NMR Analytical Approach to Clarify the Molecular Mechanisms of the Antioxidative and Radical-Scavenging Activities of Antioxidants in Tea Using 1,1-Diphenyl-2-picrylhydrazyl

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(+)-Catechin, ethyl gallate, ascorbic acid, and α-tocopherol were reacted with 1,1-diphenyl-2-picrylhydrazyl (DPPH), and the reaction mixtures were subjected to ^{13}C-nuclear magnetic resonance (NMR) analyses to clarify the molecular mechanisms of the antioxidative and radical-scavenging activities of each antioxidant. When ascorbic acid was reacted with DPPH, it was oxidized to dehydroascorbic acid by DPPH. When a mixture of ascorbic acid and (+)-catechin was reacted with DPPH, ascorbic acid scavenged DPPH radical faster than (+)-catechin. Ascorbic acid also scavenged DPPH radical faster than ethyl gallate and α-tocopherol. When (+)-catechin was reacted with DPPH, the B-ring of (+)-catechin changed to an *o*-quinone structure. However, it was reduced to (+)-catechin by ethyl gallate or α-tocopherol. α-Tocopherol and ethyl gallate had almost identical antioxidative activities. Therefore, the order of radical-scavenging ability (speed) suggested by our ^{13}C NMR study was as follows: ascorbic acid > α-tocopherol = ethyl gallate > (+)-catechin.

Keywords: *Antioxidation mechanism; NMR; DPPH; catechins; antioxidants*

INTRODUCTION

Tea (*Camellia sinensis*) leaves contain various antioxidants such as ascorbic acid, tocopherol, and tea catechins (Goto et al., 1996; Yamamoto et al., 1996). Tea catechins (flavan-3-ol derivatives) are known to possess potent antioxidative activities (Matsuzaki and Hara, 1985). The pyrogallol structure in the B-ring and the galloyl moiety at the 3 position of the flavan-3-ol skeleton are important for the radical-scavenging activity (Nanjo et al., 1996; Nanjo et al., 1999). We have already demonstrated differences in antioxidation mechanisms between the catechol and pyrogallol structures of tea catechins using the stable radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), by nuclear magnetic resonance (NMR) analysis (Sawai and Sakata, 1998). In this study, we used a new NMR analytical approach to clarify the molecular mechanisms of the antioxidative and radical-scavenging activities of several kinds of antioxidants using DPPH. We directly compared the reactivities of (+)-catechin, ethyl gallate, ascorbic acid, and α-tocopherol against radicals by ^{13}C NMR analysis.

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 min [125 MHz; 5 mm cell; spectral width, 33 898 Hz; 45° pulse; pulse repetition time 1 s; 4000 times except for dehydroascorbic acid (20 000 times)]. Chemical shifts were expressed as δ values using tetramethylsilane as an internal standard.

Sample Preparation for NMR Measurement. *(1) Reaction of Ascorbic Acid with DPPH.* Ascorbic acid (0.015 mmol) in methanol-d_4 (0.7 mL) was subjected to ^{13}C NMR analysis. Ascorbic acid (0.015 mmol) dissolved in methanol-d_4 (0.35 mL) was also reacted with DPPH (0.030 mmol) in acetone-d_6 (0.35 mL) in an NMR cell. These ^{13}C NMR spectra were compared with that of dehydroascorbic acid (0.015 mmol) in methanol-d_4 (0.7 mL). Ascorbic acid: ^{13}C NMR (methanol-d_4, 125 MHz) δ 173.6 (C-1), 155.1 (C-3), 119.9 (C-2), 77.0 (C-4), 70.8 (C-5), 63.7 (C-6) (Nishikawa and Kurata, 1998) (Figure 1A). Dehydroascorbic acid: ^{13}C NMR (methanol-d_4, 125 MHz, dehydroascorbic acid was solvated at C-2 to afford enantiomeric bicyclic dehydroascorbic acids) δ 172.5 (C-1), 107.4 (C-3), 95.2 (C-2), 89.5 (C-4), 77.1 (C-6), 74.8 (C-5); other form, 171.6 (C-1), 106.9 (C-3), 94.7 (C-2), 89.3 (C-4), 77.0 (C-6), 74.7 (C-5) (Nishikawa and Kurata, 1998) (Figure 1D).

(2) Reaction between Ascorbic Acid, (+)-Catechin, and DPPH. (+)-Catechin (0.015 mmol) was added to the reaction mixture of ascorbic acid (0.015 mmol/0.35 mL methanol-d_4) and DPPH (0.030 mmol/0.35 mL acetone-d_6) as described above. On the other hand, ascorbic acid (0.015 mmol) in methanol-d_4 (0.35 mL) was reacted with the reaction mixture of (+)-catechin (0.015 mmol) and DPPH (0.030 mmol) in acetone-d_6 (0.35 mL) to compare ^{13}C NMR spectral pattern when the reaction order among the compounds was changed. DPPH (0.030 mmol) was also added to the mixture of ascorbic acid (0.015 mmol) in methanol-d_4 (0.35 mL) and (+)-catechin (0.015 mmol) in acetone-d_6 (0.35 mL). (+)-Catechin: ^{13}C NMR (acetone-d_6, 125 MHz) δ 157.7, 157.2, 156.9 (C-5, -7, -8a), 145.7, 145.6 (C-3′, -4′), 132.2 (C-1′), 120.1 (C-6′), 115.7 (C-5′), 115.2 (C-2′), 100.7 (C-4a), 96.1 (C-6), 95.4 (C-8), 82.7 (C-2), 68.3 (C-3), 28.9 (C-4) (Porter et al., 1982) (Figure 2A).

