Novel Des-Fatty Acyl-Polymyxin B Derivatives with *Pseudomonas aeruginosa*-Specific Antimicrobial Activity

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Polymyxin B (PMB) is a cationic cyclic decapeptide antibiotic with a fatty acyl (FA) modification at the α-amino group of Dab[1] (Dab: L-α,γ-diaminobutyric acid). In this study, which is part of a series of PMB structure–activity relationship investigations focused on identifying clinically useful peptide antibiotics, we synthesized ten des-FA PMB derivatives whose N-terminal moieties were changed to basic or hydrophilic amino acids. The antimicrobial and lipopolysaccharide (LPS) binding activities of these synthetic analogs were tested. The analogs showed more potent antimicrobial activity against *Pseudomonas aeruginosa* (*P. aeruginosa*) compared with the PMB nonapeptide. In particular, [Ser[2]-Dap[3]]-PMB(2—10), Guanyl-[Thr[2]-Dab[3]]-PMB(2—10), Guanyl-[Dab[1]-Thr[2]-Dab[3]]-PMB(1—10), and $N^{\alpha,\gamma}$-diguanyl-[Dap[3]]-PMB(3—10) had antimicrobial activity equivalent to PMB. In LPS binding assays, the displacement curves shifted in a manner proportional to the number of positive charges available to bind to *Escherichia coli* (*E. coli*) and *P. aeruginosa*. Furthermore, peptides with basic side chains were comparable to PMB in binding activity assays against *E. coli* and *P. aeruginosa*. The acute toxicities of the peptides were evaluated by intravenously administering the peptides to mice through the tail vein. The toxicities of [Ser[2]-Dap[3]]-PMB(2—10), [Dap[3]]-PMB(3—10), and [Ser[3]]-PMB(3—10) were lower that of PMB (LD_{50}, 4.8 μmol/kg).

Key words polymyxin B analog; antimicrobial activity; lipopolysaccharide binding activity; *Pseudomonas aeruginosa* specificity; toxicity; des-fatty acyl-polymyxin B

Polymyxin B (PMB) is a mixture of cationic cyclic decapeptide antibiotics isolated from *Bacillus polymyxia*.[1,2] PMB contains six L-α,γ-diaminobutyric acid (Dab) residues, where the γ-amino group of the Dab[4] residue is acylated by the C-terminal Thr[10] to form a 23-member lactam ring. The N-terminus of a PMB molecule is acylated with a fatty acid such as 6-methyloctanoic acid (PMB_1), 6-methylheptanoic acid (PMB_2) or octanoic acid (PMB_3), which forms a long hydrophobic tail.[3,4] PMB exhibits potent antimicrobial activity against Gram-negative bacteria. This potency is due to the ability of PMB to neutralize endotoxins and change the permeability of the outer membrane of Gram-negative bacteria. However, PMB peptides induce serious side effects such as nephrotoxicity and neuromuscular blockade, so their therapeutic applications are limited.[3] Therefore, various PMB derivatives have been synthesized and evaluated for biological activity.[5—8] It is well known that enzymatic removal of the fatty acyl (FA)-Dab[1] from PMB by ficin yields PMB nonapeptide (PMBN),[9,10] which retains considerable lipopolysaccharide (LPS)-binding activity[11] but has reduced toxicity.[12] However, PMBN has a lower antimicrobial activity, with an minimum inhibitory concentration (MIC) value of 256 nmol/ml towards *Escherichia coli* (*E. coli*).[10] Our previous studies on the structure–antimicrobial activity relationships of PMB showed that des-FA-PMB (Des-FA-[Dab[1]]-PMB) had a considerable antimicrobial activity, with MIC values of 8, 16, and 4 nmol/ml against *E. coli*, *Salmonella* Typhimurium (*S.* Typhimurium), and *Pseudomonas aeruginosa* (*P. aeruginosa*), respectively.[13—17] N-terminal analogs of Des-FA-[X[1]]-PMB (X=any one of a number of amino acids or peptides) were synthesized and examined for their antimicrobial activity. Des-FA-[Dap[1]]-PMB (Dap; L-α,β-diaminopropionic acid) and Des-FA-[Ser[1]]-PMB N-terminal analogs of Des-FA-[X[1]]-PMB showed potent and specific antimicrobial activity against *P. aeruginosa* (MIC: 0.5—1 nmol/ml), comparable to PMB. In the present study, we synthesized more compact des-FA-PMB derivatives. We predicted that these compact derivatives would have selective and potent antimicrobial activity against *P. aeruginosa* and low acute toxicity by virtue of their smaller amino acid side chain (Ser, Dap) and shortened length of the linear peptide portion of des-FA-PMB. Previously, we reported that basic PMB decapeptides containing a basic tripeptide ([Dab-Dab-Dab[1]]- or [Arg-Arg-Arg[1]]-) in place of the Dab[1] in des-FA-PMB also had potent activity against *P. aeruginosa*, but their toxicity was significantly higher than that of PMB (data not shown). Therefore, we synthesized compact and basic des-FA-PMB analogs with guanyl-Dab or $N^{\alpha,\gamma}$-diguanyl-Dap in place of Dab in order to reduce toxicity.

