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Very-long-chain secondary alcohols and alkanediols in cuticular waxes of *Pisum sativum* leaves

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Abstract

In cuticular waxes from leaves of *Pisum sativum*, 19 secondary alcohols, 10 primary/secondary alkanediols and three secondary/secondary alkanediols were identified by various chemical transformations with product assignment employing GC–MS. The homologous series of C_{29}–C_{33} secondary alcohols (1.1 μg/cm^2) was dominated by hentriacontanol isomers (94%). Only octacosanediols and trace amounts of hexacosanediols (<1%) were detected in the primary/secondary alkanediol faction (0.7 μg/cm^2). The secondary/secondary alkanediols (0.12 μg/cm^2) contained a single homologue with chain length C_{31}. All three compound classes showed characteristic isomer distributions with secondary functional groups predominantly located between C-14 and C-16. Based on the isomer compositions, the sequence of biosynthetic steps introducing the hydroxyl functions is discussed.

Keywords: Leaf surface; Chain length; Pea; GC–MS; Biosynthetic pathways

1. Introduction

The surfaces of leaves, flowers, fruits and nonwoody stems are covered with a cuticle that consists of cutin and waxes (Walton, 1990). The primary physiological function of the cuticle is to limit nonstomatal water loss, but it is also of ecological importance as it forms the outermost layer of the plant organs. The water-repellent surfaces, formed by the cuticular waxes, guard leaf surfaces from accumulation of particles, keep leaf surfaces dry and, thus, prevent the germination of pathogen spores.

The specific functions of plant cuticles can only be understood on the basis of their characteristic wax composition and biosynthetic origin. Some plant wax mixtures contain compounds with potential isomerism, including secondary alcohols, alkanediols, ketones, ketols and alkyl esters. All these compound classes are biosynthesized in various species with characteristic product chain lengths and positions of the functional groups. For example, nonacosan-10-ol, nonacosane-4,10-diol and nonacosane-5,10-diol typically accumulate in gymnosperm needle waxes (Franich et al., 1979; Riederer, 1989; Wen et al., 2006) and on leaves of *Nelumbo nucifera* (Barthlott et al., 1996), nonacosan-14-ol, nonacosan-15-ol and nonacosan-15-one are found on leaves of *Brassica oleracea* (Holloway and Brown, 1977) and on stems of *Arabidopsis thaliana*; hentriacontan-12-ol has been described together with corresponding secondary/secondary diols for *Myricaria germanica* (Jetter, 2000); primary/secondary alkanediol isomers are associated with primary and secondary alcohols in Papaveraceae (Jetter and Riederer, 1996).

Based on isomer similarities between all the compounds co-occurring in the same species, it has been postulated that secondary alcohols serve as precursors for the biosynthesis of both corresponding ketones and secondary/secondary alkanediols (Franich et al., 1979). While the biosynthetic relationship between wax secondary alcohols and ketones have been verified biochemically, direct evidence for the

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pathways leading to wax alkanediols is lacking to date. In particular, the order in which both hydroxyl functions are introduced remains to be determined. The hydroxyl functions might be introduced at two stages in the biosynthetic pathway, either relatively early in the course of elongation of fatty acyl chains, or later in the process through hydroxylation of CH_2 groups (likely catalyzed by P450-dependent enzymes). Complete chemical analyses of the alcohol and alkanediol isomers in diverse plant species are necessary to further substantiate the hypothetical hydroxylation steps and to identify suitable model systems for molecular genetic and biochemical investigations.

Recently, the chemical composition of waxes on the leaf surfaces of four pea (*Pisum sativum*) cultivars has been investigated in much detail (Gniwotta et al., 2005). The adaxial wax consisted mainly of primary alcohols (71%), with strong predominance of hexacosanol and octacosanol, while the abaxial wax was characterized by very high amounts of alkanes (73%), in particular hentriacontane. In addition, small amounts of fatty acids, aldehydes, and alkyl esters were detected on both sides of the leaf. Secondary alcohols with unbranched, fully saturated very-long-chain structures accumulated in the abaxial wax. Hentriacontan-16-ol and hentriacontan-15-ol were reported to dominate this compound class in a 2:1 ratio, with trace amounts of hentriacontan-14-ol and of nonacosan-13-ol, -14-ol, and -15-ol being present. A number of compounds in the pea wax mixtures remained unidentified and were suspected to be very-long-chain alcohols and alkanediols.

The objective of the present work was to identify these hydroxy compounds, to compare their isomer patterns with the composition of other wax constituent classes, and to infer biosynthetic relationships between them. In particular, we wanted to address the question whether the chemical structure of the pea alcohols and alkanediols would point towards introduction of hydroxyl groups during elongation or by alkyl hydroxylation. In order to elucidate the structure of the wax alcohols and alkanediols, they were transformed into various derivatives and investigated by GC–MS.

2. Results and discussion

The mixture of cuticular wax was prepared by surface extraction of pea leaves with $CHCl_3$. TLC separation yielded a number of fractions with all the previously reported wax constituents and two bands containing unidentified compounds. One of them, designated as compound class **A** (R_f 0.79), co-migrated with a secondary alcohol standard. Another band (R_f 0.16), migrating between primary alcohols and fatty acids, appeared to contain two compound classes **B1** and **B2** according to their GC-retention behaviour and MS characteristics.

