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ARTICLE

Nematodetoxic Aurovertin-Type Metabolites from a Root-Knot Nematode Parasitic Fungus *Pochonia chlamydosporia*

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Chemical investigation of one fungal strain *P. chlamydosporia* YMF 1.00613 isolated from root knots of tobacco infected by *Meloidogyne incognita* led to the isolation and identification of four aurovertin-type metabolites, which include a new compound, aurovertin I (**A1**), and three known metabolites, aurovertins E, F and D (**A2**−**A4**). Their structures were established by spectroscopic studies such as 1D- and 2D-NMR and MS analysis. Aurovertin I (**A1**) is the first natural product with an aurovertin skeleton with one less carbon. Compounds **A3** and **A4** showed the toxicity to the worms of the free-living nematode *Panagrellus redivevus* with the LC_{50} values 88.6 and 41.7 μg/mL at 48 h, respectively. All four aurovertins did not show obvious inhibitory effects on egg hatch of root knot nematode *Meloidogyne incognita*. The results suggested that the aurovertin-type metabolites produced by *P. chlamydosporia* might be one of the pathogenic factors involved in the suppression of nematodes.

KEYWORDS: ***Pochonia chlamydosporia*; aurovertin I; aurovertins; nematodetoxic; root-knot nematode; *Panagrellus redivevus***

INTRODUCTION

The anamorphic parasitic fungus *Pochonia chlamydosporia* (Goddard) Zare & W. Gams, formerly known as *Verticillium chlamydosporium* Goddard (*1*), has been extensively studied as a potential biological control agent and as an egg parasite of root knot (*Meloidogyne* spp.), false root knot (*Nacobbus* spp.) and cyst (*Heterodera* spp. and *Globodera* spp.) nematodes (*2*). The parasitism of nematode eggs by the fungus may significantly reduce multiplication of the pest and lead to smaller population of their subsequent generations (*3*, *4*). Previous biochemical research suggested that extracellular enzymes produced by the fungal parasites during infection serve as pathogenic factors (*5*). For example, in 1994, a serine protease VCP1 was identified from *P. chlamydosporia*, and reported to play a key role in penetrating the nematode cuticle (*6*). In 2003, an extracellular chitinase CHI43 was found to have nematicidal activity in infecting nematode eggs (*7*). Investigation of the chemical components of the genus *Pochonia* (*Verticillium*) so far led to the isolation of more than 80 compounds, which consisted mainly of aromatic compounds (including resorcyclic acid lactone type monorden and pochonins) (*8*), cyclopeptides (including epidithiodioxopiperazine-type verticillins (*9*) and bassianolide(*10*)), nonaromatic polyketides (including bisvertinols (*11*), bisvertinoqinol (*12*), lowdenic acid (*13*), and vertinolide(*14*)), β-carotene-type neurosporaxanthin (*15*), aphidicolane-type diterpenoid (*16*), and pentanorlanostane triterpenoids (*17*). Many of these chemical compounds have been found to have a myriad of biological activities, including antibacterial, antifungal, antioxidative activities, antivirus and antitumor (*8*−*17*). However, studies with regard to the nematicidal activities of the natural compounds from this fungus have been rarely reported and discussed. Up to now, only one nematicidal compound, phomalactone, was reportedly obtained by bioassay-directed fractionation from the fungus *Verticillium chlamydosporium* (*18*). Since the production of secondary metabolites can also be a strategy used by nematophagous fungi to infect or kill nematode, the evaluation and commercial development of natural chemicals from nematophagous fungi have attracted considerable interest (*8*).

In the course of screening for antinematocidal substances from nematode pathogenic fungi, one strain of *P. chlamydosporia* YMF 1.00613 obtained from root-knot nematodes exhibited significant pathogenic activity toward nematode juveniles and adults. A preliminary experiment has indicated that the chloroform fraction of the fermentation of this fungal strain demonstrated good nematocidal activity. Further examination of bioactive secondary metabolites of the fungus *P. chlamydosporia* cultured on a small scale (1 L) revealed the existence of several similar metabolites, which showed a yellow-green fluorescence on TLC under UV 365 nm, and purple color after treatment with 20% H_2SO_4 in ethanol followed by being heated at 120 °C. During our detailed investigation of the fermentation on a large scale (20 L), four aurovertin-type metabolites, including one new aurovertin I (**A1**), and three known aurovertins, were for the first time isolated from culture broth and mycelia of the fungus *P. chlamydosporia*. All the structures of these aurovertin-type metabolites were resolved fully by MS and NMR spectroscopy including 2D NMR data. The new compound (**A1**) is the first

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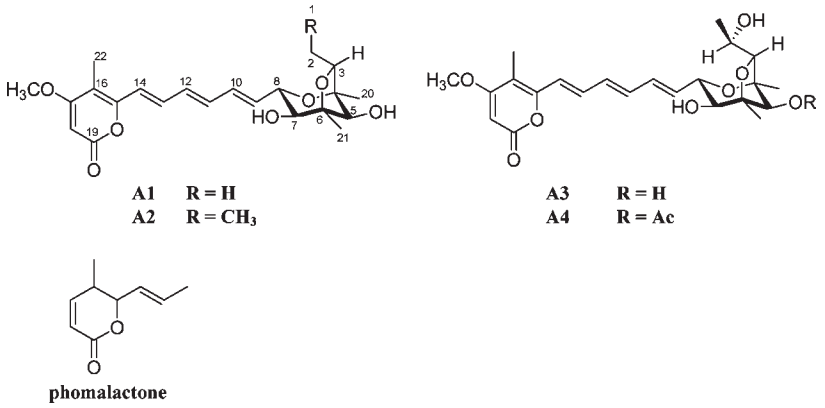

Figure 1. Structures of compounds **A1**−**A4** and phomalactone from *P. chlamydosporia*.

natural product with an aurovertin skeleton with one less carbon.

