Lipopolysaccharide (LPS), an integral part of the outer membrane of Gram-negative bacteria, is involved in a variety of biological processes including inflammation, septic shock, and resistance to host-defense molecules. LPS also provides an environment for folding of outer membrane proteins. In this work, we describe the structure-activity correlation of a series of 12-residue peptides in LPS. NMR structures of the peptides derived in complex with LPS reveal boomerang-like β-strand conformations that are stabilized by intimate packing between the two aromatic residues located at the 4 and 9 positions. This structural feature renders these peptides with a high ability to neutralize endotoxicity, >80% at 10 nm concentration, of LPS. Replacements of these aromatic residues either with Ala or with Leu destabilizes the boomerang structure with the concomitant loss of antiendoxotive and antimicrobial activities. Furthermore, the aromatic packing stabilizing the β-boomerang structure in LPS is found to be maintained even in a truncated octapeptide, defining a structured LPS binding motif. The mode of action of the active designed peptides correlates well with their ability to perturb LPS micelle structures. Fourier transform infrared spectroscopy studies of the peptides delineate β-type conformations and immobilization of phosphate head groups of LPS. Trp fluorescence studies demonstrated selective interactions with LPS and the depth of insertion into the LPS bilayer. Our results demonstrate the requirement of LPS-specific structures of peptides for endotoxin neutralizations. In addition, we propose that structures of these peptides may be employed to design proteins for the outer membrane.

LPS² or endotoxin, a major component of the outer leaflet of the outer membrane of Gram-negative bacteria, is critically involved in health and diseases of humans (1, 2). LPS is essential for bacterial survival through establishing an efficient permeability barrier against a variety of antimicrobial compounds including hydrophobic antibiotics, detergents, host-defense proteins, and antimicrobial peptides (3, 4). Several studies have demonstrated that LPS catalyzes folding of outer membrane proteins as a chaperone (5–7).

LPS, a potent inducer of innate immune systems, hence called endotoxin, is primarily responsible for lethality in sepsis and septic shock syndromes associated with serious Gram-negative infections (8–10). Circulating LPS in bloodstream is intercepted by the phagocytic cells of the innate immune system. Once induced by LPS, these phagocytes produce proinflammatory cytokines, e.g. tumor necrosis factor-α, interleukin-6, and interleukin-1β, through the activation of a Toll-like pattern recognition receptor (11, 12). The release of cytokines in response to microbial invasion is a natural function of the innate immunity. However, an uncontrolled and overwhelming production of these cytokines may cause “endotoxic shock” or septic shock, typified by endothelial tissue damage, loss of vascular tone, coagulopathy, and multiple organ failure, often resulting in death (9, 10). Sepsis is the major cause of mortality in the intensive care unit, accounting for 200,000 deaths every year in the United States alone (13). It was demonstrated that release of LPS from antibiotic-treated Gram-negative bacteria can indeed enhance sepsis (14). Therefore, an effective antibiotic should not only exert antibacterial activities but also have the ability to sequester LPS and ameliorate its toxicity. Therefore, an amalgamated property of LPS-neutralizing and antimicrobial activity would be highly desirable for antimicrobial agents. Polymyxin B is a prototypical antimicrobial and antiendoxotive antibiotic; however, its neurotoxicity and nephrotoxicity limit its application to topical use (15). The increasing emergence of bacterial strains that are resistant to conventional antibiotics has initiated vital structure/function studies of membrane-perturbing cationic antimicrobial peptides (16–20). More recent studies have been conducted to understand interactions between antimicrobial peptides with LPS to gain insights into the mechanism of outer membrane perturbation, antibacterial activities, and LPS neutralization (21–26). These studies have delineated the role of amino acid sequence properties, LPS-peptide interactions by biophysical methods, and global structural parameters, obtained by CD and FTIR.

Designing synthetic peptides and elucidation of three-dimensional structures in complex with LPS would be useful for the purpose of rational development of non-toxic antisepsis and antimicrobial therapeutics. Such studies will also be poten-
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...tially instructive in establishing rules by which folded structures can be stabilized in the LPS surface. Extensive work in the field of peptide design primarily focuses on mimicking secondary structures and tertiary folds of proteins. Usually, short linear peptides are often structurally flexible; however, the functions of these peptides are highly dependent on their ability to adopt folded structures upon complex formation with their cognate receptors. In this regard, designed peptides that would yield high resolution structures in complex with LPS have not been well pursued. LPS, being a negatively charged amphiphilic molecule, interacts with naturally occurring peptides or protein fragments containing basic/polar and hydrophobic amino acids, although there are considerable variations in lengths, sequences, and amino acid compositions among these peptides (27, 28).

