Transformation of Arctiin to Estrogenic and Antiestrogenic Substances by Human Intestinal Bacteria

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After anaerobic incubation of arctiin (1) from the seeds of *Arctium lappa* with a human fecal suspension, six metabolites were formed, and their structures were identified as (−)-arctigenin (2), (2*R*,3*R*)-2-(3′,4′-dihydroxybenzyl)-3-(3″,4″-dimethoxybenzyl)butyrolactone (3), (2*R*,3*R*)-2-(3′-hydroxybenzyl)-3-(3″,4″-dimethoxybenzyl)-butyrolactone (4), (2*R*,3*R*)-2-(3′-hydroxybenzyl)-3-(3″-hydroxy-4″-methoxybenzyl)butyrolactone (5), (2*R*,3*R*)-2-(3′-hydroxybenzyl)-3-(3″,4″-dihydroxybenzyl)butyrolactone (6), and (−)-enterolactone (7) 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 the time course of the transformation. Enterolactones obtained from the biotransformation of arctiin and secoisolariciresinol diglucoside (SDG, from the seeds of *Linum usitatissium*) by human intestinal bacteria were proved to be enantiomers, with the (−)-(2*R*,3*R*) and (+)-(2*S*,3*S*) configurations, respectively. Compound 6 showed the most potent proliferative effect on the growth of MCF-7 human breast cancer cells in culture among 1 and six metabolites, while it showed inhibitory activity on estradiol-mediated proliferation of MCF-7 cells at a concentration of 10 μM. These results indicate that the transformation of 1 by intestinal flora might be essential for the manifestation of the estrogenic and antiestrogenic activity of 1.

Key words arctiin; human intestinal bacteria; enterolactone; mammalian lignan; MCF-7 cell; phytoestrogen

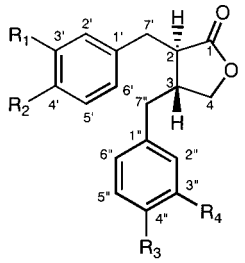
In 1980, two lignan compounds, enterodiol and enterolactone, were reported from human and animal species.[1)] They were given the name mammalian lignans, because unlike plant lignans they carry phenolic hydroxy groups only in the *meta* position of the aromatic ring and they were assumed to play an important role in the prevention of hormone-dependent diseases, such as breast cancer and prostate cancer.[1—3)] The origins of enterodiol and enterolactone were later found to be plant lignans, such as secoisolariciresinol and matairesinol, in vegetarian food, and to be transformed by gut microflora in the proximal colon.[4,5)] As enterodiol and enterolactone are similar in partial structure to estradiol, they were expected to have estrogenic or antiestrogenic activity and so far to influence hormone-dependent diseases.[6—8)] Recently, arctiin, which is a glycoside of arctigenin and abundant in the seeds of *Arctium lappa*, was reported to show a protective effect against 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP)-induced mammary carcinogensis in female rats with oral administration.[9)] Arctiin was shown to be transformed to two metabolites (arctigenin and AM2) by rat intestinal flora.[10)]

The transformation of secoisolariciresinol diglucoside (SDG) to enterodiol and enterolactone by human intestinal bacteria was studied in our department, and two bacterial strains, *Peptostreptococcus* sp. SDG-1 and *Eubacterium* sp. SDG-2, responsible for the conversion of SDG were isolated.[11)] Knowing the differences in bacterial species and their numbers between human and rat intestinal flora, we assumed that the respective transformations of arctiin (**1**) would be different. In the present paper, we report the transformation of **1** by human intestinal bacteria and the influence of its metabolites on the growth of human breast cancer MCF-7 cells.

Results

Transformation of Arctiin (1) by Human Intestinal Flora After anaerobic incubation of arctiin (**1**) with a bacterial mixture of human feces, the culture was extracted with acidified *n*-BuOH and the extract was subjected to Diaion HP-20, Sephadex LH-20, silica gel, and RP-18 column chromatography. Six metabolites (**2**—**7**) (Fig. 1) were isolated and identified by electron impact mass (EI-MS), one dimensional (1D) and two dimensional (2D)-NMR, and circular dichroic (CD) spectroscopy.

Compound **2** was detected as the first metabolite from **1** by thin-layer chromatography (TLC). The EI-MS showed a molecular ion peak at *m*/*z* 372, 162 mass units ($C_6H_{10}O_5$) less than that of **1**. The ^{1}H- (see Experimental) and ^{13}C-NMR



	R_1	R_2	R_3	R_4
1	OCH_3	OGlc	OCH_3	OCH_3
2	OCH_3	OH	OCH_3	OCH_3
3	OH	OH	OCH_3	OCH_3
4	OH	H	OCH_3	OCH_3
5	OH	H	OCH_3	OH
6	OH	H	OH	OH
7	OH	H	H	OH

Fig. 1. Structures of Arctiin (**1**) and Its Metabolites (**2**—**7**)

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spectra (Table 1) were in good agreement with those reported for (2*R*,3*R*)-(−)-arctigenin.[12]

The ^{1}H-NMR spectrum of compound **3** showed two methoxy signals in contrast with the three methoxy signals of **2**. Therefore **3** was assumed to be a demethylation product of **2**. This was confirmed by the presence of a molecular ion peak at *m*/*z* 358 in the EI-MS spectrum and ^{13}C-NMR spectral evidence (two methoxy signals, δ_C 56.0, 55.9, Table 1). The CD spectrum of **3** showed two negative Cotton effects around 282 and 233 nm, similar to that reported for (2*R*,3*R*)-2,3-dibenzylbutyrolactone.[13] The ^{1}H- and ^{13}C-NMR spectral data agreed with those of a metabolite of arctiin (**1**) transformed by rat intestinal flora.[10] The structure of **3** was thus determined to be (2*R*,3*R*)-2-(3′,4′-dihydroxybenzyl)-3-(3″,4″-dimethoxybenzyl)butyrolactone.

