Contribution of Each Amino Acid Residue in Polymyxin B_3 to Antimicrobial and Lipopolysaccharide Binding Activity

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This study on the structure–activity relationship of polymyxin B, a cyclic peptide antibiotic, used sixteen synthetic polymyxin B_3 analogs including alanine scanning analogs to elucidate the contribution of the side chains to antimicrobial activity and lipopolysaccharide (LPS) binding. Of these analogs, [Ala^5]-polymyxin B_3 showed greatly reduced antimicrobial activity against *Escherichia coli* (*E. coli*), *Salmonella* Typhimurium (*S.* Typhimurium) and *Pseudomonas aeruginosa* (*P. aeruginosa*) with MIC values of 4—16 nmol/ml, suggesting that the Dab (α,γ-diaminobutyric acid) residue at position 5 is the most important residue contributing to bactericidal activity. The antibacterial contribution of Dab when located within the lactam ring (positions 5, 8 and 9) was greater than when located outside the ring (positions 1 and 3). [D-Ala^6]-, [L-Phe^6]-, [Ala^7]-, and [Gly^7]-polymyxin B_3 analogs retained potent antimicrobial activity, indicating that neither the reduction of hydrophobic character of the D-Phe^6-Leu^7 region nor the D-configuration at position 6 is indispensable for antimicrobial activity. LPS binding studies showed that decreased hydrophobicity of the lactam ring had little effect, but the N^γ-amino function of the Dab residues at position 1, 3, 5, 8 and 9 greatly affected LPS binding, with the contribution of Dab^5 being the most significant.

Key words polymyxin B; alanine scanning; antimicrobial activity; lipopolysaccharide binding activity; diaminobutyric acid

Polymyxin B is an *N*-terminally fatty acylated peptide antibiotic isolated from *Bacillus polymyxa*.[1,2] Polymyxin B contains six α,γ-diaminobutyric acid (Dab) residues. The γ-amino group of Dab^4 is acylated by *C*-terminal Thr^{10} to form a 23-member lactam ring[3,4] resulting in a peptide that has antimicrobial activity against Gram-negative bacteria. The mechanism of action of Polymyxin B is believed to be due to the amphiphilic character of the molecule, with the basic Dab side chains interacting with the negative charges of the lipid A portion of lipopolysaccharides (LPS) on the cell surface of bacteria, and the hydrophobic D-Phe-Leu in the lactam ring and the fatty acyl group at the *N*-terminus interacting with the lipophilic part of LPS.[5,6] These interactions lead to disordering of Gram-negative bacterial cell membranes, resulting in cell death.[7] Since the total solid phase synthesis of polymyxin B_1 was first reported by Sharma in 1999,[8] various polymyxin B analogs have been synthesized and evaluated for biological activity.[9—17] However, the structure–activity relationship of polymyxin B peptides is not understood in detail, due to the lack of extensive works employing highly pure peptides. We previously reported a study aimed at clarifying the contribution of the *N*-terminal fatty acyl groups of various polymyxin B family peptides to biological activity, as well as the development of *N*-terminal analogs without fatty acyl groups.[16,18,19] The aim of the present study was to clarify the structural requirements of the side chain of each amino acid residue by means of alanine scanning, and to further examine the role of the hydrophobic portion (D-Phe-Leu) of the polymyxin B_3 lactam ring in antimicrobial activity and LPS binding, employing sixteen synthetic peptides (Fig. 1).

Experimental

General HPLC was performed using two 510 pumps (Waters Corp., Milford, MA, U.S.A.), a U6K injector (Waters), an S310 model II UV detector (Soma Optics Ltd., Tokyo, Japan), 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). Amino acid analysis of peptide acid hydrolysates was conducted on a model 7300 amino acid analyzer (Beckman Instruments Ltd., Fullerton, CA, U.S.A.). The optical rotations of the peptides were measured with a DIP-370 digital polarimeter (Nippon Bunko Co., Ltd., Tokyo, Japan). Deprotection reaction of protected peptides 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, solvent used for peptide synthesis and Fmoc-amino acids were obtained from Watanabe Chem. Ind. Ltd., Hiroshima, Japan.

