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A Novel Oxidative Dimer from Protocatechuic Esters: Contribution to the Total Radical Scavenging Ability of Protocatechuic Esters

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A novel oxidative dimer was isolated as a major product from a reaction mixture of methyl protocatechuate and DPPH radical in methanol. Its unusual benzobicyclo[3.2.1]octane structure was elucidated by extensive spectral analysis. This result suggests that the regeneration of catechol structures by the nucleophilic addition of an alcohol molecule on *o*-quinones and subsequent dimerization is one of the key reactions in the high radical-scavenging activity of protocatechuic esters in an alcoholic solvent.

Key words: antioxidant; protocatechuic acid; DPPH radical; radical-scavenging mechanism

Protocatechuic acid (3,4-dihydroxybenzoic acid) and its esters are *o*-diphenols ubiquitously found in edible plants, vegetables, and fruits. They are known to exhibit potent antioxidant activities.[1–3] It has been found that protocatechuic acid has preventive effects in carcinogenesis and cardiovascular diseases that are associated with free radicals.[4–6] We have reported that in non-alcoholic solvents, methyl protocatechuate (**1**) scavenges two equivalents of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical and is converted to the corresponding *o*-quinone (**2**).[7] In alcoholic solvents, **1** rapidly scavenged five equivalents of the radical to form **2**, its 3-hemiacetal (**3**), and further oxidized products.[8] *In situ* NMR analysis of the reaction mixture of **1** and DPPH radical in methanol revealed that **2** underwent a nucleophilic attack by a methanol molecule at C-2 to form C-2 alcohol adduct (**4**), which scavenged two additional radicals to yield *o*-quinone (**5**) and its 3-hemiacetal (**6**).[9] Furthermore, **5** underwent a second nucleophilic attack by a methanol molecule at C-6 to form a 2,6-bis-alcohol adduct (**7**), which scavenged additional radicals to form its *o*-quinone (**8**) and 3-hemiacetal (**9**).[10] It has thus been found that the nucleophilic addition of a solvent molecule on *o*-quinone is a crucial reaction in the high radical-scavenging activity of protocatechuic esters in alcoholic solvents.[9,10] However, to represent the whole picture of the radical-scavenging mechanism, it is necessary to clarify unidentified intermediates and oxidation products. In this paper, we report the identification of a novel dimer formed by the oxidation of **1** with DPPH radical in methanol, and we propose a radical scavenging mechanism in alcoholic solvents.

Materials and Methods

Chemicals. Methyl protocatechuate (**2**) and methyl 3,4-dihydroxy-2-methoxybenzoate (**4**) were prepared by methods described previously.[7] DPPH radical and other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). All solvents used were of reagent grade.

Apparatus. NMR spectra were recorded on a Bruker AMX500 spectrometer (Bruker, Tsukuba, Japan, ^{1}H, 500 MHz; ^{13}C, 125 MHz). Chemical shifts were expressed relative to the residual signals of methanol-d_4 (δ_H 3.30, δ_C 49.0 ppm). Electron ionization (EI) and Fast atom bombardment (FAB) mass spectra were obtained with a Jeol AX500 instrument (Jeol, Tokyo, Japan). Optical absorbance was acquired using a Hitachi U-3210 spectrophotometer (Hitachi, Tokyo, Japan). Analytical thin-layer chromatography was performed on silica gel plates Merck 60 F_{254} (Merck Ltd., Tokyo, Japan, 0.25 mm thickness). Ordinary phase column chromatography was performed with silica gel, Wakogel C-300 (Wako).

Colorimetric radical scavenging tests. DPPH radical-scavenging activity was measured as described previously.[7,9] 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 with a Vortex mixer and transferred to a cuvette. An absorbance reading at 517 nm was taken at 30 min after initial mixing. Acetonitrile and methanol were chosen as inert non-alcoholic and nucleophilic alcoholic solvents respectively. A solution of DL-α-tocopherol at the same concentration was measured as a positive

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control. A reduction in absorbance of 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.

