Oxidative Metabolites of the Mammalian Lignans Enterodiol and Enterolactone in Rat Bile and Urine

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Recent studies have shown that the mammalian lignans enterodiol (END) and enterolactone (ENL) are biotransformed in vitro by hepatic microsomes from rats and humans to various metabolites carrying one additional hydroxy group either at the aromatic or at the aliphatic moiety. To clarify whether these metabolites are also formed in vivo, each lignan was administered intraduodenally at a dose of 10 mg/kg of bw to bile duct-catheterized female Wistar rats and the 6 h bile analyzed by HPLC and GC-MS. With END-dosed rats, three products of aromatic and two of aliphatic monohydroxylation were found, whereas six aromatic and five aliphatic monohydroxylated biliary metabolites were detected after administration of ENL. The metabolites hydroxylated at the aromatic rings were unequivocally identified by comparison with synthetic reference compounds. The structures of the in vivo metabolites arising from aliphatic hydroxylation could not be completely elucidated; they were identical with some of the formerly reported microsomal products according to GC retention times and mass spectra. Significant amounts of most of the metabolites of the mammalian lignans identified in bile were also found in the urine of female rats after oral administration of 10 mg/kg of bw END or ENL and in the urine of female and male Wistar rats after they had been fed a diet containing 5% flaxseed. Thus, the mammalian lignans END and ENL give rise to several hydroxylated metabolites in vivo, which may contribute to the biological effects of these important food constituents.

Keywords: *Mammalian lignans; enterodiol; enterolactone; biliary metabolites; urinary metabolites; mass spectrometry*

INTRODUCTION

Phytoestrogens of the lignan type are found in a variety of foods and are of increasing interest due to their putative anticarcinogenic, antiestrogenic, and estrogenic effects (Murkies et al., 1998; Rickard and Thompson, 1997). In particular, flaxseed has been shown to contain several plant lignans, with secoisolariciresinol representing the major and matairesinol, isolariciresinol, and pinoresinol constituting minor lignan components (Meagher et al., 1999). Secoisolariciresinol and matairesinol are converted by intestinal bacteria to the mammalian lignans enterodiol (END, Figure 1) and enterolactone (ENL), which are excreted in the urine of rats and humans (Stitch et al., 1980; Setchell et al., 1980, 1983; Axelson and Setchell, 1981; Axelson et al., 1982). It is notable that high levels of mammalian lignans are reached in various body fluids in humans and animals after prolonged intake of even moderate amounts of flaxseed. For example, ingestion of 13.5 g of flaxseed per day for 6 weeks has been reported to lead to micromolar concentrations of END and ENL in the plasma of humans (Atkinson et al., 1993). This is 1000−10000 times the plasma level of the circulating endogenous steroidal estrogens (Rickard and Thompson, 1997).

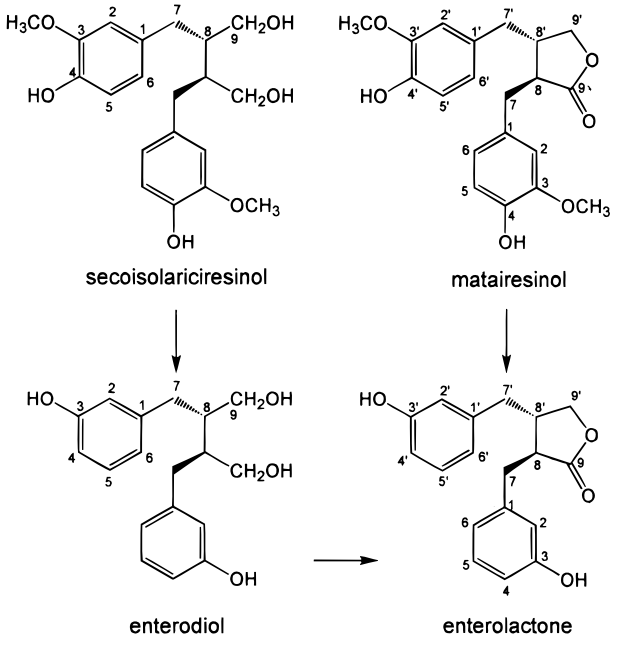

Figure 1. Chemical structures of secoisolariciresinol, matairesinol, enterodiol (END), and enterolactone (ENL). Nomenclature follows that of Ayres and Loike (1990).

To date, little is known about the biotransformation of lignans in the mammalian organism. Due to their two phenolic groups, both END and ENL are readily con-

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jugated with glucuronic and sulfuric acid prior to excretion (Adlercreutz et al., 1995). In a recent study from our laboratory, it has been reported that END gives rise to seven monohydroxylated metabolites upon incubation with microsomes from rat, pig, and human liver (Jacobs and Metzler, 1999). Three of these oxidative END metabolites are hydroxylation products of the aromatic rings, whereas the other four arise through aliphatic hydroxylation. Likewise, ENL is biotransformed by rat and human liver microsomes to six aromatic and six aliphatic monohydroxylation products (Jacobs and Metzler, 1999). The microsomal metabolites of END and ENL containing an additional aromatic hydroxyl group could be unequivocally identified by comparison with synthetic reference compounds as monohydroxylated in the para and both ortho positions of the parent phenolic group. These metabolites were subsequently also detected in the urine of female and male humans after ingestion of flaxseed for 5 days (Jacobs et al., 1999).

In the present study, we have searched for monohydroxylated metabolites of END and ENL in the bile and urine of Wistar rats after administration of the mammalian lignans per se or after the feeding of flaxseed. Our results clearly demonstrate that both aromatic and aliphatic hydroxylation reactions take place in the biotransformation of END and ENL in the rat in vivo.

MATERIALS AND METHODS

Chemicals and Materials. ENL and END were synthesized in our laboratory according to a modification of the tandem addition procedure (Pelter et al., 1983) as described by Jacobs and Metzler (1999). The purity of ENL and END was >99% according to GC-MS analysis. For the identification of oxidative metabolites, three monohydroxylated derivatives of END and seven of ENL were synthesized as reference compounds and characterized by ^{1}H and ^{13}C NMR and MS (Jacobs and Metzler, 1999). Flaxseed (*Linum usitatissimum* L., small-grained) was purchased from Klenk, Germany (1998, Ch. 9710081) and shredded prior to feeding. All other chemicals were purchased from Sigma, Aldrich, Fluka, Merck, Serva, Pharmacia, or Boehringer (Mannheim) and were of the highest purity available. Microsomes were prepared from the liver of untreated male Wistar rats and incubated with END or ENL as previously reported (Jacobs and Metzler, 1999). Metabolites were extracted from the incubation mixture with ethyl acetate, evaporated to dryness under reduced pressure, dissolved in methanol, and purified by HPLC as described below.

