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Metabolism of Paeoniflorin and Related Compounds by Human Intestinal Bacteria[1)]

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In the course of studies on the metabolism of active components of crude drugs by intestinal bacteria, paeoniflorin, oxypaeoniflorin, and benzoylpaeoniflorin isolated from the roots of *Paeonia albiflora* PALLAS (Paeoniaceae) were incubated with a human fecal suspension. Paeoniflorin, as well as oxypaeoniflorin and benzoylpaeoniflorin, was converted to three metabolites (paeonimetabolines I, II and III) by intestinal bacteria. The structure of the major metabolite (paeonimetaboline I) was elucidated by spectroscopic methods. Through screening of various strains of intestinal bacteria from human feces, *Peptostreptococcus anaerobius* was shown to have potent ability to transform paeoniflorin to paeonimetaboline I.

Keywords——*Paeonia albiflora*; paeoniflorin; benzoylpaeoniflorin; oxypaeoniflorin; metabolism; intestinal bacteria; paeonimetaboline; *Peptostreptococcus anaerobius*

Paeoniae Radix is one of the most important constituents of traditional Chinese prescriptions for treatment of abdominal pain and syndromes such as stiffness of abdominal muscles. Paeoniflorin (**1**),[2a-c)] oxypaeoniflorin (**2**),[2d)] benzoylpaeoniflorin (**3**),[2d)] albiflorin,[2d)] paeoniflorigenone (**5**)[2e,f)] and gallotannins[2g-i)] were isolated from Paeoniae Radix as physiologically active principles. Among these, **1** has been reported to have hypotensive, vasodilative, and anti-oxytocic actions as well as relaxative and inhibitory actions on the movement and tonus of smooth muscle.[3)] However, studies have not been performed on the absorption, organ-distribution, metabolism and excretion of these active principles in humans and animals.

In the present paper, we report the metabolism of **1** and related compounds by human intestinal bacteria, as a part of a series of studies on the metabolism of components of crude drugs.[4)]

Materials and Methods

Instruments——Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Specific rotations $[\alpha]_D$ were measured with a JASCO DIP-4 automatic polarimeter. Ultraviolet (UV) spectra were taken on a Shimadzu UV-210A spectrophotometer and infrared (IR) spectra with a Hitachi 260-10 infrared spectrophotometer. Nuclear magnetic resonance (NMR) spectra were measured with JEOL JNM-GX 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz), Varian XL 200 (^{1}H, 200 MHz) and JEOL JNM-FX 90Q (^{1}H, 89.5 MHz; ^{13}C, 22.5 MHz) spectrometers, and chemical shifts are presented as δ values relative to the peak observed for tetramethylsilane as an internal standard. Mass spectra (MS) were measured with a JEOL JMS D-200 mass spectrometer at an ionization voltage of 70 eV. Densitometric profiles were recorded on a Shimadzu CS-910 dual wavelength thin layer chromatoscanner (TLC-scanner).

Materials——Paeoniae Radix (the dried roots of *Paeonia albiflora* PALLAS) was purchased from Tochimoto Tenkaido Co. (Osaka). According to the method of Kaneda *et al.*,[2d] **1**, **2** and **3** were isolated in yields of 5.1 g, 1.5 g and 30 mg, respectively, from 300 g of the Paeoniae Radix. In addition, **5** was isolated in a yield of 340 mg from 500 g of the fresh Paeoniae Radix by the procedures of Shimizu *et al.*[2e] and 6-*O*-acetylpaeoniflorigenone (**6**) was prepared by the reaction of **5** with acetic anhydride in pyridine.[2e,f] Desbenzoylpaeoniflorin (**4**) and product F (**11**) were synthesized by the methods of Shibata *et al.*[2a] and Aimi *et al.*[2c] A dilution medium was prepared according to the procedure of Mitsuoka;[5] it consisted of 37.5 ml of solution A (0.78% K_2HPO_4), 37.5 ml of solution B (0.47% KH_2PO_4–1.18% NaCl–1.20% $(NH_4)_2SO_4$–0.12% $CaCl_2$–0.25% $MgSO_4 \cdot H_2O$), 1 ml of 0.1% resazurine, 0.5 g of L-cysteine·HCl·H_2O, 2 ml of 25% L-ascorbic acid, 50 ml of 8% Na_2CO_3 and water to give a final volume of 1.0 l.

Chromatography of Metabolites——For column chromatography, Wakogel C-200 was used as an absorbent. Thin-layer chromatography (TLC) was performed on Merck Kieselgel 60 F_{254}, Merck HPTLC-Fertigplatten RP-2 F_{254} (reversed phase) and Merck PSC-60 F_{254} (for preparative purposes) plates. The following solvent systems were used: A, $CHCl_3$–MeOH–benzene (5 : 1 : 1); B, AcOEt–CH_2Cl_2 (3 : 2); C, MeOH–H_2O (55 : 45). Spots on the plates were detected by exposure to iodine vapor or by spraying with an anisaldehyde–H_2SO_4 reagent, followed by heating.

