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Chemical Characterization of a Red Pigment (5,8-Dihydroxy-2,7-Dimethoxy-1,4-Naphthalenedione) Produced by *Arthrographis cuboidea* in Pink Stained Wood

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Summary

A red pigment associated with pink stain of angiosperm and gymnosperm woods caused by the fungus *Arthrographis cuboidea* was isolated, purified to crystalline form, spectroscopically characterized and identified as 5,8-dihydroxy-2,7-dimethoxy-1,4-naphthalenedione. Results also suggest that other naphthoquinone pigments may be associated with the pink stain. This is the first report on the chemical nature of the red pigment responsible for pink stain in wood.

Introduction

Stain fungi are opportunistic microorganisms that rapidly colonize the sapwood of freshly cut wood. Fungi that cause blue stain are the most frequently found organisms in cut logs and timber but there are many different sap-staining fungi that impart red, green, brown or other colors to the wood (Blanchette *et al.* 1992; Boyce 1961; Hedgcock 1906; Scheffer and Lindgren 1932). The cause of stains varies among fungi. Melanin-like pigments found within hyphae of blue stain fungi, such as *Ophiostoma* and *Ceratocystis* species, are responsible for the blue to black discoloration associated with infected wood (Zink and Fengel 1988). Other stains, however, are due to extracellular compounds produced by the fungus. Green stain of hardwoods caused by *Chlorociboria* is caused by a naphthoquinone pigment identified as xylindein (Blackburn *et al.* 1965). This unusual stain in wood has had a long history of use by wood workers and artists who utilized the extraordinary verdigris of greenish-blue color in 15–16th century Intarsia panels, 16–18th century inlaid furniture and 19–20th century Tunbridge ware (Blanchette *et al.* 1992).

Another curious stain in wood is the red to pink stain found in several timber species. Although several different fungi have been recognized as causing the stain, no work has been reported to identify the compounds responsible and the chemical nature of the red pigment. One fungus isolated repeatedly from red stained deciduous and coniferous woods by the authors is *Arthrographis cuboidea* (Sacc. et Ellis) Sigler. This fungus has been previously reported to cause a pink stain in species of *Pinus, Picea, Cupressus, Quercus, Betula,* and other woods (Chidester 1940; Schmidt and Dietz 1985). Unlike other stain fungi, *A. cuboidea* can colonize and stain heartwood as well as sapwood, and also stains wood treated with sodium pentachlorophenoxide (Schmidt and Dietz 1985). The fungus apparently does not cause significant wood cell wall decay (Schmidt and Dietz 1985). In addition to the red pigmentation, the fungus has been found to produce a tyrian blue pigment in culture media (Chidester 1940).

This study was done to elucidate the chemical nature of a red pigment produced by *A. cuboidea* within wood and other substrates.

Materials and Methods

Wood chips of southern yellow pine (*Pinus taeda* L.) were inoculated with a spore suspension of an isolate of *A. cuboidea* obtained from pink stained wood of Acer. After 4 weeks of incubation, fungus-infected woods were stained pink. Also the same isolate was cultured on whole rice kernels at 24C for 4 weeks. A sample of 100 g of pink pigmented wood or the fungus cultured on rice was milled, blended with chloroform and filtered through Whatman #4 filter paper. The filtrate was evaporated to dryness under reduced pressure on a rotary evaporator. The extract was dissolved in a small volume of chloroform and applied to the top of a column (1 × 15 cm) filled with silica gel (70–270 mesh, Aldrich #28, 861-6). The pigment was eluted with a mixture of chloroform and methanol (95 : 5 v/v). The red fraction was collected and concentrated to a 0.5 ml volume, then applied on two TLC plates (20 × 20 cm) coated with 0.25 mm layer of silica gel (Merck #5715). The chromatograms were developed with a mix-

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ture of chloroform, hexane and ethyl acetate (5 : 4 : 1 v/v/v). A red band of silica gel with R_f value 0.23 was scraped off the plate and the pigment was extracted with chloroform. After the solvent was evaporated, the extract was taken up in hexane and recrystallized from acetone or chloroform.

The Fourier-transformed infrared (FT-IR) spectrum was recorded with a Nicolet 170-SX FT-IR spectrometer (Madison, WI, USA). The IR spectrum was run neat with approximately 100 µg of sample on a KBr plate taken at a 4 cm^{-1} resolution.

The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra recorded with a Nicolet NT 300 WB FT-NMR (Fermont, CA, USA) spectrometer equipped with decoupler and 5 mm ^1H and ^{13}C probes at 300.066 MHz and 75.46 MHz, respectively. The purified sample (2 mg) was dissolved in $CDCl_3$ (99.8 atom % D, Sigma). Chemical shifts are reported on the scale using tetramethyl silane as an internal standard.

