Hydrogen-Donating Mechanism of Rosmariquinone, an Antioxidant Found in Rosemary

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ABSTRACT: Rosmariquinone (RQ), an *ortho*-quinone diterpenoid found in rosemary, was shown to act as a hydrogen-donating antioxidant. The proposed mechanism is based on the isolation of the catechol intermediate arucadiol (AD) in methyl oleate test systems. AD was also observed in a bulk soybean oil oxidation experiment, which supports the observation that RQ is converted to AD during oxidation of the oil. Because AD was found in both light-induced oxidation and autoxidation test systems, the antioxidant mechanism proceeds in a similar manner. The antioxidant activities of RQ and AD were not significantly different in the autoxidation experiments, while AD was a significantly better ($P < 0.05$) antioxidant than RQ in the light-induced oxidation.

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KEY WORDS: Arucadiol, autoxidation, hydrogen-donating mechanism, light-induced oxidation, rosmariquinone.

Rosmariquinone (RQ) belongs to a family of compounds known as phenanthrenediones (Scheme 1) and was first isolated from the roots of *Salvia miltiorrhiza* Bunge but was labeled miltirone (1). Houlihan *et al.* (2) later extracted miltirone from *Rosmarinus officinalis* L. (rosemary) and named the compound RQ, unaware of the previous isolation and identification. Formation of RQ in the plant is believed to occur through oxidation of 6,7-dehydrocarnosic acid (3). This mechanism involves NAD^+ oxidation of the phenolic hydrogen of carnosic acid to a semiquinone molecule, followed by decarboxylation and subsequent oxidation (3). The extent to which this conversion occurs can be demonstrated by the small quantities (0.0016–0.023%) (4) of RQ identified in commercial rosemary oleoresin as compared to 2.7–26% carnosol and carnosic acid (5), indicating that the oxidation occurs at a minimal rate. Because RQ has been shown to have good antioxidant activity (AOA) in prime steam lard (2,6) and soybean oil (SBO) (4), a need for identification of the antioxidant (AO) mechanism exists for the purpose of developing better and safer AO.

Weng and Gordon (6) studied a series of Tanshen quinones, including RQ, and found that the isopropyl group was essential for maximizing AOA in lard systems. These authors carried out mechanistic studies on 9,10-phenanthrenedione and concluded, based on this compound, that RQ is a chain-breaking acceptor antioxidant (CB-A AO). Although 9,10-phenanthrenedione is structurally similar to RQ, it lacks the bulky *ortho* isopropyl group found in RQ, and its three-ring system is completely aromatic. Although this mechanism (6) cannot be completely discarded, the possibility that RQ functions by a CB-A mechanism in a food system is remote. The reason for this is that a CB-A mechanism relies on the AO's ability to interact or intercept the carbon-centered free radical prior to its interaction with oxygen. At atmospheric oxygen concentration, oxygen interacts with carbon free radicals at a much faster rate than the reaction between an AO and carbon free radicals (7). For this reason, the CB-A mechanism is probably not relevant in food systems but may contribute to the AOA under reduced oxygen systems (i.e., biological system).

Because RQ is structurally similar to a variety of tanshinone compounds (Scheme 1), the AO mechanism can be determined from the general oxidation of a tanshinone, provided that the C ring contains a C_{11}, C_{12} *o*-quinone system and that the A ring contains a saturated carbon at the C-1 position. Tanshinone II_A, a structure similar to RQ but containing a furan ring rather than an isopropyl group, was studied in a photooxidation system (8) where tanshinone II_A was subjected to intense light or sunlight for various time periods. Kusumi *et al.* (8) postulated that tanshinone II_A first absorbed ultraviolet (UV) energy, resulting in a loss of the proton at C_1 and a rearrangement to a semiquinone intermediate (Scheme 2). Further oxidation resulted in the formation of a peroxide, which rearranged into a hydroperoxide at C-1, and the final stage was the addition of a second tanshinone II_A molecule to the hydroperoxide to give a polymeric peroxide intermediate that was broken into a hydroxy tanshinone II_A compound and an anhydride compound (Scheme 2). Chang *et al.* (9) supported this mechanism by isolating several anhydride compounds from the oxidation of tanshinone II_A. A similar biochemical mechanism was reported by Gonzalez *et al.* (3) for the isolated hydroxy miltirone (10) and the C-1 carbonyl compound arucadiol (10), also labeled miltiodiol (11). Be-