Preparation of a Human Intestinal Bacterial Mixture——Fresh feces obtained from a healthy man were immediately transferred into a vinyl bag filled with oxygen-free carbon dioxide. The bag was then pressed by hand to uniformly mix the contents. The feces were suspended in twenty-five volumes of an anaerobic diluant.[5] The fecal suspension thus obtained was used in the following experiments as an intestinal bacterial mixture.

Incubation of 1 with an Intestinal Bacterial Mixture——**1** (800 mg) was added to an intestinal bacterial mixture (800 ml). The mixture was incubated at 37 °C for 24 h in an anaerobic jar in which the air had been replaced with oxygen-free carbon dioxide in the presence of activated steel wool (steel wool method),[5] and then extracted four times with ethyl acetate (AcOEt, 800 ml each). The solution was evaporated *in vacuo* to give an oily residue (*ca.* 3 g). The residue was applied to a column (3 cm i.d. × 47 cm) of silica gel (180 g). The column was thoroughly washed with benzene and then eluted with $CHCl_3$ to yield an oil (90 mg) (paeonimetaboline I, **8**), having an *Rf* value of 0.52 on TLC in solvent system A (a reddish-violet color by spraying with an anisaldehyde–H_2SO_4 reagent). Crude **8** was further applied to a column of silica gel (2 cm i.d. × 26 cm), which was washed with benzene, followed by CH_2Cl_2 and then eluted with CH_2Cl_2–AcOEt (100 : 3) to yeild a colorless oil (42 mg, 13%). This oil was chromatographically pure and had the following properties. High-resolution MS: Calcd for $C_{10}H_{14}O_4$ (M^+) 198.0892. Found: 198.0892. $[\alpha]_D^{28}$ +4.1° (c=1.5, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3410 (OH), 1710 (C=O). ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 1.12 (3H, d, J= 7.5 Hz, CH_3–CH–), 1.29 (3H, s, CH_3–C–), 1.90 (1H, br q, J=7.5, –CH–CH_3), 2.04 and 2.44 (each 1H, dd, J=13.7, 2.5 Hz and J=13.7, 3.7 Hz, –CH_2–CH–), 2.49 (1H, m, –CH–CO–), 2.69 (2H, s, –CO–CH_2–), 5.17 (1H, br s, –O–CH–O–). MS *m/z*: 198 (M^+), 180, 152, 98, 69, 28 (base peak).

Incubation of 2 with an Intestinal Bacterial Mixture——**2** (600 mg) dissolved in EtOH (4 ml) was added to an intestinal bacterial mixture (600 ml). The mixture was anaerobically incubated at 37 °C for 24 h and extracted three times with AcOEt (600 ml each). The AcOEt phase was concentrated to a small volume *in vacuo*. The mixture thus obtained was applied to a silica gel column (68 g, 2.4 cm i.d. × 27 cm). The column was washed with benzene and then eluted with $CHCl_3$. Fractions of 50 ml/flask were collected and monitored by silica gel TLC. Fractions 15—24 were pooled and evaporated to dryness *in vacuo*. The residue (68 mg) was purified by rechromatography (silica gel, 30 g; column size, 1.9 cm i.d. × 16 cm). A pure oily compound was eluted with CH_2Cl_2–AcOEt (100 : 3); yield, 32 mg (13%). This compound was identical with **8** obtained from **1** based on a comparison of the *Rf* values in various solvent systems and the IR spectra.

Incubation of 3 with an Intestinal Bacterial Mixture——**3** (10 mg) was added to an intestinal bacterial mixture (10 ml). The products were anaerobically incubated at 37 °C for 24 h, then the metabolites were extracted in the same fashion as described above and analyzed by TLC in solvent systems A, B and C. A major metabolite had *Rf* values of 0.52, 0.38 and 0.62 (reverse-phase) in solvent systems A, B and C, respectively, and was identical with **8**.

Incubation of 4 with an Intestinal Bacterial Mixture——**4** (8 mg) was added to each of three tubes containing an intestinal bacterial mixture (10 ml). The tubes were incubated for 6, 12 and 24 h, respectively, and the products extracted as described above were analyzed by TLC-densitometry. Paeonimetabolines I—III were detected in all the tubes.

Incubation of Product F (11) with an Intestinal Bacterial Mixture——**11** (30 mg)[2c] was added to an intestinal bacterial mixture (30 ml) and anaerobically incubated at 37 °C for 24 h in an anaerobic jar. After the same procedure as described above, a crude extract was obtained and purified further by repeated preparative TLC with solvent system A to give an oily compound (**12**, 4 mg). ^{1}H-NMR ($CDCl_3$) δ: 1.38 (3H, s, CH_3–C–), 1.60 and 1.84 (each 1H, dd, J=10.2, 10.8 Hz and J=10.2, 5 Hz, –CH_2–CH–), 1.98 (2H, s, –C–CH_2–C–), 2.54 (1H, dd, J=10.8, 5 Hz, –CH_2–CH–), 3.40 (3H, s, –OCH_3), 4.06 (2H, d, J=3 Hz, –C–CH_2–OH), 5.44 (1H, s, –O–CH–O–). Based on the ^{1}H-NMR spectral data, this compound was identified as PF-S (**12**), a product formed by soil bacterial hydrolysis of **11**.

