Development of Des-Fatty Acyl-Polymyxin B Decapeptide Analogs with *Pseudomonas aeruginosa*-Specific Antimicrobial Activity

Naoko KATSUMA, Yuki SATO, Kazuhiro OHKI, Keiko OKIMURA, Kuniharu OHNISHI, and Naoki SAKURA*

Faculty of Pharmaceutical Sciences, Hokuriku University; Kanazawa 920–1181, Japan.
Received September 30, 2008; accepted January 23, 2009; published online January 26, 2009

Twelve N-terminal analogs of des-fatty acyl-polymyxin B (Des-FA-[X^1]-PMB, X=various amino acids or peptides) were synthesized and examined for their antimicrobial activity against *Escherichia coli* (*E. coli*), *Salmonella* Typhimurium (*S.* Typhimurium) and *Pseudomonas aeruginosa* (*P. aeruginosa*). It was found that Des-FA-[Dap^1]-, Des-FA-[Ser^1]-, Des-FA-[Dab-Dab-Dab^1]- and Des-FA-[Arg-Arg-Arg^1]-PMB had potent activity only against *P. aeruginosa*, with MIC values of 0.5—1 nmol/ml. Analogs in which X was Lys, Arg, Leu or Ala did not have increased antimicrobial activity against the three bacterial species tested compared with the lead compounds Des-FA-[Dab^1]-PMB and polymyxin B (PMB). Des-FA-[Trp^1]-PMB and Des-FA-[Phe^1]-PMB had reduced activity against *P. aeruginosa*. The results indicate that compact hydrophilic amino acids (C3) or basic tripeptides at the N-terminal provide specificity for bactericidal activity towards *P. aeruginosa*. For LPS-binding activity, Des-FA-[Dab-Dab-Dab^1]-PMB and Des-FA-[Arg-Arg-Arg^1]-PMB showed activity comparable to PMB, while Des-FA-[Ala-Ala-Ala^1]-PMB showed very low activity. Reduced acute toxicity of Des-FA-[Dap^1]-PMB and Des-FA-[Trp^1]-PMB was demonstrated by a mouse tail intravenous administration test, with LD_{50} values of 23.5 and 19.0 μmol/kg, respectively, in contrast to 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

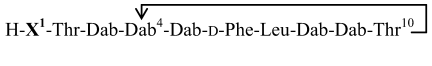
Polymyxin B (PMB) is a cyclic decapeptide antibiotic, and the N-terminal α,γ-diaminobutyric acid residue (Dab^1) is N^{α}-acylated by fatty acids such as 6-methyloctanoic acid (PMB_1), 6-methylheptanoic acid (PMB_2) and octanoic acid (PMB_3).[1,2] PMB has potent antimicrobial activity against Gram-negative bacteria, but the toxicity of PMB limits its therapeutic application.[1] It is well-known that the enzymatic removal of the fatty acyl-Dab^1 of PMB with ficin yields PMB nonapeptide (PMBN),[3,4] which retains considerable lipopolysaccharide (LPS)-binding activity[5] and has reduced toxicity.[6] However, PMBN has low bactericidal activity, with a minimum inhibitory concentration (MIC) value of >256 nmol/ml towards *Escherichia coli* (*E. coli*).[4] Thus, it appears that the fatty acyl-Dab^1 moiety of PMB greatly affects antimicrobial activity and toxicity, and has some effect on LPS binding activity.[7] Our previous studies on the structure–antimicrobial activity relationship of PMB showed that des-fatty acyl-PMB (Des-FA-[Dab^1]-PMB) has 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*).[7—9] Apparently, the introduction of Dab at the N-terminal of PMBN greatly increases its activity towards *E. coli*, from an MIC value of 256 to 8 nmol/ml. We therefore selected the cyclic decapeptide Des-FA-[Dab^1]-PMB as a lead compound for developing a peptide antibiotic with increased antimicrobial activity and decreased toxicity. In the present study, synthetic peptides with bactericidal activity specific towards *P. aeruginosa* were obtained by introducing compact amino acids with two or more basic or hydrophilic functions into the X position of des-FA-[X^1]-PMB (Fig. 1).

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 column chromatography was carried out using Toyopearl HW-40-S (Tosoh Corporation, Tokyo, Japan). Fast-atom bombardment mass spectra (FAB-MS) were obtained on a JMS-DX300 mass spectrometer (JEOL Ltd., Tokyo, Japan). The optical rotations of the peptides were measured with a DIP-370 digital polarimeter (Nippon Bunko Co., Ltd., Tokyo, Japan). Peptide deprotection with anhydrous HF was carried out in a Teflon HF apparatus (Peptide Institute Inc., Osaka, Japan). HP-TLC was performed on precoated silica gel plates (Kieselgel 60; Merck, Darmstadt, Germany). All reagents, peptide synthesis solvents and 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were purchased from Watanabe Chem. Ind. Ltd. (Hiroshima, Japan).

