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Very-long-chain hydroxyaldehydes from the cuticular wax of *Taxus baccata* needles

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Abstract

In the cuticular wax of *Taxus baccata* needles, homologous series of very-long-chain 1,5-alkanediols and 5-hydroxyaldehydes were identified by various chemical transformations with product assignment using GC–MS. The 1,5-alkanediols had chain lengths ranging from C_{28} to C_{38}, with strong predominance of even carbon numbers and a maximum at C_{32} (29%). The series of 5-hydroxyaldehydes comprised chain lengths C_{24} and C_{26}–C_{36}, and showed a pronounced prevalence of even-numbered homologues. 5-Hydroxyoctacosanal was the most abundant compound of the series (42%). The 5-hydroxyaldehydes together amounted to 0.4 μg/cm^2, corresponding to 1.2% of total wax of the needles. A polyketide-like biosynthetic pathway is proposed based on the (similar) chain length distributions and functional group patterns for both compound classes.

Keywords: *Taxus baccata*; Taxodiaceae; Yew; Alkanediols; Aldehydes; Chain length; GC–MS

1. Introduction

The surfaces of aboveground, non-woody, plant organs are covered with a cuticle that consists of cutin and waxes (Jetter et al., 2006). The primary physiological function of the cuticle is to limit non-stomatal water loss, and it is also of ecological importance as it represents the outermost layer of the plant organs. The cuticular waxes render plant surfaces water repellent, thereby keeping them dry, guarding them from accumulation of particles, and preventing 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. Cuticular waxes typically consist of complex mixtures of very-long-chain fatty acids, aldehydes, primary alcohols, esters and alkanes. It is generally accepted that these (monofunctional) compounds are biosynthesized by elongation of acyl-CoA precursors, and modification either by reduction to alcohols or by decarbonylation to alkanes (Kunst and Samuels, 2003). Some plant waxes contain compounds with two functional groups in primary and/or secondary positions. Most wax components with secondary/secondary functional groups have hydrocarbon backbones with odd numbers of carbons (Holloway and Brown, 1977; Franich et al., 1979), while the compound classes with primary/secondary functional groups have predominantly even-numbered chains (Jetter and Riederer, 1999a,b; Vermeer et al., 2003).

There are two fundamentally different pathways for the introduction of the secondary functional groups in wax components: (1) direct hydroxylation has been proposed with functional groups on/near the central carbon, e.g., nonacosane-14,15-diol in *Brassica oleracea* (Holloway and Brown, 1977), and octacosane-1,14-diol as well as hentriacotane-9,16-diol in *Pisum sativum* (Wen et al., 2006a); (2) polyketide biosynthetic pathways are leading to β-diketones (von Wettstein-Knowles, 1995) and to wax

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components with 1,3-, 1,5-, 1,7-, 1,9-substitution patterns (Jetter and Riederer, 1999a,b; Vermeer et al., 2003).

To further substantiate these pathways, potential intermediates and corresponding products have to be identified. Therefore, the objective of the present work was to elucidate the structure of candidate wax components that appeared to have two functional groups. A number of previously unidentified compounds in the cuticle of *Taxus baccata* (yew) were targeted here. In order to identify them, the cuticular wax mixture was extracted from yew needles and separated by TLC. The fractions containing unknown structures were transformed into various derivatives and analyzed by GC–MS.