Experimental

General HPLC was performed on an apparatus equipped with two 510 pumps (Waters Corp., Milford, MA, U.S.A.), a U6K injector (Waters), a Lambda-Max Model 481 LC Spectrophotometer (Waters), a 680 Automated Gradient Controller (Waters), and a Chromatocorder 21 (System Instruments Co., Ltd., Tokyo, Japan). Gel filtration column chromatography was carried out using Toyopearl HW-40-S (Tosoh Corp., Tokyo, Japan). Fast-atom bombardment mass spectrometry (FAB-MS) was conducted on a JMS-DX300 mass spectrometer (JEOL Ltd., Tokyo, Japan). All reagents, peptide synthesis solvents, and 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were purchased from Watanabe Chem., Ind., Ltd. (Hiroshima, Japan).

Synthesis of Peptides PMB analogs ((**1**)—(**10**)) were synthesized according to the route representatively shown for [Ser[2]-Dap[3]]-PMB(2—10) (Fig. 1) (**2**). The synthetic strategy was essentially as reported previously.[13] In brief, the protected peptide was constructed on 4-hydroxylmethylphenoxymethyl-resin (HMP-resin or Wang-resin, Novabiochemläufelfingen, Switzerland) via standard Fmoc solid phase chemistry using an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA, U.S.A.). Protected amino acids used were Fmoc-Dab(2-ClZ)-OH, Fmoc-Dab(Boc)-OH,

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	Peptides	1	2	3	4	5	6	7	8	9	10
	Polymyxin B (PMB)	FA-	Dab-	Thr-	Dab-	Dab*-	Dab-	D-Phe-	Leu-	Dab-	Dab-Thr*-
	Polymyxin B nonapeptide (PMBN)	H-	Thr-	Dab-	Dab*-	Dab-	D-Phe-	Leu-	Dab-	Dab-	Thr*-
(1)	Des-FA-[Dap^1-Ser^2-Dap^3]-PMB(1-10)	H-	Dap-	Ser-	Dap-	Dab*-	Dab-	D-Phe-	Leu-	Dab-	Dab-Thr*-
(2)	[Ser^2-Dap^3]-PMB(2-10)	H-	Ser-	Dap-	Dab*-	Dab-	D-Phe-	Leu-	Dab-	Dab-	Thr*-
(3)	[Dap^3]-PMB(3-10)	H-	Dap-	Dab*-	Dab-	D-Phe-	Leu-	Dab-	Dab-	Thr*-	
(4)	[Ser^3]-PMB(3-10)	H-	Ser-	Dab*-	Dab-	D-Phe-	Leu-	Dab-	Dab-	Thr*-	
(5)	Guanyl-[Dab^1-Thr^2-Dab^3]-PMB(1-10)	Guanyl-	Dab-	Thr-	Dab-	Dab*-	Dab-	D-Phe-	Leu-	Dab-	Dab-Thr*-
(6)	Guanyl-[Thr^2-Dab^3]-PMB(2-10)	Guanyl-	Thr-	Dab-	Dab*-	Dab-	D-Phe-	Leu-	Dab-	Dab-	Thr*-
(7)	Guanyl-[Dab^2-Dab^3]-PMB(2-10)	Guanyl-	Dab-	Dab-	Dab*-	Dab-	D-Phe-	Leu-	Dab-	Dab-	Thr*-
(8)	Guanyl-[Dab^3] -PMB(3-10)	Guanyl-	Dab-	Dab*-	Dab-	D-Phe-	Leu-	Dab-	Dab-	Thr*-	
(9)	Guanyl-PMB(4-10)	Guanyl-	Dab*-	Dab-	D-Phe-	Leu-	Dab-	Dab-	Thr*-		
(10)	$N^{\alpha,\gamma}$-diguanyl-[Dap^3]-PMB(3-10)	Guanyl-	Dap(Guanyl)-	Dab*-	Dab-	D-Phe-	Leu-	Dab-	Dab-	Thr*-	

FA of PMB indicated various fatty acids such as 6-methyloctanoic acid (PMB_1), 6-methylheptanoic acid (PMB_2), and octanoic acid (PMB_3). *: amide linkage between the γ-NH_2 of Dab^4 and the α-COOH of Thr^{10}

Fig. 1. Structure of Polymyxin B, Polymyxin B Nonapeptide, and Synthetic Peptides ((**1**)—(**10**))

Fmoc-Thr(Bzl)-OH, Fmoc-D-Phe-OH, Fmoc-Leu-OH, Fmoc-Ser(Bzl)-OH, and Fmoc-Dap(2-ClZ)-OH. The Fmoc group was removed with 20% piperidine in *N*-methylpyrolidone (NMP). As an example, the synthesis of [Ser^2-Dap^3]-PMB(2—10) (**2**) and guanyl-[Dab^1-Thr^2-Dab^3]-PMB(1—10) (**5**) are described below.

