Metabolism of Rosmarinic Acid in Rats

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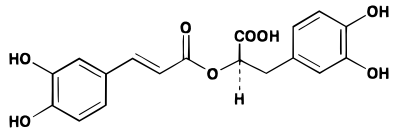
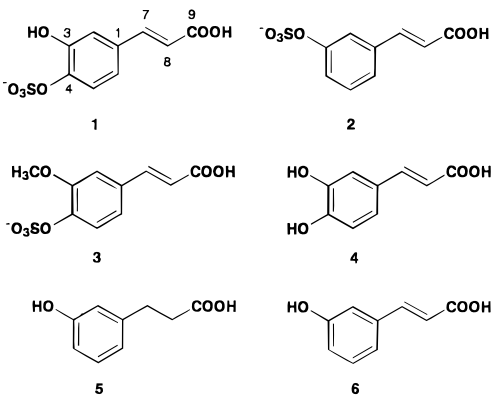
The urine of rats administered rosmarinic acid (**7**) orally contained seven metabolites, which were identified as *trans*-caffeic acid 4-*O*-sulfate (**1**), *trans*-*m*-coumaric acid 3-*O*-sulfate (**2**), *trans*-ferulic acid 4-*O*-sulfate (**3**), *trans*-caffeic acid (**4**), *m*-hydroxyphenylpropionic acid (**5**), *trans*-*m*-coumaric acid (**6**), and unchanged rosmarinic acid (**7**) by spectroscopic and chemical data. The total cumulative amount of **1**−**7** excreted in the urine 48 h after the oral administration of rosmarinic acid was approximately 31.8% of the dose administered. On the other hand, the metabolites attributed to rosmarinic acid could not be found in the bile. Orally administered rosmarinic acid may thus be concluded to be excreted in the urine rather than in the bile, with cleavage of ester bonds, selective *para*-dehydroxylation, methylation, and sulfate-conjugation. Metabolites **2**, **3**, **5**, and **6** were also detected in the plasma.

Perillae Herba, consisting of the aerial parts of *Perilla frutescens* Britton var. *acuta* Kudo (Labiatae), has been used mainly to treat inflammatory diseases and anxiety neurosis.[1] Although many components such as essential oils,[2−4] flavones,[5] and phenylpropanoids and related compounds[6,7] have been identified in Perillae Herba, the metabolic fate of these compounds has not yet been studied. Rosmarinic acid, a major constituent of the crude drug, has been shown to inhibit 5-lipoxygenase,[8] 3α-hydroxysteroid dehydrogenase,[9] and lipid peroxidation[10] and to have antiinflammatory activity.[11] Furthermore, it is reported that rosmarinic acid content gives a good correlation for the quality of the crude drug.[12] Rosmarinic acid is thus considered to be one of the most important constituents in Perillae Herba.

In this study, rosmarinic acid was administered orally to rats, and the chemical structures of the urinary metabolites derived were identified and the amounts estimated.



Rosmarinic Acid (7)



Results and Discussion

By using HPLC equipped with photodiode array detection techniques, seven metabolites, **1** (t_R 49 min), **2** (t_R 59 min), **3** (t_R 63 min), **4** (t_{RA} 66 min), **5** (t_R 78 min), **6** (t_R 88 min), and **7** (t_R 107 min), were detected in the urine of rats administered rosmarinic acid orally. Metabolites **2**, **3**, **5**, and **6** were also found in the plasma. However, the metabolites attributed to rosmarinic acid could not be found in the bile.

By enzymatic hydrolysis with arylsulfatase, **1** gave a product that was identified as *trans*-caffeic acid by comparison of t_R and UV spectrum on HPLC with those of an authentic sample. The IR spectrum of **1** showed absorption bands at 3406, 1670, and 1620, 976 cm^{-1} due to hydroxyl, conjugated carboxyl, and trans double-bond functions, respectively. Intense absorption at 1051 cm^{-1} suggested a sulfate-conjugated structure for **1**. Negative FABMS of **1** showed a molecular ion at *m*/*z* 259 $(M - H)^-$ along with a fragment ion at *m*/*z* 179 $(M - H - SO_3)^-$ corresponding to monosulfate, indicating the presence of one sulfate group in **1**. A comparison of the ^{13}C NMR spectrum of **1** with that of *trans*-caffeic acid indicated the C-4 signal of **1** to have shifted 7.0 ppm upfield, accompanied by downfield shifts of C-3 (3.5 ppm) and C-5 (7.7 ppm), indicating the sulfate group to be situated at C-4. The structure of **1** was concluded to be *trans*-caffeic acid 4-*O*-sulfate.

