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METABOLISM OF HOMOORIENTIN BY HUMAN INTESTINAL BACTERIA

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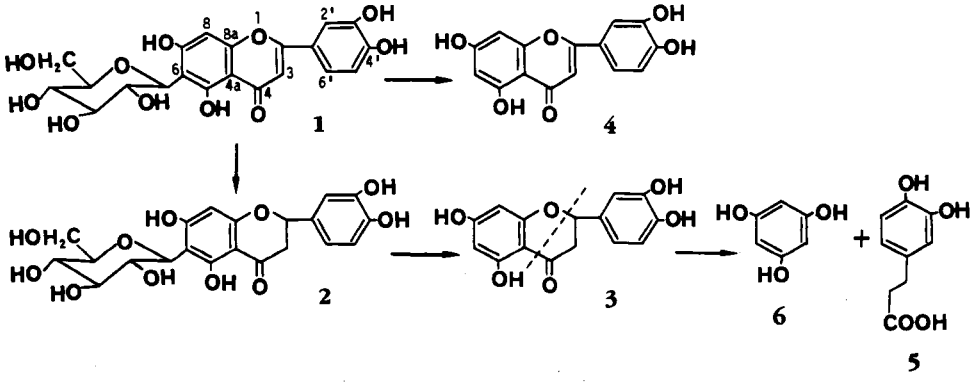
ABSTRACT.—As a part of our studies on the metabolism of bioactive compounds from oriental medicines by intestinal flora, homoorientin [**1**], a *C*-glycosylflavonoid, was anaerobically incubated with a human intestinal bacterial mixture. Homoorientin [**1**] was transformed to 6-*C*-glucosyleriodictyol [**2**], (±)-eriodictyol [**3**], luteolin [**4**], 3,4-dihydroxyphenylpropionic acid [**5**], and phloroglucinol [**6**]. A novel cleavage of the *C*-glycosyl bond was discovered for the first time by using intestinal bacteria.

A number of studies have been conducted on the metabolism of naturally occurring flavonoids, and recent studies are especially focused on the role of the intestinal flora in the metabolic transformation of flavonoids (1). Most flavonoids are reported to be metabolized to various phenolic acid derivatives through ring fission, and these transformations are mediated mainly by intestinal bacteria. However, only a few studies have been performed on the metabolism of *C*-glycosylflavonoids (2).

In the present study, we report the metabolism of a *C*-glycosylflavone, homoorientin [**1**] (isoorientin) by human intestinal bacteria.

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURES.—Mp's were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Specific rotations were measured with a Jasco DIP-4 automatic polarimeter. Uv spectra were taken on a Shimadzu UV-210A spectrophotometer and ir spectra with a Hitachi 260-10 ir spectrophotometer. ^{1}H- and ^{13}C-nmr spectra were measured with JEOL JNM-FX 270 (^{1}H, 270 MHz) and JEOL JNM-FX 90Q (^{1}H, 89.55 MHz; ^{13}C, 22.5 MHz) spectrometers, and chemical shifts are presented as δ values relative to the peak of TMS as an internal standard. Mass spectra were measured with a JEOL JMS D-200 mass spectrometer at an ionization voltage of 70 eV. Cd spectra were taken on a Jasco J-500 spectropolarimeter. For cc, Wakogel C-200 was used as an absorbent. Tlc was performed on Merck Kieselgel 60 F_{254} with solvent system A, $CHCl_3$-MeOH-HOAc-H_2O (18:5:0.75:0.5). Spots on



SCHEME 1. Metabolism of homoorientin [**1**] by human intestinal bacteria.

the plates were detected under uv lamp or by spraying with $FeCl_3$ reagent. Hplc was carried out on a Shimadzu LC-4A hplc system with a column of Develosil ODS-5 (4.5 mm i.d. × 15 cm, Nomura Chem. Co.) and with an SPD-MIA multi-channel spectrophotometric detector. Densitometric profiles were recorded on a Shimadzu CS-910 dual wavelength tlc scanner.