Synthesis of Peptides Des-FA-[X^1]-PMB analogs (**1**—**12**, Fig. 1) were synthesized as reported previously.[7] In brief, the protected peptide was constructed on 4-hydroxylmethylphenoxymethyl-resin[10] (HMP-resin, 0.74 mmol/g, Novabiochem, Läufelfingen, Switzerland) by a solid phase methodology[11] using an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA, U.S.A.). Protected amino acids used were Fmoc-Thr(Bzl)-OH, Fmoc-Dab(2-ClZ)-OH, Fmoc-Dab(Boc)-OH, Fmoc-D-Phe-OH, Fmoc-Leu-OH, Fmoc-Lys(2-ClZ)-OH Fmoc-Arg(Tos)-OH, Fmoc-Dap(2-ClZ)-OH (Dap; L-α,β-diaminopropionic acid), Fmoc-Ser(Bzl)-OH, Fmoc-Phe-OH, Fmoc-Trp(Boc)-OH, Fmoc-Ala-OH and Fmoc-Glu(OcHex)-OH. The Fmoc groups were removed with 25% piperidine in *N*-methylpyrolidone (NMP).

1) Preparation of Fmoc-[X^1]-Thr(Bzl)-Dab(2-ClZ)-Dab-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-OH (1_L—12_L) Starting from Fmoc-Thr(Bzl)-*O*-HMP-resin (1.132 g, 0.6 mmol eq), Fmoc-amino acids (1.5 mmol) and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluro-



peptide	X	peptide	X
1	Lys	7	Leu
2	Arg	8	Ala
3	Dap	9	Glu
4	Ser	10	Dab-Dab-Dab
5	Phe	11	Arg-Arg-Arg
6	Trp	12	Ala-Ala-Ala

Fig. 1. Structures of the Synthetic Peptides, Des-FA-[X^1]-PMB

* To whom correspondence should be addressed. e-mail: n-sakura@hokuriku-u.ac.jp

nium hexafluorophosphate (HATU) (1.5 mmol) were dissolved in NMP (10 ml), and *N*,*N*-diisopropylethylamine (3.0 mmol, 523 μl) was added. The mixture was transferred to a reaction vessel containing the resin and allowed to react for 2—5 h prior to acetylation with acetic anhydride (3 ml)–pyridine (3 ml) in NMP (10 ml). Coupling of Fmoc-Thr(Bzl)-OH at position 2 followed by deprotection with piperidine yielded H-Thr(Bzl)-Dab(2-ClZ)-Dab(Boc)-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-*O*-HMP-resin, which was divided into four portions and stored in NMP at 4 °C until coupling with various protected N-terminal amino acids (Fmoc-X). After introduction of Fmoc-X to this protected nonapeptide resin (0.15 mmol eq), the resin was treated with trifluoroacetic acid (TFA)–H_2O (95 : 5, 3 ml) for 1.5 h at room temperature to simultaneously cleave the peptide from the HMP-resin support and the Boc-protecting group(s) from Dab[4] or Trp[1]. TFA was removed *in vacuo* and the residue was lyophilized from dioxane. The crude product was partially purified by column chromatography on a Toyopearl HW-40-S column (1.6×95 cm, Tosoh Co.) using dimethylformamide (DMF) : H_2O (9 : 1). Fractions containing the main product were combined, evaporated and lyophilized from dioxane to give Fmoc-[X^1]-Thr(Bzl)-Dab(2-ClZ)-Dab-Dab(2-Cl)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-OH [X=Lys(2-ClZ) (**1**$_L$), Arg(Tos) (**2**$_L$), Dap(2-ClZ) (**3**$_L$), Ser(Bzl) (**4**$_L$), Phe (**5**$_L$), Trp (**6**$_L$), Leu (**7**$_L$), Ala (**8**$_L$), Glu(OcHex) (**9**$_L$), Dab(2-ClZ)-Dab(2-ClZ)-Dab(2-ClZ) (**10**$_L$), Arg(Tos)-Arg(Tos)-Arg(Tos) (**11**$_L$) and Ala-Ala-Ala (**12**$_L$)]. The structures of **1**$_L$—**12**$_L$ were confirmed by FAB-MS (Table 1). The purity of the products **1**$_L$—**12**$_L$ was 80—90% as determined by HPLC analysis, monitoring at 210 nm. The partially purified peptides (**1**$_L$—**12**$_L$) were used without further purification for the cyclization reaction.

