Oxidative Metabolism of the Mammalian Lignans Enterolactone and Enterodiol by Rat, Pig, and Human Liver Microsomes

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Hepatic microsomes from aroclor-treated male Wistar rats biotransform enterolactone to 12 metabolites, six of which carry an additional hydroxy group at the aromatic and six at the aliphatic moiety according to HPLC/MS and GC/MS analysis. The aromatic hydroxylation products were identified with the help of synthesized reference compounds as enterolactone monohydroxylated in the para position and in both ortho positions of the original phenolic hydroxy group of either aromatic ring. The synthesis of the reference compounds and their spectroscopic characterization is described. Enterodiol is metabolized by hepatic microsomes from aroclor-treated male rats to three aromatic and four aliphatic monohydroxylated metabolites. Aromatic hydroxy group. Most of the metabolites of enterolactone and enterodiol were also formed with microsomes from uninduced rat, pig, and human liver, suggesting that oxidative metabolism is a common feature in the disposition of these lignans in the mammalian organism.

Keywords: Mammalian lignans; enterolactone; enterodiol; microsomal metabolism

INTRODUCTION

The mammalian lignans enterolactone (ENL) and enterodiol (END) are weakly estrogenic compounds formed by intestinal bacteria from the plant lignans matairesinol and secoisolariciresinol (Axelson and Setchell, 1981; Setchell et al., 1982), which are constituents of flaxseed, whole-grain products, vegetables, and fruit (Thompson et al., 1991). Once generated in the intestine, ENL and END are absorbed and reach the liver where they are conjugated mainly with glucuronic acid or sulfate (Adlercreutz et al., 1995a) before entering the circulation (Axelson and Setchell, 1981). ENL and END appear in various body fluids in humans and animals (Morton et al., 1994; Atkinson et al., 1993; Adlercreutz et al., 1995b) and may reach up to 10 000-fold the concentration of normal circulating endogenous estrogens (Rickard and Thompson, 1997).

Epidemiological studies suggest that high levels of lignans may protect against hormone-dependent cancers, especially breast cancer (Rose, 1992; Adlercreutz, 1995). This is supported by data from in vivo and in vitro studies. Flaxseed was found to inhibit mammary and colon carcinogenesis in rats (Serraino and Thompson, 1992; Jenab and Thompson, 1996). The lignans also inhibited the growth of different human tumor cells in culture (Sung et al., 1996). In addition, lignans compete with estradiol for the type II estrogen receptor and stimulate the synthesis of sex hormone binding globulin (Adlercreutz et al., 1992). ENL inhibits placental aromatase (Adlercreutz et al., 1993; Wang et al., 1994). These data imply that lignans may play an important role in the prevention of cancer. However, little is known about the biotransformation of these compounds and the



Figure 1. Synthetic route for enterolactone (ENL) and derivatives monohydroxylated at the aromatic rings.

effects of their metabolites. We have therefore studied the oxidative metabolism of ENL and END with rat, pig, and human liver microsomes.

MATERIALS AND METHODS

Synthesis of Enterolactone, Enterodiol, and Standards for Metabolites. ENL was synthesized as depicted in Figure 1 by a modification of the tandem addition procedure described by Pelter et al. (1983). The starting materials were obtained by standard methods (Enders et al., 1979; Seebach and Corey, 1974; Näsman and Pensar, 1985; Feringa et al., 1994). Briefly, 5.6 mmol of n-butyllithium (3.5 mL of a 1.6 M solution in hexane) was added to a solution of 5 mmol of 3-benzyloxybenzaldehyde bis(phenylthio)acetal (2.07 g) in 10 mL of dry tetrahydrofurane (THF) at -40 °C. The mixture was stirred for 1.5 h at this temperature and then cooled to -85°C, and a solution of 5 mmol of 2-buten-4-olide (0.42 g) in 1.5 mL of dry THF was added. The mixture was stirred for another 2.5 h at -85 °C and thereafter treated dropwise with a solution of 5 mmol of 3-benzyloxybenzylbromide (1.39 g) in 2.5 mL of dry THF and 0.6 mL of N,N-dimethyl-2-imidazolidinone. The reaction mixture was kept at -30 °C overnight and then allowed to warm to room temperature. Subsequently, water (20 mL) was added, the aqueous phase was extracted with ethyl acetate (3 \times 20 mL), and the extract was washed with water and dried over magnesium sulfate. Chromatography on silica gel with methylene chloride yielded 2.1 mmol (1.46 g,

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42%) of an orange gum. The product was refluxed with Raney nickel (made of 13.6 g of nickel–aluminum alloy) in absolute ethanol (55 mL) for 5 h to yield 625 mg of *trans*-3,4-bis-(3-hydroxybenzyl)- γ -butyrolactone (ENL; quantitative yield). After crystallization from chloroform, the purity of ENL was >99% according to GC/MS.

