SYNTHESIS OF LIGNANOLACTONES, HAPTON DERIVATIVES AND DEUTERIUM LABELLING STUDIES

Eija Leppälä

Academic dissertation

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Helsinki 2018
Abstract

Lignans are naturally occurring compounds, ubiquitous in edible plants. Some plant lignans metabolize in man forming mammalian lignans. Both plant and mammalian lignans have shown to possess several biological activity and the research interest in lignans has grown due to the possible health effects.

The literature review in this work discusses the biosynthesis of plant lignans, their bioavailability from edible plants, metabolism in man and the health effects of lignans. Also the analytical methods for lignans are discussed and the synthetic approaches to lignanolactones, lignan haptens and labelled lignans are reviewed.

The experimental work focused on the synthesis of lignanolactones and lignanediols. Characterised lignanolactone reference molecules are needed for the analytical and metabolic studies of lignans. In this work eleven new lignano-9,9'-lactones and six lignano-9,9'-diols, possible metabolic products, were synthesised via the Michael-addition alkylation reaction. All the structures were fully characterised using MS, 1H NMR, 13C NMR and 2D NMR (HSQC, HMBC and COSY). The synthesised molecules were used as authentic reference materials and also for the deuteration study.

Eight lignanolactones were deuterated using D₃PO₄·BF₃/D₂O as the deuteration reagent to achieve stable D-labeled molecules. The aromatic protons of the lignans were exchanged in the deuteration reaction and labile deuteriums were back exchanged by treatment with MeOH/CH₃COCl in order to form stable deuterated compounds. The meta-substituted hydroxy or methoxy groups activate the 2,4,6 aromatic sites which led to the back exchange. The isotopic purities of the stable deuterated molecules were determined by using MS and were found to be 85 - 99%. The deuteration reaction was studied in more detail by comparing the order of deuteration/dedeuteration, monitored with NMR, to the theoretically calculated ESP values of the aromatic protons. The observations and theoretical calculations were in good agreement. The orientation, the reactivity order and stability of deuterium labels in the lignan molecules were determined.
Four enterolactone haptens, namely 3’-O-carboxymethyl-ENL, 3-O-carboxymethyl-ENL, 5-carboxymethoxy-ENL and 5’-carboxymethoxy-ENL were synthesised in order to find good immunogens for the development of the ELISA analytical method for enterolactone. The reactions for 3-O-carboxymethyl-ENL and 5-carboxymethoxy-ENL were improved, and new haptens 3’-O-carboxymethyl-ENL and 5’-carboxymethoxy-ENL were fully characterised using MS, ¹H NMR, ¹³C NMR and 2D NMR.
Acknowledgements

This study was carried out in the Laboratory of Organic Chemistry, Department of Chemistry, University of Helsinki. I wish to offer my sincere thanks to a number of people, who have supported me to complete the work. I am grateful to my supervisor, Professor Kristiina Wähälä, for introducing me to the chemistry of lignans, giving me her endless support, encouragement and advices during the project. Emeritus Professor Tapio Hase is warmly appreciated of his advices and help solving the organic problems.

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And finally my dearest ones, Matias, Emilia and Santeri. Matias, you have helped me so many ways, believed in me and arranged quiet moments for me to concentrate on finalising this work. Thank you for your patience.

Vantaa, August 2018
Eija Leppälä
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This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.


IV  Leppälä, E., Linnala, A. and Wähälä, K. Expedient quantitative enzyme immunoassay for plasma enterolactone. *Manuscript*

Abbreviations

Ac    acetyl
AIBN  azobisobutyronitrile
ARC   arctigenin
ASECO anhydrosecoisolariciresinol
Bn    benzyl
BSA   bovine serum albumine
BSTFA $N,O$-bis(trimethylsilyl)-trifluoroacetamide
Bu    butyl
CEAD  coulometric electrode array detection
COSY  correlation spectroscopy
CRP   C-reactive protein
DABCO 1,4-diazabicyclo[2.2.2]octane
DAD   diode array detection
DMF   $N,N$-dimethylformamide
DMI   1,3-dimethyl-2-imidazolidinone
DMPU  $N,N'$-dimethylpropyleneurea
DMSO  dimethylsulfoxide
EI     electron impact
ELISA enzyme-linked immunosorbent assay
END   enterodiol
ENF   enterofuran
ENL   enterolactone
ESI   electrospray ionisation
ESP   electrostatic potential
Et    ethyl
FDA   Food and Drug Administration
FIA   fluoroimmunoassay
GC    gas chromatography
HENL  7'-hydroxyenterolactone
HPLC  high performance liquid chromatography
HMDS hexamethyldisilazane
HMAT  7'-hydroxymatairesinol = 7'-HMAT
HMPA  hexamethylphosphoramide
ID    isotope dilution
IUPAC International Union of Pure and Applied Chemistry
Ip    isotopic purity
isoLAR isolariciresinol
LAH   lithium aluminium hydrate
LAR   lariicesinol
LDA   Lithium diisopropylamide
LHMDS lithium hexamethyl disilazane
LC    liquid chromatography
MAT   matairesinol
Me    methyl
MED   medioresinol
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NMO</td>
<td>N-methylmorpholine-N-oxide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOESY</td>
<td>nuclear overhouse effect spectroscopy</td>
</tr>
<tr>
<td>Oxo-MAT</td>
<td>oxo-matairesinol</td>
</tr>
<tr>
<td>PAD</td>
<td>photodiode array</td>
</tr>
<tr>
<td>PDC</td>
<td>pyridinium dicromate</td>
</tr>
<tr>
<td>Ph</td>
<td>phenyl</td>
</tr>
<tr>
<td>PIN</td>
<td>pinoresinol</td>
</tr>
<tr>
<td>ql</td>
<td>quantitation limit</td>
</tr>
<tr>
<td>QTOF</td>
<td>quadrupole time-of-flight</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>rf</td>
<td>reflux</td>
</tr>
<tr>
<td>SECO</td>
<td>secoisolariciresinol</td>
</tr>
<tr>
<td>SES</td>
<td>sesamin</td>
</tr>
<tr>
<td>SHBG</td>
<td>sex hormone-binding globuline</td>
</tr>
<tr>
<td>SIM</td>
<td>selected ion monitoring</td>
</tr>
<tr>
<td>SYR</td>
<td>syringaresinol</td>
</tr>
<tr>
<td>TBAF</td>
<td>tetra-n-butylammonium fluoride</td>
</tr>
<tr>
<td>TBDMCS</td>
<td>tert-butyldimethylchlorosilane</td>
</tr>
<tr>
<td>TPAP</td>
<td>tetrapropylammonium perruthenate</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMCS</td>
<td>tetramethylchlorosilane</td>
</tr>
<tr>
<td>TMS</td>
<td>tetramethylsilane</td>
</tr>
<tr>
<td>TR</td>
<td>time resolved</td>
</tr>
<tr>
<td>UPLC</td>
<td>ultra performance liquid chromatography</td>
</tr>
<tr>
<td>UHPLC</td>
<td>ultra-high performance liquid chromatography</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cells = leucocytes</td>
</tr>
</tbody>
</table>
1. Introduction

This work is concentrated on the synthesis of *trans* lignanolactone and lignanodiol structures, their deuteration and hapten synthesis. These molecules are useful as standards in analysing lignans from biological samples. The literature review concerns the structure of edible lignans and their metabolites, nomenclature, biosynthesis, availability from food items, metabolism of the lignanolactones in man and the health effects.

Research work in the lignan field is active and new findings concerning the metabolism and health effects are published continuously.

1.1. Structure and nomenclature of lignans

The name lignane, which was later shortened to lignan, was first used by Haworth in 1936. The name covers a large group of natural products where two C6C3 units are joined via the carbons 8 and 8’. Lignans can be further divided into subclasses: eg. furofurans, furans, dibenzylbutanes, dibenzylbutyrolactones, arylnaphthalenes, aryltetralins and dibenzocyclooctadienes (Figure 1).2,3,4 If coupling appears in some other way in the molecule (8-3’, 8-5, 3-O-4’ etc) compounds are referred to as neolignans (Figure 2.).

\[
\text{Furofurans} \quad \text{Furans} \quad \text{Dibenzylbutanes} \quad \text{Dibenzylbutyrolactones} \\
\text{Arylnaphthalenes} \quad \text{Aryltetralins} \quad \text{Dibenzocyclooctadiene}
\]

*Figure 1. Examples of lignan structures.*

2
The naming of lignans is variable (Table 1). Most lignans have trivial names such as enterolactone 1 (ENL), secoisolariciresinol 2 (SECO) or matairesinol 3 (MAT) that are frequently used. Besides trivial names semi-systematic names are also commonly used. In semi-systematic naming the lignanolactone skeleton is referred to as butyrolactone. Also the numbering of the lignane skeleton carbons has been variable (see Table 1).