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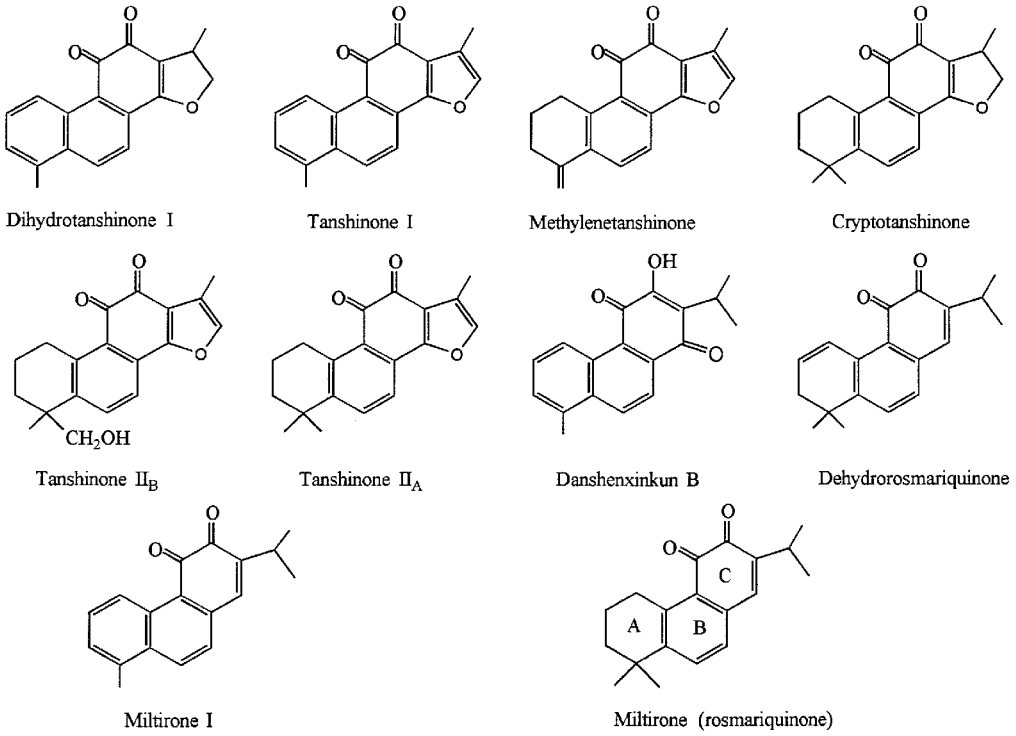


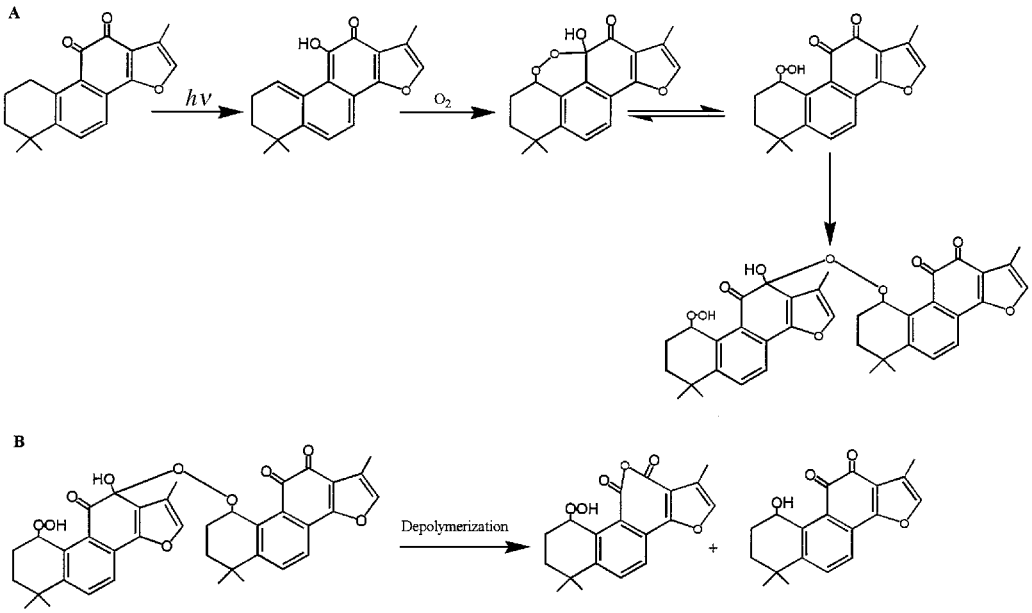
SCHEME 1

cause hydroxy miltirone and arucadiol were isolated from plant material and hypothesized to result from a biological oxidation (10), isolation of these compounds in test systems would support the AO mechanism for RQ.

The objectives of this study were to identify potential mechanisms of RQ in light-induced oxidation and autoxidation systems containing either stripped SBO or purified methyl oleate. Another goal was to determine whether isolated intermediates were antioxidants.

EXPERIMENTAL PROCEDURES

RQ model studies. RQ (100 mg; 0.35 mmol) was added to methyl oleate (5 g; 16.86 mmol) and subjected to one of the



SCHEME 2

following conditions: (i) fluorescent radiation, (ii) rose bengal (1.5 mmol; a photosensitizer) and fluorescent radiation, or (iii) rose bengal (1.5 mmol), molecular oxygen and fluorescent radiation. A second model study included the addition of RQ (500 mg; 1.77 mmol) to methyl oleate (5 g; 16.86 mmol) and was stored in a 60°C oven for 65 d.

