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Endophytic Fungus *Trichothecium roseum* LZ93 Antagonizing Pathogenic Fungi *In Vitro* and Its Secondary Metabolites

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The endophytic fungus *Trichothecium roseum* LZ93 from *Maytenus hookeri* was found to antagonize other pathogenic fungi *in vitro*. To identify which compound contributed substantially to the antagonism, we fermented the strain and purified its fermentation products. Eleven compounds were obtained, including two trichothecenes, five rosenonolactones, two cardiotonic cyclodepsipeptides, and two sterols. Compound 11β-hydroxyrosenonolactone (1) was assigned according to 1D and 2D-NMR data for the first time. At the same time, the ^{1}H and ^{13}C-NMR assignments for 6β-hydroxyrosenonolactone (2) were revised. Of all of them, only trichothecin (6) showed strong antifungal activity. Based on our observations of the antagonistic activity and the other experimental results, we suggest that the antifungal compound trichothecin was the main contributor to the antagonistic action of *T. roseum* LZ93.

***Keywords*:** antifungal, antagonism, endophytes, pathogenic fungi, trichothecin

Plant endophytes are a group of microorganisms, including fungi and bacteria, which not only live within plant internal tissues or organs without causing any apparent symptoms or diseases in the host plants, but also serve as important sources of bioactive compounds, presumably due to the symbiotic relationship with their hosts (Sturz *et al*., 2000; Owen and Hundley, 2004). Endophytic microorganisms take up residence in the tissues of almost all plants. The endophyte/host relationship is believed to be complex and probably varies from host to host and microbe to microbe (Sturz *et al*., 2000). Many endophytes are able to synthesize bioactive compounds that can be used by the plant for defense against pathogenic microorganisms (Owen and Hundley, 2004). Production of some secondary metabolites has been shown to correlate with the virulence or pathogenicity toward microorganisms. In the course of our earlier studies on chemical constituents derived from endophytic microorganisms of medicinal plants, a series of new compounds was previously isolated (Zhao *et al*., 2005, 2006, 2007).

Maytenus hookeri Loes. is a well-known medicinal plant with anticancer properties. New experimental models are currently being tested in which the cytotoxic activity of maytansine is being used to develop new drugs (Liu *et al*., 1996). In the present study, an endophytic fungal strain LZ93, which can antagonize some pathogenic fungi (*Typhula incarnata*, *Gaeumannomyces graminis*, *Phytophthora infestans*, *Alternaria solani*, and *Phyricularia oryzae*) *in vitro*, was isolated from a stem of *M. hookeri*. In order to identify which compound contributed substantially to the antagonism, we further investigated the metabolites produced by strain LZ93 and assayed their antifungal, nematicidal and antitumor activities.

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Materials and Methods

Precoated plates (Silica gel G), Silica gel G (200-300 mesh) and H from the Qingdao Marine Chemical Factory, Qingdao, China. Sephadex LH-20 from Pharmacia. NMR spectra were measured on Bruker AM-400 and Bruker DRX-500 NMR spectrometers. MS spectra were measured on Finnigan LCQ-Advantage and VG Auto-Spec-3000 mass spectrometers; values are in m/z. Optical rotations were measured on a Jasco P-1020 polarimeter.

Microbial material

Stems of *M. hookeri* were collected at Xishuangbanna, Yunnan, China, in April 2003. The stems were washed in running tap water and sterilized successively with 75% ethanol for 1 min and 1.2% sodium hypochlorite for 8 min, then rinsed five times in sterile water and cut into small pieces. These small pieces were incubated at 25°C on PDA media [consisting of potato (200 g/L), dextrose (20 g/L), and agar (15 g/L)] and cultured until colonies or mycelia appeared surrounding the segments. After culturing about one month, a strain named LZ93 appeared and was isolated. Five pathogenic fungi (*Typhula incarnata*, *Gaeumannomyces graminis*, *Phytophthora infestans*, *Alternaria solani*, and *Phyricularia oryzae*) were provided by Dr. Fan L M at Yunnan Agricultural University.

Assay for phytopathogenic fungal antagonism

The assay for antagonism of nonvolatile metabolites to fungi was performed on PDA medium by a dual culture method as described by Skidmore and Dickinson (1976). The *in vitro* antagonistic properties of strain LZ93 were investigated against five phytopathogenic fungi by the co-inoculation of 9-cm petri dishes with the strain and phyto-

pathogens, 2.5-3.0 cm apart, onto PDA medium. The petri dishes were incubated at 22°C. After 4 days incubation, the diameters of the colonies of phytopathogenic fungi were measured.

