Chapter 1

General Introduction

1 Why do we need a change in plant protection?

All over the world, farmers utilize synthetic pesticides to protect crop plants against all kinds of pests. Their application is controversial. It ensures high crop yields, which are necessary to feed the world, but the development of the active ingredient aims in harming living systems, making it a hazard to human health and wildlife (Osman, 2011). One of the main objectives of organic farming is the protection of humans and nature against these negative effects. The European Union regulation No. 834/2007 only permits the use of synthetic pesticides if it is compatible with the objectives and principles of organic processing. Their utilization is restricted to the application after an identified threat and to a positive list of registered products (article 16). In general, suitable prevention measures like crop rotation, fertilization, tillage, or selection of species should control pests. Nevertheless, this cannot always prevent the spread of plant diseases (Julius Kühn-Institut, Bundesforschungsinstitut für Kulturpflanzen). This particularly concerns infections with phytopathogenic fungi, which occur predominantly in warm and humid summers and can cause enormous crop losses. Suitable measures against the spread of such phytopathogens are still lacking in organic farming, resulting in the continuing permission of, e.g., copper-containing plant protection products (PPP). These fungicides are highly effective and act as inhibitors of essential enzymatic reactions of the phytopathogenic fungi. Their application leads to an accumulation of copper in the soil, which is regarded as a hazard, in particular for microorganisms and mollusks. In order to minimize the impact on nature, the EU Eco-regulation permits a maximum amount of copper but there are endeavors to reduce the applied amounts

further. A complete prohibition fails due to lacking alternatives which organic farming urgently needs. (Kuehne et al., 2017)

Investigation and utilization of the natural defense mechanisms of plants may be a starting point. Such systems are often based on secondary plant metabolites, which are already applied as bioactive substances in the pharmaceutical, cosmetics, and food industries. The use of secondary plant metabolites as active ingredients in a pesticide formulation necessitates research in different disciplines. This includes analytical chemistry to characterize the isolated compounds from plant extracts, followed by microbiological evaluations to detect possible target organisms and to determine effective concentrations, and finally, the integration of the active compound(s) in a pesticide formulation. All these steps were part of this thesis and the fundamental background is briefly reviewed in the following, focusing on two phenolic classes 5-*n*-alk(en)ylresorcinols (AR) and ferulic acid (FA), which are both found in species of the family of the grasses (Poaceae).

2 Phenols – a heterogeneous group of secondary plant metabolites

The distribution of secondary plant metabolites in the plant kingdom is ubiquitous. Plants only contain small quantities of this extremely diverse substance group in comparison to the primary metabolites - mainly carbohydrates, amino acids, and lipids. Therefore, these compounds were partly regarded as a caprice of nature before different scientific disciplines, e.g., medicine, pharmacology, and nutritional science, focused on them. It was realized that the key to the survival of plants in an environment full of enemies lies in the defense mechanisms of the secondary metabolites. Because of their fixed location and the lack of an immune system, plants need to protect themselves by using antigens and semiochemicals. The activities and interactions of these compounds are diverse due to over 200,000 described secondary plant metabolites. Humans have utilized the benefit of these compounds since the year one, be it toxics for arrow poison, the healing activity of plant extracts, or the effect of intoxicants. Secondary plant metabolites are classified in nitrogen-containing and nitrogen-free groups with a variety of subgroups. The work focuses on the group of phenols, which belongs to the nitrogen-free secondary metabolites and is present in