Enzymatic hydrolysis of **2** with arylsulfatase gave a product identified as *trans*-*m*-coumaric acid based on HPLC features. The IR spectrum of **2** showed absorption bands at 1670 and 1624, 980 cm^{-1} due to conjugated carboxyl and trans double-bond functions. Intense absorption at 1051 cm^{-1} indicated a possible sulfate-conjugated structure. Metabolite **2** exhibited a molecular ion at *m*/*z* 243 $(M - H)^-$ along with a fragment ion at *m*/*z* 163 $(M - H - SO_3)^-$ corresponding to a monosulfate in negative FABMS. A sulfate group is thus shown present in **2**. A comparison of the ^{13}C NMR spectrum of **2** with that of *trans*-*m*-coumaric acid indicated the C-3 signal of **2** to have shifted 4.5 ppm upfield, accompanied by downfield shifts of C-2 (6.2

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Table 1. Cumulative Urinary Excretion of the Metabolites in Rats After Oral Administration of 200 mg/kg of Rosmarinic Acid[a]

	cumulative mean % of dose						
compound	0−4 h	0−8 h	0−12 h	0−24 h	0−36 h	0−48 h	0−72 h
1	0.038 ± 0.003	0.124 ± 0.023	0.139 ± 0.027	0.149 ± 0.029	0.154 ± 0.031	0.154 ± 0.031	0.154 ± 0.031
2	n.d.[b]	n.d.	0.174 ± 0.385	7.456 ± 1.001	11.778 ± 2.711	12.137 ± 2.805	12.137 ± 2.805
3	0.081 ± 0.005	0.284 ± 0.029	0.462 ± 0.048	0.538 ± 0.066	0.559 ± 0.069	0.581 ± 0.070	0.581 ± 0.070
4	0.010 ± 0.001	0.027 ± 0.004	0.037 ± 0.005	0.063 ± 0.007	0.086 ± 0.009	0.117 ± 0.017	0.117 ± 0.017
5	n.d.	0.949 ± 0.106	5.252 ± 1.352	14.527 ± 2.560	17.430 ± 3.322	18.357 ± 3.499	18.357 ± 3.499
6	n.d.	0.015 ± 0.002	0.100 ± 0.026	0.333 ± 0.052	0.399 ± 0.072	0.413 ± 0.077	0.413 ± 0.077
7	0.041 ± 0.007	0.077 ± 0.013	0.077 ± 0.013	0.077 ± 0.013	0.077 ± 0.013	0.077 ± 0.013	0.077 ± 0.013
total	0.170 ± 0.016	1.476 ± 0.177	6.241 ± 1.856	23.143 ± 3.728	30.483 ± 6.227	31.836 ± 6.512	31.836 ± 6.512

[a] Data are expressed as mean ± S. E. ($n = 5$). [b] n.d. = not detected.

ppm) and C-4 (5.4 ppm). These shifts suggest the sulfate moiety in **2** to be situated at C-3. Metabolite **2** may thus be concluded to be *trans*-*m*-coumaric acid 3-*O*-sulfate.

Enzymatic hydrolysis of **3** with arylsulfatase gave a product identified as *trans*-ferulic acid based on HPLC features. The IR spectrum of **3** showed absorption bands at 1648 and 1592, 984 cm^{-1} due to conjugated carboxyl and trans double-bond functions. Intense absorption at 1050 cm^{-1} indicated **3** to have possibly a sulfate-conjugated structure. Negative FABMS of **3** exhibited a molecular ion at *m*/*z* 273 $(M - H)^-$ along with a fragment ion at *m*/*z* 193 $(M - H - SO_3)^-$ corresponding to monosulfate. A sulfate group is thus shown to be present in **3**. A comparison of the ^{13}C NMR spectrum of **3** with that of *trans*-ferulic acid indicated the C-4 signal of **3** to have shifted 6.0 ppm upfield, accompanied by downfield shifts of C-3 (2.8 ppm) and C-5 (5.4 ppm). These shifts indicate the sulfate group to be attached to C-4, and, accordingly, **3** may be concluded to be *trans*-ferulic acid 4-*O*-sulfate.

