Plant Science 151 (2000) 67–73

New bioactive metabolites produced by *Colletotrichum* sp., an endophytic fungus in *Artemisia annua*

Hong Lu [a], Wen Xin Zou [a], Jun Cai Meng [a,c], Jun Hu [b], Ren Xiang Tan [a,*]

[a] *State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing, University, Nanjing 210093, PR China*
[b] *State Key Laboratory of Co-ordination Chemistry, Nanjing University, Nanjing 210093, PR China*
[c] *National Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou, PR China*

Received 30 April 1999; received in revised form 24 September 1999; accepted 28 September 1999

Abstract

In addition to ergosterol (**I**), 3β,5α,6β-trihydroxyergosta-7,22-diene (**II**), 3β-hydroxy-ergosta-5-ene (**III**), 3-oxo-ergosta-4,6,8(14),22-tetraene (**IV**), 3β-hydroxy-5α,8α-epidioxy-ergosta-6,22-diene (**V**), 3β-hydroxy-5α,8α-epidioxy-ergosta-6,9(11),22-triene (**VI**) and 3-oxo-ergosta-4-ene (**VII**), a plant hormone indole-3-acetic acid (IAA) and three new antimicrobial metabolites were characterized from the culture of *Colletotrichum* sp., an endophyte isolated from inside the stem of *Artemisia annua*. The structures of the new metabolites were elucidated by a combination of spectroscopic methods (IR, MS, ^{1}H and ^{13}C NMR) as 6-isoprenylindole-3-carboxylic acid (**1**), 3β,5α-dihydroxy-6β-acetoxy-ergosta-7,22-diene (**2**) and 3β,5α-dihydroxy-6β-phenylacetyloxy-ergosta-7,22-diene (**3**), respectively. The compounds **1**–**3** and **III**–**V** inhibited the growth of all the tested bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina lutea* and *Pseudomonas* sp.) with minimal inhibitory concentrations (MICs) ranging from 25 to 75 μg/ml. Moreover, metabolites **2** and **3**, together with the known sterols **III** and **V**, were inhibitory against the fungi *Candida albicans* and *Aspergillus niger* with MICs between 50 and 100 μg/ml. At 200 μg/ml, compounds **1**–**3**, **III** and **IV** were shown to be fungistatic to the crop pathogenic fungi *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia cerealis*, *Helminthosporium sativum* and *Phytophthora capisici*. This is the first report on the endophytic fungus from *A. annua* and the bioactive metabolites thereof.

Keywords: *Artemisia annua*; Asteraceae; Endophyte; Metabolites; Auxin; Antimicrobial

1. Introduction

Endophytes are microorganisms that live in the intercellular spaces of stems, petioles, roots and leaves of plants causing no discernible manifestation of their presence and have typically gone unnoticed [1]. The symbiosis between plant and endophyte was ascertained, namely, the former protects and feeds the latter which produces 'in return' bioactive (plant growth regulatory, antibacterial, antifungal, antiviral, insecticidal, etc.) substances to enhance the growth and competitiveness of the host in nature [2]. Accordingly, some endophytes could be reliable sources of materials of the agricultural and/or pharmaceutical potential as exemplified by taxol [3], subglutinol A and B [4], and peptide leucinostatin A [5] (all could be produced by both endophytes and the hosts).

Artemisia annua (*A. annua*) L. (Asteraceae), a traditional Chinese medicinal herb well recognized for its synthesis of artemisinin (an antimalarial drug), was found to be a widespread species that can thrive in many geographically different areas. In addition to the remarkable ecological adaptability, this plant is strongly resistant to insects and pathogens. The study was thus undertaken in order to ascertain the presence of endophytes inside the plant, and if any the potential for synthesizing bioactive compounds. We wish hereby to report that *Colletotrichum* sp., an endophyte in *A.*

* Corresponding author. Tel.: +86-25-359-3201; fax: +86-25-359-3201.
E-mail address: rxtan@netra.nju.edu.cn (R.X. Tan)

0168-9452/00/$ - see front matter
PII: S0168-9452(99)00199-5

annua can produce in vitro metabolites that were shown to be antimicrobial. Others are known to be plant growth regulatory.

