A New Tetrahydrofuran Derivative from the Endophytic Fungus *Chaetomium* sp. Isolated from *Otanthus maritimus*

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A hitherto unidentified endophytic strain of the genus *Chaetomium*, isolated from the medicinal plant *Otanthus maritimus*, yielded a new tetrahydrofuran derivative, aureonitolic acid (**1**), along with 5 known natural products, **2**–**6**. The structure of **1** was determined by extensive spectroscopic analysis and comparison with reported data. Extracts of the fungus, grown either in liquid culture or on solid rice media, exhibited considerable cytotoxic activity when tested *in vitro* against L5178Y mouse lymphoma cells. Compounds **2** and **6** showed significant growth inhibition against L5178Y cells with EC_{50} values of 7.0 and 2.7 μg/mL, respectively, whereas **1** was inactive.

Key words: Endophytes, *Chaetomium*, Natural Products, Structure Elucidation

Introduction

Fungal endophytes are microorganisms that colonize living, internal tissues of plants without causing immediate, overt negative effects (Bacon and White, 2000). They have proven to be promising sources of new and biologically active natural products which are of interest for specific medicinal or agrochemical applications (Zhang *et al.*, 2006).

In continuation of our studies on endophyte-derived natural products (Aly *et al.*, 2008a, b) we investigated the fungal endophyte *Chaetomium* sp. isolated from stems of *Otanthus maritimus* growing wild on sandy beaches along the Mediterranean coast in Egypt. The genus *Otanthus*, found mainly in the Mediterranean region, belongs to the family Asteraceae and is represented by a single species. *O. maritimus* has been reported to exhibit a significant array of biological and pharmacological activities and is used in the treatment of dysentery and inflammation of the urinary bladder (Muselli *et al.*, 2007). Dry specimens of *O. maritimus* have been traditionally used as decoration, and at the same time, as means of repelling flying insects from household areas (Christodoulopoulou *et al.*, 2005).

The genus *Chaetomium* is a member of the subphylum Ascomycotina, family Chaetomiaceae. Members of this family are cellulolytic and occur naturally on paper and cotton fabrics (Alexopoulous *et al.*, 1996). *Chaetomium* species are reported to be widespread in soil and plant debris, where they are important agents of cellulose degradation (Carlile *et al.*, 2001). Several endophytic *Chaetomium* strains were previously found to suppress the growth of bacteria and fungi through direct competition, mycoparasitism and antibiosis (Park

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et al., 2005). Furthermore, *Chaetomium* species are known sources of bioactive compounds like the antibacterial furano-polyene 3-*epi*-aureonitol (Marwah *et al.*, 2007), as well as cochliodinol and related quinonoid metabolites (Brewer *et al.*, 1984). In addition, cytotoxic alkaloids like chaetominine (Jiao *et al.*, 2006) and chaetoglobosin U (Gang *et al.*, 2006) have been also reported from endophytic *Chaetomium* species.

Results and Discussion

Repeated column chromatography including preparative HPLC of the EtOAc extract of *Chaetomium* sp. liquid cultures yielded the new natural product aureonitolic acid (**1**), together with the known compounds cochliodinol (**2**), isocochliodinol (**3**), indole-3-carboxylic acid (**4**), cyclo(alanyltryptophane) (**5**) and orsellinic acid (**6**).

Compound **1** was isolated as a viscous colourless oil. The HRESI-mass spectrum exhibited a strong peak at *m/z* 259.0940 $[M+Na]^+$ indicating a molecular formula of $C_{13}H_{16}O_4$. The ^1H and ^{13}C NMR spectroscopic data of **1** (see Table I) closely resembled those of aureonitol (**1a**) (Abraham and Arfmann, 1992; Bohlmann and Ziesche, 1979). The core structure of **1** was basically evident from the COSY and HMBC correlations which established two polyene side chains, from C-2 to C-5 and from C-9 to C-12, connected to a central hydroxytetrahydrofuran nucleus at positions 6 and 8, respectively. However, a carboxylic group of **1** replacing the terminal methyl group in aureonitol (**1a**) was observed from the ^{13}C NMR signal at δ 174.5, which was confirmed by the HMBC correlations of H-2 and H-3 to C-1. On the basis of the observed coupling constants the geometries of all double bonds were assigned to be the same as in aureonitol (**1a**), that was previously isolated from *C. cochlioides* (Abraham and Arfmann, 1992). The NOE of H-6 with H-8, and, correspondingly, H-9 with H-7 revealed a *syn* configuration of the two carbon chains and a *trans* configuration of the hydroxy group at the ether ring. Furthermore, nearly similar $[\alpha]_D$ values of **1** ($[\alpha]_D^{20}$ –5.0°) and **1a** ($[\alpha]_D^{27}$ –7.8°) indicate that both compounds have the same absolute configuration as established for **1a** using the revised Mosher procedure (Abraham and Arfmann, 1992). Compound **1** was identified as a new natural product for which we propose the name aureonitolic acid (Fig. 1).

