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Review

Enterolignans

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Abstract

A review with 114 references about mammalian lignans (enterolignans). Several aspects have been reviewed: the precursors of mammalian lignans and their biosynthesis, biological activities and health effects, metabolism (in vivo and in vitro) in human and animals, some synthetic strategies to obtain enterolignan skeleton types, including the synthesis of haptens and deuterated lignans, and finally an overview of the analytical methods to detect and quantify lignans in biological matrices and foods.

Keywords: Reviews; Enterolignans

Contents

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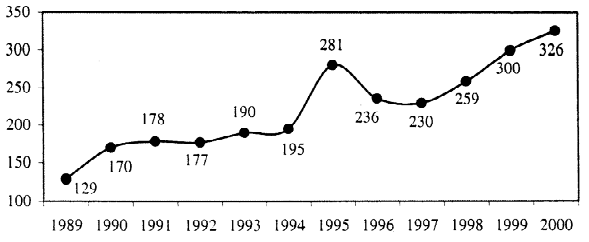


Fig. 1. Number of published papers per year about lignans from 1989 (Data from SciFinder Scholar, May 2001, Keyword: lignan).

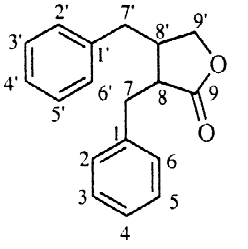
1. Introduction

Lignans are a class of secondary metabolites widely encountered in the plant kingdom. The term lignan, originally introduced by Harworth [1], covers dimeric natural products derived by the combination of two phenylpropanoid C_6–C_3 units at the β and β′ carbon atoms. In spite of their extensive distribution, their biological purpose in nature is still unclear.

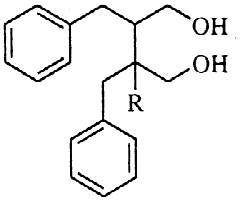
Several hundred individual lignans have been discovered in many plant species, including the wooden parts, roots, leaves, flowers, fruit and seeds. In trees, the accumulation of lignans in the core is important for the durability and longevity of the species. Lignans are also assumed to function as phytoalexins, that is to provide protection for the plants against diseases and pests such as wood rot fungi. Additionally, lignans may participate in controlling the growth of the plants.

Among the plant lignans, the podophyllotoxin-type structures have aroused widespread interest because of their antitumor properties [2]. After mammalian lignans were first reported simultaneously in Finland and UK in 1979, a flurry of research activity was created almost overnight [3,4]. No longer a subject just for botanists and wood and natural products chemists, the lignans now attract the interest of food chemists and nutrition scientists, researchers in various branches of medicine, and the food and pharmaceutical industry. During the last year alone, more than 300 papers appeared in print (Fig. 1).

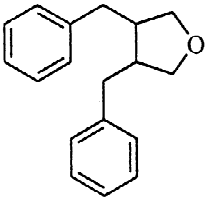
Although lignans in general combine a diversity of carbon skeletal types, the known mammalian lignans occur in only three groups which differ in their oxidation level: the dibenzylbutyrolactones (type 1), dibenzylbutanediols (type 2) and dibenzyltetrahydrofurans (type 3) (Fig. 2). Mammalian lignans (Fig. 3), also called enterolignans, were first detected in urine of man [4,5] and monkeys [6] and their definitive identification in 1981 [7] proved their structures as *trans*-3,4-bis(3-hydroxybenzyl)-dihydro-2-furanone (**1**, enterolactone, ENL, type 1) and 2,3-bis(3-hydroxybenzyl)-butane-1,4-diol (**2**, enterodiol, END, type 2). ENL and END have also been found in human plasma, saliva and feces[8–16]. It is interesting that ENL is a normal, relatively abundant constituent of human semen and prostatic fluid [17,18]. Also *trans*-3-(3-hydroxybenzyl)-4-[(hydroxy-(3-hydroxyphenyl)-methyl]-dihydro-2-furanone (**3**, 7′-hy-



Type 1:
Dibenzylbutyrolactones
1-a: 7=7'=8=8'=H (34)
1-b: 7=8=8'=H, 7'=OH (5)
1-c: 7'=8=8'=H, 7=OH/OMe (6)
1-d: 7=7'=8'=H, 8=OH (12)
1-e: 7=7'=H, 8=8'=OH (5)



Type 2:
Dibenzylbutanediols
2-a: R=H (14)
2-b: R=OH (1)



Type 3:
Dibenzyltetrahydrofurans (6)

Fig. 2. Mammalian lignan-type skeletons, example of numbering, lignan subtypes classified according to the aliphatic substitution pattern and number of naturally occurring lignans (in parentheses) for each subtype.

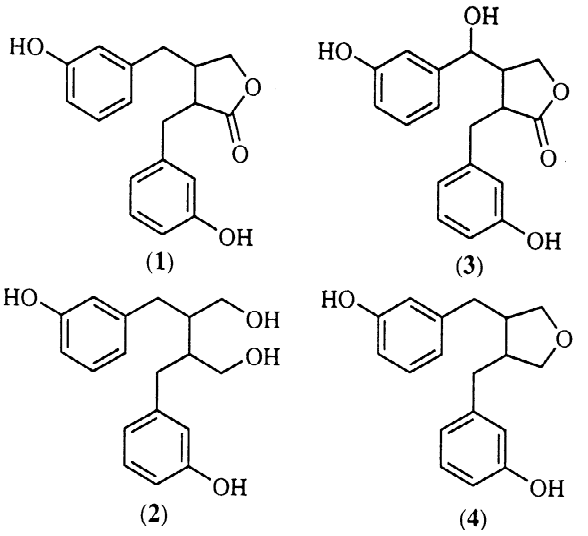


Fig. 3. Mammalian lignans (enterolignans). (**1**) Enterolactone (ENL), (**2**) enterodiol (END), (**3**) 7′-hydroxyenterolactone (HENL), (**4**) enterofuran (ENF).

droxyenterolactone, HENL) and *trans*-3,4-bis(3-hydroxybenzyl)-tetrahydro-furan (**4**, enterofuran, ENF, type 3) have been tentatively identified in human urine [19,20]. Mammalian lignans have also been detected in cow milk [21].