Compound **4** showed a molecular ion peak at *m*/*z* 342 in its EI-MS spectrum, 16 mass units less than that of **3**, suggesting that **4** is a dehydroxylation product of **3**. This was further confirmed by the presence of an additional aromatic proton signal at δ 6.70 in the ^{1}H-NMR spectrum. In the ^{13}C-NMR spectrum, a C-4′ signal appeared upfield by 28.9 ppm, while C-3′, C-5′, and C-1′ signals appeared downfield by 12.2, 14.6, and 8.9 ppm, respectively, compared with those of **3**, which indicated that dehydroxylation had occurred in the *para* position. Compound **4** also showed negative Cotton effects around 280 and 231 nm. The structure of **4** was consequently concluded to be (2*R*,3*R*)-2-(3′-hydroxybenzyl)-3-(3″,4″-dimethoxybenzyl)butyrolactone. The racemic compounds of **4** were previously synthesized by Eich *et al.*[14]

Table 1. ^{13}C-NMR (100 MHz) Spectral Data of Compounds **2**—**7**

C	**2**[a]	**3**[a]	**4**[a]	**5**[a]	**6**[b]	**7**[a]
1	178.7	179.5	178.9	178.7	181.4	179.0
2	46.6	46.5	46.3	46.3	47.5	46.3
3	41.0	41.0	41.3	41.2	42.8	41.0
4	71.3	71.6	71.4	71.3	72.8	71.4
1′	129.5	130.6	139.5	139.5	140.9	139.4
2′	111.6	116.1	116.2	116.2	117.3	116.3
3′	146.7	143.9	156.1	155.9	158.6	155.91
4′	144.6	142.9	114.0	114.0	114.7	114.1
5′	114.1	115.2	129.8	130.0	130.6	130.0
6′	122.1	121.6	121.6	121.7	121.7	121.8
7′	34.5	34.1	34.8	34.8	35.8	34.8
1″	130.5	130.1	130.5	131.2	131.4	139.8
2″	111.9	112.0	112.0	114.8	116.8	115.7
3″	149.1	149.1	149.1	145.6	146.4	155.87
4″	147.9	147.9	147.9	145.4	145.0	113.9
5″	111.4	111.5	111.5	110.8	116.4	130.0
6″	120.6	120.8	120.7	120.2	121.0	121.1
7″	38.2	38.2	38.2	37.9	38.6	38.3
-Me	55.9	56.0	56.0	56.0×2		
	55.8×2	55.9				

Measured in *a*) $CDCl_3$ and *b*) MeOH-d_4.

Compound **5** (*m*/*z* 328 $[M]^+$) in the EI-MS spectrum was 14 mass units (CH_2) less than that of **4**, indicating that **5** is a demethylation product of **4**. Proton and carbon NMR signals due to a 3′-hydroxybenzyl group were almost the same as those of **4**, while signals due to a methoxyl-bearing benzyl group were changed; a signal of C-3″ was shifted upfield by 3.5 ppm, while that of C-2″ was shifted downfield by 2.8 ppm, indicating that demethylation had occurred at C-3″ in the 3″,4″-dimethoxyphenyl ring system. This was further confirmed by the heteronuclear multiple-bond coherence (HMBC) experiment; a signal of C-4″ showed correlations to proton signals of MeO-4″, H-2″, and H-6″ (Fig. 2). The CD spectrum of **5** showed negative Cotton effects near 279 and 229 nm. The structure of **5** was consequently determined to be (2*R*,3*R*)-2-(3′-hydroxybenzyl)-3-(3″-hydroxy-4″-methoxybenzyl)butyrolactone.

Compound **6** was deduced to be a further demethylation product of **5** on the basis of its molecular ion peak (*m*/*z* 314 $[M]^+$) in the EI-MS spectrum, 14 mass units (CH_2) less than that of **5**, and no signal assignable to methoxy protons in the ^{1}H-NMR spectrum. The ^{13}C-NMR data agreed with those of a synthetic lignan reported as an oxidative metabolite of enterolactone by human liver microsomes.[15] The CD spectrum of **6** also showed negative Cotton effects around 281 and 230 nm. Therefore **6** was identified as (2*R*,3*R*)-2-(3′-hydroxybenzyl)-3-(3″,4″-dihydroxybenzyl)butyrolactone.

Compound **7** was detected as the final metabolite of arctiin (**1**) by anaerobic incubation with a human fecal suspension. It showed a bright red spot on the TLC plates after spraying with anisaldehyde/H_2SO_4 followed by heating. The ^{1}H- and ^{13}C-NMR spectra of **7** agreed well with those reported for enterolactone.[16] Negative Cotton effects were observed around 280 and 220 nm in its CD spectrum. Compound **7** was thus determined to be (2*R*,3*R*)-2-(3′-hydroxybenzyl)-3-(3″-hydroxybenzyl)butyrolactone, or (−)-enterolactone.