2.1. Secondary alcohols

It appeared likely that the constituents of compound class **A** had one secondary hydroxyl function, as they co-migrated with a secondary alcohol standard. Within fraction **A**, five distinct peaks were separated by GC after TMSi-derivatization with bis-(*N*,*N*-trimethylsilyl)-trifluoroacetamide (BSTFA). All five MS showed fragments characteristic for alcohol TMSi ethers at *m*/*z* 73, 75 and 103, but lacked a diol signal *m*/*z* 147, indicating the presence of only one hydroxyl function (Table 1). The five peaks were further characterized by molecular ions $[CH_3(CH_2)_nCH_2OTMSi]^+$ differing by 14 mass units, and by corresponding fragments $[M-15]^+$ indicating the loss of a methyl group from the TMSi derivatives. All the present evidence taken together, fraction **A** was identified as a homologous series of secondary alcohols with chain lengths C_{29}–C_{33}. GC-FID quantification (Table 2) showed that the C_{31} homologue dominated (94%), while secondary alcohols with other chain lengths contributed only minor amounts (1.4–2.2%). This result is in good accordance with

Table 1
Mass spectral data of secondary alcohol TMSi ethers

Compound	Common fragments (relative intensity)			Characteristic fragments (relative intensity)	
Nonacosan-13-ol	73 (25.6)	75 (16.1)	103 (6.8)	271 (2.3)	327 (2.2)
Nonacosan-14-ol	481 (4.7)			285 (50.7)	313 (51.9)
Nonacosan-15-ol				299 (100)	
Triacontan-13-ol	73 (19.5)	75 (12.1)	103 (6.8)	271 (1.1)	341 (1.3)
Triacontan-14-ol	495 (5.1)	510 (0.2)		285 (10.4)	327 (10.8)
Triacontan-15-ol				299 (100)	313 (97.3)
Dotriacontan-14-ol	73 (15.7)	75 (13.1)	103 (3.1)	285 (5.7)	355 (5.8)
Dotriacontan-15-ol	523 (3.8)			299 (12.0)	341 (15.8)
Dotriacontan-16-ol				313 (100)	327 (94.6)
Tritriacontan-13-ol	73 (6.4)	75 (14.2)	103 (7.4)	257 (0.2)	383 (4.5)
Tritriacontan-14-ol	537 (6.0)			285 (18.2)	369 (14.4)
Tritriacontan-15-ol				299 (25.2)	355 (39.7)
Tritriacontan-16-ol				313 (36.9)	341 (50.8)
Tritriacontan-17-ol				327 (100)	

Table 2
Relative composition (%) of the secondary alcohols, secondary/secondary alkanediols, primary/secondary alkanediols, primary alcohols and alkanes in the total wax of *Pisum sativum* leaves

Chain length	Homologue composition	Isomer composition						Homologue composition[a]
		C-12	C-13	C-14	C-15	C-16	C-17	
Secondary alcohols								*Alkanes*
C_{29}	1.9 ± 0.6		2.7 ± 0.3	48.1 ± 0.8	49.2 ± 0.9			1.4 ± 0.3
C_{30}	2.2 ± 0.8		1.0 ± 0.2	10.6 ± 0.3	88.5 ± 0.2			1.2 ± 0.04
C_{31}	93.9 ± 2.3	0.2 ± 0.01	0.2 ± 0.02	2.0 ± 0.1	35.6 ± 0.2	62.0 ± 0.3		94.7 ± 0.4
C_{32}	1.4 ± 0.7			4.0 ± 0.3	13.8 ± 1.0	82.2 ± 0.9		1.0 ± 0.1
C_{33}	1.4 ± 0.6		1.7 ± 0.7	13.3 ± 1.3	23.9 ± 0.8	41.0 ± 1.5	20.0 ± 0.7	1.6 ± 0.05
					C-8[b] C-10[b,c]	C-9[b]		
Secondary/secondary alkanediols								
C_{31}	100				8.4 ± 0.4 7.0 ± 0.3	84.6 ± 0.5		
Primary/secondary alkanediols								*Primary alcohols*
C_{26}	tr[d]	tr	tr	tr	tr			56.6 ± 3.3
C_{28}	100	0.82 ± 0.1	15.8 ± 0.6	53.3 ± 0.7	24.6 ± 0.2	4.8 ± 0.2	0.7 ± 0.1	34.2 ± 2.7

Mean values ($n = 5$) and SE are given for the percentage (%) of isomers within individual homologues.
[a] Calculated from coverages (μg/cm^2) reported by Gniwotta et al. (2005).
[b] Position of functional group introduced in second hydroxylation step.
[c] Hentriacontane-10,17-diol is shown under C-15 as it is identical with "hentriacontane-15,22-diol".
[d] Traces, i.e. less than 0.1% of the fraction detectable.

previous reports on the chain length distribution of secondary alcohols in pea wax (Gniwotta et al., 2005; Holloway et al., 1976).