2) Preparation of Fmoc-[X^1]-Thr(Bzl)-Dab(2-ClZ)-cyclic-[Dab*-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)*-] (*–*; Amide Bond between the γ-NH_2 of Dab[4] and α-COOH of Thr[10])] (1$_C$—12$_C$) The linear partially protected peptide (**1**$_L$—**12**$_L$, 0.02—0.07 mmol) was dissolved in ice-cold DMSO (1 ml)–DMF (2 ml), and diphenyl phosphorazidate[12] (DPPA; 0.1—0.35 mmol, 5-fold eq) and 4-methylmorpholine (0.2—0.70 mmol, 10-fold eq) were added. The mixture was allowed to react overnight at 4 °C to form the lactam ring, then the cyclized products (**1**$_C$—**12**$_C$) were purified on a Toyopearl HW-40-S column (1.6×95 cm) as described above for **1**$_L$—**12**$_L$. Fractions containing the main product were combined, evaporated, and the product was lyophilized from dioxane. Yields of all cyclized products (**1**$_C$—**12**$_C$) were almost quantitative (>90%). FAB-MS data are shown in Table 1; the purity of the products was shown to be 85—90% by HPLC analysis. The partially purified peptides (**1**$_C$—**12**$_C$) were used without further purification for the deprotection reaction.

3) Preparation of [X^1]-Polymyxin B (1—12) Each cyclic protected [X^1]-peptide (**1**$_C$—**12**$_C$, 40—70 mg) was dissolved in DMF (2 ml), and piperidine (0.5 ml) was added. The mixture was stirred for 5 min at room temperature and evaporated. The piperidine treatment was repeated three times and the product was lyophilized from dioxane. Lyophilized product was treated with anhydrous HF (2 ml)–anisole (0.2 ml) for 1 h on ice, then excess HF was removed *in vacuo*. The residue was dissolved in H_2O (15 ml), washed with three portions of ether (15 ml) and lyophilized. The crude product was purified by HPLC employing a CAPCELL PAK C_{18} UG-80 5 μm (2×15 cm, Shiseido Co., Ltd.) using acetonitrile–0.1% TFA. The main product peak was collected, combined, evaporated and lyophilized. The product was chromatographed on a Toyopearl HW-40-S column (1.5×57 cm) using 25% CH_3CN in 5 mmol/l HCl. Purified product was obtained as the hydrochloride salt by lyophilization. Yield of **1**—**12** was 30—45% (calculated from partially purified **1**$_C$—**12**$_C$). FAB-MS data and characteristics of the synthetic peptides are shown in Table 2. The purity of the products was >98% as determined by HPLC analysis, monitoring at 210 nm. A representative analytical HPLC chromatogram of Des-FA-[Dap^1]-PMB (**3**) is shown in Fig. 2.

Bacteria, and Bactericidal Test *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.

Table 1. FAB-MS Analysis of Linear Partially Protected Peptides (**1**$_L$—**12**$_L$) and Cyclic Protected Peptides (**1**$_C$—**12**$_C$)

Linear protected [X^1]-peptide		Found	
X	Formula	$[M+H]^+$	$[M+Na]^+$
1$_L$	$C_{118}H_{135}N_{16}O_{25}Cl_5$	2356.8	2378.8
2$_L$	$C_{117}H_{136}N_{18}O_{25}S_1Cl_4$	2369.8	2391.8
3$_L$	$C_{115}H_{129}N_{16}O_{25}Cl_5$	2314.8	2336.8
4$_L$	$C_{114}H_{129}N_{15}O_{24}Cl_4$	2234.8	2256.8
5$_L$	$C_{113}H_{127}N_{15}O_{23}Cl_4$	2214.8	2227.8
6$_L$	$C_{115}H_{128}N_{16}O_{23}Cl_4$	2244.8	2266.8
7$_L$	$C_{110}H_{129}N_{15}O_{23}Cl_4$	2171.8	2193.8
8$_L$	$C_{107}H_{123}N_{15}O_{23}Cl_4$	2129.8	2151.8
9$_L$	$C_{115}H_{135}N_{15}O_{25}Cl_4$	2269.9	2291.9
10$_L$	$C_{140}H_{157}N_{20}O_{31}Cl_7$	2864.9	2886.9
11$_L$	$C_{143}H_{172}N_{26}O_{31}S_3Cl_4$	2991.1	3013.1
12$_L$	$C_{113}H_{132}N_{17}O_{25}Cl_4$	2271.9	2293.9

