Bioorganic & Medicinal Chemistry Letters 19 (2009) 1572–1574

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Bioactive metabolites produced by *Chaetomium globosum*, an endophytic fungus isolated from *Ginkgo biloba*

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ARTICLE INFO

Article history:
Received 1 December 2008
Revised 31 January 2009
Accepted 6 February 2009
Available online 11 February 2009

Keywords:
Ginkgo biloba
Endophytic fungus
Chaetomium globosum
Chaetomugilin D
Azaphilones
Toxic activity

ABSTRACT

A novel cytotoxic chlorinated azaphilone derivative named chaetomugilin D (**1**), together with three known metabolites, chaetomugilin A (**2**), chaetoglobosins A (**3**) and C (**4**), has been isolated by a bioassay-guided fractionation from the EtOAc extract of the cultures of *Chaetomium globosum*, an endophytic fungus found in the leaves of *Ginkgo biloba*. Structure of **1** was established by analyses of spectroscopic methods, including 2D-NMR experiments (COSY, NOESY, HMQC, and HMBC). Compounds **1**–**4** displayed significant growth inhibitory activity against the brine shrimp (*Artemia salina*) and *Mucor miehei*.

Endophytes are bacteria or fungi that live in the intercellular spaces of the tissues of host plants without causing discernible manifestation of disease,[1] they can be found in virtually all terrestrial plants and play an important role for the growth of hosts.[2,3] Recently, endophytes have been recognized as important sources of a variety of structurally novel active secondary metabolites with anticancer, antimicrobial and other biological activities.[4,5] Recent studies on biologically active metabolites from endophytic microorganisms residing in the medicinal plant *Ginkgo biloba* have shown that the cultures of *Colletotrichum* sp. contain flavones that exhibited potent anticancer and antioxidation activities.[6,7]

In previous studies, several new metabolites have been reported from strains of *Alternaria alternata* and rhizobial *Mesorhizobium* sp. CCNWGX022, which were isolated from the plants *Maytenus hookeri* and *Glycyrrhiza uralensis*,[8,9] respectively. In our continuous screening for biologically active secondary metabolites from endophytic microorganisms, we investigated the secondary metabolites produced by cultures of the endophytic fungus *Chaetomium globosum* present in the leaves of *G. biloba*. Activity-directed fractionation of the ethyl acetate extract of the cultures of this fungus with good cytotoxic activity against the brine shrimp (*Artemia salina*) led to the isolation of four metabolites: two azaphilones chaetomugilins D (**1**) and A (**2**), and two cytochalasan alkaloids chaetoglobosins A (**3**) and C (**4**), in which compound **1** is a newly discovered product. In this Letter, we herein describe the isolation and structure elucidation of **1** and biological activity of these isolated products.

The fungal strain *C. globosum* was separated from the sterilized leaves of *G. biloba*, a medicinal plant growing in Linyi, Shandong province, China, and was characterized based on morphological studies and has been deposited at Center for Experiments and Education Technology, Linyi Normal University. The fungus *C. globosum* was cultivated on PDA medium for 5 days at 28 °C to provide the culture broth (30 L), which was filtered to give the mycelium and culture filtrate. The culture filtrate obtained was subsequently extracted with ethyl acetate. The mycelium was dried at 50 °C for 10 h, and ultrasonically extracted three times by ethyl acetate and acetone, respectively. The combined organic layers were defatted with cyclohexane and then dissolved in methanol to provide another crude extract (8.9 g).The results of bioassay indicated that the mycelium extract showed marked growth inhibitory activity against the brine shrimp and *Mucor miehei* at the concentration of 1 mg/ml, but the activity of the culture filtrate extract is weaker. The ethyl acetate extract (2.83 g) of culture filtrate was

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doi:10.1016/j.bmcl.2009.02.025

fractionated on a silica gel column, followed by separation on Sephadex LH-20 (CH_2Cl_2/MeOH = 6:4 and MeOH), normal and reverse phase column chromatography and preparative TLC using CH_2Cl_2/MeOH (20:1) to afford chaetomugilin D (**1,** 4.4 mg) and chaetomugilin A (**2,** 2.7 mg). Similarly, multiple fractionation of the mycelium crude extract (8.9 g) gave chaetoglobosins A (**3**, 5.9 mg) and C (**4,** 2.1 mg).