Herein, we report the isolation of aurovertin-type metabolites from the nematode pathogenic fungus *P. chlamydosporia* and the evaluation of their possible ecological role of major aurovertins in the infection of nematodes. The NMR data of known aurovertins E and F recorded in acetone-d_6 were also first described in this research.

MATERIALS AND METHODS

General Experimental Procedures. Column chromatography was performed on 200−300 mesh silica gel (Qingdao Marine Chemical Factory, Qingdao, P. R. China). Optical rotations were obtained on a Horiba-SEAP-300 spectropolarimeter. UV spectroscopic data were measured on a Shimadzu-210A double-beam spectrophotometer. IR spectra of samples in KBr disks were recorded on a Bruker-Tensor-27 spectrometer with KBr pellets. NMR spectra were carried out on either a Bruker AM-400 or a DRX-500 spectrometer with respect to solvent as internal standard. MS were recorded on a VG-Auto-Spec-3000 spectrometer.

Strain and Cultivation. Tobacco roots infected by *Meloidogyne incognita* were collected from a field in Songming prefecture, Yunnan Province, People's Republic of China, in November 2003. The infected juveniles of *M. incognita* were collected from the roots using the Baermann funnel technique (*19*). Three hundred grams of galled roots with a length of 3−5 cm were collected and placed on a Baermann funnel apparatus. A rubber tubing and collecting tube was attached to the funnel and then filled with water. In the funnel, the nematodes moved through the tissue and sank to the bottom of the funnel due to gravity. After 24 h, a pinch clamp was applied on the rubber tubing allowing for the collecting tubing containing infected nematodes to be removed. The nematode suspension was concentrated to 1 mL by centrifuge at 8000 rpm, and then 0.2 mL of the resulting suspension containing about 200 active and immobile juveniles was poured onto five Petri dishes containing 2% water agar (1 L of water containing 20 g of agar). After incubation of 10−20 days at 28 °C, mycelial tips that emerged from naturally infected juveniles were transferred to fresh Petri dishes containing potato dextrose agar (PDA medium) and recultured at least three times to obtain genetically identical cultures.

After purification by the above procedure, a strain YMF 1.00613 was obtained. The colonies of this strain were considered fast growing on PDA medium (attaining 8 cm diameter in 7 days) at 28 °C, and were suedelike to downy, white to pale yellow in color, becoming pinkish brown, red, green or yellow with a colorless, yellow or reddish brown reverse. Conidiophores were usually well differentiated and erect, verticillately branched over most of their length, bearing whorls of slender awl-shaped divergent phialides. Conidia were hyaline or brightly colored, subovate to ellipsoidal, one-celled, 3−4.5 × 1.5−2.2 μm, and were usually borne in slimy heads. Chlamydospores were pale yellow and multicellular, netted wall like, 20−25 μm in diameter. DNA extraction of *P. chlamydosporia* YMF 1.00613 and PCR experiment were carried out according to the literature (*20*). A characteristic fragment about 270 bp (**Figure 2**) was amplified by PCR using the species specific primers tub1f and tub1r, and the genome DNA of YMF 1.00613 as template (*21*). According to the macroscopic and microscopic characteristics, and PCR diagnosis, the strain No. YMF 1.00613 was identified as *P. chlamydosporia* (= *V. chlamydosporium* Goddard) (*21*).

The isolate was deposited as YMF 1.00613 in the strain collection of Laboratory for Conservation and Utilization of Bio-Resources & Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University, Kunming, P. R. China. After the conidia had developed on PDA slants in test tubes at 25 °C, the strain was kept at −30 °C as a stock culture. The strain *P. chlamydosporia* (YMF 1.00613), cultured on a PDA medium for 6 days, was inoculated into 500 mL flasks each containing 200 mL of production medium consisting of potato (peel off) 20% and glucose 2%. The pH of the medium was natural at 7. The inoculated flasks were cultured on a rotary shaker (180 rpm) at 28 °C for 12 days.

