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

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S Supporting Information

ABSTRACT: The high-temperature treatment of caffeic acid by a model reaction for the processing of foods by roasting enhanced its xanthine oxidase (XO) inhibitory activity. The thermal reaction products included various oligomeric compounds, whose structures were determined as being produced via the intermediate 4-vinylcatechol. Measurements of their XO inhibitory activities were also carried out. Among the identified oligomers, the coupling products of caffeic acid and vinylcatechol, which were mainly produced at 140–170 °C, presented stronger XO inhibitory activities than the other types of oligomers produced. Further reacted compounds, which were mainly formed at 200 °C by the addition or elimination of catechol unit in the oligomers, displayed weaker activities. These results indicated that thermal enhancement of the XO inhibitory activity of caffeic acid can be explained by the differences in the XO inhibitory activities of the various constituents of the thermal reaction products. Caffeic acid and its derivatives are polyphenols found widely distributed in foods. Moreover, XO inhibition is closely related to the prevention of the life-style-related disease gout. The results suggest that a simple roasting process (170 °C) can lend useful human-health-related functionalities to caffeic acid containing foods such as coffee.

KEYWORDS: thermal reaction, caffeic acid, xanthine oxidase inhibition, roasting temperature, structure identification

INTRODUCTION

Roasting is a high-temperature cooking method (130–230 °C),¹ that is applied to grains, vegetables, meats, and fish in both industrial food processing and home cooking.² Roasting can cause various thermal reactions of the food's chemical constituents, thereby developing the flavors and color.^{1,3} Caffeic acid is a polyphenol that is widely distributed in plant foods in its free or bound forms. Coffee beans contain free caffeic acid as well as a large amount of chlorogenic acid, a major caffeic acid derivative.^{4,5} The roasting of coffee beans at approximately 200 °C is indispensable for making coffee beverages. During the roasting process, caffeic acid derivatives are degraded into various compounds, including disassembled caffeic acid.^{6,7} The degraded products react with each other to afford more complex substances,⁸ which affect the flavor and taste as well as the human health functions of roasted coffee beverages.⁹ To date, many thermal reaction products have been identified in roasted coffee beans, including volatile flavoring compounds,¹⁰ Maillard reaction products,¹¹ bitter-tasting chlorogenic lactones,¹² and phenylindane derivatives.¹³ Recently, several epidemiological studies by Choi et al.¹⁴ and other groups¹⁵ have reported that coffee drinking reduces the risk of gout. Additionally, we recently discovered that roasted coffee bean extract exhibits xanthine oxidase (XO) inhibitory activity.¹⁶ We also reported the presence of several inhibitors in roasted coffee beans.¹⁷ XO is a key enzyme in the catabolism of purine to uric acid, the substance that causes gout when it overaccumulates in plasma.¹⁸ Stadler et al.¹⁹ were the first to

report that phenylindanes were produced from the thermal treatment of caffeic acid via a vinylcatechol intermediate. These compounds were observed in roasted coffee beans and play a role in the bitter taste of coffee.¹³ We also reported moderate XO inhibitory activities for some phenylindanes.²⁰

In this investigation, we found maximum XO inhibitory activity for the caffeic acid products of thermal treatment at around 170 °C. These conditions were used as a model reaction of the roasting process, and notably, differences in the thermal reaction products were found between 110 and 290 °C. Herein, we report the structures of the constituent compounds of the thermal reaction products (140–200 °C) and their XO inhibitory activities.

MATERIALS AND METHODS

Chemicals and Instruments. Caffeic acid was obtained from Kanto Chemicals (Tokyo, Japan). Xanthine oxidase (XO) was purchased from Wako Pure Chemicals (Osaka, Japan). Allopurinol was obtained from Sigma-Aldrich (St. Louis, MO). All other reagents and solvents were purchased from Nacalai Tesque (Kyoto, Japan). NMR spectra were obtained from a JNM-ECZ400S (400 MHz, JEOL, Tokyo, Japan) or ARX600 (600 MHz, Bruker Japan, 70 Yokohama, Japan) instrument. MS spectra were measured using a JMS-T100 spectrometer (JEOL) with direct analysis at real time

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(DART) or electrospray ionization (ESI) and time-of-flight (TOF) measuring mode under the following conditions: DART (positive mode): orifice 1, 10 V; orifice 2, 5 V; ring lens, 10 V; helium gas pressure, 0.5 MPa; flow rate, 10 L/min; ion resource temperature, 350 °C; grid voltage, 400 V. ESI (positive mode): orifice 1, 85 V and 80 °C; orifice 2, 5 V; ring lens, 15 V; helium gas pressure, 0.7 MPa; needle, 2000 V; desolvating chamber temperature, 250 °C. Elemental compositions of compounds were calculated using MassLynx 81 software, version 4.1 (Waters Japan, Tokyo, Japan) from high-resolution MS (HR-MS) data. Analytical HPLC was performed using 82 a PU-4180 RHPLC quaternary gradient pump (JASCO, Tokyo, Japan) equipped with an MD-4015 photodiode array detector 85 (JASCO) and a CTO-10AS column oven (Shimadzu, Kyoto, Japan). Data were analyzed using the ChromNAV software (v. 2, 87 JASCO). Preparative HPLC was performed using an LC-6AD pump 88 (Shimadzu) equipped with an SPD-6A UV detector (Shimadzu).

