Gigantenone, a novel sesquiterpene phytohormone mimic [1]

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Received 13 July 1988; accepted 21 April 1989

Summary. Gigantenone, a new eremophilane diepoxide, was isolated from the fungal plant pathogen *Drechslera gigantea*. It displays unique biological activity on higher plants. On most graminaceous species, the application of 18 nanomoles to a leaf surface results in the formation of 'green islands' – localized areas of chlorophyll retention. Gigantenone is structurally unrelated to the cytokinins, yet induces the green island effect associated with these phytohormones at comparable concentrations. However, on dicotyledonous species it generally causes necrotic lesions. On host plants of *D. gigantea*, gigantenone induced lesions closely resembling those appearing in natural infections. Gigantenone also induces root formation in mung bean hypocotyls and shows a high level of activity in several plant tissue culture systems.
Key words. Phytohormone; fungal plant pathogen; chlorophyll retention; green island effect; senescence.

The fungus *Drechslera gigantea* causes zonate eyespot disease on numerous grasses[3]. This leaf disease is a serious problem in many commonly cultivated turfgrasses and also occurs in weeds such as crabgrass (*Digitaria* spp.), quackgrass (*Agropyron repens*), and Bermuda grass (*Cynodon dactylon*). The lesions of zonate eyespot first appear as brown flecks. These flecks eventually enlarge and coalesce, although they are sometimes surrounded by reddish brown or dark green borders. The dark green borders are reminiscent of the 'green island' effect that commonly occurs in mildew and leaf rust infections[4]. Green islands have been attributed to the action of the cytokinin family of plant hormones. Since many phytohormones are produced by plant-pathogenic fungi, we investigated the possible link between *D. gigantea* metabolites, disease symptoms, and phytohormone activity.

Isolates of *D. gigantea*, kindly supplied by E. S. Luttrell of the University of Georgia, were maintained on potato dextrose agar containing 15% (v/v) V-8 juice. The fungus was grown in a defined liquid medium[5] at room temperature for 4 weeks. The culture was filtered through 4–6 layers of cheesecloth and the filtrate extracted with ethyl acetate (3 times with 1/3 the volume of filtrate). The extract was dried under vacuum at 40 °C. The residue was further purified by flash chromatography on silica gel with chloroform/methanol, 9/1, followed by chromatography on Sephadex LH-20 with methanol. The final chromatography was HPLC on a RP-18 (4 × 250 mm) column using acetonitrile/water, 1/1, with a flow rate of 1 ml/min. The column eluant was monitored at 254 nm to detect fractions, and biological activity was monitored by a needle-puncture-droplet overlay technique on leaves of various plant species[5]. Specifically, 5 µl of 5% ethanolic H_2O containing 10–30 nmoles of compound were placed over a fresh needle puncture on the adaxial surface of test leaves. This was the optimum range for observing biological effects. Control leaves were treated with 4 µl of 5% ethanolic H_2O and never exhibited any adverse effects. Leaves were placed in petri dishes over moistened filter paper and incubated under intermittent light at 7 $\mu E/m^2 \cdot s$ at 25–27 °C.

Extracts of *D. gigantea* grown in liquid culture yielded several biologically active eremophilanes[3], and one, trivially named gigantenone, is the subject of this report. Gigantenone was tentatively identified as a highly oxygenated eremophilane sesquiterpene on the basis of the following data: HRMS obsd. m/z 264.1359, calcd. for $C_{15}H_{20}O_4$ 264.1361; m.p. 140 °C; $[\alpha]_D = +160°$ (C0.4, $CHCl_3$); uv 242 nm (ε = 10,600); ^1H NMR δ5.73 (d, 1.9, 1H), 3.65 (s, 1H), 3.61 (ddd, 4.5, 9.5, 10.5, 1H), 2.84 (d, 4.6, 1H), 2.60 (d, 4.6, 1H), 2.48 (dddd, 1.9, 4.6, 14.4, 14.6, 1H), 2.34 (dddd, 2.7, 4.6, 4.6, 12.5, 1H), 1.81 (dq, 6.8, 10.5, 1H), 1.58 (s, 3H), 1.42 (m, 1H), 1.27 (d, 6.8, 3H), 1.10 (s, 3H); ^{13}C NMR δ189.7 (s), 165.0 (s), 120.9 (d), 70.9 (d), 64.5 (d), 59.9 (s), 55.1 (s), 51.9 (t), 44.4 (d), 41.5 (s), 35.1 (t), 30.8 (t), 18.8 (q), 18.4 (q), 11.3 (q).

