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pH Modulation of Zopfiellin Antifungal Activity to *Colletotrichum* and *Botrytis*

MITSUGU FUTAGAWA,[†] AGNES M. RIMANDO,[‡] MARIO R. TELLEZ,[‡] AND DAVID E. WEDGE*,[‡]

Shiraoka Research Station of Biological Science, Nissan Chemical Industries, Ltd., 1470 Shiraoka, Minamisaitama, Saitama 349-0294, Japan, and Natural Products Utilization Research Unit, USDA-ARS, P.O. Box 8048, University, Mississippi 38677

Zopfiellin, a novel cyclooctanoid natural product isolated from *Zopfiella curvata* No. 37-3, was evaluated in a 96-well microtiter assay for fungicidal activity against *Botrytis cinerea*, *Colletotrichum acutatum*, *Colletotrichum fragariae, Colletotrichum gloeosporioides*, and *Fusarium oxysporum*. Zopfiellin exhibited pH-dependent activity, with the most mycelial growth inhibition demonstrated at pH 5.0. Mass spectrometry and nuclear magnetic resonance spectroscopy studies indicated that zopfiellin undergoes structural changes with changes in pH. At pH 5.0, zopfiellin showed the greatest activity against *B. cinerea* (IC_{80} = 10 μM), *C. gloeosporioides* (IC_{80} = 10 μM), and *C. fragariae* (IC_{80} = 10 μM) and intermediate activity against *C. acutatum* (IC_{80} = 30 μM), and was not active against *F. oxysporum* (IC_{80} > 100 μM).

KEYWORDS: Fungicide; natural products; zopfiellin; *Zopfiella curvata*; *Botrytis cinerea*; *Colletotrichum acutatum*; *Colletotrichum fragariae*; *Colletotrichum gloeosporioides*; *Fusarium oxysporum*

1. INTRODUCTION

Certain species of filamentous fungi of the genera *Botrytis*, *Colletotrichum*, and *Fusarium* are considered major plant pathogens worldwide (*1*). Failure to control these fungi can result in serious economic losses to U.S. and worldwide agriculture (*2*). Anthracnose, caused by *Colletotrichum* spp., and *Botrytis* flower and fruit blights are serious diseases of strawberry (*Fragaria* x *ananassa* Duch.) fruit and plant production in many areas of the world, but they are especially serious in the southeastern United States (*3*, *4*). *Fusarium oxysporum*, which infects orchids and other ornamental crops, is drawing attention as a serious fungal pathogen as shorter crop production cycles become more prevelant to meet the rapidly expanding market for orchids and other ornamental crops (*5*). At the USDA-ARS, Natural Products Utilization Research Unit, research is ongoing to discover natural product-based control agents against these pathogens (*6*, *7*).

Strobilurins, a new class of fungicides available commercially as azoxystrobin and kresoxim-methyl, have received much attention as important fungicidal agents for the control of agricultural pathogenic fungi (*8*−*10*). However, the appearance of resistant strains in several countries including the United States and Mexico has raised concerns about the use of strobilurins. The increasing occurrence of plant pathogens that are insensitive to commercial fungicides continues to fuel the exploration of new natural product-based fungicides. Particularly desirable is the discovery of novel prototypes that exert activity by modes of action different from those of existing antifungal agents (*11*−*13*).

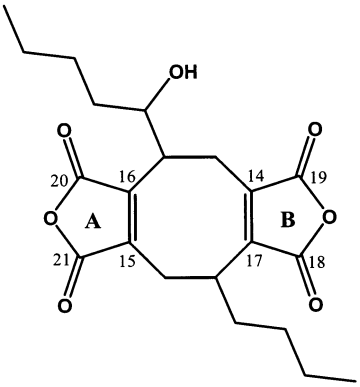

Figure 1. Zopfiellin.

