Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 8291–8294, December 1985
Chemistry

Bipolaroxin, a selective phytotoxin produced by *Bipolaris cynodontis*

(fungal metabolites/host selectivity/weed pathogen/sesquiterpene)

F. SUGAWARA*†, G. STROBEL*‡, L. E. FISHER§, G. D. VAN DUYNE§, AND J. CLARDY§

*Department of Plant Pathology, Montana State University, Bozeman, MT 59717; and §Department of Chemistry–Baker Laboratory, Cornell University, Ithaca, NY 14853-1301

Communicated by Jerrold Meinwald, August 16, 1985

ABSTRACT **Two sesquiterpenes have been isolated from the fungal pathogen of Bermuda grass *Bipolaris cynodontis*. Chemical, spectral, and x-ray diffraction studies have led to the characterization of these as bipolaroxin and dihydrobipolaroxin, highly oxygenated members of the eremophilane family. Bipolaroxin is phytotoxic to some but not all of the plants tested. To our knowledge, a phytotoxin with host selectivity isolated from a weed pathogen has not been reported previously.**

Though most well-described phytopathogenic microorganisms are parasitic on crop plants, weeds are also attacked by various fungi, bacteria, and viruses. The use of pathogenic microbes for the biological control of economically important weeds is of considerable current interest. One possible approach would be the direct application of the pathogen's phytotoxin(s) or derivatives to the noxious plant (1). Since this control method would take advantage of the presumed selectivity of the phytotoxin(s), demonstrating such selectivity is a crucial issue. Bermuda grass [*Cynodon dactylon* (L.) Pers.] is a notorious weed¶ in the grass family (2). More than 80 countries have reported it as a weed problem in at least 40 different crops. It is also listed as an important contributor to the "hay fever" problem (3). The fungus *Bipolaris cynodontis* is a natural pathogen on Bermuda grass and produces leaf blight. The zonate lesions and spots resulting from *B. cynodontis* infection suggest that a phytotoxin is involved in the disease symptomatology. We report here the isolation, characterization, and remarkable selectivity of bipolaroxin (**1**), a phytotoxin from the pathogenic fungus *B. cynodontis*.

OBSERVATIONS AND DISCUSSION

B. cynodontis was maintained on potato dextrose/agar plates containing V-8 juice (18% by volume). The fungus used for toxin production was grown at 26°C in 2-liter Erlenmeyer flasks containing 1 liter of modified M-1-D medium (4). The medium was adjusted to pH 5.5 with 0.1 M HCl, sterilized by autoclaving, and then inoculatd by mycelium from a culture plate. It was shaken at 200 rpm at 26°C for 2–3 weeks under luminescence in an incubator. Most plants used in these experiments were grown in a greenhouse maintained on the Montana State University campus. The plants used were 2–4 weeks old after germination. Seeds were planted in plastic pots containing Batco potting soil (Michigan Peat Co.) and grown in an environmentally controlled greenhouse at 22–24°C. The following plant assay was used to guide the isolation of the suspected phytotoxin. A droplet (1–5 μl) of a 2% ethanol solution was placed on a leaf blade. Usually the solution was placed over a puncture wound to enhance access to the leaf tissue. The leaves were placed on moist filter paper in a sealed Petri dish at 27–29°C for 48 hr under a "sun" light (25 microeinsteins $M^{-2}\cdot sec^{-1}$). The effects depended on the plant species and varied from no symptoms, to a slight chlorosis, to a marked reddish-brown lesion with an accompanying streak or runner extending vertically in both directions from the point of application (Fig. 1). In no cases were symptoms observed with 2% ethanol control solutions.

