In Vitro Metabolism of Plant Lignans: New Precursors of Mammalian Lignans Enterolactone and Enterodiol

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The metabolism of the plant lignans matairesinol, secoisolariciresinol, pinoresinol, syringaresinol, arctigenin, 7-hydroxymatairesinol, isolariciresinol, and lariciresinol by human fecal microflora was investigated to study their properties as mammalian lignan precursors. The quantitative analyses of lignan precursors and the mammalian lignans enterolactone and enterodiol were performed by HPLC with coulometric electrode array detector. The metabolic products, including mammalian lignans, were characterized as trimethylsilyl derivatives by gas chromatography−mass spectrometry. Matairesinol, secoisolariciresinol, lariciresinol, and pinoresinol were converted to mammalian lignans only. Several metabolites were isolated and tentatively identified as for syringaresinol and arctigenin in addition to the mammalian lignans. Metabolites of 7-hydroxymatairesinol were characterized as enterolactone and 7-hydroxyenterolactone by comparison with authentic reference compounds. A metabolic scheme describing the conversion of the most abundant new mammalian lignan precursors, pinoresinol and lariciresinol, is presented.

Keywords: *Phytoestrogens; lignans; matairesinol; secoisolariciresinol; lariciresinol; pinoresinol; syringaresinol; isolariciresinol; arctigenin; 7-hydroxymatairesinol; mammalian lignans; enterolactone; enterodiol*

INTRODUCTION

Experimental and epidemiological studies suggest that high plasma and urinary concentrations of phytoestrogens, including lignans, are associated with decreased risk for hormone-dependent diseases, such as breast cancer, and coronary heart disease (*1*−*3*). The mammalian lignans, enterolactone and enterodiol, are considered to be products of colonic bacterial metabolism of the plant-derived precursors matairesinol and secoisolariciresinol, respectively (*4*) (Figure 1). The metabolism of secoisolariciresinoldiglucoside by human intestinal microflora was recently studied by Wang et al. (*5*). Two bacterial strains capable of transforming secoisolariciresinoldiglucoside to enterolactone and enterodiol were isolated, and four new intermediates were presented.

Mammalian lignan production from various foods, using in vitro fermentation with human fecal microbiota, has been studied (*6*). Flaxseed was reported to be the richest source of precursors of mammalian lignans. However, the lignan contents of the foods were not determined, and the relationship between the amount of plant precursors and mammalian lignans produced was not measured. Ingestion of increasing amounts of flaxseed has been reported to cause a linear increase in the urinary excretion of lignans (*7*, *8*). The lignan composition of flaxseed meal was recently characterized (*7*), and the major lignan was identified as secoisolariciresinol, whereas matairesinol, pinoresinol, and isolariciresinol (Figure 1) were identified as minor lignan components.

Among grains, rye has a relatively high content of the plant lignans matairesinol and secoisolariciresinol (*9*). After rye bread consumption, urinary enterolactone and enterodiol excretion increases ∼7.3-fold (mean value), with a large variation from 1.2 to 10.9, relative to the intake of the plant lignans secoisolariciresinol and matairesinol (*10*−*12*) measured by isotope dilution gas chromatography−mass spectrometry (GC-MS) (*13*). Calculating the fecal proportion of the total mammalian lignan excretion in urine and feces from the published values in the same subjects (*14*, *15*), we found that 34−35% (mean) of the mammalian lignans are excreted by the fecal route in both omnivores and vegetarians. The excretion of the precursors is negligible in individuals not treated with antibiotics. This means that the estimated total excretion of lignans exceeds the amount of secoisolariciresinol and matairesinol in food by 10−12-fold (mean). This observation suggests that there must be some other precursors that can be converted to enterolactone by the action of intestinal bacteria.

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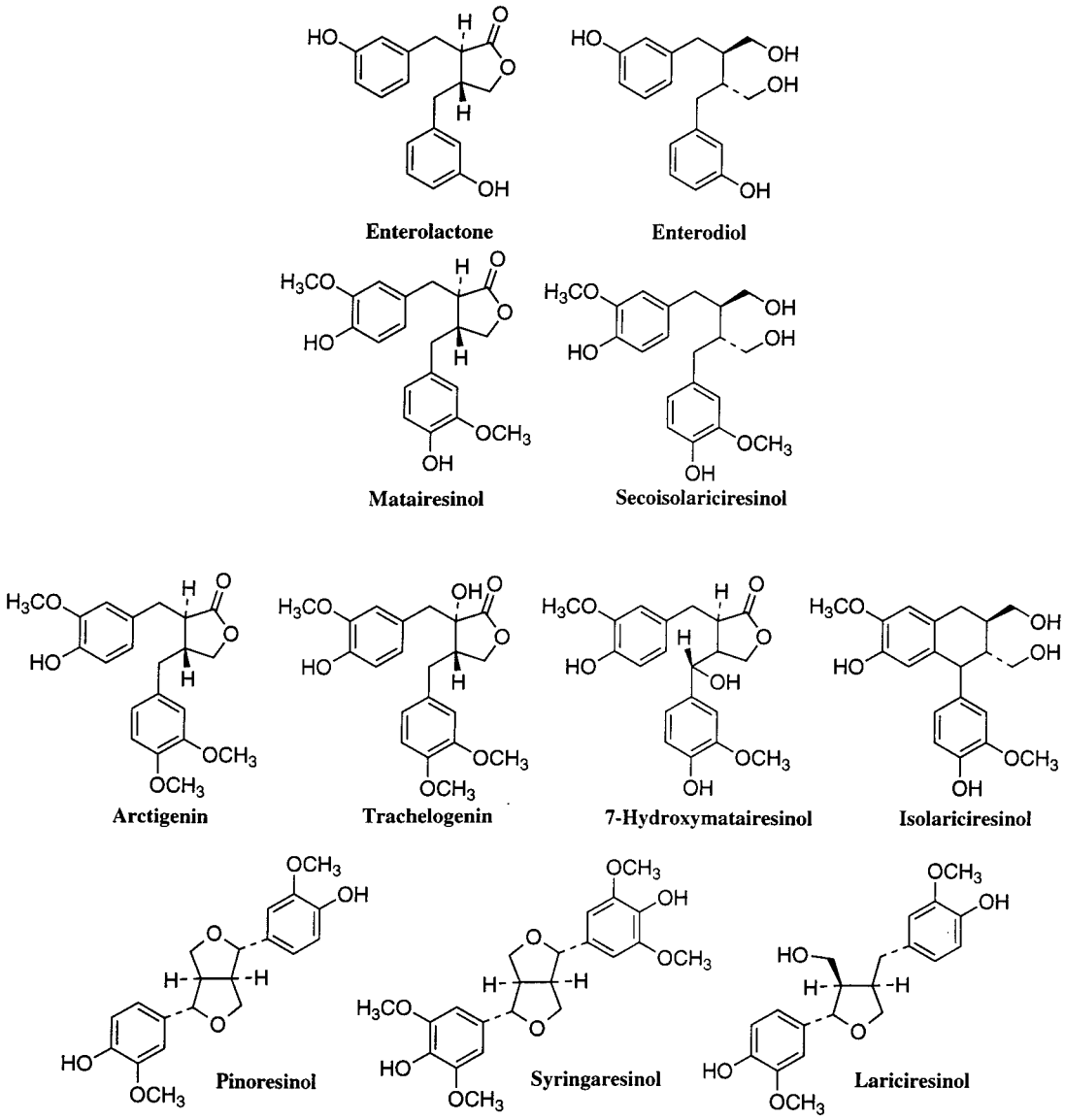

