Isolation and Characterization of Fungal Inhibitors from *Epichloë festucae*

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A series of studies was conducted to test the antifungal activity of clavicipitaceous endophytes and to identify potential fungal inhibitors in this symbiotic infection. A diverse group of endophytes was screened for antifungal activity using organic extracts from liquid fermentation cultures. Fungal inhibitors were purified from fermentation cultures of *Epichloë festucae* using a bioassay-directed extraction with *Cryphonectria parasitica* as the test organism. Compounds shown to have antifungal activity were subsequently identified using NMR and GC-MS. Extracts from a wide range of fungal isolates had various degrees of antifungal activity, but the greatest antifungal activity was observed in *E. festucae* and *Neotyphodium tembladerae*. Three types of inhibitors were isolated from a batch culture of *E. festucae*, including several indole derivatives, a sesquiterpene, and a diacetamide. Among the indole derivatives, indole-3-acetic acid and indole-3-ethanol were identified as the major indoles. These compounds were previously reported in endophytic fungi, and this study suggests a role in host disease resistance against other pathogens. The diversity in fungal inhibitors produced by this endophyte also suggests that fungal inhibitors may act additively or synergistically to reduce colonization of endophyte-infected hosts by potential fungal competitors.

Keywords: *Endophyte; grass; disease resistance; indole; sesquiterpene; diacetamide*

INTRODUCTION

Considerable research has focused on fungal endophytes and epibionts classified in tribe Balansieae of the ascomycete family Clavicipitaceae (White, 1987; Bacon and Battista, 1990; Siegel et al., 1990). These fungi form a range of symbiotic relationships with the plant families Poaceae and Cyperaceae (Clay, 1988). The relationship between plant and fungus is generally classified as mutualistic, but some members of the Balansieae are pathogenic against their host. Although the presence of endophytes in grasses often produces no visible signs on the plants, these fungi have proven to have a wide range of influences over their host, especially as it responds to the environment. In addition, endophytes may be toxic to animals when they infect grasses that are used to feed livestock. Tall fescue (*Festuca arundinacea* L. Schreb) toxicity is an endophyte-induced condition that can lead to hyperthermia, lower feed consumption, and subsequent weight loss, lower pregnancy rates, gangrene, agalactia, and other maladies in livestock. This toxicity is commonly attributed to the indole ergovaline, produced by the endophyte *Neotyphodium coenophialum* (Lyons et al., 1986; Bacon, 1988). Another condition, ryegrass staggers, is related to the alkaloid lolitrem B and is produced by the endophyte *Neotyphodium lolii* (Bacon, 1988; Porter, 1995). This toxin causes uncontrollable tremors and lack of coordination in exposed animals. Many toxic

syndromes associated with grasses throughout the world are caused by endophyte-produced toxins (White, 1987).

A broad spectrum of grasses is known to harbor *Epichloë/Neotyphodium* endophytes, including species of *Agrostis, Bromus, Cinna, Elymus, Festuca, Hordyelymus, Lolium, Poa,* and *Stipa* (White, 1987; Petroski et al., 1992). Grasses infected with endophytes have also shown increased resistance to stresses, such as drought, heat, and weed encroachment, and various degrees of resistance to insects and nematodes (Clay, 1989; Latch, 1993; Bacon, 1993; Clarke et al., 2000). The widespread abundance of plant−endophyte associations, as well as the vigor and resistance to stresses of grasses with endophytes, has prompted increased research into these interactions. Past and ongoing research has demonstrated the potential use of endophytes as biological control agents and as sources of nematicides, insecticides, and pharmaceuticals (Clay, 1989; Funk and White, 1997).

A limited number of studies have investigated the impact of endophytes on other fungi. Koshino et al. (1987) demonstrated enhanced resistance of Timothy (*Phleum pratense*) plants infected by *Epichloë typhinum* against *Cercospora* leaf spot. Stovall and Clay (1991) found that sedges infected by *Balansia cyperi* had increased resistance to fungal infections, and Burpee and Bouton (1993) also demonstrated that *Neotyphodium coenophialum* increases resistance of tall fescue to *Rhizoctonia* blight. Clarke et al. (2000) recently found that infection of fine fescue grasses by *E. festucae* enhanced resistance to dollar spot disease caused by *Sclerotinia homeocarpa*.