Extraction and Isolation. A 12-day-old fermentation broth of strain YMF 1.00613 (200 mL × 100 flasks) was filtered to separate the mycelia from the culture. The culture filtrate was concentrated in vacuo and partitioned with $CHCl_3$ (800 mL × 4), and the organic part was evaporated to dryness to give an oily residue 5.2 g. The $CHCl_3$ fraction was subjected to a Sephadex LH-20 column, developing with $CHCl_3$: MeOH (1:1 v/v) to give five fractions. Fractions were monitored by TLC, and spots were visualized by spraying with 10% H_2SO_4 in EtOH. Fraction 2 (0.5 g) was then chromatographed over a silica gel column, eluting with $CHCl_3$−MeOH (7:1, 1 L) to afford six subfractions. Subfraction 2 was repeatedly subjected to a silica gel column eluting with $CHCl_3$:MeOH (50:1) to yield compound **A4** (2.9 mg) (**Figure 1**). Subfraction 4 was rechromatographed over a silica gel column eluting with $CHCl_3$:MeOH (30:1) to give compound **A3** (25.3 mg). Subfraction 3 was repeatedly chromatographed over a silicon gel column washing with $CHCl_3$:MeOH (40:1) to offer compounds **A1** (4.1 mg) and **A2** (2.5 mg). Isolation of compounds **A1**−**A4** from the methanol extract of mycelium was similar to the above process.

The mycelium of strain YMF 1.00613 was macerated in methanol (3 × 5 L). The extract was filtered, concentrated under reduced pressure, and partitioned between water and $CHCl_3$ (800 mL × 4). The organic part was evaporated to dryness to give an oily residue 9.8 g. The $CHCl_3$ fraction was subjected to a Sephadex LH-20 column, developing with chloroform: methanol (1:1 v/v) to give five fractions. Fraction 2 (0.5 g) was then chromatographed over a silica gel column, eluting with $CHCl_3$−MeOH (7:1, 1 L) to afford six subfractions. Subfraction 2 was repeatedly subjected to a silica gel column eluting with $CHCl_3$:MeOH (50:1) to yield compound **A4** (23.8 mg) (**Figure 1**). Subfraction 4 was rechromatographed over a silica gel column eluting with $CHCl_3$:MeOH (30:1) to give compound **A3** (5.2 mg). Subfraction 3 was repeatedly chromatographed over a silica gel column washing with $CHCl_3$:MeOH (40:1) to offer compounds **A1** (1.2 mg) and **A2** (2.2 mg).

Aurovertin I (***A1***). Yellow oil; $[\alpha]_D^{23.5}$ −39.9° (*c* 0.1, MeOH); UV (MeOH) λmax (log ε) 257 (3.92), 269 (4.06), 277 (4.07), 358 (3.91); IR (KBr) ν_{max} 3425, 2975, 2935, 1694, 1632, 1640, 1554, 1560, 1455, 1407, 1379, 1310, 1252, 1191, 1149, 1130, 1088, 1040, 1016, 943, 911, 891, 822, 758, 731, 697, 661, 589, 479 cm^{-1}; ^1H NMR and ^{13}C NMR see **Table 1**; EI-MS *m*/*z* 404 $[M]^+$; positive HRESI-MS: *m*/*z* 405.1906 $[M + H]^+$ (calculated for $C_{22}H_{29}O_7$, 405.1913).

Aurovertin E (***A2***). Yellow oil; $[\alpha]_D^{23.5}$ −45.3° (*c* 0.1, MeOH); ^{1}H NMR and ^{13}C NMR see **Table 1**; EI-MS *m*/*z* 418 $[M]^+$.

Aurovertin F (***A3***). Yellow oil; $[\alpha]_D^{23.5}$ −36.5° (*c* 0.1, MeOH); ^{1}H NMR and ^{13}C NMR see **Table 1**; EI-MS *m*/*z* 434 $[M]^+$.

Aurovertin D (***A4***). Yellow oil; EI-MS *m*/*z* 448 $[M]^+$. The ^{1}H NMR and ^{13}C NMR spectroscopic data were consistent with those of aurovertin D reported in the literature (*22*).

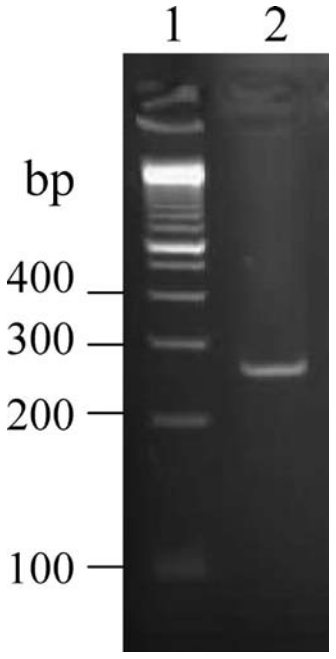

Figure 2. DNA from *P. chlamsporia* amplified with primers tub1f and tub1r. Track 1: DNA side marker (100 bp ladder). Track 2: *P. chlamsporia* YMF 1.00613.

