NMR Analytical Approach To Clarify the Antioxidative Molecular Mechanism of Catechins Using 1,1-Diphenyl-2-picrylhydrazyl

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Each tea catechin was reacted with 1,1-diphenyl-2-picrylhydrazyl (DPPH), and the reaction mixture was subjected to NMR analysis. The antioxidation mechanism of (+)-catechin [(+)-C] is considered to be due to the change of the B-ring to an *o*-quinone structure at first because of the appearance of two carbonyl signals. This is substantiated by trapping the compound as an adduct of a 1,2-phenylenediamine to an *o*-quinone. (−)-Epicatechin [(−)-EC] was also confirmed to give a similar result, but in the case of (−)-epigallocatechin [(−)-EGC] and ethyl gallate (EG) no carbonyl signals were observed. The antioxidation mechanisms of (−)-EGC and EG are different from those of (+)-C and (−)-EC. This may be one of the reasons for the differences of the antioxidative activities between the two types of catechins.

Keywords: *Antioxidation mechanism; NMR; DPPH; (+)-catechin; tea catechins*

INTRODUCTION

Tea catechins (flavan-3-ol derivatives) are known to possess potent antioxidative activities. Several investigations on the activities of tea catechins have been attempted (Matsuzaki and Hara, 1985; Hirose et al., 1990). The antioxidation mechanism is considered to be due to their radical scavenging ability. Oxidation products of (+)-catechin [(+)-C] have been identified by Nakayama and Hirose (1994) and Kobayashi (1994). Recently Nanjo et al. (1996) reported that the B-ring is important in the antioxidative activity of (+)-C. We tried to clarify the antioxidation mechanism using a free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), on a molecular basis.

MATERIALS AND METHODS

^{1}H- and ^{13}C-NMR Spectral Measurement. ^{1}H- and ^{13}C-NMR spectra of the reaction mixtures and some authentic specimens were measured with a JEOL JSX-270 FT-NMR spectrometer. Chemical shifts are expressed as δ values using TMS as an internal standard.

Sample Preparation for NMR Measurement. *(1) Reaction of (+)-C, (−)-Epicatechin [(−)-EC], and (−)-Epigallocatechin [(−)-EGC].* After the ^{1}H- and ^{13}C-NMR spectral measurement of (+)-C (4.35 mg, 0.015 mmol) in acetone-d_6 (0.7 mL), DPPH (11.82 mg, 0.030 mmol) was added into the NMR cell. The purple color of DPPH faded gradually. The reaction mixture was then subjected to ^{1}H- and ^{13}C-NMR analyses. (−)-EC and (−)-EGC were treated in the same way.

(2) Reaction of Ethyl Gallate (EG). EG (2.97 mg, 0.015 mmol) in acetone-d_6 (0.7 mL) was reacted with DPPH (5.91 mg, 0.015 mmol). The same treatment was repeated three times. Total amount of added DPPH was 0.045 mmol.

Isolation of Compound A (1). (+)-C (43.5 mg, 0.15 mmol in acetone) was reacted with DPPH (118.2 mg, 0.30 mmol), and after the color faded, the mixture was further reacted with 1,2-phenylenediamine (16.2 mg, 0.15 mmol). The reaction mixture was chromatographed on a silica gel column ($CHCl_3$/MeOH). Each product showing R_f value higher than that of (+)-C was subjected to NMR analysis to find a phenylenediamine adduct: compound A (**1**) [4.0 mg; R_f = 0.4, silica gel GF_{254} (Merck), $CHCl_3$/MeOH = 9:1] EI-MS, *m*/*z* (%) 360 (M^+, 100), 222 (95), 193 (44), 169 (53), 139 (25); HR-EIMS, *m*/*z* 360.1090 (M^+, −2.0 mmu for $C_{21}H_{16}N_2O_4$); ^{13}C NMR (acetone-d_6, 67.5 MHz) δ 158.0, 157.3, 156.5 (C-5,-7,-8a), 127.6−131.5 (phenazyl), 96.6 (C-6), 95.5 (C-8), 82.4 (C-2), 68.3 (C-3) (Figure 3); ^{1}H NMR (acetone-d_6, 270 MHz) δ 7.80−8.50 (phenazyl), 6.12 (H-8), 6.05 (H-6), 5.09 (H-2), 4.35 (H-3).

