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New lignan metabolites in rat urine

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Abstract

Ten potential lignan metabolites were quantified in rat urine extracts using liquid chromatography–tandem mass spectrometry. The rats were orally administered with the plant lignans 7-hydroxymatairesinol, matairesinol, lariciresinol or secoisolariciresinol, or with the mammalian lignan enterolactone. The samples were enzymatically hydrolysed and solid-phase extracted before analysis. Of the analysed compounds, only trace amounts of 7-oxoenterolactone could be detected in the urine extracts before administration, but after administration of any of the lignans, the excretion of 7-oxoenterolactone increased and monodemethylated matairesinol and 4,4′-dihydroxyenterolactone could be detected. In addition, other novel lignan metabolites were detected, i.e., 7-oxomatairesinol, α-conidendrin, and α- and β-conidendric acid.
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Keywords: 7-Hydroxymatairesinol; Matairesinol; Monodemethylated matairesinol; Secoisolariciresinol; Lariciresinol; Enterolactone; Isohydroxymatairesinol; 4,4′-Dihydroxyenterolactone; 7-Oxoenterolactone; 7-Hydroxysecoisolariciresinol; 7-Oxomatairesinol; α-Conidendrin; α-Conidendric acid; β-Conidendric acid

1. Introduction

The metabolism of some plant lignans such as matairesinol (MR), 7-hydroxymatairesinol (HMR), lariciresinol (LAR), and secoisolariciresinol (SECO) (both as an aglycon and as a diglycoside) has been studied both in vivo and in vitro. They have been shown to convert to the mammalian lignans enterodiol (END) and enterolactone (ENL) by the action of intestinal microbiota [1–9]. Lignans, especially ENL, have been suggested to induce a wide range of biological effects, such as antioxidant [3,10–13], antitumour [3,5,14–17], estrogenic and anti-estrogenic activities in vitro [18–21], and to protect against coronary heart disease [22,23].

Also other metabolites of plant lignans than END and ENL have been identified recently. 7-Hydroxy-ENL (7-OH-ENL) was found to be a metabolite of several plant lignans [7,9]. Several lignans have previously been detected in body fluids from humans consuming their habitual diet: ENL, END, MR, SECO, LAR, and cyclolariciresinol (CLAR) [24–26] and very recently pinoresinol and syringaresinol [27]. We showed that blood plasma from humans consuming their habitual diet may also contain 7-OH-ENL and the plant lignans HMR, 7-oxo-MR, and α-conidendrin (α-CON) [28].

Very recently we identified new plant lignans, laricirecinol-type butyrolactone lignans called isohydroxymatairesinol (iso-HMR) and *epi*-iso-HMR (Fig. 1) in alkaline solutions of HMR [29]. Iso-HMR and its 8′-

Abbreviations: bw, bodyweight; CLAR, cyclolariciresinol; CON, conidendrin; CONA, conidendric acid; DDQ, 2,3-dichloro-5,6-dicyano-l,4-benzoquinone; 4,4′-diOH-ENL, 4,4′-dihydroxyenterolactone; END, enterodiol; ENL, enterolactone; GC–MS, gas chromatography–mass spectrometry; HAc, acetic acid; HMR, hydroxymatairesinol; HPLC, high-performance liquid chromatography; HPLC–MS/MS, high-performance liquid chromatography–tandem mass spectrometry; LAR, lariciresinol; LOD, limit of detection; MeOH, methanol; MR, matairesinol; MR-CH_3, monodemethylated MR; MRM, multiple-reaction monitoring; 7-OH-ENL, 7-hydroxyenterolactone; 7-oxo-ENL, 7-oxoenterolactone; 7-OH-SECO, 7-hydroxysecoisolariciresinol; 7-oxo-MR, 7-oxomatairesinol; SECO, secoisolariciresinol; QC, quality control

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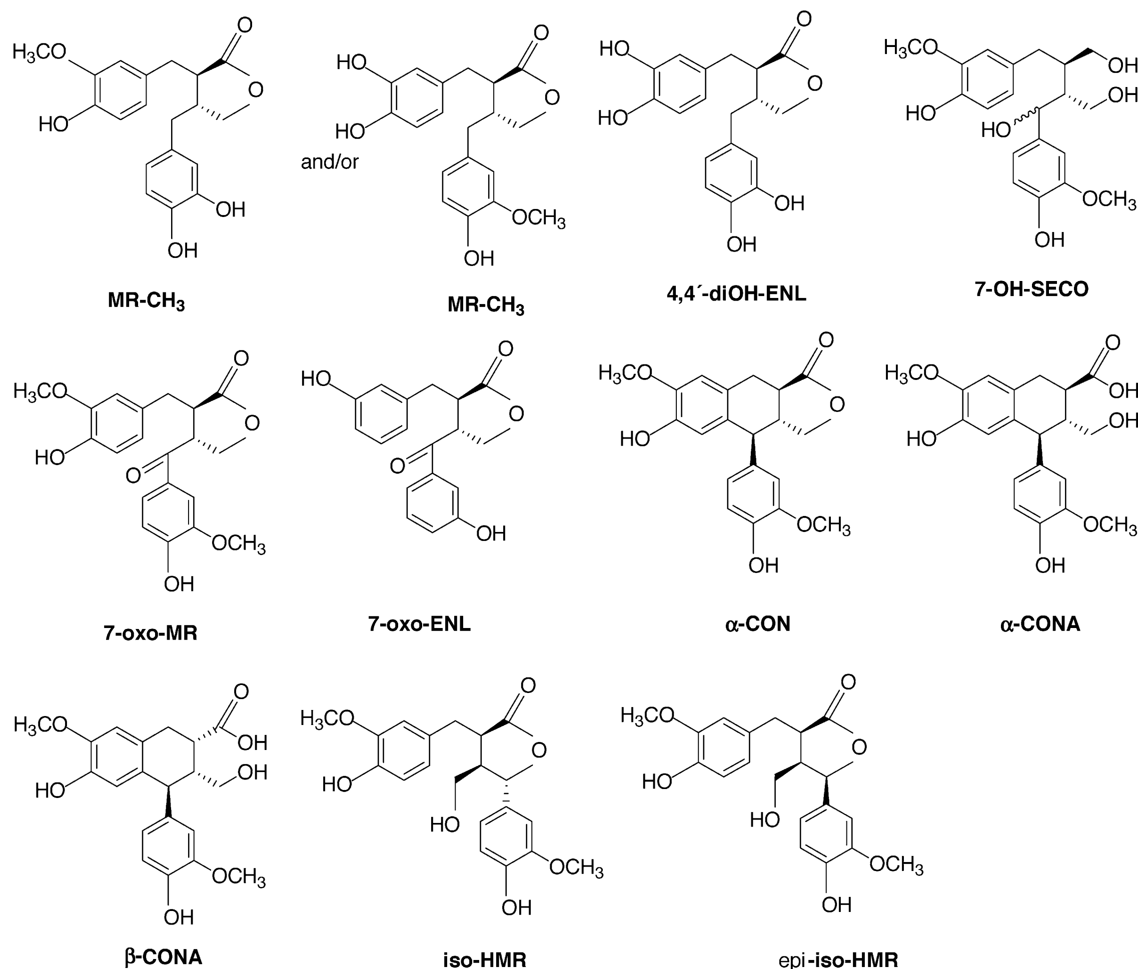

Fig. 1. Structures of the analysed lignans.

epimer were also identified in knotwood of Norway spruce [29]. Iso-HMR was shown to be present in purified HMR preparations administered to rats, and was absorbed and excreted in the urine similarly as HMR, however, it seemed not to be a metabolite of HMR [29]. Also *epi*-iso-HMR was identified in the urine of female rats administered with HMR and seems therefore to be either a metabolite of iso-HMR or formed by isomerisation of this compound in vivo. The possibility that iso-HMR could be a metabolite of other plant lignans than HMR, e.g., of LAR, cannot be excluded.

