Original 和漢医薬学会誌 4, 82－87, 1987

Metabolism of paeoniflorin and related compounds by human intestinal bacteria III.[1] Metabolic ability of intestinal bacterial strains and fecal flora from different individuals

Yue-Zhong SHU,[a] Masao HATTORI,[a] Teruaki AKAO,[b]
Kyoichi KOBASHI[b] and Tsuneo NAMBA*[a]

[a] *Department of Development for Natural Drug Resources, Research Institute for Wakan-Yaku (Oriental Medicines), Toyama Medical and Pharmaceutical University*
[b] *Department of Biochemistry, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University*

(Received April 14, 1987. Accepted July 29, 1987.)

Abstract

Various defined strains of human intestinal bacteria showed the ability to transform paeoniflorin into an epimeric mixture of paeonimetaboline I in a short period of incubation. The ratio of the 7*R*- and 7*S*-epimers was determined by proton nuclear magnetic resonance spectroscopy ; One *Bacteroides*, two *Bifidobacterium*, one *Clostridium*, five *Lactobacillus* and one *Streptococcus* species converted paeoniflorin to the epimeric mixture of paeonimetaboline I in high yields. Among these, *Bacteroides fragilis* ss. *thetaotus* formed preferably the 7*S*-epimer, while *Lactobacillus xylosus* and *L. acidophilus* formed preferably the 7*R*-epimer. The other bacteria formed both epimers with almost equal amounts. Similarly, fecal flora from different subjects showed potent metabolic ability but predominantly produced the 7*S*-epimer.

Key words human intestinal flora, individual difference in metabolism, intestinal bacteria, metabolism, *Paeonia albiflora*, paeoniflorin, paeonimetaboline

Abbreviations GAM, general anaerobic medium ; ^{1}H-NMR, proton nuclear magnetic resonance ; TLC, thin-layer chromatography

Introduction

The intestinal flora plays an important role in the metabolism of compounds administered orally or excreted into bile.[2,3] Besides endogeneous compounds (biliary components *etc.*), xenobiotic compounds (drugs, food constituents, *etc.*) undergo metabolic transformation by intestinal microorganisms. Some of these transformations provide insights into the mechanism of the therapeutic benefits or adverse effects of drugs. From this viewpoint, we have studied the metabolism of active components from Chinese medicines, which are taken orally in general, by intestinal bacteria.[4-11] These experiments have been carried out by mixed bacterial populations present in intestinal contents or feces and by identified strains from human intestine. Along this line of study, some enzymes responsible for the metabolic processes have been isolated from bacteria having a potent metabolic activity.[6,8]

In previous papers,[9,10] we have reported the structures of 7*R*- and 7*S*-paeonimetabolines I (**2** and **3**) and II (**4**) which are transformed from paeoniflorin (**1**), an active principle of peony roots (*Paeonia albiflora* PALLAS), by human intestinal bacteria, and the anti-convulsive action of 7*S*-paeonimetaboline I (**3**) on the pentylenetetrazole-treated rats.[12,13]

*〒 930-01 富山市杉谷 2630
富山医科薬科大学和漢薬研究所資源開発部門 難波恒雄
2630 Sugitani, Toyama 930-01, Japan

Journal of Medical and Pharmaceutical Society for WAKAN-YAKU 4, 82－87, 1987

In the present paper, we report the ability of various intestinal bacterial strains and fecal flora from different healthy subjects to transform paeoniflorin (**1**) to paeonimetabolines, and the ratio of 7*R*- and 7*S*-epimers of the major metabolite, which was determined by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR).

Materials and Methods

Instruments : NMR spectra were measured with a JEOL-FX 90Q (^{1}H, 90MHz) NMR spectrometer using tetramethylsilane as an internal standard. Densitometric profiles were recorded on a Shimadzu CS-910 dual wavelength thin layer chromatoscanner (TLC scanner) at 560 nm relative to a reference wavelength of 780 nm with a Zig-Zag reflection photometric mode.

Chemicals : General anaerobic medium (GAM) broth was a product of Nissui Seiyaku Co., Ltd., Tokyo. EG agar was purchased from Eiken Chemical Co., Ltd., Tokyo. 7*R*-and 7*S*-Paeonimetabolines I and II were obtained according to the method reported previously.[9,10] All chemicals used were of analytical reagent grade available.

Chromatography of metabolites : Thin layer chromatography (TLC) was performed on Merck Kieselgel 60 F_{254} plates (layer thickness 0.25 mm) with $CHCl_3$-MeOH-benzene (5 : 1 : 1). Spots on the plates were visualized by exposure to iodine vapor or by spraying with an anisaldehyde-H_2SO_4 reagent followed by heating.

