Antioxidant Mechanism of Carnosic Acid: Structural Identification of Two Oxidation Products

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To determine the antioxidant mechanism of food phenolics against the oxidation of food components, the reaction of carnosic acid, an antioxidative constituent of the popular herbs sage and rosemary, was investigated in the presence of ethyl linoleate and the radical oxidation initiator 2,2′-azobis-(2,4-dimethylvaleronitrile). During this process, carnosic acid was oxidized to an *o*-quinone and a hydroxy *p*-quinone, the chemical structures of which were confirmed by physical and chemical techniques. From a quantitative time course analysis of the production of these quinones, an antioxidant mechanism of carnosic acid is proposed, consisting of the oxidative coupling reaction with the peroxyl radical at the 12- or 14-position of carnosic acid and subsequent degradation reactions.

Keywords: *Carnosic aid; antioxidant mechanism; radical termination; sage; Labiatae; o-quinone*

INTRODUCTION

Phenolic compounds in foods have received much attention as powerful antioxidants to protect against the oxidative deterioration of food components such as polyunsaturated fatty acids. A primary mechanism for a phenolic antioxidant includes the trapping and stabilizing of species generated from the radical chain oxidation of food components. The chain-breaking antioxidation process is thought to be divided into two stages as follows:

radical trapping stage

$$S{-}OO^{\bullet} + AH \rightleftarrows S{-}OOH + A^{\bullet} \quad (1)$$

radical termination stage

$$A^{\bullet} \rightarrow \text{non-radical materials such as A}{-}\text{A, SOOA, and Aox} \quad (2)$$

(S, oxidation substrate; AH, antioxidant; A$^{\bullet}$, antioxidant radical; A−A, dimer of A; SOOA, substrate antioxidant peroxide; Aox, oxidized A). Although the first stage is a reversible process, the second stage is irreversible and must produce stable radical termination compounds. If known, the structure of the termination compound would afford important information to reveal the antioxidant mechanism of the phenolic antioxidant.

Carnosic acid (**1**) is an abietane diterpene found in the popular Labiatae herbs sage and rosemary (*1*, *2*), and it is regarded as a precursor of other diterpenoid constituents in the herbs (*3*). Carnosic acid and related diterpenes such as carnosol and rosmanol are known to have powerful antioxidant activities (*5*), and carnosic acid has the most powerful antioxidant potency among these diterpenes (*4*). Carnosic acid has a typical *o*-diphenol structure and is easily oxidized. Most diphenol compounds show potent chain-breaking antioxidant activity in food systems (*6*). Wenkert et al. (*7*) suggested an oxidation and isomerization pathway to afford carnosol from carnosic acid via an *o*-quinone intermediate. Oxidative conversion pathways to other related diterpenes have also been investigated by many groups, as well reviewed by Hall and Cuppett (*8*). They also suggested that these oxidation pathways were closely related to their antioxidant mechanism, but this has not yet been confirmed by any experimental proof. In research on the antioxidant mechanism of food phenols (*9*), we have investigated the antioxidant mechanism of carnosic acid. We now propose an antioxidant mechanism for carnosic acid, based on the chemical structures of the oxidation products, which are produced as radical termination compounds in the antioxidation process of carnosic acid.

MATERIALS AND METHODS

Materials. The dried leaves of sage (*Salvia officinalis*), which were harvested in Albania in 1998, were purchased from Mikuni Co. Ltd. (Osaka, Japan). 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN) was obtained from Wako Pure Chemicals (Osaka, Japan). Ethyl linoleate was obtained from Kanto Chemicals (Tokyo, Japan) and used after purification by silica gel chromatography. All solvents and other reagents were obtained from Nacalai Tesque (Kyoto, Japan).

Instruments. The NMR spectra were measured with an EX-400 spectrometer (JEOL, Tokyo, Japan) using the manufacturer's supplied pulse sequences [^{1}H, ^{13}C, COSY (correlated spectroscopy), and NOEDIF (nuclear Overhauser effect differential spectrum)]. Mass spectra were measured with an SX-102A spectrometer (JEOL) in the EI mode. The IR spectra were measured with an FT-IR-400 spectrometer (JASCO, Tokyo, Japan). The UV spectra were obtained with a V-520 spectrophotometer (JASCO). An LC 6A system (Shimadzu, Kyoto, Japan) equipped with a photodiode array detector (Shimadzu, SPD-M10AP) and a Gulliver Gradient LC system (JASCO) were used for the analytical and preparative HPLC, respectively.

