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PYRENOLINE-A AND -B, TWO NEW PHYTOTOXINS FROM *PYRENOPHORA TERES*

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Abstract: Two novel isoquinoline fungal derivatives, pyrenoline-A and -B, were isolated from the culture fluid of *Pyrenophora teres*, a pathogen of barley, and characterized by spectroscopic and X-ray diffraction techniques.

Pyrenophora teres, the causative fungus of net blotch disease, is an important pathogen of barley (*Hordeum vulgare*). Net blotch disease occurs wherever barley is grown in temperate and humid regions of the world.[1] Due to the increasing popularity of barley, diseases such as net blotch have ever greater significance.[2] Several groups have previously isolated bioactive compounds from *P. teres*.[3,4] Smedegard-Peterson isolated aspergillomarasmine-A and -B.[5] The aspergillomarasmines were first described by Haenni and coworkers as metabolites of *Aspergillus oryzae* and *A. flavus*.[6] They have also been isolated from other plant pathogens including *Fusarium oxysporum F sp melonis*[7] and *Colletotrichum glocosporoides*.[8,9] Pyrenolide-A, a ten-membered macrolide, has also been reported from *P. teres*.[3] In the present study we report the isolation, structure characterization, and biological activity of two novel phytotoxic isoquinoline derivatives, pyrenoline-A (1) and -B (2).

Pyrenophora teres F sp teres, isolated from infected barley plants, was provided by A. Scharen of Montana State University. The fungus was maintained on tomato juice/potato dextrose agar plates. A highly virulent isolate (Pt WPb) was used to inoculate 2 L of a modified M-1-D[10] medium in a 4 L Erlenmeyer flask. Culture flasks were typically shaken at 200 rpm at 26-28 °C for 2-3 weeks under fluorescent light. A leaf spot assay,[11] which consists of puncturing a detached leaf and overlaying the wound with 5-10 µl of test solution, was used to guide the isolation of bioactive compounds. Treated leaves were kept in a moist chamber for 42-72 hr, and the appearance and size of lesions used to measure activity. No activity was observed in extracts from uninoculated culture medium.

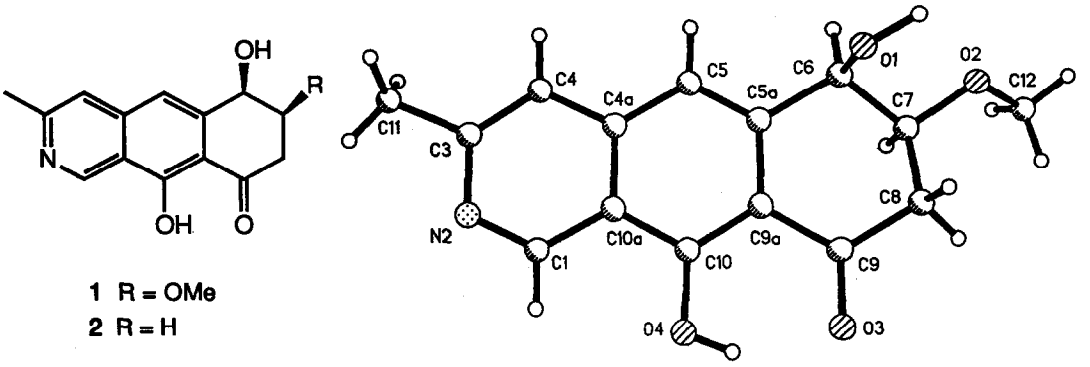

Figure 1. Computer generated perspective drawing of 1 indicating relative stereochemistry only.

