Diketopiperazine from marine bacterium *Pseudoalteromonas ruthenica* KLPp3

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Abstract

Sub-minimum inhibitory concentration levels of *Pseudoalteromonas ruthenica* KLPp3 extract showed antibiofilm activity against *Vibrio alginolyticus* and *Serratia marcescens*. The KLPp3 crude extract was fractionated by thin layer chromatography, flash chromatography, solid phase extraction and semi-preparative high performance liquid chromatography. The pure compounds were then identified using H-nuclear magnetic resonance, C-nuclear magnetic resonance, 2D-nuclear magnetic Resonance and mass spectrometry. Nine fractions were collected from purification with two active fractions. One fractions were identified belong to the family of diketopiperazine.

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Key words: Diketopiperazine; Sub-minimum inhibitory concentrations; Crude extract; Antibiofilm; *Pseudoalteromonas ruthenica*.

Contributions: FS is the student who has undertaken all the research work; AA is the lecturer who provided designs and approved the protocols to be followed in the study and the intellectual input about isolation and characterization of bacteria; GU is the lecturer who provided the intellectual input about marine environment and extraction of natural products from bacteria; LCK is the lecturer who provided the high performance liquid chromatography and nuclear magnetic resonance machines and the intellectual input about compounds identification.

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Introduction

Diketopiperazine are the smallest cyclic peptides known and they are biosynthesized from amino acids by a large variety of organisms including Gram-negative and Gram-positive bacteria, fungi and higher marine organisms.[1-4] 90% of Gram-negative bacteria produce diketopiperazine,[5] making it one of the sources for diketopiperazine isolation. Since the first report in 1924, a large number of bioactive diketopiperazine was discovered spanning activities as antitumor, antifungal, antibacterial, antiviral, antihyperglycemic and antiprion.[5]

Biofilms in general are considered as surface-associated microorganism communities enclosed in an extracellular polymeric substances.[6] Marine environment has an optimum condition for formation of biofilm. Bacterial biofilms, frequently in association with diatoms, algae, protozoa and fungi, are found on all submerged man-made structures as well as natural surfaces in the marine environment. Marine bacteria as primary colonizers are known to be responsible for the initiation of biofouling and may cause various damages in maritime activities and industries.[7] Thus, the need of novel bioactive compounds, which enhance or inhibit the settlement of organisms, is increasing. Marine bacteria are a possible source of such compounds. Indeed, previous studies have demonstrated that a high proportion of microbes isolated from the surfaces of macro-organisms produce diverse bioactive compounds.

Pseudoalteromonas ruthenica KLPp3 (GenBank accession no: KT071710) is a surface-associated bacterium recently isolated by our group from the surface of the marine crab, *Scylla serrate*.[8] The aim of this study was to evaluate the effect of *P. ruthenica* extract on the biofilm production of *Vibrio alginolyticus* and *Serratia marscescens* at sub-minimum inhibitory concentration (MIC) levels and to identify the pure active compounds.

Materials and Methods

Bacteria isolation and identification

Marine bacterium *P. ruthenica* KLPp3 (GenBank accession no: KT071710) was isolated from surface of a marine crab in Pulau

Perhentian of Malaysia. The surface of a marine crab from was swabbed using sterile Dacron swabs. The swabs were placed into a 15 mL sterile plastic tube and the swabs stem were snapped approximately 1 inch from the top of the tube. The isolation of bacteria was done by using serial dilution method. Dilutions were prepared using sterile water with 3% sodium chloride, and 0.1-mL aliquots were surface spread on Marine agar (MA) (Oxoid). After incubation at 30°C for 24 h, the bacterial cultures were transferred to fresh MA for a second incubation.

KLPp3 isolate was identified from the 16S ribosomal DNA sequence determination. DNA was extracted using the DNeasy tissue kit (QIAGEN GmbH, Hilden, Germany). PCR amplification using universal primers forward (5' AGA GTT TGA TCC TGG CTC AG 3') and reverse (5' GGT TAC CTT GTT ACG ACT 3') amplified approximately 1,500 bp of the 16S ribosomal DNA. Sequencing of the amplified DNA was performed by First Base Laboratories Sdn Bhd.

