Novel Oxidations of (+)-Catechin by Horseradish Peroxidase and Laccase

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Horseradish peroxidase (HRP; EC 1.11.1.7) catalyzed the H_2O_2-dependent oxidative coupling of (+)-catechin **1** to form three different biphenyl C–C dimers **2**–**4**, whereas *Rhus vernicifera* laccase catalyzed the formation of two new catechin–hydroquinone adducts **5** and **6**. Spectroscopic evidence showed that HRP dimers were linked through position 8 of the A-ring of one catechin moiety to C-5′ of ring B in **2** and **4** and to C-2 of ring C in **3**. The unusual catechin dicarboxylic acid dimer **4** was obtained by ortho cleavage of the E-ring. Hydroquinone served as both a shuttle oxidant and a reactant by coupling at C-2′ and C-5′ of the catechin B-ring during laccase oxidations. HRP and laccase oxidation products were compared to D,L-α-tocopherol and (+)-catechin for their abilities to inhibit iron-induced lipid peroxidation in rat brain homogenates and Fe^{3+}-ADP/NADPH in rat liver microsomes, as measured by the intensity of thiobarbituric acid reactive substance. All metabolites exhibited anti-lipid peroxidation with IC_{50} values ~2–8 times higher than those of standard compounds. Characteristic reaction products may prove to be novel markers for (+)-catechin antioxidant reactions in living systems.

KEYWORDS: (+)-Catechin; oxidations; HRP/H_2O_2; laccase/hydroquinone; antioxidant; lipid peroxidation

INTRODUCTION

Catechins are the basic structural units of condensed tannins. They belong to the class of flavan-3-ols and are found in a wide variety of vegetables, herbs, and teas (*1*). Catechins are protective against cancer and inflammatory and cardiovascular diseases (*2*), mainly because of their antioxidant activities and abilities to scavenge free radicals (*3*).

As polyphenols, catechins are susceptible to enzymatic and nonenzymatic oxidations giving rise to a variety of dimeric, oligomeric, and polymeric products (*4, 5*). Oxidations of phenols by polyphenol oxidase (PPO) (*6*), hydrogen peroxide (H_2O_2)-dependent peroxidases (*7, 8*), and copper oxidases (*7, 8*) have been described. PPO, which is involved in the biosynthesis of lignin, tannin, and melanin (*9*) catalyzes the oxygen-dependent coupling of phenols responsible for browning and undesirable hazes in fruit juices (*10*), where semi-quinone radicals are common initial phenolic oxidation products (*8–10*).

Characterization of catechin oxidation products has revealed their reactive structural features and permitted the deduction of pathways for their formation. PPO oxidation (*6*) and autoxidation (*11*) of (+)-catechin both gave biaryl and biaryl ether dimers. Polymers of (+)-catechin are usually formed by couplings between the A-ring of one unit and the B-ring of another in a so-called "head to tail" polymerization process (*6*). 8-Hydroxy-(+)-catechin (*12*) and catechin dimers (dehydrodicatechins) were obtained by both H_2O_2-dependent peroxidase (*13*) and chemical oxidation of (+)-catechin under alkaline conditions (*14*). Structurally, dehydrodicatechins differ from one another by the biphenyl or phenyl ether (C–C or C–O–C) types of linkage, the position of the interflavan linkage, and the relative conformations of biphenyl or biphenyl ether moieties (*6*). When co-oxidations of phloroglucinol and (+)-catechin were conducted with mushroom tyrosinase, a novel carbon–carbon-coupled adduct was obtained with linkages of phloroglucinol to the 6′-position of (+)-catechin (*15*).

As an extension of our ongoing studies on the chemical, enzymatic, and microbiological transformations of natural antioxidants (*16–20*), we investigated the oxidation of (+)-catechin by horseradish peroxidase (HRP)–hydrogen peroxide and laccase with hydroquinone as a shuttle oxidant (*21*). We describe five new enzyme oxidation products **2**–**6** that were isolated from preparative scale reactions and spectroscopically characterized. The antioxidant properties of these compounds versus lipid peroxidation in rat liver homogenate and rat liver microsomes were determined.

MATERIALS AND METHODS

General Experimental Procedures. (+)-Catechin, HRP [donor; hydrogen peroxide oxidoreductase, EC 1.11.1.7, type VIA, Sigma, 300 purpurogallin units/mg (*22*)], laccase [benzenediol:oxygen oxidoreductase, EC 1.10.3.2, from *Rhus vernicifera*, 130 units/mg of protein) (*23*)], hydrogen peroxide 30% w/v solution, 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), adenosine 5′-diphosphate sodium salt (ADP), nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), sodium dodecyl sulfate (SDS), $FeCl_3$, and $FeSO_4$ were

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purchased from Sigma Chemical Co. (St. Louis, MO). The purity of (+)-catechin was determined by TLC and ^{1}H NMR spectral analysis. Coomassie Plus protein assay reagent and albumin standard (bovine serum album fraction V, 2 mg/mL in a 0.9% aqueous NaCl solution containing sodium azide) were obtained from Pierce (Rockford, IL). D,L-α-Tocopherol was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals used were of analytical reagent grade.

Flash column liquid chromatography was performed with 40-μm silica gel (Baker) and Sepralyte C_{18} (40 μm) as the stationary phase. TLC was carried out on precoated silica gel 60 F_{254} (Merck) plates. TLC plates were developed using one of the following systems (prepared in volumetric ratios): (A) $CHCl_3$/MeOH/H_2O (80:20:20), (B) Me_2CO/toluene/HCOOH (4:3:1), or (C) benzene/EtOAc/HCOOH (1: 7:1). Developed chromatograms were visualized by fluorescence quenching under 254-nm UV light and by spraying with methanolic $FeCl_3$ or anisaldehyde–sulfuric acid and warming with a heat gun for 3 min.

