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Structure–activity relationships of bacterial outer-membrane permeabilizers based on polymyxin B heptapeptides

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ABSTRACT

A series of cationic cyclic heptapeptides based on polymyxin B have been synthesized for use as permeabilizers of the outer membrane of Gram-negative bacteria. Only analogs with the Dab^2-D-Phe^3-Leu^4-Xxx^5 sequence (Xxx = Dab or Orn) showed a synergistic bactericidal effect when combined with conventional antibiotics, indicating that the Dab^2 residue plays a critical role in permeation of the outer membrane of Gram-negative bacteria.

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The global spread of multidrug-resistant (MDR) bacteria is a growing threat to human health. Antimicrobial and host defense peptides are promising agents for new anti-infective chemotherapy. Polymyxins and collistins are cyclic peptides which exhibit strong antimicrobial activity against Gram-negative bacteria.[1] They can bind to the outer membrane of such bacteria, namely lipopolysaccharide (LPS) or Gram-negative endotoxin. Although polymyxins have fallen out of favor since their use in the 1960s due to nephrotoxicity and neurotoxicity, they have re-emerged as a promising antibiotic to combat against MDR pathogens despite the toxicity. In fact, polymyxin B (PMB, Fig. 1) in combination with other antimicrobials is considered a reasonable and safe treatment option of MDR Gram-negative pathogens[2] and endotoxin shock.[3]

Two N-terminal truncated forms of PMB, namely PMB nonapeptide (PMBN, Fig. 1) and PMB heptapeptide (PMBH, **1**) (Fig. 2), are not bactericidal to Gram-negative bacteria but still can bind to LPS and increase the permeability of their outer membrane to hydrophobic antibiotics.[4] These features make them clinically important leads for the development of outer-membrane permeabilizers[5] and endotoxin-neutralizing agents.[6]

The L-2,4-diaminobutyric acid (Dab) residues in PMB are known for their important role in binding to LPS, which increases the outer-membrane permeability of the antibiotic. To date, many PMB analogs have been designed and synthesized to investigate the association between PMB and LPS. Replacement of the Dab residues with Lys in the cyclic heptapeptide moiety of PMBN has been reported to drastically reduce its outer-membrane permeability.[6] To further clarify this functional loss, we synthesized a series of PMBH analogs possessing various basic amino acid residues through systematic alternations of the Dab residues at the 2-, 5- and 6-positions for determining the essential Dab residues in PMBH for high outer-membrane permeability (Fig. 2).

Compound **1** was previously prepared by enzymatic hydrolysis of PMB[4] or a solution-phase method.[7] As illustrated in Scheme 1, we synthesized PMBH analogs using solid-phase synthesis and cyclization-cleavage (SPS–CC) using Kaiser–Oxime resin,[8] which is known to be efficient for cyclic peptide synthesis.

Based on a previous report for Fmoc-solid-phase peptide synthesis (SPPS) of PMB and PMBN, with cycling between γ-NH_2(Dab) and CO_2H(Thr),[9] we cyclized the linear heptapeptides between α-NH_2(Dab^5) and CO_2H(Leu^4), as Thr(Bzl) is not a suitable C-terminal residue for segment condensation.[10] Boc-Leu-OH (substitution: 0.3–0.5 mmol/g resin) was attached to the resin using dicyclohexylcarbodiimide in CH_2Cl_2. The protected Dab derivatives, Boc-Dab(Z)-OH, and Z-Dab(Boc)-OH, were synthesized as previously reported.[7] The Boc group was removed by treatment with 25% trifluoroacetic acid (TFA)/dichloromethane at room temperature for 30 min. Stepwise elongation of the protected amino acid residues using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU)/diisopropylethylamine (DIEA) gave the protected linear peptidyl resin. The protected

