PERGAMON

Journal of Steroid Biochemistry and Molecular Biology 68 (1999) 211–218

The Journal of
Steroid Biochemistry
&
Molecular Biology

Novel metabolites of the mammalian lignans enterolactone and enterodiol in human urine

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Received 18 August 1998; accepted 8 January 1999

Abstract

Enterolactone (ENL) and enterodiol (END) are found in high concentrations in human body fluids after ingestion of flaxseed and whole-grain products. Although much interest is presently focused on these mammalian lignans because of their putative beneficial health effects, little is known about their metabolic fate in humans. We have now identified nine novel metabolites of ENL and END in the urine of female and male humans ingesting flaxseed for five days. The chemical structures of six ENL metabolites and of three END metabolites were elucidated by GC/MS analysis and comparison with authentic reference compounds obtained by chemical synthesis. The six identified metabolites of ENL were the products of monohydroxylation at the para-position and at both ortho-positions of the parent hydroxy group of either aromatic ring. Likewise, the three END metabolites were formed through aromatic monohydroxylation at the para- and ortho-positions. The biological significance of these metabolites remains to be established.

1. Introduction

The mammalian lignans enterolactone (ENL) and enterodiol (END) are diphenolic compounds formed by intestinal bacteria from the plant lignans matairesinol and secoisolariciresinol, which are ingested with numerous cereals, vegetables and fruits [1–3]. The richest source of plant lignans is flaxseed; ingestion of 13.5 g flaxseed per day for 6 weeks has been reported to lead to micromolar concentrations of ENL and END in the plasma of humans [4]. In another study, the daily consumption of 10 g flaxseed caused a steep increase in the urinary excretion of mammalian lignans, reaching a plateau at about 4 mg of ENL plus END in the 24 h-urine after three days [2]. ENL and END are weak estrogens and constitute one of several classes of so-called phytoestrogens [5,6]. Much interest has recently been focused on lignans and also on isoflavone phytoestrogens because of their possible protective role against several diseases predominant in Western countries, e.g. cancer of the breast, prostate and colon, as well as cardiovascular diseases [7,8]. However, little is known about the metabolic fate of lignans in the human body, except that they are conjugated with glucuronic acid and sulfate [9]. We have recently disclosed that hepatic microsomes from rats and humans are able to hydroxylate ENL and END both at aliphatic and aromatic positions: six products of aromatic monohydroxylation of ENL were detected by HPLC/MS and GC/MS and unambiguously identified by cochromatography with synthetic reference compounds as metabolites carrying the additional hydroxy group in the para-position and in both ortho-positions of the existing phenolic hydroxy group on either ring [10]. Likewise, three microsomal metabolites of END were identified as para- and ortho-hydroxylated products [10]. In order to see whether these in

Abbreviations: 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; HPLC, high performance liquid chromatography; HPLC/MS, high performance liquid chromatography/mass spectrometry; TMS, trimethylsilyl.

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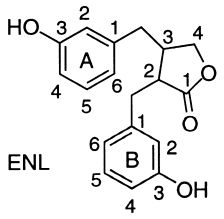
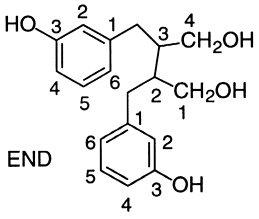
0960-0760/99/$ - see front matter
PII: S0960-0760(99)00033-3

Table 1
Synthetic derivatives of ENL and END monohydroxylated at the aromatic rings





