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Long-chain alkanediols, ketoaldehydes, ketoalcohols and ketoalkyl esters in the cuticular waxes of *Osmunda regalis* fronds

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Abstract

In the frond waxes of *Osmunda regalis* four homologous series of previously unknown compounds were detected. In order to assign their structures various derivatives were prepared and analyzed by GC-MS. The wax components were identified as C_{28} to C_{32} alkanediols, C_{26} to C_{36} ketoalcohols, C_{28} to C_{36} ketoaldehydes and long-chain fatty acid esters of C_{28} to C_{36} ketoalcohols. All fractions showed strong prevalence of even numbered homologues with a maximum for C_{30}. Isomer distributions were in all cases dominated by 1,9, 1,11-, 1,13- and 1,15-compounds, their relative abundances shifting towards the latter with increasing homologue chain length. A total of 77 (69 novel) compounds were identified, partially as minor components in unresolved isomer mixtures. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Osmundaceae; Ferns; Cuticular wax; Alcohols; Diols; Esters; Ketones

1. Introduction

The genus *Osmunda* (Osmundaceae) comprises 10 species occurring in the temperate zone of the northern hemisphere. The royal fern, *Osmunda regalis* L., is the only representative of the family Osmundaceae in Europe, and is distributed throughout the Old and New World reaching south into Africa and South America. The plant is cultivated as an ornamental, parts of it are locally eaten, its fibers are exploited for orchid-growing and the hairs of young leaves are used for textile production.

In the course of an investigation into the chemical composition of epicuticular wax crystals we initiated a comparative analysis of surface lipids from diverse plant species (Jetter & Riederer, 1996; Jetter & Riederer, 1998; Neinhuis & Jetter 1995). Such crystals occur in only a few fern genera but these presumably are of special interest for understanding the evolution of the cuticle and its function. Crystals on aerial fern surfaces had been described as platelets or filaments (Barthlott & Wollenweber, 1981), *Osmunda regalis* being an exception with rodlet-shaped crystals (Neinhuis & Barthlott, 1997). An analysis of the surface lipids from this fern species revealed the presence of typical wax constituents, e.g. alkanes, primary and secondary alcohols, aldehydes, fatty acids, alkyl esters and ketones (manuscript in preparation). Besides, four series of homologues with hitherto unknown spectral properties were encountered. In order to identify these compounds in the present study various chemical transformations were employed and product structures were assigned by GC-MS.

2. Results and discussion

The $CHCl_3$ surface extracts from fronds of *Osmunda regalis* were separated into 8 distinct fractions by TLC on silica gel with CCl_3CH_3. Among these, bands with R_f 0.56, 0.51, 0.40 and 0.10 were found to each contain one homologous series of unknown compounds designated as series A, B, C and D. TLC on silica gel

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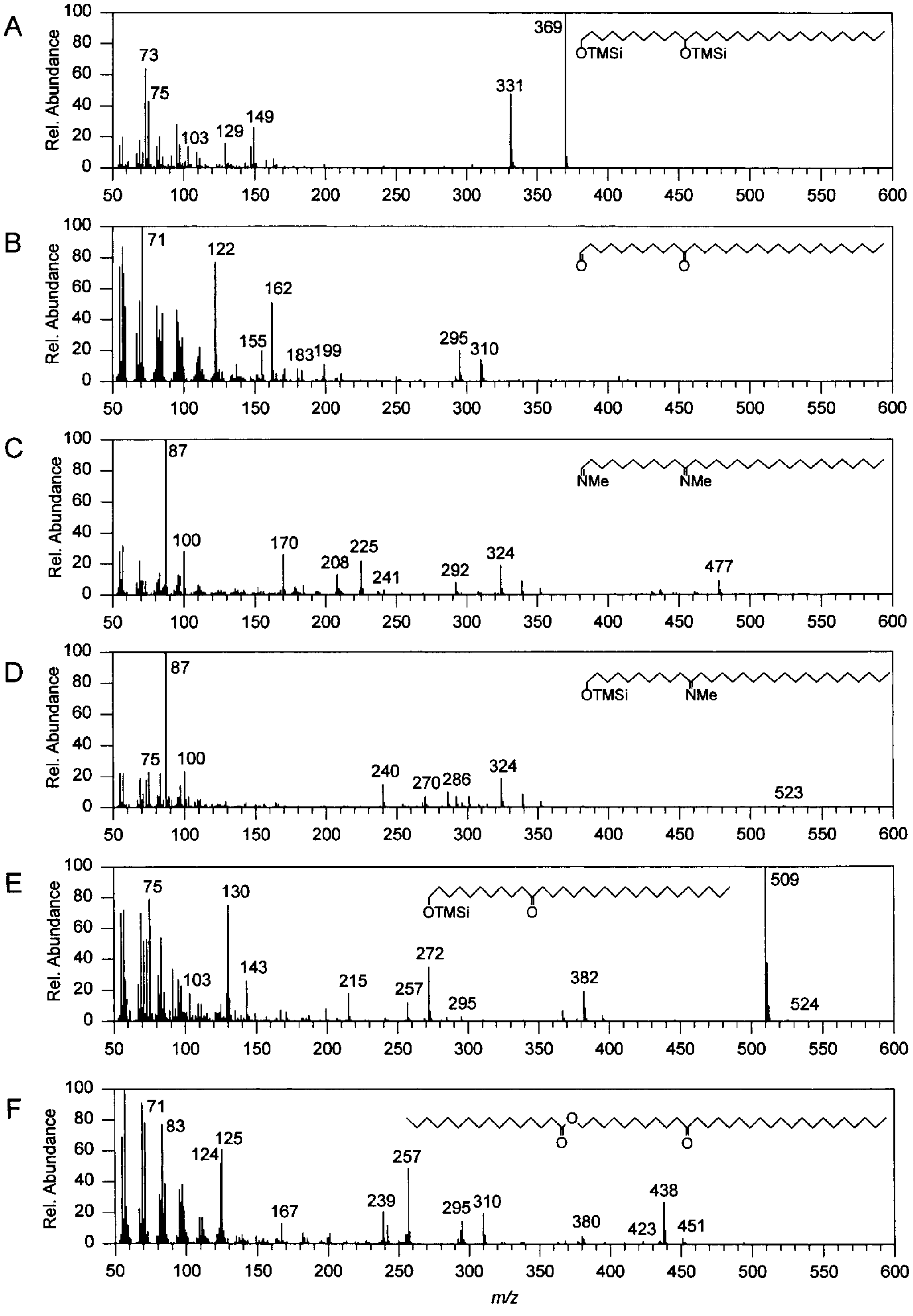

