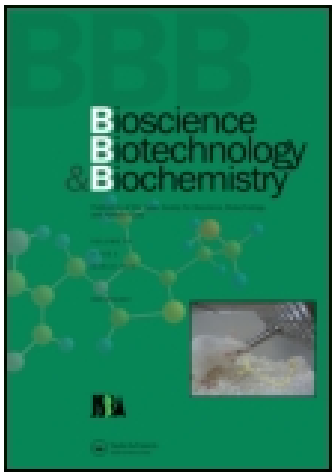

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Antioxidative Activity of Tetrahydrocurcuminoids

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Antioxidative Activity of Tetrahydrocurcuminoids

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In order to develop a new type of antioxidative compound which has both the phenolic and β-diketone moiety in the same molecule, we converted three known curcuminoids, curcumin (diferuloylmethane, U1), (4-hydroxy-3-methoxycinnamoyl)methane (U2), and bis-(4-hydroxycinnamoyl)methane (U3), which are the natural antioxidants of *Curcuma longa* L. (turmeric), to tetrahydrocurcuminoids (THU1, THU2, and THU3, respectively) by hydrogenation, and evaluated their antioxidative activity by using linoleic acid as the substrate in an ethanol/water system. Further, we used the rabbit erythrocyte membrane ghost and rat liver microsome as *in vitro* systems and determined the antioxidative activity of these curcuminoids. When we evaluated their antioxidative activity by these assays, it was found that THU1 had the strongest antioxidative activity among all curcuminoids in each assay system. THU1 has been reported to be one of the main metabolites of U1 *in vivo* [Holder *et al.*, *Xenobiotica*, 8, 761–768 (1978)]. These results suggest that THU1 must play an important role in the antioxidative mechanism of U1 *in vivo* by converting U1 into THU1.

Lipid peroxidation is known to be a free-radical chain reaction taking place in both *in vitro* and *in vivo* systems that may lead to destabilization and disintegration of cell membranes, to many age-related diseases, to aging and to susceptibility to cancer.[1] Strong attention has recently been focused on the importance of the protective defence systems in living cells against damage caused by active oxygen and free radicals. Several endogenous antioxidants such as vitamin E, vitamin C, β-carotene, urice acid, bilirubin, carnosine, and ubiquinols have been found to play an important role in nonenzymatic protection. In addition to these natural defence systems, there is increasing interest in the protective biochemical function of dietary antioxidants, which are candidates for cancer prevention and for extending the life span of animals.[2,3]

In the course of our investigation into novel types of antioxidative substances in plant materials, it was found that most natural antioxidative components can be classified into two types: phenolic and β-diketone antioxidative substances. Of the former type of natural antioxidative substances, the lignan group of sesamolinol,[4] sesaminol,[5] several pinoresinol glucosides,[6,7] and sesaminol glucosides[8] were isolated from sesame seed and sesame oil. A novel type of γ-tocopherol derivative, named prunusol A and B, both being γ-tocopherol conjugates with *p*-coumaric acid, was recently isolated from *Prunus* leaf wax.[9] In addition, several of the β-diketone type of natural antioxidative substances, *n*-tritriacontan-16,18-dione[10] and 4-hydroxy-tritriacontan-16,18-dione,[11] have been isolated from the leaf wax of *Eucalyptus* and identified as a novel class of natural antioxidant. However, few antioxidative substances that have both phenolic and β-diketone groups in the same molecule have been found. This background prompted us to develop a new type of antioxidative compound which has both functional groups in the same molecule,[12] and we thus focused on three known curcuminoids, including curcumin.

