J. Antibiot. 60(6): 357–363, 2007

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

Lariatins, Novel Anti-mycobacterial Peptides with a Lasso Structure, Produced by *Rhodococcus jostii* K01-B0171

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Received: March 8, 2007 / Accepted: May 23, 2007

Abstract Two anti-mycobacterial peptides with a lasso structure, named lariatins A and B, were separated by HP-20 and ODS column chromatographies and purified by HPLC from the culture broth of *Rhodococcus jostii* K01-B0171, which was isolated from soil aggregates collected in Yunnan, China. Lariains A and B showed growth inhibition against *Mycobacterium smegmatis* with MIC values of 3.13 and 6.25 μg/ml in agar dilution method, respectively. Furthermore, lariatin A inhibited the growth of *Mycobacterium tuberculosis* with an MIC of 0.39 μg/ml in liquid microdilution method.

Keywords lariatin A, lariatin B, lasso structure, anti-mycobacterial peptide, tuberculosis, *Rhodococcus jostii*, soil aggregates

Introduction

Our research group has focused on discovery of anti-infectives from microbial metabolites [1~3]. Tuberculosis (TB) is still the greatest single infectious cause of mortality in the world, together with HIV and malaria [4]. Moreover, the spread of the HIV promoted to increase the number of tuberculosis patients [5]. However, powerful anti-TB drugs with a new mechanism of action have not been developed in last over thirty years, and only 5 anti-TB drugs can be clinically used still now. Since isoniazid and ethambutol, first-line anti-TB drugs, show specific inhibition against *Mycobacteria*, we have screened for new agents from microbial metabolites having specific inhibition against *Mycobacterium smegmatis* among 16 test microorganisms including Gram-positive and -negative bacteria, fungi and yeasts. As a part of this program, we discovered novel

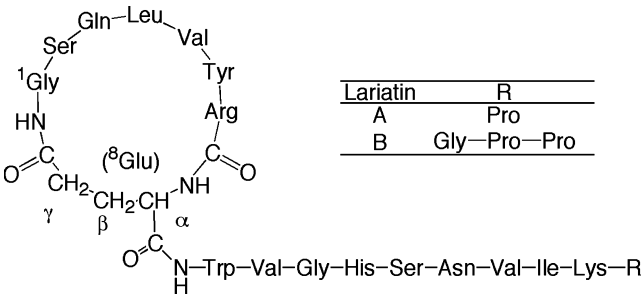

Fig. 1 Structures of lariatins A and B.

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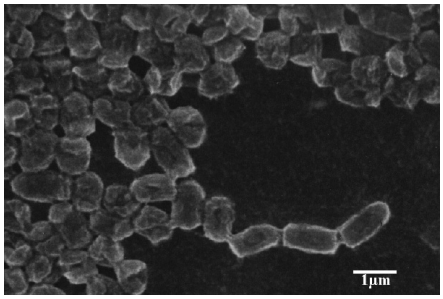


Fig. 2 Scanning electron micrograph of *Rhodococcus jostii* K01-B0171.

Fig. 3 Lasso structure of lariatin A.

compounds designated lariatins A and B (Fig. 1), unique cyclic peptides produced by *Rhodococcus jostii* K01-B0171 (Fig. 2). These peptides consist of 18 and 20 amino acid residues with an internal linkage between the γ-carboxyl group of Glu8 and the α-amino group of Gly1, and the tail region passes through the ring region. (Fig. 3). The structure elucidation was published elsewhere [6]. In this paper, the taxonomy, fermenation, isolation and physico-chemical and biological properties of lariatins are described. Lariatin A showed growth inhibition against not only *M. smegmatis* but also *Mycobacterium tuberculosis*.

Materials and Methods

Isolation of Strain K01-B0171

Soil was collected in Yunnan, China. Isolation method of strain K01-B0171 from soil aggregates was reported in detail elsewhere [7]. In brief, soil aggregates with diameters of 160～990 μm were collected by using filters, and washed with acidic electrolyzed water and sterilized water. The aggregates suspended in sterilized water were then disrupted by a sonicater, and the mixture were spread on Waksman plates to isolate colonies of microorganisms.

