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METABOLITES OF Gremmeniella

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ABSTRACT

Gremmeniella abietina was grown on a medium of 10% V-8 juice containing added glucose, and the metabolites produced were isolated by extraction with ethyl acetate or methylene dichloride. Chromatographic separation of the crude metabolites gave a blue compound, a strongly fluorescent compound, a red compound, a dark purple compound, and a yellow compound. The structures of these compounds will be reported, along with some of their biological activities. A comparison of the metabolite spectra of various North American and European strains of G. abietina will be presented.

INTRODUCTION

The metabolites produced when the fungus Gremmeniella abietina is grown in liquid culture have not previously been identified. In order to determine what compounds are responsible for symptom expression and to see whether there is a correlation between metabolites produced and the virulence of particular strains, we have studied the metabolites produced by liquid cultures of the fungus.

RESULTS AND DISCUSSION

The first strain of G. abietina studied (Canadian Forestry Service (CFS) strain C-699) was isolated from New Brunswick pine trees. It was grown at 16-17C on a medium of 10% V-8 juice containing 1% added glucose (Dorworth 1972), in still culture and in a stirred an aerated fermentation apparatus. Best yields of metabolites were obtained from the still cultures. Extraction of the filtered culture broth yielded ca. 0.04 g/l of crude metabolites, while Soxhlet extraction of the mycelium provided substantially more material (ca. 0.45 g from the mycelium growing in 1 liter of medium. Separation of the crude metabolites by flash chromatography gave, in order of elution (solvent system

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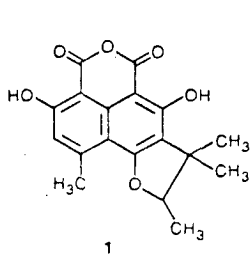
$CHCl_3$, Skellysolve B, HOAc (10:10:1)), a blue compound, a colorless strongly fluorescent compound, a red compound, a dark purple compound, a yellow compound, and a mixture of brownish materials which have not yet been completely separated and identified.

The colorless, strongly fluorescent compound, mp 250-253C, $C_{18}H_{16}O_2$, shows carbonyl absorption in the IR at 1705 and 1665 cm^{-1} and readily forms a diacetyl derivative absorbing at 1778, 1760, and 1724 cm^{-1}. The ^1H NMR spectrum showed clearly defined signals for all sixteen protons: methyl singlets at δ1.30 and 1.54; a secondary methyl doublet (δ1.50) coupled to a methine hydrogen (δ 4.80) geminal to oxygen; an aromatic methyl at δ 2.80; an aromatic hydrogen (δ 6.80); and phenolic hydrogens at δ 11.37 and 11.60. A search of the literature revealed that this compound is the anhydride 1, first isolated from oxidation of atrovenetin (2) (Barton et al. 1959), but later isolated from cultures of Penicillium herquei (Narasimhachari and Vining 1963) and subsequently from other fungi. The anhydride 1 isolated from G. abietina is enantiomeric with that previously isolated.

The dark burgundy compound, which causes a green discoloration on contact with the skin and a blue-green discoloration of paper, was shown by high resolution electron impact mass spectrometry (EIMS) and chemical ionization mass spectrometry (CIMS) to possess the molecular formula $C_{19}H_{16}O_6$. The ^1H NMR spectrum was virtually identical with that of the anhydride 1. The ^{13}C NMR spectrum, determined in DMSO-d_6, shows carbonyl peaks at δ 198 and 196 (those of the anhydride 1 appear at ~ δ 166) and a signal at δ 88 which is not present in the spectrum of the anhydride. When the spectrum is determined in CD_2Cl_2, an additional carbonyl signal appears at δ 176 and the intensity of the signal at δ88 is diminished. The data suggested that the compound is the triketone 3, present mainly as the hydrate 4. This compound, atrovenitinone, has previously been prepared (Narasimhachari and Vining 1963), by oxidation of atrovenitin (2) with benzoquinone. It was characterized as the crystalline ethanolate 5. Crystallization of the dark purple compound from ethanol provided pale yellow needles of 5, identical in all respects with an authentic sample provided by Prof. Vining. Compound 4 has not previously been isolated from natural sources. When the solvent system used for the separation of 3 contained acetone, the acetone adduct 6 (as a mixture of epimers) was obtained instead of compound 3. Treatment of 3 with acetone in the presence of a catalytic amount of acetic acid also provided 6. When a solution of compound 3 was placed on freshly peeled pine, the characteristic blue-green color of Scleroderris infected wood developed.

The blue compound proved rather unstable and difficult to purify, but after repeated silica gel chromatography and crystallization from ethanol, deep blue crystals were obtained. Efforts to determine the molecular formula of this compound, which we believe to be responsible, at least in part, for the bluish-green coloration of the wood of Scleroderris infected pine, were initially unsuccessful. The highest mass peak in the EIMS appeared at m/z 337 and corresponded to the ion $C_{19}H_{18}O_5$. Another prominent peak occurred at m/z 326 ($C_{19}H_{18}O_5$). The CIMS did not afford a recognizable parent ion. However field desorption mass spectrometry (performed by Prof. G. Wood, University of Windsor) suggested a molecular weight of 663 amu. Addition of the two high mass EIMS fragments then suggested the molecular formula is $C_{38}H_{33}NO_{10}$ (MW 663). The ^1H NMR spectrum is similar to that of the anhydride 1 and the triketone hydrate 4 with most signals doubled. The ultraviolet-visible spectrum



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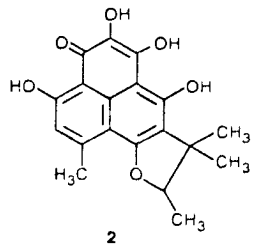