Collection of Rat Bile and Urine. The in vivo experiments with rats were carried out at Knoll AG (Ludwigshafen, Germany) and at the Institute of Toxicology of the University of Würzburg. Animals used for each study were of comparable age and body weight (bw) and were kept under controlled conditions of temperature, humidity, and light (12 h light/12 h dark cycle).

For the collection of bile, four female Wistar rats (age 5 weeks, bw ~200 g) were anesthetized with pentobarbital (40 mg/kg of bw, injected intraveneously in saline), and the bile ducts were catheterized with a polyethylene tube of 0.5 mm inner diameter. Under continued anesthesia, bile was collected for 1 h prior to the administration of the compounds and used as control. Ten milliliters of a 0.9% saline solution containing 2 mg of END or ENL was then injected into the duodenum, and bile was collected for another 6 h from each rat and stored at −20 °C until analyzed.

For the collection of urine, groups of two female Wistar rats (age 5 weeks) received 1 mL of corn oil containing 2 mg of END or ENL (dissolved in 25 μL of DMSO) by gavage, and the urine was collected at 0 °C for 24 h. Control animals received 1 mL of corn oil containing 25 μL of DMSO. After the addition of ascorbic acid (0.2%) and sodium azide (0.2%), the urine was stored at −80 °C until analyzed. In another experiment, groups of five female and five male 5-week-old Wistar rats were fed lab chow (catalog no. 3433.0.25, Kliba, Germany) containing 5% shredded flaxseed for 7 days, and the 24 h urine of each group was collected on day 8 and stored as described above. Urine collected from another five female and five male animals fed a diet without flaxseed was used as control.

Isolation of Lignan Metabolites from Rat Bile and Urine. For the cleanup of rat bile, the method described by Jacobs et al. (1999) for human urine was modified according to that of Sfakianos et al. (1997): 0.5 mL of bile mixed with 0.5 mL of ammonium acetate buffer (0.05 M, pH 5.0) was applied onto an RP-18 cartridge (9 × 10 mm, short body, Waters) preconditioned with the same buffer for solid-phase extraction. The cartridge was washed with 5 mL of ammonium acetate buffer (0.01 M, pH 5.0) prior to the elution of lignans and their metabolites with 4 mL of methanol. The eluent was diluted with water to 70% methanol and applied to a DEAE-Sephadex A 25 column (12 × 33 mm, acetate form) conditioned with 70% aqueous methanol. Bile contents were separated into three fractions using 8 mL of 70% aqueous methanol to elute unconjugated lignans, 20 mL of 0.2 M acetic acid in 70% aqueous methanol to elute an intermediate fraction, and 20 mL of 0.3 M lithium chloride in 70% aqueous methanol to elute conjugated lignans. The fractions containing the unconjugated and conjugated material were combined, concentrated to a volume of ~6 mL, mixed with 1 mL of ammonium acetate buffer (0.05 M, pH 5.0), and purified on a second RP-18 cartridge as described above. The methanol eluate was evaporated to dryness, and the residue was dissolved in 2.5 mL of ammonium acetate buffer (0.05 M, pH 5.0), mixed with 9 mg of ascorbic acid, and incubated with 4500 Fishman units of β-glucuronidase/aryl sulfatase (from *Helix pomatia*) overnight at 37 °C for hydrolysis of the conjugates. Lignans and metabolites were extracted from the incubation by another RP-18 cartridge, which was washed with 5 mL of water prior to elution of the unconjugated compounds with 4 mL of methanol. After evaporation to dryness, the residue was dissolved in 400 μL of 80% aqueous methanol with ultrasonic treatment for 10 min. Insoluble material was then removed by centrifugation at 3000 rpm for 10 min and the supernatant used for HPLC purification.

The cleanup of rat urine was carried out as described by Jacobs et al. (1999) for human urine, starting with 8 mL of urine and yielding 400 μL of a methanol solution.

Purification of Lignan Metabolites by HPLC. One hundred microliters of the final methanol solutions from the cleanup procedures of rat bile and urine was further purified by HPLC on an RP-18 HPLC column (250 × 4.6 mm, Phenomenex Prodigy 5 μm ODS) using a linear solvent gradient for elution. Solvent A was water/methanol 84:16 (v/v) adjusted to pH 2.8 with formic acid, and solvent B was methanol. The gradient changed from 25% B to 30% B in 55 min with a flow rate of 1 mL/min. UV absorbance at 275 nm was used for detection. Aliquots of 20−40 μL of the respective methanolic solution of biliary or urinary metabolites were injected per HPLC run. The fraction containing END metabolites was collected between 8 and 48 min (fraction 1), whereas unchanged END was collected between 48 and 55 min (fraction 2). Likewise, ENL metabolites were collected between 12.5 and 55 min, and ENL was collected from 55 to 60 min. Metabolites from microsomal incubations were purified accordingly.

GC-MS Analysis. For GC-MS analysis, the residues of the HPLC fractions were treated with 30 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) overnight at room temperature. HPLC fractions 2 were then diluted 1:100 with *n*-heptane. GC-MS was carried out using a GCQ ion trap mass detector (Finnigan MAT) with the following conditions: MDN-5S fused silica capillary column (29.6 m × 0.25 mm; 0.25 μm, Supelco); flow of He, run pressure from 10 to 20 psi; column temperature, 1 min isothermic at 60 °C, from 60 to 250 °C at 30 °C/min, 10 min isothermic at 250 °C, from 250 to 275 °C at 1 °C/min; injector temperature, from 50 to 275 °C at 8 °C/s; transfer line temperature, 275 °C; ion source temperature, 200 °C; ionization energy, 70 eV; injection volume, 1 μL. Full scan

Table 1. Ions in the Mass Spectra of the TMS Derivatives of END, ENL, and Their Monohydroxylated Metabolites Used for Their Detection in Selected Ion Mode

	m/z
END	500, 410, 231
aromatic hydroxylation products	678, 268, 231
aliphatic hydroxylation products	588, 408, 395
ENL	442, 263, 180
aromatic hydroxylation products	530, 351, 268
aliphatic hydroxylation products	440, 425, 307

mass spectra (mass range of 50−750 amu) were recorded at a rate of two spectra per second. Detection of END, ENL, and their metabolites was achieved by selecting appropriate ions from the full scan spectra using the Finnigan GCG 2.0 data analysis software.