Gigantenone's retention time on HPLC was 3.2–3.5 min. On silica gel TLC $CHCl_3$/MeOH, 9/1, it had an R_f of 0.40 and in EtOAc/$PhCH_3$, 1/2, an R_f of 0.33 as detected with the universal spray reagent sulfuric acid-anisaldehyde.

The HPLC purified gigantenone was recrystallized from ethyl acetate and the yield of crystalline material was approximately 1 mg/l of culture fluid. The complete stereostructure was elucidated by single crystal x-ray diffraction. Gigantenone has a space group $P2_1$ with z = 4, so two molecules of $C_{15}H_{20}O_4$ form the asymmetric unit. Cell constants were a = 11.9596(3), b = 9.8154(2), c = 12.6007(3) Å, and β = 105.89(2)°. Final agreement factor is 0.072 for the 1287 (63%) observed reflections, and both independent molecules had the same stereostructure. The absolute configuration of gigantenone shown in **1** was selected on the basis of its optical rotation ($[\alpha]_D + 160°$) compared with that of sporogen AO1 ($[\alpha]_D + 214°$). The absolute configuration of sporogen

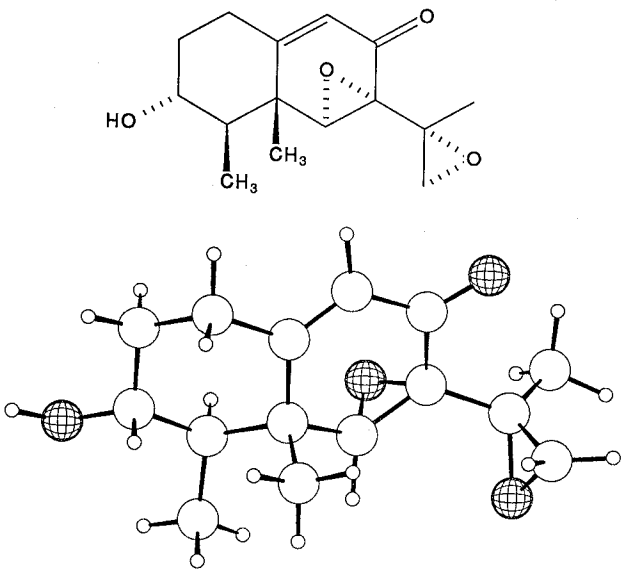

Figure 1. Two representations of gigantenone. On the top is a conventional chemical drawing and on the bottom is a computer generated perspective drawing of the final X-ray model.

AO1 was in turn based on its CD spectrum and application of the exciton chirality method to its p-bromobenzoate[6]. A computer generated perspective drawing of the final X-ray model is given in figure 1.

Although gigantenone **1** is a structurally novel eremophilane, the most interesting feature is its biological activity on higher plants. An initial survey of gigantenone's activity was made on excised leaves from a variety of plant species using a needle-puncture technique[5]. Upon treatment with gigantenone (18 nmoles/leaf), 12 of 14 monocots developed a green island after 1–2 days. These twelve include *Zea mays* 'W64A-N' and 'W64A-T', *Saccharum officinarum* '51NG100', *Avena sativa* 'Park' and 'Otana', *A. fatua, Agropyron repens, A. intermedium, A. cristatum, Cynodon dactylon, Digitaria sanguinalis, Sorghum halepense, Cyperus esculentus*, and *Rhoeo discolor*. Both *S. officinarum* 'H50-7209' and *Sorghum vulgare* 'Milo' developed necrotic lesions. After 8–10 days, those green islands which did form faded and were indistinguishable from the rest of the leaf. Interestingly, gigantenone applied to leaves of the variegated bromeliad *Ananas comosus* 'bracteatus' caused green islands in the chlorophyll-containing tissue, but only water soaking in the achlorophyllous tissue. In contrast, gigantenone caused necrosis or chlorosis on six dicotyledonous plants – *Heliothus annuus, Solanum tuberosum* 'Burbank', *Lycopersicon esculentum, Glycine max* 'Harasoy', *Vicia faba*, and *Euphorbia esula*. The species *Cirsium arvense, Citrus limon* 'Meyer', and *Taraxacum officinale* failed to respond to gigantenone while *Cucumis sativa* 'National Pickling' developed a green island surrounded by chlorotic halo. Although the green island effects were observed on many of the regular hosts of *D. gigantea*, the plant species that could be best manipulated for aging studies was the 'Park' cultivar of oats (*Avena sativa*). Gigantenone-treated 'Park' oats showed localized chlorophyll retention around the site of application (fig. 2). Quantitative measurement of the green island effect on 'Park' oats was performed by placing droplets of test solutions on leaf surfaces and then incubating these in a moist chamber for 3 days at 25–27 °C in intermittent light (7 $\mu E/m^2 \cdot s$). As measured from the site of application, 1-cm sections were cut from the leaves as follows: base = 0.5 to 1.5 cm basipetal to punctures; and tip 0.5 to 1.5 cm acropetal to punctures. Chlorophyll content was determined by extraction with 3 ml of DMSO and the samples read at 665 nm. The experiment was repeated 5 times and all data were subjected to the Neuman Kuels test for statistical significance. There was a significant reduction in chlorophyll content distally and basally to the application site which is visually observed in figure 2. In contrast, when the same concentration of kinetin, a typical cytokinin-like compound, was applied it caused the entire leaf to remain green. In a standard bioassay for cytokinin-like activity, gigantenone differed from kinetin by failing to stimulate synthesis of chlorophyll in etiolated cotyledons of cucumbers. Gigantenone was also compared to kinetin for its ability to delay senescence when leaves of 'Park' oats were floated in solutions of either compound (4.3×10^{-5} to 4.3×10^{-7} M). Kinetin-treated leaves retained significantly more chlorophyll than control leaves, while gigantenone-treated leaves did not. Thus, it is quite unlikely that gigantenone is acting through a cytokinin-like response since the biological effects are markedly different.