Screening of intestinal bacteria capable of metabolizing paeoniflorin (1) : Through the experiment, all manipulations were done under an oxygen-free CO_2 gas. All air in the medium and flasks (tubes) was also replaced by the same gas.

Twenty-four species of stock strains of intestinal bacteria kindly provided by Prof. T. Mitsuoka (The University of Tokyo) had been maintained on EG agar slants in a refrigerator at 4℃ prior to use. The bacterium was inoculated to GAM broth (10 ml), followed by 10 hr anaerobic cultivation at 37℃. The bacterial culture was diluted 10-fold with the same broth and cultivated for 16 hr. The culture was centrifuged at 7000 rpm for 10 min. The precipitates were washed with 0.9% NaCl solution (10 ml), collected by centrifugation and suspended in 0.1 M phosphate buffer (10 ml, pH *ca.* 7.4). A 200 μl aliquot of paeoniflorin (**1**, 12 mg), which had previously been passed through a sterile membrane filter (0.45 μm, FP 030/2, Scheicher and Schüll, Dessel, West Germany), was added to the bacterial suspension. After 4 hr incubation at 37℃, the products were extracted twice with AcOEt (10 ml each) and analyzed quantitatively by TLC-densitometry as described in our previous paper.[9]

Incubation of paeoniflorin (1) with a human fecal suspension : Fresh feces obtained from healthy subjects (3 g each, 2 females and 13 males) were suspended in 0.1 M phosphate buffer (pH 7.4, 30 ml). Paeoniflorin (**1**) (30 mg in 0.3 ml of H_2O) was added to the suspension (20 ml) and incubated anaerobically for 27 hr at 37℃. The mixture was then extracted with AcOEt and the products were analyzed by TLC-densitometry.[9]

Determination of the ratio of 7R- and 7S-epimers of paeonimetaboline I : Each AcOEt extract obtained as described above was re-extracted with benzene (2 ml each, 3 times) by ultra sonication. The combined solutions were loaded on a Sep-Pak cartridge (Waters Assoc., Milford, MA, U.S.A.) by a glass injector. The cartridge was successively washed with benzene (20 ml), benzene-dichloromethane (1 : 1, 20 ml ; 1 : 2, 20 ml) and benzene-chloroform (1 : 1, 15 ml). 7*R*- and 7*S*-Epimers of paeonimetaboline I (**2** and **3**, respectively) were eluted from the cartridge with benzene-chloroform (1 : 3, 20 ml). After the solvent was evaporated *in vacuo*, the residue was dissolved in $CDCl_3$, and the ratio of the epimers was determined by ^{1}H-NMR.

Results and Discussion

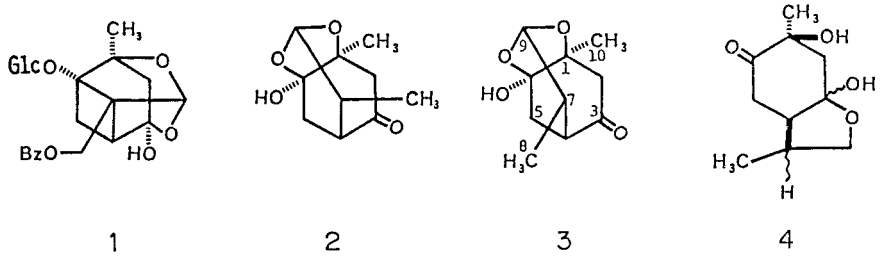
Transformation of paeoniflorin (1) by intestinal bacterial strains

Fourteen species among the bacterial strains examined had ability to transform paeoniflorin (**1**) to paeonimetabolines in a short period of incubation (4 hr). *Bacteroides fragilis* ss. *thetaotus, Bifidobacterium adolescentis, Bifidobacterium longum,*

Clostridium innocuum, Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus xylosus and *Streptococcus faecalis* produced paeonimetaboline I in high yields (more than 40%) when the products were analyzed by TLC-densitometry. A few bacterial strains including *B. fragilis* ss. *thetaotus, B. longum, Fusobacterium nucleatum, L. acidophilus, L. fermentum, L. xylosus* and *Ruminococcus* sp. produced peaonimetaboline II (**4**) in low yields (2−8%), in addition to paeonimetaboline I. Other bacteria including some *Bifidobacterium, Clostridium, Gaffkya, Peptostreptococcus* and *Proteus* species consumed paeoniflorin (**1**) but produced no detectable amounts of paeonimetabolines under these conditions.