Nematode Worm Bioassay. Since the free-living nematode *P. redivevus* was easy to cultivate, it was chosen as the test nematode in our study. *P. redivevus* was cultured on oatmeal medium (oatmeal, 20 g; water, 80 mL) at 28 °C for 7 days. The cultured nematodes (mixed stage) were obtained from culturing medium using the Baermann funnel technique. The nematodes were sterilized by 1% NaOCl solution for two minutes and enumerated under optical microscope (40×). An aqueous suspension of nematode (ca. 6000 nematodes per mL) was prepared for inoculation. Test samples (compound **A3** and **A4**) were dissolved in 600 μL of acetone, respectively, and then diluted with sterilized water containing 0.3% (v/v) Tween-20 to prepare a stock solution 400 μg/mL. The same amount of acetone dissolved in water containing 0.3% (v/v) Tween-20 was established as control. Three milliliters of tested concentrations of 400, 200, 100, 50, 25 μg/mL of sample solution prepared by the stock solution were added to clean Petri dishes (6 cm diameter), respectively. Three hundred microliters of the nematode suspension was then transferred to each Petri dish and gently mixed. All dishes were incubated at 28 °C. Nematode mortality was assessed at 6 h, 12 h, 18 h, 24 h, 36 h, 48 h exposure periods using a normal binocular microscope, and four visions in each Petri dish were picked at random for counting. Nematodes were considered to be dead when their bodies were straight and they failed to move on physical stimuli with a fine needle. Toxicity was evaluated according to the mean corrected percentage of dead nematodes (*23*). All tests were repeated three times, and data obtained were statistically analyzed. The mean corrected mortality rate (*M*%) was assessed using the following formula:

$$\mathrm{M}\ (\%) = \frac{(N_t - N_{ts})}{N_t} \times 100\% - \frac{(N_c - N_{cs})}{N_c} \times 100\%$$

where N_t is the total number of nematodes in the test, N_{ts} is the number of surviving nematodes in the test, while N_c is the total number of total nematodes in the control, and N_{cs} is the number of surviving nematodes in the control.

Nematode Egg Hatch Bioassay. Infected tobacco roots (having root galls) were collected at Yanglin prefecture, Yunnan, People's Republic of China, in November 2008. A bioassay previously reported was applied with some modifications (*24*). The roots were washed thoroughly with tap

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of Compounds **A1**−**A3**[a]

	A1		A2		A3	
no.	δ_C	δ_H mult (*J*)	δ_C	δ_H mult (*J*)	δ_C	δ_H mult (*J*)
1			12.11 q	0.88 t (9.3)	22.43 q	1.25 d (7.5)
2	12.18 q	1.17 d (6.7)	20.81 t	1.52 m	64.87 d	4.15 (overlap)
				1.43 m		
3	78.81 d	4.13 q (6.7)	84.82 d	3.76 overlap	86.86 d	3.73 d (9.3)
4	84.51 s		84.45 s		84.64 s	
5	80.61 d	3.39 d (5.2)	80.99 d	3.39 d (5.2)	81.57 d	3.36 d (5.1)
6	85.40 s		85.21 s		84.95 s	
7	77.13 d	3.11 t (8.7)	77.19 d	3.12 t (8.7)	77.07 d	3.13 t (8.6)
8	77.89 d	4.22 t (6.8)	77.92 d	4.20 t (6.8)	77.64 d	4.17 (overlap)
9	137.63 d	6.02 dd (5.8, 15.0)	137.67 d	5.98 dd (7.3, 14.9)	137.52 d	5.99 dd (5.8, 15.0)
10	130.81 d	6.45 dd (10.9, 15.0)	130.78 d	6.43 dd (11.2, 14.9)	130.79 d	6.43 dd (10.6, 15.0)
11	138.42 d	6.64 dd (10.9, 15.0)	138.43 d	6.63 dd (11.2, 14.9)	138.34 d	6.62 dd (10.7, 14.8)
12	132.03 d	6.49 dd (11.4, 15.0)	132.02 d	6.47 dd (11.2, 14.9)	131.99 d	6.48 dd (10.9, 14.8)
13	135.87 d	7.06 dd (11.4, 15.0)	135.88 d	7.05 dd (11.1, 14.9)	135.85 d	7.05 dd (11.2, 15.0)
14	120.53 d	6.59 d (15.0)	120.54 d	6.57 d (14.9)	120.43 d	6.57 d (15.0)
15	154.88 s		154.89 s		154.82 s	
16	108.54 s		108.54 s		108.55 s	
17	171.13 s		171.14 s		171.13 s	
18	89.32 d	5.51 s	89.33 d	5.51 s	89.25 d	5.51 s
19	162.79 s		162.77 s		162.96 s	
20	16.48 q	1.15 s	16.83 q	1.16 s	18.17 q	1.40 s
21	15.08 q	1.26 s	15.15 q	1.26 s	15.21 q	1.27 s
22	8.92 q	1.96 s	8.92 q	1.96 s	8.92 q	1.95 s
23	56.90 q	3.89 s	56.90 q	3.88 s	56.89 q	3.88 s
2-OH						3.69 d (6.1)
5-OH		4.64 d (5.2)		4.61 d (5.2)		4.66 d (5.2)
7-OH		3.91 d (8.7)		3.86 d (8.7)		3.99 d (8.9)

[a] Data were measured in acetone-d_6 at 400 MHz for ^{1}H and 100 MHz for ^{13}C with reference to the solvent signals, δ in ppm and *J* in Hz.

