NONAN-2-OL ESTERS IN SORGHUM LEAF EPICUTICULAR WAX AND THEIR COLLECTION BY PREPARATIVE GAS CHROMATOGRAPHY

by

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Epicuticular wax from sorghum seedling leaves was isolated and analyzed by TLC and GC. Its composition was remarkably similar to that of waxes on many other Gramineae leaves with the exception that three alkan-2-ol esters (nonan-2-ol docosanoate, nonan-2-ol tetracosanoate, and nonan-2-ol hexacosanoate) were present. This was unexpected as such esters have hitherto only been reported in waxes of plants with the capability of synthesizing β -diketone lipids. The C_{31} - C_{33} alkan-2-ol esters were separated from the C_{18} - C_{26} alkan-1-ol esters by preparative GC. The column effluent was passed through a small Dexsil-300 trap maintained at 150 °C, and the esters eluted therefrom with chloroform. This technique gave better than 90% recovery of a $[1-^{14}C]$ - C_{32} ester.

1. INTRODUCTION

Disclosing the β -diketone (primarily, hentriacontan-14,16-dione and 25-hydroxyhentriacontan-14,16-dione) and esterified alkan-2-ol (primarily tridecan-2-ol and pentadecan-2-ol) biosynthetic pathways and their genetic control is our primary goal. As biological material we have used barley spikes of the *eceriferum* mu-

tants which determine synthesis and deposition of the very long chain lipids on the external surface of epidermal cell walls. Since large amounts of material would spur attempts to isolate the enzymes involved, we have been looking for a plant which synthesizes one or more of the specified lipids on rapidly growing seedling leaves. Herein we identify nonan-2-ol

Abbreviations: GC = gas chromatography; MS = mass spectrometry; TLC = thin layer chromatography; TMS = trimethylsilyl.

esters on primary sorghum (Funk G251) leaves and describe a technique for collecting them by preparative gas chromatography (GC).

2. MATERIALS AND METHODS

2.1. Materials and chemical analyses

Seeds of sorghum (*Sorghum bicolor*, Funk G251) were planted in vermiculite and germinated in the light at 23 °C (19). Ten or 12 days later the epicuticular wax was isolated from seedling leaves as described previously (34). The wax was separated into its component lipid classes by thin layer chromatography (TLC) using silica gel H (Merck) plates and developing them first with hexane and then with amylene stabilized chloroform or only with the second solvent. Lipid classes were visualized and recovered from the TLC plates as detailed earlier (42). The isolated lipid classes and/or their derivatives (42) were quantitatively analyzed via GC using Hewlett-Packard instrumentation and 3% SP-2100 columns (42). The retention times of the esters were compared with those of C₂₄, C₂₆ and C₂₈ methyl esters and C₂₆, C₂₈ and C₂₉ esters (dodecyl, tetradecyl and pentadecyl myristate) purchased from Analabs (North Haven, Conn., USA).

To ensure that after transmethylation (23) none of the moieties of the three unknown trapped esters (section 2.2) would be lost, a modified procedure for their isolation was developed. Five g of 100-200 mesh Florisil (Koch-Light Laboratories Ltd., Colnbrook Bucks, England) was added to a 1 cm (i.d.) glass column and rinsed with 50 ml redistilled diethyl ether:hexane (50:50, v/v) followed by 100 ml hexane. Subsequent to transmethylation the ester moieties were extracted with 4 × 3 ml hexane, and loaded directly onto the column. Twenty ml hexane, 20 ml 5% diethyl ether in hexane and 20 ml 20% diethyl ether in hexane were passed through the column and collected separately. All the fatty acid methyl esters were eluted with 5% diethyl ether in hexane, and the alcohols with 20% diethyl ether in hexane. The fraction containing the alcohols was concentrated to about 1 ml at 0 °C under N₂, transferred to a small vial and concentrated to 100 µl at 0 °C before preparing trimethylsilyl (TMS) deriva-

tives (25). Qualitative GC analyses were performed on a Packard 437 instrument with a single 2 mm (i.d.) × 1 m glass column containing 10% SE-30 on Chromosorb W, HP 80-100 mesh using conditions similar to those for the Hewlett-Packard instrument (42). The reported percent distributions of the trapped esters and their fatty acid moieties were estimated by triangulation.

GC-mass spectrometry (MS) analyses of the esters, the ester fatty acid methyl esters and alcohol-TMS derivatives were carried out (40). For confirmation of the alcohol moiety structure, TMS derivatives (40) of decan-1-ol, dodecan-1-ol and tetradecan-1-ol (from Applied Science, State College, PA, USA) and nonan-2-ol were prepared and subjected to GC-MS. Nonan-2-ol was synthesized by reaction of heptylmagnesium bromide with acetaldehyde.