Cyclic protected [X^1]-peptide		Found	
X	Formula	$[M+H]^+$	$[M+Na]^+$
1$_C$	$C_{118}H_{133}N_{16}O_{24}Cl_5$	2338.8	2360.8
2$_C$	$C_{117}H_{134}N_{18}O_{24}SCl_4$	2351.8	2373.8
3$_C$	$C_{115}H_{127}N_{16}O_{24}Cl_5$	2296.8	2318.8
4$_C$	$C_{114}H_{127}N_{15}O_{23}Cl_4$	2216.8	2238.8
5$_C$	$C_{113}H_{125}N_{15}O_{22}Cl_4$	2187.8	2209.8
6$_C$	$C_{115}H_{126}N_{16}O_{22}Cl_4$	2226.8	2248.8
7$_C$	$C_{110}H_{127}N_{15}O_{22}Cl_4$	2153.8	2175.8
8$_C$	$C_{107}H_{121}N_{15}O_{22}Cl_4$	2111.8	2133.8
9$_C$	$C_{115}H_{133}N_{15}O_{24}Cl_4$	2251.9	2273.9
10$_C$	$C_{140}H_{155}N_{20}O_{30}Cl_7$	2846.9	2868.9
11$_C$	$C_{143}H_{170}N_{26}O_{30}S_3Cl_4$	2972.1	2995.1
12$_C$	$C_{113}H_{131}N_{17}O_{24}Cl_4$	2253.8	2275.8

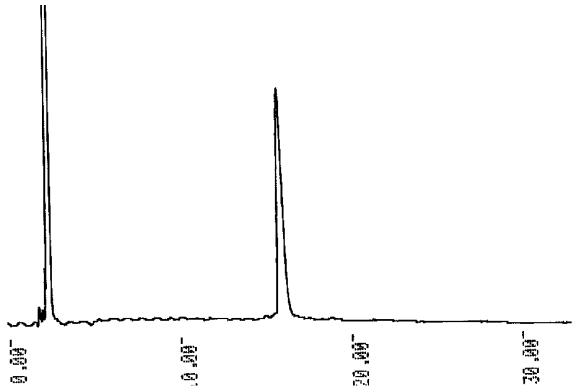
Table 2. Characterization of Synthetic Peptides **1**—**12**

Peptide	$[\alpha]_D^{20}$ (c=0.5, 12% AcOH)	FAB-MS			RP-HPLC[a)]	HP-TLC[c)]	
		Formula	$[M+H]^+$	$[M+Na]^+$	t_R[b)] (min)	Rf^1	Rf^2
1	−63.6°	$C_{49}H_{86}N_{16}O_{12}$	1092.7	1114.7	17.0	0.14	0.09
2	−64.0°	$C_{49}H_{86}N_{18}O_{12}$	1120.7	1142.7	17.0	0.09	0.05
3	−70.3°	$C_{46}H_{80}N_{18}O_{12}$	1050.6	1072.6	16.7	0.07	0.06
4	−65.2°	$C_{49}H_{79}N_{15}O_{12}$	1050	1072	16.3	0.25	0.12
5	−59.3°	$C_{52}H_{83}N_{15}O_{12}$	1111.6	1133.6	21.7	0.34	0.14
6	−52.9°	$C_{54}H_{84}N_{16}O_{12}$	1150.7	1172.7	25.5	0.42	0.15
7	−65.3°	$C_{49}H_{85}N_{15}O_{12}$	1077.6	1099.6	19.2	0.35	0.09
8	−74.7°	$C_{49}H_{79}N_{15}O_{12}$	1035.7	1057.7	16.3	0.28	0.04
9	−67.3°	$C_{48}H_{81}N_{15}O_{14}$	1093.6	1115.6	16.3	0.29	0.06
10	−78.1°	$C_{55}H_{98}N_{20}O_{14}$	1264.8	1286.8	16.6	0.08	0.05
11	−62.7°	$C_{61}H_{110}N_{26}O_{14}$	1432.9	1454.9	18.0	0.06	0.04
12	−92.8°	$C_{52}H_{89}N_{17}O_{14}$	1177.7	1199.7	16.7	0.29	0.08

a) Conditions: column, CAPCELL PAK C_{18} UG120 (6.0×150 mm); elution, a linear gradient from 12 to 20% MeCN in 0.1% TFA over 30 min; flow rate, 1.5 ml/min; detection, 210 nm. *b*) Retention time. *c*) Rf^1; *n*-butanol : pyridine : AcOH : H_2O (30 : 20 : 6 : 24). Rf^2; *n*-butanol : AcOH : AcOEt : H_2O (1 : 1 : 1 : 1).