Here, we have determined the three-dimensional structures of a series of 12-residue peptides in the context of LPS. To the best of our knowledge, these results show, for the first time, that atomic resolution structures of designed peptides obtained in LPS could be correlated with their antiendotoxic activities. Furthermore, the LPS-induced structures of active, inactive, and short peptide motif, presented here, may provide building blocks for the designing novel proteins for the outer membrane.

**EXPERIMENTAL PROCEDURES**

**Reagents**—LPS of *Escherichia coli* 0111:B4 and fluorescein isothiocyanate (FITC)-conjugated lipopolysaccharide from *E. coli* 055:B5 and spin-labeled lipids 5-doxyl-stearic acid (5-DSA) and 16-doxyl-stearic acid (16-DSA) were purchased from Sigma. Peptides were synthesized commercially by GL Biochem (Shanghai, China) and further purified by a reverse-phase HPLC, Waters™, using a C18 column (300 Å pore size, 5-μm particle size) by a linear gradient of acetonitrile/water mixture. The molecular weight of the peptides was confirmed by mass spectrometry.

**LPS Neutralization Assay**—The ability of the designed peptides to neutralize or inhibit LPS was assessed using a quantitative chromogenic limulus amoebocyte lysate (LAL) with a QCL-1000 (Cambrex) kit. Endotoxin neutralization experiments were carried out following the protocols provided by the vendor and published elsewhere (26, 27). Stock solutions of peptides were prepared in pyrogen-free water provided with the kit. Peptides at concentrations of 0.01, 0.05, 0.1, 5, and 10 μM were incubated with three different endotoxin units (EU) of LPS, namely 1, 3, and 8 EU/ml (1 EU ~0.1 ng of LPS), in a flat bottom nonpyrogenic 96-well tissue culture plate, at 37 °C for 30 min to allow peptide binding to LPS (26). A total of 50 μl of this mixture was then added to equal volume of LAL reagent, and the mixture was further incubated for 10 min followed by the addition of 100 μl of chromogenic substrate (Ac-Ile-Ala-Arg-p-nitroaniline). The reaction was terminated by the addition of 25% acetic acid, and the yellow color that developed due to cleavage of the substrate was measured spectrophotometrically at 410 nm using a Benchmark plus microplate spectrophotometer (Bio-Rad). The reduction of A410 as a function of peptide concentrations is directly proportional to the inhibition of LPS by the peptide (29, 30). All assays were repeated twice, and average values are reported.

**Determination of Minimum Inhibitory Concentration**—Antimicrobial activities of the designed peptides were determined following a previously reported method (31). Briefly, bacterial cells used for this assay, e.g. *E. coli* DH5α, *Bacillus subtilis*, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923, were cultured in Luria-Bertani (LB) media at 37 °C overnight. Cells were centrifuged and washed with the assay buffer (10 mM sodium phosphate buffer, pH 7.4) and diluted to an A600 of 0.2. About 50 μl of these bacterial cell suspensions were incubated, in a sterile 96-well microtiter plate, with the same volume of peptides at various concentrations, ranging from 1 to 200 μM, diluted from a stock solution of 0.3 mM (prepared in the same buffer) at 37 °C for 2 h. The cell suspensions were then plated onto Mueller-Hinton agar plates and incubated overnight. The minimum inhibitory concentration was expressed as the lowest concentration of the peptide where there was no visible growth of the bacteria.

**Fluorescence Studies**—All of the fluorescence experiments were performed using a Cary Eclipse fluorescence spectrophotometer (Varian, Inc.). To study the interactions of peptides with FITC-conjugated LPS, 0.5 μM FITC-LPS samples were excited at 480 nm, and change in the emission of FITC at 515 nm was monitored with various concentrations (0.01, 0.02, 0.1, 0.5, 1.0, 5.0, and 10 μM) of peptides. Samples were prepared in 10 mM phosphate buffer, pH 6.0. For the intrinsic Tryp fluorescence studies of peptides, 5 μM of each peptide was titrated with varying concentrations of LPS or DPC in a 10 mM sodium phosphate buffer at pH 6.0. The intrinsic tryptophan fluorescence emission spectra of the peptides in their free or lipid-bound forms were acquired by exciting samples at 280 nm using band passes of 5 nm for both the excitation and the emission monochromators in a 0.1-cm path length quartz cuvette. Quenching of tryptophan fluorescence was examined following sequential additions of various concentrations (0.02–3 m) of acrylamide into solutions containing peptide (5 μM) in its free and lipid-bound forms. The results of the quenching reactions were analyzed according to the Stern-Volmer equation, F0/F = 1 + Ksv[Q], where F0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, Ksv is the Stern-Volmer quenching constant, and [Q] is the molar quencher concentration.

