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Bostrycin and 4-Deoxybostrycin: Two Nonspecific Phytotoxins Produced by *Alternaria eichhorniae*†

R. CHARUDATTAN[1]* AND K. V. RAO[2]

Plant Pathology Department, Institute of Food and Agricultural Sciences,[1] and Department of Medicinal Chemistry, College of Pharmacy,[2] University of Florida, Gainesville, Florida 32611

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Two crystalline red pigments with phytotoxic activity were isolated from culture filtrates of *Alternaria eichhorniae*, a pathogen of the water hyacinth *Eichhornia crassipes*. The pigments were present in the ratio of 4:1 and were identified as bostrycin and 4-deoxybostrycin, respectively. This is the first isolation of 4-deoxybostrycin from a natural source. Bostrycin, 4-deoxybostrycin, and their isopropylidene derivatives induced necrosis on tested plant leaves comparable to the *A. eichhorniae*-induced necrosis on water hyacinth. The lowest phytotoxic concentrations of crystalline bostrycin and 4-deoxybostrycin on water hyacinth leaves were about 7 and 30 μg/ml, respectively. Both substances were inhibitory to *Bacillus subtilis* but were inactive against the fungus *Geotrichum candidum*.

The fungus *Alternaria eichhorniae* Nag Raj & Ponnappa causes a leaf blight on the water hyacinth *Eichhornia crassipes* (Mart.) Solms (5). It was described as a new species partly because it produces a bright-red, water-soluble pigment in vitro (5). A cell-free culture filtrate of the fungus was reported to induce necrosis on water hyacinth leaves (5). Our objectives were to identify the phytotoxin and evaluate its host specificity and potential as a herbicide.

MATERIALS AND METHODS

Culture. *A. eichhorniae* was isolated from diseased water hyacinth in India (1) and has been maintained at 5°C alternately on potato dextrose agar and corn meal agar tubes. For toxin production, stationary cultures were grown from mycelial transfers in potato dextrose broth in 3- or 6-liter Erlenmeyer flasks containing, respectively, 1 or 2 liters of medium per flask. The cultures were grown for 3 to 4 weeks under a 12-h photoperiod (900 lx) at 25 ± 2°C. The culture fluid was filtered through eight layers of cheesecloth, then through Whatman no. 1 filter paper, and finally through a 0.45-μm filter. Pigmentation and toxicity of the culture fluid appeared to be related; when *A. eichhorniae* was grown submerged, it did not produce pigmentation in vitro and the culture fluid was not phytotoxic.

Toxin assay. The culture fluid and its fractions were tested for phytotoxic activity against detached plant leaves or shoots in comparison with uninoculated, filtered potato dextrose broth or other appropriate controls. The toxic fluid was applied to adaxial leaf surfaces along three scratch marks (1 to 2 mm long) per leaf, using six leaves per treatment. A 12-μl drop was applied per scratch mark. Entire leaves, leaflets, or 2- by 5-cm portions of leaves (referred to as leaves) were incubated for 48 h over moist filter papers in petri plates under a 12-h photoperiod (900 lx) at 25 ± 4°C. Necrosis was rated on a scale of 0 to 3: 0, no damage; 1, necrosis up to 1 mm around the spot of application; 2, necrosis up to 2 mm; and 3, necrosis extending 3 mm and beyond. Assays were conducted twice or thrice.

Antibacterial activity of the culture fluid and purified compounds was tested against *Bacillus subtilis* ATCC 6633 by the disk-plate method (3). Filter paper disks dipped in solutions of various concentrations of compounds were placed on the seeded agar plates and incubated at 30°C for 16 to 20 h. The concentration at which a barely visible zone of inhibition was found was defined as the minimum inhibitory concentration.

Antifungal activity of the culture fluid and the purified compounds was tested against *Geotrichum candidum* by applying droplets of various concentrations of toxins to potato dextrose agar plates, allowing the droplets to dry overnight, and spraying with a 24-h-old potato dextrose broth shake culture of the fungus. After standing overnight at 25°C, plates were observed for zones of inhibition of fungal growth.

Recovery and purification. The culture fluid at pH 2 to 4 was extracted twice with *n*-butanol (0.3 volume each), the combined extract was concentrated under reduced pressure to near dryness, the residue was triturated with diethyl ether, and the dark-red microcrystalline solid was filtered (yield, 0.15 g/liter). For larger batches, an alternative procedure was used in which the culture fluid was passed through a column of DEAE-cellulose (Cellex D, Bio-Rad Laboratories; 25 g/liter of culture fluid). The dark-red pigment was absorbed on the column, and, with the passage of additional culture fluid, the band gradually moved down the column. After passage of all of the culture fluid, the column was washed with water and eluted with 1% sodium chloride–1% acetic acid in water. The eluate (ca. 5 to 10% of the volume of the culture fluid)

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was extracted with ethyl acetate in the presence of 10% sodium chloride. The extract was concentrated to dryness, and the solid was suspended in ether, filtered, and washed with ether.