Preparation of Carnosic Acid. For the preparation of carnosic acid (**1**), Richheimer's method (*10*), which was used for the isolation of carnosic acid from rosemary, was employed with modifications. Briefly, the finely ground leaves of the sage (3 kg) were extracted with methanol (2 × 9 L) containing acetic

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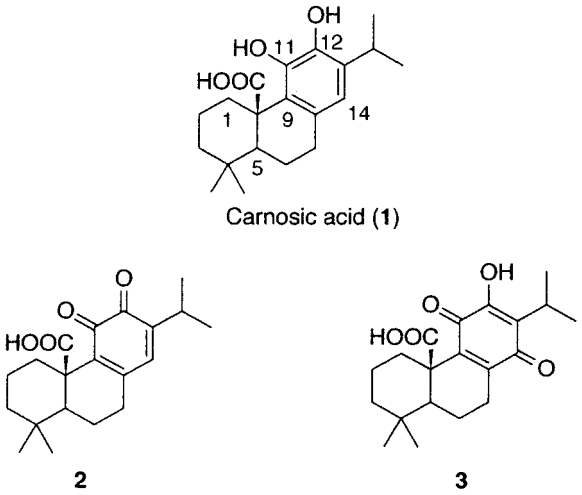

Figure 1. Chemical structures of carnosic acid (**1**) and oxidation products (**2** and **3**).

acid (1%) at room temperature for 20 h each. After filtration, the filtrate was evaporated to give a crude extract (660 g). To the extract was added hexane (6.6 L), which was stirred for 5 h, and the mixture was then kept for 16 h at room temperature. After filtration, the filtrate was concentrated to 2.5 L. The concentrate was extracted twice with a 5% aqueous $NaHCO_3$ solution. The water layer obtained was adjusted to pH 2 with 6 N HCl and then extracted with hexane (2 × 2.8 L). The hexane layer was dried over anhydrous Na_2SO_4 and evaporated. The residue was crystallized with a small amount of hexane to give 3.05 g of crude carnosic acid. Final purification was carried out by preparative HPLC under the following conditions: column, Daisopak ODS-120-AP (250 × 20 mm i.d.) (Daiso, Osaka, Japan); solvent, methanol/H_2O/acetic acid (89.1:10:0.9, v/v); flow rate, 9.5 mL/min; detection, 284 nm. The peak eluted at a the retention time of 14 min was collected and evaporated to give pure carnosic acid (1.8 g) as pale yellow needles from water/acetic acid [mp 194.5−195.0 °C, lit. 190 °C (*10*)].

Analysis of Conversion Products and Linoleate Hydroperoxide from Antioxidation Reaction of Carnosic Acid. To 34 μL of ethyl linoleate in a 10 mL screw-capped tube were successively added 4 mM carnosic acid solution (125 μL, CH_3CN), 0.3 M AMVN solution (100 μL, CH_3CN), and CH_3CN (1.75 mL) to make a reaction solution. After being well stirred by a Vortex (Scientific Industry, Bohemia, NY), the solution was incubated at 37 °C with shaking (100 min^{-1} speed) in the dark by a water bath shaker 11SD (Taitec, Koshigaya, Japan). A 20 μL aliquot was removed from the solution at 1 h intervals and diluted with 380 μL of methanol. Ten microliters of the diluted solution was injected into the HPLC to analyze the ethyl linoleate hydroperoxides under the following conditions: column, YMC-ODS-A (150 × 4.6 mm i.d.) (YMC, Tokyo, Japan); solvent, CH_3CN/H_2O (9:1, v/v); flow rate, 1.0 mL/min; detection, 234 nm. At the same intervals, an additional 5 μL aliquot was removed from the reaction mixture and injected through a injection valve with a 20 μL loop (Reodyne 7725i, Cotati, CA) into the HPLC to analyze the conversion products from the carnosic acid using the following conditions: column, Daisopak ODS-AP (250 × 4.6 mm i.d); solvent, methanol/H_2O/acetic acid (90:10:1, v/v); flow rate, 0.5 mL/min; detection, 284 nm. The concentration of each compound was calculated from the peak area using a calibration curve determined for the pure compounds [**1**, $Y = (1.07 \times 10^{-6})X + (2.10 \times 10^{-2})$ (range for $X = 7 \times 10^4$−9×10^5); **2**, $Y = (1.41 \times 10^{-6})X + (3.25 \times 10^{-3})$ (range for $X = 6.9 \times 10^4$−7.0×10^5); **3**, $Y = (1.68 \times 10^{-7})X + (9.35 \times 10^{-4})$ (range for $X = 3.8 \times 10^4$−5.9×10^5), where Y is the concentration of each compound (mM) and X is the observed peak area of each compound] (structures of **1**−**3** are given in Figure 1).

