

# Modulation of the Hydrophobic Domain of Polymyxin B Nonapeptide: Effect on Outer-Membrane Permeabilization and Lipopolysaccharide Neutralization

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## ABSTRACT

Polymyxin B nonapeptide (PMBN), a cationic cyclic peptide derived from the antibacterial peptide polymyxin B, is capable of specifically increasing the permeability of the outer membrane (OM) of Gram-negative bacteria toward hydrophobic antibiotics. In this study, we evaluated the contribution of the hydrophobic segment of PMBN (i.e., D-Phe<sup>5</sup>-Leu<sup>6</sup>) to this activity. Accordingly, we synthesized four analogs of PMBN by replacing D-Phe<sup>5</sup> with either with D-Trp or D-Tyr and Leu<sup>6</sup> with Phe or Ala and evaluated their ability to bind cell-free lipopolysaccharide (LPS) and increase bacterial OM permeability. Com-

pared with PMBN, [D-Tyr<sup>5</sup>]PMBN and [Ala<sup>6</sup>]PMBN possessed reduced LPS affinity (IC<sub>50</sub> = 2.5, 25, and 12  $\mu$ M, respectively) and significantly reduced OM permeability and LPS neutralization activity. [Phe<sup>6</sup>]PMBN exhibited rather similar affinity to cell-free LPS (IC<sub>50</sub> = 5  $\mu$ M) and the same OM permeability capacity as PMBN. However, [D-Trp<sup>5</sup>]PMBN, despite its similar affinity to cell-free LPS (IC<sub>50</sub> = 4  $\mu$ M), had moderately reduced OM permeability capacity. These results demonstrate the significant role of the PMBN hydrophobic segment in promoting biological activity.

The bacterial endotoxin lipopolysaccharide (LPS) is the major antigen of the outer membrane (OM) of Gram-negative bacteria. The presence of LPS in circulation induces uncontrolled activation of immune cells followed by cytokine-mediated damage to blood vessels and a decrease in vascular resistance, frequently leading to collapse of organs and death (Karima et al., 1999). Hence, neutralization of the devastating effects of LPS is a major target in combating endotoxemia (Lynn and Cohen, 1995). LPS is composed of three major parts, one of which is lipid A, a highly conserved hydrophobic region. Lipid A is a phosphoglycolipid molecule composed of  $\beta$ -(1,6)-linked D-glucosamine disaccharide substituted by charged phosphate groups at positions 1 and 4 and 3-hydroxy saturated fatty acids of 12 to 16 carbon atoms (Zahringer et al., 1994). Lipid A, the toxic part of LPS, is a target for cationic proteins and peptides such as polymyxin B (Rietschel et al., 1987).

Polymyxin B (PMB) is a mixture of naturally occurring cationic cyclic decapeptide derivatives isolated from *Bacillus polymyxa* (Ainsworth et al., 1947; Benedict and Langlykke 1947; Stanly et al., 1947). PMB is highly bactericidal to Gram-negative bacteria and considered one of the most efficient cell-permeabilizing compounds (Evans et al., 1999),

largely because of its high-affinity binding to lipid A (Moore et al., 1986). Although PMB is bactericidal to multidrug-resistant Gram-negative bacteria and able to neutralize the toxic effects of released LPS, its therapeutic applications are very limited because of its relative high toxicity (Vinnicombe and Stamey, 1969; Kunin and Bugg, 1971). Because most of the toxic activity of PMB resides at the N-terminal fatty amino acid 6-methylheptanoic/octanoic-Dab, the removal of this segment by proteolytic cleavage, using ficin or papain, generated a nontoxic peptide named polymyxin B nonapeptide (PMBN) (Chihara et al., 1973; Duwe et al., 1986) (Fig. 1, Table 1). Although PMBN is an extremely poor antimicrobial compound, it is still capable, like PMB, of binding to LPS (Vaara and Viljanen, 1985) and preserving a significant OM-permeabilizing action, thus rendering Gram-negative bacteria susceptible to various hydrophobic antibiotics (Vaara and Vaara, 1983; Viljanen and Vaara, 1984). Such susceptibility was evidenced by a drastic sensitization of bacteria toward hydrophobic antibiotics such as rifampin, erythromycin, clindamycin, fusidic acid, and novobiocin against which the intact OM is an effective barrier (Vaara and Vaara, 1983). PMBN was able to protect mice challenged with *Klebsiella pneumoniae* in combination with erythromycin (Ofek et al.,

**ABBREVIATIONS:** LPS, lipopolysaccharide; OM, outer membrane; PMB, polymyxin B; PMBN, polymyxin B nonapeptide; Dab, 2,4-diaminobutyric acid; Cbz, benzyloxycarbonyl; DMF, dimethylformamide; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; GRAVY, grand average of hydropathicity; ISB, Isotonic Sensitest Broth; CFU, colony forming unit; MIC, minimal inhibitory concentration; CD, circular dichroism; TNF, tumor necrosis factor; IL, interleukin; RP-HPLC, reversed phase-high-performance liquid chromatography; MM6, MONO-MAC-6.

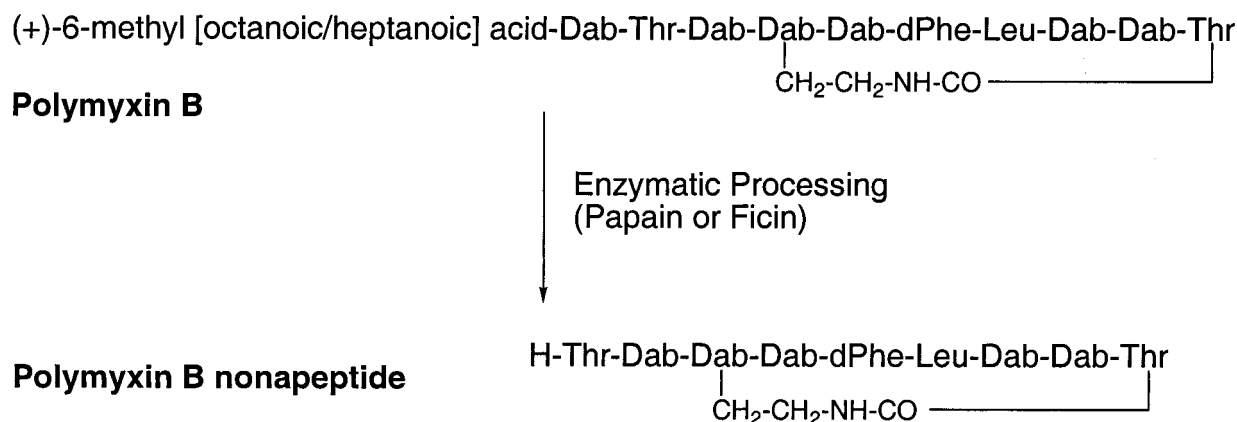


Fig. 1. The structures of polymyxin B and polymyxin B nonapeptide.

