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Antioxidation mechanism of rosmarinic acid, identification of an unstable quinone derivative by the addition of odourless thiol

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ABSTRACT

The antioxidation mechanism of rosmarinic acid was investigated. An AIBN-induced oxidation reaction of linoleate in the presence of rosmarinic acid produced one main product. The DPPH reaction of rosmarinic acid also gave two products, one of which corresponded to the product of the lipid system. Direct isolation of the products failed because of their instability. To the DPPH reaction, the addition of 1-dodecanethiol afforded three isolable adducts. Their structures revealed that the reaction produced quinone derivatives on either the caffeoyl or 2-oxyphenylpropanoyl moieties. However, the lipid oxidation afforded one quinone, which could convert to a thiol adduct. The HPLC of the reaction clarified that the antioxidation product of rosmarinic acid was the unstable quinone on the 2-oxyphenylpropanoyl moiety. This quinone formation was the first stage of the antioxidation of rosmarinic acid in the lipid system. The odourless 1-dodecanethiol is a useful reagent to identify the unstable quinone from polyphenol oxidation.

1. Introduction

Rosmarinic acid (**1**) is a potent antioxidative polyphenol widely distributed in Lamiaceae herbs (Petersen & Simmonds, 2003). The Lamiaceae herbs, such as rosemary, sage, and melissa, contain a large amount of rosmarinic acid and are used as a food additive and herbal tea for multifunctional purposes in worldwide food cultures. Many beneficial functionalities of rosmarinic acid, which include anti-inflammation (Huang et al., 2009), anti-mutagenicity (Furtado, de Almeida, Furtado, Cunha, & Tavares, 2008), the photoprotection of keratinosytes (Psotova, Svbodova, Kolarova, & Walterova, 2006), reduction of atopic dermatitis (Lee, Jung, Kog, Kim, & Park, 2008), prevention of Alzheimer's disease (Hamaguchi, Ono, Murase, & Yamada, 2009), apoptosis induction of colorectal cancer cells (Xavier, Lima, Fernandes-Ferreira, & Pereira-Wilson, 2009), etc., have been recently reported in addition to its potent antioxidant activity (Nakatani, 1996). Rosmarinic acid is now recognised to be one of the promising food-functional polyphenols. With respect to its chemical structure, rosmarinic acid has two catechol moieties. Catechol is an important sub-structure for the potent antioxidant activity of phenolic antioxidants. In rosmarinic acid, one catechol structure exists in the caffeoyl moiety and the other is in the 2-oxyphenylpropanoyl moiety. It is well known that caffeic acid has been recognised as a very strong antioxidant and its antioxidant mechanism has already been intensively investigated (Chen & Ho, 1997; Masuda et al., 2008; Moon & Terao, 1998). Although various antioxidative caffeic acid derivatives, including chlorogenic acid and caffetaric acid, are contained in various edible plants and plant-derived foods (Shahidi & Naczk, 2004), their antioxidation mechanism has not yet been fully understood, because of their structural complexity compared to that of caffeic acid.

For the general antioxidation mechanism of phenolic antioxidants, the process is thought to be divided into two stages as shown by the following schemes (Frankel, 2005):

(1) radical trapping stage

$$\text{R} - \text{OO radical} + \text{AH} \rightarrow \text{R} - \text{OOH} + \text{A radical}$$

(2) radical termination stage

$$\text{A radical} \rightarrow \text{non-radical materials}$$

where R is the substance for oxidation, the R-OO radical is the peroxyl radical of R, AH is the antioxidant, and the A radical is the antioxidant radical. Although the first stage is a reversible process, the second stage is irreversible and must produce a non-radical product. The structure of the product would afford important information about the antioxidation mechanism of the phenolic antioxidants (Masuda et al., 2010). The antioxidation process of rosmarinic acid against biomolecule oxidation, such as unsaturated lipid oxidation, is very attractive for clarifying not only its antioxidation mechanism of caffeic antioxidants, but also its relation to its various

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bioactivities. In this study, the antioxidation product from rosmarinic acid in a lipid oxidation system was investigated. To clarify the antioxidation product from rosmarinic acid, a useful and generally applicable method to identify the produced unstable quinone derivative was newly developed using an odourless thiol reagent.

