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HISPIDIN BIOSYNTHESIS IN CULTURES OF *POLYPORUS HISPIDUS*

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Key Word Index—*Polyporus hispidus*; (Polyporaceae); hispidin biosynthesis; bis-noryangonin; hydroxycinnamic acids; ^{14}C studies.

Abstract—Cultures of *Polyporus hispidus* grown on a liquid medium with glucose as the principal carbon source produced the yellow pigment hispidin (4-hydroxy-6-(3,4-dihydroxystyryl)-2-pyrone) (II). Tracer studies showed that DL-phenylalanine, DL-tyrosine, cinnamate, *p*-coumarate and caffeate were incorporated into the hispidin molecule without scrambling of the label. Good incorporation of acetate and malonate into the pyrone portion of the molecule was observed. The related styrylpyrone, bis-noryangonin (4-hydroxy-6-(4-hydroxystyryl)-2-pyrone) (I) was also detected in extracts of cultured mycelium and a cell-free enzyme preparation was obtained which catalyzed the hydroxylation of bis-noryangonin to hispidin.

INTRODUCTION

HISPIDIN (II) has been reported from many fungi including *Polyporus hispidus* Fr.[1,2] *Polyporus schweinitzii* Fr.[3] and several species of *Gymnopilus*.[4] While bis-noryangonin (I) is the only other styrylpyrone reported from fungi,[4-7] many compounds of this type are known from higher plants. Yangonin, 4-methoxy-6-(4-methoxystyryl)-2-pyrone, first isolated from *Piper methysticum* Forst.[8] is probably the most extensively studied of this class of compounds. Styrylpyrones have also been isolated from *Aniba*,[9] *Alpinia*[10] and *Ranunculus*.[11]

It has been suggested that hispidin may be oxidized to serve as a toughening polymer in the formation of the polypore basidiocarp.[12,13] No direct evidence has been presented for this, and the presence of the compound in other non-woody Basidiomycetes suggests that this might not be so. Nevertheless, the biosynthesis of the molecule, whose structure suggests a derivation from a hydroxycinnamic acid and two acetate units, has been of interest for some time. Cortisalin from *Cytidia salicina*[14] and chlorflavonin from *Aspergillus*

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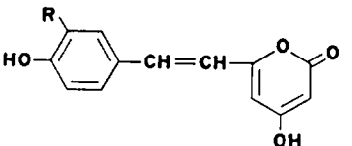
candidus[15] are the only other compounds isolated from fungi which appear to arise in this manner, although such compounds are widespread in higher plants.



(I) R = H Bis-noryangonin
(II) R = OH Hispidin

Cinnamic acid metabolism in cultures of *P. hispidus* has been investigated[16] and this work provided a good basis for the examination of hispidin biosynthesis. A recent investigation[17] of the biosynthesis of hispidin in cultures of *P. schweinitzii* has shown the incorporation of acetate-1-^{14}C and L-phenylalanine-U-^{14}C indicating a $C_9 + 2C_2$ derivation of the molecule.

RESULTS

Six-week-old cultures of *P. hispidus* grown on agar medium in darkness showed no development of yellow pigmentation characteristic of hispidin production. Two-week-old, dark-grown cultures developed colour within 24 hr after exposing them to fluorescent light. It was observed that blue light (430–530 nm) stimulated pigment formation best. Pigment production in relation to dry weight was monitored over 34 days growth of the cultures. Maximum rate of hispidin production occurred near the end of the logarithmic phase of growth and the highest yields of hispidin were obtained from 25-day-old cultures (5 mg/g dry wt mycelium) after which time the yields decreased rapidly.

TABLE 1. INCORPORATION OF VARIOUS PRECURSORS INTO HISPIDIN BY 17-DAY-OLD CULTURES OF *P. hispidus*

Compound administered	Sp. act. precursor (μCi/μM)	Sp. act. TMH* (Ci/M)	Dilution	Sp. act. veratric (μCi/μM)	$\frac{\text{Sp. act. veratric}}{\text{Sp. act. TMH}} \times 100$
Phenylalanine-2-^{14}C	2·5	$9{\cdot}8 \times 10^{-4}$	2600	—	—
Phenylalanine-3-^{14}C	3·9	$1{\cdot}1 \times 10^{-3}$	3500	$1{\cdot}1 \times 10^{-3}$	100
Tyrosine-3-^{14}C	6·9	$1{\cdot}1 \times 10^{-3}$	6300	$1{\cdot}0 \times 10^{-3}$	91
Cinnamic acid-2-^{14}C	20·0	$6{\cdot}9 \times 10^{-4}$	29 000	$3{\cdot}3 \times 10^{-5}$	4·8
p-Coumaric acid-2-^{14}C	0·18	$8{\cdot}0 \times 10^{-4}$	200	$2{\cdot}4 \times 10^{-5}$	3·0
Caffeic acid-2-^{14}C	0·16	$5{\cdot}8 \times 10^{-4}$	310	$3{\cdot}0 \times 10^{-5}$	5·2
Sodium acetate-2-^{14}C	2·0	$1{\cdot}8 \times 10^{-3}$	1100	$6{\cdot}6 \times 10^{-5}$	2·8
Malonic acid-2-^{14}C	7·4	$4{\cdot}5 \times 10^{-4}$	16 000	—	—

* TMH = Trimethylhispidin.

Two μCi of each of several specifically-labelled molecules were incubated for 24 hr with 17-day-old cultures of *P. hispidus*. The isolated hispidin was methylated, its specific activity determined and oxidized to veratric acid, the specific activity of which was also determined.