2. Materials and methods

2.1. General

IR spectra were recorded in KBr disks on a Perkin–Elmer 577 instrument. All NMR experiments were performed on a Bruker AM 500 FT-NMR spectrometer using TMS or solvent signals as the internal standard. Mass spectra were run on a VG-ZAB-HS mass spectrometer. Silica gel (200–300 mesh) for column chromatography and GF_{254} (30–40 μm) for TLC were produced by Qingdao Marine Chemical Factory, Qingdao, China. Sephadex LH-20 was purchased from Pharmacia Biotech, Sweden. All other chemicals used in this study were of analytical grade.

2.2. Source and selection of Colletotrichum sp.

Fresh stems of *A. annua* were collected from apparently healthy plants from May to October 1997 in the suburb of Nanjing, China. The stems were cut into rods ($\approx$ 10 cm in length), and rinsed in running water. After successive surface sterilization in 75% ethanol and 40% formalin (3 min each) [6], the stem rods were rinsed three times in sterilized distilled water, and cleaved aseptically into small segments. The effectiveness of the sterilizing procedure was reinforced by the vitality test as described elsewhere [6]. The flat sides of the segments were carefully placed onto potato dextrose agar (PDA) plates (supplemented with 100 mg/l ampicilin and 150 mg/l streptomycin sulphate to suppress the bacterial growth), and incubated at 28°C until the outgrowth of endophytes was discerned. Hyphal tips originating from segments were transferred to petri dishes containing PDA medium free of antibiotics. Each isolate was then grown and examined to ascertain that it originated from a single organism. Thus, a total of 178 fungal strains belonging to 32 taxa were obtained.

All of the isolated filamentous endophytic fungi from *A. annua* were inoculated on PDA, corn meal agar (CMA), oatmeal agar (OA), water agar (WA) and Czepak–Dox agar. Incubation under different conditions induced sporulation followed by identification according to the morphology of the fungal culture, the mechanism of spore production, and the characteristics of the spores. Among these fungal strains, *Colletotrichum* sp. was more frequently isolated. According to the accepted definition of endophyte [7], the result of the vitality test and the higher isolation frequency (18 out of 178 fungal strains), the microorganisms were considered as endophytes. A strain of the *Colletotrichum* sp. designated as B501 was selected for further study because of its greater potential for producing antimicrobial and plant growth regulatory substances. Living culture of *Colletotrichum* sp. B501 has been deposited under the number AF99008 in China Center for Type Culture Collection (CCTCC). Other strains were stored presently on PDA slants at 4°C and in 40% glycerol at −70°C in the Herbarium of Nanjing University.

2.3. Cultivation

The fresh mycelium grown on PDA medium at 28°C for 5 days was inoculated into 500 ml Erlenmeyer flasks containing 100 ml PD medium. After 2 days of the incubation at 28°C on rotary shaker at 150 rpm, a 40-ml culture liquid was transferred as seed into each of a total of 250 1000-ml Erlenmeyer flask containing 400-ml PD medium. The cultivation that followed was kept for 10 days at 28°C and 150 rpm on a rotary shaker.

2.4. Extraction and fractionation

The culture filtrate (total volume 110 l) and mycelium were extracted exhaustively with ethyl acetate. Evaporation of the solvent from the extract in vacuo gave a residue (35 g) which was chromatographed on a silica gel column (700 g) eluting successively with petroleum ether (1.5 l) and a petroleum ether–acetone gradient (50:1 → 1:50, 5 l) and acetone (1.5 l). Based on the TLC monitoring, the collected fractions (300 ml each) were combined into six parts (E-1: 28 g, E-2, 2.3 g; E-3, 0.5 g; E-4, 1.0 g; E-5, 0.7 g; E-6, 2.5 g). E-1 contained mainly lipids of no biological interest. CC (column chromatography) of E-2 over silica gel (150 g) with petroleum ether–ethyl acetate (50:1 → 1:1, 4 l) gave **I** (72 mg), and two mixtures (E-2/1 and E-2/2). E-2/1 was subjected to further CC fractionation over silica gel with a petroleum

