

The Contribution of the *N*-Terminal Structure of Polymyxin B Peptides to Antimicrobial and Lipopolysaccharide Binding Activity

Naoki Sakura,* Tatsuya Itoh, Yoshiki Uchida,[1] Kazuhiro Ohki, Keiko Okimura, Kenzo Chiba, Yuki Sato, and Hiroyuki Sawanishi

Faculty of Pharmaceutical Sciences, Hokuriku University, Kanagawa-machi, Kanazawa 920-1181

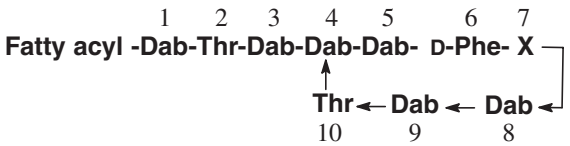
[1]Department of Food Science and Nutrition, Osaka Shoin Women's University, Hishiyanishi, Higashi-Osaka 577-8550

Received April 22, 2004; E-mail: n-sakura@hokuriku-u.ac.jp

To elucidate the *N*-terminal structure–activity relationships of polymyxin B peptides, seven polymyxin B component peptides, the structures of which having been elucidated, and seven *N*-terminal fatty acid and/or amino acid deletion analogs were synthesized, and their antimicrobial activities determined. The lipopolysaccharide (LPS) binding activities of synthetic peptides were evaluated using [Dab(Dansyl-Gly)[1]]-polymyxin B_3 (Dab; L-α,γ-diaminobutyric acid) as a fluorescent probe. The results indicated that the fatty acyl moiety was not indispensable for LPS binding, but the C_9 fatty acyl groups of polymyxin B peptides contributed to the binding affinity to a slightly greater extent than C_8 or C_7. The fatty acyl moieties of polymyxin B contributed greatly to the antimicrobial activity, while the distinct *N*-terminal structures of polymyxin B_1–B_6, bearing *normal*-, *iso*-, or *anteiso*-fatty acids, or 3-hydroxy-fatty acid with chain lengths between C_7 and C_9, did not affect bactericidal potency.

Polymyxin B family peptides, isolated from *Bacillus polymyxa*,[1,2] have potent antimicrobial activity against Gram-negative bacteria and a high binding ability to the lipid A portion of lipopolysaccharides (LPS), which is the active principle of endotoxins. One of the characteristics of these peptide antibiotics is that they contain six L-α,γ-diaminobutyric acid (Dab) units, among which the γ-amino group of the Dab at position 4 (Dab^4) is acylated by a *C*-terminal Thr^{10} to form a 23-member lactam ring.[3,4] Commercially available polymyxin B is a mixture composed of numerous closely related peptides, the *N*-terminals of which are acylated with various fatty acids (Fig. 1). Polymyxin B_1 is the main component in peptide mixtures bearing (6*S*)-6-methyloctanoic acid as the fatty acyl moiety. The minor components, i.e., polymyxin B_2, B_3, and B_4, have been shown to have 6-methylheptanoyl, octanoyl, and heptanoyl fatty acyl groups, respectively.[3,5] [Ile^7]-polymyxin B_1 might be included in this peptide family, because a minor component was reported[6] to contain L-Ile instead of L-Leu, and Leu at position 7 of polymyxin B_1 was deduced to be replaced by Ile, as determined structurally in the case of colistin peptides.[7–9] Polymyxin B_5 and B_6 were recently reported to have the same structure as polymyxin B_1, except that the fatty acyl moieties were nonanoyl and 3-hydroxy-6-methyloctanoyl, respectively, though the configuration of 3-hydroxy-6-methyloctanoic acid has not been determined.[10]

The chemical syntheses of polymyxin B peptides by classical methods[11,12] and the application of solid-phase syntheses[13–15] have been performed to confirm the structure or clarify



Polymyxin B (PMB) peptides		Fatty acyl	X
Polymyxin B_1	Ia	(6*S*)-6-methyloctanoyl	Leu
Polymyxin B_2	Ib	6-methylheptanoyl	Leu
Polymyxin B_3	Ic	octanoyl	Leu
Polymyxin B_4	Id	heptanoyl	Leu
Polymyxin B_5	Ie	nonanoyl	Leu
Polymyxin B_6	If	(3*RS*,6*S*)-3-hydroxy-6-methyloctanoyl	Leu
[Ile^7]-Polymyxin B_1	Ig	(6*S*)-6-methyloctanoyl	Ile

Fig. 1. Structures of polymyxin B peptides.

Published on the web October 9, 2004; DOI 10.1246/bcsj.77.1915

and alter the biological activities of polymyxin B. The present investigation was undertaken to determine the antimicrobial activities of each polymyxin B component peptide (Fig. 1) and the *N*-terminally deleted-analogs (Fig. 4) employing synthetic peptides. We report here the contributions of the *N*-terminal structure of polymyxin B to the LPS binding activity by LPS binding assay using mono-dansylated polymyxin B_3 as a fluorescent probe.

Results and Discussion

Synthesis of Dab Derivatives and Fatty Acids. Fmoc-Dab(2-ClZ)-OH (N^{α}-(9-flluorenylmethyloxycarbonyl)-N^{γ}-(2-chlorobenzyloxycarbonyl)-L-α,γ-diaminobutyric acid) and Fmoc-Dab(Boc)-OH were prepared from Fmoc-Dab-OH, which was obtained by a Hofmann rearrangement[16] of Fmoc-Gln-OH. Among the *N*-terminal fatty acyl moieties, *S*-(+)-6-methyloctanoic acid for polymyxin B_1 and 6-methylheptanoic acid for polymyxin B_2, were synthesized starting with *S*-(−)-2-methylbutanol and 4-methylpentanol, respectively, according to the methods described previously.[17,18] As the configuration of 3-hydroxy-6-methyloctanoic acid for polymyxin B_6 was not determined, (3*RS*, 6*S*)-3-hydroxy-6-methyloctanoic acid was synthesized as shown in Fig. 2 and used in this study.

Peptide Synthesis. The synthesis of polymyxin B component peptides was carried out as shown in Fig. 3. Starting from a hydroxymethylphenoxymethyl resin (HMP-resin) anchored with Fmoc-Thr(Bzl), the peptide chain was elongated by solid-phase synthesis using Fmoc-amino acids bearing benzyl-type protecting groups on the side chain functional groups, except Dab at position 4, which was protected by Boc. After introduction of all of the amino acids and the *N*-terminal fatty acid, cleavage of the HMP-resin support was achieved by TFA (trifluoroacetic acid), and the Boc protecting group of Dab^4 was removed simultaneously to give a linear partially protected peptide (**III**).

The cyclization reaction of **III** was carried out using DPPA (diphenyl phosphorazidate) reagent[19] between α-COOH of $Thr(Bzl)^{10}$ and γ-NH_2 of Dab^4 at 4 °C in minimal amounts of DMSO–DMF to dissolve the starting materials. Interestingly, in the lactam formation reaction, the intramolecular reaction mediated by DPPA proceeded quantitatively in the presence of a high concentration of a linear partially protected peptide (**III**). Although the reason for this cannot be explained, no polymer formation was observed at peptide concentrations as high as 0.01 mmol/mL. Neither polymer production nor β-elimination[20,21] was observed as reported previously.[14] Gel filtration on a Toyopearl HW-40 column was carried out for purification of protected peptides (**III** and **IV**) using DMF–H_2O (9:1).

For the LPS binding assay, a mono-dansylated polymyxin B_3 analog bearing a dansyl fluorescent probe on the side chain of Dab at position 1 through Gly as a spacer was designed. According to the synthetic route shown in Fig. 3, Fmoc-Dab-(ivDde)-OH (ivDde; 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl) was introduced at position 1 of the protected peptide resin, and then octanoyl was substituted for the N^{α}-Fmoc to yield Octanoyl-Dab(ivDde)-Thr(Bzl)-Dab-(2-ClZ)-Dab(Boc)-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-O-HMP-resin (**IIh**). The ivDde protecting group on N^{γ} was then removed selectively by hydrazine treatment,[22] and Fmoc-Gly-OH was coupled, followed by dansyl chloride to give Octanoyl-Dab(Dansyl-Gly)-Thr(Bzl)-Dab-(2-ClZ)-Dab(Boc)-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-O-HMP-resin (**IIh′**). TFA treatment of **IIh′** followed by a cyclization reaction with DPPA yielded Octanoyl-Dab(Dansyl-Gly)-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}) (**IVh**).