Metabolites **4**−**6** were identified as *trans*-caffeic acid, *m*-hydroxyphenylpropionic acid, and *trans*-*m*-coumaric acid, respectively, by comparison with authentic samples. Compound **7** was identified as unchanged rosmarinic acid by direct comparisons of its t_R and UV spectral features with an authentic sample on HPLC.

The urinary excretion of the metabolites and unchanged rosmarinic acid after oral administration of rosmarinic acid is shown in Table 1. The total cumulative amounts of six metabolites and rosmarinic acid excreted after 48 h corresponded to 31.836 ± 6.512% (mean ± S. E., $n = 5$) of rosmarinic acid administration. Compounds **2** (12.137 ± 2.805%) and **5** (18.357 ± 3.499%) were the major metabolites, while **1** (0.154 ± 0.031%), **3** (0.581 ± 0.070%), **4** (0.117 ± 0.017%), **6** (0.413 ± 0.077%), and unchanged rosmarinic acid (**7**) (0.077 ± 0.013%) were minor metabolites in the urine. Metabolite **6**, which possesses a hydroxy group in the structure, was considered to be easily subjected to sulfate-conjugation, followed by excretion into urine. In fact, however, **6** was present to a much lesser extent than the nonhydroxylated **2** (**2**/**6** 95:5), but it is interesting that **5** occurred as the free form in urine in spite of its possessing a hydroxyl group.

Rosmarinic acid administered orally was thus concluded to be excreted in the urine with cleavage of the ester bonds, selective *para*-dehydroxylation, methylation, and sulfate-conjugation. This *para*-dehydroxylation has been reported for *trans*-caffeic acid, *trans*-ferulic acid[13] and hesperidine, diosmin, and the aglycones,[14] and thus the gut flora would appear to be the cause for biotransformation, which is consistent with the work of Goodwin *et al*.[15]

Examination of plasma concentrations and the pharmacodynamics of the metabolites and rosmarinic acid is in progress in order to elucidate the pharmacological effects of Perillae Herba.

Experimental Section

General Experimental Procedures. Melting points were determined on a Yanagimoto micromelting point apparatus and not corrected. IR spectra were measured with a Perkin−Elmer FT-IR 1725X spectrometer. Optical rotations were in EtOH using a JASCO DIP-360 digital polarimeter at 26−28 °C. NMR spectra were recorded on a JEOL JNM-EX 270 (^1H, 270; ^{13}C, 65 MHz) spectrometer. Chemical shifts are given in δ values (ppm) downfield from tetramethylsilane. EIMS and FABMS were measured with a JEOL JMS-DX 303 mass spectrometer. The HPLC system was composed of a CCMP pump, CO-8010 column oven (Tosoh, Tokyo, Japan), and model MCPD-3600 photodiode array detector (Otsuka, Osaka, Japan). (*R*)-(+) Rosmarinic acid was isolated from Perillae Herba according to Okuda *et al*.,[7] and an authentic sample was obtained from Funakoshi Industry Co., Ltd. (Tokyo, Japan). *m*-Hydroxyphenylpropionic acid was synthesized in the usual way. The identity of these compounds was confirmed by $[\alpha]_D$, ^1H NMR, or ^{13}C NMR spectroscopy before use.[16−18] *trans*-Caffeic acid was from Wako Pure Chemical Industries, Ltd. (Osako, Japan) and *trans*-coumaric and *trans*-ferulic acids were from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Arylsulfatase was from Sigma (St. Lous, MO). MeCN, EtOH, MeOH, and trifluoroacetic acid (TFA) of special grade were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Four column chromatography, Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden), Toyopearl HW-40 F (Tosoh, Tokyo, Japan), and Kieselgel 60 silanisiert (Merck, Darmstadt, F.D., Germany) were used.

