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HERBICIDAL EFFECTS OF GELDANAMYCIN AND NIGERICIN, ANTIBIOTICS FROM *STREPTOMYCES HYGROSCOPICUS*[1]

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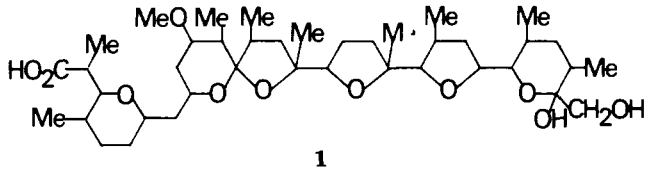
ABSTRACT.—Geldanamycin (**2**) and nigericin (**1**) (primarily the Na^+ salt) are responsible for the phytotoxicity of a *Streptomyces hygroscopicus* strain found to be highly active in our screens for producers of herbicidal antibiotics. After extraction from the cells, the compounds were purified with column and thin layer layer chromatography on silica gel, bioassayed for inhibition of garden cress (*Lepidium sativum* L.) radicle elongation, and identified with ms, ir, nmr, and cochromatography with authentic standards. Both caused 50% reduction in garden cress radicle growth at concentrations of 1-2 ppm and nearly complete inhibitions at 3-4 ppm. Symptoms of toxicity differed markedly for the two compounds. Geldanamycin at high concentrations caused radicles to turn brown and disintegrate, whereas, nigericin did not cause visible necrosis. Geldanamycin is structurally similar to the herbimycins, which are also produced by *S. hygroscopicus* and have been reported to have herbicidal activity. The phytotoxicity of geldanamycin and nigericin has stimulated investigation of their potential for use as natural product herbicides.

Streptomyces strains isolated from soil produce many secondary metabolites with potent biological activity. Some of these compounds, such as anisomycin and toyocamycin (1), bialaphos (2, 3), cycloheximide (2), glufosinate (4), herbicidins A and B (5, 6), and herbimycins A and B (7, 8) are phytotoxic. Their discovery suggests the possibility of using microbial culture to produce new herbicides (9, 10). As a result, screening programs are in progress to test microorganisms for production of herbicidal compounds (2, 4).

This paper reports the isolation, identification, and herbicidal activity of geldanamycin (**2**) and nigericin (**1**) produced by a wild strain of *Streptomyces hygroscopicus*. To the best of our knowledge, herbicidal activity on seeds and intact plants has not previously been reported for these compounds. The producer organism, designated "V9" in an earlier publication (11), was discovered in our screening program for microorganisms that produce herbicidal compounds. It caused striking inhibition of seed germination and seedling growth.

Geldanamycin (**2**) (mw 560) was first reported in 1970 as the product of *S. hygro-*



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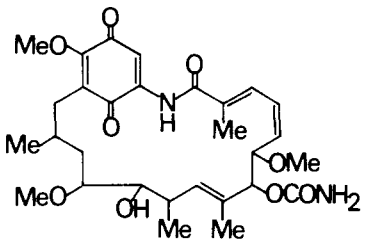

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scopicus var. *geldanus* (12, 13). It is a brilliant yellow, ansamycin antibiotic and is structurally identical to herbimycin B except for the presence of a methoxyl group on carbon 17 (8, 14). The herbimycins, also ansamycin compounds produced by a strain of *S. hygroscopicus*, have strong herbicidal activity against certain monocots and dicots (7, 8). Geldanamycin is moderately active against protozoa, fungi, and bacteria; strongly active against tumor cells; and is a potent inhibitor of DNA synthesis (12, 15). Its acute oral toxicity to rats is 2500-5000 mg/kg, whereas the intraperitoneal LD_{50} for mice is approximately 1 mg/kg (12).

