Biological Characterization of Fusapyrone and Deoxyfusapyrone, Two Bioactive Secondary Metabolites of *Fusarium semitectum*

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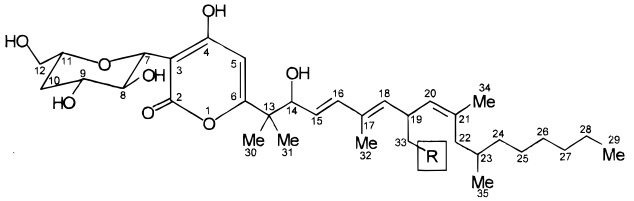
Fusapyrone (**1**) and deoxyfusapyrone (**2**), two α-pyrones originally isolated from rice cultures of *Fusarium semitectum*, were tested in several biological assays. Compounds **1** and **2** showed considerable antifungal activity against several plant pathogenic and/or mycotoxigenic filamentous fungi, although they were inactive toward yeasts isolated from plants and the Gram-positive bacterium *Bacillus megaterium* in disk diffusion assays. Compound **1** was consistently more active than **2**. Among the tested fungi, *Fusarium* species were the least sensitive to the two pyrones, while *Alternaria alternata*, *Ascochyta rabiei*, *Aspergillus flavus*, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Phoma tracheiphila*, and *Penicillium verrucosum* were the most sensitive. Compounds **1** and **2** also showed good inhibitory activity toward agents of human mycoses. Aspergilli were the most sensitive, while some species-specific variability was found among the *Candida* spp. In an *Artemia salina* larvae bioassay, **1** was not toxic at the highest concentration tested (500 μM), whereas the LC_{50} of **2** was 37.1 μM (21.8 μg/mL). Neither **1** nor **2** was phytotoxic in a panel of assays that monitored plant-cell toxicity, as well as wilt-, chlorosis-, and necrosis-inducing activity. Moreover, **2** stimulated the root elongation of tomato seedlings at doses of 10 and 100 μM. In consideration of the biological activities evidenced in this study, **1** and **2** appear to be potential candidates for biotechnological applications, as well as good models for studies on mechanism(s) of action and structure−activity relationships.

Members of the genus *Fusarium* are widespread throughout the world as soil inhabitants, plant pathogens, and food and feed contaminants.[1,2] Many *Fusarium* species have been investigated for their capability to produce bioactive secondary metabolites, and a number of molecules exhibiting a variety of structures as well as chemical and biological properties have been described so far.[3,4] Because of the obvious health and economic implications, *Fusarium* metabolites toxic to animals (mycotoxins) or plants (phytotoxins) have been the focus of most of our research. However, a number of bioactive compounds whose ecological significance and natural occurrence are not yet completely understood have also been isolated and structurally characterized.

Fusapyrone (FP) (**1**) {3-(4-deoxy-*β*-*xylo*-hexopyranosyl)-4-hydroxy-6-[2-hydroxy-7-hydroxymethyl-1,1,5,9,11-pentamethyl-3,5,8-heptadecatrienyl]-2H-pyran-2-one} and deoxyfusapyrone (DFP) (**2**), its 6-[2-hydroxy-1,1,5,7,9,11-hexamethyl] analogue, are two bioactive metabolites whose structures and chemical properties were reported by Evidente et al.[5] Structurally, **1** and **2** are 3-substituted-4-hydroxy-6-alkyl-2-pyrones that consist of a highly functionalized aliphatic chain and a 4-deoxy-*β*-*xylo*-hexopyranosyl C-glycosyl moiety bound, respectively, to the C-6 and C-3 of the 2-pyrone ring. In 1989, Chaiet et al. reported the production of a new antifungal α-pyrone by a *Fusarium* strain (*Fusarium* sp. ATCC 20883).[6] Based on the spectroscopic data (IR, UV, ^1H and ^{13}C NMR, and EI mass spectra) and the proposed structure of the antibiotic,[7] this compound might be identical to **1**, although the identity of the compounds cannot be established in the absence of stereochemical data. Other secondary metabolites containing the pyran-2-one moiety (α-pyrones) have been reported to be produced by fungi belonging to several genera, including *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, and *Trichoderma*, and exhibit a wide range of biological activities, such as antibiotic, antifungal, cytotoxic, neurotoxic, and phytotoxic.[8] Members of this class of compounds have also been investigated for their potent antitumor[9,10] and HIV protease-inhibiting[11−13] properties, and for their plant growth-regulating activity.[14−16] The chemistry, biochemistry, and toxicology of microbial α-pyrones have been reviewed by Dickinson.[8] Besides FP and DFP, other α-pyrones isolated and characterized from *Fusarium* cultures include fusalanipyrone,[17] acuminatopyrone,[18] the mycotoxin chlamidosporol[19] and its analogues,[20] and the phytotoxins poaefusarin and sporofusarin.[8]



