Available online at www.sciencedirect.com
SCIENCE DIRECT
Tetrahedron
Tetrahedron 60 (2004) 6015–6019

Cytotoxic sesterterpenes, 6-epi-ophiobolin G and 6-epi-ophiobolin N, from marine derived fungus *Emericella variecolor* GF10

Hong Wei,[a] Takuya Itoh,[a] Masahiro Kinoshita,[a] Yasuhide Nakai,[a] Mineko Kurotaki[b] and Motomasa Kobayashi[a,*]

[a]*Graduate School of Pharmaceutical Sciences, Osaka University, Yamada-oka 1-6, Suita, Osaka 565-0871, Japan*
[b]*R&D Division, Nippon Kayaku Co., Ltd, Shimo 3-31-12, Kitaku, Tokyo 115-8588, Japan*

Received 22 March 2004; accepted 6 May 2004

Available online 5 June 2004

Abstract—Two new sesterterpenes, 6-epi-ophiobolin G (**1**) and 6-epi-ophiobolin N (**3**), and six known ophiobolins were isolated from the extracts of the fungus, *Emericella variecolor* GF10, which was separated from marine sediment. The planar structures of the new compounds were deduced from analysis of the 2D NMR spectra, and the stereochemistry was determined by extensive examination of the NOESY spectrum. Additionally, the configuration of the C-6 proton in ophiobolin G (**2**) was revised from α to β, and the unsolved stereochemistry of ophiobolin H (**4**) was determined by its physicochemical evidence and the chemical correlation with ophiobolin K (**8**). Ophiobolin K (**8**) showed cytotoxic activity against various tumor cell lines, including adriamycin-resistant mouse leukemia cells (P388), with IC_{50} of 0.27–0.65 μM.

1. Introduction

Ophiobolins are a group of naturally occurring sesterterpenes with an unusual tricyclic or tetracyclic structure showing a broad spectrum of inhibitory activity against nematodes, fungi, and bacteria, and cytotoxic activity against cancer cells.[1] Ophiobolin A (**9**) is the first known member of this family, and its absolute structure was determined by X-ray crystallography of the bromo-methoxy derivative.[2] This compound was reported to inhibit calmodulin-activated cyclic nucleotide phosphodiesterase[3] and induce apoptotic cell death in the L1210 cell line.[4] Although the mechanisms of these activities remain unclear, these findings imply ophiobolin's potential for biological and pharmaceutical uses.

During our search for bioactive substances from marine microorganisms, we previously reported a novel anthracycline, komodoquinone A, from the solid-state fermentation of a marine *Streptomyces* sp. KS3.[5,6] Further study led us to the isolation of two new ophiobolins, 6-epi-ophiobolin G (**1**) and 6-epi-ophiobolin N (**3**), and six known ophiobolins, ophiobolin G (**2**),[7] ophiobolin H (**4**),[7] 6-epi-ophiobolin C (**5**),[8] ophiobolin C (**6**),[8,9] 6-epi-ophiobolin K (**7**),[10] and ophiobolin K (**8**)[10] from the culture broth of the marine derived fungus, *Emericella variecolor* GF10. This paper presents the isolation of these compounds and the structural elucidation of 6-epi-ophiobolin G (**1**), ophiobolin G (**2**), 6-epi-ophiobolin N (**3**), and ophiobolin H (**4**).

2. Results and discussion

The fungus strain of *E. variecolor* GF10 was separated from marine sediment collected at 70 m depth in the Gokasyo Gulf, Mie Prefecture, Japan. The GF10 strain was cultured at 30 °C for 2 weeks in MG medium (malt extract 20 g, glucose 20 g, bact peptone 1 g, artificial seawater 1000 mL) or barley solid medium (barley 15 g, artificial seawater 25 mL). The EtOAc soluble portion of the 2-butanone extracts of these cultures were fractionated by silica gel column chromatography and purified by reversed-phase HPLC to obtain two new sesterterpenes named 6-epi-ophiobolin G (**1**) and 6-epi-ophiobolin N (**3**) together with six known sesterterpenes. The structures of the six known sesterterpenes were identified as ophiobolin G (**2**),[7] ophiobolin H (**4**),[7] 6-epi-ophiobolin C (**5**),[8] ophiobolin C (**6**),[8,9] 6-epi-ophiobolin K (**7**),[10] and ophiobolin K (**8**)[10] by comparison of the MS and NMR data with those of the authentic compounds.