Within the C_{31} homologue peak, the isomer hentriacontan-16-ol could be unambiguously identified due to the presence of an α-fragment at *m/z* 299 ($[CH_3(CH_2)_{14}CHOTMSi]^+$), a molecular ion of *m/z* 524 and the corresponding fragment $[M-15]^+$ at *m/z* 509. This finding confirms previous reports describing hentriacontan-16-ol as the most abundant secondary alcohol in the wax of pea leaves (Gniwotta et al., 2005; Holloway et al., 1976). The MS from the same GC peak contained a series of other α-fragments $[CH_3(CH_2)_nCHOTMSi]^+$, suggesting the presence of various other C_{31} alcohol isomers. Pairs of these fragments, resulting from the cleavage of the α-bonds on both sides of the functionality, were used to identify hentriacontan-12-ol, -13-ol, -14-ol and -15-ol. In this way, all the major fragments in the mixed MS of this GC peak could be assigned. While hentriacontan-14-ol and -15-ol had previously been identified in pea leaf wax (Gniwotta et al., 2005; Holloway et al., 1976), the other two isomers were only known to occur in Barley spike wax (von Wettstein-Knowles and Netting, 1976). The other four secondary alcohol homologues in fraction **A** were also found to contain multiple isomers. They were identified, according to the α-fragments of their TMSi derivatives, as nonacosan-13-ol, -14-ol and -15-ol, triacontan-13-ol, -14-ol and -15-ol, dotriacontan-14-ol, -15-ol and -16-ol, as well as tritriacontan-13-ol, -14-ol, -15-ol, -16-ol and 17-ol. Among these secondary alcohols, dotriacontan-14-ol, -15-ol and -16-ol, as well as tritriacontan-15-ol, -16-ol and 17-ol have not been described before.

Due to the relatively small differences in molecular geometry, the various isomers within each secondary alcohol homologue could not be separated by GC. Therefore, the relative amounts of isomers had to be quantified by MS using the intensity of α-ions (Table 2). In the total wax extract isomers predominated that carried the hydroxyl functions on the central carbon of the alkyl chain or on the adjacent carbon. Within the different homologues, the C_{30} and C_{32} secondary alcohols contained mainly one isomer, triacontan-15-ol (88%) and dotriacontan-16-ol (82%), respectively. In contrast, the homologues with odd-numbered chain lengths had more even distributions of various isomers. Among the C_{31} alcohols, hentriacontan-16-ol dominated (62%), while the C_{33} alcohols contained tritriacontan-15-ol, -16-ol and 17-ol in a ratio of approximately 1:2:1, and the C_{29} alcohols contained nearly equal amounts of nonacosan-14-ol and -15-ol. The secondary alcohol fraction accounted for 4.7 ± 0.1% of the total wax extract from *P. sativum* leaves (24.3 ± 2.9 μg/cm^2; Gniwotta et al., 2005). Adaxial and abaxial waxes, amounting to 16.9 ± 1.0 μg/cm^2 and 25.3 ± 1.2 μg/cm^2 (Gniwotta et al., 2005), were found to contain 0.3 ± 0.01% and 7.3 ± 0.5% of secondary alcohols, respectively. Wax extracts from both surfaces of the leaves had very similar secondary alcohol compositions (data not shown).

2.2. Secondary/secondary alkanediols

Fraction **B** migrated between primary alcohols and fatty acids on TLC (R_f 0.16), and was therefore expected to contain compounds with two (or more) functional groups of intermediate polarity (Jetter and Riederer, 1999). In the GC trace of fraction **B**, one peak **B1** had special retention behaviour and could hence not be assigned to any homologous series. The MS of its TMSi ether (Fig. 1a) showed alcohol fragments *m/z* 75 and 103 together with a fragment *m/z* 147 that indicates the presence of two (or more) OTMSi