All reactions were monitored by thin-layer chromatography (TLC) and exposed to the appropriate conditions until TLC indicated that RQ had been consumed by the reaction conditions. The methyl oleate and RQ mixtures were separated using flash chromatography on silica gel, eluted with hexane/ethyl acetate (100:0; 98:2; 95:5; 90:10; 80:20) and methanol (100%), and collected in 25-mL fractions. This method proved successful for the separation of RQ, arucadiol (AD), and the oxidized methyl oleate.

^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded on a 300-MHz NMR instrument (Oxford Instruments, Palo Alto, CA; 300 MHz for ^{1}H, 75 MHz for ^{13}C). Chemical shift data were reported as parts per million (δ) and referenced to the residual chloroform peak of deuteriochloroform (δ 7.25 ppm) for ^{1}H NMR and to the center peak of the residual chloroform triplet (δ 77.0 ppm) for ^{13}C NMR. All OH functional groups were confirmed using deuterium oxide. Infrared (IR) absorption spectra were recorded on an Analect model RFX-30 FT-IR (Analect Instruments, Inc., Irvine, CA) spectrophotometer with a potassium bromide cell. UV data were collected using a Milton Roy Genesys 5 UV/VIS spectrophotometer [Spectronic Instruments, Inc. (formerly Milton Roy Co.), Rochester, NY]. Mass spectra were obtained using a Hewlett-Packard (Wilmington, DE) 5790 gas chromatography–mass spectrometry (GC–MS) system and a Chiral-val column (Alltech, State College, PA) using both chemical and electron impact ionization. Uncorrected melting point determinations were completed in open pyrex capillary tubes and a Mel-Temp apparatus (Fisher Scientific, St. Louis, MO). All flash column chromatography was completed using E. Merck silica gel 60 (Aldrich, Milwaukee, WI) and TLC on commercial silica gel plates (Analtech Silica HLF 250 m; Analtech, Inc., Newark, DE).

Analysis of soybean oil components. Tocopherol (α, δ), β-carotene, and chlorophyll standards were obtained from Sigma Chemical Co. (St. Louis, Mo). Gamma (γ)-tocopherol was obtained from Eastman Chemical Products Inc. (Kingsport, TN). The tocopherols were determined by high-performance liquid chromatography (12). Chlorophyll and β-carotene were spectrophotometrically analyzed by Association of Official Analytical Chemists (AOAC) methods (13,14) using the modifications of Hall and Cuppett (15).

Antioxidants. Tertiary butylhydroquinone (TBHQ) was obtained from Eastman Chemical Products Inc. (Kingsport, TN). RQ was synthesized in this laboratory by a modified method of Lee *et al.* (16), which included optimization of the reactants. Arucadiol, the red crystalline compound from the model systems, isolated as previously described, was also tested. All compounds were tested at the 200 ppm level.

Stripping of SBO. Commercial SBO was purchased from a local supermarket and stored in the dark at −18°C until needed. The oil was stripped using the modified method of Hall *et al.* (4), which included a batch process rather than a column (17). The stripping process continued until tocopherols, chlorophyll, and carotenoids were no longer detected. Solvent was evaporated under vacuum at 30°C, and the stripped SBO was stored at −18°C for no longer than four weeks.

Oxidation of bulk soybean oils. Stripped SBO (100 g) was weighed into 110-mL glass jars. Rosmariquinone, arucadiol, and TBHQ were added separately to stripped SBO at a concentration of 200 ppm (0.02%). Each sample was thoroughly mixed to assure complete dispersion of the antioxidants. TBHQ served as the positive control, and an untreated sample served as the negative control.

The jars were randomly placed under two, 15-W cool fluorescent lamps at an illuminance of 4200 lx of fluorescent radiation at 25 ± 1°C, or they were placed in an oven (60°C). Peroxide values (PV) were analyzed every 24 h (12 h when necessary), until a peroxide level of 20 meq/kg was reached in the stripped oil, using the American Oil Chemists' Society (18) official methods.

Statistical Analysis. All studies were completed in triplicate and analyzed by analysis of variance (ANOVA) with Statistical Analysis System (SAS) software (19). Least significant differences (20) were determined between the mean values of the treatments at the 95% confidence level ($P < 0.05$).

