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Articles

The Functional Association of Polymyxin B with Bacterial Lipopolysaccharide Is Stereospecific: Studies on Polymyxin B Nonapeptide†

Haim Tsubery,‡,§ Itzhak Ofek,§ Sofia Cohen,§ and Mati Fridkin*,‡

Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot, 76100, Israel, and Department of Human Microbiology, Sackler Faculty of Medicine, Tel Aviv University, Tel-Aviv, 69978, Israel

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ABSTRACT: The Gram-negative bacterial endotoxin lipopolysaccharide (LPS) is a major inducer of sepsis. The natural cyclic peptide polymyxin B (PMB) is a potent antimicrobial agent, albeit highly toxic, by virtue of its capacity to neutralize the devastating effects of LPS. However, the exact mode of association between PMB and LPS is not clear. In this study, we have synthesized polymyxin B nonapeptide, the LPS-binding cyclic domain of PMB, and its enantiomeric analogue and studied several parameters related to their interaction with LPS and their capacity to sensitize Gram-negative bacteria toward hydrophobic antibiotics. The results suggest that whereas the binding of the two enantiomeric peptides to *E. coli* and to *E. coli* LPS is rather similar, functional association with the bacterial cell is stereospecific. Thus, the L-enantiomer is capable of synergism with the hydrophobic antimicrobial drugs novobiocin and erythromycin, whereas the D-enantiomer is devoid of such activity. The potential of understanding and consequently utilizing the PMB−LPS association for novel, nontoxic PMB-derived drugs is discussed.

The rapid increase of bacterial resistance toward currently employed antibiotics has led to intensive efforts toward developing new families of antibiotics, among them the antimicrobial cationic peptides (*1*). These latter molecules have been found in almost any living organism including mammals, plants, insects, and bacteria (*2*). They all share a common amphipathicity feature despite diversity in their primary and secondary structures (*1*). A common mode of action of most cationic antimicrobial peptides involves initial electrostatic interaction of the positively charged peptide residues with the negatively charged components of the bacterial membrane (*3*, *4*). This interaction leads to a massivedisorganization of the bacterial membrane and eventually to bacterial cell death.

The bacterial endotoxin lipopolysaccharide (LPS)[1] is the major antigen of the outer membrane (OM) of Gram-negative bacteria, and is essential for bacteria survival through establishing an effective permeability barrier (*5*). Viable Gram-negative bacteria lacking LPS have not been, as yet, isolated (*6*). LPS is considered a predominant cause of sepsis (*7*). Recently, it has been shown that LPS released from antibiotic-treated Gram-negative bacteria can lead to septic shock (*8*). Thus, an efficient antibiotic should not only kill

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* Corresponding author. Tel: 972-8-9342505, Fax: 972-8-9344142.
‡ The Weizmann Institute of Science.
§ Tel Aviv University.

[1] Abbreviations: Cbz-OSu, *N*-(benzyloxycarbonyl)succinimide; CD, circular dichroism; CFU, colony forming unit; Dab, 2,4-diaminobutyric acid; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; ESMS, electrospray mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HOBT, 1-hydroxybenzotriazole; MIC, minimal inhibitory concentration; NMM, 4-methylmorpholine; NMR, nuclear magnetic resonance; LPS, lipopolysaccharide; OM, outer membrane; PMB, polymyxin B; PMBN, polymyxin B nonapeptide; PyBOP, benzotriazol-1-yl-oxy-tris-pyrolidino-phosphonium hexafluorophosphate; tBoc, *tert*-butyloxycarbonyl; TES, triethylsilane; TFA, trifluoroacetic acid.

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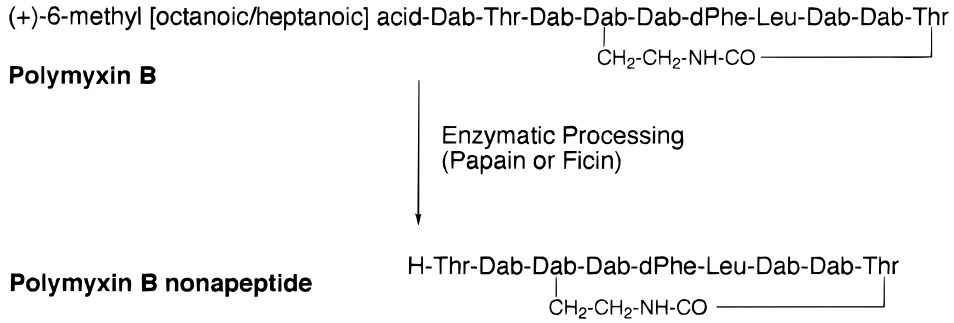

FIGURE 1: Structures of polymyxin B and polymyxin B nonapeptide.

the bacteria but also be capable of preventing other derived toxic effects. One of the most efficient OM-disorganizing and LPS-binding agents is polymyxin B (PMB) (*9*).