MATERIALS.—Homoorientin [**1**] was isolated from the dried herbs of *Swertia japonica* Makino (Gentianaceae), cultivated in Nagano Prefecture in Japan, according to the methods of Komatsu *et al.* (3). (2*S*)-Eriodictyol [**3**] and luteolin [**4**] were purchased from Carl Roth Inc. and phloroglucinol [**6**] from Wako Pure Chem. Co. 3,4-Dihydroxyphenylpropionic acid [**5**] was prepared by the catalytic reduction of 3,4-dihydroxycinnamic acid (Aldrich Chem. Co.) on 10% Pd/C. A dilution medium for an anaerobic incubation was prepared according to the procedures of Mitsuoka (4); the medium contains the following: 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 H_2O to give a final volume of 1000 ml.

PREPARATION OF AN INTESTINAL BACTERIAL MIXTURE.—Fresh feces obtained from a healthy man were thoroughly suspended in 30 volumes of the anaerobic dilution medium (4) by bubbling with oxygen-free CO_2 gas, then filtered through a piece of gauze to eliminate the residue. The fecal suspension thus obtained was used in the following experiments as an intestinal bacterial mixture.

INCUBATION OF HOMOORIENTIN [**1**] WITH AN INTESTINAL BACTERIAL MIXTURE.—Homoorientin [**1**] (400 mg) dissolved in DMSO (3 ml) was added to an intestinal bacterial mixture (420 ml). The mixture was incubated for 36 h at 37° in an anaerobic jar in which the air had been replaced with oxygen-free CO_2 in the presence of activated steel wool (steel wool method) (5). The mixture was adjusted to pH ca. 3 with 3 N HCl in an ice bath and extracted three times with EtOAc (700 ml each). The EtOAc phase was washed with H_2O, then concentrated in vacuo to give a residue (ca. 1 g). The residue was applied to a column of Si gel (90 g, 2.6 cm i.d. × 45 cm). The column was washed with C_6H_6 and eluted with C_6H_6-Et_2O (5:1 and 3:1) to afford fractions of metabolite **3** (ca. 50 mg) and metabolite **5** (5 mg), respectively. Elution with $CHCl_3$-MeOH (100:1 to 10:1) gave a fraction of metabolite **6** (13 mg). The crude metabolites **3** and **6** were further purified by repeated crystallization from C_6H_6/MeOH and MeOH/$CHCl_3$, respectively. The crude metabolite **5** was purified by preparative tlc.

Metabolite **3** *{(±)-eriodictyol}*.—Yield 38 mg (14.8%); colorless needles, mp 281–282° (decomp.); $[\alpha]^{25}D$ −1.6° (*c* = 0.7, EtOH); uv λ max (MeOH) nm 230, 289, λ sh 334; ir ν max (KBr) cm^{-1} 3360 (OH), 1640 (conjugated C=O), 1605 (Ar ring); ^1H nmr (270 MHz, acetone-d_6) δ 2.73 (1H, dd, *J* = 17.2, 3.0 Hz, 3-H_{cis}), 3.15 (1H, dd, *J* = 17.2, 12.7 Hz, 3-H_{trans}), 5.41 (1H, dd, *J* = 12.7, 3.0 Hz, H-2), 5.94 (1H, d, *J* = 2.1 Hz, H-6), 5.96 (1H, d, *J* = 2.1 Hz, H-8), 6.85 and 6.88 (each 1H, ABq, *J* = 7.0 Hz, H-5′,6′), 7.04 (1H, br s, H-2′), 8.50 (1H, br s, OH), 12.20 (1H, s, 5-OH); ^{13}C nmr (acetone-d_6) δ 43.0 (t, C-3), 79.3 (d, C-2), 95.2 (d, C-8), 96.2 (d, C-6), 102.7 (s, C-4a), 114.1 (d, C-2′), 115.4 (d, C-5′), 118.6 (d, C-6′), 131.0 (s, C-1′), 145.3 (s, C-3′), 145.7 (s, C-4′), 163.7 (s, C-8a), 164.7 (s, C-5), 166.7 (s, C-7), 196.5 (s, C-4); hrms found 288.0676, calcd for $[M]^+$, $C_{15}H_{12}O_6$, 288.0635; ms *m/z* $[M]^+$ 288, $[M - H_2O]^+$ 270, 179, 166, 153 (base peak), 136, 124, 110.

