Gloeosporone – A Macrolide Fungal Germination Self-Inhibitor Total Synthesis and Activity

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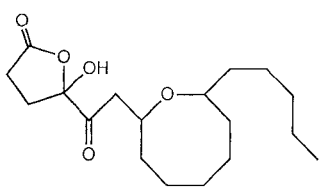
Starting with (*S*)- or (*R*)-4-Bromo-1,2-epoxybutane [commercially available or readily made from (*S*)- or (*R*)-malic acid], both enantiomers of the germination self-inhibitor gloeosporone were synthesized. The absolute configuration of the natural product was thus proven to be 4*S*,7*R*,13*R*. Microbiological studies showed that *both* enantiomers of the compound cause inhibition of germination in spores of *Colletotrichum gloeosporioides*, and that (–)-gloeosporone is also active against a variety of other fungi. In order to gain some insight into the mode of action, ^{23}Na NMR measurements and ion-selectivity studies were undertaken; none of these experiments provided evidence for a gloeosporone–metal ion interaction.

Gloeosporon – Ein Pilzkeimungs-Selbsthemmer mit Macrolidstruktur. – Totalsynthese und Aktivität

Ausgehend von (*S*)- oder (*R*)-4-Brom-1,2-epoxybutan [kommerziell erhältlich oder leicht und in hoher Ausbeute aus (*S*)- bzw. (*R*)-Äpfelsäure zugänglich] wurden die beiden Enantiomeren des Keimungs-Selbstinhibitors Gloeosporon hergestellt. Die absolute Konfiguration des Naturstoffes wurde damit als 4*S*,7*R*,13*R* bewiesen. Mikrobiologische Tests zeigen, daß *beide* Enantiomere von Gloeosporon die Keimungshemmung der Sporen von *Colletotrichum gloeosporioides* bewirken, und daß (–)-Gloeosporon gegenüber einer Reihe von anderen Pilzen aktiv ist. Zur Aufklärung des Wirkungsmechanismus wurden ^{23}Na-NMR- sowie Ionenselektivitätsmessungen durchgeführt; es konnten keine Gloeosporon–Metallion-Wechselwirkungen nachgewiesen werden.

Introduction and Retrosynthetic Analysis

In 1983, Meyer[4)] and co-workers reported the isolation of a compound from the fungus *Colletotrichum gloeosporioides* collected from leaf lesions of winged water primrose [*Jussiaea decurrens* (Walt.) DC.]. This natural substance, produced by the organism, acts to retard, decrease, or inhibit the growth of the organism producing it and as such is termed a "germination self-inhibitor". This phenomenon is important to the species to prevent crowding and destruction and to ensure a spatial distribution of the species[5)]. The natural product was given the trivial name gloeosporone.



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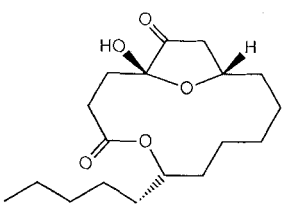

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The original structure **1** was assigned without regard to configuration and based solely on spectroscopic data[6)]. It was only after the first total synthesis of *cis*-**1** by Holmes[7)] and *trans*-**1** by Kocienski[8)] that the proposed structure of gloeosporone was found to be incorrect. In 1986 the correct structure of gloeosporone (**2**) was determined by single-crystal X-ray analysis[9)]. The absolute configuration, however, was not established at that time.

Our goals in synthesizing gloeosporone were twofold: to assign the absolute configuration as well as to provide ample quantities for biological testing[10)]. It was important to determine if the activity of the synthetic material is qualitatively similar to that of the natural inhibitor thereby defining the structure responsible for self-inhibition in the fungus. It has been suggested that gloeosporone may complex with certain metal ions and that their transport is involved in the inhibition of germination[6)]. The full account of our synthesis of both enantiomers of gloeosporone as well as some activity studies are reported herein[11)].

Our retrosynthetic analysis (*Scheme 1*) takes advantage of the known preference of gloeosporone to exist in its closed

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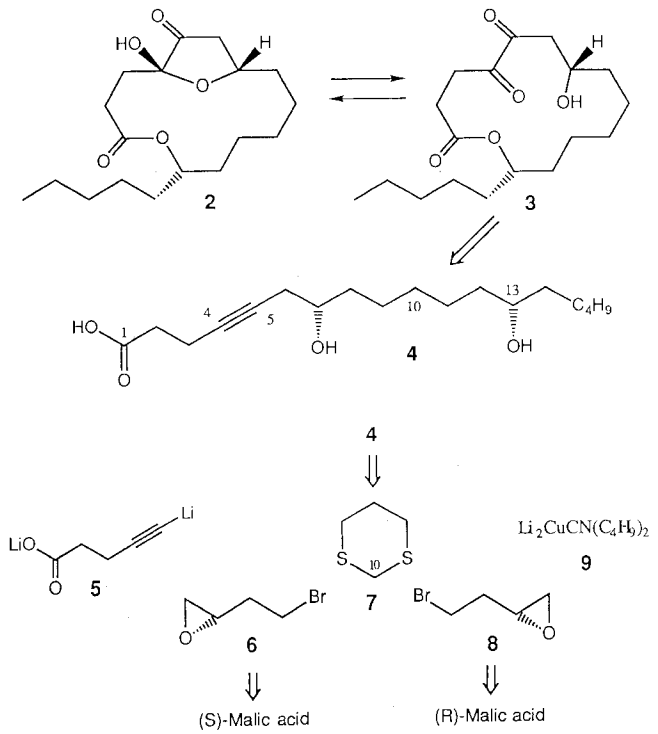
Scheme 1. Retrosynthetic analysis of gloeosporone (**2**)