RESULTS AND DISCUSSION

Oxidative Metabolites of END and ENL in Rat Bile. Bile duct-catheterized female Wistar rats kept under anesthesia were intraduodenally injected with 10 mg/kg of bw of END or ENL, and their bile was collected for 6 h. The fractions containing unconjugated material and conjugates were isolated from the bile by solid-phase extraction and ion exchange chromatography, pooled, and treated with β-glucuronidase/aryl sulfatase for the hydrolysis of the conjugates. Following cleanup by solid-phase extraction and HPLC, the fractions containing the parent lignans, that is, unconjugated and nonhydroxylated END and ENL (HPLC fraction 2), and their oxidative metabolites (HPLC fraction 1) were trimethylsilylated and analyzed by GC-MS. From the previous studies on the microsomal metabolism of END and ENL (Jacobs and Metzler, 1999) and on the urinary metabolites of lignans in humans (Jacobs et al., 1999), the mass spectra of several metabolites resulting from monohydroxylation at the aromatic or aliphatic moiety were known. Table 1 exhibits ions in the mass spectra of END and ENL and their two types of monohydroxylated metabolites that proved to be best suited for the detection of these compounds in the selected ion mode.

When the GC-MS runs of the rat biliary metabolites of END were searched for these ions (Figure 2), three metabolites of the aromatic hydroxylation type (peaks 2, 3, and 5) and two of the aliphatic hydroxylation type (peaks 1 and 4) were detected, which were not present in the control bile (Table 2, columns 1 and 3). As three synthetic reference compounds were available for the aromatic hydroxylation products of END (Jacobs and Metzler, 1999), carrying the additional hydroxy group in the para position and in either ortho position of the parent phenolic group, cochromatography of these standards with the biliary metabolites was carried out by GC-MS. The results unequivocally showed that peak 2 in Figure 2 is 6-hydroxy-END, peak 3 is 2-hydroxy-END, and peak 5 is 4-hydroxy-END. Their mass spectra are depicted in Figure 3 together with those of the aliphatic hydroxylation products peaks 1 and 4 found in rat bile. No reference compounds were available for END monohydroxylated at the aliphatic moiety. The similarity of the mass spectra implies that peaks 1 and 4 are stereoisomers of one hydroxylation product, and the major fragment ions in the mass spectra are compatible with the structure of 7-hydroxy-END and 8-hydroxy-END. A mechanism for the formation of these fragment ions is proposed in Figure 4. However, the unequivocal determination of the chemical structures of metabolites

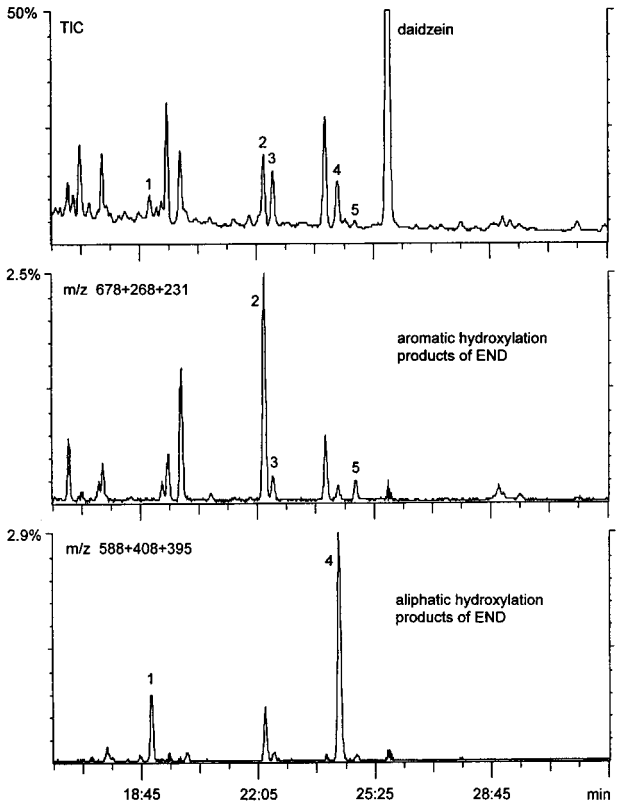

Figure 2. Capillary GC-MS analysis of bile from END-dosed rats (HPLC fraction 1 after trimethylsilylation): (top) total ion current (TIC); (middle) ion current reconstructed from three typical ions of aromatic monohydroxylation products of END; (bottom) ion current reconstructed from three typical ions of aliphatic monohydroxylation products of END. The numbered peaks represent END metabolites.

1 and 4 has to await confirmation by other spectroscopic data, for example, from NMR analysis or the comparison with synthetic reference compounds.

Cochromatography by GC-MS of the rat biliary metabolites with the products of microsomal biotransformation of END (Jacobs and Metzler, 1999) showed that peaks 1 and 4 had the same retention times and mass spectra as the two major aliphatic hydroxylation products of the microsomal incubation. Likewise, peaks 2, 3, and 5 coincided with the corresponding microsomal aromatic hydroxylation products (data not shown).

As two of the aromatic hydroxylation products of END are catechols, it is likely that these metabolites undergo further biotransformation in vivo by catechol-*O*-methyltransferase. In fact, a peak containing ions that are expected to be characteristic for END carrying one aromatic methoxy group (*m/z* 620, 530, 440, and 210) was found in the GC-MS run at a retention time of 24.0 min (data not shown). However, this metabolite could not be further characterized because no reference compounds for the methylated catechols of END were available.

When the typical ions for monohydroxylated metabolites of ENL were searched for in the GC-MS analysis of the bile of ENL-treated rats (Figure 5), six products of aromatic hydroxylation (peaks 5, 7/8, 9, 10, and 11) and five of aliphatic hydroxylation were clearly detected, most of which were not present in bile from untreated rats (Table 2, columns 2 and 3). Six synthetic reference compounds were available for the aromatic hydroxylation products of ENL (Jacobs and Metzler, 1999),