In IUPAC names the numbering of the lignan skeleton starts from ring A and continues toward the C3 unit (see Table 1). The B ring is denoted with primed numbers (Table 1). The basic skeleton, where the bond between C8-C8′ attaches two C6C3 units, is considered as lignan. The lignan diols, such as enterodiol 4 (END), are indicated by the suffix -ol. The positions of the hydroxy groups and the multiplying affixes are placed before the suffix. The lignan lactones are named using the word ‘lignano’ followed by the locant of the oxygen in the lactone ring (9,9′ for example) and the ending lactone. Substituents are indicated as prefixes. In case of the labelled compounds the positions of the labels are first marked in brackets followed by the type of the label and the number of the labels as subscript. More complicated structures are not taken for consideration in this thesis.

Figure 2. Examples of neolignans.3
**Table 1.** Selected examples of the lignan nomenclature and structures used in this work (carbon atom numbering according to IUPAC rules).

<table>
<thead>
<tr>
<th>Trivial name</th>
<th>IUPAC name</th>
<th>Semi-systematic name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterolactone 1 (ENL)</td>
<td>3,3´-Dihydroxylignano-9,9´-lactone</td>
<td>2,3-<em>bis</em>(3-hydroxybenzyl)–γ-butyrolactone or 3,4-<em>bis</em>(3-hydroxybenzyl)–γ-butyrolactone</td>
</tr>
<tr>
<td>Enterodiol 4 (END)</td>
<td>Lignane-3,3´,9,9´-tetraol</td>
<td>2,3-<em>bis</em>(3-hydroxybenzyl)–γ-butane-1,4-diol</td>
</tr>
<tr>
<td><em>Trans</em>-5´-Carboxymethoxy-enterolactone 133</td>
<td><em>Trans</em>-5´-Carboxymethoxy-3,3´-dihydroxylignano-9,9´-lactone</td>
<td>(±-<em>trans</em>-2-(3-Hydroxy-5-carboxymethoxybenzyl)-3-(3´-hydroxybenzyl)butyrolactone</td>
</tr>
<tr>
<td>D8-Enterolactone</td>
<td>[2,4,5,6,2´,4´,5´,6´]-H8-3,3´-Dihydroxylignano-9,9´-lactone</td>
<td>D8-2,3-<em>bis</em>(3-hydroxybenzyl)–γ-butyrolactone</td>
</tr>
</tbody>
</table>

1.2. Biosynthesis of lignans

Most often plant lignans exist in plants in an enantiomerically pure form. These enantiomers are formed through biosynthetic pathways, which may be distinct in different plant species. In case of *Forsythia intermedia* the biosynthetic pathway of lignans has been studied since 1990. The proposed biosynthetic pathway of (+)-MAT 3 is a consequence of enzymatic activity. The precursor of the plant lignan MAT is formed through the shikimate pathway forming phenylalanine 5 followed by the phenylpropanoid pathway to afford coniferyl alcohol 6 (Scheme 1). Stereoselective coupling of two coniferyl alcohol molecules provides (+)-pinoresinol 7 (PIN) followed by sequential conversion to (+)-lariciresinol 8 (LAR), (-)-SECO 2 and (-)-MAT 3 (Scheme 2).
Scheme 1. The phenylpropanoid pathway.\textsuperscript{18,19}

Scheme 2. The biochemical pathway to (-)-MAT \textsuperscript{3} in Forsythia intermedia.\textsuperscript{9,18}
1.3. Lignans in plants and in food

Lignans are widely found in nature. Plant lignans have been found in all parts of the plant and the amounts vary in different plants and different parts of the plants. Lignans exist in plants as sugar conjugates, but also to some extent as oligomers or as free compounds. For example, the heartwood of Norway spruce is a rich source of 7’-hydroxymatairesinol (HMAT) in the free form. The biological role of the plant lignans is not fully known but they are thought to participate in the defence mechanism of the plant as antifungicides, antimicrobials, antioxidants, insecticides, nematocides etc. For example, in spruce (Picea abies) the fungus Fomes annosus enhances the formation of MAT and HMAT which restrict the growth rate of the fungus. Another examples are MAT and bursehernin, isolated from the plant Bupleurum salicifolium, which have been shown to inhibit the hatching of the nematode Globodera pallida. Antioxidant properties have been detected eg. of sesamolin, sesamin (SES), sesamolinol from sesamin (Sesamum indicum), nordihydroguaiaretic acid from creosote bush (Larrea tridentata) and actaealactone from black cohosh (Actaea racemose).

Plant lignans can be found in various dietary plants (Table 2). Lignan contents of several food items have been assembled to food composition databases which are used to estimate lignan exposure in population-based studies. The most studied plant lignans have been SECO and MAT because they were assumed to be the precursors that could be converted to mammalian lignans ENL and END in the human body. However more recent studies have shown that also other plant lignans were converted to mammalian lignans in the gut (more details in section 1.4). Therefore, intense investigations arose to analyse all PIN, LAR, medioresinol (MED) and syringaresinol (SYR) in food. The best-known dietary source for lignans is so far flaxseed that contains SECO over 300 000 μg in 100g of flaxseeds. Other good sources for lignans are sesame seeds, cereals and whole grain products (especially rye), nuts, berries and also certain vegetables and fruits (Table 2). In the Finnish diet, most important lignan sources are whole grain products, especially rye, and berries. The average intake of plant lignans (MAT and SECO) in the Finnish population is 434 μg/day. In a Dutch population study the intake of plant lignans MAT and SECO
was similar to the Finnish population study. The total intake of plant lignans (MAT 3, SECO 2, PIN 7 and LAR 8) was determined as high as 1241 μg/day in this Dutch study.\textsuperscript{35}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Examples of dietary plant lignans.}
\end{figure}

A comparison of the amounts of the plant lignans reported in different research articles is sometimes very difficult. Earlier papers concern only the total amount of MAT 3 and SECO 2 in plants but since 2001 PIN 7, LAR 8, syringaresinol 12 (SYR), arctigenin 13 (ARC) and HMAT 9 were proven to be also precursors of mammalian lignans.\textsuperscript{34} Therefore the lignan intake has been underestimated earlier.\textsuperscript{35} On the other hand, the analytical methods and the prehandling methods are continuously under development and the results are becoming more reliable. The treatment methods may have an influence on the analytes in the sample. For example under acidic conditions some SECO may convert to anhydroSECO 14 (ASECO) while LAR 8 converts to isoLAR 15.\textsuperscript{36,27} In addition, the food samples may be used in different forms – the analysed food items might be dried or fresh.
Furthermore, the environmental factors such as farming and climate play a role in the concentration of the lignans in the plant.\textsuperscript{37}

### Table 2. Plant lignan contents in selected food items (\(\mu g/100g\) or mg/kg\(^a\)).

<table>
<thead>
<tr>
<th>FOOD ITEM</th>
<th>SECO</th>
<th>MAT 3</th>
<th>PIN 7</th>
<th>LAR 8</th>
<th>MED 11</th>
<th>SYR 12</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flaxseed</td>
<td>323670</td>
<td>5202</td>
<td>2460</td>
<td>3670</td>
<td>0</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>375322</td>
<td>153</td>
<td>730</td>
<td>2808</td>
<td>-</td>
<td>-</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>0.21\textsuperscript{a}</td>
<td>0.45\textsuperscript{ad}</td>
<td>0.45\textsuperscript{ad}</td>
<td>3.38\textsuperscript{a}</td>
<td>0.09\textsuperscript{a}</td>
<td>2.15\textsuperscript{a}</td>
<td>38</td>
</tr>
<tr>
<td>Sesame seeds</td>
<td>66</td>
<td>481</td>
<td>29331</td>
<td>9470</td>
<td>-</td>
<td>-</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>123</td>
<td>6815</td>
<td>1052</td>
<td>-</td>
<td>-</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>1137</td>
<td>47136</td>
<td>13060</td>
<td>4153</td>
<td>205</td>
<td>28\textsuperscript{c}</td>
</tr>
<tr>
<td></td>
<td>0.21\textsuperscript{a}</td>
<td>17.46\textsuperscript{ad}</td>
<td>17.46\textsuperscript{ad}</td>
<td>2.74\textsuperscript{a}</td>
<td>7.55\textsuperscript{a}</td>
<td>4.73\textsuperscript{a}</td>
<td>38</td>
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<tr>
<td>Rye bread</td>
<td>13</td>
<td>14</td>
<td>172</td>
<td>122</td>
<td>-</td>
<td>-</td>
<td>33</td>
</tr>
<tr>
<td>Rye bran</td>
<td>462</td>
<td>729</td>
<td>1547</td>
<td>1503</td>
<td>858</td>
<td>3540</td>
<td>28\textsuperscript{c}</td>
</tr>
<tr>
<td>Wheat bread (whole grain)</td>
<td>15</td>
<td>0</td>
<td>33</td>
<td>73</td>
<td>-</td>
<td>-</td>
<td>33</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>868</td>
<td>410</td>
<td>138</td>
<td>672</td>
<td>232</td>
<td>882</td>
<td>28\textsuperscript{c}</td>
</tr>
<tr>
<td>Cashew nuts</td>
<td>133</td>
<td>0</td>
<td>0</td>
<td>496</td>
<td>-</td>
<td>-</td>
<td>33\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td>316</td>
<td>55</td>
<td>19</td>
<td>307</td>
<td>-</td>
<td>26</td>
<td>28\textsuperscript{c}</td>
</tr>
<tr>
<td>Salad ru cola</td>
<td>106</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>Blackberry (dried)</td>
<td>3718</td>
<td>23</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>26</td>
</tr>
<tr>
<td>Broccoli</td>
<td>38</td>
<td>0</td>
<td>315</td>
<td>972</td>
<td>-</td>
<td>-</td>
<td>33</td>
</tr>
<tr>
<td>Garlic</td>
<td>42</td>
<td>5</td>
<td>482</td>
<td>54</td>
<td>-</td>
<td>-</td>
<td>31</td>
</tr>
<tr>
<td>Asparagus</td>
<td>743</td>
<td>14</td>
<td>122</td>
<td>92</td>
<td>3</td>
<td>58</td>
<td>39</td>
</tr>
<tr>
<td>Apricot</td>
<td>31</td>
<td>0</td>
<td>105</td>
<td>314</td>
<td>-</td>
<td>-</td>
<td>33</td>
</tr>
<tr>
<td>Lemon</td>
<td>4</td>
<td>0</td>
<td>185</td>
<td>25</td>
<td>64</td>
<td>57</td>
<td>27</td>
</tr>
<tr>
<td>Olive oil</td>
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<td>0</td>
<td>243</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>33</td>
</tr>
<tr>
<td>Black tea (Ceylon)</td>
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<td>1</td>
<td>41</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>33</td>
</tr>
<tr>
<td>Red wine</td>
<td>29</td>
<td>0</td>
<td>0.4</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>31</td>
</tr>
</tbody>
</table>

