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Fusaristatins A and B, Two New Cyclic Lipopeptides from an Endophytic *Fusarium* sp.

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Abstract Two new cyclic lipopeptides, fusaristatins A (**1**) and B (**2**) were isolated from rice cultures of a *Fusarium* sp. YG-45 in the course of a screening of endophytic fungi. Their structures of **1** and **2** were determined by spectroscopic methods. **2** showed a moderate inhibitory effect on topoisomerases I (IC_{50}: 73 μM) and II (IC_{50}: 98 μM) without cleavable complexes. Furthermore, **1** and **2** showed the growth-inhibitory activity toward lung cancer cells LU 65 with IC_{50} values of 23 and 7 μM, respectively.

Keywords lipopeptides, *Fusarium* sp., topoisomerase inhibitor, fusaristatins A and B

Introduction

Endophytic fungi living within plant tissues have proven to be a promising source for the production of structurally novel and pharmacologically active secondary metabolites [1]. As a result of our ongoing efforts to identify new compounds from endophytes, new metabolites were reported from strains of *Xylaria* sp. YUA-026 [2, 3] and *Anthracobia* sp.YST-55 [4], which were isolated from unidentified dead branches. In continuing this research, two new cyclic lipopeptides, fusaristatins A (**1**) and B (**2**), were isolated from the extract of a rice culture of an endophytic fungus, *Fusarium* sp. YG-45, which was isolated from the stem of *Maackia chinensis* (common name: Chinese maackia, family: *Leguminosae*) collected in Germany. In this report, we describe the characteristics and fermentation of the producing strain, the isolation, structure elucidation and biological characterization of the two compounds.

Results and Discussion

The producing strain *Fusarium* sp. YG-45 was grown on white rice under static conditions for three weeks and the culture was then extracted with methanol. The organic extract was concentrated and the aqueous residue extracted with ethyl acetate. This extract was subjected to silica gel and Sephadex LH-20 column chromatography to afford two compounds, **1** and **2** (Fig. 1).

1 was obtained as colorless oil. The molecular formula of **1** was determined as $C_{36}H_{58}N_4O_7$ on the basis of HR-FABMS, suggesting the presence of ten double bond equivalents. Absorptions at 1727, 1660 and 1531 cm^{-1} in

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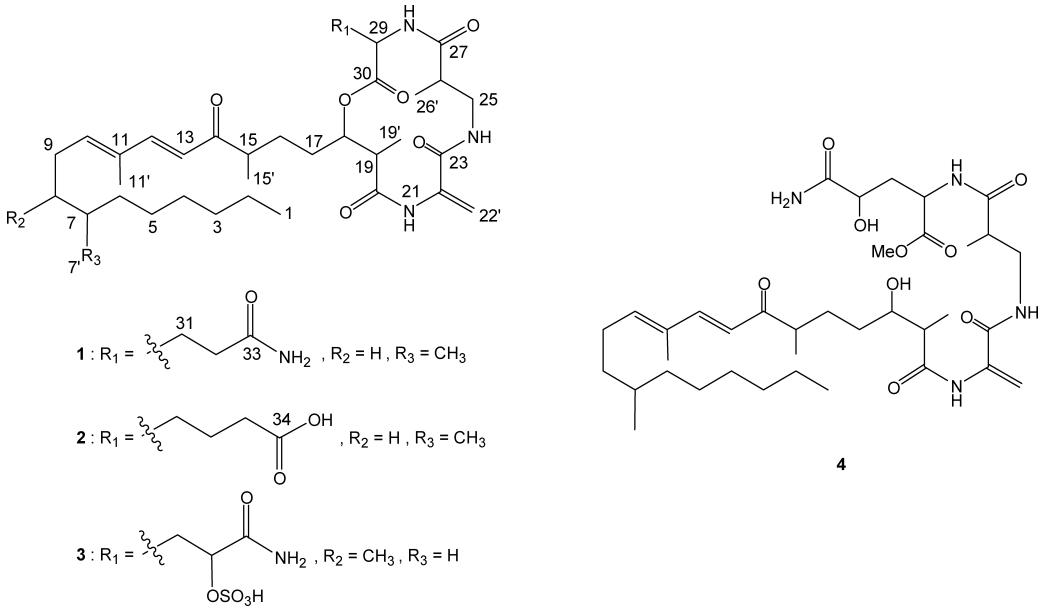