(3) Reaction between Ascorbic Acid, Ethyl Gallate, and DPPH. Ascorbic acid (0.015 mmol) in methanol-d_4 (0.35 mL) was reacted with the reaction mixture of ethyl gallate (0.015 mmol) and DPPH (0.030 mmol) in acetone-d_6 (0.35 mL). DPPH (0.030 mmol) was added to the mixture of ascorbic acid (0.015 mmol) in methanol-d_4 (0.35 mL) and ethyl gallate (0.015 mmol) in acetone-d_6 (0.35 mL). Ethyl gallate: ^{13}C NMR (acetone-d_6,

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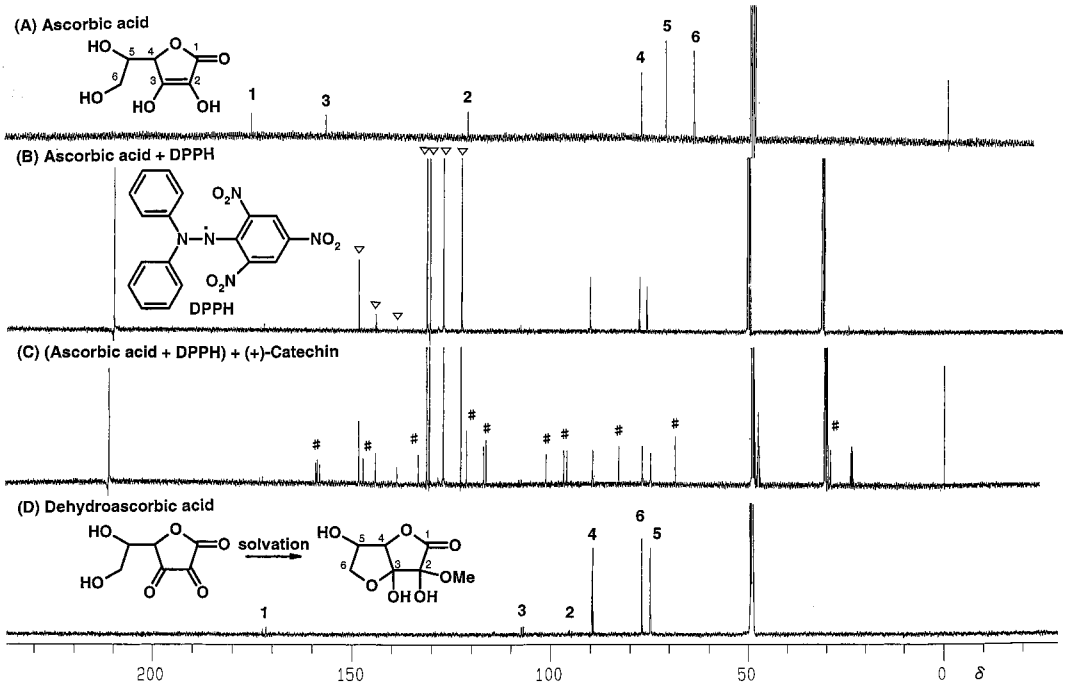

Figure 1. ^{13}C NMR analyses [acetone-d_6:methanol-d_4 = 1:1 (v/v), 125 MHz] to examine reactivity between ascorbic acid, (+)-catechin, and DPPH. (A), ascorbic acid (0.015 mmol); (B), reaction mixture of ascorbic acid (0.015 mmol) and DPPH (0.030 mmol); (C), (+)-catechin (0.015 mmol) was added to the reaction mixture of ascorbic acid (0.015 mmol) and DPPH (0.030 mmol); (D), dehydroascorbic acid (0.015 mmol). ▽, 1,1-diphenyl-2-picrylhydrazine; #, (+)-catechin.