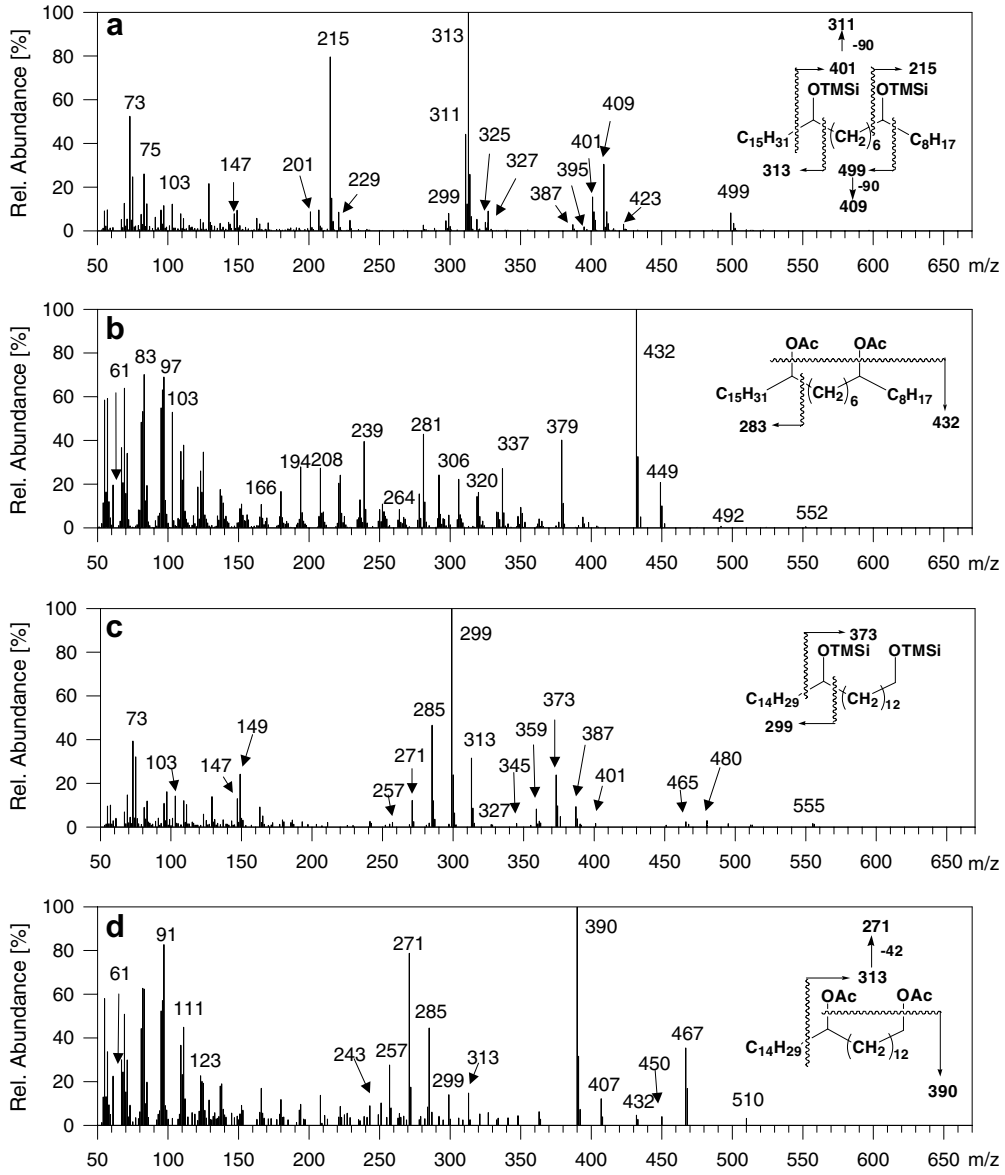

Fig. 1. Mass spectra of representative derivatives of alkanediols in the leaf wax of *Pisum sativum*. (a) bis TMSi ether of secondary/secondary hentriacontanediol isomers, (b) bis acetate of secondary/secondary hentriacontanediol isomers, (c) bis TMSi ether of primary/secondary octacosanediol isomers, and (d) bis acetate of primary/secondary octacosanediol isomers.

groups (Richter and Burlingame, 1968). Assuming an alkanediol structure, characteristic mass differences between four ions belonging to two types of ions $[C_nH_{2n}OTMSi]^+$ and $[C_mH_{2m-1}(OTMSi)_2]^+$ allowed the assignment of the low abundance signals for the molecular ion at *m/z* 612, and corresponding fragments $[M-CH_3]^+$ at *m/z* 597, $[M-TMSiOH]^+$ at *m/z* 522, and $[M-(TMSiOH)_2]^+$ at *m/z* 432. The alkanediol structure of the compound was further verified by daughter ions $\Delta m/z$ −90 $[-TMSiOH]^+$ accompanying the four α-fragments. **B1** was therefore tentatively identified as a C_{31} alkanediol, giving rise to a bis TMSi ether derivative $C_{31}H_{62}(OTMSi)_2$.

The α-ions *m/z* 215 and 313 matched the compositions of $[C_9H_{18}OTMSi]$ and $[C_{16}H_{32}OTMSi]$, respectively, thus indicating the presence of hydroxyl groups on carbons C-9 and C-16 of the hentriacontanediol backbone. This isomer assignment was confirmed by the two other α-fragments at *m/z* 401 $[C_{16}H_{31}(OTMSi)_2]^+$ and 499 $[C_{23}H_{45}(OTMSi)_2]^+$. All the data taken together, this compound was identified as the secondary/secondary diol hentriacontane-9,16-diol.

Alternative structures for **B1**, containing either additional hydroxyl or carbonyl groups, could also give rise to the fragmentation characteristics described so far for this fraction. However, these other structures can be excluded for two reasons: (i) the presence of additional hydroxyl groups seems unlikely, as ions $[C_nH_{2n-2}(OTMSi)_3]^+$ could not be detected in the MS of the TMSi derivative and (ii) the presence of a carbonyl group can also be excluded, because $LiAlH_4$ treatment left fraction

B1 unchanged (identical MS of the resulting BSTFA derivative, data not shown).

The presence of several other α-ions indicated that fraction **B1** also contained a number of C31 alkane diol isomers besides hentriacontane-9,16-diol. Based on the signals at *m/z* 201, 327, 387 and 513 and at *m/z* 229, 299, 415 and 485, hentriacontane-10,17-diol and hentriacontane-8,15-diol could be identified, respectively. To corroborate the assignment of all the different α-fragments to the various C31 alcohol isomers, all the relevant single ion traces were calculated for multiple GC–MS runs of **B1**. Each resulting extracted chromatogram showed a single peak, with those of ions that had been assigned to the same isomer coinciding. Overall, three partially separated peaks were detected, indicating that hentriacontane-10,17-diol eluted shortly before, and hentriacontane-8,15-diol shortly after the predominant isomer of hentriacontane-9,16-diol. This result matches previous reports, for example demonstrating similar partial GC separation of isomers for C_{33} secondary alcohols in *Myricaria germanica* (Jetter et al., 2006).