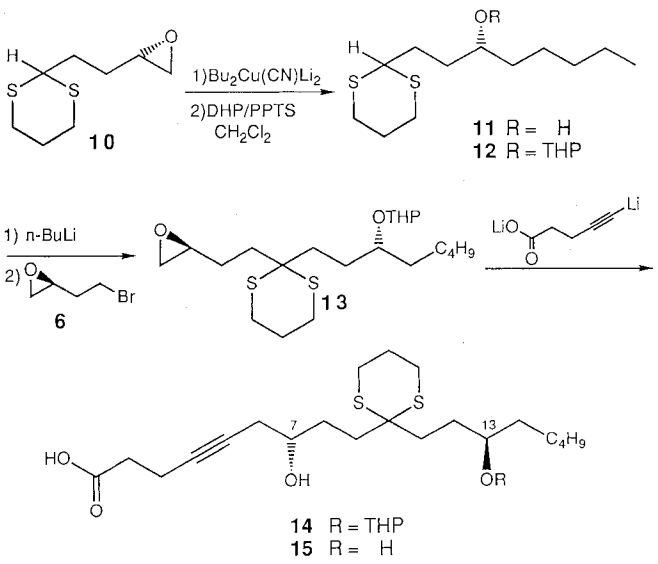


form[6]: we assumed that the hydroxy-1,2-diketone **3** would spontaneously cyclize to the correct C(4) epimer. Given that the dicarbonyl functionality would be derived from a C(4)–C(5) acetylene, opening of the lactone leads to dihydroxy acid **4**. On examining the C(6) to C(14) segment, a mirror plane containing C(10) is detected. Within this segment, the stereogenic centers at C(7) and C(13) are enantiotopic. For the construction of diol **4** the enantiomeric bromo epoxides **6** and **8** [available from (*S*)- and (*R*)-malic acid][12] could be linked together at C(10) utilizing the dithiane method[13]. The sequence of events must however prevent the formation of an intermediate *meso* compound. In the forward sense, lithio dithiane would be alkylated with bromide **8** followed by addition of the higher order[14] cuprate **9**. Then, alkylation with bromide **6** and introduction of the acetylene would lead to dihydroxy acid **4** in anticipation of macrolactonization. As depicted in Scheme 1, lactonization was planned to occur through carboxylic acid activation[15] utilizing the C(13)-hydroxyl as nucleophile. This would provide gloeosporone with the correct relative configuration. However, an alternative to this plan involved the use of *two* molecules of epoxide **6** [available from the more common (*S*)-malic acid] to produce a dihydroxy carboxylic acid that was epimeric to **4** at C(13). This would necessitate macrolactonization with inversion of configuration [at C(13)] by employing the well-known Mitsunobu reaction[16]. Since the absolute configuration of gloeosporone was unknown, we chose to prepare the enantiomer shown in Scheme 1 utilizing the same building block for incorporation of both stereogenic centers: (*S*)-(+)-4-bromo-1,2-epoxybutane[12] [commercially available (Fluka); derived from (*S*)-malic acid].

Total Synthesis of Both Enantiomers of Gloeosporone

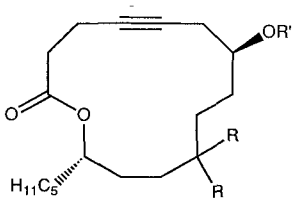
Our synthesis began with the known[17] epoxy dithiane **10** (Scheme 2), prepared from lithio dithiane and epoxy bromide **6**. The pentyl side chain was then created by epoxide-ring opening at the terminal carbon atom with lithio butyl cyano cuprate[14] (1.5 eq. THF, −20 °C). Protection of the newly formed hydroxy group of **11** as the THP ether [1.3 eq. DHP, PPTS (cat.), 4 h][18] gave the monosubstituted dithiane **12** in 83% overall yield from dithiane itself. The stereogenic center at C(7) was introduced by alkylation of the lithio derivative of **12** (1.1 eq. *n*-BuLi, THF, 5 h, −20 °C) with epoxy bromide **6**. We now focused our attention on incorporation of the C(1)–C(5) acetylenic carboxylic acid unit by reaction of doubly deprotonated 4-pentynoic acid[19] with epoxide **13**. Several counterions were examined for this transformation (Li, Na, Mg, Al, Zn) as well as a variety of solvent systems (HMPT, THF, Et_2O, DME, DMSO). Most conditions led to recovered starting materials or to very low yields of the desired product **14** (below 5%). Optimum conditions were found when 4-pentynoic acid was dissolved in neat HMPT at 0 °C, followed by the addition of 2.2 eq. BuLi in hexane. The crucial factor was to maintain the HMPT/hexane ratio at 2:1 or greater. Addition of epoxide **13** then gave (24 h, room temp.) the ring-opening product **14**, the THP group of which was removed [HOAc/THF/H_2O (4:2:1), 50 °C][20] to provide diol **15** in 40% yield (calcd. from **12**). With the complete carbon skeleton of gloeosporone established, the stage was set for exploring the macrolactonization.

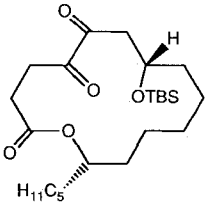
Scheme 2. Synthesis of dihydroxy acid **15**



We reasoned that under high-dilution conditions, the intramolecular esterification process would be the major reaction pathway. In fact, lactonization occurred, using the Mitsunobu reaction[16] (2 eq. DEAD, 2 eq. PPh_3, benzene, 0.005 M, 10 min), to provide **16** as the only detectable product in 67% yield. As predicted, no intermolecular esterification of the C(7)-hydroxyl group was observed.

Two major synthetic transformations remained in the gloeosporone synthesis, reductive removal of the dithiane moiety and oxidation of the acetylene to a 1,2-diketone. However, these presented a serious conflict because conventional methods for reductive removal of the dithiane (cf. Raney nickel) would destroy the triple bond and likely reduce a 1,2-diketone, whereas initial oxidation of the acetylene could alter the dithiane through sulfoxide or sulfone formation. A solution was found in the following two-step procedure for removal of the dithiane. Hydrolysis[13,21] of the dithiane in **16** (20 eq. MeI, aq. acetone, 4 h, 50 °C) provided ketone **17** in 92% yield. Then, formation of the tosyl hydrazone of **17** [1.1 eq. p-$CH_3C_6H_4SO_2NHNH_2$, p-TsOH, sulfolane/DMF (1:1), 100 °C, 15 min] followed by reduction (in one pot) with sodium cyanoborohydride[22] (4 eq. 3 h, 100 °C) gave macrolide **18** in 37% yield. In order to oxidize the acetylene without competing ketone formation at C(7) it was necessary to introduce a protecting group. We chose the *tert*-butyldimethylsilyl group since we planned to effect its removal and generate gloeosporone in one step. Alcohol **18** was allowed to react with *tert*-butyldimethylsilyl chloride [2 eq. NEt_3, 4-pyrrolidinopyridine (cat.), DMF][23] to give in 88% yield compound **19**. Oxidation of the acetylene occurred efficiently[24] using sodium periodate (4 eq.), catalytic ruthenium dioxide[25] [$CH_3CN/CCl_4/H_2O$ (2:2:3)] to give the brightly yellow dicarbonyl compound **20** (74%). Treatment of **20** with pyridine · $(HF)_x$ effected deprotection[26] of the C(7)-hydroxyl with concomitant disappearace of the yellow color.





