Fatty Acids and Sterols of *Cronartium fusiforme* Basidiospores

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ABSTRACT

The hydrocarbons, fatty acids, and sterols of *Cronartium fusiforme* basidiospores were examined by gas chromatography and mass spectrometry. Trace quantities of aliphatic hydrocarbons were detected, but these were probably not fungal products. Principal nonsubstituted fatty acids were palmitic (11.7%), linoleic (17.2%), and linolenic (16.1%) acids; the predominant acid was 9,10-*cis*-epoxyoctadecanoic acid (40.7%). Four sterols were detected, three of which were identified as stigmast-7-enol (57.6%), stigmasta-5,7-dienol (22.7%), and ergost-7-enol (16.0%). The fourth sterol (4%) is a C_{28} diene. Lipids of the aeciospores and basidiospores of *C. fusiforme* are compared.

INTRODUCTION

Chemical analyses of rust fungi have been restricted to the various spore types (uredospores, teliospores and aeciospores) because, until recently, rusts could not be cultured in the laboratory. Although they can now be cultured, axenic cultures of rust fungi show very sparse growth and do not produce spores characteristic of those that occur in the natural life cycle. In nature, frequently one spore type is produced abundantly, or at least in sufficient quantity to be collected for study, whereas the other spore forms of that species are produced in relatively limited amounts. This is the case with *Cronartium fusiforme* Hedge and Hunt ex Cumm., which causes fusiform rust of pines in the southern United States. Large numbers of aeciospores are produced in galls on the pine host each spring; its other spore forms are produced in only limited amounts, making it difficult or impossible to collect them from naturally infected materials in quantities sufficient for analysis. However, as part of a program for screening pine trees for resistance to fusiform rust, USDA Forest Service personnel, Forest Resource Protection in Ashville, NC are obtaining *C. fusiforme* basidiospores from an oak alternate host (*Quercus rubra*) artificially inoculated with aeciospores. Small quantities of basidiospores were made available to us and this note reports their fatty acid and sterol composition. To our knowledge, this is the first report of the lipid composition of basidiospores of a rust fungus.

EXPERIMENTAL PROCEDURES

Basidiospores were collected by suspending oak leaves with germinating teliospores over acidified water in petri dishes. Liberated basidiospores were concentrated by filtration on millipore filter pads and stored at about 5 C. Before shipping, the spores were frozen and packed in dry ice. Upon arrival, the spores were thawed, washed from the filter pad with distilled water, concentrated by low-speed centrifugation, refrozen, and dried by lyophilization. Dry basidiospores, 0.2 to 0.4 g, were weighed and then ground in a mortar with an equal amount of neutral alumina. The ground mixture was then added to 150 ml chloroform-methanol (2:1, v/v), warmed briefly, and then stirred magnetically for 2 hr. The cell fragments and alumina were removed by centrifugation and washed with 50 ml chloroform. Cell fragments and alumina were removed as before and the extract plus wash was concentrated under nitrogen. The extraction was considered complete since virtually all of the carotenoid pigment was removed from the spores.

The extract was analyzed for hydrocarbons, fatty acids, and sterols. A portion of the extract was placed on a 6 cm x 2 cm silica gel (60-200 mesh) column and the hydrocarbons were eluted with petroleum ether. All solvents used in this study were of nanograde quality (Mallinkrodt, Inc.). Total fatty acids were analyzed as their methyl esters, which were prepared by transesterification of the total lipid extract using 0.5 N sodium methoxide in methanol (Applied Science Laboratories, State College, PA). Free sterols were isolated from the total lipid by digitonin precipitation. One drop of 10% acetic acid and 1.5 ml of 0.5% digitonin were added to the total lipid fraction in 3 ml acetone-ethanol (1:1, v/v) and the mixture was stirred on a Vortex mixer prior to storing overnight at room temperature. The digitonide was collected by low-speed centrifugation and washed first with acetone-diethylether (1:2, v/v) and then two times with diethylether. Free sterols were recovered by dissolving the digitonides in 10 ml dimethyl sulfoxide in a 40 ml centrifuge tube which was

placed in boiling water bath for 1 hr (1). The solution was cooled to room temperature and washed three times with 5 ml of petroleum ether. The combined washes which contained the free sterols were taken to dryness under nitrogen. Sterols were analyzed as free alcohols and as acetate and trimethylsilyl ether (TMS) derivatives. Acetate derivatives were prepared by dissolving the free sterols in pyridine-acetic anhydride (1:1, v/v) and warming for 10 min. TMS derivatives were prepared with Tri-Sil (Applied Science Laboratories).