Based on the structures of the isolated metabolites, a possible metabolic process of arctiin (**1**) by human intestinal bacteria was deduced, as shown in Fig. 2. The glucose moiety

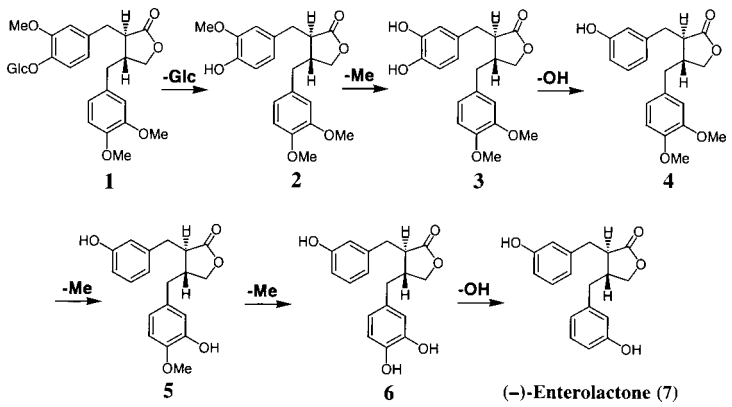

Fig. 2. Possible Pathway for the Transformation of Arctiin (**1**) by Human Intestinal Bacteria

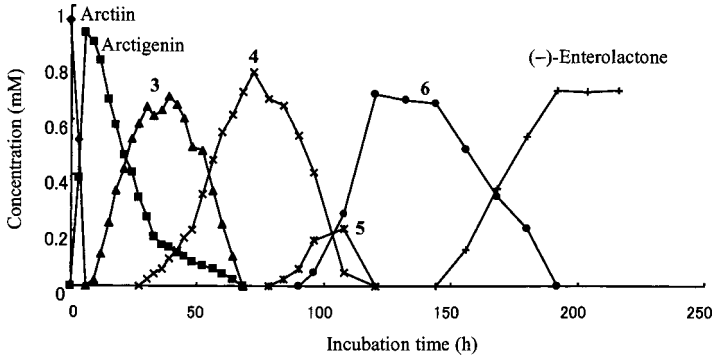

Fig. 3. Time Course for Metabolism of Arctiin (**1**) by a Human Intestinal Bacterial Mixture

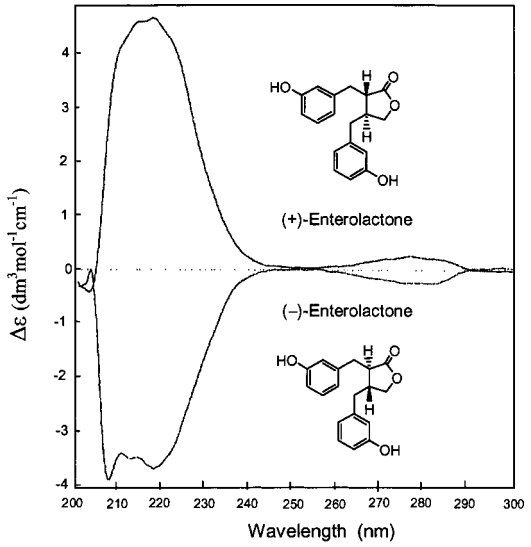

Fig. 4. CD Spectra of (−)- and (+)-Enterolactones

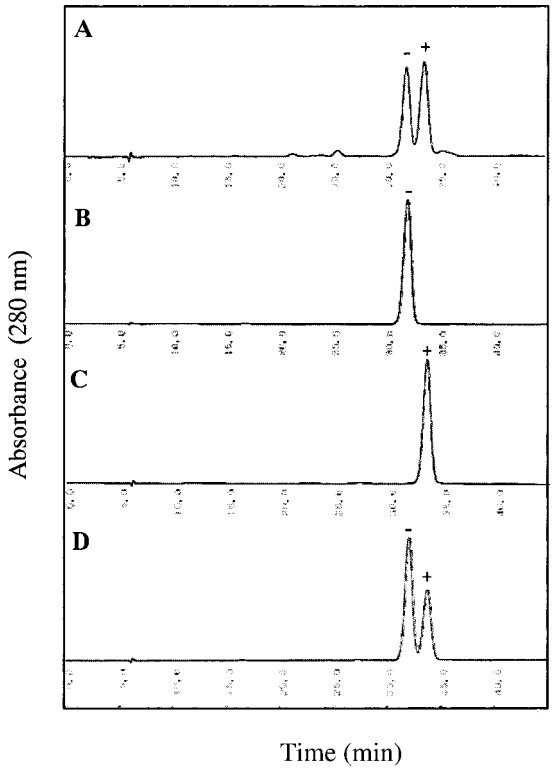

Fig. 5. Chiral HPLC Elution Profiles of (−)- and/or (+)-Enterolactones

A, synthetic (±)-enterolactone; B, (−)-enterolactone transformed from arctiin; C, (+)-enterolactone transformed from SDG; D, a mixture of (−)- and (+)-enterolactones. Column, chiral CD-Ph, S-5 μm, 4.6 mm i.d.×250 mm, Shiseido. Eluent, $CH_3CN : H_2O = 33 : 77$, 0.5 ml/min.

of **1** is first cleaved to form arctigenin (**2**). Although there are three methoxy groups in **2**, a methoxy group adjacent to a hydroxy group is easily demethylated, while two *ortho* methoxy groups are rather resistant to demethylation. A *para*-hydroxy group (not a *meta*-hydroxy group) of **3** is dehydroxylated to form **4**. Of the two methoxy groups in **4**, the one at the *meta* position is first demethylated to give **5**. Compound **5** is subsequently demethylated to give **6**. Here again, dehydroxylation of **6** gives (−)-enterolactone (**7**), which carries two phenolic hydroxy groups in the respective *meta* positions.

These metabolic processes are further supported by the time course experiment, monitored by HPLC (Fig. 3). A stepwise conversion from arctiin (**1**) to arctigenin (**2**), **3**, **4**, **5**, **6**, and (−)-enterolactone (**7**) occurs, while **7** appears to be stable even after prolonged incubation.

(−)-Enterolactone and (+)-Enterolactone Enterolactone (**7**) obtained from arctiin (**1**) showed a minus optical rotation, while that from SDG showed a plus optical rotation. The CD spectrum of (−)-enterolactone (**7**) showed two negative Cotton effects around 280 and 220 nm, in contrast with the positive Cotton effects of (+)-enterolactone at the corresponding wavelengths (Fig. 4). They were well separated by HPLC with the use of a chiral column (Fig. 5). A synthetic enterolactone was confirmed to be racemic, without any optical rotation.