Fig. 1 Structures of fusaristatins A (**1**), B (**2**), topostatin (**3**) and YM-170320 (**4**).

the IR spectrum were characteristic of esters and amide functions. The UV spectrum of **1** revealed an absorption at 285 nm, which suggested a chromophore of conjugated double bonds or a benzene ring in the molecule.

The ^{13}C NMR spectrum (C_5D_5N) showed 36 resolved peaks (Table 1), which were classified into six methyls, twelve sp^3 methylenes, one sp^2 methylene, six sp^3 methines, three sp^2 methines, two sp^2 quaternary carbon atoms, and six carbonyl groups by analysis of the DEPT spectra. The connectivity of carbon and proton atoms was established by the HMQC spectrum. The ^1H NMR spectrum also supported the molecular formula. It displayed 58 proton signals (Table 1), four signals of low-field resonances at δ_H 7.63～7.69 (1H, m), 8.17 (2H, br s), 8.80 (1H, d, J=7.6 Hz) and 10.18 (1H, br s) with no HMQC correlations with any carbon signal, which were assignable to amide protons. The ^1H NMR spectrum of **1** also revealed a large number of aliphatic signals, suggesting the possibility of a lipopeptide-related compound. Analysis of the ^1H,^1H-COSY data revealed five partial structures (I～V, Fig. 2). A combination of HMQC and HMBC experiments established the presence of three amino acids, glutamine, dehydroalanine and β-aminoisobutyric acid. In addition, a β-hydroxy C_{24} fatty acid was derived from the NMR data.

A downfield signal (NH-28) of an amide proton showed a COSY correlation with a methine proton signal at δ_H 5.05 (H-29), which in turn coupled with methylene proton resonances at δ_H 2.54～2.59 (H_2-31). These signals further coupled with methylene signals at δ_H 2.66～2.71 (H_2-32). The methylene protons H_2-31 correlated with three carbons (C-30, C-32 and C-33), and the amide protons NH_2-34 correlated with the methylene carbons C-32 and C-33 in the HMBC spectrum (Table 1), suggesting that fragment V was comprised of a glutamine.

A second amino acid residue was assigned as dehydroalanine by the following evidence. The HMBC correlations of the *exo*-methylenes H_2-22′ andx the amide proton NH-21 to C-23 indicated the *exo*-methylene to be located at C-22.

The last amino acid unit was assigned as β-aminoisobutyric acid by a methyl doublet [δ_H 1.30 (3H, d, J=7.1 Hz, H_3-26′)], which showed HMBC correlations to the β-methylene C-25 and the amide carbonyl carbon C-27. In addition, correlations were visible between the β-methylene protons H_2-25 and the carbonyl carbon C-27 in the HMBC spectrum (Table 1).

The remaining part of the molecule, $C_{24}H_{40}O_3$, was elucidated as an unsaturated fatty acid derivative by the following NMR data. HMBC correlations from H_3-11′ to C-10, C-11 and C-12, suggested a connection between fragments I and II *via* an olefinic carbon C-11. The connections between fragments II and III through C-14 were deduced from HMBC correlations from H-13 to C-15, and H-15′ to C-14, respectively. The (*E*) configurations of two internal double bonds at C-10–C-11 and C-12–C-13 in **1** were determined by the large coupling constant ($J_{12,13}$=15.7 Hz) and NOE signals between H-13 and H_3-11′, and between H-10 and H-12.

