Stereochemistry and Putative Origins of Flavanones Found in Post-administration Urine of the Traditional Chinese Remedies Shosaiko-to and Daisaiko-to

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Optically active flavanones, dihydrowogonin and dihydrooroxylin A, were found in the urine of healthy volunteers who orally received the traditional Chinese remedies Shosaiko-to and Daisaiko-to on separate occasions. These remedies, which consisted of dried extracts of Scutellariae Radix and other herbs, contained the metabolic precursors of the flavanones, but not the flavanones themselves, in stoichiometrically sufficient amounts. Structures and stereochemistry of the flavanones were elucidated by UV, circular dichroism (CD), electron impact (EI)-MS and ^{1}H-NMR analyses, showing that the biotransformations from the corresponding flavones, wogonin and oroxylin A, were stereoselective with a preference for the *S*-enantiomers. The putative origins of the flavanones were confirmed in terms of pharmacokinetics. Renal excretion–time data of the flavanones and the flavones suggested that the stereoselective transformations might have occurred in the intestinal tract as a result of microfloral metabolism before absorption.

Key words Shosaiko-to; Daisaiko-to; dihydrowogonin; dihydrooroxylin A; biotransformation

Traditional herbal remedies have been used for healing purposes for thousands of years in China. After their introduction into Japan in the sixth century, Chinese remedies were modified into Kampo remedies, and dried herb extract formulations were permitted under the Japanese national health insurance system two decades ago. In most cases, these herbal remedies are orally administered so that the chemical components present in the herbal remedies may be manipulated by intestinal microflora before being absorbed into the body. The glycosides present are usually absorbed after being hydrolyzed by bacterial enzymes to the corresponding aglycones.[1] Anaerobic hydrogenation can also be seen, *e.g.*, sennoside in Sennae Folium to give rheinanthrone.[2] Some of the bacterially generated products are not found in the medicines. Thus, to evaluate the therapeutic effects of the remedies, investigation of the compounds actually absorbed into the body is indispensable.

In our studies of traditional Chinese remedies, we have identified nine herbal compounds[3] in human urine following oral administration of the remedies Shosaiko-to (TJ-9, Tsumura, Tokyo, Japan) and Daisaiko-to (TJ-8) (see Table 1 for the herb materials). The two herbal remedies are used for the treatment of chronic hepatitis[4] and hyperlipidemia,[5] respectively. We have reported preliminarily[6] that dihydrooroxylin A found in post-administration timed urine might be a metabolite formed from oroxylin A, a major flavone in the remedies. An additional unknown flavanone, which could not be detected in the remedies, showed a similar pharmacokinetic profile to that of dihydrooroxylin A. In our present paper, we describe the structure elucidation and putative origins of these urinary flavanones. The chemical and pharmacokinetic investigations of both flavanones and flavones suggested that the stereoselective hydrogenations of flavones gave predominantly the *S*-flavanones and the site of metabolism might be the intestinal microflora, rather than the liver.

As shown in the chromatograms (Fig. 1) of post-administration urine of TJ-9 and TJ-8, we found four flavonoid peaks in the group. Compounds **1** and **2** were already identified as wogonin (5,7-dihydroxy-8-methoxyflavone) and oroxylin A (5,7-dihydroxy-6-methoxyflavone).[3] In our present study, we will report the stereochemistry and putative origins of **3** and **4**.