The rhizome of *Curcuma longa* L. (turmeric) has long been widely used in indigenous medicine for treating sprains and inflammation.[13] Curcumin (diferuloylmethane, U1†) is the main yellow pigment of turmeric, which can be extracted with diethyl ether together with two minor yellow pigments, (4-hydroxy-3-methoxycinnamoyl)methane (U2) and bis-(4-hydroxycinnamoyl)methane (U3), and has been reported to possess both antioxidative and anti-inflammatory activity.[14–18] However, there are some limitations in utilizing U1 for food and medicinal purposes because of its yellow color. This prompted us to obtain more potent and colorless antioxidants, tetrahydrocurcuminoids (THU1, THU2, and THU3, respectively), by hydrogenating the two double bonds conjugated to β-diketones in three curcuminoids (U1, U2, and U3) (Fig. 1).

Materials and Methods

Materials. U1, U2, and U3 were obtained, after purifying by preparative silica gel TLC (5% MeOH in $CHCl_3$, Merck Art. 13895), from turmeric, which was presented by Daiwa Kasei Co. (Saitama, Japan). The yields of U1, U2, and U3 were 76.0%, 19.8%, and 8.6%, respectively. Linoleic acid, platinum oxide (PtO_2†) and ammonium thiocyanate were purchased from Wako Pure Chemical Industries (Osaka, Japan), and 2-thiobarbituric acid (TBA) was purchased from Merck (Darmstadt, Germany). Commercially available rabbit blood was obtained from Japan Biotest Laboratories, and Wistar rats (8 weeks old, 250–300 g) were obtained from Chubu Kagaku Shizai Co. *tert*-Butyl-hydroperoxide (*t*-BuOOH) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Preparation of the curcuminoid derivatives. The three curcuminoids (U1, U2, and U3) were converted to their tetrahydro-forms (THU1, THU2, and THU3, respectively) by hydrogenation with PtO_2 as the catalyst according to the method of Uehara *et al.*[19] After hydrogenation, their derivatives were purified by preparative TLC (5% MeOH in $CHCl_3$; $R_f = 0.86$, 0.57, and 0.34, respectively) with yields of 42.5%, 42.5%, and 55.7%, respectively, of the original curcuminoids. Spectroscopic analyses of THU1, THU2, and THU3 were conducted by measuring MS, IR, UV, and NMR spectra.

THU1. FAB-MS *m/z* 395 $(M+Na)^+$; IR (KBr) ν_{max} cm^{-1}: 3430 (OH), 3060–2840 (CH), 1603 (C=O), 1033 (OCH_3); UV (EtOH) λ_{max} (ε) nm: 225 (1.5×10^4), 282 (1.7×10^4); ^{1}H-NMR ($CDCl_3$) δ: 2.54 (4H, t, *J*=

Abbreviations: U1, curcumin; U2, (4-hydroxy-3-methoxycinnamoyl)methane; U3, bis-(4-hydroxycinnamoyl)methane; THU1, tetrahydrocurcumin; THU2, tetrahydro-(4-hydroxy-3-methoxycinnamoyl)methane; THU3, tetrahydro-bis-(4-hydroxy-3-methoxycinnamoyl)methane; PtO_2, platinum oxide; TBA, 2-thiobarbituric acid; *t*-BuOOH, *tert*-butyl-hydroperoxide; TBARS, TBA-reacting substance; DMSO, dimethyl sulfoxide; HHU1, hexahydrocurcumin.

8.1 Hz, 1.7), 2.74–2.88 (4H, m, 2, 6), 3.49 (2H (keto), s, 4), 3.83 (6H, s, OCH_3), 5.42 (1H (enol), s, 4), 5.65 (H, broad, OH), 6.65 (2H, d, J=7.8 Hz, 6′, 6″), 6.67 (2H, s, 2′, 2″), 6.81 (2H, d, J=7.8 Hz, 5′, 5″).