Table 2. Hydroxylated Metabolites of END and ENL Identified in the Bile and Urine of Wistar Rats in Vivo following Various Treatments[a]

	bile			urine							
compound identified by GC-MS	female rats dosed with END	female rats dosed with ENL	untreated female rats	female rats dosed with END	female rats dosed with ENL	untreated female rats	female rats dosed with corn oil	female rats fed flaxseed	male rats fed flaxseed	untreated female rats	untreated male rats
END	++++	t	t	+++++	++	++	++	+++	++	+	+
2-HO-END (peak 3)[b]	+	−	−	++	t	−	−	+	t	−	−
4-HO-END (peak 5)[b]	+	−	−	++	t	t	−	+	t	−	−
6-HO-END (peak 2)[b]	++	−	−	++	−	−	−	+	t	−	−
aliphatic HO-END (peak 1)[b]	+	−	−	+	−	−	−	−	−	−	−
aliphatic HO-END (peak 4)[a]	++	−	−	+	−	−	−	−	−	−	−
ENL	+	++++	+	+++	+++++	++++	++++	+++++	++++	++++	+++
2-HO-ENL (peak 8)[c]	−	+	−	+	++	+	−	++	+	+	t
4-HO-ENL (peak 10)[c]	t	+	t	+	++	+	−	++	+	+	t
6-HO-ENL (peak 5)[c]	t	++	−	+	++	+	+	++	+	+	t
2′-HO-ENL (peak 9)[c]	−	+	−	+	+	+	−	++	+	+	t
4′-HO-ENL (peak 11)[c]	−	+	−	+	++	+	−	++	+	+	t
6′-HO-ENL (peak 7)[c]	t	++	−	+	++	+	+	++	+	+	t
aliphatic HO-ENL (peak 1)[c]	−	+	−	−	−	−	−	t	−	−	−
aliphatic HO-ENL (peak 2)[c]	−	+	−	−	−	−	−	−	−	−	−
aliphatic HO-ENL (peak 3)[c]	−	+	−	t	+	t	−	+	t	t	t
aliphatic HO-ENL (peak 4)[c]	−	+	−	−	+	+	−	+	t	t	t
aliphatic HO-ENL (peak 6)[c]	t	++	−	t	+	+	t	+	t	t	t
daidzein	++++	+++	++	++	+++	++++	++	++	++	+++	+++
equol	+++++	+++++	+++++	+++	+++	+++++	+++++	+++++	+++++	+++++	+++++
5-HO-ENL (peak *x*)[c]	+	+	t	+	+	+	+	?	?	?	?

[a] Rough estimate of the amounts of metabolites is a relative quantification within each sample compared to the compound with the highest concentration. It is based on the intensities of typical ions in the mass spectra and ranges from high (+++++) to trace (t). A comparison is possible only within each column. [b] Peak number according to Figures 2 and 7. [c] Peak number according to Figures 5 and 8.

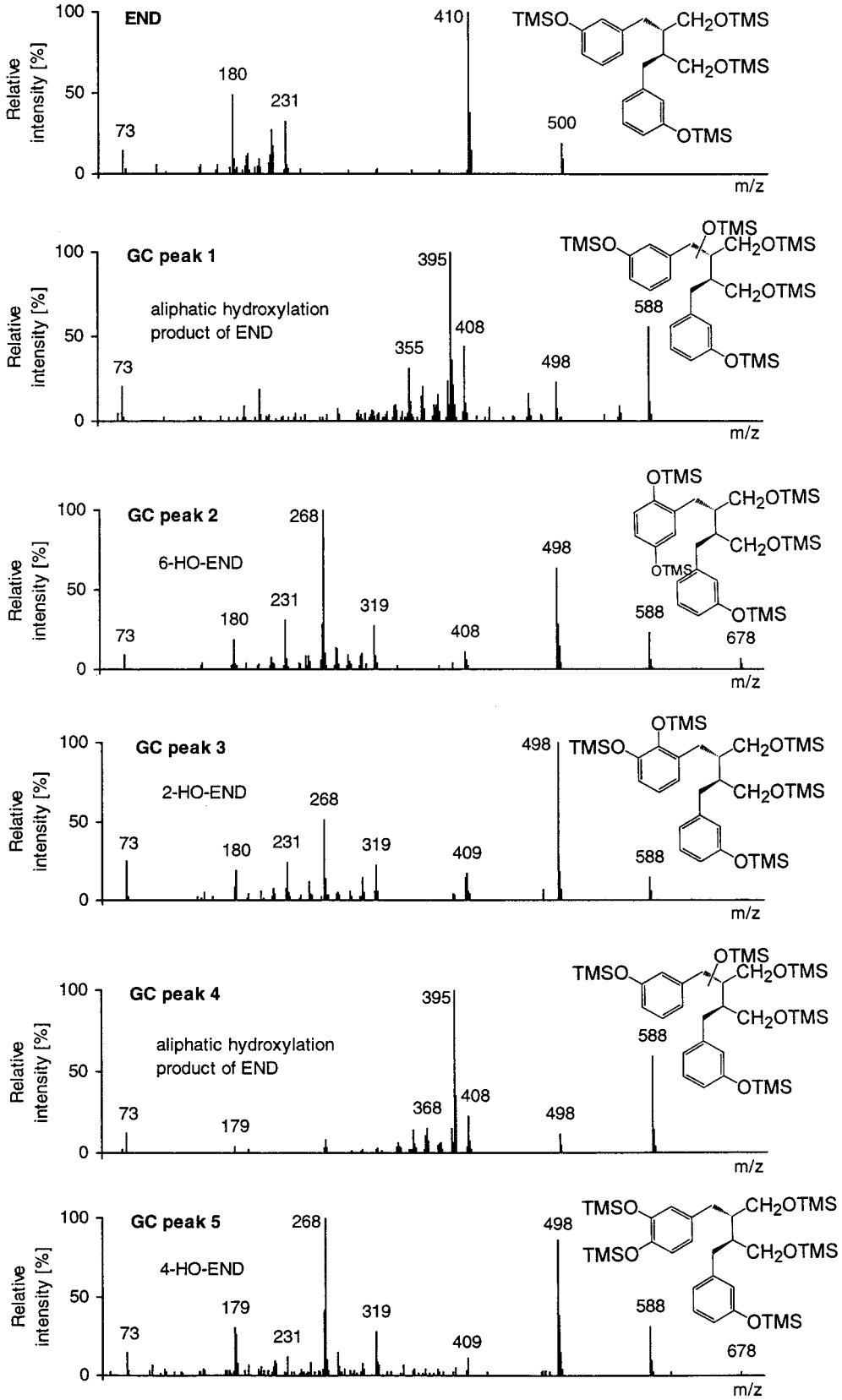

Figure 3. Mass spectra of the aromatic and aliphatic monohydroxylation products of END (trimethylsilyl derivatives). The GC peak numbers refer to Figure 2.

carrying the additional hydroxy group in the para position and in either ortho position of the parent phenolic group at either aromatic ring of ENL. Cochromatography of the bile sample with these synthetic standards revealed that peak 5 in Figure 5 is 6-hydroxy-ENL, peak 7/8 contains 6′-hydroxy-ENL and 2-hydroxy-ENL, peak 9 is 2′-hydroxy-ENL, peak 10 is 4-hydroxy-ENL, and peak 11 is 4′-hydroxy-ENL. Peaks 7 and 8 did not separate on the GC column; however, changes of the mass spectra within this unresolved peak were observed by a detailed analysis, indicating the presence of the two aromatic hydroxylation products 6′-hydroxy-

