J. Antibiot. 61(6): 387–393, 2008

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

Pargamicin A, a Novel Cyclic Peptide Antibiotic from *Amycolatopsis* sp.

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Received: February 29, 2008 / Accepted: June 13, 2008

Abstract A novel cyclic peptide antibiotic, pargamicin A was isolated from the culture broth of an actinomycete strain. The producing organism, designated ML1-hF4, was identified as a member of the genus *Amycolatopsis*. Pargamicin A was identified as a novel cyclic hexapeptide antibiotic containing piperazic acid by various spectroscopic analyses. Pargamicin A showed potent antibacterial activity against *Staphylococcus aureus* strains including MRSA and *Enterococcus faecalis*/*faecium* strains including VRE.

Keywords pargamicin A, cyclic peptide, *Amycolatopsis* sp., MRSA, VRE

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis*/*faecium* (VRE) are among the most problematic pathogens in hospital-acquired infections. Moreover, two clinical isolate strains of vancomycin-resistant *S. aureus* (VRSA) have been isolated from patients in USA since 2002 [1]. Today, only a few drugs such as linezolid [2], quinupristin/dalfopristin [2] and daptomycin [2] are clinically used for both VRE and MRSA infections. Therefore, the development of novel drugs effective for VRE and MRSA (VRSA) is greatly desired.

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In the course of screening for new antimicrobial substances from microorganisms, we found that an actinomycete strain isolated from a soil collected in Tokyo, Japan, produces a new cyclic peptide antibiotic, which was named pargamicin A (**1**, Fig. 1). Compound **1** showed strong antibacterial activity against MRSA and VRE.

In this paper, we describe the identification of the producing organism, isolation, fermentation, structure elucidation and biological activities of **1**.

Materials and Methods

General

Optical rotation was measured with a Perkin-Elmer model 241 polarimeter. UV spectra were recorded with a Hitachi 557 spectrophotometer. The IR spectrum was recorded with a Horiba FT-210 Fourier transform infrared spectrometer. The ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL JNM-A500 spectrometer at 24°C using TMS as an internal

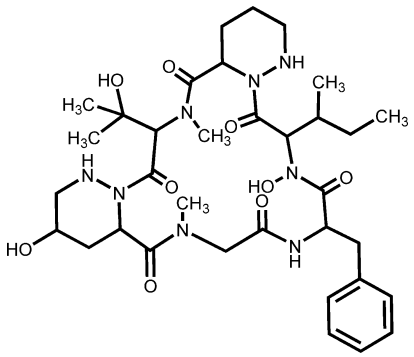

Fig. 1 Structure of pargamicin A (**1**).

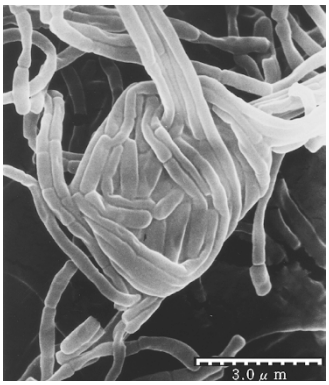

Photo 1 Scanning electron micrograph of strain ML1-hF4 grown on sucrose-nitrate agar for 12 days at 27°C.

reference. The mass spectrum was recorded with a JEOL JMS-SX102 mass spectrometer.

Taxonomy

The pargamicin A-producing organism, strain ML1-hF4 was isolated from a soil sample collected at Shinagawa, Tokyo, Japan. Morphological, cultural and physiological properties of the strain ML1-hF4 were examined according to the methods described by SHIRLING and Gottlieb [3], and Waksman [4]. Detailed observation of mycelial morphologies was performed with the use of a scanning electron microscope (Model S-570, Hitachi) after strain ML1-hF4 was incubated on sucrose-nitrate agar at 27°C for 12 days. Chemical analysis of the cell wall was performed using TLC according to the method of Staneck and Roberts [5]. Whole-cell sugars were determined by the methods of Lechevalier and Lechevalier [6]. Menaquinones were extracted following the method of Collins *et al.* [7], and analyzed by LC-MS (model M-1200H, Hitachi) with a CAPCELL PAK AG, C_{18} column (4.6 mm×150 mm, Shiseido Co., Ltd., Japan) using MeOH - isopropanol (2 : 1, v/v) as the mobile phase. Phospholipids and mycolic acids were analyzed by the procedures of Minnikin *et al.* [8, 9]. A total DNA sample of strain ML1-hF4 was prepared as reported [10]. The 16S ribosomal RNA gene (16S rDNA) was amplified by polymerase chain reaction (PCR) using genomic DNA of strain ML1-hF4 and sequenced [11]. A homology search for the most related sequences was performed using the BLAST algorithm on the DDBJ/Genebank/EMBL.