The secondary/secondary alkanediol structure of fraction **B1** was finally corroborated by transforming the hydroxyl groups into acetate esters. The reaction product was subjected to BSTFA derivatization after acetylation, and the resulting MS indicated complete acetylation, as it lacked OTMSi signals at *m/z* 73 and 75 while showing a prominent OAc ion *m/z* 61 (Fig. 1b). Interestingly, a prominent ion at *m/z* 103 was detected in the acetate MS, even though it is known to be characteristic for TMSi ethers. To verify that this fragment was due to acetate fragmentation, the MS of acetates of three standard compounds were acquired (data not shown). The bis acetates of nonacosane-5,10-diol and octacosane-1,9-diol both showed significant ion counts for *m/z* 103, while this was not observed for nonacosan-11-ol acetate. Hence, this fragment can be considered an elimination product $[AcOHAc]^+$ characteristic for alkanediol bis acetates. In the high mass region of the acetate MS of **B1**, a series of ions *m/z* 552, 492, 449 and 432 was detected, and can be interpreted as M^+, $[M-AcOH]^+$, $[M-AcOH-Ac]^+$ and $[M-(AcOH)_2]^+$, respectively, of hentriacontanediol bis acetate. This experiment thus confirms both the alkanediol structure and the C_{31} carbon backbone of **B1**. The presence of α-fragments *m/z* 269 $[CH_3(CH_2)_{11}CHOAc]^+$, 283 $[CH_3(CH_2)_{12}CHOAc]^+$ and 297 $[CH_3(CH_2)_{13}CHOAc]^+$ further corroborated the presence of multiple isomers hentriacontane-9,16-diol, -8,15-diol and -10,17-diol.

Due to the poor separation of the alkanediol isomers, they could not be quantified by GC-FID. Hence, similar to the quantification of secondary alcohol isomers, the percentage of the various hentriacontanediols in the total leaf extract was calculated by normalizing the relative intensity of α-fragments. Hentriacontane-9,16-diol accounted for 85% of the three isomers (Table 2), while hentriacontane-8,15-diol and -10,17-diol accounted for 8% and 7%, respectively. The secondary/secondary diol fraction accounted for $0.5 \pm 0.1\%$ of the total wax extract from pea leaves. Adaxial and abaxial waxes contained $0.2 \pm 0.1\%$ and $0.7 \pm 0.1\%$ of secondary/secondary alcohols, respectively, and had very similar secondary/secondary diol compositions (data not shown).

All three hentriacontanediol isomers described here have not been reported for plant cuticular waxes before. Most of the wax alkanediols previously described had chain length C_{29}, and were characterized by the presence of one functional group either on C-10 or on C-15. As examples for the first group, nonacosane-4,10-diol and -5,10-diol are typically found as wax constituents in gymnosperms (Riederer, 1989; Franich et al., 1979). Very recently, direct sampling of epicuticular wax provided evidence that these two constituents were the major compounds in tubular crystals on *Taxus baccata* needles (Wen et al., 2006). As examples for the second group of secondary/secondary alkanediols, nonacosane-14,15-diol had been identified in leaf wax of *Brassica* species together with its ketol derivative 14-hydroxy-nonacosan-15-one (Holloway and Brown, 1977).

2.3. Primary/secondary alkanediols

Fraction **B2** contained two homologues, likely of compounds with two hydroxyl functions as they co-migrated with the hentriacontanediols **B1** on TLC. The TMSi derivative of the more abundant homologue (Fig. 1c) accordingly showed MS fragments at *m/z* 73, 75, 103 and 147 characteristic for alkanediols. Moreover, the prominent signal *m/z* 149 indicated a diol geometry with one primary and one secondary functional group (Jetter and Riederer, 1999). The primary/secondary alkanediol structure was further supported by the presence of (only) two α-fragments $[C_nH_{2n}OTMSi]^+$ and $[C_mH_{2m-1}(OTMSi)_2]^+$ (Jetter and Riederer, 1999). To test for the presence of carbonyl groups, the constituents of fraction **B2** were treated with $LiAlH_4$, followed by BSTFA derivatization. The resulting MS data were identical to the TMSi ethers without $LiAlH_4$ treatment, showing that no functions other than hydroxyl groups were present. In conclusion, a molecular ion and corresponding fragment $[M-15]^+$ could be assigned, and the predominant homologue in fraction **B2** was identified as primary/secondary octacosanediol (C_{28}).

The TMSi MS data showed several α-fragments within each of the two groups $[C_nH_{2n}OTMSi]^+$ and $[C_mH_{2m-1}(OTMSi)_2]^+$ differing by 14 mass units, indicating the presence of several isomers. The two types of α-ions represented opposite ends of the alkanediol molecules, as they originated from cleavage of the two C–C bonds adjacent to the secondary functional group. Therefore, pairs of α-ions from both types belonged to the same isomer and could be recognized based on their identical relative abundance. Matching pairs confirmed the molecular weight of the alkanediol homologues, and accounted for all the α-fragments detected. Thus, the α-ions of the TMSi ethers provided independent confirmation for the

alkanediol structures of **B2**. Overall, isomeric primary/secondary octacosanediols with secondary hydroxyl groups between C-12 and C-17 were identified. Although the second homologue was present at relatively low concentration, its TLC and GC behaviour together with the characteristic fragmentation pattern of its TMSi ether (data not shown) allowed an unambiguous structure assignment. The overall chain length was found to be C_{26} and isomers ranging from hexacosane-1,12-diol to hexacosane-1,15-diol were identified.