Optical rotations were measured with a JASCO P-1020 polarimeter. UV spectra were determined with a Hitachi 340 spectrophotometer. IR spectra (cm^{-1}) were obtained using a Nicolet 205 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were obtained with a Bruker NMR 400 (Bruker Instruments, Billerica, MA), operating at 400 MHz (^{1}H) and 100 MHz (^{13}C). DQF-COSY, ROESY, HMBC, and HMQC NMR experiments were carried out using a Bruker AMX-600 high-field spectrometer equipped with an IBM Aspect-2000 processor and with software VNMR version 4.1. All NMR spectra were obtained in acetone-d_6 using TMS as the internal standard, with chemical shifts expressed in parts per million (δ) and coupling constants (J) in hertz. Electron impact mass spectra (EIMS) were obtained with a Voyager MD (ThermoQuest, Manchester, U.K.). FABMS spectra were performed with a Fisons VG-ZAB-HF reversed geometry (BE configuration, where B is a magnetic sector and E is an electrostatic analyzer) mass spectrometer (MS) (VG Analytical, Manchester, U.K.).

Enzyme Reactions. Analytical scale incubations of (+)-catechin **1** were carried out in 10 mL of pH 7.4, 0.1 M KH_2PO_4 buffer, in stainless steel-capped, 125-mL DeLong flasks containing 5 mg of (+)-catechin, 1 mg of HRP, and 0.8 mL of 0.4 mM H_2O_2. Incubations of H_2O_2 and **1** and incubations of H_2O_2 and HRP without **1** were used as controls. The reaction mixtures were incubated with shaking at 250 rpm at 28 °C, and 1-mL samples were withdrawn at 15-min intervals. After determination of pH, they were acidified to pH 3.0 with 6 N HCl and extracted with equal volumes of EtOAc/n-BuOH 9:1. Aliquots of 30 μL of extracts were spotted on TLC plates developed with solvent systems A, B, and C, and chromatograms were visualized by fluorescence quenching under 245-nm UV light and spraying developed plates with methanolic $FeCl_3$ or anisaldehyde–sulfuric acid and warming with a heat gun for 3 min. Under these conditions **1** was completely converted to **2**, **3**, and **4** within 3 h. Analytical scale formation of **5** and **6** with *R. vernificera* laccase involved incubations of 5 mg of **1** and 5 mg of hydroquinone with 2.5 units of laccase in 10 mL of pH 7.4, 0.2 M KH_2PO_4 buffer at room temperature. Incubations of hydroquinone and **1** with boiled enzyme and incubations of hydroquinone and laccase without **1** were used as the experimental controls. Incubations were conducted as above; **1** was completely converted to **5** and **6** within 1 h.

Preparative Scale Oxidations of (+)-Catechin with HRP and Laccase. A total of 250 mg of **1** and 50 mg of HRP (15000 units) were added to 500 mL of pH 7.4, 0.1 M KH_2PO_4 buffer together with 40 mL of 0.4 mM H_2O_2. The reaction mixture was divided among 20 125-mL DeLong flasks and incubated with shaking for 3 h. The reaction mixtures were combined, adjusted to pH 3.0 with 6 N HCl, and extracted three times each with 500 mL of a mixture of EtOAc/n-BuOH, 9:1. The organic extracts were washed with distilled water, dried over anhydrous sodium sulfate, and vacuum-concentrated to yield brown residues of 220 mg consisting of a mixture of **2**, **3**, and **4**.

Reactions with laccase were conducted by suspending 250 mg of (+)-catechin and 250 mg of hydroquinone in 500 mL of pH 7.4, 0.1 M KH_2PO_4 buffer with 3125 units of laccase and dividing the reaction mixtures among 20 125-mL DeLong culture flasks. Reactions were incubated for 1 h. The contents of reaction flasks were combined as before, adjusted to pH 3.0 with 6 N HCl, and extracted three times

each with 500 mL of a mixture of EtOAc/n-BuOH, 9:1. After a washing with distilled water and drying of the organic phase with anhydrous Na_2SO_4, the solvent was removed under vacuum to afford 201 mg of a brown residue containing **5** and **6**.

Isolation of Enzyme Products. The extracts were separately subjected to column chromatography over a 50 cm × 1.5 cm high-porosity polystyrene gel, Diaion HP-20 (Mitsubishi Kasei Co., Ltd., Tokyo, Japan). Elution was started with H_2O and then continued with 50% MeOH and then finally with MeOH. Chromatography of the 50% MeOH eluates (166 mg from HRP and 132 mg from laccase) over a 65 cm × 1.5 cm Sephadex LH-20 (25–150 μm) column (Pharmacia Fine Chemical Co.) was accomplished by using $CHCl_3$/MeOH (90:10 → 30:70). Three samples, **A** (6 mg), **B** (86 mg), and **C** (55 mg), from HRP and two samples, **E** (17 mg) and **F** (96 mg), from laccase were obtained by combining chromatographically similar column fractions. Subsequent purification of chromatographically similar fractions was accomplished by a sequence of reversed phase, Sepralyte C_{18} flash column chromatography (1.5 × 50 cm) using an H_2O/MeOH gradient solvent system (30 → 60%) at a flow rate of 3 mL/min; Si gel, flash column chromatography using $CHCl_3$/MeOH/H_2O (80:20:2 → 60:40: 10); and finally Sephadex LH-20 column chromatography using gradients of $CHCl_3$ in MeOH ranging from 30 to 70% to afford **2** (11.5 mg, 4.6% yield), **3** (13 mg, 5.2% yield), and **4** (10 mg, 4% yield) from the HRP preparative scale reaction and **5** (15 mg, 6% yield) and **6** (13.5 mg, 5.4% yield) from the laccase preparative scale reaction.