Influence of Arctiin (1) and Its Metabolites on the Growth of Human Breast Cancer MCF-7 Cells Since enterolactone was reported to have estrogenic activity, we examined original arctiin (**1**) and metabolites for activity using human breast cancer MCF-7 cells.[6,7] In addition to the positive control (estradiol), compound **6** showed the most potent proliferative activity among the metabolites (Fig. 6). (−)-Enterolactone also stimulated cell growth at a high concentration of 10 μM. Arctigenin (**2**) inhibited the growth, probably due to its cytotoxic property as has been reported by Moritani *et al.*[17]

Influence of Compound 6 and (−)-Enterolactone (7) on Estradiol-Mediated Proliferation of MCF-7 Cells If phytoestrogens are capable of reducing the human risk of hormone-dependent diseases, they are expected to inhibit (not stimulate) the growth of breast cancer cells. When cell

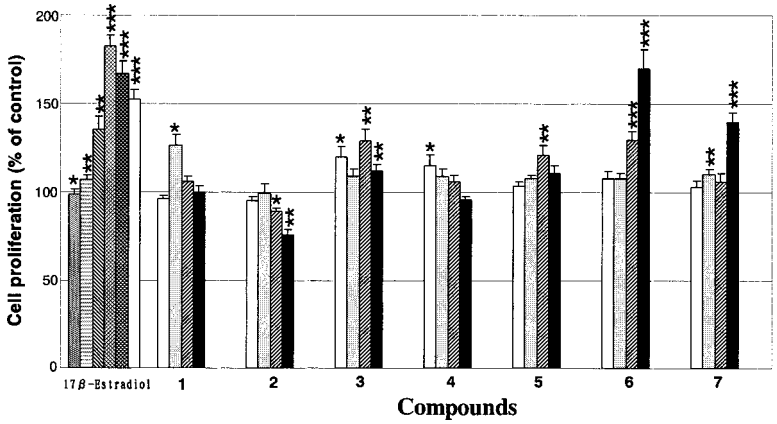

Fig. 6. Effect of Arctiin (**1**) and Its Metabolites on the Growth of Breast Cancer MCF-7 Cells in Culture

17β-Estradiol: 10^{-13} M, 10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M, and 10^{-8} M (from left to right). Compounds **1**—**7**: 10^{-8} M, 10^{-7} M, 10^{-6} M, and 10^{-5} M (from left to right). All values are expressed as the mean±S.E. (n=6). $*p<0.05$, $**p<0.01$, $***p<0.001$ *vs.* control.

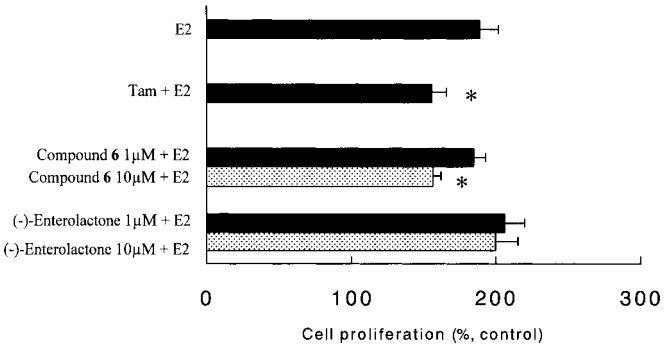

Fig. 7. Influence of Compound **6** and (−)-Enterolactone (**7**) on the Proliferation of Human Breast Cancer MCF-7 Cells in the Presence of 17β-Estradiol

E2, 17β-Estradiol (10^{-10} M); Tam, tamoxifen (1 μM), $*p<0.05$ *vs.* E2 group (n=6).

growth was stimulated in the presence of estradiol 10^{-10} M, compound **6** at a concentration of 10 μM inhibited the estradiol-mediated proliferative effect. Tamoxifen, an antiestrogenic agent used for the treatment of breast cancer, was used as a positive control in this experiment and also inhibited the proliferative effect of estradiol (Fig. 7).

Discussion

Using a GC-MS technique, the transformation of arctigenin (**2**) by human fecal microflora was recently studied by Heinonen *et al.*[18] In addition to enterolactone, two intermediates were detected and tentatively identified as 2-(3′,4′-dihydroxybenzyl)-3-(3″,4″-dimethoxybenzyl)butyrolactone and 2-(3′-hydroxybenzyl)-3-(3″,4″-dimethoxybenzyl)butyrolactone on the basis of MS evidence. Our metabolic study was performed on arctiin (**1**), a glucoside of arctigenin, by a human intestinal bacterial mixture. Seven metabolites, including those proposed by Heinonen *et al.*,[18] were isolated and identified by EI-MS, 1D- and 2D-NMR, and CD spectroscopy. Furthermore, based on the time course experiment, a possible metabolic pathway was proposed (Fig. 2). On the other hand, incubation of arctiin (**1**) with a rat intestinal bacterial mixture led to two metabolites, **2** and **3**, similar to those reported by Nose *et al.*,[10] which may reflect the difference in bacterial flora between rats and humans.

The present transformation of **1** by human intestinal bacteria includes three types of reactions: hydrolysis of glucoside; demethylation of methoxy groups; and dehydroxylation. Similar to the transformation of SDG,[11] demethylation and dehydroxylation of lignans by human intestinal bacteria are quite specific biological reactions, in contrast with chemical reactions. A methoxy group adjacent to a hydroxy group is easily demethylated, while the two vicinal methoxy groups are rather resistant to demethylation. A methoxy group at C-3 in the 3,4-dimethoxyphenyl ring system appears to be preferably converted to a hydroxy group compared with that at C-4. In the 3,4-dihydroxyphenyl ring system, a hydroxy group at C-4 is exclusively eliminated. A 3-hydroxy group on the phenyl ring appears to be stable, since this group was not eliminated further even after prolonged incubation with human intestinal bacteria.