To further corroborate the alkanediol structures, compounds in **B2** were treated with acetic anhydride. The resulting products had a prominent MS fragment *m*/*z* 61, interpreted as $[AcOH_2]^+$ and therefore diagnostic for acetates. Furthermore, the fragments *m*/*z* 467 [M−43], *m*/*z* 450 [M−60], *m*/*z* 432 [M−43−60] and *m*/*z* 390 [M−120], involving the loss of one and two acetyl moieties, were characteristic for bis acetates of alkanediols. Based on these series of fragments the ions at *m*/*z* 482 and *m*/*z* 510 in the two homologues of **B2** could be interpreted as the molecular ions of bis acetates of hexacosanediol and octacosanediol, respectively, confirming the presence of exactly two hydroxyl functions (Fig. 1d). Different from the fragmentation pattern of secondary/secondary diol acetates, the MS data showed prominent daughter fragments Δ*m*/*z* −42 below the α-ions. The acetate of an octacosane-1,9-diol standard showed a very similar fragmentation pattern (*m*/*z* 201), whereas no corresponding fragments were observed for the acetate of a nonacosane-5,10-diol standard (data not shown). Hence, the daughter fragments Δ*m*/*z* −42 are diagnostic for α-ions containing a terminal OAc group, probably due to loss of $CH_2{=}C{=}O$. Finally, octacosane-1,14-diol acetate was synthesized to verify the structure assignment. Both the GC characteristics and MS fragmentation pattern were identical to one isomer in the higher homologue of compound class **B2** (data not shown).

The total leaf wax contained 0.3 ± 0.03% primary/secondary alkanediols, corresponding to a coverage of 0.7 μg/cm^2 (Gniwotta et al., 2005). Based on the relative abundance of α-fragments in the MS of TMSi ethers of both homologues, the percentages of the alkanediol isomers could be assessed (Table 2). Within the C_{28} homologue, octacosane-1,14-diol was the most prominent isomer (53%), while octacosane-1,13-diol and -1,15-diol contributed another 16% and 25%, respectively. Due to the very low amount of hexacosanediols, their isomers could not be quantified. To our knowledge, hexacosane-1,12-diol, -1,15-diol and octacosane-1,16-diol have not been reported previously. Primary/secondary alcohols amounted to 0.6 ± 0.1% of the adaxial wax mixture, while they could not be detected in extracts from the abaxial side of the leaf.

2.4. Biosynthesis of secondary alcohols and alkanediols

It is generally accepted that in *Brassica oleracea* and *Arabidopsis thaliana* the secondary alcohol nonacosan-15-ol is biosynthesized by direct hydroxylation of the corresponding alkane. Analogously, the secondary alcohols in *Pisum sativum* leaf wax are very likely biosynthesized from corresponding alkanes, as the chain length distributions of alkanes (Gniwotta et al., 2005) and secondary alcohols were found to be very similar (Table 2). Both compound classes showed complete homologous series C_{29}–C_{33} with dominant chain length C_{31}. Further evidence supporting a biosynthetic relationship of these two compound classes comes from the finding that both alkanes and secondary alcohols accumulate to relatively high concentrations in the abaxial wax, but not in the adaxial wax. It can be concluded that an alkane hydroxylase, utilizing the available alkane substrates with little chain length specificity, is involved in wax formation of pea leaves. This implies that the secondary hydroxyl function is introduced relatively late on the biosynthetic pathway (Fig. 2), similar to secondary alcohol biosynthesis in Brassicaceae (Kolattukudy et al., 1973).

As an alternative hypothesis, it has been suggested that the secondary functional groups of some wax constituents, e.g. β-diketones, are introduced during acyl chain elongation on a polyketide pathway (von Wettstein-Knowles, 1995). Due to the repeated elongation by C_2 units, the resulting structures must carry functional groups on specific carbon atoms and have characteristic distances (odd numbers of methylene units) between them. However, all the *P. sativum* wax compounds with secondary functional groups occurred as isomeric mixtures with hydroxyl groups on various adjacent carbons, and the diols had hydroxyl groups in distances other than those characteristic for polyketides. The current chemical results thus exclude the possibility that the secondary hydroxyl function is introduced during elongation.

In vitro assays using *Brassica oleracea* leaf discs showed that alkane hydroxylation in this system occurs with only limited regioselectivity, as substantial amounts of both isomeric secondary alcohols nonacosan-14-ol and nonacosan-15-ol were formed (Kolattukudy et al., 1973). In this context, it is noteworthy that the diverse secondary alcohol homologues in pea leaf wax had a relatively narrow isomer distribution. This result suggests that the hydroxylase(s) in *Pisum sativum* is (are) able to selectively hydroxylate alkane substrates at certain carbon positions. Based on relative isomer abundances, we propose two factors contributing to the product regiospecificity of the hydroxylase: (i) it shows high preference for the central carbon in odd-numbered homologues, e.g. for hentriacontan-16-ol (62%), or the carbon next to the centre of even-numbered chains, e.g. for triacontan-15-ol (88%) and dotriacotan-16-ol (82%) and (ii) hydroxylation occurs preferentially on C-16, e.g. for nonacosan-14-ol (alias "nonacosan-16-ol") and tritriacontan-16-ol. Future enzymatic studies will have to determine the relative importance of these factors.