Table 1 ^{1}H- and ^{13}C-NMR data of fusaristatin A (**1**)

No.	δ_C	δ_H	HMBC
1	14.2 q	0.85 (3H, t, 7.0)	2, 3
2	22.9 t	1.20~1.33*	
3	32.1 t	1.20~1.33*	
4	27.2 t	1.20~1.33*	
5	29.9 t	1.20~1.33*	
6	37.1 t	1.07~1.11 (1H, m)	
		1.20~1.33*	
7	32.8 d	1.36~1.44*	
7′	19.7 q	0.87 (3H, d, 6.4)	6, 7, 8
8	36.5 t	1.20~1.33*	
		1.36~1.44*	
9	26.9 t	2.13~2.22 (2H, m)	7, 8, 10, 11
10	143.9 d	6.00 (1H, t, 7.2)	8, 12, 11′
11	133.5 s		
11′	12.2 q	1.80 (3H, s)	10, 11, 12
12	147.9 d	7.50 (1H, d, 15.7)	10, 14, 11′
13	123.5 d	6.36 (1H, d, 15.7)	11, 14, 15
14	203.3 s		
15	44.3 d	2.95~3.04 (1H, m)	14, 17
15′	17.2 q	1.09 (3H, d, 6.9)	14, 15, 16
16	28.3 t	1.49~1.61 (1H, m)	15, 15′
		1.88~2.00*	
17	30.1 t	1.79~1.85 (1H, m)	
		1.88~2.00*	
18	77.1 d	5.36~5.41 (1H, m)	16, 20, 30
19	44.2 d	2.80~2.86*	
19′	15.3 q	1.28 (3H, d, 7.1)	19, 20
20	173.2 s		
21-NH		10.18 (1H, br s)	20, 22, 23, 22′
22	138.9 s		
22′	114.1 t	5.58 (1H, s)	22, 23
		6.18 (1H, s)	22, 23
23	164.8 s		
24-NH		7.63~7.69 (1H, m)	23
25	42.5 t	3.70~3.79 (1H, m)	23, 27, 26′
		3.82~3.90 (1H, m)	23, 27, 26′
26	42.3 d	2.80~2.86*	
26′	15.0 q	1.30 (3H, d, 7.1)	25, 26, 27
27	174.5 s		
28-NH		8.80 (1H, d, 7.6)	27, 29
29	53.2 d	5.05 (1H, dd, 14.0, 7.6)	27, 30, 32
30	171.9 s		
31	27.4 t	2.54~2.59 (2H, m)	30, 32, 33
32	32.4 t	2.66~2.71 (2H, m)	29, 33
33	175.4 s		
34-NH_2		8.17 (2H, br s)	32, 33

Measured in C_5D_5N, and values in parentheses are coupling constants in Hz.

* Overlapping signals.

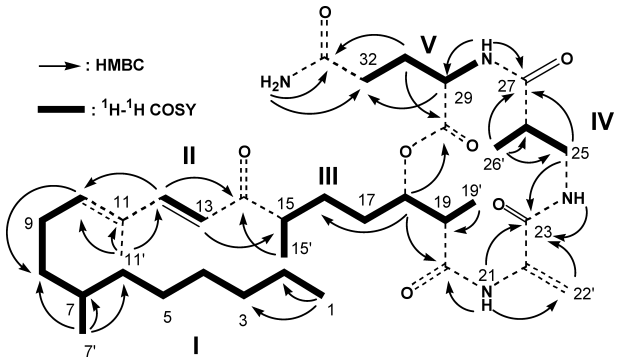

Fig. 2 Selected ^{1}H-^{1}H COSY and HMBC correlations for **1**.

The sequence of the three amino acid residues and the C_{24} unsaturated fatty acid chain unit was determined by HMBC correlations between the amide proton NH-28 of the glutamine and a carbonyl (C-27) of β-aminoisobutyric acid, between the amide proton NH-24 of β-aminoisobutyric acid and a carbonyl (C-23) of dehydroalanine, the amide proton (NH-21) of dehydroalanine and a carbonyl (C-20) of the C_{24} unsaturated fatty acid, and between the oxymethine proton H-18 and the ester carbonyl C-30. Therefore, the structure of **1** was assembled as shown in Fig. 1.