Acetylation of 8 with Acetic Anhydride–Pyridine——**8** (30 mg) was reacted with acetic anhydride (0.8 ml) in pyridine (0.8 ml) at 37 °C for 20 h. Ice-chilled water was added to the reaction mixture, which was then extracted with ether (5 ml × 8). The solution was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to yield a residue. The residue was applied to a column of silica gel (1.5 cm i.d. × 23 cm). After being washed with benzene, the column was

eluted with benzene–CH_2Cl_2 (1 : 1) to give an oily substance, 6-*O*-acetylpaeonimetaboline I (**9**), in a yield of 25 mg (69%). This monoacetate ($C_{12}H_{16}O_5$) had the following properties. $[\alpha]_D^{28}$ +24.8° (c=0.5, MeOH). IR ν_{max}^{KBr} cm^{-1}: 1740 (ester C=O), 1715 (sh, C=O). ^{1}H-NMR ($CDCl_3$) δ: 1.16 (3H, d, J=7.5 Hz, CH_3–CH–), 1.28 (3H, s, CH_3–C–), 1.95 (1H, br q, J=7.5 Hz, CH_3–CH–), 2.18 (3H, s, CH_3–CO–), 2.45—2.79 (2H, m, –CH_2–CH–), 2.50 (1H, m, –CH_2–CH–), 2.98 (2H, AB q, J=17.5, –CH_2–CO–), 5.27 (1H, br s, –O–CH–O–). MS m/z: 240 (M^+), 197, 180, 152, 98, 72, 69, 55 (base peak), 43.

Reduction of 9 with $NaBH_4$——$NaBH_4$ (8 mg) was added in portions to a solution of **9** (22 mg) in EtOH–CH_2Cl_2 (2 : 1, 2 ml). The mixture was then stirred at room temperature for 1 h. A few drops of AcOH and then CH_2Cl_2 (7 ml) were added, then the solution was washed with water (1 ml), dried over Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on a column of silica gel (7 g, 1.5 cm i.d. × 14 cm) with benzene–$CHCl_3$ (1 : 1) as an eluant. An oily substance (**10**) was obtained in a yield of 12 mg (55%) after evaporation of the solvent and had the following properties. $C_{12}H_{18}O_5$; $[\alpha]_D^{28}$ +59.1° (c=1.0, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 1720 (C=O). ^{1}H-NMR ($CDCl_3$) δ: 1.11 (3H, d, J=7.5 Hz, CH_3–CH–), 1.19 (3H, s, CH_3–C–), 1.92 and 2.64 (each 1H, d, J=16 Hz, dd, J=16, 8 Hz, –CH(OH)–CH_2–), 2.09 and 2.36 (each 1H, m, dd, J=12.8, 4.4 Hz, –CH_2–CH–), 2.14 (3H, s, CH_3–CO–), 2.26 (1H, m, –CH–CH(OH)–), 2.29 (1H, m, –CH–CH_3), 4.16 (1H, t, J=8 Hz, –CH(OH)–), 5.25 (1H, br s, –O–CH–O–). MS m/z: 242 (M^+), 182, 154, 135, 69, 43, 32 (base peak).

Reduction of 6 with $NaBH_4$——**6** (34 mg) was reduced with $NaBH_4$ (10 mg) in EtOH–CH_2Cl_2 (2 : 1, 2.5 ml) at room temperature for 1 h. After the same procedure as described above, the product was purified by preparative TLC with a solvent system of $CHCl_3$–MeOH (10 : 1) to give an alcoholic compound (**7**) in a yield of 21 mg. $[\alpha]_D^{28}$ +69.6° (c=1.4 MeOH). UV λ_{max}^{MeOH} nm: 229, 26*i* (sh), 273, 282 (sh). IR ν_{max}^{KBr} cm^{-1}: 3510 (OH), 1720 (C=O), 1600 (arom. C=C), 1580 (arom C=C). ^{1}H-NMR ($CDCl_3$) δ: 1.22 (3H, s, CH_3–C–), 2.10 (3H, s, CH_3–CO–), 2.12 and 2.40 (each 1H, dd, J=12.8, 2.2 Hz; J=12.8, 4.4 Hz, –CH_2–CH–), 2.48 (1H, m, –O–CH_2–CH–), 1.96 and 2.58 (each 1H, d, J=16 Hz; dd, J=16, 8 Hz, –CH(OH)–CH_2–), 2.76 (1H, m, –CH–CH(OH)–), 4.34 (1H, t, J=8 Hz, –CH(OH)–), 4.62 (2H, m, –O–CH_2–CH–), 5.56 (1H, s, –O–CH–O–), 7.26—7.64 (3H, m, arom. protons), 8.02 (2H, dd, J=8, 2 Hz, arom. protons). MS m/z: 362 (M^+), 152, 105 (base peak), 83, 69, 43.

Quantitative Analysis of Metabolites——Metabolites were extracted with AcOEt from the incubation mixture and applied to a silica gel TLC plate (Merck Kieselgel 60 F_{254}), which was then developed with solvent system A. Spots were visualized by uniformly spraying the plate with an anisaldehyde–H_2SO_4 reagent, followed by heating at 120 °C for 10 min; the plate was then covered with another glass plate of the same size, and all the edges were sealed with vinyl tape. The resulting reddish-violet color of metabolites was stable at room temperature for approximately 2 h. The metabolites were quantitatively analyzed with a TLC-scanner at 560 nm relative to a reference wavelength of 780 nm by using calibration lines obtained with authentic **1** and paeonimetabolines (I—III). The calibration lines were linear in the range of 1—50 μg/spot.

