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SURFACE LIPIDS OF *SPHAEROTHECA FULIGINEA* SPORES

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Key Word Index—*Sphaerotheca fuliginea*; marrow powdery mildew; fungal spores; surface lipid; alkyl, methyl and diol esters; free fatty acids; Δ^{2t} compounds.

Abstract—The major components (50%) of the surface lipid extract of fungal spores (5.6% of dry spore wt) of *Sphaerotheca fuliginea* are esters of primary alcohols and fatty acids. Esters (15%) of primary alcohols and a Δ^{2t} acid are present. The major acid moieties of the alkyl esters are C_{22} and C_{24} and of the Δ^{2t} alkyl ester is Δ^{2t} C_{22}; for both classes eicosanol is the major primary alcohol. The major ester of each class was concluded to be eicosanyl docosanoate and eicosanyl *trans*-2-docosenoate. Minor components are saturated and Δ^{2t} methyl and diol diesters and free fatty acids. The major acid moieties of the diol diesters are C_{22} and C_{24} and the major diol is 1,12-dodecanediol.

INTRODUCTION

The partitioning of fungicides into fungal spores [1] might play an important part in fungicidal activity and may be influenced by the composition of the spore surface lipid. The function of these surface lipids is a subject of conjecture but it has been suggested that they are involved in the prevention of desiccation, providing a barrier to chemical penetration, and in the germination and infection processes [2, 3].

Although the total lipid content of some fungal spores has been studied [4], especially with emphasis on hydrocarbon and fatty acid composition [5, 6], little attention has been given to surface lipids. The majority of reports have used either C_6H_6–$CHCl_3$ [7, 8], $CHCl_3$–MeOH [7] or C_6H_6–MeOH [9, 10] for extraction and the possibility that internal lipids were also removed cannot be ignored since, recently, Johnson *et al.* [5] showed that by extracting spores with heptane followed by C_6H_6–$CHCl_3$ (3:1), lipid was removed from the spore interior. One of the first reports of surface lipid was by Baker and Strobel [11] who used *n*-hexane for the extraction of uredospores of *Puccinia striiformis*. Several classes of compounds were identified but only the hydrocarbon fraction was studied in detail showing an homologous series from C_{19} to C_{31} with C_{29}, C_{31} and C_{27} predominating. Jackson *et al.* [2] extended this work and identified the major classes as β-diketone, hydrocarbon and free alcohol respectively and found a qualitative similarity between leaf and fungal surface lipids.

In the present investigation we have shown by extraction with *n*-hexane the presence of surface lipid and 82% of this wax has been identified.

RESULTS AND DISCUSSION

The extraction and separation methods used gave both the optimal yield of surface wax and efficiency of separation of the saturated and unsaturated components. The results of two extractions obtained from spores collected in successive years are given in Table 1 and the differences between them are possibly due to differences in spore age or time of year when harvested. The results obtained from spores collected in 1976–77 are those used for discussion and quantitation.

Hexane extraction yielded 5.6% of the dry spore wt. Contents of the extract were almost certainly only surface lipid since triglycerides (R_f^1 0.23) and sterols (R_f^1 0.05), commonly found in fungal spore total lipid extracts [4], were not present in the wax extract. A total lipid extract showed a component with an R_f value corresponding to a triglyceride standard (R_f 0.56) and a large residue on the baseline possibly composed of internal phospholipids. This difference further confirmed that internal lipids were not removed by washing intact spores with *n*-hexane.

Comparison of marrow leaf and *S. fuliginea* fungal spore surface waxes by TLC (C_6H_6) showed no similarity. The major leaf wax components were hydrocarbons (R_f^1 0.83) and primary alcohols (R_f^1 0.11) which were not present in the fungal spore surface wax. Preparative-TLC (50% recovery) was carried out on the alkali-washed extract and the individual fractions checked for purity using the analytical TLC solvent systems given in the Experimental.

Table 1. Composition*, yields and R_f values of fungal spore surface wax fractions from *S. fuliginea*

Component	1974–75	1976–77	R_f^1	R_f^2
Alkyl esters	36	52	0.71	0.50
Δ^{2t} alkyl esters	12	16		0.42
Diol esters	27	7	0.49	0.05
Methyl esters	tr	4		0.29
Δ^{2t} methyl esters	tr	tr		0.22
Free fatty acids	11	3	0.00	0.00
Unidentified	14	18	—	—

* % Calculated from wts obtained by prep-TLC.
R_f^1 values obtained from analytical TLC: Si gel G, C_6H_6.
R_f^2 values obtained from prep-TLC: Si gel G, 2 developments, CCl_4.