Zopfiellin, a novel cyclooctanoid natural product isolated from the ascomycete *Zopfiella curvata* (Fuckel) Winter, produced by isolate No. 37-3, emerged as a promising natural antifungal agent in a fungicide discovery program undertaken by Nissan Chemical Industries Ltd. (*14*) (**Figure 1**). Preliminary in vitro studies performed at Nissan Chemical laboratories indicated that zopfiellin possessed selective activity against *Botrytis cinerea* (IC_{50} = 2.0 μM), *Sclerotinia sclerotiorum* (IC_{50} = 4.0 μM), and *Aspergillus niger* (IC_{50} = 8.0 μM) (*15*). In this study, we evaluated zopfiellin for its potential use as a control agent against agriculturally important pathogenic *Colletotrichum* and *Fusar-*

* Address correspondence to David E. Wedge, USDA-ARS-NPURU, The National Center for Natural Products Research, University, MS 38677. Tel.: 662-915-1137. Fax: 662-915-1035. E-mail: dwedge@olemiss.edu.
[†] Nissan Chemical Industries, Ltd.
[‡] USDA-ARS.

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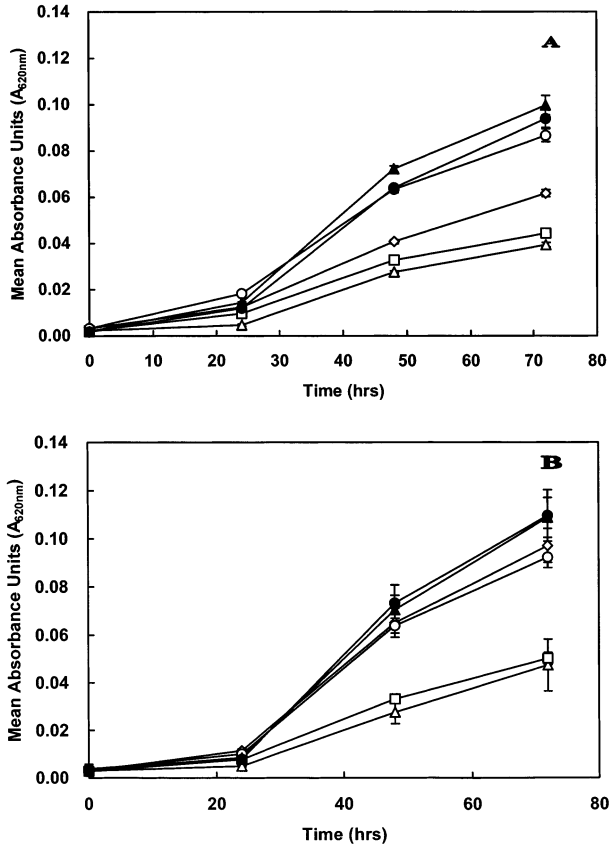

Figure 2. Effect of pH on growth of *Botrytis cinerea* in PDB (A) and RPMI (B) culture media in 96-well microtiter plates. In both media, growth, measured photometrically, was most favorable at pH between 6.0 and 7.0 at 48 and 72 h. (△, pH 4.5; □, pH 5.0; ◇, pH 5.5; ○, pH 6.0; ●, pH 6.5; and ▲, pH 7.0)

ium species. In the process, we found that the activity of zopfiellin was pH dependent. We report on the pH-dependent activity of zopfiellin against *B. cinerea*, *C. gloeosporioides*, and *C. fragariae*. We also present spectroscopic data in support of changes in the structure of zopfiellin with changes in pH.

2. MATERIALS AND METHODS

2.1. Pathogen Production and Inoculum Preparation. Isolates of *Colletotrichum acutatum* Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. were obtained from B. J. Smith, USDA, ARS, Small Fruit Research Station, Poplarville, MS. These *Colletotrichum* species were isolated from strawberry (*Fragaria* x *ananassa* Duchesne). *Botrytis cinerea* Pers.:Fr. isolated from strawberry was obtained from F. J. Louws, Department of Plant Pathology, North Carolina State University, Raleigh, NC. *Fusarium oxysporum* Schlechtend:Fr. was isolated from orchid (*Cynoches* sp.) by D. E. Wedge and identified to species by W. H. Elmer, Department of Plant Pathology and Ecology, The Conneticut Agricultural Experiment Station, New Haven, CT. Fungal cultures were grown on potato dextrose agar (PDA, Difco, Detroit, MI) in 9-cm Petri dishes and incubated in a growth chamber at 25 ± 2 °C with a 12-h photoperiod under 55 ± 5 μmol light·m^2·s^{-1}.