PMB is a mixture of naturally occurring cationic cyclic decapeptide derivatives isolated from *Bacillus polymyxa* (*10*−*12*) (Figure 1). PMB is highly bactericidal to Gram-negative bacteria and considered one of the most efficient cell-permeabilizing compounds (*9*). Its charge and unique structural features permit competitive displacement of divalent cations from their negatively charged binding sites on bacterial surface LPS. Since PMB is a far larger molecule than a divalent cation, and considering its multicharged nature, it disorganizes the bacterial OM, thereby enabling its hydrophobic N-terminal tail to penetrate through the cytoplasmic membrane. The thus produced OM perturbation causes a leak of cytoplasmic components, leading to bacterial death. This capacity of PMB stems from its high binding affinity to lipid A, the highly conserved hydrophobic domain of LPS (*5*, *13*). Although PMB inhibits the biological effects of LPS—notably TNF and IL-1 release from immune cells—its therapeutical applications are very limited because of high toxicity (*14*, *15*).

Polymyxin B nonapeptide (PMBN) is a cyclic peptide obtained from PMB by proteolytic cleavage of its amino-terminal hydrophobic acyl residue (*16*) (Figure 1). PMBN is an extremely poor antimicrobial compound (*17*), but still capable of binding, like its parental compound, to LPS, rendering Gram-negative bacteria susceptible to various hydrophobic antibiotics currently employed against Gram-positive bacteria (*18*). This latter synergistic antimicrobial capacity, often referred to as "sensitizing activity", may point to new therapeutic directions (*18*, *19*). In addition, PMBN is also capable of inhibiting some of the deleterious effects of LPS (*9*). Thus, the study of structure−function relationship of PMBN may lead to the design of potent antimicrobial peptides.

The overall structure of the PMBN peptide is the most important factor in determining its activity. Various linear analogues of PMBN were found inactive, suggesting that the cyclic structure is of major significance (*20*). Furthermore, we have recently accomplished the synthesis and structure−activity relationships of 11 cyclic analogues of PMBN and suggested that intactness of the unique architecture of the peptide is required both for efficient LPS binding and for maximal sensitization capacity (*21*).

In the present study, we aimed at elucidating several features pertaining to the PMBN−LPS interaction, particularly with regard to the possible stereospecific nature of this interaction. Accordingly, we have synthesized and studied a PMBN analogue in which every single amino acid residue of PMBN was replaced by its enantiomeric counterpart. This peptide, ['all d']PMBN (dPMBN), is a mirror image of PMBN. We found that whereas the 'all D' enantiomer binds LPS, similarly to PMBN, it poorly sensitizes the bacteria to hydrophobic antibiotics.

MATERIALS AND METHODS

All protected amino acids, coupling reagents, and polymers were obtained from Nova Biochemicals (Laufelfingen, Switzerland) or Bachem (Bubendorf, Switzerland). Synthesis grade solvents were obtained from Labscan (Dublin, Ireland).

*Synthesis of Fmoc(*D*)Dab(Cbz)-OH.* Fmoc-(D)Dab-OH (1.0 g, 2.94 mmol) was suspended in water (20 mL) containing Na_2CO_3 (0.623 g, 5.88 mmol). A solution of *N*-(benzyloxycarbonyl)succinimide (Cbz-OSu; 1.1 g, 4.40 mmol) in acetonitrile (20 mL) was added, and the homogeneous mixture was stirred overnight at room temperature (RT). The acetonitrile was removed in high vacuum, the remaining aqueous solution was extracted with ether (× 3) and acidified with HCl (to pH 2), and the white precipitate was extracted with ethyl acetate (× 3). The organic solution was washed with 1 N HCl (× 2) and then with water (× 2), dried over anhydrous Na_2SO_4, and concentrated in high vacuum, and the product was precipitated with petroleum ether (30−40 °C). The white solid obtained was filtered and dried in a vacuum [yield 93%, 1.3 g, 2.74 mmol; ESMS: *m*/*z* calcd 474.5, found 475.6 $[M + H]^+$; thin-layer chromatography: R_f (chloroform/methanol/acetic acid, 9:1:0.1) = 0.38].

Synthesis of Polymyxin B Nonapeptide Enantiomers (sPMBN and dPMBN). Linear peptide chains were assembled by conventional solid phase synthesis, using an ABIMED AMS-422 automated solid phase multiple peptide synthesizer (Langenfeld, Germany). Fmoc strategy was employed throughout the peptide chain assembly (*22*) following the company's protocol. Synthesis was initiated by using Fmoc-Thr(tBu)-Wang resin (0.7 mmol/1 g) and was performed on a 25 μmol scale. Side-chain amino protecting groups for 2,4-diaminobutyric acid (Dab) were *tert*-butyloxycarbonyl (tBoc) and benzyloxycarbonyl (Cbz). Fmoc-Thr(tBu)-OH was employed as the final building unit. Coupling was achieved using 4 equiv of benzotriazol-1-yl-oxy-tris-pyrolidino-phosphonium hexafluorophosphate (PyBOP) as a coupling agent and 8 equiv of 4-methylmorpholine (NMM), all dissolved in dimethylformamide (DMF). The fully protected peptide-bound resin was treated with piperidine (20% in DMF) for 20 min and then washed (DMF), and the free N-terminal amino moiety was reacted with 4 equiv of Cbz-OSu and 4 equiv of *N*,*N*-diisopropylethylamine (DIEA) in DMF for 3

