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Radical scavenging activity of dicaffeoyloxycyclohexanes: Contribution of an intramolecular interaction of two caffeoyl residues

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Abstract—Six regio- and stereoisomers of dicaffeoyloxycyclohexanes and 2,4-di-*O*-caffeoyl-1,6-anhydro-β-D-glucose were synthesized as model compounds of dicaffeoylquinic acids, and their radical scavenging activity was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt) radical scavenging tests. Both DPPH and ABTS radical scavenging reactions of these compounds consisted of two different steps. In the first step, catechol moieties of the caffeoyl residues were rapidly converted to *o*-quinone structures and no significant difference in the reactivity was observed among the tested compounds. In the second step, however, the rate of the reaction increased as the intramolecular distance of the two caffeoyl residues decreased. A novel intramolecular coupling product, which could scavenge additional radicals, was isolated from the reaction mixture of *trans*-1,2-dicaffeoyloxycyclohexane and DPPH radical. The result suggests that the second step of the radical scavenging reaction is arising from an intramolecular interaction between the two caffeoquinone residues to regenerate catechol structures, and that the closer their distance is, the more rapidly they react. The radical scavenging activity of natural dicaffeoylquinic acids in a biological aqueous system might also depend on the positions of caffeoyl ester groups.

1. Introduction

Caffeoylquinic acids are widely distributed in the plant kingdom and known as potent antioxidants.[1–6] In a previous paper, we reported the antioxidant activity of mono-, di-, and tricaffeoylquinic acids isolated from burdock.[1] Their activity increased in proportion to the number of caffeoyl residues. It has also been reported that three dicaffeoylquinic acids (1,3-, 3,4-, and 4,5-di-*O*-caffeoylquinic acid) showed similar antioxidant activity using the β-carotene/linoleate method.[2] However, since caffeic acid is known to be quite prone to oxidative dimerization,[7–12] intramolecular coupling reactions between two adjacent caffeoyl residues may occur during the oxidation reactions of dicaffeoylquinic acids and could influence their total antioxidant potency. Thus, we have been interested in the relationships between the esterified position of caffeoyl residues in dicaffeoylquinic acids and their radical scavenging activity.

In the present study, we synthesized six regio- and stereoisomers of dicaffeoyloxycyclohexanes and 2,4-di-*O*-caffeoyl-1,6-anhydro-β-D-glucose as model compounds. Their radical scavenging activity was compared with that of cyclohexyl caffeate by DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt) radical scavenging tests to examine whether the distance of intramolecular caffeoyl residues would affect the total radical scavenging activity. In addition, we report the isolation of an intramolecular coupling product formed by the oxidation of *trans*-1,2-dicaffeoyloxycyclohexane with DPPH radical, and propose the radical scavenging mechanism of dicaffeoyloxycyclohexanes via an intramolecular coupling reaction of the caffeoyl residues.

2. Results and discussion

Six regio- and stereoisomers of dicaffeoyloxycyclohexanes (**3**–**8**), cyclohexyl caffeate (**9**), and 2,4-di-*O*-caffeoyl-1,6-anhydro-β-D-glucose (**10**) were synthesized.

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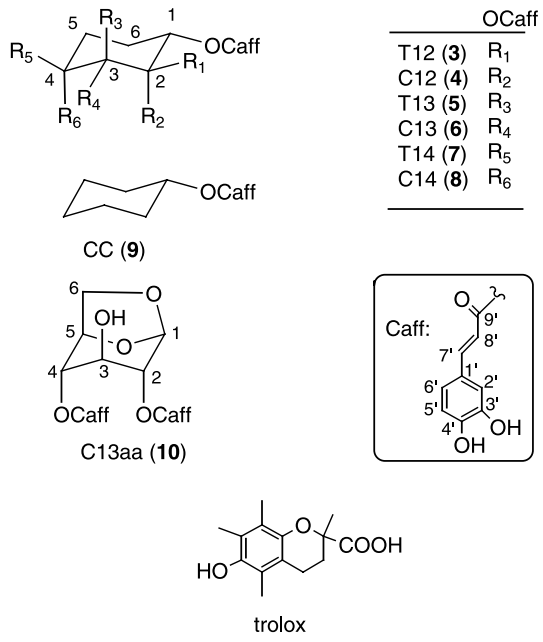

Figure 1. Structures of dicaffeoyloxycyclohexanes, 2,4-di-*O*-caffeoyl-1,6-anhydro-β-D-glucose, cyclohexyl caffeate and trolox.

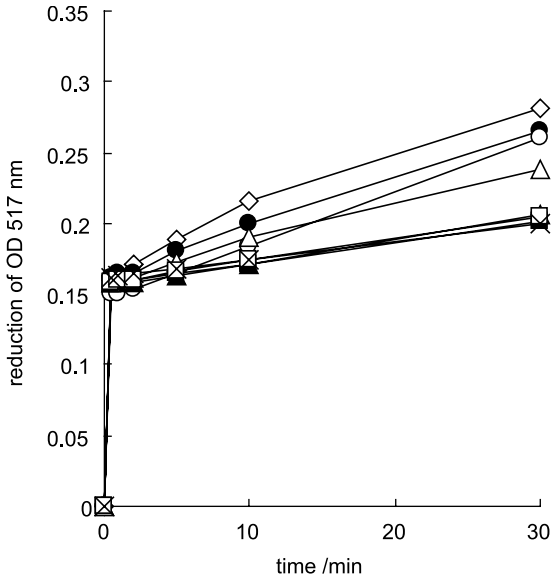

Figure 2. Time course of reduction of DPPH radical at OD 517 nm by C13aa (◇), T12 (●), C12 (○), T13 (▲), C13 (△), T14 (■), C14 (□) and CC (×). Concentrations of tested compounds are 4 μM except for CC (8 μM).

Compound **10** was prepared as a model compound of 1,3-di-*O*-caffeoylquinic acid (cynarin), which bears two caffeoyl residues in 1,3-diaxial orientation.[13] The structures of dicaffeoyloxycyclohexanes were confirmed by the comparison of the spectral data with those in the literature.[14] Compound **10** was synthesized from 1,6-anhydro-β-D-glucose. From the HMBC spectrum of **10**, H-2 (δ_H 4.66) and H-4 (δ_H 4.76) of the glucose correlated with each carbonyl carbon in acyl residues. Therefore, the caffeoyl residues were determined to be esterified with 2 and 4 position of 1,6-anhydro-β-D-glucose. The structures of these compounds are shown in Figure 1.

The time-dependent decay of the absorbance of the DPPH radical by an addition of the test compounds in 40% buffer/EtOH is presented in Figure 2. The DPPH radical scavenging reactions of all test compounds consisted of two successive but different steps. The absorbance rapidly decayed over 0–0.5 min in the first step followed by a much slower decrease of the absorbance in the second step (5–30 min). There was no significant difference among the tested compounds in the reactivity of the first step. In the second step, however, the rate of the reaction decreased in the order of C13aa > T12, C12 > C13 > T13, C14, T14. Total radical consumption of C13aa, T12, C12, and C13 was apparently larger than that of 2 equiv of CC. On the other hand, T13, C14, and T14 only scavenged DPPH radical in the same rate as 2 equiv of CC. Interestingly, the second step of the radical scavenging reaction accelerates in the presence of water (Fig. 3).