Extraction and isolation

Solid-state fermentation (10 L) was carried out on PDA, and the cultures were incubated at 28°C for 10 days. After 10 days of cultivation, the cultures were extracted exhaustively five times by ethyl acetate (EA)/methanol (MeOH)/acetic acid (80:15:5) to obtain extracts (11.7 g). The residue was chromatographed on silica gel (silica gel G, 80 g) and eluted with petroleum ether (PE)/acetone (A) (10:1 to 7:3) and then eluted with chloroform (CH)/MeOH(10:1 to 7:3) to afford 8 fractions (Fr-1 to Fr-8). Fr-1(121 mg) was subjected on silica gel column (silica gel G, 50 g) eluted with PE/A (100:5) and purified by Sephadex LH-20 (CH/MeOH 1:1) to obtain compound 3 (40 mg). Fr-2 (630 mg) was subjected on a silica gel column (silica gel G, 16 g), eluted with PE/EA (15:1 to 8:2) and then subjected on another silica gel column (silica gel H, 5 g) and eluted with PE/A (100:5) to provide compound 11 (7 mg). Fr-3 (660 mg) was chromatographed on silica gel (silica gel G, 20 g) then eluted with PE/EA (100:3 to 10:1) to produce 2 fractions (Fr-3-1 and Fr-3-2). Fr-3-1 (92 mg) was purified on a silica gel column (silica gel H, 5 g) eluting with PE/A (100:2 to 100:5) to achieve compound 5 (7 mg). Fr-3-2 (46 mg) was passed through Sephadex LH-20 (acetone) to obtain compound 6 (28 mg). Fr-4 (315 mg) was loaded on Sephadex LH-20 (CH/MeOH 1:1) and then on a silica gel column (silica gel G, 20 g) and eluted with PE/A (100:1 to 8:2) to give compound 2 (30 mg). Fr-5 (385 mg) was subjected on Sephadex LH-20 (CH/MeOH 1:1) and then on silica gel (silica gel H, 5 g) and eluted with CH to obtain compound 4 (22 mg). Fr-6 (283 mg) was loaded on Sephadex LH-20 (CH/MeOH 1:1) and then on a silica gel column (silica gel G, 3 g) and eluted with PE/EA (8:2) to produce compound 1 (60 mg). Fr-7 (980 mg) was loaded on Sephadex LH-20 (CH/MeOH 1:1) and then was chromatographed on a silica gel column (silica gel G, 10 g), eluting with PE/EA (8:2) to produce 2 fractions (Fr-7-1 and Fr-7-2). Fr-7-1 (73 mg) was loaded onto a silica gel column (silica gel H, 10 g) and eluted with PE/A (10:1 to 9:1) to give compounds 8 (10 mg) and 9 (16 mg). Fr-7-2 (39 mg) was passed through Sephadex LH-20 (CH/MeOH 1:1) to obtain compound 7 (20 mg). Fr-8 (210 mg) was passed through Sephadex LH-20 (CH/MeOH 1:1) and then fractionated on a silica gel column (silica gel H, 5 g) and eluted with CH/MeOH (100:1) to obtain compound 10 (2 mg). All compounds were obtained as colorless powder, whose other physico-chemical data are listed in Table 1.

Antifungal activity testing

A number of potential mechanisms of antagonism were possible, such as production of pigments, antibiotics, siderophores, cyanide and endochitinase. The major constituent compounds (1, 2, 3, 4, 6, 7, and 9) from *T. roseum* LZ93 were assayed for antifungal activities. The minimum inhibitory concentrations (MICs) of antifungal activities were determined by a method modified from the standardized microdilution method (Rex *et al*., 2001). Five phytopathogenic fungi were incubated on PDA at 22°C for 7-10 days. Fungal hyphae (fresh weight 600 μg) were ground and suspended into 10 ml of SD broth (0.67% yeast nitrogen base, 2.0% glucose). The fungal solutions were then diluted with SD broth (1:1000) (Amiguet *et al*., 2006) for use. All of the tested compounds were dissolved in DMSO to make stock solutions of 20 mg/ml. Different doses of test compounds (ranging from 5-300 μg/ml) were added to each well (Deepwell Plates 96, Eppendorf) containing fungal solution and maintained at a final volume of 0.2 ml (The final concentration of DMSO in each medium was not more than 3%). DMSO was used as negative control and the fungal solution without the compound was used as a positive comparison. The Deepwell Plates were then incubated at 22°C for 48 h. The MIC is the lowest concentration of test compound that demonstrated no visible growth. Experiments were performed in triplicate.

Nematicidal activity assay

The nematodes *Panagrellus redivivus* and *Caenorhabditis elegans* were cultured on oatmeal medium (20 g of oatmeal in 80 ml of H_2O) at 25°C for 7 days. The culture method of the pine wood nematode *Bursaphelenchus xylophilus* was based on Li *et al.* (2005). The cultured nematodes were separated from the culture medium using the Baerman funnel technique. Test samples (compounds 1, 2, 3, 4, 6, 7, and 9) were dissolved in acetone or methanol respectively, and then diluted to different concentrations with sterilized water for the assay. The method of nematicidal activity was based on Li *et al.* (2005). Experiments were performed in triplicate, using 5% acetone and 5% methanol as negative controls, and the mean percentage mortality was calculated. The median lethal concentrations (LC_{50}) were also calculated (Hong *et al*., 2007).