Figure 1. Structures of mammalian lignans enterolactone and enterodiol and plant lignans matairesinol, secoisolariciresinol, arctigenin, trachelogenin, 7-hydroxymatairesinol, isolariciresinol, pinoresinol, syringaresinol, and lariciresinol.

Nose et al. studied the metabolism of arctiin and tracheloside, the glycosides of arctigenin and trachelogenin (Figure 1), respectively, using rat gastric juice and intestinal microflora. During the 24-h reaction time the sugar moieties were cleaved after 5 h and some demethylation of the substituted phenolic ring was observed (*16*, *17*). The metabolism of arctigenin has been studied also with genetically engineered cell lines for the stable expression of selected rat and human cytochromes. Demethylation was favored in the ring substituted with hydroxyl and methoxy groups, producing 3′-*O*-desmethylarctigenin (*18*).

7-Hydroxymatairesinol (Figure 1), the most abundant single component of spruce (*Picea abies*) lignans, has been reported to be metabolized mainly to enterolactone in rats (*19*). Two isomers of 7-hydroxymatairesinol have been identified in human urine together with 7-hydroxyenterolactone, the metabolite of 7-hydroxymatairesinol (*20*).

We have identified and preliminarily quantified pinoresinol, syringaresinol, isolariciresinol, and lariciresinol (Figure 1) from rye bran. If some of these lignans result in enterolactone formation, it would explain the discrepancy between the plant lignan intake and the mammalian lignan production during rye consumption experiments. The results presented here indicate that pinoresinol, lariciresinol, and syringaresinol found in rye are the precursors of enterolactone and enterodiol. Quantitative information about mammalian lignan production from these new precursors is presented, and some new metabolites formed during the 24-h fecal incubation of lignans are also described.

MATERIALS AND METHODS

Compounds. Pinoresinol, pinoresinol diglucoside, syringaresinol, and syringaresinol diglucoside were isolated as described by Nishibe et al. (*21*). Dry powdered stem bark of *Acanthopanax senticosus* (Siberian ginseng) (3 kg) was extracted three times with 15 mL of hot MeOH. The MeOH solution was evaporated to a small volume under reduced pressure, diluted with water, and filtered. The filtrate was extracted successively with ether, $CHCl_3$, and *n*-BuOH. The ether extract (6.1 g) was subjected to column chromatography on silica gel (100 mesh, Mallinckrodt), eluting with a $CHCl_3$/EtOAc (99:1) solvent system with a gradually increasing proportion of EtOAc (1% continuous/stepwise 5−15%) to give syringaresinol (30 mg) and pinoresinol (17 mg), respectively.

The *n*-BuOH extract (34.8 g) was subjected to column chromatography on silica gel, eluting with a $CHCl_3$/EtOH solvent system (99:1) with a gradually increasing proportion of EtOH (1% continuous/stepwise 5−30%) to give syringaresinol diglucoside (180 mg) and pinoresinol diglucoside (70 mg), respectively. These lignans were also isolated from the bark of *Eucommia ulmoides* according to the same treatment as described for *A. senticosus*.

Arctigenin and arctiin were isolated as described by Chiba et al. (*22*). Powdered fruit of *Forsythia viridissima* (21 g) was extracted three times with 100 mL of hot MeOH. The MeOH solution was evaporated to a small volume under reduced pressure, diluted with water, and filtered. The filtrate was extracted successively with ether, $CHCl_3$, and *n*-BuOH. The ether extract was subjected to column chromatography on silica gel, eluting with a $CHCl_3$/EtOAc solvent system with a gradually increasing proportion of EtOAc (1% continuous/stepwise 5−15%) to give arctigenin (24 mg). The $CHCl_3$ extract was subjected to column chromatography on silica gel, eluting with a $CHCl_3$/EtOH solvent system with a gradually increasing proportions of EtOH (1% continuous/stepwise 5−20%) to give arctiin (98 mg). These lignans were also isolated from the fruit of *Arcticum lappa* as described for *F. viridissima*.

All of the butyrolactone type lignans (matairesinol, enterolactone, and arctigenin) were synthesized by a one-pot three-component Michael addition/alkylation reaction (*23*) in good yields. Secoisolariciresinol was obtained by $LiAlH_4$ reduction of matairesinol as was enterodiol of enterolactone (*24*). Secoisolariciresinol was converted via a perchloroacid treatment to anhydrosecoisolariciresinol. Isolariciresinol was prepared as reported (*25*). The synthesis of 7-hydroxyenterolactone will be published elsewhere. Dr. S. Ozawa and Dr. S. Nishibe (Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Japan) kindly provided lariciresinol. 7-Hydroxymatairesinol was a gift from Dr. L. Kangas (Hormos-Medical, Ltd., Finland).

