Metabolic Fate of Orally Administered Phyllodulcin in Rats

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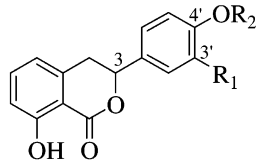
Naturally occurring phyllodulcin (**1**) was orally administered to rats to investigate its metabolic fate. Urinary metabolites were analyzed by three-dimensional HPLC. Phyllodulcin-3′-*O*-sulfate (**2**), phyllodulcin-3′-*O*-*β*-glucuronide (**3**), 2-[2-(3,4-dihydroxyphenyl)ethyl]-6-hydroxybenzoic acid (**4**), and one novel bibenzyl derivative, 2-[2-(3-hydroxy-4-methoxyphenyl)ethyl]-6-hydroxybenzoic acid (**5**), together with thunberginol G (**6**) and hydrangenol (**7**) were isolated from the phyllodulcin-treated urine. **1** was extensively metabolized to **4**−**6** by a rat fecal suspension after incubation for 24 h. Urinary excretion of **4**−**6** in rats administered phyllodulcin orally was substantially reduced when the rats were treated with antibiotics to suppress their intestinal flora. On the other hand, the incubation of **1** with rat liver S-9 mix showed the presence of **7** together with **4** and **5**.

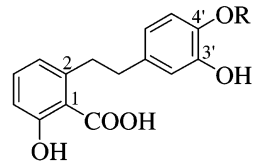
Hydrangeae folium (Amacha in Japanese) is made from the fermented and dried leaves of *Hydrangea macrophylla* Seringe var. *thunbergii* Makino (Saxifragaceae) grown in Japan. The plant contains a sweetening compound (**1**), which is a dihydroisocoumarin derivative. Phyllodulcin is well known in Japan as an oral refrigerant and as a sweetener, since it is 600−800 times sweeter than sucrose. It is also used to make tea served during the Hanamatsuri (birth of Buddha) celebration. Several biological activities have been observed for phyllodulcin, including antiallergic effects on the Schultz−Dale reaction,[1] inhibition of microsomal lipid peroxidation induced by NADPH and the Fenton-type reaction,[2] and inhibition of ACTH- and forskolin-induced steroidogenesis in the presence of Ca^{2+} in the incubation media.[3] Although some biochemical properties of **1** have been described, the metabolic fate of this compound has yet to be reported. In our continuing study on the metabolism of naturally occurring compounds, we investigated the metabolic fate of **1** in rats.

Here we describe the identification and structural elucidation of urinary metabolites from rats administered **1** orally. In addition, we investigated the biotransformation of **1** using antibiotic-treated rats, rat fecal suspension, and rat liver S-9 mix, to study the site of metabolism.

Two distinct HPLC peaks, **2** and **3**, together with unchanged **1** were detected in the *β*-glucuronidase/arylsulfatase nontreated urine of rats administered **1** orally. When the urine was treated with *β*-glucuronidase/arylsulfatase, four peaks were detected: **4**, **5**, **6**, and **7**. Compounds **2**−**7** were isolated from the urine sample by chromatography as described in the Experimental Section. Metabolite **2** was obtained as a white powder. Enzyme hydrolysis of **2** with arylsulfatase yielded **1**, confirmed by t_R agreement using HPLC. The intense absorption at 1051 cm^{-1} in the IR spectrum and SO_4^{2-} formation on carbonization suggested a sulfate-conjugated structure for **2**. Negative-ion FABMS of **2** showed a base ion peak corresponding to $(M - H - SO_3)^-$ at *m*/*z* 285 along with fragment ion peaks at *m*/*z* 387 $(M - H + Na)^-$ and 365 $(M - H)^-$, thus indicating one sulfate group in **2**. A comparison of the ^{13}C NMR spectrum of **2** with **1** showed that the C-3′ signal of **2** had shifted 3.9 ppm upfield, accompanied by downfield shifts of C-2′ (7.5 ppm) and C-4′ (3.0 ppm). These shifts indicated one sulfate group at C-3′. On the basis of these data, **2** was identified as phyllodulcin-3′-*O*-sulfate.