Synthesis of Peptides. 1) Solid Phase Synthesis of Protected Peptide-Resins ($\mathbf{1_R}$—$\mathbf{16_R}$) Polymyxin B_3 analogs (**1**—**16**, Fig. 1) were synthesized according to the route representatively shown for [Ala^3]-polymyxin B_3 (**3**, Fig. 2). The synthetic strategy was essentially as reported previously.[16] In brief, the protected peptide was constructed on 4-hydroxylmethylphenoxymethyl-resin (HMP-resin or Wang-resin, 0.74 mmol/g, Novabiochem-läufelfingen, Switzerland) by a solid phase methodology 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, Fmoc-Dab(Ac)-OH, Fmoc-Thr(Bzl)-OH, Fmoc-Phe-OH, Fmoc-D-Phe-OH, Fmoc-Leu-OH, Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Trp(Boc)-OH and Fmoc-D-Trp(Boc)-OH. Starting from Fmoc-Thr(Bzl)-O-HMP-resin (0.2 mmol, 196 mg), the Fmoc group was removed with 20% piperidine in *N*-methylpyrolidone (NMP), and the peptide chain was sequentially elongated with the appropriate Fmoc-amino acid (1.0 mmol), *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (1.0 mmol) and diisopropylethylamine (2.0 mmol) in NMP. Representatively, for the preparation of [Ala^3]-polymyxin B_3 (**3**), after the introduction of octanoic acid to the α-amino function of Dab(2-ClZ) at position 1, the protected peptide-resin was washed successively with three portions of dimethylformamide (DMF), dichloromethane, MeOH and ether, and dried *in vacuo* to give octanoyl-Dab(2-ClZ)-Thr(Bzl)-Ala^3-Dab(Boc)-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-O-HMP-resin ($\mathbf{3_R}$). Yield, 511 mg. For the preparation of the other analogs (**1**, **2** and **4**—**16**), various protected peptide-resins ($\mathbf{1_R}$, $\mathbf{2_R}$ and $\mathbf{4_R}$—$\mathbf{16_R}$) were constructed in the same manner as described for $\mathbf{3_R}$.

2) Preparation of Linear Partially Protected [Ala^3]-Polymyxin B_3 ($\mathbf{3_L}$) and Various Linear Partially Protected Polymyxin B_3 Analogs ($\mathbf{1_L}$, $\mathbf{2_L}$ and $\mathbf{4_L}$—$\mathbf{16_L}$) Protected peptide-resin ($\mathbf{3_R}$, 0.2 mmol eq) was treated

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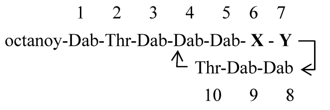
A) Alanine-scanning analogs (**1-9**)

		1 2 3 4 5 6 7 8 9 10
1.	[Ala^{1}]-polymyxin B$_3$	octanoyl-**Ala**- Thr-Dab-cyclic[Dab*-Dab-D-Phe-Leu-Dab- Dab-Thr*]
2.	[Ala^{2}]-polymyxin B$_3$	octanoyl-Dab-**Ala**-Dab-cyclic[Dab*-Dab-D-Phe-Leu-Dab- Dab-Thr*]
3.	[Ala^{3}]-polymyxin B$_3$	octanoyl-Dab-Thr-**Ala**-cyclic[Dab*-Dab-D-Phe-Leu-Dab- Dab-Thr*]
4.	[Ala^{5}]-polymyxin B$_3$	octanoyl-Dab-Thr-Dab-cyclic[Dab*-**Ala**-D-Phe-Leu-Dab- Dab-Thr*]
5.	[D-Ala^{6}]-polymyxin B$_3$	octanoyl-Dab-Thr- Dab-cyclic[Dab*-Dab-**D-Ala**-Leu-Dab- Dab-Thr*]
6.	[Ala^{7}]-polymyxin B$_3$	octanoyl-Dab-Thr- Dab-cyclic[Dab*-Dab-D-Phe-**Ala**-Dab- Dab-Thr*]
7.	[Dab(Ac)^{8}]- polymyxin B$_3$	octanoyl-Dab-Thr- Dab-cyclic[Dab*-Dab-D-Phe-Leu-**Dab(Ac)**-Dab-Thr*]
8.	[Ala^{9}]- polymyxin B$_3$	octanoyl-Dab-Thr- Dab-cyclic[Dab*-Dab-D-Phe-Leu-Dab- **Ala**-Thr*]
9.	[Ala^{10}]- polymyxin B$_3$	octanoyl-Dab-Thr- Dab-cyclic[Dab*-Dab-D-Phe-Leu-Dab- Dab-**Ala***]

(*, *) lactam ring between γ-amino function of Dab^{4} and carboxylic function of Thr^{10}

B) Analogs substituted for D-Phe^{6} and / or Leu^{7} (**10-16**)



Polymyxin B$_3$ (X, D-Phe; Y, Leu)