Assay for antitumor activity

Antitumor activity was measured by the microculture tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazalium bromide, MTT, Sigma] assay (Mosmann, 1983). Six cell lines were selected for testing (leukemia cell line HL-60, hepatocarcinoma cell line SMMC-7721,

Table 1. The Physico-chemical properties of compounds from LZ93

No	Optical rotation $[\alpha]_D^{20}$	MS	NMR
1	+27 (*c* 0.06, $CHCl_3$)	ESIMS *m/z*: 333 $[M + H]^+$, 687 $[2M + H]^+$.	Table 2
2	-112 (*c* 0.62, $CHCl_3$)	EIMS (70 eV) m/z (%): 316 ($[M]^+$, 70), 301 ($[M - Me]^+$, 30), 273 (50).	Table 2
3	+8.8 (*c* 0.68, $CHCl_3$)	EIMS (70 eV) m/z (%): 302 ($[M]^+$, 80), 287 ($[M - Me]^+$, 28), 259 (100).	Table 3
4	-94 (*c* 0.34, $CHCl_3$)	EIMS (70 eV) m/z (%): 332 ($[M]^+$, 55), 317 ($[M - Me]^+$, 75).	Table 3
5	-17.9 (*c* 0.78, $CHCl_3$)	ESIMS *m/z*: 341 $[M+ Na]^+$, 659 $[2M + Na]^+$.	Table 3
6	-21.0 (*c* 0.28, $CHCl_3$)	ESIMS *m/z*: 333 $[M + H]^+$.	Loukaci *et al*. (2000)
7	-20.0 (*c* 0.44, $CHCl_3$)	ESIMS *m/z*: 287 $[M + Na]^+$.	Loukaci *et al*. (2000)
8	-98.0 (*c* 0.64,$CHCl_3$)	ESIMS *m/z*: 608 $[M + H]^+$, 630 $[M + Na]^+$.	Tsunoo *et al*. (1997)
9	-90.9 (*c* 0.22, $CHCl_3$)	ESIMS *m/z*: 592 $[M + H]^+$, 614 $[M + Na]^+$.	Engstrom *et al*. (1975)
10	-48.0 (*c* 0.34, $CHCl_3$)	EIMS (70 eV) m/z (%): 412 $[M]^+$.	Bok *et al*. (1999)
11	-55 (*c* 0.4, $CHCl_3$)	ESIMS *m/z*: 429 $[M + H]^+$.	Yue *et al*. (2001)

lung adenocarcinoma A-549, breast cancer cell lines SK-BR-3 and MCF-7, pancreatic carcinoma cell line PANC-1, and colon cancer cells SW480). The antitumor activities of compounds (1, 2, 3, 4, 5, 6, 7, 8, and 9) against these cell lines were assayed in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Results

Isolation and identification the endophytic fungus LZ93

The stems of *M. hookeri* were incubated at 25°C on PDA medium after sterilizing. After culturing about one month, a strain named LZ93 appeared, which was identified as *Trichothecium roseum* based on traditional morphology by Prof. Liu Yun-Long (at Yunnan Agricultural University), and deposited in Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China.

Phytopathogenic fungal antagonism

T. roseum strain LZ93 caused antagonism against several of the fungal pathogens. The *in vitro* antagonistic properties of the strain were investigated against five phytopathogenic fungi. *T. roseum* LZ93 and the phytopathogenic fungi grew rapidly when cultured individually on PDA medium; however, when co-cultured with strain LZ93, *Typhula incarnata*, *Gaeumannomyces graminis*, *Phytophthora infestans*, and *Phyricularia oryzae* showed very limited growth. Their hyphal growth was obviously retarded and inhibited by LZ93. Therefore, the endophytic fungus LZ93 exhibited antagonism toward these four phytopathogenic fungi (Fig. 1). The strain *Alternaria solani* grew and spread on the plate slowly. There was no clear antagonism effect after dual culture with *T. roseum* LZ93 for 4 days. The experiments were repeated in triplicate and the results were consistent.

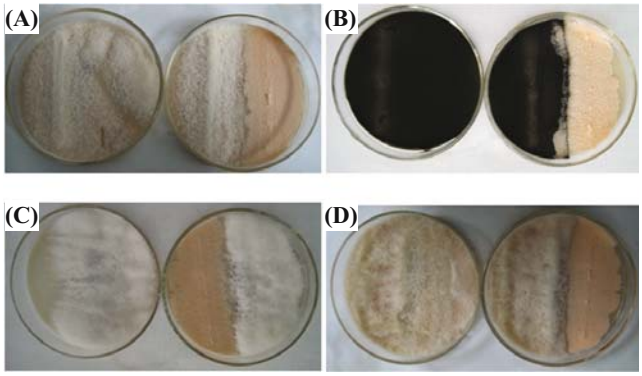

Fig. 1. Photographs showing LZ93 antagonism against pathogenic fungi. In each photograph the pathogenic fungus cultured individually is on the left and the dual culture with LZ93 is on the right, (A) *G. graminis*, (B) *Phyricularia oryzae*, (C) *Phytophthora infestans*, (D) *Typhula incarnate*

Identification of compounds

11β-hydroxyrosenonolactone (1) was previously identified by X-ray analysis (Guttormson *et al*., 1970; Kiriyama *et al*., 1971), but no NMR data were reported. Here ^{1}H and ^{13}C NMR data were assigned based on 2D-NMR experiments (Table 2). The NMR data of compound 1 were very similar to those of desoxyrosenonolactone (4) and 2α-hydroxyrosenonolactone (Hanson *et al*., 2003), but after careful analysis we found four