2. Results and discussion

TLC separation of the wax mixture from needles of *T. baccata* yielded 12 fractions with all the reported wax constituents and two bands of previously unidentified compounds, designated as compound classes **A** and **B**. Compound class **A** ($R_f = 0.05$), migrating between secondary/secondary alkanediols ($R_f = 0.08$) and fatty acids ($R_f = 0.03$), likely contained either a primary and a secondary hydroxyl group, or else three or more secondary hydroxyl and carbonyl groups. The TMSi derivative of the most prominent compound in **A** showed a MS fragment *m/z* 85, which had been interpreted as $[C_5H_9O]^+$ and found to be characteristic for 1,5-alkanediols (Jetter and Riederer, 1999b). The TMSi derivative was further characterized by an α-fragment *m/z* 247 $[C_5H_9(OTMSi)_2]^+$ together with its daughter ion *m/z* 157 $[C_5H_8OTMSi]^+$, a second α-fragment *m/z* 425 $[C_{24}H_{48}OTMSi]^+$, a fragment *m/z* 555 $[M-CH_3]^+$ and molecular ion *m/z* 570. Taken together, these data suggested that the compound was octacosane-1,5-diol. The GC profile of fraction **A** showed 10 more peaks, all with matching MS characteristics of TMSi ethers of primary/secondary diols (Table 1). Based on the chain length specific α-fragments and molecular ions, the compounds were identified as a homologous series of 1,5-alkanediols ranging from C_{28} to C_{38}.

Compound class **B** ($R_f = 0.14$) migrated between primary alcohols ($R_f = 0.17$) and secondary/secondary alkanediols ($R_f = 0.08$) on TLC, and therefore likely contained compounds with a (secondary) hydroxyl group and one (or more) carbonyl group(s). Further GC separation resulted in 12 distinct peaks with similar mass spectral characteristics, indicating that compound class **B** was a homologous series. Combining all the mass spectral data from different derivatives of all 12 compounds, it was eventually concluded that compound class **B** represented a homologous series of 5-hydroxyaldehydes comprising chain lengths C_{24} and C_{26}–C_{36}. This was corroborated by further GC–MS comparisons with a synthetic standard of 5-hydroxyoctacosanal. Since this is also the most abundant homologue in the wax, the structure elucidation will be described using it as an example.

After derivatization with bis-(*N*,*N*-trimethylsilyl)-trifluoroacetamide (BSTFA), the MS of the most prominent compound in **B** showed fragments *m/z* 73, 75 and 103 characteristic for an OTMSi group (Fig. 1a). While this indicated the presence of one hydroxyl group, the lack of a diol signal *m/z* 147 made it unlikely that a second hydroxyl group was present. Instead, the characteristic base peak *m/z* 119, which had previously been reported as a characteristic fragment for non-vicinal ketols (Jetter and Riederer, 2000; Shanker et al., 2007), indicated the presence of one or more carbonyl groups in the molecule. The fragment *m/z* 173 could be interpreted as an α ion $[C_5H_8O_2TMSi]^+$ and thus pointed to the 1,5-geometry between a carbonyl and a hydroxyl group. The compound was further characterized by another α ion *m/z* 425 $[C_{24}H_{48}OTMSi]^+$, a fragment *m/z* 481 $[M-CH_3]^+$ and the molecular ion *m/z* 496. In the spectra of all the other compounds in **B** only the latter three signals differed by 14 mass units (Table 1), indicating that they were homologous ketols. It can be concluded that these homologues differed in the number of CH_2 groups in a hydrocarbon chain attached on one side of the 1,5-bifunctionality, rather than between both functional groups.

The number and position of carbonyl groups in the compounds was assessed by reduction of the homologous mixture with LAH, followed by BSTFA derivatization (Fig. 1b). The fragments *m/z* 147 and 149 showed that the reduced derivatives were diols, and that they contained one primary and one secondary hydroxyl group, respectively (Jetter and Riederer, 1999a). All the characteristic signals for 1,5-alkanediols (see above, Jetter and Riederer, 1999b) were detected in the MS of all 12 homologues, including the fragments *m/z* 85, *m/z* 247 $[C_5H_9(OTMSi)_2]^+$ and its daughter ion *m/z* 157 $[C_5H_8OTMSi]^+$. The major compound in the mixture of reduction products showed fragments *m/z* 425 and 555 $[M-CH_3]^+$, thus confirming its structure as octacosane-1,5-diol, and the structure of the other compounds as homologous 1,5-alkanediols (Table 1). Taken together, this experiment confirmed the 1,5-ketol geometry of compounds in **B** and excluded the presence of any further functional groups.

