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BRANCHED-CHAIN CONSTITUENTS OF BRUSSELS SPROUT WAX

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Key Word Index—*Brassica oleracea* var. *gemmifera*; Cruciferae; Brussels sprout; epicuticular wax; subglaucous mutants; branched primary alcohols; branched fatty acids; branched alkanes; structural determination.

Abstract—The presence of a substantial proportion of branched, in addition to normal compounds, was confirmed in the alkyl ester and primary alcohol components present in the epicuticular wax of Brussels sprout leaves. Branched homologues with an odd carbon number belonged exclusively to the *anteiso* (ω3) series and those with an even carbon number to the *iso* (ω2) series. The main components of the esterified fatty acid fraction were *n*-C_{16} (26%), *anteiso*-C_{17} (17%), *iso*-C_{18} (3%), *n*-C_{18} (19%), *anteiso*-C_{19} (19%) and *n*-C_{20} (7·4%). The esterified primary alcohols comprised *iso*-C_{26} (10%), *n*-C_{26} (18%), *anteiso*-C_{27} (41%), *n*-C_{27}(7%), *iso*-C_{28} (3%), *n*-C_{28} (5%) and *n*-C_{29} (8%); all assignments were verified by mass spectrometry after conversion of the alcohols to the corresponding alkanes and methyl ethers. The free primary alcohol fraction had a similar qualitative composition to that of the esterified alcohols but it had a lower proportion of *br*-homologues and contained in addition a triterpenol fraction (α-amyrin 1%, β-amyrin 7%). Similar *br*-chain homologues were found in the ester and primary alcohol fractions from the mutant gl_3 but they comprised a much smaller proportion of the alcohol fractions than those found in the wax from normal plants. The structure of the major branched alkane in the wax of mutant gl_4 was also confirmed as *anteiso*-C_{30} after urea complex formation and mass spectrometry.

INTRODUCTION

The epicuticular wax from the leaves of the various cultivars of *Brassica oleracea* has been the subject of intensive study and investigations have been carried out on its chemical composition, ultrastructure [1–7], biosynthesis [review 8], physiological role [9,10] and on the influence of environment [6,7,11,12], applied chemicals [13–15] and genetic factors [3,4,6,7,16–18]. The wax is a complex mixture of aliphatic compounds in which C_{29} compounds predominate, viz. nonacosane [4,6,7,16,17,19–25], nonacosan-15-one [4,6,7,16, 17,19–22,24,25,26] (and nonacosan-14-one [26,27]), nonacosan-15-ol [6,7,16,17,20,21,24,26] (and nonacosan-14-ol [26,27]), and these together usually comprise about 60% of the wax with the alkane making up 30–40%. The remainder is composed of alkyl esters (C_{40}-C_{48}) [6,7,16, 17,22,24,28], aldehydes (mainly C_{28} and C_{30}) [6,7,16,17,29], primary alcohols (C_{22}-C_{30}) [6,7,16,17,20,28] and fatty acids (C_{14}-C_{30}) [6,7,16,17,25,28]. A minor ketol fraction is also present, the principal components being 16-hydroxynonacosan-14-one and 13-hydroxynonacosan-15-one [30]. Earlier reports [21] of the occurrence of nonacosan-10-ol and 10-hydroxynonacosan-15-one and 15-hydroxynonacosan-10-one have not been substantiated by later workers using MS. The waxes of glossy and subglaucous mutants have an altered ultrastructure and usually show a reduction in the relative amounts of C_{29} compounds with a consequent enhancement in the proportions of aldehydes (Brussels sprout gl_2 [6,7]), alkyl esters (Brussels sprout gl_3 [6,7]) or primary alcohols (cauliflower gl_5 [16,17]).

Br-chain compounds are also reported in *Brassica* waxes but structural assignments have only

been tentatively made on the basis of GLC R_t data. *Br*-homologues occur in the alkyl ester fraction (C_{43}, C_{45} and C_{47}) [7] and in its constituent fatty acids (C_{15}-C_{20}) and primary alcohols (C_{25}-C_{29}) [16–18]. The same *br*-alcohols are also found in the free primary alcohol fraction [6,7,16–18]. In the Brussels sprout mutant gl_4 additional *br*-homologues occur in the alkane fraction (mainly C_{30}) [6] and a similar compound is found in the kale mutant gl_6* [18] where branching also occurs in the aldehyde and free fatty acid fractions.

The present paper describes further work on the structural elucidation of the *br*-compounds in the wax from normal Brussel sprout leaves and their unequivocal identification by MS; a comparison is also made with the *br*-compounds present in the mutant gl_3. The structure of the *br*-alkanes from the mutant gl_4 is also deduced from MS.