The molecular formula of compound **1** was determined as $C_{25}H_{34}O_2$ by HRFABMS in conjunction with NMR analysis. The ^1H NMR spectrum of **1** showed the signals

Keywords: Ophiobolin; Marine fungus; *Emericella variecolor*; Cytotoxic; Sesterterpene.
* Corresponding author. Tel.: +81-6-6879-8215; fax: +81-6-6879-8219; e-mail address: kobayasi@phs.osaka-u.ac.jp

0040–4020/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2004.05.021

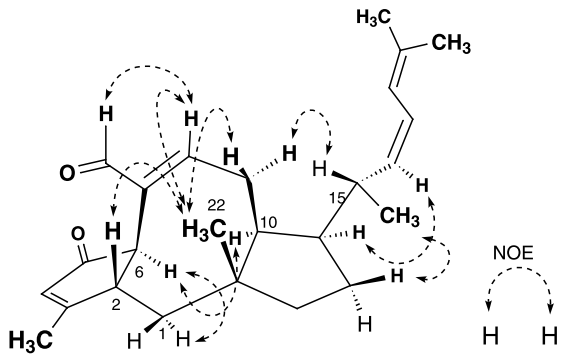


Figure 1. Key NOE correlations of 6-epi-ophiobolin G (**1**).

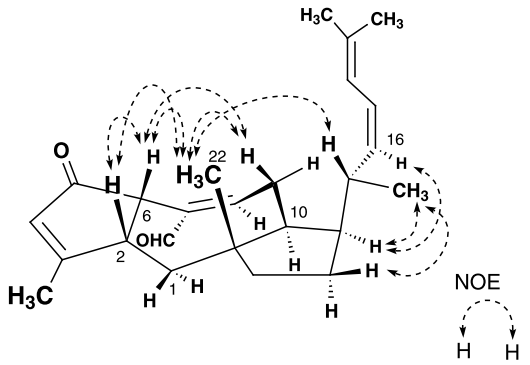


Figure 2. Key NOE correlations of ophiobolin G (**2**).

due to one aldehyde proton, five olefinic protons, four singlet methyl protons (three for vinylic methyls and one for angular methyl), and one doublet methyl protons. Detailed interpretation of a combination of $^1H-^1H$ COSY, HMQC, and HMBC spectral data of **1** revealed that **1** is a sesterterpene having a tricyclic ophiobolane skeleton,[1] which consists of one each of ketone and aldehyde, eight olefinic carbons (five of them are carbons bearing a proton, and other three are quaternary carbons), one quaternary carbon, five methine carbons, four methylene carbons, and five methyl carbons. The ^1H and ^{13}C NMR data of compound **1** closely resembled those of ophiobolin G (**2**). These evidences indicated that compound **1** is a stereoisomer of ophiobolin G (**2**). The stereostructure of **1** was elucidated by detailed analysis of the NOESY spectra of **1** and **2**. Thus, the NOESY spectrum of **1** showed the NOE correlations from H-6 (δ_H 3.38, brs) to H-10 (δ_H 2.61, m) and H-1α (δ_H 1.15, t); H_3-22 (δ_H 0.85, s) to H-2 (δ_H 2.65, m), H-8 (δ_H 6.79, d), and H-9β (δ_H 2.20, m), and the lack of NOE from H-6 to H-2 gave a clear indication that the A/B ring is *trans*-fused and the H-2 proton is on the same side with the C-22 methyl group (Fig. 1). On the other hand, the A/B-*cis* ring structure in ophiobolin G (**2**) was deduced from the strong NOESY correlation between H-2 (δ_H 3.11, brs) and H-6 (δ_H 4.16, brs) (Fig. 2). These evidences indicated that compound **1** is the 6-epi isomer of ophiobolin G (**2**). It has been reported that the C-1 carbon of ophiobolin having an A/B-*cis* ring structure resonates at higher field in comparison with the A/B-*trans* ophiobolin[11] and the proton signal at C-2 of the 6-epi isomer having H-6α is shielded by ca. 0.2–0.3 ppm in comparison with the A/B-*cis* isomer having H-6β.[12] These phenomena were also observed in this study (Tables 1 and 2); thus, the C-1 carbon and the H-2 proton of compound **1** and ophiobolin G (**2**) were observed at δ_C 46.1 and δ_C 35.7; δ_H 2.65 (m) and δ_H 3.11 (brs), respectively. The orientation of H-6 in ophiobolin G (**2**) has been previously reported to be α. Based on the above findings, the orientation of the H-6 proton in ophiobolin G