Fig. 1. Mass spectra of representative homologues from the compound classes identified in *Osmunda regalis* frond wax. (A) Triacontane-1,11-diol bis TMSi ether (fraction D), (B) 11-ketotriacontanal (fraction B), (C) 11-ketotriacontanal bis methyl oxime (fraction B), (D) hydroxytriacontan-11-one methyl oxime TMSi ether (fraction C), (E) hydroxytriacontan-11-one TMSi ether (fraction C), (F) 11-ketotriacontyl hexadecanoate (fraction A).

Table 1
MS of novel prim./sec. alkanediol TMSi ethers and hydroxyketone esters. Mass and relative abundance (in parentheses) are given for selected fragments. Ions generated by cleavage of α-bonds of secondary functional groups are highlighted in bold face

Compound	Derivative	M^+	$M\text{-}15^+$	α- and related fragments				Other fragments														
Prim./sec alkanediols																						
Octacosane-1,11-diol	bis TMSi ether	571 (1)	556 (2)	481 (1)	**341** **(81)**	**331** **(41)**	241 (3)	149 (27)	147 (30)	129 (52)	103 (10)	75 (66)	73 (100)									
Dotriacontane-1,11-diol	bis TMSi ether	miss.[a]	612 (0.1)	538 (1)	**397** **(83)**	**331** **(47)**	241 (4)	149 (40)	147 (25)	129 (29)	103 (29)	75 (60)	73 (100)									
Hydroxyketone esters																						
11-ketooctacosyl hexadecanoate	–	n.d.[b]	–	451 (3)	438 (18)	**423** **(2)**	380 (4)	282 (15)	**267** **(11)**	266 (9)	257 (37)	242 (10)	239 (17)	167 (13)	125 (54)	124 (47)	97 (45)	83 (77)	71 (76)	69 (93)	57 (100)	55 (76)
11-ketotriacontyl hexadecanoate	–	n.d.	–	451 (4)	438 (27)	**423** **(2)**	380 (5)	310 (20)	**295** **(15)**	294 (15)	257 (49)	242 (12)	239 (21)	167 (13)	125 (61)	124 (52)	97 (38)	83 (77)	71 (78)	69 (91)	57 (100)	55 (69)
11-ketodotriacontyl hexadecanoate	–	n.d.	–	451 (3)	438 (26)	**423** **(1)**	380 (5)	338 (16)	**323** **(12)**	322 (7)	257 (45)	242 (11)	239 (19)	167 (12)	125 (56)	124 (50)	97 (23)	83 (76)	71 (79)	69 (89)	57 (100)	55 (66)

[a] Miss.: missing.

[b] n.d.: not detectable due to restricted mass range of spectrometer.

with $CHCl_3$–EtOH 99:1 yielded 13 fractions, with compound class A at R_f 0.91, compound classes B and C separated into pairs of bands B1/B2 with R_f 0.67/0.61 and C1/C2 R_f 0.34/0.30, respectively, and compound class D at R_f 0.21.

Compounds in fraction D were identified as alkanediols with one hydroxyl function on a primary and the other on a secondary carbon. MS of the TMSi ethers (Fig. 1(A), Table 1) showed a set of fragments $m/z = 73$, 75, 103, 147 and 149 that had previously been recognized as characteristic for this combination of functional groups (Jetter, Riederer, Seyer & Mioskowski, 1996)). The position of the secondary hydroxyl function could be calculated from its α-fragments belonging to two series $[C_nH_{2n}OTMSi]^+$ and $[C_mH_{2m-1}(OTMSi)_2]^+$. The latter α-peak was accompanied by a secondary fragment that was probably generated by additional loss of HOTMSi ($\Delta m/z = 90$) (Franich, Gowar & Volkman, 1979). The total number of carbons of each homologue was assessed from the combination of both secondary α-fragments and in each case supported by the presence of peaks $[M\text{-}15]^+$. Thus, a homologous series comprising octacosane-1,11-diol, triacontane-1,11-diol and dotriacontane-1,11-diol was identified (Table 2). Only the C_{30} compound had been described previously as a constituent of *Papaver* leaf waxes (Jetter et al., 1996). In total wax mixtures of *O. regalis* fronds this compound class was detectable only in trace amounts. Hence, the absolute configuration of the asymmetric carbon atom carrying the secondary hydroxyl group could not be determined.

