Lipids (2016) 51:1407–1420
DOI 10.1007/s11745-016-4208-z

ORIGINAL ARTICLE

Identification of Polyketides in the Cuticular Waxes of *Triticum aestivum* cv. Bethlehem

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Received: 8 June 2016 / Accepted: 10 October 2016 / Published online: 28 October 2016

Abstract Cuticular waxes are complex mixtures consisting mostly of very-long-chain aliphatics with single, primary functional groups. However, the waxes of many plant species also include aliphatics with one or more functional groups residing on subterminal or mid-chain carbons. In the present work, the cuticular wax mixtures from flag leaf blades and peduncles of *Triticum aestivum* cv. Bethlehem were analyzed in a search for novel wax constituents with in-chain functionalities, potentially of polyketide origin. The structures of compounds belonging to six different compound classes were elucidated using gas chromatography–mass spectrometry of various derivatives. Among them, a series of 2,4-ketols was identified, with odd carbon numbers ranging from C_{25} to C_{37} and peaking at C_{33}. The analogous C_{33} 2,4-diketone was identified as well, together with a pair of co-eluting C_{31} mid-chain β-ketol isomers (16-hydroxyhentriacontan-14-one and 14-hydroxyhentriacontan-16-one), a pair of co-eluting C_{30} mid-chain α-ketol isomers (15-hydroxytriacontan-14-one and 14-hydroxytriacontan-15-one), the corresponding C_{30} 14,15-diketone, and a pair of co-eluting C_{31} ketones (hentriacontan-14-one and hentriacontan-16-one). All newly discovered structures contain ketone functional groups, with similar $C_{13}H_{27}$ and $C_{15}H_{31}$ alkyl chains on either side of the functionalities, thus resembling the previously reported very-long-chain β-diketones dominating the wheat wax mixtures. Based on these structural characteristics, possible biosynthetic pathways leading to the newly identified polyketide-like compounds are proposed.

Keywords Wax · Wheat · *Triticum aestivum* · Cuticle · Ketones · Ketols · Diketones · Mass spectrometry · Fragmentation patterns

Abbreviations

Ac	Acetyl
ACP	Acyl carrier protein
BSTFA	Bis-*N,O*-(trimethylsilyl)trifluoroacetamide
CoA	Coenzyme A
EAR	Enoyl-ACP reductase
ECR	Enoyl-CoA reductase
FAE	Fatty acyl elongase
FAR	Fatty acyl-CoA reductase
FAS	Fatty acid synthase
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
LAH	Lithium aluminum hydride
LC	Long-chain
MAH	Mid-chain alkane hydroxylase
MS	Mass spectrum/mass spectrometry
NMR	Nuclear magnetic resonance
PKS	Polyketide synthase
prim	Primary

Electronic supplementary material The online version of this article (doi:10.1007/s11745-016-4208-z) contains supplementary material, which is available to authorized users.

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sec	Secondary
TLC	Thin layer chromatography
TMS	Trimethylsilyl
VLC	Very-long-chain
VLCFA	Very-long-chain fatty acid
WS	Wax synthase

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) [1].

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 [2]. 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 [3]. In many species, the wax mixtures also comprise alicyclics, including a wide variety of triterpenoids [4–8], and in some species also aromatics such as 5-alkylresorcinols [9, 10], benzyl and phenethyl esters [11–15], and 4-hydroxyphenylpropyl, 3,4-dihydroxyphenylpropyl and 3,4-dihydroxyphenylbutyl esters [16, 17].

Aliphatic cuticular wax compounds 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_{16} and C_{18} fatty acids formed de novo in epidermal plastids [18, 19]. 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_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 before. 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 [18, 19]. 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 affects 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.

Over the past two decades, numerous new wax structures have been identified with one functional group on the end of the aliphatic chain, similar to the ubiquitous wax compounds described above, and additional mid-chain functionalities. Such primary/secondary bifunctional compounds were reported, for example, as alkanediols, ketoaldehydes, ketoalcohols and ketoalkyl esters in the wax of *Osmunda regalis* fronds [20], or δ-lactones in leaf wax of *Cerinthe minor* [21]. 1,3-Alkanediols and 3-hydroxyaldehydes were detected in leaf wax of *Ricinus communis* [22], further alkanediols in *Pisum sativum* leaves [23], 1,5-alkanediols and 5-hydroxyaldehydes in *Taxus baccata* needles [16], 1,3- and 1,2-alkanediol acetates in *Cosmos bipinnatus* petals [24], 3-hydroxyacid derivatives in *Aloe arborescens* [25], and 3-hydroxyacid and alkanediol esters in *Funaria hygrometrica* [26].

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 [27] and in various gymnosperm needle waxes [28]. Most prominently, sec/sec diketones accumulate to relatively high concentrations in wax mixtures of diverse plant species, in most cases as single compounds (rather than homologous series) with odd carbon numbers and β-constellation of the two carbonyl groups (i.e., with one methylene unit between them). For example, β-diketones such as nonacosane-6,8-dione, hentriacontane-8,10-dione and tritriacontane-10,12-dione are found in *Buxus sempervirens* wax [29], tritriacontane-16,18-dione in *Eucalyptus globulus* [30], nonacosane-10,12-dione and hentriacontane-10,12-dione in the *Hosta* cultivar 'Krossa Regal' [31], and nonacosane-8,10-dione, nonaconsane-12,14-dione, hentriacontane-10,12-dione and hentriacontane-14,16-dione in various species of *Rhododendron* [32]. The cuticular waxes of many Poaceae contain particularly high concentrations of β-diketones, most frequently hentriacontane-14,16-dione. Accordingly, this C_{31}

compound dominates the waxes, for example, of *Agropyron dasystachyum, A. riparium and A. elongatum* [33], *Hordeum vulgare* [34], and several species of wheat [35–37].

Among the Poaceae, wheat is of particular interest due to its world-wide role as a primary staple crop. There are many reports on the composition of cuticular wax mixtures on various wheat species, cultivars and organs [35–41], documenting the presence of ubiquitous compound classes such as fatty acids, aldehydes, alkanes, primary alcohols and alkyl esters, together with β-diketones such as hentriacontane-14,16-dione and its hydroxylated derivatives. Recently, the *Triticum aestivum* cultivar Bethlehem was selected for genetic investigations into β-diketone biosynthesis [42], 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 peduncles of wheat cv. Bethlehem was performed, confirming the presence of the typical wheat wax compounds, most prominently the mid-chain β-diketone hentriacontane-14,16-dione together with 8- and 9-hydroxy-hentriacontane-14,16-dione [43]. Several classes of aliphatic and aromatic esters were identified that had not been described in wheat wax before, and detailed quantification of their homolog and isomer compositions enabled predictions regarding the ester synthase enzymes involved in their biosynthesis [43].