The mammalian lignans have only two phenolic hydroxy groups, at the *meta* position of each aromatic ring. Plant lignans, metabolic precursors of the mammalian lignans, usually carry a number of hydroxy, methoxy or methylenedioxy groups in the aromatic rings. Altogether there are 62 lignan representatives found in nature belonging to the dibenzylbutyrolactone class (type 1), 15 of the dibenzylbutanediol class (type 2), and six of the less common dibenzyltetrahydrofuran class (type 3) (Fig. 2, Table 1). Normally lignans exist in nature as glycosides.

1.1. Precursors of mammalian lignans in food

So far only few precursors of mammalian lignans are known. The plant lignans matairesinol (**5**, MAT), secoisolariciresinol (**6**, SECO), 7′-hydroxymatairesinol (**7**, HMR) lariciresinol (**8**) and isolariciresinol (**9**) have been found in human urine [8,9,22–24].

The richest source of plant lignans is flaxseed (linseed, *Linum usitatissimum*) [25,26] which contains the highest concentrations of SECO of any food (28–369 mg/100 g). In addition, pinoresinol (**10**), isolariciresinol and MAT are reported to exist in flaxseed [27]. However, the most important sources for lignans in human diet are different varieties of cereal, particularly rye and barley. Since most of the lignans are located in the outer layer of the seeds (aleuronic layer) they are most abundant in wholegrain products as the aleuronic layer is usually eliminated in the milling processes along with the outer fibre layer. Lignans have also been found in other seeds, berries, soy beans, other legumes, vegetables and fruits, which contain mostly SECO and only occasionally low amounts of MAT. Recently SECO and MAT were reported also from beverages such as tea, coffee and wine (Table 2).

Table 1
Examples of naturally occurring lignans

3	**4**	**5**	**7**	**8**	**3′**	**4′**	**7′**	**8′**	Name	Skeleton type
OMe	OH				OMe	OH			Matairesinol	1-a
OMe	OH				OMe	OMe			Arctigenin	
OMe	OH				OMe	OH	OH		7′-Hydroxymatairesinol	1-b
OMe	OH		OMe		OMe	OH			7-Methoxymatairesinol	1-c
OMe	OMe	OMe	OH		OCH_2O				Podorhizol	
OCH_2O				OH	OCH_2O				Meridinol	1-d
OMe	OMe			OH	OMe	OH			Trachelogenin	
OMe	OH			OH	OMe	OH		OH	Thujastandin	1-e
OMe	OH				OMe	OH			Secoisolariciresinol	2-a
OCH_2O					OCH_2O				Dihydrocubebin	
OMe	OH			OH	OMe	OH			Carinol	2-b
OMe	OH				OMe	OH			Shonanin	3
OMe	OMe	OMe			OCH_2O				Burseran	

Numbering according to Fig. 2.

1.2. Bioavailability

There are only a few studies on the bioavailability of plant lignans. It has been found that rats with a normal gut microflora produce ENL and END which were absent from the urine of germ-free rats. It is clear that gut bacteria play an important role in the formation of mammalian lignans from their plant precursors [31–33]. Lignan metabolism seems to vary between individuals. Nesbitt et al. [9], Lampe et al. [34] and Rowland et al. [8] have reported wide variations in the excretion of ENL and END in humans. Lignans are mainly excreted as mono-glucuronides in urine but small amounts of di-glucuronides and sulfates also occur. In feces-free phytoestrogens are excreted [35]. ENL and END are found mainly conjugated to glucuronic acid in portal venous blood [31]. Lignan glycosides are poorly absorbed from the small intestine because of their hydrophobic nature, and being β-glycosides they resist hydrolysis by mammalian enzymes but are readily cleaved by bacterial enzymes [8,30]. In premenopausal women consuming 10 g of ground linseed per day for three menstrual cycles both urinary [34] and fecal [36] lignan excretion increased and varied greatly among subjects (3- to 285-fold increase). Morton [16] reported plasma levels of lignans for males consuming a cake containing 15 g cracked linseed and 15 g soybean flour. There was no increase in plasma ENL until 8.5 h after consuming the cake. The metabolism and bioavailability of lignans seem to be sensitive to many modulating factors. It is assumed that the variation in microflora composition plays an important role in the inter-individual variation of metabolism.

Table 2
Amount of lignans in selected food items

Sample	SECO[a]	MAT[a]	Ref.
Bramble	3718	23	[28]
Strawberry	1505	78	[28]
Lingonberry	1510	0	[28]
Blackcurrant	388	10	[28]
Rye	47	65	[29]
Barley	58	0	[29]
Soy beans	273	–	[30]
Flaxseed	369 900	1087	[29]
Broccoli	414	23	[29]
Garlic	379	4	[29]
Carrot	192	3	[29]
Pepper	117	7	[29]
Prince of Wales black tea	2420	305	[29]
China green tea	2890	195	[29]
Maxwell coffee	500	nd	[29]
Chianti, reserve (Italy)	1280	98	[29]
Chardonnay (France)	174	22	[29]

nd = not detected.
[a] Units are μg of lignans per 100 g dried food.

2. Biological activities and health effects

Lignans possess a range of biological activities in animals and in vitro systems, including antioxidant, antitumor, weakly estrogenic, and anti-estrogenic properties, and inhibition of enzymes involved in the metabolism of sex hormones (e.g. SHBG) [37–48]. ENL is a moderate inhibitor of placental aromatase and competes with the natural substrate androstenedione for the enzyme [49,50]. ENL also inhibits 5-α-reductase and 17β-hydroxysteroid dehydrogenase in human tissues [39]. Due to such inhibitory effects, lignans probably reduce the plasma levels of free estradiol and testosterone and may affect the development of hormone dependent diseases [37,49]. ENL inhibits the proliferation of estradiol-stimulated MCF-7 human breast cancer cells in vitro [40]. Interestingly, women with breast cancer or with a high risk for breast cancer excrete lower amounts of lignans in urine compared to healthy women, even if they have the same diet [51].

