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Fellutamides A and B, Cytotoxic Peptides from a Marine Fish-Possessing Fungus *Penicillium fellutanum*

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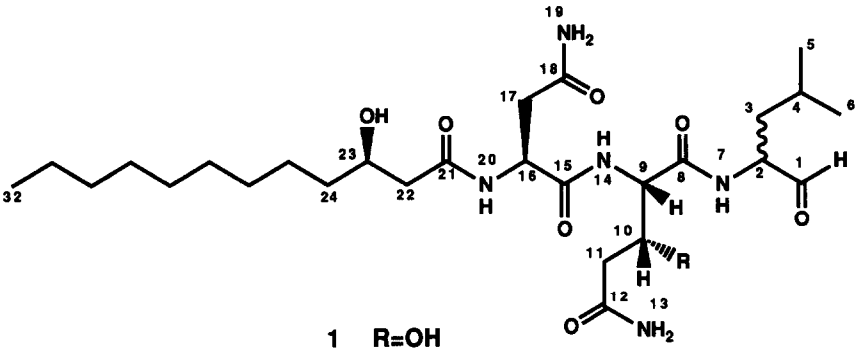
Abstract: Two cytotoxic peptides, fellutamides A and B, have been isolated from the cultured fungus *Penicillium fellutanum* Biourge which was isolated from the gastrointestine of the marine fish *Apogon endekataenia* Bleeker, and the structures were elucidated to be **1** and **2** by two-dimensional NMR and FAB MS/MS data

Recently increasing attention has been paid to marine microorganisms since they are potential source of bioactive substances for the development of new pharmaceutical agents [1] Several bioactive secondary metabolites have been isolated from marine bacteria,[2] fungi,[3] dinoflagellates,[4] or cyanophytes [5] During our studies on bioactive compounds from marine microorganisms,[6] fellutamides A (**1**) and B (**2**), two cytotoxic peptides have been isolated from a marine fish-possessing fungus *Penicillium fellutanum*[7] In this paper we describe the isolation and structure elucidation of **1** and **2**

The fungus *Penicillium fellutanum* was initially isolated from the gastrointestine of the marine fish *Apogon endekataenia* collected off Manazuru beach, Kanagawa, and grown in PYG broth (see Experimental Section) at 25 °C for 11 days The mycelium (480 g, wet weight from 6 L of culture) were extracted with methanol, and the extracts were partitioned between hexane and methanol The methanol soluble fraction was partitioned between ethyl acetate and water. The ethyl acetate soluble material was subjected to MPLC (medium pressure liquid chromatography) on ODS with MeOH/H_2O (70·30) followed by HPLC on ODS with MeOH/H_2O (70 30) to give fellutamides A (**1**, 0 006 %, wet weight) and B (**2**, 0 006 %)

Fellutamide A (**1**) was obtained as colorless amorphous powder The molecular formula was established to be $C_{27}H_{49}N_5O_8$ by HRFABMS [*m/z* 572 3664 $(M+H)^+$ for $C_{27}H_{50}N_5O_8$, Δ -0 5 mmu] From this molecular formula as well as the IR (3300, 1660, and 1540 cm^{-1}) and ^1H NMR data (Table 1), **1** was evident to be a peptide Compound **1** was, however, negative to ninhydrin test, thus suggesting to be a cyclic or an N-terminus-



1 R=OH
2 R=H

Table 1 ^{1}H and ^{13}C NMR Data of Fellutamide A (1) in DMSO-d_6

position		^{1}H		J (Hz)	^{13}C	
Leucinal						
	CHO	9 38	s		201 8	d
	NH	8 15	d	7 1		
	α	4 04	m		57 0	d
	β	1 49	t	7 3	36 4	t
	γ	1 65	m		23 7	d
	Me	0 86			22 9	q
					21 3	q
β-Hydroxyglutamine						
	CO				171 7	s
	NH	7 74	d	7 3		
	α	4 24	m		56 7	d
	β	4 38	m		67 5	d
	β-OH	5 11	m			
	γ	2 22	m		39 6	t
	$\underline{C}ONH_2$				172 3	s
	$CONH_2$	7 12	brs			
		6 79	brs			
Asparagine						
	CO				171 0	s
	NH	8 15	d	7 7		
	α	4 58	m		49 8	d
	β	2 58	m		36 9	t
		2 49	m			
	$\underline{C}ONH_2$				171 8	s
	$CONH_2$	7 44	brs			
		6 91	brs			
3-Hydroxydodecanoic acid						
	CO				171 2	s
	22	2 22	m		43 3	t
	23	3 79	m		67 4	d
	24~31	1 2~1 4			36 8	t
					31 2 (2C)	t
					29 0 (2C)	t
					28 9	t
					28 6	t
					22 0	t
	32	0 86			13 8	q