Iron Ion-Catalyzed Oxidation of Carnosic Acid. To a solution of carnosic acid (1.0 mg) in CH_3CN (0.9 mL) was added 0.1 mL of 0.38 mM $FeCl_3$ aqueous solution. The mixture was incubated at 37 °C in air with shaking. At 1 h intervals, a 5 μL aliquot was removed and analyzed by HPLC under the same conditions as that for the antioxidation reaction.

Table 1. ^{1}H and ^{13}C NMR Data of Compounds 2 and 3 (400 MHz for ^{1}H; 100 MHz for ^{13}C)

	2[a] (acetone-d_6)		**3**[a] (DMSO-d_6)	
position	^{1}H	^{13}C	^{1}H	^{13}C
1α	0.97 (dt, 4.5, 12.8)	35.0	1.00 (dt, 3.3, 13.0)	33.5
β	2.99 (dt, 12.8, 2.3)		2.85 (brd, 13.0)	
2α	1.38−1.50 (overlapped)	20.9	1.43 (overlapped)	19.5
β	2.29 (tq, 13, 3.3)		2.23 (m)	
3α	1.23 (dt, 4.8, 13.8)	42.6	1.43 (overlapped)	40.0
β	1.38−1.50 (overlapped)		1.19 (m)	
4		34.7		33.2
5	1.38−1.50 (overlapped)	53.0	1.31 (d, 12.0)	51.4
6α	1.84 (m)	18.7	1.76 (dd, 11.5, 6.3)	16.5
β	2.16 (m)		1.88 (dq, 11.5, 4.5)	
7α	2.50−2.68 (overlapped)	33.9	2.29 (m)	25.5
β	2.50−2.68 (overlapped)		2.59 (dd, 20.5, 4.5)	
8		151.4		142.7[c]
9		141.0		145.1[c]
10		47.2		45.9
11		182.3[b]		186.9[d]
12		181.4[b]		153.0
13		147.9		123.7
14	6.69 (s)	139.1		189.8[d]
15	2.81 (sep. 6.8)	28.2	3.07 (sep, 6.8)	23.4
16	1.06 (d, 6.8)	22.1	1.09 (d, 6.8)	19.9[e]
17	1.06 (d, 6.8)	22.1	1.12 (d, 6.8)	19.8[e]
18	0.92 (s)	33.2	0.90 (s)	32.1
19	0.83 (s)	20.2	0.79 (s)	19.4
20		176.2		175.3
OH			12.4 (brs)	

[a] Coupling pattern and constant (J in Hz) are in parentheses. [b−e] Assignment may be interchangeable.

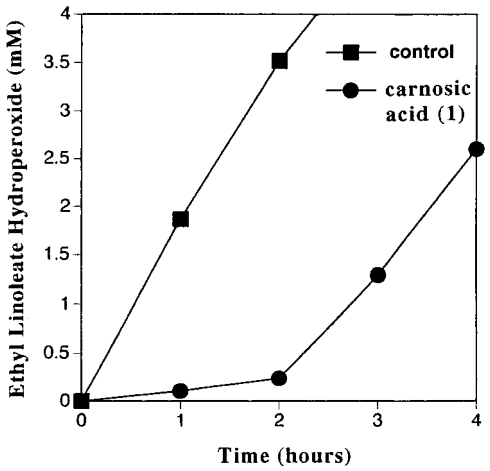

Figure 2. Antioxidant activity of carnosic acid against AMVN-induced linoleate oxidation (carnosic acid, 0.24 mM; AMVN, 15 mM; ethyl linoleate, 50 mM).