		X	Y
10.	[D-Trp^{6}]-polymyxin B$_3$	**D-Trp**	Leu
11.	[L-Trp^{7}]-polymyxin B$_3$	D-Phe	**Trp**
12.	[Gly^{6}]- polymyxin B$_3$	**Gly**	Leu
13.	[Gly^{7}]-polymyxin B$_3$	D-Phe	**Gly**
14.	[D-Ala^{6}, Ala^{7}]-polymyxin B$_3$	**D-Ala**	**Ala**
15.	[Gly^{6}, Gly^{7}]-polymyxin B$_3$	**Gly**	**Gly**
16.	[L-Phe^{6}]-polymyxin B$_3$	**L-Phe**	Leu

Fig. 1. Synthetic Polymyxin B$_3$ Analogs Used in This Study

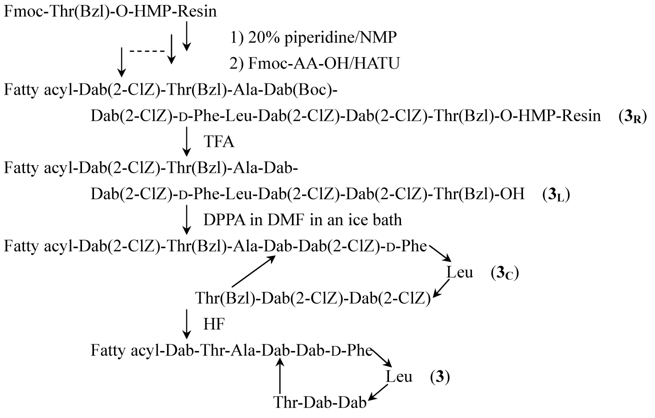


Fig. 2. Synthetic Route of [Ala^{3}]-Polymyxin B$_3$ (**3**)

with trifluoroacetic acid (TFA)–H_2O (95 : 5, 5 ml) for 1.5 h at room temperature, cleaving the peptide from the HMP-resin and the Boc protecting group from Dab^{4} and yielding octanoyl-Dab(2-ClZ)-Thr(Bzl)-Ala^{3}-Dab^{4}-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-OH ($\mathbf{3}_{\mathbf{L}}$). The excess TFA was evaporated *in vacuo* and the residue was lyophilized from dioxane. The products were dissolved in dimethylsulfoxide (DMSO) (2 ml) and fractionated on a column (1.6×95 cm) of Toyopearl HW-40-S using DMF : H_2O (9 : 1) as eluent. Fractions containing the main product ($\mathbf{3}_{\mathbf{L}}$) were combined, evaporated, and the partially purified product (the purity: 82% by HPLC analysis measured at 210 nm) was lyophilized from dioxane. Yield, 170 mg (41.8% calculated from Fmoc-Thr(Bzl)-O-HMP-resin). FAB-MS; Found (for the most abundant isotopic variant of $\mathbf{3}_{\mathbf{L}}$; formula $C_{100}H_{127}N_{15}O_{22}Cl_4$); 2032.8 $[M+H]^+$, 2054.7 $[M+Na]^+$.

The other linear partially protected peptides ($\mathbf{1}_{\mathbf{L}}$, $\mathbf{2}_{\mathbf{L}}$ and $\mathbf{4}_{\mathbf{L}}$—$\mathbf{16}_{\mathbf{L}}$) were obtained in the same manner as described for $\mathbf{3}_{\mathbf{L}}$ and their FAB-MS data (not shown) confirmed the structure of the main products. The partially purified peptides ($\mathbf{1}_{\mathbf{L}}$, $\mathbf{2}_{\mathbf{L}}$ and $\mathbf{4}_{\mathbf{L}}$—$\mathbf{16}_{\mathbf{L}}$) were used without further purification for the cyclization reaction.

