Soybean Charcoal Rot Disease Fungus *Macrophomina phaseolina* in Mississippi Produces the Phytotoxin (−)-Botryodiplodin but No Detectable Phaseolinone

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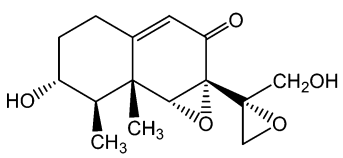
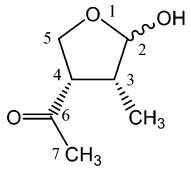
Received September 29, 2006

Research on charcoal rot disease in soybeans, and approximately 500 other plant diseases caused by the fungus *Macrophomina phaseolina*, has been severely hampered by unavailability of phaseolinone (**1**), an eremophilane sesquiterpenoid phytotoxin proposed to facilitate initial infection. Phytotoxin produced in cultures of disease-causing *M. phaseolina* isolated in Mississippi, and purified in a manner similar to that reported for **1**, was shown to be (−)-botryodiplodin (**2**), a readily synthesized mycotoxin previously isolated from *Botryodiplodia theobromae* cultures. Phaseolinone was not detected, suggesting that **2** may be the phytotoxin that facilitates infection. The availability of **2** should facilitate studies on its role in plant disease.

Charcoal rot disease, one of about 500 plant diseases caused by the fungus *Macrophomina phaseolina*,[1] is responsible for major economic losses to soybean producers in dry years.[2] The fungus is also an important reason for the failure of semiarid agriculture with crops such as guayule.[3] Research on charcoal rot and other plant diseases caused by *M. phaseolina* has been severely hampered by the unavailability of phaseolinone (**1**), a phytotoxin believed to play a role in the initial infection of roots, although this role remains to be established experimentally. The phytotoxin from *M. phaseolina* was first identified by Siddiqui et al.[4] in culture filtrates of the fungus and reported by Dhar et al.[5] to be **1**, an eremophilane sesquiterpenoid, specifically an epoxidized analogue of phomenone.[6] Phaseolinone, **1**, has been prepared semisynthetically by Kitahara et al.[7] in nine steps from (+)-sporogen-AO.



Phaseolinone (**1**)



(-)-Botryodiplodin (**2**)

A sample of the *M. phaseolina* phytotoxin was sought for studies aimed at developing resistance to charcoal rot in soybeans. Given the complexity of the Kitahara semisynthesis[7] and the unavailability of starting material, production by fermentation was undertaken using an *M. phaseolina* isolate from Mississippi, which had been shown by the method of Pearson et al.[8] to cause charcoal rot disease in soybean. Culture filtrate (8 L) was extracted and purified by the method of Siddiqui et al.[4] followed by repeated preparative TLC guided by phytotoxicity measurements in duckweed (*Lemna pausicostata*) cultures. The procedure yielded 8 mg of pure phytotoxin, which was shown by predominantly spectroscopic methods to be (−)-botryodiplodin (**2**). This is the first report of **2** being produced by *M. phaseolina* and the first report of **2** being phytotoxic. Compound **2** was first isolated from cultures of *Botryodiplodia theobromae*,[9] a fungus that causes rot in tropical fruits. The structure of **2** has been determined[10−13] and numerous syntheses of (±)- or (-)-**2** have been reported.[14−17]

Phaseolinone (**1**) was not detected in culture filtrates of *M. phaseolina* examined either in crude form or after fractionation by the method of Siddiqui et al.[4] using either HPLC/MS or TLC with 4-(*p*-nitrobenzyl)pyridine treatment, which detects epoxide moieties in mycotoxins.[18] The predicted chemical properties of **2** fit the chemical properties reported by Siddiqui et al.[4] for crude phytotoxin from *M. phaseolina* better than do the properties of **1**, because **2** contains a functional group (hemiacetal) able to give a positive test with Somogyi's reagent,[19] whereas **1** does not. Explanations for different toxin profiles in different isolates include (i) geographical and/or temporal variation in toxin production by *M. phaseolina* and (ii) multiple toxins (which is typical for mycotoxin producers) produced by the isolate in earlier studies.[4] The observation that charcoal rot can be produced in soybean by an *M. phaseolina* isolate that produces phytotoxin **2** but apparently not **1** suggests that **2** may play a role in initial infection by the fungus. Because **2** is readily prepared, the additional research needed to determine its role in diseases caused by *M. phaseolina* should be facilitated.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on an Autopol III automatic polarimeter (Rudolph Research, Flanders, NJ). UV−vis absorption spectra were obtained on a Beckman DU-7000 instrument (Beckman, Fullerton, CA). One-dimensional NMR (^{1}H, ^{13}C, and DEPT) and two-dimensional NMR (COSY, TOCSY, HMQC, and HMBC) spectroscopy were carried out on a Varian Inova 300 MHz instrument (Varian, Inc., Palo Alto, CA). High-resolution electrospray ionization MS was recorded on a ThermoFinnigan LCQ Classic ion-trap instrument (Finnigan MAT/Thermoquest, San Jose, CA).

Organisms. *Macrophomina phaseolina* isolates were cultured[20] from soybean plants with charcoal rot disease in Mississippi during 2000−2003, identified by culture characteristics including dark mycelia and black, spherical microsclerotia 75 to 90 μm in diameter, and stored in the mycological culture collection, Mississippi State University, Mississippi State, MS. A selected isolate (#124), which was shown to cause charcoal rot disease in soybean by the method of Pearson et al.,[8] was used in the present study.