Taxonomic Studies

The cultural and physiological characteristics of the strain were determined following the methods recommended by the International *Streptomyces* Project (ISP [8]) and Waksman [9]. The utilization of carbon sources was tested by growth on Pridham and Gottlieb's medium containing 1.0% carbon at 27°C [10]. The morphological properties were observed with a scanning electron microscope (model JSM-5600, JEOL). Isomers of diaminopimelic acid (DAP) in whole-cell hydrolysates were determined by the standard methods using TLC [11, 12], and the *N*-acyl types of muramic acid were determined by the method of Uchida and Aida [13]. Whole-cell sugars were analyzed [11], presence of mycolic acids was examined by the TLC method [14], and phospholipids were extracted and identified by the method of Minnikin *et al.* [15]. Menaquinones were extracted and purified by the method of Collins *et al.* [16], then analyzed by HPLC (model 802-SC, JASCO) on a chromatograph equipped with a CAPCELL PAK C18 column (Shiseido Co.) [17]. The G+C content (mol%) of chromosomal DNA was determined by the HPLC method of Tamaoka and Komagata [18]. 16S rDNA was amplified by PCR and was sequenced directly on an ABI model 377A automatic DNA sequencer using a PRISM Ready Reaction Dye Primer Cycle Sequencing Kit (Applied Biosystems). The CLUSTAL W software package (Thompson *et al.* [19]) was used for multiple-alignment with selected sequences for calculating evolutionary distances (Kimura [20]) and similarity values and for constructing a phylogenetic tree based on the neighbor-joining method (Saitou *et al.* [21]).

Analysis of Lariatin Production by HPLC

The production of lariatins was measured by analytical HPLC under the following conditions: column, Symmetry C18 (2.1×150 mm, Waters Co.); mobile phase, acetonitrile - water with 0.05% phosphoric acid, 0～40% (20 minutes); UV detection at 210 nm; flow rate of 0.2 ml/minute. Lariatins A and B were eluted with retention times of 12.3 and 12.5 minutes, respectively.

General Procedures

The mass spectra were measured with a JOEL JSM-700 MS station and a JOEL JSM-AX 505 HA in thioglycerol. IR spectra were run in KBr on a HORIBA FT-210. The UV spectra were recorded on a HITACHI 340. The optical rotations were measured on a JASCO DIP-1000 Digital Polarimeter with a 5 cm cell. The melting points were measured on a Yanagimoto Micro Melting Point Apparatus MP-S3.

Assay for Antimicrobial Activities

Antimicrobial activity was measured by paper disk method (6 mm, ADVANTEC) containing a test sample [22]. The culture conditions were followed; *Bacillus subtilis* ATCC6633 [Davis synthetic medium (0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.5% sodium citrate, 0.1% ammonium sulfate, 0.2% glucose, 0.01% $MgSO_4 \cdot 7H_2O$ and 0.8% agar), 1.0% inoculation, 37°C, 24 hours], *Staphylococcus aureus* ATCC6538P [Nutrient agar (0.5% peptone, 0.5% meat extract and 0.8% agar), 0.2% inoculation, 37°C, 24 hours], *Micrococcus luteus* ATCC9341 [Nutrient agar, 0.2% inoculation, 37°C, 24 hours], *Mycobacterium smegmatis* ATCC607 [Waksman agar (0.5% peptone, 0.5% meat extract, 0.3% NaCl, 1.0% glucose and 0.8% agar), 1.0% inoculation, 37°C, 24 hours], *Escherichia coli* NIHJ [Nutrient agar, 0.5% inoculation, 37°C, 24 hours], *Pseudomonas aeruginosa* IFO3080 [Nutrien agar, 1.9% inoculation, 37°C, 24 hours], *Xanthomonas camestris* KB88 [Nutrient agar, 1.0% inoculation, 37°C, 24 hours], *Bacteroides fragilis* ATCC23745 [GAM medium (5.0% GAM broth and 1.5% agar), 2.0% inoculation, 37°C, 24 hours], *Acholeplasma laidlawii* KB174 [Ala medium (3.0% PPLO broth, 0.2% phenol red (5.0 mg/ml), 0.1% glucose, 1.5% agar, 15.0% Horse serum and 1.0% penicillin G), 20% inoculation, 37°C, 24 hours], *Pyricularia orizae* KF180 [GY agar (1.0% glucose, 0.5% yeast extract and 0.8% agar adjusted in pH 6.0, 2.0% inoculation, 37°C, 24 hours), *Aspergillus niger* ATCC9642 [GY agar, 0.3% inoculation, 27°C, 48 hours], *Mucor racemosus* IFO4581 [GY agar, 0.3% inoculation, 27°C, 48 hours], *Candida albicans* ATCC64548 [GY agar, 0.2% inoculation, 27°C, 24 hours] and *Saccharomyces cerevisiae* KF26 [GY agar, 0.3% inoculation, 27°C, 24 hours].