2 was obtained as a colorless amorphous powder (11.5 mg): $[\alpha]^{22}_D$ +64.9 (c 1.00, MeOH); UV (MeOH) λ_{max} (log ϵ) 280 (4.52), 330 (4.22) nm; ^{1}H NMR (acetone-d_6, 600 MHz) δ 5.23 (1H, J = 7.9 Hz, H-2), 4.69 (1H, m, H-3), 2.97 (1H, dd, J = 16.2, 5.8 Hz, H-4$_{eq}$), 2.74 (1H, dd, J = 16.2, 9.2 Hz, H-4$_{ax}$), 6.19 (1H, d, J = 2.5 Hz, H-6), 5.98 (1H, d, J = 2.5 Hz, H-8), 6.96 (1H, d, J = 1.1 Hz, H-2′), 7.12 (1H, d, J = 1.1 Hz, H-6′), 5.06 (1H, J = 7.5 Hz, H-2″), 4.29 (1H, m, H-3″), 2.87 (1H, dd, J = 16.0, 5.7 Hz, H-4″$_{eq}$), 2.71 (1H, dd, J = 16.0, 8.7 Hz, H-4″$_{ax}$), 6.29 (1H, s, H-6″), 7.43 (1H, d, J = 2.0 Hz, H-2‴), 7.20 (1H, d, J = 8.0 Hz, H-5‴), 7.26 (1H, dd, J = 8.0, 2.0 Hz, H-6‴); ^{13}C (acetone-d_6, 100 MHz) δ 81.74 d (C-2), 68.39 d (C-3), 29.60 t (C-4), 157.33 s (C-5), 96.47 d (C-6), 158.55 s (C-7), 96.0 d (C-8), 156.10 (C-9), 102.35 (C-10), 134.20 s (C-1′), 113.92 d (C-2′), 145.85 s (C-3′), 149.40 s (C-4′), 126.74 s (C-5′), 119.22 d (C-6′), 82.18 d (C-2″), 69.50 d (C-3″), 28.78 t (C-4″), 156.55 s (C-5″), 96.88 d (C-6″), 157.10 s (C-7″), 110.80 s (C-8″), 152.78 s (C-9″), 100.83 s (C-10″), 134.35 s (C-1‴), 113.55 d (C-2‴), 146.60 s (C-3‴), 146.72 s (C-4‴), 117.00 d (C-5‴), 118.10 d (C-6‴); HRFABMS, m/z 579.1507 [M + H]$^+$ (calcd for $C_{30}H_{27}O_{12}$, 579.1505).

3 was obtained as a colorless amorphous powder (13 mg): $[\alpha]^{22}_D$ +86.2 (c 0.88, MeOH); UV (MeOH) λ_{max} (log ϵ) 283 (4.66), 332 (4.28) nm; ^{1}H NMR (acetone-d_6 600 MHz) δ 4.19 (1H, m, H-3), 3.02 (1H, dd, J = 15.8, 5.5 Hz, H-4$_{eq}$), 2.70 (1H, dd, J = 15.8, 9.0 Hz, H-4$_{ax}$), 6.27 (1H, d, J = 2.2 Hz, H-6), 6.13 (1H, d, J = 2.2 Hz, H-8), 6.94 (1H, d, J = 1.7 Hz, H-2′), 6.80 (1H, d, J = 8.2 Hz, H-5′), 6.74 (1H, dd, J = 8.2, 1.7 Hz, H-6′), 5.10 (1H, d, J = 7.5 Hz, H-2″), 4.35 (1H, m, H-3″), 2.87 (1H, dd, J = 16.5, 5.8 Hz, H-4″$_{eq}$), 2.65 (1H, dd, J = 16.5, 9.3 Hz, H-4″$_{ax}$), 6.32 (1H, s, H-6″), 7.25 (1H, d, J = 1.5 Hz, H-2‴), 6.87 (1H, d, J = 8.0 Hz, H-5‴), 7.11 (1H, dd, J = 8.0, 1.5 Hz, H-6‴); ^{13}C (acetone-d_6, 100 MHz) δ 86.58 s (C-2), 67.79 d (C-3), 28.82 t (C-4) 158.30 s (C-5), 96.95 d (C-6), 159.48 s (C-7), 96.18 d (C-8), 157.70 (C-9), 103.95 (C-10), 132.83 s (C-1′), 114.80 d (C-2′), 145.64 s (C-3′), 146.62 s (C-4′), 115.74 d (C-5′), 118.12 d (C-6′), 83.33 d (C-2″), 68.25 d (C-3″), 29.18 t (C-4″), 157.13 s (C-5″), 97.58 d (C-6″), 158.50 s (C-7″), 109.65 s (C-8″), 153.70 s (C-9″), 101.96 s (C-10″), 133.10 s (C-1‴), 114.28 d (C-2‴), 146.18 s (C-3‴), 146.95 s (C-4‴), 116.60 d (C-5‴), 118.58 d (C-6‴); HRFABMS, m/z 579.1508 [M + H]$^+$ (calcd for $C_{30}H_{27}O_{12}$, 579.1505).

4 was obtained as a colorless amorphous powder (10 mg): $[\alpha]^{22}_D$ +102.5 (c 0.74, MeOH); UV (MeOH) λ_{max} (log ϵ) 286 (4.52), 335 (4.12) nm; ^{1}H NMR (acetone-d_6, 600 MHz) δ 5.31 (1H, d, J = 7.3 Hz, H-2), 4.29 (1H, m, H-3), 2.99 (1H, dd, J = 15.6, 5.5 Hz, H-4$_{eq}$), 2.70 (1H, dd, J = 15.6, 9.0 Hz, H-4$_{ax}$), 6.30 (1H, d, J = 2.0 Hz, H-6), 6.26 (1H, d, J = 2.0 Hz, H-8), 6.92 (1H, d, J = 1.0 Hz, H-2′), 7.16 (1H, d, J = 1.0 Hz, H-6′), 5.11 (1H, J = 7.3 Hz, H-2″), 4.18 (1H, m, H-3″), 3.12 (1H, dd, J = 16.0, 5.7 Hz, H-4$_{eq}$), 2.62 (1H, dd, J = 16.0, 8.8 Hz,

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H-4$_{ax}$), 6.41 (1H, s, H-6″), 6.56 (1H, s, H-2‴), 7.34 (1H, d, J = 11.7 Hz, H-5‴), 6.82 (1H, d, J = 11.7 Hz, H-6‴); ^{13}C (acetone-d_6, 100 MHz) δ 82.19 d (C-2), 68.66 d (C-3), 28.90 t (C-4), 156.54 s (C-5), 96.12 d (C-6), 157.10 s (C-7), 95.36 d (C-8), 156.40 (C-9), 100.21 (C-10), 132.90 s (C-1′), 114.15 d (C-2′), 145.92 s (C-3′), 148.95 s (C-4′), 128.55 s (C-5′), 120.13 d (C-6′), 82.85 d (C-2″), 69.15 d (C-3″), 29.17 t (C-4″), 156.92 s (C-5″), 97.52 d (C-6″), 157.38 s (C-7″), 110.0 s (C-8″), 156.70 s (C-9″), 102.0 s (C-10″), 134.28 s (C-1‴), 121.23 d (C-2‴), 172.78 s (C-3‴), 176.20 s (C-4‴), 129.65 d (C-5‴), 138.12 d (C-6‴); HRFABMS, m/z 611.1403 [M + H]$^+$ (calcd for $C_{30}H_{27}O_{14}$, 611.1399).