The structures of chaetomugilin A (**2**), chaetoglobosins A (**3**) and C (**4**) were determined on the basis of ESI-MS and ^{1}H, ^{13}C, and 2D NMR data. These data were identical to those previously reported in the literature.[10–13] More interestingly, compound **2** was previously obtained from a strain of *C. globosum* originally isolated from the marine fish *Mugil cephalus*.[10]

Compound **1**[14] was obtained as an optically active yellow gum. The molecular formula $C_{23}H_{27}O_6Cl$ was determined by the $[M-H]^-$ peak at *m/z* 434.1427 in negative mode HR-ESI-MS and ^{13}C NMR spectra. The 3:1 ratio of isotope peak intensities of $(MH^-/[MH+2]^-)$ indicated the presence of chlorine atom in the molecule. The molecular formula indicated 10° of unsaturation in the molecule. The appearance of resonances for 10 sp^2 carbons in the ^{13}C NMR spectrum indicated that 6° of unsaturation were attributed to the presence of 6 double bonds and that the remaining degrees could be satisfied by the assignment of four rings. The IR spectrum of **1** showed absorption bands at 3407, 1719, and 1619 cm^{-1}, indicating the existence of hydroxyl, ester carbonyl, and conjugated carbonyl functionalities. Characteristic UV spectral data at λ 283, 384, 402, 429 nm also revealed the presence of a highly extended conjugation system in this molecule.

Analysis of the ^{1}H and ^{13}C NMR spectra (Table 1) of **1** in conjunction with DEPT experiments disclosed the presence of 23 carbon signals that resulted from five methyl groups (δ_C 19.4, 11-CH_3; 11.7, C-13; 8.8, 4′-CH_3; 18.7, C-6′; and 23.3, 7-CH_3) containing one tertiary methyl (7-CH_3), one sp^3-hybridized methylene (δ_C 29.2, C-12), four sp^2-methines (δ_C 145.6, C-1; 104.9, C-4; 120.2, C-9; and 146.9, C-10) containing oxygen-bearing carbon C-1, five sp^3-hybridized methines (δ_C 50.6, C-8; 38.9, C-11; 58.3, C-2′; 44.9, C-4′; and 76.9, C-5′) containing one oxygenated methine carbon C-5′, two quarternary oxygenated sp^3 carbons (δ_C 84.0, C-7 and 104.2, C-3′) containing a hemiacetal carbon C-3′, four quarternary sp^2 carbons (δ_C 157.7, C-3; 140.4, C-4a; 110.1, C-5; and 114.3, C-8a) and two carbonyl carbons (δ_C 189.2, C-6; and 170.5, C-1′).

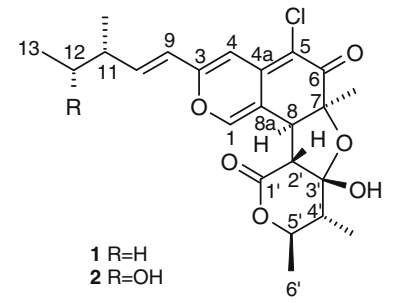
Table 1
^{1}H and ^{13}C NMR data of compound **1** (500 MHz, $CDCl_3$)