THU2. FAB-MS m/z 365 $(M+Na)^+$; IR (KBr) ν_{max} cm^{-1}: 3420 (OH), 3010–2840 (CH), 1603 (C=O); UV (EtOH) λ_{max} (ε) nm: 224 (1.4×10^4), 282 (1.0×10^4); ^{1}H-NMR ($CDCl_3$) δ: 2.54 (4H, t, J=8.2 Hz, 1, 7), 2.74–2.88 (4H, m, 2, 6), 3.50 (2H (keto), s, 4), 3.85 (3H, s, OCH_3), 5.41 (1H (enol), s, 4), 5.60 (H, broad, OH), 6.66 (1H, d, J=8.0 Hz, 5′), 6.68 (1H, s, 2′), 6.74 (2H, d, J=8.6 Hz, 3″, 5″), 6.84 (1H, d, J=8.0 Hz, 6′), 7.02 (2H, d, J=8.6 Hz, 2″, 6″).

THU3. FAB-MS m/z 313 $(M+H)^+$; IR (KBr) ν_{max} cm^{-1}: 3314 (OH), 3020–2850 (CH), 1604 (C=O); UV (EtOH) λ_{max} (ε) nm: 224 (1.5×10^4), 279 (1.6×10^4); ^{1}H-NMR ($CDCl_3$) δ: 2.53 (4H, t, J=8.3 Hz, 1, 7), 2.72–2.87 (4H, m, 2, 6), 3.49 (2H (keto), s, 4), 4.70 (2H, broad, OH), 5.38 (1H (enol), s, 4), 6.74 (4H, d, J=8.6 Hz, 3′, 5′, 3″, 5″), 7.04 (4H, d, J=8.6 Hz, 2′, 6′, 2″, 6″).

Antioxidative assay

Linoleic acid autoxidation model. Autoxidation of linoleic acid in a water/alcohol solution was assayed by the thiocyanate and TBA methods as shown in a previous report.[10] Each curcuminoid dissolved in ethanol was put into a solution of linoleic acid (0.13 ml), 99.5% ethanol (10 ml) and a 0.05 M phosphate buffer at pH 7.0 (10 ml), and the total volume was adjusted to 25 ml with distilled water. The mixed solution in a conical flask was incubated at 40°C, and the peroxide value was determined at 500 nm after a color reaction with $FeCl_2$ and thiocyanate (thiocyanate method). After the reaction, the formation of TBA-reacting substance ($TBARS^+$) was measured at 532 nm with TBA (TBA method).

Rabbit erythrocyte membrane ghost system. Commercially available rabbit blood (100 ml) was diluted with 300 ml of an isotonic buffer solution (10 mM phosphate buffer at pH 7.4/152 mM NaCl). After centrifuging the solution (3500 rpm for 20 min), the blood was lysed in 300 ml of a 10 mM phosphate buffer at pH 7.4. The erythrocyte membrane ghosts were pelleted by centrifuging (11,500 rpm for 40 min), and the precipitate was diluted to give a suspension (1.0 mg of protein/ml). Peroxidation of the erythrocyte membrane ghosts induced by *t*-BuOOH was carried out by the method described previously.[20] Curcuminoids were prepared by dissolving them in dimethyl sulfoxide ($DMSO^+$) and after incubating at 37°C for 20 min, TBARS was determined at 532 nm.

Rat liver microsome system. Wistar rats (8 weeks old, 250–300 g) were sacrificed, and their livers were perfused with 0.9% ice-cold NaCl and removed. A 0.3 M mannitol/10 mM Tris–HCl buffer at pH 7.4 was added to the livers, which were then homogenized. Microsomal fractions were obtained by differential centrifugation (8000 g × 20 min and then 105,000 g × 60 min). Fresh microsomes, in a 0.1 M phosphate buffer at pH 7.4, were prepared each time at the concentration of 1.0 mg of protein/ml. The curcuminoids were dissolved in DMSO and added to the microsomes. Peroxidation of the microsomes induced by *t*-BuOOH was carried out by the method of Cadenas *et al.*[21] and after incubating at 37°C for 30 min, TBARS was determined at 532 nm for each.