water, and the root galls then were cut away and washed with tap water and sterile water. All of the galls were cut into pieces and stirred in a beaker, then rinsed with 1% NaOCl solution for 2 min. After 2 min, nematode egg extract of *M. incognita* was sieved through a 100 mesh sieve nested on top of a 500 mesh sieve. The 100 mesh sieve was then turned, and the opposite side was washed with a gentle stream of sterile water. A 30−40 mL egg extract was collected into a 100 mL beaker. The beaker content was refrigerated at 4 °C prior to use. Test samples (compound **A1**−**A4**) were dissolved in 600 μL of acetone, respectively, and then diluted with sterilized water containing 0.3% (v/v) Tween-20 to prepare a stock solution 400 μg/mL. The same amount of acetone dissolved in water containing 0.3% (v/v) Tween-20 was established as control. Four milliliters of tested concentrations of 400, 200, 100, 50, 25 μg/mL of sample solution prepared by the stock solution was added to a clean Petri dish (6 cm diameter), respectively. An estimated 200 eggs in 80 μL of egg suspension were transferred to clean Petri dishes (6 cm diameter) containing 4 mL of tested solution and gently mixed. The upper cover of each dish contained a 6 cm diameter wet filter paper soaked with 0.5 mL of sterile water to keep moist, and 0.6 mL of sterile water was added to the papers daily during the assay. All dishes were incubated at 28 °C. Nematode egg hatch inhibition rate was assessed at 7, 8, 9, 10, 11, 12, and 13 day exposure periods using a normal binocular microscope. All tests were repeated three times, and data obtained were statistically analyzed. The mean corrected egg hatch inhibition rate (E%) was assessed using the following formula:

$$E(\%) = \left(1 - \frac{E_{th}}{E_t} \times 100\%\right) - \left(1 - \frac{E_{ch}}{E_c} \times 100\%\right)$$

where E_t is the total number of eggs in the test, E_{th} is the number of hatched eggs in the test, while E_c is the total number of eggs in the control, and E_{ch} is the number of hatched eggs in the control.

Statistical Analysis. To quantify the nematicidal effects of the compounds against *P. redivivus*, the median lethal concentrations LC_{50} values were calculated using *Microsoft Excel* (version 2003 software, USA). Regression analysis were also conducted using the linear regression model implemented in Excel. In the regression analysis, nematode mortalities were transformed into probit value, and concentrations (C) of compounds were transformed using lg(C) before analysis. The percentage comparisons were made using the chi-square test at the $P < 0.05$ significance level.

RESULTS AND DISCUSSION

Compounds **A1**−**A4** were obtained as yellowish oil in this study. Among them, compound **A3** was obtained as the first compound from the culture broth of the *P. chlamydosporia* strain. Because compound **A3** was well dissolved in acetone, instead of chloroform as previously reported, acetone was chosen as solvent for its NMR measurement. NMR experiments of compounds **A1** and **A2** isolated from the culture broth of the fungus, were also measured in the same solvent for comparison. Since there has been no previous report on the NMR data of aurovertins E and F recorded in acetone-d_6, here we described not only the data of the new aurovertin, but also those of the two known compounds.

Compound **A1** displayed an $[M]^+$ ion peak at m/z 404 in its EI mass spectrum, indicating a molecular formula of $C_{22}H_{29}O_7$, which was confirmed by positive HRESI-MS. UV absorptions at λmax 257, 269, 277, 358, in combination with the yellow color, suggested that the compound contains one large conjugated system. Absorption bands at 3425, 1694, 1632, 1640, 1554, 1560, 1455, 1407 cm^{-1} observed in the IR spectrum suggested the presence of hydroxyl group, carbonyl group, and double bonds. The ^1H NMR spectrum of **A1** (**Table 1**) revealed the presence of a secondary methyl, two quaternary methyl, and a methyl attached to a double bond, one oxygenated methyl, four oxymethines, a conjugated system with three double bonds consisting of six olefinic methines, and an olefinic methine with a highfield shifted signal at δ 5.51 (1H, s, 7.6, H-18). The ^{13}C NMR and DEPT spectra of **A1** (**Table 1**) showed 22 carbon signals, which were classified as four methyls, one methoxy, four oxygen-bearing methines, two oxygenated quaternary carbons, seven olefinic carbons (including one at δ 89.32), one olefinic quaternary carbon, and three oxygenated olefinic quaternary carbons (including a conjugated carbonyl group of an ester) at δ 154.88, 162.79, and 171.13. The above data suggested that compound **A1** was an aurovertin-type compound. This type of metabolite, a large conjugated system of a moiety of 4-methoxy-5-methyl-2*H*-pyran-2-one with a segment of (*E*)-hexa-1,3,5-triene, was confirmed through correlations detected in the $^1H-^1H$ COSY, HSQC and HMBC experiments. Careful comparison of the NMR data of **A1** with those of the known compound (**A2**) revealed that the structures of the two compounds were similar except that **A2** had one more methylene group (CH_2-2) than **A1**, and the chemical value of the C-3 at δ 84.82 (d) in **A2** was reduced to δ 78.81 (d) in **A1**. This indicated that the ethyl group attached to C-3 in **A2** was substituted by a methyl group in **A1**, since the chemical value of the C-3 neighboring the methyl group tended to shift in the high field. Further support came from analysis of correlations observed in the $^1H-^1H$ COSY and HMBC spectra (**Figure 3**). The proton signal of the methyl at δ 1.17 (3H, d, 6.7, H_3-2) showed a $^1H-^1H$ COSY relation with H-3 at δ 4.13 (1H, q, 6.7), and $^1H-^{13}C$ long-range correlations with C-3 and C-4 at δ 78.81 (d) and 84.51 (s) respectively, indicating that the methyl group was assignable to C-3. The key NOESY correlations of Me-2 with H-8, and Me-20 with H-3, were observed in the NOESY spectrum of compound **A1** (**Figure 3**), which established the relative stereochemistry of the methyl at C-3 as that of compound **A2**. A recent study determined the absolute configuration of aurovertin F by analysis of the CD spectrum (*25*). Since the optical rotation value of aurovertin F (**A3**) isolated in this experiment is identical to that of the reported one, together with the fact that the aurovertin-type of compounds obtained in this study probably share the same absolute configuration on biosynthetic grounds, we deduced that **A1** has the same absolute configuration as aurovertin F. All physicochemical data are in full agreement with the proposed structure of **A1** as described (**Figure 1**), given trivial name aurovertin I.