Preparation of [Ser^2-Dap^3]-PMB(2—10) (2) The peptide chain was elongated on Fmoc-Thr(Bzl)-O-HMP-resin (0.25 mmol, 100—200 mesh) and Fmoc-amino acid derivatives (1.0 mmol) were successively introduced on the peptide chain using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (1.0 mmol) as the coupling reagent. After construction of the desired sequence, the protected peptide-resin, Fmoc-Ser(Bzl)-Dap(2-ClZ)-Dab(Boc)-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-O-HMP-resin (685 mg) was treated with trifluoroacetic acid (TFA) (9.5 ml) in the presence of H_2O (0.5 ml) for 1 h at room temperature, cleaving the peptide from the HMP-resin and removing the N^{γ}-Boc group from Dab^4 to yield Fmoc-Ser(Bzl)-Dap(2-ClZ)-Dab^4-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-OH. The excess TFA was evaporated *in vacuo* and the residue was lyophilized from dioxane. The product (433 mg) was dissolved in dimethylformamide (DMF) and purified by gel filtration on a column (1.6×95 cm) of Toyopearl HW-40-S using DMF : H_2O (9 : 1) as the eluent. Fractions containing the desired product were combined, DMF was evaporated, and the peptide was dissolved in dioxane and lyophilized. The linear, partially protected [Ser^2-Dap^3]-PMB(2—10) (381 mg, 0.19 mmol) was dissolved in ice-cold DMF–dimethylsulfoxide (DMSO) (1 : 1), and then diphenyl phosphorazidate (DPPA) (161 μl, 0.75 mmol) and 4-methylmorpholine (NMM) (150 μl, 1.50 mmol) were added. The mixture was reacted for 18 h at 4 °C to form the lactam ring. The cyclized product Fmoc-Ser(Bzl)-Dap(2-ClZ)-Dab^4*-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-$Thr(Bzl)^{10}$* (*–*: amide bond between the α-COOH of Thr^{10} and the γ-NH_2 of Dab^4) was purified on a Toyopearl HW-40-S column (1.6×95 cm). Fractions containing the designed product (362 mg) were combined, the solvent was evaporated, and the peptide was re-solubilized in dioxane and lyophilized. The Fmoc group was removed with 20% piperidine in DMF for 5 min at room temperature, the solvent was evaporated, and the peptide was again re-solubilized in dioxane and lyophilized. The obtained product was treated with anhydrous HF (5 ml) in the presence of anisole (500 μl) for 1 h at 0 °C. After evaporation of the HF, the residue was dissolved in H_2O, washed with three portions of ether, then lyophilized. The crude product (175 mg) was further purified by HPLC employing a Capcell Pak C_{18} UG-80 column (2×15 cm, Shiseido Co., Ltd., Tokyo, Japan) using an acetonitrile (CH_3CN)–0.1% TFA solvent system. The main product was collected, the fractions were combined, the solvent was evaporated, and the product was re-solubilized in water and lyophilized. The product (121 mg) was chromatographed on a Toyopearl HW-40-S column (1.5×57 cm) using 25% CH_3CN in 5 mmol/l HCl to afford the final preparation of compound (**1**) (108 mg, 0.11 mmol).

The other PMB derivatives ((**1**), (**3**) and (**4**)) were synthesized in a manner similar to that described in (**2**).

Preparation of Guanyl-[Dab^1-Thr^2-Dab^3]-PMB(1—10) (5) Guanyl-[Dab^1-Thr^2-Dab^3]-PMB(1—10) was synthesized essentially as described above on Fmoc-Thr(Bzl)-O-HMP-resin (0.14 mmol, 100—200 mesh) using appropriate Fmoc-derivatives. The desired protected peptide-resin, Fmoc-Dab(2-ClZ)-Thr(Bzl)-Dab(2-ClZ)-Dab(Boc)-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-O-HMP-resin (785 mg) thus obtained was treated with TFA (9.5 ml) in the presence of H_2O (0.5 ml) at room temperature for 1 h to give Fmoc-Dab(2-ClZ)-Thr(Bzl)-Dab(2-ClZ)-Dab^4-Dab(2-ClZ)-d-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-OH (271 mg), which was then chromatographed on a Toyopearl HW-40-S column (1.6×95 cm) using DMF : H_2O (9 : 1). The obtained linear, partially protected peptide (221 mg, 0.095 mmol) was cyclized with DPPA (103 μl, 0.48 mmol) and NMM (97 μl, 0.95 mmol) in the same manner as described above to yield Fmoc-Dab(2-ClZ)-Thr(Bzl)-Dap(2-ClZ)-Dab^4*-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-$Thr(Bzl)^{10}$* (*–*: amide bond between the α-COOH of Thr^{10} and the γ-NH_2 of Dab^4) (173 mg). The Fmoc group of the protected cyclic peptide was removed with 20% piperidine in DMF.