One compound each in fraction B2 and C2 was tentatively identified as 11-ketooctacosanal and 11-ketooctacosanol, respectively, by comparison of their (TMSi derivative) mass spectra with those published previously (Buckner, Nelson, Hakk & Pomonis, 1984). Compound classes B and C were accordingly suspected to consist of series of ketoaldehydes and ketoalcohols, respectively. In order to corroborate this assignment, the number and position of functional groups involved were first determined by reduction of the compounds to the corresponding diols and inspection of their TMSi derivative MS. The carbonyl nature of the original functional groups was then assessed by formation of the corresponding methyl oximes.

Compounds in fraction B yielded $LiAlH_4$ reduction products that had TMSi ether MS typical for prim./sec. alkanediols (Table 3). Thus, 8 homologous alkanediols with chain lengths C_{28} to C_{36} were identified. As the C_{28} isomers were partially separated by GC, distinct MS could be acquired and octacosane-1,7-diol, -1,9-diol, -1,11-diol and -1,13-diol were identified ('D' in Table 4). Fractions B1 and B2 contained predominantly 12-/13-isomers and 10-/11-isomers, respectively, and hence pairs of isomers could be separately ident-

Table 2
Homologous composition [%] of *Osmunda regalis* frond cuticular wax fractions: ketoaldehydes, ketoalcohols, ketoalkylesters and ketoalcohols released from these esters by transesterification

Carbon number	Prim./sec. alkanediols	Free ketoaldehydes	Free ketoalcohols	Keto-alkylesters	Ester ketoalcohols
26	–	–	tr[a]	–	–
28	34	2	2	–	7
29	–	tr	Tr	–	–
30	51	63	49	–	53
31	–	tr	Tr	–	–
32	16	16	26	–	27
33	–	tr	Tr	–	–
34	–	16	16	–	10
35	–	tr	Tr	–	–
36	–	3	7	–	2
44	–	–	–	19	–
46	–	–	–	36	–
48	–	–	–	33	–
50	–	–	–	12	–
52	–	–	–	Tr	–

[a] tr: Traces, i.e. less than 0.5% detectable.

ified for most of the homologues ('D' in Table 4). Besides, the presence of additional α-fragments in given GC-peaks indicated that respective homologues contained further isomers ('d' in Table 4) (Franich et al., 1979).

In a second experiment the compounds in fraction B were transformed into the methyl oximes in order to verify the original presence of two carbonyl groups (Knapp, 1979). In all cases, pairs of (probably *cis–trans* isomeric) compounds were formed that could only be partially separated by GC and had identical MS (Vogh, 1971). Corresponding MS (Fig. 1(C), Table 3) showed prominent fragments m/z = 87 and 100 (indicative of at least one oxime group) and the expected bis-oxime molecular ions and/or peaks for $[\mathrm{M}\text{-}31]^+$ and $[\mathrm{M}\text{-}72]^+$ (Prahl & Pinto, 1987). Besides, each spectrum contained two α-fragments $[\mathrm{C}_n\mathrm{H}_{2n-1}\mathrm{NOMe}]^+$ and $[\mathrm{C}_m\mathrm{H}_{2m-3}(\mathrm{NOMe})_2]^+$ together with two series of peaks $\Delta m/z = +28, +15, -16, -32$. The α-fragments served to calculate the carbon numbers of both chain ends and hence allowed the assignment of homologues and positional isomers. Fragments m/z = 142, 170, 184, and 198 were found to be characteristic for 1,9-, 1,11-, 1,12-, and 1,13-bis methyloximes, respectively. Thus, the bis-oxime derivatives of 9-ketooctacosanal, 11-ketotriacontanal and 11-ketodotriacontanal were identified in fraction B2, and those of 13-ketotriacontanal, 12-ketohentriacontanal, 13-ketodotriacontanal, 12-ketotritriacontanal and 13-ketotetratriacontanal were identified in fraction B1 ('B' in Table 4).

The MS (Fig. 1(B), Table 3) of the underivatized compounds in fraction B showed characteristics of long-chain ketones (Sharkey, Schultz & Riedel, 1956): a base peak at m/z = 71, α-fragments $[\mathrm{C}_n\mathrm{H}_{2n-1}\mathrm{O}]^+$ and related β-fragments $[\mathrm{C}_{n+1}\mathrm{H}_{2n+2}\mathrm{O}]^+$ and $[\mathrm{C}_{n+1}\mathrm{H}_{2n+3}\mathrm{O}]^+$. Besides, a small molecular ion indicated the formula $\mathrm{C}_n\mathrm{H}_{2n-2}\mathrm{O}_2$ and a fragment $[\mathrm{M}\text{-}18]^+$ supported the presence of an aldehyde function (Christiansen, Mahadevan, Viswanathan & Holman, 1969). A second α-fragment $[\mathrm{C}_n\mathrm{H}_{2n-3}\mathrm{O}_2]^+$ together with its β-fragments was used to locate the secondary functional group relative to the carbonyl terminus. A series of smaller fragments were found to be determined by the relative position of both functional groups, pairs of prominent peaks m/z = 122 and 162, 136 and 176, or 150 and 190 being characteristic for 1,11-, 1,12- or 1,13-dicarbonyl isomers, respectively.