Nigericin (**1**) (mw 725), a polyether antibiotic, was first reported in 1949 (16) and was later found to be identical to polyetherin A produced by *S. hygroscopicus* (17-19). It is an ionophore, influencing the transport of alkali cations, particularly K^+, across mitochondrial, erthyrocyte, chloroplast, microsomal, and artificial membranes (20-24). It also impedes photophosphorylation (20). Nigericin inhibits gram-positive bacteria, mycobacteria, and certain phytopathogenic fungi (17, 25). It is patented as a cattle feed additive to enhance efficiency of feed conversion (26, 27). The intraperitoneal LD_{50} for mice ranges from 2.5 to 10-15 mg/kg (17, 25).

MATERIALS AND METHODS

ISOLATION AND CULTURE.—The producer organism was isolated from Edgemont very stony loam (organic layer) collected at the base of a hackberry (*Celtis occidentalis* L.) tree in Lancaster Co., Pennsylvania, as previously described (11). It is deposited in our culture collection at the Pesticide Research Center, Michigan State University. Liquid cultures of the isolates were grown in half-strength A-9 medium (28)(2.5 g peptone, 5 g glucose, 10 g "Brer Rabbit" green-label molasses, 250 ml tap H_2O, 750 ml distilled H_2O, pH adjusted with KOH to 6.9-7.1 before autoclaving). Seed cultures were started from colonies growing on solid medium (containing 15 g agar/liter) and were transferred two or three times to fresh medium (5% v:v) at 2-day intervals. Cultures for toxin production were grown in baffled, 2-liter Erlenmeyer flasks containing 0.5 liters of medium inoculated with 25 ml of seed culture. Liquid cultures were grown on an orbital shaker at 100-120 rpm and 28°.

TIME-COURSE STUDY.—This investigation determined how long to incubate cultures to achieve maximum toxicity. Broth and cells were aseptically sampled from four culture flasks (8 ml/flask) at 1- or 2-day intervals for 15 days and immediately frozen. At termination of the experiment, the samples were thawed and centrifuged (15 min, 14500×*g*), and pH was measured. Dilutions containing 11, 22, 33, and 83 μl of supernatant broth per 1.5 ml were prepared with distilled H_2O. Non-inoculated broth was used to standardize the total broth content of all dilutions at 0.15 ml per 1.5 ml of solution.

BIOASSAYS.—Biological activity was followed with assays of radicle growth of garden cress (*Lepidium sativum* L.) seeds (11, 29). Bioassays were used to determine the dose per petri dish causing 50% reduction of radicle growth (ID_{50}). Samples to be tested were applied to filter paper disks as broth or in an organic solvent. When samples were applied in organic solvent, the solvent was removed by air-drying. The paper disks were placed in 1.5×6 cm plastic petri dishes and moistened with 1.5 ml of distilled H_2O or, in tests of culture broth, a mixture of broth and H_2O. Controls were identical to treatments except they received distilled H_2O instead of broth or pure solvent instead of extracts. Distilled H_2O was used in controls instead of noninoculated culture broth because the broth itself had an effect on radicle growth (e.g., Figure 2). Treatments and controls consisted of three or four replicate dishes containing ten cress seeds each. Bioassays were incubated in the dark for 69-75 h at 28°. Radicle length of the seeds was then measured, non-germinating seeds being assigned a value of zero. Treatment means were expressed as a percentage of control radicle length.

EXTRACTION OF TOXINS.—Microbial cells were centrifuged (15 min, 19600×*g*) from 7 liters of the *S. hygroscopicus* broth after 10 days of culture. The supernatant was filtered (Whatman 1), and a sample was frozen for later bioassay. The remainder was partitioned twice with half-volumes of CH_2Cl_2. The resulting broth extract was brownish-yellow after evaporation of the solvent. A sample of the CH_2Cl_2-extracted broth was placed on a rotary evaporator (10 min, ≤30°) to remove residual solvent and was frozen for later bioassay. The microbial cells (75 g wet wt) were frozen, thawed, suspended in 40 ml distilled H_2O, and extracted six times in a separatory funnel with 500 ml of CH_2Cl_2 each time. The resulting cell extract was golden-yellow after evaporation of the solvent.

