#### Food and Beverage Chemistry/Biochemistry

# Temperature Effects on the Composition and Xanthine Oxidase Inhibitory Activity of Caffeic Acid Roasting Products

Toshiya Masuda, Yuya Fukuyama, Sayaka Doi, Akiko Masuda, Sinichiro Kurosawa, and Shigeyoshi Fujii J. Agric. Food Chem., Just Accepted Manuscript • DOI: 10.1021/acs.jafc.9b03633 • Publication Date (Web): 23 Jul 2019 Downloaded from pubs.acs.org on July 23, 2019

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1	Temperature Effects on the Composition and Xanthine Oxidase Inhibitory
2	Activity of Caffeic Acid Roasting Products
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17	

#### 18 ABSTRACT

19The high-temperature treatment of caffeic acid by a model reaction for the roasting processing of foods enhanced its xanthine oxidase (XO) inhibitory activity. The thermal 2021reaction products included various oligomeric compounds, whose structures were 22determined as produced via the intermediate 4-vinylcatechol. And their XO inhibitory activity measurement were also carried out. Among the identified oligomers, the 23coupling products of caffeic acid and vinylcatechol, which were mainly produced at 24140-170 °C, presented stronger XO inhibitory activity than the other types of produced 25oligomers. Further reacted compounds, which were mainly formed at 200 °C by the 26addition or elimination of a catechol unit in the oligomers, displayed weaker activity. 27These results indicated that thermal enhancement of the XO inhibitory activity of 28caffeic acid can be explained by the difference in the XO inhibitory activity of the 29various constituents of the thermal reaction products. Caffeic acid and its derivatives are 30 polyphenols found widely distributed in foods. Moreover, XO inhibition is closely 31related to the prevention of the life-style related disease, gout. The results suggest that a 32simple roasting process (170 °C) can lend useful human health-related functionalities to 33 34caffeic acid-containing foods such as coffee.

 $\mathbf{2}$ 

35	Keywords: Thermal reaction, Caffeic acid, Xanthine oxidase inhibition, Roasting
36	temperature, Structure identification
37	

### 38 INTRODUCTION

39

40	Roasting is a high-temperature cooking method (130–230 °C), <sup>1</sup> that is applied to grains,
41	vegetables, meats, and fish in both industrial food processing and home cooking. <sup>2</sup>
42	Roasting can cause various thermal reactions of the food chemical constituents, thereby
43	developing the flavors and color. <sup>1,3</sup> Caffeic acid is a polyphenol that is widely
44	distributed in plant foods in its free or bound forms. Coffee beans contain free caffeic
45	acid as well as a large amount of chlorogenic acid, a major caffeic acid derivative. <sup>4,5</sup>
46	The roasting of coffee beans at approximately 200 °C is indispensable for making coffee
47	beverages. During the roasting process, caffeic acid derivatives are degraded into
48	various compounds including disassembled caffeic acid. <sup>6,7</sup> The degraded products react
49	with each other to afford more complex substances, <sup>8</sup> which affect the flavor and taste as
50	well as human health functions of roasted coffee beverages. <sup>9</sup> To date, many thermal
51	reaction products have been identified in roasted coffee beans, including volatile
52	flavoring compounds, <sup>10</sup> Maillard reaction products, <sup>11</sup> the bitter-tasting chlorogenic
53	lactones, <sup>12</sup> and phenylindane derivatives. <sup>13</sup> Recently, several epidemiological studies by
54	Choi et al. <sup>14</sup> and other groups, <sup>15</sup> have reported that coffee drinking reduces the risk of
55	gout. Additionally, we recently discovered that roasted coffee bean extract exhibits

56	xanthine oxidase (XO) inhibitory activity. <sup>16</sup> We also reported the presence of several
57	inhibitors in roasted coffee beans. <sup>17</sup> XO is a key enzyme in the catabolism of purine to
58	uric acid, the substance that causes gout when it over-accumulates in plasma. <sup>18</sup> Stadler
59	et al. <sup>19</sup> were first to report that phenylindanes were produced from the thermal treatment
60	of caffeic acid via a vinylcatechol intermediate. These compounds were observed in
61	roasted coffee beans and play a role in the bitter taste of coffee. <sup>13</sup> We also reported
62	moderate XO inhibitory activity for some phenylindanes. <sup>20</sup>
63	In this investigation, we found maximum XO inhibitory activity for the caffeic
64	acid products of thermal treatment at around 170 °C. These conditions were used as a
65	model reaction of the roasting process and, notably, differences in the thermal reaction
66	products were found between 110 and 290 °C. Herein, we report the structures of the
67	constituent compounds of the thermal reaction products (140-200 °C) and their XO
68	inhibitory activities.
69	
70	MATERIALS AND METHODS
71	Chemicals and instruments. Caffeic acid was obtained from Kanto Chemicals (Tokyo,
72	Japan). Xanthine oxidase (XO) was purchased from Wako Pure Chemicals (Osaka,

73 Japan). Allopurinol was obtained from Sigma-Aldrich (St. Louis, MO). All other

74	reagents and solvents were purchased from Nacalai Tesque (Kyoto, Japan). NMR
75	spectra were obtained from a JNM-ECZ400S (400 MHz, JEOL, Tokyo, Japan) or
76	ARX600 (600 MHz, Bruker Japan, Yokohama, Japan). MS spectra were measured
77	using a JMS-T100 spectrometer (JEOL) with direct analysis at real time (DART) or
78	electro spray ionization (ESI), and time of flight (TOF) measuring mode under the
79	following conditions: DART (positive mode), orifice 1, 10 V; orifice 2, 5 V; ring lens,
80	10 V; helium gas pressure, 0.5 MPa; flow rate of 10 L/min; ion resource temperature,
81	350 °C; grid voltage, 400 V and ESI (positive mode), orifice 1, 85 V and 80 °C; orifice
82	2, 5 V; ring lens, 15 V; helium gas pressure, 0.7 MPa; needle, 2000 V; desolvating
83	chamber temperature, 250 °C. Elemental compositions of compounds were calculated
84	using MassLynx software, version 4.1 (Waters Japan, Tokyo, Japan) from high
85	resolution MS (HR-MS) data. Analytical HPLC was performed using a PU-4180
86	RHPLC quaternary gradient pump (JASCO, Tokyo, Japan) equipped with an MD-4015
87	photodiode array detector (JASCO) and a CTO-10AS column oven (Shimadzu, Kyoto,
88	Japan). Data were analyzed using the ChromNAV software (v. 2, JASCO). Preparative
89	HPLC was performed using an LC-6AD pump (Shimadzu) equipped with an SPD-6A
90	UV detector (Shimadzu).

