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ARTICLE

Isolation and structure determination of a new antibacterial peptide pentaminomycin C from *Streptomyces cacaoi* subsp. *cacaoi*

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Abstract

A new antibacterial peptide named pentaminomycin C was isolated from an extract of *Streptomyces cacaoi* subsp. *cacaoi* NBRC 12748^T, along with a known peptide BE-18257A. Pentaminomycin C was determined to be a cyclic pentapeptide containing an unusual amino acid, *N*δ-hydroxyarginine (5-OHArg), by a combination of ESI-MS and NMR analyses. The structure of pentaminomycin C was determined to be cyclo(–L-Leu–D-Val–L-Trp–L-5–OHArg–D-Phe–). Pentaminomycin C exhibited antibacterial activities against Gram-positive bacteria including *Micrococcus luteus*, *Bacillus subtilis*, and *Staphylococcus aureus*. The biosynthetic gene cluster for pentaminomycin C and BE-18257A was identified from the genome sequence data of *S. cacaoi* subsp. *cacaoi*.

Introduction

Many bioactive peptides have been isolated from microorganisms including bacteria and fungi [1]. Among them, several peptides, for example vancomycin, are used as therapeutic agents to treat bacterial infection. The peptide aglycone of vancomycin is biosynthesized by a nonribosomal peptide synthetase (NRPS) system, which consists of seven modules on three NRPSs (VcmA, VcmB, and VcmC) [2, 3]. Moreover, many commercial peptide antibiotics (chloramphenicol [4], daptomycin [5], teicoplanin [6], actinomycin D [7], bleomycins A2 and B2 [8], and cyclosporine A [9]) have been reported to be biosynthesized by NRPS system [10].

Cyclic peptides derived from microorganisms have been reported to exhibit a wide variety of biological activities. Malformins are a group of cyclic pentapeptides derived from fungus *Aspergillus niger* as inducers for malformations of bean plants and curvatures of corn roots [11, 12]. Among malformins, malformins A1 and C were reported to have potent cytotoxicity [13, 14]. Kakeromamide A is a cyclic pentapeptide isolated from the marine cyanobacterium, which possesses inducing activity for differentiation of neural stem cells into astrocytes in vitro [15–17]. The cyclic pentapeptides BE-18257A and B (**1** and **2** in Fig. 1) were isolated from *Streptomyces misakiensis* BA18257 as endothelium-derived vasoconstrictor factor binding inhibitors [18, 19]. Recently, cyclic pentapeptides, pentaminomycins A and B (**3** and **4** in Fig. 1), which contain *N*δ-hydroxyarginine (5-OHArg), were isolated from *Streptomyces* sp. RK88-1441 [20]. Pentaminomycin A showed an antimelanogenic activity against alpha-melanocyte stimulating hormone (α-MSH)-stimulated B16F10 melanoma cells [20].

Based on this background, we performed chemical screening for new peptides in extracts of actinobacteria using ESI-MS and HPLC (data not shown). As a result, we isolated a new antibacterial cyclic pentapeptide named pentaminomycin C (**5** in Fig. 1) from the MeOH extract of *Streptomyces cacaoi* subsp. *cacaoi* NBRC 12748^T, along with a known peptide BE-18257A (**1** in Fig. 1). Here we

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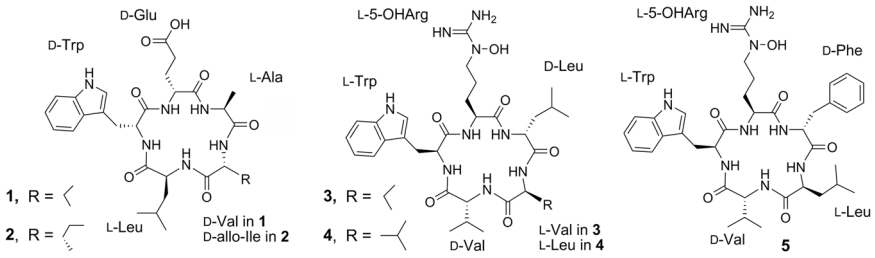

Fig. 1 Chemical structures of BE-18257A (**1**) and B (**2**), and pentaminomycins A (**3**), B (**4**), and C (**5**)

describe the isolation and structure determination of **5** from *S. cacaoi* subsp. *cacaoi*. In addition, we found the putative biosynthetic gene cluster containing two NRPSs for **1** and **5** in the genome data of *S. cacaoi* subsp. *cacaoi* [21].

