Metabolic Fate of Fraxin Administered Orally to Rats

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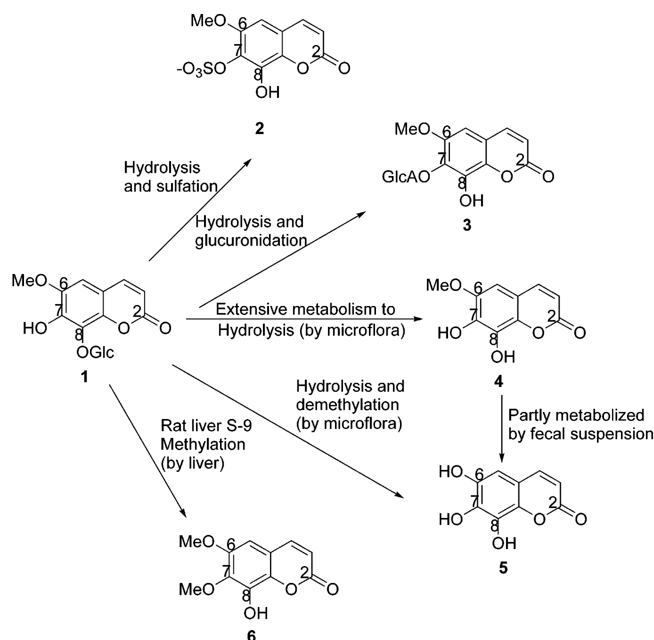
Naturally occurring fraxin (**1**) was administered orally to rats to investigate its metabolism. Urinary metabolites were analyzed by three-dimensional HPLC, and fraxetin-7-*O*-sulfate (**2**), fraxetin-7-*O*-β-glucuronide (**3**), fraxetin (**4**), 6,7,8-trihydroxycoumarin (**5**), and fraxidin (**6**) were isolated. Fraxin (**1**) was extensively metabolized to **4**, which was partly metabolized to **5** in a rat fecal suspension after incubation for 24 h. Urinary excretion of **4** and **5** in rats administered orally with **1** was substantially reduced when the rats were treated with antibiotics to suppress their intestinal flora. Incubation of **1** with a rat liver S-9 mixture yielded **6**. These results suggest that hydrolysis and demethylation of **1** are performed by intestinal microflora, while methylation occurs in the liver.

The dried bark of *Fraxinus japonica* Seringe Blume (Oleaceae) is currently sold as the Kampo medicine "shinpi" in Japan. It has traditionally been used as a diuretic, an antifebrile, an analgesic, and an antirheumatic agent.[1] The isolation of various coumarins, such as fraxin (**1**), fraxetin (**4**), escletin, and esculin, from the bark has been reported.[2,3] Among these compounds, **1** is known to be responsible for the diuretic and anti-inflammatory action of "shinpi".[4−6] Although some biological effects of **1** have been described, the metabolic fate of this compound has yet to be reported. Here, we describe the identification of urinary metabolites of fraxin (**1**) in rats. In addition, we report the biotransformation of **1** using antibiotic-treated rats, rat fecal suspension, and a rat liver S-9 mixture.

Scheme 1. Structure of **1** and Its Proposed Metabolic Pathway in Rats



Results and Discussion

Two distinct HPLC peaks due to metabolites **2** and **3** were detected in the untreated urine of rats orally administered with fraxin (**1**), whereas three distinct HPLC peaks due to **4**, **5**, and **6** were detected in β-glucuronidase/arylsulfatase-treated urine of rats orally administered with **1**. Unchanged **1** was not detected in either urine sample. Compounds **2**−**6** were isolated from urine samples by chromatography, as described in the Experimental Section.