To further corroborate the 5-hydroxyaldehyde structure, compounds in **B** were reduced with LAH and then further derivatized with acetic anhydride. The resulting products (Fig. 1c) had a prominent MS fragment *m/z* 61, interpreted as $[AcOH_2]^+$ diagnostic for acetates. Furthermore, the fragments *m/z* 450 $[M-60]^+$, *m/z* 407 $[M-43-60]^+$ and *m/z* 390 $[M-120]^+$, involving the loss of one and two acetyl moieties, were characteristic for bis acetates of alkanediols (Wen et al., 2006a). The molecular ion *m/z* 510 of octacosanediol bis acetate confirmed the presence of exactly two hydroxyl functions. Different from the fragmentation patterns of primary/secondary diol acetates from pea wax (Wen et al., 2006a), the daughter fragment Δ*m/z* 42 of the α-ion (*m/z* 334) had relatively low

Table 1
MS spectral data of 1,5-alkanediol TMSi ethers, 5-hydroxyaldehyde derivatives, and underivatized 5-hydroxyaldehydes

Compounds	Relative intensity (%)													
	Fragments characteristic for the compound class[a]							Fragments characteristic for the homologue						
TMSi ether of 1,5-alkanediols	**73**	**85**	**103**	**147**	**149**	**157**	**247**	**M**		**M-15**		**$C_nH_{2n}OTMSi$**	
Octacosane-1,5-diol	40	83	25	23	10	9	5	**570**	–	**555**	2	**425**	100
Nonacosane-1,5-diol	–	–	–	–	–	3	2	**584**	–	**569**	2	**439**	100
Triacontane-1,5-diol	45	90	30	25	10	10	7	**598**	0.5	**583**	2	**453**	100
Hentriacontane-1,5-diol	32	87	27	20	9	9	8	**612**	–	**597**	1	**467**	100
Dotriacontane-1,5-diol	32	86	7	13	8	8	7	**626**	0.2	**611**	0.4	**481**	100
Tritriacontane-1,5-diol	36	93	9	15	8	8	7	**640**	–	**625**	0.6	**495**	100
Tetratriacontane-1,5-diol	25	95	6	12	8	7	7	**654**	–	**639**	0.5	**509**	100
Pentatruacontane-1,5-diol	26	100	6	12	9	8	7	**668**	–	**653**	0.5	**523**	95
Hexatriacontane-1,5-diol	22	100	3	10	9	9	8	**682**	–	**667**	0.6	**537**	94
Heptatriacontane-1,5-diol	24	100	6	12	8	9	7	**606**	–	**681**	0.6	**551**	96
Octatriacontane-1,5-diol	25	100	4	14	9	8	7	**710**	–	**695**	0.7	**565**	95
TMSi ether of 5-hydroxyaldehydes	**73**	**103**	**119**	**173**				**M**		**M-15**		**$C_nH_{2n}OTMSi$**	
5-Hydroxytetracosanal	8	27	100	29				**440**	2	**425**	22	**369**	19
5-Hydroxyhexacosanal	9	25	100	25				**468**	2	**453**	23	**397**	12
5-Hydroxyheptacosanal	10	27	100	24				**482**	1	**467**	20	**411**	13
5-Hydroxyoctacosanal	11	22	100	16				**496**	1	**481**	13	**425**	9
5-Hydroxynonacosanal	12	21	100	17				**510**	1	**495**	13	**439**	9
5-Hydroxyteiacontanal	12	21	100	17				**524**	1	**509**	13	**453**	10
5-Hydroxylhentriacontanal	11	22	100	16				**538**	1	**523**	12	**467**	9
5-Hydroxydotriacontanal	11	19	100	18				**552**	1	**537**	12	**481**	10
5-Hydroxytritriacontanal	13	23	100	18				**566**	2	**551**	14	**495**	10
5-Hydroxytetratriacontanal	14	19	100	17				**580**	1	**565**	11	**509**	10
5-Hydroxypentatriacontanal	12	20	100	16				**594**	1	**579**	12	**523**	9
5-Hydroxyhexatriscontanal	13	20	100	19				**608**	1	**593**	13	**537**	9
Acetates of 1,5-alkanediols	**61**	**85**						**M-60-43**		**M-120**			
Tetracosane-1,5-diol	8	100						**365**	19	**348**	30		
Hexacosane-1,5-diol	8	100						**393**	17	**376**	28		
Heptacosane-1,5-diol	9	100						**407**	19	**390**	30		
Octacosane-1,5-diol	8	100						**421**	18	**404**	29		
Nonacosane-1,5-diol	9	100						**435**	15	**418**	30		
Triacontane-1,5-diol	9	100						**449**	14	**432**	28		
Hentriacontane-1,5-diol	10	100						**463**	16	**446**	29		
Detriacontane-1,5-diol	9	100						**477**	14	**460**	32		
Tritriacontane-1,5-diol	9	100						**491**	14	**474**	29		
Tetratriacontane-1,5-diol	10	100						**505**	13	**488**	35		
Pentatriacontane-1,5-diol	9	100						**519**	15	**502**	32		
Hexatriacontane-1,5-diol	8	100						**533**	14	**516**	29		
5-Hydroxyaldehydes	**98**	**111**						**M-18**					
5-Hydroxytetracosanal	100	36						**350**	7				
5-Hydroxyhexacosanal	100	30						**378**	7				
5-Hydroxyheptacosanal	100	29						**392**	4				

