ANTIOXIDATIVE LONG CHAIN ALKYLRESORCINOLS. SYNTHESIS AND DEUTERIUM LABELLING OF BIOACTIVE COMPOUNDS PRESENT IN WHOLE GRAINS

Kirsti Parikka

University of Helsinki
Faculty of Science
Department of Chemistry
Laboratory of Organic Chemistry
Finland

ACADEMIC DISSERTATION

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Supervisor
Professor Kristiina Wähälä
Laboratory of Organic Chemistry
Department of Chemistry
University of Helsinki
Finland

Reviewers
Docent Tuulikki Seppänen-Laakso
VTT Technical Research Centre of Finland
Finland

Professor Chris Willis
School of Chemistry
University of Bristol
United Kingdom

Opponent
Docent Afaf Kamal-Eldin
Department of Food Science
Swedish University of Agricultural Sciences
Sweden

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TABLE OF CONTENTS

ABSTRACT

The first synthesis of long chain 5-*n*-alkylresorcinols (C_{15}-C_{25}) in whole grains and whole grain products by a novel modification of Wittig reaction is described. 5-*n*-Alkylresorcinols are phenolic lipids that have various effects on biological systems, such as antioxidant activity and interaction with biological membranes. These compounds are considered as biomarkers of whole grain intake, which is connected with reduced risk of cardiovascular diseases and certain cancers. Novel hapten derivatives of 5-*n*-alkylresorcinols, potential compounds for immunoanalytical techniques, are prepared by the same procedure utilizing microwave catalysed aqueous Wittig reaction as the key step. The synthesised analogues are required by various analytical, metabolism and bioactivity investigations.

Four alternative strategies for producing deuterium polylabelled 5-*n*-alkylresorcinols are explored. Ring-labelled D_3-alkylresorcinols were synthesized by acidic H/D exchange. Side chain -labelled D_4-derivative was prepared by a total synthesis approach utilizing D_2 deuterogenation of a D_2-alkene derivative, and deuterogenation of alkynes was investigated in another total synthesis approach. An ω-D_3-labelled alkylresorcinol is isotopically pure and completely stable under all relevant conditions encountered during analytical work. The labelling of another phenolic component of whole grains was explored. The preparation of D_3-ferulic acid and related compounds by way of selective methylation of the precursors is described. The deuterated compounds are useful as standards in the quantification of these natural products in various substances, such as food and human fluids.

The pure 5-*n*-alkylresorcinol analogues prepared were used in *in vitro* experiments on alkylresorcinol antioxidant activity and antigenotoxicity. The *in vitro* experiments show that alkylresorcinols act as antioxidants, especially when incorporated into biological systems, but possess lower activity in chemical tests (FRAP and DPPH assay). Whole grain alkylresorcinols are shown for the first time to have a protective effect against copper induced oxidation of LDL, and H_2O_2 or genotoxic faecal water induced damage on HT29 cells.

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Helsinki, October 2007
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles. They are referred to by their Roman numerals (**I**-**V**).

I Linko, A.-M., Parikka, K., Wähälä, K. and Adlercreutz, H. Gas chromatographic–mass spectrometric method for the determination of alkylresorcinols in human plasma. *Anal. Biochem*. **2002**, *308*, 307-313.

II Parikka, K., Rowland, I. R., Welch, R. W. and Wähälä, K. In vitro antioxidant activity and antigenotoxicity of 5-*n*-alkylresorcinols. *J. Agric. Food Chem*. **2006**, *54*, 1646-1650.

III Parikka, K. and Wähälä, K. Expedient synthesis of 5-*n*-alkylresorcinols and their novel hapten derivatives. *J. Agric. Food Chem*. **2007**, submitted.

IV Parikka, K. and Wähälä, K. Synthesis of deuterated of 5-*n*-alkylresorcinols. *J. Label. Compd. Radiopharm*. **2007**, in press.

V Parikka, K. and Wähälä, K. Deuteration of dietary antioxidants: ferulic acid derivatives and α-tocopherol. *J. Label. Compd. Radiopharm*. **2007**, *50*, 475-476.

ABBREVIATIONS

AR	5-alkylresorcinol
BBN	borabicyclononane
Bu	butyl
DPPH	2,2-diphenyl-1-picrylhydrazyl
DMSO	dimethylsulfoxide
EI	electron impact
ESI	electrospray ionisation
Et	ethyl
FA	ferulic acid
FAB	fast atom bombardment
FID	flame ionisation detector
FRAP	ferric reducing potential
GC	gas chromatography
HPLC	high performance liquid chromatography
ID	isotope dilution
LC	liquid chromatography
LDL	low density lipoprotein
Me	methyl
MS	mass spectrometry
MW	microwave
NMR	nuclear magnetic resonance
SC	supercritical
SIM	selected ion monitoring
Tf	triflate
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	trimethylsilyl

1. INTRODUCTION

1.1. Alkylresorcinols present in various plant families

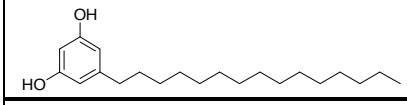
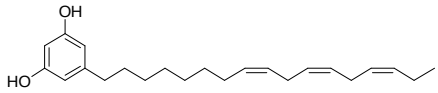
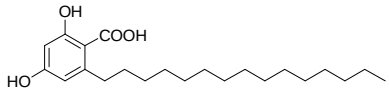
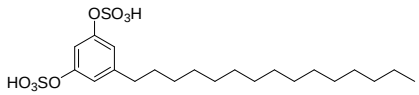
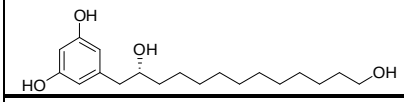
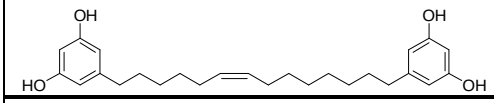
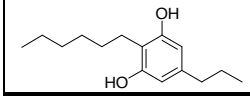
1.1.1. Structures and nomenclature

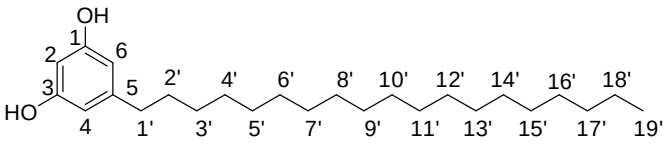
5-Alkylresorcinols and related compounds are non-isoprenoid phenolic lipids present in several families of higher plants (e.g. Anacardiaceae, Ginkgoaceae, Gramineae, Leguminoseae) and in some families of algae, mosses, fungi, bacteria and even in a marine sponge.[1] Resorcinolic lipids were first found in maidenhair tree (*Ginkgo biloba,* Ginkgoaceae). Cashew nut (*Anacardium occidentale,* Anacardiaceae) soon became an important source of alkylresorcinols, in addition to other types of phenols, which were isolated for applications in chemical industry.[1] As the functional food movement and the positive health effects of whole grains have gained attention, the Gramineae family has become a focus of interest.

Alkylresorcinols with a wide structural variety are found in various plant families. The structure is dependent on the plant source. These compounds include 5-*n*-alkyl-, 5-alkenyl-, 5-oxoalkyl-, and 5-hydroxyalkylresorcinols, and alkylresorcinols with an alkyl chain at various positions on the benzene ring, often at either C-2 or C-4, or sometimes at both positions. In bisalkylresorcinols (or 5,5'-[alkanediyl]diresorcinols) there are two benzene rings connected via a saturated or unsaturated alkyl chain. Alkylresorcinol sulfates also occur in nature. The 5-alkylresorcinols found in rye and wheat are mainly mixtures of saturated carbon chain compounds, whereas other higher plants produce mixtures of analogues with double bonds or, for example, hydroxyl groups in the alkyl chain. The alkylresorcinol sulfates are produced by fungi and dialkylresorcinols by bacteria and mosses. Examples of the different structures are shown in Table 1.

The 5-alkylresorcinols (AR) are commonly given abbreviations which describe the alkyl chain length and its degree of unsaturation, for example C19:0, C19:1 and C19:2, indicating a saturated chain of 19 carbon atoms, an unsaturated chain of 19 carbons with one double bond and an unsaturated chain of 19 carbons with two double bonds, respectively (Figure 1, **1**-**3**). However, these symbols do not carry any indication of the location of the double bonds. Names based on the resorcinol structure (e.g. 5-nonadecylresorcinol, **1**) are more commonly used than IUPAC nomenclature (5-nonadecylbenzene-1,3-diol). Trivial names are also commonly used for particular compounds, which can cause confusion (Adipostatin A = Hydrobilobol = Cardol = 5-*n*-pentadecylresorcinol **4**, Table 1).

Table 1. Examples of 5-alkylresorcinol structures and examples of their plant origin.

Structure	Side chain Length	Source	Family
4 	C_{15}	Rye (*Secale cereale*)	Gramineae
		Cashew nut (*Anacardium occidentale*)[2]	Anacardiaceae
5 	C_{17}	Heartleaf philodendron (*Philodendron scandens*)[3]	Araceae
		Marine sponge (*Haliclona* sp.)[4]	Haliclonidae
6 	C_{15}	Maidenhairtree (*Ginkgo biloba*)[5]	Ginkoaceae
		Loosestrife (*Lysimachia Japonica*)[6]	Primulaceae
		Cashew nut (*Anacardium occidentale*)[2]	Anacardiaceae
7 	C_{15}	*Streptomyces* (sp. OH-5186)[7]	Basidiomycetes
8 	C_{13}	Restharrow (*Ononis viscosa*)[8]	Leguminoseae
9 	C_{14}	Two-leaf hakea (*Hakea trifurcata*)[9]	Proteaceae
10 	C_3, C_6	*Pseudomonas* (sp. B-9004)[10]	Pseudomonales

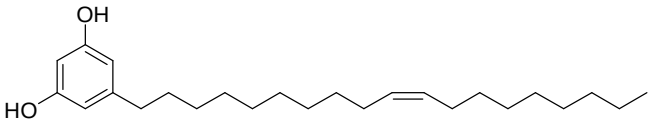


1

1,3-Dihydroxy-5-nonadecylbenzene

5-*n*-Nonadecylresorcinol

C19:0

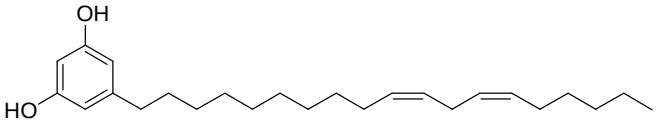


2

1,3-Dihydroxy-5-(nonadec-10'*Z*-enyl)benzene

10'(*Z*)-5-Nonadecenylresorcinol

C19:1



3

1,3-Dihydroxy-5-(nonadec-10'*Z*,13'*Z*-dienyl)benzene

10'(*Z*),13'(*Z*)-5Nonadecadienylresorcinol

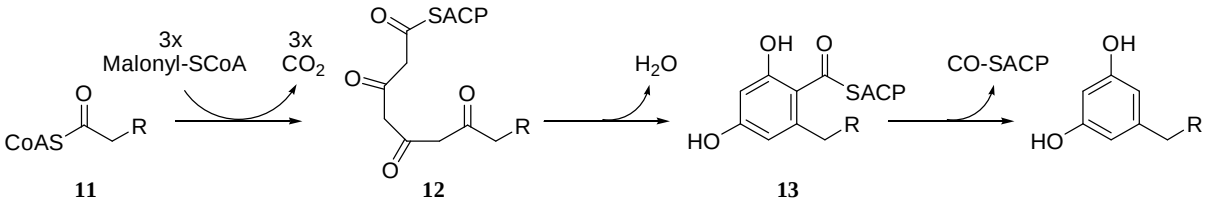
C19:2

Figure 1. Nomenclature and numbering of 5-alk(en)ylresorcinols.

1.1.2. Biosynthetic pathway and location in the plants

Alkylresorcinols are synthesized by the polyketide pathway in plants. The biosynthesis of long chain ARs (maximum length C_{19}, both even and odd chain) has been investigated in the 5-alk(en)ylresorcinol biosynthesis system of rice. The feeding of fatty acid substrates shows that they are so-called starter units in the system, since etiolated rice seedling will produce ARs when fed fatty acids, although the rice seeds from which they grow do not contain ARs. When the fatty acid provided is labelled with ^{13}C, the AR produced is also ^{13}C labelled, confirming that the fatty acid is the starter unit.[11] The study confirms the results of the earlier investigations on the biosynthetic route, which dealt with shorter alkyl chain ARs and their analogues[12-13] as well as orsellinic acid derivatives (C15:0) which have been prepared using potential biosynthetic precursors.[14]

Biosynthesis of AR starts by the condensation of malonyl coenzyme A (Malonyl-SCoA) and the fatty acid unit **11** (Scheme 1). This reaction is repeated thrice. Ring closure and dehydration of product **12** gives the 6-alkylresorcinolic acid derivative **13**. 5-Alkylresorcinol is formed by decarboxylation of the acid. The fatty acid unit which starts the reaction is a product of fatty acid biosynthesis in the plant or can be added to the system as mentioned above.[11] Orsellinic acids (6-alkylresorcinolic acids, e.g. **6**, Table 1) are produced the same way except for the decarboxylation.



Scheme 1. Biosynthetic route to 5-alkylresorcinols starting from a fatty acid.

In cereal grains, alkylresorcinols can be found in the bran (outer layers) of the grains (Figure 2), which explains their presence in whole grains and whole grain products. It is possible that their amounts are the highest in young developing grains, and when the grains mature, the levels of alkylresorcinols decrease. In mango fruit and cashew nut alkylresorcinols are present in the peel[15] and in the shell,[2] respectively. ARs are able to protect plants from mould and other harmful organisms, such as bacteria, attacking from outside, and the location of AR in the outer layers of plants enhances these protective effects.

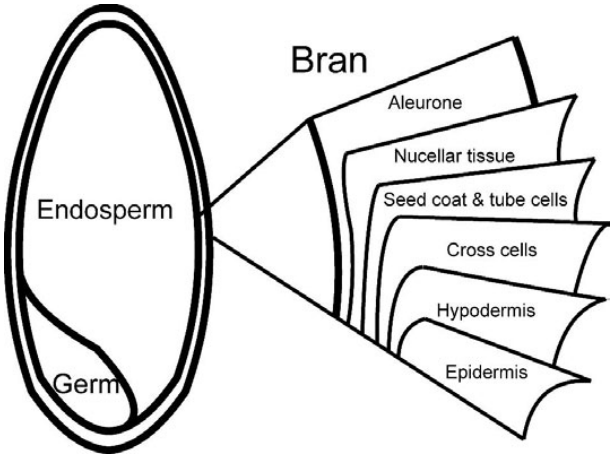


Figure 2. Cross-section of a grain.

1.1.3. Analysis of cereal grains

In the literature, several studies have described the isolation of alkylresorcinols from different plant matrices and the analysis of the extracts by various techniques. These studies were comprehensively reviewed in 1999.[1] The chromatographic techniques used in AR analysis were reviewed in 2004.[16] This thesis is concerned with the 5-alkylresorcinols that are present in cereal grains and all the different techniques that have been used to analyse and characterise them.