*Isolation of oxidation products of **1***. To a solution of compound **1** (67 mg, 0.40 mmol) in methanol (1.6 liters) was added DPPH radical (0.95 g, 2.4 mmol, 6.0 equiv.), and the mixture was stirred for 1 h at room temperature. A solution of sodium dithionite (0.40 g, 2.4 mmol, 6.0 equiv.) in water (24 ml) was added to the reaction mixture, and this was stirred for 30 min to reduce unstable *o*-quinones to their catechol forms. The resulting mixture was concentrated under reduced pressure, and the residue was dissolved in acetone and filtered. The filtrate was concentrated under reduced pressure. The residue was subjected to silica gel column chromatography and eluted first with chloroform to remove DPPH radical and its reduced hydrazine, and then with methanol. The resulting methanol eluate was concentrated under reduced pressure. The crude product was further purified by preparative HPLC (column, ODS-Prep, 20 × 250 mm, GL-Sciences, Tokyo, Japan; mobile phase, 40% acetonitrile containing 0.1% formic acid; flow rate, 5.0 ml/min; detection, UV 254 nm) to afford **4** (0.9 mg, 2.1%, t_R 12.4 min)[7] and **13** (7.0 mg, 8.8%, t_R 15.0 min). **13**: a yellow oil. ^{1}H-NMR (methanol-d_4): 3.29 (3H, *s*, 2′-OCH_3), 3.53 (3H, *s*, 2′-OCH_3), 3.73 (3H, *s*, 7′-OCH_3), 3.76 (3H, *s*, 7-OCH_3), 5.81 (1H, *d*, J = 9.8 Hz, H-5′), 7.11 (1H, *s*, H-2), 7.73 (1H, *d*, J = 9.8 Hz, H-6′). ^{13}C-NMR (methanol-d_4): 52.2 (7-OCH_3), 52.5 (2′-OCH_3), 52.8 (7′-OCH_3), 53.9 (2′-OCH_3), 65.3 (C-1′), 93.4 (C-3′), 116.9 (C-2), 119.0 (C-2′), 119.6 (C-5), 123.4 (C-1), 124.4 (C-5′), 137.7 (C-6), 145.8 (C-3), 148.1 (C-4), 150.0 (C-6′), 168.1 (C-7), 171.5 (C-7′), 196.5 (C-4′). HMBC correlation peaks: H-2/C-4, C-6, H-5′/C-1′, C-3′, H-6′/C-6, C-2′, C-4′, 2′-OCH_3/C-2′, 7-OCH_3/C-7, 7′-OCH_3/C-7′. HR-FAB-MS m/z $(M-H)^-$: calcd. for $C_{18}H_{17}O_{10}$, 393.0822; found, 393.0827.

*Isolation of compound **14***. Compound **14** was isolated from a reaction mixture of **4** and DPPH radical by the method described for **13** (15% from **4**, t_R 17.2 min). Compound **14**: a yellow oil. ^{1}H-NMR (methanol-d_4): 3.29 (3H, *s*, 2′-OCH_3), 3.53 (3H, *s*, 2′-OCH_3), 3.73 (3H, *s*, 7-OCH_3), 3.76 (3H, *s*, 7′-OCH_3), 3.80 (3H, *s*, 2-OCH_3), 5.81 (1H, *d*, J = 9.8 Hz, H-5′), 7.73 (1H, *d*, J = 9.8 Hz, H-6′). ^{13}C-NMR (methanol-d_4): 52.1 (7-OCH_3), 52.4 (2′-OCH_3), 53.0 (7′-OCH_3), 53.8 (2′-OCH_3), 62.1 (2-OCH_3), 65.5 (C-1′), 93.0 (C-3′), 116.3 (C-1), 118.8 (C-5), 135.7 (C-3), 139.4 (C-6), 147.8 (C-4), 148.1 (C-2), 119.0 (C-2′), 124.4 (C-5′), 150.3 (C-6′), 167.2 (C-7), 171.2 (C-7′), 196.3 (C-4′). HMBC correlation peaks: H-5′/C-1′, C-3′, H-6′/C-6, C-2′, C-4′, 2-OCH_3/C-2, 2′-OCH_3/C-2′, 7-OCH_3/C-7, 7′-OCH_3/C-7′. HR-FAB-MS m/z $(M-H)^-$: calcd. for $C_{19}H_{19}O_{11}$, 423.0927; found, 423.0951.

Molecular orbital calculations. The electron density and energy of LUMO were calculated by the AM1 method using the MOPAC 2000 program in the Chem3D package (CambridgeSoft, Cambridge, MA).