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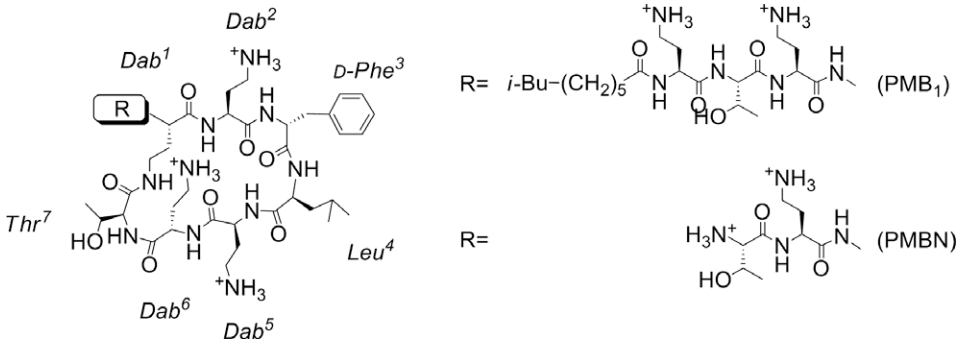
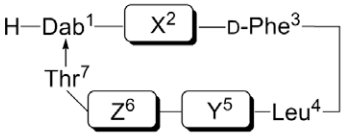
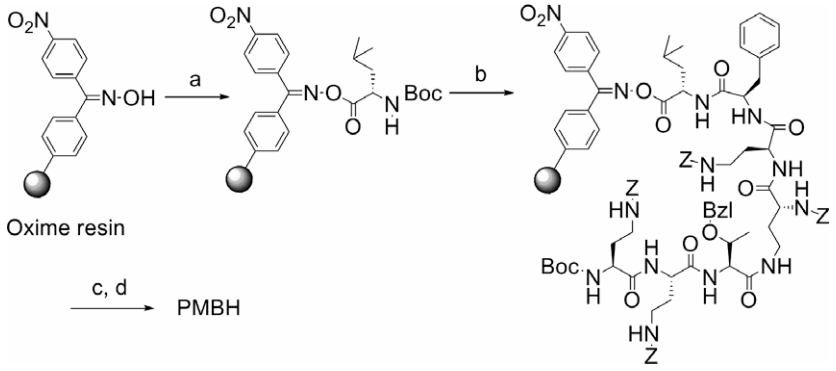


Figure 1. Structure of polymyxin B1 and polymyxin B nonapeptide (PMBN).



	X^2	Y^5	Z^6
PMBH (**1**)	Dab	Dab	Dab
[$Orn^{2,5,6}$]PMBH (**2**)	Orn	Orn	Orn
[$Dap^{2,5,6}$]PMBH (**3**)	Dap	Dap	Dap
[Orn^{2}]PMBH (**4**)	Orn	Dab	Dab
[Orn^{5}]PMBH (**5**)	Dab	Orn	Dab
[Orn^{6}]PMBH (**6**)	Dab	Dab	Orn

Figure 2. Primary structure of PMBH and analogs in this study.



Scheme 1. Solid-phase synthesis of PMBH. Reagents and conditions: (a) Boc-Leu-OH (3 equiv), DCC (3 equiv) in DCM 24 h; (b) Boc-amino acid (3 equiv), HBTU (3 equiv), HOBt (3 equiv), DIEA (5 equiv) in DMF 45 min, deprotection: 25% TFA/CH_2Cl_2, 30 min; (c) DIEA (3 equiv), AcOH (3 equiv) in DMF, 24 h; and (d) H_2, 10% Pd/C, MeOH–H_2O–TFA (40:10:2 (v/v/v)), 5 h.

peptides underwent cleavage from the resin via on-resin macrocyclization[8] and then catalytic hydrogenation in TFA/water/methanol (0.2:1:4, v/v/v) to give the desired PMBH analogs in fair yield; their structures were confirmed by ^{1}H NMR and electrospray ionization-MS. ^{1}H NMR chemical shifts and temperature coefficients of the amide protons of **1**–**6** in DMSO-d_6 were summarized in Table 1.

The ability of cationic peptides to destabilize the outer membrane of Gram-positive bacteria can be evaluated using conventional antibiotics that are only active against these microbes;[11] in the present study, novobiocin and erythromycin were used as the probe antibiotics. The MIC values of these antibiotics in the presence of **1** and its analogs are shown in Table 2. PMBN was used as a positive control. As expected, the antibiotics inhibited the growth of *Escherichia coli* upon the addition of **1** and PMBN. As the concentration of **1** was increased, the antibiotics exhibited higher activity, confirming that PMBH can destabilize the outer membrane of *E. coli*.