RESULTS AND DISCUSSION

First of all, (+)-C (0.015 mmol) was reacted with DPPH (0.030 mmol) in acetone-d_6 (0.7 mL) as a model reaction of catechins. The purple color of DPPH faded gradually. The reaction mixture was subjected to ^{1}H- and ^{13}C-NMR analyses. The spectra were compared with those of (+)-C (Porter et al., 1982). The characteristic signals due to H-2′, -5′, and -6′ (δ 6.75−6.96) (B-ring) decreased, although those signals ascribable to H-6 (δ 5.86) and H-8 (δ 6.02) (A-ring) remained unchanged. The characteristic signals due to C-2′ (δ 115.3) and C-5′ (δ 115.7) (B-ring) decreased, although the signals ascribable to C-6 (δ 96.0) and C-8 (δ 95.5) (A-ring) remained unchanged (Figure 1). These strongly suggest that the two hydroxyl groups of the B-ring are more important as radical scavengers among the four phenolic hydroxyl groups of (+)-C, in good accordance with the conclusion by Nanjo et al. (1996). They obtained peracetylated (+)-C and its glycoside and measured their radical scavenging ability using ESR. The importance of the B-ring was also mentioned in their paper.

The B-ring has two hydroxyl groups. A 2-fold amount of DPPH (0.030 mmol) completely reacted with 0.015

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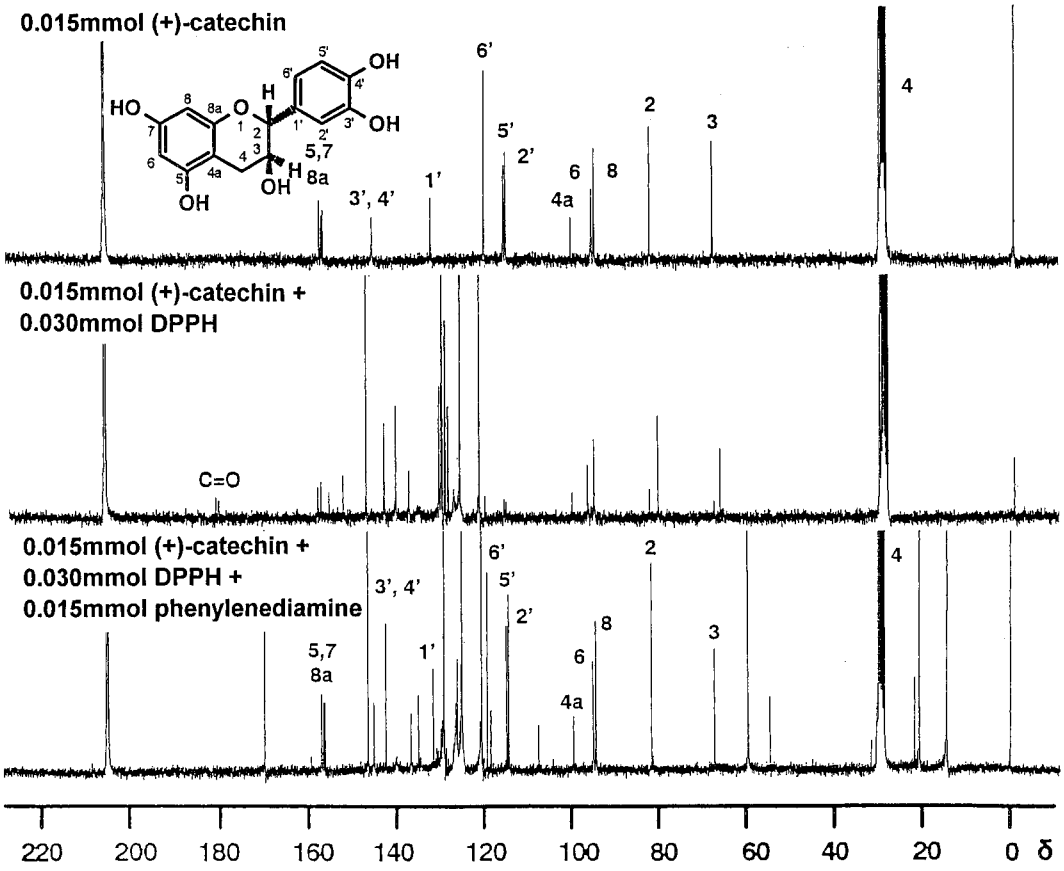

Figure 1. ^{13}C-NMR spectra of (+)-C and its reaction mixtures (acetone-d_6, 67.5 MHz).

