Biotransformation of Pinoresinol Diglucoside to Mammalian Lignans by Human Intestinal Microflora, and Isolation of *Enterococcus faecalis* Strain PDG-1 Responsible for the Transformation of (+)-Pinoresinol to (+)-Lariciresinol

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By anaerobic incubation of pinoresinol diglucoside (1) from the bark of *Eucommia ulmoides* with a fecal suspension of humans, eleven metabolites were formed, and their structures were identified as (+)-pinoresinol (2), (+)-lariciresinol (3), 3′-demethyl-(+)-lariciresinol (4), (−)-secoisolariciresinol (5), (−)-3-(3″,4″-dihydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl)butane-1,4-diol (6), 2-(3′,4′-dihydroxybenzyl)-3-(3″,4″-dihydroxybenzyl)butane-1,4-diol (7), 3-(3″-hydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl)butane-1,4-diol (8), 2-(3′,4′-dihydroxybenzyl)-3-(3″-hydroxybenzyl)butane-1,4-diol (9), (−)-enterodiol (10), (−)-(2*R*,3*R*)-3-(3″,4″-dihydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl)butyrolactone (11), (−)-(2*R*,3*R*)-2-(3′,4′-dihydroxybenzyl)-3-(3″,4″-dihydroxybenzyl)butyrolactone (12), (−)-(2*R*,3*R*)-3-(3″-hydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl)butyrolactone (13), 2-(3′,4′-dihydroxybenzyl)-3-(3″-hydroxybenzyl)butyrolactone (14), 2-(3′-hydroxybenzyl)-3-(3″,4″-dihydroxybenzyl)butyrolactone (15) and (−)-(2*R*,3*R*)-enterolactone (16) by various spectroscopic means, including two dimensional (2D)-NMR, mass spectrometry and circular dichroism. A possible metabolic pathway was proposed on the basis of their structures and time course experiments monitored by thin-layer chromatography. Furthermore, a bacterial strain responsible for the transformation of (+)-pinoresinol to (+)-lariciresinol was isolated from a human fecal suspension and identified as *Enterococcus faecalis* strain PDG-1.

Key words pinoresinol diglucoside; (+)-pinoresinol; (+)-lariciresinol; enterolactone; human intestinal microflora; *Enterococcus faecalis*

Asian people who are vegetarian or semi-vegetarian show a low incidence of and mortality from hormone-dependent diseases, such as breast and prostate cancers, compared with Western people, whose diets are rich in animal protein and fats.[1] Consumption of fiber-rich whole grain food elevates a serum mammalian lignan, enterolactone (ENL).[2,3] Furthermore, both very low and very high plasma concentrations of ENL are associated with an increased breast cancer risk,[4] and urinary excretion of mammalian lignans [enterodiol (END) and ENL] is low in patients with breast cancer.[5,6] All these findings suggest a close relationship between vegetarian diet, mammalian lignans and hormone-dependent diseases. The origins of END and ENL found in human biological fluids and in feces are plant lignans contained in fibre-rich whole grain food, which are transformed by intestinal microflora in the proximal colon.[7,8]

Until recently, secoisolariciresinol and matairesinol are the only known precursors of END and ENL.[7—9] Pinoresinol, lariciresinol, syringaresinol, 7-hydoxymatairesinol and arctigenin are newly identified enterolactone precursors,[10,11] and a possible metabolic pathway for pinoresinol (formation of mammalian lignans *via* lariciresinol and secoisolariciresinol) was proposed based on the biosynthetic pathway of secoisolariciresinol and matairesinol in plants.[10] Pinoresinol diglucoside is a major antihypertensive principle of Tu-Chung (the bark of *Eucommia ulmoides*), used in traditional Chinese medicine,[12] and pinoresinol is contained in cereals, particularly in whole-grain rye products,[1] olive oil,[13] and in various *Picea*, *Pinus* and *Abies etc*.[14] The present study was designed for a better understanding of the metabolism of pinoresinol diglucoside by human intestinal microflora, as well as isolation of the bacterial strains responsible for the respective reactions in the transformation.

Results

Transformation of Pinoresinol Diglucoside (PDG, 1) by a Human Intestinal Bacterial Mixture After anaerobic incubation of pinoresinol diglucoside (**1**) with a bacterial mixture of human feces, the culture was extracted with *n*-BuOH and the extract was subjected to Diaion HP-20, Sephadex LH-20, preparative thin-layer chromatography (TLC) and RP-18 column chromatography. Fifteen metabolites (**2**—**16**) were isolated and identified by electron impact mass (EI-MS), one dimensional (1D) and 2D-NMR, and circular dichroic (CD) spectroscopy.

Compound **2** was detected as a major metabolite after 6 h of incubation of **1** with a human fecal suspension. The $[\alpha]_D^{25}$ value measured in MeOH was +69°. The ^{1}H- and ^{13}C-NMR spectra (Table 1) were in good agreement with those reported for (+)-pinoresinol, bearing a (7*S*,7′*S*,8*S*,8′*S*)-configuration (Fig. 1).[15,16]

Compound **3** was a second metabolite when the reaction mixture was monitored by TLC. Its molecular ion peak (*m*/*z* 360 $[M]^+$) in the EI-MS spectrum was 2 mass units (2H) higher than that of **2**, suggesting that **3** is a reduced product of **2**. The $[\alpha]_D^{25}$ value in MeOH was +30°.[17] Compound **3** was determined to be (+)-lariciresinol, the assignment of each proton and carbon signal being confirmed by ^{1}H-detected multiple quantum coherence (HMQC) and heteronuclear multiple-bond coherence (HMBC) experiments (Table 1, Fig. 2).

The molecular ion peak of compound **4** at *m*/*z* 346 in the

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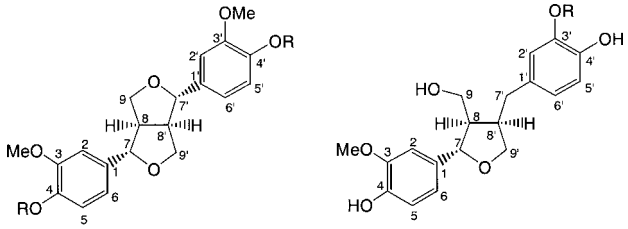
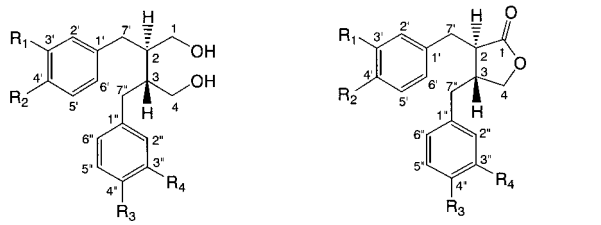
EI-MS spectrum was 14 mass units (CH_2) less than that of **3**, indicating that **4** is a demethylation product of **3**. This was supported by its ^{1}H- and ^{13}C-NMR (Table 1): in contrast to two methoxy groups in **3**, only one signal of the methoxy group was observed in **4**. Furthermore, a signal of C-3′ was shifted upfield by 2.7 ppm, while that of C-2′ was shifted downfield by 3.3 ppm, indicating that demethylation had taken place at C-3′. This was further proven by the HMBC experiment (Fig. 2), in which a signal of H-7′ was correlated to signals of C-1′, C-2′ and C-6′, and a signal of H-5′ to signals of C-1′, C-3′ and C-4′. The methoxy group was concluded to be located at C-3, since HMBC correlations were observed between signals of methoxy protons and C-3, between signals of H-5 and C-3 or C-1, between signals of H-2 and C-4 or C-6, and between signals of H-7 and C-2 or C-6. Both the optical rotation and the proton signals (including