2. Materials and methods

2.1. Chemicals and instruments

Rosmarinic acid (**1**) was purchased from Aldrich Japan (Tokyo, Japan). 2,2′-Azobis(isobutyronitrile) (AIBN) and 1-dodecanethiol were obtained from Tokyo Kasei (Tokyo, Japan). Ethyl linoleate was purchased from Kanto Chemicals (Tokyo, Japan) and used after purification by silica gel (silica 60, Merck, Darmstat, Germany) chromatography developed with 2.5% ethyl acetate in hexane. All solvents and other reagents were obtained from Nacalai Tesque (Kyoto, Japan). The NMR spectra were measured using an ECS-400 spectrometer (JEOL, Tokyo, Japan) with the manufacturer-supplied pulse sequences [^{1}H, ^{13}C, correlated spectroscopy (HH-COSY) and nuclear Overhauser effect spectroscopy (NOESY)]. The mass spectra were measured with XEVO Qtof spectrometers (Waters Japan, Tokyo, Japan) in the negative ESI mode. A LC-20ATvp low pressure gradient system (Shimadzu, Kyoto, Japan) equipped with a photodiode array detector (SPD-M20AVP, Shimadzu) and a DGU-12A degasser was employed for the analytical HPLC. A PU-980 pump equipped with a UV-975 detector (JASCO, Tokyo, Japan) was used for analysis of ethyl linoleate hydroperoxides. A LC-6AD recycling system (Shimadzu) equipped with a UV-970 detector (JASCO) was used for preparative HPLC.

2.2. HPLC Detection of the antioxidation products from rosmarinic acid against ethyl linoleate oxidation

Rosmarinic acid (0.4 mg) in CH_3CN solution (4 ml) and AIBN (158 mg) were added to 61 mg of ethyl linoleate in a straight vial (75 mm height, 40 mm diameter). The control vial was also prepared in a similar manner, but without addition of rosmarinic acid. Both solutions were well stirred and then incubated at 37 °C for 6 h with shaking (100 times min^{-1}) by a water bath shaker. Twenty microlitres of the reaction solution were injected into the analytical HPLC system under the following conditions, column, Cosmosil 5C18-AR-II (4.6 × 250 mm, Nacalai Tesque); solvent system, 1% acetic acid in H_2O (solvent A) and CH_3CN (solvent B); elution, linear gradient from 5% of solvent B to 100% of solvent B for 40 min, and then isocratic mode of 100% solvent B for 10 min; flow rate, 1.0 ml min^{-1}; detection, 280 nm. At intervals for both solutions, a 20 μl-aliquot was removed from the solution and diluted with 380 μl of methanol. Ten microlitres of the diluted solution was injected into the HPLC to analyse the ethyl linoleate hydroperoxides using a ODS-A column (4.6 × 150 mm, YMC, Tokyo, Japan) eluted with CH_3CN/H_2O (9:1, v/v) at 1.0 ml min^{-1}; with detection at 234 nm.

2.3. Preparation procedure for thiol adducts (*4*–*6*)

To a solution of rosmarinic acid (150 mg) in CH_3CN (40 ml) was added DPPH (2,2-diphenyl-1-picrylhydrazyl, 574 mg) at 23 °C with stirring. After the reaction mixture was allowed to stand for 10 min, 1-dodecanethiol (253 mg) was added to the mixture. The mixture was allowed to stand for 1 h at 23 °C and then evaporated to dryness *in vacuo* to give a residue (1 g). A part of the residue (0.4 g) was separated by preparative HPLC under the following conditions, column, Cosmosil 5C18-AR-II (20 × 250 mm, Nacalai Tesque); solvent, 1% acetic acid in H_2O/CH_3OH = 9:1, flow rate, 9.5 ml min^{-1}; detection, 280 nm, to give fraction 1 (14 mg) at the retention time (9 min) and fraction 2 (82 mg) at the retention time (11 min). Fraction 1 was purified again by preparative HPLC under the following new conditions, column, Cosmosil 5C18-AR-II (20 × 250 mm); solvent, 1% acetic acid in H_2O/CH_3OH = 85:15, flow rate, 9.5 ml min^{-1}; detection, 280 nm, to give compound **4** (8 mg). Fraction 2 (82 mg) was purified by a recycle preparative HPLC under the following new conditions, column, Cosmosil 5C18-AR-II (20 × 250 mm); solvent, 1% acetic acid in H_2O/CH_3OH = 70:30, flow rate, 9.5 ml min^{-1}; detection, 280 nm, recycle **5** (5 mg) and **6** (27 mg).

