JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Structure on Radical-Scavenging Abilities and Antioxidative Activities of Tea Polyphenols: NMR Analytical Approach Using 1,1-Diphenyl-2-picrylhydrazyl Radicals

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Tea (*Camellia sinensis*) leaves contain various antioxidants such as ascorbic acid (**1**) and polyphenols. This study tries to clarify the molecular mechanisms underlying the antioxidative and radical-scavenging activities of these antioxidants, and the reactivities of each antioxidant have been compared against that of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH, **2**) using nuclear magnetic resonance (NMR) analysis. Catechol (**3**) and (+)-taxifolin (**4**) were oxidized to *o*-quinone by **2**. However, ethyl protocatechuate (**5**) and quercetin (**6**) were not oxidized to *o*-quinone, even though they possess a catechol structure. The radical-scavenging ability of *o*-dihydroxyl phenolic compounds with a conjugated olefinic double bond (e.g., **6**) was superior to that of compounds without this bond (e.g., **4**), whereas the ability of *o*-dihydroxyl phenolic compounds possessing a conjugated carbonyl bond (**5**) was inferior to that of compounds lacking this bond (**3**). Vicinal trihydroxyl phenolic compounds with a conjugated olefinic double bond [e.g., myricetin (**7**)] had an inferior scavenging ability as compared with compounds lacking this bond [e.g., pyrogallol (**8**)], but **7** was a better scavenger than compounds with a conjugated carbonyl double bond [e.g., ethyl gallate (**9**)]. In addition, vicinal trihydroxyl phenolic compounds (e.g., **9**) were superior to *o*-dihydroxyl phenolic compounds (e.g., **6**). Finally, **1** scavenged radicals more quickly than **8**.

KEYWORDS: Antioxidation mechanism; NMR; 1,1-diphenyl-2-picrylhydrazyl; polyphenol; conjugated double bond

INTRODUCTION

Tea (*Camellia sinensis*) leaves contain various polyphenols and possess potent antioxidative activities (*1*). Tea catechins (flavan-3-ol derivatives) are major constituents of tea polyphenols. In recent years, they have attracted much attention for their potential beneficial effects in disease prevention (*2*−*9*). In particular, the antioxidant activities of catechins seem to be most important in terms of their physiological function (*9*, *10*), and there have been several studies of the antioxidant abilities of catechins against the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH, **2**) (*1*, *11*, *12*). However, the antioxidation mechanism of catechins is not fully characterized as yet.

We have developed a new nuclear magnetic resonance (NMR) analysis method, which uses **2**, to elucidate the molecular mechanisms underlying the antioxidative and radical-scavenging activities of antioxidants (*13*). Using this method, we have previously demonstrated that the antioxidation mechanism of (−)-epigallocatechin (**10**), which has a pyrogallol structure in the B-ring, differs from those of (+)-catechin (**11**) and (−)-epicatechin (**12**), which have a catechol structure in the B-ring (*13*). In brief, **11** and **12** are oxidized to *o*-quinone by **2**, whereas **10** is not oxidized to *o*-quinone. We have also compared the relative radical-scavenging activities between antioxidants [ascorbic acid (**1**), α-tocopherol, and catechins] by this new NMR analysis method (*14*).

In the current study, we have tried to clarify the molecular mechanisms underlying the antioxidative and radical-scavenging activities of other polyphenols by using ^{13}C NMR analysis coupled with **2**. Moreover, we have directly compared the reactivities of each polyphenol against the radical **2** by NMR analysis and have tried to clarify the influence of the number of hydroxyl groups and the conjugated double bond on the antioxidative activity of polyphenols.

MATERIALS AND METHODS

^{13}C NMR Spectroscopy. ^{13}C NMR spectra were measured with a JEOL JNM-LA 500 FT-NMR spectrometer at 30 °C for 3 h and 20

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10.1021/jf040423a CCC: $30.25 © 2005 American Chemical Society
Published on Web 03/31/2005