		R
1	PDG	Glc
2	(+)-Pinoresinol	H

		R
3	(+)-Lariciresinol	CH_3
4		H



	R_1	R_2	R_3	R_4
5	OCH_3	OH	OH	OCH_3
6	OCH_3	OH	OH	OH
7	OH	OH	OH	OH
8	OCH_3	OH	H	OH
9	OH	OH	H	OH
10	OH	H	H	OH

	R_1	R_2	R_3	R_4
11	OCH_3	OH	OH	OH
12	OH	OH	OH	OH
13	OCH_3	OH	H	OH
14	OH	OH	H	OH
15	OH	H	OH	OH
16	OH	H	H	OH

Fig. 1. Structures of Pinoresinol Diglucoside (**1**) and Its Metabolites (**2**—**16**)

Table 1. ^{13}C-NMR (100 MHz) Spectral Data of Compounds **2**—**4**

C	**2**	**3**	**4**
1	133.8	135.8	135.9
2	111.0	110.7	110.7
3	149.1	149.0	149.0
4	147.3	147.1	147.0
5	116.1	116.0	116.0
6	120.1	119.8	119.8
7	87.5	84.1	84.1
8	55.4	54.0	53.9
9	72.6	60.5	60.5
1′	133.8	133.6	133.6
2′	111.0	113.5	116.8
3′	149.1	149.0	146.3
4′	147.3	145.8	144.6
5′	116.1	116.2	116.4
6′	120.1	122.2	121.0
7′	87.5	33.7	33.4
8′	55.4	43.9	43.7
9′	72.6	73.5	73.6
-Me	56.4×2	56.4×2	56.4

Chemical shifts in δ ppm, measured in MeOH-d_4.

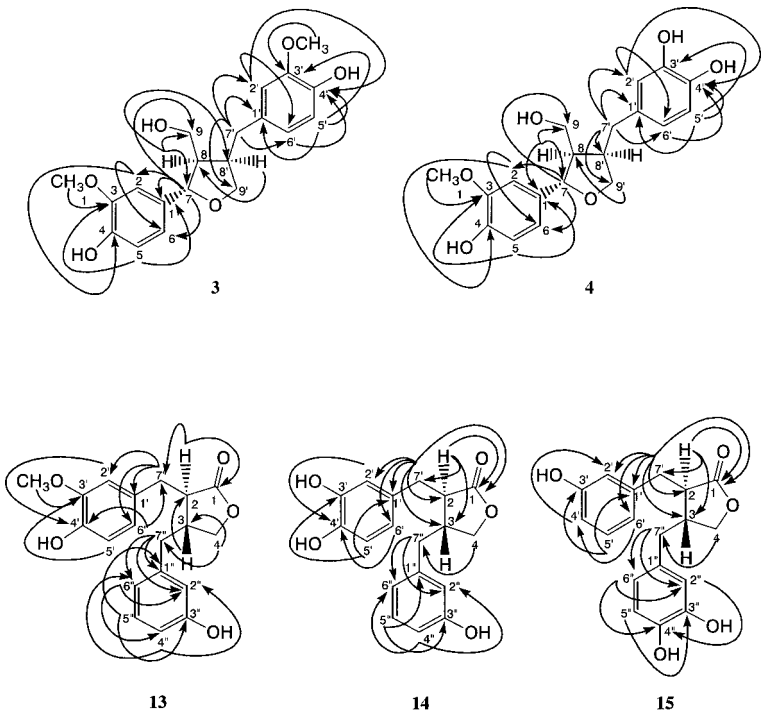


Fig. 2. HMBC Correlations of Compounds **3**, **4**, **13**, **14**, and **15**

Table 2. ^{13}C-NMR (100 MHz) Spectral Data of Compounds **5**—**10**

C	5	6	7	8	9	10
1	62.1	62.1	61.9	62.0	61.9	61.8
2	44.1	44.2	44.7	44.3	44.7	44.5
3	44.1	44.5	44.7	44.3	44.5	44.5
4	62.1	62.0	61.9	61.9	61.8	61.8
1′	133.9	133.9	133.9	133.8	133.9	144.0
2′	113.4	113.5	117.2	113.5	117.2	117.0
3′	148.8	148.8	146.3	148.8	146.1	158.3
4′	145.5	145.5	144.5	145.5	144.3	113.8
5′	115.8	115.9	116.2	115.9	116.2	130.2
6′	122.7	122.7	121.3	122.6	121.4	121.4
7′	36.0	36.0	35.7	35.9	35.6	36.2
1″	133.9	134.0	133.9	144.0	144.0	144.0
2″	113.4	117.3	117.2	117.0	117.0	117.0
3″	148.8	146.0	146.3	158.3	158.3	158.3
4″	145.5	144.2	144.5	113.7	113.8	113.8
5″	115.8	116.2	116.2	130.2	130.2	130.2
6″	122.7	121.4	121.3	121.5	121.5	121.4
7″	36.0	35.7	35.7	36.3	36.3	36.2
-Me	56.2×2	56.3		56.3		

Chemical shifts in δ ppm, measured in MeOH-d_4.

Table 3. ^{13}C-NMR (100 MHz) Spectral Data of Compounds **11**—**16**

C	11	12	13	14	15	16
1	181.6	181.6	181.4	181.4	181.4	181.2
2	47.9	47.8	47.9	47.8	47.5	47.5
3	42.4	42.6	42.2	42.4	42.9	42.6
4	72.8	72.8	72.8	72.7	72.8	72.7
1′	130.7	130.8	130.6	130.8	140.9	140.9
2′	114.0	117.5	114.0	116.6	117.3	117.3
3′	149.0	146.4	149.0	146.4	158.7	158.7
4′	146.4	145.2	146.4	145.2	114.7	114.8
5′	116.2	116.4	116.2	116.4	130.6	130.6
6′	123.1	121.9	123.1	121.9	121.7	121.7
7′	35.2	35.2	35.2	35.2	35.8	35.8
1″	131.5	131.5	141.5	141.5	131.4	141.4
2″	116.9	116.8	116.7	117.6	116.8	116.6
3″	146.4	146.4	158.7	158.7	146.4	158.7
4″	145.0	145.0	114.5	114.5	145.0	114.5
5″	116.4	116.5	130.6	130.6	116.5	130.7
6″	121.1	121.1	121.0	121.0	121.0	120.9
7″	38.7	38.6	39.3	39.2	38.6	39.2
-Me	56.4					

Chemical shifts in δ ppm, measured in MeOH-d_4.

chemical shifts and coupling constants) due to the furan ring in the ^{1}H-NMR spectrum were similar to those of (+)-lariciresinal (**3**), suggesting a similar stereochemistry at C-7, C-8 and C-8′. The structure of **4** was consequently determined to be 3′-demethyl-(+)-lariciresinol.

The ^{1}H- and ^{13}C-NMR (Table 2) data of compound **5** agreed well with those reported for secoisolariciresinol.[18] The structure of **5** was identified as (−)-secoisolariciresinol (Fig. 1), since the minus optical rotation is diagnostic for (2*R*,3*R*)-secoisolarisiresinol.[19] In the CD spectrum, two negative Cotton effects at around 228 and 289 nm were observed for **5**, in contrast with the two positive Cotton effects of (+)-secoisolariciresinol at the same wavelengths.

The ^{1}H- and ^{13}C-NMR (Table 2) data of compound **6** were in good agreement with those reported for 3-demethylsecoisolariciresinol.[9] Similar to **5**, compound **6** also showed a minus optical rotation and two negative Cotton effects at around 229 and 281 nm in the CD spectrum. The structure of **6** was thus determined to be (−)-3-(3″,4″-dihydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl)butane-1,4-diol (Fig.1).