Results and discussion

A chemical investigation of *S. cacaoi* subsp. *cacaoi* using HPLC and ESI-MS analyses was conducted. As a result, a new peptide named pentaminomycin C (**5** in Fig. 1) was detected by subjecting MeOH extract of *S. cacaoi* subsp. *cacaoi* to HPLC analysis (Fig. S1). *S. cacaoi* subsp. *cacaoi* was cultured using 5 L of modified ISP2 agar medium [22] and the extract was subjected to repeated HPLC separation to obtain **5**, along with a known peptide BE-18257A (**1** in Fig. 1).

The molecular formula of **5** was established as $C_{37}H_{51}N_9O_6$ by HR-ESI-MS, since an ion peak $[M + H]^+$ was observed at *m/z* 718.4010 (calculated *m/z* value: 718.4040). To determine the chemical structure of **5**, NMR analyses including ^1H, ^{13}C, DEPT-135, DQF-COSY, TOCSY, NOESY, HMBC, HSQC, and ^{15}N–^1H HSQC were performed. The assignment of the constituent five amino acids including leucine (Leu), valine (Val), tryptophan (Trp), *N*δ-hydroxyarginine (5-OHArg), and phenylalanine (Phe) was achieved using spin-system identification (Table 1). Regarding assignment of an unusual amino acid 5-OHArg, HMBC correlation was detected from hydrogen (*δ* H 10.52) to carbon of C=N bond (157.4). In addition, the chemical shifts of hydrogens (*δ* H 3.40) and carbon at *δ* position (*δ* C 50.6) in 5-OHArg were shifted to downfield due to presence of hydroxy residue of δ-amine. The chemical shifts were similar to the literature values of 5-OHArg previous reported for the peptides pentaminomycins A and B [20]. The sequence of amino acids was determined by interpretation of HMBC data. The HMBC correlations from the α-hydrogen and the amide hydrogen to the same carbonyl carbon were observed (half ended arrows in Fig. 2), indicating the sequence of 5-OHArg–Phe–Leu–Val–Trp. HMBC correlation from an α-hydrogen (*δ* H 4.27) to the carbonyl carbon (*δ* C 171.9) in Trp was not observed. However a NOESY correlation was observed between α-hydrogen of Trp (*δ* H 4.27) and amide hydrogen of 5-OHArg (*δ* H 7.31) as shown by double ended arrow in Fig. 2, which indicated the connection between Trp and 5-OHArg. Pentaminomycin C was shown to have structure of cyclo(–Leu–Val–Trp–5-OHArg–Phe–). Similar ESI-MS and NMR analyses were performed on **1**. The chemical shifts were similar to the reported literature values of BE-18257A [19] (data not shown). In addition, we confirmed the planer structure of **1** by 2D NMR analyses in the same manner with **5**.

The absolute configurations of the amino acids (Leu, Val, Trp, 5-OHArg, and Phe) in **5** were determined by modified Marfey's analysis [23]. To convert 5-OHArg to Arg, compound **5** (1.0 mg) was hydrolyzed in hydriodic acid (HI) according to a previous report [24]. To analyze Trp, compound **5** (1.0 mg) was hydrolyzed in 6 N HCl containing 3% phenol to recover Trp [25]. Each hydrolysate was derivatized using L-FDLA, followed by comparative analysis by HPLC using standard amino acids derivatized with L- or D-FDLA. The results indicated that the absolute configurations of amino acids were L-Leu, D-Val, L-Trp, L-5-OHArg, and D-Phe. The structure of pentaminomycin C was therefore determined to be cyclo(–L-Leu–D-Val–L-Trp–L-5–OHArg–D-Phe–). For the stereochemistries of the constituent amino acids in **1**, the same method was applied (data not shown). We confirmed the stereochemistries of constituent amino acids (L-Leu, D-Trp, D-Glu, L-Ala, D-Val) in **1**, which were identical with the previous report [19].

Compounds **1** and **5** were tested against *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus, Escherichia coli*, and *Pseudomonas aeruginosa* to determine the minimum inhibitory concentration (MIC), along with positive control compound tetracycline (Table S1) [26]. As a result, compound **5** showed antibacterial activities against Gram-positive bacteria including *B. subtilis*, *M. luteus*, and *S. aureus* with MIC of 16 μg ml^{-1}, but no activity for Gram-negative bacteria including *E. coli* and *P. aeruginosa* at the concentration of 64 μg ml^{-1} (Table S1). On the other hand, compound **1** did not show any antibacterial activity against any of the test bacteria at the concentration of 64 μg ml^{-1}.