2.2. Collection of esters by preparative GC

The method previously employed in this laboratory of trapping the effluent of a GC column in Dexsil 300 at -196 °C (24) gave only a 5% yield with the unknown sorghum esters. This was unexpected as 96% of injected methyl (1-¹⁴C)-palmitate was collectable (24). The poor trapping efficiency with the sorghum esters is presumably due to formation of an aerosol (17). To circumvent aerosol formation the eluate from the GC column must not be cooled below the melting point of the compound of interest. Since the C₃₂ ester hexadecyl palmitate has a melting point of 53-54 °C, a minimum temperature of 100 °C was desired. Thus, the major arm from the column splitter of our radio-GC system (24) was disconnected after passing through the combustion oven reduced to 150 °C, and the 0.55 cm (i.d.) × 15.5 cm piece of quartz tubing containing copper oxide emptied and refilled with the trapping material, 21 mg 1.5% Dexsil 300 on Chromosorb W AW, 60/80 mesh (Supelco, PA, USA). Esters trapped by the Dexsil were recovered by passing 3 × 3 ml of chloroform through the quartz tubing removed from the oven and cooled to room temperature. To test the efficiency of this technique a labelled C₃₂ ester, namely [1-¹⁴C]-docosanyl caprylate, was synthesized (28). Injection of known amounts of this ester followed by liquid scintillation counting of

Figure 1. Compositions of the epicuticular waxes on seedling leaves of Funk G251 sorghum (S) and spikes of Bonus barley (B) as revealed by TLC.

The lipid classes were visualized by spraying with 55% H_2SO_4 containing 0.6% (w/v) $\text{K}_2\text{Cr}_2\text{O}_7$, followed by charring. HC = hydrocarbons, EST = esters, ALD = aldehydes, POH = primary alcohols, FFA = free fatty acids, PL = polar lipids, β -DIK = β -diketones, OH- β -DIK = hydroxy- β -diketones.

the chloroform extractable radioactivity gave an average trapping efficiency of 92% for four separate trials.

3. RESULTS

Figure 1 shows that the sorghum seedling leaf wax studied is composed principally of primary alcohols, esters and aldehydes in order of decreasing importance plus minor amounts of alkanes, free fatty acids and/or polar lipids plus several other unidentified compounds with R_f values between those of the primary alcohols and the free fatty acids. The slightly greater R_f of the sorghum than the barley esters suggests they have relatively more of the longest chain homologues. While p-hydroxybenzaldehyde is a

prominent constituent of some sorghum waxes (17), it can not amount to more than a trace in the present wax as shown by co-TLC with an authentic standard. EBERT and RAMSTEINER (12) who also analyzed Funk G251 seedling wax report from MS of total wax a weight % distribution of primary alcohols = 65.9, free fatty acids = 6.6, aldehydes = 2.2, esters = 5.7 and unknowns = 19.6%. From our results (Figure 1) we can not guess what the unknowns are, but we believe that the different growing conditions (6, 39) and analytical methods used have given rise to the discord between their results and ours for the identified lipid classes.

Preparative TLC was used to isolate the lipid classes which were then analyzed by GC. The normal alkanes amounting to at least 1% of the total wax were C_{25} = 13.9, C_{27} = 14.4, C_{29} = 20.3, C_{31} = 39.9 and C_{33} = 2.6. Table I presents the compositions of five additional wax fractions. The alcohol and free acid distributions are reasonably similar to those reported by EBERT and RAMSTEINER (12), whereas those of the other three lipid fractions are not, as Table I shows. We are unable to explain the discrepancy in the results, but again suggest that a difference in growing conditions may be partly responsible (6, 39). GC of the esters (Figure 2) gave the puzzling result that the presence of the three shortest components could not be accounted for by combining observed ester alcohol and acid moieties (Table I). This problem does not arise for the C_{38} to C_{56} esters, although the hypothesis of completely random esterification does not seem justifiable at first glance.

As a first step toward identifying the three unknowns, semilog plots of apparent carbon number vs retention time were constructed after isothermal GC with methyl and myristate ester standards. The sorghum unknowns eluted distinctly later than C_{28} , $_{30}$ and $_{32}$ methyl esters, and just before C_{30} , $_{32}$ and $_{34}$ myristate esters. Since the retention times of the sorghum unknowns relative to the myristate esters was reminiscent to that of the alkan-2-ol vs alkan-1-ol esters of barley (41), a coinjection of sorghum and barley spike esters was made. The three unknowns eluted together with the barley C_{31} , $_{33}$ and $_{35}$ alkan-2-ol esters. Separate injections followed by construction of semilog plots, how-

Table I
Compositions of five lipid fractions from sorghum (Funk G251) seedling leaf wax (weight %)^a.

