Metabolism of albiflorin by human intestinal bacteria

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Abstract

In the course of studies on the metabolism of the components of Chinese crude drugs by intestinal flora, albiflorin from the roots of *Paeonia albiflora* Pallas was incubated with a human fecal suspension. Albiflorin was converted to two metabolites (A1 and A2) by intestinal bacteria. A1 and A2 were identified as paeonilactone-A and paeonilactone-B, both of which had been isolated from fresh paeony roots, by spectroscopic methods.

Key words albiflorin, intestinal bacteria, metabolism, Paeonia albiflora, paeonilactone-A, paeonilactone-B

Abbreviations IR ; infrared, mp ; melting point, ^{1}H-NMR ; proton nuclear magnetic resonance, ^{13}C-NMR ; carbon nuclear magnetic resonance, MS ; mass spectra, TLC ; thin-layer chromatography

Introduction

In our previous paper,[1] we have reported that paeoniflorin (**1**), as well as oxypaeoniflorin and benzoylpaeoniflorin, isolated from Paeoniae Radix is metabolized to paeonimetabolines I (**2**), II and III by human intestinal bacteria including *Peptostreptococcus anaerobius*. In the present paper, we report the metabolism of albiflorin (**3**), a minor component of Paeoniae Radix, which posseses a cage-like pinane skeleton similar in structure to paeoniflorin (**1**) except for a lactone bridge instead of a hemi-ketalacetal system, by human intestinal bacteria, as a part of a series of studies on the metabolism of bioactive components of crude drugs.[2-6]

Materials and Methods

Instruments : Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Infrared (IR) spectra were measured with a Hitachi 260-10 infrared spectrophotometer. Nuclear magnetic resonance (NMR) spectra were measured with JEOL JNM-GX 400 (^{1}H, 400 MHz), Varian XL 200 (^{1}H, 200 MHz) and JEOL JNM-FX 90Q (^{1}H, 89.5 MHz ; ^{13}C, 22.5 MHz) equipped with an FG/BG CMT data system, and chemical shifts are presented as values relative to the peak observed for tetramethylsilane as an internal standard. Mass spectra (MS) were measured with a JEOL D-200 mass spectrometer at an ionization voltage of 70 eV. Densitometric profiles were

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recorded on a Shimadzu CS-910 dual wavelength thin-layer chromatoscanner (TLC-scanner) with a zig-zag reflection mode.

Materials : Albiflorin (**3**) was isolated from Paeoniae Radix (*Paeonia albiflora* Pallas) according to the method of Kaneda *et al.*[7] An anaerobic dilution medium was prepared by the procedure of Mitsuoka[8] : 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-1.2 % $(NH_4)_2SO_4$-0.12 % $CaCl_2$-0.25 % $MgSO_4 \cdot H_2O$), 1 ml of 0.1% resazurine, 0.5 g of L-cysteine H_2O, 2 ml of 25 % L-ascorbic acid, 50 ml of 8 % Na_2CO_3 and water to give a final volume of 1.0l.

Chromatography of metabolites : Wakogel C-200 was used as an absorbent for column chromatography. Merck Kieselgel 60 F_{254}, Merck HPLC-Fertigplatten RP-2 F_{254} (reversed phase) and Merck PSC-60 F_{254} (preparative) were used for thin-layer chromatography (TLC) with solvent system A : $CHCl_3$-MeOH-benzene (5 : 1 : 1). A chromatogram was visualized by exposing the plate to iodine vapor or by spraying it with an anisaldehyde-H_2SO_4 reagent, followed by heating.

Preparation of a fecal suspension : A fecal suspension was prepared from feces of a healthy man as described in the previous paper.[1]

Incubation of albiflorin (**3**) *with a fecal suspension* : Albiflorin (**3**, 85 mg) was added to a fecal suspension (100 ml). The mixture was incubated for 30 hrs at 37℃ 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), and then extracted four times with ethylacetate (AcOEt, 100 ml each). The solution was evaporated *in vacuo* to give an oily residue (*ca.* 600 mg). The residue was applied to a column of silica gel (40 g, 1.9 cm i.d.× 24 cm). The column was washed with benzene (500 ml) and benzene-$CHCl_3$ (1 : 1, 500 ml), and then eluted with $CHCl_3$. The fractions (50 ml each) containing metabolites were pooled and evaporated to dryness *in vacuo*. The crude metabolites were further purified by repeated crystallization and preparative TLC. A1 and A2 were obtained in yields of 11 mg (29 %) and 1 mg (2 %), respectively.