Thermal Reaction of Caffeic Acid and HPLC Analysis of the Reaction Products. In a screw-capped test tube (i.d. of 8 mm and 91 length of 100 mm) were placed 10 mg of caffeic acid in methanol 92 (200 μ L) and 400 μ L of phosphate buffer (500 mmol/L, pH 6.0, 93 Na_2HPO_4 , and KH_2PO_4). After removal of the solvent in vacuo, the 94 tube was heated in a metal block bath (MB-1H-UII, KPI, Itami, 95 Japan) at the appropriate temperatures (110–290 °C) for 0.5 h. After 96 the tube cooled, methanol (1 mL) was added, and the mixture was 97 centrifuged at 2000 rpm for 5 min at 25 °C. Subsequently, the 98 supernatant was collected, and the solvent was removed by 99 evaporation to give the thermal reaction products. A portion of the 100 reaction products was used for the XO inhibitory assay. The 101 constituents in the products were analyzed by HPLC (Cosmosil 102 SC18-AR-II column, Nacalai, Kyoto, Japan; 250 $\times$ 4.6 mm i.d., 5 μ m; 103 flow rate, 1.0 mL/min; solvent A, 1% acetic acid in H_2O ; solvent B, 104 CH_3CN ; linear gradient from 5% solvent B to 45% solvent B for 40 105 min, then from 45% solvent B to 100% solvent B for 10 min, and then 106 100% solvent B for 5 min; and detection at 280 and 320 nm).

Gel Permeation Chromatography (GPC) Analysis of the Thermal Reaction Products. The thermal reaction products 109 (weight corresponding to 6 mg of initial caffeic acid) were treated 110 with acetic anhydride (0.5 mL) and pyridine (0.5 mL) at 25 °C for 1 111 h. After removal of acetic acid and pyridine in vacuo, the residue was 112 dissolved in THF to produce an analytical solution (0.4 mg/mL, 113 corresponding to the initial caffeic acid concentration). The solution 114 (15 μ L) was injected into a GPC column (GPC KF-803, 300 $\times$ 8 mm 115 i.d., Shodex, Tokyo, Japan) for analysis (solvent, HPLC-grade THF; 116 flow rate, 1.0 mL/min; temperature, 30 °C; and detection at 264 nm). 117 Molecular size calibration was performed under the same conditions 118 using polystyrene standards (266, 2280, 6540, and 25 500 Da 119 polystyrene; Ready Cal-Kit Poly(styrene) Low, Sigma-Aldrich, St. 120 Louis, MO).

Isolation of the Thermal Reaction Product Constituents from the Large-Scale Thermal Reactions of Caffeic Acid at 140–200 °C. For thermal treatment at 140 °C, caffeic acid (10 g) 124 was dissolved in 100 mL of methanol and 400 mL of 500 mmol/L 125 Na_2HPO_4 – KH_2PO_4 (pH 6.0) and subsequently evaporated to 126 dryness. The solid residue was heated at 140 °C for 90 min. After 127 cooling, the reaction mixture was extracted twice with 1 L of 128 methanol, and this procedure was repeated 10 times. The methanol 129 extracts were combined, and the solvent was removed. The resultant 130 residue was further purified to isolate the constituent compounds. A 131 portion (84 g) of the residue was subjected to Amberlite XAD-7 132 (Organo, Tokyo, Japan) column chromatography and eluted with 133 increasing concentrations of methanol (50 to 100%) in water to 134 produce eight separate fractions. Fraction 3 (208 mg of 6.5 g), which 135 was eluted with 60% methanol in water, was purified by preparative 136 HPLC (Cosmosil SC18-AR-II column, 250 $\times$ 20 mm i.d.; solvent, 1% 137 acetic acid in 85:15 H_2O – CH_3CN ; flow rate, 9.6 mL/min; and 138 detection at 280 nm) to yield compounds 1–4 in 2, 6, 3, and 3 mg 139 yields, respectively. Fraction 6 (14.3 g), which was eluted with 75% 140 methanol in water from the XAD-7 column, was subjected to 141 Sephadex LH-20 (GE Healthcare, Hino, Japan) column chromatog- 142 raphy (1.5 L column) and eluted with methanol to produce 5 g of

residue, which was collected from 1.5 to 1.65 L of total elution 143 volume. A part of the residue (656 mg) was purified by preparative 144 HPLC (Cosmosil SC18-AR-II column, 250 $\times$ 20 mm i.d.; solvent, 1% 145 acetic acid in 75:25 H_2O – CH_3CN ; flow rate, 9.6 mL/min; and 146 detection at 280 nm) to produce compounds 5–8 in 5, 4, 87, and 134 147 mg yields, respectively, along with 52 mg of a mixture of compounds 9 148 and 10. This mixture was separated by HPLC in recycle mode (11 149 times, under the following conditions: Cosmosil SC18-AR-II column, 150 250 $\times$ 20 mm i.d.; solvent, 1% acetic acid in 45:55 H_2O – CH_3OH ; 151 flow rate, 9.6 mL/min; and detection at 280 nm) to produce pure 9 152 and 10 in 5 mg yields. 153