Compound **7** was deduced to be a demethylation product of **6**, as its molecular weight (*m*/*z* 334 $[M]^+$) was 14 mass units (CH_2) less than that of **6**, and no signal assignable to a methoxy group was observed in its ^{1}H- and ^{13}C-NMR (Table 2) spectra. Its NMR data agreed well with those of an intermediate compound in the transformation of secoisolariciresinol to enterodiol and enterolactone.[9] Therefore, **7** was identified as 2-(3′,4′-dihydroxybenzyl)-3-(3″,4″-dihydroxybenzyl)butane-1,4-diol.

Compound **8** was determined as a dehydroxylation product of **7**. Its molecular ion peak at *m*/*z* 332 $[M]^+$ in the EI-MS spectrum was 16 mass units less than that of **7**. The ^{1}H- and ^{13}C-NMR (Table 2) spectra were in good agreement with those of a metabolite reported by Wang *et al.*[9] Compound **8** was consequently determined to be 3-(3″-hydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl)butane-1,4-diol.

The ^{1}H- and ^{13}C-NMR (Table 2) spectra of **9** were similar to those of 2-(3′-hydroxybenzyl)-3-(3″,4″-dihydroxybenzyl)butane-1,4-diol, a precursor of enterodiol, as reported by Wang *et al.*[9] The structure of **9** was finally determined to be 2-(3′,4′-dihydroxybenzyl)-3-(3″-hydroxybenzyl)butane-1,4-diol on the basis of the HMQC and HMBC experiments.

Compound **10** was identified as (−)-END by comparing its spectral data with those reported.[9,20]

Compound **11** showed a molecular ion peak at *m*/*z* 344 $[M]^+$, 4 mass units (4H) less than that of **6**. In contrast to 7 carbon signals in the high field and no signals at above δ 150 in the ^{13}C-NMR spectrum of **6**, only 6 carbon signals were detected in the high field in that of **11**, while a carbonyl signal appeared at δ 181.6. Compound **11** was determined to be an oxidized product of **6**, its proton and carbon signals being well assigned by HMQC and HMBC experiments. The methoxy group was assigned at C-3 on the basis of the HMBC experiment; the following correlations were observed between signals of methoxy protons and C-3′; H-5′ and C-3′ or C-1′; H-7′ and C-1′, C-6′, C-1 or C-2. Similar to those of 2*R*,3*R*-dibenzylbutyrolactones,[11,21] compound **11** showed a minus optical rotation, indicating a (2*R*,3*R*) configuration. The structure of **11** was consequently determined to be (−)-(2*R*,3*R*)-3-(3″,4″-dihydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl)butyrolactone. This compound was previously obtained through the demethylation of (−)-matairesinol by $AlCl_3$/pyridine in CH_2Cl_2.[22]

The molecular ion peak of compound **12** at *m*/*z* 330 $[M]^+$, 14 mass units (CH_2) less than that of **11**, suggested it is a demethylation product of **11**. This was confirmed by its ^{1}H- and ^{13}C-NMR (Table 3) spectra, in which no signal assignable to a methoxy group was detected, and a signal of C-3′ was shifted 2.6 ppm upfield, while that of C-2′ was shifted 3.5 ppm downfield. The HMQC and HMBC experiments supported that **11** was a dibenzylbutyrolactone possessing four hydroxy groups at C-3′, C-4′, C-3″ and C-4″. The two negative Cotton effects at around 230 and 280 nm in the CD spectrum indicated a (2*R*,3*R*)-configuration in dibenzylbutyrolactones.[21] Therefore, **12** was determined to be (−)-(2*R*,3*R*)-2-(3′,4′-dihydroxybenzyl)-3-(3″,4″-dihydroxybenzyl)butyrolactone, which was previously obtained by demethylation of (−)-matairesinol.[22]

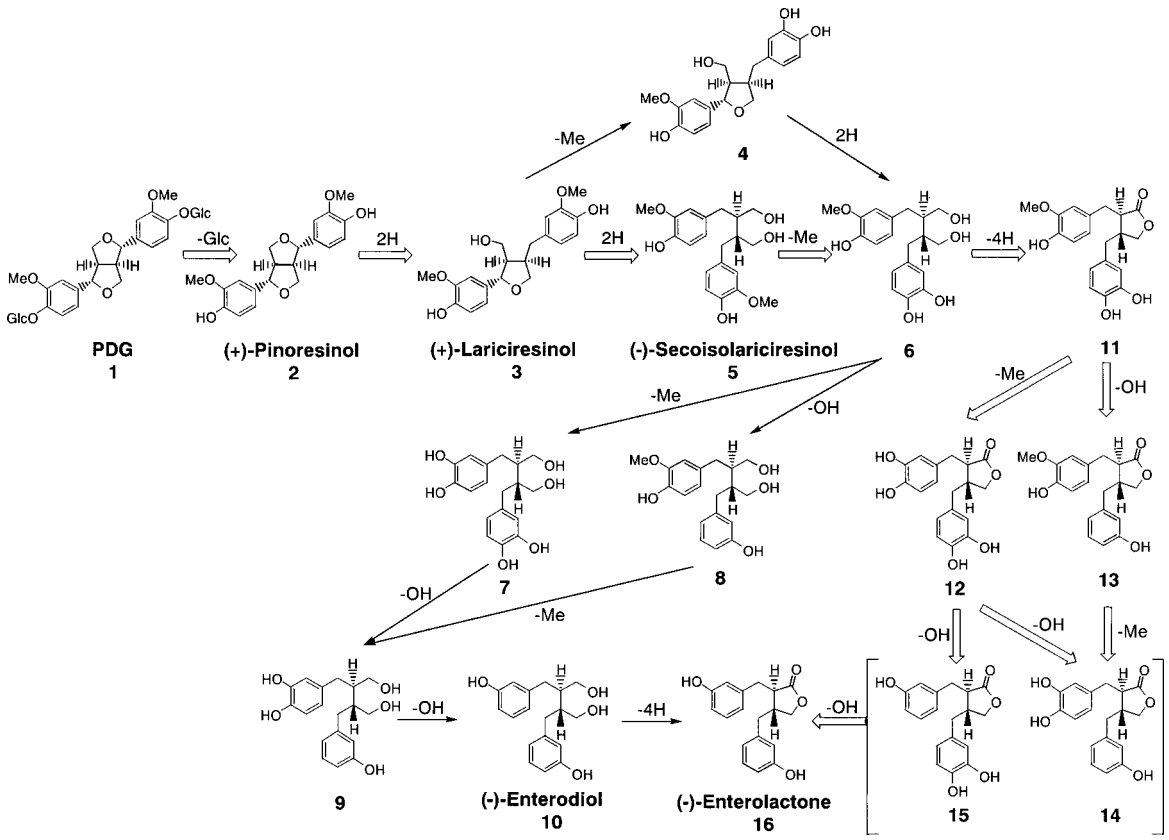


Fig. 3. Possible Pathway for the Transformation of Pinoresinol Diglucoside (**1**) by Human Intestinal Microflora

⇒ Major pathway; → Minor pathway
Conversions: **7**→**12**; **8**→**13**; **9**→**14** or **15** are also considered to be possible.

Compound **13**, EI-MS *m/z*: 328 $[M]^+$, was a deduced to be a dehydroxylation product of **11**, as carbon NMR signals due to a 4′-hydroxy-3′-methoxybenzyl group were similar to those of **11**, while the signal of C-4″ was shifted 30.5 ppm upfield, and signals of C-3″, C-5″ and C-1″ were shifted downfield by 14.2, 12.3 and 10.0 ppm, respectively, suggesting that the dehydroxylation had taken place at the 4″ position. This was confirmed by the HMBC experiment, in which there were correlations between signals of H-4″ and C-2″ or C-6″, of H-5″ and C-1″ or C-3″, and of H-7″ and C-2″ or C-6″. The HMBC experiment also proved that a methoxy group was located at the C-3′ position on the basis of correlations between signals of the methoxy protons and C-3′, of H-5′ to C-3′ or C-1′, and of H-7′ to C-1′ and C-1. Like **11** and **12**, compound **13** also showed a minus optical rotation. Therefore, the structure of **13** was concluded to be (−)-(2*R*,3*R*)-3-(3″-hydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl)butyrolactone (Fig. 2).