**Measurement of Depth of Insertion of the Peptides into LPS Vesicles**—Quenching of Trp fluorescence by spin-labeled lipids (5-DSA and 16-DSA) was used to estimate the depth of insertion of the peptide into LPS bilayer or vesicle by parallax method (32). LPS bilayer was prepared by dissolving the appropriate amount of LPS in 2:1 chloroform/methanol solution. The organic solvent was evaporated to dryness under vacuum. The lipid film was hydrated with 10 mM phosphate buffer, pH 6.0, at 60 °C and vortexed briefly. This mixture was frozen and thawed five times and extruded through a 0.1-μm membrane with the extruder (Avanti Polar Lipids, Alabaster, AL). Various concentrations of spin-labeled lipids, 5-DSA or 16-DSA, were added, from a stock solution of 1 mM (prepared in methanol) into solutions containing 5 μM peptides and 40 μM LPS vesicle. The location of the tryptophan into LPS bilayer was determined by comparing the extent of...
quenching observed from shallow (5-DSA) and deep (16-DSA) quenchers following the equation (29)

\[ Z_{1f} = \left( \frac{1}{-\pi C} \ln \frac{F_1}{F_2} - L_{21}^2 \right) / 2L_{21} \]  

(Eq. 1)

where \( Z_{1f} \) is the difference in depth between the shallow quencher and the tryptophan residue, and \( F_1 \) and \( F_2 \) are the difference between the tryptophan fluorescence intensities in the presence and absence of shallow and deep quenchers, respectively. Assuming the usual surface area of the lipid to be 70 Å, \( C \) is the quencher mole fraction in unit area. \( L_{21} \) is the difference in depth between the two quenchers. Once \( Z_{1f} \) is calculated, the distance of tryptophan from the center of the bilayer was calculated from \( Z_{CS} = Z_{1f} + L_{q} \), where \( L_{q} \) is the distance from the center of the bilayer to the shallow quencher.

** Isothermal Titration Calorimetry (ITC)**—ITC experiments were performed using a VP-ITC microcalorimeter (MicroCal Inc., Northampton, MA). All samples, dissolved in 10 mM phosphate buffer, pH 6.0, were degassed prior to use. LPS at a concentration of 0.05 mM was loaded into the sample cell (volume 1.4359 ml), and the reference cell was filled with the above mentioned buffer. Peptides, at a concentration of 1 mM, were placed into the injection. A typical titration involved 35 injections of 2.5-μl aliquots of YI12 peptides into the sample cell, at an interval of 4 min, at 25 °C. The reaction cell was stirred continuously at 300 rpm. Raw data were collected and integrated using the MicroCal Origin 5.0 software supplied with the instrument. A single site binding model was fitted to the data by non-linear least square analysis to yield the association constant (\( K_a \)) and enthalpy change (\( \Delta H \)). \( \Delta G \) and \( \Delta S \) were calculated using the fundamental equations of thermodynamics: \( \Delta G = -RT \ln K_a \) and \( \Delta S = (\Delta H - \Delta G)/T \), respectively.

** Dynamic Light Scattering**—To obtain information on the ability of designed peptides to dissociate LPS aggregates, dynamic light-scattering measurements were carried out in an BI-9000AT with digital autocorrelator (Brookhaven Instruments Corp., Holtsville, NY). The peptide and buffer solutions were filtered through 0.45-μm filters (Whatman Inc.). Measurements were made for 1 μM LPS (without any peptides), and upon incubation with 2 μM peptides, the scattering data were collected at 90°. The data were analyzed using the standard CONTIN method using the dynamic light-scattering software supplied with the instrument.

** NMR Experiments**—All of the NMR spectra were recorded on a Bruker DRX 600 spectrometer, equipped with cryo-probe and pulse field gradients. Data acquisition and processing were performed with the Topspin software (Bruker) running on a Linux workstation. Sequence-specific resonance assignments of the peptides were achieved from two-dimensional total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOE) spectra acquired in aqueous solution containing 10% D₂O at pH 4.8, 298 K. The peptide concentrations were 0.6 mM, and mixing times were 80 and 400 ms for TOCSY and NOESY, respectively. The interactions of the designed peptides with LPS were examined by recording series of one-dimensional proton NMR spectra whereby 0.6 mM peptides were titrated with various concentrations, 5, 10, and 16 μM LPS. Tr-NOESY spectra were typically obtained either at 10 μM or at 13 μM LPS, which generated a large number of Tr-NOE cross-peaks. The two-dimensional Tr-NOESY spectra were recorded at three different mixing times: 100, 150, and 200 ms with 512 increments in \( t_1 \) and 2048 data points in \( t_2 \). Tr-NOESY spectra were also obtained in D₂O for unambiguous assignments of aromatic/aromatic or aromatic/aliphatic NOEs. The spectral width was normally 12 ppm in both dimensions. After 16 dummy scans, 72 scans were recorded per \( t_1 \) increment. NMR data analyses were carried out using the program SPARKY (T. D. Goddard and D. G. Kneller, University of California, San Francisco, CA). ³¹P NMR spectra of LPS were recorded on a Bruker DRX 400 spectrometer at 298 K. Data acquisition and processing were performed with the Topspin software (Bruker) suite. The interactions of the designed peptides with LPS were examined by recording series of one-dimensional ³¹P NMR spectra whereby 0.2 mM LPS in water (pH 4.5) was titrated with various concentrations (0.1, 0.2, and 0.4 mM) of designed peptides from a stock solution that was prepared in an unbuffered water (pH 4.5).