Arctigenin, pinoresinol, syringaresinol, 7-hydroxymatairesinol, isolariciresinol, secoisolariciresinol, anhydrosecoisolariciresinol, matairesinol, enterodiol, enterolactone, and 7-hydroxyenterolactone, used as standards for quantitative analyses, were dissolved in MeOH and diluted with mobile phase prior to HPLC analysis. Anhydrosecoisolariciresinol, formed under acidic conditions from secoisolariciresinol (*13*, *26*), was included with the standards to confirm that it was not formed during the fecal incubations. 7-Hydroxyenterolactone was identified and quantified to determine whether it is a metabolite of some of the lignans investigated, especially the metabolite of 7-hydroxymatairesinol.

Incubation with Human Fecal Inoculum. The incubation method of Karppinen et al. (*27*) was modified as follows: A carbonate−phosphate buffer solution with trace elements was held in an anaerobic chamber for 2 days prior to the fermentation. Feces were collected from three healthy human volunteers, who ingested a Western diet, presented no digestive disease, and had not received antibiotics for at least 3 months. Freshly passed feces were immediately taken in an anaerobic chamber, pooled, and homogenized at the same time with an equal weight of culture medium using a Waring blender. The slurry was diluted to 16.7% (w/w) with culture medium, filtered through a 1 mm sieve, and used immediately as inoculum.

A 1−2 mg quantity of substrates (matairesinol, secoisolariciresinol, pinoresinol diglucoside, syringaresinol diglucoside, arctigenin glucoside, 7-hydroxymatairesinol, and isolariciresinol), corresponding to 2−3.5 μmol, was weighed into 50 mL glass vials, and 10 mL of fecal suspension was added in a 30 °C anaerobic chamber. The vials were sealed with rubber stoppers and shaken in a 37 °C water bath. Duplicate incubations were carried out for each substrate. Also, duplicate blanks, containing only culture medium and inoculum, were incubated for 0 and 24 h. The fermentation was stopped by plunging the vials into iced water, after which the vial contents were freeze-dried.

Lariciresinol was incubated separately at another time to confirm the results obtained with pinoresinol. The fecal suspension used for the incubation of lariciresinol was obtained from other volunteers who fulfilled the criteria described earlier. Collection of feces, incubation procedures, and analysis of the samples were carried out in the same way as described for the other plant lignans.

Table 1. Retention Times, Detection Potentials, and Molecular Weights of Lignans

compound	retention time, min	detection potential, mV	mol wt
isolariciresinol	22.72	420	360
7-hydroxymatairesinol[a]	23.93/25.15	420	374
7-hydroxyenterolactone	31.82	575	314
lariciresinol	32.39	420	360
secoisolariciresinol	32.81	420	362
syringaresinol	39.89	420	418
pinoresinol	41.21	420	358
enterodiol	43.03	575	302
matairesinol	44.50	420	358
enterolactone	50.03	575	298
arctigenin	55.70	420	372

[a] Analyte appears in two isomers, which are completely separated.

HPLC Analyses. Quantitative analyses were conducted using HPLC with the coulometric electrode array detection (CEAD), as described previously (*28*). The detection potentials, retention times, and molecular weights of the analytes are presented in Table 1.

The freeze-dried feces incubation samples were weighed (15−20 mg), 0.5 mL of water was added, and the samples were acidified with 10 μL of 6 M HCl to improve the extraction efficiency. The samples were extracted twice with 2.0 mL of diethyl ether. The extracts were combined and evaporated to dryness under N_2 flow. The samples were dissolved in 0.5 mL of MeOH and diluted with the mobile phase prior to HPLC analysis. Triplicate samples were analyzed for each incubation sample. Hydrolysis of fecal samples was omitted, because, in general, <10% of the total amount of mammalian lignans occurs conjugated in feces (*14*). Furthermore, the in vitro test system did not provide conditions for the conjugate formation. Mammalian lignans were not detected in the water phase residue after the ether extraction.

Enterolactone and enterodiol production in fecal blanks was measured from 0- and 24-h incubations. The amounts of enterolactone and enterodiol produced in 24 h fecal blanks were subtracted from results obtained from the incubations carried out with the pure compounds of the potential mammalian lignan precursors.

GC-MS Analyses. Cholesterol and other nonpolar compounds in combined ether extracts interfered with the analysis by GC-MS; thus, further purification of the samples was needed. Chromatography on Lipidex 5000 (Packard Bioscience Co.) in MeOH/H_2O/$CHCl_3$ (8:2:2) was used for this purpose. The combined ether extracts, obtained as described in the previous section, were evaporated to dryness under N_2 flow. The extracts were applied on the Lipidex 5000 column (0.5 × 5.0 cm) in 2 × 200 μL of MeOH/H_2O/$CHCl_3$ (8:2:2). Lignans and their metabolites were eluted with 4 mL of the same eluent. The fraction was dried under N_2 flow and derivatized with 100 μL of QSM (pyridine/HMDS/TMCS, 9:3:1). The silylating agent was evaporated, and the trimethylsilylated samples were dissolved in 100 μL of hexane prior to GC-MS.

GC-MS analyses were carried out with a Fisons Instrument MD 1000 quadrupole mass spectrometer coupled to a Fisons GC 8000 gas chromatograph, equipped with a BP-1 capillary column (12 m × 0.22 mm × 0.25 μm) from SGE. The flow rate of helium carrier gas was 1 mL/min. The oven temperature was programmed as follows: 150 °C (for 1 min) raised at 50 °C/min to 250 °C (for 15 min) and then at 50 °C/min to 290 °C (for 5 min). The temperature of the injection port was 280 °C. The temperatures of the ion source and interface were 200 and 250 °C, respectively. A mass range from 100 to 900 mu was scanned using an electron energy of 70 eV.