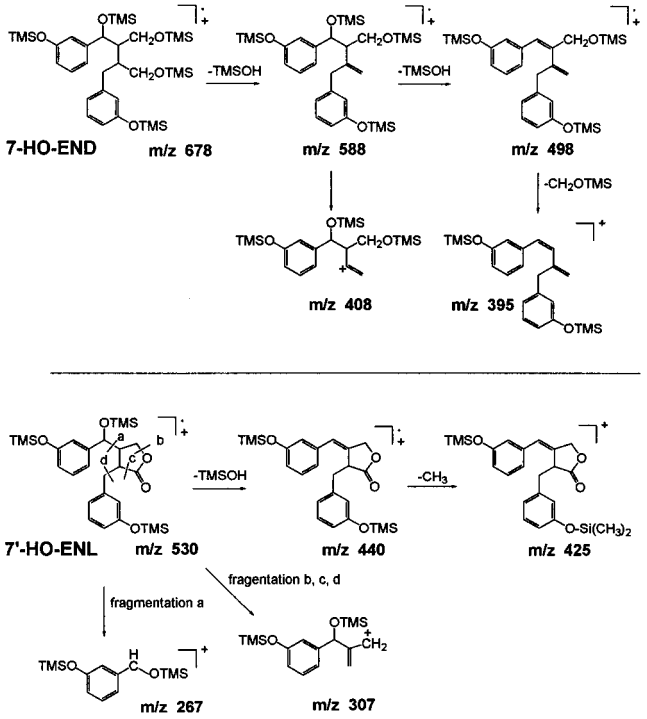

Figure 4. Proposed schemes for the mass spectrometric fragmentation of aliphatic hydroxylation products of END and ENL.

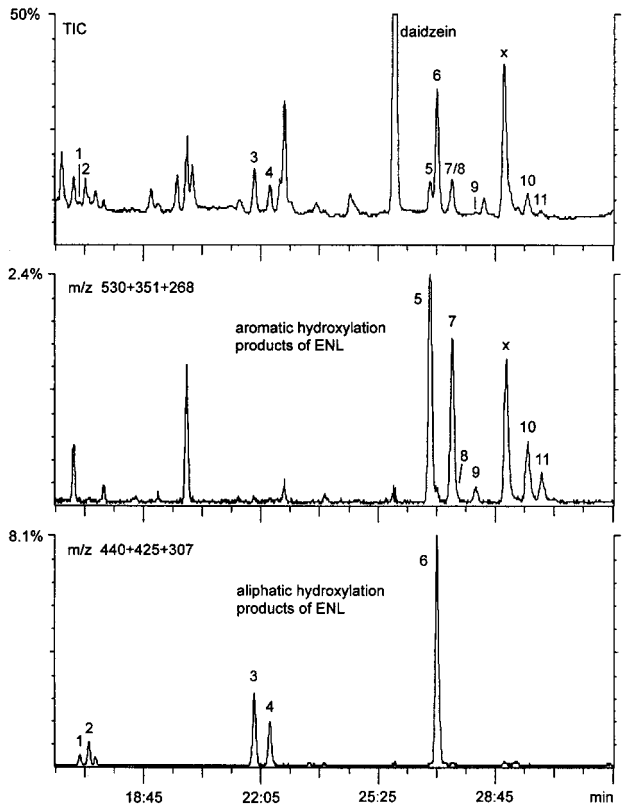

Figure 5. Capillary GC-MS analysis of bile from ENL-dosed rats (HPLC fraction 1 after trimethylsilylation): (top) total ion current (TIC); (middle) ion current reconstructed from three typical ions of aromatic monohydroxylation products of ENL; (bottom) ion current reconstructed from three typical ions of aliphatic monohydroxylation products of ENL. The numbered peaks represent ENL metabolites.

ENL and 2-hydroxy-ENL. Identical chromatographic behavior was noted with the synthetic reference compounds.

The mass spectra of all the aromatic hydroxylation products were identical with those observed in the microsomal metabolism of ENL (Jacobs and Metzler, 1999) and in the urine of humans fed flaxseed (Jacobs et al., 1999). They are depicted in the latter paper. Peaks 1−4 and 6 in Figure 5 represent products of aliphatic hydroxylation of ENL and cochromatographed in GC-MS with the corresponding microsomal metabolites of ENL. The mass spectra of their TMS derivatives are depicted in Figure 6. Whereas all spectra display intense $[M - 90]^+$ ions at *m/z* 440, only peaks 1 and 2 have an additional strong ion at *m/z* 307. The latter may be explained by fragmentation of the lactone ring of a 7′-hydroxy-ENL (Figure 4), which may exist as a pair of stereoisomers. Peaks 3−5, which lack the ion at *m/z* 307 (Figure 6), may arise from metabolites in which the aliphatic hydroxy group is located at the other benzylic position (C-7) or at C-8 or C-8′. In these molecules, the initial loss of TMSOH during mass spectrometric fragmentation may be favored as compared to 7′-hydroxy-ENL due to the formation of a double bond in conjugation with the carbonyl group, thereby preventing the fragmentation of the molecular ion at the lactone ring. However, the final allocation of the structures requires further spectroscopic information or reference compounds.

When the GC-MS run of rat bile after dosing of ENL was searched for ions typical for monomethylated catechol metabolites (*m/z* 472 and 210), three such products with retention times of 19.5, 18.3, and 29.0 min were detected (data not shown) but not further characterized due to the lack of synthetic standards.

Trace amounts of several of the hydroxylated ENL metabolites were also detected together with some ENL in the bile of END-dosed rats (Table 2, column 1). This is not surprising as END is known to be partly oxidized to ENL by intestinal bacteria (Axelson et al., 1982; Borriello et al., 1985). However, in addition to the metabolites of END and ENL arising from aromatic monohydroxylation in the para and ortho positions described above, another aromatic monohydroxylation product of ENL was observed in the GC-MS run of bile from both lignan-treated and control animals. It eluted between 2′-hydroxy-ENL and 4-hydroxy-ENL (peak *x* in Figure 5) and had the same mass spectrum and GC retention time as synthetic 5-hydroxy-ENL. As discussed later, 5-hydroxy-ENL may represent a metabolite of ENL or originate from a hitherto unknown lignan in the rat chow. The animal feed is also assumed to be the source of the isoflavone phytoestrogen daidzein (in HPLC fraction 1) and its bacterial metabolite equol (in HPLC fraction 2) observed in considerable amounts in the bile of control and lignan-treated animals (Table 2, columns 1−3).