ether–ethyl acetate (20:1, 2 l) mixture yielding **V** (50 mg) and **VI** (6 mg) as needles. Gel filtration of E-3 over Sephadex LH-20 with $CHCl_3$–MeOH (1:1) gave acid **1** (10 mg). Preparative TLC of E-4 with petroleu methyl acetate mixture (15:1, developed twice) yielded **IV** (15 mg) and a fraction that gave **VII** (7 mg) by gel filtration over Sephadex LH-20 with $CHCl_3$–MeOH (1:1). E-2/2 was combined with E-5, and the mixture was separated by CC over silica gel (60 g) with $CHCl_3$–MeOH gradient (50:1 → 1:1, 1.2 l) to give **2** (15 mg) and two mixtures (E-5/1 and E-5/2). Gel filtration of E-5/1 over Sephadex LH-20 with $CHCl_3$ to yield **3** (20 mg). E-6, combined with E-5/2, was subjected to further CC fractionation over silica gel (130 g) with $CHCl_3$–MeOH gradient (30:1 → 1:1, 1.7 l) to yield **III** (10 mg) as white needles, and two gums (E-6/1 and E-6/2). Repeated gel filtration of E-6/1 over Sephadex LH-20 with $CHCl_3$–MeOH mixture (1:1) afforded IAA (Indole acetic acid) (55 mg), and treatment of E-6/2 in the same manner gave **II** (20 mg).

2.5. Antimicrobial activity

The minimal inhibitory concentrations (MICs) were determined by paper–disk assay on LB (yeast extract 5, peptone 10, NaCl 5 and agar 20 g/l, pH 7.0) and/or PDA plates seeded with 10^6 cells (and/or spores)/ml suspension of tested bacteria and fungi, followed by incubation at 37°C for bacteria (48 h) and at 28°C for fungi (96 h), respectively. All metabolites isolated from the culture were dissolved in ethanol and applied to disks at different concentrations. The test microorganisms were *Bacillus subtilis* (*B. subtilis*); *Staphylococcus aureus* (*S. aurens*); *Sarcina lutea* (*S. lutea*); *Pseudomonas* sp.; *Candida albicans* (*C. albicans*); *Aspergillus niger* (*A. niger*); *Trichophyton rubrum* (*T. rubrum*) and *Cunninghamella elegans* (*C. elegans*).

The fungistatic action was evaluated on separate PDA plates containing metabolites at concentrations 0 (control), 50, 100 and 200 μg/ml, respectively. The crop pathogenic fungi *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia cerealis*, *Helminthosporium sativum* and *Phytophthora capisici* were inoculated thereon. Each treatment had three replicates. After 5 days of incubation at 28°C, colony diameter was measured and the effect of each compound was evaluated based on fungal growth.

3. Results

3.1. Identification of the endophytic fungus

Colletotrichum sp. was isolated frequently from older stems of *A. annua*. Newly isolated mycelium grew well on PDA, and easily produced fruiting bodies. Colonies with a regular margin attained 40–50 mm in diameter after incubation on PDA at 28°C for 5 days. The aerial mycelia where white to grey, and grey to darkish green on the reverse side of the plate. Conidia form in armeniaceous masses from abundant dark acervuli. Sclerotia are black, globose and sparse. Setae, grown out of the conidiophores, are dark brown with five septa (3.7×114.8 μm). Ellipsoid to cylindrical conidia are straight, nonseptate, and rounded at ends, $4.0–5.1 \times 11.8–15.2$ μm in size. Appressoria, typical of the genus *Colletotrichum*, are unicellular, dark brown, irregular, often lobed, and sized $6.4–6.8 \times 12.8–16.3$ μm. These morphological characteristics led to the identification of the endophytic fungus as a *Colletotrichum* sp. [8].

3.2. Analyses of the metabolites

The IAA produced by the endophytic fungus was readily identified by co-TLC and -HPLC with the authentic sample, and by comparing its H′H NMR and EIMS data with those in the literature [9]. The molecular formula of the new metabolite **1** was analyzed to be $C_{14}H_{15}O_2N$ by its spectral data (EIMS, DEPT, ^1H and ^{13}C NMR). The IR absorption band at 1661 cm^{-1} indicated the presence of a carboxyl group conjugating presumably to an aromatic nucleus. In the ^1H-NMR spectrum of **1**, the presence of an isoprenyl group was revealed by typical signals at δ1.77, 1.78 (each 3H, s), 3.48 (2H, d, $J = 7.6$ Hz) and 5.39 (1H, t, $J = 7.6$ Hz). Furthermore, a set of signals at δ7.16 (1H, br d, $J = 8.0$ Hz), 7.24 (1H, br s) and 8.13 (1H, d, $J = 8.0$ Hz), 8.54 (1H, br s), and 7.97 (1H, d, $J = 2.7$ Hz) suggested that it was an isoprenylated indole-3-carboxylic acid [10]. This proposal was reinforced by its ^{13}C NMR spectrum, in which the isoprenyl group gave characteristic carbon resonance lines at δ17.9 (CH_3), 25.8 (CH_3), 34.4 (CH_2), 123.5 (CH) and 132.5 (C). All ^1H and ^{13}C NMR data of compound **1** were assigned by comparing with those of structurally related compounds [10,11]. As anticipated, the attachment of iso-