A second, more detailed analysis of wheat cv. Bethlehem flag leaf and peduncle waxes identified 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 [44].

However, several compounds in the wheat wax mixtures and their TLC fractions could not be identified based on the preliminary mass spectral information available so far. Due to their very small quantities within the complex mixture, involving compound classes, homologs and isomers with very similar physico-chemical properties, isolation of single compounds in sufficient quantities for NMR analyses was not feasible. Therefore, the goal of the present work was to elucidate the structures of previously unknown wheat wax constituents using combined evidence from chromatographic behaviour, to assess compound polarities, and multiple lines of mass spectral information for each compound, to assess chain lengths and functional group geometries along the hydrocarbon chains. Accordingly, the wax mixtures were separated by preparative TLC to obtain large enough quantities, diverse micro-scale derivatization reactions were carried out to probe for particular functional groups, and detailed GC–MS analyses comparing multiple derivatives of each compound then allowed unambiguous structure assignments.

Materials and Methods

Plant Material and Wax Sampling

Triticum aestivum cv. Bethlehem plants were grown in greenhouses at the Weizmann Institute of Science (Rehovot, Israel) in a mix of 50% peat and 50% turf watered every 3–4 days, with light/dark cycles of 12–14 h/10–12 h, a photon flux during light cycles of 180 μmol m^{-2} s^{-1}, and temperatures of 24–26 °C/17–18 °C. For chemical analyses, ten flag leaf blades with an area of 40–50 cm^2 each (measured using ImageJ) and ten peduncles with a diameter of 2 mm and a length of 15–25 cm were harvested from mature wheat plants using clean razor blades.

Leaves or peduncles were submerged into 10 mL $CHCl_3$ (Aldrich, ≥99%, with 0.75% ethanol as stabilizer) at room temperature for 30 s, with agitation. The wax solution was transferred to another vial, and the plant material was extracted again with 10 mL $CHCl_3$ for 30 s. The two extracts were combined and the solvent evaporated under a stream of N_2 (Praxair, ≥99.998%) at 50 °C. The dry wax mixtures were stored at room temperature until fractionation by TLC.

Preparative Thin Layer Chromatography

The cuticular wax mixtures were fractionated by preparative TLC using the sandwich technique [45] on SiO_2-coated glass plates (Uniplate Analtech, silica gel 60 F_{254} layer thickness: 1 mm, size: 20 × 20 cm, with 4 cm concentrating zone) with $CHCl_3$:EtOH 98:2 (v/v) as mobile phase. Following separation, plates were sprayed with a solution of 5 mg primuline (Aldrich, 50% dye content) in 100 mL $(CH_3)_2CO$:H_2O 80:20 (v/v), and compound bands were visualized under 365 nm UV light. Silica bands of interest were scraped off the plates with spatulas and collected into 20 mL scintillation vials, where they were extracted twice with 10 mL $CHCl_3$ at room temperature, for 30 s each. After filtration through glass wool (Supelco), the combined extracts were concentrated under N_2 at 50 °C and transferred to 2 mL GC autosampler vials. Finally, the solvent was evaporated and samples stored at room temperature.

Derivatization Reactions

Acetylation of (some of the) hydroxyl-containing fractions was performed by refluxing the dry wax in 10 μL acetic anhydride (Aldrich, ≥98%) and 10 μL pyridine (Aldrich, ≥99.8%) at 70 °C for 5 min, followed by overnight stirring at room temperature. After evaporation of excess reagents under N_2, silylation was carried out as described below. For reduction, wax samples were dissolved in 50 μL $(CH_3CH_2)_2O$, before 0.1 mg $LiAlH_4$ (Aldrich, ≥95%) were added. The mixture was left to react overnight at 70 °C, with the vial cap closed, and then quenched with 10% H_2SO_4 and extracted three times with 60 μL $(CH_3CH_2)_2O$. The organic solutions were combined, and the solvent was evaporated prior to silylation as described below. Conversion of (some of the) carbonyl-containing specimens into the corresponding methoximes was accomplished by heating with 20 μL of a saturated solution of *O*-methylhydroxylamine hydrochloride (Aldrich, ≥98%) in pyridine:$CHCl_3$ 7:3 (v/v) at 70 °C for 30 min. After partitioning between 50 μL distilled H_2O and 50 μL $CHCl_3$, the organic fraction was collected and the aqueous phase extracted once more with 50 μL fresh $CHCl_3$. Both organic phases were combined, and the solvent evaporated. All samples were subjected to silylation before injection into the GC–MS, by refluxing in a mixture of 10 μL *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA, Aldrich, GC grade) and 10 μL pyridine at 70 °C for 20 min. Excess reagents were then evaporated under N_2 and the silylated wax mixtures re-dissolved in 50 μL $CHCl_3$.

Gas Chromatography-Mass Spectrometry

All analyses were performed using a GC instrument (6890 N, Agilent, Avondale PA, USA) equipped with capillary column (HP-1 100% PDMS; length: 30 m; inner diameter 0.32 mm; film thickness 0.1 μm), on-column injector and MS detector (5973 N, Agilent, EI-70 eV, ionization source temperature: 240 °C, *m*/*z* 50–750). It employed helium (Praxair, ≥99%) as a carrier gas at a flow rate of 1.4 mL min^{-1}, with an oven temperature program of 2 min at 50 °C, ramping at 40 °C min^{-1} to 200 °C, constant for 2 min, ramping at 3 °C min^{-1} to 320 °C and constant for 30 min. Aliquots of 1–2 μL were injected for GC–MS analyses.

Results

This study aimed at a detailed analysis of previously unidentified compounds 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, and fractions of interest were converted into various derivatives for comparative GC–MS analyses.

Based on their TLC behaviour and common MS fragmentation patterns, the unknown wheat wax constituents could be grouped into six different compound classes. 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 **A** co-eluted with wax γ-lactones and keto-alkan-2-ol esters (R_f 0.44), **B** with β-diketones (R_f 0.86), and **C** with prim/sec alkanediol esters (R_f 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 (R_f 0.86) and **E** with hydroxy-β-diketones (R_f 0.33). Finally, class **F** was isolated as a fraction of its own, with polarity (R_f 0.91) between those of β-diketones and alkyl esters.

Initially, the β-diketones contained in the same fraction as classes **B** and **D** were investigated for reference. As previously described [43], the β-diketone fraction was largely dominated by one compound, hentriacontane-14,16-dione (ca. 97% of the β-diketone class). It was identified by the characteristic MS fragmentation pattern of the underivatized compound (data not shown) and its trimethylsilyl (TMS) enol ether derivative (Fig. 1a), both matching previously reported data [46]. Two more aliquots of the same TLC fraction were subjected to derivatization with *O*-methylhydroxylamine and lithium aluminum hydride (LAH), 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. 1b, 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 LAH reduction mixtures identified the C_{29} structure nonacosane-14,16-dione (<0.5% of the compound class) and two isomers of the C_{33} homolog, tritriacontane-14,16-dione and tritriacontane-16,18-dione (ca. 1 and 2% of the compound class, respectively).

