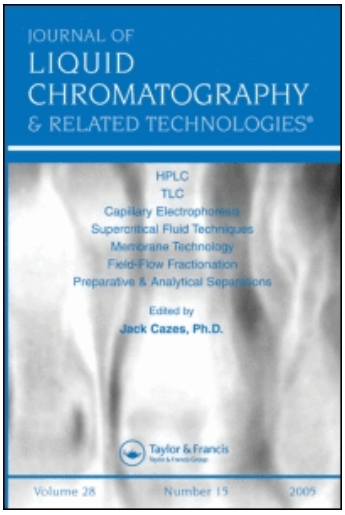

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HPLC ANALYSIS OF FUNGAL MELANIN INTERMEDIATES AND RELATED METABOLITES

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ABSTRACT

A convenient method to concentrate and separate intermediates and related secondary products of the pentaketide pathway leading to melanin biosynthesis has been developed. Twelve of the fourteen known compounds were separated using reverse phase high performance liquid chromatography with a 12 to 42% linear gradient of acetonitrile in 2% acetic acid. The remaining two, highly water soluble isomers, were isocraticly separated on the same column with 5% acetonitrile in 2% acetic acid. Solid phase extraction and concentration of representitive compounds in acidified aqueous brine was accomplished with reverse phase Sep-Pak cartridges. The effectiveness of the method was evaluated by determining the pentaketides produced by cell-free homogenates of Verticillium dahliae supplied with scytalone as the substrate and by cultures of two V. dahliae mutants. The method has the potential for quantifying all known pentaketide melanin metabolites and thus will allow for more comprehensive studies of fungal melanin biosynthesis.

INTRODUCTION

Melanin occurs in the cell walls of many fungi. Melanized fungal cells survive dessication and ultraviolet irradiation appreciably better than their hyaline counterparts (1, 2). They also are more persistant in soil,

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apparently because of melanin's ability to denature degradative exoenzymes produced by other microorganisms (1, 3). The ability of certain fungi to produce melanin also appears to be an important determinant of pathogenicity. Rice blast caused by Pyricularia oryzae can be controlled by application of tricyclazole [5-methyl-1,2,4-triazolo-(3,4-b) benzothiazole], a systemic inhibitor of melanin biosynthesis (4). Apparently, melanization of the fungal appressorium is a necessary prerequisite for the successful invasion of rice (5). The extent of such a phenomenon is just being investigated but has also been found with Colletotrichium lagenarium and C. lindemuthianum which cause anthracnose of cucumbers and beans, respectively (6, 7).

Biochemical characterization of the pentaketide melanin pathway has been obtained using mutants (8, 9, 10) and cell-free homogenates (11) of the fungus Verticillium dahliae. Known intermediates in the pathway include 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN), formed from acetate via pentaketide cyclization (12), scytalone, 1,3,8-trihydroxynaphthalene (1,3,8-THN), vermelone and 1,8-dihydroxynaphthalene (DHN) (Figure 1). Presumably DHN polymerizes to form melanin. Cultures of various melanin deficient mutants and tricyclazole treated wild types accumulate flaviolin, 2-hydroxyjuglone (2-HJ) and other related secondary metabolites, indicating that synthesis of these compounds is intimately connected to the melanin pathway (4). The pentaketide pathway of melanin biosynthesis has been shown to occur in a number of imperfect and ascomycetous fungi but has not been demonstrated in any basidiomycetes. They cannot metabolize the various intermediates to melanin and melanin biosynthesis in these fungi is not inhibited by tricyclazole (13).