Conidial suspensions were prepared according to published procedures (*16*). Conidial concentrations were determined photometrically (*16*, *17*), and suspensions were adjusted, based on a standard curve, with sterile distilled water to a stock concentration of 1.0×10^6 conidia/mL.

2.2. Fungicide-Amended Agar Assay. The antifungal activity of zopfiellin was evaluated in a preliminary study using a simple amended agar method (*18*, *19*). *Botrytis cinerea* was grown on 1/10 strength potato dextrose agar (PDA; 2.4 g/L DIFCO, diluted 1:10 v/v with demineralized water) amended with two levels of zopfiellin (0 and 25 mg/mL) of in 9-cm Petri dishes. The pH of the medium was adjusted to either 4.8 or 7.0 using 0.02 M potassium phosphate. Cultures inoculated from spores were incubated for 2 weeks in a growth chamber at 25 ± 2 °C under cool white fluorescent lamps at 55 ± 5 μmol light·m^2·s^{-1} with 12-hr photoperiods. The activity of zopfiellin was evaluated visually after 2 weeks by comparing the mycelial growth between the control and the 25 mg/mL treatment.

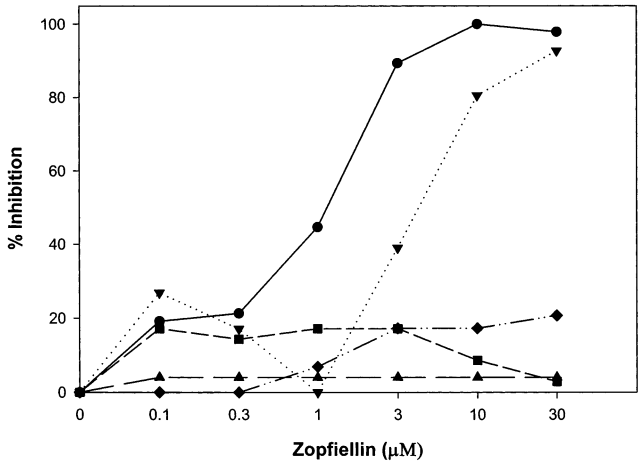

Figure 3. Effect of pH on the activity of zopfiellin against *Botrytis cinerea*. Percent growth inhibition is reported at 72 h. Zopfiellin exhibited pH-dependent activity at pH 5.0 (●) and 5.5 (▼), with greater mycelial growth inhibition observed at pH 5.0 (LC_{50} = 1.0 μM). At pH 6.0 (■), 6.5 (◆), and 7.0 (▲), the activity of zopfiellin appeared to be the same across the range of concentrations tested.

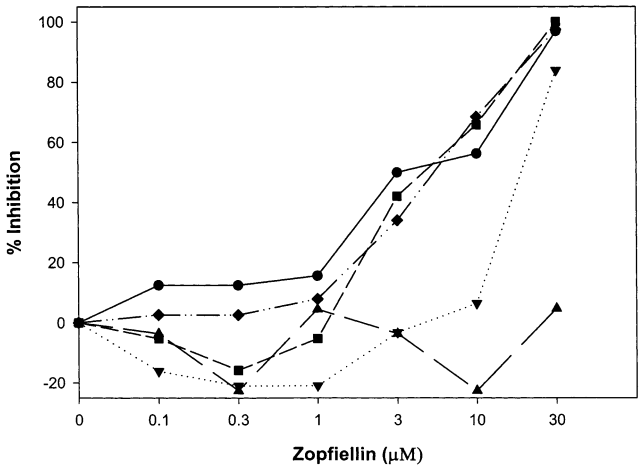

Figure 4. Activity of zopfiellin against *Fusarium oxysporum* (▲), *Colletotrichum acutatum* (▼), *C. fragariae* (■), *C. gloeosporioides* (◆), and *Botrytis cinerea* (●). Percent growth inhibition is reported for all fungi at 48 h. IC_{80} = 10 μM for *B. cinerea*, *C. gloeosporioides*, and *C. fragariae*; 30 μM for *C. acutatum*, and >100 μM for *F. oxysporum*.