(*continued on next page*)

Table 1 (*continued*)

Compounds	Relative intensity (%)		Fragments characteristic for the homologue	
	Fragments characteristic for the compound class[a]			
5-Hydroxyoctacosanal	100	28	**406**	5
5-Hydroxynonacosanal	100	29	**420**	6
5-Hydroxyteiacontanal	100	27	**434**	5
5-Hydroxylhentriacontanal	100	27	**448**	5
5-Hydroxydotriacontanal	100	26	462	5
5-Hydroxytritriacontanal	100	27	**476**	5
5-Hydroxytetratriacontanal	100	28	**490**	6
5-Hydroxypentatriacontanal	100	28	**504**	5
5-Hydroxyhexatriscontanal	100	27	**518**	4

Throughout this table, results for ion masses (m/z) are shown in bold face while the corresponding relative abundances (%) of individual ions are shown in normal font.

[a] In addition to the characteristic fragments common to all compounds of one homologous series, fragments *m/z* 55, 57, 71 were also found in all mass spectra of all the compounds.

abundance. Since the base peak *m/z* 85 was found for both the bis acetates and for the corresponding bis TMSi ethers, it is clearly diagnostic for 5-hydroxyalkanediols irrespective of the hydroxyl derivatives.

The MS of underivatized 5-hydroxyoctacosanal (Fig. 1d) showed prominent fragments *m/z* 98 and *m/z* 111 that can be interpreted as $C_6H_{10}O^+$ resulting from water elimination from the molecular ion followed by McLafferty rearrangement, and $C_7H_{11}O^+$ resulting from water elimination followed by β-fragmentation, respectively. Due to the presence of a hydroxyl group, water can be easily eliminated from the molecular ion, resulting in the fragment *m/z* 406 $[M-H_2O]^+$ with relatively high abundance. For the other homologues, this fragment differed by 14 mass units, whereas the other fragments *m/z* 98 and *m/z* 111 stayed constant (Table 1).

In summary, the spectral information indicated the presence of one hydroxyl and one carbonyl group with primary/secondary 1,5-geometry, narrowing the candidate structures of the prevalent homologue to the isomers 5-hydroxyoctacosanal and 1-hydroxyoctacosan-5-one. The latter structure seemed highly unlikely because: (1) the presence of a primary hydroxyl group and a carbonyl group would give rise to a polarity higher than that observed in our TLC experiments; (2) the α-fragment of the mid-chain functional group (*m/z* 425 for the TMSi derivatives) was not affected by LAH treatment, showing that this functionality cannot be reduced and therefore likely is a hydroxyl group; (3) the MS of the TMSi ether of 1-hydroxyoctacosan-5-one would very likely give an α-fragment *m/z* 351 (instead of *m/z* 425), which could not be detected. Since the spectral interpretation strongly favoured a 5-hydroxyaldehyde structure for compounds in **B**, one representative of the homologous series was synthesized for final proof of structure (Njardarson et al., 2002). It was found that the GC characteristics and MS fragmentation pattern of authentic 5-hydroxyoctacosanal were identical to those of one homologue in compound class **B** (data not shown). The compounds in the needle wax of *T. baccata* were thus identified as a series of 5-hydroxyaldehydes comprising chain lengths C_{24} and C_{26}–C_{36}.