Base molecular masses of *m/z* 286 were common to **3** and **4**, and greater than the values of **1** and **2** by 2 mass units. The similarity observed in their UV spectra in Fig. 1 also suggested that **3** and **4** were isomers related to **1** and **2**. The UV and ^{1}H-NMR spectra of **3** and **4** were almost superimposable on the reported data for dihydrowogonin[7] and dihydrooroxylin A,[8] respectively. Final assignment of their structures was made on the basis of an apparent difference in the chemical shifts of methoxyl protons (3.88 and 3.95 ppm for **3** and **4**, respectively). Another distinction was observed in their melting points, where the value of 152—154 °C for **3** was in accordance with the reported value for dihydrowogonin and was considerably lower than that of **4** (166—168 °C), which was consistent with the known value for dihydrooroxylin A.[7,8]

The herbal origins of **3** and **4** in TJ-9 and TJ-8 were confirmed by analyzing urine samples collected after oral administration of the aqueous extract of Scutellariae Radix, one of the herbal ingredients in both of the medicines (Table 1). According to our analysis, this herb contained substantial amounts of **1** and **2** and a small amount of **4**, but no **3**. Although there are many reports dealing with other botanic sources of dihydrowogonin, such as *Prunus avium*,[9] *Prunus cerasus*,[10] *Pyracantha coccinea*,[11] *Helichrysum cymosum*,[12] and *Chenopodium procerum*,[13] none of them are herb materials of both TJ-9 and TJ-8. In our present studies, however, we detected considerable amounts of **3** and **4** in urine obtained after administration of the extract of *Scutellaria* (*S.*) *baicalensis*. Renal excretion profiles of **3** and **4** were similar to those

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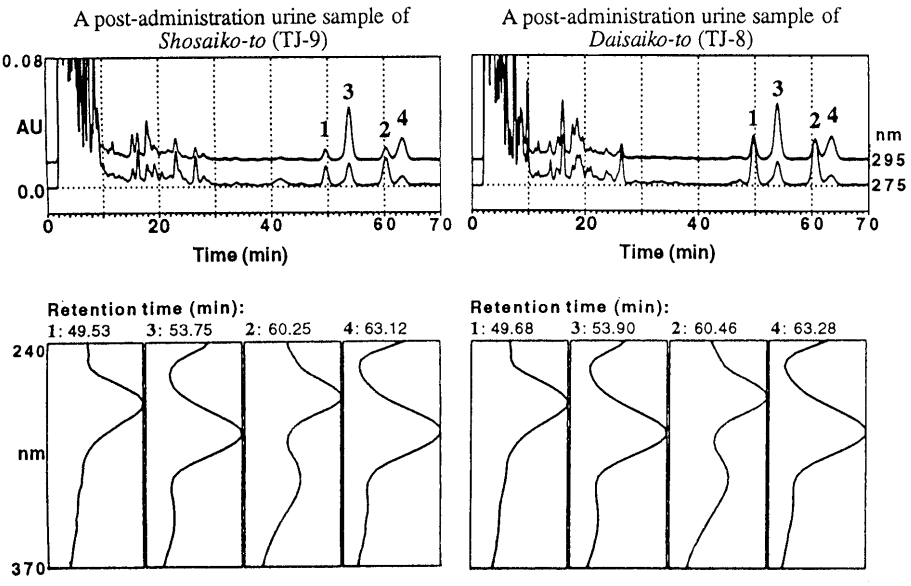

Fig. 1. Typical Chromatograms of the Enzyme-Treated Urine Samples Collected at 24—36 h Following Oral Administration of the Traditional Chinese Remedies to One of Our Subjects

The ODS column was maintained at 27 °C and H_2O–CH_3CN–CH_3COOH (69 : 30 : 1, v/v) was delivered at 1 ml/min. The UV spectra were determined with a diode-array detector. Key: **1**, wogonin; **2**, oroxylin A; **3**, dihydrowogonin; **4**, dihydrooroxylin A.