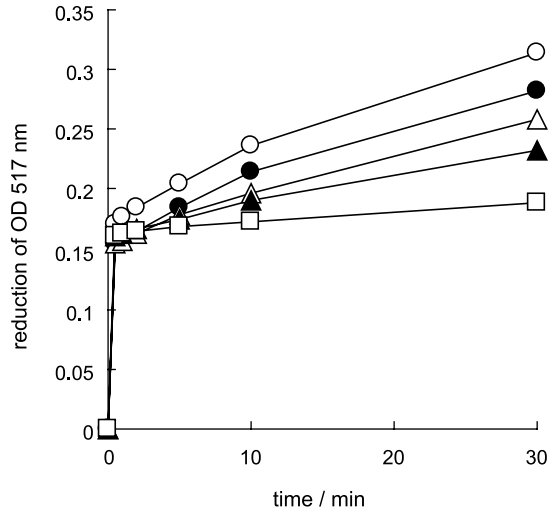

Figure 3. Time course of reduction of DPPH radical at OD 517 nm by T12 in aqueous ethanol. 60% water in ethanol (○), 40% water in ethanol (●), 20% water in ethanol (△), 10% water in ethanol (▲) and ethanol (□).

ABTS radical scavenging activity was tested in the aqueous solution, and the result is shown in Figure 4. Trolox, which is known to scavenge two radicals, was used as a positive control (Fig. 1). As was seen with DPPH radical, the ABTS radical scavenging reaction of the tested compounds also consisted of two steps. The tendency of the reactivity of the isomers was quite similar to that obtained in the DPPH radical scavenging reaction. The decrease in the absorbance of ABTS radical by the dicaffeoyl derivatives in the first step was same as 2 equiv of trolox, indicating the quantitative formation of bis-*o*-quinone in this step.

The formation of bis-*o*-quinone (**11**) from T12 was confirmed by ^{1}H NMR analysis of the DPPH radical and T12 mixture in acetonitrile-d_3. The coupling constant of H-5′ (δ_H 6.42, d, J = 10.3 Hz) and H-6′ (δ_H 7.39, d, J = 10.3 Hz) of **11** was larger than that of H-5′

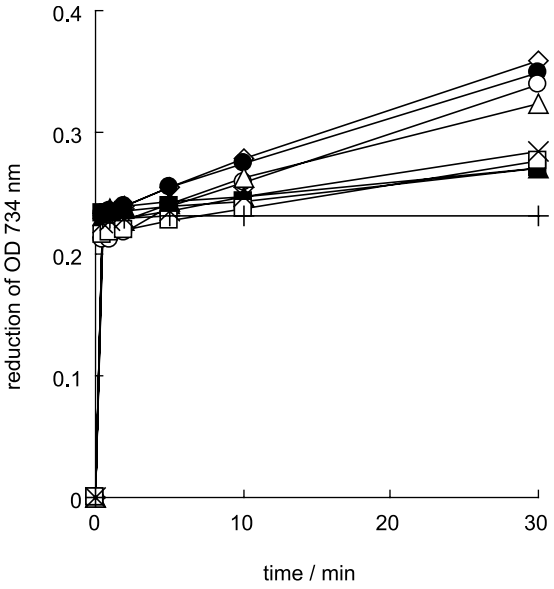

Figure 4. Time course of reduction of ABTS radical at OD 734 nm by C13aa (◇), T12 (●), C12 (○), T13 (▲), C13 (△), T14 (■), C14 (□), CC (×) and trolox (+). Concentration of tested compounds are 4 μM except for CC and trolox (8 μM).

(δ_H 6.73, d, J = 8.2 Hz) and H-6′ (δ_H 6.88, dd, J = 8.2, 2.0 Hz) of T12, as well as the low-field shift of H-6′, and the high-field shift of H-2′ and H-5′ in **11** compared to T12 indicated the formation of the *o*-quinone moiety in **11**.

These results indicate that the reaction rate of the second step increases as the intramolecular distance of the two caffeoyl residues decreases. Thus, the second step seems to arise from an intramolecular interaction of the two caffeoyl residues. There is no difference in the distance of the intramolecular caffeoyl residues in C12 and T12, since two caffeoyl residues are oriented in the *gauche* conformation in both isomers. On the other hand, C13, T13, C14, and T14 seem to exist as their extended conformers of diequatorial (C13, T14) and axial-equatorial (T13, C14) orientations. However, when the cyclohexane ring flips in C13, the intramolecular caffeoyl residues become close enough to interact with each other. In contrast, the caffeoyl residues of T13, C14, and T14 are too far to interact with each other even if the ring flipping occurs, which accounts well for the similar reactivity of these isomers as 2 equiv of CC. The highest radical scavenging activity of C13aa would reasonably be explained by the fixed conformation of the two caffeoyl residues into 1,3-diaxial manner in which they could interact with each other easily.

To confirm that intramolecular couplings of the caffeoyl residues actually occur, we attempted to isolate the oxidation products from the reaction mixture. Compounds **12** and **13** were isolated from the mixture of T12 and DPPH radical in 40% H_2O/MeOH and 40% H_2O/EtOH, respectively.

Compound **12** showed the $[M]^+$ peak at *m*/*z* 468 in FD-MS. Since the molecular weight of T12 bis-*o*-quinone (**11**) is 436, **12** is indicated to be a methanol adduct of T12. Appearance of a singlet signal at δ_H 3.48 in the ^1H NMR spectrum supports the presence of an additional methoxy group. Two sets of doublet signals due to trans double bonds at δ_H 5.91 and 7.87 (J = 15.4 Hz) and 6.35 and 7.20 (J = 16.1 Hz) indicate that the side chains of the two caffeoyl residues remained unchanged. The HMBC spectrum showed correlations between C-2′ and H-5″, and C-3′ and H-5″, suggesting that the two caffeoyl residues are linked at C-2′ and C-5″ in two aromatic rings. On the basis of ^1H and ^{13}C NMR, HMQC, and HMBC spectral data, the structure of **12** was deduced to be as shown in Figure 5. Stereochemistry of **12** was tentatively determined by the NOESY experiment.

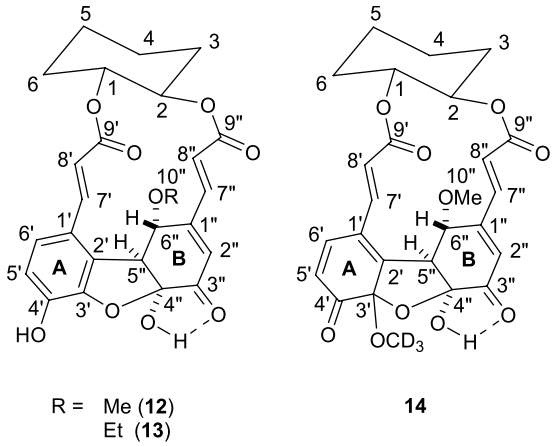

Figure 5. Structures of compounds **12**, **13**, and **14**.