Thin-layer chromatography-grade silica gel (100 g) was stirred in chloroform (500 ml) with water (4 ml) and acetic acid (4 ml), and the homogeneous slurry was poured into a column (35-mm diameter). A solution of the crude pigment (1.5 g) in chloroform (150 ml) was applied to the column, and the column was eluted with chloroform and then with 2% methanol in chloroform with a flow rate of 0.6 to 0.8 ml/min. A minor band (B) was eluted first, followed by the major band (A). Fractions corresponding to each were combined and concentrated to dryness, and the solids were crystallized from chloroform-methanol (1:3).

Isopropylidene derivatives. An alternative and more convenient procedure for the separation of the two components is through conversion to the respective isopropylidene derivatives, which are more readily soluble and sharply separated from each other by chromatography. The mixture containing components A and B (1 g) was boiled under reflux for 20 h in acetone (150 ml) with Dowex 50 X-4 resin (200 to 400 mesh), H^+ form (2 g). The reaction mixture was filtered, and the filtrate was concentrated to near dryness. The residual solid was dissolved in benzene and added to a column of silica gel (20 g). Elution with 2% acetone and 5% acetone in benzene gave two bands, B and A, as before. Corresponding fractions from each band were combined and concentrated to dryness, and the solids were crystallized from ether-hexane.

Reduction of compound A. The procedure for reduction of compound A was similar to that described by Noda et al. (6). A solution of bostrycin (component A, 0.1 g) in pyridine (8 ml) was hydrogenated in the presence of 5% palladium on carbon for 1 h. The mixture was filtered and the filtrate was concentrated to dryness. The solid was crystallized from methanol-ether.

Reduction of isopropylidene derivative of compound A. Hydrogenolysis of the isopropylidene derivative of compound A (0.2 g) was carried out as described above. Filtration of the reaction mixture and concentration to dryness gave a solid which was crystallized from ether-hexane.

Hydrolysis of the isopropylidene derivative. A solution of the isopropylidene derivative of component A (0.4 g) in aqueous ethanolic HCl (1:1; 0.2 N) was heated under reflux for 1 h. It was cooled, diluted with water, and extracted with chloroform. Concentration of the solvent extract to dryness and crystallization from chloroform-methanol gave pure component A.

Instrumentation. Melting points were determined on a Fisher-Johns apparatus and were uncorrected. The various spectra were obtained with the following instruments: UV, Beckman 25; infrared, Beckman Acculab 3; nuclear magnetic resonance, Varian T-60 with tetramethyl silane as internal standard; and mass spectra, Hitachi/Perkin-Elmer MS-30 double-focusing spectrometer. Thin-layer chromatography was performed with silica gel (Merck HF 254 + 366) with glass slides (25 by 76 and 50 by 76 mm), and the compounds were detected by their own color. Column chromatography was conduced with silica gel, 200 to 400 mesh (Fisher Scientific Co.).

RESULTS

Purification and characterization. The principle responsible for the red color and the phytotoxic and antibacterial activities of the culture fluid was readily extracted into organic solvents such as ethyl acetate or *n*-butanol at pH 2 to 4 but only partially at pH 8 to 10.

The pigment recovered by solvent extraction at pH 2 to 4 was purified by chromatography on silica gel and separated into components designated A and B which were present in the ratio of 4:1. Both were obtained as maroon crystalline solids.

The following analyses were obtained:

(i) Major component (A): yield, 0.1 g/liter; mp, 228 to 230°C; infrared, ν_{max} 3,516, 3,490, 3,360, and 1,601 cm^{-1}; UV, λ_{max} (log ε) 542 (3.73), 505 (3.93), 478 (3.87), 306 (3.95), and 230 (4.53); M^+, 336.

Analysis: calculated for $C_{16}H_{16}O_8$: C, 57.14; H, 4.80. Found: C, 56.92; H, 4.84.

(ii) Minor component (B): yield, 0.025 g/liter; mp, 200 to 202°C; infrared ν_{max} 3,400, 1,650, and 1,590 cm^{-1}; UV, λ_{max} (log ε) 542 (3.73), 505 (3.92), 478 (3.86), 306 (3.94), and 230 (4.51); M^+, 320.

Analysis: calculated for $C_{16}H_{16}O_7$: C, 60.00; H, 5.04. Found: C, 60.23; H, 5.24.