1 Fusapyrone R = OH ($C_{34}H_{54}O_9$, MW 606)

2 Deoxyfusapyrone R = H ($C_{34}H_{54}O_8$, MW 590)

FP (**1**) and DFP (**2**) were originally isolated from rice cultures of *F. semitectum* Berk. & Rav. (strain ITEM-393) by using the growth inhibition of the fungus *Geotrichum candidum* Link ex Pers. as a bioassay to guide the isolation process. However, a more in-depth investigation of their

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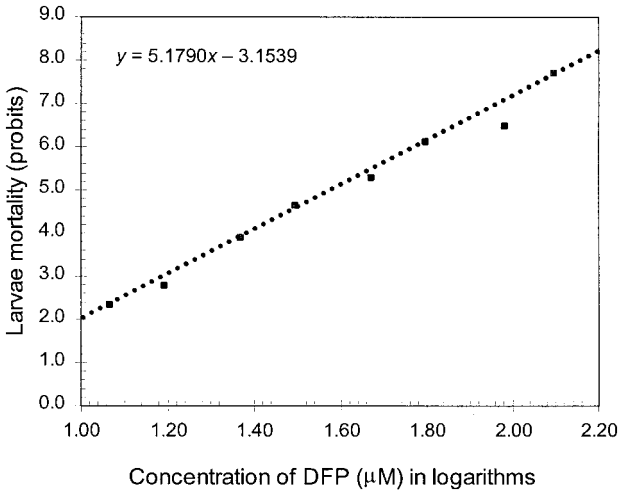

Figure 1. Regression line of the transformed dosage−mortality curve of *A. salina* larvae exposed to DFP (**2**). Mortality frequencies in terms of probits are plotted vs. logarithms of **2** concentrations. No significant differences in mortality of larvae exposed to **2** for 24 or 36 h were observed (data not shown), hence only the 24-h data sets were used for calculation of the dosage−mortality curve. Data shown are the means of six independent experiments. LC_{50} = 37.1 μM (21.8 μg/mL).

biological properties seems to be desirable. In this paper, we report a wider biological characterization of these compounds resulting from evaluation of the antimicrobial activities of **1** and **2** toward filamentous fungi and yeasts, as well as their zootoxic and phytotoxic activities.

Results and Discussion

The zootoxicities of **1** and **2** were evaluated using the brine shrimp (*Artemia salina* L.) larvae mortality bioassay. This assay has long been utilized as a simple, rapid, and reliable method to detect antitumor or cytotoxic activity.[21] Compounds **1** and **2** were tested in concentrations ranging from 11.7 to 500 μM. Compound **1** was not toxic to *A. salina* larvae at the highest concentration tested. The LC_{50} value of DFP (**2**) was 37.1 μM (21.8 μg/mL) (Figure 1).

