Recovery Mechanism of the Antioxidant Activity from Carnosic Acid Quinone, an Oxidized Sage and Rosemary Antioxidant

TOSHIYA MASUDA,*,†,‡ YUZURU INABA,† TOMOMI MAEKAWA,§ YOSHIO TAKEDA,†
HIROTOSHI TAMURA,# AND HIDEMASA YAMAGUCHI§

Faculty of Integrated Arts and Sciences, University of Tokushima, Tokushima 770-8502, Japan,
Tropical Biosphere Research Center, University of Ryukyus, Taketomi, Okinawa 907-1541, Japan,
Graduate School of Human Life Science, Osaka City University, Sumiyoshi, Osaka 558-8585, Japan,
and Faculty of Agriculture, Kagawa University, Miki, Kagawa 761-0701, Japan

A solution of carnosic acid quinone, which is a radical chain-termination product having no antioxidant activity in the antioxidant reaction of carnosic acid, recovers potent antioxidant activity upon standing. The HPLC analysis of an aged solution of carnosic acid quinone revealed that several antioxidants are produced in the solution. From the time-course and quantitative analyses of the formation of the products and their structural analysis, an antioxidant mechanism from carnosic acid quinone is proposed that includes a redox reaction of carnosic acid quinone in addition to the isomerization to lactone derivatives. In the first stage of antioxidation, carnosic acid, the reduction product from carnosic acid quinone, contributes to the potent antioxidant activity of the solution. This proposed mechanism can explain one of the reasons for the strong antioxidant activity of the extract of the popular herbs sage and rosemary.

INTRODUCTION

Sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*) are popular Labiatae herbs used as spices and folk medicines around the world. These plants show potent antioxidant activity, and their extracts are widely used commercially to increase the shelf life of foods (*1*). Carnosic acid is a major phenolic constituent of these plants (**Figure 1**) and has potent antioxidant activity. It is well-known that sage and rosemary also contain other diphenolic abietane diterpenes (*2*), which may come from the oxidation cascade of carnosic acid in the plants in a manner suggested by Wenkert (*3*). This oxidation cascade is very interesting from the viewpoint of the powerful antioxidant activity of the plants, because new antioxidative phenols, such as carnosol and rosmanol, are produced during the cascade (*4*). Researchers have also observed that carnosic acid is unstable upon standing in solution. This degradation reaction may be induced by air oxidation (*5*, *6*). Some of the products of the degradation have antioxidant activity. Antioxidant activity, especially the chain-breaking type, is a typical activity based on a chemical reaction in which the antioxidant is sacrificially oxidized to prevent oxidation of the biological substance. Generally, an antioxidant loses its activity after this reaction.

The proposed oxidation cascade from carnosic acid and the observation of new antioxidant production from carnosic acid in solution show the possibility that carnosic acid still contributes to the antioxidant activity after its oxidation. This interesting phenomenon is one of the reasons why sage and rosemary extracts are such powerful antioxidants. The oxidation and conversion pathway from carnosic acid with respect to the antioxidant activity has been a challenging research target (*7*); however, the pathway is very complicated to solve, and experimental proof for the pathway is very limited. Recently, we succeeded in clarifying the first stage of the antioxidant mechanism of carnosic acid (*8*). This mechanism revealed that carnosic acid produced two quinone derivatives during its antioxidation. The main quinone of the reaction is the ortho-quinone derivative of carnosic acid (**Figure 1**), which coincided with a key compound of the cascade suggested by Wenkert et al. (*3*) and Richheimer et al. (*9*). Carnosic acid quinone has no antioxidant activity; however, we found that its solution began to show potent antioxidant activity upon standing. We now wish to report results of our investigation on this recovery mechanism of the antioxidant activity of a solution of carnosic acid quinone, which is based on the analysis of the conversion products from carnosic acid quinone in solution.

MATERIALS AND METHODS

Chemicals and Instruments. 2,2′-Azobis(isobutyronitrile)(AIBN) was purchased from Tokyo Kasei (Tokyo, Japan). 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN) was purchased from Wako Pure

* Corresponding author. Fax: +81-88-656-7244. E-mail: masuda@ias.tokushima-u.ac.jp.
† University of Tokushima.
‡ University of Ryukyus.
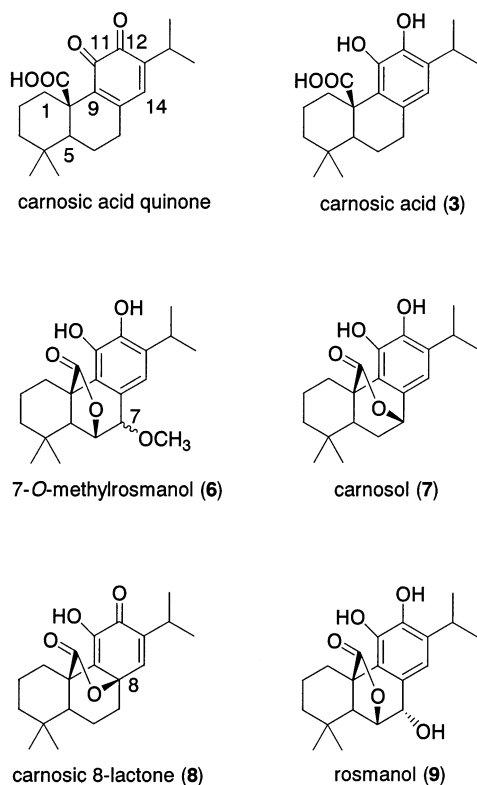§ Osaka City University.
Kagawa University.

Figure 1. Chemical structures of carnosic acid quinone and the identified products produced from carnosic acid quinone in solution in this study [carnosic acid (**3**), 7-*O*-methylrosmanol (**6**), carnosol (**7**), carnosic 8-lactone (**8**), and rosmanol (**9**)].