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 (R_f 0.44). Based on MS similarity with published data for C_{29} β-ketols in Brassicaceae waxes [47, 48], **A** was hypothesized to have mid-chain β-ketol structure. 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 [48], while lacking a fragment *m*/*z* 147 indicative of a second hydroxyl function (Fig. 2a). Two more fragments, M-15 (due to loss of methyl radical) and

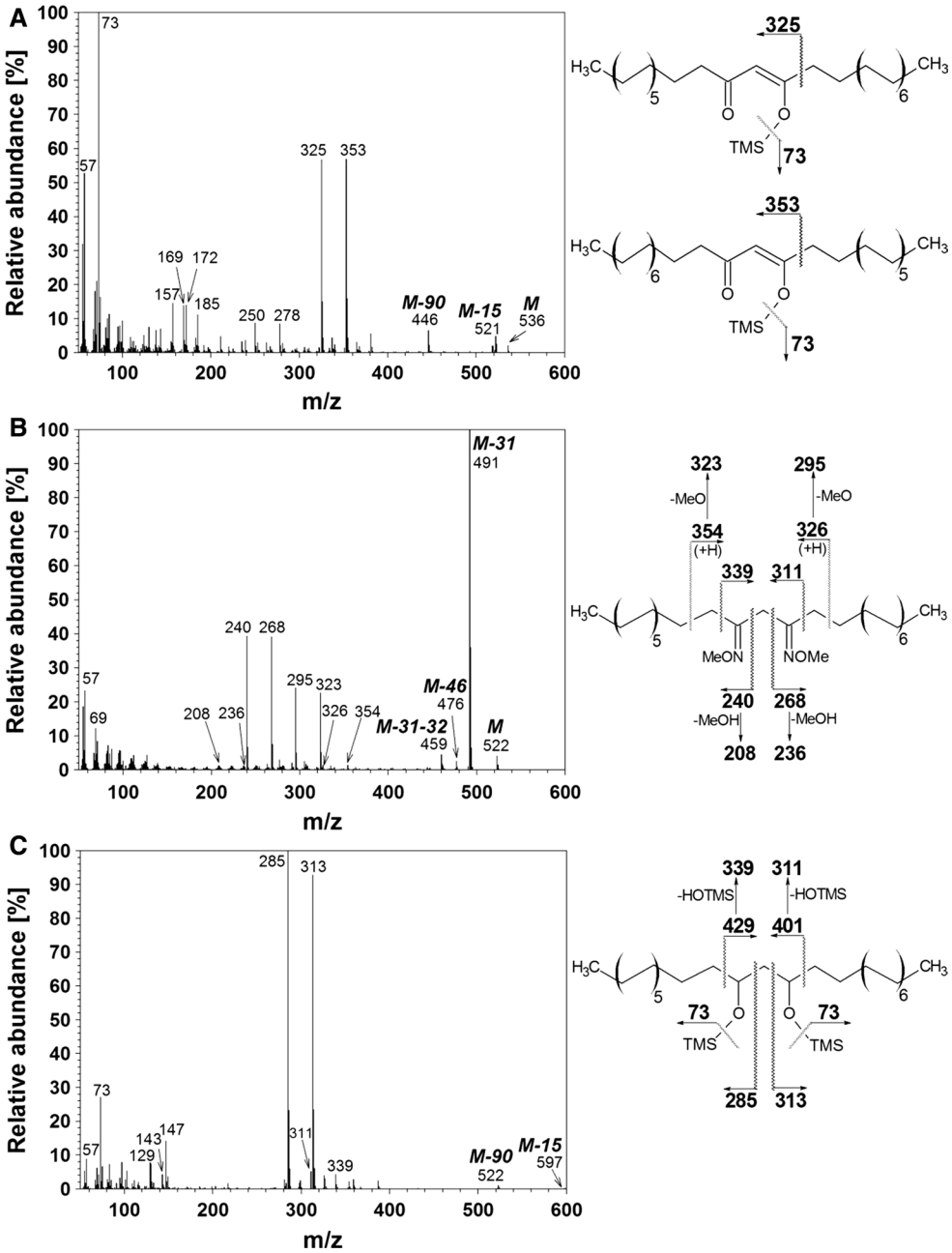

Fig. 1 Identification of mid-chain β-diketone in wheat leaf and peduncle wax. **a** Mass spectrum and major fragmentations of the TMS derivatives obtained from the two enol tautomers of hentriacontane-14,16-dione. **b** Mass spectrum and major fragmentations of the *O*-methylhydroxylamine derivative of hentriacontane-14,16-dione. **c** Mass spectrum and major fragmentations of the TMS derivative of the diol resulting from LAH reduction of hentriacontane-14,16-dione

M-90 (due to loss of $(CH_3)_3SiOH$), further confirmed the ketol structure and indicated an overall chain length of C_{31}. Finally, the α-fragments of the OTMS (*m/z* 327 and *m/z* 313) and carbonyl groups (*m/z* 211 and 355) identified one ketol isomer 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 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 LAH, 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 [49], together with M-15 and M-90 ions indicative of chain length (Fig. 2b). 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 $(CH_3)_3SiOH$ (*m/z* 339 and *m/z* 311) corroborated the presence of two OTMS groups. The TMS derivative of the LAH reduction product, thus, had MS characteristics matching those of β-diols [48], unambiguously identifying it as hentriacontane-14,16-diol. Taking this result together with the TLC behaviour and

