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ARTICLE

Paenialvin A–D, four peptide antibiotics produced by *Paenibacillus alvei* DSM 29

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Abstract

Four peptide antibiotics, named paenialvin A–D, were isolated from *Paenibacillus alvei* DSM 29. Mass spectrum analysis determined the molecular masses of paenialvin A–D to be 1891, 1875, 1877, and 1923 Da, respectively. Tandem mass spectra and nuclear magnetic resonance (NMR) were used to elucidate their chemical structures. Paenialvin A–D showed antimicrobial activity against most strains that were tested, including methicillin-resistant *Staphalococcus aureus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Loktanella hongkongensis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. In particular, the minimum inhibitory concentration of paenialvins against *Staphalococcus aureus* reached 0.8–3.2 μg/mL. Although they were cytotoxic against HeLa cells at a concentration of 50 μg/mL, the lack of hemolysis by paenialvins confirmed that they are potential candidates for anti-tumor drugs.

Introduction

Overuse of antibiotics has led to the current antibiotic resistance crisis [1, 2]. For example, a number of bacteria, such as methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, are difficult to treat in both hospitals and communities because they are resistant to one or more antibiotics [3]. The resistance of bacteria to multiple drugs poses a serious challenge to medicine. In fact, the rate of discovery of new antimicrobial agents is slower than the rate at which currently used antibiotics are becoming ineffective. The search for new, potent, and safe antimicrobial agents is urgent [4].

Paenibacillus is already well-known as a source of new antimicrobials [5]. Originally designated to the genus *Bacillus*, *Paenibacillus* was first defined as a new genus with 11 species in 1993 [6–8]. *Paenibacillus* species are widely distributed in the natural environment, and they produce diverse antimicrobial agents that are active against multiple pathogens [5, 9]. To date, the antimicrobial agents isolated from *Paenibacillus* species include peptide and polyketide antibiotics [6, 10, 11]. Peptide antibiotics, including polymyxin, fusaricidin, and polypeptin, exhibit a broad inhibition spectrum against bacteria [12–14]. In particular, polymyxins B and E have been used as a treatment of last resort for Gram-negative bacterial infections [15, 16]. *Paenibacillus* produces fewer polyketide antibiotics than polypeptide antibiotics. One of the polyketides produced by *Paenibacillus* is called paenimacrolidin, a very unstable compound. Another is a hybrid polyketide called paenilamicin with excellent antibiotic activities against fungi and *Bacillus* spp. Many antibiotics from *Paenibacillus* have strong biological activity, so *Paenibacillus* seemed to be a good subject for the search of new antibiotics.

Based on analysis of its genome sequence, *Paenibacillus alvei* DSM 29 was reported to have the potential to produce diverse polyketides and nonribosomal peptides [17]. Here, we report the discovery of four novel polypeptides, named paenialvin A–D (Fig. 1), produced by *Paenibacillus alvei* DSM 29. Paenialvin A–D exhibit antibiotic activity against most tested strains, including methicillin-resistant *Staphylococcus aureus* (MRSA), *S. aureus*, *Bacillus*