Fermentation

A slant culture of the pargamicin-producing organism was inoculated into a 500-ml baffled Erlenmeyer flask containing 110 ml of a seed medium consisting of glycerol 1.0%, dextrin 1.0%, Soytone Peptone (Difco) 0.5%, yeast extract 1.5%, $(NH_4)_2SO_4$ 1.0% and $CaCO_3$ 1.0% in deionized water (pH 7.4 before sterilization). The culture was incubated on a rotary shaker (180 rpm) at 30°C for 3 days. A 2.5 ml aliquot of the seed culture were transferred into a 500-ml Sakaguchi flask containing 125 ml of a producing medium consisting of galactose 2.0%, dextrin 2.0%, glycerol 1.0%, Soytone Peptone (Difco) 0.5%, corn steep liquor 0.5% and $CaCO_3$ 0.2% in deionized water. The fermentation was carried out on a reciprocal shaker (130 rpm) at 27°C for 5 days.

Analytical Procedure

The content of **1** in the fermentation broth and the various purification steps was monitored with reversed-phase HPLC and silica gel TLC. HPLC was performed with a CAPCELL PAK, AG, C_{18} column (4.6×150 mm, Shiseido Co., Ltd., Japan; mobile phase, $CH_3CN : H_2O = 35 : 65$; flow rate, 2.0 ml/minute; column temperature, 50°C; detection, UV at 238 nm). Compound **1** was eluted at 7.5 minutes. The antibiotic was detected on TLC plates with a solution of mixture of molybdophosphoric acid and sulfuric acid in MeOH and UV light (254 nm). Pargamicin A showed TLC (Kieselgel 60 F_{254}, Art. No. 1.05715, Merck) Rf values of 0.82 and 0.44 with solvent system of $CHCl_3$: MeOH = 19 : 1 and EtOAc : MeOH = 4 : 1, respectively.

Biological Activity

The MIC of pargamicin A were examined by serial agar dilution method using Mueller-Hinton agar (Difco) for bacteria including *Enterococci* and *Staphylococcus aureus* [12]. The MIC was observed against bacteria and yeast after incubation for 18 hours at 37°C.

Results and Discussion

Taxonomic Features of Strain ML1-hF4

Strain ML1-hF4 produced well-branched substrate mycelia. Aerial hyphae were straight or flexuous. Both substrate and aerial hyphae exhibited nocardioform fragmentation. The spore was cylindrical with a smooth surface and 0.4~0.6×0.8~1.6 μm in size (Fig. 1). No synnemata, sclerotia, sporangia or motile spores were observed.

The taxonomic properties of strain ML1-hF4 are shown in Table 1. Whole-cell hydrolysates of strain ML1-hF4 contained *meso*-diaminopimelic acid of the cell wall and galactose and arabinose. The predominant menaquinone was MK-9(H_4). The phospholipid pattern was type PII: phosphatidylethanolamine was present, but neither phosphatidylcholine nor unknown glucosamine-containing

Table 1 Taxonomic characteristics of strain ML1-hF4

Characteristics	Strain ML1- hF4
Amino acid of cell wall	*meso*-diaminopimelic acid
Whole cell sugar pattern	A (arabinose, galactose)
Major menaquinones	MK-9(H_4)
Phospholipid composition	PII
Mycolic acids	None
Fragmentation of hyphae	Positive
Color of aerial mycelium	White
Color of substrate mycelium	Pale yellow～pale brown
Soluble pigment	None

phospholipids were found. Mycolic acids were absent. The aerial mycelia of strain ML1-hF4 were white on various agar media. The substrate mycelia were pale yellow to pale brown. Soluble pigments were not produced. Permissive temperature ranges for growth of the strain were 20～37°C. The optimal temperature for growth of the strain ML1-hF4 was 30°C.