4. HR–ESI-MS (*m/z*) $[M-H]^-$ calcd for $C_{30}H_{39}O_8S$, 559.2366; found, 559.2387; ^{1}HNMR (400 MHz, CD_3OD) δ 5.16 (1H, brd, *J* = 7.3 Hz, H2), 3.00(1H, brd, *J* = 13.7 Hz, H3a), 2.99 (1H, dd, *J* = 13.7 and 7.3 Hz, H3b), 6.75 (1H, brs, H2′), 6.68 (1H, d, *J* = 8.2 Hz, H5′), 6.65 (1H, brd, *J* = 8.2 Hz, H6'), 6.33 (1H, d, *J* = 16.0 Hz, H2″), 8.43 (1H, d, *J* = 16.0 Hz, H3″), 6.83 (1H, d, *J* = 8.8 Hz, H5‴), 7.23 (1H, d, *J* = 8.8 Hz, H6‴), 2.69 (2H, t, *J* = 6.8 Hz, 2‴-SCH_2), 1.1–1.4 (20H, m), 0.88 (3H, t, *J* = 6.4 Hz, CH_3); Important correlations observed in the NOESY, H2″-H6‴, H3″-2‴-SCH_2; ^{13}CNMR (100 MHz, CD_3OD) δ 38.2 (C3), 130.7 (C1′), 117.5 (C2′), 146.0 (C3′), 145.2 (C4′), 116.3 (C5′), 121.9 (C6′), 168.5 (C1″), 116.7 (C2″), 146.1 (C3″), 120.0 (C1‴), 129.7 (C2‴), 148.1 (C3‴), 148.6 (C4‴), 117.0 (C5‴), 123.0 (C6‴).

5. HR-ESI-MS (*m/z*) $[M-H]^-$ calcd for $C_{30}H_{39}O_8S$, 559.2366; found, 559.2355; ^{1}HNMR [400 MHz, CD_3OD-acetone-d_6(2:1, v/v) δ 5.16 (1H, dd, *J* = 9.6 and 3.4 Hz, H2), 3.41 (1H, dd, *J* = 14.0 and 3.4 Hz, H3a), 3.19 (1H, dd, *J* = 14.0 and 9.6 Hz, H3b), 6.64 (1H, s, H2′), 6.66 (1H, s, H5′), 6.10 (1H, d, *J* = 16.0 Hz, H2″), 7.39 (1H, d, *J* = 16.0 Hz, H3″), 6.92 (1H, d, *J* = 1.6 Hz, H2‴), 6.67 (1H, d, *J* = 8.0 Hz, H5‴), 6.83 (1H, dd, *J* = 8.0 and 1.6 Hz, H2‴), 2.68 (2H, t, *J* = 7.6 Hz, 6′-SCH_2), 0.78 (3H, t, *J* = 7.2 Hz, 6′-$SC_{11}H_{22}$-CH_3); ^{13}CNMR (100 MHz, CD_3OD) δ 37.1 (C3), 122.4 (C1′), 121.5 (C2′), 147.3 (C3′), 145.0 (C4′), 115.1 (C5′), 133.1 (C6′), 168.6 (C1″), 115.1 (C2″), 147.9 (C3″), 127.7 (C1‴), 114.8 (C2‴), 146.8 (C3‴), 149.6 (C4‴), 116.4 (C5‴), 123.0 (C6‴), Signals for C1 and C2 were not identified for their low intensity.

6. HR-ESI-MS (*m/z*) $[M-H]^-$ calcd for $C_{30}H_{39}O_8S$, 559.2366; found, 559.2366; ^{1}HNMR (400 MHz, CD_3OD) δ 5.18 (1H, brd, *J* = 9.2 Hz, H2), 3.09 (1H, d, *J* = 13.6 Hz, H3a), 2.97 (1H, dd, *J* = 13.6 and 9.2 Hz, H3b), 6.69 (1H, brs, H2′), 6.78 (1H, brs, H6′), 6.25 (1H, d, *J* = 16.0 Hz, H2″), 7.54 (1H, d, *J* = 16.0 Hz, H3″), 7.03 (1H, brs, H2‴), 6.76 (1H, d, *J* = 8.4 Hz, H5‴), 6.93 (1H, brd, *J* = 8.4 Hz, H6‴), 2.73 (2H,t, *J* = 7.6 Hz, 5′-SCH_2), 1.1–1.5 (20H, m), 0.88 (3H, t, *J* = 7.6 Hz, 5′-$SC_{11}H_{22}$-CH_3); Important correlations observed in the NOESY, H6′- 5′-SCH_2; ^{13}CNMR (100 MHz, CD_3OD) δ 174.5 (C1), 75.3 (C2), 31.8 (C3), 129.7 (C1′), 116.7 (C2′), 146.0 (C3′), 144.8 (C4′), 125.4 (C5′), 122.4 (C6′), 168.5 (C1″), 115.2 (C2″), 147.5 (C3″), 127.7 (C1‴), 114.7 (C2‴), 146.8 (C3‴), 149.7 (C4‴), 116.4 (C5‴), 123.1 (C6‴), 34.8 (5′-SCH_2), 14.5 (CH_3).

