Byssotoxin A, a Secondary Metabolite of Byssochlamys fulva

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Byssochlamys fulva, isolated from corn, was grown on nutrient-amended shredded wheat medium for 14 days at 25 C. Crude solvent extract from these cultures was toxic to brine shrimp, chicken embryos, and rats. The extract was slightly inhibitory to the germination of pea seeds, but was nontoxic to ten species of bacteria and one of yeast. One metabolite was isolated, given the trivial name byssotoxin A, and partially characterized chemically and physically.

Incidents of food spoilage caused by Byssochlamys fulva were first reported by Olliver and Smith (16). B. fulva was thought to be confined to Great Britain (15, 18), but has since been reported from Switzerland (14), Canada (21), Australia (20), the United States (19), and Brazil (11). B. fulva, described as a saprophyte easily cultured on laboratory media, also grows well on acidic fruit juices of approximately 10% sugar content (15).

B. fulva and its imperfect state Paeclomyces varioti have been isolated from processed fruits (9, 10, 12, 15, 16), sorghum brandy (3, 19), Manitoba butter (1), and cassava bread (11). Various metabolites of B. fulva (P. varioti) and the closely related B. nivea have been reported, among them the mycotoxins byssochlamyic acid (17), patulin (6), and an unknown substance toxic to chickens (5). Variotin and uastic acid have been reported from P. varioti (3).

In our research, extracts from culture media of B. fulva were bioassayed and found to be toxic to brine shrimp and chicken embryos in preliminary experiments. Known toxic metabolites were not present in the extracts. This communication reports the isolation and partial characterization of a new toxic metabolite given the trivial name byssotoxin A.

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MATERIALS AND METHODS

Organism. B. fulva Westling AUA 583, isolated from corn in this laboratory, was used throughout the investigation. Cultures were maintained at 25 C on Czapek solution agar (3). Incubum was produced on test tube slants containing the following medium, in grams per liter: dextrose, 20; KH2PO4, 5; MgSO4, 0.5; yeast extract, 7; and agar, 25.

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Culture medium. B. fulva was grown on a medium consisting of 100 g of shredded wheat and 200 ml of nutrient solution per 2.8-liter Fernbach flask as previously described (7). Flasks were stoppered with cotton plugs, foil covered, and autoclaved for 15 min at 121 C, twice within 24 h. The medium was inoculated with conidia from a 7-day-old culture of B. fulva. Incubation was at 25 C and 95 to 100% relative humidity as stationary cultures for 2 weeks.

Preparation of crude extract. The culture in each flask was extracted with a total of 500 ml of chloroform-ethanol (4:1). Approximately 150 to 175 ml of solvent was added to each flask through a funnel without removing the cotton plug and heated on a steam bath for 2 to 3 min. The contents of the flask were transferred to a Waring blender, and an additional 75 to 100 ml of solvent was added and blended for 1 min. The extracts were filtered through Whatman no. 2 filter paper. The residue was reblended with 250 ml of solvent and refiltered, and the filtrates were combined. Extracts were filtered again through Whatman no. 42 filter paper to remove any remaining residue.

Extraction and isolation of the metabolite. The chloroform-ethanol extract was evaporated to dryness at room temperature overnight under an air stream, and the residue was redissolved in approximately 25 ml of 95% ethanol solution. A 5-ml sample was spotted on a thin-layer chromatography plate (20- by 20-cm glass plate coated with a 500-μm layer of Brinkman Instruments MN Silica Gel G-HR). Extracts of uninoculated medium were spotted as controls. Plates were developed in toluene-ethyl acetate-90% formic acid (5:4:1, vol/vol/vol). Following development, plates were air-dried and divided into five equal zones under ultraviolet (UV) light. Each band was eluted with 95% ethanol and bioassayed for toxicity to brine shrimp. Toxic extracts were retained for further extraction and purification.

Crude extracts in a minimum volume of hot ethanol were placed on a Sephadex LH-20 column (25 by 2.5 cm, ID), equilibrated, and packed with absolute ethanol-chloroform (90:10). Five fractions were obtained as visibly distinct bands on elution with...
absolute ethanol-chloroform (90:10). Fractions 3 and 4 were bioassayed and found to contain an orange-red substance toxic to the brine shrimp. These fractions were further purified by recycling through a Sephadex LH-20 column (80 by 2.5 cm, ID). Fraction volume was reduced under an air stream, and flasks were placed in a freezer for 30 min, where small orange-red needles crystallized.