Table 1. ^1H NMR data for 6-epi-ophiobolin G (**1**), ophiobolin G (**2**), 6-epi-ophiobolin N (**3**), ophiobolin H (**4**), 6-epi-ophiobolin C (**5**), ophiobolin C (**6**), 6-epi-ophiobolin K (**7**), and ophiobolin K (**8**). (600 MHz in $CDCl_3$, δ_H (mult., *J* (Hz)))

	1	2	3	4	5	6	7	8
1α	1.15 (t, 13.2)	1.34 m	1.16 (t, 13.0)	1.42 m	1.56 m	1.23 m	1.51 m	1.21 m
1β	2.03 m	1.89 m	2.04 m	1.52 m	1.76 m	1.78 m	1.83 m	1.77 m
2	2.65 m	3.11 brs	2.68 m	2.24 m	2.15 m	2.36 m	2.13 m	2.36 m
4	6.02 s	6.05 s	6.04 s	2.06 (d, 13.5)	2.42 (d, 16.5)	2.47 (d, 20.1)	2.44 (d, 16.4)	2.51 (d, 18.9)
				2.15 (d, 13.5)	3.08 (d, 16.5)	2.77 (d, 20.1)	3.04 (d, 16.4)	2.78 (d, 18.9)
6	3.38 brs	4.16 brs	3.45 (d, 4.3)	3.15 m	3.35 (d, 10.3)	3.24 (d, 9.6)	3.24 (d, 11.0)	3.26 (d, 10.3)
8	6.79 (d, 6.1)	7.06 m	6.84 (d, 4.4)	5.62 m	6.89 (d, 5.5)	7.18 (t, 8.5)	6.80 (d, 4.9)	7.11 (t, 8.5)
9α	2.92 (d, 20.6)	2.88 (d, 19.1)	2.71 m	2.46 m	2.61 m	2.28 m	2.79 m	2.11 m
9β	2.20 m	2.30 m	2.23 m	1.69 m	2.21 m	2.42 m	2.36 (d, 17.1)	2.95 m
10	2.61 m	1.90 m	2.71 m	1.59 m	2.61 m	2.63 m	2.49 m	1.59 m
12	1.44 m	1.34 m	1.43 m	1.36 m	1.47 m	1.39 m	1.40 m	1.40 m
	1.52 m	1.40 m	1.51 m	1.42 m	1.47 m	1.43 m	1.45 m	1.40 m
13α	1.28 m	1.27 m	1.25 m	1.27 m	1.16 m	1.43 m	1.18 m	1.25 m
13β	1.67 m	1.66 m	1.61 m	1.73 m	1.56 m	1.52 m	1.61 m	1.61 m
14	1.88 m	1.83 m	1.74 m	2.06 m	1.76 m	2.36 m	1.82 m	2.07 m
15	2.58 m	2.51 m	1.43 m	2.67 m	1.47 m	1.62 m	2.47 m	2.71 m
16	5.10 (t, 10.5)	5.11 m	0.99 m	5.19 m	0.98 m	1.15 m	5.06 (t, 11.2)	5.18 (t, 9.7)
			1.43 m		1.47 m	1.23 m		
17	6.09 (t, 10.5)	5.98 m	1.93 m	5.98 m	1.91 m	1.95 m	6.02 (t, 11.2)	6.01 m
			1.93 m		2.07 m	2.07 m		
18	6.00 (d, 10.5)	5.98 m	5.12 (t, 6.9)	5.98 m	5.12 (t, 6.9)	5.07 (t, 6.9)	5.95 (d, 11.2)	5.97 m
20	2.06 s	2.21 s	2.07 s	1.22 s	1.44 s	1.33 s	1.37 s	1.34 s
21	9.27 s	9.38 s	9.31 s	4.59 (d, 12.1)	9.20 s	9.20 s	9.09 s	9.21 s
				4.78 (d, 12.1)				
22	0.85 s	0.78 s	0.86 s	0.89 s	0.84 s	0.88 s	0.76 s	0.95 s
23	0.96 (d, 6.6)	0.86 (d, 6.6)	0.90 (d, 6.6)	0.87 (d, 6.6)	0.89 (d, 6.6)	0.76 (d, 6.6)	0.91 (d, 6.6)	0.92 (d, 6.6)
24	1.76 s	1.74 s	1.61 s	1.72 s	1.60 s	1.58 s	1.69 s	1.74 s
25	1.82 s	1.82 s	1.69 s	1.80 s	1.69 s	1.66 s	1.76 s	1.81 s