3) Preparation of Cyclic Protected [Ala^{3}]-Polymyxin B$_3$ ($\mathbf{3}_{\mathbf{C}}$) and Various Cyclic Protected Polymyxin B$_3$ Analogs ($\mathbf{1}_{\mathbf{C}}$, $\mathbf{2}_{\mathbf{C}}$ and $\mathbf{4}_{\mathbf{C}}$—$\mathbf{16}_{\mathbf{C}}$) Linear partially protected [Ala^{3}]-polymyxin B$_3$ ($\mathbf{3}_{\mathbf{L}}$, 142 mg, 0.07 mmol) was dissolved in ice-cold DMSO (1 ml)-DMF (2 ml), then diphenyl phosphorazidate (DPPA)[20] (66 μl, 0.35 mmol) and 4-methylmorpholine (72 μl, 0.70 mmol) were added. The mixture was allowed to react overnight at 4 °C to form the lactam ring. The reaction mixture containing the cyclization product N^{α}-octanoyl-Dab(2-ClZ)-Thr(Bzl)-Ala^{3}-cyclic[Dab^{4}*-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)^{10}*] (*–*: amide bond between α-COOH of Thr^{10} and γ-NH_2 of Dab^{4}) ($\mathbf{3}_{\mathbf{C}}$) was fractionated on a Toyopearl HW-40-S column (1.6×95 cm) as described above for $\mathbf{3}_{\mathbf{L}}$. Fractions containing the main product were combined, evaporated, and the partially purified product $\mathbf{3}_{\mathbf{C}}$ (the purity: 86% by HPLC analysis measured at 210 nm) was lyophilized from dioxane. Yield, 133 mg (94%). FAB-MS; Found (for the most abundant isotopic variant of $\mathbf{3}_{\mathbf{C}}$; formula $C_{100}H_{125}N_{15}O_{21}Cl_4$); 2014.8 $[M+H]^+$, 2036.8 $[M+Na]^+$. The other cyclic protected peptides ($\mathbf{1}_{\mathbf{C}}$, $\mathbf{2}_{\mathbf{C}}$, $\mathbf{4}_{\mathbf{C}}$—$\mathbf{16}_{\mathbf{C}}$) were obtained almost quantitatively in the same manner as described for $\mathbf{3}_{\mathbf{C}}$ and their FAB-MS data (not shown) confirmed the structure of the main products. The partially purified peptides ($\mathbf{1}_{\mathbf{C}}$, $\mathbf{2}_{\mathbf{C}}$, $\mathbf{4}_{\mathbf{C}}$—$\mathbf{16}_{\mathbf{C}}$) were used without further purification for the deprotection reaction with HF.

4) Preparation of [Ala^{3}]-Polymyxin B$_3$ (3) and Various Polymyxin B$_3$ Analogs (1, 2, 4—16) Cyclic protected [Ala^{3}]-polymyxin B$_3$ ($\mathbf{3}_{\mathbf{C}}$, 101 mg, 0.05 mmol) was treated with ice-cold anhydrous HF (2 ml)–anisole (0.2 ml) for 1 h, then excess HF was removed *in vacuo*. The residue was dissolved in H_2O (15 ml), washed with three portions of ether and lyophilized. The crude [Ala^{3}]-polymyxin B$_3$ (**3**) was purified by HPLC on a CAPCELL PAK C_{18} UG-80 5 μ (2×15 cm) column using acetonitrile–0.1% TFA as eluent. The main peak was collected and the combined eluents were 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 as eluent. Purified product **3** was obtained by lyophilization as a hydrochloride salt; yield 29 mg (44.4%). The other polymyxin B$_3$ analogs, **1**, **2** and **4**—**16**, were prepared in the same manner except for the L-Trp or D-Trp-containing peptides (**14**, **15**), which were obtained by treating $\mathbf{14}_{\mathbf{C}}$ and $\mathbf{15}_{\mathbf{C}}$ with HF (2 ml) containing anisole (0.2 ml) and ethanedithiol (0.2 ml). For amino acid analysis of synthetic peptides, acid hydrolysis was carried out with vapor of 6 mol/l HCl containing 3% phenol at 130 °C for 3 h. The peak of Dab on the analysis was determined as Lys. Data of amino acid analysis are shown in Table 1. Characterization and analytical data of synthetic peptides **1**—**16** are shown in Table 2.

Bacteria and Susceptibility Test *Escherichia coli* (*E. coli*) IFO 12734, *Salmonella* Typhimurium (*S.* Typhimurium) IFO 12529 and *Pseudomonas aeruginosa* (*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).

Antibacterial activity of the synthetic peptides was evaluated by comparison with commercially available polymyxin B (Sigma Chemical Co., St. Louis, MO, U.S.A.). Minimum inhibitory concentrations (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 serially diluted peptide in distilled water (0.25—512 nmol/ml) was added to a mixture of 10 μl bacterial suspension (approximately 10^6 CFU/ml) and 90 μl 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 then 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 to LPS Following a method described previously,[16] a solution of [Dab(N^{γ}-dansyl-Gly)^1]-polymyxin B_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., St. Louis, MO, U.S.A.) in H_2O (3 mg/ml) (10 μl, 30 mg). The solution was kept at 30 °C for 60 min until the fluorescence intensity of [Dab(N^{γ}-dansyl-Gly)^1]-polymyxin B_3 plateaued. 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 with a fluorescence spectrophotometer (F-850, Hitachi Instrument Co., Tokyo, Japan) using an excitation wavelength of 330 nm and an emission wavelength of 490 nm. The initial intensity of fluorescence was taken as 100%. The percent fluorescence intensity was plotted as a function of peptide concentration. The binding experiments were repeated at least three times for each peptide to test the reproducibility of the results. The concentrations required for 50% quenching of [Dab(N^{γ}-dansyl-Gly)^1]-polymyxin B_3 bound to LPS (IC_{50}) were derived from the quenching curves of the synthetic peptides (Figs. 4, 5, Table 3).