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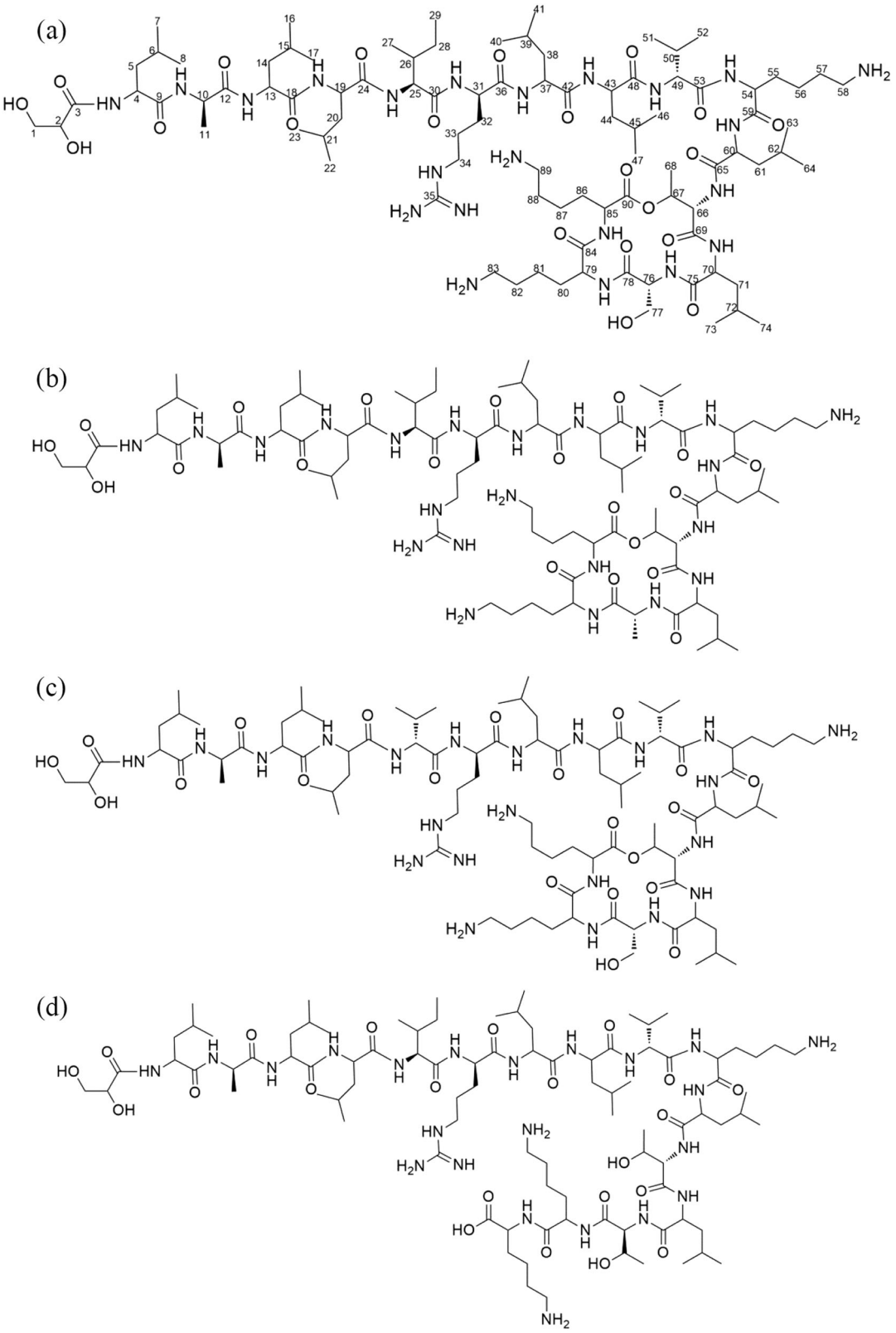

Fig. 1 Chemical structures of paenialvin A–D: **a** paenialvin A, **b** paenialvin B, **c** paenialvin C, and **d** paenialvin **D**

subtilis, and *E. coli*. In this study, we describe the cultivation, isolation, chemical identification, antimicrobial efficacy, hemolysis, and toxicity of paenialvin A–D.

Materials and methods

Strains

Paenibacillus alvei DSM 29 was isolated from foulbrood-diseased honeybees [17]. *S. aureus* (ATCC 25923 and UST 950701-005), Methicillin-resistant *S. aureus* (ATCC 43300), *E. coli* K-12 MG1165, *B. subtilis* zk31, *P. aeruginosa* PAO1, and *Loktanella hongkongensis* UST950701-009 were used to perform antimicrobial assays.

Isolation and purification of antagonistic compounds

Paenibacillus alvei DSM 29 was cultivated in 50-ml sterile Falcon tubes at 37 °C and 200 r.p.m. for 12 h. Ten milliliters of each preculture was then inoculated into 1.0 L of GSC medium (glucose 20 g, starch 20 g, $(NH_4)_2SO_4$ 20 g, yeast extract 10 g, K_2HPO_4 2.6 g, $FeSO_4{\cdot}7H_2O$ 0.1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, NaCl 0.25 g, $CaCO_3$ 9.0 g) [13]. Bacteria were grown at 30 °C and 160 r.p.m. for 72 h. A total of 10 L of cultivation medium was first centrifuged at 4.2×10^3 r.p.m. for 40 min to isolate new compounds with potential antibacterial activity. Supernatant was collected and 500 g of Diaion® HP-20 (Sigma) was added. The mixture was then shaken for 30 min at 160 r.p.m. Resin was separated and collected from the mixture, and then washed with 20% isopropanol. A crude extract was obtained by eluting the resin with 80% isopropanol. This crude extract was then subjected to flash C18 chromatography and elution with 20, 60, and 100% methanol, respectively, yielding three fractions. The antimicrobial activity of these fractions was tested against several microbial strains. Only the fraction eluted with 60% methanol exhibited good antimicrobial activity. The fraction with 60% methanol was dried in a rotor vacuum evaporator for further analysis. To obtain pure active compounds, the residue was dissolved in 1 mL of methanol and subjected to reverse phase semi-preparative high performance liquid chromatography (HPLC) with a gradient mobile phase at a flow rate of 3 mL min^{-1} in 20 min (solution A: acetonitrile with 0.5‰ trifluoroacetic acid (TFA); solution B: Milli-Q water with 0.5‰ TFA. 40% A over 5 min, 40–65% A from 5 to 25 min). The eluate was monitored at 210 nm. All the separable peaks were collected, dried, and re-dissolved in dimethyl sulfoxide (DMSO) for antimicrobial assessment. All fractions were stored at −20 °C for further use.