The metabolic pathway leading to the formation of pentaketide melanin was determined using solvent partitioning and thin-layer or column chromatography. Precise methods were not developed to quantitate the

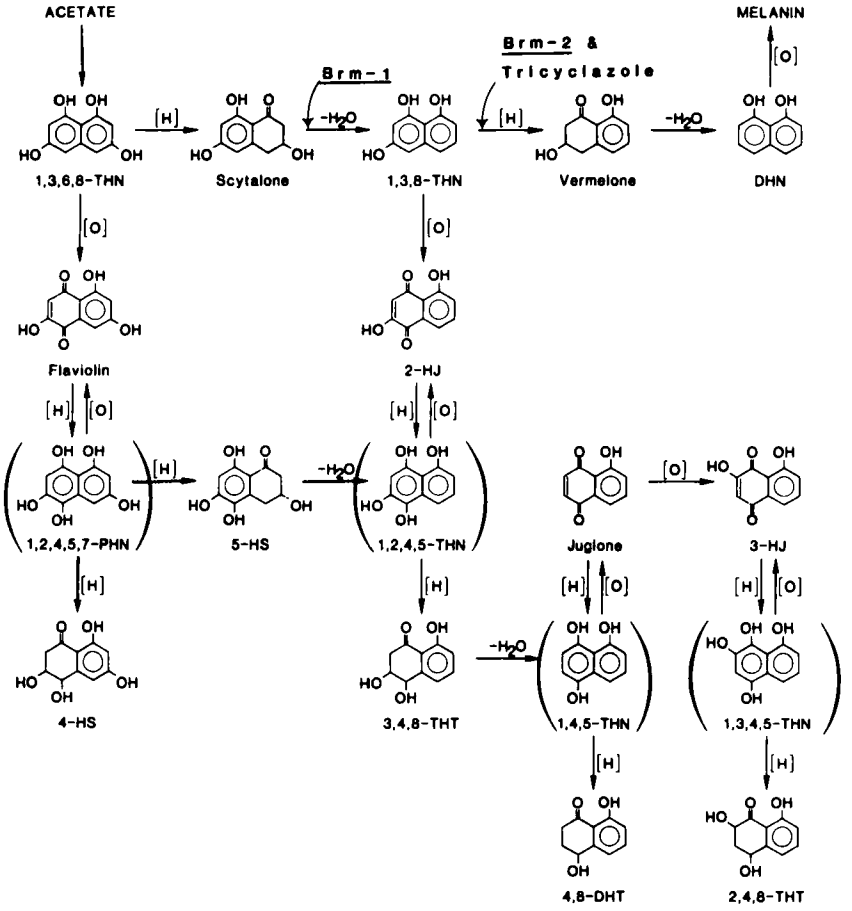

FIGURE 1. Pentaketide pathway of melanin biosynthesis and the flaviolin and 2-HJ branch pathways (17). Postulated intermediates are in brackets. Blockage sites for the two mutants and tricyclazole are indicated.

compounds. Thus accurate determinations of the effects of environmental factors and melanin inhibitors on the pathway have not been possible. Progress towards elucidating the pentaketide pathway of melanin biosynthesis depends upon a method to quantitate the various metabolic intermediates. This paper describes such a method.

MATERIALS AND METHODS

Chemicals

(+)-Scytalone and 4-hydroxyscytalone (4-HS) were purified from cultures of the brm-1 mutant of V. dahliae (9), and (-)-vermelone was purified from cultures of the brm-1 mutant supplemented with 1,3,8-THN (14). DHN, 1,3,8-THN and 1,3,6,8-THN were synthesized by the method of Lurie et al. from 1-naphthol-8-sulphonic acid, 1-naphthol-3,8-disulphonic acid and 1,8-dihydroxynaphthalene-3,6-disulphonic acid, respectively (15). Flaviolin was made from 1,3,6,8-THN by autoxidation in 0.1 M phosphate buffer (pH 8); 2-HJ and 3-hydroxyjuglone (3-HJ) were synthesized by the method of Singh et al. (16); and 3,4,8-trihydroxytetralone (3,4,8-THT), 4,8-dihydroxytetralone (4,8-DHT) and 2,4,8-trihydroxytetralone (2,4,8-THT) were prepared as described by Stipanovic and Bell (10). 5-Hydroxyscytalone (5-HS) was produced from flaviolin by Wangiella dermatitidis (17). Juglone was purchased from Sigma, Inc., St. Louis, MO.

Technical grade tricyclazole was obtained from Lilly Laboratories, Greenfield, IN; organic solvents were either filtered A.C.S. grade or HPLC grade (Omnisolv, E. Merck, Inc., Cincinati, OH) and purified water was obtained from a Milli-Q system (Millipore Corp., Bedford, MA) (18).

