IDENTIFICATION AND QUANTIFICATION OF SURFACE WAX COMPOUNDS
COVERING AERIAL ORGANS OF SELECTED PLANT SPECIES

by

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Abstract

The cuticle is an external barrier of aerial plant organs that prevents dessication. It is composed of hydrophobic waxes, i.e. complex mixtures of very-long-chain aliphatics, alicyclics and aromatics, lying on top of (epicuticular) and in between (intracuticular) a polyester matrix known as cutin. Wax compositions vary greatly between plant species, organs and tissues, both qualitatively and quantitatively. This thesis describes the identification and quantification of cuticular waxes of three plant species, including structure elucidation of novel compounds, chain length profiling, and wax compound distributions between intracuticular and epicuticular compartments for the first two species.

Leaves of *Aloe arborescens* were found covered with 15 μg/cm² wax on the adaxial side and 36 μg/cm² on the abaxial side, with 3:2 and 1:1 ratios between epicuticular and intracuticular wax layers on each side, respectively. Along with ubiquitous wax compounds, three homologous series were identified as novel 3-hydroxy fatty acids (predominantly C<sub>28</sub>), their methyl esters (predominantly C<sub>28</sub>), and 2-alkanols (predominantly C<sub>31</sub>), and their biosynthetic pathways were hypothesized based on structural similarities and homolog distributions.

The adaxial side of young and old *Phyllostachys aurea* leaves was found covered with 1.7 to 1.9 μg/cm² each of epicuticular and intracuticular waxes. In addition to typical aliphatics and alicyclics, novel primary amides were identified, with their chain length profile peaking at C<sub>30</sub>, and found exclusively in the epicuticular waxes, hence near the true plant surface.

Flag leaves and peduncles of *Triticum aestivum* cv. Bethlehem were found covered with 16 and 49 μg/cm² wax, respectively, dominated by 1-alkanols in the case of the former and β-diketones and hydroxy-β-diketones for the latter. Along with previously reported wax classes, numerous
new classes were identified as homologous series: 2-alkanol esters, benzyl esters, phenethyl esters, \( p \)-hydroxyphenethyl esters, secondary alcohols, primary/secondary diols and their esters, hydroxy- and oxo-2-alkanol esters, 4-alkylbutan-4-olides, internally methyl-branched alkanes, and 2,4-ketols. Other new compounds were found as single homologs: \( C_{33} \) 2,4-diketone, \( C_{31} \) mid-chain \( \beta \)-ketols, \( C_{30} \) mid-chain \( \alpha \)-ketols and \( \alpha \)-diketone, as well as \( C_{31} \) mid-chain ketones. Biosynthetic pathways are proposed in the thesis for the new compounds, based on common structural features and matching chain length patterns between related compound classes.
Preface

A version of Chapter 2 has been published: Racovita, R.C., Peng, C., Awakawa, T., Abe, I., Jetter, R. *Phytochemistry* **2015**, *113*, 183-194. Reproduced with permission from Elsevier. I conducted all experiments, interpreted results and wrote most of the manuscript; co-authors helped with species selection (T.A., I.A.), synthesized prior standards (C.P.), and contributed to data interpretation and writing (R.J.).

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A version of Chapter 5 has been submitted for publication. I performed the experiments, interpreted results and wrote most of the manuscript; the co-author, Dr. Reinhard Jetter, contributed to figure editing, data interpretation and writing.

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Certain paragraphs from Chapter 7 are concluding remarks from the manuscripts mentioned above. These paragraphs were written by me and edited by Dr. Reinhard Jetter.

The experiments, data analysis, interpretation and writing in the remaining chapters were my own contribution in close consultation with my supervisor, Dr. Reinhard Jetter.
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List of abbreviations

Ac: acetyl
ACP: acyl carrier protein
br: broad
BSTFA: bis-N,O-(trimethylsilyl)trifluoroacetamide
CER: Eceriferum
CoA: coenzyme A
d: doublet
dd: doublet of doublets
EAR: enoyl-ACP reductase
ECR: enoyl-CoA reductase
ER: endoplasmic reticulum
FA: fatty acid
FAE: fatty acid elongase
FAME: fatty acid methyl ester
FAR: fatty acyl-CoA reductase
FAS: fatty acid synthase
FATB: fatty acyl-ACP thioesterase B
FDR: false discovery rate
FID: flame ionization detection
GC: gas chromatography
HAD: β-hydroxyacyl-ACP dehydratase
HCD: β-hydroxyacyl-CoA dehydratase
KAR: β-ketoacyl-ACP reductase
KAS: β-ketoacyl-ACP synthase
KCR: β-ketoacyl-CoA reductase
KCS: β-ketoacyl-CoA synthase
LACS: long-chain acyl-CoA synthetase
LC: long-chain
m: multiplet
MAH1: mid-chain alkane hydroxylase 1
MS: mass spectrum/mass spectrometry
OS: (S)-2,3-oxidosqualene
OSC: oxidosqualene cyclise
PKS: polyketide synthase
prim: primary
Rf: retardation factor
s: singlet
sec: secondary
t: triplet
TLC: thin layer chromatography
TMS: trimethylsilyl
VLC: very-long-chain
WS: wax (ester) synthase
WSD1: wax ester synthase/acyl-coenzyme A : diacylglycerol acyltransferase 1
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Dedication

To my beloved father and mother
Chapter 1: Introduction to the plant cuticle

1.1. The plant cuticle: An adaptation to a dry environment

When plants expanded their natural habitat beyond the planetary ocean about 500 million years ago, they had to undergo adaptations allowing them to retain their water reserves for longer times, essential to their survival (Schreiber, 2005). By far the most important among these is the development of a lipid coating over most aerial organs, known as the plant cuticle – a complex biocomposite, with many fine intricacies still undetermined to this day.

1.2. Chemical diversity of cuticle constituents

There are two main cuticle components: a polymer matrix (cutin or cutan) and molecular waxes embedded in the cutin (intracuticular waxes) or atop of the cutin (epicuticular waxes).

1.2.1. Cutin/Cutan

Unlike cuticular waxes, which are monomeric lipids soluble in a wide range of non-polar solvents, cutin is a polymer insoluble in all common solvents, which greatly limits the analytical tools available for its characterization. However, it can be depolymerized into the corresponding monomers by saponification, for example NaOCH₃ (Graça et al., 2002) or BF₃-catalyzed (Leide et al., 2011) methanolysis, and its chemical structure can be inferred thereof. The general consensus is that cutin is a three-dimensional polyester composed mainly of long-chain (LC, i.e. C₁₆ and C₁₈) saturated and unsaturated ω-hydroxy fatty acids, often with additional mid-chain epoxy, keto, and/or hydroxyl functionalities from which the 3D structure emerges. Other minor
constituents are long-chain mono- and di-carboxylic acids (with or without mid-chain hydroxy-functionalities) and phenolics of the flavonoid family (Laguna et al., 1999). The structures of the most important $C_{16}$ and $C_{18}$ monomers are shown in Fig. 1.1. Glycerol has also been identified in the monomer mixture as 1- and 2-monoacyl glycerides by partial saponification with CaO/CH$_3$OH (Graça et al., 2002) and is believed to form cross-links between polyester chains (Pollard et al., 2008).

Figure 1.1. Structures of the most common monomers identified in the structure of cutin.
Cutin is the dominant constituent of the plant cuticle, accounting for 40-80% of total cuticle mass (Heredia, 2003), but in most – although not all plant species (e.g. Ginkgo biloba) (Briggs, 1999) – a second lipophilic polymer has been identified in the residue left from the saponification of delipidated cuticles. This polymer is named cutan and even less is known about it, although it can sometimes account for even more mass than cutin (Pollard et al., 2008). One hypothesis is that it is a polyether, with C-O bonds emerging from ring-opening of highly reactive epoxy groups (found within some of the cutin monomers described above) in the presence of nucleophiles, such as OH groups from nearby sugars or cutin fragments (Jeffree, 2006). This biochemical maturation of cutin into cutan is supported in part by the fact that the mass of the non-saponifiable leaf polymer fraction increases six times as the leaf matures in the case of Clivia miniata, a species for which epoxy-ω-hydroxy-fatty acids represent a major cutin monomer (Schmidt, 1982). However, C-C bond cross-linkages and polyester monomers atypical for cutin, such as resorcinols and α-resorcinic acid, have also been described as part of cutan structure (Deshmukh et al., 2005).

1.2.2. Cuticular waxes

Cuticular waxes exhibit an even greater variety and can be chemically classified in three main categories: aliphatics, alicyclics, and aromatics.

1.2.2.1. Aliphatics

The aliphatics are represented by very-long-chain fatty acids (VLCFA, predominantly with even carbon-chain lengths ranging from C20 to C34) and other mono-, bi-, and even tri-functional compounds biosynthetically derived from them (in fact, from their thioesters with
coenzyme A) (Samuels et al., 2008). Some of these derivatives are also dominated by even chain lengths $>C_{20}$: VLC aldehydes (Samuels et al., 2008), VLC alcohols (Samuels et al., 2008), their esters with either LC fatty acids (most commonly) or VLC fatty acids (less commonly) (Lai et al., 2007; Samuels et al., 2008), as well as acetates of such alcohols (Jetter et al., 2000). Other derivatives are dominated instead by odd chain lengths (typically $C_{23}$-$C_{35}$): mainly alkanes (dominant cuticular wax components in many species, including the model plant *Arabidopsis thaliana*) (Jetter and Kunst, 2008; Samuels et al., 2008) and various mid-chain oxygenated derivatives of them, such as secondary alcohols (Greer et al., 2007; Wen and Jetter, 2009; Wen et al., 2006a), ketones (Greer et al., 2007; Wen and Jetter, 2009), $\alpha$- and $\beta$-diols (Wen and Jetter, 2009; Wen et al., 2006a), $\alpha$- and $\beta$-ketols (Holloway and Brown, 1977; Wen and Jetter, 2009), $\beta$-diketones (Evans et al., 1975a; von Wettstein-Knowles, 1976) and hydroxy-$\beta$-diketones (Tulloch and Weenink, 1969). The structures of most commonly encountered cuticular waxes are shown in Fig. 1.2. Very small amounts of compounds with carbon chain lengths of opposite parity than shown in the figure are also encountered in wax mixtures.

**Figure 1.2. Structures of the most common plant cuticular wax compounds.** Typical ranges are: $n=11, \ldots, 16$; $m+n=16, \ldots, 25$; $p+q=12, \ldots, 14$. 
Sometimes, VLC aldehydes are not found in their free monomeric form in cuticular wax, but rather as trimers (2,4,6-trialkyl-1,3,5-trioxanes), as is the case of three species of rose flowers \((Rosa\ americana, R.\ imperial, R.\ virgo)\) (Mladenova, 1976). A series of other bifunctional VLC compounds, with even carbon-chain lengths, have also been described in the surface wax of various plant species: 1,2- and 1,3-diols, along with 1,2-diol monoacetates in \(Cosmos\ bipinnatus\) petal wax (Buschhaus et al., 2013a), 1,5-diols and 5-hydroxyaldehydes in \(Taxus\ buccata\) needles (Wen and Jetter, 2007), and 1,9 / 1,11 / 1,13 / 1,15-diols, ketols, keto-aldehydes, and ketol esters of LC fatty acids in \(Osmunda\ regalis\) fronds (Jetter and Riederer, 1999a). The analogous 5-hydroxyfatty acids have been found in the form of their δ-lactones as the major waxes in \(Cerithe\ minor\) (Jetter and Riederer, 1999b). Furthermore, there are some monofunctional compounds of odd chain length in cuticular waxes with functional groups other than mid-chain: 10-nonacosanol in the waxes of numerous gymnosperms (Franich et al., 1978; Osborne and Stevens, 1996; Alexander P Tulloch and Bergter, 1981; Tulloch, 1987) and some angiosperm species (Koch et al., 2006; Tulloch and Hoffman, 1982), 10-nonacosanone in the wax crystals on \(Osmunda\ regalis\) fronds (Jetter and Riederer, 2000), and a number of long-chain fatty acid esters of \(C_{13}-C_{19}\) 2-alkanols found in the wax of fruit capsules of Papaveraceae (Jetter and Riederer, 1996) or spikes of barley (von Wettstein-Knowles, 1976).

1.2.2.2. Alicyclics

The alicyclics are represented by triterpenoids, which are typically minor components of cuticular wax, but in some species, for example \(Olea\ europaea\) (Bianchi et al., 1993), can make up the bulk of waxes. Triterpenoids are divided into 2 main classes: phytosterols and nonsterol
triterpenoids. Both of these are derived from the cytoplasmic mevalonate biosynthetic pathway, which generates their common precursor (3S)-2,3-oxidosqualene (OS) (Guhling et al., 2006; Stiti and Hartmann, 2012). This can then either be converted to sterols via cycloartenol (formed by cycloartenol cyclase), or to nonsterol triterpenoids by a wide range of other highly versatile oxidosqualene cyclases (OSCs) (Xu et al., 2004). For example, the genome of Arabidopsis thaliana encodes no less than 13 such enzymes (Phillips et al., 2006), and one of these (baruol synthase) can produce, on its own, 23 triterpenoids (Lodeiro et al., 2007). It follows naturally that the diversity of alicyclics in plant cuticular waxes is even greater than that of VLCFA derivatives and it would be nearly impossible to achieve a truly comprehensive review. Nonetheless, the most ubiquitous phytosterols are β-sitosterol, stigmasterol, campesterol, brassicasterol, and to a lesser extent cholesterol (Johnson et al., 1963). Their common structural motif is the tetracyclic cholestane ring with an OH group at position 3 (Fig. 1.3).

Figure 1.3. 3β-Hydroxycholestane, the structural motif common to all sterols.

Nonsterol triterpenoids are classified based on their parent carbon skeleton. Most common are pentacyclics belonging to the oleanane and ursane families (Stiti and Hartmann, 2012) such as β-amyrin, erythrodiol, and oleanolic acid of the former, and α-amyrin, uvaol, and ursolic acid of the latter family (Fig. 1.4).
While triterpenoids are found most often in free form in cuticular waxes, in some species alcohol triterpenoids (whether sterols or nonsterols) have been identified as either esters of LCFA (e.g. amyrin palmitates and stearates on the adaxial side of *Rosa canina* leaves) (Buschhaus et al., 2007a), as acetates (Meusel et al., 1994), or as acetal-bound aglycons of a special class of glycosides known as saponins (Vincken et al., 2007). Triterpenoid acids can also serve as sapogenins (i.e. aglycons in saponins), forming ester linkages with carbohydrate hydroxyl functions (Vincken et al., 2007).

### 1.2.2.3. Aromatics

Aromatics are the least common of all cuticular wax classes, but they have been reported to occur in low amounts in selected species. Examples include VLC 5-alkylresorcinols in *Secale cereale* (Ji and Jetter, 2008) and *Triticum aestivum* (Adamski et al., 2013a), VLC 5-alkyl-m-guaiacols in *Tamarix canariensis* (Basas-Jaumandreu et al., 2014), VLC benzyl esters in *Rosa canina* (Buschhaus et al., 2007a) and *Olea europaea* (Bianchi et al., 1993), VLC 2-phenylethyl esters in *Olea europaea* (Bianchi et al., 1993) and *Ligustrum vulgare* (Buschhaus et al., 2007b), 4-hydroxyphenylpropyl, 3,4-dihydroxyphenylpropyl, and 3,4-dihydroxyphenylbutyl esters in *Taxus baccata* (Jetter et al., 2002; Wen and Jetter, 2007), as well as flavonoids in Pteridaceae.
(Wollenweber, 1989), Myrtaceae (Wollenweber et al., 2000) and three species of *Chrysothamnus* (Stevens et al., 1999).

**1.3. Stratification of cuticle constituents**

It is well acknowledged that some cuticular waxes are embedded within the amorphous cutin matrix (*intracuticular waxes* ICW) and can only be accessed by extraction with a nonpolar solvent (e.g. CHCl₃), while other waxes lie atop of cutin (*epicuticular waxes* ECW) and can be selectively peeled off using adhesives (e.g. gum Arabic or frozen glycerol or water) (Buschhaus and Jetter, 2011). Thus, in cross-section, the cuticle appears layered into a *cuticle proper* made of an exterior ECW film, from which crystals may or may not protrude out of the surface (depending on species/organ/tissue) and an interior layer of cutin+ICW, as well as an additional *cuticular layer* containing cutin, ICW, and fibrillar extensions of epidermal cell wall sugars (Fig. 1.5). Stacking many such cross-sections into the third dimension would generate a 3D model of the cuticle.

![Figure 1.5. Cartoon of hypothetical cross-section through the cuticle](modified from (Bargel et al., 2006)].
To date, there have been a number of GC analysis reports delineating quantitative and even qualitative compositional differences between ICW and ECW in various species (Buschhaus and Jetter, 2012; Buschhaus et al., 2007a, 2007b; Gniwotta et al., 2005; Guhling et al., 2005; Jetter et al., 2000; Ji and Jetter, 2008; van Maarseveen and Jetter, 2009; Wen et al., 2006b), as well as a recent review identifying certain common trends across these reports and speculating on some of the possible causes (Buschhaus and Jetter, 2011). The most important finding of the review was that alicyclic and aromatic wax compounds almost always accumulate preferentially (often exclusively) in the ICW. In addition, a similar preference, albeit less obvious, was noted for VLC primary alcohols and alkanediols, while the opposite seemed true for alkanes, fatty acids, and mid-chain secondary alcohols. Also noted were a few examples where homologs of same compound class having longer carbon chain predominated in the ECW, while shorter ones preferred the ICW. Overall, it was concluded that ICW incorporates preferentially more polar classes, presumably capable of stronger intermolecular bonding to cutin polar sites (e.g. hydrogen bonds), and in particular more compact, cyclic molecules and the shorter of VLC aliphatic homologs, since each of these will exclude from their own phases the other (longer) waxes due to shape/size mismatch. The seemingly contradictory epicuticular accumulation of fatty acids was easily explained by their solid state head-to-head dimerization (Bond, 2004; Leiserowitz, 1976; Moreno et al., 2006), causing their exclusion by shorter ICWs. A few exceptions where polar triterpenoids were abundant in ECW were noted (Markstadter et al., 2000; van Maarseveen and Jetter, 2009). A plausible explanation would be that there is a saturation limit of cutin with ICW beyond which even compact alicyclics are excluded into the ECW. Furthermore, specific wax interactions with the cell wall must be considered. Since
carbohydrates are capable of H-bonding even more than cutin, it seems reasonable to assume that the most polar of intracuticular waxes (compact triterpenoids with oxygenated functions like COOH or OH) will segregate in the intracuticular layer ICL, in part buffering the transition from hydrophilic cell wall to lipophilic cutin. In support of this view, oleanolic and ursolic acids, which do not form carboxy/carboxy dimers due to steric hindrance (Casado and Heredia, 1999), have only been reported as 100% intracuticular in all literature reports so far (Buschhaus et al., 2007a, 2007b; Jetter et al., 2000).

1.4. Functions of the plant cuticle

As briefly mentioned before, the most important function of the cuticle is to prevent excessive astomatous transpiration, i.e. loss of water through the plant-atmosphere interface itself as opposed to the stomatal pores carefully controlled by guard cells (Buschhaus and Jetter, 2011). It has been described that water vapor diffuses through the cuticle via a hopping mechanism (Matas and Heredia, 1999), following two possible paths: the lipophilic path – which is the main path, passes straight through the numerous amorphous portions of the cuticle, and is also the trajectory followed by many other non-electrolytes (e.g. pesticides) (Buchholz, 2006), as well as the less important polar (“aqueous”) path – which is represented for the most part by the polysaccharide microfibrils cutting through the cuticular layer and also serves as the only access way for highly polar and charged compounds (e.g. inorganic ions) (Schreiber, 2005). These latter tracks appear to be located preferentially around stomata, along and at the base of trichomes, as well as over anticlinal epidermal cell walls (i.e. cell walls between cells and therefore perpendicular to the plant surface) (Schreiber, 2008). The path that is totally inaccessible to water and thus acts as the best cuticular barrier is represented by highly ordered crystalline areas
of the cuticle, most often resulting from alignment of the very long aliphatic chains of VLCFA and their derivatives (Fig. 1.6) (Riederer and Schneider, 1990). It follows from here that the permeability of cuticular membranes has little to do with the amount of wax, cutin, or the cuticle thickness, but is rather linked to the nature and relative amounts of individual wax compounds and their self-assembly (Kerstiens, 2006). For example, a dominance of VLC alkanes within a very narrow range of chain lengths will generate the most effective water-diffusion barrier via segregation into highly crystalline condensed phases (Vioque and Pastor, 1994), whereas having additional contributors with very different chain lengths (e.g. alkyl esters combining VLC alcohols with LC acids) (Leide et al., 2011) or with completely different molecular geometries (e.g. alicyclics and aromatics) (Buschhaus and Jetter, 2012; Leide et al., 2011) will decrease resistance to non-stomatal water loss.

Figure 1.6. Cuticular crystallites formed by hydrocarbon chain alignment [modified from (Riederer and Schneider, 1990)].
But the cuticle is more than just a mere barrier for water. It is also blocking damaging UV-B and UV-C radiation from reaching the epidermis when aromatic absorbents are present (Markstadter and Riederer, 1997). Due to wax crystals formed at the surface, it effects a change in reflectance and thus a shift from an appealing glossy green to a whitish glaucous appearance, which may be less attractive to herbivores (Eigenbrode and Espelie, 1995). A similar insect herbivore or pathogen deterrent effect has been associated with increased contents of triterpenoids (e.g. α- and β-amyrins) (Balsdon et al., 1995) when the insect probes into the plant surface, or, on the contrary, with reduced levels of a certain class in the cuticular wax mixture (e.g. VLC aldehydes) (Hansjakob et al., 2011), which would otherwise direct oviposition or fungal pre-penetration. In carnivorous plants of the genus Nepenthes, cuticular aldehydes actually assist with their feeding process, by forming slippery crystals on the inner side of their pitchers and thus directing insect prey into the digestive pool (Riedel et al., 2007, 2003). In myrmecophyte Macaranga plants, a more complicated plant defense system operates: mainly two triterpenoids, epi-taraxerol and taraxerone, generate wax surface crystals that are slippery to generalist ants, but not to symbiotic ants, which are thus provided shelter by the plant and in return protect it by deterring insects and other pests (Markstadter et al., 2000). Furthermore, in general, regardless of their chemical nature, wax crystals have the merit of creating a hydrophobic and sometimes superhydrophobic plant surface (especially when combined with anatomic surface protuberances, e.g. papillose cells on lotus leaves) (Barthlott et al., 1997) that triggers a self-cleaning effect by rolling off rain or dew droplets along with dust, spores and other contaminants. The cuticle is also the first shield against mechanical damage (e.g. wind-carried particulates) and, by developing early during ontogenesis, it prevents organ fusions (Weng et al., 2010).
1.5. Biosynthesis of VLC aliphatic cuticular waxes

1.5.1. Biosynthesis of ubiquitous aliphatic waxes

The biosynthesis of aliphatic cuticular waxes has been studied extensively using the model plant species *Arabidopsis thaliana* and is known to occur in three stages: *de novo* synthesis of LC fatty acid, elongation to VLC fatty acyl-coenzyme A (acyl-CoA) intermediates and, finally, modification of the latter into final wax products (Kunst et al., 2006). The first step takes place in epidermal plastids, while the last two steps occur in the endoplasmic reticulum (ER), from where waxes are then exported through the plasma membrane and cell wall to the cuticle.

The *de novo* synthesis begins with acetyl-CoA as a C₂ starter unit which is elongated two carbons via condensation with malonyl-acyl carrier protein (malonyl-ACP) catalyzed by the β-ketoacyl-ACP synthase III (KASIII) enzyme (Clough et al., 1992). The resulting C₄ β-ketoacyl-ACP is reduced to β-hydroxyacyl-ACP, then dehydrated to trans-Δ²-enoyl-ACP and, finally, reduced again to the C₄ acyl-ACP (i.e. butanoyl-ACP), all steps being catalyzed by dedicated enzymes: two reductases and a dehydratase. Further elongation from C₄ to C₁₆ is effected by sequential condensations with malonyl-ACP catalyzed by KASI, while elongation from C₁₆ to C₁₈ acyl-ACP is afforded by KASII (Shimakata and Stumpf, 1982). The same two reductases and dehydratase are shared by all KAS enzymes in the repeated steps of reduction/dehydration/reduction that follow each condensation, such that all enzymes taken together are known as the enzymatic complex fatty acid synthase (FAS). This complex ultimately produces saturated C₁₆ and C₁₈ acyl-ACP intermediates, which are then hydrolyzed by
a fatty acyl-ACP thioesterase (FATB) to C\textsubscript{16} and C\textsubscript{18} free fatty acids, before export to the ER (Bonaventure et al., 2003).

Upon export from plastid, free fatty acids undergo thioesterification with coenzyme A (CoASH), catalyzed by a long-chain acyl-CoA synthetase (LACS), of which nine have been detected in \textit{A. thaliana} (Shockey et al., 2002).

Similar to FAS-catalyzed elongation, elongation of C\textsubscript{16} and C\textsubscript{18} acyl-CoA to VLC acyl-CoA thioesters also requires cycles of four consecutive enzyme-catalyzed reactions (Fig. 1.7):

1) condensation with malonyl-CoA to give β-ketoacyl-CoA, catalyzed by a condensing enzyme known as β-ketoacyl-CoA synthase (KCS), of which 21 have been identified in Arabidopsis (Blacklock and Jaworski, 2006);

2) reduction to a β-hydroxyacyl-CoA by the β-ketoacyl-CoA reductase (KCR) (Beaudoin et al., 2002);

3) dehydration to \textit{trans}-Δ\textsubscript{2}-enoyl-CoA by a putative β-hydroxyacyl-CoA dehydratase;

4) reduction to the two-carbon longer acyl-CoA by the enoyl-CoA reductase (ECR) (Kohlwein et al., 2001).

Together, these enzymes form an enzymatic complex known as the fatty acyl elongase (FAE) and the only product released is the elongated acyl-CoA, whose final chain length seems to be dictated by the condensing enzyme (KCS) in the complex (Blacklock and Jaworski, 2006; Franke et al., 2009; Lee et al., 2009; Paul et al., 2006; Trenkamp et al., 2004).
The modification of acyl-CoAs into final wax products takes place according to one of two main pathways (Fig. 1.7): the acyl reduction pathway, producing primary alcohols and alkyl esters, and the decarbonylation pathway, yielding aldehydes, alkanes, secondary alcohols, ketones, and other minor hydroxylation products of alkanes (e.g. α-/β-diols and α-/β-ketols).
Primary alcohols are formed from VLC acyl-CoA substrates by a fatty acyl reductase (FAR), typically without release of the intermediate aldehyde (Fig. 1.7). In Arabidopsis, this FAR has been identified as ECERIFERUM4 (CER4) (Rowland et al., 2006). Primary alcohols are either exported to the cuticle, or are esterified with long-chain acyl-CoAs into alkyl esters by a wax (ester) synthase (WS), which in A. thaliana has been characterized and named wax ester synthase/acyl-coenzyme A : diacylglycerol acyltransferase (WSD1) (Li et al., 2008).

An alternative reduction of VLC acyl-CoA by a different reductase yields VLC aldehydes, which are then decarbonylated by a decarbonylase to VLC alkanes (Fig. 1.7). In Arabidopsis, these two steps are catalyzed by the CER3 and CER1 enzymes, which form a heterodimer (Bernard et al., 2012). Presumably, the CER3 enzyme carries the reductase activity, while CER1 acts as the decarbonylase.

Alkanes are either exported to the cuticle, or undergo single or multiple hydroxylations by a hydroxylase of the cytochrome P450 family of enzymes, to yield primarily secondary alcohols and ketones, but also diols and ketols to lesser extent (Wen and Jetter, 2009). In A. thaliana, this hydroxylase targets specifically carbons around the middle of the chain and has been named mid-chain alkane hydroxylase 1 (MAH1) (Greer et al., 2007).

### 1.5.2. Biosynthesis of β-diketones in Poaceae

In the cuticular waxes of various species of Poaceae (Gramineae), the predominant compound class is often represented by β-diketones, whose biosynthesis is not well understood and is thus still the object of recent investigations (Hen-Avivi et al., 2016b; Schneider et al., 2016). However, a biosynthetic pathway has been proposed (von Wettstein-Knowles, 2012) and is depicted in Fig. 1.8 for the most commonly encountered homolog, hentriacontan-14,16-dione.
**de novo fatty acid biosynthesis**

![Diagram of fatty acid biosynthesis](image)

Figure 1.8. *Proposed biosynthesis of hentriacontan-14,16-dione.*

The main difference versus the Arabidopsis pathway is that the C$_{16}$ acyl-CoA, instead of undergoing elongation by the FAE enzymatic complex, undergoes two condensations with malonyl-CoA catalyzed by a type III polyketide synthase (PKS), an enzyme that no longer removes the β-oxygen functionality. This results in a triketide, which then undergoes six 2-carbon extensions by the FAE complex. Finally, the terminal carbon is removed by either the combination of reductase and decarbonylase enzymes, very similar to alkane formation, or alternatively by a combination of thioesterase and decarboxylase enzymes (the exact mechanism is not known).

1.6. Objectives

As mentioned in section 1.2.2., past work exploring cuticular wax compositions of plant species other than *A. thaliana* has revealed novel cuticular wax constituents, some of which have
provided new insights into wax biosynthesis beyond the Arabidopsis model (Buschhaus et al., 2013a; Busta et al., 2016; Jetter and Riederer, 2000, 1999a, 1999b; Jetter, 2000; Vermeer et al., 2003; Wen and Jetter, 2009, 2007; Wen et al., 2006a). The prospect that previously unexplored plant species could harbour novel wax compounds, with divergent biosynthetic pathways from those well established in Arabidopsis, was the driving hypothesis of the work presented in this thesis.

The main objective of the thesis was to identify and, where possible, quantify novel cuticular wax constituents from the wax mixtures coating select vegetative organs of three plant species, and thus expand cuticular wax diversity even further.

An ancillary objective of the thesis was to provide insights into the potential biosynthetic pathways leading to these novel wax constituents. Several aspects of wax biosynthesis described for Arabidopsis may not apply to other plant species, so that new questions arise:

- Can FAE elongation intermediates be intercepted by other enzymes (hydrolases, reductases, etc.) in species other than Arabidopsis?
- Is there a pathway leading to fatty acid derivatives containing nitrogen operational in other species?
- Are aldehydes released as intermediates en route to primary alcohols during wax biosynthesis in other plant species?
- Do wax ester synthases exhibit substrate specificities for one or both of their acyl-CoA and alcohol substrates in species other than Arabidopsis?
- Are there cytochrome P450 hydroxylases targeting positions other than mid-chain operational in other species?
• What types of polyketide-like structures are yielded by polyketide synthases in species other than Arabidopsis?

Due to a current scarcity of cuticular wax composition studies on monocotyledons, the three plant species chosen were all monocots. Furthermore, the species were chosen to span a diverse range of habitats, as well as practical applications. The three chosen species were: candelabra aloe (*Aloe arborescens*), a medicinal plant species endemic to the dry regions of Southern Africa (Chapter 2); fishpole bamboo (*Phyllostachys aurea*), an ornamental species originating from the subtropical areas of South-East China (Chapter 3); and bread wheat (*Triticum aestivum*), a major crop species that is cultivated in temperate climates worldwide (Chapters 4-6).
Chapter 2: Very-long-chain 3-hydroxy fatty acids, 3-hydroxy fatty acid methyl esters and 2-alkanols from cuticular waxes of *Aloe arborescens* leaves

2.1. Introduction

Land plants have lipid coatings over all their non-woody aerial parts, enabling prolonged retention of water, essential to their survival. Known as the cuticle, this coating consists of a cutin polyester matrix and complex mixtures of solvent-soluble cuticular waxes (Schreiber, 2005). In most plant species, the predominant wax constituents are very-long-chain (VLC) saturated aliphatic compounds, either with a single functional group or no functionality at all. All these compounds occur as mixtures of homologs, with chain lengths commonly ranging from $C_{24}$ to $C_{34}$. The series of the constituent fatty acids, primary alcohols, esters and aldehydes are typically dominated by even-numbered chain lengths, while alkanes, secondary alcohols and ketones are dominated by odd-numbered homologs. In a few species, alicyclic compounds such as triterpenoids can be quite abundant, sometimes even more than VLC aliphatics (Manheim Jr. and Mulroy, 1978; van Maarseveen et al., 2009).

The chemical structures and amounts of cuticular wax compounds can vary greatly between plant species, between organs of the same species, between tissues of the same organ, and sometimes even between different compartments of the cuticle covering the same tissue. Thus, it has been shown that, in certain species, wax constituents with somewhat higher polarity (e.g. alcohols) or with a more compact molecular geometry (e.g. triterpenoids) accumulate to greater extent within the more polar cutin matrix (in the intracuticular wax compartment), while
others with lower polarity (e.g. alkanes) accumulate instead outside of cutin (in the epicuticular wax layer deposited atop the matrix). In a few instances, subtle chain length partitioning effects have also been reported, where shorter homologs of fatty acids or alcohols accumulated more in the intracuticular wax and longer ones in the epicuticular wax (Buschhaus and Jetter, 2011).

The wax VLC homolog profiles are the consequence of divergent biosynthetic pathways leading to the different compound classes (Jetter et al., 2006; Kolattukudy, 1970). It is well established that wax biosynthesis proceeds by elongation of long-chain (C\textsubscript{16} and C\textsubscript{18}) fatty acyl precursors in fatty acyl elongase (FAE) complexes to VLC fatty acyl-CoAs. This elongation involves a sequence of four reactions, each catalyzed by a dedicated enzyme: 1) a decarboxylative Claisen condensation with malonyl-CoA, catalyzed by the β-ketoacyl-CoA synthase (KCS), which extends the carbon chain by two carbons; 2) a reduction of the resulting β-ketoacyl-CoA to β-hydroxyacyl-CoA by the β-ketoacyl-CoA reductase (KCR); 3) a dehydration of β-hydroxyacyl-CoA to an α,β-unsaturated acyl-CoA by a β-hydroxyacyl-CoA dehydratase (HCD); and finally 4) a second reduction to saturated acyl-CoA effected by the enoyl-CoA reductase (ECR). Typically, none of the above-mentioned intermediates are released by the enzyme complex, such that the only products of elongation are saturated acyl-CoAs with even carbon chain lengths of C\textsubscript{20} and higher. These acyl-CoA products are then either hydrolyzed to fatty acids and exported to the plant surface, or further processed via one of two parallel biosynthetic pathways: the acyl-reduction pathway, which converts them into even-numbered primary alcohols and esters, or the decarbonylation pathway, which, after a first reduction to still even-numbered aldehydes, then converts these into odd-numbered alkanes (which may, in some species, be transformed into mid-chain secondary alcohols and ketones) (Kunst et al., 2006; Samuels et al., 2008).
While much of current knowledge on wax formation stems from molecular genetic investigations using *Arabidopsis thaliana*, further insights can be expected from exploring the much greater chemical diversity of wax mixtures in other species as well. Accordingly, several recent publications described novel compounds in the waxes of diverse species, often with two functional groups in characteristic arrangements. Among these are VLC 1,3-diols and their monoacetates in petal wax of *Cosmos bipinnatus* (Buschhaus et al., 2013a), VLC 1,3-diols and 3-hydroxyaldehydes in leaf wax of *Ricinus communis* (Vermeer et al., 2003), VLC 1,5-diols and 5-hydroxyaldehydes in *Taxus baccata* needle wax (Wen and Jetter, 2007), VLC δ-lactones in *Cerinthe minor* leaf wax (Jetter and Riederer, 1999b), and a diverse range of 1,9-, 1,11-, 1,13- and 1,15-bifunctional VLC aliphatics identified in the wax of *Osmunda regalis* fronds, including ketols, ketoaldehydes, diols, and ketoalkyl esters (Jetter and Riederer, 2000, 1999a). In all these studies, the nature and the relative position of functional groups suggested biosynthetic relationships both between the compounds and with standard wax biosynthetic pathway intermediates. In line with these previous studies, the objective of the present work was to search for, identify and quantify further novel multifunctional compounds that would corroborate and expand the models for wax biosynthetic pathways. In this context, a further goal of this study was to localize potentially new wax compounds in the intracuticular or epicuticular layer, on both sides of the leaf, as a prerequisite for further studies into their biological functions.

To complement previous information on dicotyledonous species, the present investigation aimed to analyze the leaf cuticular waxes of a monocot, *Aloe arborescens*. This species is of special interest, because its leaves are known to be rich in phenolic secondary metabolites with important therapeutical applications, such as 6-phenylpyrone 2’- and 4’-O-glucosides (Beppu et al., 2004; Gutterman and Chauser-Volfson, 2000; Okamura et al., 1996; Park et al., 1998), which
have anti-histamine and anti-inflammatory activity (Bastian et al., 2013) or have led to derivatives with promising cytotoxicity against human colorectal and hepatoma cancer cells (Jin et al., 2005), 2-alkylchromone 8-C-glucosides (Gutterman and Chauser-Volfson, 2000; Okamura et al., 1996; Park et al., 1998), some of which have been shown to be efficient inhibitors of UV-induced hyperpigmentation (Choi and Chung, 2003; Choi et al., 2002) or to have anti-inflammatory properties (Bastian et al., 2013; Speranza et al., 2005), and anthraquinones and anthrone 10-C-glucosides (Beppu et al., 2004; Gutterman and Chauser-Volfson, 2000; Okamura et al., 1996; Park et al., 1998), with a wide range of medical usages from laxative to antitumor agents (Choi and Chung, 2003; Srinivas et al., 2007).