blocked peptide. The standard amino acid analysis of the hydrolysate of **1** with 6N HCl gave aspartic acid (Asp) and β-threo-hydroxyglutamic acid (βHGlu). These Asp and βHGlu proved to be generated from an asparagine (Asn) and a β-threo-hydroxyglutamine (βHGln) residues, which was revealed by the ^{1}H NMR data (Table 1) of **1** [δ 7.44 brs and 6.91 brs, NH_2-19; δ 7.12 brs and 6.79 brs, NH_2-13, ^{1}H-^{1}H COSY correlations· H-16/H_2-17 NH-20/H-16, H-9/H-10, H-10/H_2-11, and NH-14/H-9; HMBC[8] correlations: NH_2-19/C-17, NH_2-13/C-11, and OH-10/C-11] A β-hydroxy fatty acid was isolated from an etherial extract of acid hydrolysate of **1**. This fatty acid was identified as 3-hydroxydodecanoic acid (HDA) on the basis of ^{1}H NMR (δ 0 86, 3H, t, δ 1 2~1 4, 16H, δ 2.22, 2H, m; δ 3 79, 1H, m) and EIMS (*m/z* 198, M^+-H_2O) spectra. The presence of 2-amino-4-methylpentanal (leucinal) was suggested by the ^{1}H-^{1}H COSY (cross peaks. H-2/H_2-3, H_2-3/H-4, H-4/H_3-5, and H-4/H_3-6) and the HMBC (H-1/C-2, H-2/C-3, and H_2-3/C-2) spectra and confirmed by the fact that leucine was obtained on treatment of **1** with $KMnO_4$ followed by acid hydrolysis. From these results compound **1** was defined to consist of Asn, βHGln, leucinal, and HDA. Evidences for the linkage of these segments were provided by the HMBC correlations (see Experimental Section) to give rise to the structure of **1** [(HDA) - (Asn) - (βHGln) - (Leucinal)] The structure for fellutamide A (**1**) was substantiated by FAB MS/MS data (Fig 1) The spectrum (*m/z* 572, parent ion) gave daughter ion peaks (*m/z* 457, 313, and 198) due to ions generated by fission at the three amide bonds to confirm clearly the peptide sequence. The chiral GC analysis (Chirasil-Val, Alltech) clarified that the Asn residue in **1** was L-configuration and the Leu residue generated by $KMnO_4$ oxidation of **1** was DL-mixture The stereochemistry of βHGln was determined to be L-threo by chiral HPLC analysis The absolute configuration of C-3 in HDA was assigned as R by the optical rotation ($[\alpha]_D^{27}$ -17 0°, lit.[9] $[\alpha]_D^{20}$ -17.2°) The structure of fellutamide A was thus concluded to be **1**.

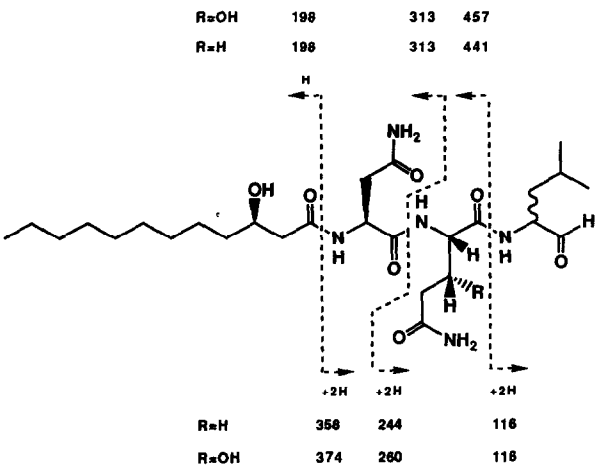


Fig 1 FAB MS/MS fragmentations of fellutamides A (1 R=OH) and B (2 R=H)

Fellutamide B (**2**) was obtained as colorless amorphous powder. The HRFABMS of **2** confirmed the molecular formula of $C_{27}H_{49}N_5O_7$ [*m/z* 556.3705 $(M+H)^+$, Δ+0.5 mmu], indicating that **2** may be a deoxygenated form of **1**. The ^1H NMR spectrum of **2** was identical with that of **1** except for the observation that β-proton (δ 4.48) of βHGln residue in **1** was replaced by two proton signals at δ 1.76 and 1.97. Amino acid analysis of acid hydrolysate of **2** gave one mole each of Asp and Glu The chiral GC analysis revealed that both of the Asp and Glu residues also bore the L configurations. From the NMR as well as the FAB MS/MS data (Fig. 1), fellutamide B (**2**) was elucidated to be a peptide[10] containing Gln instead of βHGln in **1**.

