t_{inh}). In the presence of α-Toc, which is a well-known radical scavenger, the rate of oxygen uptake was suppressed. When the induction period was over, the oxidation proceeded at the same rate as that in the absence of a radical scavenger. The addition of dihydrocoumarin **1**, which lacks the alkyl substituent on the aromatic ring, showed no appreciable effect on the oxygen absorption, while the addition of **2**–**7** significantly decreased the rate of oxygen absorption. However, the rate of oxygen absorption with **2**–**7** was faster than that of α-Toc, and lasted longer. In all cases except for **1** and **7**, the values of t_{inh} were almost the same. Catechol-type hydrocoumarin **7** had the highest antioxidative activity as measured by t_{inh}. Comparisons between **2**–**7** and α-Toc showed t_{inh} values for **2**–**7** 1.2–1.5 times larger than that for α-Toc.

Figure 3 shows oxygen absorption patterns obtained by the oxidation of linoleic acid in chlorobenzene initiated by AIBN in the presence of **2**–**7** and α-Toc. As shown in Fig. 3, 7-allylhydrocoumarin **5** had an induction period which was much longer than those of the other hydrocoumarins and α-Toc, the t_{inh} value for **5** being about 2.2 times larger than that for α-Toc.

Discussion

Antioxidant activity of hydrocoumarins toward the oxidation of tetralin initiated by AIBN

Efficient phenolic antioxidants (ArOH) are well known to terminate free radical chain peroxidation according to eqs. 1 and 2.

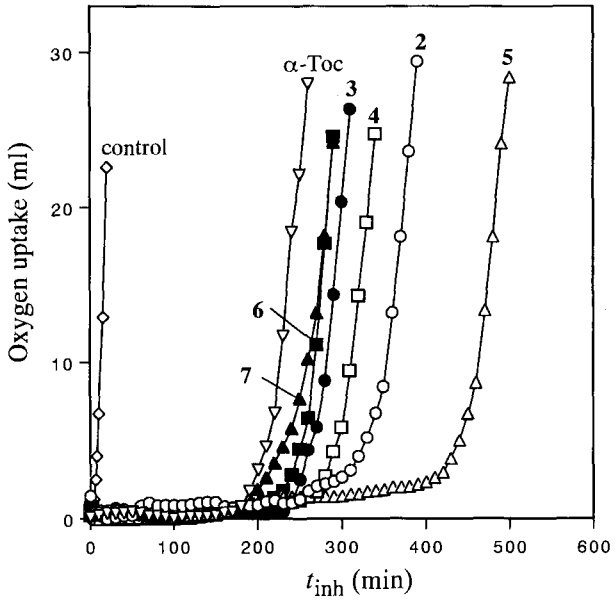

Fig. 3. Rate of Oxygen Uptake in the Oxidation of 1.5 M of Linoleic Acid in Chlorobenzene Initiated by 10 mM AIBN in the Absence and Presence of 1 mM of an Antioxidant at 61 °C under Oxygen.

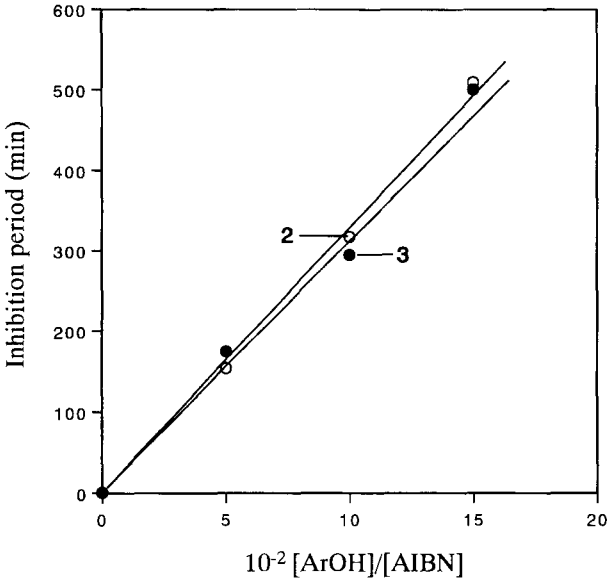

Fig. 4. Inhibition Period Produced by **2** and **3** in the Oxidation of Tetraline Induced by 10 mM AIBN at 61 °C.

$$ROO^{\cdot} + ArOH \xrightarrow{k_{inh}} ROOH + ArO^{\cdot} \quad (1)$$

$$ROO^{\cdot} + ArO^{\cdot} \xrightarrow{fast} \text{nonradical products.} \quad (2)$$

