Fusaricidin A, a New Depsipeptide Antibiotic Produced by *Bacillus polymyxa* KT-8

Taxonomy, Fermentation, Isolation, Structure Elucidation and Biological Activity

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Fusaricidin A, a new depsipeptide antibiotic, was isolated from the culture broth of *Bacillus polymyxa* KT-8 obtained from the rhizosphere of garlic suffering from the basal rot caused by *Fusarium oxysporum*. The structure of fusaricidin A was determined by 1D and 2D NMR and MS experiments coupled with amino acid analysis to be a hexadepsipeptide containing 15-guanidino-3-hydroxypentadecanoic acid as a side chain. The absolute configuration of each amino acid residue was determined by chiral HPLC. Fusaricidin A is active against fungi and Gram-positive bacteria.

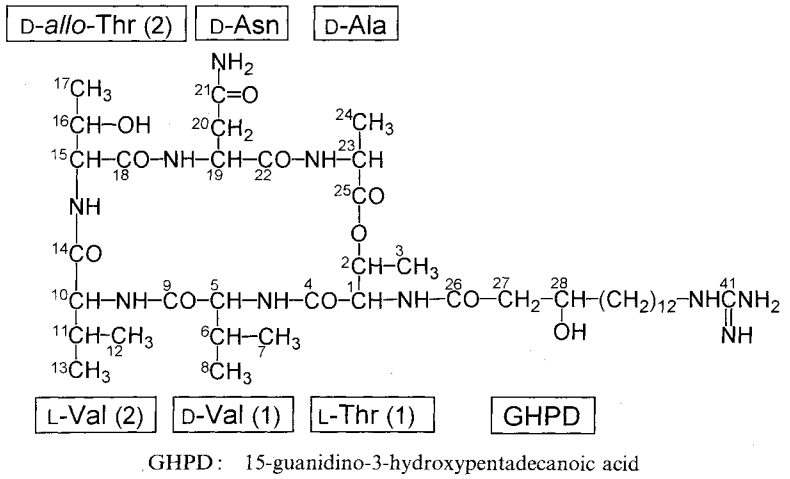
Biological control using microorganisms to suppress soil-borne plant pathogens has been successfully carried out in several recent studies[1,2]. How the diseases under studies were controlled by the microorganisms remained unclear but the antibiotics[3] elaborated by the microorganisms used were thought to play an important role in the suppression of the pathogens. These findings prompted us to search for microorganisms capable of controlling basal rot of garlic, a disease caused by *Fusarium oxysporum*. We have isolated several strains of bacteria from the rhizosphere of garlic[4,5] suffering from the disease. Among them, a *Bacillus subtilis* strain, FR-2, was found to produce new antifungal antibiotics, bacillopeptins A, B and C[6]. They are cyclic lipopeptides similar to bacillomycin L[7]. Another bacterial strain *Bacillus polymyxa* KT-8 has been shown to produce a new antibiotic complex which is more potent than bacillopeptins in its antimicrobial activity. At present, only the major component, designated fusaricidin A (**1**), has been isolated in pure form. This report describes the taxonomy of the producing organism, fermentation, isolation, structure elucidation and biological activities of fusaricidin A.

Results

Taxonomy of the Producing Organism

The producing organism, strain KT-8, was isolated from the rhizosphere of garlic cultured in Fukagawa city, Hokkaido, Japan. The taxonomic studies were carried out according to BERGEY's Manual of Systematic Bacteriology[8]. The taxonomic characteristics of the strain are given below.

Fig. 1. Structure of fusaricidin A.



GHPD: 15-guanidino-3-hydroxypentadecanoic acid

Table 1. Physiological characteristics of strain KT-8.

Temperature (°C) range for growth	10~37
pH range for growth	5~9
Relation to oxygen	Facultative anaerobic
OF test (Hugh Leifson method)	Fermentative
Nitrates reduction to nitrites	Positive
Denitrification test	Negative
Voges-Proskauer reaction	Positive
Methyl red test	Negative
Citrate utilization	Negative (KOSER's media) Weakly growth (CHRISTENSEN's media)
Indole production	Negative
H_2S production	Negative
Starch hydrolysis	Positive
Casein hydrolysis	Positive
Gelatin liquefaction	Slowly liquefied
Litmus milk	Peptonized slowly
Oxidase reaction	Negative
Catalase reaction	Positive
Urease activity	Negative
NaCl broth	No growth in 5% NaCl
Carbohydrate cleavage	
Acid and gas formations	L-Arabinose, D-glucose, D-xylose, D-mannose, D-fructose, D-galactose, maltose, sucrose, trehalose, lactose
Neither acid nor gas	D-Sorbitol, inositol, glycerol, D-mannitol

Morphological Characteristics

1) Vegetative cells on nutrient agar (30°C, 2 days): shape and size, rods and 0.6~0.7 × 2.3~3.4 μm; motility, positive; polymorphism, negative.