5 was obtained as an off-white powder (15 mg): [α]$^{22}_D$ +51.6 (c 0.50, MeOH); UV (MeOH) λ_{max} (log ϵ) 2.82 (4.82), 3.30 (4.33) nm; ^{1}H NMR (acetone-d_6, 600 MHz) δ 5.17 (1H, J = 7.7 Hz, H-2), 4.49 (1H, m, H-3), 2.93 (1H, dd, J = 15.2, 5.5 Hz, H-4$_{eq}$), 2.74 (1H, dd, J = 15.2, 9.0 Hz, H-4$_{ax}$), 6.48 (1H, d, J = 2.2 Hz, H-6), 6.36 (1H, d, J = 2.2 Hz, H-8), 7.34 (1H, d, J = 1.3 Hz, H-2′), 7.48 (1H, d, J = 1.3 Hz, H-6′), 6.78 (1H, d, J = 8.5 Hz, H-3″), 6.62 (1H, dd, J = 8.5, 2.5 Hz, H-4″), 6.67 (1H, d, J = 2.5 Hz, H-6″); ^{13}C (acetone-d_6, 100 MHz) δ 82.54 d (C-2), 68.70 d (C-3), 29.24 t (C-4), 157.78 s (C-5), 96.50 d (C-6), 158.25 s (C-7), 96.13 d (C-8), 156.48 (C-9), 100.15 (C-10), 131.85 s (C-1′), 115. 0 d (C-2′), 145.25 s (C-3′), 148.72 s (C-4′), 124.63 s (C-5′), 118.83 d (C-6′), 122.56 s (C-1″), 148.30 s (C-2″), 116.90 d (C-3″), 116.18 d (C-4″), 150.10 d (C-5″), 117.20 d (C-6″); HRFABMS, m/z 399.1083 (M + H)$^+$ (calcd for $C_{21}H_{19}O_8$, 399.1080).

6 was obtained as an off-white powder (13.5 mg): [α]$^{22}_D$ +58.2 (c 0.56, MeOH); UV (MeOH) λ_{max} (log ϵ) 280 (4.65), 330 (4.50) nm; ^{1}H NMR (acetone-d_6, 600 MHz) δ 5.13 (1H, J = 7.3 Hz, H-2), 4.27 (1H, m, H-3), 2.90 (1H, dd, J = 16.5, 5.8 Hz, H-4$_{eq}$), 2.65 (1H, dd, J = 16.5, 9.2 Hz, H-4$_{ax}$), 6.78 (1H, d, J = 2.3 Hz, H-6), 6.63 (1H, d, J = 2.3 Hz, H-8), 6.95 (1H, d, J = 8.6 Hz, H-5′), 7.14 (1H, d, J = 8.6 Hz, H-6′), 6.86 (1H, d, J = 8.2 Hz, H-3″), 6.72 (1H, dd, J = 8.2, 2.6 Hz, H-4″), 6.55 (1H, d, J = 2.6 Hz, H-6″); ^{13}C (acetone-d_6, 100 MHz) δ 83.30 d (C-2), 69.56 d (C-3), 29.75 t (C-4), 156.49 s (C-5), 97.28 d (C-6), 157.0 s (C-7), 96.75 d (C-8), 155.90 (C-9), 102.18 (C-10), 134.60 s (C-1′), 122. 40 s (C-2′), 147.72 s (C-3′), 146.55 s (C-4′), 115.36 d (C-5′), 119.11 d (C-6′), 122.15 s (C-1″), 149.0 s (C-2″), 117.81 d (C-3″), 117.40 d (C-4″), 152.18 d (C-5″), 118.60 d (C-6″); HRFABMS, m/z 399.1083 [M + H]$^+$ (calcd for $C_{21}H_{19}O_8$, 399.1080).

Lipid Peroxidation in Rat Brain Homogenates. Lipid peroxides produced in rat brain homogenates in the presence of iron were measured by the thiobarbituric acid method (*24*) with slight modifications. Male Wistar rats (250−300 g) were sacrificed, and the whole brains minus cerebella were rapidly removed and homogenized in a glass−Teflon homogenizer (Schütt Labortechnik GmbH, Göttingen, Germany) in 10 volumes of pH 7.4, 40 mM phosphate buffer. Homogenates were centrifuged at 3000g for 10 min at 4 °C to give supernatants containing 1.2 mg of protein/mL as determined by the Bradford method (*25*).

To measure malondialdehyde formed as a result of lipid peroxidation, 500-μL samples of supernatant were mixed with equal volumes of the same buffer containing 1−10 μM test samples added in 10 μL of DMSO. Samples were incubated at 37 °C for 10 min, after which time 200 μM FeSO$_4$ was added. Controls consisted of rat brain supernatant mixed with 10 μL of DMSO and FeSO$_4$. Reactions were terminated by adding 500 μg of thiobarbituric acid in 100 μL of pH 3.5, 50% v/v acetic acid buffer, followed by heating at 90 °C for 20 min. After cooling, each sample was extracted with 2 mL of n-BuOH and centrifuged at 10000g for 10 min. The absorbance of the n-BuOH extract containing thiobarbituric acid reacting substance was measured at 532 nm in a multiwell scanning spectrophotometer (Dynex MR 5000, Chantilly, VA), using an extinction coefficient (ϵ) of 18000 M^{-1} cm^{-1}. The amount of thiobarbituric acid reactive substance (TBARS) present in rat brain supernatants was determined by linear regression analysis after addition of 1,1,3,3-tetraethoxypropane (TEP). The results were expressed as nanomoles of MDA equivalents per milligram of protein of rat brain homogenate (**Table 1**). The concentration causing 50% inhibition (IC$_{50}$ value) was calculated, and all measurements were in triplicate.