Metabolite **5** *(3,4-dihydroxyphenylpropionic acid)*.—Yield 2 mg (1.2%); uv λ max (EtOH) nm 224, 283, 340; ir ν max (KBr) cm^{-1} 3350 (OH), 1705 (COOH), 1600 (Ar ring), 1520 (Ar ring); ^1H nmr (90 MHz, CD_3OD) δ 2.30–2.90 (4H, m, H_2-2 and H_2-3), 6.48 (1H, dd, *J* = 7.0, 1.5 Hz, H-6′), 6.60 (1H, d, *J* = 1.5 Hz, H-2′), 6.64 (1H, d, *J* = 7.0 Hz, H-5′); ms *m/z* $[M]^+$ 182, 136, 123 (base peak), 77.

Metabolite **6** *(phloroglucinol)*.—Yield 7 mg (7%); white powder, mp 192–194°; uv λ max (EtOH) nm 226, 267, 273, λ sh 278; ir ν max (KBr) cm^{-1} 3180 (OH), 1615 (Ar ring), 1495 (Ar ring); ^1H nmr (90 MHz, CD_3OD) δ 5.82 (3H, br s, H-2,4,6); ^{13}C nmr (CD_3OD) δ 86.1 (d, C-2,4,6), 150.4 (s, C-1,3,5); ms *m/z* $[M]^+$ 126 (base peak).

TIME COURSE OF THE METABOLISM OF HOMOORIENTIN [**1**].—Tubes containing homoorientin [**1**] (5 mg) and an intestinal bacterial mixture (5 ml) were incubated at intervals at 37° in an anaerobic jar. The mixture was adjusted to pH ca. 3 and extracted successively with EtOAc and *n*-BuOH (5 ml each). The products were then roughly separated by preparative tlc with solvent system A, and the bands corresponding to the metabolites were extracted with MeOH (2 ml, twice). After filtration through a Millex-SR filter unit (0.5 μm, Millipore Corp.), the metabolites were quantitatively analyzed by hplc under the following conditions: mobile phase, MeOH-H_2O-HOAc-5%H_3PO_4 (33:67:2:0.2) for compounds **1** and **2**, and MeOH-H_2O-HOAc-5%H_3PO_4 (48:52:2:0.2) for compounds **3**–**6**; flow rate, 1.0 ml/min; detection, 280 nm. The calibration lines of the metabolites were prepared with authentic samples.

TIME COURSE OF THE METABOLISM OF ERIODICTYOL [**3**].—Tubes containing eriodictyol [**3**] (1.5 mg) and an intestinal bacterial mixture (3 ml) were anaerobically incubated at intervals at 37°. The mixture

was adjusted to pH ca. 3 and extracted twice EtOAc (3 ml each). The products were loaded onto a Si gel tlc plate, which was then developed with solvent system A. The metabolites were separated on the plate and quantitatively analyzed with a tlc scanner at 240 nm to a reference wavelength of 550 nm by using calibration lines of authentic samples. The calibration lines were linear in the range of 1–40 μg/spot.

RESULTS

Homoorientin [**1**] was anaerobically incubated with a bacterial suspension obtained from fresh human feces. Tlc of the metabolic mixture showed three spots with R_f values of 0.61 (metabolite **3**), 0.48 (metabolite **5**), and 0.37 (metabolite **6**) under uv light. These metabolites were isolated and purified by cc, preparative tlc, and repeated crystallization. The structures of metabolites **3**, **5**, and **6** were determined as below.