The final removal of the benzyl-type protecting groups of the protected cyclic polymyxin B peptide (**IV**) was performed by HF treatment in the presence of anisole. The product was purified by RP-HPLC and highly purified polymyxin B peptide (**I**) was obtained as a hydrochloride form after gel filtration using a 5 mmol/L HCl solution containing 25% acetonitrile,

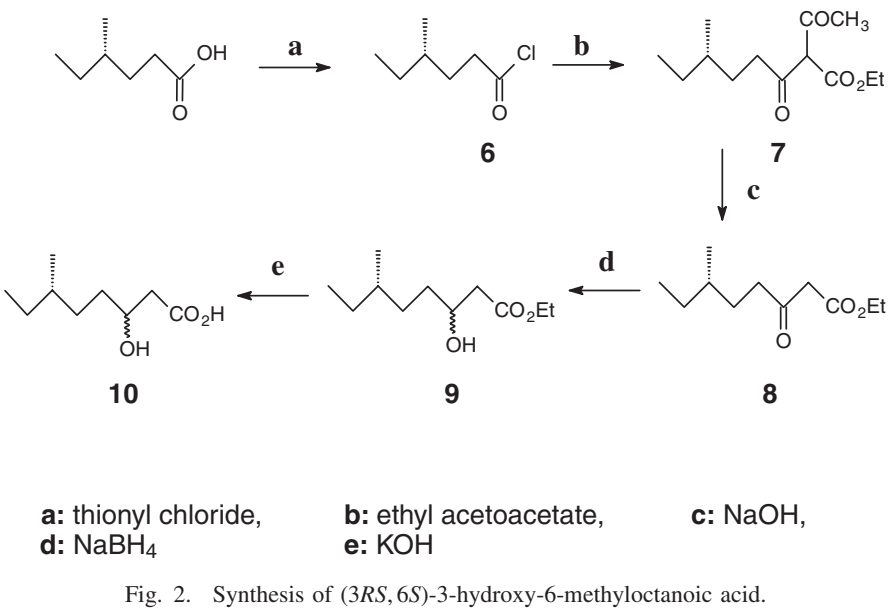


Fig. 2. Synthesis of (3*RS*, 6*S*)-3-hydroxy-6-methyloctanoic acid.

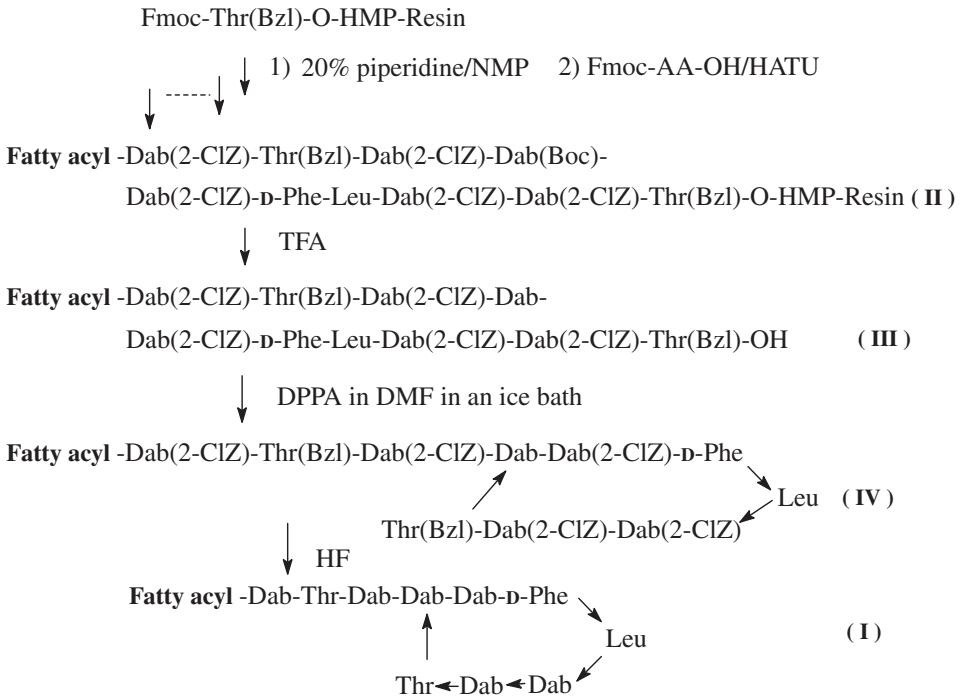


Fig. 3. Synthesis of polymyxin B peptides.

Table 1. Characteristics of Synthetic Polymyxin B (PMB) Peptides

Peptide	$[\alpha]_D^{29}$	FAB-MS			HPLC[a]	HP-TLC[b]	
	($c = 0.5$, 12% AcOH)	Formula	$[M + H]^+$	$[M + Na]^+$	t_R/min	R_f^1	R_f^2
PMB_1 (**Ia**)	−68.2°	$C_{56}H_{98}N_{16}O_{13}$	1203	1225	29.01	0.37	0.52
PMB_2 (**Ib**)	−68.2°	$C_{55}H_{96}N_{16}O_{13}$	1189	1211	25.90	0.42	0.50
PMB_3 (**Ic**)	−72.5°	$C_{55}H_{96}N_{16}O_{13}$	1189	1211	26.66	0.46	0.52
PMB_4 (**Id**)	−77.4°	$C_{54}H_{94}N_{16}O_{13}$	1175	1197	23.11	0.43	0.41
PMB_5 (**Ie**)	−73.5°	$C_{56}H_{98}N_{16}O_{13}$	1203	1225	30.08	0.48	0.47
PMB_6 (**If**)	−71.3°	$C_{56}H_{98}N_{16}O_{14}$	1219	1241	24.67 and 24.83	0.45	0.43
[Ile^7]-PMB_1 (**Ig**)	−63.6°	$C_{56}H_{98}N_{16}O_{13}$	1203	1225	27.81	0.48	0.42
[Dab(Dansyl-Gly)[1]]-PMB_3 (**Ih**)	−54.4°	$C_{69}H_{110}N_{18}O_{16}S_1$	1479	1501	38.68	0.46	0.64
Des-FA-PMB (1–10) (**Ii**)	−59.5°	$C_{47}H_{82}N_{16}O_{12}$	1063	1085	12.93	0.09	0.03
Des-FA-PMB (2–10) (**Ij**)	−46.1°	$C_{43}H_{74}N_{14}O_{11}$	963	985	12.79	0.28	0.05
Des-FA-PMB (3–10) (**Ik**)	−49.2°	$C_{39}H_{67}N_{13}O_9$	862	884	13.32	0.15	0.06
Des-FA-PMB (4–10) (**Il**)	−49.7°	$C_{35}H_{59}N_{11}O_8$	762	784	12.41	0.46	0.13
Octanoyl-PMB (2–10) (**Im**)	−66.1°	$C_{51}H_{88}N_{14}O_{12}$	1089	1111	31.14	0.59	0.43
Octanoyl-PMB (3–10) (**In**)	−74.2°	$C_{47}H_{81}N_{13}O_{10}$	988	1010	27.16	0.61	0.44
Octanoyl-PMB (4–10) (**Io**)	−66.5°	$C_{43}H_{73}N_{11}O_9$	888	910	28.22	0.66	0.48

a) HPLC conditions: Column, Capcell Pak C_{18} ACR (4.6 × 250 mm); Elution, a linear gradient from 16 to 32% CH_3CN in 0.1% TFA (30 min) followed by maintaining final condition; Flow rate, 1 mL/min; Detection, 210 nm. b) TLC solvent systems: R_f^1; BuOH:Pyridine:AcOH:H_2O (30:20:6:24). R_f^2; BuOH:AcOH:AcOEt:H_2O (1:1:1:1).

followed by lyophilization. The structure and purity of the synthetic polymyxin B component peptides (**Ia–Ig**) were confirmed by FAB-MS analysis, amino acid analysis of acid hydrolysates, high-performance thin-layer chromatography (HP-TLC), and analytical HPLC (Table 1). Individual synthetic polymyxin B peptides appeared as single peaks on analytical RP-HPLC, except for polymyxin B_6 (**If**). As **If** contained (3*RS*, 6*S*)-3-hydroxy-6-methyloctanoyl as the fatty acyl group, the diastereomeric mixture appeared as split peaks on analytical HPLC.