Oxidative Metabolites of END and ENL in Rat Urine. When the 24 h urine of female Wistar rats given a single oral dose of END was analyzed by GC-MS (Figure 7), three products of aromatic monohydroxylation (peaks 2, 3, and 5) and two of aliphatic monohydroxylation (peaks 1 and 4) were noted (Table 2, column 4). Moreover, one putative methylated catechol of END (*m/z* 620, 530, 440, and 210) was detected with a retention time of 24.0 min. These urinary END metabolites cochromatographed with the respective metabolites of END in rat bile (see Figure 2) in GC-MS and had identical mass spectra (see Figure 3). In addition, ENL and small amounts of several monohydroxylated me-

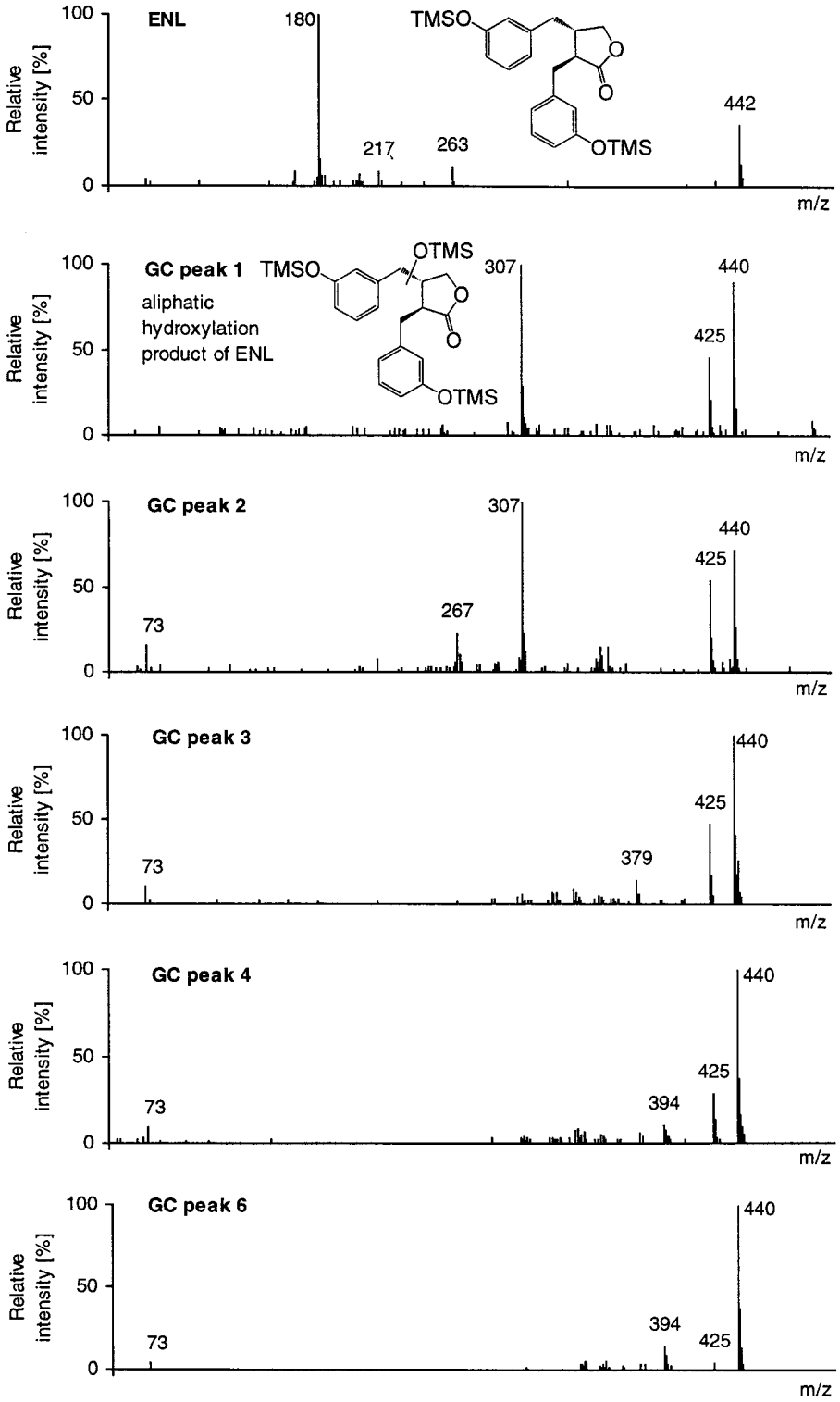

Figure 6. Mass spectra of the aliphatic monohydroxylation products of ENL (trimethylsilyl derivatives). The GC peak numbers refer to Figure 5.

tabolites of ENL were detected in the urine of END-dosed rats (Table 2, column 4), most likely arising from bacterial oxidation of END to ENL as discussed above.

The 24 h urine of female Wistar rats dosed with ENL contained the same six metabolites with an additional aromatic hydroxy group (Figure 8, peaks 5, 7/8, 9, 10, and 11) and three putative metabolites with an additional methoxy group as rat bile (data not shown). In addition, three aliphatic monohydroxylation products of ENL (Figure 8, peaks 3, 4, and 6) were clearly identified (Table 2, column 5). Thus, with the exception of two minor aliphatic hydroxylation products (peaks 1 and 2 in Figure 5), the urinary metabolites of ENL were identical with the biliary metabolites.

Two types of control experiments were conducted in the study on the urinary lignan metabolites: one was the collection of urine prior to the application of the lignans (Table 2, column 6), and the other was the collection of 24 h urine after administration of corn oil only (Table 2, column 7). In both control urines, END and ENL as well as several aromatic and aliphatic monohydroxylation products of ENL were detected by

