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PARTIAL CHARACTERIZATION OF A PHENOLIC PIGMENT FROM SPOROCARPS OF *PHELLINUS IGNIARIUS*

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Abstract—A dark brown phenolic polymer, isolated from mature sporocarps of *Phellinus igniarius* (DC. ex Fr.) Quél., is responsible for the darkening of sporocarp tissue when a drop of base is applied. The pigment is rich in aliphatic as well as phenolic hydroxyl groups, and also contains significant numbers of carboxyls and carbonyls. The presence of 3,4-dihydroxyphenyl moieties was documented by the identification of veratric and metahemipinic acids from oxidative degradation of methylated samples. Despite a 2% methoxyl content, the polymer contains no guaiacyl or syringyl moieties and therefore contains no lignin. It appears to be formed by the oxidative polymerization of a molecule containing a 3,4-dihydroxystyryl moiety. Hispidin was identified as its trimethyl ether in methylated extracts of pigmented mycelial mats of *Phellinus igniarius* grown in liquid culture.

INTRODUCTION

Chemical tests have been widely used in the study of fungi, although in most instances the underlying chemical phenomena are not understood, and, as a result, their subsequent taxonomic interpretations and applications remain points of conjecture and uncertainty. The purpose of this investigation was to examine in some detail the nature of one of these widely used chemotaxonomic criteria, namely the KOH test of xanthocroic Hymenomycetes, whose tissues darken noticeably when directly exposed to aqueous alkali solutions. One of the most widely distributed species in which this reaction is demonstrable is *Phellinus* (*Fomes*) *igniarius*, and it is the context tissues from sporophores of this species that were subjected to our analyses.

RESULTS AND DISCUSSION

Dioxane–water extracts of ground sporophores were dark brown, becoming almost black when made basic. Preliminary study indicated that a polymeric material in the extracts was responsible for the dark color with alkali. The pigment did not move from the origin on silica gel TLC plates, even with polar solvents, and the bulk of it was excluded from Sephadex G-25 packed in dioxane–water (1:1). Consequently the polymer was isolated by separating out the high molecular weight portion with a Sephadex G-25 gel column. Chromatography in this system also serves to remove sugars, soluble salts and other materials of low molecular weight [1].

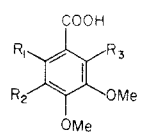
Analysis of the pigment gave: C, 55·5; H, 4·5; N, 0·7; O, 36·4; ash, 2·0 and methoxyl, 1·9%. It was high in hydroxyl content (1·0 per MW of 100), of which half was phenolic. Carboxyl groups were also present, but were less abundant (0·1 per MW of 100). The spectrum of a weakly acidic solution had a peak at 375 nm; on basification the pigment exhibited enhanced absorption in the long wavelength region. A maximum absorbance at 520 nm was exhibited by the difference curve obtained by subtracting the two spectra. Reduction with $NaBH_4$ greatly diminished the UV-visible absorption, and almost decolored the pigment; however, the polymer still became dark brown on addition of base. Methylated pigment was not darkened by base.

The IR spectrum further confirmed the high hydroxyl content (band centered at $\sim 3400\ cm^{-1}$), and also supported the presence of carboxyl groups (C=O stretching band at $\sim 1695\ cm^{-1}$,

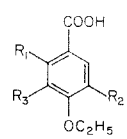
* Maintained in cooperation with the University of Wisconsin.

which persisted after $NaBH_4$ reduction, together with the absorbance due to carboxyl O–H stretching in the 2900–3300 cm^{-1} region [2]). The existence of considerable reducible carbonyl was indicated by a large decrease in the C=O stretching band in the 1650 cm^{-1} region on treatment with $NaBH_4$.

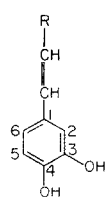
The PMR spectrum of the acetylated, methylated pigment had strong, broad peaks in both the aliphatic acetyl and phenolic acetyl regions (centered at δ2·13 and δ2·30, respectively; cf. Ref. [3]). Methyl ester and methoxyl protons gave a broad peak centered at δ3·7, and aromatic protons to a broad absorption centered at δ7·3. Reduction with $NaBH_4$ had little effect on the spectrum except to shift a small proportion of the aromatic protons upfield, which may indicate some aromatic protons *ortho* to α-carbonyl substituents [3].