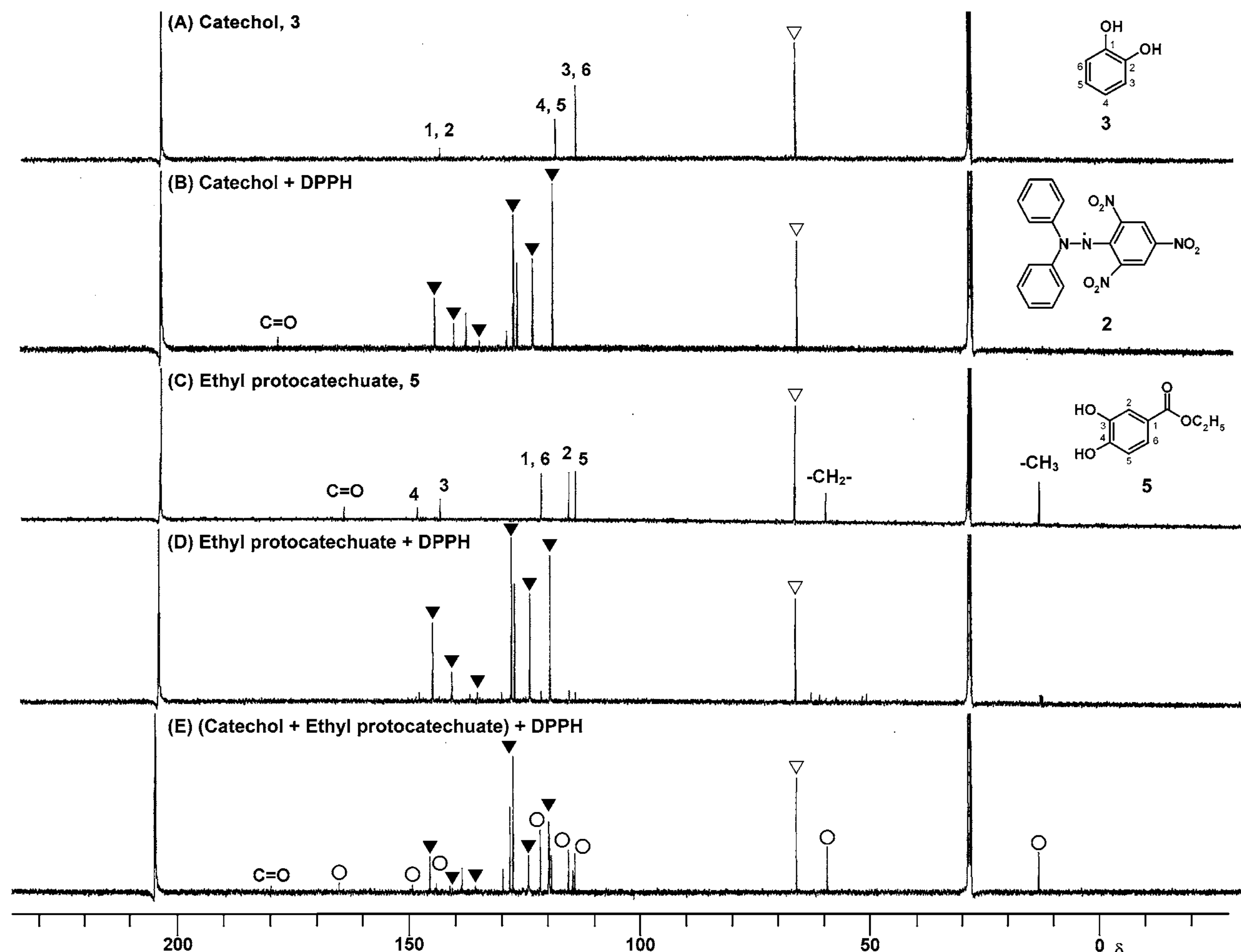

Figure 1. ^{13}C NMR analyses (acetone-d_6, 125 MHz) to examine the reactivity of catechol (**3**) and ethyl protocatechuate (**5**) with DPPH (**2**): (**A**) **3** (0.015 mmol); (**B**) reaction mixture of **3** (0.015 mmol) and **2** (0.030 mmol); (**C**) **5** (0.015 mmol); (**D**) reaction mixture of **5** (0.015 mmol) and **2** (0.030 mmol); (**E**) **2** (0.030 mmol) added to a mixture of **3** (0.015 mmol) and **5** (0.015 mmol); (▽) 1,4-dioxane (external standard); (▼) 1,1-diphenyl-2-picrylhydrazine; (○) remaining peaks of **5**.

min (proton decoupling; 125 MHz; 5 mm cell; 32768 data points; spectral width, 33898 Hz; 45° pulse; pulse repetition time, 1 s; 4000 times) with an external standard containing 1,4-dioxane in acetone-d_6 (0.015 mmol/0.06 mL) in a sealed tube with coaxial inserts (Wilmad, Buena, NJ).

1,4-Dioxane: ^{13}C NMR (acetone-d_6, 125 MHz) δ 67.6 (C-2, -3, -5, -6).

Methyl Ester of Caffeic Acid and Hydrocaffeic Acid. Thirty milligrams each of caffeic and hydrocaffeic acids were individually dissolved in 1 mL of 5% HCl in methanol (Nacalai Tesque, Inc., Kyoto, Japan) and kept at 70 °C for 1 h. The mixtures were purified by silica gel column chromatography ($CHCl_3$/MeOH) to yield the methyl esters.

Sample Preparation for NMR Measurement. *Reaction of Polyphenols with 2.* After the ^{13}C NMR spectroscopic measurement of catechol (**3**, 0.015 mmol) in acetone-d_6 (0.54 mL), **2** (0.030 mmol) was added to the NMR cell. The mixture was kept for 10 min at room temperature, and after the purple color of **2** faded, it was subjected to ^{13}C NMR analysis. Ethyl protocatechuate (**5**), quercetin (**6**), (+)-taxifolin (**4**), luteolin (**13**), eriodictyol (**14**), methyl caffeate (**15**), methyl hydrocaffeate (**16**), pyrogallol (**8**), and myricetin (**7**) were treated in the same way. Carbon signals of each polyphenol were assigned by the comparison of ^{13}C NMR spectral data with literature values (*15–20*).

3: ^{13}C NMR (acetone-d_6, 125 MHz) δ 146.0 (C-1, -2), 120.8 (C-4, -5), 116.2 (C-3, -6) (*15*) (**Figure 1A**).

5: ^{13}C NMR (acetone-d_6, 125 MHz) δ 166.5 (C=O), 150.7 (C-4), 145.6 (C-3), 123.3 (C-1, -6), 117.2 (C-2), 115.7 (C-5), 60.9 ($-CH_2-$), 14.6 ($-CH_3$) (*15*) (**Figure 1C**).

6: ^{13}C NMR (acetone-d_6, 125 MHz) δ 176.6 (C-4), 165.0 (C-7), 162.3 (C-5), 157.8 (C-8a), 148.3 (C-4′), 146.9 (C-2), 145.8 (C-3′), 136.8 (C-3), 123.8 (C-1′), 121.5 (C-6′), 116.2 (C-5′), 115.8 (C-2′), 104.2 (C-4a), 99.2 (C-6), 94.5 (C-8) (*16*) (**Figure 2A**).

4: ^{13}C NMR (acetone-d_6, 125 MHz) δ 198.2 (C-4), 167.8 (C-7), 165.0 (C-5), 164.2 (C-8a), 146.6 (C-4′), 145.7 (C-3′), 129.8 (C-1′), 120.8 (C-6′), 115.9, 115.8 (C-2′, -5′), 101.6 (C-4a), 97.1 (C-6), 96.0 (C-8), 84.5 (C-2), 73.2 (C-3) (*16*) (**Figure 2C**).