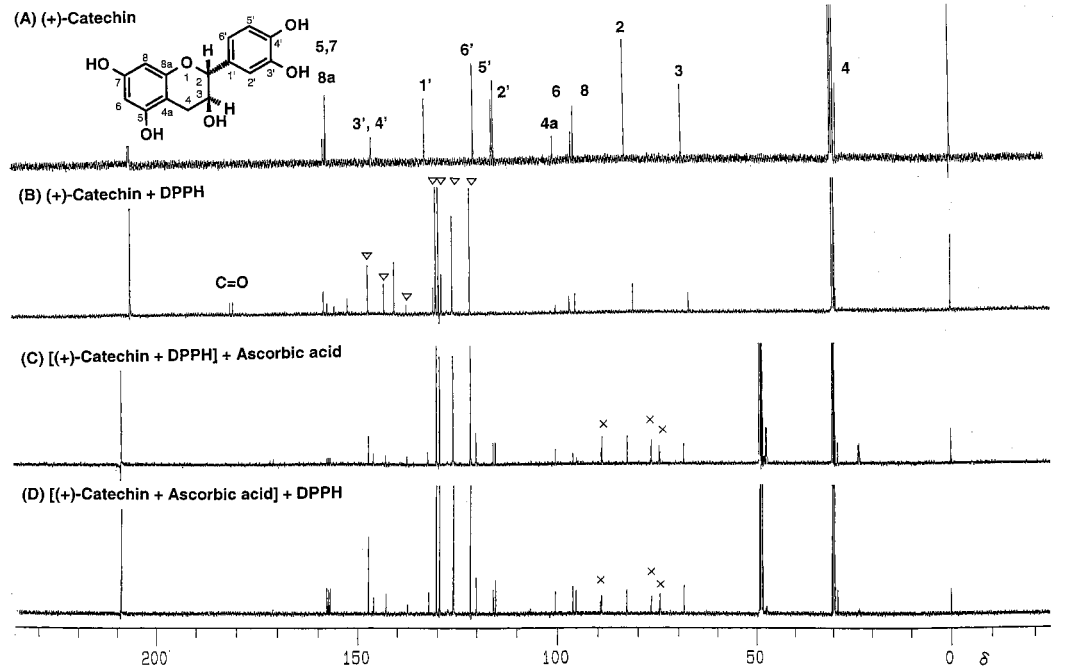

Figure 2. ^{13}C NMR analyses [acetone-d_6:methanol-d_4 = 1:1 (v/v), 125 MHz] to examine reactivity between ascorbic acid, (+)-catechin, and DPPH. (A), (+)-catechin (0.015 mmol); (B), reaction mixture of (+)-catechin (0.015 mmol) and DPPH (0.030 mmol); (C), ascorbic acid (0.015 mmol) was added to the reaction mixture of (+)-catechin (0.015 mmol) and DPPH (0.030 mmol); (D), DPPH (0.030 mmol) was added to the mixture of (+)-catechin (0.015 mmol) and ascorbic acid (0.015 mmol). ▽, 1,1-diphenyl-2-picrylhydrazine; ×, C-2 solvated bicyclic dehydroascorbic acids.

125 MHz) δ 166.7 (C=O), 146.1 (C-3, -5), 138.6 (C-4), 122.1 (C-1), 109.7 (C-2, -6), 60.9 ($-CH_2-$), 14.6 ($-CH_3$) (Nonaka et al., 1983) (Figure 3A).

(4) Reaction between Ethyl Gallate, (+)-Catechin, and DPPH. Ethyl gallate (0.015 mmol) was added to the reaction mixture of (+)-catechin (0.015 mmol) and DPPH (0.030 mmol) in acetone-d_6 (0.7 mL).

(5) Reaction between α-Tocopherol, Ascorbic Acid, and DPPH. α-Tocopherol (0.015 mmol) in acetone-d_6 (0.7 mL) was subjected to ^{13}C NMR analysis and reacted with DPPH (0.030 mmol). A mixture of α-tocopherol (0.015 mmol) and DPPH (0.030 mmol) in acetone-d_6 (0.35 mL) was reacted with ascorbic acid (0.015 mmol) in methanol-d_4 (0.35 mL). DPPH (0.030 mmol) was added to a mixture of ascorbic acid (0.015 mmol) in methanol-d_4 (0.35 mL) and α-tocopherol (0.015 mmol) in acetone-d_6 (0.35 mL). α-Tocopherol: ^{13}C NMR (acetone-d_6, 125 MHz) δ 146.2 (C-8a), 145.9 (C-6), 122.8 (C-8), 122.4 (C-7), 120.4 (C-5), 117.7 (C-4a), 74.8 (C-2), 40.3 (C-1′, -11′), 38.1 (C-3′, -5′, -7′, -9′), 33.3 (C-4′, -8′), 32.4 (C-3), 28.6 (C-12′), 25.5 (C-10′),

