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Lys17 in the 'lasso' peptide lariatin A is responsible for anti-mycobacterial activity

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ABSTRACT

C-terminal-lacking fragments of the anti-mycobacterial peptide lariatin A were obtained by hydrolysis using carboxypeptidase P and their anti-mycobacterial activities were evaluated. Lys17 was found to be essential for their antimicrobial activity. A molecular dynamics simulation, with explicit water molecules, helped determine the structural characteristics of Lys17 of lariatin A. The simulation revealed the dynamic formation and deformation of a salt bridge between the N_{ξ} atom of Lys17 and the carboxyl group of C-terminal Pro18, which is deemed to be crucial for the compound's anti-mycobacterial activity.
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Two anti-mycobacterial peptides, named lariatins A and B (Fig. 1A), were isolated from the culture broth of *Rhodococcus jostii* K01-B0171.[1] Both showed selective growth inhibition against several Mycobacteria species, including *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. The compounds are unique cyclic peptides, consisting of 18 and 20 L-amino acid residues with an internal linkage between the α-amino group of Gly1 and the γ-carboxyl group of Glu8.[2] In our previous study, the three-dimensional structure of lariatin A was determined using NMR data, indicating that the tail segment (Trp9-Pro18) passes through the ring segment (Gly1-Glu8) to form a 'lasso' structure (Fig. 1B). We showed that this lasso structure is responsible for the anti-mycobacterial activity because synthetic non-cyclic peptide possessing a similar sequence to lariatin A has no activity. However, other critical residues relevant to bioactivity remained to be defined. Recently, the C-terminal structure was found to be important for the RNA-polymerase inhibition activity of microcin J25,[3] which possesses a similar 'lasso' structure. Therefore, we investigated whether the C-terminal of the lariatins was also essential for their antimicrobial properties.

We prepared C-terminal cleavage fragments from lariatin A using an enzymatic method and evaluated their respective anti-mycobacterial activity. Residue Lys17 proved to be essential for the antimicrobial property. A molecular dynamics (MD) simulation with explicit water molecules using lariatin A was subsequently carried out to illustrate structural features of Lys17, which were disordered in NMR structures.

As previously reported,[2] early attempts of enzymatic digestion of lariatin A with arginylendopeptidase, *acromobacter* protease I, V8 protease, endoprotease Asp-N, trypsin, and chymotrypsin were failed and only the use of carboxypeptidase P was succeeded. Lariatin A (0.5 mg) was hydrolyzed by carboxypeptidase P (fin. 100 mU/mL) at 37 °C for 80 min. After the reaction, the mixture was purified by RP-HPLC[4] (Fig. 2). The retention times of lariatins A, A-ΔP and A-ΔKP were 14.1, 13.2 and 19.0 min, respectively. After collection, each fraction was applied to a solid-phase extract cartridge, followed by washing and eluting by CH_3CN, to yield lariatin A-ΔP (212 μg) and lariatin A-ΔKP (148 μg). Assignment of the compounds was performed by measuring their molecular weights on MALDI-TOF-MS.[5]

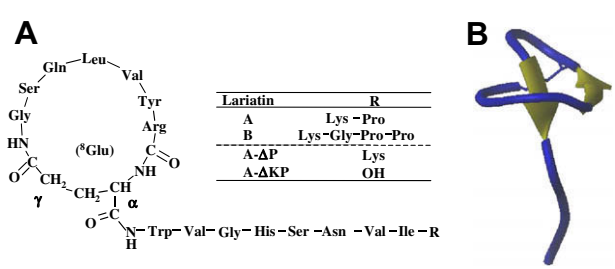

Figure 1. Structure of lariatins (A) and 3D structure of lariatin A (B).

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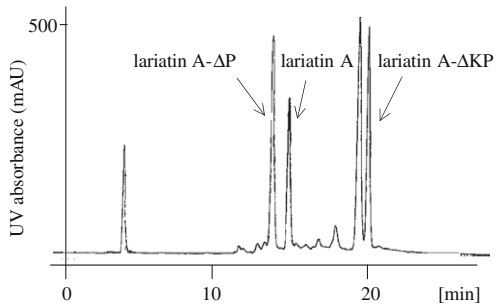

Figure 2. Purification of fragments of lariatin A by HPLC.

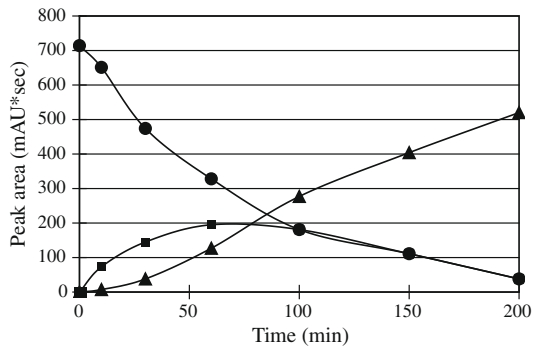

Figure 3. Time course of hydrolysis of lariatin A by carboxypeptidase P: Lariatin A (closed circle), lariatin A-ΔP (closed square), and lariatin A-ΔKP (closed triangle).