almost all kinds of plants (Wink, 2015). Phenolic compounds are often located in the outer layers of fruits and vegetables. Due to their exposure to abiotic and biotic stressors, they are assumed to offer a protective function for the plant. During food processing, the outer layers of fruits and vegetables are often removed and occur as by-products. Phenolic compounds accumulate in such side stream products, which offer an inexpensive source for the recovery of these valuable compounds. A large number of these secondary plant metabolites is consumed on a regular basis and is, therefore, regarded as safe, but toxicological assays with pure and higher concentrated compounds need to confirm their safety (Schieber, 2017). The food and dietary supplement industries often advertise products with high contents of phenols as antioxidant, but a supposed health benefit is debatable. While beneficial health effects of phenol-containing foods are generally uncontroversial, the mode of action of phenolic compounds is not fully understood. Most phenolic compounds reach the bloodstream only in limited amounts, due to their low bioavailability. The metabolism of phenols in the intestine seems to be crucial for their bioactivity, making the metabolites the effective compounds rather than the phenolic parent substances (Kuhnert, 2013). While research on the mode of action of phenols in the human body is still at the beginning, the bioactivities regarding the protection of plants against pests have already been investigated in more detail - such as antifeedants against insects and other animals or the protection against phytopathogens. Studies on the antimicrobial activities of such compounds provide fundamental knowledge on the natural protection system of plants and their potential application as natural preservatives or active ingredients in pesticides. In plant extracts, the synergistic interaction of secondary plant metabolites increases the activity against pests and reduces the probability of developing resistance against these multi-target compounds. In contrast, the utilization of mono-target compounds in synthetic pesticides increases the number of resistant microorganisms. Furthermore, it is assumed that breeding intentionally reduced the amount of secondary plant metabolites, whereby the plants lost their natural defense mechanism (Wink, 2015).

2.1 5-*n*-Alk(en)ylresorcinols

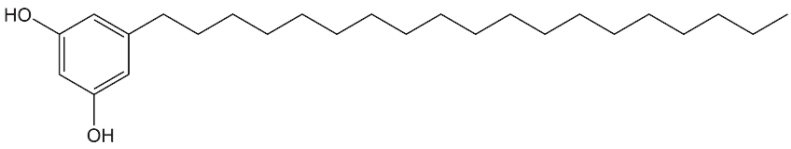

Figure 1.1: 1,3-Dihydroxy-5-nonadecylbenzene (AR C19:0)

From a chemical point of view, 5-*n*-alk(en)ylresorcinols (1,3-dihydroxy-5-*n*-alkylbenzenes) possess an amphiphilic character due to a benzene ring with two hydroxy groups (position 1 and 3) and a lipophilic alk(en)ylchain substituted on position 5 of the benzene ring. Figure 1.1 shows the structural formula of an AR C19:0. Comparable to fatty acids, the alk(en)ylchain varies in its length, the degree of saturation, and the presence of hydroxy or methoxy groups. AR are widely distributed in nature and are found in several plant families, some microorganisms, mosses, and marine sponges (Kozubek & Tyman, 1999; Landberg et al., 2014). For the recovery of these phenolic lipids, the family of the grasses is a valuable source, where biosynthesis is derived predominantly from type III polyketide synthase-associated pathways (Baerson et al., 2010). The AR in the Poaceae family constitute a mixture of numerous homologues, with the alkyl chain length ranging from 15 to 27 C-atoms and with up to three double bonds and the occurrence of monounsaturated hydroxylated derivatives (Zimmermann et al., 2017). The influence of the hydrophobic part of the molecules is predominant, making AR basically water-insoluble (Ross et al., 2004). The amounts of AR in rye (*Secale cereal* L.) and wheat (*Triticum* L.) kernels vary strongly between 268–1444 $\mu g \cdot g^{-1}$ and 220–943 $\mu g \cdot g^{-1}$ dry matter, respectively. Species-dependency but also environmental factors, such as soil composition, fertilization, and treatment with pesticides cause differences in the AR concentration (Landberg et al., 2014). The AR profiles of different cereals serve as their fingerprints, but a congruent profile within the genus *Triticum* - for example, wheat and spelt - makes chemotaxonomic differentiation difficult (Knödler et al., 2008; Zarnowski et al., 2003). Only rye contains considerable amounts of minor AR (20%) - unsaturated and hydroxylated AR - compared to wheat (7%), triticale (6%), and only trace amounts in barley cultivars (Ross et al., 2003). Figure 1.2 shows the AR profiles of wheat and rye.