2.2. Experimental

2.2.1. Preparation of total leaf wax extracts

*Aloe arborescens* plants were maintained in growth chambers at the University of British Columbia, under the following conditions: 16 h light / 8 h dark, 21°C / 19°C, ~150 μE m⁻² s⁻¹ photosynthetically active radiation. The type of soil used was Sunshine Mix No. 4 (JVK and Crofton Grower Service) and it was always allowed to dry before re-watering (typically, watering every 10-12 days). Top halves from mature leaves were excised with clean razor blades and submerged in 10 mL CHCl₃ (Aldrich, ≥99%, 0.75% ethanol as stabilizer) at room temperature for 30 s. To ensure exhaustive extraction, this step was repeated with a fresh portion of 10 mL CHCl₃ for another 30 s and the two extracts were combined. Care was taken to avoid contact between chloroform and the open cut or any aqueous gel or latex occasionally leaking through it. To this end, leaf halves were dipped into the solvent only up to a set mark and any
portions beyond the mark were cut off afterwards, such that only the extracted leaf areas remained to be photographed and then measured using the ImageJ program. One leaf half was used to prepare one total wax replicate for GC analysis. Ten leaf halves were used to prepare the extract for TLC analysis.

2.2.2. Preparation of epicuticular and intracuticular wax extracts

The epicuticular and intracuticular waxes were sampled separately using procedures described in detail elsewhere (van Maarseveen and Jetter, 2009). In short, gum arabic was applied to one side of the leaf only, the resulting polymer films were peeled off, and the polymer and adhering epicuticular waxes were partitioned between water and chloroform, respectively. After the removal of epicuticular wax, the remaining intracuticular wax was extracted with chloroform using glass cylinders pressed gently onto the leaf surface. The same procedures were followed for both the adaxial and abaxial leaf surfaces.

2.2.3. Qualitative and quantitative analyses of wax extracts

For structure elucidation, total wax mixtures were fractionated by preparative TLC as described by Wen et al. (2006a), except that a mixture of CHCl₃ and EtOH in a 98:2 ratio (v/v) was used as mobile phase. The resulting fractions were characterized by their retardation factor \( R_f \), i.e. the ratio between the distance travelled on the TLC plate by the corresponding fraction and the distance travelled by the eluent front.

Prior to GC analysis, all samples were transferred into autosampler vials where the solvent was removed at 50°C under a stream of N₂ (Praxair, ≥99.998%). Waxes were then
derivatized by refluxing in 10 μL N,O-bis(trimethylsilyl)trifluoroacetamide (Aldrich, GC grade) and 10 μL pyridine (Aldrich, ≥99.8%, anhydrous) at 70°C for 30 min. Excess reagents were removed at 50°C under a stream of N₂ (Praxair, ≥99.998%), and a known amount (10.2 μg) of n-tetracosane (Alfa Aesar, ≥99%) was added as internal standard, to which the vast majority of wax compound classes had been shown to have relative response factors of 1.00 under almost identical GC-FID conditions (Riederer and Schneider, 1989). Finally, samples were dissolved in a known volume of CHCl₃ for GC analysis.

Two different GC instruments were used for separation and detection of wax constituents, both equipped with the same type of capillary GC column (6890N, Agilent, Avondale PA, USA; 30 m long; type HP-1: 100% PDMS; 0.32 mm i.d.; df=0.1 μm), using on-column injection and following the same temperature program (2 min at 50°C, ramp 40°C/min to 200°C, constant for 2 min, ramp 3°C/min to 320°C, constant for 30 min). The first employed He gas (Praxair, ≥99%) as mobile phase, at a flow rate of 1.4 mL/min, and was equipped with MS detector (5973N, Agilent, EI-70 eV), serving primarily the purpose of qualitative identification of separated wax compounds. The second used H₂ carrier gas (Praxair, ≥99.95%) at 2.0 mL/min and an FID detector, for quantification of individual wax homologs based on normalization of peak areas against that of the internal standard. Quantitative ester isomer compositions were determined from GC-MS data as described by Lai et al. (2007).

2.2.4. Synthesis of reference compounds

The condensation of octacosanoic acid with Meldrum’s acid under Steglich esterification reaction conditions and subsequent methanolysis yielded methyl 3-oxotriacontanoate, which was
split into two portions used further in two separate reaction sequences. One portion was subjected to decarboxylative hydrolysis using aqueous base in the presence of a phase transfer catalyst, then the resulting methyl ketone was reduced with sodium borohydride to produce 2-nonacosanol reference standard. A second portion was directly reduced with sodium borohydride to give methyl 3-hydroxytriacontanoate, part of which was acid hydrolyzed to yield the 3-hydroxytriacontanoic acid standard. Detailed experimental protocols and $^1$H-NMR characterization data for all reaction products are described in Appendix A.

2.3. Results

This study aimed to provide a comprehensive chemical analysis of the lipid mixtures coating *Aloe arborescens* leaves. To this end, we first elucidated the structures of all major wax constituents, then quantified all compounds in the overall leaf wax mixture and, finally, sampled and analyzed the epicuticular and intracuticular wax layers selectively on both the adaxial and abaxial sides of the leaf.

2.3.1. Structure elucidation of unknown compounds in *A. arborescens* leaf cuticular wax

Preliminary analyses had shown that *A. arborescens* waxes contained many ubiquitously found compounds (Fig. 2.1) readily identified by comparing gas chromatography-mass spectrometry (GC-MS) data with previously published information. However, the wax mixtures also contained a number of unusual compounds whose identification required in-depth analysis based on partial purification by thin layer chromatography (TLC), MS signal assignments and structure confirmation using synthetic standards.
Figure 2.1. *Compounds identified in the total wax mixture of A. arborescens leaves.*
To enable structure elucidation of unknowns, the cuticular wax mixture extracted from *A. arborescens* leaves was separated by preparative TLC on silica gel (mobile phase CHCl₃:EtOH 98:2, v/v). The five resulting fractions A – E were further analyzed by GC-MS and found to contain known VLC fatty acids 2.1 (A, R$_f$ 0.31), VLC 1-alkanols 2.3 and terpenols 2.11 (C, R$_f$ 0.40), VLC fatty acid methyl esters (FAMEs) 2.2 and aldehydes 2.5 (D, R$_f$ 0.93), as well as VLC esters 2.4 and alkanes 2.6 (E, R$_f$ 1.00). In addition, the three most polar fractions A, B (R$_f$ 0.36) and C each contained a major homologous series of unknown compounds, based on GC-MS characteristics. Notably, all three compound classes appeared to share a common structural motif characterized by homologous fragments m/z 117+28n in their trimethylsilyl (TMS) derivative mass spectra (n being a homolog-dependent integer).

The three unknown compounds in fraction A were tentatively assigned to a homologous series of 3-hydroxy fatty acids 2.9. The TMS-derivatives of all three homologs had a prominent MS fragment m/z 147 [(CH$_3$)$_2$SiOSi(CH$_3$)$_3$]$^+$ indicating the presence of two hydroxyl groups in the native compounds (Jetter et al., 1996; Richter and Burlingame, 1968; Rontani and Aubert, 2004). The formation of a fragment m/z 117 [(CH$_3$)$_3$SiOCO]$^+$ together with the absence of a fragment m/z 103 [(CH$_3$)$_3$SiOCH$_2$]$^+$ indicated that one of the OH groups was located on a terminal carbon, not as a primary alcohol, but rather in a carboxyl group (Buschhaus et al., 2013; Jetter and Riederer, 1999b). The other functional group was recognized to be a non-terminal hydroxyl, based on one TMS-ether α-fragment m/z 233 common to all homologs and another α-fragment varying between homologs (m/z 481 in Fig. 2.2A). The fractionation of this unknown series with free fatty acids 2.1 was also suggestive of a functionalized carboxylic acid structure, however an alternative 2,4-diol structure could not be entirely ruled out thus far. We therefore synthesized C$_{30}$ 3-hydroxy fatty acid 2.9c (see Appendix A), and tested its GC-MS behaviour...
against the unknown series in fraction A. The TMS derivative of the standard showed matching MS fragmentation patterns (Fig. 2.2A-C) and GC retention time (Fig. 2.2D) with the longest homolog in the unknown series, thus confirming that the *A. arborescens* wax compounds are indeed C_{26}, C_{28} and C_{30} 3-hydroxy fatty acids 2.9.

Figure 2.2. *Structure elucidation of unknown compound series in fraction A of A. arborescens wax.* Mass spectra of (A) C_{30} 3-hydroxy fatty acid 2.9c isolated from *A. arborescens* leaf wax, and (B) synthetic C_{30} 3-hydroxy fatty acid 2.9c. (C) Major fragmentations of C_{30} 3-hydroxy fatty acid 2.9c; note that fragment m/z 597 can also originate from loss of any of the other five TMS-bound methyl groups in the molecular ion. (D) Overlay of selected ion m/z 233 chromatograms of the 3-hydroxy fatty acid series 2.9 in *A. arborescens* wax and of synthetic C_{30} 3-hydroxy fatty acid 2.9c; this fragment is distinctive for the 3-hydroxy fatty acid series 2.9 in fraction A.
Similarly, the five compounds in fraction B were tentatively assigned to a homologous series of 3-hydroxy FAMEs 2.8. The absence of fragment $m/z$ 147 and the presence of fragments $m/z$ 73 [(CH$_3$)$_3$Si]$^+$ and $m/z$ 89 [(CH$_3$)$_2$SiOCH$_3$]$^+$ indicated that the compounds contained only one OTMS group (Vermeer et al., 2003; Wen and Jetter, 2007). A TMS-ether α-fragment varying between homologs ($m/z$ 481 in Fig. 2.3A) suggested a secondary OH group in the same position as in the A series, while a second α-fragment $m/z$ 175 common to all homologs indicated an additional terminal methoxycarbonyl group. All this information, together with the TLC behaviour of B indicating polarities between 3-hydroxy fatty acids 2.9 and primary alcohols 2.3, pointed to 3-hydroxy FAME 2.8 structures. To test this assignment, we synthesized C$_{30}$ 3-hydroxy FAME 2.8d (see Appendix A) and compared its GC-MS behaviour against compounds B (Fig. 2.3A-D). Based on the match between standard and wax characteristics, we conclude that the second unknown series is a series of 3-hydroxy FAMEs 2.8.
Figure 2.3. *Structure elucidation of unknown compound series in fraction B of A. arborescens wax.* Mass spectra of (A) C₃₀ 3-hydroxy fatty acid methyl ester (FAME) 2.8d isolated from A. arborescens leaf wax, and (B) of synthetic C₃₀ 3-hydroxy FAME 2.8d. (C) Major fragmentations of C₃₀ 3-hydroxy FAME 2.8d; note that fragment m/z 539 can also originate from loss of either of the other two TMS-bound methyl groups in the molecular ion. (D) Overlay of selected ion m/z 175 chromatograms of the 3-hydroxy FAME series 2.8 in A. arborescens wax and of synthetic C₃₀ 3-hydroxy FAME 2.8d; this fragment is distinctive for the 3-hydroxy FAME series 2.8 in fraction B.
Fraction C comprised six homologs tentatively identified as 2-alkanols 2.10 based on MS similarity to data previously reported for wax from one other plant species, *Solanum tuberosum* (Szafranek and Synak, 2006a). The diagnostic α-fragment $m/z$ 117 common to all homologs in C, together with a chain length-variable α-fragment [M-15]$^+$, indicated the presence of a 2-hydroxyl group in the native homologous series (Fig. 2.4A). This structure assignment was confirmed via preparation of C$_{29}$ 2-alkanol 2.10d (see Appendix A), whose characteristic MS fragments and GC retention time matched those of one compound in C (Fig. 2.4A-D). Thus, all odd-numbered 2-alkanol 2.10 homologs from C$_{23}$ to C$_{33}$ were identified in *A. arborescens* leaf wax.
Figure 2.4. Structure elucidation of unknown compound series in fraction C of A. arborescens wax. Mass spectra of (A) C_{29} 2-alkanol 2.10d isolated from A. arborescens leaf wax, and (B) synthetic C_{29} 2-alkanol 2.10d. (C) Major fragmentations of C_{29} 2-alkanol 2.10d; note that in (C), fragment m/z 481 can also originate from loss of any of the three TMS-bound methyl groups in the molecular ion. (D) Overlay of selected ion m/z 117 chromatograms of the 2-alkanol series 2.10 in A. arborescens wax and of synthetic C_{29} 2-alkanol 2.10d; this fragment is distinctive for the 2-alkanol series 2.10 in fraction C.
2.3.2 Quantitative compositional analysis of total cuticular wax of A. arborescens leaves

After establishing the various compound classes and specific homologs present in A. arborescens cuticular wax, we used GC with flame ionization detection (FID) to quantify all compounds in the overall wax mixture extracted from both sides of the leaf. The total leaf wax coverage was 25.6±0.9 μg/cm², of which 95% was identified (1.1±0.2 μg/cm² unidentified compounds). *n*-Alkanes 2.6 were by far the most abundant compound class (8.8±0.4 μg/cm²) in the wax mixture, accompanied by lesser amounts of 1-alkanols 2.3, esters 2.4, aldehydes 2.5 and acids 2.1 (Fig. 2.4). Interestingly, the novel 3-hydroxy FAMEs 2.8 were relatively abundant (1.1±0.2 μg/cm²), whereas the 3-hydroxy acids 2.9, 2-alkanols 2.10 and non-hydroxylated FAMEs 2.2 were detected only in trace amounts (below 0.1 μg/cm²). The only branched-chain compounds were *iso*- and *anteiso*-alkanes 2.7 (below 0.1 μg/cm²). Cyclic compounds comprising both triterpenoids 2.11 (mainly lupeol 2.11d, along with β- 2.11b and α-amyrin 2.11c) and γ-tocopherol 2.11a constituted only 1% of the wax mixture (0.3±0.1 μg/cm²) (Fig. 2.5).
Figure 2.5. *Compound class composition of total leaf wax mixture.* Total coverages (μg/cm²) of compound classes within *A. arborescens* leaf wax. The inset shows an enlarged view of the area enclosed by dashed lines. Bars represent mean ± standard deviation (n = 5).
Within all aliphatic compound classes, homologous series were present. The free fatty acid series 2.1 had the widest chain length range of all fractions, varying from C_{20} to C_{34} (including trace levels of odd-numbered homologs). The acid mixture 2.1 was further distinguished by a bimodal homolog distribution dominated by chain lengths C_{28} and C_{32} (Fig. 2.5). All other compound classes had narrower homolog distributions around only one maximum, with FAMEs 2.2, 1-alkanols 2.3, 3-hydroxy acids 2.9 and 3-hydroxy FAMEs 2.8 all dominated by respective C_{28} homologs and aldehydes 2.5 dominated by C_{32}. The only compound classes with predominant odd-numbered homologs, n-alkanes 2.6 as well as 2-alkanols 2.10, had chain length profiles both peaking at C_{31}. Similarly, the two branched alkanes 2.7 identified in A. arborescens leaf wax also had C_{31} chains (but 32 carbons overall). Finally, the fatty acid alkyl esters 2.4 had chain lengths ranging from C_{42} to C_{52}, dominated by the C_{46} homolog 2.4c (Fig. 2.6).
Figure 2.6. *Relative compositions of each compound class in the total leaf wax mixture.* Relative abundances (%) of individual homologs or isomers (in the case of branched alkanes and terpenoids) from each compound class in the composition of *A. arborescens* leaf wax. Numbers on the x-axis indicate homolog chain length. Bars represent mean ± standard deviation (n = 5). Each group of bars adds up to 100%.
Each of the wax ester homologs 2.4 was found composed of several metamers, i.e. isomers with different combinations of acid 2.1 and 1-alkanol 2.3 chain lengths. Because our GC experimental conditions did not allow the separation and individual quantification of such isomers, their quantities were determined by MS instead. To this end, the same total wax extracts used for acquiring the GC-FID data described above were further analyzed by GC-MS, and the relative abundances of all $M_{\text{acid}}+1$ product ions were used to measure relative quantities of esterified acids as previously described (Gülz et al., 1994; Jetter and Riederer, 1999a, 1996; Lai et al., 2007; Reiter et al., 1999; Shepherd et al., 1995). The esters contained mainly C$_{16}$, C$_{18}$ and C$_{20}$ acids, with lesser amounts of C$_{14}$ as well as C$_{22}$-C$_{26}$ acids (Fig. 2.7A). All esterified alcohols taken together had a chain length profile ranging from C$_{20}$ to C$_{34}$, with strong predominance of the C$_{28}$ homolog 2.3b (Fig. 2.7B). The shorter esters (C$_{42}$ 2.4a and C$_{44}$ 2.4b) contained mainly C$_{16}$ acid bonded to C$_{26}$ 2.3a and C$_{28}$ 2.3b alcohols, respectively. The predominant ester homolog (C$_{46}$ 2.4c) contained both C$_{16}$ and C$_{18}$ acid (esterified with C$_{30}$ 2.3c and C$_{28}$ 2.3b alcohols, respectively). In contrast, the longer ester homologs (C$_{48}$-C$_{52}$ 2.4d-f) all had C$_{20}$ acid 2.1a as the major constituent, esterified with C$_{28}$-C$_{32}$ alcohols 2.3b-d. Thus, the variation in overall ester chain length was due to both varying alcohol and acid chain lengths, with acid diversity driving the formation of central ester homologs C$_{44}$-C$_{48}$ 2.4b-d and alcohol diversity driving the formation of all other ester homologs.
Figure 2.7. *Relative compositions of esterified acids and alcohols in the total leaf wax mixture.*

(A) Relative abundances (%) of individual isomers (indicated by chain length of their acid moiety) of each ester homolog in *A. arborescens* leaf wax, and (B) resulting relative total abundances (%) of ester-bound alcohol homologs. Bars represent mean ± standard deviation (*n* = 5). Each group of bars in (A) adds up to 100%.
2.3.3. Distribution of *A. arborescens* leaf cuticular waxes between adaxial and abaxial surfaces, as well as epicuticular and intracuticular layers

To assess how the compounds identified and quantified in the overall wax mixture are distributed across various wax layers on both sides of the *A. arborescens* leaf, the epicuticular and intracuticular waxes from the adaxial and abaxial surfaces were sampled separately. Gum arabic was used as a glue for selective sampling of only the epicuticular wax layer from one side of the leaf, similar to previous studies (Jetter and Schäffer, 2001; Ji and Jetter, 2008; Wen et al., 2006b). To ensure complete removal of epicuticular waxes, four consecutive applications of the adhesive were performed on each side of each leaf. The remaining intracuticular waxes were extracted with chloroform applied in a glass cylinder of known diameter pressed against the leaf, a method also employed previously in the aforementioned studies.

The epicuticular wax coverage on the adaxial side of the *A. arborescens* leaf was 9.0±0.9 μg/cm², while the corresponding intracuticular wax amounted to 5.9±0.2 μg/cm². In contrast, the abaxial epicuticular wax accumulated to 18.9±0.9 μg/cm², and the abaxial intracuticular wax to 17.2±0.8 μg/cm². Taken together, our results show an uneven wax distribution between the abaxial (70%) and the adaxial (30%) sides of the leaf (p<0.01). Based on the epicuticular and intracuticular coverages on both sides, an average total leaf coverage of 26 μg/cm² was calculated, thus independently confirming the result of the total leaf wax extraction (see above). The total (epi- plus intracuticular) wax mixtures had very similar relative compositions (expressed in %) on both sides of the leaf, except for a slightly higher concentration of fatty acids in the adaxial wax (p<0.01).
In order to establish whether any wax constituents accumulate preferentially in a certain wax layer, the relative compound class compositions of the epicuticular and intracuticular wax mixtures were determined. Free fatty acids 2.1 and FAMEs 2.2 were found in higher concentrations in the epicuticular waxes than in the intracuticular layers on both leaf sides (adaxial and abaxial p<0.01), while 1-alkanols 2.3 (adaxial and abaxial p<0.01) and terpenoids 2.11 (adaxial and abaxial p<0.01) showed the opposite trends and preferentially accumulated in the intracuticular layer (Fig. 2.8A-B). The adaxial wax had relatively high concentration of \( n \)-alkanes 2.6 in the epicuticular (adaxial p<0.01) and 3-hydroxy acids 2.9 in the intracuticular wax (adaxial p<0.01), while the abaxial waxes had no corresponding gradients. Aldehyde 2.5 levels also differed between the wax layers, albeit with opposing gradients on the two leaf sides (adaxial and abaxial p<0.01). All other compound classes did not show preferential accumulation in either layer on either leaf side (Fig. 2.8A-B). The coverages of all individual compounds in the epicuticular and intracuticular compartments on the adaxial and abaxial leaf surfaces of \( A. \) arborescens are reported in Table 2.1. However, from this data, no apparent accumulation of wax homologs with either short or long chain lengths is noticeable for either compartment on either leaf surface.
Figure 2.8. Relative compound class compositions of wax layers on adaxial and abaxial leaf sides. Relative abundances (%) of compound classes in epi- and intracuticular layers of (A) adaxial and (B) abaxial wax of A. arborescens leaf. The insets show enlarged views of the areas enclosed by dashed lines. Bars represent mean ± standard deviation (n = 5). Asterisks mark significant differences between the arcsin-transformed epi- and intracuticular abundances of the respective compound class (two-tailed Student’s t test, p<0.01).
Table 2.1. Composition of epi- and intracuticular waxes on the adaxial and abaxial sides of A.
arborescens leaves. Mean coverages ± standard deviations (n = 5) are given in µg/cm2.
Compound Class

Chain Length /
Isomer

Adaxial Wax (µg/cm2)
Epicuticular
Intracuticular

Abaxial Wax (µg/cm2)
Epicuticular
Intracuticular

Fatty acids 2.1

20
22
24
26
28
30
32
34

0.0042±0.0008
0.0028±0.0002
0.011±0.001
0.034±0.007
0.33±0.05
0.11±0.01
0.26±0.04
0.017±0.004

0.0012±0.0003
0.0003±0.0001
0.0025±0.0008
0.013±0.005
0.03±0.01
0.09±0.02
0.03±0.03
0.0017±0.0007

0.0099±0.0009
0.0052±0.0006
0.015±0.002
0.07±0.02
0.62±0.08
0.23±0.04
0.33±0.03
0.06±0.02

0.009±0.002
0.004±0.001
0.019±0.006
0.022±0.009
0.18±0.07
0.20±0.03
0.07±0.07
0.003±0.001

Fatty acid methyl esters 2.2
(FAMEs)

24
26
28
30
32

0.008±0.002
0.015±0.003
0.016±0.004
0.013±0.002
0.0004±0.0001

0.0032±0.0009
0.003±0.001
0.002±0.002
0.0009±0.0006
0.0002±0.0001

0.009±0.005
0.025±0.007
0.037±0.008
0.0096±0.0009
0.0020±0.0008

0.005±0.001
0.005±0.002
0.01±0.01
0.002±0.001
0.0004±0.0002

1-Alkanols 2.3

26
28
30
32
34

0.009±0.001
0.50±0.08
0.24±0.04
0.23±0.04
0.04±0.01

0.03±0.01
0.11±0.03
0.93±0.08
0.5±0.1
0.024±0.004

0.044±0.009
1.5±0.3
0.7±0.2
0.56±0.06
0.10±0.01

0.04±0.02
2.0±0.5
0.49±0.04
1.1±0.2
0.14±0.02

Esters 2.4

42
44
46
48
50
52

0.22±0.06
0.29±0.06
0.48±0.09
0.26±0.04
0.29±0.05
0.049±0.008

0.09±0.03
0.20±0.06
0.3±0.1
0.27±0.06
0.19±0.06
0.03±0.03

0.47±0.02
0.66±0.06
1.1±0.1
0.6±0.1
0.44±0.08
0.08±0.01

0.24±0.09
0.7±0.2
1.0±0.4
0.9±0.2
0.5±0.2
0.06±0.06

Aldehydes 2.5

28
30
32
34

0.11±0.02
0.11±0.02
0.33±0.06
0.26±0.06

0.25±0.08
0.019±0.005
0.6±0.2
0.05±0.02

0.3±0.1
0.4±0.1
1.2±0.1
0.8±0.2

0.25±0.09
0.4±0.1
0.31±0.08
0.2±0.1

n-Alkanes 2.6

27
29
31
33
35

0.014±0.002
0.025±0.006
3.8±0.3
0.20±0.01
0.010±0.001

0.003±0.001
0.05±0.02
0.9±0.1
0.05±0.01
0.0032±0.0005

0.047±0.006
0.09±0.01
6.0±0.6
0.39±0.06
0.023±0.003

0.004±0.002
0.03±0.01
5.0±0.6
0.5±0.1
0.0016±0.0002

Branched alkanes 2.7

32 iso
32 anteiso

0.012±0.005
0.04±0.02

0.013±0.004
0.015±0.007

0.032±0.009
0.09±0.04

0.03±0.01
0.04±0.02

3-Hydroxy fatty acid methyl esters 2.8
(3-Hydroxy FAMEs)

24
26
28
30
32

0.016±0.004
0.039±0.004
0.21±0.07
0.06±0.01
0.006±0.003

0.017±0.003
0.05±0.01
0.05±0.01
0.04±0.02
0.005±0.005

0.04±0.01
0.18±0.05
0.40±0.09
0.14±0.02
0.07±0.02

0.024±0.004
0.15±0.03
0.31±0.04
0.13±0.06
0.01±0.01

3-Hydroxy fatty acids 2.9

26
28
30

0.0007±0.0001
0.0037±0.0009
0.0005±0.0001

0.0011±0.0002
0.0046±0.0008
0.0011±0.0006

0.0026±0.0004
0.015±0.004
0.0023±0.0007

0.0036±0.0002
0.012±0.002
0.002±0.001

2-Alkanols 2.10

31
33

0.006±0.003
0.0034±0.0009

0.004±0.002
0.0024±0.0006

0.004±0.002
0.0008±0.0002

0.019±0.008
0.0017±0.0009

Terpenoids 2.11

γ-tocopherol
β-amyrin
α-amyrin
lupeol

not detected
not detected
not detected
not detected

0.008±0.002
0.017±0.008
0.011±0.002
0.09±0.01

not detected
not detected
not detected
not detected

0.009±0.002
0.11±0.03
0.04±0.01
0.3±0.1

43


2.4. Discussion

Our analyses of *A. arborescens* leaf waxes led to three major results: (1) the wax mixtures contained typical aliphatic constituents in characteristic chain length distributions; (2) we identified unusual constituents with 2- and 3-hydroxy functionalities; (3) the epicuticular and intracuticular layers on the adaxial and abaxial leaf sides differed both in their relative compound class compositions and their absolute wax amounts. All three findings have implications for the formation and accumulation of cuticular waxes in this species that will be discussed below.

2.4.1. Chain length distributions of ubiquitous wax constituents

Various Aloe species (including *A. arborescens*) were investigated before, but the analyses has been restricted to acids and alkanes so far. Our results are similar to the literature reports, however with two notable exceptions. First, fewer homologs have been detected in previous reports, with fatty acid chain lengths ranging only up to C$_{31}$ (Herbin and Robins, 1969). Second, the C$_{29}$ homolog has been reported to dominate the preeminent wax class of *n*-alkanes (Herbin and Robins 1968a, 1969), instead of C$_{31}$ in our results (89%, see Fig. 2.6). It is noteworthy that both these discrepancies involve the longer homologs in respective compound classes, pointing to chain length bias originating from the very different methods used for sample preparation (filtration, distillation, decantation, solvent changes, fractionation) and analysis (desorption from celite, packed-column GC, isothermal elution). This is confirmed by Feakins and Sessions (2010), who, using protocols very similar to ours, found the homolog distribution of *Echeveria runyeonii* leaf wax alkanes to be shifted towards higher chain lengths than reported
before (Herbin and Robins, 1968b). We conclude that our findings of relatively high concentrations of longer homologs reflect the true composition of *A. arborescens* leaf wax more accurately than the earlier reports.

Overall, the wax composition described here for *A. arborescens* is similar to those of other monocots and dicots. Previously reported wax coverages varied from ~1 μg/cm² and ~15 μg/cm² on *Arabidopsis thaliana* leaves and stems, respectively (Pascal et al., 2013), to ~600 μg/cm² on *Fuyu* persimmon fruit (Tsubaki et al., 2013). *A. arborescens* leaves had an intermediate wax coverage (~26 μg/cm²), similar to the unrelated succulent *Kalanchoe daigremontiana* (~20 μg/cm², van Maarseveen and Jetter, 2009). In particular, the aliphatic compound classes in *A. arborescens* leaf waxes had homolog distributions similar to those reported for many other species before, with predominantly odd-numbered chain lengths for alkanes (branched and unbranched), and mainly even-numbered acids, FAMEs, primary alcohols, aldehydes as well as esters. Such profiles are typical for plant cuticular waxes and are thought to originate from the different biosynthetic pathways leading to the various compound classes (Kolattukudy, 1970; Samuels et al., 2008).

Wax biosynthesis is known to occur in two parallel branch pathways, leading from acyl CoA precursors either to primary alcohols and their alkyl esters or to aldehydes and on to alkanes one carbon shorter. These biosynthetic relationships between compound classes, mostly established in studies on model species including *Arabidopsis thaliana*, may help to interpret the homolog distribution of respective fractions of the *A. arborescens* leaf wax. The products of the two branch pathways have pairwise similarities in chain length profiles, with C₃₂ and C₃₁ predominating for aldehydes and (branched and unbranched) alkanes, respectively (see Fig. 2.6), and C₂₈ for alcohols and ester alkyl moieties (compare Figs. 2.6 and 2.7). Thus, those compounds
on the same branch pathway have clearly matching chain length profiles, whereas the homolog patterns differed drastically between pathways. In contrast, the fatty acids have a bimodal distribution combining the predominant chain lengths of both branch pathways, likely reflecting the composition of the acyl CoA pool that serves as common precursors for both modification pathways. Therefore, our chemical data on acid, aldehyde, alkane, primary alcohol and ester profiles strongly suggest that the same biosynthetic pathways previously established for a few model species also operate in the distantly related *A. arborescens*. This species is characterized by a strong selectivity of the alcohol-forming branch pathway for C_{28} substrates, and an equally strong preference of the alkane-forming branch for C_{32} substrates instead.

Further substrate specificity can be inferred for the enzyme(s) forming the alkyl esters, based on isomer profiles of the six ester homologs (Fig. 2.7). On the one hand, the matching chain length distributions of all esterified 1-alkanols (Fig. 2.7B) and free 1-alkanols (Fig. 2.6) suggests that esters are formed directly from the pool of free alcohols, without enzyme specificity towards the alcohol substrate. On the other hand, the predominance of C_{16}, C_{18} and C_{20} acyl moieties in the overall ester mixture points to strong selectivity for acyl CoA substrates with these chain lengths. Therefore, our detailed homolog and isomer analyses suggest that *A. arborescens* wax esters are formed by an enzyme with access to the primary alcohols (formed on one wax biosynthetic branch pathway) and certain acyl CoA intermediates (of the fatty acid elongation machinery), albeit with drastically differing degrees of selectivity for both its substrates. It should be noted that FAMEs, as another set of esters, have a chain length distribution more similar to free acids (although not bimodal), and are therefore likely derived from them rather than from acyl CoA intermediates.
Our second major result was the identification of three unusual compound classes with 2- and 3-hydroxyl groups. Similar medium- and long-chain 2-alkanols had been reported for waxes of various plant species, albeit in esterified form: odd-numbered homologs C₉-C₁₅ in cuticular waxes of *Eucalyptus* species (Horn et al., 1964), C₁₁-C₁₇ in waxes of *Papaver* species (Jetter and Riederer, 1996), and primarily C₁₃ and C₁₅ homologs in waxes from a wide range of *Poaceae* species (Tulloch, 1983; von Wettstein-Knowles and Netting, 1976a, 1976b; von Wettstein-Knowles, 1976), together with C₉ (von Wettstein-Knowles et al., 1984). Odd-numbered C₂₃-C₃₃ 2-alkanol homologs had also been found esterified in estolides from *Juniperus scopulorum* (A P Tulloch and Bergter, 1981), but in free form had only been reported once before, in *Solanum tuberosum* leaf wax, spanning all chain lengths from C₂₅ to C₃₀ (Szafranek and Synak, 2006a).

VLC 3-hydroxy fatty acids have not been reported before, in either free or esterified form. However, there are numerous accounts involving shorter 3-hydroxy acids and their derivatives. Acid hydrolysates of glycolipids from various bacterial strains had revealed normal, as well as anteiso and iso methyl-branched C₉ to C₁₆ 3-hydroxy acids in *Coxiella burnetti* (Wollenweber et al., 1985), C₁₂ to C₁₈ in several *Bacteroides* species (Mayberry, 1980), and mostly n-C₁₄ in *Salmonella* lipopolysaccharides (Rietschel et al., 1972). Even organic household dust had been found to contain primarily even-numbered C₁₀-C₁₈ 3-hydroxy fatty acids of the same bacterial (Mielniczuk et al., 1993) or, alternatively, fungal origin (Saraf et al., 1997). Also, several species of *Rhodotorula* red yeast had been shown to produce extracellular glycolipids incorporating C₁₆ and C₁₈ 3-hydroxy fatty acids (Tulloch and Spencer, 1964). In higher plants, certain long-chain 3-hydroxy fatty acids had been described in floral oils of various species,
either as mono- or di-glycerides, very often acetylated at the 3-OH group, and much less frequently in free form. Vogel was the first to identify C\textsubscript{14}, C\textsubscript{16} and C\textsubscript{18} 3-acetoxy fatty acids as glycerides in *Calceolaria* floral oils (Vogel, 1971). Several reports followed shortly, describing C\textsubscript{16}, C\textsubscript{18} and C\textsubscript{20} 3-acetoxy fatty acids in *Krameria* spp., both in free form and in glycerides (Seigler et al., 1978; Simpson et al., 1979, 1977). Unsaturated C\textsubscript{16} and C\textsubscript{18} 3-acetoxy acids were also detected in *Lysimachia ciliata* floral oil, either free or as glycerides (Cane et al., 1983). Finally, 3-hydroxy fatty acids (typically C\textsubscript{14}, C\textsubscript{16} and C\textsubscript{18}) have recently been reported in free form (as well as acetylated and bound in glycerides) in *Diascia* spp. (Kanchana et al., 2008) and *Malpighia coccigera* floral oils (Seipold et al., 2004).

The strong structural resemblance between 3-hydroxy fatty acids, 3-hydroxy FAMEs and 2-alkanols (Figs. 2.2-2.4) co-occurring in the leaf wax mixture of *A. arborescens* suggests that these compounds are biosynthetically related. Furthermore, the very similar chain length profiles of 3-hydroxy FAMEs and 3-hydroxy acids (Fig. 2.6) makes it very plausible that these two compound classes are derived one from the other, probably in the same way that FAMEs are derived from fatty acids. Thus, a methyl transferase might convert 3-hydroxy acids into 3-hydroxy FAMEs (possibly an \textit{S}-adenosylmethionine-dependent methyltransferase). The 3-hydroxy acids in turn may be formed in one step from corresponding 3-hydroxy acyl CoA intermediates of the fatty acid elongation cycle. To intercept these intermediates from the elongase complex, a thioesterase is required. We thus propose a simple two-step biosynthetic pathway (Fig. 2.9, center), leading from known wax biosynthesis intermediates to 3-hydroxy acids and 3-hydroxy FAMEs. Considering the predominant C\textsubscript{28} chain lengths we observed for these novel compounds as well as for free acids and corresponding FAMEs in *A. arborescens* wax, it is even possible that the same thioesterase is involved in forming both hydroxy acids and
normal acids, and the same methyl transferase may be involved in converting them into hydroxy FAMEs and normal FAMEs, respectively.

For the third class of unusual wax compounds, the 2-alkanols, three alternative pathways may be postulated based on their structure and homolog distribution: firstly, they could be formed by hydrolysis of 3-keto acyl CoA intermediates of elongation, decarboxylation to 2-ketones and reduction to the corresponding 2-alcohols (Fig. 2.9, top-right). A similar biosynthetic pathway has been formulated for (esterified) medium-chain 2-alkanols of Poaceae (Kunst et al., 2006; von Wettstein-Knowles, 2012, 1987). No additional enzymes have to be invoked for this pathway, since the decarboxylation step may occur spontaneously, and both reductase and thioesterase activities with fairly similar substrate requirements are present in the form of KCR and the esterase putatively involved in 3-hydroxy acid formation, respectively. 2-Ketones would be expected as intermediates of such a pathway, however they were not detected in A. arborescens wax. Both 2-alkanols and 2-ketones have to date only been found co-occurring in potato leaf wax, but with very different chain length profiles. Therefore, a biosynthetic relationship between them has been discussed very critically (Szafranek and Synak, 2006a).