Guanylation of the cyclic partially protected peptide was performed in the following manner.[18] To a solution of the cyclic, partially protected peptide (73 mg, 0.035 mmol) in DMF (1 ml), NMM (7.9 μl, 0.077 mmol), $HgCl_2$ (10.5 mg, 0.039 mmol) and 1,3-bis(benzyloxycarbonyl)-2-methyl-2-thiopseudourea (13.8 mg, 0.039 mmol) were added at 0 °C. The reaction mixture was stirred for 18 h at 4 °C, and then centrifuged. The supernatant was purified by gel filtration on a column (1.6×95 cm) of Toyopearl HW-40-S using DMF as the eluent. Fractions containing the designed peptide were collected and lyophilized. The resulting product (55 mg) was treated with anhydrous HF (3 ml) containing anisole (0.3 ml) for 1 h at 0 °C. The crude peptide (29 mg) thus obtained was purified by HPLC on a Capcell Pak C_{18} UG-80 column (2×15 cm) using a CH_3CN–0.1% TFA solvent system. The product (20 mg) was chromatographed on a Toyopearl HW-40-S column (1.5×57 cm) using 25% CH_3CN in 5 mM HCl to yield the desired compound (**5**) (16 mg, 0.014 mmol).

The other guanyl PMB derivatives ((**6**)—(**10**)) were synthesized in a similar manner as described above. The purity of each synthetic peptide was confirmed by analytical HPLC and FAB-MS analysis (Table 1).

Bacteria, and Antimicrobial Assay *E. coli* IFO 12734, *S.* Typhimurium IFO 12529 and *P. aeruginosa* IFO 3080 were purchased from the Institute for Fermentation, Osaka (IFO), Japan. These bacterial strains were grown overnight at 37 °C on nutrient agar medium and harvested in sterile saline. The densities of the bacterial suspensions were determined at 600 nm using a standard curve relating absorbance to the number of colony-forming units (CFU). The antibacterial activity of each synthetic peptide was evaluated by comparing its activity with that of commercially available PMB (Sigma Chemical Co., St. Louis, MO, U.S.A.). The MIC of each synthetic peptide against each of the bacterial strains was determined using a standard microplate dilution method (n=6—8). One hundred microliters of each pep-

Table 1. FAB-MS Data of the Synthetic Peptides ((**1**)—(**10**))

		Formula	$[M+H]^+$, *m/z*	
			Calculated	Found
(**1**)	Des-FA-[Dap^1-Ser^2-Dap^3]-PMB(1—10)	$C_{44}H_{76}N_{16}O_{12}$	1021	1021
(**2**)	[Ser^2-Dap^3]-PMB(2—10)	$C_{41}H_{70}N_{14}O_{11}$	935	935
(**3**)	[Dap^3]-PMB(3—10)	$C_{38}H_{65}N_{13}O_9$	848	848
(**4**)	[Ser^3]-PMB(3—10)	$C_{38}H_{64}N_{12}O_{10}$	849	849
(**5**)	Guanyl-[Dab^1-Thr^2-Dab^3]-PMB(1—10)	$C_{48}H_{84}N_{18}O_{12}$	1105	1105
(**6**)	Guanyl-[Thr^2-Dab^3]-PMB(2—10)	$C_{44}H_{76}N_{16}O_{11}$	1005	1005
(**7**)	Guanyl-[Dab^2-Dab^3]-PMB(2—10)	$C_{44}H_{77}N_{17}O_{10}$	1004	1004
(**8**)	Guanyl-[Dab^3] -PMB(3—10)	$C_{40}H_{69}N_{15}O_9$	904	904
(**9**)	Guanyl-PMB(4—10)	$C_{36}H_{61}N_{13}O_8$	804	804
(**10**)	$N^{\alpha,\gamma}$-diguanyl-[Dap^3]-PMB(3—10)	$C_{40}H_{69}N_{17}O_9$	932	932

Table 2. Antimicrobial Activity of the Synthetic Peptides ((**1**)—(**10**))

		MIC (nmol/ml)		
		E. coli	*S.* Typhimurium	*P. aeruginosa*
	Polymyxin B (PMB)	1	0.5	1
	Polymyxin B nonapeptide (PMBN)	>256	>256	128
(**1**)	Des-FA-[Dap^1-Ser^2-Dap^3]-PMB(1—10)	16	32	2
(**2**)	[Ser^2-Dap^3]-PMB(2—10)	64	64	1
(**3**)	[Dap^3]-PMB(3—10)	256	256	4
(**4**)	[Ser^3]-PMB(3—10)	>256	>256	2
(**5**)	Guanyl-[Dab^1-Thr^2-Dab^3]-PMB(1—10)	4	2	1
(**6**)	Guanyl-[Thr^2-Dab^3]-PMB(2—10)	2	2	1
(**7**)	Guanyl-[Dab^2-Dab^3]-PMB(2—10)	8	8	2
(**8**)	Guanyl-[Dab^3] -PMB(3—10)	16	16	2
(**9**)	Guanyl-PMB(4—10)	>256	>256	4
(**10**)	$N^{\alpha,\gamma}$-diguanyl-[Dap^3]-PMB(3—10)	16	32	0.5

tide serially diluted with distilled water to 0.25—256 nmol/ml was added to a mixture of 10 μl of bacterial suspension (approximately 10^6 CFU/ml) and 90 μl of Mueller–Hinton broth (Becton Dickinson and Company Sparks, Cockeysville, MD, U.S.A.) in each well of a flat-bottom microplate (Corning Inc., Corning, NY, U.S.A.). The plates were incubated overnight at 37 °C to allow evaluation of the MIC. The MIC value was expressed as the lowest final concentration (nmol/ml) at which no growth was observed (Table 2).

