Identification of Potent Xanthine Oxidase Inhibitor from Oxidation of Caffeic Acid

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Keywords: antioxidant, caffeic acid, polyphenol oxidation, xanthine oxidase inhibitor, structure determination

ABSTRACT

Inhibitory activity of Fe-ion-catalysed radical oxidation products from 22 types of phenolic compounds toward xanthine oxidase (XO) was investigated. Phenols are readily oxidizable compounds in nature, and thus, showed potent antioxidant activities. Among the phenols screened in this study, noticeable activity was observed in the oxidation product of caffeic acid, whereas almost no XO-inhibitory activity of caffeic acid was observed. Assay-guided purification of the oxidation product of caffeic acid afforded a highly potent XO inhibitor, with an IC_{50} value that was calculated to be 60 nmol L^{-1} , which indicated XO-inhibitory activity much stronger than that of allopurinol ($IC_{50} = 1 \text{ } \mu\text{mol L}^{-1}$), a potent XO inhibitor and excellent medicine for the treatment of gout. The chemical structure of this new XO inhibitor was investigated by 1D and 2D NMR and HR-ESI-MS analyses, and the unique tetracyclic structure was confirmed by synthesis starting from commercially available 1,2,4-trimethoxybenzene and 3,4-dimethoxybenzoyl chloride.

53
54 Natural polyphenols have attracted considerable attention as
55 biologically active constituents in plants, plant-derived foods, and food
56 supplements. Some polyphenols exhibit strong radical-trapping ability as
57 potent antioxidants, which can prevent or ameliorate diseases initiated by
58 oxidative stress [1]. Antioxidation is a chemical reaction, and efficient
59 antioxidants are more easily oxidized than other biomolecules via radical
60 intermediates during the antioxidation process [2]. High reactivity of radical
61 oxidation exhibited by polyphenols may also lead to the efficient generation of
62 their final oxidation products in biological systems such as foods and living
63 cells [3-11]. However, the functionality arising from these newly oxidized
64 compounds has not yet been extensively examined.

65 Recently, we have observed cytotoxic activity of a new oxidation
66 product derived from sesamol, an antioxidant of sesame oil [12],
67 tyrosinase-inhibitory activity of an oxidative ring-expansion product of
68 rosmarinic acid (a polyphenol common in Labiatae plants) [13], and potent
69 lipoxygenase-inhibitory activity of oxidation-derived dimers of resveratrol
70 [14]. In the present study, we have screened the oxidation products of several
71 polyphenols and related antioxidative phenols to examine their xanthine

oxidase (XO)-inhibitory activity. XO is a key enzyme in the catabolism of purine-based nucleic acids, and produces uric acid from xanthine and hypoxanthine. The regulation of XO helps preventing hyperuricemia and gout, which are now recognized as life style-related diseases [15]. We have found that the oxidation product of caffeic acid showed remarkable XO inhibition, whereas caffeic acid (a well-known potent antioxidative polyphenol widely distributed in plants) did not [16]. In this investigation, we succeeded in isolating an active compound resulting from the oxidation of caffeic acid and determining its chemical structure via its instrumental analysis and synthesis.

Materials and methods

Materials

Caffeic acid (purity $\geq 98\%$), sinapic acid (purity $\geq 98\%$), gallic acid (purity $\geq 98\%$), resveratrol (purity $\geq 98\%$), hydroxytyrosol (purity $\geq 98\%$), and propyl gallate (purity $\geq 98\%$) were purchased from Tokyo Kasei (Tokyo, Japan). Chlorogenic acid (purity $\geq 95\%$), rosmarinic acid (purity $\geq 97\%$), siringic acid (purity $\geq 98\%$), taxifolin (purity $\geq 90\%$ as hydrate), nordihydroguaiaretic acid (purity $\geq 90\%$), 1,2,4-trimethoxybenzene,

3,4-dimethoxybenzoyl chloride, lithium diisopropylamide, pyridinium chlorochromate, and BBr_3 , and Chelex 100 resin were purchased from Sigma-Aldrich (St. Louis, USA). (-)-Epicatechin (purity 97%), ellagic acid (purity $\geq 90\%$), xanthine oxidase, xanthine, anhydrous tetrahydrofuran, NaH, and AlCl_3 were obtained from Wako Pure Chemicals (Osaka, Japan). Luteolin was purchased from Kanto Chemicals (Tokyo, Japan). Piceatannol (purity $\geq 95\%$) and secoisolariciresinol (purity $\geq 95\%$) were synthesized [17]. Carnosol (purity $\geq 95\%$) and carnosic acid (purity $\geq 95\%$) were isolated from Sage [8]. Silica gel for column chromatography (No. 1.09385) and that for thin layer chromatography (TLC, No. 1.05744) were purchased from Merck (Darmstadt, Germany). Protocatechuic acid (purity $\geq 97\%$), quercetin (purity not specified), (+)-catechin (purity 98% as hydrate), α -tocopherol (purity $\geq 96\%$), gentisic acid (purity $\geq 98\%$), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, γ -butyrolactone, methoxymethyl chloride, and thionyl chloride, and all other reagents and solvents were obtained from Nacalai Tesque (Kyoto, Japan).