Alkylresorcinols in rye were described in 1967 as a mixture of homologues with C_{15}-C_{25} carbons in the aliphatic chain (Figure 3).[17] Since then the analysis and isolation of AR of cereal grains has been performed using a variety of techniques. Unsaturated 5-alkylresorcinols, hydroxyalkylresorcinols, oxoalkylresocinols and bisalkylresorcinols have also been identified from cereal grains, but in small concentrations compared to the saturated analogues. For example, the AR content of whole grain rye bread varies from 500 to 700 μg/g.[18-19] The average of wheat and rye consumption in Finland is ca. 160 g/day based on a yearly estimation.[20] Thus the average daily intake of AR would be ca. 100 mg if only whole grain rye bread was consumed. A summary of the alkylresorcinol content of cereal grains is presented in Table 2 (page 15).

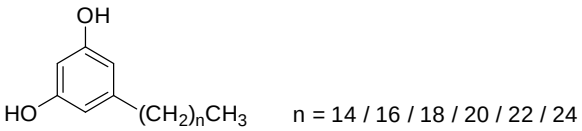


Figure 3. Major 5-*n*-alkylresorcinols found in rye.

1.1.3.1. Extraction

Extraction methods for alkylresorcinol isolation have been developed from the 1960's onward. Various organic solvents have been used to separate AR from grains, including, for example, acetone, 80% ethanol and diethyl ether.[21] Soxhlet extraction with cyclohexane has also been shown to be suitable for AR isolation.[22] Recently, the alkylresorcinol content of processed whole grain products has been found to be the same as in the raw unprocessed grain. This important observation was made after baked or processed products were extracted with hot 1-propanol.[19] Other extraction methods have indicated considerably smaller amounts of AR in the processed products.

A new extraction method, which does not use organic solvents, was developed in 2005. Supercritical CO_2 (SC-CO_2) was found to extract ARs efficiently. When compared to acetone, SC-CO_2 used with co-solvents (ethanol and methanol) yielded 8-80% more alkylresorcinols.[23] The effects of temperature, flow rate, co-solvent percentage and extraction time were investigated, and long extraction times as well as high CO_2 flow rates were found to enhance the yields of ARs. This method also made it possible to separate low and high molecular weight alkylresorcinols from each other.[24]

1.1.3.2. Fluorometric determination

Alkylresorcinols were previously considered to be growth inhibitors of livestock fed with rye, causing slower weight gain compared to animals fed with other cereals. A colorimetric method was developed for the screening of AR from rye aiming to find rye lines and cultivars with the lowest possible concentrations of AR.[17,25] The method is based on measuring the fluorescence spectrum after the reaction of alkylresorcinols with KOH in $CHCl_3$ and EtOH. The amount of AR detected in rye by this method was found to vary from 0.036% to 0.322% (w/w) in a study comparing 397 different rye varieties.[25] Other fluorometric determinations of the AR content of rye have been approximately in the same range, indicating that ARs are present in higher amounts in rye than in other cereals.[21,26-30] However, the possible presence of other co-extracted phenols might be responsible for the increase in the observed fluorescence.

1.1.3.3. Thin layer and paper chromatography

Cereal grains have been analysed by thin layer chromatography (TLC) using silica gel[31-35] and alumina.[36] After the chromatographic run, the TLC plate is stained with a staining reagent, for example Fast Blue B, which reliably indicates the presence of AR. The advantage of TLC

techniques is that they are relatively simple and easily performed without special instruments. Paper chromatography has also been used.[37] It is, however, clear that more accurate chromatographic methods have to be used for quantitative determinations.

1.1.3.4. HPLC

High performance liquid chromatography (HPLC) with reversed phase columns has been used in the quantitation and identification of alkylresorcinol homologues.[18,28,32,34,38-41] The examples in Table 2 (page 15) show that HPLC has not been used as extensively for analytical work as GC. However, 5-*n*-alkylresorcinols in cereal grains have been identified by HPLC as C15:0, C17:0, C19:0, C21:0 C23:0 and C25:0. The quantities found in rye vary from 524 μg/g, found in whole grain bread, to 4108 μg/g, found in bran.[18] A gradient elution system[18,38,40] has been found to be faster and give better separation than an isocratic system.[38] Both a photodiode array detector[28,34,41] and a UV detector set at 280 nm[18,38,40] have been used for detection.

Unsaturated alkenylresorcinols can be separated from the saturated analogues based on small differences in the retention times.[38,40-41] The major alkylresorcinol present in rye, triticale and millet has been found to be C19:0, whereas C21:0 is predominant in wheat.[40] Some previous results have been criticised because they lacked the validation needed for quantitative analysis.[16] To address this issue, in a recent study 5-pentylresorcinol was used as a reference compound.[18]

1.1.3.5. GC and GC-MS

Gas chromatography (GC)[28,42-45] or GC combined with mass spectrometry (GC-MS)[19,34,38,46] seem to be efficient methods for the determination of AR content from different matrices. Even the earlier studies performed with packed column worked quite well, successfully separating the saturated ARs from each other,[43] and even separating to some extent saturated and unsaturated analogues.[44] Usually a non-polar stationary phase has been used in the GC column. A flame ionisation detector (FID) has been used as the detector in GC, and electron impact (EI) as the ionisation technique for the MS stage of the procedure.

If alkylresorcinols are analysed by GC without derivatisation, high oven temperatures are needed.[28,38,45] Trimethylsilylated (TMS)[43] and ethyl ethers[46] of ARs, for example, have shorter retention times than underivatized analogues and lower temperatures are possible. Alkylresorcinols with different alkyl chain lengths respond similarly in GC-FID.[19] In the GC-MS studies, the m/z 124 ion (Figure 4), or m/z 268 ion for di-TMS AR, have been used to aid

the identification of alkylresorcinol peaks. The amounts of ARs vary from 720-761 μg/g (rye) to 489-1429 μg/g (wheat) according to a recent GC-MS study.[19] GC-MS has also been successfully used in the analysis of plasma, for example, as described for the first time in the study **I**, using a C20:0 analogue as an internal standard.

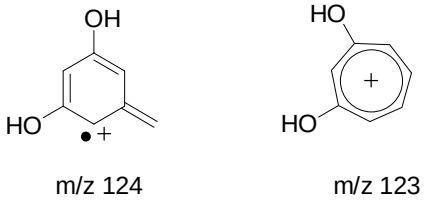


Figure 4. Typical MS fragments of ARs.

1.1.3.6. FAB and ESI MS

Mass spectrometry, including fast atom bombardment (FAB) and electrospray ionization (ESI) techniques, has been used to analyse ARs.[47-50] For example, in the analysis of two different hydroxyalkylresorcinol analogues and 5-(2'-oxo)alkylresorcinol analogues, present in very small amounts in rye (2 μg/g and 20.4 μg/g, respectively), their FAB-MS $[M–H]^-$ and $[M+Li]^+$ ions have been used in the calculation of the molecular formula, and CAD (collision-activated dissociation) spectra have been used to determine the position of the hydroxyl and carbonyl groups in the alkyl chain.[48] For example, the position of a hydroxyl group is determined by the size, as indicated by the ion peaks, of fragments cleaved off of either side of the OH group, which itself shows a "window" of 30 amu (CHOH).[48] The same technique has been utilized for determining the positions of the double bonds in rye and wheat 5-alkenylresorcinols ("window" of 54 amu, $CH_2CH=CHCH_2$).[50]

1.1.3.7. NMR

Nuclear magnetic resonance (NMR) has been used in several studies to obtain information on alkylresorcinol structures.[34,47-48,50] The proton NMR spectra of 5-*n*-alkylresorcinols are very simple. The advantage of NMR spectrometry in the analysis of compounds and mixtures extracted from plants has been the ability to observe characteristic peaks of different analogues in an unknown AR sample[48] or to check the purity of a particular AR fraction.[50] Proton NMR has provided evidence, for example, of the presence of 5-(2'-oxo)alkylresorcinols in wheat and rye extracts by way of the characteristic chemical shifts of the protons on both sides of the carbonyl group.[34,48] We have characterised the synthesized AR derivatives by NMR in study **III**.

1.1.4. Whole grain products as the dietary source of alkylresorcinols

Whole grain products are the most important dietary source of 5-*n*-alkylresorcinols. Other edible plants or products that contain significant amounts of ARs are, for example, cashew nut[2] and mango,[15] but alkylresorcinols are not present in their edible inner parts. Rice is one of the plants that contains a mixture of unsaturated and saturated homologues of alkylresorcinols (chain lengths C_{13}, C_{15} and C_{17}).[51-52] ARs have been isolated from etiolated rice seedlings[51] and rice root exudates[52] but it seems that the mature rice grains and processed rice do not contain alkylresorcinols.[19] Thus, whole grain products from the family Gramineae might be considered to be the only significant dietary source of 5-*n*-alkylrersorcinols, at least in a typical European or western diet.

Table 2. Examples of the measured amounts of 5-alk(en)ylresorcinols present in cereal grains of selected varieties and different whole grain products in μg/g.

	Total amount of AR (GC)[a]	**Total amount of AR (HPLC)[a]**	**Predominant 5-*n*-AR, proportion**	**Proportion of 5-alkenylresorcinols**
Rye grain	761[19]	900[28]	C19:0, 31%[19]	18.2%[40]
Wheat grain	642[19]	2290[28]	C21:0, 51%[19]	0.09%[40]
Barley grain	51[19]	-	C25:0, 37%[19] or 43%[42]	0.99%[40]
Triticale grain	647[19]	-	C21:0, 34%[19]	9.5%[40]
Whole rye flour	865[19]	927[18]	C19:0, 28%[18] or 31%[19]	-
Whole rye bread	707[19]	524[18]	C19:0, 28%[18] or 33%[19]	-
Rye bran	2758[42]	4108[18]	C19:0, 32%[18,42]	-
Rye bran pasta	262[19]	-	C19:0 & C21:0, 29%[19]	-
Whole wheat flour	550[19]	759[18]	C21:0, 38%[18] or 44%[19]	-
Wheat bran	2672[19]	3225[18]	C21:0, 46%[18] or 49%[19]	-

a) including saturated and unsaturated analogues

Doughs and baked products have been investigated by the same analytical techniques as raw grains. Previously, alkylresorcinol concentrations were believed to decrease during processing.[27,29-30] The recent analyses show this to be untrue. For example, the use of hot 1-propanol extraction of processed whole grain products has enhanced the yields.[19] Baking

seems to affect the alkylresorcinol concentrations little, if at all, as the amounts remain approximately the same, in the fermented and baked products as in the raw grains, depending on the method of analysis (Table 2). It is obvious that the various bran based products would be richest in alkylresorcinols, as ARs are produced in the bran of grains.

Alkylresorcinols have recently been proposed for use as biomarkers of whole grain intake.[53-54] The possibility to use biomarkers is important because an exact determination of the amounts of whole grains consumed can be problematic if only food diaries are used. Epidemiological and experimental studies suggest that whole grain products possess protective activity against cardiovascular diseases and some forms of cancer, especially colorectal and breast cancer.[55-56] Determination of the possible role of alkylresorcinols in these protective effects will require, for example, *in vivo* studies; nonetheless ARs are already known to clearly possess some biological activities, which are discussed in the next section.

1.2. BIOACTIVITY OF ALKYLRESORCINOLS

Alk(en)ylresorcinols and related compounds have been reported to have various biological activities. This thesis emphasizes the investigation of biological effects of saturated alkyl chain 5-*n*-alkylresorcinols. Many of the activities or effects of ARs might be based on their ability to interact with biological membranes.

1.2.1. Antibacterial and antifungal activity

5-*n*-Alkylresorcinols can be considered antifungal and antibacterial agents, protecting plants from harmful diseases and damage. The 5-*n*-alkylresorcinol fraction of the epicuticular waxes of barley inhibits the growth of pathogenic fungi (e.g. *Aspergillus niger*).[35] Pentadecylresorcinol (C15:0, **4**, Table 1, page 9) is one of the antibacterial compounds present in cashew nut shell oil. Cashew nut shell oil also contains an orsellinic acid C_{15} derivative (**6**, Table 1) which has a higher antibacterial activity for a broad selection of microbes than pentadecylresorcinol.[57] However, the study indicates that pentadecylresorcinol with two hydroxyl groups has higher antibacterial activity (for Gram-positive bacteria) than compounds with short alkyl chains and only one hydroxyl group. Nonetheless, a long alkyl chain is not always necessary – a short alkyl chain hexylresorcinol (C6:0) destroys dental plaque bacteria.[58]

1.2.2. Effects on enzymes

The various structural analogues of 5-alkylresorcinols interact with several enzymes.[1] The interactions of 5-*n*-alkylresorcinols with enzymes are particularly interesting. Very short alkyl chain ARs (max. C_3) are potent inhibitors of prostaglandin-H_2-synthetase.[59] Long alkyl chain ARs present in whole grains prevent the accumulation of triacylglycerol and effectively inhibit glycerol-3-phosphate dehydrogenase,[60-61] which synthesises triacylglycerol in adipocytes. Heneicosylresorcinol (C21:0) is the strongest inhibitor. Based on these observations, it is possible that ARs may have a role in the prevention of corpulence.

ARs from cereal grains are inhibitors of erythrocyte membrane acetylcholinesterase at 18-90 μM concentrations, the strength of the effect again depending on the alkyl chain length.[62] Cereal ARs at 50 μM concentration have an opposite effect on another membrane enzyme, Ca^{2+}-calmodulin-ATPase, which is stimulated. Only weak inhibition was detected at higher concentrations of ARs.[62] In the literature, there are contradictory reports concerning inhibition of soybean lipoxygenase-1 by pentadecylresorcinol (C15:0). In contrast to the other cashew nut shell oil compounds, C15:0 was found not to be an inhibitor.[63] However, when the lipids of cereal origin were investigated, C15:0 showed inhibiting activity but the most efficient inhibitor was tricosylresorcinol C23:0.[64] The disulfate of C15:0 and its isopentadecyl derivative are components of a product of *Streptomyces*, the enzyme inhibitor panosialin, which inhibits enzymes such as acid phosphatase, polygalacturonase and sialidase (also called neuraminidase).[7,65]

1.2.3. Effects on DNA

Cereal alkylresorcinols have been found to decrease the number of mutations induced in cultured lymphocytes with known mutagens (benzo[a]pyrene and mitomycin C), ARs protecting the lymphocytes more effectively than anthocyanins in the thioguanine-resistance test, cytokinesis-blocked micronucleus assay and sister chromatid exchange test.[66] The mutagenic activity of both direct and indirect acting mutagens is also reduced in the Ames test in the presence of ARs.[67] According to a study based on Comet assay analysis of DNA damage caused by H_2O_2 in lymphocytes, ARs seem to have some repair enhancing ability.[68] In an earlier study concerning DNA strand scission by two-leaf hakea, an Australian bush (*Hakea trifurcata*), extract, a mixture of 5-alkylresorcinols containing C13:0 was found to be responsible for the scission, but the mechanism was unclear. An oxidation product of AR was suggested to be the active component, and signs of a relationship between the alkyl chain length and activity were found.[9]

In our experiments concerning the antigenotoxic effect protecting HT29 cells against oxidative damage, the protective effect decreased along with the growing chain length (the shortest length tested was C15:0). However, we propose that the reason might be that solubility decreases as the chain length grows and therefore all the molecules in the mixture tested cannot participate in the system.[II]

1.2.4. Antioxidant activity

An antioxidant is defined as any substance that prevents or delays oxidation when present in low concentrations compared to the oxidisable substrate.[69] Oxidative damage is caused by reactive oxygen species, "free radicals", which are produced by aerobic metabolism.

