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ORIGINAL ARTICLE

Absolute stereostructures of chaetomugilins G and H produced by a marine-fish-derived *Chaetomium* species

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Chaetomugilins G and H were isolated from a strain of *Chaetomium globosum* that was originally isolated from the marine fish, *Mugil cephalus*, and their absolute stereostructures were elucidated on the basis of spectroscopic analyses, including 1D and 2D NMR techniques, and chemical transformation. In addition, the absolute configuration of chaetoviridin C was established by derivatization from chaetomugilin A. These compounds exhibited a growth inhibitory activity against cultured P388, HL-60, L1210 and KB cells.

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Keywords: azaphilones; *Chaetomium* sp.; cytotoxicity; chaetomugilin; chaetoviridin; fungus; marine fish

INTRODUCTION

Marine microorganisms are potentially prolific sources of highly bioactive secondary metabolites that might serve as useful leads in the development of new pharmaceutical agents. On the basis of the fact that some of the bioactive materials isolated from marine animals have been produced by bacteria, we focused our attention on new antitumor agents from microorganisms separated from marine organisms.[1–5] As part of this endeavor, we conducted a search for antitumor compounds from a strain of *Chaetomium globosum* OUPS-T106B-6 that was originally isolated from the marine fish, *Mugil cephalus,* and reported six new cytotoxic metabolites designated as chaetomugilins A (**1**), B, C, D (**2**), E and F from the culture broth of this fungal strain.[6,7] These compounds are azaphilones and they have various bioactivities, including antimicrobial, nitric oxide inhibitory, gp120-CD4-binding inhibitory, monoamine oxidase inhibitory and platelet-derived growth factor-binding inhibitory activities.[7] An examination of a disease-oriented panel of 39 human cancer cell lines (HCC panel)[8,9] suggested the possibility that the mode of action of chaetomugilins A (**1**), C and F might be different from that shown by any other anticancer drugs developed to date.[7] Our continuing search for cytotoxic metabolites from this fungal strain yielded two new azaphilones designated as chaetomugilins G (**3**) and H (**4**), along with the known compound, chaetoviridin C (**5**)[10] (Figure 1). These compounds exhibited moderate cytotoxic activity against the murine P388 leukemia cell line, the human HL-60 leukemia cell line, the murine L1210 leukemia cell line and the human KB epidermoid carcinoma cell line. We describe herein the absolute stereostructures and biological activities of these compounds.

RESULTS AND DISCUSSION

The microorganism from *M. cephalus* fish was cultured at 27 °C for 6 weeks in a medium (100 l) containing 1% soluble starch and 0.1% casein in 50% artificial seawater adjusted to pH 7.5. This fermentation was carried out on twice the volume than that used in previous reports[6,7] to obtain minor metabolites. After incubation, the AcOEt extract of the culture filtrate was purified by bioassay-directed fractionation (cytotoxicities against the P388 cell line) using a stepwise combination of Sephadex LH-20, silica gel column chromatography and reversed-phase HPLC to afford chaetomugilins G (**3**), H (**4**) and chaetoviridin C (**5**), together with chaetomugilins A–F. The physicochemical properties of chaetomugilins G (**3**) and H (**4**) are summarized in Table 1.

Chaetomugilin G (**3**) had the molecular formula, $C_{24}H_{29}ClO_7$, which was established from the $[M+H]^+$ peak in high-resolution fast atom bombardment mass spectrometry and from the ratio of the intensity of isotope peaks (MH^+/$[MH+2]^+$). Its IR spectrum exhibited bands at 3423, 1700 and 1687 cm^{-1}, which are characteristic of hydroxyl, ester and conjugated carbonyl groups, respectively. A close inspection of the ^1H and ^{13}C NMR spectra (Table 2) of **3** by DEPT and heteronuclear multiple quantum coherence (HMQC) experiments revealed the presence of four secondary methyls (11-CH_3, C-13, 4′-CH_3 and C-6′), one tertiary methyl (7-CH_3), one ester methyl (1′-OCH_3), four sp^2-hybridized methines (C-1, C-4, C-9 and C-10) including oxygen-bearing carbon (C-1), five sp^3-methines (C-8, C-11, C-12, C-4′ and C-5′) including two oxymethines (C-12 and C-5′), one quaternary oxygen-bearing sp^3-carbon (C-7), six quaternary sp^2-carbons (C-3, C-4a, C-5, C-8a, C-2′ and C-3′)