The mechanism of increased fungal disease resistance in endophyte-infected plants is unknown; however, on

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Table 1. Inhibition of *C. parasitica* by Crude Extracts of Clavicipitaceae Fungi Fermented in M104T Medium for a Period of 8 Weeks[a]

			diameter of the inhibition zone (mm)		
host	endophyte	collection location	hexane	chloroform	ethyl acetate
Agrostis sp.	*Epichloë amarillans*	Maryland	nd[b]	10.0 ± 1.0	7.0 ± 1.0
Agrostis sp.	*Epichloë amarillans*	Maryland	8.3 ± 1.5	8.0 ± 0.0	0.0
Arundinarea sp.	*Echinodothes tuberiformis*	Alabama	nd	7.7 ± 0.6	8.3 ± 0.6
Cenchrus	*Balansia obtecta*	Florida	8.0 ± 1.0	8.0 ± 1.0	8.7 ± 1.5
Festuca argentina	*Neotyphodium tembladerae*	Argentina	9.0 ± 1.0	15.3 ± 0.6	0.0
Festuca arundinacea	*Neotyphodium coenophialum*	Oregon	9.7 ± 0.6	8.3 ± 1.5	9.0 ± 1.0
Festuca ovina ssp. *glauca*	*Epichloë festucae*	England	0.0	0.0	0.0
Festuca rubra	*Epichloë festucae*	England	17.0 ± 0.0	19.7 ± 1.6	19.3 ± 1.2
Festuca rubra	*Epichloë festucae*	New Jersey	0.0	8.3 ± 0.6	5.0 ± 1.7
Festuca rubra	*Epichloë festucae*	New Jersey	7.3 ± 0.6	10.7 ± 0.6	0.0
Festuca rubra	*Epichloë festucae*	New Jersey	8.3 ± 0.6	8.3 ± 0.6	0.0
Lolium perenne	*Neotyphodium lolii*	New Jersey	7.3 ± 0.6	0.0	7.3 ± 0.6
Poa ampla	*Neotyphodium* sp.	Argentina	nd	7.7 ± 0.6	5.7 ± 2.5
Poa ampla	*Neotyphodium* sp.	Alaska	nd	4.3 ± 0.6	5.3 ± 0.6
Poa autumnalis	*Neotyphodium* sp.	Alabama	10.0 ± 0.0	10.7 ± 0.6	10.7 ± 0.6
Poa hueca	*Neotyphodium tembladerae*	Argentina	14.7 ± 1.2	21.0 ± 2.6	0.0
Poa hueca	*Neotyphodium tembladerae*	Argentina	nd	0.0	0.0
Poa interior	*Neotyphodium* sp.	Alaska	8.7 ± 0.6	5.7 ± 1.2	3.3 ± 0.6
Poa palustris	*Neotyphodium* sp.	Pennsylvania	nd	9.7 ± 1.5	8.0 ± 0.0
Poa rigidifolia	*Neotyphodium* sp.	Argentina	nd	12.3 ± 0.6	8.0 ± 1.0
Poa sp.	*Neotyphodium tembladerae*	Argentina	16.3 ± 1.5	0.0	8.3 ± 2.1
Poa sp.	*Neotyphodium* sp.	New York	9.0 ± 1.0	0.0	0.0
Poa sp.	*Neotyphodium* sp.	New York	nd	10.3 ± 0.6	16.3 ± 0.6
Poa sylvestris	*Neotyphodium* sp.	Alabama	nd	9.0 ± 1.0	5.7 ± 0.6

[a] Sequential extraction with ethyl acetate, chloroform, and hexane was used to produce extracts of various polarities. [b] nd, not determined due to insufficient extract.