Chemicals (Osaka, Japan). Acetonitrile was obtained from Kanto Chemical (Tokyo, Japan) as anhydrous grade. Ethyl linoleate was purchased from Kanto Chemical and used after purification by silica gel (silica 60, Merck, Darmstat, Germany) chromatography developed with 2.5% ethyl acetate in hexane. All solvents and other reagents were obtained from Nacalai Tesque (Kyoto, Japan). The NMR spectra were measured using a Unity Plus 500 spectrometer (500 MHz, Varian, Palo Alto, CA) or an EX-400 spectrometer (400 Hz, JEOL, Tokyo, Japan). The mass spectra were measured using an SX-102A spectrometer (JEOL). The IR spectra were measured using an FT-IR-400 spectrometer or an FT-IR-8400 spectrometer (Shimadzu, Kyoto, Japan) by the dry film method. Analytical HPLC was performed with an LC-10 low-pressure gradient system (Shimadzu) that consisted of an LC-10ATvp pump, CTO-10Avp column oven, and SPD-M10Avp photodiode array detector or an isocratic system that consisted of a PU-980 pump (JASCO, Tokyo, Japan) and a UV-970 detector (JASCO). Preparative HPLC was performed using an LC-6AD recycle system (Shimadzu) equipped with a UV-8011 detector (Tosoh, Tokyo, Japan).

Preparation of Carnosic Acid Quinone. To a solution of carnosic acid (103 mg) in acetonitrile (9 mL) was added 1 mL of ferric chloride solution [0.27 g FeCl$_3$·6H$_2$O in distilled water (1 mL)] at room temperature. After standing for a few minutes, the solution was poured into water (10 mL) and extracted twice with CH$_2$Cl$_2$ (10 mL each). The CH$_2$Cl$_2$ layer was dried over anhydrous Na$_2$SO$_4$ and evaporated. The residue was quickly crystallized in a mixture of hexane and ether to give carnosic acid quinone (85 mg), an orange powder (mp 129 °C). This powder was stored at −30 °C until needed.

Quantitative Analysis of the Conversion Products from Carnosic Acid Quinone. To a screw-capped tube (10 mL) was added 2.76 mL of acetonitrile or acetonitrile/H$_2$O (9:1), 190 μL of a 4.0 mM acetonitrile solution of carnosic acid quinone, and 52 μL of ethyl linoleate, and the mixture was stirred by a Voltex mixer (Scientific Industry, Bohemia, NY). When using methanol as the reaction solvent, a stock solution of carnosic acid quinone was also made in methanol. The solution was incubated at 37 °C. For the analysis of the antioxidation reaction, 1.00 mL of the solution was transferred to a new tube, and the appropriate

amount of AMVN or AIBN was added to the tube with stirring. The tube was incubated at the same temperature with shaking (100 rpm) by a water bath shaker 11SD (Taitech, Koshigaya, Japan) in the dark in air. At 1- or 0.5-h intervals, a 5-μL aliquot was taken from the solution and injected into the HPLC. The conversion products were quantitatively analyzed under the following conditions: column, 250 mm × 4.6 mm i.d. Daisopak ODS-AP (Daiso, Osaka); solvent, methanol/H$_2$O/acetic acid (90:10:1); flow rate, 0.5 mL/min; and detection, 284 or 254 nm. The concentration of each compound was calculated from the peak area using a calibration curve for each compound, which was obtained using each isolated compound [carnosic acid, $Y = 9.03 \times 10^{-7}X + 9.39 \times 10^{-4}$ (range for $X = 3.2 \times 10^3$ to 3.6×10^5); 7-*O*-methylrosmanol, $6.93 \times 10^{-7}X + 1.13 \times 10^{-3}$ (range for $X = 5.3 \times 10^3$ to 2.0×10^5); carnosol, $Y = 6.04 \times 10^{-7}X + 8.39 \times 10^{-4}$ (range for $X = 3.8 \times 10^3$ to 5.5×10^4) or $Y = 6.16 \times 10^{-7}X + 2.54 \times 10^{-3}$ (range for $X = 2.3 \times 10^4$ to 3.9×10^5); rosmanol, $Y = 5.13 \times 10^{-7}X + 9.33 \times 10^{-4}$ (range for $X = 2.2 \times 10^3$ to 4.2×10^4) or $Y = 5.35 \times 10^{-7}X - 1.29 \times 10^{-3}$ (range for $X = 4.3 \times 10^4$ to 4.1×10^5); carnosic 8-lactone, $Y = 2.12 \times 10^{-7}Z + 2.72 \times 10^{-4}$ (range for $Z = 1.05 \times 10^4$ to 2.18×10^5); where X is the peak area value of the 284-nm detection, Z is the peak area value of the 254-nm detection, and Y is the concentration of each compound (mM).]

Quantitative Analysis of Ethyl Linoleate Hydroperoxide. At the same intervals, an additional 20-μL aliquot from the solution mentioned above was diluted with 380 μL of methanol. Ten microliters of the solution was injected into the HPLC to analyze the ethyl linoleate hydroperoxides under the following conditions: column, 150 × 4.6 mm i.d.YMC-ODS-A (YMC, Tokyo, Japan); solvent, CH$_3$CN/H$_2$O (9:1); flow rate, 1.0 mL/min; and detection, 234 nm. Concentration of the hydroperoxide was calculated using the calibration curve: $Y = 2.29 \times 10^{-6}X - 4.38 \times 10^{-4}$ (range for $X = 9.8 \times 10^3$ to 4.8×10^6). X is the peak area value of the 234-nm detection, and Y is the concentration of the hydroperoxide (mM).

Isolation and Identification of the Conversion Products from Carnosic Acid Quinone. *Isolation of Carnosic Acid.* The solution of carnosic acid quinone (10 mg) in CH$_3$CN (10 mL) was allowed to stand at 60 °C for 4 h. After concentration of the solution to 1 mL, 200 μL of the concentrate was injected into the preparative HPLC and purified under the following conditions: column, 250 mm × 20 mm i.d. Daisopak ODS-AP; flow rate, 9.5 mL/min; solvent, CH$_3$OH/H$_2$O (9:1) containing acetic acid (1%); and detection, 254 nm. A peak at the retention time of 14.9 min, which was identical to the 7.7-min peak in the analytical HPLC, was collected. This purification was repeated 5 times. The eluates of all of the purification runs were combined and evaporated to give a colorless solid (1.6 mg), which was identical to carnosic acid according to its ^{1}H NMR and MS data (*10*).