In case of colon, the mammalian lignans have been shown to exhibit anticancer effects in vitro systems and to reduce the proliferation of colon tumor cells [41]. In rats, diets containing high amounts of lignans seem to protect against colon cancer [52,53].

Saarinen et al. [42] have shown that HMR, extracted from spruce (*Picea abies*), is a stronger antioxidant in vitro than ENL or END but has no significant estrogen-like effect. HMR suppressed the growth of established induced mammary tumors in rats and the total tumor volume was reduced after 4 weeks of treatment.

It has been suggested that lignan-rich diets could also have a protective effect against estrogen-related diseases e.g. osteoporosis [54]. Epidemiological studies indicate that high urinary concentrations of

lignans are associated with a decreased risk of coronary heart disease [55,56].

3. Biosynthesis of lignans

Lignans are presumed to be related to lignins, the second most abundant biopolymer in nature, because of the precursors and processes involved in the biogenesis of both groups. Nevertheless there are differences in the two biosynthetic pathways. For one thing, lignans are chiral almost without exception whereas the structural units in lignin are racemic.

The first steps in the biosynthesis are common to the two classes. A sequence of five enzymatic reactions—deamination, aromatic hydroxylation, *O*-methylation, CoA-mediated ligation and NADPH mediated reduction—converts phenylalanine to monolignols such as coniferyl alcohol (**11**) [43,57]. From here on the two biosynthetic routes diverge. The phenylpropanoid monolignols are thought to dimerize to C_{18} structures via oxidative phenolic coupling reactions followed by various oxidation, reduction or alkylation steps to give the lignans.

From 1990 on Lewis and coworkers [58–61] have studied lignan biosynthesis in *Forsythia* species, and were the first to clarify the formation of certain dibenzylbutyrolactone lignans, (−)-MAT in particular. It was demonstrated [58] that a 78 000 dalton ''dirigent protein'' together with a non-specific oxidase (e.g. laccase) is required for the stereoselective coupling of two molecules of (**11**) (Scheme 1; a). In vitro experiments established that laccase alone converts (**11**) into three different racemic lignan dimers whereas (+)-pinoresinol (**10**) only is obtained if laccase and the dirigent protein are simultaneously present. Subsequent enantiospecific reductions of (**10**) give first (+)-lariciresinol (**8**, Scheme 1; b and c) and then (−)-SECO (**6**). The latter undergoes enantiospecific reoxidation to (−)-MAT (**5**, Scheme 1; d) [59,60] which is methylated to give arctigenin (**12**, Scheme 1; e) [61].

According to Lewis and co-workers [58–61], these results suggest the existence of a new class of proteins responsible for the formation of different enantiomers of lignans. It is also expected that further proteins, involved in the biosynthesis of lignan skeletons other than that in the dibenzylbutyrolactone group, will be found in the future.

4. Metabolism of lignans

In humans, SECO diglucoside (**13**) and MAT are hydrolysed and metabolized by colonic microflora to the mammalian lignans ENL and END (Scheme 2) [33].

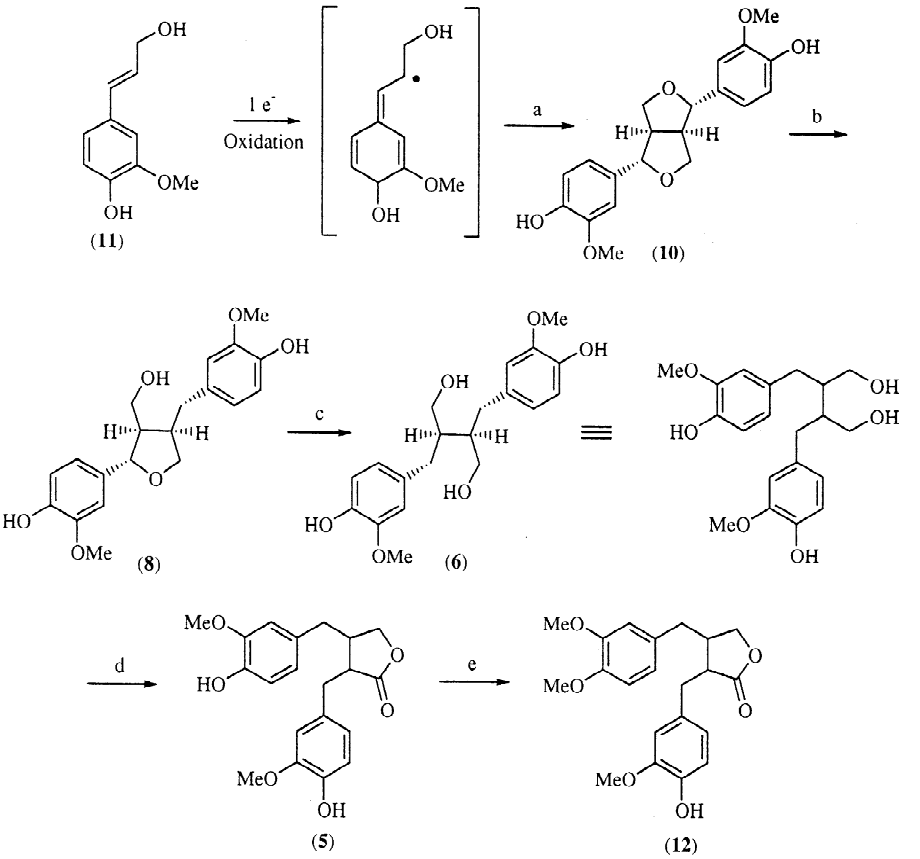
Wang et al. [62] have isolated from human feces two bacterial strains (*P.* sp. SDG-1 and *E*. sp. SDG-2) capable of demethylation and dehydroxylation of (**13**) after anaerobic incubation in vitro. Demethylation was suggested to occur prior to dehydroxylation in these studies (Scheme 3).