**NMR-derived Structure Calculation**—NMR structures were calculated using the DYANA program, version 1.5 (33). NOE intensities were qualitatively categorized as strong, medium, and weak based on cross-peak intensities in the Tr-NOE spectra obtained at a mixing time of 150 ms. The NOE cross-peaks were further translated to upper bound distance limits of 2.5, 3.5, and 5.0 Å, corresponding to strong, medium, and weak intensities, respectively. Only the φ dihedral angles were constrained between −30° and +180° to maintain a good stereochemistry of the calculated structures. Out of the 100 structures generated, the 20 lowest energy structures were used for more analysis.

** Infrared Spectroscopy**—FTIR spectra were recorded on a Nicolet Nexus 560 spectrometer (Thermo Fisher Scientific, Inc.) purged with \( N_2 \) and equipped with a mercury-cadmium-telluride (MCT/A) detector cooled with liquid nitrogen. Attenuated total reflection (ATR) spectra were measured with a 25-reflections ATR accessory from Graseby Specac (Kent, UK) and a wire grid polarizer (0.25 mm, Graseby Specac). Approximately 200 μl of a D₂O solution of LPS alone or in the presence of peptide in a 20:1 lipid/peptide molar ratio were applied onto a trapezoidal (50 × 2 × 20 mm) germanium internal reflection element. A dry, or D₂O-saturated, \( N_2 \) stream flowing through the ATR compartment was used to remove bulk water (low hydration) or to fully hydrate the sample (high hydration), respectively. A total of 200 scans were collected at a resolution of 4 cm⁻¹, averaged, and processed with one-point zero filling and Happ-Genzel apodization.

**RESULTS**

**Design of Peptides**—In an earlier study, we have determined LPS-bound structure and antiendotoxic activity of a 12-residue synthetic peptide (34). This peptide, YVLWKRKRMIFI, was designed using a co-crystal structure of LPS/FhuA (1 QFG) an outer membrane protein. The outer membrane proteins of Gram-negative bacteria are rich in β-sheet, assuming a β-barrel topology (35). The folding and stability of these proteins are maintained by a specific environment of LPS (5–7). In complex
**Designed β-Boomerang Antiendotoxic Peptides**

### Table 1

| Peptide Name | Sequence | Retention time<sup>a</sup> | >80% neutralization of LPS (at 1 and 3 EU/ml) | E. coli ATCC27853 | P. aeruginosa ATCC25923 | S. aureus ATCC25923 | B. subtilis |
|--------------|----------|----------------------------|--------------------------------------------|-----------------|------------------|-----------------|-------------|
| YI12WF       | YVLWLRKRKAFPI | 26.17                      | 0.01                                       | 10              | 30               | 10              | 20          |
| YI12WW       | YVLWLRKRKAFPI | 26.07                      | 0.01                                       | 60              | 150              | 40              | 5           |
| YI12WY       | YVLWLRKRKAFPI | 24.60                      | 0.01                                       | 6               | 15               | 3               | 6           |
| YI12FF       | YVLWLRKRKAFPI | 26.07                      | 10<sup>b</sup>                              | 50              | >200             | 200             | >200         |
| YI12LL       | YVLWLRKRKAFPI | 24.82                      | ND<sup>c</sup>                              | >200            | >200             | >200            | >200        |
| YI12AA       | YVLWLRKRKAFPI | 21.13                      | ND<sup>c</sup>                              | >200            | >200             | >200            | >200        |
| GGWF         | GWMRKRKAFPI | 16.50                      | ND<sup>c</sup>                              | >200            | >200             | >200            | >200        |

<sup>a</sup> A C<sub>18</sub> reverse phase semi-preparative column was used. The peptides were eluted using a 60-min linear gradient of acetonitrile (10%) and water (90%) containing 0.1% trifluoroacetic acid (v/v).

<sup>b</sup> YI12FF peptide shows <40% neutralization of LPS at 10 μM concentration.