92	Thermal reaction of caffeic acid and HPLC analysis of the reaction products
93	In a screw-capped test tube (i.d. 8 mm and length, 100 mm) were placed 10 mg
94	caffeic acid in methanol (200 $\mu L)$ and 400 $\mu L$ phosphate buffer (500 mmol/L, pH 6.0,
95	Na <sub>2</sub> HPO <sub>4</sub> and KH <sub>2</sub> PO <sub>4</sub> ). After removing the solvent in vacuo, the tube was heated in a
96	metal block bath (MB-1H-UII, KPI, Itami, Japan) at the appropriate temperatures
97	(110–290 °C) for 0.5 h. After cooling the tube, methanol (1 mL) was added and the
98	mixture was centrifuged at 2000 rpm for 5 min at 25 °C. Subsequently, the supernatant
99	was collected and the solvent was removed by evaporation to give the thermal reaction
100	products. A portion of the reaction products was used for the XO inhibitory assay. The
101	constituents in the products were analyzed by HPLC [Cosmosil 5C18-AR-II column
102	(Nacalai, Kyoto, Japan), 250 mm $\times$ 4.6 mm i.d., 5 $\mu m$ ; flow rate, 1.0 mL/min; solvent
103	A, 1% acetic acid in H <sub>2</sub> O; solvent B, CH <sub>3</sub> CN; linear gradient from 5% solvent B to 45%
104	solvent B for 40 min, then from 45% solvent B to 100% solvent B for 10 min, and
105	100% solvent B for 5 min; detection at 280 and 320 nm].
106	
107	Gel Permeation Chromatography (GPC) analysis of the thermal reaction products
108	The thermal reaction products (weight corresponding to 6 mg of initial caffeic
109	acid) were treated with acetic anhydride (0.5 mL) and pyridine (0.5 mL) at 25 °C for 1

110	h. After removal of acetic acid and pyridine in vacuo, the residue was dissolved in THF
111	to produce an analytical solution (0.4 mg/mL, corresponding to the initial caffeic acid
112	concentration). The solution (15 $\mu L)$ was injected into a GPC column (GPC KF-803,
113	300 mm $\times$ 8 mm i.d., Shodex, Tokyo, Japan) for analysis (solvent, HPLC grade THF;
114	flow rate, 1.0 mL/min; temperature, 30 °C; detection at 264 nm). Molecular size
115	calibration was performed under the same conditions using polystyrene standards [266,
116	2280, 6540, and 25500 Da polystyrene; Ready Cal-Kit Poly(styrene) Low, Sigma-
117	Aldrich, St. Louis, MO].
118	
119	Isolation of the thermal reaction product constituents from the large-scale thermal
120	reactions of caffeic acid at 140–200 °C
121	For thermal treatment at 140 °C, caffeic acid (10 g) was dissolved in 100 mL
121 122	For thermal treatment at 140 °C, caffeic acid (10 g) was dissolved in 100 mL methanol and 400 mL of 500 mmol/L Na <sub>2</sub> HPO <sub>4</sub> –KH <sub>2</sub> PO <sub>4</sub> (pH 6.0) and subsequently
121 122 123	For thermal treatment at 140 °C, caffeic acid (10 g) was dissolved in 100 mL methanol and 400 mL of 500 mmol/L Na <sub>2</sub> HPO <sub>4</sub> –KH <sub>2</sub> PO <sub>4</sub> (pH 6.0) and subsequently evaporated to dryness. The solid residue was heated at 140 °C for 90 min. After cooling,
121 122 123 124	For thermal treatment at 140 °C, caffeic acid (10 g) was dissolved in 100 mL methanol and 400 mL of 500 mmol/L Na <sub>2</sub> HPO <sub>4</sub> –KH <sub>2</sub> PO <sub>4</sub> (pH 6.0) and subsequently evaporated to dryness. The solid residue was heated at 140 °C for 90 min. After cooling, the reaction mixture was extracted twice with 1 L methanol and this procedure was
<ol> <li>121</li> <li>122</li> <li>123</li> <li>124</li> <li>125</li> </ol>	For thermal treatment at 140 °C, caffeic acid (10 g) was dissolved in 100 mL methanol and 400 mL of 500 mmol/L Na <sub>2</sub> HPO <sub>4</sub> –KH <sub>2</sub> PO <sub>4</sub> (pH 6.0) and subsequently evaporated to dryness. The solid residue was heated at 140 °C for 90 min. After cooling, the reaction mixture was extracted twice with 1 L methanol and this procedure was repeated ten times. The methanol extracts were combined and the solvent was removed.
121 122 123 124 125 126	For thermal treatment at 140 °C, caffeic acid (10 g) was dissolved in 100 mL methanol and 400 mL of 500 mmol/L Na <sub>2</sub> HPO <sub>4</sub> –KH <sub>2</sub> PO <sub>4</sub> (pH 6.0) and subsequently evaporated to dryness. The solid residue was heated at 140 °C for 90 min. After cooling, the reaction mixture was extracted twice with 1 L methanol and this procedure was repeated ten times. The methanol extracts were combined and the solvent was removed. The resultant residue was further purified to isolate the constituent compounds. A
<ol> <li>121</li> <li>122</li> <li>123</li> <li>124</li> <li>125</li> <li>126</li> <li>127</li> </ol>	For thermal treatment at 140 °C, caffeic acid (10 g) was dissolved in 100 mL methanol and 400 mL of 500 mmol/L Na <sub>2</sub> HPO <sub>4</sub> –KH <sub>2</sub> PO <sub>4</sub> (pH 6.0) and subsequently evaporated to dryness. The solid residue was heated at 140 °C for 90 min. After cooling, the reaction mixture was extracted twice with 1 L methanol and this procedure was repeated ten times. The methanol extracts were combined and the solvent was removed. The resultant residue was further purified to isolate the constituent compounds. A portion (84 g) of the residue was subjected to Amberlite XAD-7 (Organo, Tokyo,

128	Japan) column chromatography and eluted with increasing concentrations of methanol
129	(50 to 100%) in water to produce eight separate fractions. Fraction 3 (208 mg of 6.5 g),
130	which was eluted with 60% methanol in water, was purified by preparative HPLC
131	(Cosmosil 5C18-AR-II column, $250 \times 20$ mm i.d.; solvent, 1% acetic acid in 85:15
132	$H_2O-CH_3CN$ ; flow rate, 9.6 mL/min; detection at 280 nm) to yield compounds 1–4 in 2,
133	6, 3, and 3 mg yields, respectively. Fraction 6 (14.3 g), which was eluted with 75%
134	methanol in water from the XAD-7 column, was subjected to Sephadex LH-20 (GE
135	Healthcare, Hino, Japan) column chromatography (1.5 L column) and eluted with
136	methanol to produce 5 g residue, which was collected from 1.5 to 1.65 L of the total
137	elution volume. A part of the residue (656 mg) was purified by preparative HPLC
138	(Cosmosil 5C18-AR-II column, 250 mm × 20 mm i.d.; solvent, 1% acetic acid in 75:25
139	$H_2O-CH_3CN$ ; flow rate, 9.6 mL/min; detection at 280 nm) to produce compounds <b>5–8</b>
140	in 5, 4, 87, and 134 mg yields, respectively, along with 52 mg of a mixture of
141	compounds 9 and 10. This mixture was separated by HPLC in recycle mode (eleven
142	times) under the following conditions: Cosmosil 5C18-AR-II column, 250 mm $\times$ 20 mm
143	i.d.; solvent, 1% acetic acid in 45:55 H <sub>2</sub> O–CH <sub>3</sub> OH; flow rate, 9.6 mL/min; detection at
144	280 nm, to produce pure 9 and 10 in 5 mg yields.
145	For thermal treatment at 200 °C, 40 mL phosphate buffer (pH 6.0; 0.5 mol/L)

146	was added to caffeic acid (1 g) in methanol (20 mL) and mixed well. After removal of
147	the solvent from the mixture in vacuo, the residual solid was heated at 200 °C for 30min.
148	The resulting dark-colored residue was extracted twice with methanol (50 mL) to
149	produce 0.76 g extract after evaporation. The extract was fractionated by XAD-7
150	column chromatography eluting with increasing concentrations of methanol (10 to
151	100%) in water. Two fractions, eluted with 50 and 75% methanol, were evaporated to
152	produce fractions I and II in 74 and 86 mg yields, respectively. A portion (32 mg) of the
153	residue of fraction I was purified by HPLC (Cosmosil 5C18-AR-II column, 250 mm $\times$
154	20 mm i.d.; solvent, 1% acetic acid in 90:10 H <sub>2</sub> O-CH <sub>3</sub> CN; flow rate, 9.6 mL/min;
155	detection at 280 nm) to give compound 11 in 1 mg yield. Likewise, the residue of
156	fraction II (37 mg) was separated by HPLC (Cosmosil 5C18-AR-II column, 250 mm $\times$
157	20 mm i.d.; solvent, 1% acetic acid in 70:30 H <sub>2</sub> O-CH <sub>3</sub> CN; flow rate, 9.6 mL/min;
158	detection at 280 nm) to produce compounds 5–8 and 12 in 1, 5, 1, 2, and 2 mg yields,
159	respectively. Another reaction product (570 mg), which was obtained from the thermal
160	treatment of caffeic acid (1 g) under the same conditions, was fractionated by XAD-7
161	column chromatography using the same protocol as described above. The fraction
162	eluted with 90% methanol (195 mg) was separate d by HPLC (Cosmosil 5C18-AR-II
163	column, 250 mm $\times$ 20 mm i.d.; solvent, 1% acetic acid in 45:55 H <sub>2</sub> O–CH <sub>3</sub> CN; flow