One liter of the *P. teres* culture was filtered through cheese cloth and the aqueous filtrate extracted three times with 300 mL of ethyl acetate. The ethyl acetate extracts were combined, washed once with brine, and then dried over sodium sulfate. Evaporation of the ethyl acetate under vacuum yielded 52 mg of residue. This material was subjected to preparative thin layer chromatography (TLC) on a 20 x 20 cm silica plate (500 μm) with 9:2 chloroform/methanol (v/v) as the eluant. A broad band centered at R_f 0.57 yielded 7.2 mg of yellow residue. The yellow residue was again subjected to preparative TLC on a 20 x 20 cm silica plate (250 μm) with 9:2 chloroform/methanol. A band at R_f 0.59 yielded 1.4 mg of pyrenoline-A, while a band at R_f 0.53 yielded 1.0 mg of pyrenoline-B. Both pyrenoline-A and -B are readily visualized on TLC plates under UV light as bright blue bands or spots.

The high resolution mass spectrum of pyrenoline-A[12] indicated the molecular formula $C_{15}H_{15}NO_4$. The ^{13}C NMR spectrum showed the presence of nine aromatic carbons, which in conjunction with the presence of nitrogen, suggested a quinoline or isoquinoline aromatic nucleus. In the ^1H NMR spectrum there are only four proton signals which display any proton-proton coupling. Two geminally coupled protons (CH_2-8) at δ 2.86 (dd J = 3.3, 17.8 Hz) and 3.17 (dd J = 5.8, 17.8 Hz), were each coupled to a methine multiplet at δ 3.99 (H-7). This methine proton is also coupled, by less than 1 Hz, to a one proton broad singlet at δ 4.97 (H-6).

The high resolution mass spectrum of pyrenoline-B[13] indicated the molecular formula $C_{14}H_{13}NO_3$, which suggested pyrenoline-B lacked a methoxy group relative to pyrenoline-A. This was confirmed by the ^1H NMR spectrum of pyrenoline-B which showed no methoxy signal in the vicinity of δ 3.35, as was observed in the spectrum of pyrenoline-A. In place of the missing methoxy, and its adjacent methine proton, are signals for two additional methylene protons (CH_2-7) at δ 2.34 (m) and 2.24 (m). These new methylene protons both show coupling to the geminally coupled methylene protons (CH_2-8) at δ 2.71 (ddd J = 4.9, 8.3, 18.1 Hz) and 3.04 (ddd J = 4.9, 7.9, 18.1 Hz), and also to the hydroxymethine proton at δ 4.99 (H-6). Pyrenoline-B thus lacks the C-7 methoxy present in A. Pyrenolines A and B were otherwise spectroscopically identical. The small

yields of **1** and **2** discouraged complete structure elucidation by chemical or spectroscopic methods prompting us to pursue crystallization of **1** or **2** for X-ray analysis.

Crystals of pyrenoline-A were obtained by dissolution in methanol/dichloromethane, and allowing benzene to vapor diffuse into the solution. The crystals belonged to space group $P2_1$ (Z = 2) with $a = 5.145(6)$, $b = 19.267(3)$, $c = 6.626(8)$ Å, and $\beta = 104.65°$. All unique intensity data with $2\theta \leq 110°$ were collected using CuKα radiation. This gave 824 observed reflections. The structure was solved uneventfully using direct methods and refined to a conventional crystallographic residue of 3.84%.[14] A drawing of the final X-ray model, indicating relative stereochemistry only, is shown in Figure 1. The structure of pyrenoline-B was deduced by comparison of its spectral data to those of pyrenoline-A.

Pyrenolines A and B represent a new class of bioactive metabolites bearing no resemblance to other *P. teres* metabolites such as pyrenolide-A and the aspergillomarasamines. Pyrenoline-A was assayed on both monocots and dicots and showed no host specificity (Table 1). Pyrenoline-B is also active on different plant species, but at higher concentrations than A. Interestingly, *Hibiscus sabdariffa* showed no response to **1**, but is clearly the most susceptible species towards **2** (Table 2). Pyrenolines A and B may therefore be good candidates for a structure/activity relationship investigation.