Chemical name	Number[a]	Position of hydroxylation[b]
trans-2-(2,5-dihydroxybenzyl)-3-(3-hydroxybenzyl)-γ-butyrolactone	*1*	para in ring B of ENL
trans-3-(2,5-dihydroxybenzyl)-2-(3-hydroxybenzyl)-γ-butyrolactone	*2*	para in ring A of ENL
trans-2-(2,3-dihydroxybenzyl)-3-(3-hydroxybenzyl)-γ-butyrolactone	*3*	ortho in ring B of ENL (2,3-catechol)
trans-3-(2,3-dihydroxybenzyl)-2-(3-hydroxybenzyl)-γ-butyrolactone	*4*	ortho in ring A of ENL (2,3-catechol)
trans-2-(3,4-dihydroxybenzyl)-3-(3-hydroxybenzyl)-γ-butyrolactone	*5*	ortho in ring B of ENL (3,4-catechol)
trans-3-(3,4-dihydroxybenzyl)-2-(3-hydroxybenzyl)-γ-butyrolactone	*6*	ortho in ring A of ENL (3,4-catechol)
2-(2,5-dihydroxybenzyl)-3-(3-hydroxybenzyl)-butane-1,4-diol	*7*	para in either ring of END
2-(2,3-dihydroxybenzyl)-3-(3-hydroxybenzyl)-butane-1,4-diol	*8*	ortho in either ring of END (2,3-catechol)
2-(3,4-dihydroxybenzyl)-3-(3-hydroxybenzyl)-butane-1,4-diol	*9*	ortho in either ring of END (3,4-catechol)

vitro metabolites of ENL and END are also formed in humans in vivo, we have now analyzed the urine of males and females challenged with a diet containing flaxseed.

2. Materials and methods

2.1. Lignans, reference compounds, and chemicals

ENL and six derivatives of ENL monohydroxylated at various positions of the two aromatic rings (Table 1) were synthesized in our laboratory using a modification of the procedure reported by Pelter et al. [11]. The syntheses and characterization of these compounds by ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy and mass spectrometry is described in detail elsewhere [10]; their mass spectra are depicted in Fig. 1. END and three derivatives monohydroxylated at the aromatic moiety (Table 1, mass spectra Fig. 2) were obtained by reduction of the appropriate ENL derivatives [10]. All chemicals, reagents and solvents were purchased from Sigma, Aldrich or Fluka and were of the highest purity available.

2.2. Participants, collection and clean-up of urine

The four healthy human subjects ingesting one muffin containing 16 g of shredded flaxseed per day for 5 days were SK (female, 30 years old), SM (female, 30), RF (male, 25) and BH (male, 30). Urine samples were collected in the morning of day 1 (prior to the first muffin) and day 6, and stored at –20°C after the addition of 0.1% ascorbic acid and 0.1% sodium azide to prevent autoxidation and bacterial growth. For clean-up, a modification of the method described by Adlercreutz et al. [12] was used. Briefly, 10 ml of the urine was mixed with 1 ml of 1.5 M acetate buffer pH 3.0 and solid-phase extracted on a RP-18 cartridge rinsed with 10 ml of 0.15 M acetate buffer and eluted with methanol. The eluate was diluted with water (final concentration 70% methanol) and subsequently chromatographed on DEAE sephadex A25 (acetate form), eluted with 70% methanol for unconjugated material, 0.2 M acetic acid in 70% methanol for intermediate fraction, and 0.3 M lithium chloride in 70% methanol for conjugates of lignans, isoflavones and steroidal estrogens. The fraction containing conjugated material was concentrated to about 5 ml, diluted with 10 ml 0.15 M acetate buffer pH 3.0 and purified on a RP-18 cartridge as described above. The methanol eluate was evaporated to dryness and the residue dissolved in 2.5 ml of 0.15 M acetate buffer pH 5.0, mixed with 9 mg ascorbic acid and 9000 U arylsulfatase/β-glucuronidase (from Helix pomatia) and incubated at 37°C for 14 h. The complete incubation was again applied onto a RP-18 phase from which the deconjugated material was eluted with 3 ml methanol after rinsing with 10 ml water. The methanol fraction was evaporated to dryness and derivatized with 30 μl of a solution of *N*,O-bis-(trimethylsilyl)-acetamide in *n*-heptane (1:10 v/v) prior to GC/MS analysis.

2.3. GC/MS analysis

GC/MS was carried out using a GCQ ion trap mass detector (Finnigan MAT) with the following conditions: SE-30 fused silica column (30 m × 0.25 mm;