For thermal treatment at 200 °C, 40 mL of phosphate buffer (pH 154 6.0, 0.5 mol/L) was added to caffeic acid (1 g) in methanol (20 mL) 155 and mixed well. After removal of the solvent from the mixture in 156 vacuo, the residual solid was heated at 200 °C for 30 min. The 157 resulting dark-colored residue was extracted twice with methanol (50 158 mL) to produce 0.76 g of extract after evaporation. The extract was 159 fractionated by XAD-7 column chromatography eluting with 160 increasing concentrations of methanol (10 to 100%) in water. Two 161 fractions, eluted with 50 and 75% methanol, were evaporated to 162 produce fractions I and II in 74 and 86 mg yields, respectively. A 163 portion (32 mg) of the residue of fraction I was purified by HPLC 164 (Cosmosil SC18-AR-II column, 250 $\times$ 20 mm i.d.; solvent, 1% acetic 165 acid in 90:10 H_2O – CH_3CN ; flow rate, 9.6 mL/min; and detection at 166 280 nm) to give compound 11 in a 1 mg yield. Likewise, the residue 167 of fraction II (37 mg) was separated by HPLC (Cosmosil SC18-AR-II 168 column, 250 $\times$ 20 mm i.d.; solvent, 1% acetic acid in 70:30 H_2O – 169 CH_3CN ; flow rate, 9.6 mL/min; and detection at 280 nm) to produce 170 compounds 5–8 and 12 in 1, 5, 1, 2, and 2 mg yields, respectively. 171 Another reaction product (570 mg), which was obtained from the 172 thermal treatment of caffeic acid (1 g) under the same conditions, was 173 fractionated by XAD-7 column chromatography using the same 174 protocol as described above. The fraction eluted with 90% methanol 175 (195 mg) was separated by HPLC (Cosmosil SC18-AR-II column, 176 250 $\times$ 20 mm i.d.; solvent, 1% acetic acid in 45:55 H_2O – CH_3CN ; 177 flow rate, 9.6 mL/min; and detection at 280 nm) to produce 178 compounds 13, 14, and 16–18 in 5, 4, 3, 2, and 2 mg yields, 179 respectively. The product (17.1 g) of a third thermal reaction of 180 caffeic acid (20 g) was also fractionated into five fractions by XAD-7 181 column chromatography under the same conditions. The fraction 182 eluted with 70% methanol (2 g) produced four subsequent fractions 183 (4-1 to 4-4) in 523, 61, 80, and 47 mg yields, respectively (retention 184 times: fraction 4-1, 9–24 min; fraction 4-2, 27 min; fraction 4-3, 33 185 min; and fraction 4-4, 40 min) under the following conditions: 186 Cosmosil SC18-AR-II column, 250 $\times$ 20 mm i.d.; solvent, 1% acetic 187 acid in 70:30 H_2O – CH_3CN ; flow rate, 9.6 mL/min; and detection at 188 280 nm. Compound 13 (15 mg) was isolated from fraction 4-1 (487 189 mg) by HPLC with a retention time of 41 min (Cosmosil SC18-AR-II 190 column, 250 $\times$ 20 mm i.d.; solvent, 1% acetic acid in 60:40 H_2O – 191 CH_3OH ; flow rate, 6.7 mL/min; and detection at 280 nm). 192 Compound 15 (7 mg) was isolated from fraction 4-2 (20 mg) by 193 HPLC with a retention time of 37 min (Cosmosil SC18-AR-II 194 column, 250 $\times$ 20 mm i.d.; solvent, 1% acetic acid in 45:55 H_2O – 195 CH_3OH ; flow rate, 4.8 mL/min; and detection at 280 nm). 196 Compounds 14 (10 mg) and 17 (14 mg) were obtained from 197 fraction 4-3 (70 mg) by HPLC with retention times of 43 and 45 min, 198 respectively (Cosmosil SC18-AR-II column, 250 $\times$ 20 mm i.d.; 199 solvent, 1% acetic acid in 45:55 H_2O – CH_3OH ; flow rate, 4.8 mL/ 200 min; and detection at 280 nm). Finally, compound 18 (6 mg) was 201 isolated from fraction 4-4 (33 mg) by HPLC with a retention time of 202 50 min (Cosmosil SC18-AR-II column, 250 $\times$ 20 mm i.d.; solvent, 1% 203 acetic acid in 45:55 H_2O – CH_3OH ; flow rate, 4.8 mL/min; and 204 detection at 280 nm). 205

Analytical Data for the Structures of Compounds 5, 6, and 11–18. Chemical structures of the isolated products, except for the 207 previously identified phenylindanes (compounds 1–4, 7, and 8), were 208 elucidated by using the NMR and MS data on their intact or 209 acetylated forms, which were derived by acetylation with acetic 210 anhydride in pyridine. The data are listed as follows, and position 211

numbering of each compound is tentative, as shown in the structures in Figure 3.

Compound 5. HR-ESIMS (m/z) $[M + Na]^+$ calcd for $C_{16}H_{16}O_4Na$ 295.0946, found 298.0969, and $[2M + Na]^+$ calcd for $C_{32}H_{32}O_8Na$ 567.1995, found 567.2003; 1H NMR (CD_3OD) δ 6.69 (4H, s, H3, H6, H3', and H6'), 3.75 (2H, q, $J = 7.4$ Hz, H7 and H7'), 2.18 and 1.43 (6H, d, $J = 7.4$ Hz, H8 and H8').

Compound 6. HR-ESIMS (m/z) $[M + Na]^+$ calcd for $C_{14}H_{14}O_4Na$ 269.0790, found 269.0817, and $[2M + Na]^+$ calcd for $C_{28}H_{28}O_8Na$ 515.1682, found 515.1690; 1H NMR (CD_3OD) δ 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, $J = 8.2$ 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, H8).

