Notes

Metabolism of Paeonol in Rats

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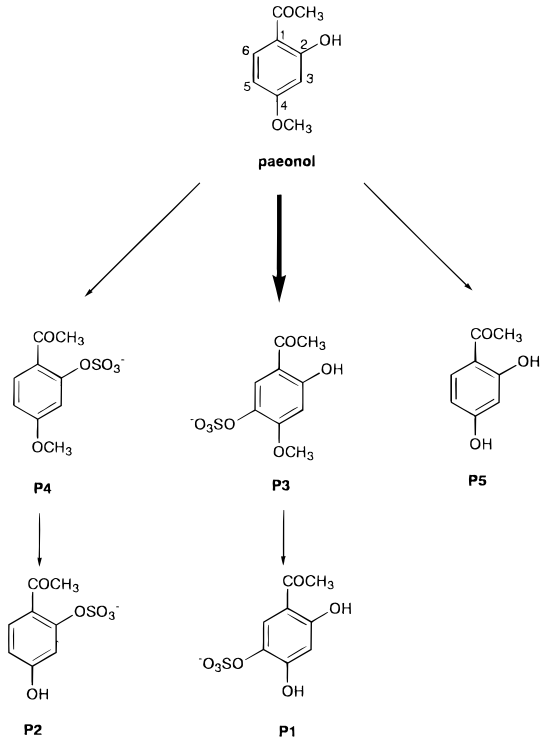
As a part of our studies on the metabolism of active components from traditional Chinese medicines, paeonol was orally administered to rats. The urinary metabolites were analyzed by 3D HPLC, and their structures were determined to be 2,4-dihydroxyacetophenone-5-*O*-sulfate (**P1**), resacetophenone-2-*O*-sulfate (**P2**), 2-hydroxy-4-methoxyacetophenone-5-*O*-sulfate (**P3**), paeonol-2-*O*-sulfate(**P4**), resacetophenone (**P5**), and unchanged paeonol, on the basis of their chemical and spectral data. Among these metabolites, **P2**−**P4** and paeonol were detected in the plasma after the oral administration of paeonol. Furthermore, the bile of rats given paeonol orally was found to contain **P3**, suggesting the enterohepatic circulation of paeonol.

Paeonol (2-hydroxy-4-methoxyacetophenone) is a major phenolic component of Moutan bark, the root bark of *Paeonia suffruticosa* Andrews (Paeoniaceae), which has been shown to exhibit antipyretic, sedative, antiinflammatory,[1] and antibacterial[2] activities. Yokoyama et al.[3] investigated the metabolism of paeonol in rats by ^{14}C tracer experiments in which 2,5-dihydroxy-4-methoxyacetophenone, resacetophenone, and unchanged paeonol were identified as the urinary metabolites after hydrolysis. Gjertsen et al.[4] studied the metabolism of paeonol in rats by the GLC−MS method in which dihydroxymethoxyacetophenone, trihydroxyacetophenone, and hydroxymethyl-2-hydroxy-4-methoxyphenyl ketone were additional metabolites. Additionally, they showed that the metabolites were mainly excreted as glucuronide and/or the sulfate conjugates. However, no report has appeared about the conjugated position and manner of their metabolism. In addition to the attention focused on drug−drug interactions in multiple-drug therapy, drug−metabolite interactions should be noted in pharmacokinetic mass−balance studies, especially in the case in which the regeneration of a drug from the conjugated metabolites is significant. Precise information regarding the pharmacokinetics of the conjugated metabolite itself is indispensable for the kinetic investigation of such a drug−conjugated-metabolite interaction study. In the present paper, as a part of our studies on the metabolism of active components from traditional Chinese medicines, the metabolites in urine, plasma, and bile as the conjugated form themselves not treated with the enzyme were examined after oral administration of paeonol in rats.

By using HPLC equipped with photodiode array detection techniques, five metabolites (**P1**−**P5**) and unchanged paeonol were detected in the urine of rats administered paeonol orally. **P2**−**P4** and unchanged paeonol were also detected in blood samples after oral administration of paeonol. Furthermore, **P3** was also detected in bile samples.

