http://dx.doi.org/10.5012/bkcs.2011.32.10.3817

A Cytotoxic Fellutamide Analogue from the Sponge-Derived Fungus *Aspergillus versicolor*

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Received August 11, 2011, Accepted August 30, 2011

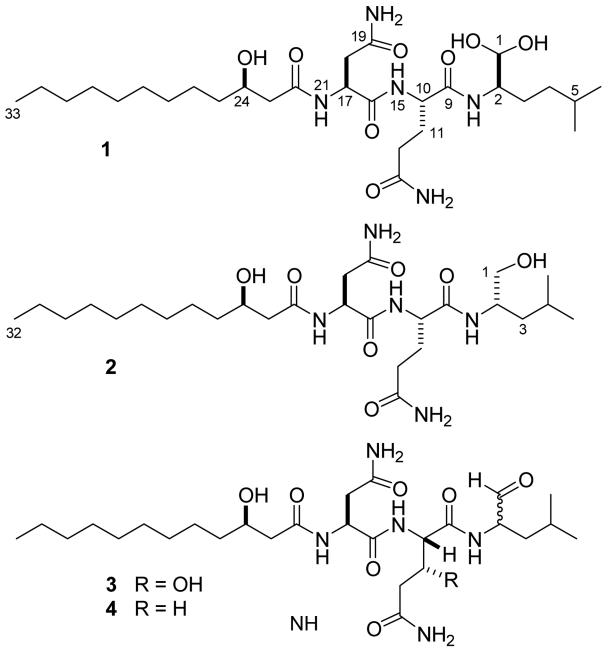
Key Words : Cytotoxicity, *Aspergillus versicolor*, Sponge-derived fungus, Fellutamide

Recently, a series of linear lipopeptides containing unusual amino acid residues have been reported from marine fungi, and these serve as a valuable source of potential drug leads. These lipopeptides were found to have interesting bioactivities, including, anti-inflammatory, antimalarial properties, antimicrobial, antileishmanial, cytotoxic and neurotoxic activities.[1-9] Fellutamides A and B are lipopeptides first isolated from the fish-derived fungus *Penicillium fellutanum*. Fellutamides exhibit cytotoxic properties,[10] and fellutamide A has been shown to stimulate the synthesis and secretion of nerve growth factor (NGF) *in vitro*.[11] Because of the pharmacological potential of this class of compounds, these lipopeptides were synthesized and their pharmacological properties were further investigated. Synthetic fellutamide B and its analogs were verified to be cytotoxic and to induce NGF secretion in L-M murine fibroblast cells.[12] Previous studies have suggested that fellutamide B inhibits the mammalian 20S proteasome, leading to increased NGF gene expression and secretion.[13] Fellutamide B was also shown to potently inhibit 20S proteasomes from *Mycobacterium tuberculosis* (Mtb) and humans.[14] An additional congener fellutamide C was isolated from the marine sponge-derived fungus *Aspergillus versicolor* as a potent cytotoxin to human cancer cells.[15] Recently, two new analogs were isolated from the soil-derived fungus *Metulocladosporiella* sp. They showed antimicrobial activity against *Candida albicans* and *A. fumigatus* and inhibited the fungal proteasome.[16]

In our continuing study on cytotoxic compounds isolated from the sponge-derived fungus *A. versicolor*, a new lipopeptide named fellutamide F (**1**) was isolated by bioactivity-guided fractionation. In this paper, the isolation, structure elucidation, and cytotoxicity evaluation of this lipopeptide are described.

The fungal strain was isolated from a marine sponge *Petrosia* sp., and it was identified as *A. versicolor* by morphological analysis. The EtOAc extract from the fungal culture caused 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 (1.27 g), 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 subjected to reversed-phase HPLC to yield fellutamide F (**1**).

Fellutamide F (**1**) was isolated as a light violet, amorphous powder. Its molecular formula was defined as $C_{28}H_{53}N_5O_8$ on the basis of HRFABMS and NMR data. The exact mass of the $[M + Na]^+$ ion (*m/z* 610.3801) matched well with the expected formula $C_{28}H_{53}N_5O_8Na$ (Δ + 0.9 mmu). General analyses of ^1H and ^{13}C NMR spectra of **1** revealed characteristic peptide resonances, including five carbonyl carbon signals at δ_C 176.0, 173.3, 173.2, 173.0, and 172.0. Additionally, two NH_2 groups were observed as two pairs of broad singlets (δ_H 7.46/6.95 and δ_H 7.23/6.72). In the ^1H NMR spectrum using DMSO-d_6 as a solvent, three NH protons arising from peptide bonds were observed, each presenting as doublets (δ_H 8.25, 8.15, and δ_H 7.45). This proved to be a convenient starting point for the identification of the individual amino acid units by COSY, HMBC, TOCSY, and HSQC experiments (Figure 1). Further analysis of the NMR