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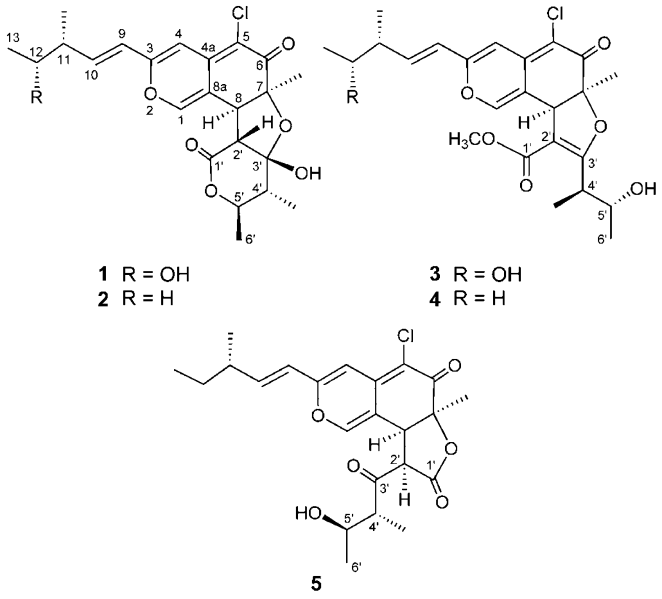


Figure 1 Structures of chaetomugilins A (**1**), D (**2**), G (**3**) and H (**4**) and chaetoviridin C (**5**).

Table 1 Physico-chemical properties of chaetomugilins G (3) and H (4)

	3	*4*
Appearance	Yellow powder	Yellow powder
M.p.	165–167 °C	119–121 °C
$[a]_D^{22}$	−80.1 (*c* 0.13, EtOH)	−175.0 (*c* 0.09, EtOH)
HRFAB-MS		
Found:	465.1679 $(M+H)^+$	449.1729 $(M+H)^+$
Calcd:	465.1685 (for $C_{24}H_{30}{}^{35}ClO_7$)	449.1731 (for $C_{24}H_{30}{}^{35}ClO_6$)
Molecular formula	$C_{24}H_{29}ClO_7$	$C_{24}H_{29}ClO_6$
UV λ_{max} (EtOH) nm (log ε)	255 (3.91), 292 (3.86), 391 (3.93), 408 (3.90)	252 (3.90), 290 (3.86), 392 (3.94), 408 (3.94)
IR ν_{max} (KBr) cm^{-1}	3423,1700, 1687, 1613, 1564, 1522	3444, 1717, 1700, 1686, 1622, 1561, 1523
TLC Rf[a]	0.47	0.65
Solubility		
Soluble	DMSO, MeOH, $CHCl_3$	DMSO, MeOH, $CHCl_3$
Insoluble	H_2O	H_2O

[a]Silica gel (10% MeOH in $CHCl_3$).

including one oxygen-bearing carbon (C-3′) and two carbonyls (C-6 and C-1′). The ^1H-^1H COSY analysis of **3** revealed two partial structural units as shown by bold-faced lines in Figure 2. The geometrical configuration of the double bond moiety (C-9–C-10) was deduced to be *trans* from the coupling constants of the olefinic protons ($J_{9,10}$=15.8 Hz). The connection of these units and of the remaining functional groups was determined on the basis of the key heteronuclear multiple-bond connectivity (HMBC) correlations summarized in Figure 2. The connection of a chlorine atom to C-5 was reasonable from its chemical shift (δ_C 110.43). Thus, the planar structure of **3** was elucidated as shown in Figure 2.