The three secondary/secondary hentriacontanediols identified in *P. sativum* wax had striking similarity to the secondary alcohols, sharing the predominant C_{31} chain

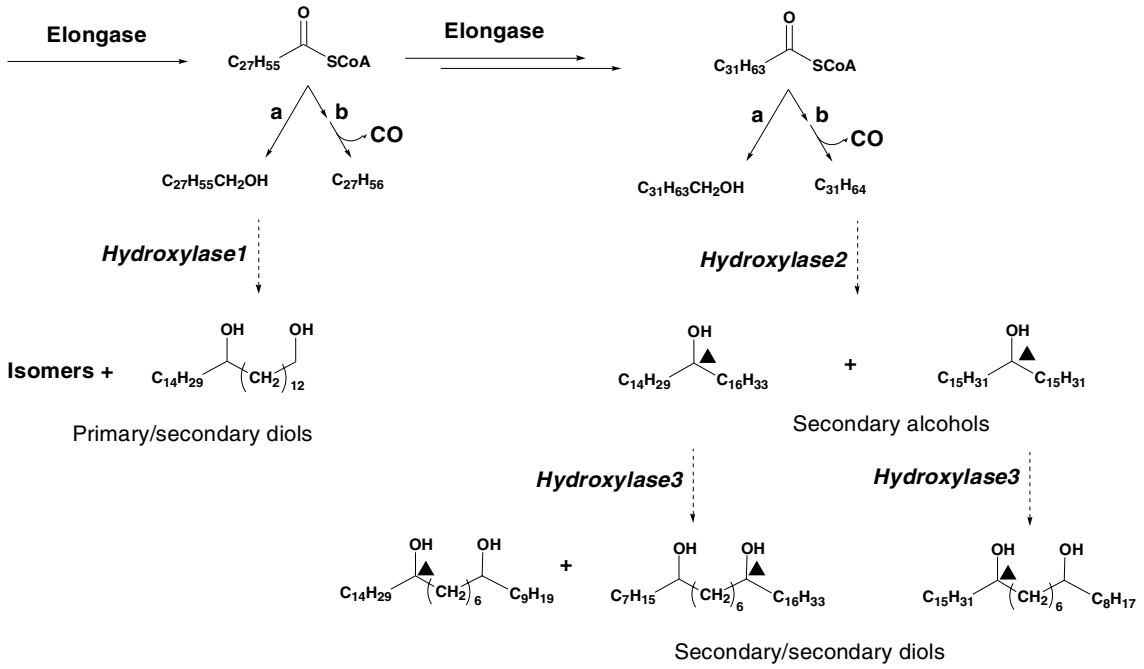

Fig. 2. Proposed biosynthetic pathways leading to secondary alcohol, primary/secondary and secondary/secondary alkanediol isomers in the leaf wax of *Pisum sativum*. Only octacosanediols and hentriacontanediols are depicted as examples. After the acyl CoAs are elongated to certain chain lengths, primary alcohols and alkanes are produced through the (a) acyl reduction and (b) decarbonylation pathways (arrows), respectively. Primary/secondary alkanediol isomers and secondary alcohol isomers are proposed to result from hydroxylation of primary alcohols and alkanes by hydroxylase1 and hydroxylase2, respectively (dashed arrows). The resulting secondary alcohols may then be further functionalized by hydroxylase3 to secondary/secondary alkanediols. The carbons with hydroxyl groups originating from secondary alcohols are highlighted by black triangles. Based on the present results, it cannot be decided whether the three hydroxylase activities shown here are due to identical or distinct but similar enzymes.

length and one hydroxyl function on either the central carbon (C-16) or the carbon next to it. Furthermore, these diols and the secondary alcohols were found at relatively high concentrations only in the wax on the abaxial side of the leaves. It is therefore very plausible that both compound classes are biosynthetically related, the alcohols serving as precursors for direct hydroxylation to the corresponding diols (Fig. 2). Accordingly, hentriacontan-15-ol likely is the precursor for the formation of hentriacontane-8,15-diol and -10,17-diol, while hentriacontan-16-ol is the intermediate en route to hentriacontane-9,16-diol. This hypothesis implies that both hydroxyl functions of the secondary/secondary diols are introduced directly into a pre-existing alkyl chain, the first hydroxylation taking place on C-15/C-16 and a second hydroxylation occurring in a distance of eight methylene units away from it. The alternative sequence of hydroxylation steps seems unlikely as no hentriacontan-8-ol, -9-ol or -10-ol isomers were found. Our wax chemical data suggest that the second hydroxylation step occurs with substrate- and regioselectivity. Hentriacontan-16-ol and hentriacontan-15-ol occurred in the wax at a ratio of 2:1, while the isomer ratio of the corresponding diol products was 5:1, indicating a preference for hentriacontan-16-ol as a substrate. The constant distance between both OH functions in the diols further suggests that the hydroxylating enzyme is able to selectively introduce the second function depending on the position of the first hydroxyl group. Future biochemical experiments will show whether both consecutive hydroxylation steps are carried out by one or two enzymes.

