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Involvement of the β-Diketone Moiety in the Antioxidative Mechanism of Tetrahydrocurcumin

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ABSTRACT. We examined the inhibitory effects of curcumin and tetrahydrocurcumin (THC), one of the major metabolites of curcumin, on the lipid peroxidation of erythrocyte membrane ghosts induced by *tert*-butylhydroperoxide. The results demonstrated that THC showed a greater inhibitory effect than curcumin. To investigate the mechanism of antioxidative activity, we examined the effects of several inhibitors, such as antioxidant enzymes, hydroxyl radical scavengers, $^1O_2$ quencher, and chelating agents for metal ions. Given that all inhibitors failed to inhibit membrane peroxidation, THC must scavenge radicals such as *tert*-butoxyl radical and peroxyl radical. To clarify the antioxidative mechanism of THC, in particular the role of the β-diketone moiety, dimethylated THC was incubated with peroxyl radicals generated by thermolysis of 2,2′-azobis(2,4-dimethylvaleronitrile). Four oxidation products were detected, three of which were identified as 3,4-dimethoxybenzoic acid, 3′,4′-dimethoxyacetophenone, and 3-(3,4-dimethoxyphenyl)-propionic acid. The fourth oxidation product seems to be an unstable intermediate, and its detailed structure has not been determined. These results suggest that the β-diketone moiety of THC must exhibit antioxidative activity by cleavage of the C—C bond at the active methylene carbon between two carbonyls in the β-diketone moiety. Because THC is one of the major metabolites of curcumin, it may also exhibit the same physiological and pharmacological properties as the active form of curcumin *in vivo* by means of the β-diketone moiety as well as phenolic hydroxy groups. BIOCHEM PHARMACOL 52;4:519–525, 1996.

KEY WORDS. *Curcuma longa* Linn; curcumin; tetrahydrocurcumin; β-diketone; antioxidant; radical scavenger

The rhizome of *Curcuma longa* Linn (turmeric) has been widely used as a yellow coloring agent and spice in many foods, and it has also been used in indigenous medicine for the treatment of inflammatory and other diseases [1]. Curcumin (diferuloylmethane, Fig. 1) has been identified as the major pigment in turmeric and has been reported to possess both antioxidative and antiinflammatory activities [2–6]. Recent studies indicate that curcumin inhibits the microsome-mediated mutagenicity of benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene [7], and that it also acts as a strong inhibitor of tumor promotion in mouse skin by 12-O-tetradecanoylphorbol-13-acetate [8].

Several studies on the absorption and metabolism of curcumin have been reported. Ravindranath and Chandrasekhara have reported on the absorption and tissue distribution of curcumin in rats [9, 10] and its *in vivo* absorption after oral administration using [^{3}H]curcumin [11]. Their results show that curcumin is transformed during absorption from the intestines and that the transformed product, which is a less polar and colorless compound than curcumin, enters the serosal side. On the other hand, Holder *et al.* [12] did not find any free or conjugated curcumin in the bile after intravenous administration of [^{3}H]curcumin; according to them, one of the major metabolites in the bile is the glucuronide conjugate of THC† (Fig. 1). THC, a colorless compound less polar than curcumin, seems to be the transformed product of curcumin during absorption from the intestines. THC may be involved in the physiological and pharmacological properties of curcumin.

In the course of our investigation to find novel types of antioxidative substances in plant materials, two β-diketone-type antioxidants, TTAD and 4-hydroxytritriacontan-16,18-dione, were isolated and identified as novel natural antioxidants from *Eucalyptus* leaf wax [13, 14]. Because curcumin also has a β-diketone moiety, this moiety must play an important role in the antioxidative activity of curcumin *in vivo*. However, the antioxidative mechanism of β-diketone-type antioxidants is not clear.

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† *Abbreviations*: THC, tetrahydrocurcumin; TTAD, *n*-tritriacontan-16,18-dione; CLA, conjugated dienoic derivatives of linoleic acid; *t*-BuOOH, *tert*-butylhydroperoxide; PtO_2, platinum oxide; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); AAPH, 2,2′-azobis(2-amidinopropane)-dihydrochloride; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; DABCO, 1,4-diazabicyclo-[2,2,2]octane; TBA, 2-thiobarbituric acid; SOD, superoxide dismutase; DMTHC, dimethoxytetrahydrocurcumin; and TBARS, TBA-reacting substance.