Isolation of 7-O-Methylrosmanol, Carnosic 8-lactone, and Carnosol. Carnosic acid quinone (101 mg) was dissolved in methanol (100 mL). After standing at 60 °C for 2 h, the solution was concentrated to 10 mL. A 200-μL portion of the concentrate was injected into the preparative HPLC and purified under the following conditions: column, 250 mm × 20 mm i.d. Daisopak ODS-AP; flow rate, 9.5 mL/min; solvent, CH$_3$OH/H$_2$O (9:1) containing acetic acid (1%); and detection, 254 nm. Three peaks eluted at the retention time of 8.3, 9.0, and 12.2 min, which were identical to the 4.1-, 4.5-, and 6.0-min peaks, respectively, in the analytical HPLC, were collected. This procedure was repeated 50 times. Each eluate was then combined and evaporated to give 7-*O*-methylrosmanol from the 8.3-min peak, carnosol (42 mg) from the 9.0-min peak, and 8-lactone derivative (12 mg) from the 12-min peak. The identification of 7-*O*-methylrosmanol and carnosol was carried out by comparison of their ^{1}H NMR and MS data with those in the literature (*10, 11*).

Analytical data for carnosic 8-lactone: HR-MS (*m/z*) found, 330.1822 [M]$^+$; Calcd for C$_{20}$H$_{26}$O$_4$, 330.1831. IR (dry film) ν_{max} 3392 (OH), 2958 (CH). 1781 (γ-lactone), 1649 (CO), 1626 cm^{-1}, UV (CH$_3$CN/H$_2$O) λ_{max} 247, 309 nm; ^{1}H and ^{13}C NMR, see **Table 1**. The acetate of carnosic 8-lactone, which was obtained by acetic anhydride and pyridine treatment: UV (CH$_3$CN/H$_2$O) λ_{max} 245, 280 (sh) nm; ^{13}C NMR (acetone-d_6) δ 27.9 (C-1), 18.5 (C-2), 41.9 (C-3), 34.8 (C-4), 51.7 (C-5), 21.2 (C-6), 39.0 (C-7), 80.1 (C-8), 136.2 (C-9), 52.3 (C-10), 149.3 (C-11), 178.7 (C-12), 147.1 (C-13), 137.1 (C-14), 27.6 (C-

Antioxidant Mechanism of Carnosic Acid Quinone

J. Agric. Food Chem., Vol. 50, No. 21, 2002   **5865**

Table 1. ^{1}H and ^{13}C NMR Spectral Data of Carnosic 8-Lactone[a]

position	[13]C	correlated protons in HMBC	[1]H[b]	correlated protons in NOESY
1	28.4	H-2β, H-3α	α: 2.53 (dt, 4.5, 14.0) β: 1.98 (brd, 15.5)	H-1β, H-3α, H-5
2	18.7	H-1α, H-1β, H-3α	α: 1.49–1.58 (overlapped) β: 2.11 (m)	H-1β, H-19
3	42.1	H-1α, H-1β, H-2β, H-18, H-19	α: 1.35 (dt, 4.0, 13.5) β: 1.49–1.58 (overlapped)	H-1α, H-5, H-18
4	34.6	H-2β, H-3α, H-5, H-6β, H-18, H-19		
5	50.9	H-3α, H-6β, H-7β, H-18, H-19	1.72 (dd, 12.5, 4.0)	H-1α, H-6α, H-18
6	21.3	H-7β	α: 2.01 (ddd, 14.0, 4.0, 1.2) β: 1.61 (dq, 5.5, 14.0)	H-5, H-7β, H-18 H-7β, H-19
7	38.7	H-1β, H-6β, H-14	α: 1.49–1.58 (overlapped) β: 2.45 (ddd, 12.5, 5.0, 1.5)	H-6β, H-14
8	79.8	H-6α, H-7β		
9	132.8	H-1α, H-5, H-7β, H-14		
10	51.6	H-1α, H-5		
11	151.5			
12	182.0	H-14, H-15		
13	146.0	H-14, H-15, H-16, H-17		
14	139.2	H-7β, H-15	6.98 (d, 0.7)	H-7β, H-15, H-16, H-17
15	27.6	H-14, H-16, H-17	2.94 (dsept, 0.5, 7.0)	
16	21.4[c]	H-15	1.08 (d, 7.0)[d]	H-14, H-15
17	21.9[c]	H-15	1.10 (d, 7.0)[d]	H-14, H-15
18	32.6	H-3α, H-5	0.96 (s)	H-3α, H-5, H-6α
19	20.6	H-5	0.96 (s)	H-2β, H-6α
20	177.2	H-1α, H-5		
11-OH			7.77 (brs)	

[a] 500 MHz for ^{1}H and 125 MHz for ^{13}C in acetone-d_6. [b] Coupling pattern and constant (*J* in Hz) are in parentheses. [c,d] Assignments may be interchangeable.

15), 21.5 (C-16 or C-17), 21.7 (C-17 or C-16), 20.7 (C-18 and C-19), 175.5 (C-20), 168.7 (CH₃*C*O), 20.2 (*C*H₃CO).

Isolation of Rosmanol. Carnosic acid quinone (21 mg) was dissolved in a mixture of CH₃CN (215 mL) and H₂O (22 mL). The solution was incubated at 37 °C for 5 h. The reaction mixture was concentrated to 3 mL, and 200 μL was injected into the preparative HPLC. Separation was carried out under the following conditions: column, 250 mm × 20 mm i.d. Daisopak ODS-AP; flow rate, 9.5 mL/min; solvent, CH₃OH/H₂O (9:1) containing acetic acid (1%); and detection, 254 nm. The peak eluted at a retention time of 6.6 min, which was identical to the 3.3-min peak in the analytical HPLC, was collected. This procedure was repeated 15 times. The eluates of all of the purification runs were combined and evaporated to give a colorless solid (1.8 mg), which was identical to rosmanol based on the MS and ^{1}H NMR data (*12*).