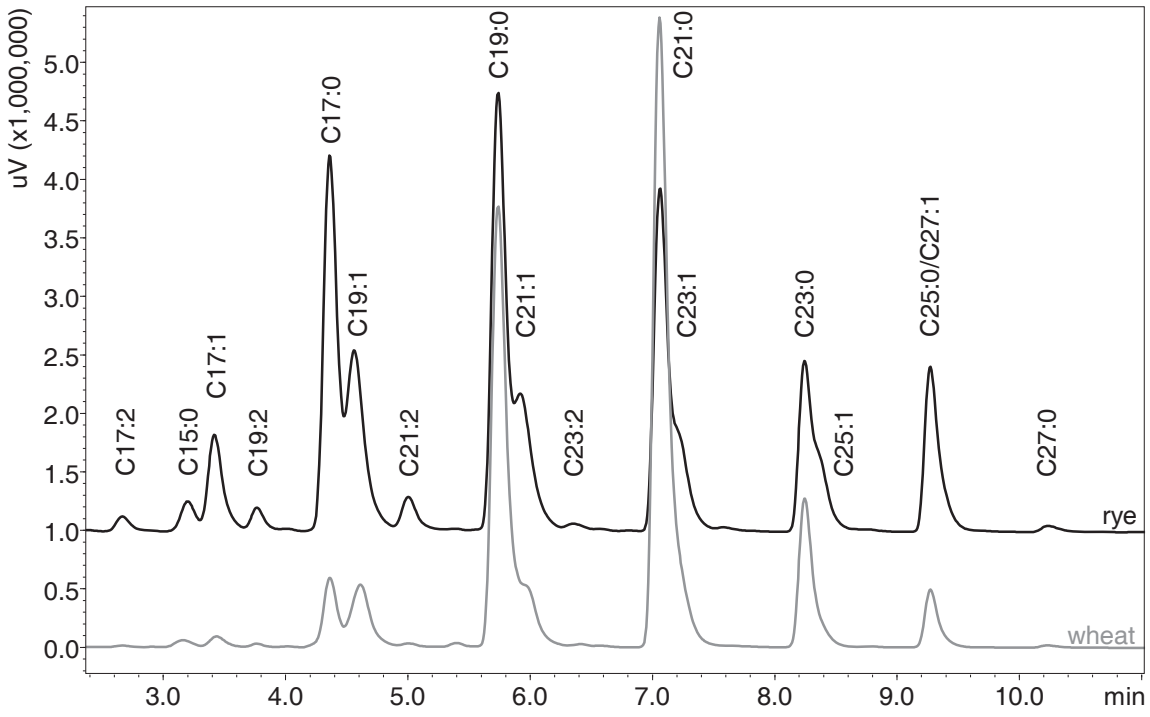

Figure 1.2: Separation of 5-*n*-alk(en)ylresorcinols from wheat and rye acetone extracts by UHPLC with fluorescence detection (excitation 276 nm, emission 306 nm).

In countries where the consumption of wholegrain rye and wheat is common, the dietary intake is high, e.g., up to 18 mg AR per day in Sweden (Ross et al., 2005). The human body absorbs AR and Landberg et al. (2014) detected low micro-molar amounts of the intact form in the blood plasma, erythrocytes, and the adipose tissue. The catabolic pathway is expected to be similar to the metabolism of tocopherols, and the main metabolites 3,5-dihydroxybenzoic acid and 3-(3,5-dihydroxyphenyl)-1-propanoic acid were found in human urine. AR and their metabolites are promising biomarkers for whole grain intake (Landberg et al., 2014). AR are supposed to induce skin irritation or even dermatitis but mainly alk(en)ylcatechols cause these problems and it requires several oxidation steps to reveal an allergenic *o*-quinonic form from AR (Knödler et al., 2009; Musehold, 1979).