The molecular formula of **2** was elucidated to be $C_{37}H_{59}N_3O_8$ from the HRFABMS. Inspection of ^{1}H and ^{13}C NMR spectra of **2** indicated that most signals in **2** were the same as those of **1** except for the signals corresponding to the glutamine residue (Tables 1 and 2). In the HMBC spectrum (Table 2) of **2**, correlations from H_2-31 to C-30, and H_2-33 to C-31 and C-34 suggested the presence of an α-aminoadipic acid residue instead of glutamine residue in **1**. The unambiguous assignments of the signals in the ^{1}H and ^{13}C NMR spectra of **2** (Fig. 1) were finally based on HMBC experiments.

The antimicrobial activities of **1** and **2** were tested by the agar dilution method. At a concentration of 100 μg/ml, **1** and **2** were inactive against *Staphylococcus aureus* NBRC 13276, *Pseudomonas aeruginosa* ATCC 15442, *Candida albicans* ATCC 2019, and *Aspergillus clavataus* F 318a.

1 and **2** were closely related to topostatin (**3**), a cyclic lipopeptide previously isolated from cultures of *Thermomonospora alba* strain No. 1520 [5]. It was reported that **3** inhibited the activities of DNA topoisomerases I and II, without cleavable complexes, and was a new type of topoisomerase I and II inhibitors having different structure compared with known topoisomerase I and II inhibitors [6, 7].

DNA topoisomerases I and II catalyze the breakage and rejoining of DNA strands to interconvert different topological forms of DNA. Topoisomerases are activated in growing cancer cells, and thus are important targets of anticancer chemotherapeutic agents [8]. Based on the structural analogy with **3**, we investigated also **1** and **2** for their topoisomerases I and II inhibitory activities (Table 3). The inhibitory activities of both compounds on topoisomerase I were observed through relaxation assays with supercoiled plasmid DNA. Camptothecin was used as a positive control (IC_{50}: 17 μM). In the presence of **1** (100 μM), a relaxation effect was not observed. However, we have found a moderate relaxation inhibitory effect of **2** (IC_{50}: 73 μM) on topoisomerase I from calf thymus glands. The inhibitory effects on the human topoisomerase II were evaluated in the decatenation assay using kinetoplast DNA. Etoposide, a known topoisomerase II inhibitor, was used as a positive control (IC_{50} 30 μM). While **1** did not show inhibitory activity against topoisomerase II, **2** presented a weak inhibition of IC_{50} 98 μM (Table 3). **3** exhibited inhibition of topoisomerases I and II (IC_{50}: 17 μM for topoisomerase I and IC_{50}: 4 μM for topoisomerase II), which was stronger than that of the positive control with etoposide. Comparisons of the inhibitory activities of **3** with **1** and **2** suggests that the presence of sulfate ($-OSO_3H$) at the 32-position of **3** may be associated with the potent inhibition of topoisomerases I and II.

Topoisomerase inhibitors of the cleavable complex-forming type such as camptothecin and etoposide stabilize the cleavable complex (topoisomerase-DNA reaction intermediate) and inhibit the DNA rejoining reaction of topoisomerase, which is the inhibitory mechanism of the inhibitors, therefore the inhibitors induce nicked or linearized DNA in the cleavage assay [5, 9, 10]. To determine whether **2** is an inhibitor of the cleavable complex-forming type or not, cleavage assays were carried out. Camptothecin and etoposide were used as the controls of cleavable complex-forming inhibitors against topoisomerases I and II, respectively. Camptothecin or etoposide induced nicked or linerized DNA with increasing concentrations. On the other hand, **2** could not induce nicked or linearized DNA even at 200 μM. These results suggest that **2** is an inhibitor of the cleavable-nonforming type as well as **3** [5]. **2** may directly act on topoisomerase I and II molecules in earlier step than the formation of the topoisomerase-DNA complex and inhibit the DNA breaking and rejoining reactions by the enzymes.