Once generated ENL and END are absorbed from the gastrointestinal tract and reach the liver where they are conjugated mainly with glucuronic and also sulfuric acid probably by hepatic phase II enzymes (UDP glucuronosyltransferases and sulfotransferases) before entering circulation [32,35,63]. The mammalian lignans are found in plasma mainly as free compounds, mono- and disulfates [12], in urine as monoglucuronides [11] and in feces as free compounds [15].

Recently Heinonen et al. [64] found new precursors for the mammalian lignans after human fecal incubation. The plant lignans syringaresinol (**14**), pinoresinol, lariciresinol and SECO were metabolized mainly to ENL and END. Arctigenin, HMR and MAT were metabolized mostly to ENL. HMR was converted also to HENL. Small amounts of ENF were found after incubation of SECO and pinoresinol [64].

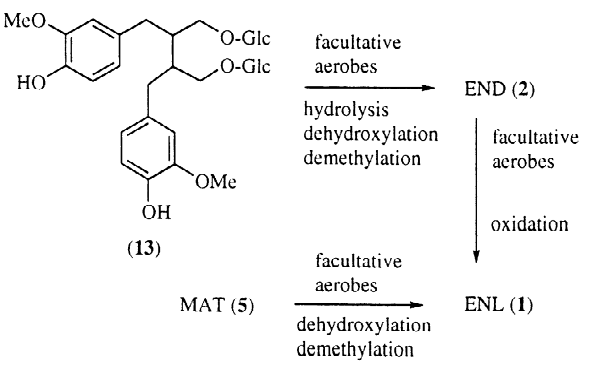
ENL and END are the main lignan metabolites in human plasma, prostate fluids, urine and feces [8–16]. Also HENL and ENF have been tentatively identified in human urine [19,20]. Small amounts of unchanged dietary lignans MAT, SECO, HMR, lariciresinol and isolariciresinol have been found in human urine [8,9,22–24].

The excretion and metabolism of mammalian lignans have been studied in several animals, for example, rats, pigs, chimpanzees and rabbits [42,65–70]. Recent research [65,71] has shown that hepatic



Scheme 1. Biosynthesis of lignans in *Forsythia* species [58–61].

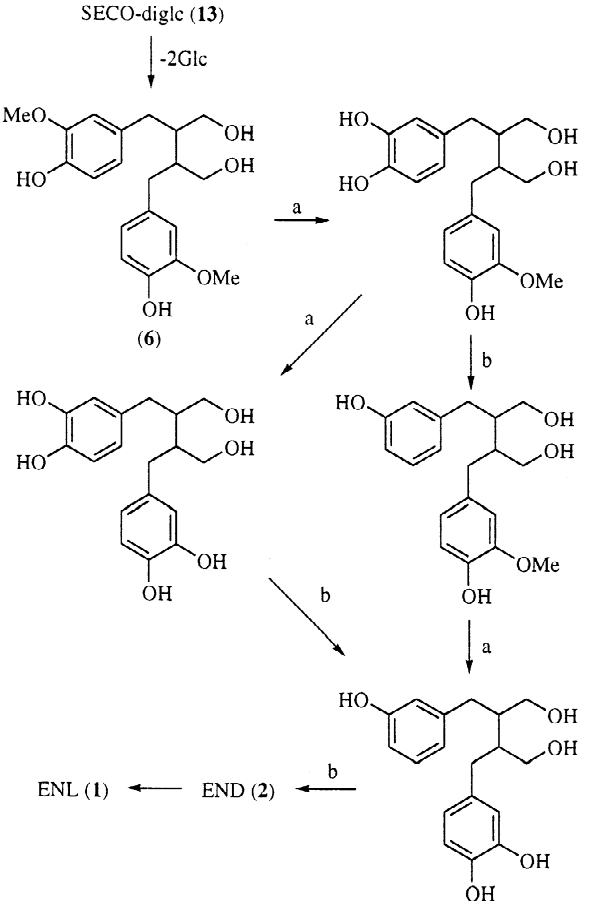
microsomes from either human or rat liver are able to hydroxylate ENL and END at the aromatic positions. Nine similar lignan metabolites (**15**–**23**, Figs. 4 and 5), having an additional hydroxy group at the *para* or the *ortho* positions of either aromatic ring, were identified from human urine after a flaxseed diet [72].



Scheme 2. Metabolic pathway for ENL formation in man [33].

The interindividual variation in lignan metabolism could be a consequence of differences in gut microflora. The functions of the intestinal bacteria are influenced by diet [8]. It has been shown that avid consumers of plant-based food have the highest lignan concentrations in plasma and urine [24,51,73] and there is a linear dose–response in urinary excretion of lignans with ingestion of increasing quantities of flaxseed or rye [9,74].

Administration of antibiotics nearly stops the ENL and END production in the gut [13,51] and it leads, after an initial rapid lowering of the lignan levels in urine, to an increase of the END/ENL ratio. The effects of antibiotics last more than 6 weeks. It has been suggested that there should be an interval of at



Scheme 3. Possible metabolism pathway for mammalian lignans by intestinal bacteria [62]. a=*P.* sp. SDG-1 (–OMe); b=*E.* sp. SDG-2 (–OH).

least 3 months after administration of antibiotics to get a correct result in the analysis.

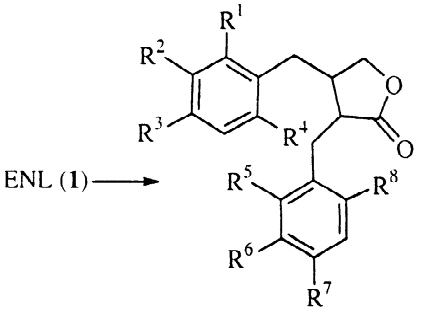
5. Synthesis of lignans

5.1. Dibenzylbutyrolactones (type 1)

One of the most common synthesis routes is the tandem Michael addition–alkylation sequence (Scheme 4) introduced by Ziegler and Schwartz [75]. The lithiated dithioacetal (**24**), formed by treatment with *n*-BuLi in THF, reacts with the butenolide (**25**) to give the Michael adduct (**26**), alkylated in situ by the benzylic chloride (**27,** *X*=Cl). The protected *trans*-3,4-dibenzylbutyrolactone (**28**) is obtained in yields in excess of 60%.