ISOLATION AND IDENTIFICATION OF TOXINS.—A sample of cell extract was dissolved in 10%

Me_2CO/hexane and loaded onto silica gel "Sep-Paks" (Waters Associates). Eleven fractions were sequentially eluted with 10-ml volumes of MeCN/CH_2Cl_2 increasing from 10 to 100% MeCN in 10% steps, followed by 10 ml of MeOH. The fractions were compared in tlc by spraying the developed plates with 5% vanillin/H_2SO_4 (30) to visualize compounds. Several fractions were combined on the basis of similar appearance. Fraction 7, eluted with MeOH, was most toxic and was applied to tlc plates for further purification. The plates were developed twice with Me_2CO-EtOAc-CH_2Cl_2 (1:1:1). Their edges were sprayed with 5% vanillin and used as guides to divide the unsprayed area into four fractions for elution. The most toxic fraction was rechromatographed with 7% MeOH/CH_2Cl_2. Compound **1**, the major band on these plates, was eluted.

Fraction 4 eluted from the Sep-Paks with 20% and 30% MeCN/CH_2Cl_2 and was also very toxic initially. Tlc revealed only traces of **1** but a large band of brilliant yellow material, which we designated **2**. During 3 months of storage in MeCN/CH_2Cl_2, however, the specific activity of Fraction 4 declined. Therefore, we chromatographed cell extract on preparative tlc plates with 7% MeOH/CH_2Cl_2 to isolate **2**. The developed plates were divided into four zones and eluted. The yellow band containing **2** was rechromatographed on preparative plates with 5% MeOH/CH_2Cl_2. Fractions were eluted and bioassayed. The fraction consisting primarily of **2** was loaded onto silica gel Sep-Paks and sequentially eluted with 50 ml each of $CHCl_3$, 5% MeCN/$CHCl_3$, and MeOH. The $CHCl_3$ fraction was rechromatographed on Sep-Paks. Nearly pure **2** was obtained by elution with 200 ml of 10% MeCN/CH_2Cl_2.

Larger amounts of both compounds were subsequently obtained by column chromatography of cell extract on silica gel. Compound **2** eluted with 20-30% MeCN/CH_2Cl_2 after removal of the less polar compounds with hexane, CH_2Cl_2, and 10-20% MeCN/CH_2Cl_2. Compound **1** was more polar and eluted with ≥50% MeCN/CH_2Cl_2 and MeOH. The fractions containing **1** were combined, loaded onto a second silica gel column, and sequentially eluted with EtOAc, Me_2CO-EtOAc-CH_2Cl_2 (1:1:1, 2:1:1, 6:1:1, 8:1:1), Me_2CO, and MeOH. Fractions containing **1** were combined and subjected to tlc with Me_2CO-EtOAc-CH_2Cl_2 (1:1:1). The band containing **1** was eluted and chromatographed on silica gel Sep-Paks with CH_2Cl_2, Me_2CO-EtOAc-CH_2Cl_2 (1:1:1), Me_2CO, and MeOH.

GENERAL ANALYTICAL PROCEDURES.—Samples were dried at ≤50° with rotary evaporation in vacuo and/or under a stream of N_2. They were routinely stored in the dark at −10°. Tlc was on Whatman LK6, LK6D, or PLK5 silica gel plates. Commonly used solvents were Me_2CO-EtOAc-CH_2Cl_2 (1:1:1) and CH_2Cl_2 containing 5-10% MeOH. Mass spectra were taken on a Hewlett-Packard 5985 electron impact spectrometer with direct probe. Ir spectra, with samples in $CHCl_3$, were obtained on a Perkin-Elmer 337 spectrophotometer. Nmr spectra of geldanamycin, with samples in DMSO-d_6, were taken on a JEOL FX90Q Fourier transform spectrometer at field strength of 22.5 MHz for ^{13}C and 90 MHz for ^1H. Authentic geldanamycin was obtained from W. Snyder, Department of Chemistry, University of Illinois, and authentic nigericin (a mixture of Na^+ and K^+ salts) from Sigma Chemical Company.