Assignment of ^{13}C signals of **4**–**6** were based on the reported data for rosmarinic acid (Kikuzaiki & Nakatani, 1989).

2.4. HPLC analysis of the thiol adduct from the antioxidation product of rosmarinic acid in a lipid oxidation system

One ml of the reaction solution was taken from the antioxidation vial of rosmarinic acid against ethyl linoleate oxidation to a new vial 1 h after the reaction started. To the new vial was added 1-dodecanethiol (0.6 μl) in CH_3CN (10 μl) and subsequently triethylamine (3.5 μl) at 23 °C. After standing for 1 h at 23 °C, 20 μl of phosphoric acid was added to acidify the solution and then 10 μl of the solution was analysed by HPLC under the following conditions: column, Cosmosil 5C18-AR-II (4.6 × 250 mm); solvent system, 1% acetic acid in H_2O (solvent A) and CH_3CN (solvent B); elution, linear gradient from 5% of solvent B to 100% of solvent B for 40 min, and then isocratic

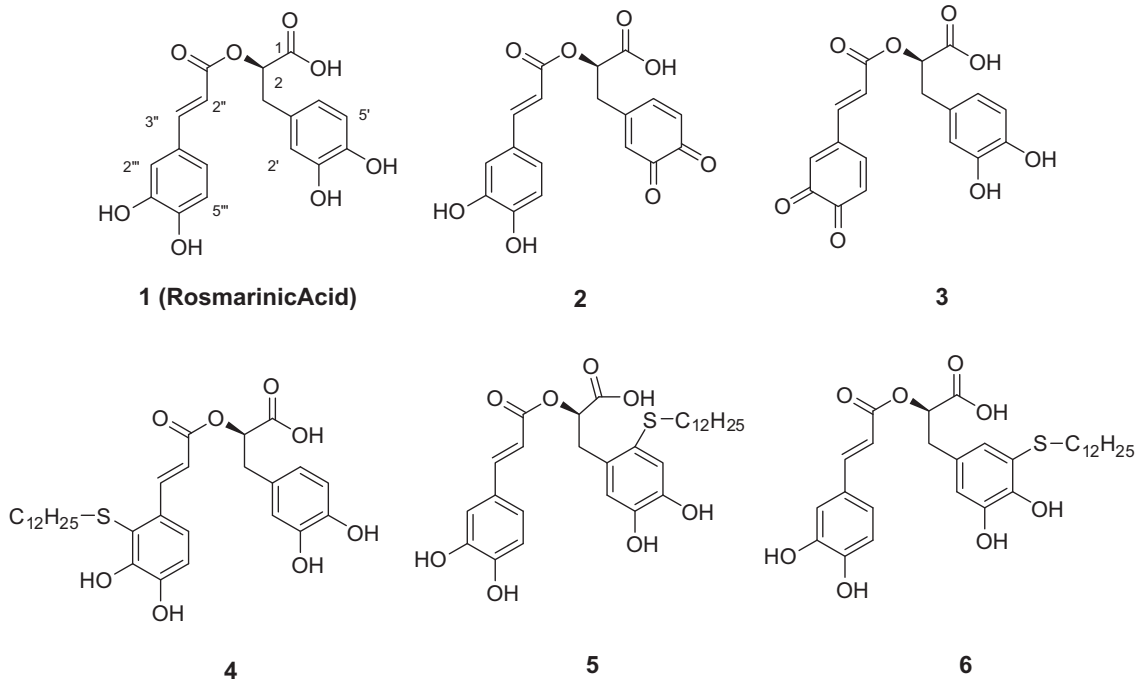

Fig. 1. Chemical structures of rosmarinic acid (**1**), its quinone derivatives (**2** and **3**), and thiol adducts (**4**–**6**). [Numbering system was tentatively used for all compounds according to a previous publication (Kikuzaiki & Nakatani, 1989)].

mode of 100% solvent B for 10 min; flow rate, 1.0 ml min^{-1}; detection, 280 nm. An additional 1 ml solution was taken from the antioxidation reaction and treated by the same procedure without addition of 1-decanethiol to obtain the reference HPLC data.

