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NOTE

Novel Xanthine Oxidase (XO) Inhibitory Phenylindanes Produced by Thermal Reaction of Caffeic Acid.

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ABSTRACT

The products from the thermal reaction of chlorogenic and caffeic acids, which is a model process of roasting coffee beans, exhibited xanthine oxidase (XO) inhibitory activity. From caffeic acid, six inhibitory phenylindanes were identified, and a new phenylindane displayed the highest inhibitory activity among them. The activity of these phenylindanes may contribute to XO inhibition-related functions of roasted coffee beverages.

Key words

Phenylindane; xanthine oxidase inhibition; caffeic acid; roasted coffee

Coffee beans are known to contain large amounts of chlorogenic acid and its isomers (3~8% of green beans) as polyphenol constituents, which exert various biological activities including antioxidant, hepatoprotective, and hypoglycemic activities,[1)] and might be responsible for health promoting effects of coffee beverages.[2)] It should be noted that coffee beverages consumed by humans are made from roasted coffee beans and not from raw beans (green beans), which are dry seeds of the tropical *Rubiaceae* plant. The roasting of coffee beans consists of a high-temperature thermal treatment at around 200 °C. During roasting, the characteristic color, aroma, and taste of coffee are developed. This suggests that the constituents of green coffee beans are converted to other compounds under the thermal process. We previously found xanthine oxidase (XO) inhibitory activity, which may relate to the prevention of gout in coffee consumers by reducing uric acid in their plasma,[3)] only in roasted coffee beans[4)] where several XO inhibitors were identified.[5,6)] However, other non-polar inhibitors, which were suggested to exist in roasted coffee beans,[5)] have not yet been identified because of the high complexity of the non-polar constituents of roasted coffee beans. Stadler and coworkers[7)] identified two phenylindanes from the thermal treatment of caffeic acid. Later, Frank and coworkers[8)] found that such phenylindanes existed in roasted coffee beans. These phenylindanes should be characteristic non-polar compounds of roasted

coffee beans. Therefore, we attempted to isolate such phenylindane derivatives from the thermal reaction of coffee bean constituents and examine their XO inhibitory activity.

In a screw-capped test tube (i.d. 8 mm, L. 100 mm) were placed 10 mg of chlorogenic acid (Carbosynth, Compton, UK), caffeic acid (Kanto Chemicals, Tokyo, Japan), or quinic acid (MilliporeSigma, St. Louis, USA) with methanol (200 μL) and 400 μL of phosphate buffer (500 mmol/L, pH 6.0, from Na_2HPO_4 and KH_2PO_4). After removing the solvent *in vacuo*, the tube was heated in a metal block bath. After cooling the tube, methanol (1 mL) was added and the mixture was centrifuged at 2000 rpm for 5 min at 25 □ to give a supernatant. After evaporation of the solvent from the supernatant, the XO inhibitory activity was measured using a previously reported method.[4)] Figure 1A shows the XO inhibitory activity of the products from the thermal reaction at 200 °C of chlorogenic acid, caffeic acid, and quinic acid. The product mixture obtained from heating chlorogenic acid expressed XO inhibitory activity at the concentration of 0.3 mg/mL, whereas that obtained from quinic acid, whose structure is contained in chlorogenic acid, did not show significant XO inhibitory activity for 1 h of reaction time (X-axis of Fig. 1A) under employed conditions. Although caffeic acid, which is also a structure contained in chlorogenic acid, displayed weak XO inhibitory activity, the product mixture obtained from its thermal treatment exhibited enhanced inhibitory

activity and the activity was stronger than that of the product mixture of chlorogenic acid (Fig. 1A). These results indicate that the thermal reaction of caffeic acid (140 □ ~) produces efficient XO inhibitors, which are expected to contain phenylindanes according to Stadler.[7] Figure 1B shows the XO inhibitory activity of the products obtained from thermal reaction of caffeic acid at three different temperatures (reaction time is expressed in X-axis) . The 170 °C reaction showed maximal XO inhibition efficiency at short time within 30 min, and then the activity gradually decreased. In contrast, the 140 °C reaction increased XO inhibition continuously for 1 h until almost the same maximal activity. Therefore, the temperature of 140 °C was chosen for the large-scale reaction because it was easy to monitor the reaction progress by HPLC analysis.