Biological assays. (i) Brine shrimp bioassay. The crude extract and the purified compound were bioassayed with brine shrimp using a method previously described (7). Ethanol solutions (0.02 and 0.04 ml) were pipetted into the wells of a Pyrex 7220 spot plate, and the wells subsequently were filled with brine shrimp-salt water solution to give a 1-ml final volume. Appropriate 2 and 4% ethanol-salt water-shrimp controls were also run, as were shrimp-salt water controls. Plates were incubated at 25 to 28°C and 98 to 100% relative humidity. Mortality was checked and recorded as the percentage of deaths after 1, 2, and 4 h.

(ii) Chicken embryo bioassay. Crude extract in chloroform-ethanol (4:1) was assayed by adding 2 ml of peanut oil to 25 ml of the extract in 50-ml Erlenmeyer flasks. Purified compound was assayed by adding 2 ml of peanut oil to 25- and 50-ml samples in 50-ml flasks. Flasks were stoppered with cotton and covered with cheesecloth secured with a rubber band. Flasks were gently heated on a steam bath to vaporize the chloroform-ethanol and sterilize the solution and were later placed in a vacuum oven overnight at 50°C to remove the residual solvent. The crude extract and purified compound were bioassayed using a modified chicken embryo test as previously described (7). Data were taken as the percentage of mortality at 2, 4, and 8 days after injection.

(iii) Rat intraperitoneal bioassay. The compound was bioassayed using a procedure previously reported (7). The test product was placed under an air stream and evaporated to dryness, and 3 ml of Me₂SO was added to the 125-ml Erlenmeyer flask. Charles River (CD), 21-day-old weanling rats were given water but no food for 24 h prior to treatment. Five rats were swabbed on the abdomen with iodine solution and injected with 0.5 ml of an approxi-mately 40-mg compound Me₂SO solution. Following injection rats were given food and water ad libitum. Rats were marked and weighed before injection and again before autopsy. Observations were made for several hours after injection and daily during the duration of the experiment. The procedure was repeated using 5, 20, and 40 mg of the compound per ml of Me₂SO. The previously described procedures were used, and weight gain relative to the control treatment was calculated for each group. Me₂SO controls consisting of extracts of uninoculated medium were bioassayed in the same manner.

(iv) Microbiological bioassay. Organisms tested included the bacteria Streptococcus pyogenes, Staphylococcus aureus (strain 80), Bacillus subtilis, Bacillus cereus mycoides, Neisseria sicca, Esche- richia coli, Salmonella typhosa, Klebsiella pneumo-niae (NWQL 33-300), Pasteurella multicolor, the yeast Saccharomyces cerevisiae ellipsoideus, and the actinomycete Nocardia sp. Incubation temperature was 25 to 28°C. The method used was essentially that of Broce et al. (2).

Filter-paper disks (6 mm in diameter) were sterilized by autoclaving for 30 min at 121°C. Dilutions were made of suspensions of bacterial cells, and 0.1 ml of the suspension was transferred to each of three petri dishes containing either tryptose or blood agar. The microorganisms were then distributed uniformly over the surface of the plate using a glass rod and a rotating motion. The filter disks were soaked overnight in methanol solutions containing 1 and 0.1 mg of the compound per ml. Blank filter-paper disks were soaked overnight in methanol for use as controls. Disks were air-dried and placed on the agar surface aseptically, and the plates were incubated at 25 to 28°C for 24 to 48 h. Bacterial and fungal inhibition zones were measured and recorded.

(v) Pea seedling bioassay. The purified compound was tested for inhibition against a dwarf variety of Little Dwarf Marvel peas (Pisum sati-vum), using the method of Burmeister and Hessel-tine (4). Seeds were soaked overnight in 50-ml aqueous solutions containing 10 μg and 0.1, 1, and 10 mg of the compound per 40 ml of water. Ten peas were soaked in water overnight as controls for each treatment. Seeds were placed in vermiculite contained in a pan (50 by 35 by 8 cm). Germination was recorded after 4 days, and plant height, root length, and fresh weight were determined after 2 weeks of growth.