1: R_1=OH, R_2=CH_3
2: R_1=O-SO_3^-, R_2=CH_3
3: R_1=O-GlcUA, R_2=CH_3
6: R_1=OH, R_2=H
7: R_1=H, R_2=H



4: R=H
5: R=CH_3

Metabolite **3** was obtained as a white powder. Enzyme hydrolysis of **3** with *β*-glucuronidase yielded **1**. The ^1H NMR spectrum showed one anomeric proton at *δ* 4.91 (d, *J* = 7.2 Hz), and negative-ion FABMS showed a molecular ion peak at *m*/*z* 461 $(M - H)^-$ corresponding to a monoglucuronide. A comparison of the ^{13}C NMR spectrum of **3** with **1** indicated that the C-3′ signal of **3** had shifted 0.13 ppm upfield, accompanied by downfield shifts of C-2′ (0.45 ppm) and C-4′ (1.0 ppm). These shifts indicated a glucuronide group at C-3′. Heteronuclear multiple-bond correlation spectroscopy (HMBC) data revealed a correlation between the anomeric proton and C-3′. Thus, **3** was identified as phyllodulcin-3′-*O*-*β*-glucuronide.

Metabolite **4** was identified as 2-[2-(3,4-dihydroxyphenyl)ethyl]-6-hydroxybenzoic acid, by comparison with reference data.[4] Furthermore, direct comparison of **4** with an authentic sample prepared from phyllodulcin by EIMS and ^1H NMR spectra supported this observation.

Metabolites **6** and **7** were identified as thunberginol G and hydrangenol, respectively, by direct comparison of EIMS and ^1H NMR spectral data with authentic samples.[5]

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Metabolite **5** was obtained as a white powder. The ^{1}H and ^{13}C NMR spectral data of **5** were similar to those of **4**, except that one of the three hydroxyl group signals in **4** had been replaced by a single methoxy group [δ 3.73 (1H, s, $-OCH_3$)]. It appeared that **5** possessed the same oxygenation pattern as **4**. A NOE was observed between the methoxy proton and H-5, indicating that the methoxyl group was attached to C-4. Thus, **5** was concluded to be 2-[2-(3-hydroxy-4-methoxyphenyl)ethyl]-6-hydroxybenzoic acid. Confirmation of the structure of **5** was made by direct comparison with an authentic sample, which was prepared from phyllodulcin by Pd−C treatment, using EIMS and ^{1}H NMR spectral data. Since the yields of **2**, **3**, **6**, and **7** were very low, the stereochemistry at C-3 could not be clarified.

To determine the contribution to the formation of **4**−**7** made by the intestinal microflora, we examined changes in urinary metabolite excretion when rats were treated with antibiotics. The urinary excretion of **4**−**6** significantly decreased over 24 h after administration of **1** in rats treated with antibiotics (**4** and **6**, $p < 0.01$; **5**, $p < 0.05$), while the excretion of **7** significantly increased ($p < 0.05$) when compared with the nontreated rats. This result indicated the importance of the gut flora in generating the urinary metabolites **4**−**6**. On the other hand, the main site of formation of **7** was assumed to be the liver enzymes. These findings prompted us to investigate the biotransformation of **1** by the intestinal microflora and rat liver S-9 fraction. During the metabolite formation and anaerobic incubation of **1** with rat fecal microflora, **1** was almost completely consumed after 36 h of incubation, and metabolites **4**−**6** were produced. Metabolite **4** increased progressively and reached a maximum concentration at 24 h, then decreased slightly on prolonged incubation. On the other hand, metabolite **6** was produced, reached its maximum at 6 h, and then decreased appreciably on prolonged incubation. During the incubation period, only traces of **5** were detected. These results suggested that **5** and **6** are metabolic intermediates. As expected, after incubation under anaerobic conditions for 12 and 6 h, compounds **5** and **6**, respectively, were almost completely converted to **4** in rat fecal suspension.

During metabolite formation of **1** by rat liver S-9 fraction, **1** decomposed in a time-dependent manner and after 2 h treatment was partly converted to **7**, which at its maximum accounted for 1.9% of the total **1**. It was interesting to note that **1** was also partly converted to **4** and **5**, accounting for maxima of 5.0% at 4 h and 3.0% at 6 h, respectively. This conversion by rat liver S-9 was not substantially inhibited by SKF 525A, the nonspecific cytochrome P450 inhibitor (data not shown).