(iii) Isopropylidene derivative of the major component (A): maroon crystalline solid; mp, 200 to 201°C; ^{1}H-nuclear magnetic resonance ($CDCl_3$), τ−3.07 (s, 1H); τ−2.05 (s, 1H); τ3.90 (s, 1H); τ4.55, 4.60, 5.60, and 5.65 (AB-Q, 2H); τ6.07 (s, 3H); τ6.44, 6.72, 7.23, and 7.50 (AB-Q, 2H); τ7.03 (s, 1H); τ8.37 (s, 3H); τ8.65 (s, 3H); and τ9.05 (s, 3H); M^+, 376.

Analysis: calculated for $C_{19}H_{20}O_8$: C, 60.63; H, 5.36. Found: C, 60.78; H, 5.46.

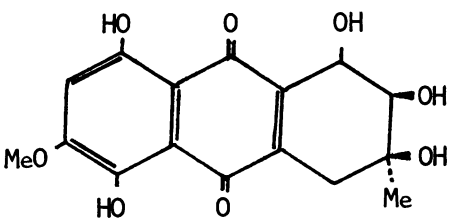
(iv) Isopropylidene derivative of the minor component (B): maroon crystalline solid; mp, 183 to 184°C; ^{1}H-nuclear magnetic resonance ($CDCl_3$), τ−3.03 (s, 1H); τ−2.63 (s, 1H); τ3.90 (s, 1H); τ5.63 (t, J = 2 Hz, 1H); τ6.07 (s, 3H); τ6.33 to 6.83 (m, 2H); τ7.30 to 8.00 (m, 2H); τ8.50 (s, 3H); τ8.63 (s, 3H); and τ8.93 (s, 3H); M^+, 360.1197. Calculated for $C_{19}H_{20}O_7$: 360.1198.

Analysis: calculated for $C_{19}H_{20}O_7$: C, 63.33; H, 5.59. Found: C, 63.20; H, 5.60.

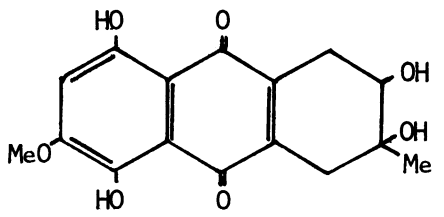
The hydrogenolysis product of component A was identical to component B by melting point, mixed melting point, thin-layer chromatographic, and spectral comparisons.

The hydrogenolysis product of the isopropylidene derivative of component A was identical to the isopropylidene derivative of component B by melting point, chromatographic, and spectral comparisons.

The analytical and spectral data of component A agreed with those described for bostrycin (Fig. 1) described by Noda et al. (6). The minor



BOSTRYCIN



4-DEOXYBOSTRYCIN

FIG. 1. Structural formulas of bostrycin and 4-deoxybostrycin (7).

component (B) showed spectral properties very similar to those of component A. The possibility of its identity with 4-deoxybostrycin (Fig. 1) (7) was considered, but the ^{1}H-nuclear magnetic resonance spectrum was difficult to match with that described in the literature because of the low solubility of the compound and the presence of several hydroxyl groups which caused peak broadening. The melting point was also not quoted in the original description. However, the experiments on the hydrogenolytic conversion of component A to component B and that of isopropylidene A to isopropylidene B, as well as the analytical and spectral data, clearly indicate that component B is identical to 4-deoxybostrycin. This compound, although described earlier as a derivative of bostrycin, has not been previously isolated from a natural source, and it was not produced as an artifact during our purification.

Phytotoxicity and antibacterial and antifungal activities. The culture fluid of *A. eichhorniae* was nonspecifically phytotoxic to plant species tested, although differences in sensitivity of the plant leaves to the culture fluid were evident (Table 1). On water hyacinth leaves, damage ratings of 2.2, 2.0, 0.3, and 0 were obtained with culture fluid concentrations of 4-, 1-, 0.1-, and 0.01-fold, respectively. The culture fluid had antibacterial activity against *B. subtilis* but no antifungal activity against *G. candidum*.

Due to poor solubility, crystalline compounds A and B and their isopropylidene derivatives were dissolved in dimethyl sulfoxide and made up in 0.025 M citrate buffer, pH 5.0; the final dimethyl sulfoxide concentration was 5% (by volume). Concentrations of 1 to 1,000 μg of purified bostrycin and its derivatives per ml were tested. Such solutions also were nonspecifically toxic to the test plant leaves at higher concentrations, although the leaves were differentially sensitive to the toxins. The sensitivities of the plant leaves to a fourfold concentration of the culture fluid and purified toxins at 250 μg/ml are compared in Table 1. The lowest toxic concentrations of the purified compounds to water hyacinth leaves were (approximately) 7 (bostrycin), 30 (4-deoxybostrycin), 7 (isopropylidenebostrycin), and 125 (isopropylidenedeoxybostrycin) μg/ml. At the lowest toxic concentrations, isopropylidenebostrycin caused a more severe necrosis on water hyacinth leaves than did bostrycin (damage ratings, 0.7 and 0.1, respectively). Purified bostrycin, 4-deoxybostrycin, and their isopropylidene derivatives also had antibacterial activity against *B. subtilis* but no antifungal activity against *G. candidum*.