Seven monohydroxylation products of ENL, viz., trans-2-(2,3-dihydroxybenzyl)-3-(3-hydroxybenzyl)-γ-butyrolactone (yield 15%, purity 97%), trans-2-(3,4-dihydroxybenzyl)-3-(3-hydroxybenzyl)-γ-butyrolactone (yield 21%, purity 96%), trans-2-(3,5dihydroxybenzyl)-3-(3-hydroxybenzyl)-y-butyrolactone (yield 15%, purity 97%), trans-2-(2,5-dihydroxybenzyl)-3-(3-hydroxybenzyl)-γ-butyrolactone (yield 29%, purity 98%), trans-3-(2,3dihydroxybenzyl)-2-(3-hydroxybenzyl)-y-butyrolactone (yield 7%, purity 58%), trans-3-(3,4-dihydroxybenzyl)-2-(3-hydroxybenzyl)-γ-butyrolactone (yield 23%, purity 96%), and *trans*-3-(2,5-dihydroxybenzyl)-2-(3-hydroxybenzyl)-y-butyrolactone (yield 21%, purity 94%) were synthesized in the same manner (Figure 1) from the respective bis(phenylthio)acetals and benzyl bromides, which were prepared from the respective benzylated hydroxybenzaldehydes purchased from Fluka (Deisenhofen, Germany), Lancaster (Mühlheim, Germany), or Aldrich (Steinheim, Germany). These and the other chemicals used were of the highest purity available.

The synthesis and characterization of the materials by ¹H and ¹³C NMR spectroscopy and by GC/MS (after derivatization with *N*, *O*-bis(trimethylsilyl)acetamide, BSA) are described in detail elsewhere (Jacobs, 1998). A brief account of the spectroscopic data of the monohydroxylated ENL compounds is given below. NMR signals for ENL were assigned according to Cooley et al. (1984); see Figure 4 for the numbering of positions.

trans-3,4-Bis(3-hydroxybenzyl)- γ *-butyrolactone (ENL).* Mass spectrum (EI, 70 eV) *m/z* (% relative abundance): 442 (67, M⁺), 263 (10), 217 (8), 205 (5), 180 (100, McLafferty rearrangement product), 165 (6), 73 (7). ¹H NMR (CDCl₃/MeOD) δ (ppm): 2.40–2.65 (4H, m, 7, 8, 8'), 2.90 (1H, dd, 7'A), 3.00 (1H, dd, 7'B), 3.85 (1H, dd, 9A), 4.15 (1H, dd, 9B), 6.45–6.76 (6H, m), 7.11–7.20 (2H, m). ¹³C NMR (CDCl₃/MeOD) δ (ppm): 35.35 (t, 7'), 38.73 (t, 7), 41.85 (d, 8), 46.94 (d, 8'), 72.20 (t, 9), 114.12 (d), 114.39 (d), 116.05 (d), 116.66 (d), 120.48 (d), 121.20 (d), 130.22 (2d), 139.85 (s), 140.36 (s), 157.72 (2s), 180.58 (s, 9').

trans-2-(2,3-Dihydroxybenzyl)-3-(3-hydroxybenzyl)-γ-butyrolactone. Mass spectrum (EI, 70 eV) m/z (% relative abundance): 530 (15, M⁺), 515 (5), 351 (100), 335 (5), 179 (12, benzylic fragmentation), 73 (6). ¹H NMR (MeOD) δ (ppm): 2.40–2.87 (6H, m, 7, 7',8, 8'), 3.47 (1H, dd, 9A), 3.87 (1H, dd, 9B), 6.43–7.12 (7H, m).

trans-2-(3,4-Dihydroxybenzyl)-3-(3-hydroxybenzyl)-\gamma-butyrolactone. Mass spectrum (EI, 70 eV) *m/z* (% relative abundance): 530 (100, M⁺), 515 (5), 267 (46, benzylic fragmentation), 205 (6), 179 (15, benzylic fragmentation), 73 (6). ¹H NMR (MeOD) δ (ppm): 2.27–2.79 (6H, m, 7, 7', 8, 8'), 3.75 (1H, dd, 9A), 3.92 (1H, dd, 9B), 6.38–7.01 (7H, m). ¹³C NMR (MeOD) δ (ppm): 39.14 (t, 7'), 39.52 (t, 7), 42.40 (d, 8), 47.74 (d, 8'), 72.75 (t, 9), 113.25 (d), 114.54 (d), 116.61 (d), 117.52 (d), 120.94 (d), 121.94 (d), 130.75 (d), 141.46 (s), 145.12 (s), 146.32 (s), 158.61 (s), 158.69 (s), 181.52 (s, 9').