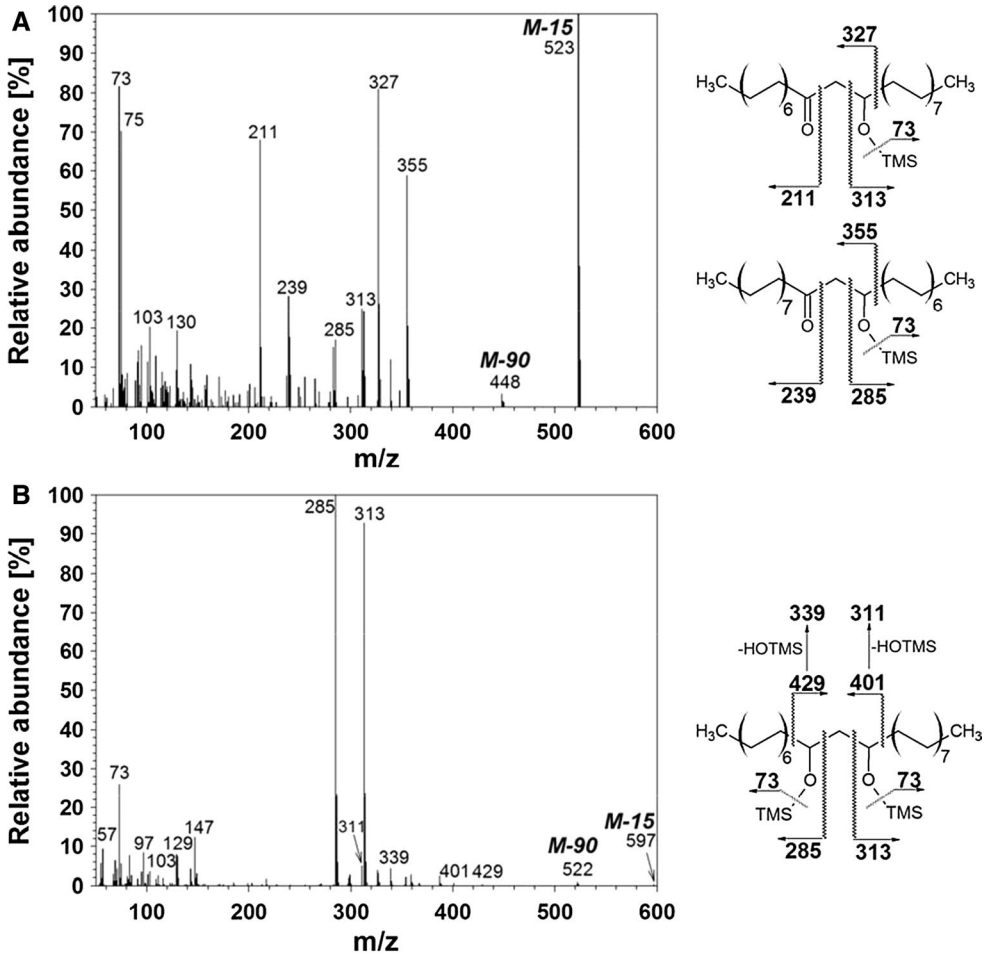

Fig. 2 Structure elucidation of mid-chain β-ketols **A** in wheat leaf and peduncle wax. **a** Mixed mass spectrum and major fragmentations of the co-eluting TMS derivatives of 16-hydroxyhentriacontan-14-one and 14-hydroxyhentriacontan-16-one. **b** Mass spectrum and major fragmentations of the TMS derivative of the diol resulting from LAH reduction of 16-hydroxyhentriacontan-14-one and 14-hydroxyhentriacontan-16-one

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 and 14-hydroxyhentriacontan-16-one, found in a ratio of ca. 1:1. 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, 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.

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. 3a) 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 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 Da higher than corresponding α-fragments [50].

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 and *m/z* 268) (Fig. 3b), together indicating the presence of at least one carbonyl function. Finally, reduction of **B** with excess LAH 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. 3c). All the MS characteristics of the LAH product (TMS derivative) thus matched those of α-diols reported before [48], identifying it 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. It is interesting to note that the two diols resulting from reduction of the α-diketone triacontane-14,15-dione (Fig. 3c) and of the β-diketone

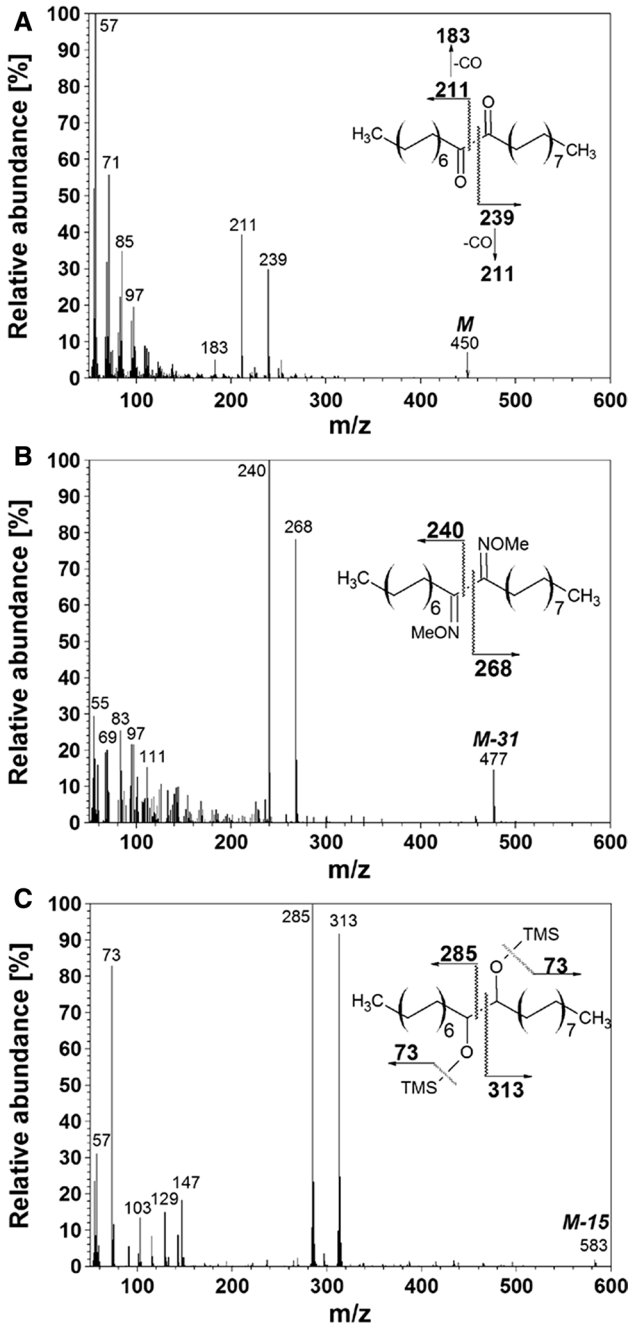

Fig. 3 Structure elucidation of mid-chain α-diketone **B** in wheat leaf and peduncle wax. **a** Mass spectrum and major fragmentations of triacontane-14,15-dione. **b** Mass spectrum and major fragmentations of the *O*-methylhydroxylamine derivative of triacontane-14,15-dione. **c** Mass spectrum and major fragmentations of the TMS derivative of the diol resulting from LAH reduction of triacontane-14,15-dione

hentriacontane-14,16-dione (see Fig. 1c) share many prominent features; however, both compounds are distinguished by their molecular ions as well as fragments *m/z* 522, 311 and 339 characteristic of the β-diketone-derived diol.