Table 1. Amino Acid Analysis of Synthetic Peptides

Peptide	Thr	Dab	Leu	Phe	Ala	Gly
1	1.99 (2)	4.83 (5)	1.00 (1)	0.99 (1)	1.19 (1)	—
2	0.99 (1)	5.73 (6)	1.06 (1)	1.04 (1)	1.19 (1)	—
3	1.98 (2)	4.65 (5)	1.05 (1)	1.05 (1)	1.28 (1)	—
4	2.04 (2)	4.77 (5)	1.01 (1)	1.01 (1)	1.18 (1)	—
5	2.04 (2)	5.75 (6)	1.01 (1)	—	1.20 (1)	—
6	2.02 (2)	5.73 (6)	—	1.04 (1)	1.21 (1)	—
7	1.98 (2)	5.93 (6)	1.03 (1)	1.05 (1)	—	—
8	2.00 (2)	4.68 (5)	0.99 (1)	1.13 (1)	1.20 (1)	—
9	1.01 (1)	5.77 (6)	0.99 (1)	0.98 (1)	1.23 (1)	—
10	2.05 (2)	6.02 (6)	0.94 (1)	—	—	—
11	1.71 (2)	6.20 (6)	1.08 (1)	—	—	—
12	1.77 (2)	6.03 (6)	1.28 (1)	—	—	0.91 (1)
13	1.69 (2)	6.18 (6)	—	1.11 (1)	—	1.01 (1)
14	1.77 (2)	6.27 (6)	—	—	1.96 (2)	—
15	1.96 (2)	6.07 (6)	—	—	—	1.97 (2)
16	1.93 (2)	5.88 (6)	1.08 (1)	1.08 (1)	—	—

Hydrolysis was carried out with vapor of 6 mol/l HCl containing 3% phenol at 130 °C for 3 h. Number in parentheses are theoretical values.

Results and Discussion

Synthesis The synthesis of polymyxin B_3 analogs (**1**—**16**) shown in Fig. 1 was carried out as reported previously.[16] The peptide chain was constructed by solid-phase synthesis using Fmoc-amino acids bearing benzyl-type protecting groups on the side chain functional groups, except for the Dab at position 4, whose γ-amino function was protected by Boc. After the cleavage of the solid support and the Boc protecting group on Dab^4 with TFA, the linear partially protected peptides ($\mathbf{1_L}$—$\mathbf{16_L}$) were cyclized between the α-COOH of $Thr(Bzl)^{10}$ and the γ-NH_2 of Dab^4 using DPPA[20] at 4 °C in minimal amounts of DMSO–DMF (1 : 1). The cyclization reaction proceeded quantitatively in a high concentration (*ca.* 0.01 mmol/ml) solution of $\mathbf{1_L}$—$\mathbf{16_L}$ to yield $\mathbf{1_C}$—$\mathbf{16_C}$, which were treated with HF to yield polymyxin B

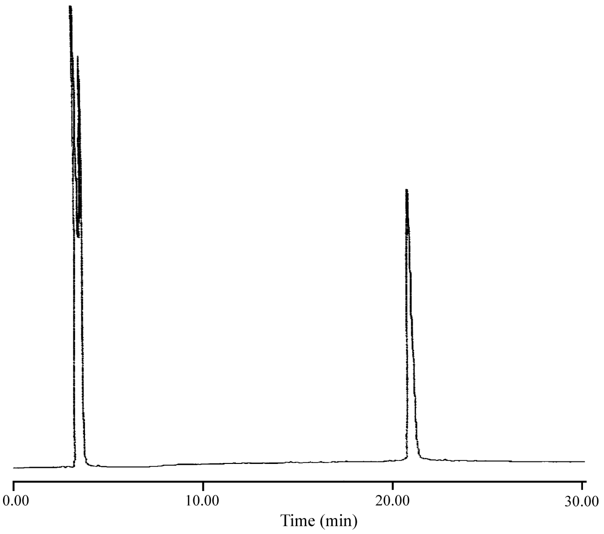
Table 3. Antimicrobial Activity and LPS-Binding Activity of Synthetic Peptides **1**—**16**

	MIC (nmol/ml)			IC_{50} (nmol/ml)
	Escherichia coli	*Salmonella* Typhimurium	*Pseudomonas aeruginosa*	
Polymyxin B (Sigma)	1	0.5	1	
1	2	2	2	20
2	8	4	2	12
3	1	1	2	19
4	16	16	4	—
5	1	1	1	6
6	1	1	1	5
7	2	4	2	26
8	4	4	2	19
9	2	1	1	5
10	2	2	2	6
11	2	2	2	5
12	2	16	1	11
13	1	2	2	11
14	2	32	4	8
15	256	>512	>512	20
16	2	2	2	10