We studied the anti-tumor effects of **1** and **2** on human cancer cells, LU 65 and COLO 201. **1** and **2** inhibited the cell growth activity against lung cancer cells LU 65 with IC_{50} values of 23 μM and 7 μM, respectively. In colon cancer cells COLO 201, both compounds did not have cytotoxic effect at the concentrations up to 100 μM. Further

Table 2 ^{1}H- and ^{13}C-NMR data of fusaristatin B (**2**)

No.	δ_C	δ_H	HMBC
1	14.2 q	0.88 (3H, t, 7.0)	2, 3
2	22.9 t	1.22～1.29*	
3	32.1 t	1.22～1.29*	
4	27.2 t	1.22～1.29*	
5	29.9 t	1.22～1.29*	
7	37.1 t	1.10～1.12 (1H, m)	
		1.22～1.29*	
7	32.7 d	1.40～1.43*	
7′	19.6 q	0.88 (3H, d, 6.4)	6, 7, 8
8	36.4 t	1.22～1.29*	
		1.40～1.43*	
9	26.8 t	2.17～2.22*	
10	144.0 d	6.03 (1H, t, 7.3)	8, 12, 11′
11	133.5 s		
11′	12.2 q	1.82 (3H, s)	10, 11, 12
12	147.8 d	7.53 (1H, d, 15.7)	10, 14, 11′
13	123.1 d	6.38 (1H, d, 15.7)	11, 14, 15
14	203.1 s		
15	44.1 d	3.04 (1H, m)	
15′	17.1 q	1.11 (3H, d, 7.0)	14, 15, 16
16	28.1 t	1.60～1.64 (1H, m)	
		1.96～2.01*	
17	29.7 t	1.81～1.87 (1H, m)	
		1.96～2.01*	
18	76.7 d	5.43～5.46 (1H, m)	16, 20, 30
19	44.1 d	2.86 (1H, m)	17, 19′
19′	15.4 q	1.30 (3H, d, 7.1)	18, 19, 20
20	173.7 s		
21-NH		10.38 (1H, br s)	20, 23, 22′
22	139.2 s		
22′	113.7 t	5.60 (1H, s)	22, 23
		6.18 (1H, s)	22, 23
23	164.8 s		
24-NH		7.78 (1H, t, 5.9)	23, 25
25	42.5 t	3.74 (1H, ddd, 13.7, 7.6, 6.0)	23, 26, 27, 26′
		3.94 (1H, ddd, 13.7, 6.2, 4.2)	23, 26, 27, 26′
26	42.4 d	2.89 (1H, m)	
26′	15.3 q	1.34 (3H, d, 7.2)	
27	174.5 s		
28-NH		8.69 (1H, br s)	27, 29
29	52.9 d	5.02～5.06 (1H, m)	27, 30, 31, 32
30	171.9 s		
31	31.1 t	2.17～2.22*	
		2.26～2.29 (1H, m)	30, 32
32	22.1 t	1.96～2.01*	
		2.04～2.10 (1H, m)	34
33	34.4 t	2.53～2.59 (2H, m)	31, 32, 34
34	175.6 s		

Measured in C_5D_5N, and values in parentheses are coupling constants in Hz.
* Overlapping signals.

Table 3 Inhibitory activities of **1**, **2**, **3**, camptothecin and etoposide against topoisomerases I and II (IC_{50}, μM)

Compound	Topoisomerase I		Topoisomerase II
	Calf thymus glands	Human placenta	Human placenta
1	>100	>100	>100
2	73	>100	98
3	17	—	4
Camptothecin	17	17	>100
Etoposide	>100	>100	30

study for the mechanism of the action by **2** could be needed to clarify the correlations between growth inhibitory activity in tumor cells LU 65 and the inhibitory activity of topoisomerases I and II *in vitro*.

An unidentified fungal strain YL-03706F produces compound YM-170320 (**4**) (Fig. 1) [11], which is an acyclic analogue of **1** and **3**. Interestingly, whereas **4** induced moderate morphological changes of a *Candida tropicalis* mutant, YL-03706F has no inhibitory activity against filamentous fungi. In addition, the strain YL-03706F has not previously been reported to produce a cyclic congener, and furthermore, an acyclic analogue such as **4** has not been isolated so far from the culture broth of **3** producing strains. However, in the biogenesis of the cyclic compounds **1**, **2** and **3**, an acyclic form such as **4** could be a precursor. In order to clarify the biosynthetic pathway, we are now further studying these new fusaristatin analogues.