Metabolite **2** was obtained as a white powder. Enzymatic hydrolysis of **2** with arylsulfatase yielded **4**, as confirmed by t_R agreement using HPLC. Intense absorption at 1049 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 $(M - H)^-$ at *m/z* 287 along with fragment ion peaks at *m/z* 207 $(M - H - SO_3)^-$, thus indicating one sulfate group in **2**. A comparison of the ^{13}C NMR spectrum of **2** with that of **4** showed that the C-7 signal of **2** has been shifted 6.6 ppm upfield, accompanied by downfield shifts of C-6 (4.1 ppm) and C-8 (5.7 ppm). These shifts indicated a sulfate group at C-7. On the basis of these data, **2** was determined to be fraxetin-7-*O*-sulfate.

Metabolite **3** was obtained as a white powder. Enzymatic hydrolysis of **3** with β-glucuronidase yielded **4**. The ^1H NMR spectrum of **3** showed one anomeric proton at δ 4.94 (d, $J = 9.6$ Hz), and negative-ion FABMS showed a molecular ion at *m/z* 383 $(M - H)^-$ corresponding to a mono-glucuronide. A comparison of the ^{13}C NMR spectrum of **3** with that of **4** showed that C-7 of **3** was shifted 3.0 ppm upfield, accompanied by downfield shifts of C-6 (3.6 ppm) and C-8 (2.9 ppm). These shifts indicated a glucuronide group at C-7. HMBC revealed a correlation between the anomeric proton and C-7. Thus, **3** was determined to be fraxetin-7-*O*-β-glucuronide.

Metabolite **4** was identified as fraxetin, by direct comparison of **4** with an authentic sample by EIMS and ^1H NMR data.

Metabolite **5** was obtained as a white powder. The ^1H and ^{13}C NMR data of **5** were similar to those of **4**, except for the disappearance of an *O*-methyl resonance. These data indicate that **5** was a demethylated derivative of **4**. Thus, **5** was 6,7,8-trihydroxycoumarin. Confirmation of the structure of **5** was made by direct comparison using EIMS and ^1H NMR data with an authentic sample, which was prepared from **4** by BBr_3 treatment.

Metabolite **6** was identified as fraxidin by direct comparison of EIMS and ^1H NMR spectral data with authentic samples.

To determine the contribution of the intestinal microflora to the formation of **4**−**6**, we examined the changes in urinary metabolite excretion when rats were treated with antibiotics. Urinary excretion of **4** and **5** was significantly decreased at 24 h after administration of **1** in rats treated with antibiotics (**4**, $p < 0.05$; **5**, $p < 0.001$), while the excretion of **6** was not significantly different when compared with nontreated rats (Figure 1). These results indicate the importance of gut flora in generating urinary metabolites **4** and

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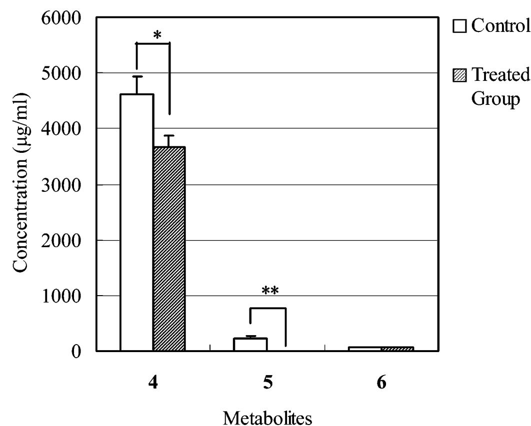

Figure 1. Effects of antibiotics on urinary excretion from rats orally administered **1** (100 mg/kg). The antibiotics phthalylsulfathiazole, kanamycin sulfate, tetracycline hydrochloride, and bacitracin were orally administered for 4 days before administration of **1**. Statistical analysis was carried out using the student's t-test ($n = 6$). $^{**}p < 0.001$, $^{*}p < 0.05$.