h. The fully protected peptide-bound resin was treated with trifluoroacetic acid (TFA)/water/triethylsilane (TES) (95:2.5:2.5; v/v/v) for 1 h at RT and filtered. The solution containing the cleavage mixture was cooled to 4 °C, and the partially protected linear peptide was precipitated with ice-cold di-*tert*-butyl 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 lyophilized. Cyclization was then performed in DMF at a peptide concentration of 1 mM using PyBOP/1-hydroxybenzotriazole (HOBT)/NMM (4:4:8, equiv) as reagents for 2 h at RT (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 catalytic (Pd/C) hydrogenation in acetic acid/methanol/water (5:4:1, v/v/v).

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, Federal Republic of 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), employing the following binary gradient: at $t = 0$ min, B = 10%; at $t = 40$ min, B = 60%; and at $t = 50$ min, B = 100%, using a flow rate of 0.8 mL/min. Separations were performed using a Spectra-Physics SP8800 liquid chromatography system equipped with an Applied Biosystems 757 variable-wavelength absorbance detector. The column effluents were monitored by the UV absorbance at 220 nm. The corresponding fractions were collected, lyophilized, and analyzed, followed by exhaustive acid hydrolysis and precolumn reaction with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) to ascertain amino acid composition (Waters 2690 Separations Module, Milford, MA). Mass spectra analysis was performed to determine molecular weights (VG-platform-II electrospray single quadropole mass spectrometer; Micro Mass, U.K.). The purity of the peptides was >98% (yield 35−40%).

Polymyxin B Nonapeptide (pPMBN). pPMBN was prepared by proteolysis of PMB with Papain or Ficin as described elsewhere (*9*) (PMB, Papain, and Ficin were purchased from Sigma Chemical Co., St. Louis, MO). The crude product was purified (>98%) by HPLC, and then analyzed and characterized as described above.

Dansyl-PMBN. Dansyl-labeled PMBN was prepared as follows: resin-bound linear peptide (25 μmol) (see above) was treated with piperidine (20% v/v, in DMF) in order to remove the Fmoc protecting group from the N-terminal α-amino function of threonine. The resin-bound peptide was then reacted with dansyl chloride (4 equiv, 27 mg) and NMM (8 equiv, 22 μL) in DMF for 2 h. The labeled linear peptide was removed from the resin, cyclized, purified (>98%), and analyzed as described above (yield 40%; ESMS: *m*/*z* calcd 1197.2, found 1198.6 $[M + H]^+$; Thr, 1.1; Dab, 4.9; DPhe, 1; Leu, 1; t_R 33.54 min).

Determination of Minimal Inhibitory Concentration (MIC). Clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, obtained as described elsewhere, were employed (*19*). The Gram-negative bacteria were grown on nutrient agar plates (Difco Laboratories, Detroit, MI) and kept at 4 °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 0.2 μm Acrodisc. An overnight culture in Isotonic Sensitest Broth (ISB, Oxoid) was adjusted to 1×10^5 CFU/mL and inoculated onto microtiter plate wells, each containing 100 μL of a serial 2-fold dilution (1000−0.5 μg/mL) of the tested antibiotics/peptides in ISB. The MIC was defined as the lowest concentration at which there was no visible bacterial growth after incubation for 20 h, at 37 °C. The results are reported for 3−5 separate tests, which varied by no more than one dilution.

Sensitizing Activity. Bacterial suspension (10 μL, 1×10^5 CFU) was inoculated onto microtiter plate wells containing 100 μL of a serial 2-fold dilution (1000−0.5 μg/mL) of novobiocin or erythromycin (Sigma Chemical Co.) in ISB. To each well was added 10 μL of the test peptide to a final concentration of 50 μg/mL. The fold decrease in MIC for novobiocin and erythromycin between wells, in the presence or absence of the test peptides, was calculated and designated as sensitizing activity.

Dansyl-PMBN Binding and Displacement Assay (*23*). The fluorescence of dansyl-PMBN bound to *E. coli* LPS was measured using an MC200 monochromator (SLM AMINCO, SLM Instruments, INC.) set at an excitation wavelength of 340 nm and at an emission wavelength of 485 nm. Binding was performed by recording the fluorescence after the addition of small portions (5−10 μL) of dansyl-PMBN solution (1×10^{-6}−1×10^{-3} M) in water to a quartz cuvette containing LPS solution (2 mL, 3 μg/mL, ~2×10^{-7} M) in *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid buffer (HEPES; 5 mM, pH 7.2). The fluorescence was measured 5 min after each addition. Binding assays with intact bacteria were performed as described above for soluble LPS with overnight bacterial suspension (adjusted to $OD_{600} = 0.2$) in HEPES buffer (5 mM, pH 7.2) containing sodium azide (5 mM). Fluorescence was measured 10 min after each addition.