Table 1. Ingredient Herbs in Shosaiko-to (TJ-9) and Daisaiko-to (TJ-8)

Herb	Family	TJ-9	TJ-8
		%	
Bupleuri Radix	Umbelliferae	29.2	26.1
Scutellariae Radix	Labiatae	**12.5**	**13.0**
Pinelliae Tuber	Araceae	20.8	17.4
Zingiberis Rhizoma	Zingiberaceae	4.2	4.4
Zizyphi Fructus	Rhamnaceae	12.5	13.0
Ginseng Radix	Araliaceae	12.5	
Glycyrrhizae Radix	Leguminosae	8.3	
Rhei Rhizoma	Polygonaceae		4.4
Aurantii Fructus Immaturus	Rutaceae		8.7
Paeoniae Radix	Paeoniaceae		13.0

Table 2. Quantitative Comparison of Flavonoid Contents between Remedies and Renal Excretion

Compound	Molar amount[a] present in the remedy administered		Cumulative molar amount[b] excreted after administration of remedies	
	TJ-9	TJ-8	TJ-9	TJ-8
Wogonin (**1**)	49.98×10^{-6}	47.81×10^{-6}	3.32×10^{-6}	4.54×10^{-6}
Oroxylin A (**2**)	27.42×10^{-6}	25.46×10^{-6}	8.16×10^{-6}	9.63×10^{-6}
Dihydrowogonin (**3**)	N.D.[c]	N.D.[c]	7.00×10^{-6}	9.10×10^{-6}
Dihydrooroxylin A (**4**)	1.04×10^{-6}	1.15×10^{-6}	4.38×10^{-6}	6.53×10^{-6}

a) Aglycone + glycosides. *b*) Free + conjugated. The urine samples were collected over 0—48 h following administration of the remedies. *c*) Not detected.

after the administration of TJ-9 and TJ-8.

We further conducted quantitative analysis of **1**—**4** present in the dosed TJ-9 and TJ-8, as well as excreted in urine samples collected up to 48 h following administration. The results are shown in Table 2. Dihydrowogonin (**3**) and its glucoside were not detected in the remedies at all, while small amounts of dihydrooroxylin A (**4**) and its glucoside were detected in the remedies, as expected from the reported data on *S. baicalensis*.[8,14] We also found considerable amounts of **1** and **2** in the remedies.

Although **3** was not detected in the remedies, considerable amounts of **3** were found in the urine samples. In the case of **4**, its cumulative excretion amounts were 4.2 and 5.7 times greater than the dosed amounts in TJ-9 and TJ-8, respectively. In contrast, dosed amounts of **1** and **2**, including their glycosides, were much greater than their cumulative excretion amounts, respectively. In the case of **1**, the dosed molar amounts in TJ-9 and TJ-8 were 15.1 and 10.5 times greater than the corresponding amounts excreted and also greater than the sum of **1** and **3** excreted in the urine. In the case of **2**, the dosed molar amounts in TJ-9 and TJ-8 were 3.4- and 2.6-fold greater than the excreted amounts and also greater than the sum of **2** and **4** excreted. These stoichiometric results suggested strongly that the whole of **3** and the majority of **4** excreted must be metabolites produced in the body from the flavones *via* some biotransformation mechanisms.

We also examined the excretion rate–time curves of **3** and **4**, comparing them with those of their putative precursors, **1** and **2**, respectively. As shown in Fig. 2, **3** exhibited an appreciable lag time for excretion, that is, the time between the administration of remedies and the start of excretion of **3**. Additionally, renal excretion rate peaks for **3** were observed at 27.5 and 22.25 h following administration of TJ-9 and TJ-8, respectively, which were after those of **1** had reached a maximum value. In the case of **4**, its excretion rate–time curves were trimodal with peaks at 0.5, 7.5 and 27.5 h. The first peak was attributed to the excretion of dihydrooroxylin A aglycone in the remedies, the second peak was derived from dihydrooroxylin A glucoside *via* the intestinal hydrolysis, and the third one was the hydrogenated metabolite of oroxylin A. The third peak of **4** was similar to the excretion rate peak of **3**. Thus, we conclude that **1** and **2** are the most possible precursors of **3** and **4**, respectively. Flavones can be hydrogenated to the corresponding flavanone and the