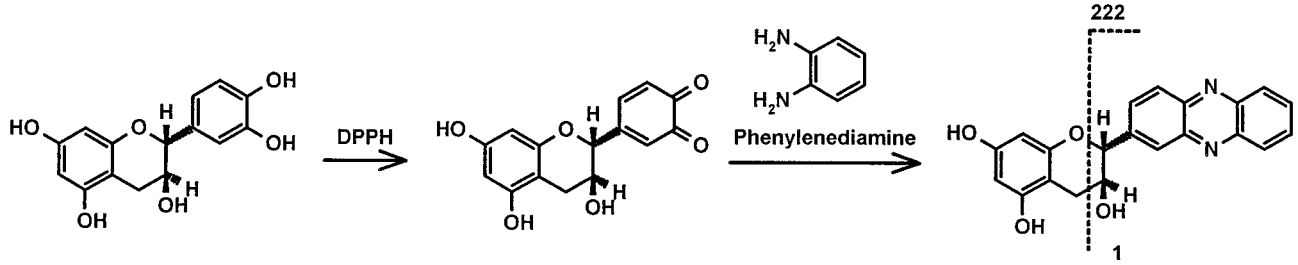

Figure 2. Reactions of (+)-C with DPPH and then with 1,2-phenylenediamine.

mmol of (+)-C. The appearance of two carbonyl signals (δ 180.5 and 181.2) suggested that the B-ring has been changed to a quinone structure (Figure 2). To substantiate this, (+)-C in acetone was reacted with DPPH and then with 1,2-phenylenediamine. The reaction mixture was subjected to ^{13}C-NMR measurement (the bottom of Figure 1). Typical signals ascribable to (+)-C (C-1′, -2′, -3′, -4′, -5′, -6′, etc.) reappeared, indicating that most *o*-quinones were reduced to (+)-C. However, it is possible that a small amount of compound A (**1**, a phenylenediamine adduct to an *o*-quinone) has been formed in this reaction mixture.

The mixture was then chromatographed on silica gel. Each of the products, which appeared at a higher R_f region than did (+)-C on silica gel TLC, was subjected to ^{1}H- and ^{13}C-NMR analyses and compared with phenazine [^{13}C NMR (acetone-d_6, 67.5 MHz) δ 144.9, 131.5, 130.6 (Figure 3); ^{1}H NMR (acetone-d_6, 270 MHz) δ 7.94−8.32 (8H)]. The ^{13}C-NMR spectrum (Figure 3) of a product, compound A (**1**), strongly suggests that **1** is a phenazine derivative produced by a condensation reaction with phenylenediamine and the diketone (Figure 2) formed by DPPH oxidation of (+)-C. The EI mass spectrum of **1** showed its molecular ion peak at *m/z* 360 as a base peak. A major fragment at *m/z* 222 (95%) is reasonably ascribed to the phenazyl moiety yielded by the cleavage shown in Figure 2. HR-EIMS analyses gave $C_{21}H_{16}N_2O_4$ for the M^+ [*m/z* 360.1090 (−2.0 mmu)] to confirm the proposed structure.

On the basis of the foregoing we showed here that the antioxidation mechanism of (+)-C is due to the change of the B-ring to an *o*-quinone structure (Figure 2). (−)-EC was also confirmed to give a similar result. The appearance of two carbonyl signals (δ 180.4 and 181.4 in this case) indicates the same conclusion.

(−)-EGC was reacted with DPPH, but no carbonyl signals appeared (Figure 4), although many original carbon signals decreased. The antioxidation mechanism of (−)-EGC (pyrogallol structure) is likely to be different from those of (+)-C and (−)-EC (catechol structures). (−)-EGC seems to give a rather stable radical (Figure 5) and not to be oxidized to an *o*-quinone. Matsuzaki and Hara (1985) reported that the antioxidative activity of catechins increased in molarity in the following

