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*Note*

# Radical-capturing Reaction of 5,7,3′,4′-Tetramethylquercetin with the AIBN Radical Initiator*

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**In order to clarify the mechanism for the radical-capturing reaction which is initiated at the $C_3$-hydroxyl group of flavonols, 5,7,3′,4′-tetramethylquercetin (TMQ) was reacted with the 2,2′-azobis-isobutyronitrile (AIBN) radical initiator in benzene. Six products, one depside and its two hydrolytic products, one nitrile adduct, and two others, were isolated from the reaction mixture, and their structures were determined by instrumental analyses. The quantitative change to the four main products against the reaction time was measured by an HPLC method. The radical-capturing reaction pathway for TMQ with AIBN is proposed from these products and their quantitative changes. The pathway dividing into two clearly reveals that one sub-path formed the depside and its hydrolytic products, while the other formed the nitrile adduct. The reactivity of each two sub-path was nearly the same, different from the case of TMQ and the 2,2′-azobis-2,4-dimethylvaleronitrile (AMVN) radical initiator.**

**Key words:** antioxidative activity; radical-capturing ability; quercetin; 2,2′-azobis-isobutyronitrile (AIBN); reaction mechanism

Studies on the antioxidative activity of naturally occurring compounds have been undertaken in various research fields, being based on accumulated experimental and epidemiological evidence suggesting the involvement of free radical-mediated lipid oxidation in a variety of disease, aging and carcinogenesis cases.[1–6] We have focused our attention on the antioxidative activity of natural compounds for more effective use of plant resouces. In our comparison and evaluation of natural compounds, many promising compounds containing flavonoids have been identified.[7,8] By considering such evidences as the necessity for free hydroxyl groups on flavonoids and the effect of the number of hydroxyl groups for exhibiting activity,[2,3,5,9] a strict comparison with a set of natural flavonoids having the most common 5,7,3′,4′-tetrahydroxylated pattern by the stopped-flow spectroscopic method revealed quercetin as the flavonol with the strongest activity.[10–12] A similar comparison with the partial methyl ethers of quercetin revealed that the $C_3$-hydroxyl group exhibited strong activity, and together with the vicinal hydroxyl group had the strongest activity.[12,13] Pioneering work on the reaction mechanism for radical-capturing of $\alpha$-tocopherol[14–16] was in progress during out experiment with flavonols, and one possible reaction pathway initiated at the $C_3$-hydroxyl group has been proposed from results with the reaction system of 5,7,3′,4′-tetramethylquercetin (TMQ, a typical flavonol) with the 2,2′-azobis-2,4-dimethylvaleronitrile (AMVN) radical initiator.[12]

In the present paper, we describe the radical-capturing reaction mechanism for TMQ and the 2,2′-azobis-isobutyronitrile (AIBN) radical initiator with weak reactivity for comparison with the case of TMQ and the AMVN radical initiator with strong reactivity.

$^1$H- and $^{13}$C-NMR spectra were obtained with Varian Inova 400 (400 MHz) and/or Inova 500 (500 MHz) FT-NMR spectrometers. MS spectra were determined with a Shimadzu GCMS-QP 5000 mass spectrometer. HPLC was performed with a system of JASCO PU-980 pumps and a JASCO UV-970 UV/VIS detector, using columns of Waters $\mu$ Bondasphere 5 $\mu C_{18}$ 100 Å (3.9 i.d. × 150 mm and 19.0 i.d. × 150 mm). The solvent systems were methanol (MeOH):$H_2O$ = 7:3, 6:4 and 5:5, v/v. UV spectral data were recorded with a JASCO V550 UV/VIS spectrophotometer. TLC and preparative TLC (PTLC) were conducted on silica gel (Merck, Kieselgel 60 $F_{254}$), and column chromatography (CC) was also run on silica gel (Fuji Silysia, BW-200). TLC, PTLC and CC used a solvent system of chloroform ($CHCl_3$):acetone:benzene = 9:1:0.5, v/v. Rutin, AIBN and AMVN were purchased from Sigma, and TMQ was prepared from rutin by the method described in the previous paper.[12]