Electrospray ionization mass spectrometry (ESI–MS), MS^2, and nuclear magnetic resonance (NMR) analysis of Panialvin A–D

ESI-MS analyses were performed on a Bruker ultra-performance liquid chromatography-time of fly-mass spectrometer in positive ion scanning mode, with a mass range of 100–2000 Da and a voltage of 4.5 kV. Two microliters of compound solution at a concentration of 0.5 mg/mL in methanol was injected into a waters BEH C18 reversed-phase UPLC column (1.7 µm, 150 mm × 2.1 mm inner diameter). MS^2 spectra were acquired by an LTQ Velos dual-pressure ion trap mass spectrometer (Thermo Fisher Scientific, USA). 1D NMR was measured on a Bruker 600 MHz NMR spectrometer and 2D NMR was performed on a Varian Inova 500 MHz NMR spectrometer. All samples were dissolved in DMSO-d_6, and chemical shifts were measured in ppm relative to TMS.

Marfey's analysis of amino acid configurations

The amino acid configurations of these fractions were determined by Marfey's method with LC/MS [18–20]. Standard L-configured amino acid solutions were prepared at a concentration of 50 mM in Milli-Q water. A mixture of 50 µL of specific amino acid aqueous solution, 10 µL of 1 M $NaHCO_3$, and 100 µL of 1% acetone solution of Marfey's reagent (Sigma) was prepared. The Marfey's reagent could be N_α-(2,4-Dinitro-5-fluorophenyl)-L-alaninamide (FDAA), 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) or D-FDLA. The combination of amino acid and Marfey's reagent in the mixture are shown in Table 1. The mixture was incubated in the dark at 80 °C for one hour, then cooled to room temperature with 20 µL of 3 M HCl added to stop the reaction. Then, the mixture was dried using nitrogen and the residue was re-dissolved in 100 µL of methanol before analysis in an ultra performance liquid chromatography-mass spectrometry (1.7 µm BEH C18 column, 0.25 mL min^{-1}, gradient from 5 to 50% CH_3CN with 0.1% formic acid, 50 min) to set up standards. One microliter of an antagonistic compound at a concentration of 50 mg mL^{-1} was hydrolyzed in 1 mL of 6 M HCl at 110 °C for 12 h. Hydrochloric acid in the hydrolysate was then removed by evaporation and the residue was re-dissolved in 100 µL of water. This aqueous solution was processed following the procedure to prepare standards as described above, and the final residues in methanol solution were subjected to UPLC–MS.

Antibacterial assays

The antibacterial activity of paenialvins were evaluated by minimum inhibitory concentration (MIC) assay against

Table 1 The retention time of products of Marfey's method. Paenialvins were totally hydrolyzed to amino acids and then reacted with FDLA or FDAA

	Time (min)				
	Standard	Paenialvin A	Paenialvin B	Paenialvin C	Paenialvin D
L-Ala-L-FDLA	32.3				
L-Ala-D-FDLA	36.6	36.8	36.6	36.6	36.8
L-Arg-L-FDLA	26.1				
L-Arg-D-FDLA	24.9	25.1	25.1	25.0	25.0
L-Val-L-FDLA	35.4				
L-Val-D-FDLA	43	43.1	43.0	43.0	43.0
L-Thr-L-FDLA	28.1	28.2	28.1	28.2	28.2
L-Thr-D-FDLA	33.6				
L-Ser-L-FDLA	29.2				
L-Ser-D-FDLA	29.8	29.9		29.9	
L-Lys-L-FDLA	25.8	25.9	25.8	25.8	25.9
L-Lys-D-FDLA	24.3	24.4	24.4	24.3	24.3
L-Ile-L-FDAA	31.7	31.8	31.8		31.8
L-Leu-L-FDAA	32.5	32.5	32.5	32.5	32.5
D-Leu-L-FDAA	37	37.0	37.0	37.0	37.0

S. aureus, Methicillin-resistant *S. aureus*, *L. hongkongensis*, *E. coli*, *B. subtilis*, and *P. aeruginosa* [21]. Briefly, the strains were inoculated in LB broth. Then the culture was incubated at 37 °C and 250 r.p.m. for 12 h, followed by dilution 10^4 times in Mueller–Hinton broth. Stock solutions of antagonistic compounds and reference antibiotics were prepared at 50 mg/mL in DMSO and further diluted to varying concentrations in 96-well plates. Each plate well contained 80 µL of microbial strain and 20 µL of diluted compound solution. The plates were then incubated at 30 °C for 20 h. The bacterial growth in each well was evaluated by measuring the absorbance of the well at 595 nm using a Thermo scientific Multiskan FC multiplate photometer.

