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Synergistic Effects of Thiols and Amines on Antiradical Efficiency of Protocatechuic Acid

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DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of protocatechuic acid and its structural analogues (methyl protocatechuate, 3′,4′-dihydroxyacetophenone, 3,4-dihydroxybenzaldehyde, and 3,4-dihydroxybenzonitrile) were examined in aprotic and protic solvents. In aprotic acetonitrile, all test compounds scavenged two radicals. In protic methanol, however, these compounds rapidly scavenged five radicals except for protocatechuic acid, which consumed only two radicals. The result indicated that higher radical scavenging activity in methanol than in acetonitrile was due to a nucleophilic addition of the methanol molecule on the oxidized quinones, which led to a regeneration of catechol structures. To investigate the importance of the nucleophilic addition on the quinones for the high radical scavenging activity, DPPH radical scavenging activity of protocatechuic acid and its analogues was examined in the presence of a variety of nucleophiles. The addition of a strong nucleophile such as a cysteine derivative significantly increased the radical scavenging equivalence. Furthermore, thiol adducts at C-2 and C-2,5 of protocatechuic acid and its analogues were isolated from the reaction mixtures. These results strongly suggest that the quinone of protocatechuic acid and its analogues undergo a nucleophilic attack at C-2 to yield 2-substituted-3,4-diols. Then, a regenerated catechol moiety of adducts scavenge two additional radicals by reoxidation into quinones, which undergo the second nucleophilic attack at the C-5. This mechanism demonstrates a possibility of synergistic effects of various nucleophiles on the radical scavenging ability of plant polyphenols containing a 3,4-dihydroxy substructure like protocatechuic acid and its analogues.

KEYWORDS: Protocatechuic acid; radical scavenging mechanism; antioxidant; DPPH radical; synergistic effect

INTRODUCTION

Phenolic acids and their esters are widely distributed as plant constituents that are known for their antioxidant activities (*1*−*14*). The radical scavenging activity of phenolic acids depends on the number and arrangement of phenolic hydroxyl groups in the molecule (*1*, *2*). Catechol-type *o*-diphenols such as protocatechuic acid (3,4-dihydroxybenzoic acid, **1**) and caffeic acid are typical molecules that show potent antiradical activity (*1*−*12*). Although many studies have been reported on the formation of *o*-quinones, little has been known about the reaction mechanism beyond quinones. In the previous paper, we reported the solvent dependency of radical scavenging activity of protocatechuic acid and its esters (*3*). In aprotic acetone, protocatechuic acid and its esters consumed two radicals and were converted to their quinones. In protic methanol or ethanol, however, protocatechuic esters rapidly scavenged more than four radicals with a concomitant conversion to quinones, their 3-hemiacetals, and further oxidative products (*15*). Alcohol-adducts at the 2-position of the ring were identified in the reaction mixtures. We found that high radical scavenging activity of protocatechuic esters in protic solvents is due to the regeneration of their catechol structures by a nucleophilic addition of the alcohol molecule on the quinones (*3*).

In this study, to examine the effect of the electron-withdrawing substituents on the catechol ring, the DPPH radical scavenging activity of protocatechuic acid analogues such as 3′,4′-dihydroxyacetophenone (**3**), 3,4-dihydroxybenzaldehyde (**4**), and 3,4-dihydroxybenzonitrile (**5**) in aprotic and protic solvents was evaluated. Furthermore, to confirm the importance of the nucleophilic addition on quinone for further antiradical reaction, we examined the radical scavenging activity of protocatechuic acid and its analogues in the presence of a nucleophile such as thiols and amines. Although conjugation of thiols with quinones of phenolic acids is a well-known phenomenon, few study reported on synergistic effects of thiols and amines on the antioxidant activity of phenolic compounds. The aim of the study is to examine the changes in the radical scavenging equivalence of protocatechuic acid and its analogues by the addition of

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various nucleophiles and to propose the radical scavenging mechanism of these catechol derivatives in the presence of a nucleophile.

MATERIALS AND METHODS

Chemicals. 3,4-Dihydroxybenzaldehyde and 3′,4′-dihydroxyacetophenone were purchased from Tokyo Kasei Kogyo Co. 3,4-Dihydroxybenzonitrile and 1-dodecanethiol were obtained from Aldrich Chemical Co. and protocatechuic acid from Sigma Chemical Co. Methyl protocatechuate (**2**) was prepared by the method described previously (*1*). *N*-(Carbobenzyloxy)cysteine benzyl ester (ZCysOBn) and N^{α}-(carbobenzyloxy)histidine benzyl ester (ZHisOBn) were prepared by the method of Feldman and Shields (*16*, *17*). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical, benzylamine, and other reagents were purchased from Wako Pure Chemical Industries. All solvents used were of reagent grade.

Apparatus. NMR spectra were recorded on a Bruker AMX-500 spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz); chemical shifts are expressed relative to the residual signals of chloroform-*d* (δ_H 7.24, δ_C 77.0), methanol-d_4 (δ_H 3.30, δ_C 49.0), acetone-d_6 (δ_H 2.04, δ_C 29.8), and pyridine-d_5 (δ_H 8.71, δ_C 123.5). Field desorption mass spectra (FD-MS) were obtained with a JEOL JMS-SX102A instrument. Optical absorbance was acquired using a HITACHI U-3210 spectrophotometer. Preparative and analytical thin-layer chromatography was performed on silica gel plates Merck 60 F_{254} (0.5 and 0.25 mm thickness), respectively.