Table 2. Characterization of Synthetic Peptides **1**—**16**

	$[\alpha]_D^{20}$ (c=0.5, 12% AcOH)	FAB-MS			HPLC[a)]	HP-TLC[b)]	
		Formula	$[M+H]^+$	$[M+Na]^+$	t_R/min	Rf^1	Rf^2
1	−75.2°	$C_{54}H_{93}N_{15}O_{13}$	1160	1182	23.1	0.47	0.45
2	−64.4°	$C_{54}H_{94}N_{16}O_{12}$	1159	1181	19.1	0.45	0.38
3	−78.8°	$C_{54}H_{93}N_{15}O_{13}$	1160	1182	21.7	0.49	0.47
4	−65.2°	$C_{54}H_{93}N_{15}O_{13}$	1160	1182	22.4	0.49	0.47
5	−65.6°	$C_{49}H_{92}N_{16}O_{13}$	1113	1135	14.9	0.21	0.30
6	−62.0°	$C_{52}H_{90}N_{16}O_{13}$	1147	1169	16.0	0.21	0.30
7	−63.4°	$C_{57}H_{98}N_{16}O_{14}$	1231	1253	21.8	0.51	0.46
8	−66.0°	$C_{54}H_{93}N_{15}O_{13}$	1160	1182	23.5	0.49	0.47
9	−70.0°	$C_{54}H_{94}N_{16}O_{12}$	1159	1181	20.8	0.42	0.43
10	−57.7°	$C_{57}H_{97}N_{17}O_{13}$	1228	1250	20.6	0.47	0.43
11	−50.7°	$C_{60}H_{95}N_{17}O_{13}$	1262	1284	21.7	0.47	0.43
12	−45.1°	$C_{48}H_{90}N_{16}O_{13}$	1099	1121	15.2	0.16	0.32
13	−43.6°	$C_{51}H_{88}N_{16}O_{13}$	1133	1155	15.1	0.17	0.37
14	−52.0°	$C_{46}H_{86}N_{16}O_{13}$	1071	1093	12.5	0.07	0.24
15	−40.7°	$C_{44}H_{82}N_{16}O_{13}$	1043	1065	11.5	0.07	0.19
16	−54.0°	$C_{55}H_{96}N_{16}O_{13}$	1189	1211	17.2	0.06	0.44

a) HPLC conditions: column; YMC-Pack pro C18 (4.6×250 mm), elution; linear gradient elution from 16 to 32% MeCN in 0.1% TFA (20 min), flow rate; 1 ml/min, detection; 210 nm. *b*) TLC solvent systems: Rf^1; BuOH : Pyridine : AcOH : H_2O (30 : 20 : 6 : 24). Rf^2; BuOH : AcOH : AcOEt : H_2O (1 : 1 : 1 : 1).



HPLC conditions: column; YMC-Pack Pro C18 (4.6×250mm), elution; linear gradient elution from 16 to 32% MeCN in 0.1% TFA (20min), flow rate; 1 ml/min, detection; 210nm

Fig. 3. Analytical HPLC of [Ala^3]-Polymyxin B_3 (**3**)

analogs (**1**—**16**). The purity of the synthetic peptides was >97% as shown representatively in Fig. 3 for analog **3**. The results demonstrated the usefulness of the synthetic protocols described herein. However, during the present Ala-scanning study, it was noted that no cyclization product was obtained from octanoyl-Dab(2-ClZ)^1-Thr(Bzl)-Dab(2-ClZ)-Dab^4-Dab(2-ClZ)-D-Phe-Leu-Ala^8-Dab(2-ClZ)-Thr(Bzl)^{10}-OH ($7'_L$), which was prepared as an intermediate for the synthesis of [Ala^8]-polymyxin B_3 ($7'$). No considerable decease of the starting material ($7'_L$) was proved by HPLC analysis of the reaction mixture in the presence of a large excess of DPPA for 48 h. When HATU was used as a cyclization reagent, the decrease of $7'_L$ peak was observed without the appearance of desired product peak (data not shown), suggesting that polymer formation occurred under the reaction condition. Then an N^γ-acetylated peptide at Dab^8 residue (**7**) was designed to evaluate the biological role of the side chain. When Dab(Ac) was introduced at position 8, the intra-molecular reaction of $\mathbf{7}_L$ proceeded smoothly to give a lactam $\mathbf{7}_C$. It can therefore be assumed that Ala^8-analog ($7'_L$) has an exceptionally special conformation under the reaction condition, by which the lactam formation is completely disturbed.

