Metabolic activation of lignans to estrogenic and anti-estrogenic substances by human intestinal bacteria

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The role of human intestinal bacteria is indispensable for the metabolic activation of phytoestrogenic precursor lignans. This review summarized recent researches on the *in vitro* intestinal bacteria transformation of lignan precursors, secoisolariciresinol diglucoside (SDG), arctiin, pinoresinol diglucoside (PDG), and their aglycones, to mammalian lignans enterodiol (END) and enterolactone (ENL). The isolation and characterization of several bacteria from human feces, responsible for the related reactions were also described.

Key words Phytoestrogen, lignan, mammalian, metabolism, intestinal bacteria.

Abbreviations ENL, enterolactone; END, enterodiol; SECO, secoisolariciresinol; MAT, matairesinol; SDG, secoisolariciresinol diglucoside; PDG, pinoresinol diglucoside.

1. Introduction

Intestinal flora takes part in an important role for the activation of orally ingested substances. Depending on the metabolic action of the bacteria which makes its domicile in the gastrointestinal tract, some precursory compounds are transformed into various metabolites. To date, it has been discovered that one after another, after being absorbed, the metabolites showed more potential physiological activity than their precursors. Among them, phytoestrogens are the most typical examples.

2,3-Bis(3-hydroxybenzyl)-γ-butyrolactone and 2,3-bis (3-hydroxybenzyl)butane-1,4-diol, known respectively as enterolactone (ENL, **1**) and enterodiol (END, **2**),[1,2] are two most famous phytoestrogenic lignans (Chart 1). They were identified 25 years ago firstly in serum, urine and bile of humans and several animals,[2-6] and given the name mammalian lignans to distinguish them from plant-derived lignans since they carry phenolic hydroxy groups only in the *meta* position of the aromatic rings, differing from the plant-derived lignans. As being structurally similar with several estrogen-like substances, such as dienoestrol, diethylstilboestrol and its dihydroderivative hexoestrol, they received increasing attention on their estrogen-like biological properties later.[7-11] Moreover, from many epidemiological studies, the evidence that high concentrations of ENL (**1**) and END (**2**) in body fluids correlated with a low incidence of hormone-mediated diseases, in particular breast cancer, has been provided.[12-15] These compounds have been considered to be potential anti-cancer agents by the United States National Cancer Institute.[16]

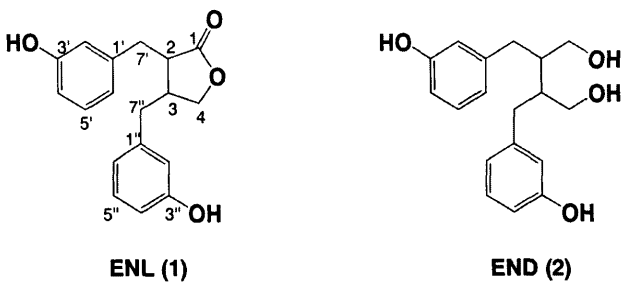

Chart 1 Structures of mammalian lignans ENL (**1**) and END (**2**).

The origins of mammalian lignans found in human and animal biological fluids are considered to be plant lignans existed in whole-grain cereals, seeds, nuts, vegetables, and some herbs,[17-20] which are finally transformed to mammalian lignans by intestinal microflora in the proximal colon. According to epidemiological studies, people living in areas with a higher phytoestrogen consumption and a higher plasma concentration of ligans was found to have a lower cancer risk.[21-23] On the other hand, antibiotic treatment can decrease the serum concentration and urinary excretion of ENL (**1**) and END (**2**) in human subjects.[1,12,24] All these findings emphasize the importance of intestinal bacteria for the metabolic activation of orally consumed plant lignans.

Till now at least 13 plant lignans have been identified as the precursors of mammalian lignans and their *in vivo* or/and *in vitro* metabolism are also under investigation by many research groups. This review chiefly describes about the *in vitro* transformation of lignan precursors (Chart 2) by intestinal microflora, as well as isolation and characterization of some bacteria responsible for the related reactions.

2. Secoisolariciresinol and secoisolariciresinol diglucoside

The pioneer metabolic studies on the production and metabolism of mammalian lignans from plant precursors by intestinal microflora were carried out by Borriello *et al.* in 1985, using flaxseed and its components secoisolariciresinol

