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NOTE

Structure and antibacterial activities of new cyclic peptide antibiotics, pargamicins B, C and D, from *Amycolatopsis* sp. ML1-hF4

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Infectious diseases caused by drug-resistant bacteria are one of the most serious problems currently facing the medical field, as warned by the CDC[1] and the WHO.[2,3] Among these drug-resistant bacteria, methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis/faecium* (VRE) are two of the most intractable nosocomial pathogens that show broad-range drug resistance. Vancomycin has been used as the last resort for the treatment of infectious diseases caused by *S. aureus* and enterococci; however, since 1986, VRE strains have emerged and become widespread.[4,5] Since the beginning of this century, vancomycin intermediate-resistant *S. aureus* have been frequently isolated[6] and 33 strains of vancomycin high-level resistant *S. aureus* have also emerged in Asian countries and in the United States.[7] Effective drugs against these pathogens are urgently needed.

During the course of our screening for antibiotics active against both methicillin-resistant *S. aureus* and VRE, pargamicin A (PRG-A, Figure 1) was discovered from the fermentation broth of soil actinomycete strain *Amycolatopsis* sp. ML1-hF4.[8] PRG-A is a structurally unique cyclic peptide consisting of *N*-methyl-3-hydroxy valine, 4-hydroxy piperazic acid (4-OH-Pip), sarcosine, phenylalanine, *N*-hydroxy isoleucine (*N*OH-Ile) and piperazic acid (Pip), which shows excellent *in vitro* antibacterial activity that is either comparable to, or more potent than, that of other currently available drugs, including vancomycin.[8] Our previous studies revealed that PRG-A exerted rapid bactericidal activity against staphylococci and enterococci via a mechanism that involved disruption of the membrane integrity of the target cells,[9] a mode of action distinct from that of daptomycin.[9,10] During the process of optimization of PRG-A production, we discovered new active components PRG-B, -C and -D (Figure 1) in the culture broth of the PRG-A-producing strain *Amycolatopsis* sp. ML1-hF4. This present study investigates the fermentation, isolation, structural elucidation and antimicrobial activities of these new PRG-A-analogs and discusses the structure–activity relationship of PRGs.

The PRG-A producing strain was cultured for 3 days at 30 °C in liquid media consisting of 2% galactose, 2% dextrin hydrate, 1% soytone, 0.5% corn steep liquor, 0.2% ammonium sulfate and 1.0% calcium carbonate (pH 7.0 before sterilization). This seed culture was inoculated (3%) into media consisting of 0.33% galactose, 0.33% dextrin hydrate, 0.17% glycerol, 0.17% soytone, 0.083% corn steep liquor, 0.033% ammonium sulfate and 0.2% calcium carbonate (pH 7.0 before sterilization), and cultivated for 7 days at 27 °C with shaking (180 r.p.m.). This media was sixfold diluted compared with that reported at the time of PRG-A discovery.[8] The resulting 2.5 l culture broth was then centrifuged (3000 *g*, 10 °C, 15 min) and the supernatant was applied to a Diaion HP-20 column (Mitsubishi Chemical Co., Tokyo, Japan, 300 ml wet volume). The column was washed with 1 liter each of distilled water, 40% aqueous methanol and was eluted with 90% aqueous methanol. The eluted fraction was dried

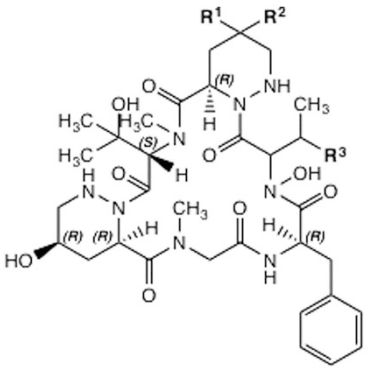

Figure 1 Structure of PRGs A, B, C and D.

