JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 393–400 **393**

Identification of Urinary and Intestinal Bacterial Metabolites of Ellagitannin Geraniin in Rats

HIDEYUKI ITO,* AYUMU IGUCHI, AND TSUTOMU HATANO

Department of Pharmacognosy, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Tsushima, Okayama 700-8530, Japan

Hydrolyzable tannins, including ellagitannins, occur in foods such as berries and nuts. Various biological activities, including antioxidant, antiviral, and antitumor activities, have been noted and reported for ellagitannins, but the absorption and metabolism of purified ellagitannins are poorly understood. We describe herein the characterization of urinary and intestinal microbial metabolites in rats after the ingestion of ellagitannins. Urine samples were collected after oral administration of ellagitannins such as geraniin (**1**), corilagin (**2**), and their related polyphenols. The suspension of rat intestinal microflora was anaerobically incubated with ellagitannins. Each sample was separated by column chromatography and/or preparative HPLC to give seven metabolites, **M1**−**M7**. The structures of these metabolites were determined on the basis of spectroscopic data and chemical evidence. These compounds, except for **M1**, were characterized as ellagitannin metabolites for the first time. Furthermore, among four major metabolites (**M1**–**M4**) in urine, **M2** showed an antioxidant activity comparable to intact geraniin and related polyphenols.

KEYWORDS: Ellagitannin; metabolites; urine; intestinal microflora; geraniin; corilagin; ellagic acid; gallic acid; brevifolincarboxylic acid; radical scavenger

INTRODUCTION

Tannins are widespread constituents of the vegetable kingdom and are classified into two groups, hydrolyzable and condensed tannins (*1*). Ellagitannins belong to the hydrolyzable tannin group, which occur in foods such as raspberries, strawberries, blackberries, pomegranates, almonds, and walnuts (*2*). In vitro and in vivo studies of ellagitannins have demonstrated various biological activities including antioxidant (*3*), antiviral (*4*), antimutagenic (*5*), antimicrobial (*6, 7*), and antitumor promotion activities (*8, 9*), suggesting that the consumption of ellagitannins may play a role in human health benefits. Nevertheless, bioavailability after the ingestion of purified ellagitannins is not fully understood. Ellagic acid is one of the hydrolysates of ellagitannins, the metabolism of which has previously been studied. The metabolite from urine and feces after oral administration of ellagic acid to rats was characterized as 3,8-dihydroxy-6*H*-dibenzo[*b,d*]pyran-6-one (urolithin A) (*10*). Recently, the absorption and metabolism of punicalagin from pomegranate have been reported in rats (*11*) and humans (*12–14*). The metabolism of ellagitannins from several foodstuffs has also been demonstrated in humans (*15*). Although the presence of various metabolites in plasma or urine after the ingestion of juice from fruits rich in ellagitannins has been suggested, the structures of these metabolites have not been clearly characterized to date, except for urolithin A. The aim of the present study was to investigate the metabolic fate of ellagitannins through the isolation and structural elucidation of possible urinary and intestinal microbial metabolites in rats after the ingestion of purified ellagitannins, geraniin (**1**). A typical ellagitannin, geraniin, composed of all common acyl units such as a galloyl, hexahydroxydiphenoyl, and dehydrohexahydroxydiphenoyl groups, was used in this study. Moreover, the identified metabolites were evaluated for in vitro antioxidant activity in comparison with intact ellagitannins and related polyphenols.

* Corresponding author. Fax: +81 86 251 7926; e-mail: hito@cc.okayama-u.ac.jp.

MATERIALS AND METHODS

General experiments. ESIMS and high-resolution (HR) ESIMS were performed with a Micromass AutoSpec OA-Tof using 50% aqueous MeOH containing 0.1% NH_4OAc as a solvent. ^1H and ^{13}C NMR spectra were recorded on a Varian INOVA AS600 (600 MHz for ^1H and 150 MHz for ^{13}C) and Varian Mercury 300 (300 MHz for ^1H NMR), and chemical shifts are given in δ (ppm) values relative to that of the solvent [CD_3OD (δ_H 3.35; δ_C 49.0)] on a tetramethylsilane scale. The standard pulse sequences programmed for the instrument (INOVA AS600) were used for each 2D measurement (COSY, NOESY, HSQC, and HMBC). J_{CH} were set at 8 Hz in the HMBC spectra. Normal-phase HPLC was conducted on a 250 × 4.6 mm i.d. YMC-Pack SIL A-003 column (YMC Co., Ltd., Kyoto, Japan) developed with *n*-hexane/MeOH/tetrahydrofuran/formic acid (55:33:11:1) containing oxalic acid (450 mg/L) at a flow rate of 1.5 mL/min and with detection at 280 nm, at ambient temperature. Reversed phase HPLC was performed on a 250 × 4.6 mm i.d. Inertsil ODS-3 column (GL Sciences, Tokyo, Japan) developed with $H_2O/CH_3CN/HCOOH$ (74:25:1 or 72:25:3) at a flow rate of 1.0 mL/min and with detection at 280 or 305 nm, at 40 °C. Preparative reversed-phase HPLC was performed with a 250 × 4.6 mm i.d. Inertsil ODS-3 column with H_2O/

10.1021/jf0726942 CCC: $40.75
Published on Web 12/29/2007

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for **M1–M7** in CD_3OD

	M1		M2		M3		M4		M5		M6		M7	
position	δ_H[a]	δ_C[b]	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	7.98 d (9.0)[c]	124.6	7.87 d (9.0)	124.6	8.03 d (8.4)	125.0	7.90 d (9.0)	125.0	8.46 d (9.0)	119.0	8.94 d (9.0)	129.8	8.88 d (9.0)	129.8
2	6.86 dd (2.4, 9.0)	114.2	6.85 dd (2.4, 9.0)	114.1	6.87 dd (2.4, 8.4)	114.2	6.86 dd (2.4, 9.0)	114.2	6.79 d (9.0)	112.2	6.80 dd (2.4, 9.0)	113.2	6.79 dd (2.4, 9.0)	113.3
3		160.1		160.2		160.3		160.6		146.3		158.9		158.5
4	6.76 d (2.4)	104.1	6.74 d (2.4)	104.0	6.75 d (2.4)	104.0	6.75 d (2.4)	104.0		133.2	6.73 d (2.4)	103.7	6.75 d (2.4)	103.7
4a		152.7		153.2		153.3		153.4		140.9		152.4		152.0
6		163.5		163.6		163.5		163.6		164.7		164.2		163.8
6a		121.8		112.7		113.8		111.9		110.5		112.2		123.1
7	7.64 d (2.4)	114.9	7.62 s	115.3	7.61 s	115.1	7.70 s	111.5	7.37 s	107.6	7.40 s	108.1	7.29 d (2.4)	107.3
8		158.5		147.4		148.3		149.6		147.4		146.5		158.8
9	7.36 dd (2.4, 9.0)	125.3		155.0		156.3	7.49 s	155.7		148.3		141.9	6.85 d (2.4)	111.0
10	8.06 d (9.0)	124.3	7.46 s	107.4	7.59 s	103.8		107.6		144.6		143.8		157.6
10a		129.2		131.7		131.5		132.9		117.1		118.2		117.2
10b		111.8		111.8		112.4		111.5		113.5		112.2		112.0
8-OCH_3					4.12 s	56.8	4.02 s	56.6						
9-OCH_3														

[a] 600 MHz. [b] 150 MHz. [c] *J* values (Hz) are in parentheses.