Thus, a large-scale caffeic acid-phosphate buffer salt mixture was prepared as described [caffeic acid (10 g) was dissolved in 100 mL of methanol and 400 mL of 500 mmol/L Na_2HPO_4-KH_2PO_4 (pH 6.0) and then evaporated to dryness]. The solid mixture was heated in a stainless reactor (i.d. 14 cm; h. 15 cm) under a N_2 atmosphere at 140 °C for 90 min. After cooling, the reaction mixture was extracted twice with 1L of methanol. This procedure was repeated ten times (in total 100 g of caffeic acid were treated). After removal of the methanol from the extract, the residue was used for the isolation of the

products. Part of the residue (84 g) was subjected to Amberlite XAD-7 column chromatography eluted with increasing percent of methanol (50% to 100%) in water, which produced 8 separate fractions. Fraction 3 (208 mg out of 6.5 g), which was eluted with 60% methanol in water, was purified by preparative HPLC under the following conditions [column, Cosmosil 5C18-AR-II (250x20 mm i.d.); solvent, 1% acetic acid in H_2O–CH_3CN = 85:15; flow rate, 9.6 mL/min; detection, 280 nm]. Products **1** (3 mg), **2** (3 mg), **3** (62 mg), and **4** (6 mg) were isolated from the peaks at retention times: 34 min, 39 min, 24 min, and 28 min, respectively. Products **5** (87 mg) and **6** (134 mg) were isolated from fraction 6 (an eluted fraction with 75% methanol in water) using Sephadex LH-20 column chromatography and subsequent HPLC purification [column, Cosmosil 5C18-AR-II (250x20 mm i.d.); solvent, 1% acetic acid in H_2O–CH_3CN = 75:25; flow rate, 9.6 mL/min; detection, 280 nm; collected peaks, retention time 43min (product **5**) and 47 min (product 6)].

Product **5** showed a molecular-related ion peak at *m/z* 295 in the ESI-MS. The ^1H NMR of **5** showed two sets of aromatic protons, one at 6.48 (dd, *J*=7.8 and 1.8 Hz), 6.53 (d, *J*=1.8 Hz), and 6.69 (d, *J*=7.8 Hz) ppm, and another one at 6.68 (brs) and 6.42 (brs) ppm, indicating the presence of a tri-substituted and a tetra-substituted benzene rings. Geminal coupled protons were observed at 2.09 and 2.19 ppm, which were both

coupled with the protons at 4.16 and 3.22 ppm. The proton at 3.22 ppm was also coupled with the methyl protons at 1.23 ppm. These data indicated that **5** is a phenyl-substituted indane derivative. From the comparison of the ^{1}H NMR analytical data (chemical shifts and coupling constants), we concluded that **5** is the 1,3-*trans* isomers of Stadler's phenylindanes.[7)] Product **6** showed the same molecular-related ion at *m/z* 295 and very similar ^{1}H NMR data to those of **5**. Typical differences were observed in the chemical shifts and coupling constants of the protons at 1-, 2-, and 3-positions of the indane structure, which indicate that **6** is the *cis* isomer of **5** [7)] as shown in Fig. 2.

Products **3** and **4** showed similar ^{1}H NMR data to those of **5** and **6**. The comparison of the ^{1}H NMR spectra of **3** and **4** revealed that one proton signal at corresponding to the 2-methylene was lacking and another proton signal was shifted to higher frequency (3.29 ppm) in the spectrum of **3**. The negative ESI-MS showed a molecular-related ion at *m/z* 271.0986, which indicated that **3** had the molecular formula $C_{17}H_{16}O_6$. These data suggested the presence of a carboxylic acid group at the 2-positon. The *m/z* value of 653.1634 observed in the ESI-MS was assigned to a characteristic carboxylic acid cluster ion $[2M-2H+Na]^-$. The relative stereochemistry of the three substituted non-aromatic carbons of the indane was determined to be relative *1S, 2R, 3S* according

to an NOE observed from 1-methyl protons to the proton at the 3-proton and a very strong NOE observed from 2-H to the proton at the 2-position of 3-phenyl group in the NOE differential spectra of **3**. Although product **4** shows a similar ^{1}H NMR spectrum of **3**, some differences are observed in the chemical shifts and coupling constants of protons at 1-, 2-, and 3-positons. Moreover, the observed NOEs from 1-methyl protons to the protons at 2- and 2′-positions suggested that relative stereochemistry is *1S, 2S, 3R*. The structures of **3** and **4** are shown in Fig.2. The planar structure of **3** and **4** was already reported as a forming aid obtained from coffee in a US patent by Martine and coworkers.[9)]

The ESI-MS data showed peaks at *m/z* 315.0897 ($C_{17}H_{15}O_6$ $[M-H]^-$) and 653.1628 ($C_{34}H_{30}O_{12}Na$ $[2M-2H+Na]^-$) for product **1**, and 315.0902 ($C_{17}H_{15}O_6$ $[M-H]^-$) and 653.1629 ($C_{34}H_{30}O_{12}Na$ $[2M-2H+Na]^-$) for product **2**. Moreover, similar ^{1}H NMR data for both compounds indicated that they were stereoisomers of each other. The ^{1}H NMR of **2** revealed the presence of a 1,3,4-tri-substituted benzene and a 1,3,4,6-tetra-substituted benzene similar to other isolated products. A proton network ($CH-CH_2-CH-CH_2$), which was identified from the COSY, suggested a two-substituted indane structure similar to that of **5** and **6**. The chemical shift of a terminal proton at 4.03 ppm was assigned to a methine proton signal between two benzene rings, while the