trans-2-(3,5-Dihydroxybenzyl)-3-(3-hydroxybenzyl)-γ-butyrolactone. Mass spectrum (EI, 70 eV) *m/z* (% relative abundance): 530 (51, M⁺), 515 (8), 293 (3), 268 (100, McLafferty rearrangement product), 253 (3), 73 (3). ¹H NMR (MeOD) δ (ppm): 2.34–2.98 (6H, m, 7, 7', 8, 8'), 3.88 (1H, dd, 9A), 4.55 (1H, dd, 9B), 6.17–7.10 (7H, m). ¹³C NMR (MeOD) δ (ppm): 35.96 (t, 7'), 39.16 (t, 7), 42.70 (d, 8), 47.41 (d, 8'), 72.75 (t, 9), 102.09 (d), 108.91 (d), 108.91 (d), 114.52 (d), 116.57 (d), 120.96 (d), 130.69 (d), 140.91 (d), 141.50 (s), 141.57(s), 158.64 (s), 159.76(s), 181.35 (s, 9').

trans-2-(2,5-Dihydroxybenzyl)-3-(3-hydroxybenzyl)-\gamma-butyrolactone. Mass spectrum (EI, 70 eV) *m/z* (% relative abundance): 530 (71, M⁺), 515 (5), 351 (100), 335 (16), 267 (6, benzylic fragmentation), 73 (6). ¹H NMR (CDCl₃/MeOD) δ (ppm): 2.34 (1H, dd, 7'A), 2.61–2.82 (4H, m, 7, 8, 8'), 3.24 (1H, dd, 7'B), 3.89 (1H, dd, 9B), 4.13 (1H, dd, 9A), 6.52–6.70 (6H, m), 7.05–7.08 (1H, m). ¹³C NMR (CDCl₃/MeOD) δ (ppm): 30.22 (t, 7'), 38.71 (t, 7), 42.46 (d, 8), 45.75 (d, 8'), 72.32 (t, 9), 113.86 (d), 115.01 (d), 115.92 (d), 116.31 (d), 118.07 (d), 120.43 (d), 125.72 (s), 130.03 (d), 140.70 (s), 149.03 (s), 150.11 (s), 157.43 (s), 181.18 (s, 9').

trans-3-(2,3-Dihydroxybenzyl)-2-(3-hydroxybenzyl)-\gamma-butyrolactone. Mass spectrum (EI, 70 eV) *m/z* (% relative abundance): 530 (100, M⁺), 515 (25), 351 (4), 335 (16), 268 (45, McLafferty rearrangement product), 179 (12, benzylic fragmentation), 73 (9). ¹H NMR (MeOD) δ (ppm): 2.46–2.98 (6H, m, 7, 7', 8, 8'), 3.92 (1H, dd, 9A), 4.07 (1H, dd, 9B), 6.48–7.02 (7H, m). ¹³C NMR (MeOD) δ (ppm): 33.95 (t, 7'), 35.04 (t, 7), 42.78 (d, 8), 46.82 (d, 8'), 71.92 (t, 9), 112.88 (d), 114.63 (d), 120.15 (d), 120.34 (d), 122.40 (d), 125.35 (d), 130.46 (d), 145.00 (s), 146.37 (s), 153.02 (s), 153.44 (s), 157.21 (s), 180.36 (s, 9').

trans-3-(3,4-Dihydroxybenzyl)-2-(3-hydroxybenzyl)-γ-butyrolactone. Mass spectrum (EI, 70 eV) *m/z* (% relative abundance): 530 (85, M⁺), 515 (4), 293 (21), 268 (100, McLafferty rearrangement product), 179 (16, benzylic fragmentation), 73 (31). ¹H NMR (MeOD) δ (ppm): 2.24–2.70 (4H, m, 7, 8, 8'), 2.79–2.99 (2H, m, 7'), 3.87 (1H, dd, 9A), 4.06 (1H, dd, 9B), 6.37–7.14 (7H, m). ¹³C NMR (MeOD) δ (ppm): 35.81 (t, 7'), 38.61 (t, 7), 42.90 (d, 8), 47.54 (d, 8'), 72.84 (t, 9), 114.76 (d), 116.84 (d), 117.31 (d), 121.06 (d), 121.76 (d), 130.62 (d), 131.45 (s), 140.94 (s), 145.01 (s), 146.32 (s), 158.71 (s), 181.44 (s, 9').

trans-3-(2,5-Dihydroxybenzyl)-2-(3-hydroxybenzyl)-γ-butyrolactone. Mass spectrum (EI, 70 eV) *m/z* (% relative abundance): 530 (100, M⁺), 515 (5), 335 (63), 293 (7), 268 (64, McLafferty rearrangement product), 180 (10, McLafferty rearrangement product), 180 (10, McLafferty rearrangement product), 73 (9). ¹H NMR (CDCl₃/MeOD) δ (ppm): 2.55–2.95 (6H, m, 7, 7', 8, 8'), 3.97 (1H, dd, 9B), 4.11 (1H, dd, 9A), 6.43–6.84 (6H, m), 7.10–7.14 (1H, m). ¹³C NMR (CDCl₃/MeOD) δ (ppm): 32.98 (t, 7'), 35.08 (t, 7), 40.54 (d, 8), 47.08 (d, 8'), 72.48 (t, 9), 114.31 (d), 114.79 (d), 116.42 (d), 116.97 (d), 117.74 (d), 121.63 (d), 126.97 (s), 130.14 (d), 139.86 (s), 148.70 (s), 150.09 (s), 157.34 (s), 180.99 (s, 9').