TMQ (1.0 mM) and AIBN (10 mM) were dissolved in benzene (500 ml), and the solutions kept at 37°C

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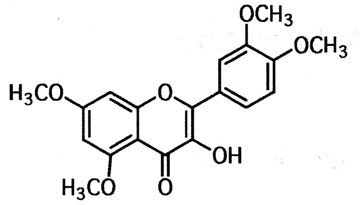
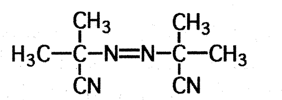
**Substrate**



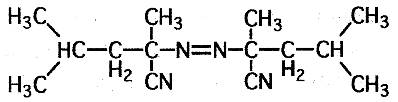
5,7,3',4'-Tetramethylquercetin (TMQ)

**Radical initiator**



$H_3C-\underset{CN}{\overset{CH_3}{C}}-N=N-\underset{CN}{\overset{CH_3}{C}}-CH_3$

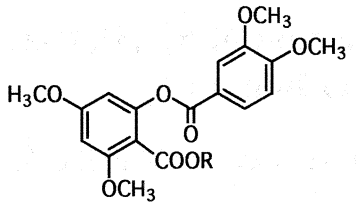
2,2'-Azobis-isobutyronitrile (AIBN)



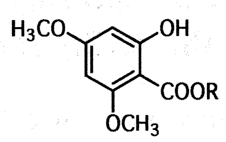
$\begin{matrix}H_3C\\H_3C\end{matrix}HC-\underset{H_2}{C}-\underset{CN}{\overset{CH_3}{C}}-N=N-\underset{CN}{\overset{CH_3}{C}}-\underset{H_2}{C}-CH\begin{matrix}CH_3\\CH_3\end{matrix}$

2,2'-Azobis-(2,4-dimethylvaleronitrile) (AMVN)

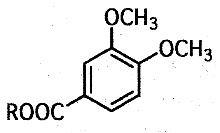
**Products**



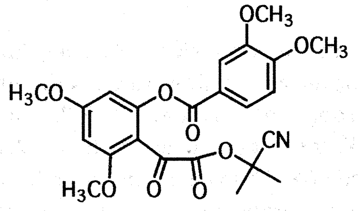
Product 1 ($R=CH_3$)
Product 1' ($R=H$)



Product 2 ($R=CH_3$)
Product 2' ($R=H$)
Product 5' ($R=CH_2CH_3$)



Product 3 ($R=CH_3$)
Product 3' ($R=H$)
Product 6' ($R=CH_2CH_3$)



Product 4

for 43, 75 or 83 h in the dark. The end point of the reaction was determined as the disappearance of the TMQ spot by TLC monitoring. The reaction mixture was concentrated *in vacuo* to yield an oily residue. This residue was dissolved in a small amount of ethanol (EtOH), and after keeping overnight at room temperature, the deposited AIBN was filtered off. After evaporating the filtrate, the residue was dissolved in ethyl acetate (EtOAc), and the solution was successively washed with $NaHCO_3$ and $Na_2CO_3$ solutions (3 times each). The combined washings were acidified with 8 N HCl and then extracted with EtOAc (3 times). Part of the evaporated EtOAc layer was treated with diazomethane, the methylated fraction being submitted to CC, preparative HPLC and recrystallization, and products 1′, 2′ and 3′ were isolated. After instrumental analyses, these products were identified as methyl 4,6-dimethoxy-2-(3,4-dimethoxybenzoyloxy)-salicylate, methyl 4,6-dimethoxysalicylate and methyl veratrate by direct comparison with authentic samples that had respectively been isolated from the reaction mixture of TMQ and AMVN. Detailed data by instrumental analyses have been presented in our previous paper.[12] Accordingly, original products 1-3 were 4,6-dimethoxy-2-(3,4-dimethoxybenzyloxy)-salicylic acid, 4,6-dimethoxysalicylic acid and veratric acid. The original 4,6-dimethoxysalicylic acid and veratric acid must have been produced from depside product 1 by a further hydrolytic reaction. The isolation of three products shows that the radical-capturing reaction pathway for TMQ with AIBN was similar to the main route for the reaction of TMQ with AMVN,[12] and for the photosensitized oxygenation reaction of quercetin in the presence of rose bengal.[17]