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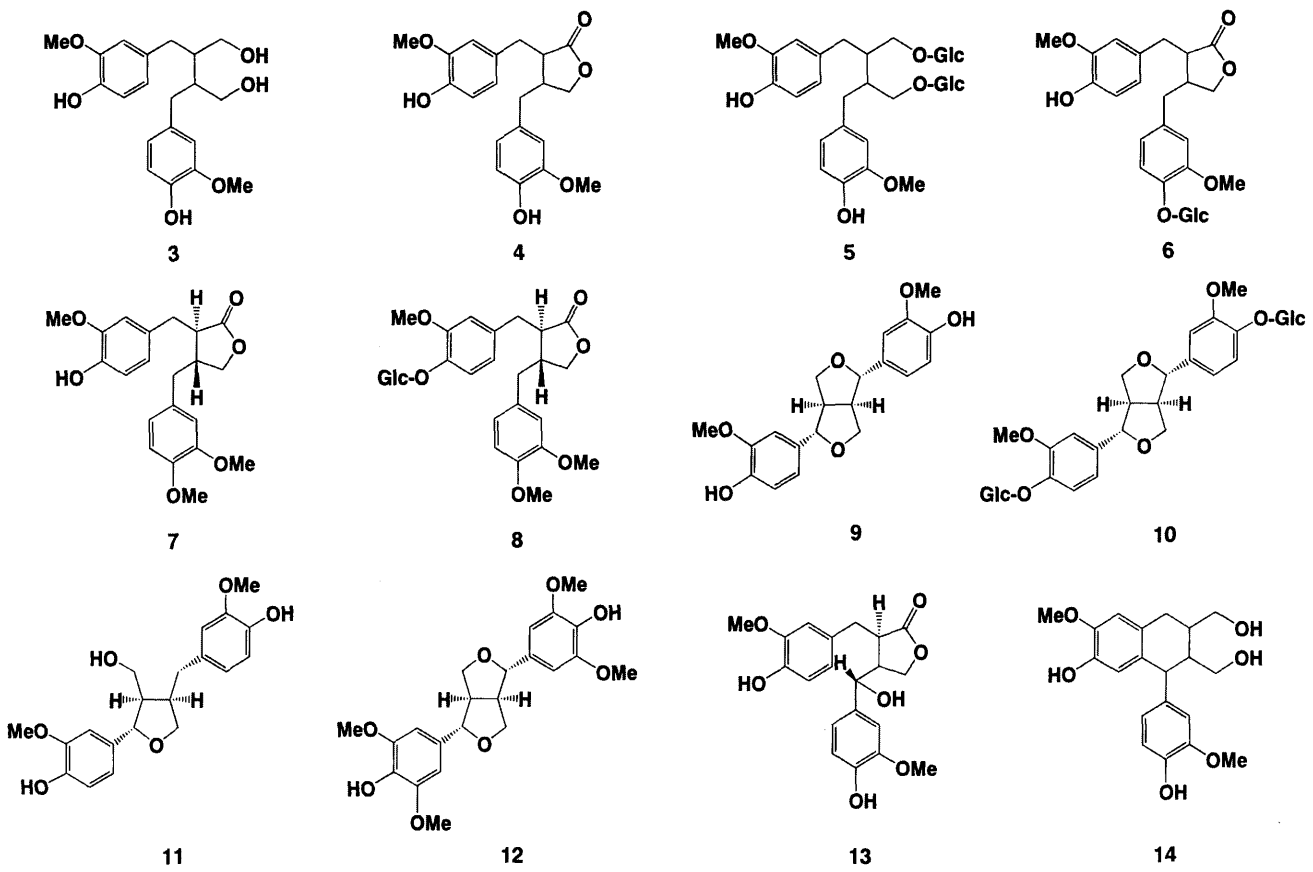

Chart 2 Structures of plant precursors of ENL (**1**) and END (**2**) mentioned in this review.
3, SECO; **4**, MAT; **5**, SDG; **6**, matairesinoside; **7**, arctigenin; **8**, arctiin; **9**, (+)-pinoresinol; **10**, PDG; **11**, (+)-lariciresinol; **12**, syringaresinol; **13**, 7-hydroxymatairesinol; **14**, isolariciresinol.

(SECO, **3**) and matairesinol (MAT, **4**), aglycones of secoisolariciresinol diglucoside (SDG, **5**) and matairesinoside (**6**), respectively.[25] SECO (**3**) was an initially known lignan precursor of mammalian lignans.[17] By co-incubating original substances with a human fecal suspension, they found that human fecal microflora was able to produce END (**2**) and ENL (**1**) from SECO (**3**) and other plant lignans contained in flaxseed. As for the metabolic reactions, they speculated that SECO (**3**) was metabolized to END (**2**) probably through dehydroxylation and then demethylation by facultative bacteria; END (**2**) was subsequently oxidized by facultative bacteria to yield ENL (**1**). In addition, they deduced a similar metabolism for MAT (**4**), which was transformed to ENL (**1**) through dehydroxylation and demethylation by facultative bacteria.[25]

It is noteworthy that, though some information was obtained as mentioned above, the metabolic pathways to ENL (**1**) and END (**2**) from lignan precursors were not clarified until 2000, when Hattori's group carried out an *in vitro* biotransformation of SDG (**5**) using human intestinal bacteria.[26] SDG is a main component in flaxseed,[27] which is the richest known source of phytoestrogenic lignans.[20] After anaerobic incubation of SDG (**5**) with a human fecal suspension in GAM broth, seven metabolites including four new ones were isolated by silica gel, Sephadex LH-20 and RP-18 chromatography. They were identified as (+)-SECO (**3**), 3-demethyl-(+)-secoisolariciresinol (**15**), 2-(3-hydroxylbenzyl)-3-(4-hydroxy-3-methoxylbenxyl)butane-1,4-diol (**16**), didemethylsecoisolariciresinol (**17**), 2-(3-hydroxylbenzyl)-3-(3,4-dihydroxybenzyl)butane-1,4-diol (**18**), (+)-END (**2**) and (+)-ENL (**1**) (Chart 3). Furthermore, two bacterial strains, SDG-1 and SDG-2, capable of the demethylation and dehydroxylation, respectively, in the transformation of metabolite **15** to END (**2**), were isolated from human feces after repeated culture in GAM broth containing a compound **3** or **17** under anaerobic conditions, followed by colonization. According to comparative biochemical and morphological studies with those reported for *P. productus* isolated from human fecal samples, strain SDG-1 was characterized as a *Peptostreptococcus* sp. and tentatively named strain SDG-1. Similarly, strain SDG-2 was determined to be an *Eubacterium* genus and given a name *E.* sp. strain SDG-2.