Two bacterial strains, *Peptostreptococcus* sp. SDG-1 and *Eubacterium* sp. SDG-2, responsible for demethylation and dehydroxylation of SDG, were isolated from a human fecal suspension.[11] However, the two bacteria failed to perform demethylation in **2** or dehydroxylation in **3**, suggesting the presence of other bacteria responsible for the transformation of **1** in human fecal flora. Dehydroxylation by *Eubacterium* sp. SDG-2 was found to be stereospecific on (3*R*)-flavan-3-ols.[19] We assume that different types of bacteria are responsible for the demethylation or dehydroxylation in compounds **2** and **3**, and the reactions may be carried out by those bacterial enzymes.

The metabolism of lignans in animals or humans may be intriguing and complex. Based on the *in vitro* transformation of SDG and arctiin (**1**) by intestinal bacteria, these compounds may be demethylated and dehydroxylated in the gastrointestinal tract. However, the metabolites may be subjected to methylation and hydroxylation in the liver, and returned to the precursor to some extent.[15,20] Pharmacokinetic studies are necessary to determine which metabolite are predominant in the serum at intervals after oral administration of **1**.

During the course of experiments on the transformation of SDG and arctiin (**1**), we demonstrated that enterolactone from **1** has a (−)-(2*R*,3*R*) configuration, while that from SDG had a (+)-(2*S*,3*S*) configuration. Arctiin was transformed to (−)-(2*R*,3*R*)-enterolactone because arctigenin has a (−)-(2*R*,3*R*) configuration. On the other hand, SDG was transformed to (+)-(2*S*,3*S*)-enterolactone because secoiso-

lariciresinol is in a (+)-(2*S*,3*S*) form, and this form was maintained during the metabolic transformations including oxidation of butane-1,4-diol to a lactone. Thus the absolute configurations at C-2 and C-3 in the respective enterolactone molecules originate from those of the precursor. Although intestinal bacteria perform a variety of reactions, such as hydrolysis, demethylation, dehydroxylation, and oxidation, the absolute configuration of lignans examined did not change during the transformation while the $[\alpha]_D$ values and CD spectra of the metabolites were monitored.

Compound **6** showed both estrogenic and antiestrogenic activity on the growth of MCF-7 cells. Although there is one case report of an inhibitory effect of enterolactone on estradiol-mediated proliferation of MCF-7 cells, we did not observe such a result with (−)-enterolactone (**7**).[7] Considering the stereochemistry of enterolactone, we do not know the absolute structure of enterolactone the previous group used. To our knowledge, this is the first report of a study of enterolactone with a (2*R*,3*R*) configuration for estrogenic activity. Estrogenic and antiestrogenic assays of (−)- and (+)-enterolactones will be performed in our laboratory to determine whether the stereostructure determines the activity.

As (−)-arctigenin (**2**) was reported to be a lead structure for inhibitors of HIV-1 integrase,[14] all the metabolites from arctiin (**1**) and SDG will be studied for anti-HIV and estrogenic activity. The structure–activity relationship will also be investigated.

Phytoestrogens, including isoflavones and lignans, have been suggested to have a protective effect against hormone-dependent diseases such as breast cancer and prostate cancer. Since the first report of enterodiol and enterolactone, many studies have been performed, especially by the group of Adlercreutz,[21] to elucidate the relationship between phytoestrogens and human health. It is still not clear whether phytoestrogens have beneficial or deleterious effects, and pharmacological studies of enterolactone and enterodiol are insufficient.[21,22] Although chemists have synthesized enterodiol and enterolactone, it is difficult to obtain sufficient amounts for *in vivo* studies.[23,24] Our results may contribute to the study of mammalian lignans in three ways. 1) Using naturally abundant arctiin (**1**) as a precursor for the synthesis of (−)-enterolactone makes it realistic to obtain sufficient amounts of metabolites for *in vivo* studies. 2) This is the first report on obtaining optically pure mammalian lignans from natural precursors, (−)-(2*R*,3*R*)-enterolactone from arctiin and (+)-(2*S*,3*S*)-enterolactone from SDG. The absolute configuration of mammalian lignans should be taken into consideration in future studies of their activity. 3) Compound **6**, capable of inhibiting the proliferative activity of estradiol in MCF-7 cells, is promising and worthy of further study.

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, Japan) at 25 °C, CD spectra with a JASCO J 805 spectropolarimeter (Jasco Co.), UV spectra with a Shimadzu UV-2200 recording spectrophotometer (Shimadzu Co., Kyoto, Japan), 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), ^{1}H-detected multiple quantum coherence (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, Japan), Sephadex LH-20 (Pharmacia Co., Tokyo, Japan), silica gel BW-820 MH, and ODS (Fuji Silysia Chemical Co., Nagoya, Japan). TLC was carried out on precoated silica gel 60 F_{254} plates (0.25 mm, Merck Co., Darmstadt, Germany), with the solvent system $CHCl_3$–MeOH (10 : 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 (Shimadzu) equipped with a SIL-9A auto injector, two LC-6A liquid chromatographys, a SPD-6A UV spectrophotometric detector, and a C-R8A chromatopac under the following conditions: column, YMC pack ODS-AP 302 (S-5 μm, 300 A, 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 17.5% isocratic for 41 min, A 17.5—30% from 41 to 55 min, A 30—100% from 55 to 56 min); flow rate, 1.0 ml/min; injection volume, 5 μl; and detection, UV 280 nm.

Plant Materials The seeds of *A. lappa* L. and *L. usitatissium* L. were purchased from Tochimoto Tenkaido Co. (Osaka, Japan).