the basis of the abundance of mycotoxins produced by these fungi, it is hypothesized that compounds produced by endophytes may at least play a partial role in this phenomenon. Although the chemical basis is unknown, cultures of some endophytes produce antifungal effects when cocultured with pathogenic fungi (White and Cole, 1986; Siegel and Latch, 1991), supporting the mycotoxin hypothesis. Yoshihara et al. (1985) identified fungitoxic sesquiterpenes and Koshino et al. (1987) identified fungitoxic fatty acids in stromata of *Epichloë typhina*, a tissue that is composed of a combination of plant and fungal tissues. However, no attempts have been made to confirm that inhibitory compounds isolated from stromata are fungus-produced or to identify similar antifungal compounds in monocultures of endophytic fungi. It is also unclear how widespread the phenomenon of antifungal activity is among clavicipitaceous endophytes. The objectives of the following research were to assess the scope of in vitro fungal inhibition across a wide sampling of fungal endophytes and to isolate and identify potential antifungal compound(s) from cultures of endophytic fungi.

MATERIALS AND METHODS

Screening of Endophytes for Antifungal Compounds. Endophytes were isolated from a wide range of grasses (Table 1). *Epichloë/Neotyphodium* spp. were isolated from leaf sheaths, culms, or seeds and cultured on potato dextrose agar (PDA) after the procedures of Bacon (1988). The antifungal activity of the various isolates was assessed from liquid fermentation cultures, using a medium (M104T) previously described by Bacon (1988) as suitable for ergot alkaloid fermentation. Flasks containing 125 mL of M104T medium were inoculated with three small pieces (5 mm in diameter) of actively growing mycelium on PDA and maintained at 25 °C for 4 weeks with gentle agitation on a rotary shaker. Cultures were then moved to a dark incubator and held stationary at 25 °C for 4 weeks. At harvest, the cultures were thoroughly homogenized with a Polytron homogenizer and centrifuged at 12000 rpm for 25 min, and the supernatant was collected with the use of vacuum

filtration. The aqueous fraction was sequentially extracted with 2 volumes each of hexane, chloroform, and ethyl acetate, and the organic phases within each solvent were combined. The organic fractions were dried using a rotary evaporator, the weight of extract was determined, and the extracts were stored under N_2 at −80 °C until bioassays were conducted. Cultures without endophyte were maintained and extracted in the same fashion and served as controls.

Fungal Bioassay. A specific fungal bioassay was developed for these experiments in which *Cryphonectria parasitica*, the causal agent in chestnut blight, was used as the test organism. Agar diffusion assays using this organism had been developed previously (Bradley L. Hillman, Rutgers University, personal communication), but the assay used in this study was an agar overlay procedure similar to those described by Rahalison et al. (1994). *C. parasitica* possesses characteristics that make it useful for this type of assay. First, the fungus produces copious amounts of conidia within 3−4 weeks after culturing on a standard medium, and it is therefore easy to maintain uniform seeding stock. Second, the cultures turn a bright orange upon germination of the condia, which allows visualization of an inhibition zone without stains, as with other methods (Paxton, 1991). Conidial suspensions (5×10^6 conidia mL^{-1}) were used to seed the agar overlays to a uniform inoculum density. Conidia were obtained initially from 1-month-old cultures of *C. parasitica* on PDA by flooding the plates with sterile water containing Tween 20 at 25 mL L^{-1} and disrupting the conidia from the surface of the medium with a sterile glass rod. The spore concentration of the conidial suspension was determined by serial dilution and subsequently used to obtain the final spore concentration of 5×10^6 conidia mL^{-1}.