To clarify the biosynthetic pathway, we searched the biosynthetic gene cluster for pentaminomycin C and BE-18257A in draft genome sequence of *S. cacaoi* subsp. *cacaoi* NRRL B-1220^T (MUBL01000001–MUBL0100981) isogenic to NBRC 12748^T. As pentaminomycin C and BE-

Table 1 NMR chemical shift values of **5** in DMSO-d_6

AA	Position	δ H (J = Hz)	δ C	δ N
Leu	CO		172.4	
	NH	7.52 (d, 9.0)		122.0
	α	4.37 (m)	50.6	
	β	1.32 (m)	41.1	
	γ	1.40 (m)	24.4	
	δ1	0.79 (d, 6.5)	22.1	
	δ2	0.82 (d, 6.6)	22.9	
Phe	CO		170.8	
	NH	8.80 (d, 8.2)		127.4
	α	4.45 (m)	53.9	
	β	2.78 (d, 14.0, 9.4)	34.5	
		2.93 (d, 14.0, 5.9)		
	γ		138.0	
	δ	7.21 (m)	129.2	
	ε	7.21 (m)	128.2	
	ζ	7.16 (m)	126.4	
OHArg	CO		170.6	
	NH	7.31 (d, 7.2)		115.3
	α	4.14 (m)	52.9	
	β	1.51 (m)	28.2	
	γ	1.16 (m), 1.33 (m)	22.2	
	δ	3.40 (m)	50.6	
	N-OH	10.52 (s)		
	C=NH	ND	157.4	
	NH_2	ND		
Trp	CO		171.9	
	NH	8.55 (d, 7.8)		125.8
	α	4.27 (m)	55.5	
	β	2.89 (dd, 14.6, 11.3)	27.1	
		3.17 (dd, 14.6, 3.6)		
	ε1	10.75 (s)		130.8
	δ1	7.15 (m)	124.0	
	γ		110.2	
	ε3	7.50 (d, 7.9)	118.1	
	ζ3	6.96 (m)	118.5	
	η2	7.03 (m)	121.1	
	ζ2	7.29 (d, 8.1)	111.5	
	ε2		136.3	
	δ2		127.0	
Val	CO		171.6	
	NH	8.37 (d, 7.5)		123.3
	α	3.67 (dd, 9.9, 7.5)	60.1	
	β	1.62 (m)	28.7	
	γ1	0.31 (d, 6.7)	18.6	
	γ2	0.73 (d, 6.6)	19.2	

ND not detected

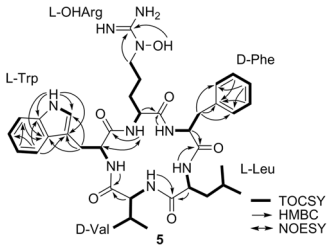

Fig. 2 Key 2D NMR correlations of pentaminomycin C (**5**)

18257A are composed of five amino acid residues comprising two to three D-amino acids, we looked for NRPS genes harboring five modules and comprising two to three epimerase (E) domains. Consequently, two NRPS genes were found as shown in Fig. 3. The gene cluster includes regulatory genes, transport-related genes, and biosynthetic genes including *mtb*, putative hydrolase, and P450 genes in addition to two NRPS genes. The gene BZY53_RS10935 was considered as the NRPS gene responsible for the synthesis of BE-18257A, because the second, third, and fifth modules include E domain that isomerizes L-amino acid to D-configuration (Fig. 3). Our bioinformatic analysis using online analysis tools [27, 28] suggested the substrates of the first, fourth, and fifth adenylation (A) domains were Leu, Ala, and Val, respectively, which are in accordance with constituent amino acid residues (L-**Leu**–D-Trp–D-Glu-L-**Ala**–D-**Val**) in BE-18257A as indicated by bold letters (Fig. 3). Another NRPS gene (BZY53_RS10880 to BZY53_RS01305) was considered responsible for pentaminomycin C synthesis, because the second and fifth modules contain E domain, and second, fourth, and fifth A domains were predicted to incorporate Val, Arg, and Phe as the substrate, respectively, which account for the amino acid sequence of pentaminomycin C (L-Leu–D-Val–L-Trp–L-Arg–D-Phe). P450 enzyme encoding gene(s) of BZY53_RS10990 and/or BZY53_RS10985 will hydroxylate the Arg residue to form 5-OHArg in pentaminomycin C. In NRPS pathways, elongated peptide chains are released from the peptidyl carrier protein (PCP) in the last module of NRPSs by the thioesterase (TE) domain next to the PCP, but no TE domains are present in both the NRPSs for pentaminomycin C and BE-18257A. Recently, putative hydrases, stand-alone enzymes belonging to the penicillin-binding family, such as SurE, MppK, and DsaJ, were shown to be involved in the release of peptide chains from the PCP and its macrocyclization to afford cyclic peptides [29, 30]. A putative hydrolase adjacent BZY53_RS10935 showed homology to these enzymes (about 40% similarities and 50% identities in amino acid sequence). As SurE has been reported as a trans-acting TE cyclizing two distinct non-ribosomal peptides encoded in a single gene cluster [29, 30], the putative hydrolase (BZY53_RS10940) will also act to form two cyclic peptides pentaminomycin C and BE-18257A. Taken together, we herein propose the biosynthetic pathways of pentaminomycin C and BE-18257A in Fig. 4. Each A domain converts each amino acid building block into aminoacyl adenylate and transfers them to the PCP within each module to form the corresponding aminoacyl thioester. E domain within each module epimerize α-carbon of Trp, Glu, and Val loaded on modules 2, 3, and 5