Chemicals and Media General anaerobic medium (GAM) broth was purchased from Nissui Co. (Tokyo, Japan). Arctiin (**1**) was isolated from the seeds of *A. lappa* L. as follows. The pulverized seeds (2.5 kg) were extracted two times with MeOH (7 l) by soaking overnight. The combined solutions were evaporated under reduced pressure to give a residue. The residue was suspended in H_2O and extracted with *n*-hexane. The H_2O fraction was applied to a column of Diaion HP-20 ion-exchange resin and the column was eluted with H_2O, 50% MeOH and MeOH. The 50% MeOH fraction was subjected to silica gel column chromatography with $CHCl_3$–MeOH (10 : 1→9 : 1) to give arctiin (**1**) in a yield of 26.8 g as a colorless powder by lyophilization.

(±)-Enterolactone was prepared according to a modified method of Makela *et al.*[23]: To 1 g of a 3-(benzyloxy)-1-[bis(phenylthio)methyl]-benzene[24] in THF (15 ml) at −60 °C was added a solution of *n*-butyl lithium in *n*-hexane (1.6 M, 5 ml). The solution was stirred for 2 h at the same temperature, and then a solution of 2-butenolide (200 mg) in THF (2 ml) was added. The reaction was stirred for further 3 h at the same temperature and then 1 ml of TMEDA was added, followed by immediate addition of 0.67 g of 3-(benzyl)-1-(bromomethyl)benzene[24] in THF (2 ml). The reaction mixture was left overnight, and the reaction stopped by the addition of water, then extracted with $CHCl_3$, washed with water, and dried over Na_2SO_4. After evaporation of the solvent, the residue was purified by silica gel column chromatography to give 3-{[3-(benzyloxy)phenyl]methyl}-4-{[3-(benzyloxy)phenyl]bis(phenylthio)methyl}-dihydro-2(3*H*)-furanone (0.4 g). A solution of the dihydro-2(3*H*)-furanone in ethanol, together with Raney Ni (W-2) was refluxed for 3 h. The catalyst was removed by filtration. After evaporation of the solvent, the residue was purified by silica gel column chromatography to give (±)-enterolactone, which was identified by a comparison of spectral data with those reported.[16,24]

(+)-Enterolactone was obtained from SDG, from the seeds of *L. usitatissium* by anaerobic incubation with human intestinal bacteria according the method of Wang *et al.*[11]

Tamoxifen was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 17β-Estradiol was purchased from Caliches Bioscience, Inc. (La Jolla, CA, U.S.A.). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was purchased from Wako Co. (Osaka, Japan).

Preparation of a Human Intestinal Bacterial Mixture Fresh feces (5 g) obtained from a healthy subject was homogenized in 100 ml of GAM broth and the sediments were removed by decantation to give a 5% human intestinal bacterial (HIB) mixture.

Preparation of a Rat Intestinal Bacterial Mixture Fresh feces (5 g), obtained from male Wistar strain rats (9—10 weeks old, Sankyo Laboratory. Service, Tokyo, Japan), was homogenized in 100 ml of GAM broth and the sediments were removed by decantation to give a 5% rat intestinal bacteria (RIB) mixture.

Incubation of Arctiin (1) with HIB Mixture and Isolation of Metabolites An HIB mixture (77 ml) and arctiin (**1**, 374 mg) in 2 ml of MeOH were added to 700 ml of GAM broth and anaerobically incubated at 37 °C for 24 h. The reaction mixture was extracted three times with 777 ml of *n*-BuOH (saturated with H_2O, containing 0.1% acetic acid). After evaporation of *n*-BuOH *in vacuo*, the H_2O suspension was applied to a column of ion-exchange resin Diaion HP-20 and the column was eluted with H_2O, 50% aqueous MeOH, and MeOH. The MeOH fraction was further chromatographed two times on a Sephadex LH-20 column eluted with MeOH–H_2O (6 : 4→8 : 2→10 : 0) and a silica gel column eluted with $CHCl_3$–MeOH (100 : 0→

100 : 4) to afford compounds **2** (105.8 mg) and **3** (97.2 mg).

Arctiin (**1**, 748 mg) and 140 ml of an HIB mixture were added to 1400 ml of GAM broth and the mixture was anaerobically incubated at 37 °C. At intervals, 100 ml reaction mixture was removed and extracted three times with *n*-BuOH (saturated with H_2O, containing 0.1% acetic acid). The *n*-BuOH solutions were combined and evaporated to give an H_2O suspension. The H_2O suspension was chromatographed on a Diaion HP-20 column eluted with H_2O, 50% aqueous MeOH, and MeOH. The 50% aqueous MeOH and MeOH fractions were combined and applied to a Sephadex LH-20 column eluted with MeOH–H_2O (6 : 4). Of the 18 fractions collected, fractions 4—10 were subjected to silica gel column chromatography eluted with $CHCl_3$–MeOH (100 : 0→100 : 10) and ODS column chromatography eluted with 50% aqueous MeOH to afford compounds **4** (69.7 mg) and **5** (5.2 mg). Fractions 11—16 were chromatographed on a silica gel column eluted with $CHCl_3$: MeOH (100 : 2→100 : 5) and then a Sephadex LH-20 column to give compounds **6** (79.3 mg) and **7** (48 mg).

Incubation of Arctiin (1) with an RIB Mixture A 0.5 ml portion of the RIB mixture and 50 μl of 100 mM arctiin (**1**) in MeOH were added to 5 ml of GAM broth and anaerobically incubated at 37 °C for 1 week. The reaction mixture was removed at intervals and extracted with *n*-BuOH (saturated with H_2O, containing 0.1% acetic acid). The *n*-BuOH solution was checked by TLC, as described above.