164	rate, 9.6 mL/min; detection at 280 nm) to produce compounds 13, 14, and 16–18 in 5, 4,
165	3, 2, and 2 mg yields, respectively. The product (17. 1 g) of a third thermal reaction of
166	caffeic acid (20 g) was also fractionated into five fractions by XAD-7 column
167	chromatography under the same conditions. The fraction eluted with 70% methanol (2
168	g) produced the subsequent four fractions 4-1 to 4-4 in 523, 61, 80, and 47 mg yields,
169	respectively, (retention times: fraction 4-1, 9–24 min, fraction 4-2, 27 min, fraction 4-3,
170	33 min, and fraction 4-4, 40 min) under the following conditions: Cosmosil 5C18-AR-II
171	column, 250 mm $\times$ 20 mm i.d.; solvent, 1% acetic acid in 70:30 H <sub>2</sub> O–CH <sub>3</sub> CN; flow
172	rate, 9.6 mL/min; detection at 280 nm. Compound 13 (15 mg) was isolated from
173	fraction 4-1 (487 mg) by HPLC with a retention time of 41 min (Cosmosil 5C18-AR-II
174	column, 250 mm $\times$ 20 mm i.d.; solvent, 1% acetic acid in 60:40 H <sub>2</sub> O–CH <sub>3</sub> OH; flow
175	rate, 6.7 mL/min; detection at 280 nm). Compound 15 (7 mg) was isolated from fraction
176	4-2 (20 mg) by HPLC with a retention time of 37 min (Cosmosil 5C18-AR-II column,
177	250 mm $\times$ 20 mm i.d.; solvent, 1% acetic acid in 45:55 H <sub>2</sub> O–CH <sub>3</sub> OH; flow rate, 4.8
178	mL/min; detection at 280 nm). Compounds $14 (10 \text{ mg})$ and $17 (14 \text{ mg})$ were obtained
179	from fraction 4-3 (70 mg) by HPLC with retention times of 43 and 45 min, respectively
180	(Cosmosil 5C18-AR-II column, 250 mm × 20 mm i.d.; solvent, 1% acetic acid in 45:55
181	H <sub>2</sub> O–CH <sub>3</sub> OH; flow rate, 4.8 mL/min; detection at 280 nm). Finally, compound <b>18</b> (6

182	mg) was isolated from fraction 4-4 (33 mg) by HPLC with a retention time 50 min
183	(Cosmosil 5C18-AR-II column, 250 mm × 20 mm i.d.; solven, 1% acetic acid in 45:55
184	H <sub>2</sub> O–CH <sub>3</sub> OH; flow rate, 4.8 mL/min; detection at 280 nm).
185	
186	Analytical data for the structures of compounds 5, 6, and 11–18.
187	Chemical structures of the isolated products, except previously identified
188	phenylindanes (compounds 1–4, 7, and 8), were elucidated by NMR and MS data of
189	their intact or acetylated forms, which were derived by acetylation with acetic anhydride
190	in pyridine. The data were listed as follows and position numbering of each compound
191	is tentative as shown in the structures in Fig. 3.
192	5. HR-ESIMS (m/z) $[M{+}Na]^{+}$ calcd for $C_{16}H_{16}O_{4}Na$ 295.0946 , found
193	298.0969, and $[2M+Na]^+$ calcd for $C_{32}H_{32}O_8Na$ 567.1995, found 567.2003; <sup>1</sup> H NMR
194	(CD <sub>3</sub> OD) δ 6.69 (4H, s, H3, H6, H3', and H6'), 3.75 (2H, q, J=7.4 Hz, H7 and H7'), and
195	1.43 (6H, d, J=7.4 Hz, H8 and H8').
196	6. HR-ESIMS (m/z) $[M+Na]^+$ calcd for $C_{14}H_{14}O_4Na$ 269.0790, found 269.0817
197	and $[2M\text{+}Na]^{\text{+}}$ calcd for $C_{28}H_{28}O_8Na$ 515.1682, found 515.1690; $^1H$ NMR (CD_3OD) $\delta$
198	6.65 (2H, d, J=1.6 Hz, H3 and H2'), 6.70 (2H, d, J=8.2 Hz, H6 and H5'), 6.57 (2H, dd,
199	J=8.2 and 1.6 Hz, H5 and H6'), 3.87 (1H, q, J=7.2 Hz, H7), and 1.52 (3H, d, J=7.2 Hz,

- 200 H8).
- 201 **9**. HR-DARTMS (m/z) [M+H]<sup>+</sup> calcd for C<sub>24</sub>H<sub>25</sub>O<sub>6</sub> 409.1651, found 409.1656;
- 202 NMR, See Table S1 in supporting information.
- 203 **10**. HR-DARTMS (m/z) [M+H]<sup>+</sup> calcd for C<sub>24</sub>H<sub>25</sub>O<sub>6</sub> 409.1651, found
- 409.1652; NMR, See Table S1 in supporting information.
- 205 **11**. DARTMS (m/z)  $[M+H]^+$  111; <sup>1</sup>H NMR (acetone-d<sub>6</sub>)  $\delta$  6.65 (2H, AA'BB'
- coupling, H3 and H6) and 6.79 (2H, AA'BB' coupling, H4 and H5).
- 207 **12**. DARTMS (m/z) [M+H]<sup>+</sup> 139; <sup>1</sup>H NMR (CD<sub>3</sub>OD) δ 6.65 (1H, d, J=1.9 Hz,
- 208 H3), 6.69 (1H, d, J=8.0 Hz, H5), 6.53 (1H, dd, J=8.0 and 1.9 Hz, H6), 2.52 (2H, q, J=7.4
- 209 Hz, 4-CH<sub>2</sub>-CH<sub>3</sub>), 1.20 (3H, t, J=7.4 Hz, 4-CH<sub>2</sub>-CH<sub>3</sub>).
- 210 **13**. HR-ESIMS  $[M+Na]^+$  calcd for C<sub>14</sub>H<sub>14</sub>O<sub>4</sub>Na 269.0790, found 269.0816, and
- 211 [2M+Na]<sup>+</sup> calcd for C<sub>28</sub>H<sub>28</sub>O<sub>8</sub>Na 515.1682, found 515.1667; <sup>1</sup>H-NMR (CDCl<sub>3</sub>) of
- 212 tetraacetate (**13a**) δ 6.99–7.03 (2H, complex, H3 and H5), 7.06–7.12 (3H, complex, H6,
- 213 H4', and H6'), 4.25 (1H, q, J=7.0 Hz, H7), 1.57 (3H, d, J=7.0 Hz, H8), 7.23 (1H, dd,
- 214 J=8.6 and 8.2 Hz, H5'), 2.18 (3H, s, 1, 2, 2' or 3'-CH<sub>3</sub>CO), 2.25 (3H, s, 1, 2, 2' or 3'-
- 215  $CH_3CO$ , 2.26 (6H, s, 1, 2, 2' or 3'- $CH_3CO$ ).
- 216 **14**. HR-DARTMS  $[M+H]^+$  calcd for C<sub>10</sub>H<sub>13</sub>O<sub>2</sub> 165.0916, found 165.0914; <sup>1</sup>H-
- 217 NMR (CDCl<sub>3</sub>) of diacetate of 14, δ 3.17 (1H, sext, J=7.6 Hz, H1), 1.64 (1H, dq, J=12.4