Table 2. Composition* of fungal spore surface wax fractions from *S. fuliginea*

Number of C atoms	Hydrolysis products of diol esters: diol acid		alkyl esters: alcohol acid		Δ^{2t} alkyl esters†: alcohol acid		Δ^{2t} alkyl esters	alkyl esters	Δ^{2t} methyl esters	methyl esters	free acids
10	1	—	—	—	—	—	—	—	—	—	—
11	20	—	—	—	—	—	—	—	—	—	—
12	79	—	—	—	—	—	—	—	—	—	—
18	—	—	2	—	2	—	—	—	—	—	—
20	—	tr	75	3	84	—	—	—	—	—	4
20: Δ^{2t}	—	tr	—	—	—	—	—	—	tr	—	—
22	—	41	23	75	14	29	—	—	—	13	57
22: Δ^{2t}	—	49	—	—	—	58	—	—	30	—	8
24	—	10	—	22	—	13	—	—	—	81	31
24: Δ^{2t}	—	—	—	—	—	—	—	—	70	—	—
26	—	—	—	—	—	—	—	—	—	6	tr
38	—	—	—	—	—	—	—	tr	—	—	—
40	—	—	—	—	—	—	—	8 5	—	—	—
40: Δ^{2t}	—	—	—	—	—	—	5	—	—	—	—
42	—	—	—	—	—	—	—	57	—	—	—
42: Δ^{2t}	—	—	—	—	—	—	67	—	—	—	—
44	—	—	—	—	—	—	—	30	—	—	—
44: Δ^{2t}	—	—	—	—	—	—	11	—	—	—	—
46	—	—	—	—	—	—	—	4.5	—	—	—
Unidentified	—	2	—	—	—	—	17	—	—	—	

* In %, obtained by GLC.
tr = less than 1 %: † = high % of C_{22} and C_{24} acids possibly due to unidentified material in Δ^{2t} alkyl ester fraction.

The major fraction, which comprises 50 % of the surface lipid extract, contained even chain alkyl esters (C_{38} to C_{46}). Reaction with NaOMe/MeOH followed by GC–MS indicated that the fatty acid components were C_{20}, C_{22} and C_{24}, and the primary alcohols C_{18}, C_{20} and C_{22}. The relative amounts of each of these lipids and all other identified components are given in Table 2. The major ester was concluded to be eicosanyl docosanoate. Alkyl esters have been detected previously in the surface lipid of fungal spores [2] but only as minor components.

A second ester fraction, 15 % of the total extract, showed one major peak on GLC. PMR of this fraction (Table 3) showed Δ^{2t} unsaturation, indicated by two doublets of triplets centred at 5.80 δ (proton e) and 6.96 δ (proton f) which are due to coupling of the individual olefinic protons to each other. These are further coupled to the methylene protons (c), in one case weakly 5.80 δ (J_2 = 1.5 Hz) and in the other strongly 6.96 δ (J_2 = 6.8 Hz). The large J_1 value, 15.5 Hz, indicated *trans* unsaturation. Further, using additive substituent rules the predicted values for the olefinic protons were 5.83 and 6.88 δ [12], in close agreement with those obtained. Confirmation of the structure was obtained by reacting the ester with NaOMe/MeOH and the resultant acid characterised by GC–MS as 3-methoxydocosanoic acid by comparison with a reported spectrum of the C_{18}Me ester homologue [13]. This indicated the parent acid to be 2-docosenoic since the methoxide ion attacks preferentially at the 3-position. The ester was then hydrolysed using aq. KOH to obtain the unsaturated acid, the Me ester of which gave a spectrum (GC–MS) identical to that obtained for a pure sample of Me *trans*-2-docosenoate; this spectrum was also comparable to that reported for the C_{18} homologue [14]. The presence of Δ^{2t} unsaturation was further confirmed since the spectra of the *cis* and *trans* isomers are significantly different [14]. GLC R_ts of both the alkyl ester and the acid Me ester were longer than those of the corresponding saturated esters which is also characteristic of Δ^{2t} esters [14]. The major primary alcohol was shown by GC–MS to be eicosanol and the major ester was concluded to be eicosanyl *trans*-2-docosenoate. This Δ^{2t} ester fraction is the first to be reported in fungal spore surface lipid but similar esters have been detected in leaf surface waxes [15, 16].

A further ester fraction, 7 % of surface lipid extract, contained diol diesters. After reaction with NaOMe/MeOH and hydrolysis by aq. KOH the diols, as TMSi ethers, were characterised by GC–MS as C_{10}, C_{11} and C_{12} α, ω homologues by comparison with published spectra [17]. The fatty acid components were C_{22} and C_{24} and also *trans*-2-docosenoic acid which was confirmed by GC–MS of the unsaturated and methoxylated derivative. Although diol diesters have been found in leaf surface wax [18] they have not, previously, been found in fungal spore surface wax.

Table 3. PMR* peak assignments for eicosanyl *trans*-2-docosenoate

$$\underset{a}{Me}\text{—}\underset{b}{(CH_2)_{17}}\text{—}\underset{c}{CH_2}\text{—}\underset{f}{CH}\text{=}\underset{e}{CH}\text{—}\underset{}{CO_2}\text{—}\underset{d}{CH_2}\text{—}\underset{b}{(CH_2)_{18}}\text{—}\underset{a}{Me}$$

Proton	Chemical shift (δ, ppm)	No. of protons
a	0.88 *t*	6
b	centred at 1.26 *s*	80 (±4)
c	2.22 *m*	2
d	4.08 *t*	2
e	5.80 *dt* (J_1 = 15.5 Hz; J_2 = 1.5 Hz)	1
f	6.96 *dt* (J_1 = 15.5 Hz; J_2 = 6.8 Hz)	1

* 100 MHz, $CDCl_3$, TMS as reference.