The displacement assay was performed as follows: to a quartz cuvette containing either LPS solution (2 mL, 3 μg/mL, ~2×10^{-7} M) in HEPES buffer (5 mM, pH 7.2) or bacterial suspension in the same HEPES buffer containing sodium azide (5 mM) was added dansyl-PMBN (0.55 or 10 μM, respectively) and allowed to equilibrate at RT for 10−15 min. Subsequently, small portions (5−10 μL) of peptide solutions (1×10^{-5}−1×10^{-3} M) were added. The inhibition of fluorescence was measured 5 min after each addition. The percent inhibition was plotted as a function of the peptide concentration, and IC_{50} values were calculated from the maximal specific displacement (I_{max}).

Circular Dichroism (CD) Studies. CD spectra were recorded on a Jasco Model J-500C spectrophotometer equipped with a Data Processor station Model DP-500N. Duplicate scans over a wavelength range of 190−250 nm were taken at a chart speed of 10 nm/min in a 0.1 cm path length quartz cell at RT. Peptides were dissolved in 5 mM potassium phosphate buffer, pH 7.2, at a final concentration

Table 1: Peptide Primary Structure and Identification

peptide	structure[a]	t_R (min)[b]	ESMS (*m*/*z*)[c]	amino acid analysis[d]
pPMBN	TX*cyclo*[XX**F**LXXT]	25.1	963.6 (962.57)	T,1.92; X,4.98; L,1.0; F,1.0
sPMBN	TX*cyclo*[XX**F**LXXT]	25.1	963.6 (962.57)	T,1.86; X,4.82; L,1.0; F,1.0
dPMBN	**TX***cyclo*[**XX**F**LXXT**]	25.1	963.6 (962.57)	T,1.91; X,4.87; L,1.0; F,1.0

[a] Boldface amino acids are the D-enantiomers; X = 2,4-diaminobutyric acid. [b] t_R, retention time on analytical RP-18 HPLC column (see Materials and Methods). [c] Numbers in parentheses refer to calculated values. [d] The calculated values are T = 2, X = 5, L = 1, and F = 1.

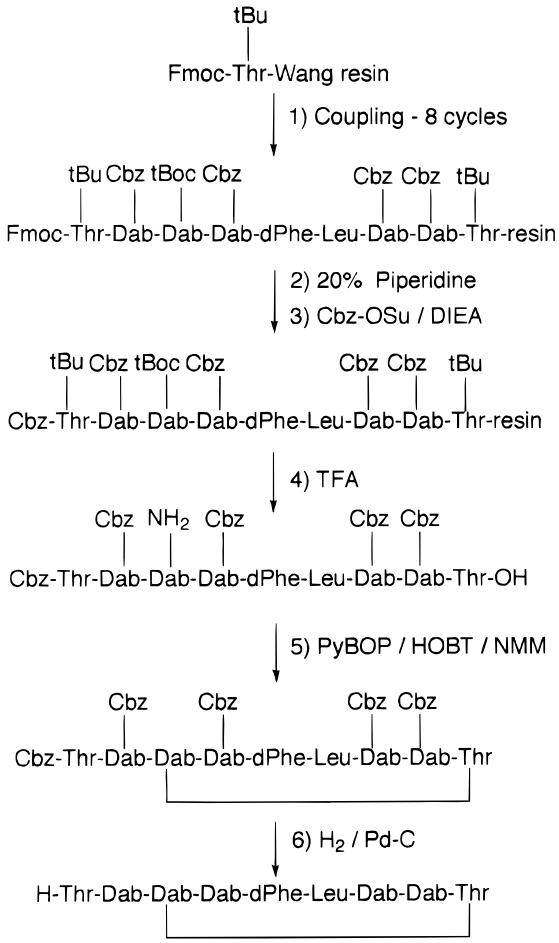

FIGURE 2: Synthesis of sPMBN and dPMBN.

of 0.1 mM. Measurements were taken either in potassium phosphate buffer or in buffer containing 50 and 90% trifluoroethanol (TFE, v/v).

NMR Studies (24).^{1}H NMR spectra were obtained on a Bruker AVANCE 400 spectrometer (400 MHz). To evaluate the binding of peptides to LPS, line-broadening experiments were performed by successively adding small aliquots of concentrated stock solution of LPS (12 mg/mL in deuterium oxide) to peptide solutions (5 mM) in water/deuterium oxide (9:1) (pH 2.3) up to a final concentration of 0.6 mg/mL.

RESULTS

Polymyxin B nonapeptide (sPMBN) and its D-enantiomer—an analogue in which all amino acid residues are replaced, correspondingly, by their optical isomers ([‘all d’]PMBN; dPMBN)—were synthesized by employing a combination of solid phase linear chain assembly and a subsequent cyclization in solution (Figure 2). Recently, a related approach for the total synthesis of polymyxin B1 was reported (*25*). The peptides were purified to homogeneity (>98%) by HPLC,