13: ^{13}C NMR (acetone-d_6, 125 MHz) δ 181.8 (C-4), 163.7 (C-7), 162.2 (C-5), 157.5 (C-8a), 148.9 (C-4′), 146.9 (C-2), 145.3 (C-3′), 122.6 (C-1′), 119.0 (C-6′), 115.4 (C-5′), 112.9 (C-2′), 104.2 (C-4a), 103.0 (C-3), 98.4 (C-6), 93.4 (C-8) (*17*).

14: ^{13}C NMR (acetone-d_6, 125 MHz) δ 196.0 (C-4), 166.2 (C-7), 164.1 (C-5), 163.2 (C-8a), 145.2 (C-4′), 144.8 (C-3′), 130.4 (C-1′), 118.1 (C-6′), 114.9 (C-5′), 113.5 (C-2′), 102.1 (C-4a), 95.6 (C-6), 94.7 (C-8), 78.8 (C-2), 42.4 (C-3) (*18*).

15: ^{13}C NMR (acetone-d_6, 125 MHz) δ 166.6 (C-9), 147.7 (C-7), 145.2 (C-4), 144.5 (C-3), 126.3 (C-1), 121.3 (C-6), 115.1 (C-5), 114.0 (C-2), 113.9 (C-8), 50.2 ($-CH_3$) (*19*).

16: ^{13}C NMR (acetone-d_6, 125 MHz) δ 172.3 (C-9), 144.4 (C-4), 142.9 (C-3), 132.2 (C-1), 119.1 (C-6), 114.9 (C-5), 114.8 (C-2), 50.3 ($-CH_3$), 35.3 (C-7), 29.8 (C-8) (*19*).

8: ^{13}C NMR (acetone-d_6, 125 MHz) δ 146.8 (C-1, -3), 133.8 (C-2), 120.0 (C-5), 108.1 (C-4, -6) (*15*) (**Figure 3A**).

7: ^{13}C NMR (acetone-d_6, 125 MHz) δ 176.5 (C-4), 165.0 (C-7), 162.3 (C-5), 157.7 (C-8a), 146.9 (C-2), 146.4 (C-3′, -5′), 136.9 (C-4′), 136.4 (C-3), 122.8 (C-1′), 108.3 (C-2′, -6′), 104.1 (C-4a), 99.1 (C-6), 94.4 (C-8) (*20*) (**Figure 3C**).

