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A Cytotoxic Lipopeptide from the Sponge-Derived Fungus *Aspergillus versicolor*

Yoon Mi Lee,[a] Hung The Dang,[a] Jongki Hong,[†] Chong-O. Lee,[‡] Kyung Sook Bae,[§] Dong-Kyoo Kim,[#] and Jee H. Jung[*]

College of Pharmacy, Pusan National University, Busan 609-735, Korea. [*]E-mail: jhjung@pusan.ac.kr
[†]College of Pharmacy, Kyung Hee University, Seoul 130-701, Korea
[‡]Korea Research Institute of Chemical Technology, Daejon 305-343, Korea
[§]Korea Research Institute of Bioscience and Biotechnology, Daejon 305-333, Korea
[#]Biohealth Product Research Center, Inje University, Kimhae 621-749, Korea
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Sponge-derived fungi are a prolific source of bioactive secondary metabolites. To date, more than 134 metabolites from over 34 sponge-derived fungal strains have been reported. Although the nature of the associations between sponges and fungi is not completely understood, sponge-associated fungi are an interesting source of new bioactive natural products.[1,2]

In our previous study on cytotoxic compounds from the sponge-derived fungus *Aspergillus versicolor*, three polyketides were isolated.[3] In a subsequent study, a new peptide (**1**) was isolated by bioactivity-guided fractionation. Peptides are well-established bioactive metabolites from marine sources. Approximately 20 peptides have been reported from sponge-derived fungi, including efrapeptins Eα, H, RHM3, RHM4 (from two fungi, *Acremonium* sp. and *Metarrhizium* sp),[4] homodestcardin (from *Fusarium graminearum*),[5] clonostachysins A and B (from *Clonostachys rogersoniana*),[6] a cyclodepsipeptide (from a *Clonostachys* sp),[7] linear octapeptides (from an *Acremonium* sp),[8] petrosifungins A and B (from *Penicillium brevicompactum*),[9] fellutamides A and B (from *Penicillium fellutanum*),[10] and halovirs (from a *Scytalidium* sp).[11] This paper describes the isolation, structure elucidation, and cytotoxicity evaluation of a new lipopeptide from the fungus *Aspergillus versicolor*.

The fungal strain was isolated from a marine sponge *Petrosia* sp., and identified as *Aspergillus versicolor* by morphological analysis. The fungal culture was extracted with EtOAc at room temperature. The EtOAc extract showed significant toxicity to brine shrimp larvae (LC_{50} 32 μg/mL). This EtOAc extract was partitioned between *n*-hexane and 90% aqueous MeOH. The latter layer, which was toxic to brine shrimp larvae (LC_{50} 0.4 μg/mL), was subjected to reversed-phase MPLC to afford 13 fractions. Fraction 7 (LC_{50} < 0.1 μg/mL), one of the bioactive fractions, was further subjected to reversed-phase HPLC to yield compound **1**, which was named fellutamide C. Fellutamide C (**1**) is structurally similar to fellutamides A (**2**) and B (**3**), which were first isolated from the fish-derived fungus *Penicillium fellutanum*.[10] If only the functional group of the terminal unit is considered, fellutamide C (**1**) is a reduced alcohol form of fellutamide B (**3**).

Fellutamide C (**1**) was isolated as a light violet, amorphous powder. Its molecular formula was defined as $C_{27}H_{51}N_5O_7$ on the basis of HRFABMS and NMR data. The exact mass of the [M + Na]$^+$ ion (*m/z* 580.3694) matched well with the expected formula (Δ + 0.8 mmu). General analyses of the ^{1}H and ^{13}C NMR spectra of compound **1** revealed characteristic peptide resonances, including five amide carbon signals at δ 176.8, 173.3, 173.0, 172.3, and 172.0. Four proton signals for two amino groups (NH_2) were observed as two pairs of broad singlets (δ 7.44/6.95 and δ 7.22/6.75). In the ^{1}H NMR spectrum using DMSO-d_6 as the solvent, three NH protons of peptide bonds were observed as doublets (δ 8.15, 8.02, and 7.43). This proved to be a convenient starting point for identifying the individual amino acid unit by COSY, HSQC, and HMBC experiments (Figure 1). Further analysis of the NMR data of compound **1** revealed the presence of an amino alcohol unit (Lol, leucine-derived leucinol), common amino acid units (asparagine and glutamine), and a fatty acyl chain (3-hydroxydodecanoic acid). The presence of a leucinol (Lol) moiety was apparent from the COSY correlation of the hydroxymethylene protons at δ 3.46 (H-β'), with the *N*-methine proton signal at δ 3.94 (H-α). The presence of a gluta-