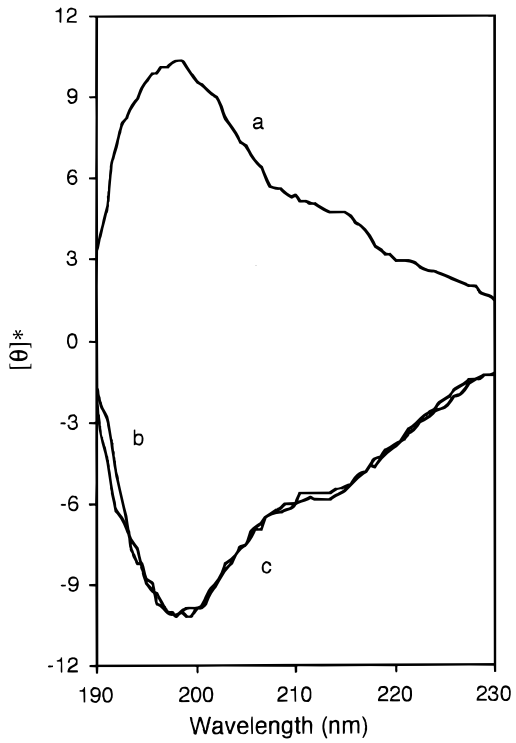

FIGURE 3: Circular dichroism spectra of PMBN peptides (0.1 mM) in PB/TFE (1:1, v/v). [dPMBN (a), sPMBN (b), and pPMBN (c).] $[\theta]^* = \theta_{obs} \times 10^{-3}$ deg cm^2 dmol^{-1}.

and their correct amino acid composition and calculated molecular weights were ascertained by amino acid analysis and electrospray mass spectrometry (Table 1). pPMBN, the proteolytic product of natural PMB, was prepared as previously described (*9*). The expectation that the two enantiomers of PMBN would exhibit mirror-image conformations was confirmed by CD measurements (Figure 3). As shown, the L-enantiomers, both the one obtained from proteolysis of polymyxin B (pPMBN) and the synthetic one (sPMBN), have identical ellipticity patterns which are equal in amplitude but opposite in sign to that of the D-enantiomer (dPMBN). This finding suggests that not only the backbone chiral center but also the chiral centers formed in the “folding” are mirror images. The recorded CD points to a rather random structure in both aqueous and TFE solutions (not shown). Recent ^{1}H NMR studies suggested that the conformation of PMBN is of type II′ β-turn centered on the DPhe-Leu segment for the free peptide (*26*), and an envelope-like fold of the peptide ring for the LPS-bound peptide (*27*).

For further structural comparison, the ^{1}H NMR spectra of the enantiomeric peptides were studied. Thus, both enantiomers exhibited identical patterns in the presence or absence of *E. coli* LPS, the molecular target of PMBN (and PMB) on the bacterial cell surface (Figure 4). The addition of LPS to the peptides resulted in a concentration-dependent line