1994). Structure-function analysis of PMBN has revealed the significance of the unique structural architecture of the naturally derived PMBN molecule (Tsubery et al., 2000a,b). The interaction of PMB as well as of PMBN with LPS may be mediated by two processes: electrostatic interactions between the positive charges of the peptidic Dab residues and the negative charges of the phosphates of LPS, and hydrophobic contacts of the N-terminal fatty acid 6-methylheptanoic/octanoic and D-Phe<sup>5</sup>-Leu<sup>6</sup> of PMB with the hydrophobic core of the lipid A moiety of LPS (Srimal et al., 1996; Surajit et al., 1997; Pristovsek and Kidric, 1999). Several structural aspects pertaining to the contribution of the positive charges of PMBN to its interaction with LPS were recently reported (Tsubery et al., 2000a).

To better understand the contribution of the hydrophobic segment D-Phe<sup>5</sup>-Leu<sup>6</sup> to the peptide-LPS association, the D-Phe<sup>5</sup> residue was replaced either by D-Trp or D-Tyr and Leu<sup>6</sup> by Phe or Ala. The interaction of the resultant PMBN analogs with LPS and their biological activities were investigated.

## Materials and Methods

**Synthesis of PMBN Analogs.** All protected amino acids, coupling reagents, and polymers were obtained from Nova Biochemicals (Laufelfingen, Switzerland) and Bachem (Bubendorf, Switzerland). Synthesis-grade solvents were obtained from Labscan (Dublin, Ireland). Linear peptide chains were assembled by conventional solid phase synthesis, using an automated solid phase multiple peptide synthesizer (AMS-422; ABIMED, Langenfeld, Germany). 9-Fluorenylmethoxycarbonyl strategy was employed throughout the peptide-chain assembly (Atherton and Sheppard, 1989) following the company's protocol. Synthesis was initiated by using 9-fluorenylmethoxycarbonyl-Thr(tBu)-Wang resin (0.7 mmol/g) and performed on a 25-μmol scale. Side-chain amino protecting groups for 2,4-diaminobutyric acid were *tert*-utyloxycarbonyl and benzyloxy-

carbonyl (Cbz). Coupling was achieved using 4 equivalents of benzotriazole-1-yl-oxy-tris-pyrolidino-phosphonium hexafluorophosphate as a coupling agent in presence of 8 equivalents of 4-methylmorpholine, all dissolved in dimethylformamide (DMF). The fully protected peptide-bound resin was treated with piperidine (20% in DMF) for 20 min, and the free N-terminal amino moiety of Thr<sup>9</sup> reacted with 4 equivalents of *N*-(benzyloxycarbonyloxy)succinimide and 4 equivalents of *N,N*-diisopropylethylamine in DMF for 3 h. The fully protected peptide-bound resin was treated with trifluoroacetic acid (TFA)/water/triethylsilane (95:2.5:2.5; v/v/v) for 1 h at room temperature and the reaction mixture was filtered. The solution was cooled down to 4°C and the partially protected linear peptide was precipitated with ice-cold di-*tert*-utyl methyl ether/petroleum ether (30–40°C; 1:3, v/v) and centrifuged. The pellet was washed with the same mixture, dissolved in water/acetonitrile (2:3, v/v) and the solution was lyophilized. Cyclization was performed in DMF at peptide concentrations of 1 mM using benzotriazole-1-yl-oxy-tris-pyrolidino-phosphonium hexafluorophosphate/1-hydroxybenzotriazole/4-methyl morpholin (4:4:8 equivalents) as reagents for 2 h at room temperature (yield >95% according to analytical HPLC). The reaction mixture was concentrated in high vacuum and the cyclic peptidic product was precipitated by treatment with water. Final deprotection, i.e., removal of Cbz, was achieved by a mixture of TFA/bromotrimethylsilane/thioanisole/ethanedithiol/*m*-cresol (58:10:19:10:3, v/v/v/v/v) at 0°C for 1 h. The product was precipitated by the addition of cold *tert*-utylmethyl ether, centrifuged, washed with cold *tert*-utylmethyl ether, and lyophilized from water.

**Reversed-Phase HPLC Purification and Analyses.** The crude synthetic peptides were purified with a prepacked LichroCart RP-18 column (250 × 10 mm; 7-μm bead size; E. Merck, Darmstadt, Germany) employing a binary gradient formed from 0.1% TFA in water (solution A) and 0.1% TFA in 75% acetonitrile in water (solution B). The column was eluted at *t* = 0 min, B = 0%, and at *t* = 48 min, B = 60%, using a flow rate of 5 ml/min. For purity evaluation, analytical reversed-phase HPLC was performed using a prepacked Lichrospher-100 RP-18 column (250 × 4 mm, 5-μm bead size; E. Merck)

TABLE 1  
Peptide primary structure and identification

Peptide	Sequence <sup>a</sup>	ESMS ( <i>m/z</i> )	Amino Acid Analysis <sup>b</sup>
PMBN	TXcyclo[XXFLXXT]	963.6 (962.6)	T,1.92; X,4.98; L,1.0; F,1.0
[Phe <sup>6</sup> ]PMBN	TXcyclo[XXFFXXT]	998.0 (997.1)	T,1.89; X,4.95; F,2.0
[Ala <sup>6</sup> ]PMBN	TXcyclo[XXFAXXT]	921.9 (920.2)	T,1.93; X,5.09; A,1.0; F,1.0
[D-Trp <sup>5</sup> ]PMBN	TXcyclo[XXWLXXT]	1002.6 (1001.4)	T,1.78; X,4.88; L,1.0
[D-Tyr <sup>5</sup> ]PMBN	TXcyclo[XXYLXXT]	979.4 (978.6)	T,1.71; X,4.67; L,1.0; Y,1.0

ESMS, electrospray ionization mass spectrometry.