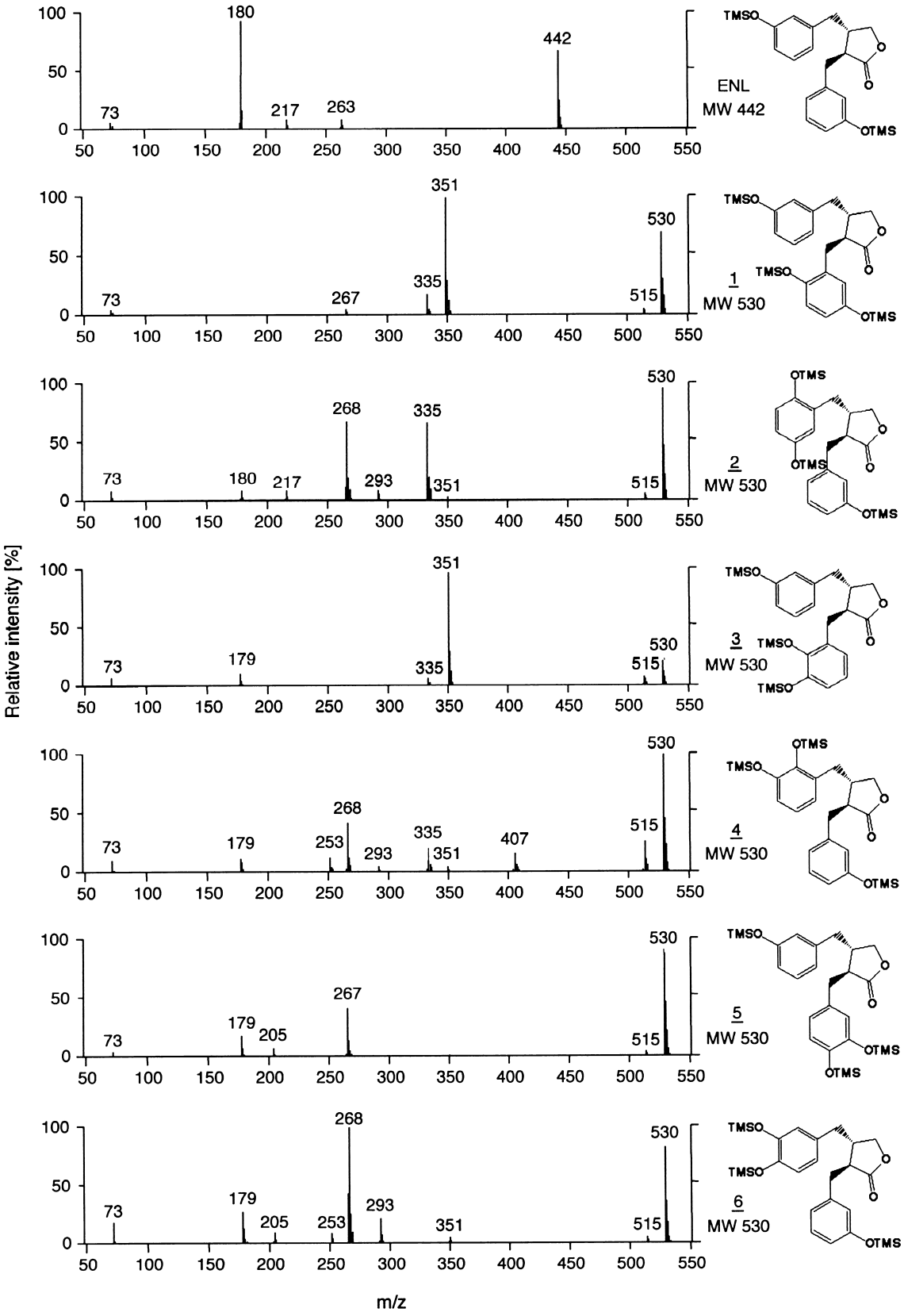


Fig. 1. Mass spectra of ENL and six synthetic reference compounds monohydroxylated at various positions of the two aromatic rings of ENL. Electron impact (70 eV) mass spectra of the trimethylsilylated compounds were recorded with a GCQ ion trap mass detector. The numbers of the compounds refer to Table 1.