-10	and 7.6 Hz, H2a), 2.29 (1H, ddt, J=12.4, 7.6, and 4.0 Hz, H2b), 2.77–2.92 (2H, m, H3),
219	6.99 (1H, s, H4 or H7), 6.95 (1H, s, H4 or H7), 1.26 (3H, d, J=7.6 Hz, 1-C <u>H</u> <sub>3</sub> ), 2.28
220	(6H, s, 5- and 6-C <u>H</u> <sub>3</sub> CO ).
221	<b>15</b> . HR-ESIMS $[M+Na]^+$ calcd for $C_{16}H_{16}O_4Na$ 295.0946, found 295.0986, and
222	$[2M+Na]^+$ calcd for $C_{32}H_{32}O_8Na$ 567.1995, found 567.2003; <sup>1</sup> H NMR (CD <sub>3</sub> OD) $\delta$ 3.21
223	(1H, dquint, J=8.8 and 7.0 Hz, H1), 1.99 – 2.08 (1H, m, H2a), 2.20 (1H, ddd, J=12.0,
224	7.0 and 1.2 Hz, H2b), 4.38 (1H, brd, J=8.0 Hz, H3), 6.60 (1H, d, J=8.0 Hz, H6), 6.73
225	(1H, d, J=8.0 Hz, H7), 1.25 (1H, d, J=7.0 Hz, 1-C <u>H</u> <sub>3</sub> ), 6.49 (1H, d, J=1.7 Hz, H2'), 6.66
226	(1H, d, J=8.0 Hz, H5'), 6.44 (1H, dd, J=8.0 and 1.7 Hz, H6').
227	<b>16</b> . HR-ESIMS $[M+Na]^+$ calcd for $C_{16}H_{16}O_4Na$ 295.0946, found 295.0964; <sup>1</sup> H
227 $228$	<b>16</b> . HR-ESIMS [M+Na] <sup>+</sup> calcd for $C_{16}H_{16}O_4Na$ 295.0946, found 295.0964; <sup>1</sup> H NMR (CD <sub>3</sub> OD) $\delta$ 3.08 (1H, sext, J=7.5 Hz, H1), 1.55 (1H, dt, J=12.4 and 7.5 Hz, H2a),
227 228 229	<ul> <li>16. HR-ESIMS [M+Na]<sup>+</sup> calcd for C<sub>16</sub>H<sub>16</sub>O<sub>4</sub>Na 295.0946, found 295.0964; <sup>1</sup>H</li> <li>NMR (CD<sub>3</sub>OD) δ 3.08 (1H, sext, J=7.5 Hz, H1), 1.55 (1H, dt, J=12.4 and 7.5 Hz, H2a),</li> <li>2.75 (1H, dt, J=12.4 and 7.5 Hz, H2b), 4.24 (1H, t, J=7.5 Hz, H3), 6.61 (1H, d, J=8.0</li> </ul>
227 228 229 230	<b>16</b> . HR-ESIMS [M+Na] <sup>+</sup> calcd for $C_{16}H_{16}O_4$ Na 295.0946, found 295.0964; <sup>1</sup> H NMR (CD <sub>3</sub> OD) $\delta$ 3.08 (1H, sext, J=7.5 Hz, H1), 1.55 (1H, dt, J=12.4 and 7.5 Hz, H2a), 2.75 (1H, dt, J=12.4 and 7.5 Hz, H2b), 4.24 (1H, t, J=7.5 Hz, H3), 6.61 (1H, d, J=8.0 Hz, H6), 6.73 (1H, d, J=8.0 Hz, H7), 1.26 (3H, d, J=7.5 Hz, 1-C <u>H<sub>3</sub></u> ), 6.60 (1H, d, J=2.0
227 228 229 230 231	<b>16</b> . HR-ESIMS [M+Na] <sup>+</sup> calcd for C <sub>16</sub> H <sub>16</sub> O <sub>4</sub> Na 295.0946, found 295.0964; <sup>1</sup> H NMR (CD <sub>3</sub> OD) δ 3.08 (1H, sext, J=7.5 Hz, H1), 1.55 (1H, dt, J=12.4 and 7.5 Hz, H2a), 2.75 (1H, dt, J=12.4 and 7.5 Hz, H2b), 4.24 (1H, t, J=7.5 Hz, H3), 6.61 (1H, d, J=8.0 Hz, H6), 6.73 (1H, d, J=8.0 Hz, H7), 1.26 (3H, d, J=7.5 Hz, 1-C <u>H<sub>3</sub></u> ), 6.60 (1H, d, J=2.0 Hz, H2'), 6.70 (1H, d, J=8.0 Hz, H5'), 6.55 (1H, dd, J=8.0 and 2.0 Hz, H6').
227 228 229 230 231 232	<b>16</b> . HR-ESIMS $[M+Na]^+$ calcd for C <sub>16</sub> H <sub>16</sub> O <sub>4</sub> Na 295.0946, found 295.0964; <sup>1</sup> H NMR (CD <sub>3</sub> OD) $\delta$ 3.08 (1H, sext, J=7.5 Hz, H1), 1.55 (1H, dt, J=12.4 and 7.5 Hz, H2a), 2.75 (1H, dt, J=12.4 and 7.5 Hz, H2b), 4.24 (1H, t, J=7.5 Hz, H3), 6.61 (1H, d, J=8.0 Hz, H6), 6.73 (1H, d, J=8.0 Hz, H7), 1.26 (3H, d, J=7.5 Hz, 1-C <u>H<sub>3</sub></u> ), 6.60 (1H, d, J=2.0 Hz, H2'), 6.70 (1H, d, J=8.0 Hz, H5'), 6.55 (1H, dd, J=8.0 and 2.0 Hz, H6'). <b>17</b> . HR-ESIMS [M+Na] <sup>+</sup> calcd for C <sub>18</sub> H <sub>18</sub> O <sub>4</sub> Na 321.1103, found 321.1137, and
<ol> <li>227</li> <li>228</li> <li>229</li> <li>230</li> <li>231</li> <li>232</li> <li>233</li> </ol>	16. HR-ESIMS [M+Na] <sup>+</sup> calcd for $C_{16}H_{16}O_4Na$ 295.0946, found 295.0964; <sup>1</sup> H NMR (CD <sub>3</sub> OD) $\delta$ 3.08 (1H, sext, J=7.5 Hz, H1), 1.55 (1H, dt, J=12.4 and 7.5 Hz, H2a), 2.75 (1H, dt, J=12.4 and 7.5 Hz, H2b), 4.24 (1H, t, J=7.5 Hz, H3), 6.61 (1H, d, J=8.0 Hz, H6), 6.73 (1H, d, J=8.0 Hz, H7), 1.26 (3H, d, J=7.5 Hz, 1-CH <sub>3</sub> ), 6.60 (1H, d, J=2.0 Hz, H2'), 6.70 (1H, d, J=8.0 Hz, H5'), 6.55 (1H, dd, J=8.0 and 2.0 Hz, H6'). 17. HR-ESIMS [M+Na] <sup>+</sup> calcd for $C_{18}H_{18}O_4Na$ 321.1103, found 321.1137, and [2M+Na] <sup>+</sup> calcd for $C_{36}H_{36}O_8Na$ 619.2308, found 619.2301; <sup>1</sup> H-NMR (CDCl <sub>3</sub> ) of
<ol> <li>227</li> <li>228</li> <li>229</li> <li>230</li> <li>231</li> <li>232</li> <li>233</li> <li>234</li> </ol>	16. HR-ESIMS [M+Na] <sup>+</sup> calcd for C <sub>16</sub> H <sub>16</sub> O <sub>4</sub> Na 295.0946, found 295.0964; <sup>1</sup> H NMR (CD <sub>3</sub> OD) $\delta$ 3.08 (1H, sext, J=7.5 Hz, H1), 1.55 (1H, dt, J=12.4 and 7.5 Hz, H2a), 2.75 (1H, dt, J=12.4 and 7.5 Hz, H2b), 4.24 (1H, t, J=7.5 Hz, H3), 6.61 (1H, d, J=8.0 Hz, H6), 6.73 (1H, d, J=8.0 Hz, H7), 1.26 (3H, d, J=7.5 Hz, 1-CH <sub>3</sub> ), 6.60 (1H, d, J=2.0 Hz, H2'), 6.70 (1H, d, J=8.0 Hz, H5'), 6.55 (1H, dd, J=8.0 and 2.0 Hz, H6'). 17. HR-ESIMS [M+Na] <sup>+</sup> calcd for C <sub>18</sub> H <sub>18</sub> O <sub>4</sub> Na 321.1103, found 321.1137, and [2M+Na] <sup>+</sup> calcd for C <sub>36</sub> H <sub>36</sub> O <sub>8</sub> Na 619.2308, found 619.2301; <sup>1</sup> H-NMR (CDCl <sub>3</sub> ) of tetraacetate of 17 $\delta$ 2.81 (1H, dq, J=7.8 and 6.8 Hz, H1), 2.55 (1H, dt, J=1.6 and 7.8 Hz,