The total needle wax contained approximately 0.4 μg/cm^2 of 5-hydroxyaldehydes. The homologous series was dominated by even-numbered compounds, containing 42% of 5-hydroxyoctacosanal, 22% of 5-hydroxytriacontanal, 19% of 5-hydroxydotriacontanal, and 17% of the other homologues (Fig. 2a). The 5-hydroxylaldehydes could be detected in a wax sample isolated from the abaxial side of the needle, but not in the wax from the adaxial side. It is not clear whether they were absent from this side of the needle, or not detectable due to the lower total wax amounts on the adaxial surface (Wen et al., 2006b). The 1,5-alkanediols were present in the needle wax only at trace levels. Interestingly, this homologous series was also dominated by even-numbered chains (Fig. 2b).

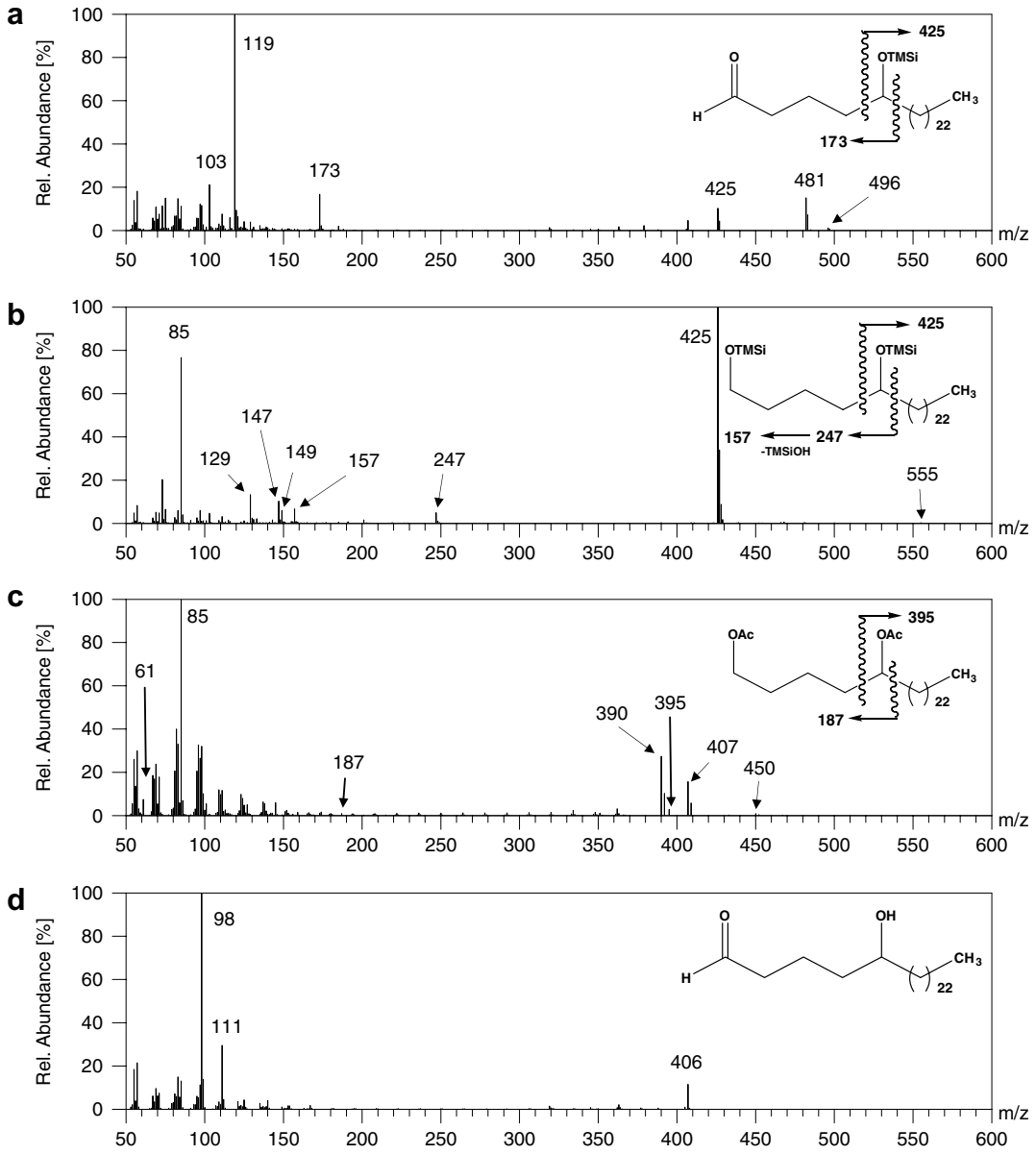

Fig. 1. Mass spectra of representative derivatives of 5-hydroxyaldehydes in the needle wax of *Taxus baccata*. (a) TMSi ether of 5-hydroxyoctacosanal, (b) bis TMSi ether of octacosane-1,5-diol, (c) bis acetate of octacosane-1,5-diol, and (d) underivatized 5-hydroxyoctacosanal.

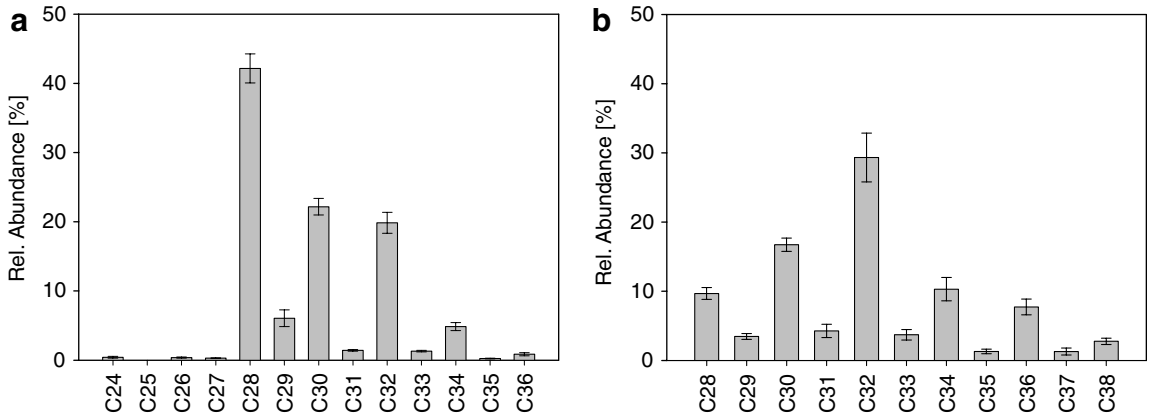

Fig. 2. Chain length distribution (%) of (a) 5-hydroxyaldehydes ($n = 5$, ±SE) and (b) 1,5-alkanediols ($n = 3$, ±SE) in *T. baccata* needle wax.