In the present study of in vivo metabolism of **1**, six urinary metabolites including sulfate and glucuronide conjugates of **1** were detected in rat urine by HPLC. To our knowledge, metabolite **5** is a novel bibenzyl derivative. Furthermore, it was demonstrated that **4** and **5** were produced by the intestinal microflora and rat liver S-9 fraction, while **6** or **7** were produced only by the intestinal microflora or in the intact liver. These data were derived from the use of antibiotic-treated rats and incubation of **1** with rat fecal microflora and rat liver S-9 fraction, respectively. With HPLC analysis of rat fecal incubation mixtures, a substantial amount of **4** was found, while a small amount of **6** and only a trace of **5** were detected. This suggested that intermediates **5** and **6** were easily and immediately converted to **4** by intestinal bacteria through reductive cleavage and demethylation reactions, respectively. This observation may be explained by the differences in activities of bacterial enzymes in the demethylation and reductive cleavage reaction pathways. This hypothesis was confirmed by examination of the incubations of **5** and **6** with rat fecal suspensions. In fact, the incubations of **5** and **6** were shown to be almost completely converted to **4** in rat fecal suspension under anaerobic conditions for 12 and 6 h, respectively. The metabolic pathways deduced from the present study demonstrate that **1** was demethylated to produce **6**, reductively cleaved to produce **5**, and a combination of both or either process to produce **4**. On the other hand, **1** appeared to be converted to **7** via direct demethylation and dehydroxylation in the liver. The identified phyllodulcin metabolites were all products of demethylation, dehydroxylation, and reductive cleavage reactions. Since aromatic compounds such as flavonoids and coumarins are partly metabolized to phenolic acids by intestinal bacteria as well as by plants and aerobes,[6−8] it appears that ring fission in oxygen-containing heterocyclic compounds through reductive or oxidative cleavage is a common reaction of living organisms. In general, orally administered phenolic compounds, especially those with a low polarity, undergo hydroxylation and/or glucuronide and sulfate conjugation primarily by the intestinal microflora and secondarily in the liver and other tissues. On the other hand, there are reports on the dehydroxylation and demethylation in rat liver for naturally occurring compounds, such as the 7β-hydroxy-C_{27} plant sterol,[9] nobiletin,[10] and tangeretin.[11] Therefore, it is not surprising that phyllodulcin could be directly converted to **7** by the liver and associated enzymes. Furthermore, the conversion of **1** to **7** by rat liver S-9 fraction was not significantly inhibited by SKF 525A (data not shown), suggesting that a rat liver enzyme other than cytochrome P450 was involved in the conversion.

Experimental Section

General Experimental Procedures. Phyllodulcin was isolated from Hydrangeae dulcis folium, the fermented and dried leaves of *Hydrangea macrophylla* Seringe var. *thunbergii* Makino. Authentic phyllodulcin as well as kanamycin sulfate, tetracycline hydrochloride, and bacitracin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phthalylsulfathiazole was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Arylsulfatase (type H-1), β-glucuronidase (type H-2), glucose-6-phosphate, and proadifen were purchased from Sigma (St. Louis, MO). Glucose-6-phosphate dehydrogenase was from Oriental Yeast Co., Ltd. (Tokyo, Japan). For column chromatography, Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) and Wakosil C-200 (Wako Pure Chemical) were used. All other reagents were of the highest purity commercially available. Uncorrected melting points were determined on a Yanagimoto micro melting point apparatus. Infrared (IR) spectra were measured with a Perkin-Elmer FT-IR1725X spectrometer. NMR spectra were recorded on JEOL JNM-EX 270 (^{1}H, 270; ^{13}C, 67.8 MHz), JNM-EX 400 (^{1}H, 400; ^{13}C, 100 MHz), and JNM-LA 600 (^{1}H, 600; ^{13}C, 150 MHz) spectrometers. Chemical shifts are given in δ values (ppm) downfield relative to tetramethylsilane. Electron impact (EI) and fast atom bombardment (FAB) MS were measured on a JEOL JMS-DX 303 mass spectrometer. The HPLC system was comprised of a CCMP-II pump, CO-8020 column oven (Tosoh, Tokyo, Japan), and model MCPD-3600 photodiode array detector (Otsuka, Osaka, Japan). HPLC conditions for analysis of metabolites were as follows: column, CAPCELL-PAK C_{18} (5 μm, 3.0 mm i.d. × 250 mm, Shiseido, Tokyo, Japan); column temperature, 40 °C; flow rate, 0.5 mL/min; detection, 200−400 nm; mobile phase, a linear gradient system with 0.1% trifluoroacetic acid in H_2O (A) and CH_3CN (B), A/B = 95/5 (0 min) → 40/60 (50 min).