\textsuperscript{b} \(\mu g/100\,g\) fresh edible weight
\textsuperscript{c} \(\mu g/100\,g\) concentrations of lignans in extracts
\textsuperscript{d} MAT/ Pin is reported as combined content
1.4. Metabolism

In early 1980, the first enterolignans or mammalian lignans ENL 1 and END 4 were identified from human urine.\textsuperscript{40,41} These enterolignans were found to be metabolites of the plant lignans MAT 3 and SECO 2.\textsuperscript{42,43} Heinonen \textit{et al.} discovered in a human fecal incubation study that PIN 7, LAR 8, SYR 12, ARC 13, HMAT\textsuperscript{34} 9 and SES\textsuperscript{44} 10 may also convert to mammalian lignans. Later Jin \textit{et al.} demonstrated in incubation studies that the furofuran-type asarin\textsuperscript{45} and trachelogenin\textsuperscript{46} are also precursors of ENL 1.

Besides new precursors also several new minor metabolites of plant lignans have been found due to the development of detection methods such as GC-MS. The first minor metabolites, found in human urine, were 7'-hydroxyenterolactone (HENL) and enterofuran (ENF)\textsuperscript{34}. Later, several other minor metabolites, such as HMAT\textsuperscript{34} 9, series of ENL 1 and END 4 oxidative metabolites\textsuperscript{47,48,49} and metabolites of SYR\textsuperscript{34} 12 and SES 10 were found in \textit{in vitro} and \textit{in vivo} studies.\textsuperscript{50,51,52} The significance and the biological activity of these new metabolites is not known. As these minor metabolites exist in very low concentrations in man, authentic reference compounds are required for the urine and plasma sample analyses.

\textbf{Scheme 3.} Proposed metabolic pathway from an \textit{in vitro} study by Heinonen \textit{et al.}\textsuperscript{34}
Intensive studies are going on regarding the metabolism and metabolic pathways of plant lignans because of the possible health effects. Several intestinal bacterial strains have been identified taking part by converting the plant lignans. Wang et al. and later Clavel et al. identified from human faeces several bacterial species that take part in the conversion of SECO-diglucoside and also PIN, LAR and MAT to enterolignans. So far, it is known that consumed MAT and SECO glycosides are first hydrolysed by human intestinal microflora to release the corresponding aglycones. Bacterial strains, such as Clostridium sp., Bacteroides fragilis and Bacteroides distasonis have been identified to participate in the deglycosidation forming SECO. These aglycones are further biotransformed by intestinal bacteria to END in the colon via demethylation and dehydroxylation. Several bacterial strains have been shown to participate in the biotransformation step such as Butyribacterium methylotrophicum, Eubacterium callanderi and Eubacterium limosum. Demethylation and dehydroxylation have been observed to occur for example to SECO, MAT, LAR, PIN, SES and ARC. Many of the biotransformations were found to occur in a stereoselective manner.

After aglycone formation and absorption in the intestinal track, glucuronic and to a lesser extent sulfuric acid derivatives of enterolignans are mainly formed in liver. This phase II metabolic product formation (glucuronic and sulfuric acid derivatives) may take place to some extent already during the uptake in the colon in the epithelial cells according to a study by Jansen et al. Most likely enterolignans are taking part in the hepatic circulation. In plasma, ENL, END and MAT are excreted as glucuronide or sulfate conjugates and as free compounds, in urine mainly as monoglucuronides, and in faeces mostly in the free form. Setchell et al. observed in a human dose response study that the plasma SECO concentration peak occurs 7.2 ± 1.0 h after SECO diglucoside intake. Furthermore the END concentration peak in serum was observed after 19.2 ± 2.5 h and the ENL concentration peak after 26.7 ± 2.3 h.

The metabolic action is not complete in the human body and therefore certain plant lignans such as MAT, LAR, isoLAR, SECO, SYR and PIN have been detected from human urine. In human intestinal Caco-2 cell studies it was shown that plant lignans such as PIN and SECO might be absorbed also from the small intestine.
The ENL plasma concentrations among European adults (Denmark, France, Germany, Greece, Italy, Spain, Sweden, The Netherlands and UK) have been studied by Peeters et al. The geometric mean of plasma ENL was found to be 9.0 nmol/L, and the median was correspondingly 10.6 nmol/L. Highest concentration values were in Denmark (27 nmol/L, geometric mean). Bolvig et al. measured ENL plasma concentration from 2237 Danes and found the median for women to be 21.3 nmol/L and for men 18.6 nmol/L. In a Finnish population study the median serum ENL concentrations were found to be 13.8 nmol/L in men and 16.6 nmol/L in women. The variation of ENL concentrations between individuals was huge. The range in men was 0-95.6 nmol/L and in women 0-182.6 nmol/L. Knudsen et al. analysed 3956 human plasma samples (Danish epidemiological study) with a direct quantitation method of ENL-glucuronide, ENL-sulfide and free ENL. They measured ENL-glucuronide concentration 0.2-650 nM (mean 28.5 nM) for the major component, ENL-sulfide 0-30 nM (mean 1.3 nM) and free ENL only in a few samples.

The intake of lignan rich food explains partly the variation of ENL and also END concentrations in plasma but not totally. It is thought that variations in the intestinal microflora are affect strongly the ENL and END concentrations. Thus for example, antibiotics drastically decrease the plasma ENL concentration values and the effect can be seen up to 12-16 months. The antibiotic effect was found to be stronger for women than for men. Recently Bolvig et al. demonstrated in a pig study the correlation of dietary plant lignan concentration and plant lignan and enterolignan concentrations in plasma and in urine. They were also able to identify the direct link between antibiotic use and lower ENL concentration. Also age, sex, body weight, smoking, intake of dietary fibre, transit time, alcohol, caffeine and the frequency of defecation are correlated to the ENL concentrations but they do not explain the entire variation. On the whole, there are several and complex issues that have an effect on the colonic environment and further affect the ENL and END concentrations in man.
1.5. Health effects

The interest in lignans has risen because of the possible health effects. Diseases such as breast cancer, prostate cancer, colon cancer, cardiovascular diseases and also diabetes are associated strongly with diet. There are also large regional differences in mortality rates. Several review articles have been written about the subject and these have been summarised briefly.

Enterolignans are assumed to have an effect on hormone dependent cancers such as breast cancer and prostate cancer. The breast cancer risk has been shown to correlate with a low concentration of plasma ENL in some studies. One possibility for this phenomenon is connected to the enterolignan structure that is similar to that of estradiol (17-β-estradiol). Enterolignans might block receptor sites against estrogen and have an effect on the hormonal balance in the human body and therefore prevent hormone dependent cancers. Several studies have attempted to clarify the role of lignans and the possible mechanism of the action: eg. ENL inhibition to 5α-reductase, lignan inhibition to aromatase, plasma ENL correlation to the SHBG (sex hormone-binding clobulin) level, the binding of ENL to the estrogen receptor (ERα and ERβ), lignan bindings to the estradiol nuclear binding type II, and ENL and END effects on the MCF-7 breast cancer cell proliferation. Recently in a breast cancer cell line study (ER positive and ER negative) ENL was found to enhance radiosensitivity. Therefore ENL might be a potential candidate for the combined therapy to decrease the radiation doses in the breast cancer patient treatments.