Test microorganisms

The biofilm-forming bacteria *Serratia marcescens* SM-11, *V. alginolyticus* PPjt4, and *V. alginolyticus* PPbp4 were maintained on nutrient agar (NA; Difco, USA) slants with 3% sodium chloride at 4°C and discarded monthly. Primary stock cultures are stored in glycerol stock at -80°C.

Extraction of *Pseudoalteromonas ruthenica* KLPp3 crude extract

P. ruthenica KLPp3 was grown in total of 10L LB broth by shaking for 90 hours and was extracted using ethyl acetate. After the incubation, the bacterial culture was centrifuged at 4000 rpm for 15 minutes and the supernatant was filtered through a 0.22 µm membrane. The supernatants were extracted with the equal volumes of ethyl acetate. The extraction process was repeated three times and then the extract was concentrated in a rotary evaporator. The crude extracts were stored at 4°C and must be analyzed within 2 days. For long-term storage, the crude extracts are maintained at -80°C.

Minimum inhibitory concentration

All bacterial strains were subjected to the antimicrobial tests by using the disc diffusion method on the Mueller-Hinton agar. An aliquot of 0.1 mL of 0.5 McFarland equivalents, approximately from an exponentially growing culture was spread on the agar for the development of a bacterium strain lawn at 30°C. Next, on the lawn-agar of each plate, eight discs of crude extracts with concentration of 0.1 mg/mL, 0.2 mg/mL, 0.5 mg/mL and 1 mg/mL were placed individually at equal distances from one another. The plates were incubated for 18 hours at 30°C and were examined for size-measurements of inhibition zones around each disc.

Antibiofilm activity assay

The biofilm formation of *V. alginolyticus* PPjt4, *V. alginolyticus* PPbp4 and *S. marcescens* SM-11 was evaluated in the presence of the crude extract and purified compounds. The quantification of the *in vitro* biofilm production was based on the method previously reported.[9] Briefly, the wells of a sterile 96-well flat-bottomed polystyrene plate were filled with 100 uL of the appropriate medium. A 1/100 dilution of overnight bacterial culture was added into each well. The first row contained the untreated bacteria, while the second row contained 0.1 mg/mL of the crude extract. The plates were incubated for 24 h at 30°C. After rinsing with 0.9% saline, the adhered cells were stained with 0.1% crystal violet, rinsed twice with 0.9% saline and thoroughly dried. The dye bound to the adherent cells was resolubilized with 80% (v/v) ethanol *per* well. The OD of each well was measured at 590 nm.

Fractionation and compound isolation

The crude extract was fractionated on a solid phase extraction (SPE) column (C18). Water and Methanol (MeOH) were used as solvent with ratio 3:7, 5:5, 9:1 and 10:0. High performance liquid chromatography (HPLC) purification was accomplished by reverse phase RP18 column (Waters, 5µm, 4.6×250mm, 5 µm particle size). The elution was at a flow rate of 1 mL min^{-1} with two solutions: methanol and distilled water, starting with 5% MeOH for 10 min, which was increased linearly to 20% AcN in 20 min and held for 5 min at this level. Then MeOH was increased linearly to 100% and held for 5 min at this level before returning to 5% MeOH in 5 min, and then equilibrated for 15 min. HPLC analyses were performed using photodiode array detector with a wavelength range 200-800nm.

Spectroscopic analysis

The nuclear magnetic resonance (NMR) spectra were measured on a Varian Mercury Plus 400 FT-NMR at 400 MHz for ^{1}H and ^{13}C, in $CDCl_3$, respectively. As for 2D NMR (COSY, HMBC, HSQC), the spectra were measured at 600 MHz in $CDCl_3$. Silica gel (Merck, 230–400 mesh) and polygoprep gel were used for column chromatography. Mass spectrometry was recorded with Quattro Ultima triple quadrupole mass spectrometer (Waters, Manchester, UK) equipped with an electrospray interface.