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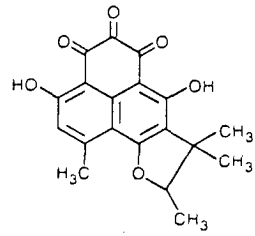

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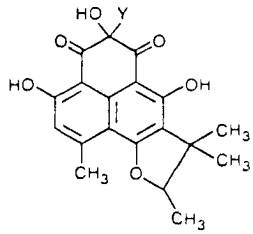
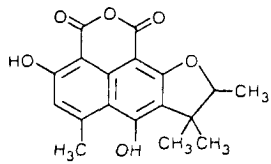


4 Y = OH
5 Y = OCH_2CH_3
6 Y = CH_2COCH_3



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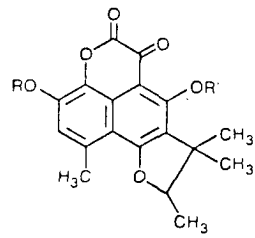
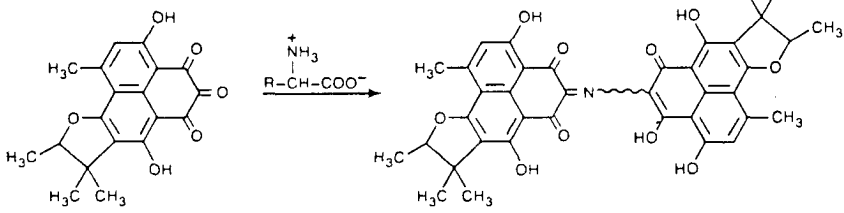


9 R = R' = H
9a R = Ac, R' = H
9b R = R' = Ac

$(CHCl_3)$ shows λ_{max} 268, 368, 405 (sh) and 608 nm. Narasimhachari and Vining (1963) had previously suggested that the characteristic green pigment of Penicillium herquei might be formed by a ninhydrin-like reaction between atrovenitenone (4) and amino acids, but they were unable to completely characterize the pigment. In our hands, treatment of a buffered solution of glycine (see Scheme 1).

SCHEME 1



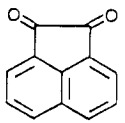
4

7

With atrovenitinone (4) in aq. dioxane and purification of the product by flash chromatography gave blue crystalline material identical in all respects with the compound isolated from G. abietina. The pigment, for which we suggest the trivial name Scleroderris blue, thus possesses structure 7 (Scheme 1).

The yellow compound, mp 239-240C, is isomeric with the anhydride 1 ($C_{18}H_{16}O_6$) and initially we suspected it to be the isomeric ether 8. The ^1H NMR spectrum was very similar to that of 1, and treatment with acetic anhydride-pyridine afforded a monoacetyl derivative. The ^{13}C NMR spectrum, however, shows carbonyl absorption at δ 186.2 and 170.0, which is not in good agreement with the anhydride structure. Also, the IR spectrum shows carbonyl absorption at 1750 cm^{-1}, unlike that of the anhydride. An X-ray crystallographic study of the monoacetate, performed by J. Clardy and E. Arnold of Cornell University, revealed that the yellow compound possesses the biogenetically novel internal phenylglyozylate lactone structure 9 (the monoacetate is 9a), the ketonic carbonyl accounting for the δ 186.2 signal in the ^{13}C NMR spectrum. Treatment of 9 with acetic anhydride-sulfuric acid at 60C furnished the diacetyl derivative 9b.

The red compound, mp 210-212°, $C_{18}H_{16}O_5$, has one oxygen less than the anhydride 1 and the yellow compound 9. The ^1H NMR spectrum is very similar to that of 1, but the ^{13}C NMR spectrum shows ketonic carbonyls at δ 189.6 and 186.2. The IR spectrum shows carbonyl absorption at 1714 and 1690 cm^{-1}. This compound readily forms a diacetyl derivative, the IR of which shows carbonyl absorption at 1780 and 1740 cm^{-1}, simlar to that of acenaphthenquinone 10. This evidence suggested that the red compound has structure 11 (the diacetate is 11a) and this was confirmed by alkaline peroxide oxidation to give, after



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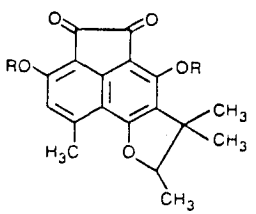
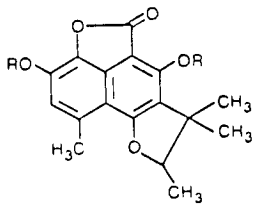


11 R = H
11a R = Ac



12 R = H
12a R = CH_3

acidification, the anhydride 1, along with the lactone 12, the product of Dakin oxidation of 11. Treatment of 12 with CH_2N_2 provides the ether 12a.

The red compound represents the first acenaphthenquinone isolated from natural sources. We believe that red compound 11 may be the biogenetic precursor of the anhydride 1 and the yellow compound 9.

We have compared, using high pressure liquid chromatography (HPLC), the metabolites of three other strains of G. abietina with those of strain C-699. The metabolites of strain C-659 ("North American strain", from Ontario, SC-1 of Dorworth and Krywienczyk 1975) are very similar to C-699. However, the metabolite spectra produced by strain C-706 ("European strain" from Sweden, SS-1 of Dorworth and Krywienczyk, 1975) and strain C-707 ("New York strain", from New York, SUS-8 of Dorworth et al. 1977) are quite different from that of strain C-699. It is interesting to note that strain C-699 (SC-73 of Dorworth) was isolated from New Brunswick and serologically reacted positively to "European strain" similar to "New York strain". The anhydride 1 is produced by all four

strains. Identification of the other metabolites produced by the different strains is in progress.

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Scleroderris canker of conifers

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