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16 R,R = -S(CH$_2$)$_3$S- ; R' = H
17 R,R = O ; R' = H
18 R,R = H,H ; R' = H
19 R,R = H,H ; R' = TBS

Synthetic (+)-gloeosporone (**2**) directly isolated (80%) was identical to the natural product by spectroscopic comparison (500-MHz ^{1}H-NMR, 75-MHz ^{13}C-NMR, IR, MS). The synthetic material had m.p. 117–118 °C (hexane) whereas the melting point of the natural product was reported to be 108–110 °C. The optical rotation $[\alpha]_D$ of synthetic gloeosporone was +52 (c = 0.71 in $CHCl_3$). The rotation increased to +58 (c = 0.48) in acid-free chloroform, and in benzene it was measured to be +79 (c = 0.40). The optical rotation of the natural material was reported to be −14 (c = 0.28 in $CHCl_3$)[9]. We were led to believe that our synthetic material, with the absolute configuration 4*R*,7*S*,13*S*, was in fact the enantiomer of natural gloeosporone. The lack of agreement in the value of optical rotation, however, remained disturbing. In order to resolve the question of absolute configuration, we synthesized (−)-gloeosporone with configuration 4*S*,7*R*,13*R*, ultimately starting from (*R*)-malic acid. This enantiomer of gloeosporone had optical rotation of −61 (c = 0.34) in acid-free $CHCl_3$ and −72 (c = 0.34) in benzene. Activity studies of both enantiomers of gloeosporone showed that (−)-gloeosporone is in fact the natural product (see also the activity studies described below).

In conclusion, the first[11,27] total synthesis of (+)- and (−)-gloeosporone has been achieved in eleven steps (4.2% overall yield) from readily available starting materials, and the natural product was proved to have 4*S*,7*R*,13*R* configuration (enantiomer of **2**, i.e. *ent*-**2**).

Activity Studies on Gloeosporone

1) *Microbiological tests:* Like the natural product, synthetic (−)-gloeosporone (*ent*-**2**) inhibits germination of spores of *Colletotrichum gloeosporioides* (Penz.) *Sacc. f. sp. jussiaea.*, the synthetic and natural materials being active at comparable concentrations (4 μg · ml^{-1} for 50% inhibition in the present test vs. 2 μg · ml^{-1} reported for natural gloeosporone under slightly different conditions[5,28]). This confirms that it is indeed gloeosporone rather than an undetected contaminant in the natural samples which acts as the germination self-inhibitor.

Interestingly, germination is also reduced in the presence of synthetic (+)-gloeosporone, although its activity is somewhat smaller than that of the (−)-enantiomer. At a concentration of 4 μg · ml^{-1} (12 μM), synthetic *ent*-**2** reduced germination by one-half compared to control samples, whereas the (+)-enantiomer caused only a 30% reduction. Inhibition by both enantiomers diminishes upon progressive dilution (Table 1), disappearing at 2 μg · ml^{-1} for the (+)-enantiomer but remaining significant through 0.4 μg · ml^{-1} (1.2 μM) for (−)-gloeosporone.

Table 1. Germination of *C. gloeosporioides* spores in the presence of synthetic (−)- and (+)-gloeosporone, and ethanol controls[a]

Concentration of **2** and *ent*-**2** [μg ml^{-1}]	Conc. of EtOH [%]	% Germination EtOH control	*ent*-(−)-**2**	(+)-**2**
4	0.2	68	35	48
2	0.1	73	64	69[b]
0.4	0.02	90	86	91[b]
0.04	0.002	93	92[b]	93[b]
0.004	0.0002	89	92	93
0	0	91	–	–

[a] Spore concentration 10^5 · ml^{-1}, incubation for 5.5 h at 28 °C, four-day-old spores; average of two independent determinations. – [b] Not statistically different from control by chi-square test ($P > 0.05$).

This result suggests that gloeosporone might not interfere with the active site of an enzyme, since the two enantiomers would be expected to be differentiated more efficiently in the chiral environment of such a site.

(−)-Gloeosporone was also screened against a variety of other microorganisms. It was found that the natural enantiomer is active against some fungi (listed in Table 2), but does not show any influence on the growth of bacteria.

Part of the activity studies were performed in vitro, where the influence of gloeosporone on the growth of fungal mycelium was verified. In addition, fungi growing on plants were treated with aqueous solutions of gloeosporone (in vivo tests). For comparison, the results of these screening tests are shown with the data of a commercially available fungicide (Vitavax®, Table 2). (−)-Gloeosporone showed interesting activity against bean rust (*Uromyces appendiculatus*) and inhibited mycelial growth of several fungi in vitro including *Rhizoctonia solani* and *Ustilago maydis*. At higher dosages, also *Pyricularia oryzae*, *Pleospora betae*, *Moniminia fructigena*, and *Phytophtora cinnamoli* were inhibited in vitro, whereas no other disease besides bean rust could be controlled in vivo.

The inhibition of the most sensitive fungi was both during spore germination and vegetative growth. The most sensitive fungi belong to the class of Basidiomycetes including filamentous (*Rhizoctonia*) as well as yeast (*Ustilago*) fungi growing saprobically or biotrophically (*Uromyces*).

2) *Studies involving gloeosporone–metal interactions:* As mentioned above, inhibition of spore germination by both natural gloeosporone and its enantiomer suggests that the biological activity does not involve a direct gloeosporone/enzyme interaction, which is expected to be more highly enantioselective. One alternative possibility is that gloeosporone is involved in a process of ion transport across cell membranes that is key to germination or lack thereof, as has been proposed for some other fungal germination self-inhibitors[29].

To investigate the possibility that gloeosporone can influence transport of ions across biological membranes, the ability of synthetic *ent*-**2** to transport Na^+ across lipid bilayers of large unilamellar vesicles (LUV) was examined by ^{23}Na NMR. The general principles and technique, described in detail elsewhere[30], involve preparation of the LUV (which mimic the biomembranes) in the presence of the ionic species to be detected, so that part of the ionic system is encapsulated within the vesicles while the rest remains outside. Under these conditions the relative concentrations of $ions_{in}$ and $ions_{out}$ are at equilibrium, so there is no inside/outside ionic potential to influence transport. Addition of a lanthanide shift reagent, in this case dysprosium(III) tris(polyphosphate) (DyPPP), induces a high-field shift of the NMR signals of the ions with which it has contact, those *outside* the vesicles. With the signals of $ions_{in}$ and $ions_{out}$ thus distinguished, one can add samples of a potential transport agent and detect transport by line broadening or coalescence of the signals in a typical dynamic NMR experiment. Quantitative evaluation of line broadening in the range of intermediate NMR exchange times affords the transport rates, and examination of these as a function of the concentration of transport agent can be indicative of the molecularity of the transport process with respect to the agent.