Colorimetric Radical Scavenging Tests. DPPH radical scavenging activity was measured as described previously (*1*, *3*). To a solution of a test compound (12.5 μM, 4 mL) was added 1 mL of DPPH radical (500 μM) in a test tube. The solution was immediately mixed vigorously for 10 s by a Vortex mixer and transferred to a cuvette. The absorbance reading at 517 nm was taken at 0.5, 1, 2, 5, 10, and 30 min after initial mixing. Acetonitrile and methanol were chosen as inert and nucleophilic solvents, respectively. A solution of *dl*-α-tocopherol in the same concentration was measured as a positive control. A reduction of the absorbance, 0.228, by the positive control was regarded as corresponding to the consumption of two molecules of DPPH radical. All experiments were performed in triplicate.

Colorimetric Radical Scavenging Tests in the Presence of a Nucleophile. To a solution of a test compound (12.5 μM) and a nucleophile (50 or 500 μM) in acetonitrile (4 mL) was added 1 mL of DPPH radical (500 μM) in a test tube. The solution was immediately mixed vigorously for 10 s by a Vortex mixer and transferred to a cuvette. The absorbance reading at 517 nm was taken at 0.5, 1, 2, 5, 10, and 30 min after initial mixing. ZCysOBn, 1-dodecanethiol, benzylamine, and ZHisOBn were used as nucleophiles. A solution of *dl*-α-tocopherol in the same concentration was measured as a positive control. A reduction of the absorbance, 0.228, by the positive control was regarded as corresponding to the consumption of two molecules of DPPH radical. All experiments were performed in triplicate.

NMR Measurements of the Reaction Mixture of 1 (or 2) and DPPH Radical in the Presence of a Nucleophile. To DPPH radical (30 μmol, 3.0 equiv) was added **1** (or **2**) (10 μmol) and ZCysOBn (10 μmol, 1.0 equiv) in acetone-d_6 (0.4 mL). The mixture was immediately transferred to a NMR tube and mixed vigorously. ^{1}H NMR spectra were recorded at 10 min after mixing.

NMR Measurements of the Reaction Mixture of 6 (or 7) and DPPH Radical. To DPPH radical (30 μmol, 3.0 equiv) was added **6** (or **7**) (10 μmol) in acetone-d_6 (0.4 mL). The mixture was immediately transferred to a NMR tube and mixed vigorously. ^{1}H NMR spectra were recorded at 10 min after mixing.

Isolation of ZCysOBn Adducts (General Method). To a solution of a catechol (1.5 mmol) in acetone (50 mL) was added DPPH radical (1182 mg, 3.0 mmol, 2.0 equiv). After 10 min, ZCysOBn (518 mg, 1.5 mmol, 1.0 equiv) was added to the reaction mixture and stirred for 1 h at room temperature. The reaction mixture was evaporated under the reduced pressure. The residue was subjected to preparative TLC to afford ZCysOBn adducts (compounds **6**−**15**).

Isolation of Compounds 6 and 11. Compounds **6** and **11** were isolated from the reaction mixture of **1**, ZCysOBn, and DPPH radical. **6**: 27%; dark brown oil; FD-HR-MS, *m/z* $[M]^+$ 497.1182, calcd $C_{25}H_{23}NO_8S$, 497.1145; ^{1}H NMR δ (methanol-d_4) (*J* in Hz) 3.31−3.42 (2H, m, H-3′), 4.40 (1H, m, H-2′), 4.92−5.08 (5H, m, NH and Bn-CH_2), 6.79 (1H, d, J = 8.4, H-5), 7.25 (1H, d, J = 8.4, H-6), 7.28−7.32 (10H, m, Bn); ^{13}C NMR δ (methanol-d_4) 37.5 (C-3′), 55.6 (C-2′), 67.8, 68.1 (Bn-CH_2), 115.7 (C-5), 123.0 (C-2), 123.7 (C-6), 128.9 (C-1), 128.7−129.5 (Bn), 148.5 (C-3), 149.6 (C-4), 170.9 (C-7), 172.0 (C-1′); HMBC correlation peaks, H-5/C-1, 3, H-6/C-2, 4, 7, H-3′/C-2, 1′. **11**: 14%; dark brown oil; FD-HR-MS, *m/z* $[M]^+$ 840.2047 (calcd for $C_{43}H_{40}N_2O_{12}S_2$, 840.2047); ^{1}H NMR δ (chloroform-*d*) (*J* in Hz) 3.27−3.37 (4H, m, H-3′ and 3″), 4.54 and 4.61 (2H, br s, H-2′ and 2″), 4.95−5.08 (8H, m, Bn-CH_2), 5.74 and 5.89 (2H, br s, NH), 7.23−7.30 (20H, m, Bn), 7.72 (1H, s, H-6); ^{13}C NMR δ (chloroform-*d*) 39.7 (C-3′ and 3″), 54.1 and 54.5 (C-2′ and 2″), 67.4−67.8 (Bn-CH_2), 119.0 (C-2), 120.4 (C-5), 128.1−128.6 (Bn), 148.4 (C-4), 169.0 (C-7), 169.9 and 170.1 (C-1′ and 1″); HMBC correlation peaks, H-6/C-2, 4, 7, H-3′ and 3″/C-2, 5, 1′, 1″.