Bipolaroxin. Bipolaroxin was isolated from the broth of 3-week-old cultures of *B. cynodontis* grown in shake culture (200 rpm, 26°C, 2-liter flasks) on a modified M-1-D medium (4). The fungal mycelium was removed from the culture broth by centrifugation for 15 min at 7000 rpm. The supernatant liquid was concentrated to half volume with a rotary evaporator at 40°C, and then it was extracted with ethyl acetate (three times, 200 ml each). All of the phytotoxic activity was in the organic fraction. The organic fraction was washed with water and evaporated under reduced pressure to give an oily residue (≈0.2 g). The residue was further purified, guided by plant assays, by using flash chromatography (5) on Merck silica gel 60 (230–400 mesh) packed and eluted with chloroform/methanol, 9:1 (vol/vol). This was followed by preparative TLC on Merck silica gel (60 F-254). Bipolaroxin had R_f values of 0.49 in chloroform methanol, 9:1 (vol/vol) and 0.28 in toluene/ethyl acetate, 1:1 (vol/vol). It could be visualized on TLC plates with the following reagents: anisaldehyde/sulfuric acid/phosphomolybdic acid in methanol, antimony trichloride solution, and ferric chloride followed by heating. It was also visible by its absorbance at 254 nm. The next stage of the isolation involved gel-permeation chromatography on Pharmacia LH-20 (65 g) using methanol elution and HPLC using Merck Lichrosorb RP-18 (10 mm × 250 mm), UV (280 nm) detector, a flow rate of 5.0 ml/min, and acetonitrile/water, 65:35 (vol/vol). Analytical HPLC used Merck Lichrosorb RP-18 (4.6 mm × 250 mm), UV (280 nm) detector, a flow rate of 0.5 ml/min, and two different solvent systems. The first (solvent system A) was acetonitrile/water, 65:35 (vol/vol), and the second (solvent system B) was methanol/water, 80:20 (vol/vol). Bipolaroxin had retention times (R_t) of 5.3 min in the first system and 5.6 min in the second. The yield of pure bipolaroxin was 1–2 min/liter of culture broth. The bipolaroxin isolated in this fashion could be crystallized from ethanol (mp, 163–165°C).

Bipolaroxin was characterized by a single crystal x-ray diffraction analysis. Crystals formed in the orthorhombic space group $P2_12_12_1$ with $a = 7.628(1)$, $b = 9.710(2)$, and $c = 18.556(3)$ Å. All unique diffraction maxima with $2\theta \leq 114°$ were collected on a computer-controlled four-circle diffractometer by using variable-speed 1° ω-scans and graphite monochromated CuK$\bar{\alpha}$ radiation (1.54178 Å). Of the 1073

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†Present address: Laboratory of Herbicide and Plant Growth Regulators, RIKEN, Wako-shi, Saitama 351, Japan.
‡To whom reprint requests should be addressed.

¶Distinguishing a plant as a weed reflects a judgment about what should be growing in a given area. Some cultivars of Bermuda grass are used to prevent soil erosion, whereas others make excellent lawns and playing fields.

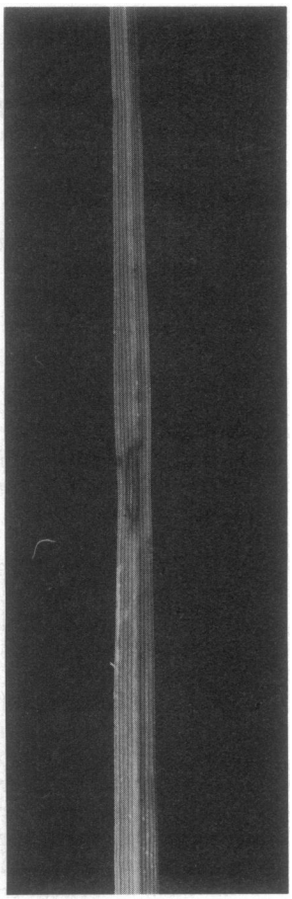


FIG. 1. Effects of bipolaroxin on a leaf of Bermuda grass 48 hr after the application of a 5-μl droplet containing 0.38 mM bipolaroxin to a puncture wound. Note the zonate lesion around the point of application and the flecking above and below this point. A leaf, serving as a control with 5 μl of a 2% ethanol solution, showed no observable effects.

reflections measured, 936 (87%) were judged observed [$F_o \geq 3\sigma(F_o)$] after correction for Lorentz, polarization, and background effects.[‖] A phasing model, which revealed all of the nonhydrogen atoms, was found by using a multisolution weighted tangent formula approach. Block diagonal least-squares refinements employing anisotropic nonhydrogen atoms and isotropic hydrogens converged to a standard crystallographic residual of 0.062.[**] The result of the x-ray analysis is given in Fig. 2 and a conventional structural