Since peonimetaboline I, as well as paeonimetaboline II, is a mixture of 7*R*- and 7*S*-epimers (**2** and **3**, respectively) which are unable to separate each other on TLC as reported previously,[10] the ratio of the epimers was directly analyzed by ^{1}H-NMR. Prior to analysis, the AcOEt extract was treated with a Sep-pak silica cartridge to eliminate impurities from bacteria and culture medium. The ratio of 7*R*- and 7*S*-epimers of paeonimetaboline I was determined on the basis of relative intensities of the methylene protons at C-2 (**2**, δ2.61 ; **3**, δ2.69) or the *sec*-methyl protons (**2**, δ0.90, d, J=7.3 Hz ; **3**, δ1.12, d, J=7.5 Hz) in the ^{1}H-NMR spectrum of the epimeric mixture (Fig. 2).

The results are summarized in Table I. Most



1 2 3 4

Fig. 1 Stuctures of paeoniflorin and paeonimetabolines.

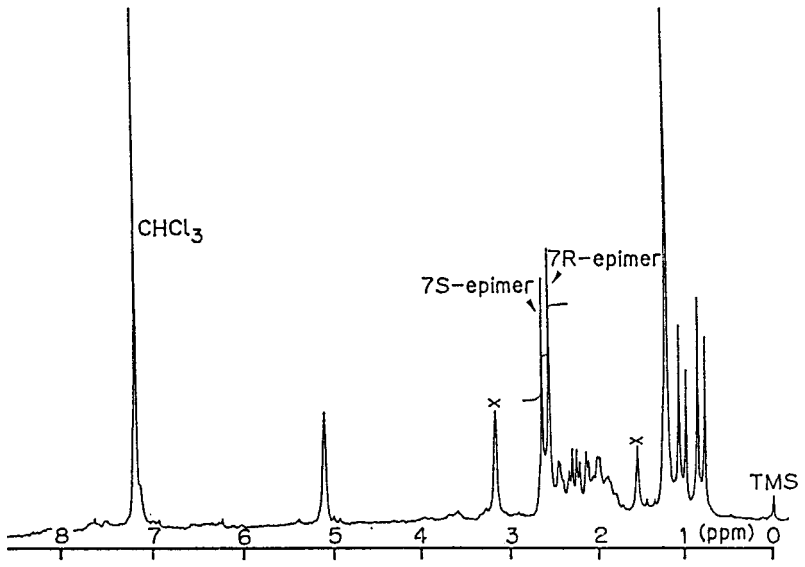


Fig. 2 ^{1}H-NMR spectrum (90 MHz) of a mixture of 7*R*- and 7*S*-paeonimetaboline I (**2** and **3**) transformed by *Lactobacillus brevis*.

of strains possessing potent transforming activity were shown to yield the epimeric mixtures consisting of almost equal amounts of the 7*R*- and 7*S*-epimers. *B. fragilis* ss. *thetaotus* formed preferably the 7*S*-epimer with a ratio of 5 : 2 (7*S* : 7*R*), whereas *L. acidophilus* and *L. xylosus* formed preferably the 7*R*-epimer with a ratio of 1 : 2 (7*S* : 7*R*). However, the ratio of a small amount of the metabolite (less than 10% in yields) produced by bacterial strains having weak activities could not be determined due to the detectable amount of both epimers being more than 0.6 mg (3 μmol)/^{1}H-NMR tube. For the same reason, the ratio of the epimeric mixture of paeonimetaboline II was not determined.

Since no epimerization between 7*R*- and 7*S*-paeonimetaboline I (**2** and **3**) was observed under the incubation conditions, the variations in the ratio of the epimers may be due to the difference in bacterial enzymes which take part in the reduction of the metabolic intermediate as proposed in the previous paper.[10]

Trasformation of paeoniflorin (1) by fecal flora

Similarly, fecal flora from healthy human subjects were examined for ability to transform paeoniflorin (**1**) to paeonimetabolines and the metabolites were analyzed by TLC-densitometry and ^{1}H-NMR (Table II). All the fecal specimens consumed 61−100% of added paeoniflorin (**1**) and produced paeonimetaboline I in yields of 4−75% in a 27 hr incubation. Some specimens produced a small amount of paeonimetaboline II. Nine of the specimens predominantly produced the 7*S*-epimer of paeonimetaboline I with ratios of 6 :

Table I Metabolites of paeoniflorin by defined bacterial strains from human intestine.