3. Results and discussion

3.1. HPLC analysis of the radical reaction products of rosmarinic acid

The antioxidant activity of rosmarinic acid (1, Fig. 1) and its antioxidation products were investigated by HPLC. A mixture of rosmarinic acid (0.25 mM) and ethyl linoleate (25 mM) in acetonitrile (4 ml) was oxidised by a radical initiator, AIBN (158 mg), at 37 °C. The oxidation of ethyl linioleate was monitored by the concentration of its hydroperoxides using HPLC with detection at 234 nm (Fig. 2).

The antioxidation reaction product produced from rosmarinic acid was analysed at one-hour intervals by another HPLC under gradient conditions. Fig. 3 shows that peak 2 was observed at 19.2 min as the major antioxidation product of rosmarinic acid. The time-course analyses of peaks 1 (rosmarinic acid) and 2 were carried out, and the data are summarized in Fig. 4. As shown in Fig. 4, the concentration of rosmarinic acid decreased during the antioxidation period for 4 h. After 4 h, rosmarinic acid does not show any antioxidant activity, as shown in Fig. 2. The reaction product, which was observed as peak 2, first increased, then slowly decreased up to 4 h (Fig. 4). After 4 h, no typical product could be observed in the HPLC data (data not shown). These data indicated that the peak compound **2** should be the initial product produced by the antioxidation reaction of rosmarinic acid (1) in the lipid oxidation system. Our attempt to isolate compound **2** failed, because **2** was too unstable to be purified by any chromatographic techniques. Direct LC–ESI-MS analysis of peak 2 was, however, successful and the obtained data [m/z 357.0602 $(M-H)^-$] indicated that the peak 2 compound (**2**) lost two hydrogens from rosmarinic acid, revealing that **2** was a quinone derivative of rosmarinic acid.

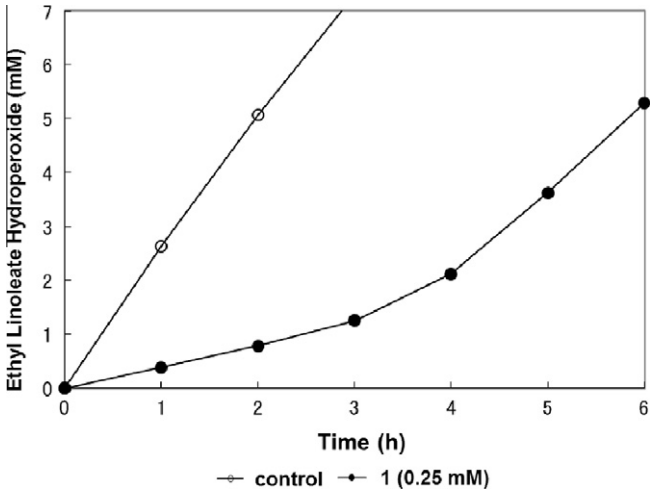

Fig. 2. Antioxidant activity of rosmarinic acid against AIBN-induced ethyl linoleate oxidation. [Concentration of reactants: ethyl linoleate (50 mM), AIBN (0.24 M), rosmarinic acid (0.25 mM or 0 M(control))].

Rosmarinic acid has two catechol sub-structures, one of which is in the caffeoyl moiety and the other is in the 2-oxyphenylpropanoyl moiety. A question arose as to which catechol the quinone structure was derived from. Antioxidative polyphenols possess many phenol groups in their structures. For understanding the antioxidation reaction of the polyphenols, an efficient method to identify the most reactive position among the many phenol groups that exist in the polyphenol compounds should be acquired. For identification of the radical oxidation position of the polyphenols, Sawai and coworkers (Sawai & Moon, 2000) reported direct NMR measurements of the polyphenol in the presence of DPPH in a deuterium solvent. They revealed quinone formation for the polyphenols by observation of the quinone carbonyl signals in the ^{13}CNMR. This method is very simple, however, no position information of