1.2.4.1. In vitro antioxidant activity assays

In the so-called ferric reducing ability of plasma (FRAP) assay, ferric ions (Fe^{III}) are reduced to ferrous (Fe^{II}) ions by antioxidants.[70] In this context, antioxidants are considered as reductants and the oxidant is modelled by Fe^{III}. The oxidizing species reacts with the antioxidant instead of the possible substrate and is reduced to a harmless species. Different mixtures and solutions of antioxidants can be investigated (not necessarily solutions of plasma). In the reduction, an intensely coloured ferrous-tripyridyltriazine complex is formed and the absorbance of the solution is measured. The values of absorbance of the test mixtures are compared to the values of solutions of known concentrations of Fe^{II} in control samples.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical. Thus it is a practical reagent for investigating the free radical scavenging ability of antioxidants.[71] Due to its stability, no radical generation under the experimental conditions is required. In the DPPH assay, the antioxidant being tested reduces the DPPH radical and a change in the absorption of the test solution is measured.

Oxidized low density lipoprotein (LDL) particles take part in the early stages of atherosclerosis.[72-73] Thus, limiting the oxidation of LDL might prevent the development of the initial lesions in artery walls. LDL oxidation is studied *in vitro* by isolating LDL fraction from plasma and adding copper ions and the studied compounds to the solution. The copper ions induce oxidation of LDL, which can be followed by observing the formation of conjugated dienes in the solution.[74]

1.2.4.2. Activity of ARs

In the 5-*n*-alkylresorcinol structure, there are two hydroxyl groups at positions C-1 and C-3 of the aromatic ring, and a lipophilic, saturated alkyl chain at the C-5 position. Thus the structure has a dual nature, one end being hydrophilic and the other end hydrophobic, which causes a variety of effects in different environments. The presence of the two hydroxyl groups suggests that ARs have radical scavenging and hydrogen donation abilities. Previous research on the antioxidant properties of ARs includes studies on pentadecylresorcinol (C15:0), which has been found to slow the rate of oil and lipid oxidation.[75] Lipids and phospholipid bilayers are protected from ferrous ions induced oxidation by a mixture of rye ARs.[76] Pentadecylresorcinol has been found to be a stronger inhibitor of H_2O_2-induced oxidation of erythrocyte membranes than nona- or tridecylresorcinols (C19:0 or C23:0, respectively), showing that the activity is dependent on the chain length, and all these long alkyl chain ARs were more active than olivetol (C5:0), which is essentially inactive.[77] This difference is explained by the ability of the long chain analogues to incorporate into membranes. We described the ability of ARs to inhibit the oxidation of LDL for the first time in study **II**.

However, the radical scavenging and hydrogen donation power of C15:0 is low according to the triacylglyceride oxidation model and the DPPH assay.[78] Our experiments agree with these results (DPPH and FRAP assays, study **II**). A known antioxidant, such as ferulic acid (FA) for example, is highly active in these assays. This may indicate that ARs participate in the prevention of oxidative damage only if there is a lipophilic interaction in the system.

1.2.5. Interaction with membranes

Lipophilicity and the ability to interact with biological membranes are probably the most important properties of ARs. The other properties, the antioxidant activity for example, are enhanced or enabled by the lipophilic properties. When the lipophilic alkyl chain can interact with the system, the antioxidant activity increases.[77] A biological membrane can be defined as a lipid bilayer that surrounds, for example, the cytoplasm of cells. It usually consists of a double phospholipid layer where the hydrophobic tails of the lipids face each other, forming the inside of the bilayer, and the hydrophilic heads of the lipids point toward either the outside or the inside of the cell. Proteins are embedded in or attached to the membranes of cells and act as, for example, transporters.

1.2.5.1. Participation in transport systems

Alkylresorcinols affect the transport systems of various bilayers. In relation to this, erythrocyte membranes have been the focus of various investigations. The effects of ARs were studied in the beginning of the 1980's when the permeability of erythrocytes and liposomes was found to change after incorporation of alk(en)ylresorcinols, enabling small molecules to pass through the membrane,[79-80] and again very recently, when ARs were found to incorporate into human erythrocytes.[81] ARs can take part in the red cell membrane–water transport system, increasing water permeability.[82] In addition, the mobility of 5-doxyl and 12-doxylstearate spin probes is increased in erythrocytes and liposomes in the presence of C19:0 (and C19:1).[83]

1.2.5.2. Stable bilayers

5-*n*-Alkylresorcinols are present in the membranes of the cysts (specialized resting cells) of bacteria (e.g. *Azotobacter*), protecting the cysts more efficiently than plain phospholipid bilayers.[84] These effects have been investigated by modelling the bacterial membrane with planar bilayers synthesized from ARs and typical bacterial phospholipids (Figure 5).[85-86] Various experiments on bilayer formation with different mixtures show that liposomes incorporating ARs are more stable and homogenous than liposomes formed from phospholipids only and the encapsulation of water-soluble solutes is enhanced.[87] In contrast to this, but in agreement with the observations discussed above, an increase in permeability is observed when ARs are present only in the medium outside these vesicles. The encapsulating effect appears when ARs are introduced into the medium before formation of the membrane.[87-88] This enhancement of encapsulation has generated the idea of utilizing ARs in liposomal drug delivery.[88]

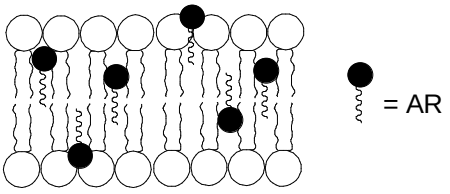


Figure 5. Hypothetical structure of membrane with AR incorporated into the bilayer.

1.3. ABSORPTION AND METABOLISM OF ALKYLRESORCINOLS IN ANIMALS AND HUMANS

1.3.1. Gastro-intestinal absorption

The first studies on the gastro-intestinal absorption of alkylresorcinols were published in recent years using pigs[89] and rats.[89-90] The ileal effluents of pigs fed different rye fractions gave 21-40% recovery of ARs, containing the same ratios of AR analogues as in the rye samples.[89] The experiments with rats, in which they were fed with a ^{14}C labelled C21:0, gave 34% recovery as measured from urine. The results show that ARs are absorbed in the small intestine and excreted in metabolised forms in the urine.[89] ARs have also been found in the perirenal tissue of rats fed an AR rich diet, which suggests that ARs might be stored in fat tissues in the body.[90]

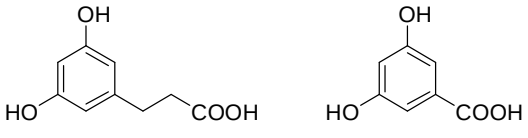
ARs were quantified from human plasma for the first time using a C20:0 analogue as an internal standard.[I] The amounts of AR analogues found in plasma seem to correlate with the AR in the whole grain products consumed,[91] which might allow the use of ARs as biomarkers. Ileostomy samples obtained from patients who had undergone ileostomy were analysed and give similar information on absorption as the experiments on animals, indicating that ca. 60% of alkylresorcinols are absorbed or converted to metabolites.[92]

As an analytical technique, GC-MS requires often time-consuming sample preparation such as derivatisation, extraction, chromatographic purification, and additionally special instrumentation. In comparison, immunochemical techniques would be relatively simple. They are rapid and suitable for screening purposes in large populations. In developing immunoanalytical methods, haptens that can form a peptide bond are needed for the preparation of immunogens capable of producing specific antisera. Immunoassays have not yet been used in the analysis of alkylresorcinols due to the lack of required haptens. We present here the preparation of novel AR haptens with varying alkyl chain lengths, which are ready for immunochemical experiments, in study **III**.

1.3.2. Metabolism

Little is currently known about the metabolism of ARs. The amount of intact ARs in the urine and ileostomy samples described in the absorption studies is approximately 30% of the amount consumed. The remaining ARs are apparently absorbed from the small intestine or converted to metabolites. So far, only two possible metabolites have been found from urine samples. They have been identified as 3-(3,5-dihydroxyphenyl)propanoic acid **14** and 3,5-

dihydroxybenzoic acid **15** (Figure 6).[93] It is suggested that these metabolites are formed by β-oxidation of the alkyl chain of AR.[93] These metabolites also might possess biological activity. According to a DPPH assay, the antioxidant activity of **15** is moderate, and between the activities of *ortho* dihydroxybenzoic acids (stronger effect) or monohydroxybenzoic acids (weaker effect).[94] Similar types of compounds, for example phenylpropanoic, phenylacetic and benzoic acids, are known metabolites of polyphenols such as flavonoids and anthocyanins.[95-96]



14 **15**

Figure 6. Proposed metabolites of 5-*n*-alkylresorcinols.[93]

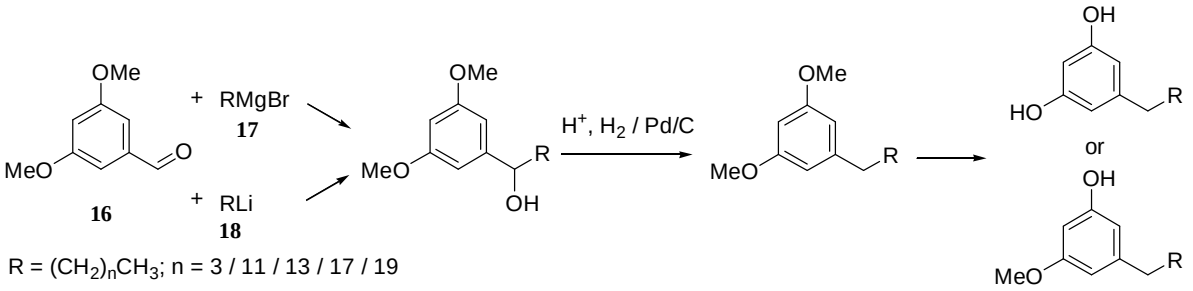
1.4. PREPARATION OF ALKYLRESORCINOLS AND THEIR DERIVATIVES

1.4.1. Different strategies for synthesis of 5-*n*-alkylresorcinols

The major part of the literature on 5-*n*-alkylresorcinols concerns their isolation from plants, identification of the extracted compounds, and their biological effects. However, some synthetic approaches have been published. The main issue for planning a synthesis of AR is how to form the carbon-carbon bond between the aromatic ring and the alkyl chain. This depends, for example, on the desired chain length in the product. In addition to the Grignard reaction, which has been used commonly to prepare long alkyl chain analogues, several other types of syntheses are described for one homologue only or very short alkyl chain ARs.

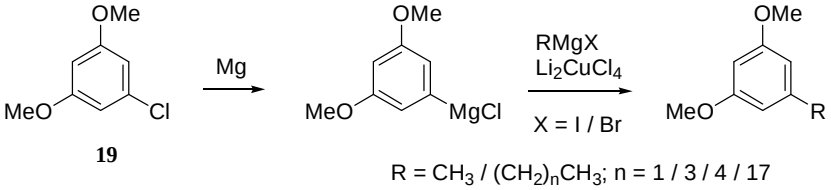
1.4.1.1. Grignard and related reactions

Grignard and related reactions used in the preparation of 5-*n*-alkylresorcinols and the subsequent reaction steps are presented in Scheme 2. The starting materials used in these reactions are 3,5-dimethoxybenzaldehyde **16** and alkylmagnesium bromides **17**[97-102] or alkyllithiums **18**[103] with various alkyl chain lengths. When it is reported, the overall yield of the Grignard approach is generally 18-48%[97-99] and in one case 59% (C13:0).[100]



Scheme 2. Grignard approaches in the synthesis of ARs.[97-101]

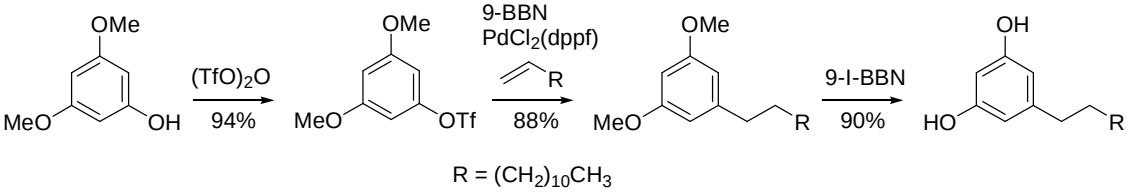
Variations on Grignard reactions have also been used to produce ARs. For example, alkyllithium reacts with 3,5-dimethoxyfluorobenzene to produce C15:0 and short chain length ARs (C3:0-C7:0), giving the short chain products in 52-64% yields in the Grignard step.[104] Another Grignard application uses dilithium tetrachlorocuprate catalysed coupling. Both short[105] and long alkyl chain[86] ARs have been prepared (Scheme 3) using this method. C19:0 and C21:0 were synthesized using 3,5-dimethoxybenzyl bromide as the starting material instead of the 3,5-dimethoxychlorobenzene **19** but the yield was not mentioned.[86] The yield of short chain C5:0 is 66%, which does not differ from the yields of the other reaction routes.[105]



Scheme 3. Li_2CuCl_4-catalysed Grignard coupling.[86,105]

1.4.1.2. Suzuki coupling

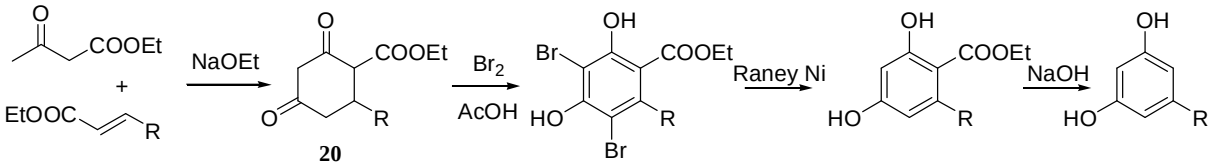
Grevillol (C13:0) has been prepared by a palladium-catalysed Suzuki coupling in a good yield (Scheme 4). The necessity for terminal alkene as the starting material for the reaction limits the number of compounds available for this kind of approach to AR synthesis in general. An unusual demethylation by 9-iodo-borabicyclononane (9-I-BBN) is reported in the same study.[106]



Scheme 4. Suzuki type coupling as the key step in the synthesis of C13:0.[106]

1.4.1.3. Aromatization of cyclohexane derivatives

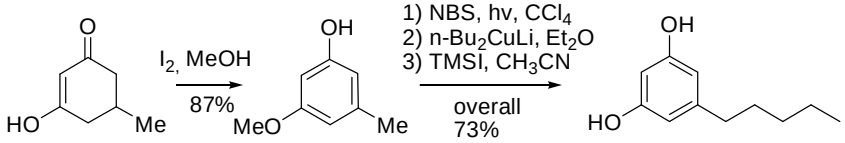
6-Alkyl-2,4-dioxocyclohexanecarboxylates **20** have been obtained from the reaction of acetoacetic ester and α,β-unsaturated esters prepared from corresponding aldehydes. The products were aromatised by treatment with bromine, debrominated and finally decarboxylated, giving ARs (Scheme 5).[107-108] The overall yields of C6:0 C11:0 were reported to be 61 and 66%, respectively.[108] When bromine in acetic acid failed to give a good yield for the intermediate of pentylresorcinol, monobromination with cupric bromide in 1,2-dimethoxyethane in the second step was also investigated.[108] The purpose of the preparation of short alkyl chain analogues was to use them as starting materials in cannabinol synthesis.[108]