The known compounds **2**–**6** (Fig. 1) were identified based on their UV, ^1H , ^{13}C NMR and mass spectral data and by comparison with reported data (Jerram *et al.*, 1975; Sekita, 1983; Aldrich, 1992; Marchelli *et al.*, 1975; Evans and Staunton, 1988).

Compounds **1**–**6** were tested for cytotoxicity toward L5178Y mouse lymphoma cells. Orsellinic acid (**6**) exhibited significant growth inhibition with an EC_{50} value of 2.7 μg/mL, followed by EC_{50} = 7.0 μg/mL for cochliodinol (**2**). **2** and related quinoid metabolites were previously reported to inhibit the growth and metabolism of a range of bacterial genera (Brewer *et al.*, 1984). Interestingly, isocochliodinol (**3**), a positional isomer of **2**, showed only weak activity toward L5178Y mouse lymphoma cells. The new compound **1** showed no cytotoxic activity.

Table I. NMR data of **1** at 500 (^1H) and 125 (^{13}C) MHz (in MeOH-d_4).

Position	δ_H	COSY	NOE	HMBC	δ_C
1					174.5
2	5.89, d (15.1)	3	4	1,3,4	128.0
3	7.00, dd (15.1, 10.7)	2,4	5	1,4,5	139.0
4	6.30, dd (15.3, 10.7)	3,5		2,3,6	132.5
5	5.89, dd (15.3, 8.5)	4,6		3,4,6,7,13	138.1
6	2.82, q (7.8)	5,7,13A,13B	4,8,13A>>5,7	4,5,7,13	52.4
7	3.80, dd (7.8, 6.8)	6,8		5,6,8,9	82.3
8	4.05, dd (6.9, 6.8)	7,9		7,10	86.2
9	5.70, dd (14.8, 6.9)	8,10	7,11	8,11	133.1
10	6.28, dd (14.8, 10.5)	9		8,12	134.1
11	6.29, ddd (16.1, 10.5, 9.4)	12A,12B	9	12	137.7
12A	5.21, d (16.1, 1.7)	11,12B		10	118.0
12B	5.08, d (9.4, 1.7)	11,12A		10,11	
13A	4.05, dd (8.4, 8.4)	6,13B		5,7,8	71.7
13B	3.80, dd (8.4, 8.4)	6,13A		5,7,8	

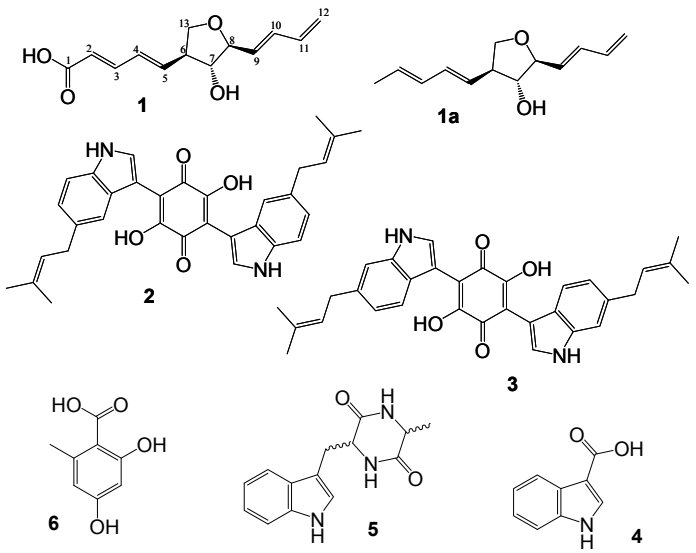

Fig. 1. Chemical structures of compounds **1**–**6**.

Experimental

General experimental procedures

Optical rotations were determined on a Perkin-Elmer-241 MC polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on ARX 500 and AVANCE DMX 600 NMR spectrometers. ESI-MS was conducted on a Finnigan LC Q-Deca mass spectrometer and HRESI- mass spectra were obtained on a Micromass Qtof 2 mass spectrometer. Solvents were distilled before use, and spectral grade solvents were used for spectroscopic measurements. HPLC analysis was performed using a HPLC (Dionex P580) system coupled to a photodiode array detector (UVD340S). Routine detection was at 235, 254, 280, and 340 nm. The separation column (125 × 4 mm, L × ID) was prefilled with Eurospher-10 C_{18} (Knauer, Germany) using the following gradient (MeOH, 0.02% H_3PO_4 in H_2O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100% MeOH.