RESULTS AND DISCUSSION

Spectral data. Arucadiol (miltiodiol) was purified from the crude reaction mixtures of the model test systems to give a red-orange crystal: IR (KBr) λ_{max}: 3471, 2957, 2925, 2866, 1633, 1590, 1506, 1461, 1416, 1320, 1177, 1031, 965 cm^{-1}; ^{1}H NMR: δ 10.65 (*s*, 1 H, OH), 7.95 (*d*, *J* = 7.97 Hz, 1H), 7.32 (*d*, *J* = 7.97 Hz, 1 H), 7.25 (*s*, 1 H), 6.90 (*s*, 1 H, OH), 3.24 (*sept*, *J* = 6.82, 1 H), 2.90 (*t*, *J* = 6.82 Hz, 2 H), 2.10 (*t*, *J* = 6.82 Hz, 2 H), 1.45 (*s*, 6 H), 1.35 (*d*, *J* = 7.97, 6 H), (Fig. 1B); ^{13}C NMR: δ 204.3, 158.2, 144.9, 138.1, 137.7, 136.5, 127.8, 125.3, 120.3, 120.2, 118.6, 36.2, 35.9, 35.4, 29.6 (2 C), 27.6, 22.2 (2 C) (Fig. 2B); low-resolution MS (chemical ionization, methane): *m/z* 298.1 (M), 299.1 (M + 1), 300.1 (M + 2); UV-Vis (methanol): λ_{max} (ε) 275 (17,730), 410 (2184).

Evaluation of model test systems. All methyl oleate–RQ model systems gave two major oxidation compounds: several oxidized methyl oleate products and AD. Two minor compounds were also isolated but not characterized. The AD structure was characterized by IR, UV/Vis, NMR, and mass spectrometry and compared to the spectral data of RQ. The spectral data of AD were identical to previously reported data (10,11). The infrared spectrum of AD had a broad peak at 3471 cm^{-1}, indicating the presence of a hydroxyl group. This peak was lacking, as expected, for RQ, and the increase in the molecular weight by 16 *m/z* on the mass spectrum indicated the addition of an oxygen atom. UV/Vis λ_{max} had shifted from 257 nm in RQ to 275 nm in AD, indicating the presence of phenolic hydrogens at C-11 and C-12 positions. The phe-