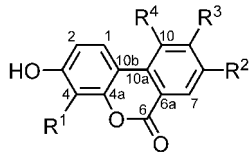
CH_3CN/HCOOH (74:25:1) [system A] and/or a 300 × 10 mm i.d. YMC-Pack ODS A-324 (YMC Co., Ltd.) with H_2O/MeOH (55:45) containing 1% HCOOH [system B]. Column chromatography was conducted on MCI GEL CHP-20P (75–150 μm) (Mitsubishi Kasei Co., Tokyo, Japan) and Sephadex LH-20 (Pharmacia).

Chemicals. Geraniin (**1**) and brevifolin (**6**) were isolated from *Geranium thunbergii* (*16–18*), and corilagin (**2**) and brevifolincarboxylic acid (**5**) were isolated from *Phyllanthus flexuosus* (*19*). Gallic acid (**3**), ellagic acid (**4**), and β-glucuronidase (G7896) were purchased from Sigma-Aldrich Japan K.K.

Animal Experiments. Male SD rats, 6 weeks of age, weighing ca. 180 g, were obtained from Charles River Laboratories, Yokohama, Japan. The rats were kept at a controlled temperature of 23 °C under a 12 h light/dark cycle. Each rat was placed in a metabolic cage (Natsume Seisakusho, Tokyo, Japan) with free access to tap water and a type MF standard diet (Oriental Yeast, Tokyo, Japan) for one week before the experiments. The individual ellagitannins and related polyphenols, **1**, **2**, **4**, and **5** (each 20 mg/head) were suspended in 2 mL of 5% gum acacia solution. Each suspension was orally administered by direct stomach intubation. Control rats were administered a 5% gum acacia solution as a vehicle. Urine for analysis was collected at −24–0, 0–12, 12–24, 24–48, and 48–72 h in a 100 mL Erlenmeyer flask containing 5 mL toluene. Urine for the isolation of metabolites was collected over 48 h. All samples of urine with removed toluene were stored at − 20 °C until use. The experimental protocol was approved by the animal research control committee of Okayama University.

Isolation and Structural Elucidation of Metabolites from Urine and Intestinal Bacteria in Rats. Urine (250 mL) collected from four rats after oral administration of **1** was acidified with 0.58 M acetic acid (20 mL) and incubated with β-glucuronidase (1×10^5 units) containing sulfatase activity for 1 h at 37 °C (*20, 21*). After the addition of 1 M HCl (10 mL), the sample was subjected to column chromatography over a 30 × 1.1 cm i.d. MCI GEL CHP-20P column developed with H_2O and increasing amounts of MeOH ($H_2O \rightarrow 10 \rightarrow 20 \rightarrow 30 \rightarrow 40 \rightarrow 50 \rightarrow 60 \rightarrow$ 100% MeOH) and 70% aqueous acetone. The combined eluates of 60% MeOH (20.0 mg) and 100% MeOH (13.7 mg) portions were purified by preparative reversed HPLC [system A] to give **M1** (2.6 mg), **M2** (2.3 mg), **M3** (0.8 mg), and **M4** (0.6 mg). **M1**: a white powder, ^{1}H and ^{13}C NMR data, see **Table 1**; ESIMS *m/z* 229 $[M + H]^+$; High resolution (HR)ESIMS *m/z* 229.0495 $[M + H]^+$, $C_{13}H_8O_4$ + H, requires 229.0501. **M2**: a white powder, ^{1}H and ^{13}C NMR data, see **Table 1**; ESIMS *m/z* 245 $[M + H]^+$; HRESIMS *m/z* 245.0449 $[M + H]^+$, $C_{13}H_8O_5$ + H, requires 245.0450. **M3**: a white powder, ^{1}H and ^{13}C NMR data, see **Table 1**; ESIMS *m/z* 259 $[M + H]^+$; HRESIMS *m/z* 259.0603 $[M + H]^+$, $C_{14}H_{10}O_5$ + H, requires 259.0606. **M4**: a white powder, ^{1}H and ^{13}C NMR data, see **Table 1**; ESIMS *m/z* 259 $[M + H]^+$; HRESIMS *m/z* 259.0590 $[M + H]^+$, $C_{14}H_{10}O_5$ + H, requires 259.0606.

A fecal suspension (10% w/v) from four rats was prepared by homogenizing rat feces with a medium (7.5 g of $Na_2HPO_4 \cdot 12H_2O$, 2.25 g of KH_2PO_4, 0.4 g of Tween 80, 0.3 g of $Na_2S \cdot 9H_2O$, and 0.25 g of agar in 500 mL of distilled water and autoclaved at 121 °C for 15 min prior to use). After centrifugation at 1,000 rpm for 5 min, the supernatant was used as a microflora suspension. An aliquot (80 mL) of the suspension was incubated with a solution (800 μL) of 10% (w/v) **1** in DMSO at 37 °C with AnaeroPack disposable O_2-absorbing and CO_2-generating agent (Mitsubishi Gas Chemical Co, Inc., Tokyo, Japan) in an anaerobic jar. After incubation for 96 h, the reaction mixture was separated by column chromatography over MCI GEL CHP-20P in the same manner as above. **M5** (9.4 mg) was furnished from the 40% MeOH eluate. The combined eluates of 50% and 60% MeOH were further purified by preparative reversed HPLC [system A] to afford **M6** (7.6 mg) and **M7** (1.2 mg). **M5**: a white powder, ^{1}H and ^{13}C NMR data, see **Table 1**; ESIMS *m/z* 277 $[M + H]^+$; HRESIMS *m/z* 277.0343 $[M + H]^+$, $C_{13}H_8O_7$ + H, requires 277.0348. **M6**: a white powder, ^{1}H and ^{13}C NMR data, see **Table 1**; ESIMS *m/z* 261 $[M + H]^+$; HRESIMS *m/z* 261.0398 $[M + H]^+$, $C_{13}H_8O_6$ + H, requires 261.0399. **M7**: a white powder, ^{1}H and ^{13}C NMR data, see **Table 1**; ESIMS *m/z* 245 $[M + H]^+$; HRESIMS *m/z* 245.0449 $[M + H]^+$, $C_{13}H_8O_5$ + H, requires 245.0450.