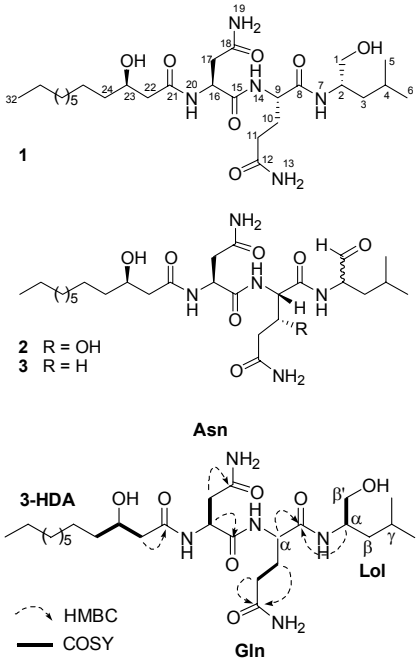

Figure 1. Key COSY and HMBC correlations of compound **1**.

[a]These authors equally contributed to this work.

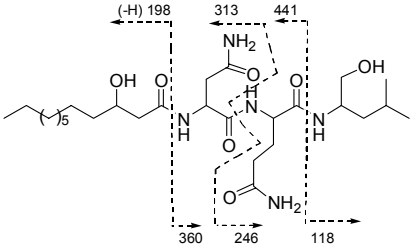

Figure 2. Key FAB-CID-MS/MS fragmentations of the $[M + H]^+$ ion of compound **1**.

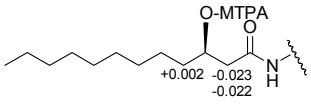

Figure 3. $\Delta\delta$ (Δ_S - Δ_R) values for the MTPA esters of compound **1**.

mine (Gln) moiety was suggested by the COSY correlations of the α, β, and γ protons in an isolated C_3H_5 unit, which was expanded to an aminodicarboxyl unit on the basis of the HMBC correlations of the α proton to a carbonyl carbon (δ 172.0) and the γ proton to another carbonyl carbon (δ 176.8). The connectivity of the Lol and Gln moieties was confirmed by the HMBC correlation of the α-proton of the Lol unit with the carbonyl carbon (δ 172.0) of the Gln residue. The asparagine (Asn) unit was suggested by the HMBC correlations of the α-methine proton at δ 4.63 with the carbonyl carbon signal at δ 172.3, and the β-methylene protons at δ 2.80/2.72 with another amide carbonyl carbon signal at δ 173.0. Finally, the presence of a fatty acyl moiety 3-hydroxydodecanoic acid (3-HDA) at the *N*-terminal of the peptide was confirmed by NMR and MS. In the HMBC spectrum, there were correlations between the two methylene proton signals (H-22) at δ 2.43 and 2.32 and the carbonyl carbon signal at δ 173.3 (C-21). The FAB-CID-MS/MS spectrum of compound **1** corroborated the planar structure assigned by NMR spectroscopy. Fragmentations of the $[M + H]^+$ ion (*m/z* 558) at the peptide (amide) bonds were expected as fragment ion pairs at *m/z* 441/118 (3-HDA-Asn-Gln/Lol), 313/246 (3-HDA-Asn/Gln-Lol), and 199/360 (3-HDA/Asn-Gln-Lol) (Figure 2). Except the fragment ion pair of 3-HDA/Asn-Gln-Lol, other fragmentations were observed as expected. The fragment on pair of 3-HDA/Asn-Gln-Lol was observed at *m/z* 198/360. The fragment of *m/z* 199 had apparently undergone the further loss of a proton.