The FD-MS of **13** exhibited the $[M]^+$ peak at *m*/*z* 482, indicating that the molecular weight of **13** was 14 mass units larger than that of **12**. The ^1H NMR spectrum of **13** was very similar to that of **12**, except for the presence of signals of an ethoxy group at δ_H 1.11 (3H, t, J = 7.0 Hz), 3.62 (1H, dq, J = 9.0, 7.0 (q) Hz) and 3.87(1H, dq, J = 9.0, 7.0 (q) Hz) in place of the methoxy group at δ_H 3.48 in **12**. Hence, the structure of **13** was concluded to be the ethoxy analog of **12**.

The DPPH radical scavenging activity of T12 and **12** in 40% H_2O/MeOH was compared with DL-α-tocopherol, which scavenges two molecules of DPPH radical. The decrease in the absorbance of DPPH radical by T12 in the first step corresponded to that by 2 equiv of DL-α-tocopherol. In the second step, radical scavenging reaction reached plateau at 2 h and the decrease in the absorbance coincided with 2.5-fold of that by DL-α-tocopherol. The result suggests that T12 rapidly scavenged four radicals in the first step, and then slowly consumed additional five radicals in the second step. On the other hand, **12** consumed DPPH radicals corresponding to 1 equiv of DL-α-tocopherol. It seems that **12** is derived from an addition of methanol to T12 bis-*o*-quinone (**11**) as discussed later, and hence, 4 equiv of DPPH radicals should be consumed during its production from T12 in the first step. Compound **12** scavenged approximately two radicals, which suggests that **12** partly contributes

to the second step of the radical scavenging reaction of T12.

To elucidate the radical scavenging reaction after the formation of **12**, the reaction mixture of **12** and DPPH radical was directly analyzed by NMR. The ^{1}H NMR spectrum of the mixture of **12** and DPPH radical in methanol-d_4/acetone-d_6 (3:2) showed the disappearance of signals due to **12**, and the appearance of signals of a closely related compound to **12**. In situ 2D NMR analyses of the reaction mixture suggested that two propenoate parts and B-ring in **12** remained unchanged. However, the coupling constant of the doublet signals of H-5′ and H-6′ (J = 10.3 Hz) was larger than that of H-5′ and H-6′ of **12** (J = 8.4 Hz), indicating that the catechol structure of A-ring in **12** was oxidized to the quinone form. This was supported by the appearance of an additional carbonyl carbon at δ_C 193.1. In addition, high-field shift of C-3′ (δ_C 100.3) of A-ring compared to C-3′ (δ_C 146.4) in **12**, suggests the formation of acetal via a nucleophilic addition of methanol-d_4 at C-3′ quinone carbonyl. Therefore, the structure of **14** was determined to be as shown in Figure 5.

A plausible radical scavenging mechanism of T12 in water–methanol is outlined in Figure 6. In the first step, the caffeoyl residues rapidly scavenge four radicals and are converted to the corresponding *o*-quinone structures (**11**). The resultant *o*-quinone (**11**) undergoes a nucleophilic attack by a methanol molecule at C-6″ of the B-ring to yield an enolate intermediate, which spontaneously attacks C-2′ of A-ring quinone. It is well documented that caffeoquinone is susceptible to nucleophilic attack by thiol compounds such as glutathione and cysteine at C-2.[15,16] On the other hand, it was speculated that a water molecule attacks at C-6 of caffeoquinone.[17] In this study, only the C-6 alcohol adduct was isolated, and neither C-2 nor C-5 adduct was detected. However, the reason why the position of nucleophilic attack differs by nucleophiles remains to be clarified. The stereoselective introduction of the C-6″ methoxy group is probably a result from the conformation of T12 in the reaction solution and from the relative stability of the resultant functionalized macrocyclic ring. Previously, we showed that protocatechuquinone methyl ester undergoes a nucleophilic attack by alcohol molecules and thiol compounds, followed by a regeneration of the catechol structure.[18,19] However, in the case of T12, an intramolecular Michael addition preferentially occurs due to the presence of a reactive quinone in the vicinity, without reproducing the catechol structure. Then subsequent aromatization leads to a regeneration of the catechol structure of the A-ring to form **12a**, which is equilibrated with more stable **12** in the reaction solution. The regeneration of the catechol structure of the B-ring of **12a** is unlikely to occur by steric disfavor of the generation of the highly functionalized[12] metacyclophan ring. When an excess amount of radical exists in the reaction mixture, **12a** would scavenge two additional radicals to yield **12b**. Then, a methanol molecule attacks C-3′ quinone carbonyl of **12b** to form the corresponding 3-hemiacetal by a similar mechanism described for protocatechuquinone 3-hemiacetal.[20] The resultant alkoxide ion in the A-ring attacks adjacent C-4″ carbonyl of the B-ring to form more stable cyclic hemiacetal **14**. Although the formation of **14** from **12** was confirmed by NMR in methanol-d_4/acetone-d_6 = 3:2, compound **12** would also be converted to **14** in H_2O/MeOH, since **12** consumed