Isolation of Compounds 7 and 12. Compounds **7** and **12** were isolated from the reaction mixture of **2**, ZCysOBn, and DPPH radical. **7**: 22%; dark brown oil; FD-HR-MS, *m/z* $[M]^+$ 511.1289, calcd $C_{26}H_{25}NO_8S$, 511.1302; ^{1}H NMR δ (chloroform-*d*) (*J* in Hz) 3.24 (1H, dd, J = 7.4 and 14.1, H-3′), 3.43 (1H, m, H-3′), 3.82 (3H, s, H-7(OMe)), 4.55 (1H, br s, H-2′), 5.00−5.13 (4H, m, Bn-CH_2), 5.82 (1H, br d, NH), 6.91 (1H, d, J = 8.4, H-5), 7.26−7.33 (10H, m, Bn), 7.44 (1H, d, J = 8.4, H-6); ^{13}C NMR δ (chloroform-*d*) 40.3 (C-3′), 52.1 (C-7(OMe)), 54.5 (C-2′), 67.3, 67.8 (Bn-CH_2), 115.2 (C-5), 118.1 (C-2), 124.5 (C-6), 126.5 (C-1), 128.1−128.7 (Bn), 147.6 (C-3), 147.7 (C-4), 166.6 (C-7), 170.0 (C-1′); HMBC correlation peaks, H-5/C-1, 3, H-6/C-2, 4, 7, H-7(OMe)/C-7, H-3′/C-2, 1′. **12**: 8%; dark brown oil; FD-HR-MS, *m/z* $[M]^+$ 854.2159 (calcd $C_{44}H_{42}N_2O_{12}S_2$, 854.2181); ^{1}H NMR δ (chloroform-*d*) (*J* in Hz) 3.26−3.39 (4H, m, H-3′ and 3″), 3.80 (3H, s, H-7(OMe)), 4.50 and 4.62 (2H, br s, H-2′ and 2″), 4.93−5.08 (8H, m, Bn-CH_2), 5.75 and 5.97 (2H, br s, NH), 7.24−7.53 (20H, m, Bn), 7.53 (1H, s, H-6); ^{13}C NMR δ (chloroform-*d*) 37.4 and 39.5 (C-3′ and 3″), 52.2 (C-7(OMe)), 54.0 and 54.4 (C-2′ and 2″), 67.2−67.6 (Bn-CH_2), 118.7 (C-2), 120.2 (C-5), 128.0−128.6 (Bn), 147.6 (C-4), 166.2 (C-7), 169.8 and 170.0 (C-1′ and 1″); HMBC correlation peaks, H-6/C-2, 4, 7, H-7(OMe)/C-7, H-3′and 3″/ C-2, 5, 1′, 1″.

Isolation of Compounds 8 and 13. Compounds **8** and **13** were isolated from the reaction mixture of **3**, ZCysOBn, and DPPH radical. **8**: 21%; dark brown oil; FD-HR-MS, *m/z* $[M]^+$ 495.1342, calcd $C_{26}H_{25}NO_7S$, 495.1353; ^{1}H NMR δ (chloroform-*d*) (*J* in Hz) 2.51 (3H, s, H-7(Me)), 3.21 (1H, dd, J = 7.2 and 14.1, H-3′), 3.37 (1H, dd, J = 4.1 and 14.1, H-3′), 4.53 (1H, br s, H-2′), 4.99−5.13 (4H, m, Bn-CH_2), 5.84 (1H, br d, NH), 6.92 (1H, d, J = 8.4, H-5), 7.20 (1H, d, J = 8.4, H-6), 7.25−7.32 (10H, m, Bn); ^{13}C NMR δ (chloroform-*d*) 29.2 (C-7(Me)), 40.6 (C-3′), 54.6 (C-2′), 67.3, 67.7 (Bn-CH_2), 115.1 (C-5), 116.4 (C-2), 123.0 (C-6), 128.1−128.6 (Bn), 134.7 (C-1), 146.2 (C-3), 147.4 (C-4), 170.1 (C-1′), 199.6 (C-7); HMBC correlation peaks, H-5/C-1, 3, H-6/C-2, 4, 7, H-7(Me)/C-1, 6, 7, H-3′/C-2, 1′. **13**: 10%; dark brown oil; FD-HR-MS, *m/z* $[M]^+$ 838.2203 (calcd for $C_{44}H_{42}N_2O_{11}S_2$, 838.2232); ^{1}H NMR δ (chloroform-*d*) (*J* in Hz) 2.45 (3H, s, H-7(Me)), 3.19−3.34 (4H, m, H-3′ and 3″), 4.50 and 4.58 (2H, br s, H-2′ and 2″), 4.91−5.10 (8H, m, Bn-CH_2), 5.68 and 5.81 (2H, br s, NH), 7.27 (1H, s, H-6), 7.23−7.31 (20H, m, Bn); ^{13}C NMR δ (chloroform-*d*) 29.6 (C-7(Me)), 37.4 (C-3′), 53.8 (C-2′), 67.3−67.7 (Bn-CH_2), 117.0 (C-2), 119.7 (C-5), 128.1−128.7 (Bn), 136.0 (C-1), 147.4 (C-4), 169.9 and 170.0 (C-1′ and 1″), 199.4 (C-7); HMBC correlation peaks, H-6/C-2, 4, 7, H-7(Me)/C-1, 7, H-3′and 3″/C-2, 5, 1′, 1″.