<sup>a</sup> D-amino acid are in boldface. X = Dab.

<sup>b</sup> Trp destroyed upon hydrolysis procedure.

with the following binary gradient: at  $t = 0$  min,  $B = 10\%$ , and at  $t = 40$  min,  $B = 60\%$  at a flow rate of 0.8 ml/min.

HPLC separations and analyses were performed using a liquid chromatography system (SP8800; Spectra-Physics, Stahnsdorf, Germany) equipped with a variable-wavelength absorbance detector (ABI 757; Applied Biosystems Foster City, CA). The column effluents were monitored by UV absorbance at 220 nm. Purity of peptides was  $>98\%$  (yields, 30–45%). The corresponding fractions were collected, lyophilized, and analyzed after exhaustive acid hydrolysis and pre-column reaction with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate to ascertain amino acid composition (2690 separations Module; Waters, Milford, MA). Mass spectra analysis was performed to determine molecular weights (VG-platform-II electrospray single quadrupole mass spectrometer; Micro Mass, Manchester, UK).

**PMBN.** PMBN was prepared by proteolysis of PMB (Sigma, St. Louis, MO) with papain (Sigma) as described elsewhere (Danner et al., 1989). The crude product was purified ( $>98\%$ ) by HPLC, then analyzed and characterized as described above.

**Dansyl-PMBN.** Dansyl-PMBN was synthesized as described elsewhere (Tsubery et al., 2000a).

**Calculation of Peptides Hydropathicity (GRAVY).** Peptides grand average hydropathicity (GRAVY) was calculated using the Web-available program developed by Kyte and Doolittle (1982) (<http://www.expasy.ch/cgi-bin/protscale.pl>). To calculate relative hydropathicity values of the peptides, the Dab residues, (i.e., the unnatural amino acid) were replaced by Lys residues and the peptides were considered linear. Each amino acid was assigned a hydropathy index, a value reflecting its relative hydrophilicity and hydrophobicity. The sum of the hydropathy indices of a given sequence divided by the number of residues in the sequence generates the GRAVY score. In our case, the difference is in only one amino acid; hence, the GRAVY score reflects the different hydropathy indices of the residues at positions 5 and 6.

**Molecular Modeling.** The coordinates of PMB were obtained from Pristovsek and Kidric (1999). The model structure of LPS was constructed using the MSI package (MSI Inc., San Diego, CA). The complex PMB-lipid A was assembled in a way that the interactions were as described by Pristovsek and Kidric (1999). The model structure of the complex was energy minimized in vacuum. Initially, only the lipid A molecule was allowed to change, whereas the PMB molecule was fixed at the NMR structure. Subsequently, the restrictions were removed and both molecules were allowed to change. The complex PMBN-LPS was modeled by removal of residues -1 (6-methyl heptanoic acid) and 0 (Dab) from the above PMB-LPS complex and the energy was minimized again. The energy minimization was performed using the consistent-valence force field within the Discover module of the MSI package (MSI, Inc.). The convergence requirement was for the maximum derivative to be less than 0.001.

**Determination of Minimal Inhibitory Concentration.** The employed clinical isolates of *Escherichia coli*, *K. pneumoniae*, and *Pseudomonas aeruginosa* were obtained as described elsewhere (Ofek et al., 1994). The Gram-negative bacteria were grown on nutrient agar plates (Difco Laboratories, Detroit, MI) and kept at  $4^{\circ}\text{C}$ . Lyophilized aliquots of peptides (2 mg, determined by weight and ascertained by amino acid composition analysis) were dissolved in sterile double distilled water and filtered using a  $0.2\text{-}\mu\text{m}$  Acrodisc filter (Gelman Sciences, Ann Arbor, MI). An overnight culture in Isotonic Sensitest Broth (ISB; Oxoid, Basingstoke, Hampshire, England) was adjusted to  $1 \times 10^5$  CFU/ml and inoculated onto microtiter plate wells, each containing  $100\text{ }\mu\text{l}$  of a serial 2-fold dilution ( $1000\text{--}0.5\text{ }\mu\text{g/ml}$ ) of the tested antibiotics/peptides in ISB. The MIC was defined as the lowest concentration at which no visible bacterial growth was detected after incubation for 20 h, at  $37^{\circ}\text{C}$ . Results are reported for three to four separate tests.

**Outer Membrane Permeabilizing Activity.** Bacterial suspension ( $10\text{ }\mu\text{l}$ ,  $1 \times 10^5$  CFU) was inoculated onto microtiter plate wells containing  $100\text{ }\mu\text{l}$  of a serial 2-fold dilution ( $1000\text{--}0.5\text{ }\mu\text{g/ml}$ ) of novobiocin (Sigma) in ISB. To each well,  $10\text{ }\mu\text{l}$  of the test peptide was

added to a final concentration of  $50\text{ }\mu\text{g/ml}$ . The MIC was defined as the lowest concentration at which there was no visible bacterial growth after incubation for 20 h, at  $37^{\circ}\text{C}$ . Results are reported for three to four separate tests.

**Dansyl-PMBN Binding and Displacement Assay.** The displacement assay was performed as follows:  $0.55\text{ }\mu\text{M}$  dansyl-PMBN was added to a quartz cuvette containing LPS solution (2 ml,  $3\text{ }\mu\text{g/ml}$ ,  $\sim 2 \times 10^{-7}\text{ M}$ ) in 5 mM HEPES, pH 7.2, and allowed to equilibrate at room temperature for 10 to 15 min. Subsequently, small portions ( $5\text{--}10\text{ }\mu\text{l}$ ) of peptide solutions ( $1 \times 10^{-6}\text{--}1 \times 10^{-3}\text{ M}$ ) were added. Inhibition of fluorescence was measured 5 min after each addition of peptides. Percent inhibition was plotted as a function of the added peptide concentration and  $\text{IC}_{50}$  values were calculated from maximal specific displacement ( $I_{\text{max}}$ ).

**Circular Dichroism (CD) Studies.** CD spectra were recorded on an Aviv-202 circular dichroism spectrometer (Lakewood, NJ). Duplicate scans over a wavelength range of 190 to 250 nm were taken at a chart speed of 12 nm/min in a 0.1-cm path-length quartz cell at room temperature. Peptides were dissolved in 5 mM phosphate buffer, pH 7.2, at a final concentration of 0.2 mM. A baseline was recorded and subtracted after each spectrum. Ellipticity is reported as the mean residue ellipticity  $[\theta]$  in degrees  $\text{cm}^2\text{ dmol}^{-1} \times 10^{-3}$ .