Animal Experiments. Male Sprague-Dawley rats (6 weeks, 140−150 g) were purchased from Japan SLC, Inc. These animals were housed at 22 ± 2 °C, humidity 55 ± 10%, in a light (9:00−21:00)-controlled room with free access to water and commercial rodent chow (CE-2, Clea Japan Inc., Tokyo, Japan). After 3 days of feeding, food was withheld for 18 h, and thereafter phyllodulcin (100 mg/kg body weight) uniformly dispersed in 0.5% Tween 80 (ICN Pharmaceuticals, Ltd.) was administered orally by direct stomach intubation. The animals had free access to water and sugar during the experiments.

Preparation of Urine Samples. The urine samples were collected over 24 h using a metabolic cage. The urine was filtered through a 0.45 μm membrane filter, and then 20 μL of the sample was injected into the HPLC.

Enzymatic Hydrolysis of Urine Samples. A urine sample (20 mL) was transferred to a test tube to which was added 5.0 mL of citrate buffer (pH 5.2) and 150 μL of β-glucuronidase solution followed by incubation at 37 °C for 24 h. The incubated solution was extracted three times with AcOEt (40 mL). The organic layer was dried with anhydrous Na_2SO_4 overnight and dried at 40 °C. The residue was dissolved in MeOH, and a 20 μL aliquot was injected into the HPLC.

Isolation of Metabolites. The urine sample (80 mL) obtained from rats after oral administration of **1** (260 mg) that had been incubated with β-glucuronidase/arylsulfatase was extracted three times with AcOEt. The organic layer was dried with anhydrous Na_2SO_4 for 24 h and evaporated to dryness at 40 °C. The residue was dissolved in a small amount of MeOH and chromatographed on Sephadex LH-20 with MeOH. The fractions containing metabolites **3**−**6** were subjected to preparative HPLC. The HPLC conditions were as follows: column, Wakosil-II 5C18 (5 μm, 7.5 mm i.d. × 300 mm, Wako Pure Chemicals Industries Ltd., Osaka, Japan); mobile phase, H_2O (A) and MeOH (B); linear gradient system, A/B = 20/80 (0 min) → 80/20 (120 min); flow rate, 1.0 mL/min; detective wavelength, 310 nm. Each metabolite fraction was evaporated to dryness at 40 °C in vacuo to afford **4** (5 mg), **5** (0.5 mg), **6** (0.3 mg), and **7** (0.8 mg), respectively.

Compound 2: white powder; mp 118−119 °C; IR (KBr) ν_{max} 3469, 1674, 1618, 1516, 1231, 1051 cm^{-1}; ^{1}H NMR (270 MHz, DMSO-d_6) δ 3.15 (1H, dd, J = 3.4, 16.6 Hz, H-4 cis), 3.35 (1H, J = 3.4, 11.9 Hz, H-4 trans), 3.76 (3H, s, OCH_3), 5.69 (1H, dd, J = 3.4, 11.9 Hz, H-3), 6.90 (2H, d, J = 8.4 Hz, H-5, -7), 7.01 (1H, d, J = 8.4 Hz, H-5′), 7.16 (1H, dd, J = 2.1, 8.4 Hz, H-6′), 7.52 (1H, dd, J = 8.4, 8.4 Hz, H-6), 7.61 (1H, d, J = 2.1 Hz, H-2′), 10.92 (1H, s, 8-OH); ^{13}C NMR (67.8 MHz, DMSO-d_6) δ 33.2 (C-4), 55.2 (OCH_3), 79.6 (C-3), 108.0 (C-8a), 111.9 (C-5′), 114.9 (C-5 or -7), 117.9 (C-5 or -7), 119.0 (C-2′), 121.1 (C-6′), 129.3 (C-1′), 135.7 (C-6), 139.9 (C-4a), 142.1 (C-3′), 150.4 (C-4′), 160.4 (C-8), 168.8 (C-1); FABMS m/z 387 (M − H + Na)$^-$, 365 (M − H)$^-$, 285 (M − H − SO_3)$^-$.