Table 2. Quantitative HPLC Results of the Plant Lignan Conversion to Mammalian Lignans Enterolactone and Enterodiol

compound	precursor, nmol	ENL, nmol	ENL, CV[a]	END, nmol	END, CV[a]	conversion % in 24 h
matairesinol	3073	1973	4.5	0		62
secoisolariciresinol	3260	676	3.2	1643	3.9	72
pinoresinol diglucoside	3504	664	3.8	1112	7.6	55
syringaresinol diglucoside	2803	82	2.6	10	8.1	4.0
arctigenin glucoside	3745	198	2.2	0		5.5
7-hydroxymatairesinol	2353	343	1.7	4	13	15
isolariciresinol	2722	0		0		
0-h feces incubation	0	8	15	3	15	
24-h feces incubation	0	16	5.2	1	6.4	
lariciresinol[b]	2778	1290	8.2	1524	6.7	101
0-h feces incubation[b]	0	2	1.8	1	1.1	
24-h feces incubation[b]	0	17	5.7	1	13	

[a] CV calculated for triplicate determination. [b] Incubated separately.

RESULTS

The quantitative results of enterolactone and enterodiol, produced during the fecal incubation of each lignan precursor, are summarized in Table 2. The conversions of the precursors in the 24-h incubation ranged from 4.0% (syringaresinol) to 72% (secoisolariciresinol). Lariciresinol, for which a different fecal inoculum was used, was completely (101%) converted to enterolactone and enterodiol. After 24 h of incubation, no added plant lignans were detected in the respective incubation extracts, except isolariciresinol, which was not converted to enterolactone or enterodiol. Variation between duplicate incubations ranged from 1.4 to 9.2% for enterolactone and from 1.5 to 11% for enterodiol. The coefficients of variation (CV) for triplicate analyses for each incubation sample are presented in Table 2. The values ranged from 1.7 to 15% for enterolactone and from 3.9 to 15% for enterodiol. The highest variation was for 0 h of incubation, when the concentrations of the analytes were very low.

The GC-MS total ion chromatographic (TIC) profiles of the incubation samples and the 24-h blank samples are presented in Figure 2. The metabolites formed from the lignan precursors were absent from the chromatograms of the blank samples. The mass spectra of some metabolites are listed in Table 3.

Matairesinol. Enterolactone was the major compound identified after the fecal incubation of matairesinol. Of the added matairesinol, 62% was recovered as enterolactone. Some minor metabolites were also formed, but the structures of these compounds were not elucidated.

Secoisolariciresinol. Secoisolariciresinol was converted to enterodiol and enterolactone, which accounted for 72% of the added secoisolariciresinol. The mass spectrum of the compound eluting at the retention time of 5.51 min was identical to that reported for enterofuran (*29*). The structure of the compound was confirmed with the authentic reference compound synthesized as described by Liggins et al. (*29*).

Pinoresinol. Conversion of pinoresinol to enterolactone and enterodiol was almost as effective (55%) as for the known plant precursors, secoisolariciresinol and matairesinol (72 and 62%, respectively). Enterolactone and enterodiol were the only major metabolites detected during the incubation. The TIC profile produced was similar to that produced after the incubation of secoisolariciresinol. A minor metabolite, enterofuran, was detected also in the incubation extract of pinoresinol.

Syringaresinol. Syringaresinol was partly converted to enterodiol and enterolactone during the incubation (4%). Several other metabolites (S1−S10; Figure 2) were isolated and tentatively identified by the interpretation of their mass spectra. The postulated structures of the identified metabolites, S1−S7, are presented in the Figure 3. Because no authentic reference compounds were available, the quantitative analysis of these metabolites was not possible.

The mass spectrum of metabolite S1, eluting at the retention time of 11.41 min, gave a molecular ion at *m*/*z* 530 and a base peak at *m*/*z* 268, which corresponds to the structure of enterolactone with one trimethylsilylated hydroxyl group attached. The hydroxyl group is attached to the phenolic ring, because an ion at *m*/*z* 470 that would arise from the loss of an aliphatic hydroxyl group, $[M - TMSOH]^+$, is absent in the spectrum. The mass spectrum of metabolite S2, at the retention time of 11.79 min, was identical to that of metabolite S1.

Metabolite S3 (retention time of 11.99 min) showed a molecular ion of *m*/*z* 592. The base peak at *m*/*z* 210 suggests that one of its phenolic rings bears one methoxy and one hydroxyl group. The other phenolic ring has two methoxy groups attached because it has an abundant fragment at *m*/*z* 152. The fragment ions at *m*/*z* 502 and 412 are formed by the loss of two aliphatic hydroxyl groups (M − TMSOH and M − TMSOH − TMSOH, respectively).

Metabolite S4 (retention time of 12.47 min) had the same molecular ion as secoisolariciresinol (*m*/*z* 650). The base peak of the spectrum is at *m*/*z* 268, which suggests that one of the phenolic rings has two trimethylsilylated hydroxyl groups attached. Two methoxy groups are attached to the other ring, confirmed by the presence of the ion at *m*/*z* 152. The successive loss of two aliphatic hydroxyl groups produces ions at *m*/*z* 560 and 470, respectively.

Another isomer of secoisolariciresinol, metabolite S5, eluted at a longer retention time, 12.71 min. The compound gave a molecular ion and base peak at *m*/*z* 650 and 210, respectively. The earlier retention time relative to that of secoisolariciresinol suggests that neither of the para positions of the compound are substituted. Furthermore, the abundant ion at *m*/*z* 209, which is typical for the trimethylsilylated lignans having *m*-methoxy-*p*-hydroxy substitution, such as secoisolariciresinol, matairesinol, and isolariciresinol, is absent in the mass spectrum. Possibly this metabolite is 5,5′-dimethoxyenterodiol.