Cytotoxicity assay

HeLa cells were used to assess the cytotoxicity of antagonistic compounds [22]. Briefly, the stock solutions of compounds (50 mg mL^{-1} in DMSO) were diluted in the assay media Dulbecco's modified Eagle medium (DMEM). Ninety microliters with 1×10^5 cells/mL were planted into 96-well plates and incubated at 37 °C for 12 h, followed by the addition of compounds into the wells at various concentrations. After the cells were incubated at 37 °C for 48 h, the supernatant was removed and 20 µL of MTT (2.5 mg/mL) in PBS buffer was added into each well. The cells were further incubated at 37 °C for 3 h, followed by the addition of 80 µL of DMSO to each well and incubating for another 15 min. The absorbance of each well was measured at 570 nm by a Thermo scientific Multiskan FC multiplate photometer.

Hemolysis assay

2% fresh rabbit red blood cells were washed with PBS until the upper phase was clear after centrifugation. Two hundred microliters of pellet was re-suspended into 10 mL of PBS for further experiments. 0.5 mL of compound at a concentration of 100 µg/mL in PBS was mixed with 0.5 mL of 2% cell solution and incubated at 37 °C for 3 h. As a positive control and a negative control, 0.5 mL of water and 0.5 mL of PBS were mixed with 0.5 mL of 2% cell solution, respectively. After centrifuging at 15,000 rpm min^{-1}, 100 µL of the upper phase was added to a 96-well plate for measurement. The absorbance of each well was measured at 570 nm by a Thermo scientific Multiskan FC multiplate photometer [23].

Results and discussion

Isolation and purification

Antimicrobial agents were harvested by extracting the culture medium of *Paenibacillus alvei* DSM 29 with resin. After evaporation of the organic solvent, the residue consisting antimicrobial agents was separated by reverse phase C18 flash chromatography. The fraction with 60% methanol at a concentration of 50 µg mL^{-1} in DMSO showed antimicrobial activity against *S. aureus* and methicillin-resistant *S. aureus*. Based on the bioactivity of this fraction, it was further purified by HPLC, yielding several peaks on an HPLC profile (Figure S1). Four of the peaks were

determined to represent new compounds, which were named paenialvin A–D (Fig. 1). The final yields of paenialvin A–D from 10 L cultivation medium were ~5.4 mg of paenialvin A, 1.1 mg of paenialvin B, 1.5 mg of paenialvin C, and 0.3 mg of paenialvin D.

Determination of structure

Based on the ESI–MS analysis, the molecular masses of paenialvin A–D were 1891, 1875, 1877, and 1923 Da, respectively (Figure S2). To determine the amino acid sequences of paenialvin A, MS–MS analysis was performed. The MS–MS spectrum of the triply charged ion of paenialvin A at *m/z* 631.7 is shown in Fig. 2a. Successive fragmentation from one terminus of paenialvin A resulted in b-type ions at 667.85, 1221.62, 994.52, 881.52, 768.43, 612.35, 499.76, 386.26, 273.09, and 202.00, along with the related a-type ions at 558.26, 671.35, 799.44, 898.52, 1011.53, 562.76, 640.85, 697.43, 753.93, 810.44, 846.52, and 602.35. These ion fragments were assigned to the following sequence: Leu/Ile-Ala-Leu/Ile-Leu/Ile-Leu/Ile-Arg-Leu/Ile -Leu/Ile-Val-Lys-Leu/Ile (Fig. 2b). The amino acid sequence in the lactonic ring of paenialvin A cannot be determined from the MS–MS spectrum of paenialvin A, because the ion fragments of the lactonic ring were missing. In MS–MS spectrum, the fragment at *m/z* 697.4 had highest signal among all signals. The ion fragment at *m/z* 697.4 with double charge was then chosen to perform MS^3. The MS^3 spectrum of the ion at *m/z* 697.4 is shown in Fig. 3a. Ion fragments at 836.52, 937.61, 1050.61, 569.26, and 633.35, together with others at 558.26, 457.17, 344.25, and 257.17 allowed the following sequence to be assigned: Thr-Leu/Ile-Ser-Lys-Lys (Fig. 3b). Based on the sequences of these two fragments, Paenialvin A should have a sequence of Ala-Leu/Ile-Leu/Ile-Leu/Ile-Arg-Leu/Ile-Leu/Ile-Val-Lys-Leu/Ile-Thr-Leu/Ile-Ser-Lys-Lys. In the NMR spectra, two characteristic methyls (δ_H 0.79 3H, overlapped, δ_C 15.4; δ_H 0.84 3H, overlapped, δ_C 18.2) and a specific tertiary carbon atom (δ_H 1.74 H, m, δ_C 36.5) (Table 2) indicated the presence of one isoleucine module in this compound. In addition, based on heteronuclear multiple bond correlation (HMBC) data, the hydrogen atom (δ_H 1.74) on the specific tertiary atom of isoleucine module is correlated with the carbonyl carbon atom (δ_C 171.15), and the carbonyl carbon atom (δ_C 171.15) is correlated with the hydrogen atom (δ_H 8.09) on the nitrogen atom of arginine module (Figure S12). The isoleucine module should be located in the N-terminal of the arginine module (Fig. 1a). The signal in HMBC spectrum (Figure S12) also showed the correlation between C-90 (δ_C 170.82) and H-67 (δ_H 5.30), indicating that the