The antifungal activities of **1** and **2** were tested on 18 species (24 strains) of plant pathogenic and/or mycotoxigenic filamentous fungi, 11 strains of yeasts isolated from plants, and 10 agents of human mycoses. Both **1** and **2** showed considerable antifungal activity against several filamentous fungi but were inactive against yeasts at the assay dose of 15 μg/disk (Table 1). A wide variability of susceptibilities to the two pyrones was observed, hence their activities seemed to be species- and strain-specific. *Fusarium* species, with the exception of *F. graminearum*, were the least sensitive, while *Alternaria alternata*, *Ascochyta rabiei*, *Aspergillus flavus*, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Phoma tracheiphila*, *Penicillium verrucosum*, and *Penicillium brevi-compactum* were the most sensitive species. Compound **1** was consistently more active than **2**. In several cases, **1** was as active or more active than nystatin against *Ph. tracheiphila* and *P. brevi-compactum*. Compound **1** was mostly fungistatic at 5 μg/disk and fungicidal at 15 μg/disk. However, *Ph. tracheiphila* ITEM 1605, *P. verrucosum* ITEM 439, and *B. cinerea* strains were killed after exposure to 5 μg/disk of **1**. Both **1** and **2** were fungicidal to *Asc. rabiei* at 5 μg/disk (data not shown).

Among the agents of human mycoses that were examined, *Aspergilli* were the most sensitive to **1** and **2**, while some species-specific variability was found among the yeasts (Table 2). In particular, *Cryptococcus neoformans* was inhibited by both **1** and **2**, *Candida kefyr* only by **1**, and *Candida albicans* and *Candida glabrata* were inhibited by neither compound at doses as high as 50 μg/mL. Although **1** was more active than **2**, particularly toward *C. kefyr*, the difference in the activity of the two compounds for human pathogens was less marked than that observed for phytopathogenic fungi.

Table 1. Antifungal Activity of FP (**1**) and DFP (**2**) in Comparison with Nystatin Toward Some Filamentous Fungi and Yeasts (paper disk assay [15 μg/disk])

species and strain[b]	diameter of the inhibition zone (mm)[a] FP	DFP	nystatin
Filamentous fungi			
Alternaria alternata ITEM 468	18.8 ++	10.8 ++	30.2 +++
A. alternata ITEM 511	18.0 +++	11.0 +	32.2 +++
A. alternata ITEM 526	20.3 +++	11.7 +	28.7 +++
A. alternata ITEM 750	18.7 ++	10.5 +	29.2 +++
A. citri ITEM 466	18.0 ++	9.2 +	30.0 +++
Ascochyta rabiei ITEM 1067	35.8 +++	12.0 +++	38.5 +++
Aspergillus flavus ITEM 9	27.3 ++	12.4 ++	29.0 +++
A. parasiticus ITEM 11	27.5 ++	12.7 ++	28.7 +++
Botrytis cinerea ITEM 966	29.3 +++	13.5 +++	31.2 +++
B. cinerea	30.0 +++	17.0 +	31.0 +++
Cladosporium cladosporioides ITEM 2079	28.7 ++	16.2 +	37.3 +++
C. cucumerinum ITEM 2095	33.3 +++	13.5 ++	32.7 +++
Colletotrichum gloeosporioides ITEM 1729	31.8 +++	13.8 ++	38.2 +++
Fusarium acuminatum ITEM 795	17.8 +	0	20.8 +++
F. graminearum ITEM 2	26.5 +++	10.8 ++	25.9 +++
F. moniliforme ITEM 1497	20.1 +	0	18.7 +++
F. oxysporum ITEM 149	27.7 +	4.0 +	19.5 +++
F. oxysporum ITEM 1462	28.3 +	0	20.0 +++
F. oxysporum f. sp. lycopersici ITEM 1586	30.0 +	4.0 +	21.7 +++
F. subglutinans	6.0 +	0	24.0 +++
F. semitectum ITEM 393	28.7 ++	8.0 +	27.0 +++
Phoma tracheiphila ITEM 1605	40.0 +++	16.3 +++	31.8 +++
Penicillium verrucosum ITEM 439	29.7 +++	11.0 ++	28.2 +++
P. brevi-compactum ITEM 449	30.0 +++	10.5 ++	18.3 +++
Yeasts			
Candida guilliermondii ITEM 1638	0	0	20.0 +++
C. maltosa ITEM 1639	0	0	20.0 +++
Kluyveromyces fragilis ITEM 1657	0	0	19.5 +++
K. lactis ITEM 1656	0	0	18.0 +++
Pichia anomala ITEM 1625	0	0	18.8 +++
P. anomala ITEM 1661	0	0	15.0 +++
P. dryadoides ITEM 1663	0	0	19.0 +++
P. guilliermondii ITEM 1644	0	0	18.5 +++
P. kluyveri ITEM 1649	0	0	16.5 +++
Saccharomyces cerevisiae ITEM 1633	0	0	21.0 +++
Rhodotorula pilimanae	0	0	28.5 +++