Isolation of Oxidation Products. To a solution of carnosic acid (50 mg) in CH_3CN (45 mL) was added 5 mL of aqueous $FeCl_3$ solution, which was prepared with 10 mg of $FeCl_3 \cdot 6H_2O$ and 100 mL of H_2O at room temperature with stirring. The mixture was shaken at 40 °C in air for 6 h. After concentration to ∼5 mL, the concentrate was injected into the MPLC [column, Develosil LOP ODS 24S (30 × 2.4 cm i.d.) (Nomura Chemical, Seto, Japan); solvent, CH_3OH/H_2O/acetic acid (90:10:1, v/v); flow rate, 5 mL/min; detection, 284 nm]. The peaks observed at retention times of 36 and 46 min were collected. The eluate at 36 min was evaporated in vacuo (44 mg) and crystallized with ether/hexane to give **2** (8 mg) as an orange powder. The eluate at 46 min was evaporated to give **3** (4 mg) as a yellow powder.

2: mp 129.0 °C; UV (CH_3OH), λ_{max} 421.5 nm (ϵ 1760); HR-EIMS, (m/z) $[M]^+$ calcd for $C_{20}H_{26}O_4$ 330.1831, found 330.1861;

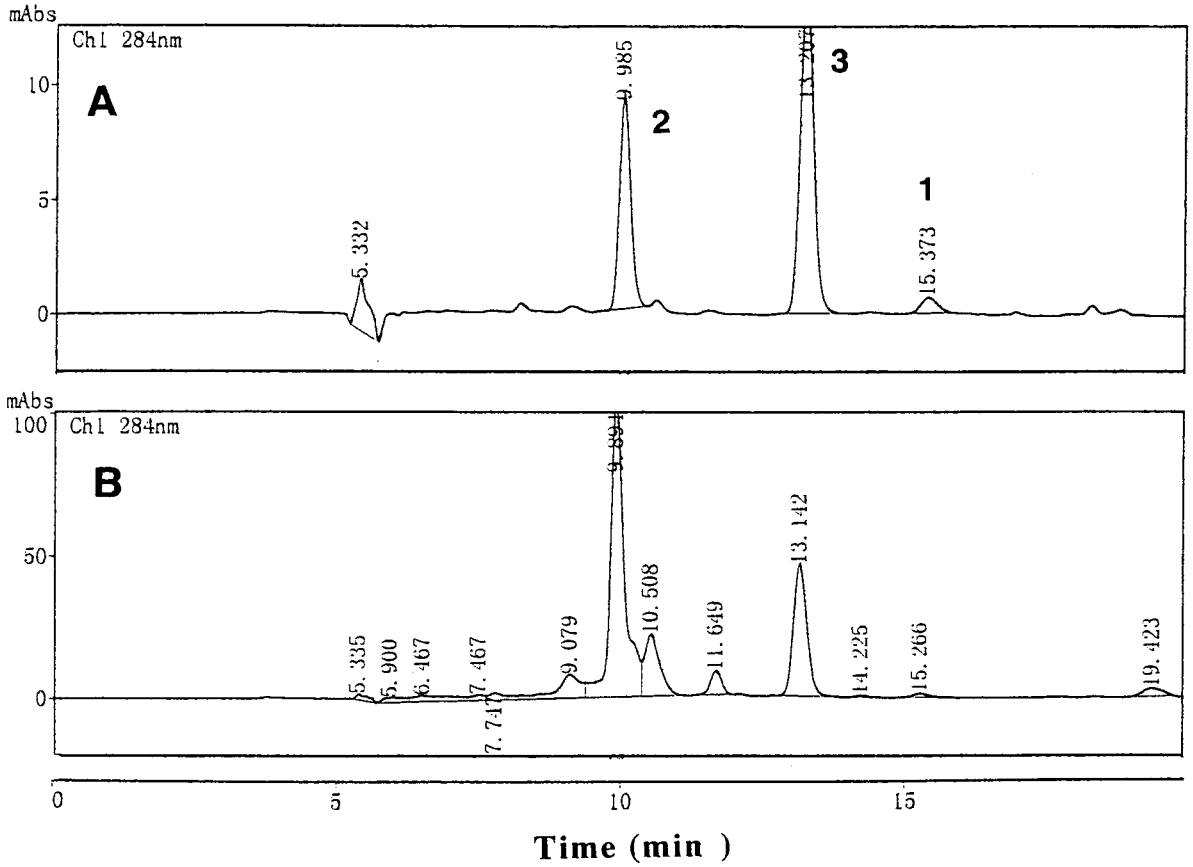