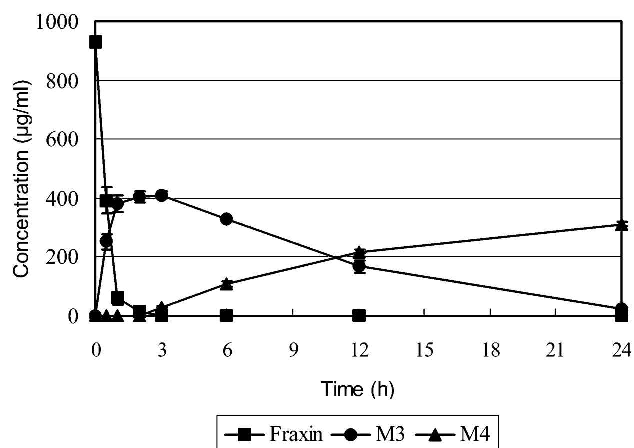

Figure 2. Time course of metabolite formation by bacterial mixture from rat feces.

5. On the other hand, the main site of formation of **6** was thought to be the liver. These findings prompted us to investigate the biotransformation of **1** by intestinal microflora and that of **4** by rat liver S-9 fraction. During anaerobic incubation of **1** with rat fecal microflora, **1** was almost completely consumed after 24 h, and metabolites **4** and **5** were produced (Figure 2). Metabolite **4** increased progressively and peaked at 3 h, then decreased gradually with prolonged incubation. Metabolite **5** was also produced, peaking at 24 h and increasing with prolonged incubation. During incubation of **4** with rat liver S-9 fraction in the presence of an NADPH-generating system and *S*-adenosyl-L-methionine, **4** decomposed in a time-dependent manner and after 8 h treatment was partly converted to **6**, which at its peak accounted for 29.1% of the initial amount of **4** (Figure 3). Incubation of **4** without *S*-adenosyl-L-methionine did not produce **6**, suggesting that methylation of **4** occurred by catechol-*O*-methyltransferase (COMT) (data not shown).

In the present study of in vivo metabolism of **1**, five urinary metabolites including sulfate and glucuronide conjugates of **4** were detected in rat urine by HPLC. Furthermore, it was shown that **4** and **5** were produced by intestinal microflora, while **6** was produced by methylation in the S-9 fraction. These data were obtained through the use of antibiotic-treated rats and incubation of **1** with rat fecal microflora and rat liver S-9 fraction, respectively. The following metabolic pathways were deduced from the results of the present study: **1** was hydrolyzed to produce **4**, followed by demethylation to produce **5** in the intestinal tract; **4** was partly methylated to **6** in the liver.

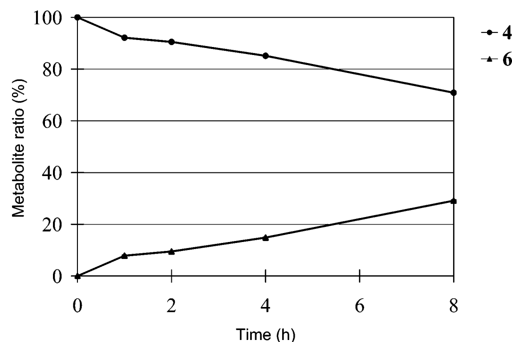

Figure 3. Time course of metabolite formation by rat S-9 mixture.

In general, orally administered phenolic compounds, particularly those with low polarity, undergo hydroxylation and/or glucuronide and sulfate conjugation primarily by intestinal microflora and secondarily in the liver and other tissues. It is well known that phenolic methoxyl groups of aromatic compounds, such as flavonoids and coumarins, are partly demethylated in the liver.[7−11] However, it is interesting to note that both methylated and demethylated metabolites of **4** were produced after hydrolysis of **1** in the rat body.