Alternatively, the 2-alkanols might be formed by decarboxylation of 3-hydroxy acids, likely under enzymatic control (Fig. 2.9, center-right). This pathway would require a decarboxylase for which little precedence exists, and very strong chain length specificity would have to be invoked for the implicated enzyme to explain the different homolog distributions of its substrates and products (predominantly C_{28} hydroxy acids and C_{31} 2-alkanols, respectively).

Finally, 2-alkanols might also be formed by reduction of 3-hydroxy acyl CoAs and decarbonylation of the 3-hydroxy aldehyde intermediates (Fig. 2.9, bottom-right). It should be noted that these intermediates were not detected in A. arborescens wax, but they have been
described in (not closely related) *Ricinus communis* (Vermeer et al., 2003). This pathway is analogous to formation of alkanes and might involve the same enzymes, likely homologs of the Arabidopsis CER3 and CER1 proteins (Bernard et al., 2012). The involvement of these enzymes could in fact explain the very similar chain length profiles of alkanes and 2-alkanols. Furthermore, this pathway would resemble the one proposed above leading to 3-hydroxy acids, in that both require intercepting the same elongation intermediates, thus together suggesting an unusual leak of the elongase enzyme complex. Similar interception of elongation intermediates by thioesterases has been reported, however only for medium-chain acyl-ACP intermediates from FAS complexes in plant plastids (Yu et al., 2010) and the cytosol of sponge-associated bacteria (He et al., 2012). All arguments taken together, the third pathway seems to be most plausible based on our chemical evidence alone. However, the models discussed above (and possibly others) must be tested using genetic and biochemical tools, either in *A. arborescens* or in comparable models.
Figure 2.9. *Proposed biosynthetic pathways to 3-hydroxy acids, 3-hydroxy FAMEs and 2-alkanols.* Full arrows describe the biosynthetic pathways most consistent with our *A. arborescens* leaf wax analysis results. Broken arrows describe two alternative pathways towards 2-alkanols that are neither supported, nor can be ruled out solely on the basis of our results herein. Intermediates shown in square brackets are expected to be unstable and thus undetectable in surface wax. Enzymes shown in round brackets denote situations where the respective reactions can also proceed non-enzymatically.
2.4.3. Gradients between epi- and intracuticular wax layers

Substantial gradients were found in the chemical composition of the epicuticular and intracuticular wax layers on both sides of the A. arborescens leaf. The differences between adjacent layers were most pronounced for alicyclic compounds, both triterpenoids and tocopherols. This result is similar to results for many other species, where terpenoids had consistently been found accumulating predominantly (or exclusively) in the intracuticular wax layer (Buschhaus and Jetter, 2012; Buschhaus et al., 2007a, 2007b; Guhling et al., 2005; Jetter and Schäffer, 2001; van Maarseveen and Jetter, 2009).

Some of the aliphatic compound classes in A. arborescens also exhibited gradients between the epicuticular and the intracuticular wax mixtures, although only with relatively small differences in percentages between the layers. Acids were found at slightly higher concentrations in the epicuticular wax and primary alcohols in the intracuticular wax, similar to some other species investigated before (Buschhaus and Jetter, 2011). Our results on A. arborescens waxes thus help to further corroborate the previous notion that partitioning between both wax layers may be driven by differences in compound polarity (where acids must be considered fairly nonpolar due to formation of hydrogen-bonded head-to-head dimers). All other compounds showed either inconsistent effects between both sides of the A. arborescens leaf, or no gradients at all. Such conflicting behaviours for wax classes have been reported before (Buschhaus and Jetter, 2011). Interestingly, the unusual 3-hydroxy FAMEs and 2-alkanols did not partition differentially between wax layers on either leaf side, and 3-hydroxy acids showed a gradient between layers only on the adaxial side (see Fig. 2.8). Thus, these compounds with additional secondary functional groups show partitioning trends similar to those of relatively nonpolar mono-functional wax constituents.
Chapter 3: Composition of the cuticular waxes coating the adaxial side of *Phyllostachys aurea* leaves: Identification of very-long-chain primary amides

3.1. Introduction

In the evolutionary transition from aquatic to terrestrial environments, plants developed a lipid coating to protect their non-woody organs against excessive water loss (Schreiber, 2005). This skin, the cuticle, consists of the polymer cutin and a mixture of solvent-extractable waxes (Kolattukudy, 1970). Cutin is a cross-linked polyester incorporating mainly saturated and unsaturated long-chain (LC, C\(_{16}\)-C\(_{18}\)) hydroxy-fatty acids, epoxy-fatty acids, diacids, as well as glycerol (Pollard et al., 2008). Cuticular waxes are complex mixtures of very-long-chain (VLC, typically C\(_{20}\)-C\(_{34}\)) aliphatics, with no functionality (alkanes), a single terminal oxygen functional group (fatty acids, aldehydes, primary alcohols), or an in-chain group (secondary alcohols, ketones, alkyl esters). Most of the resulting compound classes occur as series of homologs with predominantly even-numbered hydrocarbon chains (acids, aldehydes, primary alcohols, esters), while some others have mainly odd-numbered homologs (alkanes, secondary alcohols, ketones) (Jetter et al., 2006). In many species, alicyclic compounds such as triterpenoids and tocopherols are present along with VLC aliphatics, in some species at fairly high concentrations (Bianchi et al., 1992; Markstadter et al., 2000; Nordby and McDonald, 1994).
Genetic and biochemical investigations have led to a detailed understanding of wax biosynthesis in *Arabidopsis thaliana* (Jetter et al., 2006; Li-Beisson et al., 2010; Samuels et al., 2008). In this model species, long-chain fatty acids are produced *de novo* in the plastids of epidermal cells and then transferred to the endoplasmic reticulum (ER) for further elongation to VLC acyls by a four-enzyme fatty acyl-CoA elongase (FAE) complex. The array of acyl-CoA homologs serves as precursors for modification into the wax products, along (i) an acyl reduction pathway yielding primary alcohols and alkyl esters, (ii) a decarbonylation pathway to aldehydes, alkanes, secondary alcohols and ketones, or (iii) hydrolysis to corresponding free fatty acids. Much evidence has been provided over several decades, showing that parts or all of the biosynthesis pathways thus defined for Arabidopsis may also be operational in diverse other species.

However, wax composition varies substantially between plant species, and thus further biosynthetic pathways, or variations of the Arabidopsis pathways, must lead to compounds not present in this model species. Therefore, further insights into wax composition, biosynthesis and function can be obtained from investigations into diverse plant lineages. For example, recent studies have revealed novel multi-functionalized wax compounds such as hydroxy alkyl esters in *Funaria hygrometrica* (Busta et al., 2016), 1,3-diol monoacetates in *Cosmos bipinnatus* wax (Buschhaus et al., 2013b). As another case point, 3-hydroxy fatty acids and their methyl esters were recently discovered in the leaf wax of the monocot *Aloe arborescens* (Racovita et al., 2015), in contrast to many earlier reports that had identified mostly standard wax compounds in Poaceae monocots (von Wettstein-Knowles, 2012). In all cases, comparisons between the chemical diversity within and between taxa led to new insights into wax biosynthesis. Thus,
detailed chemical investigations of diverse lineages have the potential to reveal further variation of wax compositions and to reveal the presence of new biosynthetic mechanisms.

Among the wax compound classes, the esters formed by combination of VLC alcohols and acids are of particular interest. Due to their extra-long hydrocarbon chains and molecular weights almost double that of other wax constituents, the esters may contribute substantially to the physiological function of the cuticle as transpiration barrier (Riederer and Schreiber, 1995). Their molecular properties also make them high-value industrial chemicals, for application as specialty lubricants, cosmetic and pharmaceutical products (Jetter and Kunst, 2008). Plant wax esters vary both in their overall chain length and in the chain lengths of their acyl and alkyl moieties, thus giving rise to homology and isomerism. The wax ester mixtures of a few plant species have been investigated in detail, and they were found to have characteristic patterns of overall ester homologs and acid/alcohol combinations. For example, *Arabidopsis thaliana* stem wax esters are formed mainly by C\textsubscript{16} acid and C\textsubscript{20}-C\textsubscript{32} alcohols (Lai et al., 2007). The esters in *Camelina sativa* leaf wax had C\textsubscript{16} and C\textsubscript{20}-C\textsubscript{22} acyl moieties combined with C\textsubscript{22}-C\textsubscript{26} alkyls (Razeq et al., 2014), while those of *Aloe arborescens* had C\textsubscript{16}-C\textsubscript{20} acyls and C\textsubscript{20}-C\textsubscript{34} alkyls (Racovita et al., 2015), and those on barley *cer-u^69* spikes had C\textsubscript{16} and C\textsubscript{20} acyls connected to C\textsubscript{22}-C\textsubscript{26} alkyls (von Wettstein-Knowles and Netting, 1976a). The compositions in some species resembled the homolog patterns of accompanying free alcohols, suggesting that the same alcohol pool may serve either for direct export to the plant surface or for formation of wax esters while still inside the epidermis cell. However, characteristic differences between chain length profiles of alcohols or acids before and after esterification further suggest substrate specificities of the ester synthase enzymes involved (Lai et al., 2007; Li et al., 2008).
Recent studies have revealed a stratification with substantial compositional gradients between the wax embedded within the cutin matrix (intracuticular wax) and that lying atop of it (epicuticular wax) (Buschhaus and Jetter, 2011). For many plant species, the somewhat more polar wax constituents (e.g., alcohols) and those with a more compact molecular geometry (e.g., terpenoids) were found in higher concentrations in the intracuticular wax. The least polar compound classes (e.g., alkanes) often accumulated preferentially in the epicuticular wax mixtures, where they affect the hydrophobicity of plant surfaces (Holloway, 1969) or may play a role in plant-pathogen (Gniwotta et al., 2005) and plant-insect interactions (Udayagiri and Mason, 1997). Depending on the species, both the intracuticular and the epicuticular wax layers may contribute to the transpiration barrier function of the cuticle (Jetter and Riederer, 2015). Overall, it is important to determine the chemical compositions of both the epi- and intracuticular wax mixtures towards a deeper understanding of their respective biological functions.

Leaves of the bamboo Phyllostachys aurea exhibit interesting surface properties (Badyal, pers. commun.), and this monocot species was hence chosen here for detailed wax analyses. No Phyllostachys wax compositions had been reported before, and studies of other bamboo genera (Bambusa, Dendrocalamus) had been restricted to the composition of one compound class, n-alkanes, for chemotaxonomy purposes (Li et al., 2012). Therefore, the present study aimed at a comprehensive analysis of the leaf cuticular waxes of Phyllostachys aurea, to identify all major compounds and to quantify respective compound classes, homolog distributions and isomer patterns in the epicuticular and intracuticular wax mixtures of young and old leaves.
3.2. Experimental

3.2.1. Plant material and preparative thin layer chromatography (TLC)

Leaves were harvested using clean metal tweezers and scissors from outdoor beds of old and young *Phyllostachys aurea* plants in June 2015 (Vancouver, British Columbia, Canada). Plant age was assessed based on the color of the culm: plants with a golden brown culm were designated as “old”, while those with a green culm as “young”.

For a first experiment, ten young leaves were submerged in 10 mL CHCl$_3$ (Aldrich, ≥99%, 0.75% ethanol as stabilizer) and vigorously shaken for 30 seconds. The CHCl$_3$ extract was transferred to another vial and the leaves were shaken with another portion of 10 mL CHCl$_3$ for another 30 seconds. The second extract was added to the first one and the total volume reduced at 50°C under a gentle stream of N$_2$ (Praxair, ≥99.998%). Compound classes in the resulting total wax extract were fractionated by preparative TLC, using the sandwich technique (Tantisewie et al., 1969), glass plates coated with silica gel 60 F$_{254}$ as stationary phase (Uniplate™, Analtech Inc., layer thickness: 1 mm, size: 20x20 cm, without concentrating zone), and a mixture of hexane:methanol:ethyl acetate 85:10:5 (v/v/v) as mobile phase (Hexane: Aldrich, anh., ≥99%; Methanol: Aldrich, for HPLC, ≥99.9%; Ethyl acetate: Aldrich, anh., ≥99.8%). TLC bands were visualized under 365 nm UV light after spraying the plates with primuline (5 mg in 100 mL acetone/water 80/20, v/v), scratched off with clean spatulas into separate glass vials, and extracted twice with 10 mL each of fresh CHCl$_3$ for 30 seconds, with agitation. The extracts were filtered through glass wool (Supelco), concentrated under N$_2$ at 50°C, transferred to GC autosampler vial inserts, evaporated to dryness and stored until GC-MS analysis.
3.2.2. Preparation of epicuticular and intracuticular wax extracts

In a second experiment, four sets of leaves were sampled from each of young and old culms. To obtain the epicuticular wax extracts, each set of leaves was first painted with an aqueous solution of gum arabic (1.5 g/mL) over the full adaxial area of the leaves, using a paintbrush. After about 30 minutes, a solid film of gum arabic and adhering epicuticular waxes were lifted with clean tweezers and transferred to a glass vial containing 7 mL each of distilled water and CHCl$_3$. The gum arabic application was repeated twice more and the resulting films added to the same vial and partitioned between water and CHCl$_3$. The organic layer was carefully transferred to another glass vial using a Pasteur pipette; the aqueous layer was extracted once more with a fresh portion of 7 mL CHCl$_3$, which was then combined with the first organic extract. The combined CHCl$_3$ extracts were concentrated at 50°C under a stream of N$_2$, quantitatively transferred to GC autosampler vial inserts, evaporated to dryness and stored until GC analysis. Typically, one biological replicate consisted in a set of four leaves, each with an adaxial area of ~8 cm$^2$. Exact leaf areas were computed using the ImageJ software, after photographing the set of leaves with their adaxial side up on a white paper background in the presence of a ruler.

To obtain the intracuticular wax extracts, a glass cylinder (9.6 mm in diameter) was gently pressed onto the adaxial surface of leaves, following epicuticular wax removal, and 1.5 mL fresh CHCl$_3$ were loaded inside it and agitated to improve extraction efficiency by bubbling air from a Pasteur pipette for 30 seconds. The CHCl$_3$ extract of intracuticular waxes was then transferred to a clean glass vial, and the extraction was repeated twice more with another two
portions of 1.5 mL fresh CHCl₃. Typically, the extracts from eight leaf locations (two per leaf) were pooled together and represented one replicate. The resulting extracts were concentrated at 50°C under N₂, quantitatively transferred to GC autosampler vial inserts, evaporated to dryness and stored until GC analysis.

3.2.3. Derivatization reactions

In preparation for GC-MS/FID analysis, wax extracts were spiked with 10 μL of CHCl₃ containing 1.02 mg/mL internal standard n-tetracosane (Alfa Aesar, ≥99%) and subjected to silylation with 10 μL N,O-bis(trimethylsilyl)trifluoroacetamide BSTFA (Aldrich, GC grade) in 10 μL pyridine (Aldrich, anh., ≥99.8%) for 30 min at 70°C. Then, samples were taken to dryness at 50°C under N₂ and re-dissolved in 20 μL CHCl₃. Under these conditions, amides did not undergo silylation, resulting in broad GC peaks and poor resolution for all homologs in this class. Therefore, after re-evaporation to dryness, samples were derivatized using 20 μL benzyl bromide (Aldrich, ≥98%) in the presence of 0.1 mg NaH (Aldrich, dry, ≥95%) at 70°C for 1 hour. After aqueous work-up, products were partitioned into CHCl₃, the organic layer was removed and the solvent evaporated to dryness. Lastly, the residue was subjected to silylation with BSTFA/pyridine again, re-dried, and then re-dissolved in 20 μL CHCl₃.

3.2.4. Gas chromatography (GC) analysis of wax extracts

Two GC instruments were used for the qualitative and quantitative analysis of wax constituents, respectively. Shared characteristics of both instruments were the type of capillary
GC column (6890N, Agilent, Avondale PA, USA; 30 m long; type HP-1: 100% PDMS; 0.32 mm i.d.; df=0.1 µm) and the oven temperature program (on-column injection at 50ºC, constant for 2 min, ramp 40ºC min⁻¹ to 200ºC, constant for 2 min, ramp 3ºC min⁻¹ to 320ºC, constant for 30 min). The first instrument was operated with He gas (Praxair, ≥99%) as mobile phase at a flow rate of 1.4 mL/min, and was equipped with an MS detector (5973N, Agilent, EI-70 eV, m/z 50-750) for qualitative identification of the separated wax compounds. The second GC employed H₂ as carrier gas (Praxair, ≥99.95%) at 2.0 mL/min and an FID detector for the quantification of individual wax constituents, based on normalization of their peak areas against that of the internal standard. It had been determined previously that most plant wax compounds have relative response factors of 1.00 with respect to n-tetracosane under almost identical GC-FID conditions (Riederer and Schneider, 1989). It should be noted that the exact FID response factors of the newly discovered acyl amide are currently unknown, and thus their quantification against the tetracosane standard had to be based on the assumption of response factors similar to other wax compounds. The current amide quantification may therefore slightly under- or overestimate the absolute amide amounts. The relative metamer compositions of alkyl ester were determined from GC-MS data as described elsewhere (Lai et al., 2007).

For statistical analysis, percentage values describing wax composition were arcsin-transformed, and then pair-wise comparisons were performed simultaneously on the entire dataset using Student’s t-tests (two-tailed, alpha = 0.05) and raw p values adjusted using a False Discovery Rate (FDR) equal to 5% with GraphPad Prism v6.0 software.
3.2.5. Synthesis of C$_{30}$ amide standard

9.0 mg (0.02 mmol) triacontanoic acid (Aldrich, 98%) were dissolved in 1.00 mL freshly distilled dichloromethane (Fisher, 99.9%) in a 5 mL glass vial, with warming. 0.03 mL (0.41 mmol) of thionyl chloride (Aldrich, 97%) were added, the vial was sealed and the mixture was stirred for four hours with occasional heating up to 70°C to keep the triacontanoic acid dissolved. The resulting mixture was dried under a gentle stream of nitrogen at 50°C and re-dissolved in 1.00 mL dichloromethane. 0.50 mL (12.55 mmol) aqueous ammonia 28.0-30.0% (Aldrich) was added, and the two-phase system was vigorously stirred overnight. The organic layer was removed, and the aqueous phase extracted with 0.50 mL of fresh dichloromethane. Both organic solutions were combined, concentrated under nitrogen and loaded onto a preparative TLC glass plate coated with silica gel 60 F$_{254}$ (layer thickness: 1 mm, size: 20x20 cm, with 4 cm concentrating zone), which was then developed with hexane:methanol:ethyl acetate 85:10:5 (v/v/v) as mobile phase. A white solid was obtained (1.0 mg, 11% yield) and found to contain >99% triacontanoic acid amide by GC-MS. $^1$H NMR (400 MHz, CDCl$_3$): δ 5.31 (s, 2H, CONH$_2$), 2.36 (t, 2H, $J$=7.6 Hz, CH$_2$CONH$_2$), 1.22-1.38 (br m, 54H, aliphatic CH$_2$), 0.89 (t, 3H, J=6.7 Hz, CH$_3$).

3.3. Results and discussion

The work herein aimed to compare the chemical composition of different layers within the cuticular waxes on the adaxial side of Phyllostachys aurea leaves on young and old culms. First, the structures of all major wax constituents were established by gas chromatography-mass
spectrometry (GC-MS; 3.3.1.), then the epicuticular and intracuticular wax layers were selectively sampled and their compound class composition (3.3.2.) and homolog patterns determined by GC with flame ionization detection (FID; 3.3.3.). Finally, the wax ester isomer distributions were quantified for each layer in further GC-MS analyses using signal ratios of specific acyl fragments (3.3.4.).

3.3.1. Identification of very-long-chain (VLC) fatty acid amides (3.1) in the adaxial cuticular waxes of P. aurea leaves

Qualitative GC-MS analysis of cuticular wax mixtures from the adaxial leaf side of both young and old bamboo plants identified homologous series (Fig. 3.1) of free fatty acids 3.2, primary alcohols 3.3, alkyl esters 3.4, aldehydes 3.5 and alkanes 3.6. They were accompanied by terpenoids 3.7, including several isomers of tocopherols, triterpenols and triterpenyl palmitates, all of which were identified by GC-MS comparison with authentic standards, or by MS characteristics matching those reported in the literature or mass spectral databases.

However, five small, evenly spaced GC peaks with MS features unprecedented in the wax literature warranted further investigation. All members of this compound class (series A) had identical, abundant fragment ions and molecular ions differing by multiples of 28 amu between them, indicating homologous compounds. For further structure elucidation, the P. aurea leaf wax mixture was fractionated by thin layer chromatography (TLC) using SiO$_2$-coated glass plates and hexane:methanol:ethyl acetate 85:10:5 (v/v/v) as mobile phase (Nordby and McDonald, 1994). The fraction containing series A ($R_f$ 0.06) was separated from free fatty acids
3.2 (Rf 0.23), primary alcohols 3.3 and most of the terpenols 3.7e-h (Rf 0.32), glutinol 3.7c and epifriedelanol 3.7d (Rf 0.38), aldehydes 3.5, alkyl esters 3.4 and terpenoid esters 3.7i-l (Rf 0.44), and alkanes 3.6 (Rf 1.00).

Figure 3.1. Compounds identified in the adaxial wax mixture of P. aurea leaves.
Compounds A had parent ions M with odd m/z, for example m/z 451 for the predominant homolog (Fig. 3.2A), indicating the presence of nitrogen. Taken together with the TLC results, this suggested fatty acid amide structures 3.1 for compounds A. This hypothesis was supported by M-43 fragments for all homologs, most likely resulting from loss of isocyanic acid (HNCO) from M. It was further confirmed by fragments m/z 59, 72 and 128 common to all compounds A, reminiscent of ions m/z 74, 87 and 143 characterizing fatty acid methyl esters, but 15 amu lighter due to the presence of –NH₂ instead of –OMe. It seems likely that the signature fragments of compounds A thus originate via mechanisms similar to those described before for fatty acid methyl esters (Härtig, 2008). Overall, compounds A were thus far confirmed as a homologous series of VLC fatty acid amides 3.1 with even-numbered chain lengths ranging from C₂₆ to C₃₄.

To further test this structure assignment, the fraction enriched in series A was benzylated with excess benzyl bromide in the presence of excess NaH, resulting in a mixture of singly and doubly N-benzylated derivatives, both with MS features fully consistent with the primary amide parent structure (Fig. 3.2B and 3.2C). Finally, the C₃₀ amide homolog 3.1c was synthesized from commercially available C₃₀ fatty acid 3.2h via a Schotten-Baumann reaction. The synthetic standard showed identical MS features and GC retention time as one compound in series A, confirming its structure to be C₃₀ amide 3.1c (Fig. 3.2D). It should be noted that the N-benzylated derivatives gave sharper GC peaks than the underivatized amides and were therefore used for GC-MS peak identification.
Figure 3.2. *Structure elucidation of compound series A from the adaxial wax mixture of P. aurea leaves.* (A) Mass spectrum and fragmentation schemes of plant C_{30} amide 3.1c. (B) Mass spectrum and fragmentation schemes of N-benzylated plant C_{30} amide. (C) Mass spectrum and fragmentation schemes of N,N-dibenzylated plant C_{30} amide. (D) Overlay of extracted ion chromatograms (m/z 149) of the monobenzylated amide series from *P. aurea* adaxial leaf wax and the monobenzyl derivative of synthetic C_{30} amide; the fragment m/z 149 is distinctive for the monobenzyl derivatives of aliphatic amides.
Fatty acid amides had not been described in plant cuticular waxes before. More generally, nitrogen-containing compounds had been reported in only a few instances as constituents of plant wax mixtures, specifically in the form of monoterpenoid indole alkaloids, such as catharanthine, in the surface waxes of *Catharanthus roseus* leaves (Roepke et al., 2010), and of *N*-permethylated alkaloids in the waxes of several Papaveraceae species (Jetter and Riederer, 1996). In both cases, the *N*-containing compounds were found in surface extracts from species with fairly high concentrations of alkaloids in the underlying tissues, raising the possibility that these compounds were present due to contamination of wax samples (extracted from interior parts of respective organs). The discovery of fatty acid amides in bamboo wax may now raise similar questions. However, it should be noted that other internal lipids often found as contaminants of cuticular wax mixtures, such as acyl glycerides, were not detected in our bamboo wax samples, and that the acyl amides were found exclusively in the epicuticular waxes (see section 3.3.2.). Therefore, the fatty acid amides must be regarded as true components of the bamboo cuticle rather than contamination from internal tissues.

Interestingly, a compound with similar VLC acyl and amide feature, *N*-2-(*p*-hydroxyphenyl)ethyl C$_{30}$ amide, had previously been isolated from macerated, dried leaves of *Pseudaranthemenum carruthersii* (Nga et al., 2012). Unfortunately, the localization of this compound within the plant tissues was not studied. However, its acyl chain length and structural resemblance to 2-(*p*-hydroxyphenyl)ethyl esters of VLC fatty acids, which are fairly common cuticular wax constituents (Acevedo et al., 2000; Alfatafa et al., 1989; Oksuz and Topcu, 1992), both suggest that it may be formed in epidermal tissues (along with cuticular waxes). It is thus plausible that this amide too is accumulating in the cuticular wax of *P. carruthersii*. 
Shorter-chain acyl amides are of pharmacological importance, for example oleamide present in human plasma and involved in the sleep/wake cycle (Mendelson and Basile, 2001) and vasodilation (Hiley and Hoi, 2007), linoleamide mediating Ca$^{2+}$ flux (Lo et al., 2001), erucamide modulating water balance (Hamberger and Stenhagen, 2003), and elaidamide potentially functioning as an endogenous inhibitor of epoxide hydrolase (Morisseau et al., 2001). In this context, it is interesting to speculate whether the VLC primary amides occurring on the leaf surfaces of the *P. aurea* bamboo may also have biological functions, either affecting the physiological properties of the cuticle as a transpiration barrier or its ecological functions in interactions with microorganisms and herbivores.

It should also be noted that primary amides of unsaturated C$_{20}$ and C$_{22}$ fatty acids, such as erucamide, are produced on an industrial scale from seed oils of *Limnanthes alba* (Burg and Kleiman, 1991) and *Crambe abyssinica* (Nieschlag and Wolff, 1971), and serve as slip agents and antiblock agents for low-density polyethylene sheets (Shuler et al., 2004). Saturated amides, like stearamide and behenamide, have been used in the fabrication of water-repellent textiles (Nieschlag and Wolff, 1971).

While the biosynthetic pathways leading to fatty acid amides in plants are currently unknown, the biosynthesis of mammalian fatty acid amides has been investigated (Farrell and Merkler, 2008). There is experimental support for three proposed mammalian biosynthesis pathways: (1) direct amidation of fatty acyl-CoA intermediates by ammonia, catalyzed by cytochrome c (Driscoll et al., 2007), (2) two-step oxidation of *N*-acylethanolamines to *N*-$
acylglycines (Chaturvedi et al., 2006; Prusakiewicz et al., 2002) and their cleavage by peptidylglycin α-amidating monooxygenase (PAM), and (3) a combined pathway involving cytochrome c-mediated production of N-acylglycines followed by PAM oxidation to the primary fatty acid amide (Mueller and Driscoll, 2007). It is tempting to speculate that the bamboo acid amides are formed on a pathway similar to (1) above, where ammonia directly amidates VLC acyl-CoA thioesters generated in the epidermal ER by the fatty acyl elongase (FAE) enzymatic complex. This scenario is assuming the presence of ammonia in or near the ER, possibly released during amino acid metabolism (Rosler et al., 1997). However, a plant pathway involving N-acylglycine intermediates must also be considered.

3.3.2. Compound class gradients between the epicuticular and intracuticular layers lining

*P. aurea* leaves

In a second set of experiments, all compounds were quantified within the epicuticular and intracuticular wax layers of both young and old bamboo leaves. Based on previous experience with diverse other plant species (Buschhaus et al., 2007a, 2007b; Jetter and Schäffer, 2001), three consecutive treatments with gum Arabic were used to selectively remove the epicuticular wax layer, followed by extraction with CHCl₃ to sample the intracuticular wax.

The epicuticular wax on the adaxial side of young leaves amounted to 1.81 ± 0.08 μg/cm², while the adjacent intracuticular wax had a significantly lower coverage of 1.69 ± 0.06 μg/cm² (p<0.05). In comparison, the epicuticular wax load on old leaves was 1.92 ± 0.06 μg/cm².
again accompanied by significantly lower amounts of intracuticular wax at \(1.71 \pm 0.09 \, \mu g/cm^2\) (p<0.01).

The relative amounts of almost all compound classes differed between the epicuticular and intracuticular wax layers on young and old leaves (Fig. 3.3A/B). On the adaxial side of young leaves, free fatty acids \(3.2\), alkyl esters \(3.4\), aldehydes \(3.5\), alkanes \(3.6\) and amides \(3.1\) accumulated preferentially in the epicuticular layer, while primary alcohols \(3.3\) and terpenoids \(3.7\) were found at higher concentrations in the intracuticular wax (Fig. 3.3A). On old leaves, similar gradients were observed (Fig. 3.3B), except that alkanes \(3.6\) were found evenly distributed between layers. Interestingly, fatty acid amides \(3.1\) were found exclusively in the epicuticular wax of both young and old leaves.

Overall, our findings on compound class gradients between epi- and intracuticular wax layers on bamboo leaves are in accordance with reports from other plant species, with higher concentrations of primary alcohols \(3.3\) in the intracuticular compartment, and preferential accumulation of alkanes \(3.6\), aldehydes \(3.5\) and alkyl esters \(3.4\) in the epicuticular wax (Buschhaus and Jetter, 2012; Buschhaus et al., 2007a; Gniwotta et al., 2005; Racovita et al., 2015; Riedel et al., 2007; Wen et al., 2006). It has been speculated that such gradients may be due to the higher polarity of alcohols \(3.3\) and their hydrogen-bonding capability towards oxygen atoms of cutin (Buschhaus and Jetter, 2011), and our results thus support this hypothesis.
Figure 3.3. *Compound class distribution within the epicuticular and intracuticular wax mixtures coating the adaxial side of P. aurea leaves.* Relative abundances (mass %) of compound classes in each wax layer on leaves of (A) young and (B) old plants. Bars represent mean ± standard deviation ($n = 4$). Asterisks mark discovery of significant differences between arcsin-transformed percentages based on Student’s t-test ($p < 0.05$, FDR 5%).
Fatty acids 3.2 accumulated in the epicuticular wax of bamboo leaves, again in accordance with previous reports on other species (Jetter and Schäffer, 2001; Racovita et al., 2015; Wen et al., 2006b). This finding is of special interest, since fatty acids 3.2 are known to form H-bonded head-to-head dimers in the solid state, resulting in relatively low overall polarities similar to alkyl esters 3.4 (Bond, 2004; Leiserowitz, 1976; Moreno et al., 2006). The accumulation of fatty acids 3.2 together with esters 3.4 in the epicuticular wax thus further underpins the hypothesis that epi-/intracuticular partitioning is driven by polarity of wax compounds.

Interestingly, the bamboo fatty acid amides 3.1 accumulated exclusively in the epicuticular wax layer, an extreme partitioning unprecedented in the plant wax literature. This behaviour again suggests relatively low polarity, and might be explained by hydrogen-bonded molecular associations in the solid state, as described for crystals of tetradecanamide (Turner and Lingafelter, 1955) and decanamide (Brathovde and Lingafelter, 1958), as well as monolayers of dodecanamide (Bhindé et al., 2010a) and hexadecanamide (Bhindé et al., 2010b) deposited on graphite substrates. Of note, it has been shown that fatty acid amides 3.1, added as slip/antiblock agents to bulk polyolefin matrices, spontaneously migrate to the polymer surface (similar to those in the bamboo cuticle), where they serve to reduce the coefficient of friction and thus prevent the adherence of polymer sheets to one another (Dragnevski et al., 2009; Ramirez et al., 2002).
3.3.3. Individual compound gradients between the epicuticular and intracuticular layers lining *P. aurea* leaves

The chain length profiles of aliphatic compound classes were similar between the epicuticular and intracuticular wax mixtures, for both young and old plants (Fig. 3.4A/B). In particular, the primary alcohol 3.3 and aldehyde 3.5 fractions each had similar homolog distributions in the epicuticular and intracuticular waxes of both young and old leaves. In contrast, free fatty acids 3.2 showed bimodal chain length distribution with maxima at C_{16} and C_{28} in the epicuticular leaf wax of old leaves, whereas the adjacent intracuticular acids exhibited a trimodal profile peaking at C_{16}, C_{22} and C_{30}. For young bamboo leaves, only the C_{18} and C_{22} acids showed significantly higher relative amounts in the intracuticular compartment compared with the epicuticular wax, while on old leaves C_{16}-C_{22} acids accumulated in the intracuticular layer, and C_{24} and C_{28} acids in the epicuticular wax. Similar patterns have been reported for fatty acids on *Taxus baccata* needles, where acid homologs up to C_{24} accumulated preferentially in the intracuticular layer, and those longer than C_{24} in the epicuticular layer (Wen et al., 2006b).

The homolog distributions of alkyl esters 3.4 and alkanes 3.6 were fairly similar between the epicuticular and intracuticular layers on both young and old leaves (Fig. 3.4A/B). The primary amides 3.1, found only in the epicuticular wax, were dominated by the C_{30} homolog 3.1c. The shorter homologs (C_{26} and C_{28}) were more abundant than the longer ones (C_{32} and C_{34}) on the adaxial surface of young leaves, while old leaves showed the opposite pattern.
Figure 3.4. *Single constituent distribution within compound classes in the epicuticular and intracuticular wax layers on the adaxial side of P. aurea leaves.* Relative abundances (mass %) of individual VLC homologs or terpenoid isomers within each compound class on (A) young and (B) old leaves. Bars represent mean ± standard deviation ($n = 4$). Asterisks mark discovery of significant differences between arcsin-transformed percentages based on Student’s t-test ($p < 0.05$, FDR 5%). Minor odd-numbered acid, alcohol and aldehyde homologs and even-numbered alkane homologs were omitted for clarity (and thus respective groups of bars do not add up to 100%).
Most of the cyclic compounds also showed concentration gradients between the wax layers. In particular, γ-tocopherol 3.7a was found in greater proportion in the epicuticular layer of old leaves, while it was evenly distributed on young ones (Fig. 3.4A/B). α-Tocopherol 3.7b was also found in greater proportion in the epicuticular wax of old leaves. Glutinol 3.7c, β-amyrin 3.7e, and α-amyrin 3.7f accumulated preferentially in the intracuticular layer on both young and old leaves. In contrast, isomultiflorenol 3.7g and epifriedelanol 3.7d were found mainly in the epicuticular wax compartment on old leaves. Lupeol 3.7h was found evenly distributed between compartments regardless of plant age. Finally, glutinyl palmitate 3.7i and isomultiflorenyl palmitate 3.7l exhibited preferential accumulation in the epicuticular layer of young and old leaves.

Overall, terpenoids 3.7 thus accumulated to higher concentrations in the intracuticular layer, likely due to their more compact molecular geometry and polar functional groups, again consistent with many previous studies (Buschhaus and Jetter, 2012; Buschhaus et al., 2007a, 2007b; Racovita et al., 2015; Riedel et al., 2007, 2003). A notable exception to this pattern were triterpenoid esters 3.7i-1, with partitioning behaviour opposite to that of free triterpenols. This new finding suggests that the presence of an alkyl side chain, masking the polar functionality and substantially increasing the molecular volume, may drastically decrease their overall polarity and make the terpenoid esters 3.7i-1 partition into the epicuticular layer (together with the alkyl esters 3.4).
3.3.4. Ester isomer profiles in the epicuticular and intracuticular layers on *P. aurea* leaves

In a separate experiment, the distribution of alkyl and acyl moieties within each of the alkyl ester homologs was determined. Since the ester metamers, comprising various combinations of acyls and alkyls all with the same total chain length, could not be resolved chromatographically, they had to be profiled based on mass spectral information. Average mass spectra were acquired from each ester homolog peak from epicuticular and intracuticular wax at both leaf ages, and relative abundances of product ions produced by McLafferty rearrangement with double hydrogen transfer (R-CO₂H₂⁺) were used to calculate isomer distributions, as reported before (Lai et al., 2007; Racovita et al., 2015; Razeq et al., 2014).

The ester homologs 3.4 within the epicuticular wax on young bamboo leaves had isomer compositions grouping them according to total ester chain length (Fig. 3.5A). The short ester homologs, with total carbon numbers C₃₆-C₄₀ 3.4a-c, were mainly formed by acids with gradually increasing chain lengths (C₁₆-C₂₀) in combination with the same alcohol (C₂₀). In contrast, the long ester homologs, C₄₆-C₅₂ 3.4f-i, incorporated primarily one acid (C₂₂) linked to various alcohols (C₂₄-C₃₀). The most abundant ester homolog, C₄₈ 3.4g, was thus formed mainly by combination of C₂₂ acid and C₂₆ alcohol. The mid-range ester homologs, C₄₂-C₄₄ 3.4d-e, had isomer compositions transitioning between the patterns of the shorter and longer esters, on the one hand comprising relatively large percentages of C₂₂ acid and on the other hand C₂₀ alcohol, accompanied by various other combinations of acid and alcohols chain lengths. The epicuticular esters on old leaves had isomer compositions very similar to those on young leaves (Fig. 3.5B).
Figure 3.5. *Relative isomer compositions of ester homologs in epicuticular and intracuticular waxes on the adaxial side of young and old P. aurea leaves.* Relative abundances (mass %) of individual metamers (indicated by their acyl moiety chain length) within each ester homolog in leaf wax from (A) young and (B) old plants. Bars represent mean ± standard deviation (*n* = 4, except for old leaf intracuticular *n* = 3).