236	$C\underline{H}_{3}$ ), 7.15 (1H, s, H2'), 7.00 (1H, s, H5'), 3.23 (1H, dd, J=16.2 and 7.8 Hz, 7' $\beta$ - $C\underline{H}_{2}$ ),
237	2.82 (1H, brd, J=16.2 Hz, $7'\alpha$ -C <u>H</u> <sub>2</sub> ), 2.25 (6H, s, 5-, 6-, 3'- or 4'-C <u>H</u> <sub>3</sub> CO), 2.27 (3H, s,
238	5-, 6-, 3'- or 4'-C <u>H</u> <sub>3</sub> CO), 2.30 (3H, s, 5-, 6-, 3'- or 4'-C <u>H</u> <sub>3</sub> CO). Observed NOESY
239	correlations from H2 to $1-C\underline{H}_3$ , $3-C\underline{H}_3$ $7'\alpha-C\underline{H}_2\alpha$ , and $7'\beta-C\underline{H}_2$ , from H7 to $1-C\underline{H}_3$ , from
240	1-C <u>H<sub>3</sub></u> to H2, and H7, from $7'\beta$ -C <u>H<sub>2</sub></u> to 3-C <u>H<sub>3</sub></u> , from 3-C <u>H<sub>3</sub></u> to H2, $7'\beta$ -C <u>H<sub>2</sub></u> , H4, and
241	H2', and from H2' to $3-C\underline{H}_{\underline{3}}$ .
242	<b>18</b> . HR-ESIMS $[M+Na]^+$ calcd for $C_{18}H_{18}O_4Na$ 321.1103, found 321.1133, and
243	$[2M+Na]^+$ calcd for $C_{36}H_{36}O_8Na$ 619.2308, found 619.2295; <sup>1</sup> H-NMR (CDCl <sub>3</sub> ) of
244	tetraacetate of <b>18</b> , 3.04 (2H, dq, J=4.8 and 7.0 Hz, H1 and H7'), 2.61 (1H, dt, J=8.6 and
245	4.8 Hz, H2), 4.64 (1H, d, J=8.6 Hz, H3), 6.95 (2H, s, H4 and H2'), 7.11 (2H, s, H7 and
246	H5'), 1.32 (6H, d, J=7.0 Hz, 1-C <u>H</u> <sub>3</sub> and 7'-C <u>H</u> <sub>3</sub> ), 2.26 (6H, s, 5-, 6-, 3'-, or 4'-C <u>H</u> <sub>3</sub> CO),
247	2.28 (6H, s, 5-, 6-, 3'-, or 4'-C <u>H</u> <sub>3</sub> CO). Observed NOE from 1- and 7'-C <u>H</u> <sub>3</sub> to H2 and H3
248	in NOE difference spectra.
249	
250	Measurement of the xanthine oxidase inhibitory activity of the isolated compounds
251	The xanthine oxidase inhibition assay was performed according to a
252	previously reported protocol. $^{17}$ Briefly, a reaction medium consisting of 10 $\mu L$ of 1
253	mmol/L xanthine in DMSO, 10 $\mu L$ of the test sample (6 mg/mL for thermal products

and 4 mmol/L for isolated compounds) in DMSO, and 160 µL of 12.5 mmol/L

phosphate buffer (pH 7.4) was pre-incubated at 37 °C for 5 min. To this solu
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- of 0.027 unit/mL XO buffer solution was added. After incubation at 37 °C for 10 min,
- 257 3% HClO<sub>4</sub> aq (25 µL) was added to quench the reaction. An aliquot of the solution (20
- 258 μL) was injected into the HPLC column to quantify the uric acid production [Mightysil
- 259 RP-18 GP Aqua column (Kanto Chemical, Tokyo Japan), 250 mm × 4.6 mm i.d., 5 μm;
- flow rate, 1.0 mL/min; solvent, 2.5:97.5 methanol–0.1% phosphoric acid in water;
- detection at 290 nm; temperature, 35 °C]. The percent inhibition was calculated
- according to the following equation: inhibition (%) = [(peak area of uric acid in control)]
- 263 experiment) (peak area of uric acid in sample experiment)]  $\times$  100 / (peak area of uric
- acid in control experiment).

statistically significant.

265

#### 266 Statistical analysis

Activity data obtained from the experiments in triplicate are presented as mean  $\pm$ standard error (SE). The differences of the data between sample and control experiments were evaluated by the Dunnett's test. A p-value < 0.05 was considered

271

#### 272 RESULTS AND DISCUSSION

273

#### 274 HPLC analysis of the thermal reaction products of caffeic acid at various

#### 275 temperatures

In our previous work, we observed the XO inhibitory activity of a model 276277roasting reaction product of chlorogenic acid while intact chlorogenic acid did not inhibit XO activity. We also examined the XO inhibitory activity of the thermally 278treated caffeic and quinic acids, which are both components of chlorogenic acid.<sup>20</sup> The 279results revealed that the XO inhibitory activity of the thermal reaction product of caffeic 280acid was greater than that of chlorogenic acid, while no XO inhibitory activity was 281observed for the product of quinic acid. Therefore, in this investigation the thermal 282reaction of caffeic acid was further investigated for the production of XO inhibitors and 283enhanced XO inhibitory activity. The high-temperature thermal treatment of caffeic acid 284was carried out through a model roasting reaction according to a previously reported 285method [caffeic acid : solid support (a phosphoric salt)] =  $10:28.^{20}$  The XO inhibitory 286activities of the thermal treatment products at different temperatures are illustrated in 287288Fig. 1. Intact caffeic acid has weak XO inhibitory activity (22% inhibition) at the examined concentration (0.3 mg/mL), while the XO inhibitory activities of the thermal 289