The fungus *Penicillium fellutanum* is not a marine specific one, since the same species have been also isolated from terrestrial sources[7]. Fellutamides A (**1**) and B (**2**) were potently cytotoxic against murine leukemia P388 (IC_{50}, 0 2 and 0.1 μg/mL, respectively) and L1210 (IC_{50}, 0 8 and 0 7 μg/mL, respectively) cells, and human epidermoid carcinoma KB (IC_{50}, 0.5 and 0.7 μg/mL, respectively) cells in vitro.

Experimental Section

General procedure. Optical rotations were determined on a JASCO DIP-370 polarimeter IR spectrum were measured on a JASCO IR Report-100 spectrometer. ^1H and ^{13}C NMR spectra were recorded on a JEOL EX-400 spectrometer in DMSO-d_6 The 2 49 ppm resonance of residual DMSO and 39 5 ppm of DMSO-d_6 were used as internal references, respectively Mass spectra were obtained on an HX-110 spectrometer by using glycerol as a matrix Wako C-200 silica gel (Wako Pure Chemical) was used for glass column chromatography and TLC was carried out on Merck silica gel GF_{254}.

Cultivation. The fungus *Penicillium fellutanum* Biourge was isolated from the gastrointestine of the fish *Apogon endekataenia* Bleeker (Kosuji-ishimochi in Japanese), which was collected off Manazuru beach, Kanagawa, Japan Cultures of *P fellutanum* were grown in PYG broth [peptone 1%, yeast extract 0 5%, glucose 2% in ASW (Jamarin S, Jamarin Laboratory), pH 7 0] Cultures were incubated at 25 °C for 11 days

Isolation. The harvested cells (480 g, wet weight) from 6L of culture were extracted with methanol (500 mL x 3), and the extracts were partitioned between hexane (500 mL) and methanol (500 mL) The methanol-soluble fraction was partitioned between EtOAc (300 mL) and H_2O (300 mL). The EtOAc layer was evaporated under reduced pressure. The residue was washed with EtOAc and the EtOAc-insoluble material was subjected to the reversed-phase MPLC [(μ-bondapak C_{18}, Waters, 15 x 100 mm, 10 μm, H_2O/MeOH (30 70); flow rate, 2 5 mL/min] and then purified by HPLC [Capcell Pak C_{18}, Shiseido, 10 x 250 mm, 5 μm; H_2O/MeOH (30 70), flow rate 1 0 mL/min] to afford fellutamides A (**1**, 32 mg) and B (**2**, 34 mg)

Fellutamide A (1). Colorless amorphous powder, $[\alpha]_D^{22}$ -12 7° (*c* 1 0, MeOH); IR (KBr) ν_{max} 3400, 1660, and 1540 cm^{-1}; ^1H and ^{13}C NMR (Table 1), FABMS (positive ion,

glycerol matrix) *m/z* 572 $(M+H)^+$, HRFABMS found *m/z* 572.3664 $(M+H)^+$, calcd for $C_{27}H_{50}N_5O_8$ 572.3669; ^1H-^{13}C long-range correlations obtained by the HMBC spectrum (H/C) H-1/C-2, H-2/C-3, H_2-3/C-2, NH-7/C-2, NH-7/C-8, H-9/C-10, H_2-11/C-10, OH-10/C-11, NH_2-13/C-11, NH-14/C-8, NH-14/C-15, H-16/C-17, H_2-17/C-16, H_2-17/C-18, NH_2-19/C-17, NH-20/C-15, NH-20/C-17, NH-20/C-21, OH-23/C-22, and H-23/C-24