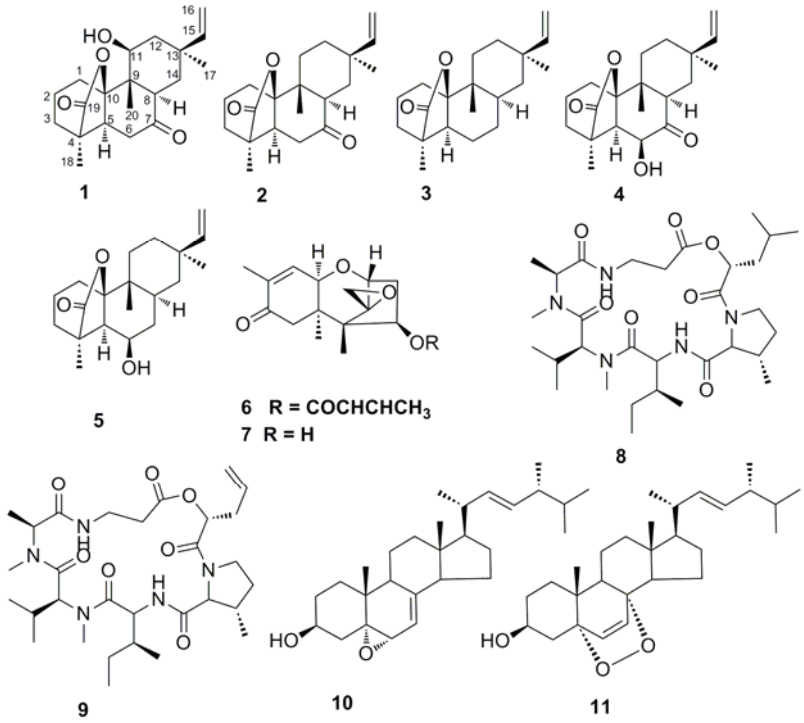

Fig. 2. Structure of compounds from *Trichothecium roseum* LZ93.

obvious dissimilarities between compounds 1 and 4. Thus, we think the OH group position is different in the two compounds. The ^{1}H, ^{13}C, HMQC, and HMBC spectra (Table 2) revealed the detail structure. In the HMBC spectrum, one vinyl group at δ_H 5.83 (1H, dd, J_1=10.7, J_2=17.4 Hz) and 5.02 (2H, dd, J_1=10.7, J_2=17.4 Hz) was assigned to C-15 and C-16 by the HMBC correlations from H-15 to C-13 (δ 35.8) and C-14 (δ 31.3) and H-16 to 13 (δ 35.8), 15 (δ 148.3), and 17 (δ 23.0). Three methyls were placed at C-4, C-9, and C-13 on the basis of HMBC correlations from H-18 (δ 1.13, s) to C-3 (δ 35.5), C-4 (δ 46.9), C-5 (δ 51.2), C-19 (δ 179.1), from H-20 (δ_H 0.91 and δ_C 10.1) to the C-8 (δ 47.9), C-9 (δ 44.1), C-10 (δ 86.4), C-11 (δ 68.6), and from H-17 (δ_H 1.02 and δ_C 23.0) to the C-12 (δ 40.3), C-13 (δ 35.8), C-14 (δ 31.3). The OH group was assigned to C-11 by the HMBC correlations from H-11 (δ_H 4.15 and δ_C 68.6) to the C-9 (δ 44.1), C-10 (δ 86.4), C-20 (δ 10.1). Together with other NMR data (Table 2), compound 1 was identified to be as 11β-hydroxyrosenonolactone (Fig. 2).

Compound 2 was identified as 6β-hydroxyrosenonolactone but according to references (Kiriyama *et al*., 1971; Dockerill *et al*., 1978; Loukaci *et al*., 2000; Hanson *et al*., 2003) and the data for compound 1 (Table 2), the chemical shifts of two carbons may previously have been assigned incorrectly. Hence, the 2-D NMR data were recorded. The ^{1}H, ^{13}C, HMQC, and HMBC spectra (Table 2) revealed that the chemical shifts of CH_2-6 should interconvert with those of CH_2-11. Methyl (C-20) was placed at C-9 on the basis of HMBC correlations from H-20 (δ 0.92, s) to C-10 (δ 87.0), C-8 (δ 47.4), C-9 (δ 38.9), C-11 (δ 30.7). The H-6 (δ 2.35-2.41 and 2.13) correlated with C-5 (δ 50.8), C-4/8 (δ 47.2 and/or 47.3), C-7 (δ 210.2), C-10 (δ 87.0). Moreover, the ^{1}H,^{1}H-COSY spectra showed that H-5 correlated with H-6. Together with other NMR data (Table 2), compound 2 was identified as rosenonolactone (Fig. 2). Our results were consistent with the data reported in references (Dockerill *et al*., 1978; Hanson *et al*., 2003) except for the two positions at CH_2-6 and CH_2-11 (Table 2).

