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PHYTOTOXICITY OF THREE LACTONES FROM *NIGROSPORA SACCHARI*

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Key Word Index—*Nigrospora sacchari*; metabolite; phytotoxin; (+)-phomalactone; electrolyte leakage.

Abstract—A culture broth of *Nigrospora sacchari* showed strong herbicidal activity in treatment of intact greenhouse-grown plants. Three lactones were isolated from the culture broth. The major compound, which exhibited the most significant effect of the fungal metabolites in the assay procedures, was identified as (+)-phomalactone, 6-(1-propenyl)-5,6-dihydro-5-hydroxy-2*H*-pyran-2-one. Others were 5-[1-(1-hydroxybut-2-enyl)]-furan-2-one and 5-[1-(1-hydroxybut-2-enyl)]-dihydrofuran-2-one. These fungal metabolites were investigated for their herbicidal effects in an electrolyte leakage assay and the results obtained appeared to indicate that the herbicidal damage was caused by cellular disruption. Phomalactone caused remarkable and rapid electrolyte leakage on cucumber cotyledon discs at concentrations higher than 50 ppm.

INTRODUCTION

Diseased leaves of wild plants are collected to isolate toxigenic fungi and bacteria which may produce natural compounds for potential use as herbicides or herbicidal adjuvants. During our screening studies to find novel phytotoxins from microorganisms, we found that a culture broth of *Nigrospora sacchari* (JT910831-1) showed remarkable herbicidal activity against seedlings in a greenhouse test, and isolated (+)-phomalactone (**1**) as a causative phytotoxin. Phomalactone is a member of a 6-substituted-5,6-dihydro-2*H*-pyran-2-one group (others include asperlin [1] and goniothalamine [2]) which has been reported to exhibit broad biological activities. In 1969, Evans *et al.* first isolated phomalactone from *Nigrospora* sp. and elucidated its chemical structure including its absolute configuration on the basis of ^{1}H NMR spectroscopy and circular dichroism studies [3]. In 1970, Yamamoto *et al.* reported the isolation of phomalactone from *Phoma* sp. and detailed its antibacterial activity [4]. In 1994, Krasnoff *et al.* identified an insecticidal metabolite from several strains of *Hirsutella thompsonii* var. *synnematosa* which are entomopathogenic fungi of the apple maggot as phomalactone [5].

Evans also first isolated **3** from *Nigrospora* sp. and elucidated its planar structure [3], while its biological activities have not been reported previously. Compound **2** was isolated for the first time as a natural product.

In this paper, the first details of the phytotoxicity and the herbicidal action of these fungal lactones are reported.

RESULTS AND DISCUSSION

Isolation of bioactive substances

A crude EtOAc extract, which exhibited strong herbicidal activity in a whole plant assay, from 10-day-old culture broth of *Nigrospora sacchari* was used for the identification of bioactive substances. Subsequent isolation of the causal phytotoxins was carried out by a simple leaf-puncture assay [6]. The phytotoxic metabolites were purified by column chromatography on silica gel, followed by HPLC, giving **1** as a major component. Further repeated chromatography with a HPLC recycling system gave **2** and **3** from the lower polar fraction.

Chemical characterization of the isolated compounds

The high resolution FAB mass spectrum of **1** indicated a molecular formula of $C_8H_{10}O_3$ that was supported by ^{1}H, ^{13}C and DEPT NMR spectra. In the IR spectrum, the absorption peaks at 3404 and 1717 cm^{-1}

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indicated the presence of a hydroxyl group and a carbonyl group, respectively. The carbonyl group was also suggested from a signal at δ 163.6 in the ^{13}C NMR spectrum. On the basis of ^{1}H–^{1}H COSY spectrum, the correlation of all the protons could be explained with the structure of **1**. Furthermore, the ^{1}H NMR spectrum and optical rotation of **1** were identical with previous data [3, 5], which confirmed it as (+)-phomalactone.