Figure 3. HPLC analysis of the conversion products from carnosic acid under linoleate antioxidation conditions (2 h) (A) and under $FeCl_3$-catalyzed oxidation conditions (6 h) (B).

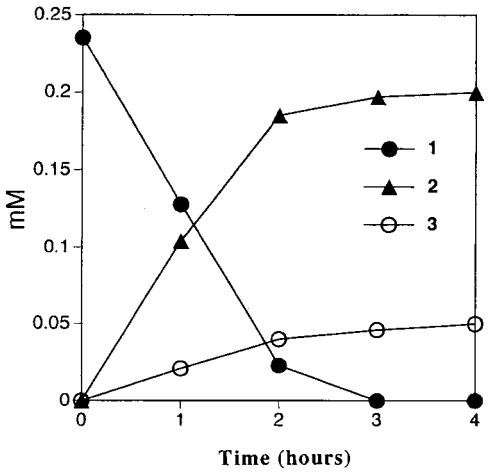

Figure 4. Time course analytical data of carnosic acid (**1**) and compounds **2** and **3** in the antioxidation reaction.

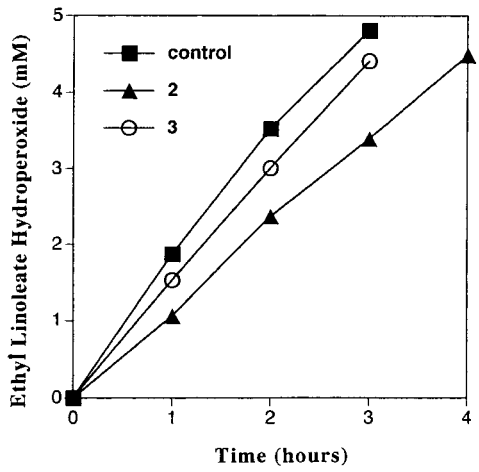

Figure 5. Antioxidant activity of compounds **2** and **3** (0.24 mM) against AMVN-induced linoleate oxidation (AMVN, 15 mM; ethyl linoleate, 50 mM).

EI-MS, *m/z* 330 $[M]^+$ (15%), 286 (100%), 284 (45%), 215 (65%); IR (KBr), ν_{max} 3100, 1698, 1661 cm^{-1}; ^1H NMR and ^3C NMR, see Table 1.

3: mp 172.0−173.0 °C (dec); UV (CH_3OH), λ_{max} 278.5 (ϵ 11020), 406 (ϵ 530); HR-EIMS, (*m/z*) $[M]^+$ calcd for $C_{20}H_{26}O_5$ 346.1780, found 346.1766; EI-MS, *m/z* 346 $[M]^+$ (20%), 300 (100%), 285 (15%), 257 (15%); IR (KBr), ν_{max} 3362, 3100, 1702, 1652, 1629, 1614, 1252 cm^{-1}; ^1H NMR and ^{13}C NMR, see Table 1.