This technique has been used successfully in studies of a variety of transport agents, both ion carriers (monensin[31], nigericin[32], valinomycin[33], etc.) and channel-formers (gramicidin D[30]). Typically, the transport of ^{23}Na, ^{7}Li, and/or ^{39}K has been followed. In those cases transport which is readily observable on the NMR time scale occurs with substrate concentrations of 0.5 mM or less, often even at the 1–10 μM level.

Examination of synthetic (−)-gloeosporone (*ent*-**2**) by this technique showed no ^{23}Na line broadening even with the concentration of *ent*-**2** at 3.6 mM, which is at least 10 times greater than concentrations at which transport is detectable with the ion carriers that have been investigated. Accordingly, (−)-gloeosporone does not serve as an ion carrier for Na^+ under these conditions.

To test whether gloeosporone serves as a ligand for other metal ions in solution, synthetic *ent*-**2** (1% by weight) was dissolved in a membrane consisting of 33% polyvinyl chloride (by weight), 66% *o*-nitrophenyl octyl ether (by weight) and 50 mol-% potassium tetrakis(4-chlorophenyl) borate[34,35]. This membrane was then used to study the ability of gloeosporone to selectively complex with metal ions (Figure 1). For better comparison, the ion selectivities (for a definition see ref.[36]) of a "blank" membrane and a membrane containing the Mg^{++}-selective ligand ETH 5282[37] are shown.

Figure 1 clearly shows that gloeosporone does not selectively complex with metal ions under the conditions studied.

Thus, the activity of gloeosporone may have to do with its hidden 1,2-diketone functionality: 1,2-Dicarbonyl compounds are used in peptide chemistry as specific reagents

Table 2. Microscreening of (−)-gloeosporone and Vitavax® against fungi in vivo (leaves) and in vitro (petri dishes)

Compound	Concentration[a]	Fungal disease intensity (in vivo)[b]						Fungal growth (in vitro)						
		PiL	BcV	CsH	SfC	EgH	UaP	Pc	Pu	Mf	Pb	Rs	Um	Po
ent-**2**	1000	1	1	3	1	0	9							
	100	1	1	1	1	0	3	9	7	9	9	9	9	9
	10	1	1	1	1	0	1	1	1	3	1	9	9	9
	1							1	1	1	1	7	7	3
Vitavax®	1000	7	7	3	9	0	9							
	100	1	1	1	1	0	9	1	1	1	9	9	9	9
	10	1	1	1	1	0	7	1	1	1	7	9	9	1
	1							1	1	1	3	3	9	3

[a] mg active ingredient per liter agar (in vitro) or per liter spray solution (in vivo). – [b] For abbreviations of the organisms and the definition of the values (1–9) see experimental part.

for arginine[38]. Among the compounds used are diketones like 1,2-cyclohexanedione and α-keto aldehydes such as phenylglyoxal. Our speculation, of whether the masked 1,2-dicarbonyl functionality of gloeosporone may participate in a similar reaction, is under investigation.

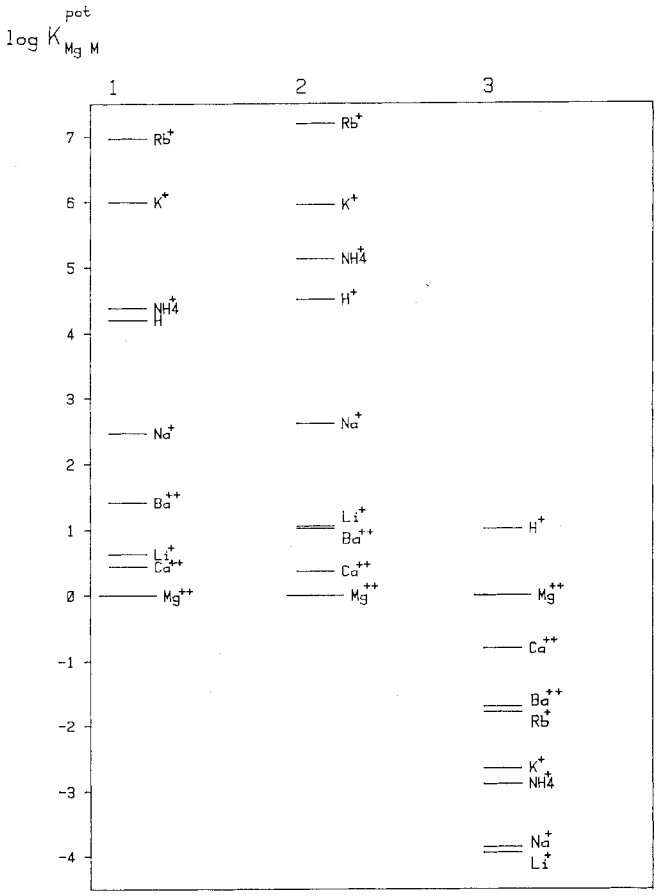

Figure 1. Comparison of the ion-selectivity of a membrane-containing synthetic (−)-gloeosporone (1), a ligand-free membrane (2) and a membrane containing the Mg^{++}-selective ligand ETH 5282 (3)

We gratefully acknowledge receipt of stipends from the *Fonds der Chemischen Industrie* (Germany) given to G. A., and from the *American Cancer Society, Inc.*, granted to R. Z.

Experimental

1) *General:* Melting points were determined with a Büchi-510 melting point apparatus with 50°C range thermometers (Anschütz). – Distillations were done using a Kugelrohr oven (Büchi GKR-50); the temperature of the air bath is given. – Optical rotations were done on a Perkin-Elmer 241 Polarimeter (1 ml solution in a 10-cm cell). – IR spectroscopy used a Perkin-Elmer spectrophotometer (values given in cm^{-1}). – ^1H NMR spectra were recorded on a Bruker WM 300 spectrometer; chemical shifts are given in δ values relative to tetramethylsilane as the internal standard (0 ppm), coupling constants J in Hertz; multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. – ^{13}C-NMR spectra were recorded on a Varian CFT-20 (25.5 MHz) spectrometer; chemical shifts are given in δ values relative to tetramethylsilane as the internal standard (0 ppm). – Mass spectroscopy utilized a Hitachi-Perkin-Elmer RMU-6M; intensities m/z are given in % to the base peak; [M] = molecular ion. – Thin-layer chromatography was done on TLC plates silica gel 60 F_{254}, 0.25 mm thick (Merck, Darmstadt), detection was by ultraviolet light (254 nm) and/or treatment with a solution of 9 ml *p*-methoxybenzaldehyde, 4 ml glacial acetic acid and 13 ml conc. sulfuric acid in 340 ml of ethanol, followed by heating. – Flash-column chromatography was performed according to ref.[39].