11-Ketooctacosanal and 11-ketotriacontanal were identified in fraction B2, 13-ketotriacontanal, 13-ketodotriacontanal and 13-ketotetratriacontanal were identified in fraction B1 ('A' in Table 4). Additionally, 10-ketononacosanal, 7-, 9- and 15-ketotriacontanal, 10- and 12-ketohentriacontanal, 7-, 9-, 11- and 15-ketodotriacontanal, 12-ketotritriacontanal, 7-, 9-, 11-, 15- and 17-ketotetratriacontanal, 7-, 9-, 13-, 15- and 17-ketohexatriacontanal were detected as minor compounds and identified according to the characteristic fragments described above ('a' in Table 4). All 26 structures are in agreement with the assignments for the corresponding derivatives formed by reduction and oximation of fraction B. In these derivatization experiments 7-, 9- and 13-ketooctacosanal were detected as additional components of *Osmunda* frond waxes. Among the above mentioned compounds only 11-ketooctacosanal had previously been identified (Buckner et al., 1984), the other 28 representing new structures.

Ketoaldehydes constituted ca 4% of the 22.5 μg of cuticular waxes per cm^2 leaf area of *Osmunda*. Among them homologues with even carbon numbers were pre-

Table 3
MS of novel ketoaldehydes and their derivatives

Compound	**Derivative**	M$^+$	M-x^{+a}		α- and related fragments									Other fragments					
7-Keto-octacosanal	diol bis TMSi ether	miss.	556 (0.2)		481 (1)	**397 (100)**	**275 (20)**	185 (1)						149 (31)	147 (22)	129 (25)	103 (23)	75 (58)	73 (72)
9-Keto-octacosanal	bis methyloxime	miss.	449 (2)	408 (3)			**324 (4)**			241 (2)		213 (0.5)					142 (3)	100 (28)	87 (100)
9-Keto-octacosanal	diol bis TMSi ether	miss.	556 (0.2)		481 (1)	**369 (100)**	**303 (47)**	213 (1)						149 (26)	147 (14)	129 (22)	103 (19)	75 (41)	73 (63)
13-Ketooctacosanal	diol bis TMSi ether	miss.	556 (0.2)		481 (1)	**313 (100)**	**359 (20)**	269 (1)						149 (26)	147 (14)	129 (22)	103 (19)	75 (41)	73 (63)
10-Ketononacosanal	diol bis TMSi ether	miss.	570 (0.1)		495 (0.3)	**369 (100)**	**317 (76)**	227 (1)						149 (25)	147 (19)	129 (23)	103 (24)	75 (47)	73 (59)
11-Ketotriacontanal	–	451 (0.3)	433 (0.3)		407 (3)		311 (11)	310 (14)	**295 (20)**	211 (5)	199 (11)	198 (3)	**183 (7)**	180 (8)	162 (51)	155 (17)	122 (77)	99 (28)	71 (100)
11-Ketotriacontanal	bis methyloxime	508 (0.1)	477 (9)	436 (3)	352 (4)	339 (9)	**324 (19)**	308 (2)	292 (8)	269 (1)	256 (0.3)	**241 (3)**	225 (22)	224 (3)	209 (4)	208 (13)	170 (26)	100 (28)	87 (100)
13-Ketotriacontanal	–	451 (0.3)	433 (0.6)		407 (3)		283 (9)	282 (8)	**267 (21)**	239 (3)	227 (4)	226 (3)	**211 (9)**	208 (9)	190 (12)	183 (18)	150 (7)	127 (13)	71 (100)
13-Ketotriacontanal	bis methyloxime	508 (0.1)	477 (1)	436 (2)	324 (7)	311 (5)	**296 (13)**	284 (1)	280 (2)	297 (3)	284 (6)	**269 (1)**	253 (4)	252 (7)	237 (5)		198 (6)	100 (28)	87 (100)
13-Ketotriacontanal	diol bis TMSi ether	miss.	584 (0.2)		510 (0.4)	341 (94)	359 (22)	269 (2)						149 (52)	147 (25)	129 (29)	103 (27)	75 (75)	73 (100)
10-Ketohentriacontanal	diol bis TMSi ether	miss.	598 (1)	524 (1)	495 (1)	**397 (46)**	**317 (36)**	227 (2)						149 (10)	147 (3)	129 (33)	103 (33)	75 (99)	73 (73)
12-Ketohentriacontanal	bis methyloxime	miss.	491 (0.5)	450 (1)	352 (1)	339 (5)	**324 (13)**	308 (1)	292 (5)			**255 (0.3)**	239 (2)	238 (8)	223 (6)		184 (4)	100 (29)	87 (100)
12-Ketohentriacontanal	diol bis TMSi ether	miss.	598 (4)			**369 (100)**	**345 (27)**	255 (3)						149 (31)	147 (27)	129 (29)	103 (15)	75 (41)	73 (15)
11-Ketodotriacontanal	bis methyloxime	miss.	505 (1)	464 (2)	380 (2)	367 (5)	**352 (11)**	336 (1)	320 (5)	269 (1)		**241 (1)**	225 (4)	224 (10)	209 (5)	208 (3)	170 (7)	100 (25)	87 (100)
13-Ketodotriacontanal	–	479 (0.3)	461 (0.3)		435 (2)		311 (13)	310 (12)	**295 (24)**	239 (5)	227 (8)	226 (4)	**211 (9)**	208 (9)	190 (29)	183 (25)	150 (12)	127 (16)	71 (100)
13-Ketodotriacontanal	bis methyloxime	536 (0.2)	505 (2)	464 (3)	352 (4)	339 (8)	**324 (17)**	308 (2)	292 (7)	297 (1)	284 (2)	**269 (3)**	253 (9)	252 (9)	237 (6)	208 (3)	198 (14)	100 (29)	87 (100)
13-Ketodotriacontanal	diol bis TMSi ether	627 (0.1)	612 (0.1)		538 (0.4)	**369 (100)**	**359 (25)**	269 (1)						149 (30)	147 (11)	129 (14)	103 (12)	75 (37)	73 (51)
12-Ketotritriacontanal	bis methyloxime	miss.	519 (1)	478 (1)	380 (2)	367 (5)	**352 (10)**	336 (1)	320 (4)			**255 (1)**	239 (5)	238 (9)	223 (4)		184 (7)	100 (26)	87 (100)
12-Ketotritriacontanal	diol bis TMSi ether	miss.	626 (2)		552 (3)	**397 (92)**	**345 (36)**	255 (8)						149 (51)	147 (21)	129 (44)	103 (34)	75 (42)	73 (100)
13-Ketotetratriacontanal	–	miss.	489 (0.3)		463 (2)		339 (13)	338 (9)	**323 (21)**	267 (1)	227 (10)	226 (5)	**211 (10)**	208 (2)	190 (30)	183 (28)	150 (11)	127 (16)	71 (100)
13-Ketotetratriacontanal	bis methyloxime	564 (0.1)	533 (3)	492 (2)	380 (3)	367 (8)	**352 (17)**	336 (2)	320 (6)	297 (1)	284 (2)	**269 (3)**	253 (15)	252 (9)	237 (6)		198 (16)	100 (29)	87 (100)
13-Ketotetratriacontanal	diol bis TMSi ether	n.d.	640 (0.1)	610 (0.1)	566 (0.1)	**397 (100)**	**359 (31)**	269 (2)						149 (33)	147 (12)	129 (15)	103 (13)	75 (39)	73 (56)
15-Ketohexatriacontanal	diol bis TMSi ether	n.d.	n.d.	638 (3)	594 (5)	**397 (99)**	**387 (20)**	297 (7)						149 (53)	147 (16)	129 (2)	103 (10)	75 (73)	73 (100)