ENL have shown to inhibit prostate cancer growth and development. The mechanism for the phenomenon is not known. On the other hand, contradictory results have been published. Olsen et al. found out that the mortality of Danish men with prostate cancer and ENL plasma concentrations were not related.

In case of cardiovascular diseases in man, whole-grain cereals have shown to have a protective role. In several studies, high fiber intake has been associated with a lower coronary heart disease risk factor. Lower coronary heart disease risk and cardiovascular disease risk has also been associated with a high ENL serum concentration. Beside risk factors lignan rich fiber may slightly reduce total cholesterol in man.
Anti-inflammatory properties have been associated with a possible cardioprotective role. In a cell line study HMAT 9 has been shown to exhibit anti-inflammatory activity in human aortic endothelial cells.\textsuperscript{96} In a human study, concerning over 4000 participants, urinary ENL 1 and END 4 concentrations were shown to have a significant inverse relation to CRP (C-reactive protein) and WBC (white blood cells).\textsuperscript{97} The result may suggest a possible inverse relation between the intake of enterolignans and inflammation.

A similar situation is seen in colorectal cancer where fiber rich food is associated with a protective effect against colorectal cancer.\textsuperscript{81} Colon cancer cell (SW480) treatment with ENL 1 and END 4 resulted in a decrease of cell numbers. On the whole, the protective mechanism of lignan rich fiber is not very well known but lignans may participate in the protection.\textsuperscript{88}

The most recent findings of the effect of enterolignan and plant lignans are from the lung cancer field. High concentrations of ENL 1 have been found to reduce the size and number of the lung cancer cells in a cell line study.\textsuperscript{96} In an asbestos-exposed mice study flaxseed diet was found to reduce the acute inflammatory response.\textsuperscript{99}

Diabetes and body weight are also associated with lignans. In an investigation among US women higher urinary ENL 1 and END 4 concentrations were reported to have significant association with a lower risk of development of type 2 diabetes.\textsuperscript{100} Also among US women Hu \textit{et al}. reported the higher ENL 1 urinary concentration with a low body mass correlation.\textsuperscript{101} In the case of men, the association seems to be different. Xu \textit{et al}. reported that among US men obesity was strongly associated with the highest concentrations of urinary ENL 1 in the study, but in addition, a high concentration of ENL 1 was linked to a low risk of the metabolic syndrome.\textsuperscript{102}

Many plant lignans and enterolignans act as antioxidants.\textsuperscript{103,38,104} One of the newest findings is that due to this effect some lignans might have an inhibitory effect on the Alzheimer disease\textsuperscript{105}. Also ARC 13 and MAT 3 have been reported to act as possible anti-aging agents because of the antioxidant effect.\textsuperscript{106}

The results of these studies are not always clear and some similar studies show controversial results, possibly due to the heterogeneity of the studies. The variability of the methods to determine lignan intake or lignan concentration in man (serum, plasma, urine) and the diversity of polyphenols might lead to the inconsistent results. Also the
study designs of the anticancer effects are varied and for example the cancer types, cell lines and doses are not always comparable.

On the whole, additional and more precise studies should be done in order to evaluate the evidence to the effect of lignans eg. on cancer risk\textsuperscript{107} Therefore, the true effects of lignans still remain unclear.

1.6. Analytical methods

Recently several review articles\textsuperscript{108,109,110,111,112,113} have appeared concerning the methods for analysing lignans from plants and from biological samples. There may be difficulties for detecting lignans from food and biological samples because of low concentrations. Lignans occur mostly in conjugated forms; in food as glycosides and in the most often analysed biological fluids urine and blood as glucuronides or sulfates. The plant lignan samples are most often treated with acid or enzymatically hydrolysed to get the free lignan. From biological fluids, lignan glucuronides or sulfates are usually enzymatically hydrolysed using β-glucuronidase/sulfatase. Thus, requirements for the analytical methods used are demanding. The detection limits should be low and separation capacity high to get reliable results. Reference molecules are widely used for taking into account the losses of analytes during prehandling procedures.
<table>
<thead>
<tr>
<th>Matrix</th>
<th>Method</th>
<th>Analyte</th>
<th>Limit of detection</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human plasma</td>
<td>HPLC-MS-MS</td>
<td>HMR, HENL, Oxo-MAT, MAT, END, ENL</td>
<td>0.13-267 pg/mL</td>
<td>114</td>
</tr>
<tr>
<td>Rats urine</td>
<td>HPLC-MS-MS</td>
<td>HMR, HENL, LAR CLAR, SECO, MAT, END, ENL</td>
<td>0.040-2.02 nM</td>
<td>115</td>
</tr>
<tr>
<td>Food</td>
<td>HPLC-MS-MS</td>
<td>SECO, MAT, SES, PIN, LAR, HMAT, MED, Oxo-MAT, SYR</td>
<td>Not mentioned</td>
<td>28</td>
</tr>
<tr>
<td>Plasma Urine</td>
<td>LC-MS/MS</td>
<td>ENL, END, MAT, SECO, LAR, isoLAR</td>
<td>0.024-100 ng/mL (quantitation limit)</td>
<td>78</td>
</tr>
<tr>
<td>Plasma</td>
<td>LC-MS/MS</td>
<td>ENL glucuronide</td>
<td>26 pM</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ENL sulfat</td>
<td>16 pM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ENL</td>
<td>86 pM</td>
<td></td>
</tr>
<tr>
<td>Plasma Milk Mammary gland</td>
<td>UPLC-QTOF-MS</td>
<td>END glucuronide, ENL glucuronide, SECO glucuronide</td>
<td>END 0.13 nM, ENL 0.05 nM, SECO 2.80 nM</td>
<td>117</td>
</tr>
<tr>
<td>Food (seeds)</td>
<td>UHPLC-ESI-QTOF-MS</td>
<td>MAT, MED, SYR, LAR, ARC, PIN/MAT</td>
<td>0.05 mg/kg (from seeds)</td>
<td>38</td>
</tr>
</tbody>
</table>
1.6.1. GC-MS

GC-MS has been the most popular analytical method for lignans in recent years. The first analysing methods for ENL 1 and END 4 from urine were GC\(^40\) and GC-MS.\(^41\) These methods have been most popular even though much pre-handling and many derivatisations are required. Lignan samples are usually hydrolysed enzymatically using β-glucuronidase/sulfatase or by acidic treatment followed by the purification of the sample most often by solid phase extraction. Because most of the analysed lignans contain one or more hydroxyl groups derivatisation is required to get more volatile analytes for GC. In most cases lignans are silylated to form volatile derivatives.

In quantitative analysis, internal standards are used to correct for the losses during sample pre-treatment.

Adlercreutz et al. introduced the ID-GC-MS-SIM (isotope dilution GC-MS selected-ion–monitoring) method to determine lignans in human urine, plasma, feces, and also in food samples to determine MAT 3, SECO 2 and ASECO 14.\(^36,118,119,120\) This method can be used to determine even a low concentration of a lignan. Morton et al. have presented an ID-GC-MS method for the detection of ENL 1 and END 4 from plasma and food samples.\(^121\) They used β–glucuronidase/aryl sulphatase (Helix pomatia) for the hydrolysis of conjugated lignans, a column of Sephadex LH-20 for the purification and reaction with BSTFA to form trimethylsilyl derivatives of ENL 1 and END 4 as pre-treatment of the samples prior to GC.

1.6.2. HPLC/LC

The popularity of the HPLC analytical methods for lignans has risen and the methods to analyse and detect lignans from urine and blood samples have improved. The prehandling extractions may have an influence on the results especially for plant samples and therefore it is important to plan what lignans will be analysed and which prehandling methods are suitable for that purpose. For example, basic and acidic conditions may cause HMAT 9 to undergo several reactions.\(^122,123\) The pH used may lead to the reactions of HMAT 9, while under mildly basic aqueous conditions some epimerisation occurs yielding mainly allo-HMR 16 and HMAT 9. More strongly basic conditions (pH 10) lead to intramolecular nucleophilic addition followed by lactone ring
opening yielding mainly α-conidendric acid 17 and β–conidendric acid 18. Also HMAT 9 may transform into isoHMAT 19. Scheme 4 shows some examples of the transformations under alkaline conditions.

Scheme 4. Reactions of HMAT 9 under basic conditions.¹²²

The reversed-phase columns are widely used for the analysis of lignans because many lignans have polar functional groups. Several detecting methods have been used in the lignan analyses such as HPLC-UV, HPLC-DAD/PAD, HPLC-CEAD, HPLC-MS and HPLC-MS-MS.

Horn-Ross¹²⁴ et al. used HPLC for determing ENL 1 and END 4 in human urine. Lignans were hydrolysed by glucuronidase and sulfatase followed by solid-phase extraction with C¹₈ Sep Pak of the urine sample and by HPLC (reversed –phase)-MS analysis.