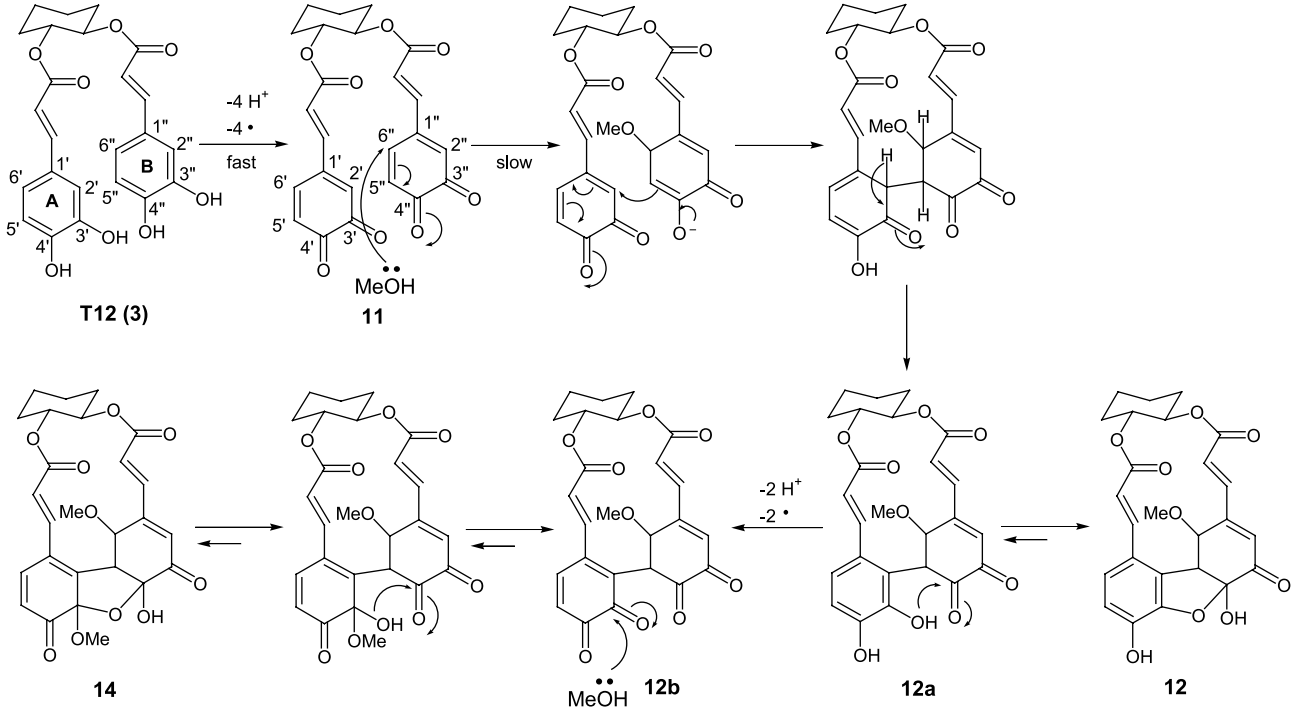

Figure 6. Plausible radical scavenging mechanism of T12 in water–methanol.

approximately two radicals in both 40% H_2O/MeOH and 40% acetone/MeOH (methanol/acetone = 3:2).

It is suspected that a water molecule may also attack T12 bis-*o*-quinone (**11**) in water/MeOH or water/EtOH solution, but the formation of a water adduct was not confirmed in the present study. The reason that alcohol adducts form preferentially might be explained by the higher nucleophilicity of alcohol than water. However, the presence of water may play a crucial role in the second step, since the DPPH radical scavenging reaction accelerates by the increase of water content in the solvent. In addition, the fact that **12** was not detected in the reaction mixture of T12 and DPPH radical in methanol also supports the importance of water. The increasing amount of water results in higher polar nature of the solution, which might be advantageous for closer stacking of hydrophobic caffeoyl or caffeoquinone groups.

Although the two caffeoyl residues need to be close to each other during the intramolecular coupling reactions, T13, C14, T14, and CC slowly consumed radicals after the first step. The result suggests that intermolecular dimerizations might also occur, which could contribute to the radical scavenging activity. However, in a dilute solution, intramolecular couplings of the caffeoyl residues would effectively increase the rate of the second step of the radical scavenging reactions.

3. Conclusion

Radical scavenging reactions of dicaffeoyloxycyclohexanes in an aqueous alcohol consisted of two different steps. In the first step, catechol moieties of the caffeoyl residues were rapidly converted to *o*-quinone structures. The second step was triggered by the addition of an alcohol molecule to the quinone followed by an intramolecular coupling of the two caffeoquinone residues to regenerate catechol structures, which then react with additional radicals, especially in the aqueous solution. The total radical scavenging potency was thus affected by the intramolecular distance of the two caffeoyl residues in the molecule. The antiradical activity of natural dicaffeoylquinic acids might also be dependent on positions of caffeoyl ester groups in the biological aqueous system.

4. Experimental

4.1. General

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), dimethyl sulfoxide (DMSO)-d_6 (δ_H 2.49), acetone-d_6 (δ_H 2.04, δ_C 29.8) and acetonitrile-d_3 (δ_H 1.93). Field desorption mass spectra (FD-MS) and fast atom bombardment mass spectra (FAB-MS) were obtained with JEOL JMS-SX102A and JEOL JMS-AX-500 instruments, respectively. Optical absorbance was acquired using a UV-1600 spectrophotometer (Shimadzu). Preparative and analytical thin-layer chromatography was performed on silica gel plates Merck 60 F_{254} (0.5 mm and 0.25 mm thickness), respectively. Column chromatography was performed with silica gel, Wakogel C-300 (Wako Pure Chemical Industries) and Diaion HP 20 (Mitsubishi Chemical Co.).

trans-1,2-Cyclohexanediol, 1,3- and 1,4-cyclohexanediols (*cis* and *trans* mixture) and 1,6-anhydro-β-D-glucose were purchased from Tokyo Kasei Kogyo Co. *cis*-1,2-Cyclohexanediol and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Aldrich Chemical Co. and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) from Sigma Chemical Co. Caffeic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, potassium peroxodisulfate (potassium persulfate), and other reagents were purchased from Wako Pure Chemical Industries. All solvents used were of reagent grade.

4.2. Colorimetric radical scavenging tests

4.2.1. DPPH radical scavenging activity. To a solution of a test compound in ethanol (2 mL) and 100 mM acetate buffer (pH 5.5, 2 mL) was added 1 mL of DPPH radical (500 μM, in ethanol) in a test tube. In the different water–ethanol ratio experiments, the amount of water and ethanol was adjusted so as to match the required final ratio. Ethanol was used in the place of antioxidant solution as a control. The solution was immediately mixed vigorously for 10 s by a Vortex mixer and transferred to a cuvette. The absorbance reading was taken 0.5, 1, 2, 5, 10, and 30 min after initial mixing. The radical scavenging activity of T12 (**3**) and **12** was compared with DL-α-tocopherol in 40% H_2O/MeOH as described previously. The radical scavenging equivalence was expressed as the values relative to that of DL-α-tocopherol as 2.0.[21] All experiments were performed in triplicate.

4.2.2. ABTS radical scavenging activity. ABTS radical scavenging activity was measured using a modified Re et al.[22] method. ABTS radical cation ($ABTS^{+\cdot}$) was produced by reacting a 7 mM aqueous solution of ABTS with 2.45 mM potassium persulfate (final concentration). The reaction mixture was allowed to stand in the dark at room temperature for 12–16 h prior to use. $ABTS^+$ solution was diluted with distilled water to an absorbance of 0.70 (±0.02) at 734 nm. To a diluted $ABTS^+$ solution (2.97 mL) was added 0.03 mL of a test compound in ethanol. The solution was immediately mixed vigorously for 10 s by a Vortex mixer and transferred to a cuvette. The absorbance reading was taken 0.5, 1, 2, 5, 10, and 30 min after initial mixing. Ethanol was used in place of antioxidant solution as a control. An ethanol solution of trolox was measured as a positive control. All experiments were performed in triplicate.

4.3. Chemistry

4.3.1. 3,4-Di-*O*-(methoxycarbonyl)caffeic acid (1).[14] To a solution of caffeic acid (9.0 g, 50 mmol) in 1 M NaOH (110 mL) was added methyl chloroformate (23 mL,

300 mmol) at 0 °C. The reaction mixture was stirred for 1 h. The precipitate was collected by filtration and washed with water. The crude product was recrystallized from ethanol to afford **1** as a white powder (13.7 g, 93%); FD-MS *m/z*: 296 $[M]^+$; ^{1}H NMR (methanol-d_4): δ 3.80 (6H, s, OCH_3), 6.42 (1H, d, J = 16.0 Hz, H-8), 7.28 (1H, d, J = 8.4 Hz, H-5), 7.47 (1H, dd, J = 8.4, 2.0 Hz, H-6), 7.52 (1H, d, J = 2.0 Hz, H-2), 7.56 (1H, d, J = 16.0 Hz, H-7).