Anti-mycobacterial activity was measured by agar dilution method or liquid microdilution method. Agar dilution method: Test organisms were adjusted to approximately 1.0×10^6 CFU/ml, and inoculated using multipoint inoculator (Sakuma). Middlebrook 7H11 agar plates with or without the test compounds were inoculated with the test organisms and incubated for 14 days at 37°C and then examined to determine the MIC for each organism.

Liquid microdilution method: *M. tuberculosis* H37Rv was adjusted to approximately 1.0×10^6 CFU/ml in Middlebrook 7H9 broth containing 0.05% Tween 80 and 0.5% glycerol. The culture broth (200 μl) was added to each well of a 96-well microplate (Corning) with or without the test compounds. After incubation for 5 days at 37°C, 12.5% Tween 80 (20 μl) and alamarblue (20 μl, Biosource) were added to each well. After overnight incubation at 37°C, the measurement of A_{570} and A_{600} was carried out to determine the MIC.

Results

Taxonomy of the Producing Strain K01-B0171

Strain K01-B0171 was isolated from a soil sample collected in Yunnan Province, China using the ultrasonic processor from the inside of soil aggregates [7]. Good growth occurred on yeast extract-malt extract agar, tyrosine agar, nutrient agar and others, and no aerial mycelia grew. Color of colonies was orange to brown. Cells were short rods or coccus and were 1.1～1.4×0.7～0.8 μm in size (Fig. 2). Growth temperature range was 6°C to 37°C. D-Glucose, D-mannitol, D-fructose, L-rhamnose, *myo*-inositol and sucrose were used but L-arabinose, raffinose, melibiose, and xylose were not used as sole carbon. No melanoid pigment was produced.

Strain K01-B0171 contained *meso*-DAP, arabinose and galactose in whole-cell hydrolysates. The acyl type of the peptidoglycan was glycolyl. The predominant menaquinone was MK-8(H_2). Mycolic acids were detected and phosphatidylethanolamine was detected as the phospholipid. The G+C content (mol%) of the DNA was 66%. These results indicated strain K01-B0171 belongs to the genus *Rhodococcus*. 16S rDNA sequence (1513 nucleotides) was determined for strain K01-B0171 and the DDBJ accession number is AB204817. The phylogenetic analysis with 16S rDNA database sequences revealed that strain K01-B0171 branched deeply within a member of the genus *Rhodococcus* and most closely related to *Rhodococcus jostii* IFO 16295^T [24] (Fig. 4). As the sequence similarity was high value (99.5%), the strain K01-B0171 should be identified with *Rhodococcus jostii*.

Fermentation

Strain K01-B0171 was grown and maintained on an agar slant consisting 1.0% starch, 0.3% NZ amine, 0.1% yeast extract, 0.1% meat extract, 1.2% agar and 0.3% $CaCO_3$. For all liquid cultures, a medium was used consisting of 3.0% mannitol. 1.0% glucose, 0.5% yeast extract, 0.5% ammonium succinate, 0.1% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0001% $MgCl_2 \cdot 4H_2O$, 0.0001% $ZnSO_4 \cdot 7H_2O$, 0.0001% $CuSO_4 \cdot 5H_2O$ and 0.0001% $CoCl_2 \cdot 6H_2O$ (pH 7.0 before sterilization). A loopful of spores of *Rhodococcus* sp. was inoculated into 200 ml of medium in two 500-ml Erlenmeyer flasks on a rotary shaker (210 rpm). The inoculated flasks were incubated at 27°C for 3 days. A 200 ml portion of the culture was transferred to a 30-liter jar fermenter containing 20 liters of the same medium and the fermentation was carried out at