Compounds **14** and **15** were obtained as an isomeric mixture with a ratio of about 45 : 55, and were not satisfactorily separated, even by HPLC. The EI-MS spectrum showed a molecular ion peak at *m/z* 314 $[M]^+$, 16 mass units less than that of **12**, suggesting that both compounds are a mixture of two dehydroxylation isomers from **12**. Compounds **14** and **15** were identified as 2-(3′,4′-dihydroxybenzyl)-3-(3″-hydroxybenzyl) butyrolactone and 2-(3′-hydroxybenzyl)-3-(3″,4″-dihydroxybenzyl) butyrolactone, respectively, in which the latter was identical to one of the metabolites of (−)-arctigenin by human intestinal bacteria.[11] The proton and carbon signals of **14** and **15**, respectively, were assigned by the HMBC experiment (Fig. 2). Both compounds having a (2*S*,3*S*)-configuration were reported as oxidative metabolites of enterolactone by human liver microsomes.[23]

Compound **16** was identified as ENL, a final metabolite of PDG after a week of incubation with human intestinal microflora. It showed two negative Cotton effects at 220 and 277 nm in the CD spectrum. Therefore, compound **16** was determined to be (−)-(2*R*,3*R*)-enterolactone. A compound having the same configuration was obtained as the final metabolite of (−)-arctigenin.[11]

A Possible Transformation Pathway of PDG by Human Intestinal Microflora Based on the structures of metabolites of PDG and the time course experiments monitored by TLC, a possible metabolic pathway was proposed (Fig. 3). As metabolites **4** and **7**—**10** were isolated in quite small yields compared with other metabolites, and they were not well detected during the time course experiments, we speculated that the major pathway may be that indicated with a bold arrow in Fig. 3, while the minor pathway is shown with a fine arrow.

Isolation of a Bacterial Strain Capable of Transforming (+)-Pinoresinol (2) to (+)-Lariciresinol (3) from a Human Fecal Suspension After repeated culture of a human fecal flora in GAM broth containing 0.5 mM compound **2**, and screening of colonies on BL agar plates for (+)-pinoresinol-metabolizing activity, a bacterial strain (strain PDG-1) capable of transforming **2** to **3** was isolated. Strain PDG-1 was a facultative anaerobe, but its growth was

Table 4. Comparative Biochemical Characteristics of Strain PDG-1, *Enterococcus faecalis* and *Enterococcus faecium*

Characteristics	Strain PDG-1	*E. faecalis*[a)]	*E. faecium*[a)]
Motile	−	−	−
Hemolysis	−	−	α
Growth in 0.1% methylene blue milk	+	+	+
Growth in 0.1% thallous acetate	+	+	+
Growth in 0.02% sodium azide	+		
Reduction of tetrazolium	+	+	−
Reduction of potassium tellurite	−	+	−
Ammonia from arginine	+	+	+
Starch hydrolysis	−		
Hippurate hydrolysis	+	+	+
Esculin hydrolysis	+	+	+
Acid from L-arabinose	+	−	+
Acid from D-arabinose	−		+
Acid from arbutin	+	−	+
Acid from melezitose	+	+	−
Acid from melibiose	−	−	+
Acid from sorbitol	±	+	−
Acid from L-sorbose	+	−?	+?
Acid from amygdalin	+	+	+
Acid from cellobiose	+	+	+
Acid from D-fructose	+	+?	+?
Acid from galactose	+	+	+
Acid from D-glucose	+	+	+
Acid from glycerol	±	+	+
Acid from lactose	+	+	+
Acid from maltose	+	+	+
Acid from D-mannose	+	+?	+?
Acid from mannitol	+	+	+
Acid from ribose	+	+	+
Acid from salicin	+	+	+
Acid from sucrose	±	+	+
Acid from erythritol	−	−	−

a) Data of "Bergey's Manual of Systematic Bacteriology".[24)] Symbols: +, positive (pH 5.0 or below); −, negative (pH 5.5 or above); ±, weak (pH, 5.0—5.5); α, α-hemolysis on blood agar; ?, D- or L- not mentioned.

slower under aerobic conditions than under anaerobic conditions. Colonies on BL agar plates were circular, smooth and cream and Gram positive. Cells were ovoid, 0.5—1 μm in diameter, mostly in pairs or short chains under an electron microscope. The predominant product of glucose fermentation was lactic acid. Strain PDG-1 could initiate growth under critical conditions such as 10 °C and 45 °C, 6.5% NaCl, pH 9.6 and 40% bile. It also survived by heating at 60 °C for 30 min. Based on these characteristics, strain PDG-1 was assigned to be the genus *Enterococcus*, where the genus of enterococci is classified into four species, *Enterococcus faecalis*, *E. faecium*, *E. avium* and *E. gallinarum*.[23)] Among them, *E. faecalis* and *E. faecium* are residents of the intestinal tracts of humans and most animals. The distinguishing features of strain PDG-1 and the two species are shown in Table 4. Strain PDG-1 fermented melezitose but could not ferment melibiose. Furthermore, it was able to reduce tetrazolium, showing obscure-dull-red colonies on an EF agar plate (*E. faecium* shows yellow colonies). Since characteristics of strain PDG-1 are quite similar to those of *E. faecalis*, the bacterial strain was named *Enterococcus faecalis* strain PDG-1.[23)] After anaerobic incubation of (+)-pinoresinol

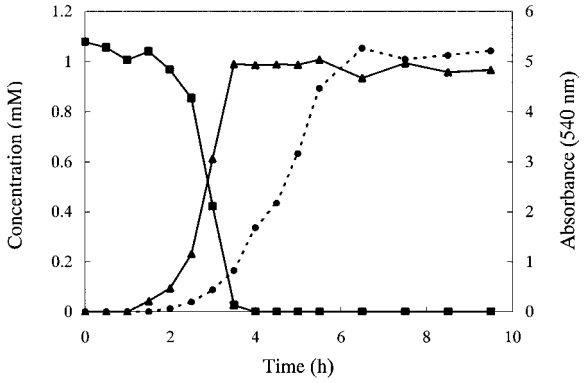


Fig. 4. Time Course of the Transformation of (+)-Pinoresinol (**2**, ■) to (+)-Lariciresinol (**3**, ▲) by *Enterococcus faecalis* Strain PDG-1

Bacterial growth (·····) was monitored by measuring turbidity at 540 nm.

(35 mg) with strain PDG-1 in 200 ml GAM broth for 24 h, a metabolite was isolated in a yield of 26 mg by repeated column chromatography, and the metabolite was identified as (+)-lariciresinol by direct comparison of its optical rotation value and proton NMR data with those of an authentic sample of **3**.

Transformation of (+)-Pinoresinol (2) to (+)-Lariciresinol (3) by *Enterococcus faecalis* Strain PDG-1 The isolated *E. faecalis* strain PDG-1 showed potent ability to transform **2** to **3**. Figure 4 shows the time courses of the formation of **3**, consumption of **2** and the bacterial growth in GAM broth. Compound **3** was formed starting from 1.5 h incubation, accompanied by the consumption of **2** and bacterial growth. After 4 h incubation, **2** was not detected anymore, while **3** reached the maximum concentration. The time course experiment clearly showed a correlation between bacterial growth and transforming activity, and confirmed that the bacterial strain PDG-1 is responsible for the transformation of **2** to **3**, but not for the transformation of **3** to (−)-secoisolariciresinol (**5**).