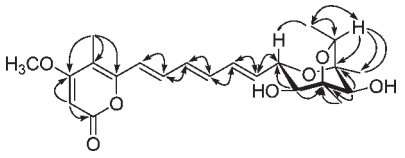

Figure 3. Significant correlations of compound **A1** in $^1H-^1H$ COSY (H↔H), HMBC (H→C) and ROESY (H∪H) spectra.

The first four aurovertin-type compounds, aurovertin A−D, are secondary metabolites initially isolated as toxic substances in fermentation broth of the fungus *Calcarisporium arbuscula* NRRL 3705 (*26*). The structure of aurovertin B was proposed on the basis of its chemical properties and both ^{13}C and ^1H NMR spectroscopic data (*27*), and characterized by an unusual 6-ethyl-1,5-dimethyl-4,7-dioxabicyclo[3.2.1]octane ring system with a conjugated α-pyrone moiety. The absolute configuration of aurovertin B was finally determined by total synthesis (*28*). Structures of aurovertins A and D were determined on the basis of spectroscopic comparison with aurovertin B. Aurovertin E was isolated from the fungus Basidiomycete *Albatrellus confluen*, as well as aurovertin B (*29*). Another three new aurovertins (F, G and H), together with aurovertin D, were obtained from the entomopathogenic fungus *Metarhizium anisopliae* (*25*). Among all the reported eight aurovertin-type metabolites to date, aurovetin C was much different from the others from a structural

point of view because it lacked one carbon in its skeleton. It should be noticed that the ethyl side in the moiety of 6-ethyl-1, 5-dimethyl-4,7-dioxabicyclo[3.2.1]octane in aurovertin B was substituted with a methyl group in aurovetin C. However, the structure of aurovetin C still remained unidentified to date since it appeared without comment or any data. Therefore, the novel compound (**A1**), determined to possess a moiety of 1,5,6-trimethyl-4,7-dioxabicyclo[3.2.1]octane ring, and identified as an analogue of aurovetin C, can be said to be the first natural product with an aurovertin skeleton with one less carbon. Compounds **A2**−**A4** were elucidated as aurovertins E, F and D, respectively, on the basis of their NMR data and comparison with the data in the literature.

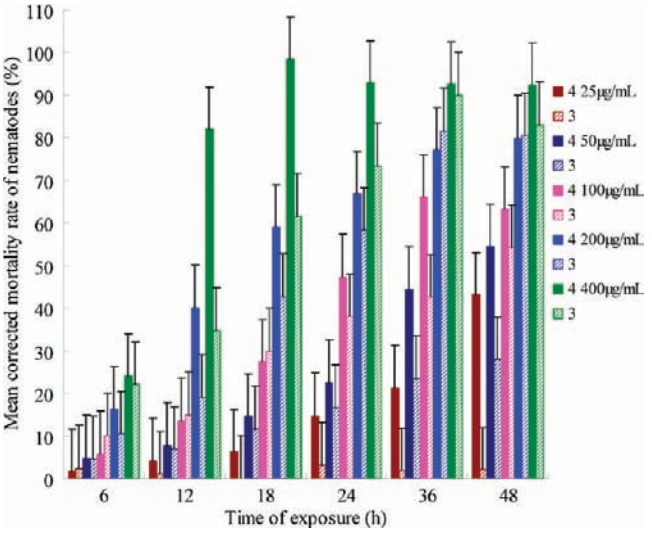

Figure 4. Nematodexic effect of compounds **A3** and **A4** on *P. redivivus* juveniles and adults. Mean corrected mortality rate (%) of *P. redivevus* according to the concentrations (μg/mL) of compounds **A3** and **A4** and exposure time.