The partial 16S rDNA sequence (457 bp, positions 27～510, *Escherichia coli* numbering system [13]) of strain ML1-hF4 showed high similarity value with members of the genus *Amycolatopsis*, such as *Amycolatopsis albidoflavus* AB327251 (444/457, 97%), *A. halotorelans* DQ000196 (443/458, 96%), *A. echigoensis* AB248535 (443/459, 96%) and *A. niigatensis* AB248537 (441/458, 96%).

These phenotypic and genotypic properties suggested that strain ML1-hF4 belonged to the genus *Amycolatopsis* [14].

Therefore, the strain was identified as an *Amycolatopsis* sp. and designated *Amycolatopsis* sp. ML1-hF4. Detailed taxonomic study of strain ML1-hF4 is in progress.

Fermentation and Isolation

The fermentation broth (5.0 liters) was separated to the mycelial cake and supernatant by centrifugation. The supernatant was applied on a Diaion HP20 (Mitsubishi Chemical Co.) column (38×155 mm). The column was washed with deionized water (450 ml) and 50% aq MeOH (450 ml). The active principle was eluted with 80% aq MeOH (450 ml) and 80% aq Me_2CO (450 ml). The active solutions were collected and concentrated *in vacuo* to yield a brown oil (576 mg). The brown oil containing the active substance was chromatographed on a silica gel column with stepwise development comprising successive 300 ml aliquots of EtOAc : MeOH mixtures of compositions 10 : 1, 9 : 1, 4 : 1 and 2 : 1, respectively. The active fractions were collected and concentrated *in vacuo* to give a pale brown powder (158 mg). The active material was further purified by reversed phase HPLC developing with CH_3CN : H_2O=35 : 65 (CAPCELL PAK C18 UG120, 4.6×150 mm, Shiseido Co., Ltd. Japan, flow rate, 8.0 ml/minute). The active fractions were collected and concentrated *in vacuo* to give **1** (78 mg, Fig. 2) as colorless powder.

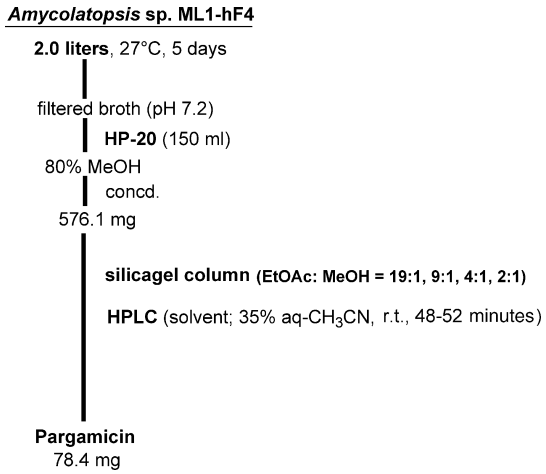

Fig. 2 Isolation procedure of pargamicin A (**1**).

Table 2 Physico-chemical properties of pargamicin A (**1**)

Molecular formula	$C_{34}H_{52}N_8O_9$
FAB-MS (*m/z*)	717 $(M+H)^+$
	715 $(M-H)^-$
HRFAB-MS Calcd:	717.3936 (as $C_{34}H_{53}N_8O_9$)
Found:	717.3917 $(M+H)^+$
$[\alpha]_D^{25}$	+11.3° (*c* 0.927, MeOH)
UV $\lambda_{max}^{MeOH\ nm\ (\varepsilon)}$:	end
IR ν_{max} (KBr) cm^{-1}	3600～3200, 2968, 2933, 1624, 1545, 1493, 1464, 1406, 1275, 1255, 1190, 1153, 1128, 1092, 964, 920
Color reaction	I_2, molybdophosphoric acid-sulfuric acid
positive	$FeCl_3$, Rydon-Smith