Epicuticular wax of oak leaves was also examined. Wax was removed by dipping the leaves in chloroform for 15 sec (2). The chloroform was evaporated under nitrogen and the hydrocarbons were isolated by silica gel column chromatography as described above.

Hydrocarbons, fatty acids, and sterols were analyzed by gas chromatography (GLC) using either a Varian Aerograph 2440 or 1400 gas chromatograph equipped with flame ionization detectors. Hydrocarbons and sterols were separated on a 3 m x 2 mm ID glass column packed with 3% SE-30 on Chromosorb Q. Column temperature for hydrocarbon anaylses was programmed from 150 to 270 C at 4 C/min; for sterol analyses the temperature was 250 or 270 C isothermal. Fatty acids were separated on a 3 m x 2 mm ID stainless steel column packed with 12% DEGS (diethylene glycol succinate) on Gas Chrom P. Column temperature for fatty acids analyses was 180 C isothermal and the injector and detector temperatures for all GLC analyses were 270 C. Identification of individual lipids was made by comparison of GLC retention times with those of authentic standards and/or by GLC-mass spectrometry. Mass spectrometric (MS) analyses were made on a DuPont 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 of 70 eV.

RESULTS AND DISCUSSION

The total lipid content of *C. fusiforme* basidiospores was ca. 31%, which is high compared to most fungal spores (3). Rust spores generally have a high lipid content, ranging between 4 and 20% of the spore weight. Aeciospores of *C. harknessii, C. ribicola,* and *C. commandrae* contained 12.5, 18, and 17% (4,5) total lipid, respectively, while *C. fusiforme* aeciospores contained 3.7% (6).

Rust and smut spores 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} predominating (3,7). Basidiospores of *C. fusiforme* contained only trace quantities of aliphatic hydrocarbons ranging in chain-length from about C_{21} to C_{36}. However, there was no odd over even carbon chain predominance characteristic of hydrocarbons from fungal and higher plant materials (7,8). The typical alkane pattern was not observed with *C. fusiforme* aeciospores (6). Since it was not possible to see that precautions against contamination by petroleum products were taken during spore collection, we cannot be certain that the hydrocarbons detected were fungal products. Although the hydrocarbon pattern of the basidiospores was not typical of higher plants, alkanes from the host epicuticular wax were determined. The oak hydrocarbon pattern was not similar to that of the basidiospores, but was typical of higher plant epicuticular wax (8). Alkanes ranged from C_{23} to C_{33}, with C_{27}, C_{29} and C_{31} being the predominant homologues.

TABLE I

Fatty Acids and Sterols of *C. fusiforme* Basidiospores

Fatty acids	%	Sterols	%
C_{14}	1.2	stigmast-7-enol	57.6
C_{15}	0.3		
C_{16}	11.7	stigmasta-5,7-dienol	22.7
$C_{16:1}$	0.7		
C_{17}	1.3	ergost-7-enol	16.0
C_{18}	3.2		
$C_{18:1}$	3.5	C_{28} diene	4.0
$C_{18:2}$	17.2		
C_{20}	4.1		
$C_{18:3}$	16.1		
epoxy C_{18}	40.7		