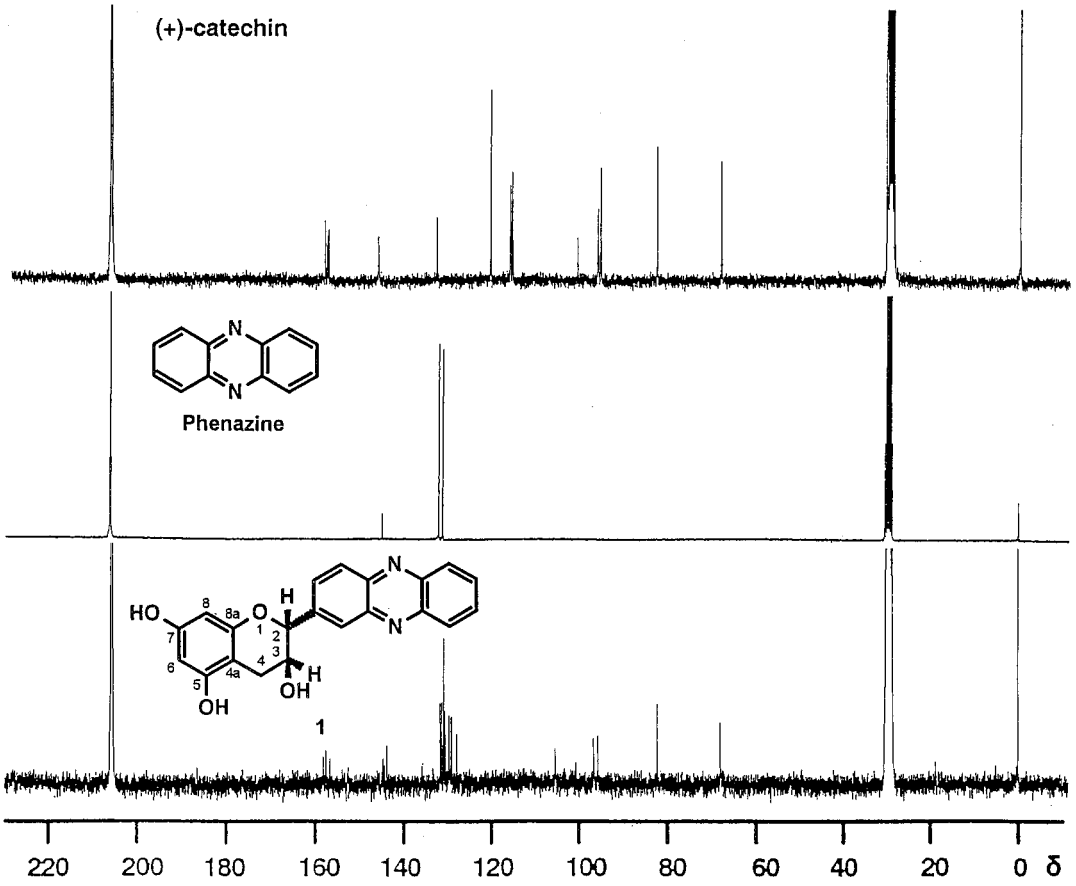

Figure 3. ^{13}C-NMR spectra of (+)-C, phenazine, and compound A (**1**) (acetone-d_6, 67.5 MHz).