**Inhibition of Cytokine Release.** Peptide solutions (1, 10, and  $100\text{ }\mu\text{M}$ , final concentration) were incubated (10 min,  $37^{\circ}\text{C}$ ) with *E. coli*-LPS (20 ng/ml, final concentration) in an assay medium (RPMI medium/10% newborn calf serum, 1 mM sodium pyruvate, 1% non-essential amino acids, and  $9\text{ }\mu\text{g/ml}$  insulin) in polypropylene tubes. MONO-MAC-6 (MM6) cells ( $5 \times 10^5$ /tube) were added and tubes were incubated for 4 h for TNF $\alpha$  production and 18 h for IL-6 production. Cytokine levels were determined using matched antibody pairs according to the manufacturer's guide to custom enzyme-linked immunosorbent assay development protocol (Endogen, MA).

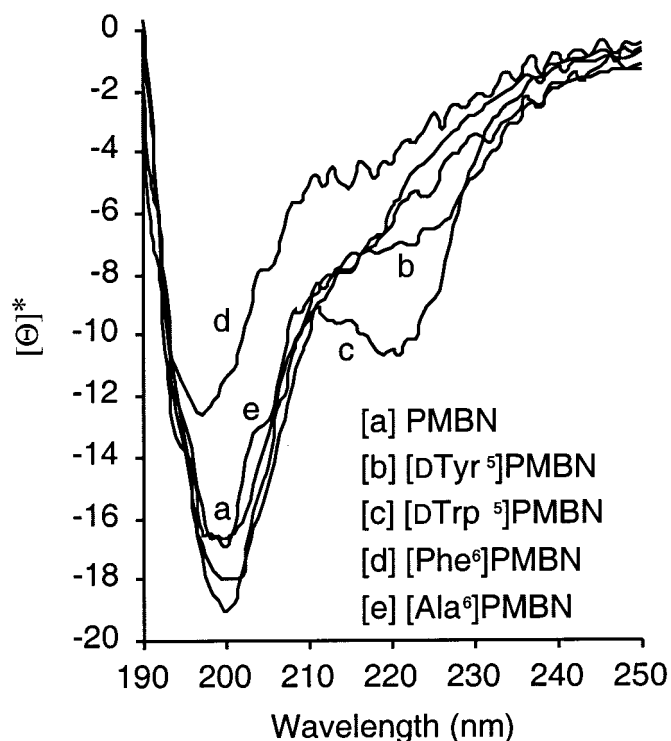
## Results

Four PMBN analogs, [D-Trp<sup>5</sup>]PMBN, [D-Tyr<sup>5</sup>]PMBN, [Phe<sup>6</sup>]PMBN, and [Ala<sup>6</sup>]PMBN were synthesized using a combination of linear peptide synthesis and cyclization in solution. The peptides were purified to homogeneity ( $>98\%$ ) by HPLC and their correct amino acid composition and calculated molecular weights were ascertained by amino acid analysis and electrospray mass spectrometry, respectively (Table 1). D-Phe<sup>5</sup> was replaced either with D-Trp or D-Tyr, whereas Leu<sup>6</sup> was replaced with Phe or Ala (Fig. 1, Table 1). Considering the hydrophobicity on the basis of relative retention time on a RP-18 column, [D-Trp<sup>5</sup>]PMBN and [Phe<sup>6</sup>]PMBN were equally hydrophobic to PMBN whereas [D-Tyr<sup>5</sup>]PMBN and [Ala<sup>6</sup>]PMBN were less hydrophobic (Table 4). The hydropathicity scale (Kyte and Doolittle, 1982), however, indicated that [D-Trp<sup>5</sup>]PMBN was much less hydrophobic than PMBN (Table 4).

The peptides' structure was evaluated using CD measurements. [D-Tyr<sup>5</sup>]PMBN and [Ala<sup>6</sup>]PMBN (0.2 mM) in phosphate buffer exhibited a random structure similar to 0.2 mM PMBN. A minor difference in the CD pattern was observed at 218 to 230 nm. [D-Trp<sup>5</sup>]PMBN at the same concentration (0.2 mM) exhibited a maximal negative ellipticity at 200 nm and an additional maximal negative ellipticity at 220 nm. [Phe<sup>6</sup>]PMBN exhibited reduced ellipticity compared with PMBN and a maximal negative ellipticity at 297 nm (Fig. 2).

**Antimicrobial and OM Permeabilization Activities of PMBN Analogs.** Unlike PMBN, none of the analogs was active against *P. aeruginosa* ( $\text{MIC} > 250\text{ }\mu\text{g/ml}$ , Table 2). The peptides' ( $50\text{ }\mu\text{g/ml}$ ) potency to increase the bacterial OM permeability toward novobiocin was evaluated. [Phe<sup>6</sup>]PMBN





**Fig. 2.** Circular dichroism spectra of PMBN and its two analogs. Measurements were taken with peptide solutions (0.2 mM) in phosphate buffer (5 mM, pH 7.5) at 25°C.  $[\Theta]$  is the mean residue ellipticity  $\times 10^{-3}$  in degrees  $\text{cm}^2 \text{dmol}^{-1}$ .

was as potent as PMBN, whereas [Ala<sup>6</sup>]PMBN was 4- to 8-fold less active than PMBN (Table 3). [D-Trp<sup>5</sup>]PMBN was 8-fold less potent in the OM permeabilization assay compared with PMBN. The activity of [D-Tyr<sup>5</sup>]PMBN, however, was very weak (Table 3).

**LPS Binding.** The interaction of the PMBN analogs with the bacterial cell-free LPS was quantified using the dansyl-PMBN displacement assay. Table 4 shows that the peptide concentrations required for 50% displacement were 4 and 5  $\mu\text{M}$  for [D-Trp<sup>5</sup>]PMBN and [Phe<sup>6</sup>]PMBN, respectively, similar to PMBN ( $\text{IC}_{50} = 2.5 \mu\text{M}$ ). However, [D-Tyr<sup>5</sup>]PMBN and [Ala<sup>6</sup>]PMBN exhibited significantly lower potency compared with PMBN (Table 4).