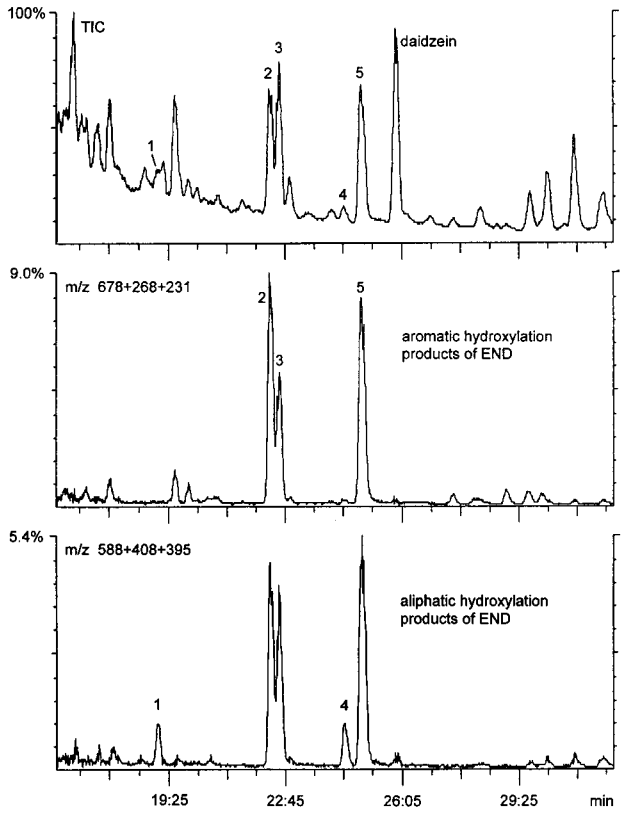

Figure 7. Capillary GC-MS analysis of urine from END-dosed rats (HPLC fraction 1 after trimethylsilylation): (top) total ion current (TIC); (middle) ion current reconstructed from three typical ions of aromatic monohydroxylation products of END; (bottom) ion current reconstructed from three typical ions of aliphatic monohydroxylation products of END. The numbered peaks represent END metabolites.

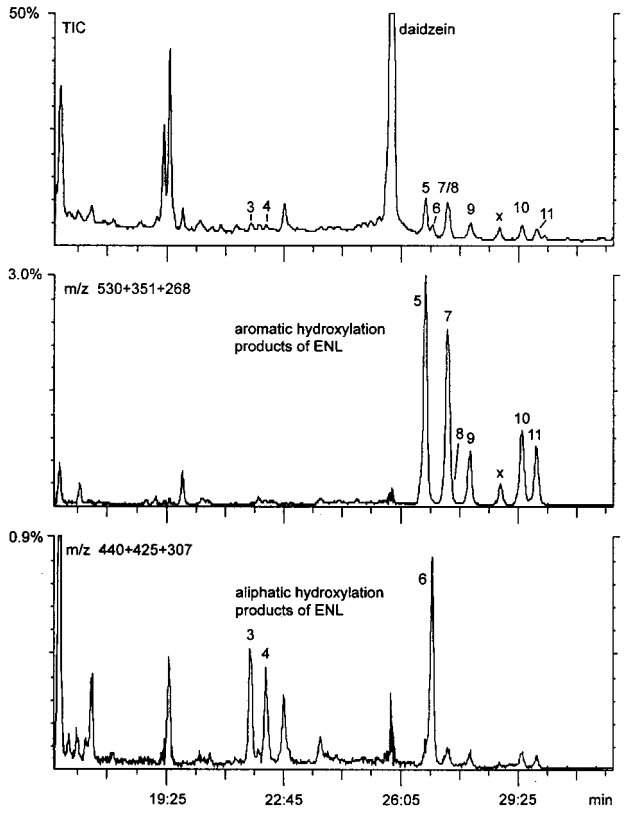

Figure 8. Capillary GC-MS analysis of urine from ENL-dosed rats (HPLC fraction 1 after trimethylsilylation): (top) total ion current (TIC); (middle) ion current reconstructed from three typical ions of aromatic monohydroxylation products of ENL; (bottom) ion current reconstructed from three typical ions of aliphatic monohydroxylation products of ENL. The numbered peaks represent ENL metabolites.

GC-MS analysis, but at much lower concentrations than in the urine of the rats dosed with END or ENL. Again, these lignans and their metabolites as well as the isoflavones daidzein and equol are assumed to arise from the rat feed.

In another experiment, groups of five female and five male Wistar rats were fed a diet supplemented with 5% shredded flaxseed for 7 days and their 24 h urine samples collected after that 1-week feeding period. GC-MS analysis of this urine revealed the presence of large amounts of END and ENL and the same patterns of aromatic and aliphatic monohydroxylation products of END and ENL (Table 2, columns 8 and 9) as observed after administration of the mammalian lignans described above. Lower amounts of ENL metabolites were again observed in the urine of control animals receiving only normal rat chow (Table 2, columns 10 and 11). In the blood serum of rats fed flaxseed, but not in the serum of control animals, very low amounts of most of the aromatic monohydroxylated metabolites of ENL and END could be detected by GC-MS analysis (data not shown).

In a recent paper, we have reported that the mammalian lignans END and ENL are biotransformed by hepatic microsomes from rats and humans (Jacobs and Metzler, 1999). The in vitro metabolites comprised several products arising through monohydroxylation of the parent lignans at the aromatic and aliphatic moiety. The present study clearly shows that oxidative biotransformation of the mammalian lignans also occurs in the rat in vivo, as numerous hydroxylated metabolites of END and ENL are detectable in the bile and urine after administration of the lignans per se or after the feeding of flaxseed. Of the five hydroxylated END metabolites detected by GC-MS, three carry the additional hydroxy group at the aromatic and two at the aliphatic part of the molecule. The products of aromatic hydroxylation were unequivocally identified by chromatographic and spectroscopic comparison with synthetic reference compounds, whereas the exact structures of the aliphatic hydroxylation remain to be established, although hydroxylation at the benzylic position is conceivable on the basis of the mass spectra. The metabolic pathways of END are depicted in Figure 9. Moreover, preliminary evidence was obtained for at least one metabolite carrying an additional aromatic methoxy group and most likely arising from methylation of a catechol metabolite of END.

For ENL, a total of 11 monohydroxylated metabolites were detected in rat bile and urine in vivo. Six of these metabolites arise through aromatic and five through aliphatic hydroxylation. The higher number of oxidative ENL metabolites as compared to END metabolites is due to the fact that END but not ENL is a symmetrical molecule. The six aromatic hydroxylation products of ENL were again identified with the help of synthetic reference compounds. Their structures are given in the scheme of oxidative ENL metabolism (Figure 10). As with END, the exact positions of aliphatic hydroxylation of ENL remain unknown but, according to the mass spectra, may involve hydroxylation of the benzylic carbon atoms. Furthermore, three metabolites possibly

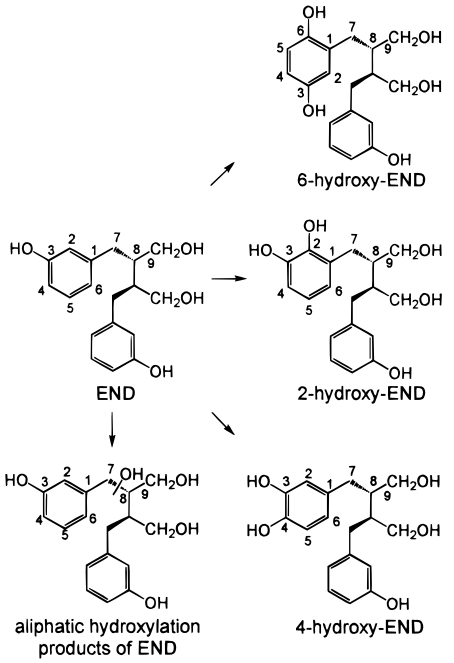

Figure 9. Oxidative metabolism of END in the rat in vivo.