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Table 1 NMR data of PRGs A, B, C and D

		PRG-A					PRG-B					PRG-C					PRG-D		
		^{13}C (p.p.m.)	^{1}H (p.p.m.)	Multiplicity J Hz			^{13}C (p.p.m.)	^{1}H (p.p.m.)	Multiplicity J Hz			^{13}C (p.p.m.)	^{1}H (p.p.m.)	Multiplicity J Hz			^{13}C (p.p.m.)	^{1}H (p.p.m.)	Multiplicity J Hz
4-OH-Pip	α	48.1 d	5.26	d 6.2	4-OH-Pip	α	48.0 d	5.25	d 6.0	4-OH-Pip	α	48.1 d	5.26	d 6.3	4-OH-Pip	α	47.9 d	5.27	d 6.2
	β	31.4 t	2.11	m		β	31.4 t	2.12	ddd 2.1,6.0,14.5,		β	31.3 t	2.13	m		β	31.3 t	2.14	m
			2.51	d 14.3				2.51	brd 14.5				2.51	brd 14.6				2.49	m
	γ	61.89 d	3.93	m		γ	61.9 d	3.93	brs		γ	61.9 d	3.95	brs		γ	61.8 d	3.96	brs
	γ-OH		5.11	brs		γ-OH		5.01	brs							γ-OH		5.09	brs
	δ	52.9 t	2.95	m		δ	52.9 t	2.94	brd 15.3		δ	52.8 t	2.94	brd 15.1		δ	53.0 t	2.93	brd 15.5
			3.04	d				3.03	brdd 12.8, 15.3				3.07	brt 13.8				3.08	dt 1.6,14.1
	CO	170.9 s				CO	170.9 s				CO	170.9 s				CO	171.4 s		
	NH		4.57	dd 2.5, 13.0		NH		4.58	d 12.8		NH		4.59	dd 2.1,13.2		NH		4.69	d 1.5,13.2
NMe-3-OH-Val	α	55.8 d	5.55	s	NMe-3-OH-Val	α	55.9 d	5.55	s	NMe-3-OH-Val	α	57.1 d	5.53	s	NMe-3-OH-Val	α	57.6 d	5.41	s
	β	73.4 s				β	73.4 s				β	73.4 s				β	73.5 s		
	β-OH		4.98	s		β-OH		4.97	brs		β-OH		4.92	brs		β-OH		4.85	brs
	γ	25.5 q	1.12	s		γ	25.6 q	1.12	s		γ	25.7 q	1.13	s		γ	25.9 q	1.08	s
	δ	28.7 q	1.32	s		δ	28.7 q	1.32	s		δ	28.7 q	1.33	s		δ	28.5 q	1.31	s
	CO	173.7 s				CO	173.7 s				CO	173.1 s				CO	173.2 s		
	NMe	33.6 q	3.24	s		NMe	33.6 q	3.24	s		NMe	34.5 q	3.33	s		NMe	34.3 q	3.21	s
Pip	α	43.0 d	5.63	dd 2.0, 5.2	Pip	α	43.0 d	5.65	d 5.3	4-OH-Pip2	α	43.5 d	5.71	d 7.6	O-Pip	α	48.6 d	6.30	t 3.8
	β	25.0 t	1.85	m		β	25.0 t	1.85	m		β	30.2 t	1.94	brd 15.1		β	41.3 t	2.49	d 3.8
													2.13	m					
	γ	18.8 t	1.47	brd 13.0		γ	18.9 t	1.47	m		γ	59.8 d	3.72	brs		γ	202.2 s		
			2.38	m				2.38	m										
	δ	48.1 t	2.62	dt 3.1, 13.0		δ	47.7 t	2.69	dq 3.4,13.4							δ	57.2 t	3.40	dd 11.3,16.9
			3.11	m				3.11	brd 13.4		δ	54.9 t	2.84	dt 2.2,13.7				3.46	dd 3.8,16.9
	CO	171.3 s				CO	171.4 s						3.11	brd 1.6,10.9		CO	169.0 s		
	NH		5.12	m		NH		5.12	d 12.7		CO	172.4 s				NH		5.08	dd 3.8,11.2
											NH		5.02	d 13.2					

Table 1 (Continued)