Instrumentation

A modular HPLC system consisting of a #950 pump, #985 controller and #970 variable wavelength detector (Tracor Instruments, Inc., Austin, TX); 100 µl loop injector, #7125 (Rheodyne, Inc., Cotati, CA); and a #3390A recording integrator (Hewlett-Packard, Inc., Palo Alto, CA) was used for this study. The column was a prepacked 4.6 mm X 150 mm LC-18 (Supelco, Inc., Bellefonte, PA), protected with an inlet filter and a guard column. System development was performed at room temperature using a mobile phase flow rate of 1 ml/min. Detection was carried out at 254 nm.

Chromatography

Retention times for selected pentaketide intermediates were first determined isocraticly using aqueous acetonitrile. Some of the

intermediates exhibited peak tailing which was most effectively controlled with 2% acetic acid. Methanol and tetrahydrofuran were not satisfactory substitutes for acetonitrile. This information was used to design a gradient using acetonitrile and 2% acetic acid. The solvent mixtures were: A, acetonitrile-water-acetic acid (5:93:2, v/v) and B, acetonitrile-water-acetic acid (75:23:2, v/v). Optimum separation was obtained with an 18 min. linear gradient from 90% A to 47% A (12 to 42% acetonitrile) followed by a 4 min. isocratic step at 47% A. The column was then washed for 5 min. at 28% A (55% acetonitrile) and equilibrated. The only isomers not separated in this gradient system, 4-HS and 5-HS, were resolved with an isocratic method (acetonitrile-water-acetic acid; 5:93:2, v/v).

Solid Phase Extraction

Scytalone, flaviolin and juglone with capacity factors (k_i) of 2.2, 4.5, and 7.2, respectively, were dissolved in water which was then fortified with NaCl (20% w/v) and adjusted to pH 3.5 with acetic acid. This solution was applied to a set of twin C^{18} Sep-Pak cartridges (Waters Assoc., Milford, MA), prepared according to the manufacturer's instructions. The cartridges were separated, washed with water (10 ml), and eluted with a sufficient volume of acetonitrile-water-acetic acid (55:43:2, v/v) to collect two 5 ml samples from the first and one 5 ml sample from the second cartridge. An aliquot of each sample was diluted with 6 volumes of water and chromatographed.

Biological Applications

The effectiveness of the complete technique was judged by qualitatively evaluating pentaketide metabolites produced by three strains of V. dahliae: a wild type, T9(ATCC 44574) and two melanin deficient mutants, brm-1 (ATCC 44571) and brm-2 (ATCC 44572). Detailed descriptions of these isolates have been published (8,9,10). Stock cultures were grown on potato-carrot-dextrose agar (PCDA) (19) and transferred at 2- to 3-week intervals. Inoculations were made by spreading conidial suspensions over the surface of

the solid medium (20 ml) in 100 x 15 mm Petri plates. The cultures were then incubated in the dark at 24° C for 7 days.

The entire contents of plates containing brm-1 or brm-2 were diced and extracted twice for 2 hrs. with 100 ml acetone. The combined extracts were filtered and the acetone removed in vacuo. The resulting aqueous solutions were acidified (pH 3.5), salted (NaCl, 20% w/v), and each was passed through a C^{18} Sep-Pak cartridge. The cartridges were processed and the eluate appropriately diluted with water and chromatographed.

Cell-free homogenates were prepared from plates of the wild type strain as previously described (11). The homogenates, with and without tricyclazole (8 ppm), were fortified with scytalone and incubated anaerobically at room temperature. After 10 hrs, each reaction mixture was acidified, salted, processed and chromatographed in a manner identical to that used to extract the metabolites from the plate cultures.

RESULTS AND DISCUSSION

The retention times (t_r), k_i and resolution factors (R_s) of compounds in the melanin pathway and the two branch pathways are given in Table 1.