Fe^3-ADP/NADPH-Dependent Lipid Peroxidation in Rat Liver Microsomes. The influence of catechin metabolites **2**−**6** on lipid

Table 1. Anti-Lipid-Peroxidation Effects of **2**–**6** Expressed as IC$_{50}$ Inhibition Values for TBARS Formation in Rat Brain Homogenates and Rat Liver Microsomes

metabolite	brain homogenate IC$_{50}$ (μM)^b	liver microsomes IC$_{50}$ (μM)^c
2	1.36 ± 0.05	2.26 ± 0.48
3	1.43 ± 0.10	2.45 ± 0.52
4	1.60 ± 0.16	3.97 ± 0.85
5	1.82 ± 0.19	6.12 ± 1.14
6	1.73 ± 0.22	6.65 ± 1.23
D,L-α-tocopherol	5.30 ± 0.3	17.30 ± 2.32
(+)-catechin	3.80 ± 0.2	9.50 ± 1.47

^{a} Values are presented as mean ± SE of three-test sample observation. P < 0.05 for all values. ^{b} MDA production in controls was 14.8 ± 1.7 nmol/mg protein). ^{c} MDA production in controls was 13.2 ± 1.2 nmol/mg protein).

peroxidation induced by Fe^3-ADP/NADPH in rat liver microsomes was examined using the procedure described by Hino et al. (*26*) with minor modifications. Livers from male Wistar rats (as above) were homogenized in 5 volumes (w/v) of cold 0.25 M sucrose containing pH 7.4, 100 mM Tris-HCl, 5 mM dithiothreitol (DTT), and 10 mM MgCl$_2$ using a Potter−Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 400g at 4 °C for 10 min to give a supernatant that was centrifuged at 10000g for 10 min. The 10000g supernatant was again centrifuged at 105000g for 1 h to produce microsomal pellets that were resuspended in pH 7.4, 100 mM Tris-HCl containing 0.25 M sucrose, 5 mM DTT, and 10 mM MgCl$_2$ to give a preparation containing 2.5 mg/mL protein (*25*).

To measure by Fe^3-ADP−NADPH-dependent MDA in rat liver microsomes, reaction mixtures consisted of 0.8 mL of pH 7.4, 50 mM Tris-HCl containing 100 μL of microsomal suspension and 1−10 μM concentrations of products **2**−**6** or standards added in (10 μL) DMSO. Reactions were preincubated at 37 °C for 10 min before receiving 1 mM ADP, 10 mM FeCl$_3$, and 20 mM NADPH. Reaction mixtures were incubated for 20 min at 37 °C before lipid peroxidation inhibition was measured. Reactions were terminated by the addition of 1.5 mL of 0.8% TBA, 200 μL of 8.1% SDS, and 1.5 mL of pH 3.3, 20% acetic acid and by heating at 90 °C for 20 min. Reactions were cooled on ice, 2 mL of n-BuOH was added, and tubes were vigorously shaken. Centrifugation at 1000g for 10 min gave a colored organic layer for visible absorption measurement at 532 nm.

Statistical Analysis. All experimental data are shown as means ± SD and accompanied by the number of observations. Statistical analysis was performed using Student's t test, and the significant difference was set at P < 0.05.

RESULTS AND DISCUSSION

Incubations of (+) catechin **1** with HRP/H$_2$O$_2$ gave dimeric products **2**−**4** (**Figure 1**), whereas oxidations of (+)-catechin with laccase/hydroquinone gave products **5** and **6** (**Figure 2**). Products were observed only in complete reactions containing enzymes plus their reaction mediators. Thus, the enzyme oxidation products were not artifacts caused under conditions of incubation. Metabolites were extracted from preparative-scale enzyme reactions, and products were purified by extensive chromatography. The UV−vis spectra of **2**−**6** all resembled that for **1**, with maximum absorbances near 280 and 325 nm.

Horseradish Peroxidase Products. HRP product **2** gave m/z 579.1507, (M + H)$^+$ for $C_{30}H_{27}O_{12}$, by positive-ion HRFABMS, suggesting a dimeric structure. The EIMS molecular ion and highly characteristic fragment ions at m/z 289 (30%) and 259 (83%) derive from retro-Diels−Alder-type cleavages of the C- and F-rings, indicating that the two (+)-catechin units were likely carbon−carbon-linked through the B- and D-rings.

The ^{1}H NMR spectrum of **2** clearly showed its dimeric nature by the presence of duplicate signals typically found in flavanols
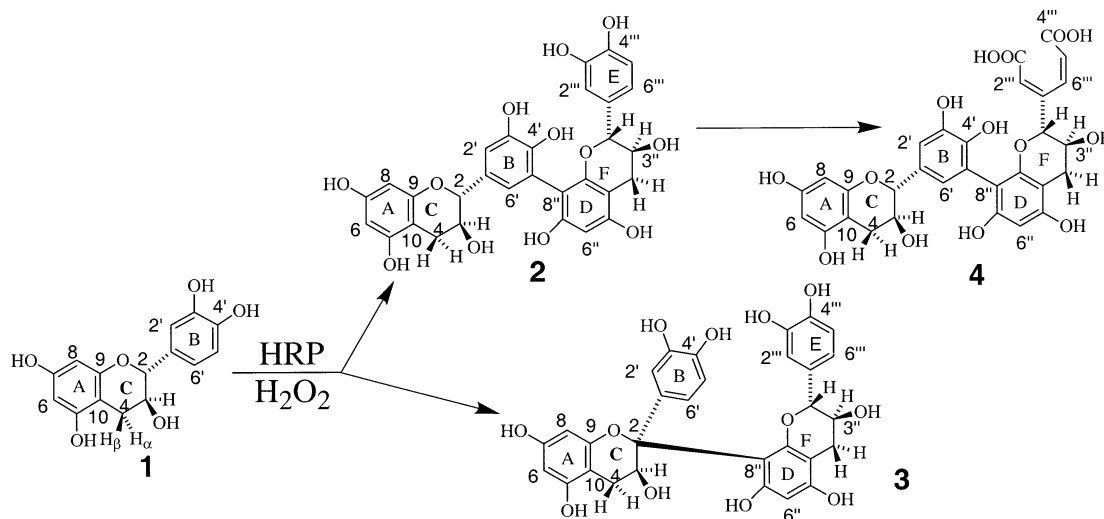
Figure 1. Products obtained by the H_2O_2-dependent horseradish peroxidase oxidation of (+)-catechin.
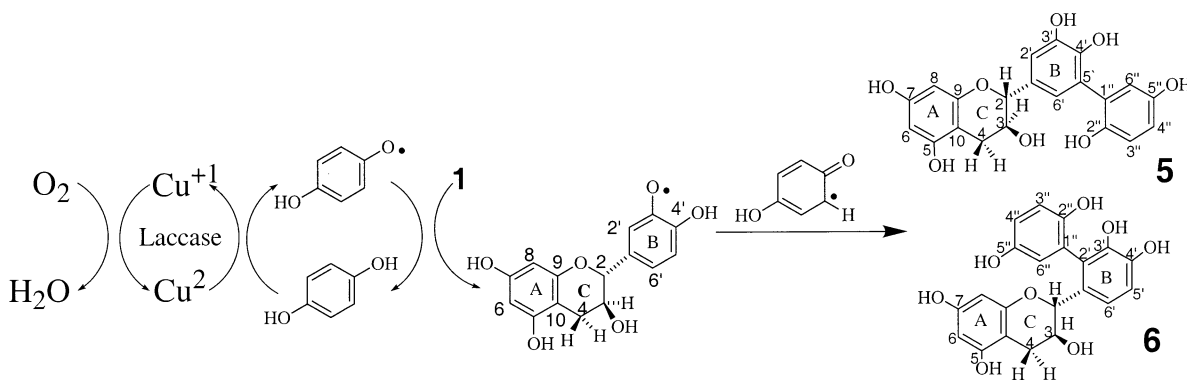