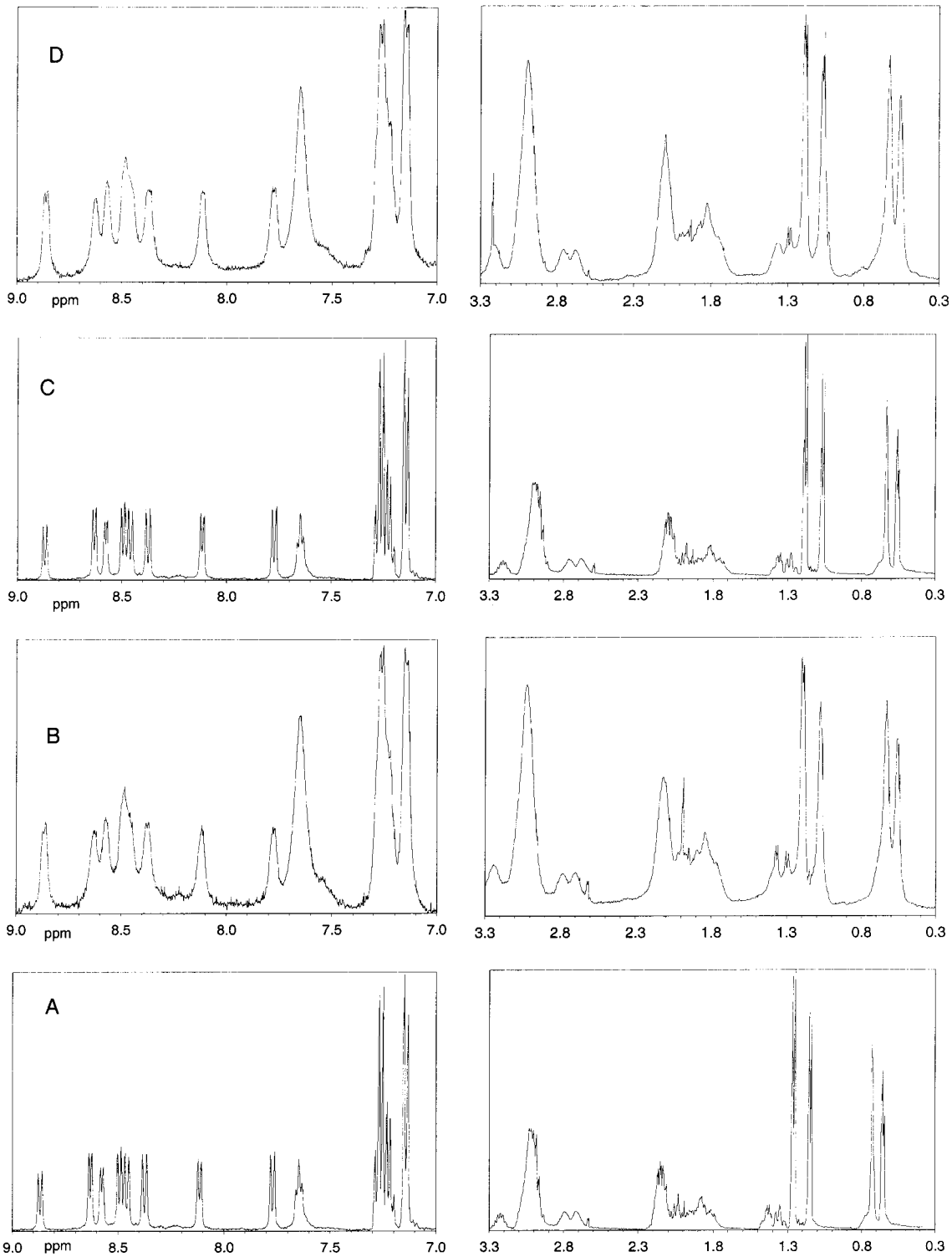

FIGURE 4: ^{1}H NMR spectra of pPMBN and dPMBN in the presence and absence of LPS. ^{1}H NMR spectra were obtained on a Bruker AVANCE 400 spectrometer (400 MHz) by successively adding small aliquots from a concentrated stock solution of LPS (12 mg/mL in D_2O) to peptide solutions (5 mM) in H_2O/D_2O (9:1) (pH 2.3) to a final concentration of 0.6 mg/mL LPS. (A) dPMBN. (B) dPMBN + LPS (0.6 mg/mL). (C) pPMBN. (D) pPMBN + LPS (0.6 mg/mL).

broadening of most peptide-related resonance, presumably due to an enhanced exchange between free and LPS-bound molecules (*26*). As shown, the line broadening effect is observed in both the backbone ($C^{\alpha}H$ and NH) and the side-chain resonances. An identical effect was observed for both enantiomers. The ^{1}H NMR data suggest that the two mirror-image peptides interact with cell-free LPS in a rather similar manner.

The peptides pPMBN, sPMBN, and dPMBN were tested for their potential to inhibit the growth of different species of Gram-negative bacteria. Their MIC values toward clinical isolates of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* are shown in Table 2. As expected, all three peptides had practically no bactericidal activity against *E. coli* and *K. pneumoniae* (*17*). However, both pPMBN and sPMBN exhibited growth-inhibition against *P. aeruginosa* (8 μg/mL).

Table 2: MIC Values (μg/mL) of PMBN Peptides

peptide	*E. coli*	*K. pneumoniae*	*P. aeruginosa*
pPMBN	1000	>1000	8
sPMBN	>500	>1000	8
dPMBN	>1000	>1000	>1000
PMB	3	4	3

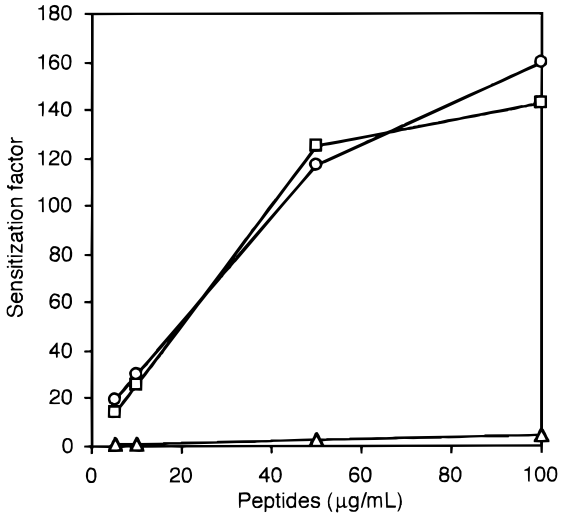

FIGURE 5: Sensitization of *E. coli* toward novobiocin by the three PMBN peptides. The relative potency is shown as the ratio of novobiocin MIC in the absence and presence of the PMBN peptides (5−100 μg/mL) that reduces the MIC toward *E. coli* from 62.5 down to 0.5 μg/mL. pPMBN (□), sPMBN (○), and dPMBN (△).

Table 3: MIC Values (μg/mL) of Erythromycin

antibiotic	*E. coli*	*K. pneumoniae*
erythromycin	125	125
Erythro+pPMBN[a]	2	8
Erythro+dPMBN[a]	125	125

[a] The peptides were at a final concentration of 50 μg/mL.

The enantio analogue, dPMBN, had no antimicrobial activity against any of these species (≥1000 μg/mL).