Fungal material

The fungus *Chaetomium* sp. was isolated from fresh healthy stems of wildly growing *Otanthus maritimus*, which was collected in April 2004 from the Mediterranean coast of Alexandria, km 21, Egypt. A voucher specimen is deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University, Egypt. Following surface sterilization with 70% EtOH for 1 min the stems were rinsed in sterile water. To distinguish the remaining epiphytic fungi from endophytic fungi, an imprint of the stem surface on biomalt agar was done. Small tissue samples from inside the stems were aseptically cut and pressed onto agar plates containing an antibiotic to suppress bacterial growth (composition of isolation medium: 15 g/L malt extract, 15 g/L agar, and 0.2 g/L chloramphenicol in distilled water, pH 7.4–7.8). After incubation at room temperature the fungal strain under investigation was found to grow exclusively on the plant tissue, but not on the agar plates taken from the imprint of the stem surface. From the growing cultures pure strains of *Chaetomium* sp. were isolated by repeated re-inoculation on malt agar plates.

Identification of fungal cultures

The fungus (strain no. V2S1) was identified using a molecular biological protocol by DNA amplification and sequencing of the ITS region as described previously (Wang *et al.*, 2006). This fungal strain was identified as *Chaetomium* sp.; however, due to the lack of similar sequences in GenBank, identification of the strain to the species level was not possible. A voucher strain is kept at one of the authors' laboratory (P. P.).

Cultivation

Mass growth of the fungus was carried out in Erlenmeyer flasks (1 L each), in liquid Wickerham medium (Wickerham, 1951) (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, distilled water added up to 1000 mL, pH 7.2–7.4, adjusted with 10% NaOH or 36.5% HCl, liquid medium/flask = 300 mL, 20 flasks) and on rice solid medium (to 100 g commercially available rice 100 mL of distilled water was added and the mixture kept overnight prior to autoclaving, 6 flasks) at room temperature under static conditions for 21 and 30 d, respectively.

Extraction and isolation

Mycelia and culture filtrates were collected and successively extracted with EtOAc. The EtOAc extract (0.5 g) was taken to dryness and then partitioned between *n*-hexane and 90% MeOH. The 90% MeOH crude fraction (0.4 g) was chromatographed over Sephadex LH-20 using MeOH as eluting solvent. Fractions were monitored by TLC on silica gel F_{254} plates (Merck, Darmstadt, Germany) using UV light (254 and 366 nm) and anisaldehyde spray reagent for detection. Further purification was achieved by preparative HPLC (Varian, PrepStar 218) on a Microsorb 60–8 C_{18} column (250 × 21.4 mm, L × ID) using AcN/H_2O gradient as the mobile phase, as well as semi-preparative HPLC (Merck Hitachi L-7100) on an Eurosphere 100–10 C18 column (300 × 8 mm, L × ID, Knauer) using MeOH/H_2O gradient as the mobile phase. The rice culture was extracted with EtOAc, and the concentrated residue (1.1 g) partitioned between *n*-hexane and 90% MeOH. The 90% MeOH-soluble material (0.8 g) was then fractionated by vacuum-liquid chromatography (VLC) on silica gel 60 using *n*-hexane/EtOAc/MeOH gradient elution. Yields of compounds were as follows: **1**, 1.7 mg (from liquid culture); **2**, 255.7 mg (from liquid culture, 55.7 mg; from rice culture, 200 mg); **3**, 18.8 mg (from liquid culture, 8.8 mg; from rice culture, 10 mg); **4**, 20.1 mg (from liquid culture); **5**, 5.4 mg (from liquid culture); **6**, 49.6 mg (from liquid culture).

Aureonitolic acid (**1**): Viscous colourless oil; $[\alpha]_D^{20}$ −5.0° (*c* 0.5, MeOH). – UV: λ_{max} (PDA) = 225.2, 263.1 nm. – ^{1}H and ^{13}C NMR: see Table I. – ESI-MS, negative: *m/z* = 471.5 $[2M–H]^-$ (65), 281.4 $[M+HCOO]^-$ (100), 235.6 $[M–H]^-$ (55). – HRESI-MS: *m/z* = 259.0940 $[M+Na]^+$ (calcd. for $NaC_{13}H_{16}O_4$, 259.0946).

Cell proliferation assay

Cytotoxicity was tested against L5178Y mouse lymphoma cells using a microculture tetrazolium (MTT) assay and compared to that of untreated controls as described previously (Ashour *et al.*, 2006). All experiments were carried out in triplicate and repeated three times. As controls, media with 0.1% EGMME/DMSO were included in the experiments. The depsipeptide kahalalide F isolated from *Elysia grandifolia* (Ashour *et al.*, 2006) was used as positive control.

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