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A stable quinone identified in the reaction of carnosol, a major antioxidant in rosemary, with 2,2-diphenyl-1-picrylhydrazyl radical

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Abstract

Carnosol is known as a major antioxidant in rosemary (*Rosmarinus officinalis* L.). The reaction of carnosol with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was carried out and a novel product was identified as a quinine of carnosol, carnosylquinone. A mechanism for the carnosol-DPPH reaction is proposed.
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1. Introduction

Oxidation, the main cause of food deterioration, occurs spontaneously in lipids or lipid-containing foods. It can result in organoleptic rancidity in the finished products, generating undesirable flavour and colour, deterioration of food quality, such as vitamin destruction, and nutritional loss (Sherwin, 1978). Antioxidants are added to foods for retarding the oxidation. Antioxidants terminate free radical reactions, principally by functioning as reducing agents or peroxyl radical chain interrupters (Menschikova & Zenkov, 1993). Antioxidants may react with highly reactive radical species to yield a second, less reactive free radical, or a non-radical species. This secondary radical is sufficiently stable to preferentially undergo chain termination rather than initiate further radical formation. Both synthetic and natural antioxidants typically include, as part of their molecular structure, an aromatic ring to delocalize the free electron of a radical and one or more hydroxyl groups to provide labile hydrogen atoms. Many of the natural antioxidants are derivatives of flavonoids, coumarins, tocopherols, hydroxycinnamic acids and carotenoids.

Natural antioxidants can be found in almost every vegetable oil, mostly as tocopherols. Ascorbic acid, isoascorbic acid and their ester derivatives, such as ascorbyl palmitate, are used in many foods as antioxidants. A great number of spices are known for their excellent antioxidant properties, e.g., rosemary and sage. Rosemary (*Rosmarinus officinals* L.) and sage (*Salvia officinals* L.) leaves are commonly used as spices and flavouring agents. They have also attracted much attention since Chipault, Mizuno, Hawkin, and Lundberg (1955) reported their antioxidant activity.

It has been reported that rosemary extract is effective for retarding the rancidity development in potato chips which have been processed under deep-fat frying conditions and its antioxidative activity is comparable to that of most synthetic antioxidants (Chang, Ostric-Matijasevic, Hsieh, & Huang, 1977). At least 12 diterpene phenols have been isolated from rosemary (Cuvelier, Richard, & Berset, 1996; Ho et al., 1998; Houlihan, Ho, & Chang, 1984, 1985; Inatani, Nakatani, Fuwa, & Seto, 1982; Nakatani & Inatani, 1981, 1984; Wu, Lee, Ho, & Chang, 1982). Several of them, such as carnosol,

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carnosic acid, rosmanol, rosmaridiphenol, rosmadial and miltirone have been shown to have antioxidant activity.

2,2-Diphenyl-1-picryhydrazyl is a stable free radical. It has been widely used for antioxidant determinations. Chen and Ho (1995) demonstrated the radical-scavenging ability of carnosol. However, the antioxidation mechanism is still not clear. It is important to clarify the stable radical termination products from carnosol and examine the antioxidation process of carnosol on the basis of products. Reaction products of DPPH radicals with other phenolic antioxidants, such as resveratrol, catechin, epicatechin, epigallocatechin and epigallocatechin gallate have been reported (Sang et al., 2002; Wang, Jin, & Ho, 1999; Zhu et al., 2001).