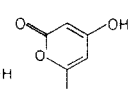


(1), $R_1 = R_2 = R_3 = H$
(2), $R_1 = COOH$, $R_2 = R_3 = H$
(3), $R_1 = R_3 = H$, $R_2 = COOH$
(4), $R_1 = R_2 = H$, $R_3 = COOH$



(5), $R_1 = R_3 = H$, $R_2 = OC_2H_5$
(6), $R_1 = R_3 = H$, $R_2 = OMe$
(7), $R_1 = H$, $R_2 = R_3 = OMe$





(8), R = H
(9), R =
(10), R = COOH

Two major products were obtained on oxidative degradation of methylated samples: veratric acid (**1**), in a 3·1% yield, and metahemipinic acid (**2**), in a 6·9% yield. Only comparatively minor amounts of additional products were seen by GLC. Isohemipinic (**3**) and hemipinic acid (**4**) were not detected. Oxidative degradation of ethylated samples yielded 2·6% of protocatechuic acid diethyl ether (**5**) and 6·7% of an unidentified product, presumably the ethyl analog of metahemipinic acid, i.e. 4,5-diethoxyphthalic acid. Vanillic acid ethyl ether (**6**) and syringic acid ethyl ether (**7**) were not detected.

Thus the extracted pigment is a phenolic polymer, and ionization of phenolic groups produces the extended chromophores responsible for the dark color with base—the positive KOH test. Further, the pigment is formed by the oxidative polymerization of a molecule containing the 3,4-dihydroxyphenyl moiety. The polymerization of radicals formed by oxidation of phenols in biological systems is well documented [4], the most studied case being the oxidative polymerization of *p*-hydroxycinnamyl alcohols to form lignins [5].

When the material is methylated and then oxidatively degraded, the major (aromatic) product is metahemipinic acid (**2**), showing that a major linkage is between an alkyl moiety and the 6-position of the 3,4-dihydroxyphenyl portion. This linkage indicates that one important mesomeric form of the radical intermediate in biosynthesis is with the unpaired electron on an alkyl carbon atom, and in turn indicates that the pigment is formed by polymerization of a molecule in which an alkyl radical is an important mesomer, namely one containing the 3,4-dihydroxystyryl moiety (**8**). Lignin formation is similar, since *p*-hydroxycinnamyl alcohols contain the 4-hydroxystyryl moiety.

A possible candidate for a precursor molecule is hispidin [4-hydroxy-6-(3,4-dihydroxystyryl)-2-pyrone], (**9**), which has been identified in fungi related to *Phellinus igniarius*, viz. *Inonotus* (*Polyporus*) *hispidus* (Bull. ex Fr.) Karst. and *Phaeolus* (*Polyporus*) *schweinitzii* (Fr.) Pat. [6,7]. It has even been suggested [8] that hispidin is oxidized in maturing sporocarps of *Inonotus hispidus* to produce a structural polymer. Consequently, we examined extracts of mature sporocarps and also of pigmented mycelial mats of *Phellinus igniarius* for hispidin, and identified the compound in the latter, but not in the former. Its absence in sporocarps is evidently due to its oxidative polymerization.

While we cannot conclude that our pigment is formed by the oxidative polymerization of hispidin, several points are in accord with this: (a) both pigment and hispidin are produced by the organism; (b) phenol-oxidizing enzymes, which would readily polymerize hispidin, are produced by *Phellinus igniarius* [9]; (c) both hispidin and pigment contain the 3,4-dihydroxyphenyl moiety, and hispidin contains the 3,4-dihydroxystyryl structure (**8**) that seems to be the salient portion of the pigment precursor; (d) both have similar ali-

phatic hydroxyl contents; (e) the elemental compositions are consistent with the pigment being formed from hispidin (hispidin: $C_{13}H_{10}O_5$; pigment: $C_{13}H_{12}O_6$) (cf. lignins, Ref. [5]); and (f) hydrolysis of the lactone function in some of the hispidin moieties would account for the carboxyl groups found in the pigment.