Phytotoxicity Assay. Phytotoxicity of pure compounds and purification fractions was estimated in a series of eight dilutions in triplicate in axenic cultures of duckweed (*Lemna pausicostata* Helgelm. 6746)

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10.1021/np060480t CCC: $37.00
Published on Web 01/06/2007

according to the method of Tanaka et al.[21] using increased electrolyte leakage, reduction of DMSO-extractable chlorophyll levels, and reduction of wet weight gain relative to vehicle controls as measures of toxicity.

Phytotoxin Production. *M. phaseolina* isolate #124 was initially cultured on potato dextrose agar. Several 1 cm^2 pieces of agar medium with associated mycelium were used to inoculate 2 L flasks containing 500 mL aliquots of potato dextrose broth (PDB) prepared from components using fresh potatoes. Use of fresh potatoes rather than dehydrated medium was found to be necessary to get production of pigment and phytotoxin. Five flasks were inoculated for each culture and shaken on a rotary shaker at 125 rpm at 24 °C for 2 weeks. Culture materials were filtered through cheesecloth and pooled, and a 200 mL aliquot was passed through a membrane filter (250 μm, Millipore Corp., Billerica, MA) to obtain a cell-free filtrate, which was bioassayed[21] for phytotoxicity.

Phytotoxin Extraction and Isolation. Extraction and initial purification of phytotoxic culture filtrates used the method of Siddiqui et al.,[4] specifically, adding 20−30 g/L fine, powdered charcoal, shaking for 1 h, collecting the charcoal by centrifuge at 10 000 rpm for 10 min, air drying, weighing, eluting with $CHCl_3$ until no more color was removed, and evaporating the eluate. The residue was dissolved in a minimum of EtOH, diluted with H_2O, filtered, and defatted by extraction with an equal volume of diethyl ether, and the aqueous phase was evaporated to dryness under reduced pressure at 30 °C to give 0.6 g of crude yellow extract. The crude extract was fractionated by preparative TLC (silica gel, 0.5 mm thick layer) in $CHCl_3$−MeOH (95:5) as the eluting solvent system. One edge of each plate was sprayed with anisaldehyde in sulfuric acid solution and heated for 5 min at ∼120 °C. Four bands (R_f = 0.1, 0.32, 0.55, and 0.9) were scraped from the plates, eluted with 50% aqueous EtOH, and assayed for phytotoxicity with *L. pausicostata*. The active fraction (R_f = 0.55) comigrated with a brown-colored spot formed with 4-(*p*-nitrobenzyl)pyridine, a chromogenic reagent that gives blue spots with epoxides and other alkylating agents.[18] Repeated preparative TLC under the same conditions guided by color formation in guide strips treated with 4-(*p*-nitrobenzyl)pyridine treatment gave 8 mg of pure phytotoxin from 8 L of *M. phaseolina* culture medium. No material giving a blue spot with the 4-(*p*-nitrobenzyl)-pyridine color reagent was detected on TLC in the region where phaseolinone would be expected to run. Also, no phytotoxic substance with the M_r of phaseolinone (M + H = 281) could be detected using high-resolution mass spectrometry with electrospray ionization to examine crude extracts or eluates from all segments of TLC sheets developed with *n*-BuOH−HOAC−H_2O (4:1:1)[4] and other solvent systems.

(−)-Botryodiplodin (2): colorless oil; $[\alpha]^{25}_D$ −69 (*c* 0.188, $CHCl_3$); UV ($CHCl_3$) λ_{max} 278 nm; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.18 (s, 1H, HOCHCHMe), 4.84 (d, J = 12.0 Hz, OH′), 4.28 (t, 1H, J = 8.8 Hz, H5), 4.02−4.09 (m, H5 and H5′), 3.67 (q, 1H, J = 7.2 Hz, H4′), 3.42 (dt, 1H, J = 2.8, 7.6 Hz, H4), 3.04 (bd, J = 1.8, 1H. OH), 2.61 (quint, 1H, J = 7.2 Hz, H3), 2.44−2.49 (m, 1H, H3′), 2.30 (s, 3H, CH_3CO), 2.21 (s, 3H, CH'_3CO), 1.06 (d, 3H, J = 7.2 Hz, $CHCH_3$), 0.87 (d, 3H, J = 7.2 Hz, $CHCH'_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 213.7 (s), 206.6 (s), 104.3 (d), 100.6 (d), 70.0 (t), 66.6 (t), 53.3 (d), 53.2 (d), 42.8 (d), 41.5 (d), 32.7 (q), 30.6 (q), 12.8 (q), 9.9 (q); HRESIMS *m*/*z* 167.0691 (calcd for $C_7H_{12}O_3Na$, 167.0684).

Acknowledgment. This work was supported in part by the Mississippi Soybean Promotion Board and the Mississippi State University Research Initiatives grants program. The authors thank K. Tucker and R. M. Kersh for valuable technical assistance.

Supporting Information Available: NMR assignments for **2**, isolation flow diagram, one-dimensional ^{13}C and ^{1}H NMR spectra, two-dimensional (TOCSY, HMBC) NMR spectra, key TOCSY and HMBC interactions, and structures of species present in preparations of **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP060480T