Isolation of Compounds 9 and 14. Compounds **9** and **14** were isolated from the reaction mixture of **4**, ZCysOBn, and DPPH radical. **9**: 27%; dark brown solid; FD-HR-MS, *m/z* $[M]^+$ 481.1189, calcd $C_{25}H_{23}NO_7S$, 481.1196; ^{1}H NMR δ (pyridine-d_5) (*J* in Hz) 3.85−3.89 (1H, m, H-3′), 4.01−4.05 (1H, m, H-3′), 5.05−5.25 (6H, m, H-2′, NH, Bn-CH_2), 7.16 (1H, d, J = 8.1, H-5), 7.24−7.39 (10H, m, Bn), 7.77 (1H, d, J = 8.1, H-6), 11.08 (1H, s, H-7); ^{13}C NMR δ (pyridine-d_5) 36.9 (C-3′), 55.4 (C-2′), 66.7, 67.2 (Bn-CH_2), 116.3 (C-5), 121.6 (C-6), 124.2 (C-2), 130.5 (C-1), 149.1 (C-3), 153.1 (C-4), 128.1−128.8 (Bn), 171.5 (C-1′), 191.3 (C-7); HMBC correlation peaks, H-5/C-1, 3, H-6/C-2, 4, 7, H-7/C-1, 6, H-3′/C-2, 1′. **14**: 14%; dark brown oil; FD-HR-MS, *m/z* $[M]^+$ 824.2047 (calcd $C_{43}H_{40}N_2O_{11}S_2$, 824.2075); ^{1}H NMR

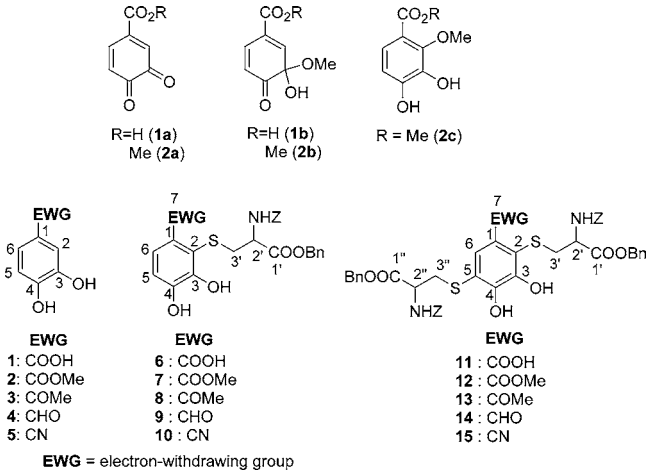

Figure 1. Chemical structures of protocatechuic acid and its related compounds.

δ (pyridine-d_5) (J in Hz) 3.71−3.76 and 3.85−3.89 (4H, m, H-3′ and 3″), 5.06−5.28 (12H, m, H-2′, NH, Bn-CH_2), 7.22−7.41 (20H, m, Bn), 8.06 (1H, s, H-6), 10.95 (1H, s, H-7); ^{13}C NMR δ(pyridine-d_5) 35.2 (C-3′ and 3″), 55.1, 55.4 (C-2′ and 2″), 66.7, 66.8, 67.2 (Bn-CH_2), 122.4 and 122.8 (C-2 and -6 interchangeable), 124.4 (C-5), 128.1−128.9 (Bn), 130.1 (C-1), 152.7 (C-4), 171.3 (C-1′ and 1″), 190.8 (C-7); HMBC correlation peaks, H-6/C-2, 4, 7, H-7/C-1, 2, 6, H-3′and 3″/C-2, 5, 1′, 2′, 1″, 2″.