2.3. Microtiter Assay. A 96-flat-bottom-well microtiter plate (untreated; Nunc Micro Well, Roskilde, Denmark) was used in initial experiments to determine the optimum pH for growth using *B. cinerea*, and in subsequent assays to evaluate the effectiveness of zopfiellin in inhibiting spore germination and mycelial growth of *B. cinerea*, *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, and *F. oxysporum*. Technical-grade benomyl, vinclozolin, and iprodione (Chem Service, West Chester, PA) were used as internal standards. Technical-grade zopfiellin was obtained from Nissan Chemical Industries Ltd. (Minamisaitama, Saitama, Japan). All media were filter-sterilized using a 500-mL, 0.2-μm filtration unit (Nalge Nunc International Corp., Rochester, NY).

Each test well received 90 μL of buffered growth medium, 100 μL of conidia at 1.0×10^4/mL, and 10 μL of zopfiellin solution. The final concentration of conidia in each well was 5×10^3/mL. The test

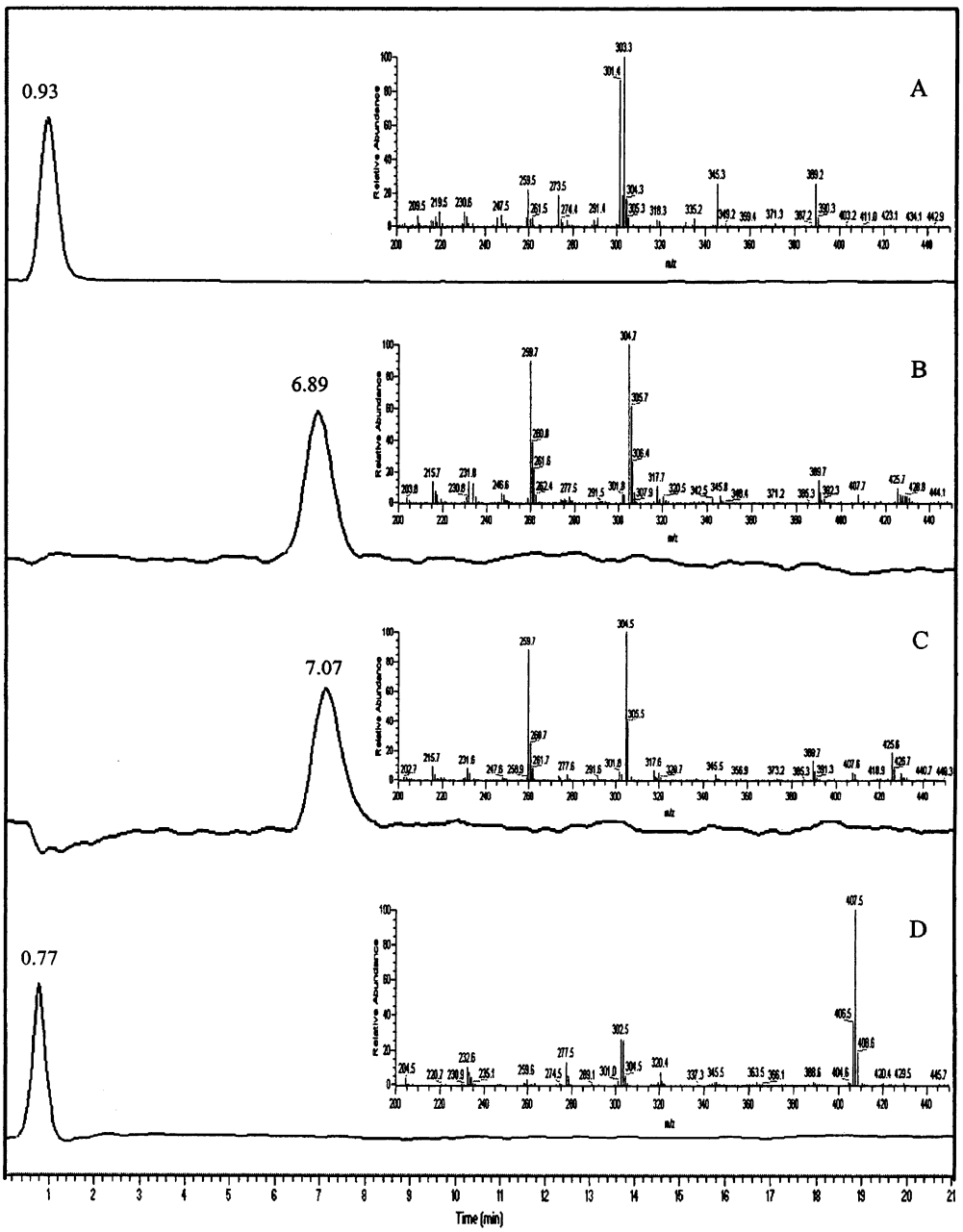

Figure 5. LC–MS analysis of zopfiellin. Chromatogram of zopfiellin (A) dissolved in and eluted with CH_3CN; (B) dissolved in CH_3CN and eluted with 1:1 CH_3CN:H_2O; (C) dissolved in 1:1 CH_3CN:1% aqueous CH_3COOH and eluted with 1:1 CH_3CN:H_2O; and (D) dissolved in 1:1 CH_3CN:1% aqueous NH_4OH and eluted with 1:1 CH_3CN:H_2O.

organisms were treated with increasing doses of zopfiellin, where the final concentrations in the well were 0.0, 0.1, 0.3, 1.0, 3.0, 10.0, and 15.0 μM. The microtiter plates were incubated in a growth chamber at 25 °C under cool fluorescent lights as described in section 2.1. Growth, in the form of fungal biomass, was evaluated at 0, 24, 48, and 72 h by measuring the absorbance at 620 nm using a Packard SpectraCount microplate photometer (Packard Instrument Co., Meriden, CT).