M1: $R^1=R^3=R^4=H$, $R^2=OH$
M2: $R^1=R^4=H$, $R^2=R^3=OH$
M3: $R^1=R^4=H$, $R^2=OH$, $R^3=OCH_3$
M4: $R^1=R^4=H$, $R^2=OCH_3$, $R^3=OH$
M5: $R^1=R^2=R^3=R^4=OH$
M6: $R^1=H$, $R^2=R^3=R^4=OH$
M7: $R^1=R^3=H$, $R^2=R^4=OH$

Figure 1. Structures of ellagitannin metabolites identified from rat urine and suspension of rat intestinal microflora.

HPLC Analysis of Metabolites. Each collected urine sample (1 mL) from four rats per group, following oral administrations of individual **1**, **2**, **4**, and **5**, was acidified with 0.58 M acetic acid (50 μL) and incubated with β-glucuronidase (20 μL). After acidification with 1 M HCl (40 μL), the reaction mixture was extracted with ethyl acetate (1 mL). The organic layer was evaporated to dryness under nitrogen gas, redissolved in MeOH, filtered through a 0.20 μm, Minisart N filter (Sartorius AG., Goettingen, Germany), and injected (3 μL) into the HPLC system. For quantification of geraniin metabolites, urine (1 mL) postadministration of **1** (50 mg) was acidified with 0.58 M acetic acid (5 μL) containing quercetin (5 mM) in DMSO (5 μL) as an internal standard, with the subsequent deconjugation and extraction steps performed as described above. Urinary metabolites were quantified by comparison with a standard curve obtained using known concentrations (10, 20, 50, and 100 μM) of **M1**. The limits of detection (signal-to-noise = 3) and quantification (signal-to-noise = 10) were 1.5 and 5 μM, respectively. The range of the coefficient of variation and the accuracy for each concentration (n = 3) were calculated to be 2.50–9.57% and 0.79–5.79%, respectively.

Synthesis of Metabolites M1−M4. A solution of 2-bromo-5-methoxybenzoic acid (5 g, Wako Pure Chemical Industries, Osaka, Japan) and $AlCl_3$ (15 g) in chlorobenzene (150 mL) was refluxed for 2.5 h. After cooling, the reaction mixture was added to ice and extracted with diethyl ether (250 mL × 3). The extract of diethyl ether was evaporated to yield 2-bromo-5-hydroxybenzoic acid (4.2 g), which was identified by comparison of the reported physicochemical data (*22*). 2-Bromo-5-hydroxybenzoic acid (3.9 g) and resorcinol (3.9 g, Tokyo Chemical Industry Co., Tokyo, Japan) in a 4 M NaOH solution (9 mL) were heated at 60 °C in a water bath for 30 min. After the addition of 10% aqueous $CuSO_4$ (1.8 mL), the reaction mixture was heated again at 80 °C for 10 min. The precipitate was filtered to give **M1** (1.9 g) as a white powder.

Similar condensation of 2-bromo-4, 5-dimethoxybenzoic acid (3.9 g) with resorcinol (3.3 g) in alkaline solution and $CuSO_4$ catalyst formed 3-hydroxy-8, 9-dimethoxy-6*H*-dibenzo[*b*,*d*]pyran-6-one (**7**, 2.6 g) as a white powder, ^{1}H NMR (300 MHz, CD_3OD) δ: 3.95, 4.06 (3H each, s, $-OC\underline{H}_3$), 6.77 (1H, d, J = 2.4 Hz, H-4), 6.86 (1H, dd, J = 2.4, 8.7 Hz, H-2), 7.61, 7.68 (1H each, s, H-7, 10), 8.10 (1H, d, J = 8.7 Hz, H-1). Demethylation of **7** (2.25 g) with $AlCl_3$ (6.75 g) in chlorobenzene (67.5 mL) at 100 °C for 6 h afforded a mixture of **M2**−**M4**. The mixture was subjected to chromatography over a 43 × 1.1 cm i.d. Sephadex LH-20 column developed with EtOH → EtOH/MeOH (1:1) → MeOH, to furnish **M2** (89.2 mg) from eluates of EtOH and EtOH/MeOH (1:1). The MeOH eluate was further purified by preparative HPLC [system B] to yield **M2** (38.2 mg), **M3** (14.1 mg), and **M4** (29.4 mg).

Radical Scavenging Activity of Superoxide Anion Activity Generated by Xanthine/XOD System. The reaction mixture consisted of the sample in 24% aqueous DMSO (200 μL), 0.2 M phosphate buffer (pH 7.5, 100 μL), 0.75 mM NBT in water (600 μL), 0.1 mM xanthine in water (400 μL), and 5 mM diethylenetriaminepentaacetic acid (DTPA) in phosphate buffer (100 μL). After preincubation at 25 °C for 10 min, XOD (80 U/mL) in phosphate buffer (200 μL) was added to the reaction mixture. The final mixture was incubated at 25 °C for 20 min, and then the absorbance was recorded at 560 nm. A blank was measured in the same way with no enzyme. Aqueous DMSO (24%) was used as a control. The IC_{50} value is the concentration of an antioxidant quenching 50% radicals in the reaction mixture under the assay condition. Triplicate reactions were carried out for each level of every individual sample.

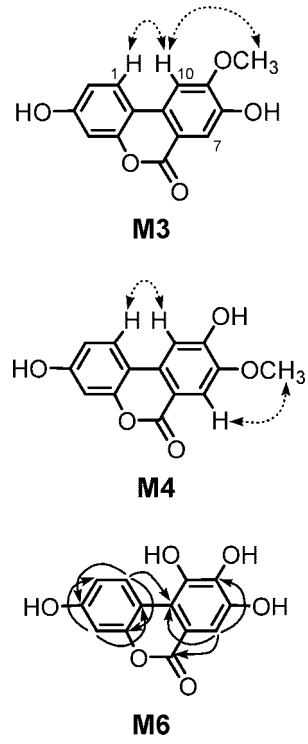


Figure 2. Key NOE correlations (dashed arrows) of **M3** and **M4**, and HMBC correlations (arrows point from proton to carbon) of **M6**.

