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A novel ring-expanded product with enhanced tyrosinase inhibitory activity from classical Fe-catalyzed oxidation of rosmarinic acid, a potent antioxidative Lamiaceae polyphenol

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ABSTRACT

The iron-ion catalyzed oxidation of the ethanol solution of rosmarinic acid, a potent antioxidant polyphenol of Lamiaceae (Labiatae) plants, afforded a highly tyrosinase-inhibitory active product. The structure of the active product in the oxidation product mixture was determined using extensive NMR spectroscopy to have a novel oxygen-containing seven-membered ring system. The formation mechanism of the unique ring structure from the catechol part of the rosmarinic acid was proposed.

Natural polyphenol has currently attracted much attention as an active constituent in functional foods and food supplements, which promises to improve human health. The polyphenol shows a very potent antioxidant activity, and this activity is closely linked to various beneficial actions, including anti-aging, prevention of cancer, cardiovascular disease, etc.[1] The antioxidant efficiency of the polyphenolic antioxidant depends on its oxidizable property. The potent antioxidant is oxidized much faster than other biomolecules, thus providing the potent antioxidant activity.[2] This easily oxidizable property of the polyphenol may lead to new oxidation products in foods and in the human body.[3–8] However, the functionality, regardless of being beneficial or non-beneficial, of the oxidation products has not yet been intensively examined. Recently, we examined the chemical structures and cytotoxic property of the oxidation products from sesamol, a sesame antioxidant.[9] We have now screened tyrosinase inhibitory activity of the oxidation products from various polyphenols. Tyrosinase is widely distributed in plants, microorganisms, and animals. In food, tyrosinase accelerates the browning of fresh food, which results in a loss of its market value. In humans, tyrosinase is responsible for the melanization of skin. Therefore, tyrosinase inhibitors have attracted strong interest in food and cosmetic sciences.

Rosmarinic acid (**1**) is a potent antioxidative polyphenol widely distributed in Lamiaceae (Labiatae) herbs.[10] The Lamiaceae herbs such as rosemary, sage, and melissa contain a large amount of rosmarinic acid and they are used as a food additive and herb tea for multifunctional purposes. Many beneficial activities of rosmarinic acid, which include antiinflammation,[11] antimutagenicity,[12] photoprotection of keratinocytes,[13] reduction of atopic dermatitis,[14] prevention of Alzheimer's disease,[15] apoptosis induction of colorectal cancer cell,[16] etc., have been recently reported in addition to its potent antioxidant activity.[17] Rosmarinic acid

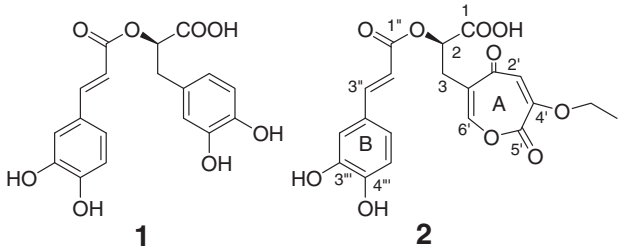

Figure 1. The structure of rosmarinic acid (**1**) and its oxidation product (**2**).

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Table 1
^{1}H, ^{13}C and 2D NMR data for **2**[a]