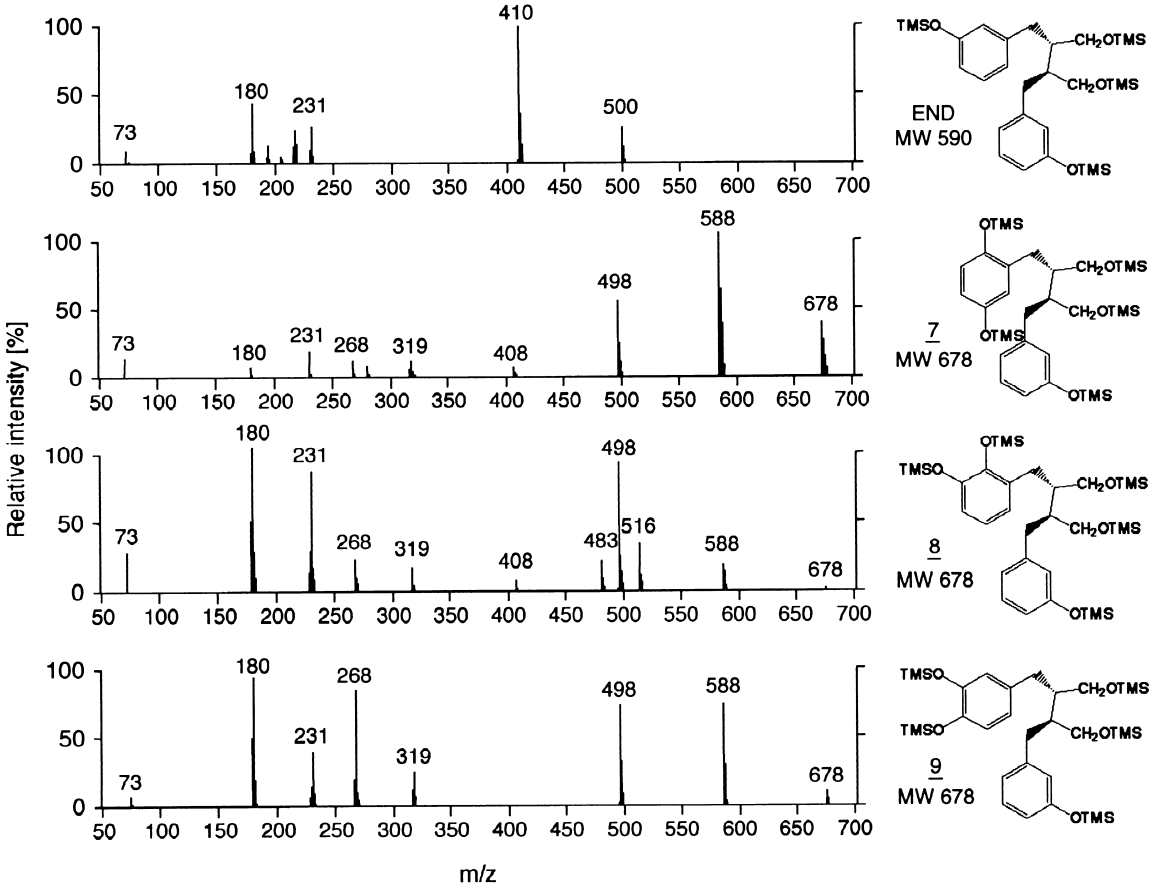


Fig. 2. Mass spectra of END and three synthetic reference compounds monohydroxylated at various positions of the aromatic rings of END. Electron impact (70 eV) mass spectra of the trimethylsilylated compounds were recorded with a GCQ ion trap mass detector. The numbers of the compounds refer to Table 1.

Table 2
GC retention times (RT), molecular weight (MW) and major ions (relative intensity) in the mass spectra of ENL and END and their urinary metabolites monohydroxylated at the aromatic rings

Compound[a]	RT (min)	MW[b]	Characteristic ions (m/z) in mass spectrum[c]
ENL	15.85	442	442 (15), 263 (15), 217 (7), 180 (100), 73 (13)
ENL-M6	16.43	530	530 (52), 351 (100), 335 (24), 267 (8), 73 (13)
ENL-M7	16.68	530	530 (100), 335 (71), 268 (60), 180 (15), 73 (11)
ENL-M8	17.50	530	530 (8), 351 (100), 335 (9), 179 (27), 73 (15)
ENL-M9	17.80	530	530 (81), 335 (39), 268 (100), 179 (41), 73 (57)
ENL-M11	18.67	530	530 (40), 267 (100), 205 (19), 179 (69), 73 (22)
ENL-M12	18.85	530	530 (84), 293 (27), 268 (100), 179 (47), 73 (35)
END	11.13	500	500 (16), 410 (100), 180 (88), 73 (25)
END-M4a	11.75[c]	678	678 (35), 588 (100), 498 (67), 268 (17), 231 (14), 73 (18)
END-M4b	11.75[c]	678	678 (12), 588 (65), 498 (100), 268 (34), 231 (10), 180 (17), 73 (14)
END-M5	12.57	678	678 (17), 588 (75), 498 (54), 268 (100), 231 (17), 180 (9), 73 (4)
SEC[d]	15.35	650	650 (25), 560 (91), 470 (100), 261 (73), 209 (57), 73 (19)