DISCUSSION

Bostrycin has been previously isolated from *Bostryconema alpestre* Cesati (6), *Nigrospora oryzae* (Berk. & Br.) Petch (2), and *Arthrinium phaeospermum* (Corda) M. B. Ellis (10). Its antibacterial activity has been fully described by these authors. Because of the low solubility of these compounds in common chromatographic solvents, separation by silica gel chromatography is difficult, especially when gram quantities are involved. The alternative method, based on conversion to isopropylidene derivatives, offers a significant improvement. The isopropylidene derivatives are more stable and can be readily converted back to the original compounds by treatment with dilute ethanolic acid.

The possibility of using host-specific phytotoxins as herbicides prompted this study. Tentoxin, a cyclic tetrapeptide produced by *Alternaria tenuis* Auct., which selectively disrupts the chloroplast function in certain specific higher plant species (8, 9), has been tested as a potential herbicide (G. E. Templeton, personal communication). Although differences in sensitivity to bostrycin, 4-deoxybostrycin, and their isopropylidene derivatives were noted among the test plants (Table 1), there was no evidence that these compounds were specific. Therefore, a previous claim (4) of some degree of host specificity for a partially purified toxin from the culture fluid of *A. eichhorniae* could not be supported by our study.

Bostrycin and 4-deoxybostrycin have not been implicated previously as phytotoxins. The mimicking of the pathogenic, necrotic symptom

TABLE 1. Phytotoxicity of *A. eichhorniae* culture filtrate, bostrycin, 4-deoxybostrycin, isopropylidene-bostrycin, and isopropylidenedeoxybostrycin

Test plant	Damage rating (±SE)[a]				
	Culture filtrate (4× concn)	Bostrycin (250 μg/ml)	4-Deoxybostrycin (250 μg/ml)	Isopropylidene-bostrycin (250 μg/ml)	Isopropylidene-deoxybostrycin (250 μg/ml)
Capsicum annuum (pepper)	1.0 ± 0	2.9 ± 0.1	2.2 ± 0.4	2.9 ± 0.1	0.5 ± 0.2
Carica papaya (papaya)	0.5 ± 0	1.9 ± 0.3	1.9 ± 0.1	2.2 ± 0.4	0.7 ± 0.2
Eichhornia crassipes (water hyacinth)	2.2 ± 0.4	1.9 ± 0.1	1.2 ± 0.1	2.9 ± 0.1	1.2 ± 0.1
Hydrilla verticillata[b] (hydrilla)	0	0	0	NT[c]	NT
Lycopersicon esculentum (tomato)	0.8 ± 0.3	2.0 ± 0.4	1.3 ± 0.2	2.4 ± 0.3	0.6 ± 0.1
Morrenia odorata (milkweed vine)	2.0 ± 0	1.8 ± 0.2	1.4 ± 0.4	3.0 ± 0	0.7 ± 0.1
Nicotiana tabacum (tobacco)	NT	2.5 ± 0.2	2.9 ± 0.1	2.4 ± 0.2	1.0 ± 0.1
Phaseolus vulgaris (bush bean)	0.8 ± 0.2	2.8 ± 0.2	1.8 ± 0.3	2.8 ± 0.3	0.4 ± 0.2
Pontederia lanceolata (pickerelweed)	1.3 ± 0.3	1.2 ± 0.1	1.2 ± 0.1	1.6 ± 0.1	0.3 ± 0.1
Triticum aestivum (wheat)	2.0 ± 0	2.6 ± 0.2	3.0 ± 0	3.0 ± 0	1.1 ± 0.3
Zea mays (corn)	2.0 ± 0	0.9 ± 0.1	1.6 ± 0.1	2.7 ± 0.1	0.3 ± 0.1

[a] Controls, which rated 0, included sterilized filtered potato dextrose broth at a fourfold concentration and 5% dimethyl sulfoxide in 0.025 M citrate buffer, pH 5.0. Damage rating: 0, no damage; 1, necrosis up to 1 mm around the application spot; 2, necrosis up to 2 mm; 3, necrosis extending 3 mm and beyond. Given are the average values from 6 to 18 spots per treatment and two or three trials.

[b] Phytotoxicity was assayed with a 10-cm-long shoot of this aquatic plant incubated in the toxic fluid; detached leaves were used for other plant species.

[c] NT, Not tested.

produced by *A. eichhorniae* on water hyacinth by the purified compounds suggests a phytotoxic role for bostrycin and 4-deoxybostrycin in water hyacinth blight. Bostrycin and its derivatives may have potential as broad-spectrum herbicides.

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