Time Course of the Metabolism of 1——Tubes containing **1** (5 mg), fresh feces (200 mg wet weight) and an anaerobic dilution medium (5 ml)[6] were incubated at 37 °C for the indicated time intervals in an anaerobic jar. The mixture was extracted twice with AcOEt (5 ml each) and analyzed by TLC densitometry as described above.

Screening of Intestinal Bacterial Strains for the Ability to Metabolize 1——Each precultured bacterial strain was inoculated into GAM broth (10 ml) and cultivated at 37 °C for 24 h. The cultured medium was immediately centrifuged at 3500 rpm for 20 min. The precipitate was suspended in an anaerobic dilution medium (5 ml).[6] An aliquot (100 μl) of a solution of **1** (80 mg/ml) which had been passed through a sterile membrane filter (0.45 μm, FP 0.30/2, Schleicher and Schuell, Dessel, West Germany) was added to the bacterial suspension. The mixture was incubated at 37 °C for 24 h and extracted with AcOEt (5 ml). The metabolites thus obtained were analyzed quantitatively by TLC-densitometry.

Results and Discussion

Structure of a Major Metabolite

Through anaerobic incubation with a human intestinal bacterial mixture, **1** was transformed to three metabolites (named paeonimetabolines, I, II and III) which gave a reddish purple color on a TLC plate with an anisaldehyde–H_2SO_4 reagent (Fig. 1). A major metabolite (paeonimetaboline I, **8**, Rf=0.52 on silica gel TLC in solvent system A) showed a chemical composition of $C_{10}H_{14}O_4$ on high-resolution mass spectrometry, and contained two methyl, two methylene, three methine, two quaternary and one carbonyl carbons as shown by the ^{13}C-NMR data (Table I). Compound **8** showed neither characteristic ^{1}H- and ^{13}C- NMR signals (Fig. 2 and Table I) due to a glucose moiety nor UV absorption due to a benzoyl group present in the original **1**, indicating that these groups had been hydrolyzed in the metabolic processes. Further, it showed no ^{1}H-NMR signals due to two protons attached to a hydroxy-

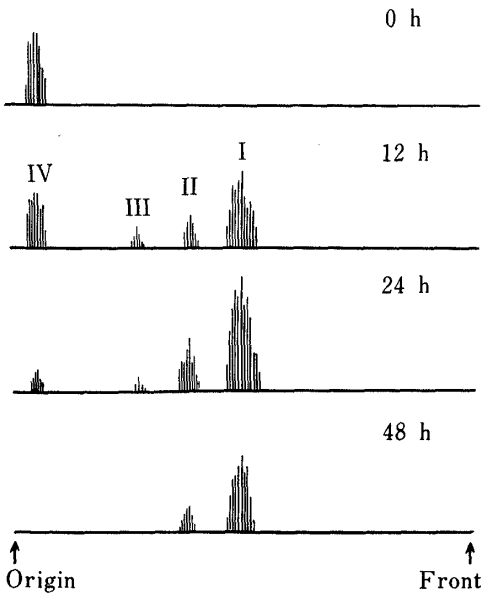


Fig. 1. Densitometric Profiles of the Metabolites of Paeoniflorin

The metabolites were chromatographed on a silica gel TLC plate in solvent system A and the plate was sprayed with anisaldehyde-H_2SO_4 reagent, followed by heating at 120 °C for 10 min. Quantitative analysis was carried out by TLC-densitometry at 560 nm and 780 nm (reference wavelength). I (Rf=0.52), paeonimetaboline I; II (Rf=0.42), paeonimetaboline II; III (Rf=0.30), paeonimetaboline III; IV (Rf=0.08), paeoniflorin.

TABLE I. ^{13}C-NMR Data for Paeoniflorigenone, Paeonimetaboline I and Their Derivatives

Carbon No.	Compounds 5	6	7	8[a]	9	10
C_1	78.5 (s)	80.7 (s)	82.7 (s)	77.4 (s)	79.6 (s)	81.4 (s)
C_2	46.7 (t)	47.4 (t)	41.9 (t)	47.5 (t)	48.2 (t)	42.1 (t)
C_3	210.2 (s)	208.3 (s)	69.7 (d)	210.9 (s)	210.2 (s)	68.1 (d)
C_4	46.2 (d)	45.7 (d)	36.5 (d)	50.2 (d)	49.6 (d)	39.6 (d)
C_5	34.1 (t)	31.4 (t)	32.5 (t)	30.4 (t)	27.6 (t)	28.2 (t)
C_6	101.5 (s)	106.1 (s)	106.1 (s)	101.6 (s)	106.5 (s)	106.8 (s)
C_7	43.0 (d)	43.2 (d)	48.6 (d)	37.9 (d)	38.2 (d)	32.5 (d)
C_8	62.5 (t)	62.2 (t)	64.5 (t)	14.6 (q)	14.6 (q)	14.6 (q)
C_9	99.6 (d)	100.8 (d)	100.4 (d)	103.1 (d)	104.2 (d)	103.6 (d)
C_{10}	21.0 (q)	20.9 (q)[b]	21.4 (q)[c]	20.9 (q)	20.9 (q)[d]	21.4 (q)[e]
CH_3–CO–		168.6 (s)	168.4 (s)		168.6 (s)	168.4 (s)
		21.2 (q)[b]	21.8 (q)[c]		21.2 (q)[d]	21.6 (q)[e]
C_6H_5–CO–	128.2 (d)	128.3 (d)	128.2 (d)			
	129.5 (d)	129.5 (d)	129.5 (d)			
	130.4 (s)	130.1 (s)	130.1 (s)			
	133.4 (d)	132.9 (d)	132.8 (d)			