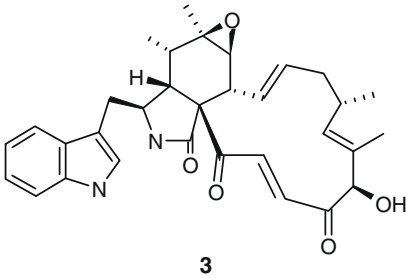
No.	δ_c	δ_H (*J* in Hz)	Selected HMBC
1	145.6 d	7.27 (1H, s)	C-8
3	157.7 s		C-4,1,9,10
4	104.9 d	6.55 (1H, s)	C-9
4a	140.4 s		C-1,8
5	110.1 s		C-4,
6	189.2 s		C-8,7-Me
7	84.0 s		C-8,7-Me
8	50.6 d	2.97 (1H, d, 10.1)	C-1,2′,7-Me
8a	114.3 s		C-8,1,4,2′
9	120.2 d	6.04 (1H, d, 15.6)	C-4,11
10	146.9 d	6.52 (1H, dd, 15.6, 6.5)	C-9,11,12
11	38.9 d	2.23 (1H, m, 6.8)	C-9,10,12,13,11-Me
12	29.2 t	1.42 (2H, m)	C-11,10,13,11-Me
13	11.7 q	0.87 (3H, t, 7.3)	C-11,12
7-Me	23.3 q	1.39 (3H, s)	C-8
11-Me	19.4 q	1.10 (3H, d, 6.4)	C-10,11,12
1′	170.5 s		C-8, 2′
2′	58.3 d	3.06 (1H, d, 10.1)	C-8
3′	104.2 s		C-4′,2′,5′,4′-Me
4′	44.9 d	1.87 (1H, dq, 10.1, 6.9)	C-6′,4′-Me
5′	76.9 d	4.28 (1H, dq, 10.1, 6.4)	C-4′, 6′, 4′-Me
6′	18.7 q	1.40 (3H, d, 6.4)	
4′-Me	8.8 q	1.11 (3H, d, 6.2)	C-4′

The ^{1}H and ^{13}C NMR and UV spectral data of **1** were similar to those of chaetomugilin A (**2**), suggesting that they possess the same azaphilone skeleton. The distinct difference in NMR spectra between compounds **1** and **2** was that an oxomethine signal at C-12 (δ_C 70.9, d; δ_H 3.81, br s) in **2** was replaced by a methylene (δ_C 29.2, t; δ_H 1.42, m) in **1.** Furthermore, the position of a chlorine atom was determined to be at C-5 for its chemical shift value (δ_C 110.1).[10]

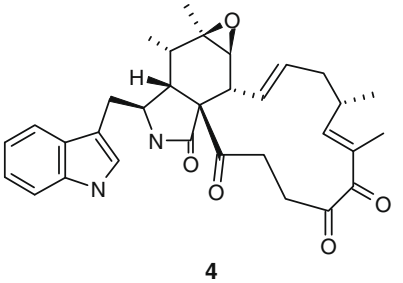
From a large vicinal coupling constants ($J_{9,10}$ = 15.6 Hz) between two olefinic protons, the configuration of the double bond at C-9 was deduced to be of the *trans* form. These facts suggested that **1** was a 12-deoxo analogue of chaetomugilin A (**2**), as further substantiated by a ^{1}H–^{1}H COSY experiment (three partial structural portions: from H-9 to H-13, and 11-CH_3, from H-2′ to H-8, from H-6′ to H-5′, H-4′ and 4′-CH_3 depicted as a bold line in Figure 2 and the key HMBC correlations (from H-10 to C-3, from H-4 to C-4a and C-5, from H-1 to C-3, C-4a, C-8 and C-8a, from H-8 to C-6, C-7, 7-CH_3, C-3' and C-1', from H-5' to C-3′ and C-1′ (Fig. 2).

The relative stereochemistry of **1** was deduced from the distinct NOE enhancement of H-5′/H-8, H-5′/7-CH_3, H-2′/H-4′ and H-4′/H-6′ observed in the NOESY spectra (Fig. 3). The absolute configurations for **1** were not established, but they are assumed to be as given above by comparison of their negative optical rotations with those of chaetomugilin A (**2**), whose absolute configuration has been determined as 7*S*, 8*S*, 11*R*, 12*R*, 2′*R*, 3′*R*, 4′*R* and 5′*R*.[10] On the other hand, biogenetically, compounds **1** and **2** have similar biosynthetic pathway in *C. globosum*. Thus, the absolute stereochemistry of **1** is likely the same as in **2** except for C-12. Consequently, the structure of **1**, whose absolute configuration was





3



4

Figure 1. Structures of compounds **1**–**4**.

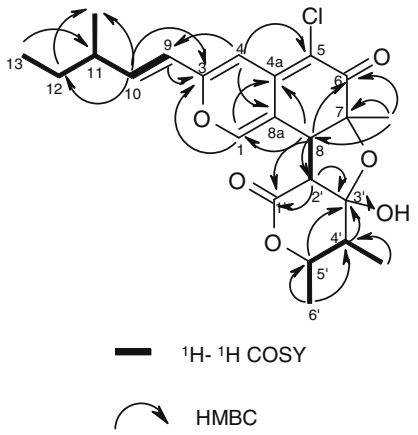


Figure 2. Key HMBC and ^{1}H–^{1}H COSY correlations for compound **1**.