For the fatty acyl deletion analogs (**Ii–Il**), *N*-terminally Fmoc-protected cyclic peptides (**IVi–IVl**) were prepared. The Fmoc was removed by piperidine prior to the final deprotection with HF. Thus, the Fmoc strategy in combination with the benzyl-type protection for side chain functional groups was adopted because it was a versatile synthetic method for structurally complicated peptides, such as polymyxin B, composed of six diamino acids, Dab, and a lactam ring. In this study, not only the polymyxin B component peptides, but also [Dab(Dansyl-Gly)[1]]-polymyxin B_3 (**Ih**) were successfully synthesized

		1 2 3 4 5 6 7 8 9 10
Des-FA-PMB (1-10)	**Ii**	**H-Dab-Thr-Dab-Dab*-Dab-D-Phe-Leu-Dab-Dab-Thr*-**
Des-FA-PMB (2-10)	**Ij**	**H-Thr-Dab-Dab*-Dab-D-Phe-Leu-Dab-Dab-Thr*-**
Des-FA-PMB (3-10)	**Ik**	**H-Dab-Dab*-Dab-D-Phe-Leu-Dab-Dab-Thr*-**
Des-FA-PMB (4-10)	**Il**	**H-Dab*-Dab-D-Phe-Leu-Dab-Dab-Thr*-**
Octanoyl-PMB (2-10)	**Im**	**C_7H_{15}CO-Thr-Dab-Dab*-Dab-D-Phe-Leu-Dab-Dab-Thr*-**
Octanoyl-PMB (3-10)	**In**	**C_7H_{15}CO-Dab-Dab*-Dab-D-Phe-Leu-Dab-Dab-Thr*-**
Octanoyl-PMB (4-10)	**Io**	**C_7H_{15}CO-Dab*-Dab-D-Phe-Leu-Dab-Dab-Thr*-**

*** : amide linkage between γ-NH_2 of Dab^4 and α-COOH of Thr^{10}**

Fig. 4. Structure of *N*-terminal deletion polymyxin B-related peptides.

Table 2. Antimicrobial and LPS Binding Activities of Synthetic Polymyxin B (PMB) Peptides

Peptide	Minimum inhibitory concentration (MIC)/nmol mL^{-1}			LPS binding
	Escherichia coli	*Salmonella typhimurium*	*Pseudomonas aeruginosa*	IC_{50}[a] /nmol mL^{-1}
PMB (Sigma)	1.0	0.5	1.0	4
PMB_1 (**Ia**)	1.0	1.0	1.0	4
PMB_2 (**Ib**)	1.0	1.0	1.0	9
PMB_3 (**Ic**)	0.5	0.5	1.0	8
PMB_4 (**Id**)	0.5	0.5	0.5	10
PMB_5 (**Ie**)	1.0	1.0	1.0	6
PMB_6 (**If**)	0.5	0.5	0.5	7
[Ile^7]-PMB_1 (**Ig**)	0.5	0.5	0.5	5
[Dab(Dansyl-Gly)^1]-PMB_3 (**Ih**)	1.0	1.0	1.0	—
Des-FA-PMB (1–10) (**Ii**)	8.0	16	4.0	13
Des-FA-PMB (2–10) (**Ij**)	>256	>256	128	19
Des-FA-PMB (3–10) (**Ik**)	128	256	64	21
Des-FA-PMB (4–10) (**Il**)	>256	>256	128	>32
Octanoyl-PMB (2–10) (**Im**)	2.0	2.0	2.0	12
Octanoyl-PMB (3–10) (**In**)	16	16	8.0	14
Octanoyl-PMB (4–10) (**Io**)	128	128	128	30

a) The concentrations required for 50% inhibition of **Ih**–LPS binding were derived from the quenching curves of synthetic peptides in Fig. 6.

using the four different amino protecting groups, Fmoc, ivDde, Boc, and ClZ, followed by their selective removal during the synthetic procedure.

Antimicrobial Activities of Synthetic Polymyxin B Peptides. The antimicrobial activities of fifteen synthetic peptides (**Ia**–**Io**) were estimated by the standard micro plate dilution method. The minimum inhibitory concentration (MIC) values of synthetic polymyxin B component peptides (**Ia**–**Ig**) were 0.5–1.0 mol/mL toward *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* (Table 2). No significant differences in antimicrobial potency were observed among the five component peptides (**Ia**–**Ie**) despite their distinct fatty acyl structures. Synthetic polymyxin B_6 (**If**), bearing (3*RS*, 6*S*)-3-hydroxy-6-methyloctanoic acid as the *N*-terminal fatty acid, was also as active as the other component peptides. Based on the above results, it was difficult to determine whether one of the diastereomeric mixtures of **If** would be active, while the counterpart would be inactive. Thus, the structural differences between *normal*-, *iso*-, and *anteiso*-fatty acids, or 3-hydroxy fatty acid with chain lengths of C_7, C_8, and C_9 did not affect the antimicrobial potency. The contribution of the various fatty acyl groups of colistin (polymyxin E) derivatives was examined by Chihara,[23,24] however, the synthetic peptides, prepared directly from colistin nonapeptide, were heterogeneous, making it difficult to evaluate the results. It was shown that substitution of Ile for Leu^7 in polymyxin B_1 did not have any significance with respect to biological function, and a similar result was recently reported regarding [Ile^7]-polymyxin E_1.[25] The results outlined above indicated that all polymyxin B molecules isolated and structurally elucidated to date retain similar antimicrobial activity. The results indicated that any polymyxin B molecule could be selected for further study of the structure–activity relationships, with deletion, replacement, or modification of the amino acid residues. Therefore, polymyxin B_3 (**Ic**), with octanoyl at the *N*-terminal, was selected as a lead compound for the following advanced study using synthetic peptides. [Dab(Dansyl-Gly)^1]-polymyxin B_3 (**Ih**), a key compound for the LPS binding assay, retained potent bactericidal activity with MIC of 1 nmol/mL.

Octanoyl-polymyxin B (4–10) (**Io**), devoid of the *N*-terminal tripeptide Dab-Thr-Dab in polymyxin B_3 and instead composed of a cyclic heptapeptide and an octanoyl group at N^{α} of Dab^4, retained some antimicrobial activity, although it was less than 1/100 of that of polymyxin B_3 (**Ic**). Elongation of the peptide chain from the N^{α} of Dab^4 increased the potency in a stepwise manner, and octanoyl-polymyxin B (2–10) (**Im**) had almost full activity with an MIC of 2.0 nmol/mL. However, deletion of the octanoyl group from **Im** resulted in des-fatty acyl-polymyxin B (2–10) (**Ij**), which showed very little activity. These results were in agreement with the lack of antimicrobial activity of polymyxin B nonapeptide, prepared by enzymatic treatment of native polymyxin B with papain or ficin.[26,27] It was shown in this study that a shorter peptide, des-fatty acyl-polymyxin B (3–10) (**Ik**), had greater bactericidal activity than that of **Ij**, although its activity was very low.

In a recent synthetic study of polymyxin B nonapeptide analogs, it was reported that elongation from polymyxin B nonapeptide with tri-alanine (Ala_3-polymyxin B nonapeptide) or hexa-alanine (Ala_6-polymyxin B nonapeptide) resulted in a complete loss of antimicrobial activity.[28] However, des-fatty acyl-polymyxin B (1–10) (**Ii**) showed considerable activity with MIC values of 8.0, 16, and 4.0 nmol/mL against *E. coli*, *S. typhimurium*, and *P. aeruginosa*, respectively. Thus, it was of interest that the elongation of des-fatty acyl-polymyxin B (2–10) (**Ij**) with a hydrophilic and basic amino acid, Dab, regained the antimicrobial activity. However, the *N*-terminal fatty acyl group was demonstrated to make a greater contribution to the bactericidal activity, as the antibacterial potency of **Ii** was still low similar to that of octanoyl-polymyxin B (3–10) (**In**).

LPS Binding Activity. Dansyl-polymyxin B is usually prepared from native commercially available polymyxin B by coupling with dansyl chloride.[29] The reaction yields heterogeneous products because of the existence of five free amino functions of Dab residues at positions 1, 3, 5, 8, and 9 in the molecule. In this study, a structurally well-defined mono-dansylated fluorescent probe, [Dab(Dansyl-$Gly)^1$]-polymyxin B_3 (**Ih**), was prepared and used to establish an LPS binding assay system. As **Ih** showed almost full antimicrobial activity, it may bind to LPS of the living bacterial membrane in the same way as polymyxin B itself. Furthermore, [Ala^1]-polymyxin B_3 retained full antimicrobial activity with an MIC value of 1 nmol/mL,[30] suggesting that a basic amino acid residue at position 1 is not a structural requirement for bactericidal activity. That is, modification of the side chain amino function of Dab^1 was assumed not to disturb the interaction of polymyxin B with LPS. The maximum fluorescence spectrum of **Ih** was shifted from 560 to 505 nm, and the intensity was greatly enhanced at 505 nm by titration with LPS (Fig. 5).