For the synthesis of 2,3-bis-(3-hydroxybenzyl)-butane-1,4diol (END) according to Groen and Leemhuis (1980), 0.77 mmol (230 mg) of ENL dissolved in 5 mL of dry THF was added to 5.7 mmol (216 mg) of lithium aluminum hydride in 30 mL of dry THF and stirred for 1 h at room temperature before the mixture was refluxed for 2.5 h. The reduction gave END in quantitative yield with a purity >99% according to GC/MS.

The same procedure was used at a 1 mg scale for the chemical reduction of *trans*-2-(2,3-dihydroxybenzyl)-3-(3-hydroxybenzyl)- γ -butyrolactone, *trans*-3-(3,4-dihydroxybenzyl)-2-(3-hydroxybenzyl)- γ -butyrolactone, and *trans*-2-(2,5-dihydroxybenzyl)-3-(3-hydroxybenzyl)- γ -butyrolactone to obtain 2-(2,3-dihydroxybenzyl)-3-(3-hydroxybenzyl)-butane-1,4-diol, 2-(3,4-dihydroxybenzyl)-3-(3-hydroxybenzyl)-butane-1,4-diol, and 2-(2,5-dihydroxybenzyl)-3-(3-hydroxybenzyl)-butane-1,4-diol, respectively.

2,3-Bis(3-hydroxybenzyl)-butane-1,4-diol (END). Mass spectrum (EI, 70 eV) m/z (% relative abundance): 500 (27, M⁺ – TMSOH), 410 (100, M⁺ – 2TMSOH), 231 (25), 217 (22), 205 (4), 194 (10), 180 (48, McLafferty rearrangement product), 73 (11). ¹H NMR (DMSO- d_6) δ (ppm): 1.90 (2H, m, 8), 2.37–2.63 (4H, m, 7), 3.37 (4H, m, 9), 6.54–6.57 (6H, m), 6.99–7.06 (2H, m), 9.20 (2H, s). ¹³C NMR (DMSO- d_6) δ (ppm): 33.86 (t, 7), 42.43 (d, 8), 59.99 (t, 9), 112.38 (d), 115.78 (d), 119.49 (d), 128.80 (d), 142.92 (s), 156.99 (s).

2-(2,3-Dihydroxybenzyl)-3-(3-hydroxybenzyl)-butane-1,4diol. Mass spectrum (EI, 70 eV) m/z (% relative abundance): 678 (3, M⁺), 588 (22, M⁺ – TMSOH), 498 (95, M⁺ – 2TMSOH), 268 (26), 231 (87), 180 (100), 73 (10).

 $2\mathcal{-}(3,4\mathcal{-}Dihydroxybenzyl)\mathcal{-}3\mathcal{-}(3\mathcal{-}hydroxybenzyl)\mathcal{-}butane\mathcal{-}1,4\mathcal{-}diol.$ Mass spectrum (EI, 70 eV) m/z (% relative abundance): 678 (11, M⁺), 588 (72, M⁺ - TMSOH), 498 (70, M⁺ - 2TMSOH), 268 (90), 231 (41), 180 (100), 73 (4).

2-(2,5-Dihydroxybenzyl)-3-(3-hydroxybenzyl)-butane-1,4diol. Mass spectrum (EI, 70 eV) m/z (% relative abundance): 678 (41, M⁺), 588 (100, M⁺ - TMSOH), 498 (55, M⁺ -2TMSOH), 268 (14), 231 (16), 180 (6), 73 (15). **Microsomal Preparations and Incubations.** Microsomes were prepared from the liver of untreated or aroclor-treated male Wistar rats and untreated pigs. Treatment with Aroclor 1254 (one intraperitonal injection on day 1 of a dose of 500 mg/kg body weight dissolved in sesame oil at 100 mg/mL, sacrifice on day 6) was carried out to induce hepatic cytochrome P-450-dependent monooxygenases. Microsomes of human liver were kindly provided by Dr. W. Dekant, University of Würzburg. All microsomes were prepared according to the method of Lake (1987). Protein concentration was determined by the protein assay of Pierce based on copper ions and bicinchoninic acid (Smith et al., 1985). The concentration of cytochrome P-450 was measured by the method of Omura and Sato (1964). All chemicals used for microsomal preparations and incubations were of the highest purity available.