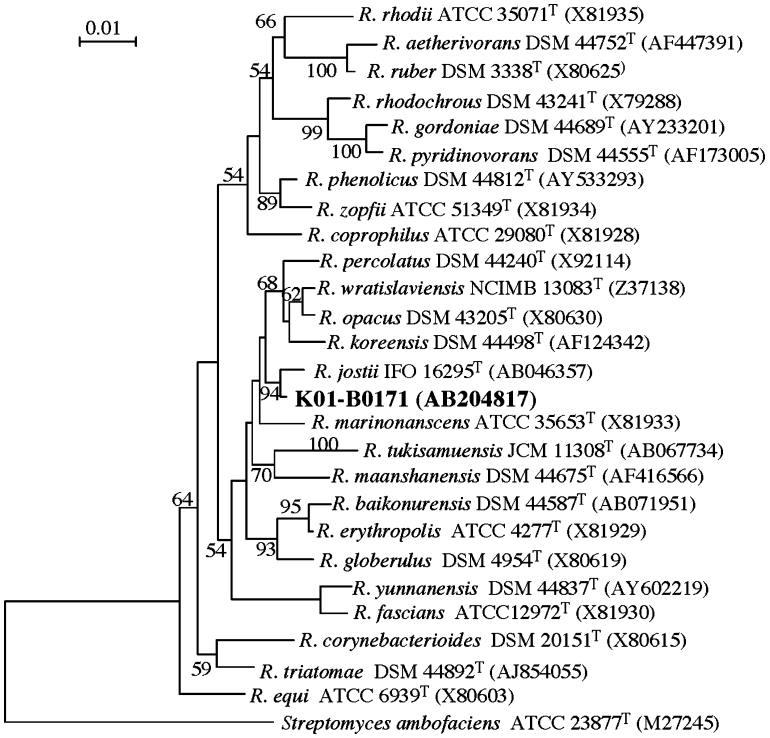


Fig. 4 Phylogenetic tree showing the position of strain K01-B0171 based on 16S rDNA analysis.

The numbers at the nodes indicate the level (%) of bootstrap support based on neighbor-joining analysis of 1000 resampled date sets. Only value grater than 50% are shown.

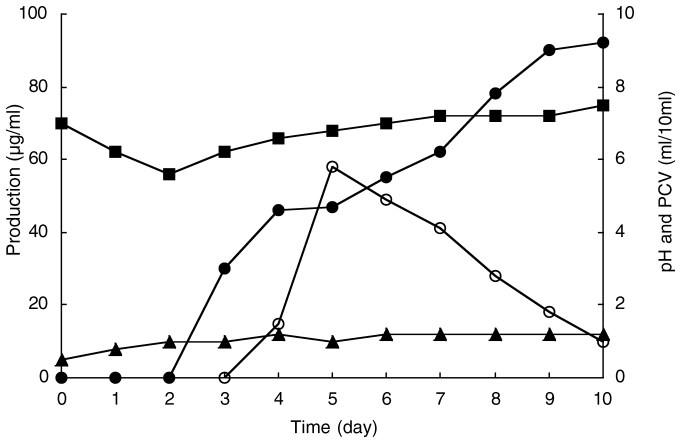
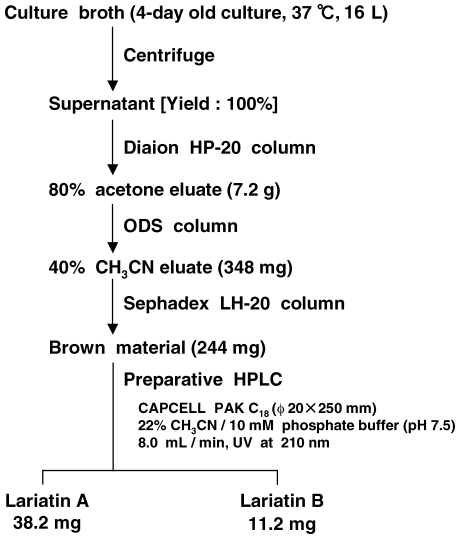


Fig. 5 Typical time course of lariatin production by *Rhodococcus jostii* K01-B0171.