All of the compounds had k_i values within the optimum range ($1<ki<10$). The area/height ratios (peak width at half height) for all compounds except 1,3,6,8-THN were less than 0.3 min. The calculated R_s values for nearest neighbor compounds were thus always greater than 1 (<2% band overlap). Figure 2 is a typical chromatographic trace of eight standards.

The system can resolve three of the four sets of isomeric intermediates (2,4,8-THT, 3,4,8-THT, and scytalone; vermelone and 4,8-DHT; and 2-HJ and 3-HJ). The isocratic retention times of 4-HS and 5-HS were 8.2 and 9.0 min. respectively, with an R_s of 1.

Scytalone, flaviolin and juglone in acidified brine were completely bound to the first of the twin C^{18} cartridges. They were entirely eluted from the cartridge with the first 5 ml of solvent (acetonitrile-water-acetic acid; 55:43:2, v/v). The capacity factors of these three compounds are

TABLE 1

Retention Times (t_r) and Capacity (k_i) and Resolution (R_s) Factors of Pentaketide Melanin Intermediates and Branch Pathway Products.

Compound	t_r(min)	k_i	R_s
4-HS	5.0±.1 *	1.1	0
5-HS	5.0±.1	1.1	3.2
2,4,8-THT	6.6±.1	1.8	1.2
3,4,8-THT	7.2±.2	2.0	1.3
Scytalone	7.7±.2	2.2	1.5
1,3,6,8-THN	8.8±.3	2.7	4.1
4,8-DHT	11.6±.2	3.8	1.5
Vermelone	12.5±.3	4.2	2.0
Flaviolin	13.2±.2	4.5	1.6
3-HJ	13.8±.2	4.8	1.1
1,3,8-THN	14.3±.3	5.0	4.2
2-HJ	16.6±.2	5.9	6.2
Juglone	19.7±.2	7.2	4.4
DHN	21.6±.2	8.0	

* Standard deviation of the mean; n=3

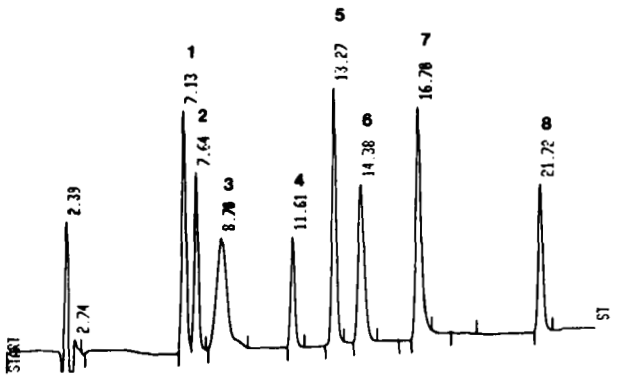

FIGURE 2. Gradient separation of eight melanin intermediates and secondary metabolite standards. The compounds and the amounts injected are: 1. 3,4,8-THT (250 ng); 2. Scytalone (250 ng); 3. 1,3,6,8-THN (250 ng); 4. 4,8-DHT (250 ng); 5. Flaviolin (125 ng); 6. 1,3,8-THN (250 ng); 7. 2-HJ (1 ug); 8. DHN (1 ug). AUFS = 0.16. Experimental conditions described in text.

