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Oxidative Dimers Produced from Protocatechuic and Gallic Esters in the DPPH Radical Scavenging Reaction

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DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging reactions of protocatechuic and gallic acids, and their methyl esters, have been investigated by NMR. In acetone, methyl protocatechuate was gradually converted to a Diels−Alder adduct of two molecules of the intermediate quinone in the reaction with DPPH radical, whereas methyl gallate rapidly gave a symmetrical dimer via a putative quinone precursor. Both dimers are rather unstable and their structures have been deduced by in situ NMR measurements of the reaction mixtures. Gallic acid also gave a corresponding symmetrical dimer in the same reaction as methyl gallate, although protocatechuquinone produced from protocatechuic acid did not yield a Diels−Alder adduct, unlike its methyl ester. Interestingly, these dimer formations were not observed in methanol solution.

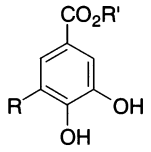
KEYWORDS: Protocatechuic acid; gallic acid; DPPH radical; antiradical activity; ortho-quinone; dimerization

INTRODUCTION

Phenolic acids and their esters are common plant constituents that are known for their antioxidant activities (*1*−*10*). The radical scavenging abilities of these acids depend greatly on the number and arrangement of phenolic hydroxyl groups. Thus, gallic acid (3,4,5-trihydroxybenzoic acid) possesses a higher antiradical activity than protocatechuic acid (3,4-dihydroxybenzoic acid), whereas 4-hydroxybenzoic acid shows little radical scavenging activity (*1*, *2*, *5*, *6*, *8*). Hence, pyrogallol-type triphenols carrying three adjacent hydroxyl groups on a benzene ring effectively scavenge more radicals than catechol-type *o*-diphenols. Although gallic and protocatechuic acids show notable radical scavenging activities, the reaction mechanism and difference in antiradical reactivities between these pyrogallol- and catechol-type phenolic acids are still unclear. In the course of our research on the radical scavenging mechanism of pyrogallol and catechol type polyphenols, we have found by in situ NMR measurements (*11*, *12*) that protocatechuic and gallic esters (**Figure 1**) gave oxidative dimers with different connectivities in the reaction with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (*13*, *14*) in an aprotic solvent.

MATERIALS AND METHODS

Reagents. Protocatechuic acid (**1**) (Sigma Chemical Co.), gallic acid (**3**) and methyl gallate (**4**) (Tokyo Kasei Kogyo Co., Ltd.), and DPPH radical (Wako Pure Chemical Industries, Ltd.) were purchased from the indicated supplier. Methyl protocatechuate (**2**) was prepared from protocatechuic acid by heating it with methanol containing 10% hydrogen chloride. All solvents used were of technical grade. Deuterated solvents used in NMR were obtained from Aldrich Chemical Co.



	R	R'
1	H	H
2	H	Me
3	OH	H
4	OH	Me

Figure 1. Structures of phenolic acids and methyl esters.

Colorimetric Radical Scavenging Tests. To a solution of DPPH radical (500 μM, 1 mL) was added a phenol solution (12.5 μM, 4 mL) in a test tube. Final molar ratio of the radical and phenols was 10:1. The solution was immediately mixed vigorously for 10 s by a Vortex mixer and transferred to a cuvette. After the solution in the cuvette sat 30 min at room temperature, the absorbance at 517 nm was measured by a Hitachi U-3210 instrument. Acetone and ethanol were chosen as aprotic and protic solvents, respectively. An ethanol solution of 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 (*13*, *15*).

NMR Measurements. NMR spectra were determined with a Bruker AMX500 instrument (^{1}H, 500 MHz; ^{13}C, 125 MHz). Acetone-d_6 and methanol-d_4 were used as aprotic and protic solvents, respectively, while no significant difference in the spectral patterns was observed in methanol and ethanol. For the purpose of identification of the reaction products, chemical shifts were calculated from the residual solvent signals of δ_H 2.04 and δ_C 29.8 ppm in acetone-d_6. 2D COSY, HMQC, and HMBC spectra were obtained using Bruker programs. All assignments of signals, including those buried under strong peaks of 2,2-diphenyl-1-picrylhydrazine produced by the reduction of DPPH radical,