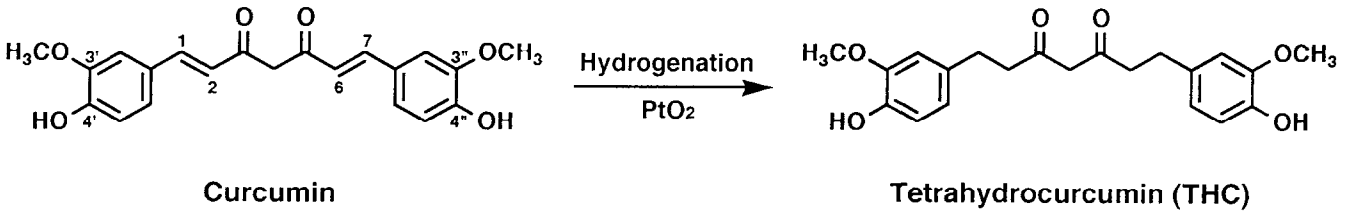


FIG. 1. Preparation of tetrahydrocurcumin (THC) from curcumin by hydrogenation with PtO_2.

Recently, Hirose *et al.* [15] reported that TTAD strongly inhibits hepatic and pancreatic carcinogenesis. Moreover, Pariza and Ha [16] reported that CLA are effective in inhibiting benzo[*a*]pyrene-induced forestomach neoplasia in mice and also in suppressing the process of tumor promotion in the mouse forestomach [16]. One of the possible mechanisms for the anticarcinogenicity of CLA is thought to be that an oxidized derivative of CLA must be the actual ultimate antioxidant form rather than CLA itself, although the structure of the active form has not been determined. However, it has been suggested that the introduction of the β-diketone moiety into the CLA molecule is the most likely candidate as the active form of CLA [17]. Therefore, it is very important to investigate the antioxidative mechanism of β-diketone-type antioxidants.

In this study, we examined the inhibitory effects of curcumin and THC on *t*-BuOOH-induced lipid peroxidation. To investigate the mechanism of antioxidative activity, we examined the contribution of oxidizing species in the peroxidation system. This paper also gives details on the antioxidative mechanism at the β-diketone moiety of THC and reports on an investigation of the metabolic pathway of THC in reaction with peroxyl radicals.

MATERIALS AND METHODS

Materials

Curcumin was obtained after purification by preparative silica gel TLC (5% MeOH in $CHCl_3$, Merck Art. 13895) from turmeric, which was a gift from the Daiwa Kasei Co., Ltd., Saitama, Japan. The yield of curcumin was 76.0%. PtO_2, AMVN, AAPH, mannitol, DMSO, DTPA, DABCO, 3,4-dimethoxybenzaldehyde, and acetyl acetone were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. TBA was purchased from Merck (Darmstadt, F.R.G.). Egg yolk phosphatidylcholine, SOD (from bovine erythrocytes), and catalase (from bovine liver) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Commercially available rabbit blood was obtained from Japan Biotest Laboratories Inc. (Kokubunji, Tokyo, Japan). *t*-BuOOH was purchased from the Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan.

Preparation of Curcuminoid Derivatives

THC. Curcumin was converted to THC by hydrogenation with PtO_2 as the catalyst according to the method of Uehara *et al.* [18]. After hydrogenation, THC was purified by preparative TLC (5% MeOH in $CHCl_3$, R_f = 0.86) with a yield of 42.5%. The identity and purity of THC were confirmed by using MS, IR, UV, and NMR spectra. THC: FAB-MS *m/z* 395 $(M + Na)^+$; IR (KBr) ν_{max} 3430 (OH), 3060–2840 (CH), 1603 (C=O), 1033 (OCH_3)cm^{-1}; UV(EtOH) λ_{max}(log ε) 225(4.16), 282(4.24) nm; ^1H NMR ($CDCl_3$) δ 2.54 (4H, t, *J* = 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 (2H, 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″).