Plant lignans are transformed to mammalian lignans in the intestine by demethylation and dehydroxylation. It has been shown that the demethylation occurs before dehydroxylation, e.g., mono- and didemethylated SECO could be isolated after anaerobic incubation of SECO with human fecal suspension [30]. Analogously, MR may be transformed to the demethylated forms, i.e., monodemethylated MR (MR-CH_3) and 4,4′-dihydroxyenterolactone (4,4′-diOH-ENL). Niemeyer et al. [6] have shown that plant lignans may be hydroxylated or transformed to other plant lignans through oxidative metabolism occurring in rat liver microsomes. They showed, e.g., that SECO is transformed to 7-hydroxysecoisolariciresinol (7-OH-SECO) in vitro. We showed very recently that a part of ingested plant lignans might be transformed to other plant lignans also in vivo (in rats) [9]. For example, administration of MR increased the urinary excretion of HMR and administration of SECO increased the excretion of LAR. The same conversions have also been shown to take place in vitro [6]. It has been shown that also the mammalian lignans END and ENL are further biotransformed by hydroxylation both in vitro with liver microsomes [31] and in vivo [32].

It is possible that lignans possessing a hydroxyl group at C-7 may be oxidised, e.g., HMR may be transformed to 7-oxo-MR and 7-OH-ENL to 7-oxo-ENL. The in vitro oxidation of HMR to 7-oxo-MR has been reported [33,34].

HMR may be dehydrogenated, i.e., transformed to α-CON, which happens in vitro under acid conditions [35,36], alkaline conditions [36], or by irradiation with light [33]. The lactone ring in α-CON may then be hydrolysed to give α-conidendric acid (α-CONA), which also happens in vitro under alkaline conditions [37,38]. α-CON can also be transformed to β-CON through enolisation [38], and β-CON can then be hydrolysed to β-conidendric acid (β-

CONA). Very recently we showed that α- and β-CON and -CONAs can be detected in an aqueous alkaline solution of HMR [36].

Thus, recent studies have shown that many other metabolites are formed from plant lignans than END and ENL. These other metabolites may have biological effects and therefore they are important to characterise. In a previous work we analysed mammalian lignans, CLAR, and the administered plant lignans in the urine of rats that had been administered with HMR, MR, SECO or LAR and showed that plant lignans may be transformed to other plant lignans [9]. In this work we analysed 10 potential lignan metabolites (the structures of which are shown in Fig. 1) in the same rat urine samples. The rats had been orally administered with a single dose (25 mg/kg of body weight, bw) or multiple doses (25 mg/kg bw/day for 10 days) of (−)-HMR, (−)-MR, (+)-LAR or (−)-SECO. The dose 25 mg/kg bw was chosen on the basis of our previous experiments [2,9], which show that only a small percentage of the administered plant lignan is converted to mammalian lignans in rats. As the potential metabolites analysed in this work were studied for the first time, we wanted to administer the parent lignans in sufficient doses to be able to identify and quantify these metabolites. We analysed these potential metabolites also in the urine of rats that had been orally administered with racemic ENL (in a dose of 10 mg/kg bw/day for 60 days).

2. Experimental

2.1. General

2.1.1. Chemicals

Chemicals used in sample preparation and liquid chromatography were essentially the same as described in a previous work [2]. The administered plant lignans HMR, LAR, and SECO were in the enantiomeric form found in the softwood species from which they were isolated. (−)-HMR was isolated from knots of Norway spruce and (−)-MR was prepared from (−)-HMR by modifications of methods described previously [35]. Also the methods for isolation of (−)-SECO from knots of *Araucaria angustifolia* [39] and (+)-LAR from knots of *Pinus cembra* [9] have been described previously. The HMR preparation used was a mixture of two diastereomers, (−)-HMR (major isomer) and (−)-*allo*-HMR (minor isomer) with an isomeric ratio of approximately 70/30 and it contained approximately 2% iso-HMR. ENL was prepared using total synthesis at the Technical Research Centre of Finland. The method for preparation of d_6-MR, which was used as internal standard in the HPLC–MS/MS analyses, has been described previously [40]. All analysed lignans were prepared in our laboratory except for α-CON, which was prepared at Hormos Medical Corp. (Oulu, Finland) as described by Freudenberg and Knof [35]. 7-oxo-ENL was prepared using total synthesis. The CONAs were prepared by storing α-CON in an aqueous alkaline solution at pH 10 in room temperature for several days. Due to the instability (i.e., ring closure to α-CON of the solid compounds), they could not be isolated from the reaction mixture. 7-OH-SECO was prepared from HMR as described previously [41]. 7-oxo-MR was prepared by oxidation of HMR with 2,3-dichloro-5,6-dicyano-l,4-benzoquinone (DDQ) [34]. MR-CH_3 and 4,4′-diOH-ENL were prepared by reaction of MR with $AlCl_3$ and pyridine according to a modification of a previously described method [42]. MR-CH_3 and 4,4′-diOH-ENL were separated from each other by flash chromatography (elution with $CHCl_3$:MeOH 98:2, v/v). MR-CH_3 was an approximately 1:1 mixture of the two demethylated forms at C-3 and C-3′ which could not be separated from each other chromatographically. (+)-Iso-HMR and (+)-*epi*-iso-HMR were isolated from an alkaline solution of HMR [29].

All the lignans prepared in our laboratory (except CONAs) were purified on silica gel and vacuum-dried. The purity of the lignans was checked using ^{1}H NMR spectroscopy and GC–FID and GC–MS analyses, and all these three methods showed that the amount of impurities was some per cent. As determined by GC–MS by dividing the peak area of an analyte with the total peak area the purities were: d_6-MR 98%, 7-oxo-ENL 93%, 4,4′-diOH-ENL and 7-OH-SECO 94%, α-CON and 7-oxo-MR 98%, MR–CH_3 99%, iso-HMR 98% and *epi*-iso-HMR 95%.