RESULTS AND DISCUSSION

TOXICITY OF BROTH AND EXTRACTS.—The biological activity of broth became apparent after 3 days of incubation, peaked between 7 and 11 days, and declined thereafter (Figure 1). At 22, 33, and 83 μl supernatant broth per petri dish, cress radicle growth was inhibited, whereas at 11 μl per dish stimulation occurred. Stimulation followed a pattern nearly identical, but opposite that of inhibition, with greatest effect from broth incubated 9 days. The standard incubation period for toxin production was thus set at 10 days. The pH of the culture broth rose steadily during incubation from 6.3 on the first day to 7.7 on day 9 and 8.0 by days 13 and 15.

The phytotoxic compounds were almost completely extracted from the culture broth with CH_2Cl_2 (Figure 2). Before extraction, 21 μl of broth (cells removed) per petri dish caused 50% reduction of cress radicle growth, compared to the distilled H_2O control. Stimulation occurred at 10 μl, whereas nearly complete inhibition occurred at 60 μl per dish, the highest dose tested. In contrast, CH_2Cl_2-extracted broth was not strongly inhibitory, even at 60 μl. Noninoculated broth stimulated radicle growth at 30 μl and was not appreciably inhibitory up to 60 μl per dish.

Cell and broth extracts were similar in specific activity. Both stimulated radicle growth at 1-4 μg, caused 50% inhibition at 5-6 μg, and reduced radicle growth to ≤15% at 10 μg per dish. Because 4.0 g of cell extract and 1.1 g of broth extract were recovered from the 7 liters of culture extracted, 79% of the total activity was associated with the microbial cells and only 21% with the broth.

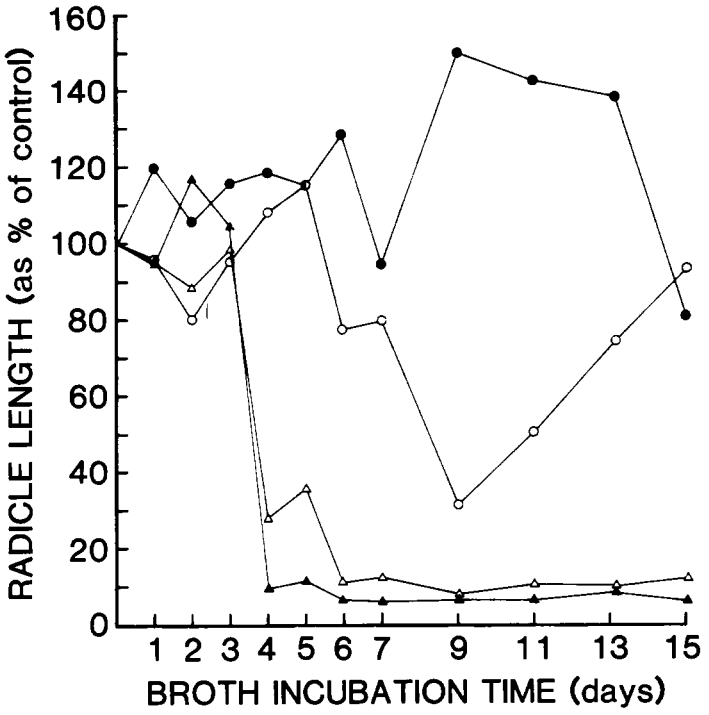

FIGURE 1. Effect of incubation time (at 28°) and concentration of *Streptomyces hygroscopicus* culture broth (cells removed) on radicle growth of garden cress seeds. Quantity of broth applied per bioassay dish: 11 (●), 22 (○), 33 (△), and 83 (▲) μl.