Based on the structures of seven metabolites and the time course experiments using two isolates and a human intestinal bacteria mixture monitored by HPLC, a possible metabolic process was deduced for the transformation of SDG (**5**) to ENL (**1**) (Chart 3). The transformation included four types of reactions, hydrolysis of glucoside, demethylation of a methoxy group, dehydroxylation of a 4-hydroxy group in the 3,4-dihydroxyphenyl moiety, and oxidation of dibenzylbutanediol to dibenzylbutyrolactone. Since mono- and di-demethylated metabolites **15** and **17**

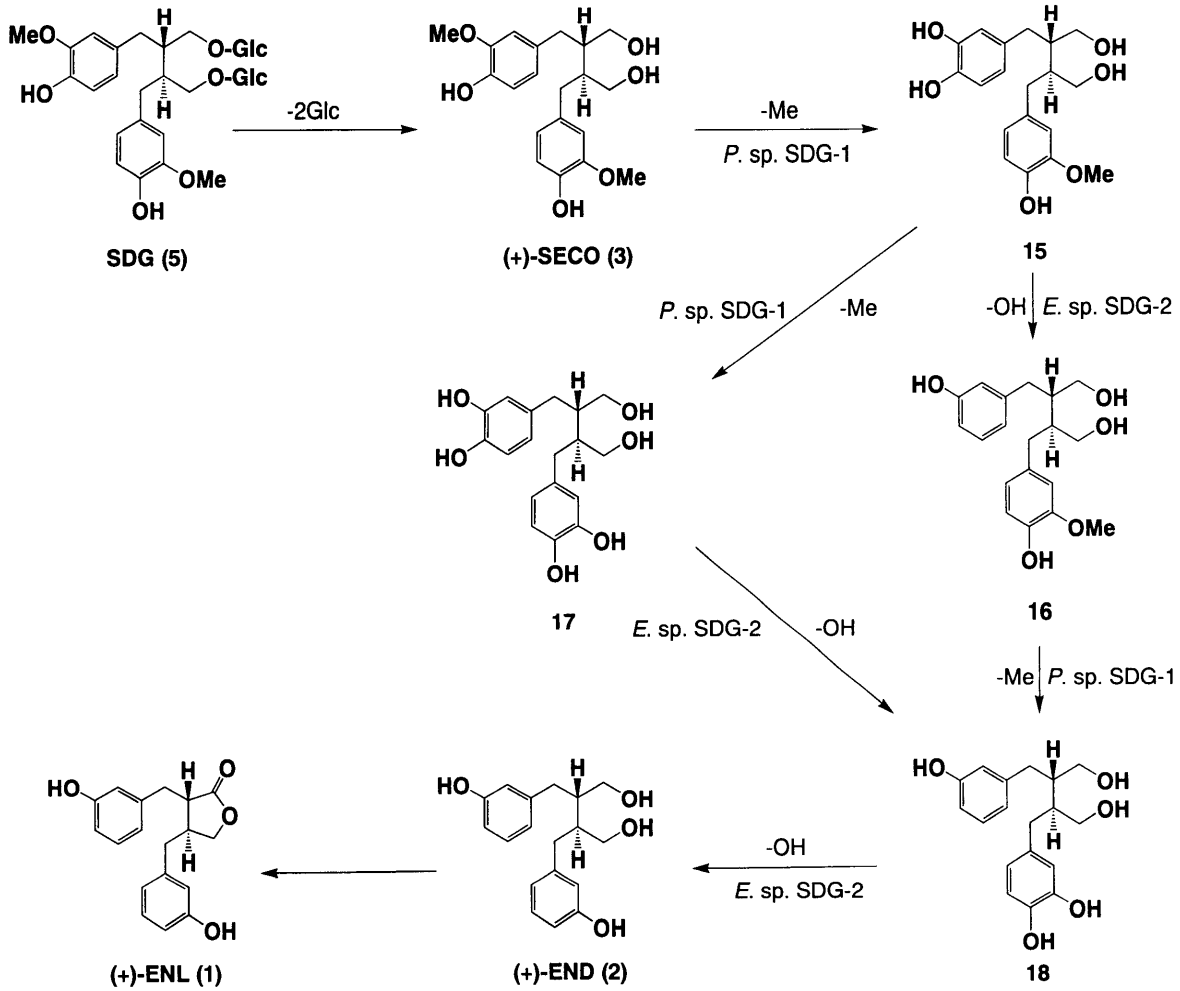

Chart 3 Possible pathway for the transformation of SDG (**5**) by *P.* sp. strain SDG-1, *E.* sp. strain SDG-2 and human intestinal bacteria (from Ref. 26).

were firstly formed by incubation with strain SGD-1 in a short period of time, in contrast with dehydroxylated ones **18** and END (**2**) with strain SGD-2, the authors concluded that the demethylation probably took place prior to dehydroxylation. Their findings provided an unequivocal and concrete *in vitro* evidence for the role of human intestinal bacteria in the metabolism of plant-derived lignans to mammalian lignans.

The metabolic action of a human intestinal bacterium, *E.* sp. strain SDG-2, on *para*-dehydroxylation of the aromatic ring in the metabolic processes, deeply aroused the authors' interests to investigate whether this bacterium was able to catalyze *p*-dehydroxylation of the catechol moiety of catechines, a group of polyphenolic flavan-3-ols, which they have previously found to be converted to *para*-dehydroxylated metabolites by human and rat fecal bacteria *in vitro*.[28] As shown in Chart 4, they found interesting findings when various (3*R*)- and (3*S*)-flavan-3-ols were anaerobically incubated with *E.* sp. strain SDG-2: this bacterium was capable of cleaving the heterocyclic ring (C-ring) for all six catechins [(+)-catechin (**19**), (+)-epicatechin (**20**), (-)-catechin (**21**), (-)-epicatechin (**22**), (-)-gallocatechin (**23**), and (-)-epigallocatechin (**24**)], but not their gallates; the further conversion, *p*-dehydroxylation of B-ring, only occurred in (3*R*)-flavan-3-ols (**21** and **22** *via* **26** to **27**, **23** and **24** *via* an intermediate to **28**), but not (3*S*)-flavan-3-ols (**19** and **20** to **25** only).[29] It seems reasonable that, the stereochemistry of a free hydroxy group at C-3 is closely related with the ring fission and subsequent dehydroxylation of a *para*-hydroxy group by *E.* sp. strain SDG-2.