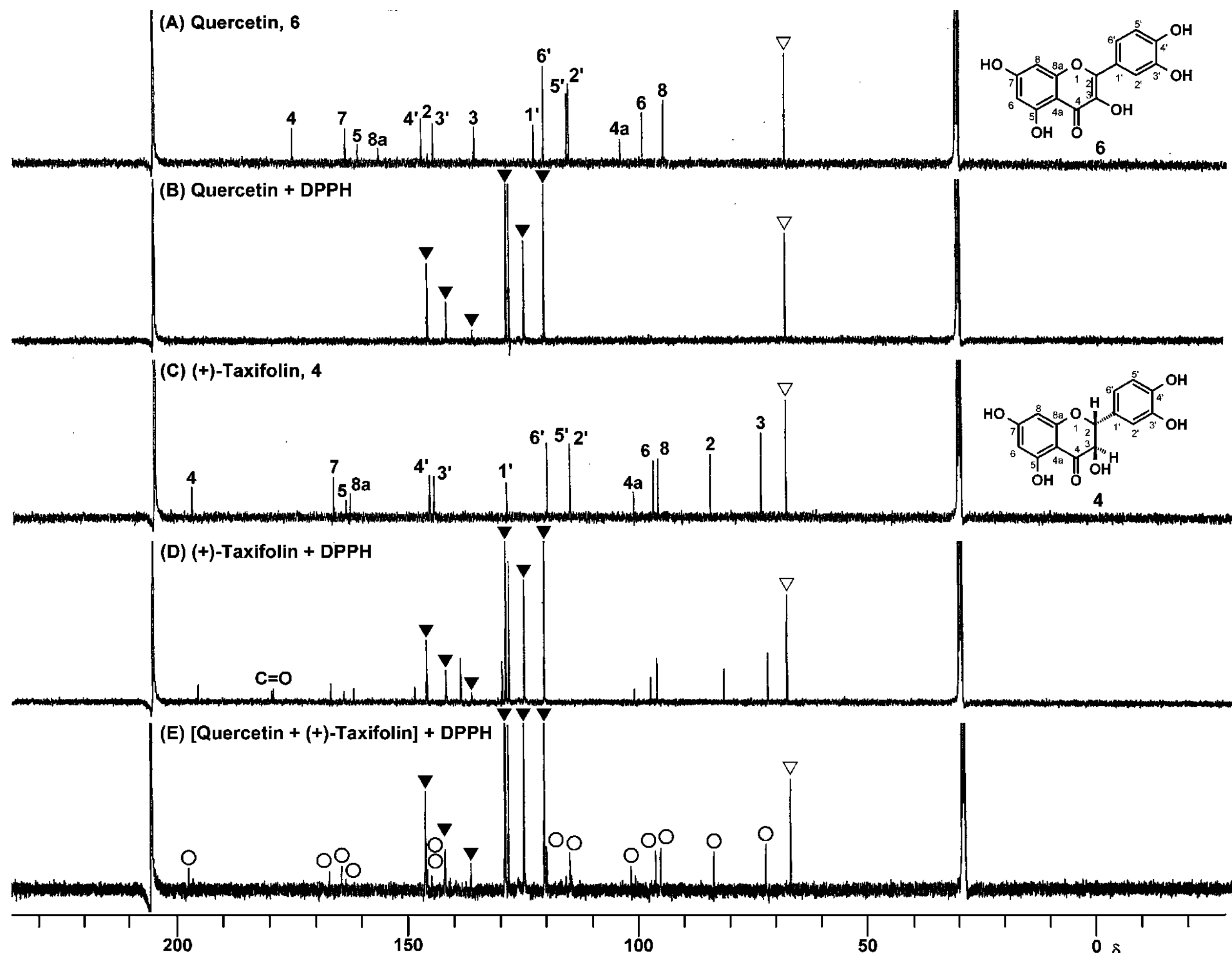

Figure 2. ^{13}C NMR analyses (acetone-d_6, 125 MHz) to examine the reactivity of quercetin (**6**) and (+)-taxifolin (**4**) with DPPH (**2**): (**A**) **6** (0.015 mmol); (**B**) reaction mixture of **6** (0.015 mmol) and **2** (0.030 mmol); (**C**) **4** (0.015 mmol); (**D**) reaction mixture of **4** (0.015 mmol) and **2** (0.030 mmol); (**E**) **2** (0.030 mmol) added to a mixture of **6** (0.015 mmol) and **4** (0.015 mmol); (▽) 1,4-dioxane (external standard); (▼) 1,1-diphenyl-2-picrylhydrazine; (○) remaining peaks of **4**.

Comparison of the Antioxidative Abilities of Polyphenols. A mixture of two antioxidants (**3** and **5**, **6** and **4**, **13** and **14**, **15** and **16**, **7** and **9**, **9** and **6**, or **8** and **7**; 0.015 mmol each) in acetone-d_6 (0.54 mL) was reacted with **2** (0.030 mmol for the first five combinations; 0.045 for the last two combinations). Alternatively, a mixture of **1** (0.015 mmol) in methanol-d_4 (0.27 mL) and **8** (0.015 mmol) in acetone-d_6 (0.27 mL) was reacted with **2** (0.030 mmol).

All of the mixtures were kept for 10 min at room temperature, and after the purple color of **2** faded, they were subjected to ^{13}C NMR analyses.

RESULTS AND DISCUSSION

Reaction of *o*-Dihydroxyl Phenolic Compounds with DPPH (2). *Reaction of Catechol (3) with DPPH (2).* ^{13}C NMR analysis was carried out to clarify the molecular mechanisms underlying the antioxidative effects of tea polyphenols. **3** was used as a model compound for polyphenols such as **11** or **12** that possess a catechol structure. **3** (0.015 mmol) was reacted with **2** (0.030 mmol), the reaction mixture was subjected to ^{13}C NMR analysis (**Figure 1B**), and the resulting spectrum was compared with that of **3** (**Figure 1A**). The characteristic signals of **3** (δ 116.2, 120.8, and 146.0) had completely disappeared, and new signals (δ 131.5, 140.2, and 181.2) were clearly detected (**Figure 1B**). The appearance of a carbonyl signal (δ 181.2) suggested that **3** was oxidized to *o*-quinone by **2**, similar to the B-ring of **11** (*13*). Other signals (δ 121.4, 125.8, 129.2, 129.9, 137.3, 142.9, and 147.1) were ascribable to **2** (δ 129.2) and to 1,1-diphenyl-2-picrylhydrazine marked with ▼ produced by the reduction of **2** (**Figure 1B**).

Reaction of Ethyl Protocatechuate (5) with DPPH (2). Protocatechuic and gallic acids are constituents of tannin in plants. We have previously described the mechanism underlying the antioxidative effects of **9** (*13*, *14*). When **9** was reacted with **2**, most of the signals of **9** decreased and no carbonyl signals appeared (*13*, *14*). In the current study, we used **5** as a model compound of *o*-dihydroxyl phenolic acids in ester form. When **5** (0.015 mmol) was reacted with **2** (0.030 mmol) and subjected to ^{13}C NMR analysis (**Figure 1D**), the intensities of the original carbon signals ascribable to **5** decreased, and the characteristic signal due to the ester carbonyl (δ 166.5) completely disappeared. Although **5** possesses a catechol structure, no carbonyl signals ascribable to an *o*-quinone moiety appeared. These observations indicate that the mechanism underlying the antioxidative effect of **5** is similar to that of **9** (*13*) and that the radical may be stabilized including ester carbonyl group not to yield an *o*-quinone.

Reaction of Catechol (3) and Ethyl Protocatechuate (5) with DPPH (2). A mixture of **3** (0.015 mmol) and **5** (0.015 mmol) was reacted with **2** (0.030 mmol) and subjected to ^{13}C NMR analysis (**Figure 1E**). Their relative radical-scavenging abilities were directly assessed by comparing the resulting spectrum with those of **3** (**Figure 1A**) and **5** (**Figure 1C**). The intensities of