ISOLATION.—Fractions 4 and 7 from Sep-Pak chromatography of cell extract were most inhibitory, 20 μg per dish reducing cress radicle growth to 28% and 23% that of the control. Although similar in specific activity, the fractions differed considerably in tlc (MeCN-CH_2Cl_2, 1:1). Fraction 4 contained an array of compounds having Rf values of 0 to 0.44, with most of the material between 0.24 and 0.44. Compound **2**, the major component, occurred between 0.31 and 0.44, was bright yellow, and turned brown on reaction with 5% vanillin/H_2SO_4. The compounds in Fraction 7 were more polar, with Rf values of 0 to 0.34 and the bulk of material <0.16. Compound **1** predominated, turned bright red on reaction with 5% vanillin/H_2SO_4, and streaked from the origin to 0.16. Further purification of Fraction 7 by tlc produced nearly pure **1**; 20 μg per dish reduced cress radicle growth to 7% of the control. Preparative tlc of cell extract followed by chromatography on Sep-Paks produced nearly pure **2**; 10 μg per dish inhibited radicle growth to 7%.

CHARACTERIZATION.—The mass and ir spectra of **1** were similar to the spectra of authentic nigericin (Na^+-K^+ salts) and to published data (17, 31). Nigericin exists as a free acid and as metallic salts (32). The spectra indicated our sample was mainly the Na^+ salt. Co-chromatography of **1** with authentic nigericin (Na^+-K^+ salts) in three different solvents (MeOH-MeCN, 1:2; MeOH-CH_2Cl_2, 1:9; Me_2CO-EtOAc-CH_2Cl_2, 1:1:1), showed both had identical Rf values and color reaction with 5% vanillin/H_2SO_4. These results support the identity of **1** as nigericin.

The mass spectrum of **2** was similar to that published for geldanamycin (12). The ir, ^{1}H-nmr, and ^{13}C-nmr spectra were essentially identical to those of authentic gel-

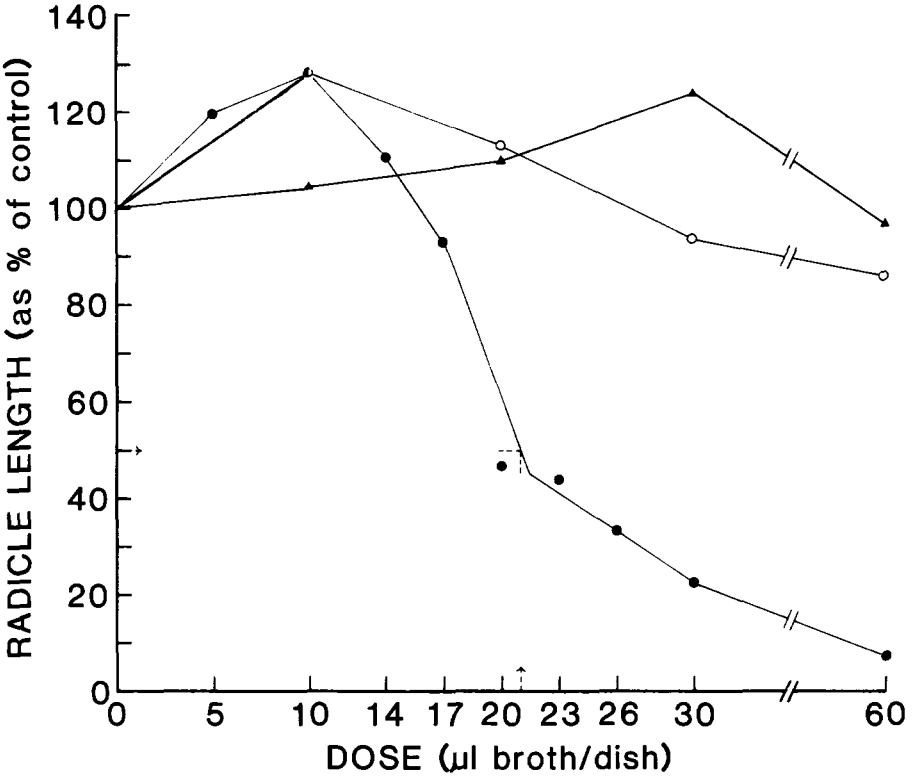

FIGURE 2. Effect of *Streptomyces hygroscopicus* culture broth (cells removed) (●), CH_2Cl_2-extracted culture broth (cells removed) (○), and noninoculated broth (▲) on radicle growth of garden cress seeds. Arrows at 50% on ordinate and corresponding position on abscissa indicate ID_{50} for culture broth.

danamycin. Compound **2** and authentic geldanamycin had identical Rf values and color reaction with 5% vanillin/H_2SO_4. Therefore, we conclude Compound **2** is geldanamycin (**2**).