General procedure

NMR spectra were recorded using an ECS-400 spectrometer (400

MHz for ^1H , JEOL, Tokyo, Japan). ESI-MS spectra were measured using a XEVO-QTOF spectrometer (Waters Japan, Tokyo, Japan). IR spectra were obtained using an FTIR-8400 spectrometer (Shimadzu, Kyoto, Japan). Analytical HPLC was performed using an LC-10 low-pressure gradient system (Shimadzu) equipped with an SPD-M20A photodiode array detector (Shimadzu). Preparative HPLC was carried out with an LC-6A pump (Shimadzu) equipped with a UV-975 detector (Jasco, Tokyo, Japan). Melting points were measured with an MP-J3 micro melting point apparatus (Yanako, Kyoto, Japan) and the values were uncorrected.

Oxidation of antioxidative phenolics.

Phenolic oxidation was carried out according to previously reported methods [14]. Briefly, to an ethanol solution (10 mL) of each phenol (0.5 mmol L⁻¹) in a screw-capped vial [i.d. 35 mm × 78 mm (h)] was added a 50-μM FeCl₃ aq. solution (100 μL). The vial was incubated under an oxygen atmosphere at 40 °C, and the oxidation process was monitored using HPLC [column, 250 mm × 4.6 mm i.d., 5 μm, Cosmosil 5C18-AR-II (Nacalai Tesque)]. At the time when almost all of the phenol was consumed or after

incubation for 19–20 d, the solution was passed through Chelex 100 (2–4 g) to remove the iron ions. The solvent was removed *in vacuo* to afford an oxidation product for XO-inhibitory assay.

Xanthine oxidase (XO) inhibition assay

Xanthine-oxidase-inhibition assay was carried out according to a previously reported method with slight modification [18]. The reaction medium [consisting of 110 $\mu\text{mol L}^{-1}$ xanthine aq. (90 μL), the test sample in ethanol (10 μL), and 12.5 $\mu\text{mol L}^{-1}$ phosphate buffer (pH 7.8, 80 μL)] was pre-incubated at 37 °C for 10 min. To this solution, freshly prepared 0.027 units mL^{-1} of an XO buffer solution (20 μL) was added. After incubation at 37 °C for 10 min, 3% HClO_4 aq. (25 μL) was added to quench the reaction. An aliquot (20 μL) of the solution was injected into the HPLC column to quantify the uric acid produced under the following conditions: [column, Daisopak SP-120-5-ODS-BP (150 $\times$ 4.6 mm i.d., Daiso, Osaka, Japan); solvent, CH_3OH –1% phosphoric acid aq. = 5:9 (v/v); flow rate, 0.5 mL min^{-1} ; detection, 290 nm.] 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)] $\times 100$ / (peak area of uric acid in control experiment).

Oxidation of caffeic acid and isolation of XO inhibitor 1

A 500-mL screw-capped glass bottle was charged with caffeic acid (500 mg) and ethanol (50 mL). Then, 0.56 mol L⁻¹ FeCl₃ aq. solution (500 μ L) was added, and the solution was allowed to stand at 40 °C under an O₂ atmosphere for 14 d. The solution from two bottles was diluted with EtOAc, washed three times with saturated NaCl aq., dried over anhydrous Na₂SO₄, and evaporated to obtain the oxidation product (0.98 g). The oxidation product (0.5 g) was subjected to gel permeation chromatography using Toyopearl HW-40F gel (Tosoh, Tokyo, Japan). Methanol was used as the eluent, and 10 fractions were separated (column size, 50 $\times$ 3 cm i.d.; fractionation volume, 25–50 mL). After the fractions were evaporated to dryness (yield: fractions 1, 27 mg; 2, 34 mg; 3, 59 mg; 4, 14 mg; 5, 104 mg; 6, 98 mg; 7, 54 mg; 8, 32 mg; 9, 12 mg; 10, 7 mg), their XO-inhibitory activities were measured. Potent inhibitory fraction 8 (30 mg) was further purified using preparative HPLC to obtain peak 1 compound as XO inhibitor **1** (0.9 mg) under the following