For the agar overlay technique, a 10×10 cm aluminum-backed silica gel chromatography plate (EM Science silica gel 60) was gridded into nine equal squares using a soft lead pencil, sterilized with 95% EtOH for 20 min, and dried under the laminar flow hood. Exactly 200 μg of crude extract from each solvent fraction was applied in 20 μL of the extracting solvent to the center of each square. Each sample was replicated three times, with solvent alone serving as the control. Fractions that yielded <1 mg of crude extract were not tested. After application of test materials, plates were placed under a stream of air in the laminar flow hood to facilitate evaporation of the solvent. At dryness, assay plates

Table 2. ^{1}H NMR (Acetone-d_6), ^{13}C NMR (Acetone-d_6, 100 MHz), and MS Analysis of Compounds Isolated from a Fermentation Culture of *E. festucae*

compound	^{1}H (ppm, J in Hz)	^{13}C (ppm)	EI-MS
indole-3-acetic acid	7.59 (d, 8.0), 7.37 (d, 8.0), 7.08 (t, 8.0), 6.98 (t, 8.0), 7.27 s, 3.70 s	171.27, 134.06, 124.53, 122.27, 119.82, 119.56, 112.22, 109.55, 30.63	175 (M$^+$) 130 (bp)
indole-3-ethanol[a]	7.56 (d, 7.6), 7.36 (d, 7.4), 7.06 (t, 8.0), 7.00 (t, 8.0), 7.08 s, 3.78 (d, 6.2), 2.96 (d, 6.4)	134.50, 125.10, 121.96, 120.62, 117.97, 117.93, 110.70, 110.67, 61.82	161 (M$^+$) 130 (bp)
methylindole-3-carboxylate	8.27 (d, 9.0), 7.54 (d, 9.0), 7.25 m, 8.32 s, 4.70 s	174.69, 133.59, 126.75, 124.26, 123.16, 122.64, 114.2, 113.09, 65.98	175 (M$^+$) 144 (bp)
indole-3-carbox-aldehyde	8.21 (d, 8.0), 7.53 (d, 8.0), 10.02 s, 8.18 s, 7.25 m	183.98, 136.50, 133.50, 123.62, 123.12, 121.78, 121.13, 117.10, 111.58	145 (M$^+$) 144 (bp)
N,N-diacetamide[b]	12.13 brs, 2.05 s	175.18, 30.27	101 (M$^+$)
cyclonerodiol[c]	5.11 (t, 7.0), 2.05 m, 1.68 s, 1.62 s, 1.25 s, 1.16 s, 1.04 (d, 7.2)	131.35, 124.04, 80.85, 74.44, 53.85, 43.78, 40.00, 25.65, 25.28, 24.62, 23.90, 22.22, 17.25, 14.10, 13.31	222 (M − H$_2$O)$^+$, 127, 109, 95, 43

[a] Signal of the second carbon of CH$_2$ was overlapped with signals of the solvent. [b] DMSO-d_6 was used as the solvent. [c] CDCl$_3$ was used as the solvent, and some signals of the protons were overlapped.

were sprayed uniformly with an autoclaved agar (1%) solution that had been cooled to near solidification (45 °C) and aseptically inoculated with the *C. parasitica* conidial suspension to obtain the final concentration of 5×10^6 conidia mL^{-1}. The thickness of the agar film was between 0.5 and 1.0 mm. Assay plates were incubated at 30 °C in sterile glass Petri dishes for 5 days, at which time the diameter of the inhibition zone was measured and recorded for each extract. Each compound by concentration treatment was replicated three times, and treatment effects were tested by analysis of variance procedures. Mean separations were conducted using a protected LSD at the 0.05 level of probability.

Extraction and Isolation of Antifungal Compounds. To isolate and identify potential fungal inhibitors, an isolate of *E. festucae* (BM7, M. D. Richardson) from a clone of *Festuca rubra* was cultured in 12, 2-L flasks, each containing 1000 mL of M104T medium. After continuous agitation on a rotary shaker for 4 weeks (25 °C), cultures were held stationary in the dark for 4 weeks (25 °C). At the end of the fermentation period, cultures were pulverized with a Polytron homogenizer and centrifuged at 2800 rpm for 30 min, and the supernatant was collected (pH 6.8) and extracted with 2 volumes of ethyl acetate. The crude extract was then subjected to column chromatography (silica gel, chloroform/methanol = 100:1−1:1 as eluent). Fifteen fractions were collected from the silica column and tested for bioactivity using the bioautography agar overlay method as previously described. Of the 15 fractions, those that demonstrated antifungal activity were further purified and identified.