PCV: ▲, pH: ■, lariatin A: ● and lariatin B: ○.



Scheme 1. Isolation procedures of lariatins A and B.

37°C for 4 days. The typical time course of fermentation is shown in Fig. 5. The production of lariatin A was slowly increased and reached a maximum level of about 90 μg/ml on day 10 after inoculation. The production of lariatin B reached a maximum level of about 60 μg/ml on day 5, thereafter the amount slowly decreased.

Isolation

The procedure for the isolation of lariatins A and B is summerized in Scheme 1. The 4-day old culture broth (16 liters) was centrifuged to separate mycelium and supernatant. The supernatant was passed through a column of Diaion HP-20 (75×220 mm, Nihon Rensui Co.)

previously equilibrated with water. After washing with water (3 liters) and 20% acetone (3 liters), the active materials were eluted with 80% acetone (3 liters). The whole eluate was concentrated *in vacuo* and lyophilized to dryness to yield a brown material (7.2 g). The material was dissolved in water and applied on an ODS column (35×270 mm, Senshu Scientific Co.) previously equilibrated with water. After washing with water (600 ml) and 20% acetonitrile (600 ml), the active materials were eluted with 40% acetonitrile (600 ml). The whole eluate was concentrated *in vacuo* to dryness to yield a brown material (348 mg). The material was dissolved in a small amount of methanol and passed through a Sephadex LH-20 column (25×1100 mm, Amersham Biosciences Co.). The active fractions were concentrated *in vacuo* to dryness to yield a brown material (244 mg). The active material was purified by HPLC on a CAPCELL PAK C_{18} column (20 i.d.×250 mm, Shiseido Co.) with 22% acetonitrile containing 10 mM phosphate buffer (pH 7.5) at 8 ml/minute detected at UV 210 nm. The retention times of lariatins A and B were 44 and 56 minutes, respectively (Fig. 6). The active fractions were desalted on an OASIS HLB column (60 mg, Waters Co.) previously equilibrated with water. After washing with water (3 ml), lariatins were recovered with 80% acetonitrile (2 ml), each of which was concentrated *in vacuo* to dryness to afford lariatin A (38.2 mg) and lariatin B (11.2 mg) as pale yellow powders [6].

Physico-chemical Properties

The physico-chemical properties of lariatins A and B are summarized in Table 1. Lariatins A and B had molecular formula of $C_{94}H_{143}N_{27}O_{25}$ and $C_{101}H_{153}N_{29}O_{27}$ respectively, established on the basis of HRFAB-MS 2051.0764

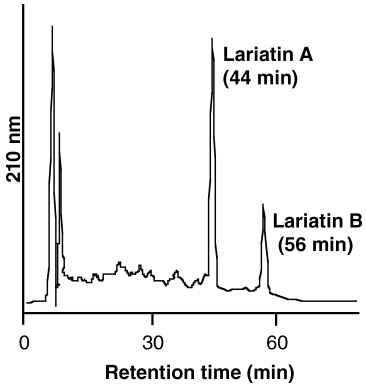


Fig. 6 Purification of lariatins A and B by HPLC.
The detail condition of HPLC was described in "Results".