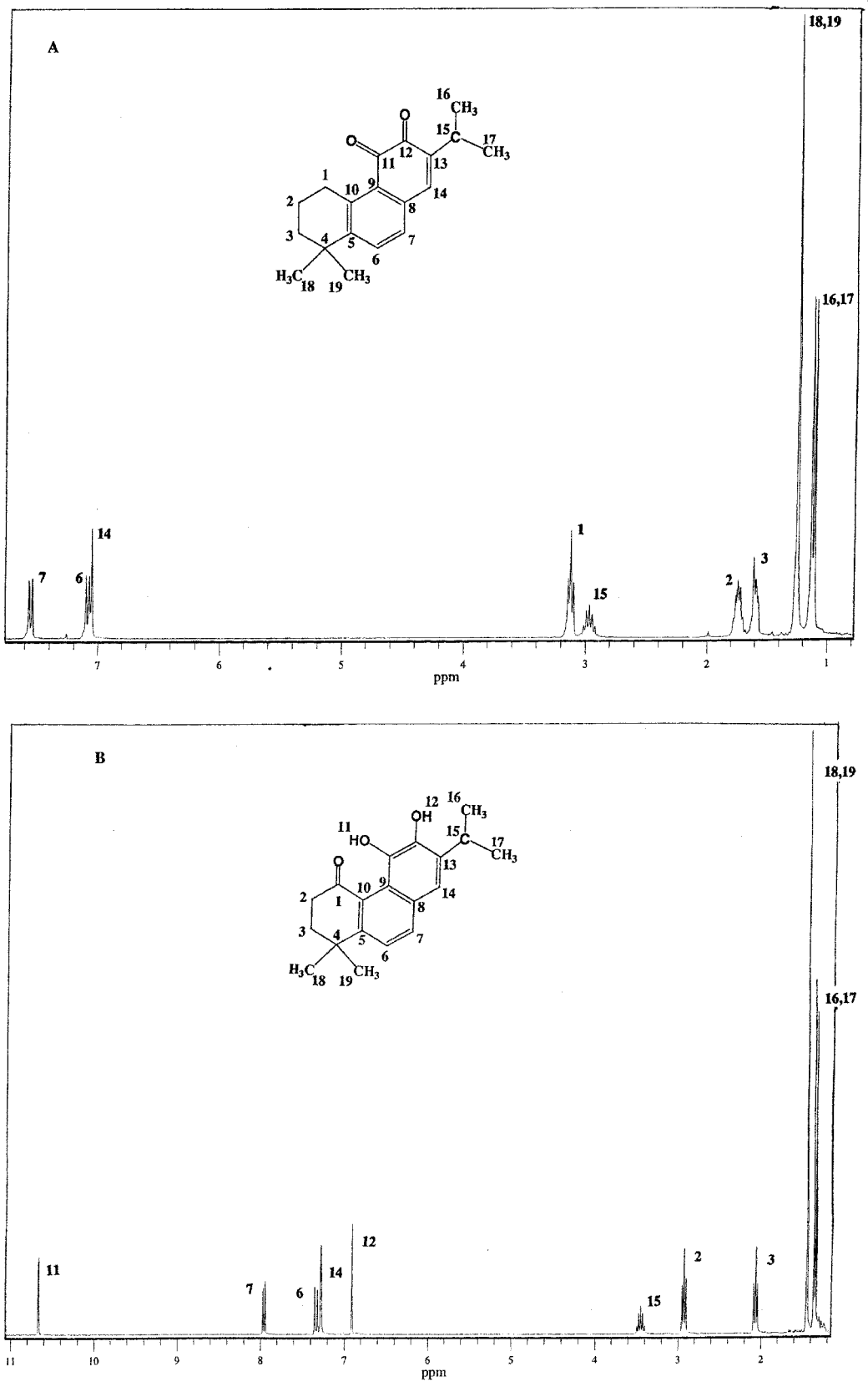


FIG. 1. ^{1}H nuclear magnetic resonance (NMR) spectra for rosmariquinone (A) and arucadiol (B).

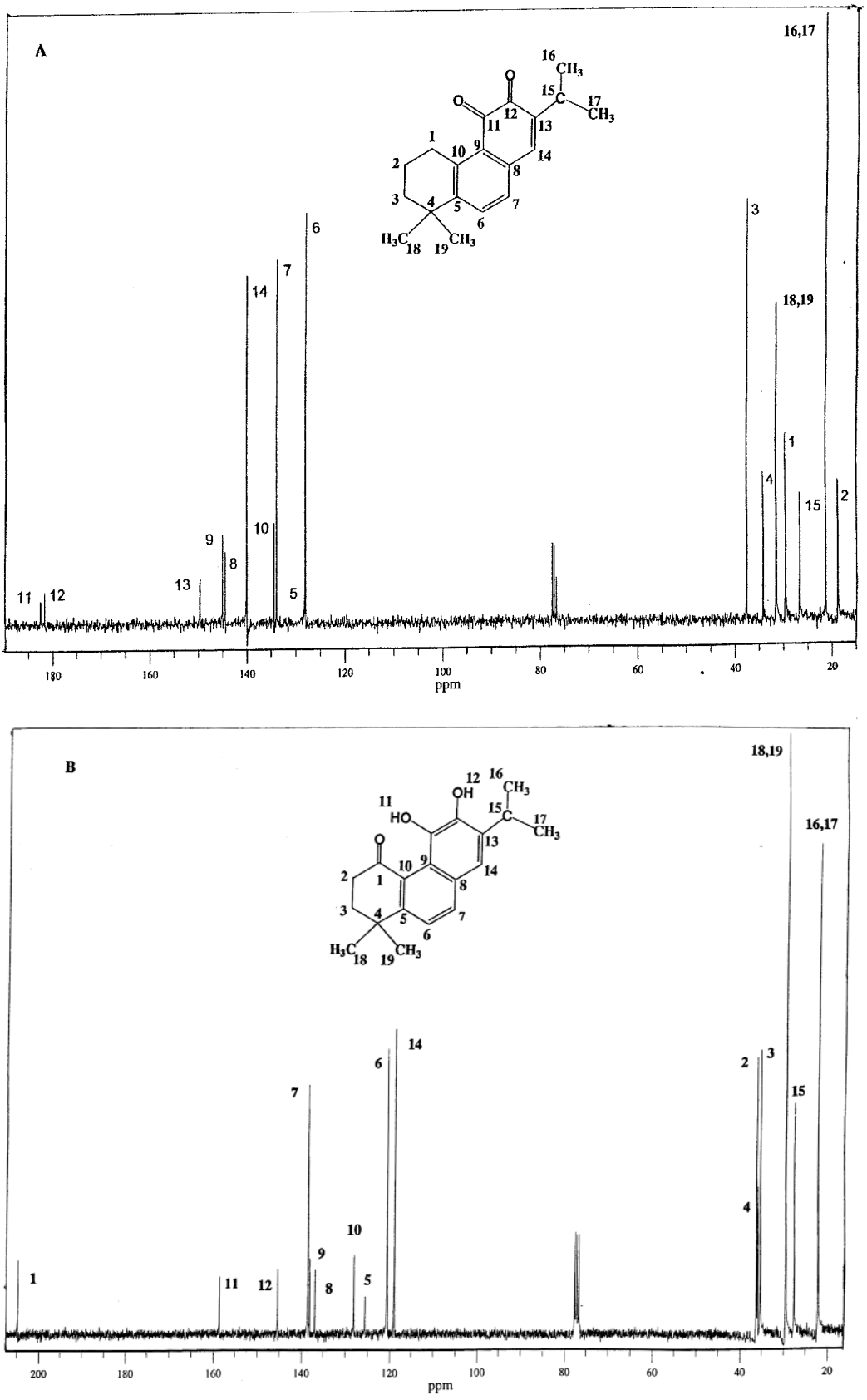


FIG. 2. ^{13}C NMR spectra for rosmariquinone (A) and arucadiol (B). See Figure 1 for abbreviation.