2.3.1. Effect of pH on Growth of and Antifungal Activity of Zopfiellin against *Botrytis.* Initial studies to determine the effect of pH on growth of *B. cinerea* were performed using two different culture media, i.e., potato dextrose broth (PDB; 2.4 g/L DIFCO, diluted 1:10 v/v with demineralized water) and Roswell Park Memorial Institute mycological media (RPMI; 16.2 g/L, Life Technologies, Grand Island, NY). The pH of the culture medium was adjusted from pH 4.5 to 7.0 in increments of 0.5 using either 0.02 M lactate buffer (for pH 4.5−6.0) or 0.02 M phosphate buffer (for pH 6.5−7.0).

In subsequent experiments, using PDB as the culture medium, the effect of pH on the activity of zopfiellin (at concentrations of 0.0, 0.1, 0.3, 1.0, 3.0, 10.0, and 15.0 μM) against *B. cinerea* was investigated.

2.3.2. Antifungal Activity of Zopfiellin against *Colletotrichum* and *Fusarium* spp. The antifungal activity of zopfiellin was evaluated for *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, and *F. oxysporum*. In these experiments, the pH of the culture medium was adjusted to 5.0 (0.02 M phosphate buffer). Zopfiellin was tested at final concentrations of 0.0, 0.1, 0.3, 1.0, 3.0, 10.0, and 15.0 μM in the wells.

Each microtiter plate experiment consisted of two replications with four observations each, unless indicated otherwise. Mean absorbance values and standard errors were used to evaluate fungal growth at 48 and 72 h. Data represent means pooled over two experiments ± standard deviations ($n = 8$).

2.4. High-Performance Liquid Chromatography−Mass Spectrometry (LC−MS) and Nuclear Magnetic Resonance (NMR) Spectroscopy Analyses. LC−MS was carried out using an LCQ system (Thermoquest Corp., Schaumburg, IL). Zopfiellin (0.5 mg/mL) dissolved in CH_3CN, CH_3CN:H_2O: (1:1), CH_3CN:1% aqueous HOAc (1:1), or CH_3CN:1% aqueous NH_4OH (1:1) was analyzed using electrospray ionization, operated in the negative mode. The HPLC column was a Luna C18 (Phenomenex, Torrance, CA) 3 μm, 100 × 1