Isolation of Compounds 10 and 15. Compounds **10** and **15** were isolated from the reaction mixture of 5, ZCysOBn, and DPPH radical. **10**: 35%; brown solid; FD-HR-MS, m/z $[M]^+$ 478.1219, calcd $C_{25}H_{22}N_2O_6S$, 478.1200; ^{1}H NMR δ (pyridine-d_5) (J in Hz) 3.92 (1H, dd, J = 7.6 and 13.3, H-3′), 4.06 (1H, dd, J = 5.2 and 13.3, H-3′), 5.15∼5.26 (6H, m, H-2′, NH, Bn-CH_2), 7.04 (1H, d, J = 8.1, H-5), 7.22∼7.38 (11H, m, H-6, Bn); ^{13}C NMR δ (pyridine-d_5) 36.8 (C-3′), 55.5 (C-2′), 66.7, 67.3 (Bn-CH_2), 109.0 (C-1), 116.5 (C-5), 117.8 (C-7), 122.5 (C-2), 126.1 (C-6), 128.1−128.9 (Bn), 150.5 (C-3), 152.1 (C-4), 171.4 (C-1′); HMBC correlation peaks, H-5/C-1, 3, H-6/C-2, 4, 7, H-3′/C-2, 1′. **15**: 6%; brown oil; FD-HR-MS, m/z $[M]^+$ 821.2078 (calcd $C_{43}H_{39}N_3O_{10}S_2$, 821.2079); ^{1}H NMR δ (chloroform-d) (J in Hz) 3.19−3.35 (4H, m, H-3′ and 3″), 4.49 and 4.57 (2H, br s, H-2′ and 2″), 5.01−5.13 (8H, m, Bn-CH_2), 5.60 and 5.79 (2H, br s, NH), 7.27 (1H, s, H-6), 7.30−7.34 (20H, m, Bn); ^{13}C NMR δ (chloroform-d) 37.7 (C-3′), 53.7 (C-2′), 67.4−68.0 (Bn-CH_2), 117.0 (C-7), 121.6 (C-2 and 5), 128.2−128.7 (Bn), 149.2 (C-4), 169.5 and 169.7 (C-1′ and 1″); HMBC correlation peaks, H-6/C-2, 4, 7, H-3′and 3″/C-2, 5, 1′, 1″.

RESULTS AND DISCUSSION

In the previous paper, we reported the interesting solvent dependency of the DPPH radical scavenging reaction of protocatechuic esters (*3*). In aprotic solvents such as acetone and acetonitrile, protocatechuic acid (**1**) and its esters scavenged two radicals in 30 min. In contrast, in protic solvents such as methanol, ethanol, and 1-propanol, protocatechuic esters were rapidly oxidized by five radicals, whereas **1** consumed approximately two radicals. An NMR analysis of the reaction mixture of methyl protocatechuate (**2**) and DPPH radical in methanol showed that **2** was rapidly converted to *o*-quinone (**2a**) and its 3-hemiacetal (**2b**) (*15*). In addition, a methanol-adduct at the 2-position of **2**, 2-methoxyprotocatechuic acid methyl ester (**2c**), was formed (**Figure 1**). These results demonstrated that in alcoholic solvents, **2a** underwent nucleophilic attack by the solvent alcohol molecule at the 2-position of the ring, leading to a regeneration of the catechol structure that could scavenge two additional radicals. This mechanism accounts well for the higher DPPH radical scavenging activity of protocatechuic esters in alcohols than in aprotic solvents.

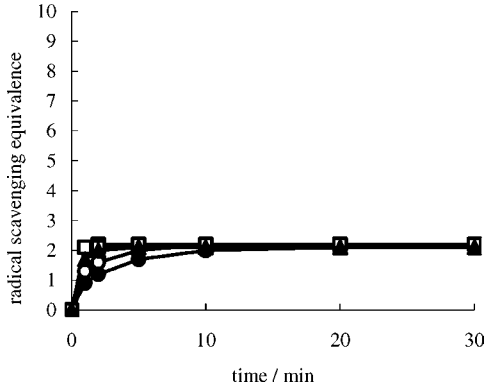

Figure 2. Time course of DPPH radical scavenging activity of protocatechuic acid (●), its methyl ester (○), 3′,4′-dihydroxyacetophenone (■), 3,4-dihydroxybenzaldehyde (□), and 3,4-dihydroxybenzonitrile (▲) in acetonitrile. The equivalence is expressed as the values relative to that of dl-α-tocopherol as 2.0.

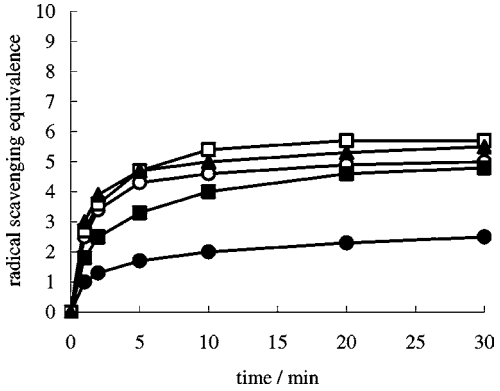

Figure 3. Time course of DPPH radical scavenging activity of protocatechuic acid (●), its methyl ester (○), 3′,4′-dihydroxyacetophenone (■), 3,4-dihydroxybenzaldehyde (□), and 3,4-dihydroxybenzonitrile (▲) in methanol. The equivalence is expressed as the values relative to that of dl-α-tocopherol as 2.0.