Table 2. ^{13}C NMR data for 6-epi-ophiobolin G (**1**), ophiobolin G (**2**), 6-epi-ophiobolin N (**3**), ophiobolin H (**4**), 6-epi-ophiobolin C (**5**), ophiobolin C (**6**), 6-epi-ophiobolin K (**7**), and ophiobolin K (**8**) (150 MHz in $CDCl_3$, δ_C)

	1	2	3	4	5	6	7	8
1	46.1	35.7	45.8	35.9	41.6	36.1	41.4	35.1
2	49.3	48.8	49.0	50.9	49.7	50.9	49.6	50.3
3	177.6	177.4	177.5	80.0	76.7	76.8	76.7	76.9
4	130.5	130.7	130.2	50.8	55.2	54.8	55.0	54.9
5	207.7	207.1	207.4	116.5	217.1	217.4	216.9	217.2
6	50.2	48.3	50.0	52.8	49.1	48.5	48.9	48.6
7	140.2	137.7	140.4	138.8	142.0	141.5	141.4	141.4
8	158.1	160.3	156.9	123.3	159.8	164	160.5	163.9
9	31.1	29.6	31.0	24.9	31.2	24.8	30.8	25.5
10	44.0	46.4	43.1	55.0	43.3	53.5	43.8	53.6
11	45.6	46.0	45.0	43.5	44.6	43.9	44.7	43.9
12	44.5	40.3	44.6	42.9	45.7	42.6	45.7	42.6
13	27.9	27.8	27.1	26.6	27.4	22.9	27.7	26.5
14	52.3	46.4	51.5	47.1	51.8	45.3	52.1	47.2
15	32.9	35.3	31.8	35.5	32.2	32.8	32.6	35.3
16	135.9	137.4	37.2	137.9	37.2	37	135.6	137.1
17	124.2	122.3	25.7	121.7	26.0	26.0	124.0	122.5
18	120.1	120.2	124.4	120.3	124.7	124.5	120.0	120.0
19	136.7	135.7	131.6	135.0	131.7	131.4	136.4	136.1
20	17.4	18.7	17.2	25.6	26.1	25.5	25.9	25.7
21	193.2	194.9	193.0	71.5	194.5	196.2	194.7	196.2
22	23.1	24.5	23.1	18.7	23.7	19.0	23.3	18.7
23	21.4	20.6	18.6	20.2	18.9	16.5	21.3	20.4
24	18.2	18.1	17.7	18.1	18.0	17.6	18.2	18.1
25	26.7	26.4	25.7	26.4	25.9	25.7	26.5	26.6

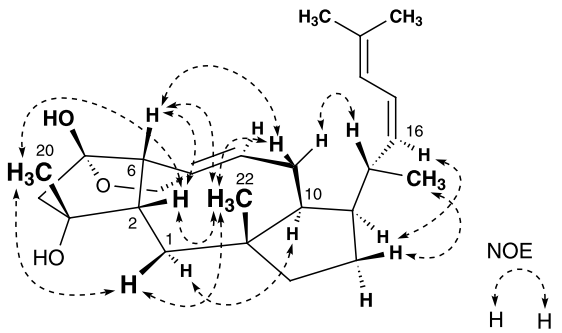
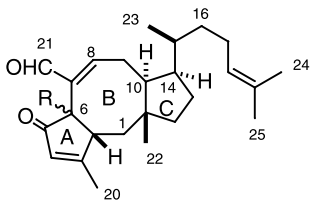


Figure 3. Key NOE correlations of ophiobolin H (**4**).