Posn.	δ_H (m, Hz)	Correlated H in long range COSY[b]	Correlated H in NOESY	δ_C	Correlated H in HMBC
1				173.0	H2, H3ab
2	5.31 (dd, 8.5, 4.5)	H3ab	H3ab, H3′, H6′	71.2	H3ab
3a	2.88 (dd, 14.0, 8.5)	H2, H6′, H3b	H2, H3′, H6′	28.5	H2, H6′
3b	3.13 (dd, 14.0, 4.5)	H2, H6′, H3a	H2, H6′		
1′				127.0	H3ab, H3′, H6′
2′				180.6	H3ab, H3′, H6′
3′	7.03 (br s)	H6′	H2, 4′-O$CH_2$$CH_3$	118.6	
4′				160.2	H3′, 4′′-O$CH_2$$CH_3$
5′				155.2	H3′, H6′
6′	8.22 (s)	H3ab,H3′	H2, H3ab	156.7	H3ab
4′-O$CH_2$$CH_3$	4.38 (q, 7.0)	4′-OCH$_2$$CH_3$	H3′, 4′-OCH$_2$$CH_3$	64.0	
4′-OCH$_2$$CH_3$	1.36 (t, 7.0)	4′-O$CH_2$$CH_3$	4′-O$CH_2$$CH_3$	14.2	
1″				168.2	H2, H2″, H3″
2″	6.26 (d, 15.5)	H3″		114.1	H3″
3″	7.56 (d, 15.5)	H2″, H2‴, H5‴, H6‴		148.0	H2″, H2‴, H6‴
1‴				127.6	H2″, H3″, H2‴, H5‴
2‴	7.03 (br s)	H3″, H5‴, H6‴	H2″, H3″	115.2	H5‴, H6‴
3‴				146.8	H2‴, H5‴
4‴				149.8	H2‴, H5‴, H6‴
5‴	6.77 (d, 8.0)	H3″, H2‴, H6‴	H6‴	116.7	H2‴, H6‴
6‴	6.95 (dd, 8.0, 2.0)	H3″, H2‴, H5‴	H2″, H3″, H5‴	123.2	H3″, H5‴

[a] Recorded in CD_3OD at 400 or 500 MHz for ^{1}H and 125 MHz for ^{13}C.
[b] Used delay time was 200 ms.

should be one of the promising food-functional polyphenols (Fig. 1).

To obtain the oxidation products of rosmarinic acid, we employed the Fe-catalyzed oxidation as the possible oxidation reaction in nature including foods. To an ethanol solution of rosmarinic acid was added a catalytic amount of $FeCl_3$, and stored under an oxygen atmosphere until almost all the rosmarinic acid was consumed. The resulting oxidation mixture was found to show a remarkable tyrosinase inhibitory activity compared to that of the oxidation products from other antioxidative polyphenols and related phenolics (caffeic acid, catechin, carnosic acid, carnosol, chlorogenic acid, ellagic acid, epicatechin, eugenol, gallic acid, gentisic acid, gingerol, hydroxytyrosol, nordihydroguaiaretic acid, piceatannol, protocatechuic acid, propyl gallate, quercetin, resveratrol, sinapic acid, syringic acid, secoisolariciresinol, taxifolin, and α-tocopherol). Although the oxidation product from rosmarinic acid contained various constituents, our assay-guided purification of the active mixture resulted in the isolation of one active compound having a novel oxidized structure. In this communication, we report the unique chemical structure and its potent tyrosinase inhibitory activity.