<sup>c</sup> ND: no detectable inhibition.

with LPS, the designed peptide assumed a novel amphipathic structure whereby the cationic and hydrophobic residues were segregated into distinctly different regions (34). The C terminus of the peptide showed two consecutive β-turns, whereas the N terminus appeared to be extended. In LPS inhibition assays, the peptide showed relatively weak activity (IC<sub>50</sub> ~ 10 μM). Interestingly, in the LPS-bound state, residues Trp<sup>4</sup> and Met<sup>9</sup> of the designed peptide showed NOE contacts, indicating that they are within ~5 Å apart. The Met residue was introduced into the primary sequence as an NMR chemical shift marker (34). Here, we have replaced Met<sup>9</sup> with aromatic residues, Trp, Phe, and Tyr, to enhance packing interactions with Trp<sup>4</sup> in complex with LPS. Such substitution may result in a defined folded structure with a large hydrophobic surface of the peptide in the context of LPS with a plausible enhancement in endotoxin neutralization and antimicrobial activities (Table 1). To underscore the role of presumable aromatic-aromatic interactions, peptides containing Ala<sup>4</sup>/Ala<sup>9</sup> and Leu<sup>4</sup>/Leu<sup>9</sup> were also prepared (Table 1). The Leu residues were particularly introduced to determine correlation of the hydrophobicity and aromatic-aromatic packing to the folding and activities of these peptides. To understand the specific role of Trp in the structure/activity, another peptide containing Phe at positions 4 and 9 has also been made (Table 1). In addition, an octapeptide was prepared to elucidate the role(s) of the hydrophobic residues at the N and C termini (Table 1).

**LPS Neutralization and Antimicrobial Activities of the Designed Peptides**—To determine the ability of the peptides to inhibit or neutralize LPS, sensitive chromogenic LAL assays were conducted (see “Experimental Procedures”). This assay can detect endotoxin at very low concentrations down to ~1 pm. LAL assays were conducted at three different LPS concentrations, 1, 3, and 8 EU/ml, with six different concentrations of peptides. As can be seen, peptides containing aromatic residues at positions 4 and 9, i.e., YI12WF, YI12WW, YI12WY, and YI12FF (Table 1), demonstrated the inhibition of LPS-mediated activation of LAL enzyme (Fig. 1). Peptides YI12WF, YI12WW, and YI12WY inhibited ≥80% endotoxin even at a concentration of 10 nM at 1 EU/ml (Fig. 1, *top*) and 3 EU/ml (Fig. 1, *middle*). At these LPS concentrations, ≥95% inhibition is observed at 100 nM concentrations for YI12WF, YI12WY, and YI12WW peptides (Fig. 1, *top* and *middle*). The YI12FF peptide shows a weak inhibitory activity, only ≤40% at 10 μM concentration at 1- and 3-EU/ml doses of LPS (Fig. 1, *top* and *middle*).
Even at a much higher concentration of LPS (8 EU/ml), YI12WF, YI12WY, and YI12WW peptides demonstrate ~80% neutralization of endotoxin at 10 μM concentrations (Fig. 1, bottom). No detectable inhibition of LPS was found for the YI12LL or Y12AA peptides even at a higher concentration of ≥100 μM (data not shown). The truncated 8-residue GG8WF peptide (Table 1) also lacks antiendotoxic activity (data not shown).

We have also examined antimicrobial activities of these peptides against two Gram-negative and two Gram-positive bacterial strains (Table 1). The order of antimicrobial activities follows YI12WY > YI12WF > YI12WW (Table 1). Other peptides are found to be rather inactive against these bacteria, although Y112FF and YI12LL showed some antibacterial activity only against the E. coli strain (Table 1). Peptide hydrophobicity has been used as a parameter to explain the antibacterial and hemolytic activities of antimicrobial peptides (36). The hydrophobicity of peptides can easily be measured from their retention time in a C18 column. A longer retention time is indicative of a higher hydrophobicity. However, the lack of LPS neutralization properties of the YI12LL and YI12AA peptides or the diminished activity of the YI12FF peptide cannot be simply explained by the hydrophobicity of these peptides (Table 1). The HPLC retention time of the active peptide YI12WY is similar to the largely inactive peptide YI12LL (Table 1). Furthermore, the Y112FF peptide, showing reduced LPS-neutralizing activity, also has a similar hydrophobicity to that of YI12WW (Table 1). One would find a positive correlation between LPS-neutralizing activities and hydrophobicity by comparing HPLC retention time for the inactive Y12AA peptide with the active peptides (Table 1). However, the lack or reduced activities of the YI12LL and YI12FF peptides clearly indicate the requirement of more specific interactions. These data suggest that rather than a global hydrophobicity, an explicit involvement of aromatic residues at positions 4 (Trp) and (Phe, Tyr, or Trp) 9 are important for LPS inhibitory activities of the designed peptide.