NMR and GC-MS Analyses. NMR spectra (400 MHZ ^{1}H and 100 MHZ ^{13}C) were performed in acetone-d_6 on a Varian 400 FT-NMR spectrophotometor. The GC-MS was performed on a Finnigan MAT 8230 spectrophotometer with GC separation using a glass column (SE-100), helium 30 mL/min, column temperature from 70 to 350 °C at 10 °C/min with a 5 min delay to program, injector and FID temperatures; MS, EI probe, 70 eV.

Confirmation of Inhibitory Activity of Selected Indoles. A second bioassay was used to confirm the antifungal activity of indole-3-acetic acid (IAA) and indole-3-ethanol (IEtOH) (Aldrich Chemical Co.). IAA and IEtOH were dissolved in 1 mL of 95% ethanol, filter sterilized (0.25 mm), and incorporated into 200 mL of autoclaved PDA cooled to 45 °C to yield final concentrations of 10, 100, 500, and 1000 mg mL^{-1}. Approximately 15 mL of each amended medium was poured into plastic Petri dishes (85 mm diameter). Control plates containing 95% ethanol were prepared in the same fashion. A 3-mm plug of *C. parasitica*, *Lactisaria fusiformis*, *Magnaporthe poae*, or *Rhizoctonia solani* was placed in the center of each plate and placed in an incubator at 25 °C for 7 days. Colony diameter was measured and used to determine percent inhibition of indoles relative to control using the following formula: % inhibition = 100(diameter of control − diameter of treatment)/diameter of control. Each fungus by compound concentration was replicated three times.
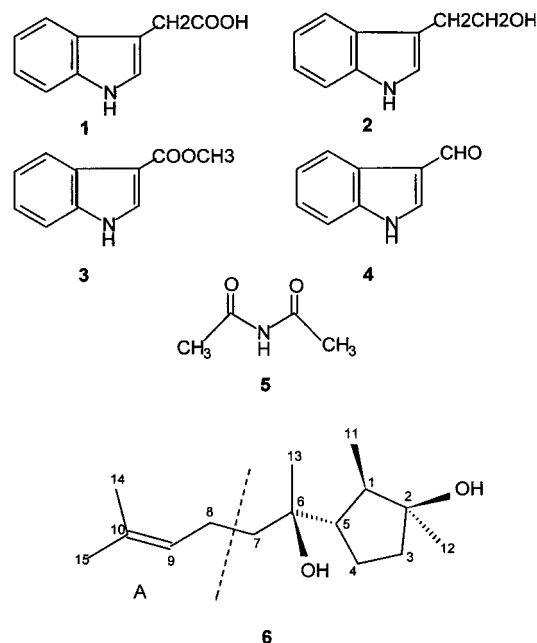

Figure 1. Lewis structures for fungal inhibitors isolated from fermentation cultures of *E. festucae*. The structures are identified as follows: **1**, indole-3-acetic acid (IAA); **2**, indole-3-ethanol (IEtOH); **3**, methylindole-3-carboxylate; **4**, indole-3-carboxaldehyde; **5**, diacetamide; **6**, cyclonerodiol.

RESULTS

Twenty-two of the endophytes tested in this study demonstrated antifungal activity in at least one of the fractions (Table 1). The greatest inhibition was observed with an isolate of *E. festucae* from *F. rubra* and an isolate of *Neotyphodium tembladerae* from *Poa hueca*. The high antifungal activity from the *E. festucae* isolate prompted its use in chemical isolation studies. The only endophyte that did not exhibit any inhibitory activity in this test was isolated from *Festuca ovina* ssp. *glauca*. Interestingly, this endophyte/host combination was shown previously to be devoid of ergot alkaloids (Tredway et al., 1999), one of the primary indole derivatives associated with these fungi.