The time course of hydrolysis of lariatin A by carboxypeptidase P is shown in Figure 3.[7] The amount of lariatin A slowly decreased, while that of lariatin A-ΔP increased, reaching a maximum level of about 200 mAU s 60 min after incubation. Thereafter the amount slowly decreased. The amount of lariatin A-ΔKP increased slowly and almost all lariatin A was hydrolyzed into lariatin A-ΔKP some 200 min after incubation. During longer incubation, the fragments shorter than lariatin A-ΔKP could not be obtained because they were hydrolyzed easily. This suggested that the C-terminal residues of lariatin A might be important factors in resistance to hydrolysis.

Anti-*M. smegmatis* activity of all fragments, determined by the conventional paper disk assay[6], is summarized in Table 1. Lariatin A-ΔP showed activity as potent as lariatin A, while lariatin A-ΔKP showed no activity. This result clearly indicates Lys17 is vital for antimicrobial activity.

We recently reported the three-dimensional structure of lariatin A determined through NMR study.[2] However, it was difficult to speculate the structural features of Lys17, as the C-terminal residues were disordered in the NMR structures due to the absence of medium- and long-range NOEs.[2] Therefore, we performed MD simulation, with explicit water[8], in order to obtain structural and dynamic characteristics of the C-terminal residues of laratin A. Since the MD simulation uses standard van der Waals and electrostatic interactions, which are included as purely repulsive term and excluded in building up NMR structures, respectively, it can provide more reliable solution structures of biomolecules.[9,10]

In the time-evolution of the RMS deviation on all heavy atoms between the initial structure and the trajectory structures, the RMSD values reached stable states after about 1–2 ns, and converged to around 3.0 Å through the MD simulation.[11] The last 25 ns trajectories were considered as representative solution structures of lariatin A and used for analyses. The average distance and dihedral violations from NMR-derived constraints over 10–35 ns were 0.084 ± 0.020 Å and 0.12 ± 0.63°, respectively, indicating that the solution structures obtained by the MD simulation satisfied the experimental data. The distribution of the RMS difference of all heavy atoms over 10–35 ns about the mean structure as a function of the residue number,[12] which was similar to that on NMR structures, suggested that the C-terminal two residues, Lys17 and Pro18, do not have a fixed conformation, even in the MD simulation. However, we found an interesting dynamic behavior variation between these two residues. Figure 4A plots distances between the N_ζ atom of Lys17 and the backbone carboxyl carbon atom of Pro 18 along the MD trajectory. Figure 4B shows the distribution of distances between these two atoms. A large population around the distance of 3–4 Å was observed. This clearly indicates dynamic formation and deformation of a salt bridge between the N_ζ atom of Lys17 and the carboxyl group of Pro18.[13] Thus, the MD simulation with explicit water revealed interesting structural and dynamic characteristics of the C-terminal residues, Lys17 and Pro18. The presence of a salt bridge between these two residues may explain their resistance to hydrolysis. Lariatins A-ΔP probably forms a similar salt bridge between Lys17 and the C-terminal carboxyl group.

On the basis of the results obtained in the MD simulation, we can consider two possibilities of Lys17 for the antimicrobial prop-

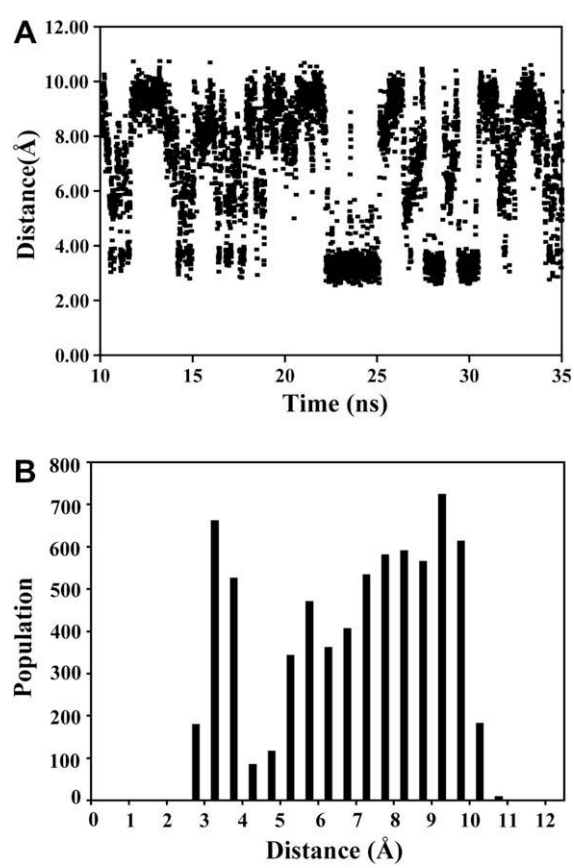

Figure 4. (A) Distances between the N_ζ atom of Lys17 and the backbone carbonyl carbon atom of Pro 18 along the MD trajectory. (B) Distribution of distances between these two atoms.