RESULTS AND DISCUSSION

Antioxidant Activity of the Stock Solutions of Carnosic Acid Quinone. Carnosic acid quinone is a radical chain-termination compound accumulated during the antioxidation of carnosic acid (*8*). Our previous study reached the conclusion that carnosic acid quinone had no reactivity toward the peroxyl radical; therefore, it showed no chain-breaking antioxidant activity (*8*). However, during additional investigations, we found that a solution of carnosic acid quinone began to show potent antioxidant activity upon standing. **Figure 2** shows the antioxidant activity of acetonitrile and methanol solutions of carnosic acid quinone (initial concentration, 0.25 mM), which were allowed to stand for 4 h at 37 °C. In **Figure 2A**, the acetonitrile solution of carnosic acid quinone shows strong antioxidant activity for 0.5 h. The methanol solution of carnosic acid quinone shows longer activity than the acetonitrile solution, and its induction period is about 4 h, as shown in **Figure 2B**. In contrast, the fresh solution of carnosic acid quinone showed very weak activity, and no induction period was observed (**Figure 2C**).

Analysis of the Conversion of Carnosic Acid Quinone in Acetonitrile. The HPLC analysis of the 0.25 mM acetonitrile solution of carnosic acid quinone was carried out. **Figure 3A** shows the HPLC analytical result of the 4-h solution of carnosic acid quinone. This result indicated that carnosic acid quinone was unstable in the solvent and produced several new products. As previously mentioned, this 4-h solution had a strong antioxidant activity, suggesting that the newly produced compounds contributed to the strong activity of the solution. Typical large peaks, which are indicated as **1**, **2**, **3**, **4**, and **5** in **Figure 3A**, were selected and analyzed versus time. **Figure 4** shows that these new peaks increased for 8 h, whereas carnosic acid quinone decreased from 0.25 mM to 0.15 mM, which indicates that these new compounds came from carnosic acid quinone by a degradation reaction in solution. To reveal the contribution of these new compounds to the strong antioxidant activity of the solution, we analyzed the concentration change of each new compound induced by a radical oxidation inducer, AIBN. It is well-known that a chain-breaking antioxidant must be oxidatively consumed by the radical oxidation reaction. If the solution contains a potent antioxidative compound, the compound should decrease as a result of the oxidation, and then its oxidized product would accumulate in the system. After standing at 37 °C for 4 h, the solution of carnosic acid quinone was divided into 2 tubes, and AIBN was added to one of the tubes at a concentration of 15 mM. **Figure 4** shows the time-dependent change on the concentration of the new compounds **1**−**5** and carnosic acid quinone with or without AIBN. Peaks of compounds **3** and **5** were obviously decreased after the addition of AIBN, whereas there was almost no change in peaks **1** and **2**. Interestingly, the decreasing rate of carnosic acid quinone slightly slowed after the addition of AIBN. These results indicated that compounds **3** and **5** have antioxidant activity and the others have no or only weak activity. The decreased rate of carnosic acid quinone was also affected by the radical conditions used. Carnosic acid quinone might be a termination product of the radical oxidation reaction from the newly produced antioxidants.
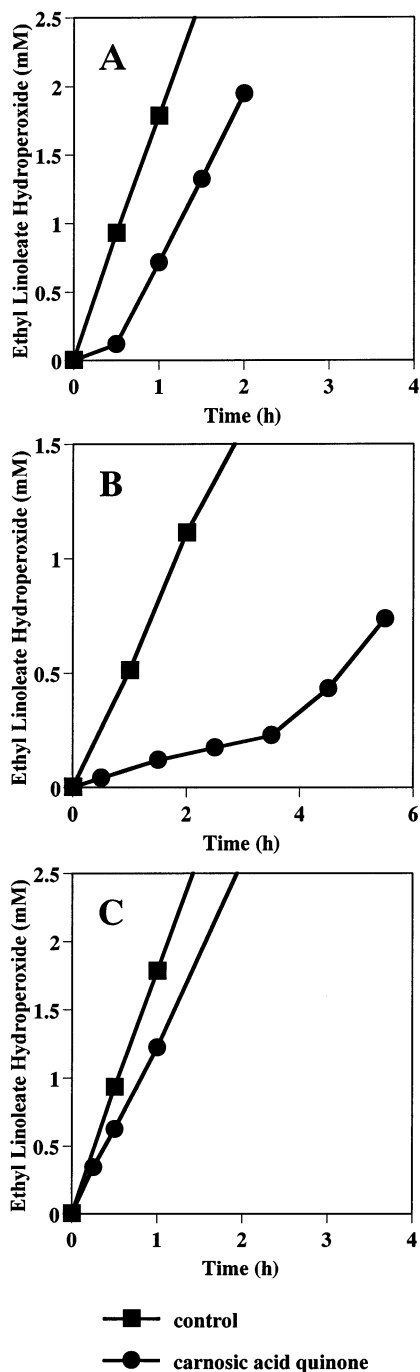
5866 *J. Agric. Food Chem.,* Vol. 50, No. 21, 2002

Masuda et al.

Figure 2. Antioxidant activity of carnosic acid quinone solutions (0.25 mM). Panel A, the 4-h solution of carnosic acid quinone in acetonitrile; panel B, the 4-h solution of carnosic acid quinone in methanol; panel C, the fresh solution of carnosic acid quinone in acetonitrile. Oxidation of ethyl linoleate (50 mM) was induced by AMVN (15 mM) at 37 °C.