R = CH_3 / $(CH_2)_nCH_3$; n = 4 / 5 / 8 / 10 / 12

Scheme 5. Synthesis of ARs by way of aromatisation of cyclohexanes.[107]

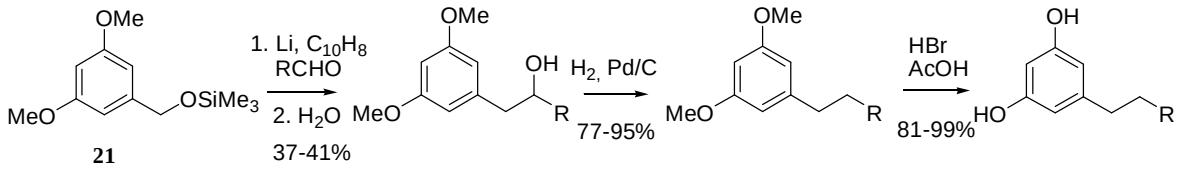
The proposed precursors of AR biosynthesis in plants are similar to the intermediates of the reaction in Scheme 5. The synthesis of orsellinic acid derivatives (for example **6**, Table 1, page 9), which are thought to be of similar origin to ARs, has been studied using the same intermediates.[14] Olivetol (C5:0) has been prepared by another approach utilizing 1,3-cyclohexanedione as shown in Scheme 6.[109] Olivetol dimethyl ether has been prepared in a similar way from the corresponding benzyl tosylate and lithium di-*n*-butyl cuprate.[110]



Scheme 6. Preparation of olivetol.[109]

1.4.1.4. Arene-catalysed lithiation

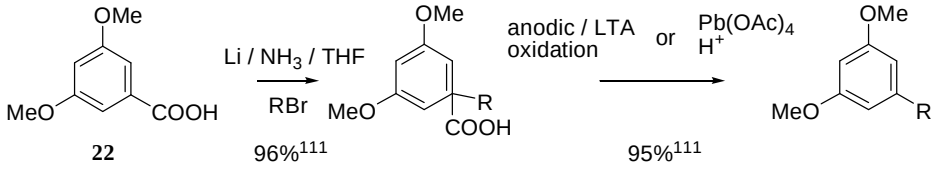
The reaction of 3,5-dimethoxybenzyl trimethylsilyl ether **21** and aldehydes, in the presence of Li powder and catalytic amounts of naphthalene, has been reported to give grevillol (C13:0) and olivetol (C5:0) in moderate overall yield after hydrolysis, catalytic hydrogenation and demethylation (Scheme 7).[111]



Scheme 7. Arene-catalysed lithiation.[111]

1.4.1.5. Reductive alkylation and oxidative decarboxylation

Short alkyl chain derivatives of AR have been prepared by the reductive alkylation of 3,5-dimethoxybenzoic acid **22** followed by oxidative decarboxylation (Scheme 8).[112-113] These short chain analogues (C_2 or C_4) with different functional groups in the chain have been used as starting materials for A-ring aromatic steroids[112] or the phytoalexin orchinol.[113]

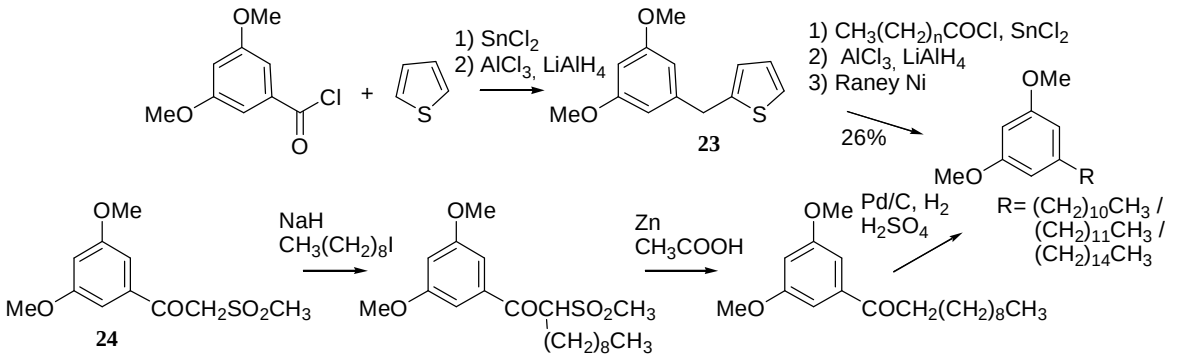


Scheme 8. Reductive alkylation-oxidative decarboxylation.[112-113]

Reductive alkylation-oxidative decarboxylation is claimed to be superior, in terms of yield and reliability, to the Grignard reaction with corresponding starting materials.[112-113] Thus this process could also be an option for the preparation of long alkyl chain ARs, but the required odd carbon chain bromides are not commercially available.

1.4.1.6. Thiophene and β-keto sulfone intermediates

Along with the early attempts to prepare 1,14-bis(3,5-dimethoxyphenyl)tetradecane, two methods to prepare AR dimethyl ethers were found. Desulfurisation of **23**[114] or alkylation of **24**[115] followed by reduction gives AR derivatives with a C_{15} maximum chain length (Scheme 9).



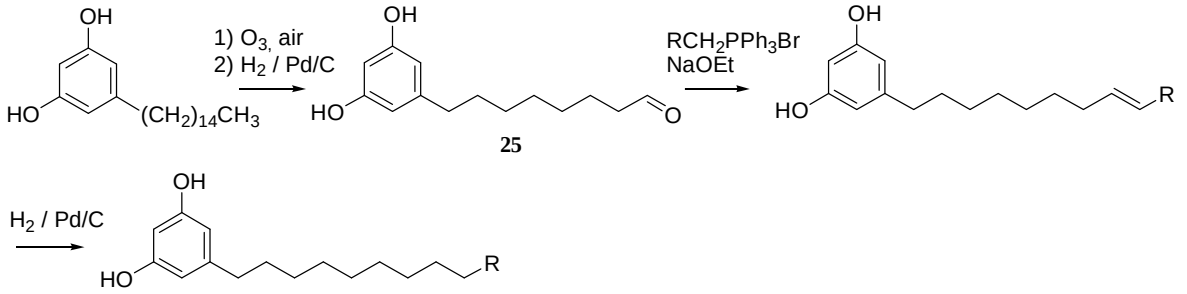
Scheme 9. Thiophene and β-keto sulfone intermediates in the preparation of ARs.[114-115]

1.4.1.7. Wittig reaction

The Wittig reaction[116] is one of the most important reactions in the preparation of alkenes. In a typical Wittig reaction, a phosphonium ylide reacts with an aldehyde or ketone, giving *Z* and/or *E* alkenes and phosphine oxide as the products. The reaction conditions, the type of ylide and the carbonyl compound affect the stereoselectivity for *Z* or *E* alkenes.[117] The Wittig reaction might also be considered an important C-C bond formation reaction, giving alkanes after reduction of the double bond.

We have investigated the Wittig reaction in an aqueous media as the key step in preparation of ARs.**[III]** In the literature, the use of a mixture of organic solvent and water in the Wittig reaction has been described in the reactions of unstabilized short alkyl chain triphenylphosphoranes, for example.[118-119] Water as a solvent has been described only a few times. The existing examples include the reactions of stabilized ylides with aryl aldehydes or short alkyl chain alkanals.[120-122] A benzylic semi-stabilized ylide has been used in the preparation of *o*- and *p*-nitrostyrenes from formaldehyde, which is highly reactive.[123] Reactions with aryl or short alkyl chain alkanals in the presence of LiCl have also been reported and give good yields.[124-125] It has been thought that the reagents of the Wittig reaction need to be more water soluble, and to provide solubility, aryl modified phosphonium salts carrying a COOH group[126] or PEG attachments[127] have been developed.

Recently, the Wittig reaction has been found promising in the preparation of ARs starting from an ozonolysis product **25** of C15:0 isolated from cashew shell nut liquid (CSNL).[128] CNSL is produced in large amounts in the processing of cashew nuts for the food industry. It contains a mixture of C15:0 and alkylresorcinol type compounds (e.g. anacardic acid).[2] The reaction of the ozonolysis product **25** with different alkylphosphoranes gives unsaturated analogues of ARs (Scheme 10).[128] However, once again the lack of availability of the odd alkyl chain bromides is problematic and limits the use of this reaction in the production of ARs. In our Wittig strategy, we have utilized the commercially available even chain alkyl bromides.**[III]**



Scheme 10. Preparation of ARs starting from ozonolysis of C15:0.[128]

To sum up, some of the reactions giving short chain analogues might be suitable for the production of long chain ARs, however, the lack of availability of all the starting materials might cause problems. The Grignard reaction seems to give moderate overall yields but requires moisture-free conditions and possibly suffers from the formation of side products. We have found the Wittig reaction described in study **III** to be the most practical strategy to produce ARs with either a short or long alkyl chain.

1.4.2. Enhancement of reactions with MW irradiation

The utilization of microwave (MW) irradiation (0.3-300GHz) in heating and catalysis of organic reactions is based on the ability of molecules present in the reaction mixture to absorb MW energy and convert it to heat by dipole rotation. Very efficient heating is thus achieved compared to conventional heating where the heat is conducted to the reaction from outside by way of baths or electric mantles. The ability of substances to absorb MW depends on their dielectric properties. Polar molecules such as H_2O and DMSO absorb MW energy and convert it to heat efficiently. Solvents with less dipolar character or solvent-free conditions can also be used in the reactions, depending on the conditions in question.[129]

The first applications of MW in organic synthesis are from the 1980´s.[130-131] Since then, MW equipment has developed from ordinary kitchen ovens to laboratory instruments and several reviews describe the use of MW catalysis in various reactions.[129,132-133] The advantages of MW heating are dramatically shortened reaction times and increased yields. Sometimes a decrease in the formation of side products or new selectivity has been described. At the moment it is generally accepted that the enhancement of the reaction is based only on thermal and kinetic effects.[134-135] However, the possibility of the existence of a so-called microwave effect catalysing the reactions by some other, special mechanism has been investigated.[136]

Previously, MW techniques have not been applied in the preparation of ARs. We have shown MW catalysis to be very useful in the key step of AR preparation.[III]

1.4.3. Isotopically labelled alkylresorcinols

1.4.3.1. Isotopic labelling

Metabolic and analytical studies often require isotopically labelled analogues of the compounds that are being investigated. Using these internal standards all the losses during sample preparation can be corrected and the analytes reliably identified and quantified. For example, deuterium labelled internal standards of isoflavonoids, lignans and estrogens have

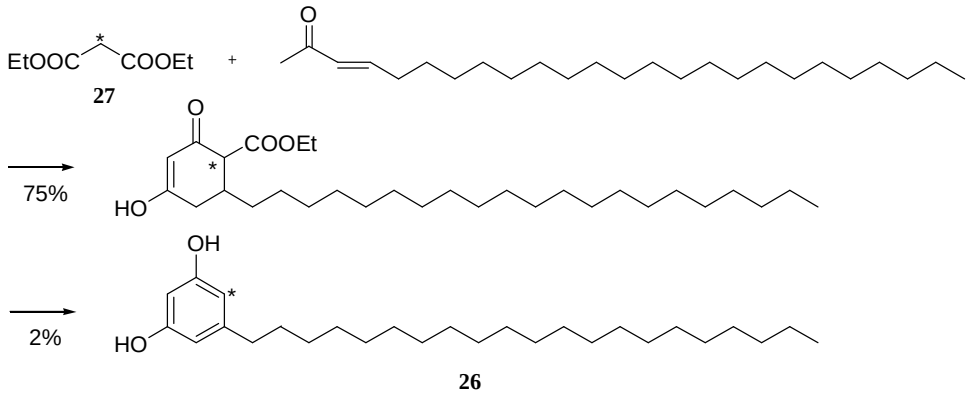
been used successfully in isotope dilution GC-MS selected ion monitoring (ID-GC-MS-SIM) measurements.[137-138] In the ion chromatogram, the compound to be analysed and its deuterium labelled analogue usually have the same retention time. The difference in the molecular weight of the analyte and the internal standard should be at least 3 amu to avoid overlap of their mass spectra peaks.

Early investigations on hydrogen/deuterium exchange of aromatic protons showed that the reaction mechanism is electrophilic aromatic substitution.[139] Mineral acids such as DCl and D_2SO_4 are commonly used as reagents in the exchange as well as NaOD in base catalysed deuterations. Labelling with the non-radioactive isotope of carbon, ^{13}C, is very expensive compared to deuterium labelling due to the high price of ^{13}C-labelled starting materials. The various deuteration reagents and solvents such as D_2O, MeOD, DCl, D_2SO_4 and NaOD for deuterium exchange and e.g. the reducing agents $LiAlD_4$ and $NaBD_4$, used in total synthetic approaches, are readily available and much less costly.

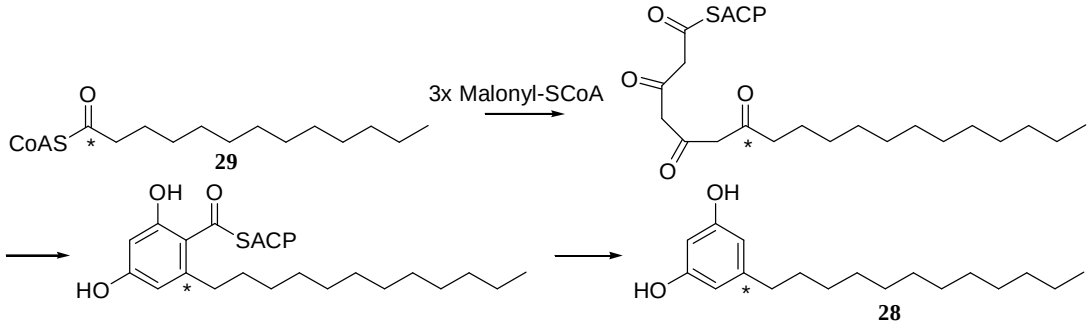
The quantitative and qualitative measurement of 5-*n*-alkylresorcinols from biological fluids has been performed using an even-chain analogue of AR as an internal standard,[I] even though such even-chain ARs have been detected from grains.[40,47] Labelled 5-*n*-alkylresorcinol analogues with varying alkyl chain lengths would allow a more reliable estimation of losses during sample preparation in various quantitative measurement procedures. In the literature, the preparation of labelled alkylresorcinols has been reported only a few times. We discuss the preparation of new deuterium labelled long alkyl chain 5-*n*-alkylresorcinols in study **IV.**

1.4.3.2. Carbon isotopes

Preparation of an isotopically labelled long chain 5-*n*-alkylresorcinol has been described only twice. ^{14}C-Heneicosylresorcinol **26** (C21:0) was synthesized for a metabolism study. Rats were fed with the labelled C21:0 whose absorption and metabolism were followed. The isotopically pure compound was prepared using a ^{14}C-labelled diethylmalonate **27** (Scheme 11).[89] A ^{13}C-dodecylresorcinol (C12:0) **28** has been obtained by biosynthesis starting from a ^{13}C-labelled tridecanoate substrate **29** by a special AR production system in etiolated rice seedlings (Scheme 12). The isotopic purity and the localisation of the label were determined by MS and NMR. Several even and odd chain ARs are produced by the system.[11]



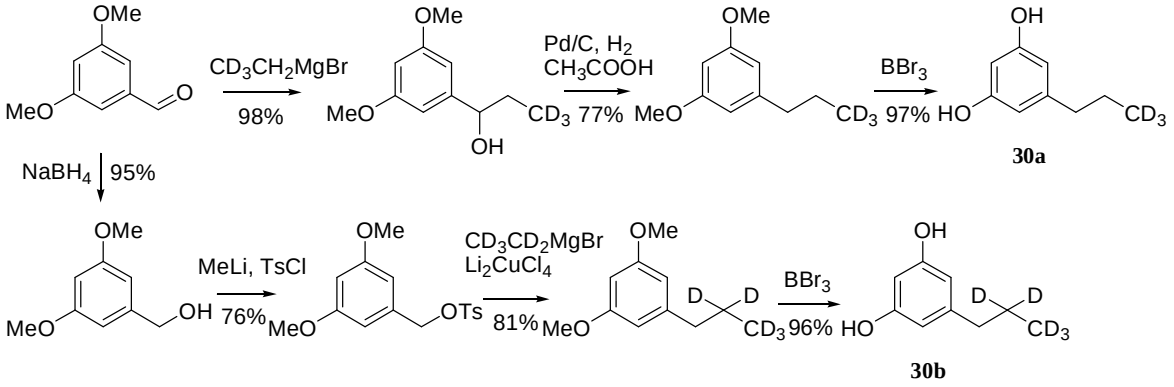
Scheme 11. Preparation of ^{14}C-5-heneicosylresorcinol.[89]



Scheme 12. Biosynthesis of ^{13}C-5-dodecylresorcinol.[11]

1.4.3.3. Deuterium labelled short alkyl chain ARs

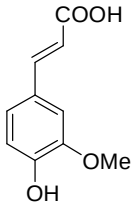
Deuterium labelling of short saturated alkyl chain AR analogues has been reported twice. The compounds such as **30a-b** are substructures of tetrahydrocannabinols and their metabolites, and have been used as starting materials in the preparation of labelled cannabivarins and cannabinols. The D labels have been introduced into the alkyl chain by way of Grignard reactions (Scheme 13).[140] Labelled C5:0 (D_3, D_5) has been prepared by the same strategy starting from 3-(3,5-dimethoxyphenyl)propanal.[141] The products are isotopically pure and overall yields are good.