**Neutralization of LPS Toxic Effects.** The ability of PMBN and its analogs to neutralize stimulatory effects of LPS on the human monocyte cell line MM6 was tested. Peptides were preincubated with *E. coli*-LPS and the mixture was allowed to interact with the MM6 cells. Levels of released TNF $\alpha$  and IL-6 were measured after 4 and 18 h, respectively, using enzyme-linked immunosorbent assay. As shown in Fig. 3, stimulation of MM6 with LPS (20 ng/ml) triggered the release of  $\sim 40$  and  $\sim 70$  ng/ml of IL-6 and

**TABLE 2**  
MIC values of PMBN peptides

Peptide	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>
		$\mu\text{g/ml}$	
PMBN	>500	>500	8
[Phe <sup>6</sup> ]PMBN	>500	>500	>250
[Ala <sup>6</sup> ]PMBN	>500	>500	>250
[D-Trp <sup>5</sup> ]PMBN	500	>500	>500
[D-Tyr <sup>5</sup> ]PMBN	>500	>500	>500

**TABLE 3**  
MIC values of novobiocin  
Peptides were at a final concentration of 50  $\mu\text{g/ml}$ .

Antibiotic	<i>E. coli</i>	<i>K. pneumoniae</i>
	$\mu\text{g/ml}$	
Novobiocin	62	250
Novobiocin + PMBN	1	4
Novobiocin + [Phe <sup>6</sup> ]PMBN	2	4
Novobiocin + [Ala <sup>6</sup> ]PMBN	8	16
Novobiocin + [D-Trp <sup>5</sup> ]PMBN	8	31
Novobiocin + [D-Tyr <sup>5</sup> ]PMBN	31	125

**TABLE 4**  
Peptide affinity to LPS and relative hydrophobicity

Displacement of LPS-bound dansyl-PMBN by PMBN analogs. Increasing concentrations of PMBN peptides were added to *E. coli*-LPS solution (3  $\mu\text{g/ml}$ ) bound to dansyl-PMBN (0.55  $\mu\text{M}$ ). The fluorescence inhibition was measured 5 min after each addition at excitation and emission wavelengths of 340 and 485 nm, respectively. Data represent the mean derived from four experiments. Analytical HPLC was performed on Vydac<sup>TM</sup> RP-18 column using a linear gradient of  $t = 0$  min,  $B = 0\%$ ;  $t = 55$  min,  $B = 80\%$ . Grand average of hydropathicity (GRAVY) was calculated using the Web-available program developed by Kyte and Doolittle (<http://www.expasy.ch/cgi-bin/protscale.pl>).

Peptide	$\text{IC}_{50}$	$t_R$	Hydropathicity (GRAVY)
	$\mu\text{M}$	min	
PMBN	2.5	21.3	-1.589
[Phe <sup>6</sup> ]PMBN	5	21.3	-1.700
[Ala <sup>6</sup> ]PMBN	12	15.7	-1.811
[D-Trp <sup>5</sup> ]PMBN	4	21.7	-2.000
[D-Tyr <sup>5</sup> ]PMBN	25	16.4	-2.044

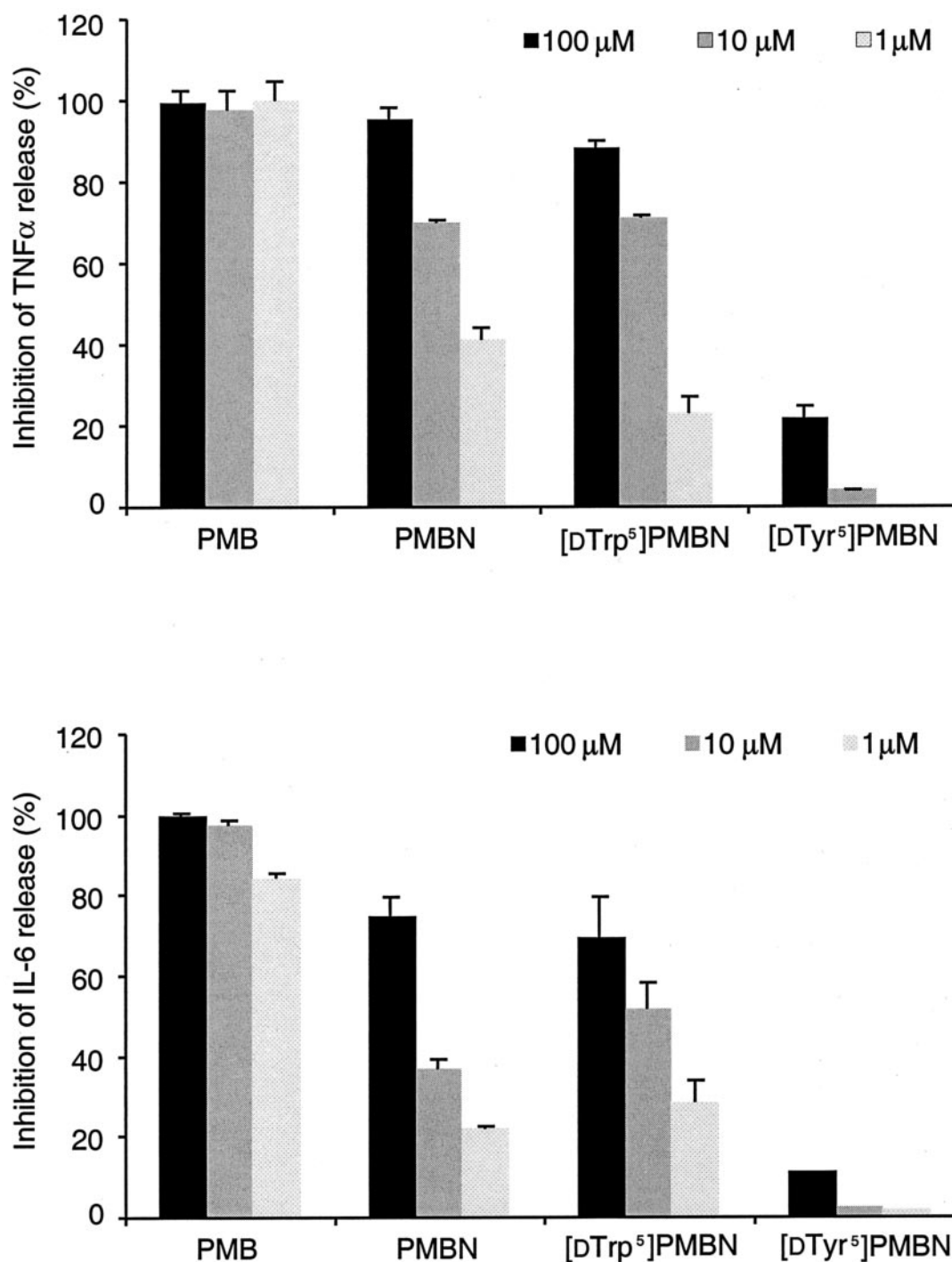
TNF $\alpha$ , respectively. PMBN and [D-Trp<sup>5</sup>]PMBN were equally potent inhibitors of TNF $\alpha$  release (95–23%). A similar effect was observed for the inhibition of IL-6 release. PMBN and [D-Trp<sup>5</sup>]PMBN inhibited IL-6 release at the range of 75–20% in a dose-dependent manner (1–100  $\mu\text{M}$ ). However, [D-Tyr<sup>5</sup>]PMBN was a much weaker inhibitor, causing no significant inhibition either in the TNF $\alpha$  or the IL-6 assay (20 and 10%, respectively). As shown, PMB exhibited maximal inhibition capacity even at 1  $\mu\text{M}$  concentration.