LPS binding activity was examined according to the method reported by Moore.[31] The fluorescence of the LPS–**Ih** complex, measured at 490 nm, was quenched by the addition of synthetic peptides in a concentration-dependent manner. The apparent binding affinities of various polymyxin B component peptides to LPS and of *N*-terminal deletion analogs were estimated from their quenching curves (Figs. 6A and 6B), from which the peptide concentration to inhibit 50% binding of **Ih** to LPS (IC_{50} value) was obtained (Table 2). Synthetic polymyxin B_1, B_5, B_6, and [Ile^7]-polymyxin B_1 (**Ia**, **Ie**, **If**, and **Ig**) bearing a C_9 fatty acyl showed almost as potent LPS binding activity as native polymyxin B, while polymyxin B_2, B_3, and B_4 (**Ib**, **Ic**, and **Id**) bearing a C_8 and C_7 fatty acyl showed slightly but significantly lower binding activity. Among the *N*-terminal deletion analogs, des-fatty acyl-polymyxin B (1–10) (**Ii**), octanoyl-polymyxin B (2–10) (**Im**), and octanoyl-polymyxin B (3–10) (**In**) showed high binding activities, but their activities were lower than those of polymyxin B component peptides. The IC_{50} values of des-fatty acyl-polymyxin B (2–10) (**Ij**) and des-fatty acyl-polymyxin B (3–10) (**Ik**) were 2–5 fold higher than those of polymyxin B component peptides. Octanoyl-polymyxin B (4–10) (**Io**) had a further reduced binding activity to LPS, and des-fatty acyl-polymyxin B (4–10) (**Il**) had a greatly reduced binding activity. Introduction of an octanoyl group into these des-fatty acyl-polymyxin B peptides caused increases in the LPS binding activity to the same degree as introduction of one amino acid, i.e., the IC_{50} value of octanoyl-polymyxin B (2–10) (**Im**) was similar to that of des-fatty acyl-polymyxin B (1–10) (**Ii**), and that of octanoyl-polymyxin B (3–10) (**In**) was similar to des-fatty acyl-polymyxin B (2–10) (**Ij**). However, although octanoyl-polymyxin B (3–10) (**In**) and des-fatty acyl-polymyxin B (2–10) (**Ij**) showed similar binding activities, this was not reflected in their antimicrobial activities, as LPS binding activity did not parallel antimicrobial activity.

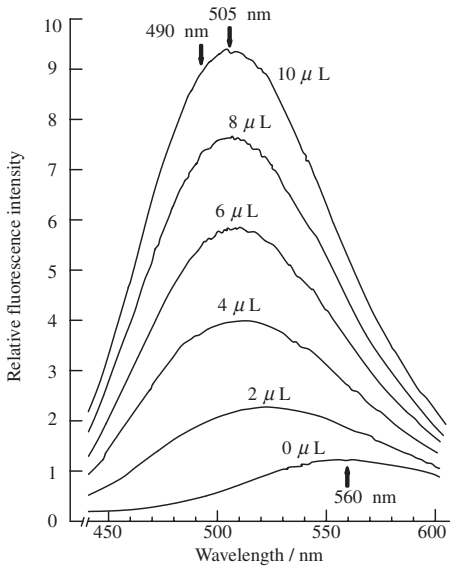


Fig. 5. Fluorescence emission spectra of [Dab(Dansyl-$Gly)^1$]-polymyxin B_3 (**Ih**). A solution of **Ih** (10 nmol/mL) in HEPES buffer (5 mmol/L, pH 7.2) was titrated with a solution of *E. coli* LPS (3 mg/mL) (2 μL each) in a quartz cuvette. Excitation was at 330 nm. The arrows labeled 505 nm and 490 nm indicate the maximum wavelength of fluorescent enhancement and the wavelength used for LPS binding assay of various synthetic peptides, respectively.

Conclusion

Seven polymyxin B component peptides and seven *N*-terminal fatty acid and/or amino acid deletion analogs were synthe-

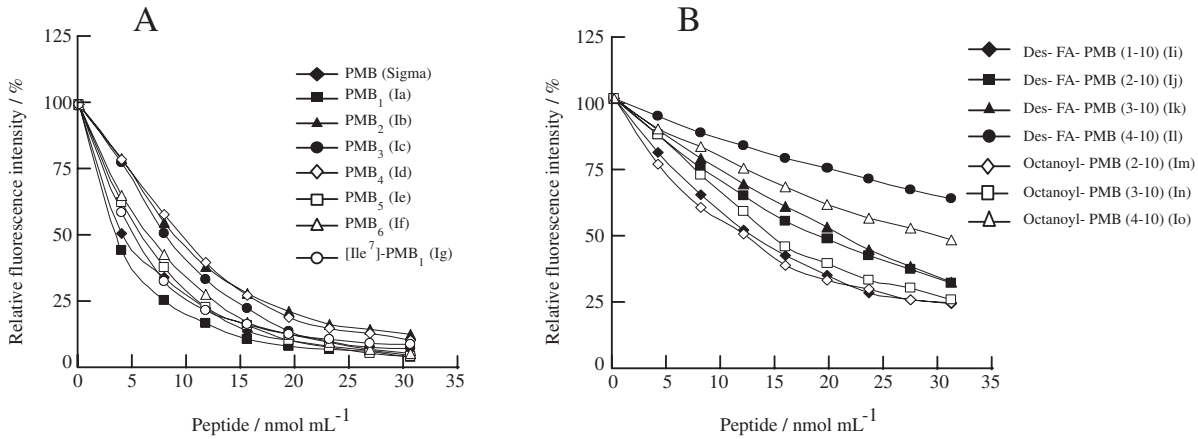


Fig. 6. Quenching curves of [Dab(Dansyl-Gly)[1]]-polymyxin B_3 (**Ih**) caused by addition of various polymyxin B (**A**) and *N*-terminal deletion polymyxin B peptides (**B**).

sized, and their antimicrobial activities were determined. The LPS binding activities of the synthetic peptides were evaluated using [Dab(Dansyl-Gly)[1]]-polymyxin B_3 as a fluorescent probe. The results revealed that the fatty acyl moiety was not indispensable in terms of LPS binding, but the C_9 fatty acyl groups of the polymyxin B peptides did contribute to the binding affinity to a slightly greater extent than the C_8 or C_7. The fatty acyl moieties of polymyxin B contributed greatly to the antimicrobial activity, while the distinct *N*-terminal structures of polymyxin B_1–B_6, bearing *normal*-, *iso*-, and *anteiso*-fatty acids, or a 3-hydroxy-fatty acid with chain lengths between C_7 and C_9, did not change the bactericidal potency. The introduction of an octanoyl group into the amino termini of shorter polymyxin B peptides demonstrated the contribution of the fatty acyl group to the antimicrobial activity, as well as to the increases in the LPS binding activity.

Experimental

General. HPLC was performed on an apparatus equipped with a 590 and 510 pump (Waters Corp., Milford, MA, USA), 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 with a Toyopearl HW-40S (Tosoh Corporation, Tokyo, Japan). Amino acid analysis of the acid hydrolysate was conducted on a model 7300 amino acid analyzer system (Beckman Instruments Ltd., Fullerton, CA USA). HF cleavage reactions were carried out in a Teflon HF apparatus (Peptide Institute Inc., Osaka, Japan). Fast-atom bombardment mass spectra (FAB-MS) were obtained on a JMS-DX300 mass spectrometer (JEOL Ltd., Tokyo, Japan). The ^{1}H NMR spectra were recorded in $CDCl_3$ as a solvent using TMS as an internal standard on a JEOL-PMX 60-SI spectrometer. The optical rotations of peptides were measured with a DIP-370 digital polarimeter (Nippon Bunko Co., Ltd., Tokyo, Japan). HP-TLC was performed on precoated silica gel plates (Kieselgel 60; Merck, Darmstadt, Germany). All reagents, solvents, and Fmoc-amino acids were obtained as reagent grade products from Watanabe Chem. Ind. Ltd. (Hiroshima, Japan) or Wako Pure Chem. Ind. Ltd. (Tokyo, Japan). Octanoic acid, heptanoic acid, and nonanoic acid were from Aldrich Chemical Company Inc. (Milwaukee, WI, USA).

Fmoc-Dab-OH (1). Fmoc-Gln-OH (36.8 g, 0.10 mol) was subjected to a Hofmann rearrangement with *I*,*I*-bis(trifluoroacetoxy)iodobenzene[16] (47.3 g, 0.11 mol) in DMSO (225 mL)–H_2O (25 mL). After 20 h, the reaction mixture was diluted with cold H_2O (300 mL) and washed with ether. The pH was then adjusted to 6.5 with 4 mol/L NaOH to yield a precipitate, which was collected and washed with boiling ethanol (200 mL). Yield 30.22 g (88.9%). mp 169.5–171.7 °C, $[\alpha]_D^{29}$ −4.0° (*c* 1.0, 50% AcOH), high-resolution FAB-MS (JMS-DX300 mass spectrometer, JEOL Ltd.): 341.1502 $[M + H]^+$, (calcd for $C_{19}H_{21}N_2O_4$; 341.1501). Found: C, 65.64; H, 6.14; N, 8.02%. Calcd for $C_{19}H_{20}N_2O_4 \cdot 1/2H_2O$: C, 65.30; H, 6.14; N, 7.71%.