The isolation of **P1**-**P5** and unchanged paeonol from the urine was performed by chromatographic separation on a Sephadex LH-20 column and repeated preparative HPLC, as described in the Experimental Section. The structures of **P1**−**P5** and unchanged paeonol were determined as below. Unchanged paeonol was identified as paeonol by direct comparison with an authentic sample.

Scheme 1. Proposed Pathways of Metabolism of Paeonol in Rat[a]



[a] The thickness of the arrows indicates the relative importance of the pathways.

P1−**P4** had intense absorption bands at 1043−1050 cm^{-1}, indicative of a sulfate group, but at different positions of the ring. The enzymatic hydrolysis of **P1**−**P4** with arylsulfatase gave 2,4,5-trihydroxyacetophenone, resacetophenone, 2-hydroxy-4-methoxyacetophenone, and paeonol, which were identified by direct comparison with

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authentic samples (MS, NMR, and t_R on HPLC data), respectively.

P1 showed a pseudomolecular ion peak at *m*/*z* 247[M − H]$^-$ in the negative FABMS. A comparison of the ^{13}C NMR spectrum of **P1** with that of 2,4,5-trihydroxyacetophenone showed the C-5 signal of **P1** to have shifted 4.3 ppm upfield, accompanied by downfield shifts of C-4 (5.6 ppm) and C-6 (8.8 ppm). These shifts indicate a sulfate group at C-5. Based on these data, **P1** was concluded to be 2,4-dihydroxyacetophenone-5-*O*-sulfate.

P2 showed a pseudomolecular ion peak at *m*/*z* 231[M − H]$^-$ in the negative FABMS. The ^{1}H NMR spectrum of **P2** exhibited aromatic ABX-type signals at δ6.72 (dd, J = 8.7, 2.3 Hz), 6.79 (d, J = 2.3 Hz), and 7.82 (d, J = 8.7 Hz) assignable to H-5, -3, and -6, respectively. A comparison of the ^{13}C NMR spectral data with those of resacetophenone indicated the sulfation at C-2. Thus, **P2** was determined as resacetophenone-2-*O*-sulfate.

P3 exhibited a pseudomolecular ion peak at *m*/*z* 261-[M − H]$^-$ in the negative FABMS. A comparison of the ^{13}C NMR spectral data with those of 2,5-dihydroxy-4-methoxyacetophenone suggested the sulfation at C-5. Thus, **P3** was determined as 2-hydroxy-4-meyhoxyacetophenone-5-*O*-sulfate.

P4 showed a pseudomolecular ion peak at *m*/*z* 245[M − H]$^-$ in the negative FABMS. The ^{1}H NMR spectrum of **P4** exhibited signals for three ABX-type aromatic signals at δ6.75 (dd, J = 8.7, 2.5 Hz, H-5), 7.05 (d, J = 2.5 Hz, H-3), and 7.61 (d, J = 8.7 Hz, H-6). A comparison of the ^{13}C NMR spectral data with those of paeonol indicated the sulfation at C-2. Thus, **P4** was determined as paeonol-2-*O*-sulfate.

P5 was identified as resacetophenone by direct comparison with an authentic sample (MS, NMR, and t_R on HPLC data). The assignment of carbon signals were determined from the ^{13}C−^{1}H shift correlation spectroscopy (^{13}C−^{1}H COSY) and HMBC spectra.

Paeonol is known to undergo rapid absorption and excretion after its oral dosage to rats.[3] Additionally, it has been shown to be hydroxylated to 2,5-dihydroxy-4-methoxyacetophenone and demethylated to resacetophenone, both being excreted in the conjugated form. More extensive studies by Baba et al.[8] in mice, rats, guinea pigs, rabbits, and humans confirmed and extended these findings. Urine was shown to be the main excretory route in all species, and small amounts of paeonol itself (as well as larger amounts of the two aforementioned metabolites) were detected. The metabolites were excreted as the free, glucuronide, and sulfate conjugates and as the enzyme-resistant conjugates.