2.1.2. Equipment

The HPLC system and conditions were the same as described previously [2,28], except that an XTerra MS C_8 HPLC column (2.1 mm × 150 mm, 3.5 μm particle size) equipped with a guard column (XTerra C8 2.1 mm × 10 mm, particle size 3.5 μm) (Waters Corp., Milford, MA, USA) were used. The eluents used were 0.1% HAc (acetic acid)/isopropanol (99/1, v/v) (A) and methanol (MeOH)/0.1% HAc/isopropanol (90.0:9.9:0.1, v/v) (B). A 16 min linear gradient from 22% to 73% B was used; the total analysis time was 25 min and the flow rate 0.20 ml/min. The HPLC–MS/MS method using a Micromass Quattro Micro triple-quadrupole mass spectrometer equipped with an electrospray source was essentially the same as previously described [9]. Negative ions were acquired in the MRM (multiple reaction monitoring) mode.

2.1.3. Sample preparation

The urine samples (0.50 ml) were enzymatically hydrolysed at pH 4.0 with *Helix pomatia* enzyme mixture at 37 °C overnight and then cleaned-up using solid-phase extraction (Sep-Pak tC18 columns, 1 cm^3) as described previously [2]. d_6-MR was added as an internal standard in an amount of 0.97 μg after the hydrolysis but before the extraction. The lignans were eluted with 2.0 mL of MeOH. The extracts were stored at −20 °C. Prior to analysis they were gently evaporated to dryness under a nitrogen flow at +45 °C, and then reconstituted in MeOH/0.1% HAc 20/80 (v/v).

2.2. Animal experiments

Forty male Sprague–Dawley rats were orally administered (by gavage) with the (−)-HMR preparation, (−)-SECO, (−)-MR or (+)-LAR in a dose of 25 mg/kg bw. The experimental procedure has been described in detail in a previous work [9]. All male rats were fed with a semipurified lignan-poor C1000 basal diet starting 1 week before the administration of lignans until the end of the experiment.

Racemic ENL was administered to nine Sprague–Dawley female rats (18 weeks old) in a dose of 10 mg/kg bw for 60 days. The rats were part of a mammary cancer study and thus gavaged with 12.0 mg of dimethyl benz[*a*]-anthracene at the age of 50 days. Starting at the age of 43 days the rats were fed with a C1000 basal diet until the end of the experiment.

From the male rats, 24 h urine samples were collected immediately after the first lignan administration and after daily administration (one dose/day) for 10 days. From the female rats, urine samples were collected after 60 days of daily administration (one dose/day). The rat urine collections and lignan administrations to rats were done essentially in the same way as described in our previous work [2]. Individual 24 h urine samples were collected immediately before lignan administration ("baseline" samples) and after varying times of lignan exposure. Blank urine for the standard and quality control (QC) samples was collected from adult male rats, fed at least 1 week with the C1000 diet.

2.3. HPLC–MS/MS analyses

The limits of detection (LODs) and linear ranges of the compounds in spiked mobile phase (MeOH/0.1% HAc 20/80, v/v) were determined by injecting pure compounds at several different concentrations through the guard column. The LODs in urine were determined by adding the most diluted standard solution to solid-phase extracted matrix (unhydrolysed pooled blank urine) and injecting the sample through the analytical column. The signal-to-noise ratio (S/N) was determined and the LODs were calculated as the concentrations at which S/N is 3.0. The linear ranges in urine were determined on the basis of the calibration curves.

The ion suppression due to matrix components was determined by analysing a standard solution (a) dissolved in mobile phase and (b) spiked into solid-phase extracted matrix (unhydrolysed pooled blank urine). Each sample was analysed three times.

The transitions monitored were the deprotonated molecular ions to the predominant fragment ions of m/z 343, 340, 329, 314, 159, 123, 108, and 83 for iso-HMR and *epi*-iso-HMR, α-CON, 7-OH-SECO, α- and β-CONA, 7-oxo-ENL, 4,4′-diOH-ENL, 7-oxo-MR, and MR-CH_3 and d_6-MR, respectively. The cone voltages ranged from 20 to 40 V and collision energy voltages from 8 to 38 eV. The conditions were optimised by syringe infusion of the pure compound (except for the CONAs, which were optimised using a solution of α-CON stored at alkaline pH, which contained the CONAs as products).

The standard samples and the real samples contained the same amount of internal standard. The quantitation was done by using calibration curves of standard samples consisting of unhydrolysed, solid-phase extracted (as the real samples), pooled blank rat urine spiked with pure compounds.

The relative amounts of CONAs were calculated by dividing the peak area response (CONA/d_6-MR) in each sample with the response in the sample showing the lowest response. The most concentrated standard solution contained iso-HMR, *epi*-iso-HMR, 7-OH-SECO, 7-oxo-MR, α-CON, 4,4′-diOH-ENL, and 7-oxo-ENL in concentrations of 70.7, 126.8, 65.5, 14.2, 13.8, 35.2, and 5.95 μM, respectively (in MeOH). This solution was diluted in the proportions 1:4, 1:49, 1:499, and 1:4999 (standard solution/MeOH, v/v) for the calibration curve. MR-CH_3 was analysed at a later occasion and a separate standard solution for this compound was prepared containing 14.5 μM MR-CH_3. This solution was diluted as the standard solution containing the other lignans. The concentrations of the compounds in the most diluted standard sample ranged from 0.59 to 12.7 nM. For each sample batch, three QC samples consisting of the 1:49 dilution were prepared in the same way as the standard samples.

2.4. Statistical analyses

The analyses were performed using Statistica version 5.1 software for Windows. The data distribution (Normality) was tested using Shapiro–Wilk's W-test. Normally distributed data were analysed with one-way analysis of variance followed by Tukey's least significance test. Non-normally distributed data were analyzed with the Kruskal–Wallis median test followed by the Mann–Whitney U-test. The acceptable level of significance was set at $p \leq 0.05$. Results are expressed as mean ± standard deviation.

3. Results and discussion

3.1. Analytical method

The LODs and the linear ranges of the lignans in spiked extracted urine and in spiked mobile phase are listed in Table 1. The concentrations at the upper part of the linear range represented the most concentrated standard solution included in the calibration curve.