Results and Discussion

Curcumin (U1) and two minor components (U2 and U3) (Fig. 1) were obtained from commercially available turmeric after purification by preparative silica gel TLC (5% MeOH in $CHCl_3$; R_f=0.80, 0.49, and 0.28, respectively). Two double bonds conjugated to β-diketones in the curcuminoids were reduced to tetrahydro-forms by hydrogenating with PtO_2 as the catalyst (Fig. 1), and their derivatives were purified by preparative TLC (5% MeOH in $CHCl_3$; R_f=0.86, 0.57, and 0.34, respectively). All the olefinic protons in the ^{1}H-NMR spectra of the original curcuminoids were found to have disappeared in the ^{1}H-NMR spectrum of the tetrahydrocurcuminoids. All the spectroscopic data for THU1 were identical with those described in the previous paper for this compound.[22] On the other hand, no spectroscopic data for THU2 and THU3 have been previously reported in the literature, although all data from the instrumental analyses for the two tetrahydrocurcuminoids, THU2 and THU3, are reasonable.

The antioxidative activity of these curcuminoids was evaluated by using linoleic acid as the substrate in an ethanol/water system, and was determined by the thiocyanate and TBA methods. As shown in Fig. 2A, U1 had the strongest antioxidative activity among the natural curcuminoids (U1, U2, and U3) when determined by the thiocyanate method,

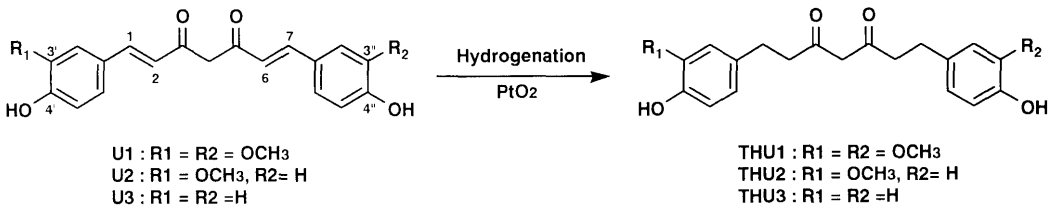

Fig. 1. Preparation of Tetrahydrocurcuminoids (THU1–3) from Curcuminoids (U1–3) by Hydrogenating with PtO_2.

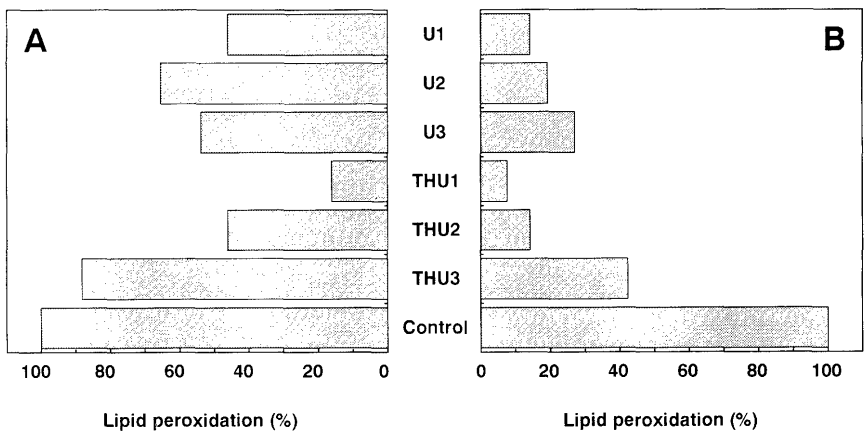

Fig. 2. Antioxidative Activity of Curcuminoids in the Linoleic Acid Autoxidation Model Determined by the Thiocyanate (A) and TBA (B) Methods.