Next, we synthesized analogs in which all Dab residues except the 1-position in PMBH were replaced by Orn ([$Orn^{2,5,6}$]PMBH, **2**) or L-2,3-diaminopropionic acid (Dap) ([$Dap^{2,5,6}$]PMBH, **3**) to evaluate their membrane permeability. Interestingly, both analogs were less active than **1**. Furthermore, the PMB_3 analog that was derived from **2** ([$Orn^{5,8,9}$]PMB_3, Fig. 3) exhibited weaker antimicrobial activity against *E. coli* and was inactive against Gram-positive *Staphylococcus aureus* (Table 3). These results indicate that replacement of the basic amino acid residues in PMBH reduces outer-membrane permeability.

Thus, we synthesized three additional PMBH analogs containing only one Orn residue, namely [Orn^2]PMBH (**4**), [Orn^5]PMBH (**5**), and [Orn^6]PMBH (**6**), to further evaluate membrane permeability. Interestingly, only **4** did not induce membrane permeability of

Table 1
^{1}H NMR chemical shifts (δ [ppm] at 20 °C) and temperature coefficients ($\Delta\delta/\Delta T$ [ppb/K]) of the amide protons of **1**–**6** in DMSO-d_6

Residue		**1** X=Dab Y=Dab Z=Dab	**2** X=Orn Y=Orn Z=Orn	**3** X=Dap Y=Dap Z=Dap	**4** X=Orn Y=Dab Z=Dab	**5** X=Dab Y=Orn Z=Dab	**6** X=Dab Y=Dab Z=Orn
Dab[1]	δ(γ-NH)	7.47	7.67	8.53	7.36	7.40	7.31
	$\Delta\delta/\Delta T$	−0.5	—[a]	−5.0	−0.8	−0.6	—[b]
X[2]	δ(α-NH)	8.94	8.27	9.06	8.83	8.74	8.92
	$\Delta\delta/\Delta T$	−3.8	−3.6	−3.3	−5.6	—[c]	−3.2
D-Phe[3]	δ(α-NH)	8.79	8.66	8.62	8.80	8.77	8.78
	$\Delta\delta/\Delta T$	−4.8	−4.7	−3.6	−3.7	—[d]	−4.7
Leu[4]	δ(α-NH)	8.37	8.27	7.93	8.44	8.45	8.41
	$\Delta\delta/\Delta T$	−3.2	−3.5	−1.4	−3.4	−2.9	−3.1
Y[5]	δ(α-NH)	7.78	7.35	9.36	7.81	7.53	7.73
	$\Delta\delta/\Delta T$	−1.2	−1.6	−7.0	−1.2	−0.6	−1.3
Z[6]	δ(α-NH)	8.51	8.66	8.32	8.68	8.79	8.68
	$\Delta\delta/\Delta T$	−3.4	−4.7	−0.9	−3.7	—[e]	−3.2
Thr[7]	δ(α-NH)	7.53	6.99	7.54	7.57	7.31	7.34
	$\Delta\delta/\Delta T$	−1.6	0.02	−0.3	−1.6	−0.4	−0.6

[a] Overlaped with the signal of γ-NH(Dab[1]).
[b] Overlaped with the signal of arom. (D-Phe[3]).
[c] Overlaped with the signal of α-NH(D-Phe[3] and Dab[6]).
[d] Overlaped with the signal of α-NH(Dab[2,6]).
[e] Overlaped with the signal of α-NH(Dab[2] and D-Phe[3]).

Table 2
Minimum Inhibitory Concentrations (MIC, μg/ml) of antibiotics against *E. coli* K12 W3110 in the presence of PMB analogs[a]

Polymyxin B analog[b]	Antibiotic[d]	MIC of antibiotic in the presence of the indicated concentration (μg/ml) of polymyxin B analog						
		0.5	1	2	4	8	16	32
PMBN[c]	Novobiocin	16	8	4	4	2	2	1
	Erythromycin	32	16	8	8	8	8	4
PMBH (**1**)	Novobiocin	64	32	16	8	8	8	4
	Erythromycin	64	32	16	16	8	8	4
[Orn[2,5,6]]PMBH (**2**)	Novobiocin	—[e]	128	64	64	32	32	16
	Erythromycin	64	64	64	64	64	64	32
[Dap[2,5,6]]PMBH (**3**)	Novobiocin	64	64	32	32	16	16	8
	Erythromycin	64	32	32	16	16	16	16
[Orn[2]]PMBH (**4**)	Novobiocin	64	64	64	64	32	32	32
	Erythromycin	64	64	64	64	64	32	32
[Orn[5]]PMBH (**5**)	Novobiocin	32	32	16	16	8	8	4
	Erythromycin	32	16	8	8	8	4	4
[Orn[6]]PMBH (**6**)	Novobiocin	32	32	32	16	8	8	8
	Erythromycin	32	32	16	16	8	8	8