Structure Elucidation of Pargamicin

The physico-chemical properties of pargamicin are summarized in Table 2. The molecular formula of pargamicin was determined to be $C_{34}H_{52}N_8O_9$ by HRFAB-MS. Its characteristic IR absorbances at 1624 and 1545 cm^{-1} and the positive color reaction with Rydon-Smith reagent revealed the presence of amide moiety, and the positive reaction in the $FeCl_3$ color test also indicated the presence of an *N*-hydroxy moiety. The relationships between specific proton and carbon signals in the ^1H- and

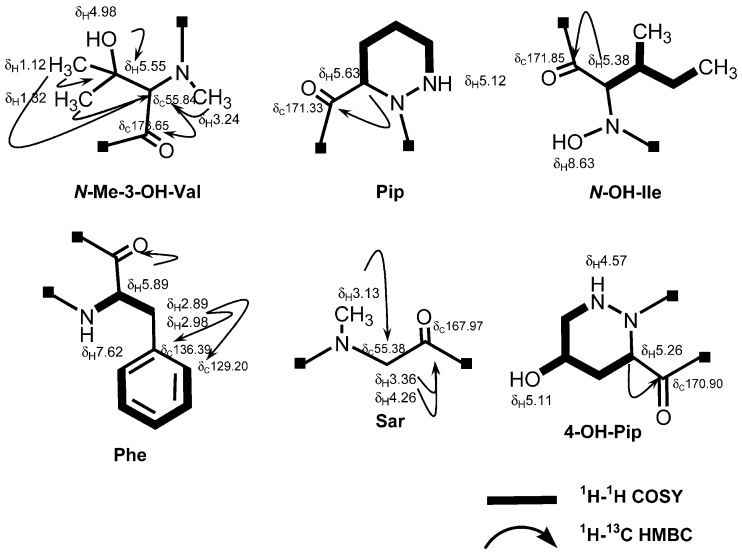

Fig. 3 Partial structures of pargamicin A (**1**).

^{13}C-NMR data of pargamicin were established by DEPT and HMQC spectra. These spectra revealed the presence of six carbonyls, six sp^2 phenyl, twelve sp^3 methines, eight methylenes and six methyl groups. Analyses of ^{1}H-^{1}H COSY and HMBC spectra revealed six partial structures, as shown in Fig. 3. The ^{1}H connection from NH (δ_H 7.62) to methylene protons (δ_H 2.89 and 2.98) and long range correlation from these methylene protons to phenyl carbons at δ_C 136.29 and 129.20, together with that of an α-methine (δ_H 5.89) to δ_C 172.74 showed the presence of a phenylalanine (Phe) moiety. The long range HMBC couplings between an *N*-methyl (δ_H 2.89) and a methylene at δ_C 55.38 and the methylene protons (δ_H 3.36, 4.26) and δ_C 167.97 revealed the presence of *N*-methylglycine (sarcosine: Sar). The ^{1}H-^{1}H spin network from an α-methine (δ_H 5.26) to NH (δ_H 4.57) as well as hydroxyl proton (δ_H 5.11) and the long range coupling between the α-methine and δ_C 170.90 suggested the presence of 4-hydroxypiperazic acid (4-OH-Pip) residue. The long range correlations from dimethyl protons (δ_H 1.12, 1.32) and hydroxyl proton (δ_H 4.98) to a quartanary carbon (δ_C 73.42) and α-methine (δ_C 55.84), together with those from the methine (δ_H 5.55) to δ_C 173.65 and from *N*-methyl group (δ_H 3.24) to the methine indicated the presence of an *N*-methyl-3-hydroxyvaline (*N*-Me-3-OH-Val) residue. The proximity of an α-methine (δ_H 5.63) to an NH proton (δ_H 5.12) and the long range correlations between α-methine and δ_C 171.33 showed the presence of a piperazic acid residue (Pip). Lastly, COSY correlation indicated a connection between branched methyl protons (δ_H 1.33 t, 0.80 d) to an α-methine (δ_H 5.38, d) while a long range HMBC correlation from the same α-methine to a carbonyl