The intracuticular esters 3.4 on both young and old leaves closely resembled those in the adjacent epicuticular layers (Fig. 3.5), with only a few shifts in the chain length profiles of esterified acid and alcohol moieties. In particular, the intracuticular C₄₀ ester 3.4c contained higher proportions of C₁₆, C₂₂ and C₂₄ acids than its epicuticular counterpart, irrespective of plant age. In contrast, the C₄₂ 3.4d and C₄₄ 3.4e esters had much lower percentages of C₁₆ acid metamers than respective epicuticular ester homologs. Finally, the longest ester homologs, C₄₆-C₅₀ 3.4f-h, comprised relatively large amounts of esterified C₂₄ acid as well. Overall, the wax
esters 3.4 in the intracuticular wax were thus characterized by a slightly broader chain length distribution than those in the epicuticular layer. It should be noted that the isomer composition of the least abundant ester homologs (C$_{36}$ 3.4a, C$_{38}$ 3.4b and C$_{52}$ 3.4i) could not be determined, as their concentrations were below the MS detection limit.

Overall, our ester isomer analyses suggest that P. aurea leaves may harbor up to three wax ester synthases with different substrate chain length preferences to produce most of the ester isomers and homologs. Namely, one synthase may prefer C$_{22}$ and (to lesser extent) C$_{24}$ acyl-CoA for formation of C$_{42}$-C$_{52}$ esters 3.4d-i, a second enzyme may prefer C$_{20}$ primary alcohol to form C$_{36}$-C$_{40}$ esters 3.4a-c, and a third enzyme C$_{16}$ acyls for formation of C$_{42}$ 3.4d and C$_{44}$ 3.4e esters. However, the amounts and isomer composition of the latter two ester homologs may also be explained by availability of substrates (rather than enzyme specificity), as C$_{26}$ and C$_{28}$ alcohols had relatively high concentrations (compare Fig. 3.3A/B) and C$_{16}$ acyl-CoA, as the first precursor common to many ER-localized lipid pathways (Lai et al., 2007; Ohlrogge and Browse, 1995), may also be expected to be abundantly available at the site of biosynthesis in the epidermal ER.

The cumulative amounts of esterified acids could be determined by pooling the percentages of all acyl moieties in individual ester homologs, taking coverages (µg/cm$^2$) of individual homologs into account and including triterpenoid esters. Independent of plant age, both the epicuticular and intracuticular esters were dominated by C$_{16}$ acid, primarily due to the large amounts of triterpenoid palmitates (Fig. 3.6A/B). There were no significant differences between epicuticular and intracuticular amounts of esterified C$_{16}$ acid at both ages. In contrast, most of the other acids accumulated in substantially lower amounts in the esters of the intracuticular layer of both old and young leaves.
Figure 3.6. Profile of total esterified acids in the epicuticular and intracuticular waxes on the adaxial side of young and old *P. aurea* leaves. Relative abundance (mass %) of each esterified acid homolog on the adaxial side of (A) young and (B) old *P. aurea* leaves. Bars represent mean ± standard deviation (*n* = 4). Asterisks mark discovery of significant differences between arcsin-transformed percentages based on Student’s t-test (*p* < 0.05, FDR 5%).
Similarly, the chain length profiles of total esterified primary alcohols and triterpenols could be calculated, and were also found to be very similar between young and old leaves (Fig. 3.7A/B). Both the epicuticular and intracuticular esterified alcohol profiles were bimodal, peaking at C_{26}/C_{24} and, to a lesser degree, at C_{20}. The majority of alcohol homologs accumulated preferentially in the epicuticular waxes. Among the triterpenols, only glutinol was found more in the epicuticular esters than the intracuticular.
Figure 3.7. Profile of total esterified alcohols in the epicuticular and intracuticular waxes on the adaxial side of young and old P. aurea leaves. Relative abundance (mass %) of each esterified VLC alcohol homolog and terpenol isomer on the adaxial side of (A) young and (B) old P. aurea leaves. Bars represent mean ± standard deviation (n = 4). Asterisks mark discovery of significant differences between arcsin-transformed percentages based on Student’s t-test (p < 0.05, FDR 5%).
Chapter 4: Composition of cuticular waxes coating flag leaf blades and peduncles of *Triticum aestivum* cv. Bethlehem

4.1. Introduction

The leaves, non-woody stems, flowers and fruit of terrestrial plants are coated by cuticles to prevent uncontrolled water loss. This lipid layer is composed of the polyester cutin and a mixture of cuticular wax, some of which is embedded in the cutin matrix (intracuticular wax) and some of which lies on top of it (epicuticular wax) (Buschhaus and Jetter, 2011). Cuticular wax composition varies both qualitatively and quantitatively between plant species, and in some cases between organs or even tissues of the same species.

Waxes are complex mixtures commonly composed of monofunctional derivatives of very-long-chain (VLC, i.e. >C$_{20}$) fatty acids, including primary alcohols (1-alkanols), alkyl esters, aldehydes, alkanes, secondary alcohols, ketones, and free fatty acids (Jetter et al., 2006). Alicyclic compounds, such as triterpenoids, are also often found mixed together with VLC aliphatics in cuticular waxes, sometimes in greater amount than the latter (Belge et al., 2014; Bianchi et al., 1993; Markstadter et al., 2000; Nordby and McDonald, 1994; van Maarseveen et al., 2009). Lastly, in several plant species, aromatic wax compounds have been reported as well, such as alkylresorcinols (Adamski et al., 2013; Ji and Jetter, 2008), alkyl *m*-guaiacols (Basas-Jaumandreu et al., 2014), benzyl and phenethyl esters (Gülz and Marner, 1986; Jetter and Riederer, 1996; Rapley et al., 2004).
VLC aliphatic waxes occur as homologous series, typically with consecutive homologs separated by two methylene (CH$_2$) units as a consequence of their biosynthesis. Extensive genetic and biochemical studies have led to a comprehensive understanding of cuticular wax biosynthesis in the model plant species *Arabidopsis thaliana* (Kolattukudy, 1970; Li-Beisson et al., 2010; Samuels et al., 2008). Accordingly, plant wax biosynthesis begins in the plastids of epidermal cells, where long-chain (LC, C$_{16}$-C$_{18}$) fatty acids are synthesized *de novo*. Next, these are transferred to the endoplasmic reticulum (ER), where a fatty acyl-CoA elongase (FAE) enzymatic complex extends them in increments of two carbons to VLC fatty acyl-CoA thioesters, which are then processed via two major pathways. On the *acyl reduction pathway*, fatty acyl-CoA reductase (FAR) enzyme(s) generate(s) fatty 1-alkanols, by reduction via fatty aldehyde intermediates that are not released, and wax ester synthase(s) link these fatty 1-alkanols with other (V)LC acyl-CoAs into alkyl esters. On the *decarbonylation pathway*, another reductase transforms fatty acyl-CoA substrates into aldehydes, which are then decarbonylated to $n$-alkanes. In some species (including Arabidopsis), alkanes can be further hydroxylated by cytochrome P450 enzymes to secondary alcohols, ketones, diols and ketols (Greer et al., 2007; Wen and Jetter, 2009).

However, the Arabidopsis model of wax biosynthesis does not account for the whole range of VLC aliphatic wax structures encountered in diverse species. Valuable insight can thus be obtained by exploring biosynthetic pathways operating in other plant species. In particular, Poaceae are thought to have a third wax biosynthetic pathway leading to β-diketones (Kunst et al., 2006; von Wettstein-Knowles, 2012, 1976). Based on genetic and biochemical evidence for
barley (*Hordeum vulgare*), it was inferred that this pathway comprises three enzyme activities (CER-Q, CER-C, CER-U) residing on a single multi-domain protein (von Wettstein-Knowles and Sogaard, 1981, 1980), including at least one polyketide 3-ketoacyl-CoA synthase (pkKCS) and a cytochrome P450 hydroxylase. However, a detailed understanding of the \( \beta \)-diketone pathway is currently missing. Substantial progress on wax biosynthesis can thus be expected in the near future based on the recently assembled genome information for the Poaceae crops barley and wheat. The molecular genetic investigations will have to build on reliable data on the chemical composition of the waxes of these model species.

Numerous reports have described wax compositions of wheat over more than four decades (Bianchi et al., 1980; Tulloch and Weenink, 1969; Wang et al., 2015a, 2015b). On the one hand, these previous studies all gave very similar qualitative results, describing very similar sets of typical wax constituents matching those on most other plant species, together with one to four \( \beta \)-diketones. However, the large majority of the investigations were carried out fairly long ago, with analytical equipment far inferior to modern instruments. Accordingly, many important wax components may have been missed, and specialty wax components that could inform speculations on wheat wax biosynthesis pathways and specific enzymes have not been described to date.

On the other hand, several previous reports suggested much quantitative variation in the relative amounts of ubiquitous wax compounds within respective wheat wax mixtures. Some of this variation has been ascribed to differences between wheat species or cultivars with different genetic backgrounds. Substantial variation was also linked to plant age, likely due to changes in
plant architecture and organ composition during plant ontogeny. However, many of the previous wheat wax studies focused either only on leaves or analyzed whole plants without distinguishing organs (Bianchi and Corbellini, 1977; Bianchi et al., 1980; Tulloch and Hoffman, 1973, 1971; Tulloch and Weenink, 1969; Tulloch, 1973). Only a few, mostly recent studies showed distinct wax compositions, in particular between leaves, stems and inflorescence parts (Adamski et al., 2013b; Wang et al., 2015a, 2015b). The findings thus far match those carried out in much more detail for barley (Lundqvist and von Wettstein, 1962; Lundqvist et al., 1968; von Wettstein-Knowles, 1969) pointing to particular differences between the lower and the upper portions of the wheat plants. Thus, the (lower) leaves seem to have wax mixtures dominated by 1-alkanols, while the inflorescence waxes contain relatively large percentages of β-diketones. The organs in the transition zone are of particular interest, including the flag leaf (i.e. the top-most leaf directly below the inflorescence) and the peduncle (i.e. the stem segment between flag leaf and inflorescence).

In the light of the scattered information on wheat cuticle chemistry, the goal of the present investigation was to provide a comprehensive wax analysis of a wheat cultivar that is concurrently used in molecular genetic and biochemical investigations into the biosynthesis of wheat waxes (Hen-Avivi et al., 2016). To this end, the bread wheat (*Triticum aestivum* L.) cultivar Bethlehem was chosen, and wax analyses were performed using GC-MS to elucidate wax component structures, GC-FID for accurate quantification of individual compounds, and further quantification based on characteristic MS fragments to also assess isomer profiles within some of the compound classes. We concentrated our analyses on the blade of the uppermost leaf.
(the flag leaf) and the top stem portion (the peduncle), as two organs expected to have 1-alkanol- and β-diketone-dominated waxes, respectively.

4.2. Experimental

4.2.1. Plant material and preparation of total wax extracts

*Triticum aestivum* cv. Bethlehem plants were grown continuously in a greenhouse at the Weizmann Institute of Science (Rehovot, Israel) in a 12-14 h / 10-12 h light/dark cycle at 24-26°C / 17-18°C, respectively. A mix of 50% peat and 50% turf was used for plant growth, and plants were watered every 3-4 days with 300-500 mL water per 5 L pot. Flag leaves and peduncles were excised from mature plants using clean razor blades during the month of August 2013. Typically, one flag leaf blade with a total area of 40-50 cm² (both sides) and, respectively, one peduncle with a cylindrical area of approx. 20 cm² represented one biological replicate. Exact areas of leaf blades were determined by photographing them and using the ImageJ software to calculate the area of one side, then multiplying by 2 to account for both leaf sides. Exact areas of peduncles were calculated using the formula for lateral surface of a cylinder (π x D x L), after measuring the diameter D and length L of peduncles with a ruler.

Leaf specimens were rolled up and peduncles cut into smaller pieces such as to be fully submerged into 10 mL CHCl₃ (Aldrich, ≥99%, 0.75% ethanol as stabilizer) containing also 5 μg of *n*-tetracosane (Alfa Aesar, ≥99%) as internal standard. After stirring for 30 s at room temperature, the CHCl₃ was transferred to another vial, and a fresh portion of 10 mL CHCl₃ was
added to the plant material and the extraction repeated for another 30 s. The two combined extracts were evaporated at 50°C under a stream of N₂ (Praxair, ≥99.998%), until the volume was low enough for transfer to 2 mL GC autosampler vials.

4.2.2. Derivatization reactions

In preparation for GC analysis, all samples were derivatized by silylation as follows: samples were taken to dryness under N₂, then 10 μL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Aldrich, GC grade) and 10 μL pyridine (Aldrich, ≥99.8%, anhydrous) were added, the vial was sealed and the mixture was refluxed at 70°C for 20 min. Then, excess reagents were completely removed under N₂ and the sample re-dissolved with 50 μL CHCl₃. Under such conditions, acidic OH groups underwent silylation, but the enol tautomers of β-diketones did not to any significant extent. By increasing derivatization time to 30-120 min, some of the β-diketones formed trimethylsilyl ethers of their enol tautomers, but no complete conversion was achieved for any of the derivatization times tested. Thus, the 20 min derivatization time was chosen in all quantitative experiments to prevent silylation of β-diketones, as recommended by other authors (Stefan Schulz et al., 2000).

For further confirmation of hydroxyl group location in hydroxy-β-diketones, one specimen was acetylated prior to silylation and GC-MS analysis, as follows: to the dry wax mixture dissolved in 10 μL pyridine, 10 μL acetic anhydride (Aldrich, ≥98%) were added and the mixture was refluxed for 5 min at 70°C, then was stored overnight at room temperature, before evaporating excess reagents and performing silylation as described above. For the same scope, a
second specimen was fully reduced, as follows: to the dry wax mixture dissolved in 50 μL diethyl ether (Aldrich, ≥99.7%, anhydrous, 1 ppm BHT as inhibitor), 0.1 mg LiAlH₄ (Aldrich, ≥95%) were added, the vial was sealed and left to react at 70°C overnight. Then, the reaction mixture was quenched with 10% aqueous H₂SO₄ and extracted three times with 60 μL each of fresh diethyl ether, the ether extracts were combined and evaporated under N₂, and silylation was performed as described above.

4.2.3. GC-MS and GC-FID analysis

Two different GC instruments were used for separation, identification and quantification of wax constituents, both equipped with the same type of capillary GC column (6890N, Agilent, Avondale PA, USA; 30 m long; type HP-1: 100% PDMS; 0.32 mm i.d.; df=0.1 μm), both with on-column injection at 50°C and programmed to follow the same temperature program (2 min at 50°C, ramp 40°C/min to 200°C, constant for 2 min, ramp 3°C/min to 320°C, constant for 30 min). The first GC instrument employed He (Praxair, ≥99%) as carrier gas, at a flow rate of 1.4 mL/min, and was equipped with MS detector (5973N, Agilent, EI-70 eV, ionization source temperature 240°C), serving primarily the purpose of identification of compounds in the plant wax mixtures. The second used H₂ (Praxair, ≥99.95%) as mobile phase at 2.0 mL/min and was equipped with an FID detector, for quantification of individual wax homologs based on normalization of peak areas against that of the internal standard. The relative response factors of all cuticular wax classes with respect to the internal standard were approximated to 1.00, in agreement with past reports using the same GC operation conditions (Riederer and Schneider, 1989).
4.3. Results

The present work aimed to provide a comprehensive analysis of the wax mixtures coating select vegetative organs of mature wheat. Specifically, the blade of the flag leaf and the peduncle were to be investigated, as examples of surfaces characterized by the presence of platelet-shaped wax crystals and tubule-shaped wax crystals, respectively (Wang et al., 2015a). In the following, the results will be described in direct comparisons between both organs, first for the overall wax composition (2.1) and the proportions of acyl monomers contained in the mixtures (2.2), then the chain length distribution (2.3) and isomer composition of the ester-linked dimers (2.4), and finally the compositions of the polyketide and terpenoid wax constituents (2.5).

4.3.1. Overall composition of wheat flag leaf blades and peduncles

Preliminary screening by gas chromatography-mass spectrometry (GC-MS) revealed well over 100 different compounds in each of the two wax mixtures extracted from wheat flag leaf blades and peduncles. The large majority of these were present in both wheat wax mixtures, and they were identified as compounds falling into 14 different classes (Fig. 4.1). Four of these constituent classes were recognized as free fatty acids, aldehydes, \( n \)-alkanes, and \( 1 \)-alkanols, hence compounds found ubiquitously in the wax of many species. Five other compound classes found on both wheat organs were identified as esters combining acyl moieties with various aliphatic or aromatic alcohols. Further wheat wax constituents were assigned polyketide structures, two of them purely aliphatic (\( \beta \)-diketone and hydroxy-\( \beta \)-diketones) and two combining aliphatic and aromatic moieties (alkylresorcinols and methyl alkylresorcinols). Finally, a few compounds found only in peduncle wax were identified as terpenoids.
Figure 4.1. Compounds identified in the total wax mixtures of T. aestivum cv. Bethlehem flag leaf blades and peduncles.
While flag leaf blade and peduncle waxes were found to share most compound classes, they differed drastically in the relative proportions of these constituents (Fig. 4.2). The mixture extracted from flag leaves was dominated by 1-alkanols 4.2, representing 55% of the GC-detected compounds (8.82 ± 0.73 μg/cm²) (Fig. 4.2A). They were accompanied by substantial amounts of alkanes 4.4 (9%; 1.46 ± 0.15 μg/cm²), 1-alkanol esters 4.5 (9%; 1.51 ± 0.14 μg/cm²), β-diketone 4.10 (6%; 0.92 ± 0.25 μg/cm²) and hydroxy-β-diketones 4.11 (8%; 1.22 ± 0.40 μg/cm²). Relatively small portions of acids 4.1 (1%; 0.15 ± 0.03 μg/cm²), aldehydes 4.3 (3%; 0.40 ± 0.07 μg/cm²), benzyl esters 4.7 (0.2%; 0.03 ± 0.004 μg/cm²), phenethyl esters 4.8 (0.1%; 0.02 ± 0.004 μg/cm²), p-hydroxyphenethyl esters 4.9 (1%; 0.18 ± 0.06 μg/cm²), 2-alkanol esters 4.6 (1%; 0.16 ± 0.03 μg/cm²), alkylresorcinols 4.12 (2%; 0.25 ± 0.02 μg/cm²) and methyl alkylresorcinols 4.13 (2%; 0.33 ± 0.02 μg/cm²) were present. Taken together, 96% of the wax mass detected by GC could be identified. The wheat flag leaf had a total wax coverage of 16 ± 1 μg/cm², including all identified and unidentified compounds.

In contrast to the flag leaf wax, the mixture extracted from wheat peduncles was dominated by β-diketone 4.10 (37%; 17.91 ± 1.92 μg/cm²) and hydroxy-β-diketones 4.11 (44%; 21.20 ± 2.38 μg/cm²) (Fig. 4.2B). Alkanes 4.4 (7%; 3.40 ± 0.77 μg/cm²), 1-alkanols 4.2 (2%; 1.13 ± 0.14 μg/cm²) and 1-alkanol esters 4.5 (2%; 1.13 ± 0.09 μg/cm²) were present at much lower concentrations than in the leaf wax mixture. Relatively small portions of acids 4.1 (1%; 0.44 ± 0.05 μg/cm²), aldehydes 4.3 (1%; 0.46 ± 0.06 μg/cm²), benzyl esters 4.7 (0.2%; 0.10 ± 0.02 μg/cm²), phenethyl esters 4.8 (0.1%; 0.05 ± 0.01 μg/cm²), p-hydroxyphenethyl esters 4.9 (2%; 0.84 ± 0.23 μg/cm²), 2-alkanol esters 4.6 (1%; 0.51 ± 0.06 μg/cm²), alkylresorcinols 4.12 (0.3%;
0.17 ± 0.03 μg/cm²) and methyl alkylresorcinols 4.13 (0.3%; 0.16 ± 0.01 μg/cm²) were detected in the peduncle wax, thus in relative amounts similar to those in flag leaf wax. Terpenoids 4.14 accumulated to 0.6% (0.27 ± 0.07 μg/cm²) in the peduncle wax mixture, leaving only 2% of the wax mixture unidentified. The wheat peduncle had a total wax coverage of 49 ± 5 μg/cm², and thus more than triple the wax quantity covering the flag leaf blade.
Figure 4.2. Compound class compositions of total wax mixtures of T. aestivum cv. Bethlehem. Coverages (μg/cm²) of compound classes within wax mixtures covering the (A) flag leaf blade and (B) peduncle. Bars represent mean ± standard deviation (n = 5).
4.3.2. Chain length distributions of common wheat compound classes

The compound classes typically found in plant cuticular waxes (fatty acids, 1-alkanols, aldehydes, alkanes) were all present in the wheat wax mixtures as extended series of homologs (Fig. 4.3). The acid fraction 4.1 of the flag leaf wax was characterized by the presence of even-numbered homologs, ranging from C\textsubscript{20} to C\textsubscript{32} and with a bimodal distribution, having a broad maximum between C\textsubscript{26} and C\textsubscript{30} and a second maximum at C\textsubscript{22}. The peduncle wax contained the same acid homologs 4.1, largely dominated by C\textsubscript{28} acid alone and with a relatively even distribution across all other chain lengths. The primary alcohol (1-alkanol) fraction 4.2 within the wheat flag leaf wax mixture comprised even-numbered homologs between C\textsubscript{24} and C\textsubscript{32}, with a very strong predominance of C\textsubscript{28}. The peduncle wax contained the 1-alkanol homologs 4.2 from C\textsubscript{20} to C\textsubscript{34}, albeit with a more even distribution peaking both at C\textsubscript{24} and at C\textsubscript{30}/C\textsubscript{32}.

The flag leaf wax comprised even-numbered aldehydes 4.3 ranging from C\textsubscript{24} to C\textsubscript{34}, with C\textsubscript{28} aldehyde dominating and accompanied by significant amounts of C\textsubscript{30} and C\textsubscript{32} aldehydes. The peduncle wax again contained the same homologs 4.3, but with relatively little C\textsubscript{28} aldehyde and predominantly C\textsubscript{30} and C\textsubscript{32} aldehydes instead. Finally, the alkane fractions 4.4 in the wax mixtures from both wheat organs were characterized by odd-numbered homologs from C\textsubscript{25} to C\textsubscript{33}, with very similar chain length distributions in the flag leaf and peduncle waxes peaking at C\textsubscript{31} alkane. Odd-numbered acids, 1-alkanols, aldehydes, and even-numbered alkanes were only found in traces in both the flag leaf and peduncle waxes.
Figure 4.3. *Chain length distributions within common compound classes of T. aestivum cv.* Bethlehem waxes. Relative abundances (%) of individual homologs from each of four common compound classes in the wax mixtures covering the (A) flag leaf blade and (B) peduncle. Numbers on the x-axis indicate homolog chain length. Bars represent mean ± standard deviation \((n = 5)\). Not all bar groups add up to 100% because minor homologs of opposite parity have been omitted for clarity.
4.3.3. Chain length distributions of wheat wax esters

Five of the compound classes found equally in wheat flag leaf and peduncle wax mixtures were characterized by ester linkages between (very-) long-chain fatty acids and various alcohols in extended series of homologs (Fig. 4.4). The 1-alkanol esters 4.5, resulting from esterification of acyls with primary alcohols, had overall carbon numbers ranging from C\textsubscript{38} to C\textsubscript{54} in both wheat wax mixtures. In the flag leaf wax, 1-alkanol esters 4.5 were found to have a bimodal chain length distribution peaking at C\textsubscript{44} and C\textsubscript{50}, whereas those in the peduncle wax were dominated by the C\textsubscript{44} ester homolog alone. The esters 4.6 formed by acyls and 2-alkanols had total carbon numbers ranging from C\textsubscript{29} to C\textsubscript{37} in both wheat wax mixtures, with similar homolog compositions culminating at C\textsubscript{35} in flag leaf and peduncle waxes alike.

The three remaining classes of esters were formed by (very-) long-chain fatty acids and aromatic alcohols. Representative mass spectra for one homolog from each class (4.7b, 4.8d and 4.9c) are shown in Fig. 4.5, and the corresponding fragmentation mechanisms in Fig. 4.6. One of the aromatic alcohols, benzyl alcohol, was esterified with C\textsubscript{28}, C\textsubscript{30} and C\textsubscript{32} fatty acids to form esters with overall carbon numbers of C\textsubscript{35}, C\textsubscript{37} and C\textsubscript{39}, respectively (Fig. 4.4). Among these homologs, the one comprising C\textsubscript{30} acid dominated in both flag leaf and peduncle waxes. Broader ranges of acyl chain lengths were found esterified with phenethyl and \( p \)-hydroxyphenethyl alcohols, with apparent preference for C\textsubscript{24}-C\textsubscript{28} and C\textsubscript{32} acyl chain lengths, respectively (Fig. 4.4).
Figure 4.4. *Chain length distributions within wax ester classes in T. aestivum cv. Bethlehem waxes.* Relative abundances (%) of individual homologs from each of five wax ester classes in the wax mixtures covering the (A) flag leaf blade and (B) peduncle. Numbers on the x-axis indicate total homolog chain length. For aromatic esters, the individual chain lengths of their acyl part plus alcohol part are specified in round brackets. Bars represent mean ± standard deviation *(n = 5).* All bar groups add up to 100%.
Figure 4.5. *Representative mass spectra of aromatic esters.*

Mass spectra of (A) benzyl triacontanoate 4.7b, (B) phenethyl octacosanoate 4.8d, and (C) trimethylsilyl derivative of *p*-hydroxyphenethyl dotriacontanoate 4.9c from the cuticular wax mixture of *T. aestivum* cv. Bethlehem. Insets show 10-fold enhancements of the 400-650 amu mass range.
Figure 4.6. Fragmentation schemes of aromatic esters.

Major MS fragmentations of (A) benzyl triacontanoate 4.7b, (B) phenethyl octacosanoate 4.8d, and (C) trimethylsilyl derivative of p-hydroxyphenethyl dotriacontanoate 4.9c from the cuticular wax mixture of *T. aestivum* cv. Bethlehem.
4.3.4. Chain length distributions of esterified alcohols and acids: ester isomer compositions

The three ester classes comprising simple aromatic alcohols occurred as homologous series due to variation of acyl chain lengths (as described in 4.3.3.), but did not exhibit isomerism. In contrast, each homolog within the fractions of 1-alkanol 4.5 and 2-alkanol esters 4.6 occurred as a complex mixture of metamers, i.e. isomers fusing complementary chain lengths of the acyl and alkyl moieties (1-alkyl and 2-alkyl, respectively). Metamers of the same 1-alkanol ester homolog co-eluted under the current GC conditions, so their relative composition had to be determined based on mass spectral rather than chromatographic information. To this end, the relative intensities of all M_{acid}+1 product ions detected within one GC-MS peak were quantified and used to calculate metamer percentages, as described previously (Lai et al., 2007; Racovita et al., 2015; Razeq et al., 2014; von Wettstein-Knowles and Netting, 1976a).

In the wheat flag leaf wax, the C_{44}-C_{54} 1-alkanol esters 4.5d-i comprised predominantly C_{28} alcohol, whereas C_{38}-C_{42} 1-alkanol esters 4.5a-c incorporated mainly C_{22} alcohol along with C_{24} alcohol (Fig. 4.7A). A greater diversity of alcohol chain lengths was found within the peduncle 1-alkanol esters 4.5, where the C_{38}-C_{44} ester homologs 4.5a-d contained predominantly C_{22} alcohol, the C_{46}-C_{52} homologs 4.5e-h predominantly C_{24} and C_{26} alcohols, and the C_{54} ester 4.5i predominantly C_{26} and C_{32} alcohols (Fig. 4.7B).
Figure 4.7. Relative isomer compositions of 1-alkanol ester homologs in T. aestivum cv. Bethlehem waxes. Relative abundances (%) of individual metamers (indicated by chain length of their 1-alkanol moiety) within each ester homolog in the composition of wax mixtures covering the (A) flag leaf blade and (B) peduncle. Numbers on the x-axis indicate total homolog chain length. Bars represent mean ± standard deviation ($n = 5$). All bar groups add up to 100%.
The current GC conditions led to partial separation of metamer peaks for some 2-alkanol ester homologs, while others remained ill-separated. Therefore, the overall isomer composition of this ester class had to be determined from their mass spectra averaged over multiple GC peaks encompassing one homolog (Fig. 4.8). The relative quantification of 2-alkanol ester isomers 4.6 was based on summation of intensities of the three most intense product ions, M\text{acid}, M\text{acid}+1, and M\text{alkyl}-1, again as previously described (Aasen et al., 1971; von Wettstein-Knowles and Netting, 1976a). For both organs studied, the C\text{29} 2-alkanol ester 4.6a comprised mainly C\text{7}, C\text{9} and C\text{13} 2-alkanols, the C\text{31}-C\text{33} esters 4.6b-c mainly C\text{7} and C\text{13} 2-alkanols, and the C\text{35}-C\text{37} esters 4.6d-e primarily C\text{7} and C\text{15} 2-alkanols (Fig. 4.9). Thus, all 2-alkanol ester homologs incorporated 2-alkanols with fairly similar bimodal distributions.
Figure 4.8. Identification of C_{31} 2-alkanol ester metamers. Mass spectra of the esters consisting of (A) C_{16} acid and C_{15} 2-alkanol, (B) C_{18} acid and C_{13} 2-alkanol, (C) C_{20} acid and C_{11} 2-alkanol, (D) C_{22} acid and C_{9} 2-alkanol, and (E) C_{24} acid and C_{7} 2-alkanol. (F) Single ion chromatograms of fragments characteristic of fatty acid and 2-alkanol homologs constituting the C_{31} esters 4.6b, showing (partial) GC separation of the metamers.
Figure 4.9. *Relative isomer compositions of 2-alkanol ester homologs in T. aestivum cv. Bethlehem waxes.* Relative abundances (%) of individual metamers (indicated by chain length of their 2-alkanol moiety) within each ester homolog in the composition of wax mixtures covering the (A) flag leaf blade and (B) peduncle. Numbers on the x-axis indicate total homolog chain length. Bars represent mean ± standard deviation ($n = 5$). All bar groups add up to 100%.
Combining the quantitative information on the isomer compositions of all aliphatic esters (Figs. 4.4, 4.7 and 4.9), the aromatic esters (Fig. 4.4), and the composition of the two isomeric amyrenyl behenates (see below), the overall profile of ester-bound alcohols could be calculated for the flag leaf and peduncle wax mixtures (Fig. 4.10). The series of esterified 1-alkanols were dominated by the C_{28} homolog in the leaf wax mixture, similar to the chain length profile of the free (i.e. non-esterified) 1-alkanols in this wax mixture (compare Fig. 4.3A). In contrast, the esterified 1-alkanols of the peduncle wax had a broad distribution around C_{24}, with only minor resemblance to the homolog profile of the accompanying free alcohols (compare Fig. 4.3B). The esterified 2-alkanols had overall chain length profiles peaking at C_{7} and at C_{13}/C_{15}, reflecting the bimodal distributions within all homologs of this ester class (compare Fig. 4.9). Among the esterified aromatic alcohols, p-hydroxyphenethyl alcohol was by far the most abundant in both wheat wax mixtures. It should be noted that, in the peduncle wax, this aromatic alcohol was found esterified in quantities comparable to those of individual homologs of primary alcohols. Finally, relative minor amounts of α-4.14c and β-amyrin 4.14b were found esterified in the peduncle wax mixture (see below).
Figure 4.10. *Relative compositions of esterified alcohols in T. aestivum cv. Bethlehem waxes.* Relative total abundances (%) of all types of ester-bound alcohol homologs in the composition of wax mixtures covering the (A) flag leaf blade and (B) peduncle. Bars represent mean ± standard deviation (*n* = 5).
Similar to the profiles of esterified alcohols, the overall chain length profiles could be calculated for all ester-bound fatty acids taken together (Fig. 4.11). In both wheat wax mixtures, the total esterified acids had bimodal homolog distributions with maxima at C\textsubscript{22} and C\textsubscript{32}. However, the absolute maxima were reversed, with predominance of C\textsubscript{22} acid in the flag leaf esters and C\textsubscript{32} in the peduncle esters. The latter finding is mostly due to the much higher \textit{p}-hydroxyphenethyl ester 4.9 load on peduncles (compare Figs. 4.2 and 4.4).

![Graph showing relative composition and total esterified acid coverage for flag leaf and peduncle esters.]

Figure 4.11. \textit{Relative compositions of esterified acids in T. aestivum cv. Bethlehem waxes.} Relative total abundances (%) of ester-bound fatty acid homologs in the composition of wax mixtures covering the (A) flag leaf blade and (B) peduncle. Bars represent mean ± standard deviation (\(n = 5\)).
4.3.5. Composition of wheat wax polyketides and terpenoids

Finally, the wheat wax mixtures contained several classes of polyketides and terpenoids, some of them comprising series of homologs and others isomers. One of the aliphatic polyketide fractions consisted of a single compound, hentriacontane-14,16-dione 4.10a, in both flag leaf and peduncle waxes. In contrast, two hydroxy-β-diketones 4.11a-b with differing hydroxyl group positions were identified based on characteristic MS fragments of various derivatives (Figs. 4.12 and 4.13). The relative amounts of the two isomers could be assessed from the mass spectra of their co-eluting TMS derivatives, by summing intensities of their characteristic α-fragment pairs m/z 201 and 435, or m/z 215 and 421, respectively (note that the larger ions 435 and 421 are due to α-fragmentation and loss of H₂O). The results showed that 8- 4.11a and 9-hydroxy-β-hentriacontane-14,16-dione 4.11b were present in roughly equal amounts within the flag leaf wax mixture, and also in the peduncle wax.

The aromatic polyketides, alkylresorcinols 4.12 and methyl alkylresorcinols 4.13, occurred as series of homologs with mainly odd-numbered side-chains. Their chain length profiles were very similar, both between the two fractions and between the wheat organs, in all cases dominated by the C₂₃ homolog (Fig. 4.14).

Terpenoids were detected only in the peduncle wax mixture. Among them, the most (and equally) abundant were two isomeric alcohols, α- 4.14c and β-amyrin 4.14b (Fig. 4.14). They were accompanied by lupeol 4.14d (a third isomer), and by esters of the amyrins with behenic (i.e. C₂₂) acid 4.14e-f. α-Tocopherol 4.14a was present in amounts comparable to lupeol 4.14d.
Figure 4.12. Mass spectra of various derivatives of 8- and 9-hydroxyhentriacontane-14,16-diones. Mass spectra of the mixture of 8-4.11a and 9-hydroxyhentriacontane-14,16-dione 4.11b from the cuticular wax of *T. aestivum* cv. Bethlehem that was (A) trimethylsilylated on the 8/9-hydroxy group, (B) trimethylsilylated on both the 8/9-hydroxy group and the β-diketone enol functionality, (C) acetylated on the 8/9-hydroxy group, and (D) fully reduced with lithium aluminum hydride and then fully trimethylsilylated on all hydroxyl groups of the resulting triol. Insets show 10-fold enhancements of the 400-620 amu mass range.
Figure 4.13. *Fragmentation schemes of various derivatives of 8-hydroxyhentriacontane-14,16-dione.* Major MS fragmentations of the 8-hydroxyhentriacontane-14,16-dione isomer 4.11a from the cuticular wax of *T. aestivum* cv. Bethlehem that was (A) trimethylsilylated on the 8 hydroxyl group, (B) trimethylsilylated on both the 8-hydroxyl group and both tautomeric β-diketone enol functionalities, (C) acetylated on the 8-hydroxyl group, and (D) fully reduced with lithium aluminum hydride and then fully trimethylsilylated on all hydroxyl groups of the resulting triol.
Figure 4.14. *Relative compositions of polyketide and terpenoid compound classes in T. aestivum cv. Bethlehem waxes.* Relative abundances (%) of individual homologs from each of four polyketide compound classes and of individual terpenoid isomers in the wax mixtures covering the (A) flag leaf blade and (B) peduncle. x-Axis labels indicate total homolog chain length or the name of the terpenoid isomer. For alkylresorcinols 4.12 and methyl alkylresorcinols 4.13, the individual chain lengths of their alkyl side chains plus the aromatic ring plus (if applicable) the methyl group are specified in round brackets. Bars represent mean ± standard deviation (n = 5). All bar groups add up to 100%.
4.4. Discussion

The chromatographic analyses of cuticular waxes from *T. aestivum* cv. Bethlehem revealed three major results: (i) the composition and relative amounts of the various wax compounds differed dramatically between flag leaf blade and peduncle, including several compound classes that had not been described for wheat cuticular wax mixtures before; (ii) most compound classes occurred as homologous series with highly characteristic chain length profiles in both wheat organs; (iii) 1-alkanol and 2-alkanol esters occurred as complex mixtures of metamers, with characteristic chain length patterns of their esterified acid and alkanol moieties, respectively. All of these aspects will be discussed in light of the underlying wax biosynthetic mechanisms.