Part of the EtOAc solution obtained from the reaction mixture was applied to CC, and the elutaes gave product 4 by repetitive HPLC and recrystalization. The MS spectrum was obtained, showing characteristic fragment ions of $C_6H_3(OCH_3)_2C\equiv O^+(A^+)$ ($m/z$ 165) and $C_6H_2(OCH_3)_2(OH)C\equiv O^+(B^+)$ ($m/z$ 181), and the molecular ion ($m/z$ 457) of an odd number which suggested the presence of one nitrogen atom. The $^1$H-NMR spectrum showed five proton signals of 1,3,4-tri- and 1,2,4,6-tetrasubstituted aromatic rings, 12 protons of four methoxyl groups, and six aliphatics of an isobutyronitrile moiety. The $^{13}$C-NMR spectrum confirmed the presence of 23 carbon signals, these being assigned to 12 carbons of two aromatic rings, four carbons of four methoxyl groups, four carbons of one isobutyronitrile group and three carbons of three carbonyl groups. The carbonyl carbons were divided into two ester carbons and one ketone carbon, based on their chemical shifts. The evaluation of these carbonyl carbons is remeniscent of the result for product 5, 1-(2-veratroyloxy-4,6-dinaethoxyphenyl)-2-(2,4-dimethylvareronitrile-2-oxy)-ethane-1,2-dione that havs been isolated from the reaction of TMQ and AMVN.[12] The$^1$H and $^{13}$C-NMR spectra of product 4 were also similar to those of product 5, except for the iso-butyronitrile moiety. Therefore, product 4 was elucidated to be 1-(2-veratroyloxy-4,6-dimethoxyphenyl)-2-(isobutyronitrile-2-oxy)-ethane-1,2-dione with the following analytical data:

DI-MS *m/z* (%): 457 (2.1, M$^+$), 346 (3.2), 345 (15.4), 181 (2.0, $B+H^+$), 165 (100, $A^+$), 152 (4.8), 137 (8.3, $(A-CO)^+$), ($A$ minus CO)$^+$ 122 (3.0), 107 (2.5), 79 (5.9), 77 (7.5) & 69 (3.4). $^1$H-NMR ($CDCl_3$) $\delta$: 1.81 (3H×2, each s, $C-(CH_3)_2$), 3.89, 3.95, 3.96, 3.96 (3H×4, each s, $Ar-OCH_3$), 6.49 (1H, d, $J$=2.20 Hz, $C_5$–H), 6.44 (1H, d, $J$=2.20 Hz, $C_3$–H), 6.94 (1H, d, $J$=8.42 Hz, $C_5$–H), 7.64 (1H, d, $J$=2.20 Hz, $C_2$–H) & 7.84 (1H, d.d, $J$=2.20 & 8.42 Hz, $C_6$–H). $^{13}$C-NMR ($CDCl_3$) $\delta$: 26.5 ($C-(\underline{C}H_3)_2$), 56.0, 56.2, 57.0 ($Ar-O\underline{C}H_3$),69.5 ($\underline{C}-(CH_3)_2$), 96.4 ($C_3$), 102.4 ($C_5$), 109.5 ($C_1$), 110.5 ($C_2$), 112.4 ($C_5$), 119.7 (CN), 122.0 ($C_1$), 124.4 ($C_6$), 148.4, 152.9, 154.2, 162.0, 163.0 ($C_{2,4,6\&3,4}$), 164.5 (C=O), 166.2 (C=O) & 180.5 (C=O).