[a] $x = 15$ for TMSi ethers, $x = 18$ for underivatized aldehydes, $x = 31$, 72 for oximes.

Table 4
Isomer composition [%] of ketoaldehydes in *Osmunda regalis* frond waxes calculated from abundances of corresponding keto α-fragments $[C_nH_{2n-1}O]^+$. Derivatives used for identification are given (capitals = full MS available, small letters = characteristic fragments detected in isomer mixture, a = ketoaldehyde, d = diol bis TMSi ether, b = bis methyl oxime, lit = previously identified)

Ketoaldehyde carbon number	Keto group position							
	7	9	10	11	12	13	15	17
28	0.2, D	0.5, DB	–	1, ADB lit	–	0.1, D	–	–
29	–	–	tr, aD	–	–	–	–	–
30	2,a	3,a	–	52, ADB	–	4, ADB	1, ad	–
31	–	–	tr, AD	–	tr, aDB	–	–	–
32	0.2,a	0.4,a	–	3, aDB	–	11, ADB	2, ad	–
33	–	–	–	–	tr, aDB	–	–	–
34	0.2,a	0.1,a	–	0.5, ad	–	12, ADB	3, ad	0.5, ad
36	0.1,a	0.1,a	–	–	–	0.1, ad	2,aD	0.6, ad

vailing, especially C_{30} and to a lesser degree C_{32} and C_{34} (Table 2). In the C_{28}/C_{30}, C_{32}/C_{34} and C_{36} homologues the isomers with 1,11-, 1,13-, and 1,15-structures were found to predominate, respectively (Table 4). Ketoaldehyde homologues with odd carbon numbers were detected only as trace components and their isomer compositions could therefore not be quantified. It is noteworthy that only 1,10- and 1,12-isomers were identified for the C_{29} and C_{33} homologues, respectively. Thus, the position of the secondary functional group in odd-numbered ketoaldehydes differs from that in the comparable even-numbered homologues.

Compounds in series C yielded $LiAlH_4$ reduction products that again had TMSi ether MS typical for prim./sec. alkanediols (Table 5). Homologues with chain lengths between C_{26} and C_{36} were detected (Table 2). Fraction C2 was found to contain the 1,11-isomers of even-numbered and 1,10-isomers of odd-numbered homologues while fraction C1 was dominated by 1,13- and 1,12-isomers, respectively. It is noteworthy that similar TLC separation of isomers had been achieved- for both the ketoaldehydes (fraction B) and the ketoalcohols (fraction C). This effect had been described by Buckner et al., 1984 and could be due to the reversible formation of intramolecular hemiacetals. Besides, C_{26}- and C_{28}-isomers were partially GC-resolved and thus 16 different diols were identified ('D' in Table 6). More isomers were detected by the presence of characteristic α-fragments $[C_nH_{2n-1}(OTMSi)_2]^+$ ('d' in Table 6). The reduction products of fractions B and C were predominated by the same diol homologues and isomers but the latter fraction additionally contained small amounts of 15 novel compounds.