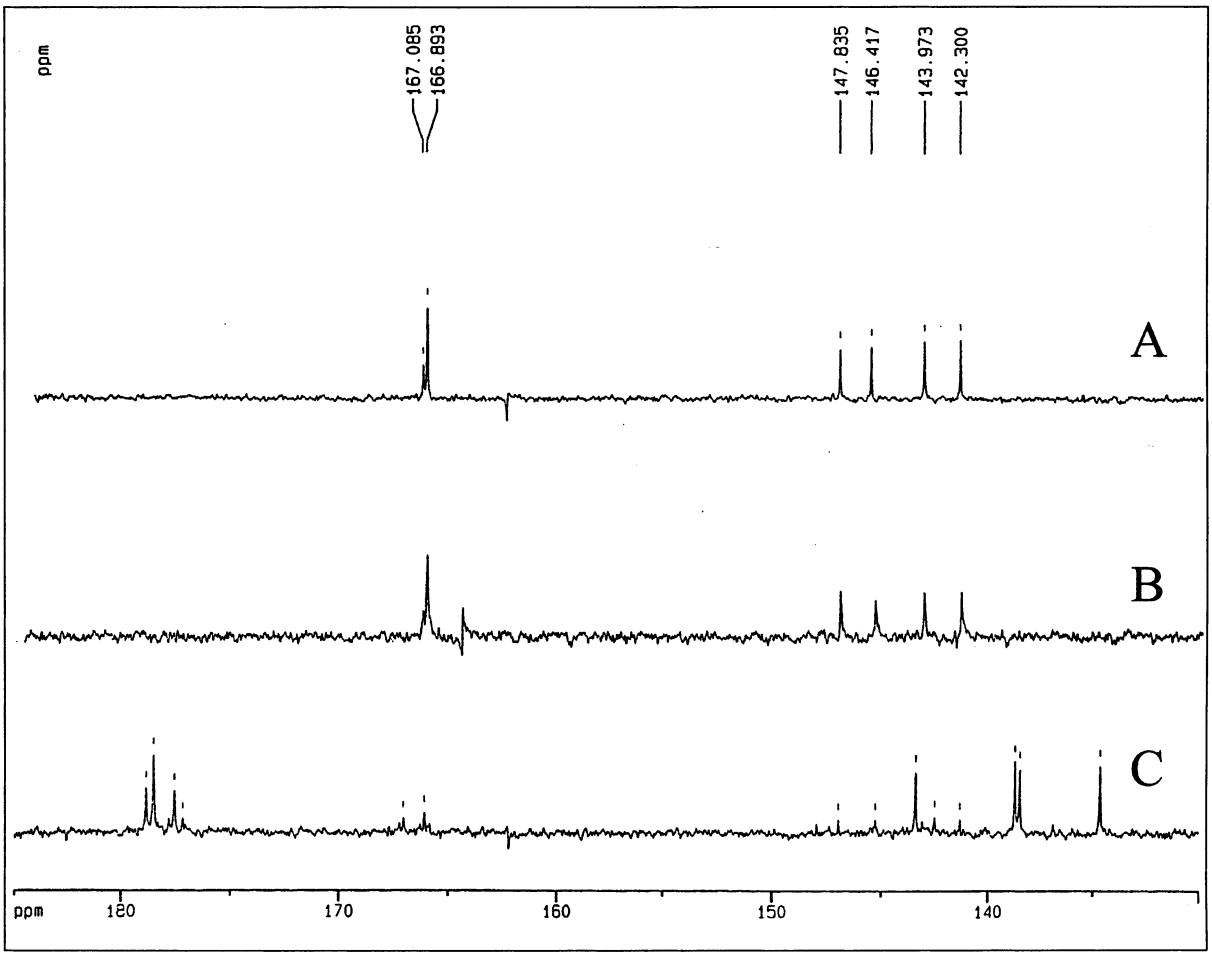

Figure 6. ^{13}C NMR spectra of zopfiellin between 130 and 185 ppm. Zopfiellin dissolved in (A) CD_3CN, (B) 1:1 CD_3CN:D_2O, and (C) 1:1 CD_3CN:1% ND_4OD in D_2O showing changes in the chemical shifts of the carbonyl and olefinic carbons.

mm; the mobile phase [CH_3CN or CH_3CN:H_2O: (1:1)] flow rate was 0.2 mL/min. NMR spectra were obtained on a Bruker Avance DPX 300 instrument (Billerica, MA). ^{13}C NMR spectra were obtained for zopfiellin in D_2O, CD_3CN, D_2O:CD_3CN (1:1), and 1% ND_4OD in D_2O:CD_3CN (1:1). Deuterated NMR solvents were purchased from Sigma-Aldrich (St. Louis, MO).