Table 1
Anti-*M. smegmatis* activity of lariatin A and its fragments.

Compounds	Inhibition zone (mm)*		
	1 μg	3 μg	10 μg
Lariatin A	16.4	20.3	22.4
Lariatin A-ΔP	16.0	19.8	22.8
Lariatin A-ΔKP	–	–	–

*Paper disks (ϕ 8 mm) were used.

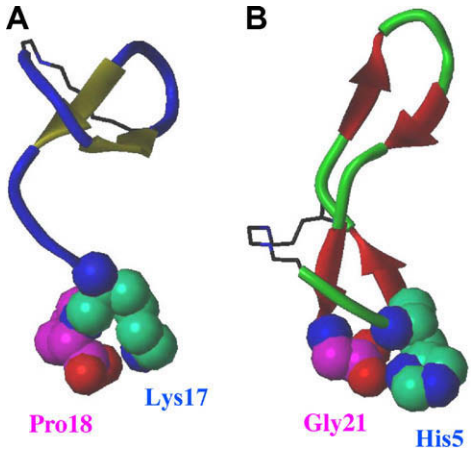

Figure 5. Comparison of the MD structure (28 ns) of lariatin A (A) and the NMR structure of microcin J25 (B). Residues related to salt bridge structure are displayed.

erty of lariatin A. First, the distorted Lys17 may be important to recognize a target molecule of the mycobacteria. For example, it may form a direct hydrogen-bond interaction with the target molecule. Second, the salt bridge structure between Lys17 and Pro18 may be critical to bind with the target molecule. In order to obtain further information on the latter possibility, we have compared the 3D structure of lariatin A with that of microcin J25, which is also a microbial polypeptide with antibacterial activity.[14] Lariatin A and microcin J25 belong to the same subfamily of microbial polypeptides with a 'lasso' structure, although their antibacterial spectra are different.[2] Therefore, we wondered whether the salt bridge structure is also common between them. Figure 5 shows the comparison of the solution structure of lariatin A obtained by the MD simulation and the representative NMR structure of microcin J25. Interestingly, we can see that microcin J25 also possesses a salt bridge structure around the C-terminal region, which is formed between the C-terminal carboxyl group and His5. In addition, recent chemical modification experiments with microcin J25 revealed that the C-terminal carboxyl group is critical for its RNA-polymerase inhibition activity, indicating the importance of the salt bridge structure.[3] These results suggested that the salt bridge structure of lariatin A might be also relevant to its anti-mycobacterial activity. In this study, we could reveal that Lys17 is essential for the antimicrobial property of laratin A by the enzymatic method, and discuss its structural features using the MD simulation. The information will be useful for the rational design of promising drug lead compounds based on lariatins.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.033.