prenyl group on C-6 was required by splitting pattern of H-4 at δ8.13 (1H, d, $J = 8.0$ Hz) and the signal of C-7 at δ110.6 [10,11]. The structure of metabolite **1** was thus established as 6-isoprenyl-indole-3-carboxylic acid.

The identification of ergosterol (**I**) was established on its spectral data (^{1}H and ^{13}C NMR, DEPT, and ^{1}H-^{1}H COSY) [12]. So identified were 3β,5α,6β-trihydroxyergosta-7,22-diene (**II**) [13], 3β-hydroxy-ergosta-5-ene (**III**) [14], 3-oxo-ergosta-4,6,8(11),22-tetraene (**IV**) [15], 3β-hydroxy-5α,8α-epidioxy-ergosta-6,22-diene (**V**) [16], 3β-hydroxy-5α,8α-epidioxy-ergosta-6,9(11),22-triene (**VI**) [16] and 3-oxo-ergosta-4-ene (**VII**) [17].

The ^{1}H and ^{13}C NMR spectra of compound **2** were closely similar to those of **II** suggesting that it was presumably a derivative of 3β,5α,6β-trihydroxyergosta-7,22-diene [13]. A pair of three-proton singlets at δ0.46 and 0.91, along with four methyl doublets ($J = 6.4$ Hz) at δ0.72, 0.74, 0.81 and 0.92, and double doublets at δ5.12 ($J = 15.4$, 7.2 Hz) and 5.05 ($J = 15.4$, 8.0 Hz) was indicative of its ergosta-22-ene skeleton. However, an acetate singlet at δ2.03 in its ^{1}H NMR spectrum of **2** and an IR ester absorption band at 1738 cm^{-1} indicated the presence of an acetoxy group. Furthermore, a pair of mutually coupled proton signals at δ4.80 and 5.16 (each 1H, d, $J = 5.0$ Hz) ascribed to H-6 and H-7 suggested that sterol **2** was 6-*O*-acetyl derivative of 3β,5α,6β-trihydroxyergosta-7,22-diene [18]. This proposal was confirmed by its ^{13}C NMR data, which were assigned by comparing them with those of **II** [13]. All these evidence established the structure of **2** as 3β,5α-dihydroxy-6β-acetoxy-ergosta-7,22-diene, a hitherto unreported ergosterol derivative.

The ^{1}H and ^{13}C NMR spectra of compound **3** were similar in part to those of **2** indicating that it was also a 3β,5α,6β-trihydroxy-ergosta-7,22-diene derivative. However, the acetate singlet at δ2.03 in the ^{1}H-NMR spectrum of **2** was replaced by a set of signals including a methylene singlet at δ3.69 and five proton multiplet centered at δ7.33, presumably ascribable to a phenylacetyl group. The presence of this group was further confirmed by the base peak at m/z 91 in its EI mass spectrum, and a group of carbon resonance lines at δ170.7 (C), 41.5 (CH_2), 132.8 (C), 128.6 (2 × CH), 129.3 (2 × CH), 127.5 (CH) in the ^{13}C NMR spectrum of **3**. Furthermore, a pair of doublets ($J = 5.0$ Hz) at δ4.87 and 5.25 arising from H-6 and H-7 demonstrated that the phenylacetyl group was anchored on C–6 [18]. Therefore, the structure of the new sterol **3** was determined as 3β,5α-dihydroxy-6β-phenylacetyloxy-ergosta-7,22-diene.