2) Spores (30°C, 1~3 days): shape, elliptical; position, central; sporangia, swollen at spore site.

3) Gram-stain, prevailing negative.

Cultural Characteristics

1) Colony on nutrient agar plate (30°C, 1~3 days) and slant (30°C, 1~7 days): pale brown, regular margin, smooth surface. Diffusible pigments were not observed.

2) Nutrient broth (30°C, 1~7 days): uniform, moderate growth, turbid with sediment.

Physiological Characteristics

The physiological characteristics are summarized in Table 1.

Based on the above characteristics, the microorganism was classified as a strain of *Bacillus polymyxa* and designated *Bacillus polymyxa* KT-8.

Fermentation

The growth of *B. polymyxa* KT-8 on nutrient agar slant culture for 2 days at 25°C was used to inoculate 500-ml Sakaguchi flasks each containing 125 ml of a production medium having the following composition: glucose 15 g, Kyoleopin (garlic extract, Wakunaga Pharmaceutical Co., Ltd.), 100 ml, and potato extract 1000 ml. The potato extract was prepared by extracting 200 g of fresh potato with 1000 ml of water for 1 hour at 100°C, filtering off the solid and adding water to 1000 ml. The flasks were shaken on a reciprocal shaker at 110 strokes per minute for 72 hours at 25°C. The production of the antibiotics was monitored by a disk method using *Fusarium oxysporum* HF 8801[4] as the test organism. The antibiotic concentration reached a maximum 3 days after inoculation.

Fig. 2. Isolation procedure for fusaricidin A.

Culture broth (8 liters)
↓ centrifuged at 8,000 rpm
Supernatant
↓ extracted with *n*-BuOH
n-BuOH extract
↓
Silica gel column chromatography
↓ eluted with $CHCl_3$-MeOH (4:1~2:1)
Sephadex LH-20 column chromatography
↓ eluted with MeOH
Preparative HPLC (ODS)
↓ eluted with a gradient of CH_3CN in 0.1% TFA
Preparative HPLC (ODS)
↓ eluted with 32% CH_3CN in 0.1% TFA
Fusaricidin A (20 mg)

Isolation and Purification

The isolation procedure for fusaricidin A is outlined in Fig. 2. Activity against *F. oxysporum* HF 8801 was monitored as described above during the isolation. The cultured broth (8 liters) was centrifuged at 8000 rpm and the supernatant was extracted twice with the same volume of *n*-BuOH. The *n*-BuOH extracts were combined and concentrated *in vacuo* to give an oily residue. This residue was mixed with 60 ml of silica gel and slurried with MeOH. After evaporating the solvent, the resulting dry powder was applied to a silica gel column (40 × 400 mm) pre-packed with $CHCl_3$-MeOH (4:1). The column was eluted stepwise with a mixture of $CHCl_3$-MeOH from 4:1 to 2:1. The active fractions were combined and concentrated *in vacuo* to afford a crude powder. This powder was dissolved in 20 ml of MeOH and applied to a Sephadex LH-20 column (40 × 400 mm) pre-packed with MeOH. The column was

eluted with MeOH. Evaporation of the solvent in the active fractions gave a crude mixture of antibiotics as colorless powder. This material was then purified by reverse phase preparative HPLC using a Capcell Pak C_{18} SG-120 column (4.6×250 mm, Shiseido Co., Ltd.) with 1% CH_3CN in 0.1% TFA as mobile phase A and 80% CH_3CN in 0.1% TFA as mobile phase B with a flow rate of 1 ml/minute. A gradient was run from 30% to 70% phase B in 10 minutes with UV detection at 215 nm to give crude fusaricidin A. For further purification of the crude fusaricidin A, preparative HPLC using the same column was repeatedly carried out under an isocratic condition with 32% CH_3CN in 0.1% TFA. Concentration and lyophilization of the eluates yielded pure fusaricidin A (20 mg).