Assay of LPS Binding of the Synthetic Peptides As described previously,[13] a solution of [Dab(N^γ-dansyl-Gly)^1]-PMB_3 in H_2O (1 μmol/ml) (4 μl, 4 nmol) was added to a quartz cuvette containing *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) buffer (HEPES; 5 mmol/l, pH 7.2) (1 ml), followed by a solution of *E. coli* LPS (serotype 055:B5) (Sigma) in H_2O (3 mg/ml) (10 μl, 30 μg). The solution was kept at 30 °C for 60 min, by which time the fluorescence intensity of [Dab(N^γ-dansyl-Gly)^1]-PMB_3 reached a plateau. A solution of each PMB_3 analog (1 μmol/ml) (4 μl aliquots) was added cumulatively to the quartz cuvette at 5-min intervals to obtain eight data points (4—32 nmol). The change in fluorescence intensity was measured after each addition at an excitation wavelength of 330 nm and an emission wavelength of 490 nm using an F-850 fluorescence spectrophotometer (Hitachi Instrument Co., Tokyo, Japan). The initial intensity of fluorescence was taken as 100%. The percent fluorescence intensity was plotted as a function of the concentration of peptide. The binding experiments were repeated at least three times for each peptide to establish the reproducibility of the results. To study the inhibitory effect on *P. aeruginosa* LPS (Sigma), a larger volume of [Dab(N^γ-dansyl-Gly)^1]-PMB_3 in H_2O (1 μmol/ml) (20 μl, 20 nmol) was added to the quartz cuvette.

Acute Toxicity The LD_{50} of each synthetic peptide (PMB, PMBN, (**2**)—(**6**) and (**10**)) was determined in male ddY mice (4 weeks old; Japan SLC, Hamamatsu, Japan). The following peptide solutions in saline were prepared: PMB sulfate (Sigma); 0.25, 0.50, 0.75, and 1.0 μmol/ml, and PMBN; 2.0, 2.5, 3.0, 3.5, and 4.0 μmol/ml. The concentration of each synthetic peptide solution was determined from the results of a pretest. The solutions were injected through the lateral tail vein at a rate of 0.1 ml/30 s to a volume of 0.1 ml/10 g body weight. Five to ten mice were used per dose. The LD_{50} was determined by the Litchfield–Wilcoxon method[19] from the number of mice dying following each dose.

Table 3. Acute Toxicity of the Synthetic Peptides ((**2**)—(**6**) and (**10**))

		LD_{50} value (μmol/kg)	95% confidence interval (μmol/kg)
	Polymyxin B (Sigma)	4.8	4.3—5.3
	Polymyxin B nonapeptide (PMBN)	31.5	25.5—39.0
(**2**)	[Ser^2-Dap^3]-PMB(2—10)	40.9	36.0—49.7
(**3**)	[Dap^3]-PMB(3—10)	>50	—
(**4**)	[Ser^3]-PMB(3—10)	>50	—
(**5**)	Guanyl-[Dab^1-Thr^2-Dab^3]-PMB(1—10)	5.4	2.8—12.1
(**6**)	Guanyl-[Thr^2-Dab^3]-PMB(2—10)	7.0	4.3—11.5
(**10**)	$N^{\alpha,\gamma}$-diguanyl-[Dap^3]-PMB(3—10)	10.4	6.2—16.7

Analogs (**1**) and (**7**)—(**9**) were not evaluated.

All animal procedures were approved by the Animal Care and Use Committee of Hokuriku University.

Results and Discussion

Synthesis of Peptides Synthesis of des-FA-PMB analogs ((**1**)—(**10**)) was carried out as reported previously (Fig. 1).[13,17] The product was extensively purified by HPLC and gel-filtration prior to examination of its biological activity. The purity of the synthetic peptides was >98%.

Antimicrobial Activity The antimicrobial activity of the synthetic peptides against *E. coli*, *S.* Typhimurium and *P. aeruginosa* is summarized in Table 2.

The MIC values of Des-FA-[Dap^1-Ser^2-Dap^3]-PMB(1—10) (**1**), [Ser^2-Dap^3]-PMB(2—10) (**2**), [Dap^3]-PMB(3—10)