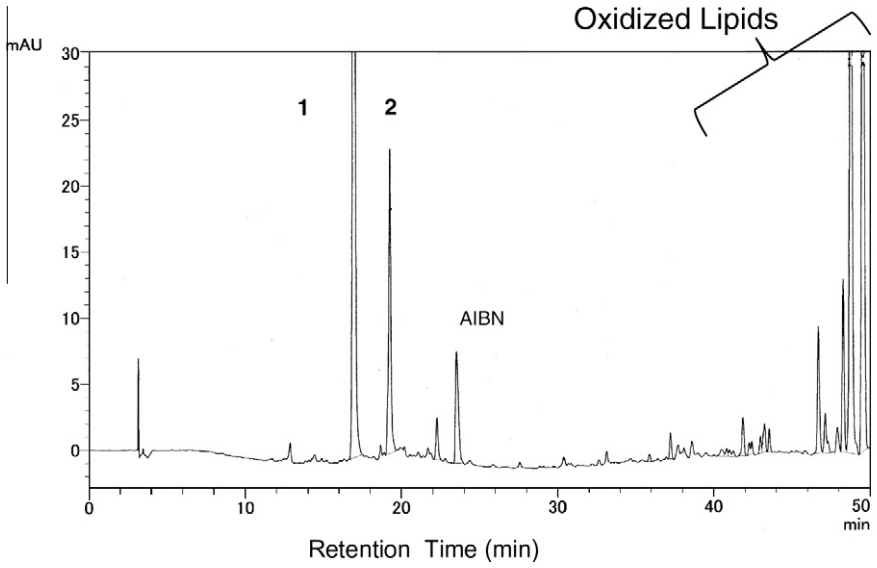

Fig. 3. HPLC analytical data of AIBN-induced oxidation products in ethyl linoleate with rosmarinic acid (**1**) (reaction time: 1 h); HPLC conditions: column, Cosmosil 5C18-AR-II (4.6 × 250 mm); solvent system, 1% acetic acid in H_2O (solvent A) and CH_3CN (solvent B); elution, linear gradient from 5% of solvent B to 100% of solvent B for 40 min, and then isocratic mode of 100% solvent B for 10 min; flow rate, 1.0 ml min^{-1}; detection, 280 nm.

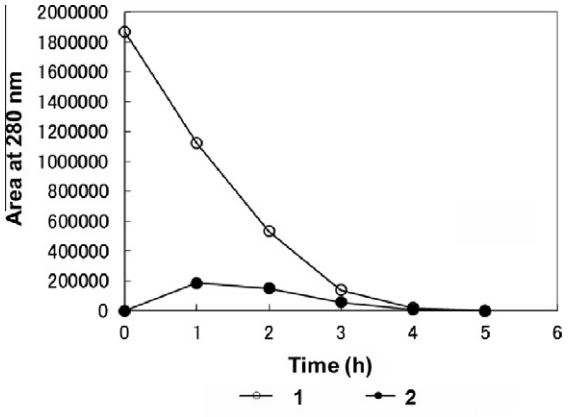

Fig. 4. Time-course analytical data of rosamrinic acid (**1**) and its antioxidation product (**2**) by gradient HPLC.

the quinone in multi-catechol compounds was obtained. Kondo and coworkers (Kondo, Kurihara, Miyata, Suzuki, & Toyota, 1999) reported an antioxidation mechanism of tea catechins based on an MS fragment consideration and MO calculations. Yoshida et al. (Yoshida et al., 1989) employed ESR for identification of reacted phenol in tannins, which are typical multi-catechol compounds in nature. They observed a radical signal for one of the galloyl groups in a tannin compound. When the oxidation product of the antioxidative polyphenol is stable, the chemical structure of the product would afford important information about the reacted position in its antioxidation (Masuda, Inaba, & Takeda, 2001; Krishnamachari, Levine, & Pare, 2002; Wei & Ho, 2006). Chemically, quinone reacts with nucleophilic substances such as amines and thiols (Bitter, 2006), and the thiol is the more reactive reagent (Grant et al., 1986), therefore, several thiol reagents have been employed for the thiolysis reaction in the structure determination of some oligomeric biomolecules (Haslam, 1977). In foods, various thiol adducts to phenolics have also been found (Cejudo-Bastane et al., 2010). This information indicated that some thiols can be used to identify the position of the quinone structure that was produced by the antioxidation reaction of the catechol moiety. It should be noted that most thiol compounds have a very unpleasant smell and this smell has prevented researchers from employing thiol compounds as a reagent. Recently, Node and coworkers (Node, 2007) developed several odourless thiol compounds, and they succeeded in applying them for various syntheses instead of ordinary thiols. Therefore, we planned to employ one of their odourless thiols, 1-dodecanethiol, to determine the quinone position of the antioxidation product of rosmarinic acid.