DMTHC. DMTHC was prepared synthetically by condensation of 3,4-dimethoxybenzaldehyde and acetyl acetone by the method of Pabon [19]. The identity of the synthetic compound was established by using MS, IR, UV, and NMR spectra. Dimethoxycurcumin was reduced to DMTHC by hydrogenation with PtO_2 as described above. DMTHC was purified by preparative TLC (*n*-hexane:ethyl acetate = 1:1, R_f = 0.53) with a yield of 52.0%. The identity and purity were confirmed by using MS, IR, UV, and NMR spectra. DMTHC: EI-MS *m/z* 400 (M^+); IR (KBr) ν_{max} 2937 (CH), 1592 (C=O), 1029 (OCH_3) cm^{-1}; UV (EtOH) λ_{max}(log ε) 228(4.23), 280(4.26) nm; ^1H NMR ($CDCl_3$) δ 2.57 (4H, t, *J* = 7.8 Hz, 1,7), 2.82–2.91 (4H, m, 2,6), 3.53 (2H(keto), s, 4), 3.866–3.871 (12H, m, OCH_3), 5.46 (1H(enol), s, 4), 6.72–6.82 (6H, m, 2′,5′,6′,2″,5″,6″).

Antioxidative Assay

Commercially available rabbit blood (100 mL) was diluted with 300 mL of isotonic buffer solution (10 mM phosphate buffer, pH 7.4/152 mM NaCl). After centrifugation (1500 *g*, 20 min), the blood was lysed in 300 mL of 10 mM phosphate buffer, pH 7.4. Erythrocyte membrane ghosts were pelleted by centrifugation (20,000 *g*, 40 min), and the precipitate was diluted to give a suspension (1.0 mg protein/mL). Peroxidation of the erythrocyte membrane ghosts induced by *t*-BuOOH was carried out by a method described previously [20]. After incubation at 37° for 20 min, the formation of TBARS was determined at 532 nm.

Preparation of Liposomes

Liposomes were prepared from egg yolk phosphatidylcholine. Egg yolk phosphatidylcholine was suspended in 10

mM phosphate buffer (pH 7.4) and vortexed. Liposomes were always handled in an atmosphere of nitrogen, to prevent auto-oxidation.

Oxidation of DMTHC

DMTHC (100 μmol) and AMVN (3 mmol) were dissolved in oxygen-saturated acetonitrile and incubated in a screw-cap test tube at 37° according to the method of Liebler *et al.* [21]. AMVN was used in order to replicate the method of Liebler *et al.* [21] on a larger scale. DMTHC (4 μmol) incorporated in the liposomes (10 mg/mL phosphate buffer) and AAPH (200 μmol) were incubated in a screw-cap test tube at 37°. AAPH was used instead of AMVN in the liposome experiment because AMVN is insoluble in phosphate buffer. Oxidation products were analyzed by reverse-phase HPLC on a Develosil ODS-5 column (4.6 × 150 mm) (Nomura Chemical Co., Ltd.). An aliquot of the reaction mixture was eluted with a linear gradient of a two-solvent system at a flow rate of 1.0 mL/min. Solvent A (0.1% trifluoroacetic acid:methanol 8:2) and solvent B (100% methanol) were used for the gradient. The gradient employed was as follows: 100 to 0% A in 30 min, isocratic at 0% A for 10 min, 0 to 100% A in 10 min. The elution was monitored by absorbance at 280 nm. The peak area was determined by use of a Shimadzu C-R3A Chromatopac.