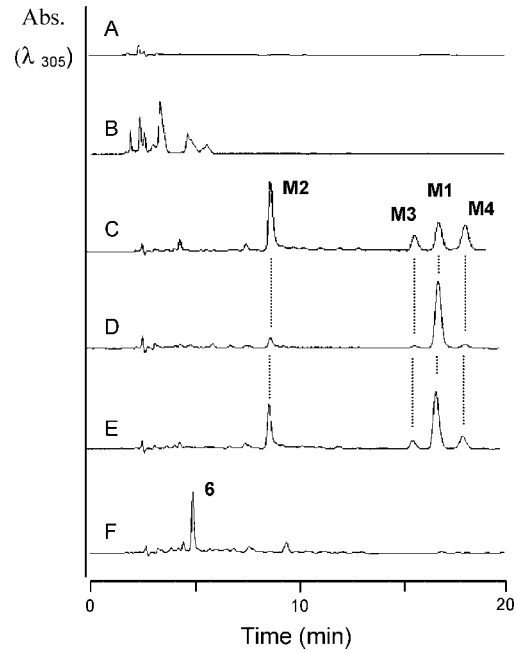


Figure 3. HPLC profiles of metabolites in rat urine collected 24–48 h after oral administration of ellagitannins or related polyphenols. (**A**) Profile of blank urine sample; (**B**) profile of enzyme-untreated urine after intake of **1**; (**C**) profile of enzyme-treated urine after intake of **1**; (**D**) profile of enzyme-treated urine after intake of **2**; (**E**) profile of enzyme-treated urine after intake of **4**; (**F**) profile of enzyme-treated urine, collected 0–12 h after intake of **5**.

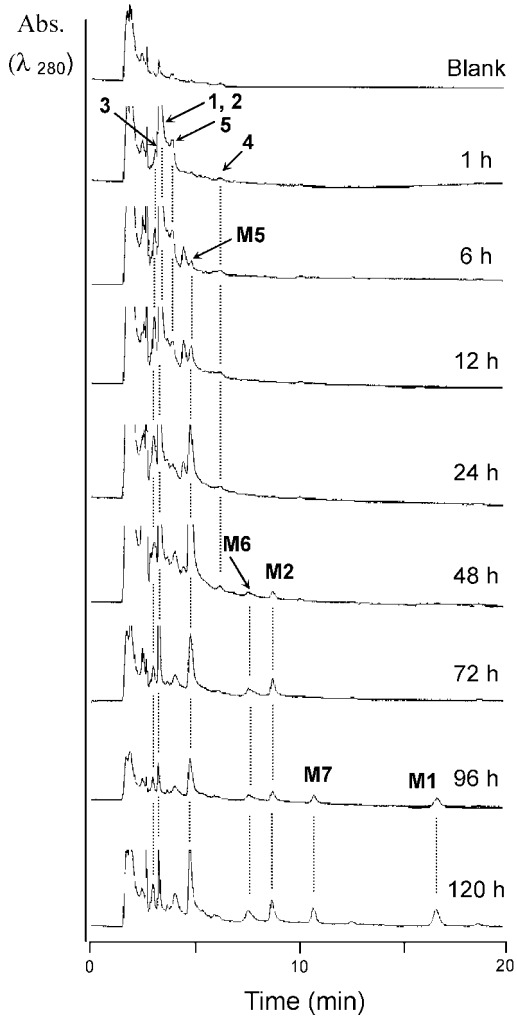


Figure 4. HPLC profiles of metabolites formed by anaerobic incubation of **1** with intestinal microflora suspension from rat feces.

Radical Scavenging Activity in Superoxide Anion Activity Generated by the PMS/NADH System. Superoxide anions were generated nonenzymatically by the PMS/NADH system (*23*). A mixture of sample solution dissolved in 24% aqueous DMSO (200 μL), 5 mmol/L DTPA (360 μL), 0.2 M phosphate buffer (pH 7.5, 200 μL), 0.38 mM NBT in water (200 μL), 0.24 mM NADH in phosphate buffer (200 μL), and 57 μM PMS in water (40 μL) was kept for 20 min at ambient temperature. Absorbance at 560 nm was measured against control 24% aqueous DMSO. Radical scavenging activity (IC_{50}) was expressed as a percent compared to superoxide anion generation of the control solution. Reactions were carried out in triplicate.

Radical Scavenging Activity on DPPH. EtOH solutions (500 μL) of each compound at various concentrations (0.2–100 μM) were added to a solution of DPPH (Nacalai Tesque, Kyoto, Japan) (30 μM) in EtOH (500 μL), and the reaction mixture was shaken vigorously. After holding at ambient temperature for 5 min, the remaining DPPH was determined by colorimetry at 520 nm, and the radical scavenging activity of each compound was expressed using the ratio of the absorption of DPPH (%) relative to the control EtOH (100%) in the absence of a sample. The IC_{50} value was calculated by comparing the concentrations of control and tested compounds. All tests were performed in triplicate.

RESULTS AND DISCUSSION

Structural Elucidation of Metabolites. Urine samples were collected for 48 h following oral administration of **1**, and the samples were incubated with β-glucuronidase and separated by repeated column chromatography to afford four major metabolites (**M1**−**M4**). Geraniin was anaerobically incubated with a suspension of rat intestinal microflora. After 96 h of incubation, the suspension was filtered and then subjected to column chromatography and/or preparative HPLC, to give five metabolites (**M1**, **M2**, **M5**−**M7**).

Structures of **M1** (urolithin A; 3,8-dihydroxy-6*H*-dibenzo[*b,d*]pyran-6-one) (*24*), **M2** (3,8,9-trihydroxy-6*H*-dibenzo[*b,d*]pyran-6-one) (*25*), **M4** (3,9-dihydroxy-8-methoxy-6*H*-dibenzo[*b,d*]pyran-6-one) (*24*), **M5** (3,4,8,9,10-pentahydroxy-6*H*-dibenzo[*b,d*]pyran-6-one) (*26*), and **M7** (3,8,10-trihydroxy-6*H*-dibenzo[*b,d*]pyran-6-one) (*27*) were identified by comparison of spectroscopic data with those reported in the literature (**Figure 1**). ^{1}H and ^{13}C NMR data for these metabolites are summarized in **Table 1**.

The molecular formula of **M3** was established as $C_{14}H_{10}O_5$ by an $[M + H]^+$ peak in the HRESIMS, which agreed with that of **M4**. The ^{1}H NMR spectrum of **M3** showed signals of an ABX type [δ 8.03 (d, J = 8.4 Hz), δ 6.87 (dd, J = 2.4, 8.4 Hz), δ 6.75 (d, J = 2.4 Hz)], and two 1H-singlet signals (δ 7.61, 7.59), besides a methoxyl proton signal (δ 4.12). Resonances due to lactone carbonyl (δ 163.5) and methoxyl (δ 56.8) carbons along with 12 sp^2 carbons were observed in the ^{13}C NMR spectrum of **M3**. The NMR feature of **M3** was similar to that of **M4**, as shown in **Table 1**, suggesting that a methoxyl group in **M3** is located at C-3 or -9 positions, differing from the C-8 methoxyl position in **M4**. The methoxyl position in **M3** was established by the NOESY experiment. The H-1 proton at δ 8.03 displayed NOE correlation with one (δ 7.59) of the two 1H-singlet signals, which in turn was associated with the methoxyl proton signal at δ 4.12 (**Figure 2**), establishing that the methoxyl group was located at the C-9 position. The structure of **M4** was confirmed by a similar NOESY experiment (**Figure 2**). On the basis of these findings, the structure of **M3** was determined as 3,8-dihydroxy-9-methoxy-6*H*-dibenzo[*b,d*]pyran-6-one.