Results

Bacteria identification

The partial 16S rDNA sequence from the isolate was used for searching in Basic Local Alignment Search Tool and showed 96% homology to the corresponding sequence of *P. ruthenica* (reference no: AB617563.1)

Minimum inhibitory concentration

Preliminary experiments were carried out to assess the effects of crude extract on the growth of *V. alginolyticus* PPbp4, *V. alginolyticus* PPjt4 and *S. marcescens* SM-11 (Table 1). The

Table 1. Minimum inhibitory concentration of crude extracts.

Tested bacteria	Inhibition (mm) 0.1 mg/mL	0.2 mg/mL	0.5mg/mL	1 ml/mL	Negative Control	Positive control
S. marcescens	6	6	7	7	6	13 (streptomycin)
V. algynolyticus PPbp4	6	6	7	7	6	11 (streptomycin)
V. algynolyticus PPjt4	6	6	7	7	6	11(streptomycin)

bacterial cultures were separately treated with the crude extract at a concentration of 0.1 mg/mL, 0.2 mg/mL, 0.5 mg/mL and 1 mg/mL. The inhibitory effect started at the concentration of 0.5 mg/mL. The MIC of the crude extract was 0.5 mg/mL. There were no inhibitory effects at the concentration of 0.1 mg/mL and 0.2 mg/mL. This result has shown that the concentration of 0.1 mg/mL and 0.2 mg/mL did not prevent the growth of bacteria. The sub-MIC, 0.1 mg/mL, was chosen for further tests.

Effects of *Pseudoalteromonas ruthenica* KLPp3 crude extract on the biofilm formation

The effects of sub-MIC crude extract of *P. ruthenica* KLPp3 on biofilm-forming bacteria were examined. It inhibited 59.71%, 45.02% and 56.4% of the biofilm production in *V. alginolyticus* PPbp4, *V. alginolyticus* PPjt4 and *S. marcescens* SM-11 respectively (Figure 1A).

Effects of *Pseudoalteromonas ruthenica* KLPp3 crude extract on the initial attachment

Initial attachment of the bacteria to a solid surface is the first step in biofilm formation. As reported in Figure 1B, *P. ruthenica* KLPp3 crude extract reduced the attachment of *V. alginolyticus* PPbp4, *V. alginolyticus* PPjt4 and *S. marcescens* SM-11.

Purification and characterization of antibiofilm compounds

Antibiofilm activity of *P. ruthenica* KLPp3 crude extract resulting from growth of *P. ruthenica* KLPp3 in LB broth was recovered in the 90% methanol phase after SPE on a C18 column. After further separation by HPLC using a RP18 column (Figure 2), each fraction was tested for antibiofilm activity and only two fractions have antibiofilm activity (Figure 3). One compound, KLPP3-4.3a.9 was identified using NMR.

The pure compound KLPP3-4.3a.9 was colourless and dissolved in methanol. The 1HNMR spectrum showed a broad 1H singlet of an amide at δ 5.66 ppm (4NH), 1H triplet at δ 4.56 ppm (H8), five doublet of doublets at δ 4.44 (H6), δ 4.30 (H3), δ 3.76 (H9), δ 3.60 (H10) and δ 2.76 (H10) ppm, two multiplet at δ 2.02 (H7) and δ 2.33 (H7), and three doublet at δ 3.55. In addition, aromatic protons consist of two multiplet, two doublet of doublets and a triplet between δ 7.20-7.35 (H12, H13, H14, H15, H16) ppm. KLPP3-4.3a.9 had the molecular formula $C_{14}H_{16}N_2O_3$, as determined by ESMS 261 [$M^+ + H^+$]. All of NMR spectrums are shown on Figures 4 and 5. Data from the experiments elucidating the chemical structures of compound KLPP3-4.3a.9 is summarized and compared with two reference compound cyclo(L-Phe–trans-4-OH-L-Pro)[10] and cyclo(L- phenylalanyl-4-hydroxy-L-prolin)[11] in