Analyses. (i) Chromatography. Three solvent systems were used to determine the Rf values of the new metabolite: toluene-ethyl acetate-90% formic acid (5:4:1, vol/vol/vol); chloroform-acetone (85:15), and methanol-chloroform (60:40). A 5-μl sample in ethanol was spotted on a thin-layer chromatography plate as previously described, developed in the respective solvent systems, dried, and examined under long- and short-wave UV light.

(ii) Spot tests and spray reagents. Spot tests and spray reagents referred to below are as detailed by Krebs et al. (13) and Fieg and Anger (8).

(iii) Melting point determinations. An electro-thermal melting-point apparatus (Electrothermal Engineering Ltd., London) was used to determine corrected temperatures for signs of first change, melting, and decomposition.

(iv) Optical rotation. Determinations were carried out using a 1% solution in methanol with a Steeg and Reuter SR5 polarimeter.

(v) Percentage of nitrogen by Kjeldahl method. Nitrogen content of the compound was determined in our laboratory using a semimicro Kjeldahl method and also by Galbraith Laboratories, Inc., Knoxville, Tenn.

(vi) UV and visible absorbance. Initial UV-visible spectra were made using a Beckman DK-2 spectrophotometer. The UV and visible absorbances were determined on methanol solutions (5 × 10⁻⁴ M concentration at 356, 285, and 247 nm, and 5 × 10⁻⁴ M concentration at 472 nm) using a Perkin-Elmer (Coleman 139) spectrophotometer. Molar extinction coefficients were calculated for each wavelength.
(vii) Infrared analysis. A 3-mg sample of purified compound was placed in 300 mg of KBr. Samples and KBr were heated overnight at 105°C to remove moisture. A KBr pellet was prepared, and absorbance was determined using a Beckman model IR-33 spectrophotometer.

(viii) Nuclear magnetic resonance analysis. A 10-mg sample of purified compound was dissolved in 0.3 ml of deuterated Me$_2$SO containing 5% tetramethylsilane as the internal standard. Data were recorded using a Hitachi Perkin-Elmer R24 model spectrometer.

(ix) Mass spectral analysis. Low-resolution mass spectra were determined in our laboratory using a DuPont 21-490 mass spectrometer with a heated solid-sample probe inlet. Analysis was made of the trimethylsilyl derivative of byssotoxin A. High-resolution mass spectra were obtained by the high-resolution mass spectrometer of the Department of Chemistry, Florida State University, Tallahassee, Fla.

RESULTS

Biological assays. Toxicity of the crude extract and purified byssotoxin A to brine shrimp is shown in Table 1. The crude extract caused 90 to 100% mortality in 1 h. Byssotoxin A toxicity ranged from 0 to 15% in 1 h to 30 to 40% in 4 h. The possibility of the toxicity of the crude extract being due to fatty acids occurring at the same $R_f$ value was removed by purification of the toxin by crystallization from methanol.

Table 2 shows the toxicities of byssotoxin A and the crude extract to chicken embryos. Crude extract toxicity ranged from 80 to 100% by day 8. Byssotoxin A caused 20% mortality in 25-mg doses and 20 to 40% mortality in 50-mg doses in 8 days.

In one series of experiments rats were injected with pure byssotoxin A and crude byssotoxin in amounts varying from 2.5 to more than 30 mg per dose dissolved in Me$_2$SO. Crude extracts killed four of five rats within 3 days at the higher doses. Byssotoxin A killed two of five rats. Me$_2$SO controls did not kill any of the rats, nor did 2.5 mg or less of toxin per rat.

The effect of various concentrations of byssotoxin A on weight gains of Charles River (CD) rats is presented in Table 3. The weight gains for the rats ranged from 56 to 96% of the control for the 20- and 10-mg doses, respectively.

Byssotoxin A was tested for its inhibitory effect toward ten bacteria and one yeast. Growth of the microorganisms was not inhibited at concentrations of 100 to 1,000 $\mu$g/ml. Some activity of byssotoxin A was observed on Little Marvel pea seedlings. Germination of seeds treated with solutions of toxin of 0.001 to 10.0 mg/50 ml of water was 40 to 70% of that of untreated controls. Also, the height of plants was reduced 21 to 52%, root length was reduced 50%, and dry weight of seedlings was reduced 63% by a 59% solution of byssotoxin A.