[a] metabolite numbers refer to Figs. 3–5.
[b] trimethylsilyl derivatives.
[c] can only be separated on new column.
[d] SEC, secoisolariciresinol.

Table 3
Effect of a diet containing 16 g flaxseed per day on the urinary level of lignans after 5 days. Data are relative units for the peak areas in GC/MS analysis of the same aliquot from a 10 ml urine sample after clean-up

			Before start of diet		5 days after start of diet	
Subject	Gender	Age	ENL	END	ENL	END
SK	Female	30	4647	4529	292,665	39,639
SM	Female	30	368	n.d.[a]	16,638	47,856
RF	Male	25	n.d.	n.d.	31,921	77,383
BH	Male	30	995	310	34,201	20,618

[a] n.d., not detectable (< 100).

0.25 μm); 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; injection volume, 1 μl.

3. Results

In order to analyze human urine by capillary GC/MS for the presence of ENL and END and their putative aromatic hydroxylation products, a clean-up procedure published by Adlercreutz's laboratory was used except that the last two steps, i.e. the separations of isoflavones and steroidal estrogens, were omitted [12]. Control experiments showed that these compounds did not interfere with the detection of the mammalian lignans and their metabolites by GC/MS using specific ions. In another control experiment, the overall recovery of ENL added to blank urine was found to be 30%. In all experiments, 10 ml of urine was used and the same aliquot of the purified extract was analyzed by GC/MS.

For the location of ENL, END and their metabolites in the total ion chromatogram obtained by GC/MS of the trimethylsilylated urine samples, the GC retention times and major ions of the mass spectra were used, which were known from the synthetic reference compounds (Figs. 1 and 2). After location, final identification was achieved by comparing the complete mass spectrum of the metabolite with that of the authentic reference compound, and by cochromatography. The respective data for the detected metabolites are summarized in Table 2.

The urine of the four volunteers was first analyzed for the presence of ENL and END and their monohydroxylated metabolites prior to the onset of the flaxseed-containing diet. Only trace amounts of ENL and END, and none of the metabolites, were detected in the urine of subjects SM, RF, and BH, whereas the urine of SK contained significant levels of END and ENL (Table 3). This is in agreement with the fact that SK has a higher proportion of vegetarian food in her normal diet than the other subjects.

After 5 days on a diet containing flaxseed, the lignan content of the urine of all four subjects had increased ten- to seventy-fold (Table 3). Two of the urines (BH and SK) contained more ENL while the other two had more END. This difference does not correlate with gender and is probably caused by variations of the intestinal bacterial flora, possibly due to recent intake of antibiotics.

When the GC/MS runs were retrieved for ions specific for monohydroxylated metabolites of ENL