4.4.1. Wax composition differences between organs and new compound classes

There are several literature reports on the composition of cuticular waxes extracted from either whole plants (Bianchi and Corbellini, 1977; Bianchi et al., 1980) or only leaf blades (Tulloch and Hoffman, 1973; Tulloch, 1973; Wang et al., 2015b) of various wheat cultivars. Fairly recently, the compositions of waxes covering flag leaf blades and peduncles of the wheat cvs. Shango and Shamrock (Adamski et al., 2013b) and Ming 988, respectively (Wang et al., 2015a) were reported. Only the latter report quantified wax loads per surface area, thus enabling direct comparisons of wax amounts with our results. It should be noted that cv. Ming 988 had very similar total wax loads between organs (8.2 ± 0.2 μg/cm² on flag leaf blades leaves and 8.8 ± 0.2 μg/cm² on peduncles), while our analyses showed three times larger wax loads on peduncles than flag leaves for cv. Bethlehem. The wax load on flag leaves of cv. Bethlehem was
in turn twice that of Ming 988, overall suggesting higher wax biosynthetic activity in cv. Bethlehem.

Some of the wax compound classes reported here have not been detected in other wheat cultivars, such as 2-alkanol esters, esters of aromatic alcohols and, in the case of peduncles, aldehydes and terpenoids. While aldehydes and terpenoids are ubiquitous wax compounds, 2-alkanol esters are infrequently found in cuticular wax mixtures. However, they have been described as side products of the β-diketone pathway in other Poaceae, for example in the waxes of barley (Mikkelsen, 1984; von Wettstein-Knowles and Netting, 1976a, 1976c; von Wettstein-Knowles, 2012, 1987, 1976) and sorghum (Penny von Wettstein-Knowles et al., 1984). Esters of aromatic alcohols had not been identified before in Poaceae waxes, but in many other unrelated species. For example, benzyl esters have been found in cuticular waxes of *Eucalyptus globulus* (Jones et al., 2002; Rapley et al., 2004; Steinbauer et al., 2004), *Fagus sylvatica* (Gülz et al., 1989), *Humulus lupulus* (Güll et al., 1993), *Acer pseudoplatanus* (Prasad and Gülz, 1990), *Simmondsia chinensis* (Gülz and Marner, 1986), as well as *Papaver rhoes* and *P. somniferum* (Jetter and Riederer, 1996). Phenethyl esters have been reported in cuticular waxes of *Eucalyptus globulus* (Jones et al., 2002; Rapley et al., 2004; Steinbauer et al., 2004), *Simmondsia chinensis* (Gülz and Marner, 1986), *Solanum tuberosum* (Szafranek and Synak, 2006b), as well as *P. rhoes* and *P. somniferum* (Jetter and Riederer, 1996). Finally, p-hydroxyphenethyl esters have been found in *Buddleja cordata* (Acevedo et al., 2000), *Inula graveolens* (Oksuz and Topcu, 1992) and *Bongardia chrysogonum* (Alfatafta et al., 1989). It is noteworthy that alkylresorcinols and methyl alkylresorcinols have been detected on both flag leaves and peduncles of the wheat cvs. Shango and Shamrock (Adamski et al., 2013b).
The major differences between our results and previous wheat wax analyses are in the much higher loads of 1-alkanols and 1-alkanol esters on flag leaf blades, and of β-diketone and hydroxy-β-diketones on peduncles of cv. Bethlehem (see Fig. 4.2). However, these findings are similar to those for cv. Ming 988 and various barley lines (von Wettstein-Knowles, 1969; Wang et al., 2015a), confirming that the acyl reduction wax biosynthetic pathway is strongly dominating wax production in flag leaves, while the β-diketone pathway dictates wax production in peduncles. Both pathways are thus differentially regulated to a high degree, and cv. Bethlehem will be an ideal tool to study biosynthetic mechanisms in comparisons between both organs.

4.4.2. Chain length distributions of ubiquitous wax compound classes

All ubiquitous compound classes were found as series of homologs with distinctive chain length profiles in the waxes of wheat cv. Bethlehem. The bimodal profile of flag leaf fatty acids, peaking at C28 and C22, was described before for T. aestivum cvs. Selkirk and Manitou (Tulloch and Hoffman, 1973), whereas cvs. Ming 988 (Wang et al., 2015a) and Xinong 2718 (Wang et al., 2015b) showed similar predominance of C28 fatty acid but not C22. In contrast, the cv. Bethlehem peduncle wax showed a single maximum at C28 fatty acid (see Fig. 4.3), in this characteristic resembling cv. Ming 988 (Wang et al., 2015a). All taken together, the wheat cultivars and organs differentially accumulate two acid chain lengths, thus pointing to differences either in substrate pool compositions or enzyme preferences. This finding may spur further studies into the biosynthesis of VLC free fatty acids, the mechanisms of which are not understood at present.
The cv. Bethlehem flag leaf wax contained high amounts of 1-alkanols, in which the C_{28} homolog strongly dominated, similar to diverse other wheat cultivars and organs (Adamski et al., 2013b; Bianchi and Corbellini, 1977; Bianchi et al., 1980; Tulloch and Hoffman, 1973; Tulloch, 1973; Wang et al., 2015a, 2015b). The matching 1-alkanols and acid profiles on cv. Bethlehem flag leaves, both peaking at C_{28}, suggest preferential accumulation of the common precursor C_{28} acyl-CoA. It seems likely that at least one fatty acyl-CoA reductase (FAR) uses this substrate to form wax alcohols in leaf epidermal cells. The TaFAR5 enzyme has recently been found to convert C_{28} upon heterologous expression in tomato (Wang et al., 2015b), and to be highly expressed in leaves. Hence, it may well be involved in the formation of this central wax constituent.

The peduncle wax 1-alkanols of various wheat cultivars are dominated by the C_{24} homolog (Adamski et al., 2013b; Wang et al., 2015a), thus in part matching the bimodal profile with maxima at C_{24} and C_{30}/C_{32} 1-alkanols of cv. Bethlehem. Overall, this may suggest the presence of two FARs with substrate specificities different from the corresponding leaf enzyme. Interestingly, the wheat TaFAR1 and TaFAR5 enzymes have recently been found to exhibit some preference for C_{22/24} and C_{20} (as well as C_{28}) substrates, respectively, but they are expressed at relatively low levels in peduncles (Wang et al., 2015a, 2015b). Therefore, our chemical results suggest involvement of other FARs in 1-alkanol formation in wheat peduncles, three more of which have been annotated in the genome.

The wheat cv. Bethlehem organs had very different wax aldehyde profiles, the C_{28} homolog dominating in flag leaves and the C_{30} and C_{32} homologs in peduncles. The leaf results match
previous reports, where aldehydes of various wheat cultivars were also dominated by the C\textsubscript{28} homolog (Bianchi and Corbellini, 1977; Bianchi et al., 1980; Wang et al., 2015a, 2015b). Thus, the aldehyde distribution matches that of the accompanying 1-alkanols, and not that of the alkanes peaking at C\textsubscript{29} in some cultivars and at C\textsubscript{31} in cv. Bethlehem. Taken together, these results suggest a biosynthetic relationship between aldehydes and 1-alkanols in wheat leaves, likely involving step-wise reduction of acyl-CoA precursors (by one or two reductases) and release of aldehyde intermediates. This hypothesis contrasts with evidence from Arabidopsis, where 1-alkanols are largely formed without release of aldehyde intermediates by the FAR CER4 (Kunst and Samuels, 2003), while the parallel decarbonylation pathway leads via aldehydes to alkanes. Interestingly, the peduncle aldehyde pattern did resemble that of the accompanying alkanes, suggesting that in this organ the aldehydes are intermediates en route to alkanes. Consequently, peduncles (but not flag leaves) likely harbour an alkane-forming pathway similar to Arabidopsis, possibly involving decarbonylases with different substrate preference for C\textsubscript{32} and C\textsubscript{30} aldehyde substrates in cv. Bethlehem and other cultivars, respectively.

4.4.3. Chain length and isomer distributions of aliphatic and aromatic esters

The cv. Bethlehem 1-alkanol esters had even-numbered chain lengths, with a bimodal distribution on leaves (peaking at C\textsubscript{44} and C\textsubscript{50}) and a unimodal distribution on peduncles (peaking only at C\textsubscript{44}), very similar to other wheat cultivars (Tulloch and Hoffman, 1973; Tulloch, 1973; Wang et al., 2015a). In contrast, the 2-alkanol esters had odd-numbered chain lengths and unimodal distributions on both organs (peaking at C\textsubscript{35}), a pattern closely resembling
that reported for the wax mixtures of cer-\textit{u}69 barley spikes without awns (von Wettstein-Knowles and Netting, 1976a).

The chain length profiles of esterified acids (see Fig. 4.11A) in flag leaves of cv. Bethlehem suggested the presence of two wax ester synthases with high substrate specificity for C\textsubscript{22} and C\textsubscript{16} acyl-CoA, respectively. In contrast, the 1-alkanol substrates appeared to be used proportional to their availability and without specificity (compare Figs. 4.10A and 4.3A). Thus, esters C\textsubscript{50} (C\textsubscript{22} acyl + C\textsubscript{28} alkyl) and C\textsubscript{44} (C\textsubscript{16} acyl + C\textsubscript{28} alkyl) were most abundant, leading to a characteristic bimodal distribution. For peduncles, less substrate specificity was apparent for acyl-CoAs, although C\textsubscript{22} acyl-CoA was still the predominantly incorporated substrate (see Fig. 4.11B). Instead, additional specificity for C\textsubscript{22}-C\textsubscript{24} 1-alkanol substrates could be inferred, based on the ester metamer profile quite different from that of free 1-alkanols (compare Figs. 4.10B and 4.3B).

The 2-alkanol ester compositions were nearly identical between cv. Bethlehem organs (compare Figs. 4.9A and B), and so were the resulting profiles of esterified 2-alkanols (compare Figs. 4.10A and B). The C\textsubscript{15} and C\textsubscript{13} 2-alkanols dominated, similar to reports on cuticular waxes from barley (von Wettstein-Knowles and Netting, 1976a). However, on both wheat flag leaves and peduncles also C\textsubscript{7} 2-alkanol was incorporated in substantial amounts, leading to bimodal distribution. Of note, the C\textsubscript{7} 2-alkanol moiety had not been reported before in wax of any plant species. Overall, it appears that two 2-alkanol ester synthases may be active in wheat: one with high substrate specificity for the C\textsubscript{7} 2-alkanol and no acyl-CoA specificity, and another with high
specificity for the C_{18}-C_{22} acyl-CoAs (and especially for C_{20} acyl-CoA) using the available 2-alkanol pool to produce the full array of 2-alkanol esters (especially C_{35} and C_{33}).

The characteristic chain length distributions of aromatic esters, which were very similar between cv. Bethlehem leaves and peduncles (see Fig. 4.4), suggest the presence of several additional ester-forming enzymes with distinct acyl substrate chain length preferences. Thus, benzyl esters were formed preferentially from C_{30} acyl-CoA, phenethyl esters from C_{24}-C_{28} acyl-CoAs, and \( \rho \)-hydroxyphenethyl esters from C_{32} acyl-CoA substrate. Lastly, triterpenoid esters were likely formed by a further enzyme fusing C_{16} acyl-CoA with the two most available triterpenol substrates, \( \beta \)- and \( \alpha \)-amyrin, albeit only in the peduncles (compare Figs. 4.10B and 4.14B).

4.4.4. Chain length distributions of polyketide wax compound classes

The most abundant polyketides, \( \beta \)-diketones and hydroxy-\( \beta \)-diketones, occurred as single homologs with 31 carbons in cv. Bethlehem waxes, as described previously for almost all other wheat cultivars (Adamski et al., 2013b; Bianchi and Corbellini, 1977; Bianchi et al., 1980; Tulloch and Hoffman, 1973; Tulloch, 1973; Wang et al., 2015a, 2015b). The only notable exception to this pattern is cv. Demar 4, where small amounts of the C_{29} homolog were detected as well (Bianchi and Corbellini, 1977). The 1:1 isomer ratio between 8-hydroxy- and 9-hydroxy-\( \beta \)-diketones found here had also been reported before for cvs. Selkirk and Manitou (Tulloch and Hoffman, 1973). The current findings thus are in accordance with the \( \beta \)-diketone and hydroxy-\( \beta \)-diketone biosynthesis pathways outlined before (von Wettstein-Knowles, 2012).
Finally, the phenolic polyketide classes of alkylresorcinols and methyl alkylresorcinols were found in wheat cv. Bethlehem waxes as series of odd-numbered homologs with alkyl side chains from C_{19} to C_{29}, peaking at C_{23}. The distributions were thus very similar to those of the cvs. Shamrock and Shango (Adamski et al., 2013b), with the addition of C_{29} homologs not reported before. The biosynthesis of plant alkylresorcinols is known to be effected by a type-III polyketide synthase, an alkylresorcinol synthase, that first performs the extension of an acyl-CoA substrate with three malonyl-CoAs to a tetraketide intermediate, and then its intramolecular aldol cyclization and decarboxylation to the final alkylresorcinol (Baerson et al., 2010). How the additional methyl group in methyl alkylresorcinols is introduced is currently unknown, but the matching side-chain homolog profiles of the two types of phenolic lipids suggest an at least partially shared pathway, perhaps with methylmalonyl-CoA replacing malonyl-CoA in one of the extension steps or a subsequent C-methylation of alkylresorcinols by a dedicated enzyme. In both wheat organs investigated here, it would appear that the alkylresorcinol synthase has a chain length preference for C_{24} acyl-CoA substrate, resulting in a predominance of homologs with C_{23} side chains.
Chapter 5: Novel oxidized compounds and internally methyl-branched alkanes from cuticular waxes of *Triticum aestivum* cv. Bethlehem

5.1. Introduction

Most above-ground organs of land plants are covered by a hydrophobic coating, known as the cuticle, which is sealing them against uncontrolled loss of water. The cuticle is composed of the polyester cutin and cuticular wax that can easily be extracted with organic solvents (Jetter et al., 2006). Cutin consists of saturated and unsaturated long-chain (LC, C\textsubscript{16} and C\textsubscript{18}) hydroxy or epoxy fatty acids, linked via ester bonds either directly between fatty acids or via glycerol (Nawrath, 2006; Pollard et al., 2008). Cuticular waxes are typically very-long-chain (VLC, \(\text{C}_{20}\)) saturated aliphatic compounds, bearing one functional group or no functionality. Most commonly encountered are homologous series of even-numbered fatty acids, primary alcohols, alkyl esters and aldehydes, as well as odd-numbered alkanes, secondary alcohols and ketones (Jetter et al., 2006; Kolattukudy, 1970). In the wax mixtures of some plant species, alicyclics (e.g., triterpenoids) and aromatics (e.g., alkylresorcinols) can also be quite abundant (Basas-Jaumandreu et al., 2014; Bianchi et al., 1992; Ji and Jetter, 2008; Manheim Jr. and Mulroy, 1978; Nordby and McDonald, 1994).

The characteristic mixtures of various aliphatic wax constituents result from biosynthetic pathways that are fairly well understood, mainly due to extensive studies in the model species *Arabidopsis thaliana* (Kunst et al., 2006; Samuels et al., 2008). Wax biosynthesis begins in the epidermal plastids, where the fatty acid synthase (FAS) elongates acetyl-CoA into LC fatty
acids. These are then transferred to the endoplasmic reticulum (ER), where fatty acid elongase (FAE) complexes extend their chain length two carbons at a time to VLC acyls. The resulting VLC fatty acyl-CoAs can then be further processed into final wax compounds, by (1) reduction to primary alcohols which may be esterified with (V)LC fatty acids; or (2) partial reduction to aldehydes, their subsequent decarbonylation to alkanes, and, in some species, hydroxylation of the alkanes to secondary alcohols and ketones.

While the Arabidopsis model has proved invaluable for our understanding of cuticular wax biosynthesis, much can be learned from studying the diversity of wax structures in other plant species as well. Thus, numerous novel wax compounds were discovered recently in various plant species, suggesting divergence from the traditional pathways in Arabidopsis. Among these are many compounds with multiple functional groups, including diols (Buschhaus et al., 2013b; Jetter, 2000; Wen and Jetter, 2009; Wen et al., 2006a), hydroxyaldehydes (Vermeer et al., 2003; Wen and Jetter, 2007), ketols (Wen and Jetter, 2009), ketoaldehydes (Jetter and Riederer, 1999a), hydroxyacids (Racovita et al., 2015), hydroxyesters (Busta et al., 2016; Racovita et al., 2015), and ketoesters (Jetter and Riederer, 1999a). In many of these studies, the nature of the functional groups, their relative positions and the chain length profiles within respective homologous series could be used to infer the biosynthetic origin of functional groups or entire molecules. The work herein is an extension of these studies, with the objective of seeking, identifying and quantifying novel wax compounds to further our understanding of wax biosynthesis beyond the well-known constituents of the Arabidopsis wax mixture.
Bread wheat (*Triticum aestivum*) is rapidly becoming a new model species for wax biosynthesis studies (Adamski et al., 2013b; Hen-Avivi et al., 2016b; Millet et al., 2013; Wang et al., 2015a, 2015b), due to its importance as a major staple crop world-wide and its susceptibility to drought, combined with the recognized role of cuticular waxes in conferring drought resistance in this species. In a previous analysis of flag leaf blade and peduncle waxes of *T. aestivum* cv. Bethlehem by gas chromatography-mass spectrometry (GC-MS), we quantified various VLC fatty acids, primary alcohols, aldehydes, alkanes and 1-alkanol esters common to most plant species (Racovita et al., 2016). Furthermore, benzyl esters, phenethyl esters, p-hydroxyphenethyl esters were identified for the first time in wheat wax, together with various terpenoids. Finally, several compounds characteristic of Poaceae waxes were reported, including β-diketones, hydroxy-β-diketones, alkylresorcinols, methyl alkylresorcinols and 2-alkanol esters. However, numerous compounds in the cuticular wax mixtures of flag leaf blades and peduncles remained unidentified. To elucidate the molecular structures of these additional wheat wax constituents, we have now performed in-depth mass spectrometric analyses, using various derivatives of each novel structure for comparison of fragmentation patterns. Finally, the homolog and isomer patterns of the novel compound classes were assessed, to enable conclusions on their biosynthetic origins.
5.2. Experimental

5.2.1. Plant material

Flag leaves and peduncles were harvested from mature *Triticum aestivum* cv. Bethlehem plants during the months of August 2013 for total wax specimens and August 2014 for specimens for preparative thin layer chromatography (TLC). Plants were grown in greenhouses at Weizmann Institute of Science (Rehovot, Israel) on 50% peat – 50% turf, with watering every 3-4 days (~400 mL per 5 L pot). Growth conditions were: 12-14 h / 10-12 h light / dark cycles (180 μmol m⁻² s⁻¹ light), with temperatures of 24-26°C / 17-18°C, respectively. For total wax samples, one leaf blade with total area of 40-50 cm² (both sides) and one peduncle with a projected surface area of ~20 cm² were used per biological replicate. For preparative TLC samples, ten leaf blades and ten peduncles of the same size were used. Exact areas for leaf blades were determined by capturing them in photographs and using the ImageJ software to measure the area of one side, then multiplying by a factor of 2. For peduncles, areas were determined by measuring the length L and diameter D of the peduncle and then calculating the area of specimen with the formula: π x D x L.

5.2.2. Chemicals

The following chemicals were acquired from Sigma-Aldrich (Oakville ON, Canada) and used without further purification: chloroform (≥99%, with 0.75% ethanol as stabilizer), ethanol (≥99.8%, HPLC grade), pyridine (≥99.8%, anhydrous), N,O-bis(trimethylsilyl)trifluoroacetamide
5.2.3. Preparation of wax extracts

Rolled-up leaves and peduncle pieces were extracted for 30 s at ambient temperature with 10 mL chloroform, to which 5 μg n-tetracosane were added prior to extraction, as an internal standard. The chloroform was then transferred to another vial and the extraction repeated for another 30 s with a fresh portion of 10 mL chloroform. The combined chloroform extracts were then evaporated to dryness under a stream of N₂ at 50°C, leaving behind the wax mixtures for either preparative TLC or GC analysis.

5.2.4. Preparative thin layer chromatography

Fractionation of compound classes in the total wax extracts was carried out by preparative TLC, using the sandwich technique (Tantisewie et al., 1969). Glass plates coated with silica gel 60 F₂₅₄ (Uniplate, Analtech, layer thickness: 1 mm, size: 20x20 cm, with 4 cm
concentrating zone) served as stationary phase, and a mixture of chloroform:ethanol 98:2 (v/v) served as mobile phase. At the end of separation, TLC plates were sprayed with primuline (5 mg in 100 mL acetone/water 80/20, v/v) and bands were visualized under 365 nm UV light. All bands were removed from the plate with clean spatulas into several glass vials, and each extracted twice with 10 mL portions of fresh chloroform for 30 s, at ambient temperature. Then, the combined extracts were filtered through glass wool (Supelco), partially evaporated under N₂ at 50°C, transferred to 2 mL GC vials, evaporated to dryness and stored until GC-Mass Spectrometry (MS) analysis.

5.2.5. Derivatization reactions

Prior to GC analysis, all samples were silylated by refluxing with 10 μL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 10 μL pyridine at 70°C for 20 min. Excess reagents were then removed under a gentle stream of N₂ and the silylated waxes re-dissolved in 50 μL CHCl₃.

Acetylation was carried out by refluxing a mixture of dry wax, 10 μL pyridine, and 10 μL acetic anhydride at 70°C for 5 min, then allowing it to stir overnight at ambient temperature. After removal of excess reagents under N₂, silylation was carried out as described above.

Complete reduction of carbonyl and ester groups was achieved by dissolving the wax sample in 50 μL diethyl ether and adding 0.1 mg LiAlH₄, then allowing the mixture to react
overnight at 70°C. After quenching with 10% H$_2$SO$_4$, followed by three sequential extractions with 60 μL diethyl ether each, the combined extracts were evaporated to dryness and silylated as described above.

Carbonyl-containing compounds were transformed into their methoximes by heating with 20 μL of a saturated solution of O-methylhydroxylamine hydrochloride in pyridine:chloroform 7:3 (v/v) for 30 min at 70°C. The resulting mixture was partitioned between 50 μL distilled water and 50 μL chloroform and the chloroform fraction retained. After extracting the aqueous phase one more time with 50 μL fresh chloroform, the chloroform extracts were combined, evaporated to dryness and silylated as described above.

Transesterification was achieved by heating the waxes in 100 μL of 14% BF$_3$-methanol solution at 70°C for 2 hours. Then, the products were isolated by partitioning between 50 μL distilled water and 50 μL diethyl ether and repeating the extraction two more times with fresh portions of 50 μL ether. After evaporation to dryness, silylation was carried out as described above.

5.2.6. Gas chromatography

Two Gas Chromatography (GC) instruments were used for identification and quantification of wax constituents, respectively, both equipped with the same type of capillary GC column (6890N, Agilent, Avondale PA, USA; length: 30 m; type: HP-1 100% PDMS; i.d.: 0.32 mm; df: 0.1 μm), both equipped with on-column injector and programmed to follow the same
temperature program (2 min at 50°C, ramp 40°C min⁻¹ to 200°C, constant for 2 min, ramp 3°C min⁻¹ to 320°C, constant for 30 min). One GC instrument employed helium as mobile phase, at a flow rate of 1.4 mL/min, and was equipped with MS detector (5973N, Agilent, EI-70 eV, m/z 50-750). The other used hydrogen as carrier gas at 2.0 mL/min and was equipped with a flame ionization detector (FID). Wax compounds were quantified by normalizing their GC-FID peak areas against that of the internal standard, added in known amount. The relative response factors of all wax compound classes with respect to the internal standard were approximated to 1.00, in agreement with literature reports using the same GC-FID operation conditions (Riederer and Schneider, 1989).

5.3. Results

The principal goals of the work herein were to identify novel compounds in the cuticular waxes of the bread wheat (*Triticum aestivum*) cultivar Bethlehem (sections 5.3.1. – 5.3.4.) and to determine their relative quantities within the wax mixtures covering flag leaf blades and peduncles (section 5.3.5.).

5.3.1. TLC separation of cuticular waxes of *T. aestivum* flag leaf blades and peduncles

Preliminary experiments showed that the unknown wheat wax constituents belonged to seven different compound classes A-G (Fig. 5.1). Each of these comprised a series of compounds separated into equally spaced GC peaks with shared characteristic MS fragmentation patterns, and thus each class was recognized as a homologous series of compounds. To enable
their structure elucidation, the compound classes were separated and concentrated by preparative thin layer chromatography (TLC) using silica gel as stationary phase and CHCl₃:EtOH 98:2 (v/v) as mobile phase.

Figure 5.1. *Oxidized compounds and internally methyl-branched alkanes identified in the wax mixtures of T. aestivum cv. Bethlehem.* For compound classes where several co-eluting isomers were detected per homolog, only the major isomer is shown for each homolog in each series.

The flag leaf blade wax mixture was separated into eleven fractions, which were analyzed individually by GC-MS. Among them, seven fractions were found to contain previously identified compound classes, namely 5-alkylresorcinols and methyl 5-alkylresorcinols (R_f 0.09), 2-(p-hydroxyphenyl)ethyl esters of VLC fatty acids (R_f 0.27), free VLC fatty acids (R_f 0.30), hydroxy-β-diketones (R_f 0.33), 1-alkanols (R_f 0.38), β-diketones along with small amounts of aldehydes (R_f 0.86), and several very non-polar wax classes such as n-alkanes, iso- and anteiso-
alkanes, esters of 1- and 2-alkanols, benzyl esters and 2-phenylethyl esters (Rf 1.00). The unknown compound classes were found to have widely varying TLC behaviour, with series A (Rf 0.72), C (Rf 0.54), D (Rf 0.35), as well as E and F (Rf 0.44) in fractions of their own, and series B co-eluting with free VLC fatty acids (Rf 0.30) and series G with the non-polar wax classes (Rf 1.00). The peduncle wax mixture yielded nine fractions, with identical Rf values and compositions very similar to corresponding leaf wax fractions. However, series A, B and C were not found in peduncle wax.

5.3.2. Structure elucidation of compound classes A - C

Based on TLC behaviour, fraction A exhibited polarity intermediate between aldehydes and primary alcohols, and it was thus hypothesized to contain VLC secondary alcohols 5.1. All six compounds in A yielded TMS derivatives with common diagnostic MS fragment m/z 73 [(CH3)3Si]+, homolog-dependent M-15 and M-90 ions, due to loss of methyl radical and (CH3)3SiOH, respectively, and no m/z 147 [(CH3)2SiOSi(CH3)3]+ (Fig. 5.2A), together confirming the secondary alcohol structure (Diekman et al., 1967; Racovita et al., 2015; Wen et al., 2006a). Each homolog, in its TMS derivative mass spectrum, also displayed a pair of α-fragments, with one ion m/z 257 common to all homologs indicative of a hydroxyl group on C-12, and a second fragment varying with chain length (m/z 397 for the C33 homolog in Fig. 5.2A). These fragments were accompanied by further pairwise combinations of α-fragments differing by 14 Da (e.g., C-10 m/z 229, C-11 m/z 243, C-13 m/z 271, C-14 m/z 285). A summary of all identified homologs and regioomers within them together with their diagnostic MS fragments is presented in Table 5.1. Extracted ion chromatograms (EICs) of the shorter α-fragments in these
pairs revealed small retention time differences between them, but all well within the overall GC peak of the respective homolog. Taken together, the GC and MS information thus revealed the presence of positional isomers (regiomers) of secondary alcohols that were only partially GC-separated. All six homologs were found dominated by the C-12 isomer, accompanied by others bearing hydroxyls on neighbouring carbon atoms (Fig. 5.2B).

For structure confirmation, acetyl (Ac) derivatives of compounds A were prepared and analyzed by GC-MS, revealing a characteristic fragment $m/z$ 61 [CH$_3$COOH$_2$]$^+$ common to all homologs indicative of a hydroxyl group (Buschhaus et al., 2013b; Wen et al., 2006a), as well as M-60 fragments varying between homologs due to elimination of acetic acid AcOH (Fig. 5.2C). α-fragments were much less prominent than for TMS derivatives, but further loss of AcOH resulted in distinct pairs of fragments confirming the presence of different regiomers, with $m/z$ 167/307 for the C-12 alcohol isomer, $m/z$ 153/321 for the C-11 isomer, $m/z$ 181/293 for C-13, etc. Taken together, the TLC behaviour and GC-MS data for TMS and Ac derivatives demonstrated that A was a series of secondary alcohol homologs 5.1, each comprising several regiomers with hydroxyls at and around C-12.
Figure 5.2. *Structure elucidation of secondary alcohols in wheat leaf wax.* (A) Mass spectrum of co-eluting TMS derivatives of C$_{33}$ sec alcohol isomers and major fragmentations of main isomer. (B) EICs showing cumulative intensity of $m/z$ 73 and 75, as well as intensities of short $\alpha$-fragments of main isomer and the four next most abundant isomers. (C) Mass spectrum of co-eluting Ac derivatives of C$_{33}$ sec alcohol isomers and major fragmentations of main isomer.
Table 5.1. *Characteristic m/z fragments of trimethylsilyl ethers of secondary alcohols in leaf wax*

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<th>Compound</th>
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<th>Fragments characteristic of isomer</th>
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<td>Pentatriacontan-18-ol</td>
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Series B consisted of three compounds found in the same fraction as free fatty acids in flag leaf wax. The TMS derivatives of all compounds in B shared diagnostic MS fragments m/z 73 [(CH$_3$)$_3$Si]$^+$ and m/z 75 [(CH$_3$)$_2$SiOH]$^+$, m/z 103 [(CH$_3$)$_3$SiOCH$_2$]$^+$, and m/z 147 [(CH$_3$)$_2$SiOSi(CH$_3$)$_3$]$^+$ and m/z 149 [(CH$_3$)$_2$SiOSi(CH$_3$)$_2$OH]$^+$, together indicating the presence of one primary and one secondary hydroxyl group (Fig. 5.3A) (Jetter and Riederer, 1999a; Jetter, 2000; McCloskey et al., 1968). They were accompanied by homolog-dependent M-15 and M-15-90 ions, due to loss of methyl radical and loss of both methyl and (CH$_3$)$_3$SiOH, respectively. Pairs of α-fragments, including one ion m/z 257 common to all homologs and a second fragment varying with chain length (m/z 415 for the C$_{28}$ homolog in Fig. 5.3A), indicated a hydroxyl group on the ω-12 carbon. Further α-fragments differing by 14 Da were present, and EICs revealed small retention time differences between them (Fig. 5.3B). The diagnostic MS fragments for all identified homologs and regiomers are presented in Table 5.2.

The Ac derivatives of compounds in fraction B showed a characteristic fragment m/z 61 [CH$_3$COOH$_2$]$^+$ confirming the presence of at least one hydroxyl group (Fig. 5.3C), as well as homolog-dependent parent ions M and daughter ions due to loss of up to two acetyl groups and acetic acid molecules (M-43, M-60, M-60-43 and M-60-60), supporting the presence of a second hydroxyl functionality (Wen et al., 2006a). Different regiomers were discerned based on loss of CH$_2$CO or AcOH from α-fragments, confirming the presence of a hydroxyl function on ω-12 (m/z 167 / 313) and, for example, ω-11 (m/z 153 / 327), ω-13 (m/z 181 / 299). Taken together, the TLC behaviour and GC-MS data for TMS and Ac derivatives demonstrated that B was a homologous series of prim/sec diols 5.2, with sec hydroxyl groups predominantly in the ω-12 position or on adjacent carbons.
Figure 5.3. *Structure elucidation of primary/secondary diols in wheat leaf wax.* (A) Mass spectrum of co-eluting TMS derivatives of C$_{28}$ prim/sec diol isomers and major fragmentations of main isomer. (B) EICs showing intensities of m/z 73 and of short α-fragments of main isomer and the four next most abundant isomers. (C) Mass spectrum of co-eluting Ac derivatives of C$_{28}$ prim/sec diol isomers and major fragmentations of main isomer.
Table 5.2. *Characteristic m/z fragments of bis(trimethylsilyl) ethers of primary/secondary diols in leaf wax*

<table>
<thead>
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<th>Compound</th>
<th>Fragments characteristic of homolog</th>
<th>Fragments characteristic of isomer</th>
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Series C comprised five compounds with very long GC retention times (>50 min), suggesting relatively high molecular weights and long carbon chains, likely in the form of esters linking two VLC moieties. Based on TLC behaviour, these compounds had polarities between primary and secondary alcohols, rendering alkyl ester structures with an additional secondary hydroxyl function plausible. The TMS derivatives of compounds C had a common diagnostic MS fragment m/z 73 [(CH$_3$)$_3$Si]$^+$, but no m/z 147 [(CH$_3$)$_2$SiOSi(CH$_3$)$_3$]$^+$ (Fig. 5.4A), indicating the presence of only one hydroxyl group in the native compounds. The five homologs also had fragments characteristic of the acid components of esters, such as acylium ions M$_{acid}$-17 and fragments formed via McLafferty rearrangement with double hydrogen transfer M$_{acid}$+1 (m/z 323 and 341 for the C$_{30}$ homolog containing C$_{22}$ acid shown in Fig. 5.4A) (Kingston et al., 1974). The hydroxy ester structure thus confirmed, further ions could be inferred to result from TMS.
transfer from the sec hydroxyl to the ester group, i.e. \( m/z \) 397 and 413 for the C\(_{50}\) homolog in Fig. 5.4A (Busta et al., 2016; von Wettstein-Knowles and Madsen, 1984), and from sequential loss of \((\text{CH}_3)_3\text{SiOH}\) and an acid molecule from M (C\(_{22}\) acid with molecular weight 340 amu in Fig. 5.4A).

The TMS derivatives of C also showed pairs of \( \alpha \)-fragments, one common to all homologs (\( m/z \) 257) indicating an \( \omega \)-12 hydroxyl group, and another one varying with chain length (\( m/z \) 665 for the C\(_{50}\) homolog in Fig. 5.4A). Other pairs of \( \alpha \)-fragments differing by 14 amu units again suggested positional isomers, and small retention time differences in EICs confirmed the presence of, among others, \( \omega \)-10 (\( m/z \) 229), \( \omega \)-11 (\( m/z \) 243), \( \omega \)-13 (\( m/z \) 271), \( \omega \)-14 (\( m/z \) 285) hydroxyls. (Fig. 5.4B; Table 5.3). Ac derivatives served to confirm the tentatively assigned structures of C, with fragments \( m/z \) 61 [\( \text{CH}_3\text{COOH}_2 \)]\(^+\) as well as M-43 and M-60 fragments due to loss of an acetate moiety corroborating the presence of one hydroxyl function in all homologs (Fig. 5.4C). Further fragments due to ester-linked acids (\( \text{M}_{\text{acid}-17} \) and \( \text{M}_{\text{acid}+1} \)), to loss of both acetic acid and the VLC acid from M (\( m/z \) 390) further confirmed the hydroxy ester structures. Lastly, \( \alpha \)-fragmentation and subsequent loss of \( \text{CH}_2\text{CO} \) gave rise to ions confirming the presence of an \( \omega \)-12 hydroxyl group (\( m/z \) 593), together with several co-eluting positional isomers (e.g., \( m/z \) 607 for \( \omega \)-11 and \( m/z \) 579 for \( \omega \)-13 hydroxyls).

Finally, reduction of a separate aliquot of series C with excess LiAlH\(_4\) yielded a single bifunctional compound, C\(_{28}\) \( \text{prim/sec} \) diol, together with C\(_{16}\) to C\(_{24}\) \text{prim} alcohols. The structure of the C\(_{28}\) \( \text{prim/sec} \) diol was assigned based on identical GC and MS behaviour (of the TMS derivative) to the corresponding homolog in series B. Thus, the LiAlH\(_4\) derivatization confirmed
that compounds C were esters of prim/sec C$_{28}$ diols 5.3. Taken together, our TLC and GC-MS data demonstrate that fraction C was a homologous series of esters 5.3 containing C$_{16}$ to C$_{24}$ fatty acids linked to the terminal hydroxyl of prim/sec C$_{28}$ diols. Each ester homolog comprised several isomers, with sec hydroxyl groups at and around the $\omega$-12 carbon.