In our study, compounds **A3** and **A4**, the major aurovertin constituents produced by the nematode-parasitic fungus *P. chlamydosporia*, were evaluated for their nematode toxicity activities against *P. redivivus* (**Figure 4**). Both compounds showed nematode toxicity activities toward the worms. The LC_{50} values of **A3** and **A4** against *P. redivivus* were measured as 88.6 and 41.7 μg/mL, respectively, at 48 h. It was observed that the internal structures of worms were disintegrated and a lot of vacuoles formed within their body during the experiments (**Figure 5**). After 48 h, only residual empty somatocysts of the dead worms could be observed (**Figure 5e**). All the aurovertins **A1**−**A4** were tested for their inhibitory effect on nematode egg hatch of *P. redivivus* (**Figure 6**). The egg hatch inhibitory rates for all the compounds were not more than 5% at concentrations of 25−400 μg/mL. It could be concluded that this aurovertin-type metabolite did not exert any inhibitory effect on egg hatch of root-knot nematode *P. redivivus*.

It has been proposed that the nematode parasitic fungus *P. chlamysporia* could produce extracellular proteases to infect nematode eggs or penetrate nematode cuticles, which eventually lead to the death of nematode (*5*). Our result suggested that a series of aurovertin-type metabolites produced by the nematode-parasitic fungus could be also involved in killing the nematodes.

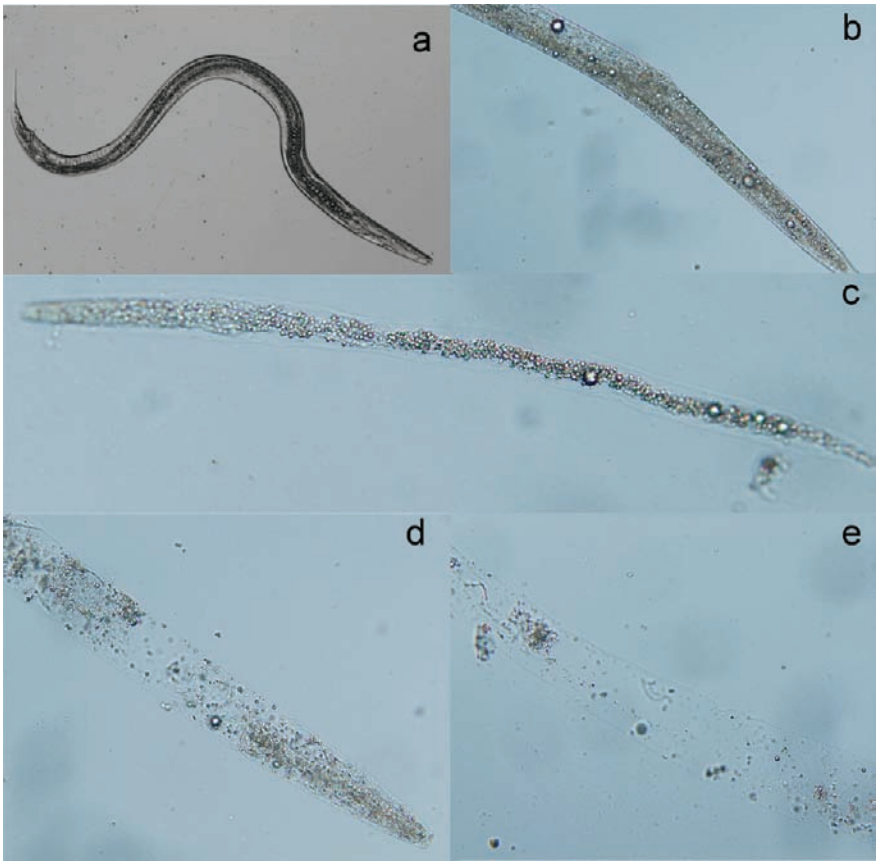

Figure 5. Nematode *P. redivivus* juveniles and adults of *P. redivivus* treated with water (**a**) and with aurovertins (**b**−**e**). A healthy, active nematode (**a**). Aurovertins treated nematode with disrupted internal structures (**b**−**d**), vacuolated nematode (**c**−**e**), and an almost empty somatocysts of the dead nematode after 48 h (**e**). Nematode lengths ca. 400 μm.

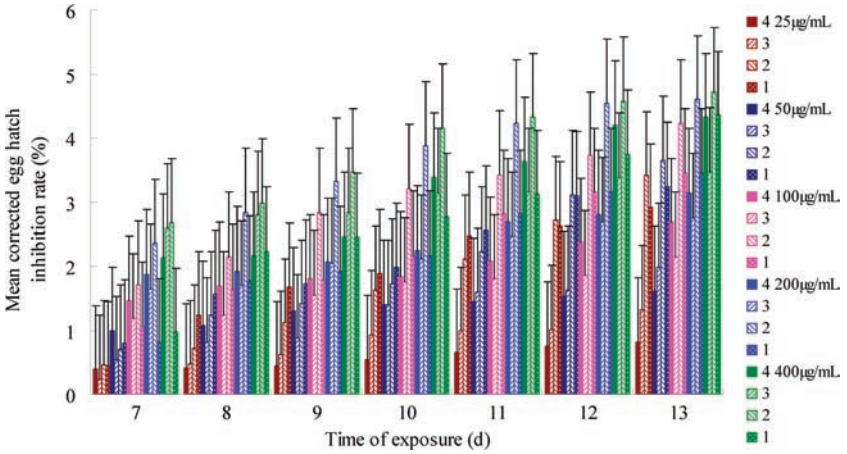

Figure 6. Nematodexic effect of compounds **A1**−**A4** on egg hatch of *Meloidogyne incognita*. Mean corrected egg hatch inhibition rate (%) of *M. incognita* according to the concentrations (μg/mL) of compounds **A1**−**A4** and exposure time.