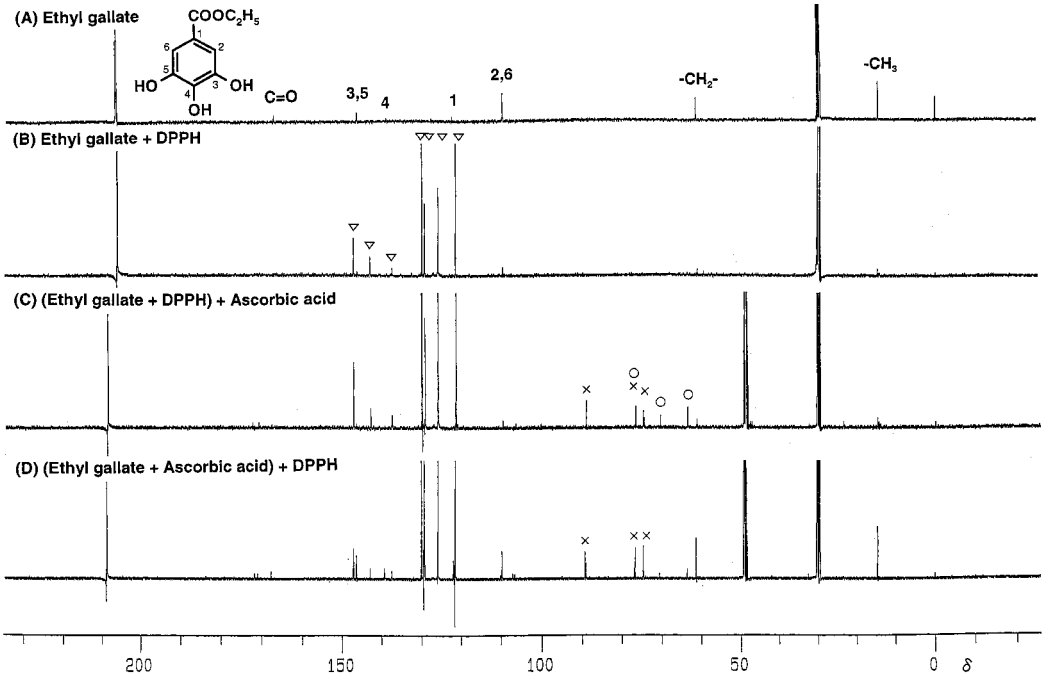

Figure 3. ^{13}C NMR analyses [acetone-d_6:methanol-d_4 = 1:1 (v/v), 125 MHz] to examine reactivity between ethyl gallate, ascorbic acid, and DPPH. (A), ethyl gallate (0.015 mmol); (B), reaction mixture of ethyl gallate (0.015 mmol) and DPPH (0.030 mmol); (C), ascorbic acid (0.015 mmol) was added to the reaction mixture of ethyl gallate (0.015 mmol) and DPPH (0.030 mmol); (D), DPPH (0.030 mmol) was added to the reaction mixture of ethyl gallate (0.015 mmol) and ascorbic acid (0.015 mmol). ▽, 1,1-diphenyl-2-picrylhydrazine; ○, ascorbic acid; ×, C-2 solvated bicyclic dehydroascorbic acids.

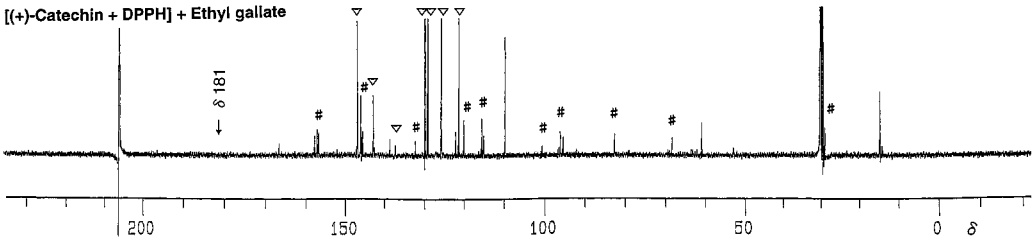

Figure 4. ^{13}C NMR spectrum (acetone-d_6, 125 MHz) after ethyl gallate (0.015 mmol) was added to the reaction mixture of (+)-catechin (0.015 mmol) and DPPH (0.030 mmol). ▽, 1,1-diphenyl-2-picrylhydrazine; #, (+)-catechin.

25.0 (C-6′), 24.1 (C-2a), 22.9 (C-12′a, -13′), 21.7 (C-4, -2′), 20.0 (C-4′a, -8′a), 12.7 (C-7a), 12.0 (C-8b), 11.8 (C-5a) (Matsuo, 1983) (Figure 5A).

(6) Reaction between α-Tocopherol, (+)-Catechin, and DPPH. The mixture of (+)-catechin (0.015 mmol) and DPPH (0.030 mmol) in acetone-d_6 (0.7 mL) was further reacted with α-tocopherol (0.015 mmol).

(7) Reaction between α-Tocopherol, Ethyl Gallate, and DPPH. Ethyl gallate (0.015 mmol) was added to the reaction mixture of α-tocopherol (0.015 mmol) and DPPH (0.030 mmol) in acetone-d_6 (0.7 mL). Another mixture of ethyl gallate (0.015 mmol) and DPPH (0.030 mmol) in acetone-d_6 (0.7 mL) was further reacted with α-tocopherol (0.015 mmol).

All mixtures were reacted for more than 10 min, and after the purple color of DPPH faded, they were subjected to ^{13}C NMR analyses.

RESULTS AND DISCUSSION

Reaction of Ascorbic Acid with DPPH. ^{13}C NMR analysis was carried out to clarify the molecular mechanisms of the antioxidative effects of (+)-catechin, ethyl gallate, ascorbic acid, and α-tocopherol using the stable radical, DPPH. First, we compared the differences in ^{13}C NMR spectra obtained by changing the reaction order among (+)-catechin, ascorbic acid, and DPPH (Figures 1 and 2). After reaction of ascorbic acid and 2 molar equiv of DPPH, the signals of ascorbic acid (δ 63.7, 70.8, 77.0, 119.9, 155.1, and 173.6) completely disappeared, and new signals (δ 74.6, 74.8, 76.7, 76.9, 89.2, 89.3, 106.8, and 171.1) appeared (Figure 1B). These new signals were ascribed to the enantiomers of bicyclic dehydroascorbic acid solvated at C-2 position (Nishikawa and Kurata, 1998) (Figure 1D). It was reported that the C-2 solvated bicyclic dehydroascorbic acid is formed by the solvation of the C-2 carbonyl group of dehydroascorbic acid in aqueous solution such as methanol and ethanol. Therefore, this result indicates that the C-2 solvated bicyclic dehydroascorbic acid was produced from dehydroascorbic acid formed after oxidation of ascorbic acid by DPPH. In addition, it was suggested that one molecule of ascorbic acid scavenges two (or more) molecules of DPPH radical. Other signals (δ 121.5, 126.0, 129.3, 130.1, 137.2, 142.9, and 147.2) were ascribable to 1,1-diphenyl-2-picrylhydrazine (marked with ▽) produced by reduction of DPPH (Figure 1B).