Figure 3. HPLC analytical data for the 4-h solution of carnosic acid quinone in acetonitrile (panel A), the 4-h solution of carnosic acid quinone in methanol (panel B), and the 4-h solution of carnosic acid quinone in the water/acetonitrile (panel C).

Identification of New Compounds Produced in Acetonitrile Solution of Carnosic Acid Quinone. To identify the chemical structures of compounds **3** and **5**, the isolation of these compounds was attempted. Unfortunately, compound **5** was very unstable and could not be isolated by our technique. On the other hand, the isolation of compound **3** was successful using preparative HPLC. A spectroscopic structural analysis of compound **3** was carried. Interestingly, the analytical data revealed that compound **3** was identical to carnosic acid. It should be noted that carnosic acid is a reduced compound of carnosic acid quinone and has potent antioxidant activity. There is no reducing reagent added to the solution; therefore, the self-
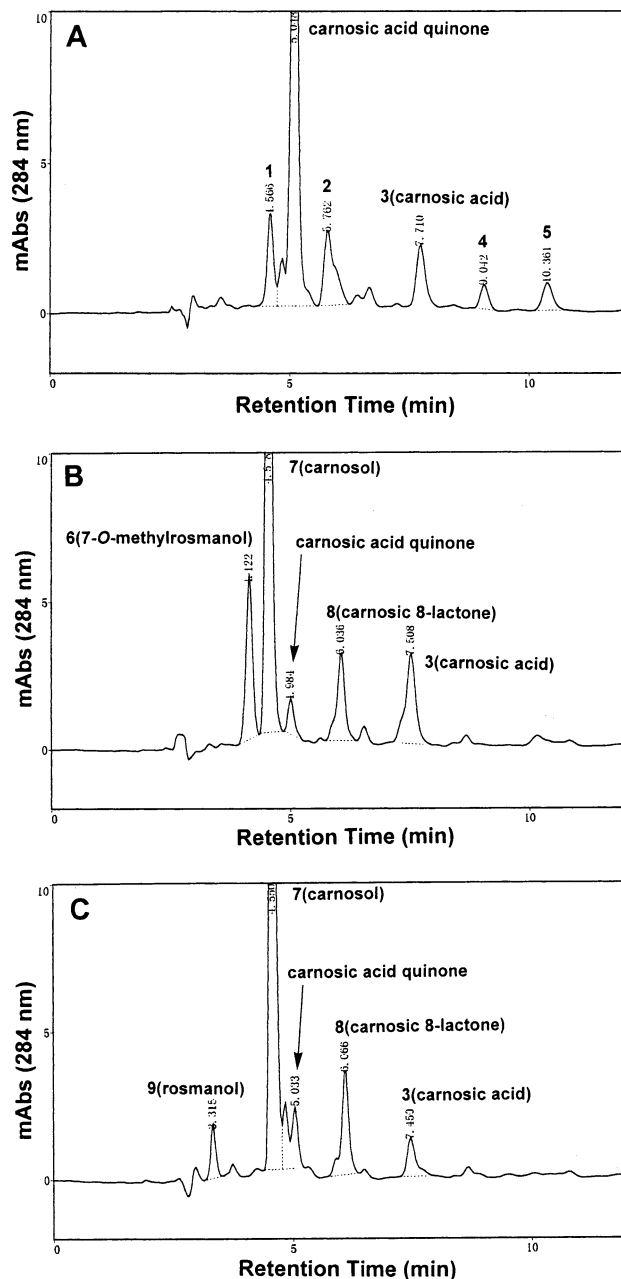
redox reaction (disproportionation) of carnosic acid quinone should be considered. On the basis of the redox principle, if carnosic acid was produced from carnosic acid quinone by such a reaction, the same amount of an oxidized compound from carnosic acid quinone must be produced. During the time-course analysis results (**Figure 4**), peak **4** increased faster in the presence of AIBN than in the experiment without AIBN. The compound corresponding to peak **4** might be an oxidation product from carnosic acid quinone in the redox reaction. To obtain structural information, we tried to isolate **4**. The HPLC separation of **4** was successful and yielded a purple solution; however, concentration of the solution failed, and no spectroscopic analysis could be performed, because **4** was completely degraded during the concentration. It is well-known that the intensive oxidation of a phenolic compound often gives colored products. Frampton et al. (*13*) reported that the oxidation of
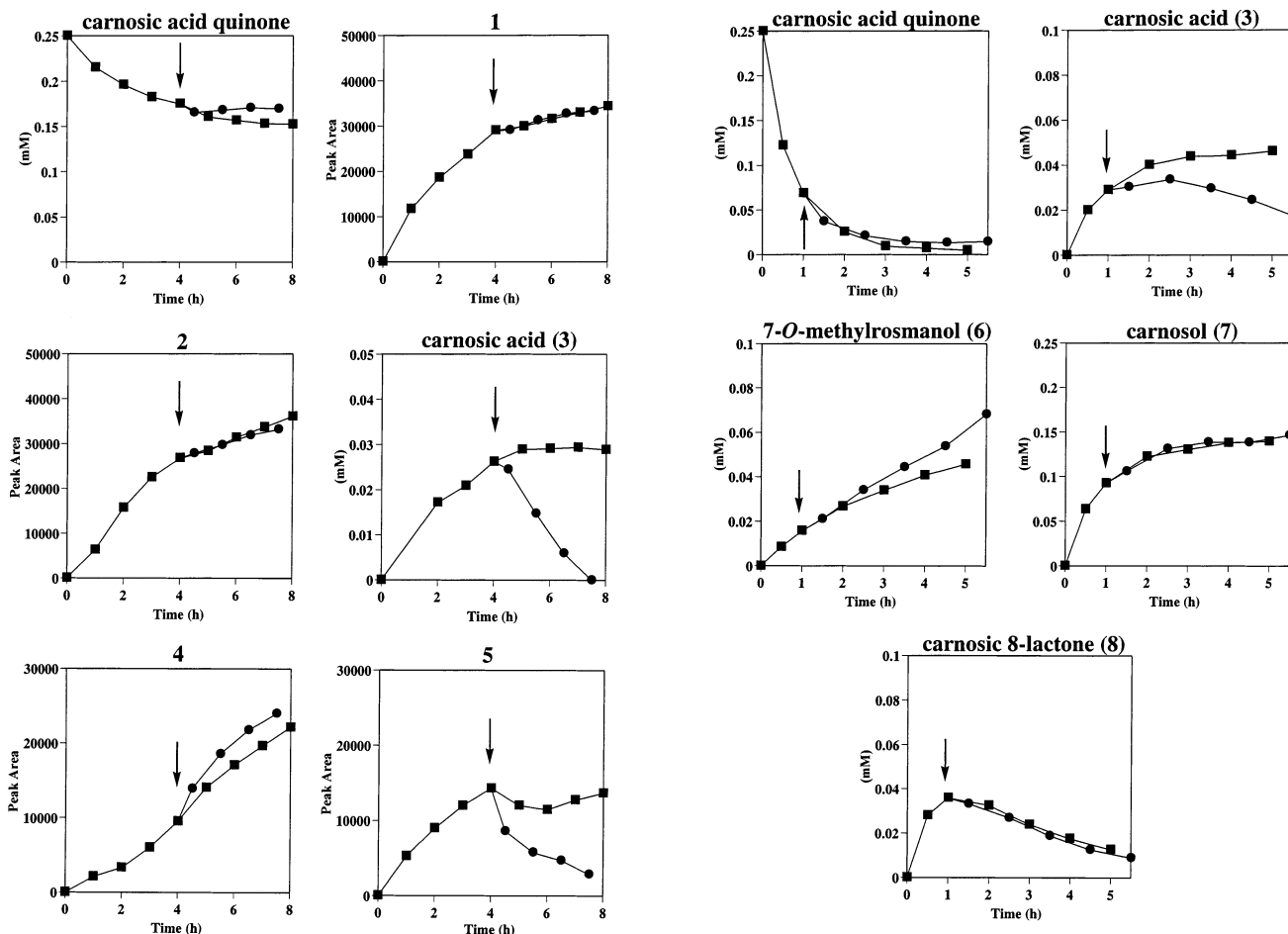
Antioxidant Mechanism of Carnosic Acid Quinone