121 signals at 2.37 and 2.83 ppm, with coupling constants characteristic germinal protons,
122 were assigned to protons adjacent to a carboxylic acid. The assignments were confirmed
123 by the HMBC correlation between the methylene protons and a carbonyl carbon at 179
124 ppm (this carbon chemical shift was obtained from the F1-projection of the HMBC
125 spectrum). Taking into consideration the above data, structure **2** was assigned as a newly
126 identified compound:
127 1-hydroxycarbonylmethyl-3-(3,4-dihydroxy)phenyl-5,6-dihydroxyindane. The relative
128 stereochemistry of the acetic acid group at the 1-position and the dihydroxylphenyl
129 group at the 3-position was determined to be *cis* (structure **2** in Fig. 2) from the NOESY
130 of **2** (one NOE correlation between 1-CH_2 and 2′-H, and other between 1-H and 3-H).
131 The ^{1}H NMR spectral data of **1** indicated that **1** is a stereoisomer of **2** concerning the 1-
132 and 3-positions of the indane scaffold, which was deduced from a clear NOE correlation
133 observed between the 1-methylene protons and the proton at 3-position. Thus, **1** was
134 identified as a new compound with *trans* stereochemistry of the
135 1-hydroxycarbonylmethyl and 3-dihydroxyphenyl groups as shown in Fig. 2.
136 The XO inhibitory activity of the isolated phenylindanes (concentration: 200
137 μmol/L) was measured by a previously reported procedure,[4)] which is based on the
138 quantitative HPLC analysis of produced uric acid, the data are summarized in Table 1.

While caffeic acid showed almost no activity at the measured concentration, isolated phenylindanes exerted stronger activity than caffeic acid. Especially newly identified phenylindane **1** had the most potent activity (62 % inhibition at 200 μmol/L) among them. Pyrogallol (IC_{50} 0.73μmol/L) and chlorogenic acid 1,5-lactones (IC_{50} 210~360 μmol/L) isolated from roasted coffee beans have been identified as XO inhibitors. Although the phenylindanes identified in this work have moderate XO inhibitory activity comparing with potently active pyrogallol, they are non-polar inhibitors produced from caffeic acid, which may play a role in the XO inhibitory activity exerted by roasted coffee.

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Table 1 XO inhibitory activity of identified phenylindanes (200 μmol/L) from thermal reaction product of caffeic acid

compound	% inhibition (mean±SD, n=3)
1	61.9 ± 0.6
2	47.0 ± 0.6
3	18.8 ± 3.5
4	20.4 ± 2.6
5	25.5 ± 2.3
6	35.5 ± 2.6
Caffeic acid	4.0 ± 2.2
Allopurinol (0.5 μmol/mL)[a]	51.9 ± 1.8

[a] 0.5 μmol/mL of allopurinol was employed as positive control.

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Figure Legends

Figure 1. Panel A, XO inhibitory activity of thermal reaction products (0.3 mg/mL) from chlorogenic acid, caffeic acid and quinic acid at 200 °C. Scale of X-axis expresses reaction time. Data are expressed at the mean ± SD (n=3)

Panel B, XO inhibitory activity of thermal reaction product (0.3 mg/mL) from caffeic acid at the different temperatures. Scale of X-axis expresses reaction time. Data are expressed at the mean ± SD (n=3)

Figure 2. Structures of identified phenylindanes from the thermal reaction of caffeic acid