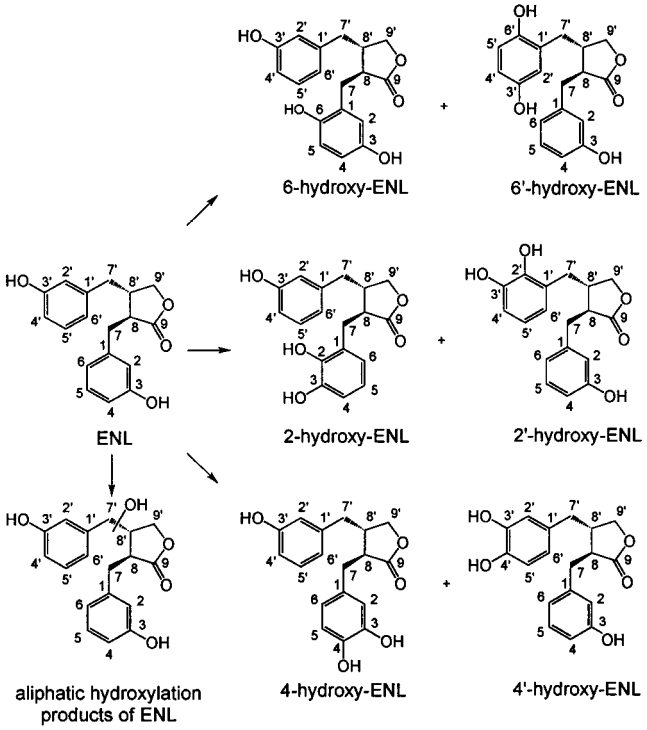

Figure 10. Oxidative metabolism of ENL in the rat in vivo.

arising from monomethylation of ENL catechols were tentatively identified.

Small amounts of several oxidative metabolites were found together with significant amounts of END and ENL in the excreta of untreated control rats (Table 2). It is proposed that the mammalian lignans detected in controls are derived from plant lignans present in the animal feed. The chow is also assumed to provide the isoflavone phytoestrogens daidzein and its bacterial metabolite equol found in the excreta of both lignan-dosed and control rats (Table 2).

The rat feed may also be the source of 5-hydroxy-ENL, although metabolic formation in the rat from ENL cannot be ruled out. If 5-hydroxy-ENL originates from rat feed, it may be present in free form or as a glycoside, or it may be derived from a hitherto unknown plant lignan by bacterial metabolism (see Note Added in Proof). Alternatively, metabolic formation could occur by hydroxylation of ENL in the meta position to the existing hydroxy group. At present, it is unclear to what extent the diet or the mammalian metabolism of ENL accounts for 5-hydroxy-ENL, although the following facts appear to argue against its metabolic formation: (i) aromatic hydroxylation in the meta position is an uncommon metabolic reaction; (ii) 5′-hydroxy-ENL was not formed with hepatic microsomes in vitro, whereas all other hydroxylated ENL metabolites were (Jacobs and Metzler, 1999); (iii) there was no evidence for the formation of the isomeric 5′-hydroxy-ENL, whereas the hydroxylation reactions at other aromatic positions occurred to a comparable extent at both rings of ENL in vivo or in vitro; and (iv) 5-hydroxy-ENL occurred in the excreta of both control rats and rats dosed with END or ENL.

This study has for the first time demonstrated the in vivo formation of hydroxylated metabolites of END and ENL in the rat. Although no attempt of quantification was made, it can be roughly estimated from the intensities of major characteristic ions in the mass spectra that the oxidative metabolites account for <3% of the parent lignans END and ENL. Small amounts of hydroxylated END and ENL have recently been identified in the urine of humans after the ingestion of flaxseed (Jacobs et al., 1999). Interestingly, only products of aromatic hydroxylation of END and ENL were detected in human urine, whereas both aromatic and aliphatic monohydroxylation products were observed in rat excreta. In the urine of humans ingesting flaxseed, 7-hydroxy-ENL and 7-hydroxymatairesinol have previously been tentatively identified (Adlercreutz et al., 1995).

At present, it is unknown whether the hydroxylated metabolites of the mammalian lignans are biologically active or not. END and ENL are known to be weak estrogens and also to act as antioxidants (Adlercreutz, 1984; Prasad, 1997; Harper et al., 1999; Kitts et al., 1999). It has recently been reported that END and ENL are devoid of genotoxic potential in several in vitro assays including the induction of micronuclei and gene mutations in cultured Chinese hamster V79 cells and the disruption of cell-free microtubule assembly (Kulling et al., 1998). As V79 cells lack cytochrome P-450 activity and no external metabolizing system was used in these studies, it remains unknown whether the hydroxylation products of the mammalian lignans are also devoid of genotoxicity. Studies on the hormonal, antioxidant, and genotoxic potential of the oxidative END and ENL metabolites are currently in progress in our laboratory.

ABBREVIATIONS USED

BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; bw, body weight; DEAE, diethylaminoethyl; DMSO, dimethyl sulfoxide; END, enterodiol, 2,3-bis(3-hydroxybenzyl)butane-1,4-diol; ENL, enterolactone, *trans*-2,3-bis(3-hydroxybenzyl)-γ-butyrolactone; GC, gas chromatography; GC-MS, gas chromatography−mass spectrometry; HO, hydroxy; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; ODS, octadecylsilyl; RP, reverse phase; TMS, trimethylsilyl; UV, ultraviolet.

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NOTE ADDED IN PROOF

Dr. N. G. Lewis (Washington State University, Pullman, WA) has kindly suggested that possible precursors of 5-hydroxy-ENL are the plant lignans thujaplicatin (5-hydroxymatairesinol) or thujaplicatin methyl ether (5-methoxymatairesinol), which have already been described in the literature from Western red cedar (MacLean, H.; K. Murakami, K. *Can. J. Chem.* **1966**, *44*, 1541−1545) and which may be present in rat feed or bedding material.

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