The oxidation of rosmarinic acid was carried out as follows. To 100 mg of rosmarinic acid in ethanol (10 mL) was added 0.05 M $FeCl_3$ (0.05 mL) in a straight screw-capped vial (i.d. 35 × *h* 78 mm). Ten such vials were incubated at 40 °C for 5 days under an oxygen atmosphere. The solutions were combined and then treated with Chelex 100™ (20 g) to remove the Fe ions and evaporated. The residue was fractionated by column chromatography using an ODS gel (Cosmosil-140C18-OPN) eluted with 20–70% methanol in H_2O containing 0.1% TFA. The 30% methanol-eluted fraction was purified by HPLC (Column: Cosmosil 5C18-AR-II, 20 × 250 mm, Solvent: methanol–1% AcOH aq = 1:1) to afford an active compound (**2**, 32 mg, t_R 12 min). The HR-ESI-MS of **2** (*m/z* 417.0818 $[M-H]^-$) suggested $C_{20}H_{18}O_{10}$ as the molecular formula of **2**. Although the ^{1}H NMR of **2** was very similar to that of rosmarinic acid (**1**), the signal set due to an ethoxy group was additionally observed, indicating that **2** was a reacted compound with the solvent from the original rosmarinic acid. The reacted position of the ethanol was indicated to be on the aromatic ring A because the caffeoyl (including aromatic ring B) and 2-oxypropionic acid moieties were intact from their proton and carbon NMR signals very similar to those of the rosmarinic acid. In the ^{1}H NMR of **2** (Table 1), only two singlet signals were observed as assignable to the ring A protons, both of which were correlated in long range COSY spectrum of **2**. The chemical shift of the benzylic proton signals of ring A were slightly changed from that of rosmarinic acid. These results indicated that a new ring system was produced on the original ring A of rosmarinic acid by this Fe-catalyzed oxidation. The fine structure of the ring A was mainly deduced by the C–H long range coupling connectivities observed in the HMBC spectra of **2** (Fig. 2). Two aromatic proton signals (δ 8.22 and 7.03), and two benzylic proton signals (δ 2.88 and 3.13) were correlated with the carbon at 180.6 ppm, which indicated that the 2-position of ring A should be a carbonyl group. The aromatic proton at 7.03 ppm was also correlated with the carbons at 160.2 and 155.2 ppm, one of which (160.2 ppm) was adjacent to the ethoxyl group which was revealed by an HMBC correlation with the methylene protons (δ 4.38). The other aromatic proton at 8.22 ppm was also correlated with the 1-position carbon (127.0 ppm), a benzylic carbon (28.5 ppm), and the carbon at 155.2 ppm in the HMBC spectrum (Fig. 2) and with the carbon at 156.7 ppm in the HMQC spectrum. The HR-MS results revealed that ring A contained $C_8H_7O_4$ in the molecular formula ($C_{20}H_{18}O_{10}$). These results strongly indicated that the ring A has a unique oxygen-contained seven-membered ring structure as shown in Figure 1. A very upfield shifted carbonyl carbon at 155.2 ppm of **2** should be assignable to the 5-position of ring A by the HMBC correlations and comparison of the typically upfield shifted signal of the 2-carbonyl carbon observed in 3-oxygenated coumarins (ex. 158.5 ppm), which had the similar conjugated system to that of **2**.[18]

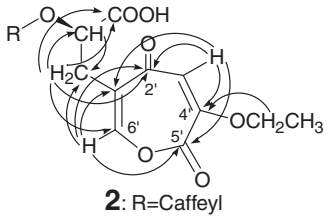

Figure 2. Proton-carbon long range correlations (arrows) around the A ring of **2** in HMBC spectrum.