Smeds et al. have developed and validated an HPLC-MS-MS method to detect HMAT and its potential metabolites from human plasma. The limits of detections were found HMAT I 9 (8.0 pg), HENL (0.27 pg), 7-oxo-MAT 20 (0.37 pg), α-conidendrin (0.37 pg), MAT 3 (0.63 pg), END 4 (0.08 pg), ENL 1 (0.004 pg).¹¹⁴,¹²⁵,¹¹⁵
Knudsen et al. have validated an LC-MS/MS method for the quantitation of MAT 3, HMAT 9, SECO 2, LAR 8, isoLAR 15, SYR 12, MED 11, PIN 7, END 4 and ENL 1 in both human and pig plasma and urine. The method allowed the quantitation of lignans in the range 0.024-100 ng/mL and with run time of 4.8 min per sample. Furthermore ENL-glucuronide, ENL-sulfate and free ENL 1 LC-MS/MS methods were developed and validated according to FDA guidelines. The human plasma samples were prepared by solid phase extraction before analysis. The run time was 2.6 min.

The UHPLC-ESI-QTOF-MS (ultra-high performance liquid chromatography - electrospray ionisation - quadrupole time-of-flight- mass spectrometry) method has been used to determine the lignan profile in five different dry seeds: sunflower, flaxseed, poppy, sesame and pumpkin seeds. Separation for UHPL was achieved with a reverse-phase column using gradient elution and MS was run in the positive mode. Detectable amounts from seeds were over 0.05 mg/kg. Also Jansen et al. used LC/QTOF MS/MS for the analysis of glucuronide and sulfate conjugated ENL and glucuronide conjugated END (in vivo study). They used also a reverse-phase column with gradient elution and MS was run in the negative mode.

HPLC with coulometric electrode array detection (CEAD) was used by Gamache et al. for the detection of ENL 1 and END 4 from plasma, tissue and urine. The limit of detection was 1.32 ng/mL for both ENL 1 and END 4. Also, Nurmi et al. used coulometric array detection for a wider range of lignans: SECO 2, MAT 3, ASECO 14, END 4, ENL 1, isoLAR 15, LAR 8, SYR 12, PIN 7, HMAT I and II 9 and MED 11. The method was validated and the limits of detection were 0.71-1.55 nmol/L.

LC-PDA-MS (photodiode array) has been used by Franke et al. for the detection of ENL and END from human urine and plasma. The method was validated and recoveries and limits of quantitation from urine and plasma were determined. Limits of quantitation for urine were: ENL 1 11 pg, END 4 13 pg, plasma: ENL 1 3 pg, END 4 11 pg.
1.6.3. MS fragmentations

Mass spectrometry is widely used in lignan analysis. As seen from previous chapters, MS is combined in many cases with GC and LC analysis. The MS fragmentations of several whole-grain lignans such as MAT, LAR, PIN, HMAT, hydroxyMED and SECO have been recently presented in a review article concerning the MS based analysis of phytochemicals.\textsuperscript{130}

Eklund \textit{et al.} used ESI-ion-trap for the fragmentation study of plant lignans and mammalian lignans.\textsuperscript{131} Several structures of butyrolactones, butanediols and furofuran type lignans were analysed and possible fragmentation pathways were proposed. Phenolic and non-phenolic structures were included in the study and also positive and negative ion modes. Similar results were observed by Hanhineva \textit{et al.} in the fragmentation of MAT, SECO, PIN and LAR using ESI (-) MS.\textsuperscript{132}

In ESI(-) characteristic main fragments for the butyrolactone lignans were the ions [M-H-44]\textsuperscript{-}, probably due to the loss of CO\textsubscript{2} and the formation of ions [M-H-15]\textsuperscript{-} which are probably due to the loss of CH\textsubscript{3} (Scheme 5). Several other ions were found depending of the molecular structure.

The main fragment in the negative ion mode from the butanediol lignans was found to be [M-H-48]\textsuperscript{-} which could be due to the loss of formaldehyde and water. Other fragmentations due the β-cleavage, loss of CH\textsubscript{3} and loss of CH\textsubscript{2}O (Scheme 6) were also proposed.

\begin{center}
\textbf{Scheme 5. MS ESI(-), observed fragmentations of phenolic butyrolactones.}\textsuperscript{131}
\end{center}
1.6.4. TR-FIA

Time-resolved fluoroimmunoassay (TR-FIA) has been developed for fast screening of ENL \( \text{1} \) from several plasma and urine samples.\textsuperscript{133,134} It is a useful method especially for epidemiological studies. In this method 5-carboxymethoxyenterolactone \( \text{21} \) is coupled to BSA (bovine serum albumin) and used as an antigen in the immunisation of rabbits and formation of antiserum. 5-CarboxymethoxyENL \( \text{21} \) was also labelled with europium chelate to form a tracer for analysis. Before analysis urine or plasma samples are treated with \( \beta \)-glucuronidase/sulfatase to hydrolyse conjugated ENL to the aglycone and extracted with ether. Analytical plates were coated with diluted antiserum and pre-treated samples added followed by the addition of a tracer. ENL \( \text{1} \) in the sample is first attached to antigens. After that the tracer is attached to the free antigens and the fluorescence is measured and compared to a calibration curve. The lesser amount of fluorescence is correlated to a higher amount of ENL \( \text{1} \) in the sample. Working range for ENL \( \text{1} \) was established to 1.5-540 nmol/L.
1.6.5. ELISA

Enzyme-linked immunosorbent assay (ELISA) method was developed for podophyllotoxin by Porter et al. (1993) for the rapid analysis of podophyllotoxin in plants.\(^{135}\) They used an ‘antibody-sandwich’ ELISA approach. The hapten was formed by treating podophyllotoxin with succinic anhydride. The formed hapten was further coupled with BSA (bovine serum albumin) or KLH (keyhole limpet hemocyanin) to get the antigen. The antigens were injected to rabbits to form antibodies, that were isolated from serum. Antibodies were used for coating the ELISA plates. The plates were treated with a standard or sample followed by diluted antibody and antibody-enzyme conjugate treatments and finally with \(p\)-nitrophenyl phosphate substrate. The amount of hydrolysed phosphate substrate, which is equal to the bound antibody, was measured with a plate reader.

An ELISA method has been developed by Attoumbre et al. in order to quantitate the amount of SECO 2 from plants.\(^{136}\) \textit{Para}-aminohippuric acid was used for hapten formation and then coupled with BSA. Indirect ELISA technique was used for analysing SECO 2 from different parts of flaxseed.

Shrinkaruk et al. have developed and validated a competitive ELISA method for the ENL 1 assay of mice plasma.\(^{137}\) ENL haptens, 5-carboxymethoxyenterolactone 21 and 5-carboxypropoxyenterolactone 22 (page 39), were coupled with BSA in order to obtain the antigen and furthermore the antibodies.

1.6.6. Requirements for labelled internal standards in quantitative analysis

When lignans are analysed from food/plant samples or from human body fluids prehandling methods are most often required. Prehandling methods may cause losses of the lignans from the sample, leading to problems in quantitative analyses. In quantitative analysis, it is essential to be able to take into account the losses of the analytes in the sample. Reliable results are achieved by using internal standards. The most suitable standards are reference molecules that have same retention time in GC or in HPLC but have a different molecular mass. Carbon and silicon have fairly abundant M\(^{+1}\) and M\(^{+2}\) isotopes and therefore especially in GC methods where silylated derivatives are often used the M\(^+\) (m/z) value should differ by at least three units from the analyte to
avoid interference. Additionally, no unlabelled species should be present and the standards should be stable and withstand the prehandling methods used. The most often used labels in lignan analyses have been $^2\text{H}$ and $^{13}\text{C}$. Labelling methods are discussed in more detail in section 2.3.

2. Synthesis

2.1 Synthesis of lignans

The latest development area in the synthesis of lignans is asymmetric synthesis and there are several articles on the synthesis of pure diastereomeric or enantiomeric lignanolactones. There are reported methods for the preparation of cis-lignanolactones$^{138,139,140}$, several articles about optically active trans forms$^{141,142,143,144,145,146,147,148,149,150,151,152,153,154,155,156}$ and chemoenzymatic methods for 3- and 2-component.$^{157,158,159}$ Also HMAT 9, isolated from Norway spruce (Picea abies), has been used as a starting material for the asymmetric synthesis of (-)-MAT 3, (-)-ENL 1 among others.$^{160,161}$ There are also several methods for the synthesis of 2-component products via chiral succinic acid derivaties$^{162}$

This work concerns the synthesis of trans (±)-lignanolactones and therefore also the literature review of synthesis concerns these structures. Two most popular methods to synthesise lignanolactones and diols are the Stobbe condensation and tandem Michael addition alkylation reactions but also methods such as oxidative coupling, radical cyclisation and Baylis-Hillman reactions have been used.