The absolute configuration of the three amino acids of compound **1** were determined using Marfey's method.[12] Three pairs of amino acid standards, including D-/L-aspartic acid, D-/L-glutamic acid, and D-/L-leucine were derivatized with Marfey's reagent's (FDAA) to establish the reference R_f values of the corresponding amino acid-FDAA derivatives. The amino acid-FDAA derivatives were characterized by reversed-phase TLC (D-Asp-FDAA derivative: R_f 0.76; L-Asp-FDAA derivative: R_f 0.84; D-Glu-FDAA derivative: R_f 0.67; L-Glu-FDAA derivative: R_f 0.73; D-Leu-FDAA derivative: R_f 0.08; L-Leu-FDAA derivative: R_f 0.23). As fellutamide C (**1**) contained a leucinol moiety, it was first oxidized to convert the leucinol to a leucine moiety before hydrolysis.[10] After oxidation of compound **1** using the Jones reagent, followed by acid hydrolysis and derivatization with Marfey's reagent, the resulting mixture clearly showed three spots on the TLC with R_f values of 0.84, 0.73, and 0.23, which showed a good match to the corresponding L-Asp-FDAA, L-Glu-FDAA, and L-Leu-FDAA derivatives, respectively.

The absolute configuration of the 3-hydroxy group in the acyl moiety of compound **1** was defined by the modified Mosher's method.[13] A positive $\Delta\delta$ ($\delta_S - \delta_R$) value was observed for H-24

Table 1. ^{1}H and ^{13}C NMR Data of Compound **1**

unit	position	δ_C[b]	δ_H (*J* in Hz)[a]	HMBC
Lol	NH[c]		8.02, d (7.0)	
	α	48.8, d	3.94, m	β, γ, CO
	β	38.6, t	1.42, m	α, γ, δ, Me
			1.33, m	
	β'	64.0, t	3.46, d (5.0)	α, β, CO
	γ	25.3, d	1.62, m	
	CH_3	22.0, q	0.89, d (6.8)	β, γ
		23.5, q	0.92, d (6.8)	
Gln	NH[c]		7.43, d (7.0)	
	CO	172.0, s		
	α	52.9, d	4.27, dd (15.5, 7.0)	γ, CO
	β	27.9, t	2.15, m	α, γ, CO
			1.90, m	
	γ	31.5, t	2.30, m	α, β, $\underline{CO}NH_2$
	$\underline{CO}NH_2$	176.8, s		
	$CO\underline{NH_2}$[c]		7.22, br s	
			6.75, br s	
Asn	NH[c]		8.15, d (7.0)	
	CO	172.3, s		
	α	50.2, d	4.63, t (7.0)	β, CO
	β	36.2, t	2.80, dd (15.5, 7.0)	α, $\underline{CO}NH_2$
			2.72, dd (15.5, 7.0)	
	$\underline{CO}NH_2$	173.0, s		
	$CO\underline{NH_2}$[c]		7.44, br s	
			6.95, br s	
3-HDA	21	173.3, s		
	22	43.6, t	2.43, dd (15.5, 4.5)	1, 3, 4
			2.32, d (15.5)	
	23	67.5, d	3.97, m	2, 4
	24	37.1, t	1.48, m	3, CH_2
	25 - 30	31.4 (2C), t	1.29 - 1.36	4, CH_2
		29.2, t		
		29.0, t		
		28.9 (2C), t		
	31	22.3, t		
	32	14.1, q	0.88, t (7.0)	

[a]Measured in CD_3OD (500 MHz), [b]Measured in CD_3OD (100 MHz), [c]Measured in DMSO-d_6 (500 MHz). [d]HMBC correlations are from proton stated to the indicated carbon(s).

Table 2. Cytotoxicity Data of Compound **1**[a]

compound	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
1	33.1	23.8	5.1	3.9	3.1
doxorubicin	0.02	0.11	0.07	0.22	0.33

[a]Data expressed in ED_{50} values (μM). A549, human lung cancer; SK-OV-3, human ovarian cancer; SK-MEL-2, human skin cancer; XF498, human CNS cancer; HCT15, human colon cancer.

(+0.002), while a negative value was observed for H-22 (− 0.023), indicating that the configuration of the hydroxy group is *R* (Figure 3). Therefore, the absolute configuration of compound **1** was established as (2*S*, 9*S*, 16*S*, 23*R*).

Since the structure of fellutamide C is closely related to fellutamides A (**2**) and B (**3**), it might be supposed that this fungus also produces **2** and **3**. However, we could not detect or isolate these analogues except an additional unknown fellutamide C analogue from the broth.