**LPS Binding Affinity of the Peptides**—ITC is used to determine binding interactions of the designed peptides with LPS (Fig. 2). Binding parameters can be obtained for all but GG8WF peptide as a result of the unsaturable binding. Table 2 summarizes thermodynamic parameters of the interactions. The LPS-peptide interactions are characterized by an endothermic heat released or entropy-driven process, as indicated by upward trends of the ITC profiles (Fig. 2). The entropy-driven complex formation at 25 °C between LPS and peptide had been reported earlier (37, 38). In this work, the temperature was kept below the phase transition temperature (~37 °C) of LPS favoring endothermic binding reactions. As can be seen, all peptides interact with LPS at μM affinities with $K_d$ ranging from as high as 5.9 μM to 1 μM (Table 2). These data indicate that mere LPS binding of peptides do not correlate with LPS neutralization or antimicrobial activities. Interestingly, the inactive peptides, YI12AA and YI12LL, show a somewhat high affinity binding to LPS as compared with the active peptides (Table 2).

**Effect of Designed Peptides on LPS Micelles**—We have probed structural perturbations of LPS micelles upon interactions with designed peptides using fluorescence of FITC-

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**FIGURE 2. Binding affinity of the designed peptides with LPS.** Top panel, isothermal titration calorimetry of YI12WF peptide; middle panel, YI12FF peptide; and bottom panel, YI12LL peptide. ITC experiments were carried out in 10 mM sodium phosphate buffer, pH 6.0, at 25 °C. Aliquots (2.5 μl) from 1 mM peptide were injected into solution containing 0.05 mM LPS, and the heat exchange was measured. Molar concentrations of LPS were estimated assuming molecular mass of 10 kDa (30, 48).
conjugated LPS, dynamic light scattering (Fig. 3), and 31P NMR of LPS (Fig. 4). As proposed earlier, LPS molecules form soluble aggregates, causing quenching of the fluorescence intensity of FITC (39). Binding of peptides or proteins with LPS may cause an enhancement of the FITC fluorescence as a result of the plausible dissociation of LPS aggregates (21, 39, 40). This observation has been correlated with the ability of the LPS-interacting peptides with their endotoxin neutralization activities (21, 40). It has been shown that the aggregated forms of LPS or lipid A are biologically more potent than the monomeric forms (41).

Fig. 3 demonstrates changes in the intensity of FITC-LPS fluorescence as a function of concentrations of different peptides. Additions of active peptides, YI12WW, YI12WF, and YI12WY, had caused an enhancement in FITC-LPS fluorescence, indicating the probable dissociation of LPS aggregates. The moderately active peptide YI12FF also demonstrated a dequenching of FITC-LPS fluorescence only at higher concentrations (Fig. 3A). There was no increase in FITC fluorescence in the presence of inactive peptides, YI12LL, YI12AA, or GG8WF. Interestingly, additions of the YI12AA peptide in FITC-LPS solutions appeared to cause a quenching of FITC fluorescence (Fig. 3A), indicating a different structural change of LPS aggregates (see below). The disaggregation of LPS by active peptides, YI12WF, YI12WY, and YI12WW, is also seen from dynamic light-scattering studies (Fig. 3B). The LPS alone produces a polydisperse sample with a diameter centered at 7000 nm (Fig. 3B). There is a dramatic shift of the average size of LPS toward the lower values in the presence of YI12WF and YI12WW peptides (Fig. 3B). Similar results were also obtained for the active peptide YI12WY (data not shown). The YI12FF peptide, showing a reduced LPS neutralization, disaggregates LPS at a lower extent as compared with the highly active peptides (Fig. 3B). On the other hand, the inactive peptide, YI12AA, does not show any dissociation of LPS aggregates (Fig. 3B). Rather, in the presence of YI12AA, LPS becomes more polydisperse with populations having larger sizes (Fig. 3B). This observation may explain the quenching of FITC fluorescence detected in the case of the YI12AA peptide (Fig. 3A) because more aggregation of LPS will reduce the fluorescence intensity of FITC (Fig. 3A).

We have utilized 31P NMR of LPS to study its interactions with the active peptide YI12WF and the inactive peptide YI12AA. LPS produces two well separated 31P resonances at −2.00 and −0.72 ppm (Fig. 4). By comparison with 31P NMR