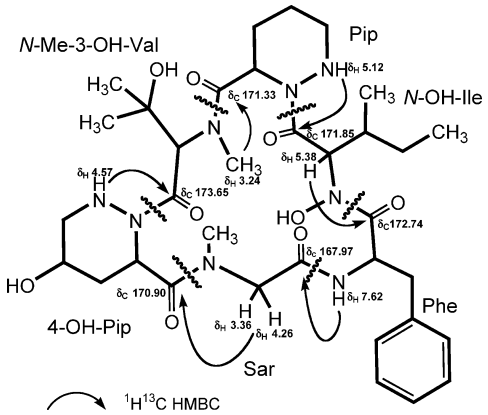

Fig. 4 Selected HMBC correlations of pargamicin A (**1**).

at δ_C 167.97, revealed the presence of an *N*-substituted isoleucine moiety. The remaining exchangeable proton (δ_H 8.63, s) did not show any correlations in a ^{1}H-^{15}N HMQC experiment, suggesting the presence of an *N*-hydroxyl group. This amino acid residue was therefore postulated to be *N*-hydroxy-isoleucine (*N*-OH-Ile).

The connectivity between these residues was established by the key HMBC correlations illustrated in Fig. 4 as follows; NH proton (δ_H 7.62) of Phe to C=O carbon (δ_C 167.97) of Sar, methylene protons (δ_H 3.36, 4.26) of Sar to C=O carbon (δ_C 170.90) of 4-OH-Pip, NH proton (δ_H 4.57) of 4-OH-Pip to C=O carbon (δ_C 173.65) of *N*-Me-3-OH-Val, *N*-Me protons (δ_H 3.24) of *N*-Me-3-OH-Val to C=O carbon (δ_C 171.33) of Pip, NH proton (δ_H 5.12) of Pip to C=O (δ_C 171.85) of *N*-OH-Ile and α-methine proton (δ_H 5.38) of *N*-OH-Ile to C=O carbon (δ_C 172.74) of Phe, by HMBC spectrum. These results revealed that

Table 3 ^{1}H- and ^{13}C-NMR data for Pargamicin A (**1**) in $CDCl_3$

Position	δ_C	δ_H	*J* (Hz)		Position	δ_C	δ_H		*J* (Hz)
4OHPip	α	48.06 d	5.26 d	6.2	NOHIle	α	60.97 d	5.38 d	6.0
	β	31.40 t	2.11 m			β	34.91 d	2.11 m	
			2.51 d	14.3		β-Me	15.33 q	0.59 t	6.9
	γ	61.89 d	3.93 m			γ	25.43 t	1.33 m	
	γ-OH		5.11 br s			δ	12.92 q	0.80d	7.4
	δ	52.85 t	2.95 m			CO	171.85 s		
			3.04 m			NOH		8.63 s	
	CO	170.90 s							
	NH		4.57 dd	2.5, 13.0	Phe	α	47.71 d	5.89 ddd	6.0, 10.4, 10.4
NMe_3OHVal	α	55.84 d	5.55 s			β	37.61 t	2.89 dd	6.0, 13.3
	β	73.42 s						2.98 m	10.4, 13.3
	β-OH		4.98 s			1	136.39 s		
	γ	25.54 q	1.12 s			2/6	129.20 d	7.22 m	
	γ	28.71 q	1.32 s			3/5	128.37 d	7.22 m	
	CO	173.65 s				4	126.65 d	7.17 m	
	NMe	33.62 q	3.24 s			CO	172.74 s		
Pip	α	43.00 d	5.63 dd	2.0, 5.2		NH		7.62 d	10.4
	β	25.04 t	1.85 m		Sar	α	55.38 t	3.36 d	16.3
	γ	18.84 t	1.47 br d	13.0				4.26 d	16.3
			2.38 m			CO	167.97 s		
	δ	48.06 t	2.67 ddd	3.1, 13.0, 13.0		NMe	38.07 q	3.13 s	
			3.11 m						
	CO	171.33 s							
	NH		5.12 m						

Chemical shifts are given in ppm adjusted with TMS as internal standard.

pargamicin is a new cyclic hexapeptide consisting of cyclo(Phe-Sar-(4-OH-Pip)-(*N*-Me-3-OH-Val)-Pip-(*N*-OH-Ile)).