The bioassay-directed separation of the *E. festucae* fermentation culture yielded six compounds in relatively high concentration that possessed antifungal activity, including several indole derivatives (**1**−**4**), a sesquiterpenoid (**6**), and a diacetamide (**5**) (Table 2; Figure 1). Among the indoles were four, 3-substituted indole derivatives as follows: (**1**) 12 mg of IAA, (**2**) 5 mg of

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Table 3. Inhibition of *C. parasitica* by Compounds Isolated from a Fermentation Culture of *E. festucae*

compound	diameter of inhibition zone (mm)[a] at concn of			
	100 µg	50 µg	25 µg	12.5 µg
indole-3-acetic acid	21	16	14	12
indole-3-ethanol[a]	14	11	9	7
methylindole-3-carboxylate	11	8	6	4
indole-3-carboxaldehyde	15	13	10	7
N,N-diacetamide[b]	14	10	7	3
cyclonerodiol[c]	13	0	0	0

[a] Data from three replicates.

IEtOH, (**3**) 4 mg of methylindole-3-carboxylate, and (**4**) 10 mg of indole-3-carboxaldehyde. The purity of each compound was >95%, as confirmed by TLC separation with multiple mobile phases and by recrystallization. In addition, the NMR signals for each compound were free from interfering noise. The mass spectra of six additional trace but readable peaks could be elucidated respectively as 1,3-dihydroindole-2-one (17.349 min), 1*H*-indole (14.527 min), 3-acetylindole (23.272 min), and 3-propionylindole (23.770 min). The ^{1}H NMR spectra of all of the compounds showed the typical characteristic of a 3-substituted indole chromophore, aromatic proton signals of 1,2-disubstituted benzene (two doublets at 7.30−8.30 ppm assigned to H-5 or H-8 and two triplets at 6.90−7.30 ppm due to H-6 or H-7). The resonance of an olefinic methine proton at C-2 appeared as a singlet, which shifted from 7.10 to 8.30 ppm depending on the property of the 3-substituted group. The signal of methylene in **1** was observed at 3.70 ppm as a singlet. A pair of coupled triplets at 2.60 and 2.95 ppm in the ^{1}H NMR of **2** were attributable to the protons on the side chain. In compound **3** the signal of methyl appeared at 4.70 ppm and the aldehyde proton in **4** resonated at 9.77 ppm. In the mass spectra of these compounds, two series were grouped. In one series the base peak appeared at *m*/*z* 130 such as in **1** and **2**, having a methylene attached at C-3. In the other series the fragment *m/e* 144 is the base peak such as in **3** and **4**, having a carbonyl at C-3. The trace 3-substituted indole derivatives were detected using GC-MS.

When tested against *C. parasitica*, the activities of the various indoles were similar at high concentrations, but IAA had the greatest activity at lower concentrations (Table 3). Both the sesquiterpenoid and diacetamide exhibited antifungal activities similar to the those of the indoles when tested in this assay. IAA and IEtOH were further evaluated for antifungal activity against some common grass pathogens (Figure 2) and were found to demonstrate inhibitory activity against several additional fungi, including *R. solani*, *M. poae*, and *L. fusiformis*. The most sensitive grass pathogen to indole derivatives was *L. fusiformis*, but all species responded to higher concentrations of the compounds by increased inhibition.

Compound **5** was identified as diacetamide on the basis of EI-MS, ^{1}H NMR, and ^{13}C NMR (Table 2). This compound was abundant in the fermentation flasks, with 25 mg being recovered. Cyclonerodiol (**6**; Figure 1), a sesquiterpene, was obtained as a colorless syrup (8 mg). EI-MS gave a fragment ion peak at *m*/*z* 222, which seems to represent $[M − H_2O]^+$. Its molecular formula was established as $C_{15}H_{28}O_2$ on the basis of EI-MS, ^{1}H NMR, and ^{13}C NMR. The ^{1}H NMR spectrum of cyclonerodiol showed five methyl signals at 1.04 ppm (d, $J = 7.0$ Hz), 1.25 ppm, 1.16 ppm, 1.68 ppm, and 1.62
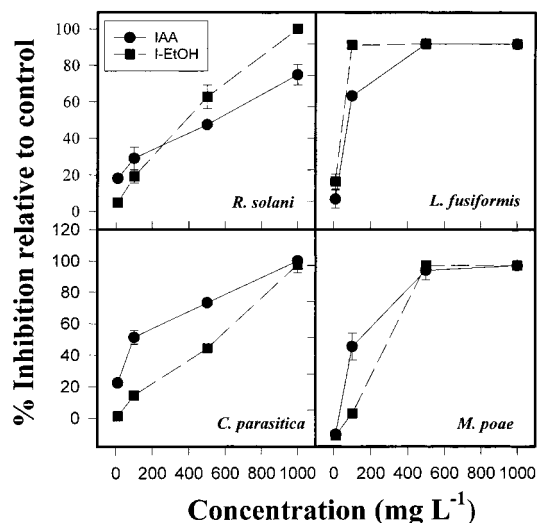