The relative stereochemistry of **3** was examined by conducting NOESY experiments (Table 2). NOE correlations (6′-H/8-H and 6′-H/7-CH_3) implied that 8-H is oriented *cis* to the 7-methyl group. However, the relative configuration of C-11, C-12, C-4′ and C-5′ could not be elucidated. Treatment with *p*-TsOH of chaetomugilin A (**1**) in MeOH gave chaetomugilins B and C, as reported previously.[6,7] This time, the above reaction was carried out on the condition that more *p*-TsOH was used, which then resulted in **3** together with chaetomugilins B and C (Scheme 1). Product **3** was confirmed to be identical to natural **3** on the basis of IR, UV and NMR spectra and optical rotations. This result allowed us to assign the absolute configuration of all the asymmetric centers (7*S*, 8*R*, 11*R*, 12*R*, 4′*R* and 5′*R*) in chaetomugilin G (**3**).

Chaetomugilin H (**4**), which contained one oxygen atom less than **3**, was assigned the molecular formula, $C_{24}H_{29}ClO_6$. The general features of its UV, IR and NMR spectra (Table 3) closely resembled those of **3**, except that the proton signals for H-11 (δ_H 2.25, sept), H-12 (δ_H 1.43, quint) and H-13 (δ_H 0.90, t), and the carbon signals for C-10 (δ_C 146.04), C-11 (δ_C 38.76), C-12 (δ_C 29.18), C-13 (δ_C 11.68) and 11-CH_3 (δ_C 19.35) in **4** revealed a chemical shift difference relative to those of **3**. The above evidence implied that the hydroxyl methine at C-12 in **3** was replaced with a methylene in **4**. The planar structure of **4** was confirmed by analyzing ^1H-^1H COSY correlations and HMBC correlations (Table 3). In NOESY experiments, the same NOE correlations (6′-H/8-H and 6′-H/7-CH_3) as those of **3** were observed. As in compound **3**, treatment with *p*-TsOH of chaetomugilin D (**2**) in MeOH gave product **4** (Scheme 1), which was confirmed to be identical to natural **4** on the basis of IR, UV and NMR spectra and optical rotations. The above lines of evidence revealed the absolute stereostructure of chaetomugilin H (**4**). Treatment of chaetomugolins C and F with *p*-TsOH also gave **3** and **4**, respectively (Scheme 1). This fact implied that the transformation from **1** and **2** to **3** and **4** proceeded through chaetomugolins C and F, respectively.

The planar structure of chaetoviridin C (**5**), which was confirmed to be identical in terms of IR, UV and NMR spectra, as well as optical rotation, has already been reported by Natori and co-workers,[10] but the stereochemistry has remained undecided. To determine the absolute configuration of **5**, the derivatization from chaetoviridin C (**5**) to chaetomugilin D (**2**) was carried out. Treatment with *p*-TsOH of **5** in MeOH gave product **2**, which was confirmed to be identical to natural **2** on the basis of IR, UV and NMR spectra and optical rotations.[7] This result allowed us to assign the absolute configuration of all the asymmetric centers (7*S*, 8*S*, 11*S*, 2′*R*, 4′*R* and 5′*R*) in chaetoviridin C (**5**).

Chaetomugilins A (**1**) and D (**2**) were stable in the mixture, MeOH/$CHCl_3$, for several days. In the process of isolation, the time that **1** was exposed to MeOH on the silica gel column chromatography was very short (6–7 h, the longest time). In addition, **2** was not exposed to MeOH in this procedure. These results suggested that chaetomugilins G (**3**) and H (**4**) were not artifacts of chaetomugilins A (**1**) and D (**2**), respectively.

Assays for the growth inhibitory activity of other azaphilones using various cancer cell lines are rarely reported. As a primary screen for antitumor activities, the cancer cell growth inhibitory activities of chaetomugilins G (**3**) and H (**4**) were examined using the murine P388 leukemia cell line, the human HL-60 leukemia cell line, the murine L1210 leukemia cell line and the human KB epidermoid carcinoma cell line. Compound **3** exhibited significant cytotoxicity to P388 and HL-60 cell lines. In addition, compound **4** also showed moderate activity against KB cell lines (Table 4). These results implied that the presence of the hydroxyl group at C-12 reduced activity.