Physico-chemical Properties

Fusaricidin A (**1**) was obtained as colorless amorphous powder, and its physico-chemical properties are summarized in Table 2. It is easily soluble in MeOH and DMSO, slightly soluble in water, and insoluble in $CHCl_3$ and *n*-hexane. It gave positive reaction with Biuret reagent and Sakaguchi reagent, but was negative in ninhydrin test. The color reactions and ^1H and ^{13}C NMR data suggested that **1** is a peptide having a guanidino group.

Table 2. Physico-chemical properties of fusaricidin A.

Appearance	Colorless amorphous powder
MP (°C)	201~219 (dec)
Molecular formula	$C_{41}H_{74}N_{10}O_{11}$
Molecular weight	882
FAB-MS (*m/z*)	883 $(M+H)^+$
HRFAB-MS (*m/z*)	
Calcd :	883.5617 (as $C_{41}H_{75}N_{10}O_{11}$)
Found:	883.5598 $(M+H)^+$
$[\alpha]_D^{21}$ (*c* 0.5, MeOH)	+12.8°
UV in MeOH	End absorption
Color reaction	
Positive :	Biuret, Sakaguchi
Negative:	Ninhydrin
Solubility	
Soluble :	MeOH, DMSO
Insoluble:	$CHCl_3$, *n*-hexane

Structure Elucidation

The molecular formula of fusaricidin A (**1**) was determined to be $C_{41}H_{74}N_{10}O_{11}$ from HRFAB-MS and ^{13}C NMR data. Complete acid hydrolysis of **1** was performed, and the resulting hydrolysate was extracted with $CHCl_3$. Amino acid analysis of the water layer of the hydrolysate showed the presence of two moles each of threonine and valine, and one mole each of alanine and aspartic acid (or asparagine). The Asp was revealed to have come from Asn residue in **1** as described below. The stereochemistries of the amino acids in the hydrolysate were examined by chiral HPLC using a SUMICHIRAL OA-5000 column. The results proved the presence of L-Val, D-Val, L-Thr, D-*allo*-Thr, D-Asn and D-Ala residues in **1**. The $CHCl_3$ layer of the hydrolysate contained a lipophilic compound, which gave a molecular ion peak at *m/z* 298 in the positive FAB-MS. This lipophilic compound gave a negative reaction with ninhydrin but exhibited positive Sakaguchi reaction. These results suggest the presence of a guanidino group in the molecule. Fusaricidin A itself also showed a negative ninhydrin test and did not react with diazomethane. These facts suggest that **1** is a cyclic lipopeptide composed of six amino acid residues and a lipophilic moiety which has a guanidino group.

The ^1H and ^{13}C NMR spectrum of **1** were measured in DMSO-d_6 and are shown in Figs. 3 and 4, respectively. The DEPT, ^1H-^1H COSY, HSQC, ROESY, HMBC spectra of **1** were also measured in DMSO-d_6 and carefully examined. Long-range ^1H-^1H and ^1H-^{13}C correlations observed in the ROESY and HMBC spectra are shown in Figs. 5 and 6, respectively. One guanidino carbon at δ_C 156.7, eight carbonyl carbons at δ_C 172.9, 172.4, 171.9, 170.8, 170.5, 170.2, 169.6, 168.3, and no olefinic carbons, were observed in the ^{13}C NMR spectrum of **1** (Fig. 4), indicating that **1** possesses one ring in the molecule since ten degrees of unsaturation are required from its molecular formula. Among nine proton signals observed in the low-field region of the ^1H NMR spectrum of **1** (Fig. 3), six protons at δ_H 8.43, 8.37, 8.24, 8.07, 7.26, 7.23 could be assigned to the α amide protons of the six amino acid residues by the analysis of ^1H-^1H COSY data. The two singlet protons at δ_H 7.45 and 7.03 were attributed to the terminal amide protons ($CONH_2$) of the Asn residue by the ROESY and HMBC experiments, establishing that the Asp in the hydrolysate derived from the Asn residue in **1**. One remaining triplet-like signal at δ_H 7.60 was ascribed in the similar manner to the guanidino ($NHC(=NH)NH_2$) proton. Seven methyl signals in the ^1H NMR spectrum of **1** were assigned as follows based on the above 2D NMR data. That is, the two doublet methyl signals at δ_H 0.82 and 0.76 were assigned to the methyls of one Val residue, which was temporarily named Val(1). The two doublet methyls at δ_H 0.87 and 0.85 were assigned to those of the other Val residue named Val(2), and the doublet methyl signal at δ_H 1.10 was ascribed to that of one Thr

Fig. 3. ^{1}H NMR spectrum of fusaricidin A (**1**) (400 MHz, DMSO-d_6).