		PRG-A				PRG-B				PRG-C				PRG-D		
		^{13}C (p.p.m.)	^{1}H (p.p.m.)	Multiplicity J Hz		^{13}C (p.p.m.)	^{1}H (p.p.m.)	Multiplicity J Hz		^{13}C (p.p.m.)	^{1}H (p.p.m.)	Multiplicity J Hz		^{13}C (p.p.m.)	^{1}H (p.p.m.)	Multiplicity J Hz
*N*OH-Ile	α	61.0 d	5.38	d 6.0	*N*OH-Val	60.5 d	5.37	d 6.2	*N*OH-Ile	60.8 d	5.46	d 5.9	*N*OH-Ile	58.6 d	5.69	d
	β	34.9 d	2.11	m		27.9 d	2.41	m		35.0 d	2.09	m		33.6 d	2.15	m
	β-Me	15.3 q	0.59	t 6.9		18.9 q	0.64	d 6.9		15.2 q	0.55	d 6.8		15.6 q	0.79	d 6.9
	γ	25.4 t	1.33	m		18.7 q	0.96	d 7.2		25.4 t	1.27	m		25.2 t	0.74	m
	δ	12.9 q	0.80	d 7.4		171.9 s					1.39	m			0.88	m
	CO	171.9 s				NOH	8.56	s		13.0 q	0.79	t 7.5		11.0 q	0.64	t 7.4
	NOH		8.63	s						172.1 s				172.6 s		
										NOH	8.53	brs		NOH	8.54	brs
Phe	α	47.7 d	5.89	dt 6.0, 10.4	Phe	48.0 d	5.88	dt 6.0,10.1	Phe	47.8 d	5.86	dt 5.9,10.2	Phe	48.1 d	5.85	ddd 5.8,10.6
	β	37.6 t	2.89	dd 6.0, 13.3		37.5 t	2.89	dd 6.0,13.6		37.5 t	2.90	dd 5.8,13.4		37.5 t	2.90	dd 5.6,13.6
			2.98	m			2.99	dd 11.0,13.6			2.99	dd 11.2,13.7			2.99	dd 11.1,13.3
	1	136.4 s				136.5 s				136.3 s				136.0 s		
	2	128.4 d	7.22	m		128.4 d	7.23	m		128.4 d	7.24	m		128.5 d	7.26	m
	3	129.2 d	7.22	m		129.2 d	7.23	m		129.2 d	7.22	m		129.3 d	7.23	m
	4	126.7 d	7.17	m		126.7 d	7.16	m		126.7 d	7.17	m		126.8 d	7.19	m
	5	129.2 d	7.22	m		129.2 d	7.23	m		129.2 d	7.22	m		129.3 d	7.23	m
	6	128.4 d	7.22	m		128.4 d	7.23	m		128.4 d	7.24	m		128.5 d	7.26	m
	CO	172.7 s				172.8 s				172.4 s				171.8 s		
	NH		7.62	d 10.4		NH	7.60	d 10.1		NH	7.57	d 10.0		NH	7.50	d 10.0
Sar	α	55.4 t	3.36	d 16.3	Sar	55.4 t	3.35	d 16.2	Sar	55.4 t	3.35	d 16.2	Sar	55.4 t	3.38	d 6.0
			4.26	d 16.3			4.28	d 16.2			4.26	d 16.0			4.19	d 16.0
	CO	168.0 s				167.9 s				168.1 s				168.3 s		
	NMe	38.1 q	3.13	s		38.1 q	3.12	s		38.0 q	3.14	s		38.0 q	3.15	s

Abbreviations: 4-OH-Pip, 4-hydroxy piperazic acid; NMe-3-OH-Val, *N*-methyl-3-hydroxy valine; NOH-Ile, *N*-hydroxy isoleucine; NOH-Val, *N*-hydroxy valine; O-pip, 4-oxo piperazic acid; Phe, phenylalanine; Pip, piperazic acid; PRG, pargamicin; Sar, sarcosine.
^{1}H (600MHz) and ^{13}C (150 MHz) chemical shifts are adjusted with TMS as an internal standard.