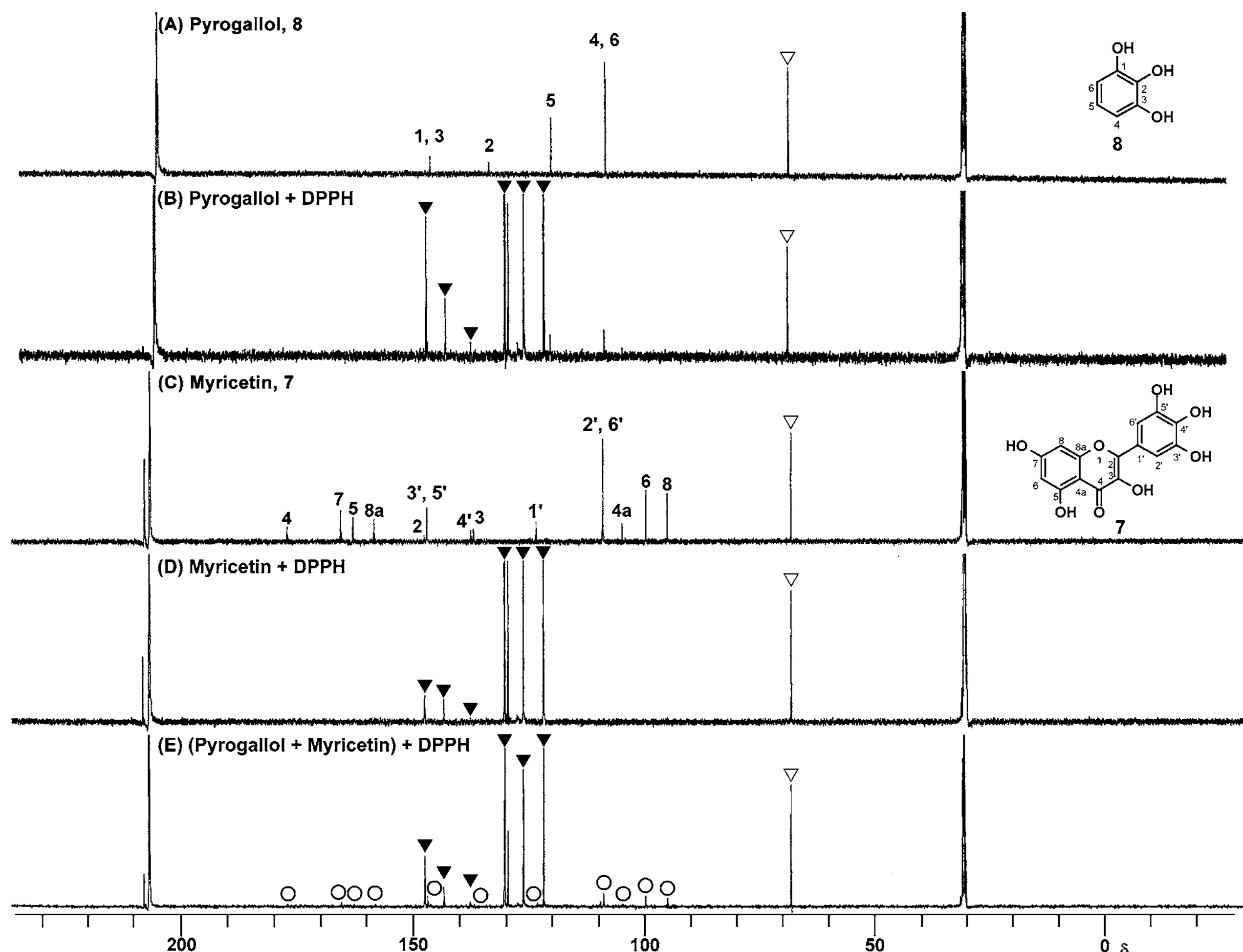

Figure 3. ^{13}C NMR analyses (acetone-d_6, 125 MHz) to examine the reactivity of pyrogallol (**8**) and myricetin (**7**) with DPPH (**2**): (**A**) **8** (0.015 mmol); (**B**) reaction mixture of **8** (0.015 mmol) and **2** (0.045 mmol); (**C**) **7** (0.015 mmol); (**D**) reaction mixture of **7** (0.015 mmol) and **2** (0.030 mmol); (**E**) **2** (0.045 mmol) added to a mixture of **8** (0.015 mmol) and **7** (0.015 mmol); (▽) 1,4-dioxane (external standard); (▼) 1,1-diphenyl-2-picrylhydrazine; (○) remaining peaks of **7**.

most of the original carbon signals ascribable to **3** [C-3 and 6 (δ 116.2), C-4 and 5 (δ 120.8), C-1 and 2 (δ 146.0)] decreased, and a new carbonyl signal (δ 181.2) was clearly detected. However, the signals of **5** [$-CH_3$ (δ 14.6), $-CH_2-$ (δ 60.9), C-5 (δ 115.7), C-2 (δ 117.2), C-1, 6 (δ 123.3), C-3 (δ 145.6), C-4 (δ 150.7), C=O (δ 166.5) (marked with ○)] remained. Therefore, this observation suggests that radicals of DPPH are scavenged more rapidly by **3** than by **5**, indicating that the presence of a conjugated carbonyl group decreases the antioxidative activities of *o*-dihydroxyl phenolic compounds.

*Reaction of Quercetin (**6**) with DPPH (**2**).* We previously described the mechanism underlying the antioxidative effects of **11** and **12** (*13*). When **11** was reacted with **2**, the B-ring of **11** was oxidized to an *o*-quinone structure (*13*). **6** is a constituent of tea flavonol and, like **11**, has an *o*-dihydroxyl group in the B-ring. Therefore, the appearance of dicarbonyl carbon signals from the B-ring of oxidized **6** was expected from the ^{13}C NMR spectrum (**Figure 2B**) after the reaction of **6** (0.015 mmol) and **2** (0.030 mmol). However, the original carbon signals ascribable to **6**, not only those in the B-ring but also those in the A-ring, completely disappeared and no new signals appeared, even though **6** possesses a catechol moiety. This suggests that the mechanism underlying the antioxidative effect of **6** does not involve the formation of *o*-quinone and differs from that of **11**. Thus, the difference in the antioxidation mechanisms of **11** and **6** may depend on the presence or absence of the olefinic double bond between the C-2 and C-3 conjugated with the B-ring.

*Reaction of (+)-Taxifolin (**4**) with DPPH (**2**).* The structure of **4**, dihydroquercetin, is the same as that of **6** except that it lacks an olefinic double bond between C-2 and C-3 in the C-ring. Therefore, to confirm whether the difference in antioxidation mechanisms is due to the presence or absence of a double bond between C-2 and C-3 in the C-ring of flavonoids, we selected **4** as a model compound. **4** (0.015 mmol) was also reacted with **2** (0.030 mmol) and subjected to ^{13}C NMR analysis (**Figure 2D**). The characteristic signals of the B-ring [C-2′, 5′ (δ 115.8, 115.9), C-6′ (δ 120.8), C-1′ (δ 129.8), C-3′ (δ 145.7), C-4′ (146.6)] disappeared, and two carbonyl signals (δ 180.5 and 180.8) were clearly detected in the ^{13}C NMR spectrum (**Figure 2D**). This observation confirmed that the *o*-quinone structure was produced after oxidation of the B-ring of **4** similar to **11** (*13*). Taken together, these results clearly confirm that structural differences in **6** and **4**, namely, whether they possess a conjugated double bond at the 2,3-position, affect the mechanism underlying the antioxidative effects of these molecules.