Reaction between Ascorbic Acid, (+)-Catechin, and DPPH. (+)-Catechin (0.015 mmol) was added to the reaction mixture of ascorbic acid (0.015 mmol) and DPPH (0.030 mmol), and the mixture was subjected to ^{13}C NMR analysis (Figure 1C). The signals of C-2 solvated bicyclic dehydroascorbic acids still remained after addition of (+)-catechin. Moreover, two carbonyl

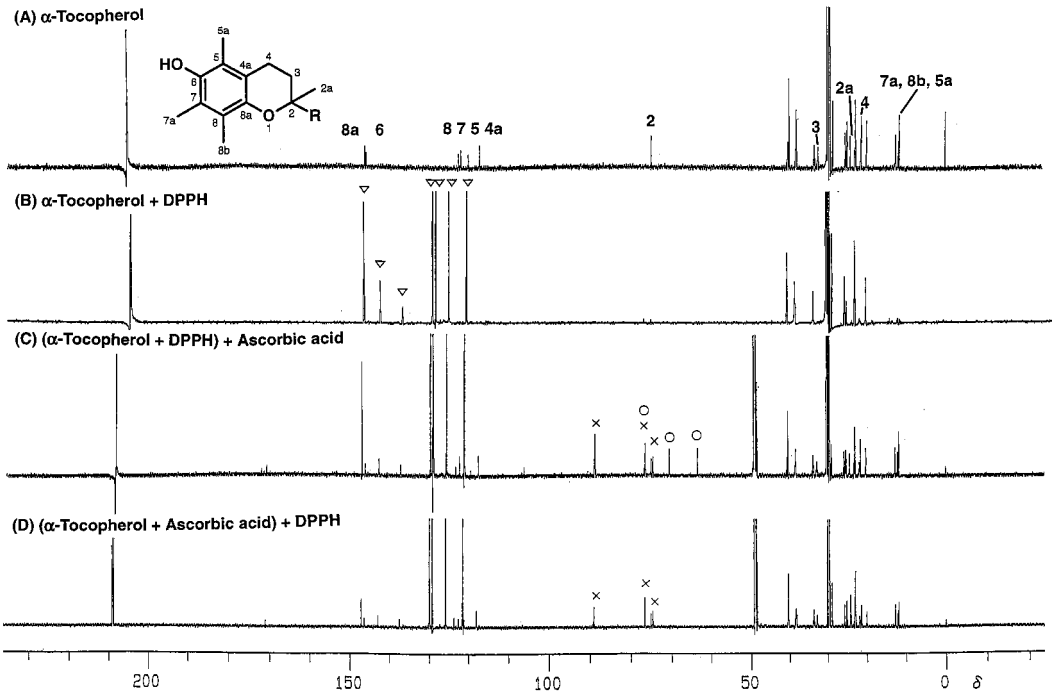

Figure 5. ^{13}C NMR analyses [acetone-d_6:methanol-d_4 = 1:1 (v/v), 125 MHz] to examine reactivity between α-tocopherol, ascorbic acid, and DPPH. (A), α-tocopherol (0.015 mmol); (B), reaction mixture of α-tocopherol (0.015 mmol) and DPPH (0.030 mmol); (C), ascorbic acid (0.015 mmol) was added to the reaction mixture of α-tocopherol (0.015 mmol) and DPPH (0.030 mmol); (D), DPPH (0.030 mmol) was added to the reaction mixture of α-tocopherol (0.015 mmol) and ascorbic acid (0.015 mmol). ▽, 1,1-diphenyl-2-picrylhydrazine; ○, ascorbic acid; ×, C-2 solvated bicyclic dehydroascorbic acids; R, $C_{16}H_{32}$.

signals, the representative signals (δ 180.5 and 181.2) of (+)-catechin in the oxidation form (Sawai and Sakata, 1998), were also not detected. These results suggested that ascorbic acid cannot be regenerated from dehydroascorbic acid by (+)-catechin.