The primary/secondary alkanediols in *P. sativum* had exclusively even-numbered chain lengths matching the homologue pattern of the primary alcohols that mainly co-occurred on the adaxial side of the leaf (Gniwotta et al., 2005). These results suggest that the primary alcohols serve as the substrates for further hydroxylation towards those diols (Fig. 2). The primary function originates from the acyl group generated by elongation, while the secondary function is only later introduced by oxidation of a methylene unit. This hydroxylation step has a preference for carbons near the centre of the substrate chain, and might thus be catalyzed by a hydroxylase similar or identical to the enzyme involved in secondary alcohol synthesis. The alternative order of steps, invoking secondary alcohols as precursors for chain-end hydroxylation, is very unlikely as both secondary alcohols with chain lengths C_{26} or C_{28} and primary/secondary alkanediols corresponding to the C_{29}–C_{33} secondary alcohols were absent.

In conclusion, secondary alcohols and secondary/secondary alkanediols of pea leaf wax are likely biosynthetically related with alkanes. We hypothesize that both functional groups are introduced sequentially and with certain specificity first near the centre of the alkyl chain, and then in a constant distance from it. Our results further suggest that the primary/secondary alkanediols of *P. sativum* leaf wax are biosynthesized independently using primary

alcohols as substrates. The secondary functional group of these diols might be introduced by an enzyme similar or identical to the one involved in secondary alcohol biosynthesis.

3. Experimental

3.1. Wax extracts

Seeds of *P. sativum* cv Avanta were obtained from IPK Gatersleben Genebank, Germany. Plants were grown in soil:sand (1:1) mixed with 50% vermiculite in plastic pots (diameter 9 cm). The plants were kept in growth chambers under the following conditions: day/night 14 h/10 h; light intensity 300–400 μmol photons/m^2s; temperature 22 °C/18 °C; relative humidity 70%. For analysis, fully expanded 13 d-old leaves were harvested randomly (BBCH macro stage 1, Code 15; Lancashire et al., 1991). Total leaf wax extracts were obtained by dipping entire leaves twice for 30 s into 100 ml $CHCl_3$ that had been heated to approximately 40 °C. The resulting leaf extracts were used for identification of compounds and for quantification of isomers and homologues within compound classes. Percentages of compound classes in the total wax mixture, extracted either from both sides of the leaves together or from a single side only, were calculated after assigning novel compounds in a previously acquired dataset (Gniwotta et al., 2005). In both experiments, identical conditions were used for plant growth and wax sampling.

3.2. Wax qualitative and quantitative analyses

Compound classes were separated by TLC (sandwich technique (Tantisewie et al., 1969), silica gel, mobile phase $CHCl_3$) and localized by staining with primuline and UV-light. Bands were removed from the plates, eluted with $CHCl_3$, filtered, concentrated in a stream of N_2 and stored at 4°C. Two fractions **A** (R_f 0.79) and **B** (R_f 0.16) contained unknown compounds and were subjected to detailed qualitative analyses. To this end, constituents were studied with capillary GC (5890N, Agilent, Avondale, PA, USA; column 30 m HP-1, 0.32 mm i.d., df = 0.1 μm) with He carrier gas inlet pressure programmed for constant flow of 1.4 ml/min and mass spectrometric detector (5973N, Agilent). GC was carried out with temperature programmed injection at 50 °C, oven 2 min at 50 °C, raised by 40 °C/min to 200 °C, held for 2 min at 200 °C, raised by 3 °C/min to 320 °C and held for 30 min at 320 °C.

Relative compositions (weight%) both of homologues and of isomers within individual homologues were quantified based on GC runs of five independently prepared samples from one leaf wax extract. Homologues were quantified by GC-FID (same GC conditions as described above). Isomers were quantified based on relative abundance of characteristic fragments of MS.

3.3. Derivatization reactions

Constituents of fractions **A** and **B** were derivatized in three alternative reactions: (1) compounds containing free hydroxyl groups were transformed into TMSi ethers by reaction with bis-(*N*,*N*-trimethylsilyl)-trifluoroacetamide (BSTFA) in pyridine for 30 min at 70 °C; (2) hydroxyl groups were acetylated by adding pyridine and Ac_2O to the dried fraction, heating the mixture to 70 °C for 5 min, keeping it at RT overnight. The products were isolated by addition of H_2O and extraction with $CHCl_3$; and (3) the unknown constituents were subjected to reduction by excess of $LiAlH_4$ in refluxing tetrahydrofuran overnight, hydrolysis with 10% H_2SO_4, and extraction of the solution with $CHCl_3$.

3.4. Synthesis of reference compound octacosane-1,14-diol

1,14-Tetradecanediol (Fluka, Buchs, Switzerland) was oxidized into tetradecanedial by pyridinium chlorochromate at room temperature for 1 h in anhydrous CH_2Cl_2. Tetradecanedial was purified on TLC, and then reacted with tetradecylmagnesium chloride (1.0 M solution in tetrahydrofuran, Sigma–Aldrich, MO, USA) at 50 °C for 4 h. The reaction was quenched by addition of water. The product mixture was isolated by extraction with $CHCl_3$, reduced with $LiAlH_4$, and then acetylated as described above with acetic anhydride (Fluka, Buchs, Switzerland).

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