**Molecular Modeling of Peptide-LPS Complex.** The structure of PMB bound to LPS was determined by NMR spectroscopy (Pristovsek and Kidric, 1999). Based on the coordinates provided for PMB, a model corresponding to 1:1 PMB/lipid A complex was generated (Fig. 4). The complex is characterized by electrostatic interactions between four of the five positive side chains of PMB (positions 0, 4, 7, and 8, Fig. 4) and two of the negative phosphate groups of the phosphorylated lipid A head-groups. The hydrophobic side chains at the N terminus and at positions 5 and 6 of PMB interact with the aliphatic chains of lipid A. The PMBN-LPS complex was generated upon removal of residues -1 (6-methyl heptanoic acid) and 0 (Dab) from the PMB molecule in the PMB-LPS complex and energy minimization. The minimized structure suggests that the interaction between the positive Dab moiety at position 0 and the phosphate group in lipid A is replaced by an electrostatic interaction between Dab<sup>2</sup> of PMBN (numbering as for PMB) and the same phosphate group. The positive side chain of Dab<sup>2</sup> in PMB is exposed to the solvent in the PMB-LPS complex (Fig. 4). The hydrophobic segment D-Phe<sup>5</sup>-Leu<sup>6</sup> still interacts with the aliphatic chains of lipid A.

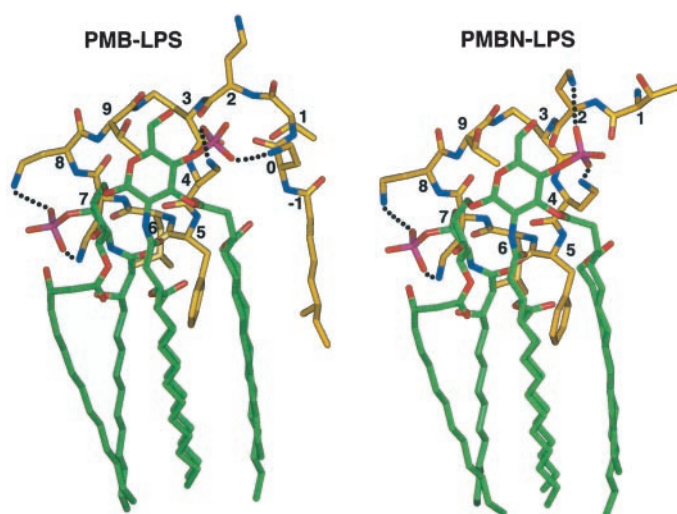
## Discussion

The interaction of PMB and PMBN with their bacterial target molecule, LPS, was extensively studied in recent years. The intermolecular peptide-lipid associations were attributed to the amphiphilic features of PMB, involving primarily the hydrophobic face of the peptide enforced into an appropriate alignment by the positively charged side chains of the 2,4-diaminobutyric acid residues (Srimal et al., 1996). Similar structural considerations were deduced and elabo-

rated from two-dimensional NMR and molecular dynamics studies of PMBN and *E. coli*-LPS (Bhattacharjya et al., 1997; Pristovsek and Kidric, 1999). According to the latter study, PMB and PMBN share a type II'  $\beta$ -turn structure for the free peptide and an envelope-like fold of the peptide ring for the LPS-bound peptide. The present structure-function study of PMBN focused on the D-Phe<sup>5</sup>-Leu<sup>6</sup> segment, the peptide ring hydrophobic domain of PMBN. As shown in our previous study, the substitution of D-Phe<sup>5</sup> with L-Phe (i.e., construc-



**Fig. 3.** Inhibition of TNF $\alpha$  (A) and IL-6 (B) release from LPS-stimulated MM6 cells. Peptides (1–100  $\mu$ M) were incubated (10 min) with LPS (20 ng/ml) and MM6 cells ( $0.5 \times 10^6$ ) were added. Levels of TNF $\alpha$  and IL-6 were detected after 4 and 18 h, respectively. Data are expressed as the percentage from baseline (20 ng/ml LPS, normalized to 100%) and represented as mean  $\pm$  S.D. derived from three experiments.



**Fig. 4.** The structure of complexes PMB-LPS and PMBN-LPS. The PMB-LPS complex was generated based on coordinates provided elsewhere (Pristovsek and Kidric, 1999). The PMBN-LPS complex was assembled by removal of residues -1 and 0 from the above PMB-LPS complex and energy minimization.

tion of [L-Phe<sup>5</sup>]PMBN) resulted in loss of activity (Tsubery et al., 2000a). This finding is in line with the notion that position  $i + 1$  of type II'  $\beta$ -turn is generally occupied by a D-amino acid (or Gly) (Venkatachalam, 1968). Indeed, position 2 of all naturally occurring polymyxins is occupied by the D-form of Phe or Leu, whereas position 6 is occupied by the L-form of either Leu, Ile, or Thr. In the present study, PMBN was modified so that the configuration at positions 5 and 6 was retained but the hydrophobicity was changed.