Compound **2** [(−)-Arctigenin]: Colorless prisms (MeOH). mp 100.5—101.5 °C. $[\alpha]_D^{25}$ −25.8° (c=0.20, MeOH). UV λ_{max}^{MeOH} (ε): 231 (8800), 281 (4000) nm. IR (KBr) ν_{max}: 3424 (OH), 1762 (γ-lactone CO) cm^{-1}. EI-MS *m/z*: 372 $[M]^+$. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 2.45—2.66 (4H, m, H-2, 3, 7″), 2.92 (2H, m, H-7′), 3.81 (3H, s, –OC$\underline{H}_3$), 3.82 (3H, s, –OC$\underline{H}_3$), 3.85 (3H, s, –OC$\underline{H}_3$), 3.88 (1H, dd, J=9.18, 7.28 Hz, H_a-4), 4.13 (1H, dd, J=9.18, 7.24 Hz, H_b-4), 6.46 (1H, d, J=1.94 Hz, H-2″), 6.55 (1H, dd, J=8.19, 1.94 Hz, H-6″), 6.61 (1H, dd, J=7.99, 1.94 Hz, H-6′), 6.64 (1H, d, J=1.94 Hz, H-2′), 6.75 (1H, d, J=8.19 Hz, H-5″), 6.83 (1H, d, J=7.99 Hz, H-5′). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{282}$ −0.102, $\Delta\varepsilon_{233}$ −3.06 ($dm^3\,mol^{-1}\,cm^{-1}$).

Compound **3** [(2*R*,3*R*)-2-(3′,4′-Dihydroxybenzyl)-3-(3″,4″-dimethoxybenzyl)butyrolactone]: Amorphous powder. $[\alpha]_D^{25}$ −42.8° (c=0.13, MeOH). UV λ_{max}^{MeOH} (ε): 228 (12000), 281 (5800) nm. IR (KBr) ν_{max}: 3421 (–OH), 1751 (γ-lactone CO) cm^{-1}. EI-MS *m/z*: 358 $[M]^+$. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 2.48—2.64 (4H, m, H-2, 3, 7″), 2.85 (2H, d, J=5.88 Hz, H-7′), 3.81 (3H, s, –OC$\underline{H}_3$), 3.85 (3H, s, –OC$\underline{H}_3$), 3.88 (1H, dd, J=8.94, 7.24 Hz, H_a-4), 4.14 (1H, dd, J=8.94, 7.00 Hz, H_b-4), 6.48 (1H, d, J=1.94 Hz, H-2″), 6.51 (1H, dd, J=8.19, 1.94 Hz, H-6′), 6.57 (1H, dd, J=8.19, 1.94 Hz, H-6″), 6.63 (1H, d, J=1.94 Hz, H-2′), 6.75 (1H, d, J=8.19 Hz, H-5′), 6.76 (1H, d, J=8.19 Hz, H-5′). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{282}$ −0.65, $\Delta\varepsilon_{233}$ −3.08 ($dm^3\,mol^{-1}\,cm^{-1}$).

Compound **4** [(2*R*,3*R*)-2-(3′-Hydroxybenzyl)-3-(3″,4″-dimethoxybenzyl)-butyrolactone]: Amorphous powder. $[\alpha]_D^{25}$ −51.1° (c=0.12, MeOH). UV λ_{max}^{MeOH} (ε): 224 (11000), 276 (5000) nm. IR (KBr) ν_{max}: 3309 (–OH), 1747 (γ-lactone CO) cm^{-1}. EI-MS *m/z*: 342 $[M]^+$. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 2.47—2.51 (2H, m, H-3, H_a-7″), 2.59—2.62 (2H, m, H-2, H_b-7″), 2.89 (1H, dd, J=14.01, 6.77 Hz, H_a-7′), 2.98 (1H, dd, J=14.01, 5.30 Hz, H_b-7′), 3.82 (3H, s, –OC$\underline{H}_3$), 3.85 (3H, s, –OC$\underline{H}_3$), 3.88 (1H, dd, J=9.18, 7.72 Hz, H_a-4), 4.14 (1H, dd, J=9.18, 7.24 Hz, H_b-4), 6.48 (1H, d, J=2.18 Hz, H-2″), 6.56 (1H, dd, J=7.99, 1.94 Hz, H-6″), 6.61 (1H, t, J=2.18 Hz, H-2′), 6.68 (1H, br d, H-6′), 6.71 (1H, dd, H-4′), 6.76 (1H, d, J=7.99 Hz, H-5″), 7.14 (1H, t, J=7.72 Hz, H-5′). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{280}$ −0.46, $\Delta\varepsilon_{231}$ −3.11 ($dm^3\,mol^{-1}\,cm^{-1}$).

Compound **5** [(2*R*,3*R*)-2-(3′-Hydroxybenzyl)-3-(3″-hydroxy-4″-methoxybenzyl)butyrolactone]: Amorphous powder. $[\alpha]_D^{25}$ −30.7° (c=0.10, MeOH). UV λ_{max}^{MeOH} (ε): 220 (10000), 279 (4100) nm. IR (KBr) ν_{max}: 3450 (–OH), 1747 (γ-lactone CO) cm^{-1}. EI-MS *m/z*: 328 $[M]^+$. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 2.43—2.48 (2H, m, H-3, H_a-7″), 2.56—2.59 (2H, m, H-2, H_b-7″), 2.90 (1H, dd, J=13.77, 6.77 Hz, Ha-7′), 2.97 (1H, dd, J=13.77, 5.30 Hz, H_b-7′), 3.85 (1H, dd, J=8.94, 7.72 Hz, H_a-4), 3.87 (3H, s, –OC$\underline{H}_3$), 4.10 (1H, dd, J=8.94, 7.24 Hz, H_b-4), 6.50 (1H, dd, J=7.95, 2.18 Hz, H-6″), 6.59 (1H, d, J=2.18 Hz, H-2″), 6.61 (1H, t, J=2.0 Hz, H-2′), 6.72 (1H, m, H-6′), 6.74 (1H, m, H-4′), 6.74 (1H, d, J=7.95 Hz, H-5″), 7.17 (1H, t, J=7.72 Hz, H-5′). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{279}$ −0.46, $\Delta\varepsilon_{229}$ −3.10 ($dm^3\,mol^{-1}\,cm^{-1}$).