During the induction period, the rate of oxidation can be represented as follows by eq. 3:[12,13]

$$-d[O_2]/dt = R_{inh} = k_p R_i[RH]/nk_{inh}[ArOH] \quad (3)$$

where k_p is the propagation rate constant of the chain reaction, k_{inh} is the rate constant of inhibition, R_i is the rate of chain initiation, n is the stoichiometric factor, and RH represents the organic substrate. The induction period, t_{inh}, can be represented as follows by eq. 4:

$$t_{inh} = n[ArOH]/R_i \quad (4)$$

These equations enable the peroxyl radical-trapping activities of antioxidants to be expressed by three values: t_{inh}, n, and k_{inh}. The value of k_{inh} can be determined by carefully measuring the oxygen uptake during inhibition, provided that k_p is known and R_i is controlled by using eq. 3. However, the value of k_{inh} is difficult to obtain experimentally. Consequently, the relative antioxidative activity was calculated as the ratio of k_{inh}/k_p,[14] since k_p was not known. Equations 3 and 4 give eq. 5 as follows:

$$k_{inh}/k_p = [RH]/t_{inh}R_{inh} \quad (5)$$

Equation 4 suggests that the induction period would be proportional to the antioxidant concentration. Figure 4 shows that the induction period produced by the addition of **2** and **3** for the oxidation of tetralin initiated with AIBN was proportional to [ArOH]/[AIBN].

Table 1. Inhibition of the Oxidation of Tetralin by 1 mM of an Antioxidant Initiated by 10 mM α,α′-Azobisisobutyronitril (AIBN) at 61 °C

Compd. No.	t_{inh} min	R_{inh} $\times 10^7$ M/s	k_{inh}/k_p	n
1	99	7.82	1508	0.8
2	317	1.87	2064	2.5
3	294	0.75	5548	2.4
4	316	1.25	3097	2.5
5	309	1.02	3882	2.5
6	303	2.00	2019	2.4
7	378	0.72	4495	3.0
α-Toc	247	0.29	17081	(2.0)

The stoichiometric factor for the antioxidants tested was first obtained from equation 4. The rate of initiation (R_i) was determined from the induction period measured in the presence of α-Toc under the same oxidation conditions, in which $n = 2.0$ was assumed. The stoichiometric factors for hydrocoumarins **1**–**7** and α-Toc are listed in Table 1. Less-hindered hydrocoumarin **1** showed a low n value, presumably being related to an ArO$^{\cdot}$ wasting reaction that resulted from a chain-transfer reaction with the substrate and a bimolecular self-reaction.[15] However, the n value for other hydrocoumarins **2**–**7** was larger than that of α-Toc. In particular, catechol-type hydrocoumarin **7**, which has two hydroxy groups on the aromatic ring, had a much higher n value. This suggests that **7** can trap three peroxyl radicals per molecule.

The antioxidative activities as measured by the ratio of rate constant k_{inh}/k_p for **1**–**7** and α-Toc in tetralin are also listed in Table 1, the antioxidative activity

decreasing in the order α-Toc > **3** > **7** > **5** > **4** > **2**,**6** > **1**. It is clear from Table 1 that α-Toc had the highest antioxidative activity as measured by k_{inh}/k_p. α-Toc scavenged the peroxyl radicals generated from tetralin more quickly than hydrocoumarins **1**–**7**, but this lowered the value of n. α-Toc exhibited a k_{inh}/k_p value 3 times that of hydrocoumarin **3** which has the same substituent as α-Toc on the aromatic ring. By comparing the alkyl substituents on the aromatic ring between **1**, **2** and **3**, two methyl groups *ortho* to the phenolic OH group gave a higher k_{inh}/k_p value than that with an unsubstituted or methyl group *ortho*. Bulky substituents such as propyl or allyl groups gave a lower k_{inh}/k_p value than that with a methyl group. Hydrocoumarins **4**–**6** each exhibited a lower k_{inh}/k_p value than that of **3**. Burton *et al.*[4] have reported similar results that k_{inh} of 2,6-di-*t*-butylphenol was about 8 times smaller than that of 2,6-di-methylphenol in the oxidation of styrene. Judging from the k_{inh}/k_p and n values, it can be said that catechol-type hydrocoumarin **7** and tetramethylhydrocoumarin **3** were better chain-breaking antioxidants for the autoxidation of tetralin than the other hydrocoumarins. The strong reactivity of catechol-type hydrocoumarin **7** was due to its *o*-dihydroxy structure that made oxidation intermediate *o*-semiquinone fairly stable and easily oxidized to form the *o*-quinone.[7]