Compound class **C** was found in a single GC peak, detected in a fraction (R_f 0.54) slightly less polar than that containing the β-ketols (class **A**). Again, MS similarity with previously reported *Brassica* and *Arabidopsis* wax ketols [47, 48] suggested that **C** was a mixture of isomeric α-ketols (acyloins). 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. 4a). 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 C_{31} ketol structure, and two α-fragments at *m/z* 211 and 239, interpreted as C_{14} and C_{16} acylium ions indicative of a carbonyl group. **C** was, thus, recognized as an α-ketol mixture 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. 4b), thus confirming the presence of two isomeric α-ketol structures. A second aliquot of the TLC fraction was reduced with LAH, resulting in the same α-diol as from **B** (data not shown), and, therefore, further corroborating the C_{30} 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. 4c), 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 $(CH_3)_3SiOH$), 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 C_{30} mid-chain α-ketols, 15-hydroxytriacontan-14-one and 14-hydroxytriacontan-15-one. Based on relative abundances of specific MS fragments (e.g., *m/z* 285/313 for the TMS derivatives), both isomers were present in a ratio of ca. 1:1. Interestingly, these structures are closely related to those of compound classes **A** and **B**, the former identified as mid-chain β-ketols differing from the α-ketols by the presence

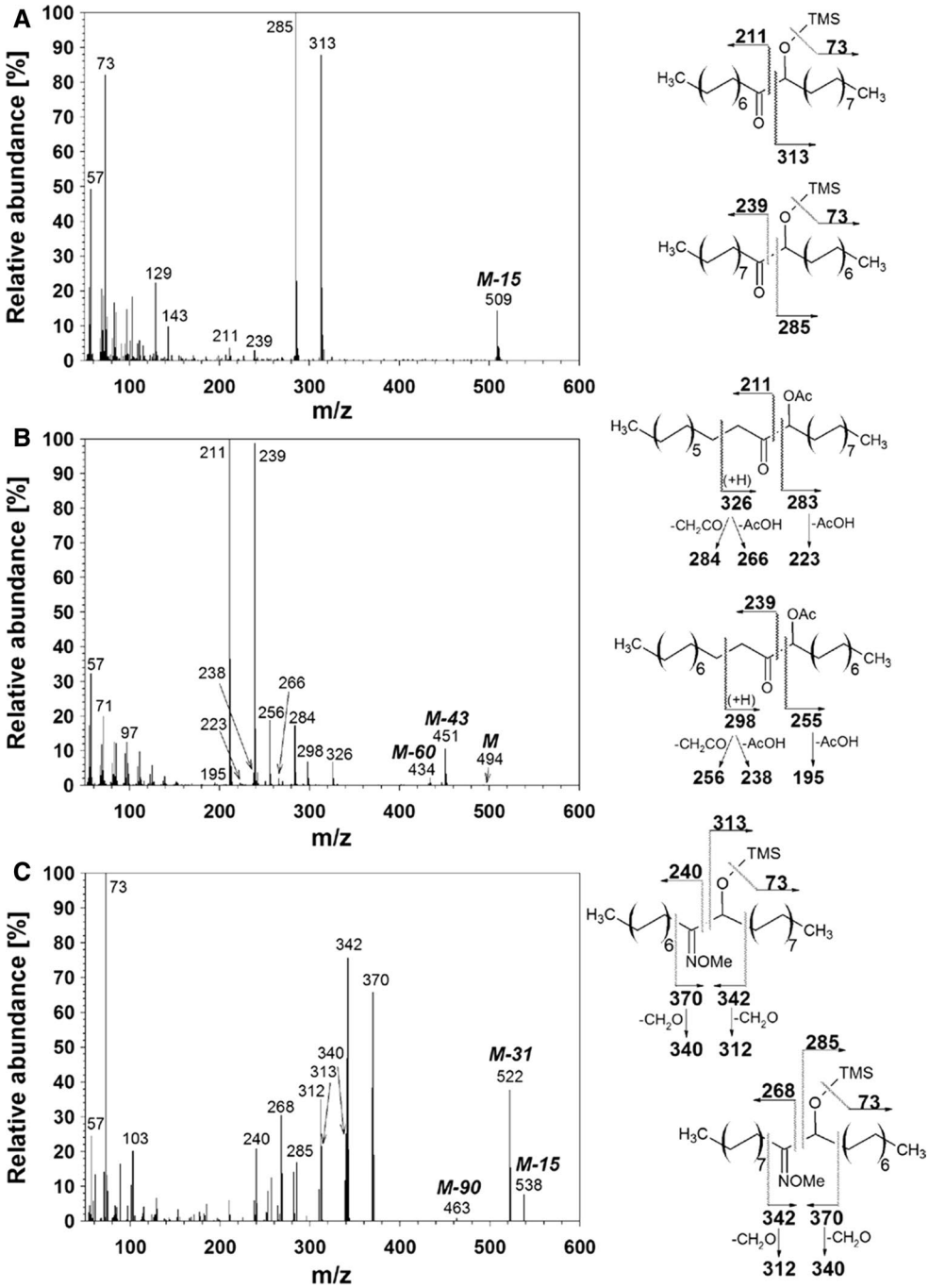

Fig. 4 Structure elucidation of mid-chain acyloins **C** in wheat leaf and peduncle wax. **a** Mass spectrum and major fragmentations of co-eluting TMS derivatives of 15-hydroxytriacontan-14-one and 14-hydroxytriacontan-15-one. **b** Mass spectrum and major fragmentations of co-eluting acetate esters of 15-hydroxytriacontan-14-one and 14-hydroxytriacontan-15-one. **c** Mass spectrum and major fragmentations of co-eluting *O*-methylhydroxylamine/TMS derivatives of 15-hydroxytriacontan-14-one and 14-hydroxytriacontan-15-one

of one methylene unit between functional groups, and the latter identified as a mid-chain α-diketone differing from the α-ketols only in functional group oxidation state.

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. 5a). 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, indicating 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