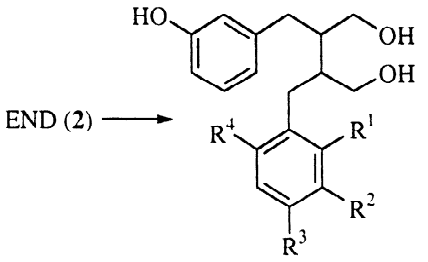
Schlessinger et al. [76] improved the yield to 85% (after desulfurization) simply by replacing the benzylic chloride with the corresponding bromide (**27**, *X*=Br). Iodides have also been used [77]. An aryl aldehyde *S,S′*-dimethyldithioacetal [76] or 1,3-dithiane [75,78–80], or more commonly *S,S′*-diphenyldithioacetal [67,77,81–84] may serve as the acyl anion equivalent source. A complexing agent such as hexamethylphosphoric triamide (HMPA), *N,N,N′,N′*-tetramethylethylene diamine (TMEDA), or 1,3-dimethyl-2-imidazolidone (DMI) is frequently added to assist the alkylation step. Reactions are commonly run at −78 °C and after the addition of the benzylic halide, the reaction mixture is allowed to reach ambient temperature overnight. Yields depend largely on the aromatic substitution, with R^1 and R^2 being almost any combination of allyl [84], methoxy, benzyloxy, methylenedioxy or silyloxy groups.

The protected dibenzylbutyrolactone (**28**) is converted to a lignan (**29**) by desulfurization with Raney nickel [67,76,81,83,84]. Yields are mostly quantitative, and the benzyl ether protections are removed at the same time. Instead of desulfurization, the dithioacetal moieties may be hydrolyzed to furnish the 7′-oxolignans (**30**) by a variety of reagents, for



(**16**) R^2=R^6=R^8=OH, R^1=R^3=R^4=R^5=R^7=H
(**17**) R^2=R^4=R^6=OH, R^1=R^3=R^5=R^7=R^8=H
(**18**) R^1=R^2=R^6=OH, R^3=R^4=R^5=R^7=R^8=H
(**19**) R^2=R^3=R^6=OH, R^1=R^4=R^5=R^7=R^8=H
(**20**) R^2=R^6=R^7=OH, R^1=R^3=R^4=R^5=R^8=H
(**21**) R^2=R^5=R^6=OH, R^1=R^3=R^4=R^7=R^8=H

Fig. 4. Microsomal metabolites of ENL in vitro [72].

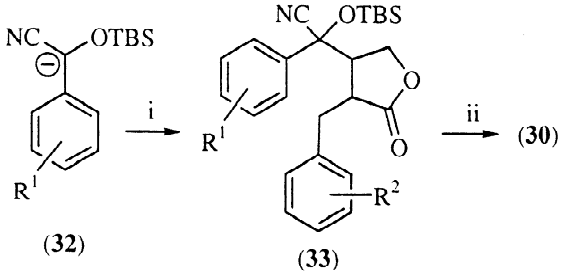


(**21**) R^2=R^4=OH, R^1=R^3=H
(**22**) R^2=R^3=OH, R^1=R^4=H
(**23**) R^1=R^2=OH, R^3=R^4=H

Fig. 5. Microsomal metabolites of END in vitro [72].

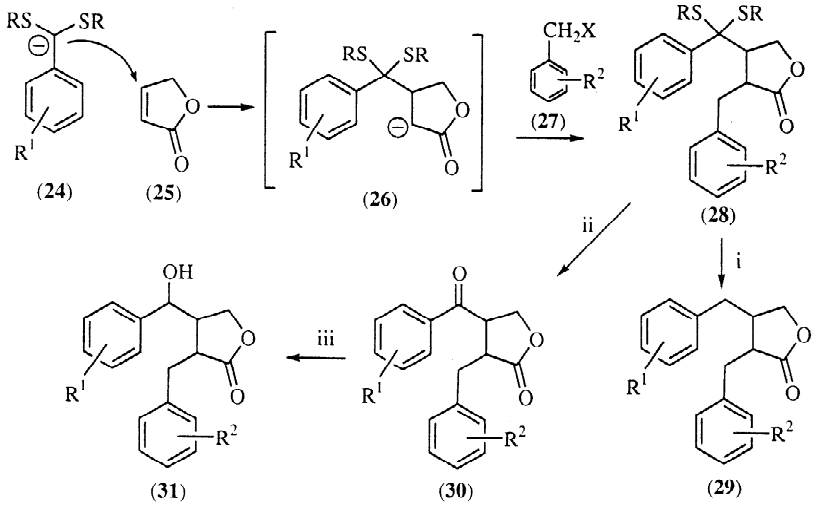
example mercuric oxide and boron trifluoride etherate [75,78]. The ketones (**30**) may be carried further to the secondary alcohols (**31**) by hydride reduction, usually in high yield (Scheme 4).

Instead of dithioacetal-based approaches, other acyl carbanion equivalents [85] may be used. Thus, Iwasaki et al. [86] have reacted the LDA-generated protected cyanohydrin anion (**32**) with (**25**) (Scheme 5) followed by alkylation to complete the dibenzylbutyrolactone synthesis. The keto group was then exposed by treatment of (**33**) with tetrabutylammonium fluoride (TBAF).

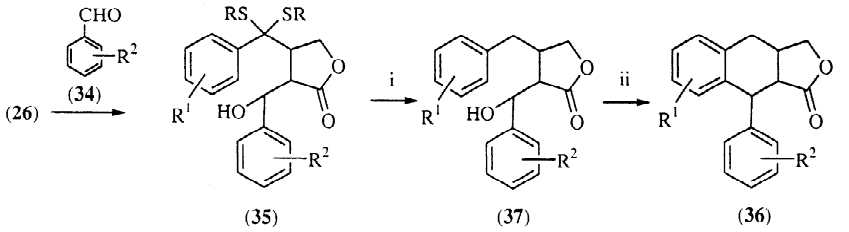


Scheme 5. Reagents: i=(**25**), (**27**); ii=TBAF.