Metabolite **M6** exhibited an $[M + H]^+$ peak in the HRESIMS, corresponding to the molecular formula $C_{13}H_8O_6$. The ^{1}H NMR spectrum revealed signals due to an ABX type [δ 8.94 (d, J = 9.0 Hz), δ 6.80 (dd, J = 2.4, 9.0 Hz), δ 6.73 (d, J = 2.4 Hz)], and a 1H-singlet signal at δ 7.40. The presence of 13 sp^2 carbons, including a lactone carbonyl carbon (δ 164.2) and five oxygen-bearing carbons (δ 158.9, 152.4, 146.5, 143.8, 141.9), was indicated in ^{13}C NMR and HSQC spectra of **M6** (**Table 1**). MS and NMR data implied that **M6** is a dibenzopyran derivative with an extra hydroxyl group as compared with **M7**. The location of the extra hydroxyl group was established by an HMBC experiment (**Figure 2**). The 1H-singlet signal at δ 7.40 was correlated with lactone carbonyl carbon resonance at δ 164.2 through three-bond long-range coupling, indicating that the 1H-singlet was assigned at H-7. Furthermore, the H-7 signal showed the three-bond correlation with the oxygen-bearing carbon resonance at δ 141.9 (C-9), indicating that the extra hydroxyl group was located in the C-9 position. On the basis of these findings, the structure of **M6** was determined as 3,8,9,10-tetrahydroxy-6*H*-dibenzo[*b,d*]pyran-6-one.

Among ellagitannin metabolites, **M1** has been isolated and characterized from rat urine and feces after oral administration of ellagic acid (*10*) and has been identified as a metabolite in sheep serum and urine after the consumption of *Terminalia oblongata* leaves, which contain abundant ellagitannins (*1*). Metabolite **M1** has been also characterized as castoreum pigment I from the scent gland of beavers (*28*),

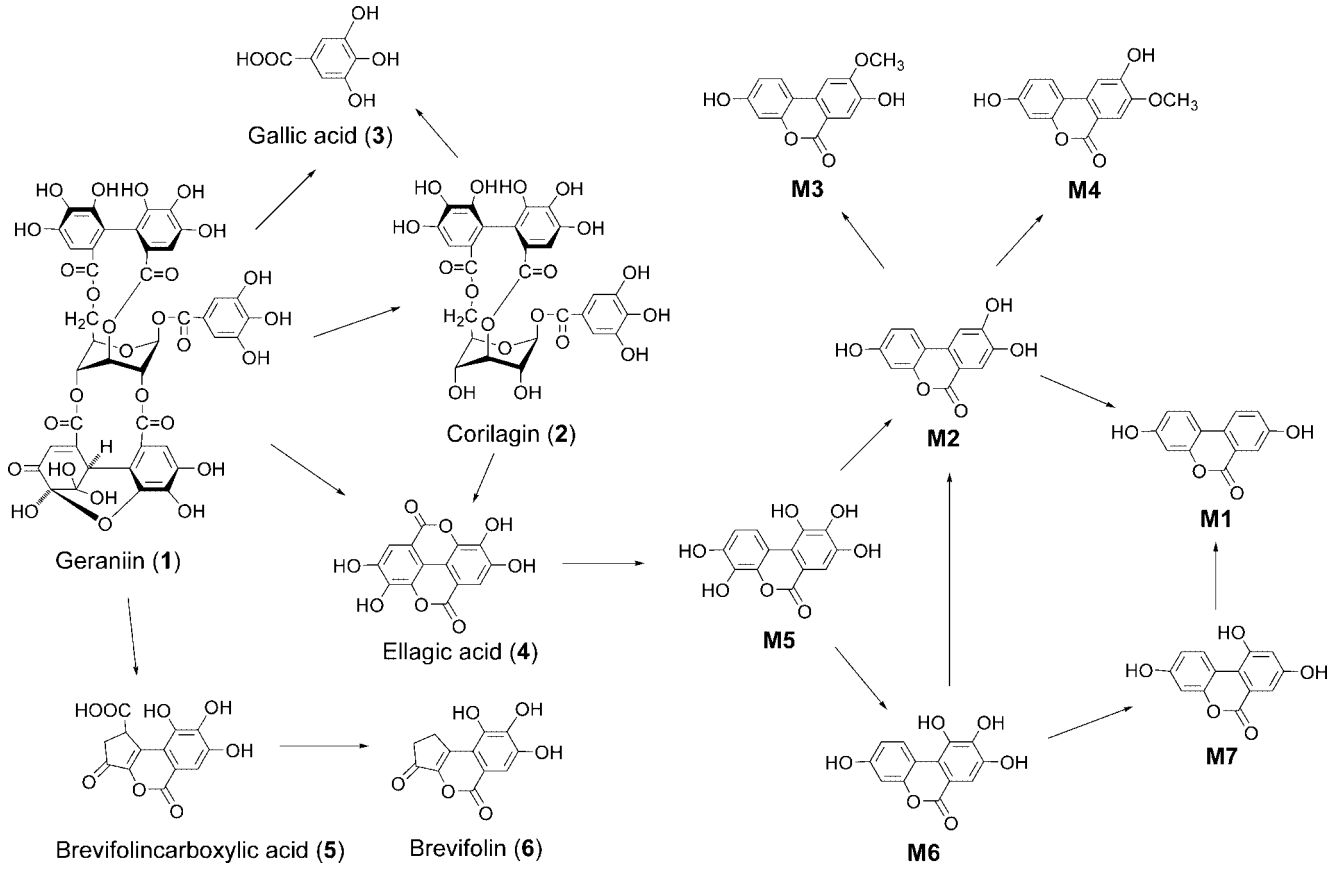


Figure 5. Proposed metabolic pathway for the formation of metabolites that originated from **1**.