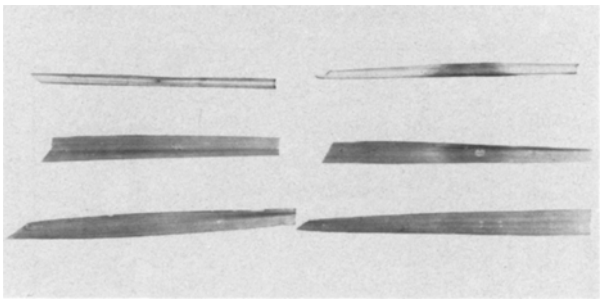

Figure 2. *A. sativa* ('Park' oats) leaves treated with 18 nmoles of gigantenone in 4 µl of 5% ethanol. The leaves were 10 days old and held at 23–27 °C in intermittent light (7 $\mu E/m^2 \cdot s$). The left column shows leaves (top to bottom) that were aged for 24 h, 48 h and 120 h. Note the formation of the green islands. The column of leaves on the right side served as controls and were treated with 4 µl of 5% ethanol.

While gigantenone-induced green islands contained greater amounts of chlorophyll than did senescing tissue, it was not known if this tissue functioned in CO_2 fixation. We examined the spectral characteristics of chlorophyll extracted from gigantenone-induced green islands and found no difference from chlorophyll in healthy green tissue. To test the ability of green islands to fix CO_2, leaves of 'Park' oats displaying well-developed green islands were placed in a small chamber containing $^{14}CO_2$