The microsomal incubations were carried out in a final volume of 2 mL containing 1 mg of microsomal protein, $25 \,\mu$ M test compound dissolved in DMSO (final concentration 2.5%), and a NADPH-generating system (0.9 U isocitrate dehydrogenase, 9.4 mM isocitrate, 1.21 mM NADP⁺, and 4.3 mM MgCl₂) in phosphate buffer (0.1 M, pH 7.4). After a preincubation period of 2 min at 37 °C, the reaction was initiated by the addition of 70 μ L of the NADPH-generating system and terminated after 30 min at 37 °C by extraction with 3 × 2 mL of ethyl acetate. This extract was evaporated to dryness and dissolved in 100 μ L of methanol for HPLC analysis.

Analytical Methods. HPLC analysis was carried out on a Polygosil RP-18 column ($250 \times 4 \text{ mm}$; 5 μ m) with a linear gradient program from 25% A (methanol)/75% B (16% methanol/84% water, adjusted to pH 2.8 with formic acid) to 30% A/70% B in 55 min. The flow rate was 1 mL/min; the UV absorbance was monitored at 275 nm or recorded with a diode array detector.

For HPLC/MS analysis, the same HPLC conditions were used, but the gradient started at 15% A/85% B and ended at 20% A/80% B. Two ion source types were used: (i) atmospheric pressure chemical ionization (APCI) with the following conditions: corona current, 49.8 μ A; corona voltage, 6.232 kV; vaporizer temperature, 544 °C; 0.1 mL/min H₂O/25% aqueous NH₃ (1/1, v/v) was added to the eluate prior to the ion source; (ii) electrospray ionization (ESI) with conditions as follows: spray voltage, 2.72 kV; spray current, 99.9 μ A; capillary temperature, 200 °C; 0.1 mL/min H₂O was added to the eluate.

For GC/MS analysis, the sample was evaporated to dryness under reduced pressure, and the residue was trimethylsilylated with 30 μ L of BSA (1:10 in heptane). Conditions were as follows: SE-30 fused silica column (30 m × 0.25 mm; 0.25 μ m); flow of He, 40 cm/s; column temperature, from 70 to 250 °C at 30 °C/min, from 250 to 270 °C at 1 °C/min; injector, 275 °C; transfer line, 275 °C; ion source, 150 °C; ionization energy, 70 eV (ion trap mass detector); injection volume, 1 μ L.

RESULTS

Microsomal Metabolism of Enterolactone. Pilot studies with hepatic microsomes from untreated and aroclor-treated rats, untreated pigs, and untreated humans showed that liver microsomes of aroclor-treated rats metabolized ENL to the greatest extent. The extract from this microsomal incubation gave rise to nine metabolite peaks in HPLC analysis using an UV detector (Figure 2, peaks A-I). When the HPLC eluate containing all the metabolites was trimethylsilylated and subject to GC/MS, 12 compounds were found (Figure 3, peaks 1-12) that were absent in the respective eluate obtained from control incubations without ENL. ENL itself coelutes with peak 5 in GC/MS analysis and therefore had to be separated from the metabolites by HPLC. By analyzing each HPLC metabolite peak by GC/MS, the 12 ENL metabolite peaks of the GC profile were correlated with the nine metabolite peaks observed in HPLC. This correlation and the molecular ions of the





Figure 2. HPLC profile of the enterolactone (ENL) metabolites from aroclor-induced rat liver microsomes.



Figure 3. GC profile of the enterolactone (ENL) metabolites from aroclor-induced rat liver microsomes.

 Table 1. Chromatographic and Spectroscopic Data of

 Enterolactone (ENL) and Its Metabolites

HPLC peak	<i>RT</i> in HPLC (min)	UV maxima (nm)	[M – H] [–] (HPLC/MS)		corresponding	M+
			ESI	APCI	GC peak	(GC/MS)
Α	16.4	280	295	295	1, 2 (dp) ^a	440
B/C	19.4/20.4	284, 294	313	313	6	530
					7	530
D	28.2	279	313	313	11	530
					12	530
Ε	32.4	nm ^b	313	295	5 (dp)	440
F	35.5	279, 244	313	295	4, 5 (dp)	440
G	38.6	280, 242	313	295	4, 10 (dp)	440
Н	41.6	286, 244	295	295	10 (dp)	440
Ι	47.0	nm	\mathbf{nd}^{c}	nd	3, 4, 5 (dp)	440
					8	530
					9	530
ENL	51.4	277, 283	297	297		442

 a dp, daughter product. b nm, not measurable (intensity too low for UV spectrum). c nd, not detected.

12 ENL metabolites are summarized in Table 1 together with the HPLC/MS data and the UV maxima as obtained with a diode array detector.

