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# Hydrocarbons, fatty acids and sterols of *Cronartium fusiforme* aeciospores†

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Hydrocarbons, fatty acids and sterols of *Cronartium fusiforme* aeciospores were determined using gas chromatographic and mass spectrometric techniques. Low levels of aliphatic hydrocarbons ($C_{20}$ to $C_{34}$) were detected in the spore extracts, but were probably contaminants from the host tissue. Stigmasta-$\Delta^{7,24(28)}$-dienol was the principal sterol component of the aeciospore oil and represented about 0·3% of the spore weight. Fatty acids ranged from $C_{12}$ to $C_{22}$ in chain-length and *cis*-epoxyoctadecanoic acid was the predominant acid (49·4%). Other major fatty acid components were $C_{16}$ (12·3%) and $C_{18:3}$ (9·2%).

## INTRODUCTION

Fusiform rust is caused by *Cronartium fusiforme* Hedge and Hunt ex Cumm and is an important fungal disease of pine in the southern United States. Although this fungus has been axenically cultured [*3*] the only ready available tissue for study are aeciospores collected from the pine hosts. Germination of aeciospores prior to infection seems to be independent of factors from the alternate host (Red oak). Lipid appears to be the storage material used as an energy source during spore germination in rust fungi. Rust spores generally have a relatively high lipid content, ranging between 7 and 24% of the spore mass [*19*, *20*]. As part of our studies on the host–parasite relationship of *C. fusiforme* and its hosts, this paper describes the hydrocarbon, fatty acid and sterol content of dormant aeciospores of this fungus.

## MATERIALS AND METHODS

### *Spore collection and preparation*

Aeciospores of *C. fusiforme* were collected from mature fusiform rust galls on loblolly pine trees (*Pinus taeda* L.) located in Auburn, Alabama, during the spring of 1975. Spores were separated from debris by passing them through a 62-μm sieve. Spores were dried over $CaCl_2$ in a vacuum desiccator at 5 °C for 2 days and stored in a screw cap vial at the same temperature. Immature gall tissue was also collected for analysis. Galls were considered immature if the peridium had not ruptured. Immature

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gall tissues were divided into three groups ranging from almost mature with the aecium containing loose spores and just prior to peridium rupture, to very immature with the aecia in the very early stages of development. Because of the difficulty in removing aecia from host tissue, extracts of immature gall tissues contained fatty acids from both the fungal and host tissue.

*Lipid extraction and analysis*

Spores (1 to 2 g) were ground in a mortar and pestle with 2 to 3 times their weight of lipid-free neutral alumina or broken with a B. Braun mechanical cell homogenizer (Quigbey-Rochester, Inc., Rochester, New York). Lipids were then extracted with chloroform : methanol (2 : 1 v/v) for 1 h. The extraction was aided by initially warming the mixture and by magnetic stirring throughout the extraction period. The extraction was considered essentially complete, since virtually all of the carotenoid pigment was removed from the spore fragments. The spore fragments and alumina were removed by centrifugation and the extract was taken to dryness under nitrogen.

Individual fatty acids in the form of methyl esters were separated by gas–liquid chromatography (g.l.c.). Total fatty acid methyl esters were prepared by either transmethylation using sodium methoxide in methanol (Applied Science Laboratories, Inc., State College, Pennsylvania) or after alkaline hydrolysis (see below) with boron trifluoride in methanol [*15*]. A portion of the total lipid extract was separated into three fractions using silica gel (60 to 200 mesh) column chromatography (12 × 3 cm). The hydrocarbon fraction was obtained by passing *n*-hexane through the column, followed by benzene for the neutral lipids and methanol for the polar lipids. Fatty acid methyl esters were prepared from the neutral and polar lipid fractions by transmethylation as described above.