Experimental

General

Optical rotations were measured with a Horiba model SEPA-300 polarimeter, whereas IR and UV spectra were recorded with JASCO J-20A and Shimadzu UV mini-1240 spectrophotometer, respectively. Mass spectra were obtained using a Finngan MAT 95 spectrometer, and ^{1}H and ^{13}C NMR spectra were acquired with a Varian Unity 400 spectrometer, at 400/100 MHz, respectively. Chemical shifts are given on a δ (ppm) scale with TMS as an internal standard. Column chromatography was conducted on silica gel 60 (Merck) and Sephadex LH-20 (GE Healthcare). TLC was carried out using precoated silica gel plates (0.5 mm; Merck and Macherey-Nagel & Co.), and spots were detected by spraying with 10% vanillin in H_2SO_4 followed by heating, or by UV irradiation. Topoisomerase I from the calf thymus gland and supercoiled pBR322 DNA from *Escherichia coli* were purchased from MBI Fermentas. Topoisomerase II from human placenta and kinetoplast DNA from *Crithidia fasciculata* were purchased from TopoGEN. Camptothecin and etoposide were obtained from Aldrich and Calbiochem, respectively.

Isolation of the Producing Strain

The fungal strain YG-45 was isolated from the stem of *Maackia chinensis* collected in Oct. 2005 in the botanical garden in Göttingen, Germany. A twig segment from *Maackia chinensis* was surface sterilized by successively submersion in 70% EtOH for 1 minute, 5.0% sodium hypochlorite for 5 minutes and 70% EtOH for 1 minute, and then rinsed twice with sterile water. The sterilized samples were dried on sterilized paper and cut into 1-cm pieces. The pieces were placed on plates of potato dextrose agar (PDA) containing chloramphenicol (100 mg/l). Successive subculturing of the outgrowing fungi resulted in a pure culture initially coded YG-45. This strain was identified as a *Fusarium* species by Centraalbureau voor Schimmelcultures (The Netherlands). The strain *Fusarium* sp. YG-45 has been deposited at the laboratory of the Faculty of Agriculture, Yamagata University, Yamagata, Japan.

Fermentation, Extraction and Isolation

The fungal strain YG-45 was cultivated on sterilized rice (total 400 g, 40 g/500-ml Erlenmeyer flask ×10) at 25°C for 3 weeks. The moldy rice was extracted MeOH, and the MeOH extract was concentrated. The resulting aqueous phase was extracted first with *n*-hexane and then EtOAc. The EtOAc extract (5.1 g) was chromatographed on a silica gel column using a stepwise gradient starting from 10% EtOAc in hexane to 100% EtOAc. Purification of the eluates was monitored by the characteristic intense blue coloration with 10% vanillin in H_2SO_4 on TLC plates. The 100% EtOAc fraction (50.4 mg) was further subjected to

Sephadex LH-20 column chromatography by eluting with MeOH to afford fractions 1 through 15. Fraction 4 (22.4 mg) was chromatographed on a silica gel column ($CHCl_3$ - MeOH) to yield **1** (7.2 mg) and **2** (2.2 mg).

1: oil; $[\alpha]_D^{20}$ −35° (*c* 0.11, MeOH); UV λ_{max}^{MeOH} (ε) 285 (25,000); IR ν_{max} (KBr) cm^{-1} 3382, 2927, 1727, 1660, 1531, 1238, 1172; FAB-MS: 659 $[M+H]^+$. HR-FAB-MS: 659.4378 ($[M+H]^+$, $C_{37}H_{59}N_4O_7$, calcd. 659.4385).