4.3.2. 3,4-Di-*O*-acetylcaffeic acid (2). To a solution of caffeic acid (9.0 g, 50 mmol) in 1 M NaOH (110 mL) was added acetic anhydride (19 mL, 200 mmol) at 0 °C. The reaction mixture was stirred for 1 h. The precipitate was collected by filtration and washed with water. The crude product was recrystallized from ethanol to afford **2** as a white powder (12.3 g, 93%); FD-MS *m/z*: 264 $[M]^+$; ^{1}H NMR (acetone-d_6): δ 2.28 (6H, s, $COCH_3$), 6.52 (1H, d, J = 16.0 Hz, H-8), 7.30 (1H, d, J = 8.1 Hz, H-5), 7.59 (1H, d, J = 2.0 Hz, H-2), 7.60 (1H, dd, J = 8.1, 2.0 Hz, H-6), 7.65 (1H, d, J = 16.0 Hz, H-7).

4.3.3. 3,4-Di-*O*-(methoxycarbonyl)caffeoyl chloride (1a) and 3,4-di-*O*-acetylcaffeoyl chloride (2a).[14] To a solution of **1** (2.96 g, 10 mmol) in toluene (20 mL) was added oxalyl chloride (5.2 mL, 60 mmol, 6 equiv). After stirring for 1.5 h at 60 °C, toluene and unreacted oxalyl chloride were removed under the reduced pressure. Compound **2a** was prepared using the same method described for **1a**. The crude products **1a** and **2a** were used immediately without purification.

4.3.4. *trans*-1,2-Bis[3,4-di-*O*-(methoxycarbonyl)caffeoyloxy]cyclohexane (3a).[14] *trans*-1,2-Cyclohexanediol (1.7 g, 15 mmol) was reacted with **1a** (12.6 g, 40 mmol, 2.7 equiv) without solvent in an oil bath at 150 °C for 20 min. The residue was subjected to silica gel column chromatography (hexane/ethyl acetate = 2:1) to afford **3a** (4.8 g, 47%) as a yellow oil; FD-MS *m/z*: 672 $[M]^+$; ^{1}H NMR (chloroform-*d*): δ 1.35–1.39 (2H, m, H-4_{eq} and 5_{eq}), 1.41–1.47 (2H, m, H-3_{eq} and 6_{eq}), 1.74–1.75 (2H, m, H-4_{ax} and 5_{ax}), 2.09–2.11 (2H, m, H-3_{ax} and 6_{ax}), 3.85 (6H, s, OCH_3), 3.86 (6H, s, OCH_3), 4.97 (2H, m, H-1 and 2), 6.29 (2H, d, J = 16.0 Hz, H-8′), 7.24 (2H, d, J = 8.5 Hz, H-5′), 7.34 (2H, dd, J = 8.5, 2.0 Hz, H-6′), 7.38 (2H, d, J = 2.0 Hz, H-2′), 7.53 (2H, d, J = 16.0 Hz, H-7′).

4.3.5. *trans*-1,2-Dicaffeoyloxycyclohexane (T12, 3).[14] To a solution of **3a** (4.8 g, 7.1 mmol) in tetrahydrofuran (50 mL) and methanol (200 mL) was added 2% Na_2CO_3 (100 mL) and stirred at room temperature for 12 h. After acidification with 10 M HCl to pH 1–2, the mixture was diluted with water and extracted with diethyl ether. The organic layer was dried over anhydrous magnesium sulfate and evaporated under the reduced pressure. The residue was subjected to silica gel column chromatography (hexane/ethyl acetate = 1:2). The crude solid from the eluate was recrystallized from ethyl acetate/hexane to afford **3** (2.8 g, 90%) as a white powder; FD-MS *m/z*: 440 $[M]^+$; ^{1}H NMR (methanol-d_4): δ 1.41–1.45 (2H, m, H-4_{eq} and 5_{eq}), 1.49–1.54 (2H, m, H-3_{eq} and 6_{eq}), 1.78–1.79 (2H, m, H-4_{ax} and 5_{ax}), 2.08–2.11 (2H, m, H-3_{ax} and 6_{ax}), 4.95 (2H, m, H-1 and 2), 6.18 (2H, d, J = 15.9 Hz, H-8′), 6.73 (2H, d, J = 8.2 Hz, H-5′), 6.88 (2H, dd, J = 8.2, 2.0 Hz, H-6′), 6.98 (2H, d, J = 2.0 Hz, H-2′), 7.49 (2H, d, J = 15.9 Hz, H-7′).

4.3.6. *cis*-1,2-Dicaffeoyloxycyclohexane (C12, 4).[14] *cis*-1,2-Cyclohexanediol was converted to *cis*-1,2-dicaffeoyloxycyclohexane (**4**) by the same method as **3**. **4**: pale yellow powder; FD-MS *m/z*: 440 $[M]^+$; ^{1}H NMR (acetone-d_6): δ 1.48–1.50 (2H, m), 1.69–1.76 (4H, m), 1.91–1.95 (2H, m), 5.16 (2H, m, H-1 and 2), 6.28 (2H, d, J = 15.9 Hz, H-8′), 6.84 (2H, d, J = 8.2 Hz, H-5′), 7.01 (2H, dd, J = 8.2, 2.0 Hz, H-6′), 7.15 (2H, d, J = 2.0 Hz, H-2′), 7.55 (2H, d, J = 15.9 Hz, H-7′).

4.3.7. *trans*- and *cis*-1,3-Bis[3,4-di-*O*-(methoxycarbonyl)caffeoyloxy]cyclohexanes (5a and 6a).[14] A mixture of *cis*- and *trans*-1,3-cyclohexanediols (ca. 1:1) was reacted with **1a** by the same method as described for **3a**. A stereoisomeric mixture of 1,3-bis[3,4-di-*O*-(methoxycarbonyl)caffeoyloxy]cyclohexanes (**5a** and **6a**) was subjected to preparative TLC (chloroform/methanol/acetic acid = 100:1:0.1) to afford *trans*-form (**5a**) (R_f = 0.37, 20%) and *cis*-form (**6a**) (R_f = 0.29, 23%). **5a**: yellow oil; FD-MS *m/z*: 672 $[M]^+$; ^{1}H NMR (chloroform-*d*): δ 1.25–1.99 (8H, m, H-2, 4, 5 and 6), 3.92 (12H, s, OCH_3), 5.28 (2H, br s, H-1 and 3), 6.41 (2H, d, J = 16.0 Hz, H-8′), 7.32 (2H, d, J = 8.4 Hz, H-5′), 7.44 (2H, dd, J = 8.4, 2.0 Hz, H-6′), 7.47 (2H, d, J = 2.0 Hz, H-2′), 7.62 (2H, d, J = 16.0 Hz, H-7′) **6a**: yellow oil; FD-MS *m/z*: 672 $[M]^+$; ^{1}H NMR (chloroform-*d*): δ 1.25–2.19 (8H, m, H-2, 4, 5 and 6), 3.91 (12H, s, OCH_3), 4.95 (2H, br s, H-1 and 3), 6.37 (2H, d, J = 16.0 Hz, H-8′), 7.30 (2H, d, J = 8.6 Hz, H-5′), 7.39 (2H, dd, J = 8.6, 2.0 Hz, H-6′), 7.45 (2H, d, J = 2.0 Hz, H-2′), 7.60 (2H, d, J = 16.0 Hz, H-7′).