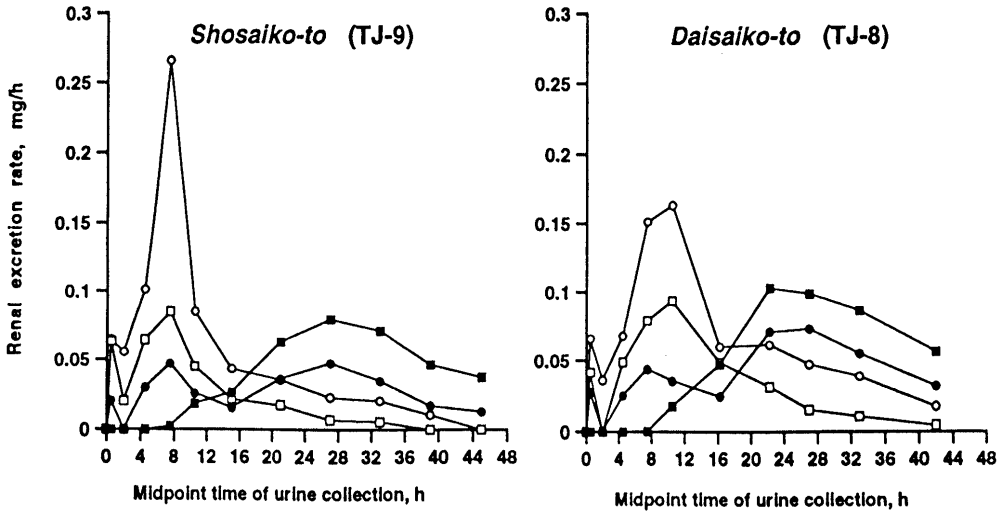

Fig. 2. Renal Excretion Rate–Time Profiles of Flavonoids (Free+Conjugated) Following Oral Administration of the Traditional Chinese Remedies Shosaiko-to (TJ-9) and Daisaiko-to (TJ-8) on Separate Occasions to One of the Subjects

Key: —□—, wogonin (**1**); —○—, oroxylin A (**2**); —■—, dihydrowogonin (**3**); —●—, dihydrooroxylin A (**4**).

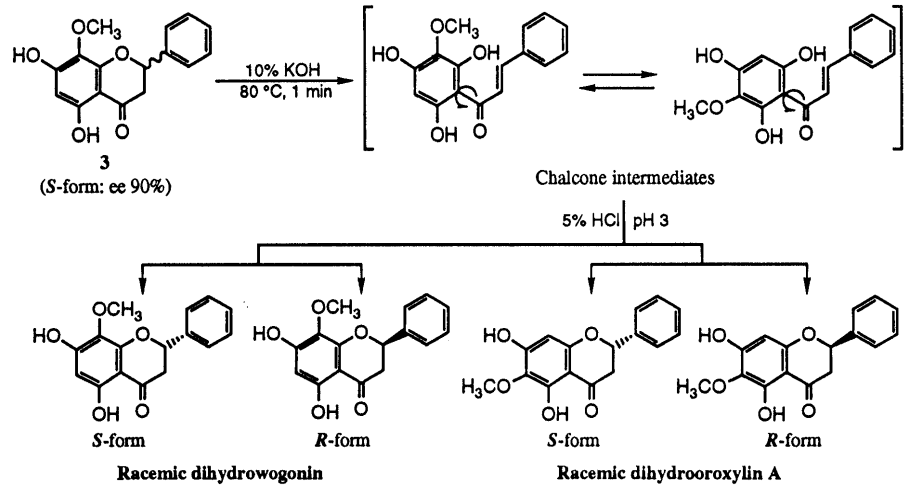

Chart 1. Chemical Transformation of Urinary Dihydrowogonin (**3**) Simultaneously into Racemic Dihydrowogonin and Dihydrooroxylin A

biotransformations are mediated mainly by the human intestinal microflora. An example of the biotransformation of flavone to flavanone by human intestinal bacteria has been reported by Hattori *et al.*[15] From our observation of the above excretion profiles, we inferred that the biotransformations of **1** and **2** might also predominently occur in the intestinal tract.