2. Materials and methods

2.1. Preparation of carnosol

The ground dried leaves of rosemary (*Rosmarinus officinalis* L.) (300 g) were extracted three times, for 2–3 h, with 750 ml hexane at 25 °C in a 2-litre stainless steel vessel fitted with a mechanical stirrer. The solvent was evaporated under vacuum with a rotary evaporator to yield 11–13 g of extract. The powdered rosemary extract was dissolved in 200 ml of methanol and allowed to stand at room temperature for 1 week. After removing the methanol under vacuum with a rotary evaporator, 0.5 g of the dry residue was dissolved in 2 ml hexane:ether (3:1) and was injected into a preparative column (550 × 25-mm internal diameter) packed with activated silicic acid (Mallinckrodt, St. Louis, MO). The mobile phase, delivered by a piston pump, was hexane:ether (3:1), and the flow rate was 2.5 ml/min. The eluent was monitored by a UV detector at 254 nm. The carnosol fraction, which eluted between 12.5 and 14.5 min, was collected, and the solvent was evaporated. After removal of solvent, the carnosol fraction was recrystallized from methanol to yield 70–90 mg of carnosol. The PB-LC/MS mass spectrum of carnosol showed a molecular ion at *m/z* 330 (11%) and major fragmentation ions at *m/z* 286 (100%), 284 (32%), 215 (62%) and 269 (23%).

2.2. Structural elucidation of carnosol by PB-LC/MS

The identity of carnosol was confirmed by particle beam liquid chromatography/mass spectrometry on a HP 5989A Mass Engine and a HP1090 HPLC coupled by a HP 59980B particle beam interface. The column was a Supelco Supelcosil LC-18 HPLC column (particle size 5 μm, 2.1 × 150 mm); 1 mg of carnosol were dissolved in 1 ml of acetonitrile, and then 10 μl was injected. The mobile phase was 30% of acetonitrile and 70% of water, and the flow rate was 0.4 ml/min. The temperature of the particle beam interface was 65 °C. Mass spectra were obtained by EI at 70 eV, and scanned from 45 to 450 amu.

2.3. The reaction of carnosol and 2,2-diphenyl-1-picrylhydrazyl (DPPH)

Carnosol (20 mg) was dissolved in 2 ml of acetonitrile and 50 mg of DPPH was dissolved in 5 ml of acetonitrile. The DPPH solution was added dropwise to the carnosol solution. The reaction mixture was checked by TLC until all of the carnosol spot had disappeared.

2.4. Isolation and purification of reaction products

2.4.1. Thin-layer chromatography

Sigma–Aldrich silica gel TLC plates (250-μm thickness, 2–25-μm particle size) was employed for TLC analysis. The elution solvent was hexane and ethyl acetate (7:3).

2.4.2. Silica gel column chromatography

The reaction mixture was coated onto 0.7 g of silica gel. A glass column (450 × 22-mm internal diameter) packed with 50 g of silica gel (Fisher Scientific, Springfield, NJ) was used. The column was developed with 100 ml of hexane, followed by 150 ml of hexane:ethyl acetate (9:1), and then 300 ml of hexane:ethyl acetate (7:3 v/v). Every fraction was monitored by TLC. The fraction size was 20 ml.

2.4.3. High performance liquid chromatography

A green product, obtained from silica gel column chromatography, was dissolved in acetonitrile and subjected to HPLC separation. HPLC analysis was performed with an HP 1090 liquid chromatograph. The column was a Waters Nova-Pak C18 column (3.9 × 300 mm). The mobile phase was 30% of acetonitrile and 70% of water. The flow rate was 1 ml/min. An UV detector monitoring at 285 nm was employed.

2.5. Structural Elucidation of Reaction Products

2.5.1. Desorption chemical ionization mass spectrometry (DCI/MS) analysis

DCI/MS experiments were performed on a HP 5989A Mass Engine. The DCI probe was a Direct Exposure Probe, 4500 Series (Finnigan MAT, CA). Ammonia was used as the reagent gas, and the ion source pressure was 0.7 Torr. The sample was air-dried on a platinum emitter; the emitter was heated by a separated power supply at a heating rate of 100 mA/s until the maximum current, 1.3 A, was reached. The mass scanned was from 50 to 450 amu. The ion-source

temperature was set at 250 °C, and the analyzer temperature was 100 °C.

2.5.2. Nuclear magnetic resonance

^{1}H NMR and ^{13}C NMR experiments were performed on a Bruker AM-500.