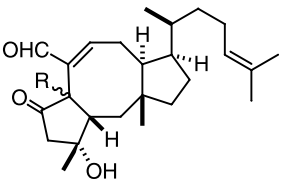
(**2**) should be revised to be β and compound **1** should be 6-epi-ophiobolin G having H-6α.

Compound **3** named 6-epi-ophiobolin N has a molecular formula of $C_{25}H_{36}O_2$ as determined by HRFABMS. The proton and carbon signals ascribable to the A and B rings in **3** were closely similar to those of **1**, while other signals were almost identical to those of 6-epi-ophiobolin C (**5**) (Tables 1 and 2). This suggested that **3** has a hybrid structure of **1** and **5**. NOE correlations from H-6 (δ_H 3.45, d) to H-10 (δ_H 2.71, m) and H-1α (δ_H 1.16, t); H_3-22 (δ_H 0.86, s) to H-1β (δ_H 2.04, m), H-2 (δ_H 2.68, m), H-8 (δ_H 6.84, d), and H-9β (δ_H 2.23, m) in the NOESY spectrum of **3** indicated that **1** and **3** share the same stereostructure. Consequently, the structure of compound **3**, 6-epi-ophiobolin N, was clarified to be a 16,17-dihydro analogue of 6-epi-ophiobolin G (**1**), and also the 6-epi isomer of the previously reported congener named anhydrozizanin A.[8]

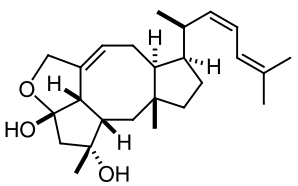
The physical data of compound **4** were identical with those of ophiobolin H.[7] The unsolved stereochemistry of ophiobolin H (**4**) led us to do further structural examination of this compound. The NOESY spectrum of **4** exhibited NOE correlations from H-6 (δ_H 3.15, m) to H-2 (δ_H 2.24, m), H-9β (δ_H 1.69, m), and H_3-22 (δ_H 0.89, s); H-10 (δ_H 1.59, m) to H-1α (δ_H 1.42, m) and H-14 (δ_H 2.06, m) indicating a *cis* fusion of the ring A/B. Further observation of the NOE correlations from H_3-20 (δ_H 1.22, s) to H-2 (δ_H 2.24, m) and H-1β (δ_H 1.52, m) suggested that the methyl group at C-3 is β-orientation (Fig. 3). This was corroborated by the fact that the reduction of ophiobolin K (**8**)[10] with $CeCl_3/6H_2O$ and $NaBH_4$ afforded a single product, which was identical with ophiobolin H (**4**) on the basis of HPLC, TLC, ^1H NMR, and HRFABMS comparison. Furthermore, the orientation of the hydroxyl group at C-5 could be deduced as β, since the stereostructure having 5β-hydroxyl group, which was shown in Figure 3, is only reasonable one to be able to explain the presence of the above NOE correlations. Based on these findings, the unsolved



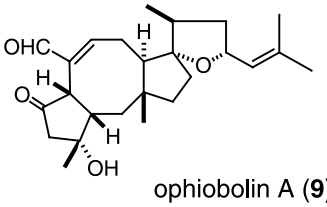
R = α-H, 16-*cis* 6-epi-ophiobolin G (**1**)
R = ß-H, 16-*cis* ophiobolin G (**2**)
R = α-H 6-epi-ophiobolin N (**3**)



R = α-H 6-epi-ophiobolin C (**5**)
R = ß-H ophiobolin C (**6**)
R = α-H, 16-*cis* 6-epi-ophiobolin K (**7**)
R = ß-H, 16-*cis* ophiobolin K (**8**)



ophiobolin H (**4**)



ophiobolin A (**9**)

Chart 1.