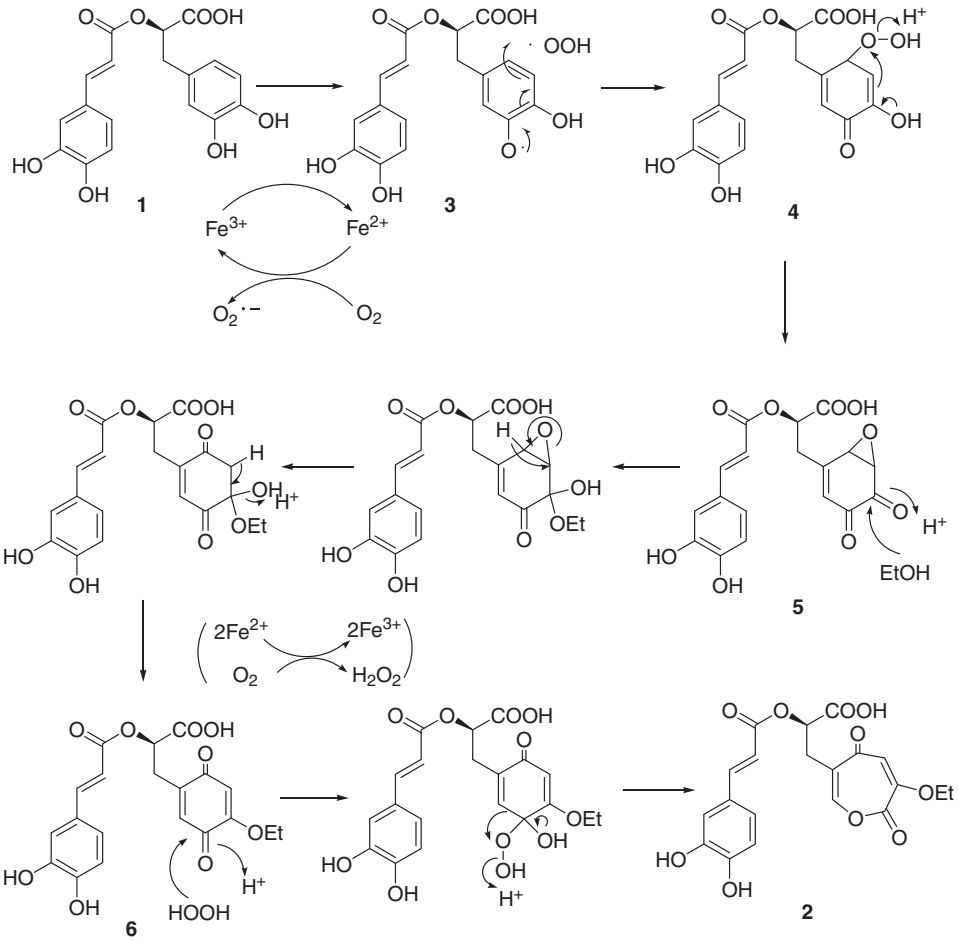

Figure 3. A proposed mechanism for formation of **2** from rosmarinic acid (**1**) in the presence of Fe ion.

The Fe-catalized oxidation afforded the unique ring-expanded compound from rosmarinic acid, however, its isolation yield was very low. Therefore we explored the possibilities that this reaction generally occurred in the catechol structure. Ethyl 3-(3,4-dihydroxyphenyl)-2-hydroxypropionate, which was a simplified ester derivative of rosmarinic acid by removing oxidatively sensitive caffeyl moiety of rosmarinic acid, was oxidized under the same conditions, yielding the similar seven-membered ring compound[19] in over 30% yield without any adjustment of the reaction conditions. Further investigation to record higher yield of the oxidation is now in progress. This oxidation also took place in 4-methyl catechol and the produced the seven-membered compound[20] showed two carbonyl absorptions at 1651 cm^{-1} (conjugated carbonyl part) and 1747 cm^{-1} (ester part) in IR spectrum[21] and two carbonyl signals at 181.8 ppm and similar upfield-shifted 154.8 ppm in ^{13}C NMR to that of **2**, supporting the seven-membered structure of **2** and its generality of the reaction of catechol compounds. It should be noted that this type of ring-expanding oxidation of catechol is proposed in the reaction of human homogentisate dioxygenase by Titus and co-workers.[22] Our found reaction maybe a biomimetic oxidation and should support Titus' proposed enzymatic reaction mechanism. In Figure 3, one of the possible formation mechanisms of the unique structure of **2** from rosmarinic acid (**1**) was illustrated. At first, the catechol structure of the ring A of rosmarinic acid was oxidized by ferric ion to produce the phenolic radical **3**. The produced ferrous ion reduces oxygen to produce a superoxide anion. In the second step, the rosmarinic acid radical **3** is reacted with a protonated superoxide radical to afford a hydroperoxide **4**. This hydroperoxide is unstable and then converted to diketoepoxide **5** by a mechanism similar to the epoxidation reaction of an en-

Table 2
Tyrosinase inhibitory activity of rosmarinic acid (**1**), oxidation mixture from rosmarinic acid, and compound **2**[a]