Compound 9. HR-DARTMS (m/z) $[M + H]^+$ calcd for $C_{24}H_{25}O_6$ 409.1651, found 409.1656. For NMR results, see Table S1 in the Supporting Information.

Compound 10. HR-DARTMS (m/z) $[M + H]^+$ calcd for $C_{24}H_{25}O_6$ 409.1651, found 409.1652. For NMR results, see Table S1 in the Supporting Information.

Compound 11. DARTMS (m/z) $[M + H]^+$ 111; 1H NMR (acetone- d_6) δ 6.65 (2H, AA'BB' coupling, H3 and H6) and 6.79 (2H, AA'BB' coupling, H4 and H5).

Compound 12. DARTMS (m/z) $[M + H]^+$ 139; 1H NMR (CD_3OD) δ 6.65 (1H, d, $J = 1.9$ Hz, 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, $J = 7.4$ Hz, 4- CH_2 - CH_3), 1.20 (3H, t, $J = 7.4$ Hz, 4- CH_2 - CH_3).

Compound 13. HR-ESIMS $[M + Na]^+$ calcd for $C_{14}H_{14}O_4Na$ 269.0790, found 269.0816, and $[2M + Na]^+$ calcd for $C_{28}H_{28}O_8Na$ 515.1682, found 515.1667; 1H NMR ($CDCl_3$) of tetraacetate (13a) δ 6.99–7.03 (2H, complex, H3 and H5), 7.06–7.12 (3H, complex, H6, 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, $J = 8.6$ and 8.2 Hz, H5'), 2.18 (3H, s, 1-, 2-, 2', or 3'- CH_3 CO), 2.25 (3H, s, 1-, 2-, 2', or 3'- CH_3 CO), 2.26 (6H, s, 1-, 2-, 2', or 3'- CH_3 CO).

Compound 14. HR-DARTMS $[M + H]^+$ calcd for $C_{10}H_{13}O_2$ 165.0916, found 165.0914; 1H NMR ($CDCl_3$) of diacetate of 14, δ 3.17 (1H, sext, $J = 7.6$ Hz, H1), 1.64 (1H, dq, $J = 12.4$ 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), 6.99 (1H, s, H4 or H7), 6.95 (1H, s, H4 or H7), 1.26 (3H, d, $J = 7.6$ Hz, 1- CH_3), 2.28 (6H, s, 5- and 6- CH_3 CO).

Compound 15. HR-ESIMS $[M + Na]^+$ calcd for $C_{16}H_{16}O_4Na$ 295.0946, found 295.0986, and $[2M + Na]^+$ calcd for $C_{32}H_{32}O_8Na$ 567.1995, found 567.2003; 1H NMR (CD_3OD) δ 3.21 (1H, dq, $J = 8.8$ and 7.0 Hz, H1), 1.99–2.08 (1H, m, H2a), 2.20 (1H, ddd, $J = 12.0$, 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 (1H, d, $J = 8.0$ Hz, H7), 1.25 (1H, d, $J = 7.0$ Hz, H1- CH_3), 6.49 (1H, d, $J = 1.7$ Hz, H2'), 6.66 (1H, d, $J = 8.0$ Hz, H5'), 6.44 (1H, dd, $J = 8.0$ and 1.7 Hz, H6').

Compound 16. HR-ESIMS $[M + Na]^+$ calcd for $C_{16}H_{16}O_4Na$ 295.0946, found 295.0964; 1H NMR (CD_3OD) 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- CH_3), 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').

Compound 17. HR-ESIMS $[M + Na]^+$ calcd for $C_{18}H_{18}O_4Na$ 321.1103, found 321.1137, and $[2M + Na]^+$ calcd for $C_{36}H_{36}O_8Na$ 619.2308, found 619.2301; 1H NMR ($CDCl_3$) of tetraacetate of 17, δ 2.81 (1H, dq, $J = 7.8$ and 6.8 Hz, H1), 2.55 (1H, dt, $J = 1.6$ and 7.8 Hz, H2), 6.92 (1H, s, H4), 6.90 (1H, s, H7), 1.35 (3H, d, $J = 6.8$ Hz, 1- CH_3), 1.54 (3H, s, 3- CH_3), 7.15 (1H, s, H2'), 7.00 (1H, s, H5'), 3.23 (1H, dd, $J = 16.2$ and 7.8 Hz, 7' β - CH_2), 2.82 (1H, brd, $J = 16.2$ Hz, 7' α - CH_2), 2.25 (6H, s, 5-, 6-, 3', or 4'- CH_3 CO), 2.27 (3H, s, 5-, 6-, 3', or 4'- CH_3 CO), 2.30 (3H, s, 5-, 6-, 3', or 4'- CH_3 CO). Observed NOESY correlations from H2 to 1- CH_3 , 3- CH_3 , and 7' α - CH_2 ; and 7' β - CH_2 ; from H7 to 1- CH_3 ; from 1- CH_3 to H2 and H7; from 7' β - CH_2 to 3- CH_3 ; from 3- CH_3 to H2, 7' β - CH_2 , H4, and H2'; and from H2' to 3- CH_3 .