Discussion

At least seven plant lignans are now thought to be precursors of mammalian lignans, END and ENL.[10)] These precursors may be divided into four types: dibenzylbutanes (secoisolariciresinol), dibenzylbutyrolactones (matairesinol, 7-hydroxymatairesinol and arctigenin), furano lignans (lariciresinol) and furofuran lignans (pinoresinol and syringaresinol). Of these plant lignans, secoisolariciresinol diglucoside (SDG) and arctiin (the glucoside of arctigenin) are the only two, of which the transformation pathways by human intestinal microflora were extensively studied.[9,11)]

The transformation of SDG by human intestinal microflora includes four types of reactions, hydrolysis of glucoside, demethylation of a methoxy group, elimination of a 4-hydroxy group in the 3,4-dihydroxyphenyl moiety, and oxidation of dibenzylbutanediol to dibenzylbutyrolactone.[9)] Meanwhile, the transformation of arctiin, already bearing a butyrolactone structure, consists of the first three types of reactions as mentioned above for SDG.[11)] The present study of PDG (Fig. 1) may give us a much clearer understanding of the bacterial biotransformation of plant lignans to mammalian lignans, since the conversion passes through all three other types of precursors (compounds **3** and **4** are furano lignans,

compounds **5** and **6** are dibenzylbutanes, and compound **11** is a dibenzylbutyrolactone). Five types of reactions were involved in the conversion of PDG to END and ENL: reductive cleavage of furofuran rings, in addition to those reactions mentioned for the transformation of SDG.

The transformations of PDG, SDG and arctiin by human intestinal microflora show some similarities, and some of the reactions are quite specific in contrast with chemical reactions. First, hydrolysis of lignan glucosides to their aglycones is the first step of transformation, and it is generally finished within 12 h (under the conditions used in our experiments).[9,11] Second, demethylation of a methoxy group adjacent to a hydroxy group easily takes place, compared with that of two vicinal methoxy groups.[9,11] Third, dehydroxylation always takes place after a 4-hydroxy-3-methoxyphenyl moiety has been demethylated to a 3,4-dihydroxyphenyl moiety, and a 4-hydroxy group is eliminated to leave another 3-hydroxy group intact, which is resistant to further dehydroxylation.[9,11] Fourth, the oxidation of dibenzylbutanediols to dibenzylbutyrolactone may occur at each step, such as **6** to **11**, **7** to **12**, **8** to **13**, **9** to **14** or **15**, and **10** to **16**, in which the oxidation seems to proceed with no change in the absolute configuration [(−)-(2*R*,3*R*)-enterodiol to (−)-(2*R*,3*R*)-enterolactone, (+)-(2*S*,3*S*)-enterodiol to (+)-(2*S*,3*S*)-enterolactone].[9,11] Last, the reductive cleavage of the furofuran rings, **2** to **5** *via* **3** (furofurans to dibenzylbutanes *via* furans), proceeds with the retention of stereochemistry at C-8 and C-8′ in lariciresinol or at C-2 and C-3 in secoisolariciresinol, as shown in the case of (+)-pinoresinol to (−)-secoisolariciresinol *via* (+)-lariciresinol (Fig. 3). A similar transformation has been reported in the biosynthesis of plant lignans in *Forsythia intermedia* and *Thuja plicata* (western red cedar).[25—27]

In the transformations of PDG, SDG and arctiin by human intestinal microflora, the absolute configuration at the adjoining positions of two phenylpropanol moieties seems to be retained; PDG and arctiin give (−)-ENL, while SDG gives (+)-ENL.[9,11]

Furthermore, a bacterial strain responsible for the transformation of (+)-pinoresinol to (+)-lariciresinol was isolated from a human fecal flora and characterized as *Enterococcus faecalis* strain PDG-1. Unlike plant enzymes,[25,27] this bacterial strain does not perform the further transformation of (+)-lariciresinol to (−)-secoisolariciresinol. *E. faecalis* strain PDG-1 showed high capability for transforming (+)-pinoresinol to (+)-lariciresinol, in which the conversion (starting a (+)-pinoresinol concentration at about 1 mM) was almost completed after 3.5 h of incubation, when the bacteria were still in the lag phase (16% of its maximum growth, Fig. 4). To our knowledge, this is the first example of reductive cleavage of an ether linkage of furofuran lignans by a defined bacterial strain. It is also worthwhile investigating the bacterial strains responsible for the conversion of (+)-lariciresinol to (−)-secoisolariciresinol.

Pinoresinol, lariciresinol and syringaresinol are present in cereals, and the amounts of them in rye bran are 10 to 50 times higher than those of secoisolariciresinol and matairesinol.[1,10] Therefore, the proposed metabolic pathway for pinoresinol diglucoside, from pinoresinol to END and ENL *via* lariciresinol and secoisolariciresinol, may take place in the gastrointestinal tract of most healthy persons consuming an Asian diet consisting of cereals.

Experimental

General An anaerobic incubator, EAN-140 (Tabai Co., Osaka, Japan), was used for incubation with intestinal bacteria. Melting points were measured on a Yanagimoto micro hot stage melting point apparatus. Optical rotations were measured with a DIP-360 automatic polarimeter (Jasco Co., Tokyo) at 25 °C, CD spectra with a JASCO J 805 spectropolarimeter (Jasco Co.), UV spectra with a Shimadzu UV-2200 spectrophotometer (Shimadzu Co., Kyoto), and IR spectra with a FT/IR-230 infrared spectrometer (Jasco Co.). EI-MS was measured with a JMS-GC mate mass spectrometer at an ionization voltage of 70 eV (JEOL Co., Akishima, Japan). ^{1}H- and ^{13}C-NMR, ^{1}H–^{1}H-correlated spectroscopy (COSY), HMQC and HMBC experiments were performed on a JNM LA 400 NMR spectrometer (^{1}H, 400 MHz; ^{13}C, 100 MHz, JEOL Co.).

Chromatography Column chromatography was carried out on Diaion HP-20 (Mitsubishi Chemical Co., Tokyo), Sephadex LH-20 (Pharmacia Co., Tokyo), Silica gel BW-820 MH and ODS (Fuji Silysia Chemical Co., Nagoya, Japan). TLC was carried out on pre-coated silica gel 60 F_{254} plates (0.25 mm and 0.5 mm, Merck Co., Darmstadt, Germany), with a solvent system of $CHCl_3$–MeOH (9 : 1), and spots were detected under a UV lamp or after spraying with 5% anisaldehyde/H_2SO_4 reagent, followed by heating. HPLC was performed on a Shimadzu SCL-6B system (Kyoto) equipped with an SIL-9A auto injector, two LC-6A liquid chromatograph pumps, an SPD-6A UV spectrophotometric detector and a C-R8A Chromatopac under the following conditions: column, YMC pack ODS-AP 302 (S-5 μm, 300A, 150×4.6 mm I.D., YMC Co., Kyoto); mobile phase, CH_3CN (solvent A) and H_2O containing 0.1% TFA (solvent B) in a gradient mode (A 15—33% from 0 to 15 min); flow rate, 1.0 ml/min; injection volume, 5 μl; detection, UV 280 nm.

Preparation of a Human Intestinal Bacterial (HIB) Mixture Feces (5 g) were obtained from two healthy subjects, who ingested an Asian diet and had not received antibiotics for at least 3 months. Fresh feces were homogenized in 100 ml of GAM broth (Nissui Co., Tokyo) under anaerobic conditions, and the sediments were removed by decantation to give a 5% HIB mixture.