Animals. Male Sprague−Dawley rats (150−300 g) were purchased from Japan SLC, Inc. These animals were specifically pathogen-free and kept in our own environmentally controlled quarters (temperature: 22 ± 2 °C, humdity: 55 ± 10%, 12 h dark−light cycle) for at least 1 week before use. Normal food (CLEA Japan Inc., Tokyo, Japan) and H_2O were available at all times, except that the food was withdrawn 18 h prior to experimentation. Rosmarinic acid was administered orally at 200 mg/kg (as 20% EtOH solution).

Preparation of Urine Samples for HPLC Analysis. Two rats were kept in one metabolic cage in the breeding room. Urine (1 mL), collected for 24 h after

oral administration of rosmarinic acid, was added to 5 mL of MeOH. The solution was filtered through a 0.45-μm membrane filter; 20 μL of the filtered solution was injected into HPLC.

Preparation of Bile Samples for HPLC Analysis. A polyethylene tube was inserted into the rat bile duct under pentobarbital sodium anesthesia. Bile was collected into MeOH with cooling for 12 h after oral administration of rosmarinic acid. The solution (20 μL), filtered through a 0.45-μm membrane filter, was injected into HPLC.

Preparation of Plasma Samples for HPLC Analysis. Rosmarinic acid was administered orally to rats. After 12 h, the animals were anesthetized with pentobarbital sodium, and blood (5 mL from each animal) was collected from the inferior vena cava with a heparinized tube and immediately centrifuged at 3000 rpm for 10 min at room temperature. Individual plasma (2 mL) was added to 12 mL of MeOH and stirred well. The mixture was centrifuged at 3000 rpm for 10 min at room temperature, and the supernatant was evaporated to dryness at 40 °C in vacuo. The residue was dissolved in 0.2 mL H_2O, and 50 μL of solution, filtered through a 0.45-μm membrane filter, was injected into HPLC.

HPLC Conditions. The HPLC conditions for qualitative and quantitative analysis of metabolites were as follows: column, TSK gel ODS-120T (Tosoh, Tokyo, Japan, 250 mm × 4.6 mm i.d.); column temperature, 40 °C; flow rate, 1 mL/min; detection, UV at 190−400 nm. The mobile phases were a gradient system with 0.1% TFA in H_2O (A) and MeCN (B). The gradient system was A/B = 100/0 (0 min) → 95/5 (60 min) → 80/20 (100 min) → 80/20 (120 min). Preparative HPLC conditions were as follows: column, TSK gel ODS-120T (Tosho, Tokyo, Japan, 300 mm × 7.6 mm i.d.); column temperature, room temperature; flow rate, 2 mL/min; detection, UV at 280 nm. The mobile phases were a gradient system with 0.05% TFA in H_2O (A) and MeCN (B). The gradient system was A/B = 95:5 (0 min) → 85:15 (30 min).

Isolation of Metabolites from the Urine. For isolation of metabolites, urine (about 1.2 L) after rosmarinic acid administration was collected in a similar manner for the HPLC sample and was chromatographed on Sephadex LH-20 successively with H_2O and MeOH as eluents. The H_2O eluate fractions containing urinary metabolites (**1**, **2**, **3**, and **5**) were lyophilized, and the residue was subjected to preparative HPLC. Fractions **4** and **6**, eluted with MeOH, were evaporated to dryness, followed by preparative HPLC. Each eluate containing metabolites **1** (14 mg), **2** (30 mg), **3** (10 mg), **4** (4 mg), **5** (30 mg), or **6** (8 mg) was freeze-dried.