(**3**), and [Ser^3]-PMB(3—10) (**4**) were respectively 16, 64, 256, and >256 nmol/ml against *E. coli* and 32, 64, 256, and >256 nmol/ml against *S.* Typhimurium. Compared with these hydrophilic small des-FA-PMB analogs containing Ser and/or Dap ((**1**)—(**4**)), the peptides with shorter chain lengths were less active against *E. coli* and *S.* Typhimurium, but retained potency against *P. aeruginosa* (MIC: 1—4 nmol/ml). Des-FA-[Dap^1-Ser^2-Dap^3]-PMB(1—10) (**1**) has lower antimicrobial activity (MIC: 2 nmol/ml) than PMB against *P. aeruginosa*. [Ser^2-Dap^3]-PMB(2—10) (**2**) retained the same antimicrobial activity against *P. aeruginosa* (MIC: 1 nmol/ml) as PMB (MIC: 1 nmol/ml), but showed less activity against *E. coli* and *S.* Typhimurium (MIC: 64 nmol/ml). Despite having the same number of amino acids, the antimicrobial activity of [Ser^2-Dap^3]-PMB(2—10) (**2**) was different from that of PMBN against the three bacteria. [Ser^2-Dap^3]-PMB(2—10) had 64-fold higher activity than PMBN against *P. aeruginosa*. We reported earlier that PMB octapeptide (PMB(3—10)) has low antimicrobial activity against *E. coli*, *S.* Typhimurium, and *P. aeruginosa* (MIC values: 128, 256, and 64 nmol, respectively).[13] [Dap^3]-PMB(3—10) (**3**) and [Ser^3]-PMB(3—10) (**4**) had high antimicrobial activity (MIC values of 4 and 2 nmol/ml, respectively) compared with PMB octapeptide against *P. aeruginosa*, whereas the MIC values of these analogs were very low against *E. coli* and *S.* Typhimurium. These results show that small hydrophilic amino acids (Dap, Ser) at the N-terminal are essential for antimicrobial specificity towards *P. aeruginosa*.[17] Shortening the side chain by a single methylene results in increased activity.

In PMB derivatives with an introduced guanyl group, Guanyl-[Dab^1-Thr^2-Dab^3]-PMB(1—10) (**5**) retained antimicrobial activity against *E. coli*, *S.* Typhimurium, and *P. aeruginosa* (MIC: 4, 2, and 1 nmol/ml, respectively), and its activity was higher than that of des-FA-PMB decapeptide (MIC: 8, 16, and 4 nmol/ml against the same three bacteria, previously reported).[13] Guanyl-[Thr^2-Dab^3]-PMB(2—10) (**6**) and Guanyl-[Dab^2-Dab^3]-PMB(2—10) (**7**) have much higher activity than PMBN (MIC values of >256, >256, and 128 nmol/ml, respectively) against the three bacteria. Guanyl-[Dab^3] -PMB(3—10) (**8**) and $N^{\alpha,\gamma}$-diguanyl-[Dap^3]-PMB(3—10) (**10**) also have higher activity (MIC: 16, 16, and 2 nmol/ml and 16, 32, and 0.5 nmol/ml, respectively) than Des-FA-PMB octapeptide (MIC: 128, 256, and 64 nmol/ml, respectively, against the same three bacteria).[9] Guanyl-PMB (4—10) (**9**) has less activity (MIC: >256 nmol/ml), similar to that of Des-FA-PMB(4—10) (MIC: >256 nmol/ml) against *E. coli* and *S.* Typhimurium, but retains its potency against *P. aeruginosa* (MIC: 4 nmol/ml). Their guanylated analogs exhibited reduced antimicrobial activity against *E. coli* and *S.* Typhimurium as compared with PMB, but their activities were higher than those of the Ser and Dap analogs. The guanylated peptides showed potent antimicrobial activity against *P. aeruginosa* (MIC: 0.5—4 nmol/ml). $N^{\alpha,\gamma}$-diguanyl-[Dap^3]-PMB(3—10) (**10**) was 2-fold more potent than PMB against *P. aeruginosa*. The MIC value of Guanyl-PMB(4—10) (**9**) against *P. aeruginosa* was 4 nmol/ml. In a previous study we and Urakawa *et al.* found that PMB heptapeptide and its derivatives have no significant antimicrobial activity against Gram-negative bacteria, but the binding activity to LPS is retained.[13,22] Therefore, PMB heptapeptide analogs with better permeability into the outer membranes were designed. Only Guanyl-PMB(4—10) (**10**), which is the lowest molecular weight analog studied, had antimicrobial activity against *P. aeruginosa*.

The difference of their antimicrobial specificity against the three bacterial species tested can be largely explained by structural differences in the bacterial cell membranes, especially the lipid A portions, which anchor the LPS molecule to the lipid layer of the membranes.[23] Lipid A of *E. coli* and *S.* Typhimurium is largely composed of five C14 fatty acids and one C12 fatty acid, whereas that of *P. aeruginosa* is composed of four C12 and two C10 fatty acids. The membrane lipid components of *P. aeruginosa* are less hydrophobic than those of the two other bacteria, so the cell membrane structure of *P. aeruginosa* is less compact. This allows for hydrophobic interactions between the D-Phe and Leu side chains of PMB analogs and the outer membrane, resulting in membrane disorder that leads to cell death. Absence of the N-terminal FA group in PMB is apparently not essential for antimicrobial activity towards *P. aeruginosa*.

Tsubery *et al.*[7] showed that the structures of PMB and PMBN bound to LPS were modeled based on coordinates provided for PMB by Pristovšek and Kidrič.[24] Elimination of the positively charged Dab^1 residue, which interacts with the negatively charged phosphate group in the PMB-LPS complex, is not likely to significantly weaken the LPS-peptide interaction. This is because of the conformational change of the side chain at the Dab^3 residue, allowing for the efficient interaction of the Dab^3 residue with the negatively charged phosphate group. We considered that our synthetic analogs induce a conformational change of the LPS-peptide interaction.