2.1.1 Recovery and isolation

Determining the bioactivity of a compound and its mode of action in detail requires the previous isolation or synthesis of the active compound. The availability of unsaturated

AR reference substances is lacking and, therefore, the application of expensive synthesis or the isolation from crude extracts is necessary (Ross et al., 2004). The latter was part of this thesis, which enabled the recovery of adequate amounts of highly purified saturated and unsaturated AR from rye bran for further bioassays (see Chapter 3). This section briefly reviews the state-of-the-art methods for the extraction and separation of AR, with particular attention being paid to preparative applications and techniques for the fractionation of saturated and unsaturated AR homologues.

Whole grains and brans of the Poaceae family have commonly been used as a source for the extraction of AR. Milling of kernels prior to extraction leads to a decrease in extraction time and an increase in total extract weight while the amount of AR remains constant (Ross et al., 2004; Landberg et al., 2007). Since co-extracted materials might interfere with subsequent chromatographic analysis, Landberg et al. (2007) recommend the extraction of AR from intact grains. The application of organic solvents such as acetone, ethyl acetate, methanol, ethanol, diethyl ether, chloroform, cyclohexane, or hexane is necessary, due to the poor water solubility of AR from cereal grains (Ross et al., 2004). The use of acetone and ethyl acetate is common and provides the highest AR yields, whereas *n*-hexane only extracts small amounts of AR (Ziegler et al., 2015; Zarnowski & Suzuki, 2004). Extraction with methanol leads to some discrimination against longer AR (Ross et al., 2004). Without further purification, extracts contain low amounts of AR but an excess of co-extracted ballast substances (Zarnowski & Suzuki, 2004). The extraction process is generally conducted in flasks, partly with stirring or shaking, at room temperature for up to 72 h (Zarnowski & Suzuki, 2004). Agil et al. (2012) identified a temperature of 24 °C, a duration between 16 and 24 h, and a solid-to-solvent (acetone) ratio of 1:40 as optimum conditions for AR extraction from triticale bran. Further applied techniques are Soxhlet extraction (Zarnowski & Suzuki, 2004), pressurized liquid extraction (Holt et al., 2012), and ultrasound-assisted extraction (Geerkens et al., 2015). Supercritical CO_2 ($scCO_2$) (Athukorala et al., 2010; da Cruz Francisco et al., 2005; Rebolleda et al., 2013) yielded an AR profile and amount comparable to those obtained by conventional extractions (Landberg et al., 2007). For purification of AR, a two-step approach was useful (Athukorala et al., 2010). A first extraction step with $scCO_2$ led to the removal of non-polar lipid components like mono-,

di-, or triglycerides and phospholipids, which constitute the major components in organic solvent extracts from grains or brans (Athukorala et al., 2010; da Cruz Francisco et al., 2005). In a second step, the addition of co-solvents like ethanol or methanol yielded in high amounts of AR due to their amphiphilic character (Athukorala et al., 2010).

Liquid chromatography (LC) has most commonly been used for the separation of AR, for both analytical and semi-preparative purposes. (U)HPLC systems are used for rapid routine analysis of AR. The analysis time was reduced from 90 min in HPLC to 14 min in UHPLC (Ross, 2012). Whereas separation was previously carried out using normal-phase columns (Mullin et al., 1992), nowadays only reversed-phase columns are used. Column temperature varies from room temperature to 60 °C (Ross, 2012). Due to the lipophilic structure of AR, eluents need a high portion of organic solvents. First published gradient programs started with 100% water and increased up to 100% methanol (Ross et al., 2004). However, shorter programs require high levels of organic solvent in the starting conditions of the gradient, e.g., 89% methanol in a UHPLC method. In comparison to gas chromatography (GC), an LC system can be coupled to a wider range of detectors, namely diode array, fluorescence, or electrochemical detectors. The latter detector provides a higher sensitivity than UV/Vis detectors (Ross & Sunil, 2009). Absorption spectra of AR show three local maxima between 203–210 nm, 275–281 nm, and 280–283 nm (Knödler et al., 2008). Although (U)HPLC is suitable for the separation of AR, complex AR profiles lead to co-elution of saturated and unsaturated homologues on C18 columns, resulting in an incomplete separation of extracts with high amounts of unsaturated AR (e.g., rye). Thus, quantification of the minor AR derivatives should be handled with caution (Ross & Sunil, 2009). Zimmermann et al. (2017) developed a UHPLC method based on an octyl phase that provides an enormous improvement in the separation efficiency of AR and the quantification of minor AR. It is assumed that the use of semi-preparative HPLC systems for the isolation of individual AR homologues is unfeasible for complex extracts or might lead to suitable results only when several purification steps are applied. Until now, only argentation chromatography allowed the recovery of individual AR on semi-preparative scale (Kozubek & Tyman, 1995; Gubernator et al., 1999). This technique is adapted from the separation of fatty acid mixtures. The double bonds of the alkyl chain