290	reaction products were greater than that of caffeic acid. The activity increased with
291	increasing reaction temperature up to 170 °C, after which it decreased gradually up to
292	290 °C. Figure 2A (Figure S1A in supporting information) displays the HPLC data
293	representing the constituents of the thermal reaction products obtained by gradient
294	elution on an octadecylsilyl silica gel (ODS) analytical column with 5 to 100%
295	acetonitrile. At the reaction temperature 110 °C, the only noticeable peak was that of the
296	starting material at the retention time 15.5 min. When the temperature was increased to
297	140 and 170 °C, numerous peaks were observed in the retention time range of 19 to 42
298	min, along with the peak corresponding to caffeic acid (retention time 15.5 min), with
299	very complex peaks observed at 28-42 min. With a further increase in temperature to
300	200 °C, the caffeic acid peak and peaks at 19–22 min disappeared. Moreover, the
301	intensity of the complex peaks at 30–42 min were lower than those observed at 170 °C.
302	On the other hand, a new peak appeared at the retention time 11.6 min for the reactions
303	at temperatures of 200 °C or greater. At temperatures over 230 °C, almost all peaks
304	gradually disappeared, except for that of the major peak at 11.6 min and a minor peak at
305	27.0 min. Figure 2B (Figure S1B in supporting information) presents GPC data of the
306	acetylated products from the thermal reactions of caffeic acid. Under the employed GPC
307	conditions, acetylated caffeic acid (0.26 kDa), which was obtained from caffeic acid by

308	treatment with acetic anhydride and pyridine, was detected at the retention time 10.8
309	min. The area of the earlier eluting peak increased with increasing reaction temperature
310	up to 200 °C. This peak was clearly observed at 10.1 min (corresponding to 0.7 kDa:
311	calculated from the top of the peak) for the reaction at 170 °C and broadened to the
312	retention time 9 min at 200 °C, which indicates that higher weight molecules (up to ca.
313	0.3 kDa) were produced by the elevation of the reaction temperature. In the GPC data of
314	the reactions at 230 °C or above, the peak was further broadened between the retention
315	times 8.5 and 12 min, while two new peaks at retention times 11.1 and 11.5 min were
316	also observed, which indicates that low-molecular-weight degradation products (below
317	0.3 kDa) were produced along with higher weight compounds (up to ca. 6 kDa).
318	Considering the data of constituent analysis (Fig. 2A and S1A), molecular size analysis
319	(Fig. 2 B and S1B), and XO inhibitory activities of the products from the reactions at
320	various temperatures (Fig. 1), it was determined that the XO inhibitory activity of
321	moderate-molecular-weight compounds (3-0.3 kDa by GPC analysis), which may be
322	coupling products of caffeic acid, should exhibit stronger than that of caffeic acid.
323	Additionally, these compounds might contribute towards the enhanced XO inhibitory
324	activity of the products obtained at 170 °C. On the other hand, higher molecular weight
325	products, produced in the reactions at above 200 °C and eluted earlier from the GPC

326	column, might exhibit weak XO inhibition. Finally, the low-molecular-weight products,
327	observed as peaks eluted later from the GPC column of the reactions at high
328	temperatures, may also present weak XO inhibitory activity. Thus, the accumulation of
329	both the higher and lower molecular weight products should reduce the XO inhibitory
330	activity of the reaction products produced at above 200 °C. From the constituent
331	analysis data of the products obtained at various temperatures (Fig. 2A and S1A),
332	moderate-molecular-weight products, which are predicted to be strong XO inhibitors,
333	should correspond to the peaks in the retention time range of 19 to 42 min in the HPLC
334	data of the reactions at 140–200 °C.
335	
336	Isolation and structure identification of the constituent compounds obtained from
337	thermal reactions at 140–200 °C
338	To identify the compounds that correspond to the peaks observed in the HPLC
339	constituent analysis of the products obtained at 140-200 °C, isolation and structure
340	identification of the constituents of the thermal reaction products were carried out.
341	Multiple large scale thermal reactions of caffeic acid were performed at 140 and 200 °C;
342	in total, 18 compounds were purified from the reaction products as described in the
343	Materials and Methods section. The isolated compounds 1, 2, 3, 4, 7, and 8 were

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344	identical to the previously identified phenylindanes according to their NMR and MS
345	data (Fig. 3.) <sup>20</sup> Compounds <b>15</b> and <b>16</b> were determined to be other phenylindane
346	compounds that had been previously identified by Frank et al. <sup>13</sup> Compounds 11 and 12
347	were determined to be catechol and 4-ethylcatechol, which are reportedly found in
348	roasted coffee. <sup>21</sup> The structures of compounds 5, 6, and 14 were also identified as shown
349	in Fig. 3 by comparing the NMR and MS data to previously reported data. <sup>22–24</sup> Notably,
350	compounds 9, 10, 13, 17, and 18 were compounds newly identified in this work and
351	their structures were elucidated by NMR and MS analyses.
352	High resolution mass spectrometry (HR-MS) data of compounds 9 and 10
353	indicated that they were constitutional isomers with the molecular formula $C_{24}H_{24}O_6$ (9:
354	m/z 409.1656 [M+H] <sup>+</sup> and 10: m/z 409.1652 [M+H] <sup>+</sup> ). The <sup>1</sup> H-NMR spectra of 9 and
355	10 were also very similar, indicating that they were stereoisomers as summarized in
356	Table S1 (supporting information). The <sup>1</sup> H-NMR spectrum of <b>9</b> revealed seven aromatic
357	proton signals at 6.60, 6.67, and 6.72 ppm in the same coupling system and at 6.19,
358	6.20, 6.66, and 6.77 ppm as singlet shape. The former peaks indicated the existence of a
359	tri-substituted benzene ring with protons arranged in a 1,2,4 configuration while the
360	latter peaks revealed the presence of two tetra-substituted benzene rings with protons
361	oriented para to one other; these systems were derived from the aromatic moiety of

362	caffeic acid. The HH-COSY data of $9$ clarified two coupling systems, consisting of $CH_3$
363	(δ 1.21, 1-CH <sub>3</sub> )–CH (δ 3.19, H1)–CH <sub>2</sub> (δ 2.05, H2)–CH (δ 4.49, H3), and CH <sub>3</sub> (δ 1.56,
364	1"-CH <sub>3</sub> )–CH ( $\delta$ 4.29, H1"). The HMBC spectrum of <b>9</b> showed correlations from C1a to
365	H1, from C3a to H3, from C1 to H7, from C3 to H4 and H2', from C4 to H3, from C7 to
366	H1, and from C1' to H3 as shown in Fig. 4, indicating the existence of a phenylindane
367	structure consisting of two tetra-substituted benzene rings and the CH <sub>3</sub> -CH-CH <sub>2</sub> -CH
368	system. Other C-H long-range correlations were observed from C1" to H5', H3", H7",
369	and 1"-CH <sub>3</sub> ; from C2" to H1" and 1"-CH <sub>3</sub> ; from C7" to H1"; and from 1"-CH <sub>3</sub> to H1"
370	between the CH <sub>3</sub> -CH system and a tri-substituted benzene. Correlations were also
371	observed from C1' to H1"; and C6' to H1" and 1"-CH <sub>3</sub> between the CH <sub>3</sub> -CH system and
372	one of the tetra-substituted benzenes as shown in Fig. 4. These results indicated that an
373	ethylcatechol structure was attached to the 6'-position of the tetra-substituted benzene at
374	the 3-position of the indane sub-structure. The stereochemistry of the three chiral
375	carbons was deduced from the observed NOE correlations in the NOESY spectrum of 9.
376	Strong correlations between H1 and H2' and between H3 and 1-CH <sub>3</sub> revealed the <i>trans</i> -
377	relationship of the methyl group at the 1-position and the phenyl group at the 3-position.
378	The stereochemistry of C1" could not be deduced from its NMR data due to its flexible
379	structure. Thus the structure of <b>9</b> was determined as depicted in Fig. 3.