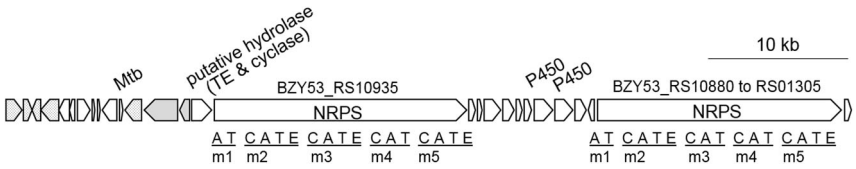

Fig. 3 Biosynthetic gene cluster for pentaminomycin C (**5**) and BE-18257A (**1**). ORFs of BZY53_RS11015 to BZY53_RS10895, BZY53_R10880 plus BZY53_RS01305, and BZY53_RS35155 are indicated. BZY53_RS11015 to BZY53_R10880 are encoded in MUBL01000147, while BZY53_RS01305 and BZY53_RS35155 are in MUBL01000010. The two contig sequences were assembled via 78 bp overlapping between 3′-terminal of MUBL01000010 and 5′-terminal of MUBL01000147. As the sequence of the right NRPS is divided into BZY53_R10880 and BZY53_RS01305, the ORF was indicated as BZY53_R10880 plus BZY53_RS01305. Domain organizations are shown under NRPS genes and each module is underlined. A adenylation domain; C condensation domain, E epimerase domain, T peptidyl carrier protein, which is also called thiolation domain; m module. TE thioesterase. Regulatory and transport-related genes are dotted and gray-colored, respectively. The same gene cluster is also present in the genome of strain NBRC 12748^{T} (BJMM010000001-BJMM010000197) but its sequence is fragmentated in to so many contigs (BJMM010000017, BJMM010000182, BJMM010000163, BJMM010000158, BJMM01000085, BJMM01000063, etc) that whole the sequence could not be determined