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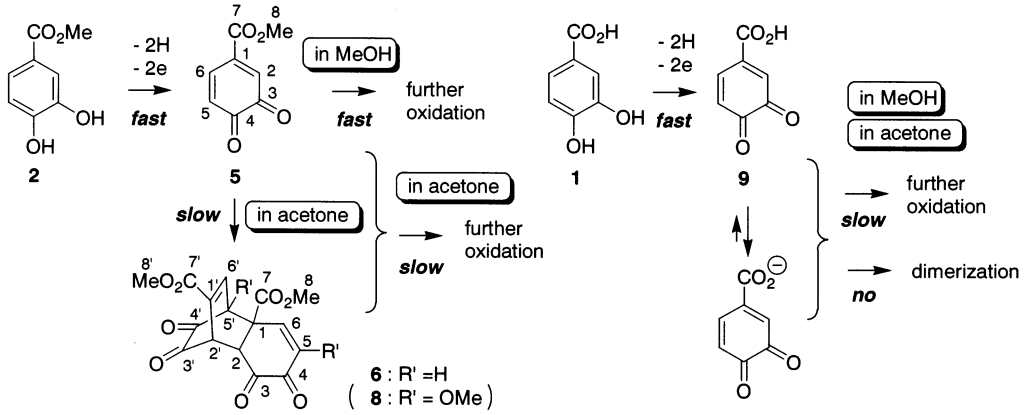

Figure 2. Oxidation of methyl protocatechuate (**2**) and protocatechuic acid (**1**) with DPPH radical.

were done by the 2D spectral data. Peak areas were standardized against that of the residual peak of acetone-d_6 for determining the conversion rate.

Reaction of Methyl Protocatechuate (2) and DPPH Radical. To DPPH radical (14.5 mg, 37 μmol, 2.8 equiv) was added a solution of methyl protocatechuate (**2**, 2.1 mg, 13 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 periodically. ^{13}C NMR, COSY, HMQC, and HMBC spectra were consecutively measured from 3 h after mixing.

Protocatechuquinone methyl ester (**5**). ^{1}H NMR δ (acetone-d_6): 3.91 (3H, s, H-8), 6.47 (1H, d, J = 10.3 Hz, H-5), 6.89 (1H, d, J = 2.0 Hz, H-2), 7.54 (1H, dd, J = 10.3, 2.0 Hz, H-6). ^{13}C NMR δ (acetone-d_6): 53.6 (C-8), 131.2 (C-5), 132.7 (C-2), 137.6 (C-6), 139.7 (C-1), 165.3 (C-7), 179.8 (C-4), 181.4 (C-3).; HMBC correlation peaks: H-8/C-7, H-5/C-1, 3, H-2/C-4, 6, 7, H-6/C-2, 4.

Protocatechuquinone methyl ester dimer (**6**). ^{1}H NMR δ (acetone-d_6): 3.72 (3H, s, H-8′), 3.76 (3H, s, H-8), 4.32 (1H, d, J = 2.4 Hz, H-2), 4.34 (1H, d, J = 6.9 Hz, H-5′), 4.50 (1H, dd, J = 2.4, 2.1 Hz, H-2′), 6.58 (1H, d, J = 10.7 Hz, H-5), 7.37 (1H, d, J = 10.7 Hz, H-6), 7.42 (1H, dd, J = 6.9, 2.1 Hz, H-6′). ^{13}C NMR δ (acetone-d_6): 51.9 (C-2′), 53.0 (C-8′), 53.1 (C-2), 54.4 (C-8), 55.5 (C-1), 58.5 (C-5′), 132.0 (C-5), 135.8 (C-1′), 139.3 (C-6′), 149.7 (C-6), 163.0 (C-7′), 172.3 (C-7), 178.9 (C-4), 185.8 (C-3′), 186.0 (C-4′), 190.1 (C-3). HMBC correlation peaks: H-8′/C-7′, H-8/C-7, H-2/C-2′, 1′, 7, 3, H-5′/C-2, 1, 1′, 6′, 6, 3′ and/or 4′, H-2′/C-1, 1′, 6′, 3′ and/or 4′, H-5/C-1, 3, H-6/C-2, 5′, 4, H-6′/C-2′, 5′, 7′, 3′ and/or 4′.