380	The <sup>1</sup> H and <sup>13</sup> C NMR data, including correlations in the COSY and HMBC, of
381	10 were very similar to those of 9 (Table S1 in supporting information and Fig. 4),
382	indicating that the planar structure of 10 was the same as that of 9. Differences in the
383	stereochemistry of 10 were deduced by NOESY. The observed NOE between H1 and
384	H3 in the NOESY spectrum of 10 revealed that the methyl group at the 1-position and
385	the phenyl group at the 3-position were <i>cis</i> relative to one another. Similar to 9, the
386	stereochemistry at the 1"-position could not be elucidated; thus, the structure of
387	compound 10 was determined as depicted in Fig. 3.
388	The HR-MS data of compound 13 revealed a molecular-related ion at $m/z$
389	269.0816 (Calcd for $C_{14}H_{14}O_4Na$ , 269.0790), indicating the molecular formula
390	$C_{14}H_{14}O_4$ , which is the same as that of compound <b>6</b> . The NMR data of the tetra-
391	acetylated derivative (13a) of 13 showed the presence of a $CH_3$ -CH unit [4.25 (1H, q, J
392	= 7.0 Hz), 1.57 (3H, d, J = 7.0 Hz)], and six aromatic protons, which were very similar
393	to those of <b>6</b> . A signal for an aromatic proton having two <i>ortho</i> -couplings [7.23 (1H, dd,
394	J = 8.6 and $8.2$ Hz)] and signals of four phenolic acetyl groups indicated that 13 was a
395	regioisomer of <b>6</b> in reference to the attached position of the CH <sub>3</sub> –CH unit as shown in
396	Fig. 3.

The HR-MS data of compounds 17 and 18 indicated that both molecular

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398	formulas were the same $C_{18}H_{18}O_{14}$ (17: m/z 321.1137 [M+Na] <sup>+</sup> , 18: m/z 321.1133
399	$[M+Na]^+$ , both calculated for $C_{18}H_{18}O_4Na$ , 321.1103). The <sup>1</sup> H-NMR spectrum of <b>17a</b> ,
400	which was obtained from 17 by reaction with acetic anhydride in pyridine, showed four
401	signals for phenolic acetyl groups at 2.25 (3H, s $\times$ 2), 2.27 (3H, s), and 2.30 (3H, s) ppm
402	and four singlet aromatic proton signals at 6.90, 6.92, 7.00 and 7.15 ppm, indicating the
403	presence of two sets of 1,2,4,5-tetra-substituted benzene rings derived from two
404	molecules of caffeic acid. The HH-COSY of $17a$ revealed the presence of a CH <sub>3</sub> (1.35
405	ppm)–CH (2.81 ppm)–CH (2.55 ppm)–CH <sub>2</sub> (2.82 and 3.23 ppm) coupling system,
406	which was adjacent to the aromatic rings. Another singlet signal (1.54 ppm) due to a
407	methyl group adjacent to a quaternary carbon was also observed. These data indicated
408	that 17 comprosed a bicyclo[3.3.0]octane structure appended with two
409	dihydroxybenzene rings and two methyl groups. The stereochemistry around the
410	bicylooctane rings was deduced by NOE correlations from H2 to the 1- and 3-methyl
411	protons in the NOESY spectrum of 17a, revealing the presence of <i>cis</i> relations. Thus,
412	the structure of 17 was determined as depicted in Fig. 3.
413	Although 17 and 18 presented the same molecular-related peak in their MS
414	data, the <sup>1</sup> H NMR data of <b>18a</b> (acetylated derivative of <b>18</b> ) were much simpler than
415	those of 17a, indicating that 18 was a symmetrical isomer of 17. The HH-COSY of 18a

416	indicated the presence of a symmetrical coupling system, [CH <sub>3</sub> (1.32 ppm)–CH (3.04
417	ppm)] <sub>2</sub> CH (2.61 ppm)CH (4.64 ppm), which was attached to two tetra-substituted
418	benzene rings. In addition to the above data, proton signals due to four phenolic acetyl
419	groups [2.26 (3H $\times$ 2) and 2.28 (3H $\times$ 2) ppm] and two sets of <i>para</i> -substituted aromatic
420	protons [6.95 (1H $\times$ 2) and 7.11 (1H $\times$ 2)] indicated that 18 had a symmetrical
421	bicyclo[3.3.0]octane structure similar to that of 17. NOEs were observed from 1- and 7'-
422	$CH_3$ to H2 and H3 in the NOE difference spectra of <b>18</b> . Thus, the structure of <b>18</b> was
423	determined as depicted in Fig. 3.
424	
425	Structure-based classification of the thermal reaction products
425 426	<b>Structure-based classification of the thermal reaction products</b> The high-temperature treatment of caffeic acid stimulates its decarboxylation to
425 426 427	Structure-based classification of the thermal reaction products The high-temperature treatment of caffeic acid stimulates its decarboxylation to afford 4-vinylcatechol, which is significantly more reactive than the parent caffeic
<ul><li>425</li><li>426</li><li>427</li><li>428</li></ul>	Structure-based classification of the thermal reaction products The high-temperature treatment of caffeic acid stimulates its decarboxylation to afford 4-vinylcatechol, which is significantly more reactive than the parent caffeic acid. <sup>25</sup> Therefore, 4-vinylcatechol presents an important intermediate of the thermally
<ul> <li>425</li> <li>426</li> <li>427</li> <li>428</li> <li>429</li> </ul>	Structure-based classification of the thermal reaction products The high-temperature treatment of caffeic acid stimulates its decarboxylation to afford 4-vinylcatechol, which is significantly more reactive than the parent caffeic acid. <sup>25</sup> Therefore, 4-vinylcatechol presents an important intermediate of the thermally mediated reactions of caffeic acid. By considering the structural differences of the
<ul> <li>425</li> <li>426</li> <li>427</li> <li>428</li> <li>429</li> <li>430</li> </ul>	Structure-based classification of the thermal reaction products The high-temperature treatment of caffeic acid stimulates its decarboxylation to afford 4-vinylcatechol, which is significantly more reactive than the parent caffeic acid. <sup>25</sup> Therefore, 4-vinylcatechol presents an important intermediate of the thermally mediated reactions of caffeic acid. By considering the structural differences of the constituent compounds obtained from the thermal reactions, the identified compounds
<ul> <li>425</li> <li>426</li> <li>427</li> <li>428</li> <li>429</li> <li>430</li> <li>431</li> </ul>	Structure-based classification of the thermal reaction products The high-temperature treatment of caffeic acid stimulates its decarboxylation to afford 4-vinylcatechol, which is significantly more reactive than the parent caffeic acid. <sup>25</sup> Therefore, 4-vinylcatechol presents an important intermediate of the thermally mediated reactions of caffeic acid. By considering the structural differences of the constituent compounds obtained from the thermal reactions, the identified compounds were classified into three categories based on their vinylcatechol substructure: category
<ul> <li>425</li> <li>426</li> <li>427</li> <li>428</li> <li>429</li> <li>430</li> <li>431</li> <li>432</li> </ul>	Structure-based classification of the thermal reaction products The high-temperature treatment of caffeic acid stimulates its decarboxylation to afford 4-vinylcatechol, which is significantly more reactive than the parent caffeic acid. <sup>25</sup> Therefore, 4-vinylcatechol presents an important intermediate of the thermally mediated reactions of caffeic acid. By considering the structural differences of the constituent compounds obtained from the thermal reactions, the identified compounds were classified into three categories based on their vinylcatechol substructure: category I (compounds 1–4) consists of the coupling products of vinylcatechol and caffeic acid,