2.1.1 Stobbe condensation

In Stobbe condensation a diethylsuccinate derivative reacts with an aldehyde under basic conditions. The lactone 23 formed by elimination is transformed to the carboxylate anion which can be alkylated to (±)-trans-lignanolactone 24 (Scheme 7).$^{163,164}$ Cooley et al. used a synthetic route where the last two steps were in reversed order, first alkylation, followed by reduction and (±)-trans-lignanolactone 25 formation.
Bambagiotti-Alberti et al. have used the Stobbe condensation as the first step to synthesise butyrolactone lignans with identical aromatic substituents (Scheme 8).\textsuperscript{166,167} Stobbe condensation afforded dibenzylidene succinic acid intermediates 26, 27 followed by reaction with H\textsubscript{2} over Ru\textsubscript{4}H\textsubscript{4}(CO)\textsubscript{8}(PBu\textsubscript{3})\textsubscript{4} catalyst to form the γ-lactone ring directly. The method gives circa 10:1 \textit{trans}:\textit{cis} ratio depending on the molecule’s structure 28-30. The \textit{cis} form can be transformed to \textit{trans} by treatment with methanolic potassium hydroxide solution.

\begin{align*}
\text{Scheme 7. Synthesis of lignanolactones by Brown \textit{et al.}, Loriet \textit{et al.} and Cooley \textit{et al.}}^{163,164,165}
\end{align*}

\begin{align*}
\text{Scheme 8. Synthesis of lignanolactones by Bambaggiotti-Alberti \textit{et al.}}^{166,167}
\end{align*}
2.1.2 Tandem Michael addition alkylation and aldolisation

Ziegler introduced the Michael addition reaction for the synthesis of (±)-trans-lignanolactones 31. In the Michael addition reaction 2-butenolide 32 reacts with an aryl dithioacetal enolate 33 formed in basic conditions. The base is usually LDA or n-BuLi and the reaction temperature is -78°C. The anion 34 formed is further reacted with an aryl halide 35 while HMPA or similarly acting reagents such as DMI or TMEM are added. The alkylation is performed in temperatures between -78°C - rt. Pelter et al. reported in 1981 a Michael addition reaction to ENL 1 skeleton. Instead of alkylation also aldolisation can be performed by using an aryl aldehyde 36 instead of an aryl halide to form 7-hydroxylignanolactones 37 (Scheme 9).

![Scheme 9. Tandem Michael addition alkylation and aldolization reactions](image)

After forming the skeleton of the lignanolactone, thio groups and other possible protection groups such as benzylic ethers are cleaved using Raney nickel. The formed lignanolactone 38 can be reduced further to a diol 39 (Scheme 10) and followed by treatment with percloric acid to form the anhydro 40. Another approach to cleave the thio groups from a lignan skeleton 31 is to use HgO-BF₃ which gives keto lactones 41. The keto group can be reduced to 7’-hydroxylignanolactone 42.
The 8-hydroxylignanolactone 44 structure can be formed by an oxidation reaction of molecule 43.\textsuperscript{174}

\[ \text{Scheme 10. Synthesis of butyrolactone lignan derivatives} \]

Instead of aryl dithio derivatives also \( O \)-silylated cyanohydrins 45 have been used for Michael addition reactions.\textsuperscript{175,176} The base used here was LDA and reaction temperature was \(-78^\circ\text{C}\). The 2-butenolide 32 and the aryl aldehyde 46 were added after 7h, whose treatment with \( t \)-butylammoniumfluoride gave a protected keto lactone 47 (Scheme 11). This method has also applications for controlling the stereochemistry at C-7 and C7’ position.\textsuperscript{176,177,178,179}

\[ \text{Scheme 11. Synthesis of keto lactone structure 47 (yields 61-94%).} \textsuperscript{175,176} \]
A different type of Michael addition alkylation has been used by Bella et al. affording predominantly trans lignanolactones such as (±)-hinokinin 48.\textsuperscript{180} The starting material 49 was first treated with acid followed by tandem Michael addition alkylation forming a lignanolactone with a trans-cis 50, 51 ratio of >10:1 in 70-45% yield. The final step was elimination of the phenylselenyl group with NiCl\textsubscript{2}/ NaBH\textsubscript{4} in THF affording mainly the trans isomer of lignanolactone 52 with 90-95% yield (Scheme 12).

Scheme 12. Synthesis of trans-lignanolactone 52.\textsuperscript{180}

### 2.1.3 Oxidative coupling

Oxidative coupling has been used by Belletiere et al. to prepare lignanolactone type molecules such as methylated ENL 53 and benzylated MAT 54.\textsuperscript{181,182,183} Starting materials are quite simple, for example m-methoxycinnamic acid 55 for ENL and cinnamic acid 56 for MAT. After reduction and in certain cases also after benzylation of a phenolic OH, LDA is used for dianion formation. Oxidative coupling occurs after treating the reaction mixture with I\textsubscript{2} followed by acidification forming structures 57, 58. Acetic anhydride is used for anhydride 59, 60 formation followed by conversation to the acid esters 61, 62 by methanol. After reduction and acidification protected trans-lignanolactones 53, 54 are formed.
Scheme 13. Oxidative coupling reaction for protected ENL 53 and MAT 54. Kamlage et al. have used palladium catalysed addition of tributylstannane to 2-butyne-1,4-diol 63 as the first step followed by TPAP-catalysed oxidation step to form lactone structures 64, 65. Stille coupling was performed with arylbromide followed by hydrogenation with Pd/C or Raney-Ni as catalyst and finally alkylation with arylbromide and LHMDS as base to form 66 (Scheme 14).

Scheme 14. Reaction conditions and yields of trans-lignanolactone 66. Radial cyclisation

A radical cyclisation approach was used by Srikrishna et al. to form butyrolactone products (Scheme 15). Cinnamyl alcohol 67 is converted to the bromoacetal
derivative 68 followed by cyclisation using tributyltin chloride, sodium cyanoborohydride and azobisisobutyronitrile (AIBN). The formed acetal 69 was treated with the Jones reagent to give the butyrolactone 70.

![Scheme 15. Reaction conditions and yields of 70.](image)

Belletire et al. used a similar approach, a radical cyclisation method to form trans-lignanolactones 71a and cis-lignanolactones 71b in 4:1 ratio with 46% overall yield.

![Scheme 16. Radical cyclisation for trans- and cis-lignanolactones 71a, 71b.](image)

Radical cyclization has been used also by Singh et al. for synthesising lignanolactones (Scheme 17). The first step is to prepare a mixture of substituted alkenylsilanol 72/disiloxane 73 followed by the Heck reaction and hydrolysis to form the racemic acid 74. The acid is converted to the phenylselenomethyl ester 75 and finally cyclised providing trans and cis lactones 76 in a 78/22 ratio. A different route is used for isomeric lignanolactones. The racemic acid is reduced to alcohol 77 and further converted to a phenylselenocarbonate 78. After radical cyclcation lignanolactone is formed trans and cis 79 forms (78/22).
2.1.5 Lignanolactones via Baylis-Hillman reaction

Lignan intermediates can be synthesised by the Baylis-Hillman method starting from an aromatic aldehyde 80 and suitable activated alkene 81. After the protection of the hydroxy group a cyanide group is attached to the double bond followed by the reduction of the ester group. The diastereoisomeric alcohols 82a, 82b formed are separated by column chromatography and hydrolysed followed by cyclisation to form the (±)-lactone 83 (Scheme 18).

Scheme 17. Radical cyclisation for trans- and cis-lignanolactones by Singh et al.\textsuperscript{187}

Scheme 18. Baylis-Hillman reaction.\textsuperscript{187,188}
2.1.6 Synthesis of haptens

In the literature, there is so far only few articles concerning the synthesis of lignanolactone haptens. Haptens are derivatived molecules which can be coupled with carrier protein. Mäkelä et al. have synthesised four ENL acid chain derivatives (haptens) with two different approaches.\(^{190}\) For 3-carboxymethylENL 84, 5-carboxymethoxyENL 21 and 4-carboxyethoxyENL 85 the lignan skeleton 86, 87, 88 was constructed using the tandem Michael addition alkylation. The silylic protection group was cleaved using tetrabutylammonium fluoride and the acid chain was attached to the lignan framework as ethyl ester (Scheme 19).

![Scheme 19. Synthesis of lignanolactone haptens 21, 84, 85.\(^{190}\)](image)

A different method was used for synthesis of 4-carboxyethylENL 89. The side chain was attached to the benzylic bromide 90 which was further used for the tandem Michael addition-alkylation reaction. The formed lignanolactone was oxidised to form the carboxylic acid chain (Scheme 20).
Shinkaruk et al. have synthesised 5-carboxypropoxyenterolactone \( \text{22} \) using 3-hydroxybenzaldehyde \( \text{91} \) and benzylic bromide \( \text{92} \) as the starting materials (Scheme 21).\(^{137} \) The Stobbe condensation was used to form the hydroxybenzylbutyrolactone intermediate \( \text{93} \) followed by the alkylation reaction with protected benzylic bromide. The TBDPS protection group was cleaved using tetrabutylammonium fluoride followed by attachment of the acid chain to the C-5 position under alkaline conditions. To form the 5-carboxypropoxyenterolactone \( \text{22} \) the benzylic protection groups were cleaved by catalytic hydrogenation followed by base catalysed ester hydrolyse. The yields of the reaction steps are presented in the Scheme 21.