Our previous study shows that PMB octapeptide (PMB(3—10)) has low antimicrobial activity against *P. aeruginosa*, with an MIC value of 64 nmol/ml.[13] [Dap^3]-PMB(3—10) (**3**) and [Ser^3]-PMB(3—10) (**4**) showed high antimicrobial activities, with MIC values of 4 and 2 nmol/ml, respectively, compared with PMB octapeptide against *P. aeruginosa*. These results show that small hydrophilic amino acids (Dap, Ser) at the N-terminal are essential for antimicrobial specificity towards *P. aeruginosa*.[17] We consider that shortening a single methylene in the side chain of Dab and/or Thr allows for the efficient interaction of the linear portion in PMB analogs with the negatively charged phosphate group. The differences may be related to the distance between the phosphate group in LPS and the linear portion of the PMB analog. The guanyl group is a strong basic group. The strong interaction between the negatively charged phosphate group in LPS and the positively charged portion of the guanyl PMB derivative influences the antimicrobial activity.

LPS Binding Activity The LPS-binding activities of the synthetic peptides were evaluated by measuring the displacement of [Dab(N^{γ}-dansyl-Gly)^1]-PMB$_3$ bound to *E. coli* and *P. aeruginosa* LPS, using the methodology reported previously.[13] The mechanism by which these synthetic peptides exclude the fluorescent probe bound to LPS could be explained as follows.[25] First, the N-terminal cationic portion of the peptide binds to the phosphate anions of lipid A and/or the carboxylate anions of the adjacent 3-deoxy-D-manno-octulosonic acid (Kdo) portion of the LPS molecule. This ionic binding causes structural changes in LPS, resulting in the loss of hydrophobic binding between the FA group(s) of the

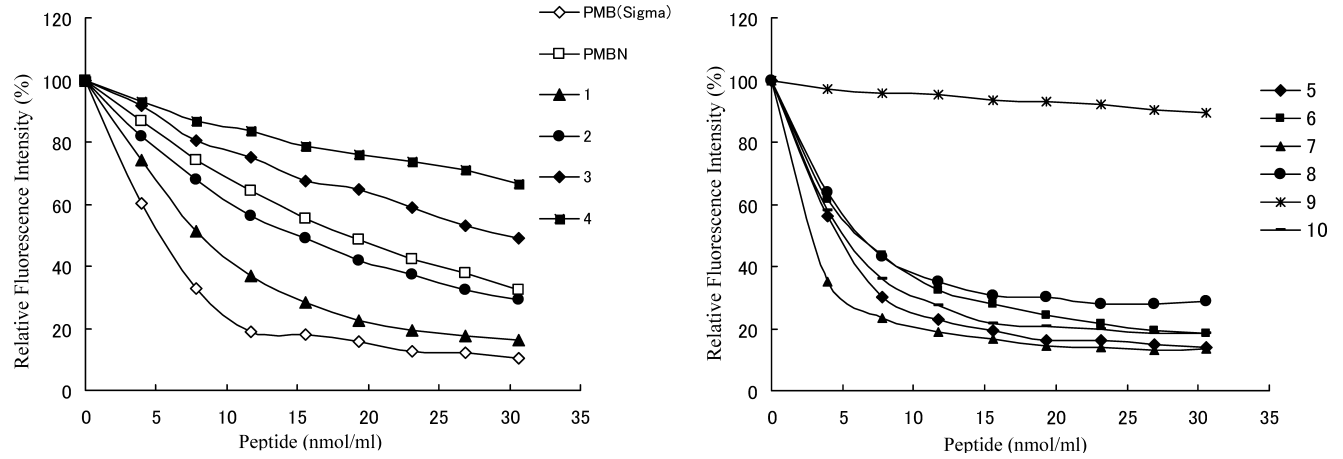

Fig. 2. Displacement of [Dab(N^{γ}-dansyl-Gly)^{1}]-PMB_3 Bound to *E. coli* LPS by Synthetic Peptides ((**1**)—(**10**))