Fmoc-Dab(2-ClZ)-OH (2). To a solution of **1** (17.02 g, 0.050 mol) and TEA (triethylamine) (7.0 mL, 0.05 mol) in H_2O (100 mL)–DMSO (30 mL) was added a solution of Z(2-Cl)-OSu (15.60 g, 0.055 mol) in DMSO (70 mL). The mixture was stirred for 3 h at room temperature while keeping the pH at 8 with TEA. The mixture was diluted with H_2O (150 mL)–TEA (7.0 mL), washed three times with ether (200 mL each), and then acidified with 6 mol/L HCl. The product was extracted three times with AcOEt (150 mL) and washed three times with 0.5 mol/L HCl and saturated NaCl. The combined organic phases were dried over Na_2SO_4 and evaporated to give an oil, which was solidified with isopropyl alcohol (150 mL). The product was collected and washed with boiling AcOEt (50 mL). Yield 21.80 g (85.7%). mp 134.6–136.2 °C, $[\alpha]_D^{29}$ −13.0° (*c* 0.5, methanol), high-resolution FAB-MS: 509.1477 $[M + H]^+$, (calcd for $C_{27}H_{26}ClN_2O_6$; 509.1476). Found: C, 63.66; H, 5.04; N, 5.59%. Calcd for $C_{27}H_{25}ClN_2O_6$: C, 63.72; H, 4.95; N, 5.50%.

Fmoc-Dab(Boc)-OH (3). To a solution of **1** (10.21 g, 0.030 mol) and TEA (4.20 mL, 0.03 mol) in H_2O (60 mL)–DMSO (30 mL) was added a solution of Boc_2O (8.51 g, 0.039 mol) in DMSO (30 mL). The mixture was treated in the same manner as described above for **2**. The product was purified with AcOEt (25 mL)-MeOH (5 mL)–petr. ether (60 mL). Yield 9.80 g (74.2%). mp 102.0–103.7 °C, $[\alpha]_D^{29}$ −13.4° (*c* 0.5, methanol), high-resolution FAB-MS: 441.2026 $[M + H]^+$, (calcd for $C_{24}H_{29}N_2O_6$; 441.2026). Found: C, 65.49; H, 6.42; N, 6.33%. Calcd for $C_{24}H_{28}N_2O_6$: C, 65.44; H, 6.41; N, 6.36%.

(6*S*)-6-Methyloctanoic Acid (4) and 6-Methylheptanoic Acid (5). The fatty acids used for the preparation of polymyxin B_1 and B_2 were synthesized by methods reported previously,[17,18] starting with (2*S*)-2-methylbutanol and 4-methylpentanol, respectively. **4**:

High-resolution FAB-MS: 159.1387 $[M + H]^+$, (calcd for $C_9H_{19}O_2$; 159.1385). **5**: MS (m/z): 145 $[M + H]^+$.

(4*S*)-4-Methylhexanoyl Chloride (6). A mixture of (4*S*)-4-methylhexanoic acid (33.7 g, 0.26 mol) and thionyl chloride (83.3 g, 0.70 mol) was refluxed for 5 h. The reaction mixture was distilled to give **6** (22.9 g, 59.1%; bp 94 °C/63 mm). ^{1}H NMR ($CDCl_3$) δ 0.78–1.00 (6H, m, C**H**$_3$ × 2), 0.98–1.90 (5H, m, CH_3C**H**$_2$(CH_3)C**H**C**H**$_2$), 2.87 (2H, t, J = 8.0 Hz, C**H**$_2$CO). IR (liquid) cm^{-1}: 1803. FAB-MS (m/z): 149 $[M + H]^+$.

(6*S*)-Ethyl 2-Acetyl-6-methyl-3-oxooctanoate (7). Ethyl acetoacetate (40.6 g, 0.31 mol) was added in a dropwise manner to a solution of clean sodium (3.6 g, 0.15 g atom) in dry diethyl ether (470 mL) at room temperature, followed by stirring for 12 h. To the above solution was added **6** (22.9 g, 0.155 mol) in a dropwise manner at room temperature. The mixture was then refluxed for 10 h. Water was added to the reaction mixture. The ether layer was extracted and washed with brine, dried, and concentrated. The residue was distilled to give **7** (22.15 g, 59.0%; bp 106–109 °C/4 mm). ^{1}H NMR ($CDCl_3$) δ 0.78–1.00 (6H, m, C**H**$_3$ × 2), 0.98–1.90 (5H, m, CH_3C**H**$_2$(CH_3)C**H**C**H**$_2$), 1.30 (3H, t, J = 8.0 Hz, C**H**$_3$$CH_2$), 2.30 (3H, s, COC**H**$_3$), 2.63 (2H, t, J = 8.0 Hz, C**H**$_2$CO), 4.00 (1H, s, Ac(CO)C**H**CO_2Et), 4.23 (2H, d, J = 8.0 Hz, CH_3C**H**$_2$). IR (liquid) cm^{-1}: 1762, 1716. High-resolution MS (m/z): 242.1521 (Calcd for $C_{13}H_{22}O_4$: 242.1518).

(6*S*)-Ethyl 6-Methyl-3-oxooctanoate (8). To a solution of sodium hydroxide (1.79 g, 0.045 mol) in water (142 mL) was added **7** (0.045 mol). The solution was heated at 100 °C for 45 min. After cooling rapidly in an ice bath, the solution was extracted twice with ether. The ether solution was dried and distilled in vacuo to give **8** (4.72 g, 52.4%; 92–94 °C/4 mm). ^{1}H NMR ($CDCl_3$) δ 0.78–l.00 (6H, m, C**H**$_3$ × 2), 0.98–1.90 (5H, m, CH_3C**H**$_2$C**H**-(CH_3)C**H**$_2$$CH_2$), l.25 (3H, t, J = 8.0 Hz, C**H**$_3$$CH_2$), 2.50 (2H, t, J = 8.0 Hz, C**H**$_2$CO), 3.40 (2H, s, COC**H**$_2$COEt), 4.20 (2H, q, J = 8.0 Hz, OC**H**$_2$$CH_3$). IR (liquid) cm^{-1}: 1747, 1718. High-resolution MS (m/z): 200.1414 (Calcd for $C_{11}H_{20}O_3$: 200.1413).

(3*RS*, 6*S*)-Ethyl 3-Hydroxy-6-methyloctanoate (9). To a solution of **8** (4.72 g, 24 mmol) in ethanol (100 mL) and water (2 mL) was added sodium tetrahydroborate (445 mg, 12 mmol) at room temperature. The mixture was then stirred for 2 h. After removal of the solvent under reduced pressure, the residue was extracted with ether. The ether layer was washed with brine, dried, and evaporated. The oily residue was purified by column chromatography [SiO_2, CH_2Cl_2] to give **9** (viscous oil: 3.66 g, 75.1%). ^{1}H NMR ($CDCl_3$) δ 0.78–1.00 (6H, m, C**H**$_3$ × 2), 0.98–1.90 (5H, m, CH_3C**H**$_2$(CH_3)C**H**C**H**$_2$), 1.23 (3H, t, J = 8.0 Hz, OCH_2-C**H**$_3$), 2.42 (1H, d, J = 8.0 Hz, one of CH(OH)C**H**$_2$COOEt), 2.46 (1H, d, J = 6.0 Hz, one of CH(OH)C**H**$_2$COOEt), 3.10 (1H, br s, O**H**, exchangeable by D_2O), 4.0 (1H, m, C**H**(OH)), 4.13 (2H, q, J = 8.0 Hz, OC**H**$_2$$CH_3$). IR (liquid) cm^{-1}: 3452, 1735. High-resolution FAB-MS (m/z): 203.1644 $[M + H]^+$, (Calcd for $C_{11}H_{23}O_3$: 203.1647).