The densities of bacterial suspensions were determined at 600 nm, using a standard curve relating absorbance to the number of colony forming units (CFU). Antibacterial activity of the synthetic peptides was evaluated in comparison with that of commercially available PMB (Sigma Chemical Co., St Louis, MO., U.S.A.). MIC of the synthetic peptides against the bacterial strains were determined using a standard microplate dilution method ($n=6$—8). One hundred microliters of each peptide 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-bottomed microplate (Corning Inc., Corning, NY, U.S.A.). The plates were incubated overnight at 37 °C for MIC evaluation. The MIC value was expressed as the lowest final concentration (nmol/ml) at which no growth was observed (Table 3).

LPS Binding Assay of Synthetic Peptides As described previously,[7] 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* (serotype 055:B5) lipopolysaccharide (LPS, Sigma Chemical Co.) 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 polymyxin B_3 analog (1 μmol/ml) (4 μl each) was added cumulatively to the quart 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 at 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 concentration required for 50% quenching of [Dab(N^{γ}-dansyl-Gly)^1]-PMB_3 bound to LPS (IC_{50}) was derived from the quenching curve of each synthetic peptide. The binding experiments were repeated at least three times for each peptide to obtain reproducible results.



Conditions: column, CAPCELL PAK C_{18} UG120 (6.0×150 mm); elution, a linear gradient from 12 to 20% MeCN in 0.1% TFA over 30 min; flow rate, 1.5 ml/min; detection, 210 nm

Fig. 2. Analytical HPLC of Des-FA-[Dap^1]-Polymyxin B (**3**)

Acute Toxicity of Des-FA-[Dap^1]-PMB (3) Des-FA-[Trp^1]-PMB (6) The lethal dose (LD_{50}) of synthetic peptides (PMB, PMBN, Des-FA-[Dap^1]-PMB (**3**) and Des-FA-[Trp^1]-PMB (**6**)) were determined in male ddY mice (4 weeks old; Japan SLC, Hamamatsu, Japan). The following peptide solutions in saline (μmol/ml) were prepared: PMB sulfate (Sigma); 0.25, 0.50, 0.75, and 1.0. PMBN; 2.0, 2.5, 3.0, 3.5, and 4.0. Des-FA-[Dap^1]-PMB (**3**); 1.0, 1.5, 2.0, 2.5, and 3.0. Des-FA-[Trp^1]-PMB (**6**); 1.0, 1.5, 2.0, 2.5, and 3.0. The rate and volume of injection through the lateral tail vein were 0.1 ml/30 s and 0.1 ml/10 g body weight, respectively. Five to ten mice per dose were used. The LD_{50} was determined by the Litchfield–Wilcoxon method[13] from the number of mice dying following each dose.

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

Results and Discussion

Synthesis of N-terminal fatty acyl-free and substitution analogs of N-terminal Dab^1 of PMB, Des-FA-[X^1]-PMB, was readily achieved using the synthetic route reported previously.[7] Fmoc-[X^1]-Thr(Bzl)-Dab(2-ClZ)-Dab(Boc)^4-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)^{10}-*O*-HMP-resin was synthesized by solid phase techniques using Fmoc-amino acids and HATU as the coupling reagent. Treatment of the protected peptide resin with TFA simultaneously cleaved the peptide from the solid support and the Boc group from Dab^4. Lactam formation on the resulting linear partially protected peptide was achieved with DPPA reagent,[12] yielding cyclic protected Des-FA-[X^1]-PMB quantitatively. Final deprotection was performed by piperidine treatment, followed by HF. 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%, as shown representatively by the HPLC chromatogram of Des-FA-[Dap^1]-PMB (**3**) (Fig. 2).

The antimicrobial activity of synthetic peptides against *E. coli*, *S.* Typhimurium and *P. aeruginosa* is summarized in Table 3. When the N-terminal Dab of the key compound, Des-FA-[Dab^1]-PMB, was replaced by Dap or Ser, the antimicrobial activity against *P. aeruginaosa* increased four times. The activity of Des-FA-[Dap^1]-PMB (**3**) and Des-FA-[Ser^1]-PMB (**4**) was as potent as that of PMB. However, these analogs did not have increased activity against *E. coli* and *S.* Typhimurium, *i.e.*, **3** showed the same moderate bactericidal activity as Des-FA-[Dab^1]-PMB, and **4** showed low

Table 3. Antimicrobial Activity and LPS-Binding Activity of Des-FA-[X^1]-PMB (**1**—**12**)