conditions: column, Cosmosil 5C18-AR-II (250 × 20 mm i.d.); solvent, CH₃CN–1% acetic acid in H₂O = 3:7 (v/v); flow rate, 5 mL min⁻¹; collected peak retention time, 30 min. From fraction 9, the other remaining peak compounds **2–4** (0.2, 0.3, and 0.3 mg, respectively) were purified under the following conditions: column, Cosmosil 5C18-AR-II (250 × 20 mm i.d.); solvent, CH₃CN–1% acetic acid in H₂O = 25:75 (v/v); flow rate, 5 mL min⁻¹. Inhibitor **1** (1.6 mg) was prepared again from caffeic acid (3 g) by using the same procedure for structural analysis.

Preparation of 5

Compound **5** was prepared according to the method reported by Donnelly et al. [19]. To a solution of 1,2,4-trimethoxybenzene (10.0 g) in dry Et₂O (150 mL), powdered AlCl₃ (23.8 g) and a solution of 3,4-dimethoxybenzoyl chloride (14.4 g) in Et₂O (150 mL) were added under stirring at 0 °C. After stirring for 44 h at 23 °C, ice was added to the mixture. The mixture was extracted three times with CHCl₃, dried over anhydrous Na₂SO₄, and evaporated to dryness. The residue was recrystallized using CH₃OH to afford **5** (15.5 g) in 42% yield. **5**: yellow crystals from methanol;

m.p. 147.5–148.0 °C (lit 145–146 °C [19]); HR-ESI-MS m/z found, 319.1184
[M+H]⁺; Calcd for C₁₇H₁₉O₆, 319.1182; ¹H NMR (CDCl₃): δ 12.50 (1H, s,
2-OH), 7.32 (1H, dd, J = 8.4 and 1.7 Hz, H-6□), 7.28 (1H, d, J = 1.7 Hz,
H-2□), 7.11 (1H, s, H-6), 6.96 (1H, d, J = 8.4 Hz, H-5□), 6.57 (1H, s, H-3),
3.98 (3H, s, OCH₃), 3.95 (3H, s, OCH₃), 3.94 (3H, s, OCH₃), 3.76 (3H, s,
OCH₃).

Synthesis of 6

To hexane-washed NaH (0.58 g) and **5** (5.0 g), dry tetrahydrofuran
(130 mL) was added under a N₂ atmosphere. Methoxymethyl chloride (1.6
mL) was added to the mixture under stirring at 23 °C. After stirring for 1 h,
acetic acid (1 mL) was added. The solution was poured into saturated
NaHCO₃ aq., extracted two times with CHCl₃, dried over anhydrous Na₂SO₄,
and evaporated to dryness. The residue was crystallized with Et₂O–hexane to
afford **6** (5.3 g) in 99 % yield. **6**: pale yellow powder from Et₂O–hexane; m.p.
106.5–107.0 °C; HR-ESIMS m/z found, 385.1245 [M+Na]⁺; Calcd for
C₁₉H₂₂O₇Na, 385.1263; ¹H NMR (CDCl₃): δ 7.53 (1H, d, J = 1.8 Hz, H-2□),
7.34 (1H, dd, J = 8.0 and 1.8 Hz, H-6□), 6.92 (1H, s, H-6), 6.84 (1H, d, J =

205 8.0 Hz, H-5 $\square$), 6.81 (1H, s, H-3), 4.98 (2H, s, 2-OCH $\underline{2}$ OCH $\underline{3}$), 3.95 (3H, s,
206 OCH $\underline{3}$), 3.94 (6H, s, OCH $\underline{3}$ $\times$ 2), 3.85 (3H, s, OCH $\underline{3}$), 3.35 (3H, s,
207 2-OCH $\underline{2}$ OCH $\underline{3}$); IR (KBr) ν cm $^{-1}$: 1655 (C=O), 1269 (C-O).