Fellutamide C (**1**) was evaluated for its cytotoxicity against a panel of five human solid tumor cell lines (Table 2). The compound showed cytotoxiciy to most of the five human solid tumor cell lines. The skin cancer (SK-MEL-2), CNS cancer (XF498), and colon cancer (HCT15) cells were more sensitive to this peptide. Fellutamides A and B were reported to be potent cytotoxins with IC_{50} values ranging from 0.1 to 0.8 μg/mL against murine leukemia P388 and L1210, and human epidermoid carcinoma KB cells.[10] However, the activity of these analogues cannot be directly compared because different cell lines were employed for fellutamides A and B and a positive control was not included in the test.

Experimental

General Procedure. The optical rotations were measured using a JASCO P-1020 digital polarimeter. The ^1H and ^{13}C NMR spectra were recorded on Varian Inova 500 MHz and Varian Unity 400 MHz spectrometers, respectively. The chemical shifts are reported with reference to the respective residual solvent or deuterated solvent peaks (δ_H 3.30 and δ_C 49.0 for CD_3OD, δ_H 2.50 and δ_C 39.5 for DMSO). The FABMS data was obtained on a JEOL JMS SX-102A spectrometer. The HRFABMS data was obtained on a JEOL JMS SX-101A spectrometer. HPLC was performed on a C8-5E Shodex packed column (preparative, 250 × 10 mm, 5 μm, 100 Å), and Shodex C18E (preparative, 250 × 10 mm, 5 μm, 100 Å) columns using a Shodex RI detector.

Animal Material. The sponge was collected by hand using SCUBA (20 m in depth) in 2004 off the coast of Jeju Island, Korea. The collected sample was frozen immediately. This specimen was identified as *Petrosia* sp. and the morphology of the sponge specimen was described elsewhere.[14]

Fungal Strain. The fungal strain was isolated from the marine sponge *Petrosia* sp. After rinsing in sterile sea water, small pieces of the surface and inner tissue of the sponge were homogenized and inoculated on malt extract agar (MEA) petri dishes. The sterilized MEA medium (prepared with 75% sea water, obtained locally) contained glucose (20 g/L), malt extract (20 g/L), agar (20 g/L), peptone (1 g/L), and antibiotics (10,000 units/mL penicillin and 10 mg/mL streptomycin, 5 mL/L). The emerging fungal colonies were transferred to the same media in a petri dish and incubated at 25 °C for 10 - 14 days to allow colony development.[15] The pure fungal strain, which is designated as PF-10M, was identified as *Aspergillus versicolor* by morphological analysis. The strain was deposited at KRIBB, Korea.

Extraction and Isolation. Fermentation was performed in 250 mL malt media in 500 mL Erlenmeyer flasks for subculturing. For a large scale culture, 250 mL of the subculture was transferred to a 2 L Erlenmeyer flask each containing 20 g/L malt media, and fermentation was carried out on a rotary shaker (32 °C, 150 rpm, 21 days). The cultured fungus (8 L) was extracted with 16 L of EtOAc, to afford the EtOAc extract (2.0 g, brine shrimp lethality, LC_{50} 32 μg/mL), which was partitioned between *n*-hexane (0.7 g, LC_{50} 51 μg/mL) and 90% aq MeOH (1.27 g, LC_{50} 0.4 μg/mL). The 90% MeOH layer was subjected to a step-gradient MPLC (ODS-A, 120 Å, S-30/50 mesh) eluting with 50% to 100% MeOH to afford 13 fractions. Fraction 7, which is one of the bioactive fractions (LC_{50} < 0.1 μg/mL), was subjected to reversed-phase HPLC (C8 Shodex Pack ODS, 250 × 10 mm, 5 μm, 100 Å) eluting with 75% aq MeOH to afford 5 subfractions. Compound **1** (4.8 mg) was obtained by the purification of subfractions 3 and 4, by reversed-phase HPLC (C8 Shodex Pack ODS, 250 × 10 mm, 5 μm, 120 Å).