The radical scavenging activity of phenolic compounds depends on several factors such as number of hydroxyl groups and electron-donating or -withdrawing substituents on the benzene ring (*1*, *2*). In this study, to examine the effect of electron-withdrawing groups (−CO_2H, −CO_2Me, −COMe, −CHO, −CN) on the catechol skeleton, the DPPH radical scavenging activity of protocatechuic acid (**1**), methyl protocatechuate (**2**), 3′,4′-dihydroxyacetophenone (**3**), 3,4-dihydroxybenzaldehyde (**4**), and 3,4-dihydroxybenzonitrile (**5**) was evaluated by the colorimetric method. Time course of the radical scavenging activity of **1**−**5** in acetonitrile as an inert solvent and methanol as a nucleophilic solvent is shown in **Figures 2** and **3**, respectively. After 30 min, the relative radical scavenging equivalence of each compound, when that of dl-α-tocopherol as standard was designated as 2, was **1**, 2.2; **2**, 2.2; **3**, 2.2; **4**, 2.2; and **5**, 2.1 in acetonitrile and **1**, 2.5; **2**, 5.0; **3**, 4.8; **4**, 5.7; and **5**, 5.5 in methanol. The radical scavenging activity of **1**, **2**, and **5** in methanol was in accordance with the result examined in ethanol (*4*). The DPPH radical scavenging activity of **3**−**5** was comparable to that of protocatechuic esters, both in methanol and in acetonitrile. The result suggests that the radical scavenging reactions of **3**−**5**, which carry electron-withdrawing groups at the C-4 of the catechol ring, are similar to that of protocatechuic esters. In contrast, **1** showed low activity even in nucleophilic methanol unlike protocatechuic esters. To examine the effect of methanol, DPPH radical scavenging activity of **1** and **2** in the different methanol−acetonitrile ratio

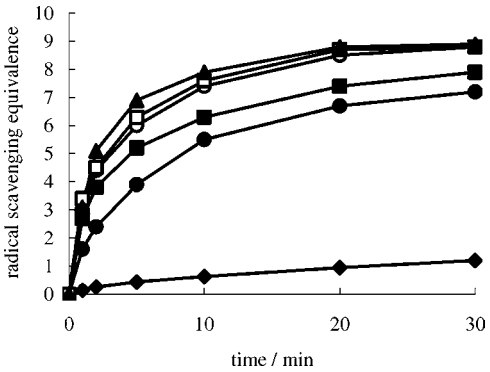

Figure 4. Time course of DPPH radical scavenging activity of protocatechuic acid (●), its methyl ester (○), 3′,4′-dihydroxyacetophenone (■), 3,4-dihydroxybenzaldehyde (□), and 3,4-dihydroxybenzonitrile (▲) in acetonitrile in the presence of ZCysOBn (40 μM, 4 equiv, ◆). The equivalence is expressed as the values relative to that of dl-α-tocopherol as 2.0.

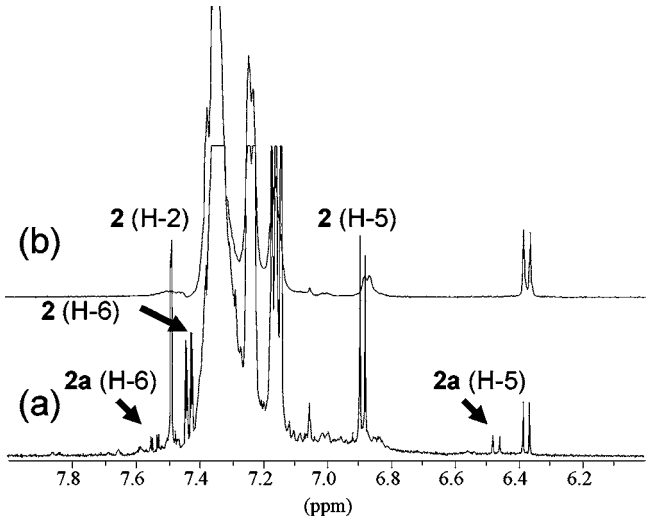

Figure 5. ^{1}H NMR spectra of the reaction mixtures of methyl protocatechuate, ZCysOBn, and DPPH radical (**a**) and of compound **7** and DPPH radical (**b**) in acetone-d_6 10 min after being mixed. The intense signals in the range of 7.1–7.4 ppm are due to DPPH hydrazine.

was determined. The radical scavenging equivalence in 30 min of **1** and **2** was as follows: **1**, 2.2 (0% methanol); 2.1 (10% methanol); 2.2 (20% methanol); 2.4 (50% methanol); 2.5 (100% methanol); and **2**, 2.2 (0% methanol); 2.9 (10% methanol); 4.0 (20% methanol); 4.7 (50% methanol); 5.0 (100% methanol). The radical scavenging activity of **2** considerably increased as the ratio of methanol increased. This result corroborates the mechanism that the high radical scavenging activity of protocatechuic esters in methanol is due to their regeneration of catechol structures by nucleophilic addition of methanol on *o*-quinones. On the other hand, **1** showed no significant effect of methanol, indicating that the nucleophilic addition of methanol hardly occurs on **1a**. This difference in reactivity might be due to the acidity of the carboxyl group. Strong electron-withdrawing property of the quinone carbonyls enhances the dissociation of carboxylic group to the carboxylate ion, which has a relatively electron-releasing nature as compared to other electron-withdrawing groups (−CO_2Me, COMe, −CHO, −CN) (*1*, *3*).