Table 2 NMR spectral data of chaetomugilin G (3) in $CDCl_3$

Position	δ_H[a]		*J/Hz*	*^{1}H-^{1}H COSY*	*NOE*	δ_C		*HMBC (C)*[b]
1	7.55	s			8, 1′-OCH_3	147.4	(CH)	3, 4a, 8, 8a
3						156.6	(C)	
4	6.52	s			9	105.3	(CH)	3, 5, 8a, 9
4a						141.2	(C)	
5						110.4	(C)	
6						185.0	(C)	
7						87.9	(C)	
8	4.19	s			1, 7-CH_3, 6′	47.6	(CH)	1, 4a, 6, 7, 8a, 7-CH_3, 1′, 2′, 3′
8a						113.5	(C)	
9	6.13	d	15.8 (10)	10	4, 11, 13, 11-CH_3	122.2	(CH)	3, 4, 10, 11
10	6.60	dd	15.8 (9), 7.0 (11)	9, 11	11, 12, 13, 11-CH_3	141.8	(CH)	3, 11, 12, 11-CH_3
11	2.45	sex	7.0 (10, 12, 11-CH_3)	10, 12, 11-CH_3	9, 10, 12, 13, 11-CH_3	44.1	(CH)	9, 10, 12, 13, 11-CH_3
12	3.82	quint	7.0 (11, 13)	11, 13	10, 11, 13, 11-CH_3	70.9	(CH)	10, 11, 13, 11-CH_3
13	1.20	d	7.0 (12)	12	9, 10, 11, 12	20.4	(CH_3)	11, 12
7-CH_3	1.62	s			8, 6′, 4′-CH_3	22.9	(CH_3)	6, 7, 8
11-CH_3	1.13	d	7.0 (11)	11	9, 10, 11, 12	14.8	(CH_3)	10, 11, 12
1′						165.3	(C)	
2′						104.7	(C)	
3′						173.9	(C)	
4′	3.33	quint	6.8 (5′, 4′-CH_3)	5′, 4′-CH_3	5′, 4′-CH_3, 6′, 1′-OCH_3	40.7	(CH)	2′, 3′, 5′, 6′, 4′-CH_3
5′	3.92	quint	6.8 (4′, 6′)	4′, 6′	4′, 6′, 4′-CH_3	69.7	(CH)	3′, 4′, 6′, 4′-CH_3
6′	1.22	d	6.8 (5′)	5′	8, 7-CH_3, 4′, 5′, 4′-CH_3	21.5	(CH_3)	4′, 5′
4′-CH_3	1.13	d	6.8 (4′)	4′	7-CH_3, 4′, 5′, 6′	14.8	(CH_3)	3′, 4′, 5′
1′-OCH_3	3.71	s			1′, 4′	51.1	(CH_3)	1′

[a]^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constants (*J*/Hz). Figures in parentheses indicate the proton coupling with that position.
[b]Long range ^{1}H-^{13}C correlations from H to C observed in the HMBC experiments.

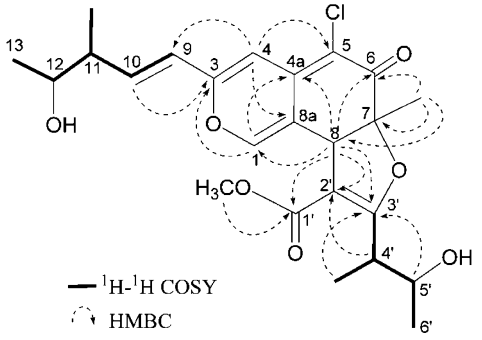


Figure 2 Selected ^{1}H-^{1}H COSY and HMBC correlations of chaetomugilin G (**3**).