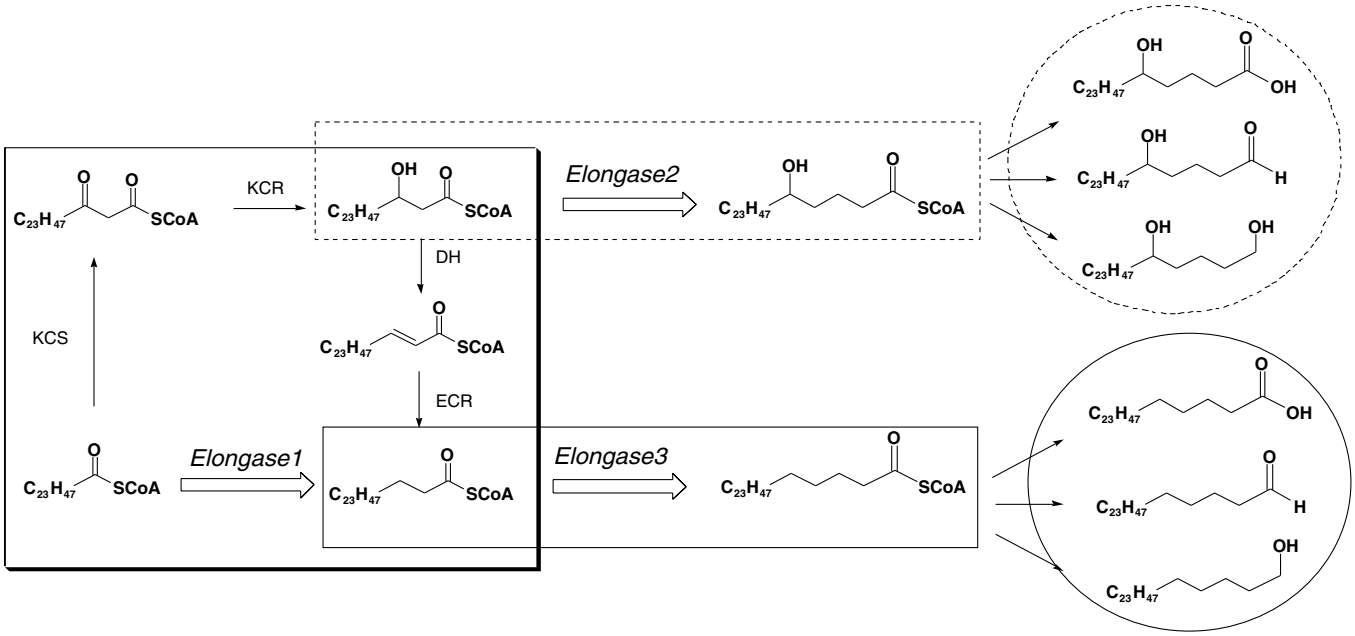

Fig. 3. Proposed biosynthetic pathway leading to 5-hydroxyaldehydes and 1,5-alkanediols in the needle wax of *Taxus baccata*. Only the C_{28} homologues are depicted as an example. In normal wax biosynthesis (shown in boxes with black lines), acyl CoA chains are elongated by elongase complex(es) containing 3-ketoacyl CoA synthase (KCS), 3-ketoacyl CoA reductase (KCR), dehydratase (DH) and enoyl CoA reductase (ECR) (shown in box with shade). Multiple rounds of elongation lead to C_{28} acyl CoA, which is further modified into the corresponding free fatty acid, aldehyde and alcohol (circled with black line). A polyketide-like pathway is proposed for biosynthesis of 5-hydroxyaldehydes and 1,5-alkanediols (shown in box with dashed lines). A hydroxacyl CoA intermediate is elongated directly and the resulting 5-hydroxyacyl CoA is further modified (shown in dashed cycle). Two or more of the elongase complexes shown may be identical.

3. Conclusions

The similar chain length distributions of 5-hydroxyaldehydes and 1,5-alkanediols suggest a biosynthetic relationship between both compound classes. Furthermore, the predominance of even-numbered homologues in both series makes it very likely that both compound classes are formed, in analogy to other common wax components, on pathways involving acyl reduction rather than decarbonylation. We therefore conclude that the primary function of both compound classes originates by reduction of a derivative generated by fatty acid elongation. In contrast, the constant 1,5-geometry of all alkanediols and hydroxyaldehydes detected here suggests that the secondary functional group is introduced during elongation. In analogy to polyketide biosynthesis, one round of elongation may start with 3-hydroxyacyl CoA intermediates instead of the corresponding acyl CoAs (Fig. 3). The resulting 5-hydroxyacyl CoAs could be modified into 5-hydroxyacids, 5-hydroxyaldehydes, or 1,5-alkanediols. 5-Hydroxy fatty acids were not detected in yew needle wax, but their δ-lactones have been identified in leaf cuticular wax of *Cerinthe minor* (Jetter and Riederer, 1999b).