Results and Discussion

Compound **13**, together with the C-2 methanol adduct (**4**), was isolated as an oxidation product from the reaction mixture of **1** and DPPH radical in methanol. Its molecular formula was identified as $C_{18}H_{18}O_{10}$ from the HR-FAB-MS result, indicating that **13** was a dimeric product of **1**. The ^{1}H-NMR spectrum showed a pair of doublet signals at δ 5.81 (H-5′) and δ 7.73 (H-6′), with a larger coupling constant (J = 9.8 Hz) than that found in **4** ($J_{H\text{-}5/H\text{-}6}$ = 8.6 Hz). H-6′ showed $^3J_{CH}$ HMBC correlations with an acetal carbon at δ 119.0 (C-2′) and a carbonyl carbon at δ 196.5 (C-4′), indicating that **13** was a quinone acetal-like product. Additionally, HMBC correlations between C-2′ and two methoxy groups suggested the formation of dimethyl acetal at C-2′. H-5′ was correlated with two sp^3 carbons of C-1′ and C-3′. Moreover, the HMBC spectrum showed correlations of H-2/C-6 and H-6′/C-6. These results suggest that the two protocatechuoyl residues were linked at C-6 and C-1′. The other interunit C-C linkage was between a quaternary benzene carbon assignable to C-5 (δ 119.6) and a downfield-shifted quaternary oxycarbon of C-3′ (δ 93.4). On the basis of ^{1}H and ^{13}C-NMR, HMQC, and HMBC spectral data, the structure of **13** is proposed to be as depicted in Fig. 1.

From a reaction mixture of **4** and DPPH radical in methanol, compound **14** was isolated by the procedure described for **13**. The negative FAB-MS of **14** exhibited a $[M-H]^-$ peak at m/z 423, indicating that the molecular weight of **14** was 30 mass units larger than that of **13**. The ^{1}H-NMR spectrum of **14** was very similar to that of **13**, except for the disappearance of a singlet signal of H-2 and the presence of the signal of an additional methoxy group. Furthermore, the 2D NMR spectra were consistent with those of **13**. Hence it was concluded that the structure of **14** was the 2-methoxy derivative of **13**.

A plausible formation mechanism for **13** is outlined in Scheme 1. First, **5** undergoes nucleophilic attack by a methanol molecule at C-2′ to yield dimethyl acetal (**10**). Since the resulting dimethyl acetal (**10**) does not have a proton at C-2′, regeneration of a catechol structure cannot occur. Instead, ketonization of C-4′ generates an

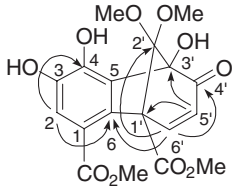
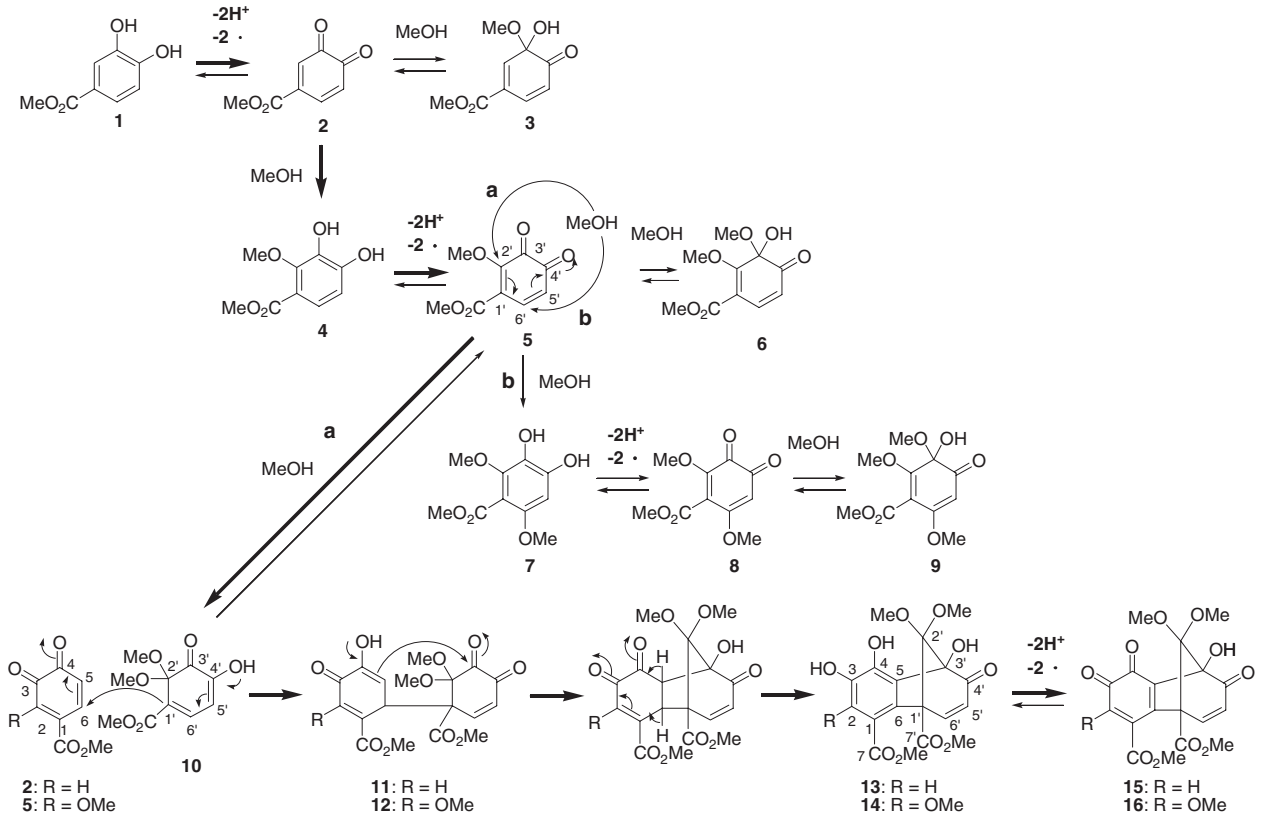