Table 2. Identification of ENL Metabolites Monohydroxylated at the Aromatic Rings

GC peak	position of hydroxylation ^a	chemical name of metabolite
6	para in ring B	<i>trans</i> -2-(2,5-dihydroxybenzyl)-3-(3-hydroxybenzyl)-γ-butyrolactone
7	para in ring A	trans-3-(2,5-dihydroxybenzyl)-2-(3-hydroxybenzyl)-γ-butyrolactone
8	ortho in ring B (2,3-catechol)	trans-2-(2,3-dihydroxybenzyl)-3-(3-hydroxybenzyl)-γ-butyrolactone
9	ortho in ring A (2,3-catechol)	<i>trans</i> -3-(2,3-dihydroxybenzyl)-2-(3- hydroxybenzyl)-γ-butyrolactone
11	ortho in ring B (3,4-catechol)	trans-2-(3,4-dihydroxybenzyl)-3-(3-hydroxybenzyl)-y-butyrolactone
12	ortho in ring A (3,4-catechol)	$trans$ -3-(3,4-dihydroxybenzyl)-2-(3-hydroxybenzyl)- γ -butyrolactone

^a Relative to existing hydroxy groups in ENL.



Figure 4. Possible positions of enterolactone (ENL) and enterodiol (END) for aliphatic and aromatic hydroxylation. Positions are numbered according to Cooley et al. (1984).

The mass spectrum of the parent compound ENL in its trimethylsilylated form exhibited a strong molecular ion at m/z 442 (17%, data not shown). Six of the 12 ENL metabolites analyzed by GC/MS (viz., peaks 6-9, 11, and 12) had their molecular ions at m/z 530, which implied one additional hydroxy group. The mass spectra of the other six GC peaks (i.e., peaks 1-5 and 10) exhibited their highest m/z at 440, which suggested the introduction of one additional double bond into the ENL molecule. This double bond might have been formed through elimination of a trimethylsilylated hydroxy group during GC/MS. As aromatic and aliphatic hydroxylation can occur at various sites of the ENL molecule (Figure 4) it was assumed that the metabolites with $m/z 53\overline{0}$ represent products of aromatic hydroxylation whereas those with m/z 440 were less stable products of aliphatic hydroxylation.

This proposition was corroborated by the mass spectra obtained by HPLC/MS (Table 1). ENL exhibited a [M - H] ion at m/z 297 both with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The HPLC peaks B-D, corresponding to GC/ MS peaks 6, 7, 11, and 12 and thought to represent metabolites hydroxylated at the aromatic rings, displayed [M - H] ions at m/z 313 in HPLC/MS with both ESI and APCI. In contrast, HPLC peaks E-G, corresponding to GC/MS peaks 4, 5, and 10, exhibited [M -H] ions at m/z 313 only with the more gentle ESI but gave rise to [M - H] ions at m/z 295 with APCI, indicating the elimination of water. It is suggested that HPLC peaks E-G are aliphatic hydroxylation products of ENL sufficiently stable for ESI but not for APCI. HPLC peaks A and H displayed [M - H] ions at m/z



Figure 5. Microsomal metabolites of enterolactone (ENL) formed through aromatic hydroxylation. The GC peak numbers refer to Figure 3.

295 with both EPI and APCI and may therefore represent highly unstable aliphatic hydroxylation products eliminating water readily with any ionization method. Elimination from some of the aliphatic positions can lead to multiple olefinic daughter products (Figure 4).

For further identification of the aromatic hydroxy metabolites, six derivatives of ENL with an additional hydroxy group in ring A or B in para position or in the two ortho positions of the existing phenolic hydroxy group were synthesized. In addition, an ENL derivative hydroxylated in the meta position of ring B was prepared by chemical synthesis. With these reference compounds, the ENL metabolites represented by GC peaks 6–9, 11, and 12 were unequivocally identified as shown in Table 2 by cochromatography in HPLC and GC as well as identical UV and mass spectrum. The complete pattern of these metabolites is depicted in Figure 5.

Microsomal Metabolism of Enterodiol. When incubations of END with hepatic microsomes from untreated and aroclor-treated rats, untreated pigs, and untreated humans were analyzed by HPLC with UV detector, the highest turnover was observed with aroclor-treated rat liver microsomes, but the number and amounts of metabolites of END were markedly lower than with ENL. Six metabolite peaks of END were detectable by HPLC analysis (Figure 6, peaks A–F). The eluate containing the metabolites was then trimethyl-silylated and analyzed by GC/MS, which gave rise to seven metabolite peaks as shown in Figure 7. Peaks 4a and 4b could only be separated on a new GC column. The correlation of HPLC and GC peaks, the UV maxima

OD 275 nm



Figure 6. HPLC profile of the enterodiol (END) metabolites from aroclor-induced rat liver microsomes.



Figure 7. GC profile of the enterodiol (END) metabolites from aroclor-induced rat liver microsomes.

data, and the MS data of the END metabolites are summarized in Table 3.