Table 3. Cytotoxic activity of ophiobolin K (**8**) against various cultured tumor cells

Compound	IC_{50} value (μM)							
	T-47D	MDA-MB-231	HOP18	NCI-H460	HCT116	ACHN	P388	P388/ADR[a]
Adriamycin	0.048	0.095	0.11	0.0061	0.055	0.048	0.012	2.56
Ophiobolin K (**8**)	0.35	0.57	0.65	0.57	0.33	0.27	0.51	0.36

[a] Adriamycin-resistant cells.

stereochemistry at C-3, C-5, and C-6 of ophiobolin H (**4**) was determined to be as depicted in Chart 1.

The compounds obtained here are well correlated, and all can be considered as congeners of ophiobolin A (**9**). The stereochemistry at C-14 and C-15 and the absolute stereostructure were deduced from those of ophiobolin A,[2,11] whose absolute stereostructure was determined by X-ray crystallography of its bromo-methoxy derivative, and ophiobolin C,[9] of which asymmetric total synthesis has been accomplished.

These compounds showed cytotoxicity against the neuroblastoma cell line, Neuro 2A. The treatment of 1–3 μM of these compounds induced cell death accompanied by shrinkage in cell soma and chromatin condensation at 12 or 24 h after drug application. Ophiobolin K (**8**) was further tested with various cultured cell lines. As showed in Table 3, **8** showed seven times stronger cytotoxic activities against P388/ADR tumor cells than adriamycin.

The liquid culture of the GF10 strain in the MG medium produced ophiobolins in poor yield (0.1–0.6 mg/L for compounds **1**–**4**, 2–3 mg/L for compounds **5**–**8**). On the other hand, the culture in the solid-state medium based on cereals produced ophioblins in higher yield. In the case of the rice medium or soybean medium, 0.5–1.5 mg of **1**–**4** and 5–10 mg of **5**–**8** were produced in both 100 g medium, while in the cases of the barley medium, corn medium, or potato medium, 1–5 mg of **1**–**4** and 10–30 mg of **5**–**8** were produced in each 100 g medium, respectively.

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded on a Varian Unity Inova 600 (600 MHz) spectrometer using the solvent peak as the internal standard. Spots on TLC were detected by spraying 1% $Ce(SO_4)_2$/10% H_2SO_4 [1 g $Ce(SO_4)_2$, 100 mL 10% aq. H_2SO_4] with subsequent heating. Artificial seawater was prepared by Aquamarine (Yashima Pure Chemical Co. LTD, Japan). Other instruments used to obtain physical data and the experimental conditions for chromatography were the same as in our previous paper.[5]

3.2. Fungus material, culture conditions, and extraction

The *E. variecolor* GF10 strain was separated from the marine sediment collected from a depth at 70 m in Gokasyo Gulf, Mie Prefecture, Japan, in 2002 and deposited in our laboratory. The GF10 strain was classified as *E. variecolor* from its cultural characteristics and 16S rDNA sequence. MG medium (malt extract: 20 g, glucose: 20 g, bact peptone: 1 g, artificial seawater: 1000 mL) was used as seed medium and liquid medium. Rice solid medium (rice: 25 g, artificial seawater: 50 mL, in a 500 mL flask), barley solid medium (barley: 15 g, artificial seawater: 25 mL, in a 500 mL flask), soybean solid medium (soybean: 50 g, artificial seawater: 75 mL, in a 500 mL flask), corn solid medium (canned corn: 100 g, solid Aquamarine: 1.4 g, liquid Aquamarine: 1 mL, in a 500 mL flask), and potato solid medium (sliced fresh potato: 100 g, solid Aquamarine: 1.4 g, liquid Aquamarine: 1 mL, in a 500 mL flask) were used as solid medium. They were all autoclaved before use. The GF10 strain was cultured in the seed medium at 30 °C for 5 days. Then, the broth of the strain was inoculated into the production medium and cultured under static conditions at 30 °C for 2 weeks. The culture of the MG medium was filtered, and then the filtrate was partitioned with 2-butanone, and the residue was extracted with acetone. The organic extracts were combined and evaporated under reduced pressure to give an extract, which was further partitioned into an EtOAc–H_2O mixture. The EtOAc layer was evaporated under reduced pressure to give an EtOAc extract. For solid-state fermentation, the culture was extracted with acetone and a mixed solvent (EtOAc–MeOH–acetone, 1:2:4), and then the organic solvent was combined and evaporated under reduced pressure to give an extract. The extract was partitioned into an EtOAc–H_2O mixture, and the EtOAc layer was evaporated under reduced pressure to afford an EtOAc extract.