BIOLOGICAL ACTIVITY.—Nigericin (**1**) and geldanamycin (**2**) were comparable in their reduction of cress radicle growth (Figure 3). For both, 2-3 µg per dish, which is 1-2 ppm in the 1.5 ml of H_2O added, reduced cress radicle growth to 50%. Radicle growth was almost totally suppressed by 5 µg per dish (3-4 ppm). The effects of our purified compounds on cress seeds were nearly identical to those obtained for the authentic samples.

Nigericin inhibited cress radicle growth at all doses tested (Figure 3a). Geldanamycin, in contrast, stimulated at the lowest dose and inhibited at higher ones (Figure 3b). A pattern of stimulation at low doses and inhibition at higher ones is typical of many herbicidal compounds (33).

The symptoms of geldanamycin injury differed markedly from those of nigericin. Geldanamycin did not entirely inhibit cress germination or radicle elongation, even at the highest dose tested. Soon after emergence, however, radicles exposed to high geldanamycin concentrations turned brown and began to disintegrate, indicating a post-germination effect. Nigericin inhibited radicle growth, but it did not cause browning or tissue necrosis.

Further study is underway to determine the potential of geldanamycin and nigericin for use as herbicides.

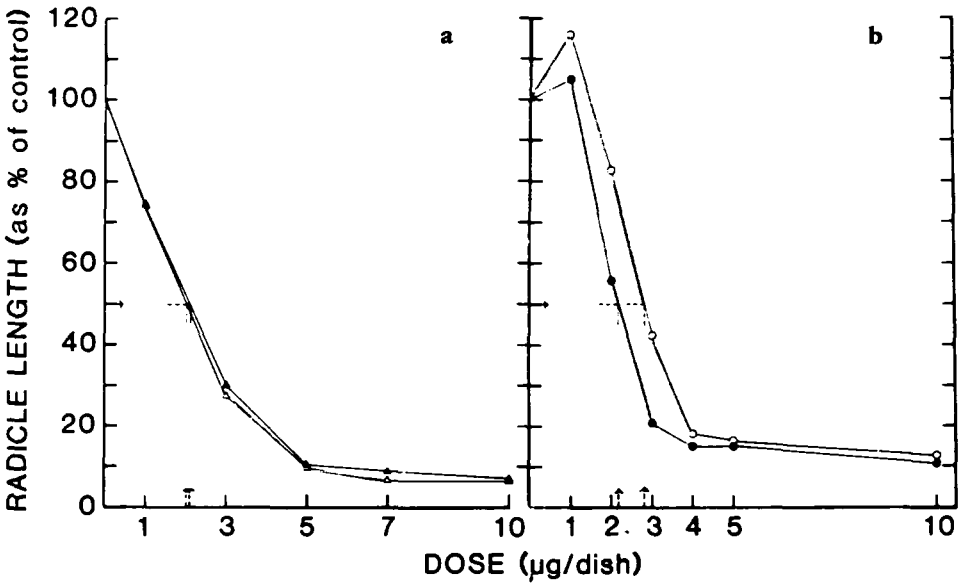

FIGURE 3. Effect of nigericin (**1**) and geldanamycin (**2**) on radicle growth of garden cress seeds.
a. Nigericin, primarily Na^+ salt, isolated from *Streptomyces hygroscopicus* (△) vs. authentic nigericin Na^+-K^+ salts (▲).
b. Geldanamycin isolated from *S. hygroscopicus* (○) vs. authentic geldanamycin (●). Arrows at 50% on ordinate and corresponding position on abscissa indicate ID_{50}.

ACKNOWLEDGMENTS

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