*Reaction of Luteolin (**13**) and Eriodictyol (**14**) with DPPH (**2**).* To verify this, we widened our investigation to other flavonoids and selected **13** and **14** as model compounds. The structure of **13** is the same as that of **14** except that it has an olefinic double bond between C-2 and C-3 in the C-ring. Therefore, these compounds are suitable for comparing differences in the antioxidation mechanisms caused by the presence or absence of a double bond between C-2 and C-3 in the C-ring

of flavonoids. **13** (0.015 mmol), which has a double bond between C-2 and C-3, was reacted with **2** (0.030 mmol) and subjected to ^{13}C NMR analysis (data not shown). Similar to the findings for **6**, the original carbon signals ascribable to **13** disappeared, and no new carbonyl signals appeared. **14** (0.015 mmol), which does not have this double bond, was also reacted with **2** (0.030 mmol) (data not shown). Similar to the findings for **4** and **11**, the B-ring of **14** was oxidized to an *o*-quinone structure. The hydroxyl bond at the 3-position in **6** and **4** seems to have no effect on the differences in the antioxidation mechanisms of these molecules.

*Reaction of Methyl Caffeate (**15**) with DPPH (**2**).* To clarify further the effect of the conjugated olefinic double bond, we investigated model compounds with simpler structures. Caffeic acid is also widely distributed in the plant kingdom and occurs in the form of an ester of quinic acid [chlorogenic acid (3-caffeoylquinic acid)] in tea. We used caffeic acid methyl ester (**15**) as a model compound of the partial B- and C-ring structures in **6**. **15** (0.015 mmol) was reacted with **2** (0.030 mmol) and subjected to ^{13}C NMR analysis (data not shown). The intensities of most of the original carbon signals ascribable to **15** decreased, but no new carbon signals corresponding to *o*-quinone were observed, even though **15** possesses a catechol structure. This observation indicates that the mechanism underlying the antioxidative effect of **15** is also similar to those of **5**, **6**, and **13**. Taken together, these results suggest that the differences in the two antioxidation mechanisms observed for polyphenols possessing a catechol structure are due to the existence of either a conjugated olefinic double bond or a carbonyl bond in the side chain of the catechol group (*21*).

*Reaction of Methyl Hydrocaffeate (**16**) with DPPH (**2**).* To verify this hypothesis, hydrocaffeic acid methyl ester (**16**) (0.015 mmol), which has no conjugated double bond at the 2-position, was also reacted with **2** (0.030 mmol) and subjected to ^{13}C NMR analysis. Most of the original carbon signals ascribable to **16** [C-8 (δ 29.8), C-7 (δ 35.3), C-2 (δ 114.8), C-5 (δ 114.9), C-6 (δ 119.1), C-1 (δ 132.2), C-3 (δ 142.9), C-4 (144.4)] disappeared, and two carbonyl signals (δ 179.0 and 179.8) were clearly detected in the ^{13}C NMR spectrum (data not shown). This observation confirms that an *o*-quinone structure is produced after oxidation of the catechol moiety of **16**, similar to the oxidation of **3**, **4**, **11**, **12**, and **14**. These results strongly support the suggestion that structural differences in **15** and **16**, namely, the presence of a conjugated double bond at the 2-position, are responsible for the differences in their antioxidation mechanisms.

*Reaction of Quercetin (**6**) and (+)-Taxifolin (**4**) with DPPH (**2**).* To compare directly the relative radical-scavenging activities between a compound with and a compound without this conjugated double bond, a mixture of **6** (0.015 mmol) and **4** (0.015 mmol) was reacted with **2** (0.030 mmol) (**Figure 2E**), and the resulting the ^{13}C NMR spectrum was compared with those of **6** (**Figure 2A**) and **4** (**Figure 2C**). The intensities of most of the original carbon signals ascribable to **6** [C-8 (δ 94.5), C-6 (δ 99.2), C-4a (δ 104.2), C-2′ (δ 115.8), C-5′ (δ 116.2), C-6′ (δ 121.5), C-1′ (δ 123.8), C-3 (δ 136.8), C-3′ (δ 145.8), C-2 (δ 146.9), C-4′ (δ 148.3), C-8a (δ 157.8), C-5 (δ 162.3), C-7 (δ 165.0), C-4 (δ 176.6)] decreased. However, the signals of **4** [C-3 (δ 73.2), C-2 (δ 84.5), C-8 (δ 96.0), C-6 (δ 97.1), C-4a (δ 101.6), C-2′, -5′ (δ 115.8, 115.9), C-6′ (δ 120.8), C-1′ (δ 129.8), C-3′ (δ 145.7), C-4′ (δ 146.6), C-8a (δ 164.2), C-5 (δ 165.0), C-7 (δ 167.8), C-4 (δ 198.2)], not only those of the A-ring but also those of the B-ring, remained. In addition, the two carbonyl signals (δ 180.5 and 180.8), which were representative of **4** in its oxidized form (**Figure 2D**), were not detected, indicating that **4** did not scavenge **2**. Therefore, this observation suggests that radicals of DPPH are scavenged more rapidly by **6** than by **4**. Similarly, radicals of DPPH were scavenged more rapidly by **13** than by **14** and more rapidly by **15** than by **16** (data not shown). These results indicate that *o*-dihydroxyl phenolic compounds that possess a conjugated olefinic double bond (e.g., **6**) have a superior radical-trapping ability as compared with those that do not possess this type of double bond (**3**, **4**, **11**, and **12**), whereas *o*-dihydroxyl phenolic compounds that possess a conjugated carbonyl bond (e.g., **5**) have an inferior radical-trapping ability as compared with those that lack this bond.