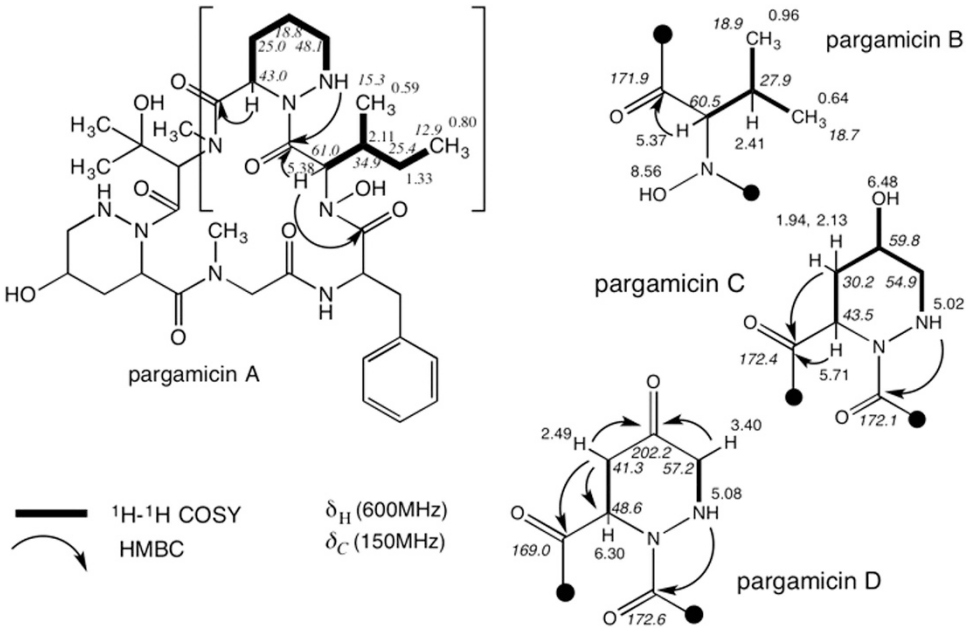

Figure 2 Structural difference of PRGs by NMR analyses.

on a rotary evaporator (733.2 mg) and then purified by reverse-phase HPLC. The HPLC conditions were as follows: column, Shiseido CAPCELL PAK UG-120 (Tokyo, Japan, 30 × 250 mm); flow rate, 10 ml min^{-1}; solvent, 38–47% CH_3CN aq+0.01% trifluoro acetic acid, linear gradient; column oven, 48 °C; detection, 210 nm. PRG-B (5.1 mg), PRG-C (24.9 mg), PRG-D (50.1 mg) and PRG-A (26.4 mg) were eluted at 25–26, 44–45, 47–48 and 70–71 min, respectively.

The physicochemical properties of PRG-A, B, C and D are summarized in the Supplementary Material (Supplementary Table S1). The molecular formulae of PRG-A, B, C and D were $C_{34}H_{52}N_8O_9$, $C_{33}H_{50}N_8O_9$, $C_{34}H_{52}N_8O_{10}$ and $C_{34}H_{50}N_8O_{10}$, respectively, as determined by HR–ESI–MS and NMR spectra (^{1}H and ^{13}C NMR spectra, Supplementary Figures S1–S6; 2D-COSY spectra, Supplementary Figures S7–S9; and HR–ESI–MS, Supplementary Figures S10–S12). NMR data are summarized in Table 1. The molecular formulae, IR spectra, optical rotations and NMR spectral data of the PRGs indicated that they are closely related in structure, except for the Pip or *N*OH-Ile moieties. The structures of PRG-B, C and D were determined by comparison with the NMR data for PRG-A and the structural differences are shown in Figure 2.

The molecular formula of PRG-B was $C_{33}H_{50}N_8O_9$, which was smaller by one CH_2 unit than PRG-A. The ^{1}H correlations of PRG-B changed the signals at δ 5.37, 2.41, 0.64 and 0.96 compared with those in PRG-A at δ 5.38, 2.11, 0.59, 1.33 and 0.80, as determined by the analysis of the ^{1}H-^{1}H COSY spectrum for PRG-B. These findings indicated that PRG-B possessed a *N*-hydroxyvaline (*N*OH-Val) moiety instead of the *N*OH-Ile moiety present in PRG-A. The order of connectivity of these Pip and *N*OH-Val moieties was determined to be the same as that observed for PRG-A by analysis of the HMBC spectrum (from δ 5.37 (*N*OH-Val, H-α) and δ 5.12 (Pip, NH) to 171.9 (*N*OH-Val, CO); 5.65 (Pip, H-α) and 3.24 (*N*-methyl-3-hydroxy valine, *N*Me) to 171.4 (Pip, CO)). The other NMR data and the molecular formula ($C_{33}H_{50}N_8O_9$) were all consistent with this change. Thus, the structure of PRG-B was determined as shown in Figure 1.