Total sterols were isolated from the non-saponifiable fraction after alkaline hydrolysis of the total lipid for 12 h with 10% KOH in ethanol : water (9 : 1 v/v). Sterols were analyzed as free alcohols and as acetate and trimethylsilyl ether (TMS) derivatives. Acetate derivatives were prepared by dissolving the non-saponifiable lipid fraction in pyridine : acetic anhydride (1 : 1 v/v) and heating for 10 min. TMS derivatives were prepared with Sil-Prep (pyridine : hexamethylethyldisilazane : trimethylchlorosilane; 9 : 3 : 1) (Applied Science Laboratories). One milliliter Sil-Prep 50 $mg^{-1}$ sterol was added to the sample and allowed to stand at room temperature for at least 30 min prior to analysis by g.l.c.

Hydrocarbons, fatty acid methyl esters and sterols were analyzed by g.l.c. using either a Varian Aerograph 2440 or 1400 gas chromatograph equipped with flame ionization detectors. Hydrocarbons and sterols were separated on a 3 m × 2 mm i.d. glass column packed with either 0·5 or 3% GE SE-30 on Chromosorb Q. Column temperature for hydrocarbon analyses was programmed from 150 to 270 °C at 4 °C/min and for sterol analyses the temperature was 250 °C isothermal. Fatty acids were separated on a 3 m × 2 mm i.d. stainless steel column packed with 12% DEGS (diethylene glycol succinate) on Gas-Chrom P. Column temperature for fatty acid analyses was 180 °C and the injector and detector temperatures for all g.l.c. analyses were 250 °C. Identifications of the individual lipids were made by comparison of g.l.c. retention times with those of authentic standards and by g.l.c.–mass spectrometry (g.l.c.–ms). Mass spectrometric (ms) analyses were made on a Du Pont

21–490 single-focusing mass spectrometer linked to a Varian Aerograph 1400 gas chromatograph. All ms analyses were conducted with the source temperature at 200 °C and an ionizing voltage at 70 eV.

## RESULTS AND DISCUSSION

### *Total lipids*

The lipid content of *C. fusiforme* aeciospores represented 3·7% of the spore dry weight, which is low compared to that of spores of other rust fungi investigated, including aeciospores of three other *Cronartium* species. Tulloch & Ledingham [*19, 20*] reported that the total lipid content of aeciospores of *C. harknessii* [*16*], *C. ribicola* [*20*] and *C. commandrae* [*20*] represented 12·5, 18 and 17%, respectively of the spore weight.

### *Hydrocarbons*

Spores of rust and smut fungi generally contain a homologous series of *n*-alkanes ranging in chain-lengths from $C_{16}$ to $C_{36}$, with $C_{27}$, $C_{29}$ and $C_{31}$ as the predominant homologues [*21, 23*]. The hydrocarbon fraction from 2 g of *C. fusiforme* aeciospores contained only trace quantities of hydrocarbons. While the chain-lengths ranged between $C_{20}$ and $C_{34}$, the chain-length distribution was not typical of that normally found in rust spores [*21, 23*]. Although major aliphatic hydrocarbons of most biological materials have odd-numbered carbon chains, hydrocarbons of this type were not predominant in *C. fusiforme* aeciospores. The hydrocarbon fraction of uninfected host tissue was very similar to that of the aeciospores, so we conclude that hydrocarbons of the spore extracts may not be entirely of fungal origin and were contaminants from the host tissues. To our knowledge, the hydrocarbon content of only the rusts *Puccinia graminis* [*21*] and *P. striiformis* [*1, 6*] (uredospores) have been reported, but it was not unequivocably shown that the hydrocarbons of these spores are fungal products. Evidence for the presence of hydrocarbons in aeciospores has not been reported. Jackson *et al.* [*6*] showed that additional material was present in *P. striiformis* uredospore hydrocarbon fractions compared to similarly extracted leaf surface wax of the host plant (wheat). However, the predominant hydrocarbons in *P. graminis* [*21*] and *P. striiformis* [*1, 6*] were $C_{29}$ followed by $C_{31}$ and $C_{27}$ in decreasing relative proportions and this is the same as that reported for several wheat varieties [*6, 17, 18*].