Compounds 3-5 were determinated as desoxyrosenonolactone (Dockerill *et al*., 1978), 6β-hydroxyrosenonolactone (Dockerill *et al*., 1978; Hanson *et al*., 2003) and rosololactone (Dockerill *et al*., 1978) (Table 3), and compounds 6 and 7 were determinated as trichothecin and trichothecolone (Loukaci *et al*., 2000) on the basis of their NMR data and comparison with the data given in references (Fig. 2). Compounds 8 and 9 were two cyclodepsipeptides and were identified as roseocardin

Table 2. The NMR data of compounds 1 and 2

Position	1			2		
	^{1}H	^{13}C	HMBC	^{1}H	^{13}C	HMBC
1	2.61 (1H, m) 1.84 (1H, m, overlap)	30.2	2, 3, 5, 10 3	2.25 (1H, m, overlap) 1.70 (1H, m)	30.2	2 2
2	2.00 (1H, m) 1.84 (1H, m, overlap)	19.8	1, 3, 4, 10 3	1.95 (1H, m, overlap) 1.76 (1H, m, overlap)	19.8	3,10 \
3	1.79 (1H, m) 1.69 (1H, m)	35.5	1, 2, 4, 5,18 \	1.76 (1H, m, overlap) 1.60 (1H, m)	35.4	1, 5 4, 5,19
4	\	46.9	\	\	47.2	\
5	2.28 (1H, m)	51.2	3, 4, 1, 7	2.25 (1H, m, overlap)	50.8	3, 4, 7,10,19
6	2.43 (1H, m, overlap) 2.18 (1H, m)	35.7	\ 4, 5, 10, 7	2.35-2.41 (1H, m) 2.13 (1H, m)	35.8	5, 4/8, 7, 10 5, 4/8, 7, 10
7	\	209.4	\	\	210.2	\
8	2.43 (1H, m, overlap)	47.9	7, 9, 10, 11, 20	2.37-2.42 (1H, m)	47.3	7, 9, 10, 14, 20
9	\	44.1	\	\	38.9	\
10	\	86.4	\	\	87.0	\
11	4.15 (1H, brd, 11.6)	68.6	20, 9, 10	1.94 (1H, m, overlap) 1.46 (1H, m)	30.7	\ 8, 9, 10,13
12	1.55(1H, m) 1.45 (1H, m, overlap)	40.3	17, 9 \	1.30 (1H, m) 1.46 (1H, m, overlap)	31.3	9, 14, 17 \
13	\	35.8	\	\	35.0	\
14	1.65 (1H, m) 1.45 (1H, m, overlap)	31.3	9, 8 \	1.65 (1H, m) 1.53 (1H, m)	31.6	8, 13, 17 7, 15,17
15	5.83 (1H, dd, J_1 =10.7, J_2 = 17.4 Hz)	148.3	12, 13, 14, 17	5.85 (1H, dd, J_1 = 10.8 Hz, J_2 = 17.5 Hz)	149.5	13, 14, 17
16	5.02 (2H, dd, J_1 = 10.7, J_2 = 17.4 Hz)	110.3	17, 13,15	5.00(2H, dd, J_1 = 10.8, J_2 = 17.5 Hz)	109.9	13, 15
17	1.02 (3H, s)	23.0	12, 13, 14	0.96 (3H, s)	21.8	13, 14, 15
18	1.13 (3H, s)	16.9	3, 4, 5, 19	1.10 (3H, s)	16.8	3, 4, 5, 19
19	\	179.1	\	\	179.3	\
20	0.91 (3H, s)	10.1	8, 9, 11, 10	0.92 (3H, s)	16.9	8, 9, 10, 11

(Tsunoo *et al*., 1997) and roseotoxin B (Engstrom *et al*., 1975). In addition, compounds 10 and 11 were identified as 5α,6α-epoxy-24(*R*)-methylcholesta-7,22-dien-3β-ol (Bok *et al*., 1999) and 5α,8α-epidioxyergosta-6,22-dien-3β-ol (Yue *et al*., 2001) on the basis of their NMR data and comparison with the data given in references (Fig. 2).

Antifungal activity of compounds

The compounds 1, 2, 3, 4, 6, 7, and 9 were assayed for antifungal activity, and only compound 6 showed good inhibition of the phytopathogenic fungi such as *Typhula incarnate* (MIC 50 μg/ml), *G. graminis* (MIC 30 μg/ml), *P. infestans* (MIC 30 μg/ml), *A. solani* (MIC 5 μg/ml), *Phyricularia oryzae* (MIC 20 μg/ml). The experiments were repeated in triplicate and the results were consistent. Compounds 1, 2, 3, 4, 7, and 9 did not show inhibition of the tested phytopathogenic fungi at 300 μg/ml. To our surprise, although compound 7 was very similar to compound 6, it did not show inhibition of phytopathogenic fungi at 300 μg/ml. It is clear that the substituent at 4-OH was the key group for antifungal activities.

Nematicidal activity of compounds

In our study, compounds 2, 3, 6, 7, 8, and 9 were evaluated for their nematicidal activities against *Panagrellus redivivus*, *C. elegans* and *B. xylophilus* (Table 4). Compounds 2, 3, 7, and 9 did not show obvious activity against the tested nematodes. The LC_{50} values of compound 6 against *Panagrellus redivivus*, *C. elegans*, and *B. xylophilus* were 88.7, 105.4, and 167.1 μg/ml respectively at 72 h. Compound 8 exhibited activity against *C. elegans* with a LC_{50} value of 141.25 μg/ml at 72 h, but had no activity against *Panagrellus redivivus* and *B. xylophilus* at the tested dose (Table 4).