The recovery of the detector response due to ion suppression caused by matrix components was the following (%): iso-HMR 6.47 ± 0.55, *epi*-iso-HMR 6.48 ± 1.12, d_6-MR 5.20 ± 0.54, MR-CH_3 13.4 ± 0.83, 7-oxo-MR 12.9 ± 0.91, α-CON 2.28 ± 0.13, 7-OH-SECO 6.30 ± 0.18, 4,4′-diOH-ENL 45.3 ± 2.90, and 7-oxo-ENL 30.6 ± 1.43. These values were very similar to those determined previously for other lignans [9]. However, the extensive ion suppression is not expected to affect the reliability of the method to any higher

Table 1
LODs and linear ranges of lignans in spiked mobile phase (MeOH/0.1% HAc, 20/80, v/v) and in spiked extracted blank urine

Compound	LOD (nM)		Linear range (nM)	
	Mobile phase	Extracted urine	Mobile phase	Extracted urine
7-Hydroxysecoisolariciresinol	0.76	12.5	0.8–7600	20 to ≥33,700
Isohydroxymatairesinol	1.52	9.7	1.6–5600	12 to ≥10,200
epi-Isohydroxymatairesinol	0.56	12.6	7.6–7600	23 to ≥94,100
4,4′-Dihydroxyenterolactone	0.40	3.0	0.4–8000	5.0 to >7050
7-Oxomatairesinol	0.38	2.9	0.4–14,200	3.4 to ≥14,200
7-Oxoenterolactone	0.08	0.40	0.16–2500	0.76 to >1190
Monodemethylated matairesinol	3.72	28.1	3.7–7400	29 to ≥14,500
α-Conidendrin	0.82	27.5	0.8–11,000	33 to ≥13,800

Injection volume 50 μl.

degree because the variability in the composition of the rat urine is expected to be small. This is because the urine is collected from a relatively large group of animals of the same strain, age, and sex that have been housed at standardised conditions and fed the same diet.

The linearity of the calibration curves was >0.998 of all compounds. Of iso-HMR, 4,4′-diOH-ENL, and 7-oxo-ENL, the most concentrated standard solution was omitted from the calibration curve because it exceeded the linear range, and of 7-OH-SECO and α-CON, the most diluted standard solution was omitted because it was below the linear range.

The average deviation from the expected concentrations of the compounds in the QC samples ranged from 2.5% (α-CON) to 14.8% (7-oxo-ENL) ($n = 9$, three determinations); for MR-CH_3 4.9% ($n = 3$, one determination).

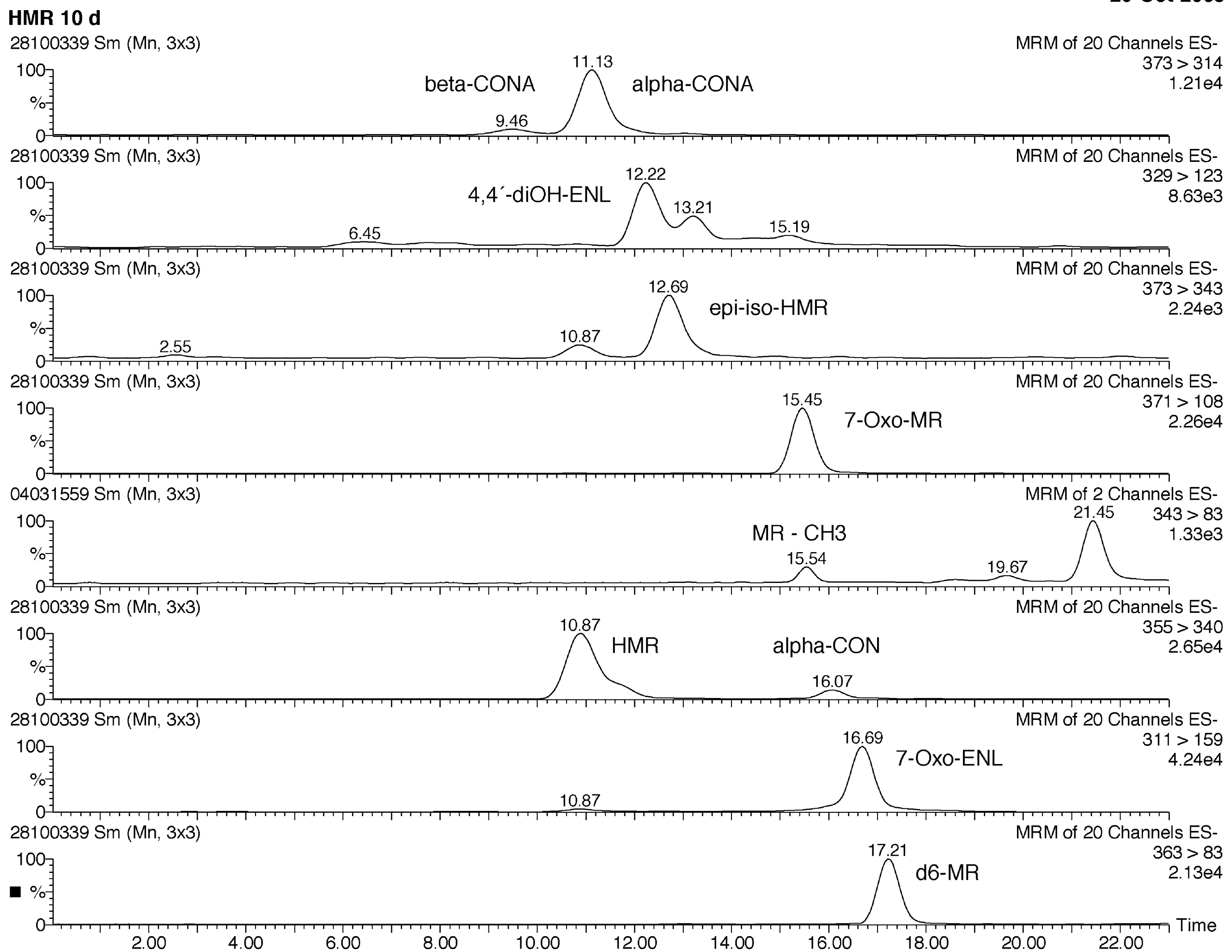

Fig. 2. MRM chromatograms of the analysed lignans in a urine sample of a rat administered with multiple doses of HMR (25 mg/kg bw/day for 10 days).

The solid-phase extraction recoveries, calculated by dividing the calibration curve slope of spiked urine extract with the slope of pure compounds in mobile phase were (mean values, $n = 3$): iso-HMR 74%, *epi*-iso-HMR 79%, 7-OH-SECO 50%, and α-CON, MR-CH_3, 7-oxo-MR, 7-oxo-ENL, and 4,4′-diOH-ENL 100%.

It should be noted that these parameters could not be determined for the CONAs, as they were not available as pure compounds.

The unhydrolysed blank urine contained 7-oxo-MR and 7-oxo-ENL in detectable but unquantifiable amounts.

The HPLC retention times relative to d_6-MR were: 7-OH-SECO 0.50, β-CONA 055, α-CONA 0.65, iso-HMR 0.70, 4,4′-diOH-ENL 0.71, *epi*-iso-HMR 0.74, 7-oxo-MR 0.90, MR-CH_3 0.90, α-CON 0.93, and 7-oxo-ENL 0.97.

Fig. 2 shows typical MRM chromatograms of the analysed lignans in a urine sample of a rat that had been administered with multiple doses of HMR. In the MRM chromatogram of 4,4′-diOH-ENL, other peaks can also be seen. These are probably peaks of dihydroxylated ENL with the hydroxyls at other positions.