According to the hydrophobicity scale the amino acids follow the order Phe > Leu > Trp > Ala > Tyr (Kyte and Doolittle, 1982; Engelman et al., 1986). The hydrophobicity of the various newly synthesized PMBN analogs was evaluated experimentally using RP-HPLC as well as calculated based on the grand average of hydrophobicity (GRAVY) program developed by Kyte and Doolittle (1982). According to RP-HPLC, the peptides hydrophobicity follow the order [D-Trp<sup>5</sup>]PMBN > PMBN = [Phe<sup>6</sup>]PMBN > [Ala<sup>6</sup>]PMBN > [D-Tyr<sup>5</sup>]PMBN. However, the GRAVY scale generated the following order: PMBN > [Phe<sup>6</sup>]PMBN > [Ala<sup>6</sup>]PMBN > [D-Trp<sup>5</sup>]PMBN > [D-Tyr<sup>5</sup>]PMBN (Table 4). The difference might perhaps be caused by the actual overall structures that the peptides assume in the aqueous-organic milieu (acetonitrile-water).

The present CD measurement show that [D-Trp<sup>5</sup>]PMBN has a relative minor negative shoulder at 218 to 240 nm compared with PMBN and exhibits a rather marked negative band centered at 222 nm. This additional maximal negative ellipticity, however, could be attributed to the contribution of the indole moiety of Trp (Atkinson and Pelton, 1992). The CD spectra of [Ala<sup>6</sup>]PMBN was identical to that of PMBN, whereas [Phe<sup>6</sup>]PMBN exhibited a CD pattern with reduced ellipticity. However, no major peptide structural changes were found among the four peptides and all displayed random coil structure.

Although most bacteria are resistant to the bactericidal activity of PMBN, *P. aeruginosa* is an exception. None of the newly synthesized PMBN analogs was able to inhibit the growth of *P. aeruginosa*. So far, any modification made in

PMBN resulted in loss of direct bactericidal activity toward *P. aeruginosa* (Tsubery et al., 2000a,b). When the peptides were evaluated for their ability to permeate the bacterial OM, [D-Tyr<sup>5</sup>]PMBN was found to be inactive, whereas [D-Trp<sup>5</sup>]PMBN and [Ala<sup>6</sup>]PMBN exhibited reduced OM permeabilizing activity compared with PMBN. [Phe<sup>6</sup>]PMBN, however, was as active as PMBN. This loss and reduced activities of the respective peptides might perhaps be explained by their GRAVY (Kyte and Doolittle, 1982) (that is, the lower the hydrophobicity, the lower the antibacterial activity). Indeed, [Phe<sup>6</sup>]PMBN has an OM permeabilization ability identical to that of PMBN and the values of their hydrophobicity index are close. These observations are consistent with the finding that, as with other antimicrobial peptides, amphipathicity is an essential parameter for the activity of PMB and its analogs (Liao et al., 1995; Srimal et al., 1996; Bhattacharya et al., 1997; Hancock, 1997; Pristovsek and Kidric, 1999).

However, the affinity of the peptides to cell-free LPS as evaluated by the Dansyl-PMBN displacement assay had greater correlation with the hydrophobicity scale drawn by RP-HPLC (Table 4). Thus, the interaction of the peptides with cell-free LPS is somehow different from their interaction with cell-bound LPS.

The interaction of LPS with immune cells via its receptor (CD 14) results in stimulatory effects leading, among others, to enhanced release of cytokines such as TNF $\alpha$  and IL-6 (Viriyakosol and Kirkland, 1995). The ability of PMBN and its analogs [D-Trp<sup>5</sup>]PMBN and [D-Tyr<sup>5</sup>]PMBN to neutralize the stimulatory effect of LPS was evaluated. This capacity relates to the binding of the peptides to the LPS molecule and impairing its association with cognate immune cell receptors. Thus, both PMBN and [D-Trp<sup>5</sup>]PMBN bound to LPS and prevented its interaction with the LPS-receptor on MM6 cells in a similar dose-dependent manner. [D-Tyr<sup>5</sup>]PMBN, however, was not able to inhibit the release of TNF $\alpha$  and IL-6. These results are in good correlation with the affinity of the peptides to cell-free LPS and to the hydrophobic scale drawn by RP-HPLC.

The structures of PMB and PMBN and its analogs bound to LPS were modeled based on coordinates provided for PMB (Pristovsek and Kidric, 1999) (Fig. 4). Elimination of the positively charged residue Dab<sup>0</sup>, which interacts with the negatively charged phosphate group in the PMB-LPS complex, is not likely to significantly weaken the LPS-peptide interaction. This is partly because of the conformational change of the side chain at position 2, allowing for the efficient interaction of the Dab<sup>2</sup> residue with the negatively charged phosphate group and partly because the exposed phosphate can interact with water (Fig. 4). Thus, modifications at position 2 may lead to the weakening of the interaction. This assumption is supported by our previous observation that substitution at position 2 with Lys reduces the peptide OM permeabilizing activity as well as its affinity to LPS (Tsubery et al., 2000a). Preservation of the cationic nature of the peptides is of great significance in relation to their ability to enhance penetration through biological membrane. However, it was shown that various linear cationic analogs of PMBN analogs are devoid of activity, suggesting that the cyclic structure is of major significance (Vaara, 1991).

The hydrophobic interaction of PMBN with the bacterial



membrane is of great significance for its OM permeabilization activity. Indeed, the parent PMB molecule has two hydrophobic regions, the fatty acid moiety at the N terminus and the D-Phe<sup>5</sup>-Leu<sup>6</sup> segment in the peptide ring. The removal of the fatty tail abolished its direct antimicrobial activity. Modulation of the hydrophobic segment of PMBN reduced its OM permeabilization activity. The proximity between the aromatic ring of D-Phe<sup>5</sup> and the side chain of Leu<sup>6</sup> promotes the formation of the  $\beta$ -turn in the peptide (Pristovsek and Kidric, 1999). Thus, substitution at these positions in PMBN with less hydrophobic residues may impair the stability of this  $\beta$ -turn. Such a structural change may affect the amphiphilic nature of the peptide and, in turn, weaken its LPS binding and activity.