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2. Iwatsuki, M.; Tomoda, H.; Uchida, R.; Gouda, H.; Hirono, S.; Ōmura, S. *J. Am. Chem. Soc.* **2006**, *128*, 7486.
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4. *Preparation of hydrolysates of lariatin A.* Examined peptide (500 μg) was dissolved in 400 μL of 50 mM acetate-buffer (pH 3.7), followed by adding 100 μL of carboxypeptidase P (lariatin A: 500 mU/mL) in 50 mM acetate-buffer (pH 3.7) and incubated at 37 °C for 80 min. After treatment with carboxypeptidase P, reaction mixture was purified by RP-HPLC (column: CAPCELL PAK C18 (4.6ϕ × 250 mm, Siseido), 10–50% CH_3CN/0.05% H_3PO_4 (40 min), 1.0 mL/min, UV 210 nm, room temperature). The retention times of lariatins A, A-ΔP and A-ΔKP were 9.1, 8.0 and 15.0 min, respectively. All fractions with a peak observed at 210 nm were collected. After removing CH_3CN in vacuo, each fraction was applied to solid-phase extract cartridge OASIS HLB (30 mg, Waters), followed by washing and eluting by CH_3CN to yield lariatins A-ΔP (212 μg) and A-ΔKP (148 μg).
5. Mass spectra were acquired on a Shimadzu-Biotech AXIMA-CFR MALDI-TOF instrument (Shimadzu-Biotech). MALDI targets were prepared by depositing an aqueous solution of the sample (0.5 μL) onto the MALDI plate with the matrix solution (0.5 μL, see below) and allowing the mixture to dry under ambient conditions. Targets prepared from 2,5-dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxycinnamic acid (α-CHCA) as matrix solution were recrystallized from ethanol. Data acquisition and processing were controlled by Kratos Launchpad software. All spectra recorded for this work were obtained with the standard instrument settings for optimum transmission at medium and high mass. The TOF was externally calibrated using angiotensin II ($[M+H]^+$ 1046.5418) and insulin chain B ($[M+H]^+$ 3494.6508) deposited directly onto the sample stage. The molecular weights of lariatin A, A-ΔP and A-ΔKP were determined as 2050, 1953 and 1825 Da, respectively.
6. Method of measuring anti-*M. smegmatis* activity is described in Ref. 1.
7. *Time course of hydrolysis by carboxypeptidase P.* Lariatin A (50 μg) was dissolved in 40 μL of 50 mM acetate-buffer (pH 3.7), followed by adding 10 μL of carboxypeptidase P (500 mU/mL, EC number 3.4.16.1, Takara Bio Inc.) in 50 mM acetate-buffer (pH 3.7) and incubated at 37 °C. After treatment (0, 10, 30, 60, 100, 150 and 200 min) with carboxypeptidase P, 2 μL of reaction mixture was transferred to 18 μL of methanol to stop the enzyme reaction. Source/Product ratio of examined peptides were determined by comparing the peaks area in reverse-phase high-performance liquid chromatography (RP-HPLC) analyses (column: Symmetry C18 (2.1ϕ × 150 mm, Waters), 10–30% CH_3CN/0.05% H_3PO_4 (20 min), 0.2 mL/min, UV 210 nm, 40 °C) before and after treatment with carboxypeptidase P.
8. *Molecular dynamics (MD) simulation with the explicit solvent water*: The MD simulation was performed using the AMBER7.0 simulation package (Case, D. A.; Pearlman, D. A.; Caldwell, J. W.; Cheatham, T. E.; Wang, J.; Ross, W. S.; Simmerling, C. L.; Darden, T. A.; Merz, K. M.; Stanton, R. V.; Cheng, A. L.; Vincent, J. J.; Crowley, M.; Tsui, V.; Gohlke, H.; Radmer, R. J.; Duan, Y.; Pitera, J.; Massova, I.; Seibel, G. L.; Singh, U. C.; Weiner, P. K.; Kollman, P. A. AMBER7; University of California: San Francisco, 2002.) and the Cornell et al. force field, that is, parm94.dat, (Cornell, W. D.; Cieplak, P.; Bayly, C. I.; Gould, I. R.; Merz, K. M. Jr.; Ferguson, D. M.; Spellmeyer, D. C.; Fox, T.; Caldwell, J. W.; Kollman, P. A. *J. Am. Chem. Soc.* **1995**, *117*, 5179). Among a total of 81 NMR structures obtained in the previous paper,[2] the one with the lowest energy value was used as an initial structure for the MD simulation. The initial structure was solvated in a box of TIP3P water molecules with a margin of 10 Å along each dimension. A total of two Cl^- ions was added to neutralize the system. The total number of atoms was 9209. The system was equilibrated as follows; in order to relax the water around the solute, the water was minimized for 1000 steps, followed by 35 ps of 300 K MD with 100 kcal mol^{-1} $Å^{-2}$ restraints on all solute atoms. This was followed by the second energy minimization until the root-mean-square (RMS) value of the potential gradient was below 0.05 kcal mol^{-1} $Å^{-1}$. Finally, the system was heated from 0 to 300 K in 40 ps. After this equilibration phase, the production phase followed at 300 K for up to 35,000 ps (35 ns). We added potential functions for the NMR-derived restraints to the standard AMBER potential functions in order to make the trajectory satisfy these experimental data more closely. The potential energy term was quadratic for the distance and dihedral angle violations smaller than 3 Å and 30°, respectively, and was linear beyond these values. The force constants were 10 kcal mol^{-1} $Å^{-2}$ and 50 kcal mol^{-1} rad^{-2} for distance and dihedral angle restraints, respectively. The MD simulation was carried out at constant pressure (1 atm) and temperature (300 K), under periodic boundary conditions, and with particle-mesh Ewald treatment of electrostatics (Darden, T.; York, D.; Pedersen, L. *J. Chem. Phys.* **1993**, *98*, 10089). SHAKE (Ryckaert, J. P.; Ciccotti, G.; Berendsen, H. J. C. *J. Comput. Phys.* **1977**, *23*, 327) was applied to all bonds involving hydrogen, and a time step of 1 fs was used. An 8-Å cutoff was used for the non-bonded interactions.
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11. See Supplementary Figure 1.
12. See Supplementary Figure 2.
13. Several snapshots illustrating lariatin A's dynamic variable are shown in Supplementary Figure 3.
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