Next, we focused on the peptides' ability to sensitize Gram-negative bacteria toward the hydrophobic antibiotics novobiocin and erythromycin, presumably by augmenting penetration of the antibiotic through the OM into the bacteria (*18*, *19*). This activity was evaluated in terms of the peptides' ability to reduce the MIC values of novobiocin and erythromycin toward *E. coli* and *K. pneumoniae*. pPMBN and sPMBN showed a very high capacity to render the bacteria susceptible to novobiocin (Figure 5). However, the enantio analogue, dPMBN, exhibited significantly reduced potency to increase novobiocin penetration into the bacteria, even at a concentration as high as 100 μg/mL (0.1 mM) (Figure 5). Similar results were obtained in studies with erythromycin (Table 3).

The interaction of PMB (and PMBN) with the bacterial cell-bound LPS, as well as with cell-free LPS and its lipid A domain, was quantified using the dansyl-PMB binding assay (*23*). The method is based upon enhancement of the dansyl fluorescence with increments of LPS or lipid A−peptide association. Binding experiments with PMBN were performed by a modification of the above method, using dansyl-PMBN rather than dansyl-PMB. Dansyl-PMBN was synthesized by reacting the free N-terminal α-amino function

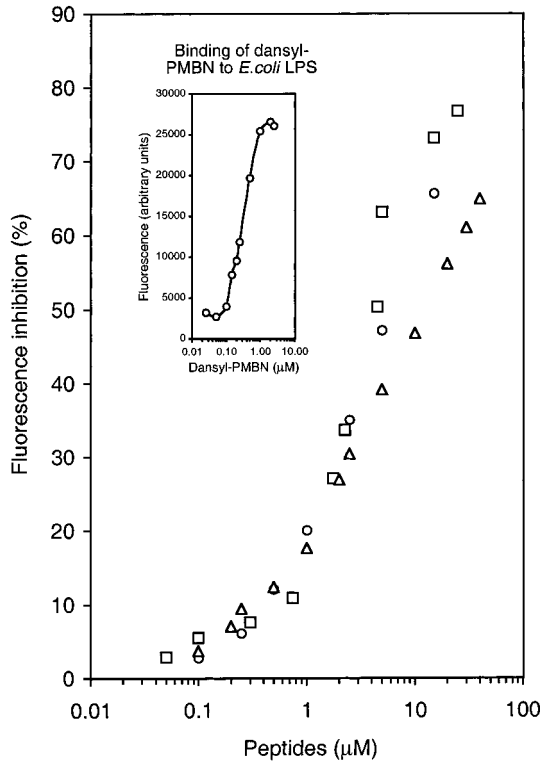

FIGURE 6: Displacement of LPS-bound dansyl-PMBN by PMBN enantiomers. Increasing concentrations of PMBN peptides [pPMBN (□), sPMBN (○), and dPMBN (△)] were added to *E. coli* LPS solution (3 μg/mL) bound to dansyl-PMBN (0.55 μM). The fluorescence inhibition was measured 5 min after each addition at excitation and emission wavelengths of 340 and 485 nm, respectively.

of the peptide, prior to cleavage of the peptide from the polymeric support, with dansyl chloride, as detailed under Materials and Methods. A plot of enhanced fluorescence as a function of dansyl-PMBN added to LPS is illustrated in Figure 6 (inset). The ability of the peptides pPMBN, sPMBN, and dPMBN to displace dansyl-PMBN (0.55 μM) from cell-free LPS (3 μg/mL) was evaluated (Figure 6). Related calculations revealed that the concentrations of these peptides required for 50% displacement are 2.5 and 3 μM for pPMBN and sPMBN (maximal inhibition observed, I_{max} = 80%), respectively, and 2.5 μM for dPMBN (I_{max} = 65%). In addition, the ability of these peptides to displace dansyl-PMBN (10 μM) from whole bacteria was evaluated. The concentrations required for 50% displacement are 25 and 20 μM (I_{max} = 75% and 60%) for pPMBN and dPMBN, respectively (Figure 7).

DISCUSSION

The binding of PMB to the bacterial LPS through the latter's highly conserved lipid core, i.e., lipid A, is the major event leading to toxin neutralization by the peptide. The exact nature of this association is, as yet, enigmatic and rather controversial. Several studies have been recently directed toward understanding the nature of PMB−LPS association (*24*, *26*−*28*). Achieving this goal may lead to novel therapeutic routes for combating Gram-negative-induced sepsis.

Studies employing isothermal titration calorimetry suggest that the binding of PMB to *E. coli* LPS and to lipid A is stoichiometric and noncooperative, with affinity in the