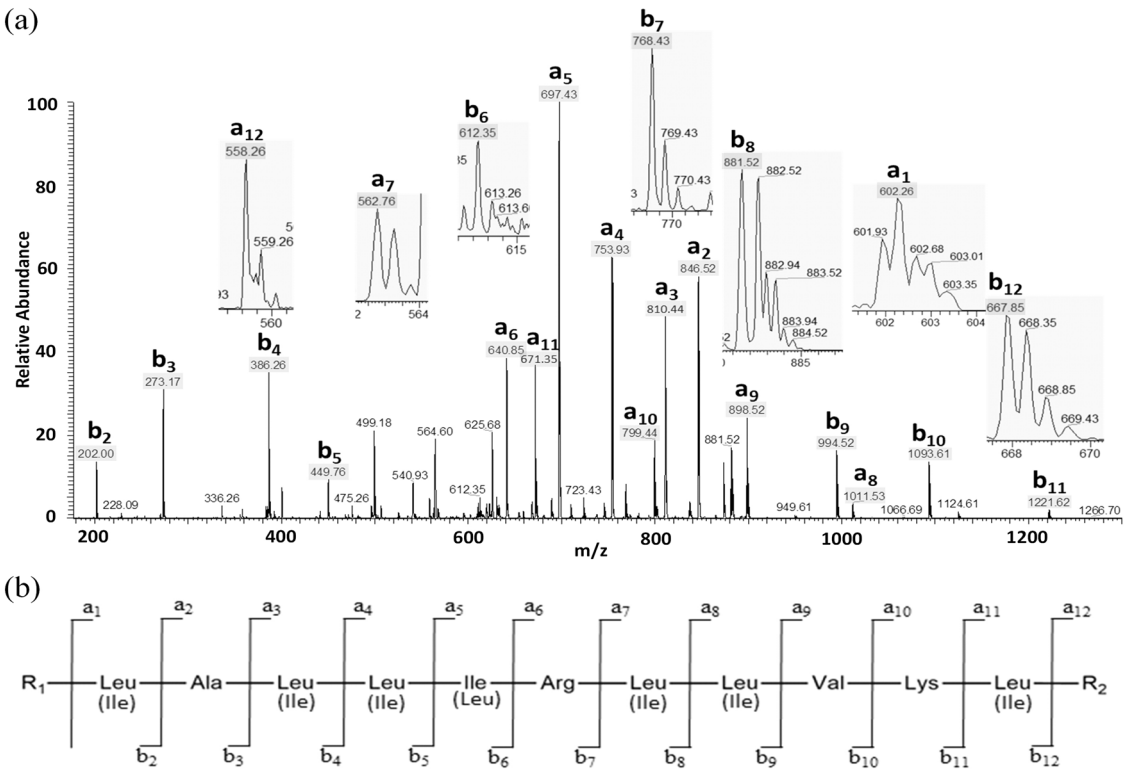

Fig. 2 **a** Tandem mass spectrum of paenialvin A at *m/z* 631.7. **b** The amino acid sequence of chain part of paenialvin A was confirmed by these a and b type fragment ions