Compound 18. HR-ESIMS $[M + Na]^+$ calcd for $C_{18}H_{18}O_4Na$ 321.1103, found 321.1133, and $[2M + Na]^+$ calcd for $C_{36}H_{36}O_8Na$ 619.2308, found 619.2295; 1H NMR ($CDCl_3$) of tetraacetate of 18, δ 3.04 (2H, dq, $J = 4.8$ and 7.0 Hz, H1 and H7'), 2.61 (1H, dt, $J = 8.6$ and 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 H5'), 1.32 (6H, d, $J = 7.0$ Hz, 1- CH_3 and 7'- CH_3), 2.26 (6H, s, 5-, 6-, 3', or 4'- CH_3 CO), 2.28 (6H, s, 5-, 6-, 3', or 4'- CH_3 CO). Observed NOE from 1- and 7'- CH_3 to H2 and H3 in NOE difference spectra.

Measurement of the Xanthine Oxidase Inhibitory Activities of the Isolated Compounds.

The xanthine oxidase inhibition assay was performed according to a previously reported protocol.¹⁷ Briefly, a reaction medium consisting of 10 μ L of 1 mmol/L xanthine in DMSO, 10 μ L of the test sample (6 mg/mL for thermal products and 4 mmol/L for isolated compounds) in DMSO, and 160 μ L of 12.5 mmol/L phosphate buffer (pH 7.4) was preincubated at 37 $^{\circ}$ C for 5 min. To this solution, 20 μ L of 0.027 unit/mL XO buffer solution was added. After incubation at 37 $^{\circ}$ C for 10 min, 3% $HClO_4$ (aq) (25 μ L) was added to quench the reaction. An aliquot of the solution (20 μ L) was injected into the HPLC column to quantify the uric acid production (Mightysil RP-18 GP Aqua column, Kanto Chemical, Tokyo Japan; 250 $\times$ 4.6 mm i.d., 5 μ m; flow rate, 1.0 mL/min; solvent, 2.5:97.5 methanol–0.1% phosphoric acid in water; detection at 290 nm; temperature, 35 $^{\circ}$ C). The percent inhibition was calculated according to the following equation: inhibition (%) = [(peak area of uric acid in control experiment) – (peak area of uric acid in sample experiment)]/(peak area of uric acid in control experiment) $\times$ 100.

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

RESULTS AND DISCUSSION

HPLC Analysis of the Thermal Reaction Products of Caffeic Acid at Various Temperatures.

In our previous work, we observed a model roasting reaction product of chlorogenic acid having XO inhibitory activity, whereas intact chlorogenic acid did not inhibit XO activity. We also examined the XO inhibitory activities of the thermally treated caffeic and quinic acids, which are both components of chlorogenic acid.²⁰ The results revealed that the XO inhibitory activity of the thermal reaction product of caffeic acid was greater than that of chlorogenic acid, whereas no XO inhibitory activity was observed for the product of quinic acid. Therefore, in this investigation the thermal reaction of caffeic acid was further investigated for the production of XO inhibitors and enhanced XO inhibitory activity. The high-temperature thermal treatment of caffeic acid was carried out through a model roasting reaction according to a previously reported method with 10:28 caffeic acid–solid support (a phosphoric salt).²⁰ The XO inhibitory activities of the thermal treatment products at different temperatures are illustrated in Figure 1. Intact caffeic acid has weak XO inhibitory activity (22% inhibition) at the examined concentration (0.3 mg/mL), whereas the XO inhibitory activities of the thermal reaction products were greater than those of caffeic acid. The activity increased with increasing reaction temperature up to 170 $^{\circ}$ C, after which it decreased gradually up to 290 $^{\circ}$ C. Figure 2A (Figure S1A in the Supporting Information) displays the HPLC data representing the constituents of the thermal reaction products obtained by gradient elution on an octadecylsilyl silica gel (ODS) analytical column with 5 to 100% acetonitrile. At a reaction temperature of 110 $^{\circ}$ C, the only noticeable peak was that of the starting material at a retention time of 15.5 min.

Figure 1. Xanthine oxidase inhibitory activities of the thermal reaction products (0.3 mg/mL) of caffeic acid. Data are expressed as the means $\pm$ SE ($n = 3$).

GPC column, might exhibit weak XO inhibition. Finally, the low-molecular-weight products, observed as peaks eluted later from the GPC column from the reactions at high temperatures, may also present weak XO inhibitory activities. Thus, the accumulation of both the higher- and lower-molecular-weight products should reduce the XO inhibitory activities of the reaction products produced at and above 200 °C. From the constituent analysis data of the products obtained at various temperatures (Figures 2A and S1A), moderate-molecular-weight products, which are predicted to be strong XO inhibitors, should correspond to the peaks in the retention time range of 19 to 42 min in the HPLC data of the reactions at 140–200 °C.

Isolation and Structure Identification of the Constituent Compounds Obtained from Thermal Reactions at 140–200 °C.

To identify the compounds that correspond to the peaks observed in the HPLC constituent analysis of the products obtained at 140–200 °C, isolation and structure identification of the constituents of the thermal reaction products were carried out. Multiple large scale thermal reactions of caffeic acid were performed at 140 and 200 °C; in total, 18 compounds were purified from the reaction products, as described in the Materials and Methods section. The isolated compounds 1–4, 7, and 8 were identical to the previously identified phenylindanes according to their NMR and MS data (Figure 3).²⁰ Compounds 15 and 16 were determined to be other phenylindane compounds that had been previously identified by Frank et al.¹³ Compounds 11 and 12 were determined to be catechol and 4-ethylcatechol, which are reportedly found in roasted coffee.²¹ The structures of compounds 5, 6, and 14 were also identified, as shown in Figure 3, by comparing the NMR and MS data to previously reported data.^{22–24} Notably, compounds 9, 10, 13, 17, and 18 were compounds newly identified in this work, and their structures were elucidated by NMR and MS analyses.