2: oil; $[\alpha]_D^{20}$ −37° (*c* 0.12, MeOH); UV λ_{max}^{MeOH} (ε) 285 (25,000); IR ν_{max} (KBr) cm^{-1} 3417, 2927, 1733, 1658, 1531, 1205; FAB-MS: 674 $[M+H]^+$. HR-FAB-MS: 674.4375 ($[M+H]^+$, $C_{36}H_{60}N_3O_8$, calcd. 674.4380).

Relaxation and Cleavage Assays of Topoisomerase I

The activity of compounds on the relaxation of DNA topoisomerase I (human plasenta) was determined by the measuring the conversion of the supercoiled pBR322 DNA to relaxed form, as described previously [5]. Briefly, each reaction mixture has a total volume of 20 μl containing 1 unit topisomerase I (20 units for the cleavage assay) and supercoiled pBR 322 DNA (0.4 μg). First, 10×buffer (2.0 μl) [0.5 M Tris-HCl, (pH 7.5), 1.2 M KCl, 0.1 M $MgCl_2$, 5 mM EDTA, and 5 mM DTT], 0.6 μg BSA, test compounds with different concentrations, 1 unit of topoisomerase I (0.05 μl), and 0.15 μg pBR 322 DNA were diluted with ultrapure water to keep the total volume for 20 μl. Then, this mixture was incubated at 37°C for 40 minutes and terminated by adding 5.0 μl loading buffer [200 mM Tris (pH 7.5), 200 mM boric acid, 5 mM EDTA (pH 7.5), 50% glycerol, and 10% bromophenol blue]. 15 μl of the mixture was analyzed on a 1.0% agarose-gel by running at 50 V for 1 hour in TBE buffer [100 mM Tris-borate buffer (pH 8.5) containing 2.5 mM EDTA]. After electrophoresis, gels were dyed in EtBr solution (0.5% mg/ml ethidium bromide) for 30 minutes and the supercoiled pBR322 DNA on the gel was measured by a densitometer. For the cleavage assay [5, 10], the reaction mixture was terminated by the addition of 5.0 μl of the stop solution containing 5.0% SDS and 12.5 μg proteinase K, thereafter incubated for an additional 30 minutes at 37°C. Loading buffer was added and the mixture was run into 1.0% agarose gel containing EtBr (0.5 μg/ml) at 50 V for 2 hours. After agarose gel electrophoresis, the nicked pBR322 DNA on the gel was measured by a densitometer.

Decatenation and Cleavage Assays of Topoisomerase II

The inhibitory activities of the compounds with DNA topoisomerase II was assessed by a decatination reaction of kinetoplast DNA [5]. The reaction mixture contained 50 mM Tris-HCl, (pH 7.5), 0.5 mM ATP, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 0.6 μg BSA, 12.8 μl test compounds with different concentrations, 0.325 μg catenated kDNA, and 1 unit of topoisomerase II in a total volume of 20 μl. After incubation at 37°C for 40 minutes, the mixtures were subjected to electrophoresis on 1.0% agarose gel (50 V) for 1 hour. After electrophoresis, the gels were stained with EtBr solution and decatenated kDNA on the gel was measured by a densitometer. For the cleavage activity [5, 11], 10 units topoisomerase II and supercoiled pBR 322 DNA as substrate were used. After electrophoresis (1.2% agarose gel containing 0.1% SDS), the increase of linearized pBR322 DNA was estimated as the stabilizing of cleavable complex by an inhibitor.

Assay for Antimicrobial Activity

The minimal inhibition concentration (MIC) was determined by the agar dilution method, using nutrient agar for bacteria (*Staphylococcus aureus* NBRC 13276 and *Pseudomonas aeruginosa* ATCC 15442), Sabouraud agar for *Candida albicans* ATCC 2019 and PD agar for *Aspergillus clavatus* F 318a. Bioassay procedure was the same as in article [2].

Cell Lines and Cell Culture

The COLO 201 human colon carcinoma cell line and human lung carcinoma cell line LU 65 were obtained from Japanese Cancer Research Resources Bank. Cells were cultured in medium with RPMI 1640 (Nissui Co., Japan) containing 10% fetal bovine serum (HyClone Inc., USA) and penicillin/streptomycin (Invitrogen Co., USA) at 37°C in a humidified atmosphere of 5.0% CO_2 in air.