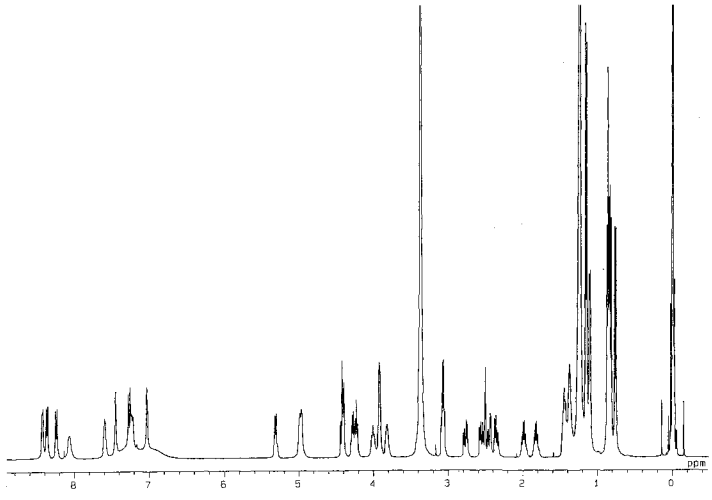
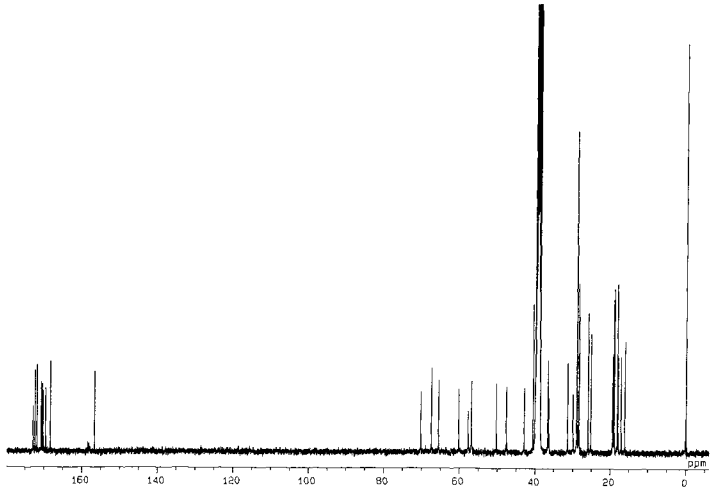


Fig. 4. ^{13}C NMR spectrum of fusaricidin A (**1**) (100 MHz, DMSO-d_6).



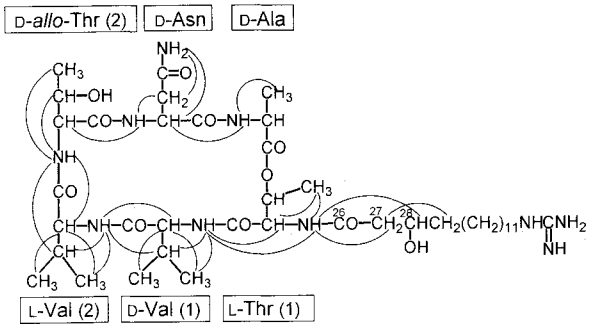
(or *allo*-Thr) residue named Thr(2). The doublet signal at δ_H 1.15 which corresponded to two methyls was attributed to that of Ala residue and that of the other Thr (or *allo*-Thr) residue named Thr(1). These results indicated that there is no methyl group in the lipophilic compound residue. The full assignments of all protons and carbons of the six amino acid residues in **1** were obtained as shown in Table 3 by detailed analyses of the DEPT, ^{1}H-^{1}H COSY, HSQC, ROESY and HMBC data of **1**.