3. Arctigenin and arctiin

Arctigenin (**7**) and its glucoside arctiin (**8**) are lignans bearing a butyro-lactone residue. They are abundant in the seeds of *Arctium lappa* Linne, which are used in traditional Chinese medicine and folk medicine in Japan. Nose *et al.* reported the metabolism of arctiin (**8**) using both rat gastric juice and rat intestinal microflora *in vitro*.[30] Arctiin (**8**) was rapidly transformed to arctigenin (**7**) and 2-(3",4"-dihydroxybenzyl)-3-(3',4'-dimethoxylbenzyl)butyrolactone (AM2, **29**) with a rat intestinal suspension, but not converted to any metabolites in a rat gastric juice (Chart 5). They further analyzed the metabolites of orally administrated arctiin (**8**) in rats.[31] However, AM2 (**29**) was not detected in rat sera. Since all the metabolites were found in the large intestine, they speculated that the metabolite **29** would be returned to the original compound (**8**) through

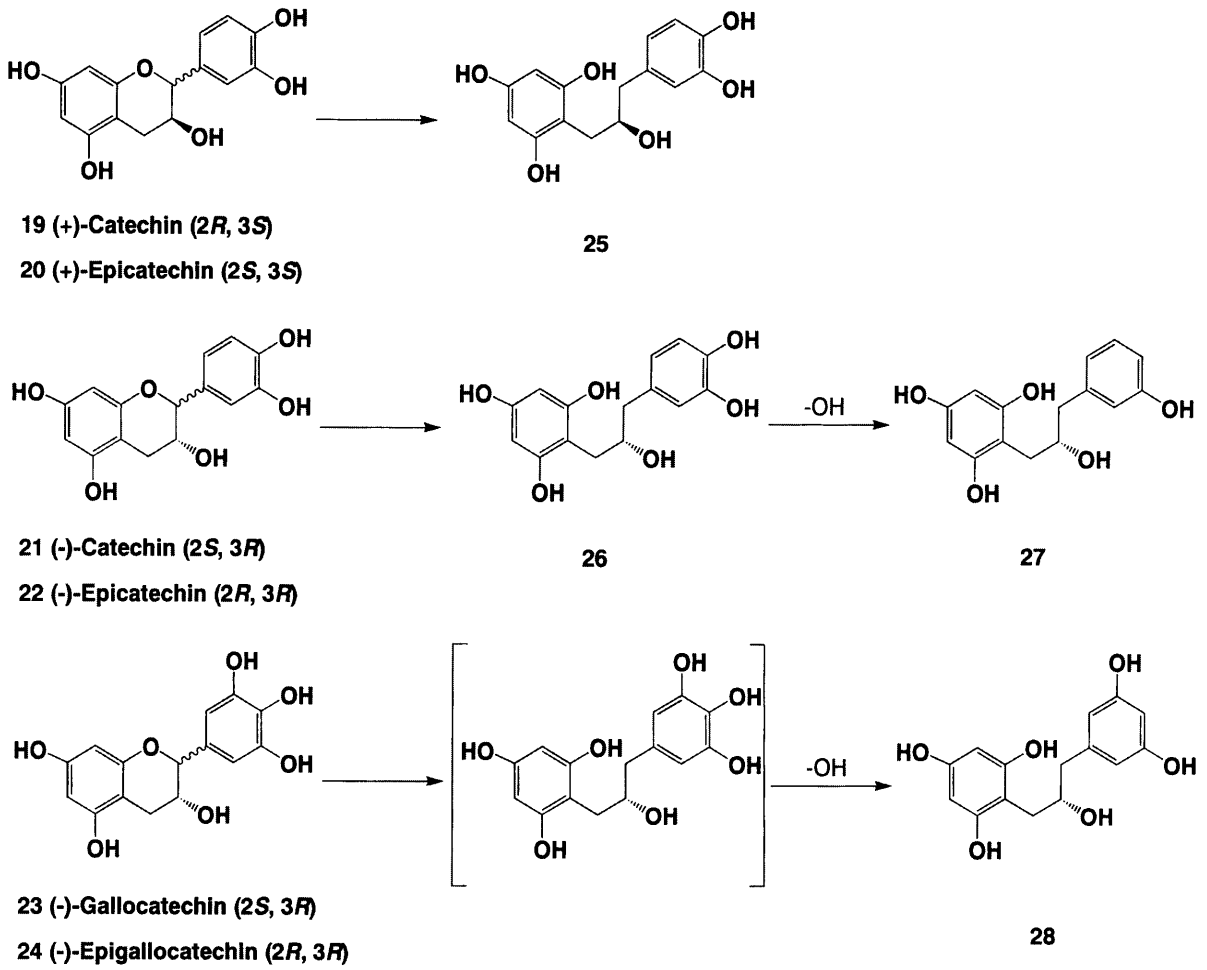

Chart 4 Possible metabolic pathway of catechins (**19-24**) by *E.* sp. strain SDG-2 (from Ref. 29).