(3*RS*, 6*S*)-3-Hydroxy-6-methyloctanoic Acid (10). A mixture of **9** (1.65 g, 8.2 mmol), potassium hydroxide (0.82 g, 15 mmol), water (2 mL), and ethanol (9 mL) was refluxed for 90 min. After ethanol was removed by distillation, water (3 mL) was added to the residue, neutralized with hydrochloric acid, and then extracted with dichloromethane. The dichloromethane layer was washed with brine, dried, and concentrated. The residue was purified by column chromatography [SiO_2, CH_2Cl_2] to give **10** (viscous oil: 1.31 g, 91.2%). ^{1}H NMR ($CDCl_3$) δ 0.70–l.10 (6H, m, C**H**$_3$ × 2), 0.98–l.90 (5H, m, CH_3C**H**$_2$(CH_3)C**H**C**H**$_2$). 2.50 (lH, d, J = 8.0 Hz, one of CH(OH)C**H**$_2$COOH), 2.53 (1H, d, J = 6.0 Hz, one of CH(OH)C**H**$_2$COOH), 4.03 (1H, m, C**H**(OH)), 6.87 (2H, br s, O**H**, COO**H**, exchangeable by D_2O), IR (liquid) cm^{-1}: 3423, 1712. High-resolution FAB-MS (m/z): 175.1334 $[M + H]^+$, (Calcd for $C_9H_{19}O_3$: 175.1331). Total yield of **10** was 13.0% from (4*S*)-4-methylhexanoic acid.

Protected Polymyxin B$_1$ Peptide Resin [(6*S*)-6-Methyloctanoyl-Dab(2-ClZ)-Thr(Bzl)-Dab(2-ClZ)-Dab(Boc)-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-OMP-Resin] (IIa) and Related Compounds (IIb–IIg). General Procedure 1: The protected amino acids used were Fmoc-Dab(2-ClZ)-OH, Fmoc-Dab(Boc)-OH, Fmoc-Thr(Bzl)-OH, Fmoc-D-Phe-OH, and Fmoc-Leu-OH. Fmoc-Thr(Bzl)-O-HMP-resin was prepared by the coupling of Fmoc-Thr(Bzl)-OH (8 mol equiv.) to 4-hydoxymethylphenoxymethyl-resin (HMP-resin or Wang-resin, 0.74 mmol/g, Novabiochem, Läufelfingen, Switzerl) with DCC (4 mol equiv.) in the presence of 4-dimethylaminopyridine (DMAP, 0.1 mol equiv.). Starting from Fmoc-Thr(Bzl)-O-HMP-resin (344 mg, 0.2 mmol), the peptide chain was elongated through a solid-phase methodology using a peptide synthesizer (ABI 433A; Applied Biosystems, Foster City, CA, USA). Deprotection of the Fmoc group was performed by treatment with 20% piperidine in *N*-methylpyrolidone (NMP). Fmoc-amino acids (0.5 mmol), HATU (0.5 mmol), and *N*,*N*-diisopropylethylamine (1.0 mmol) were stirred with the resin for 1 h in NMP for each coupling reaction. After introduction of (6*S*)-6-methyloctanoic acid (**4**) to *N*-terminal of Dab[1], the peptide resin was washed consecutively three times with DMF, dichloromethane, MeOH, and ether, and then dried in vacuo to yield **IIa** (686 mg).

The other protected peptide resins (**IIb–IIg**) were prepared in the same manner as described in General procedure 1 using 6-methylheptanoic acid (**5**), octanoic acid, heptanoic acid, nonanoic acid (Wako Pure Chem. Ind. Ltd., Osaka, Japan), or (3*RS*, 6*S*)-3-hydroxy-6-methyloctanoic acid (**10**) as fatty acids.

Linear Partially Protected Polymyxin B$_1$ [(6*S*)-6-Methyloctanoyl-Dab(2-ClZ)-Thr(Bzl)-Dab(2-ClZ)-Dab-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-OH] (IIIa) and Related Compounds (IIIb–IIIg). General Procedure 2: TFA–H_2O (95:5) (6 mL) was added to **IIa** (630 mg) on ice and then stirred for 90 min at room temperature. After filtration to remove the resin, the TFA solution was evaporated in vacuo, and the residue was lyophilized from dioxane. The crude product (240 mg) was dissolved in DMSO (1 mL)–DMF (1 mL) and applied to a column (1.6 × 95 cm) of Toyopearl HW-40S (Tosoh Co., Tokyo, Japan) using DMF:H_2O (9:1) as the eluent. Fractions containing the main product were combined, evaporated, and lyophilized from dioxane to give **IIIa** 175 mg (39.0% from Fmoc-Thr(Bzl)-OHMP-resin). $[\alpha]_D^{28}$ −20.8° (*c* 0.5, DMF), FAB-MS; Found (for the most abundant isotopic variant); 2244.85 $[M + H]^+$, Calcd for $C_{110}H_{137}Cl_5N_{16}O_{24}$, HPLC: t_R 34.3 min [Column, YMC-Pack A-803 C_4 (4.6 × 250 mm); Elution, a linear gradient (30 min) from 38 to 76% MeCN in 0.1% TFA; Flow rate, 1 mL/min; Detection, 210 nm].

The other linear partially protected polymyxin B peptides (**IIIb–IIIg**) were prepared in the same manner as described in General procedure 2.

Linear Partially Protected Polymyxin B$_2$ (IIIb). $[\alpha]_D^{28}$ −22.7° (*c* 0.5, DMF), FAB-MS; Found; 2230.83 $[M + H]^+$, Calcd for $C_{109}H_{135}Cl_5N_{16}O_{24}$, HPLC: t_R 33.5 min.

Linear Partially Protected Polymyxin B$_3$ (IIIc). $[\alpha]_D^{28}$ −33.7° (*c* 0.5, DMF), FAB-MS; Found; 2230.82 $[M + H]^+$, Calcd for $C_{109}H_{135}Cl_5N_{16}O_{24}$, HPLC: t_R 33.5 min.

Linear Partially Protected Polymyxin B$_4$ (IIId). $[\alpha]_D^{28}$

−45.8° (*c* 0.5, DMF), FAB-MS; Found; 2217.81 $[M + H]^+$, Calcd for $C_{108}H_{133}Cl_5N_{16}O_{24}$, HPLC: t_R 33.1 min.

Linear Partially Protected Polymyxin B$_5$ (IIIe). $[\alpha]_D^{28}$ −15.5° (*c* 0.5, DMF), FAB-MS; Found; 2244.85 $[M + H]^+$, Calcd for $C_{110}H_{137}Cl_5N_{16}O_{24}$, HPLC: t_R 34.8 min.

Linear Partially Protected Polymyxin B$_6$ (IIIf). $[\alpha]_D^{28}$ −7.8° (*c* 0.5, DMF), FAB-MS; Found; 2261.86 $[M + H]^+$, Calcd for $C_{110}H_{137}Cl_5N_{16}O_{25}$, HPLC: t_R 32.7 and 33.2 min.

Linear Partially Protected [Ile^7]-Polymyxin B$_1$ (IIIg). $[\alpha]_D^{28}$ −15.5° (*c* 0.5, DMF), FAB-MS; Found; 2244.84 $[M + H]^+$, Calcd for $C_{110}H_{137}Cl_5N_{16}O_{24}$, HPLC: t_R 34.0 min.

Cyclic Protected Polymyxin B$_1$ [(6*S*)-6-Methyloctanoyl-Dab(2-ClZ)-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})] (IVa). General Procedure 3: To an ice-cold solution of **IIIa** (0.03 mmol, 67 mg) in DMSO (1 mL)–DMF (2 mL) was added diphenyl phosphorazidate (DPPA)[19] (0.12 mmol, 33 mg) and 1-methylmorpholine (0.30 mmol, 30 μL). The mixture was stirred at 4 °C for 24 h and evaporated to half the original volume. The solution was applied to a Toyopearl HW-40S column in the same manner as described in General procedure 2. The fractions containing the product were combined, evaporated, and lyophilized from dioxane to give **IVa**: 63.5 mg (95.1%). $[\alpha]_D^{28}$ −18.2° (*c* 0.5, DMF), FAB-MS; Found (for the most abundant isotopic variant); 2227.85 $[M + H]^+$, Calcd for $C_{110}H_{135}Cl_5N_{16}O_{23}$. HPLC; t_R 37.7 min (under the same conditions as described above for **IIIa**).

The other cyclic protected polymyxin B peptides (**IVb–IVg**) were prepared in the same manner as described in General procedure 3. The yields of **IVb–IVg** were almost quantitative.

Cyclic Protected Polymyxin B$_2$ (IVb). $[\alpha]_D^{28}$ −18.6° (*c* 0.5, DMF), FAB-MS; Found; 2213.83 $[M + H]^+$, Calcd for $C_{109}H_{133}Cl_5N_{16}O_{23}$, HPLC: t_R 36.9 min.

Cyclic Protected Polymyxin B$_3$ (IVc). $[\alpha]_D^{28}$ −16.2° (*c* 0.5, DMF), FAB-MS; Found; 2213.83 $[M + H]^+$, Calcd for $C_{109}H_{133}Cl_5N_{16}O_{23}$, HPLC: t_R 36.9 min.