Figure 2. Products obtained by the laccase/hydroquinone-mediated oxidation of (+)-catechin.

such as (+)-catechin. A pair of ABMX spin patterns characteristic of the protons of the heterocyclic C- and F-rings at δ 5.23 (d, J = 7.9 Hz, H-2), 5.06 (d, J = 7.5 Hz, H-2″), 4.69 (m, H-3), 4.29 (m, H-3″), 2.97 (dd, J = 16.2, 5.8 Hz, H-4$_{eq}$), 2.74 (dd, J = 16.2, 9.2 Hz, H-4$_{ax}$), 2.87 (dd, J = 16.0, 5.7 Hz, H-4″$_{eq}$), and 2.71 (dd, J = 16.0, 8.7 Hz, H-4″$_{ax}$) supported the presence of two (+)-catechin units in **2**. Suitably large coupling constants for H-2 and H-4 protons of both dimer units reflected unchanged 2,3-trans stereochemistries in the C- and F-rings (*27*). Additionally, from the ROESY spectrum, the H-2 and H-2″ resonances showed a scalar peak correlation with H-4$_{eq}$ and H-4″$_{eq}$. Only the H-4$_{eq}$ and 4″$_{eq}$ are in position for long-range coupling with H-2 and H-2″. Therefore, signals for H-4 (δ 2.74) and H-4″ (δ 2.71) were assigned to the axial protons. Volume integration of rotating-frame Overhauser correlations arising from H-3 revealed that its distance to H-4$_{ax}$ was smaller than the distance to H-4$_{eq}$. This observation demonstrates that both C- and F-rings have 2,3-trans stereochemistries (*27*).

Signals for eight of the nine aromatic protons in the spectrum of **2** form two sets of AX spin patterns, one characteristic of catechin at δ 6.19 (d, J = 2.5 Hz, H-6) and 5.98 (d, J = 2.5 Hz, H-8) and the other corresponding to B-ring with meta coupling at δ 6.96 (d, J = 1.1 Hz, H-2′) and 7.12 (d, J = 1.1 Hz, H-6′). This result confirmed the (+)-catechin B-ring linkage through C-5′. A set of ABX-type signals for an E-ring were observed at δ 7.43 (d, J = 2.0 Hz, 2‴), 7.20 (d, J = 8.0 Hz, H-5‴), and 7.26 (dd, J = 8.0, 2.0 Hz, H-6‴). The remaining aromatic proton at δ 6.29 was a singlet in a pentasubstituted D-ring. The ^{13}C NMR spectrum also showed that **2** was a catechin dimer showing numerous duplicate signals, particularly

in the high-field region (δ 28−82), associated with the heterocyclic C-ring where the C-2, C-3, and C-4 carbon resonances showed up as twin peaks of comparable intensities. The remaining carbon signals were more or less consistent with the chemical shift values for the phloroglucinol-type A- and D-rings and the catechol B- and E-rings of flavan-3-ol. The presence of two quaternary carbons at δ 126.74 (C-5′) and 110.80 (C-8″) was established by DEPT. The presence of an unsubstituted aromatic carbon in each of the B- and D-rings suggested a biphenyl type of linkage between the two catechin moieties (*6*). All connectivities of A-, C-, F-, and E-rings of **2** were confirmed by HMBC, DQF-COSY, and ROESY analysis. Confirmation of ring-B substitution as shown for **2** was clear by HMBC correlation of H-2′ to C-2, C-4′, and C-6′ and H-6′ correlation to C-2, C-2′, C-4′, C-6′, and C-8″ in the D-ring. The DQF-COSY spectrum showed unambiguous spin−spin couplings of both H-6′ and H-2′ to H-2. HMQC and HMBC spectra also showed cross-peaks between H-6″ (δ 6.29, s), C-5″ (δ 156.55), C-7″ (157.10), and C-8″ (δ 110.80) showing that **2** is a novel B-ring functionalized (+)-catechin dimer, (+)-catechin (5′→8″) catechin.

HRP product **3** gave *m/z* 579.1508, (M + H)$^+$ for $C_{30}H_{27}O_{12}$, by positive-ion HRFABMS, identical to the MS for **2** and indicative of a different dimeric product. ^{1}H and ^{13}C NMR spectra for **3** were closely comparable to those for **2** except for the presence of two aromatic ABX-type signals for the B- and E-rings at δ 6.94 (d, J = 1.7 Hz, 2′), 7.25 (d, J = 1.5 Hz, 2‴), 6.80 (d, J = 8.2 Hz, 5′), 6.87 (d, J = 8.0 Hz, H-5″) 6.74 (dd, J = 8.2, 1.7 Hz, 6′), and 7.11 (dd, J = 8.0, 1.5 Hz, H-6‴). HMBC cross-peaks for H-2′/H-6′ and C-2 and H-2‴/H-6‴ and