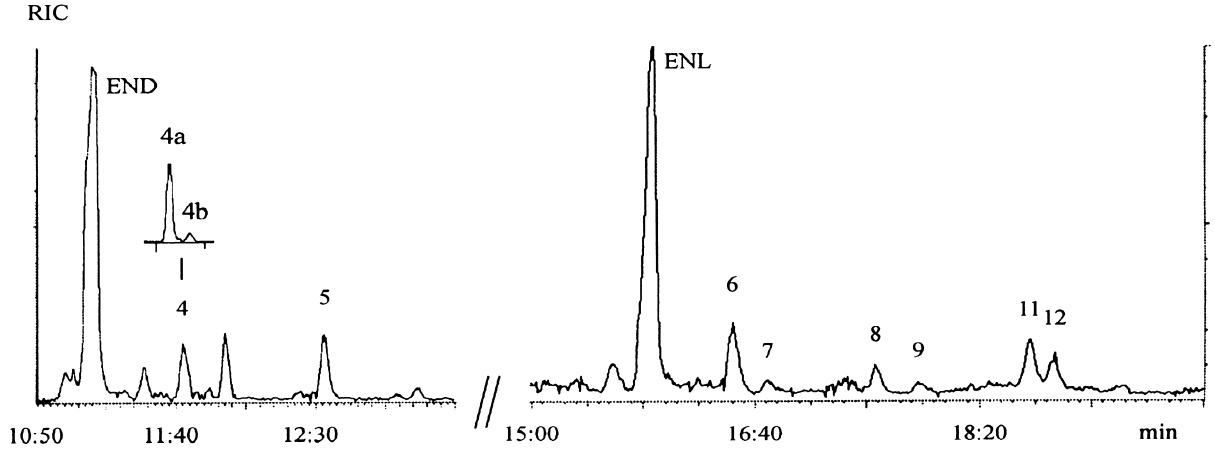


Fig. 3. Capillary-GC/MS analysis of the urine of subject RF after ingestion of flaxseed. The urinary sample was analyzed after clean-up and trimethylsilylation. The figure represents the segments of the chromatogram containing END and its metabolites (peaks 4 and 5) and ENL and its metabolites (peaks 6–12). The peaks are numbered according to the system used for microsomal metabolites of ENL and END [10]. The reconstructed ion current (RIC) was obtained by adding the intensities of ions 588, 498 and 410 for END and its monohydroxylation products (see Fig. 2), and of ions 530, 442 and 351 for ENL and its monohydroxylation products (see Fig. 1).

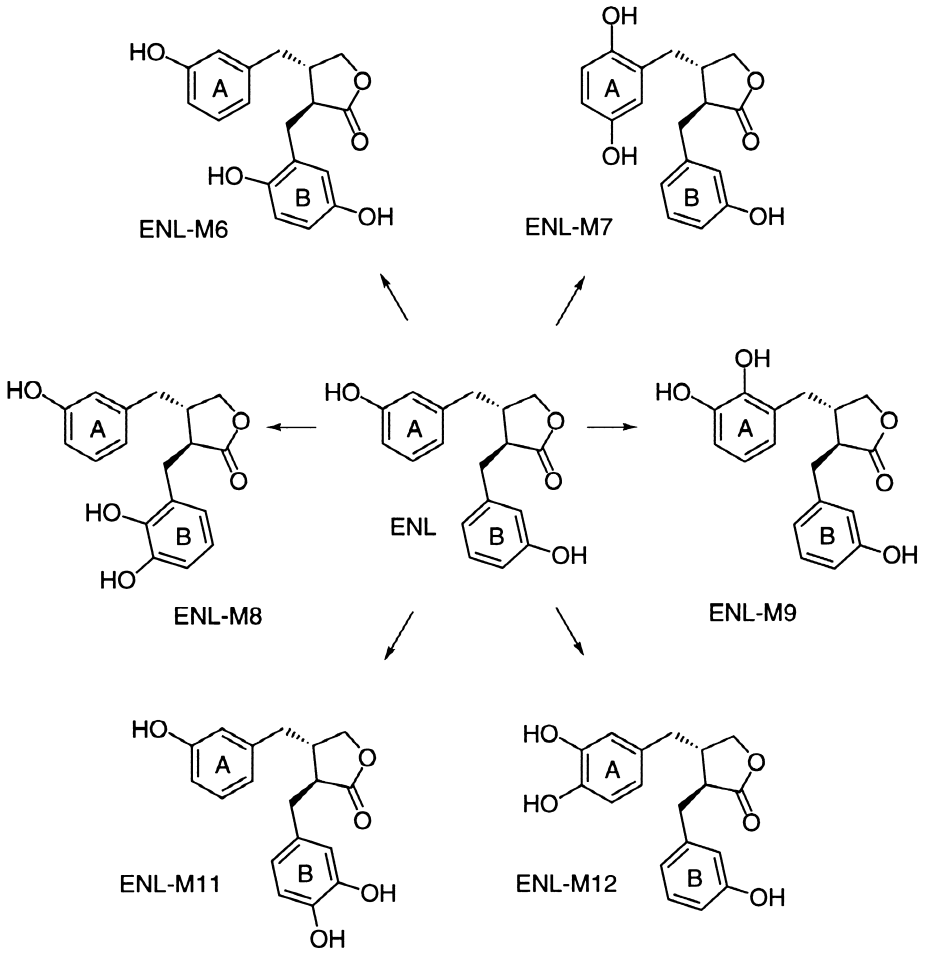


Fig. 4. Metabolites of ENL in human urine after ingestion of flaxseed. Numbers refer to GC peaks in Fig. 3.