Fatty acids of rust spores are generally similar to those of other fungi and of higher plants (3); however, rust fungi are distinguished from almost all other fungi by the presence of 9,10-*cis*-epoxyoctadecanoic acid (epoxy C_{18}) which comprises between 1.7 and 78% of the total fatty acids in the spores studied (3). Fatty acids, as methyl esters, of *C. fusiforme* basidiospores were identified by comparison of GLC retention times with those of authentic standards and fatty acid methyl esters previously identified by GLC-MS prepared from *C. fusiforme* aeciospores (6). Nonsubstituted fatty acids are similar to those of other rust spores (4,5) and aeciospores of *C. fusiforme* (6) (Table I). Chain-lengths ranged from C_{14} to C_{18}, with C_{16} (11.7%) being the predominant saturated acid and $C_{18:2}$ (17.2%) and $C_{18:3}$ (16.1%) being the most abundant unsaturated acids. Like all uredospores, aeciospores, and teliospores of rust fungi previously reported (3,9), basidiospores of *C. fusiforme* contain high rela-

TABLE II

Major High Mass Ion Fragments in the Mass Spectra of Principal Sterols from *C. fusiforme* Basidiospores

Sterol	Molecular ion (m/e)	Base peak (m/e)	Major high mass fragments (m/e)
Stigmast-7-enol			
Free	414	414	399,379,353,314,299,271,255,246,231, 229,213
Acetate	456	313	441,439,396,394,381,379,356,341,327, 296,273,255,229,213
TMS	486	386	471,469,371,343,281,255,229,213
Stigmasta-5,7-enol			
Free	412	412	397,394,379,353,271,253
Acetate	454	394	439,379,356,353,313,253
TMS	484	484	469,396,386,381,379,343,281,253,299, 213
Ergost-7-enol			
Free	400	400	385,365,339,273,255,231,229,213
Acetate	442	442	427,382,367,315,255,229,213
TMS	472	472	457,382,367,281,255,229,213

tive proportions of the epoxy C_{18} acid (40.7%) characteristic of this group of fungi. While the fatty acid composition of aeciospores (6) and basidiospores of this species are very similar, there are certain qualitative and quantitative differences between them. C_{22}, $C_{22:1}$ and $C_{24:1}$ were tentatively identified as minor components of *C. fusiforme* aeciospores, but they were not detected in the basidiospores. Also, C_{18} and $C_{18:1}$ were present in basidiospores in approximately one-half the relative proportions found in aeciospores, whereas $C_{18:2}$ and $C_{18:3}$ were present in 1.8 to 2.7 times higher relative concentrations, respectively, than in aeciospores. Basidiospores contain about 10% less of the epoxy C_{18} acid than aeciospores.

Ergosterol is generally considered the principal fungal sterol; however, it has not been detected in rust spore extracts (3,9). Δ^7C_{29} sterols are predominant in rust fungi (9). Digitonin precipitable sterols of *C. fusiforme* basidiospores were analyzed as the free alcohol and as the acetate and TMS derivatives by GLC-MS. The principal sterol represents 56.7% of the total, and was identified as stigmast-7-enol. Molecular ions for the free alcohol and the acetate and TMS derivatives were m/e 414, 456, and 486, respectively, which suggests a monounsaturated C_{29} sterol. Ion fragments at m/e 255 in each spectra and 296 and 356 in that of the acetate derivative suggest that the single double bond is located in the ring system of the sterol (10). Two probable locations for the single double bond of the sterol ring system are the Δ^5 or Δ^7 positions. The absence of M^+-129 in the mass spectrum of the TMS derivative suggests that the double bond is not in the Δ^5 position (10,11). According to Knights (10), Δ^7 sterols seem to have no unique ions, but may be characterized by m/e 213 [M^+-(side-chain + 42 + ROH)] and m/e 229 [M^+-(side-chain + 27 + RO)], and Δ^7 sterols show a strong M^+-(side chain + 2 x H). This is true for the principal sterol of *C. fusiforme* basidiospores with m/e 271, 313, and 343 as prominent ion fragments in the spectra of the free alcohol and acetate and TMS derivatives, respectively. Also, m/e 213 and 229 are present in the spectra of this sterol.