4.3.8. *trans*- and *cis*-1,3-Dicaffeoyloxycyclohexanes (T13, 5 and C13, 6).[14] Compounds **5** and **6** were prepared by selective hydrolysis of **5a** and **6a**, respectively, by the same method as described for **3** except for using 5% $NaHCO_3$ in place of 2% Na_2CO_3. The crude product was subjected to preparative TLC (hexane/ethyl acetate = 1:2) to afford **5** (R_f = 0.21, 76%) and **6** (R_f = 0.38, 51%). **5**: yellow oil; FD-MS *m/z*: 440 $[M]^+$; ^{1}H NMR (methanol-d_4): δ 1.65–2.00 (8H, m, H-2, 4, 5 and 6), 5.21 (2H, br s, H-1 and 3), 6.26 (2H, d, J = 16.0 Hz, H-8′), 6.77 (2H, d, J = 8.4 Hz, H-5′), 6.95 (2H, dd, J = 8.4, 2.0 Hz, H-6′), 7.04 (2H, d, J = 2.0 Hz, H-2′), 7.54 (2H, d, J = 16.0 Hz, H-7′) **6**: yellow oil; FD-MS *m/z*: 440 $[M]^+$; ^{1}H NMR (methanol-d_4): δ 1.30–2.30 (8H, m, H-2, 4, 5 and 6), 4.90 (2H, br s, H-1 and 3), 6.22 (2H, d, J = 15.9 Hz, H-8′), 6.73 (2H, d, J = 8.2 Hz, H-5′), 6.89 (2H, dd, J = 8.2, 2.0 Hz, H-6′), 7.02 (2H, d, J = 2.0 Hz, H-2′), 7.53 (2H, d, J = 15.9 Hz, H-7′).

4.3.9. *trans*- and *cis*-1,4-Bis[3,4-di-*O*-(methoxycarbonyl)caffeoyloxy]cyclohexanes (7a and 8a).[14] A mixture of *trans*- and *cis*- 1,4-cyclohexanediols (ca. 1:1) was reacted with **1a** by the same method as described for **3a**. A stereoisomeric mixture of 1,4-bis[3,4-di-*O*-(methoxycar-

bonyl)caffeoyloxy]cyclohexanes (**7a** and **8a**) was subjected to preparative TLC (chloroform/methanol/acetic acid = 100:1:0.1) to afford *trans*-form (**7a**) (R_f = 0.69, 17%) and *cis*-form (**8a**) (R_f = 0.57, 16%). **7a**: white powder; FD-MS *m*/*z*: 672 $[M]^+$; ^{1}H NMR (chloroform-*d*): δ 1.65 (4H, m), 2.07 (4H, m), 3.90 (12H, s, OCH_3), 4.95 (2H, br s, H-1 and 4), 6.38 (2H, d, J = 16.0 Hz, H-8′), 7.31 (2H, d, J = 8.4 Hz, H-5′), 7.41 (2H, dd, J = 8.4, 2.0 Hz, H-6′), 7.45 (2H, d, J = 2.0 Hz, H-2′), 7.59 (2H, d, J = 16.0 Hz, H-7′) **8a**: colorless crystal; FD-MS *m*/*z*: 672 $[M]^+$; ^{1}H NMR (chloroform-*d*): δ 1.80 (4H, m), 1.91 (4H, m), 3.90 (12H, s, OCH_3), 5.00 (2H, br s, H-1 and 4), 6.40 (2H, d, J = 16.0 Hz, H-8′), 7.31 (2H, d, J = 8.5 Hz, H-5′), 7.42 (2H, dd, J = 8.5, 2.0 Hz, H-6′), 7.46 (2H, d, J = 2.0 Hz, H-2′), 7.62 (2H, d, J = 16.0 Hz, H-7′).

4.3.10. *trans*- and *cis*-1,4-Dicaffeoyloxycyclohexanes (T14, 7 and C14, 8).[14] Compounds **7** and **8** were prepared by selective hydrolysis of **7a** and **8a**, respectively, by the same method as described for **3**. The crude product was subjected to preparative TLC (hexane/ethyl acetate = 1:2) to afford **7** (R_f = 0.31, 38%) and **8** (R_f = 0.25, 39%). **7**: yellow oil; FD-MS *m*/*z*: 440 $[M]^+$; ^{1}H NMR (DMSO-d_6): δ 1.57 (4H, m), 1.97 (4H, m), 4.82 (2H, br s, H-1 and 4), 6.24 (2H, d, J = 15.8 Hz, H-8′), 6.74 (2H, d, J = 8.1 Hz, H-5′), 7.00 (2H, dd, J = 8.1, 2.0 Hz, H-6′), 7.03 (2H, d, J = 2.0 Hz, H-2′), 7.46 (2H, d, J = 15.8 Hz, H-7′) **8**: yellow oil; FD-MS *m*/*z*: 440 $[M]^+$; ^{1}H NMR (acetone-d_6) ppm (J in Hz): δ 1.82 (4H, m), 1.90 (4H, m), 4.95 (2H, br s, H-1 and 4), 6.31 (2H, d, J = 15.9 Hz, H-8′), 6.86 (2H, d, J = 8.2 Hz, H-5′), 7.06 (2H, dd, J = 8.2, 2.0 Hz, H-6′), 7.17 (2H, d, J = 2.0 Hz, H-2′), 7.56 (2H, d, J = 15.9 Hz, H-7′).

4.3.11. Cyclohexyl caffeate (CC, 9). Cyclohexanol was converted to cyclohexyl caffeate (**9**) by the same method as **3**. **9**: yellow solid; mp 151–155 °C; FD-HR-MS *m*/*z*: 262.1211 $[M]^+$; calcd for $C_{15}H_{18}O_4$, 262.1205; ^{1}H NMR (acetone-d_6): δ 1.27–1.32 (1H, m, H-4_{ax}), 1.35–1.49 (4H, m, H-2_{ax}, 3_{eq}, 5_{eq} and 6_{ax}), 1.52–1.56 (1H, m, H-4_{eq}), 1.73–1.75 (2H, m, H-3_{ax} and 5_{ax}), 1.85 (2H, m, H-2_{eq} and 6_{eq}), 4.78 (1H, m, H-1_{ax}), 6.25 (1H, d, J = 15.9 Hz, H-8′), 6.85 (1H, d, J = 8.2 Hz, H-5′), 7.02 (1H, dd, J = 8.2, 2.0 Hz, H-6′), 7.15 (1H, d, J = 2.0 Hz, H-2′), 7.51 (1H, d, J = 15.9 Hz, H-7′).