The stereochemistry of urinary **3** and **4** was investigated by means of chiral HPLC and circular dichroism (CD) spectrophotometry. The CD spectra exhibited a negative $\pi \rightarrow \pi^*$ Cotton effect and a positive $n \rightarrow \pi^*$ Cotton effect, indicating that both urinary metabolites preferentially possessed *S*-configurations.[16] To obtain racemic derivatives of **3** and **4**, we conducted a racemization reaction as described in the Experimental section. Chemical treatments of optically active urinary **3** in 10% KOH solution followed by acidification of the resulting mixture with 5% HCl to pH 3 gave racemic dihydrowogonin and dihydrooroxylin A simultaneously. The chemical transformation is considered to involve isomerization between two tautomers of ring-opened chalcone structures (Chart 1). The resulting racemic mixtures showed two paired chromatographic peaks (Fig. 3.) Paired peaks showed superimposable UV spectra. Since both **3** and **4** exhibited *S*-optical activity, the enantiomeric elution orders were easily determined by comparing the chiral chromatograms of the *S*-rich urinary flavanones with those of the racemic mixtures. The *R*-enantiomer of dihydrowogonin eluted first, while the *S*-antipode did so in the case of dihydrooroxylin A. The urinary products also showed paired peaks with the peak area being strongly perturbed in favor of the *S*-enantiomers. Enantioexcesses of the *S*-enantiomers calculated from the peak area were 90% and 80% in urinary **3** and **4**, respectively.

Finally, we conclude that dihydrowogonin (**3**) excreted into the urine was produced from wogonin. In the case of urinary dihydrooroxylin A (**4**), the majority was derived from oroxylin A (**2**) and the remainder was of herbal origin. It is worth mentioning that baicalein (5,6,7-trihydroxyflavone), another major flavone present in *S. baicalensis*, was also transformed into its dihydro derivative (data not shown). However, the rate of the transformation was very low. Taking this into consideration, we anticipate in general that flavones present in herbal medicines could be more or less transformed into the corresponding flavanones in the body. The chemistry and

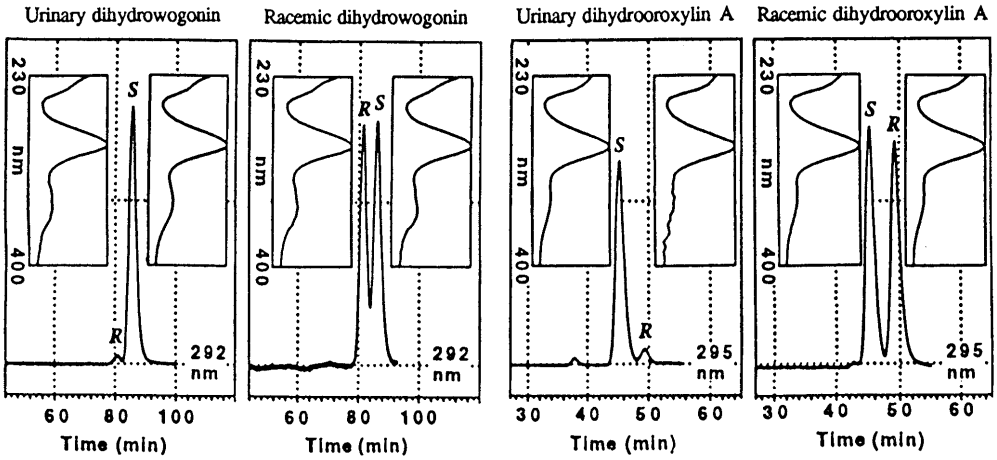

Fig. 3. Enantiomeric Resolution of the Urinary Flavanones and Their Corresponding Racemic Modifications

The Chiralcel OD column was maintained at 25 °C, and UV detection was set over 230—400 nm. The mobile phases delivered at 0.4 ml/min were *n*-hexane–EtOH–CH_3COOH (959 : 40 : 1, v/v) for urinary/racemic dihydrowogonin and *n*-hexane–EtOH–CH_3COOH (919 : 80 : 1, v/v) for urinary/racemic dihydrooroxylin A.

biochemistry of the biotransformed flavanones may play important roles in the pharmacological effects of herbal medicines.