Mass spectrometry was done with a Kratos MS-25 GC/MS (Kratos Analytical, Ramsey, NJ, USA). An ionization potential of 70 eV was used for electron impact (EI) spectra. Isobutane was the reagent gas for spectra obtained in the positive chemical ionization (CI) mode. Samples were introduced through either direct insertion probe or coupled gas chromatography – mass spectrometry (GC/MC) using a DB-5 (30 m × 0.25 mm id., 0.25 µ film thickness); the temperature was programmed from 60 to 250 °C at 10 °C/min (J & W, Folsom, CA, USA).

Results and Discussion

Analysis of the pigment

Inoculation of either wood or rice *A. cuboidea* produced the same red pigment. However, the compound extracted from wood was of higher purity than from rice (lower amount of accompanying impurities). After purification, about 2 mg of pure, feather-shaped crystals of the metabolite were obtained. The red color of the pigment resembled that of the naphthoquinones formed by *Fusarium moniliforme*. Also, when the compound was dissolved in solvents of higher polarity (methanol for example) and in basic solutions, its color changed to blue which is similar to that of the pigments reported earlier by Golinski and Vesonder (1991). The infrared spectrum (Fig. 1A) contained no free hydroxyl bands and exhibited strong C=O stretch which is consistent with a naphthoquinone compound. In the proton NMR spectrum, six hydrogen atoms (two methoxyl groups) at 3.929 ppm, two aromatic hydrogens at 6.385 ppm and two phenolic hydrogens at 12.708 ppm were observed. The last of the above mentioned signals were of low intensity because of ion exchange. The position of phenolic hydrogens is also indicative of a naphthoquinone structure.

High resolution MS in an electron impact analysis gave a molecular weight of 250.0452 amu corresponding to an empirical formula of $C_{12}H_{10}O_6$. The EI and CI mass spectra (Fig. 1B & 1C respectively) gave a fragmentation pattern that corresponds to a compound described as a naphthoquinone red pigment 5,8-dihydroxy-2,7-dimethoxy-1,4-naphthalenedione (#2808-46-0) McLafferty and Stauffer 1989) (Fig. 2). The molecular ion in electron impact mode is 250 m/z (100); major ions are: 220 (17.5); 207 (17.0); 179 (14.1); 151 (14.8); 108 (7.6); 95 (14.8); 69 (40.8); 53 (21.0); 44 (27.2).