Fig. 1. Key HMBC Correlations of **13**.



Scheme 1. Possible Formation Mechanism for Compounds **13** and **14**.

anion at C-1′, which attacks C-6 of **2** to form **11** *via* Michael addition. Then intramolecular aldol condensation and subsequent aromatization give **13**. Similarly, dimerization of **5** and **10** forms **14**.

To substantiate this preference in the positions of the nucleophilic attacks, the electron density of LUMO of **5** was calculated by the semi-empirical method. The electron densities of the carbons of **5** were as follows: C-1′, 0.20; C-2′, 0.38; C-3′, 0.29; C-4′, 0.16; C-5′, 0.14; and C-6′, 0.17. Since the LUMO electron density of C-2′ of **5** was much higher than that of C-6′, the nucleophilic addition on C-2′ of **5** to form **10** (reaction **a**, Scheme 1) should be a kinetically preferred reaction. However, we have found that **5** underwent a nucleophilic attack at C-6′ (reaction **b**, Scheme 1), rather than at C-2′, by a second methanol molecule to yield the 2,6-bis-methanol adduct (**7**) in methanol.[10] Considering that reaction **a** is reversible and that dimerization occurs only when a reactive C-1′ anion produced is caught by a strong electrophile such as an *o*-quinone, that exists in the near environment, a nucleophilic addition on C-6′, a less likely but irreversible reaction, should occur in part and then rapidly convert to a thermodynamically stable catechol (**7**). It is interesting that a similar benzobicyclo[3.2.1]octane dimer was isolated as a coupling product between 4-methyl-*o*-benzoquinone and 5-methylpyrogallol.[11] This dimerization reaction might be a common oxidation mechanism of polyphenol molecules. Indeed, HPLC analysis of reaction mixtures of **1** and **4** with DPPH radical in methanol revealed that these dimers, **13** and **14** respectively, were the predominant products.

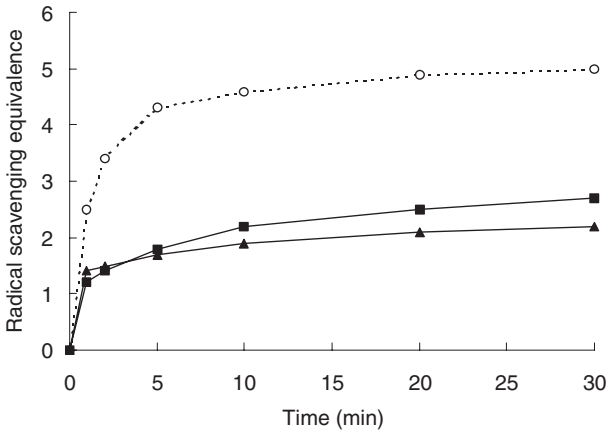


Fig. 2. Time Courses of DPPH Radical Scavenging Activity of Methyl Protocatechuate (**1**, ○), and Its Oxidative Dimers **13** (■) and **14** (▲) in Methanol.