3.2. Identification and quantity of novel metabolites

Of the analysed compounds, only 7-oxo-ENL could be detected in the baseline samples; the average excretion of the male rats was 0.0031 nmol/24 h (quantified in six samples out of 20) and of female rats 0.32 nmol/24 h (quantified in all five baseline samples).

7-OH-SECO could not be detected in any sample. This may partly be due to the poor solid-phase extraction recovery (50%), which is probably due to the high hydrophility of the compound, causing a poor adsorption to the solid phase (C18). Niemeyer et al. [6] identified this compound as an oxidative metabolite of SECO in rat liver microsomes, but not in vivo (in rat bile or urine) after administration of SECO, and their findings are supported in the present study. Another compound that could not be detected in any of the samples in this study was *epi*-iso-HMR. Recently we showed that *epi*-iso-HMR was detectable in the urine of HMR administered female rats [29], but the overall concentration of HMR was higher in those urine samples as compared to the male urine samples analysed in the present study. It is possible that the concentration of *epi*-iso-HMR was too low in the male urine samples to enable detection. The detection of *epi*-iso-HMR in the female urine samples suggests that this compound can be a metabolite of HMR or that it is formed by isomerisation of iso-HMR in vivo. Iso-HMR was detectable in the urine only after HMR administration, i.e., LAR does not seem to be oxidised to iso-HMR. The excretion of iso-HMR was 5.1 nmol/24 h after a single dose and 6.9 nmol/24 h after administration of one dose/day for 10 days. The excretion yield

Table 2
Excretion of 7-oxomatairesinol, monodemethylated matairesinol, 4,4′-dihydroxyenterolactone, 7-oxoenterolactone, and α-conidendrin in rat urine after administration of a single oral dose (25 mg/kg bw) and after multiple doses of 25 mg/kg bw/day for 10 days of plant lignans

Administered lignan, mean dose (nmol/24 h)	Urinary excretion (nmol/24 h)[a]				
	7-Oxomatairesinol	Monodemethylated matairesinol	4,4′-Dihydroxyenterolactone	7-Oxoenterolactone	α-Conidendrin
HMR					
27632 (single)	13.1 ± 4.14a	11.2 ± 9.94c	5.15 ± 6.68c,d	0.319 ± 0.241c	3.34 ± 1.24b
$n_{quant.}/n_{anal.}$	10/10	10/10	10/10	10/10	10/10
27738 (multiple)	12.9 ± 5.19a	10.3 ± 5.29c	6.74 ± 4.10b,c	2.88 ± 0.522b	3.84 ± 0.942b
$n_{quant.}/n_{anal.}$	10/10	10/10	10/10	10/10	10/10
SECO					
27675 (single)	nd	1.50 ± 2.19d	1.97 ± 2.66d	0.0311 ± 0.0219d	nd
$n_{quant.}/n_{anal.}$		5/11	10/11	9/11	
27696 (multiple)	nd	10.4 ± 9.97c	6.06 ± 5.29b,c	5.82 ± 2.37a	nd
$n_{quant.}/n_{anal.}$		9/11	11/11	11/11	
MR					
28314 (single)	0.418 ± 0.458[c]	33.5 ± 14.6b	14.2 ± 16.7b,c	0.277 ± 0.162c	33.4 ± 13.9a
$n_{quant.}/n_{anal.}$	4/6	6/6	6/6	5/6	6/6
28307 (multiple)	6.01 ± 4.06[b]	157 ± 151a	109 ± 126a	6.52 ± 4.83a,b	1.22 ± 0.340b
$n_{quant.}/n_{anal.}$	6/6	6/6	6/6	6/6	6/6
LAR					
26768 (single)	nd	1.27 ± 1.77d	0.502 ± 1.07e	0.0204 ± 0.0313d	1.97 ± 1.53b
$n_{quant.}/n_{anal.}$		4/10	2/10	5/10	10/10
27066 (multiple)	nd	11.3 ± 4.39c	12.9 ± 13.0b	3.92 ± 1.24a	2.05 ± 1.54b
$n_{quant.}/n_{anal.}$		10/10	10/10	10/10	10/10

Values are mean ± standard deviation, nd: not detected in any sample; $n_{quant.}/n_{anal.}$: number of samples in which the lignan could be quantified/number of analysed samples. Different letters (a–e) indicate statistically significant concentration differences ($p < 0.05$) of a certain analysed lignan between groups of animals administered with different lignans or with single or multiple doses of the same lignan.

[a] The urinary excretion of the respective administered plant lignan itself was (single and multiple dose, respectively): HMR 553 ± 181 and 725 ± 203, SECO 51.3 ± 21.3 and 111 ± 42.5, MR 38.5 ± 22.4 and 73.4 ± 87.9, LAR 696 ± 156 and 891 ± 222. The excretion of CLAR after LAR administration was 735 ± 338 and 863 ± 388 (single and multiple dose, respectively) [9].

(average excreted amount/administered amount) of both iso-HMR and 7-*allo*-HMR was about 1% (both after a single dose and after 10 days of exposure). In female rats, the excretion yield of iso-HMR in the urine 48 h after a single dose of HMR (50 mg/kg bw) was similar to that of the major isomer of HMR, i.e., about 6% [29]. The reason for this difference in excretion yield between the sexes is unknown. However, iso-HMR does not seem to be a metabolite of HMR in neither male nor female rats, as in urine the proportion of this compound of the total HMR amount is smaller than in the administered HMR preparation.

In Table 2 the urinary excretion of 7-oxo-MR, MR-CH_3, 4,4′-diOH-ENL, 7-oxo-ENL, and α-CON is presented and the excretion of administered lignans determined in a previous work [9] are listed in a footnote of the Table. All the administered lignans are also excreted as such, which is an indication of the absorption of the plant lignan. The plant lignan absorption cannot be used as a marker of overloading the mammalian lignan production capacity of rats. Plant lignan absorption and excretion is not strictly dependent on mammalian lignan production—prolonged exposure of HMR, LAR or SECO causes an increase in the excretion of both plant and mammalian lignans [9]. 7-oxo-MR could be detected in the urine after HMR or MR administration, but not after administration of SECO, LAR (Table 2) or ENL.

The excretion of 7-oxo-ENL increased after administration of the studied plant lignans (Table 2) and ENL. The excretion after ENL administration was 16.8 ± 8.06 nmol/24 h (quantified in all analysed samples). Unfortunately, the ENL administration experiment cannot be compared to the plant lignan administration experiment on a quantitative basis because of different doses and sexes in the two experiments.

Also MR-CH_3 and 4,4′-diOH-ENL could be detected in urine after administration of all the plant lignans (Table 2) and ENL. After ENL administration, the excretion of MR-CH_3 was 1.50 ± 1.17 nmol/24 h (quantified in eight samples out of nine) and of 4,4′-diOH-ENL 12.7 ± 6.09 nmol/24 h (quantified in all analysed samples). The excreted amount of 4,4′-diOH-ENL was higher than that of 7-oxo-ENL both after a single dose and after prolonged exposure to all the administered plant lignans.