Cyclic Protected Polymyxin B$_4$ (IVd). $[\alpha]_D^{28}$ −19.4° (*c* 0.5, DMF), FAB-MS; Found; 2199.81 $[M + H]^+$, Calcd for $C_{108}H_{131}Cl_5N_{16}O_{23}$, HPLC: t_R 36.1 min.

Cyclic Protected Polymyxin B$_5$ (IVe). $[\alpha]_D^{28}$ −20.0° (*c* 0.5, DMF), FAB-MS; Found; 2227.83 $[M + H]^+$, Calcd for $C_{110}H_{135}Cl_5N_{16}O_{23}$, HPLC: t_R 37.7 min.

Cyclic Protected Polymyxin B$_6$ (IVf). $[\alpha]_D^{28}$ −12.7° (*c* 0.5, DMF), FAB-MS; Found; 2243.84 $[M + H]^+$, Calcd for $C_{110}H_{135}Cl_5N_{16}O_{24}$, HPLC: t_R 35.6 and 36.0 min.

Cyclic Protected [Ile^7]-Polymyxin B$_1$ (IVg). $[\alpha]_D^{28}$ −12.7° (*c* 0.5, DMF), FAB-MS; Found; 2227.84 $[M + H]^+$, Calcd for $C_{110}H_{135}Cl_5N_{16}O_{23}$, HPLC: t_R 37.5 min.

Polymyxin B$_1$ [(6*S*)-6-Methyloctanoyl-Dab-Thr-Dab-*cyclic*-(Dab*-Dab-D-Phe-Leu-Dab-Dab-Thr*) (* Amide Bond between the γ-NH$_2$ of Dab^4 and α-COOH of Thr^{10})] (Ia) and Related Compounds (Ib–Ig). General Procedure 4: Cyclic protected polymyxin B$_1$ (**IVa**) (44.5 mg, 0.02 mmol) was treated with anhydrous HF (2 mL)–anisole (0.2 mL) for 1 h in an ice bath. The excess HF was then removed in vacuo. The residue was dissolved in H_2O (15 mL), washed three times with ether, and lyophilized. The crude product was purified by HPLC employing a Capcell Pak C_{18} (1 × 25 cm). The eluate containing the main product was lyophilized. The product was passed through a Toyopearl HW-40 column (1.5 × 57 cm) using 25% CH_3CN in 5 mmol/L HCl as the eluent to give purified polymyxin B$_1$ as a hydrochloride salt (**Ia**): 14.5 mg (52.3%). Amino acid analysis of the acid hydrolysate: Thr 2.12 (2), Dab 5.94 (6), Phe 0.96 (1), Leu 0.97 (1).

The other polymyxin B peptides **Ib–Ig** were prepared from **IVb–IVg** in the same manner as described in General procedure 4, and the characteristics of the synthetic polymyxin B peptides are shown in Table 1.

[Dab(Dansyl-Gly)^1]-polymyxin B$_3$ [Octanoyl-Dab(Dansyl-Gly)-Thr-Dab-*cyclic*-(Dab*-Dab-D-Phe-Leu-Dab-Dab-Thr*) (* Amide Bond between the γ-NH$_2$ of Dab^4 and α-COOH of Thr^{10})] (Ih). Octanoyl-Dab(ivDde)-Thr(Bzl)-Dab(2-ClZ)-Dab-(Boc)-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-O-HMP-resin (**IIh**) (0.1 mmol) was prepared in the same manner as described in General procedure 1, except that Fmoc-Dab-(ivDde)-OH (Watanbe Chem. Ind. Ltd., Hiroshima, Japan) was introduced to position 1. After the incorporation of octanoic acid, **IIh** was stirred for 10 min in 2% hydrazine in DMF. Hydrazine treatment was repeated three times to remove the ivDde protecting group.[22] The amino function of the side chain of Dab^1 was then acylated with Fmoc-Gly-OH, followed by treatment with 20% piperidine in DMF and coupling with dansyl chloride to give Octanoyl-Dab(Dansyl-Gly)-Thr(Bzl)-Dab(2-ClZ)-Dab(Boc)-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-O-HMP-resin (**IIh′**). According to General procedure 2, **IIh′** was treated with 95% TFA to yield a linear partially protected peptide, Octanoyl-Dab(Dansyl-Gly)-Thr(Bzl)-Dab(2-ClZ)-Dab-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-OH (**IIIh**). FAB-MS; Found (for the most abundant isotopic variant); 2352.9 $[M + H]^+$, Calcd. for $C_{115}H_{144}Cl_4N_{18}O_{25}S$. According to General procedure 3, **IIIh** was reacted with DPPA to yield protected cyclic [Dab(Dansyl-Gly)^1]-polymyxin B$_3$ (**IVh**). FAB-MS; Found (for the most abundant isotopic variant); 2334.9 $[M + H]^+$, Calcd for $C_{115}H_{142}Cl_4N_{18}O_{24}S$. According to General procedure 4, **IVh** was treated with HF to yield **Ih**. Yield: 14 mg. The characteristics of **Ih** are shown in Table 1.

Des-Fatty Acyl-Polymyxin B (1–10), [H-Dab-Thr-Dab-*cyclic*-(Dab*-Dab-D-Phe-Leu-Dab-Dab-Thr*) (* Amide Bond between the γ-NH$_2$ of Dab^4 and α-COOH of Thr^{10})] (Ii) and Related Compounds (Ij–Il). General Procedure 5: 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 (**IIi**), obtained as an intermediate in General procedure 1, was treated with 95% TFA according to General procedure 2 to yield a linear partially protected peptide, Fmoc-Dab(2-ClZ)-Thr(Bzl)-Dab(2-ClZ)-Dab-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-OH (**IIIi**). FAB-MS; Found (for the most abundant isotopic variant); 2327.8 $[M + H]^+$, Calcd for $C_{116}H_{131}Cl_5N_{16}O_{25}$. The DPPA cyclization of **IIIi** according to General procedure 3 yielded Fmoc-Dab(2-ClZ)-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}) (**IVi**). FAB-MS; Found (for the most abundant isotopic variant); 2309.8 $[M + H]^+$, Calcd for $C_{116}H_{129}Cl_5N_{16}O_{24}$. **IVi** was treated with 20% piperidine in DMF for 30 min to remove the Fmoc group. The solution was evaporated in vacuo, and the residue was lyophilized from dioxane. The product was treated with HF and purified according to General procedure 4 to yield **Ii**. The characteristics of **Ii** are shown in Table 1.

Des-Fatty Acyl-Polymyxin B (2–10) (Ij), Des-Fatty Acyl-Polymyxin B (3–10) (Ik), and Des-Fatty Acyl-Polymyxin B (4–10) (Il). In the same manner as described in General procedure 5, **Ij**, **Ik**, and **Il** were prepared. Their characteristics are shown in Table 1.

Octanoyl-Polymyxin B (2–10) [Octanoyl-Thr-Dab-*cyclic*-

(Dab*-Dab-D-Phe-Leu-Dab-Dab-Thr*) (* Amide Bond between the γ-NH_2 of Dab^4 and α-COOH of Thr^{10})] (Im). General Procedure 6: The coupling of octanoic acid with 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, obtained as an intermediate in the General procedure 1, yielded Octanoyl-Thr(Bzl)-Dab(2-ClZ)-Dab(Boc)-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab-(2-ClZ)-Thr(Bzl)-O-HMP-resin (**IIm**). Treatment of **IIm** with 95% TFA according to General procedure 2 yielded Octanoyl-Thr(Bzl)-Dab(2-ClZ)-Dab-Dab(2-ClZ)-D-Phe-Leu-Dab(2-ClZ)-Dab(2-ClZ)-Thr(Bzl)-OH (**IIIm**). FAB-MS; Found (for the most abundant isotopic variant); 1961.9 $[M + H]^+$, Calcd for $C_{97}H_{122}Cl_4N_{14}O_{21}$. The DPPA cyclization reaction of **IIIm** according to General procedure 3 yielded Octanoyl-Thr(Bzl)-Dab-(2-ClZ)-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}) (**IVm**). FAB-MS; Found (for the most abundant isotopic variant); 1943.9 $[M + H]^+$, Calcd for $C_{97}H_{120}Cl_4N_{14}O_{20}$. Treatment of **IVm** with HF followed by purification of the product according to General procedure 4 yielded **Im**. The characteristics of **Im** are shown in Table 1.

Octanoyl-Polymyxin B (3–10) (**In**) and Octanoyl-Polymyxin B (4–10) (**Io**) were prepared in the same manner as described in General procedure 6 and the characteristics are shown in Table 1.