3.3. Spectroscopic data of the new metabolites

3.3.1. Isoprenylindole-3-carboxylic acid (*1*)

White needle; $C_{14}H_{15}O_2N$; m.p. 54–55°C; IR ν_{max} (cm^{-1}): 1661, 1466, 720; EIMS m/z (rel. int.): 229 (M^+) (7), 212 (23), 184 (32), 57 (100); ^{1}H NMR ($CDCl_3$, 500 MHz), 8.54 (1H, br s, N–H), 7.97 (1H, d, $J = 2.7$ Hz, H-2), 8.13 (1H, d, $J = 8.0$ Hz, H-4), 7.16 (1H, br d, $J = 8.0$ Hz, H-5), 7.24 (1H, br s, H-7), 3.48 (2H, d, $J = 7.6$ Hz, H-1′), 5.39 (1H, t, $J = 7.6$ Hz, H-2′), 1.77, 1.78 (3H each, s, H-4′ and H-5′); ^{13}C NMR ($CDCl_3$, 500 MHz): 131.7 (C-2), 124.1 (C-3), 121.4 (C-4), 121.5 (C-5), 137.5 (C-6), 110.6 (C-7), 129.0 (C-8), 137.5 (C-9), 34.4 (C-1′), 123.5 (C-2′), 132.5 (C-3′, 17.9 (C-4′), 25.8 (C-5′).

3.3.2. 3β,5α-Dihydroxy-6β-acetoxy-ergosta-7,22-diene (*2*)

Needles, $C_{30}H_{48}O_4$; m.p. 89–90°C; IR ν_{max} (cm^{-1}): 3441, 2956, 1738, 1380, 1272, 1029, 969; EIMS m/z (rel. int.): 472 (M^+) (1.2), 412 (20.8), 394 (51.8), 377 (66.2), 252 (100), 174 (15.6), 69 (86.5); ^{1}H NMR ($CDCl_3$, 500 MHz): 3.91 (1H, m, H-3), 4.80 (1H, d, $J = 5.0$ Hz, H-6), 5.16 (1H, d, $J = 5.0$ Hz, H-7), 0.46 (3H, s, H-18), 0.91 (3H, s, H-19), 0.92 (3H, d, $J = 6.4$ Hz, H-21), 5.12 (1H, dd, $J = 15.4$, 7.2 Hz, H-22), 5.05 (1H, dd, $J = 15.4$, 8.0 Hz, H-23), 0.72 (3H, d, $J = 6.4$ Hz, H-26), 0.74 (3H, d, $J = 6.4$ Hz, H-7), 0.81 (3H, d, $J = 6.4$ Hz, H-28), 2.03 (3H, s, acetyl); ^{13}C NMR ($CDCl_3$, 500 MHz): 31.3 (C-1), 30.7 (C-2), 67.3 (C-3), 40.3 (C-4), 74.0 (C-5), 75.1 (C-6), 115.0 (C-7), 144.4 (C-8), 43.3 (C-9), 38.9 (C-10), 22.6 (C-11), 39.1 (C-12), 43.8 (C-13), 54.7 (C-14), 21.2 (C-15), 28.0 (C-16), 55.8 (C-17), 12.2 (C-18), 17.9 (C-19), 39.4 (C-20), 21.2 (C-21), 135.4 (C-22), 132.1 (C-23), 42.8 (C-24), 33.1 (C-25), 20.0 (C-26), 19.6 (C-27), 17.6 (C-28), 170.7 and 20.0 (acetyl).

3.3.3. 3β,5α-Dihydroxy-6β-phenylacetyloxy-ergosta-7,22-diene (*3*)

Colorless gum; $C_{36}H_{52}O_4$; IR ν_{max} (cm^{-1}): 3414, 2955, 1732, 1603, 1497, 1455, 1370, 1251, 1159,

Table 1
Antimicrobial activities of metabolites from the endophyte culture

Test microbes	MICs (μg/ml)					
	1	**2**	**3**	**III**	**IV**	**V**
Bacillus subtilis	25	75	50	75	25	75
Staphylococcus aureus	50	*	75	*	75	75
Sarcina lutea	75	50	*	*	*	75
Pseudomonas sp.	50	75	50	75	50	50
Candida albicans	*	100	75	75	*	50
Aspergillus niger	*	100	50	50	*	75

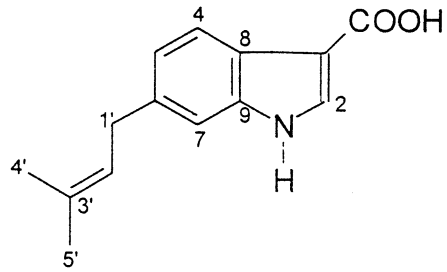
* Inactive at 200 μg/ml.