3.2. Isolation and structure determination of peak compounds **4**–**6**

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable radical dehydrogenation reagent (Fieser & Fieser, 1967). Hence, we employed DPPH to produce all the quinone derivatives from rosmarinic acid. The treatment of rosmarinic acid with *ca.* 1.5 equivalents of DPPH in an acetonitrile solution gave two quinone derivatives (**2** and **3**), which were detectable by HPLC at the retention times of 19.1 and 16.5 min, respectively [the retention time of rosmarinic acid (**1**):16.7 min] (Fig. 5A). The former corresponded to peak 2 that was observed in the lipid antioxidation reaction of rosmarinic acid. To the DPPH reaction mixture 1-dodecanethiol (three equivalents) was added as a nucleophilic and odourless thiol reagent. One hour later, the HPLC analysis of the reaction mixture revealed that the two quinone peaks (2 and 3) disappeared and three peaks (4–6, retention times: 36.0, 36.9, and 37.9, respectively) were produced (Fig. 5B). These new peak compounds were stable during purification, therefore, isolation of the three compounds (**4**–**6**) was successful as pure forms by a preparative HPLC technique.

Compounds **4**–**6** were all isolated as colourless oils. Their molecular formulas were estimated to the same $C_{30}H_{40}O_8S$ based on their HR–ESI-MS results. The MS results indicated that **4**–**6** were adducts of one molecule of 1-dodecanethiol to the rosmarinic acid quinones. The added position of the thiol in each compound was analysed by NMR. In the ^{1}H-NMR of **4**, a proton signal due to the aromatic ring of the caffeoyl moiety of **4** disappeared compared to that of rosmarinic acid. This lost proton position should be at the 2-position of the aromatic ring because the two remaining proton signals (5- and 6-positions) showed an *ortho*-coupling relation (J = 8.2 Hz). These results indicated that the thiol was attached at the 2‴-position, which was supported by two NOE correlations

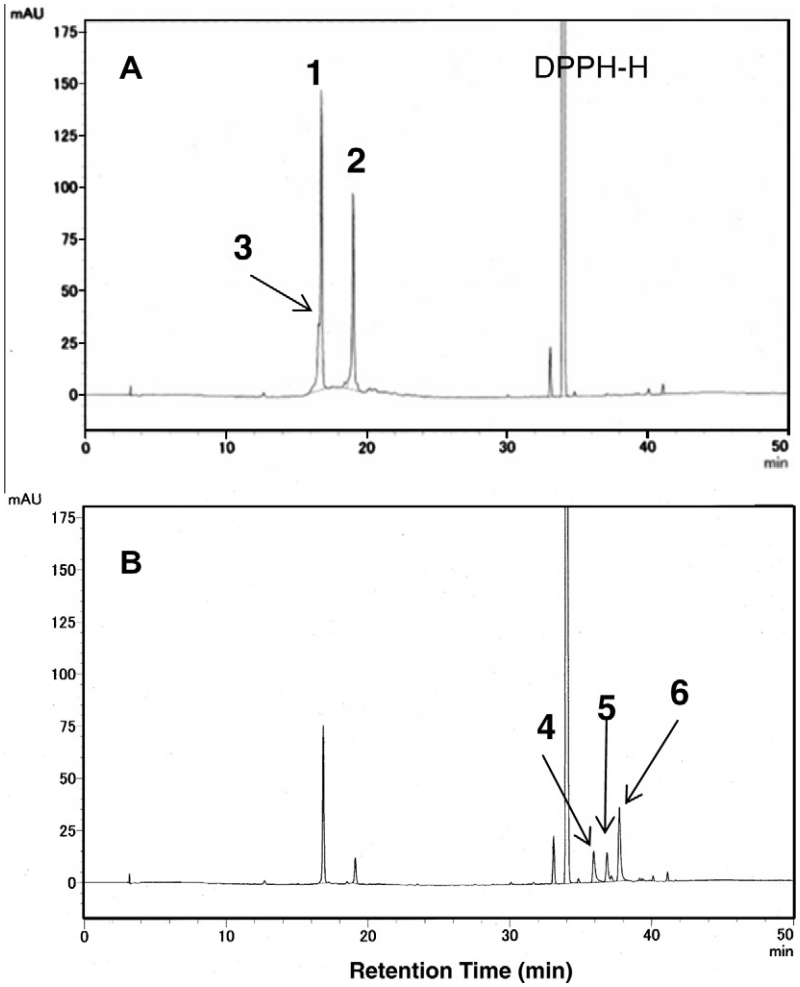

Fig. 5. HPLC analytical data of DPPH reaction of rosmarinic acid (**1**) (panel A) and of 1-dodecanethiol adducts from the DPPH reaction products (panel B); HPLC conditions are the same as in Fig. 3.