3. Results

3.1. The reaction of carnosol and 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The carnosol was reacted with DPPH in acetonitrile. The purple colour of DPPH faded gradually and turned into orange colour. After the reaction was completed, only two spots were observed on the TLC plate, one with an orange colour ($R_f = 0.8$) the other with a green colour ($R_f = 0.35$). The green compound was believed to be a product generated from carnosol-radical reaction. The mixture was then chromatographed on silica gel. An orange-coloured compound and 15 mg of a green-coloured compound were obtained as expected. The green-coloured compound was then injected into the HPLC for further purification.

3.2. DCI/MS analysis of reaction products

The DCI–MS suggested that the molecular weight of the green-coloured compound was 328 (Fig. 1). The *m/z* 329 is called quasimolecular ion $(M + H)^{+}$ which is an even-electron protonated ion with greater stability than the radical molecular ion $(M^{+\cdot})$ (Fig. 2).

3.3. ^{13}C and ^{1}H NMR analysis

The ^{13}C NMR spectrum of the carnosol–DPPH reaction product is shown in Table 1 and ^{1}H NMR spectrum in Table 2. Three of the 20 carbons are carbonyl carbons. This suggests that decarboxylation does not occur when carnosol reacts with DPPH; the carbon skeleton is intact. The reaction product was identified as a quinone with structure shown in Fig. 3 and named carnosylquinone.

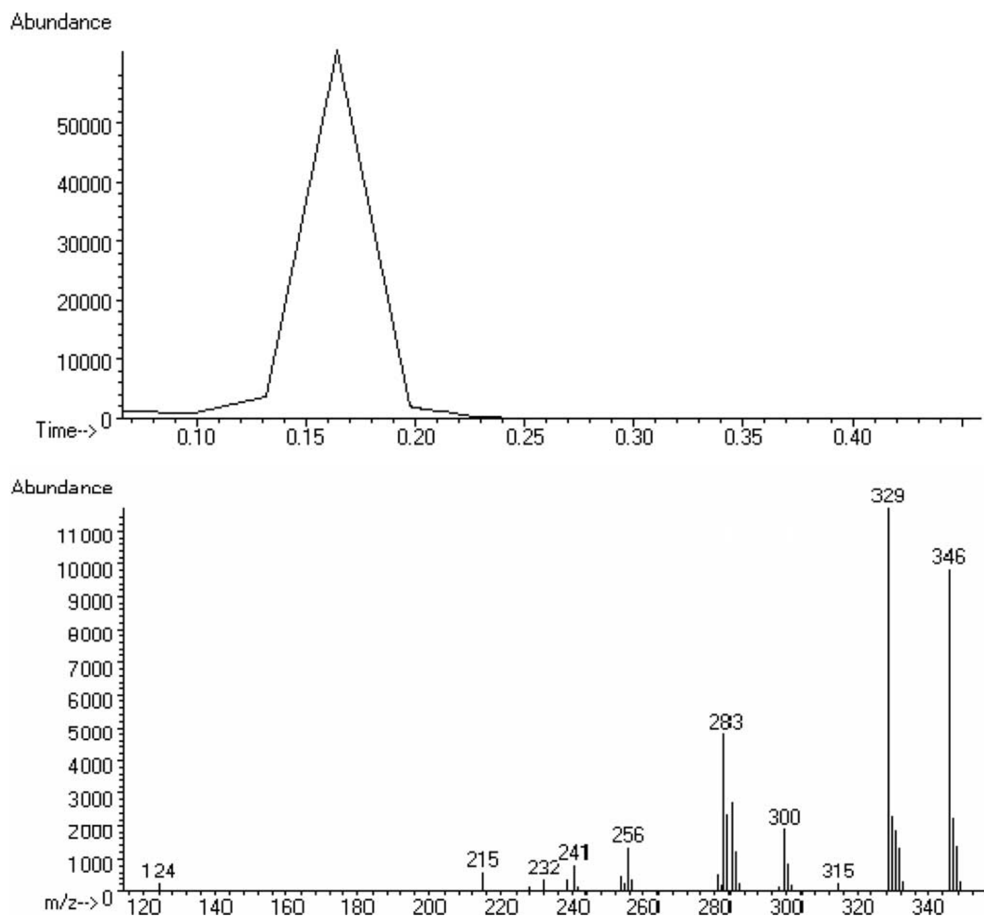

Fig. 1. DCI-MS spectrum of carnosol–DPPH reaction product (top: TIC).

