New nematicidal azaphilones from the aquatic fungus *Pseudohalonectria adversaria* YMF1.01019

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Pseudohalonectria adversaria; pseudohalonectrin; *Bursaphelenchus xylophilus*; nematicidal.

Abstract

Two new azaphilone metabolites, named pseudohalonectrin A (**1**) and B (**2**), were isolated from the culture of the aquatic fungus *Pseudohalonectria adversaria* YMF1.01019, originally separated from submerged wood in Yunnan Province, China. Pseudohalonectrin A and B were assessed for their nematicidal activity against the pine wood nematode *Bursaphelenchus xylophilus* and their structures were defined after spectral analysis. This is the first report of secondary metabolites from any member of the genus *Pseudohalonectria*.

Introduction

Today, the search for new producers of biologically active compounds is actively underway among fungi growing under extreme conditions, because the synthesis of new secondary metabolites and potential biologically active compounds that help such fungi to survive and adapt to these conditions can be expected with the greatest probability (Gloer, 1995, 1997; Grabley *et al*., 1999). Because of this reason, the aquatic fungi are of special interest. Recently, many new bioactive compounds have been reported from the five aquatic fungi *Anguillospora longissima* (Harrigan *et al*., 1995), *Annulatascus triseptatus* (Li *et al*., 2003), *Dendrospora tenella* (Oh *et al*., 1999b), *Massarina tunicate* (Oh *et al*., 1999a, 2001, 2003) and *Paraniesslia* sp. (Dong *et al*., 2005).

During an ongoing screening for biologically active aquatic fungi, we have found that the mycelial cultures of fungal strain YMF1.01019 exhibited potent nematicidal activity against pine wood nematodes, *Bursaphelenchus xylophilus* (Dong *et al*., 2004). This fungus was isolated from a submerged woody substrate collected in a freshwater habitat, and identified as *Pseudohalonectria adversaria* of the subfamily *Magnaporthaceae* (Cai *et al*., 2002). Although 14 species of the genus *Pseudohalonectria* were already reported, no phytochemical studies have appeared in the literature to the present. This prompted us to investigate its bioactive secondary metabolites. Bioassay-directed fractionation of the aliphatic extracts yielded two new mixed pyrone-quinone skeleton metabolites, pseudohalonectrin A (**1**) and B (**2**), which belong to ascochitine-type azaphilone antibiotics. The present paper is concerned with the isolation, identification and nematicidal activity of these two compounds.

Materials and methods

Culture and fermentation of *P. adversaria* YMF1.01019

The fungal strain of *P. adversaria* YMF1.01019 was initially isolated from a submerged woody substrate collected from the freshwater habitat in Yunnan Province, China and deposited in the Laboratory for Conservation and Utilization of Bioresources, Yunnan University, Yunnan Province, China (culture collection number YMF1.01019). The strain was maintained on PDA medium (potato 200 g, sucrose 20 g, agar 18 g, and water 1000 mL) and fermented with

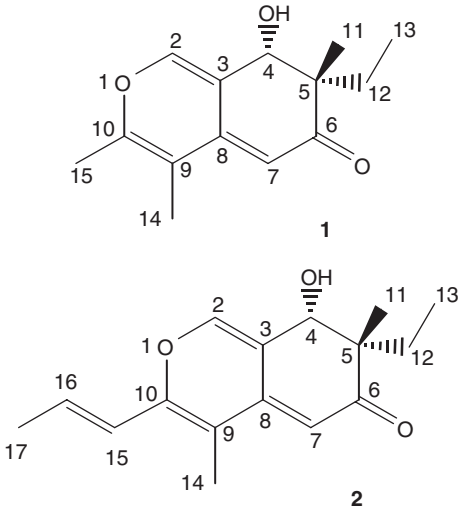

Fig. 1. Structures of pseudohalonectrin A (1) and B (2).

solid Sabouraud's medium (peptone 10 g, glucose 40 g and water 1000 mL) for 6 days at 25%.