Antitumor activities of compounds

The inhibitory effects of compounds 4, 6, 7, 8, and 9 on growth of various human tumor cell lines are shown in Table 5. Compound 6 showed strong inhibiting activities to all the tested cell lines, with an IC_{50} of about 0.18-1.55 μM. Compounds 1, 2, 3, and 5 did not show any inhibitory effect at 40 μM. The remaining compounds showed various dose effects against the cell lines.

Discussion

Fungal endophytes infect their hosts without causing visible disease symptoms and have been isolated from almost every host thus far studied (Tan and Zou, 2001). Although it has long been known that fungal secondary metabolites are crucial

Table 3. The NMR data of compounds 3-5

Position	3		4		5	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	1.70 (1H, m) 1.50 (1H, m)	30.9	2.10 (1H, m) 2.35 (1H, m)	31.6	1.75 (1H, m) 1.56 (1H, m)	31.7
2	1.65 (1H, m) 1.43 (1H, m)	19.9	1.80 (1H, m) 1.95 (1H, m)	19.7	1.70 (1H, m) 1.85 (1H, m)	19.7
3	1.85 (1H, m) 1.68 (1H, m)	35.7	1.60 (1H, m) 1.80 (1H, m)	36.4	1.75 (1H, m) 1.60 (1H, m)	35.4
4	\	47.4	\	45.6	\	45.6
5	2.09 (1H, m)	52.4	2.61 (1H, d, J = 4.8, Hz)	54.9	2.19 (1H, m)	55.5
6	1.25∼1.50 (2H, m)	18.1	3.96 (1H, d, J = 4.8 Hz)	68.5	4.22 (1H, brd, J = 8.3 Hz)	63.8
7	1.25 ∼1.50 (2H, m)	25.6	\	212.8	1.90∼2.0 (2H, m)	36.4
8	1.30 ∼1.60 (1H, m)	30.8	2.83 (1H, brs)	47.1	1.30-1.80 (1H, m)	32.0
9	\	38.2	\	40.6	\	37.9
10	\	88.1	\	86.5	\	87.7
11	1.33 ∼1.50 (2H, m)	29.7	1.28 ∼1.80 (2H, m)	31.3	1.34 ∼1.55 (2H, m)	31.0
12	1.41 ∼1.80 (2H, m)	32.1	1.30 ∼1.80 (2H, m)	31.4	1.40 ∼1.80 (2H, m)	32.2
13	\	35.9	\	35.0	\	36.0
14	1.20 (1H, m) 1.40 (1H, m)	40.3	1.20 (1H, m) 1.40 (1H, m)	31.6	1.20 (1H, m) 1.40 (1H, m)	39.9
15	5.80 (1H, dd, J_1 = 10.7, J_2 = 17.5 Hz)	150.5	5.85 (1H, dd, J_1 = 10.8, J_2 = 17.5 Hz)	149.5	5.83 (1H, dd, J_1 = 10.7, J_2 = 17.5 Hz)	150.4
16	4.93 (2H, dd, J_1 = 10.7, J_2 = 17.5 Hz)	109.1	5.00 (2H, dd, J_1 = 10.8, J_2 = 17.5 Hz)	109.9	4.89 (1H, dd, J_1 =10.7, J_2 = 17.5Hz),	109.2
17	0.96 (3H, s)	22.2	0.97 (3H, s)	21.8	0.99 (3H, s)	22.4
18	1.06 (3H, s)	17.0	1.40 (3H, s)	17.0	1.31 (3H, s)	16.9
19	\	180.8	\	179.7	\	181.1
20	0.97 (3H, s)	14.7	1.10 (3H, s)	16.4	1.24 (3H, s)	13.4

Table 4. Effect of compounds 6 and 8 on the proof mortality of nematodes *in vitro* (%)*

Nematode	Concentration (μg/ml)	compound 6	compound 8
C. elegans	400	83.1	84.3
	200	72.1	67.2
	100	48.9	35.5
	50	27.0	14.1
	LC50	105.4	141.25
P. redivivus	400	92.6	-
	200	80.7	-
	100	57.5	-
	50	26.2	-
	LC50	88.7	-
B. xylophilus	400	76.2	-
	200	58.7	-
	100	34.1	-
	50	13.3	-
	LC50	167.1	-

* Experiments were made in triplicate, and mean percentage mortality was calculated.
-, Proof mortality <10%.