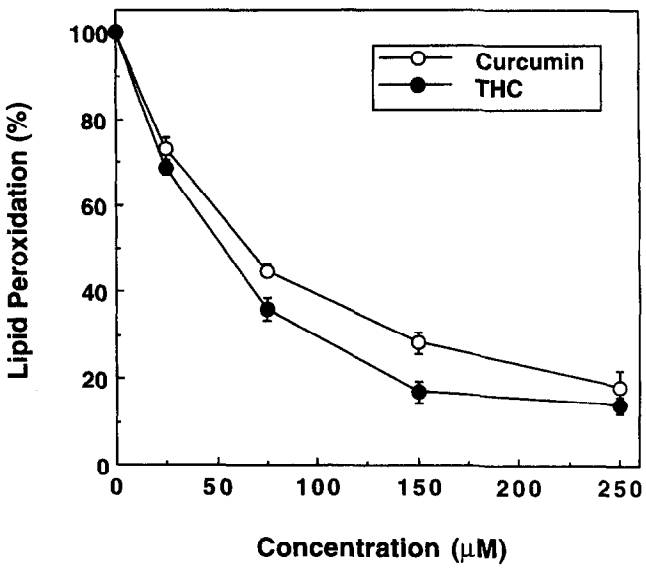


FIG. 2. Inhibitory effects of curcumin and THC on the lipid peroxidation of erythrocyte membrane ghosts induced by *t*-BuOOH. After erythrocyte membrane ghosts at a concentration of 1.0 mg protein/mL were incubated with 2.0 mM *t*-BuOOH for 20 min in the presence or absence of each curcuminoid, TBARS formation was determined at 532 nm. A control containing no added curcuminoids represented 100% lipid peroxidation (1.66 μM MDA equivalents). Results are the means ± SD from four separate experiments.

RESULTS

THC was prepared by hydrogenation of curcumin with PtO_2 as the catalyst (Fig. 1). All olefinic protons in the ^1H NMR spectra of curcumin were found to disappear in the ^1H NMR spectrum of THC. All spectroscopic data of THC were identical with those described in a previous paper on this compound [22].

We examined the inhibitory effects of curcumin and THC, which is one of the major metabolites of curcumin, on the lipid peroxidation of erythrocyte membrane ghosts induced by *t*-BuOOH. Concentration-dependent inhibition was observed, and THC exhibited a greater inhibitory effect than curcumin, especially at 150 μM (Fig. 2). To investigate the mechanism of antioxidative activity, we examined what kind of oxidizing species are involved in the lipid peroxidation. We investigated the effects of inhibitors of active oxygen species, such as antioxidant enzymes, hydroxyl radical scavengers (mannitol and DMSO), $^1O_2$ quencher (DABCO), and chelating agents for metal ions (DTPA), on the lipid peroxidation of erythrocyte membrane ghosts by determining TBARS formation. As shown in Fig. 3, all inhibitors failed to inhibit membrane peroxidation. This result supported the previously reported result that *tert*-butoxyl radical may be the oxidizing species in lipid peroxidation [23]. THC must scavenge radicals, such as alkoxyl radical or peroxyl radical, and these effects are superior to curcumin.

The antioxidative mechanism of THC, especially at the β-diketone moiety, was also investigated. To obtain more information on the role of the β-diketone moiety of THC, the antioxidative effect of the phenolic hydroxyl groups was blocked by methylation of the groups. The dimethylated THC, DMTHC, was obtained by hydrogenation of the synthetic compound with PtO_2, after dimethoxycurcumin was prepared as shown in Materials and Methods. The identity and purity of the synthesized DMTHC were confirmed by using MS, IR, UV, and NMR spectra. DMTHC was incubated with peroxyl radicals, generated by the thermolysis of AMVN at 37° in oxygen-saturated acetonitrile. The time-dependent reaction of DMTHC with AMVN was examined by reverse-phase HPLC on a Develosil ODS-5 column. Four oxidation products were detected as shown in Fig. 4A. Peaks 1, 2, and 3 were found to increase with incubation time. On the other hand, peak 4 decreased gradually after 33 hr of incubation (Fig. 5). This result suggests that peak 4 must be an unstable intermediate in the reaction of DMTHC with AMVN. The reaction mixture was rapidly chilled by immersion in ice at 33 hr, and the reaction was stopped to determine the structure of peak 4. These four products were isolated and characterized. The proposed structures of the isolated peaks 1–3 are shown in Table 1; identification was performed by ^1H NMR and EI-MS. Isolated peak 4 was also investigated. The ^1H NMR spectrum of the product suggested the possibility of the presence of hydroperoxide, although the detailed structure has not been determined. We also used the liposome model in order to mimic the erythrocyte membranes. DMTHC incorporated in the liposomes was incubated with AAPH, which generates peroxyl radicals similarly to AMVN. As a result