J. Agric. Food Chem., Vol. 50, No. 21, 2002 **5867**

Figure 4. Time-course analytical data of carnosic acid quinone and compounds **1**–**5** typically observed in the HPLC analysis of the reaction with (●) or without (■) AIBN in acetonitrile (15 mM). The scale on the *Y* axis is the concentration in the solution (isolated compounds) or peak area for the analysis (unidentified compounds). The arrow indicates the addition time of AIBN to the solution.

Figure 5. Time-course analytical data of carnosic acid quinone and compounds **6**–**8** typically observed in the HPLC analysis of the reaction with (●) or without (■) AIBN in methanol (15 mM). The arrow indicates the addition time of AIBN to the solution.

vitamin E, a potent antioxidative phenol, gave a purple material. This purple compound **4** is possibly an oxidized compound produced during the redox reaction of carnosic acid quinone.

Conversion of Carnosic Acid Quinone in Methanol. The methanol solution of carnosic acid quinone showed a much longer induction period than that of the acetonitrile solution (**Figure 2**). We also analyzed the materials produced from carnosic acid quinone in methanol by the HPLC technique. **Figure 3B** shows the analytical result of the 4-h methanol solution. Although this HPLC profile looks different from that of the acetonitrile solution (**Figure 3A**), carnosic acid production is clearly observed at the retention time of 7.5 min, which is similar to that in the acetonitrile solution. The time-course analysis of each produced peak was conducted, and the data are shown in **Figure 5**. In methanol, the decrease of carnosic acid quinone was much faster than in acetonitrile solution, and most of carnosic acid quinone was consumed within 3 h of its dissolution. In addition to the peaks of carnosic acid and carnosic acid quinone, three typical peaks, **6**, **7**, and **8**, were observed at retention times of 4.1, 4.5, and 6.0 min, respectively. To identify these peak compounds, we carried out their isolation.

Identification of the Compounds Produced in the Methanol Solution of Carnosic Acid Quinone. The isolation of compounds **6**−**8** was successful by using preparative HPLC. Compound **7** was isolated as a colorless powder. Its [^1]H NMR

and MS data revealed that **7** was identical to carnosol (**Figure 1**). Compound **6** was isolated as a colorless solid. Its [^1]H NMR indicated that **6** consisted of two stereoisomeric compounds in a ratio of 4:1. Intensive analysis of the [^1]H NMR data of **6** revealed that **6** was identical to an epimeric mixture of 7-*O*-methylrosmanol (**Figure 1**). Its MS data also supported these structures. Compound **8** was isolated as a colorless viscous oil. Its molecular formula was determined to be $C_{20}H_{26}O_4$ from the high-resolution mass spectrum result, which indicated that **8** was an isomer of carnosic acid quinone. The [^1]H NMR data of compound **8** were similar to those of carnosic acid; however, several carbon chemical shifts were very different from those of carnosic acid. In particular, a carbon signal assignable to the 8-position was quite upfield-shifted to 79.8 ppm, as compared with that of carnosic acid (129.7 ppm). From the chemical shifts for the carbons at the 9-, 11- and 12-positions (δ 132.8, 151.5, and 182.0 ppm, respectively), the presence of an enolated α-diketone structure was suggested. These data indicated that **8** was a substance substituted at the 8-position of the benzene ring of carnosic acid quinone by an addition of an oxygen function. The IR spectrum of **8** showed a typical peak at 1781 cm^{-1}, which indicated the presence of a γ-lactone structure, and therefore, the oxygen function should be the carboxylic acid at the 20-position. Thus, compound **8** was determined as depicted in **Figure 1** and named carnosic 8-lactone.