208

209 *Synthesis of 7 and 8*

210

211 A lithium enolate solution of γ -butyrolactone was prepared by the very
212 slowly addition of γ -butyrolactone (1.3 mL) in dry tetrahydrofuran (30 mL) to
213 1 mol L $^{-1}$ lithium diisopropylamide (8.3 mL) in dry tetrahydrofuran (60 mL)
214 at -78 °C; then, **6** (3.0 g) in dry tetrahydrofuran (30 mL) was added dropwise
215 to this lithium enolate solution of γ -butyrolactone under stirring for 0.5 h at
216 -78 °C. The resulting mixture was stirred at -78 °C for another 1.5 h and it was
217 then added dropwise to a solution of acetic acid (3 mL) in tetrahydrofuran (45
218 mL) at -78 °C. The resulting solution was poured into saturated NaHCO $\underline{3}$ aq.,
219 extracted two times with CHCl $\underline{3}$, dried over anhydrous Na $\underline{2}$ SO $\underline{4}$, and
220 evaporated to dryness. The residue was purified by silica-gel column
221 chromatography (ethyl acetate–hexane=1:1) to afford the major isomer **7** (2.1
222 g) in 53% yield, the minor isomer **8** (0.24 g) in 14% yield, and the recovered **6**
223 (0.88 g) in 29% recovered yield. Major isomer **7**: colorless powder from

224 Et₂O–hexane; m.p. 147.0 °C; HR-ESI-MS *m/z* found, 453.1546 [M+Na]⁺;
 225 Calcd for C₂₃H₂₈O₉Na, 453.1525; ¹H NMR (CDCl₃): δ 7.11 (1H, s, H-6),
 226 6.95 (1H, d, *J* = 2.0 Hz, H-2□), 6.79 (1H, dd, *J* = 8.0 and 2.0 Hz, H-6□), 6.75
 227 (1H, d, *J* = 8.0 Hz, H-5□), 6.71 (1H, s, H-3), 4.83 (1H, d, *J* = 7.0 Hz,
 228 2-OCH₂OCH₃), 4.81 (1H, brs, OH), 4.67 (1H, d, *J* = 7.0 Hz, 2-OCH₂OCH₃),
 229 4.26 (1H, dt, *J* = 15.3 and 8.6 Hz, H-4a□), 4.15 (1H, dd, *J* = 15.3 and 8.2 Hz,
 230 H-4b□), 3.87 (6H, s, OCH₃), 3.83 (6H, s, OCH₃ × 2), 3.81 (3H, s, OCH₃), 3.64
 231 (1H, brt, *J* = 8.6 Hz, H-2□), 3.25 (3H, s, 2-OCH₂OCH₃), 2.39 (1H, m, H-3a□),
 232 2.01 (1H, m, H-3b□); IR (KBr) ν cm⁻¹: 3416 (OH), 1736 (C=O), 1255, 1217,
 233 1148 (C-O). Minor isomer **8**: colorless cubes from CHCl₃–Et₂O–hexane; m.p.
 234 130.0–131.0 °C; HR-ESI-MS *m/z* found, 453.1539 [M+Na]⁺; Calcd for
 235 C₂₃H₂₈O₉Na, 453.1525; ¹H NMR (CDCl₃): δ 7.27 (1H, s, H-6), 6.99 (1H, d,
 236 *J* = 2.2 Hz, H-2□), 6.94 (1H, dd, *J* = 8.4 and 2.2 Hz, H-6□), 6.76 (1H, d, *J* =
 237 8.4 Hz, H-5□), 6.68 (1H, s, H-3), 4.66 (1H, d, *J* = 6.8 Hz, 2-OCH₂OCH₃),
 238 4.60 (1H, d, *J* = 6.8 Hz, 2-OCH₂OCH₃), 4.27 (1H, m, H-4a□), 4.21 (1H, m,
 239 H-4b□), 4.00 (1H, brt, *J* = 9.0 Hz, H-2□), 3.91 (3H, s, OCH₃), 3.88 (1H, brs,
 240 OH), 3.84 (6H, s, OCH₃ × 2), 3.81 (3H, s, OCH₃), 3.23 (3H, s, 2-OCH₂OCH₃),
 241 2.37 (1H, m, H-3a□), 2.15 (1H, m, H-3b□); IR (KBr) ν cm⁻¹: 3422 (OH),

1767 (C=O), 1263 (C-O).