The second most abundant sterol in *C. fusiforme* basidiospores is stigmasta-5,7-dienol, comprising 22.7% of the total. This sterol had a shorter retention than stigmast-7-enol as would be expected on a SE-30 GLC column, but was only partially resolved from it. Molecular ions from the mass spectra of the free alcohol and the acetate and TMS derivatives were 412, 454, and 484, respectively, which correspond to a C_{29} sterol with two double bonds. Both double bonds may be located in the ring system, one in the ring system and the other in the side-chain or both in the side-chain. Based on structures of known diunsaturated sterols of plant or fungal origin, the most probable structure for a sterol with two double bonds in the ring would be $\Delta^{5,7}$, or if one double bond was in the side chian it may be located in either the Δ^{22} or $\Delta^{24(28)}$ position. To our knowledge, a naturally occurring sterol with no double-bonds in the ring system and two in the side-chain has not been reported. The presence of a significant peak at m/e 253 [M^+-side-chain + ROH)] rather than m/e 255 in each spectrum suggests that both double bonds are located in the ring system. The presence of significant peak at m/e 353 in the spectrum of the free alcohol is evidence for the $\Delta^{5,7}$ structure which, according to Smith and Korn (12), is produced by the splitting of ring A [M^+-(C-1 to C-3)] and seems

to be characteristic of $\Delta^{5,7}$ sterols. This fragment is absent from the corresponding $\Delta^{7,24(28)}$ C_{29} isomer (6).

The third most abundant sterol comprising 16.0% of the total is ergost-7-enol (fungisterol). The molecular ions in mass spectra of the free alcohol and the acetate and TMS derivatives are m/e 400, 442, and 472, respectively, which are indicative of a C_{28} sterol with a single double bond in the ring structure. The absence of M^+-129 in the spectrum of the TMS derivative suggests that the double bond is not in the Δ^5 position. The presence of significant peaks at m/e 213 and 229 in each spectrum suggests that the double bond is in the Δ^7 position.

The fourth sterol of *C. fusiforme* basidiospores comprised 4% of the total and had a shorter retention time than ergost-7-enol, but was only partially resolved from it. Due to the small sample size, a definitive mass spectrum was not obtained for this sterol. However, it was tentatively identified as a C_{28} diene, with two double bonds in the ring system (m/e 253). Major high mass ion fragments of the three principal sterols of *C. fusiforme* are summarized in Table II.

Sterols of several rust species have been reported. Three of these reports concerned uredospores of *Puccinia graminis* (13-15). Weete and Laseter (15) confirmed the presence of stigmast-7-enol by mass spectrometry as the most abundant sterol of *P. graminis* uredospores, which was accompanied by a C_{29} diene and ergost-7-enol. Jackson and Frear (16) identified the Δ^7 and $\Delta^{7,24(28)}$ C_{29} sterols in flax rust (*Melampsora lini*) uredospores which were accompanied by small amounts of the $\Delta^{5,7}$ isomer. Lin et al. (17,18) identified (24Z) stigmast-7,24(28)-dienol as the principal sterol of *Uromyces phaseoli* uredospores which was accompanied by low relative amounts of the corresponding Δ^7 C_{29} isomer. At this time, it appears that rust uredospores are of two types based on sterol composition; one type contains stigmast-7-enol as the principal sterol, accompanied by ergost-7-enol, and another group has stigmasta-7,24(28)-dienol as the major sterol.

These data, combined with our previous report of *C. fusiforme* aeciospore sterols (6), represents the first comparison of the sterol composition of two different spore types of a single rust species. Aeciospores of *C. fusiforme* contains stigmasta-7,24(28)-dienol which represents ca. 98% of the total sterols, whereas the basidiospores of this species contain stigmast-7-enol, stigmasta-5,7-dienol, and ergost-7-enol, as principal sterols. The sterol composition of basidiospores is very similar to uredospores of *P. graminis* and *M. lini*. From the standpoint of host damage, basidiospores are formed on the alternate host, *Q. rubra*; aeciospores infect oak where uredia and then telia are subsequently produced. Teliospores germinate to form basidiospores, which infect primary hosts, *Pinus taeda* or *P. elliottii*. The similarity of the sterol composition between basidiospores of *C. fusiforme* and the uredospores of other rust species may be linked to the fact that uredospores and basidiospores of *C. fusiforme* are formed on the same host (oak). Both spore types are haploid, but basidiospores contain a single nucleus whereas the uredospores are dikaryotic.

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