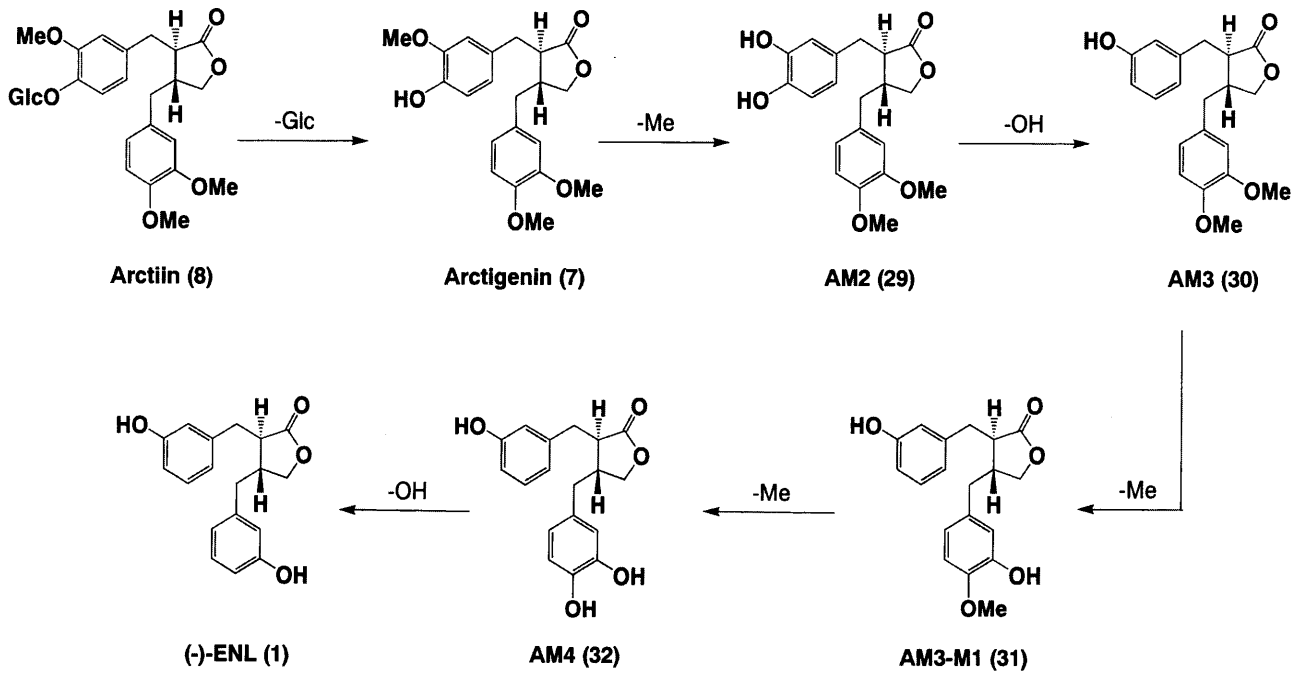

Chart 5 Possible pathway for the transformation of arctiin (**8**) by human intestinal bacteria (from Ref. 33).

methylation by catechol-*O*-methyltransferase in the liver.

Using a GC-MS technique, Heinonen *et al.* found that 4% of arctigenin (**7**) was metabolized to ENL (**1**) after a 24h fecal incubation.[32] In addition to ENL (**1**), AM2 (**29**) and a new metabolite 2-(3'-hydroxybenzyl)-3-(3",4"-dimethoxylbenzyl)butyrolactone (AM3, **30**) were tentatively identified on the basis of MS spectral analysis. No intermediates from AM3 (**30**) to ENL (**1**) were detected.

Since the metabolic pathway to ENL (**1**) from arctigenin (**7**) was not clearly explained, Hattori's group recently performed a metabolic study on arctiin (**8**) using a human intestinal bacterial mixture.[33] After anaerobic incubation, six metabolites including those proposed by Heinonen *et al.*[32] were isolated and identified as (-)-arctigenin (**7**), AM2 (**29**) and AM3 (**30**), and (2*R*,3*R*)-2-(3'-hydroxybenzyl)-3-(3"-hydroxy-4"-methoxybenzyl)butyrolactone (AM3-M1, **31**), (2*R*,3*R*)-2-(3'-hydroxybenzyl)-3-(3",4"-dihydroxybenzyl)butyrolactone (AM4, **32**), and (-)-ENL (**1**) by EI-MS, NMR and CD spectral means (Chart 5). Interestingly, on the basis of the $[\alpha]_D$ and CD spectral evidence, it was shown that ENL (**1**) obtained from arctiin (**8**) had a (-)-(2*R*,3*R*) configuration, while that from SDG (**5**) had a (+)-(2*S*,3*S*) configuration. Accordingly, the authors indicated that the intestinal bacteria did not change the absolute configuration of lignans examined during the transformation of precursor lignans to ENL (**1**) and END (**2**), which includes a variety of reactions.

With six metabolites, the possible pathway for the transformation of arctiin (**8**) by human intestinal bacteria was proposed as shown in Chart 5.[33] Arctiin (**8**) underwent the first three steps of reactions mentioned above for SDG (**5**) to eventually give a mammalian lignan, (-)-ENL (**1**). In comparison with the case of SDG (**5**), the authors drew the inference that a methoxy group adjacent to a hydroxy group seemed to be easily demethylated in contrast with two vicinal methoxy groups; in the 3,4-dihydroxyphenyl ring, demethylation preferentially occurs on the methoxy group at C-3.

More consequentially, using human breast cancer MCF-7 cells, they examined the estrogenic and antiestrogenic activity of parent compound arctiin (**8**) and its metabolites. As a result, (-)-ENL (**1**) stimulated cell growth at a high concentration of 10 μM, and meaningfully, compound **32** potentially showed both estrogenic activity on the tumor growth and antiestrogenic activity on the estradiol-mediated proliferation of MCF-7 cells (Fig. 1 and 2). It was considered to be a biphasic effect of phytoestrogens.[34] Their study is the first example of ENL(**1**) with a (2*R*,3*R*) configuration for estrogenic activity.