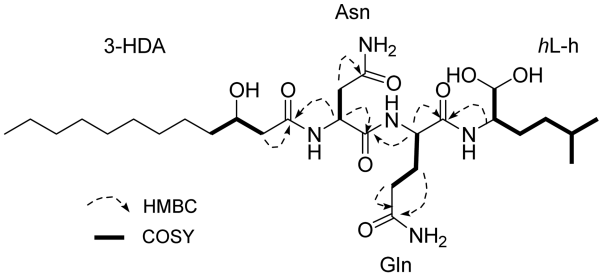


Figure 1. Key HMBC and COSY correlations of **1**.

data of compound **1** revealed the presence of a homoleucinal-hydrate (*h*L-h) moiety, two amino acid residues (asparagine [Asn] and glutamine [Gln]), and an acyl chain (3-hydroxydodecanoic acid [3-HAD]). The ^{1}H NMR spectrum of **1** was very similar to that of fellutamide C (**2**), which had previously been isolated from the same strain.[15] The only notable difference between **1** and **2** was the replacement of the carbinol group (δ_H 3.94/δ_C 48.8, H-2/C-2) with an acetal group (δ_H 4.41/δ_C 99.7, H-1/C-1). A spin coupling system, including the acetal methine proton (δ_H 4.41, H-1), α proton (δ_H 3.96, H-2), and alkyl chain protons (H-3–H-7), was delineated by COSY and TOCSY experiments (Figure 1). The presence of a Gln moiety was suggested by COSY correlations of α, β, and γ protons in an isolated C_3H_5 chain, which was expanded to an amide carbonyl unit on the basis of HMBC correlations of the α (H-10) and γ (H-12) protons to carbonyl carbons at δ_C 172.0 (C-19) and δ_C 176.0 (C-13), respectively. Connectivity between the homoleucinal-hydrate (*h*L-h) moiety and Gln moiety was determined by HMBC correlation of α proton (H-2) of the *h*L-h moiety with the carbonyl carbon (δ_C 172.0) of the Gln residue. The Asn unit was suggested by HMBC correlation of the α-methine proton (δ_H 4.62, H-17) with the carbonyl carbon (δ_C 173.0, C-16) and by correlation of the β-methylene protons (δ_H 2.75/2.73, H-18) with another amide carbonyl carbon (δ_C 173.2, C-19). The presence of the acyl moiety 3-HAD at the *N*-terminal of the peptide unit was confirmed by NMR and MS analysis. In the HMBC spectrum, correlation of α-methylene proton signals (δ_H 2.43 and δ_H 2.31, H-23) with the carbonyl carbon signal at δ_C 173.3 (C-22) was observed. As expected, the FAB-CID-MS/MS fragmentations of the $[M + H]^+$ ion in compound **1** were identical to those of **2** for 3-HAD-Asn-Gln, 3-HAD-Asn, and 3-HAD units (*m/z* 441, 313, and 198, respectively). However, corresponding counter

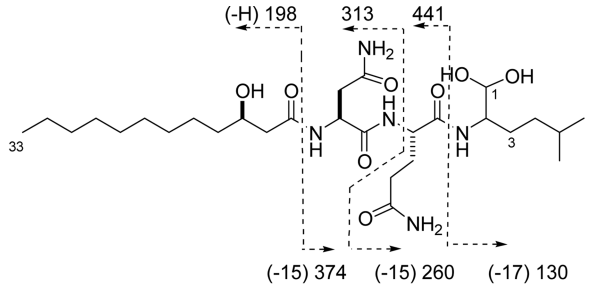


Figure 2. Key FAB-CID-MS/MS fragmentations of the $[M + H]^+$ ion of **1**.

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

Unit	Position	δ_H (m, *J* in Hz)[a]	δ_C[b]	HMBC
hL-h	NH	8.15 (d)[c]		
	α	3.96 (m)	53.1	
	β	1.47 (m)	38.5	α
	β	4.41 (d)	99.7	α
	γ	1.30 (m)	31.0	δ, CH_3
	δ	1.35 (m)	32.0	γ
	CH_3	0.86 (d, 6.8)	21.8	β, γ
		0.90 (d, 6.8)	23.7	
Gln	NH	7.45 (d)[c]		
	CO		172.0	
	α	4.31 (m)	54.6	β, γ, CO
	β	2.15 (m)	28.8	α, γ, CO, $\underline{CO}NH_2$
		1.90 (m)		
	γ	2.30 (m)	32.5	α, β, $\underline{CO}NH_2$
	$\underline{CO}NH_2$		176.0	
	$CO\underline{NH_2}$	7.23 (br s)[c]		
		6.72 (br s)[c]		
Asn	NH	8.25 (d, 7.0)[c]		
	CO		173.0	
	α	4.62 (t, 6.5)	52.0	β, CO
	β	2.75 (m)	37.3	α, $\underline{CO}NH_2$
		2.73 (dd, 6.5)		
	$\underline{CO}NH_2$		173.2	
	$CO\underline{NH_2}$	7.46 (br s)[c]		
		6.95 (br s)[c]		
3-HDA	CO		173.3	
	α	2.43 (dd, 14.5, 5.0)	44.5	β, γ, CO
		2.31 (m)		
	β	3.96 (m)	69.7	
	γ	1.48 (m)	38.0	β, δ
	CH_2	1.29-1.36	31.0	γ, CH_3
			30.7 (2C)	
			30.4	
			28.9 (2C)	
			22.1	
	CH_3	0.89 (t, 7.0)	14.4	