Metabolite A1 (**4**) : Colorless needles, mp 106℃ from acetone-cyclohexane, high resolution MS : Calcd. for $C_{10}H_{14}O_4$, M^+198.0891, Found : 198.0861 ; IR $\nu_{max}^{KBr}cm^{-1}$: 3400 (OH), 1760 (γ-lactone), 1720 (ketone), 1170 (C-O-C) ; ^{1}H-NMR ($CDCl_3$, 400MHz) : δ1.26 (3H, d, J=7Hz, CH_3-CH-), 1.46 (3H, s, CH_3-C-), 1.87 and 2.61 (each 1H, dd, J=14 and 11Hz and J=14 and 6Hz, -C-CH_2-CH-O), 2.38 (1H, dq, J=12 and 7Hz, CH_3-CH-CH-), 2.64 and 2.94 (each 1H, dd, J=15 and 2Hz and J=15 and 8Hz, -CH-CH_2-CO-), 2.75 (1H, m, -CH-CH-CH_2-), 4.90 (1H, sext, J=11, 6 and 6Hz, -CH-O-CO-), low resolution MS (m/z) : 198(M^+), 180, 170, 152, 124, 110, 87, 56, 43 (base peak).

Acetylation of A1 (**4**) *with acetic anhydride-pyridine* : Acetic anhydride (0.3 ml) was added to a solution of A1 (10 mg) in pyridine (0.5 ml). The mixture was kept overnight at 37℃. The product was then applied to a column (1.0 cm i.d.× 23 cm) of silica gel (10 g). After being washed with hexane-benzene (1 : 1 and 2 : 3), the column was eluted with benzene to yield an oily compound (**5**, 5 mg, 38%), $C_{12}H_{16}O_5$, IR ν_{max}^{KBr} cm^{-1} : 1770 (γ-lactone), 1750 (ester C=O), 1715 (ketone). ^{1}H-NMR ($CDCl_3$, 200MHz) : δ 1.32 (3H, d, J=7 Hz, CH_3-CH-), 1.45 (3H, s, CH_3-C-), 2.07 (3H, s, CH_3-CO-), 2.30 and 2.88 (each 1H, dd, J=14 and 7Hz, and J=14 and 9Hz, -C-CH_2-CH-O-), 2.54 (1H, m, CH_3-CH-CH-), 2.62 and 2.96 (each 1H, dd, J=18 and 6Hz ; m, -CH-CH_2-CO-), 2.92 (1H, m, -CH-CH-CH_2-), 4.92 (1H, sext, J=9, 6 and 6 Hz, -CH-O-CO-).

Metabolite A2 (**6**) : $C_{10}H_{12}O_4$, ^{1}H-NMR ($CDCl_3$, 400MHz) : δ 1.40 (3H, s, CH_3-C-), 1.96 and 2.53 (each 1H, dd, J=14 and 9 Hz, and J=14 ald 6Hz, -C-CH_2-CH-O), 2.79 and 2.96 (each 1H, dd, J=16 and 4Hz, and J=16 and 8Hz, -CH_2-CO-), 3.68 (1H, m, -CH-C=), 4.99 (1H, sext, J=9, 9 and 6Hz, -CH-O-CO-), 5.68 and 6.37 (each 1H, d, J= 3 Hz and J=3Hz, CH_2=).

Quantitative analysis of metabolites : Metabolites were extracted with AcOEt from the incubation mixture and applied to a TLC plate of silica gel, which was then developed with solvent system A. The plate was uniformly sprayed with an anisaldehyde-H_2SO_4 reagent, followed by heating for 10 min at 120℃, covered with another

glass plate of the same size, and all the edges were sealed with vinyl tape. The metabolites were quantitatively analyzed with a TLC-scanner at 600 nm relative to a reference wavelength of 780 nm. The calibration curves were linear in a range of 1-50 μg/spot for albiflorin (**3**), A1 and A2.