4. Pinoresinol and pinoresinol diglucoside

Pinoresinol (**9**) contained in almost all commonly consumed foods,[20] was one of the newly reported mammalian lignan precursors by Heinonen *et al.*[32] According to their report, 55% of pinoresinol (**9**) was converted to ENL (**1**) and END (**2**) during a 24h incubation with a human fecal suspension. The authors assumed that pinoresinol (**9**) was transformed to mammalian lignans *via* lariciresinol (**11**) and SECO (**3**) or/and MAT (**4**), similar to the biosynthetic pathway of compounds **3** and **4** in the plants.

More recently, Hattori's group carried out an investigation to throw light on the bacterial metabolism of pinoresinol (**9**).[35] Using pinoresinol diglucoside (PDG, **10**), a major antihypertensive principle of Du-Zhong (the bark of *Eucommia ulmoides*) used in traditional Chinese medicine,[36]

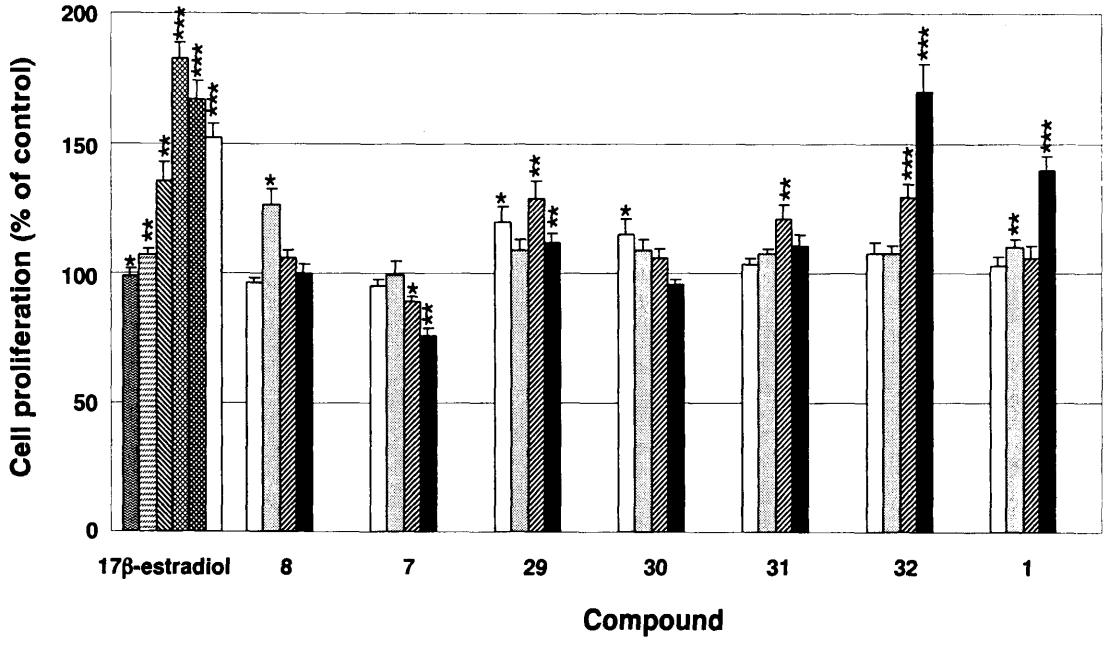

Fig. 1 Effect of arctiin (**8**) and its metabolites on the growth of breast cancer MCF-7 cells in culture (from Ref. 33).
17β-estradiol: 10^{-13} M, 10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M, and 10^{-8} M (from left to right). Compounds **8**, **7**, **29**-**32** and **1**: 10^{-8} M, 10^{-7} 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.

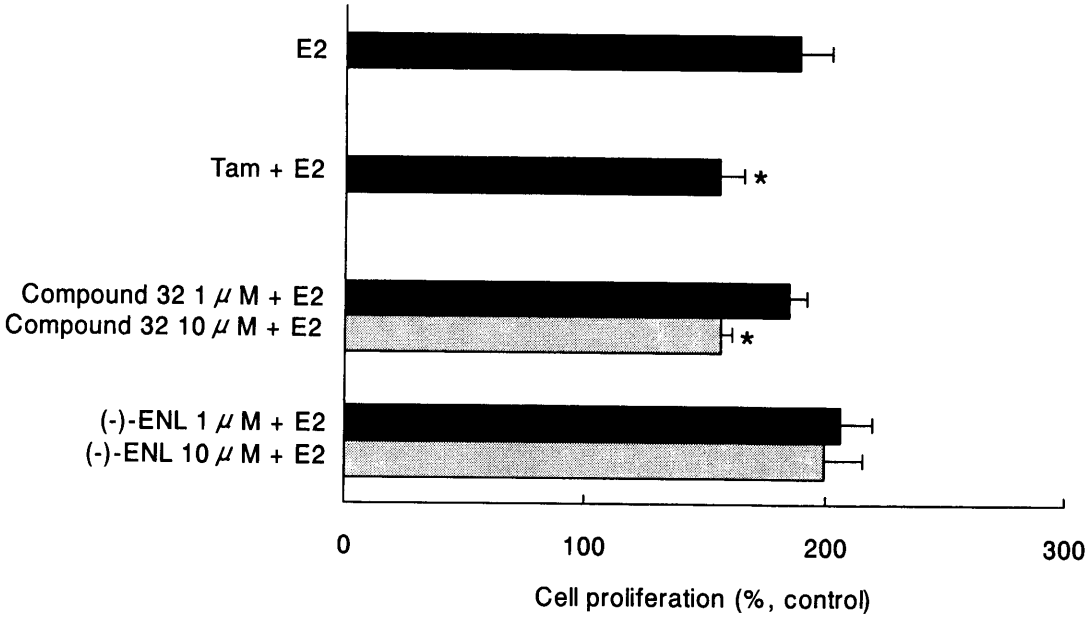

Fig. 2 Influence of compound **32** and (-)-ENL (**1**) on the proliferation of human breast cancer MCF-7 cells in the presence of 17β-estradiol (from Ref. 33).
E2, 17β-estradiol (10^{-10} M); Tam, tamoxifen (1 μM), *p<0.05 *vs.* E2 group (n=6).