Table 1 Physico-chemical properties of lariatins A and B

	Lariatin A	Lariatin B
Appearance	Pale yellow powder	Pale yellow powder
Molecular formula	$C_{94}H_{143}N_{27}O_{25}$	$C_{101}H_{153}N_{29}O_{27}$
Molecular weight	2050	2204
FAB-MS (*m/z*)		
positive	2051 $[M+H]^+$	2205 $[M+H]^+$
	2073 $[M+Na]^+$	
HRFAB-MS (*m/z*)		
calcd.	2051.0826	2205.1568
found $[M+H]^+$	2051.0764	2205.1504
$[\alpha]_D^{26}$ (*c* 0.3, 50% MeOH)	−19.6°	−26.6°
UV $\lambda_{max}^{50\%\ MeOH}$ nm (ε)	203 (182,700)	203 (225,200)
	220 (66,800)	220 (83,200)
	284 (9,800)	284 (11,900)
IR ν_{max}^{KBr} cm^{-1}	1650	1643
Melting point	240°C (dec)	240°C (dec)
Solubility		
soluble	H_2O, MeOH, DMSO	H_2O, MeOH, DMSO
insoluble	$CHCl_3$, EtOAc	$CHCl_3$, EtOAc
Color reaction		
positive	Ninhydrin	Ninhydrin
	Rydon-Smith	Rydon-Smith

Table 2 Antimicrobial activity of lariatins A and B

Test organism	Inhibition zone (mm) at 10 μg/6 mm disk		
	Lariatin A	Lariatin B	Isoniazid
Bacillus subtilis ATCC6633	—	—	—
Staphylococcus aureus ATCC6538P	—	—	—
Micrococcus luteus ATCC9341	—	—	—
Mycobacterium smegmatis ATCC607	19	18	26
Escherichia coli NIHJ	—	—	—
Pseudomonas aeruginosa IFO3080	—	—	—
Xanthomonas campestris KB88	—	—	—
Bacteroides fragilis ATCC23745	—	—	—
Acholeplasma laidlawii KB174	—	—	—
Pyricularia oryzae KB180	—	—	—
Aspergillus niger ATCC9642	—	—	—
Mucor racemosus IFO4581	—	—	—
Candida albicans ATCC64548	—	—	—
Saccharomyces cerevisiae KF26	—	—	—

$[M+H]^+$ (calcd. 2051.0826) and 2205.1504 $[M+H]^+$ (calcd. 2205.1568). It was supported by NMR analysis (date not shown). Rydon-Smith reaction was positive and the absorption at 1650 cm^{-1} was dominant in the IR spectra, indicating that these compounds are peptides. From these date, lariatins are found to be novel compounds. The structures were elucidated as shown in Fig. 1, which was published elsewhere [6].

Biological Activities

Lariatins A and B showed strong inhibition only against *M. smegmatis* among 14 microorganisms tested as well as isoniazid on the conventional paper disk assay (Table 2). Therefore, anti-mycobacterial activity using an agar dilution method was studied. The MIC values of lariatins A and B were 3.13 and 6.25 μg/ml for *M. smegmatis* Takeo, respectively. Furthermore, in liquid microdilution method, lariatin A inhibited the growth of *M. tuberculosis* with an MIC value of 0.39 μg/ml.

Discussion

Mycobacteria have a very unique cell wall structure. From the whole genome sequence [25], there are a number of enzymes involved in biosynthesis of the cell wall. For example, mycolic acids, extremely long fatty acids, form a broad family of more than 500 closely related structures and comprise about 30% of the dry weight of *M. tuberculosis*, and the microorganism has about 250 distinct enzymes involved in fatty acid metabolism (*vs.* 50 for *E. coli*). Isoniazid and ethambutol are first line tuberculosis drug, which inhibit cell wall biosynthesis; isoniazid inhibits mycolic acid synthesis by blockade of Type II fatty acid synthase [26], and ethambutol inhibits arabinogalactan mycolate synthesis by blockade of arabinosyltransferase [27]. Recently, platensimycin with a very unique structure was isolated as an inhibitor of Fab2 in Type II fatty acid synthase from *Streptomyces* starin [28]. Interestingly, lariatins show specific inhibition against mycobacterial growth. As described above, the compounds show the similar biological characteristics. They inhibited the growth of not only *M. smegmatis* but also *M. tuberculosis* (Table 2). Therefore, it might be plausible that the target molecule of lariatins lies within the cell wall biosynthetic steps in mycobacteria as well as isoniazid and ethambutol.

Acknowledgments This study was supported in part by the grant of the 21st Century COE Program and Scientific Research on Priority Areas 16073215 from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We thank to Ms. Miki Teshima, Microbiological Research Institute Otsuka Pharmaceutical Co., Ltd., for her useful advice and the measurement of MICs.

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