### TABLE 2
Thermodynamic parameters of the interactions of the designed peptides with LPS

| Peptide | YI12AA | YI12WF | YI12WW | YI12WY | YI12FF | YI12LL |
|---------|--------|--------|--------|--------|--------|--------|
| $K_a$ ($\mu M^{-1}$) | $0.65 \pm 0.03$ | $0.22 \pm 0.02$ | $0.21 \pm 0.01$ | $0.17 \pm 0.03$ | $0.23 \pm 0.02$ | $1.0 \pm 0.06$ |
| $\Delta H$ (kcal.mol$^{-1}$) | $5.0 \pm 0.04$ | $2.3 \pm 0.07$ | $1.3 \pm 0.02$ | $2.8 \pm 0.02$ | $2.2 \pm 0.04$ | $1.1 \pm 0.01$ |
| $\Delta S$ (kcal.mol$^{-1}$deg$^{-1}$) | $12.80$ | $9.47$ | $8.40$ | $9.80$ | $10.1$ | $7.8$ |
| $\Delta G$ (kcal. mol$^{-1}$) | $-7.8$ | $-7.17$ | $-7.14$ | $-7.02$ | $-7.19$ | $-6.70$ |
| $K_d$ ($\mu M$) | $1.54$ | $4.5$ | $4.7$ | $5.9$ | $4.3$ | $1.0$ |

**FIGURE 3.** Disaggregation of LPS by the designed peptides. A, difference in fluorescence intensity of FITC-labeled LPS as a function of various concentrations of YI12WW, YI12WF, YI12WY, and YI12FF peptides. The YI12AA peptide that lacks the ability to neutralize LPS showed a quenching of FITC fluorescence. The FITC-LPS samples were excited at a wavelength of 480 nm. AU, arbitrary units. B, plots showing diameter versus intensity of scattered light for LPS (in the absence of peptides) and in the presence of YI12WF, YI12WW, YI12FF, and YI12AA peptides. All experiments were carried out in 10 mM sodium phosphate buffer, pH 6.0.
Sequence-specific resonance assignments for all the amino acids were achieved by the analyses of TOCSY and NOESY spectra (data not shown) (44). The NOESY spectra of the free peptide were predominantly characterized by intraresidue and sequential NOEs between the backbone protons and the side chain proton resonances (supplemental Fig. S1), indicating that peptides are highly mobile in their free forms and do not adopt any unique conformation(s).

Additions of low concentrations of LPS into the solutions containing peptides had caused concentration-dependent broadening of almost all the proton resonances without any significant change in chemical shifts, demonstrating a fast or intermediate exchange between free and LPS-bound states at the NMR time scale (supplemental Fig. S2) (see “Experimental Procedures”). Two-dimensional $^1$H–$^1$H NOESY spectra of the active peptides, YI12WF, YI12WY, and YI12WW, were obtained in the presence of LPS at a peptide/LPS molar ratio of 35:1. A large number of NOE cross-peaks were observed as a result of Tr-NOE (45, 46) effects, implying well folded conformations of the LPS-bound states of the peptides (Fig. 5). LPS forms high molecular weight bilayers/micelles in solution at a very low concentration (14 μg/ml) (47, 48), enabling determination of LPS-bound structures of peptides by the Tr-NOE method (49–53). Analyses of Tr-NOESY spectra reveal strong sequential α$n$ (i, i+1) and weak HN/HN NOEs for the hydrophobic/aromatic residues at the N and C termini, whereas more medium range NOE contacts of the type HN/HN (i to i+2) and Ca/HN (i to i+2) were observed for the central positively charged KRKR segment (supplemental Fig. S3). Most importantly, the NOE contacts between the Trp$^4$ ring protons with the aromatic residue at position 9, i.e. Phe$^9$, Tyr$^9$, or Trp$^9$ in YI12WF, YI12WY, and YI12WW, respectively, are unambiguously assigned (Fig. 5). For example, the well separated N$^H$ proton, resonating at 10.18 ppm, of Trp$^4$ shows NOE contact with the β-protons (2.96 ppm) of Phe$^9$ residue (Fig. 5A, left panel). There were NOE contacts between W4H4 and W4H5 to F9C$^6$Hs (Fig. 5A, right panel). Similarly, in the YI12WF peptide, long range NOE contacts were observed between the ring protons and indole N$^H$ proton of Trp$^4$ with the C$^6$Hs of Tyr$^9$ (Fig. 5B). In the case of the YI12WY peptide, NOEs between the non-degenerate β-protons of Trp$^4$ and Trp$^9$ were detected (Fig. 5C, inset). In addition, NOEs involving W9H6 proton with Trp$^9$ C$^6$Hs (Fig. 5C) and N$^H$ of Trp$^4$ with Trp$^9$ C$^6$H proton were identified in complex with LPS (Fig. 5C, left panel). The YI12FF peptide also showed NOEs between residues Phe$^4$ and Phe$^9$ in complex with LPS (data not shown). Tr-NOESY spectra of the inactive peptide YI12AA showed different NOE contacts whereby long range NOEs were detected between A4C$^6$H/Phe$^1$ ring protons and Tyr$^1$ ring protons with Ile$^{12}$ C$^6$H$^3$ group (supplemental Fig. S4).