Metabolite S6 at the retention time of 13.21 min had a molecular ion at *m*/*z* 708. Two abundant fragments at *m*/*z* 210 (the base peak) and *m*/*z* 268 were dominant in the spectrum of this metabolite. The other phenolic

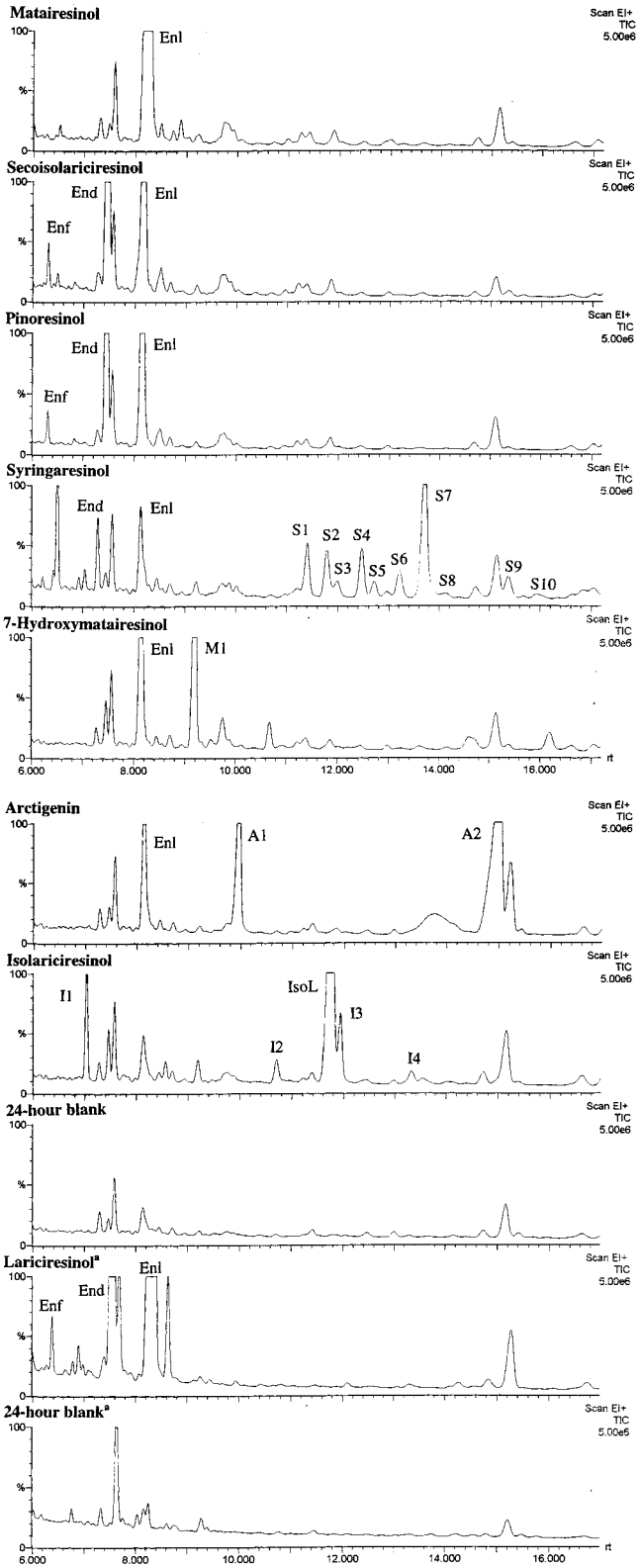

Figure 2. TIC (GC-MS) profiles of 24-h fecal incubations of plant lignans and blank samples. [a]Incubated separately with a different fecal suspension.

ring thus seems to have both methoxy and hydroxyl groups, whereas the other phenolic ring contains two trimethylsilylated hydroxyl groups.

The major metabolite, S7, eluting at the retention time of 13.71 min, gave a molecular ion of *m/z* 766. The base peak at *m/z* 268 was the only intensive peak in the mass spectrum, suggesting that both of the phenolic rings contain two trimethylsilylated hydroxyl groups.

The minor compounds at the retention times of 14.04, 15.37, and 15.93 min appeared in the extracts of fecal incubation of syringaresinol. Compounds eluting at the same retention times interfered with the interpretation of the mass spectra of these metabolites. The interfering compounds were present also in the chromatograms of blank samples. The spectra of the minor metabolites S8, S9, and S10 were generated by subtracting the ions of the interfering compounds from the mass spectrum. In the spectrum of the first compound (S8) there were ions at *m/z* 444, 429, 210, and 152 (the most abundant ion in generated spectrum). A possible structure of this compound might be the lactone metabolite of metabolite S3. The compound at a retention time of 15.37 min (S9) had ions at *m/z* 694, 604, 514, and 268 (most abundant ion); its structure has not been elucidated. The third compound at a retention time of 15.93 min (S10) had a molecular ion of *m/z* 560; other characteristic fragments of the spectrum were *m/z* 545, 268 (most abundant ion), and 210. The proposed structure for this compound is the corresponding lactone metabolite of S6.

7-Hydroxymatairesinol. 7-Hydroxymatairesinol was converted to enterolactone and 7-hydroxyenterolactone (M1 in Figure 2). Some minor metabolites were also formed, but the structures of these compounds could not be elucidated. The amounts of 7-hydroxyenterolactone and enterolactone were 180 and 343 nmol/sample, respectively. During the 24-h incubation 23% of the added 7-hydroxymatairesinol was converted to these two major metabolites; 7-hydroxyenterolactone represented 34% of the total metabolites. Minor metabolites were not included in these percentages because we were not able to quantify them.

Arctigenin. Four percent of the arctigenin was metabolized to enterolactone. Two other metabolites were formed during the incubation. The metabolite A1 (retention time of 9.97 min) gave a molecular ion at *m/z* 414, and the base peak was at *m/z* 151. The structure of the compound is tentatively identified as 3′-demethyl-4′-dehydroxyarctigenin. The other major metabolite, A2, had a retention time of 15.02 min. The compound was identified as 3′-demethylarctigenin, an isomer of matairesinol. This compound showed a molecular ion at *m/z* 502 and other abundant fragments at *m/z* 267, 179, and 151. The fragment at *m/z* 179 corresponds to the loss of one methyl group followed by the loss of a trimethylsilyl group from the fragment at *m/z* 267, that is, $[267 - (CH_3 + TMS)]^+$. The structures of the tentatively identified metabolites of arctigenin, A1 and A2, are presented in Figure 4.