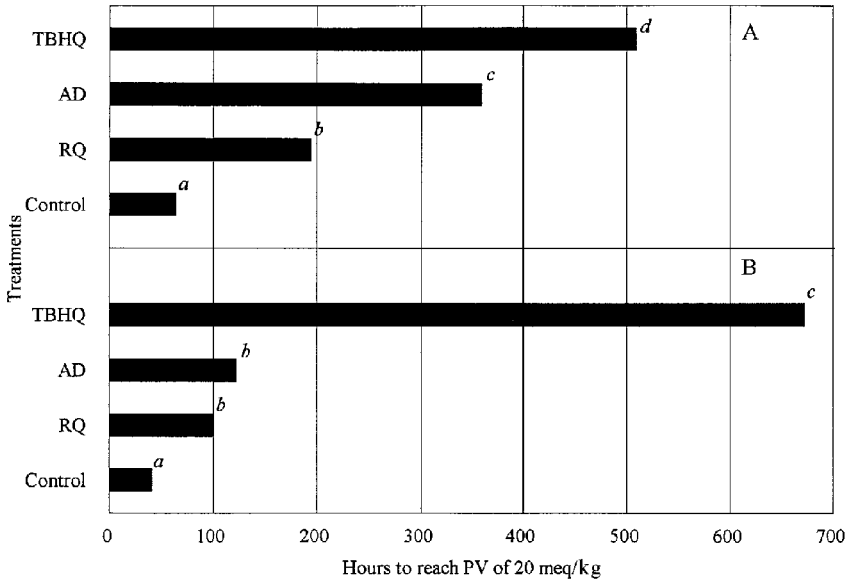


FIG. 3. Number of hours in which arucadiol (AD), rosmariquinone (RQ), and tertiary butylhydroquinone (TBHQ) delay the oxidation of stripped soybean oil to a 20 meq/kg level in photooxidation (A) or autoxidation (B). Letters *a–d* represent significant differences ($P < 0.05$) between treatments.

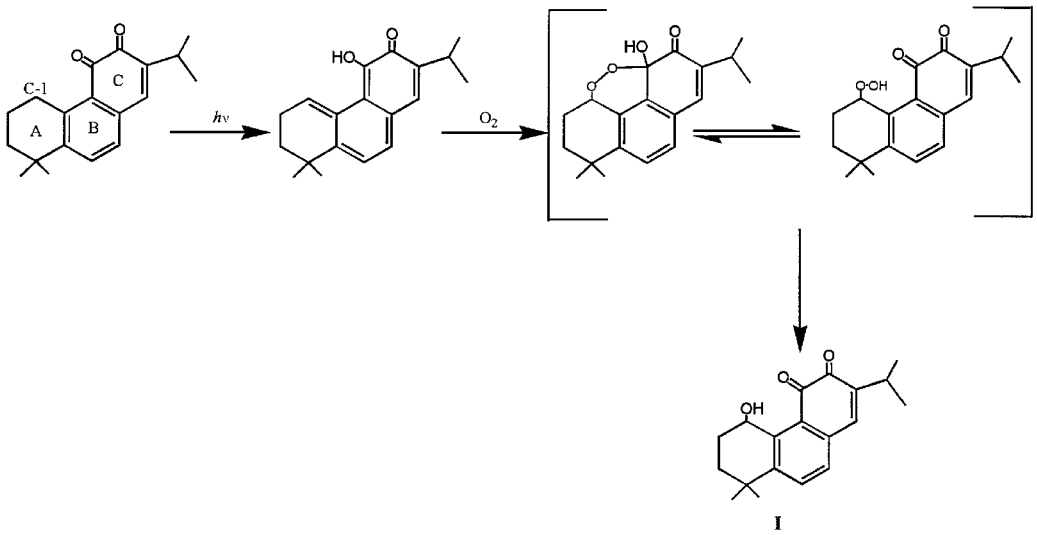
nolic property was also confirmed by the treatment of AD with sodium hydroxide and the observation that λ_{max} shifted from 275 to 254 nm.

The ^{1}H NMR (Fig. 1) of RQ and AD had several characteristic proton signal changes that supported the AD structure. The loss of the triplet signal at δ 3.14 ppm indicated that the C-1 protons were lost during the oxidation process. Because the two protons were lost at the C-1 position and the mass spectrum indicated an addition of an oxygen atom, the C-1 position can be characterized as being a carbonyl. The addition of singlets at δ 10.65 and 6.90 ppm indicated the presence of two hydroxyl protons, which was supported by the disappearance of the singlet peaks after a deuterium oxide exchange experiment (data not shown). The C-11 proton is believed to be hydrogen-bonded to the C-1 carbonyl, thus causing the downfield shift of the phenolic proton to 10.65 ppm. The C-6, C-7, and C-14 aromatic protons are also shifted downfield in comparison to their position in RQ (Fig. 1). The adjacent C-2 and C-3 protons are represented by two triplets at 2.90 and 2.10 ppm**,** respectively**.**

The ^{13}C NMR spectra of AD and RQ can also be easily distinguished. Arucadiol has only one carbonyl (δ 204 ppm), and the two hydroxyl-bearing carbons can be found at δ 158 and 145 ppm (Fig. 2). In contrast, RQ has two carbonyl carbons at δ 182 and 181 ppm and lacks the carbonyl at δ 204 ppm. All other carbons are similar between AD and RQ. The identification of AD has been clearly established by the spectral data and is supported by the literature.