RESULTS AND DISCUSSION

The constituent classes of the wax were isolated by PLC and examined by GLC (see experimental) and GC–MS for evidence of *br*-chain homologues. The relative proportions of the various classes were similar to those found by Baker [7]. Homologues which did not correspond with an *n*-series were found in substantial amounts in the primary alcohol and alkyl ester fractions, occurring in both the fatty acid and primary alcohol components obtained by hydrolysis of the ester fraction. Structural information on the unhydrolysed ester fraction could not be obtained directly from GC–MS because the system available was unsuitable for operation at the high temperatures necessary for elution of the homologues. No significant quantities of *br*-chain compounds were found in any of the remaining fractions (alkane, ketone, aldehyde, sec. alcohol and fatty acid) and all the compounds identified were in agreement with previous work.

Free primary alcohols

The underivatized fraction was analysed initially but resolution of the various constituents by GLC was poor. However, peaks corresponding with *n*-C_{24} (M^+-18 *m/e* 336), *n*-C_{26} (M^+-18 *m/e* 364), *anteiso* (ω3)-C_{27} (M^+-18 *m/e* 378, M^+-18-29 *m/e* 349, M^+-18-57 *m/e* 321), *n*-C_{28} (M^+-18 *m/e* 392), *anteiso*-C_{29} (M^+-18 *m/e* 406, M^+-18-29 *m/e* 377, M^+-18-57 *m/e* 349), *n*-C_{30} (M^+-18 *m/e* 420) alcohols and an amyrin-type triterpenol (M^+ *m/e* 426, base peak *m/e* 218) could be identified. The existence of triterpenols in the fraction was confirmed using a TLC test.

Better GLC resolution of the alcohols was obtained after conversion to the corresponding TMSi ether derivatives but little information about branching could be obtained after GC–MS. The derivatives exhibited intense M^+-15 ions and showed that both a *n*- and *br*-series of compounds was present, each series showing a similar range from C_{24}-C_{30}. The MS of *iso*-alcohol TMSi ethers, however, were indistinguishable from those of the *n*-compounds and those of *anteiso*-alcohol TMSi ethers were also similar but showed an additional ion of low rel. intensity corresponding with M^+-15-72. Two peaks in the fraction gave identical MS (base peak *m/e* 218, M^+ *m/e* 498) and were identified as α- and β-amyrin TMSi ethers, the β- eluting before the α- compound on GLC. These compounds partially co-eluted with aliphatic alcohol TMSi ethers in the range C_{29}-C_{30} on SE30 but were well resolved from them on OV210 and OV225. The quantitative composition of the fraction (Table 1) was calculated from the OV210 results; triterpenols comprised *ca* 9% of the fraction.

Unequivocal identification of the primary alcohol homologues was made by GC–MS after their conversion to the corresponding alkanes and methyl ethers, both being prepared from the mesylate derivative. *Br*-homologues with an odd carbon number belonged exclusively to the *anteiso* (ω3)-series yielding *anteiso*-alkanes (diagnostic ions M^+-29, M^+-57) and *anteiso*-methyl ethers (diagnostic ions M^+-18, M^+-18-29, M^+-18-57), and those with an even carbon number to the *iso* (ω2)-series giving *iso*-alkanes (diagnostic ions M^+-15, M^+-43) and *iso*-methyl ethers (diagnostic ions M^+-18, M^+-18-28, M^+-18-56). *Br*-compounds comprised *ca* 40% of the fraction (Table 1) and odd carbon number homologues predominated with 24-methylhexacosanol as the major component (25%). Hexacosanol (32%) and octacosanol (12%) were the principal *n*-compounds of the fraction.

* Seed for this plant was not obtainable for verification of these results. M. J. K. Macey, personal communication.

Table 1. Relative composition (%) of fractions from normal Brussels sprout wax containing branched chain and cyclic constituents

Homologues	Esterified fatty acids*	Esterified primary alcohols†	Free primary alcohols†
n-C_{14}	0·9	—	—
anteiso-C_{15}	0·4	—	—
n-C_{15}	1·5	—	—
iso-C_{16}	0·6	—	—
n-C_{16}	25·8	—	—
antesio-C_{17}	16·8	—	—
n-C_{17}	0·9	—	—
iso-C_{18}	3·0	—	—
n-C_{18}	18·7	—	—
anteiso-C_{19}	19·3	—	—
n-C_{19}	0·6	—	—
iso-C_{20}	0·4	—	—
n-C_{20}	7·4	—	—
anteiso-C_{21}	0·7	—	—
n-C_{22}	0·9	—	—
anteiso-C_{23}	0·6	—	—
iso-C_{24}	0·7	—	—
n-C_{24}	0·2	2·1	2·5
anteiso-C_{25}	—	1·0	0·1
n-C_{25}	—	1·0	0·3
iso-C_{26}	—	9·5	4·2
n-C_{26}	—	17·7	31·7
anteiso-C_{27}	—	40·8	25·0
n-C_{27}	—	6·9	0·3
iso-C_{28}	—	3·4	2·6
n-C_{28}	—	5·2	11·8
anteiso-C_{29}	—	tr	5·0
n-C_{29}	—	7·8	1·8
iso-C_{30}	—	tr	0·3
n-C_{30}	—	1·6	4·8
			α-amyrin 1·3
			β-amyrin 7·3
Unidentified	0·6	2·8	1·0

* GLC analysis as corresponding Me ester derivative on SE30 column.
† GLC analysis as corresponding TMSi ether derivative on OV210 column.