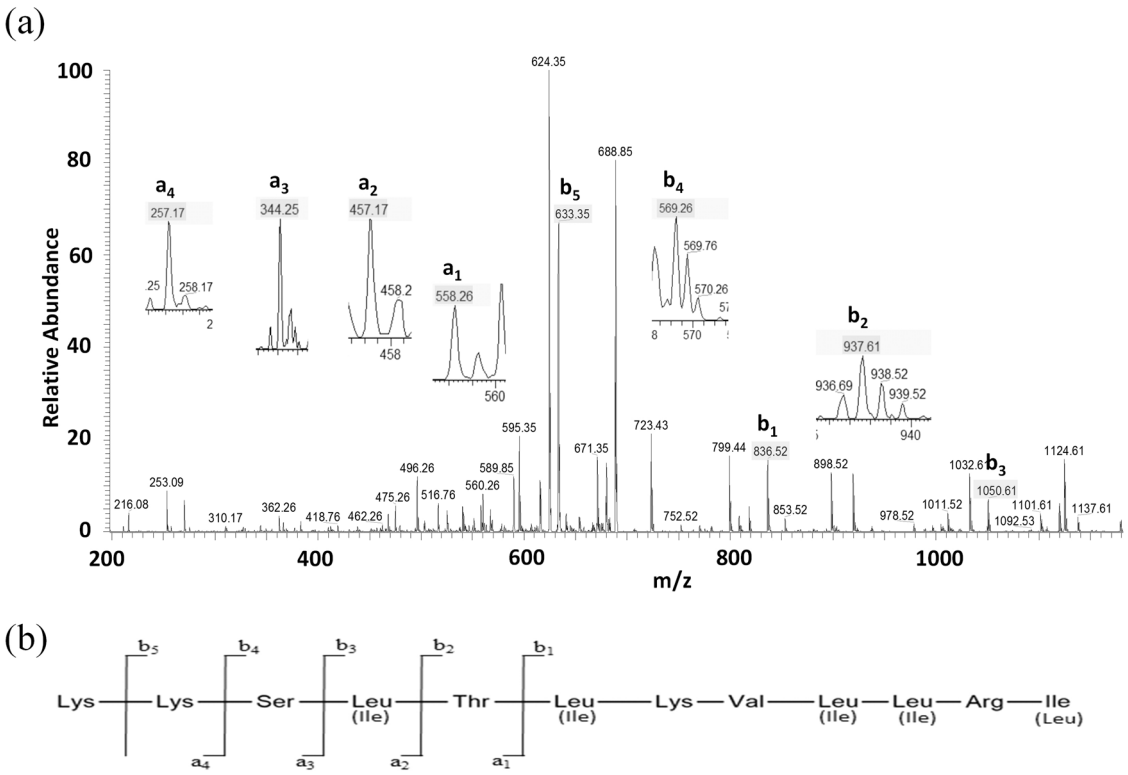

Fig. 3 **a** Tandem mass spectrum of paenialvin A at *m/z* 697.5. **b** The amino acid sequence of lactonic ring of paenialvin A was confirmed by these a and b type fragment ions

ester bond was formed between C-90 and C-67. In addition, the chemical structure of the residue in paenialvin A was determined using ^{1}H and ^{13}C chemical shifts, along with ^{1}H–^{1}H correlation (^{1}H–^{1}H COSY) and HMBC. Two alcohol functions were revealed at δ_C 64.2 (C-1) and δ_C 72.891 (C-2) (Figure S9), while C-1 and C-2 were a primary alcohol and a secondary alcohol, respectively. Signals in the ^{1}H–^{1}H correlation spectrum (Figure S11) showed the correlation between the hydrogen atoms at δ_H 3.52, 3.60 and δ_H 3.96 (Figure S8), revealing that C-1 and C-2 are connected to each other. The signal in the HMBC spectrum indicated that C-3 (δ_C 172.73) was correlated with H-2 (δ_H 3.96). Therefore, C-3 is connected with the secondary alcohol function (C-2). Signals in the ^{1}H–^{1}H correlation spectrum (Figure S11) showed the correlation between the hydrogen atoms at δ_H 4.42 and δ_H 7.94, indicating that N-4 and C-4 are connected to each other. Besides, the correlation of C-3 (δ_C 172.73) and H (δ_C 7.94) in HMBC spectrum reveals that C-3 is connected to N-4.

The chemical structures of paenialvin B, C and D are similar to paenialvin A based on tandem mass spectra. The successive ion fragments of each compound are shown in Figure S3–S7. An alanine module substituted the serine module of paenialvin A in paenialvin B, while other modules showed no difference from paenialvin A (Figure S4). Paenialvin C had one more valine module than paenialvin A, but there was no isoleucine in paenialvin C (Figure S5). In paenialvin D, a threonine module replaced the serine module of paenialvine A, and the ester bond was hydrolyzed, causing an increase of molecular weight of 32 Da (Figure S7).