Each curcuminoid was added to the solution at a concentration of 20 μM, and the mixed solution was incubated at 40°C for 4 days (A) or for 10 days (B). A control containing no added samples represents 100% lipid peroxidation. A single experiment is shown, representative of several. Each result is the average of duplicate determinations.

monitoring the formation of lipid hydroperoxide. The result is consistent with the data of Toda *et al.*[23] However, when these curcuminoids were converted to tetrahydrocurcuminoids by hydrogenation, THU1 and THU2 showed stronger antioxidative activity than the respective original curcuminoids (U1 and U2). The same profiles were observed, as shown in Fig. 2B, when their antioxidative activities were determined by the TBA method, monitoring the formation of TBARS. These results indicate U1 to be the most effective antioxidant among the three natural antioxidants in both the initiation and propagation of lipid peroxidation, and THU1 prepared by hydrogenation to have the strongest antioxidative activity among all curcuminoids in both stages. Earlier studies on the metabolism of U1, using [^{3}H]-U1, have indicated that considerable amounts of radioactivity were detectable in the blood, liver and kidney,[24] so we also tried to evaluate the antioxidative activity of curcuminoid by *in vitro* systems, using the rabbit erythrocyte membrane ghost (Fig. 3) and rat liver microsome (Fig. 4), determining the TBARS formation after inducing lipid peroxidation by *t*-BuOOH. As shown in Figs. 3 and 4, THU1 had the strongest antioxidative activity in the two assay systems, as well as in the linoleic acid autoxidation model already described above. However, in respect of the other two tetrahydrocurcuminoids (THU2 and THU3), Fig. 3 shows that these exhibited much more antioxidative activity than the original curcuminoids (U2 and U3), while Fig. 4 shows that they exhibited almost the same activity as that of their originals.

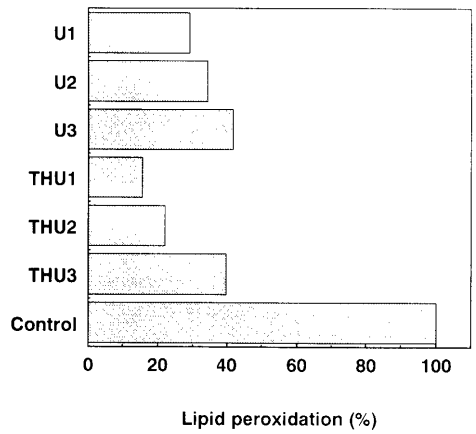

Fig. 3. Antioxidative Activity of Curcuminoids in the Rabbit Erythrocyte Membrane Ghost System Determined by the TBA Method.

Each curcuminoid was added to the solution at a concentration of 280 μM, and the mixed solution was incubated at 37°C for 20 min. A control containing no added samples represents 100% lipid peroxidation. A single experiment is shown, representative of several. Each result is the average of duplicate determinations.

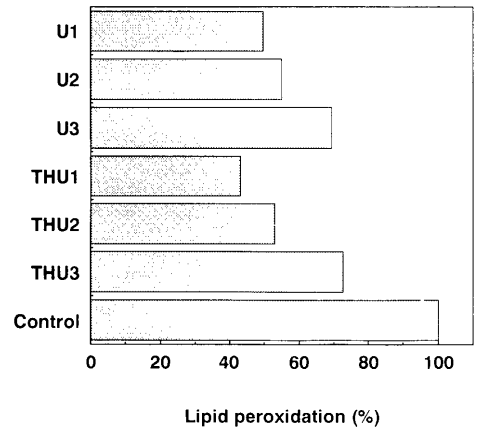

Fig. 4. Antioxidative Activity of Curcuminoids in the Rat Liver Microsome System Determined by the TBA Method.

Each curcuminoid was added to the solution at a concentration of 10 μM, and the mixed solution was incubated at 37°C for 30 min. A control containing no added samples represents 100% lipid peroxidation. A single experiment is shown, representative of several. Each result is the average of duplicate determinations.