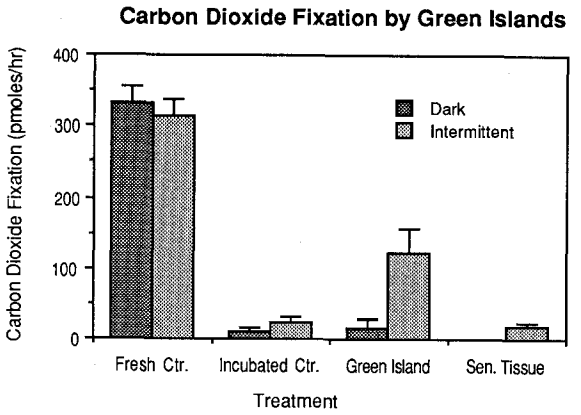

Figure 3. CO_2 fixation by green islands on 'Park' oats leaves. The apical 6 cm of 10-day-old 'Park' oats leaves were excised and placed in a moist incubator. Treatments were applied as 5 µl droplets over needle puncture wounds on adaxial leaf surfaces, and the leaves were incubated either in the dark for 4 days [Dark] or under intermittent light (80 µE/m^2 · s, 16 h day, 8 h night) for three days [intermittent]. Incubated leaves and freshly harvested leaves were then placed in small, airtight chamber (532 cm^3) under constant light (18 µE/m^2 · s) for 1 h in the presence of 0.05 mmole $^{14}CO_2$ (8.4 Ci/mole). Sections (2 cm) containing green islands were excised, diced, and placed in 0.5 ml perchloric acid: H_2O_2 (1:2) for 1 h at 100 °C [Green Island]. 2-cm sections immediately basal to green islands were similarly treated. This tissue represents senescent tissue [Sen. Tissue] from leaves containing green islands. After digestion, 10 ml aquasol was added to samples which were then counted for 50 min in Packard TriCarb 3320 Scintillation Counter. Cpm's were converted to DPM's via a quench correction curve. CO_2 fixation (pmoles CO_2/h) values were then calculated based on specific radioactivity of $^{14}CO_2$. Each treatment was carried out on five replicates, and each treatment was repeated three times. The standard deviations are shown as error bars.

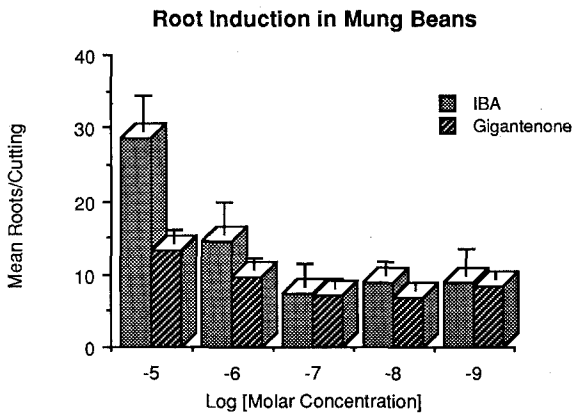

Figure 4. Mung bean seedlings (*Phaseolus aureus*), 9 days old, were harvested, cotyledons were removed, and the hypocotyl was cut to leave 3 cm of hypocotyl plus 4-cm shoot which included two primary leaves and an apical bud. Cuttings were placed in individual test tubes containing 2 ml of test solution. They were placed under constant light (18 µE/m^2 · s) at 25 °C for 24 h. The test solution was then replaced with 4 ml of distilled, deionized water. Cuttings were then incubated at the same temperature and constant light intensity. After 6 days, the number of roots exceeding 1 mm was counted. Each treatment involved five samples, and the treatments were repeated three times. The standard deviations are shown, and IBA refers to indole-3-butyric acid. Water controls had 3.6(0.9) mean roots/cutting.

and the amount of CO_2 fixed was measured according to standard procedures under varying light conditions. With intermittent light, gigantenone-induced green islands were about half as effective in CO_2 fixation as fresh control leaves (fig. 3). Little CO_2 fixation occurred in the senescing tissue next to the green island (fig. 3). Without exposure to light, even though chlorophyll was present, the gigantenone-induced green islands were essentially unable to fix CO_2 (fig. 3). Thus, while prominent green islands are produced in the dark, light is required during development for the green islands to retain an ability to fix CO_2.

Currently, green islands in plant tissue infected with various biotrophic fungi (rusts and mildews) are attributed to the cytokinin phytohormones[7]. The evidence for cytokinin involvement is presumptive since the isolation and chemical characterization of cytokinins from these fungi or infected tissues has never been reported. As this report demonstrates, other fungal products are likely to be involved in the induction of the green island effect[8]. Since gigantenone nevertheless seemed to possess properties exhibited by some phytohormones, we tested for several other activities and found the ability to activate adventitious rhizogenesis in mung bean (*Phaseolus aureus*). Gigantenone's root-inducing activity in the µM to sub-µM range is nearly equivalent to that of indole-3-butyric acid (fig. 4). In various plant tissue culture systems, gigantenone was tested at µM concentrations in the absence of added phytohormones. Initial results revealed an ability to hasten rooting in petunia (*Petunia hybrida*) explants by ten days, enhance shoot growth of asparagus (*Asparagus officinalis*) explants, and stimulate rhizogenes in *Brunfelsia* spp. explants at 1.8 µM. At 18 µM this activity was completely inhibited. In addition, the number of adventitious buds on redwood (*Sequoia sempervirens*) explants was tripled in the presence of 1.8 µM gigantenone.

The discovery that gigantenone has a number of biological activities has implications in plant physiology and plant pathology. The induction of green islands, necrogenesis, rhizogenesis in mung beans, and various tissue culture effects are all promising areas of research in pathological physiology, photosynthetic efficiency, senescence, vegetation propagation, and development of selective herbicides.

1 We thank the US-National Science Foundation (Grant DMB-8607347) for partially supporting this work.
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