Synthesis of **11** and **12**

To a solution of **7** (1.0 g) in dry pyridine (30 mL), thionyl chloride (0.19 mL) was added dropwise at 0 °C. After standing for 1 h at 0 °C, the mixture was poured into saturated NaHCO₃ aq., extracted three times with CHCl₃, dried over anhydrous Na₂SO₄, and evaporated to dryness to afford a mixture of **9** and **10**. Readable peaks in the ¹H NMR spectrum of major isomer **9** (CDCl₃): δ 6.50 (1H, s, H-3), 4.94 (2H, brs, 2-OCH₂OCH₃), 4.30 (2H, t, *J* = 7.2 Hz, H-4□), 3.90 (3H, s, OCH₃), 3.89 (3H, s, OCH₃), 3.79 (3H, s, OCH₃), 3.75 (3H, s, OCH₃), 3.32 (3H, s, 2-OCH₂OCH₃), 3.20 (2H, t, *J*=7.2 Hz, H-3□), Readable peaks in ¹H-NMR of minor isomer **10** (CDCl₃): δ 6.49 (1H, s, H-3), 4.96 (2H, brs, 2-OCH₂OCH₃), 4.31 (2H, t, *J*=7.2 Hz, H-3□), 3.87 (3H, s, OCH₃), 3.82 (3H, s, OCH₃), 3.74 (3H, s, OCH₃), 3.36 (3H, s, 2-OCH₂OCH₃), 2.90 (2H, brt, *J*=6.3 Hz, H-3□). Without any further purification, the mixture was dissolved in acetic acid–H₂O solution (1:1, 50 mL). The solution was maintained at 80 °C for 11 h and then evaporated *in vacuo*. The residue was purified by silica-gel column chromatography (ethyl acetate–hexane = 1:2) to

261 afford **11** (0.61 g) in 70% yield and **12** (0.15 g) in 16% yield. **11**: colourless
262 powder from Et₂O–hexane; m.p. 201.5–202.0 °C; HR-ESI-MS *m/z* found,
263 409.1257 [M+Na]⁺; Calcd for C₂₁H₂₂O₇Na, 409.1263; ¹H-NMR (CDCl₃): δ
264 7.02 (1H, d, *J* = 7.6 Hz, H-5□), 6.89 (1H, s, H-6), 6.82 (1H, dd, *J* = 7.6 and
265 2.0 Hz, H-6□), 6.79 (1H, d, *J* = 2.0 Hz, H-2□), 6.42 (1H, s, H-3), 3.98 (3H, s,
266 OCH₃), 3.95 (3H, s, OCH₃), 3.88 (3H, s, OCH₃), 3.79 (2H, m, H-4□), 3.66
267 (3H, s, OCH₃), 2.70 (2H, m, H-3□); IR (KBr) ν cm⁻¹: 3431(OH), 1676 (C=O),
268 1252 (C-O). **12**: pale yellow powder from Et₂O-hexane; m.p. 162.0–162.5 °C;
269 HR-ESIMS *m/z* found, 451.1363 [M+Na]⁺; Calcd for C₂₃H₂₄O₈Na, 451.1369;
270 ¹H-NMR (CDCl₃): δ 7.02 (1H, d, *J* = 8.2 Hz, H-5□), 6.88 (1H, s, H-6), 6.83
271 (1H, dd, *J* = 8.2 and 1.2 Hz, H-6□), 6.76 (1H, d, *J* = 1.2 Hz, H-2□), 6.42 (1H,
272 s, H-3), 4.22 (2H, m, H-4□), 3.98 (3H, s, OCH₃), 3.95 (3H, s, OCH₃), 3.90
273 (3H, s, OCH₃), 3.67 (3H, s, OCH₃), 2.75 (2H, m, H-3□), 1.98 (3H, s,
274 2-OCOCH₃); IR (KBr) ν cm⁻¹: 1747 (C=O), 1701 (C=O), 1225 (C-O).

275

276 *Synthesis of 13*

277

278 To a solution of **11** (20 mg) in CH₂Cl₂ (1 mL), pyridinium

chlorochromate (21 mg) was added at 23 °C under stirring. Upon the addition of Et₂O, precipitate formed, which was removed by filtration using a small silica gel column. The solution was evaporated to dryness, and the residue was purified by silica gel TLC (ethyl acetate–hexane = 1:1) to afford **13** (14 mg) in 70% yield. **13**: colourless amorphous solid; HR-ESI-MS m/z found, 407.1104 [M+Na]⁺; Calcd for C₂₁H₂₀O₇Na, 407.1107; ¹H NMR (CDCl₃): δ ppm 9.75 (1H, d, J = 0.8 Hz, H-4□), 6.99 (1H, d, J = 8.4 Hz, H-5□), 6.90 (1H, s, H-6), 6.79 (1H, dd, J = 8.4 and 1.4 Hz, H-6□), 6.75 (1H, d, J = 1.4 Hz, H-2□), 6.51 (1H, s, H-3), 3.96 (3H, s, OCH₃ × 2), 3.86 (3H, s, OCH₃), 3.69 (3H, s), 3.58 (1H, brd, J = 17.0 Hz, H-3a□), 3.48 (1H, brd, J = 17.0 Hz, H-3b□); IR (KBr) ν cm⁻¹: 2837 (CHO), 1701 (C=O), 1254 (C-O).