The molecular formula of **2** was estimated to be $C_8H_{10}O_3$, which was the same as that of **1**, according to a high resolution FAB mass spectrum. The ^{1}H and ^{13}C NMR spectra of **2** were also closely related to those of **1**. The doublet adsorption attributed to a carbonyl group was observed at 1789 and 1760 cm^{-1} in the IR spectrum, whereas the carbonyl carbon signal was observed at δ 173.0 in the ^{13}C NMR spectrum. This finding suggested the presence of an unsaturated γ-lactone, in which the double bond was conjugated with the carbonyl group [7]. Furthermore, ^{1}H–^{1}H correlation studies indicated a structure similar to that of **1**. Treatment of **1** in MeOH containing 5% HCl yielded **2** by recyclization because of the stability of the ring structure. But, in the case of treatment with a mild acid, **2** was not detected. Therefore, **2** was considered not to be an artifact produced during the process of the extraction and fractionation procedure. The optical rotation and the ^{1}H NMR spectrum of the metabolite were completely identical with those of the acid-treated product from **1**. This indicated the chemical structure including the absolute configuration was determined as shown in Fig. 1.

The molecular formula of **3** was estimated to be $C_8H_{12}O_3$, according to a high resolution FAB mass spectrum. An absorption peak at 1776 cm^{-1} in the IR spectrum and a signal at δ 177.1 in the ^{13}C NMR indicated the presence of a γ-lactone moiety. The ^{1}H and ^{13}C NMR spectra of **3**, compared to those of **2**, indicated the hydrogenation of an olefinic group in the lactone moiety. Furthermore, by the connectivity of ^{1}H–^{1}H coupling, the chemical structure of **3** was determined to be a 3,4-dihydro derivative of **2**. The absolute configuration was elucidated on the basis of optical rotation and analysis of the ^{1}H NMR spectrum. The data of a reduced product prepared by catalytic hydrogenation [8] from **3** were completely identical with those of **4** from **2**.

Phytotoxic characteristics of the three lactones

In order to clarify the phytotoxic features of the fungal metabolites, the biological activities of the three lactones were evaluated by the following bioassays.

In an initial survey, the activities of the three lactones were compared in a leaf-puncture assay. A droplet containing each compound was applied to a puncture wound on cowpea leaves. In the results, it was observed that 5 μg of **1**, **2** and **3** produced 10, 5 and 2 mm diameter necrotic lesions, respectively, within 72 h of treatment. This showed that phomalactone was the most phytotoxic of the metabolites produced by this fungus.

Moreover, the lactones were tested in whole plant assays with a variety of plant species. Phomalactone was shown to be rapid-acting and caused water-soaked lesions in the area of application within 24 h of spraying at a concentration of 1000 ppm. Further, 72 h after incubation in the greenhouse, the treated plants were constricted and desiccated with necrosis. On the other hand, treatment at 1000 ppm with **2** and **3** resulted in the following; **2** caused necrosis with leaves curling at the margins and **3** caused slight necrosis at the tips of leaves within 72 h after treatment. In addition, it was observed for these com-

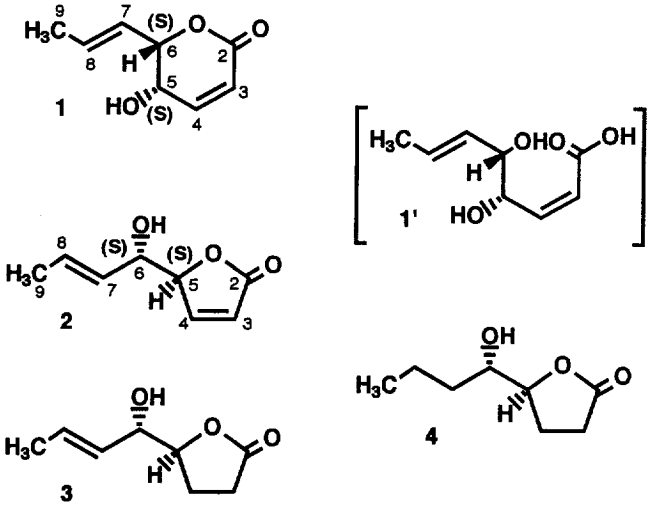

Fig. 1. Structures of **1**–**3** isolated from culture filtrate of *Nigrospora sacchari* and **4** prepared from catalytic hydrogenation of **2** and **3**. **1'** is a hypothetical compound.

pounds that there were no significant differences in terms of test plant species in this assay.