[a] Activity is classified as: 0 = no effect; + = weakly fungistatic (reduced density of fungal growth); ++ = fungistatic (no growth); +++ = fungicidal. [b] ITEM codes refer to the Istituto Tossine e Micotossine da Parassiti Vegetali Culture Collection.

The phytotoxicity of **1** and **2** to plant cells and organs was also determined and compared to fusaric acid (FA), a well-known *Fusarium* phytotoxin. The results of the cytotoxicity bioassay performed on chickpea cells are shown in Figure 2. Compounds **1** and **2** were much less phytotoxic than FA, their LC_{50} values being about one tenth that of FA (Figure 2). Neither α-pyrone was toxic at a concentration of 10^{-5} M.

In the tomato-leaf puncture assay, only **1** was active, causing necrotic spots at concentrations of 10^{-2} and 10^{-3}

Table 2. Antifungal Activity of FP (**1**) and DFP (**2**) toward Yeasts and Filamentous Fungi of Human Mycoses

species and strain		minimum inhibitory concentration (μg/mL) 24 h	48 h	72 h
Candida kefyr Y0601	FP	0.78	1.56	3.12
	DFP	>50.00		
C. albicans Y01009	FP	>50.00		
	DFP	>50.00		
C. albicans 1	FP	>50.00		
	DFP	>50.00		
C. albicans 2	FP	>50.00		
	DFP	>50.00		
C. glabrata 12	FP	50.00	>50.00	
	DFP	>50.00		
Cryptococcus neoformans 13	FP	6.25	6.25	6.25
	DFP	6.25	6.25	6.25
Cryptococcus neoformans 14	FP	3.12	3.12	3.12
	DFP	3.12	3.12	3.12
Aspergillus fumigatus 1	FP	1.56	1.56	1.56
	DFP	3.12	3.12	3.12
A. niger 2	FP	1.56	3.12	3.12
	DFP	1.56	1.56	1.56
A. flavus 3	FP	1.56	1.56	1.56
	DFP	1.56	1.56	1.56

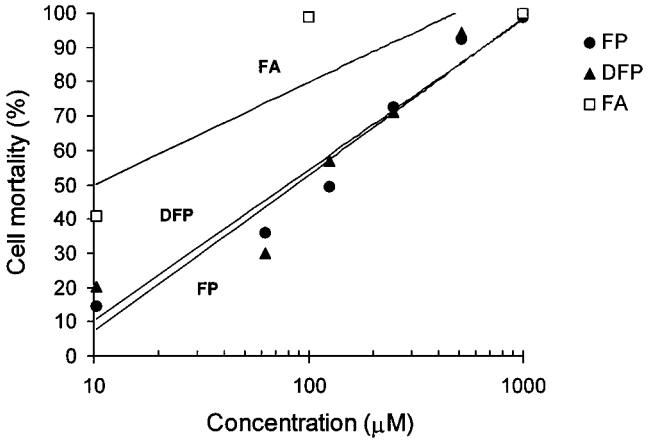

Figure 2. Toxicity of FP (**1**), DFP (**2**), and FA to chickpea cells. Data shown are the means from three independent experiments.

M (2-mm and 1-mm diameter, respectively). When the leaf-puncture assay was performed on chickpea leaves, both toxins caused chlorosis and necrosis at concentrations ≥ 10^{-3} M. By comparison, FA caused severe symptoms on both tomato and chickpea leaves at 5×10^{-4} and 10^{-4} M, respectively.