Bacteria and Susceptibility Test. *Escherichia coli* IFO 12734, *Salmonella typhimurium* IFO 12529, and *Pseudomonas aeruginosa* IFO 3080 were purchased from the Institute for Fermentation, Osaka (IFO), Japan. These bacteria were grown overnight at 37 °C on nutrient agar medium and harvested in sterile saline. The densities of 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 the synthetic peptides was evaluated in comparison with that of commercially available polymyxin B (Sigma Chemical Co., St. Louis, MO, USA). Minimum inhibitory concentrations (MIC) of the synthetic peptides against the bacteria were determined using the standard microplate dilution method ($n = 6$–8). Aliquots of 100 μL of each serially diluted peptide in the range of 0.25–512 nmol/mL with distilled water were added to a mixture of 10 μL of bacterial suspension (approximately 10^6 CFU/mL) and 90 μL of Mueller-Hinton broth (Difco Laboratories, Franklin Lakes, NJ, USA) in each well of flat-bottomed microplates (Corning Laboratory Sciences Company, Corning, NY, USA). 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 2).

Titration of Dansyl-Fluorescence of [$Dab(Dansyl\text{-}Gly)^1$]-polymyxin B_3 (Ih) with LPS. The fluorescence spectra of **Ih** were measured at 30 °C using a fluorescence spectrophotometer F-850 (Hitachi Instrument Co., Tokyo, Japan) at an excitation wavelength of 330 nm. A solution of *E. coli* (serotype 055:B_5) lipopolysaccharide (LPS) (3 mg/mL, Sigma Chemical Co.) was added cumulatively at 10 min intervals (2 μL each; 0, 6, 12, 18, 24, and 30 μg of LPS) to quartz cuvettes containing **Ih** (10 nmol) in *N*-(2-hydroxyethyl)piperazine-*N*′-ethane-1-sulfonic acid buffer (HEPES; 5 mmol/L, pH 7.2) (1 mL). The enhancement and changes in the maximum wavelength of the fluorescence caused by the binding of **Ih** to LPS were monitored after each addition of LPS solution (Fig. 5).

Binding Assay of Synthetic Peptides to LPS. A solution of [$Dab(Dansyl\text{-}Gly)^1$]-polymyxin B_3 (**Ih**) in H_2O (1 μmol/mL) (4 μL, 4 nmol) was added to quartz cuvettes containing HEPES buffer (5 mM, pH 7.2) (1 mL), followed by a solution of LPS in H_2O (3 mg/mL) (10 μL, 30 μg). The solutions were kept at 30 °C for 1 h until the fluorescence intensity reached a plateau. The intensity of the dansyl fluorescence of **Ih** was measured at an excitation wavelength of 330 nm and an emission wavelength of 490 nm. A solution of each polymyxin B component peptide (**Ia**–**Ig**) or *N*-terminal deletion analog (**Ii**–**Io**) (1 μmol/mL) (4 μL each) was added cumulatively to the quartz cuvettes at 5-min intervals to obtain 8 points (4–32 nmol). The changes in fluorescence intensity were measured after each addition. The initial intensity of fluorescence was taken as 100%. The percent fluorescence intensity was plotted as a function of the peptide concentration. The concentrations required for 50% quenching of **Ih** bound to LPS (IC_{50}) were derived from the quenching curves of synthetic peptides (Table 2 and Fig. 6). The binding experiments were repeated at least three times for each peptide to obtain reproducible results. Comparisons among the groups of the IC_{50} values of polymyxin B component peptides were made using ANOVA. The IC_{50} values of **Ib**, **Ic**, and **Id** were significantly higher than that of **Ia** ($p < 0.01$), and those of **Ia**, **Ie**, **If**, and **Ig** were not significantly different. No significant differences among the IC_{50} values of **Ib**, **Ic**, **Id**, and **If** were observed.

We thank Dr. C. Yanaihara, Osaka College of Pharmacy, for her critical reading of the manuscript. This work was partially supported by a special grant from Hokuriku University.

References

1 G. C. Ainsworth, A. M. Brown, and G. Brownlee, *Nature*, **160**, 263 (1947).

2 R. G. Benedict and A. F. Langlykke, *J. Bacteriol.*, **54**, 24 (1947).

3 D. R. Storm, K. S. Rosenthal, and P. E. Swanson, *Ann. Rev. Biochem.*, **46**, 723 (1977).

4 T. Suzuki, K. Hayashi, K. Fujikawa, and K. Tsukamoto, *J. Biochem. (Tokyo)*, **56**, 335 (1964).

5 A. H. Thomas, J. M. Thomas, and J. Holloway, *Analysis*, **105**, 1068 (1980).

6 I. Elverdam, P. Larsen, and E. Lund, *J. Chromatogr.*, **218**, 653 (1981).

7 K. Fujikawa, K. Suketa, K. Hayashi, and T. Suzuki, *Experientia*, **21**, 307 (1965).

8 Y. Ikai, H. Oka, J. Hayakawa, N. Kawamura, T. Mayumi, M. Suzuki, and K. Harada, *J. Antibiot.*, **51**, 492 (1988).

9 J. A. Orwa, C. Govaerts, R. Busson, E. Roets, A. Van Schepdael, and J. Hoogmartens, *J. Antibiot.*, **54**, 595 (2001).

10 J. A. Orwa, C. Govaerts, R. Busson, E. Roets, A. Van Schepdael, and J. Hoogmartens, *J. Chromatogr., A*, **912**, 369 (2001).

11 K. Vogler, R. O. Studer, P. Lanz, W. Lergier, and E. Böhni, *Experientia*, **20**, 365 (1964).

12 K. Vogler, R. O. Studer, P. Lanz, W. Lergier, and E. Böhni, *Helv. Chim. Acta*, **48**, 1161 (1965).

13 S. K. Sharma, A. D. Wu, N. Chandramouli, C. Fotsch, G. Kardash, and K. W. Bair, *J. Pept. Res.*, **53**, 501 (1999).

14 N. Sakura, T. Itoh, K. Ohki, S. Nagai, and Y. Uchida, "Peptide Science 1999," ed by N. Fujii, The Japanese Peptide Society (2000), pp. 413–416.

15 P. C. de Visser, N. M. A. J. Kriek, P. A. V. van Hooft, A. Van Schepdael, D. V. Filippov, G. A. van der Marel, H. S. Overkleeft, J. H. van Boom, and D. Noort, *J. Pept. Res.*, **61**,

298 (2003).

16 A. S. Radhakrishna, M. E. Parham, R. M. Riggs, and G. M. Loudon, *J. Org. Chem.*, **44**, 1746 (1979).

17 J. M. Crawforth, J. Fawcett, and B. J. Rawlings, *J. Chem. Soc., Perkin Trans. 1*, **1998**, 1721.

18 K. Vogler and L. H. Chopard-dit-Jean, *Helv. Chim. Acta*, **43**, 279 (1960).

19 K. Ozawa, T. Shioiri, and S. Yamada, *Chem. Pharm. Bull.*, **25**, 122 (1977).

20 S. Sakakibara, *Biopolymers*, **37**, 17 (1995).

21 H. Tsubery, I. Ofek, S. Cohen, and M. Fridkin, *J. Med. Chem.*, **43**, 3085 (2000).

22 S. R. Chhabra, B. Hothi, D. J. Evans, P. D. White, B. W. Bycroft, and W. C. Chan, *Tetrahedron Lett.*, **39**, 1603 (1998).

23 S. Chihara, A. Ito, M. Yahata, T. Tobita, and Y. Koyama, *Agric. Biol. Chem.*, **38**, 521 (1974).

24 S. Chihara, A. Ito, M. Yahata, T. Tobita, and Y. Koyama, *Agric. Biol. Chem.*, **38**, 1767 (1974).

25 T. Kline, D. Holub, L. Therrien, T. Leung, and D. Ryckman, *J. Pept. Res.*, **57**, 175 (2001).

26 S. Chihara, T. Tobita, M. Yahata, A. Ito, and Y. Koyama, *Agric. Biol. Chem.*, **37**, 2455 (1973).

27 M. Vaara and T. Vaara, *Antimicrob. Agents Chemother.*, **24**, 107 (1983).

28 H. Tsubery, I. Ofek, S. Cohen, and M. Fridkin, *Peptides*, **22**, 1675 (2001).

29 S. Fidai, S. W. Farmer, and R. E. W. Hancock, "Methods in Molecular Biology," ed by W. M. Shafer, Humana Press, Totowa, NJ (1997), Vol. 78, pp. 187–204.

30 K. Kanazawa, K. Ohki, S. Nagai, N. Sakura, and Y. Uchida, The 122th Annual Meeting of The Pharmaceutical Society of Japan, Chiba, March, 2002.

31 R. A. Moore, N. C. Bates, and R. E. W. Hancock, *Antimicrob. Agents Chemother.*, **29**, 496 (1986).