Synthesis of tetramethyl derivative of 1 (14)

To a solution of **13** (8 mg) in CH₂Cl₂ (1 mL), 0.1 mL of BBr₃ (1 mol L⁻¹ CH₂Cl₂ solution) was added at 0 °C. After stirring for 15 min, the mixture was poured into saturated NaHCO₃ aq., extracted three times with CHCl₃, dried over anhydrous Na₂SO₄, and evaporated to dryness. The residue was purified by silica gel TLC (1% CH₃OH in CHCl₃) to afford **14** (5 mg) in 66 %

yield. **14**: colourless powder; m.p. 253.5–254.5 °C; HR-ESI-MS m/z found, 389.1016 $[M+Na]^+$; Calcd for $C_{21}H_{18}O_6Na$, 389.1001; 1H NMR ($CDCl_3$): δ 8.22 (1H, d, $J = 8.6$ Hz, H-3 $\square$), 8.19 (1H, s, H-2 $\square$), 7.98 (1H, s, H-6), 7.77 (1H, d, $J = 8.6$ Hz, H-4 $\square$), 7.27 (1H, s, H-5 $\square$), 7.01 (1H, s, H-3), 4.09 (3H, s, OCH_3), 4.08 (3H, s, OCH_3), 4.00 (6H, s, $OCH_3 \times 2$); IR (KBr) ν cm^{-1} : 1724 (C=O), 1248 (C-O).

Total synthesis of inhibitor 1

To a solution of **14** (3.8 mg) in CH_2Cl_2 (0.8 mL), 0.18 mL of BBr_3 (1 mol L^{-1} CH_2Cl_2 solution) was added at 23 °C under a N_2 atmosphere. The mixture was stirred in a screw-capped tube at 23 °C for 12 h, after which H_2O (0.2 mL) and CH_3OH (1 mL) were added. This solution was purified by using preparative HPLC (column, Cosmosil C18 AR-II, 250 $\times$ 20 mm i.d.; CH_3CN –1% CH_3COOH in $H_2O = 3:7$; flow rate, 10 mL/min; detection, 330 nm) to collect the peak eluted at 14 min as the retention time. Removal of the solvent afforded **1** (2.8 mg) in 66% yield. The 1H NMR, HR-ESI-MS, and MS^E (an automated MS/MS) data showed that the structure of synthesized **1** was identical to that of isolated **1**. Synthesized **1**: pale yellow powder; m.p. (in

sealed tube) > 195 °C gradually decomposed; HR-ESI-MS m/z found, 309.0399 [M-H]⁻ and 311.0549 [M+H]⁺; Calcd for C₁₇H₉O₆, 309.0399 and for C₁₇H₁₁O₆, 311.0556; ¹H NMR (CD₃OD) δ : 8.26 (1H, s, H-2□), 8.01 (1H, s, H-6), 7.94 (1H, d, J = 8.4 Hz, H-3□), 7.64 (1H, d, J = 8.4 Hz, H-4□), 7.27 (1H, s, H-5□), 6.87 (1H, s, H-3); IR (KBr) ν cm⁻¹: 3472 (OH), 1684 (C=O), 1245 (C-O).

Results and discussion

For screening of XO-inhibitory activity, oxidation products derived from 22 antioxidative phenolic compounds, most of which are recognized as polyphenols, were employed (Fig. 1). These oxidation products were obtained via a radical oxidation reaction with oxygen in the presence of a ferric ion catalyst. Figure 2 shows the XO inhibition results, which present the activity of the oxidation products and their parent phenols in a concentration of 18 mg mL⁻¹. Although several phenols exhibited potent XO-inhibitory activity in their intact forms (which was reduced after their oxidation), the oxidation products from caffeic acid, resveratrol, rosmarinic acid, and taxifolin showed enhanced inhibitory activity against XO, while their corresponding original

phenols showed weak or negligible activity. These results show that some compounds produced via oxidation of the phenolic precursors can be used as powerful XO inhibitor. In this regard, apparent difference in the inhibitory activity of caffeic acid ($2 \pm 5\%$ inhibition) and its oxidation product ($31 \pm 2\%$) should be noted. These results strongly showed that highly potent XO inhibitors are present in the oxidation product derived from caffeic acid. To identify the active substances, an assay-guided purification was carried out.