Incubation of PDG (1) with HIB Mixture and Isolation of the Metabolites An HIB mixture (300 ml) and PDG (**1**, 1.9 g) dissolved in 10 ml of MeOH were added to 3.0 l of GAM broth and anaerobically incubated at 37 °C for seven days. A 125 ml portion of reaction mixture was taken out at intervals, followed by extracting 3 times with *n*-BuOH (saturated with H_2O, containing 0.1% acetic acid). The *n*-BuOH solutions were combined and evaporated *in vacuo* to give a residue. The residue was dissolved in H_2O. The H_2O suspension was chromatographed on a Diaion HP-20 column and eluted with H_2O, 50% aq. MeOH and MeOH. The 50% aq. MeOH and MeOH fractions were combined and applied to a Sephadex LH-20 column and eluted with MeOH–H_2O (6 : 4). Fractions containing metabolites were combined and chromatographed again on Sephadex LH-20 and eluted with MeOH–H_2O (1 : 1). Of the 35 fractions collected, fractions 15—35, which contained metabolites, were chromatographed on preparative TLC and purified by ODS column chromatography to afford compound **5** (14.1 mg) from fractions 15—18; compounds **2** (62.4 mg), **3** (4.1 mg), **4** (3.5 mg), **6** (6.0 mg) and **8** (1.5 mg) from fractions 19—26; compounds **7** (0.6 mg), **9** (0.3 mg), **10** (1.6 mg), **11** (11.9 mg) and **13** (10.4 mg) from fractions 27—31; compound **12** (5.9 mg), a mixture of **14** and **15** (17.4 mg), and **16** (48.6 mg) from fractions 32—35.

Compound **2** [(+)-Pinoresinol]: Amorphous powder. $[\alpha]_D^{25}$ +69° (c= 0.10, MeOH) [lit.[15] $[\alpha]_D^{24}$ +71.1° (MeOH), lit.[17] $[\alpha]_D^{20}$ +72° (MeOH)]. UV λ_{max}^{MeOH} (ε): 231 (14000), 280 (5800) nm. IR (KBr) ν_{max}: 3448 (OH), 1511 (arom. C=C) cm^{-1}. EI-MS m/z: 358 $[M]^+$. ^{1}H-NMR (CD_3OD, 400 MHz): δ 3.12 (2H, m, H-8, 8′), 3.83 (2H, dd, J=8.9, 3.6 Hz, H_a-9, 9′), 3.84 (6H, s, –OC$\underline{H}_3$), 4.21 (2H, dd, J=8.9, 6.8 Hz, H_b-9, 9′), 4.69 (2H, d, J=4.4 Hz, H-7, 7′), 6.75 (2H, d, J=8.0 Hz, H-5, 5′), 6.79 (2H, dd, J=8.0, 1.7 Hz, H-6, 6′), 6.93 (2H, d, J=1.7 Hz, H-2, 2′). ^{13}C-NMR: Table 1. CD (MeOH): CD (MeOH): $\Delta\varepsilon_{211}$ +2.67, $\Delta\varepsilon_{221}$ +1.07, $\Delta\varepsilon_{232}$ +0.64, $\Delta\varepsilon_{282}$ +0.45 (dm^3 mol^{-1} cm^{-1}).

Compound **3** [(+)-Lariciresinol]: Amorphous powder. $[\alpha]_D^{25}$ +30° (c= 0.10, MeOH) [lit.[17] $[\alpha]_D^{20}$ +32° (MeOH)]. UV λ_{max}^{MeOH} (ε): 229 (13000), 281 (5600) nm. IR (KBr) ν_{max}: 3448 (OH), 1516 (arom. C=C) cm^{-1}. EI-MS m/z: 360 $[M]^+$. ^{1}H-NMR (CD_3OD, 400 MHz): δ 2.37 (1H, m, H-8), 2.48 (1H, dd, J=13.4, 11.1 Hz, H_a-7′), 2.73 (1H, m, H-8′), 2.92 (1H, dd, J=13.4, 4.8 Hz, H_b-7′), 3.62 (1H, dd, J=10.9, 6.5 Hz, H_a-9), 3.72 (1H, dd, J=8.4, 5.8 Hz, H_a-9′), 3.82 (3H, s, –OC$\underline{H}_3$), 3.83 (1H, dd, J=10.9, 8.0 Hz, H_b-9), 3.84 (3H, s, –OC$\underline{H}_3$), 3.97 (1H, dd, J=8.4, 6.5 Hz, H_b-9′), 4.74 (1H, d,

J=7.0 Hz, H-7), 6.64 (1H, dd, J=8.0, 1.9 Hz, H-6′), 6.71 (1H, d, J=8.0 Hz, H-5′), 6.75 (1H, m, H-6), 6.76 (1H, m, H-5), 6.79 (1H, d, J=1.9 Hz, H-2′), 6.90 (1H, d, J=1.8 Hz, H-2). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{288}$ −0.26, $\Delta\varepsilon_{235}$ −1.07 (dm^3 mol^{-1} cm^{-1}).

Compound **4** [3′-Demethyl-(+)-lariciresinol]: Amorphous powder. $[\alpha]_D^{25}$ +15° (c=0.10, MeOH). UV λ_{max}^{MeOH} (ε): 221 (7800), 281 (3400) nm. IR (KBr) ν_{max}: 3448 (OH), 1520 (arom. C=C) cm^{-1}. EI-MS m/z: 346 [M]$^+$. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ 2.34 (1H, m, H-8), 2.43 (1H, dd, J=13.7, 12.1 Hz, H$_a$-7′), 2.70 (1H, m, H-8′), 2.84 (1H, dd, J=13.7, 4.9 Hz, H$_b$-7′), 3.61 (1H, dd, J=11.0, 6.5 Hz, H$_a$-9), 3.70 (1H, dd, J=8.0, 6.5 Hz, H$_a$-9′), 3.82 (1H, m, H$_b$-9), 3.84 (3H, s, –OC<u>H</u>$_3$), 3.97 (1H, dd, J=8.0, 6.8 Hz, H$_b$-9′), 4.75 (1H, d, J=6.8 Hz, H-7), 6.52 (1H, dd, J=8.0, 1.9 Hz, H-6′), 6.63 (1H, d, J=1.9 Hz, H-2′), 6.67 (1H, d, J=8.0 Hz, H-5′), 6.75 (1H, m, H-6), 6.77 (1H, m, H-5), 6.89 (1H, d, J=1.7 Hz, H-2). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{235}$ −0.4, $\Delta\varepsilon_{287}$ −0.19 (dm^3 mol^{-1} cm^{-1}).

Compound **5** [(−)-Secoisolariciresinol]: Amorphous powder. $[\alpha]_D^{25}$ −16° (c=0.10, MeOH) [lit.[28] $[\alpha]_D^{30}$ −28.2° (MeOH)]. UV λ_{max}^{MeOH} (ε): 226 (10000), 281 (4600) nm. IR (KBr) ν_{max}: 3425 (OH), 1516 (arom. C=C) cm^{-1}. EI-MS m/z: 362 [M]$^+$. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ 1.86 (2H, m, H-2, 3), 2.50 (2H, dd, J=13.8, 7.7 Hz, H$_a$-7′, 7″), 2.61 (2H, dd, J=13.8, 7.0 Hz, H$_b$-7′, 7″), 3.54 (4H, m, H-1, 4), 3.68 (3H, s, –OC<u>H</u>$_3$), 6.49 (2H, dd, J=8.0, 1.9 Hz, H-6′, 6″), 6.54 (2H, d, J=1.9 Hz, H-2′, 2″), 6.61 (2H, d, J=8.0 Hz, H-5′, 5″). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{289}$ −0.29, $\Delta\varepsilon_{228}$ −0.29 (dm^3 mol^{-1} cm^{-1}).