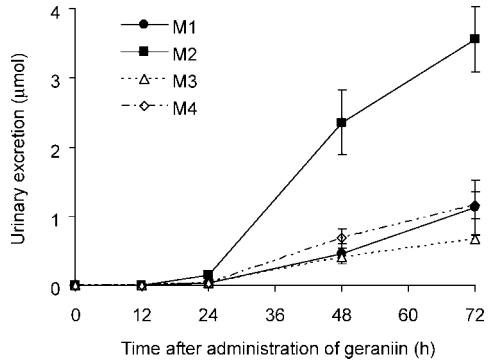


Figure 6. Cumulative urinary excretion profile of major metabolites in rats after oral administration of 50 mg/head of **1**. Urine was collected using metabolic cages 0–12, 12–24, 24–48, and 48–72 h after administration. Values are the means ± SEM ($n = 4$), represented by vertical bars.

a constituent of renal calculi in sheep (*29*), and a plant ingredient of Shilajit (*30*) and *Trapa natans* (*31*). Furthermore, **M1** has been recently found as a urinary metabolite in humans after pomegranate juice supplementation (*14*). Metabolites **M5** and **M7** have been identified as phytochemicals from *Tamarix nilotica* (*26*) and as metabolites from feces of *Trogopterus xanthipes* (*27*), respectively. In the present study, **M2**−**M7** were characterized as ellagitannin metabolites for the first time.

Detection of Metabolites of Ellagitannins in Urine and Fecal Suspension. Urinary excretions of metabolites were demonstrated by reversed phase HPLC analysis after oral administration of ellagitannins or their related polyphenols to rats. The identity of each metabolite was based on their retention times, which were compared to the respective standards. **M1**−**M4** were markedly excreted in urine after 24–48 h postconsumption of **1** (**Figure 3C**) and were unambiguously identified as major metabolites. These metabolites were also detected in urine samples after oral administration of either **2** or **4**, as shown in **Figure 3**, panels **D** and **E**, respectively. After oral dosing of **5**, which is a hydrolysate from **1**, **6**, due to the decarboxylated derivative of **5**, was observed in urine until 12 h (**Figure 3F**). No ellagitannins, or their related polyphenols and metabolites, were detected in blank urine (**Figure 3A**). No major metabolites were detected in the urine sample untreated with enzyme after geraniin ingestion, but several other weakly retained peaks were observed on reversed-phase HPLC analysis (**Figure 3B**), suggesting that the metabolites were excreted in urine as conjugate forms.

The time course profiles of the intestinal microbial transformation of **1** using reversed-phase HPLC are presented in **Figure 4**. Geraniin (**1**) started to be converted to **2**, **3**, **4**, and **5** after 1 h of anaerobic incubation with rat fecal suspension, indicating that **1** was first hydrolyzed by microflora. The production of **M5** was displayed 6 h after incubation, and then **M2** and **M6** were detected after 48 h of incubation. Upon further incubation, transformation to **M1** and **M7** occurred after 96 h. The level of each metabolite was below the detection limit in both the blank sample and the suspension incubated with killed flora. The reincubation of both **2** and **4** among intermediate hydrolysates provided a similar observation in the case of **1**. Furthermore, **5** was decarboxylated to **6** during reincubation with gut microflora (data not shown). Geraniin (**1**) was first subjected to ester hydrolysis with a rat fecal suspension, to give **2**, **3**, **4**, and **5**, resulting in the similar hydrolysis of **1** under acidic conditions (*16*). Among hydrolysates, the metabolism of **4** was expanded

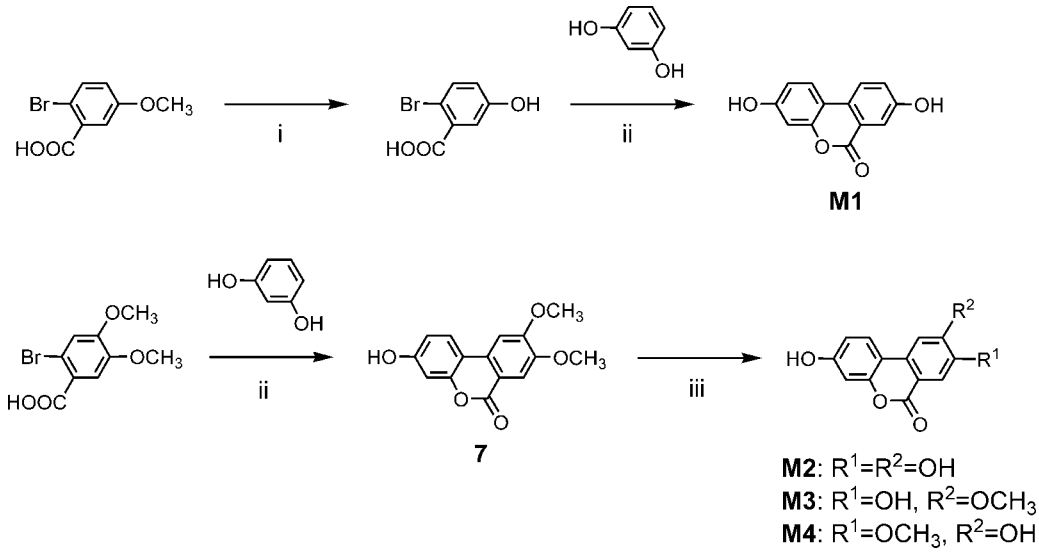


Figure 7. Preparation of **M1**−**M4**. Reagents and conditions: (i) $AlCl_3$, chlorobenzene, reflux; (ii) $CuSO_4$, NaOH, 80 °C; (iii) $AlCl_3$, chlorobenzene, 100 °C.

Table 2. DPPH Radical Scavenging and SOD-like Activities of Major Metabolites

	SOD		
compound	xanthine−XOD IC_{50} (μM)	PMS-NADH IC_{50} (μM)	DPPH IC_{50} (μM)
1	0.8	1.3	0.6
2	0.8	0.7	0.4
3	13	7.8	1.9
4	>100	21	1.7
pyrogallol	3.5	4.7	2.1
M1	>100	>100	>100
M2	>100	51	1.9
M3	>100	>100	11
M4	>100	>100	62

to **M1**, **M2**, **M6**, and **M7** through **M5** by fission of one of two lactone rings and subsequent dehydroxylation.

In the present study, transformation to both **M3** and **M4**, identified as urinary metabolites, was not observed in all rat fecal suspension with ellagitannins. The methylation of polyphenols having a catechol unit by catechol-*O*-methyltransferase (COMT) is well-known in flavonoids and catechins (*32*). The production of **M3** and **M4** was attributed to the methylation of **M2**, absorbed from intestines, by COMT in the liver or kidneys. A proposed metabolic sequence for the formation of these metabolites that originated from **1** is shown in **Figure 5**. Although 4-*O*-methylgallic acid and pyrogallol have been identified as metabolites of **3** in humans (*33*) and rats (*34*), only traces of these metabolites were detected in this study (data not shown).

Figure 6 shows the cumulative urinary excretion of major metabolites (**M1**−**M4**) after oral administration of **1**. Almost all metabolites began to be detected in rat urine after 24 h of geraniin intake, and their excretion increased until 72 h. Urinary excretion of each major metabolite was calculated relative to **M1** and was expressed as μmol **M1** over 72 h. The total excretion of major metabolites in urine over 72 h reached 12.4% of the amount administrated. Among these, **M2** was highly recovered in the urine at a level of 3.56 ± 0.48 μmol/72 h (6.8 ± 0.5% of oral dose of **1**). The urinary excretion of all metabolites persisted for more than 48 h after geraniin administration. This suggested that ellagitannin metabolites formed by microbial hydrolysis and subsequent reduction in the colon are absorbed and eliminated through enterohepatic circulation. Our data reinforced the notion that the metabolism of ellagitannins is dependent on intestinal microflora (*11, 13*).