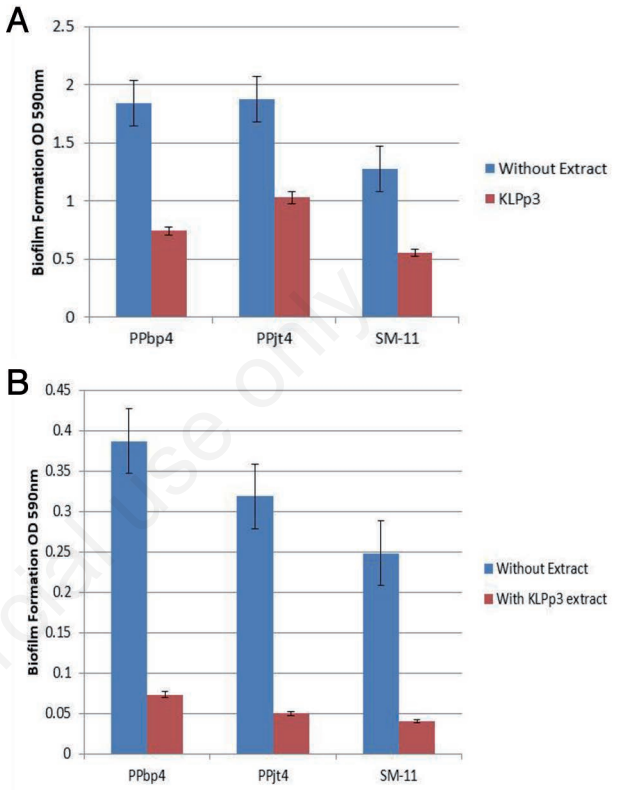

Figure 1. A) Effect of *Pseudoalteromonas ruthenica* KLPp3 crude extract on biofilm formation; B) Effect of *P. ruthenica* KLPp3 crude extract on initial attachment.

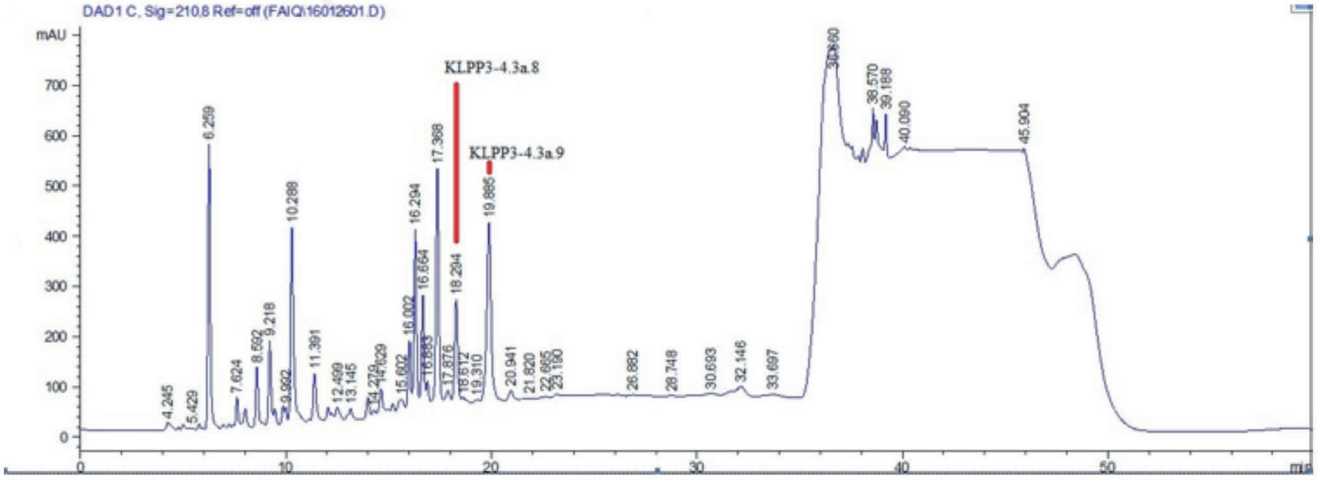

Figure 2. Fractionation of *Pseudoalteromonas ruthenica* KLPp3 crude extract using high performance liquid chromatography.

Table 2. KLPP3-4.3a.9 is identified as 3-benzyl-7-hydroxyhexahydropyrrolo[1.2a]pyrazine-1,4-dione (Figure 6).