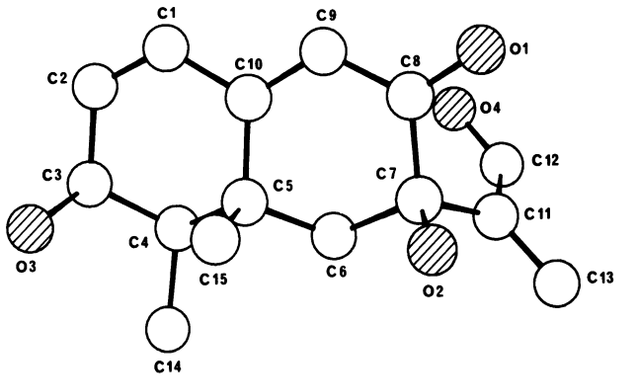
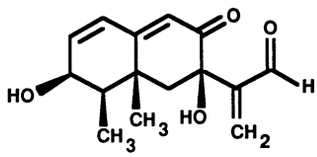


FIG. 2. A computer-generated perspective drawing of the x-ray model of bipolaroxin (**1**). Hydrogens are omitted for clarity, and oxygen atoms are designated by a series of parallel lines.

drawing is shown as **1**. It should be noted that this experiment did not define the absolute configuration of bipolaroxin. Furthermore, chiroptical data {$[\alpha]_D^{22}$ +310° (c = 0.24, $CHCl_3$)} did not allow the unambiguous assignment of an absolute stereochemistry. The enantiomer shown as **1** was arbitrarily drawn to have the same absolute stereochemistry as other fungal eremophilanes (ref. 6 and references therein).



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Spectroscopic data for bipolaroxin (**1**) were consistent with the structure derived from the x-ray analysis. The high-resolution mass spectrum gave an m/z of 262.1205 (calc. for $C_{15}H_{18}O_4$, 262.1205). The UV spectrum (EtOH) displayed maxima [λ_{max} nm (ϵ)] at 216 (3600) and 279 (9300). These peaks disappeared on hydrogenation,[††] confirming the presence of a conjugated dienone (8). The ^1H NMR spectrum (250 MHz, $CDCl_3$) showed 18 protons, including a methyl group at δ 1.48 (s); a methyl at 1.13 (d), which was coupled to a methine proton at 1.80 (dq); one hydroxylated methine proton at 4.17 (dd); and five olefinic protons: 5.98 (1H, s), 6.84 (1H, s), and a three-proton pattern between 6.31 and 6.34, which was not first-order (s = singlet; d = doublet; dd = double doublet; dq = double quartet; t = triplet; q = quartet; m = multiplet). Table 1 summarizes the complete ^1H NMR spectrum. The ^{13}C NMR spectrum (62.9 MHz, d_6-acetone) revealed 15 carbons: two carbonyl carbons at δ 195.7 (s) and 193.6 (d); six sp^2 carbons at 162.9 (s), 156.0 (s), 138.5 (d), 135.5 (t), 128.5 (d), and 123.4 (d); two oxygen substituted carbons at 75.2 (s) and 67.4 (d); three sp^3 carbons at 46.0 (t), 43.1 (d), and 36.9 (s); and two methyls at 23.0 (q) and 10.5 (q).

Dihydrobipolaroxin. A second component of the extract was produced in smaller quantities and was chromatographically quite distinct from bipolaroxin. It was isolated following the same procedure given for bipolaroxin, and the yield was 0.3–0.6 mg/liter of culture broth. In the TLC systems employed dihydrobipolaroxin had R_f values of 0.37 in chloroform/methanol, 9:1 (vol/vol), and 0.14 in toluene/ethyl acetate, 1:1 (vol/vol). On HPLC dihydrobipolaroxin had R_t