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12. Pyrenoline-A (**1**): Orange crystals, mp 80° dec;, UV (MeOH) λ_{max} (ε) 246 nm (8300), 250 8200), 356 (5800); IR ($CHCl_3$) 3220 br, 2920, 1630, 1100 cm^{-1}; $[\alpha]_D$ = - 33° (MeOH, c = 0.0016); ^1H NMR ($CDCl_3$, 400 MHz) δ 14.02 (1 H, s), 9.61 (1 H, s), 7.37 (1 H, s), 7.36 (1 H, s), 4.97 (1 H, br s), 3.99 (1 H, m), 3.45 (3 H, s), 3.17 (1 H, dd, J = 5.8, 17.8 Hz), 2.86 (1 H, dd, J = 3.3, 17.8 Hz), 1.26 (3 H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 200.8 s, 163.9 s, 157.0 s, 149.1 d, 142.0 s, 141.1 s, 118.4 d, 117.9 s, 115.2 d, 110.3 s, 78.3 d, 69.4 d, 57.1 q, 39.1 t, 24.6 q; HRMS (EI) M/Z 273.1004 daltons; LRMS M/Z (rel. int.) 274 (16), 273 (93), 255 (3), 241 (13), 239 (20), 215 (42), 188 (12), 187 (100), 186 (33), 158 (11).
13. Pyrenoline-B (**2**): light orange oil; UV (MeOH) λ_{max} (ε) 244 nm (8300), 270 (7300), 350 (7400); IR ($CHCl_3$) 3300 br, 2920, 1630, 1090 cm^{-1}; $[\alpha]_D$ = - 22° (MeOH, c = 0.0014); ^1H NMR ($CDCl_3$, 400 MHz) δ 14.29 (1 H, br s), 9.61 (1 H, s), 7.36 (1 H, s), 7.22 (1 H, s), 4.99 (1 H, m), 3.04 (1 H, ddd, J = 4.9, 7.9, 18.1 Hz), 2.71 (1 H, ddd, J = 4.9, 8.3, 8.1 Hz), 2.69 (3

H, s), 2.34 (1 H, m), 2.24 (1 H, m); HRMS (EI) M/Z 243.0905 daltons; LRMS M/Z (rel. int.) 244 (16), 243 (100), 226 (8), 225 (19), 214 (8), 197 (9), 196 (6), 158 (4).
14. Cambridge ref. Archival crystallographic data have been deposited with the Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK. Please give a complete literature citation when ordering.

Table 1. Effects of Pyrenoline A on different barley cultivars (*Hordeum vulgare)* and other plant species, using the leaf puncture assay. Toxin solutions were applied in drops on 5µl on the wounded leaf, and observations were made after 42 hrs.

	Concentration in mM			
Plant Species	8	4	0.4	0.1
Hordeum vulgare cv Compana	+	+	+/-	-
Gallatin	+	+	+	-
Clark	+	+	+/-	-
Wabet	+	+	+/-	-
Dactylon cynodon	++	+	+	+/-
Fescuta spp	+	+	-	-
Hibiscus sabdariffa	-	-	-	-
Agropyron repens	+	+	+/-	-

- = no symptoms at all; +/- = <1 mm lesion; + = 1-2.5 mm lesion; ++ = 2.5-5 mm lesion; +++ = 5-10 mm lesion. A 5% ethanol solution served as a control and did not elicit any symptoms.

Table 2. Effects of Pyrenoline B on different plant species, using the leaf puncture assay. Toxin solution was applied in drops of 5µl on the wounded leaf, and observations were made 42 hrs after treatment.

	Concentration in mM			
Plant Species	8	4	0.4	0.1
Hordeum vulgare cv Compana	+	+	-	-
Avena sativa	+	+	-	-
Esculenta esculentum	++	+	-	-
Euphorbia heterophila	+	+	-	-
Hibiscus sabdariffa	+++	+++	++	+

- = no symptoms at all; +/- = <1 mm lesion; + = 1-2.5 mm lesion; ++ = 2.5-5 mm lesion; +++ = 5-10 mm lesion. A 5% ethanol solution served as a control and did not elicit any symptoms.

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