The tandem addition–alkylation strategy is applicable also for the synthesis of 7-hydroxylignans, isomeric with **31**. To this end, the benzylic halide is replaced by an aromatic aldehyde (**34**) as the final component (Scheme 6). This gives the aldol-type addition product (**35**) in high yield and fairly short reaction time [75,76,78,87–90]. The presence of the 7-hydroxy group makes possible the synthesis of another class of lignans, namely those of the podophyllotoxin series (**36**). A C–C bond can be formed between the 7 and the 2′ positions of the deprotected lignans (**37**), creating a new six-membered ring in the lignan skeleton. For the cyclization various acidic reagents such as trifluoroacetic acid (TFA) [87,88] or $SnCl_4$ [89] have been used in high yield.



Scheme 4. Reagents: i=Raney Ni; ii=$BF_3 \cdot Et_2O$; iii=$NaBH_4$.

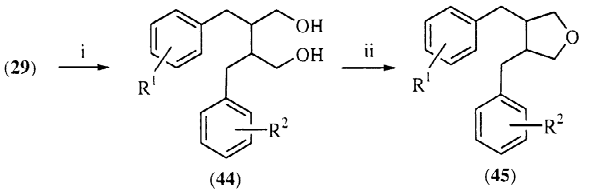


Scheme 6. Reagents: i = Raney Ni; ii = TFA.

A different approach to dibenzylbutyrolactone lignans is provided by the Stobbe condensation (Scheme 7), which takes place between an appropriately substituted aldehyde (**38**) and a succinate diester (**39**) to give the conjugated succinate monoester (**40**), reduced by catalytic hydrogenation to **41**. A reductive lactonization of the latter to the butenolide (**42**) may be performed for example with $LiBH_4$ [80] or $Ca(BH_4)_2$ [91,92]. The carbanion (**43**) obtained by treating **42** with LDA may then be alkylated with a benzyl bromide (**27**, $X = Br$) to give dibenzylbutyrolactones such as (**29**), or can be added to an aldehyde (**34**) to generate the 7-hydroxy type lignans (**37**).

5.2. Dibenzylbutanediols (type 2) and dibenzyltetrahydrofurans (type 3)

Dibenzylbutyrolactones (**29**) may also be considered precursors for dibenzylbutanediols and further for dibenzyltetrahydrofuran lignans. Thus, reduction of **29** with $LiAlH_4$ in THF gives the diols (**44**) (Scheme 8) in very high yield [83,93,94]. Compounds (**44**) when treated with $HClO_4$ in acetone undergo cyclization to give tetrahydrofuran structures (**45**).

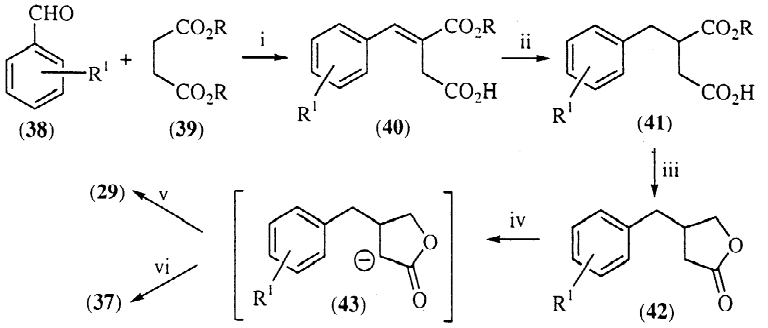


Scheme 8. Reagents: i = $LiAlH_4$; ii = $HClO_4$.

5.3. Deuterated lignans

Mention should also be made of the synthesis of deuterated lignans, which are needed as internal standards in the analysis of lignans by the isotope dilution gas chromatography–mass spectrometry technique (see later). For this use, no unlabeled species must be present and the isotope labels must remain stable under analytical conditions. Two different approaches to D-labelled lignans have been published.

In the first one by Kirk et al. [77], the label is



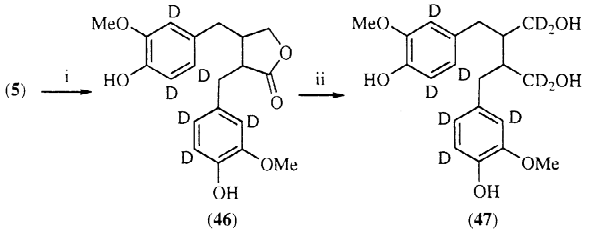
Scheme 7. Reagents: i = Base; ii = H_2, Pd/C; iii = $LiBH_4$: iv = LDA; v = (**27**); vi = (**34**).

carried along from the deuterated 2-butenolide used to perform the tandem Michael addition–alkylation. Thus the resulting dibenzylbutyrolactone skeleton carries two D atoms at the 9′ position. Subsequent reduction of the lactones with $LiAlD_4$, may be used to prepare $^2H_4$-dibenzylbutanediols.

The second method [95–97], developed in our laboratory, is based on H/D exchanges in the lignan skeleton. The dibenzylbutyrolactone is treated with $D_3PO_4 \cdot BF_3$ (Scheme 9) to give regioselectively a multiply deuterated product (**46**, six D atoms in case of MAT, for example) [98]. If required, a treatment with $LiAlD_4$ introduces two further deuteriums to the diol type lignan (**47**).

5.4. Haptens [84,99]

Synthetic lignan haptens are required for the immunoassay analysis (see later). To begin with, a suitable functional group must be appended to the particular lignan to be analysed. This derivatized lignan (hapten) is then coupled to a macromolecular carrier protein (e.g. bovine serum albumin) to form the antigen used to immunize rabbits for the production of specific antibodies. In the particular case of ENL hapten, the functional group is a carboxylic acid at the end of an alkyl chain attached to one of the phenolic hydroxyls via an ether linkage (**48**, Fig. 6). The framework is generally obtained by the tandem Michael addition–alkylation in the same way as shown previously for the synthesis of lignans of type 1. The carboxylic side chain can be added to the completed lignan skeleton or it can be already present in one of the aromatic starting materials. In the former case, silyl and benzylic protection groups are often used owing to their orthogonal deprotection. This synthetic method requires eight reaction steps for the starting materials and four steps after the tandem reaction.