Synthesis of Metabolites. Metabolite **M1** was prepared by the condensation of resorcinol with 2-bromo-5-hydroxybenzoic acid (*22*) in alkaline solution and copper catalyst (*30*). 2-Bromo-4,5-dimethoxybenzoic acid, by similar condensation with resorcinol, furnished 3-hydroxy-8,9-dimethoxy-6*H*-dibenzo[*b,d*]pyran-6-one (**7**), which was demethylated and purified by column chromatography and preparative HPLC to afford **M2**−**M4** (**Figure 7**). The structures of **M1**−**M4** were also verified by chemical synthesis. These products were used as authentic compounds for the identification of metabolites derived from rat urine and for the evaluation of antioxidant activity.

Radical Scavenging Activity of Metabolites. The antioxidant activity of the major metabolites (**M1**−**M4**) was evaluated by DPPH radical scavenging effects and two SOD-like activities. Superoxide anions were generated either by xanthine−XOD enzymatic reaction or by PMS-NADH nonenzymatic reaction. The results are summarized in **Table 2**. In both of the assays for SOD-like activity, all major metabolites exhibited no activity except for **M2** in the PMS-NADH assay (IC_{50} = 51 μM). On the other hand, among the tested metabolites, **M2** had a radical scavenging effect with IC_{50} = 1.9 μM, comparable to **1** and its related polyphenols in the DPPH assay. These data suggested that **M2** may play a role as an active substance after ellagitannin ingestion.

Ellagitannins, including **1**, have a hexahydroxydiphenoyl group in their molecules, so **4**, readily lactonized from the hexahydroxydiphenic acid, is produced upon hydrolysis of ellagitannins (*16*). The metabolism data obtained in the present study suggested that metabolites (**M1**−**M7**) were derived from **4** through the hydrolysis of ellagitannins. Because several metabolites are unidentified in human and rat biofluids after the consumption of pomegranate juice rich in ellagitannins (*11, 13*), the present data may serve to develop bioavailability studies of ellagitannins.

In summary, we isolated seven metabolites (**M1**−**M7**) from rat urine after the ingestion of ellagitannins and from rat gut microbial suspension with ellagitannins. The structures of these metabolites were elucidated on the basis of spectro-

scopic and spectrometric data. These compounds, except for **M1**, were characterized as ellagitannin metabolites for the first time. Furthermore, **M1**−**M4** were predominantly excreted in rat urine after the ingestion of **1**; among which, **M2** showed an antioxidant activity comparable to **1**. Although the metabolites are present in the circulatory system as conjugated forms, several data have been reported about biological activity of conjugates. Quercetin conjugates retain antioxidant activity in plasma (*35*–*37*). Luteolin glucuronide is deconjugated into aglycone by β-glucuronidase released from neutrophils after induction of inflammation (*38*). Thus, these findings support the idea that these metabolites were absorbed into the circulatory system and transported in urine after ellagitannin intake, which may contribute to the health benefits of ellagitannins. Further study of the bioavailability of ellagitannins is under investigation to clarify the active principles of antioxidant and cancer chemopreventive effects.

ABBREVIATIONS USED

ESIMS, electrospray ionization mass spectrometry; COSY, ^{1}H-^{1}H correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple-bond correlation; SOD, superoxide-dismutase; PMS, phenazine methoxysulfate; NBT, nitroblue tetrazolium; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

ACKNOWLEDGMENT

The authors are grateful to the SC-NMR Laboratory of Okayama University for NMR measurements.

LITERATURE CITED

(1) Okuda, T.; Yoshida, T.; Hatano, T. Hydrolyzable tannins and related polyphenols. *Fortschr. Chem. org. Naturs.* **1995**, *66*, 1–117.

(2) Clifford, M. N.; Scalbert, A. Ellagitannins, occurrence in food, bioavailability and cancer prevention. *J. Food Sci. Agric.* **2000**, *80*, 1118–1125.

(3) Okuda, T.; Yoshida, T.; Hatano, T. Ellagitannins as active constituents of medicinal plants. *Planta Med.* **1989**, *55*, 117–122.

(4) Nakashima, H.; Murakami, T.; Yamamoto, N.; Sakagami, H.; Tanuma, S.; Hatano, T.; Yoshida, T.; Okuda, T. Inhibition of human immunodeficiency viral replication by tannins and related compounds. *Antiviral Res.* **1992**, *18*, 91–103.

(5) Okuda, T.; Mori, K.; Hayatsu, H. Inhibitory effect of tannins on direct-acting mutagens. *Chem. Pharm. Bull.* **1984**, *32*, 3755–3758.

(6) Funatogawa, K.; Hayashi, S.; Shimomura, H.; Yoshida, T.; Hatano, T.; Ito, H.; Hirai, Y. Antibacterial activity of hydrolyzable tannins derived from medicinal plants against *Helicobacter pylori*. *Microbiol. Immunol.* **2004**, *48*, 251–261.

(7) Shiota, S.; Shimizu, M.; Mizusima, T.; Ito, H.; Hatano, T.; Yoshida, T.; Tsuchiya, T. Restoration of effectiveness of β-lactams on methicillin-resistant *Staphylococcus aureus* by tellimagrandin I from rose red. *FEMS Microbiol. Lett.* **2000**, *185*, 135–138.

(8) Ito, H.; Miyake, M.; Nishitani, E.; Mori, K.; Hatano, T.; Okuda, T.; Konoshima, T.; Takasaki, M.; Kozuka, M.; Mukainaka, T.; Tokuda, H.; Nishino, H.; Yoshida, T. Anti-tumor promoting activity of polyphenols from *Cowania mexicana* and *Coleogyne ramosissima*. *Cancer Lett.* **1999**, *143*, 5–13.

(9) Okabe, S.; Suganuma, M.; Imayoshi, Y.; Taniguchi, S.; Yoshida, T.; Fujiki, H. New TNF-α releasing inhibitors, geraniin and corilagin, in leaves of Acer nikoense, Megusurino-ki. *Biol. Pharm. Bull.* **2001**, *24*, 1145–1148.

(10) Doyle, B.; Griffiths, L. A. The metabolism of ellagic acid in the rat. *Xenobiotica* **1980**, *10*, 247–256.

(11) Cerda, B.; Llorach, R.; Ceron, J. J.; Espin, J. C.; Tomas-Barberan, F. A. Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice. *Eur. J. Nutr.* **2003**, *42*, 18–28.