The EtOH filtrate, after filtering off the deposited AIBN was concentrated *in vacuo*, and the residue was applied to CC. Small amounts of products 5′ and 6′ were isolated from the eluates, and purified by PTLC and recrystalization. The $^1$H-NMR spectrum of product 5′ showed the presence of 14 protons comprising one methyl group, two methoxyl groups, one methylene group, one pair of *meta*-relationship aromatic protons and one aromatic hydroxyl group. The $^{13}$C-NMR spectrum revealed 11 carbons comprising one carbon of a carbonyl group, six of an aromatic ring, two of methoxyl groups and two of an aliphatic moiety. The $^1$H- and $^{13}$C-NMR spectra of product 5′ were very similar to those of product 2′, except for the aliphatic moiety. Molecular ion M$^+$ of product 5′ was observed at *m/z* 226, and that of product 2′ gave M$^+$ at *m/z* 212. The mass difference of two molecular ions indicates that product 5′ had one ethyl moiety. Thus, the structure for product 5′ was identified as ethyl 2-hydroxy-4,6-dimethoxybenzoate. Similarly, the instrumental analysis indicated product 6′ indicated to be ethyl veratrate, and the estimated structure was confirmed by a direct comparison with the specimen prepared from purchased veratric acid. If EtOH was not used for the removal of AIBN, isolation and purification, products 5′ and 6′ were not obtained. They had also not been isolated from the reaction of TMQ with AMVN.[12)] Consequently, it is considered that products 5′ and 6′ were artifacts derived from unknown reaction intermediates; for example, they might have been formed from the intermediates and EtOH under acidic conditions by a reaction like Fischer-Speier esterification.[18)] Instrumental analysis data for product 5′: DI-MS *m/z* (%): 226 (23.2, M$^+$), 181 (24.2), 180 (100, $B^+$), 152 (27.6), 137 (29.8, $C_6H_4(OCH_3)_2^+$), 109 (5.3), 95 (9.9), 81 (6.6), 69 (19.1) & 66 (7.2). $^1$H-NMR ($CDCl_3$) $\delta$: 1.40 (3H, d, $J$=6.96 Hz, $-CH_3$), 3.82, 3.81 (3H×2, each s, $Ar-OCH_3$), 4.30 (2H, q, $J$=6.96 Hz, $-CH_2-$), 5.96 (1H, d, $J$=2.20 Hz, $C_5$–H), 6.11 (1H, d, $J$=2.56 Hz, $C_3$–H) & 12.08 (1H, s, $C_2$–OH). $^{13}$C-NMR ($CDCl_3$) $\delta$: 15.2 ($-CH_3$), 56.4, 57.0 ($Ar-O\underline{C}H_3$), 62.2 ($-CH_2-$), 92.6, 94.4 ($C_{3\&5}$), 97.9 ($C_1$), 163.2 ($C_2$), 166.1, 166.9 ($C_{4\&5}$) & 172.2 (C=O). Instrumental analysis data for product 6′: DI-MS *m/z* (%): 210 (66.1, M$^+$), 195 (7.8, $M-CH_3^+$), 182 (28.4), 166 (13.9), 165 (100, $C_6H_3(OCH_3)_2C\equiv O^+$), 137 (14.5), 121 (11.4), 107 (8.9), 95 (9.9), 79 (34.0) & 65 (9.7). $^1$H-NMR ($CDCl_3$) $\delta$: 1.39 (3H, t, $J$=7.10 Hz, $-CH_3$), 3.94 (3H×2, each s, $Ar-OCH_3$), 4.37 (2H, q, $J$=7.10 Hz, $-CH_2-$), 6.89 (1H, d, $J$=8.47 Hz, $C_5$–H), 7.55 (1H, dd, $J$=2.06 & 8.47 Hz, $C_6$–H) & 8.47 (1H, d, $J$=2.06 Hz, $C_2$–H).