Scheme 9. Reagents: i = $D_3PO_4 \cdot BF_3$; ii = $LiAlD_4$.

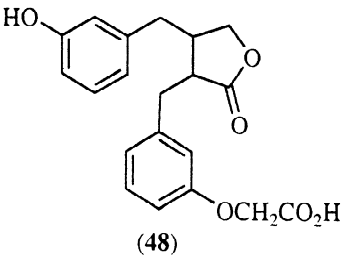


Fig. 6. Example of an ENL hapten.

6. Analysis of lignans

6.1. Quantitative analysis

Lignans, both mammalian and plant, occur naturally mostly as various conjugates. Plant lignans are glycosidically linked to a wide variety of different sugars, which poses a problem for analytical work. Depending on the analytical system samples may require quite laborious pre-treatment such as hydrolysis, pre-purification and derivatisation (GC). This also necessitates the use of various internal standards to correct for losses during the procedure.

The need for a fast and simple, yet reliable method for analysing large quantities of samples is increasing all the time. This is true especially of human samples, where quite large populations must be studied in order to evaluate the physiological effects of phytoestrogens.

6.2. Extraction

The first step in food analysis is the extraction of a known amount of dry, usually lyophilised, sample with a polar solvent such as aqueous methanol or acetonitrile. The extract may be purified further by partitioning with a non-polar solvent, for example with hexane. This is done to remove lipids and other non-polar components which may interfere with the analysis and shorten the column life.

6.3. Hydrolysis

Since glycosides cannot be analysed directly by

GC, lignans must be freed by hydrolysis and derivatised prior to the analysis. It is also unlikely that all of the possible target lignan glycosides are known. Two approaches to the hydrolysis of glycosidic bonds between lignans and carbohydrates have been utilized. The first is to break the bond enzymatically, for example with juice extracted from the snail *Helix pomatia*, β-glucosidase from almonds, or cellulase from fungus *Aspergillus niger*. Alternatively, glycosides may be hydrolyzed with hot (100 °C) 1–2 *M* HCl for about an hour. A problem with acid hydrolysis of natural products is their stability. We have reported [100] that one of the target plant lignans, SECO, is dehydrated under acidic conditions to anhydrosecoisolariciresinol (**47**, ANHSEC, also called shonanin, Table 2). ANHSEC is also a naturally occurring compound and may interfere with the analysis of SECO if present in a plant sample being analysed [20].

6.4. Analytical techniques

6.4.1. HPLC

The predominant analysis method for phytoestrogens in general is HPLC. The main advantage is the ease of use. Samples do not necessarily require time-consuming pre-treatment and may, in principle, be injected into a system right after the extraction and analysed as such. The problem is to achieve a good enough resolution and sensitivity since phytoestrogen levels are usually quite low.

Commonly UV or UV–DAD detectors are used, but recently new methods for detecting analytes have been introduced. For example coulometric electrode array detection (ECD) has been utilized for the quantitation of the mammalian lignans ENL and END from human fluids [101,102]. Also LC–MS has been used for lignan glycoside analysis [103]. Nose et al. [70,104] have used a reversed-phase HPLC–UV method for the analysis of the lignans arctiin (arctigenin-4-*O*-glc), tracheloside (trachelogenin-4-*O*-glc), and their metabolites in rat gastrointestinal tract. Free lignans as such, and those from the hydrolysis of conjugates were extracted from serum with dichloromethane. *p*-Hydroxybenzophenone was used as an internal standard. Analytes were detected with an UV detector at 280 nm and the sensitivity was 0.005–0.02 a.u.f.s.

Gamache and Acworth [101] used HPLC–ECD to analyse phytoestrogens in plasma, tissue and urine. Coulometric detection minimizes sample pre-treatment since it is sensitive, selective and also allows the resolution of co-eluting analytes based on the differences in their oxidation–reduction behaviour. A reversed-phase column (C_{18} Hypersil, 150×3 mm, 3 μm) was used together with a serial array of eight coulometric electrodes. The mobile phase consisted of gradient sodium acetate buffer, methanol and acetonitrile. For the determination of free phytoestrogens in urine, samples were diluted with mobile phase, centrifuged and analysed as such in a 30-min run. To determine total phytoestrogens samples were first hydrolysed with β-glucuronidase. Analytes were quantified using standard mixtures treated similarly. Limits of detection for END and ENL were 10 pg. Intraassay precision was calculated as the percentage relative standard deviation (% RSD) and was 1.86 for END and 1.66 for ENL.

Nurmi and Adlercreutz [102] similarly applied HPLC–ECD for the analysis of lignans. Phytoestrogen plasma samples were hydrolysed by glucuronidase/sulfatase treatment and extracted with diethyl ether. Evaporated samples were redissolved in methanol and analysed. Recoveries were determined using tritiated estradiol-17β-D-glucuronide. Separation was carried out using gradient elution with a sodium acetate buffer, methanol and acetonitrile. The column was a C_{18} reversed-phase column (Inertsil ODS-3, 150×3 mm, 3 μm). Total analysis time was 85 min including stabilizing time. Detection limits were 6.2 pg (ENL), 5.8 pg (END), 6.5 pg (MAT), 3.4 pg (SECO) and 5.4 pg (ANHSEC).

Urinary lignans have also been analysed by HPLC–APCI–MS [105]. Phytoestrogens were extracted from urine with Sep-Pak C_{18} cartridges and 4-methylumbelliferone glucuronide was added as an internal standard. After hydrolysis with glucuronidase/sulfatase aglycones were recovered by solid-phase extraction as above and subjected to HPLC–MS analysis. The column used was a C_8 reversed-phase column (4.6×150 mm, 300 Å pore size). The solvent gradient was 0–50% acetonitrile in aqueous ammonium acetate over 15 min. After chromatographic separation, the eluate stream was diluted with ammonium hydroxide and multiple reaction monitoring was carried out by selecting parent

molecular ions and specific daughter ions formed by collision with argon–10% nitrogen gas.