Caffeic acid was oxidized in the presence of a catalytic amount of ferric ions under oxygen at 40 °C for 2 weeks. The product was first partitioned with ethyl acetate and saturated NaCl aq. to remove Fe salts. The ethyl acetate-soluble material was next fractionated using gel permeation chromatography (Toyopearl HW-40F) to afford 10 fractions, and the potent activity was observed in fractions 8 and 9 (Fig. 3). HPLC analysis of the two fractions was carried out (Fig. 4). In the HPLC profile of fraction 8, three major peaks were observed at retention times of 13.1, 15.6, 19.2 min, in fraction 9, four major peaks were observed at retention times of 15.6, 16.5, 18.1, and 19.2 min. By comparing these retention times, as well as UV spectroscopic data for the peaks, the peak compound at 13.1 min was determined to correspond to caffeic acid, with the peaks at retention times

19.2, 15.6, 16.5, and 18.1 min assigned to new compounds **1–4**, respectively. Preparative HPLC was used for the purification of the two active fractions and afforded compounds **1–4** in their pure forms, thus allowing their XO-inhibitory activities to be examined (Fig. 5). The XO-inhibitory data in Fig. 5 show that compound **1** was the most active, followed by compounds **2** and **4**.

The chemical structure of compound **1**, the most potent inhibitory compound, was next elucidated. High-resolution electrospray mass spectral (ESI-MS) analysis of **1** showed a deprotonated molecular peak at m/z 309.0406 $[M-H]^-$, which confirmed that **1** has a molecular formula of $C_{17}H_{10}O_6$, indicating that **1** is a dimeric derivative of caffeic acid. In the 1H NMR of **1**, only four singlet and two coupled proton signals were observed in the aromatic region of the spectrum. We concluded that the absence of four proton signals, whose presence was predicted from the molecular formula, might be because of the presence of phenolic hydroxy groups. This was confirmed by acetylating **1**, which afforded the tetra-acetate of **1** [1H NMR: δ 4.09 (3H, s), 4.08 (3H, s) 4.00 (6H, s); MS m/z 479 $(M+H)^+$]. Unfortunately, we could not isolate a sufficient amount of **1** to obtain ^{13}C NMR data. However, we were able to employ HMQC and HMBC as proton-observed 2D

NMR techniques to analyse the proton–carbon network and estimate the ^{13}C chemical shifts from projection of these 2D spectra (Table 1). The proton–carbon long-range couplings, which were deduced from HMBC experiments (Fig. 6), revealed the unique structure of **1** bearing three benzene rings with a lactone moiety. The presence of lactone segment was confirmed by a predicted ^{13}C signal at 163 ppm and a peak at 1693 cm^{-1} in the IR spectrum. The planar structure of **1** was also confirmed by the very strong NOE correlation between protons at 8.26 (H-2') and 8.01 (H-6) ppm. A possible mechanism for the formation of **1** from caffeic acid via Fe-catalysed radical oxidation is depicted in Fig. 7.

The synthesis of **1** was investigated for confirming the elucidated structure, as well as for developing a reliable preparation method for future investigations. First, benzophenone **5** was prepared in 42% yield according to a reported method [19] from 1,2,4-trimethoxybenzene. After protecting the phenolic hydroxy group with a methoxymethyl group, a lithium enolate of γ -butyrolactone was reacted with **5** at $-78\text{ }^{\circ}\text{C}$ to afford two adducts, **7** and **8**, in 53% and 14% yield, respectively. Although these adducts were diastereomers, major isomer **7** was used for the next reaction without the absolute determination of its stereochemistry. It was treated with thionyl chloride in

pyridine at 0 °C to afford a mixture of dehydrated products **9** and **10**. Without separating and determining the stereochemistry of either product, the methoxymethyl group was removed by reaction with acetic acid–H₂O at 80 °C. Fortunately, major isomer **9** afforded desired product **11** in 70% yield (calculated on the basis of **7**), by deprotection and subsequent lactone rearrangement, along with acetate **12** in 16% yield. Acetate **12** could be formed from minor isomer **10** because the *E*-relationship of lactone and phenol in deprotected **10** did not promote lactone rearrangement. After the oxidation of the hydroxy group of **11**, the obtained aldehyde **13** was reacted with BBr₃ at 0 °C to regioselectively form cyclized compound **14** in 60% yield. BBr₃ is one of the most effective deprotection reagents for alkylated phenols [20]; however, the Lewis acid properties of BBr₃ allowed the cyclization to be achieved. Further treatment of **11** with BBr₃ at an elevated temperature, 23 °C, afforded **1** in 60% yield. Spectroscopic data obtained for synthetic **1** were identical with those obtained for isolated **1**. Thus, **1** was determined to have the structure shown in Fig. 6. The structures of moderately active **2** and **4** were not elucidated in this study.