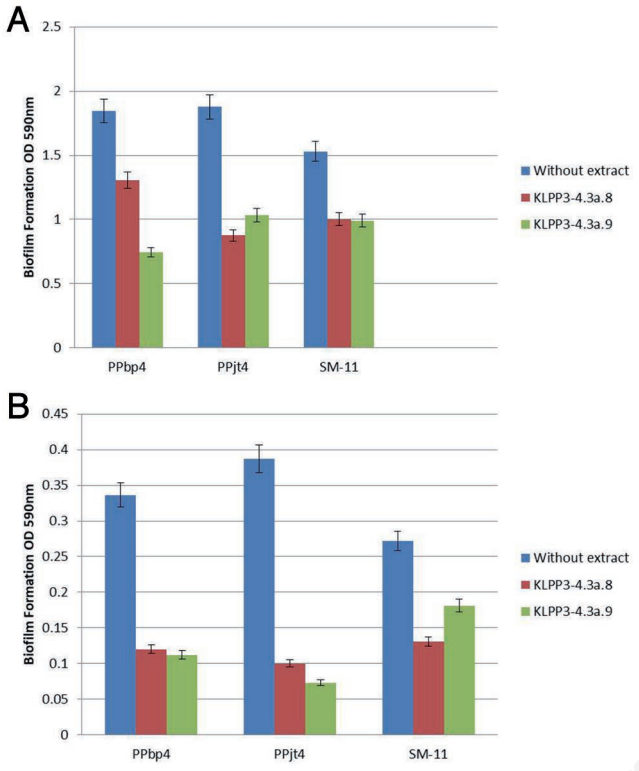

Figure 3. A) Effect of KLPP3-4.3a.8 and KLPP3-4.3a.9 on biofilm formation; B) Effect of KLPP3-4.3a.8 and KLPP3-4.3a.9 on initial attachment.

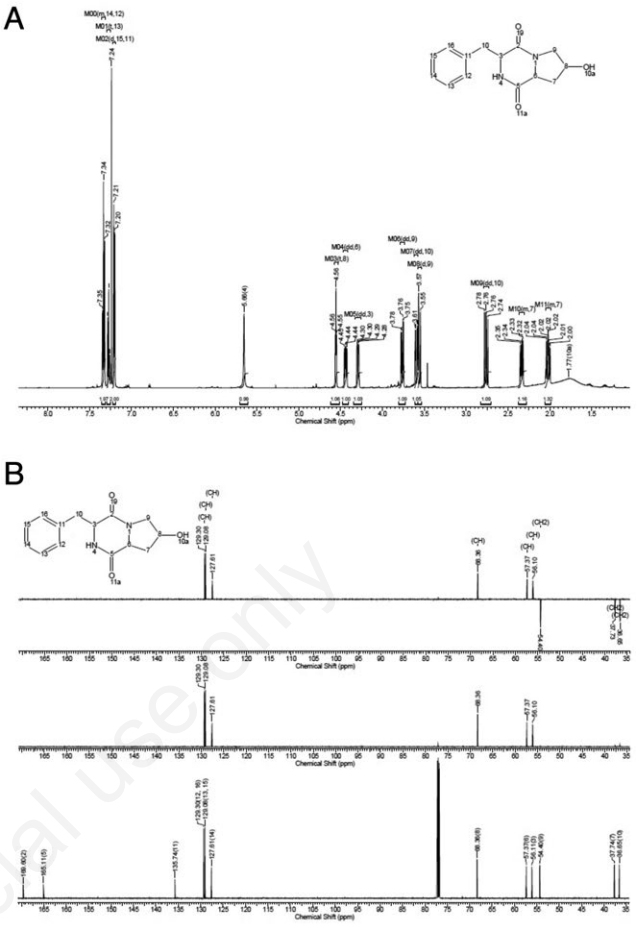

Figure 4. A) H- nuclear magnetic resonance of KLPp3-4.3a.9. B) C- nuclear magnetic resonance of KLPp3-4.3a.9.

Table 2. ^{1}H and ^{13}C nuclear magnetic resonance data for KLPP3-4.3a.9 and its nearest reference compounds.

