Note

Production of the Phytoxic Metabolite, Ferricrocin, by the Fungus Colletotrichum gloeosporioides

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A siderophore was isolated as a non-specific phytoxic compound from a culture of Colletotrichum gloeosporioides isolated from infected blackberry. This siderophore was identified as ferricrocin by NMR, IR, MS, and CD spectra. The phytoxic activities of ferricrocin and deferrifericrocin were compared.

Since it has been reported that many phytopathogens produce phytoxins,1,2 we have been searching for fungal phytoxins which could be of potential bioherbicidal use. Colletotrichum gloeosporioides was isolated from an infected blackberry (Rubus spp.) stem from North Carolina, U.S.A. Rubus spp. is known as a host of C. gloeosporioides Penzig.3 The metabolites of phytoxins by C. gloeosporioides, whose host is the olive (Olea europaea L.), has been reported,4,5 but the production of phytoxic compounds by this fungus was not described.

C. gloeosporioides was grown on a solid medium and extracted with Me2CO. We observed that C. gloeosporioides produced non-selective phytoxic substances from results obtained by a whole-plant assay on 7 different weeds, the results being as follows: jointvetch was the most severely damaged, pigweed and Florida beggarweed were severely burned and would not grow out of the damage, and johnsongrass was stunted but not killed by the extract. The Me2CO extracts showed further phytoxic activity from a leaf-wounding assay, and an active phytoxic fraction was purified. The Me2CO extracts (221.4 g) were fractionated into the H2O phase and EtOAc phase. The H2O phase (199.5 g) was purified by HP-20, silica gel column chromatography and by HPLC, and compound 1 (111.5 mg) was isolated as a phytotoxic. Compound 1 was found to contain iron when treated with color-producing iron ammonium thiocyanate and potassium ferrocyanide indicators.

The FAB-MS data for compound 1 showed a mass peak at m/z 771 [M + H] +. An amino acid analysis of compound 1 detected ornithine, glycine, and serine in a 3:2:1 ratio. These data indicated compound 1 to be ferricrocin.

Since the iron existing in compound 1 caused severe line broadening of the NMR signals, compound 3 was derived from compound 1 by the Linhas method6 to elucidate the structure of compound 1 by NMR. 1H-NMR chemical-shift assignments of compound 3 were confirmed by 1H-1H COSY as described in the experimental section,6,8 while its 13C-NMR signal assignments were confirmed by DEPT, 13C-1H COSY and HMBC as also described.7 The six carbonyl groups of amino acids in the 13C-NMR spectrum were assigned by HMBC. The 1H- and 13C-NMR signals of three ornithines became distinguishable by COSY, HMBC, and NOESY, and the sequence of the six amino acids, three ornithines, two glycines, and serine was first examined by NOESY. It was confirmed that the six amino acids were connected in a sequence (Fig.). The ligand chirality around the metal center of compound 1 was determined by its CD spectrum, which exhibited a positive CD band at 450 nm, so that it must have had a Δ configuration.8) Compound 1 was thereby confirmed to be ferricrocin from its NMR and CD spectra.

While ferricrocin has been isolated from Neurospora, Aspergillus and Epicoccum cultures,9–11 this is the first description of the production of siderophores from Colletotrichum.

Compound 1 was tested against cotyledons, the results of this assay, shown in Table 1, giving a phytoxicity from 10−2 M to 1/64 x 10−2 M.

Table 1. Results of the Cotyledon Assay on Velvetleaf

<table>
<thead>
<tr>
<th>Concentration</th>
<th>1</th>
<th>1/2</th>
<th>1/4</th>
<th>1/8</th>
<th>1/16</th>
<th>1/32</th>
<th>1/64</th>
<th>1/128</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect on 1</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Effect: +, no symptoms; +, slight; ++, moderate; ++++, severe; ++++, death.</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

This compound was dissolved in H2O and then diluted with H2O. Concentration: 1, 10−2 M; 1/2, 1/2 x 10−2 M; 1/4, 1/4 x 10−2 M; 1/8, 1/8 x 10−2 M; 1/16, 1/16 x 10−2 M; 1/32, 1/32 x 10−2 M; 1/64, 1/64 x 10−2 M; and 1/128, 1/128 x 10−2 M.
Table II. Phytotoxicity of Compounds 1 and 2 by a Leaf-wounding Assay

<table>
<thead>
<tr>
<th>Concentration</th>
<th>1</th>
<th>1/2</th>
<th>1/4</th>
<th>1/8</th>
<th>1/16</th>
<th>1/32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect on compound 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Effect on compound 2</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Effect on control*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Effect: --, no symptoms; +, 0–5 mm zone of necrosis; ++, 6–10 mm zone; +++ , 11–15 mm zone.