Reaction of Methyl Gallate (4) and DPPH Radical. To DPPH radical (14.7 mg, 37 μmol, 2.6 equiv) was added a solution of methyl gallate (**4**, 2.5 mg, 14 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 periodically. ^{13}C NMR, HMQC, and HMBC spectra were consecutively measured from 20 min after mixing. No signals from galloquinone methyl ester were observed during the reaction.

Oxidative methyl gallate dimer (**7**). ^{1}H NMR δ (acetone-d_6): 3.86 (6H, s, H-8, 8′), 4.21 (2H, d, J = 0.5 Hz, H-2, 2′), 6.17 (2H, s, OH-4, 4′), 6.98 (2H, d, J = 0.5 Hz, H-6, 6′). ^{13}C NMR δ (acetone-d_6): 53.8 (C-8, 8′), 59.3 (C-2, 2′), 91.1 (C-4, 4′), 132.6 (C-6, 6′), 146.5 (C-1, 1′), 164.8 (C-7, 7′), 194.2 (C-5, 5′), 196.9 (C-3, 3′). HMBC correlation peaks: H-8/C-7, H-2/C-4 and/or 4′, 6, 1, 7, 5′, 3 and/or 3′, OH-4/C-2′, 4, 5, 3, H-6/C-2, 4, 7.

RESULTS AND DISCUSSION

The DPPH radical scavenging abilities of protocatechuic acid (**1**), methyl protocatechuate (**2**), gallic acid (**3**), and methyl gallate (**4**) were determined by the colorimetric method. After 30 min, the relative radical scavenging equivalence of each compound, when that of α-tocopherol in ethanol as standard was designated as 2, was as follows: **1**, 2.1; **2**, 2.0; **3**, 4.7; **4**, 4.0 in acetone; and **1**, 2.3; **2**, 4.7, **3**, 4.6; **4**, 4.5 in ethanol, respectively. Interestingly, **2** showed a dramatic increase of DPPH radical consumption when changing the solvent from aprotic acetone to protic ethanol, whereas **1** as well as **3** and **4** showed little change in their antiradical activity in either solvent.

The NMR measurements of the reaction mixture of **2** and DPPH radical in acetone showed that **2** was rapidly converted to protocatechuquinone methyl ester (**5**) (*16*). The oxidation to **5** was complete in a few minutes, as the signals from the starting **2** completely disappeared within 10 min. And then, dimerization of the resultant **5** gradually occurred in the reaction mixture to give the dimer (**6**) (**Figure 2**). The yield of **6** was 4% in 15 min based on **2** and reached 50% in 6 h. After 12 h, the quinone peaks almost disappeared, whereas the peaks due to **6** still remained. The amount of **6** then decreased during prolonged standing, changing to a complex mixture indicated by the complicated methoxyl proton signals in the ^{1}H NMR spectrum. The structural determination of **6** was done by in situ NMR measurements of the reaction mixture, as attempted isolation of **6** from the reaction mixture failed because of its instability. In the reaction mixture, a characteristic doublet at δ_H 6.47 for H-5 of **5** diminished, and a new doublet at δ_H 6.58 appeared instead. In addition, three proton signals at δ_H 4.32, 4.34, and 4.50, each 1 H, also appeared. The methoxyl proton region contained two singlets of nearly similar height at δ_H 3.72 and 3.76, together with a methoxyl signal of **5** at δ_H 3.91. The 2D COSY, HMQC, and HMBC spectra of the reaction mixture unambiguously indicated **6** to be a Diels−Alder adduct of two molecules of **5** (**Figure 2**). This type of dimerization products of *o*-benzoquinone derivatives has been previously reported (*17*−*21*). Among them, a dimer **8** (*21*) produced from 5-methoxyprotocatechuquinone methyl ester by treatment with cupric acetate hydrate corresponds to a dimethoxyl derivative of **6**. The NMR data of **6** are consistent with those of the hydrate form of **8**. The relative stereochemistry of the Diels−Alder addition in **6** is presumed to be *exo* as shown because the corresponding dimethoxyl derivative **8** easily underwent an intramolecular hemiacetalization between carbonyls of C-3 and C-3′ (*21*).