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These results (Table 1) are in good agreement with the proposed biogenesis of hispidin from a cinnamic acid derivative with the addition of two acetate units.

Examination of chromatographed extracts of *P. hispidus* revealed the presence of at least four other components which fluoresced yellow- to blue-green in longwave UV light. The UV spectra of all these compounds showed a strong absorbance near 360–370 nm suggesting that they are styrylpyrones. One of these substances which was present in only trace amounts gave identical colour reactions and R_fs to an authentic sample of bis-noryangonin. An enzyme preparation was subsequently obtained which catalysed the conversion of bis-noryangonin to hispidin. Two ml of the enzyme catalysed the hydroxylation of approximately 2 μM of bis-noryangonin in 1 hr.

DISCUSSION

Although light has been shown to stimulate growth in *P. schweinitzii*, nothing has been reported concerning the effect of light on hispidin production. Our study indicates that light is required for pigment formation in *P. hispidus*.

The rapid production of hispidin during what has been referred to as the idiophase of growth[12] is typical of secondary metabolite formation in microbial cultures. With respect to the proposed route of biosynthesis it is reasonable that this should occur at a later stage of cultural maturity than does maximum phenylalanine ammonia-lyase activity which occurs at the transition from teliophase to idiophase.[16]

p-Coumaric and caffeic acids were most efficiently incorporated of all precursors examined (Table 1), indicating that hispidin can arise through hydroxylated cinnamic acid derivatives. The poor incorporation of cinnamic acid is not understood. We have observed that concentrations as low as 0·1% cinnamic acid in the medium arrest the growth of the organism. Degradative studies of methylated hispidin showed that phenylalanine and tyrosine labelled in the third carbon yielded veratric acid of approximately the same specific activity as the parent compound. The veratric acid obtained when other precursors were employed failed to show a significant incorporation of label.

Although bis-noryangonin was present in quantities too small to be critically identified, its characteristic UV fluorescence, R_fs and colour reactions with various spray reagents were considered good evidence of its identity. Furthermore, an enzyme preparation previously shown to catalyse the hydroxylation of *p*-coumaric acid,[16] even more readily catalysed the hydroxylation of bis-noryangonin to hispidin, adding further credibility to the presence of bis-noryangonin in the fungus. This suggested that hispidin could be synthesized from cinnamate or *p*-coumarate via styrylpyrone or bis-noryangonin, as well as directly from caffeic acid.

EXPERIMENTAL

Culturing. Liquid agar cultures were prepared and incubated as described earlier.[16]

Measurement of hispidin production. The medium was removed by filtration and the mycelium washed with 100 ml dist. H_2O, frozen and lyophilized. The dry mycelium was weighed and exhaustively extracted with MeOH in a mortar. The extract was reduced in vol. *in vacuo* and banded on cellulose (Avicel, 500 μ) TLC plates which were developed in toluene–HCO_2Et–HCO_2H (5:4:1). The hispidin band was eluted by powdering the cellulose, placing it in a small column and washing with MeOH. The molar extinction coefficient at 366 nm was used to calculate the yield of hispidin.

Specific activity of trimethylhispidin. Radioactive hispidin was isolated from the mycelium as described above, dried *in vacuo* and dissolved in 1 ml dry acetone. To the solution was added 200 mg anhy. K_2CO_3 and 0·1 ml of Me_2SO_4 and the mixture was refluxed for 24 hr. The reaction mixture was then reduced in vol. *in vacuo*, banded onto TLC plates and developed as described above. The recovered trimethylhispidin

was eluted in MeOH and the specific activity determined from the extinction coefficient (log ϵ 4·39) and scintillation counting.

Degradation of trimethylhispidin to veratric acid. The recovered radioactive trimethylhispidin was dissolved in 5 ml of dry acetone containing 1·4 mg unlabelled authentic trimethylhispidin. To the acetone solution was added 50 mg of $KMnO_4$ and the mixture was allowed to react at room temp. for 2 hr with occasional agitation. The acetone was then removed *in vacuo* and 1·0 ml of 1 N HCl and 1·0 ml of tech. sodium bisulphite (10% aq.) were added to obtain a colourless solution. This solution was extracted with 3 × 2 ml Et_2O and the Et_2O solution transferred to a sublimation vessel where it was taken to dryness with gentle warming and sublimed at 0·3 mm Mercury at 110°. The recovered veratric acid was further purified in the chromatographic system described above and the specific activity determined in the same manner as for the trimethylhispidin.

Preparation and administration of radioactive compounds. DL-Phenylalanine-3-^{14}C, DL-phenylalanine-2-^{14}C, sodium acetate-2-^{14}C and malonic acid-2-^{14}C were obtained from New England Nuclear Corporation. Cinnamic acid-2-^{14}C and tyrosine-3-^{14}C were obtained from International Chemical and Nuclear Corporation and *p*-coumaric acid-2-^{14}C and caffeic acid-2-^{14}C were prepared by condensation of the appropriate benzaldehyde with malonic acid-2-^{14}C in pyridine with a trace of piperidine.[18] 2 μCi of the radioactive compound was administered to the culture medium of each Roux bottle. The compound was dispersed by brief agitation and the cultures incubated with the radioisotopes for 24 hr.

Bis-noryangonin hydroxylase. A cell-free extract was prepared as described previously for *p*-coumaric acid hydroxylase.[16] 3 μM of authentic bis-noryangonin were incubated with 2 ml of crude enzyme under the conditions previously described.

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