to the pathogenicity of many fungi, only a little experimental work has been done to study the role of secondary metabolites in the endophyte-host interaction. Most investigations have concentrated on isolating secondary metabolites and characterizing their biological activity (Tan and Zou, 2001). *T. roseum* LZ93, one of the endophytes from *M. hookeri*, showed antagonism against four phytopathogenic fungi and its extracts also showed strong antifungal activities. Thus, we investigated its mechanism. *T. roseum* is a common entophytic fungus, and there are several reports about antagonism between *T. roseum* and certain plant pathogenic fungi dating as far back as 1949 (Freeman and Morrison, 1949; Rod, 1984; Al-Heeti and Sinclair, 1988; George, 1995; Lacicowa and Pieta, 1996; Mandal *et al*., 1999; Vanneste *et al*., 2002; Ajit *et al*., 2006; Huang and Erickson, 2008). Trichothecin produced by *T. roseum* is an ester of isocrotonic acid (Freeman and Morrison, 1949), which is a strong antifungal agent. In our present research, we systemically analyzed and purified *T. roseum* chemical constituents and 11 compounds were isolated from fermentation products of strain LZ93, including two trichothecenes, five rosenonolactones, two cardiotonic cyclodepsipeptide, and two sterols. Although two trichothecenes were obtained, only trichothecin, which was reported to be an antifungal agent since 1949 (Freeman and Morrison, 1949) showed antifungal activity. In our experiments, trichothecin showed multiple activities: antifungal, antitumor, and nematicidal. Trichothecene type compounds were reported to have antiviral effects on plant viral infections (Bawden and Freeman, 1952), with trichothecin being more effective in managing viral infection in bean plants than in tobacco. The main constituent of *T. roseum* was a rosenonolactone-type of diterpenoid, which only showed weak activities. Compound 4 showed certain antitumor activities, but these did not contribute to antagonism against phytopathogenic fungi.

The events associated with the antagonistic process are, generally-speaking, of three types: In the first, the bioagent acts by entwining, strangling, and penetrating the pathogen's hyphae; in the second, a biological macromolecule such as chitinase (Ajit *et al*., 2006) can inhibit and/or degrade the pathogen's hyphae,; and in the third, active metabolites inhibit and/or kill the pathogen's hyphae. In the present work, based on our observations of antagonistic activities, we suggest that the antifungal compound trichothecin contributed the principal antagonistic action. *T. roseum* can obviously inhibit the growth of pathogenic fungi, but it cannot kill or degrade the pathogenic fungi. After contact of the two fungi, the pathogen's hyphae could not diffuse. Trichothecin is a nonvolatile and lipid-soluble compound, thus it diffused slowly in solid media (which was also used to accummulate compounds during growth). The yield of trichothecin in the solid fermentation was 2.8 μg/ml and logically the content of trichothecin in the PDA medium was more than the yield. Further, in terms of the strength of the inhibition, the content in the medium may have been equal to the MIC against phytopathogenic fungi.

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Table 5. Effect of compounds on the growth of human tumor cell lines (IC_{50} μM)

Compound	HL-60	SMMC-7721	A-549	SK-BR-3	PANC-1	MCF-7	SW480
4	9.84	>[a]	23.79	19.94	17.37	-[b]	-
6	0.18	1.41	0.39	1.55	0.28	-	-
7	14.51	>	>	>	>	>	>
8	5.02	>	>	-	-	19.65	20.45
9	29.46	>	>	-	-	19.08	18.32
Control (MW300)	3.17	11.27	16.46	8.29	18.15	18.74	14.88

[a] The compound did not show an inhibitory effect at 40 μM.
[b] Not done. (The compound was not assayed against the cell line).

optical rotations, UV, IR, NMR and mass spectra.

References

Ajit, N.S., R. Verma, and V. Shanmugam. 2006. Extracellular chitinases of fluorescent pseudomonads antifungal to *Fusarium oxysporum* f. sp. *dianthi* causing carnation wilt. *Curr. Microbiol.* 52, 310-316.

Al-Heeti, M.B. and J.B. Sinclair. 1988. Antagonism between *Gliodadium roseum*, *Trichoderma harzianum*, or *Trichothecium roseum* and *Phytophthora megasperma* f. sp. *glydnea*. *Mycopathologia* 103, 135-140.

Amiguet, T.V., P. Petit, C.A. Ta, R. Nunez, P. Sanchez-Vindas, L.P. Alvarez, M.L. Smith, J.T. Arnason, and T. Durst. 2006. Phytochemistry and antifungal properties of the newly discovered tree pleodendron costaricense. *J. Nat. Prod.* 69, 1005-1009.

Bawden, F.C. and G.G. Freeman. 1952. The nature and behaviour of inhibitors of plant viruses produced by *Trichothecium roseum* Link. *J. Gen. Microbiol.* 7, 154-168.

Bok, J.W., L. Lermer, J. Chilton, H.G. Klingeman, and G.H.N. Towers. 1999. Antitumor sterols from the mycelia of *Cordyceps sinensis*. *Phytochemistry* 51, 891-898.

Dockerill, B., J.R. Hanson, and M. Siverns. 1978. The ^{13}C NMR spectra of some rosane diterpenoids. *Phytochemistry* 17, 572-573.

Engstrom, G.W., J.V. DeLance, J.L. Richard, and A.L. Baetz. 1975. Purification and characterization of roseotoxin b, a toxic cyclodepsipeptide from *Trichothecium roseum*. *J. Agric. Food Chem.* 23, 244-253.

Freeman, G.G. and R.I. Morrison. 1949. The isolation and chemical properties of trichothecin, an antifungal substance from *Trichothecium roseum* Link. *Biochem. J.* 44, 1-5.

George, D.G. 1995. Biological and chemicalcontrol of rice blast disease (*Pyricularia oryzae*) in Northern Greece. *Cah. Mediterr.* 15, 61-68.

Guttormson, R., P. Main, A.J. Allison, and K.H. Overton. 1970. Structure of rosein III: X-ray analysis without a heavy atom. *J. Chem. Soc. D* 719-720.