Subsequent oximation and silylation of fraction C was applied to elucidate the original nature of the two functional groups by MS (Fig. 1(D), Table 5). Fragments m/z = 73, 75, 87 and 100 and respective molecular ions and/or peaks for $[M\text{-}31]^+$ indicated the presence of one methyl oxime and one TMSi ether group. The spectra showed α-fragments $[C_nH_{2n-1}NOMe]^+$ surrounded by peaks $\Delta m/z = +28$, $+15$, -32 and α-fragments $[C_mH_{2m-2}(NOMe)(OTMSi)]^+$ with peaks $\Delta m/z = +15$, -16, -46. Thus, the MS data revealed the presence of a terminal hydroxyl and a mid-chain carbonyl function. In fractions C1 and C2 the oximes of 10 compounds were identified ('O' in Table 6). It is noteworthy that, in contrast to the aldoximes of ketoaldehydes (see above), *cis–trans* isomers of the ketoximes could not separated by GC (Vogh, 1971).

The derivatization experiments unambiguously confirmed the ketoalcohol nature of the homologous series in fraction C. Their isomer composition was eventually determined by GC-MS of TMSi ethers (Fig. 1(E), Table 5). Corresponding MS were characterized by prominent fragments m/z = 75, 130, 143 and $[M\text{-}15]^+$ together with two α-fragments $[C_nH_{2n-1}O]^+$ and $[C_mH_{2m-2}(OTMSi)O]^+$ and related β-fragments. Full spectra could be acquired for 8 compounds ('K' in Table 6), 19 other ketoalcohols were identified and quantified using their α-fragments ('k' in Table 6). Among the 38 ketoalcohol homologues and isomers identified (in various derivatization experiments) as *Osmunda* wax components, only 11-ketooctacosanol (Buckner et al., 1984) and a series of 1,15-, 1,17- and 1,19-isomers (Robinson, Eglinton, Cranwell & Zeng, 1989) had previously been described ('lit' in Table 6).

Ketoalcohols constituted approximately 10 mass-% of the frond waxes of *O. regalis*. The homologue and isomer distribution of ketoalcohols followed the patterns found for the corresponding ketoaldehydes. Compounds with even carbon numbers prevailed with a pronounced maximum for chain length C_{30} (Table 2). Isomers carrying the carbonyl function in ω-19 position predominated for the C_{29}-C_{32} homologues, while ω-17- and ω-21 isomers were most abundant in C_{26}–C_{28} and C_{33}–C_{36} compounds, respectively (Table 6).

For structure elucidation of compounds in fraction A transesterification with BF_3/MeOH was employed

Table 5
MS of novel ketoalcohols and their derivatives

Compound	Derivative	M^+	$M\text{-}x^+$	α- and related fragments	Other fragments
Hydroxy-hexacosan-7-one	diol bis TMSi ether	miss.	528 (0.3)	454 (0.3) **369 (78)** **275 (80)** 185 (4)	149 (30) 147 (22) 129 (28) 103 (31) 75 (55) 73 (100)
Hydroxy-hexacosan-9-one	diol bis TMSi ether	miss.	528 (0.3)	454 (0.3) **341 (73)** **303 (86)** 213 (1)	149 (30) 147 (22) 129 (28) 103 (31) 75 (55) 73 (100)
Hydroxy-nonacosan-10-one	methyl oxime TMSi ether	540 (0.2)	509 (1)	352 (3) 339 (7) **324 (17)** 292 (6) 287 (5) **272 (13)** 256 (8) 226 (13)	100 (22) 87 (100) 75 (22) 73 (22) 57 (21) 55 (22)
Hydroxy-triacontan-11-one	methyl oxime TMSi ether	554 (0.3)	523 (1)	352 (4) 339 (9) **324 (19)** 292 (7) 301 (7) **286 (10)** 270 (7) 240 (15)	100 (23) 87 (100) 75 (23) 73 (18) 57 (22) 55 (22)
Hydroxy-triacontan-11-one	TMSi ether	524 (1)	509 (100) 395 (4)	382 (19) 367 (7) **295 (3)** 285 (3) 272 (35) **257 (12)** 241 (2) 215 (18)	199 (8) 143 (26) 130 (75) 75 (79) 55 (70)
Hydroxy-triacontan-13-one	TMSi ether	524 (15)	509 (83)	354 (8) 339 (5) **267 (3)** 300 (20) **285 (9)**	143 (44) 130 (77) 111 (21) 75 (100) 55 (63)
Hydroxy-hentriacontan-12-one	methyl oxime TMSi ether	568 (0.2)	537 (1)	352 (5) 339 (7) **324 (16)** 292 (5) 315 (6) **300 (7)** 284 (6) 254 (14)	100 (23) 87 (100) 75 (24) 73 (18) 57 (24) 55 (21)
Hydroxy-dotriacontan-11-one	methyl oxime TMSi ether	582 (0.4)	551 (1)	380 (9) 367 (7) **352 (21)** 320 (5) 301 (11) **286 (12)** 270 (12) 240 (26)	100 (28) 87 (100) 75 (25) 73 (23) 57 (14) 55 (28)
Hydroxy-dotriacontan-11-one	TMSi ether	553 (2)	538 (91) 423 (3)	410 (18) 395 (8) **323 (4)** 285 (5) 272 (42) **257 (16)** 241 (3) 215 (22)	199 (9) 143 (40) 130 (100) 75 (91) 55 (82)
Hydroxy-dotriacontan-13-one	methyl oxime TMSi ether	582 (0.2)	551 (0.5)	352 (4) 339 (4) **324 (10)** 292 (4) 329 (5) **314 (3)** 298 (3) 268 (11)	100 (21) 87 (100) 75 (27) 73 (19) 57 (24) 55 (16)
Hydroxy-dotriacontan-13-one	TMSi ether	553 (9)	538 (90)	382 (19) 367 (12) **295 (3)** 300 (31) **285 (12)**	143 (55) 130 (98) 111 (14) 75 (100) 55 (57)
Hydroxy-tritriacontan-12-one	methyl oxime TMSi ether	596 (0.2)	565 (1)	380 (2) 367 (6) **352 (14)** 320 (4) 315 (7) **300 (7)** 284 (7) 254 (15)	100 (24) 87 (100) 75 (25) 73 (16) 57 (23) 55 (20)
Hydroxytetratriacontan-13-one	methyl oxime TMSi ether	610 (0.2)	579 (1)	380 (3) 367 (7) **352 (15)** 320 (5) 329 (8) **314 (6)** 298 (8) 268 (14)	100 (23) 87 (100) 75 (28) 73 (17) 57 (24) 55 (21)
Hydroxytetratriacontan-13-one	TMSi ether	581 (11)	566 (68)	410 (15) 395 (10) **323 (7)** 300 (47) **285 (16)**	143 (58) 130 (100) 111 (17) 75 (93) 55 (53)
Hydroxypentatriacontan-14-one	methyl oxime TMSi ether	624 (0.1)	593 (0.4)	380 (3) 367 (7) **352 (11)** 320 (4) 343 (6) **328 (11)** 312 (8) 282 (25)	100 (26) 87 (100) 75 (40) 73 (37) 57 (52) 55 (34)
Hydroxyhexatriacontan-15-one	methyl oxime TMSi ether	638 (0.2)	607 (1)	380 (4) 367 (7) **352 (15)** 320 (5) 357 (6) **342 (5)** 326 (9) 296 (13)	100 (23) 87 (100) 75 (32) 73 (16) 57 (28) 55 (21)