EXPERIMENTAL SECTION

General

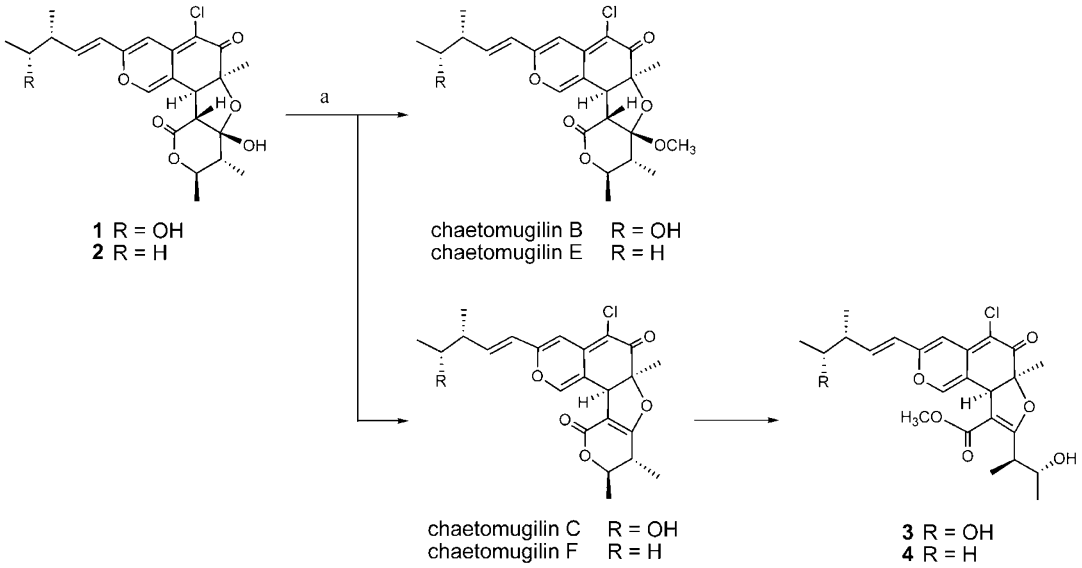
The m.ps. were determined on a Yanagimoto micro-melting point apparatus (Yanagimoto Ltd., Kyoto, Japan) and are uncorrected. UV spectra were recorded on a Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan), and IR spectra on a JASCO FT/IR-680 Plus (JASCO Corporation, Tokyo, Japan). NMR spectra were recorded at 27 °C on Varian UNITY INOVA-500 and MERCURY spectrometers (Varian Technologies Japan Ltd., Tokyo, Japan), with tetramethylsilane as an internal reference. FABMS data were obtained using a JEOL JMS-700 (Ver. 2) mass spectrometer (JEOL Ltd., Tokyo, Japan). Optical rotations were recorded on a JASCO J-820 polarimeter (JASCO Corporation). Liquid chromatography over silica gel (mesh 230–400) (NACARAI Tesque, inc., Kyoto, Japan) was performed at medium pressure. HPLC was run on a Waters ALC-200 (Nihon Waters K.K., Tokyo, Japan) instrument equipped with a differential refractometer (R 401) (Nihon Waters K.K.) and Shim-pack PREP-ODS (25 cm×20 mm i.d.) (Shimazu Corporation, Kyoto, Japan). Analytical TLC was performed on precoated Merck aluminum sheets (DC-Alufolien Kieselgel 60 F254, 0.2 mm) (Merck Ltd. Japan, Tokyo, Japan) with the solvent system, CH_2Cl_2-MeOH (9:1), and compounds were viewed under a UV lamp and sprayed with 10% H_2SO_4, followed by heating.