Compound 3: white powder; mp 173−180 °C; IR (KBr) ν_{max} 3422, 1672, 1519, 1616, 1231 cm^{-1}; ^{1}H NMR (600 MHz, CD_3-OD) δ 3.21 (1H, dd, J = 3.0, 16.5 Hz, H-4 cis), 3.37 (1H, dd, J = 3.0,12.6 Hz, H-4 trans), 3.88 (3H, s, OCH_3), 4.91 (1H, d, J = 7.2, anomeric proton), 5.63 (1H, dd, J = 3.0, 15.0 Hz, H-3), 6.86 (2H, m, H-5, -7), 7.05 (1H, d, J = 8.4 Hz, H-5′), 7.20 (1H, dd, J = 1.8, 8.4 Hz, H-6′), 7.37 (1H, d, J = 1.8 Hz, H-2′), 7.47 (1H, dd, J = 7.2, 8.4 Hz, H-6); ^{13}C NMR (150 MHz, CD_3OD) δ 35.5 (C-4), 56.8 (OCH_3), 73.6 (C-4″), 74.9 (C-2″), 76.6 (C-5″), 77.7 (C-3″), 82.2 (C-3), 103.3 (C-1″), 109.6 (C-8a), 113.7 (C-5′), 116.8 (C-5 or -7), 118.0 (C-2′), 119.5 (C-5 or -7), 122.3 (C-6′), 132.7 (C-1′), 137.6 (C-6), 141.8 (C-4a), 148.0 (C-3′), 151.5 (C-4′), 163.3 (C-8), 171.6 (C-1), 180.5 (C-6″); negative FABMS m/z 483 (M − H + Na)$^-$, 461 (M − H)$^-$, 285 (M − GlcUA)$^-$.

Compound 5: white powder; mp 142−145 °C; IR (KBr) ν_{max} 3400, 1666, 1610, 1510 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 2.73 (2H, t, J = 8.0 Hz, H-β), 3.16 (2H, t, J = 8.0 Hz, H-α), 3.74 (3H, s, OCH_3), 6.62 (1H, dd, J = 2.2, 8.4 Hz, H-6′), 6.67−6.68 (2H, m, H-3 or -5, -2′), 6.74 (1H, d, J = 8.4 Hz H-3 or -5), 6.80 (1H, d, J = 8.4 Hz, H-5′), 7.23 (1H, dd, J = 8.4, 8.4 Hz, H-4); ^{13}C NMR (100 MHz, CD_3OD) δ 39.0 (C-α), 39.5 (C-β), 56.6 (OCH_3), 112.9 (C-3), 114.6 (C-1), 116.1 (C-5′), 116.6 (C-2′), 120.6 (C-6′), 123.2 (C-5), 134.2 (C-4), 136.6 (C-1′), 146.0 (C-6), 147.3 (C-3′ or -4′), 147.4 (C-3′ or -4′), 162.6 (C-2), 174.4 (*C*OOH); FABMS m/z 287 (M − H)$^-$; HREIMS m/z 288.0998 (calcd for $C_{15}H_{16}O_5$, 288.0996).

Demethylation of 1. BBr_3 (0.7 g, 0.27 mL) was added dropwise to a solution of **1** (80 mg) in CH_2Cl_2 (5 mL) at −78 °C, then stirred at room temperature under a N_2 atmosphere for 24 h. The reaction mixture was evaporated in vacuo, and the residue was dissolved in 10% KOH. The aqueous layer was acidified with 10% H_2SO_4, extracted with AcOEt, and then dried over anhydrous Na_2SO_4 and evaporated in vacuo. The residue was dissolved in MeOH and chromatographed on Sephadex LH-20 using MeOH as the eluant, and then chromatographed on Wakosil C-200 using n-hexane−AcOEt as eluant. The fraction containing **6** was subjected to preparative HPLC to give **6** (32 mg, 42% yield). HPLC conditions: column, Wakosil-II 5C18 (5 μm, 7.5 mm i.d. × 300 mm, Wako Pure Chemicals, Osaka, Japan); mobile phase, H_2O (A) and MeOH (B), linear gradient system, A/B = 95/5 (0 min) → 40/60 (100 min); detection wavelength, 220 nm; flow rate, 1.0 mL/min at room temperature.