In this study, the structures of the urinary metabolites as the conjugated form themselves not treated with enzyme after paeonol was orally administered to rats were clearly shown. We confirmed the report of Yokoyama et al.,[3] which described the metabolic conversion of paeonol to demethylated (**P1**) and hydroxylated (**P3**) derivatives, and we also showed that these metabolites were mainly excreted in the urine as monosulfated compounds, especially as a sulfate (**P3**) of hydroxylated derivative. **P3**, the major metabolite of paeonol in rat urine was also found in rat bile, suggesting the enterohepatic circulation of paeonol. Furthermore, we confirmed the presence of **P2**−**P4** and unchanged paeonol in the plasma after paeonol was orally administered to the rats. These findings suggested that the metabolites may be the active forms when paeonol was orally administered. From the results of these studies, the major metabolic pathway of paeonol after oral administration in rats is suggested to be as follows. The paeonol was hydroxylated and subsequently sulfated at the C-5 position, and then excreted in urine. Interestingly, our results showing that the loss of the methyl ether at C-4 seems to be relatively random, while the sulfation process appears to be specific because, basically, the compounds are sulfated at position C-2 and C-5, with or without the loss of the methyl ether at C-4. Accordingly, further detailed metabolic studies will be needed to make clear the metabolism of paeonol. Because the precise information regarding the pharmacokinetics of the conjugated metabolite itself is indispensable for a kinetic investigation of such a drug−conjugated-metabolite interaction study in today's multiple-drug therapy, we are now investigating the pharmacokinetic work and the biological activities of these metabolites.

Experimental Section

Apparatus. Melting points were determined on Yanagimoto micromelting-point apparatus and are not corrected. IR spectra were measured with a Perkin−Elmer FT-IR 1725X spectrometer. NMR spectra were recorded on a JEOL JNM-EX 270 with tetramethylsilane as an internal standard, and the chemical shifts are given as δ values. MS were measured with a JEOL DX-303 mass spectrometer. HPLC system consisted of a CCPM-II pump, CO-8020 column oven (Tosoh, Tokyo, Japan), and model MCPD-3600 photodiode array detector (Otsuka Electronics, Osaka, Japan).

Reagents. Paeonol was prepared according to the method of Tahara.[5] 2,4,5-Trihydroxyacetophenone was synthesized according to the method of Daly et al.[6] 2,5-Dihydroxy-4-methoxyacetophenone was prepared according to the method of Mauthner.[7] Arylsulfatase was purchased from Sigma (St. Louis, MO). All other reagents were of special grade.

Animals. Male SD rats (Japan SLC, Inc.), 6 weeks old, were used. These animals were maintained under a constant temperature (22 ± 2 °C) and humidity (55 ± 10%), with free access to commercial food pellets (CA-1, Clea Japan, Tokyo) and tap water in a room with a 12-h light/12-h dark cycle. For 18 h prior to the experiments, they were deprived of food but had a free access to water.

Preparation of Urine, Plasma, and Bile Samples. Urine sample: Paeonol (50 mg/kg) dissolved in propylene glycol was administered orally to three rats, and urine specimens were obtained over 24 h by using a metabolic cage. MeOH (5 mL) was added to 1 mL of the urine, and the mixture was filtered through a 0.45 μm filter, and then 20 μL of the sample was injected into the HPLC column.

Plasma sample: Under light anesthesia with ether, paeonol (50 mg/kg) was administered orally to three rats, and blood specimens were drawn from the portal vein at 15, 30, and 60 min after administration with a heparin-treated syringe, respectively. The collected blood samples were centrifuged at 3000 rpm for 10 min to obtain plasma. Methanol (5 mL) was added to the plasma, and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant solution was evaporated to dryness below 40 °C under reduced pressure. The residue was dissolved in 1 mL of distilled water, and the mixture was filtered through a 0.45 μm filter, and then 20 μL of the sample was injected into the HPLC column.

Bile sample: Under light anesthesia with ether, three rats were bile-duct cannulated and administered 50 mg/kg of paeonol orally. Bile samples were collected from the rats into cooled MeOH for a 24-h period, and the bile samples were filtered through a 0.45 μm filter, and then 20 μL of the sample was injected into the HPLC column.