Number of carbons	Aldehydes	Alcohols	Free acids	Ester alcohols ^b	Ester acids
16					2.9
18					6.1
20	(0.8)		1.2	3.1 (7.5)	49.7 (19.3)
22	(1.2)		2.9	37.7 (15.3)	17.9 (25.8)
24	(1.0)		7.6	5.0 (5.1)	17.0 (17.9)
26	2.2 (2.7)	1.6	4.7	2.4 (4.1)	4.4 (5.0)
28	14.4 (35.6)	23.4	39.3	22.0 (37.1)	1.1 (12.0)
30	18.1 (30.5)	10.5	26.3	6.0 (20.4)	(13.4)
32	60.2 (28.2)	63.1	16.0	18.5 (10.2)	(9.5)
34	2.3				
Other ^c	2.7	1.3	2.0	5.5	0.9

^a Aldehydes and alcohols as acetates; acids as methyl esters. () = values reported in reference (12) for C₂₀-C₃₂ homologues; alcohols analyzed as TMS derivatives.

^b Nonan-2-ol lost in this analysis (see section 2.1).

^c Includes unknowns, odd chain homologues, plus all components amounting to less than 1%.

ever, revealed that the sorghum unknowns eluted on the tailing sides of the barley peaks.

The three unknowns whose % distribution in order of increasing retention time was 8.7, 75.2 and 16.0, respectively, were next separated from the C₃₈ to C₅₆ esters by preparative GC as described in section 2.2. GC of the trapped material revealed only the three desired components in roughly 15.4, 75.6 and 9.0%, respectively, which was considered adequate for further work. The trapped material was subjected to transesterification and the potential ester moieties prepared for GC-MS analyses as detailed in section 2.1. to avoid loss of short chain components. GC of one fraction revealed three peaks having the % distribution of approximately 13.9, 73.7 and 12.4 which by GC-MS were identified as C₂₂, C₂₄ and C₂₆ methyl esters, respectively. This composition of the acid moieties is markedly different from that of the total ester fraction in which the 20 carbon chain lengths dominate (Table I), presumably explaining in part the apparent non-random esterification alluded to above. The only peak present in the gas chromatogram of the fraction which ought to contain alcohol TMS derivatives eluted at 113 °C. The presence in its mass spectrum of a base peak at m/z 117 and a smaller one (approx. 10%) at m/z 201 are

readily interpretable as α-cleavage fragments of the TMS derivative of nonan-2-ol. The mass spectrum was identical to that of the known standard except that the latter had a trace peak at m/z 215 (M-1). The identification of a single

Figure 2. GLC trace of esters from seedling leaves of Funk G251 sorghum.

Total carbon numbers are given for the alkan-2-ol esters (C₃₁-C₃₅) and alkan-1-ol esters (C₃₈-C₅₆).

Figure 3. Mass spectrum of nonan-2-ol tetracosanoate.

alcohol combined with the similarity in % distribution of the starting unknown esters and the derived acid moieties leads to the suggestion that the three unknowns in the sorghum esters are nonan-2-ol docosanoate, nonan-2-ol tetracosanoate and nonan-2-ol hexacosanoate.

To confirm these structural allocations, GC-MS of the trapped esters was carried out. Figure 3 is a mass spectrum of the deduced C_{33} ester. The three peaks at m/z 351, 368 and 369 are characteristic for the acid moiety fragments RCO^+ , RCO_2H^+ and $RCO_2H_2^+$, respectively, where $R = C_{23}H_{47}$; while the intense peak at m/z 126 is attributable to the alcohol $[R'-1]^+$ fragment, where $R' = C_9H_{19}$ (1, 41). Molecular ion peaks were neither expected nor detected. Absence of a peak at m/z 154 indicates that C_{11} -2-ol isomers are not present in the C_{33} ester. Heptan-2-ol isomers should give a $[R'-1]^+$ fragment peak at m/z 98. Since peaks arising from the predicted C_{26} acid moiety, RCO^+ , RCO_2H^+ and $RCO_2H_2^+$ at m/z 379, 396 and 397, respectively, are not present, the existence of heptan-2-ol esters is considered unlikely.

4. DISCUSSION

Compositional analyses of cuticular waxes from a number of different types of sorghum including tropical to temperate varieties have been made (2, 4, 8, 9, 12, 43, 44, 45, 46). The waxes have been collected from various individual organs or combinations thereof present in seedlings to mature plants using techniques ranging from those that remove only the surface lipids, that is, epicuticular waxes, to those that in addition extract internal lipids, for example, fatty acids. Thereafter, anywhere from one to five wax classes (including alkanes, esters, primary alcohols, free acids, aldehydes, and p-hydroxybenzaldehyde) have been studied with the level varying from relative amount determinations to complete chain length distribution analyses of individual lipid classes or fractions. Even if allowances are made for the marked diversity of plants combined in the genus sorghum and the noted experimental differences, one is struck by the extraordinary range in wax composition reported. This includes the few cases where similarities might be expected.