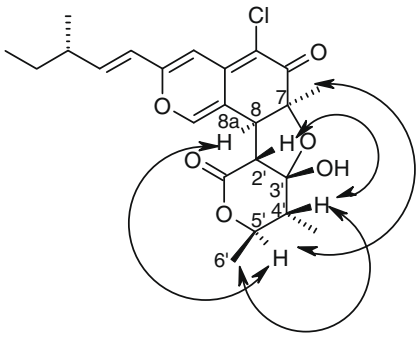


Figure 3. Key NOE correlations of compound **1**.

assigned as 7*S*, 8*S*, 11*R*, 2′*R*, 3′*R*, 4′*R* and 5′*R*, was elucidated as shown in Figure 1, designated chaetomugilin D.

Azaphilones are an important class of oxoisochromane derivatives that have been isolated from various microorganisms, mainly from fungal species, such as *Emericella falconensis*,[15] *Penicillium multicolor*,[16] *Penicillium sclerotiorum*,[17] *Monascus purpureus*,[18] *Annulohypoxylon cohaerens*,[19] *C. globosum* var. *flavo-viridae*,[20] *Chaetomium cupreum*,[21] and *Hypoxylon* sp.[22] To date, chaetomugilins A (**2**), B and C as cytotoxic fungal metabolites have been reported from a *Chaetomium* species separated from a marine fish, *Mugil cephalus*,[10] but this family of azaphilones are very rare. To the best of our knowledge, chaetomugilins D (**1**) represents the third member of a new group of chaetomugilin analogue with a double *cis*-fused hemiacetal tetrahydrofuran ring substructure at C-7 and C-8, and C-2′ and C-3′.

The growth inhibitory activity of compounds **1**–**4** was evaluated against brine shrimp (*A. salina*)[23,24] and *M. miehei*. After incubation for 24 h, new compound **1** and three reported compounds **2**–**4** showed significant toxicity toward brine shrimp larvae at a concentration of 10 μg/ml, with mortality rates (%) of 75.2, 78.3, 83.4 and 75.3, respectively. Compounds **3** and **4** also exhibited marked inhibitory effects on *M. miehei* by the agar diffusion method with observed zones of inhibition of 25 and 15 mm in diameter, respectively, at 10 μg/disk.

In conclusion, a novel chloride-containing azaphilone analogue, chaetomugilin D (**1**), together with three known metabolites **2**–**4** was isolated from the cultures of *C. globosum*, an endophytic fungus isolated from the leaves of *G. biloba*. The isolated compounds were found to show remarkable growth inhibitory activity against the brine shrimp and *M. miehei*.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 30430020), the Program for New Century Excellent Talents in University (NCET-05-0852) and the Program for Changjiang Scholars and Innovative Research Team in University (IRT0749), and the National Financial Aid for Studying Abroad (2007103088). We also thank F. Lissy for technical assistance and H. Frauendorf and R. Machinek, in the laboratory of institution for organic and biomolecular chemistry, University of Göttingen, Germany, for the MS and NMR measurements.

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14. *Data for chaetomugilin D (**1**)*: Yellow gum; $[\alpha]_D^{20} = -21$ (*c* 0.04, MeOH); IR (KBr) ν_{max} 3407 (OH), 1719 (C=O), 1619 (C=O), 1561, 1521 cm^{-1}; UV λ_{max} (EtOH)/nm 283 ($\log\varepsilon$ 3.92), 384 (4.04), 402 (3.98), 429 (3.78); HR-ESI-MS *m/z* 434.1427 $[M-H]^-$ (calcd for $C_{23}H_{27}O_6Cl$, 434.1423); ^{1}H and ^{13}C NMR data, see Table 1.
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$M = [(A - B - N)/(G - N)] \times 100$.
M = percent of the dead larvae after 24 h; *A* = number of the dead larvae after 24 h; *B* = average number of the dead larvae in the blind samples after 24 h; *N* = number of the dead larvae before starting the test; *G* = number of selected larvae for test.