α-CON and α- and β-CONA could be detected in the urine after administration of HMR, MR or LAR (Tables 2 and 3). After SECO or ENL administration, α-CON or CONAs could not be detected in any of the analysed samples. Surprisingly, after a single dose of MR, the excreted amount of both α-CON and CONAs was significantly higher than after administration of HMR or LAR (Tables 2 and 3).

In our previous work, we showed that prolonged exposure (10 days) to plant lignans causes a considerable increase of the urinary excretion of mammalian lignans in rats and a moderate increase of the excretion of some plant lignans [9]. The present study shows that prolonged exposure to all the administered plant lignans causes a significant increase in the excretion of 7-oxo-ENL (Table 2). For MR-CH_3 and 4,4′-diOH-ENL, the increase is significant for all administered plant lignans except HMR. Furthermore, prolonged exposure to HMR does not increase the excretion of 7-oxo-MR, α-CON or CONAs. The excretion of α-CON and CONAs does not increase after prolonged exposure to LAR, either, and after exposure to MR, the excretion of these compounds even decreases (Tables 2 and 3).

Table 3
Excretion of α- and β-conidendric acids in rat urine after administration of a single oral dose (25 mg/kg bw) and after multiple doses of 25 mg/kg bw/day for 10 days of plant lignans

Administered lignan	Urinary excretion per 24 h	
	α-Conidendric acid	β-Conidendric acid
HMR		
Single dose	9.05 ± 3.07b	3.30 ± 2.30b
$n_{quant.}/n_{anal.}$	10/10	9/10
Multiple doses	11.2 ± 2.63b	6.21 ± 2.44b
$n_{quant.}/n_{anal.}$	10/10	10/10
SECO		
Single dose	nd	nd
Multiple doses	nd	nd
MR		
Single dose	125 ± 42.6a	44.4 ± 11.6a
$n_{quant.}/n_{anal.}$	6/6	6/6
Multiple doses	5.56 ± 1.43c	2.70 ± 3.10b
$n_{quant.}/n_{anal.}$	6/6	3/6
LAR		
Single dose	6.15 ± 5.77b,c	nd
$n_{quant.}/n_{anal.}$	10/10	
Multiple doses	8.12 ± 6.74b,c	nd
$n_{quant.}/n_{anal.}$	10/10	

The relative values are mean ± standard deviation, nd: not detected in any sample; $n_{quant.}/n_{anal.}$: number of samples in which the lignan could be quantified/number of analysed samples. Different letters (a–c) indicate statistically significant concentration differences ($p < 0.05$) of a certain analysed lignan between groups of animals administered with different lignans or with single or multiple doses of the same lignan.

When the excreted amounts of novel metabolites (Table 2) are compared to the amounts of previously known metabolites analysed in the same samples [9], it can be noted that MR-CH_3, 7-oxo-MR, and α-CON seem to be quantitatively more important metabolites of HMR than MR and LAR. Moreover, after prolonged exposure to SECO or LAR, MR-CH_3, and 4,4′-diOH- and 7-oxo-ENL seem to be more abundant metabolites than LAR and HMR, respectively. In our previous work with male rats we noted that only a minor portion of the administered plant lignan was excreted into the urine as mammalian lignan metabolites [9]. After 10 days of exposure, 4–8% of the administered plant lignan was converted to enterolignans (ENL + END + 7-OH-ENL). The percentual conversion of plant lignans after prolonged exposure to the novel lignan metabolites (MR-CH_3 + 7-oxo-MR + 4,4′-diOH-ENL + 7-oxo-ENL + α-CON) was even smaller, as shown in Fig. 3. The total urinary excretion yield of these compounds was approximately 0.1% after SECO, LAR or HMR administration and 1% after MR administration (of which MR-CH_3 contributed for 0.55%).

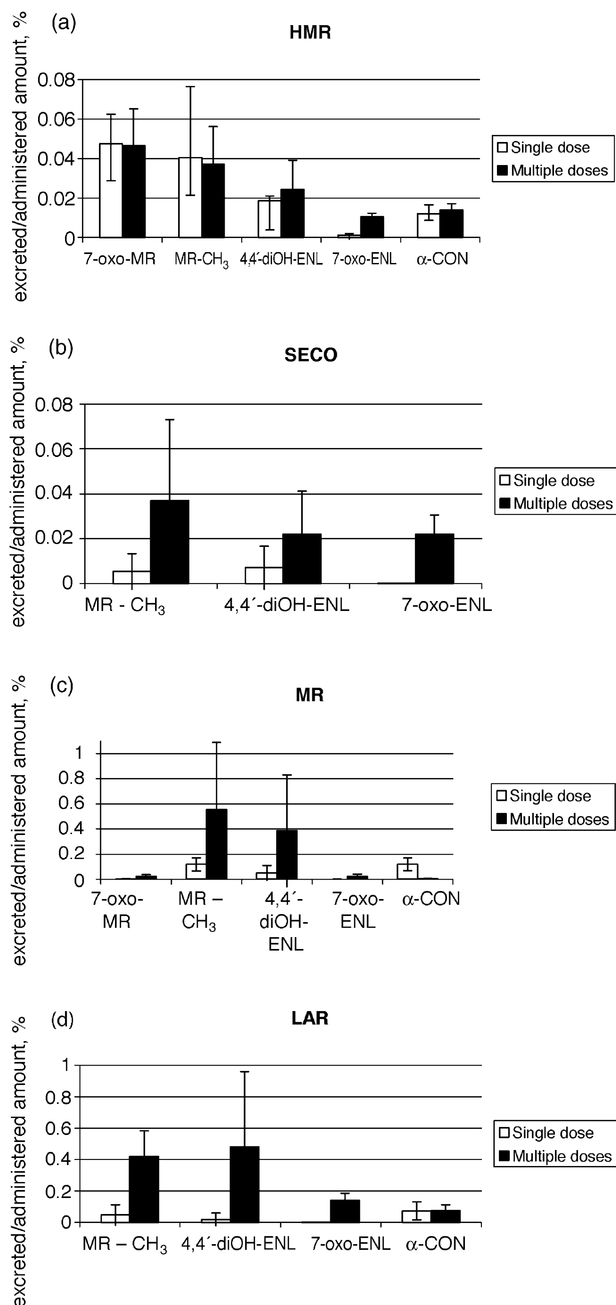

Fig. 3. Urinary excretion yields (of administered lignan amount %) of lignan metabolites. Administered lignan: (a) HMR; (b) SECO; (c) MR; and (d) LAR.