Time course of the metabolism of albiflorin (**3**) *by intestinal bacteria* : Tubes containing albiflorin (**3**, 3 mg) and a fecal suspension (5 ml) were incubated for indicated time intervals at 37℃ in an anaerobic jar. The metabolic mixture was extracted twice with AcOEt (5 ml each) and analyzed by TLC-densitometry.

Results

Metabolism of albiflorin (**3**) *by human intestinal flora*

During anaerobic incubation with a fecal suspension of humans, albiflorin (**3**) was converted to two metabolites (A1 and A2), which could be separated by TLC (Rfs, 0.43 and 0.41 for A1 and A2, respectively, in solvent system A) and gave a characteristic orange color with an anisaldehyde-H_2SO_4 reagent, followed by heating. None of these metabolites were, however, detected by anaerobic incubation of albiflorin (**3**) with a heat-treated fecal suspension or by aerobic incubation with a fecal suspension. This suggests that anaerobes present in the feces are responsible for the formation of these metabolites. The metabolites (A1 and A2) were isolated by a solvent-extraction technique and purified by repeated column chromatography and preparative TLC.

Structure of metabolite A1

A1, colorless needles, mp 106℃, $C_{10}H_{14}O_4$, showed no characteristic ^{1}H- and ^{13}C-NMR signals due to a glucose moiety, a benzoyl group and an oxymethylene group present in the original albiflorin (**3**), indicating that these were eliminated and transformed to other groups through the metabolic processes by intestinal bacteria. The IR spectrum showed strong absorption bands at 3400, 1760 and 1720cm^{-1} assignable to the stretching vibration of hydroxyl, γ-lactone and ketone groups, respectively. The ^{13}C-NMR spectrum (Table I) showed the presence of two methyl carbons (δ 13.1 and 24.8), two methylene carbons (δ 35.5 and 42.1), three methine carbons (δ 37.9, 43.8 and 73.5) including one oxygen-bearing methine carbon, one oxygen-bearing quarternary carbon (δ 73.6) along with two carbonyl carbons of γ-lactone and ketone groups (δ 177.0 and 210.4, respectively), which were assigned by means of ^{1}H-complete decoupling (COM), off-resonance decoupling (OFR), insensitive nuclei enhanced by polarization transfer (INEPT) and ^{1}H-selective decoupling (SEL). The ^{1}H-NMR (90 MHz) showed proton signals at δ 1.25 (3H, d, J=7 Hz), 1.45 (3H, s) and 4.90 (1H, sext, J=11, 6 and 6 Hz) assignable to *sec*-methyl, *tert*-methyl and oxygen-bearing methine protons. On irradiation at δ 2.38 (1H, m), the doublet of the *sec*-methyl proton (δ 1.25) and the multiplet of the methine proton (δ 2.75) changed to a singlet and an intense and sharp multiplet, respectively. On irradiation at δ 2.75 (1H, m), the sextet of the oxygen-bearing methine proton (δ 4.90) and the multiplet of the methine proton (δ 2.38) changed to a double doublet (J=11 and 6 Hz) and an intense multiplet, respectively. On irradiation at δ 4.90, the double doublet at δ 1.88 (1H, J=14 and 11 Hz) and the multiplet at δ 2.58 (1H) changed to both doublets (J=14 Hz and J=14 Hz, respectively). From this evidence, a partial structure is presented as follows :

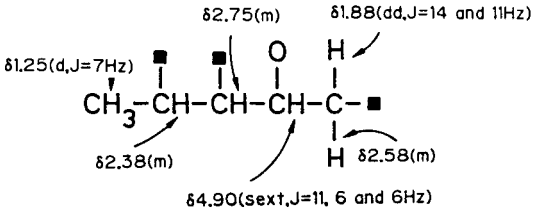

Fig. 1 A partial structure of A1.