Pargamicin has a unique cyclic peptide containing *N*-methyl- and *N* or *C*-hydroxyl-amino acids. The stereochemistry and the structures of other analogs of pargamicin will be reported later.

Biological Activity

The antimicrobial activities of **1** are shown in Table 4. Pargamicin A (**1**) showed strong antimicrobial activities against Gram-positive bacteria including MRSA and VRE strains (Table 3). Its MICs were 0.39～0.78 μg/ml for susceptible *S. aureus* and multidrug-resistant *S. aureus* including MRSA, and 0.39 μg/ml for susceptible *E. faecalis*/*faecium* (n=2) and vancomycin resistant *E. faecalis*/*faecium* (n=4). However, **1** showed no activity at 100 μg/ml against pathogenic Gram-negative bacteria and yeast such as *Salmonella enteritidis* and *Candida albicans*, respectively.

Discussion

Our screening program for new antibiotics has yielded a new anti-MRSA and anti-VRE agent designated pargamicin A (**1**) from an *Amycolatopsis* sp. Compound **1** showed strong antimicrobial activities against MRSA and VRE. It is worth noting that **1** showed more potent activity against VRE than current anti-VRE drugs. Structural studies revealed that **1** is a cyclic hexapeptide antibiotic containing unusual residues including piperazic acid and *N*-methyl and *N*- or *C*-hydroxyl amino acid. Compound **1** is a cyclic peptide antibiotic containing piperazic acid. This residue is an unusual amino acid that has been found previously in peptides or cyclic depsipeptides from actinomycetes but has previously only been described once as a component of an actinomycete-derived cyclic peptide. L 156373, discovered as oxtocin and vasopressin antagonist in 1989 by Pettibone D. J. *et al.* is the sole example from a *Streptomyces* [15]. While L 156373 shares structural similarities with **1**, it has no anti-MRSA/VRE activities. These results indicate that **1** should be an interesting lead compound for the development of novel anti-MRSA/VRE

Table 4 Antibacterial activities of pargamicin A (**1**)

		MIC(μg/ml)	
Test organisms	Strain	Pargamicin A	Vancomycin
Enterococcus faecalis	JCM 5803	0.39	0.78
E. faecalis	NCTC 12201 (VRE, vanA)	0.39	>400
E. faecalis	NCTC 12203 (VRE, vanA)	0.39	>400
E. faecium	JCM 5804	0.39	0.78
E. faecium	NCTC 12202 (VRE, vanA)	0.39	>400
E. faecium	NTCTC 12204 (VRE, vanA)	0.39	>400
Staphylococcus aureus	FDA 209P	0.78	0.39
S. aureus	Smith	0.78	0.39
S. aureus	MS 9610 (MDR)	0.78	0.78
S. aureus	MS 16526 (MRSA)	0.78	0.78
S. aureus	TY-04282 (MRSA)	0.78	0.78
Escherichia coli	NIHJ	>100	>100
Shigella dysenteriae	JS 11910	>100	>100
Salmonella enteritidis		>100	>100
Pseudomonas aeruginosa	A3	>100	>100
Klebsiella pneumonie	PCI 602	>100	>100
Candida albicans	3147	>100	>100

Muller Hinton agar, 37°C, 18 hours. VRE: vancomycin resistant *Enterococcus*. MDR: multidrug resistant, MRSA: Methicillin resistant *Staphylococcus aureus*.

drugs based on the new mode of action. Studies on the mode of action of **1** and the structure-activity relations are under investigation.

Acknowledgements We thank Mrs. Seiko Hattori for measuring the MIC and Mrs. Yumiko Kubota for measuring the NMR spectra.

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