Functional Group	Chemical Shift (ppm) KLPP3-4.3.9		Ström *et al.*[10]		Jiang *et al.*[11]	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
C=O	169.60	-	171.29		169.4	
C-H	56.11	4.30	57.65	4.48	56.0	4.30
N-H	-	5.66	-	7.95		5.65
C=O	165.11	-	167.19		165.0	
C-H	57.37	4.44	55.19	4.37	57.3	4.45
C-H2	37.74	2.33 2.02	38.76	2.07 1.39	37.6	2.35 2.08
C-H	68.36	4.56	68.65	4.28	68.3	4.58
$C-H_2$	54.40	3.76 3.55	58.35	3.71 3.29	54.3	3.78 3.57
$C-H_2$-Ph	36.65	3.60 2.76	37.95	3.17	36.5	3.64 2.76
C	135.74	-	137.51		135.6	
CH	129.30	7.20-7.21	131.14	7.24	129.2	7.25
CH	129.08	7.32-7.35	129.63	7.27	129.2	7.25
CH	127.61	7.27	128.23	7.22	129.0	7.25
CH	129.08	7.32-7.35	129.63	7.27	127.5	7.25
CH	129.30	7.20-7.21	131.14	7.24	129.0	7.25

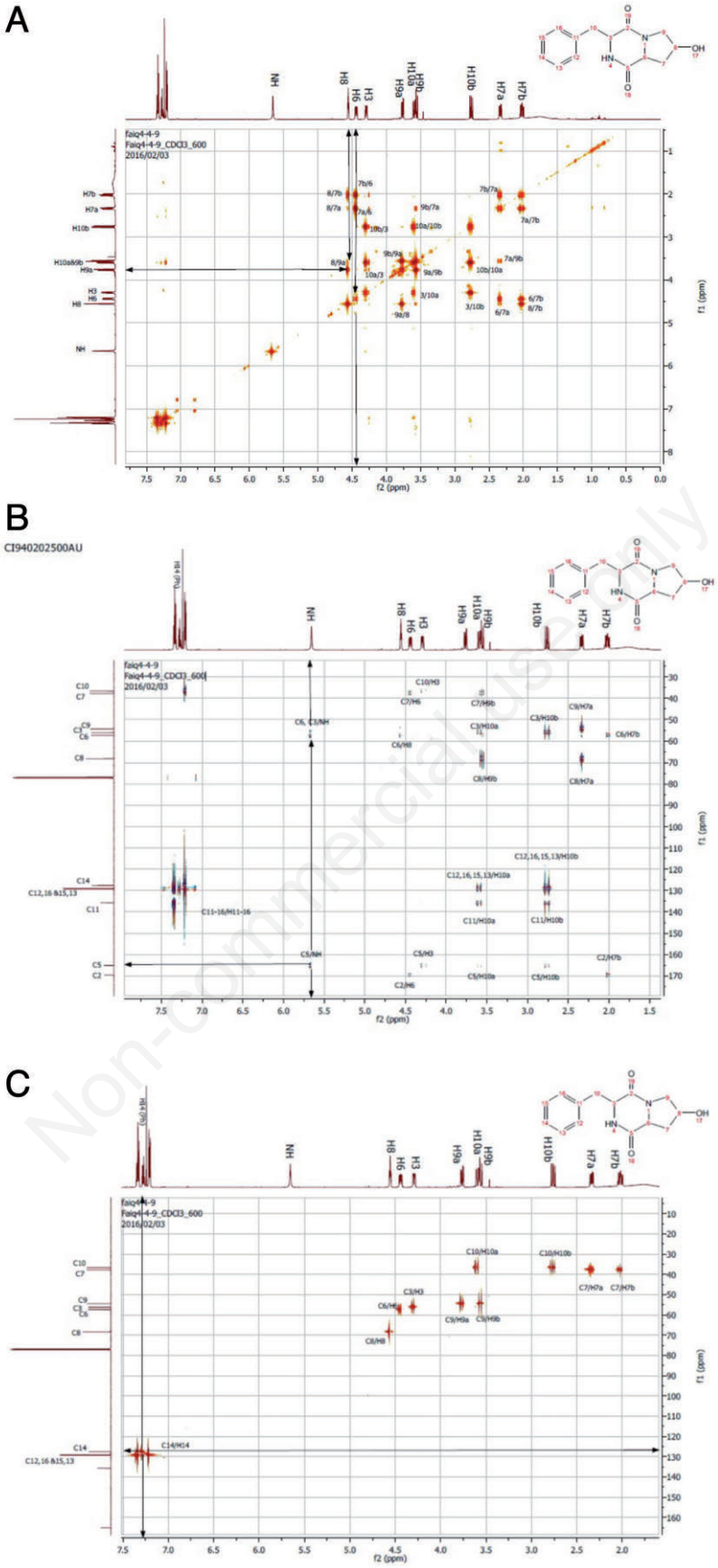

Figure 5. A) COSY nuclear magnetic resonance of KLPp3-4.3a.9; B) HMBC nuclear magnetic resonance of KLPp3-4.3a.9; C) HSQC nuclear magnetic resonance of KLPp3-4.3a.9.

Discussion

Sub-MIC of antibiotic allows susceptible strains to continue growing. Studies show that the sub-MICs of antibiotic can reduce the ability of the biofilm production without killing the targets.[12,13] Sub-MIC level of *P. ruthenica* KLPp3 crude extract could have potential as antibiofilm, reducing initial attachment and biofilm formation. When tested against matured biofilm, it did not show any effect (data is not shown). These may indicate that *P. ruthenica* KLPp3 crude extract inhibit biofilm formation by disturbing initial attachment. Previously reported that sub-MIC of antibiotics was able to inhibit the attachment of *Pseudomonas aeruginosa*.[14] It has been reported that the adherence of *Streptococcus mutans* and *Streptococcus sobrinus* to saliva-coated hydroxyapatite (sHA) discs were reduced by extract of *Withania somnifera* at sub-MIC concentrations.[15] Studies show that the sub-MICs of antibiotics and antimicrobial compounds can reduce the ability of the biofilm production without killing the targets.[14,16]