[a] Measured in Mueller–Hinton broth (see Supplementary data).
[b] MICs of PMBH and analogs alone were >128 μg/ml in all cases.
[c] PMBN was obtained from Sigma.
[d] MICs of novobiocin and erythromycin alone were 128 and 64 μg/ml, respectively.
[e] Not determined.

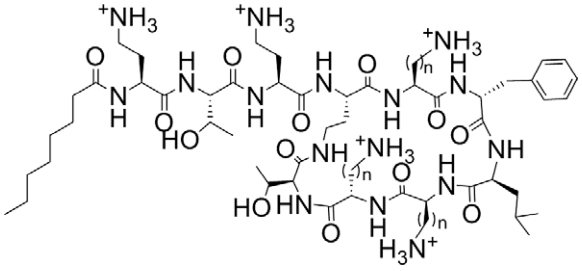


Figure 3. Structure of polymyxin B_3 (n = 2) and [Orn[5,8,9]]PMB_3 (n = 3).

the antibiotics. (Table 1) These results indicated that the basic amino acid residue at the 2-position in PMBH is important for high outer-membrane permeability.

Table 3
MICs (μg/ml) of polymyxin B_3 (PMB_3) and [Orn[5,8,9]]PMB_3[a]

Peptides	MIC (μg/ml)	
	S. aureus 209P	*E. coli* K12 W3110
PMB[b]	64	0.5
PMB_3	>128	0.5
[Orn[5,8,9]]PMB_3	>128	16

[a] Measured in Mueller–Hinton broth (see Supplementary data).
[b] PMB was obtained from Sigma.

CD spectra of **1**–**6** were measured to obtain information about conformational behavior of PMBH analogs in water and membrane-mimetic environment (Fig. 4). 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP) was used as a membrane-mimetic solvent.[12] Native PMBH