It is well-documented that *o*-quinones derived from catechins and phenolic acids conjugate with thiols such as glutathione (*18*−*25*). We assumed that an addition of a nucleophile, instead of alcohol, might enhance the radical scavenging activity of protocatechuic esters in inert solvents. Time course of DPPH radical scavenging equivalence in acetonitrile in the presence of a cysteine derivative (*N*-(carbobenzyloxy)cysteine benzyl ester, ZCysOBn) is shown in **Figure 4**. ZCysOBn was used as a nucleophile because cysteine was insoluble in acetonitrile. Although **1** and **2** scavenged only two radicals without ZCysOBn in acetonitrile (**Figure 2**), the radical scavenging activity noticeably enhanced by the addition of ZCysOBn. Similarly, the radical scavenging activity of **3**−**5** was also enhanced by the addition of ZCysOBn. This significant enhancement of the radical scavenging activity clearly indicates that the regeneration of a catechol structure also occurred in aprotic solvents via a nucleophilic attack of ZCysOBn on *o*-quinones. In addition, the result supports the idea that the radical scavenging reactions of **3**−**5**, which bear electron-withdrawing groups (−COMe, −CHO, −CN) on the C-4 of catechol ring, proceed similarly to that of protocatechuic esters. Interestingly, there was no difference in enhancement of the activity between **1** and **2** in the presence of a nucleophile, in spite of the fact that **1** showed far lower activity than **2** in methanol. It is indicated that the strong *S*-nucleophile like ZCysOBn was indiscriminate in attacking **1a** and **2a**, whereas the weaker alcoholic nucleophile could add only to more reactive **2a**.

Formation of ZCysOBn adducts was confirmed by an isolation of mono- and bis-ZCysOBn adducts from the reaction mixtures of protocatechuic acid analogues (**1**−**5**), ZCysOBn, and DPPH radical in acetonitrile. The ^{1}H NMR spectra of all mono-ZCysOBn adducts showed a pair of doublet peaks of H-5 and H-6. In addition, the HMBC correlation between H-3′ of the cysteine residue and C-2 of the catechol ring was observed. Thus, the cysteine residue was connected at C-2 of the substrates. On the other hand, only one singlet peak of an aromatic proton was observed in the spectrum of bis-adducts. In the HMBC spectra, this singlet peak showed a cross-peak with C-7 of the side chain, which indicated the proton to be H-6. Furthermore, H-3′ and H-3″ of two cysteine residues showed correlations between C-2 and C-5. Hence, the second cysteine residue should be at C-5. From these results, the position of ZCysOBn of adducts were revealed to be C-2 (**6**−**10**) and C-2,5 (**11**−**15**) of the catechol ring (**Figure 1**). TLC analyses of the reaction mixtures of catechols (**1**−**5**), DPPH radical, and ZCysOBn showed only the spots of reactants, C-2 monoadducts and C-2,5 bis-adducts. This result indicates that ZCysOBn mainly attacks at C-2 of the quinone. Furthermore, the reaction mixture of **1** (or **2**), ZcysOBn, and DPPH radical in acetone-d_6 was directly analyzed by ^{1}H NMR. The NMR spectrum of the reaction mixture of **2**, ZcysOBn, and DPPH radical is shown in **Figure 5a**. Together with the characteristic doublet signal of H-5 in the corresponding quinone (**2a**) at δ 6.47, J = 10.3 Hz (*1*), a doublet peak at δ 6.37 was observed. This latter doublet signal also had a typical large coupling constant, J = 10.1 Hz, of a quinone form. The same doublet signal appeared in the ^{1}H NMR spectrum of the reaction mixture of **7** and DPPH radical in acetone-d_6 (**Figure 5b**), and its structure was determined by in situ 2-D-NMR analyses. Both H-5 and H-6, δ 7.37, showed $^3J_{CH}$ HMBC correlation with two distinct carbonyls of C-3 (δ 176.9) and C-4 (δ 180.2), respectively, although a signal of H-6 was invisible in the 1-D spectrum by overlapping with large signals of DPPH hydrazine and benzyl groups. Therefore, the doublet signal at δ 6.37 was assigned as H-5 of a quinone-form of **7**. In addition, the corresponding doublet signal was also observed in the reaction

Scheme 1. Plausible Radical Scavenging Mechanism of Protocatechuic Acid and Its Structural Analogues in Aprotic Solvents in the Presence of a Nucleophile