Figure 5.4. *Structure elucidation of primary/secondary diol esters in wheat leaf wax.* (A) Mass spectrum of co-eluting TMS derivatives of C$_{50}$ prim/sec diol ester isomers and major fragmentations of main isomer. (B) EICs showing intensities of $m/z$ 73 and of short $\alpha$-fragments of main isomer and the four next most abundant isomers. (C) Mass spectrum of co-eluting Ac derivatives of C$_{50}$ prim/sec diol ester isomers and major fragmentations of main isomer.
Table 5.3. *Characteristic m/z fragments of trimethylsilyl ethers of primary/secondary diol esters in leaf wax*

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<th>Compound</th>
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<th>Fragments characteristic of isomer</th>
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5.3.3. Structure elucidation of compound classes D and E

The five homologs in series D were, according to relative TLC retention, more polar than primary alcohols and the hydroxy esters in fraction C. Compounds D were tentatively assigned as VLC esters of hydroxy-2-alkanols 5.4 linked through the 2-OH group, based on similarity of their TMS derivative mass spectra with that of 7-hydroxypentadecan-2-ol eicosanoate reported in the literature (von Wettstein-Knowles and Madsen, 1984). The combination of TMS derivative M-15 and ion m/z 73 [(CH$_3$)$_3$Si]$^+$ together with the lack of m/z 147 [(CH$_3$)$_2$SiOSi(CH$_3$)$_3$]$^+$ confirmed the presence of only one hydroxyl function. Two ester metamers with differing acid (and, consequently, also alcohol) chain lengths were immediately apparent for each homolog in D, based on the presence of two homologous pairs of M$_{acid}$-17 / M$_{acid}$ fragments (m/z 295 / 312 and 323 / 340 for the C$_{35}$ homolog in Fig. 5.5A). Similarly, homologous pairs of TMS transfer fragments were observed (m/z 369 / 385 and 397 / 413 in Fig. 5.5A), indicative of two co-eluting metamers for each ester homolog. Furthermore, four pairs of complementary α-fragments were observed for each ester chain length (m/z 215 / 497, 201 / 511, 187 / 525, and 173 / 539), suggesting the presence of two regioomers for each of the two metamers. Careful analysis of overlap between EIC traces of regiomer-specific α-fragments and of acid-specific fragments (Fig. 5.5C) allowed identification of four co-eluting isomers within the C$_{35}$ ester homolog, listed in decreasing relative abundance: (i) C$_{20}$ acid + C$_{15}$ 2,8-diol (m/z 385 and 201); (ii) C$_{20}$ acid + C$_{15}$ 2,7-diol (m/z 385 and 215); (iii) C$_{22}$ acid + C$_{13}$ 2,7-diol (m/z 413 and 187); (iv) C$_{22}$ acid + C$_{13}$ 2,8-diol (m/z 413 and 173). A summary of all identified homologs and isomers within them along with their diagnostic MS fragments is presented in Table 5.4.
The structures of the diol esters in D were confirmed by MS analysis of their Ac derivatives. They showed the characteristic \( m/z \) 61 [CH\(_3\)COOH\(_2\)]\(^+\) indicative of hydroxyl group presence in the native structure, as well as homolog-dependent M-60, M-M\(_{\text{acid}}\), M-M\(_{\text{acid}}\)-43, M-M\(_{\text{acid}}\)-60 fragments due to single or combined losses of acetyl- and fatty acyl-derived moieties (Fig. 5.5D). Fragments M\(_{\text{acid}}\)-17 / M\(_{\text{acid}}\)+1 further confirmed the presence of two metamers per homolog \( (m/z \) 295 / 313 and 323 / 341 in Fig. 5.5D). The \( \alpha \)-fragments had very low intensity, but their product ions resulting from loss of acetic acid confirmed the presence of the sec hydroxyl \( (m/z \) 111 and 421 in Fig. 5.5D). Finally, reduction of an aliquot of the fraction with excess LiAlH\(_4\) gave rise to two new compounds with relatively short GC retention times (as TMS derivatives). Their mass spectra were unambiguously assigned to mixtures of pentadecane-2,7-diol plus pentadecane-2,8-diol (Fig. 5.5F) and tridecane-2,7-diol plus tridecane-2,8-diol, respectively, thus confirming the presence of four isomers per ester homolog in D. Taken together, the TLC and GC-MS data identified D as a series of ester homologs formed by linking various fatty acids with 7- and 8-hydroxy-2-tridecanol as well as 7- and 8-hydroxy-2-pentadecanol.
Figure 5.5. Structure elucidation of hydroxy-2-alkanol esters in wheat leaf and peduncle wax.

(A) Mass spectrum of co-eluting TMS derivatives of C_{35} hydroxy-2-alkanol ester isomers. (B) Major fragmentations of all isomers in (A). (C) EICs showing cumulative intensity of m/z 73 and 75, as well as intensities of: metamer-shared \( \alpha \)-fragments m/z 185 and 199, regiomer-characteristic \( \alpha \)-fragments m/z 173, 187, 201 and 215, and TMS-transfer acid fragments m/z 385 and 413. (D) Mass spectrum of co-eluting Ac derivatives of C_{35} hydroxy-2-alkanol ester isomers. (E) Major fragmentations of all isomers in (D). (F) Mass spectrum and major fragmentations of TMS derivatives of co-eluting C_{15} diol isomers obtained via LiAlH_{4} reduction of the C_{35} hydroxy-2-alkanol ester isomer mixture (corresponding information for C_{13} diols not shown).
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</tbody>
</table>

The five homologs in series E, with polarity between primary alcohols and prim/sec diol esters, were tentatively assigned oxo-2-alkanol ester structures 5.5 based on similarity of their mass spectra with that of 7-oxopentadecan-2-ol eicosanoate reported before (von Wettstein-Knowles and Madsen, 1984). Treatment of E with BSTFA left the compounds in E lacking the TMS fragment m/z 73, suggesting that they did not bear hydroxyl groups (Fig. 5.6A). Instead, they had fragments indicative of the 2-alkanol ester structure, such as \( M_{\text{acid}}-17 \) (m/z 295 and 323 for the two metamers in Fig. 5.6A), \( M_{\text{acid}}+1 \) (m/z 313 and 341 in Fig. 5.6A), and \( M_{\text{alcohol}}-1 \) (m/z 225 and 197 in Fig. 5.6A). They also showed prominent homolog-independent α-fragments indicating the carbonyl position on the 2-alkanol moiety, either 7- or 8-oxo groups on C₁₅ 2-
alkanol \((m/z 141\) and \(m/z 127\), respectively), and 7- or 8-oxo groups on \(C_{13}\) 2-alkanol \((m/z 113\) and \(m/z 99\), respectively; Fig. 5.6A-C and Table 5.5). Complementary \(\alpha\)-fragments were not observed, but some of the closely-related fragments resulting from McLafferty rearrangement on the same side of the carbonyl function were sizeable \((m/z 452\) and 438 for the \(C_{35}\) homolog in Fig. 5.6A). Molecular ions \(M\) could not be detected under the current conditions.

To directly probe the presence of a carbonyl functionality, an aliquot of fraction \(E\) was derivatized with \(O\)-methylhydroxylamine and the corresponding methoximes analyzed by MS. The resulting homologs all showed fragments \(m/z 87\) and \(m/z 100\) diagnostic for methoximes (Jetter and Riederer, 1999a) (Fig. 5.6D), accompanied by prominent MS ions characterizing the 2-alkanol ester structure, such as \(M_{\text{acid}-17}\) \((m/z 295\) and 323 for the two metamers in Fig. 5.6D), \(M_{\text{acid}+1}\) \((m/z 313\) and 341 in Fig. 5.6D), and \(M_{\text{alcohol}-1}\) \((m/z 254\) and 226 in Fig. 5.6D). \(\alpha\)-Fragments and related McLafferty fragments indicative of methoxime position were 29 amu higher than corresponding signals of the underivatized carboxyls (e.g., \(m/z 156\) and \(m/z 481\) in Fig. 5.6D instead of \(m/z 127\) and \(m/z 452\) in Fig. 5.6A). Finally, fragments \(M-31\) due to loss of methoxy group indicated homolog chain lengths, while \(M\) ions were not detected.

For further structure confirmation, fraction \(E\) was subjected to reduction with \(\text{LiAlH}_4\), resulting in the same two pairs of diol isomers also formed by reduction of series \(D\). Taken together, the TLC behaviour as well as the GC-MS characteristics of diverse derivatives demonstrated that fraction \(E\) was a homologous series of esters containing various fatty acids linked to 7- and 8-oxo-2-tridecanol\(^1\) as well as 7- and 8-oxo-2-pentadecanol\(^2\).

\(^1\) IUPAC names: 2-hydroxytridecan-7-one and 12-hydroxytridecan-6-one.
\(^2\) IUPAC names: 2-hydroxypentadecan-7-one and 2-hydroxypentadecan-8-one

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Figure 5.6. Structure elucidation of oxo-2-alkanol esters in wheat leaf and peduncle wax. (A) Mass spectrum of co-eluting isomers of C\textsubscript{35} oxo-2-alkanol ester. (B) Major fragmentations of all isomers from (A). (C) EICs showing intensity of m/z 57, as well as intensities of: metamer-characteristic alkyl fragments m/z 197 and 225, regiomer-characteristic α-fragments m/z 99, 113, 127 and 141, and M\textsubscript{acid}+1 fragments m/z 313 and 341. (D) Mass spectrum of co-eluting isomers of methoxime derivatives of C\textsubscript{35} oxo-2-alkanol ester. (E) Major fragmentations of all isomers from (D).
Table 5.5. Characteristic m/z fragments of oxo-2-alkanol esters in leaf and peduncle wax

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fragments characteristic of homolog</th>
<th>Fragments characteristic of isomer type:</th>
<th>-regiomer</th>
<th>-metamer</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-oxotridecane-2-ol stearate</td>
<td>-</td>
<td></td>
<td>99 114 424</td>
<td>197 267 285</td>
</tr>
<tr>
<td>7-oxotridecane-2-ol stearate</td>
<td></td>
<td></td>
<td>113 128 410</td>
<td></td>
</tr>
<tr>
<td>8-oxopentadecane-2-ol palmitate</td>
<td></td>
<td></td>
<td>127 142 396</td>
<td>225 239 257</td>
</tr>
<tr>
<td>7-oxopentadecane-2-ol palmitate</td>
<td></td>
<td></td>
<td>141 156 382</td>
<td></td>
</tr>
<tr>
<td>8-oxotridecane-2-ol arachidate</td>
<td>-</td>
<td></td>
<td>99 114 452</td>
<td>197 295 313</td>
</tr>
<tr>
<td>7-oxotridecane-2-ol arachidate</td>
<td></td>
<td></td>
<td>113 128 438</td>
<td></td>
</tr>
<tr>
<td>8-oxopentadecane-2-ol stearate</td>
<td></td>
<td></td>
<td>127 142 424</td>
<td>225 267 285</td>
</tr>
<tr>
<td>7-oxopentadecane-2-ol stearate</td>
<td></td>
<td></td>
<td>141 156 410</td>
<td></td>
</tr>
<tr>
<td>8-oxotridecane-2-ol behenate</td>
<td>-</td>
<td></td>
<td>99 114 480</td>
<td>197 323 341</td>
</tr>
<tr>
<td>7-oxotridecane-2-ol behenate</td>
<td></td>
<td></td>
<td>113 128 466</td>
<td></td>
</tr>
<tr>
<td>8-oxopentadecane-2-ol arachidate</td>
<td></td>
<td></td>
<td>127 142 452</td>
<td>225 295 313</td>
</tr>
<tr>
<td>7-oxopentadecane-2-ol arachidate</td>
<td></td>
<td></td>
<td>141 156 438</td>
<td></td>
</tr>
<tr>
<td>8-oxotridecane-2-ol lignocerate</td>
<td>-</td>
<td></td>
<td>99 114 508</td>
<td>197 351 369</td>
</tr>
<tr>
<td>7-oxotridecane-2-ol lignocerate</td>
<td></td>
<td></td>
<td>113 128 494</td>
<td></td>
</tr>
<tr>
<td>8-oxopentadecane-2-ol behenate</td>
<td></td>
<td></td>
<td>127 142 480</td>
<td>225 323 341</td>
</tr>
<tr>
<td>7-oxopentadecane-2-ol behenate</td>
<td></td>
<td></td>
<td>141 156 466</td>
<td></td>
</tr>
<tr>
<td>8-oxotridecane-2-ol cerotate</td>
<td>-</td>
<td></td>
<td>99 114 536</td>
<td>197 379 397</td>
</tr>
<tr>
<td>7-oxotridecane-2-ol cerotate</td>
<td></td>
<td></td>
<td>113 128 522</td>
<td></td>
</tr>
<tr>
<td>8-oxopentadecane-2-ol lignocerate</td>
<td></td>
<td></td>
<td>127 142 508</td>
<td>225 351 369</td>
</tr>
<tr>
<td>7-oxopentadecane-2-ol lignocerate</td>
<td></td>
<td></td>
<td>141 156 494</td>
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</table>

5.3.4. Structure elucidation of compound classes F and G

Series F comprised four compounds, found in the same fraction as series E, tentatively identified as VLC 4-alkylbutan-4-olides (4-alkyl-γ-lactones) 5.6 based on similarity of their mass spectra with those of shorter-chain 4-alkylbutan-4-olides reported previously (McFadden et al., 1965). Similar to series E, compounds F could not be silylated (no m/z 73), suggesting that they lacked a hydroxyl group (Fig. 5.7A). All homologs had a diagnostic base peak m/z 85, likely formed via cleavage of the alkyl side chain, as well as m/z 100 formed via McLafferty
rearrangement. Chain length-dependent molecular ions were accompanied by fragments M-18, M-18-18 and M-18-44, likely due to loss of water and acetaldehyde (Table 5.6).

Transesterification of F with excess CH₃OH/BF₃ resulted in an open-chain product that could not be silylated, similar to the behaviour of 5-alkyl-δ-lactones under the same conditions (Jetter and Riederer, 1999b), albeit independent of derivatization time. The transesterification products of F were identified as methyl 4-methoxyalkanoates based on their shared α-fragment m/z 131, and a second homolog-dependent α-fragment (m/z 381 for the C₂₈ homolog in Fig. 5.7B). Molecular ions were found accompanied by M-15, M-32, M-15-32, and M-32-32 due to loss of methyl radical and/or methanol molecule(s). Lastly, reduction of F with excess LiAlH₄ followed by reaction with BSTFA resulted in silylated 1,4-diols with MS characteristics (Fig. 5.7C) indicative of two hydroxyl groups (m/z 73 [(CH₃)₂Si]⁺, m/z 75 [(CH₃)₂SiOH]⁺, m/z 103 [(CH₃)₃SiOCH₂]⁺, m/z 147 [(CH₃)₂SiOSi(CH₃)₃]⁺ and m/z 149 [(CH₃)₂SiOSi(CH₃)₂OH]⁺). Other diagnostic signals were the α-fragments m/z 233 (independent of homolog) and m/z 439 (depending on the homolog), along with the base peak m/z 143 resulting from loss of (CH₃)₃SiOH from the shorter α-fragment. Chain length-dependent M-15 (loss of methyl) and M-90 (loss of (CH₃)₂SiOH) further confirmed the 1,4-diol structures of the reduction products. All data for F taken together unambiguously established this as a homologous series of 4-alkylbutan-4-olides 5.6.
Figure 5.7. Structure elucidation of 4-alkylbutan-4-olides in wheat leaf and peduncle wax. (A) Mass spectrum and major fragmentations of C$_{28}$ 4-alkylbutan-4-olide 5.6b. (B) Mass spectrum and major fragmentations of its product of transesterification with CH$_3$OH/BF$_3$. (C) Mass spectrum and major fragmentations of TMS derivative of LiAlH$_4$ reduction product from C$_{28}$ 4-alkylbutan-4-olide.
**Table 5.6. Characteristic m/z fragments of 4-alkylbutan-4-olides in leaf and peduncle wax**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fragments characteristic of homolog</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Docosylbutan-4-olide</td>
<td>332 358 376 394</td>
</tr>
<tr>
<td>4-Tetracosylbutan-4-olide</td>
<td>360 386 404 422</td>
</tr>
<tr>
<td>4-Hexacosylbutan-4-olide</td>
<td>388 414 432 450</td>
</tr>
<tr>
<td>4-Octacosylbutan-4-olide</td>
<td>416 442 460 478</td>
</tr>
</tbody>
</table>

Series G comprised six compounds in the least polar fraction of wheat wax, tentatively assigned as internally methyl-branched alkanes 5.7 by analogy with spectra of 15-methyl-alkanes (von Wettstein-Knowles, 2007). Accordingly, compounds G did not exhibit MS fragments indicative of silylation, and other derivatization reactions (such as acetylation, methoximation, transesterification or LiAlH₄ reduction) did not alter the compounds in any way, thus confirming the pure hydrocarbon structures. Homolog-dependent molecular ions M and fragments M-15 (due to loss of the methyl branch) were observed (Fig. 5.8A/C). They were accompanied by α-fragments diagnostic for the methyl branch position, with a predominant m/z 168/169 common to all even-numbered homologs 5.7a/c/e and indicative of methyl branching at C-11 (Fig. 5.8A), and m/z 182/183 common to all odd-numbered homologs 5.7b/d/f and indicative of methyl branching at C-12 (Fig. 5.8C). Complementary homolog-dependent α-fragments were m/z 308/309 for the C₃₂ homolog 5.7e (Fig. 5.8A) and m/z 280/281 for the C₃₁ homolog 5.7d (Fig. 5.8C). Also noticeable were other α-fragments of additional, less abundant regiomers bearing methyl branches at C-9 (m/z 140/141), C-13 (m/z 196/197), or C-15 (m/z 224/225) of even-numbered homologs (Fig. 5.8B), and at C-10 (m/z 154/155) or C-14 (m/z 210/211) of odd-numbered homologs (Fig. 5.8D). A summary of all in-chain branched alkane homologs and isomers identified here is presented in Table 5.7 together with their diagnostic MS fragments.
Overall, our MS data identified compounds G as a homologous series of alkanes 5.7 bearing methyl branches primarily on C-11 and C-12, respectively.

Figure 5.8. Structure elucidation of internally branched alkanes in wheat leaf and peduncle wax. (A) Mass spectrum of co-eluting isomers of C_{32} internally branched alkane and major fragmentations of the main isomer 5.7e. (B) EICs showing intensity of m/z 85, as well as cumulative intensities of regiomer-characteristic α-fragments m/z 140+141, 168+169, 196+197 and 224+225. (C) Mass spectrum of co-eluting isomers of C_{31} internally branched alkane and major fragmentations of the main isomer 5.7d. (B) EICs showing intensity of m/z 85, as well as cumulative intensities of regiomer-characteristic α-fragments m/z 154+155, 182+183, and 210+211.
Table 5.7. Characteristic m/z fragments of internally branched alkanes in leaf and peduncle wax

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fragments characteristic of homolog</th>
<th>Fragments characteristic of isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-Methylheptacosane</td>
<td>379  394</td>
<td>140/141  280/281</td>
</tr>
<tr>
<td>11-Methylheptacosane</td>
<td>168/169</td>
<td>252/253</td>
</tr>
<tr>
<td>13-Methylheptacosane</td>
<td>196/197</td>
<td>224/225</td>
</tr>
<tr>
<td>10-Methylloctacosane</td>
<td>393  408</td>
<td>154/155  280/281</td>
</tr>
<tr>
<td>12-Methylloctacosane</td>
<td>182/183</td>
<td>252/253</td>
</tr>
<tr>
<td>14-Methylloctacosane</td>
<td>210/211</td>
<td>224/225</td>
</tr>
<tr>
<td>9-Methylnonacosane</td>
<td>407  422</td>
<td>140/141  308/309</td>
</tr>
<tr>
<td>11-Methylnonacosane</td>
<td>168/169</td>
<td>280/281</td>
</tr>
<tr>
<td>13-Methylnonacosane</td>
<td>196/197</td>
<td>252/253</td>
</tr>
<tr>
<td>15-Methylnonacosane</td>
<td>224/225</td>
<td>224/225</td>
</tr>
<tr>
<td>10-Methyltriacontane</td>
<td>421  436</td>
<td>154/155  308/309</td>
</tr>
<tr>
<td>12-Methyltriacontane</td>
<td>182/183</td>
<td>280/281</td>
</tr>
<tr>
<td>14-Methyltriacontane</td>
<td>210/211</td>
<td>252/253</td>
</tr>
<tr>
<td>9-Methylhentriacontane</td>
<td>435  450</td>
<td>140/141  336/337</td>
</tr>
<tr>
<td>11-Methylhentriacontane</td>
<td>168/169</td>
<td>308/309</td>
</tr>
<tr>
<td>13-Methylhentriacontane</td>
<td>196/197</td>
<td>280/281</td>
</tr>
<tr>
<td>15-Methylhentriacontane</td>
<td>224/225</td>
<td>252/253</td>
</tr>
<tr>
<td>10-Methyldotriacontane</td>
<td>421  436</td>
<td>154/155  336/337</td>
</tr>
<tr>
<td>12-Methyldotriacontane</td>
<td>182/183</td>
<td>308/309</td>
</tr>
<tr>
<td>14-Methyldotriacontane</td>
<td>210/211</td>
<td>280/281</td>
</tr>
</tbody>
</table>

5.3.5. Quantification of new compounds from cuticular waxes of *T. aestivum* cv. Bethlehem flag leaf blades and peduncles

Gas chromatography with flame ionization detection (GC-FID) was used in a second set of experiments to quantify the newly identified compounds in the total wax mixtures of wheat flag leaves and peduncles. They had fairly low wax coverages over both organs, ranging from 0.008 ± 0.001 μg/cm² for 4-alkylbutan-4-olides 5.6 to 0.10 ± 0.02 μg/cm² for secondary alcohols 5.1 in the flag leaf waxes, and from 0.12 ± 0.03 μg/cm² for 4-alkylbutan-4-olides 5.6 to 0.32 ±
0.08 μg/cm² for internally branched alkanes 5.7 in peduncle waxes (Fig. 5.9A/B). Neither of the oxo-2-alkanol ester homologs could be quantified reliably enough to calculate respective coverages.

Figure 5.9. Total coverages of new compound classes in wheat leaf and peduncle waxes. Coverages (μg/cm²) of new compound classes identified in the total wax mixtures covering the (A) flag leaf blade and (B) peduncle of T. aestivum cv. Bethlehem. Bars represent mean ± standard deviation (n = 5).
As all new compound classes comprised series of homologs, their characteristic chain length distributions could be further assessed. Secondary alcohols 5.1 (compounds A) were found as a homologous series with odd-numbered carbon chains from C\textsubscript{25} to C\textsubscript{35} and a bimodal distribution peaking at C\textsubscript{27} 5.1b and C\textsubscript{33} 5.1e (Fig. 5.10A). In contrast, the prim/sec diols 5.2 (compounds B) ranged from C\textsubscript{26} to C\textsubscript{30}, with only even-numbered homologs present and a single homolog, C\textsubscript{28} 5.2b, accounting for more than 90% of this fraction. Similarly, the prim/sec diol esters 5.3 (compounds C) contained the same alkyl moiety together with various fatty acids, resulting in a homologous series of even-numbered total chain lengths from C\textsubscript{44} to C\textsubscript{52} that peaked at C\textsubscript{50} 5.3d. All three compound classes were found only in flag leaf wax.

Hydroxy-2-alkanol esters 5.4 (compounds D) were present in both flag leaf and peduncle wax mixtures and had nearly identical chain length profiles in both organs, spanning the odd-numbered homologs from C\textsubscript{31} to C\textsubscript{39} with a single maximum at C\textsubscript{35} 5.4c (Fig. 5.10A/B). The fraction of oxo-2-alkanol esters 5.5 (compounds E) comprised odd-numbered C\textsubscript{31} to C\textsubscript{39} homologs, peaking around C\textsubscript{35} 5.5c.

The wheat waxes contained even-numbered C\textsubscript{26} to C\textsubscript{32} 4-alkylbutan-4-olides 5.6 (compounds F) with unimodal chain length distributions peaking at C\textsubscript{28} 5.6b in both organs, albeit with a considerably higher proportion of the C\textsubscript{30} homolog 5.6c in peduncle wax as compared to flag leaf wax (Fig. 5.10A/B). Finally, the internally branched alkanes 5.7 (compounds G) also had very similar chain length profiles ranging from C\textsubscript{28} to C\textsubscript{33} in the wax mixtures from both organs, with even-numbered homologs 5.7a/c/e considerably more abundant than odd-numbered ones 5.7b/d/f (the methyl branch is included in the carbon number). Among the even-numbered alkanes, the C\textsubscript{32} homolog 5.7e was most abundant, while C\textsubscript{31} 5.7d predominated among the odd-numbered homologs.
Figure 5.10. *Chain length distributions of new compound classes in wheat leaf and peduncle waxes.* Relative abundances (%) of each homolog from each of the six new compound classes identified in the total wax mixtures covering the (A) flag leaf blade and (B) peduncle of *T. aestivum* cv. Bethlehem. Numbers on the x-axis indicate homolog chain length. Bars represent mean ± standard deviation (*n* = 5). Each bar group adds up to 100%.
5.4. Discussion

Our in-depth analysis of the wax mixtures on flag leaf blades and peduncles of *T. aestivum* cv. Bethlehem revealed the presence of (i) homologous series of secondary alcohols, primary/secondary diols and primary/secondary diol esters, all with secondary hydroxyls on and around identical methylene units 12 carbons away from one chain end; (ii) esterified C\(_{13}\) and C\(_{15}\) 2-alkanols with hydroxy- or keto-functions on C-7 or C-8; (iii) a homologous series of \(\gamma\)-lactones, as such derived from fatty acids with hydroxyl functions on C-4; and (iv) alkanes with (total) carbon numbers ranging from \(C_{28}\) to \(C_{33}\) and methyl branches on C-11 or C-12. Both the homolog and the isomer distribution of all seven compound classes can now be used to infer potential biosynthetic pathways leading to them.

5.4.1. Secondary alcohols, primary/secondary diols and diol esters in flag leaf wax

The wax mixture on wheat flag leaves comprised homologous series of secondary alcohols, primary/secondary diols and corresponding diol esters, thus compounds characterized by secondary hydroxyls. All three compound classes were found to contain mainly regiomers with secondary hydroxyls on a methylene unit 12 carbons away from one end of the hydrocarbon chain, designated as C-12 in the secondary alcohols or as the \(\omega-12\) carbon in the diols. These major isomers in each of the three series were accompanied by further, minor regiomers, characterized by hydroxyls on methylene units in the vicinity of C-12. The finding that all three compound classes shared very similar isomer patterns around their secondary hydroxyl functions suggests that they are biosynthetically related.
The isomer distribution in all three compound classes, centred around one carbon position with minor but significant admixtures of isomers with hydroxyls on adjacent carbons is reminiscent of the isomer mixtures of wax secondary alcohols in several other species. For example, C\textsubscript{25}-C\textsubscript{33} secondary alcohols with hydroxyls on C-6 to C-14 have been reported for leaf and fruit capsule waxes of several Papaveraceae species (Jetter and Riederer, 1996), C\textsubscript{29}-C\textsubscript{33} secondary alcohols with hydroxyls on C-12 to C-17 for *Pisum sativum* leaf wax (Wen et al., 2006a), and secondary alcohols, ketones, vicinal secondary/secondary diols and ketols with functional groups between C-13 and C-15 for *Arabidopsis thaliana* stem wax (Wen and Jetter, 2009). The latter Arabidopsis compounds are known to be formed by a single enzyme, the mid-chain alkane hydroxylase MAH1 (Greer et al., 2007). This cytochrome P450-dependent monooxygenase exhibits characteristically limited regio-specificity, catalyzing the (repeat) hydroxylation of several methylene units near the centre of the hydrocarbon chain. Accordingly, it has been proposed that homologous cytochrome P450 enzymes with similarly limited regio-specificity form the broad mixtures of secondary alcohol isomers found in the waxes of other plant species (Jetter et al., 2006).

It is important to note that a second important mechanism for introducing hydroxyl functions in wax molecules exists. In many plant species, wax secondary alcohols were identified with hydroxyl groups exclusively on even-numbered carbon atoms, such as 10-nonacosanol on *Malus domestica* fruit (Dong et al., 2012), 10-heptacosanol, 10-nonacosanol and 12-nonacosanol on *Osmunda regalis* fronds (Jetter and Riederer, 2000), or C\textsubscript{23}-C\textsubscript{33} 2-alkanols from *Aloe arborescens* leaves (Racovita et al., 2015). It has been proposed that such secondary
alcohols, with functional groups on every other rather than adjacent carbons, may be derived from β-hydroxyacetyl-CoA intermediates of fatty acid elongation instead of cytochrome P450 hydroxylation (Racovita et al., 2015; Wen and Jetter, 2007).

Our current findings of broad isomer distributions for the wheat secondary alcohols suggest that they may be synthesized by a cytochrome P450 enzyme and not as derivatives of fatty acid elongation intermediates. We conclude that wheat likely possesses a MAH1-like enzyme hydroxylating preferentially on C-12 of C_{25}-C_{35} alkane substrates. Interestingly, the resulting secondary alcohols had a bimodal chain length distribution, peaking at C_{27} and C_{33} (compare Fig. 5.10A), very different from that of the corresponding alkane precursors, with a single maximum at C_{31} (Racovita et al., 2016). This suggests either an unusual chain length preference of this hydroxylase for C_{33} and C_{27} alkane substrates, or else the presence of two very similar enzymes with similar regio-specificity but different substrate chain length preference.

The broad isomer distributions of the primary/secondary diols in the wheat leaf wax suggest that they are also formed by a cytochrome P450 hydroxylase. It seems plausible that the enzyme(s) converting alkanes into secondary alcohols (see above) can also hydroxylate primary alcohols into corresponding diols. Considering the clear predominance of C_{28} diol (compare Fig. 5.10A), very similar to the profile of precursor primary alcohols (Racovita et al., 2016), it appears that the pool of alcohols is used non-discriminatively by the enzyme(s). Interestingly, both the secondary alcohols and diols had in-chain hydroxyls mainly on the methylene unit 12 carbons away from the methyl (or ω--) terminus, while the distance to the second methyl or alcohol terminus varied. We thus propose that the wheat MAH1-like enzyme may achieve its
(limited) regio-specificity by tight binding of the short alkyl moiety of the alkane or primary alcohol substrates, effectively counting carbons in from the methyl terminus. It should also be noted that the C28 alcohol thus serving as substrate has an overall molecule geometry, including the carbon chain and the oxygen atom, resembling the C29 alkane homolog against which the enzyme(s) seemed to discriminate (compare Fig. 5.10A).

Primary/secondary diols with in-chain hydroxyl groups on several adjacent carbons, similar to those in wheat wax, had been reported for several other plant species before. For example, *Pisum sativum* leaves contain C26–C28 diols with 1,12- through 1,17-functionalities (Wen et al., 2006a), while the eustigmatophyte *Nannochloropsis gaditana* has C28-C36 diols with 1,13- through 1,19-geometries (Mejanelle et al., 2003). Again it seems likely that the secondary hydroxyls of these diols may be introduced by cytochrome P450 enzymes. In contrast, other diols were detected with the secondary hydroxyls exclusively on odd-numbered carbons (when counting from the primary OH), and are thought to be formed as elongation by-products. Examples for such compounds include the C28-C38 1,5-diols in *Taxus baccata* needles (Wen and Jetter, 2007), C28-C32 1,11-diols in *Osmunda regalis* fronds (Jetter and Riederer, 1999a), C32 1,9-, 1,11- and 1,13-diols in *Myricaria germanica* leaves (Jetter, 2000), or C30 1,11-, C32 1,13-, C34 1,15- and C36 1,17-diols in *Azolla filiculoides* whole plants (Speelman et al., 2009).

Finally, esters of primary/secondary diols have scarcely been reported, including the C46-C52 esters of C30 1,11-, C32 1,13-, C34 1,15- and C36 1,17-diols of the fern *Azolla filiculoides* (Speelman et al., 2009) and C40-C52 esters of C30 1,5-, 1,7- and 1,9-diols of the moss *Funaria hygrometrica* (Busta et al., 2016). It seems plausible that such esters are acylation products of
corresponding free primary/secondary diols, formed by wax ester synthases. The diol esters found in wheat wax had nearly identical homolog and regiomer distributions as the accompanying free diols, suggesting that the responsible wax ester synthase shows no preference for the diol substrate. Instead, based on the chain length distribution of diol esters peaking at C\textsubscript{50} (compare Fig. 5.10A), it appears that the wax ester synthase shows high substrate preference for C\textsubscript{22} acyl-CoA as its second substrate. Since the same preference was noted for a wax ester synthase forming unsubstituted VLC esters in the leaves of the same wheat cultivar (Racovita et al., 2016), it is very likely that the same wax ester synthase produces esters of both primary alcohols and diols. This conclusion is in accordance with our finding that only the primary hydroxyl of the primary/secondary diols was esterified, but not the secondary group.

**5.4.2. Hydroxy-2-alkanol esters and oxo-2-alkanol esters in flag leaf and peduncle waxes**

2-Alkanol esters have been identified in several grass species, typically as minor components associated with the much more prominent wax β-diketones (Racovita et al., 2016; von Wettstein-Knowles and Netting, 1976a; von Wettstein-Knowles et al., 1984). Most previous analyses revealed only esters of 2-alkanols bearing no other functional groups, except for one report identifying 7-oxo-pentadecan-2-ol as a minor constituent of barley spike wax (von Wettstein-Knowles and Madsen, 1984). The same compound was thus now also detected in wheat waxes, together with its C\textsubscript{13} homolog and 8-oxo isomers. Furthermore, we identified the four corresponding hydroxy-2-alkanols, with chain lengths and in-chain functional group positions matching those of the keto-2-alkanols, all esterified with various fatty acids.
The common overall chain length profiles and isomer distributions of the hydroxy-2-alkanol esters and oxo-2-alkanol esters identified here suggest a biosynthetic relationship between both compound classes. Considering that regiomers with functional groups on adjacent carbons were detected for all the 2-alkanol ester derivatives, it is likely that the in-chain functionalities are introduced by a cytochrome P450 hydroxylase similar to MAH1. Accordingly, we propose that wheat has a cytochrome P450 enzyme catalyzing either a single hydroxylation leading to the hydroxy-2-alkanol esters or a double-hydroxylation to the corresponding oxo-2-alkanol esters.

The apparent regio-specificity of this enzyme, as a C-7/C-8 hydroxylase, clearly differs from that of the cytochrome P450 discussed above for the formation of secondary alcohols and diols. Interestingly, both enzymes also appear to have different expression patterns, since the secondary alcohols/diols were found only in flag leaves, whereas the oxidized 2-alkanol esters were detected in both peduncle and flag leaf waxes (even though potential precursors for both product groups were likely present in both organs). Taken together, we conclude that *T. aestivum* cv. Bethlehem has at least two distinct cytochrome P450 enzymes involved in wax biosynthesis, one being a C-12-specific hydroxylase forming secondary alcohols and diols, and the other one a C-7/8-specific hydroxylase involved in formation of oxidized 2-alkanol esters.

The matching chain length distributions of the esterified hydroxy/oxo-2-alkanols and 2-alkanols (Racovita et al., 2016), both peaking at C\textsubscript{13/15}, indicate the latter could be the substrates for hydroxylation by this second hydroxylase targeting carbons C-7 and C-8 of the 2-alkanol moiety. The C-7 and C-8 positions found preferentially hydroxylated in 2-alkanols are very
similar to those of hydroxyl groups in oxidized β-diketones (e.g., 8- and 9-hydroxy-hentriacontane-14,16-dione), relative to both ends of either the 2-alkanols or the alkyl moiety within the β-diketones (Racovita et al., 2016). It is thus plausible that the same hydroxylase is involved in the formation of hydroxy/oxo-2-alkanol esters and hydroxy-β-diketones. In fact, the geometry of the β-diketone, bearing a C_{13} alkyl tail on a -CO-CH-CO- functionality, is fairly similar to that of the (C_{15}) 2-alkanol esters, having a C_{13} alkyl tail on a –CHCH_{3}-O-CO-functionality. The same cytochrome P450 may thus accept either the β-diketone or the 2-alkanol ester as substrate for hydroxylation on C-7 or C-8. However, it cannot be ruled out that hydroxylation may occur in earlier stages of the pathways leading to 2-alkanol esters and β-diketones, rather than on the latter two products.

5.4.3. 4-Alkylbutan-4-olides and internally branched alkanes in flag leaf and peduncle waxes

While δ-lactones have been identified as prominent components of the wax mixture on leaves of *Cerinthe minor* (Jetter and Riederer, 1999b), the corresponding 4-alkylbutan-4-olides (4-alkyl-γ-lactones) have not been reported before as plant cuticular wax constituents. However, γ-lactones have been identified in several plant species, albeit without localizing them to a specific organ or tissue. For example, a homologous series of C_{24} to C_{30} γ-lactones was detected in the ground aerial parts of *Flourensia cernua* (Mata et al., 2003), the C_{32} γ-lactone in the aerial parts of *Pluchea lanceolata* (Ali et al., 2001), and an unsaturated C_{21} homolog in the stem bark of *Garcinia mannii* (Hussain and Waterman, 1982). While the biosynthetic pathways leading to these structures remain unknown, it seems possible that they are formed via α-hydroxylation of
acyl-CoA substrates similar to the reactions thought to lead to 1,2-bifunctional wax compounds (Buschhaus et al., 2013a). The resulting α-hydroxyacyl-CoA intermediates might be elongated further by the FAE complex, leading to 4-hydroxyacyl-CoAs that, upon intramolecular esterification, would yield 4-alkylbutan-4-olides.