High-resolution mass spectrometry (HR-MS) data of compounds 9 and 10 indicated that they were constitutional isomers with the molecular formula $C_{24}H_{24}O_6$ (9: m/z 409.1656 $[M + H]^+$ and 10: m/z 409.1652 $[M + H]^+$). The 1H NMR spectra of 9 and 10 were also very similar, indicating that they were stereoisomers, as summarized in Table S1 (Supporting Information). The 1H NMR spectrum of 9 revealed seven aromatic proton signals at 6.60, 6.67, and 6.72 ppm in the same coupling system and at 6.19, 6.20, 6.66, and 6.77 ppm as singlet shapes. The former peaks indicated the existence of a trisubstituted benzene ring with protons arranged in a 1,2,4 configuration, whereas the latter peaks revealed the presence of two tetra-substituted benzene rings with protons oriented *para* to one other; these systems were derived from the aromatic moiety of caffeic acid. The HH-COSY data of 9 clarified two coupling systems, consisting of CH_3 (δ 1.21, 1- CH_3)– CH (δ 3.19, H1)– CH_2 (δ 2.05, H2)– CH (δ 4.49, H3) and CH_3 (δ 1.56, 1''- CH_3)– CH (δ 4.29, H1''). The HMBC spectrum of 9 showed correlations from C1a to H1, from C3a to H3, from C1 to H7, from C3 to H4 and H2', from C4 to H3, from C7 to H1, and from C1' to H3, as shown in Figure 4, indicating the existence of a phenylindane structure consisting of two tetra-substituted benzene rings and the CH_3 – CH – CH_2 – CH system. Other C–H long range correlations were observed from C1'' to H5', H3'', H7'', and 1''- CH_3 ; from C2'' to H1'' and 1''- CH_3 ; from C7'' to H1''; and from 1''- CH_3 to H1'' between the CH_3 – CH system and a trisubstituted benzene. Correlations were also

When the temperature was increased to 140 and 170 °C, numerous peaks were observed in the retention time range of 19 to 42 min, along with the peak corresponding to caffeic acid (retention time of 15.5 min), with very complex peaks observed at 28–42 min. With a further increase in temperature to 200 °C, the caffeic acid peak and the peaks at 19–22 min disappeared. Moreover, the intensities of the complex peaks at 30–42 min were lower than those observed at 170 °C. On the other hand, a new peak appeared at the retention time of 11.6 min for the reactions at temperatures of 200 °C or greater. At temperatures over 230 °C, almost all peaks gradually disappeared, except for the major peak at 11.6 min and a minor peak at 27.0 min. Figure 2B (Figure S1B in the Supporting Information) presents GPC data on the acetylated products from the thermal reactions of caffeic acid. Under the employed GPC conditions, acetylated caffeic acid (0.26 kDa), which was obtained from caffeic acid by treatment with acetic anhydride and pyridine, was detected at a retention time of 10.8 min. The area of the earlier eluting peak increased with increasing reaction temperature up to 200 °C. This peak was clearly observed at 10.1 min (corresponding to 0.7 kDa, calculated from the top of the peak) for the reaction at 170 °C, and it broadened to a retention time of 9 min at 200 °C, which indicated that higher-weight molecules (up to ca. 0.3 kDa) were produced by the elevation of the reaction temperature. In the GPC data of the reactions at 230 °C or above, the peak was further broadened between the retention times of 8.5 and 12 min, and two new peaks at retention times of 11.1 and 11.5 min were also observed, which indicated that low-molecular-weight degradation products (below 0.3 kDa) were produced along with higher-weight compounds (up to ca. 6 kDa). Considering the data from constituent analysis (Figures 2A and S1A) and molecular size analysis (Figures 2 B and S1B) and the data on the XO inhibitory activities of the products from the reactions at various temperatures (Figure 1), it was determined that the XO inhibitory activities of moderate-molecular-weight compounds (3–0.3 kDa by GPC analysis), which may be coupling products of caffeic acid, should be stronger than that of caffeic acid. Additionally, these compounds might contribute toward the enhanced XO inhibitory activities of the products obtained at 170 °C. On the other hand, higher-molecular-weight products, produced in the reactions at and above 200 °C and eluted earlier from the

Figure 2. Selected HPLC analytical data of various thermal reaction products of caffeic acid. (A) Chromatograms obtained using an octadecylsilyl silica gel (ODS) column. (B) Chromatograms obtained using a gel permeation chromatography (GPC) column.

451 observed from C1' to H1'' and from C6' to H1'' and 1''-CH₃
 452 between the CH₃—CH system and one of the tetra-substituted
 453 benzenes, as shown in Figure 4. These results indicated that an
 454 ethylcatechol structure was attached to the 6'-position of the

tetra-substituted benzene at the 3-position of the indane 455
 substructure. The stereochemistry of the three chiral carbons 456
 was deduced from the observed NOE correlations in the 457
 NOESY spectrum of 9. Strong correlations between H1 and 458

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 and substituted benzene rings.