[a]Measured in CD_3OD (500 MHz). [b]Measured in CD_3OD (100 MHz). [c]Measured in DMSO-d_6 (500 MHz)

fragments (*m/z* 130, 260, and 374) were observed with 15 or 17 amu mass shifts from the expected mass (Figure 2). These counter fragments were supposed to be generated by loss of the hydroxyl group of the *h*L-h moiety (*m/z* 130), or by further migration of 2 protons (*m/z* 374 and 260). Geminal diol hydrates are usually unstable, but a few examples of stable-form germinal diol hydrates, such as formaldehyde hydrate and betaine aldehyde hydrate, have been encountered.[17] The resonance of a proton bound to an acetal carbon is δ_H 4.6-5 ppm upfield relative to that of an aldehydic proton. Likewise, the ^{13}C chemical shift value of the gem-diol was in good agreement with the expected chemical shift.[18,19]

The absolute configurations of the two amino acid residues (Asn and Gln) of **1** were determined by Marfey's method.[20]

Table 2. Cytotoxicity Data[a] of Compounds **1** and **2**

Compound	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
1	1.81	1.20	0.67	0.14	0.13
2[b]	18.42	13.28	2.83	2.16	1.74
doxorubicin	0.01	0.06	0.04	0.12	0.18

[a]Data expressed in ED_{50} values (μg/mL). A549, human lung cancer cells; SK-OV-3, human ovarian cancer cells; SK-MEL-2, human skin cancer cells; XF498, human CNS cancer cells; HCT15, human colon cancer cells. [b]Data cited from ref. 15. In fact, compounds **1** and **2** had been assayed in the same batch.

Fellutamide F (**1**) was subjected to acid hydrolysis and derivatization with Marfey's reagent (1-fuoro-2,4-dinitrophenyl-5-L-alanine amide [FDAA]). The resulting mixture clearly showed two spots on reversed-phase TLC, with R_f values of 0.84 and 0.73, matching well with those corresponding to standard L-Asp-FDAA and L-Glu-FDAA, respectively.

The configuration of the hydroxyl group in the acyl chain was presumed to be the same as that of co-isolated fellutamide C (**2**, 24*R*) on the basis of the identical ^{13}C NMR shifts of the carbonyl (δ_C 173.3, C-22), α (δ_C 44.5, C-23), β (δ_C 69.7, C-24), and γ (δ_C 37.4, C-25) carbons.[15] According to previous studies, the *R* form of 3-HAD seems to be the prevalent form found in nature.[21-23] The optical rotation of **1** ($[\alpha]_D$ + 163) was opposite to that of **2** ($[\alpha]_D$ - 128), implying a reversed configuration of the terminal amino acid derivative (*h*L-h).[15] Nevertheless, the definite configuration of the *h*L-h moiety remains to be determined.

Fungal metabolites structurally related to **1** and **2** are the lipopeptides fellutamides A (**3**) and B (**4**), which were isolated from the fish-derived fungus *P. fellutanum*.[10] If only the functional group of the terminal unit is considered, fellutamide C (**2**) is a reduced alcohol form, and fellutamide F (**1**) is a hydrated form of fellutamide B (**4**). The substantial stability of the acetal form (**1**) of the aldehyde is possibly due to the electron-withdrawing effect of the α nitrogen.[24] It is noteworthy that the *C*-terminal of fellutamide F (**1**) is composed of an unusual amino acid skeleton derivative, possibly derived from homoleucine.

Fellutamide F (**1**) was evaluated for cytotoxicity against a panel of five human solid tumor cell lines (Table 2). Fellutamide F (**1**) showed strong cytotoxicity toward all of the five cell lines, with higher potency than compound **2**. Human skin cancer cells (SK-MEL-2), CNS cancer cells (XF498), and colon cancer cells (HCT15) were more sensitive to this lipopeptide. Cytotoxic potencies of fellutamide F (**1**) against XF498 and HCT15 cells were comparable to those of doxorubicin.