The antioxidative mechanism of U1 (a β-diketone type of antioxidant) *in vivo* is not clear at the present stage, although many studies on the metabolite of U1 have recently appeared. Ravindranath *et al.* have reported on the absorption and tissue distribution of U1 in rats[25,26] and its *in vivo* absorption after an oral administration, using [^{3}H]-U1.[24] Their results show that U1 was transformed during absorption by the intestines and that the transfomed product entered the serosal side. On the other hand, Holder *et al.*[27] did not find any free or conjugated U1 in the bile after an intravenous administration of [^{3}H]-U1. According to them, the major metabolites in the bile were glucuronide conjugates of THU1 and hexahydrocurcumin (HHU1†). We suggest that HHU1 is a metabolite formed after the conversion of U1 into THU1, because one side of the β-diketone of THU1 can be reduced to HHU1 by hydrogenation. Further, because the metabolite of U1 obtained by Ravindranath *et al.*[26] was colorless and less polar than U1, it is thought that the product might be THU1 or HHU1. We suggest that THU1 might have been concerned in the antioxidative mechanism of U1 *in vivo* by the conversion of U1 into THU1. We have reported that THU1 was

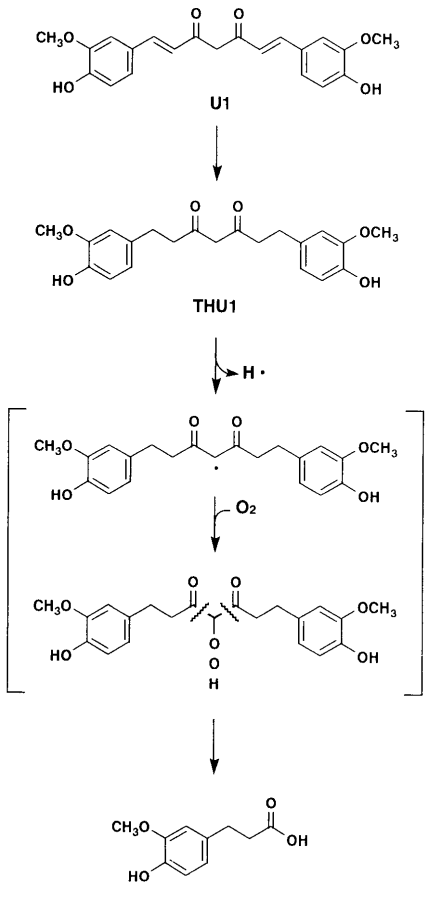

Fig. 5. Proposed Antioxidative Mechanism for U1 in a Biological System.

metabolized to oxidative degradation products during the autoxidation process in the presence of linoleic acid,[28] and the result indicates that phenolic hydroxy groups act as the radical scavenger at the first stage of the antioxidative mechanism for THU1. Further, we have recently obtained preliminary results indicating that the β-diketone structure also plays an important role in the antioxidative mechanism of THU1, because C–C bond cleavage was observed in the β-diketone moiety during the antioxidative process. A detailed examination of this is now in progress. Thus, it is thought that the β-diketone structure of THU1 might act as the radical scavenger in the intermediate or last stage of the antioxidative mechanism in THU1. Holder *et al.*[27] have also isolated dihydroferulic acid, the C–C bond-cleavage product of the β-diketone moiety of THU1, as a minor metabolite of U1 (Fig. 5); these results thus suggest that U1 might at first be metabolized to THU1, which could manifest the antioxidative activity of U1 *in vivo*, that the phenolic hydroxy groups of THU1 might act as the radical scavenger in the first stage of the antioxidative mechanism in U1 *in vivo*, and that the β-diketone structure of THU1 might act as the radical scavenger in the intermediate or last stage.

U1 has been reported to inhibit the microsome-mediated mutagenicity of benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]-anthracene,[29] and has also been reported to inhibit tumor promotion, whose effect can be explained as roughly parallel to the relative antioxidative activity.[30] More recently, Hirose *et al.* have reported that *n*-tritriacontan-16,18-dione, one of the β-diketone type of antioxidants, effectively inhibited hepatic and pancreatic carcinogenesis.[31] Therefore, it is expected that THU1 may have antimutagenicity as well as anti-tumor promoting activity. A detailed experiment to clarify this is currently in progress.

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