Esterified primary alcohols and fatty acids

The esterified alcohols of the wax had a similar qualitative composition to that found for the free alcohols, except that no triterpenols were detected (Table 1); the same methods were used for structural elucidation of the *br*-alcohols. The quantitative composition of the two fractions, however, was different and *br*-homologues comprised a larger proportion of the esterified fraction (*ca* 55%) than that found in the free fraction. The main *br*-component was 24-methylhexacosanol (41%) and significant amounts of 24-methylpentacosanol (10%) and 26-methylheptacosanol (3%) were also present. The principal *n*-alcohols were C_{26}, C_{29}, C_{27} and C_{28} respectively.

Structural assignments of the esterified fatty acids (Table 1) were made by MS and were confirmed by determination of ECL values using capillary (WCOT) GLC on a polar phase. *Br*-homologues comprised over 40% of the fraction and, as was found in the alcohol fractions of the wax, homologues with an odd carbon number belonged exclusively to the *anteiso* series and those with an even number to the *iso* series. No evidence of other monomethyl substituted or *br*-acids was obtained from capillary GLC. The major *br*-acids were 14-methylhexadecanoic (17%) and 16-methyloctadecanoic (19%); *iso*-acids made up less than 5% of the fraction with 16-methylheptadecanoic being the most abundant (3%). The principal *n*-acids were C_{16}, C_{18}, C_{20} and C_{15}, respectively.

Alkyl ester and free primary alcohol fractions from mutant gl_3

Mutant gl_3 was selected for comparison with normal plants because the major components of the wax are alkyl esters and primary alcohols [6,7]. The qualitative composition of the isolated alkyl ester (and its constituent fatty acids and primary alcohols) and primary alcohol fractions were identical to those found in normal plants but the quantitative compositions of the alcohol fractions differed from those in normal plants. The content of *br*-compounds in the free alcohol fraction was lower (12%), with 24-methylhexacosanol comprising *ca* 10% of the total alcohols. *n*-Hexacosanol was the major component of this fraction (75%) and a small triterpenol fraction (<2%) was also present. A higher proportion of *br*-homologues occurred in the esterified alcohol fraction (26%) but the proportion was again lower than that found in the same fraction from normal plants; the major components were *n*-hexacosanol (66%) and 24-methylhexacosanol (18%). The composition of the esterified fatty acids (*n*-C_{16} 25·8%, anteiso-C_{17} 16·8%, *n*-C_{18} 18·7%, *anteiso*-C_{19} 19·3%, *n*-C_{20} 6·9%) was similar to that found in the corresponding fraction from normal plants.

Br-*alkanes from mutant* gl_4

The alkane fraction was isolated from the wax by column chromatography and GLC analysis showed that *ca* 6% of the homologues were not of the *n*-series. The content of these compounds in the fraction was increased to *ca* 30% by repeated complex formation with urea and the fraction then analysed by GC–MS. 3-Methylnonacosane was identified as the major *br*-compound with much smaller amounts of the *iso*-C_{27}, *anteiso*-C_{28} and *iso*-C_{27} homologues; the principal constituent of the original fraction was nonacosane (90%).

Our results with Brussels sprout generally confirm the tentative identifications of *br*-chain compounds made by previous workers [6,7,16–18] in a range of *B. oleracea* waxes. These compounds, however, usually comprise a small proportion (*ca* 5–10%) of the wax of normal glaucous plants but they become more important constituents of the waxes from subglaucous or glossy mutant forms. The quantitative composition of the fractions containing *br*-compounds and in particular the ratio of *n* to *br*-compounds appears to vary according to cultivar and is also altered by mutation [7]. In the normal form of cauliflower, for example, the esterified acids contain greater quantities of *br*-homologues (*ca* 70%, anteiso-C_{19} 47%) [18] than those found in Brussels sprout. Also in Brussels sprout it has been demonstrated that the *n*- to *br*-homologue ratio can be influenced by growth conditions [6]. Although no GC–MS analysis was carried out on the unhydrolysed ester fraction of Brussels sprout wax previous GLC work [7] has shown the principal homologues to be *n*-C_{42}, *br*-C_{43}, *n*-C_{44}, *br*-C_{45}, *n*-C_{46} and *br*-C_{47}. However, analyses of the component fatty acid and alcohols indicate that the exact composition of the fraction must be complex because odd carbon number homologues can be derived from either *br*-acids + *n*-alcohols or *n*-acids + *br*-alcohols and the even homologues from either *br*-acids + *br*-alcohols or *n*-acids + *n*-alcohols.