434	two or three vinylcatechols, and category III (compounds 6, 13, 14, 17, and 18) is
435	composed of compounds formed by coupling with or by elimination of the catechol
436	moiety (Fig. 3). All the categorized compounds were assigned to the observed peaks in
437	the HPLC chromatograms from the reactions at 140, 170, and 200 °C as shown in Fig. 5.
438	The category I compounds were observed in the 140 °C reaction product along with
439	caffeic acid and the category II compounds. The HPLC data of the 170 °C reaction
440	product indicated a decrease in the intensities of caffeic acid and the category I
441	compounds. Additionally, the region representing the category II compounds (retention
442	time 30-40 min) became more complex compared to the same region in the
443	chromatogram of the 140 °C reaction product. This indicated that at the reaction
444	temperature 170 °C, various vinylcatechol oligomers were additionally produced instead
445	of the caffeic acid-vinylcatechol compounds. Through consideration of the GPC
446	analytical data of the 170 °C reaction product (Fig. 2 B), it was determined that the
447	oligomerization degree of the newly produced compounds might be higher than that of
448	the compounds observed in the 140 °C reaction product. At the reaction temperature
449	200 °C the category I compounds disappeared, while the category III compounds were
450	observed in addition to the vinylcatechol oligomers (category II).
451	

# 452 XO inhibitory activities of the oligomeric compounds from the thermal reactions of 453 caffeic acid

454	Figure 6 illustrates the XO inhibitory activities of the isolated constituents (200
455	$\mu$ mol/L) of the thermal reaction products. Although the starting material, caffeic acid,
456	showed very weak activity at the examined concentration, the isolated compounds
457	exerted stronger activities in the order 3 > 4 > 8, 10, 17 > 7, 9, 2, 5, 1, 6 > 16, 15, 14,
458	and 13, based on their percent inhibition values. The compounds with the highest
459	inhibition levels, caffeic acid-vinylcatechol compounds 3 and 4, were detected in the
460	products of the 140 and 170 °C reactions but were absent in the product of the 200 °C
461	reaction. There were followed by compounds 7, 8, (vinylcatechol dimers) and 10
462	(trimer), which were mainly observed in the products of the reactions at 140 and 170 °C.
463	Conversely, the weakest inhibitory compounds 13 and 14, catechol-eliminated
464	compounds belonging to category III, were both observed solely as the constituents of
465	the product of the 200 °C reaction.
466	A comparison of the results of the XO inhibitory activity of the identified
467	compounds with those of the thermal reaction products (110-290 °C, Fig. 1) indicated
468	that caffeic acid was initially converted to caffeic acid-vinylcatechol compounds, some
469	of which contributed to the enhanced inhibitory activity of the products produced at

470	170 °C or less. Subsequently, caffeic acid was converted to the moderately active
471	vinylcatechol oligomers followed by their transformation to weakly active catechol-
472	reacted compounds, the production of which decreases the XO inhibitory activity of the
473	thermal reaction products produced at the temperatures above 170 °C. Although not all
474	the produced constituents of the thermal reaction products could be identified due to
475	their complexity, the observed structural differences of the constituents might induce
476	the enhanced XO inhibitory activity of thermal reaction products. Notably, the thermal
477	treatment at 170 °C, which exhibited the maximum XO inhibitory activity, coincides
478	with the general roasting conditions of food processing. <sup>26</sup> Caffeic acid and its
479	derivatives including chlorogenic acid, are widely distributed in plant-derived
480	foodstuffs. <sup>27</sup> Therefore, the roasting processing of foodstuffs might present significant
481	XO inhibitory activity by the thermal reaction of caffeic acid derivatives. In terms of
482	coffee beverages, we previously reported that only roasted coffee beans exhibit XO
483	inhibitory activity, <sup>16</sup> which enhances the ability of coffee to prevent gout. <sup>28</sup> Frank et
484	al. <sup>13</sup> reported the existence of various vinylcatechol oligomers in roasted coffee, as
485	bitter-tasting compounds, by using LC-MS techniques. Although pyrogallol and
486	chlorogenic lactones have been identified as significant XO inhibitors present in roasted
487	coffee beans, <sup>16,17</sup> vinylcatechol oligomers and related compounds might also play a role

488	in the XO inhibitory activity. Differences in the inhibitory ability due to the different
489	reaction temperatures should also be considered when determining the conditions of the
490	roasting process of raw coffee beans. Optimized roasting conditions should be useful for
491	the production of health-functional coffee beverages that exhibit XO inhibitory activity.
492	Although the bioavailability of the identified phenylindanes and vinylcatechol
493	oligomers has not been examined to date, the ability of caffeic acid and the related
494	phenolic acids in coffee has been well studied. <sup>29,30</sup> These compounds can be absorbed in
495	humans in their conjugated and non-conjugated forms. Ota et al. <sup>31</sup> examined the
496	interaction of these phenolic acids and corresponding vinylcatechol derivatives with
497	lipid membranes. The results revealed that the more non-polar vinylcatechols displayed
498	stronger interactions with the membrane, which were related to their bioavailability.
499	The identified thermal reaction products are also non-polar substances compared to
500	caffeic acid; therefore, higher bioavailability is expected for the product constituents.
501	When the non-polar thermal reaction products of caffeic acid are absorbed by humans,
502	they might exhibit characteristic functions in the human body. XO is localized in the
503	liver and intestine and produces uric acid, <sup>32,33</sup> the over accumulation of which is
504	responsible for the development of gout. <sup>28</sup> The absorbed thermal products might play a
505	role in inhibiting XO from overworking when reaching liver or intestine, thereby

- 506 preventing the development of gout.
- 507
- 508 **Financial support**: JSPS Kakenhi (Grant No. 15H02892 for T. M.) and a collaborative
- 509 research fund between Ajinomoto-AGF (Tokyo, Japan) and Osaka City University.

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603	Figure Captions
604	
605	Figure 1. Xanthine oxidase inhibitory activities of the thermal reaction products
606	(0.3 mg/mL) of caffeic acid.
607	Data are expressed as the mean $\pm$ SE (n = 3).
608	
609	Figure 2. Selected HPLC analytical data of various thermal reaction products of
610	caffeic acid.
611	(A) Chromatograms obtained using an octadecylsilyl silicagel (ODS) column; (B)
612	chromatograms obtained using a gel permeation chromatography (GPC) column.
613	
614	Figure 3. Chemical structures of the identified constituent compounds of the
615	thermal reaction products of caffeic acid.
616	Tentative positional numbering is based on the numbering systems of indane or
617	substituted benzene rings.
618	
619	Figure 4. Selected HMBC and NOESY correlations observed in compounds 9 and
620	10.

621	Blue arrows, long-range C-H coupling and red double-headed arrows, NOE.
622	
623	Figure 5. Peak assignment of the identified constituent compounds in the HPLC
624	profiles of the thermal reaction products at 140, 170, and 200 °C.
625	Blue numbers, category I compounds (caffeic acid-vinylcatechol coupled compunds);
626	red numbers, category II compounds (vinylcatechol oligomers); and green numbers,
627	category III compounds.
628	
629	Figure 6. Xanthine oxidase inhibitory activities of identified compounds 1–18 and
630	caffeic acid (200 μmol/L).
631	Data are expressed as the mean $\pm$ SE (n = 3). *Significant differences (P < 0.05)
631 632	Data are expressed as the mean $\pm$ SE (n = 3). *Significant differences (P < 0.05) compared to of caffeic acid (Dunnett's test). Allopurinol (0.5 $\mu$ mol/L), a positive
631 632 633	Data are expressed as the mean $\pm$ SE (n = 3). *Significant differences (P < 0.05) compared to of caffeic acid (Dunnett's test). Allopurinol (0.5 µmol/L), a positive control sample, showed 52.3% inhibition under the same conditions.
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640 a GPC column.





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Figure 3. Chemical structures of the identified constituent compounds of the thermal reaction products of caffeic acid. Tentative positional numbering is based on the numbering systems of indane or substituted benzene rings.

246x150mm (300 x 300 DPI)



Figure 4. Selected HMBC and NOESY correlations observed in compounds 9 and 10. Blue arrows, long-range C–H coupling and red double-headed arrows, NOE.

126x50mm (300 x 300 DPI)