### *Sterols*

Although the spores of relatively few rust fungi have been analyzed for their sterol content, it is clear that their sterols are not typical of fungi in general [*22, 23*]. Ergosterol is the principal sterol of most higher fungi, but $\Delta^7$ $C_{29}$ sterols are predominant in rust fungi and ergosterol has not been reported as a component of rust spores [*23*]. Total sterols obtained following saponification of the total lipid extract of *C. fusiforme* represented 10·8% of the total lipids and 0·32% of the spore dry weight; similar results have been reported previously for uredospores of *Uromyces phaseoli* [*14*]. Analysis of the non-saponifiable lipid fraction by g.l.c. indicated the presence of a single major sterol, accompanied by two minor sterols ($<2\%$ of total sterols) which

were only partially resolved from the major component. Based on g.l.c.–ms analysis, the major sterol was identified as a $C_{29}$ diene, with molecular ion peaks at m/e 412, 454 and 484 for the free sterol and its acetate and TMS derivatives, respectively. Evidence for a single nuclear double bond is m/e 255 (8·8%), 296 (8·3%) and 356 (76·6%) in the mass spectrum of the acetate derivative [*8*]. Two probable locations for the nuclear double bond are the $\Delta^5$ and $\Delta^7$ positions. It is unlikely that the double bond is in the $\Delta^5$position because of the absence of $M^+$–129 in the mass spectrum of the TMS derivative(characteristic of $\Delta^5$ TMS sterols) [*2*]. The presence of both m/e 343 (96%) and 386 (100%) as the principal ion fragments in the TMS mass spectrum are indicative of a $\Delta^{7,24(28)}$ double bond in the $C_{29}$ sterol [*9*]. The presence of $\Delta^7$ and $\Delta^{24(28)}$ double bonds in the principal sterol of *C. fusiforme* are suggested by a base peak at m/e 313 and a strong m/e 356 (76·6%) peak in the mass spectrum of the acetate derivative [*14*]. Based on these data and the fact that the mass spectra of the free sterol and its derivatives closely resemble those published previously [*6*, *7*, *14*], the major sterol of *C. fusiforme* aeciospores is believed to be stigmasta-$\Delta^{7,24(28)}$-dienol. Configuration of the $\Delta^{24(28)}$ double bond was not determined, but the $\Delta^{7,24(28)}$ $C_{29}$

TABLE 1

*Relative intensities of major ion fragments in the mass spectra of stigmasta-$\Delta^{7,24(28)}$-dienol from* C. fusiforme *aeciospores and its acetate and TMS derivatives*

| m/e | Relative intensity (%) Free | Acetate | TMS |
|---|---|---|---|
| 484 | — | — | 15·5 ($M^+$) |
| 469 | — | — | 10·8 ($M^+-15$) |
| 454 | — | 6·6 ($M^+$) | — |
| 439 | — | 3·6 ($M^+-15$) | — |
| 412 | 12·8 ($M^+$) | — | — |
| 397 | 7·8 ($M^+-15$) | — | — |
| 396 | — | — | — |
| 394 | — | — | 3·7 ($M^+-90$) |
| 386 | — | — | 100·0 |
| 379 | — | 2·7 | 5·6 |
| 371 | — | — | 9·1 |
| 356 | — | 76·6 | — |
| 345 | — | — | 9·3 |
| 343 | — | — | 96·0 |
| 431 | — | 5·8 | — |
| 318 | — | — | 5·9 |
| 314 | 88·9 | 25·5 | — |
| 313 | — | 100·0 | — |
| 299 | 10·6 | 2·5 | — |
| 296 | 3·9 | 8·3 | 9·0 |
| 288 | — | 7·0 | — |
| 281 | 5·5 | 5·5 | 7·4 |
| 273 | — | — | — |
| 271 | 100·0 | 4·1 | 3·1 |
| 269 | 9·4 | — | — |
| 255 | 10·5 | 8·8 | 13·0 |
| 253 | 6·1 | 10·6 | 13·9 |
| 231 | 8·3 | — | — |
| 229 | — | 4·7 | 6·5 |
| 213 | 10·0 | 12·7 | 13·9 |

sterol of bean rust uredospores had the (Z) configuration [*14*]. Relative intensities of the major ion fragments in the mass spectra of stigmasta-$\Delta^{7,24(28)}$-dienol and its acetate and TMS derivatives are given in Table 1.