[‖]All crystallographic calculations were done on a PRIME 9950 computer operated by the Cornell Chemistry Computing Facility. Principal programs employed were REDUCE and UNIQUE, data reduction programs by M. E. Leonowicz, Cornell University, 1978; MULTAN 78, MULTAN 80, and RANTAN 80, systems of computer programs for the automatic solution of crystal structures from x-ray diffraction data (locally modified to perform all Fourier calculations including Patterson syntheses) written by P. Main, S. E. Hull, L. Lessinger, G. Germain, J. P. Declercq, and M. M. Woolfson, University of York, England, 1978 and 1980; BLS78A, an anisotropic block diagonal least-squares refinement written by K. Hirotsu and E. Arnold, Cornell University, 1980; PLUTO78, a crystallographic illustration program by W. D. S. Motherwell, Cambridge Crystallographic Data Centre, 1978; and BOND, a program to calculate molecular parameters and prepare tables written by K. Hirotsu, Cornell University, 1978.

[**]Crystallographic parameters have been deposited with the Cambridge Crystallographic Data File, University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW, England, and are available from them. Please give a complete literature citation when ordering.

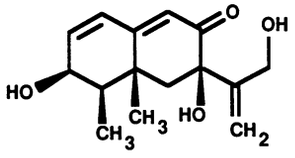
[††]Hydrogenation was carried out on 10% Pd-C in ethanol for 2 hr at room temperature. See ref. 7 for a similar case.

Table 1. ^{1}H NMR data for bipolaroxin and dihydrobipolaroxin

	Bipolaroxin (1)	Dihydrobipolaroxin (2)
H1	5.99 s	5.93 s
H2	6.33 d, J = 4.8	6.35 d, J = 3.5
H3	4.17 dd, J = 4.8, 4.8	4.18 m
H4	1.79 dq, J = 4.8, 7.2	1.77 dq, J = 3.5, 7.2
H6a	2.09 d, J = 14.1	2.01 d, J = 14.4
H6b	1.96 d, J = 14.1	1.95 d, J = 14.4
H9	6.31 s	6.40 s
H12	9.52 s	—
H12a	—	4.24 d, J = 13.3
H12b	—	4.13 d, J = 13.3
H13a	6.89 s	5.30 s
H13b	6.34 s	5.29
H14	1.48 s	1.47 s
H15	1.13 d, J = 7.2	1.18 d, J = 7.2

Values given are chemical shift (δ), multiplicity, and coupling (Hz).

values of 5.3 min with system A and 5.6 min with system B described above. The HRMS gave an m/z of 264.1362 (calc. for $C_{15}H_{20}O_4$, 264.1361), and there was a single UV absorption at 281 nm. The ^{1}H NMR spectrum was quite similar to that of bipolaroxin except for the aldehydic proton signal, which had been replaced by an AB quartet at δ 4.13 and 4.24, and exomethylene protons, which had shifted to δ 5.28 (s) and 5.30 (s). These data strongly suggested that the aldehyde had been reduced to an alcohol. On this basis, dihydrobipolaroxin is most plausibly formulated as structure **2**.



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Plant Assays. Bipolaroxin (**1**) displayed excellent levels of phytotoxicity and selectivity in the assay system, as is illustrated by the reaction of various crop and weed species given in Table 2. At an application level of 3.8 mM, it produced symptoms on 37 different plant species. The susceptible plants were 2–4 weeks old and included monocots and dicots. It produced no symptoms on 8 other species. It is interesting to note that those species that are most sensitive to bipolaroxin are also the normal hosts of *B. cynodontis*—i.e., goosegrass, Johnson grass, and Bermuda grass. Nevertheless, a number of other weedy and crop species are also sensitive to the toxin and a number are insensitive (Table 2). At an application level of 0.38 mM, it caused no symptoms on wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), cotton (*Gossypium hirsutum*), and corn (*Zea mays*). However, Bermuda grass and Johnson grass (*Sorghum halepense*) gave a reaction at a concentration of 0.038 mM. With sensitive species, applications to a leaf wound was not essential. When bipolaroxin was placed directly onto the leaf surface, characteristic symptoms were produced on Bermuda grass and Johnson grass at 0.38 mM. The potential practical significance of this observation is that both of these plants are serious weeds in sugarcane (*Saccharum* spp.) in many parts of the world and that the two clones of sugarcane tested showed no observable symptoms at 3.8 mM bipolaroxin. This is only one example of several crop–weed combinations that should lend itself to more comprehensive greenhouse and field studies. Eremophilanes have been reported recently as phytotoxins from fungi attacking economically important crops (9, 10), but there has been no indication that they display host selectivity. To our knowledge, a host-selective phytotoxin from a weed pathogen has not been reported previously.