Abbreviations given in parentheses indicate the signal patterns observed in the off-resonance and INEPT (insensitive nuclei enhanced by polarization transfer) methods. s, singlet; d, doublet; t, triplet; q, quartet. *a*) Measured at 125 Hz; the multiplicity of carbons was also confirmed by means of an INEPT experiment. *b*—*e*) Assignments may be interchanged in each column.

or a benzoyloxy-bearing carbon atom (C_8), which are observed in the spectra of paeoniflorin derivatives.[7] The ^{1}H- and ^{13}C-NMR spectra, however, showed the presence of a *sec*-methyl (^{1}H δ 1.12, J=7.5 Hz; ^{13}C δ 14.6), a *tert*-methyl (^{1}H δ 1.29; ^{13}C δ 20.9), a ketonic carbonyl (^{13}C δ 210.9) and two carbons bearing two oxygen functions, which are assignable to an acetal (^{1}H δ 5.17; ^{13}C δ 103.1) and a hemiketal (^{13}C δ 101.6). On irradiation at δ 1.90 (1H, br q, J= 7.5 Hz), the doublet of the *sec*-methyl proton at δ 1.12, the multiplet of the methine proton at δ 2.49 and the singlet of the acetal proton at δ 5.17 changed to a singlet, an intense multiplet and a sharp and intense singlet, respectively (Fig. 2). On irradiation at δ 2.49 (1H, m), a pair of double doublets at δ 2.04 (1H, J=13.7, 2.5 Hz) and 2.44 (1H, J=13.7, 3.7 Hz) changed to

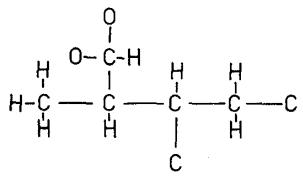


Chart 1. A Partial Structure of Paeonimetaboline I

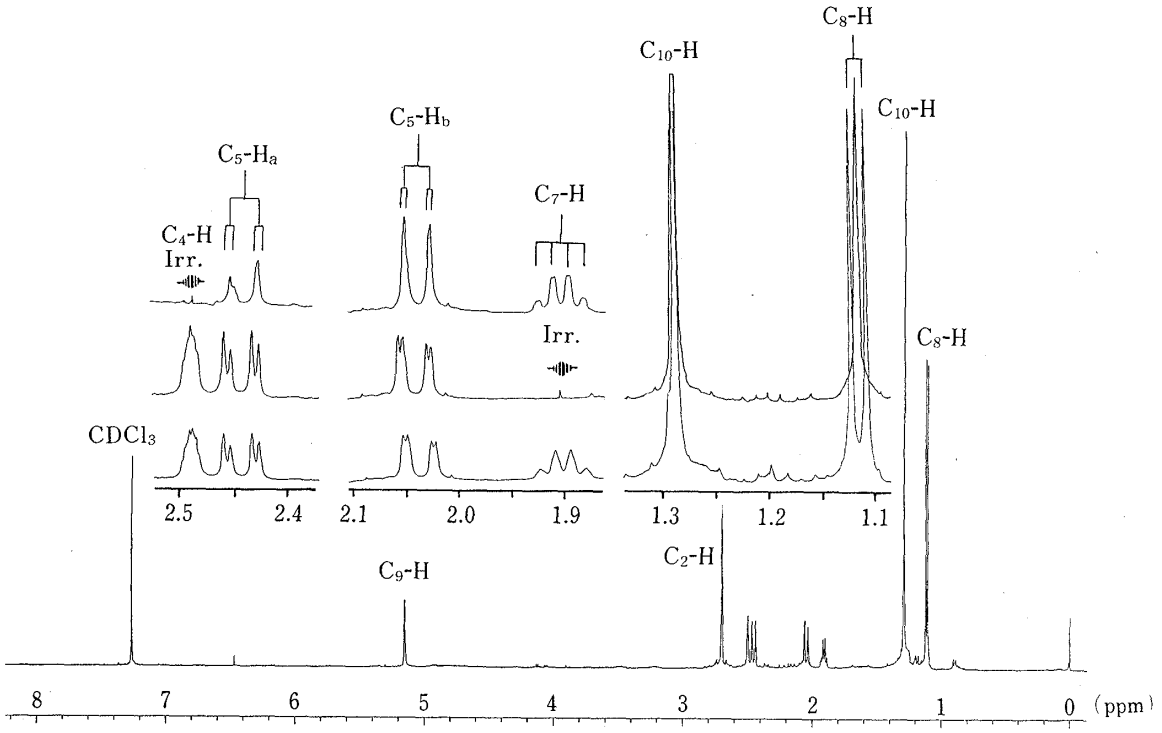
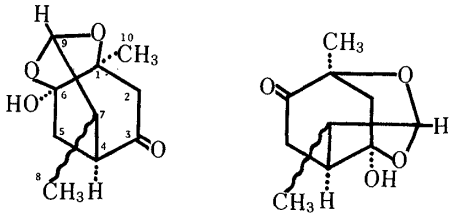