Reaction of Vicinal Trihydroxyl Phenolic Compounds with DPPH (2). *Reaction of Pyrogallol (**8**) with DPPH (**2**).* In addition, we used **8** as a model of polyphenols such as **10** that possess a pyrogallol structure. When **8** (0.015 mmol) was reacted with **2** (0.030−0.045 mmol, gradually) and subjected to ^{13}C NMR analysis, the original carbon signals ascribable to **8** decreased and no new signals appeared (**Figure 3B**). This indicates that **8** is stabilized as a radical and does not yield an *o*-quinone moiety, similar to **5**, **6**, and, **10** (*13*) after reaction with **2**.

*Reaction of Myricetin (**7**) with DPPH (**2**).* **7** is also a constituent of tea polyphenol and, like **8**, has a vicinal trihydroxyl phenolic group. When **7** (0.015 mmol) was reacted with **2** (0.030 mmol) and subjected to ^{13}C NMR analysis, the original carbon signals ascribable to **7** completely disappeared and no new signals appeared (**Figure 3D**). This suggests that, like **8**, **7** is stabilized as a radical and does not yield an *o*-quinone structure after reaction with **2**.

*Reaction of Pyrogallol (**8**) and Myricetin (**7**) with DPPH (**2**).* We compared the radical-scavenging abilities between **8** and **7** to investigate the effect of an olefinic double bond conjugated to pyrogallol. When a mixture of **8** (0.015 mmol) and **7** (0.015 mmol) was reacted with **2** (0.030−0.045 mmol, gradually), the signal intensities of **8** decreased more significantly than those of **7** (**Figure 3E**), which suggests that radicals are scavenged more quickly by **8**, and also probably by **10**, than by **7**. Vicinal trihydroxyl phenolic compounds that possess a conjugated olefinic double bond (e.g., **7**) have an inferior radical-trapping ability as compared with those that lack this type of double bond (e.g., **8**), although *o*-dihydroxyl phenolic compounds that possess a conjugated olefinic double bond (e.g., **6**) have a superior radical-trapping ability as compared with those that do not possess this bond (e.g., **3**).

*Reaction of Myricetin (**7**) and Ethyl Gallate (**9**) with DPPH (**2**).* When **9** (**Figure 4A**) was reacted with **2**, no new carbonyl signals appeared (data not shown), although the intensities of the original signals of **9** decreased. **9** is stabilized as a radical and does not yield an *o*-quinone structure after reaction with **2** (*13*, *14*). When a mixture of **7** (0.015 mmol) and **9** (0.015 mmol) was reacted with **2** (0.030 mmol), the signal intensities of **7** decreased more significantly than those of **9** (marked with ○) (**Figure 4B**). Therefore, this observation indicates that vicinal trihydroxyl phenolic compounds that possess a conjugated olefinic double bond have a superior radical-trapping ability as compared with those that possess a conjugated carbonyl bond, such as *o*-dihydroxyl phenolic compounds.

Comparison of Radical-Scavenging Abilities between *o*-Dihydroxyl and Vicinal Trihydroxyl Phenolic Compounds. *Reaction of Ethyl Gallate (**9**) and Quercetin (**6**) with DPPH (**2**).* We also compared the radical-scavenging abilities between vicinal trihydroxyl phenolic compounds and *o*-dihydroxyl