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C-2″ confirmed the two catechol moieties B and E and their attachment to rings C and F, respectively. A singlet for H-6″ (δ 6.32) of a pentasubstituted benzene ring, with HMBC correlations with C-5″ and C-7″, confirmed a (+)-catechin D-ring functionalization at C-8″ as with **2**. The lack of a prominent signal for H-2, and the presence of an isolated ABX system at δ 4.19 (m, H-3), 3.02 (dd, J = 15.8, 5.5 Hz, H-4$_{eq}$), and 2.70 (dd, J = 15.8, 9.0 Hz, H-4$_{ax}$), versus the expected ABMX system in an unsubstituted C-ring, showed that the 2-position was substituted. ^{1}H NMR signals at δ 5.10 (d, J = 7.5, H-2″), 4.35 (m, H-3″) 2.87 (dd, J = 16.5, 5.8 Hz, H-4″$_{eq}$), and 2.65 (dd, J = 16.5, 9.3 Hz, H-4″$_{ax}$) as an ABMX spin pattern showed a second, unsubstituted F-ring as in **2**. These data indicated a structure that contained an altered (+)-catechin C-ring. The proton signal for H-2 was absent, and only one proton signal for H-2″ of ring F was observed at δ 5.10 (1H, d, J = 7.5 Hz). The C-2 carbon signal appeared at δ 86.58 versus 81.74 d in **2**. This carbon resonance indicated that the two flavan-3-ol units were joined via an unusual C-2 → C-8″ carbon−carbon linkage. The chemical shifts of C-6″ (δ 109.65) and H-6″ (δ 6.32) of **3** are similar to those in spectra of related ([2′,8]- and [6′,8]-) bis(+)-catechins coupled via their respective B- and A-rings (*13, 14*).

These assignments are supported by HMQC and HMBC spectral data. By HMQC, C-3, 6′, and 6″ methine carbon signals showed [1]J_{CH} to H-3, H-6′, and H-6″, respectively. HMBC showed couplings between H-3 and C-8″ and between H-2′ and C-2, C-1′, C-4′, and C-6′, whereas H-6′ correlated well with C-2, C-2′, and C-4′. Correlations of H-6″ with C-8″ and C-9″ clearly indicated the point of the interflavanoid linkage to be at C-2 → C-8″. The stereochemistry of the attachment at position 2 is supported by the NOESY relationship of 3-H in ring C to 2′,6′-protons in ring B, a relationship that could not exist if the opposite configuration occurred. Thus, **3** is a novel C-ring functionalized (+)-catechin dimer, produced by HRP, and its structure was assigned as (+)-catechin (2→8″) catechin.

HRP oxidation product **4** gave 611.1403, (M + H)[+] for $C_{30}H_{27}O_{14}$, by HRFABMS indicating still another catechin dimer. The ^{1}H and ^{13}C NMR spectra of **4** were similar to those for **2**. A meta-coupled B-ring with signals at δ 6.92 (d, J = 1.0 Hz, H-2′) and 7.16 (d, J = 1.0 Hz, H-6′) confirmed a (+)-catechin B-ring coupling at C-5′ as in **2**. A-ring protons were clear at δ 6.30 (d, J = 2.0 Hz, H-6) and 6.26 (d, J = 2.0 Hz, H-8). Doublets at δ 5.31 and 5.11, two multiplets at δ 4.29 and 4.18, and two double doublets located at δ 2.99/2.70 and 3.12/2.62 were very similar to signals for H-2, H-3, H-4$_{eq}$, and H-4$_{ax}$, respectively, of the C-and F-rings of **2**. Supported by HMQC, HMBC, DQF-COSY, and ROESY correlations, the A-, B-, C-, D-, and F-rings of **3** were similar to those of **2**, and **4** differed from **2** only in the E-ring. The remnant of the E-ring of **4** contained signals for a pair of doublets at δ 7.34 and 6.82 for H-5‴ and H-6‴. HMQC correlated these signals to C-5‴ (δ 129.65) and C-6‴ (δ 138.12). The coupling constant (J = 11.7 Hz) for H-5‴ and H-6‴ showed a cis double bond. The H-2‴ singlet aromatic proton at δ 6.56 correlated with C-2‴ (δ 121.23) by HMQC, completing the assignments of proton signals. The ^{13}C NMR spectrum indicated the presence of two carboxylic acid carbonyl carbons at δ 172.78 and 176.20. HMBC showed [2]J_{CH} interactions between H-6‴ with C-1‴ and a [3]J_{CH} interaction with C-2‴, whereas H-5‴ showed a [2]J_{CH} interaction with C-6‴ and a [3]J_{CH} interaction with C-1‴. Additionally, HMBC correlations between carbonyl carbon-4‴ and H-5‴ and H-6‴ and between carbonyl-3‴ and H-2‴ confirmed two acid positions at C-3‴ and C-4‴. Thus,

metabolite **4** is the unusual flavan-3-ol dimer identified as 1‴-{8″-[3′,4′-dihydroxy-1′-(3,5,7-trihydroxychroman-2-yl)phenyl]-3″,5″,7″-trihydroxychroman-2″-yl}hexa-2‴,6‴-diene-2‴,5‴-dioic acid.

Laccase Products. Two similar products were obtained when hydroquinone and (+)-catechin were oxidized with laccase. FABMS gave m/z 399 for $C_{21}H_{19}O_8$ for both compounds, indicating that they were adducts apparently formed by couplings of hydroquinone with **1**. The ^{1}H NMR spectrum of **5** was similar to that of **1**, except for changes in the B-ring. In addition to a set of ABMX spin patterns for the heterocyclic C-ring aliphatic protons, seven aromatic proton signals comprising a pair of AX spin systems were observed. These were typical of A- and B-ring proton signals at δ 6.48 and 6.36 (each 1H, d, J = 2.2 Hz, H-6 and H-8) for ring A, meta-coupled doublets (J = 1.3 Hz) at δ 7.34 and 7.48 for H-2′ and H-6′ of ring B, and an ABX spin system for H-3″ (δ 6.78), H-4″ (δ 6.62), and H-6″ (δ 6.67) with ortho (J = 8.5 Hz), meta (J = 2.5 Hz), and ortho−meta (J = 8.5, 2.5 Hz) coupling constants for an attached hydroquinone ring. The number of proton signals and the coupling pattern suggested a carbon−carbon biphenyl linkage to (+)-catechin.