Peptide	X	MIC (nmol/ml)			LPS-binding IC_{50} (nmol/ml)
		Escherichia coli	*Salmonella* Typhimurium	*Pseudomonas aeruginosa*	
PMB	FA-Dab	1	0.5	1	3
	Dab	8	16	4	19
1	Lys	16	32	32	22
2	Arg	8	16	16	15
3	Dap	8	16	1	14
4	Ser	64	64	1	>32
5	Phe	8	8	16	>32
6	Trp	8	8	8	20
7	Leu	16	32	64	>32
8	Ala	32	64	32	>32
9	Glu	256	>256	256	>32
10	Dab-Dab-Dab	8	32	0.5	3
11	Arg-Arg-Arg	16	64	1	3
12	Ala-Ala-Ala	64	128	128	>32

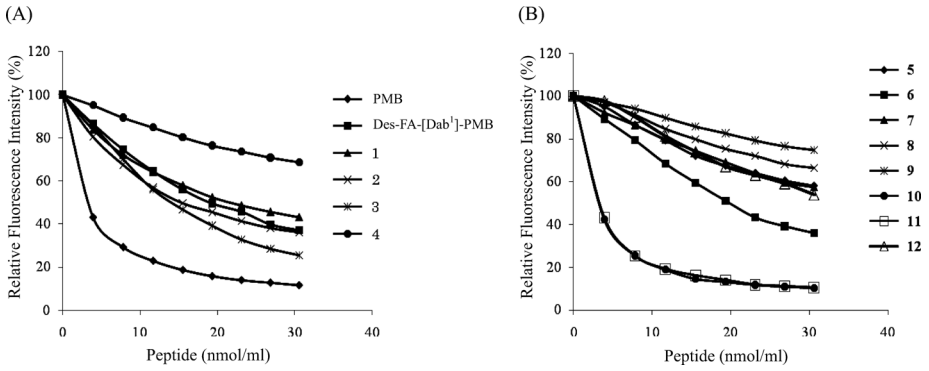


Fig. 3. Displacement of [Dab(dansyl-Gly)^1]-Polymyxin B_3 Bound to LPS by Des-FA-[X^1]-PMB (**1**—**12**)

activity with the MIC value of 64 nmol/ml. Therefore, both PMB analogs are *P. aeruginaosa*-specific antibiotics. On the other hand, the antimicrobial activity of Des-FA-[Arg^1]-PMB (**2**) against *E. coli* and *S.* Typhimurium did not change, but its activity against *P. aeruginaosa* decreased to 1/4 that of Des-FA-[Dab^1]-PMB. Des-FA-[Lys^1]-PMB (**1**) showed poor activity against the three bacterial species tested. These results suggest that small (C_3) hydrophilic amino acids (Dap and Ser) are required at the N-terminal for bactericidal specificity to *P. aeruginosa*. The hydrophilic character of the side chain is essential, since another C_3 amino acid (Ala)-analog (**8**) showed greatly decreased activity. Substitution of basic amino acids with bigger side chains [*e.g.*, C_5 (Arg) or C_6 (Lys)] for C_4 (Dab^1) resulted in poor specific activity towards *P. aeruginaosa*.

Among analogs with neutral and aromatic amino acids at the N-terminal, Des-FA-[Phe^1]-PMB (**5**) and Des-FA-[Trp^1]-PMB (**6**) retained antimicrobial activity against *E. coli* and *S.* Typhimurium, with MIC values of 8 nmol/ml; however, both analogs had reduced activity against *P. aeruginosa*. The hydrophobic and aromatic functions of Phe and Trp appear to take the place of the fatty acyl moiety of PMB and apparently contribute to the structural requirements for bactericidal activity towards *E. coli* and *S.* Typhimurium. Des-FA-[Glu^1]-PMB (**9**) had no activity against the three bacterial species tested, demonstrating that the introduction of an acidic group in the N-terminal completely abolishes the bactericidal function of the PMB molecule.