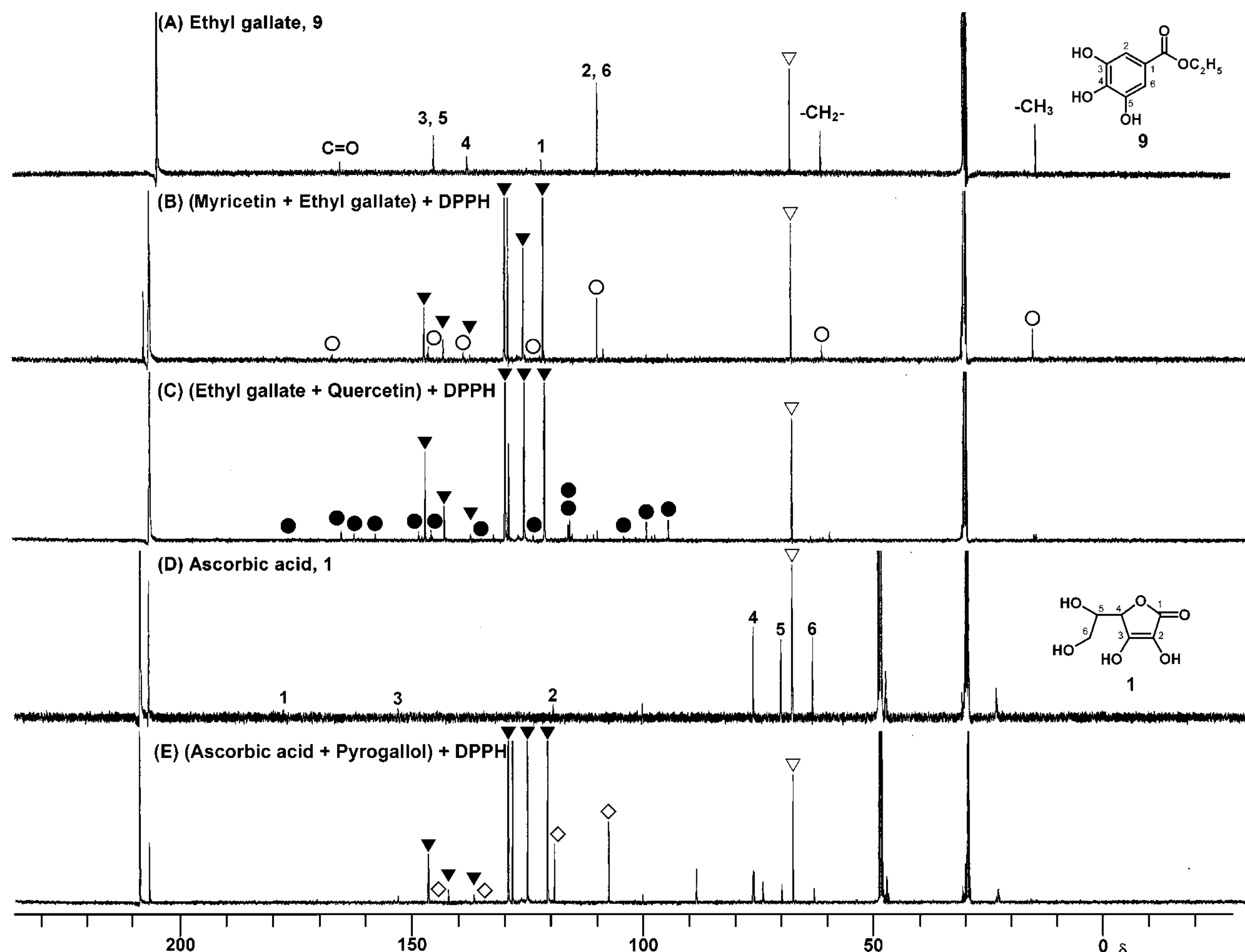

Figure 4. ^{13}C NMR analyses (acetone-d_6, 125 MHz) to examine the reactivity of ethyl gallate (**9**), myricetin (**7**), and quercetin (**6**) with DPPH (**2**) and ^{13}C NMR analyses [acetone-d_6/methanol-d_4 = 1:1 (v/v), 125 MHz] to examine the reactivity of ascorbic acid (**1**) and pyrogallol (**8**) with DPPH (**2**): (**A**) **9** (0.015 mmol); (**B**) **2** (0.030 mmol) added to a mixture of **7** (0.015 mmol) and **9** (0.015 mmol); (**C**) **2** (0.045 mmol) added to a mixture of **9** (0.015 mmol) and **6** (0.015 mmol); (**D**) **1** (0.015 mmol); (**E**) **2** (0.030 mmol) added to a mixture of **1** (0.015 mmol) and **8** (0.015 mmol); (▽) 1,4-dioxane (external standard); (▼) 1,1-diphenyl-2-picrylhydrazine; (○) remaining peaks of **9**; (●) remaining peaks of **6**; (◇) remaining peaks of **8**.

phenolic compounds. When a mixture of **9** (0.015 mmol) and **6** (0.015 mmol) was reacted with **2** (0.030−0.045 mmol, gradually) (**Figure 4C**), the signal intensities of **9** decreased, but no significant changes were observed in the signals of **6**. This observation therefore suggests that radicals of DPPH are scavenged more rapidly by **9** than by **6**. This indicates that the radical-trapping ability of the vicinal trihydroxyl group (pyrogallol structure) is superior to that of the *o*-dihydroxyl group (catechol structure) and that the gallate moiety is more important than the *o*-dihydroxyl group in the B-ring as a radical-scavenging active site in the (−)-epicatechin gallate (**17**) structure.

There have been several studies of radical-scavenging abilities of polyphenols (*1*, *11*, *12*, *22*). Their orders of radical-scavenging abilities are similar to our study on the whole. However, radical-scavenging abilities of **10** and **17** are unclear. The number of radicals of DPPH trapped by **17** is reported to be more than that by **10** at the same molar concentration. Nevertheless, **10** may scavenge radicals more rapidly than **17** when **10** and **17** are both present as in our study.

Comparison of Radical-Scavenging Abilities between Ascorbic Acid (1) and Polyphenol. *Reaction of Ascorbic Acid (1) and Pyrogallol (8) with DPPH (2).* **1** is also known to possess potent antioxidative activity. After **1** and 2 molar equiv of **2** were reacted, the signals of **1** (δ 63.4, 70.4, 76.4, 119.7, 153.4, and 178.0) decreased and new signals appeared (δ 74.6, 74.8, 76.7, 76.9, 89.2, 89.3, 106.8, and 171.1). These signals were ascribed to enantiomers of bicyclic dehydroascorbic acid solvated at the C-2 position (*23*), which would be produced from dehydroascorbic acid formed after the oxidation of **1** by **2** (*14*).

The radical-scavenging ability of **1** was compared to that of **8**, which had been found to have the most potent antioxidative activity of the compounds described above. A mixture of **1** (0.015 mmol) and **8** (0.015 mmol) was reacted with **2** (0.030 mmol), and the resulting spectrum was compared with that of **1** (**Figure 4D**). The signal intensities of **8** did not decrease. Instead, the signals of **1** decreased and the signals corresponding to C-2 solvated bicyclic dehydroascorbic acids appeared (**Figure 4E**). These results clearly show that radicals of DPPH were scavenged more rapidly by **1** than by **8**; in other words, **1** possesses a more potent antioxidative activity than all of the polyphenols described above.

There have been several studies of scavenging abilities of catechins and **1** against radicals of DPPH (*11*, *24*). The number of radicals of DPPH trapped by polyphenol is reported to be more than that by **1** at the same molar concentration. Nevertheless, **1** may scavenge radicals more rapidly than polyphenol when **1** and polyphenol are both present as in our study.

In this ^{13}C NMR study, we have directly compared the relative radical-scavenging activities among antioxidants in tea. These

results can be summarized as follows. First, the radical-trapping ability of vicinal trihydroxyl phenolic (pyrogallol-type) compounds is superior to that of *o*-dihydroxyl phenolic (catechol-type) compounds. Second, the presence of a conjugated carbonyl bond decreases the antioxidative activities of both *o*-dihydroxyl and vicinal trihydroxyl phenolic compounds. The presence of a conjugated olefinic double bond also decreases the antioxidative activities of vicinal trihydroxyl phenolic compounds, but it increases that of *o*-dihydroxyl phenolic compounds. The order of radical-scavenging ability suggested by our study is as follows: **1** > **8**, **10** > **7** > **9**, **17** > **6**, **13**, **15** > **3**, **4**, **11**, **12**, **14**, **16** > **5**.

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Received for review October 12, 2004. Revised manuscript received January 31, 2005. Accepted February 2, 2005.

JF040423A