Cell Growth Assay

The cell viability was assessed with 4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitro-phenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate sodium (WST-8), a tetrazolium salt, using a commercially available kit (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). In brief, human lung carcinoma cells (LU 65, 5×10^3 cells/ml) were seeded in 270 μl of culture medium/well in 96-well plates. Then, 30 μl of test sample dissolved in aqueous DMSO at various concentrations were added and then incubated in a 5.0% CO_2 incubator at 37°C for 72 hours. The last concentration of DMSO was 10% or less. At the end of the incubation, 100 ml of each cultured cell was transferred the other 96-well plates and 10 μl of WST-8 assay solution was added per well and incubated at 37°C in humidified air supplemented with CO_2 for an additional 4 hours. The amount of formazan formed was measured at an optical density of 450 nm using a microplate reader and the cell viability was expressed as a percentage of the untreated control. The IC_{50} values were determined

by the extrapolation of the dose-response viability curves. 5-Fluorouracil was used as positive control (IC_{50} values of 1.0 μM).

Cell Cytotoxicity Assay

Human colon carcinoma cells COLO 201 (1×10^5 cells/ml) were seeded in 270 μl of culture medium/well in 96-well plates. The cells were treated with 30 μl of different concentrations of the test compounds and were incubated at 37°C for 72 hours. Cytotoxicity was measured using WST-8 method similar to that in the case of cell growth assay.

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References

1. Gunatilaka AAL. Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. J Nat Prod 69: 509–526 (2006)
2. Shiono Y, Murayama T, Takahashi K, Okada K, Katohda S, Ikeda M. Three oxygenated cyclohexenone derivatives, produced by an endophytic fungus. Biosci Biotechnol Biochem 69: 287–292 (2005)
3. Shiono Y, Murayama T. New eremophilane-type sesquiterpenoids, eremoxylarins A and B from xylariaceous endophytic fungus YUA-026. Z Naturforsch 60b: 885–890 (2005)
4. Shiono Y. Anthracobic acids A and B, two polyketides, produced by an endophytic fungus *Anthracobia* sp. Chem Biodivers 3: 217–223 (2006)
5. Suzuki K, Nagao K, Monnai Y, Yagi A, Uyeda M. Topostatin, a novel inhibitor of topoisomerases I and II produced by *Thermomonospora alba* strain No. 1520. I. Taxonomy, fermentation, isolation and biological activities. J Antibiot 51: 991–998 (1998)
6. Suzuki K, Yahara S, Kido Y, Nagao K, Hatano Y, Uyeda M. Topostatin, a novel inhibitor of topoisomerases I and II produced by *Thermomonospora alba* strain No. 1520. II. Physico-chemical properties and structure elucidation. J Antibiot 51: 999–1003 (1998)
7. Suzuki K, Yamaizumi M, Tateishi S, Monnai Y, Uyeda M. Topostatin, a novel inhibitor of topoisomerases I and II produced by *Thermomonospora alba* strain No. 1520. III. Inhibitory properties. J Antibiot 52: 460–465 (1999)
8. Li TK, Liu LF. Tumor cell death induced by topoisomerase-targeting drugs. Ann Rev Pharmacol Toxicol 41: 53–77 (2001)
9. Yamashita Y, Kawada S, Fujii N, Nakano H. Induction of mammalian DNA topoisomerase II dependent DNA cleavage by antitumor antibiotic streptonigrin. Cancer Res 50: 5841–5844 (1990)
10. Hsiang YH, Hertzberg R, Hecht S, Lui LF. Camptothecin induces protein-linked DNA breaks *via* mammalian DNA topoisomerase I. J Biol Chem 260: 14873–14878 (1985)
11. Sugawara T, Tanaka A, Tanaka K, Nagai K, Suzuki K, Suzuki T. YM-170320, a novel lipopeptide antibiotic inducing morphological change of colonies in a mutant of *Candida tropicalis* pK233. J Antibiot 51: 435–438 (1998)