Compound **6** [(−)-3-(3″,4″-Dihydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl) butane-1,4-diol]: Amorphous powder. $[\alpha]_D^{25}$ −10° (c=0.10, MeOH) [lit.[29] $[\alpha]_D^{25}$ −29.7° (MeOH)]. UV λ_{max}^{MeOH} (ε) 221 (12000), 282 (5300) nm. IR (KBr) ν_{max}: 3425 (OH), 1516 (arom. C=C) cm^{-1}. EI-MS m/z: 348 [M]$^+$. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ: 1.90 (2H, m, H-2, 3), 2.59 (4H, m, H-7′, 7″), 3.56 (4H, m, H-1, 4), 3.76 (3H, s, –OC<u>H</u>$_3$), 6.43 (1H, dd, J=8.0, 2.2 Hz, H-6″), 6.55 (1H, dd, J=8.0, 1.9 Hz, H-6′), 6.56 (1H, d, J=2.2 Hz, H-2″), 6.63 (1H, d, J=1.9 Hz, H-2′), 6.63 (1H, d, J=8.0 Hz, H-5″), 6.66 (1H, d, J=8.0 Hz, H-5′). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{229}$ −1.26, $\Delta\varepsilon_{280}$ −0.13 (dm^3 mol^{-1} cm^{-1}).

Compound **7** [2-(3′,4′-Dihydroxybenzyl)-3-(3″,4″-dihydroxybenzyl)butane-1,4-diol]: Amorphous powder. UV λ_{max}^{MeOH} (ε): 220 (12000), 283 (4600) nm. IR (KBr) ν_{max}: 3448 (OH), 1523 (arom. C=C) cm^{-1}. EI-MS m/z: 334 [M]$^+$. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ 1.90 (2H, m, H-2, 3), 2.55 (4H, m, H-7′, 7″), 3.54 (4H, m, H-1, 4), 6.44 (2H, dd, J=8.0, 2.1 Hz, H-6′, 6″), 6.58 (2H, d, J=2.1 Hz, H-2′, 2″), 6.63 (2H, d, J=8.0 Hz, H-5′, 5″). ^{13}C-NMR: Table 1.

Compound **8** [3-(3″-Hydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl)-butane-1,4-diol]: Amorphous powder. UV λ_{max}^{MeOH} (ε): 220 (13000), 275 (6800) nm. IR (KBr) ν_{max}: 3394 (OH), 1520 (arom. C=C) cm^{-1}. EI-MS m/z: 332 [M]$^+$. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ 1.94 (2H, m, H-2, 3), 2.61 (4H, m, H-7′, 7″), 3.56 (4H, m, H-1, 4), 3.76 (3H, s, –OC<u>H</u>$_3$), 6.56 (1H, m, H-6′), 6.56 (1H, dd, J=8.1, 1.8 Hz, H-4″), 6.58 (1H, m, H-6″), 6.58 (1H, d, J=1.8 Hz, H-2″), 6.65 (1H, d, J=1.9 Hz, H-2′), 6.66 (1H, d, J=8.0 Hz, H-5′), 7.03 (1H, t, J=8.1 Hz, H-5″). ^{13}C-NMR: Table 1.

Compound **9** [2-(3′,4′-Dihydroxybenzyl)-3-(3″-hydroxybenzyl)butane-1,4-diol]: Amorphous powder. UV λ_{max}^{MeOH} (ε): 220 (7900), 280 (2300) nm. IR (KBr) ν_{max}: 3444 (OH), 1523 (arom. C=C) cm^{-1}. EI-MS m/z: 318 [M]$^+$. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ 1.94 (2H, m, H-2, 3), 2.55 (2H, m, H-7″), 2.62 (2H, m, H-7′), 3.50 (2H, m, H-1), 3.60 (2H, m, H-4), 6.45 (1H, dd, J=8.1, 1.9 Hz, H-6′), 6.57 (1H, m, 4″), 6.58 (1H, d, J=1.9 Hz, H-2′), 6.60 (1H, d, J=1.9 Hz, H-2″), 6.61 (1H, m, H-6″), 6.63 (2H, d, J=8.1 Hz, H-5′), 7.04 (1H, t, J=8.0 Hz, H-5″). ^{13}C-NMR: Table 1.

Compound **10** [(−)-Enterodiol]: Amorphous powder. $[\alpha]_D^{25}$ −12° (c=0.10, MeOH) [lit.[20] $[\alpha]_D^{23}$ −13.2° (EtOH)]. UV λ_{max}^{MeOH} (ε): 216 (13000), 274 (3700) nm. IR (KBr) ν_{max}: 3448 (OH), 1512 (arom. C=C) cm^{-1}. EI-MS m/z: 302 [M]$^+$. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ 1.97 (2H, m, H-2, 3), 2.63 (4H, m, H-7′, 7″), 3.52 (2H, dd, J=10.9, 4.6 Hz, H$_a$-1, 4), 3.60 (2H, dd, J=10.9, 3.4 Hz, H$_b$-1, 4), 6.59 (6H, m, m, H-2′, 4′, 6′, 2″, 4″, 6″), 7.03 (2H, t, J=8.0 Hz, H-5′, 5″). ^{13}C-NMR: Table 1.

Compound **11** [(−)-(2R,3R)-3-(3″,4″-Dihydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl)butyrolactone]: Amorphous powder. $[\alpha]_D^{25}$ −20° (c=0.10, MeOH). UV λ_{max}^{MeOH} (ε): 221 (12000), 282 (5500) nm. IR (KBr) ν_{max}: 3448 (OH), 1751 (γ-lactone CO), 1519 (arom. C=C) cm^{-1}. EI-MS m/z: 344 [M]$^+$. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ 2.41—2.51 (3H, m, H-3, 7″), 2.64 (1H, m, H-2), 2.84 (2H, d, J=5.8 Hz, H-7′), 3.80 (3H, s, –OC<u>H</u>$_3$), 3.88 (1H, m, H$_a$-4), 4.09 (1H, dd, J=8.7, 7.2 Hz, H$_b$-4), 6.39 (1H, dd, J=8.2, 2.0 Hz, H-6″), 6.52 (1H, d, J=2.0 Hz, H-2″), 6.58 (1H, dd, J=8.0, 1.9 Hz, H-6′), 6.65 (1H, d, J=8.2 Hz, H-5″), 6.67 (1H, d, J=1.9 Hz, H-2′), 6.70 (1H, d, J=8.0 Hz, H-5′). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{232}$ −2.93 (dm^3 mol^{-1} cm^{-1}).

Compound **12** [(−)-(2R,3R)-2-(3′,4′-Dihydroxybenzyl)-3-(3″,4″-dihydroxybenzyl) butyrolactone]: Amorphous powder. $[\alpha]_D^{25}$ −14° (c=0.10, MeOH). UV λ_{max}^{MeOH} (ε): 220 (11000), 283 (5300) nm. IR (KBr) ν_{max}: 3444 (OH), 1747 (γ-lactone CO), 1523 (arom. C=C) cm^{-1}. EI-MS m/z: 330 [M]$^+$. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ 2.36—2.39 (1H, m, H$_a$-7″), 2.42—2.54 (2H, m, H-2, H$_b$-7″), 2.57—2.62 (1H, m, H-3), 2.78 (1H, dd, J=14.0, 6.5 Hz, H$_a$-7′), 2.83 (1H, dd, J=14.0, 5.5 Hz, H$_b$-7′), 3.86 (1H, t, J=8.8 Hz, H$_a$-4), 4.03 (1H, dd, J=8.8, 7.5 Hz, H$_b$-4), 6.38 (1H, dd, J=8.0, 1.9 Hz, H-6″), 6.48 (1H, dd, J=8.1, 2.2 Hz, H-6′), 6.51 (1H, d, J=1.9 Hz, H-2″), 6.65 (1H, d, J=8.0 Hz, H-5″), 6.65 (1H, d, J=2.2 Hz, H-2′), 6.69 (1H, d, J=8.1 Hz, H-5′). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{230}$ −2.27, $\Delta\varepsilon_{280}$ −0.14 (dm^3 mol^{-1} cm^{-1}).