Analytical. Byssotoxin A was essentially insoluble in acetone, benzene, and hexane; slightly soluble in chloroform, ethanol, and water; soluble in methanol and 5% NaOH (on heating); and very soluble in Me$_2$SO. It was not optically active as determined by polarimetry.

Byssotoxin A appeared as a single orange to orange-red spot on thin-layer chromatography plates. $R_f$ values were: 0.5 in toluene-ethyl acetate-formic acid (5:4:1, vol/vol/vol), 0.3 in chloroform-acetone (85:15), and 0.9 in chloro-

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### Table 1. Toxicity of crude extract and byssotoxin A to brine shrimp

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concn (%)</th>
<th>Mortality*</th>
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</thead>
<tbody>
<tr>
<td></td>
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<tr>
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<td>0</td>
</tr>
<tr>
<td>Crude extract</td>
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<td>90</td>
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<td>100</td>
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<tr>
<td>Pure compound</td>
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</tr>
<tr>
<td>Pure compound</td>
<td>4</td>
<td>15</td>
</tr>
</tbody>
</table>

*a Data indicate the mean of three replicates of 100 brine shrimp each.

b Extract of uninoculated check media.

### Table 2. Toxicity of crude extract and byssotoxin A to chicken embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route</th>
<th>Dose (mg)</th>
<th>Mortality*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Controls*</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Crude extract</td>
<td>Air sac</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Crude extract</td>
<td>Yolk</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Pure compound</td>
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<tr>
<td>Pure compound</td>
<td>Yolk</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

*a Data indicate the mean of two replicates of five eggs each. Data was recorded 2, 4, and 8 days after injection (14-day-old eggs).

b Extract of uninoculated medium.

### Table 3. Effect of various concentrations of byssotoxin A on weight gains of Charles River (CD) rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg)</th>
<th>Wt of rats (g)*</th>
<th>Wt increase (g)</th>
<th>Proportion of control (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Me$_2$SO control</td>
<td>0.0</td>
<td>53.8</td>
<td>199.4</td>
<td>145.6</td>
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<tr>
<td>Pure compound</td>
<td>2.5</td>
<td>54.0</td>
<td>188.0</td>
<td>134.0</td>
</tr>
<tr>
<td>Pure compound</td>
<td>10.0</td>
<td>56.2</td>
<td>196.8</td>
<td>139.6</td>
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<tr>
<td>Pure compound</td>
<td>20.0</td>
<td>54.6</td>
<td>140.8</td>
<td>186.2</td>
</tr>
</tbody>
</table>

*a Data indicate the mean of five rats after 4 weeks.
form-methanol (40:60). The compound decomposed without melting at 285 to 292 C.

Byssoxin A gave negative results for ninhydrin and biuret tests both before and after hydrolysis with 6 N HCl. Ferric chloride and indole tests were also negative. The compound completely dissolved in 5% NaOH on heating to give a pale green color, but was not soluble in hot or cold 5% NaHCO₃. The compound was negative to Dragendorff reagent, p-aminobenzaldehyde, and Ehrlich reagent. A weak or mixed reaction occurred with iodoplatinate, phenol reagent, and phosphomolybdic acid. A strong blue-purple color was obtained in response to concentrated H₂SO₄ and all reagents containing the concentrated acid. A negative test was obtained for primary and tertiary amines. Results were positive for aliphatic secondary amines. Attempts to reduce byssotoxin A using sodium hydrosulfite were unsuccessful.

Physical and chemical properties. The UV spectrum of byssotoxin A showed λMeOH/max 247 nm (ε = 50,600), 285 nm (ε = 17,600), 356 nm (ε = 11,200), and 472 nm (ε = 146). The infrared spectrum showed prominent absorption of 3,279 cm⁻¹, 2,941 cm⁻¹, 2,899 cm⁻¹, 2,108 cm⁻¹, 1,608 cm⁻¹, 1,460 cm⁻¹, 1,370 cm⁻¹, and 1,285 cm⁻¹. The nuclear magnetic resonance spectrum in Me₂SO showed a complex pattern with a series of unresolved multiplets with centers at 0.9 and 1.2 µg/ml. A three-proton singlet occurred at 3.3 µg/ml, a one-proton triplet at 4.3 µg/ml, a one-proton doublet at 5.2 µg/ml, a one-proton singlet at 6.3 µg/ml, and a one-proton singlet at 12.1 µg/ml, which when deuterated disappeared.