6.4.2. GC

The main analytical method for lignan phytoestrogens, however, seems to be gas chromatography with various quantification methods. Isotope dilution gas chromatography–mass spectrometry in the selected ion monitoring (ID-GC–MS–SIM) method presented by Adlercreutz et al. [63] allows assays of phytoestrogens in food samples and human fluids. It utilizes a deuterated internal standard for each phytoestrogen to be studied thus making it possible to control for losses of the compounds before the analysis itself. For urinary samples a Sep-Pak C_{18} cartridge was used for extraction. After extraction, chromatography on DEAE-Sephadex in the hydroxyl form (DEAE-OH^-) is employed to collect free and conjugated phytoestrogens (sulfates and glucuronides) from the sample. Next, conjugates were hydrolysed (glucuronides) or solvolysed (sulfates) and deuterated internal standards added. QAE-Sephadex in the acetate form (QAE-Ac^-) was used to separate lignans from isoflavonoids other than equol which eluted with lignans. QAE-Sephadex in the carbonate form was used to separate lignans from estrogens. Samples were silylated with pyridine–hexamethyldisilazane–trimethylchlorosilane (Py–HMDS–TMSCl) (9:3:1, v/v) solution to form trimethylsilyl derivatives of the compounds. The method has been modified for the quantitation of phytoestrogens in human plasma [12], feces [15] and various foods [28,100,106,107].

A similar isotope dilution method has been used by Morton and co-workers [17,108] for the analysis of phytoestrogens from foods and human samples. Instead of using ion-exchange chromatography they first hydrolysed samples and then separated them by Sephadex LH-20. After that samples were derivatised using BSTFA (*N,O*-bis-(trimethylsilyl)-trifluoroacetic amide) and analysed by GC–MS. The internal standards were added before chromatography. This procedure is not applicable if conjugates also are analysed from the same samples since all the standards are in the aglycone form.

Thompson and co-workers [53,109,110] have used a GC–FID method for analysing lignans in various biological samples. Lignans were extracted with C_{18} cartridges and hydrolysed with β-glucuronidase. Aglycones were then further purified and isolated on Sephadex DEAE-OH^- ion-exchange column. Samples were derivatised using a Py–HMDS–TMSCl (3:2:1, v/v) solution and subjected to GC analysis.

A simple method for quantifying the lignans SECO, MAT and ANHSEC from foods was recently presented by Liggins et al. [20]. Freeze-dried food samples were first hydrolysed with hydrochloric acid. Aglycones were partitioned with ethyl acetate and derivatised after evaporation of the solvent. Samples were derivatised by Py–BSTFA–TMSCl. Analysis was carried out by gas chromatography–mass spectrometry.

6.4.3. Immunoassay

Since GC and HPLC are not very suitable for large population screening purposes and are not sensitive enough for the assay of unconjugated phytoestrogens in plasma, a new analytical method based on immunoassay was developed. In immunoassay, an antigen and an antibody interact reversibly to form a soluble antigen–antibody complex. Labelled antigen and unlabelled antigen compete for binding to the limited number of antibody binding sites. The greater the quantity of unlabelled antigen, the less labelled antigen is bound. The concentration of unlabelled antigen is derived from the extent to which it competitively inhibits the binding of the labelled antigen to a specific antibody. The method is standardised with known concentrations of unlabelled antigen. Qualitative and quantitative evaluation of an antibody used for immunoassay is based on two criteria: the specificity, determined by cross-reaction with antigen analogues, and the affinity, determined by the measurement of affinity constants [111].

A method called time-resolved fluoroimmunoassay (TR-FIA) was developed for the rapid analysis of ENL in human urine, using europium chelate as a label [84,112,113]. The ENL derivatives (haptens) carrying a carboxylic acid appendage are used for the production of antiserum and tracer. Immunoassays usually provide an increase in sensitivity compared to GC–MS or HPLC but sometimes a decrease in selectivity since cross-reactivity occurs. The greatest

Table 3
Examples of different lignan analysis methods

Method	Matrix	Analytes	Sensitivity	Precision (C.V. %)		Ref.
				Interassay	Intraassay	
ID-GC–MS–SIM	Human urine	ENL, END	3–4 nmol/24 h	ENL: 4.1 END: 8.1	4.9–10.1 6.9–8.4	[10]
ID-GC–MS–SIM	Food samples	MAT, SECO	2–3 μg/100 g	MAT: 8.5 SECO: 11.9	6.6 4.6	[100]
HPLC–UV	Rat serum	Arctiin, Tracheloside	0.005–0.02 a.u.f.s.			[70]
HPLC–ECD	Plasma	ENL, END	ENL: 5.8 pg/19.2 fmol END: 6.2 pg/20.8 fmol	ENL: 12 END: 44	1.5 42	[102]
HPLC–ECD	Human urine, rat plasma/tissue	ENL, END	ENL: 10 pg/4.42 nM END: 10 pg/4.37 nM			[101]
TR-FIA	Human plasma	ENL	2.1 pg/20 μl	5.5–9.9	4.6–6.0	[112]
TR-FIA	Human plasma	ENL	8.9 pg/20 μl	6.9–9.9	3.3–6.0	[114]

advantage is, however, the speed of the analysis. A batch of hundred samples can be completed in 4 h [112]. It has a 10- to 100-fold increase in sensitivity and assay range compared to conventional enzyme immunoassay (EIA) and fluoroimmunoassay (FIA) methods. It also seems to be highly specific since no cross reactions were observed except to a small extent with END (0.28%).

Also a nonisotopic estrogen receptor-based assay has been developed to detect phytoestrogenic compounds in foods. The assay gives a measure of the total estrogenic load of the food as genistein equivalents [113].

A summary of the analytical methods and their analytes is shown in Table 3.

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