(12) Seeram, N. P.; Lee, R.; Heber, D. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice. *Clin. Chim. Acta* **2004**, *348*, 63–68.

(13) Seeram, N. P.; Henning, S. M.; Zhang, Y.; Suchard, M.; Li, Z.; Heber, D. Pomegranate juice ellagitannin metabolites are present in human plasma and some persist in urine for up to 48 h. *J. Nutr.* **2006**, *136*, 2481–2485.

(14) Cerda, B.; Espin, J. C.; Parra, S.; Martinez, P.; Tomas-Barberan, F. A. The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy-6*H*-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans. *Eur. J. Nutr.* **2004**, *43*, 205–220.

(15) Cerda, B.; Tomas-Barberan, F. A.; Espin, J. C. Metabolism of antioxidant and chemopreventive ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in humans: identification of biomarkers and individual variability. *J. Agric. Food Chem.* **2005**, *53*, 227–235.

(16) Okuda, T.; Yoshida, T.; Hatano, T. Constituents of *Geranium thunbergii* Sieb. et Zucc. − Part 12 − Hydrated stereostructure and equilibration of geraniin. *J. Chem. Soc. Perkin Trans. I* **1982**, 9–14.

(17) Okuda, T.; Yoshida, T.; Mori, K. Brevifolin, corilagin and other phenols from *Geranium thunbergii*. *Phytochemistry* **1975**, *14*, 1877–1878.

(18) Ito, H.; Hatano, T.; Namba, O.; Shirono, T.; Okuda, T.; Yoshida, T. Constituents of *Geranium thunbergii* Sieb. et Zucc. XV. Modified dehydroellagitannins, geraniinic acids B and C, and phyllanthusiin F. *Chem. Pharm. Bull.* **1999**, *47*, 1148–1151.

(19) Yoshida, T.; Itoh, H.; Matsunaga, S.; Tanaka, R.; Okuda, T. Tannins and related polyphenols of Euphorbiaceous plants. IX. Hydrolyzable tannins with $^1C_4$ glucose core from *Phyllanthus flexuosus* Muell. Arg. *Chem. Pharm. Bull.* **1992**, *40*, 53–60.

(20) Lafay, S.; Morand, C.; Manach, C.; Besson, C.; Scalbert, A. Absorption and metabolism of caffeic acid and chlorogenic acid in the small intestine of rats. *Br. J. Nutr.* **2006**, *96*, 39–46.

(21) Ito, H.; Gonthier, M. P.; Manach, C.; Morand, C.; Mennen, L.; Remesy, C.; Scalbert, A. Polyphenol levels in human urine after intake of six different polyphenol-rich beverages. *Br. J. Nutr.* **2005**, *94*, 500–509.

(22) Tomita, M.; Kura, S.; Tanaka, S. Rearrangement of bromine atom in the demethylation of bromomethoxybenzoic acid. III. 2-Bromobenzoic acid and 3-methoxy-6-bromobenzoic acid. *J. Pharm. Soc. Jpn.* **1956**, *76*, 1122–1125.

(23) Nishikimi, M.; Appaji, N.; Yagi, K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 849–854.

(24) Pandey, J.; Jha, A. K.; Hajela, K. Synthesis and biological activities of some new dibenzopyranones and dibenzopyrans: search for potential oestrogen receptor agonists and antagonists. *Bioorg. Med. Chem.* **2004**, *12*, 2239–2249.

(25) Ghosal, S.; Lata, S.; Kumar, Y. Free radicals of shilajit humus. *Indian J. Chem.* **1995**, *34B*, 591–595.

(26) Nawwar, M. A. M.; Souleman, A. M. A. 3,4,8,9,10-Pentahydroxydibenzo[b,d]pyran-6-one from *Tamarix nilotica*. *Phytochemistry* **1984**, *23*, 2966–2967.

(27) Jeong, S. J.; Kim, N. Y.; Kim, D. H.; Kang, T. H.; Ahn, N. H.; Miyamoto, T.; Higuchi, R.; Kim, Y. C. Hyaluronidase inhibitory active 6*H*-dibenzo[*b,d*]pyran-6-ones from the feces of *Trogopterus xanthipes*. *Planta Med.* **2000**, *66*, 76–77.

(28) Lederer, E. Chemistry and biochemistry of some mammalian secretions and excretions. *J. Chem. Soc.* **1949**, 2115–2125.

(29) Pope, G. S. Isolation of two benzocoumarins from 'clover stone' a type of renal calculus found in sheep. *Biochem. J.* **1964**, *93*, 474–477.

(30) Ghosal, S.; Reddy, J. P.; Lal, V. K. Shilajit I: chemical constituents. *J. Pharm. Sci.* **1976**, *65*, 772–773.
(31) Shirataki, Y.; Yoshida, S.; Toda, S. Dibenzo-α-pyrons in fruits of *Trapa natans*. *Nat. Med.* **2000**, *54*, 160.
(32) Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747.
(33) Hodgson, J. M.; Morton, L. W.; Puddey, I. B.; Beilin, L. J.; Croft, K. D. Gallic acid metabolites are markers of black tea intake in humans. *J. Agric. Food Chem.* **2000**, *48*, 2276–2280.
(34) Yasuda, T.; Inaba, A.; Ohmori, M.; Endo, T.; Kubo, S.; Ohsawa, K. Urinary metabolites of gallic acid in rats and their radical-scavenging effects on 1,1-diphenyl-2-picrylhydrazyl radical. *J. Nat. Prod.* **2000**, *63*, 1444–1446.
(35) Manach, C.; Morand, C.; Crespy, V.; Demigne, C.; Texier, O.; Regerat, F.; Remesy, C. Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. *FEBS Lett.* **1998**, *426*, 331–336.
(36) Moon, J.; Tsushida, T.; Nakahara, K.; Terao, J. Identification of quercetin 3-*O*-*β*-D-glucuronide as an antioxidative metabolite in rat plasma after oral administration of quercetin. *Free Radic. Biol. Med.* **2001**, *30*, 1274–1285.
(37) Day, A. J.; Bao, Y.; Morgan, M. R.; Williamson, G. Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radic. Biol. Med.* **2000**, *29*, 1234–1243.
(38) Shimoi, K.; Saka, N.; Nozawa, R.; Sato, M.; Amano, I.; Nakayama, T.; Kinae, N. Deglucuronidation of a flavonoid, luteolin monoglucuronide, during inflammation. *Drug Metab. Dispos.* **2001**, *29*, 1521–1524.

Received for review September 11, 2007. Revised manuscript received November 4, 2007. Accepted November 6, 2007. This study was supported by a Grant-in-Aid for Scientific Research (No. 17604005) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by Mishima Kaiun Memorial Foundation.

JF0726942