Fellutamide B (2). Colorless amorphous powder, $[\alpha]_D^{21}$ -24 7° (*c* 0.5, MeOH); IR (KBr) ν_{max} 3400, 1660, and 1540 cm^{-1}, ^1H ($CDCl_3$) δ 0 86 (9H, H_3-5, H_3-6, and H_3-30), 1 2~1.4 (16H, H_2-24~31), 1.49 (2H, H_2-3), 1 65 (1H, H-4), 1 76 (1H, H-10), 1.97 (1H, H-10), 2.11 (2H, H_2-11), 2.22 (2H, H_2-22), 2.46 (1H, H-17), 2 56 (1H, H-17), 3.79 (1H, H-23), 4 04 (1H, H-2), 4 20 (1H, H-9), 4 59 (1H, H-16), 6 70 (1H, NH_2-13), 6 88 (1H, NH_2-19), 7.18 (1H, NH_2-13), 7.32 (1H, NH-7), 7 39 (1H, NH_2-19), 8 06 (2H, NH-14, 20), and 9 36 (1H, H-1) , ^{13}C NMR (DMSO-d_6) δ 13 8 (q, C-32), 21.3 (q, C-5 or 6), 22 0 (t), 23 0 (t, C-5 or 6), 23 9 (d, C-4), 28 6 (t), 28.7 (t, C-10), 28 9 (t), 29 0 (t), 31 2 (t), 31 3 (t, C-11), 36.8 (t), 36 9 (t, C-3 and C-17), 43 3 (t, C-22), 49.8 (d, C-16), 52 4 (d, C-9), 56.6 (d, C-2), 67 4 (d, C-23), 171.0 (s, C-15), 171 2 (s, C-21), 171 7 (s, C-8), 171 8 (s, C-18), 173 8 (s, C-11), and 201 6 (d, C-1), FABMS (positive ion, glycerol matrix) *m/z* 556 $(M+H)^+$, HRFABMS found 556 3705 $(M+H)^+$, calcd for $C_{27}H_{50}N_5O_7$ 556 3700; ^1H-^{13}C long-range correlations obtained by the HMBC spectrum (H/C)· H-2/C-1, H-2/C-3, H_2-3/C-4, H-4/C-3, H-2/C-8, H_2-10/C-9, H_2-10/C-12, H_2-11/C-12, NH-14/C-8, NH-14/C-9, NH-14/C-15, H-16/C-17, H-16/C-18, NH_2-19/C-17, NH-20/C-15, NH-20/C-16, NH-20/C-21, OH-23/C-22, and OH-23/C-24

Amino acid analysis by GC. Compound **1** or **2** (0 5 mg each) was hydrolyzed with 6 N HCl (1 mL) at 110 °C for 24 h in a sealed tube After the solvent was removed under reduced pressure, the residue was treated with 9% HCl/MeOH (1 mL) at 100 °C for 30 min in screw-capped reaction vial The reaction mixture was evaporated to dryness by a nitrogen stream, trifluoroacetic anhydride (TFAA)/CH_2Cl_2 (1 1, 1 mL) was added, and the mixture was allowed to stand at 100 °C for 5 min and evaporated The residue was dissolved in CH_2Cl_2 for GC analysis Capillary GC analyses were carried out using a Chiralsil-Val column (Alltech, 0 32 mm x 25 m) and nitrogen as a carrier gas. The program rate for the analyses was 50 to 200 °C at 4 °C/min Standard amino acids were also converted to the TFA/Me derivatives by the same procedure.

Determination of 3(R)-hydroxydodecanoic acid. Compound **1** (3 0 mg) was hydrolyzed with 6N HCl (1 5 mL) at 110 °C for 1 h in a sealed tube The hydrolysate was extracted with ether and ether-soluble fraction was evaporated under reduced pressure The residue was purified by SiO_2 column (5 x 80 mm) with $CHCl_3$/MeOH (12 1) to afford 3(R)-hydroxydodecanoic acid; $[\alpha]_D^{27}$ -17 0° (*c* 0 1, $CHCl_3$), ^1H NMR ($CDCl_3$) δ 0 86 (3H, t, J=7 1 Hz), 1 27 (14H), 1 48 (2H), 2 46 (1H, dd, J=8 8 and 16.5 Hz), 2 57 (1H, dd, J=3 3 and 16 5 Hz), and 4 03 (1H, m); EIMS *m/z* (relative intensity) 198 (M^+-H_2O, 1), 180 (3), 138 (6), 112 (6), 97 (11), 89 (100), 83 (23), 71 (41), 55 (28), 43 (29); HREIMS found m/z 198 1627 (M^+-H_2O), calcd for $C_{12}H_{22}O_2$ 198 1619.

Determination of L-threo-β-hydroxyglutamine. The standard amino acid analysis was performed with a Hitachi amino acid autoanalyzer (Model 835) by using a column (4.0 x 250 mm, #2617) at a flow rate of 0 275 mL/min with 0.2 N Na buffer and detected at 570 nm Each retention time of DL-erythro- and DL-threo-β-hydroxyglutamic acid was 23 8 and 21.3 min, respectively The chiral HPLC analysis was carried out by using Chiralpak WH (4 6 x 250 mm, Daicel Chemical Industries, Ltd.) at a flow rate of 1 0 mL/min with 1 0 mmol/L of $CuSO_4$ aqueous solution and detected at 254 nm Each retention time of L- and D-threo-β-hydroxyglutamic acid was 33 and 44 min, respectively

Oxidation of fellutamide A (1). To a suspension of **1** (1 0 mg) in water (0.5 mL), potassium permanganate (2 0 mg) was added and the mixture was vigorously stirred at room temperature After 30 min the reaction mixture was extracted with n-BuOH (10 mL x 2) and the organic layer was evaporated under reduced pressure to afford the oxidized compound (0.8 mg) of **1**, which was, after acid hydrolysis, subjected to amino acid analysis or chiral GC experiment

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