Concerning the phytotoxic activity relationship between the three lactones, it was interesting that **1** exhibited a much more significant phytotoxic effect than **2**, although it could be expected that both **1** and **2** would develop into the same chain structure: a hypothetical compound (**1'**) shown in Fig. 1, by a C—O bond cleavage of the lactone ring. This result presumes that the ring structure of the phomalactone molecule plays an important role in the phytotoxic effect. Further studies should be carried out to clarify the detail of structure-activity relationships.

Effect on plant cells

In the whole plant assay, phomalactone caused rapid chlorosis and desiccation which resembled that caused by dipyridinium herbicides and *p*-nitrodiphenyl ether herbicides. In order to clarify this phenomenon, the direct effect of the metabolites on plant cells was examined in an electrolyte leakage assay which has been utilized to determine many types of stress to plants [9–11]. In the examination of the dose-response relationship, it was revealed that a significant effect in this assay was observed for phomalactone, even at low concentrations in which there was only slight visible damage in the whole plant assay. It caused appreciable electrolyte leakage to cucumber cotyledon discs at concentrations of more than 50 ppm as shown in Fig. 2. In addition, the electrical conductivity of the bathing media finally reached maximum at a dosage of 125 ppm, but it increased in a concentration-dependent manner soon after treatment.

Figure 3 shows a continuous change in the electrical conductivity of the bathing media. Bathing media incubated with 1000 ppm of phomalactone under continuous fluorescent light showed a rise in conductivity after a three to four hour time lag. However, **2** and **3** caused little electrolyte leakage even after 24 h, reflecting their low degree of activity in the leaf-puncture and whole plant assays.

The rapid membrane damage caused by these compounds, resulting in electrolyte leakage, occurred in the same manner under light and dark conditions as shown in Fig. 3. This phenomenon was different from the light-dependent membrane damage caused by *p*-nitrodiphenyl ether [12–14] or bipyridinium herbicides [15–18]. Typical membrane damage is the result of lipid peroxidation [18]. The peroxidation of membrane lipid ultimately depends on the generation of a toxic oxygen species such as superoxide [15, 16] and singlet oxygen [12, 13]. The toxic oxygen species are generated in the light and in the presence of oxygen molecules [14, 17]. The effect of oxygen and light conditions on phomalactone and these herbicides was compared in this assay. The results, presented in Table 1, showed that there was little or no effect on the activity of phomalactone of either the light conditions or the oxygen concentration, whereas the herbicides were inactive under dark conditions.

From the above observation, it is suggested that phomalactone kills plants through cellular disruption resulting in electrolyte leakage. But the action, which does not depend on light and oxygen, is different from that of *p*-nitrodiphenyl ether or bipyridinium herbicides. The interaction of phomalactone on plant cell membranes should be studied to clarify the details of the mechanism of action.

EXPERIMENTAL

General

Chemical data were recorded on the following instruments: ^1H (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) NMR, Bruker AM-500: TMS as int. standard;