Scheme 13. Deuterium labelled substructures of tetrahydrocannabinols.[140]

1.5. FERULIC ACID

Ferulic acid **31** (FA) is the major phenolic acid present in rye and wheat bran.[142] As FA is a well-known antioxidant, we have used it for comparison in measurements of the antioxidant activity of ARs.[II] We have investigated the selective methylation of the benzaldehyde precursor of FA, resulting in selectively deuterated and stable D_3-FA and its derivatives.[V] The compounds have been prepared for use as standards in epidemiological studies on polyphenols as biomarkers for the risk of chronic diseases.



31

1.5.1. Ferulic acid in grains

Whole grain products are an important source of FA;[18,142-143] however, FA is also present in other plants and plant derived products such as berries (e.g. FA glucose ester, 11-15 mg/kg), vegetables (e.g. FA glucose ester, 42-64 mg/kg) and coffee (feruloylquinic acids, 0.36-1.175% of dry beans).[144] FA is located in bran of the grains, which limits its availability to whole grain products. The amount of FA in grains depends on the variety. A large proportion of FA is in an esterified form, bonded to the arabinopyranose C-5 hydroxyl group of arabinoxylans.[143] The concentration of the ester measured from crude extracts of wheat varies from 1.56 μg/g in wheat flour to 34.2 μg/g in wheat bran.[145] In rye, the concentration of FA ranges from 900 μg/g to 1170 μg/g of dry matter depending on the variety.[146] Other phenolic acids such as isoferulic acid (3-hydroxy-4-methoxycinnamic acid) have been detected in minor amounts in grains.[142]

1.5.2. Antioxidant activity

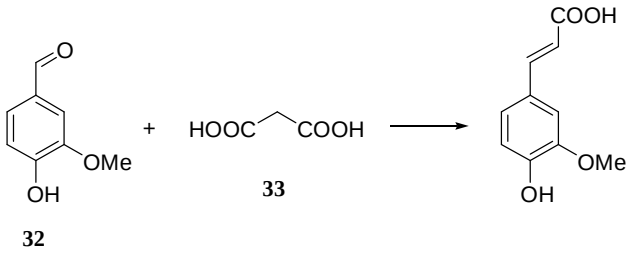
The antioxidant activity of phenolic acids depends on their structure. Due to the double bond and the methoxy group in their structures, ferulic acid and other cinnamic acids are stronger antioxidants than related compounds with simpler structures (e.g. benzoic acids).[147] Ferulic acid and its esters have various antioxidative effects.[148] FA prevents lipid oxidation and autoxidation, and when compared to related compounds such as coumaric, sinapic and caffeic acids, FA has the strongest protective effect.[149] When the oxidation level of synaptosomal membrane systems is measured in terms of protein oxidation, lipid peroxidation and reactive

oxygen species, FA is found to protect the neuronal system from all these kinds of oxidative stress. Thus FA might provide protection from neurodegenerative disorders such as Alzheimer's disease.[150] The antioxidant capacity of processed and unprocessed wheat samples has been evaluated by measuring oxygen radical absorbance capacity, inhibition of photochemiluminescence, inhibition of oxidation of low density lipoprotein (LDL) and deoxyribonucleic acid, or by using a Rancimat method (apparatus for determining oxidative stability at elevated temperature), giving high levels of antioxidant capacity by the most unprocessed samples.[145] FA is one the major phenolic acids present in these samples, and thus is presumably a potent factor in their antioxidative effects.

1.5.3. Synthetic approaches

1.5.3.1. Ferulic acid

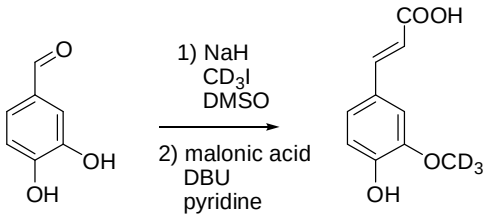
Knoevenagel condensation and its modifications, with vanillin **32** and malonic acid **33** as starting materials, has been commonly used in the preparation of FA (Scheme 14).



32 **33**

Scheme 14. Knoevenagel condensation.

MW irradiation has been used in a so-called Doebner modification of Knoevenagel condensation[151] and in solvent-free experiments using KF-Al_2O_3 as a catalyst,[152] which give FA and other cinnamic acids in good yields. Isotopic labelling of FA has been reported previously. These studies include, for example, the preparation of a ^{13}C-labelled[153] and a ^{14}C-labelled[154] FA. A D_1-labelled FA has been prepared by using deuterated malonic acid as the starting material.[155] The preparation of a D_3-FA with the labels in the methoxy group has been reported only once in the preparation of a precursor for a labelled coniferyl alcohol (Scheme 15).[156]



34 83%

Scheme 15. Preparation of D_3-FA.[156]

1.5.3.2. Selective methylation of precursors

The selective methylation of 3,4-dihydroxybenzaldehyde **34** makes available practical starting materials for synthesis of either ferulic or isoferulic acid. In addition to the reaction shown in Scheme 15, selective methylation of **34** has been described in the literature. Vanillin or D_3-vanillin has been prepared selectively by using NaOH in ethanol with CD_3I.[157] Protection of the 4-hydroxy group following the methylation and deprotection has also been described.[158] The reaction of **34** with CH_3I in NaH/DMSO gives vanillin in 52% yield.[159] The difference between the yields of combinations of KOH, $KHCO_3$, or K_2CO_3 with CH_3I has been investigated in the preparation of isovanillin (3-hydroxy-4-methoxybenzaldehyde).[160] Dimethyl sulfate has been used instead of CD_3I in the selective preparation of unlabelled isovanillin.[161-162] We describe the use of a new combination of base/solvent (*t*-BuOK/DMSO) in study **V** and present the preparation of D_3-FA and related compounds.

2. AIMS OF THE STUDY

The aim of the present study was to promote further investigations of 5-*n*-alkylresorcinols from whole grains due to their importance in nutrition and health. Synthetic organic chemistry was the primary method used to develop a number of tools for facilitating studies in this area. An important objective was to synthesise 5-*n*-alkylresorcinols present in whole grains and 5-*n*-alkylresorcinol derivatives, such as haptens, for *in vitro* and *in vivo* investigations. Similarly, analytical and metabolic studies require deuterium labelled standards. Particularly, isomerically and isotopically pure, stable polydeuterated 5-*n*-alkylresorcinols are required in development of quantitative analysis. To meet these overall goals, the specific aims of this study were as follows:

1. To develop an efficient synthesis for 5-*n*-alkylresorcinols. (**III**)

2. To develop a synthesis method for alkylresorcinol haptens, which could be used in immunoanalytical experiments. (**III**)

3. To develop deuteration methods for 5-*n*-alkylresorcinols and prepare deuterium labelled and cold standards suitable for analytical and metabolic studies. (**I**, **IV**)

4. To investigate biological properties, such as antioxidant activity and antigenotoxicity, of 5-*n*-alkylresorcinols utilizing the synthesized pure homologues with different alkyl chain lengths. (**II**, **III**)

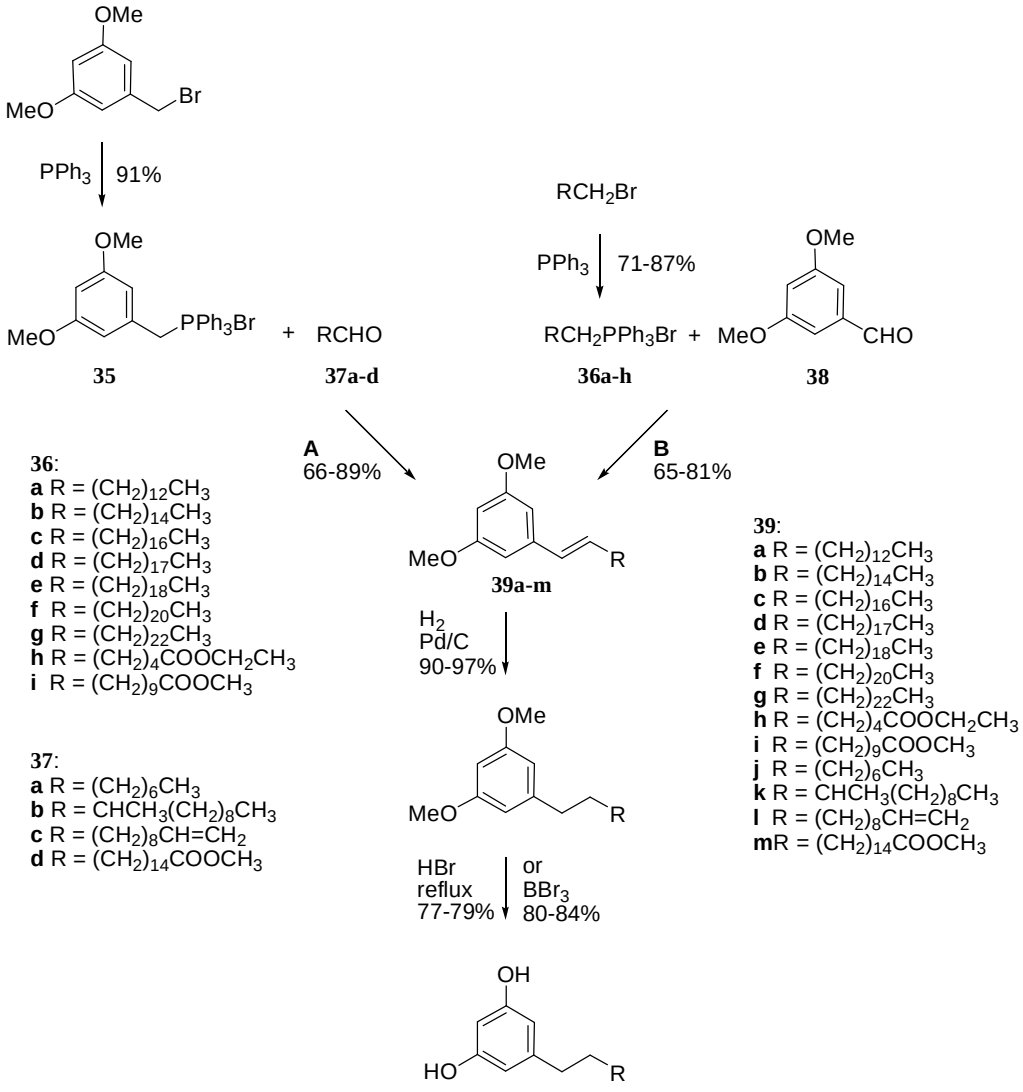
5. To study the preparation of deuterium labelled ferulic acid and related compounds. (**V**)

3. RESULTS AND DISCUSSION

3.1. SYNTHESIS

3.1.1. Synthesis of 5-*n*-alkylresorcinols and their derivatives by the Wittig reaction

We have studied the Wittig reaction as the key step in the preparation of AR and their derivatives.[III] Following the Wittig reaction, reduction of the double bond and demethylation are performed (Scheme 16) giving ARs and AR haptens in c.a. 40% overall yield.



Scheme 16. Preparation of ARs by the Wittig reaction.

3.1.1.1. Conventional methods

The conventional Wittig reaction methods include the use of strong bases such as *n*-BuLi in THF under an inert atmosphere. In the so-called salt-free conditions there is no lithium present and NaH or sodium alkoxides are commonly used as bases. We chose the salt-free system to prepare ARs, as it is a more environmentally friendly strategy than the methods utilizing lithium bases. In our approach, K_2CO_3 was found to work well in a dioxane/water mixture. The reactions required refluxing several hours to run to completion and the yields were ca. 60-75%.

3.1.1.2. Microwave assisted reactions

The use of MW heating in the Wittig reaction was found to cut down the reaction time dramatically. The MW catalysed reactions were complete in 3-10 minutes. A yield increase was obtained in the case of the C23:0 precursor, for example. Two different approaches towards AR synthesis were studied, using either a semi-stabilised benzylphosphonium ylide **35** or unstabilized alkylphosphonium ylides **36** (Scheme 16, routes A and B).

We found that an organic solvent was not necessary in the reactions of **35** and **37** (route A) even if they are not soluble in water. Different concentrations of aqueous K_2CO_3 as a solvent were examined (0.1 M/1 M/5 M/saturated) and the most dilute of them, 0.1 M, was found to be optimal. These reactions gave a good yield under the pressurized conditions, as well as in an open vessel. The pressure in the sealed vessel was usually about 9 bar during the reactions. The use of NaOH as the base was also investigated but resulted in vigorous reactions and even broken reaction vessels.

The very long alkyl chain alkanals are not commercially available but the corresponding bromides are, being thus excellent precursors for ARs (route B). The reactions of **36** and **38** required the presence of an organic solvent, as the yield was not satisfactory in the various aqueous K_2CO_3 solutions tested (0.1 M/1 M/5 M/saturated), of which the saturated solution gave less than 50% yield at best (in contrast to route A). A DMSO/H_2O mixture was found to be an optimal solvent for these reactions, giving products **39a-i** in 65-81% yield. Other solvent mixtures tested (e.g. dioxane/H_2O) gave poor yields. The reactions of route B were performed in an open flask, as the yields of the reactions in sealed vessels were not good due to instability or degradation of the ylides under pressure.