Des-FA-[Dab-Dab-Dab^1]- and Des-FA-[Arg-Arg-Arg^1]-PMB (**10**, **11**) were highly bactericidal only against *P. aeruginosa*, with MIC values of 0.5—1 nmol/ml. Substitution of the four positive charges in H-Dab-Dab-Dab- or H-Arg-Arg-Arg- for H-Dab- of Des-FA-[Dab^1]-PMB or H-Arg- of Des-FA-[Arg^1]-PMB (**2**) resulted in a 8—16 fold increase in bactericidal activity against *P. aeruginosa*. However, these analogs (**10**, **11**) had slightly decreased bactericidal activity against *E. coli* and significantly decreased activity against *S.* Typhimurium. These experimental results of the distinct antimicrobial potency against three bacteria tested could be explained mainly by the structural difference of the bacterial cell membranes especially in lipid A portions, which harbor the whole LPS molecule to the lipid layer of membrane. Lipid A of *E. coli* and *S.* Typhimurium has common components of five C14 and one C12 fatty acids, while, that of *P. aeruginosa* has four C12 and two C10 fatty acids. The membrane lipid components of *P. aeruginosa* have less hydrophobicity than those of the other two bacteria, rendering that the cell membrane structure is not so tight that the hydrophobic interaction of the side chain of D-Phe and Leu of PMB analogs with the outer membrane causes the disorder of membrane to lead the cell death. The lack of N-terminal fatty acyl group in **10** and **11** seems not to be essential as bactericidal toward *P. aeruginosa* under the presence of Dab-Dab-Dab or Arg-Arg-Arg, which may contribute to ionic binding to the inner core of LPS. On the other hand, Des-FA-[Ala-Ala-Ala^1]-PMB (**12**) had markedly reduced activity against all three bacterial species tested. Although the N-terminal tripeptide portions of **10**—**12** correspond to the fatty acyl-Dab^1 component in the PMB family in terms of size, the neutral tripeptide portion of **12** cannot contribute to retaining the bactericidal nature of the structure. Overall, the present results are consistent with the report by Tsubery *et al.*[14] that Des-FA-[Ala-Ala-Ala^1]-PMB is inactive at $>500\,\mu$g/ml against *E. coli* and *P. aeruginosa*.

Table 4. Acute Toxicity

Peptide	LD_{50} value (μmol/kg)	95% Confidence interval (μmol/kg)
PMB (Sigma)	4.8	4.3—5.3
PMBN	31.5	25.5—39
3	23.5	16.7—33.1
6	19.0	17.4—20.7

The LPS-binding activity of the synthetic peptides was evaluated by measuring the displacement of [Dab(dansyl-Gly)^1]-PMB_3 bound to *E. coli* LPS, using the methodology reported previously.[7] The displacement curve of Des-FA-[Dap^1]-PMB (**3**) shifted slightly to the left compared to Des-FA-[Dab^1]-PMB, and the LPS-binding activity of **3** was slightly higher than that of other basic amino acid analogs such as Des-FA-[Lys^1]-PMB (**1**) and Des-FA-[Arg^1]-PMB (**2**) (Fig. 3A). Des-FA-[Trp^1]-PMB (**6**) showed LPS-binding activity similar to that of Des-FA-[Dab^1]-PMB. The other neutral amino acid analogs (**4**—**8**) had poor LPS-binding activity (IC_{50} >32 nmol/ml). Des-FA-[Glu^1]-PMB (**9**) competed negligibly with dansylated PMB_3 bound to LPS. These results indicate that the LPS binding activity of hydrophilic analogs (**1**—**4**) paralleled their antimicrobial activity against *E. coli*.

Des-FA-[Dab-Dab-Dab^1]- and Des-FA-[Arg-Arg-Arg^1]-PMB (**10**, **11**) had markedly potent binding activity, similar to that of polymyxin B. Interestingly, the potent LPS binding

activity of **10** and **11** was not reflected in their bactericidal potency against *E. coli*, which was moderate, with MIC values of 8 and 16 nmol/ml, respectively (Table 3, Fig. 3B). It is evident from these experimental results that the N-terminal basic tripeptide portions of **10** and **11** contribute to binding to the LPS molecule, however, the mode of binding appears to be different from that of PMB, since the cationic characteristics of H-Dab-Dab-Dab^1- of **10** and H-Arg-Arg-Arg^1- of **11** are quite different from that of fatty acyl-Dab^1- of PMB. Therefore, the mechanism by which **10** and **11** bind to exclude the fluorescent probe bound to LPS is hypothesized to be as follows. First, the N-terminal tripeptide cationic portions of **10** and **11** bind to phosphate anions of lipid A and/or carboxylate anions of the adjacent 3-deoxy-D-*manno*-octulosonic acid (Kdo) portion in LPS molecule. Next, the ionic binding of **10** and **11** causes structural changes in LPS, resulting in the loss of hydrophobic binding between the fatty acyl group(s) of the lipid A portion of LPS and the octanoyl-Dab^1 portion of the probe, octanoyl-[Dab(N^{γ}-dansyl-Gly)^1]-PMB_3. The structural changes in the hydrophobic portions of *E. coli* LPS caused by this strong ionic binding do not participate in the destruction of the outer membrane of the living *E. coli* cell, since the bactericidal activity of **10** and **11** against *E. coli* is not improved. As discussed here, the present results demonstrate that the binding activity of **10** and **11** to *E. coli* LPS does not parallel their bactericidal activity against *E. coli*. On the other hand, Des-FA-[Ala-Ala-Ala^1]-PMB (**12**) shows very low binding activity, possibly because of the deficiency of both ionic and hydrophobic interaction with LPS.