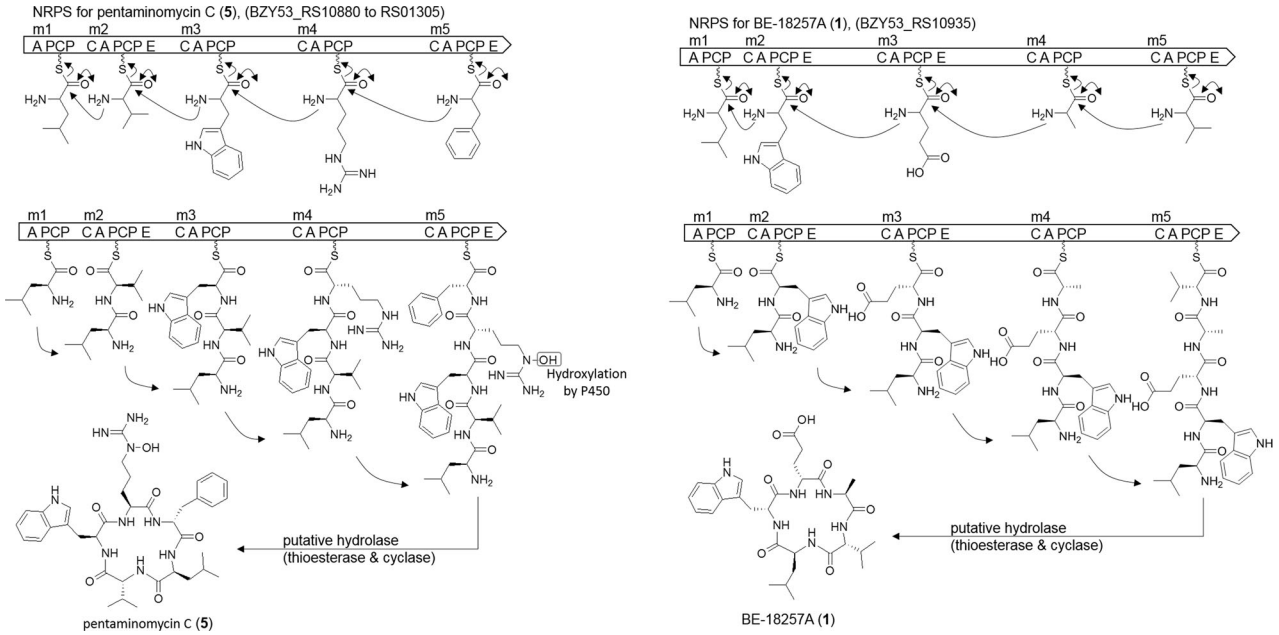

Fig. 4 Proposed biosynthetic pathways for pentaminomycin C (**5**) and BE-18257A (**1**). Letters in the figure represent as following: A adenylation domain; C condensation domain; E epimerase domain; m module; PCP peptidyl carrier protein, which is also called thiolation domain

(BE-18257A) and Val and Phe on modules 2 and 5 (pentaminomycin C) to afford D-configuration. C domains catalyze for successive *N*-acylation to yield L, D, D, L, D- and L, D, L, L, D-pentapeptidyl thioesters attached to the PCPs of module 5, respectively. The Arg residue in pentaminomycin C is hydroxylated to be 5-OHArg by P450 enzymes. The putative hydrolase releases the two linear pentapeptide chains from the PCPs and then cyclizes them to yield pentaminomycin C and BE-18257A, respectively.

Genome mining is often employed to search for novel secondary metabolites, for which accurate bioinformatic analysis of secondary metabolite-biosynthetic gene clusters is essential. Usually, a single gene cluster is believed to produce compounds containing the same backbone. However, the gene cluster that we reported here produces two types of peptides whose backbones are different. The methodology of bioinformatic to predict final product of NRPS system is based on relationship between amino acid sequence of substrate binding pocket in A domain and actual amino acid substrate. So far data accumulation is not enough to perfectly predict final product. Studies on structure determination of actual products coupled with identification of the corresponding biosynthetic gene clusters provide useful information to the research fields because accumulation of such knowledges is essential to bioinformatic analysis.

Experimental section

Microbial strains

Bacterial strains including *Streptomyces cacaoi* subsp. *cacaoi* NBRC 12748^T, *M. luteus* NBRC 3333^T, *P. aeruginosa* NBRC 12689^T, *B. subtilis* subsp. *subtilis* NBRC 13719^T, *S. aureus* subsp. *aureus* NBRC 100910^T, and *E. coli* NBRC 102203^T were obtained from the NBRC culture collection (NITE Biological Resource Center, Japan).

Isolation of peptides

S. cacaoi subsp. *cacaoi* NBRC 12748^T was cultured in 5 L of modified ISP2 agar medium [22] (2 g Leu, 4 g yeast extract, 10 g malt extract, 4 g glucose, and 15 g agar in 1 L distilled water, adjusted pH 7.3) for 9 days at 30 °C. Bacterial cells were harvested and extracted with double volume of MeOH. The mixture was filtered through filter paper (Whatman No.1, GE Healthcare Life Science, UK), followed by centrifugation at 4000 rpm for 10 min to remove insoluble compounds. The extract was concentrated by rotary evaporation and compound **5** was isolated using HPLC (Navi C30-5 column; 4.6 × 250 mm; Wakopak, Wako Pure Chemical Industries, Tokyo, Japan). The HPLC condition was set as isocratic elution mode; 37% MeCN/water containing 0.05% trifluoroacetic acid (TFA) at flow rate 1 ml/min and the UV detector was set at the wavelength of 220 nm to obtain **5** (Retention time; 26.70 min). The extract was repeatedly subjected to HPLC purification and freeze-drying to afford 3.0 mg of **5**, along with **1** (5.0 mg).

ESI-MS analysis

ESI-MS analyses were performed using a JEOL JMS-T100LP mass spectrometer. For accurate MS analysis, reserpine was used as an internal standard.