The structural characteristics of hydrocoumarins are an electron-withdrawing carbonyl group to the heterocyclic oxygen atom (lactone- type oxygen). The lone pair on the lactone-type oxygen in a position *para* to the OH group in hydrocoumarins would not contribute to stabilization of the phenoxyl radical by interaction of the unpaired electron on the lone pair with the lactone-type oxygen.[3,4] In other words, the lower reactivity of hydrocoumarins **1**–**7** than of α-Toc can be explained by the electron-withdrawing nature of the carbonyl group attached to the oxygen at the 1-position which cannot take part in stabilization of the phenoxyl radical.

We also attempted to elucidate the factors affecting hydrogen abstraction in the chain-breaking reaction of hydrocoumarin **3**, 2,2,5,7,8-pentamethyl-6-chromanol (PMC) as a reference for α-Toc and 6-hydroxy-4,5,7,8-tetramethylcoumarin (HTMC) in respect of the dissociation energy of the O-H (D(O-H)) bond which was calculated by using semiempirical MNDO-AM1. We obtained the D(O-H) values by calculating the difference between the enthalpy for phenolic antioxidants and the relative phenoxyl radical species and hydrogen radical, as shown in Fig. 5. For PMC, we calculated the D(O-H) value to be 8 and 11 kJ/mol weaker than that for hydrocoumarin **3** and HTMC, respectively. The results for the antioxidative activity and the dissociation energy suggest that the OH group of α-Toc was more effective than that of hydrocoumarins **1**–**7**.

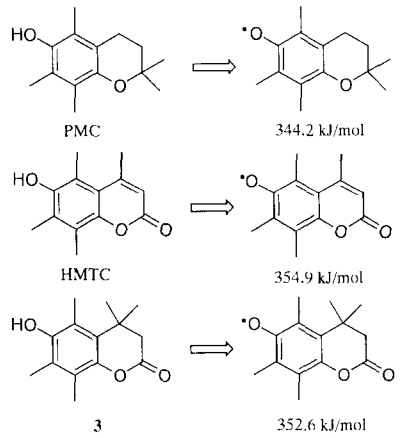

Fig. 5. Calculated Bond dissociation Energy, D(O-H), for PMC, HMC, and **3**.

Table 2. Inhibition of the Oxidation of Linoleic Acid by 1 mM of an Antioxidant in a Chlorobenzene Solution Initiated by 10 mM AIBN at 61°C

Compd. No.	t_{inh} min	R_{inh} $\times 10^7$ M/s	k_{inh}/k_p	n
2	319	2.68	300	3.2
3	251	1.96	522	2.6
4	276	1.88	495	2.8
5	437	1.96	300	4.5
6	233	5.17	213	2.4
7	231	1.21	918	2.4
α-Toc	195	0.98	1337	(2.0)

Antioxidative activity of hydrocoumarins toward the oxidation of linoleic acid initiated by AIBN

The antioxidative activities toward the autoxidation of linoleic acid in chlorobenzene are shown in Table 2. The n values for **2**–**7** are about 1–2 times larger than that for α-Toc. Among them, allylcoumarin **5** had a much higher n value of 4.5 in comparison with that of α-Toc. This suggests that hydrogen atoms not existing as a phenolic group contributed to the scavenging effect. One probable effect is that the hydrogen attached to the α-carbon of the *o*-substituent becomes a hydrogen donor to phenoxyl radicals to regenerate a phenol, because a benzyl-type radical is stable. Therefore, the regenerated O-H group and the benzyl-type radical can also trap other peroxyl radicals, enabling the value of n to be more than 2. In particular, the radical generated from **5** is more stable than other radical species, because of both allylic and benzyl radicals. We, therefore, calculated the bond dissociation energy (BDE) of the allylic hydrogen for **5** (Fig. 6) to be 13 and 15 kJ/mol weaker than that for the phenolic hydrogen and α-hydrogen to the carbonyl group, respectively. Similar results have been reported with *o*-substituted phenols.[16] However, it is not clear why the n value for allylcoumarin **5** varied according such substrates

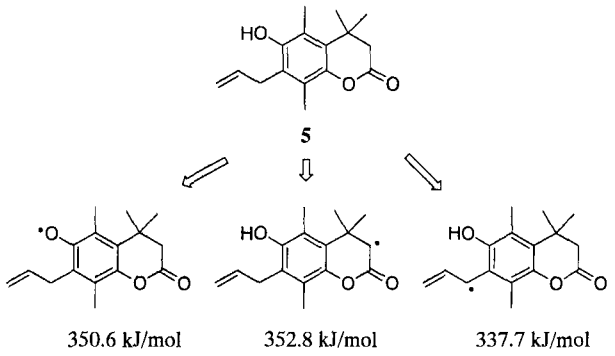

Fig. 6. Calculated Bond Dissociation Energy for **5**.