between H2″ and H6‴, and between H3″ and α-methylene protons of the thiol in the NOESY of **4**. In the ^{1}HNMR of **5** and **6**, a proton signal of the aromatic ring of the 2-oxyphenylpropanoyl moiety of **5** and **6** disappeared from the proton signals of the corresponding aromatic ring of rosmarinic acid, indicating that both **5** and **6** were added products with 1-dodecanethiol on the aromatic ring of the 2-oxyphenylpropanoyl moiety. The substituted positions of the thiol were determined to be the 6′-position for compound **5** and the 5′-position for compound **6** based on the 1D and 2D NMR analytical results. In the ^{1}HNMR of **5**, two sharp singlet protons were observed in the aromatic ring of the 2-oxyphenylpropanoyl moiety, indicating that the two protons existed *para* to each other. Therefore, the thiol should be present at the 6-position of the aromatic ring, which was strongly supported by the up-field shifted signals (δ3.41 and 3.19) of the methylene protons at the 1′-position. In the ^{1}HNMR of **6**, two singlet protons were also observed in the aromatic ring of the 2-oxylphenylpropanoyl moiety, however, these singlet signals were broad, thus indicating that these protons were *meta* to each other on the aromatic ring. These results clarified that the thiol existed at the 5-position of the aromatic ring, which was supported by an NOE observation between H6′ (δ 6.78) and the α-methylene protons of the thiol. Based on these results, compounds **4**–**6** were determined to be 1-dodecanethiol adducts at the 2‴-, 5′-, and 6′-positions, respectively, and the structures of these isomeric adducts also indicated that compound **4** was derived from the quinone **3**, and compounds **5** and **6** were from the quinone **2** (Fig. 1).

3.3. HPLC analysis of the thiol adduct from the antioxidation product of rosmarinic acid in lipid oxidation system

We succeeded in identifying the structures of the 1-dodecanethiol adducts that were produced from the corresponding two quinones of rosmarinic acid (**2** and **3**). Next, the thiol addition reaction using the odourless thiol was applied to the AIBN-induced lipid oxidation system. The addition of 1-dodecanethiol to the quinone **2** did not proceed by the direct addition of the thiol to the antioxidation reaction mixture of rosmarinic acid in the lipid oxidation system. As a disadvantage of 1-dodecanethiol, its reactivity is not very high compared to the ordinary small molecular thiol compounds. The antioxidation reaction continuously produces radical species from a radical initiator, AIBN, and the thiol compound can be easily oxidised under the stated conditions, which probably prevents the thiol addition to the quinone of rosmarinic acid. After intensive investigation of the thiol addition conditions, we found that some organic bases promoted the addition reaction of the thiol to the quinone. Fig. 6 shows the HPLC analytical results of the 1-dodecanethiol addition reaction with an excess amount of triethylamine. The HPLC profile (Fig. 6) clearly shows that the peak of the quinone **2** disappeared, and the peak of compound **6** was detected. From the production of **6**, the unstable antioxidation product **2** should be structure **2** in Fig. 1.

As a conclusion, this investigation revealed that the antioxidation reaction of rosmarinic acid should occur at the catechol position of its 2-oxyphenylpropanyl moiety during the first stage of

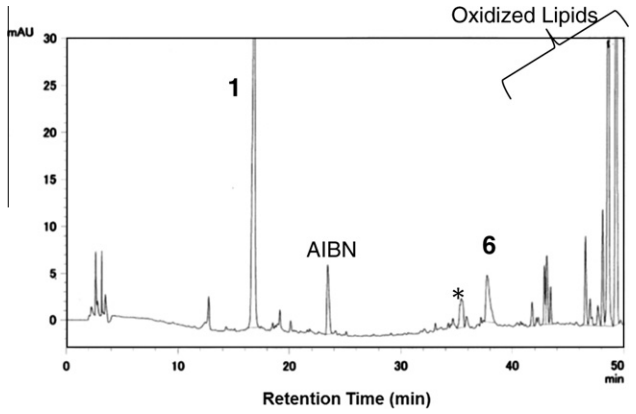

Fig. 6. HPLC analytical data of 1-dodecanethiol adduct **6** from the AIBN-induced antioxidation reaction of rosmarinic acid; *indicates impurity peak from employed triethylamine; HPLC conditions are the same as in Fig. 3.

the antioxidation against lipid oxidation. Polyphenols possessing many catechol sub-structures, such as anthocyanins, procyanidins, and tannins, are well-known as potent complex antioxidants, however, the first reaction position of most of them during their antioxidation has not yet been clarified. This odourless thiol addition technique should be useful without any unpleasant smell for identification of the unstable quinone structure as a first reacted position of multi-catechol-bearing polyphenols like rosmarinic acid in this investigation.

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