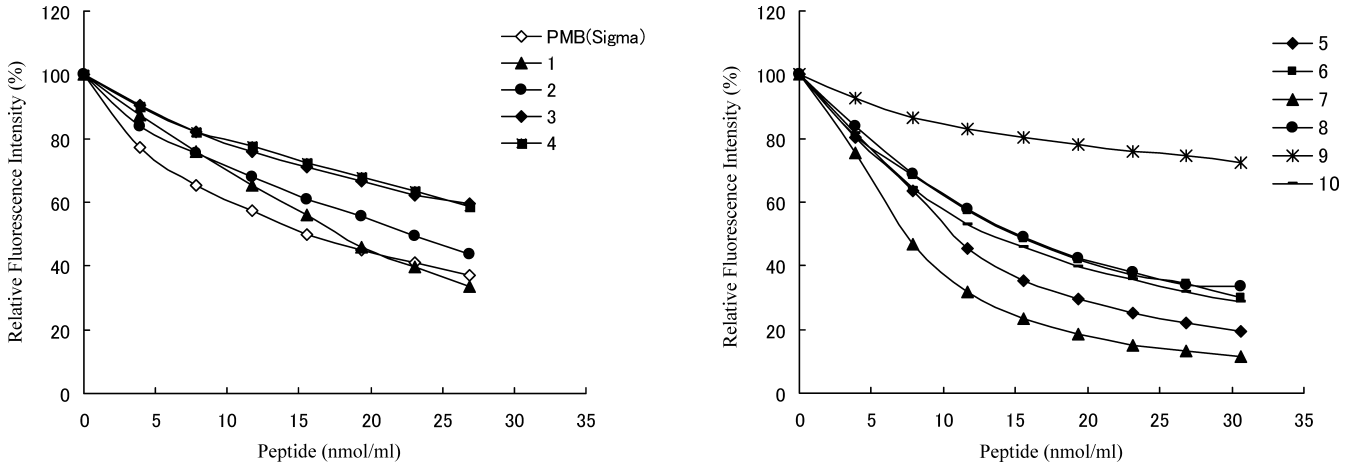

Fig. 3. Displacement of [Dab(N^{γ}-dansyl-Gly)^{1}]-PMB_3 Bound to *P. aeruginosa* LPS by Synthetic Peptides ((**1**)—(**10**))

lipid A portion of LPS and the octanoyl-Dab^{1} portion of the probe, [Dab(N^{γ}-dansyl-Gly)^{1}]-PMB_3.

N-terminal PMB analogs with Ser and/or Dap ((**1**)—(**4**)) had low *E. coli* LPS binding activity compared with PMB (Fig. 2). The binding ability was directly proportional to the number of positive charges on the peptide that could interact with the *E. coli* LPS. Furthermore, these results indicate that the LPS binding activity of compact hydrophilic analogs paralleled their antimicrobial activity against *E. coli*. Guanyl-PMB analogs ((**6**)—(**10**)) had potent LPS binding activity except for Guanyl-PMB(4—10) (**9**), and the binding activities of Guanyl-[Dab^{2}-Dab^{3}]-PMB(2—10) (**7**), Guanyl-[Dab^{1}-Thr^{2}-Dab^{3}]-PMB(1—10) (**5**), and $N^{\alpha,\gamma}$-diguanyl-[Dap^{3}]-PMB(3—10) were especially potent, equivalent to PMB binding activity against *E. coli*.

In the case of *P. aeruginosa*, N-terminal PMB analogs with Ser and/or Dap ((**1**)—(**4**)) had slightly lower LPS binding activity compared with PMB, whereas the Guanyl-PMB analogs had high LPS binding activity, except for (**9**) (Fig. 3). However, unlike with the N-terminal analogs, the binding activities of the Guanyl-PMB analogs did not correlate with their antimicrobial activities, showing that the FA portion of the molecule plays only a secondary role in LPS binding activity towards *P. aeruginosa*.

The difference between LPS binding activity and antimicrobial activity is presumed that the difference of interaction of the peptides with cell-free LPS or cell-bound LPS.

Acute Toxicity The acute toxicities of (**2**)—(**6**) and (**10**) compared with PMB and PMBN were examined by tail intravenous bolus injection in mice. These injections were lethal within 24 h. The administration of PMB and its analogs induced respiratory arrest and death within 5 min of injection. This effect may be caused by neuromuscular blockade.[20,21] The LD_{50} values of [Ser^{2}-Dap^{3}]-PMB(2—10) (**2**), [Dap^{3}]-PMB(3—10) (**3**), and [Ser^{3}]-PMB(3—10) (**4**) were 40.9 μmol/kg, $>$50 μmol/kg, and $>$50 μmol/kg, respectively. The results demonstrate that the acute toxicity of these peptides in mice was lower compared to native PMB (LD_{50}: 4.8 μmol/kg) (Table 2), and furthermore, they were less toxic than PMBN (LD_{50}: 31.5 μmol/kg). The toxicities of the guanylated peptides were higher than that of PMBN. $N^{\alpha,\gamma}$-diguanyl-[Dap^{3}]-PMB(3—10) (**10**) showed low toxicity compared with PMB.

In the present study, we synthesized hydrophilic and compact des-FA-PMB analogs (Fig. 1). We developed N-terminal analogs of PMB lacking a FA moiety and demonstrated that these analogs have antimicrobial activity specific towards *P. aeruginosa*. In [Ser^{2}-Dap^{3}]-PMB(2—10) (**2**), introduction of a small amino acid (Ser, Dap) resulted in potent antimicrobial activity specific towards *P. aeruginosa* (MIC: 1 nmol/ml,

equal to that of PMB) and very low acute toxicity (8.5-fold less toxic than PMB). The guanylated analogs maintained antimicrobial potency, especially against *P. aeruginosa*, but they were as toxic as PMB. Of particular interest is [Ser^3]-PMB(3—10) (**4**), a PMB octapeptide derivative. Our results show that it is a non-toxic, compact analog with highly selective antimicrobial activity towards *P. aeruginosa*. The improved properties of this analog provide a useful base for the development of PMB analogs with potent antimicrobial activity and low toxicity. These results provide clues for developing a potent antimicrobial analog with low acute toxicity.

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