No symptoms were observed on tomato cuttings treated with 10^{-3} M of either **1** or **2**, while the first symptom of phytotoxicity (chlorosis of leaf veins) to cuttings treated with FA was detected at the concentration of 10^{-5} M, and complete wilting (leaves and stems) was observed at 10^{-4} M.

Neither **1** nor **2** showed phytotoxic activity in the tomato-seedling germination assay. On the contrary, at the doses of 10^{-4} and 10^{-5} M, **2** stimulated the elongation of rootlets, the values being more than 120% of the control (Figure 3). Shoot length was unaffected by treatment with either **1** or **2**.

FP (**1**) and DFP (**2**) are two bioactive metabolites of *Fusarium* of which very little is known. The availability of literature data on the toxicity of *Fusarium* mycotoxins toward *A. salina* allowed us to compare the toxicological relevance of **1** and **2** with those toxins. While **1** was not toxic to *A. salina*, the toxicity of **2** was similar to fusaproliferin (LC_{50} = 23.7 μg/mL),[22] but 10- to >100-fold lower

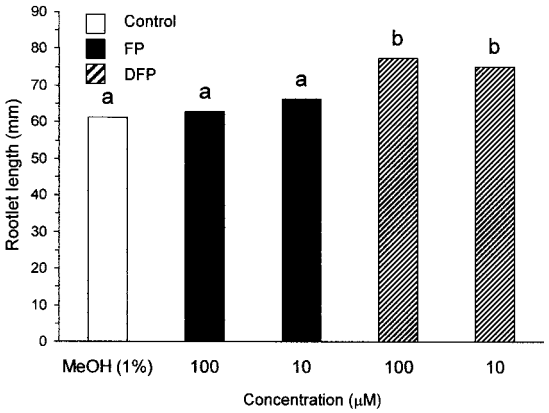

Figure 3. Tomato seedling growth assay. Bars with the same letter were not different according to Duncan's multiple range test ($p = 0.05$).

than the beauvericin[23] and the trichothecenes.[24] However, some *F. semitectum* strains have been shown to produce more than 1000 ppm of **2** when grown on autoclaved corn kernels (Altomare et al., unpublished). So far, *F. semitectum* is the only species of *Fusarium* reported to produce **2**. *F. semitectum* occurs mostly as a saprophyte in soil and on decaying plant tissues,[2,25] but it has also been found in association with cereal grains,[26] soybean seeds,[27] peanuts,[28] and bananas.[29] To evaluate the actual mycotoxicological significance of **2**, it would be useful to investigate the production of this toxin by other *Fusarium* species and its occurrence in naturally infested agricultural commodities.

According to McLaughlin,[21] compounds with LC_{50} < 1000 ppm in the brine-shrimp lethality assay are considered active and potentially cytotoxic against tumor cell lines. DFP (**2**) showed a noteworthy toxicity in this assay, indicating that it may be an interesting compound to be tested in more specific antitumor systems.

Compounds **1** and **2** showed antifungal activity against filamentous fungi, while no activity was observed against yeasts isolated from plants. In addition, in an agar diffusion assay, both **1** and **2** were inactive toward the Gram-positive bacterium *Bacillus megaterium* at the dose of 30 μg/disk (data not shown). Compounds **1** and **2** also showed a differential antifungal activity toward difficult-to-treat human pathogenic fungi such as *Aspergillus* spp. Interestingly, *C. kefyr*, an emergent opportunistic pathogen, showed a remarkable sensitivity only to **1**.

Neither **1** nor **2** were phytotoxic in a panel of assays that evaluated wilt-, chlorosis-, and necrosis-inducing activity. Furthermore, DFP (**2**) showed plant growth-regulating activity, as it stimulated the root elongation of tomato seedlings. Plant growth regulating activity has also been reported for other molecules belonging to the chemical family of α-pyrones, although with different findings. While neovasinone, a metabolite from *Neocomospora vasinfecta*, was reported to promote the root growth of lettuce seedlings,[14] 6-pentyl-α-pyrone from *Trichoderma harzianum* inhibited coleoptile elongation of etiolated wheat germlings.[30] Interestingly, the phytotoxic activity of α-pyrones is greatly affected by different moieties bound to the common active core, the α-pyrone ring.