4.3.12. 2,4-Bis-*O*-(3,4-di-*O*-acetylcaffeoyl)-1,6-anhydro-β-D-glucose (10a). To a solution of 1,6-anhydro-β-D-glucose (2.6 g, 16 mmol) in pyridine (40 mL) was added dropwise a solution of **2a** (11.3 g, 40 mmol, 2.5 equiv) in toluene (100 mL). After stirring at room temperature for 8 h, the reaction mixture was evaporated under the reduced pressure. The crude product was subjected to silica gel column chromatography (chloroform/methanol = 100:3) to afford **10a** (3.0 g, 57%) as a yellow oil; FD-MS *m*/*z*: 655 $[M+H]^+$; ^{1}H NMR (chloroform-*d*): δ 2.27 (12H, s, $COCH_3$), 3.81 (1H, dd, J = 7.1, 6.0 Hz, H-6), 3.92 (1H, m, H-3), 4.23 (1H, d, J = 7.1 Hz, H-6), 4.69 (2H, m, H-2 and 5), 4.80 (1H, m, H-4), 5.56 (1H, m, H-1), 6.44 (2H, d, J = 16.0 Hz, H-8′ and 8″), 7.17 (2H, m, H-5′ and 5″), 7.32–7.35 (4H, m, H-2′, 2″, 6′ and 6″), 7.67 (2H, d, J = 16.0 Hz, H-7′ and 7″).

4.3.13. 2,4-Di-*O*-caffeoyl-1,6-anhydro-β-D-glucose (C13aa, 10). Compound **10** was prepared by hydrolysis of **10a** with 5% $NaHCO_3$, using the same method as described for **3**. **10**: white powder; FAB-HR-MS (negative) *m*/*z*: 485.1111 $[M-H]^-$; calcd for $C_{24}H_{21}O_{11}$, 485.1083; ^{1}H NMR (acetone-d_6): δ 3.73 (1H, dd, J = 7.1, 5.9 Hz, H-6), 3.88 (1H, m, H-3), 4.25 (1H, d, J = 7.1 Hz, H-6), 4.66 (2H, m, H-2 and 5), 4.76 (1H, m, H-4), 4.89 (1H, d, J = 4.7 Hz, 3-OH), 5.43 (1H, br s, H-1), 6.32 and 6.34 (each 1H, d, J = 16.0 Hz, H-8′ and 8″), 6.82 and 6.83 (each 1H, d, J = 8.4 Hz, H-5′ and 5″), 7.01 and 7.02 (each 1H, dd, J = 8.4, 2.0 Hz, H-6′ and 6″), 7.18 and 7.19 (each 1H, d, J = 2.0 Hz, H-2′ and 2″), 7.60 and 7.62 (each 1H, d, J = 16.0 Hz, H-7′ and 7″); ^{13}C NMR (acetone-d_6): δ 66.5 (C-6), 70.0 (C-3), 73.9 (C-5), 74.7 (C-4), 75.4 (C-2), 101.0 (C-1), 115.0 (C-8′ and 8″), 115.2 (C-2′ and 2″), 116.4 (C-5′ and 5″), 122.9 (C-6′ and 6″), 127.4 (C-1′ and 1″), 146.3 (C-3′ and 3″), 146.6 (C-7′ and 7″), 149.0 (C-4′ and 4″), 166.7, 166.9 (C-9′ and 9″); HMBC correlation peaks: H-1/C-2, C-3, C-5, C-6, H-2 and H-5/C-1, C-3, C-4, C-9′, C-9″, 3-OH/C-2, C-4, H-4/C-3, C-9′, C-9″, H-6/C-1, C-4, H-2′ (2″)/C-4′ (4″), C-6′ (6″), C-7′ (7″), H-5′ (5″)/C-1′ (1″), C-3′ (3″), C-4′ (4″), H-6′ (6″)/C-2′ (2″), C-4′ (4″), C-5′ (5″), C-7′ (7″), H-7′ (7″)/C-2′ (2″), C-6′ (6″), C-8′ (8″), C-9′ (9″), H-8′ (8″)/C-1′ (1″), C-9′ (9″).

4.4. Isolation of oxidation products of *trans*-1,2-dicaffeoyloxycyclohexane (3)

4.4.1. Isolation of compound 12. To a solution of T12 (**3**, 55 mg, 125 μmol) in 40% H_2O/MeOH (3 L) was added DPPH radical (394 mg, 1.0 mmol, 8 equiv) in methanol (40 mL) and stirred for 20 min at room temperature. Sodium dithionite (400 mg, 2.3 mmol) was added to the mixture to reduce unstable *o*-quinones to their catechol forms. The reaction mixture was concentrated until methanol was completely removed. The residue was subjected to Diaion HP 20 column chromatography and washed with water, and then eluted with methanol. The methanol fraction was evaporated under the reduced pressure. The residue was subjected to silica gel column chromatography with chloroform to remove DPPH radical, and then with ethyl acetate. The ethyl acetate fraction was concentrated, and the crude product was further purified by preparative TLC (chloroform/ethyl acetate/formic acid = 50:50:1) to afford compound **12** (R_f = 0.63, 4.3 mg, 7.4%) as a yellow crystal; mp > 300 °C; FD-HR-MS *m*/*z*: 468.1404 $[M]^+$; calcd for $C_{25}H_{24}O_9$, 468.1420; ^{1}H NMR (acetone-d_6): δ 1.45 (2H, m, H-4_{ax} and 5_{ax}), 1.57 (2H, m, H-3_{ax} and 6_{ax}), 1.82 (2H, m, H-4_{eq} and 5_{eq}), 2.00 (2H, m, H-3_{eq} and 6_{eq}), 3.48 (3H, s, H-10″), 4.63 (1H, ddd, J = 11.6, 9.5, 4.7 Hz, H-2), 4.70 (1H, d, J = 4.1 Hz, H-5″), 5.06 (1H, dd, J = 4.1, 1.5 Hz, H-6″), 5.08 (1H, ddd, J = 11.6, 9.5, 4.7 Hz, H-1), 5.91 (1H, d, J = 15.4 Hz, H-8′), 6.35 (1H, d, J = 16.1 Hz, H-8″), 6.51 (1H, dd, J = 1.5, 0.7 Hz, H-2″), 6.74 (1H, d, J = 8.4 Hz, H-5′), 6.87 (1H, s, 4″-OH), 6.94 (1H, d, J = 8.4 Hz, H-6′), 7.20 (1H, dd, J = 16.1, 0.7 Hz, H-7″), 7.87 (1H, d, J = 15.4 Hz, H-7′), 8.54 (1H, s, 4′-OH); ^{13}C NMR (acetone-d_6): δ 24.5 (C-4), 24.6 (C-5), 30.7 (C-6), 31.2 (C-3), 51.5 (C-5″), 57.1 (C-10″), 74.0 (C-6″), 75.3 (C-1), 78.9 (C-2), 106.2

(C-4″), 118.2 (C-5′), 121.4 (C-6′), 122.5 (C-8′), 125.2 (C-1′), 126.5 (C-2′), 127.3 (C-8″), 132.1 (C-2″), 140.7 (C-7″), 143.7 (C-4′), 144.2 (C-7′), 146.4 (C-3′), 154.0 (C-1″), 165.7 (C-9″), 166.8 (C-9′), 190.7 (C-3″); HMBC correlation peaks: H-1/C-2, C-9′, H-2/C-1, C-9″, H-5′/C-1′, C-3′, C-4′, C-6′, H-6′/C-2′, C-3′, C-4′, C-5′, H-7′/C-1′, C-6′, C-9′, H-8′/C-1′, C-9′, H-2″/C-1″, C-4″, C-6″, C-7″, C-8″, 4″-OH/C-3″, C-4″, C-5″, H-5″/C-2′, C-3′, C-1″, C-3″, C-4″, C-6″, H-6″/C-1″, C-2″, C-4″, C-5″, C-7″, C-10″, H-7″/C-1″, C-2″, C-6″, C-8″, C-9″, H-8″/C-1″, C-2″, C-7″, H-10″/C-6″.