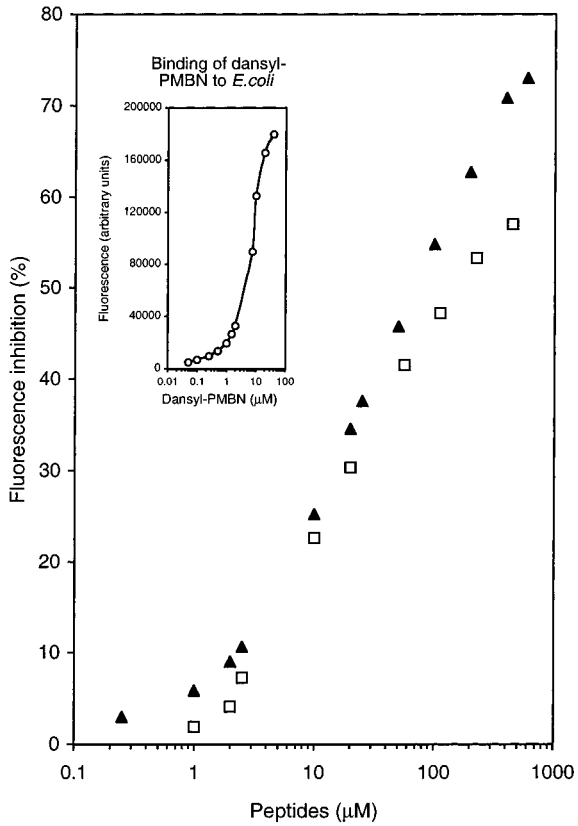

FIGURE 7: Displacement of *E. coli*-bound dansyl-PMBN by PMBN enantiomers. Increasing concentrations of PMBN peptides [pPMBN (▲) and dPMBN (□)] were added to *E. coli* suspension (OD_{600} = 0.2) bound to dansyl-PMBN (10 μM). The fluorescence inhibition was measured 5 min after each addition at excitation and emission wavelengths of 340 and 485 nm, respectively.

micromolar range (*29*). Conclusions drawn from studies dealing with binding of dansyl-PMB to LPS and lipid A, isolated from *P. aeruginosa*, suggest a cooperative peptide−LPS association involving more than a single binding site for PMB on each LPS and lipid A molecule, i.e., four and two binding sites, respectively (*13*). The binding affinity found for both is 0.38 μM, rather similar to the value (0.3 μM) found employing dansyl-labeled LPS from *S. typhimurium* (*30*). The intermolecular peptide−lipid interactions are attributed to the amphiphilic feature 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 (*29*). Similar structural considerations were further deduced and elaborated from 2D NMR and molecular dynamics studies of PMBN and *E. coli* LPS (*24*, *26*). According to the latter, PMB and PMBN have a type II′ β-turn structure for the free peptide and an envelope-like fold of the peptide ring for the LPS-bound peptide.

In the present study, the ability of the peptides pPMBN, sPMBN, and dPMBN to displace dansyl-PMBN from LPS was evaluated (Figure 6). Accordingly, 50% inhibition of fluorescence was apparent at concentrations of 2.5, 3, and 2.5 μM for pPMBN, sPMBN, and dPMBN, respectively. In this regard, it seems that the association of the two enantiomers of PMBN bears a rather close resemblance. Furthermore, our ^{1}H NMR studies suggest, as deduced from the line-broadening patterns, identical conformational changes of the peptides upon their binding to LPS. Different picture emerges, however, from the antibacterial studies. Thus, pPMBN and sPMBN are active against *P. aeruginosa* (MIC, 8 μg/mL) whereas dPMBN is inactive (MIC, >1000 μg/mL) (Table 2). Similarly, pPMBN and sPMBN enhance most substantially the sensitivity of *E. coli* and *K. pneumoniae* toward erythromycin and novobiocin, whereas dPMBN is inactive. These differences in the ability of the peptides to permeabilize the bacterial membrane cannot be explained on the basis of the binding affinities of the two enantiomers toward LPS. Moreover, the capacity of both sPMBN and dPMBN to displace dansyl-PMBN from intact *E. coli* is rather similar (Figure 7).

As expected, the functional interaction of biologically active peptides with their specific cellular receptors is stereospecific. Thus, even minor structural alternations may lead to dramatic modulation in activity. 'All D'-enantiomers of regulatory peptides have been often used as tools to understand the mode of action of the parental compound. As a rule, enantiomers of receptor-specific peptides were found to be devoid of activity [e.g., D-oxytocin, D-gastrin, and D-bradykinin (*31*−*33*)]. In contrast, enantiomers of surface-active peptides might be, theoretically, equally potent. Indeed, several 'all D'-enantiomers of antibacterial peptides, e.g., cecropin (*34*) and magainin (*35*, *36*), were found to be as potent as their 'all L' counterparts. However, the D forms of apidaecine (*37*) and drosocin (*38*), antimicrobial proline-rich peptides isolated from honeybees and *Drosophila*, respectively, are totally inactive. In the present study, the 'all d' enantiomer of PMBN lost one activity but retained another; that is, it lost its sensitizing activity, but retained its LPS binding capacity, suggesting that these two PMBN functions, although consecutive, are mediated by two different molecular mechanisms. One of these functions, sensitizing activity, requires the structural intactness of the PMBN molecule, while the other, LPS binding, is less demanding and requires a general sequence similarity to the parent PMBN molecule, and its chirality seems to be less important. This notion is supported by the finding that several cyclic analogues of PMBN, including derivatives with minor structural alterations, bind to LPS rather similarly to PMBN but are unable to sensitize Gram-negative bacteria toward hydrophobic antibiotic (*21*). Taken together, it is postulated that the permeation activity of PMBN, as well as that of PMB, is mediated by a chiral bacteria−peptide association in which the natural conformation of the peptide plays a crucial role. Therefore, the development of efficient PMB- and PMBN-related antibiotic drugs should predominantly rely on the above structural considerations.

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