In GC/MS, trimethylsilylated END does not exhibit a molecular ion at m/z 590 but a [M - 90] ion at m/z500, arising probably through the loss of TMSOH. The molecular ions of GC peaks 4a, 4b, and 5 were at m/z678, indicating an additional hydroxy group that is not eliminated. In contrast, GC peaks 1–3 and 6 displayed their highest m/z at 588, which suggests the elimination of an additional hydroxy group. Thus, the microsomal metabolites of END appear to consist of three stable aromatic hydroxylated products with molecular ion at m/z 678 and four less stable aliphatic hydroxylated products with molecular ion at m/z 588. This assignment is supported by the data from HPLC/MS with APCI (Table 3).

As the two aromatic rings of END are equivalent, there are only four possible aromatic monohydroxylation products (Figure 4). Three of them were chemically

 Table 3. Chromatographic and Spectroscopic Data of

 Enterodiol (END) and Its Metabolites

HPLC peak	<i>RT</i> in HPLC (min)	UV maxima (nm)	[M – H] [–] (HPLC) APCI	corresponding GC peak	M ⁺ (GC/MS)
А	7.8	nm ^a	299	(dp) ^{<i>b</i>,<i>c</i>}	588
В	8.9	nm	299	$(\mathbf{dp})^c$	588
С	10.6	246, 276	299	3 (dp)	588
D/E	14.9	249, 279	299	6 (dp)	588
			317	4	678
F	18.0	279	317	5	678
END	33.9	276, 283	301		590

^{*a*} nm, not measurable (intensity too low for UV spectrum). ^{*b*} dp, daughter product. ^{*c*} GC peaks 1 and 2 could not be unambiguously correlated with the HPLC peaks due to the small amounts of metabolites.



Figure 8. Microsomal metabolites of enterodiol (END) formed through aromatic hydroxylation. The GC peak numbers refer to Figure 7.

synthesized and compared with the END metabolites in HPLC and GC by cochromatography. Identical retention times as well as identical UV and mass spectra clearly showed that GC peak 4a is 2-(2,5-dihydroxybenzyl)-3-(3-hydroxybenzyl)-butane-1,4-diol (END hydroxylated at the para position), GC peak 4b is 2-(2,3dihydroxybenzyl)-3-(3-hydroxybenzyl)-butane-1,4-diol (END hydroxylated at the more sterically hindered ortho position), and GC peak 5 is 2-(3,4-dihydroxybenzyl)-3-(3-hydroxybenzyl)-butane-1,4-diol (END hydroxylated at the less sterically hindered ortho position). A metabolic scheme is depicted in Figure 8.

Pattern of ENL and END Metabolites with Microsomes from Different Species. Incubations of ENL with hepatic microsomes of untreated and aroclor-treated male rats, untreated pigs, and untreated humans showed qualitatively similar elution profiles in HPLC and GC/MS but quantitative differences in the relative amounts of the various metabolites (Table 4). Metabolites 3, 8, and 9 could only be found with microsomes from aroclor-treated rats in very low quantities (see also Figure 3) and are omitted from Table 4. Metabolites 11 and 12 were not observed with human microsomes. The other metabolites, i.e., GC peaks 1, 2, 4–7, and 10 were formed with microsomes from all three species, although in different quantities. With

 Table 4. Pattern of ENL Metabolites with Microsomes

 from Various Species (Expressed in Percent of Total

 Metabolites^a)

metabolite (GC/MS) ^b	type of hydroxylation ^c	aroclor- treated rat (1.71) ^d	untreated rat $(0.59)^d$	pig (0.54) ^d	human (0.39) ^d
1	aliphatic	1	5	6	3
2	aliphatic	2	4	13	5
4	aliphatic	8	26	13	19
5	aliphatic	19	7	11	17
6	aromatic	24	10	26	7
7	aromatic	13	10	9	6
10	aliphatic	14	29	20	43
11	aromatic	3	3	1	\mathbf{nd}^{e}
12	aromatic	16	6	1	nd

^{*a*} Calculated from the peak areas of the total ion current chromatogram obtained by GC/MS analysis (e.g., Figure 3 for aroclor-induced rat liver microsomes). ^{*b*} According to Figure 3. ^{*c*} According to tentative identification (see text). ^{*d*} Cytochrome P-450 content (nmol/mg of microsomal protein). ^{*e*} nd, not detected, <0.2%.

human microsomes, the generation of aliphatic hydroxylation products (peaks 4, 5, and 10) is preferred at the expense of aromatic hydroxylation products (peaks 6 and 7).

Incubations of END with liver microsomes from untreated rats, pigs, and humans generated only very small quantities of metabolites and showed a similar pattern as observed with aroclor-treated rat microsomes (Figure 7): the predominating END metabolites in all microsomes were GC peaks 3 and 6, although the ratio varied between species: GC peak 3 accounted for 8% of total metabolites with untreated rat, 39% with porcine, and 13% with human hepatic microsomes, whereas GC peak 6 represented 92% in untreated rat, 58% in porcine and 87% in human microsomes. GC peaks 1, 2, and 4 were not detected in untreated rat, porcine, or human microsomes.