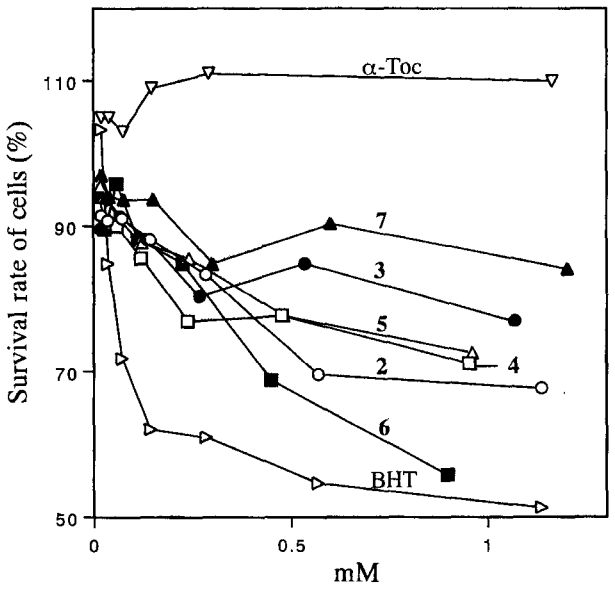

Fig. 7. Cytotoxicity of Hydrocoumarins **2-7**, α-Toc and BHT toward Normal Human Fibroblasts Measured by the MTT Method.

as tetralin and linoleic acid, but it is conceivable that these factors would arise from the effects of changing the oxidizable substrate. The transition state for intramolecular hydrogen migration of the allyl hydrogen atom to the phenoxyl radical is stabilized in a more-polar substrate such as linoleic acid than in the tetralin system.[17]

The antioxidative activities evaluated by the k_{inh}/k_p values decreased in the order α-Toc>**7**>**3**>**4**>**2,5**>**6**. The value for α-Toc was 1.5-6.3 times more than those of hydrocoumarins **2-7**. However, all the antioxidants tested exhibited a marked drop in activity by at least an order of magnitude compared to the values for k_{inh}/k_p in tetralin. This difference is probably due to the fact that linoleic acid is different from tetralin in its oxidizability. Thus, the hydrogens attached to the doubly allylic carbon of linoleic acid would be easier to abstract than the benzylic hydrogens of tetralin. When linoleic acid was used as the substrate, therefore, reaction 6 proceeded at the same time.

$$ROO^{\bullet} + RH \xrightarrow{k_p} R^{\bullet} + ROOH \qquad (6)$$

Cytotoxicity of the hydrocoumarins

The cytotoxicity of hydrocoumarins **2-7**, α-Toc and BHT on normal human fibroblasts was measured by the MTT method.[18] As shown in Fig. 7, an increase in the dose of hydrocoumarins **2-7** caused a progressive decrease in the survival rate of the cells. The cytotoxicity of **2-7** decreased in the order **6**>**2**>**4,5**>**3**>**7**. Catechol-type hydrocoumarin **7** showed the highest survival rate for the cells among compounds **2-7**. There is no clear criterion for the safety of antioxidants with the present method because interpreting the cytotoxicity test for human safety has not been done. Hence, the safety of **2-7** was estimated by a comparison with α-Toc and BHT. The cytotoxicity of BHT, which is a popular synthetic antioxidant for polymers, rubbers and lubricating oils, showed at relatively low concentrations. These results suggest that hydrocoumarins **2-7** would be safer antioxidants than BHT, although their safety would be inferior to that of α-Toc.

In conclusion, the overall efficiency of hydrocoumarins can be determined by the induction period, as well as by the ratio of k_{inh}/k_p. Judging from the t_{inh} value, it can be said that hydrocoumarins **2-7** behaved as better chain-breaking antioxidants for the autoxidation of tetralin and linoleic acid than structurally comparable α-Toc. On the other hand, α-Toc had higher antioxidative activity when evaluated by the k_{inh}/k_p value. Among these hydrocoumarins, catechol-type hydrocoumarin **7** showed less cytotoxicity toward human fibrobrast than BHT did. Therefore, hydrocoumarin **7** may be an effective antioxidant for preventing the oxidation of oils and fats.