The acute toxicity of Des-FA-[Dap^1]-PMB (**3**) and Des-FA-[Trp^1]-PMB (**6**) in comparison with PMB[15] and PMBN was examined by tail intravenous bolus injection in mice, which resulted in lethality within 24 h. The administration of PMB and its analogs induced respiratory arrest and death within 5 min after injection. This effect may be caused by neuromuscular block.[16,17] The LD_{50} values of **3** and **6** were 23.5 and 19.0 μmol/kg, respectively. The results demonstrate that the acute toxicity of **3** in mice was improved from that of native PMB (LD_{50}, 4.8 μmol/kg) (Table 4), *i.e.*, 4.90-fold less toxic than PMB, though still more toxic than PMBN (LD_{50}, 31.5 μmol/kg).

The present study of N-terminal analogs of polymyxin B lacking a fatty acyl moiety lead to the synthesis of Des-FA-[Dap^1]-PMB (**3**), Des-FA-[Ser^1]-PMB (**4**), Des-FA-[Dab-Dab-Dab^1]-PMB (**10**) and Des-FA-[Arg-Arg-Arg^1]-PMB (**11**). These compounds showed potent antimicrobial activity specifically against *P. aeruginosa* (MIC, 0.5—1 nmol/ml) comparable to PMB. The latter two peptides had the same LPS binding activity as PMB. Des-FA-[Dap^1]-PMB (**3**) showed 4.90-fold less acute toxicity than PMB. These results provide clues for developing a potent bactericidal analog specific for *P. aeruginosa* with low acute toxicity.

References

1) Storm D. R., Rosenthal K. S., Swanson P. E., *Annu. Rev. Biochem.*, **46**, 723—763 (1977).
2) Suzuki T., Hayashi K., Fujikawa K., Tsukamoto K., *J. Biochem.* (Tokyo), **56**, 335—343 (1964).
3) Chihara S., Tobita T., Yahata M., Ito A., Koyama Y., *Agric. Biol. Chem.*, **37**, 2455—2463 (1973).
4) Vaara M., Vaara, T., *Antimicrob. Agents Chemother.*, **24**, 107—113 (1983).
5) Ofek I., Cohen S., Rahmai R., Kabha K., Tamarkin D., Herzig Y., Rubinstein E., *Antimicrob. Agents Chemother.*, **38**, 374—377 (1994).
6) Danner R. L., Joiner K. A., Rubin M., Patterson W. H., Johnson N., Ayers K. M., Parrillo J. E., *Antimicrob. Agents Chemother.*, **33**, 1428—1434 (1989).
7) Sakura N., Itoh T., Uchida Y., Ohki K., Okimura K., Chiba K., Sato Y., Sawanishi H., *Bull. Chem. Soc. Jpn.*, **77**, 1915—1924 (2004).
8) Okimura K., Ohki K., Sato Y., Ohnishi K., Uchida Y., Sakura N., *Bull. Chem. Soc. Jpn.*, **80**, 543—552 (2007).
9) Okimura K., Ohki K., Sato Y., Ohnishi K., Sakura N., *Chem. Pharm. Bull.*, **55**, 1724—1730 (2007).
10) Wang S., *J. Am. Chem. Soc.*, **21**, 1328—1333 (1973).
11) Merrifield R. B., *Adv. Enzymol. Relat. Areas Mol. Biol.*, **32**, 221—296 (1969).
12) Shioiri T., Yamada S., *Chem. Pharm. Bull.*, **22**, 855—858 (1974).
13) Litchfield J. T., Wilcoxon F., *J. Pharmacol. Exp. Ther.*, **96**, 99—113 (1949).
14) Tsubery H., Ofek I., Cohen S., Fridkin M., *Peptides*, **22**, 1675—1681 (2001).
15) Lake P., DeLeo J., Cerasoli F., Logdberg L., Weetall M., Handley D., *Antimicrob. Agents Chemother.*, **48**, 2987—2992 (2004).
16) Viswanath D. V., Jenkins H. J., *J. Pharm. Sci.*, **67**, 1275—1280 (1978).
17) Singh Y. N., Marshall I. G., Harvey A. L., *Br. J. Anaesth.*, **54**, 1295—1306 (1982).