The sequence of the six amino acids in **1** was determined by ROESY and HMBC experiments. As seen in Fig. 5, ROESY correlations were observed between the α proton of Thr(1) (δ_H 4.40) and the NH of Val(1) (δ_H 7.26), between the α-H of Val(1) (δ_H 4.42) and the NH of Val(2) (δ_H 8.37), between the α-H of Val(2) (δ_H 4.23) and the NH of Thr(2) (δ_H 8.43), between the α-H of Thr(2) (δ_H 3.92) and the NH of Asn (δ_H 8.07) as well as between α-H of Asn (δ_H 4.28) and the NH of Ala (δ_H 7.23). Furthermore, an HMBC correlation was observed between the β proton of Thr(1) at δ_H 5.32 and the carbonyl carbon of Ala at δ_C 170.5 (Fig. 6). These data indicate the presence of an ester linkage between the hydroxyl group of Thr(1) and the carbonyl group of Ala resulting in a depsipeptide ring. The linkage is evident from the chemical shifts of the Thr(1) carbons. A downfield shift of the β-CH carbon (C-2) to δ_C 70.2, and upfield shifts of α-CH (C-1) and γ-CH_3 (C-3) to δ_C 56.8 and 16.2, respectively, are attributed to the acylation. The shift of these signals was examined by comparing the corresponding carbon signals of Thr residues which are linked with normal peptide bonds in peptides such as bacillopeptins[6], pneumocandins[9] and thioxamycin.[10] Similar downfield and upfield shifts

were observed for the carbon signals of Thr residues in known depsipeptides such as protactin[11], micropeptins[12], WS9326A[13] and aselacins[14] in which each Thr is involved in an ester linkage through its β-CHOH. We would like to propose the name "Threonine Shifts" for these characteristic carbon chemical shift changes of the Thr residue in the situation like this. Based on the findings above described, the sequence of the amino acids in the depsipeptide ring of **1** was determined as cyclic [Thr(1)→Val(1)→Val(2)→Thr(2)→D-Asn→D-Ala] with an ester linkage between Thr(1) and D-Ala.

In order to assign the stereochemistries of Thr(1), Thr(2), Val(1) and Val(2) residues, a partial acid hydrolysis of **1** was carried out with 6N HCl at 105°C for 6 hours in a sealed tube. The resulting hydrolysate gave one peptide spot and four amino acid spots on two dimensional TLC. The peptide spot was eluted from TLC plates, and hydrolyzed. Analysis of the hydrolysate by chiral HPLC showed the presence of L-Thr (not D-*allo*-Thr) and D-Val. This dipeptide was also dansylated and the DNS-dipeptide obtained was hydrolyzed to give on two dimensional TLC one fluorescent spot, which was identified as DNS-Thr by comparison with the chromatograms of the authentic samples of DNS-Thr and DNS-Val under the same TLC conditions. The hydrolysate of the DNS-dipeptide was further examined by chiral HPLC. Only D-Val as a free amino acid was detected. This result indicates that the *N*-terminal amino acid of the dipeptide is L-Thr, *i.e.*, this dipeptide is L-Thr→D-Val. Therefore, Thr(1) is L-Thr, Val(1) is D-Val, Val(2) is L-Val, and Thr(2) is D-*allo*-Thr. Thus the sequence and stereochemistries of all amino acid residues in **1** were established as shown in Fig. 1.

Fig. 5. ^{1}H-^{1}H correlations of fusaricidin A (**1**) by ROESY experiment.



Since the atoms, $C_{25}H_{42}N_7O_9$, were ascribed to the six amino acid residues, the atoms, $C_{16}H_{32}N_3O_2$, could be allotted to the lipophilic compound residue by taking the molecular formula, $C_{41}H_{74}N_{10}O_{11}$, of **1** into account. As shown previously, after the proton and carbon signals of the six amino acid residues have been assigned, the remaining proton and carbon signals include one carbonyl (δ_C 171.9), one oxygen substituted methine (δ_H 3.82, δ_H(OH) 4.99, δ_C 67.5), 13 methylenes and one guanidino group. The analyses of the HMBC and ROESY spectra of **1** revealed the presence of the partial structures of $-CO-CH_2-CHOH-CH_2-$ and $-CH_2CH_2-NH(C=NH)NH_2$. From these data, the lipophilic compound residue was determined to be a fatty acid, 15-guanidino-3-hydroxypentadecanoic acid (**2**). As described above, the fatty acid in the hydrolysate of **1** yielded in the positive FAB-MS a molecular ion peak of m/z 298 $(M+H)^+$ which is smaller than that of **2** by 18 mass units. This result is consistent with the explanation that during complete acid hydrolysis of **1**, **2** lost 18 mass units by dehydration.