Experimental

The HPLC system consisted of a solvent delivery pump (BIP-I, Jasco, Tokyo, Japan), a syringe loading sample injector (Model 7125, Rheodyne, Cotati, CA, U.S.A.), an octadecyl silica (ODS) analytical column (UG120, 250 × 4.6 mm, Shiseido, Tokyo, Japan) maintained at 27 °C in a column oven, and a UV/Vis multi-wavelength detector (MD-910, Jasco). The signal from the detector was collected and analyzed with a DP-L910/V system (Version 7, Jasco). A Chiralcel OD column (250 × 4.6 mm, Daicel, Tokyo, Japan) maintained at 25 °C was used in chiral HPLC analysis.

Melting points were determined by using a micro-melting point apparatus (Yanagimoto, Tokyo, Japan) without correction. UV/Vis spectra were run on a UV/Vis spectrophotometer (Ubest-30, Jasco). ^{1}H-NMR spectra were recorded in $CDCl_3$ on a NMR spectrometer (AM-400, Bruker, Germany) at 400 MHz using tetramethylsilane (TMS) as the internal standard. The electron impact MS (EI-MS) were obtained on a mass spectrometer (Model M-80, Hitachi, Tokyo, Japan). CD spectra were measured on a spectropolarimeter (J-700, Jasco).

The organic solvents and the other chemical reagents used were of analytical grade, purchased from Wako (Osaka, Japan). Diatomaceous earth granules, particle size 50—100 μm, were prepared from Celite No. 545 (Johns Manville, Denver, CO, U.S.A.). β-D-Glucuronidase (EC 3.2.1.31, from bovine liver) was supplied by Sigma (St. Louis, MO, U.S.A.), and its activity was 624000 units/g of solid. A 10-mg amount of the β-D-glucuronidase was dissolved in 1 ml of the 0.1 M acetate buffer (pH 4.7) to prepare an enzyme preparation of 6240 units/ml. β-D-Glucosidase (EC 3.2.1.21, 36000 units/g powder, from sweet almond) was purchased from Oriental Yeast (Tokyo, Japan), and a 50-mg amount was added to 300 μl of the acetate buffer solution to prepare a 6000-units/ml β-D-glucosidase preparation. Authentic specimens of wogonin and oroxylin A were purchased from Wako and Tsumura (Tokyo, Japan), respectively.

Herbal Materials Shosaiko-to (TJ-9) and Daisaiko-to (TJ-8) (Tsumura, Tokyo, Japan) were extract granule preparations for ethical use, the herbal ingredients of which are shown in Table 1. The dry root of *Scutellaria baicalensis* GEORGI was obtained from Uchida Wakanyaku (Tokyo, Japan).

The *S. baicalensis* root was mechanically powdered to 100-mesh. A 1.5-g amount of this powder was extracted twice with 100 ml of water by ultrasonication for 10 min and left standing for 1 h at ambient temperature. After filtration, the aqueous extract was concentrated to 150 ml under reduced pressure. This concentrated extract was orally administered to one of our subjects.

For analytical purposes, 20 mg of TJ-9, TJ-8 or the *S. baicalensis* powder was treated with 6 ml of 0.1 M acetate buffer (pH 4.7) by ultrasonication for 5 min at room temperature. After standing for 30 min, each mixture was centrifuged at 1550 × *g* for 10 min. The supernatants were stored at −20 °C until subjected to enzyme treatments.