Acetylation of 3. To 1.7 mg of **3** were successively added acetic anhydride (0.5 mL) and pyridine (0.5 mL). After standing for 4 h at room temperature, the mixture was evaporated in vacuo. The residue was purified by silica gel TLC [solvent, EtOAc/hexane (1:2)] to give 1.2 mg of the acetate of **3**: HR-EIMS, (*m/z*) $[M]^+$ calcd for $C_{22}H_{28}O_6$ 388.1886, found 388.1870; ^1H NMR ($CDCl_3$) δ 1.12 (td, J = 13.0 and 4.8 Hz, 1H, H-1), 2.88 (dt, J = 13.0 and 2.5 Hz, 1H, H-1), 1.53 (m, 1H, H-2), 2.23−2.42 (overlapped, 1H, H-2), 1.53 (m, 1H, H-3), 1.24 (m, 1H, H-3), 1.39 (d, J = 11.8 Hz, 1H, H-5), 1.89 (brdd, J = 12.8 and 7.3 Hz, 1H, H-6), 2.02 (m, 1H, H-6), 2.23−2.42 (overlapped, 1H, H-7), 2.78 (dd, J = 20.3 and 5.3 Hz, 1H, H-7), 3.12 (sep, J = 7.0 Hz, 1H, H-15), 1.20 (d, J = 7.0 Hz, 3H, H-16 or 17), 1.18 (d, J = 7.0 Hz, 3H, H-16 or 17), 0.95 (s, 3H, H-18), 0.84 (s, 3H, H-19), 2.30 (s, 3H, 12-CH_3CO).

RESULTS AND DISCUSSION

Antioxidant Reaction of Carnosic Acid. The antioxidant reaction conditions were designed by using