Fig. 2. ^{1}H-NMR Spectra of Paeonimetaboline I Measured in $CDCl_3$ at 500 MHz

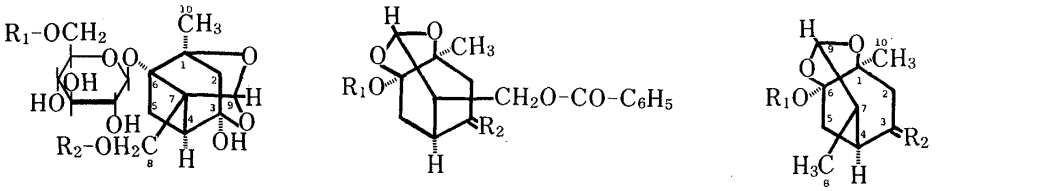
doublets. These findings revealed the presence of the following system in **8** (Chart 1). Furthermore, the IR spectra showed the presence of a hydroxyl (3410 cm^{-1}) as well as a ketonic carbonyl attached to a six-membered ring (1710 cm^{-1}). Based on these spectral data, the structure of **8** was proposed to be either A or B in Chart 2. Since structure A is quite similar to that of **5** (Chart 3) isolated from fresh Paeoniae Radix,[2e] various spectroscopic comparisons were carried out between their acetates and dihydroacetates. Both **8** and **5** gave monoacetates (**9** and **6**, respectively). On acetylation, ^{13}C-NMR signals due to C_1, C_5 and C_6 shifted similarly with differences of +2.2 (+2.2) (the value for **5** is indicated in parentheses), −2.8 (−2.7) and +4.9 (+4.6) ppm units, respectively, in both compounds, and those due to C_2 and C_4 shifted similarly with smaller differences of +0.7 (+0.7) and −0.6 (−0.5) ppm unit, respectively (Table I). Other carbon signals due to C_3, C_7 and C_9 and C_{10} were not affected by acetylation. These results suggest that the hydroxyl of the hemiketal moiety in **8** is attached at C_6, as in **5**. Furthermore, on reduction of a ketonic carbonyl[8] of both acetates with sodium borohydride, ^{13}C-NMR signals due to C_2 and C_4 were shifted to a higher field by −6.1 (−5.5) and −10.0 (−9.2) ppm, respectively, but those due to C_1 and C_5 were shifted slightly to lower field by +1.8 (+2.0) and +0.6 (+1.1) ppm, respectively, indicating that a ketonic carbonyl in the ring is located not at C_6 but at C_3 in **8**, as in **5**. Based on these data on the well-known neighboring effect in ^{13}C-NMR signals, induced by acetylation of a hydroxyl or by reduction of a carbonyl, the structure of **8** was determined to be A in Chart 2. In a comparison of the ^{13}C-chemical shifts at C_5 (δ 30.4 (t) and 34.1 (t) in **8** and **5**, respectively), the



A B

Chart 2. Possible Structures of Paeonimetaboline I

former showed signals at higher field by 3.7 ppm, suggesting a steric effect on the carbon shielding between the C_5 atom and the methyl protons attached to the C_8 atom (*γ-gauche* relative to each other).[9] Furthermore, on reduction of the acetates of **8** and **5**, ^1H- and ^{13}C-signals due to the *sec*-methyl protons (δ 1.11, d, $J=7.5$ Hz, C_8-H) and the C_8 atom (δ 14.6, q) in the former did not change at all, while those due to the methyleneoxy protons (centered at δ 4.23, each dd, C_8-H) and the C_8 atom (δ 62.2, t) which project just above the carbonyl group in the latter (Chart 3) shifted appreciably to lower field by 0.39 and 2.3 ppm, respectively. These findings indicate that the absolute configurations around the C_7 atoms are different: **8** has *S*-configuration at C_7 (Chart 3) and **5** has *R*-configuration, as demonstrated by X-ray analysis.[2f]



1: $R_1=H$, $R_2=-CO-C_6H_5$
2: $R_1=H$, $R_2=-CO-C_6H_4(OH)$
3: $R_1=R_2=-CO-C_6H_5$
4: $R_1=R_2=H$

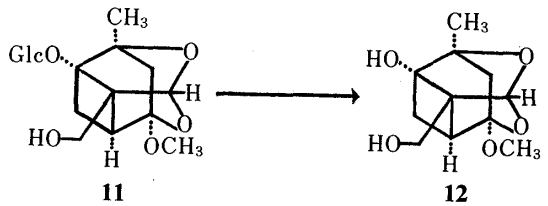
5: $R_1=H$, $R_2=O$
6: $R_1=-CO-CH_3$, $R_2=O$
7: $R_1=-CO-CH_3$, $R_2=\overset{OH}{\underset{H}{<}}$

8: $R_1=H$, $R_2=O$
9: $R_1=-CO-CH_3$, $R_2=O$
10: $R_1=-CO-CH_3$, $R_2=\overset{OH}{\underset{H}{<}}$

Chart 3. Structures of Paeoniflorin, Paeoniflorigenone, Paeonimetaboline I and Their Derivatives

Other metabolites, paeonimetabolines II and III were also isolated but their structures were not determined in this experiment due to their extreme instability and the difficulty in obtaining sufficient amounts for structure elucidation.