Culture and isolation of metabolites

A strain of *C. globosum* was initially isolated from the marine fish, *Mugil cephalus,* collected from the Katsuura Bay of Japan in October 2000. The marine fish was wiped with EtOH and its gastrointestinal tract was applied to the surface of nutrient agar layered in a Petri dish. Serial transfers of one of the resulting colonies provided a pure strain of *C. globosum*. The fungal strain was cultured at 27 °C for 6 weeks in a liquid medium (100 l) containing 1% soluble starch and 0.1% casein in 50% artificial seawater adjusted to pH 7.5. The culture was filtered under suction and extracted thrice with AcOEt. The combined extracts were evaporated *in vacuo* to afford a mixture of crude metabolites (20.3 g). The AcOEt extract was passed through Sephadex LH-20 (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA), using $CHCl_3$–MeOH (1:1) as eluent. The second fraction (7.2 g) was chromatographed on a silica gel column with a $CHCl_3$–MeOH gradient as eluent. The $CHCl_3$ eluate (1.3 g) was purified by HPLC using MeCN–H_2O (80:20) as eluent to afford Fr. 1 (160.3 mg), **2** (121.3 mg), **5** (21.7 mg), chaetomugilin E (34.1 mg) and chaetomugilin F (32.2 mg). Fr. 1 was purified by HPLC using MeCN–H_2O (60:40) as eluent to afford **4** (20.8 mg). The MeOH–$CHCl_3$ (1:99) eluate (1.8 g) was purified by HPLC using MeOH–H_2O (70:30) as eluent to afford Fr. 2 (134.7 mg), Fr. 3 (323.8 mg) and Fr.4 (122.9 mg). Fr. 2 was purified by HPLC using MeCN–H_2O (35:65) as eluent to afford **3** (22.5 mg). Fr. 3 was purified by HPLC using MeOH–H_2O (60:40) as eluent to afford **1** (220.5 mg). Fr. 4 was purified by HPLC using MeCN–H_2O (55:45) as eluent to afford chaetomugilin B (38.5 mg) and chaetomugilin C (44.1 mg).

Derivatization of 3 from 1

p-TsOH (22.7 mg) was added to a MeOH solution (5 ml) of chaetomugilin A (**1**) (27.2 mg) and the reaction mixture was left at room temperature for 6 h.



Scheme 1 Reagents and conditions: (**a**) MeOH, *p*-TsOH, 20 °C, 6 h.

Table 3 NMR spectral data of chaetomugilin H (4) in $CDCl_3$

Position	δ_H[a]	*J/Hz*	*^{1}H-^{1}H COSY*	*NOE*	δ_C		*HMBC (C)*[b]
1	7.55	s		8, 1′-OCH_3	147.4	(CH)	3, 4a, 8, 8a
3					157.2	(C)	
4	6.49	s		9	104.8	(CH)	3, 5, 8a, 9
4a					141.4	(C)	
5					110.1	(C)	
6					184.8	(C)	
7					87.9	(C)	
8	4.19	s		1, 7-CH_3, 6′	47.6	(CH)	1, 4a, 6, 7, 8a, 7-CH_3, 2′, 3′
8a					113.5	(C)	
9	6.04	d	15.8 (10)	10	4, 11, 11-CH_3	120.3	(CH)	3, 4, 10, 11
10	6.50	dd	15.8 (9), 7.5 (11)	9, 11	11, 12, 13, 11-CH_3	146.0	(CH)	3, 11, 12, 11-CH_3
11	2.25	sept	7.5 (10, 12, 11-CH_3)	10, 12, 11-CH_3	9, 10, 12, 13, 11-CH_3	38.8	(CH)	9, 10, 12, 13, 11-CH_3
12	1.43	quint	7.5 (11, 13)	11, 13	10, 11, 13, 11-CH_3	29.2	(CH_2)	10, 11, 13, 11-CH_3
13	0.90	t	7.5 (12)	12	10, 11, 12	11.7	(CH_3)	11, 12
7-CH_3	1.62	s		8, 6′, 4′-CH_3	23.0	(CH_3)	6, 7, 8
11-CH_3	1.08	d	7.5 (11)	11	9, 10, 11, 12	19.4	(CH_3)	10, 11, 12
1′					165.4	(C)	
2′					104.8	(C)	
3′					173.9	(C)	
4′	3.33	quint	6.9 (5′, 4′-CH_3)	5′, 4′-CH_3	5′, 4′-CH_3, 6′, 1′-OCH_3	40.8	(CH)	3′, 5′, 6′, 4′-CH_3
5′	3.92	quint	6.9 (4′, 6′)	4′, 6′	4′, 6′, 4′-CH_3	69.7	(CH)	3′, 4′-CH_3
6′	1.22	d	6.9 (5′)	5′	8, 7-CH_3, 4′, 5′, 4′-CH_3	21.5	(CH_3)	4′, 5′
4′-CH_3	1.13	d	6.9 (4′)	4′	7-CH_3, 4′, 5′, 6′	14.8	(CH_3)	3′, 4′, 5′
1′-OCH_3	3.71	s			1′, 4′	51.1	(CH_3)	1′

[a]As in Table 2.
[b]As in Table 2.