In the past decades, bioactive metabolites of microbial origin have been the subject of scientific research in several fields, including pharmacology, food science, mycotoxicology, and plant pathology. A relatively novel and promising field of study is the application of these compounds in agriculture, as pesticides, herbicides, or plant-growth regulators.[31] In fact, microbial metabolites are expected to overcome the resistance and pollution that have accompa-

nied the use of synthetic pesticides and can inspire the synthesis of new environmental friendly molecules. The considerable antifungal activity of FP (**1**) is of some interest for its possible use in agriculture, especially in consideration of the low phytotoxicity and mycotoxicity evidenced in this work.

Experimental Section

***Artemia salina* Bioassay.** Assays were performed in 24-well culture plates (Corning, NY). Each well contained 30−40 larvae in 500 μL of marine water [3.3% (wt/vol) marine salts in distilled water]. Both **1** and **2** were dissolved in methanol (MeOH) and serially diluted to obtain a range of concentrations. The methanolic solutions of **1** or **2** were transferred to the wells to a final solvent concentration of 1% (vol/vol). Tests were performed in quadruplicate. The percentage of larvae mortality was determined after exposure to the α-pyrones for 24 and 36 h at 27 °C. The dosage−mortality curves of **1** and **2** were calculated with data from six independent experiments by using the standard procedure of probit analysis.

Antifungal Activity of 1 and 2. An agar diffusion method was utilized to test the activities of **1** and **2** against filamentous fungi and yeasts isolated from plants. Activities of the two compounds were compared with the antifungal antibiotic nystatin (Sigma, St. Louis, MO). Three milliliters of 0.7% (wt/vol) water−agar containing 10^4 conidia/mL of the test fungus or a suspension of yeast cells were poured into 9-cm diameter Petri dishes containing 7 mL of solidified potato−dextrose−agar (PDA) (Difco, Detroit, MI). After solidification of the water−agar layer, 6-mm diameter cellulose disks (Difco), previously impregnated with methanolic solutions of **1**, **2**, or nystatin and air-dried, were laid on the agar surface. The substances were tested at the dose of 5 and 15 μg/disk. Plates were incubated at 25 °C for 24−72 h, depending on the germination time of the test fungus, and the antifungal activity was evaluated by the diameter of the growth inhibition halo of three replicated disks. Afterward, the disks were removed and small pieces of agar (about 2 × 2 mm) underneath the disks were transferred to fresh PDA in order to check the fungicidal effect of **1** and **2**. After 24−72 h of incubation at 25 °C, the PDA plates were checked for the growth of colonies. Antifungal activity was scored into four classes: fungicidal effect (+++), fungistatic effect (++), weak fungistatic effect with reduced density of growth within the inhibition zone (+), and no effect (0). The experiment was repeated twice with three replicates, and the results were averaged.

The determination of the minimal inhibitory concentration of **1** and **2** against yeast and mold clinical isolates has been achieved by a broth micromethod well assay. Briefly, 10 2-fold dilutions (50−0.097 μg/mL) of each compound were made using liquid Yeast Nitrogen Base (YNB) (Difco) with 0.5% glucose, in phosphate buffer pH 7. The medium, including the appropriate concentrations of the compounds, was distributed in aliquots of 180 μL for each concentration into the first 10 microwells of each row. Medium controls (medium without compounds) were also included. The inoculum was obtained from 24- to 48-h-old fungal cultures grown on Sabouraud agar plates. Each microwell was inoculated with 20 μL of fungal suspension to obtain a final concentration of 5 × 10^2 cells of *C. albicans* and *C. kefyr*, and 5 × 10^3 cells or conidia of *C. glabrata*, *Cryptococcus neoformans*, and *Aspergillus* spp., respectively. The plates were incubated at 35 °C for a maximum of 72 h, and growth was observed every 24 h. The complete absence of growth was considered to be related to minimum inhibitory concentration in comparison with the growth observed in the control wells.