2) *Synthesis of Gloeosporone. – 2-[(3S)-3-Hydroxyoctyl]-1,3-dithiane* (**11**): Copper(I) cyanide (4.90 g, 54 mmol), dried azeotropically with benzene (two times), was suspended in 120 ml dry THF under argon and the reaction cooled to −78°C. To this was added *n*-BuLi (67.5 ml, 108 mmol, 1.6 M in hexane) and the reaction was warmed to −25°C. After 20 min, epoxide **10** (6.75 g, 35.5 mmol) in 15 ml THF was added dropwise so that the internal temperature remained around −20°C. The reaction was kept at −20°C for 2 h and then 100 ml of satd. NH_4Cl solution/sat. NH_4OH solution (4:1) was added. The reaction was stirred for 10 h at room temperature after which time ether was added and the phases separated. The aqueous phase was extracted with two 150 ml portions of ether. The combined organic phase was washed with water and brine, dried ($MgSO_4$), and filtered. Evaporation of the solvent in vacuo gave 8.70 g (97%) of the crystalline alcohol **11** suitable for use in the next reaction. For analytical data a sample was recrystallized; m. p. 64.0–64.8°C (ether/pentane), $[\alpha]_D$ = −4.9 (c = 1, $CHCl_3$). – IR (KBr): $\tilde{\nu}$ = 3350 cm^{-1}, 3220, 2920, 2850, 1465, 1265, 1130, 905, 650. – ^1H NMR ($CDCl_3$): δ = 0.89 (t, J = 7 Hz, 3H, H_3C), 1.26–2.01 (m, 14H, HO, 5-H_{eq}, 6 H_2C), 2.06–2.16 (m, 1H, 5-H_{ax}), 2.81–2.88 (m, 4H, H_2CS), 3.62 (m, 1H, *H*COH), 4.07 (t, J = 7 Hz, 1H, HCS_2). – MS: m/z (%) = 248 (28) [M^+], 14 (33), 132 (100), 119 (53), 81 (16), 67 (11), 55 (11).

$C_{12}H_{24}S_2O$ (248.45) Calcd. C 58.01 H 9.74
Found C 58.23 H 9.73

ent-**11**: m.p. 64–65°C (ether), $[\alpha]_D$ = +4.4 (c = 1.4, $CHCl_3$).

2-〈(3S)-3-[(2RS)-Tetrahydro-2-pyranyloxy]-octyl〉-1,3-dithiane (**12**): The alcohol **11** (8.57 g, 34.5 mmol) was dissolved in 210 ml dichloromethane. To this was added 1.75 g pyridinium *p*-toluenesulfonate and 6.5 ml (71 mmol) dihydropyran. The reaction mixture was stirred for 15 h at room temperature, after which ether (200 ml) and saturated sodium hydrogen carbonate solution (150 ml) were added. The layers were separated and the organic layer washed with brine, dried ($MgSO_4$), and the solvent evaporated in vacuo. Flash column chromatography (hexane/ethyl acetate, 12:1) of the crude material gave 8.95 g (78%) of the THP compound **12** as a colorless oil. Analytical data of the diastereoisomeric mixture: IR (film): $\tilde{\nu}$ = 2940 cm^{-1}, 2860, 1425, 1275, 1130, 1075, 1020. – ^1H NMR ($CDCl_3$): δ = 0.88 (m, 3H, H_3C), 1.28–1.97 (m, 19H, 5-H_{eq}, 9 H_2C), 2.07–2.15 (m, 1H, 5-H_{ax}), 2.81–2.88 (m, 4H, H_2CS), 3.46–3.51 (m, 1H, $HCCO_2$), 3.61–3.66 (m, 1H, $HCCO_2$), 3.86–3.93 (m, 1H, *H*COH), 4.00–4.09 (m, 1H, HCS_2), 4.64–4.66 (m, 1H, HCO_2). – MS: m/z (%) = 332 (1.5) [M^+], 247 (80), 231 (18), 229 (17), 132 (68), 119 (19), 107 (15), 85 (100), 67 (15), 55 (15), 41 (17).

$C_{17}H_{32}S_2O_2$ (332.57) Calcd. C 61.40 H 9.70
Found C 61.45 H 9.73

2-[(3S)-3,4-Epoxybutyl]-2-〈(3S)-3-[(2RS)-Tetrahydro-2-pyranyloxy]octyl〉-1,3-dithiane (**13**): Dithiane **12** (1.71 g, 5.15 mmol) was dissolved in 15 ml dry THF under argon and cooled to −40°C. Then *n*-butyllithium (3.3 ml, 5.4 mmol, 1.6 M in hexane) was added dropwise. The reaction temperature was kept at −20°C for 5 h, then the mixture was cooled to −78°C and the epoxide **6** (0.75 ml, 7.5 mmol) was added quickly. The reaction was allowed to warm

to −20°C over 12 h, after which time 20 ml of saturated NH_4Cl solution were added. The aqueous layer was extracted with ether and the combined organic extracts were washed with brine, dried ($MgSO_4$) and the solvent evaporated in vacuo. Epoxide **13** was obtained as a viscous oil (1.96 g, 92%), which was unstable to chromatography and distillation. The crude product was therefore used in the next step.