(Riederer & Schönherr, 1986). The reaction product contained methyl hexadecanoate together with small amounts of methyl octadecanoate. Besides, ketoalcohols with even carbon numbers and chain lengths C_{28}–C_{36} were identified as TMSi ethers. Isomers carrying the keto function in 11-position were largely predominating. The homologue pattern of esterified ketoalcohols closely resembled those of free ketoaldehydes and ketoalcohols in *O. regalis* (Table 2).

Thus, the original constituents of fraction A were identified as esters of ketoalcohols. This interpretation was confirmed by their MS (Fig. 1(F), Table 1), fragments m/z = 239, 257 being characteristic for hexadecanoic acid esters (Wakeham & Frew, 1982), and two α-fragments together with related pairs of β-fragments indicating the presence and location of a carbonyl group (Dodova-Anghelova & Ivanov, 1973). Molecular ions lay beyond the mass range of the spectrometer. Additional peaks at m/z = 124, 125, 167 were found to be characteristic for this compound class. Hexadecanoates of 11-ketooctacosanol, 11-ketotriacontanol and 11-ketodotriacontanol were identified by their full spectra while corresponding octadecanoates and esters of 9-ketooctacosanol, 13-ketodotriacontanol, 11-ketotetratriacontanol and 13-ketotetratriacontanol were detected only by their characteristic carbonyl α-fragments. Relative portions of ester homologues (Table 2) should hence reflect the amounts of hexadecanoic acid esters of 11-ketoalcohols. Together, these esters represented approximately 2 mass-% of the *O. regalis* frond waxes.

In the present study four closely related series of bifunctional very long-chain components were identified. Due to the similarity of their homologue and isomer patterns it seems plausible that they represent subsequent steps in biosynthetic pathways. It remains to be investigated whether the terminal or the mid-chain function are generated first and whether they are formed in oxidative or reductive reaction sequences. In this context it is noteworthy that long-chain hydroxyacids with homologue and isomer distributions similar to the patterns described here have been identified as components of *Juniperus scopulorum* cuticles (Tulloch & Bergter, 1981) and microalgal compounds (Gelin, Volkman, de Leeuw & Sinninghe Damste, 1997). Also, respective keto fatty acids have been described as insect cuticular components (Meinwald, Smolanoff, Chibnall & Eisner, 1975; Cameron & Drake, 1976).