Concentration effect of **1** on XO-inhibitory activity was examined, along with that of caffeic acid and allopurinol. Allopurinol is a potent inhibitor

of XO and a medicine primarily used to treat hyperuricemia [21]. Fig. 7 shows concentration-dependent inhibition of **1**, with its IC₅₀ value determined to be 60 nmol L⁻¹ from the concentration response curve; the IC₅₀ value of **1** is much lower than those of allopurinol (1,000 nmol L⁻¹) and caffeic acid (> 10⁶ nmol L⁻¹). On the basis of the IC₅₀ values, compound **1** is estimated to be a 17-fold stronger XO inhibitor than allopurinol and also a desirable candidate for use as gout medicine.

Conclusions

Fe-catalysed radical oxidation of antioxidative polyphenols and related phenols was found to be an effective method of developing potent XO inhibitors. From the oxidation of caffeic acid, a highly potent XO inhibitor was identified, with its inhibitory activity estimated to be 17-fold stronger than allopurinol. Its unique chemical structure was determined by both instrumental analysis and synthesis.

Acknowledgements.

This work was supported by JSPS Kakenhi (No. 23500929) and JST A-step (No. AS231Z00426D).

Figure Captions

Fig. 1. Chemical structures of antioxidative phenols used for oxidation.

Fig. 2. Xanthine oxidase inhibitory activity of antioxidative phenols and their oxidation products

■ : phenols ($100 \mu\text{mol L}^{-1}$); □ : oxidized phenols ($100 \mu\text{mol L}^{-1}$ as from estimated concentration from original phenols). Allopurinol ($1 \mu\text{mol L}^{-1}$) was used for positive control. Data are expressed as mean $\pm$ SD ($n = 3$).

Fig. 3. Xanthine oxidase inhibitory activity of fractions (18 mg L^{-1}) obtained from gel permeation chromatography of oxidation product of caffeic acid.

[Data are expressed as mean $\pm$ SD ($n = 3$).]

Fig. 4. HPLC profiles of active fractions 8 and 9 obtained from the gel permeation chromatography.

[HPLC conditions: column; Cosmosil 5C₁₈-AR-II (250 × 4.6 mm i. d., Nacalai Tesque, Kyoto, Japan); solvent ; A, 1% acetic acid in H₂O(A); B, CH₃CN, linear gradient from B 5% (0 min) to 100% (40 min) and then B 100% for 10 min. flow rate; 1.0 mL min⁻¹; detection wave length, 280 nm.]

Fig. 5. Xanthine oxidase inhibitory activity of peak compounds 1–4 and caffeic acid (18 mg L⁻¹), isolated from active fractions 8 and 9.

[Data were expressed as mean ± SD (n = 3).]

Fig. 6 Chemical structure of xanthine oxidase (XO) inhibitor 1 (left) and observed C-H long range correlations in HMBC of 1 (right).

Carbon positions on the structure are temporarily numbered.

469

470 **Fig. 7.** Proposed formation mechanism of 1 from caffeic acid.

471

472

473 **Fig. 8.** Synthetic route of 1.

474

475 Carbon positions on each structure are temporarily numbered.

476 [MOM: methoxymethyl; Py: pyridine; PCC: pyridinium chlorochromate.]

477

478

479 **Fig. 9.** Concentration-depended xanthine oxidase inhibitory data of compound

480 1, allopurinol, and caffeic acid.

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550

Table 1.NMR data of xanthine oxidase inhibitor **1**

Position ^a	¹³ C chemical shift ^b	Correlated protons in HMBC	¹ H chemical shift ^b (<i>J</i> in Hz)	Correlated protons in NOESY
1 α	134	H-2', H-3''		
1	111	H-3		
2	148	H-6		
3	103		6.87 (s)	
4	142	H-3, H-6		
5	146	H-3		
6	112		8.01 (s)	H-2'
1'	116	H-4''		
2'	110		8.26 (s)	H-6
3'	149	H-2'		
4'	147	H-5'		
5'	111	H-4''	7.27 (s)	H-4''
6'	123	H-5', H-4''		
1''	163	H-3''		
2''	— ^c			
3''	122		7.95 (d, 8.4)	H-4''
4''	126	H-5'	7.64 (d, 8.4)	H-5', H-3''

(400 MHz for ¹H in CD₃OD)

^a Position numbering is tentative as shown in Fig. 6.

^b ¹³C chemical shift was estimated from F_1 projection of HMQC and HMBC of **1**.

^c Chemical shift could not be determined.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6

Fig. 8.

Fig. 9.