HPLC Conditions. A stainless steel column (250 × 4.6 mm i. d.), packed with reversed-phase TSKgel ODS-120T (5 μm, Tosoh Company Ltd., Tokyo, Japan) were used. The mobile phase was the following linear gradient system: solvent A; 0.05% trifluoroacetic acid (TFA), solvent B; 50% MeCN with 0.05% TFA, A/B = 100/0 (0 min) → 85/15 (60 min) → 30/70 (100 min) → 30/70 (110 min) → 0/100 (120 min). Flow rate 1.0 mL/min at 40 °C.

Isolation of Urinary Metabolites. Paeonol (2.8 g) was orally administered to six rats at 50 mg/kg over a period of two months, and urine samples were collected by using metabolic cages. The combined urine sample (1900 mL) collected from paeonol-treated rats was chromatographed on Sephadex LH-20 (0.05N HCl → H_2O → MeOH), and the fractions containing metabolites were subjected to preparative HPLC. The conditions were as follows: column; TSKgel ODS-120T (10 μm, 300 × 7.8 mm i. d., Tosoh Company Ltd., Tokyo, Japan), mobile phase; solvent A; 20% MeOH, solvent B; 100% MeOH, linear gradient system, A/B = 100/0 (0 min) → 40/60 (100 min). Flow rate; 2.0 mL/min at room temprature. Eachz metabolite fraction was purified by Sephadex LH-20 column chromatography to give **P1** (10 mg), **P2** (12 mg), **P3** (101 mg), **P4** (8 mg), **P5** (32 mg), and paeonol (18 mg), respectively.

Enzymatic Hydrolysis of Metabolites. P1−**P4** (each 1 mg) were incubated with crude arylsulfatase (5 units, type H-1) in 0.1M citrate buffer (pH 5.2) for 2 h at 37 °C. Each reaction mixture was extracted with ether, and the organic layer was washed with 1 N HCl and H_2O, and evaporated in vacuo to give the aglycon.

P1 (2,4-dihydroxyacetophenone-5-*O*-sulfate): white powder, mp 140−141 °C, negative FABMS *m*/*z* 269 $[M - H + Na]^-$, 247 $[M - H]^-$, 167 $(M - H - SO_3)^-$; IR (KBr) ν_{max} cm^{-1} 3506 (OH), 1649 (conjugated C═O), 1518 (arom. C═C), 1044 (−O−SO_3); ^{1}H NMR (DMSO-d_6, 270 MHz) δ 2.49 (3H, s, −COCH_3), 6.31 (1H, s, H-3), 7.67 (1H, s, H-6), 12.32 (1H, s, H-2); ^{13}C NMR (DMSO-d_6, 60 MHz) δ 26.4 (−COCH$_3$), 103.7 (C-3), 111.9 (C-1), 124.6 (C-6), 133.9 (C-5), 157.5 (C-2), 160.1 (C-4), 202.2 (−COCH$_3$).

P2 (resacetophenone-2-*O*-sulfate): white powder, mp 97−98°C; negative FABMS *m*/*z* 253 $[M - H + Na]^-$, 231 $[M - H]^-$, 151 $[M - H - SO_3]^-$; IR ν_{max} cm^{-1} 3423 (OH), 1656 (conjugated C═O), 1619 (arom. C═C), 1050 (−O−SO_3); ^{1}H NMR (DMSO-d_6, 270 MHz) δ 2.57 (3H, s, −COCH_3), 6.72 (1H, dd, J = 8.7, 2.3 Hz, H-5), 6.79 (1H, d, J = 2.3 Hz, H-3), 7.82 (1H, d, J = 8.7 Hz, H-6), 12.30 (1H, s, H-4); ^{13}C NMR (DMSO-d_6, 60 MHz) δ 26.9 (−COCH$_3$), 106.5 (C-3), 110.8 (C-5), 115.2 (C-1), 132.4 (C-6), 160.1 (C-2), 162.7 (C-4), 203.1 (−COCH$_3$).