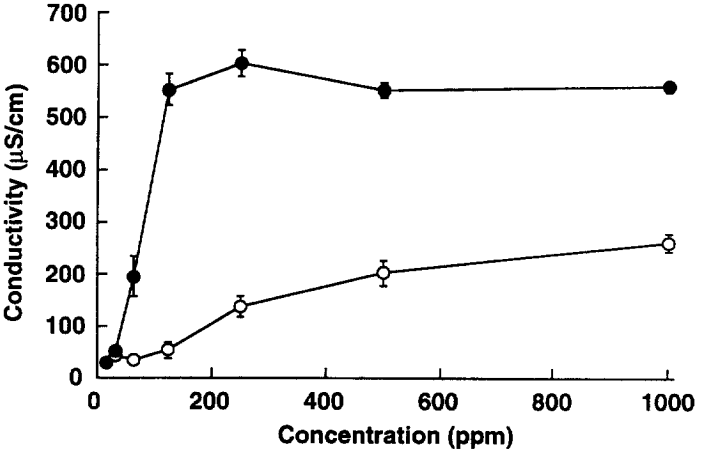

Fig. 2. Effect at various concentrations of **1** on electrolyte leakage assay. Each plot is the mean of the electrical conductivity minus that of control: within ○ 10 and within ● 20 h after incubation under continuous fluorescent light. Error bars indicate standard deviation ($n = 3$).

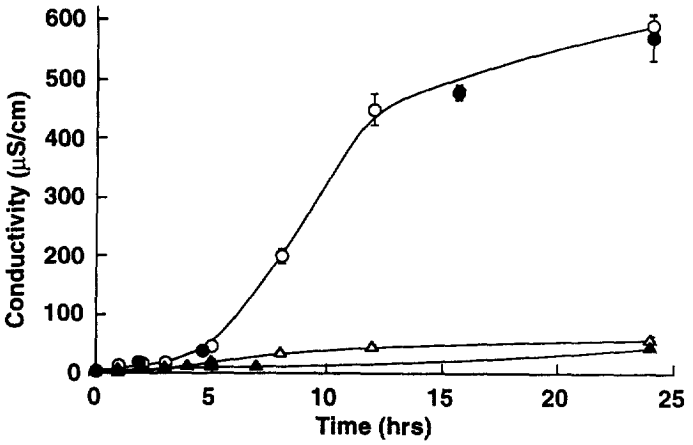

Fig. 3. Effects of three lactones on electrolyte leakage assay. Each plot is the mean of the electrical conductivity of the bathing media incubated with 1000 ppm of ○ **1**, △ **2** and ▲ **3** under continuous fluorescent light and 1000 ppm of ● **1** in the dark, minus that of control. Error bars indicate standard deviation ($n = 3$).

Table 1. Comparison of effects between phomalactone and light-dependent herbicides on electrolyte leakage assay. Each value (μS/cm) is the mean of the conductivity change for 24 h minus that of control for 24 h with ± standard deviation ($n = 3$). Phomalactone, paraquat (bipyridinium) or chlomethoxynil (*p*-nitrodiphenyl ether) was added at a final concentration of 1000 ppm

	Light/Air	Dark/Air	Light/N_2*
Phomalactone	534.3±9.3	338.0±18.8	584.1±27.8
Paraquat	535.4±30.7	44.0±22.7	578.9±36.8
Chlomethoxynil	284.0±12.6	34.6±10.4	30.3±15.2

* The oxygen concentration of the atmosphere was less than 1%.

FAB-MS: Kratos concept-2H; FT-IR: Nicolet 800 FT-IR spectrometer; Optical rotation: Jasco DIP-370 polarimeter; Conductivity: Horiba conductivity meter DS-15. Fractionation was carried out with the following systems: HPLC: Hitachi L-6000 pump and L-3300 RI monitor; CC: Kieselgel 60 (70–230 mesh, Merck); TLC: precoated Kieselgel 60 F_{254} plates (0.2 mm, Merck).

Isolation and culture of the fungus.

Nigrospora sacchari was isolated from diseased canna (*Canna indica*) leaves collected in Yokohama in 1991. The fungus was grown in a 3 l Erlenmeyer flask containing 1 l of the following medium: 30 g glucose, 3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 1 g yeast extract and water to 1 l, pH adjusted to 6 with HCl. The culture was prepared on a rotary shaker (180 rpm) at 25° for 10 days.