The fatty acid side chain was determined to attach to the L-Thr(1) residue through an amide bond by the analyses of the HMBC and ROESY spectra of **1**; that is, an ^{1}H-^{13}C correlation was observed from the NH of L-Thr(1) to the CO (C-26) of the fatty acid residue in the HMBC spectrum (Fig. 6), and this NH of L-Thr(1) was also observed to correlate with the methylene (C-27)

Fig. 6. Long-range ^{1}H-^{13}C correlations of fusaricidin A (**1**) by HMBC experiment.

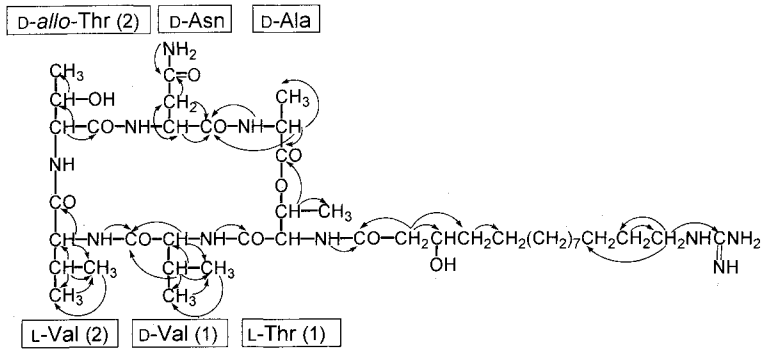


Table 3. ^{1}H and ^{13}C NMR chemical shifts of fusaricidin A (**1**) in DMSO-d_6.

Moiety	Position	δ_C	δ_H (J in Hz)
L-Thr(1)	1	56.8[a]	4.40 dd (8.6, 2.3)
	2	70.2	5.32 dq (6.2, 2.3)
	3	16.2	1.15 d (6.8)
	4	168.3	
	1-NH		8.24 d (8.3)
D-Val(1)	5	56.9[a]	4.42 dd (9.5, 8.3)
	6	31.4	1.83 m
	7	18.2	0.76 d (6.6)
	8	19.0	0.82 d (6.8)
	9	170.8	
	5-NH		7.26 d (9.0)
L-Val(2)	10	57.8	4.23 dd (7.2, 7.2)
	11	30.0	1.99 m
	12	18.0	0.87 d (6.1)
	13	19.2	0.85 d (6.1)
	14	172.9	
	10-NH		8.37 d (7.6)
D-*allo*-Thr(2)	15	60.2	3.92 br s
	16	65.6	3.93 br s
	17	19.5	1.10 d (5.8)
	18	170.2	
	15-NH		8.43 d (4.6)
	16-OH		4.97 br s
D-Asn	19	50.3	4.28 dd (13.9, 6.6)
	20	36.5	2.56 dd (15.1, 6.6)
			2.77 dd (15.0, 6.5)
	21	172.4	
	22	169.6	
	19-NH		8.07 br s
	21-NH_2		7.03 s
			7.45 s
D-Ala	23	47.7	4.01 m
	24	17.2	1.15 d (6.8)
	25	170.5	
	23-NH		7.23 d (8.3)
GHPD	26	171.9	
	27	43.0	2.36 dd (13.5, 6.8)
			2.45 dd (13.5, 5.0)
	28	67.5	3.82 br s
	29	36.7	1.38 br s
	30	25.2	~1.24 br s
	31~37	28.5	
		28.9	
		29.0	
	38	26.0	
	39	28.3	1.45 m
	40	40.6	3.07 dd (12.8, 6.7)
	41	156.7	
	40-NH		7.60 t-like
	28-OH		4.99 br s

[a] Assignments may be interchanged.
GHPD: 15-guanidino-3-hydroxypentadecanoic acid.

protons and with the methine (C-28) proton in the fatty acid residue in the ROESY spectrum (Fig. 5).

Based on these results described above, the total structure of fusaricidin A has thus been elucidated as shown in Fig. 1. The absolute configuration of C-3 of 15-guanidino-3-hydroxypentadecanoic acid remains to be determined.

Table 4. Antimicrobial activities of fusaricidin A.