Extraction and isolation of compounds

The mycelial cultures of *P. adversaria* YMF1.01019 were successively extracted with MeOH four times at room temperature. The combined extracts were concentrated to dryness in vacuo to give the residue (40 g), which showed *in vitro* nematicidal activity against pine wood nematodes *B. xylophilus*. This residue was loaded on to a silica gel column (5 cm i.d. × 120 cm) containing 1 kg of silica gel G (200–300 mesh; Qingdao Marine Chemical Ltd., Qingdao, China). The column was successively eluted with petroleum ether (bp 60–90 °C), $CHCl_3$, ethyl acetate and methanol. The fractions eluted with $CHCl_3$ and ethyl acetate, which were active, were pooled and concentrated to dryness. The active residue (4.2 g) was loaded on to a silica gel column [200 g Silica gel G (200–300 mesh), 3.6 cm i.d. × 120 cm] that was then eluted using a solvent mixture of $CHCl_3$ – CH_3OH with increasing concentrations of CH_3OH in $CHCl_3$ as eluents. The resulting fractions were monitored by TLC (Silica gel G, 0.25-mm film thickness; Qingdao Marine Chemical Ltd., Qingdao, China) and reduced to one active fraction, **F1** (400 mg). The **F1** fraction was subjected to silica gel column chromatography [20 g Silica gel G (200–300 mesh), 2.1 cm i.d. × 80 cm, petroleum ether (bp 60–90 °C) – CH_3COCH_3 (1 : 9, 1 : 4, 3 : 7, 2 : 3, V/V)] to yield 8 mg of compound **1** and 2 mg of compound **2** (Fig. 1).

Identification of compounds

The structures of pseudohalonectrins isolated from the cultures of *P. adversaria* YMF1.01019 were determined by spectroscopic analysis. Optictical rotations were measured on a Horiba SEPA-300 polarimeter (Horiba, Tokyo, Japan).

Table 1. Nuclear magnetic resonance data of pseudohalonectrin A and B (δ ppm, $CDCl_3$)

	1		2	
No.	^{13}C (mult.)	^{1}H (mult., J, Hz)	^{13}C (mult.)	^{1}H (mult., J, Hz)
2	144.4 (d)	7.29 (s)	144.3 d	7.33 s
3	120.0 (s)		120.1 s	
4	74.6 d	4.44 (s)	74.8 d	4.41 s
5	50.4 s		50.5 s	
6	201.4 s		201.4 s	
7	105.5 d	5.34 (s)	105.7 d	5.36 s
8	144.7 s		144.7 s	
9	112.2 s		112.9 s	
10	154.7 s		151.2 s	
11	18.8 s	1.14 (s)	19.0 s	1.13 s
12	24.5 d	1.61 (q, 7.6)	24.6 d	1.67 (q, 7.5)
13	9.1 s	0.85 (t, 7.6)	9.3 s	0.89 (t, 7.5)
14	13.3 s	1.85 (s)	13.8 s	
15	18.3 s	2.19 (s)	122.3 d	6.00 (dd, 1.4, 15.5)
16			136.3 d	6.55 (ddd, 7.0, 15.5)
17			18.6 s	1.95 (dd, 1,4, 7.0)

The nuclear magnetic resonance (NMR) spectra were recorded on DRX-500 NMR spectrometers (Bruker, Karlsruhe, Germany), with TMS as an internal standard and coupling constants represented in Hz. Infrared (IR) spectra were obtained in KBr pellets with a Bio-Rad FTS-135 spectrophotometer (Bio-Rad, Richmond, CA, USA). Electrospray ionization mass spectra (ESIMS) and high resolution electrospray ionization time of flight mass spectra (HRMS-ESI-TOF) data were taken on a VG Auto Spec-3000 mass spectrometer (VG, Manchester, England).

Pseudohalonectrin A (**1**): light yellow amorphous powder (CH_3COCH_3); $[\alpha]_D^{24.5}$ +66.6° (c 0.50, in acetone); UV (CH_3OH) λ_{max} (ε) 350.0 (11947), 202.5 (20879) nm; IR (film) ν_{max} 3441, 2960, 2925, 2855, 1725, 1629, 1544, 1461, 1382, 1273, 1160, 1117, 1073, 1034, 561 cm^{-1}; ESIMS *m/z* (rel. int) 235 $[M+H]^+$(100), 257 $[M+Na]^+$ (18), 491 $[2M+Na]^+$ (28); HRMS (ESI-TOF) *m/z*: 235.1335 $[M+H]^+$ (calcd for $C_{14}H_{19}O_3$, 235.1334); NMR (500 MHz, CD_3Cl) see Table 1.