Concentration: 1, $10^{-2}$ M; 1/2, $10^{-2}$ M; 1/4, $10^{-3}$ M; 1/8, $10^{-4}$ M; 1/16, $10^{-5}$ M; and 1/32, $10^{-6}$ M.

Compounds 1 and 2 were dissolved in H$_2$O and then diluted with H$_2$O.

* The control was H$_2$O.

Siderophores produced by microorganisms have been used to scavenge iron from environments suffering from iron deficiency. Siderophores have also been reported to have many biological functions, but there are no reports on their phytotoxic activity. Since the activity of compound 1 was enhanced by the removal of iron, this suggests that phytotoxic activity has some relation with chelating activity.

Experimental

Culture. Colletotrichum gloeosporioides was grown at 22°C for 7–10 days in 3-liter flasks containing a Shredded Wheat-based medium, composed of the following compounds: Shredded Wheat (Nabisco spoonsize), 100 g; sucrose, 40 g; yeast extract (Difco Laboratories), 4 g; mycelial broth (Difco Laboratories), 10 g; and distilled H$_2$O, 200 ml.

Whole plant assay. Whole plant tests were done on florida beggarweed (Desmodium tortuosum), dandelion (Taraxacum vulgarum), pigweed (Amaranthus retroflexus), johnsongrass (Sorghum halepense), tall fescue (Festuca arundinacea), velvetleaf (Abutilon theophrasti), and jointtch (Aeschynomene spp.). These plants were treated with a 2-ml solution at 1 g/ml and phytotoxicity was assessed 7 days after the treatment.

Leaf-wounding assay. Cowpea plants (2 weeks old) were grown in a greenhouse. The toxin solution to be tested was dissolved in 20% MeOH-H$_2$O, and true leaves of cowpea were injured with 2 µl of this solution. The control used was a 20% MeOH-H$_2$O solution, and phytotoxicity was assessed 5 days after the treatment.

Cotyledon assay. Velvetleaf plants (7 days old) were grown in a greenhouse, and the cotyledons were removed. The stems were trimmed, leaving 3 cm of hypocotyl below the point of cotyledon attachment plus the shoot, which included two primary leaves. The cuttings were immersed in 1-ml vials containing 750 µl of the toxic solution which had been dissolved in H$_2$O. The resulting phytotoxicity was observed 3 days after the treatment.

Compound 1 (ferrecrocin). Red amorphous substance; UV $\lambda_{max}$ (H$_2$O) nm (ε): 422 (2058); CD nm (Δε): 289 (–307), 360 (–132), 450 (+174); FAB-MS m/z: 771 [M+H]$^+$. FAB-HR-MS m/z: [M+H]$^+$. Calculated for C$_{28}$H$_{44}$N$_{8}$O$_{14}$Fe$^+$, 771.2486; found, 771.2590. IR $\nu_{max}$ (KBr) cm$^{-1}$: 3339 br, 2940, 1653, 1581, 1524.

Compound 2 (deferrferrecrocin). Crude compound 2 was derived from compound 1 (15 mg), and then purified by HPLC in a YMC C-18 AQ reverse-phase column (30% MeOH-H$_2$O) to give 12 mg of pure compound 2. FAB-MS m/z: 718.3 [M+H]$^+$. FAB-HR-MS m/z: [M+H]$^+$. Calculated for C$_{29}$H$_{46}$N$_{8}$O$_{14}$Fe$^+$, 718.3372; found, 718.3369.

Compound 3 (alamicrocin). Compound 3 (12 mg) was derived from crude compound 2, and then purified by HPLC in a YMC C-18 AQ reverse-phase column (30% MeOH-H$_2$O) to give 14 mg of pure compound 3. FAB-MS m/z: 742.3 [M+H]$^+$. Calculated for C$_{29}$H$_{44}$N$_{8}$O$_{15}$, 742.2952; found, 742.3004. 300 MHz 1H-NMR (DMSO-d$_6$) δ (ppm): 1.08 (Orn, C$_H$), 1.71 (Orn, C$_H$), 1.75 (Orn, C$_H$), 2.03, 2.06, 2.07 (COM$_3$, C$_H$), 2.80 (Orn, C$_H$), 3.41 (Gly, C$_H$), 3.50 (Ser C$_H$), 3.73 (Gly, C$_H$), 3.80 (Gly, C$_H$), 4.00 (Ser, C$_H$), 4.12 (Orn, C$_H$), 4.20 (Orn, C$_H$), 4.73 (Orn, C$_H$), 5.25 (Ser C$_O$,OH), 6.40 (Orn, NH), 6.86 (Gly, NH), 7.85 (Ser, NH), 9.08 (Gly, NH), 10.3 (Orn, NH), 11.55 (MeOH CO). FAB-HR-MS m/z: [M+H]$^+$. Calculated for C$_{30}$H$_{46}$N$_{8}$O$_{15}$Fe$^+$, 742.2952; found, 742.2954.

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References