Hanson, J.R., P.B. Hitchcock, I. Pibiri, and F. Piozzi. 2003. The biotransformation of the diterpenoid, rosenonolactone by *Mucor plumbeus. J. Chem. Research(S)* 147-149.

Hong, L.J., G.H. Li, W. Zhou, X.B. Wang, and K.Q. Zhang. 2007. Screening and isolation of a nematicidal sesquiterpene from *Magnolia grandiflora* L. *Pest Manag. Sci.* 63, 301-305.

Huang, H.C. and R.S. Erickson. 2008. Factors affecting biological control of *Sclerotinia sclerotiorum* by fungal antagonists. *J. Phytopathol.* 156, 628-634.

Kiriyama, N., Y. Yamamoto, and Y. Tsuda. 1971. Chemical correlation of rosein III (11-hydroxyrosenonolactone) and rosenonolactone. *J. Chem. Soc. D* 37-39.

Lacicowa, B. and D. Pieta. 1996. The efficiency of microbiological dressing of pea seeds (*Pisum sativum* L.) against pathogenic soilborn fungi. *Rocz. Nauk. Roln. Ser. E* 15-21.

Li, G.H., Y.M. Shen, and K.Q. Zhang. 2005. Nematicidal activity and chemical component of *Poria cocos*. *J. Microbiol.* 43, 17-20.

Liu, C., B.M. Tadayoni, L.A. Bourret, K.M. Mattocks, S.M. Derr, W.C. Widdison, N.L. Kedersha, P.D. Ariniello, V.S. Goldmacher, J.M. Lambert, W.A. Blättler, and R.V. Chari. 1996. Eradication of large colon tumor xenografts by targeted delivery of maytansinoids. *Proc. Natl. Acad. Sci. USA* 93, 8618-8623.

Loukaci, A., O. Kayser, K. Bindseil, K. Siems, J. Frevert, and P.M. Abreu. 2000. New trichothecenes isolated from *Holarrhena floribunda*. *J. Nat. Prod.* 63, 52-56.

Mandal, S., K.D. Srivatava, A. Rashmi, D.V. Singh, S. Mandal, and R. Aggarwal. 1999. Mycoparasitic action of some fungi on spot blotch pathogen (*Drechslera sorokiniana*) of wheat. *Indian Phytopathol.* 52, 39-43.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55-63.

Owen, N.L. and N. Hundley. 2004. Endophytes-The chemical synthesizers inside plants. *Sci. Prog.* 87, 79-99.

Rex, J.H., M.A. Pfaller, T.J. Walsh, V. Chaturvedi, A. Espinel-Ingroff, M.A. Ghannoum, L.L. Gosey, F.C. Odds, M.G. Rinaldi, D.J. Sheehan, and D.W. Warnock. 2001. Antifungal susceptibility testing: practical aspects and current challenges. *Clin. Microbiol. Rev.* 14, 643-658.

Rod, J. 1984. Antagonistic effects of some fungi on fungal pathogens causing storage rots of onion (*Allium cepa* L.). *Ceska Mykol.* 38, 235-239.

Skidmore, A.M. and C.H. Dickinson. 1976. Colony interaction and hyphal interference between *Septoria nodorurn* and phylloplane fungi. *Trans. Br. Mycol. Soc.* 66, 57-64.

Sturz, A.V., B.R. Christie, and J. Nowak. 2000. Bacterial endophytes: potential role in developing sustainable systems of crop production. *Crit. Rev. Plant Sci.* 19, 1-30.

Tan, R.X. and W.X. Zou. 2001. Endophytes: a rich source of functional metabolites. *Nat. Prod. Rep.* 18, 448-459.

Tsunoo, A., M. Kamijo, N. Taketomo, Y. Sato, and K. Ajisaka. 1997. Roseocardin, a novel cardiotonic cyclodepsipeptide from *Trichothecium roseum* TT103. *J. Antibiot.(Tokyo)* 50, 1007-1013.

Vanneste, J.L., R.A. Hill, S.J. Kay, R.L. Farrell, and P.T. Holland. 2002. Biological control of sapstain fungi with natural products and biological control agents: a review of the work carried out in New Zealand. *Mycol. Res.* 106, 228-232.

Yue, J.M., S.N. Chen, Z.W. Lin, and H.D. Sun. 2001. Sterols from the fungus *Lactarium volemus*. *Phytochemistry* 56, 801-806.

Zhao, P.J., L.M. Fan, G.H. Li, N. Zhu, and Y.M. Shen. 2005. Antibacterial and antitumor macrolides from *Streptomyces* sp. Is9131. *Arch. Pharm. Res.* 28, 1228-1232.

Zhao, P.J., G.H. Li, and Y.M. Shen. 2006. New chemical constituents from the endophyte *Streptomyces* species LR4612 cultivated on *Maytenus hookeri*. *Chem. Biodivers.* 3, 337-342.

Zhao, P.J., H.X. Wang, G.H. Li, H.D. Li, J. Liu, and Y.M. Shen. 2007. Secondary metabolites from endophytic *Streptomyces* sp. Lz531. *Chem. Biodivers.* 4, 899-904.