Figure 2. Inhibition of *R. solani*, *L. fusiformis*, *C. parasitica*, and *M. poae* by IAA and IEtOH at 10, 100, 500, and 1000 mg L^{-1} concentrations.

ppm, suggesting that this compound might possess an unusual structural feature as a sesquiterpene. The methyl (C-11) having a proton signal at 1.04 ppm as a doublet was deduced to connect to a methine (C-1). An olefinic proton signal appeared at 5.11 ppm (d, $J = 7.0$ Hz), indicating the existence of an isopentene fragment A. The absence of signal at the low field except the olefinic proton signal in ^{1}H NMR and the carbon signals at 74.44 and 80.85 ppm indicated the presence of two oxygenated quaternaries (C-2 and C-6). The data on cyclonerodiol in ^{1}H NMR and ^{13}C NMR revealed that a ring exists because the unsaturated degree of the molecule was two but only one double bond is indicated to be present in the molecule. On the basis of the biosynthesis principle of sesquiterpene and knowledge about cyclopentanoid sesquiterpene, the structure was determined as cyclonerodiol. Furthermore, the fragment ions in EI-MS were the same as those described by Nozoe (1970) for cyclonerodiol.

DISCUSSION

IAA is a common plant auxin and was a major secondary metabolite isolated from the culture of *E. festucae*. Previous studies have reported IAA production by grass endophytes (De Battista et al., 1990; Porter et al., 1985), but its biological role to the symbiosis has not been determined. On the basis of its well-known growth regulatory properties, it seems likely that production of this compound by an endophyte would impact the physiology of the host. Previous studies have demonstrated that endophytes can significantly alter or modify host tissues with which they associate (White et al., 1998), and IAA may play a role in triggering alterations in host tissues in the stromata of some endophytes. The *Epichloë* stroma contains the young expanding inflorescence primordium and leaves of the host plant enclosed in a fungal mycelium. To form a stroma, the mycelium of the endosymbiont grows out of plant tissues onto the inflorescence primordium and onto the leaves that surround the primordium. The primordium and leaves become permeated with mycelium, and their living tissues are integrated into the stroma, which is the reproductive structure of the endophyte. The early production of auxins and auxin-

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like compounds by the endophyte may enable the symbiont to alter development of the host tissues, preventing maturation of the epidermis and causing hypertrophy, as previously demonstrated (White et al., 1997).

The production of IEtOH by endophytic fungi was reported previously (Porter et al., 1985), but its potential role in the host is also unknown. IEtOH has also been reported in other symbiotic fungi, especially mycorrhizae, in which it has been speculated to alter growth characteristics in that symbiosis. Brown (1992) found that IEtOH produced by *Zygorrhynchus moelleri* possessed antifungal activity, supporting data in the present study. Barroso et al. (1986) demonstrated that IEtOH produced by mycorrhizae was secreted into the culture medium, suggesting that it might serve to interact with the host infection process or growth characteristics of the host. Although the effect of IEtOH on host grass tissues may also be plant growth regulatory, IEtOH can also serve as a precursor for the final synthesis of IAA by the host (Sandburg, 1984). This role suggests that the production and excretion of IEtOH into host tissues by endophytic fungi may serve as a pool for the synthesis of IAA by the host grass.