Furthermore, in comparison of A1 with its monoacetate, the ^{13}C-NMR spectra showed that the oxygen-bearing quarternary carbon and ketonic carbon signals (δ 73.6 and 210.4, respectively) present in A1 were greatly shifted with differences of δ+5.3 and −5.9, respectively, but the oxygen-bearing methine carbon signal (δ 73.5)

was not changed at all by acetylation (Table I). These findings indicate that an oxygen attached to the quarternary carbon is a free hydroxyl, which can be acetylated with acetic anhydride-pyridine at 37℃ for 28 hrs, and another oxygen attached to the methine carbon forms a γ-lactone ring in A1 and its acetate. Based on these findings, the structure of A1 was deduced as (4) in Figure 2, even though the absolute configuration around C-7 was still uncertain. In higher frequency NMR (400 MHz), all the proton signals in A1 were well separated and could satisfactorily be assigned. The proton signal attached to C-7 was observed at δ 2.38 as a double quartet ($J=12$ and 7 Hz), in which the coupling constants were ascribed to the vicinal protons between H-7 and H-4 and between H-7 and H-9. Based on the well known relationship between the dihedral angle and the coupling constant of vicinal protons, the dihedral angle between H-7 and H-4 was estimated to *ca.* 150°. This indicates that the absolute configuration around C-7 is *R*-configuration. Finally, the structure of A1 was established as (4) in Figure 2, which seemed to be identical with that of paeonilactone-A as reported by Hayashi *et al.*[9] A direct comparison of 400 MHz ^{1}H-NMR and ^{13}C-NMR spectral data of A1 and with those

Table I ^{13}C-NMR spectral data for A1 and its acetate (δ in $CDCl_3$).

Carbon No	A1 (4)	A1-acetate (5)
C-1	73.6 (s)[a]	78.9 (s)
C-2	35.5 (t)	37.6 (t)[b]
C-3	73.5 (d)	73.5 (d)
C-4	37.9 (d)	41.1 (d)[c]
C-5	42.1 (t)	38.6 (t)[b]
C-6	210.4 (s)	204.5 (s)
C-7	43.8 (d)	41.8 (d)[c]
C-8	177.0 (s)	177.7 (s)
C-9	13.1 (q)	14.8 (q)
C-10	24.8 (q)	22.3 (q)
CH_3-CO-		22.3 (q)
		169.8 (s)

a) Abbreviations given indicate the signal patterns observed in the off-resonance method. s, singlet ; d, doublet ; t, triplet ; q, quartet. b-C) Assignment may be interchanged.

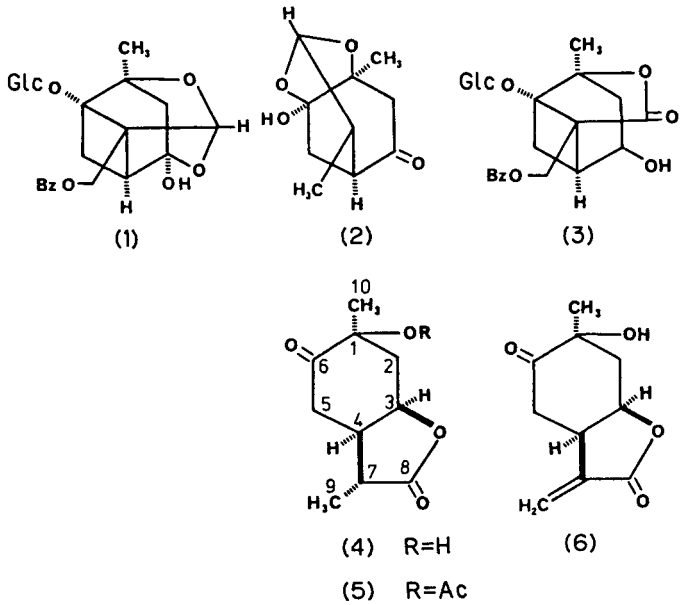

Fig. 2 Structures of paeoniflorin, albiflorin, paeonimataboline I, A1 (paeonilactone A) and A2 (paeonilactone-B).

of an authentic compound concluded that A1 was identical with paeonilactone-A (**4**).