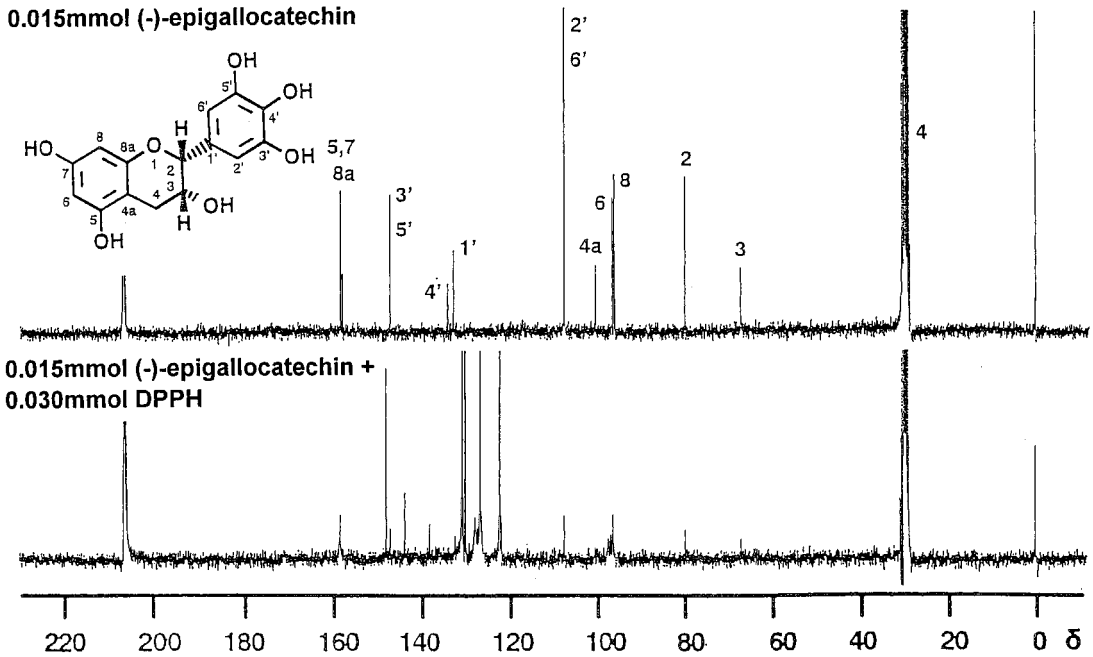

Figure 4. ^{13}C-NMR spectra of (−)-EGC and its reaction mixture with DPPH (acetone-d_6, 67.5 MHz).

order: EC < epicatechin gallate (ECg) < EGC < epigallocatechin gallate (EGCg). Especially notable, the activities of EGC and EGCg (pyrogallol type) are 3 times as high as those of EC and ECg (catechol type). Matsuzaki and Hara (1985) mentioned that a hydroxyl group at C-5′ is important in antioxidative activity. We clarified that these differences of the antioxidative activities were due to the differences of the antioxidation mechanisms.

When ethyl gallate (EG) was reacted with DPPH, the characteristic signal due to ester carbonyl (δ 166.6) decreased. This suggests that the radical may be stabilized as in the forms (Figure 6) not to yield an *o*-quinone. These observations support the more potent

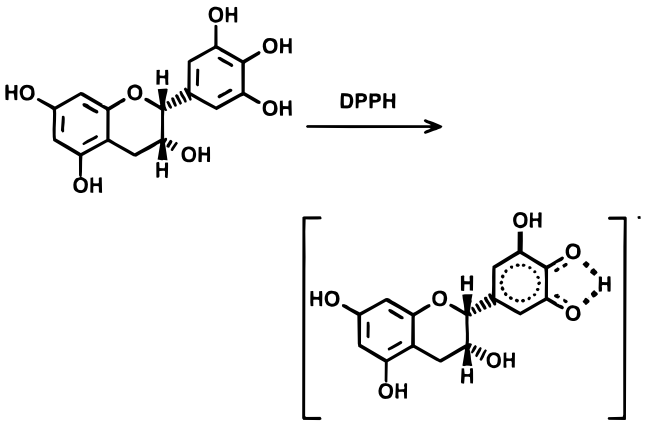

Figure 5. Plausible structure of an (−)-EGC radical generated by DPPH.

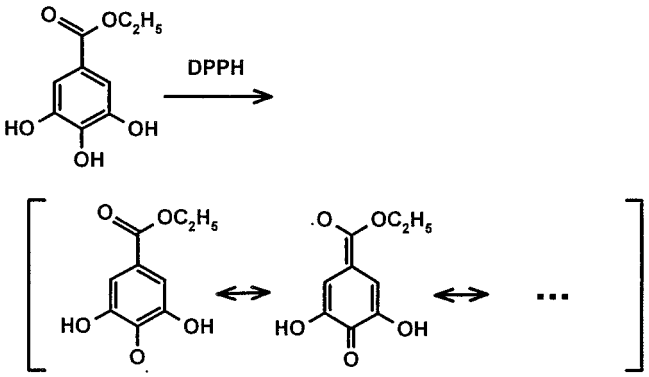

Figure 6. Plausible structures of EG radicals generated by DPPH.

antioxidative activities of (−)-epigallocatechin gallate compared with other catechins.

ABBREVIATIONS USED

DPPH, 1,1-diphenyl-2-picrylhydrazyl; (+)-C, (+)-catechin; (−)-EC, (−)-epicatechin; (−)-EGC, (−)-epigallocatechin; EG, ethyl gallate; ECg, epicatechin gallate; EGCg, epigallocatechin gallate; TMS, tetramethylsilane.

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