Antimicrobial Activity The antimicrobial activity of the synthetic peptides (**1**—**16**) was estimated by the standard micro plate dilution method. The minimum inhibitory concentration (MIC) values of peptides toward three Gram-negative bacteria, *E. coli*, *S.* Typhimurium, and *P. aeruginosa*, are summarized in Table 3. Polymyxin B (Sigma Co.) was used as a standard for antimicrobial potency. [Ala^1]-polymyxin B_3 (**1**) had slightly reduced antimicrobial potency against the three Gram-negative bacteria, with a MIC value of 2 nmol/ml, which corresponds to 1/2—1/4 the activity of native polymyxin B. [Ala^3]-polymyxin B_3 (**3**) had the same or half the antimicrobial activity as polymyxin B, suggesting that the side chain amino function of Dab^3 is not indispensable for activity. The antimicrobial activity of [Ala^2]-polymyxin B_3 (**2**) was 1/2—1/8 that of polymyxin B, demonstrating that the Thr^2 residue is more important than the basic amino acids (Dab^1 and Dab^3) in the *N*-terminal region located outside the 23-member lactam ring. On the other hand, three Dab residues in the lactam ring were found to play an important bactericidal role since the antimicrobial activities of [Ala^5]-, [Dab(Ac)^8]-, and [Ala^9]-polymyxin B_3 (**4**, **7**, **8**) were less than those of **1** and **3**. Substitution of Ala for Dab at position 5 markedly reduced the activity, to 1/16 (*E. coli*), 1/32 (*S.* Typhimurium) and 1/4 (*P. aeruginosa*) that of native polymyxin B. It should be emphasized that the side chain of Dab^5 was the most important of the five Dab amino functions in polymyxin B_3 for biological activity.

[D-Ala^6]- and [Ala^7]-polymyxin B_3 (**5**, **6**) retained the same or half the antimicrobial activity of polymyxin B toward three bacteria tested, showing that the reduction in hydrophobicity by replacing either D-Phe^6 or Leu^7 with D-Ala or Ala had little effect on reducing antimicrobial activity. Further studies revealed that even [Gly^6]- and [Gly^7]-polymyxin B_3 (**12**, **13**) retained the same or half the activity of polymyxin B against *E. coli* and *P. aeruginosa*. Thus the hydrophobic side chains in the lactam ring (D-Phe^6-Leu^7) do not appear to be essential for the antimicrobial activity of polymyxin B_3 against these two bacteria. However, *S.* Typhimurium showed low susceptibility to the Gly^6-analog (**12**), which had only 1/32 the antimicrobial activity of polymyxin B. [D-Ala^6, Ala^7]-polymyxin B_3 (**14**) showed greatly reduced activity, especially toward *S.* Typhimurium (1/64). The low susceptibility of *S.* Typhimurium to polymyxin B_3 analogs with reduced hydrophobicity at positions 6 and 7 suggests that the cell membrane of this bacterium may have different characteristics from that of *E. coli* and *P. aeruginosa*. [Gly^6, Gly^7]-polymyxin B_3 (**15**) was inactive against three bacterial species tested. The complete loss of antimicrobial activity of **15** might be explained by the complete loss of hydrophobicity simultaneously at positions 6 and 7, or by the drastic change of the whole peptide conformation, caused possibly by substitution of Gly-Gly for D-Phe-Leu. Conversely, an increase in hydrophobicity in terms of the Nozaki–Tanford hydrophobicity scale[21] at position 6 or 7 did not increase bactericidal activity, since [D-Trp^6]-polymyxin B_3 (**10**) and [Trp^7]-polymyxin B_3 (**11**) showed moderately reduced (1/2—1/4) activity, with MIC values of 2 nmol/ml, against the three bacterial species tested. A similar result was reported regarding polymyxin B nonapeptide analogs: [D-Trp^6]-polymyxin B (2—10) had moderately reduced activity and [Phe^7]-polymyxin B (2—10) had the same activity in an outer membrane permeabilizing assay.[14]

It is known that in the polymyxin B family of peptides, which includes colistin, D-amino acids are commonly located at position 6. However, both [L-Phe^6]-polymyxin B_3 (**16**) and [Gly^6]-polymyxin B_3 (**12**) showed considerable potency against *E. coli* and *P. aeruginosa* with MIC values of 1—2 nmol/ml. Therefore, the D-configuration at position 6 is not a prerequisite for the antimicrobial activity of polymyxin B_3.