Proposed Mechanism for the Production of Antioxidants from Carnosic Acid Quinone in Methanol. The chemical structures of all the detected compounds in the methanol solution
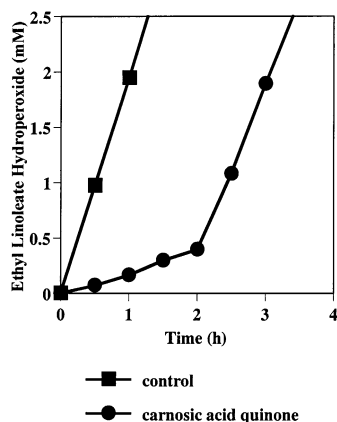
Figure 6. Antioxidant activity of the 4-h solution of carnosic acid quinone (0.25 mM) in water/acetonitrile (1:9). Oxidation of ethyl linoleate (50 mM) was induced by AMVN (15 mM) at 37 °C.

showed that carnosol and carnosic 8-lactone were lactone isomers of carnosic acid quinone and seemed not to be associated with the redox reaction of carnosic acid quinone, because their oxidation stage is the same as that of carnosic acid quinone. On the other hand, 7-*O*-methylrosmanol is obviously an oxidized derivative of carnosic acid quinone. The production amount of 7-*O*-methylrosmanol was 0.045 mM for 5 h after dissolution of the carnosic acid quinone, which was equal to that of carnosic acid (0.045 mM at 5 h). These results strongly indicated that in the methanol solution, carnosic acid quinone underwent a self-redox reaction and yielded equal amounts of the reduced and oxidized compounds, carnosic acid and 7-*O*-methylrosmanol, respectively. The addition of a radical oxidation inducer, AIBN, induced oxidative change in the concentration of each compound. The time-course data of the products for the reaction in the presence of AIBN are also shown in **Figure 5**. After the addition of AIBN (1 h after the dissolution of carnosic acid quinone) in methanol, the concentration of carnosic acid had gradually decreased, as compared with that in the experiment without AIBN. The concentrations of carnosol and carnosic 8-lactone did not change between the experiments with and without AIBN, whereas 7-*O*-methylrosmanol increased. These results indicate that carnosol and carnosic 8-lactone did not contribute to the antioxidant activity under these conditions, and 7-*O*-methylrosmanol is possibly a termination compound of this antioxidation reaction. As previously mentioned, the antioxidation of carnosic acid produced carnosic acid quinone; therefore, the relative, but slight increase of carnosic acid quinone in the presence of AIBN could be explained by the reoxidation of the produced carnosic acid. This redox cycle between carnosic acid and carnosic acid quinone would constantly produce 7-*O*-methylrosmanol as the oxidant, which could explain the relative increase in the concentration of 7-*O*-methylrosmanol in the presence of AIBN.

Production of Rosmanol from Carnosic Acid Quinone under Aqueous Conditions. As already mentioned, 7-*O*-methylrosmanol is an oxidized product of carnosic acid quinone in methanol solution. The methoxy group that appeared at the 7-position of the compound is thought to come from the solvent. Methanol has nucleophilicity and may be incorporated into an intermediate of the oxidation of carnosic acid quinone. Generally, the presence of methanol is very rare in food or a plant body, and the presence of water, which also has nucleophilicity, is more normal. Thus, we reexamined the antioxidant activity and the conversion of carnosic acid quinone in the presence of water. **Figure 6** shows the antioxidant activity of a 4-h solution
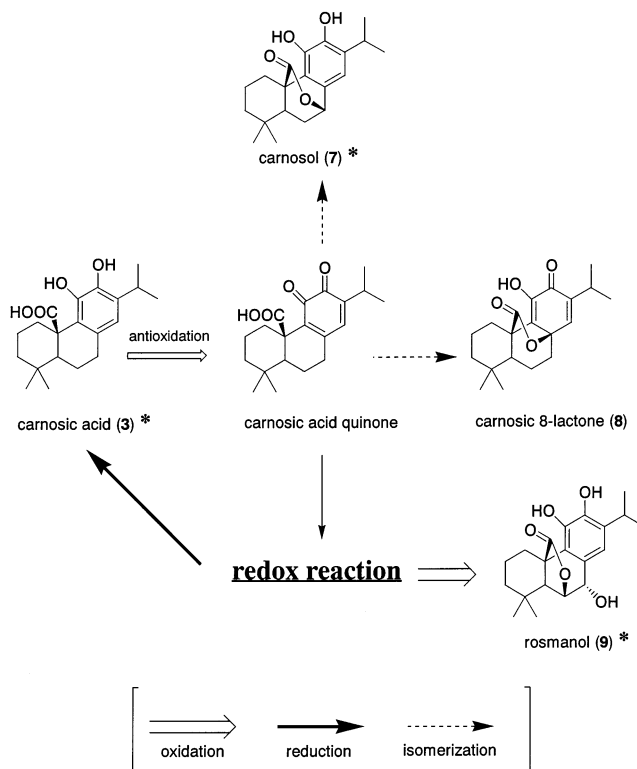

Figure 7. Proposed antioxidant mechanism from carnosic acid quinone. Different shape of arrow indicates different type of reaction occurred on carnosic acid quinone, as shown at the bottom of the figure. Asterisk (*) indicates the compound having antioxidant ability.

of carnosic acid quinone in a mixture of acetonitrile/water (9:1). A strong antioxidant activity was observed for 2 h, which was more effective than that of the acetonitrile solution. The HPLC analytical result of the 4-h solution is shown in **Figure 3C**. Although the same products, including carnosic acid, carnosol, and carnosic 8-lactone, were observed in the solution, 7-*O*-methylrosmanol, however, was not detected. Instead of the 7-*O*-methylrosmanol peak, a new peak **9** was observed at the retention time of 3.3 min. The isolation and structural analysis of the peak compound **9** clarified that it was identical to rosmanol. Rosmanol is the desmethyl derivative of 7-*O*-methylrosmanol at the 7-*O*-position and has already been isolated from rosemary as a potent antioxidant (*12*). The quantitative analysis of the concentration of rosmanol (0.01 mM) in the 4-h solution is comparable to that of carnosic acid (0.015 mM). These data indicated that rosmanol was an oxidative product from carnosic acid quinone produced by the self-redox reaction of carnosic acid quinone in the presence of water, which is similar to the 7-*O*-methylrosmanol production in methanol solution.