The free fatty acids, 3% of surface extract, contained primarily C_{22} and C_{24} and *trans*-2-docosenoic acid was also present. Two free Me ester fractions were isolated, saturated esters which were 4% of the total extract and Δ^{2t} esters which were only present in a trace amount. In each fraction C_{22} and C_{24} predominated and in both the major component was C_{24}.

Although Jackson [2] found qualitative similarities between the major components of *P. striiformis* surface lipids and its host leaf surface lipids no such similarities were found in the present investigation. Such a high concentration of ester is, at the moment, unique in fungal spore surface lipid extracts. The major surface lipids found in other species have been hydrocarbons, β-diketone and alcohols found by Jackson [2] and fatty acids by Fisher [19]. However, few reports have studied only surface lipid and a broader comparison between surface lipid extracts is not possible.

EXPERIMENTAL

Since all powdery mildews are obligate parasites a suitable host plant for obtaining spores was required. Marrow plants were selected because spores were easy to collect from them and were also used in the fungicide partitioning study [1]. Identification of the marrow powdery mildew spores used in this study is difficult. Fisher [19] using the same source of spores has described the organism as *E cichoracearum* but the conidial stage, examined microscopically, appears identical with that reported by Nagy [20], Crüger and Meyer [21] and Schlösser [22] for *S. fuliginea*. Hydrolyses were carried out using aq. KOH (1%: reflux 4 hr) and reactions using NaOMe (0.5 M: reflux 1 hr) were carried out in MeOH.

Collection. Spores were collected during 1974–75 and 1976–77 by brushing them from the surface of infected marrow leaves (cv Green Bush) grown in a glasshouse, and stored in a desiccator at 4°. Before extraction they were brushed through a 100 mesh sieve to remove plant debris and divided into 10 aliquots.

Extraction. Each aliquot (100 mg) was extracted by gently shaking with *n*-hexane (6 ml) in a glass stoppered centrifuge tube (10 ml) for 1 min. The tubes were immediately centrifuged (1 min) and the supernatant filtered through pre-washed (MeOH) glass wool. A second extraction (5 min) gave little wax and was discarded. The extract was taken to dryness, the residue dissolved in $CHCl_3$ (2 ml) and washed with cold aq. KOH (1%; 3 × 1 ml). The combined KOH washings were neutralized with conc H_2SO_4 (1 drop) and the free fatty acids extracted with Et_2O. The total lipid extract was obtained by grinding the spores in $CHCl_3$–MeOH (3:1) using a pestle and mortar. The ruptured spores (confirmed by light microscopy) were refluxed in $CHCl_3$–MeOH (3:1) for 1 hr and filtered. The filtrate was taken to dryness on a rotary evaporator, dissolved in $CHCl_3$ and used for analytical TLC. The upper surface wax of marrow leaves was obtained by washing with $CHCl_3$ from a burette.

Chromatography. Prep TLC was carried out on Si gel G plates, 20 × 20 cm, pre-washed with CCl_4. The extract was applied (20 mg per plate) and the plates developed twice in CCl_4, bands being detected using I_2 vapour and the components recovered using Et_2O. Analytical TLC was carried out on Si gel G, developing in C_6H_6, CCl_4 or petrol (60–70)–Et_2O–HOAc (80:20:1). GLC and GC–MS analysis was carried out on a 1% Dexsil 300 column (1 m × 2 mm) programmed from 120 to 250° (350° for alkyl esters) using a dual FID instrument with N_2 at 30 ml/min (He for GC–MS) as carrier gas. Acids were characterized as their Me esters prepared by esterification with CH_2N_2 [23]. Alcohols and diols were characterized as their TMSi ethers prepared by silylation using NO-*bis*(trimethylsilyl)-acetamide/Py (1:1 at 60° for 1 hr). GLC R_ts were compared to those of authentic reference compounds and quantitation was obtained by disc integration.

Identification of eicosanyl trans-*2-docosenoate.* $\lambda_{max}^{isooctane}$ nm: 214; γ_{max}^{wax} cm^{-1}: 1720 (C=O) 1650 (C=C); GC–MS 70 eV *m/e* (rel. int.). (a) NaOMe reaction product: 369 (M-15) (24), 354 (M-30) (10), 352 (M-32) (7), 320 (M-64) (7), 311 (M-73) (14), 295 (M-89) (10), 117 ($MeO_2CCH_2(OMe)CH$) (100), 75 ($C_3H_7O_2^+$) (91). (b) Hydrolysis product: 352 (M^+) (10), 321 (M-OMe) (11), 320 (M-MeOH) (16), 278 (M-74) (6), 269 (M-83) (3), 255 (M-97) (8), 236 (M-116) (5), 113 (47).

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