NMR analysis

NMR sample was prepared by dissolving **5** in 500 µl of DMSO-d_6. 1D ^{1}H, ^{13}C, DEPT-135, and all 2D NMR spectra were obtained on Bruker Avance800 spectrometer with quadrature detection following the previous report [31].

Modified Marfey's analysis

The amino acid analyses were carried out in sealed vacuum hydrolysis tube. Compound **5** (1.0 mg) was hydrolyzed in 500 µl of HI acid at 155 °C for 24 h to remove the hydroxy group of OHArg according to a previous report [24]. To recover Trp, compound **5** (1.0 mg) was hydrolyzed in 500 µl of 6 N HCl containing 3% phenol at 166 °C for 25 min [25]. After cooling to room temperature, the hydrolysate was evaporated using a rotary evaporator and the remaining solvent was completely dried under vacuum. The hydrolysate was resuspended in 200 µl of water, followed by adding 10 µl of the solution of *N*α-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (L-FDLA; Tokyo Chemical Industry Co., LTD, Tokyo, Japan) in acetone (10 µg µl^{-1}). The 1 M $NaHCO_3$ solution (100 µl) was added to the hydrolysate and the mixture was incubated at 80 °C for 3 min. The reaction mixture was cooled to room temperature and neutralized with 50 µl of 2 N HCl. The 50% MeCN/water (1 ml) was added to the mixture before subjected to HPLC. For standard amino acids, each amino acid (1 mg) was derivatized with L-FDLA and D-FDLA in the same manner. Approximately 20 µl of each FDLA derivative was analyzed by HPLC (C18 column, 4.6 × 250 mm, Wakopak Handy ODS, Wako Pure Chemical Industries, Tokyo, Japan). DAD detector (MD-2018, JASCO, Tokyo, Japan) was used for detection of the amino acid derivatives accumulating the data of absorbance from 220 to 420 nm. HPLC analysis for all standard amino acids (Leu, Val, Trp, Arg, and Phe) was performed using solvent A (distilled water containing 0.05% TFA) and solvent B (MeCN containing 0.05% TFA) at a flow rate of 1 ml min^{-1} in linear gradient mode from 0 to 70 min, increasing percentage of solvent B from 25 to 60% (HPLC condition 1). The retention times (min) for L- and D-FDLA derivatized amino acids (HPLC condition 1) were as follows; L-Arg–D-FDLA (18.56 min), L-Arg–L-FDLA (20.25 min), L-Val–L-FDLA (40.68 min), L-Leu–L-FDLA (46.51 min), L-Trp–L-FDLA (47.39 min), L-Phe–L-FDLA (48.66 min), L-Val–D-FDLA (53.76 min), L-Trp–D-FDLA (54.37 min), L-Phe–D-FDLA (59.30 min), and L-Leu–D-FDLA (62.57 min). HPLC analysis for Val was performed using solvent A (distilled water containing 0.05% TFA) and solvent B (MeCN containing 0.05% TFA) at a flow rate of 1 ml/min in isocratic mode 40% of solvent B for 55 min (HPLC condition 2). The retention times (min) for L- and D-FDLA derivatized amino acids (HPLC condition 2) were as follows: L-Val–L-FDLA (21.05 min) and L-Val–D-FDLA (40.23 min). HPLC analysis for Trp was performed using solvent A (distilled water containing 0.05% TFA) and solvent B (MeCN containing 0.05% TFA) at a flow rate of 1 ml min^{-1} in isocratic mode 45% of solvent B for 55 min (HPLC condition 3). The retention times (min) for L- and D-FDLA derivatized amino acids (HPLC condition 3) were as follows: L-Trp–L-FDLA (18.57 min) and L-Trp–D-FDLA (28.04 min).

Antibacterial activity test

The antibacterial activities of peptides were assessed using the MICs test in 96-well microplates according to the previous

report [26]. The peptides were tested against bacterial strains including *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, and *M. luteus*. Tetracycline was used as a positive control. Using the broth dilution technique with Mueller-Hinton Broth (MHB), serial twofold dilution of the compounds (**1** and **5**) and tetracycline were prepared (50 µl/well), ranging from 64 to 0.0625 µg ml^{-1}. Test bacteria were diluted with MHB to give a final inoculum of 10^5 CFU ml^{-1}. The bacterial solution (50 µl) was transferred to each well, which contain a various concentrations of testing compound. The MICs was determined as the lowest concentration that gave no visible growth after incubation of the microplates at 30 °C for 24 h.

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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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