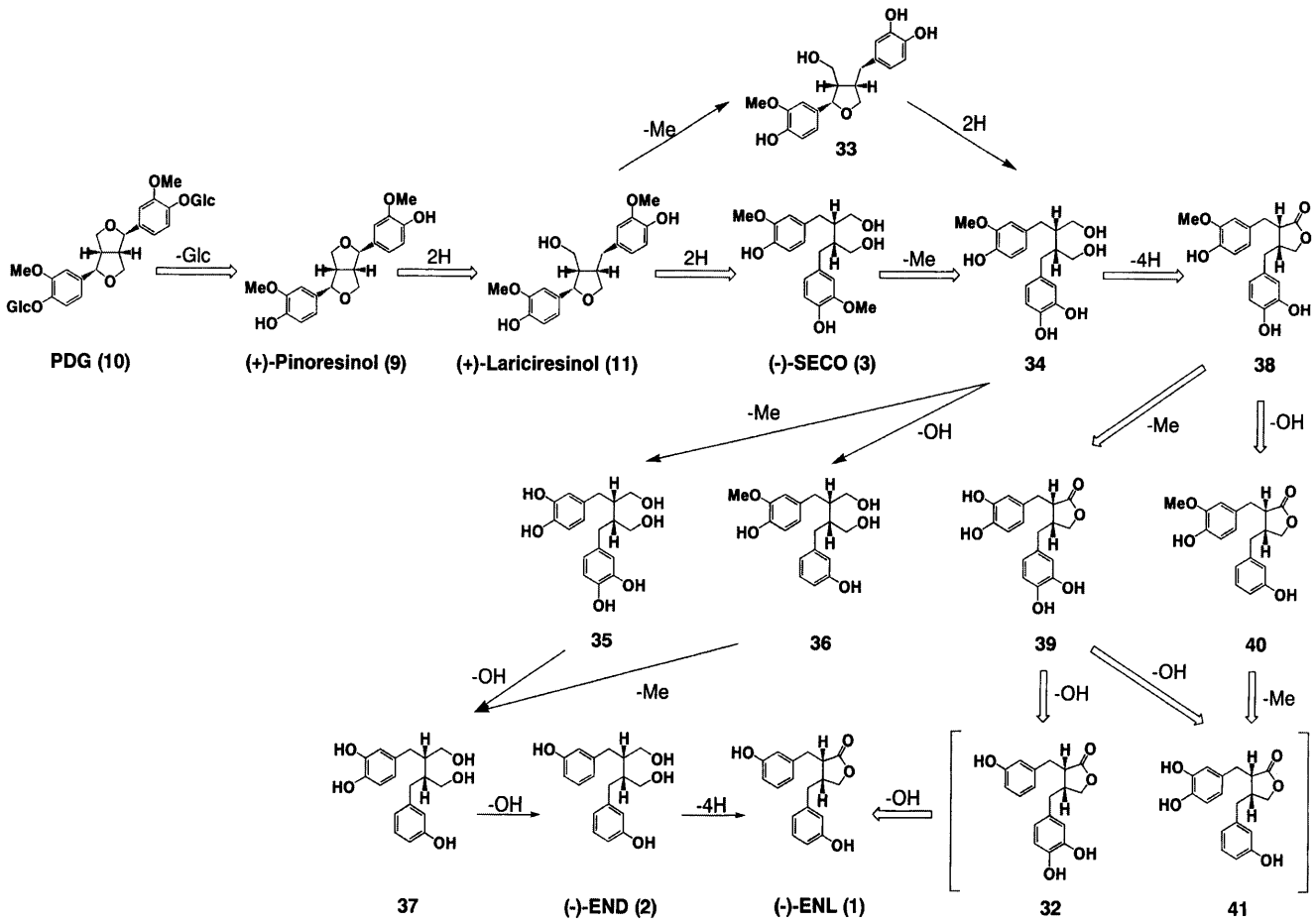

Chart 6 Possible pathway for the transformation of PDG (**10**) by human intestinal bacteria (from Ref. 35).
=> Major pathway;→ Minor pathway. Conversions: **35**→**39**: **36**→**40**; **37**→**41** or **32** are also considered to be possible.