There were a few additional observations that gave some clues as to the mode of action of bipolaroxin. The first was that increased light intensities lead to a more rapid onset of symptoms. Thus, toxin-sensitive plants treated with 3.8 mM bipolaroxin under higher light intensities (150 microeinsteins $M^{-2}\cdot sec^{-1}$, six times normal) develop symptoms within 2–4 hr rather than 48 hr. The final toxicity and selectivity of bipolaroxin were not influenced by light intensity, except for wheat and barley, in which the toxin threshold level was decreased by one order of magnitude.

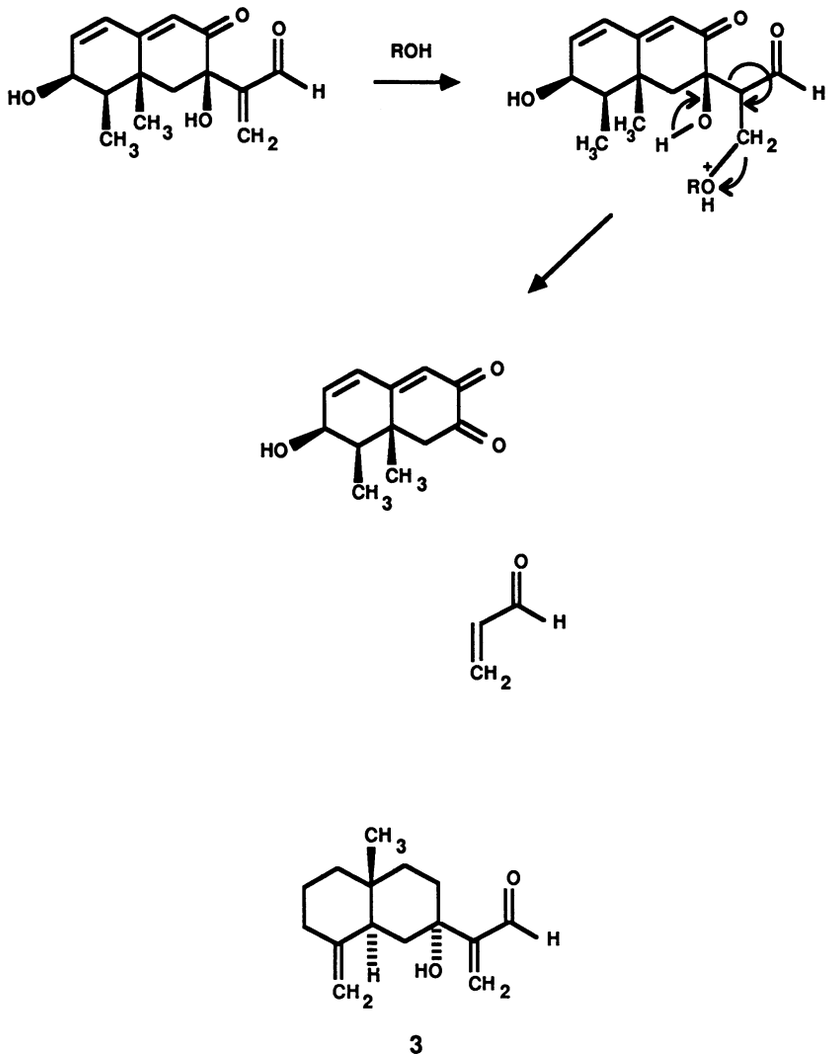
The second observation is that dihydrobipolaroxin showed no activity at 3.8 mM against Bermuda grass, goosegrass, wheat, or barley. The lack of phytotoxicity indicates that the C12 aldehyde is essential for activity. A plausible mechanism for this has been suggested by Schneider and Nakanishi (11) in their study of the phytoalexin 7-hydroxycostal (**3**, in Scheme 1), which also has a carbon bearing a hydroxy group and an enal moiety. They propose that a nucleophile adds in a conjugate manner to the enal, followed by a 1,3-diol cleavage as shown in Scheme 1. The consequence of this reaction would be the liberation of acrolein, a known plant toxin, and an α-diketone, which could also be a phytotoxin. A similar reaction could not take place with dihydrobipolaroxin because the initial conjugate addition is unlikely.