973; EIMS m/z (rel. int.): 548 (M^+)(0.9), 412 (5.57), 396 (14.9), 279 (42.9), 153 (33.3), 91 (100); ^{1}H NMR ($CDCl_3$, 500 MHz): 3.91 (1H, m, H-3), 4.87 (1H, d, $J = 5.0$ Hz, H-6), 5.25 (1H, d, $J = 5.0$ Hz, H-7), 0.55 (3H, s, H-18), 0.99 (3H, s, H-19), 1.00 (3H, d, $J = 6.4$ Hz, H-21), 5.22 (1H, dd, $J = 15.4$, 7.2 Hz, H-22), 5.13 (1H, dd, $J = 15.4$, 8.0 Hz, H-23), 0.82 (3H, d, $J = 6.4$ Hz, H-26), 0.84 (3H, d, $J = 6.4$ Hz, H-27), 0.92 (3H, d, $J = 6.4$ Hz, H-28), 3.69 (2H, s, H-2′), 7.33 (5H, m, Ar–H); ^{13}C NMR ($CDCl_3$, 500 MHz): 31.2 (C-1), 30.5 (C-2), 67.1 (C-3), 41.5 (C-4), 74.4 (C-5), 75.1 (C-6), 114.8 (C-7), 144.1 (C-8), 43.3 (C-9), 39.3 (C-10), 22.6 (C-11), 39.1 (C-12), 43.7 (C-13), 54.8 (C-14), 21.1 (C-15), 28.0 (C-16), 55.8 (C-17), 12.2 (C-18), 17.9 (C-19), 40.3 (C-20), 21.1 (C-21), 135.4 (C-22), 132.2 (C-23), 42.8 (C-24), 33.1 (C-25), 19.9 (C-26), 19.6 (C-27), 17.6 (C-28), 170.7 (C-1′), 41.5 (C-2′), 132.8 (C-3′), 128.6 (2C, C-4’ and C-8′), 129.3 (2C, C-5′ and C-7′), 127.5 (C-6′).

3.4. Antimicrobial properties

All the 11 metabolites isolated from cultures of the endophyte *Colletotrichum* sp. were subjected to antimicrobial assay. Compounds **2**, **3**, **III** and **V** exhibited antimicrobial activities against the bacteria *B. subtilis*, *S. aureus*, *S. lutea* and *Pseudomonas* sp. (MICs: 25–75 μg/ml), and against the fungi *C. albicans* and *A. niger* (MICs: 50–100 μg/ml). But none was active against *T. rubrum* and *C. elegans* even at the highest concentration (200 μg/ml). Meanwhile, the growth of those bacteria could also be inhibited by compounds **1** and **IV** (MICs: 25–75 μg/ml) (Table 1).

On the other hand, all compounds of the endopyhtic origin were tested for fungistatic activities to the crop pathogenic fungi *Phytophthora capisici* (Phc), *Rhizoctonia cerealis* (Rhc), *Gaeumannomyces graminis* var. *tritici* (Ggt), and *Helminthosporium sativum* (Hes). As summarized in Table 2, compounds **1**, **2** and **III** were fungistatic to Phc and Rhc, the sterols **2**, **3** and **IV** to Ggt, and metabolites **1** and **3** to Hes. However, no fungistasis could be discerned at 50 and 100 μg/ml.

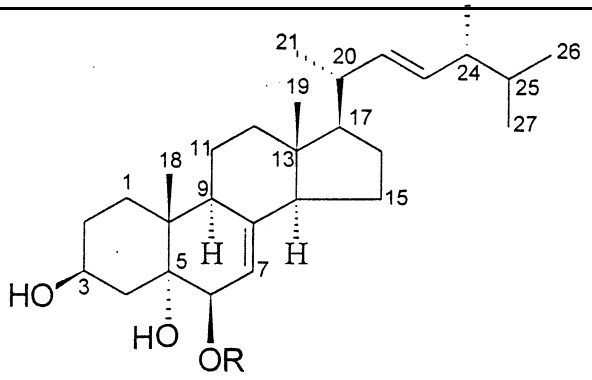


1

Table 2
Colony diameter (cm) of the tested fungi on PDA containing compounds **1**–**3**, **III** and **IV** (at 200 μg/ml)[a]