Next, we studied reduction of oxidized (+)-catechin by ascorbic acid. When (+)-catechin (0.015 mmol) was reacted with DPPH (0.030 mmol), the characteristic signals of the B-ring [C-2′ (δ 115.2), C-5′ (δ 115.7), C-6′ (δ 120.1), C-1′ (δ 132.2), C-3′, 4′ (δ 145.6, 145.7)] disappeared and two carbonyl signals (δ 180.5 and 181.2) were clearly detected in the ^{13}C NMR spectrum (Figure 2B). The two carbonyl signals were produced after oxidation of the B-ring of (+)-catechin (Sawai and Sakata, 1998). However, after ascorbic acid (0.015 mmol) was added to the reaction mixture of (+)-catechin and DPPH, the two carbonyl signals completely disappeared, and signals of C-2 solvated bicyclic dehydroascorbic acids (marked with ×) emerged. No signals of ascorbic acid were observed (Figure 2C). This result indicated that one molecule of ascorbic acid regenerates one molecule of (+)-catechin from the oxidized form. In addition, a mixture of ascorbic acid (0.015 mmol) and (+)-catechin (0.015 mmol) was reacted with DPPH (0.030 mmol) and then subjected to ^{13}C NMR analysis (Figure 2D). The two carbonyl signals ascribable to (+)-catechin in the oxidized form were not detected. Moreover, signals of C-2 solvated bicyclic dehydroascorbic acids (marked with ×), but not ascorbic acid, appeared. These results clearly showed that ascorbic acid scavenges DPPH radicals more rapidly than (+)-catechin in the organic solvent system. There have been several studies of scavenging abilities of catechins and ascorbic acid against DPPH radicals (Nanjo et al., 1996; Yoshida et al., 1989). The number of DPPH radicals trapped by (+)-catechin was reported to be more than that by ascorbic acid at the same molar concentration. Nevertheless, ascorbic acid may scavenge radicals more rapidly than (+)-catechin when ascorbic acid and (+)-catechin are both present as shown above (Figures 1 and 2). Therefore, ascorbic acid may spare (+)-catechin in DPPH radical-scavenging when both are present together.

Reaction between Ascorbic Acid, Ethyl Gallate, and DPPH. Ethyl gallate (0.015 mmol) was reacted with DPPH (0.030 mmol), and the reaction solution was subjected to ^{13}C NMR analysis (Figure 3B). No carbonyl signals appeared, although the intensities of most of the original carbon signals ascribable to ethyl gallate [$-CH_3$ (δ 14.6), $-CH_2-$ (δ 60.9), C-2 and 6 (δ 109.7), C-3 and 5 (δ 146.1), and C═O (δ 166.7)] decreased as described previously (Sawai and Sakata, 1998).

Ascorbic acid (0.015 mmol) was added to the reaction mixture of ethyl gallate (0.015 mmol) and DPPH (0.030 mmol) (Figure 3C), and its ^{13}C NMR spectrum was compared with that of the mixture of ethyl gallate and DPPH (Figure 3B). If one molecule of ethyl gallate scavenged two or more molecules of DPPH radical and one molecule of ascorbic acid regenerated one molecule of ethyl gallate, signals of ascorbic acid would not be detected in the spectrum of Figure 3C. However, carbon signals of ascorbic acid (marked with ○) as well as those of C-2 solvated bicyclic dehydroascorbic acids (marked with ×) were observed (Figure 3C). This suggested that ethyl gallate is not easily regenerated from the oxidized form generated after radical-scavenging, even if it is present with ascorbic acid. This indicated that the ethyl gallate radical (oxidized form) has a very stable structure (Sawai and Sakata, 1998; Yoshida et al., 1989).

On the other hand, when ethyl gallate (0.015 mmol) was added to the reaction mixture of ascorbic acid (0.015 mmol) and DPPH (0.030 mmol), dehydroascorbic acid, which was produced after reaction of ascorbic acid and DPPH, was not reduced to ascorbic acid (data not

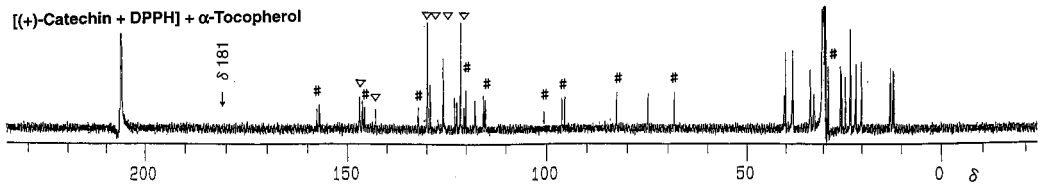

Figure 6. ^{13}C NMR spectrum [acetone-d_6, 125 MHz] after α-tocopherol (0.015 mmol) was added to the reaction mixture of (+)-catechin (0.015 mmol) and DPPH (0.030 mmol). ▽, 1,1-diphenyl-2-picrylhydrazine; #, (+)-catechin.

shown). The mixture of ascorbic acid (0.015 mmol) and ethyl gallate (0.015 mmol) was also reacted with DPPH (0.030 mmol) and subjected to ^{13}C NMR analysis (Figure 3D). The signal intensities of ethyl gallate did not decrease. Instead, the signals of ascorbic acid disappeared, and those of C-2 solvated bicyclic dehydroascorbic acids (marked with ×) appeared. These observations suggested that ascorbic acid scavenges radicals faster than ethyl gallate and has stronger reducing activity than ethyl gallate.