3. RESULTS AND DISCUSSION

Preliminary amended-agar experiments in which *B. cinerea* was grown in 9-cm Petri dishes on agar amended with 25 mg/mL zopfiellin at two different pH conditions demonstrated 100% inhibition of mycelial growth at pH 4.8, while only 50% inhibition was observed at pH 7.0. In the control treatments, in which no zopfiellin was added, no difference in the growth (rated as 100%) was observed between pH 4.8 and 7.0.

These results triggered a series of studies to investigate more thoroughly the effect of pH on the antifungal activity of zopfiellin. The use of a 96-well microtiter bioassay was deemed practical for these studies. Initial experiments were conducted to determine an appropriate liquid medium and pH that would support the most favorable growth conditions for *B. cinerea*. Growth curves showed that PDB cultures at pH 6.0, 6.5, and 7.0, and RPMI cultures at pH 5.5, 6.0, 6.5, and 7.0, produced the most growth at 48 and 72 h (**Figure 2**). In subsequent microtiter plate experiments, the effect of pH on the zopfiellin activity was further investigated. With PDB as culture medium adjusted to pH 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0, zopfiellin demonstrated significant *B. cinerea* growth inhibition in a dose-dependent manner at pH 5.0 and 5.5. No differences in the fungal growth patterns were observed at pH 6.0, 6.5, and 7.0, which indicated that zopfiellin was not biologically active (**Figure 3**) at these pH conditions. The greatest antifungal activity occurred at pH 5.0. Changes in absorbance values indicated that growth inhibition increased as the concentration of zopfiellin increased. Significant loss in the inhibitory activity was seen at concentrations below 1.0 μM, and concentrations greater than 10.0 μM had an absorbance value of ca. 0.001, which indicated 100% inhibition of *B. cinerea* growth (**Figure 4**). The IC_{50} value of zopfiellin in PDB medium was determined to be approximately 2.56 μM. Similar results were obtained in parallel experiments using RPMI culture medium, but zopfiellin appeared to be less active in this medium, with an IC_{50} value of 10.3 μM at pH 5.0 (data not shown).

Having determined the growth culture parameters at which zopfiellin exerts its greatest activity, these conditions were applied in studies that followed to determine the inhibitory activity of zopfiellin against *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, and *F. oxysporum*. *B. cinerea* was also included in these studies. At 15.0 μM, the highest concentration at which it was tested, zopfiellin showed nearly 100% growth inhibition of all fungi except *F. oxysporum* (**Figure 4**). Zopfiellin appeared to exert its greatest activity against *B. cinerea*. The therapeutic inflection threshold (IC_{80}) was 10 μM for *B. cinerea*, *C. gloeosporioides*, and *C. fragariae*. However, at concentrations below 10 μM, zopfiellin was less inhibitory to *C. gloeosporioides* and *C. fragariae* than to *B. cinerea*. *F. oxysporum* was not sensitive to zopfiellin.

Table 1. ^{13}C NMR Chemical Shift Values of the Olefinic and Carbonyl Carbons of Zopfiellin[a]

carbon no.	A[b]	B[b]	C[b]
14	142.30	142.30	134.64
15	143.97	143.97	138.47
16	146.41	146.41	138.71
17	147.83	147.83	143.39
18	167.08	167.08	178.82
19	166.89	166.89	178.48
20	166.89	166.89	177.53
21	166.89	166.89	178.48

[a] Chemical shifts in ppm, referenced to CD_3CN. [b] Zopfiellin dissolved in CD_3CN (A); 1:1 CD_3CN:D_2O (B); and 1:1 CD_3CN:1% ND_4OD in D_2O (C).