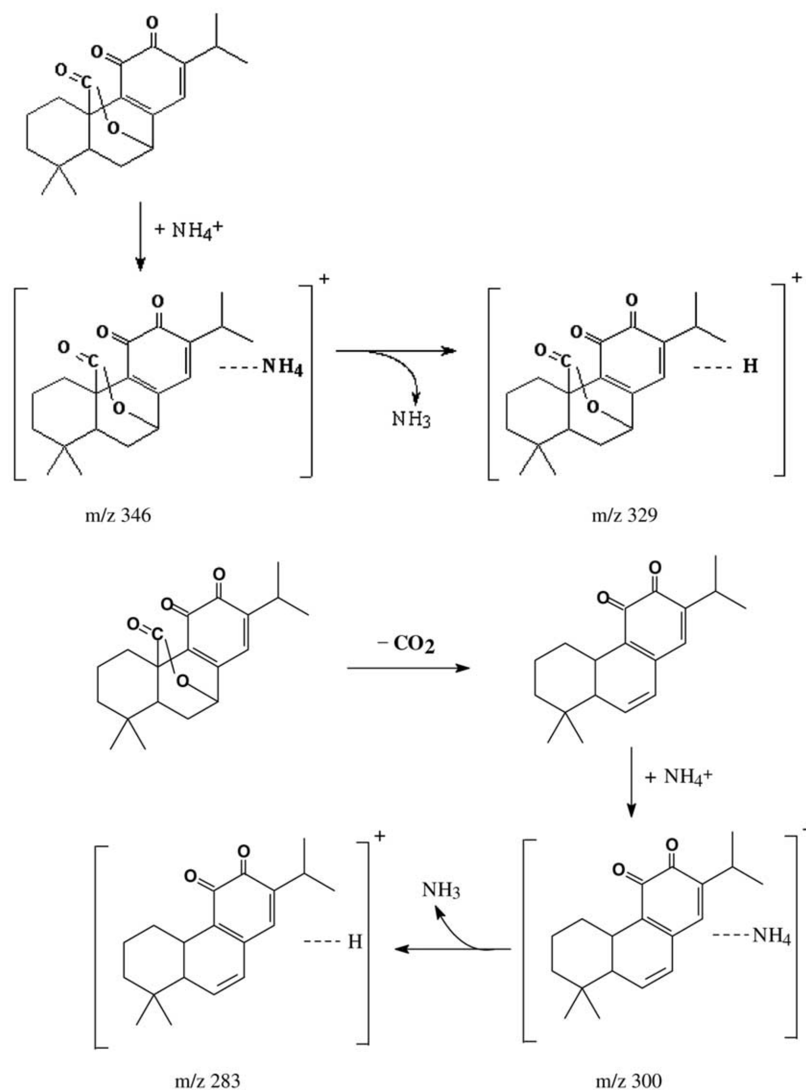

Fig. 2. The interpretation of mass spectrum of carnosol–DPPH reaction product.

4. Discussion

The primary antioxidant activity of rosemary and sage is due to the presence of carnosic acid (Wenkert, Fuchs, & McChesney, 1964). It was also reported that carnosic acid and carnosol are responsible for 90% of antioxidant activity of rosemary (Cuvelier et al., 1996).

Carnosic acid can be converted to carnosol in methanol (Brieskorn & Domling, 1969; Ho, Ferraro, Chen, Rosen, & Huang, 1994). This conversion is through a semiquinone intermediate. The conversion of carnosol to rosmanol has also been reported (Gonzalez, Andres, Aguiar, & Luis, 1992; Ho et al., 1994).