The HMBC spectrum showed cross-peaks between H-2 (δ 5.17, d, J = 7.7 Hz) and B-ring carbons C-2′ (δ 115.0) and C-6′ (δ 118.83), between H-6′ (7.48, d, J = 1.3 Hz) and C-1″ (δ 122.56), and between H-6″ δ 6.67 (d, J = 2.5 Hz) on the hydroquinone moiety and C-5′, C-1″, and C-2″. Thus, **5** is a C-1 → C-5′-linked catechin−hydroquinine adduct.

Laccase product **6** was also a hydroquinone−(+)-catechin adduct with the same A- and C-rings as **5**. By ^{1}H NMR, the splitting pattern of the B-ring protons in **5** was replaced by an AB spin system in **6**. Signals for H-5′ (δ 6.95) and H-6′ (δ 7.14) (each d, J = 8.6 Hz) confirmed that the B-ring was attached to a hydroquinone moiety through C-2′. The ^{13}C NMR spectral data for **6** were also very similar to those for **5**, except for the resonances of a methine carbon at C-5′ (δ 115.36) and a quaternary carbon signal for C-2′ (δ 122.40). HMQC and HMBC spectra with [1]J_{CH} (H-3′/C-2′) and [3]J_{CH} (H-2/C-2′/C-6′) couplings clearly showed that the hydroquinone moiety was linked to the B-ring at C-2′.

Copper oxidases and peroxidases are ubiquitous in nature (*21, 23, 28, 30*). Thus, the model studies demonstrated here with HRP/H_2O_2 and laccase/hydroquinone can be used to predict catechin oxidation products similar to **2**−**6** in plants, microorganisms, and mammals. Peroxidases are well-known for their abilities to catalyze H_2O_2-dependent one-electron oxidations of phenols. The oxidation process involves initial H_2O_2 oxidation of HRP to the catalytically active species "compound **I**" (*28*), a porphyrin cation radical, Fe(IV) species. Compound **I** abstracts hydrogen from phenolic substrates, leading to phenoxy radical intermediates (*7, 8*). As a member of the blue copper oxidases, laccase catalyzes oxidations of phenolic compounds by a one-electron abstraction mechanism that is ultimately coupled to the reduction of molecular oxygen to water (*7, 8*).

HRP oxidations of (+)-catechin gave novel coupled products containing C−C interflavan linkages, positioned between the B- and D-rings in **2** and **4** and between the C- and D-rings in **3**. We speculate that oxidation at the 7-phenol and delocalization of the resulting radical to position 8 provide one catechin radical species common to all observed HRP products. Initial oxidation of the B-ring 4′-phenol and electron delocalization to position 5′ provides the necessary components for radical coupling to **2**. Couplings between positions 8 and 2 of two radical species would provide the unusual dimer **3**. The subsequent oxidation

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of **2** to form dicarboxylic acid product **4** represents a novel catechol ring cleavage reaction. A similar intradiol oxygenolytic cleavage of 3,5-di-*tert*-butylcatechol was catalyzed by Fe^{3+} model complexes as a mimic to enzymes such as HRP (*8*). It is interesting to note that the A-ring in (−)-epigallocatechin gallate can be oxidized to form an acid product when reacted with H_2O_2 (*29*).

Laccase/hydroquinone oxidations gave catechin−hydroquinone adducts **5** and **6** both coupled to the B-ring of catechin. This type of coupling would require formation of either 3′- or 4′-phenoxy radical intermediates that could couple with a semiquinone radical. The likely process involves oxidation of hydroquinone to semiquinone radical that serves as a "shuttle oxidant" (*21*) in catalyzing radical oxidation of the B-ring of **1**. Similar shuttle oxidation processes are well-known with the copper oxidases (*30*), peroxidases (*21*), and tyrosinase (*15*).

Catechins are well-known antioxidants that quench reactive oxygen and reactive nitrogen species. Among the most important of these in biological systems are superoxide radical anion ($O_2^{\bullet-}$), hydroxyl radical (HO•), lipid peroxyl radical, nitric oxide radical (•NO), and peroxynitrite ($ONOO^-$) (*31*). Various studies have addressed the antioxidant capacities of catechins in simplified in vitro assay systems, cultured cells, perfused organs, and in vivo (*31*). Catechins effectively suppress lipid peroxidation in biological tissues and subcellular fractions such as microsomes and low-density lipoproteins (*31*). The antioxidative activities of catechins are embodied in their abilities to trap the chain-carrying peroxyl radicals by donation of phenolic hydrogens from rings A and B (*32*) or C-ring benzylic protons (*32*). We were interested in ascertaining whether catechin products **2**−**6** retained antioxidant properties, even after catechin had been oxidatively dimerized or reacted with hydroquinone. The antioxidant activities of the catechin metabolites **2**−**6** were evaluated using Fe^{2+}-induced lipid peroxidation in rat brain homogenates and $FeCl_3$−ADP in the presence of NADPH in rat liver microsomes. Well-known antioxidants D,L-α-tocopherol and (+)-catechin were used for comparison.

For rat brain homogenate alone, the amount of TBARS measured was 3.3 ± 0.2 nmol of malondialdehyde/mg of protein (MDA/mg of protein). After addition of 200 μM Fe^{2+}, the amount of TBARS was increased to 14.8 ± 0.8 nmol of MDA/mg of protein. Metabolites **2**−**6** caused no interference in absorption measurements at 532 nm. Metabolites **2**−**6** all inhibited the Fe^{2+} ion-induced lipid peroxidation in rat brain homogenates with IC_{50} values 2−4 times lower than those for D,L-α-tocopherol and catechin (**Table 1**). Addition of iron to solutions of **2**−**6** caused no spectral shifts or increases in absorbance, suggesting that these new catechin derivatives may exert their effects by scavenging free radicals rather than by functioning as iron chelators (*32*). With liver microsomal preparations, TBARS of 13.2 ± 1.2 nmol of MDA/mg of protein were measured in controls. Metabolites **2**−**6** gave IC_{50} values 3−8 times lower than that for D,L-α-tocopherol and 1.5−4 times lower than that for (+)-catechin (**Table 1**).

The results show that these metabolites have antioxidant properties slightly better than D,L-α-tocopherol and catechin themselves. This is interesting in that although the products obtained by peroxidase and laccase enzyme oxidations were formed by radical processes, they retain slightly enhanced antioxidant activities.

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JF020503J