Structure of metabolite A2

A2, $C_{10}H_{12}O_4$; The ^{1}H-NMR (400 MHz) spectrum showed that A2 had neither glucosyl and benzoyl moieties present in the original albiflorin (**3**), nor a *sec*-methyl group present in the metabolite, A1, but showed characteristic signals at δ 1.40 (3H, s), 4.99 (1H, sext. J=9, 9 and 6 Hz), and 5.68 and 6.37 (each 1H, d, J=3 Hz) due to the presence of *tert*-methyl protons, an oxygen-bearing methine proton (possibly as a part of a γ-lactone in comparison of the chemical shift with A1) and terminal vinyl protons ($-\dot{C}=CH_2$) (instead of *sec*-methyl protons in A1). This finding along with the fact the A2 was an intermediate to A1 in the metabolic processes by intestinal bacteria as will be mentioned below, reveals that the structure of A2 may be (**6**) (paeonilactone-B) in Figure 2, as reported by Hayashi *et al.*[9] Finally, A2 was determined to be identical with paeonilactone-B by direct comparison of ^{1}H-NMR spectral data (400 MHz).

Time course of the metabolism

Figure 3 shows the time course of the metabolism of albiflorin (**3**) by intestinal bacteria. During anaerobic incubation with a fecal suspension, albiflorin (**3**) was almost completely consumed in 24 hrs but two metabolites (A1 and A2) were produced instead. The amount of A1 increased progressively and reached a maximum after *ca.* 36 hrs and tended to decline gradually by prolonged incubation. On the other hand, the amount of A2 increased in the first 6-12 hrs and reached a maximum after 12-15 hrs, then disappeared in 36 hrs, suggesting that A2 is a metabolic intermediate or a chemically unstable metabolite. A similar time course was also observed in the metabolism of albiflorin (**3**) by one of the human intestinal bacteria, *Peptostreptococcus anaerobius*, which has the ability to transform paeoniflorin (**1**) to paeonimetaboline I (**2**) as reported previously.[1]

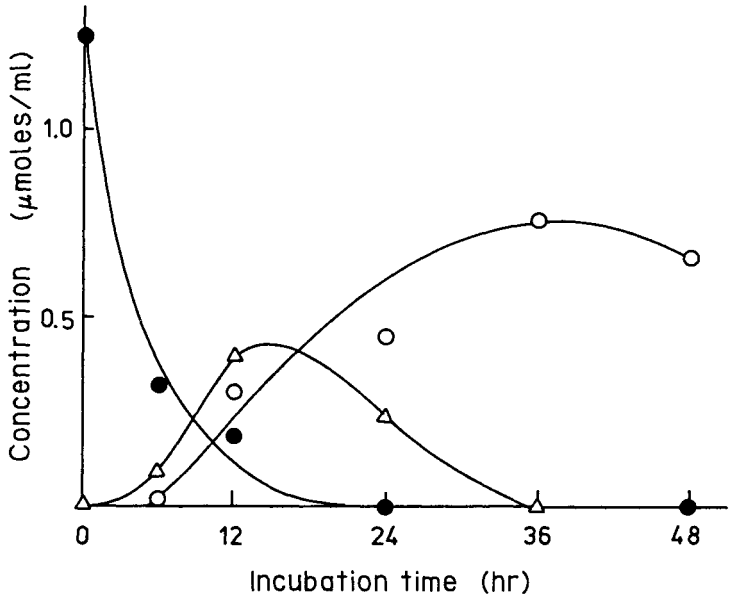

Fig. 3 Time course of the metabolism of albiflorin.
(●), albiflorin ; (○), A1 (paeonilactone-A) ;
(△), A2 (paeonilactone-B)