Experimental

General Procedures. Optical rotations were measured using a JASCO P-1020 digital polarimeter. ^1H and ^{13}C NMR spectra were recorded on Varian Inova 500 MHz and Varian Unity 400 MHz spectrometers, respectively. Chemical shifts were 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-d_6). FAB-MS data were obtained on a JEOL JMS SX-102A spectrometer. HR-FAB-MS data were obtained on a JEOL JMS SX-101A spectrometer. HPLC was performed with a C8-5E Shodex packed column (preparative, 250 × 10 mm, 5 μm, 100 Å), and Shodex C18E columns (preparative, 250 × 10 mm, 5 μm, 100 Å) using a Shodex RI detector.

Animal Material. The sponge was collected by hand in 2004 using SCUBA (20 m in depth) off the coast of Jeju Island, Korea. The collected sample was frozen immediately. This specimen was identified as *Petrosia* sp.; the morphology of the sponge specimen has been described elsewhere.[25]

Fungal Strain. The fungal strain was isolated from the marine sponge *Petrosia* sp. Following a rinse with sterile seawater, small pieces of surface and inner tissues of the sponge were homogenized and then inoculated on malt extract agar (MEA) petri dishes. The sterilized MEA medium (prepared with 75% seawater) 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). 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.[26] The pure fungal strain, designated as PF10M, was identified as *A. versicolor* by morphological analysis.

Extraction and Isolation. Fermentation was performed in 250 mL malt media in 500 mL Erlenmeyer flasks for subculture. For large scale culture, 250 mL of subculture was transferred into a 2 L Erlenmeyer flask 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 give 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 step-gradient MPLC (ODS-A, 120 Å, S-30/50 mesh), eluting with 50% to 100% MeOH to afford 13 fractions. Fraction 7, 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** (3.8 mg) was obtained by purification of subfraction 4.

Fellutamide F (1): Light violet, amorphous powder; $[\alpha]_D$ + 163 (*c* 0.13, MeOH); IR ($CHCl_3$) ν_{max} 3500, 3000, 1650, 1550 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; LRFABMS *m/z* 610 $[M + Na]^+$, *m/z* 588 $[M + H]^+$; HRFABMS *m/z* 610.3801 $[M + Na]^+$ (calculated for $C_{28}H_{53}N_5O_8Na$, 610.3792).

Evaluation of Cytotoxicity. Cytotoxicity assays against five human tumor cell lines was performed at the Korea Research Institute of Chemical Technology. Cell lines used were A549 cells derived from human lung cancer tumors; SK-OV-3 cells derived from human ovarian cancer tumors; SK-MEL-2 cells derived from human skin cancer tumors; XF498 cells derived from human CNS cancer tumors; and HCT15 cells derived from human colon cancer tumors.

Hydrolysis and Derivatization of 1. Compound **1** (0.8

mg) was 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 (RT) and evaporated with MeOH several times to remove excess HCl. The obtained mixture was dissolved in 100 μL acetone and 50 μL H_2O. The solution was added to 40 μL of 1 M $NaHCO_3$ and then to 100 μL of 1% FDAA in acetone. The reaction mixture was heated to 40 °C for 2 h and then cooled to RT. The reaction was stopped 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. Two hundred microliters of 1% FDAA in acetone and 40 μL of 1.0 M $NaHCO_3$, were added to a 2.0 mL reaction vial containing 5 μM pure amino acid standard in 100 μL H_2O. The mixture was heated at 40 °C for 1 h in a Reacti-Therm^{TM} heating module. After the mixture was cooled to RT, 20 μL of 2 N HCl was added to stop the reaction, and the resulting solution was diluted with the mobile phase to make the chromatographic sample solution for TLC analysis.[20,27,28] Accordingly, two pairs of amino acid standards, including D-/L-aspartic acid and D-/L-glutamic acid were derivatized with Marfey's reagent (FDAA) to establish the reference R_f values for the corresponding amino acid-FDAA. Amino acid-FDAA conjugates were characterized by reversed-phase TLC (RP-18F_{254s}) (D-Asp-FDAA: R_f 0.76; L-Asp-FDAA: R_f 0.84; D-Glu-FDAA: R_f 0.67; L-Glu-FDAA: R_f 0.73).

Acknowledgments. This study was financially supported by a grant from the Post-Doc Development Program (2010) of the Pusan National University and the Marine Biotechnology Program, Ministry of Land, Transport, and Maritime Affairs. We thank Prof. Chung Ja Sim (Hannam University) and Dr. Kyung Sook Bae (Korea Research Institute of Bioscience and Biotechnology) for the identification of the sponge and fungus, respectively.

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