and END, six ENL metabolites and three END metabolites were found in the urine of each of the four subjects (Table 2). A representative ion chromatogram is depicted in Fig. 3. The peaks are numbered according to the system used for microsomal metabolites of ENL and END [10]. Peaks 4a, 4b and 5 are metabolites of END, whereas peaks 6 to 12 are metabolites of ENL. Peaks 4a and 4b could only be separated on a new GC column. As the synthetic reference compounds were available for ENL and END monohydroxylated in para- and the two ortho-positions of the parent hydroxy groups (Table 1), the END metabolite 4a could be unambiguously identified as 2-(2,5-dihydroxybenzyl) - 3 - (3 - hydroxybenzyl) - butane - 1,4 - diol (compound *7* in Table 1 and Fig. 2, para-hydroxylated), the END metabolite 4b as 2-(2,3-dihydroxybenzyl)-3-(3-hydroxybenzyl)-butane-1,4-diol (compound *8*, ortho-hydroxylated), and END metabolite 5 as 2-(3,4-dihydroxybenzyl)-3-(3-hydroxybenzyl)-butane-1,4-diol (compound *9*, ortho-hydroxylated) by comparison of the complete mass spectra and cochromatography. Likewise, the ENL metabolites peaks 6–12 were identified with the help of the respective reference compounds listed in Table 1. ENL metabolite 6 is trans-2-(2,5-dihydroxybenzyl)-3-(3-hydroxybenzyl)-γ-butyrolactone (compound *1*, para-hydroxylated in ring B of ENL), metabolite 7 is trans-3-(2,5-dihydroxybenzyl)-2-(3-hydroxybenzyl)-γ-butyrolactone (compound *2*, para-hydroxylated in ring A), metabolite 8 is trans-2-(2,3-dihydroxybenzyl)-3-(3-hydroxybenzyl)-γ-butyrolactone (compound *3*, the 2,3-catechol in ring B), metabolite 9 is trans-3-(2,3-dihydroxybenzyl)-2-(3-hydroxybenzyl)-γ-butyrolactone (compound *4*, the 2,3-catechol in ring A), metabolite 11 is trans-2-(3,4-dihydroxybenzyl)-3-(3-hydroxybenzyl)-γ-butyrolactone (compound *5*, the 3,4-catechol in ring B), and metabolite 12 is trans-3-(3,4-dihydroxybenzyl)-2-(3-hydroxybenzyl)-γ-butyrolactone (compound *6*, the 3,4-catechol in ring A). A complete scheme of the identified metabolites is presented in Figs. 4 and 5. It should be noted that the peak sizes of END and ENL and their oxidative metabolites as depicted in Fig. 3 do not reflect their exact amounts, as the chromatograms were reconstructed from different ions.