To our knowledge, this is the first report of sterols in rust aeciospores and the sterol composition of *C. fusiforme* aeciospores is similar to that reported for uredospores of other rust fungi. The first report of sterols in rust spores was by Hougen *et al.* [*4*] who identified ergost-$\Delta^7$-enol by chemical methods in extracts of wheat stem rust (*P. graminis*) uredospores. Ergost-7-enol or stigmasterol was identified as a minor component of wheat stem rust uredospores by g.l.c. retention times and was accompanied by trace amounts of an unknown sterol, cholesterol and the major sterol stigmast-$\Delta^7$-enol. Weete & Laseter [*24*] confirmed the presence of stigmast-$\Delta^7$-enol as the principal sterol and ergost-$\Delta^7$-enol as a minor sterol component of *P. graminis* and *P. striiformis* uredospores by mass spectrometry. A diunsaturated $C_{29}$ sterol was also present as a minor component of those spores. Jackson & Frear [*7*] identified the $\Delta^7$ and $\Delta^{7,24(28)}$ $C_{29}$ sterols in flax rust (*Melampsora lini*) uredospores which were accompanied by small amounts of the $\Delta^{5,7}$ diene. Like *C. fusiforme* aeciospores, the principal sterol of *U. phaseoli* uredospores was identified as (24Z) stigmasta-$\Delta^{7,24(28)}$-dienol which was accompanied by the $\Delta^7$ monoene [*12*]. Although it has not been demonstrated unequivocably, the $C_{29}$ sterols reported as components of rust spores are probably fungal products and not contaminants from the host. Stigmast-$\Delta^7$-enol and stigmasta-$\Delta^{7,24(28)}$-dienol have been reported as higher plant products [*5, 8*], but the typical plant sterols sitosterol, campesterol and stigmasterol have not been reported as rust spore constituents. They were not detected in *U. phaseoli* uredospores when a direct comparison of the host and bean rust spore sterols was made [*13*]. Furthermore, germinating bean rust spores had the ability to synthesize sterols from radiolabeled precursors [*14*].

*Fatty acids*

The fatty acid composition of spores of numerous rust fungi has been reported [*19, 20*] and it is generally similar to that of other fungi [*23*]. Compared to other fungi, rust spore oils contain high relative proportions of $C_{18:3}$, but the most characteristic feature of rust spore fatty acids is *cis*-9, 10-epoxyoctadecanoic acid. The epoxy $C_{18}$ acid may constitute between 1·7 and 78% of the total fatty acids with most rust spores containing 20 to 40% [*19, 20*]. The total fatty acids of *C. fusiforme* aeciospores were very similar to that reported previously for other rust spores including three other *Cronartium* species [*19, 20*]. *Cis*-9,10-epoxyoctadecanoic acid was the principal fatty acid at 49·4% of the total fatty acids (Table 2), which is 10 to 15% higher than the values reported for the other *Cronartium* species [*19, 20*]. The epoxy $C_{18}$ acid was identified by g.l.c. by comparison to an authentic sample of the compound and also by g.l.c.–ms. The mass spectrum of the methyl ester derivative of the epoxy $C_{18}$ acid was very similar to that of the standard and that published previously [*16*]. The molecular ion was m/e 312 with the base peak at m/e 155 and other prominent ion fragments in the high mass range m/e 281 ($M^+$–31), 199 and 171. Separation of the total lipid into neutral and polar fractions by silica column chromatography and fatty acid analysis of the fractions showed that the epoxy $C_{18}$ acid was predominantly in the polar lipid fraction.