The phenomenon that orally administered purified plant lignans are only partly converted to mammalian lignans is documented before and there are several possible explanations, although all currently hypothetical. First, it is possible that the conversion rate of the plant lignans is slow and during the passage in the intestinal tract only part of the administered plant lignans will be converted to mammalian lignans and absorbed. Second, it seems evident that only part of the lignans (both mammalian and plant lignans) are absorbed. Only a few per cent of the orally administered mammalian lignan (ENL) dose was recovered into urine in rats [2]. Furthermore, as we have shown previously, all plant lignans are not absorbed equally [2,9]. It seems to be strictly dependent on the chemical structure of the lignan (and possibly dependent on the chemical properties of the compound). Third, in addition to plant lignan absorption as such, the chemical structure of the administered plant lignan seems to determine the quantity and type of mammalian lignans produced in vivo. Fourth, the fecal excretion was not measured in the above mentioned studies and it is likely that a large portion of lignans are excreted in feces. In humans, fecal excretion of lignans has been documented to be higher than excretion into urine [43]. Accordingly, in rats, fecal excretion was the most significant route of excretion after administration of the flaxseed lignan SECO diglycoside [44]. Fifth, we cannot exclude the possibility that part of the administered purified lignans could be degraded or converted to non-absorbable compounds prior to entering the absorption sites in the intestines.

3.3. Possible metabolic pathways

Not surprisingly, 7-oxo-MR seems to be more readily formed from HMR, which already has a hydroxyl group at C-7 than from MR, as the excretion was higher after administration of HMR (Table 2). Previously it has been shown that HMR is transformed to 7-oxo-MR in vitro by irradiation with light [33] or by oxidation with DDQ [34]. Obviously an oxidation at C-7 of HMR may occur also in vivo and the same seems to happen even with MR (Fig. 4). This is supported by the findings that 7-oxo-MR is present in human plasma [28] suggesting that it is an oxidative metabolite of MR, which is commonly present in the diet (in, e.g., flaxseed, whole grains, and vegetables).

7-oxo-ENL may be formed by oxidation of ENL or 7-OH-ENL (Fig. 5). We have recently shown that the urinary excretion of 7-OH-ENL increases after administration of HMR, MR, SECO, LAR [9] or ENL (unpublished results) to rats. After HMR or MR administration, the formed 7-oxo-MR may also partly be transformed to 7-oxo-ENL by demethylation and dehydroxylation (Fig. 4). However, the amount of 7-oxo-ENL excreted in urine after 10-day daily exposure to HMR and MR was significantly ($p < 0.05$) lower, not higher, than after exposure to SECO or LAR (Table 2). This may be due to the formation of large amounts of END after SECO or LAR administration [9]—it is possible that part of the formed END is oxidised to 7-OH-END and 7-oxo-END, which is then further oxidised to 7-oxo-ENL. When monitoring daughter ions of m/z 315 and 317 (deprotonated molecular ions of 7-oxo- and 7-OH-END, respectively) in urine extracts from rats dosed with SECO, peaks were found with mass spectra that could possibly originate from 7-oxo-END and 7-OH-END, respectively.

The amounts of $MR\text{-}CH_3$ and 4,4′-diOH-ENL excreted after MR administration are significantly higher than after administration of the other plant lignans studied (Table 2). This is not surprising as these compounds are expected to

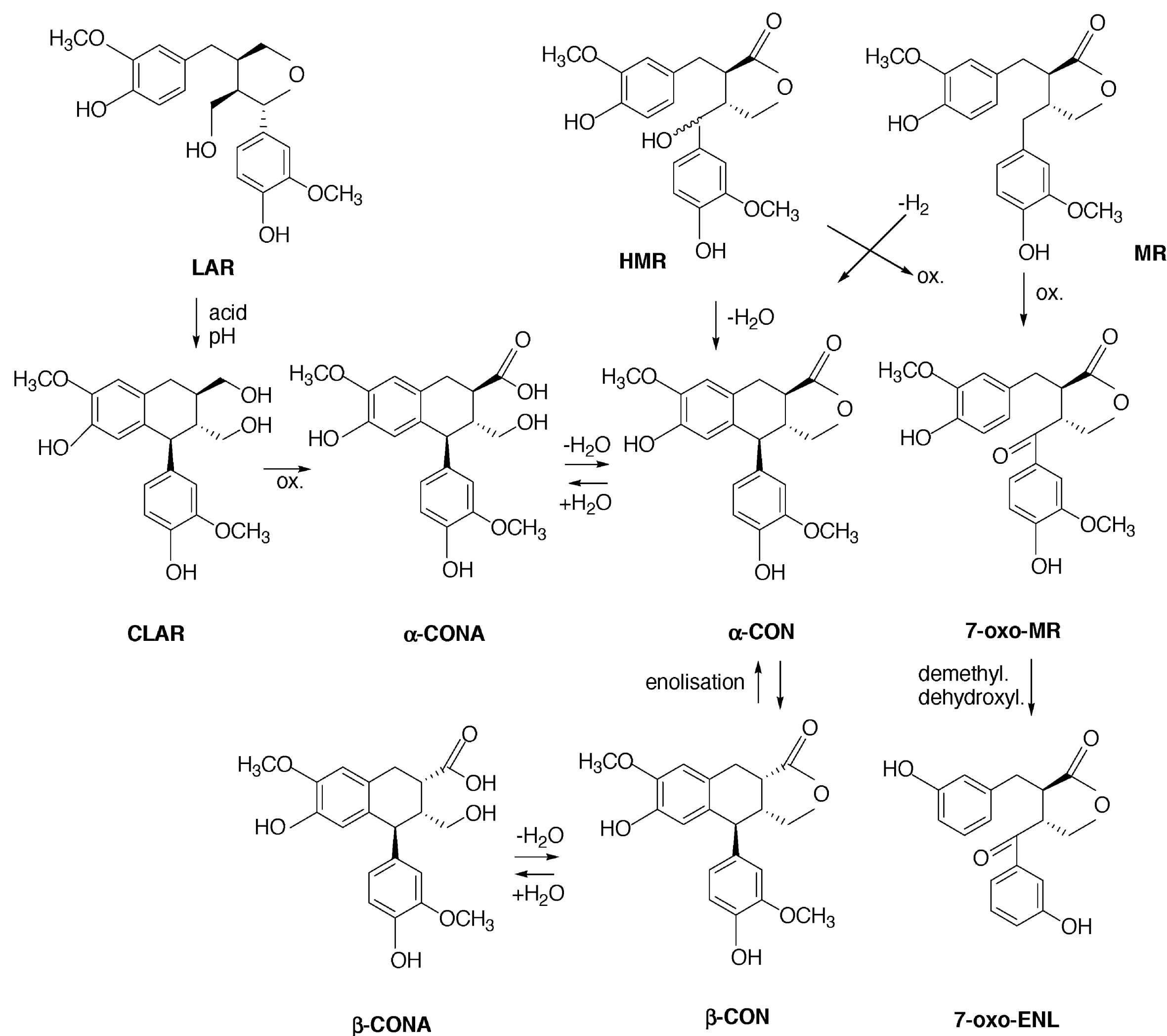

Fig. 4. Possible routes for formation of oxidative metabolites of plant lignans.

be more easily formed from MR than from the other plant lignans because only demethylation of the aromatic methoxy groups is required (Fig. 5). MR-CH_3 is probably formed from HMR via MR. The urinary excretion of MR has been shown to increase following administration of HMR to rats [9]. From LAR, MR-CH_3 is probably formed via SECO which is then demethylated and dehydrogenated and oxidised, like in the formation of ENL from END. The urinary excretion of SECO has been shown to increase after administration of LAR to rats [9]. Surprisingly, MR-CH_3 seems to be formed also following administration of ENL. Our recent studies indicate that the urinary excretion of MR increases after administration of ENL to rats (unpublished results), and if MR is formed from ENL, also MR-CH_3 may be formed. This formation would imply aromatic hydroxylation, but also methylation of the hydroxyl groups at C-3 and/or C-3′.