Other comparable series of bifunctional compounds have previously been described. In plant waxes sec./sec. diols and ketols with two secondary groups are frequently associated with corresponding secondary alcohols (Franich et al., 1979; Holloway & Brown, 1977; Holloway, Brown, Baker & Macey, 1977; Tulloch & Bergter, 1981). α,β-diols, α,γ-diols and α,ω-diols, but not their carbonyl derivatives, have been

Table 6
Isomer composition [%] of ketoalcohols in *Osmunda regalis* frond waxes calculated from abundances of corresponding keto α-fragments $[C_mH_{2m-2}O(TMSi)O]^+$. Derivatives used for identification are given (capitals = full MS available, small letters = characteristic fragments detected in isomer mixture, k = ketoalcohol TMSi ether, d = diol bis TMSi ether, o = methyl oxime TMSi ether, lit = previously identified)

Ketoalcoholcarbon number	Keto group position										
	7	9	10	11	12	13	14	15	17	19	21
26	tr, D	tr, D	–	–	–	–	–	–	–	–	–
28	tr, D	0.7, kD	–	0.7, KD lit	–	0.5, KD	–	–	–	–	–
29	–	–	tr, DO	tr, d	tr,d	–	–	–	–	–	–
30	2, kd	8, kd	–	30, KDO	–	9, KD	–	0.9, kd lit	–	–	–
31	–	–	tr, D	tr, d	tr, kDO	–	–	–	–	–	–
32	0.8, kd	3, kd	–	8, KDO	–	12, KDO	–	2, kd lit	0.8, kd	–	–
33	–	–	tr, d	tr, d	tr, DO	–	–	–	–	–	–
34	–	1, kd	–	1, kd	–	8, KDO	–	3, kd lit	1, kd lit	2, k	–
35	–	–	–	–	–	–	tr, O	–	–	–	–
36	–	–	–	–	–	1, kd	–	3, kDO	1, kd lit	0.2, kd lit	2, kd

detected both on surfaces of living plants (Akihisa et al., 1997; Dodova-Anghelova & Ivanov, 1969; Fauth et al., 1998; Tulloch, 1971) and in geologic material (Huang, Li, Lockheart, Peakman & Eglington, 1994; Pond, Langworthy & Holzer, 1986; Ten Haven, Baas, de Leeuw & Schenck, 1987). Ketoalcohols and ketoaldehydes (C_{26}–C_{28} and C_{30}, substitution on C_{10} or C_{12}) had previously been detected in the cuticular lipids of diapausing pupae of *Manduca sexta* (Buckner et al., 1984) and *Helicoverpa zea* (Buckner, 1993). Thus, the *O. regalis* compounds described here have homologue patterns similar to those of insects but their isomer distribution is very distinct. In the same insect waxes ketoalcohols had been found esterified mainly to C_2 and C_4 acids (Buckner, Nelson, Fatland, Hakk & Pomonis, 1984). Mixtures of alkanediols and ketoalcohols with one functional group each at the end and near the center of the carbon chain were detected in geologic sediments (Robinson et al., 1989) and had been attributed to bacterial (Morris & Brassell, 1988; Pond et al., 1986) and microalgal deposition (Mercer & Davies, 1979; Volkman, Barrett, Dunstan & Jeffrey, 1992).

3. Experimental

3.1. Plant material and sample preparation

Plants were grown in the Botanical Garden of the University of Würzburg in 1998. Sterile fronds were cut and immediately immersed twice for 30 s in $CHCl_3$ at room temp. The resulting solns of cuticular waxes were dried, filtered and the solvent partially removed under reduced pressure. This wax mixture was separated into compound classes (Holloway, 1984) by TLC on silica gel using $CHCl_3$–EtOH 99:1 or CCl_3CH_3 as the mobile phases (sandwich technique at room temp). Bands, as detected by staining with primuline and UV-light, were removed from the plates and eluted with $CHCl_3$.

3.2. Wax analysis

The qualitative composition of the fractions (or their derivatization products) was studied by capillary GC with on-column-injection (30 m OV-1 WCOT i.d. 320 μm) and MS detector (70 eV, m/z 50–650). GC was carried out with temperature programmed injection at 50°C, oven 2 min at 50°C, 40°C min^{-1} to 200°C, 2 min at 200°C, 3°C min^{-1} to 300°C, 30 min at 300°C and He carrier gas inlet pressures programmed 8 min at 40 kPa, 2 kPa min^{-1} to 150 kPa, 8 min at 150 kPa.

Homologue distributions were quantified within respective TLC fractions. For this purpose, GC was used under conditions as described above, but with carrier gas H_2 and FID integration. Base-line separation of all homologues was achieved. Where isomers could not be separated by GC their proportions were estimated using relative abundances of corresponding carbonyl α-fragments $[C_nH_{2n-1}O]^+$ and $[C_mH_{2m-2}O(TMSi)O]^+$ of ketoaldehydes and ketoalcohol TMSi ethers, respectively.

3.3. Derivatization reactions

Prior to GC analysis hydroxyl-containing compounds in all samples were transformed to the corresponding TMSi derivatives by reaction with bis-*N*,*N*-trimethylsilyltrifluoroacetamide in pyridine (30 min at 70°C).

Carbonyl-containing compounds were identified by reduction of appropriate fractions with $LiAlH_4$ in refluxing THF over 48 h. The mixt. of LiAl-alcoholate complexes was hydrolyzed with 10% H_2SO_4, the alcohols obtained by extraction of the soln with Et_2O and transformed into the corresponding TMSi ethers. Alternatively, carbonyl-containing compounds were identified as methyloximes after treatment with methyl hydroxylamine hydrochloride in pyridine-$CHCl_3$ 7:3 (30 min at 70°C).

For structure elucidation of novel esters the corresponding TLC fraction was treated with BF_3/MeOH (60 min at 70°C). The reaction products were isolated by addition of H_2O and extraction with Et_2O. After transformation into the corresponding TMSi ethers they were analyzed by GC-MS.

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