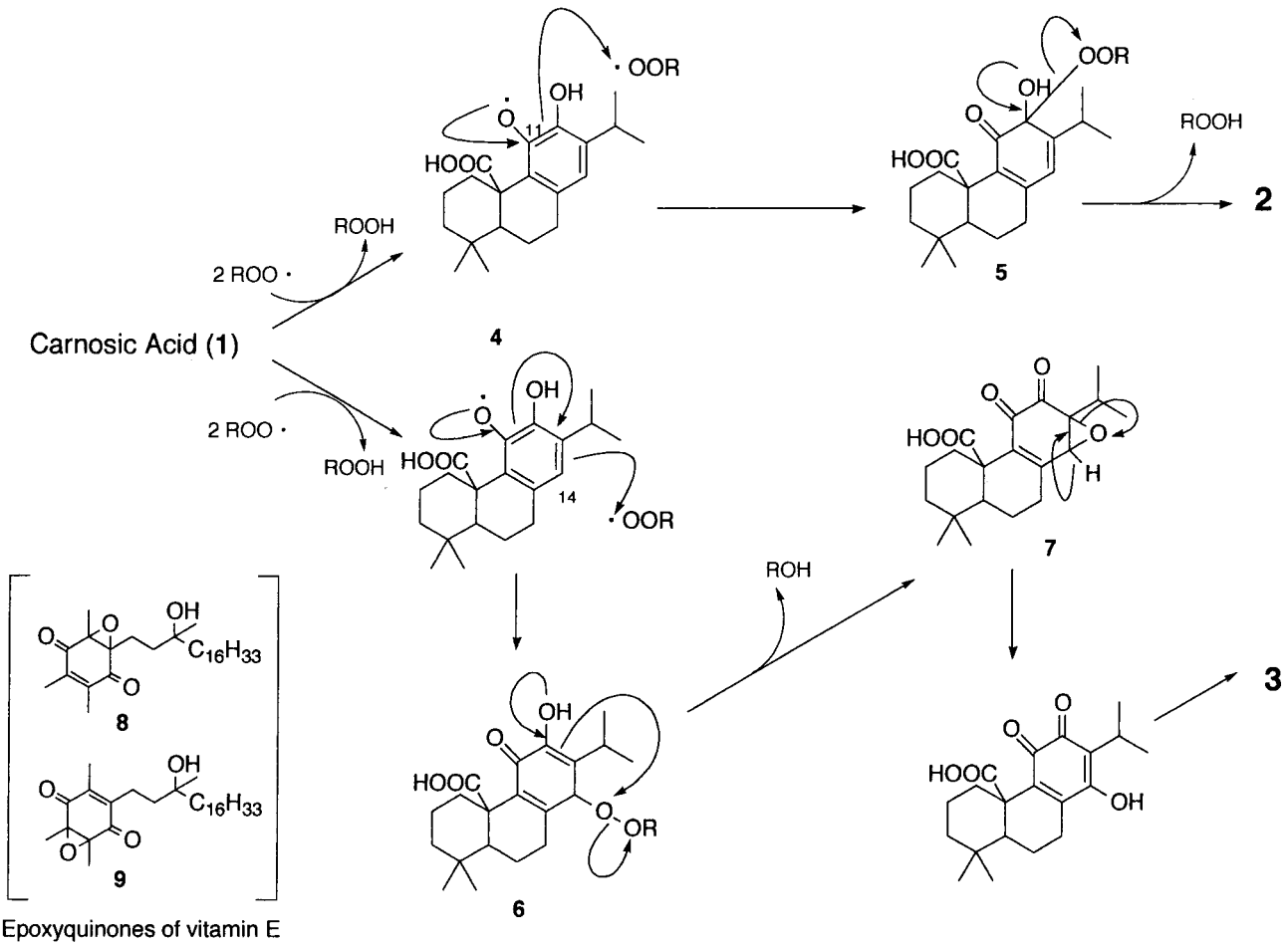

Figure 6. Proposed antioxidant mechanism of carnosic acid. ROOH, ROO•, and ROH denote hydroperoxide, peroxyl radical, and hydroxide, respectively.

ethyl linoleate and AMVN as the oxidation substrate and radical oxidation initiator, respectively. The antioxidant activity of 0.24 mM carnosic acid against 50 mM ethyl linoleate is shown in Figure 2. Figure 2 shows that carnosic acid strongly inhibited the accumulation of the hydroperoxides of ethyl linoleate for 2 h and then does not show any inhibition compared with that of the control experiment, which was carried out without carnosic acid. Figure 3A shows the HPLC analysis of the reaction mixture at 2 h. Carnosic acid (**1**) is observed as a low intensity at a retention time of 15.4 min, whereas two major peaks are observed at retention times of 9.9 and 13.2 min. From a time course analysis of these three peaks, carnosic acid was observed to constantly decrease, whereas peaks **2** and **3** increased for 2 h. After complete disappearance of the carnosic acid peak, the peak intensities of both **2** and **3** did not change (Figure 4). These results strongly indicated that compounds **2** and **3** were radical termination compounds produced from carnosic acid during its antioxidation process.

Isolation and Structure Identification of 2 and 3. To clarify the chemical structures of compounds **2** and **3**, we planned to isolate them from the antioxidation reaction mixture. Unfortunately, we failed to achive this because of difficulty in removing the large amount of coexisting lipid and radical initiator. During many experiments, we found that carnosic acid was unstable in the presence of aqueous solvents, even though it is stable in nonpolar organic solvents. This instability was also observed by Schwarz and Ternes (*11*) and Curvelier et al. (*12*), who observed the formation of several oxidation products of carnosic acid when it was dissolved in methanol. We presumed that this instability of carnosic acid is due to air oxidation catalyzed by a transition metal ion such as iron, which often exists as an impurity in polar solvents. Oszmianski et al. (*13*) oxidized catechin, which has the same *o*-diphenol structure as carnosic acid, using an iron catalyst. Thus, we tried to use ferric ion to obtain the same compounds **2** and **3**, and the addition of a catalytic amount of ferric chloride to a carnosic acid solution in the presence of air produced these compounds, as shown in Figure 3B. Although the production ratio of **2** and **3** was very different from that of the antioxidant reaction data (Figure 3A), this procedure was superior for the isolation of the reaction products because metal ion was easily removed by normal extraction techniques. Thus, we employed this method and isolated **2** (8 mg) and **3** (4 mg) in the microcrystalline state from 50 mg of carnosic acid (**1**).