LPS Binding Activity The LPS binding activity of synthetic polymyxin B_3 analogs was evaluated as reported previously.[16] All Ala-substituted analogs for Dab residues at positions 1, 3, 5, 8 and 9 showed greatly reduced binding activity to LPS, indicating the important contribution of the Dab basic side chain to binding. On the contrary, Ala-substituted analogs for the residues at positions 2, 6, 7 and 10 had the same binding activity as polymyxin B_3, showing a less stringent requirement for neutral side chains at these positions

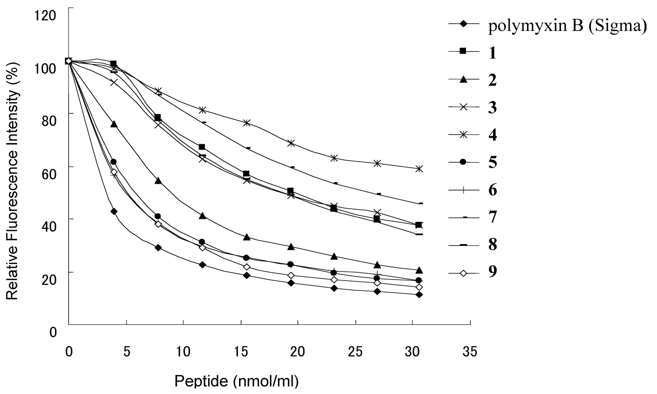


Fig. 4. Displacement of [Dab(dansyl-Gly)^1]-Polymyxin B_3 Bound to LPS by Polymyxin B_3 Analogs **1**—**9**

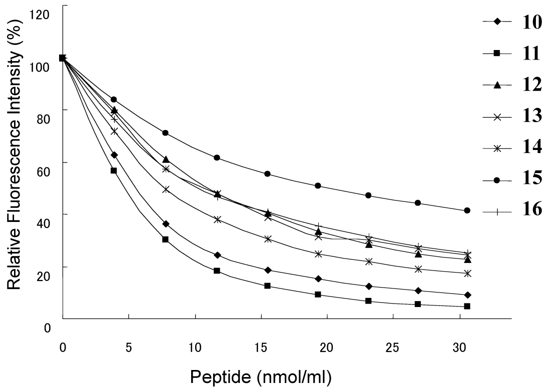


Fig. 5. Displacement of [Dab(dansyl-Gly)^1]-Polymyxin B_3 Bound to LPS by Polymyxin B_3 Analogs **10**—**16**

(Figs. 4, 5). The positive charges of the five Dab side chains in this peptide antibiotic are believed to interact with the negative charges of the phosphate groups of LPS.[5,6] The present results demonstrate that the γ-amino function of Dab^5 makes the greatest contribution to binding to LPS by electrostatic interactions. The primary role of the amino function at position 5 was so great that the IC_{50} value of [Ala^5]-polymyxin B_3 (**4**) could not be assessed under the experimental conditions of this study. The low LPS-binding activity of **4** resulted in very low antimicrobial activity, as described above. The contribution of the γ-amino function of Dab^8 was the second most important contribution to binding, with an IC_{50} value of 26 nmol/ml. The IC_{50} values of the other Ala-substitution analogs for Dab residues at position 1, 3 and 9 were 19—20 nmol/ml.

The role of the Thr residue at position 2 appears to be more important than the Thr at position 10 for LPS binding, since the displacement curve of [Ala^2]-polymyxin B_3 (**2**) shifted to the right of that of [Ala^{10}]-polymyxin B_3 (**9**). Substitution of Ala either for Phe or Leu at positions 6 and 7 did not affect binding to LPS, indicating that the slight decrease in hydrophobicity in the lactam ring was tolerated. However, the substantial loss in hydrophobicity at positions 6 and 7 resulted in a considerable decrease in binding, as seen in the displacement curve of [Gly^6,Gly^7]-polymyxin B_3 (**15**) (Fig. 5). Inversely, the increase in hydrophobicity by substitution of D-Trp or Trp for D-Phe^6 or Leu^7 resulted in the same LPS-binding activity as polymyxin B. Replacement of D-Phe^6 with the L-form lowered binding. This result is in contrast to the high antimicrobial activity of [L-Phe^6]-polymyxin B_3 (**16**) towards microorganisms.

In conclusion it was found that the Dab residues located in the lactam ring of polymyxin B_3 are indispensable for antimicrobial activity, with Dab^5 being the most important residue. The Dab residues at positions 1 and 3 are not essential for bactericidal activity. Neither the hydrophobic character of the D-Phe^6-Leu^7 region nor the D-configuration at position 6 is indispensable for antimicrobial activity. For LPS binding activity, all the Dab residues, especially Dab^5, play an important role.

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