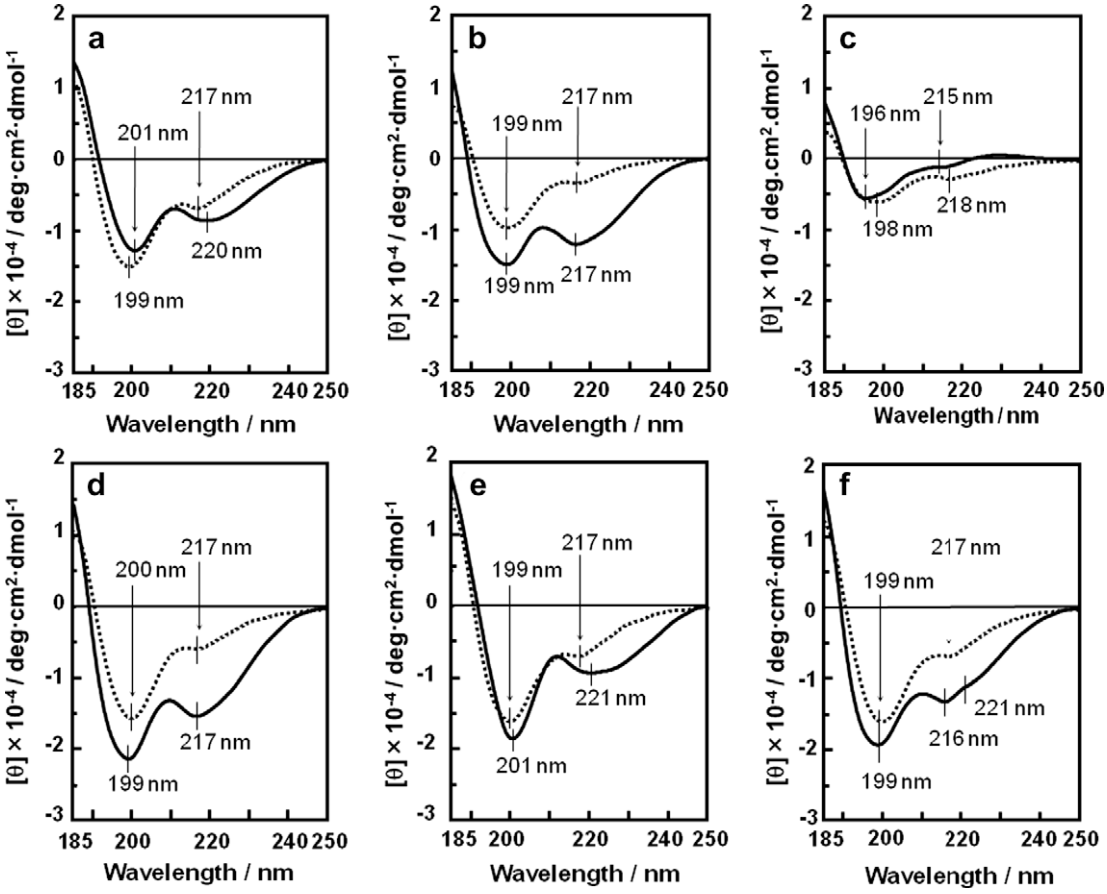


Figure 4. CD spectra of **1**–**6** in water and HFIP. (20 °C, [peptide] = 1 mg/mL) Dotted lines and solid lines indicate the spectra in water and HFIP, respectively. (a) **1**, (b) **2**, (c) **3**, (d) **4**, (e) **5**, and (f) **6**.

(**1**) has two minimum at 199 and 217 nm in water (Fig. 4a, dotted line). In HFIP, the spectrum was slightly red-shifted to 201 and 220 nm (Fig. 4a, solid line). According to the literature, cyclic heptapeptide moiety of PMBN adopts type II′ β-turn around D-Phe-Leu and γ-turn around Thr residue in water.[13] In addition, Kopple and co-workers have reported that CD spectrum of *cyclo*(Orn-D-Phe-Pro)$_2$ has two negative bands centered at 200 and 222 nm in HFIP, which is characteristic of type II′ β-turn conformation.[14] These facts suggests that **1** adopts type II′ β-turn conformation in water and HFIP. In the case of **2**, red-shift of the two negative bands was not observed by changing solvent from water to HFIP but the intensity of the spectrum was significantly increased (Fig. 4b, solid line), suggesting that **2** adopts an ordered conformation in membrane-mimicking environment but the conformation is not preferable for outer-membrane permeation. CD spectra of **3** in water and HFIP were also similar to those of **1** but with weaker intensity, indicating that the main-chain conformation of **3** is slightly distorted (Fig. 4c). We consider that this conformational destabilization reduces the outer-membrane permeability.

Among the PMBH analogs containing only one Orn residue, outer-membrane permeable analog **5** showed similar CD spectral change to native PMBH **1** (Fig. 4e). On the other hand, CD spectra of the inactive analog **4** in water and HFIP were resemble to those of Orn^{2,5,6} analog **2**. CD spectrum of the analog **6** in HFIP showed two negative bands at 199 and 216 nm with a shoulder at 221 nm (Fig. 4f). Although the CD spectrum of **6** has characteristics of both native PMBH **1** and Orn^2 analog **4** in HFIP, the outer-membrane permeability of **6** was comparable to that of **1**.

Recently, Sakura and co-workers have also reported that substitution of the Dab residue at the 5-position in PMB$_3$ by Ala decreases both antimicrobial and LPS-binding activities.[15] The Dab residues in the heptapeptide provide the critical electrostatic contacts with phosphate groups of lipid A.[16,17] In our case, replacement of the Dab residue at position 2 in PMBH by Orn significantly reduced outer-membrane permeability despite remaining cationic side chain. CD spectral comparison among native PMBH (**1**) and the inactive analogs (**2** and **4**) suggested that the conformation of **2** and **4** are somewhat different from that of **1** in membrane-mimicking environment. We consider that the conformational differences could weaken the electrostatic interaction between the cationic side chain of Orn^2 and a phosphate group of Lipid A but the differences still remain unclear. To address this issue, detailed NMR studies are in progress.

In summary, we have found that the replacement of the Dab^2 residue in PMBH by a basic amino acid reduces outer-membrane permeability of Gram-negative bacteria. Our results should contribute to designing outer-membrane permeabilizers or LPS-neutralizing agents using PMB and related peptides.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.01.040.

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