Human Urine Sampling Three healthy male volunteers from this university campus took part in the testing program. They were aged 24—29, weighed 60—71 kg, and gave their informed consent to participate. The study was approved by the ethics committee of our department. Timed urine samples were collected before and after oral administration of a 7.5- or 5-g dosage of TJ-9 and TJ-8 as described previously.[3] In order to determine the herbal origin of **3** and **4**, one of the subjects took orally 150 ml of the *S. baicalensis* extract. Urine samples were collected at 0, 2, 6, 12, 24, 30, 36 and 48 h after intake. All of the above urine samples were stored at −20 °C pending analysis.

Enzyme Treatment and Rapid Flow Fractionation (RFF) for Sample Preparation The urine samples and the herbal extracts (each 3 ml) were treated with 30 μl of the β-D-glucuronidase preparation (187 units). The urine samples had been adjusted to pH 4—5 with acetic acid before the enzyme treatment. The resulting mixtures were incubated at 37 °C for 12 h and then stored at −20 °C until RFF extraction. In addition, 3-ml aliquots of herbal extracts were also treated with 30 μl of β-D-glucosidase (180 units) and 3 μl of β-D-glucuronidase (187 units) preparations under the same incubation conditions mentioned above.

RFF[17] is a technique for liquid–liquid extraction of biological and herbal samples on a diatomaceous earth cartridge before HPLC analysis. The RFF units were purchased from Kusano Scientific (Tokyo, Japan). A 200-μl volume of the enzyme-treated sample was applied to an RFF column (10 mm i.d., 3-ml capacity), and then 7 ml of CH_2Cl_2–EtOH (90 : 10, v/v) were introduced into the column. The eluate was collected and the solvent was evaporated to dryness with the aid of a stream of dry air at 40 °C. The resulting residue was analyzed by reversed-phase HPLC, the conditions of which are shown in the legend to Fig. 1.

Preparative Isolation of 3 and 4 from Urine Urine samples collected over 18—42 h after administration of the remedies were used. A 150-ml volume of the urine pretreated with β-D-glucuronidase (EC 3.2.1.31) was extracted with 1800 ml of 1,2-dichloroethane on a diatomaceous earth column (5 × 14 cm i.d.). The eluate was evaporated to dryness under reduced pressure, and the residue was dissolved in 2 ml of ethanol and introduced into the ODS column repeatedly with a mobile phase solvent of H_2O–CH_3CN–CH_3COOH (63 : 35 : 2, v/v). At a flow rate of 1.5 ml/min, the fractions containing **3** and **4** were collected separately at t_R values of 16 and 19 min. The solvents were evaporated to dryness under reduced pressure at 40 °C. The above products of **3** and **4** exhibited *S*-enantiomeric excesses of 90% and 80%, respectively, in chiral HPLC analysis, the conditions of which are described in the legend to Fig. 3. The products were recrystallized from 10% aqueous CH_3CN to afford 1.4 mg of **3** and 0.3 mg of **4** from the urine samples.

3 was obtained as colorless needles, mp 152—154 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 2.87 (1H, dd, J = 17.2, 3.1 Hz, H-3), 3.09 (1H, dd, J = 17.2, 12.8 Hz, H-3), 3.88 (3H, s, OCH_3-8), 5.49 (1H, dd, J = 12.8, 3.1 Hz, H-2), 6.16 (1H, s, H-6), 7.40—7.49 (5H, m, H-2′—6′). UV λ_{max}^{MeOH} nm: 290; $\lambda_{max}^{MeOH+CH_3COONa}$ nm: 294, 330. EI-MS (70 eV) *m/z* (relative intensity %): 286 (M^+, 100), 271 (16), 209 (9), 167 (9). CD λ (EtOH)

nm: 287 ($\pi\rightarrow\pi^*$, negative Cotton effect), 310 ($n\rightarrow\pi^*$, positive Cotton effect).