Test organism	MIC (μg/ml)
Staphylococcus aureus FDA 209P	<0.78
S. aureus Smith	<0.78
Micrococcus luteus IFO 3333	<0.78
Bacillus subtilis ATCC 6633	3.12
Escherichia coli NIHJ	>100
Klebsiella pneumoniae KC-1	>100
Pseudomonas aeruginosa IFO 3445	>100
Serratia marcescens IFO 3736	>100
Candida albicans IFO 1594	>100
Saccharomyces cerevisiae HUT 7099	>100
Fusarium oxysporum HF 8801 (pathogenic to garlic)	1.56
F. oxysporum HF 8835 (nonpathogenic to garlic)	1.56
Aspergillus niger HUT 2016	3.12
A. oryzae IFO 4214	3.12
Penicillium thomii	3.12

Biological Activities

Antimicrobial activities of fusaricidin A (**1**) were determined by a two-fold serial agar dilution method using Mueller-Hinton media for bacteria after 24 hours incubation at 37°C, and using Sabouraud media for yeasts and fungi after 48 hours incubation at 28°C. The resulting MICs are shown in Table 4. As was expected, **1** showed strong activity against a wide variety of fungi. **1** also exhibited remarkably strong activity against Gram-positive bacteria such as *Staphylococcus aureus* and *Micrococcus luteus*. However, **1** showed no activity even at 100 μg/ml against all Gram-negative bacteria tested.

Discussion

In the present study, we have isolated a new antifungal and antibacterial antibiotic, fusaricidin A (**1**), from the culture broth of a bacterial strain *Bacillus polymyxa* KT-8. Its structure has been elucidated to be a cyclic hexadepsipeptide containing a unique 15-guanidino-3-hydroxypentadecanoic acid side chain (Fig. 1).

Among the known peptide antibiotics, fusaricidin A structurally resembles KT-6291A[15] which was isolated from an unidentified strain *Bacillus* sp. KB-291. The structure of KT-6291A was determined as cyclic [Thr (or *allo*-Thr) (1)→Val→Val→*allo*-Thr (or Thr) (2)→Asn→Ala] with an ester linkage between Ala and Thr (or *allo*-Thr) (1) and with a side chain of the same fatty acid as fusaricidin A linking to the Thr (or *allo*-Thr) (1) through an amide bond. However, the distinction of the positions of Thr and *allo*-Thr in KT-6291A was not be made, nor were reported the absolute configurations of the amino acid residues. Furthermore, KT-6291A was not active even at 100 μg/ml against *Fusarium oxysporum* f. sp. *cucumerinum*, whereas fusaricidin A showed

activity at 1.56 μg/ml against *F. oxysporum* HF 8801 or *F. oxysporum* HF 8835. Fusaricidin A displayed strong activity against not only fungi but also Gram-positive bacteria especially *Staphylococcus aureus* FDA 209P, *S. aureus* Smith and *Micrococcus luteus* IFO 3333 (Table 4). These results suggest that fusaricidin A might have activity against MRSA.

Experimental

General

Two dimensional TLC was carried out on Merck Kiesel gel 60 F-254 plates (Art. No. 5715) in *n*-BuOH-AcOH-H_2O (3:1:1) for the first dimension and in phenol-H_2O (4:1) for the second dimension. Silica gel column chromatography was performed using Wakogel C-200. Spectral data were recorded on the following instruments: ^1H and ^{13}C NMR, JEOL JNM-A400 spectrometer; low and high resolution FAB-MS, JEOL JMS-SX102 spectrometer; UV-vis, JASCO V-520 spectrophotometer; optical rotation, Union PM-101 automatic digital polarimeter.

Analysis of the Amino Acids

Fusaricidin A was completely hydrolyzed with 6N HCl at 105°C for 24 hours in a sealed tube and the reaction mixture was extracted with $CHCl_3$. The water layer of the hydrolysate was analyzed on a Hitachi L-8500 amino acid autoanalyzer.

Absolute Configuration of the Amino Acids

Fusaricidin A was completely hydrolyzed in the same manner as that for the above amino acid analysis. Amino acids in the hydrolysate were separated by two dimensional TLC, and the position of each amino acid on the TLC plate was determined by spraying with very dilute fluorescamine in acetone followed by detection of fluorescence under 360 nm UV light. The spots of amino acids on the TLC plate were scraped off and extracted with water from the silica gel. Each amino acid was analysed by chiral HPLC and its HPLC chromatogram was compared with that of the authentic chiral amino acid under the same HPLC condition. The chiral HPLC was carried out under the following conditions: column, SUMICHIRAL OA-5000, 4.6 × 150 mm; mobile phase, 1.0 mM $CuSO_4$ for Ala and Val, 0.5 mM $Cu(OAc)_2$ for Thr and *allo*-Thr, 2.0 mM $CuSO_4$ in H_2O-MeOH (85:15) for Asp; flow rate, 1.0 ml/minute for Ala, Val and Asp, 0.5 ml/minute for Thr and *allo*-Thr; detection, UV 254 nm; temperature, 35°C.