Finally, alkanes with an in-chain methyl branch had been described before as constituents of insect cuticular waxes (Nelson and Sukkestad, 1975), of wool wax (Mold et al., 1966), but also of plant cuticular waxes, namely leaf waxes of walnut tree (Stránsky et al., 1970) and spike waxes of barley (von Wettstein-Knowles, 2007). It is important to note that, different from previous reports, the in-chain-branched alkane regiomers identified here in wheat wax have methyl groups separated by two carbons, located on odd-numbered carbons in the chain of even-numbered homologs and on even-numbered carbons in odd-numbered homologs. Based on these isomer patterns, we conclude that the methyl groups are most likely introduced during FAE-catalyzed elongation of acyl-CoA precursors, possibly by incorporating a methylmalonyl-CoA extender unit in lieu of the normal malonyl-CoA. This assertion is supported by the finding that even-numbered internally branched alkanes (having odd chain lengths) were more abundant than their odd-numbered homologs (with even chain lengths), in the same way that odd-numbered n-alkanes are more abundant than those with even chain lengths.
Chapter 6: Novel polyketides and polyketide-derived compounds from cuticular waxes of *Triticum aestivum* cv. Bethlehem

6.1. Introduction

The above-ground surfaces of primary plant organs are coated with a lipidic cuticle to minimize uncontrolled water loss to the dry atmosphere. The plant cuticle is composed of a polyester matrix known as cutin and cuticular waxes lying on top of cutin (epicuticular waxes) or embedded within the cutin matrix (intracuticular waxes) (Buschhaus and Jetter, 2011).

Cutin is a biopolymer incorporating a diversity of long-chain (LC, i.e. C$_{16}$ and C$_{18}$) monomers, most commonly ω-hydroxy fatty acids or dicarboxylic acids, sometimes with additional in-chain hydroxyl, epoxy, or keto functionalities that participate in cross-linking of polyester chains (Nawrath, 2006). Cuticular waxes are complex mixtures consisting mostly of very-long-chain (VLC, i.e. >C$_{20}$) aliphatics, such as fatty acids, primary alcohols, alkyl esters, aldehydes, alkanes, secondary alcohols and ketones (Jetter et al., 2006). In many species, the wax mixtures also comprise alicyclics, including a wide variety of triterpenoids (Belge et al., 2014; Bianchi et al., 1993; Markstadter et al., 2000; Nordby and McDonald, 1994; van Maarseveen et al., 2009), and in some species also aromatics such as 5-alkylresorcinols (Adamski et al., 2013; Ji and Jetter, 2008), benzyl and phenethyl esters (Buschhaus et al., 2007a, 2007b; Gülz and Marner, 1986; Jetter and Riederer, 1996; Rapley et al., 2004), and 4-hydroxyphenylpropyl, 3,4-dihydroxyphenylpropyl and 3,4-dihydroxyphenylbutyl esters (Jetter et al., 2002; Wen and Jetter, 2007).
Aliphatic cuticular waxes typically occur as series of either even- or odd-numbered homologs, according to the biosynthetic mechanisms leading to the various product structures. From molecular genetic studies of the model plant species *Arabidopsis thaliana* it is well established that wax biosynthesis utilizes C\textsubscript{16} and C\textsubscript{18} fatty acids formed *de novo* in epidermal plastids (Kunst et al., 2006; Samuels et al., 2008). In the course of transport to the endoplasmic reticulum, these acids are activated into thioesters by long-chain acyl-CoA synthetase (LACS) enzymes. Fatty acyl elongase (FAE) complexes then extend the acyl chains with C\textsubscript{2} units, to yield mixtures of homologous acyl-CoAs with even carbon numbers. In the FAE reaction cycle, first a ketoacyl-CoA synthase (KCS) enzyme catalyzes a Claisen condensation of the acyl-CoA substrate with malonyl-CoA, and then three other enzymes perform the stepwise reduction of the β-keto group into a methylene, to yield an acyl-CoA two carbons longer than its precursor. FAE complexes with different KCS enzymes have different product chain length specificities, and the interplay of the different FAEs thus determines the overall chain length profile of the acyl-CoA product pool generated.

The elongated acyl-CoAs are finally converted into diverse end products by modification of their head-groups. On the acyl reduction pathway, a fatty acyl-CoA reductase (FAR) reduces the carboxyl functionality into primary alcohols, part of which are exported to the cuticle, while others are fused with (very-) long-chain acyl-CoAs by a wax synthase (WS) to produce alkyl esters. As neither of the reactions on this pathway affect the carbon structure of the substrates, the alcohol and ester products all have even carbon numbers. On the second pathway, a different reductase partially reduces acyl-CoAs to even-numbered aldehydes, some of which are exported
to the cuticle, while others are decarbonylated to odd-numbered alkanes. The alkanes may undergo single or double hydroxylation by a mid-chain alkane hydroxylase (MAH) to produce odd-numbered secondary alcohols, ketones, diols and ketols.

However, cuticular waxes of many plant species also include VLC aliphatics with more than one functional group. Over the past two decades, numerous new wax structures have been identified with one functional group at the end of the aliphatic chain, similar to the ubiquitous wax compounds described above, and an additional functionality inside the chain. Such primary/secondary bifunctional compounds were identified for example as alkanediols, ketoaldehydes, ketoalcohols and ketoalkyl esters in the wax of *Osmunda regalis* fronds (Jetter and Riederer, 1999a), or δ-lactones in leaf waxes of *Cerinthe minor* (Jetter and Riederer, 1999b). 1,3-Alkanediols and 3-hydroxyaldehydes were reported for leaf waxes of *Ricinus communis* (Vermeer et al., 2003), further alkanediols in *Pisum sativum* leaves (Wen et al., 2006a), 1,5-alkanediols and 5-hydroxyaldehydes in *Taxus baccata* needles (Wen and Jetter, 2007), 1,3- and 1,2-alkanediol acetates in *Cosmos bipinnatus* petals (Buschhaus et al., 2013b), 3-hydroxyacid derivatives in *Aloe arborescens* (Racovita et al., 2015), and 3-hydroxyacid and alkanediol esters in *Funaria hygrometrica* (Busta et al., 2016).

In the waxes of many other species, bifunctional compounds with two in-chain functionalities were identified. For example, both prim./sec. and sec./sec. diols were found in cuticular waxes of *Myricaria germanica* leaves (Jetter, 2000) and in the gymnosperm needle waxes (Wen et al., 2006b). Most prominently, sec./sec. diketones accumulate to relatively high concentrations in wax mixtures of diverse plant species, in most cases as single compounds with
odd carbon numbers and \( \beta \)-constellation of the two carbonyl groups (i.e., with one methylene unit between them). Specifically, \( \beta \)-diketones such as nonacosane-6,8-dione, hentriacontane-8,10-dione and tritriacontane-10,12-dione are found for example in *Buxus sempervirens* (Dierickx, 1973), tritriacontane-16,18-dione is found in *Eucalyptus globulus* (Horn et al., 1964), nonacosane-10,12-dione and hentriacontane-10,12-dione in *Hosta ‘Krossa Regal’* (Jenks et al., 2002), and nonacosane-8,10-dione, nonaconsane-12,14-dione, hentriacontane-10,12-dione and hentriacontane-14,16-dione in various species of *Rhododendron* (Evans et al., 1975b). The cuticular waxes of many Poaceae contain particularly high concentrations of \( \beta \)-diketones, most frequently hentriacontane-14,16-dione. Accordingly, this \( C_{31} \) compound dominates the waxes of *Agropyron dasystachyum, A. riparium* and *A. elongatum* (Tulloch, 1983), *Hordeum vulgare* (von Wettstein-Knowles and Netting, 1976c), and several species of wheat (Tulloch and Hoffman, 1973, 1971; Tulloch and Weenink, 1969).

Among the Poaceae, wheat is of particular interest due to its world-wide role as primary staple crop. There are many reports on the composition of cuticular wax mixtures on various wheat species, cultivars and organs (Bianchi and Corbellini, 1977; Bianchi et al., 1980; Tulloch and Hoffman, 1973, 1971; Tulloch and Weenink, 1969; Wang et al., 2015a, 2015b), documenting the presence of ubiquitous compound classes such as fatty acids, aldehydes, alkanes, primary alcohols and alkyl esters, together with \( \beta \)-diketones such as hentriacontane-14,16-dione and its hydroxylated derivatives. Recently, the *Triticum aestivum* cultivar Bethlehem was selected for genetic investigations into \( \beta \)-diketone biosynthesis (Hen-Avivi et al., 2016), and comprehensive wax analyses of this cultivar were required to establish a chemical reference dataset. Accordingly, a comparative analysis of the wax mixtures on flag leaves and
As many compounds in the wheat wax mixtures remained unidentified, a more detailed analysis of the cv. Bethlehem flag leaf and peduncle waxes was carried out, relying on the pre-separation of the complex mixture by thin layer chromatography (TLC). Several TLC fractions were found to contain novel compounds, and six of them were identified as homologous series of sec. alcohols, prim./sec. diols, esters of prim./sec. diols, esters of sec./sec. diols (hydroxy-alkan-2-ols), esters of sec./sec. ketols (oxo-alkan-2-ols) and γ-lactones. Thus, all the new compound classes were recognized as derivatives of the ubiquitous wax constituents, carrying additional secondary hydroxyl or keto functions. It was therefore hypothesized that all the novel compounds were formed by oxidation of respective wax precursors, likely mediated by P450-dependent monooxygenases (Racovita and Jetter, 2016).

While oxygenated derivatives of many ubiquitous wax compounds were thus identified, no homologs, isomers or derivatives of the predominant mid-chain β-diketones were found. However, several compounds in the wheat wax mixtures and their TLC fractions could not be identified to date, leading us to speculate that some of them might indeed represent the β-
diketone derivatives missing so far. Therefore, the goal of the present work was to search the flag leaf and peduncle waxes of the wheat cv. Bethlehem for novel β-diketone derivatives. To this end, the wax mixtures were separated by preparative TLC to obtain large enough quantities for detailed GC-MS analysis, and six TLC fractions were found to contain unknown compounds. They were transformed into various derivatives to distinguish functional groups in the novel structures and assign isomer compositions.

6.2. Experimental

6.2.1. Plant material

_Triticum aestivum_ cv. Bethlehem plants were grown in greenhouses at the Weizmann Institute of Science (Rehovot, Israel). The type of soil used was a mix of 50% peat and 50% turf and it was watered every 3-4 days, using approx. 400 mL water per 5 L pot. Growth conditions included alternating light / dark cycles of 12-14 h / 10-12 h, with temperatures of 24-26°C / 17-18°C and a photon flux during light cycles of 180 μmol m² s⁻¹. For every preparative thin layer chromatography experiment, ten flag leaf blades with an area of 40-50 cm² each (measured using ImageJ) and ten peduncles with a diameter of 2 mm and a length of 15-25 cm were harvested in August 2014 from mature wheat plants using clean razor blades.

6.2.2. Wax extracts

Leaves and peduncles were submerged into 10 mL chloroform (Aldrich, ≥99%, with 0.75% ethanol as stabilizer) at ambient temperature for 30 s, with agitation. The chloroform with
extracted waxes was transferred to another vial and the plant material was extracted a second time with another 10 mL chloroform for another 30 s. The two chloroform extracts were combined and dried under a stream of N₂ (Praxair, ≥99.998%) at 50°C. The remaining waxes were stored until fractionation by TLC.

6.2.3. Preparative thin layer chromatography

Fractionation of cuticular wax classes by preparative TLC was done using the sandwich technique (Tantisewie et al., 1969). A mixture CHCl₃:EtOH 98:2 (v/v) was employed as mobile phase, while the stationary phase was represented by SiO₂-coated glass plates (Uniplate Analtech, silica gel 60 F₂₅₄ layer thickness: 1 mm, size: 20x20 cm, with 4 cm concentrating zone). Following separation, TLC bands were visualized under 365 nm UV light after spraying the plates with a solution of 5 mg primuline (Aldrich, 50% dye content) in 100 mL (CH₃)₂CO:H₂O 80:20 (v/v). All bands were scraped off the plates with spatulas and collected into 20 mL scintillation vials, where they were extracted twice with 10 mL portions of CHCl₃ at ambient temperature, for 30 s each. After filtration through glass wool (Supelco), the combined extracts were partially evaporated under N₂ at 50°C, transferred to 2 mL GC autosampler vials, evaporated again to dryness, and stored until GC-MS analysis.

6.2.4. Derivatization reactions

Derivatization reactions in preparation for GC-MS analysis were carried out using various derivatization reagents: N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Aldrich, GC
grade) for silylation; acetic anhydride (Aldrich, ≥98%) for acetylation; lithium aluminum hydride (Aldrich, ≥95%) for reduction; and O-methylhydroxylamine hydrochloride (Aldrich, ≥98%) for conversion of carbonyls into methoximes. Specific derivatization protocols are described below.

All samples were subjected to silylation before injection into the GC-MS, by refluxing in a mixture of 10 μL BSTFA and 10 μL pyridine at 70°C, for 20 min. Excess reagents were then evaporated under N₂ and the silylated waxes re-dissolved with 50 μL CHCl₃.

Acetylation of hydroxyl-containing specimens was performed by refluxing the dry wax in a mixture of 10 μL acetic anhydride and 10 μL pyridine at 70°C, for 5 min, followed by overnight stirring at ambient temperature. Following evaporation of excess reagents under N₂, silylation was carried out as described above.

Wax samples for reduction were dissolved in 50 μL (CH₃CH₂)₂O to which 0.1 mg LiAlH₄ were added. The mixture was left to react overnight at 70°C, with the vial cap closed. Then it was quenched with 10% H₂SO₄ and extracted three times with 60 μL (CH₃CH₂)₂O each. The combined extracts were evaporated to dryness and silylated as described above.

Conversion of carbonyl-containing specimens into the corresponding methoximes was accomplished by heating them with 20 μL of a saturated solution of O-methylhydroxylamine hydrochloride in pyridine:CHCl₃ 7:3 (v/v) at 70°C, for 30 min. After partitioning between 50 μL distilled H₂O and 50 μL CHCl₃, the organic fraction was collected and the aqueous phase
extracted once more with 50 μL fresh CHCl₃. Finally, the combined chloroform extracts were evaporated to dryness and silylated as described above.

6.2.5. Gas chromatography-mass spectrometry

The GC instrument used for identification and relative quantification of wax constituents was equipped with capillary GC column (6890N, Agilent, Avondale PA, USA; length: 30 m; type: HP-1 100% PDMS; i.d.: 0.32 mm; df: 0.1 μm), on-column injector at 50°C, and MS detector (5973N, Agilent, EI-70 eV, ionization source temp.: 240°C, m/z 50-750). It employed helium (Praxair, ≥99%) as carrier gas at a flow rate of 1.4 mL min⁻¹ and was programmed to follow the following temperature program: 2 min at 50°C, ramp 40°C min⁻¹ to 200°C, constant for 2 min, ramp 3°C min⁻¹ to 320°C, constant for 30 min.

6.3. Results

This study aimed at a detailed analysis of the compounds associated with β-diketones in wax mixtures on wheat flag leaves and peduncles. To enable structure elucidation, the wax mixtures were extracted from the two organs and separated by TLC (silica, mobile phase CHCl₃:EtOH 98:2), and diverse unknown constituents were located in specific fractions. Their chemical structures were studied by inspecting MS fragmentation patterns of a variety of derivatives, in each case chosen to provide multiple lines of evidence for the presence and relative position of functional groups along the hydrocarbon backbone.
Based on their TLC behaviour and common MS fragmentation patterns, the unknown wheat wax constituents could be grouped into six different compound classes (Fig. 6.1). All six fractions, designated as A-F, were detected equally in flag leaf and peduncle waxes. Preliminary evidence suggested that compounds A-C bore structural similarities, however they were found to have widely differing polarities since class A co-eluted with wax γ-lactones and keto-alkan-2-ol esters (Rf 0.44), B with β-diketones (Rf 0.86), and C with prim./sec. alkanediol esters (Rf 0.54). Similarly, classes D and E were found structurally related to each other, again despite differing polarities, as D also co-eluted with β-diketones (Rf 0.86) and E with hydroxy-β-diketones (Rf 0.33). Finally, class F was isolated as a fraction of its own, with polarity between those of β-diketones and alkyl esters (Rf 0.91).

![Chemical structures](image)

Figure 6.1. Polyketides and polyketide-derived compounds identified in the wax mixtures of T. aestivum cv. Bethlehem.
Initially, the β-diketones 6.1 contained in the same fraction as classes B and D were investigated for reference. As previously described (Racovita et al., 2016), the β-diketone fraction was largely dominated by one compound, hentriacontane-14,16-dione 6.1b (ca. 97% of the β-diketone class). It was identified by the characteristic MS fragmentation pattern of its trimethylsilyl (TMS) enol ether derivative, exhibiting α-bond cleavage products together with a series of M, M-15 and M-90 ions (Fig. 6.2A). Two more aliquots of the same TLC fraction were subjected to derivatization with O-methylhydroxylamine and lithium aluminum hydride (LiAlH₄), and the resulting bis-methoxime and reduction products showed characteristic α- and McLafferty fragments confirming the presence of two keto groups in β-constellation (Fig. 6.2B/C). The same wax fraction contained further mid-chain β-diketones that had not been described in wheat wax before, and close inspection of the TMS derivative and LiAlH₄ reduction mixtures identified the C₂₉ structure nonacosane-14,16-dione 6.1a (<0.5% of the compound class) and two isomers of the C₃₃ homolog, tritriacontane-14,16-dione 6.1c and tritriacontane-16,18-dione (ca. 1% and 2% of the compound class, respectively).
Figure 6.2. Identification of mid-chain β-diketone in wheat leaf and peduncle wax. (A) Mass spectrum and major fragmentations of the TMS derivatives obtained from the two enol tautomers of hentriacontane-14,16-dione 6.1b. (B) Mass spectrum and major fragmentations of the O-methylhydroxylamine derivative of hentriacontane-14,16-dione 6.1b. (C) Mass spectrum and major fragmentations of the TMS derivative of the diol resulting from LiAlH₄ reduction of hentriacontane-14,16-dione 6.1b.
6.3.1. Structure elucidation of compound classes A – C

Compound class A was detected as a single GC peak in a wheat wax fraction of intermediate polarity (Rf 0.44). Based on MS similarity with published data for C29 β-ketols in Brassicaceae waxes (Holloway and Brown, 1977; Wen and Jetter, 2009), A was hypothesized to have mid-chain β-ketol structure 6.2. In particular, the TMS derivative of A had a fragment m/z 73 diagnostic for a hydroxyl group and a fragment m/z 130 indicative of a β-ketol structure (Wen and Jetter, 2009), while lacking a fragment m/z 147 indicative of a second hydroxyl function (Fig. 6.3A). Two further fragments, M-15 (due to loss of methyl radical) and M-90 (due to loss of (CH₃)₃SiOH), further confirmed the ketol structure and indicated an overall chain length of C₃₁. Finally, the α-fragments of the OTMS group (m/z 327 and m/z 313) and of the carbonyl group (m/z 211 and 355) identified one ketol isomer 6.2a with keto and hydroxyl functionalities on C-14 and C-16, while pairs of further ions (m/z 285 and 355; m/z 239 and 327) revealed the presence of a second isomer 6.2b with reversed configuration of keto and hydroxyl functions at C-16 and C-14, respectively.

For structure confirmation, the fraction containing A was treated with LiAlH₄, a derivatizing reagent that can probe keto groups by reducing them to hydroxyls. In the resulting mixture, the original compound A was replaced with a single new one. The mass spectrum of its TMS derivative showed the characteristic fragments m/z 73 and m/z 147 for diols (Jetter et al., 1996), together with M-15 and M-90 ions indicative of chain length (Fig. 6.3B). Two pairs of α-fragments (m/z 285 and m/z 313; m/z 429 and m/z 401) together with one pair of product ions
formed by loss of (CH$_3$)$_3$SiOH ($m/z$ 339 and $m/z$ 311) corroborated the presence of two OTMS groups, and the LiAlH$_4$ reduction product was thus identified as hentriacontane-14,16-diol. Taking this result together with the TLC behaviour and GC-MS data of the TMS derivative, we conclude that A is a mixture of two co-eluting C$_{31}$ mid-chain β-ketol regiomers, 16-hydroxyhentriacontan-14-one 6.2a and 14-hydroxyhentriacontan-16-one 6.2b. Thus, the compounds in A are β-ketols that are structurally related to the major β-diketone present in wheat wax, hentriacontane-14,16-dione 6.1b, with identical chain length of C$_{31}$ and position of functional groups on C-14 and C-16, but with either one of the keto groups replaced by a hydroxyl.
Figure 6.3. *Structure elucidation of mid-chain β-ketols in wheat leaf and peduncle wax.* (A) Mass spectrum and major fragmentations of co-eluting TMS derivatives of 16-hydroxyhentriacontan-14-one 6.2a and 14-hydroxyhentriacontan-16-one 6.2b. (B) Mass spectrum and major fragmentations of TMS derivative of the diol obtained via LiAlH₄ reduction of 16-hydroxyhentriacontan-14-one 6.2a and 14-hydroxyhentriacontan-16-one 6.2b.
Class B comprised a single GC peak in the TLC fraction also containing the β-diketones. Treatment of this fraction with BSTFA left B unchanged, its mass spectrum lacking features \((m/z \ 73, 75)\) characteristic of hydroxyls (Fig. 6.4A) and instead suggesting the presence of two alkyl termini without functional groups (prominent \(m/z \ 57, 71, 85\), etc.). A pair of \(α\)-fragments \((m/z \ 211 \text{ and } m/z \ 239)\) in conjunction with a molecular ion \(m/z \ 450\) further suggested a \(C_{30}\) chain bearing an \(α\)-diketo functionality on C-14 and C-15. Alternative structures, such as \(C_{29}\) or \(C_{31}\) mono-ketone isomers, seemed unlikely due to the lack of other fragmentations around the carbonyl group, such as McLafferty rearrangement with double hydrogen transfer characteristic of VLC ketones leading to fragments 16 amu higher than corresponding \(α\)-fragments (Vajdi et al., 1981).

To confirm the presence of two carbonyl groups in B, two more derivatives were generated and characterized by MS. First, condensation with \(O\)-methylhydroxylamine yielded a new compound with prominent fragment \(M-31\), due to loss of a methoxy unit \((m/z \ 477)\), and \(α\)-fragments 29 Da heavier than those of the native compound \((m/z \ 240 \text{ and } m/z \ 268)\) (Fig. 6.4B), together indicating the presence of at least one carbonyl function. Finally, reduction of B with excess LiAlH\(_4\) followed by silylation resulted in a single compound with characteristic fragments \(m/z \ 73\) and \(m/z \ 147\), prominent \(α\)-fragments \(m/z \ 285\) and 313, and an ion \(M-15\) at \(m/z \ 583\) (Fig. 6.4C), and thus identified as triacontane-14,15-diol. This finding, together with the combination of spectral features of the other derivatives, unambiguously identified compound B as the mid-chain \(α\)-diketone, triacontane-14,15-dione 6.3a. The two diols resulting from reduction of the \(α\)-diketone triacontane-14,15-dione (Fig. 6.4C) and of the \(β\)-diketone hentriacontane-14,16-dione (see Fig. 6.2C) share many prominent features, however the two compounds are distinguished by
different GC retention times, and by molecular ions as well as fragments m/z 522, 311 and 339 characteristic of the \(\beta\)-diketone-derived diol.

![Mass spectrum and major fragmentations of triacontan-14,15-dione 6.3a.](image)

![Mass spectrum and major fragmentations of O-methylhydroxylamine derivative of triacontan-14,15-dione 6.3a.](image)

![Mass spectrum and major fragmentations of TMS derivative of the diol obtained via LiAlH\(_4\) reduction of triacontan-14,15-dione 6.3a.](image)

Figure 6.4. Structure elucidation of mid-chain \(\alpha\)-diketone in wheat leaf and peduncle wax. (A) Mass spectrum and major fragmentations of triacontan-14,15-dione 6.3a. (B) Mass spectrum and major fragmentations of O-methylhydroxylamine derivative of triacontan-14,15-dione 6.3a. (C) Mass spectrum and major fragmentations of TMS derivative of the diol obtained via LiAlH\(_4\) reduction of triacontan-14,15-dione 6.3a.
Compound class C was found in a single GC peak, detected in a fraction (Rf 0.54) slightly less polar than that containing the β-ketols (class A). Again, MS similarity with previously reported Brassica and Arabidopsis wax ketols (Holloway and Brown, 1977; Wen and Jetter, 2009) suggested that C was a mixture of isomeric α-ketols (acyloins) 6.4a-b. The mass spectrum of TMS-derivatized C showed an ion m/z 73 not accompanied by m/z 147, suggesting a single hydroxyl group, and α-fragments m/z 285 and 313 suggesting OH-group location 14 and 16 carbons in from one alkyl chain end, respectively (Fig. 6.5A). While the spectrum thus far closely resembled that of a (TMS-derivatized) secondary alcohol, nonacosan-14-ol, further MS features clearly distinguished the two compounds. In particular, the TMS derivative of C exhibited a fragment M-15 characteristic of a C₃₁ ketol structure, and two α-fragments at m/z 211 and 239, interpreted as C₁₄ and C₁₆ acylium ions, showing the presence of a carbonyl group. C was thus recognized as an α-ketol mixture 6.4a-b rather than a secondary alcohol.

Further confirmation of the α-ketol structure was provided by the MS characterization of three more derivatives of C. One of them, generated by acetylation with acetic anhydride, showed very prominent acylium fragments m/z 211 and 239, together with a series of ions M, M-43 (loss of acetyl) and M-60 (loss of acetic acid) (Fig. 6.5B), thus confirming the presence of two isomeric α-ketol structures 6.4a-b. A third aliquot of the TLC fraction was reduced with LiAlH₄, resulting in the same α-diol as from B (data not shown), and therefore further corroborating the C₃₀ structure bearing two functional groups on C-14 and C-15. Finally, to also directly probe the presence of a carbonyl group, another aliquot of the fraction was derivatized with O-methylhydroxylamine and then silylated. While the resulting oxime exhibited the same hydroxyl α-fragments m/z 285 and 313 as the simple silyl derivative, the acylium ions were replaced by
fragments 29 Da heavier (m/z 240 and 268) (Fig. 6.5C), confirming the presence of one carbonyl function. This interpretation was underpinned by ions M-15 (loss of methyl), M-31 (loss of methoxy) and M-90 (loss of (CH₃)₃SiOH), and the positions of the carbonyl and hydroxyl groups on either C-14 or C-15 were again confirmed by several other α-fragments in the spectrum of the silylated oxime along with some of their product ions. Overall, the TLC behaviour of the native compound and our GC-MS results for various derivatives identified C as a mixture of two C₃₀ mid-chain α-ketols, 15-hydroxytriacontan-14-one 6.4a and 14-hydroxytriacontan-15-one 6.4b. Interestingly, these structures are closely related to those of compound classes A and B, the former identified as mid-chain β-ketols 6.2a-b differing from the α-ketols 6.4a-b by the presence of one methylene unit between functional groups, and the latter identified as a mid-chain α-diketone 6.3a differing from the α-ketols 6.4a-b only in functional group oxidation state.
6.3.2. Structure elucidation of compound classes D and E

Class D was represented by a single compound in the same TLC fraction as β-diketones and α-diketones (B), and was therefore suspected to have diketone structure as well. D was not affected by treatment with BSTFA, and its mass spectrum accordingly lacked all fragments characteristic of OH groups (m/z 73, 75) (Fig. 6.6A). Instead, it showed characteristic α-fragments m/z 85 and 435, together with a base peak m/z 100 due to a McLafferty rearrangement (without double H transfer) indicating the presence of at least one carbonyl group. The two ions M and M-18 (due to loss of water) indicated a molecular weight of 492 Da, pointing to either a C$_{33}$ diketone or C$_{34}$ monoketone structure.

To further investigate the number and relative positions of carbonyls in D, the fraction was derivatized with O-methylhydroxylamine. The product had an M ion 58 amu higher than the original compound, and product ions due to loss of a methoxy radical (M-31), dimethylether (M-46), or a methoxy radical and methanol (M-31-32), together indicating the presence of two carbonyl groups (Fig. 6.6B). The diketo structure was confirmed by a McLafferty-fragment (m/z 158) accompanied by a product ion resulting from loss of methoxy (m/z 127), thus firmly establishing that D was a C$_{33}$ diketone. This finding, taken together with the size of various α-fragments and McLafferty rearrangement products, identified D as a subterminal β-diketone, tritriacontane-2,4-dione 6.5a. Finally, this structure was confirmed by reduction with LiAlH$_4$, leading to a new compound identified as (TMS-derivatized) tritriacontane-2,4-diol (see below also for E).
Figure 6.6. *Structure elucidation of 2,4-diketone in wheat leaf and peduncle wax.* (A) Mass spectrum and major fragmentations of tritriacontan-2,4-dione 6.5a. (B) Mass spectrum and major fragmentations of O-methylhydroxylamine derivative of tritriacontan-2,4-dione 6.5a.
Compound class E consisted of seven compounds recognized as a homologous series based on their equidistant GC separation and common MS fragmentation patterns. All compounds E formed TMS derivatives with similar MS characteristics, exhibiting a fragment \( m/z \ 73 \ [(CH_3)_3Si]^+ \) but no \( m/z \ 147 \ [(CH_3)_2SiOSi(CH_3)_3]^+ \) (Fig. 6.7A), thus indicating the presence of only one hydroxyl group in the native compounds. All homologs also had a noticeable fragment \( m/z \ 130 \) diagnostic for β-ketols (Wen and Jetter, 2009), and an \( \alpha \)-fragment with TMS transfer \( (m/z \ 115) \) suggesting a 2-keto function. An \( \alpha \)-fragment base peak \( m/z \ 159 \), common to all homologs, accompanied by product ions due to loss of \( CH_4 (m/z \ 143) \) and \( CH_2CO (m/z \ 117) \), indicated the presence of a 4-hydroxy-2-keto structure. Conversely, longer \( \alpha \)-fragments were found to vary with homolog chain length \( (m/z \ 509 \) for the homolog in Fig. 6.7A), in parallel with respective M-15 ions indicative of molecular weight and thus total chain length. Given all the evidence summarized thus far, class E was tentatively identified as a homologous series of C\(_{25}\) to C\(_{37}\) 4-hydroxy-2-ketones 6.6 (i.e., subterminal β-ketols). A summary of all identified homologs and their diagnostic MS fragments is presented in Table 6.1.

To further test the structure assignment, compounds E were transformed into acetates. The resulting derivatives showed pairs of \( \alpha \)-fragments of relatively low intensity together with product ions resulting from loss of acetic acid, one set of them homolog-independent \( (m/z \ 129 \) and \( m/z \ 69) \) and the other one varying with homolog chain length \( (m/z \ 419 \) and \( m/z \ 461 \) for the C\(_{33}\) homolog 6.6e in Fig. 6.7B). Other fragments indicative of chain length were due to loss of water, an acetyl radical, and/or acetic acid from the molecular ion (M-18, M-43, M-60, and M-
60-18, respectively). The acetate spectra of compounds E thus confirmed the subterminal β-ketol structures (2,4-ketols) 6.6.

To specifically test the existence of a carbonyl functionality, a third aliquot of the fraction was derivatized with O-methylhydroxylamine and then silylated. The mass spectrum of the product (Fig. 6.7C) showed a homolog-independent α-fragment \( m/z \) 188 and its product ions due to loss of CH₄ and CH₂O \( m/z \) 172 and 158, respectively), with the first two ions shifted 29 amu higher than for the same compound without oximation, and thus confirming the presence of one carbonyl group. Other diagnostic fragments were due to loss of CH₃, CH₃O, and/or (CH₃)₃SiOH from the molecular ion (M-15, M-31, M-90 and M-31-90, respectively), and the base peak corresponded to the second α-fragment of the OTMS functionality \( m/z \) 509 for the C₃₃ homolog 6.6e in Fig. 6.7C).

Lastly, reduction of compounds E with LiAlH₄ followed by silylation resulted in TMS-derivatized 2,4-diols, in the case of the C₃₃ homolog 6.6e identical to that produced upon reduction of compound D 6.5a. Their structure was confirmed by signature MS fragments \( m/z \) 73 [(CH₃)₃Si]+ indicative of at least one hydroxyl, an ion \( m/z \) 147 [(CH₃)₂SiOSi(CH₃)₃]+ suggesting a diol, and pairs of α-fragments indicating hydroxyl groups on C-2 \( m/z \) 117 and, for the C₃₃ homolog 6.6e, \( m/z \) 625) and on C-4 \( m/z \) 233 and, for the C₃₃ homolog 6.6e, \( m/z \) 509) (Fig. 6.7D). Fragments due to loss of CH₃ and/or (CH₃)₃SiOH from the molecular ion (M-15, M-90 and M-15-90) further indicated the molecular weight and therefore chain length of the diol homologs. Taken together, the TLC behaviour of the native compounds and the GC-MS data for TMS derivatives, acetate esters, methoxime/TMS derivatives, and (TMS-derivatized) LiAlH₄
reduction products unambiguously established that E was a homologous series of odd-numbered 4-hydroxy-2-ketones 6.6, spanning chain lengths from C_{25} to C_{37}. Relative GC-MS peak areas showed that this series of subterminal β–ketols was dominated by the C_{33} homolog 6.6e.

Figure 6.7. *Structure elucidation of 2,4-ketols in wheat leaf and peduncle wax.* (A) Mass spectrum and major fragmentations of TMS derivative of C_{33} 2,4-ketol 6.6e. (B) Mass spectrum and major fragmentations of Ac derivative of C_{33} 2,4-ketol 6.6e. (C) Mass spectrum and major fragmentations of O-methylhydroxylamine/TMS derivative of C_{33} 2,4-ketol 6.6e. (D) Mass spectrum and major fragmentations of TMS derivative of the diol obtained via LiAlH_{4} reduction of C_{33} 2,4-ketol 6.6e.
Table 6.1. *Characteristic m/z fragments of trimethylsilyl ethers of 2,4-ketols in flag leaf and peduncle waxes of* *Triticum aestivum cv. Bethlehem*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fragments characteristic of homolog</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Hydroxypentacosan-2-one</td>
<td>397</td>
</tr>
<tr>
<td>4-Hydroxyheptacosan-2-one</td>
<td>425</td>
</tr>
<tr>
<td>4-Hydroxynonacosan-2-one</td>
<td>453</td>
</tr>
<tr>
<td>4-Hydroxyhentriacontan-2-one</td>
<td>481</td>
</tr>
<tr>
<td>4-Hydroxytritriacontan-2-one</td>
<td>509</td>
</tr>
<tr>
<td>4-Hydroxypentatriacontan-2-one</td>
<td>537</td>
</tr>
<tr>
<td>4-Hydroxyheptatriacontan-2-one</td>
<td>565</td>
</tr>
</tbody>
</table>

6.3.3. Structure elucidation of compound class F

Compound class F was detected as a single peak in the GC trace from a TLC fraction running between $\beta$-diketones and alkyl esters, and thus of only moderate polarity. The mass spectrum of F did not show any of the fragments characteristic of OH groups after treatment with BSTFA (such as $m/z$ 73, 75), but instead exhibited $\alpha$-fragments characteristic of ketones (Fig. 6.8A). Specifically, the set of $\alpha$-fragments $m/z$ 211 and $m/z$ 267 indicated one ketone isomer, while the corresponding ion $m/z$ 239 suggested a co-eluting isomer with keto group position shifted by two carbons. This interpretation was corroborated by McLafferty+1 fragments 16 amu higher than the $\alpha$-fragments ($m/z$ 227 and 283; $m/z$ 255). The molecular ion ($m/z$ 450) indicated a total ketone chain length of $C_{31}$. 
To further probe the presence and location of the carbonyl functionality, methoxime derivatives were prepared from F. The resulting compounds showed diagnostic methoxime fragments ($m/z$ 87 and $m/z$ 100) (Jetter and Riederer, 1999a), $\alpha$-fragments 29 amu higher than the original ketone isomers ($m/z$ 240 and $m/z$ 296; $m/z$ 268), and corresponding McLafferty rearrangement fragments as well as product ions resulting from loss of methanol (Fig. 6.8B). The oxime spectrum thus confirmed the presence of two isomeric ketones. The chain length, C$_{31}$, was corroborated by the molecular ion and its product fragment M-31 (due to loss of a methoxy unit).

Finally, the ketone mixture was reduced with excess LiAlH$_4$ and the resulting isomeric secondary alcohols subjected to silylation. Their mass spectra showed the alcohol-characteristic fragment $m/z$ 73, as well as $\alpha$-fragments indicating the location of the hydroxyl function on C-14 ($m/z$ 285 and $m/z$ 341) or on C-16 ($m/z$ 313) (Fig. 6.8C). Fragments due to loss of CH$_3$ or (CH$_3$)$_3$SiOH from the molecular ion (M-15 and M-90), confirmed the C$_{31}$ chain length. All taken together, our TLC and MS data thus unambiguously identified compound F as a mixture of mid-chain ketones, hentriacontan-14-one 6.7a and hentriacontan-16-one 6.7b.
Figure 6.8. Structure elucidation of ketones in wheat leaf and peduncle wax. (A) Mass spectrum and major fragmentations of co-eluting ketone isomers hentriacontan-14-one 6.7a and hentriacontan-16-one 6.7b. (B) Mass spectrum and major fragmentations of co-eluting O-methylhydroxylamine derivatives of hentriacontan-14-one 6.7a and hentriacontan-16-one 6.7b. (C) Mass spectrum and major fragmentations of co-eluting TMS derivatives of the LiAlH₄ reduction products of hentriacontan-14-one 6.7a and hentriacontan-16-one 6.7b.
6.4. Discussion

In this work we discovered several new classes of compounds in the cuticular waxes covering wheat flag leaf blades and peduncles, together with new homologs and isomers of the predominant class of β-diketones. Among the newly identified wheat wax constituents, only the ketones were found to have a single in-chain functionality, whereas all other new wheat wax structures featured two functional groups varying 1) in their relative configuration, 2) in their positions within the hydrocarbon chain, and 3) in their oxidation states.