Phytotoxic Activity of FP and DFP. Plant Cell Assay. Two-week-old chickpea plants were watered 30 min prior to cell isolation. Leaves were cut into small pieces that were imbued with an enzyme cocktail solution under reduced pressure until the tissues turned dark. The enzyme cocktail consisted of 2% Cellulase R10 (Kinki Jakult Co., Shingikancho Nishinomiya, Japan), 0.3% Macerozyme R10 (Kinki Jakult), and 0.07% Pectolyase Y23 (Sigma), dissolved in holding buffer (citric acid monohydrate, 10.5 g/L; glucose, 100 g/L; $MgSO_4 \cdot 7H_2O$, 1 mM; K_2HPO_4, 1 mM; $CaCl_2 \cdot 2H_2O$, 5 mM; NaOH, 6.2 g/L, adjusted to pH 5.55 with HCl 0.1 M).[32] The digested material was filtered through one layer of 200 mesh nylon, and then washed three times by centrifuging at 350 rpm for 5 min at 10 °C in the holding buffer. Cell viability was checked by vital staining with fluorescine diacetate (FDA). Solutions at different concentrations of **1** and **2** were placed in 96-well plates (50 μL/well), and 50 μL of cell suspension were transferred into each well (final assay doses were 10^{-5} to 10^{-3} M of **1** or **2** in 1% MeOH). Fusaric acid (FA) was used as a positive control at the doses of 10^{-5}, 10^{-4}, and 10^{-3} M. Cells were incubated at 25 °C for 3 h in the dark, and after incubation the cells were stained with FDA. FDA was prepared as a stock solution in acetone (5 mg/mL) and stored at −20 °C. The stock solution was diluted (1:50) with holding buffer just before use, and 50 μL were added to each well. Then, 30-μL samples of cell suspension were transferred onto a microscope slide, and the viability of 30 cells was assessed under a fluorescence microscope (cells with intact plasma membranes fluoresced yellow-green, while dead cells remained unstained). The experiment was repeated twice.

Leaf Puncture Assay. Tomato and chickpea leaves were utilized for this assay. Toxins were dissolved in a small volume of MeOH and then brought up to the assay concentration with distilled water (the final content of MeOH was 4%). Each toxin was assayed at concentrations ranging from 10^{-2} to 10^{-7} M. Fully expanded young leaves were detached from plants, and 15 μL of the test compound were applied both on the adaxial and abaxial sides of leaves that had previously been needle-punctured. Droplets of MeOH in distilled water (4%) and FA at the same concentrations of **1** and **2** were applied on leaves as controls. Each treatment was repeated three times. The leaves were then kept in a moist chamber to prevent the droplets from drying. The effect of the toxins on the leaves, consisting in chlorotic or necrotic spots surrounding the puncture, were observed after 5 days.

Tomato Cutting Assay. Compounds **1** and **2** were assayed on tomato cuttings at the stage of five true leaves. Toxins were dissolved in a small volume of MeOH and then brought up to the final concentration with distilled water. Each toxin was assayed at four different concentrations (10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M). Young plants grown in the greenhouse were cut with a razor blade. Stems were immersed in the solutions containing the toxins and kept in a growth chamber at 22 °C for 24 h under fluorescent light with a photoperiod of 12 h. Plants were then transferred to distilled water and kept in the same conditions as above for 48 hours more, and the symptoms were visually evaluated.

Tomato Seedlings Assay. Tomato seeds were surface sterilized with NaClO (4%) for 10 min, thoroughly washed with sterile distilled water, and left to germinate for 3 days in the dark onto wet filter paper in glass Petri dishes. Seeds were then transferred into 5-cm diameter Petri dishes (10 seeds/dish) containing filter paper impregnated with 2 mL of a solution of the toxin in 1% MeOH. Seeds were incubated in a growth chamber at 25 °C under a 12-h photoperiod for 4 days, and then both the shoot and the rootlet length were measured. Compounds **1** and **2** were assayed at 10^{-4} and 10^{-5} M concentrations. The experiment was carried out in triplicate and repeated once.

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