As in the case of **1**, other related compounds, **2**, **3** and **4**, were also transformed to **8** as a major metabolite along with small amounts of paeonimetabolines II and III. On the other hand, **11** was not converted to these metabolites but to **12**, a methyl ether of the aglycone of **1**, similar to the case of hydrolysis of **11** by soil bacteria as reported by Yoshioka *et al.*,[6] thus suggesting that cleavage of the hemiketal-acetal system in **1** and related compounds is essential for the formation of **8**.



11 **12**

Chart 4. Transformation of Product F to PF-S

Time Course of Metabolite Formation

Figure 3 shows the time course of metabolism of **1** by human intestinal bacterial mixture. Compound **1** was completely converted to three metabolites in 27 h and the major metabolite (**8**) reached a maximum concentration (75% of the added substrate) at 24 h, but decreased appreciably after prolonged incubation, suggesting that it was further converted to other anisaldehyde–H_2SO_4 negative compounds or incorporated into the bacterial cells. Similarly, paeonimetaboline II was also formed progressively for 24 h but then decreased gradually. Paeonimetaboline III, however, appeared in small amounts only at the early stage of incubation.

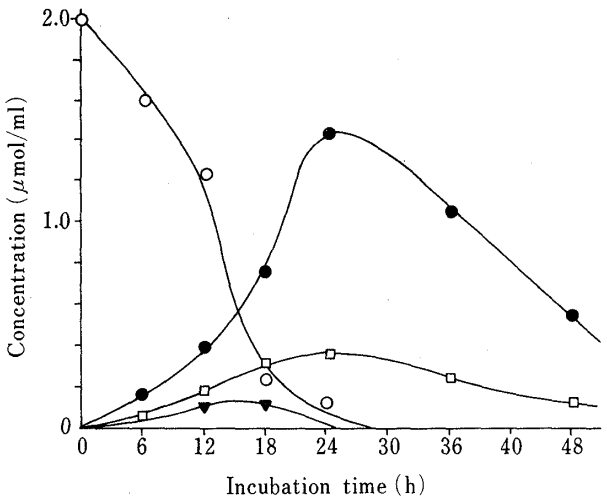


Fig. 3. Time Course of Metabolism of Paeoniflorin by a Human Intestinal Bacterial Mixture

○, paeoniflorin; ●, paeonimetaboline I; □, paeonimetaboline II; ▼, paeonimetaboline III.

TABLE II. Ability of Bacterial Strains from Human Feces and Other Sources to Metabolize Paeoniflorin

Bacterial species	Paeonimetaboline I (%)	Paeoniflorin recovered (%)
Bacteroides fragilis ss. *thetaotus*	1	0
Bifidobacterium adolescentis	0	38
Bifidobacterium bifidum a E319	0	32
Bifidobacterium breve KZ 1287	0	41
Bifidobacterium longum IV-55	0	23
Bifidobacterium pseudolongum PNC-2-9-G	0	40
Clostridium butyricum	2	0
Clostridium innocum ES 24-06	Faint	56
Clostridium innocum KZ 633	Faint	38
Clostridium perfringens To-23	0	35
Escherichia coli 0-127	0	30
Fusobacterium nucleatum G-0470	0	17
Gaffkya anaerobia G-0608	0	49
Klebsiella pneumoniae ATCC 13883	0	58
Lactobacillus acidophilus ATCC 4356	0	26
Lactobacillus brevis II-46	0	34
Lactobacillus fermentum ATCC 9338	0	0
Lactobacillus plantarum ATCC 14917	0	0
Lactobacillus xylosus ATCC 155775	0	2
Peptostreptococcus anaerobius 0240	42	38
Proteus mirabilis S2	0	35
Ruminococcus sp. P01-3	0	36
Streptococcus faecalis II-136	Faint	22
Veillonella parula ss. *paruvula* ATCC 10790	0	43

Screening of Bacterial Strains Capable of Metabolizing 1

Various bacterial strains from human feces and other sources were examined for the ability to metabolize **1** to **8** (Table II). All of the bacterial strains metabolized 42%—100% of **1** in 24 h as calculated from percentages of the substrate recovered. Among these, *Clostridium butyricum, Bacteroides fragilis, Lactobacillus fermentum* and *Lactobacillus plantarum* completely decomposed **1** but no metabolites were detected from the culture broth of the latter two strains. Only *Peptostreptococcus anaerobius* had the ability to transform **1** to **8** in relatively high yield (42% of the added amount of **1**), though *Clostridium butyricum* and *Bacteroides fragilis* had weak activity (2% and 1%, respectively).