Compound	Concentration							IC_{50} (mM)
	0.01 mM	0.03 mM	0.10 mM	0.30 mM	1.00 mM	3.00 mM	10.0 mM	
Compound **2**	10.6 ± 2.6	35.3 ± 0.9	52.5 ± 1.0	—[b]	—	—	—	0.08
Rosmarinic acid (**1**)	—	—	—	—	40.7 ± 8.2	69.8 ± 13.0	93.6 ± 2.5	1.40
Oxidation mixture from **1**[c]	—	—	32.6 ± 2.9	58.7 ± 3.1	63.8 ± 5.9	—	—	0.25
Kojic acid[d]	47.8 ± 3.4	74.1 ± 1.7	—	—	—	—	—	0.012

[a] Each value is expressed as mean ± SD ($n = 3$).
[b] — Not examined.
[c] Molar concentration was calculated as rosmarinic acid (MW 360).
[d] Positive control sample.

one by alkaline hydroperoxide. Several migration reactions with the addition of ethanol produce paraquinone **6**. The quinone **6** is then reacted with a hydroperoxide via the Baeyer–Villiger reaction to afford compound **2**.

The tyrosinase inhibitory activity of **2** was evaluated based on a previously reported procedure using mushroom tyrosinase.[23] Compound **2** showed the inhibitory activity between 0.01 and 0.1 mM to be concentration-dependent and its IC_{50} was calculated to be 0.08 mM. The IC_{50} values of rosmarinic acid and its oxidation mixture under the same conditions were obtained to be 1.4 and 0.25 mM, respectively. Therefore, compound **2** has a very strong inhibitory activity and should be chemical evidence of the very strong activity of the oxidation product of rosmarinic acid (Table 2).

Acknowledgments

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19. ^1H NMR (acetone-d_6, 400 MHz): δ 8.10 (1H, s, H6′), 6.92 (1H, s, H3′), 4.39 (2H, q, J = 7.2 Hz, 4′-OCH_2), 4.37 (1H, overlapped with the signal of 4′-OCH_2, H2), 4.13 (2H, q, J = 7.2 Hz, 1-OCH_2), 2.9 (1H, overlapped with the signal of H_2O, H3b), 2.62 (1H, dd, J = 14.0 and 8.0 Hz, H3a), 1.36 (3H, t, J = 7.2 Hz, 4′-OCH_2CH_3), 1.12 (3H, t, J = 7.2 Hz, 1-OCH_2CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 179.3 (C2′), 173.7 (C1), 159.7 (C4′), 154.0 (C6′), 152.9 (C5′), 126.4 (C1′), 118.4 (C3′), 68.7 (C2), 63.0(4′-OCH_2), 61.9 (1-OCH_2), 30.6 (C3), 14.1 (1- or 4′-OCH_2CH_3), 14.0 (1- or 4′-OCH_2CH_3); HR-ESI-MS m/z 293.0630 $[M+Na]^+$, calcd for $C_{12}H_{14}O_7Na$, 293.0637. Proton and carbon numbering system was adopted temporarily form that of **2**.
20. ^1H NMR (CD_3OD, 400 MHz): δ 8.06 (s, H6′), 6.87 (s, H3′), 4.38 (2H, q, J = 7.2 Hz, 4′-OCH_2), 1.87 (3H, s, H1), 1.36 (4′-OCH_2CH_3); ^{13}C NMR (CD_3OD, 100 MHz): δ 181.8 (C2′), 161.1 (C4′), 155.3 (C6′), 154.8 (C5′), 128.3 (C1′), 117.9 (C3′), 64.0 (4′-OCH_2), 14.3 (4′-OCH_2CH_3), 10.8 (C1); HR-ESI-MS m/z 183.0650 $[M+H]^+$, calcd for $C_9H_{11}O_4$, 183.0657. Proton and carbon numbering system was adopted temporarily form that of **2**.
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