It has been reported that the DPPH radical-scavenging effect of phenolic compounds is due to their hydrogen-donating ability (Yen & Duh, 1994). A major mechanism for a phenolic antioxidant in food is the trapping and stabilizing of free radicals, such as the lipid peroxyl radical, which is generated from radical chain oxidation of food components. This property is also important in biological systems for scavenging oxygen radicals.

Most phenolic antioxidants can donate a hydrogen radical and then be converted to non-radical species, so structural information about these non-radical products would provide important information for understanding the antioxidant mechanism.

A phenolic antioxidant bearing *ortho*-dihydroxyl groups was investigated as an efficient antioxidant, and the produced stable *o*-quinone material plays a key role in its antioxidation (Hall & Cuppett, 1997). It is reported (Natella, Nardini, Felice, & Scaccini, 1999) that the antioxidant efficiency of monophenols is enhanced by the introduction of a second hydroxy group and is increased by one or two methoxy substitutions in positions *ortho* to the hydroxy group. All of these studies have indicated that the antioxidant activity of phenolic acids is related to their structure.

Table 1
^{13}C NMR data of carnosol–DPPH reaction product

Carbon no.	δ
C-1	28.4
C-2	19.1
C-3	41.2
C-4	34.9
C-5	45.1
C-6	28.2
C-7	77.2
C-8	136.2
C-9	150.2
C-10	49.1
C-11	180.2
C-12	178.6
C-13	154.0
C-14	130.6
C-15	28.0
C-16	21.6
C-17	21.7
C-18	32.2
C-19	19.6
C-20	174.2

Table 2
^{1}H NMR data of carnosol–DPPH reaction product

Proton no.	δ
H-1α	2.24 (1H)
H-1β	2.55 (1H)
H-2α	1.56 (1H)
H-2β	1.84 (1H)
H-3α	1.24 (1H)
H-3β	1.47 (1H)
H-5	1.77 (1H)
H-6α	2.18 (1H)
H-6β	2.04 (1H)
H-7	5.42 (1H)
H-14	7.04 (1H)
H-15	2.87 (1H)
H-16	1.07 (3H)
H-17	1.09 (3H)
H-18	0.68 (3H)
H-19	0.16 (3H)

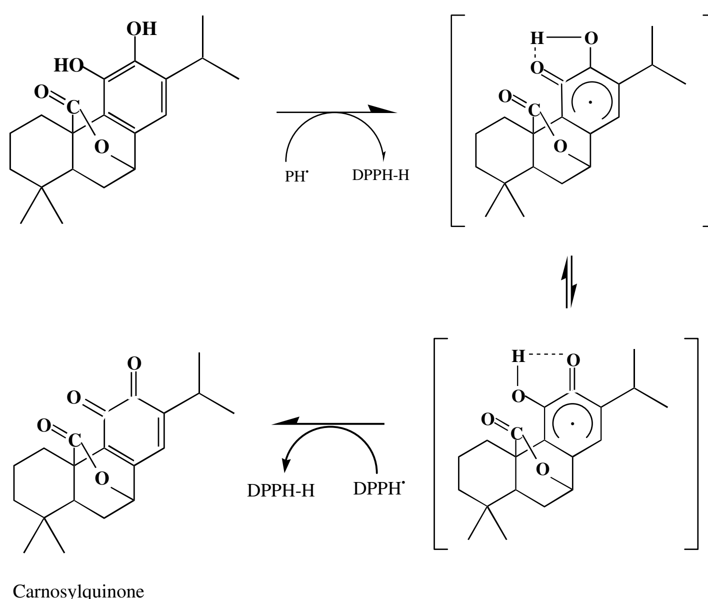

Fig. 3. The proposed mechanism of carnosol–DPPH reaction.

The proposed mechanism of carnosol-DPPH reaction is shown in Fig. 3. The B-ring has been changed to a quinone structure. It suggests that a carnosol molecule could scavenge two radicals.

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