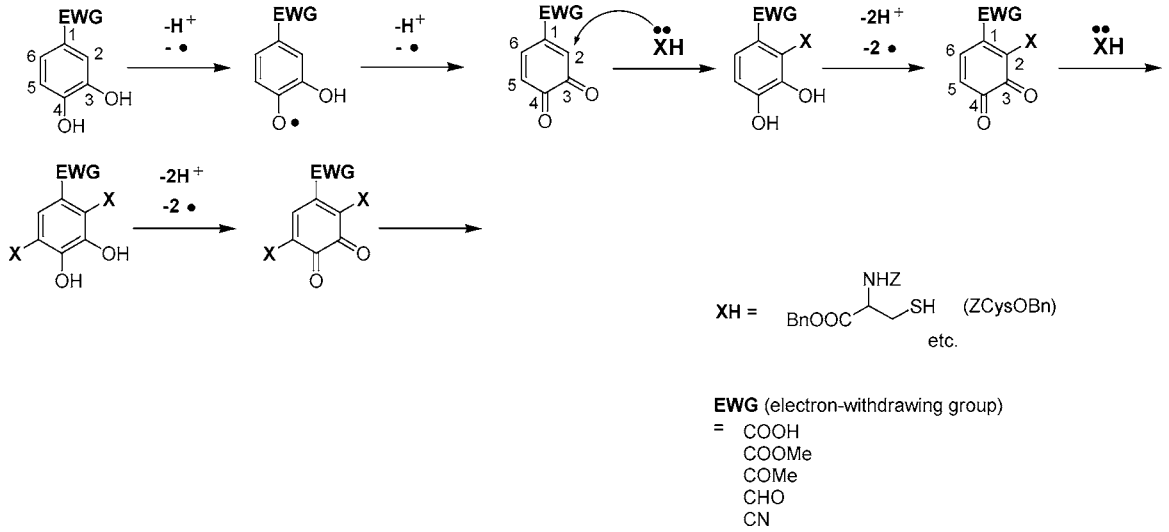

Table 1. DPPH Radical Scavenging Equivalence of Protocatechuic Acid and Its Methyl Ester in Acetonitrile in the Presence of Nucleophiles after 30 min

	radical scavenging equivalence[a]
1-dodecanethiol (40 μM)	0.2
PA + 1-dodecanethiol (40 μM)	5.6
PAMe + 1-dodecanethiol (40 μM)	3.6
benzylamine (400 μM)	0.2
PA + benzylamine (400 μM)	4.7
PAMe + benzylamine (400 μM)	4.0
ZHisOBn (400 μM)	0.1
PA + ZHisOBn (400 μM)	3.5
PAMe + ZHisOBn (400 μM)	3.6

[a] The equivalence is expressed as the values relative to that of *dl*-α-tocopherol as 2.0.

mixture of **1**, ZcysOBn, and DPPH radical in acetone-d_6. C-2 of the quinone should be the most reactive position since it is doubly activated by C-4 and C-7 carbonyls by cross conjugation. Furthermore, Cheynier et al. reported that quinone of caftaric acid, caffeoyltartaric acid, forms adduct with glutathione at C-2 of the catechol ring (*19*). These results indicate that a nucleophile first attacks at C-2, then C-5 of the quinone.

The DPPH radical scavenging equivalence of **1** and **2** in the presence of other nucleophiles such as 1-dodecanethiol, benzylamine, and ZHisOBn after 30 min is shown in **Table 1**. The radical scavenging activity of **1** and **2** was also conspicuously enhanced by the addition of 1-dodecanethiol. However, the synergistic effects of N-nucleophiles such as benzylamine and ZHisOBn were lower than those of S-nucleophiles (ZCysOBn, 1-dodecanethiol), and the outcome of the comparable enhancements to the S-nucleophiles required 10 times higher concentration of the N-nucleophiles. These results might be accounted for by the difference in nucleophilicity of the S- and N-nucleophiles.

The above results strongly suggest that the radical scavenging reaction of protocatechuic acid and its analogues in inert solvents in the presence of a nucleophile proceeds as shown in **Scheme 1**. Protocatechuic acid and its analogues are oxidized by two radicals to give the corresponding quinones. The quinones spontaneously undergo an attack by nucleophiles at the 2-position of the ring, which leads to a regeneration of a catechol structure. A regenerated catechol moiety of adducts scavenge two additional radicals by reoxidation into quinones. Then, the resultant 2-substituted quinones undergo the second nucleophilic attack at the 5-position of the ring. This mechanism could account well for the high radical scavenging activity of protocatechuic acid analogues in the presence of a nucleophile. In water, protocatechuic esters scavenge approximately six radicals similar to the reactions in alcohols (*3*). It indicates that their quinones would undergo nucleophilic attack by a water molecule as was seen in alcoholic solvents. Thus, the observed synergistic effects on antioxidant activity might also be seen between catechols and biological thiols or amines (cysteine, glutathione, and histidine etc.) in vivo and could be an important antioxidant mechanism in biological systems. Monks and Lau also reported the regeneration of hydroquinone/catechol structure from quinones by the nucleophilic addition of glutathione and the biological reactivity of the resultant conjugates (*21*).

In conclusion, protocatechuic esters and their analogues scavenge approximately five radicals in nucleophilic alcoholic solvents, whereas only two radicals are scavenged in inert solvents. The radical scavenging activity of these compounds in inert solvents could be significantly enhanced by the addition of a nucleophile. This result strongly suggests that, in the presence of a nucleophile, a regeneration of a catechol structure also occurs in inert solvents via a nucleophilic attack on quinones as shown in the reaction of methyl protocatechuate (**2**) in methanol (*3*). Finally, the radical scavenging reactions of catechol derivatives containing electron-withdrawing groups such as caffeic acid (3,4-dihydroxycinnamic acid) and luteolin (3′,4′,5,7-tetrahydroxyflavone) might also proceed in similar mechanism as protocatechuic esters.

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