Discussion

By annaerobic incubation of albiflorin with intestinal bacterial flora, albiflorin (**3**) was metabolized to paeonilactone-A (A1, **4**) and paeonilactone-B (A2, **6**), both of which had been isolated from fresh paeony roots. The possible processes and the mechanism of the formation of paeonilactones A (**4**) and B (**6**) by intestinal flora could be illustrated as follows (Figure 4) : Albiflorin (**3**) is hydrolyzed by bacterial β-glucosidase and esterase to an aglycone (**3a**), glucose and benzoic acid. Since commercially available β-glucosidase from sweet almonds hardly hydrolyzed albiflorin (**3**) as well as paeoniflorin (**1**) to an aglycone even by prolonged incubation (unpublished results), a different type of β-glucosidase seems to be responsible for the hydrolysis of the glucosidic bond. Then, a four membered ring of the aglycone may be cleaved to form a terminal double bond at C-7 and C-8, following the cleavage of a lactone ring as proposed in a previous paper.[1] The cyclization may occur at C-3 and C-8 rather than at C-1 and C-8 to from a γ-lactone ring possibly by reason of less steric hindrance in the former. Paenoilactone-B (**6**) is further reduced to paeonilactone-A (**4**) by bacterial reductase.

Since techniques of anaerobic cultivation were established in quite recent years, much attention has been focused on the metabolism of drugs by anaerobes in the gastrointestinal tract. This may be due to the fact that increases in biological activity may be brought by intestinal flora and these may have pharmacological and toxicological significance (activation of prodrugs, transformation to carcinogens *etc.*) and these reactions may be of importance in the enterohepatic circulation of drugs, in themetabolic differences among various species, strains or individuals and in the metabolic adaptation to the drugs administered repeatedly.[11] As crude drugs used in Chinese medicine are orally administered to humans in general, the components are inevitably in contact with intestinal bacteria and may be transformed to bioactive principles. This is true for sennosides A and B, the components of Rhei Rhizoma, which are stepwisely hydrolyzed and reduced to yield rheinanthrone, an active purga-

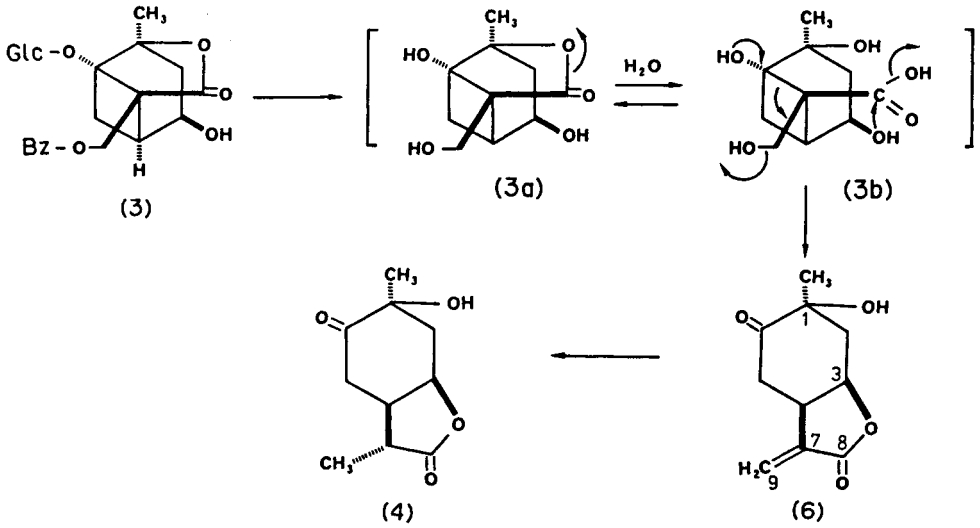

Fig. 4 Possible metabolic processes of albiflorin by intestinal bacteria.

tive-principle, by intestinal flora.[3] Paeoniflorin (**1**) and albiflorin (**3**) from Paeoniae Radix were also transformed to various metabolites by human intestinal flora as shown in this and previous[1] reports, and one of these metabolites (paeonimetaboline I, **2**) showed appreciable suppression of convulsions induced by penetetrazole in rats (unpublished results). These findings emphasize the role of intestinal flora for manifestation of pharmacological action of crude drugs administered orally. Further studies on the pharmacological action of these metabolites are now in progress.

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