form reversible π-complexes with the Ag^+-ions, which show an increased stability with an increasing number of double bonds. Argentation chromatography is based on the differing stability of the complexes and their separation with thin-layer chromatography (TLC) or column chromatography, not only enabling the fractionation of saturated from unsaturated fatty acids but also according to the number of double bonds, and even their conjugation (Belitz et al., 2008). The use of an $AgNO_3$ impregnated silica gel (12%, w/w) packed column and elution with chloroform/methanol (85:15, v/v) led to saturated, mono-, and diunsaturated AR fractions. Subsequent isolation of AR homologues was accomplished on RP18 silica gel columns with an isocratic elution using methanol/water (94:4, v/v) (Gubernator et al., 1999). Methods for large-scale isolation of individual AR homologues are currently missing (Ross et al., 2004). TLC is used for analytical and semi-preparative purposes. Like in LC, complete separation of individual AR homologues necessitates argentation chromatography (Kozubek & Tyman, 1995; Kozubek, 1984). Initially performed as a two-dimensional TLC system, the separation of homologues was later conducted on silver nitrate-impregnated silica gel plates and elution with chloroform/ethyl acetate (70:30, v/v) (Kozubek & Tyman, 1995). Fast Blue B salt is widely used as a coloring agent and forms pink to deep crimson stains depending on the chain length of the analytes (Kozubek, 1984). AR treated with a vanillin solution form bright red spots (Mullin & Collins, 1991). Application of TLC is difficult in adaptation for quantitative analysis and very time-consuming when used for semi-preparative purposes (Mullin & Collins, 1991), due to the limited amount of loaded extract. Furthermore, argentation chromatography methods are expensive (Bruce et al., 1990).

GC systems have also been applied for the quantitative analysis of AR. Compared to HPLC methods, GC provides an improved chromatographic resolution of AR in complex extracts and a more accurate quantification (Ross & Sunil, 2009). GC-flame ionization detectors with a detection limit of 5 $\mu g \cdot g^{-1}$ sample are commonly used. The stationary phase is generally non-polar, e.g., 100% dimethylpolysiloxane or 5% phenyl-methylpolysiloxane. A previous derivatization step is optional (trimethylsilyl ethers and ethylation) (Ross et al., 2004).

In this thesis, low-temperature crystallization was used as a means for the separation of AR homologues (see Chapter 2). This method adapted from fatty acids analysis allows the separation of saturated from unsaturated fatty acids. Temperature-dependent precipitation of fatty acids depends on their solubility in a solvent, which decreases with an increasing number of C-atoms and increases with the number of double bonds (Brown & Kolb, 1955). Solubility is solvent-dependent, e.g., ethanol is superior to methanol and acetone (Brown & Kolb, 1955), whereby the crystallization process may even be conducted without solvents (Strohmeier et al., 2014). The separation requires a fatty acid concentration between 5 and 15% and various filtration methods allow the recovery of the precipitate (Strohmeier et al., 2014; Fontell et al., 1960). This process is easy to handle and does not require reagents other than the solvents. The occurrence of chemical processes, e.g., oxidation, is unlikely due to the low temperature. The process enables a precise separation of saturated from unsaturated fatty acids, whereas co-crystallization affects the separation of monoene and polyene acids. The use of urea or silver ions can meet such analytical challenges (Fontell et al., 1960).