The model RQ-methyl oleate system indicates that RQ was converted to an active AO (i.e., AD) under oxidation conditions. The AOA of AD was confirmed in the light-induced oxidation of bulk SBO (Fig. 3A). Arucadiol significantly (359 h; $P < 0.05$) improved the stability of the stripped SBO when compared to RQ (194 h). However, TBHQ had the best ($P < 0.05$) AOA and delayed oxidation to the 20 meq/kg level for 500 h. Under autoxidation conditions (Fig. 3B), AD (123 h) was not significantly ($P > 0.05$) different from RQ (99 h) at controlling the oxidation of the stripped SBO. AD and RQ had AOA values that were far less than TBHQ. The isolation of AD from the methyl oleate-RQ model systems suggests that the AOA of RQ was primarily a hydrogen-donating mechanism and relied on the ability of RQ to convert to AD.

The proposed mechanism is hypothesized as being similar to that of oxidized tanshinone II_A (8,21). The suggested mechanism (Scheme 3) follows the process where the C-11 carbonyl undergoes enolization to form a semiquinone intermediate and an endoperoxide, which converts to the C-1 hydroperoxy quinone. The hydroperoxy quinone intermediate is unstable and can undergo degradation, rather than dimerization, as proposed by Kusumi *et al.* (8), to the C-1 hydroxy compound hydroxymiltirone (**I**; Scheme 3). If dimerization had occurred in the model system, a dimer product would have been detected. Also, dimerization is a relatively slow process in comparison to the attack of the hydroperoxy quinone by the more abundant and electronically similar RQ; thus, it is more likely that the C-1 hydroperoxide quinone compound interacts with RQ to create the additional hydroperoxy quinone and the C-1 hydroxy quinone (i.e., hydroxymiltirone; Scheme 3). Hydroxymiltirone can rearrange to give a C-1 hydroxyl, C-11,12-semiquinone intermediate (Scheme 4A). Subsequent loss of the C-1 hydroxyl hydrogen and rearrangement of the aromatic ring electrons and tautomerization of the C-12 quinone gives arucadiol (**II**; Scheme 4A). The final step of the suggested mechanism follows hydrogen donation to the lipid peroxy radical (Scheme 4B). A



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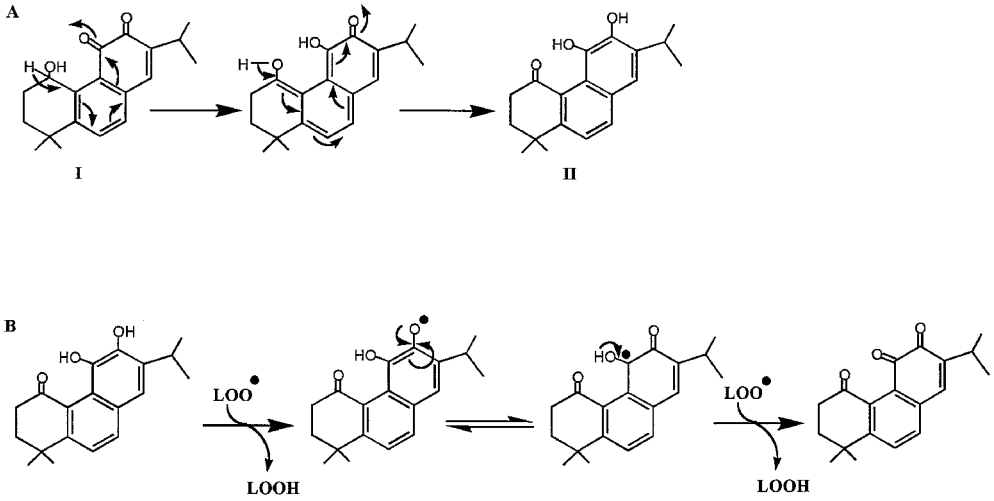

SCHEME 4

second hydrogen donation can also occur through a rearrangement process.

Although other AO mechanisms may be responsible for the AOA of RQ, this study suggests that the AOA of RQ follows a hydrogen-donating mechanism as the primary AO mechanism in both light-induced oxidation and autoxidation of bulk SBO. A previous observation (17) showed that RQ and tocopherols acted synergistically in autoxidation, and this, in combination with the observation that tocopherol regeneration also occurred in this system, suggests a hydrogen-donating mechanism (17).