In order to understand the radical-capturing reaction process, a mixture of TMQ (0.1 mM) and AIBN (1 mM) in benzene (100 ml) was kept for 108 h in the dark at 37°C. Part of the resulting reaction mixture was taken out every 12 h, the aliquot was concentrated *in vacuo*, and the resulting residue was treated with diazomethane. The treated residue was dissolved in MeOH containing an internal standard of flavone (0.125 mg/ml based on the aliquot), and after filtering with DISMIC-13JP (PTFE, 0.5 $\mu$m), the MeOH solution was subjected to an HPLC analysis under the following conditions: solvent, $MeOH:H_2O=6:4$, v/v; flow rate, 0.3 ml/min., detection, $\lambda$ 280 nm; product, retention time (min), product 1′, 21; product 2′, 17; product 3′, 11 and product 4, 26. The quantitative changes to these four main products against time were determined twice (Fig. 1). Although detailed changes were observed in all the curves, only one example is given here. TMQ disappeared after about 72 h, while AMVN and TMQ disappeared after only 10 h.[12)] It is suggested that the reactivity of AIBN was about one

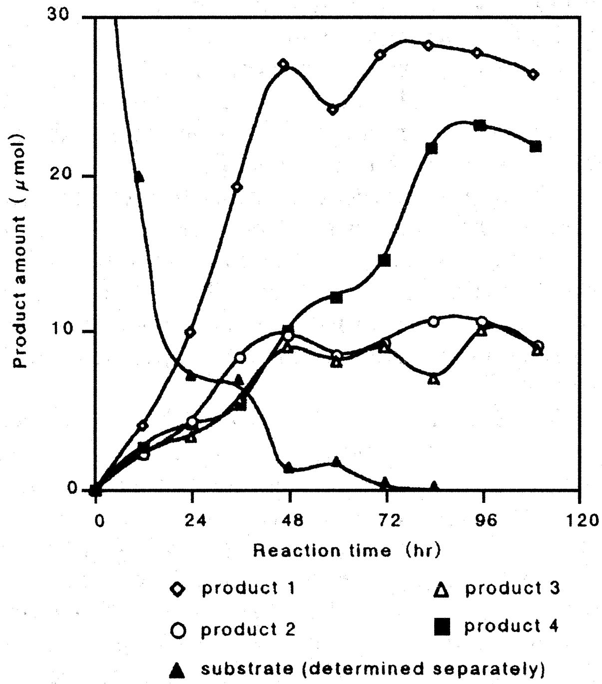


**Fig. 1.** Quantitative Changes to Products from the Reaction of TMQ with the AIBN Radical Initiator.

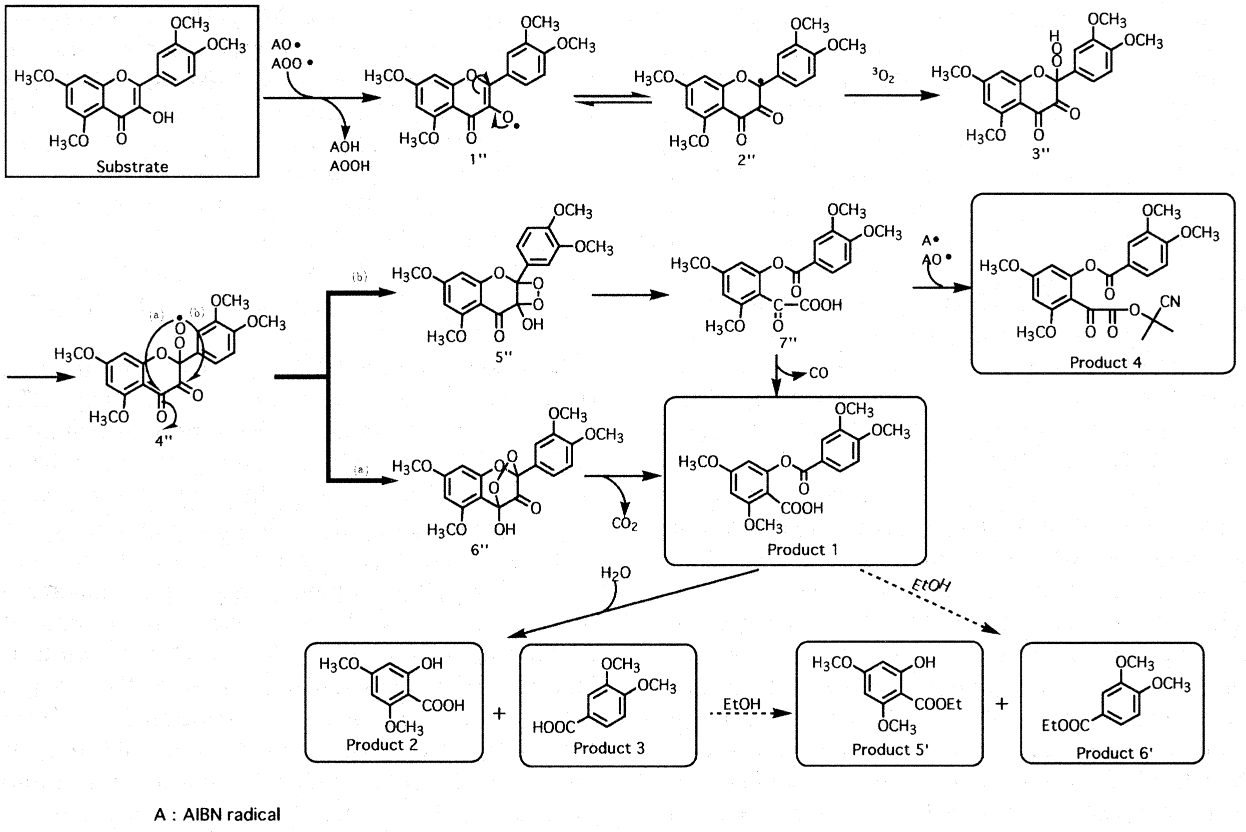


**Fig. 2.** Possible Radical-capturing Reaction Mechanism for 5,7,3′,4′- Tetramethylquercetin (TMQ) and the AIBN Radical Initiator.

seventh that of AMVN. The depside formation reached its maximum after 48 h, and a quantitative level was retained until the end of the experiment, though it decreased a little at one stage. Further degraded products 2 and 3 increased up to 48 h, and their quantities were maintained untill the end. The amount of product 2 was always little less than that of product 3. This result indicates that product 2, 4,6-dimethoxysalicylic acid with one free hydroxyl group, may function as a substrate in the