Experimental Section

General Experimental Procedures. Fraxin, arylsulfatase (Type H-1), β-glucuronidase (Type H-2), glucose-6-phosphate, and proadifen were purchased from Sigma (St. Louis, MO). 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). Glucose-6-phosphate dehydrogenase was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). For column chromatography, Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) was used. All other commercially available reagents were of the highest purity. 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 a JEOL JNM-EX 400 (^{1}H, 400; ^{13}C, 100 MHz) and JNM-LA 600 (^{1}H, 600; ^{13}C, 150 MHz) spectrometer. Chemical shifts are given in δ values (ppm) downfield relative to tetramethylsilane. Electron impact (EI) and fast atom bombardment (FAB)-MS were performed on a JEOL JMS-DX 303 mass spectrometer. The HPLC system comprised a CCMP-II pump, a CO-8020 column oven (Tosoh, Tokyo, Japan), and a model MCPD-3600 photodiode array detector (Otsuka, Osaka, Japan). HPLC conditions for analysis of metabolites were as follows: column, CAPCELLPAK C_{18} (5 μm, 3.0 mm i.d. $\times$ 250 mm, Shiseido, Tokyo, Japan); column temperature, 40 °C; flow rate, 0.5 mL/min; detection, 200−400 nm; mobile phase, linear gradient of 0.1% trifluoroacetic acid in H_2O (A) and CH_3CN (B), A/B = 95/5 (0 min) → 90/10 (80 min) → 60/40 (90 min).

Animal Experiments. Male Sprague−Dawley rats (6 weeks, 160−180 g) were purchased from Japan SLC, Inc. Animals were housed at 22 ± 2 °C and 55 ± 10% humidity in a light-controlled room (light from 9:00 to 21:00) 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 fraxin (100 mg/kg body weight) uniformly dispersed in water was administered orally by direct stomach intubation. Animals had free access to water and sugar during the experiments.

Preparation of Urine Samples. Urine samples were collected over a 24 h period using a metabolic cage. Urine was filtered through a 0.45 μm membrane filter, and 50 μL was subjected to HPLC.

Enzymatic Hydrolysis of Urine Samples. Urine (20 mL) was transferred to a test tube to which 5.0 mL citrate buffer (pH 5.2) and 150 μL of β-glucuronidase solution were added followed by incubation at 37 °C for 24 h. The incubated solution was extracted three times with EtOAc (40 mL). The organic layer was dried over anhydrous Na_2SO_4 and was evaporated to dryness at 40 °C. The residue was dissolved in MeOH, and a 10 μL aliquot was subjected to HPLC.

Isolation of Metabolites. For isolation of **2** and **3**, urine (120 mL) obtained from rats after oral administration of **1** (540 mg) was lyophilized, dissolved in purified H_2O, and centrifuged at 3000 rpm for 20 min. The supernatant was subjected to Sephadex LH-20 column chromatography ($H_2O \rightarrow$ MeOH). The H_2O-eluted fraction was again subjected to column chromatography and was lyophilized to obtain the metabolites **2** (4 mg) and **3** (2 mg). For isolation of **4**−**6**, urine (130 mL) obtained from rats after oral administration of **1** (610 mg) was incubated with β-glucuronidase/arylsulfatase and was extracted three times with EtOAc. The organic layer was dried over 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 subjected to chromatography on Sephadex LH-20 with MeOH. Fractions containing metabolites **4**−**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, A/B = 95/5 (0 min) → 80/20 (90 min) → 90/10 (100 min); flow rate, 1.0 mL/min; detection wavelength, 220 nm. Each metabolite fraction was evaporated to dryness at 40 °C in vacuo to afford **4** (58 mg), **5** (3 mg), and **6** (1 mg).

Compound 2: white powder (4 mg); IR (KBr) ν_{max} 3200, 1714, 1617, 1505, 1461, 1049 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 7.95 (1H, d, J = 9.0 Hz, H-4), 6.86 (1H, s, H-5), 6.41 (1H, d, J = 9.6 Hz, H-3), 3.78 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 159.5 (C, C-2), 149.3 (C, C-6), 143.9 (CH, C-4), 138.4 (C, C-8), 137.9 (C, C-8a), 132.6 (C, C-7), 114.9 (CH or C, C-3 or 4a), 114.8 (CH or C, C-3 or 4a), 100.1 (CH, C-5), 55.5 (CH_3, OCH_3); negative FABMS m/z 287 (M − H)$^-$, 207.