Nitrogen analysis showed that the compound contained 2 mol of nitrogen per mol of compound. High-resolution mass spectral analysis of the trimethylsilyl derivative of byssotoxin A gave a precise mass of 474.2354. Subtraction of the mass of the trimethylsilyl moiety placed the nominal mass of byssotoxin A as 402. The probable formula of byssotoxin A was thus deduced to be C₃₀H₇₆N₂O₁₀ (found, 402.1958; required, 402.1943). A reliable mass spectrum and fragmentation pattern of the parent compound was not obtained because of the high temperature (in excess of 350 C) required for volatilization and the many complex rearrangements that can occur at such elevated temperatures. The highest electronic mass ion observed for the parent compound was 358.1889, a fragment that did not satisfy the various criteria required of a molecular ion. The probable formula of this fragment was C₂₀H₇₂O₁₀ (found, 358.1889; required, 358.1887).

DISCUSSION

B. fulva (P. varioti) was shown to be slightly or moderately toxigenic to several biological systems including brine shrimp, chicken embryos, rats, and possibly pea seeds, but not to several bacteria and one yeast species. The toxic crude extract did not contain byssochlamycid acid, patulin, or variotin, which are the known mycotoxins produced by species of Byssichlamys. Three Byssochlamys secondary metabolites, all orange-red pigments, had related UV spectra and R₁ values and appeared to be similar compounds. One of these pigments was produced on a wider variety of natural and synthetic media and in greater quantity than the other two and was selected for initial investigation of this group of metabolites. The compound was given the trivial name byssotoxin A.

Bioassay data on brine shrimp, chicken embryos, and rats indicated that byssotoxin A was not extremely toxic, nor was it solely responsible for the toxic effects. Crude extracts were more toxic than the highest dose of pure toxin. Several other metabolites exhibited low levels of toxicity, an observation previously suggested by Chu (5). Thus Byssichlamys appears to produce a variety of mycotoxins, some of which are only slightly toxic, some of low to intermediate toxicity (i.e., byssotoxin A), and some of relatively high toxicity (i.e., byssichlamycid acid). Apparently, not all of these toxins are produced at the same stage of growth or to the same degree on any one substrate. This phenomenon is probably not uncommon among the fungi and may even be the rule rather than the exception. Several fungi have been bioassayed in our laboratory, in which the crude extract was toxic to brine shrimp and chicken embryos, but on further examination no single potent toxin could be isolated. Typically, toxicity of the extract diminished steadily on fractionation and partition into its component parts to a greater degree than could be explained as due to loss of material during purification procedures.

The structure of byssotoxin A was not determined. Chemical analyses and infrared and UV spectra indicated that it is neither an indole nor an alkaloid. A positive test was obtained for a secondary amine, but tests were negative for primary and tertiary amines, amino acids, and amides. A nitrosamine could not be ruled out. In fact, fragmentation analysis indicated that the compound breaks up in the mass spectrometer, losing an N₂O fragment. This is a characteristic of various compounds, including nitrosamines. Fragmentation analysis also in-
dicated that byssotoxin A consists of a large, stable ring structure plus a side chain of eight or nine carbons that terminates in an amine or nitrosamine group. Chemical and physical data do not match those of any known mycotoxin nor any other previously reported compound insofar as can be determined. Response to sulfuric acid resembled that of methoxyhydroxytoluquinone in that both compounds produce dark purple colors with the concentrated acid. The infrared spectrum of byssotoxin A did not exhibit carbonyl absorption between 5.5 and 6 \( \mu \)m, although a strong band did occur at 6.22 \( \mu \)m. Thus, a strongly bonded hydroxyquinone was not ruled out, although spot chemical tests for anthraquinones and quinones in general were either negative or inconclusive. Nuclear magnetic resonance data indicated the presence of a strongly bonded hydroxy and the absence of amino groups and amides.

ACKNOWLEDGMENTS

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LITERATURE CITED