4 was obtained as pale yellow needles, mp 166—168 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 2.83 (1H, dd, J=17.1, 3.1 Hz, H-3), 3.08 (1H, dd, J=17.1, 13.0 Hz, H-3), 3.95 (3H, s, OCH_3-6), 5.41 (1H, dd, J=13.0, 3.1 Hz, H-2), 6.13 (1H, s, H-8), 7.39—7.45 (5H, m, H-2′—6′). UV λ_{max}^{MeOH} nm: 290, $\lambda_{max}^{MeOH+CH_3COONa}$ nm: 294, 326. EI-MS (70 eV) m/z (rel. int.%): 286 (M^+, 100), 271 (17), 209 (8), 167 (12). CD λ (EtOH) nm: 290 ($\pi\rightarrow\pi^*$, negative Cotton effect), 337 ($n\rightarrow\pi^*$, positive Cotton effect).

Preparation of Racemic Dihydrowogonin and Dihydrooroxylin A As illustrated in Chart 1, the optically active **3** isolated from the post-administration urine was first treated with 10% KOH solution at 80 °C for 1 min. The resulting mixture was acidified with 5% HCl to pH 3.0. The flavanones were extracted from the resulting mixture by the RFF technique. The solvent was evaporated off and the racemic dihydrowogonin and dihydrooroxylin A were separated on the ODS column with CH_3CN–H_2O–CH_3COOH (33 : 65 : 2, v/v). Neither of the racemic mixtures exhibited optical activity in their CD spectra, but both showed paired flavanone peaks on the chiral chromatograms (Fig. 3).

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References and Notes

1) Akao T., Hattori M., Namba T., Kobashi K., *J. Med. Pharm. Soc. WAKAN-YAKU*, **9**, 1—13 (1992).
2) Akao T., Mibu K., Hattori M., Namba T., Kobashi K., *J. Pharmaco-bio Dyn.*, **8**, 800—807 (1985).
3) Li C., Homma M., Oka K., *J. Chromatogr. B*, **693**, 191—198 (1997).
4) Hirayama C., Okumura M., Tanikawa K., Yano M., Mizuta M., Ogawa N., *Gastroenterologia Japonica*, **24**, 715—719 (1989).
5) Ishigaki K., Shimizu T., Imura M., Nagai K., Mitake H., Kondo K., *J. Med. Pharm. Soc. WAKAN-YAKU*, **5**, 328—329 (1988).
6) Li C., Homma M., Ohkura N., Oka K., *Tetrahedron: Asymmetry*, **8**, 1145—1147 (1997).
7) Nagarajan G. R., Parmar V. S., *Planta Med.*, **32**, 50—53 (1997).
8) Takagi S., Yamaki M., Inoue K., *Yakugaku Zasshi*, **100**, 1220—1224 (1980).
9) Chopin J., Molho D., Pacheco H., Mentzer C., *Compt. Rend.*, **243**, 712—714 (1956).
10) Wagner H., Hörhammer L., Rüger R., Khalil E., Farkas L., *Tetrahedron Lett.*, **19**, 1471—1473 (1969).
11) Bilia A. R., Catalano S., Pistelli L., Morelli I., *Phytochemistry*, **33**, 1449—1452 (1993).
12) Jakupovic J., Zdero C., Grenz M., Tsichritzis F., Lehmann L., Hashemi-Nejad S. M., Bohlmann F., *Phytochemistry*, **28**, 1119—1131 (1989).
13) Bergeron C., Marston A., Hakizamungu E., *Int. J. Pharmacogn.*, **33**, 115—119 (1995).
14) Miyaichi Y., Tomimori T., *Nat. Med.*, **49**, 350—353 (1995).
15) Hattori M., Shu Y. Z., El-Sedawy A., Namba T., Kobashi K., Tomimori T., *J. Nat. Prod.*, **51**, 874—878 (1988).
16) Gaffield W., *Tetrahedron*, **26**, 4093—4108 (1970).
17) Oka K., Ohki N., Noguchi M., Matsuoka Y., Irimajiri S., Abe M., Takizawa T., *Anal. Chem.*, **56**, 2614—2617 (1980).