Molecules with 3,4-dihydroxystyryl moieties would be expected to polymerize very readily on oxidation, having many possibilities for radical coupling. Assuming that hispidin (or other 3,4-dihydroxystyryl-containing molecule) is the precursor for the pigment, it is interesting that coupling of aryl radicals evidently occurred in the 6-position but not significantly in the 2- or 5-positions. If coupling had occurred in the 2- or 5-positions, hemipinic acid (**4**) and isohemipinic acid (**3**), respectively, would have been formed on oxidative degradation after methylation. These products were not detected. This point was examined with a model polymer, made by oxidatively polymerizing caffeic acid (**10**), which contains the 3,4-dihydroxystyryl moiety. Like the fungal pigment, this synthetic polymer was dark brown and became nearly black with base. Also, the synthetic polymer yielded veratric (**1**) and metahemipinic acids (**2**) as major products on oxidative degradation after methylation (5·3 and 1·7% yields, respectively). This result indicates that oxidative coupling of a hispidin-like compound can occur preferentially in the 6-position and provides additional support for the hypothesis that the pigment is formed via the oxidative polymerization of a molecule containing the 3,4-dihydroxystyryl structure. The synthetic polymer differed from the natural pigment by the different relative proportions of veratric and metahemipinic acids, and, more importantly, in that isohemipinic (**3**) and hemipinic acid (**4**) were produced. The relative proportions of products **1** and **2** depend on the degree of oxidation during polymerization (i.e. how much H_2O_2 was used), but the formation of isohemipinic and hemipinic acids in addition to **1** and **2** indicates a fundamental difference in the coupling reactions between caffeic acid and the precursor of the extracted pigment.

The dark color of the fungal pigment, even without addition of base, probably reflects the presence of *o*-quinones and their condensation products. A substantial amount of carbonyl, which could be reduced by $NaBH_4$, was indicated by IR studies, and is in accord with this. The 3,4-dihydroxyphenyl moiety is readily oxidized to an *o*-quinone structure.

Analogies have been drawn between fungal sporocarp components, and the lignins of higher plants [10–12]. While our data indicate that the pigment is a product of phenol oxidation, as are lignins, it is clear that it is not a lignin and contains no lignin component. Vanillic acid ethyl ether (**4**) and syringic acid ethyl ether (**7**) were not detected among the products of oxidative degradation of ethylated samples, as one or both are from lignins. Thus the guaiacyl (4-hydroxy-3-methoxyphenyl) and syringyl (3,5-dimethoxy-4-hydroxyphenyl) moieties, characteristic of lignins, are absent. Both types of structures have been found in sporocarps of *Inonotus* (*Poria*) *obliquus* (Pers.) Pilát [11,13], and our pigment had a small number of methoxyl groups (1·9%). The detection of guaiacyl and syringyl moieties in these instances is apparently a function of the extraction of sterile forms of *I. obliquus* (and not the poroid sporocarps) which were probably contaminated with lignin-containing particulates.

Although further research is needed to determine whether or not our pigment is responsible for the positive KOH test in other major groups of xanthocroic Hymenomycetes, evidently it is *Inonotus hispidus* and *Phaeolus schweinitzii*, as well as *Phellinus igniarius*.

EXPERIMENTAL

Sporocarps. Mature, air-dried sporocarps were taken from the collections of the Center for Forest Mycology Research, U.S.D.A., Forest Service, Madison, Wisconsin.

Pigment isolation. 31 g of dry sporophore context tissue (<40 mesh) was extracted with purified dioxane–H_2O, 1:1 (400 ml) by shaking under N_2 for 40 hr. Insolubles were removed by centrifugation and solvents removed from the dark brown supernatant by vacuum evaporation. This was redissolved in dioxane–H_2O (1:1). additional insolubles removed by centrifugation, and the solution was applied to the top of a 3·3 × 36 cm column of Sephadex G-25 packed in dioxane–H_2O (1:1). Elution was with the same solvent at a flow rate of 12 ml/cm^2/hr. The pigment was monitored at 375 nm, and also at 520 nm after addition of base to a diluted sample. In this gel system an excluded polymer (spruce milled wood lignin [14]) was eluted at 135 ml, 3,4-dimethoxybenzyl alcohol at 230 ml, and NaCl at 360–440 ml. The material eluted in the volume 135–190 ml was combined and recovered by evaporation of solvents (1·5 g = 5% of sporocarp). The acetylated pigment (100 mg) (Ac_2O–pyridine) containing a little pyridine, was dissolved in 2 ml dioxane containing 0·1 ml H_2O, and added dropwise to 30 ml H_2O. Insoluble material was collected by centrifugation, redissolved in dioxane and reprecipitated into H_2O. Evaporation gave a