TABLE 2

*Total fatty acids of* C. fusiforme *aeciospores and immature fusiforme galls from loblolly pine*

| Fatty acid | Aeciospores (%)[a] | Fusiform rust gall (%)[b] 1[c] | 2[d] | 3[e] |
|---|---|---|---|---|
| $C_{14}$ | 1·0 | 1·2 | 0·6 | 0·4 |
| $C_{15}$ | 0·5 | 0·6 | 0·6 | 0·4 |
| $C_{16}$ | 12·3 | 14·7 | 9·3 | 4·8 |
| $C_{16:1}$ | 0·9 | 1·6 | 2·5 | 2·1 |
| $C_{18}$ | 4·3 | 6·0 | 5·9 | 1·8 |
| $C_{18:1}$ | 5·7 | 22·2 | 35·5 | 32·5 |
| $C_{18:2}$ | 6·2 | 16·7 | 20·0 | 17·9 |
| $C_{20}$ | 0·8 | 12·0 | 3·7 | 6·2 |
| $C_{18:3}$ | 9·2 | 10·1 | 7·8 | 6·9 |
| 9,10-Epoxy $C_{18}$ | 49·4 | 9·7 | 10·5 | 16·3 |

[a] 9·7% of the total fatty acids were comprised of trace quantities of unidentified components and $C_{12}$ (0·2%), $C_{22}$ (2·2%) $C_{22:1}$ (2·3%) and possibly $C_{24:1}$ (trace) each of which were tentatively identified only by g.l.c. retention times.

[b] 1, 2 and 3 represent stages of accial development with stage 1 being the least mature.

[c] 5·3% of the total fatty acid were comprised of trace quantities of unidentified components and $C_{12}$ (trace), $C_{22}$ (1·5%) and $C_{22:1}$ (1·4%).

[d] 3·6% of the total fatty acids were comprised of trace quantities of unidentified components and $C_{12}$ (trace), $C_{22}$ (1·9%) and $C_{22:1}$ (0·7%).

[e] 10·7% of the total fatty acids were comprised of trace quantities of unidentified components and $C_{12}$ (trace), $C_{22}$ (5·3%) and $C_{22:1}$ (2·1%).

The major non-substituted saturated acid in *C. fusiforme* aeciospore oil was palmitic ($C_{16}$) and the principal unsaturated acid was linolenic ($C_{18:3}$) (Table 2). There is generally an inverse relationship between the amounts of the epoxy $C_{18}$ acid and the $C_{18}$ unsaturated acids [*19*, *20*]. This is illustrated in Table 2 where the fatty acids of developing fusiforme rust galls are given. In contrast to the epoxy $C_{18}$ and $C_{18}$ unsaturated fatty acid content of the aeciospores, the immature galls contain 9·7 to 16·3% of the epoxy $C_{18}$ acid and correspondingly larger amounts of $C_{18:1}$ (22·2 to 35·5%) and $C_{18:2}$ (16·7 to 20·0%).

The distribution of fatty acids in the gall tissue (aecia plus woody pine tissue) was quite different from that in the aeciospores. As noted above, $C_{18:1}$ and $C_{18:2}$ were the principal fatty acids of the gall tissue (Table 2). In this study it was not possible to distinguish between the fungal and host fatty acids in the gall tissue since the major pine stem fatty acids are, with the exception of epoxy $C_{18}$, very similar to the gall tissue. The principal fatty acids of slash pine (*Pinus elliottii* Engelm.) stem tissue was $C_{16}$ (17·2%), $C_{18:2}$ (30·3%) and $C_{18:1}$ (17·9%) [*11*]. Evidence for the contribution of fungal fatty acids to the total gall fatty acids was the presence of epoxy $C_{18}$.

To summarize, the lipid content of *C. fusiforme* aeciospores is considerably lower than that of other *Cronartium* species and spore forms of other rust fungi and, hence, may not represent an important reserve material for germination. There is certainly some constituent(s) of the host that is required by this fungus, presumably some factor that can be supplied by only a limited number of pine species, and certainly the host influences the metabolism of the fungal invader. However, it is important to note that while there is an intimate association between the fungus and its host there seems to be little or no exchange of lipids between them. This is supported by the

absence of typical higher plant sterols in spore materials. Also, stigmast-$\Delta^7$-enol [*10*] and 9,10-epoxy $C_{18}$ [*11*] were present in low quantities or absent, respectively, from infected pine tissues.

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