Compound 3: white powder (2 mg); IR (KBr) ν_{max} 3416, 1714, 1617, 1507, 1466 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.93 (1H, d, J = 9.6 Hz, H-4), 6.84 (1H, s, H-5), 6.39 (1H, d, J = 9.6 Hz, H-3), 4.94 (1H, d, J = 7.6 Hz, H-1′), 3.78 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 170.7 (C, C-6′), 159.5 (C, C-2), 148.8 (C, C-6), 144.0 (CH, C-4), 137.7 (C, C-8a), 136.2 (C, C-7), 135.6 (C, C-8), 114.5 (CH and C, C-3 and 4a), 103.6 (CH, C-1′), 99.9 (CH, C-5), 75.7 (CH, C-3′), 75.1 (CH, C-5′), 72.8 (CH, C-2′), 71.1 (CH, C-4′), 55.8 (CH_3, OCH_3); negative FABMS m/z 383 (M − H)$^-$, 207, 192.

Compound 4: pale yellow powder (58 mg); IR (KBr) ν_{max} 3411, 1688, 1612, 1508, 1456 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 7.89 (1H, d, J = 9.0 Hz, H-4), 6.80 (1H, s, H-5), 6.22 (1H, d, J = 9.6 Hz, H-3), 3.82 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 160.4 (C, C-2), 145.2 (C, C-6), 144.9 (CH, C-4), 139.2 (C, C-7, 8a), 132.7 (C, C-8), 111.7 (CH, C-3), 110.1 (C, C-4a), 100.2 (CH, C-5), 55.9 (CH_3, OCH_3); EIMS m/z 208 [M]$^+$, 193, 180, 165, 137, 109; HREIMS m/z 208.0355 (calcd for $C_{10}H_8O_5$, 208.0372).

Compound 5: yellow powder (3 mg); IR (KBr) ν_{max} 3233, 1684, 1620, 1531, 1478 cm^{-1}; ^{1}H NMR (acetone-d_6, 600 MHz) δ 7.75 (1H, d, J = 9.6 Hz, H-4), 6.63 (1H, s, H-5), 6.13 (1H, d, J = 9.6 Hz, H-3); ^{13}C NMR (acetone-d_6, 150 MHz) δ 161.0 (C, C-2), 145.2 (CH, C-4), 143.7 (C, C-6), 139.1 (C, C-7 or 8a), 139.0 (C, C-7 or 8a), 133.6 (C, C-8), 113.2 (CH, C-3), 111.9 (C, C-4a), 104.3 (CH, C-5); EIMS m/z 194 [M]$^+$, 166, 138, 110; HREIMS m/z 194.0216 (calcd for $C_9H_6O_5$, 194.0215).

Compound 6: colorless needles (CH_3OH) (1 mg); mp 170−180 °C (ref 172−174 °C); IR (KBr) ν_{max} 3288, 1690, 1613, 1501, 1464 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 7.93 (1H, d, J = 9.6 Hz, H-4), 6.83 (1H, s, H-5), 6.37 (1H, d, J = 9.6 Hz, H-3), 3.82 (3H, s, 6-OCH_3), 3.77 (3H, s, 7-OCH_3); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 160.0 (C, C-2), 149.6 (C, C-6), 144.6 (CH, C-4), 140.0 (C, C-7), 138.4 (C, C-8 or 8a), 138.3 (C, C-8 or 8a), 114.4 (C, C-4a), 114.2 (CH, C-3), 100.0 (CH, C-5), 60.4 (CH_3, 7-OCH_3), 55.8 (CH_3, 6-OCH_3); EIMS m/z 222 [M]$^+$, 207, 179; HREIMS m/z 222.0538 (calcd for $C_{11}H_{10}O_5$, 222.0528).

Demethylation of 4. BBr_3 (3.85 mL) was added dropwise to a solution of **4** (200 mg) in CH_2Cl_2 (20 mL) at 0 °C and was then stirred at room temperature under N_2 for 24 h. The reaction mixture was evaporated in vacuo, and the residue was dissolved in H_2O (100 mL). The solution was extracted with EtOAc, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was dissolved in MeOH and subjected to chromatography on Sephadex LH-20 using MeOH as the eluant. The fraction containing **5** was again subjected to column chromatography to obtain **5** (160 mg, yield 86%).