DISCUSSION

The mammalian lignans ENL and END may reach considerable concentrations in body fluids after ingestion of whole-grain products, in particular flaxseed, and certain vegetables. As epidemiological and biochemical studies suggest an anticarcinogenic potential for ENL and END, these compounds are of considerable interest for cancer prevention by appropriate nutrition. However, little is known about possible health risks. We have recently reported that ENL and END as well as their plant precursors matairesinol and secoisolariciresinol lack activity at various endpoints for genotoxicity in vitro, e.g., the induction of micronuclei and gene mutations in Chinese hamster V79 cells or the interference with cell-free microtubule assembly (Kulling et al., 1998). However, there are no reports in the literature to date about the oxidative metabolism of these lignans and the biological effects of their metabolites.

The results of the present study on the metabolism of ENL and END in liver microsomes from untreated and aroclor-treated rats, untreated pigs, and untreated humans clearly show that both ENL and END undergo oxidative biotransformation. ENL is metabolized by microsomes of aroclor-treated rats to products carrying one additional hydroxy group at different aliphatic and aromatic positions. In theory, five aliphatic positions are available for the introduction of a hydroxy function into the ENL molecule (Figure 4). For racemic *trans*-ENL, each hydroxylation can lead to two diastereomers. As

we could detect six ENL metabolites with aliphatic hydroxylation (viz., HPLC peaks A and E-I) at least three aliphatic positions must have been hydroxylated by liver microsomes. The ultimate identification of the hydroxylated positions requires the synthesis of appropriate reference compounds, which is in progress in our laboratory. Aromatic hydroxylations of ENL can yield eight different metabolites as there are four positions at each of the two rings that are not equivalent (Figure 4). With a oclor-induced rat liver microsomes, hydroxylation takes place at six aromatic positions as could be shown by GC/MS analysis. All six metabolites were identified by means of synthetic reference substances as products of a ring hydroxylation in the para position and the two ortho positions of the existing phenolic hydroxy groups in both rings. The complete pattern of these metabolites is depicted in Figure 5. No evidence was obtained for the presence of ENL metabolites hydroxylated in the meta position, for which one reference compound was available; the failure to find such metabolites is not surprising as meta hydroxylation of aromatic compounds is an uncommon metabolic reaction.

In contrast to ENL, which was readily metabolized by aroclor-induced rat liver microsomes, the number and amounts of END metabolites formed by the same microsomes were much less. Aliphatic hydroxylation of END can theoretically take place at two different positions leading to four diastereomers (Figure 4). As four such metabolites (HPLC peaks A-D/E) were found, both aliphatic positions of END must be assumed to undergo metabolic hydroxylation. In addition, three of the four possible aromatic positions were hydroxylated (GC peaks 4a, 4b, and 5), although to a very small extent. These were identified with the help of synthetic reference compounds as the para and both ortho positions of END (Figure 8).

A comparison of the oxidative biotransformation of ENL and END by microsomes of untreated and aroclortreated rats, untreated pigs, and untreated humans showed similar qualitative but different quantitative patterns of metabolites. In all three uninduced species, the major metabolic route is the hydroxylation at aliphatic positions; the predominance of aliphatic over aromatic hydroxylation is most striking in human microsomes. Pretreatment of rats with aroclor, which is a mixture of chlorinated biphenyls and is known to induce several classes of cytochrome P450 isoenzymes (Ryan et al., 1982), appears to shift the metabolic pattern towards aromatic hydroxylation.

In conclusion, this study has shown that ENL and END undergo aliphatic and aromatic hydroxylation at various positions upon incubation with mammalian hepatic microsomes. We have recently observed that several of these novel metabolites of ENL and END are also excreted in the urine of human subjects fed a diet containing flaxseed (Jacobs et al., 1999). All six products of aromatic monohydroxylation of ENL (Figure 5) and all three aromatic monohydroxylated metabolites of END (Figure 8), but surprisingly none of the aliphatic hydroxylation products, could be detected in human urine. Studies are now in progress to synthesize further reference compounds for the identification of all metabolites of ENL and END and the investigation of their genotoxic and estrogenic potentials.

ABBREVIATIONS USED

APCI, atmospheric pressure chemical ionization; BSA, N, O-bis(trimethylsilyl)acetamide; DMSO, dimethyl sulfoxide; EI, electron impact; END, enterodiol, 2,3-bis(3-hydroxybenzyl)butane-1,4-diol; ENL, enterolactone, *trans*-2,3-bis(3-hydroxybenzyl)- γ -butyrolactone; ESI, electrospray ionization; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography/mass spectrometry; THF, tetrahydrofurane; TMS, trimethylsilyl.

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