Metabolite **3**, colorless needles, mp 281–282° (decomp.). The chemical composition was determined to be $C_{15}H_{12}O_6$ by hrms. The ir spectrum showed strong absorption bands at 3360, 1640, and 1605 cm^{-1}, assignable to the stretching vibration of hydroxyl, conjugated carbonyl, and aromatic ring. The uv spectrum exhibited no intense absorption band due to a cinnamoyl system present in the original compound [**1**], but the λ max shifted bathochromically to 289 nm (band II) and the shoulder appeared at 334 nm (band I), indicating the presence of a flavanone skeleton (6). The ^{1}H-nmr spectrum showed no characteristic signals due to a glucose moiety and an olefinic proton (H-3) present in homoorientin [**1**], but showed ABX type signals at δ 2.73, 3.15, and 5.41 (H_A-3, H_B-3, and H_X-2, respectively, J_{AX}=3.0 Hz; J_{BX}=12.7 Hz, and J_{AB}=17.2 Hz) and two doublet signals at δ 5.94 (1H, J=2.1 Hz, H-6) and 5.96 (1H, J=2.1 Hz, H-8) as well as three aromatic protons indicating the B ring was intact. Based on the above findings, the structure of metabolite **3** was deduced as eriodictyol (5,7,3′,4′-tetrahydroxyflavanone). By a direct comparison of the spectroscopic data, including ^{13}C nmr and ms, with those of an authentic sample, metabolite **3** was identified to be eriodictyol. Since this metabolite showed no characteristic cd bands as observed in naturally occurring (2*S*)-eriodictyol (7), which exhibits positive and negative Cotton effects at 328 nm ([θ] +505) and 293 nm ([θ] −4175), metabolite **3** was concluded to be a racemate.

Metabolite **5** was detected on tlc as a minor metabolite giving a greenish-gray color by spraying with $FeCl_3$ reagent and identified as 3,4-dihydroxyphenylpropionic acid by comparing the spectroscopic data with those of an authentic sample.

Metabolite **6** was isolated as a white powder with *m/z* 126 $[M]^+$ in the ms. The ^{1}H-nmr spectrum showed only a broad singlet at δ 5.82 due to the magnetically equivalent aromatic protons. The structure of the metabolite was identified as phloroglucinol by direct spectroscopic comparisons with an authentic sample.

Other two minor metabolites **2** and **4** were detected with a three-dimensional hplc system (data not shown), and they were identified as 6-*C*-glucosyleriodictyol and luteolin, respectively, by comparison of uv spectra and retention times with authentic samples. Geissman and Clinton (8) reported the preparation of eriodictyol [**3**] from luteolin [**4**] by catalytic hydrogenation on Adam's catalyst. Under similar conditions only traces of the C-2 epimers of 6-*C*-glucosyleriodictyol [**2**] were formed from homoorientin [**1**].

Figure 1 shows the time course of metabolism of homoorientin [**1**] by human intestinal bacterial mixture. Homoorientin [**1**] was almost completely consumed one day after the start of incubation, and many metabolites were produced. 6-*C*-Glucosyleriodictyol [**2**] increased in amount in the first 6–12 h but disappeared at ca. 20 h, suggesting that the glycosyl [**2**] is a metabolic intermediate. Another metabolic intermediate, eriodictyol [**3**], increased progressively and reached a maximum concentration at 24 h, then decreased appreciably after prolonged incubation. Luteolin [**4**], the aglycone of homoorientin [**1**], was produced in relatively low yields during the incuba-