Test Fungi	**1**	**2**	**3**	**III**	**IV**	**Control**
Ggt	2.03 ± 0.21(3)	0.50 ± 0.00(3)	0.670 ± 0.06(3)	2.10 ± 0.12(3)	0.50 ± 0.00(3)	2.00 ± 0.06(3)
Phc	0.50 ± 0.00(3)	0.50 ± 0.00(3)	2.20 ± 0.17(3)	0.50 ± 0.06(3)	2.20 ± .32(3)	2.20 ± 0.06(3)
Rhc	0.80 ± 0.15(3)	0.80 ± 0.21(3)	3.40 ± 0.40(3)	0.50 ± 0.00(3)	3.40 ± 0.38(3)	3.40 ± 0.21(3)
Hes	0.60 ± 0.12(3)	1.50 ± 0.17(3)	0.70 ± 0.06(3)	1.50 ± 0.21(3)	1.40 ± 0.32(3)	1.40 ± 0.15(3)

[a] The diameter of each inoculum is 0.5 cm. Ggt, *Gaeumannomyces graminis* var. *tritici*; Phc, *Phytophthora capisici*; Rhc, *Rhizoctonia cerealis*; Hes, *Helminthosporium sativum*.



2 R = $COCH_3$
3 R = $COCH_2C_6H_5$

4. Discussion

Previously, *Colletotrichum* spp. such as *C. gloeosporioides* [19,20], *C. magna* [21] have been reported as endophytes of some plants. To our knowledge, *Colletotrichum* sp. was ascertained for the first time to be an endophyte inside the representative of the family Asteraceae. Among the eleven metabolites characterized from the culture of *Colletotrichum* sp., the new compounds **1**–**3** and known ergosterol derivatives (**III**, **IV** and **V**) were inhibitory against Gram-negative and -positive bacteria, such as *Pseudomonas* sp. and *B. subtilis* Table 1. Among these antibacterial metabolites, the sterols **2**, **3**, **III** and **V** were antifungal, too, and compounds **1**–**3**, **III** and **IV** fungistatic to the crop pathogenic fungi at 200 μg/ml. In addition to the antimicrobial activity, sterol **V** was previously reported to be antiviral [22]. These findings suggested the possibility that the endophyte *Colletotrichum* sp. in *A. annua* could protect the host by producing metabolites, which may be toxic or even lethal to phytopathogens. IAA is a well-known important auxin that can regulate plant physiological processes at low concentrations. Sterol **III** was reported to possess plant growth stimulating activity [23]. That the endophyte *Colletotrichum* sp. can produce plant growth regulators such as IAA, raised a possibility that the presence of endophytic fungus in *A. annua* could regulate the growth of the host. In conclusion, the characterization of antimicrobial and plant growth regulatory metabolites from the culture of *Colletotrichum* sp. showed that the endophytic fungus is presumably involved in the superior adaptability and competitiveness of *A. annua* in nature.

On the other hand, the study concerned only one of the 32 endophytes isolated. Our preliminary assay also indicated that some of the other endophyte isolates are also capable of synthesizing bioactive substances. This observation suggested that *A. annua* endophytes may have pharmaceutical and/or agricultural potential. Further investigation concerning the topic is highly desired.

Acknowledgements

This work was financed by grants for RXT from NNSF (No.39725033 and 39670873) and for HL from CPSF (No. 1998-06-23).