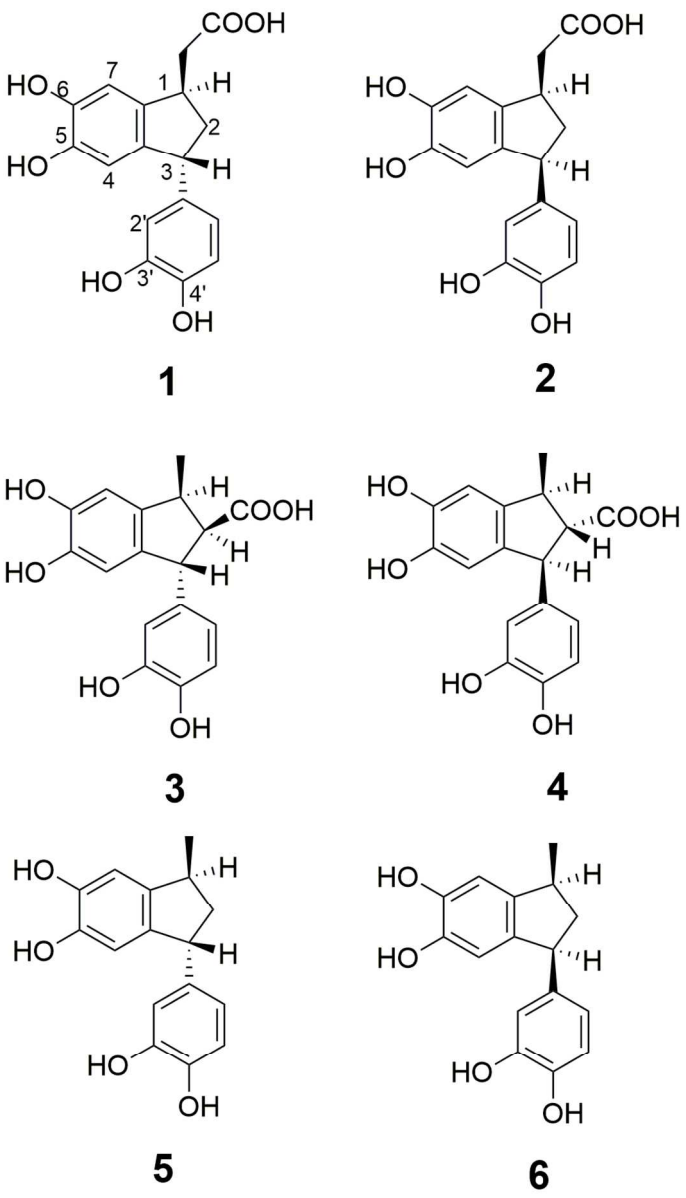

Figure 2. Structures of identified phenylindanes from the thermal reaction of caffeic acid

85x149mm (300 x 300 DPI)

Supplemental (Fukuyama et al.)

Table S1. ^{1}H-NMR Data of Products 1—6 (400 MHz for ^{1}H in CD_3OD)

	Product					
position	**1**	**2**	**3**	**4**	**5**	**6**
1	3.58 (dt, 6.4, 8.6)	3.43[f] (dt, 5.9, 8.6)	3.48 (quin, 7.4)	3.27 (dq, 7.2, 9.7)	3.22 (dquin, 6.0, 6.9)	3.05 (dquin, 7.1,10.0)
2	2.23 (m)	1.61 (dt, 10.0, 12.4)	3.29[a] (dd, 7.4, 9.8)	2.64 (t, 9.7)	2.09 (ddd, 6.0, 7.8, 12.8)	1.47 (m)
		2.71 (dt, 7.4, 12.4)				2.62 (dt, 7.1, 12.4)
3	4.18[d] (t, 7.4)	4.03[f] (dd, 7.4, 10.0)	4.51 (d, 9.8)	4.33 (d, 9.7)	4.16 (dd, 6.0, 7.8)	3.97 (dd, 7.1, 10.6)
4	6.35 (s)	6.32 (s)	6.30 (s)	6.30 (s)	6.42 (s)	6.31 (s)
7	6.75 (s)	6.72 (s)	6.68 (s)	6.66 (s)	6.68 (s)	6.67 (s)
2'	6.56 (d, 2.0)	6.64e (d, 1.8)	6.67 (d, 2.2)	6.66 (s)	6.53 (d, 1.8)	6.63 (d, 2.2)
5'	6.70 (d, 7.8)	6.73 (d, 8.0)	6.73 (d, 8.2)	6.74 (d, 7.8)	6.69 (d, 7.8)	6.73 (d, 8.0)
6'	6.51 (dd, 2.0, 7.8)	6.58 (dd, 1.8, 8.0)	6.60 (dd, 2.2, 8.2)	6.59 (dd, 2.0, 7.8)	6.48 (dd, 1.8, 7.8)	6.58 (dd, 2.2, 8.0)
1-CH_2	2.37[d] (dd, 8.6, 14.8)	2.37[e,g] (dd, 8.6, 14.8)[b]	—	—		
	2.56[d] (dd, 6.4, 14.8)	2.83[e,g] (dd, 5.9, 14.8)[b]				
1-CH_3	—	—	1.16[b] (d, 7.4)	1.42[c] (d, 7.2)	1.23 (d, 6.9)	1.32 (d, 7.1)

Coupling pattern and constants (*J* in Hz) are described in parenthesis.

[a] NOE-observed proton with 2′-H of the same compound; [b] NOE-observed proton with 3-H of the same compound; [c] NOE-observed proton with 2-H and 2′-H of the same compound; [d,e,f] The same character shows the correlated proton group in the NOESY of each compound; [g] Proton correlated with the carbonyl carbon of carboxylic acid group (δ 179) in the HMBC of the same compound