Isolariciresinol. Isolariciresinol remained mostly unchanged during the incubation. The mass spectra of the four minor metabolites are listed in Table 3. The metabolite I1 (retention time of 7.02 min) had molecular ion at *m/z* 588 with a base peak at *m/z* 395. The loss of two aliphatic hydroxyl groups can be presumed from the peaks at *m/z* 498 and 408. The metabolite was tentatively identified as 1,2,3,4-tetrahydro-6-hydroxy-1-(3-hydroxyphenyl)-2,3-naphthalenedimethanol, a metabolite of isolariciresinol corresponding to enterodiol, the metabolite of secoisolariciresinol.

The compound at the retention time of 11.93 min, metabolite I3, had a molecular ion at *m/z* 706 and a base peak at *m/z* 513. The fragments at *m/z* 616 and 526 indicate that this metabolite also has two aliphatic

Table 3. Mass Spectral Data of the Metabolites Formed during the 24-h Fecal Incubation of Lignan Precursors

metabolite[a]	retention time, min	fragment ions, *m/z* (%)
S1	11.41	530 (5), 515 (2), 268 (100), 179 (5)
S2	11.79	530 (3), 515 (3), 268 (100), 179 (3)
S3	11.99	592 (<1), 502 (<1), 412 (3), 399 (2), 268 (36), 210 (100), 152 (69)
S4	12.47	650 (<1), 560 (1), 470 (2), 457 (1), 319 (6), 268 (100), 152 (69)
S5	12.71	650 (<1), 635 (<1), 560 (<1), 470 (1), 210 (100), 180 (2), 147 (8)
S6	13.21	708 (<1), 618 (1), 528 (2), 319 (6), 268 (92), 210 (100), 180 (3)
S7	13.71	766 (<1), 676 (1), 586 (1), 573 (1), 319 (6), 268 (100)
A1	9.97	414 (35), 271 (21), 217 (17), 180 (27), 177 (42), 152 (97), 151 (100)
A2	15.02	502 (60), 487 (1), 267 (100), 179 (91), 151 (35)
M1	9.21	530 (5), 440 (5), 293 (19), 267 (100), 219 (5), 179 (12)
I1	7.02	588 (<1), 498 (21), 408 (30), 395 (100), 380 (10), 368 (6), 229 (11), 179 (24), 147 (21)
I2	10.70	576 (6), 561 (2), 486 (24), 455 (13), 396 (29), 383 (100), 365 (29), 356 (24), 179 (4)
I3	11.93	706 (10), 691 (8), 616 (82), 527 (89), 513 (100), 486 (48), 437 (84), 411 (35), 267 (48), 179 (48)
I4	13.32	576 (3), 561 (5), 486 (41), 455 (63), 396 (45), 383 (81), 365 (100), 209 (52), 179 (18)

[a] The codes refer to Figures 2−5.

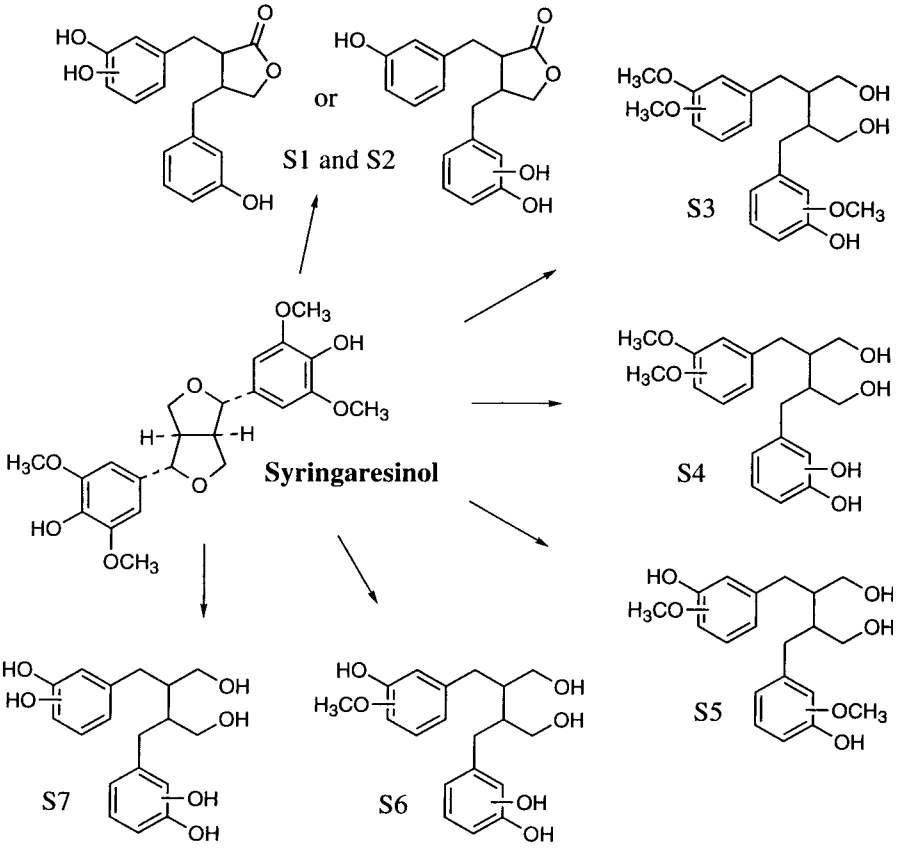

Figure 3. Structures of the tentatively identified syringaresinol metabolites.

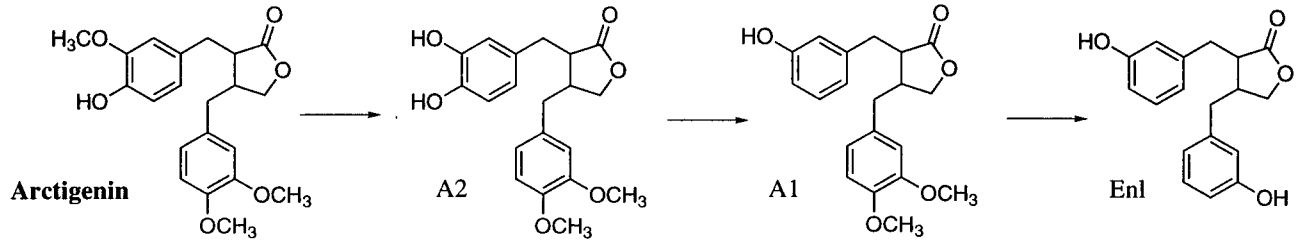

Figure 4. Structures of arctigenin metabolites and proposed metabolic pathway.

hydroxyl groups. The molecular ion suggests that one of the methoxy groups of isolariciresinol is demethylated, producing the fragment at *m/z* 267, whereas the other phenolic ring still bears one trimethylsilylated hydroxyl group and methyl group. The proposed metabolic pathway and structures of tentatively identified metabolites, I1 and I3, are presented in Figure 5. The structures of two isomeric metabolites (I2, I4) remain unknown.