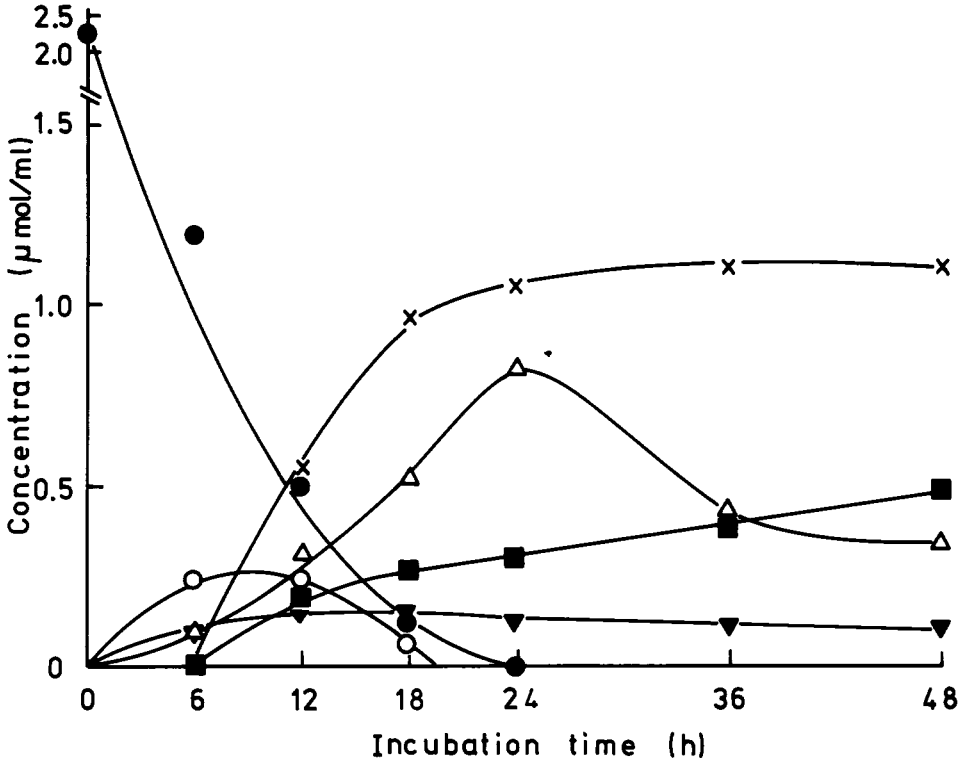


FIGURE 1. Metabolic time course of homoorientin [**1**] by human intestinal bacteria. Homoorientin [**1**] (●), 6-*C*-glucosyleriodictyol [**2**] (○), (±)-eriodictyol [**3**] (△), luteolin [**4**] (▼), 3,4-dihydroxyphenylpropoinic acid [**5**] (×), phloroglucinol [**6**] (■).

tion period, and 3,4-dihydroxyphenylpropionic acid [**5**] and phloroglucinol [**6**] were produced gradually after 6 h.

Next, eriodictyol [**3**] and luteolin [**4**], which were assumed to be metabolic intermediates as mentioned above, were anaerobically incubated with intestinal bacterial mixture under similar conditions. The extract from the incubation mixture of eriodictyol [**3**] showed two spots on tlc, and these metabolites were identical with 3,4-dihydroxyphenylpropionic acid [**5**] and phloroglucinol [**6**] by direct comparisons of the uv, ir, and ms spectra and R_f values with those of authentic samples.

Figure 2 shows the time course of metabolism of eriodictyol [**3**]. The substrate was completely converted to these two metabolites in 30 h. The concentrations of 3,4-dihydroxyphenylpropionic acid [**5**] exceeded those of phloroglucinol [**6**] during the incubation, suggesting that an A-ring part of eriodictyol [**3**] may be converted to other compounds or phloroglucinol [**6**] may be incorporated into bacterial cells.

However, luteolin [**4**] did not transform to any other metabolite under the conditions.