The molecular formula of PRG-C was $C_{34}H_{52}N_8O_{10}$, which was larger by one oxygen atom than PRG-A. Analysis of the ^{1}H-^{1}H COSY spectrum for PRG-C revealed signals at δ 5.71, 1.94, 2.13, 3.72, 2.84, 3.11, 5.02 and 6.48, compared with signals at δ 5.63, 1.85, 1.47, 2.38, 2.62, 3.11 and 5.12 for PRG-A. This indicated that PRG-C possessed a 4-OH-Pip moiety instead of the Pip moiety observed in PRG-A. The order of connectivity of these 4-OH-Pip and *N*OH-Ile moieties was determined to be the same as that seen for PRG-A by analysis of the HMBC spectrum (from δ 5.71 (4-OH-Pip, H-α) and 3.33 (*N*-methyl-3-hydroxy valine, *N*Me) to 172.4 (4-OH-Pip, C-α); 5.46 (*N*OH-Ile, H-α) and δ 5.02 (4-OH-Pip, NH) to 172.1 (*N*OH-Ile, CO)). The other NMR data and the molecular formula ($C_{34}H_{52}N_8O_{10}$) were all consistent with this change. Thus, the structure of PRG-C was determined as shown in Figure 1.

The molecular formula of PRG-D was $C_{34}H_{50}N_8O_{10}$, which was smaller by one H_2 unit than PRG-C. Analysis of the ^{1}H-^{1}H COSY spectrum revealed signals at δ 6.30, 2.49, 3.40, 3.46 and 5.08 for PRG-D, compared with signals at δ 5.71, 1.94, 2.13, 3.72, 2.84, 3.11, 5.02 and 6.46 for PRG-C. The connectivity of the moiety determined by HMBC and the cross-peaks observed from δ 3.40 and 2.49 to the carbonyl carbon at δ 202.2 indicated that PRG-D possessed a 4-oxo-piperazine (O-Pip) moiety instead of the 4-OH-Pip moiety observed in PRG-C. The order of connectivity of these O-Pip and *N*OH-Ile moieties was determined to be the same as in PRG-C, as determined by analysis of the HMBC spectrum (from δ 6.30 (O-Pip, H-α) and 3.21 (*N*-methyl-3-hydroxy valine, *N*Me) to 169.0 (O-Pip, CO); 5.69 (*N*OH-Ile, H-α) and 5.08 (O-Pip, NH) to 172.6 (*N*OH-Ile, CO)). The other NMR data and the molecular formula ($C_{34}H_{50}N_8O_{10}$) were all consistent with this change. Thus, the structure of PRG-D was determined as shown in Figure 1. Taken together, these results revealed that PRG-B, C and D are new cyclic peptide metabolites.

Table 2 Antimicrobial activities of pargamicins

	MIC ($\mu g\ ml^{-1}$)				
Test organisms	*PRG-A*	*PRG-B*	*PRG-C*	*PRG-D*	*Vancomycin*
Gram positives					
S. aureus FDA 209P	1	8	2	32	0.25
S. aureus Smith	1	8	2	32	0.5
S. aureus MS9610	1	16	4	64	1
S. aureus MRSA No. 5	1	8	4	32	1
S. aureus MRSA No. 17	1	8	4	64	1
S. aureus MS16526 (MRSA)	1	16	4	64	1
S. aureus TY-04282 (MRSA)	2	16	4	64	0.5
E. faecalis JCM 5803	1	8	0.5	8	0.5
E. faecalis NCTC12201 (VRE)	1	8	0.5	8	>128
E. faecalis NCTC12203 (VRE)	1	8	0.5	8	>128
E. faecium JCM 5804	1	8	1	8	0.5
E. faecium NCTC12202 (VRE)	1	8	0.5	8	>128
E. faecium NCTC12204 (VRE)	1	8	0.5	8	128
Micrococcus luteus FDA 16	0.5	4	1	8	0.25
M. luteus IFO 3333	1	4	1	8	0.125
Bacillus subtilis NRRL B-558	1	16	1	32	0.25
B. subtilis PCI 219	1	8	1	32	0.25
B. subtilis ATCC23857 (168)	2	8	1	32	N.T.
Bacillus cereus ATCC10702	1	8	0.5	8	1
Corynebacterium bovis 1810	1	8	0.5	16	0.125
Gram negatives					
Escherichia coli NIHJ	>64	>64	>64	>64	128
E. coli K-12	>64	>64	>64	>64	128
Shigella dysenteriae JS11910	>64	>64	>64	>64	>128
Salmonella enteritidis 1891	>64	>64	>64	>64	>128
Proteus vulgaris OX19	>64	>64	>64	>64	>128
Proteus mirabilis IFM OM-9	>64	>64	>64	>64	>128
Serratia marcescens B-0524	>64	>64	>64	>64	>128
Pseudomonas aeruginosa A3	>64	>64	>64	>64	>128
Klebsiella pneumoniae PCI 602	>64	>64	>64	>64	>128

Abbreviations: MRSA, methicillin-resistant *S. aureus*; PRG, pargamicin.