Antibiotic Treatment of Animals. Gut sterilization was performed 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. One hour after 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 EtOAc. 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 subjected to HPLC. Statistical analysis was carried out using the Student's t-test ($n = 6$). P values of less than 0.05 were considered to indicate statistical significance.

Incubation of 1 with Rat Fecal Suspension. Fresh feces (8.0 g) obtained from male SD rats was homogenized in bicarbonate buffer (pH 7.0, 25 mL) by bubbling with CO_2 gas to eliminate air, and sediment was removed by filtration through gauze. The filtrate was used as a fecal suspension.

A tube containing **1** (1.0 mg) in DMSO (30 μL) and fecal suspension (2 mL) was incubated 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 was extracted three times with EtOAc (10 mL). The EtOAc layer was concentrated to dryness in vacuo, and the residue was dissolved in MeOH (1 mL). A 20 μL aliquot of this solution was analyzed by HPLC.

Incubation of 1 with Rat Liver 9000*g* Supernatant. The S-9 fraction of male SD rats (control animal liver S9, In Vitro Technologies Inc., MD) was used to investigate the metabolism of **1** according to the method described by Cooper and Brodie,[13] with the following modifications. The incubation mixture contained S-9 fraction (8 mL), 1 mM *S*-(5′-adenosyl)-L-methionine chloride (3 mg), 20 mM $MgCl_2 \cdot 6H_2O$, and **4** (5 mg) dissolved in 0.1 M PBS (pH 7.4, 7.5 mL). Incubation was carried out at 37 °C for 0, 1, 2, 4, and 8 h. The mixture was extracted twice with EtOAc, and the organic layer was evaporated to dryness at 40 °C. The residue was dissolved in MeOH and filtered through a 0.45 μm membrane filter, and 20 μL of the sample was subjected to HPLC.

Quantitative Analysis of Metabolites. A calibration graph was prepared from peak areas obtained by subjecting 10 μL of the sample solution to HPLC over a concentration range 10−800 μg/mL for **1**, 50−5000 μg/mL for **4**, 30−500 μg/mL for **5**, and 30−250 μg/mL for **6**. The resulting calibration graphs were linear, and each quantitative value represented the mean of three experiments. Recoveries of standards added to each blank sample were 90.2−99.4%, and the relative standard deviations were 2.12−3.34%.

Supporting Information Available: Three-dimensional HPLC chromatograms of rat urine excreted for 24 h after oral administration of **1** (100 mg/kg). This information is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Kariyone, T.; Kimura, Y. *Saishin Wakanyakuyoshokubutsu*; Hirokawa Shoten: Tokyo, 1976; p 105.
(2) Shimada, G. *Yakugaku Zasshi* **1940**, *60*, 508−510.
(3) Morita, N.; Hori M. *Yakugaku Zasshi* **1953**, *73*, 771−772.
(4) Yamagami, I.; Suzuki, Y.; Itoh, K. *Folia Pharmacol. Jpn.* **1968**, *64*, 714−729.
(5) Iida, G. *Tohoku J. Exp. Med.* **1935**, *25*, 454−470.
(6) Okui, T. *Tohoku J. Exp. Med.* **1938**, *32*, 225−232.
(7) Canivenc-Lavier, M. C.; Brunold, C.; Siess, M. H.; Suschetet M. *Xenobiotica* **1993**, *23*, 259−266.
(8) Mennes, W. C.; Van Holsteijn, C. W. M.; Timmerman, A.; Noordhoek, J.; Blaauboer, B. J. *Biochem. Pharmacol.* **1991**, *41*, 1203−1208.
(9) Mennes, W. C.; Luijcky, N. B. L.; Wortelboer, H. M.; Noordhoek, J.; Blaauboer, B. J. *Arch. Toxicol.* **1993**, *67*, 92−97.
(10) Horsmans, Y.; Desager, J. P.; Harvengt, C. *Life Sci.* **1993**, *53*, 421−426.
(11) Yue, Q. Y.; Säwe, J. *Eur. J. Clin. Pharmacol.* **1997**, *52*, 41−47.

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