Pseudohalonectrin B (**2**): light yellow amorphous powder (CH_3COCH_3); $[\alpha]_D^{24.5}$ +53.6° (c 0.41, in acetone); UV (CH_3OH) λ_{max} (ε) 349.0 (10746), 202.5 (20776) nm; IR (film) ν_{max} 3441, 2963, 2926, 2857, 1725, 1631, 1548, 1464, 1383, 1273, 1160, 1119, 1076, 1039, 563 cm^{-1}; ESIMS *m/z* (rel. int) 261 $[M+H]^+$(100), 283 $[M+Na]^+$ (14), 543 $[2M+Na]^+$ (22); HRMS (ESI-TOF) *m/z*: 261.3404 $[M+H]^+$ (calcd for $C_{16}H_{21}O_3$, 261.3401); NMR (500 MHz, CD_3Cl) see Table 1.

Nematode culture and nematicidal study

Nematicidal activity was determined in a microtiter plate assay as described previously (Dong *et al.*, 2004). The test

organism was pine wood nematode (*B. xylophilus*), which was maintained in our laboratory. *Bursaphelenchus xylophilus* was grown on potato dextrose broth (PDB) agar media containing a strain of *Botrytis cinerea* in disposable Petri dishes wet with 2–4 mL of physiological saline. The cultures were stored at room temperature and subcultured prior to the assay. The assay was conducted in Corning polystyrene 96-well plates. The nematodes were added to 1 mL of physiological saline in a scintillation vial. This was diluted until the nematode counts were 20–25 in a 48-μL aliquot. A solution (48 μL) containing nematodes was delivered to each of three wells per treatment. Two microliters of DMSO (5%) or DMSO (5%) and test compounds was added to each well. The plates were covered, parafilmed and kept in a humid chamber. The numbers of live and dead nematodes were counted under a binocular microscope after different incubation times. Toxicity was estimated according to the mean percentage of dead nematodes. Nematodes were considered dead if they gave no response to physical stimuli such as mechanical stirring or pricking with the point of a needle.

Results and discussion

Structure elucidation of compounds

Pseudohalonectrin A (**1**) was obtained as a pale yellow powder. The UV spectrum of **1** displayed absorption maxima at 202.5 and 350.0 nm, with the fine structure suggesting a polyene chain. The ESI positive ion mass spectrum of **1** showed strong $[M+H]^+$ (base peak), $[M+Na]^+$ and $[2M+Na]^+$ peaks at 235, 257 and 491, respectively. High-resolution mass measurement on the $[M+H]^+$ (*m/z* 235.1335) in the ESI mass spectrum, in combination with ^1H and ^{13}C NMR data, supported a molecular formula $C_{14}H_{18}O_3$, indicating 6 degrees of unsaturation. IR absorptions implied the presence of a hydroxyl (3441 cm^{-1}), and a carbonyl (1725 cm^{-1}) moiety, and double bands (1629, 1544 cm^{-1}). NMR measurements were initially made in pyridine-d^5, but it was not possible to obtain the resolution required for the unambiguous assignment of all signals. Satisfactory resolution was obtained in CD_3Cl. The relatively few resonances that appear in the ^1H NMR spectra of **1** (Table 1) were spread over the whole spectrum without signal overlapping: three methine signals at δ_H 7.29 (s), 5.34 (s), 4.44 (s), four methyl signals at δ_H 0.85 (t), 1.14 (s), 1.85 (s), 2.19 (s), and one methene signal at δ_H 1.61 (q). The ^{13}C NMR spectrum of **1** (Table 1) showed the following resonances: (i) one carbonyl group at δ_C 201.4, accounting for the first oxygen atom of the molecular formula; (ii) six additional sp^2 carbons (between δ_C 154.7 and 105.5), building up three double bonds; (iii) one methine carbon bearing a hydroxy group appeared at δ_C 74.6, accounting for the second oxygen atom of the molecular formula; and (iv) six carbon atoms in the sp^3 region of the spectrum. Thus, to account for the remaining unsaturations and the third oxygen atom required by the molecular formula, **1** must possess two rings, one of which ought to be fused into one oxygen atom.