3.3. Isolation of ophiobolins (1–8)

The EtOAc extract (4.5 g) of the MG medium culture (1 L×10) was fractionated by SiO_2 column chromatography (*n*-hexane–EtOAc) to give five fractions (A–E). The active fraction C (70 mg) was further separated by reversed-phase HPLC (Cosmosil 5C18-AR, 10×250 mm, MeOH–H_2O=85:15) to furnish 6-epi-ophiobolin K (**7**, 12 mg), ophiobolin K (**8**, 11 mg), 6-epi-ophiobolin C (**5**, 9 mg), 6-epi-ophiobolin G (**1**, 2 mg), 6-epi-ophiobolin C (**6**, 10 mg), and 6-epi-ophiobolin N (**3**, 3 mg). The purification of the EtOAc extract (3.4 g) of the barley solid medium culture (barley 40 g×20) by the same procedure gave ophiobolin G (**2**, 15 mg), 6-epi-ophiobolin G (**1**, 9 mg), ophiobolin H (**4**, 8 mg), and 6-epi-ophiobolin N (**3**, 9 mg). For quantitative analyses, the EtOAc extracts were fractionated by SiO_2 column chromatography (*n*-hexane–EtOAc) and the fractions containing ophiobolins were analyzed by HPLC (Cosmosil 5C18-AR, 10×250 mm, MeOH–H_2O=80:20, UV 230 nm).

3.3.1. 6-epi-Ophiobolin G (1). Amorphous powder; $[\alpha]_D^{23}$=+117° (*c* 1.05, MeOH); IR (KBr) ν_{max} cm^{-1}: 2965, 1705, 1680; UV ($CHCl_3$) λ_{max} (ε): 228 nm (27000); ^1H and

^{13}C NMR data: shown in Tables 1 and 2; FABMS: *m/z* 389 [(M+Na)$^+$]; HRFABMS: found *m/z* 389.2466. Calcd for $C_{25}H_{34}O_2Na$: 389.2456.

3.3.2. Ophiobolin G (2). Amorphous powder; $[\alpha]_D^{23}$=+26° (*c* 0.88, MeOH); UV ($CHCl_3$) λ_{max} (ε): 227 nm (29700); ^{1}H and ^{13}C NMR: data shown in Tables 1 and 2; FABMS: *m/z* 367 [(M+H)$^+$]; HRFABMS: found *m/z* 367.2642. Calcd for $C_{25}H_{35}O_2$: 367.2637.

3.3.3. 6-epi-Ophiobolin N (3). Amorphous powder; $[\alpha]_D^{23}$=+88° (*c* 0.34, MeOH); IR (KBr) ν_{max} cm^{-1}: 2970, 1702, 1670; UV ($CHCl_3$) λ_{max} (ε): 226 nm (19000); ^{1}H and ^{13}C NMR: data shown in Tables 1 and 2; FABMS: *m/z* 391 [(M+Na)$^+$]; HRFABMS: found *m/z* 391.2634. Calcd for $C_{25}H_{36}O_2Na$: 391.2613.

3.3.4. Ophiobolin H (4). Amorphous powder; $[\alpha]_D^{23}$=+44° (*c* 0.12, MeOH); UV ($CHCl_3$) λ_{max} (ε): 240 nm (16800); ^{1}H and ^{13}C NMR: data shown in Tables 1 and 2; FABMS: *m/z* 409 [(M+Na)$^+$]; HRFABMS: found *m/z* 409.2717. Calcd for $C_{25}H_{38}O_3Na$: 409.2719.