The solvent was evaporated off under reduced pressure and the residue was purified by HPLC using MeCN–H_2O (55:45) as eluent to afford **3** (3.1 mg), chaetomugilin B (7.2 mg) and chaetomugilin C (6.7 mg).

Derivatization of 4 from 2

Using the same procedure as above with **1**, chaetomugilin D (**2**) (32.7 mg) was treated with *p*-TsOH (25.8 mg) in MeOH (8 ml), and the products were purified by HPLC using MeCN–H_2O (70:30) as eluent to afford **4** (4.1 mg), chaetomugilin E (8.9 mg) and chaetomugilin F (8.2 mg).

Derivatization of 2 from chaetomugilin C

Using the same procedure as above with **1**, chaetomugilin C (12.6 mg) was treated with *p*-TsOH (18.6 mg) in MeOH (3 ml) and the products were purified by HPLC using MeCN–H_2O (55:45) as eluent to afford **3** (4.8 mg).

Table 4 Cytotoxicity of the metabolites against P388, HL-60, L1210 and KB cell lines

Compound	*Cell line P388* $IC_{50}(\mu M)$[a]	*Cell line HL-60* IC_{50} (μM)[a]	*Cell line L1210* IC_{50} (μM)[a]	*Cell line KB* IC_{50} (μM)[a]
Chaetomugilin				
G (**3**)	24.1	19.8	123.6	137.8
H (**4**)	12.3	10.3	93.3	18.8
5-FU[b]	1.7	2.7	3.0	6.0

[a]DMSO was used as vehicle.
[b]Positive control.

Derivatization of 4 from chaetomugilin F

Using the same procedure as above with **1**, chaetomugilin F (15.5 mg) was treated with *p*-TsOH (20.6 mg) in MeOH (4 ml) and the products were purified by HPLC using MeCN–H_2O (70:30) as eluent to afford **4** (6.3 mg).

Derivatization of 2 from 5

Using the same procedure as above with **1**, *p*-TsOH (10.5 mg) was added to a MeOH solution (3 ml) of chaetoviridin C (**5**) (18.7 mg) and the reaction mixture was left at room temperature for 1 h. The solvent was evaporated off under reduced pressure and the residue was purified by HPLC using MeCN–H_2O (80:20) as eluent to afford **2** (12.8 mg).

Assay for cytotoxicity to P388 and HL-60 cell lines

Cytotoxicity of chaetomugilins G (**3**) and H (**4**) was examined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide method. P388, HL-60, L1210 and KB cells were cultured in Eagle's Minimum Essential Medium (10% fetal calf serum) at 37 °C in 5% CO_2. The test material was dissolved in DMSO to give a concentration of 10 mM, and the solution was diluted with the Essential Medium to give concentrations of 200, 20 and 2 μM. Each solution was combined with each cell suspension (1×10^5 cells ml^{-1}) in the medium. After incubating at 37 °C for 72 h in 5% CO_2, the grown cells were labeled with 5 mg ml^{-1} 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide in phosphate-buffered saline, and the absorbance of formazan dissolved in 20% SDS in 0.1 N HCl was measured at 540 nm using a microplate reader (Model 450) (Bio-Rad Laboratories, Inc., Tokyo, Japan). Each absorbance value was expressed as a percentage relative to the control cell suspension that was prepared without the test substance, using the same procedure as that described above. All assays were performed thrice. Semilogarithmic plots were constructed from the averaged data and the effective dose of the substance required to inhibit cell growth by 50% (IC_{50}) was determined.

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