DISCUSSION

Homoorientin [**1**] obtained from a whole plant of *S. japonica*, a bitter stomachic in the Japanese folk medicine, has been reported to have antihepatotoxic activity in D-galactosamine-induced cytotoxicity by using primary culture of rat hepatocytes (9). However, no other studies including the metabolism of homoorientin [**1**] by intestinal bacteria have been performed so far.

By anaerobic incubation with human intestinal flora, homoorientin [**1**] was metabolized to 6-*C*-glucosyleriodictyol [**2**], eriodictyol [**3**], luteolin [**4**], 3,4-dihy-

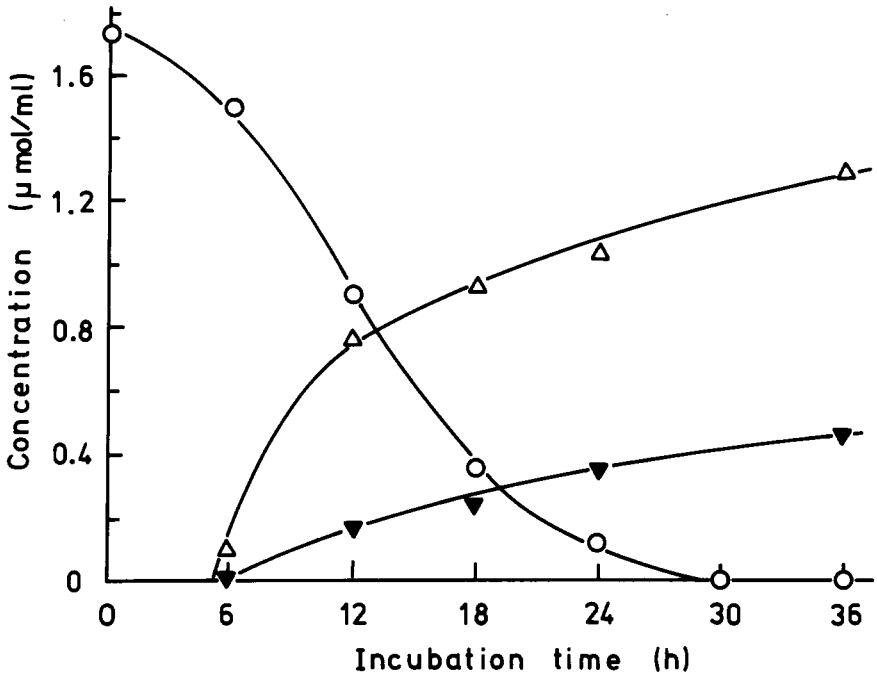


FIGURE 2. Metabolic time course of eriodictyol [**3**] by human intestinal bacteria. Eriodictyol [**3**] (○), 3,4-dihydroxyphenylpropionic acid [**5**] (△), phloroglucinol [**6**] (▼).

droxyphenylpropionic acid [**5**], and phloroglucinol [**6**]. Scheme 1 shows metabolic processes deduced from the present results; homoorientin [**1**] is reduced to a *C*-glycosylflavanone type intermediate, 6-*C*-glucosyleriodictyol [**2**]. The *C*-glycosyl bond is then cleaved to produce eriodictyol [**3**], which is subsequently converted to 3,4-dihydroxyphenylpropionic acid [**5**] and phloroglucinol [**6**] via ring fission. On the other hand, homoorientin [**1**] seems to be converted to luteolin [**4**] via direct cleavage of the *C*-glycosyl bond as a minor metabolic process. Booth *et al.* (10) reported that after oral administration of eryodictyol [**3**] to rats, *m*-hydroxyphenylpropionic acid was detected as a major metabolite in urine along with less amounts of *m*-coumaric acid and a glucuronide of eriodictyol by means of two-dimensional paper chromatography. However, these compounds were not formed by anaerobic incubation of eriodictyol [**1**] with human intestinal bacteria.

ACKNOWLEDGMENTS

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