Mixtures of *cis* and *trans* isomers of **39** were obtained in the reactions with *Z*/*E* ratios varying from ca. 40:60 (route B) to ca. 80:20 (route A, product **39m**) according to ^{1}H NMR and GC-

MS. Although stereochemical control is thus lacking, it is of no consequence as the ultimate targets of the reactions are the C=C reduced, saturated alkyl chain ARs.

The preparation of phosphoranes under MW irradiation has been described in the literature.[163-164] However, we have not succeeded with an MW catalysed one-pot technique where the triphenyl phosphonium salt **36** would be formed from the bromide and then react with the aldehyde. Preparation of the phosphoranes was investigated in various solvents (e.g. xylene, toluene) giving ca. 5% yields at best.

3.1.1.3 Haptens

We chose four different alkyl chain lengths for the haptens **40** to represent alkylresorcinols with very short, medium and long alkyl chains, of which C_{17} (**40c**) and C_{23} (**40d**) are equivalent to the alkylresorcinols present in whole grain products (Figure 7). The nonpolar and hydrophobic character of alkylresorcinols increases as the alkyl chain length becomes longer, which may lead to differences in reactivity in immunochemical experiments even if the compounds are otherwise very similar to each other.

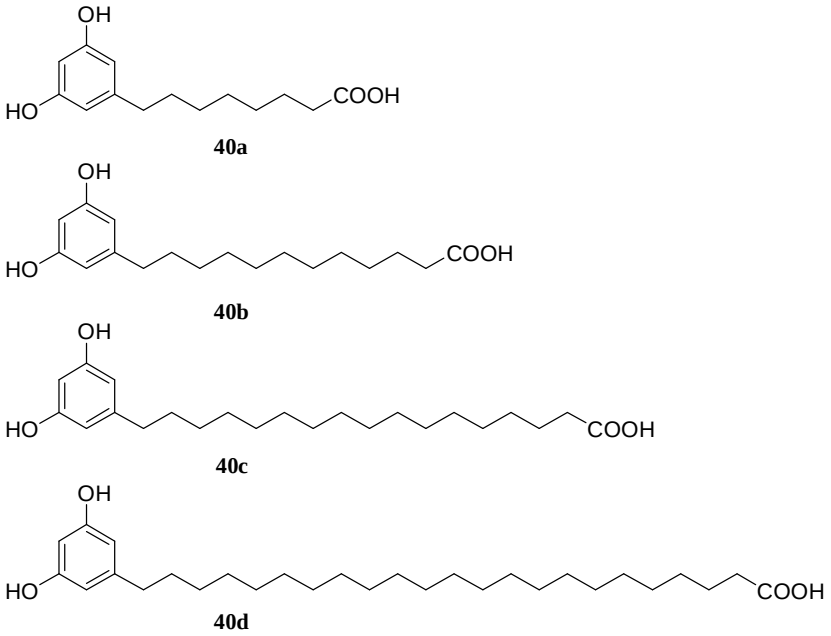
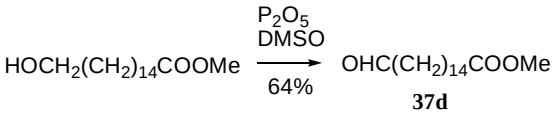


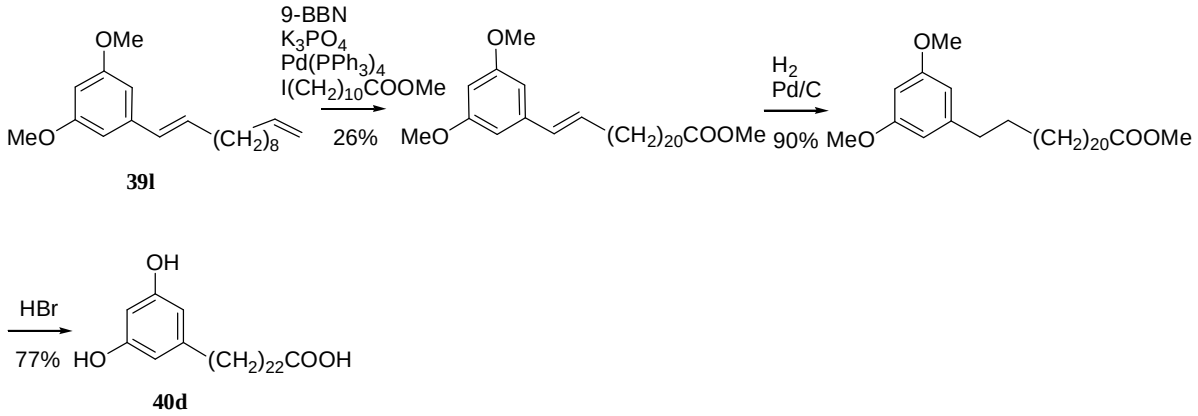
Figure 7. Novel AR haptens.

Haptens can be prepared by way of both routes A and B (Scheme 16). The best yield in the Wittig step was afforded by route A, utilizing the benzylic phosphonium salt **35** with the alkanal starting material **37d** (C_{16}) which was prepared by a Swern-type oxidation[165] (Scheme 17). Some of the alkanal precursor analogues with varying chain lengths are commercially available.



Scheme 17. Swern-type oxidation.

The Wittig product **39l** was a starting material for the C_{23} hapten **40d**, for which commercial alkanal or alkyl bromide precursors are not available (Scheme 18).

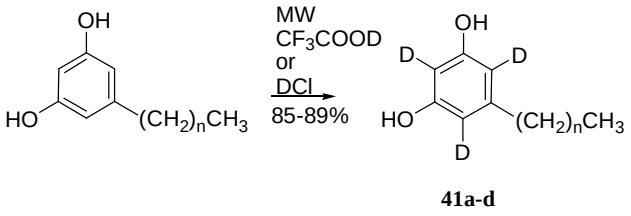


Scheme 18. The preparation of a C23:0 hapten derivative.

3.2. DEUTERIUM LABELLING

3.2.1. H/D exchange of 5-*n*-alkylresorcinols

Deuteration reagents such as DCl, CF_3COOD and $D_3PO_4*BF_3$ were explored in the acid catalysed H/D exchange experiments.[IV] The deuteration of the ARs was not complete when the reactions were done at room temperature ($D_3PO_4*BF_3$), under reflux (DCl, CF_3COOD) or under pressure ($D_3PO_4*BF_3$). Prolonged reaction times and experiments with strong acid ($D_3PO_4*BF_3$) lead to the decomposition of ARs. These problems were avoided by using MW heating. The fast MW catalysed reactions with DCl or CF_3COOD gave the D_3-alkylresorcinols **41a-d** in good yields and isotopic purity (Scheme 19).



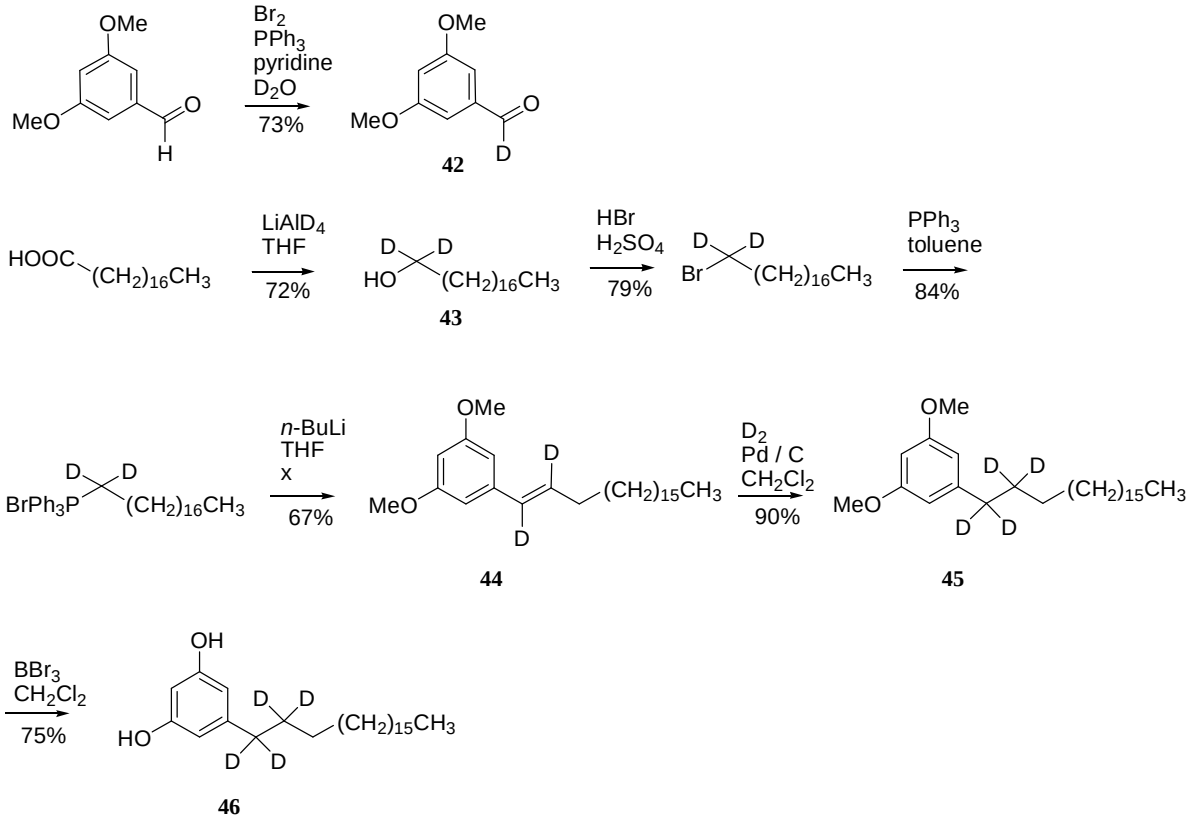
Scheme 19. H/D exchange. **a**, n=14; **b**, n=16; **c**, n=18; **d**, n=20

Scrambling problems[166] were observed when the mass spectra of the products **41** were run using EI (direct inlet). When an ESI technique (direct inlet) was used, no scrambling occurred and the mass spectra agreed with the NMR data for the products, in which no aromatic protons were seen. We suggest that these compounds should be useful as standards in LC-MS based analytical studies.

3.2.2. Total synthesis of deuterated 5-*n*-alkylresorcinols

3.2.2.1. Synthesis of D_4-nonadecylresorcinol

To avoid scrambling problems and ensure the stability of the labels in the various analytical and pretreatment operations that sample preparation may require, for example incubation under acidic conditions or derivatization, we studied the incorporation of four deuterium labels into the alkyl chain at the benzylic (α) position and the β site as shown in Scheme 20.[IV]

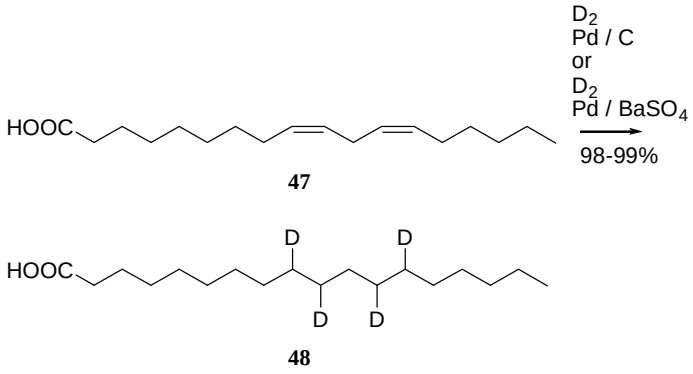


Scheme 20. Preparation of D_4-C19:0.

The labelling was done in a stepwise manner, involving the use of a C-1 deuterated aldehyde **42** (ArCDO) and a C-1 dideuterated primary alcohol **43** (RCD_2OH) as starting materials and catalytic C=C reduction using D_2 gas. The D_2-species **44** was homogenous by as determined MS (direct inlet) and NMR, but the reduced product **45** (and **46**) was contaminated by some

D_3-nonadecylresorcinol (up to 30%) and a lesser amount of D_2-nonadecylresorcinol (up to 10%). Thus it appeared that D/H scrambling occurred in the C=C reduction. The exchange and redistribution of hydrogen and deuterium has been observed previously in the deuterogenation of simple alkenes over a nickel catalyst, which gave a mixture of alkanes with varying deuterium content, however the D_4-derivative as the main product.[167-168]

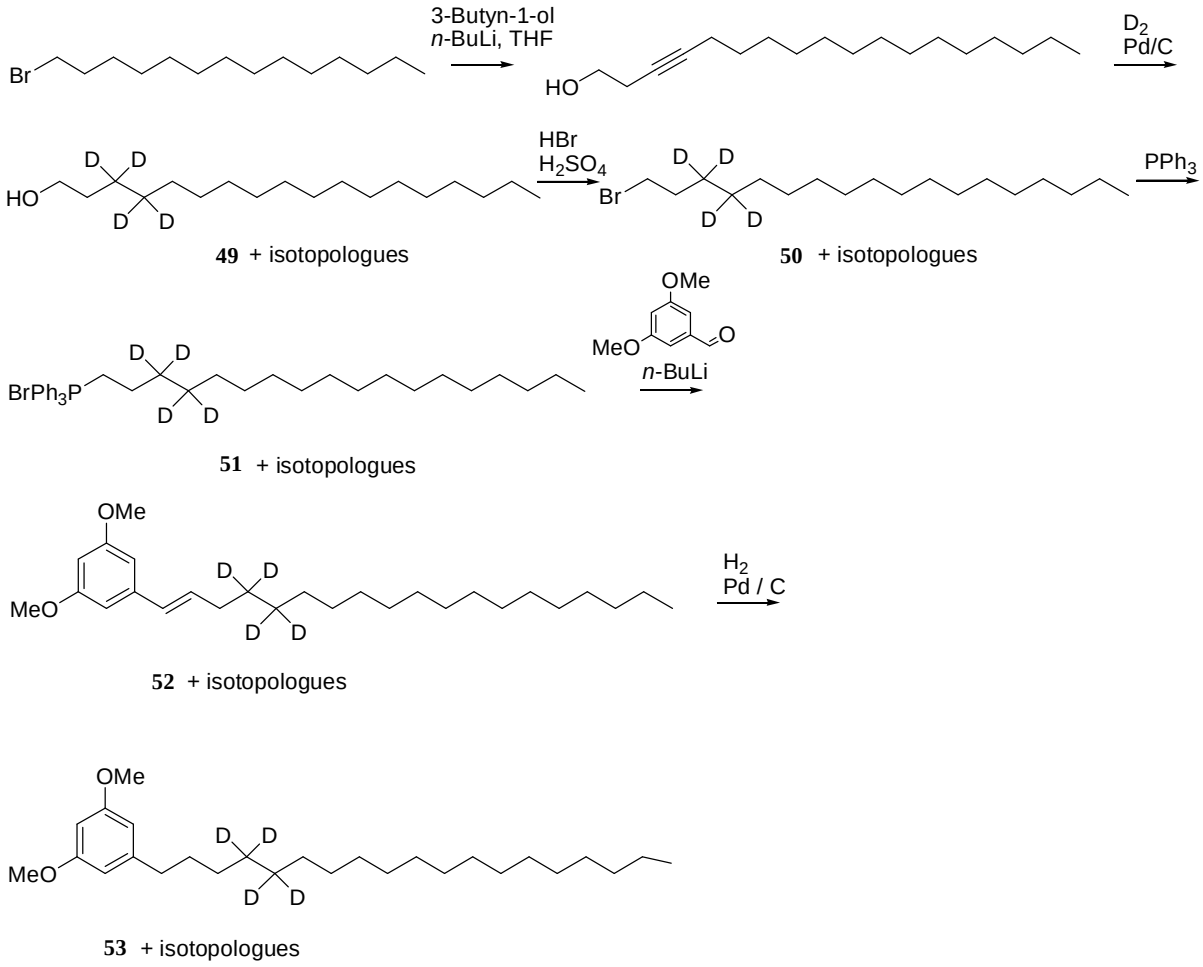
Another alkene reduction strategy was investigated.[IV] The deuterogenation of linoleic acid **47**, which is a potential starting material for alkylresorcinols, did not furnish the D_4-stearic acid **48** but gave instead a mixture of D_0 up to D_{15} isotopologues, D_4- and D_5-derivatives being the main products according to MS (Scheme 21). There were no differences between the products produced by the two catalysts used in the reaction (Pd/C or Pd/$BaSO_4$). Apparently, linoleic acid, with its methylene skipped diene structure, is much more prone to scrambling and redistribution reactions than the styrene derivative **44**.