2.1.2 Antifungal activity

Various assays have proved the bioactivity of AR (Stasiuk & Kozubek, 2010). Their localization and accumulation in the outer parts of plants led to the assumption that these compounds primarily have a defensive role (Baerson et al., 2010). Regarding the objective of this work, this section focuses on the present literature of the antimicrobial or, more specifically, the antifungal activity of AR. Different approaches have been reported, starting with the application of crude AR-rich extracts, followed by purified fractions, and finally the testing of isolated AR homologues.

Antibacterial effects against methicillin-resistant *Staphylococcus aureus* have been published for AR from the mushroom *Phlebia incarnata*, with an alkyl chain length of 15 and 17 C-atoms and a degree of saturation from 0–3. In comparison to the synthetic antibiotic ciprofloxacin (IC_{50} 0.1 $\mu g \cdot mL^{-1}$), AR C17:3 was demonstrated to be highly effective (IC_{50} 2.5 $\mu g \cdot mL^{-1}$). Other mono-, di-, and trienoic AR showed moderate inhibition (IC_{50} 5.0–15.0 $\mu g \cdot mL^{-1}$), while AR C15:0 and AR C17:0 were inactive (Jin & Zjawiony, 2006). AR C13:2 isolated from a *Lithraea molleoides* extract completely

inactivated uropathogenic *Proteus mirabilis* after 2 h through the application of 1.2 mg·mL^{-1}, whereas a saturated homologue required a longer exposure time and higher concentrations (Carpinella et al., 2011). Himejima and Kubo (1991) proved that AR from cashew nut shell oil are more effective against different Gram-positive bacteria than their corresponding cardanols (only one hydroxy group on the benzene ring) but clearly less effective than the corresponding anacardic acids (addition of a carboxyl group). All tested phenolic lipids possessed 15 C-atoms in the alkyl chain and the study confirmed that the number of double bonds increases the activity (Himejima & Kubo, 1991). These publications already provide a fundamental knowledge of a structure-depending activity of AR against bacteria. In contrast, the numerous publications dealing with antifungal effects often lack evaluation of structure-dependencies or even the detection of AR as the active substances in crude extracts. AR from mango fruits are supposed to be involved in the latency of *Alternaria alternata* - the causal organism of black spot disease - and *Colletotrichum gloeosporioides* infections in unripe fruits. The active homologues are AR C17:1 and AR C15:0 (Cojocaru et al., 1986; Droby et al., 1986, 1987; Hassan et al., 2007). However, Kienzle et al. (2014) found contradictory results which showed that AR are not a deciding factor in the fungal resistance of the fruit. Reiss et al. (1989) reported that 10 mg AR·mL^{-1} malt extract agar reduced the spreading of *Penicillium roqueforti* and *Aspergillus parasiticus*, but a reduced mold growth on rye bread compared to wheat bread was not assigned to the higher AR levels in rye (Reiss, 1989). In contrast, the AR fraction from the epicuticular waxes of barley seeds was correlated to the inborn resistance against the pathogenic fungi *Aspergillus niger* and *Penicillium chrysogenum* (Garcia et al., 1997). Zarnowski et al. (1999) demonstrated the *in vitro* activity of an AR mixture from rye against the growth of *Fusarium culmorum*, *Ceratobasidium cornigerum*, and *Rhizoctonia solani*. The effective concentration varied between 70–210 µg·mL^{-1}. Moreover, Magnucka et al. (2014) showed that the herbicide pyrazon - an inhibitor of the light reaction of photosynthesis - led to an accumulation and a homologue pattern modification of AR in rye seedlings, resulting in a drastically improved resistance of winter rye against the infection by *Ceratobasidium cornigerum*. AR-containing extracts from durum wheat whole grain had fungistatic activity against some causal agents of Fusarium head blight (FHB), *Fusarium*