REFERENCES

1. Hayashi, T., H. Kakisawa, H.-Y. Hsu, and Y. Chen, The Structure of Miltirone, A New Diterpenoid Quinone, *Chem. Comm. 1*:299 (1970).
2. Houlihan, C., C.-T. Ho, and S. Chang, The Structure of Rosmariquinone—A New Antioxidant Isolated from *Rosmarinus officinalis* L., *J. Am. Oil Chem. Soc. 62*:96–98 (1985).
3. Gonzalez, A., L. Andres, Z. Aguiar, and J. Luis, Diterpenes from *Salvia mellifera* and Their Biogenetic Significance, *Phytochemistry 31*:1297–1305 (1992).
4. Hall III, C., S. Cuppett, D. Wheeler, and X. Fu, Effects of Bleached and Unbleached Rosemary Oleoresin and Rosmariquinone on Light-Sensitized Oxidation of Soybean Oil, *J. Am. Oil Chem. Soc. 56*:668–671; *71*:533–535 (1994).
5. Cuvelier, M.-E., C. Berset, and H. Richard, Antioxidant Constituents in Sage (*Salvia officinalis*), *J. Agric. Food Chem. 42*:665–669 (1994).
6. Weng, C., and M. Gordon, Antioxidant Activity of Quinones Extracted from Tanshen (*Salvia Miltiorrhiza* Bunge), *J. Agric. Food Chem. 40*:1331–1336 (1992).
7. Bolland, J.L., and P. Ten Have, Kinetic Studies in the Chemistry of Rubber and Related Materials. IV. The Inhibitory Effect of Hydroquinone on the Thermal Oxidation of Ethyl Linoleate, *Trans. Faraday Soc. 42*:201–210 (1947).

8. Kusumi, T., T. Kishi, H. Kakisawa, and T. Kinoshita, Photooxidation of Tanshinone II (6, 7, 8, 9-Tetrahydro-1, 6, 6-trimethylphenanthro[1,2-*b*]furan-10,11-dione), *J. Chem. Soc., Perkin I.* 1716–1718 (1976).
9. Chang, H., T. Choang, K. Chui, P. Hon, C. Lee, T. Mak, and H. Wong, Novel Constituents of Chinese Drug Danshen (*Salvia miltiorrhiza* Bunge): Isolation of (6, 7, 8, 9-tetrahydro-1, 6, 6-trimethylfuro[3, 2-*c*]naphth[2, 1-e]oxepine-10,12-dione) and its Derivatives, Secondary Metabolites Produced by Photooxidation Reactions, *J. Chem. Res. (S),* 114–115 (1990).
10. Michavila, A., M. De La Torre, and B. Rodriguez, 20-Nor-abietane and Rearranged Abietane Diterpenoids from the Root of *Salvia argentea, Phytochemistry 25*:1935–1937 (1986).
11. Ginda, H., T. Kusumi, M. Ishitsuka, H. Kakisawa, Z. Weijie, C. Jun, and G.Y. Tain, Salviolone, A Cytotoxic Bisnorditerpene with a Benzotropolone Chromaphore from a Chinese Drug Dan-Shen (*Salvia Miltiorrhiza*), *Tetrahedron Letters 29*:4603–4606 (1988).
12. Carpenter, A.P., Determination of Tocopherols in Vegetable Oil, *J. Am. Oil Chem. Soc. 56*:668–671 (1979).
13. *Official Methods Analysis of the Association of Official Analytical Chemists*, edited by S. Williams, Association of Official Analytical Chemists, Washington, D.C., 1984, Method Section 3.140.
14. *Ibid.*, Method Section 43.017.
15. Hall III, C., and S. Cuppett, Effects of a Bleached and Unbleached Rosemary Oleoresin on Light-Sensitized Oxidation of Soybean Oil, *J. Amer. Oil Chem. Soc. 70*:477–482 (1993).
16. Lee, J., H. Mei, and J. Snyder, Synthesis of Miltirone by an Ultrasound-Promoted Cycloaddition, *J. Org. Chem. 55*:5013–5016 (1990).
17. Hall III, C., in *A Mechanistic Study To Determine And Define The Antioxidant Activity Of Rosmariquinone*. Ph. D. Dissertation, University of Nebraska-Lincoln, 1996, pp. 143–145.
18. AOCS Official Method Cd-8-53 Peroxide Value, *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th edn., edited by D. Firestone, American Oil Chemists' Society, Champaign, 1989.
19. Statistical Analysis System (SAS), SAS Institute, Inc., Cary, North Carolina (1985).
20. Steel, R., and J. Torrie, in *Principles And Procedures Of Statistics. A Biometrical Approach*, McGraw-Hill Book Company, New York, pp. 173–177, (1980).

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