Indole derivatives are also responsible for making some hosts unpalatable to mammals. One grass, sleepygrass (*Achnatherum robustum*), contains a *Neotyphodium* endophyte that produces lysergic acid amide, a powerful indole that can induce sleep in animals, particularly horses (Petroski et al., 1992). Lysergic acid amide acts as a mammal feeding deterrent because animals learn to avoid infected grasses (Clay, 1988, 1989). Johnson et al. (1985) and Clay and Cheplick (1989) demonstrated that endophyte-produced indole alkaloids may also play a role in defending plants from insect predators. Our study adds to the possible functions of indole derivatives the role of antifungal, defensive compound. Indole derivatives may serve multiple functions to the fungus and symbiotic system, and it is probable that specific variations of indoles are better suited for specific functions than other variants. This may account for the numerous variations in which indole alkaloids are encountered in nature (Porter, 1994).

The antifungal activity of indole derivatives, especially IAA, against our bioassay organism, as well as several important pathogenic fungi of grasses, suggests that this compound class may also play a role in fungal disease resistance of endophyte-infected grasses. Other studies have reported that this important class of compounds possesses both fungistatic (Himejima, 1993) and antibiotic activity (Kubo et al., 1993). Indoles have also been shown to act synergistically with other known antifungal compounds to enhance their biological properties. Himejima (1993) found that indole alone had fungistatic activity against yeast (*Candida albicans*) but enhanced the fungicidal activity of both anethole and polygodial, known fungicidal compounds. A synergistic model has also been proposed for several of the insecticidal compounds produced in endophyte–grass associations (Yates et al., 1989) and may also be important with respect to antifungal compounds. Studies are currently underway to test this hypothesis with the antifungal compounds identified in this study. Studies that examine the levels of indole derivatives in these grasses under disease attack are also needed to clarify what role they may play in disease resistance. DeBat-

tista et al. (1990) reported that tall fescue contained IAA levels of 0.31 μg g^{-1} of dry weight of leaf tissue when grown under optimum conditions, but there are no reports of IAA levels in endophyte-infected fine fescue grasses.

Our results suggest that sesquiterpenes are potential fungal inhibitors of endophyte origin. The sesquiterpene cyclonerodiol, which was isolated in this study, has been previously isolated from *Trichothecium* and *Gibberella* species fungi (Nozoe, 1970). Yoshihara et al. (1985) identified analogues of cyclonerodiol, which they denominated chokol A, chokol B, and chokol C, in stromata of *E. typhina* on timothy grass (*Phleum pratense*). Cyclonerodiol and the chokols are probably derived through the same biosynthetic pathway via an intermediate of nerolidyl pyrophosphate. It has been proposed that chokols may be responsible for increased fungal disease resistance of timothy plants infected by *E. typhina* (Yoshihara, 1985). However, in the present study, antifungal activity of cyclonerodiol was seen only at relatively high concentrations (Table 3). Whether the cyclopentanoid sesquiterpenes such as cyclonerodiol have other functions awaits investigation.

To our knowledge, diacetamide has not been reported as a metabolite of Clavicipitaceae. Because of the small size of this compound, it may have been overlooked by previous investigators. However, in this study, this compound was produced abundantly compared to the other fungal inhibitors isolated from broth cultures. As a potential defensive compound, diacetamide is intriguing because it would provide the fungus a biochemically inexpensive means of defending its host. In that respect, it is very different from the other two types of metabolites identified.

Sesquiterpenes, indoles, and other fungal inhibitors, such as diacetamide, may accumulate in infected host tissues, much in the same fashion as ergopeptide alkaloids (Lyons et al., 1986). The additive or synergistic impact of fungal inhibitors may be effective in reducing colonization of plant tissues by pathogenic fungi. It would also be an adaptive advantage to the endophyte to protect host resources by producing compounds that reduce the development of other competing microbes. The result is a symbiotic system that has enhanced fitness and survivability.

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