The heteronuclear multiple quantum coherence. (HMQC) data allowed the assignment of all the protons to their bonding carbons. Inspection of the ^1H-^1H COSY spectrum indicated that the molecule contains few spin systems, all being restricted to few resonances, and therefore providing scarce information about the carbon framework of **1**. However, extensive analysis of the heteronuclear multiple bond coherence (HMBC) data in $CDCl_3$ led to two quite informative partial structures (Fragment **1a** and Fragment **1b**). Fragment **1a** (C-1-C-3-C-6, C-11, C-12 and C-13) was unambiguously estabolished by the HMBC correlations (H_3-13/C-12, C-5, and H_2-12/C-4, C-5, C-6, C-11, C-13 and H-4/C-11, C-12, C-6, C-5, C-2, C-3) and the chemical shift of C-2 (δ_C 144.4, d), according to which C-2 must be an enolic carbon. This was further supported by the correlation between H-2 and H-4 in the long-range ^1H-^1H COSY spectrum. Fragment **1b** (C-8-C-9-C-10, C-14, C-15) was deduced from the HMBC correlations (H-2/C-3, C-8, C-10 and H-14/C-8, C-9, C-10 and H-15/C-8, C-9). Furthermore, the HMBC correlation between H-2 and C-10 also suggested that C-10 was connected to C-2 through an oxygen atom. Finally, the linkage of both C-8 of fragment **1b** and C-6 of fragment **1a** to the remaining olefinic carbon C-7 (δC 105.5, d) to buid up the chemical skeleton of **1** was established by the observed HMBC correlations from

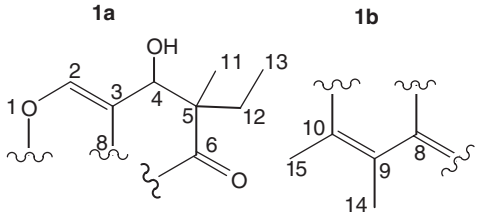

Fig. 2. Structure fragments of **1**.

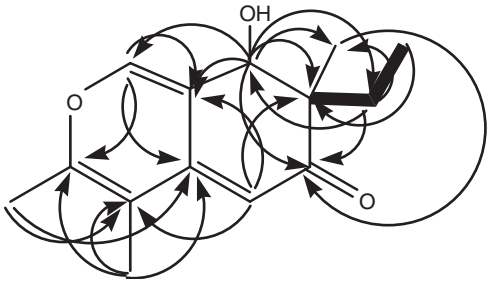

Fig. 3. COSY (-) and selected HMBC (→) correlations of **1**.

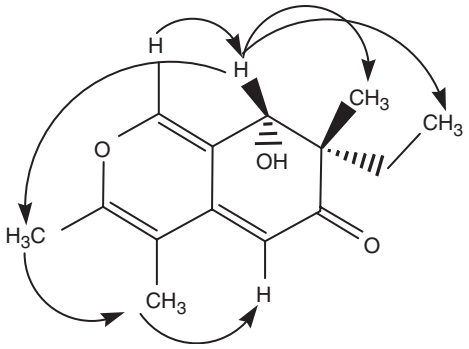

Fig. 4. Key NOESY correlations detected for **1**.

Table 2. Effect of pseudohalonectrin A and B on the mortality of *B. xylophilus in vitro*

Compounds	Concentrations (ppm)	Per cent mortality after different exposure periods (hours)		
		12	24	36
1	200	0.0	83.2	92.4
	100	0.0	53.8	82.1
	50	0.0	25.6	31.3
2	200	0.0	84.5	93.1
	100	0.0	52.7	82.8
	50	0.0	28.6	33.6
Control		0.0	1.1	1.4

H-7 (δ_H 5.34, s) to C-2, C-5 and C-9, and long-range COSY correlation between H-2 and H-7 (see Figs 1–3).