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

The HR-MS data of compound 13 revealed a molecular-related ion at m/z 269.0816 (calcd for $C_{14}H_{14}O_4Na$, 269.0790), indicating the molecular formula $C_{14}H_{14}O_4$, which is the same as that of compound 6. The NMR data of the tetra-acetylated derivative of 13 (13a) showed the presence of a CH_3 – CH unit (4.25 (1H, q, J = 7.0 Hz), 1.57 (3H, d, J = 7.0 Hz)) and six aromatic protons, which were very similar to those of 6. A signal for an aromatic proton having two *ortho*-couplings (7.23 (1H, dd, J = 8.6 and 8.2 Hz)) and signals of four phenolic acetyl groups indicated that 13 was a regioisomer of 6 in reference to the attached position of the CH_3 – CH unit as shown in Figure 3.

The HR-MS data of compounds 17 and 18 indicated that both molecular formulas were the same, $C_{18}H_{18}O_{14}$ (17: m/z 321.1137 $[M + Na]^+$, 18: m/z 321.1133 $[M + Na]^+$, both calculated for $C_{18}H_{18}O_{14}Na$, 321.1103). The 1H NMR spectrum of 17a, which was obtained from 17 by reaction with acetic anhydride in pyridine, showed four signals for phenolic acetyl groups at 2.25 (3H, s $\times$ 2), 2.27 (3H, s), and 2.30 (3H, s) ppm and four singlet aromatic proton signals at 6.90, 6.92, 7.00, and 7.15 ppm, indicating the presence of two sets of 1,2,4,5-tetra-substituted benzene rings derived from two molecules of caffeic acid. The HH-COSY of 17a revealed the presence of a CH_3 (1.35 ppm)– CH (2.81 ppm)– CH (2.55 ppm)– CH_2 (2.82 and 3.23 ppm) coupling system, which was adjacent to the aromatic rings. Another singlet signal (1.54 ppm) due to a methyl group adjacent to a quaternary carbon was also observed. These data indicated that 17 comprised a bicyclo[3.3.0]octane structure appended with two dihydroxybenzene rings and two methyl groups. The stereochemistry around the bicyclooctane rings was deduced by NOE

H2' and between H3 and 1- CH_3 revealed the *trans*-relationship of the methyl group at the 1-position and the phenyl group at the 3-position. The stereochemistry of C1'' could not be deduced from its NMR data because of its flexible structure. Thus, the structure of 9 was determined as depicted in Figure 3.

The 1H and ^{13}C NMR data of 10, including correlations in the COSY and HMBC spectra, were very similar to those of 9 (Table S1 in the Supporting Information and Figure 4), indicating that the planar structure of 10 was the same as that of 9. Differences in the stereochemistry of 10 were deduced by NOESY. The observed NOE between H1 and H3 in the NOESY spectrum of 10 revealed that the methyl group at the 1-position and the phenyl group at the 3-position were *cis* relative to one another. Similar to 9, the stereochemistry at the 1''-position could not be elucidated; thus, the structure of compound 10 was determined as depicted in Figure 3.

correlations from H2 to the 1- and 3-methyl protons in the NOESY spectrum of **17a**, revealing the presence of *cis* relations. Thus, the structure of **17** was determined as depicted in Figure 3.

Although **17** and **18** presented the same molecular-related peaks in their MS data, the ^1H NMR data of **18a** (acetylated derivative of **18**) were much simpler than those of **17a**, indicating that **18** was a symmetrical isomer of **17**. The HH-COSY of **18a** indicated the presence of a symmetrical coupling system, CH_3 (1.32 ppm)– CH (3.04 ppm) $_2$ – CH (2.61 ppm)– CH (4.64 ppm), which was attached to two tetra-substituted benzene rings. In addition to the above data, proton signals due to four phenolic acetyl groups, 2.26 (3H $\times$ 2) and 2.28 (3H $\times$ 2) ppm, and two sets of *para*-substituted aromatic protons, 6.95 (1H $\times$ 2) and 7.11 (1H $\times$ 2), indicated that **18** had a symmetrical bicyclo[3.3.0]octane structure similar to that of **17**. NOEs were observed from 1- and 7'- CH_3 to H2 and H3 in the NOE difference spectra of **18**. Thus, the structure of **18** was determined as depicted in Figure 3.

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.²⁵ 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 on the basis of their vinylcatechol substructure: category I (compounds 1–4) consists of the coupling products of vinylcatechol and caffeic acid, category II (compounds 5, 7, 8, 9, 10, 15, and 16) consists of the coupling products of two or three vinylcatechols, and category III (compounds 6, 13, 14, 17, and 18) is composed of compounds formed by coupling with or elimination of the catechol moiety (Figure 3). All the categorized compounds were assigned to the observed peaks in the HPLC chromatograms from the reactions at 140, 170, and 200 °C, as shown in Figure 5. The category I compounds were observed in the 140 °C reaction product along with caffeic acid and the category II compounds. The HPLC data of the 170 °C reaction product indicated decreases in the intensities of caffeic acid and the category I compounds. Additionally, the region representing the category II compounds (retention time of 30–40 min) became more complex compared with the same region in the chromatogram of the 140 °C reaction product. This indicated that at the reaction temperature of 170 °C, various vinylcatechol oligomers were additionally produced instead of the caffeic acid–vinylcatechol compounds. Through consideration of the GPC analytical data of the 170 °C reaction product (Figure 2 B), it was determined that the oligomerization degree of the newly produced compounds might be higher than that of the compounds observed in the 140 °C reaction product. At the reaction temperature of 200 °C, the category I compounds disappeared, whereas the category III compounds were observed in addition to the vinylcatechol oligomers (category II).