Compare the leaf blade analyses of the Italian varieties Alliance A and SD102 (8) with those of the American variety Redbine 60 and a bloomless mutant (43).

In the following we itemize what we consider the most interesting facets of this diversity. Firstly, that free acids can be predominant or major components of a plant wax (4, 8, 9, 45) is quite unusual. Likewise the presence of p-hydroxybenzaldehyde is unexpected especially in such large amounts as in cv. 65D from Botswana (44, 46). Secondly, the thick, white waxy bloom present on parts of leaf blades and sheaths is not attributable to β -diketone lipids as it is in many other Gramineae (7, 26, 36, 42). In fact to our knowledge, this lipid class has not been reported in any sorghum wax. Thirdly, a number of the analyses specify chain lengths shorter or longer than characteristic for epicuticular waxes. For example, ester alcohols from Alliance A and SD102 (8), free alcohols and alkanes in Redbine 60 and the bloomless mutant (43) and ester acids from 65D (45).

The presently investigated epicuticular wax from seedling leaves of Funk G251 lacks the special features noted above in points one and three. In fact the wax classes identified, their relative amounts and chain length compositions were all within the range of variation known from other Gramineae leaves such as barley (16, 35), corn (5), oats (31), wheat (32) and various grasses (29, 30, 33). The only exception to this generalization was the identification of three nonan-2-ol esters. Hitherto, esterified alkan-2-ols have only been identified in waxes from plants that synthesize β -diketones (18, 30, 37). This is presumably a reflection of the close biosynthetic relationship between these two wax lipids (25, 38), the ease by which such short alcohols can be lost subsequent to ester hydrolysis and the failure of esterified alkan-2-ols, in contrast to β -diketone lipids, to contribute markedly to plant phenotype. Inhibition of β -diketone synthesis by genetic modification can lead to epicuticular waxes, however, in which esterified alkan-2-ols are present in the absence of β -diketones (37). Furthermore, alkan-2-ols may be located elsewhere than in epicuticular waxes which thus far is the only site of β -diketone lipids. C_{17} - and C_{19} -2-ols are present as

monomers covalently attached to cutin and suberin polymers from corn, peas, lettuce and barley (13, 14). Of these four species only the latter is known to synthesize esterified alkan-2-ols (41), but they are shorter, primarily C_{13} and C_{15} , and are absent in epicuticular wax of the leaves which tissue was the source of the cutin polymer analyzed. In addition, both free and esterified alkan-2-ols ranging in length from 15 to 25 carbons are associated with but not covalently attached to the suberin layer of the periderm present in the underground storage organ of the parsnip (15).

Unlike many of the previous studies of sorghum waxes, the present one was not directed toward assigning the epicuticular wax or one of its constituents a specific function in, for example, drought resistance (40, 43) herbicide sensitivity (11, 12) or plant-insect interaction (2, 3, 4, 44, 45, 46). Our identification of nonan-2-ol esters, however, may be pertinent with respect to the recent reports (3, 45) that wax esters deter nymphs of *Locusta migratoria* from feeding on seedling leaves of cvs. CSH 1, IS 1082 and 65D. The ester fraction isolated from CSH1 and IS 1082 (2, 4) contained C_{40} - C_{58} esters composed of C_{16} - C_{30} alkan-1-ols and C_{14} - C_{30} acids. The data, however, are inconsistent in failing to account for the esters by combining either randomly, as the authors point out, or non-randomly the reported moieties. Esters of 65D seedling leaves were almost completely separated by TLC into two fractions (45). This implies that the esters are of one type but fall into two markedly different groups of chain lengths and/or they are of two structurally different types, for example, in having alkan-1-ol vs alkan-2-ol moieties. WOODHEAD favors the former possibility and despite the fact that both isolated ester fractions were deterrent considers the shorter esters more effective. This deduction was based on an analysis of the acid moieties of one 65D ester fraction which revealed primarily 12-20 carbons. Furthermore, nymphs were presented with $C_{36} + C_{38}$ vs $C_{46} + C_{48}$ tetracosanyl esters. The former having $C_{12} + C_{14}$ acid moieties proved deterrent, while the latter having $C_{22} + C_{24}$ acid moieties was not. Unfortunately, the complementary distributions – of acid moieties from the other isolated 65D ester fraction, alcohol moieties from both fractions,

and of the two ester fractions themselves – needed to put WOODHEAD'S speculation on a firm basis are not given. Confirmation of the structural nature behind the 65D ester detergency is thus required. In the course of such investigations, we suggest that the potential existence of alkan-2-ol esters or derivatives of (40) be considered even though cvs. 65D and Funk G251 are not closely related. This proposal is based on the literature discussed above plus the known role of acetic and propionic acid esters of 3,7-dimethylpentadecan-2-ol as sex pheromones for two genera of pine sawflies (20, 22).

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