The relative stereochemistry of **1** was fixed by a Nuclear Overhauser effect spectroscopy (NOESY) spectrum and was elucidated to be as shown in Fig. 4. There are two stereogenic centers in **1**: C-4 and C-5. The NOE correlations from H-4/H-2, and H-4/H_3-11 suggest that these protons are positioned on the same side of the molecule. Therefore, it was deduced that H-4 and H_3-11 occupy the quasi-equatorial positions of C-4 and C-5, respectively, while the quasi-axial positions of C-4 and C-5 are occupied by the hydroxy group, C-12.

The ESI positive ion mass spectrum of **2** showed strong $[M+H]^+$ (base peak), $[M+Na]^+$ and $[2M+Na]^+$ peaks at 261, 283 and 543, respectively. High-resolution mass measurement on the $[M+H]^+$ (*m/z* 261.3404) in the ESI mass spectrum, in combination with ^1H and ^{13}C NMR data, supported a molecular formula $C_{16}H_{20}O_3$, indicating 7 degrees of unsaturation. The UV, IR and NMR spectra of this compound were very similar to those obtained for **1**, indicating that they are structurally related. The most striking differences in the NMR data of **2** compared to that of **1** were the appearance of one additional double band signal [δ_C 122.3 (d, C-15) and 136.3 (d, C-16), δ_H 6.00 (dd, $J = 1.4$ and 15.5 Hz, H-15), and 6.55 (ddd, $J = 7.0$ and 15.5 Hz, H-16)]. The positioning of this between C-17 and C-10 of the C-10/C-9 double bond was shown by observed C**H**$_3$-17/H-16 and H-16/H-15 ^1H-^1H couplings, and by HMBC correlations from H-16 to C-10 and from H-15 to both C-10 and C-9. The relative stereochemistry of C-15/C-16 double bond was assigned as trans by the coupling constant value of 15.5 Hz between H-15 and H-16. These spectral changes were consistent with the existence of the one additional double band on C-15/C-16. Despite the spectral differences, however, combined 2D NMR experiments showed that **2** had the same proton-proton and proton-carbon correlations throughout the entire molecule as **1**. Thus, the structure of **2** was determined as a derivative of **1** possessing the same pyrone-quinone skeleton (see Fig. 1).

Nematicidal activity

The nematicidal activity of pseudohalonectrin A and B is shown in Table 2. The results revealed that pseudohalonectrin A and B displayed moderate nematicidal activity against *B. xylophilus*.

Discussion

The present study has demonstrated the presence in *P. adversaria* YMF1.01019 of two new nematicidal metabolites possessing the pyrone-quinone structure, which are usually called azaphilones because of the affinity of these compounds for ammonia, yielding vinylogous γ-pyridones (Park *et al*., 2005). Many metabolites of this type, including ascochitine, sclerotiorin, monascorubrin and monascoflavin, have been characterized from various fungi (Foremska *et al*., 1992; Park *et al*., 2005). They were commonly reported to be phytotoxic and to possess antibacterial and antifungal activity (Foremska *et al*., 1992; Park *et al*., 2005). These reports and our results suggested azaphilone compounds were active against several pests such as nematode, weeds, bacteria and fungi. Therefore, the azaphilone class of compounds may have the potential to be developed as effective and alternative pest-managing natural products to some of the synthetic pest-managing agents on the market. Further studies are required to determine the effective dose and potential of the azaphilone class of compounds as pest-managing activity agents under field conditions.

The present study has also demonstrated the production of nematicidal metabolites produced by the fungus inhabiting the freshwater environment. In freshwater ecosystems, submerged woody substrata are the main energy input (Wong *et al*., 1998). Wood is, however, a substrate greatly deficient in nitrogen and therefore the nitrogen utilized by freshwater fungi may be obtained from other sources. Nematodes are cosmopolitan organisms, adapted to living in soil and water. They have been shown to be an integral part of various ecosystems, serving as food for small invertebrates or fungi (Dropkin, 1980). With their high

nitrogen component, nematodes are considered as playing an important role in providing nitrogen to other organisms in freshwater ecosystems. Several nematophagous fungi have previously been reported in wood that was submerged in freshwater, e.g. *Dactylella ellipsospora* Grove (Hyde & Goh, 1998) and *Dactylella aquatica* (Ingold) Ranzoni (Kane *et al*., 2002), and these species are normally found from the dead bodies of the nematodes. It would also make sense if other wood-inhabiting fungi occurring on wood in freshwater were able to supplement their diets by obtaining nitrogen via digesting nematodes. The ability for these fungi to produce nematicides that can kill nematodes, which they can subsequently consume, would be advantageous.