Compound **2** was isolated as an orange powder. Its molecular formula was determined by high-resolution EI-MS to be $C_{20}H_{26}O_4$, which was two hydrogen atoms fewer than that of carnosic acid. ^{1}H NMR data of **2** did not show significant difference in chemical shifts and coupling constants of the nonaromatic protons when compared with those of carnosic acid, which indicated that the gross structure was similar to the latter. In its ^{13}C NMR spectrum, two new carbonyl carbons (δ 181.4

and 182.3) were observed, in addition to a carboxylate carbon (δ 176.2). The IR spectrum of **2** showed a carbonyl absorbance at 1661 cm^{-1}, which was assignable to an *o*-quinone structure. From these data, the structure of compound **2** was determined to be an *o*-quinone derivative of carnosic acid as depicted in Figure 1. This compound was identical to the quinone intermediate, noted by Wenkert et al. (*7*), in the formation of carnosol from carnosic acid, and this is the first identification of the *o*-quinone to the best of our knowledge.

Compound **3** was isolated as a pale yellow powder and its molecular formula determined to be $C_{20}H_{26}O_5$ by HR-MS. Although the patterns of the protons were very similar to those of **2**, an aromatic signal was absent in its ^1H NMR spectrum compared with that of **2**. A sharp IR absorption at 3362 cm^{-1} and oxygen count in the molecular formula strongly indicated the presence of a hydroxyl group on the aromatic moiety. The presence of the hydroxyl group was also confirmed by acetylation. The fine separated absorbances (1652, 1629, and 1614 cm^{-1}) in the carbonyl region of its IR spectrum suggested the presence of a *p*-quinone structure. Thus, the structure of compound **3** was determined to be the hydroxy *p*-quinone derivative depicted in Figure 1.

The antioxidant activity of these isolated compounds was measured according to the same procedure previously used for carnosic acid as shown in Figure 5. As expected, almost no activity was observed for either **2** and **3**, confirming that they may be regarded as termination compounds on the basis of their stability against radicals.

Proposed Antioxidant Mechanism of Carnosic Acid. An antioxidant mechanism for carnosic acid based on the chemical structures of **2** and **3** and time course analysis results (Figure 4) was considered. Although **3** appears to be an oxidation product derived from **2**, the time course analysis results show that the production of **3** has no lag time compared with that of **2**, which strongly indicates that **3** is not produced from **2** but, most likely, produced directly from **1**. From these facts and the structures of the termination compounds **2** and **3**, we propose the antioxidant mechanism for carnosic acid as illustrated in Figure 6. Carnosic acid has two reactive phenol groups in its aromatic part. Inatani et al. (*5*) suggested that the phenolic group at the 11-position was more important for its antioxidant activity, on the basis of structure−activity relationship studies using the structurally related rosmanol derivatives. Thus, we considered that the 11-phenolic group first undergoes hydrogen donation to a radical species such as a lipid peroxyl radical, to produce the carnosate radical (**4**). Radical termination of **4** takes place next with a second lipid peroxyl radical by a radical−radical coupling reaction. This coupling reaction occurs at the 12- or 14-position because they are the ortho- and para-positions, respectively, to the oxygen radical and are the radical-stabilizing positions by the captodative effect (*14*). When the coupling proceeds at the 12-position (ortho-position), a peroxyl hemiacetal **5** is formed. This acetal is unstable and affords **2** by elimination of a hydroperoxide. When the coupling proceeds at the 14-position (para-position), a peroxide **6** is produced. This peroxide is also unstable because a reactive enol at the 13-position in **6** attacks an oxygen of the peroxide to cleave the peroxide bond, affording an epoxide intermediate **7**. This type of epoxide formation accompanied by peroxide cleavage is a well-known organic oxidation reaction using organic peroxide reagent (*15*). Structurally similar epoxyquinones were also observed as the antioxidation products of vitamin E (*16*, *17*). Although the formation mechanism of these epoxyquinones of vitamin E (**8**, **9**) has not been suggested, they may be produced by a pathway similar to that for the antioxidation of carnosic acid. This epoxide intermediate **7** is not very stable because a migratory hydrogen is adjacent to the epoxide in contrast with the stable epoxyquinones of vitamin E, which has no migratory hydrogen adjacent to the epoxide. Finally, two-step isomerization steps occur in the epoxyquinone **7**, affording compound **3**. Quantitative analysis results showed that the production ratio of **2** and **3** was 4:1, which may depend on the selectivity of the radical coupling position in this proposed mechanism.

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