**Scheme 20. Synthesis of lignanolactone hapten \( \text{89} \).**\(^{190} \)

**Scheme 21. Synthesis of 5-carboxypropoxyenterolactone \( \text{22} \).**\(^{137} \)
2.1.7 Isotopic labelling of lignanolactones

According to the literature, the most commonly used isotopic label for lignanolactone is $^2$H but some papers are also published showing $^{14}$C, $^{13}$C and $^3$H labels. $^{14}$C-labelling has been performed biosynthetically$^{11}$ by feeding labelled coniferyl alcohol 6 to plants forming $^{14}$C labelled MAT and SECO.$^{12}$ Total synthesis has been used for making $^{13}$C labelled ENL, END, MAT 94 and SECO. K$^{13}$CN was used as source of the label (Scheme 22).$^{191}$ Labelled $^{13}$C lignanolactones are laborious and very expensive to synthesise, especially because a minimum of three labels is needed to avoid interference of $^{12}$C and especially in GC methods silicon isotopes from the silylated derivative of the analyte.

Scheme 22. Total synthesis of $[7,9,7'\text{-}^{13}\text{C}]-\text{MAT}$ 94.$^{191}$

Tritiated SECO$^{14}$ has been prepared starting from coniferyl aldehyde using NaB$^3$H$_4$ for the reduction. The formed $[9\text{-}^{3}\text{H}]-\text{coniferyl alcohol}$ was converted by FeCl$_3$ to $[9,9'\text{-}$
\[ ^3\text{H}_2\]-PIN and hydrogenation over Pd/C to form [9,9'-\text{H}_2]-SECO. In addition [7',3\text{H}]-7'-hydroxymatairesinol has been synthesized by a tandem Michael addition-alkylation procedure (Scheme 9, 10). Tritium was introduced to the molecule with NaB\text{\textsuperscript{3}H}_4 reduction of the 4,4'-dibenzy1-7'-oxo-MAT.\textsuperscript{9}

Deuterium labels, available from the relatively inexpensive \textsuperscript{2}H or \textsuperscript{2}H\textsubscript{2}O, have been most commonly used for lignanolactones, \textsuperscript{2}H being attached either to the aliphatic\textsuperscript{192,166,167} region of the lignan framework or to the aromatic region.\textsuperscript{193,194,173}

Kirk et al prepared \textsuperscript{2}H\textsubscript{2} ENL \textsuperscript{95} by using dideuterated 2-butenolide \textsuperscript{96} as starting material in tandem Michael addition alkylation reaction procedure (Scheme 23). [9',9'-\text{H}_2]-ENL \textsuperscript{95} was formed through multistep synthesis with 49% overall yield.\textsuperscript{192} This compound was further reduced to [9',9'-\text{H}_2]-END \textsuperscript{97} or [9,9',9'-\text{H}_4]-END \textsuperscript{98} by using correspondingly LiAlH\textsubscript{4} or LiAl\textsubscript{2}H\textsubscript{4}.

Another route, used by Bambaggiotti et al, is used for preparing butyrolactone lignans with identical aromatic substituents (Scheme 24).\textsuperscript{166,167} Deuteration was performed using \textsuperscript{2}H\textsubscript{2} over Ru\textsubscript{3}H\textsubscript{4}(CO)\textsubscript{8}(PBu\textsubscript{3})\textsubscript{4} catalyst to form the \(\gamma\)-lactone ring for structures 99-101.
Scheme 24. Synthesis of $^2$H$_6$-lignanolactones.$^{166,167}$

All deuteration methods for aromatic ring of lignanolactones are based on H/$^2$H exchange reaction under acidic conditions (Scheme 25). Wähälä et al. refluxed ENL 1 in PBr$_3$/H$_2$O to give [2,4,6,2',4',6'-2H$_6$]-ENL 102 in 35% yield and with >90% isotopic purity.$^{194}$ Neidight et al. used $^2$HClO$_4$/H$_2$O to get [2,5,6,2',5',6'-2H$_6$]-MAT 103 in 84% yield.$^{193}$ [2,5,6,2',5',6'-2H$_6$]-MAT 103 has also been made by Rasku et al. using $^2$H$_3$PO$_4$·BF$_3$/H$_2$O as the reagent. $^2$H$_6$-MAT 103 was further reduced with LiAlD$_4$ to get [2,4,6,2',4',6'-2H$_8$]-SECO. The treatment with HClO$_4$/acetone furnished [2,5,6,2',5',6'-2H$_8$]-ASECO. Deuteriums in secoisolariciresinol were chemically stable and survived the perchloric acid treatment.$^{173}$ So far the most expedient deuteronlabelling method has been reported by Hakala et al.$^{195}$ MAT 3 was deuterated using $^2$HCl/2H$_2$O in ionic liquid [bmim]Cl and under microwave irritation for 40 minutes giving [2,5,6,2',5',6'-2H$_8$]-MAT 103 in 90% yield and with >90% isotopic purity. In our approach, we have used $^2$H$_3$PO$_4$·BF$_3$/H$_2$O method for deuteration of eight lignanolactone structures in publications of I and III giving high yields and high isotopic purity.
Scheme 25. Published methods for deuteration of unsubstituted positions of aromatic rings in lignanolactones.\textsuperscript{193,194,173,195}
3 Aims of the study

The study of metabolism and new possible metabolites of plant lignans is an active topic and new findings are continuously published. The field is however lacking certain reference molecules for these studies. We thus wanted to synthesise a number of lignanolactone reference molecules for metabolic studies using Michael addition-alkylation sequences. Many of the synthesised lignanolactones were new compounds, while some structures were previously lacking certain analytical data.

In case of the haptens the aim of the study was to synthesise several different haptens for the development of an ELISA method for analysing ENL 1. Another aim was to develop a short and efficient synthetic route to haptens. Based on our haptens an ELISA kit for ENL quantitation was developed and commercialized (by Wallac).

In analytical studies, fast quantitative methods for analysing lignan contents from biological samples are needed. In these methods, stable deuterated reference samples are required. We used deuteration methods for complete lignan structures, avoiding multistep synthetical routes. The aims of this study from the chemistry point of view were:

1. To synthesise several new lignanolactone and lignanodiol molecules as reference molecules for analytical and metabolic studies, and give complete analytical data of the molecules.
2. To establish synthetic paths for lignan haptens in order to find good immunogens for the development of the ELISA analytical method for enterolactone.
3. To develop a deuteration method for several lignanolactone molecules that can be used in quantitative analysis.
4. To investigate the orientation, the reactivity order and stability of deuterium labels in the lignan molecules and compare calculated ESP values with experimental results.
4 Results and discussion

4.1 Synthesis of lignanolactones and lignanodiols (II and III)

The total synthesis of lignanolactones was performed using the tandem Michael addition – alkylation reaction giving the (±)-trans lignanolactone skeleton in one step. The yields of the reactions were lower for ortho or meta substituted aromatic starting materials, especially when benzyloxy groups were present in both meta positions. The reason was most likely the steric hindrance of the benzyloxy groups. Improved yields were obtained using more concentrated reaction solutions.

After forming the lignane skeleton, Raney nickel was used for desulfurization and concomitant debenzylation to form the lignanolactones. Diols were prepared by LiAlH₄ reduction of the corresponding lactones. On the whole, eleven new lignano-9,9'-lactones 104-108, 110-115 and six lignano-9,9'-diols 116-121 were prepared (Figure 4).

All the molecular structures were fully characterized using MS, ¹H NMR, ¹³C NMR and 2D NMR (HSQC, HMBC and COSY). The synthesised molecules were used as authentic reference materials and also for the deuteration study (II).

Scheme 26. The basic synthetic route for the lignanolactones and lignanodiols.
4.2 Synthesis of deuterated lignans (I, III and V)

Labelled reference compounds are needed for quantitative analysis as standards. In the MS methods, the reference compound’s M⁺ peak should have at least three units’ difference from the analyte. Especially in GC-MS methods where the sample compounds are silylated, the heavier isotopes of carbon and silicon give fairly intense M⁺+1 and M⁺+2 peaks.

We have used D₃PO₄·BF₃/D₂O as an effective deuteration reagent for the deuteration of several liganolactones. This deuteration reagent is strongly acidic and it allows in case of ENL 1 the deuteration of all unsubstituted positions in aromatic rings, even the less-active sites C-5 and C-5’ in 75% yield. Furthermore, the treatment of D₈-ENL 122 with LiAlD₄ gave D₁₀-END 123 in 75% yield.

The deuterated ENL was further silylated to get standards suited for GC-MS analysis (Sheme 27).
Scheme 27. Reactions forming deuterated ENL 122, END 123 and deuterated ENL-TMS 124 derivative.