Hydrogenation of 1 and 6. Phyllodulcin (**1**, 20 mg) and **6** (15 mg) were each dissolved in MeOH (14 mL) and hydrogenated over Pd−C (10%, 18 mg). The reaction mixtures were stirred at room temperature under a H_2 atmosphere for 1 h. The reaction mixtures were filtered through a 0.45 μm membrane filter and the filtrates concentrated in vacuo to give **5** (20 mg, 100% yield) and **4** (16 mg, 100% yield), respectively.

Antibiotic Treatment of Animals. Gut sterilization was done according to the method of Goodwin et al.[11] with minor modifications. Rats were given a mixture of kanamycin sulfate (45 mg), tetracycline hydrochloride (20 mg), bacitracin (1 mg), and phthalylsulfathiazole (0.5 mg) orally once daily for 4 days. After 1 h of the last dose on the fourth day, **1** (100 mg/kg) was administered. Urine samples were collected for 24 h, incubated with β-glucuronidase for 24 h, and then extracted three times with AcOEt. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness at 40 °C. The residue was dissolved in a small amount of MeOH and injected into the HPLC. Statistical analysis was carried out by means of the Student's t-test (n = 4). P values less than 0.05 were considered to indicate statistical significance.

Incubation of Phyllodulcin with Rat Fecal Suspension. Fresh feces (1.0 g) obtained from male SD rats was homogenized in 0.1 M phosphate buffer saline (PBS) (pH 7.4, 25 mL) by bubbling with CO_2 gas to eliminate air, and sediments were removed by filtration through gauze. The filtrate was used as a fecal suspension in this experiment.

Each tube containing **1** (0.5 mg), **5** (0.1 mg), and **6** (0.1 mg) in dimethyl sulfoxide (10 μL) and fecal suspension (3 mL) was incubated at intervals at 37 °C in an anaerobic jar in which air was replaced with oxygen-free CO_2. The resulting mixture was adjusted to pH ca. 3 with 0.05% trichloroacetic acid and extracted three times with AcOEt (10 mL). The AcOEt layer was concentrated to dryness in vacuo, and the residue was dissolved in MeOH (1 mL). A 20 μL sample of the solution was analyzed by HPLC.

Incubation of Phyllodulcin with Rat Liver 9000*g* Supernatant. The rats were killed by decapitation and the livers immediately removed. They were perfused in situ with 1.15% KCl solution, weighed, cut into small pieces, and then homogenized in three volumes (w/v) of 0.1 M PBS (pH 7.4). All subsequent procedures were performed at 0−4 °C. Nuclear and celluar debris and mitochondoria were sedimented by centrifugation at 9000g for 20 min at 4 °C. The supernatant (S-9 fraction) was used for metabolism of phyllodulcin according to the method described by Cooper and Brodie,[13] with the following modifications. The incubation mixture contained the S-9 fraction (10 mL), 1 mM phyllodulcin dissolved in 100 μL of MeOH, the NADPH generating system (1.3 mM $NADP^+$, 40 units of glucose-6-phosphate dehydrogenase, 0.3 mM glucose-6-phosphate), 3.3 mM $MgCl_2$, and 0.1 M PBS (pH 7.4), in a final volume of 12 mL. Protein concentration was determined by the method of Lowry et al. with bovine serum albumin as the standard protein. The amount of protein used was 350 μg/mL, and the incubation was carried out at 37 °C for 24 h. The mixture was extracted twice with AcOEt, and

the organic layer was then evaporated to dryness at 40 °C. The residue was dissolved in MeOH, the mixture was filtered through a 0.45 μm membrane filter, and then 20 μL of the sample was injected into the HPLC.

Quantitative Analysis of Metabolites. A calibration graph was prepared from peak areas obtained by injecting 20 μL of the sample solution for HPLC over the concentration range 5−500 μg/mL for **1**, 4−1580 μg/mL for **4**, 5−50 μg/mL for **5**, 50−1500 μg/mL for **6**, and 10−150 μg/mL for **7**. The resulting calibration graphs were linear, and each quantitative value represents the mean of three experiments. The recoveries of standards added to each blank sample were 88.8−96.2%, and the relative standard deviations were 1.24−3.19%.

Supporting Information Available: Isolation procedure of **2** and **3**. This information is available free of charge via the Internet at http://pubs.acs.org.

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