Purification of active principles

The culture filtrate was extracted with EtOAc (3 × 1 l) and the extract obtained was subjected to silica gel CC with C_6H_{12}–EtOAc (40:60) as an eluting solvent. The phytotoxic frs were collected and further purification was carried out by HPLC on a silica column (YMC, 6 × 250 mm) which was eluted with $CHCl_3$–*iso*-PrOH (95:5), to give **1** (106.9 mg) as a major component. Lower polar frs were combined and repeated chromatography with a HPLC recycling system eluting with $CHCl_3$–*iso*-PrOH (97:3) gave **2** (12.3 mg) and **3** (17.6 mg).

6(*S*)-(1-propenyl)-5,6-dihydro-5(*S*)-hydroxy-2H-pyran-2-one (**1**). Colorless needles, $[\alpha]_D^{25}$ +174.0° ($CHCl_3$, *c* 0.100). High resolution FAB-MS *m/z*: $[M+H]^+$ 155.07182 (calcd for $C_8H_{11}O_3$, 155.07082); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3404 (OH), 2921, 2854, 1717 (C=O), 1627, 1445, 1379, 1258, 1156, 1075, 1033, 966, 935, 892, 829, 710, 642; ^1H NMR: δ 6.99 (1H, *dd*, $J = 5.3$, 9.7 Hz, H-4), 6.13 (1H, *dd*, $J = 0.4$, 9.7 Hz, H-3), 6.01 (1H, *ddq*, $J = 1.1$, 15.3, 6.5 Hz, H-8), 5.73 (1H, *ddq*, $J = 7.0$, 15.3, 1.4 Hz, H-7), 4.83 (1H, *ddquint*, $J = 3.1$, 7.0, 0.8 Hz, H-6), 4.20 (1H, *ddd*, $J = 3.1$, 5.3, 8.0 Hz, H-5), 2.14 (1H, *d*, $J = 8.0$ Hz, OH) 1.82 (3H, *ddd*, $J = 0.8$, 1.6, 6.5 Hz, H-9); ^{13}C NMR: δ 163.6 (*s*, C-2), 144.9 (*d*, C-4), 133.1 (*d*, C-8), 124.0 (*d*, C-7), 122.7 (*d*, C-3), 81.4 (*d*, C-6), 63.2 (*d*, C-5), 16.1 (*g*, C-9).

5(*S*)-[1-(1(*S*)-hydroxybut-2-enyl)]-furan-2-one (**2**). Colorless oil. $[\alpha]_D^{25}$ −131.6° ($CHCl_3$ *c* 0.613), High resolution FAB-MS *m/z*: $[M+H]^+$ 155.07162 (calcd for $C_8H_{11}O_3$, 155.07082); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3604 (OH), 3448 (OH), 2922, 1789 (C=O), 1673, 1622, 1469, 1379, 1234, 1162, 1098, 1042, 969, 897, 830, 818; ^1H NMR: δ 7.43 (1H, *dd*, $J = 1.6$, 5.8 Hz, H-4), 6.19 (1H, *dd*, $J = 2.0$, 5.8 Hz, H-3), 5.87 (1H, *ddq*, $J = 0.9$, 15.1, 6.5 Hz, H-8), 5.51 (1H, *ddq*, $J = 7.3$, 15.1, 1.6 Hz, H-7), 5.00 (1H, *dt*, $J = 6.0$, 1.8 Hz, H-5), 4.19 (1H, *br dd*, $J = 6$, 7 Hz, H-6), 1.75 (3H, *dd*, $J = 1.6$, 6.5 Hz,

H-9); ^{13}C NMR: δ 173.0 (*s*, C-2), 153.7 (*d*, C-4), 131.6 (*d*, C-8), 127.5 (*d*, C-7), 122.9 (*d*, C-3), 86.1 (*d*, C-5), 73.4 (*d*, C-6), 17.9 (*g*, C-9).