Bacterial species	Paeonimetabolines (%) I (7*S* : 7*R*)	II	III	Paeoniflorin recovered (%)
Bacteroides fragilis ss. *thetaotus*	46 (5 : 2)	8	0	0
Bifidobacterium adolescentis	73 (1 : 1)	0	0	faint
Bifidobacterium bifidum a E319	0	0	0	21
Bifidobacterium breve KZ 1287	0	0	0	58
Bifidobacterium longum IV-55	62 (1 : 1)	3	0	30
Bifidobacterium pseudolongum PNC-2-9-G	0	0	0	39
Clostridium butyricum	0	0	0	60
Clostridium innocuum ES 24-06	0	0	2	52
Clostridium innocuum KZ 633	76 (1 : 1)	0	0	faint
Clostridium perfringens To-23	0	0	0	52
Escherichia coli 0-127	0	0	0	64
Fusobacterium nucleatum G-0470	5 (n.d.)	4	0	40
Gaffkya anaerobia G-0608	0	0	0	34
Klebsiella pneumoniae ATCC 13883	9 (n.d.)	0	0	36
Lactobacillus acidophilus ATCC 4356	69 (1 : 2)	3	0	23
Lactobacillus brevis II-46	83 (1 : 1)	0	0	0
Lactobacillus fermentum ATCC 9338	45 (1 : 1)	4	0	faint
Lactobacillus plantarum ATCC 14917	94 (1 : 1)	0	0	faint
Lactobacillus xylosus ATCC 155775	40 (1 : 2)	2	0	53
Peptostreptococcus anaerobius 0240	0	0	0	45
Proteus mirabilis S2	0	0	0	39
Ruminococcus sp. P01-3	7 (n.d.)	2	0	53
Streptococcus faecalis II-136	72 (1 : 1)	0	0	faint
Veillonella parula ss. *paruvula* ATCC 10790	4 (n.d.)	0	0	53

n.d. : not determined.

Table II Metabolites of paeoniflorin by fecal flora from different individuals.

Name	Sex	Age	Paeonimetabolines (%) I (7S : 7R)	II	Paeoniflorin recovered (%)
Y.K.[a)]	M	25	75 (4 : 3)	4	22
G.G.[b)]	M	32	72 (1 : 1)	faint	9
T.M.[b)]	M	27	68 (6 : 1)	4	12
J.Y.[b)]	M	34	55 (4 : 3)	faint	8
O.M.[a)]	M	25	52 (5 : 4)	faint	faint
M.C.[b)]	F	27	45 (1 : 1)	0	19
S.C.[b)]	M	26	36 (5 : 2)	3	13
S.Y.[a)]	F	24	36 (4 : 1)	faint	17
A.T.[a)]	M	27	24 (4 : 1)	0	22
Y.M.[b)]	M	42	23 (2 : 1)	4	0
P.H.[a)]	M	42	20 (4 : 1)	0	0
S.H.[a)]	F	25	20 (2 : 1)	2	39
E.A.[c)]	M	33	18 (6 : 1)	0	0
D.M.[a)]	M	38	15 (3 : 1)	0	11
P.N.[a)]	M	55	4 (n.d.)	4	0

F, female subject ; M, male subject ; n.d., not determined.
a) Japanese, b) Chinese, c) Egyptian.

1−2 : 1 (7*S* : 7*R*) but the others yielded almost equal amounts of the 7*R*- and 7*S*-epimers (**2** and **3**, respectively).

Although some variations in the amount of the metabolites and in the ratio of the epimers were observed among the fecal specimens from different subjects, no obvious change was observed between those from a limited number of the same subjects (n=2) in the repeated experiments. On the other hand, the metabolic activities seemed to be related to the ages of the subjects. The fecal suspensions from aged subjects (over 40 years old) consumed paeoniflorin (**1**) completely but produced relatively smaller amounts of 7*R*- and 7*S*-paeonimetaboline I (less than 25 %), compared with those from younger subjects. Since the intestinal flora in adults were reported to be influenced by age,[14)] the above observation suggests that some changes in bacterial species and population capable of transforming paeoniflorin (**1**) to paeonimetabolines are accompanied by age. However, further studies will be necessary to clarify the relationship between the metabolic activities and changes in the intestinal flora for different aged-individuals.

Acknowledgements

Partial financial support for this study from Tsumura Juntendo Co., Ltd. (Tokyo) is acknowledged.