4. Experimental

4.1. Plant material

Twigs were harvested in spring from plants of *Taxus baccata* L. growing continuously on the campus of the University of British Columbia. Mature needles were cut from the twigs using razor blades. Batches of 30–40 needles were used for total wax analysis, while 5–8 needles were used for brushing to extract waxes from abaxial and adaxial surfaces separately.

4.2. Wax extraction

For total wax extraction, cut needles were immediately immersed twice for 30 s in $CHCl_3$ at room temperature. To selectively extract waxes from the abaxial and adaxial surfaces of needles, either of the two surfaces was brushed gently with fabric glass that had been pre-extracted (in a Soxhlet apparatus) and soaked with $CHCl_3$ (Wen et al., 2006b). The resulting solutions were filtered, dried, and stored at 4 °C until they were analyzed.

4.3. Qualitative and quantitative analysis of 5-hydroxyaldehydes

Compound classes in total waxes were separated by TLC (sandwich technique (Tantisewie et al., 1969), silica gel, mobile phase $CHCl_3$) and visualized 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 contained unknown compounds and were subjected to detailed qualitative analyses. Relative compositions (wt%) of homologues were quantified based on the abundance of characteristic MS fragments. To this end, constituents were

studied with capillary GC (5890N, Agilent, Avondale, PA, USA; column 30m 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. The coverage (μg/cm^2) of the most abundant homologue, 5-hydroxyoctacosanal, was quantified by GC-FID after adding a defined amount of *n*-tetracosane into the total wax extracts as an internal standard. Inlet pressure was programmed for constant flow of 2.0 ml/min with H_2 as carrier gas. GC temperature program was the same as for MS. The coverage of all the 5-hydroxyaldehydes was then calculated based on the relative abundance of 5-hydroxyoctacosanal in the homologous series.

4.4. Derivatization reactions

The constituents of the fractions containing unknown compounds 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 at 70 °C for 5 min, and then keeping it at RT overnight. The products were isolated by addition of H_2O and extraction with $CHCl_3$; (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 $CHCl_3$.

4.5. Synthesis of reference compound 5-hydroxyoctacosanal

1-Tetracosanol (Sigma–Aldrich, Steinheim, Switzerland) was oxidized into tetracosanal by pyridinium chlorochromate at RT for 1 h in anhydrous CH_2Cl_2. Column purified tetracosanal reacted with 3-butenylmagnesium bromide (0.5 M solution in THF, Sigma–Aldrich, MO, USA) while refluxing for 1 h. The reaction was quenched by addition of water, and then extracted with $CHCl_3$. 1-Octacosen-4-ol was purified by column chromatography. 5-Hydroxyoctacosanal was then synthesized using cross metathesis followed by oxidation as described by Njardarson et al. (2002). Briefly, 4,4,5,5-tetramethyl-2-vinyl-1,3,2-dioxaborolane (3 equiv., Sigma–Aldrich, MO, USA) and Grubbs catalyst (0.1 equiv., Sigma–Aldrich, MO, USA) were added to 1-octacosen-4-ol (1 equiv.) solution in CH_2Cl_2, and the mixture was kept refluxing for 8 h. The desired cross metathesis product was purified on TLC and then oxidized by trimethyl *N*-oxide (Sigma–Aldrich, MO, USA) in refluxing THF for 4 h. The resulting synthetic 5-hydroxyoctacosanal was characterized by GC–MS as described above: TMSi ether 119 (100), 73 (25), 103 (33), 173 (18), 481 (45), 425 (22), 496 (4); free alcohol 98 (100), 111(25), 406 (7).

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