Marfey's method was used to identify the absolute configurations of the amino acids in paenialvins. Paenialvins were hydrolyzed by hydrochloric acid, and the products were reacted with L-FDLA to form complexes. These complexes were analyzed by UPLC–MS and were then compared to the standards. As shown in Table 1, all paenialvins have D-configured alanine, arginine, valine and L-configured Threonine. The serine modules in paenialvin A and C have D configurations, while the isoleucine modules in Paenialvin A, B and D have L configurations. Both leucine and lysine modules in paenialvins have two configurations, but the specific configuration of each module cannot be identified.

Antibacterial activity and cytotoxicity

Using a microdilution method, the antimicrobial activities of the purified compounds against both Gram-positive and Gram-negative bacteria were tested and compared with the antibiotic in clinical use. As shown in Table 3, paenialvin A exhibited inhibitory activity against all the

Table 2 Nuclear magnetic resonance (NMR) data for CD_3SOCD_3-dissolved paenialvin A

Pos.	δ_C[a]	δ_H[b] (mult. *J* in Hz)	Pos.	δ_C[a]	δ_H[b] (mult. *J* in Hz)
1	64.20	3.52 (1H, dd, 13.2, 3.0)	45	24.30	1.45 (1H, m)
		3.60 (1H, dd, 13.2, 3.0)	46	21.24	0.81 (1H, overlapped)
2	72.89	3.96 (1H, overlapped)	47	21.24	0.81 (1H, overlapped)
3	172.73		48	170.98	
4	52.44	4.42 (1H, overlapped)	49	58.35	4.10 (1H, overlapped)
4-NH		7.94 (1H, d, 6.6)	49-NH		7.82 (1H, overlapped)
5	40.42	1.57 (2H, overlapped)	50	30.52	1.98 (1H, m)
6	24.27	1.48 (1H, m)	51	19.23	0.83 (1H, overlapped)
7	21.18	0.78 (3H, overlapped)	52	19.23	0.83 (1H, overlapped)
8	21.18	0.78 (3H, overlapped)	53	171.25	
9	172.45		54	58.35	4.11 (1H, overlapped)
10	48.65	4.23 (1H, m)	54-NH		7.82 (1H, overlapped)
10-NH		8.09 (1H, d, 7.8)	55	30.52	1.72 (2H, m)
11	17.17	1.18 (3H, d, 8.4)	56	19.23	1.26 (2H, m)
12	172.31		57	26.47	1.51 (2H, m)
13	50.76	4.32 (1H, overlapped)	58	38.89	2.75 (2H, m)
13-NH		8.03 (1H, d, 8.4)	58-NH		7.78 (1H, overlapped)
14	40.64	1.53 (2H, overlapped)	59	172.13	
15	24.19	1.47 (1H,m)	60	53.08	4.10 (1H, overlapped)
16	21.48	0.83 (3H, overlapped)	60-NH		7.82 (1H, overlapped)
17	21.48	0.83 (3H, overlapped)	61	41.25	1.50 (2H, overlapped)
18	171.90		62	22.05	1.28 (1H, m)
19	52.44	4.42 (1H, overlapped)	63	21.79	0.85 (3H, overlapped)
19-NH			64	21.79	0.85 (3H, overlapped)
20	40.42	1.53 (2H, overlapped)	65	173.07	
21	24.27	1.47 (1H,m)	66	55.58	4.42 (1H, overlapped)
22	21.58	0.83 (3H, overlapped)	66-NH		8.34 (1H, d, 9.0)
23	21.58	0.83 (3H, overlapped)	67	71.20	5.30 (1H, m)
24	172.73		68	17.17	1.18 (3H, d, 8.4)
25	48.64	4.14 (1H, overlapped)	69	168.45	
25-NH		7.94 (1H,d, 6.6)	70	51.40	4.22 (1H, overlapped)
26	36.51	1.74 (1H, m)	70-NH		8.09 (1H, d, 7.8)
27	15.37	0.79 (3H, overlapped)	71	40.35	1.54 (2H, overlapped)
28	22.30	1.28 (2H, m)	72	22.05	1.29 (1H, m)
29	18.20	0.84 (3H, overlapped)	73	21.73	0.83 (3H, overlapped)
30	171.15		74	21.73	0.83 (3H, overlapped)
31	48.65	4.29 (1H, overlapped)	75	172.53	
31-NH		8.09 (1H, d, 7.8)	76	57.47	4.15 (1H, overlapped)
32	29.86	1.72 (2H, m)	76-NH		7.71 (1H, overlapped)
33	24.27	1.48 (2H, m)	77	60.77	3.68 (1H, overlapped)
34	40.35	3.07 (2H, m)			3.77 (1H, overlapped)
35	156.88		78	170.62	
34-Nha		7.73 (1H, overlapped)	79	53.08	4.09 (1H, overlapped)
NHb		6.92 (2H, overlapped)	79-NH		7.82 (1H,overlapped)
NHc		6.69 (1H, overlapped)	80	29.99	1.72 (2H, m)
36	172.22		81	17.47	1.26 (2H, m)
37	51.18	4.33 (1H, overlapped)	82	26.59	1.51 (2H, m)