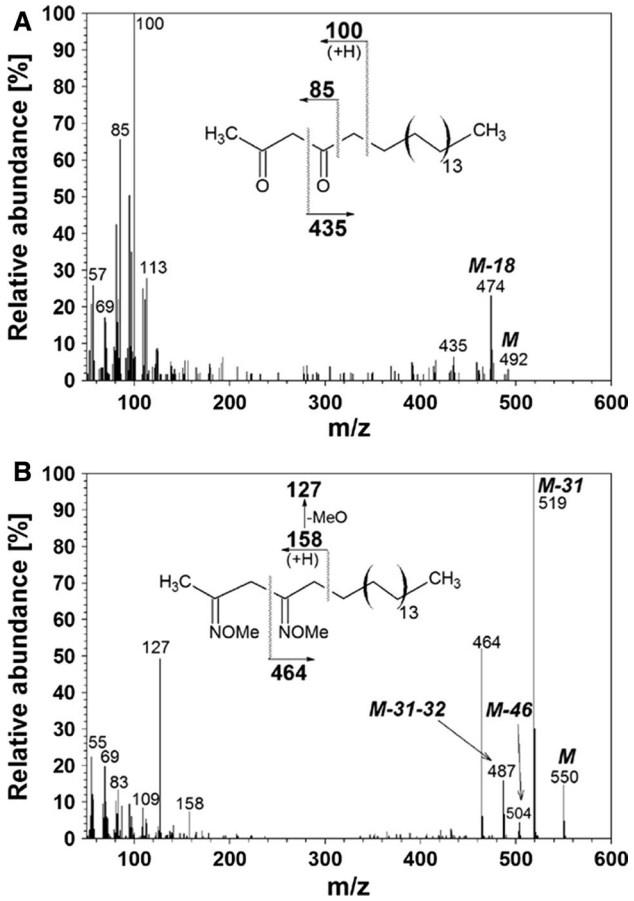

Fig. 5 Structure elucidation of subterminal β-diketone (2,4-diketone) **D** in wheat leaf and peduncle wax. **a** Mass spectrum and major fragmentations of tritriacontane-2,4-dione. **b** Mass spectrum and major fragmentations of the *O*-methylhydroxylamine derivative of tritriacontane-2,4-dione

O-methylhydroxylamine. The product had an M ion 58 Da 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. 5b). 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. Finally, this structure was confirmed by reduction with LAH, leading to a new compound identified as (TMS-derivatized) tritriacontane-2,4-diol (see below also for **E**).

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. 6a), 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 [48], and an α-fragment with TMS transfer (*m/z* 115) suggesting a 2-keto function. An α-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 α-fragments were found to vary with homolog chain length (*m/z* 509 for the homolog in Fig. 6a), 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 (i.e., subterminal β-ketols). A summary of all identified homologs and their diagnostic MS fragments is presented in Table S1.

To further test the structure assignment, compounds **E** were transformed into acetates. The resulting derivatives showed pairs of α-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 in Fig. 6b). 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).

To specifically test the existence of a carbonyl functionality, a second aliquot of the fraction was derivatized with *O*-methylhydroxylamine and then silylated. The mass spectrum of the product (Fig. 6c) showed a homolog-independent α-fragment (*m/z* 188) and its product ions due to loss of CH_4 and CH_2O (*m/z* 172 and 158, respectively), with the first two ions shifted 29 Da 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 CH_3, CH_3O, and/or $(CH_3)_3SiOH$ 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 C_{33} homolog in Fig. 6c).

Lastly, reduction of compounds **E** with LAH followed by silylation resulted in TMS-derivatized 2,4-diols, in the case of the C_{33} homolog identical to that produced upon reduction of **D**. Their structure was confirmed by signature MS fragments *m/z* 73 $[(CH_3)_3Si]^+$ indicative of at least one hydroxyl, an ion *m/z* 147 $[(CH_3)_2SiOSi(CH_3)_3]^+$ suggesting a diol, and pairs of α-fragments indicating hydroxyl groups on C-2 (*m/z* 117 and, for the C_{33} homolog, *m/z* 625) and on C-4 (*m/z* 233 and, for the C_{33} homolog, *m/z* 509)

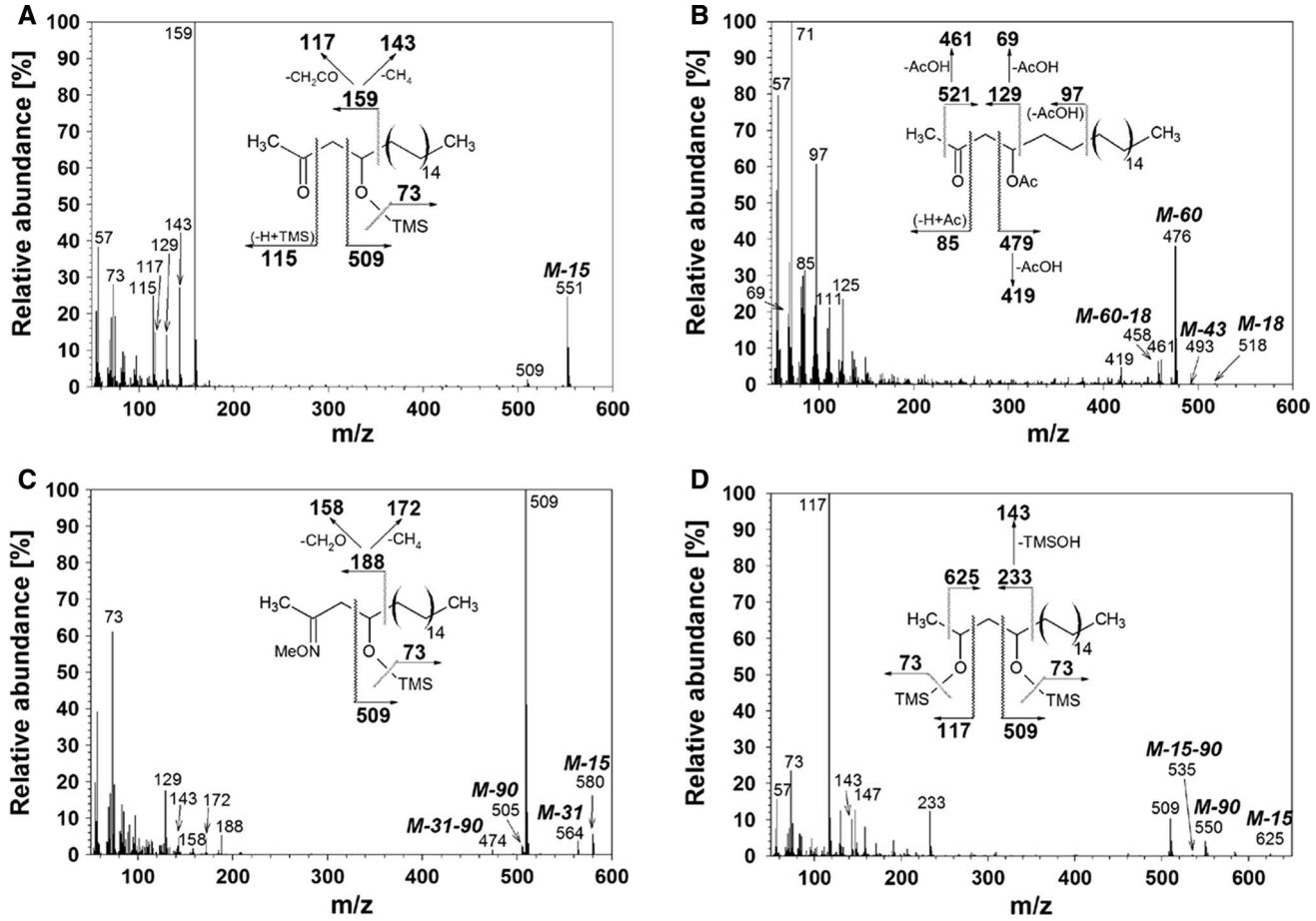

Fig. 6 Structure elucidation of subterminal β-ketols (2,4-ketols) **E** in wheat leaf and peduncle wax. **a** Mass spectrum and major fragmentations of the TMS derivative of C_{33} 2,4-ketol. **b** Mass spectrum and major fragmentations of the acetate ester of C_{33} 2,4-ketol. **c** Mass spectrum and major fragmentations of the *O*-methylhydroxylamine/TMS derivative of C_{33} 2,4-ketol. **d** Mass spectrum and major fragmentations of the TMS derivative of the diol resulting from LAH reduction of C_{33} 2,4-ketol

(Fig. 6d). Fragments due to loss of CH_3 and/or $(CH_3)_3SiOH$ 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 various derivatives unambiguously established **E** as a homologous series of odd-numbered 4-hydroxy-2-ketones, 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 (ca. 80%), lesser amounts of the C_{35} homolog (ca. 20%) and trace amounts of all other chain lengths.