5(*S*)-[1-(1(*S*)-hydroxybut-2-enyl)]-dihydrofuran-2-one (**3**). Colorless oil. $[\alpha]_D^{25}$ +51.2° ($CHCl_3$, *c* 0.760), High resolution FAB-MS *m/z*: $[M+H]^+$ 157.08763 (calcd for $C_8H_{13}O_3$, 155.08647); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3590 (OH), 3477 (OH), 2950, 2922, 1775 (C=O), 1676, 1605, 1460, 1421, 1381, 1219, 1183, 1118, 1081, 1036, 969, 917, 816; ^{1}H NMR: δ 5.86 (1H, *ddq*, *J* = 0.9, 15.4, 6.5 Hz, H-8), 5.52 (1H, *ddq*, *J* = 7.2, 15.4, 1.7 Hz, H-7), 4.44 (1H, *dt*, *J* = 5.5, 7.2 Hz, H-5), 4.09 (*br t*, *J* = 6 Hz, H-6), 2.607 (1H, *ddd*, *J* = 6.0, 9.9, 17.9 Hz, H-3), 2.52 (1H, *ddd*, *J* = 8.4, 9.4, 17.9 Hz, H-3), 2.23 (1H, *dddd*, *J* = 6.0, 7.2, 9.4, 13.1 Hz, H-4), 2.08 (1H, *dddd*, *J* = 7.2, 8.4, 9.9, 13.1, Hz, H-4), 1.74 (3H, *dd*, *J* = 1.7, 6.5 Hz, H-9); ^{13}C NMR: δ 177.1 (*s*, C-2), 131.1 (*d*, C-8), 128.0 (*d*, C-7), 82.8 (*d*, C-5), 75.1 (*d*, C-6), 28.6 (*d*, C-3), 23.9 (*d*, C-4), 18.0 (*g*, C-9).

Reaction of **1** *with HCl/MeOH*

A solution of phomalactone **1** (10 mg) in MeOH (10 ml) was added to MeOH (10 ml) containing 10% HCl. The reaction mixture was stirred for 2 h at room temp., then poured into EtOAc (100 ml) and washed with satd $NaHCO_3$ aq. After removal of the solvent, the residue was chromatographed on silica gel with $CHCl_3$–*iso*-PrOH to yield **2** (4.8 mg).

Catalytic hydrogenation of **2**

To a solution of **2** (8.4 mg) in absolute EtOH (1.0 ml) was added 5% palladium carbon catalyst (4.0 mg). The reaction mixture was stirred vigorously under 1 atm of hydrogen at room temp. for 3 h, then filtered through celite to remove the catalyst. After removal of the solvent, the residue was chromatographed on silica gel to yield **4** (5.7 mg).

(*S*)-[1-(1(*S*)-hydroxybutyl)]-dihydrofuran-2-one (**4**). Colorless oil. $[\alpha]_D^{25}$ +37.9° ($CHCl_3$ *c* 0.217); ^{1}H NMR: δ 4.41 (1H, *dt*, *J* = 4.6, 7.4 Hz, H-5), 3.59 (1H, *m*, H-6), 2.62 (1H, *ddd*, *J* = 5.3, 9.9, 17.8 Hz, H-3), 2.53 (*dt*, *J* = 17.8, 9.0 Hz, H-3), 2.25 (1H, *dddd*, *J* = 5.3, 7.3, 9.1, 12.8 Hz, H-4), 2.11 (1H, *dddd*, *J* = 7.5, 9.0, 9.9, 12.8 Hz, H-4), 1.96 (1H, *d*, *J* = 5.8 Hz, OH), 1.51 (2H, *m*, H-7), 1.6–1.4 (2H, *m*, H-8), 0.95 (3H, *t*, *J* = 7.0 Hz, H-9).

Leaf-puncture assay

This assay was carried out on the basis of a method reported by Tanaka *et al.* [6]. Extract, fractions or pure compounds were dissolved in 70% Me_2CO containing 0.1% Tween 80 and then 5 μl droplets of each were placed on puncture wounds on third leaves of 3 week-old cowpea (*Vigna sinensis*) grown in a greenhouse. After incubation for 72 h, the diameter of the resulting necrotic lesion was measured. There was no effect from the application of 70% Me_2CO containing 0.1% Tween 80 only, as a control.