References

[1] G.A. Strobel, D.M. Long, Endophytic microbes embody pharmaceutical potential, ASM News 64 (1998) 263–268.
[2] G.C. Carroll, Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont, Ecology 69 (1988) 2–9.
[3] A. Stierle, G.A. Strobel, D. Stierle, Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew, Science 260 (1993) 214–216.
[4] J.C. Lee, E. Lobkovsky, N.B. Pliam, G.A. Stroble, J. Clardy, Subglutinol A and B: immunosuppressive compounds from the endophytic fungus *Fusarium cubglutinans*, J. Org. Chem. 60 (1995) 7076–7077.
[5] G.A. Stroble, W.M. Hess, Glucosylation of the peptide leucinostatinA, produced by an endophytic fungus of European yew, may protect the host from leucinostatin toxicity, Chem. Bio. 4 (1997) 529–536.
[6] B. Schulz, U. Wanke, S. Draeger, H.J. Aust, Endophytes from herbaceous plants and shrubs, effectiveness of surface sterilization methods, Mycol. Res. 97 (1993) 1447–1450.
[7] D. Wilson, Fungal endophytes: out of sight but should not be out of mind, Oikos 68 (1993) 378–384.
[8] J.C. Wei, Manual of Fungal Identification, Shanghai Science and Technology Press, Shanghai, 1979, pp. 463–476.
[9] M.P. Ho, H.S. Chung, M.L. David, Pulsed high-pressure liquid injection of biological molecules into supersonic beam/mass spectrometry with resonant two-photon ionization detection, Appl. Spectroscopy 42 (1988) 1200–1206.
[10] H. Chiji, Y. Arakawa, S. Ueda, M. Kuroda, M. Izawa, 5,2′-dihydroxy-6,7-methylenedioxyisoflavone from seed balls of sugar beet, Phytochemistry 25 (1986) 281–282.
[11] G. Burton, A.A. Ghini, E.G. Gros, ^{13}C NMR spectra of substituted indoles, Mag. Reson. Chem. 24 (1986) 829–831.
[12] R.J. Cushley, J.D. Filipenko, ^{13}C fourier transform

N.M.R. XIII-reassignment of the ^{13}C spectrum of ergosterol, Org. Magn. Reson. 8 (1976) 308–309.
[13] M. Kobayashi, M.M. Krishira, K. Ishida, V. Anjianeyulu, Marine sterols XXIV: isolation of 24-methylenecholestane-1α,3β,5α,6β,16β-pentol from *Sinularia* sp. of soft coral, Chem. Pharm. Bull. 40 (1992) 2845–2846.
[14] L.M. Zeng, Z Zeng, J.Y. Su, Studies on the steroids of marine sponge, Chem. Res. Chin. Univ. 11 (1995) 174–177.
[15] M. Kobayashi, M.M. Krishira, K. Ishida, V. Anjianeyulu, Marine sterols XXII: occurrence of 3-oxo-4, 6, 8(14)-triunsaturated steroids in the sponge *Dysidea herbacea*, Chem. Pharm. Bull. 40 (1992) 72–74.
[16] W.G. Ma, X.C. Li, D.Z. Wang, C.R. Yang, Ergosterol peroxides from *Cryptoporus volvatus*, Yunnan Zhiwu Yanjiu 16 (1994) 196–200.
[17] W.R. Abraham, G. Schmeda-Hirschmann, 24(s)-3β-hydroxy-ergosta-5-en-6-one from *Cyttaria johowii*, Phytochemistry 36 (1994) 459–461.
[18] F. Cafieri, E. Fattorusso, M. Gavagnin, C. Santacroce, 3β, 5α, 6β-trihydroxysterols from the mediterranean bryozoan *Myriapora truncata*, J. Nat. Prod. 48 (1985) 944–947.
[19] I. Okane, A. Nakagiri, T. Ito, Preliminary study of endophytic fungi in evergreen plants from Ishigaki and Iriomote Islands, Institute for Fermentation Research Communications (Osaka) 0 (1997) 45–51.
[20] C.S. Smith, T. Chand, R.F. Harris, J.H. Andrews, Colonization of a submersed aquatic plant, eurasian water milfoil *Myriophyllum spicatum*, by fungi under controlled conditions, Appl. Environ. Microbiol. 55 (1989) 2326–2332.
[21] S. Freeman, R.J. Rodriquez, Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist, Science 260 (1993) 75–78.
[22] U. Lindequist, A. Lesnau, E. Teuscher, H. Pilgrim, Antiviral activity of ergosterol peroxide, Pharmazie 44 (1989) 579–580.
[23] A.M Rashkes, A.U Kariev, A.A Umarov, M.M Kiktev, N.K Khidyrova, Ya.V Rashkes, Kh.M Shakhidoyatov, Composition and growth-stimulating activity of secondary metabolites of cotton leaves, Khim. Prir. Soedin. 4 (1995) 614–617.