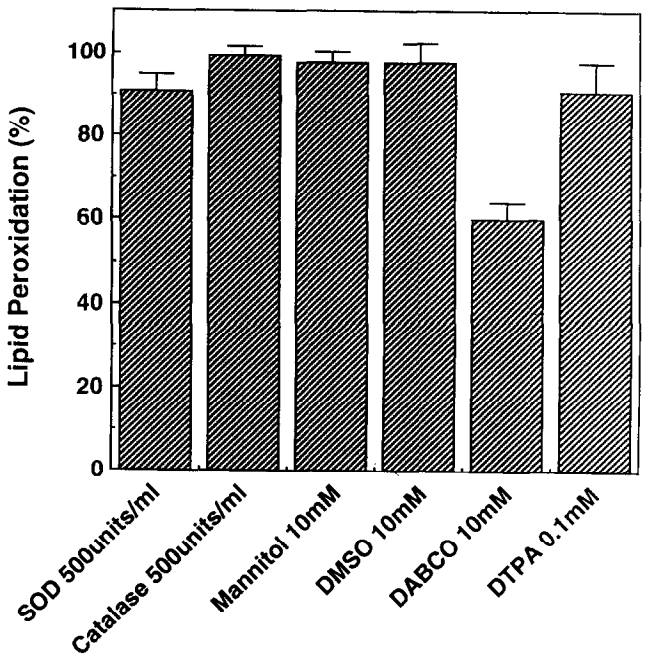


FIG. 3. Effects of inhibitors on the lipid peroxidation of erythrocyte membrane ghosts induced by *t*-BuOOH. After erythrocyte membrane ghosts at a concentration of 1.0 mg protein/mL were incubated with 2.0 mM *t*-BuOOH for 20 min in the presence or absence of various active oxygen scavengers, the formation of TBARS was determined at 532 nm. A control containing no added scavengers represented 100% lipid peroxidation (2.26 μM MDA equivalents). Results are the means ± SD from four separate experiments.

of the reaction, three oxidation products (peaks 1–3) have been identified based on similar retention times as the peaks 1–3 obtained with AMVN; however, peak 4 was not detected (Fig. 4B). DMTHC gave the same reaction products in both oxidation model systems.

DISCUSSION

Given that THC must be the transformed product of curcumin during absorption from the intestines [9–12], the transformed THC must be transported in blood and distributed in some tissues such as liver or kidney. We examined the inhibitory effects of curcumin and THC on the lipid peroxidation of erythrocyte membrane ghosts induced by *t*-BuOOH. The result demonstrated that THC showed a greater inhibitory effect that curcumin, especially at 150 μM (Fig. 2). Curcumin has been reported to act as a strong inhibitor of mutagenicity [7] and tumor promotion in mouse skin by 12-O-tetradecanoylphorbol-13-acetate [8]. Therefore, THC may have more effective antimutagenicity or antitumor activity than curcumin.

We examined oxidizing species in the lipid peroxidation of erythrocyte membrane ghosts to investigate the mechanism of antioxidative activity. We investigated the effects of several inhibitors on lipid peroxidation by determining TBARS formation. Because all inhibitors used (SOD, catalase, mannitol, DMSO, DABCO, and DTPA) failed to inhibit membrane peroxidation, *tert*-butoxyl radical may be the oxidizing species in lipid peroxidation as reported previously [23], and THC must scavenge radicals such as alkoxyl radical and peroxyl radical. Even though the concentration used was high (10 mM), the approximately 40% inhibition of lipid peroxidation by DABCO suggests that either singlet oxygen has a minor role in the *t*-BuOOH-induced lipid peroxidation process or, more likely, DABCO is also a poor scavenger of alkoxyl or peroxyl radicals.

We have found two novel β-diketone-type antioxidants, TTAD and 4-hydroxy-tritriacontan-16,18-dione [13, 14]. Recently, Hirose *et al.* [15] reported that TTAD strongly inhibits hepatic and pancreatic carcinogenesis. Several simple β-diketones such as 1,1,1-trifluoroacetylacetone,