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References

1) WELLER, D. M.: Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annual Review of Phytopathology 26: 379~407, 1988
2) PARK, C.; C. T. PAULITZ & R. BAKER: Biocontrol of *Fusarium* wilt of cucumber resulting from interactions between *Pseudomonas putida* and nonpathogenic isolates of *Fusarium oxysporum*. Phytopathology 78: 190~194, 1988
3) HOMMA, Y. & T. SUZUI: Role of antibiotic production in suppression of radish damping-off by seed bacterization with *Pseudomonas cepacia*. Ann. Phytopath. Soc. Japan 55: 643~652, 1989
4) YOO, S. J.; K. KOBAYASHI, A. OGOSHI, H. SUGIMOTO & Y. KAJIMURA: Biological control of basal rot of garlic. The suppressive effect of antagonistic bacteria and nonpathogenic *Fusarium oxysporum* in inoculated soil. Ann. Phytopath. Soc. Japan 57: 123~124, 1991
5) FURUTANI, H.; H. SATO, A. SUZUKI, M. TSUKAMOTO & Y. KAJIMURA (Wakunaga Pharmaceutical Co., Ltd.): Biocontrol agents against plant pathogens. Jpn. Kokai 253827 ('94), Sep. 13, 1994
6) KAJIMURA, Y.; M. SUGIYAMA & M. KANEDA: Bacillopeptins, new cyclic lipopeptide antibiotics from *Bacillus subtilis* FR-2. J. Antibiotics 48: 1095~1103, 1995
7) PEYPOUX, F.; M. POMMIER, B. DAS, F. BESSON, L. DELCAMBE & G. MICHEL: Structures of bacillomycin D and bacillomycin L, peptidolipid antibiotics from *Bacillus subtilis*. J. Antibiotics 37: 1600~1604, 1984
8) SNEATH, P. H. A.: Section 13. Endospore-forming Gram-positive rods and cocci. *In* BERGEY's Manual of Systematic Bacteriology. Volume 2. *Ed.*, P. H. A. SNEATH *et al.*, pp. 1104~1138, Williams & Wilkins Co., 1986
9) HENSENS, O. D.; J. M. LIESCH, D. L. ZINK, J. L. SMITH, C. F. WICHMAN & R. E. SCHWARTZ: Pneumocandins from *Zalerion arboricola*. III. Structure elucidation. J. Antibiotics 45: 1875~1885, 1992
10) YUN, B. S.; T. HIDAKA, K. FURIHATA & H. SETO: Microbial metabolites with *tipA* promoter inducing activity. III. Thioxamycin and its novel derivative, thioactin, two thiopeptides produced by *Streptomyces* sp. DP94. J. Antibiotics 47: 1541~1545, 1994
11) HANADA, M.; K. SUGAWARA, Y. NISHIYAMA, H. KAMEI, M. HATORI & M. KONISHI: Protactin, a new antibiotic metabolite and a possible precursor of the actinomycins. J. Antibiotics 45: 20~28, 1992
12) OKINO, T.; M. MURAKAMI, R. HARAGUCHI, H. MUNEKATA, H. MATSUDA & K. YAMAGUCHI: Micropeptins A and B, plasmin and trypsin inhibitors from the blue-green alga *Microcystis aeruginosa*. Tetrahedron Letters 34: 8131~8134, 1993
13) SHIGEMATSU, N.; K. HAYASHI, N. KAYAKIRI, S. TAKASE, M. HASHIMOTO & H. TANAKA: Structure of WS9326A, a novel tachykinin antagonist from a *Streptomyces*. J. Org. Chem. 58: 170~175, 1993
14) HOCHLOWSKI, J. E.; P. HILL, D. N. WHITTERN, M. H. SCHERR, R. R. RASMUSSEN, S. A. DORWIN & J. B. MCALPINE: Aselacins, novel compounds that inhibit binding of endothelin to its receptor. II. Isolation and elucidation of structures. J. Antibiotics 47: 528~535, 1994
15) TAKAYAMA, Y. & S. YAMADA (Mitsubishi Yuka Co., Ltd.): New antibiotics, KT-6291A and KT-6291B. Jpn. Kokai 275898 ('90), Nov. 9, 1990