Compound 1: obtained as white powder; mp 225−257 °C (dec.); $[\alpha]^{28}_D$ −0.12° (*c* 0.3, EtOH); UV (EtOH) λ_{max} (log ϵ) 202 (3.64), 214 (3.59), 276 (3.59), 305 (sh) (3.31) nm; IR (KBr) ν_{max} 3406, 1670, 1620, 1501, 1051, 976 cm^{-1}; ^{1}H NMR (DMSO-d_6, 270 MHz) δ 6.25 (1H, d, J = 16.0 Hz, H-8), 6.89 (1H, dd, J = 2.1, 8.2 Hz, H-6), 6.96 (1H, d, J = 2.1 Hz, H-2), 7.08 (1H, d, J = 16.0 Hz, H-7), 7.14 (1H, d, J = 8.2 Hz, H-5); ^{13}C NMR (DMSO-d_6, 65 MHz) δ 115.2 (C-2), 118.5 (C-6), 122.7 (C-5), 127.3 (C-8), 132.9 (C-1), 136.5 (C-7), 141.0 (C-4), 149.0 (C-3), 170.7 (C-9); FABMS *m/z* 281 [M − H + Na]$^-$, 259 [M − H]$^-$, 179 [M − H − SO_3]$^-$.

Compound 2: obtained as white powder; mp 282−283 °C (dec.); $[\alpha]^{28}_D$ −0.58° (*c* 0.4, EtOH); UV (EtOH) λ_{max} (log ϵ 217 (3.90), 268 (3.86) nm; IR (KBr) ν_{max} 1670, 1624, 1508, 1051, 980 cm^{-1}; ^{1}H NMR (CD_3OD, 270 MHz) δ 6.50 (3H, d, J = 16.0 Hz, H-8), 7.31−7.36 (3H, m, H-4, H-5, H-6), 7.52 (1H, d J = 1.3 Hz, H-2), 7.57 (1H, d, J = 16.0 Hz, H-7); ^{13}C NMR (CD_3OD, 65 MHz) δ 121.5 (C-2), 122.5 (C-6), 124.0 (C-4), 125.5 (C-8), 130.7 (C-5), 137.3 (C-1), 144.0 (C-7), 154.6 (C-3), 163.6 (C-9); FABMS *m/z* 265 [M − H + Na]$^-$, 243 [M − H]$^-$, 163 [M − H − SO_3]$^-$.

Compound 3: obtained as white powder, mp 226−228 °C (dec.); $[\alpha]^{28}_D$ 0.77° (*c* 0.4, EtOH); UV (EtOH) λ_{max} (log ϵ) 214 (3.87), 277 (3.86), 300 (sh) (3.96) nm; IR (KBr) ν_{max} 1665, 1632, 1516, 1050, 984 cm^{-1}; ^{1}H NMR (DMSO-d_6, 270 MHz) δ 3.78 (3H, s, −OCH_3), 6.38 (1H, d, J = 15.8 Hz, H-8), 6.98 (1H, dd, J = 2.0, 8.5 Hz, H-6), 7.09 (1H, d, J = 2.0 Hz, H-2), 7.14 (1H, d, J = 15.8 Hz, H-7), 7.44 (1H, d, J = 8.5 Hz, H-5); ^{13}C NMR (DMSO-d_6, 65 MHz) δ 55.7 (−OCH_3), 110.7 (C-2), 119.4 (C-6), 120.8 (C-5), 127.3 (C-8), 131.8 (C-1), 137.0 (C-7), 143.0 (C-4), 150.6 (C-3), 171.7 (C-9); FABMS *m/z* 295 [M − H + Na]$^-$, 273 [M − H]$^-$, 193 [M − H − SO_3]$^-$.

Compound 4: obtained as pale yellow powder; mp 181−183 °C; $[\alpha]^{26}_D$ −0.93° (*c* 0.5, EtOH); UV (EtOH) λ_{max} (log ϵ) 216 (4.04), 242 (3.92), 297 (sh) (4.02), 326 (4.11) nm; IR (KBr) ν_{max} 3436, 1659, 1620, 1500, 976 cm^{-1}; ^{1}H NMR (DMSO-d_6, 270 MHz) δ 6.17 (1H, d, J = 15.8 Hz, H-8), 6.76 (1H, d, J = 8.1 Hz, H-5), 6.96 (1H, dd, J = 2.1, 8.1 Hz, H-6), 7.02 (1H, d, J = 2.1 Hz, H-2), 7.42 (1H, d, J = 15.8 Hz, H-7); ^{13}C NMR (CD_3OD, 65 MHz) δ 114.5 (C-2), 115.0 (C-5), 115.6 (C-8), 121.0 (C-6), 125.6 (C-1), 144.4 (C-7), 145.5 (C-3), 148.0 (C-4), 167.7 (C-9); HREIMS *m/z* 180.0414 (calcd for $C_9H_8O_4$, 180.0422).