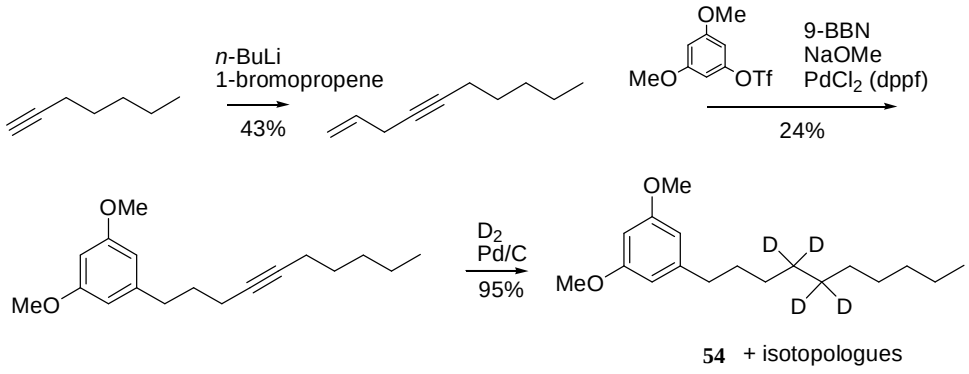
Scheme 21. Deuteration of linoleic acid and the expected D_4-stearic acid as the product.

3.2.2.2. Deuterogenation of acetylenes

D/H scrambling occurred in the reduction of the acetylenic starting materials by D_2 gas when two variations on another strategy based on the deuterogenation of an alkyne to alkane were studied (Schemes 22 and 23).[IV] Deuterium atoms were introduced not only to the acetylenic carbons but also elsewhere in the alkyl chain, resulting in a mixture of isotopologues (of **49** and the intermediates **50**-**52**) according to MS (direct inlet) and NMR. In the final alkylresorcinol derivative **53**, the D_4 species was the main product (ca. 29%) but D_2-, D_3- and at least D_5- and D_7-derivatives were present (approximately 11%, 20%, 20%, 12% and 7%, respectively). In the case of **54** the distribution of the products was different and the heavier species were more abundant compared with **53**. The D_3-, D_4-, D_5- and D_6-derivatives were the main constituents in **54** (18%, 20%, 20% and 19%, respectively).



Scheme 22. D_2 deuterogenation and the isotopologous products.



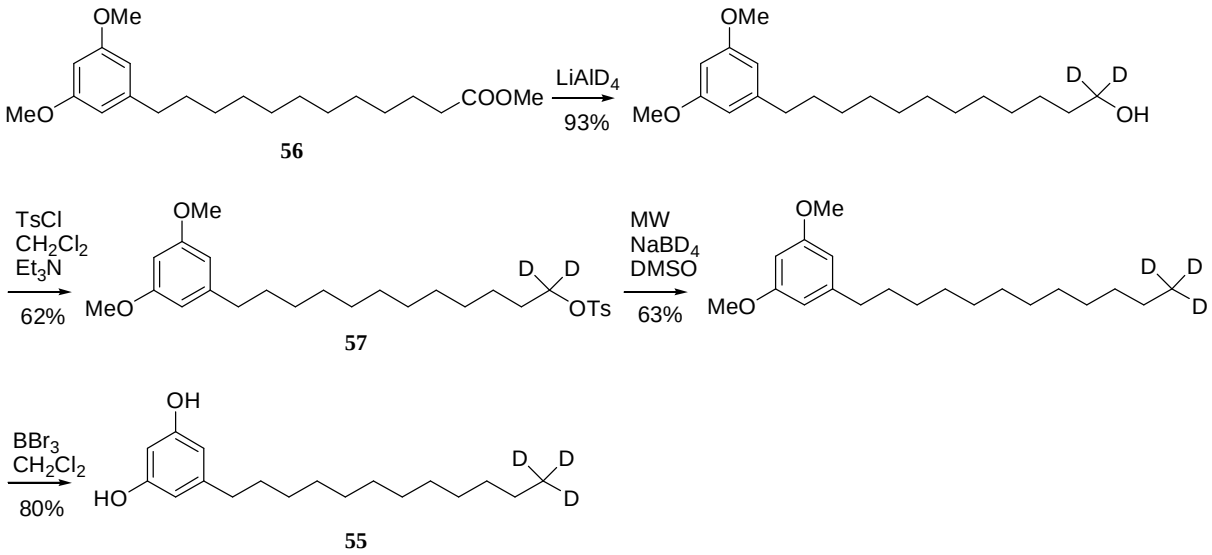
Scheme 23. D_2 deuterogenation of an alkynylresorcinol dimethyl ether.

H/D exchange and redistribution has not been reported to occur to a significant degree when alkynes are reduced selectively to alkenes[169] suggesting that in our case the scrambling occurs at the subsequent alkene reduction stage. However, in our experiments there was a difference between the products of the deuterogenation of either alkenes or alkynes. In contrast to the product **45** (and **46**), in which only D_2- and D_3-derivatives was present in addition to D_4, in

products **53** (and **49**-**52**) and **54** there were clearly significant amounts of D_5- and D_6-derivatives in the mixture. It seems that controlling the heterogeneous catalytic D_2 reduction of any olefinic or acetylenic starting material is problematic and specifically deuterated alkylresorcinols cannot be prepared in this way.

3.2.2.3. Synthesis of D_3-alkylresorcinols

Placing the D atoms at the far end of the alkyl chain in completely unactivating surroundings made available derivatives (for example, **55**) that are not vulnerable to any kind of D/H exchanges whatsoever, either in solution or in the mass spectrometer.[IV] A 5-(ω-methoxycarbonylalkyl)resorcinol dimethyl ether **56** may be converted in four steps to ω-D_3-alkylresorcinol with an isotopic purity in excess of 95% (Scheme 24). In the first step, two D atoms were introduced by $LiAlD_4$ reduction of the ester. The tosylate **57** was conveniently reduced by $NaBD_4$ in DMSO in 8 minutes under MW irradiation.

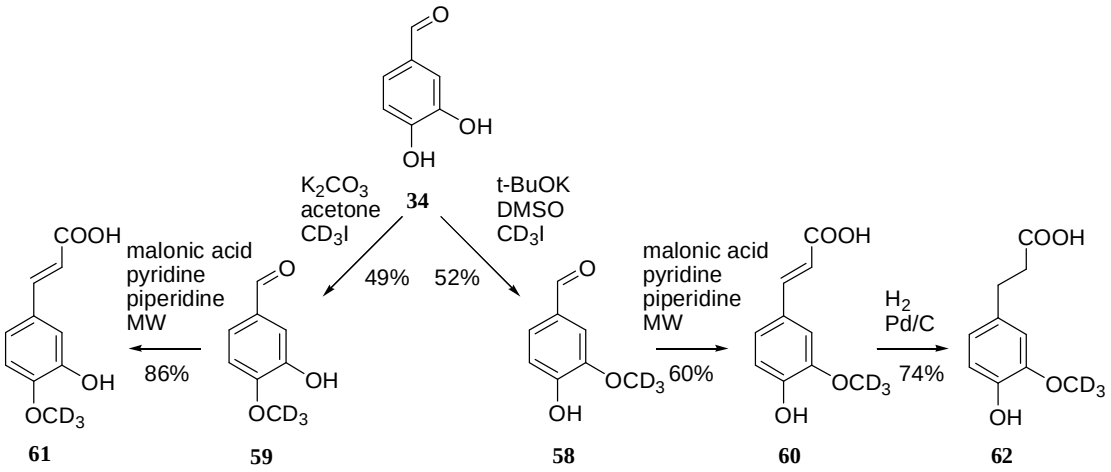


Scheme 24. Synthesis of pure and stable D_3-AR.

3.2.3. Preparation of D_3-ferulic acid and related compounds

We investigated the selective methylation of 3,4-dihydroxybenzaldehyde **34** with CD_3I.[V] The methylation of the 4-hydroxy or the 3-hydroxy group of **34** gave D_3-vanillin **58** or D_3-isovanillin **59**, respectively. Several combinations of bases and solvents were tested and both K_2CO_3/acetone and a new combination of a base and solvent, *t*-BuOK/DMSO, were found to selectively give the product (Scheme 25). Under these conditions, only one monomethylated product was formed, and the formation of minor amounts of di-*O*-methylated product (D_6-3,4-dimethoxybenzaldehyde) was not of a consequence as it was easily separated from the products by flash chromatography. The reaction of **58** or **59** with malonic acid catalysed by

MW irradiation gave D_3-ferulic acid **60** or D_3-isoferulic acid **61**, respectively. The previously described preparation of unlabelled FA catalysed by MW irradiation in a domestic MW oven[151] was not reproducible in the CEM Discover system as the yield of **60** we obtained was, at best, 60%. However, we found that the MW catalysed reaction worked very well in the preparation of **61**. Hydrogenation of **60** by H_2 gave D_3-dihydroferulic acid[170] **62** (Scheme 25).



Scheme 25. Preparation of D_3-FA and related D_3-labelled compounds.

3.3. BIOACTIVITY

We used the prepared ARs (C15:0, C17:0, C19:0, C21:0 and C23:0) to investigate bioactivity. The derivatives tested are those present in whole grain products, and the experiments in our study model some actions that these ARs might have in the human body.

3.3.1. Antioxidant activity

3.3.1.1. FRAP assay

We compared the antioxidant power of ARs to the activity of FA.[II] Alkylresorcinols gave a linear, dose-related response, but their activity was only approximately 10% of the activity of FA (Figure 8).

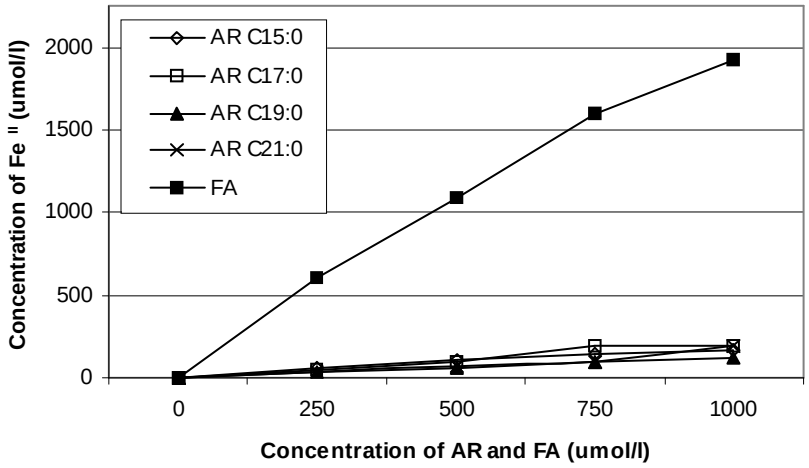


Figure 8. FRAP assay.

3.3.1.2. DPPH assay

We again compared the radical scavenging abilities of ARs and FA in the DPPH assay.[II] The results were similar to those obtained from the FRAP assay; the antioxidant power of ARs once again was lower than that of FA (Figure 9). A small difference in the activity of C15:0 and C21:0 might suggest that the alkyl chain length affects the system.

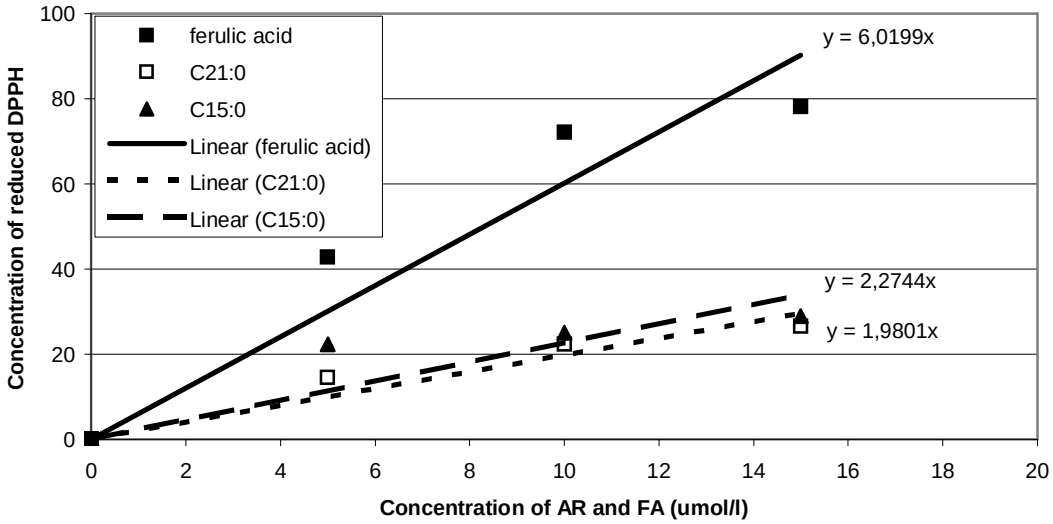


Figure 9. DPPH assay. C17:0 and C19:0 are not included in the chart but give approximately same response as C15:0 and C21:0.

3.3.1.3. Inhibition of LDL oxidation

The Cu(II)-induced oxidation of LDL was delayed by adding ARs to the LDL solutions.[II] Of the concentrations of ARs tested, the 25 µM solutions of C15:0 and C17:0 gave the best protection from oxidation, increasing the lag time by 65 min and 40 min, respectively. However, an AR concentration as low as 2.5 µM also had a protective effect, but the increase in the observed lag time was only approximately 15 min (Figure 10). The solubility of the longer alkyl chain ARs (C19:0, C21:0 and C23:0) in the test medium, 5% MeOH in H_2O, was problematic and they had to be excluded from the study. A 5% MeOH solution had to be used instead of water to enhance the solubility of the ARs tested. Since the human body is a purely aqueous environment, the lowest possible concentration of MeOH was used so that conditions would closely resemble those *in vivo*. The absorbance of a sample containing 5% MeOH only was measured and it did not differ from the control sample.