The crude extract targeted *V. alginolyticus*, one of the pioneers of biofilm formation. Huang *et al.* suggest that vibrios in the seawater tend to attach to a surface and are one of the pioneer groups in the biofilm community.[17] The molecular confirmation of dominant genera in marine biofilm subjected to16s rDNA results reveals initial marine biofilm forming bacteria was *V. alginolyticus*.[18] It had been reported that *V. alginolyticus* employed biofilm formation to survive in the marine environments and cause vibriosis in shrimp and biofouling in the process.[18,19]

One antibiofilm substances was identified. The presence of cyclic dipeptide that consists of a six-membered ring containing two amide linkages where the two nitrogen atoms and the two carbonyls are at opposite positions in the ring both suggest that the compound is diketopiperazine. The chemical structures was determined as 3-benzyl-7-hydroxyhexahydropyrrolo[1.2a]pyrazine-1,4-dione. Cyclo(L-Phe–trans-4-OH-L-Pro) has previously been reported to be both antibacterial and antifungal but never before been used as antibiofilm activity.[5,20]

Conclusions

In conclusion, 3-benzyl-7-hydroxyhexahydropyrrolo[1.2a] pyrazine-1,4-dione produced by *P. ruthenica* KLPp3 inhibits *in vitro* biofilm formation. The inhibitory effect of 3-benzyl-7-hydroxyhexahydropyrrolo[1.2a]pyrazine-1,4-dione maybe due to inhibition of pre-formed biofilms by blocking the attachment of bacteria to surfaces.

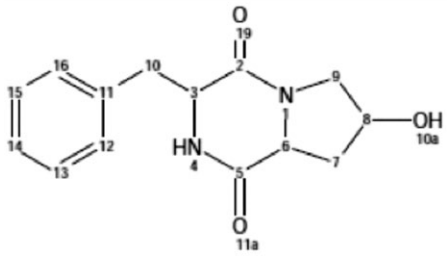

Figure 6. Chemical structure of KLPp3-4.3a.9.

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