In contrast, in a protic solvent such as methanol, the intensity of the signals of **5** generated in the reaction with DPPH radical was even smaller than those in aprotic acetone, and many other small signals were simultaneously observed, which suggests that a complex reaction proceeded compared to that of the acetone solution. The signals of **6**, however, were not observed in methanol. Hence **5**, once generated in the methanol solution, would react quickly to give further oxidation products by excess DPPH radical in the protic solvent (*7*). On the contrary, in acetone, the lifetime of **5** was longer compared to that in the methanol solution and thus dimerization could gradually occur.

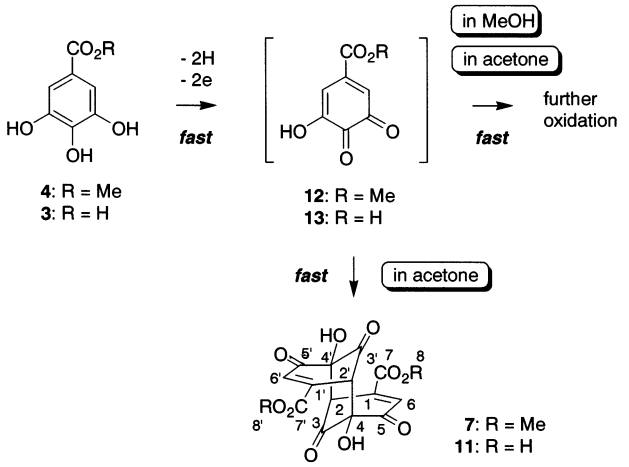

Figure 3. Oxidation of methyl gallate (**4**) and gallic acid (**3**) with DPPH radical.

In the protic solvent, the presence of solvent molecules might directly accelerate a further oxidation reaction probably due to a high reactivity of **5** against the nucleophilic attack with the alcohol molecule, resulting in a regenaration of the catechol structure, which accounts for the higher DPPH radical scavenging ability in ethanol than that in acetone as found in the colorimetric measurements.

In the case of the acid **1**, the reaction course was significantly different from that of **2**. In acetone, **1** was quickly oxidized to the quinone **9** by DPPH radical. After that, however, the signals of protocatechuquinone remained unchanged in the NMR spectrum, and no dimerization product was detected at all in several hours. This resistance to dimerization by **9** seems to arise from the difference in electronic properties of the quinones **5** and **9**. The strong electron-withdrawing nature of the quinone carbonyls in **9** might enhance the acidity of the carboxyl group conjugated to the parent quinone skeleton compared to that of **1**. As a result, the number of deprotonated molecules increases in **9**. The electronic nature of carboxylic and ester groups, and its dissociated carboxylate form is clearly different, as is reflected in substituent effects in the aromatic electrophilic substitution reaction (*22*). Deprotonation of the carboxyl group reduces its electron-withdrawing nature and hence might decrease the reactivity of **9** as a dienophile. In protic methanol, the intensity of the signals from **9** that appeared soon after mixing was larger than that of **5** from **2**. Also, the peak patterns within the reaction mixture of **1** with DPPH radical in methanol were relatively simple compared to those in **2**. Hence, further reaction with DPPH radical after conversion to **9** was considerably slower compared to the rapid and complex reaction that proceeded with **5**. This low reactivity of **9** accounts well for the difference in the relative radical scavenging equivalence of **1** and **2** after 30 min reaction in ethanol.

Methyl gallate (**4**) also gave a dimerization product **7**, when reacted with DPPH radical in acetone (**Figure 3**). Production of **7** was rapid compared to that of **6**, and the yield reached 62% in 10 min based on **4**. This dimer was relatively stable in the NMR tube and survived even after a few days, although its separation from the reaction mixture also resulted in its decomposition as with **6**. Thus, the structure of **7** was determined by direct measurements of a series of 2D NMR spectra in the reaction mixture. The NMR signal pattern of **7** was simple, consisting of three singlets at δ_H 4.21, 6.17, and 6.98, as well as that of a methoxyl at δ_H 3.86, and its symmetric structure was deduced mainly by HMBC data. Quideau and Feldman (*23*) reported the formation of a similar unstable symmetric dimer