subsequent radical-capturing reaction, but its further degraded products have not so far been detected. Product 4 also showed a marked increase up to the end point. The formation of the radical adduct was characteristic, because the formed amount is more than the corresponding amount in the experiment with AMVN.[12]

The reaction of TMQ with AMVN has afforded many noteworthy products.[12] The reaction of TMQ with AIBN gave six products, their formation pathway being summarized next (Fig. 2). After derivation of the isobutylnitrile radicals from AIBN, a similar reaction process proceeded to that described in the previous paper.[12] At the stage of ketohydroperoxy radical (4″) formation, the pathway divided into two depending upon the $C_3$ or $C_4$ addition of the peroxy radical moiety in 4″. Route (a) gave product 1 *via* intermediate 6″, and product 1 further formed products 2 and 3 by hyrolytic degradation. On the other hand, route (b) afforded product 4 *via* intermediates 5″ and 7″. According to the quantitative reaction of TMQ with AIBN, the reactivity of route (b) was nearly equal to that of the route (a), this being different from the case of AMVN (Fig. 1).[12] It is anticipated that the features of the radical-capturing reaction depend on the reactivity of the radical species, but this proposal can not be explained well at the present. The formation of ethylated products 5′ and 6′ is interesting, although the formation mechanism from the unknown parent products also can not be explained. These aspects will be subjects for further work.

In conclusion, two approaches using two species of radical initiators were established for revealing the radical-capturing reaction pathway for the flavonol group which is the most active among the flavonoids. It will thus become possible to investigate the initiation pathway from the most active vicinal hydroxyl group on quercetin for typical natural flavonol.