## References

- Ainsworth GC, Brown AM, and Brownlee G (1947) Aerosporin, an antibiotic produced by *Bacillus aerosporus* Greer, *Nature (Lond)* **160**:263.
- Atherton E and Sheppard RC (1989) *Solid Phase Peptide Synthesis—A Practical Approach*. Practical Approach Series. Oxford, UK, IRL Press.
- Atkinson RA and Pelton JT (1992) Conformational study of cyclo[D-Trp-D-Asp-Pro-D-Val-Leu], an endothelin-A receptor-selective antagonist. *FEBS Lett* **296**:1–6.
- Bhattacharjya S, David SA, Mathan VI, and Balaram P (1997) Polymyxin B nonapeptide: conformations in water and in the lipopolysaccharide-bound state determined by two-dimensional NMR and molecular dynamics. *Biopolymers* **41**:251–265.
- Benedict RG and Langlykke AF (1947) Antibiotic activity of *Bacillus polymyxa*. *J Bacteriol* **54**:24–25.
- Chihara S, Tobita T, Yahata M, Ito A, and Koyana Y (1973) Enzymatic degradation of colistin, isolation and identification of  $\alpha$ -N-acetyl  $\alpha$ ,  $\gamma$ -diaminobutyric acid and colistin nonapeptide. *Agric Biol Chem* **37**:2455–2463.
- Danner RL, Joiner KA, Rubin M, Patterson WH, Johnson N, Ayers KM, and Parrillo JE (1989) Purification, toxicity and antiendotoxin activity of polymyxin B nonapeptide. *Antimicrob Agents Chemother* **33**:1428–1434.
- Duwe AK, Rupar CA, Horsman GB, and Vas SI (1986) In vitro cytotoxicity and antibiotic activity of polymyxin B nonapeptide. *Antimicrob Agents Chemother* **30**:340–341.
- Engelman DM, Steitz TA, and Goldman A (1986) Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. *Annu Rev Biophys Biophys Chem* **15**:321–353.
- Evans ME, Feola DJ, and Rapp RP (1999) Polymyxin B sulfate and colistin: old antibiotics for emerging multi-resistant gram-negative bacteria. *Ann Pharmacother* **33**:960–967.
- Hancock RE (1997) Peptide antibiotics. *Lancet* **349**:418–422.
- Karima R, Matsumoto S, Higashi H, and Matsushima K (1999) The molecular pathogenesis of endotoxic shock and organ failure. *Mol Med Today* **5**:123–132.
- Kunin CM and Bugg A (1971) Binding of polymyxin antibiotics to tissues: the major determinant of distribution and persistence in the body. *J Infect Dis* **124**:394–400.
- Kyte J and Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**:105–132.
- Liao SY, Ong GT, Wang KT, and Wu SH (1995) Conformation of polymyxin B analogs in DMSO from NMR spectra and molecular modeling. *Biochim Biophys Acta* **1252**:312–320.
- Lynn WA and Cohen J (1995) Adjunctive therapy for septic shock: a review of experimental approaches. *Clin Infect Dis* **20**:143–158.
- Moore RA, Bates NC, and Hancock RE (1986) Interaction of polycationic antibiotics with *Pseudomonas aeruginosa* lipopolysaccharide and lipid A studied by using dansyl-polymyxin. *Antimicrob Agents Chemother* **29**:496–500.
- Ofek I, Cohen S, Rahmani R, Kabha K, Tamarkin D, Herzig Y, and Rubinstein E (1994) Antibacterial synergism of polymyxin B nonapeptide and hydrophobic antibiotics in experimental gram-negative infections in mice. *Antimicrob Agents Chemother* **38**:374–377.
- Pristovsek P and Kidric J (1999) Solution structure of polymyxins B and E and effect of binding to lipopolysaccharide: an NMR and molecular modeling study. *J Med Chem* **42**:4604–4613.
- Rietschel ET, Brade H, Brade L, Brandenburg K, Schade U, Seydel U, Zahringer U, Galanos C, Luderitz O, Westphal O, et al. (1987) Lipid A, the endotoxic center of bacterial lipopolysaccharides: relation of chemical structure to biological activity. *Prog Clin Biol Res* **231**:25–53.
- Srimal S, Surolia N, Balasubramanian S, and Surolia A (1996) Titration calorimetric studies to elucidate the specificity of the interactions of polymyxin B with lipopolysaccharides and lipid A. *Biochem J* **315**:679–686.
- Stanly PG, Shepard RG, and White HJ (1947) *Bull Johns Hopkins Hosp* **81**:43–54.
- Surajit B, Sunil AD, Mathan VI, and Balaram P (1997) Polymyxin B nonapeptide: conformations in water and in the lipopolysaccharide-bound state determined by two-dimensional NMR and molecular dynamics. *Biopolymers* **41**:251–265.
- Tsubery H, Ofek I, Cohen S, and Fridkin M (2000a) Structure-function studies of polymyxin B nonapeptide: implications to sensitization of gram-negative bacteria. *J Med Chem* **43**:3085–3092.
- Tsubery H, Ofek I, Cohen S, and Fridkin M (2000b) The functional association of polymyxin B with bacterial lipopolysaccharide is stereospecific: studies on polymyxin B nonapeptide. *Biochemistry* **39**:11837–11844.
- Vaara M (1991) The outer membrane permeability-increasing action of linear analogues of polymyxin B nonapeptide. *Drugs Exp Clin Res* **17**:437–443.
- Vaara M and Vaara T (1983) Sensitization of Gram-negative bacteria to antibiotics and complement by a nontoxic oligopeptide. *Nature (Lond)* **303**:526–528.
- Vaara M and Viljanen P (1985) Binding of polymyxin B nonapeptide to gram-negative bacteria. *Antimicrob Agents Chemother* **27**:548–554.
- Venhatachalam CM (1968) Stereochemical criteria for polypeptides and proteins. V. Conformation of a system of three linked peptide units. *Biopolymers* **6**:1425–1436.
- Viljanen P and Vaara M (1984) Susceptibility of Gram-negative bacteria to polymyxin B nonapeptide. *Antimicrob Agents Chemother* **25**:701–705.
- Vinnicombe J and Stamey TA (1969) The relative nephrotoxicities of polymyxin B sulfate, sodium sulfomethyl-polymyxin B, sodium sulfomethyl-colistin (colymycin) and neomycin sulfate. *Invest Urol* **6**:505–519.
- Viriyakosol S and Kirkland T (1995) Knowledge of cellular receptors for bacterial endotoxin—1995. *Clin Infect Dis Suppl* **2**:S190–195.
- Zahringer U, Lindner B, and Riettschel ET (1994) Molecular structure of lipid A, the endotoxic center of bacterial lipopolysaccharides. *Adv Carbohydr Chem Biochem* **50**:211–276.

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