Lariciresinol. Lariciresinol, incubated separately with a different fecal suspension, was completely converted to enterolactone and enterodiol (101%). Entero-

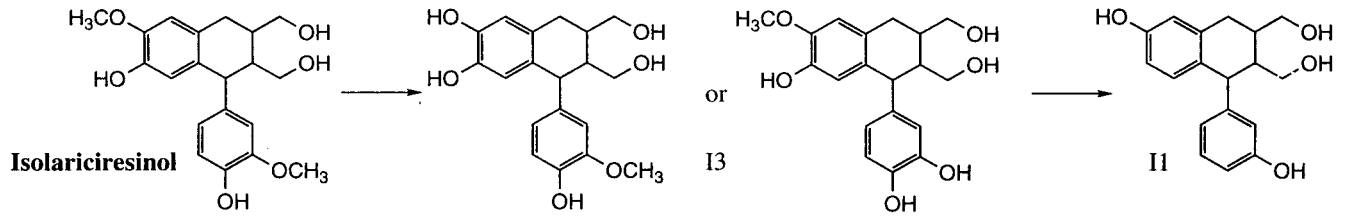

Figure 5. Minor metabolites identified in the fecal incubation extracts of isolariciresinol and proposed metabolic pathway for isolariciresinol.

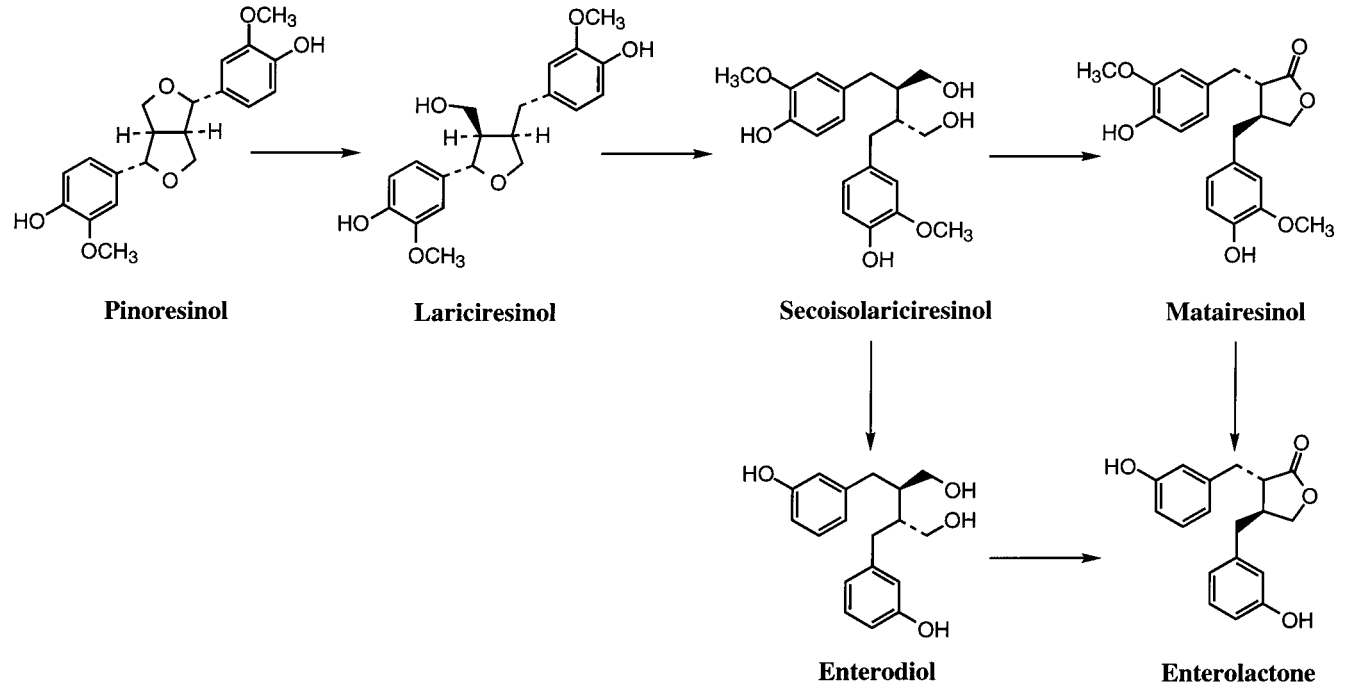

Figure 6. Proposed metabolic pathway for pinoresinol: formation of mammalian lignans via lariciresinol and secoisolariciresinol intermediates.

furan was identified as a minor metabolite of lariciresinol. The TIC profile was similar to those of secoisolariciresinol and pinoresinol.

DISCUSSION

It has been long assumed that matairesinol and secoisolariciresinol are the only plant lignans that are converted to enterolactone and enterodiol by intestinal microflora (*4*, *30*). In this study new precursors for the mammalian lignans were found. Syringaresinol and especially pinoresinol and lariciresinol were metabolized to enterodiol and enterolactone in a manner analogous to that of secoisolariciresinol. In addition to matairesinol, both arctigenin and 7-hydroxymatairesinol were metabolized to enterolactone to some extent. The formation of enterolactone from 7-hydroxymatairesinol has been previously demonstrated (*19*). Isolariciresinol was stable during the fecal incubation and was not metabolized as easily as the other lignans investigated.