(7S,13R)-7-Hydroxy-10,10-[1,3-propanediylbis(thio)]-13-[(2RS)-tetrahydro-2-pyranyloxy]-4-octadecynoic Acid (**14**): 4-Pentynoic acid (0.59 g, 6 mmol) was dissolved in dry HMPT and cooled to 0°C under argon. To this was added *n*-butyllithium (8 ml, 12.4 mmol) and the mixture stirred for 1 h. Epoxide **13** (1.86 g, 4.6 mmol) in 5 ml HMPT was added and the reaction mixture warmed to room temperature and stirred for 24 h after which time the dark brown solution was poured into water. The aqueous layer was extracted with ether in order to remove nonacidic impurities. Then the aqueous layer was acidified to pH 2 using 6 N hydrochloric acid and extracted with two 75 ml portions of ether. These extracts were washed with brine, dried ($MgSO_4$), and the solvent was evaporated in vacuo. In practice, the protecting group was immediately hydrolyzed, however, a small sample of compound **14** was purified (flash chromatography, hexane/ethyl acetate, 1:2) for analytical data (diastereoisomeric mixture). – IR (film): $\tilde{\nu}$ = 3500 cm^{-1} (OH), 3000, 2940, 2860, 1720, 1600, 1450, 1430, 1270, 1130, 1070, 1020, 990. – ^1H NMR ($CDCl_3$): δ = 0.87–0.91 (m, 3H, CH_3), 1.29–2.47 (m, 28H, 13 CH_2, COOH, OH), 2.49–2.56 (m, 4H, $C\equiv C-CH_2-CH_2-COOH$), 2.72–2.91 (m, 4H, 2 H_2CS), 3.49–3.57 (m, 1H, *H*COH), 3.62–3.72 (m, 2H, 5-H, THP), 3.87–3.97 (m, 1H, 13-H), 4.68–4.71 and 4.78–4.80 (m, 1H, 2-H, THP). – MS: *m/z* (%) = 500 (3) [M^+], 416 (5.7), 393 (1.6), 381 (1.8), 309 (100), 291 (57.1), 274 (4.6), 197 (25.4), 145 (9), 107 (11.8), 85 (85.3), 55 (62.3), 41 (42.6).

$C_{26}H_{44}O_5S_2$ (500.76) Calcd. C 62.36 H 8.86
Found C 62.30 H 8.83

(7S,13R)-7,13-Dihydroxy-10,10-[1,3-propanediylbis(thio)]-4-octadecynoic Acid (**15**): The crude hydroxy acid **14** (2.0 g) was dissolved in 28 ml AcOH/THF/H_2O (4:2:1) and heated at 50°C for 12 h. Then the reaction mixture was cooled to room temperature and toluene was added. The solvent was evaporated in vacuo and the residue chromatographed (hexane/ethyl acetate, 1:2) to give the dihydroxy acid **15**, 0.86 g (40% starting from **11**) as a colorless oil; $[\alpha]_D$ = −7.9 (*c* = 1.1, $CHCl_3$). – IR (film): $\tilde{\nu}$ = 3420 cm^{-1}, 2950, 2930, 2860, 1720, 1425, 905. – ^1H NMR ($CDCl_3$): δ = 0.89 (t, *J* = 7 Hz, 3H, H_3C), 1.27–2.55 (m, 20H, 10 CH_2), 2.49 (app. s, 4H, CH_2-CH_2-COOH), 2.71–2.97 (m, 4H, 2 H_2CS), 3.65–3.76 (m, 2H, 2 *H*COH), 3.81 (s, br., 3H, 2 OH, COOH). – MS: *m/z* (%) = 416 (2) [M^+], 309 (100), 291 (91), 197 (54), 145 (19), 137 (21), 107 (31), 99 (32), 67 (40), 55 (86), 46 (86), 29 (32).

ent-**15**: $[\alpha]_D$ = +7.0 (*c* = 1.6, $CHCl_3$).

(9S,17S)-17-Hydroxy-9-pentyl-10-oxa-1,5-dithiaspiro[5.13]nonadec-14-yn-11-one (**16**): Triphenylphosphine (1.73 g, 6.61 mmol) and dihydroxy acid **15** (1.34 g, 3.31 mmol) were dissolved in 650 ml dry benzene under argon. Diethyl azodicarboxylate (1.0 ml, 6.6 mmol) was added quickly dropwise and the reaction mixture stirred for 10 min at room temperature. The benzene was removed in vacuo and the residue adsorbed onto silica gel. Flash column chromatography (hexane/ethyl acetate, 2.5:1) afforded lactone **16** (0.88 g, 2.2 mmol, 67%) as a colorless oil. – $[\alpha]_D$ = −70.4 (*c* = 1.28, $CHCl_3$). – IR (film): $\tilde{\nu}$ = 3520 cm^{-1}, 2950, 2930, 2860, 1723, 1455, 1380, 1233, 1170, 905. – ^1H NMR (300 MHz): δ = 0.89 (t, *J* = 7 Hz, 3H, H_3C), 1.23–2.66 (m, 25H, OH, 12 CH_2), 2.73–2.90 (m, 4H, 2 H_2CS), 3.65–3.71 (m, 1H, *H*COH), 4.93–4.97 (m, 1H, HCOCO). – MS: *m/z* (%) = 398 (2) [M^+], 291 (100), 207 (7), 145 (9), 107 (11), 67 (13), 55 (22), 41 (24).

ent-**16**: $[\alpha]_D$ = +68 (*c* = 1.4, $CHCl_3$).

(8S,14S)-8-Hydroxy-14-pentyl-1-oxacyclotetradec-5-yne-2,13-dione (**17**): Macrolide **16** (155 mg, 0.39 mmol) was dissolved in 12 ml acetone/water (5:1). To this was added MeI (0.48 ml, 7.8 mmol) and the reaction kept at 50°C for 20 h. The reaction mixture was then cooled to room temperature and poured into ether/saturated sodium hydrogen carbonate solution. The layers were separated and the aqueous layer extracted with two 50 ml portions of ether. The organic layers were combined, washed with brine, dried ($MgSO_4$), and the solvent evaporated in vacuo. Flash column chromatography (hexane/ethyl acetate, 2:3) gave ketone **17** (110 mg, 0.357 mmol, 92%) as a colorless oil. – $[\alpha]_D$ = −11.5 (*c* = 0.93, $CHCl_3$). – IR (film): $\tilde{\nu}$ = 3450 cm^{-1}, 2960, 2930, 1759, 1730, 1430, 1370, 1340, 1265, 1165, 1040. – ^1H NMR ($CDCl_3$): δ = 0.88 (t, *J* = 7 Hz, H_3C), 1.22–2.13 (m, 14H, 7 CH_2), 2.34–2.68 (m, 9H, $C\equiv C-CH_2-CH_2-CO$, $CH_2-CO-CH_2$, OH), 3.73–3.76 (m, 1H, *H*COH), 4.94–5.03 (m, 1H, HCOCO). – MS: *m/z* (%) = 290 (0.1) [$M - H_2O]^+$, 233 (77), 197 (100), 179 (24), 161 (27), 105 (26), 95 (45), 91 (53), 85 (69), 55 (67), 41 (43).

$C_{18}H_{28}O_4$ (308.42) Calcd. C 70.10 H 9.15
Found C 69.98 H 9.22

ent-**17**: $[\alpha]_D$ = +10.7 (*c* = 0.95, $CHCl_3$).