Phytotoxins from weed pathogens are a poorly explored source of selective herbicides. Their potential value lies not only in their direct application but also in the chemical

Table 2. Reaction of some crop and weed plants to bipolaroxin (**1**)

	Bipolaroxin, mM					
Name	3.8 (5.0)	0.76 (1.0)	0.38 (0.5)	0.076 (0.1)	0.038 (0.05)	Control (0)
Zea mays (corn)	+	+				–
Helianthus annus (sunflower)	–					–
Saccharam spp. (sugarcane)						
51NG97	+	+	+			–
H50-7209	+					–
Cynodon dactylon (Bermuda grass)	+	+	+	+	+	–
Sorghum halepense (Johnson grass)	+	+	+	+	+	–
Eleucine indica (goosegrass)	+	+	+			–
Festuca sp. (fescue)	+					–
Avena fatua (wild oats)	+	+				–
Amaranthus arverse (pigweed)	–					–

Values in parentheses represent μg/5 μl.



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SCHEME 1. Proposed generation of acrolein from bipolaroxin (1). ROH is a general nucleophile.

information that they provide. Rational modifications of the original structure could illuminate the relations between molecular structure, biological activity, and host specificity. Bipolaroxin (1) with its readily modified functional groups, herbicidal activity, and host selectivity is an excellent candidate for such investigations.

We thank Dr. E. L. Luttrell of the Department of Plant Pathology, University of Georgia, for supplying us with a culture of *B. cynodontis*. We thank Dr. C. Woodward and Dr. W. Gallagher, Department of Biochemistry, University of Minnesota, St. Paul, for gathering the CD data. Technical support was provided by L. Harrison, J. Sears, and K. Manfredy. The work at Bozeman was funded by grants from Sandoz Co. and the Montana Agricultural Experiment Station. The work at Cornell was partially supported by National Institutes of Health Grant CA24487.

1. Robeson, D., Strobel, G., Matsumoto, G. K., Fisher, L. E., Chen, M. H. M. & Clardy, J. (1984) *Experientia* **40**, 1248–1250.
2. Holm, L. G., Pluneknett, D. L., Pancho, J. V. & Herberger, J. P. (1977) in *The World's Worst Weeds, Biology and Distribution* (University Press of Hawaii, Honolulu), pp. 25–31.
3. Stier, H. (1983) in *Plants of Allergenic Importance* (Miles Laboratories, Spokane, WA), pp. 9–10.
4. Pinkerton, F. & Strobel, G. A. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4007–4011.
5. Still, W. C., Kahn, M. & Mitra, A. (1978) *J. Org. Chem.* **43**, 2923–2925.
6. Tanaka, S., Wada, K., Maruno, S. & Hattori, H. (1984) *Tetrahedron Lett.* **25**, 5907–5910.
7. Tachibana, K., Sakaitani, M. & Nakanishi, K. (1984) *Science* **226**, 703–705.
8. Silverstein, R. M., Bassler, G. C. & Morrill, T. C. (1981) *Spectrometric Identification of Organic Compounds* (Wiley, New York), pp. 316–317.
9. Bottalio, A., Frisullo, S., Lerario, P., Randazzo, G. & Capasso, R. (1982) *Phytopathol. Mediterr.* **21**, 39–40.
10. Dahr, T. K., Siddiqui, K. A. I. & Ali, E. (1982) *Tetrahedron Lett.* **23**, 5459–5462.
11. Schneider, J. A. & Nakanishi, K. (1983) *J. Chem. Soc. Chem. Commun.*, 353–355.