Prompted by observations that the antifungal activity of zopfiellin was pH dependent, studies were conducted to investigate changes in the zopfiellin structure that might occur due to changes in pH. LC−MS studies were carried out which showed that, when zopfiellin was dissolved in acetonitrile (**Figure 5A**), a 1:1 mixture of acetonitrile:water (**Figure 5B**), and a 1:1 mixture of acetonitrile:1% aqueous acetic acid (**Figure 5C**), a molecular ion peak (*m*/*z* 390) was obtained that indicated an intact zopfiellin molecule. It should be noted that the minute peaks of *m*/*z* 408 and 426 in **Figure 5B**,**C** are due to addition of one or two molecules of water during ionization, a common phenomenon observed in LC−MS determinations where water is part of the eluent. These peaks were absent when water was absent in the sample and the eluent (**Figure 5A**). The occurrence of an intact ring is corroborated by NMR experiments, showing no changes in the chemical shifts of the carbonyl (C18, C19, C20, C21) and olefinic (C14, C15, C16, C17) carbons (**Table 1**) when zopfiellin was dissolved in 100% deuterated acetonitrile (**Figure 6A**) and in a 1:1 mixture of deuterated acetonitrile and deuterated water (**Figure 6B**). Zopfiellin dissolved in 1:1 acetonitrile:1% aqueous acetic acid showed the same retention time and mass spectrum (**Figure 5C**) as that dissolved in 1:1 acetonitrile:water (**Figure 5B**) and indicated no structural change. However, when zopfiellin was dissolved in 1:1 acetonitrile:1% aqueous ammonium hydroxide, an early-eluting peak appeared ($t_R = 0.77$ min), the mass spectrum of which showed a molecular ion peak of *m*/*z* 408 (**Figure 5D**). This peak represents zopfiellin having undergone hydrolysis at one of the anhydride rings (A or B, **Figure 1**). The ^{13}C NMR spectrum of zopfiellin obtained in a 1:1 mixture of deuterated acetonitrile and 1% deuterated ammonium hydroxide in deuterated water (**Figure 6C**) showed chemical shifts corresponding to the two rings of zopfiellin open. These results indicated that as the pH increases, zopfiellin undergoes structural changes consistent with the opening by hydrolysis of first one and then a second anhydride ring.

Our results suggest that zopfiellin activity and structural conformation are pH dependent. A hypothesis can be formulated where at low pH the acid anhydride (closed ring form) is permeable to the fungal cell membrane, and once across the membrane zopfiellin converts to the open-ring form, which is probably the biologically active form (*20*). Amagasa et al. (*21*) reported that the microbial natural product cornexistin has a similar acid anhydride conformation and is easily converted to its hydrolysis products at pH 7. Although we have not directly established that a particular structure does exert a greater inhibitory activity against *B. cinerea*, results from studies suggest that the closed-ring bis-anhydride moiety is the form present when the highest antifungal activity is observed. The form that is present under physiological conditions is not presently known.

4. CONCLUSIONS

Botrytis cinerea was found to grow best at pH 6.0−7.0 in potato dextrose broth. However, at pH levels between 6.0 and 7.0, zopfiellin demonstrated no antifungal activity across the range of concentrations tested. Zopfiellin dose-dependent antifungal activity was observed at pH 5.0 and 5.5, with the greatest growth inhibition at pH 5.0 ($IC_{50} = 2.56$ μM). The apparent pH-dependent activity of zopfiellin could be explained by changes in molecular structure: as the pH of the media increased, the anhydride closed rings were hydrolyzed to hydride open rings. Two-step ring opening and the structural conformation were confirmed by the LC−MS and ^{13}C NMR studies. At pH 5.0 and 10 μM, zopfiellin demonstrated 100% growth inhibition of *Botrytis* and *Colletotrichum* isolates known to be resistant to dicarboximide and benzimidazole fungicides. Future studies using pH-buffered diluents are essential in order to properly evaluate zopfiellin as a plant protectant for greenhouse and field applications. These studies indicate that zopfiellin may have commercial potential as a natural product fungicide for small fruits and greenhouse crops.

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