The EIMS spectra of TMS-derivatized labelled and unlabelled compounds were used for comparison to determine the isotopic purities. Also, it was shown that deuterium labels are stable in the molecules during the TMS-derivatisation reaction and are therefore applicable for use as standards. As can be seen from Figure 5 the MS spectra profiles of TMS derivatised $^{[2H_8]}$-ENL 124 (a) and ENL 1 (b) are almost identical. In the $^{[2H_8]}$-ENL 122 spectrum there are hardly even traces of ENL $^2H_7$-$^2H_0$. The isotopic purities were determined to be as high as $> 99\%$ for $^{[2H_8]}$-ENL 122 and $> 95\%$ for $^{[2H_10]}$-END 123.

Figure 5. Part of the MS spectra for TMS derivatised $^{[2H_8]}$-ENL 124 (a) and ENL 1 (b).
The deuteration sites of the molecules were established using the $^1$H NMR and $^{13}$C NMR spectra. In $^1$H NMR the particular proton signal is absent in the spectra when H/D exchange has occurred at that specific position. In Figure 6, the correlation of ENL and $[^2$H$_8$]-ENL spectra shows that ENL aromatic protons at C-2, 4, 5, 6 and 2’, 4’, 5’, 6’ are absent in the spectra. Also in the $^{13}$C NMR spectra changes are useful for the determination of the deuterium positions. The C-H signal (singlet) is changed to a low intensity triplet when deuterium has exchanged into that position (Figure 6).

**Figure 6.** Part of $^{13}$C NMR spectra (left) and $^1$H NMR spectra (right) of ENL 1 (upper) and $[^2$H$_8$]-ENL122 (lower).

The deuteration reaction was studied with seven different lignanolactone molecules containing varying methoxy or hydroxy groups in the aromatic rings. All aromatic protons were exchanged to $^2$H. It was observed that structures having two meta-substituted hydroxy or methoxy groups activated positions 2, 4 and 6 and therefore some back exchange occurred. To achieve stable deuterated labels molecules were treated with MeOH/CH$_3$COCl. The isotopic purities of the stable deuterated molecules were determined by using MS and were found to be 85%- 95%.

The NMR spectra were used to determine the sites of the deuteriums in the aromatic ring and also for monitoring the deuteration and the dedeuteration reactions (Figure 7).
Figure 7. The synthesised stable deuterated molecules 125-131 with isotopic purities and observed deuteration reactivity order.

The observations based on NMR data and theoretically calculated electrostatic potential (ESP) values were compared in order to study the relative reactivity of the aromatic protons. The ESP charges were calculated for the molecules as in sandwich conformation (Figure 8).

Figure 8. The sandwich conformation for the structure 130.
The calculated ESP values for the molecules 125-131 are shown in Figure 9. A low ESP value indicates a low electron density and therefore more labile proton. The calculated and observed values were in good agreement. The meta-substituted hydroxy or methoxy groups activated the 2,4,6 aromatic positions which led to the back exchange. The calculations gave also low ESP values for the aromatic position 2,4,6 for structures 128-131. Another finding was that meta-substituted methoxy groups (molecule 130) activate the 2’ position more than meta-substituted hydroxy groups in 130 and also methoxy groups in position 2 and 2,3 give less active 2’ position 125 and 126. The phenomenon was observed for the molecule 130 as the isotopic purity was 85% because of the tendency to back exchange at the 2’ site. Structure 128 was more stable and had the isotopic purity 92%. The ESP value was -0.42 for position 2’ in the molecule 130 and the ESP value 125, 126 and 128 were -0.35 - 0.37 which was in agreement with the observation.

![ESP values](image)

Figure 9. The calculated ESP-values for structures 125-132. ESP-values in position C-2’ in structures 125 and 130 are underlined.

4.3 Synthesis of haptens (IV)

Four different ENL haptens were synthesized (3’-O-carboxymethyl-ENL 132, 3-O-carboxymethyl-ENL 82, 5-carboxymethoxy-ENL 21 and 5’-carboxymethoxy-ENL 133 and were selected for the development of a colorimetric ELISA assay method to
determine ENL concentrations in human plasma and urine. ENL haptens were synthesized by a method of Mäkelä et al. – with two different modifications (Scheme 29). Previously unknown 3’-O-carboxymethylENL 132 and 5’-carboxymethoxyENL 133 were prepared and characterized by 2D NMR methods.

5-Carboxymethoxyenterolactone 21 was synthesized by a shorter method than previously from 3-benzyloxy-5-tert-butyldiphenylsilyloxybenzyl bromide 134 obtained in 4 reaction steps in 72% overall yield, an improvement over a previous 5 steps synthesis in 7% overall yield. 3,5-Dibenzyloxybenzaldehyde 136 was selectively debenzylated by sodium hydride and ethanethiol followed by silylation, reduction of the aldehyde and bromination (Scheme 28). The resulting benzylic bromide 134 was used for the tandem Michael addition alkylation – reaction (Scheme 29).

Scheme 28. Synthetic route for 134 and 135.

The above protected aldehyde 136 was also used for the synthesis of 5’-carboxymethoxyENL 133, by way of the corresponding aryl bis(dithio)acetal 135 which was then deprotonated for the Michael addition alkylation –reaction

3-O-CarboxymethylenL 82 was prepared by a shorter synthetic route than previously published. Silyl ether protection and deprotection steps were avoided by attaching the ethyl carboxylate side chain to 3-hydroxybenzaldehyde followed by NaBH₄ reduction and PBr₃ bromination of the corresponding alcohol. The 3-(ethoxycarbonylmethoxy)benzyl bromide 137 thus obtained was used for the tandem Michael addition alkylation sequence to form the lignan framework 141.
Desulfurization, debenzylation and ester hydrolysis afforded enterolactone equipped with a 3-O-carboxymethyl appendage 82 (Scheme 29).

Scheme 29. Synthesis routes for haptens 82, 22, 132 and 133. The R<sup>1</sup>-R<sup>4</sup> for the starting materials (134, 135, 137-140) and the intermediate molecules 141-147 is presented.

The formed four ENL haptens were further used for the development of ELISA competitive quantitation method.

The BSA protein (Bovine serum albumin) was attached to the acid chain of ENL haptens and BSA-coupling was done according to Yatsimirskaya et al. The formed four hapten-BSA conjugates were used for competitive ELISA method development and therefore were used for immunization of mice in order to form antibody against ENL.
The antibody solution was added to the prehandled plasma samples and the antibody was let to attach to the ENL in the plasma or urine sample. Samples were pipetted to the analysis kits that were beforehand covered with ENL-BSA. The unreacted antibody was attached to the analysis kit immunogens (ENL-BSA) and the amount of the ENL in the plasma or in the urine sample was calculated using calibration solutions.

The developed competitive ELISA method was compared with the TR-FIA method. According to the results the sensitivity of ELISA and TR-FIA were found to be comparable.
5 Conclusions

In this thesis work, eleven lignanolactones and six lignanodiols were prepared using a modification of the tandem Michael addition alkylation reaction. Ortho and meta substituted aromatic starting materials reacted less readily, especially when benzyloxy groups were in both meta positions, most likely due to the steric hindrance. Improvements for these syntheses were obtained with more concentrated reaction solutions. All new lignans were fully characterised by MS, $^1$H NMR, $^{13}$C NMR and 2D NMR (HSQC, HMBC and COSY).

In the deuteration studies, seven new lignanolactones and two previously known lignanolactones were deuterated using D$_3$PO$_4$·BF$_3$/D$_2$O as the deuteration reagent. The orientation, the reactivity order and stability of deuterium labels in the lignan molecules were determined. The aromatic protons of the lignans were exchanged in the deuteration reaction and labile deuteriums were back exchanged upon MeOH/CH$_3$COCl treatment. The meta-substituted hydroxy or methoxy groups activate the 2,4,6 aromatic sites which led to the back exchange. Also 3,5-dimethoxy groups activated the aromatic site 2', even more than 3,5-hydroxy groups. Observations were in agreement with the calculated ESP values of the aromatic protons. The isotopic purities of the stable deuterated molecules were determined by using MS and were found to be 85-99%.

In last part of this thesis work, four enterolactone haptens, 3'-O-carboxymethyl-ENL, 3-O-carboxymethyl-ENL, 5-carboxymethoxy-ENL and 5'-carboxymethoxy-ENL were synthesised. The reactions for 3-O-carboxymethyl-ENL, 5-carboxymethoxy-ENL were improved and the new haptens 3'-O-carboxymethyl-ENL, 5'-carboxymethoxy-ENL were fully characterised using MS, $^1$H NMR, $^{13}$C NMR and 2D NMR.

The formed four ENL haptens were further used for the development of ELISA competitive quantitation method to determine enterolactone concentration from human plasma. The developed ELISA method results were comparable in sensitivity to those obtained by TR-FIA.

On the whole, fast and accurate analytical methods are needed for the lignan research work. Depending of the analytical methods used, the synthesised labelled and unlabelled lignans are applicable for use as references. Authentic reference molecules are also required in the metabolic studies or in other biological studies.
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