和文抄録

芍薬成分 paeoniflorin はヒト腸内分離菌株と 4 時間培養することにより paeonimetaboline I の 7*R* と 7*S* の立体異性体の混合物に変換された。その異性体の生成比は ^{1}H-NMR スペクトルから求めた。*Bacteroides, Bifidobacterium, Clostridium, Lactobacillus, Streptococcus* の一部の菌株に paeoniflorin から paeonimetaboline I への強い代謝活性が認められた。*Bacteroides fragilis* ss. *thetaotus* は優先的に 7*S* 体, *Lactobacillus xylosus* 及び *L. acidophilus* は優先的に7*R*体を生成したが, 他の菌株はほぼ等量の 7*R*, 7*S* 体を生成した。15名の健常人に由来する糞便懸濁液も強い代謝活性を示し, 優先的に 7*S*-paeonimetaboline I を生成した。

References and Notes

1) A part of this study was presented at the 33rd Annual Meeting of the Pharmacognostical Society of Japan, Saitama, 1986, Abstr. p. 80

2) "Human Intestinal Flora" (Eds. by B.S. Drasar and M. J. Hill), Academic Press Inc., London, pp. 53-167, 1974

3) "Human Intestinal Microflora in Health and Disease" (Ed. by D.J. Hentges), Academic Press Inc., New York, pp. 189-263, 1983

4) Hattori, M., Kim, G., Motoike, S., Kobashi, K. and Namba, T. : Metabolism of sennosides by intestinal flora. *Chem. Pharm. Bull.* **30**, 1338-1346, 1982

5) Hattori, M., Sakamoto, T., Yamagishi, T., Sakamoto, K., Konishi, K., Kobashi, K. and Namba, T. : Metabolism of glycyrrhizin by human intestinal flora. II. Isolation and characterization of human intestinal bacteria capable of metabolizing glycyrrhizin and related compounds. *Chem. Pharm. Bull.* **33**, 210-217, 1985

6) Akao, T., Akao, T., Mibu, K., Hattori, M., Namba, T : and Kobashi, K. : Enzymatic reduction of sennidin and sennoside in *Peptostreptococcus intermedius*. *J. Pharmacobio-Dyn.* **8**, 800-817, 1985

7) Hattori, M., Endo, Y., Takebe, S., Kobashi, K., Fukasaku, N. and Namba, T. : Metabolism of magnolol from Magnoliae Cortex. II. Absorption, metabolism and excretion of (ring-^{14}C) magnolol in rats. *Chem. Pharm. Bull.* **34**, 158-167, 1986

8) Akao, T, Akao, T., Hattori, M., Namba, T. and Kobashi, K. : 3β-Hydroxysteroid dehydrogenase of *Ruminococcus* sp. from human intestinal bacteria. *J. Biochem.* **99**, 1425-1431, 1986

9) Hattori, M., Shu, Y.Z., Shimizu, M., Hayashi, T., Morita, N., Kobashi, K., Xu, G.J. and Namba, T. : Metabolism of paeoniflorin and related compounds by human intestinal bacteria. *Chem. Pharm. Bull.* **33**, 3838-3846, 1985

10) Shu, Y.Z., Hattori, M., Akao, T., Kobashi, K., Kagei, K., Fukuyama, K., Tsukihara, T. and Namba, T. : Metabolism of paeoniflorin and related compounds by human intestinal bacteria. II. Structure of 7*S*- and 7*R*-paeonimetabolines I and II formed by *Bacteroides fragilis* and *Lactobacillus brevis*. *Chem. Pharm. Bull.* **35**, 3726-3733, 1987

11) Hattori, M., Shu, Y.Z., Kobashi, K. and Namba, T. : Metabolism of albiflorin by human intestinal bacteria. *J. Med. Pharm. Soc. WAKAN-YAKU* **2**, 398-404, 1985

12) Shu, Y.Z., Hattori, M., Namba, T., Mibu, K., Akao, T. and Kobashi, K. : Abstracts of Papers, The 6th Symposium on the Development and Application of Naturally Occurring Drug Materials, Nagoya, July 1986, p. 49-51

13) Shu, Y.Z., Hattori, M., Namba, T., Mibu, K., Akao, T. and Kobashi, K. : Metabolic activation of components of oriental medicines by human intestinal flora. On paeoniflorin and albiflorin, constituents of peony roots. *J. Pharmacobio-Dyn.* **10**, s-58, 1987

14) "Human Intestinal Microflora in Health and Disease" (Ed. by D.J. Hentges), Academic Press Inc., New York, pp. 27-28, 1983