Reaction between Ethyl Gallate, (+)-Catechin, and DPPH. Moreover, we examined the differences in radical-scavenging ability between (+)-catechin and ethyl gallate against DPPH radicals (Figure 4). Ethyl gallate (0.015 mmol) was added to the reaction mixture of (+)-catechin (0.015 mmol) and DPPH (0.030 mmol) followed by ^{13}C NMR analysis. The two carbonyl signals (δ 180.5 and 181.2; Figure 2B), which appeared after reaction of (+)-catechin and DPPH, disappeared, and the characteristic signals of the B-ring [C-2′ (δ 115.2), C-5′ (δ 115.7), C-6′ (δ 120.1), C-1′ (δ 132.2), C-3′, 4′ (δ 145.6, 145.7)] reappeared by addition of ethyl gallate, suggesting that (+)-catechin in the oxidized form after radical-scavenging can be reduced to (+)-catechin by ethyl gallate.

On the other hand, when (+)-catechin (0.015 mmol) was added to the reaction mixture (Figure 3B) of ethyl gallate (0.015 mmol) and DPPH (0.030 mmol), no signals of (+)-catechin in the oxidized form were detected (data not shown). Furthermore, no significant changes were observed in signals of ethyl gallate (Figure 3B) in the oxidized form after reaction of ethyl gallate and DPPH. These observations indicated that oxidized ethyl gallate cannot be reduced by (+)-catechin. It is likely that ethyl gallate has a superior radical-trapping ability to (+)-catechin. (−)-Epicatechin gallate, a representative antioxidant of tea (Nanjo et al., 1999), possesses both a catechol structure (B-ring) and a gallate structure. The gallate moiety was confirmed to be more important than B-ring as a radical-scavenging active site in (−)-epicatechin gallate structure.

Reaction between α-Tocopherol, Ascorbic Acid, and DPPH. α-Tocopherol is regenerated by ascorbic acid from the α-tocopherol radical and/or the oxidation product (Liebler et al., 1989; Mukai et al., 1987; Niki et al., 1984; Niki, 1987; Packer et al., 1979; Sato, 1990). We directly compared the antioxidative abilities between ascorbic acid and α-tocopherol using DPPH radical and ^{13}C NMR. α-Tocopherol (0.015 mmol) was reacted with DPPH (0.030 mmol) and subjected to ^{13}C NMR analysis (Figure 5B). Signals ascribed to the chroman ring [C-5a (δ 11.8), C-8b (δ 12.0), C-7a (δ 12.7), C-4 (δ 21.7), C-2a (δ 24.1), C-3 (δ 32.4), C-2 (δ 74.8), C-4a (δ 117.7), C-5 (δ 120.4), C-7 (δ 122.4), C-8 (δ 122.8), C-6 (δ 145.9), C-8a (δ 146.2)] of α-tocopherol decreased. It was also reported that one molecule of α-tocopherol scavenges 2.0 or 2.5 molecules of radical (Burton and Ingold, 1981; Niki et al., 1984; Moon and Terao, 1998), and then the α-tocopherol is decomposed to α-tocopherylquinone in para-form (Liebler et al., 1990; Matsuo and Matsumoto, 1987; Yamauchi et al., 1990). However, the ^{13}C NMR spectrum shown in Figure 5B showed no carbonyl signals. This suggested that α-tocopherol is stabilized as α-tocopheroxyl radicals in this reaction system (Yamauchi, 1997) similarly to ethyl gallate (Sawai and Sakata, 1998; Figure 3B).

Next, ascorbic acid (0.015 mmol) was added to the reaction solution of α-tocopherol (0.015 mmol) and DPPH (0.030 mmol) followed by ^{13}C NMR analysis (Figure 5C). The decreased signals ascribed to the chroman ring of α-tocopherol after reaction with DPPH (Figure 5B) were recovered by addition of ascorbic acid. If one molecule of α-tocopherol scavenged two molecules of DPPH radical and one molecule of ascorbic acid regenerated one molecule of α-tocopherol, signals of ascorbic acid would not have been detected in the spectrum of Figure 5C. However, signals of ascorbic acid (marked with ○) and C-2 solvated bicyclic dehydroascorbic acids (marked with ×) coexisted in the spectrum. α-Tocopherol is likely to be hardly regenerated from the conjugated structure of α-tocopherol radical and/or any oxidation product from α-tocopherol after radical-scavenging, even if it is present along with ascorbic acid. In addition, α-tocopherol (0.015 mmol) was added to the mixture of ascorbic acid (0.015 mmol) and DPPH (0.030 mmol) (data not shown). The ^{13}C NMR spectrum showed that the α-tocopherol added after oxidation of ascorbic acid was not able to reduce dehydroascorbic acid. These results suggested that ascorbic acid can regenerate α-tocopherol from the radical form, but the reverse seems not to occur.

DPPH (0.030 mmol) was reacted with a mixture of ascorbic acid (0.015 mmol) and α-tocopherol (0.015 mmol) (Figure 5D). Signals of α-tocopherol did not change, and signals of C-2 solvated bicyclic dehydroascorbic acids (marked with ×), not ascorbic acid, were detected after the reaction. Therefore, ascorbic acid was confirmed to scavenge radicals more rapidly than α-tocopherol in the reaction. Ascorbic acid can act synergistically with α-tocopherol by reducing the α- tocopheroxyl radical to regenerate α-tocopherol (Packer et al., 1979; Doba et al., 1985; Sato et al., 1990). However, to our knowledge, this is the first report of the direct comparison of radical-scavenging ability between ascorbic acid and α-tocopherol using NMR.