Compound **13** [(−)-(2R,3R)-3-(3″-Hydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl)butyrolactone]: Amorphous powder. $[\alpha]_D^{25}$ −32° (c=0.10, MeOH). UV λ_{max}^{MeOH} (ε): 220 (11000), 279 (4400) nm. IR (KBr) ν_{max}: 3444 (OH), 1747 (γ-lactone CO), 1519 (arom. C=C) cm^{-1}. EI-MS m/z: 328 [M]$^+$. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ 2.48—2.58 (3H, m, H-3, 7″), 2.66 (1H, m, H-2), 2.84 (2H, d, J=6.0 Hz, H-7′), 3.80 (3H, s, –OC<u>H</u>$_3$), 3.88 (1H, m, H$_a$-4), 4.10 (1H, dd, J=8.7, 7.0 Hz, H$_b$-4), 6.53 (1H, d, J=1.1 Hz, H-2″), 6.54 (1H, m, H-6″), 6.59 (1H, dd, J=8.0, 2.0 Hz, H-6′), 6.62 (1H, ddd, J=8.0, 2.5, 1.1 Hz, H-4″), 6.69 (1H, d, J=2.0 Hz, H-2′), 6.71 (1H, d, J=8.0 Hz, H-5′), 7.06 (1H, t, J=8.0 Hz, H-5″). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{230}$ −2.50 (dm^3 mol^{-1} cm^{-1}).

Compound **14** [2-(3′,4′-Dihydroxybenzyl)-3-(3″-hydroxybenzyl)butyrolactone]: EI-MS m/z: 314 [M]$^+$. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ 2.44 (1H, m, H$_a$-7″), 2.57 (1H, m, H-3), 2.60 (2H, m, H-2, H$_b$-7″), 2.78 (1H, dd, J=14.0, 6.5 Hz, H$_a$-7′), 2.85 (1H, dd, J=14.0, 5.3 Hz, H$_b$-7′), 3.87 (1H, t, J=8.9 Hz, H$_a$-4), 4.06 (1H, dd, J=8.9, 7.5 Hz, H$_b$-4), 6.48 (1H, dd, J=8.0, 2.2 Hz, H-6′), 6.52 (1H, d, J=2.4 Hz, H-2″), 6.52 (1H, dt, J=8.2, 2.4 Hz, H-6″), 6.61 (1H, ddd, J=8.2, 2.4, 1.0 Hz, H-4″), 6.65 (1H, d, J=2.2 Hz, H-2′), 6.68 (1H, d, J=8.0 Hz, H-5′), 7.06 (1H, t, J=8.2 Hz, H-5″). ^{13}C-NMR: Table 1.

Compound **15** [2-(3′-Hydroxybenzyl)-3-(3″,4″-dihydroxybenzyl)butyrolactone]: EI-MS m/z: 314 [M]$^+$. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ 2.36 (1H, m, H$_a$-7″), 2.44 (1H, m, H-3), 2.50 (1H, m, H$_b$-7″), 2.66 (1H, m, H-2), 2.83 (1H, dd, J=13.8, 6.5 Hz, H$_a$-7′), 2.92 (1H, dd, J=13.8, 5.5 Hz, H$_b$-7′), 3.86 (1H, t, J=8.9 Hz, H$_a$-4), 4.03 (1H, dd, J=8.9, 7.2 Hz, H$_b$-4), 6.38 (1H, dt, J=8.0, 2.2 Hz, H-6″), 6.50 (1H, d, J=2.2 Hz, H-2″), 6.63—6.67 (4H, m, H-2′, H-4′, H-6′, H-5″), 7.10 (1H, t, J=8.0 Hz, H-5′). ^{13}C-NMR: Table 1.

Compound **16** [(−)-(2R,3R)-Enterolactone]: Amorphous powder. $[\alpha]_D^{25}$ −39° (c=0.15, MeOH) [lit.[11] $[\alpha]_D^{25}$ −46° (MeOH), lit.[20] $[\alpha]_D^{23}$ −43° ($CHCl_3$)]. λ_{max}^{MeOH} (ε): 216 (11000), 274 (3100), 280 (sh, 2800) nm. IR (KBr) ν_{max}: 3421 (–OH), 1747 (γ-lactone CO). EI-MS m/z: 298 [M]$^+$. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ 2.43 (1H, m, H$_a$-7″), 2.49 (1H, m, H-3), 2.56 (1H, m, H$_b$-7″), 2.67 (1H, m, H-2), 2.83 (1H, dd, J=13.8, 7.0 Hz, H$_a$-7′), 2.93 (1H, dd, J=13.8, 5.3 Hz, H$_b$-7′), 3.88 (1H, t, J=8.9 Hz, H$_a$-4), 4.06 (1H, dd, J=8.9, 7.2 Hz, H$_b$-4), 6.51 (1H, d, J=2.2 Hz, H-2″), 6.53 (1H, d, J=8.0 Hz, H-6″), 6.61 (1H, ddd, J=8.0, 2.2, 1.0 Hz, H-4″), 6.64—6.67 (3H, m, H-2′, 4′, 6′), 7.05 (1H, t, J=8.0 Hz, H-5″), 7.10 (1H, t, J=8.2 Hz, H-5′). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{277}$ −0.24, $\Delta\varepsilon_{220}$ −3.62 (dm^3 mol^{-1} cm^{-1}).

Isolation and Characterization of a Bacterial Strain Responsible for the Transformation of (+)-Pinoresinol (2) to (+)-Laricresinol (3) An HIB mixture was repeatedly cultured with GAM broth containing 0.5 mM compound **2** at 37 °C in an anaerobic incubator. (+)-Pinoresinol-metabolizing activity was monitored by TLC, as described above. A portion of the culture was seeded on BL agar (Nissui Co., Tokyo) plates and anaerobically incubated at 37 °C for 24 h. Colonies were picked up and screened for the activity of transforming **2** to **3**. Such a procedure was repeated until a pure strain possessing the activity was isolated. Characterization, including fermentation tests, was carried out according to the methods of Mitsuoka[30] and the Bergey's manual.[24]

Time Course for the Transformation of (+)-Pinoresinol (2) to (+)-Laricresinol (3) by *Enterococcus faecalis* Strain PDG-1 100 μl of 60 mM (+)-pinoresinol (**2**) in MeOH and 500 μl of precultured *Enterococcus faecalis* strain PDG-1 were added to 5 ml of GAM broth, and the mixture was incubated at 37 °C under anaerobic conditions. Every two 100 μl portions were taken out at intervals: one was used for measuring the bacterial growth and the other for HPLC analysis. The bacterial growth was monitored by measuring the absorbance at 540 nm. Transformation of **2** to **3** was monitored by HPLC: a 100 μl portion of the sample was extracted with *n*-BuOH (saturated with H_2O, containing 0.1% acetic acid, 100 μl×3). After evaporation of *n*-BuOH *in vacuo*, the residue was dissolved in 0.5 ml of MeOH. The MeOH solution was diluted with a mixed solvent of MeOH and

H_2O (1 : 1) to a volume of 2.5 ml, which was filtered through a 0.2 μm membrane filter, and a 5 μl portion was injected to a column for HPLC analysis. Substrate and metabolite were well separated and detected under the conditions mentioned above. Concentrations of **2** and metabolite **3** were calculated according to calibration curves of the respective authentic samples.

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