(8S,14S)-8-Hydroxy-14-pentyl-1-oxacyclotetradec-5-yn-2-one (**18**): Keto lactone **17** (158 mg, 0.513 mmol) was dissolved in 8 ml DMF/sulfolane (1:1) and 18 mg *p*-toluenesulfonic acid was added followed by 119 mg (0.641 mmol) tosylhydrazine. The reaction mixture was heated to 110°C and after 10 min 129 mg (2 mmol, 4 eq.) $NaBH_3CN$ was added. The reaction was kept at 110°C for 3 h, cooled to room temperature and poured into water. The aqueous layer was extracted with hexane (three times 50 ml) and the combined organic layers were washed with brine, dried ($MgSO_4$), and the solvent evaporated in vacuo. Flash column chromatography (hexane/ethyl acetate, 4:1) provided lactone **18** as a crystalline compound (55.7 mg, 37%) with m.p. 85–86.5°C (ether/pentane). – $[\alpha]_D$ = −23.9 (*c* = 0.57, $CHCl_3$). – IR (KBr): $\tilde{\nu}$ = 3350 cm^{-1}, 3005, 2930, 2860, 1745, 1460, 1430, 1375, 1165. – ^1H NMR ($CDCl_3$): δ = 0.88 (t, *J* = 7 Hz, 3H, H_3C), 1.22–1.77 (m, 16H, 8 CH_2), 2.28–2.68 (m, 5H, H_2C-H_2C-CO, OH), 3.68–3.76 (m, 1H, *H*COH), 5.05–4.97 (m, 1H, HCOCO). – MS: *m/z* (%) = 294 (0.1) [M^+], 276 (1), 183 (32), 165 (46), 112 (100), 109 (36), 95 (50), 70 (43), 55 (37), 41 (27).

$C_{18}H_{30}O_3$ (294.43) Calcd. C 73.43 H 10.27
Found C 73.50 H 10.35

ent-**18**: m.p. 84–86°C (ether/pentane), $[\alpha]_D$ = +23.4 (*c* = 0.8, $CHCl_3$).

(8S,14S)-8-[(tert-Butyldimethylsilyl)oxy]-14-pentyl-1-oxacyclotetradec-5-yn-2-one (**19**): Lactone alcohol **18** (46.4 mg, 0.158 mmol) was dissolved in 0.5 ml DMF and a few crystals 4-pyrrolidinopyridine, 48.4 µl (0.347 mmol) triethylamine, as well as 47.6 mg (0.316 mmol) *tert*-butyldimethylsilyl chloride were added. The reaction mixture was stirred at room temperature for 20 h under argon, then poured into water (50 ml) and extracted with two 30 ml portions of hexane. The combined organic layers were washed with brine, dried ($MgSO_4$), and the solvent evaporated in vacuo. Flash column chromatography (hexane/ethyl acetate, 10:1) afforded the silyl ether **19** (55.9 mg, 87%) as a colorless oil. – $[\alpha]_D$ = −6.6 (*c* = 0.85, $CHCl_3$). – IR (film): $\tilde{\nu}$ = 2930 cm^{-1}, 2860, 1750, 1460, 1255, 1165, 1090, 835. – ^1H NMR ($CDCl_3$): δ = 0.04 [s, 6H, $Si(CH_3)_2$], 0.85–0.89 (m, 12H, *t*Bu, CH_3), 1.28–1.79 (m, 18H, 9 CH_2),

2.10–2.67 (m, 6H, $CH_2-C\equiv C-CH_2-CH_2-CO$), 3.71–3.77 (m, 1H, HCOSi), 5.00–5.06 (m, 1H, HCOCO). – MS: *m/z* (%) = 409 (0.1) $[M + 1]^+$, 351 (78), 297 (23), 217 (60), 185 (25), 169 (100), 133 (20), 73 (94), 55 (19).

$C_{24}H_{44}SiO_3$ (408.7) Calcd. C 70.53 H 10.85
Found C 70.66 H 11.31

ent-**19**: $[\alpha]_D = +5.5$ ($c = 0.9$, $CHCl_3$).

(8S,14S)-8-[(tert-Butyldimethylsilyl)oxy]-14-pentyl-1-oxacyclotetradec-5-yne-2,5,6-trione (**20**): The silyl ether **19** (34 mg, 83.4 mmol) was dissolved in 7 ml $H_2O/CH_3CN/CCl_4$ (3:2:2) and sodium periodate (99 mg, 0.46 mmol) was added followed by 4–7 mg of RuO_2 hydrate. The reaction mixture was stirred vigorously for 1.5 h and then poured into water/dichloromethane, and the layers were separated. The aqueous layer was extracted with three 30 ml portions of dichloromethane, the combined organic layers were washed with brine, dried ($MgSO_4$), and filtered through an 8-cm column of celite. The solvent was removed in vacuo to give the bright yellow dicarbonyl compound **20** (30.6 mg, 69.5 μmol, 83%). – IR (film): $\tilde{\nu}$ = 2950 cm^{-1}, 2930, 2860, 1735, 1720, 1710, 1600, 1460, 1260, 1110, 1090, 930, 840. – ^1H NMR ($CDCl_3$): $\delta = 0.07$ [s, 6H, $Si(CH_3)_2$], 0.87–0.90 (m, 12H, *t*Bu, CH_3), 1.06–1.63 (m, 18H, 9 CH_2), 2.63 (*AMX*, J_{AM} = 14.3 Hz, J_{AX} = 4 Hz, 1H, 7-H), 2.10–2.67 (m, 4H, 3-H, 4-H), 3.28 (A*M*X, J_{AM} = 14.3 Hz, J_{MX} = 10.2 Hz, 1H, 7-H), 4.16–4.20 (m, 1H, HCOSi), 4.89–4.92 (m, 1H, HCOCO).