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References

Cai L, Tsui CKM, Zhang KQ & Hyde KD (2002) Aquatic fungi from lake Fuxian, Yunnan, China. *Fungal Diversity* **9**: 57–70.

Dong JY, Zhao ZX, Cai L, Liu SQ, Zhang HR, Duan M & Zhang KQ (2004) Nematicidal effect of freshwater fungal cultures against the pine-wood nematode, *Bursaphelenchus xylophilus*. *Fungal Diversity* **15**: 123–133.

Dong JY, Li R, He HP & Zhang KQ (2005) Nematicidal sphingolipids from the fresh water fungus *Paraniesslia* sp. YMF1.01400. *Eur J Lipid Sci Technol* **107**: 779–785.

Dropkin VH (1980) *Introduction to Plant Nematology, 38–44*, pp. 242–246, 256. John Wiley & Sons, New York.

Foremska E, Kostecki M & Chelkowski J (1992) Biosynthesis, preparation and properties of ascochitine. *Acta Biotechnologica* **12**: 461–465.

Gloer JB (1995) The chemistry of fungal antagonism and defense. *Can J Bot* **73**: S1265–S1274.

Gloer JB (1997) Environmental and microbial relationships. *Mycota* (Wicklow DT & Soderstrom BE, eds), pp. 249–268. Springer-Verlag, Heidelberg.

Grabley IS, Thiericke R & Zeeck A (1999) The Chemical screening approach. *Drug Discovery from Nature* (Grabley S & Thiericke R, eds), pp. 124–148. Springer Verlag, Berlin.

Harrigan GG, Armentrout BL, Gloer JB & Shearer CA (1995) Anguillosporal, a new antibacterial and antifungal metabolite from the freshwater fungus *Anguillospora longissima*. *J Nat Prod* **58**: 1467–1469.

Hyde KD & Goh TK (1998) Fungi on submerged wood in the Riviere St Marie-Louis, The Seychelles. *South African Journal of Botany* **64**: 330–336.

Kane DF, Tam WY & Jones EBG (2002) Fungi colonising and sporulating on submerged wood in the River Severn, UK. *Fungal Diversity* **10**: 45–55.

Li C, Vitka MV & Gloer JB (2003) Annularins A–H: new polyketide metabolites from the freshwater aquatic fungus *Annulatascus triseptatus*. *J Nat Prod* **66**: 1302–1306.

Oh H, Gloer JB & Shearer CA (1999a) Massarinolins A-C: new bioactive sesquiterpenoids from the aquatic fungus *Massarina tunicate*. *J Nat Prod* **62**: 497–501.

Oh H, Kwon T, Gloer JB, Marvanova L & Shearer CA (1999b) Tenellic acids A-D: new bioactive diphenyl ether derivatives from the aquatic fungus *Dendrospora tenella*. *J Nat Prod* **62**: 580–583.

Oh H, Swenson DC, Gloer JB & Shearer CA (2001) Massarilactones A and B: novel secondary metabolites from the fresh-water aquatic fungus *Massarina tunicata*. *Tetrahedron Lett* **42**: 975–977.

Oh H, Swenson DC, Gloer JB & Shearer CA (2003) New bioactive rosigenin analogs and aromatic polyketide metabolites from the freshwater aquatic fungus *Massarina tunicata*. *J Nat Prod* **66**: 73–79.

Park JH, Choi GJ, Jang KS, Lim HK, Kim HT, Cho KY & Kim JC (2005) Antifungal activity against plant pathogenic fungi of chaetoviridins isolated from *Chaetomium globosum*. *FEMS Microbiology Lett* **252**: 309–313.

Wong KMK, Goh TK, Hodgkiss IJ, Hyde KD, Ranghoo VM, Tsui CKM, Ho WH, Wong WS & Yuen TC (1998) The role of freshwater fungi in freshwater ecosystems. *Biodiversity and Conservation* **7**: 1187–1206.