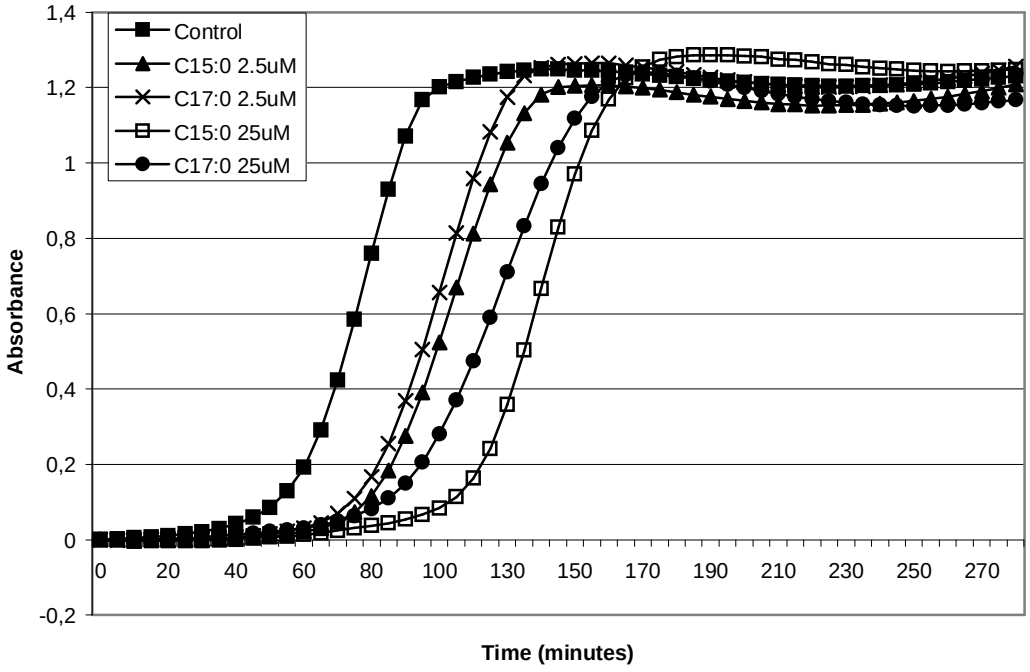


Figure 10. The effect of AR on Cu(II) induced oxidation of LDL. Absorbance of conjugated dienes as a function of time.

3.3.2. Antigenotoxicity

We chose the colorectal cell line HT29 for the antigenotoxicity tests.[II] Colorectal cancer is the most common cancer in western countries after lung and breast cancers.[171] Oxidative DNA damage is an important mutagenic and apparently carcinogenic factor.[172] The HT29 cell line has been widely used as a model for studying colon carcinogenesis in combination with the

single cell gel electrophoresis (comet) assay which measures single strand DNA damage.[173-175] We quantified the damage by measuring the length of the tail of the so-called comets, which were formed from the damaged DNA in electrophoresis. H_2O_2[176-177] and faecal water[174] have been commonly used as the DNA damaging agents.

3.3.2.1. Inhibition of H_2O_2 induced damage

In the experiments, the HT29 cells were incubated with ARs, then ARs were removed and H_2O_2 added to the cells. The protective effects were investigated using various incubation times and concentrations of ARs. A 24h incubation with a 100 µM concentration of AR (C15:0, C17:0, C19:0, C21:0 or C23:0) gave the most significant results. The protective effects of C15:0 and C17:0 were almost the same. The protective effects of C19:0 and C21:0 were also statistically significant. The inhibition of DNA damage was much lower with shorter incubation times, e.g. 1h (Figure 11).

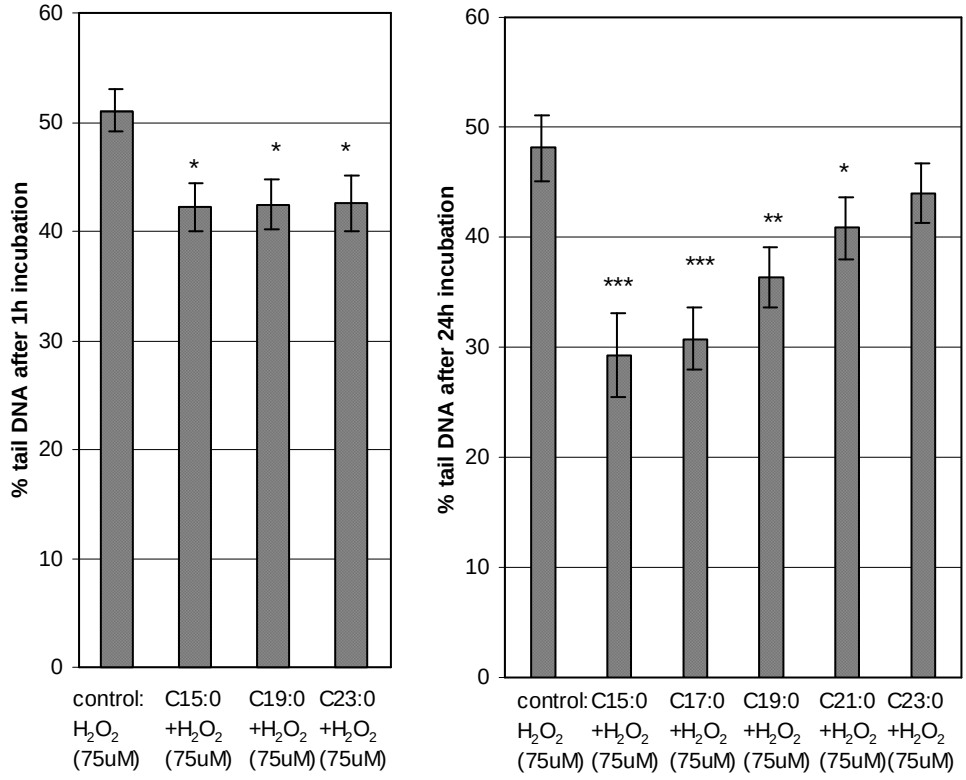


Figure 11. Inhibition of H_2O_2 induced damage at a 100µM concentration of AR. The asterisks represent the significance of the results (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$) compared with the control.

3.3.2.2. Inhibition of faecal water induced damage

Approximately 30% of all faecal water samples from human volunteers are highly genotoxic towards human colon cells.[174] Thus faecal water is highly useful in *in vitro* experiments on anticancer activity in the colon and the results should be relevant.

We investigated the antigenotoxic effect of selected ARs (C15:0, C19:0 and C23:0) on faecal water induced damage (Figure 12). The protective effect of C15:0 at a 50 μM concentration was clear and approximately the same as in the experiments with H_2O_2. The result of C15:0 was also, according to statistical calculations, the only significant result, however, C19:0 seemed to possess some activity. In this case the 100 μM concentration of AR, which was the most effective in the H_2O_2 experiments, gave very similar results to the 50 μM incubation, but the results had lower significance ($P < 0.05$). The result of the experiment with the lowest concentration of ARs tested (10 μM) was not statistically significant but indicated that also the longer alkyl chain ARs (C19:0 and C23:0) might have some protective effect toward the cells.

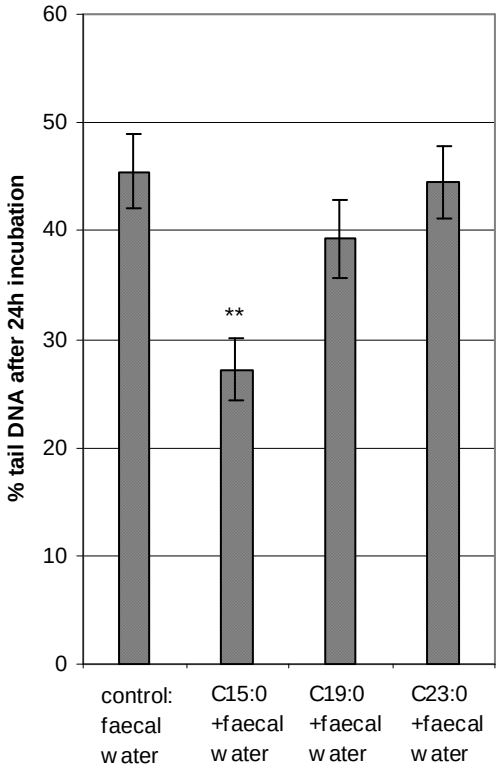


Figure 12. Inhibition of faecal water induced damage at a 50μM concentration of AR. The asterisks represent the significance of the results (**; $P < 0.01$) compared with the control.

To summarize the antigenotoxicity results, AR seem to be incorporated into cell walls because they are able to protect the cells against oxidative damage after the incubating medium, and AR in it, has been removed. The 100 µM concentrations of ARs, which were most effective in decreasing the oxidative DNA damage, are ca. 30 times higher than the concentration found in plasma.[I] It should be noted, however, that tissue concentrations might be higher than those in plasma. In the case of isoflavones, for example, the concentration in prostate tissue has been shown to be as much as 10 times higher than the plasma level.[178] The biological activity of ARs in these assays appears to be dependent on their chain length. Based on the results, the dependence may be a consequence of solubility, which is limited because of the extended nonpolar alkyl chain and decreases with increasing chain length.

4. CONCLUSIONS

5-*n*-Alkylresorcinols and their hapten derivatives were prepared by methods that utilized the microwave catalysed Wittig reaction as a key step. The reactions of a semi-stabilized ylide and alkanals gave good yields in pressurized or open systems in water without an organic solvent. Alternative microwave catalysed reactions of 3,5-dimethoxybenzaldehyde and non-stabilised alkyltriphenylphosphonium salts in DMSO/H_2O were also successful. Thus the methods are suitable for various alkylresorcinol and related compound precursors without need for solubility enhancing groups in their structure.

Various labelling techniques were investigated, including H/D exchange and total synthesis approaches. The ring-labelled D_3-ARs were obtained in approximately 90% isotopic purity. This type of compound would be practical in analytical studies that are based on electrospray ionisation MS. The D_4-AR derivative labelled at the benzylic α-site and at the β-site might be utilized as a standard, since the M^+ of the D_2-species does not overlap with any peak of the unlabelled analogue. The alkyne deuterogenation strategies investigated lead to extensive H/D scrambling along the alkyl chain. These studies on various deuteration strategies showed that the deuteration method giving the D_3-AR with the labels at the end of the alkyl chain was the most reliable. The product was obtained in good yield and its isotopic purity exceeded 95%. No H/D scrambling is expected under any conditions. Deuterated alkylresorcinols prepared by this synthesis route would constitute highly suitable standards for metabolic and analytical studies. The labelled D_3-ferulic acid and related D_3-labelled compounds were obtained in good yield. The products were pure and isotopically stable and thus suitable for analytical work.

The *in vitro* experiments on the biological activity of ARs agreed with some of the previous results, showing that ARs possess biological activity, and especially antioxidant activity, which is strengthened when ARs are incorporated into a biological system. However, substantial investigation and *in vivo* experiments still need to be done to establish how ARs behave in the human body.

5. EXPERIMENTAL

The experimental part of the work that has not been included in the studies (**I-V**) is described in this section.

5.1. General

MW catalysed reactions were performed in a CEM Discover system. NMR spectra were obtained with a Varian 300 MHz spectrometer using tetramethylsilane as an internal standard. MS spectra were obtained with a JEOL JMS SX102 mass spectrometer operating at 70 eV or with a Varian Saturn 2000 instrument.

5.2. D_3-vanillin (58)

3,4-Dihydroxybenzaldehyde **34** (14.5 mmol) and *t*-BuOK (30 mmol) were stirred in DMSO (10 ml) for 1.5h. CD_3I (14.5 mmol) was added and the stirring continued overnight. Water was added and the solution acidified with dilute HCl. The product was extracted with EtOAc. The combined extracts were washed with water and dried over $MgSO_4$. Purification by flash chromatography eluting with hexane/acetone 1:1 gave **58** in 52% yield. ^{1}H NMR (D_6-acetone): δ 7.12 (1H, d, *J* = 8.4 Hz), 7.44 (2H, m), 8.58 (1H, s), 9.82, (1H, s). MS (EI): m/z (%) 81 (20), 109 (12), 126 (10), 154 (100, M^+), 155 (60).

5.3. D_3-isovanillin (59)

3,4-Dihydroxybenzaldehyde **34** (14.5 mmol), K_2CO_3 (29 mmol) and CD_3I (14.5 mmol) were refluxed in acetone (15 ml) for 2h. Water was added and the solution acidified with dilute HCl. The product was extracted with EtOAc. The combined extracts were washed with water and dried over $MgSO_4$. Purification by flash chromatography eluting with hexane/acetone 1:1 gave **59** in 49% yield. ^{1}H NMR (D_6-acetone): δ 7.01 (1H, d, *J* = 7.8 Hz), 7.35 (1H, d, *J* = 2.1 Hz), 7.43 (1H, dd, *J* = 7.8 Hz and *J* = 2.1 Hz), 8.04 (1H, s), 9.83, (1H, s). MS (EI): m/z (%) 81 (18), 109 (14), 126 (11), 154 (100, M^+), 155 (61).

5.4. D_3-ferulic acid (60)

The benzaldehyde **58** (1.35 mmol), malonic acid (2.7 mmol), pyridine (0.5 ml) and piperidine (0.01 ml) were MW irradiated at 90ºC for 10 minutes in an open flask. Water and dilute H_2SO_4 were added and the product extracted with Et_2O. Combined extracts were dried over $MgSO_4$. Purification by flash chromatography eluting with MeOH/CH_2Cl_2 1:4 gave **60** in

60% yield. ^{1}H NMR (D_6-acetone): δ 6.36 (1H, d, *J* = 15.9 Hz), 6.87 (1H, d, *J* = 8.4 Hz), 7.11 (1H, dd, *J* = 8.4 Hz and *J* = 2.1 Hz), 7.28 (1H, d, *J* = 2.1 Hz), 7.60 (1H, d, *J* = 15.6 Hz), 7.99 (1H, s). MS (EI): m/z (%) 133 (10), 179 (13), 197 (100, M^+), 198 (17).

5.5. D_3-isoferulic acid (61)

The benzaldehyde **59** (1.87 mmol), malonic acid (3.74 mmol), pyridine (1 ml) and piperidine (0.01 ml) were MW irradiated at 90ºC for 15 minutes in an open flask. Addition of water and dilute H_2SO_4 crystallized the product. A wash with water gave pure **61** in 86% yield. ^{1}H NMR (D_6-acetone): δ 6.30 (1H, d, *J* = 15.9 Hz), 6.95 (1H, d, *J* = 8.4 Hz), 7.10 (2H, m), 7.56 (1H, d, *J* = 15.6 Hz), 7.71 (1H, s). MS (EI): m/z (%) 133 (25), 151 (15), 180 (35), 197 (100, M^+), 198 (15).

5.6. D_3-dihydroferulic acid (62)

D_3-Ferulic acid **60** (0.28 mmol) was stirred in EtOH (20 ml) with Pd/C (6 mg, 10% w/w). H_2 gas was introduced to the reaction mixture at atmospheric pressure and room temperature. Filtration of the reaction mixture through Celite® and flash chromatography eluting with MeOH/CH_2Cl_2 1:4 gave pure **62** in 74% yield. ^{1}H NMR (D_6-acetone): δ 2.55 (2H, t, *J* = 7.8 Hz), 2.82 (2H, t, *J* = 7.8 Hz), 6.70 (2H, m), 6.84 (1H, m), 7.87 (2H, br s). MS (EI): m/z (%) 83 (12), 97 (10), 122 (10), 140 (100), 199 (93, M^+), 200 (10).

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