Firstly, regarding the relative configuration of functionalities, the mid-chain β-ketols (A), subterminal β-diketone (2,4-diketone) (D) and subterminal β-ketols (2,4-ketols) (E) featured two functional groups on carbons separated by one methylene group and thus shared the relative group constellation with the previously identified β-diketones. In contrast, the α-diketones (B) and α-ketols (C) had two functional groups on directly adjacent carbons. Secondly, the mid-chain α-ketols (C), α-diketones (B), β-ketols (A) and ketones (F) shared very similar functional group positions within the hydrocarbon chains with the mid-chain β-diketones, having predominantly C_{13}H_{27} and C_{15}H_{31} alkyl chains on either side of the functionalities. Again, this characteristic contrasted with some other wheat wax constituents, where the subterminal 2,4-diketone (D) and 2,4-ketols (E) had functional groups near one chain terminus, with one alkyl residue constant as CH_{3} and the other one widely varying around C_{29}H_{59} (from C_{21}H_{43} to C_{33}H_{67}). Thirdly, all the bifunctional compounds could be divided into two distinct classes also according to functional group oxidation states, either with two carbonyls (B and D, hence designated as diketones) or with one hydroxyl and one carbonyl function (A, C and E, hence designated as ketols). In summary, each of the novel wheat wax compound classes shared some
but not all structural features with several others, suggesting that all the compound classes are biosynthetically related. Based on their structural commonalities and differences, potential biosynthetic pathways leading to them can now be outlined.

Several of the new wheat wax compound classes had polyketide-like structures, suggesting that they are biosynthesized through condensation reactions with malonyl units catalyzed by polyketide synthase (PKS) enzymes. In particular, the subterminal \(\beta-(2,4)\)-diketone (D) may be derived from \(C_{30}\) fatty acyl-CoA, occurring as an intermediate of elongation and modification towards normal wheat wax compounds such as \(C_{30}\) alcohol and \(C_{29}\) alkane (Fig. 6.9). It seems plausible that this acyl-CoA serves as substrate for a PKS catalyzing two consecutive condensation reactions with malonyl-CoA extenders, and that the resulting \(C_{34}\) triketide (3,5-diketoacyl-CoA) may be hydrolyzed and decarboxylated to the 2,4-diketone product. It should be noted that the latter two reactions, involving thioester hydrolysis and loss of CO\(_2\) from the carboxylate, may both occur spontaneously but could also be enzyme-catalyzed, similar to the formation of methylketones in tomato trichomes (Yu et al., 2010b). It should further be noted that the initial reaction towards the 2,4-diketone is identical with a condensation occurring as part of VLCFA elongation en route to other wax constituents, where a KCS enzyme in a FAE complex is thought to utilize the same \(C_{30}\) fatty acyl-CoA and malonyl-CoA substrates. It can therefore not be excluded that, instead of a PKS catalyzing two condensation rounds, the first of them may be carried out by a KCS and only the second one by a PKS. In this scenario, the ketoacyl-CoA intermediate of elongation would have to be intercepted (either directly or by a thioesterase releasing a free acid that is re-activated to a thioester) and transferred to the PKS for further condensation with a malonyl unit.
Based on the structural similarity and matching major homolog chain length, the
subterminal \( \beta-(2,4) \)-ketols also identified in wheat waxes (E) are very likely biosynthetically
related to the 2,4-diketone (Fig. 6.9). It seems plausible that either the diketone itself or an
intermediate along the pathway leading to it can serve as precursor for ketol formation, implying
that a reductase enzyme may specifically transform the 4-keto group into the corresponding
hydroxyl. Alternatively, also the 3-hydroxyacyl-CoA intermediate of the FAE complex may be
intercepted and used as starter for condensation with malonate, possibly catalyzed by the same
PKS as in (then parallel) 2,4-diketone formation. Based on the chain length profile of the wheat
2,4-ketols and 2,4-diketones, both peaking at \( C_{33} \), we conclude that this PKS may be relatively
specific for \( C_{30} \) acyl-CoA or \( C_{32} \) ketoacyl-CoA substrate. It is thus distinguished from other PKS
enzymes thought to participate in wheat wax formation, such as the PKS producing mid-chain \( \beta \)-
diketones from \( C_{14} \) and/or \( C_{16} \) acyl substrates (see below) or the one thought to be involved in
alkylresorcinol formation from \( C_{24} \) acyl-CoA and similar precursors (Racovita et al., 2016).

Interestingly, a homologous series of 2,4-diketones with chain lengths ranging from \( C_{25} \) to
\( C_{31} \) had been identified in the suberin of Ericaceae roots (van Smeerdijk and Boon, 1987), thus
spanning a fairly wide range of homologs but not including the \( C_{33} \) diketone identified in wheat
wax. In contrast, 2,4-ketols like those described here had not been reported before. However,
closely related isomers had been identified as 4,6-ketols (4-hydroxyalkan-6-ones and 6-
hydroxyalkan-4-ones) in lipid mixtures from sunflower pollen, albeit with shorter chain lengths
ranging from \( C_{19} \) to \( C_{27} \) (S Schulz et al., 2000).
Figure 6.9. Proposed biosynthetic pathway to subterminal β-diketone (2,4-diketone) and subterminal β-ketols (2,4-ketols). Normal wax biosynthesis proceeds via elongation of C28 to C30 acyl-CoA, and in repeat reaction cycles to C32 acyl-CoA and beyond, catalyzed by four enzymes (a: KCS; b: KCR; c: HCD; d: ECR) in the FAE complex (left). The β-keto intermediates may be intercepted and used as substrate(s) for a condensation reaction (center) catalyzed by a PKS enzyme (e). The resulting triketide may undergo (enzymatic or spontaneous) hydrolysis (f) and decarboxylation (g) to a 2,4-diketone three carbons longer than the original acyl substrate (right). Alternatively, the PKS may also catalyze the formation of the β-ketoacyl-CoA intermediate. Although such reaction sequences leading to C31 or C35 2,4-diketones are feasible, only the C33 2,4-diketone (shaded box) was identified in wheat wax. Corresponding reaction sequences starting with β-hydroxyacyl-CoA intermediates may lead to 2,4-ketols of various chain lengths (shaded boxes). Alternatively, either the 2,4-diketone(s) or their precursors may be enzymatically reduced (h) to the corresponding β-hydroxy intermediates.
The wheat mid-chain β-ketols strongly resembled the β-diketone dominating the wax mixtures, hentriacontane-14,16-dione, both in their chain length and functional group positions. Therefore, it may be surmised that the mid-chain β-ketols are either intermediates on the β-diketone biosynthesis pathway or side products of it. It has long been recognized that β-diketones are polyketide in nature, and it was hypothesized that their formation involves two PKS-catalyzed condensation steps (von Wettstein-Knowles, 2012). However, very recently Hen Avivi et al. (2016) showed that the synthesis of wheat mid-chain β-diketone instead proceeds via hydrolytic intercept of a thioester intermediate of fatty acid synthesis, likely C\textsubscript{16} β-ketoacyl-ACP, and only one PKS-catalyzed condensation reaction. By analogy, it may now be speculated that mid-chain β-ketols are formed by intercept of C\textsubscript{16} β-hydroxyacyl-ACP, another intermediate of fatty acid elongation, followed by a PKS-mediated condensation reaction (Fig. 6.10). Either one or both of the reactions involved, the thioester hydrolysis and the PKS reaction, may be catalyzed by the same enzyme(s) as the corresponding reactions in β-diketone synthesis. Alternatively, the wheat mid-chain β-ketols might also be formed by reduction of either the β-diketone or one of the intermediates along the pathway leading to it.

Mid-chain β-ketols with structures very similar to those observed in wheat wax had previously been reported. Namely, C\textsubscript{29} β-ketols were reported together with α-ketols, secondary alcohols and ketones in Arabidopsis thaliana stem wax (Wen and Jetter, 2009) and in the leaf wax from four Brassica species (Holloway and Brown, 1977). It was then also established that the Arabidopsis β-ketols and the accompanying compounds with secondary functionalities are all products of a cytochrome P450-dependent enzyme, mid-chain alkane hydroxylase (MAH1), which hydroxylates alkane substrates on C-13, C-14 and C-15. The somewhat variable group
positions in the Brassicaceae ketols hence contrast with the wheat mid-chain β-ketols, where functional groups were exclusively located on C-14 and C-16. Thus, the differences in isomer profiles reflect the very different biosynthetic origins of ketols in both cases, in Arabidopsis involving cytochrome P450 oxidation of alkanes and in wheat intercept of fatty acid synthesis intermediates and PKS reactions.

The formation of wheat wax mid-chain ketones may also be assessed in light of the biosynthetic pathways likely leading to the similar diketone and ketol compounds accompanying them. Due to common features in the overall molecular structures, most prominently the predominance of C_{13}H_{27} and C_{15}H_{31} alkyl chains in various homologs and isomers, it seems likely that the wheat ketones are formed as side products of the major wax polyketides (Fig. 6.10). The ketones can thus be viewed as derivatives of the mid-chain diketones, with one carbonyl group instead of two, suggesting that one of the reactions generating the diketo functionalities may be skipped. Accordingly, the β-ketoacyl intermediate of β-diketone formation might, instead of the PKS-catalyzed condensation leading to the mid-chain β-diketone, be elongated by FAE systems to give ketone products. Alternatively, the β-keto thioester hydrolysis step of β-diketone formation could be omitted, and a PKS-mediated reaction utilizing a fatty acyl instead of a β-ketoacyl would lead to the ketones. Again, the potential biosynthesis pathways described thus as variants of β-diketone formation differ from those leading to Brassicaceae wax ketones via MAH1-mediated hydroxylation (Greer et al., 2007).

Interestingly, the Brassicaceae ketones have their carbonyl group on odd- and even-numbered carbons, for example nonacosan-15-one in Arabidopsis wax, while the wheat ketones
were characterized by a functionality exclusively on even-numbered carbons. The parity of the keto group position can hence serve as a distinguishing feature for the ketone formation pathways, involving either hydroxylation of alkanes mediated by cytochrome P450 enzymes similar to MAH1 or polyketide-forming enzymes. Based on this conclusion, it may be speculated that other ketones with carbonyl groups exclusively on even-numbered carbons may also be formed by polyketide pathways. Such ketones are prominent wax constituents in several species, for example nonacosan-10-one in the waxes of *Osmunda regalis* (Jetter and Riederer, 2000) and several apple cultivars (Dodova-Anghelova and Ivanov, 1973; Dong et al., 2012), and hentriacontan-16-one in waxes of sandal (Chibnall et al., 1937), leek (Rhee et al., 1998), *Annona senegalensis* (MacKie and Misra, 1956) and beech (Gülz et al., 1989).

Finally, mid-chain α-ketols (acyloins) had also been reported before, often together with β-ketols and ketones sharing the same acyl groups, for example in the cuticular waxes of *Arabidopsis thaliana* (Wen and Jetter, 2009), four *Brassica* species (Holloway and Brown, 1977), or the fern *Osmunda regalis* (Jetter and Riederer, 2000). However, in all previous instances predominantly odd-numbered homologs of α-ketols were identified (especially C29), similar to the alkane accompanying them, and thus suggesting a biosynthetic relationship. Accordingly, an Arabidopsis enzyme, MAH1, was found to catalyze the oxidation of alkane precursors, via secondary alcohols and ketones, to α- and β-diols as well as α- and β-ketols (Greer et al., 2007; Wen and Jetter, 2009). In stark contrast, the wheat α-ketols had even carbon numbers (C30), hence making formation from alkane precursors unlikely. Instead, the alkyl moieties involved, C13H27 and C15H31, may suggest a head-to-head condensation of C14 and C16 acyl precursors via an unknown mechanism involving rarely observed C-C bond formation.
between two carbonyl carbons. Interestingly, the wheat wax α-ketols were found as mixtures of isomers with reversed positions of the hydroxyl and carbonyl groups (similar to the *Brassica* ketols). Based on this observation, it may be speculated that the mid-chain α-ketols are formed by fusion of C\textsubscript{14} and C\textsubscript{16} \textit{n}-aldehydes as novel wax biosynthesis precursors. Alternatively, reductive coupling of C\textsubscript{14} and C\textsubscript{16} acyl-CoAs or a combination of acyl-CoA and aldehyde substrates also seem feasible. Finally, a similar reaction, albeit with different redox requirements, may afford the C\textsubscript{30} α-diketone as an intermediate en route to the ketols (Fig. 6.10).
Figure 6.10. Proposed biosynthetic pathways to mid-chain ketones, α- and β-diketones, and α- and β-ketols. Fatty acid de novo synthesis proceeds via elongation of C\textsubscript{14} to C\textsubscript{16} acyl-ACP, and in a further reaction cycle to C\textsubscript{18} acyl-ACP, catalyzed by four enzymes (i: KAS; j: KAR; k: HAD; l: EAR) in the FAS complex (center). It was recently shown (Hen-Avivi et al., 2016) that mid-chain β-diketones are formed by hydrolytic intercept of β-ketoacyl-ACP intermediates of elongation (m) followed by PKS-mediated condensation (n). We propose that corresponding mid-chain β-ketols may be formed either by analogous intercept of β-hydroxyacyl-ACP intermediates of elongation, or else by reduction of the β-diketone or its precursor(s). Similarly, hydrolytic intercept of acyl-ACPs (o) or their reduction to aldehydes (p) may provide the substrate for head-to-head condensation (q) leading to α-diketones and α-ketols, respectively. Finally, either PKS-mediated condensation (r) or elongation of the β-keto intermediate (s) may lead to the ketones identified in wheat waxes.
Chapter 7: Conclusions and future research directions

7.1. Concluding remarks

The present thesis has the main merit of expanding the current knowledge about plant cuticular wax chemical diversity. Numerous new structures have been identified in the three chosen species and elucidated using primarily gas chromatography-mass spectrometry in combination with chemical derivatization, sometimes supplemented by synthesis of authentic standards. In addition, comprehensive wax compositional studies for three new plant species have been performed and are presented in the thesis. For two of the three species, the partitioning of wax compounds between the intracuticular and epicuticular compartments of the cuticle has also been studied, thus supplementing previous studies on wax partitioning using other plant species. Insights into the biosynthesis of novel waxes are also provided and often challenge previously well-established concepts, such as that FAE intermediates are not released by the FAE complex or intercepted by other enzymes, that aldehydes are not released as intermediates en route to primary alcohols, or that nitrogen-containing fatty acid derivatives are not encountered as cuticular waxes.

Summaries of the most important findings from each thesis chapter are presented below:

In chapter 2, the chemical composition of Aloe arborescens leaf cuticular wax was characterized both qualitatively and quantitatively. The total wax load on the abaxial side was
more than double that on the adaxial side, and the epicuticular waxes constituted approximately 50% of the wax load on the abaxial side and 60% on the adaxial side, respectively. The waxes were found to contain VLC compound classes very similar to other species, with distinct gradients between the intracuticular and epicuticular layers pointing to polarity-driven partitioning. The aliphatic wax constituents had very characteristic chain length distributions, highlighting the distinct specificities of the two modifying pathways involved in wax biosynthesis: the acyl reduction pathway and the alkane-forming pathway. Three homologous series of unique 1,3-bifunctional and 2-monofunctional compound classes were identified as very-long-chain 3-hydroxy fatty acids, 3-hydroxy FAMEs and 2-alkanols. The co-occurrence of these compound classes with very similar functional groups, together with characteristic chain length distributions, suggested that they are biosynthetically related. While the pathways and enzymes involved in their formation can only be speculated based on our chemical results, they point to an important new possibility: VLC fatty acyl elongation intermediates bearing a 3-hydroxyl group may be intercepted by other enzymes successfully competing for substrate with the β-hydroxyacyl-CoA dehydratase (HCD) component of the FAE complex. One such enzyme may be a thioesterase forming free acids, and another one the reductase yielding aldehydes.

In the work presented in chapter 3, the adaxial side of young and old P. aurea bamboo leaves was studied and established to be covered with about 3.5 μg/cm² of total (epicuticular and intracuticular) wax, which is a relatively low wax load fairly close to that of A. thaliana leaves (~1 μg/cm²). Epicuticular waxes represented slightly more than 50% of total wax for both plant ages. Along with ubiquitous VLC aliphatic wax classes (fatty acids, alcohols, alkyl esters, aldehydes, alkanes), substantial amounts of terpenoids were detected, which accounted for 30-
40% of the epicuticular waxes and ~50% of the intracuticular waxes, depending on plant age. In general, pronounced gradients were observed between the relative abundances of all compound classes in the epicuticular and intracuticular compartments, commensurate with their relative polarities. Most notably, fatty acid amides were identified here as a novel wax compound class and found to partition exclusively into the epicuticular wax layer, where they might have a role in plant-insect and plant-pathogen interactions, or in preventing organ fusions during ontogenesis, similar to artificial fatty amide slip agents added to polyolefine plastics to prevent sheet adhesion. The discovery of fatty acid amides also raises questions regarding the biosynthetic mechanism(s) incorporating nitrogen into VLC acyl compounds. Underlying processes can only be hypothesized at this stage, but most likely involve integration of the wax biosynthetic and aminoacid metabolic pathways.

The work presented in chapter 4 is a comprehensive compositional comparison of cuticular wax mixtures from two organs (flag leaf blade and peduncle) of *Triticum aestivum* cv. Bethlehem. The total wax coverage of peduncles was three times larger than that of flag leaf blades, and peduncle wax was dominated by C₃₁ β-diketone and 8- and 9-hydroxy-β-diketones, while flag leaf wax contained more than 50% 1-alkanols. Accordingly, the (currently unknown) biosynthetic pathway to the β-diketones and the (at least partially parallel) acyl reduction pathway to wax 1-alkanols are differentially regulated in peduncles and in flag leaves. Interestingly, the chain length profile of aldehydes closely matched that of 1-alkanols on flag leaf blades, suggesting that aldehydes may be released as intermediates of the reduction leading to alcohols, in contrast to our current understanding of wax biosynthesis in Arabidopsis. However, on peduncles, the aldehyde profile matched that of alkanes, in line with those of Arabidopsis.
Diverse classes of esters were identified in wheat cv. Bethlehem waxes as complex mixtures of metamers, necessitating detailed quantitative analysis and suggesting the presence of diverse ester synthase enzyme with distinct substrate specificities.

In the work presented in chapter 5, seven new compound classes were identified and quantified in the wax mixtures covering flag leaf blades and peduncles of *T. aestivum* cv. Bethlehem. Three of them were secondary alcohols, primary/secondary diols and their esters with (very-) long chain acids, all of which were found as homologous series only in flag leaf wax. These three classes appeared biosynthetically related based on their similar secondary hydroxyl groups (on and around C-12 from the non-functionalized chain end). The hydroxy-2-alkanol esters and oxo-2-alkanol esters found in both organs are biosynthetically related to hydroxy-β-diketones, as suggested also by a common location of hydroxyl/oxo groups. We hypothesize that both compound groups, the secondary alcohols/diols and the oxidized 2-alkanol esters/β-diketones, are formed by two distinct P450 enzymes with C-12 and C-7/8 regio-specificity, respectively. In contrast, the two other compound classes identified here, 4-alkylbutan-4-olides and internally methyl-branched may be formed via α-oxidation and through incorporation of methylmalonyl-CoA into fatty acyl-CoA intermediates, respectively. Overall, we thus propose three specific variations from the normal wax biosynthesis pathways to occur in wheat, in the form of P450 oxidation, α-oxidation and methylmalonate incorporation.

In chapter 6, six new classes of cuticular wax compounds were identified in TLC fractions of wax mixtures coating flag leaf blades and peduncles of *Triticum aestivum* cv. Bethlehem. They included a homologous series of C\(_{25}\) - C\(_{37}\) 2,4-ketols and C\(_{33}\) 2,4-diketone, whose common
structures suggested polyketide-type biosynthesis. Based on the predominance of the C₃₃ homologs in both compound classes, we conclude that the PKS likely involved in the formation of these subterminal ketols and diketones must have a preference for C₃₀ acyl-CoA or C₃₂ ketoacyl-CoA substrate, thus distinguishing it from other PKSs thought to be involved in the biosynthesis of other wheat wax constituents. Two such PKSs, forming mid-chain β-diketones and alkylresorcinols, have been recognized before. We further identified regioomers of a C₃₁ mid-chain ketone and a C₃₁ mid-chain β-ketol, which are likely side products of the β-diketone biosynthetic pathway. It seems plausible that such minor products may be formed when either a step of the β-diketone biosynthetic pathway is skipped, leading to a ketone instead, or when a reduction step is added, leading to the corresponding ketol. Finally, C₃₀ 14,15-diketone and a pair of corresponding C₃₀ mid-chain α-ketol regioomers were identified in wheat wax, clearly differing from previous literature reports of similar acyloins with odd carbon numbers. We hypothesize that the wheat α-ketols are formed by head-to-head condensation of C₁₄ and C₁₆ acyl precursors, in the form of thioesters or aldehydes, and not by hydroxylation of alkanes like the acyloins found in Brassicaceae waxes.

Overall, several of the wheat wax compounds identified in chapter 6 have very similar mid-chain-functionalized structures, characterized by C₁₃H₂₇ and C₁₅H₃₁ alkyl chains on a group of central carbons carrying functional groups. Accordingly, the potential biosynthetic mechanisms generating all these compounds also share a common characteristic, in that they branch off the normal wax biosynthesis pathway in a relatively early stage, through interception or diversion of C₁₄ and C₁₆ elongation intermediates and/or their utilization by PKS enzymes. It may be inferred that either one or more PKS enzymes in wheat must be able to handle such long-chain (C₁₄ and C₁₆) acyl substrates. In contrast, the subterminal β-diketone (2,4-diketone) and
subterminal β-ketols (2,4-ketols) found in wheat wax are likely formed by a different PKS with preference for very-long-chain (C_{30}) substrates. Another PKS is expected to be involved in the formation of alkylresorcinols from acyl substrates such as C_{24}, catalyzing three consecutive condensation reactions with malonate plus a final cyclization. This information, together with the evidence provided here, now leads us to distinguish at least three different PKSs participating in wheat wax biosynthesis, with very different biochemical properties including preference for C_{14/16}, C_{24} and C_{30} substrates.

In conclusion, the main objective of the thesis was achieved in that approximately 20 new wax compound classes were identified across the three plant species studied. Furthermore, valuable insight into cuticular wax biosynthesis was gained from exploring the potential biosynthetic origins of these compounds. Thus:

- It was suggested here that, unlike in A. thaliana, in other species (such as A. arborescens and T. aestivum cv. Bethlehem) FAE and/or FAS elongation intermediates (namely β-keto and β-hydroxyacyl thioesters) can be intercepted by other enzymes (hydrolases, reductases, PKSs) to produce interesting new wax structures with terminal (1,3-), subterminal (2,4-), or mid-chain bifunctionalities.

- A pathway to primary amides was proposed to operate in P. aurea, primary amides being the first example of nitrogen-containing fatty acid derivatives in cuticular waxes.

- In the flag leaves of T. aestivum cv. Bethlehem, the correlation between chain length profiles of primary alcohols and aldehydes suggested the latter to be intermediates en...
route to the former released by the fatty acyl reductase, again unlike in the Arabidopsis model.

- It was found that wax ester synthases exhibit widely variable substrate specificity (for either their acyl-CoA or their alcohol substrate) depending on the plant species and even on the organ of the species.

- Products of cytochrome P450 enzymes different from the Arabidopsis mid-chain alkane hydroxylase were identified in wheat, with hydroxyls at positions C-8/9 or ω-12.

- A great variety of polyketides and polyketide-like structures were identified also in wheat, including midchain and subterminal β-diketones and ketols, as well as simple ketones, suggesting the great versatility of the PKS enzymes responsible for their biosynthesis.

7.2. Future research directions

7.2.1. Search for further novel wax compounds in other plant species

The present thesis describes the identification of numerous new wax compounds, however its findings are limited by the fact that only three plant species were explored. By expanding the repertoire of plant species studied, further novel wax structures are expected to be discovered. It would be of particular interest to continue the exploration of monocotyledons, since they have proven to be so rich in new compounds in the work herein. This exploration could be targeted at entirely new families and genera, but it could equally aim at studying new species from the same
families and genera as those in the present work. For example, other species in the genus *Aloe* may reveal further bifunctional compounds with 1,3-constellation of functionalities, such as diols, ketols, hydroxy-aldehydes, keto-aldehydes, hydroxy-esters or keto-esters. Other cereal species whose cuticular waxes have been less extensively studied (sorghum, millet, oats, triticale, fonio, teff) may be a source of further polyketides or oxygenated compounds. Finally, other species in the genus *Phyllostachys* may harbour a wider range of cuticular wax compounds containing nitrogen, for example amines resulting from reduction of amides or secondary amides formed via acylation of amines similar to wax ester formation.

### 7.2.2. Investigations of some of the proposed biosynthetic pathways

Numerous biosynthetic routes have been proposed towards the novel compounds described in this thesis, but they are all hypothetical at this stage. It would thus be of interest to probe at least some of these pathways, using molecular biological and/or biochemical tools. The plant species most suitable for this kind of work is *Triticum aestivum*, whose genome has been fully sequenced (International Wheat Genome Sequencing Consortium, 2014). In particular, the genes encoding the hydrolase and polyketide synthase (PKS) responsible for the production of β-diketones and the gene encoding the cytochrome P450 enzyme responsible for the hydroxylation of β-diketones were established (Hen-Avivi et al., 2016). It would be of interest to check if the minor polyketide metabolites structurally related to β-diketones (i.e. β-ketols, ketones, α-diketones, α-ketols) are still present or not in plants with mutations of either or both of the hydrolase and PKS, thus proving or disproving the participation of these enzymes in the production of the aforementioned minor wax constituents. Also, in a P450-knock-out mutant
line, it would be interesting to search for the presence of hydroxy- and oxo-2-alkanol esters, in order to establish whether the same cytochrome P450 enzyme forming hydroxy-β-diketones is forming these oxidized 2-alkanol esters as well. Biochemically, it would be interesting to purify this enzyme and feed it 2-alkanol substrates to directly observe the hydroxylation process of these substrates, as well as β-diketone substrates. This would eliminate the alternative possibility that the hydroxylation happens earlier in the pathway, on one of the common intermediates of β-diketones and 2-alkanol esters, such as the β-ketoacids produced by the hydrolase.

Of further interest would be to identify in the wheat genome the second cytochrome P450-encoding gene responsible for the production of secondary alcohols and diols in wheat flag leaves. This gene would have to be highly expressed in the flag leaf blades, but with negligible expression level in the peduncle, a feature that could be used to select it from among multiple gene candidates. If a mutation in this gene results in loss of secondary alcohols and/or diols, its role in their biosynthesis would thus be confirmed. For the diol monoesters, the homolog of Arabidopsis WSD1 would have to be identified in the wheat genome. A mutation in this gene would result in loss of esters of primary alcohols in addition to diol esters, making it unclear whether diol esters are absent due to knock-out of the gene directly responsible for their production or due to lack of ester substrates for hydroxylation by the cytochrome P450. It would be more useful to isolate and purify the wheat wax ester synthase and feed it in vitro separately primary alcohol and primary/secondary diol substrates (each in combination with acyl-CoAs) and thus test its versatility in esterifying both types of substrates with primary OH.

While the genome of Phyllostachys aurea has not been sequenced, it would nonetheless be of great interest to identify, clone and characterize the gene encoding the cytochrome c putatively responsible for the biosynthesis of VLC primary amides. A mutation in this gene
resulting in loss of amides would clarify the participation of cytochrome $c$ in amide biosynthesis. If the cytochrome $c$ enzyme is isolated and purified, a feeding experiment with ammonia and acyl-CoA will confirm whether or not this simplest pathway is indeed the pathway leading to primary amides in $P. aurea$. 
References


Buschhaus, C., Peng, C., Jetter, R., 2013. Very-long-chain 1,2- and 1,3-bifunctional compounds from the cuticular wax of \( \textit{Cosmos bipinnatus} \) petals. Phytochemistry 91, 249–256.


Herbin, G.A., Robins, P.A., 1968b. Studies on plant cuticular waxes. II. Alkanes from members of the genus *Agave* (Agavaceae), the genera *Kalanchoe*, *Echeveria*, *Crassula* and *Sedum* (Crassulaceae) and the genus *Eucalyptus* (Myrtaceae) with an examination of Hutchinson’s sub-division of the ang. Phytochemistry 7, 257–268.


Appendices

Appendix A: Synthetic protocols for reference compounds from Chapter 2

A.1 General information

All materials, reagents and solvents were used as received from the vendor, except for tetrahydrofuran (THF) and CH$_2$Cl$_2$, which were distilled from Na/benzophenone and CaH$_2$, respectively. All reactions were performed in oven-dried flasks (150°C), without inert atmosphere protection. Analytical TLC was performed on aluminium sheets coated with silica gel 60 F254 (layer thickness 0.2 mm, pore size 60 Å). Preparative TLC was performed on glass plates coated with silica gel 60 F254 (layer thickness 0.5 mm or 1 mm, 20x20 cm, with 4 cm concentration zone). TLC plates were sprayed with a dilute primuline solution (5 mg in 100 mL acetone/water 80/20, v/v) and visualized under 365 nm UV light. Column chromatography was carried out using silica gel (pore size 60 Å, 230–400 mesh). Compound purity was assessed via GC-MS, with derivatization and GC-MS conditions as described in the experimental section. $^1$H-NMR spectra were recorded from CDCl$_3$ solutions at 25°C on a Bruker Avance 300 MHz spectrometer.

A.2 Synthesis scheme

Synthetic standards of C$_{30}$ 3-hydroxy fatty acid, C$_{30}$ 3-hydroxy fatty acid methyl ester (FAME) and C$_{29}$ 2-alkanol were needed for structure confirmation. All three compounds were synthesized from the same starting material, C$_{28}$ fatty acid, first converted in two steps to C$_{30}$ 3-keto FAME, and then either in two steps into the secondary alcohol or in one step each into the 3-hydroxy FAME and the 3-hydroxy fatty acid (Figure A1).
Figure A.1. Synthesis scheme.

Reaction sequences employed for synthesis of authentic standards of C_{29} 2-alkanol, C_{30} 3-hydroxy FAME, and C_{30} 3-hydroxy fatty acid.

A.3 Synthetic procedures and \(^1\)H NMR characterization data

5-Octacosanoyl Meldrum’s acid: 200 mg (0.471 mmol) octacosanoic acid (Aldrich, ≥98.5%), 451 mg (2.35 mmol) N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC, Aldrich, ≥98%), and 230 mg (1.88 mmol) 4-dimethylaminopyridine (DMAP, TCI, ≥99%) were dissolved together in 20 mL freshly distilled dichloromethane (Fisher, ≥99.9%). After stirring for 30 min at room temperature, 271 mg (1.88 mmol) Meldrum’s acid (TCI, ≥98%) were added, and the mixture was stirred overnight. The reaction mixture was vacuum filtered to remove precipitated urea. The organic phase was washed sequentially with diluted HCl (Fisher, 32-35%
original conc.) and NaHCO₃ (Merck, ≥99.7%), then dried over anhydrous Na₂SO₄ (Merck, ≥99%), and the solvent removed under vacuum. The residue was taken up in chloroform and purified by column chromatography (using hexane:ethyl acetate 5:1 as mobile phase) to give the final product as a white solid (235 mg, 91% yield). ¹H-NMR (300 MHz, CDCl₃): δ 3.06 (2H, t, J=7.2 Hz, CH₂CO), 1.73 (6H, s, C(CH₃)₂), 1.10-1.70 (50H, br m, aliphatic CH), 0.88 (3H, t, J=6.9 Hz, CH₃).

*Methyl 3-oxotriacontanoate*: 235 mg (0.427 mmol) 5-octacosanoyl Meldrum’s acid were dissolved in 25 mL of methanol (Fisher, ≥99.9%) and refluxed for eight hours. Then the solution was allowed to cool to room temperature, the precipitate collected by vacuum filtration and washed with cold methanol to afford the practically pure product as a white solid (202 mg, 98% yield). ¹H-NMR (300 MHz, CDCl₃): δ 3.74 (3H, s, COOCH₃), 3.44 (2H, s, COCH₃COOCH₃), 2.52 (2H, t, J=7.2 Hz, CH₂CO), 1.10-1.70 (50H, br m, aliphatic CH), 0.88 (3H, t, J=6.9 Hz, CH₃).

2-Nonacosanone: To a solution of 50 mg (0.104 mmol) methyl 3-oxotriacontanoate in 3 mL dimethylformamide (DMF, Aldrich, ≥99.8%) and 0.5 mL distilled water, 132 mg (2.35 mmol) KOH (Fisher, reagent grade, 86.6% batch assay) and a catalytic amount of tetrabutylammonium bromide (Aldrich, ≥98%) were added. The mixture was refluxed at 120°C for four hours, then quenched with dilute HCl and extracted with chloroform. The combined organic layers were dried over anhydrous Na₂SO₄, filtered and concentrated under reduced pressure. The residue was taken up in hexane (Fisher, HPLC grade, ≥99.9%) and purified by column chromatography (eluting with hexane:ethyl acetate 20:1) to afford the product as a white solid (31.5 mg, 73%
yield). \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \(\delta\) 2.41 (2H, t, \(J=7.5\) Hz, CH\(_2\)CO), 2.13 (3H, s, COCH\(_3\)), 1.10-1.90 (50H, br m, aliphatic CH), 0.88 (3H, t, \(J=6.9\) Hz, CH\(_3\)).

2-Nonacosanol: To a solution of 15 mg (0.035 mmol) 2-nonacosanone in a mixture of 1 mL CHCl\(_3\) and 1 mL THF (Aldrich, \(\geq 99.9\%\), 250 ppm BHT as inhibitor), 11 mg (0.291 mmol) of NaBH\(_4\) (Fisher, \(\geq 98\%\)) were added. After stirring overnight at room temperature, the mixture was quenched with dilute HCl and the product extracted with chloroform. The combined organic layers were dried over Na\(_2\)SO\(_4\), filtered and concentrated under reduced pressure. The residue was taken up in chloroform and purified on a preparative TLC plate (hexane:ethyl acetate 11:1) to afford the product as a white solid (11 mg, 73\% yield). \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \(\delta\) 3.80 (1H, m, CH\(_{10}OH\)), 1.20-1.50 (52H, br m, aliphatic CH), 1.18 (3H, d, \(J=6.3\) Hz, CH(OH)CH\(_3\)), 0.88 (3H, t, \(J=6.9\) Hz, CH\(_3\)).

Methyl 3-hydroxytriacontanoate: To a solution of 48 mg (0.1 mmol) methyl 3-oxotriacontanoate in 4 mL of distilled CHCl\(_3\) and 4 mL of distilled THF, 48 mg (1.27 mmol) of NaBH\(_4\) were added. The mixture was allowed to stir at room temperature overnight before being quenched with dilute HCl and extracted with fresh chloroform. The combined organic layers were dried over Na\(_2\)SO\(_4\), filtered and concentrated under reduced pressure. The residue was purified by recrystallization from MeOH to yield the product as a white solid (34.5 mg, 72\% yield). \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \(\delta\) 4.01 (1H, m, CH\(_{10}OH\)), 3.71 (3H, s, COOCH\(_3\)), 2.52 (1H, dd, \(J=16.2, 3.0\) Hz, CHHCOOCH\(_3\)), 2.40 (1H, dd, \(J=16.2, 8.7\) Hz, CHHCOOCH\(_3\)), 1.10-1.70 (52H, br m, aliphatic CH), 0.88 (3H, t, \(J=6.9\) Hz, CH\(_3\)).
**3-Hydroxytriacontanoic acid:** At room temperature, 2 mL of glacial acetic acid (Merck, ≥99.8%) were added to 20 mg (0.04 mmol) methyl 3-hydroxytriacontanoate placed in a glass vial. The vial was heated mildly to dissolve the solid. Then concentrated HCl was added to the solution until a precipitate started to persist. A few more drops of acetic acid were added to give a slightly opaque solution. The mixture was allowed to stand for three days at room temperature and then the white solid was collected by vacuum filtration. The crude product was taken up in chloroform, washed with NaHCO₃ and dried over anhydrous Na₂SO₄. The drying agent was removed by filtration, and the filtrate was dried under vacuum to afford the product as a white solid (13.8 mg, 71% yield).

\[^1\text{H-NMR (300 MHz, CDCl}_3\text{): } \delta 4.07 (1\text{H, m, CHOH}), 2.56 (1\text{H, dd, } J=16.1, 3.0 \text{ Hz, CHHCOOH}), 2.48 (1\text{H, dd, } J=16.1, 8.5 \text{ Hz, CHHCOOH}), 1.10-1.70 (52\text{H, br m, aliphatic CH}), 0.88 (3\text{H, t, } J=6.9 \text{ Hz, CH}_3)\]