Obviously, also didemethylated MR (4,4′-diOH-ENL) is a stable metabolite of MR and HMR in vivo, like didemethylated SECO, which could be isolated after incubation of SECO with human fecal suspension [30]. In one study, only the monodemethylated forms of MR could be detected after incubation of rat liver microsomes with MR [6]. From SECO and LAR, 4,4′-diOH-ENL is probably formed via END and ENL; from ENL by aromatic hydroxylation (Fig. 5). Statistical analyses showed a strong correlation ($r = 0.83$) between excretion of 4,4′-diOH-ENL and MR-CH_3 after administration of plant lignans, obviously because these compounds are subsequent steps in the metabolism (demethylation) of these. Statistical analyses also showed a weak ($r = 0.49$), but significant correlation between excretion of MR-CH_3 and 7-oxo-ENL and between excretion of 4,4′-diOH-ENL and 7-oxo-ENL ($r = 0.35$) after administration of plant lignans. This is because the excretion of 7-oxo-ENL, like of 4,4′-diOH-ENL and MR-CH_3, increases after administration of all plant lignans, probably because it is formed from ENL. α-CON has been shown to be formed from HMR in vitro under both acidic [35,36] and alkaline conditions [36] and following irradiation with light [33]. Also β-CON and α-

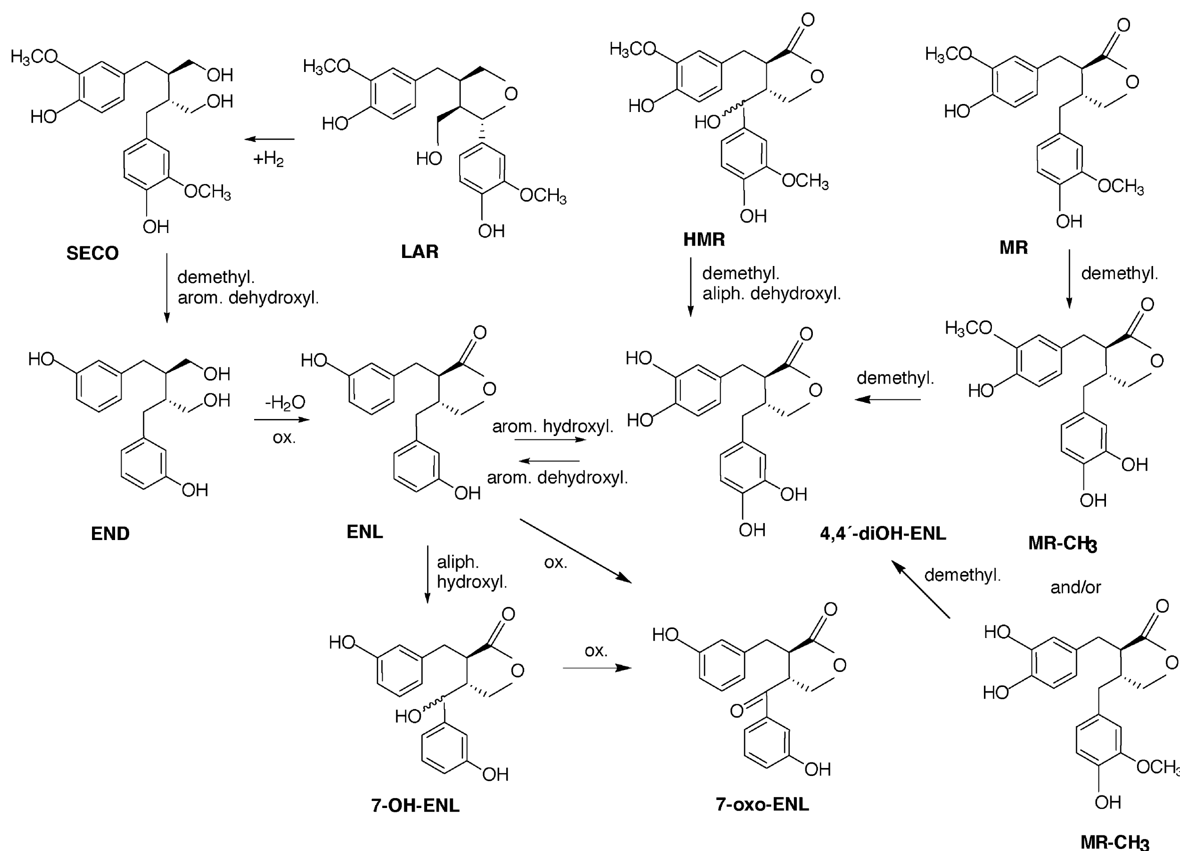

Fig. 5. Possible routes for formation of enterolignans.

and β-CONAs have been detected in an aqueous alkaline solution of HMR [36]. α-CON can be converted to α-CONA by hydrolysis of the lactone ring, which happens in vitro under alkaline conditions [37,38]. Through enolisation, α-CON can be transformed to β-CON [38], which can then be hydrolysed to β-CONA (Fig. 4). It seems therefore that once CON is formed, also CONAs may be easily formed. Statistical analyses of the excretion of CON and CONAs show, indeed, a strong correlation ($r = 0.95$) between these compounds. The fact that β-CON could not be detected in any urine sample indicates a rapid transformation to β-CONA. It seems that α-CON is even more readily formed from MR than from HMR in vivo (after a single dose) (Table 2). From LAR, α-CON is probably formed via CLAR, which is oxidised to α-CONA and then converted to α-CON by the loss of water (Fig. 4). However, no β-CONA could be detected in the urine after administration of LAR (Table 3). It is possible that the amount of β-CONA after LAR administration was too small to enable detection, taking into consideration that the formation of β-CONA from LAR requires one step more than from HMR and MR (Fig. 4). In a previous study, the oxidative metabolites of MR found in the present study (7-oxo-MR, 7-oxo-ENL, α-CON, and CONAs) could not be detected in vitro using hepatic microsomes [6]. No oxidative metabolites could be detected in vivo (in rat urine), but probably the above mentioned metabolites were not monitored in the GC–MS analyses.

In conclusion, contrary to earlier assumptions, this work shows that many oxidative metabolites of lignans may be formed in vivo. The biological importance of the novel lignan metabolites remains to be investigated.

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