References

1) Ames, B. N., Dietary carcinogens and anticarcinogens: Oxygen radicals and degenerative disease. *Science*, **221**, 1256-1264 (1983).
2) Chen, H., and Tappel, A. L., Protection of multiple antioxidants against hem protein oxidation and lipid peroxidation induced by $CBrCl_3$ in liver, lung, kidney, and spleen. *J. Agric. Food Chem.*, **44**, 854-858 (1996).
3) Burton, G. W., and Ingold, K. U., Autoxidation of biological molecules 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants *in vitro*. *J. Am. Chem. Soc.*, **103**, 6472-6477 (1981).
4) Burton, G. W., Doba, T., Gabe, E. J., Hughes, L., Lee, F. L., Prasad, L., and Ingold, K. U., Autoxidation of biological molecules. 4. maximizing the antioxidant activity of phenols. *J. Am. Chem. Soc.*, **107**, 7053-7065 (1985).
5) Tanaka, O., Nozoe, S., Aimi, N., and Nagai, M., Tennenbutsu Kagaku (in Japanese), Nankodo, Tokyo, pp. 235-242 (1998).

6) Foti, M., Piatteli, M., Baratta, M. T., and Ruberto, G., Flavonoids, coumarins, and cinnamic acids as antioxidants in a micellar system. Structure-activity relationship. *J. Agric. Food Chem.*, **44**, 497–501 (1996).
7) Yu, W., Liu, Z. Q., and Liu, Z. L., Antioxidant effect of coumarin derivatives on free radical initiated and photosensitized peroxidation of linoleic acid in micelles. *J. Chem. Soc. Perkin Trans. II.* 969–974 (1999).
8) Nishiyama, T., Sugimoto, T., Miyamoto, N., Uezono, M., and Nakajima, Y., Antioxidant activities of phenols having a fused oxygen-containing heterocyclic ring. *Polym. Deg. Stab.*, **70**, 103–109 (2000).
9) Yamamura, Y., Ishida, T., Tatara, H., and Nishiyama, T., Chain-breaking fused heterocyclic antioxidants: antioxidant activities of 9*H*-xanthene-2,7-diols and α-tocopherol upon liposomal membranes. *J. Am. Oil Chemist,s Soc.*, **74**, 739–744 (1997).
10) Yamamura, Y., Ishida, T., Ueyama, H., Hayashida, H. Ishimaru, T., and Nishiyama, T., Comparison of the efficiencies of the fused heterocyclic compounds, 9*H*-xanthene-2,7-diols, and related chain-breaking phenolic antioxidants. *Bull. Chem. Soc. Jpn.*, **69**, 1713–1717 (1996).
11) Zheng, A., Shan, D., and Warg, B., A redox-sensitive resin linker for the solid phase synthesis of *C*-terminal modified peptides. *J. Org. Chem.*, **64**, 156–161 (1999).
12) Hammond, G. S., Boozer, C. E., Hamilon, C. E., and Sen, J. A., Air oxidation of hydrocarbons III. Mechanisms of inhibitor action in benzene and chlorobenzene solutions. *J. Am. Chem. Soc.*, **77**, 3238–3234 (1955).
13) Haward, J. A., in "Free radicals," ed. by Kochi, J. K., Wiley, New York, pp. 3–62 (1973).
14) Niki, E., Saito, M., Yoshikawa, Y., Yamamoto, Y., and Kamiya, Y., Oxidation of lipids. XII. Inhibition of oxidation of soybean phosphatidylcholine and methyl linoleate in aqueous dispersions by uric acid. *Bull. Chem. Soc. Jpn.*, **59**, 471–477 (1986).
15) Burton, G. W., Page, Y., Gabe, E. J., and Ingold, K. U., Antioxidant activity of vitamin E and related phenols. Importance of stereoelectronic factors. *J. Am. Chem. Soc.*, **102**, 7791–7792 (1980).
16) Ohkatsu, Y., and Nishiyama, T., Phenolic antioxidants: effect of *o*-alkyl substituents. *Sekiyu Gakkaishi*, **41**, 251–257 (1998).
17) Howard, J. A., and Yamada, T., Absolute rate constants for hydrocarbon autoxidation. 31. Autoxidation of cumene in the presence of tertiary amines. *J. Am. Chem. Soc.*, **103**, 7102–7106 (1981).
18) Gerlier, D., and Thomasset, N., Use of MTT colorimetric assay to measure cell activation. *J. Immunol. Methods*, **94**, 57–63 (1986).