XO Inhibitory Activities of the Oligomeric Compounds from the Thermal Reactions of Caffeic Acid.

Figure 6 illustrates the XO inhibitory activities of the isolated constituents (200 $\mu\text{mol/L}$) of the thermal reaction products. Although the starting material, caffeic acid, showed very weak activity at the examined concentration, the isolated compounds exerted stronger activities in the order $3 > 4 > 8, 10, 17 > 7, 9,$

Figure 5. Peak assignments of the identified constituent compounds in the HPLC profiles of the thermal reaction products at 140, 170, and 200 °C. Blue numbers, category I compounds (caffeic acid–vinylcatechol coupled compounds); red numbers, category II compounds (vinylcatechol oligomers); green numbers, category III compounds.

2, 5, 1, 6 > 16, 15, 14, 13, on the basis of their percent inhibition values. The compounds with the highest inhibition levels, caffeic acid–vinylcatechol compounds **3** and **4**, were detected in the products of the 140 and 170 °C reactions but were absent in the product of the 200 °C reaction. These were followed by compounds **7**, **8** (vinylcatechol dimers), and **10** (trimer), which were mainly observed in the products of the reactions at 140 and 170 °C. Conversely, the weakest inhibitory compounds, **13** and **14**, catechol-eliminated compounds belonging to category III, were both observed solely as the constituents of the product of the 200 °C reaction.

A comparison of the results of the XO inhibitory activities of the identified compounds with those of the thermal reaction products (110–290 °C, Figure 1) indicated that caffeic acid was initially converted to caffeic acid–vinylcatechol compounds, some of which contributed to the enhanced inhibitory activities of the products produced at 170 °C or less. Subsequently, caffeic acid was converted to the moderately active vinylcatechol oligomers, which were then transformed into weakly active catechol-reacted compounds, the production of which decreased the XO inhibitory activities of the thermal reaction products produced at the temperatures above 170 °C. Although not all of the produced constituents of the thermal reaction products could be identified because of their complexity, the observed structural differences in the constituents might induce the enhanced XO inhibitory activities of the thermal reaction products. Notably, the thermal treatment at 170 °C, which exhibited the maximum XO inhibitory activity, coincides with the general roasting conditions of food processing.²⁶ Caffeic acid and its derivatives, including chlorogenic acid, are widely distributed in plant-derived foodstuffs.²⁷ Therefore, the processing of foodstuffs by roasting might result in significant XO inhibitory activity through the thermal reactions of caffeic acid derivatives. In terms of coffee beverages, we previously reported that only roasted coffee beans exhibited XO inhibitory activity,¹⁶ which enhanced the ability of coffee to prevent gout.²⁸ Frank et al.¹³

Figure 6. Xanthine oxidase inhibitory activities of identified compounds 1–18 and caffeic acid (200 $\mu\text{mol/L}$). Data are expressed as the means $\pm$ SE ($n = 3$). Asterisks (*) indicate significant differences ($p < 0.05$) when compared with caffeic acid (Dunnett's test). Allopurinol (0.5 $\mu\text{mol/L}$), a positive control sample, showed 52.3% inhibition under the same conditions.

reported the existence of various vinylcatechol oligomers in roasted coffee as bitter-tasting compounds by using LC-MS techniques. Although pyrogallol and chlorogenic lactones have been identified as significant XO inhibitors present in roasted coffee beans,^{16,17} vinylcatechol oligomers and related compounds might also play a role in the XO inhibitory activity. Differences in inhibitory ability due to the different reaction temperatures should also be considered when determining the conditions of the roasting process of raw coffee beans. Optimized roasting conditions should be useful for the production of health-functional coffee beverages that exhibit XO inhibitory activity.

Although the bioavailabilities of the identified phenylindanes and vinylcatechol oligomers have not been examined to date, the abilities of caffeic acid and the related phenolic acids in coffee have been well studied.^{29,30} These compounds can be absorbed in humans in their conjugated and nonconjugated forms. Ota et al.³¹ examined the interaction of these phenolic acids and corresponding vinylcatechol derivatives with lipid membranes. The results revealed that the more nonpolar vinylcatechols displayed stronger interactions with the membrane, which were related to their bioavailability. The identified thermal reaction products are also more nonpolar than caffeic acid; therefore, higher bioavailability is expected for the product constituents. When the nonpolar thermal reaction products of caffeic acid are absorbed by humans, they might exhibit characteristic functions in the human body. XO is localized in the liver and intestine and produces uric acid,^{32,33} the over accumulation of which is responsible for the development of gout.²⁸ The absorbed thermal products might play a role in inhibiting XO from overworking when reaching the liver or intestine, thereby preventing the development of gout.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b03633.

Figure S1. HPLC analytical data of various thermal reaction products (110–290 $^{\circ}\text{C}$) of caffeic acid and reference samples (caffeic acid and polystyrene marker): chromatograms obtained using (A) ODS column and (B) GPC column (PDF)

Table S1. NMR data of vinylcatechol oligomers 9 and 10 (PDF)

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Notes

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