They could manage to reach inside the prey, and then disintegrate the internal contents of nematodes and turn the interior of the prey into vacuole.

Among the aurovertin-type metabolites up to now, aurovertin B, the major component of the mixture of aurovertins produced by the strain of *C. arbuscula*, was regarded as a potent inhibitor of ATP-synthesis and ATP-hydrolysis catalyzed by mitochondria1 enzyme systems. It could bind to and inhibit mitochondrial ATPase, thereby uncoupling oxidative phosphorylation (*30*, *31*). Its biological property has led to the frequent use in studies of the mechanisms of phosphate esterification and related reactions, as well as in investigations of the structures of ATPase enzymes. Most recently, a report indicated that aurovertin B also had strong inhibition on the proliferation of several breast cancer cell lines and arrested cell cycle at the G0/G1 phase, but little influence on the normal cell line MCF-10A, which suggested that aurovertin B could be used as an antitumorigenic agent and be exploited in cancer chemotherapy (*32*). However, there were few studies involving the biological activity of other aurovertins.

Our finding was the first report that the functions of these aurovertin-type metabolites were related to the infection process of a nematode-parasitic fungus. The mechanism action of these aurovertin-type metabolites on the interior of nematode in our study is not elucidated, but it could be similar to that of aurovertin B that works as an inhibitor of ATPase-like enzymes due to their structural similarities.

The chemical structures of aurovertin-type metabolites were partly similar to the previously reported nematocidal compound phomalactone, which contains a carbon skeleton of 5-methyl-6-((*E*)-prop-1-enyl)pyran-2-one. Phomalactone, which was also found in other fungi, e.g. *Nigrospora sphaerica*, and the entomopathogenic fungi *Hirsutella thompsonii* var. *synnematosa*, showed the mortality of *M. incognita* reaching at 84% in 96 h at the concentration of 500 mg/L (*33*, *34*). Compared with phomalactone, aurovertin-type metabolites isolated in our study displayed stronger nematode toxicity activities, which suggested that the long chain attached to the moiety of 5-methyl-6-((*E*)-prop-1-enyl)pyran-2-one in aurovertin-type metabolites would increase the antinematodal activity.

The result that no tested aurovertins show any activities against nematode egg hatch could be explained with the fact that the eggshell of nematodes keeps the compounds from entering in the inside of eggs, because previous reports suggested that the significant hatch suppression by the bioactive compounds was associated with their abilities to pass through the nematode egg shell (*35*).

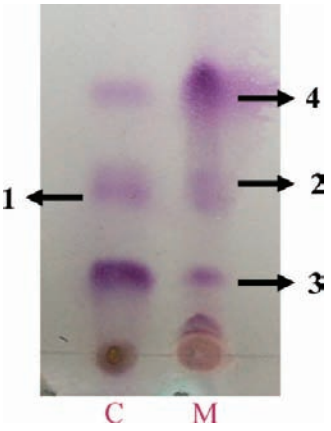

Figure 7. Aurovertin profile of the chloroform fractions of the extracts of culture broth (C) and mycelia (M) of *P. chlamydosporia* by TLC.

During the course of the aurovertin-type metabolic profiling of *P. chlamydosporia*, we noted that compound **A4** was the richest aurovertin-type component in the chloroform fraction of the extract of mycelia of *P. chlamydosporia*, while compound **A3** was the major metabolite in the corresponding part of the fermentation broth (**Figure 7**). Though compound **A4** showed a bit stronger nematode toxicity activity than **A3**, it could not be assumed that the mycelia of the fungus are more nematode toxic than the culture broth because the nematode toxicity activities of trace amounts of aurovertin-type metabolites still remain unknown. In addition, since compound **A3** was the *O*-deacetyl derivative of compound **A4**, it seemed that the fungus changed the composition of aurovertin-type metabolites before they released these secondary metabolites to the culture broth. It can be expected that acetyltransferase was possibly involved in the modifying process. It is not clear why the composition of the aurovertin-type metabolites of the mycelia of *P. chlamydosporia* is different from that of the culture broth.

In conclusion, by chemical metabolite profiling we have identified four aurovertin-type metabolites from a large-scale fermentation of a nematode-parasitic fungus *P. chlamydosporia* and solved their structures by 2D NMR spectroscopy. The new compound (**A1**), aurovertin I, is the first natural aurovertin with one less carbon. Biological evaluation of these compounds revealed their nematode toxicity activity. Our results indicated that aurovertin-type compounds would be involved in the infective process of nematode parasitic fungus *Pochonia chlamy-*

dosporia. The study would help reveal the infective mechanism of this agriculturally important biological agent, and further study may be warranted.

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This paper is dedicated to Professor Han-Dong Sun on the occasion of his 70th birthday.

Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **A1**−**A3** and 2D NMR of compound **A1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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