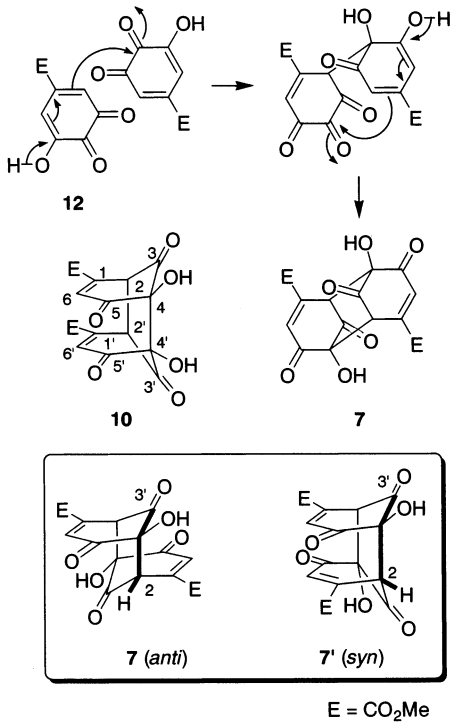

Figure 4. Formation and relative stereochemistry of galloquinone methyl ester dimer (**7**).

10 (**Figure 4**) in the acetone solution of an oxidation product prepared from **4** with *o*-chloranil in ether at −40 °C. Interestingly, the NMR data of both dimers, **7** and **10**, are very similar except those of C-2 (δ_H 4.21/δ_C 59.3 for **7** and δ_H 4.36/δ_C 53.2 for **10**) and C-4 (δ_C 91.1 for **7** and δ_C 96.0 for **10**), which suggests that the coupling manners of **7** and **10** are different. The dimer **10** is proposed to be a head-to-head coupling (2−2′ and 4−4′) product, whereas **7** should be derived through head-to-tail coupling (2−4′ and 4−2′), which was confirmed by the inter-residual HMBC correlations of H-2/C-5′ and OH-4/C-2′. This type of symmetrical dimers has been reported as an oxidation product of pyrogallol derivatives, and both head-to-head and head-to-tail structures are present (*19*, *20*, *23*, *24*). Hence, the formation of **7** and its regioselectivity might depend on the difference in structures and reaction conditions such as temperature. Although the relative stereochemistry of those symmetrical dimers has not yet been proposed in the literature, **7** is tentatively assigned as an *anti*-isomer because steric repulsion must arise in the *syn*-addition when the second C−C coupling occurs via a hypothetical quinone precursor (**Figure 4**). Furthermore, the interresidue intense HMBC cross-peak of three-bond H-2/C-3′ also supports their antiperiplanar arrangement adopted in the *anti*-isomer (**7**) in contrast to the dihedral angle of approximately 120° in the *syn*-isomer (**7′**), while a contribution of an intraresidue two-bond H-2/C-3 correlation should be weak. Sawai and Moon (*12*) reported the reaction of ethyl gallate with DPPH radical in acetone. However, signals from the corresponding dimer were not detected on their spectrum. In contrast, ethyl gallate was also converted to its symmetrical dimer through the reaction with DPPH radical in our experiment (data not shown). The apparent difference might arise from the ^{13}C measurement conditions.

In contrast to the fact that protocatechuic acid (**1**) gave no signals of the Diels−Alder dimer from the intermediate quinone in the NMR spectrum, unlike its ester **2**, gallic acid (**3**) yielded the corresponding dimer (**11**) although its conversion rate was lower than that from **4**. The oxidative coupling reaction of **4**

might start from an intermediate *o*-quinone **12** as proposed previously (*21*). In protic methanol, no dimerization product was detected from both **3** and **4** as has been seen in **1** and **2**. Protic solvents would enhance a radical scavenging reaction via the highly reactive hydroxyquinone intermediates (**12** and **13**).

In conclusion, both methyl protocatechuate and gallate gave their quinone dimers with different coupling selectivities when reacting with DPPH radical in aprotic acetone. However, protocatechuquinone was the only product from protocatechuic acid and no such dimer was produced under the same reaction condition, whereas gallic acid was converted to a dimer similar to that observed with its methyl ester. In contrast, dimer formation from both esters was not found in protic methanol. The difference in radical scavenging ability of protocatechuic acid and its methyl ester in alcoholic solvents might be partly accounted for by the reactivity of the resulting quinone toward an attack of the solvent molecule, although the direct evidence must be disclosed by further experiments. It is also unclear what causes the marked difference in radical scavenging abilities between catechol-type protocatechuate and pyrogallol-type gallate. Further efforts to identify the reaction course of these phenolic acids and esters are now in progress.

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