3.4. Reduction of ophiobolin K (8)

A solution of **8** (1 mg) in EtOH (0.2 mL) was treated with $CeCl_3/6H_2O$ (2 mg) and $NaBH_4$ (2 mg), and the mixture was stirred at 0 °C for 0.5 h. The reaction mixture was diluted with 5 mL of mixed solvent (*n*-hexane–EtOAc=2:1) and then filtered with a silica gel pad. The filtrate was evaporated under reduced pressure, and the resulting residue was purified by HPLC (Cosmosil 5SL, 10×250 mm, *n*-hexane–EtOAc, 4:1) to obtain a product (0.8 mg), which was identified with ophiobolin H (**4**) by HPLC, TLC, ^{1}H NMR, and HRFABMS.

3.5. Assay for activity in Neuro 2A cells

Neuro 2A cells were grown in Dulbecco's modified essential medium (DMEM) with 10% fetal bovine serum (FBS). The cells were kept in incubator at 37 °C with 5% CO_2. The cells were plated on 24-well plates at a density of 2×10^4 per well with 1 mL of culture medium. After 24 h cultivation, the medium was exchanged for fresh medium, and the testing sample in 10 μL of EtOH was added to each well. After 12 or 24 h incubation, morphological changes in the cells were observed under microscope.

3.6. Assay for cytotoxic activity

NCI-H460, HOP18 (human lung carcinoma), MDA-MB-231, T-47D (human breast carcinoma), ACHN (human 'renal carcinoma), HCT116 (human colon carcinoma), P388 (mouse leukemia cells) and P388/ADR (adriamycin resistant cells) were cultured in RPMI-1640 medium supplemented with 10% FBS. All cells were maintained at 37 °C with 5% CO_2. Cells were seeded into 96-well plates (1×10^4 cells/well) and incubated for 24 h. The test sample, dissolved in DMSO, was added in serial dilutions and the cells were further incubated for 72 h. In vitro cytotoxic activity was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay or WST-1 [5-(2,4-disulfophenyl)-2-(4-iodophenyl)-2H-tetrazolium, inner salt, sodium salt] assay.

Acknowledgements

This study was financially supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

References and notes

1. Au, T. K.; Chick, W. S. H.; Leung, P. C. *Life Sci.* **2000**, *67*, 733–742.
2. Morisaki, M.; Nozoe, S.; Iitaka, Y. *Acta Crystallogr.* **1968**, *B24*, 1293–1302.
3. Leung, P. C.; Taylor, W. A.; Wang, J. H.; Tipton, C. L. *J. Biol. Chem.* **1984**, *259*, 2742–2747.
4. Fujiwara, H.; Matsunaga, K.; Kumagai, H.; Ishizuka, M.; Ohizumi, Y. *Pharm. Pharmacol. Commun.* **2000**, *6*, 427–431.
5. Itoh, T.; Kinoshita, M.; Aoki, S.; Kobayashi, M. *J. Nat. Prod.* **2003**, *66*, 1373–1377.
6. Itoh, T.; Kinoshita, M.; Wei, H.; Kobayashi, M. *Chem. Pharm. Bull.* **2003**, *51*, 1402–1404.
7. Gutler, H. G.; Crumley, F. G.; Cox, R. H.; Springer, J. P.; Arrendale, R. F.; Cole, R. J.; Cole, P. D. *J. Agric. Food Chem.* **1984**, *32*, 778–782.
8. Nozoe, S.; Hirai, K.; Tsuda, K. *Tetrahedron Lett.* **1966**, *20*, 2211–2216.
9. Rowley, M.; Tsukamoto, M.; Kishi, Y. *J. Am. Chem. Soc.* **1989**, *111*, 2735–2737.
10. Singh, S. B.; Smith, J. L.; Sabnis, G. S.; Dombrowski, A. W.; Schaeffer, J. M.; Goetz, M. A.; Bills, G. F. *Tetrahedron* **1991**, *47*, 6931–6938.
11. Li, E.; Clark, A. M.; Rotella, D. P.; Hufford, C. D. *J. Nat. Prod.* **1995**, *58*, 74–81.
12. Tsipouras, A.; Adefarati, A. A.; Tkacz, J. S.; Frazier, E. G.; Rohrer, S. P.; Birzin, E.; Rosegay, A.; Zink, D. L.; Goetz, M. A.; Singh, S. B.; Schaeffer, J. M. *Bioorg. Med. Chem.* **1996**, 531–536.