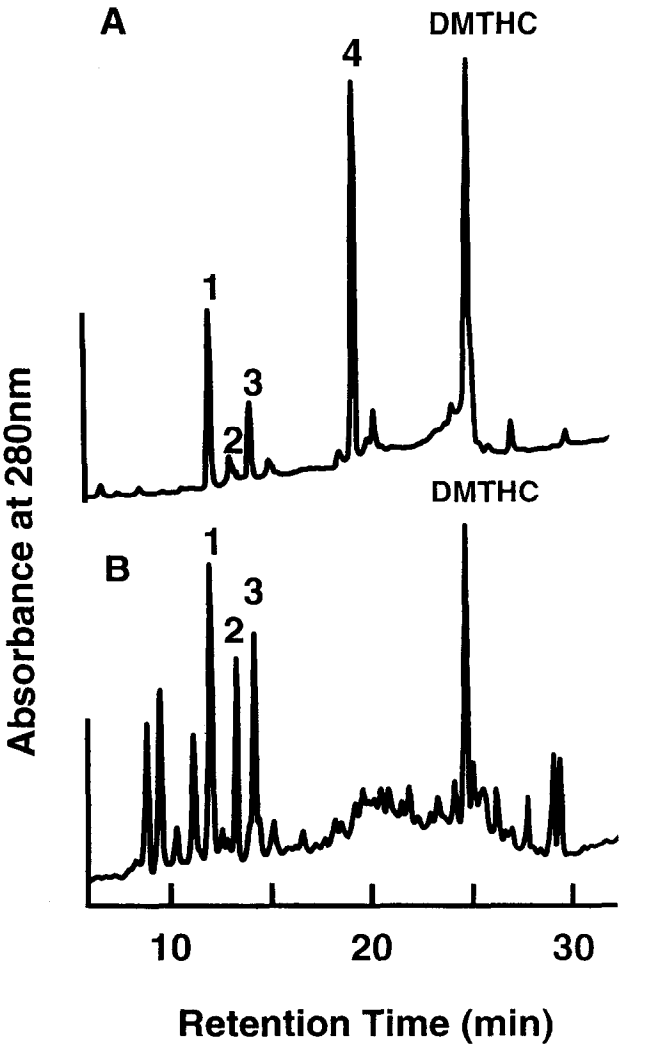


FIG. 4. HPLC profile of DMTHC oxidized with AMVN (A) and AAPH (B). DMTHC (100 μmol) was incubated with 3 mmol AMVN for 33 hr (A), and DMTHC (4 μmol) incorporated into the liposomes (10 mg/mL phosphate buffer) was incubated with 200 μmol AAPH for 94 hr (B). An aliquot of the solution was submitted for reverse-phase HPLC on a Develosil ODS-5 column (4.6 × 150 mm) as described in Materials and Methods.

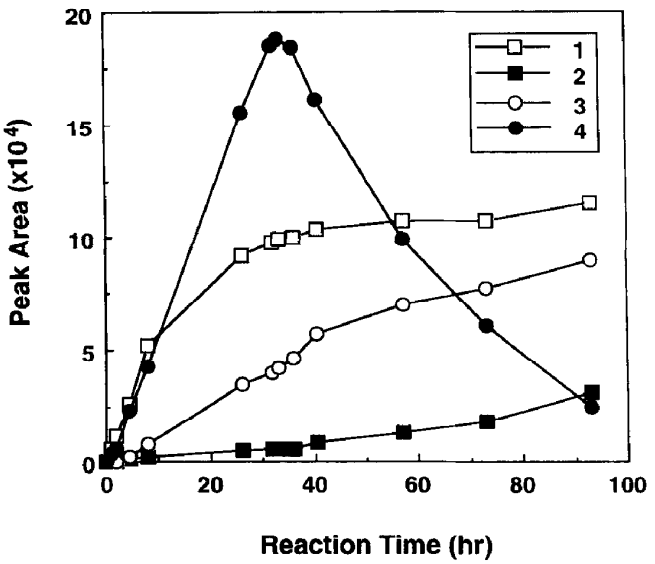
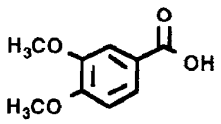
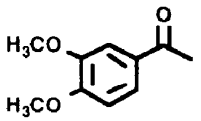
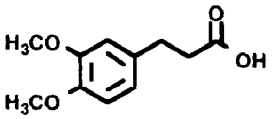


FIG. 5. Time-dependent changes of oxidation products of DMTHC during incubation with AMVN. DMTHC at a concentration of 100 μmol was incubated with 3 mmol AMVN. An aliquot of the solution was submitted for reverse-phase HPLC on a Develosil ODS-5 column (4.6 × 150 mm), and the peak area was determined by use of a Shimadzu C-R3A Chromatopac as described in Materials and Methods.

acetylacetone, benzoylacetone and dibenzoylmethane were reported to inhibit mutagenicity induced by 2-nitrofluorene using the *Salmonella typhimurium* strain [24]. Therefore, it is very important to investigate the antioxidative mechanism of β-diketone-type antioxidants. Because THC, one of the major metabolites of curcumin, also has a β-diketone moiety, which may play an important role in antioxidative activity, we examined the antioxidative mechanism of the β-diketone-type antioxidants by using THC. The antioxidative effect of the phenolic hydroxy groups of THC was blocked by methylation of the groups, and then the role of the β-diketone moiety alone on the antioxidative mechanism was investigated. Dimethylated THC, DMTHC, was prepared as described in Materials and Methods. DMTHC exhibited approximately 36% inhibition (64.15 ± 0.006% lipid peroxidation) at 150 μM on the lipid peroxidation of erythrocyte membrane ghosts induced by *t*-BuOOH.