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## References

1) Terao, J., Effects of $\alpha$-tocopherol (in Japanese). *Shokuhin to Kaihatsu*, **28**, 10–13 (1993).
2) Javanovic, S. V., Steenken, S., Tosic, M., Marjanovic, B., and Simic, M., Flavonoids as antioxidants. *J. Am. Chem. Soc.*, **116**, 4846–4851 (1994).
3) Igile, G. O., Oleszek, W., Jurzysta, M., Burda, S., Fafunso, M., and Fasanmade, A. A., Flavonoids from *Vernonia amygdalina* and their antioxidant activities. *J. Agric. Food Chem.*, **42**, 2445–2448 (1994).
4) Kawagishi, S., Research methods for bioactive compounds in foodstuffs (in Japanese). Gakkai Shuppan Center, Tokyo, pp. 7–22 (1996).
5) Gadow, A., Joubert, E., and Hansmann, F., Comparison of the antioxidant activity of aspalanthin with that of other plant phenols of Rooibos tea (*Aspalathus linearis*), $\alpha$-Tocopherol, BHT, and BHA. *J. Agric. Food Chem.*, **45**, 632–638 (1997).
6) Ogata, M., Hoshi, M., Shimotohno, K., Urano, S., and Endo, T., Antioxidant activity of magnolol, honokiol and related phenolic compounds. *J. Am. Oil Chem. Soc.*, **74**, 557–562 (1997).
7) Kawase, S., Narita, R., Watanabe, A., Kawai, S., and Ohashi, H., Antioxidative activity of asadanin and its homologs. Abstracts of the 43rd annual meeting of the Japan Wood Research Society (in Japanese), p. 344 (1993).
8) Kawase, S., Watanabe, A., Kawai, S., and Ohashi, H., Study on the antioxidative activity of tree phenols, 1: Comparison of antioxidative ability by the improved Wills method. Abstract of the 44th annual meeting of the Japan Wood Research Society (in Japanese), p. 257 (1994).
9) Zhongli, C., Yongke, H., and Jilan, W., Study of baicalin scavenging hydroxyethylperoxy radicals by radiolysis of aerated ethanol-baicalin system. *Radiat. Phys. Chem.*, **47**, 869–871 (1996).
10) Kawase, S., Watanabe, A., Kawai, S., and Ohashi, H., Study on the antioxidative activity of tree phenols, 2. Abstract of the 44th annual meeting of the Japan Wood Research Society (in Japanese), p. 258 (1994).
11) Kawase, S., Kyogoku, T., Kawai, S., and Ohashi, H., Study on the antioxidative activity of tree phenols, 3. Abstract of the 45th annual meeting of the Japan Wood Research Society (in Japanese), p. 409 (1995).
12) Ohashi, H., Kyogoku, T., Ishikawa, T., Kawase, S., and Kawai, S., Antioxidative activity of tree phenolic constituents, I. Radical capturing reaction of flavan-3-ols with radical initiator. *J. Wood Science*, **45**, 53–63 (1998).
13) Kyogoku, T., Ishikawa, T., Kawai, S., and Ohashi, H., Studies on the antioxidative activity of tree phenols, 5. Antioxidative reaction of quercetin methyl derivatives. Abstract of the 47th annual meeting of the Japan Wood research Society (in Japanese), p. 415 (1997).
14) Yamauchi, R., Matsui, T., Kato, K., and Ueno, Y., Reaction product of $\alpha$-tocopherol with methyl linoleate peroxyradicals. *Lipid*, **25**, 152–158 (1990).
15) Koga, T. and Terao, J., Phospholipids increase radical-scavenging activity of vitamin E in a bulk oil model system. *J. Agric. Food Chem.*, **43**, 1450–1454 (1995).
16) Yamauchi, R., Kato, K., and Ueno, Y., Free-radical scavenging reactions of $\alpha$-tocopherol during the antioxidation of methyl linoleate in bulk phase. *J. Agric. Food Chem.*, **43**, 1455-1461 (1995).
17) Matsuura, T., Matsushima, H., and Nakashima, R., Photoinduced reactions XXXVI, Photosensitized oxygenation of 3-hydroxyflavones as a non-enzymatic model for quercetinase. *Tetrahedron*, **26**, 435–443 (1970).
18) Gotoh, T., Kakizawa, H., and Minato, H. (translated from Japanese), Fischer esterification. In Advanced Organic Chemistry eds. Fieser, L. F. and Fieser, M. (1961) Reinhold, New York. Maruzen, Tokyo, p. 450 (1964).