The stereochemistry of hydroxyl group of 4-OH-Pip was determined by 2D ROESY spectrum (Supplementary Figure S13) of PRG-C. ROEs from H-β_{ax} (2.13 p.p.m.) to H-δ_{ax} (3.07 p.p.m.) in 4-OH-Pip indicated a 1,3-diaxial orientation, suggesting 4-OH-Pip is a chair formation and the correlation from H-α (5.26 p.p.m.) to H-β (2.13, 2.51 p.p.m.) revealed the H-α was equatorial. Furthermore, ROESY correlations from H-γ (3.95 p.p.m.) to both axial and equatorial of H-β and H$-\delta$ (2.94 and 3.07 p.p.m.) suggested that H-γ was equatorial and the γ–hydroxyl group was axial established the relative stereochemistry of 4-OH-Pip is *R**,*R**. The 4-OH-Pip2 in PRG-C was determined by the same way as *R**,*R**.

The stereochemistry of constituent amino acids of PRG A, B, C and D were determined by advanced Marfey's method using 1-fluoro-2,4-dinitrophenyl-5-D- and L-leucineamide[11] (see Supplementary Materials, Supplementary Figures S14–S17). The acid hydrolysates of PRGs were derivatized by treating with 1-fluoro-2,4-dinitrophenyl-5-D- and L-leucineamide, and analyzed using extracted ion chromatogram by LC–HR–ESI–MS. We referred the report of dentigerumycin to analyze the elution pattern of both D-Pip and (2*S*, 4*S*)-4-OH-Pip.[12] It was revealed that PRG-A contained D-Pip, D-Phe, (2*R*, 4*R*)-4-OH-Pip and *N*Me-3-OH-L-Val, PRG-B contained D-Pip, D-Phe and *N*Me-3-OH-L-Val, and PRG-C and D contained D-Phe, (2*R*, 4*R*)-4-OH-Pip and *N*Me-3-OH-L-Val, respectively. *N*OH-Val and *N*OH-Ile might be epimerized during acid hydrolysis. The 1-fluoro-2,4-dinitrophenyl-5-D- and L-leucineamide derivatives of 4-OH-Pip in PRG-B and O-Pip in PRG-D were not detectable. Collectively, the absolute structures of PRGs are shown in Figure 1.

The antimicrobial activities of the PRGs are summarized together with that of vancomycin in Table 2. MICs were determined as previously reported.[13] PRG-A and PRG-C exhibited potent antibacterial activity against Gram-positive bacteria including methicillin-resistant *S. aureus* and VRE; the antibacterial activity of PRG-B and PRG-D against these bacteria was weaker. PRG-C and PRG-D, which possess a polar group in the northern region of Pip, exhibited four- to eightfold weaker antibacterial activity against staphylococci than against enterococci, whereas PRG-A and PRG-B showed equal activity against staphylococci and enterococci. This suggested that the presence of polar groups in the northern region of Pip might be disadvantage to the interaction with staphylococcal membrane. VRE were as sensitive to all of the PRGs as vancomycin-sensitive enterococci and all of the tested compounds, including vancomycin, showed no activity against Gram-negative bacteria.

The only structural differences between the PRGs are in the Pip-(*N*OH-Ile) moiety of PRG-A. However, despite this structural similarity, the antimicrobial activities of the PRGs showed remarkable differences. This suggested that the side chain of isoleucine residues and the northern Pip structures might have an important role in the interaction with the bacterial membrane and the localization of PRGs on the bacterial surface. Further studies into Pip containing cyclic peptides, such as the PRGs, may therefore result in the discovery of new types of antibacterial drugs that selectively disturb the bacterial membrane.

DEDICATION

The authors dedicate this work to Professor Satoshi Ōmura, a distinguished Novel Prize awardee in Physiology or Medicine 2015.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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