Peroxyl radicals are known to be the chain-carrying radicals in lipid peroxidation, and their reactions with tocopherol, one of the phenolic-type antioxidants, have been studied by numerous investigators [21, 25–27]. For example, Winterle *et al.* [25] reported the reactions of tocopherol with peroxyl radicals generated from azobis[(*n*-butylcarboxyl)-propane] and azobis(isobutyronitrile). Yamauchi *et al.* [26] and Matsuo *et al.* [27] also have reported the reactions with peroxyl radicals generated from AMVN. Given this background, DMTHC was incubated with peroxyl radicals, which were generated by thermolysis of AMVN at 37° in oxygen-saturated acetonitrile, to confirm the antioxidative mechanism of β-diketone-type antioxidants. Four oxidation products were detected by reverse-phase HPLC as shown in Fig. 4A. Peaks 1, 2, and 3 were found to increase with incubation time and were identified as 3,4-dimethoxybenzoic acid, 3′,4′-dimethoxyacetophenone, and 3-(3,4-dimethoxyphenyl)-propionic acid, respectively. Peak 4 decreased gradually after 33 hr of incubation, suggesting that it must be an unstable intermediate in the reaction of DMTHC with peroxyl radicals. Although the detailed structure of isolated peak 4 has yet to be determined, the ^{1}H NMR spectrum suggests the possibility of the presence of hydroperoxide. From these data, an antioxidative mechanism of DMTHC (β-diketone moiety of THC) is proposed, as shown in Fig. 6. Both enol and keto forms of

TABLE 1. Structures and analytical data for oxidation products of DMTHC

Oxidation product	^{1}H NMR δ (ppm)	EI-MS (*m/z*)
	3.95 (3H, s)	182 (M^+)
	3.96 (3H, s)	167
	6.92 (1H, d, *J* = 8.4)	
	7.59 (1H, d, *J* = 1.9)	
3,4-Dimethoxybenzoic acid	7.77 (1H, dd, *J* = 8.4, 1.9)	
	2.58 (3H, s)	180 (M^+)
	3.95 (3H, s)	165
	3.96 (3H, s)	137
	6.90 (1H, d, *J* = 8.3)	77
	7.53 (1H, d, *J* = 2.0)	
3′,4′-Dimethoxyacetophenone	7.59 (1H, dd, *J* = 8.3, 2.0)	
	2.67 (2H, t, *J* = 7.7)	210 (M^+)
	2.92 (2H, t, *J* = 7.7)	151
	3.86 (3H, s)	121
	3.87 (3H, s)	91
3-(3,4-Dimethoxyphenyl)-propionic acid	6.74–6.82 (3H, m)	77