15 metabolites were isolated after anaerobic incubation with human intestinal bacteria (Chart 6). They were identified as (+)-pinoresinol (**9**), (+)-lariciresinol (**11**), 3'-demethyl-(+)-lariciresinol (**33**), (-)-SECO (**3**), (-)-3-(3",4"-dihydroxybenzyl)-2-(4'-hydroxy-3'-methoxybenzyl)butane-1,4-diol (**34**), 2-(3',4'-dihydroxybenzyl)-3-(3",4"-dihydroxybenzyl)butane-1,4-diol (**35**), 3-(3"-hydroxybenzyl)-2-(4'-hydroxy-3'-methoxybenzyl)butane-1,4-diol (**36**), 2-(3',4'-dihydroxybenzyl)-3-(3"-hydroxybenzyl)butane-1,4-diol (**37**), (-)-END (**2**), (-)-(2*R*,3*R*)-3-(3",4"-dihydroxybenzyl)-2-(4'-hydroxy-3'-methoxybenzyl) butyrolactone (**38**), (-)-(2*R*,3*R*)-2-(3',4'-dihydroxybenzyl)-3-(3",4"-dihydroxybenzyl)butyrolactone (**39**), (-)-(2*R*,3*R*)-3-(3"-hydroxybenzyl)-2-(4'-hydroxy-3'-methoxybenzyl) butyrolactone (**40**), 2-(3',4'-dihydroxybenzyl)-3-(3"-hydroxybenzyl)butyrolactone (**41**), AM2 (**32**), and (-)-ENL (**1**), by various spectroscopic means. With these metabolites, the authors deduced a possible metabolic pathway on the basis of the time course experiment monitored by TLC (Chart 6).[35] Five types of reactions were involved in the conversion of PDG (**10**) to ENL (**1**) and END (**2**): reductive cleavage of furofuran rings, in addition to those reactions mentioned above for SDG (**5**).

Furthermore, as the first bacterium capable of the reductive cleavage of an ether linkage of furofuran lignans, a bacterial strain with high capability and specificity for transforming (+)-pinoresinol (**9**) to (+)-lariciresinol (**11**) was isolated. According to the biochemical characteristics compared with those of *Enterococcus faecalis* and *Enterococcus faecium,* this strain was characterized as *E. faecalis* genus and named strain PDG-1.

Synthetically comparing the transformations of PDG (**10**), arctiin (**8**) and SDG (**5**), the authors concluded that the conversions of these precursors to mammalian lignans by human intestinal microflora have five similarities: 1) the first step of the transformation is hydrolysis of glucosides to their aglycones and it is generally completed within 12h; 2) the methoxy group adjacent to a hydroxy group is easily demethylated comparing with that of two vicinal methoxy group; 3) dehydroxylation always occurs after a 4-hydroxy-3-methoxyphenyl moiety has been demethylated to a 3,4-dihydroxyphenyl moiety, and a 4-hydroxy group is exclusively eliminated to leave another 3-hydroxy group, which is resistant to further dehydroxylation; 4) dibenzylbutanediols may be oxidized to dibenzylbutyrolactone at each step, in which the oxidation seems to proceed with no change in the absolute configuration; 5) the reductive ring fission of furofurans to dibenzylbutanes *via* furans (**10** to **3** *via* **11**) maintains the stereochemistry at C-8 and C-8' in furofuran and furans or at C-2 and C-3 in dibenzylbutanes.

5. Others

In addition to the above mentioned lignan precursors, of which the metabolism by human intestinal bacteria have been thoroughly studied, Heinonen *et al.* examined the *in vitro* metabolic properties as mammalian lignan precursors of syringaresinol (**12**), 7-hydroxymatairesinol (**13**), and isolariciresinol (**14**), as well as five other ones adverted in this chapter (**3**, **4**, **7**, **9**, and **11**).[32] By anaerobiclly incubating **12**－**14** separately with a human fecal suspension for 24 h, syringaresinol (**12**) was partly converted to ENL (**1**) and END (**2**) (4%), 7-hydroxymatairesinol (**13**) to ENL (**1**) and 7-hydroxyenterolactone (23%), while isolariciresinol (**14**) remained mostly unchanged. Though some metabolites were tentatively identified by the interpretation of their mass spectra, the metabolism was not studied in detail.

6. Conclusion

Since the first report of ENL (**1**) and END (**2**), many studies on phytoestrogens were conducted in regard to their source, metabolism, pharmacological effects, and relationship with human health.

The lignan precursors identified up to now may be divided into four types: dibenzylbutanes (**3**, **5**, **14** and secoisolariciresinol 4-*O*-β-D-glucopyranoside), dibenzylbutyrolactones (**4**, **6**, **7**, **8** and **13**), furano lignans (**11**), and furofuran lignans (**9**, **10** and **12**). Of them, SDG (**5**), arctiin (**8**), and PDG (**10**) are the only three, of which the transformation pathways by human intestinal microflora were extensively studied. According to their *in vitro* transformation by intestinal bacteria mentioned above, these compounds may be hydrolysed, demethylated, dehydroxylated and oxidized in the gastrointestinal tract. The metabolism of lignans *in vivo* to mammalians, however, is inevitably more complicated and intriguing. Moreover, phytoestrogenic lignans act as estrogen promoting multiplication of breast cancer cells. Thus, further studies on their absorption, distribution and excretion, as well as metabolism, are necessary to determine the amount of phytoestrogens that people should take in for maintaining their health apart from various diseases, such as breast and colon cancers and cardiopathies, associated with estrogens.

Phytoestrogens relating to human health are of increasing interest in the world. In contrast with Europe, especially Scandinavian countries, such as Finland and Sweden, where the concern about lignans are higher in relation with a large consumption of cereals as their food, the attention on phytoestrogenic lignans in Japan is little to date comparing with isoflavones, which are almost exclusively contained in soy and soy products. The purpose of this review is not only to emphasize the importance of intestinal bacteria for the activation of orally ingested drugs but also to promote the investigation on phytoestrogenic lignans.

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the meeting.

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Japanese abstract

ヒト腸内細菌は広く自然界に存在する植物エストロゲンの前駆体の代謝活性化に必須である。

本総説では *in vitro* の実験に基づいた植物エストロゲン前駆体 secoisolariciresinol diglucoside (SDG), arctiin, pinoresinol diglucoside (PDG) やそれらのアグリコンの動物リグナン, enterodiol (END), enterolactone (ENL) への変換に関する最近の研究成果を概説している。また, これら関連反応に関与する腸内細菌の単離や性質についても言及している。

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