Table 2 (continued)

Pos.	δ_C[a]	δ_H[b] (mult. *J* in Hz)	Pos.	δ_C[a]	δ_H[b] (mult. *J* in Hz)
37-NH		8.03 (1H, d, 8.4)	83	38.75	2.75 (2H, m)
38	40.77	1.46 (2H, overlapped)	84	172.22	
39	22.30	1.25 (1H, m)	85	53.08	4.09 (1H, overlapped)
40	21.36	0.84 (3H, overlapped)	85-NH		7.82 (1H, overlapped)
41	21.36	0.84 (3H, overlapped)	86	29.86	1.72 (2H, m)
42	171.01		87	17.47	1.26 (2H, m)
43	51.18	4.30 (1H, overlapped)	88	26.68	1.51 (2H, m)
43-NH		7.99 (1H, d, 9.0)	89	38.71	2.75 (2H, m)
44	40.77	1.53 (2H, overlapped)	90	170.82	

Table 3 The minimum inhibitory concentration of paenialvins and the reference antibiotic against several tested strains

Strain	MIC (μg ml^{-1})				
	Paenialvin A	Paenialvin B	Paenialvin C	Paenialvin D	Ampicillin sodium salt
S. aureus UST950701-005	0.8	3.2	3.2	1.6	6.4
MRSA ATCC 43300	3.2	3.2	25	3.2	6.4
S. aureus ATCC 25923	1.6	3.2	3.2	1.6	<0.2
Loktanella hongkongensis UST950701-009	25	>50	>50	>50	6.4
Escherichia coli K-12 MG1165	12.5	25	50	25	1.6
P. aeruginosa PAO1	25	>50	>50	>50	>50
Bacillus subtilis zk31	<0.2	0.8	1.6	0.8	<0.2

tested strains, while the other three compounds showed no activity against either *Loktanella hongkongensis* UST950701-009 or *Pseudomonas aeruginosa* PAO1. Although ampicillin sodium salt showed stronger potent activity than paenialvins against Gram-negative bacteria strains, paenialvins were slightly more active against Gram-positive bacteria strains than the clinical antibiotic tested. Interestingly, paenialvin A had great antibacterial activity against Gram-positive bacteria, especially against *Staphylococcus aureus* UST950701-005. The MIC of paenialvin A against *Staphylococcus aureus* UST950701-005 was as little as 0.8 μg/mL, which was much lower than the clinical antibiotic tested. All paenialvins showed cytotoxicity against HeLa cells at a concentration of 50 μg/mL. Nevertheless, paenialvin B, C, and D showed no cytotoxicity against HeLa cells at a concentration of 10 μg/mL. At this concentration, paenialvin B, C, and D inhibited the growth of *S. aureus* and MRSA. Paenialvin A was non-cytotoxic to HeLa cells only when the concentration was less than 2 μg/mL (Table S1). Hemolysis assay was performed using fresh rabbit red blood cells. No hemolysis was observed when paenialvins were tested at a concentration of 50 μg/mL (Table S1). Paenialvin A–D contain lysine and arginine, resulting in a net positive charge of +2, +3 and +4 in mass spectra, which could be assigned to "cationic peptide antibiotics" [24]. Cationic peptide antibiotics are well known to kill bacteria quickly, partly by physically disrupting the cell membranes. They utilize positively charged amino acids to form a polar hydrophilic surface to attach to cell membranes. The excellent antibacterial activities of positively charged paenialvins may behave as cationic peptide antibiotics to disrupt cell membranes.

In conclusion, four new polypeptides, paenialvin A–D, were discovered from *Paenibacillus alvei* DSM 29. The chemical structures of paenialvin A–D were identified by a combination of mass spectrum, tandem mass spectrum, and NMR. Antimicrobial activity, hemolysis, and cytotoxicity reveal that paenialvin B–D are potential therapeutic agents to treat infections caused by *S. aureus* and MRSA. Considering the cytotoxicity of paenialvin A, it might be used as an antitumor agent.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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