Biosynthesis of the *br*-alcohols and acids of *Brassica* waxes probably occurs by an elongation process utilizing acetate and *br*-chain amino acid precursors as proposed by Netting *et al.* [18]. Support for this pathway is found in earlier work by Kolattukudy [31] using broccoli. Although he failed to identify *br*-compounds in the wax, he found that the fatty acid synthesizing system of the leaf was capable of utilising labelled *br*-amino acid substrates for the synthesis of wax constituents. The major radioactive components were esters and primary alcohols but the production of *br*-acids was only demonstrated in the internal lipids of the plant. The free and esterified alcohols are probably derived from the same metabolic pool which obviously is a different one from that which gives rise to the free fatty acid and aldehyde components of the wax [18]. The present study, however, has also demonstrated that the esterified alcohols have a higher content of *br*-homologues than the free alcohols.

EXPERIMENTAL

Epicuticular wax was isolated using a brief immersion in $CHCl_3$ from mature leaves of *B. oleracea* var *gemmifera* 'normal' (Cambridge Special X Ashwells strain) and the subglaucous mutants gl_3 (Irish Elegance) and gl_4 (Seven Hills). The plants were grown in pots in a greenhouse. Wax (70 mg) from normal plants was fractionated by PLC on 6 × 0·75 mm thickness Si gel G plates as described in Ref. [7]. The constituent

alcohols and fatty acids of the alkyl ester fraction were obtained using NaOMe–MeOH [32] and isolated by PLC on Si gel G using C_6H_6. Isolated fractions were analysed by GLC using an FID instrument and R_t data determined on 2 mm id stainless steel columns (a) 1 m 1% SE30 (b) 1 m 1% OV17 (c) 1 m Dexsil 300 (d) 2 m 3% OV210 (e) 2 m OV225. N_2 at 30 ml/min and temp. programming at 6°/min were used with a limit of 250° on columns (d) and (e), 300° on (a) and (b) and 350° on (c). Hydroxy compounds were analysed as TMSi ethers [33] and fatty acids as Me esters [34]. The GC–MS column and conditions were those previously described [33] and MS were recorded at both 20 and 70 eV.

Identification of n-*chain components* was made by GLC co-injection with authentic compounds and by MS.

Br-*alcohols*. These were identified by MS comparison with authentic *iso*- and *anteiso*-C_{18} alcohols and with published information [35]. Mesylate derivatives were prepared from the primary alcohol fraction using $MeSO_2Cl$ and C_5H_5N [36]. Alkanes were prepared from the mesylates (1 mg) by reduction with $LiAlH_4$ in Et_2O [37] and purified by chromatography on a short column of Si gel and elution with hexane; *n*- and *br*-chain components were identified by GC–MS and comparison with the lit. [38]. Methyl ethers were also prepared from the mesylates (1 mg) by refluxing with 0·5 ml of 0·5% NaOMe–MeOH for 3 hr. The reaction mixture was taken to dryness, 1 ml satd NaCl soln added and the products recovered by extraction with 3 × 1 ml Et_2O. The derivatives were purified by preparative-TLC on Si gel G using C_6H_6 and characterised by GC–MS (20 eV) using comparison with the methyl ethers prepared from authentic *iso*- and *anteiso*-C_{18} alcohols and with published information [35].

Br-*acids*. These were identified by MS [39] and from ECL values [40] determined using a WCOT column (50 m × 0·25 mm) coated with butanediol succinate. The GLC analysis was carried out isothermally at 170° with a He flow rate of 5 ml/min using an inlet split ratio of 20:1.

Triterpenols were identified by TLC (colour response with H_2SO_4 spray at 100°), GLC R_t data and MS using authentic samples of α- and β-amyrin.

Alkyl esters and primary alcohols were isolated from mutant gl_3 wax (5 mg) by PLC as described earlier; the relative amounts of the constituent classes of the wax were similar to those previously reported [7]. Similar methods to those used for the corresponding fractions from the wax of normal plants were employed for analysis and structural elucidation.

Alkanes were obtained from mutant gl_4 wax (25 mg) after Si gel column chromatography and elution with hexane (yield 9 mg). Urea clathration was carried out in hexane soln as described in Ref. [41] and the progress of the reaction was monitored by analysing small samples (1–2 μl) of the supernatant soln by GLC. MS identification of the alkane fraction was carried out as described earlier.

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