Equivalence is expressed as values relative to that of DL-α-tocopherol as 2.0.

The DPPH radical scavenging activity of isolated **13** and **14** was examined in methanol (Fig. 2). Both **13** and **14** scavenged approximately two equivalents of the radical in 30 min, indicating that **13** and **14** were converted to the corresponding *o*-quinones (**15** and **16**).

As shown in Scheme 1, two molecules of **1** should scavenge eight equivalents of the radical to afford **15** and 10 equivalents to yield **16**, indicating consumption of four and five equivalents respectively per molecule of **1**. The fact that more than five equivalents of the radical are scavenged by **1** in methanol cannot be explained only by the formation of these dimers. The radical-scavenging reactions of polyphenols are quite complex, and hence a complete elucidation of the total reaction profile appears to be impracticable. However, in this case, considering that **13** and **14** were the predominant isolable oxidation products from **1** and **4** respectively, even in the diluted solution, the formation of these dimers evidently contributes to the total radical scavenging activity of **1**.

In conclusion, compound **13**, a novel oxidation dimer, was isolated from a mixture of **1** and DPPH radical in methanol. The result suggests that regeneration of catechol structures by the nucleophilic addition of a solvent molecule on *o*-quinones and subsequent dimerization are key reactions in the high radical-scavenging activity of protocatechuic esters in alcoholic solvents. Further investigation is needed to determine whether the radical-scavenging reaction of protocatechuic esters found in the present study occurs in aqueous biological systems.

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References

1) Robbins, R. J., Phenolic acids in foods: an overview of analytical methodology. *J. Agric. Food Chem.*, **51**, 2866–2887 (2003).
2) Natella, F., Nardini, M., Felice, M. D., and Scaccini, C., Benzoic and cinnamic acid derivatives as antioxidants: structure-activity relation. *J. Agric. Food Chem.*, **47**, 1453–1459 (1999).
3) Mattila, P., and Kumpulainen, J., Determination of free and total phenolic acids in plant-derived foods by HPLC with diode-array detection. *J. Agric. Food Chem.*, **50**, 3660–3667 (2002).
4) Nakamura, H., Nishikawa, A., Furukawa, F., Kasahara, K., Miyauchi, M., Son, H. Y., and Hirose, M., Inhibitory effects of protocatechuic acid on the post-initiation phase of hamster pancreatic carcinogenesis induced by *N*-nitrosobis(2-oxopropyl)amine. *Anticancer Res.*, **20**, 3423–3427 (2000).
5) Tseng, T. H., Hsu, J. D., Lo, M. H., Chu, C. Y., Chou, F. P., Huang, C. L., and Wang, C. J., Inhibitory effect of *Hibiscus* protocatechuic acid on tumor promotion in mouse skin. *Cancer Lett.*, **126**, 199–207 (1998).
6) Tanaka, T., Kojima, T., Kawamori, T., and Mori, H., Chemoprevention of digestive organs carcinogenesis by natural product protocatechuic acid. *Cancer*, **75**, 1433–1439 (1995).
7) Saito, S., Okamoto, Y., and Kawabata, J., Effects of alcoholic solvents on antiradical abilities of protocatechuic acid and its alkyl esters. *Biosci. Biotechnol. Biochem.*, **68**, 1221–1227 (2004).
8) Saito, S., Okamoto, Y., Kawabata, J., and Kasai, T., Quinone hemiacetal formation from protocatechuic acid during the DPPH radical scavenging reaction. *Biosci. Biotechnol. Biochem.*, **67**, 1578–1579 (2003).
9) Saito, S., and Kawabata, J., Effects of electron-withdrawing substituents on DPPH radical scavenging reactions of protocatechuic acid and its analogues in alcoholic solvents. *Tetrahedron*, **61**, 8101–8108 (2005).
10) Saito, S., Gao, H., and Kawabata, J., DPPH radical scavenging reaction of protocatechuic esters in alcohols: formation of bis-alcohol adduct. *Helv. Chim. Acta*, **89**, 821–831 (2006).
11) Yanase, E., Sawaki, K., and Nakatsuka, S., The isolation of a bicyclo[3.2.1] intermediate during formation of benzotropolones, a common nucleus found in black tea pigments: theaflavins. *Synlett*, 2661–2663 (2005).