4.4.2. Isolation of compound 13. Compound **13** was isolated from the reaction mixture of T12 (**3**) and DPPH radical in 40% H_2O/EtOH by the same method as described for **12**. **13**: 2.4 mg, 4.0%; pale yellow crystal; $R_f = 0.68$ (chloroform/ethyl acetate/formic acid = 50:50:1); mp > 300 °C; FD-HR-MS *m/z*: 482.1590 $[M]^+$; calcd for $C_{26}H_{26}O_9$, 482.1577; ^{1}H NMR (acetone-d_6): δ 1.11 (3H, t, $J = 7.0$ Hz, 10″-CH_3), 1.46 (2H, m, H-4_{ax} and 5_{ax}), 1.57 (2H, m, H-3_{ax} and 6_{ax}), 1.82 (2H, m, H-4_{eq} and 5_{eq}), 2.00 (2H, m, H-3_{eq} and 6_{eq}), 3.62 (1H, dq, $J = 9.0$, 7.0 (q) Hz, H-10″), 3.87 (1H, dq, $J = 9.0$, 7.0 (q) Hz, H-10″), 4.63 (1H, ddd, $J = 11.6$, 9.5, 4.7 Hz, H-2), 4.69 (1H, d, $J = 4.1$ Hz, H-5″), 5.08 (1H, ddd, $J = 11.6$, 9.5, 4.7 Hz, H-1), 5.13 (1H, dd, $J = 4.1$, 1.2 Hz, H-6″), 5.90 (1H, d, $J = 15.4$ Hz, H-8′), 6.32 (1H, d, $J = 16.1$ Hz, H-8″), 6.50 (1H, d, $J = 1.2$ Hz, H-2″), 6.74 (1H, d, $J = 8.4$ Hz, H-5′), 6.86 (1H, s, 4″-OH), 6.93 (1H, d, $J = 8.4$ Hz, H-6′), 7.18 (1H, d, $J = 16.1$ Hz, H-7″), 7.87 (1H, d, $J = 15.4$ Hz, H-7′), 8.53 (1H, s, 4′-OH); ^{13}C NMR (acetone-d_6): δ 15.5 (C-11″), 24.5 (C-4), 24.6 (C-5), 30.7 (C-6), 31.2 (C-3), 51.5 (C-5″), 65.2 (C-10″), 72.5 (C-6″), 75.3 (C-1), 78.9 (C-2), 106.3 (C-4″), 118.1 (C-5′), 121.3 (C-6′), 122.4 (C-8′), 125.1 (C-1′), 126.6 (C-2′), 127.1 (C-8″), 132.1 (C-2″), 140.7 (C-7″), 143.7 (C-4′), 144.2 (C-7′), 146.4 (C-3′), 154.1 (C-1″), 165.6 (C-9″), 166.8 (C-9′), 190.8 (C-3″).

4.5. NMR analyses

4.5.1. Reaction of *trans*-1,2-dicaffeoyloxycyclohexane (3) and DPPH radical. To DPPH radical (4.9 mg, 12.5 μmol, 2.5 equiv) was added a solution of *trans*-1,2-dicaffeoyloxycyclohexane (**3**, 2.2 mg, 5 μmol) in acetonitrile-d_3 (0.4 mL). The mixture was immediately transferred to an NMR tube and analyzed by ^{1}H NMR.

4.5.2. *trans*-1,2-Dicaffeoyloxycyclohexane bis-*o*-quinone (11). ^{1}H NMR (acetonitrile-d_3): δ 1.41–1.45 (2H, m), 1.52–1.54 (2H, m), 1.75–1.77 (2H, m), 2.08–2.10 (2H, m), 4.97 (2H, m, H-1 and 2), 6.42 (2H, d, $J = 10.3$ Hz, H-5′), 6.49 (2H, s, H-2′), 6.56 (2H, d, $J = 16.0$ Hz, H-8′), 7.39 (2H, d, $J = 10.3$ Hz, H-6′), 7.40 (2H, d, $J = 16.0$ Hz, H-7′).

4.5.3. Reaction of compound 12 and DPPH radical. To a solution of DPPH radical (7.0 mg, 17.8 μmol, 3.0 equiv) in acetone-d_6 (0.1 mL) was added compound **12** (2.8 mg, 5.9 μmol) in methanol-d_4/acetone-d_6 (3:1, 0.4 mL). The mixture was immediately transferred to an NMR tube and analyzed by NMR.

Compound 14. ^{1}H NMR (methanol-d_4/acetone-d_6 = 3:2): δ 1.46, 1.59, 1.85, 2.05 (each 2H, m, H-3, 4, 5 and 6), 3.42 (3H, s, H-10″), 4.28 (1H, d, $J = 3.2$ Hz, H-5″), 4.68 (1H, m, H-2), 5.04 (1H, d, $J = 3.2$ Hz, H-6″), 5.15 (1H, m, H-1), 5.94 (1H, d, $J = 10.3$ Hz, H-5′), 6.22 (1H, d, $J = 15.8$ Hz, H-8′), 6.35 (1H, d, $J = 16.0$ Hz, H-8″), 6.49 (1H, s, H-2″), 7.08 (1H, d, $J = 10.3$ Hz, H-6′), 7.27 (1H, d, $J = 16.0$ Hz, H-7″), 7.71 (1H, d, $J = 15.8$ Hz, H-7′); ^{13}C NMR (methanol-d_4/acetone-d_6 = 3:2): δ 24.8, 24.9, 30.9, 31.4 (C-3, 4, 5 and 6), 51.9 (C-5″), 57.3 (C-10″), 73.8 (C-6″), 76.3 (C-1), 79.5 (C-2), 100.3 (C-3′), 103.6 (C-4″), 125.5 (C-1′), 125.7 (C-8′), 125.9 (C-5′), 126.6 (C-8″), 132.4 (C-2″), 141.6 (C-6′), 141.7 (C-7″), 141.8 (C-7′), 148.9 (C-2′), 153.2 (C-1″), 166.6 (C-9″), 167.9 (C-9′), 191.7 (C-3″), 193.1 (C-4′); HMBC correlation peaks: H-5′/C-3′, H-6′/C-2′, C-4′, H-7′/C-9′, H-2″/C-4″, C-6″, H-5″/C-1″, H-7″/C-6″, C-9″, H-8″/C-1″, H-10″/C-6″.

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