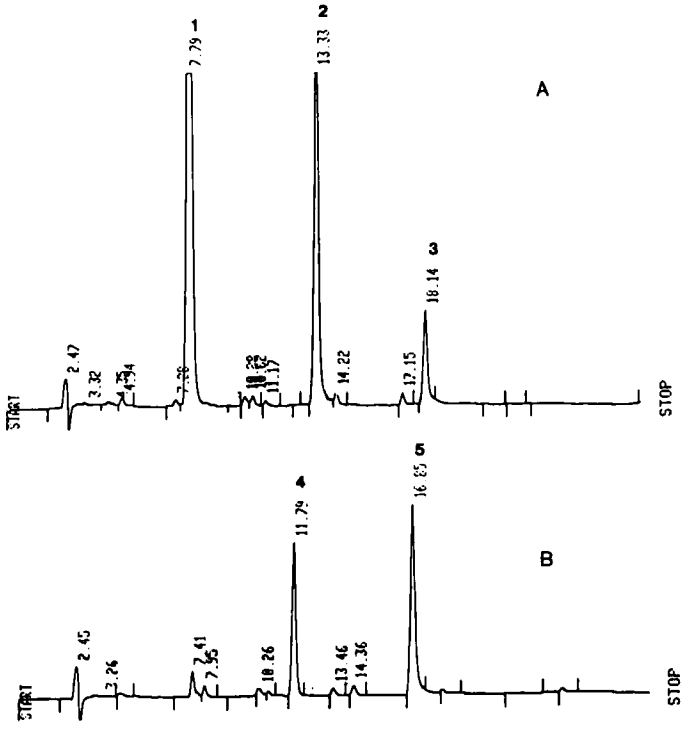

FIGURE 3. Solid phase extraction and gradient separation of melanin intermediates and secondary path products produced by the brm-1 (A) and brm-2 (B) mutants. 1. scytalone; 2. flaviolin; 3. unknown; 4. 4,8-DHT; 5. 2-HJ. AUFS = 0.32. Experimental conditions described in text.

representitive of the entire array of compounds in this study. This showed that solid phase extraction can be used to concentrate and purify these compounds prior to chromatography.

The combination of solid phase extraction and chromatography was used to analyze the melanin intermediates formed from V. dahliae brm-1 and brm-2 cultures. The technique was also used with untreated and tricyclazole treated wild type homogenates using scytalone as a substrate. The results qualitatively agree with previous work. The brm-1 strain primarily produced scytalone, flaviolin and an unknown compound at t_r = 18.1 min. and the brm-2

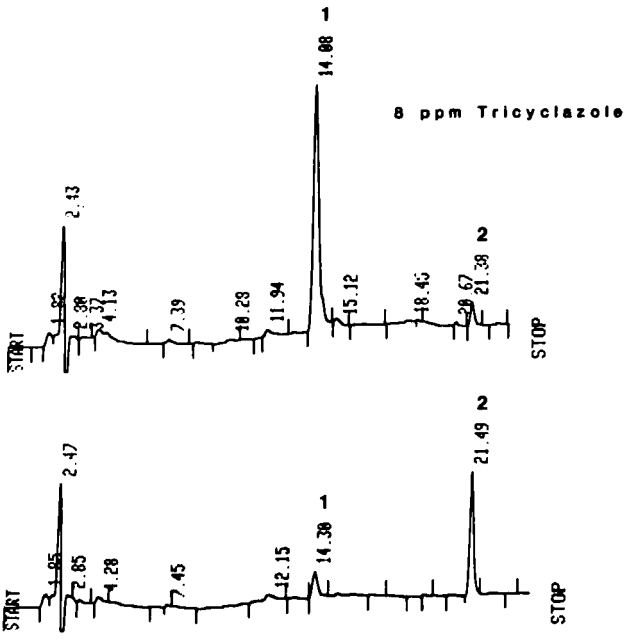

FIGURE 4. Solid phase extraction and gradient separation of melanin intermediates and secondary path products produced by anaerobically maintained cell free homogenates of the wild type fungus. The substrate was scytalone. 1. 1,3,8-THN; 2. DHN. AUFS = 0.16. Experimental conditions described in text

strain primarily produced 4,8-DHT and 2-HJ (figure 3). The cell-free homogenates converted scytalone to 1,3,8-THN and DHN (figure 4). This occurred irrespective of the addition of 8 ppm tricyclazole to the homogenates. As anticipated, the ratio of 1,3,8-THN to DHN in the untreated reaction mixture was different from the tricyclazole treated mixture. The untreated predominantly produced DHN whereas the treated predominantly produced 1,3,8-THN.

Pentaketide melanin intermediates and secondary metabolites can be analyzed with this method in a much more facile manner than past procedures. The method can separate any of the known metabolites in the pentaketide

melanin and secondary pathways. Both in vitro and in vivo studies on the effects of environmental factors and exogenous melanin inhibitors on pentaketide melanin biosynthesis can now be accomplished easily and more exactly. Such studies are in progress.

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