Fellutamide C (1). Light violet, amorphous powder; $[\alpha]_D$ - 128 (*c* 0.18, MeOH); IR ($CHCl_3$) ν_{max} 3500, 1650, 1550 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; LRFABMS *m/z* 580 $[M + Na]^+$, *m/z* 558 $[M + H]^+$; HRFABMS *m/z* 580.3694 $[M + Na]^+$(calcd for $C_{27}H_{51}N_5O_7Na$, 580.3686); FAB-CID-MS/MS *m/z* 441/118 (3-HDA-Asn-Gln/Lol), 313 /246 (3-HDA-Asn/Gln-Lol), and 198 /360 (3-HDA/Asn-Gln-Lol).

Evaluation of Cytotoxicity. The cytotoxicity assays against five human tumor cell lines (A549, human lung cancer; SK-OV-3, human ovarian cancer; SK-MEL-2, human skin cancer; XF 498, human CNS cancer; and HCT15, human colon cancer) were performed at the Korea Research Institute of Chemical Technology. The rapidly growing cells were harvested, counted, and inoculated into 96-well microtiter plates at the appropriate concentrations (1-2 × 10^4 cells/well). After incubation for 24 h, the compounds dissolved in culture medium (RPMI 1640, Gibco; 10% FBS, Gibco) were applied to the culture wells in triplicate followed by incubation for 48 h at 37 °C in a 5% CO_2 atmosphere. The culture was fixed with cold TCA and stained with 0.4% SRB (sulforhodamine B, Sigma) dissolved in 1% acetic acid. After dissolving the bound dye with 10 mM unbuffered tris base using a gyrotatory shaker, the absorbance at 520 nm was measured using a microplate reader (Dynatech Model MR 700). The 50% inhibitory concentration (ED_{50}) was defined as the concentration that reduced the absorbance by 50% compared to the control level in the untreated wells.

Preparation of the (*R*)- and (*S*)-MTPA Esters of 1. Two portions (each 0.5 mg) of compound **1** were treated overnight with (*S*)-(+)- and (*R*)-(-)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (0.6 μL) in C_5D_5N (0.5 mL) at room temperature to afford the (*R*)- and (*S*)-MTPA esters, respectively (**1r** and **1s**, respectively).[13]

(*S*)-MTPA Ester of 1 (1s): ^1H NMR (pyridine-d_5, 500 MHz) 4.302 (1H, m, H-23), δ 2.184 (1H, m, H-22b), 2.109 (1H, m, H-22a), 1.552 (2H, m, H-24).

(*R*)-MTPA Ester of 1(1r): ^{1}H NMR (pyridine-d_5, 500 MHz) 4.274 (1H, m, H-23), δ 2.207 (1H, m, H-22b), 2.131 (1H, m, H-22a), 1.550 (2H, m, H-24).

Oxidation, Hydrolysis, and Derivatization of 1. Compound **1** (0.8 mg) was dissolved in acetone and the solutions were cooled to 0 °C. Two microliter of Jones reagent (6.68 g of CrO_3 in 5.75 mL of concentrated H_2SO_4, then H_2O until volume of 25 mL) was added, and the mixture was stirred at 0 °C for 2 h. The reaction mixture was then filtered and washed with diethyl ether. The filtrate was concentrated under reduced pressure to afford a residue, which was then dissolved in 1.0 mL of 6 N HCl and hydrolyzed for 24 h at 110 °C. The reaction mixture was cooled to room temperature and evaporated with MeOH several times to remove the excess HCl. The mixture obtained was dissolved in 100 μL of acetone and 50 μL of H_2O. The solution was treated with 40 μL of 1 M $NaHCO_3$ followed by 100 μL of 1% FDAA (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) in acetone. The reaction mixture was heated to 40 °C for 2 h and then cooled to room temperature. The reaction was quenched by adding 20 μL of 2 N HCl and the sample was ready for TLC analysis.

Derivatization of Standard Amino Acids with Marfey's Reagent (FDAA) and TLC Analysis.[12,16,17] Two hundred microliters of a 1% FDAA acetone solution was added to a 2.0 mL reaction vial containing 5 μM of the pure amino acid standard in 100 μL of H_2O. Subsequently, 40 μL of 1.0 M $NaHCO_3$ was added. The mixture was maintained at 40 °C for 1 h in a Reacti-Therm heating module. After cooling the mixture to RT, 20 μL of 2 N HCl was added to quench the reaction, and the resulting solution was diluted with the mobile phase to make the chromatographic sample solution for TLC analysis (RP-18F_{254s}, 50% MeOH in water).

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