In the present study secoisolariciresinol was metabolized mainly to enterolactone and enterodiol, and no new intermediates presented by Wang et al. (*5*) could be detected. The incubation time in this study was 24 h, whereas Wang et al. incubated secoisolariciresinol for 15 h. The differing results may be due to a longer incubation time and the individual variation of the intestinal microflora. One minor metabolite of secoisolariciresinol was identified as enterofuran. This metabolite was recently introduced as the metabolite of anhydrosecoisolariciresinol, also known as shonanin (*29*). Anhydrosecoisolariciresinol and secoisolariciresinol were not detected in incubation extracts of secoisolariciresinol. Enterofuran was detected also in the incubation extracts of pinoresinol and lariciresinol. It is thus suggested that these other plant lignans may be the main precursors of enterofuran.

The biosynthesis of matairesinol and secoisolariciresinol in plants has been reported to occur by the breakdown of pinoresinol to secoisolariciresinol via lariciresinol (*31*). The intestinal bacterial metabolism of pinoresinol seems to occur in a similar way. During the incubation pinoresinol was effectively converted to enterolactone and enterodiol, which are the main metabolites of secoisolariciresinol. It is thus suggested that the metabolism of pinoresinol involves the formation of secoisolariciresinol as an intermediate (Figure 6). The results obtained with lariciresinol, which was incubated separately at another time, confirmed that it is metabolized to enterolactone and enterodiol in a similar way as secoisolariciresinol and pinoresinol. The results suggest that lariciresinol can act as an intermediate in the metabolic pathway of pinoresinol presented in Figure 6. None of the other plant lignans was as extensively converted to mammalian lignans as lariciresinol, which was incubated with a different fecal suspension (conversion = 101%).

The structure of syringaresinol differs from that of pinoresinol by two additional methoxy groups at meta positions in both phenolic rings. This relatively complex substitution pattern yielded several metabolites during the incubation. Most of the metabolites that were tentatively identified for syringaresinol share an oxydiarylbutane structure, suggesting that the breakdown of syringaresinol occurs in a similar way to that of pinoresinol, involving the corresponding lariciresinol and secoisolaricinol intermediates. The formation of the metabolites with a diarylbutyrolactone structure occurred to a lesser extent.

The *m*-methoxy-*p*-hydroxy substitution pattern of the plant lignans is known to be converted by intestinal microflora to a single *m*-hydroxy substitution of the phenolic ring of the mammalian lignans (*4*). Tentative identification of 5,5′-dimethoxyenterodiol, an isomer of secoisolariciresinol, suggests that intestinal bacteria also demethylate the additional *m*-methoxy groups of syringaresinol. The major metabolite, S7, would by analogy be 5,5′-dihydroxyenterodiol and the other metabolites *m*-substituted demethylation intermediates between S3 and S7. The tentative identification of syringaresinol metabolites S1 and S2 as 5′-hydroxyenterolactone and 5-hydroxyenterolactone, respectively, might explain the isolation of meta-hydroxylated enterolactone metabolites in the recent study of the oxidative metabolism of lignan metabolites in rats (*32*).

The two adjacent methoxy groups of arctigenin were resistant to demethylation, consistent with results from previous studies (*16*−*18*). In this investigation three metabolites were formed during the 24-h fecal incubation of arctiin. The main metabolite, 3′-demethylarctigenin, was the only metabolite previously reported (*17*). The adjacent methoxy groups of arctigenin were also unchanged in 3′-demethyl-4′-dehydroxyarctigenin, the other major metabolite of arctigenin isolated in this study. Some of the arctigenin was converted to enterolactone. No intermediates between 3′-demethyl-4′-dehydroxyarctigenin and enterolactone were detected.

Relatively higher amounts of 7-hydroxymatairesinol were metabolized to 7-hydroxyenterolactone rather than to enterolactone than reported in a recent study, in which the orally administered 7-hydroxymatairesinol was recovered mainly as enterolactone in rat urine (*19*). Rat intestinal microflora is probably very different from human microflora. Trachelogenin, which has the hydroxyl group attached at the 8′-position, has been reported to be metabolized mainly to 3′-demethyltrachelogenin, in which the aliphatic hydroxyl group is still present (*17*). The intestinal microflora is capable of eliminating the aliphatic hydroxyl group, as in this study the main metabolite of 7-hydroxymatairesinol was also enterolactone.

Isolariciresinol was stable during the incubation. It was the only lignan in this investigation that was not metabolized to enterolactone or enterodiol. The rigid fused ring structure seems to protect the lignan from metabolic reactions, because the conversion of *m*-methoxy and *p*-hydroxy substitution of the phenolic rings to *m*-hydroxy substitution does not occur as easily as with secoisolariciresinol or the other lignans investigated in the present study.

The new precursors, especially pinoresinol, syringaresinol, and later investigated lariciresinol, may provide finally the explanation for the discrepancy between plant lignan input and mammalian lignan output in the rye feeding experiments (*11*, *12*). The amounts of pinoresinol, lariciresinol, and syringaresinol in rye bran are from 10 to 50 times higher than the amounts of secoisolariciresinol and matairesinol (Heinonen and Nurmi, unpublished data). Even though syringaresinol was very slowly converted to enterolactone during the incubation, the superior concentration of the compound in rye can provide a sufficient amount of precursors together with pinoresinol and lariciresinol to produce 10−12 times more enterolactone than would be expected from the analysis of only secoisolariciresinol and matairesinol in rye.

The quantitative analysis of the new lignan precursors in different foods is being carried out. It seems that in rye pinoresinol is a quantitatively more important precursor than the previously known matairesinol and secoisolariciresinol. Rye, as well as flaxseed (*7*), still contains some other lignan-like compounds, the structures of which have not been elucidated. Whether these lignans can also act as mammalian lignan precursors is of interest for further studies.

ABBREVIATIONS USED

ENL, enterolactone; END, enterodiol; ENF, enterofuran; TMS, trimethylsilyl; GC-MS, gas chromatography−mass spectrometry; S1−S10, metabolites of syringaresinol; A1, A2, metabolites of arctigenin; M1, 7-hydroxyenterolactone; I1−I4, metabolites of isolariciresinol.

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