Compound 5: obtained as white powder; mp 113−115 °C; $[\alpha]^{26}_D$ −0.77° (*c* 0.5, EtOH); UV (EtOH) λ_{max} (log ϵ) 202 (3.99), 214 (3.76), 274 (1.15) nm; IR (KBr) ν_{max} 3356, 1687, 1510 cm^{-1}; ^{1}H NMR (DMSO-d_6, 270 MHz) δ 2.20 (1H, t, J = 7.3 Hz, H-8), 2.69 (1H, t, J = 7.3 Hz, H-7), 6.52 (1H, dd, J = 1.5, 7.7 Hz, H-4), 6.55 (1H, d, J = 7.7 Hz, H-6), 6.65 (1H, J = 1.5 Hz, H-2), 6.97 (1H, t, J = 7.7 Hz, H-5); ^{13}C NMR (CD_3OD, 65 MHz) δ 33.9 (C-7), 41.1 (C-8), 113.7 (C-4), 116.1 (C-2), 120.6 (C-6), 130.2 (C-5), 145.2 (C-1), 158.4 (C-3), 181.9 (C-9); HREIMS *m/z* 166.0666 (calcd for $C_9H_{10}O_3$, 166.0630).

Compound 6: obtained as white powder; mp 192−195 °C; $[\alpha]^{26}_D$ −0.11° (*c* 0.5, EtOH); UV (EtOH) λ_{max} (log ϵ) 215 (4.15), 231 (4.05), 274 (4.19), 311 (sh) (3.61) nm; IR (KBr) ν_{max} 3382, 1671, 1620, 1500, 986 cm^{-1}; ^{1}H NMR (CD_3OD, 270 MHz); 6.45 (1H, d, J = 15.8 Hz, H-8), 6.74 (1H, dd, J = 2.2, 7.7 Hz, H-4), 6.95 (1H, d, J = 2.2 Hz, H-2), 6.97 (1H, d, J = 7.7 Hz, H-6), 7.16 (1H, t, J = 7.7 Hz, H-5), 7.34 (1H, d, J = 15.8 Hz, H-7); ^{13}C NMR (CD_3OD, 65 MHz) δ 115.3 (C-2), 118.6 (C-4), 119.2 (C-8), 120.8 (C-6), 131.0 (C-5), 137.1 (C-1), 146.5 (C-7), 159.1 (C-3), 170.5 (C-9); HREIMS *m/z* 164.0484 (calcd for $C_9H_8O_3$, 164.0473).

Enzymatic Hydrolysis. Compounds **1**, **2**, and **3** (each 1 mg) were incubated in 0.1 M citrate buffer (pH 5.2) with arylsulfatase (5 units) for 2 h at 37 °C. The aglycons (*trans*-caffeic, *trans*-*m*-coumaric, and *trans*-ferulic acids) were identified by comparison with authentic samples on HPLC.

Quantification of Urinary Metabolites. Urine (1−8 mL) samples were diluted with MeOH to a final volume of 10 mL. An aliquot of the sample was filtered through a 0.45-μm membrane filter. A 20-μL urine sample was subjected to HPLC. Quantification of the metabolites were done by measuring peak areas. Calibration plots of the peak area of each metabolite against metabolite concentration were linear from 0.5 to 300 μg/mL for **1**−**7** in urine. The recovery of each metabolite in urine was 98.7% for **1**, 94.8% for **2**, 98.2% for **3**, 100.8% for **4**, 102.7% for **5**, 98.0% for **6** or 100.3% for **7** based on the determination of standard samples added to drug-free urine. The detection limits for **1**−**7**, at a signal-to-noise ratio of 8, were 0.132, 0.832, 0.164, 0.262, 0.428, 0.282, and 0.366 μg/mL, respectively.

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