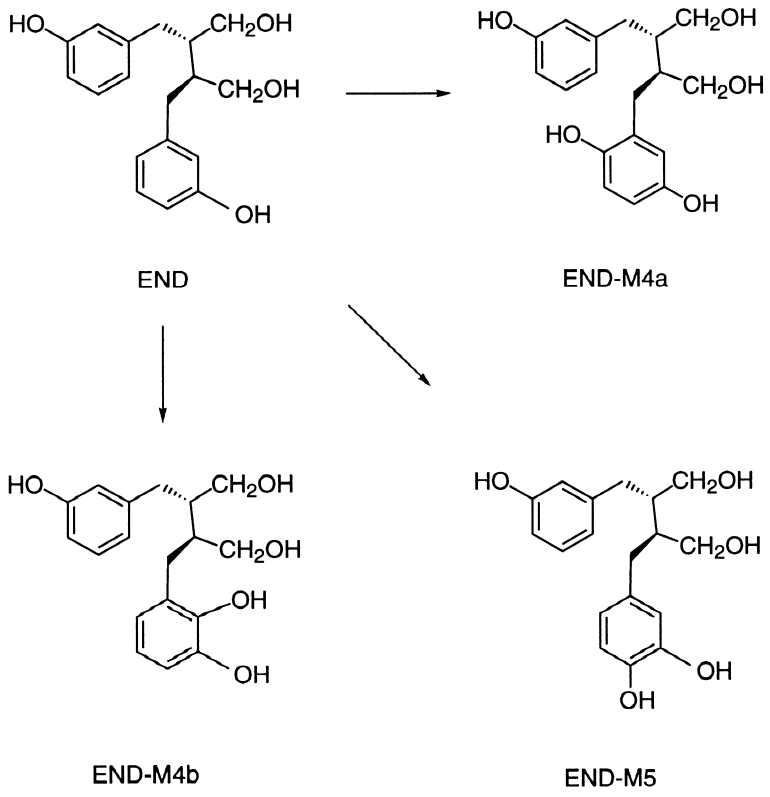


Fig. 5. Metabolites of END in human urine after ingestion of flaxseed. Numbers refer to GC peaks in Fig. 3.

4. Discussion

In the present study, nine metabolites of the mammalian lignans enterolactone (ENL) and enterodiol (END) are described for the first time in the urine of humans ingesting flaxseed. These metabolites carry one additional hydroxy group at the aromatic rings of ENL and END, and their chemical structures were unambiguously identified with the help of synthetic reference compounds. The six ENL metabolites are depicted in Fig. 4. Whereas metabolites 6, 7, 8 and 9 must arise through hydroxylation in the para- and one of the ortho-positions of the parent hydroxy groups in ring A or B which are not equivalent, metabolites 11 and 12 may either be formed through ortho-hydroxylation of ENL or may be intermediates of the bacterial conversion of secoisolariciresinol and/or matairesinol to ENL. The three END metabolites are depicted in Fig. 5 and are formed by hydroxylation in para- and ortho-position of the original hydroxy groups at the aromatic rings which are equivalent in END. Alternatively, metabolite 5 may represent an intermediate of the bacterial biotransformation of secoisolariciresinol to END.

The metabolites of ENL and END were found in the urine of each of four human subjects challenged with a diet containing flaxseed. No attempt has been made so far to quantitate exactly these metabolites. Assuming identical recovery during clean-up of the urine it can be estimated from the intensity of major characteristic ions in the mass spectra that they account for less than 5% of the total urinary lignans. The metabolites were found in the fraction of conjugated material, which contains by far the largest proportion of mammalian lignans in human urine [9]. In a previous study, a dimethoxy analogue of END has been tentatively identified in the urine of a pregnant women and two mono- and dimethoxy analogues of END and ENL were detected in the urine of an adult female vervet monkey by GC/MS analysis [13].

The formation of metabolites of ENL and END may have biological consequences, as the introduction of additional hydroxy groups can possibly affect the estrogenic and the toxicological potential of these compounds. For example, recent studies with isoflavone phytoestrogens have shown that genistein acts as a clastogen and mutagen in cultured Chinese hamster V79 cells whereas daidzein, which differs by only one hydroxy group, is devoid of such genotoxic effects [14]. We have recently assayed the mammalian lignans ENL and END as well as their plant precursors matairesinol and secoisolariciresinol at various endpoints for genetic toxicity in cultured V79 cells and found that all four lignans lack genotoxicity [15]. Analogous studies are now in progress in our laboratory with the aromatic

hydroxylation products of ENL and END shown in Figs. 2 and 3.

The monohydroxylated metabolites of ENL and END may also have an inhibitory effect on aromatase, as has been reported for para-dihydroxylated analogues of ENL and END and some of their methoxy derivatives [16,17].

In our previous investigation of the microsomal metabolism of ENL and END, aliphatic hydroxylation products were observed in addition to the aromatic ones [10]. No such products of aliphatic hydroxylation of lignans were detected in human urine so far. It remains to be shown whether they are not formed in vivo or whether they are lost during the clean-up procedure. Moreover, their estrogenic and genotoxic potentials await investigation. The metabolic disposition of lignans and biological activities of their metabolites deserve attention in view of the wide exposure to these important plant constituents and their putative beneficial effects on human health.

Acknowledgements

This study has been supported by the Deutsche Forschungsgemeinschaft (Grant Me 574/9-2).

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