Structure Elucidation of Compound Class F

Compound class **F** was represented by three homologous compounds in the TLC fraction running between β-diketones and alkyl esters, and, thus, of only moderate polarity. The mass spectra of compounds **F** lacked fragments characteristic of OH groups after treatment with BSTFA (such as *m/z* 73, 75), but instead exhibited α-fragments characteristic of ketones (Fig. 7a). Specifically, the set of α-fragments *m/z* 211 and *m/z* 267 (for the late-eluting homolog) 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 Da higher than the α-fragments (*m/z* 227 and 283; *m/z* 255). The molecular ion (*m/z* 450) indicated a C_{31} ketone chain length.

To further probe the presence and location of the carbonyl functionality, methoxime derivatives were prepared of **F**. The resulting compounds showed diagnostic methoxime fragments (*m/z* 87 and *m/z* 100) [20], α-fragments 29 Da 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. 7b). The oxime spectrum

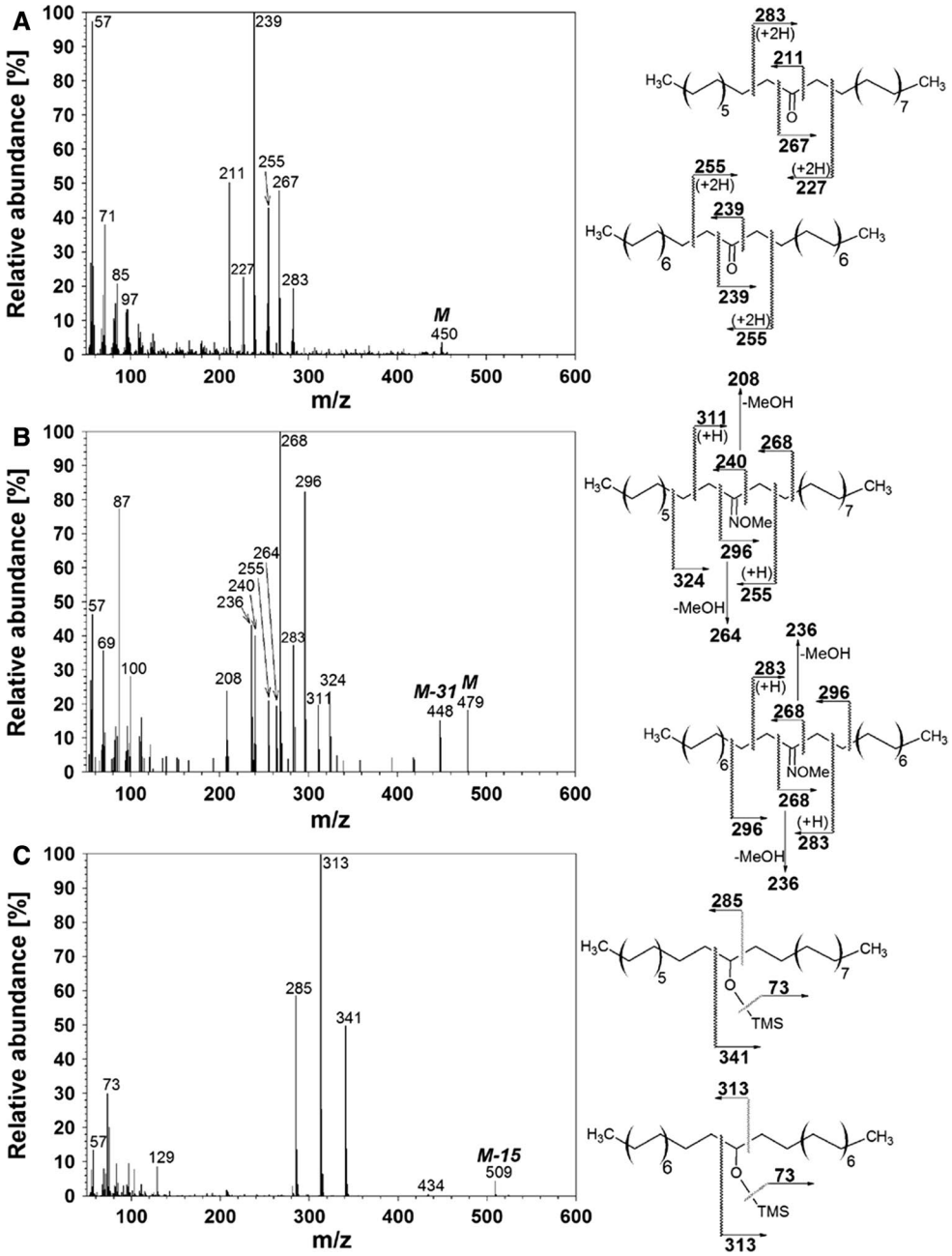

Fig. 7 Structure elucidation of mid-chain ketones **F** in wheat leaf and peduncle wax. **a** Mass spectrum and major fragmentations of the co-eluting ketone isomers hentriacontan-14-one and hentriacontan-16-one. **b** Mass spectrum and major fragmentations of the co-eluting *O*-methylhydroxylamine derivatives of hentriacontan-14-one and hentriacontan-16-one. **c** Mass spectrum and major fragmentations of the co-eluting TMS derivatives of the secondary alcohols resulting from LAH reduction of hentriacontan-14-one and hentriacontan-16-one

thus confirmed the presence of two isomeric ketones for the late-eluting homolog, and a chain length of C_{31} 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 LAH and the resulting isomeric secondary alcohols subjected to silylation. Their mass spectra closely resembled those of other secondary alcohols reported before [23], with an alcohol-characteristic fragment *m/z* 73 as well as α-fragments indicating the location of the hydroxyl function for the late-eluting homolog on C-14 (*m/z* 285 and *m/z* 341) or on C-16 (*m/z* 313) (Fig. 7c). Fragments due to loss of CH_3 or $(CH_3)_3SiOH$ from the molecular ion (M-15 and M-90) confirmed the C_{31} chain length of this homolog. All

taken together, our TLC and MS data thus unambiguously identified compounds **F** as a mixture of mid-chain ketones. Based on the relative abundances of carbonyl α-fragments from the underivatized compounds, it may be estimated that the fraction was dominated by the C_{29} ketone nonacosan-14-one (ca. 45%), accompanied by its isomer nonacosan-12-one (ca. 1%), the C_{27} ketones heptacosan-12-one (ca. 2%) and heptacosan-14-one (ca. 13%), as well as the C_{31} ketones hentriacontan-14-one (ca. 11%) and hentriacontan-16-one (ca. 28%).