P3 (2-hydroxy-4-methoxyacetophenone-5-*O*-sulfate): white powder, mp 162 °C; negative FABMS *m*/*z* 283 $[M - H + Na]^-$, 261 $[M - H]^-$, 181 $[M - H - SO_3]^-$; IR (KBr) ν_{max} cm^{-1} 3470 (OH), 1643 (conjugated C═O), 1510 (arom. C═C), 1050 (−O−SO_3); ^{1}H NMR (CD_3OD, 270 MHz) δ 2.55 (3H, s, −COCH_3), 3.87 (3H, s, −OCH_3), 6.51 (1H, s, H-3), 7.86 (1H, s, H-6); ^{13}C NMR (CD_3OD, 60 MHz) δ 26.5 (−COCH$_3$), 56.6 (−OCH$_3$), 101.5 (C-3), 113.2 (C-1), 125.6 (C-6), 135.2 (C-5), 160.9 (C-2), 163.4 (C-4), 204.5 (−COCH$_3$).

P4 (paeonol-2-*O*-sulfate): pale yellow oil; negative FABMS *m*/*z* 267 $[M - H + Na]^-$, 245 $[M - H]^-$, 165 $[M - H - SO_3]^-$; IR (NaCl) ν_{max} cm^{-1} 3529 (OH), 1666 (conjugated C═O), 1604 (arom. C═C), 1043 (−O−SO_3); ^{1}H NMR (DMSO-d_6, 270 MHz) δ 2.55 (3H, s, −COCH_3), 3.78 (3H, s, −OCH_3), 6.75 (1H, dd, J = 2.5, 8.7 Hz, H-5), 7.05 (1H, d, J = 2.5 Hz, H-3), 7.61 (1H, d, J = 8.7 Hz, H-6); ^{13}C NMR (DMSO-d_6, 60 MHz) δ 31.1 (−COCH$_3$), 55.4 (−OCH$_3$), 107.3 (C-3), 109.5 (C-5), 123.9 (C-1), 130.5 (C-6), 154.6 (C-2), 162.8 (C-4), 196.8 (−COCH$_3$).

P5 (resacetophenone): colorless needles, mp 134−135 °C; EIMS *m*/*z* 152 $[M^+]$; IR (KBr) ν_{max} cm^{-1} 3181 (OH), 1631 (conjugated C═O), 1609 (arom. C═C); ^{1}H NMR (DMSO-d_6, 270 MHz) δ 2.52 (3H, s, −COCH_3), 6.24 (1H, d, J = 2.5 Hz, H-3), 6.38 (1H, dd, J = 8.9, 2.3 Hz, H-5), 7.76 (1H, d, J = 8.9 Hz, H-6), 12.61 (1H, s, H-2); ^{13}C NMR (DMSO-d_6, 60 MHz) δ 26.3 (−COCH$_3$), 102.2 (C-3), 108.1 (C-5), 112.8 (C-1), 133.7 (C-6), 164.2 (C-4), 164.8 (C-2), 202.7 (−COCH$_3$).

Paeonol: pale yellow needles, mp 51 °C; EIMS *m*/*z* 152 $[M^+]$; IR (KBr) ν_{max} cm^{-1} 3450 (OH), 1620 (conjugated C═O), 1580 (arom. C═C); ^{1}H NMR (DMSO-d_6, 270 MHz) δ 2.56 (3H, s, −COCH_3), 3.82 (3H, s, −OCH_3), 6.47 (1H, d, J = 2.5 Hz, H-3), 6.53 (1H, dd, J = 8.9, 2.5 Hz, H-5), 7.85 (1H, d, J = 8.9 Hz, H-6), 12.64 (1H, s, H-2); ^{13}C NMR (DMSO-d_6, 60 MHz) δ 26.5 (−COCH$_3$), 55.6 (−OCH$_3$), 100.6 (C-3), 107.2 (C-5), 113.7 (C-1), 133.2 (C-6), 164.0 (C-2), 165.6 (C-4), 203.1 (−COCH$_3$).

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