Possible Metabolic Processes

Chart 5 shows possible processes leading to the formation of **8** from **1** and related compounds by intestinal bacteria. Compounds **1**, **2** and **3** are hydrolyzed to glucose and benzoic acid (or *p*-hydroxybenzoic acid), and an aglycone (i) by the action of intestinal bacterial β-glucosidase and esterase. Following the reversible conversion of the hemiketal-acetal system to ketone, hydroxyl and aldehyde groups, the four-membered ring (C_4–C_5–C_6–C_7) may be cleaved to form a new C=C bond at C_7 and C_8 (iii). This intermediate may again form a hemiketal-acetal system (iv) at C_1, C_6 and C_9 rather than at C_1, C_3 and C_9 for stereochemical reasons. Further, the terminal C=C bond may be reduced stereospecifically by bacterial enzymes to give **8**.

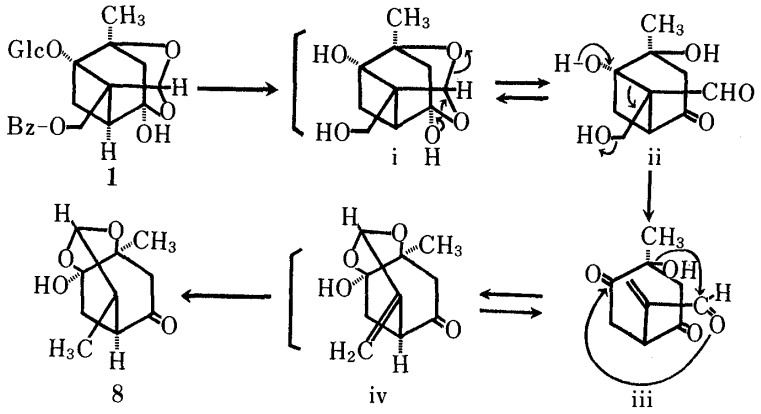


Chart 5. Possible Metabolic Processes of Paeoniflorin by a Human Intestinal Bacterial Mixture

Although it has been reported that human fecal flora differ within individuals and are influenced by various factors such as sex, age, diet, environmental stress, changes in habits and antibiotics,[10] repeated experiments on the metabolism of **1** by intestinal bacterial mixtures showed that no appreciable change in metabolites was observed under the conditions used, irrespective of the use of different fecal samples supplied by three individuals. This suggests that common bacterial species, including *Peptostreptococcus* spp.[10a] as demonstrated above, take part in the metabolic processes. Further studies, however, will be necessary to determine inter-individual or daily variation in the metabolic activity in the intestinal flora.

In conclusion, **1**, as well as **2** and **3**, was transformed to three metabolites by human intestinal bacteria. The structure of the major metabolite (paeonimetaboline I) was determined to be **8** (Chart 3) by spectroscopic methods. Only *Peptostreptococcus anaerobius* had the ability to transform **1** to **8** in relatively high yield among the various bacterial strains tested.

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References and Notes

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3) *a*) K. Takagi and M. Harada, *Yakugaku Zasshi*, **89**, 879 (1969); *b*) *Idem, ibid.*, **89**, 887 (1969); *c*) *Idem, ibid.*, **89**, 893 (1969); *d*) M. Harada, *ibid.*, **89**, 899 (1969).
4) *a*) K. Kobashi, T. Nishimura, M. Kusaka, M. Hattori and T. Namba, *Planta Medica*, **40**, 225 (1980); *b*) M. Hattori, G. Kim, S. Motoike, K. Kobashi and T. Namba, *Chem. Pharm. Bull.*, **30**, 1338 (1982); *c*) M. Hattori, T. Sakamoto, K. Kobashi and T. Namba, *Planta Medica*, **48**, 38 (1983); *d*) M. Hattori, T. Sakamoto, T. Endo, N. Kakiuchi, K. Kobashi, T. Mizuno and T. Namba, *Chem. Pharm. Bull.*, **32**, 5010 (1984); *e*) M. Hattori, T. Sakamoto, T. Yamagishi, K. Sakamoto, K. Konishi, K. Kobashi and T. Namba, *ibid.*, **33**, 210 (1985).
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6) I. Yoshioka, T. Sugawara, K. Yoshikawa and I. Kitagawa, *Chem. Pharm. Bull.*, **20**, 2450 (1972).
7) K. Yamazaki, M. Kaneda and O. Tanaka, *Tetrahedron Lett.*, **44**, 3965 (1976).
8) As in the case of **5**,[2f] the configuration of the hydroxyl at C_3 in **10** was determined to be β on the basis of the following proton spin–spin decoupling experiment: on irradiation at δ 4.16 (t, J=8 Hz, C_3-H), the double doublet at δ 2.64 (1H, J=16, 8 Hz, C_2-H) and the multiplet at δ 2.26 (1H, C_4-H) changed to a doublet and an intense multiplet, respectively. Furthermore, on irradiation at either δ 2.64 or 2.26, the triplet at δ 4.16 changed to a doublet (J=8 Hz). In the same way, the reduction product of **6** was determined to be **7** (Chart 3).
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