quantitative yield (OAc = 29·2%). The acetylated pigment was methylated by brief treatment of a dioxane–H_2O (19:1) solution with CH_2N_2 in Et_2O. The pigment (200 mg) was methylated by treatment in 3 ml $HCONMe_2$(DMF) with excess CH_2N_2 in DMF-ether at room temp. over an 8-day period. After removal of solvents the residue was dissolved in 2 ml dioxane–H_2O (19:1), and added dropwise to 20 ml of dry Et_2O. Methylated pigment was recovered quantitatively by centrifugation and held under high vacuum at 50° overnight (OMe = 18·3%). To reduce the pigment (100 mg), solid $NaBH_4$ (20 mg) was added to a vigorously stirred solution in dioxane–H_2O (1:1) at 5–10°. After 1 hr the solution was allowed to warm to room temp. A heavy foam that had formed gradually disappeared, an additional 10 mg $NaBH_4$ was added, and the solution held for 12 hr. The solution was then adjusted to pH 5 with acidic dioxane–H_2O (1:1) (N HCl) and the reduced pigment freed of salts by passage through Sephadex G-25.

Spectroscopic analyses. IR spectra of reduced and non-reduced pigment were taken as KBr pellets; the PMR spectrum of the acetylated, then methylated, pigment was in $CDCl_3$.

Functional group determinations. Carboxyl groups were determined from the amount of MeOH released on saponification of a methylated sample [14]. Total hydroxyl content was estimated from the acetyl content, phenolic hydroxyl from the gain in methoxyl on diazomethane methylation (with correction for methyl ester content), and aliphatic hydroxyl was taken as the difference between total and phenolic hydroxyl contents.

Degradative characterization. Oxidative degradations ($KMnO_4$, H_2O_2) of methylated samples of pigment were done using procedures developed for lignins [14,15]. Two major products, obtained together with much smaller amounts of several other materials, were identified as veratric acid (**1**) and *m*-hemipinic acid (**2**). They were isolated as their methyl esters from the reaction mixture by preparative TLC (silica gel HF_{254}, $CHCl_3$), and identified by comparisons with authentic samples by TLC, GLC [14], and by PMR. Similar oxidative degradation of ethylated samples [14,16] yielded protocatechuic acid diethyl ether (**3**), identified by comparison with an authentic sample by GLC [14], and an unidentified product, presumably 4,5-diethoxyphthalic acid. Only minor amounts of other products (containing single aromatic nuclei) were detected; vanillic acid ethyl ether (**6**) and syringic acid ethyl ether (**7**) were not detected.

Isolation and identification of trimethyl hispidin from mycelial mats. Phellinus igniarius (isolate FP 59071-S) was grown in still culture at 25° on the culture medium described by Hatfield and Brady [17]. 6-week-old dark-grown cultures showed yellow brown pigmentation. The thick mycelial mats were pressed free of excess water and ground to a slurry in MeOH in a blender. After 24 hr in the dark, the mycelium was removed by filtration (dry wt = 4·3 g) and solvent removed from the extract by vacuum evaporation. The dry residue was suspended in 25 ml dry acetone, and methylated by stirring 48 hr with 1 g K_2CO_3 + 1 ml of Me_2SO_4 in the dark under N_2 at 25° [17]. Trimethylhispidin (25 mg) was separated from other materials by preparative TLC (silica gel HF_{254}; 5% EtOH in $CHCl_3$, R_f 0·38; 10% EtOAc in C_6H_6, R_f 0·11). The product was identified by comparisons (IR, UV, TLC) with an authentic sample. Oxidative degradation of the isolated material with $KMnO_4$ and H_2O_2 [15] yielded veratric acid (**1**) (>40%).

Oxidative polymerization of caffeic acid [18]. 1·7 g caffeic acid and 80 mg horseradish peroxidase were dissolved in 400 ml of degassed phosphate buffer (0·01 M) at pH 6·0 (readjusted to pH 6·0 with NaOH). This solution and 400 ml 23·6 mM solution of H_2O_2 in degassed buffer were added simultaneously and separately to 200 ml of stirred degassed buffer containing 20 mg peroxidase, over a 15-hr period with a proportioning pump. All solutions were under N_2 and the reaction vessel was kept dark. 10 hr after final addition the soluble reaction mixture was freed of H_2O by vacuum evaporation, the residue was dissolved in dioxane–H_2O (1:1) and filtered. Salts and low molecular weight materials were removed by column chromatography using the same system used to purify the pigment. The yield was about 900 mg of polymeric material (excluded from the Sephadex G-25 gel).

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