Whole plant assay

Barnyardgrass (*Echinochola crus-galli* var. *crus-galli*), green foxtail (*Setaria viridis*), slender amaranth (*Amaranthus viridis*) and velvetleaf (*Abutilon theophrasti*) which were used for this assay, were grown in individual pots in a greenhouse for 10 days. Each solution (2 ml of 1000 ppm) of pure compounds dissolved in 70% Me_2CO containing 0.1% Tween 80 was sprayed over the 20 seedlings in each pot (100 × 100 mm). The treated plants were incubated in the greenhouse. There was no effect from the application of 70% Me_2CO containing 0.1% Tween 80 only, as a control.

Electrolyte leakage assay

This assay was carried out on the basis of a method reported by Duke *et al.* [11]. 6 mm diameter leaf discs were punched from expanded cotyledons of 10-day-old cucumber seedlings with a cork borer. The discs were soaked in a bathing medium containing 1% sucrose and 1 mM of 2-(*N*-morpholino)ethansulfonic acid as a non-ionic buffer and washed several times to remove cellular content which had leaked from broken cells before the experiments. 10 discs were floated on 1 ml of the bathing media in the wells (10 mm diameter) of 12 well-test plates. The testing compounds were dissolved in 10 μl of methanol, added to each well. The plates were incubated under continuous fluorescent light (12,000 lux) or in darkness at 25° in a growth chamber and the electrical conductivity of the bathing media was measured periodically using a conductivity meter. Each treatment was replicated three times.

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REFERENCES

1. Argoudelis, A. D., Coats, J. H. and Herr, R. R., *Antimicrobial Agents and Chemotherapy*, 1965, 801.
2. Hlubucek, J. R. and Robertson, A. V., *Aust. J. Chem.*, 1967, **20**, 2199.
3. Evans, R. H. Jr., Ellestad, G. A. and Kunstmann, M. P., *Tetrahedron Lett.*, 1969, **22**, 1791.
4. Yamamoto, I., Suide, H., Hemmi, T. and Yamano, T., *Takeda Kenkyusho Ho.*, 1970, **29**, 1.
5. Krasnoff, S. B. and Gupta, S., *J. Chem. Ecol.*, 1994, **20**, 293.
6. Tanaka, M., Ohra, J., Tsujino, Y., Sawaji, Y. and

Fujimori, T., *Biosci. Biotech. Biochem.*, 1994, **58**, 565.
7. Jones, R. N., Angell, C. L., Ito, T. and Smith, R. J. D., *Can. J. Chem.*, 1959, **37**, 2007.
8. Thompson, H. W., McPherson, E. and Lences, B. L., *J. Org. Chem.*, 1976, **41**, 2993.
9. Vanstone, D. E. and Stobbe, E. H., *Weed Sci.*, 1977, **25**, 352.
10. Duke, S. O., Vaughn, K. C. and Meeusen, P. L., *Pesticide Biochemistry and Physiology*, 1984, **21**, 368.
11. Duke, S. O. and Kenyon, W. H., in *Target Assays for Modern Herbicides and Related Phytotoxic Compounds*, ed. P. Boger and G. Standmann. Lewis Publishers, Boca Raton, 1991, pp. 61–66.
12. Haworth, P. H. and Hess, F. D., *Plant Physiol*, 1988, **86**, 672.
13. Scalla, R. and Matringe, M., *Rev. Weed Sci.*, 1994, **6**, 103.
14. Matsunaka, S., *J. Agr. Food Chem.*, 1969, **17**, 171.
15. Kunert, K. J. and Dodge, A. D., in *Target Sites of Herbicide Action*, ed. P. Boger and G. Standmann. CRC Press, Boca Raton, 1989, pp. 45–59.
16. Dodge, A. D., *Endeavour*, 1971, **30**, 130.
17. Mees, G. C., *Ann. Appl. Biol.*, 1960, **48**, 601.
18. Van Rensen, J. J. S., *Physiol. Plant.*, 1975, **33**, 42.