Recovery Mechanism of the Antioxidant Activity of Solution of Carnosic Acid Quinone. We now propose an antioxidant mechanism starting from carnosic acid quinone, which is supported by experimental evidence, as illustrated in **Figure 7**. Carnosic acid quinone is inactive in radical reactions, including the chain-breaking antioxidation reaction, but chemically very unstable in solution. Carnosic acid quinone in solution converts by two routes. One is the isomerization process to lactone derivatives, including carnosol and its isomer, carnosic 8-lactone, by the intramolecular addition of a 20-carboxylic acid. The other is the self-redox reaction that gives carnosic acid and rosmanol. The selection of the conversion pathway is dependent upon the solvent property. A protic solvent may enhance the

Antioxidant Mechanism of Carnosic Acid Quinone

J. Agric. Food Chem., Vol. 50, No. 21, 2002 **5869**

isomerization to carnosol; however, the self-redox route fundamentally appeared in solution. Although carnosic 8-lactone is antioxidatively inactive (data not shown), carnosic acid, carnosol, and rosmanol are active, and carnosic acid is known to be the most potent antioxidant among them (*14*). From the viewpoint of antioxidant activity, the inactive carnosic acid quinone produces the most active carnosic acid. The produced carnosic acid significantly contributes to the activity of the solution. This carnosic acid quinone−carnosic acid route produces an oxidized compound, such as rosmanol or 7-*O*-methylrosmanol, in protic solvent or an unidentified substance in acetonitrile. These substances may contribute to the antioxidant activity after carnosic acid consumption. Carnosol and rosmanol are also recognized as powerful antioxidants (*14*). Carnosol and rosmanol, if produced in solution, can also contribute to the antioxidant activity in the next stage. Following the conversion mechanism of carnosic acid to other diterpenes proposed by Wenkert et al. (*3*), a straightforward route to produce carnosol and rosmanol from carnosic acid quinone in the extracts of sage and rosemary was believed to explain their strong antioxidant activity (*15*). Now, based on the results obtained in this investigation, we present a new antioxidant mechanism, as shown in **Figure 7**, to explain the strong antioxidant activity of rosemary and sage, useful and popular herbs in food cultures around the world.

ACKNOWLEDGMENT

LITERATURE CITED

(1) Tena, M. T.; Valcarcel, M.; Hidalgo, R. J.; Ubera, J. L. Supercritical fluid extraction of natural Antioxidants from Rosemary: Comparison with liquid solvent sonication. *Anal. Chem.* **1997**, *69*, 521−526.

(2) Tada, M. Biological activities of antioxidants from herbs in Labiatae. *Foods Food Ingredients J. Jpn*. **2000**, *184*, 33−39.

(3) Wenkert, E.; Fuchs, A.; McChesney, J. D. Chemical artifacts from the family Labiatae. *J. Org. Chem.* **1965**, *30*, 2931−2934.

(4) Aruoma, O. I.; Halliwell, B.; Aeschbach, R. Antioxidant and pro-oxidant property of active rosemary constituents: carnosol and carnosic acid. *Xenobiotica* **1992**, *22*, 257−268.

(5) Schwartz, K.; Ternes, W. Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis* II. Isolation of carnosic acid and formation of other phenolic diterpenes. *Z. Lebensm. Unters. Forsch.* **1992**, *195*, 99−103.

(6) Cuvelier, M.-E.; Berset, C.; Richard, H. Antioxidant constituents in sage (*Salvia officinalis*). *J. Agric. Food Chem.* **1994**, *42*, 665−669.

(7) Hall, C. A., III; Cuppett, S. L. Structure activities of natural antioxidants. In *Antioxidant Methodology In Vivo and In vitro Concepts*; Aruoma, O. I., Cuppett, S. L., Eds; AOCS Press: Champaign, IL, 1997; pp 141−172.

(8) Masuda, T.; Inaba, Y.; Takeda, Y. Antioxidant mechanism of carnosic acid: structural identification of two oxidation products. *J. Agric. Food Chem.* **2001**, *49*, 5560−5565.

(9) Richheimer, S. L.; Bailey, D. T.; Bernart, M. W.; Kent, M.; Vininski, J. V.; Anderson, L. D. Antioxidant activity and oxidative degradation of phenolic compounds isolated from rosemary. *Recent Res. Dev. Oil Chem.* **1999**, *3*, 45−58.

(10) Richheimer, S. L.; Bernart, M. W.; King, G. A.; Kent, M. C.; Bailey, D. T. Antioxidant activity of lipid-soluble phenolic diterpenes from rosemary. *J. Am. Oil Chem. Soc.* **1996**, *73*, 507−514.

(11) Inatani, R.; Nakatani, N.; Fuwa, H.; Seto, H. Structure of a new antioxidative phenolic diterpene isolated from rosemary (*Rosemarinus officinalis* L.). *Agric. Biol. Chem.* **1982**, *46*, 1661−1666.

(12) Nakatani, N.; Inatani, R. Two antioxidative diterpenes from rosemary (*Rosmarinus officinalis* L.) and a revised structure. *Agric. Biol. Chem.* **1984**, *48*, 2081−2085.

(13) Frampton, V. L.; Skinner, W. A.; Cambour, P.; Bailey, P. S. α-Tocopurple, an oxidation product of α-tocopherol. *J. Am. Chem. Soc.* **1960**, *82*, 4632−4634.

(14) Schwart, K.; Ternes, W.; Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis* I. Determination of phenolic diterpenes with antioxidant activity amongst tocochromanols using HPLC. *Z. Lebensm. Unters. Forsch.* **1992**, *195*, 95−98.

(15) Fisher, C. Spice of life. *Chem. Brit.* **2002**, 40−42.