Discussion

In this work, the structures of previously unidentified wheat wax constituents were elucidated using multiple lines of MS evidence, also in comparison with published MS information on analogous wax compounds. We discovered several new classes of compounds featuring two functional groups, varying in their relative geometry (α-configuration in compounds **B** and **C**, β-configuration in **A**, **D** and **E**), in their positions within the hydrocarbon chain (mid-chain functions in **A**, **B** and **C**, sub-terminal functions in **D** and **E**), and functional group oxidation states (two carbonyls in **B** and **D**, a hydroxyl and a carbonyl in **A**, **C** and **E**). Thus, each of the novel wheat wax compound classes shared some but not all structural features with several others, suggesting close biosynthetic relationships between them. Based on their structural commonalities and differences, potential biosynthetic pathways leading to them can now be hypothesized.

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 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. 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. Both these latter reactions may occur spontaneously but could also be enzyme-catalyzed, similar to the formation of methylketones in tomato trichomes [51]. Instead, the initial condensation of acyl-CoA and malonyl-CoA may be carried out by a KCS, and the resulting ketoacyl-CoA intermediate could be transferred to a PKS for a second condensation with malonate to the triketide.

Based on the structural similarity and matching major homolog chain length, the subterminal β-ketols (**E**) are very likely biosynthetically related to the wheat 2,4-diketone, where (a) the ketols may be formed by reduction of one carbonyl in the diketone or a diketo-precursor, (b) the diketone may conversely be an oxidation product of the corresponding ketol (or its precursors), or (c) both may be derived in parallel reactions starting with the keto- and hydroxyacyl-CoA intermediates of FAE elongation. All three scenarios imply a PKS that, based on the chain length profile of the wheat 2,4-ketols and 2,4-diketones (both peaking at C_{33}), appears to be relatively specific for C_{30} acyl-CoA or C_{32} ketoacyl-CoA substrates. It is thus distinguished from other PKS enzymes thought to participate in wheat wax formation, such as the PKS producing mid-chain β-diketones from C_{14} and/or C_{16} acyl substrates (see below) or the one implicated in alkylresorcinol formation from C_{24} acyl-CoA and similar precursors [43].

Interestingly, a homologous series of 2,4-diketones with chain lengths ranging from C_{25} to C_{31} had been identified in the waxes associated with suberin of Ericaceae roots [52], 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} [53].

It has long been recognized that β-diketones are polyketide in nature [54], and it was recently shown that their synthesis proceeds via hydrolytic intercept of a fatty acid synthesis intermediate (likely C_{16} β-ketoacyl-ACP) and a consecutive PKS-catalyzed condensation reaction [42]. By analogy, it may now be speculated that mid-chain β-ketols are formed by intercept of C_{16} β-hydroxyacyl-ACP, another intermediate of fatty acid elongation, followed by a PKS-mediated condensation. Either one or both of the reactions involved 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.

It should be noted that mid-chain β-ketols with structures very similar to those observed in wheat wax had previously been reported. Namely, C_{29} β-ketols were reported together with α-ketols, secondary alcohols and ketones in *Arabidopsis thaliana* stem wax [48] and in the leaf waxes of four *Brassica* species [47]. 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 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, the ketones may be viewed as derivatives of the mid-chain diketones, with one carbonyl group instead of two. This suggests that only one of the two reactions generating the diketo-functionalities may be carried out, while the other one is bypassed. Accordingly, either the β-ketoacyl intermediate may be hydrolyzed but then not condensed by a PKS (and elongated by FAEs instead), or the PKS may utilize fatty acyl substrates (instead of β-ketoacyls intercepted from FAE). 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 [55].

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* [48], four *Brassica* species [47], or the fern *Osmunda regalis* [56]. Notably, in all previous instances predominantly odd-numbered homologs of α-ketols were identified (especially C_{29}), suggesting a biosynthetic relationship to the (mainly odd-numbered) alkanes accompanying them. Accordingly, MAH1-catalyzed hydroxylation of alkane precursors (in Arabidopsis) was found to lead via secondary alcohols and ketones to α- and β-ketols [48, 55]. In stark contrast, the wheat α-ketols had even carbon numbers (C_{30}), hence, making formation from alkane precursors unlikely. Instead, the alkyl moieties involved, $C_{13}H_{27}$ and $C_{15}H_{31}$, may suggest a head-to-head condensation of C_{14} and C_{16} acyl precursors via an unknown mechanism involving rarely observed C–C bond formation between two carbonyl carbons.

In conclusion, six new classes of cuticular wax compounds were identified here in TLC fractions of wax mixtures coating flag leaf blades and peduncles of *Triticum aestivum* cv. Bethlehem. They included a homologous series of 2,4-ketols and a 2,4-diketone likely formed by a PKS with preference for C_{30} acyl-CoA or C_{32} ketoacyl-CoA substrates, thus distinguishing it from other PKSs implicated in the biosynthesis of mid-chain β-diketones and alkylresorcinols [54]. We further identified mid-chain ketones, β-ketols, α-ketols and α-diketones all derived from the β-diketone biosynthetic pathway, either as side products resulting from omission or addition of reaction steps, or as product combinations resulting from parallel reactions on hydroxy- and keto-substrates. Accordingly, the newly identified compounds have very similar mid-chain-functionalized structures, characterized by $C_{13}H_{27}$ and $C_{15}H_{31}$ alkyl chains attached to a cluster of functionalized carbons. Interestingly, this finding implies that the pathways leading to them divert from normal wax formation in a relatively early stage, through interception or transfer of C_{14} and C_{16} elongation intermediates. The proposed biosynthetic pathways thus differ drastically from those leading to very similar compounds with mid-chain functionalities in Arabidopsis and other Brassicaceae waxes.

Acknowledgements The authors are grateful to Ms. Shelly Hen-Avivi and Dr. Asaph Aharoni (Weizmann Institute of Science, Israel) for supplying wax samples for this work. Financial support received from the Natural Sciences and Engineering Research Council (Canada), the Canada Research Chairs Program, and the Canada Foundation for Innovation is also gratefully acknowledged.

Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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