Compound **6** [(2*R*,3*R*)-2-(3′-Hydroxybenzyl)-3-(3″,4″-dihydroxybenzyl)-butyrolactone]: Amorphous powder. $[\alpha]_D^{25}$ −36.7° (c=0.12, MeOH). UV λ_{max}^{MeOH} (ε): 220 (12000), 281 (4500) nm. IR (KBr) ν_{max}: 3332 (–OH), 1751 (γ-lactone CO) cm^{-1}. EI-MS *m/z*: 314 $[M]^+$. ^{1}H-NMR (CD_3OD, 400 MHz): δ 2.35 (1H, m, H_a-7″), 2.47 (1H, m, H-3), 2.51 (1H, m, H_b-7″), 2.65 (1H, m, H-2), 2.83 (1H, dd, J=14.01, 7.0 Hz, H_a-7′), 2.92 (1H, dd, J=14.01, 5.30 Hz, H_b-7′), 3.85 (1H, t, J=8.94, H_a-4), 4.05 (1H, dd, J=8.94, 7.24 Hz, H_b-4), 6.37 (1H, dd, J=7.95, 2.18 Hz, H-6″), 6.50 (1H, d, J=2.18 Hz, H-2″), 6.63—6.66 (4H, m, H-2′, H-4′, H-6′, H-5″), 7.10 (1H, t, J=7.95 Hz, H-5′). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{281}$ −0.51, $\Delta\varepsilon_{230}$ −3.13 ($dm^3\,mol^{-1}\,cm^{-1}$).

Compound **7** [(−)-Enterolactone]: Amorphous powder. $[\alpha]_D^{25}$ −46.0° (c=0.15, MeOH). UV λ_{max}^{MeOH} (ε): 218 (10000), 275 (3800), 281 sh (3500) nm. IR (KBr) ν_{max}: 3394 (–OH), 1747 (γ-lactone CO) cm^{-1}. EI-MS *m/z*: 298 $[M]^+$. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 2.50 (2H, m, H_a-7″, H-3), 2.60 (2H, m, H_b-7″, H-2), 2.88 (1H, dd, J=14.01, 6.77 Hz, H_a-7′), 2.96 (1H, dd, J=14.01, 5.30 Hz, H_b-7′), 3.90 (1H, dd, J=9.18, 7.52 Hz, H_a-4), 4.12 (1H, dd, J=9.18, 7.00 Hz, H_b-4), 6.47 (1H, t, J=1.90 Hz, H-2″), 6.58 (1H, br d, J=7.76 Hz, H-6″), 6.61 (1H, t, J=1.9 Hz, H-2′), 6.69—6.75 (3H, m, H-4′, 6′, 4″), 7.13 (1H, t, J=7.90 Hz, H-5′), 7.16 (1H, t, J=7.90 Hz, H-5″). ^{13}C-NMR: Table 1. CD (MeOH): $\Delta\varepsilon_{280}$ −0.26, $\Delta\varepsilon_{220}$ −3.60 ($dm^3\,mol^{-1}\,cm^{-1}$).

Time Course for the Transformation of Arctiin (1) by HIB Sixty microliters of 100 mM arctiin (**1**) in MeOH and 600 μl of an HIB mixture were added to 6 ml of GAM broth, and the mixture was incubated at 37 °C under anaerobic conditions. A 100 μl aliquot was removed at intervals and 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 water to a volume of 1 ml and filtered through a 0.2 μm membrane filter, and a 5 μl portion was injected on a column for HPLC analysis. Metabolites were well separated and detected under the conditions mentioned above. Concentrations of arctiin (**1**) and its metabolites were calculated according to the calibration curves of the respective authentic samples.

Effects of Arctiin (1) and Its Metabolites on the Growth of Human Breast Cancer MCF-7 Cells Human breast cancer MCF-7 cells were purchased from the Institute of Physical and Chemical Research (Wako, Japan). Medium A: Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies) supplemented with 5% fetal bovine serum (FBS, Gibco BRL, Life Technologies), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Gibco BRL, Life Technologies). Medium B: Phenol red-free DMEM (Gibco BRL, Life Technologies, New York, U.S.A.) supplemented with 10% heat-inactivated charcoal/dextran-treated human serum.[25] MCF-7 cells were maintained in medium A and subcultured for 3 or 4 d. The cells were collected by trypsinization (0.25% trypsin, Nacalai Tesque, Kyoto, Japan) and suspended in medium B (5000 cells/100 μl), then seeded in a 96-well culture plate (100 μl/well). After 24 h culture, the medium was changed with 90 μl of fresh medium (medium B) and 10 μl of a sample solution. After continuous culture for 4 d, the proliferation of cells was assessed using an MTT assay.[26] Estradiol, arctiin (**1**), and its metabolites were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10^{-2} M and diluted with medium B before use.

Effects of Compound 4 and (−)-Enterolactone (7) on Estradiol-Mediated Proliferation of MCF-7 Cells Tamoxifen was dissolved in DMSO to give a concentration of 10^{-2} M and the solution was diluted with medium B before use. Either tamoxifen (final concentration 1 μM), compound **6** (final concentration 1 or 10 μM), or (−)-enterolactone (**7**) (final concentration 1 or 10 μM) was added to the culture containing 10^{-10} M estradiol as described above.

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