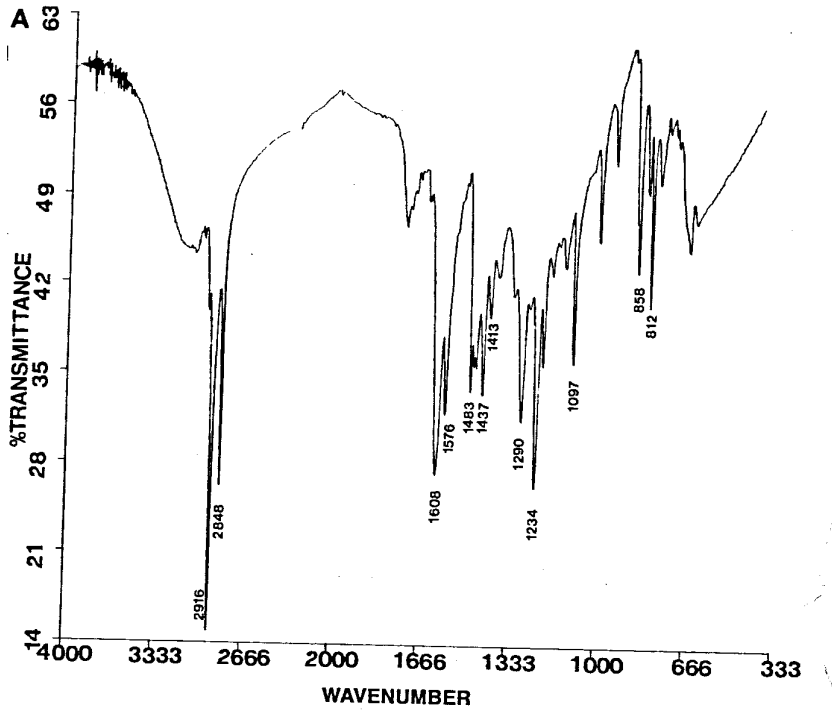

Fig. 1. Spectral characteristics of the red pigment. A: Infrared spectrum

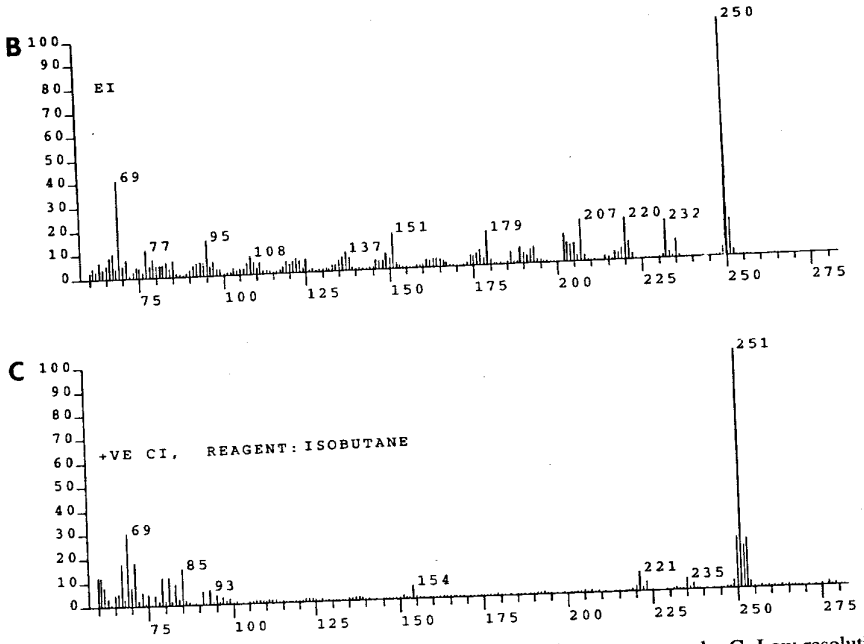

Fig. 1. Spectral characteristics of the red pigment. B: High resolution MS in electron impact mode; C: Low resolution MS in chemical ionization mode

The biosynthesis and structure of this pigment were first described by Gerber and Wieclawek (1966); they reported its production by *Streptomyces coelicolor.* Spectroscopic data, especially the mass spectral fragmentation pattern, matches the data for the red pigment produced by *A cuboidea* within red stained wood and thus confirms the identity of the red metabolite. The fungus produces the metabolite extracellularly in contrast to the many other wood staining fungi that impart stains to wood due to pigments within fungal hyphae (Eriksson *et al.* 1990).

Occurence of pigment in fungi

The naphthoquinone moiety is apparently common in natural products and especially among pigments formed by fungi of *Fusarium* species. A family of red colored metabolites with chemical structures similar to the naphthoquinone pigment described above is listed in Table 1. The 5,8-dihydroxy-2,7-dimethoxy-1,4-naphthalenedione represents the lowest molecular weight component among these pigments. *F. moniliforme* and *F. solani* are recognized as frequent producers of naphthoquinone pigments (Vesonder and Golinski 1989). Other orange, pink or crimson red stains of wood, described by several authors, could be due to a variety of related naphthoquinone pigments. Both *A. cuboidea* and *Fusarium* species appear to have similar secondary metabolites that induce pink-red stains of wood. However *A. cuboidea* appears to be widely distributed in forests and capable of staining a wide array of different woods.

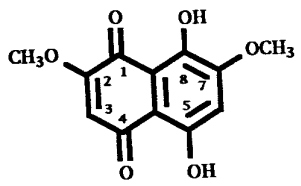

Fig. 2. The chemical structure of 5,8-dihydroxy-2,7-dimethoxy-1,4-naphthalenedione.

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Table 1. Other red colored metabolites with related chemical structures.

Compound name	Substituents at ring position				
	2	3	5	6	7
2,3-dihydro-5,8-dihydroxy-6-methoxy-2-hydroxymethyl-3(2′-hydroxypropyl)-1,4-naphthalenedione	–H –CH_2OH	–H –$CH_2CH(OH)CH_3$		–OCH_3	–H
5,8-dihydroxy-2-methoxy-6-hydroxymethyl--7-(2′-hydroxypropyl)-1,4-naphthalenedione	–OCH_3			–CH_2OH	–$CH_2CH(OH)CH_3$
Javanicin, solanione	–CH_2COCH_3	–CH_3			–OCH_3
8–0-methyljavanicin	–CH_2COCH_3	–CH_3	–OCH_3		–OCH_3
8–0-methylsolaniol	–$CH_2CH(OH)CH_3$	–CH_3	–OCH_3		–OCH_3
Norjavanicin	–CH_2COCH_3				–OCH_3
Novarubin	–$CH(CH_2OH)COCH_3$				–OCH_3
Solaniol	–$CH_2CH(OH)CH_3$	–CH_3			–OCH_3
2,5,8-trihydroxy-6-methoxy-3-(2′-oxo-propyl)--1,4-naphthalenedione	–OH	–CH_2COCH_3		–OCH_3	–H
5,8-dihydroxy-2,7-dimethoxy-1,4-naphthalenedione (red pigment associated with pink stain)	–OCH_3				–OCH_3

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