$C_{24}H_{44}SiO_5$ (440.7) Calcd. C 65.41 H 10.06
Found C 65.90 H 9.95

(1R,6S,12S)-1-Hydroxy-6-pentyl-5,15-dioxabicyclo[10.2.1]pentadecane-4,14-dione [(+)-gloeosporone, **2**]: The dicarbonyl compound **20** (38.6 μmol, 17 mg) was dissolved in 2 ml THF and cooled to 0°C. To this was added 20 drops of pyridine · $(HF)_x$ complex (Fluka). The reaction mixture was stirred at room temperature for 70 min (TLC!), after which time the yellow color had disappeared. The heterogenous solution was then poured into ether (100 ml) and washed successively with water, saturated sodium hydrogen carbonate solution and brine. The organic layer was dried ($MgSO_4$) and the solvent evaporated in vacuo. Pure synthetic gloeosporone was obtained by PTLC (Merck plates 20 × 20 cm, 0.5 mm thickness, hexane/ethyl acetate, 2:1, two developments), in 80% yield (10.1 mg). The synthetic material was identical to the natural product by IR, ^1H NMR (500 MHz), ^{13}C NMR (75 MHz), and MS. However, the optical rotation and the melting point were significantly different from the reported data[9], necessitating the synthesis of (–)-gloeosporone starting from (*R*)-malic acid. The data obtained from the two enantiomers are as follows ⟨$[\alpha]_D$ were measured in HCl-free (Alox-filtered) chloroform⟩:

Compound	Melting point	$[\alpha]_D^{\text{room temp.}}$
"natural" (–)-gloeosporone	108–110°C	–30, c = 0.08
synthetic (+)-gloeosporone	115–117°C	+58, c = 0.48
synthetic (–)-gloeosporone	117–118°C	–61, c = 0.34

Comparison of the ^{13}C NMR shift values of (+)-gloeosporone and natural (–)-gloeosporone[9] (values of the latter in parentheses): 209.0 (209.0), 174.4 (174.5), 99.0 (98.9), 74.4 (74.5), 73.5 (73.3), 40.4 (40.4), 34.6 (34.6), 32.3 (32.3), 32.2 (32.2), 31.7 (31.7), 30.0 (30.0), 29.5 (29.5), 26.0 (25.9), 25.3 (25.3), 24.9 (25.2), 22.5 (22.5), 21.2 (21.1), 14.0 (14.0).

3) *C. Gloeosporioides Spore Germination Bioassays:* The isolate of *C. gloeosporioides* was that from which gloeosporone was originally isolated[5], and had been stored in a cryofreezer since 1985. The revitalized culture demonstrated an inverse relationship between spore concentration and percent germination, confirming that the spores remained sensitive to germination self-inhibition[5]. Media, culture incubation, spore washing procedures, and assay conditions were as described earlier[5] except for the following differences:

a) spores were harvested after four days of incubation in liquid culture;

b) the amount of 95% ethanol to dissolve the enantiomers of gloeosporone was reduced by one-half (1 mg/0.5 ml), and corresponding control concentrations of ethanol were also tested;

c) a one-half dilution of inhibitor solutions and controls was added to the 10-fold dilutions described previously;

d) spores in solutions were incubated 5.5 h at 28°C before germination was stopped with lactophenol cotton blue. More than 300 spores were counted per duplicate plate. χ^2 Tests for the comparison of proportions in two independent samples were used to determine significant differences in % germination between treatments.

4) *^{23}Na- and ^7Li-NMR Measurement of Ion Transport:* LUV-containing 100 mM NaCl and buffered with K_2HPO_4/H_3PO_4 to a pH of 8.2 were prepared from 4:1 phosphatidylcholine and phosphatidylglycerol as described earlier[30]. ^{23}Na NMR showed that ca. 10–15% of the total aqueous volume was inside the vesicles. The NMR sample was prepared by adding a solution of 1.6 mg (4.9 μmol) of synthetic *ent*-**2** in 160 μl of methanol to 0.6 ml of filtered vesicles, agitating with a vortex mixer, incubating for 90 min in a 50°C circulating water bath, cooling to room temperature, and diluting 1:1 with an aqueous solution of DyPPP (synthesized as described in ref.[40]) such that the final concentration of DyPPP outside the vesicles was 5 mM and the net concentration of synthetic (–)-gloeosporone was 3.6 mM. The sample was allowed to equilibrate overnight before data aquisition. Accumulation of 2000 FIDs was conducted at probe temperatures of both 24.1 and 49.0°C. Neither set showed significant broadening of the $^{23}Na_{in}$ line compared to a control sample which was prepared identically except for the omission of *ent*-**2**.

5) *Activity of Gloeosporone Against Plant Pathogenic Fungi.* – a) *In vitro tests:* Two milliliters of cell suspension of each organism were mixed with 100 ml of liquid agar at 45°C (malt extract agar). With an Eppendorf multipipette, agar pills (100 μl) containing the organism were placed on the bottom of empty petri dishes. Three agar pills per organism and concentration of the chemical [synthetic (–)-gloeosporone or Vitavax®, respectively] were used to evaluate the fungicidal activity of the chemical. Then, 10-ml portions of ethanolic suspension of the chemical were placed on top of the agar pills after they had solidified. After the ethanol had evaporated, the agar pills were incubated in humid chambers for 7 days at 24°C in the dark.

b) *In vivo tests:* Leaf discs, 14 mm in diameter (tomato, beans, cucumber), or leaf pieces, 10 mm in length (barley), were produced from susceptible varieties grown in the greenhouse. The leaf discs were spray-treated by applying 0.2 ml of ethanolic suspension of the chemical per petri dish containing six leaf discs. The treated discs were inoculated with spore suspensions (10^4–10^5 per ml) either by applying two drops per disc, each 10 μl in quantity (*Phytophthora, Botrytis*), or by spraying spore suspension on the discs (other fungi) 24 hours after they have been treated with the chemical. The discs were incubated on water agar containing 15 mgl^{-1} benzimidazole (except for *Botrytis* and *Cochliobolus*) for seven to ten days at 18–24°C, depending on the fungus, under a 14/10 hours light/dark cycle.

c) *Evaluation:* Fungal growth in the agar pill was evaluated visually using the following rating system: 1 unchanged growth; 3 inhibited growth; 7 strongly inhibited growth; 9 no growth. Disease intensity on the leaf disc was rated visually using a similar rating system: 1 strong disease intensity; 3 medium disease intensity; 7 little disease intensity; 9 no disease.

d) *Microorganisms*

PiL	*Phytophthora infestans* on *Lycopersicon*
BcV	*Botrytis cinerea* on *Vicia*
CsH	*Cochliobolus sativus* on *Hordeum*
SfC	*Sphaerotheca fuliginea* on *Cucumis*
EgH	*Erisyphe graminis* on *Hordeum*
UaP	*Uromyces appendiculatus* on *Phaseolus*
Pc	*Phythophthora cinnamomi*
Pu	*Pythium ultimum*
Mf	*Monilinia fructigena*
Pb	*Pleospora betae*
Rs	*Rhizoctonia solani*
Um	*Ustilago maydis*
Po	*Pyricularia oryzae*

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