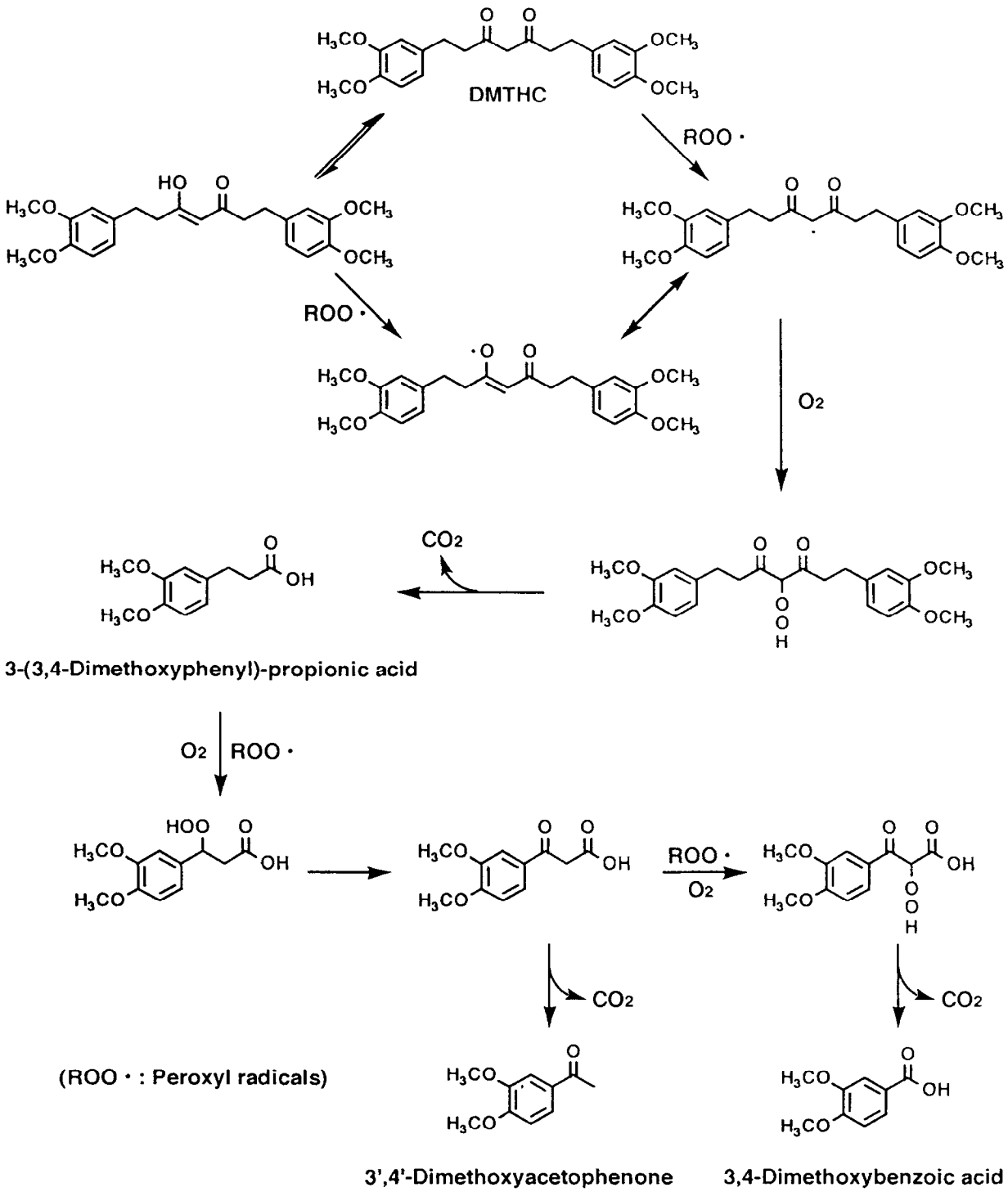


FIG. 6. Proposed antioxidative mechanism of DMTHC.

DMTHC must scavenge free radicals, and the C—C bond at the active methylene carbon between two carbonyls in the β-diketone moiety is cleaved. As a result, 3-(3,4-dimethoxyphenyl)-propionic acid was formed, and 3,4-dimethoxybenzoic acid and 3',4'-dimethoxyacetophenone were produced as secondary oxidation products. This mechanism has been confirmed by the reaction of DMTHC incorporated in the liposomes with AAPH, because the same three oxidation products have been identified (Fig. 4B). These results strongly support the conclusion that the β-diketone moiety must play an important role in the antioxidative mechanism of THC. Although many workers [21, 25–27] have reported on phenolic-type natural antioxidants including tocopherol, this is the first report to show the involvement of the β-diketone moiety in antioxidative mechanisms.

This study on the antioxidative mechanism of β-diketone-type antioxidants indicates that the β-diketone moiety may play an important role in the elucidation of antimutagenesis or anticarcinogenesis, because β-diketone-type antioxidants such as TTAD have been reported to inhibit tumor promotion and carcinogenesis [7, 8, 15]. In addition, because THC is the rapidly metabolized product of curcumin during absorption from the intestines, it is expected that THC may have the same important physiological and pharmacological properties as the active form of curcumin *in vivo* by means of the β-diketone moiety as well as phenolic hydroxy groups. A detailed experiment testing this conclusion is underway.

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