Reaction between α-Tocopherol, (+)-Catechin, and DPPH. The radical-scavenging abilities of (+)-catechin and α-tocopherol were also compared in the same manner (Figure 6). α-Tocopherol (0.015 mmol) was added to the reaction mixture (Figure 2B) of (+)-catechin (0.015 mmol) and DPPH (0.030 mmol). Two carbonyl signals (δ 180.5 and 181.2; Figure 2B) from oxidized (+)-catechin disappeared, and the typical signals of the B-ring [C-2′ (δ 115.2), C-5′ (δ 115.7), C-6′ (δ 120.1), C-1′ (δ 132.2), C-3′, 4′ (δ 145.6, 145.7)] reappeared after the addition of α-tocopherol (Figure 6).

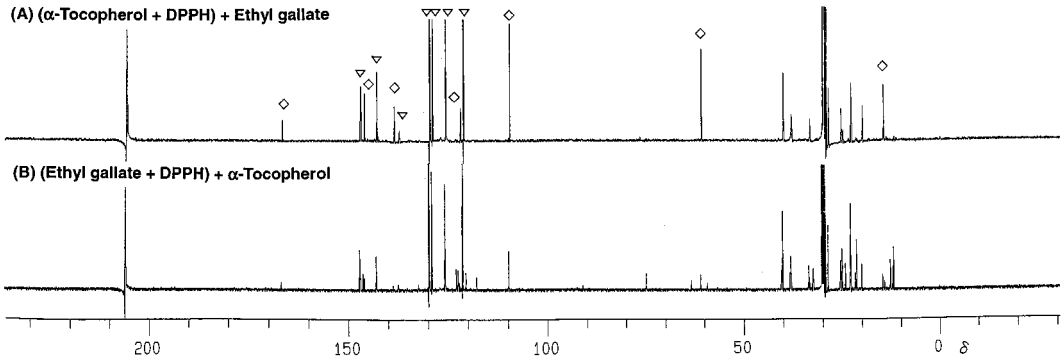

Figure 7. ^{13}C NMR analyses (acetone-d_6, 125 MHz) to examine reactivity between α-tocopherol, ethyl gallate, and DPPH. (A), ethyl gallate (0.015 mmol) was added to the reaction mixture of α-tocopherol (0.015 mmol) and DPPH (0.030 mmol); (B), α-tocopherol (0.015 mmol) was added to the reaction mixture of ethyl gallate (0.015 mmol) and DPPH (0.030 mmol). ▽, 1,1-diphenyl-2-picrylhydrazine; ◇, ethyl gallate.

Thus, α-tocopherol reduced the oxidized (+)-catechin. On the other hand, when (+)-catechin (0.015 mmol) was added to the reaction mixture (Figure 5B) of α-tocopherol (0.015 mmol) and DPPH (0.030 mmol) (data not shown), the two carbonyl signals were not detected in the ^{13}C NMR spectrum. The decreases in chroman signals of α-tocopherol after reaction with DPPH (Figure 5B) were not recovered by addition of (+)-catechin. (+)-Catechin was confirmed to not be oxidized in the reaction system until α-tocopherol was exhausted. One molecule of α-tocopherol was found to scavenge two and/or more molecules of DPPH radical, because (+)-catechin was not oxidized even after addition of (+)-catechin to the reaction mixture of α-tocopherol and DPPH (1:2; molar ratio).

Reaction between α-Tocopherol, Ethyl Gallate, and DPPH. Last, we examined the reaction between ethyl gallate, α-tocopherol, and DPPH. Ethyl gallate (0.015 mmol) was added to the reaction mixture of α-tocopherol (0.015 mmol) and DPPH (0.030 mmol) following by ^{13}C NMR analysis (Figure 7A). The decreases in chroman signals of α-tocopherol after reaction with DPPH (Figure 5B) were not recovered by addition of ethyl gallate. The signals of ethyl gallate (marked with ▽) did not show any changes. Ethyl gallate was not able to regenerate α-tocopherol from its radical form and/or any oxidation product. On the other hand, when α-tocopherol (0.015 mmol) was added to the reaction mixture of ethyl gallate (0.015 mmol) and DPPH (0.030 mmol) (Figure 7B), the decreases in intensity of ethyl gallate signals after reaction with DPPH (Figure 3B) were not recovered. The signals of α-tocopherol did not show any changes. α-Tocopherol was also not able to regenerate ethyl gallate from the oxidized form. These results indicated that α-tocopherol and ethyl gallate have similar levels of antioxidative activity.

The results of our ^{13}C NMR study of the mechanisms of antioxidative effects of several antioxidants using DPPH can be summarized as follows. First, the structural changes of antioxidants after scavenging of radicals can be directly observed at the molecular level. This allowed us to determine the active site of each antioxidant in each radical-scavenging reaction. Second, the relative radical-scavenging activity (speed) can be compared between antioxidants. Last, it may be possible to predict the number of radicals trapped by each antioxidant. Further studies of these points are currently in progress in our laboratory.

ABBREVIATIONS USED

NMR, nuclear magnetic resonance; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

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