Three-dimensional Structures of the Peptides Bound to LPS—Ensembles of high resolution structures of the active peptides, YI12WF, YI12WY, YI12WW, and the inactive YI12AA peptide, as a complex with LPS, were obtained by use of Tr-NOE-driven distance restraints (Table 3). The LPS-bound conformations of the designed peptides are well defined as determined by close superposition of the calculated structures and low root mean square deviation values (Fig. 6 and Table 3). All three active peptides, YI12WF, YI12WY, and YI12WW, acquire an
amphipathic structure in complex with LPS. The positively charged residues at the center form a loop defining the cationic face of the molecule, whereas the N and C termini hydrophobic residues adopt extended or β-strand conformations, making the hydrophobic surface (Fig. 6, A–C). In complex with LPS, the hydrophobic face of the active peptides is defined by aromatic-aromatic packing between the critical residue Trp4, with aromatic residues Phe9, Tyr9, or Trp9 in YI12WF, WY, and WW peptides, respectively (Fig. 6, A–C). In addition, in all three peptides, the N terminus residue Val2 is packed against Trp4, and the C terminus Phe11 makes facile contacts with Phe9, Tyr9, and Trp9, presumably strengthening the non-polar cluster (Fig. 6, A–C). The β-type structure with the aromatic-aromatic packing between residues 4 and 9 of the active peptides resembles a “boomerang” or β-boomerang structure. A helical boomerang structure was earlier described for the fusion domain of the influenza virus hemagglutinin protein in DPC micelles (54). The boomerang structure of the 20-residue fusion domain, defined by two helical segments, is dictated by the packing between two aromatic residues located at i and i+5 positions, i.e. Phe9 and Trp14. These residues are placed at the base of an intervening hydrophilic loop (Fig. 7). The superposition of the LPS-bound structure of the YI12WF peptide with the DPC-bound fusion domain structure shows the boomerang architecture of the designed peptide (Fig. 7). Interestingly, replacement of aromatic residue with Ala had been shown to dramatically reduce the fusion activity of the domain with the concomitant disruption of the boomerang structure (55).

The LPS-bound NMR structure of the inactive peptide YI12AA has been determined (Fig. 6D and Table 3). The YI12AA also folds into an amphipathic structure; in the context of LPS, however, the absence of the aromatic-aromatic packing at i to i+5 stabilizes a rather open struc-
The hydrophobic residues at the N and C termini are clustered into one side with aromatic residues Tyr1 and Phe9 facing each other (Fig. 6D). An LPS Binding Structured Motif Defined by GG8WF Peptide—Encouraged by the observation of a strong tendency of aromatic/aromatic packing between residues 4 and 9, we determined the LPS-bound structure of the short 8-residue peptide GG8WF (Table 1). GG8WF also yields a large number of Tr-NOE cross-peaks while bound to LPS (Fig. 8A). The indole N/H9280Ho of Trp2 shows NOE contact with C/H9252Hs of Phe7 (Fig. 8A, left panel). The C/H9252Hs of Phe7 also shows NOEs with the C6H and C2H ring protons of Trp2 (Fig. 8, right panel). Interestingly, more long range NOEs were detected between C/H9251H protons of residue Gly8 with ring protons of residue Trp2 (Fig. 8A). An ensemble of structures of GG8WF was obtained from Tr-NOE-driven distance constraints including 12 long range NOEs (Fig. 8B and Table 3). The well defined structure shows packing between Trp2/Phe7 rings with the positively charged residues segregated out at the top (Fig. 8C). The Phenyl ring of Phe7 appeared to stack over the indole ring of Trp2 (Fig. 8C).

This structural scaffold derived for the GG8WF peptide in LPS may be termed as a “structured LPS binding motif.” In principle, this independently folded sequence motif could be introduced into other peptides or proteins to develop endotoxin-neutralizing molecules or LPS targeting novel proteins (see “Discussion”).

Localization of Peptides in LPS—Intrinsic Trp fluorescence was used to determine localization of the active peptides,

| TABLE 3 | Summary of structural statistics for the 20 final structures of the designed peptides |
| --- | --- | --- | --- | --- | --- |
| Distance restraints | YII12WW | YII12WY | YII12WF | YII12AA | GG8WF |
| Sequential (| | | | | |
| Medium-range (2 ≤ | | | | | |
| Long range (i−j) | 60 | 61 | 58 | 50 | 30 |
| 4) | 58 | 59 | 58 | 30 | 21 |
| 5) | 5 | 5 | 4 | 2 | 12 |
| Angular restraints (| | | | | |
| Deviation from mean structure (Å) | 11 | 11 | 11 | 11 | 7 |
| All residues (N, Cα, C') | 0.1 | 0.1 | 0.2 | 1.3 | 0.2 |
| Heavy atoms | 0.8 | 0.6 | 0.7 | 2.0 | 0.7 |
| Ramachandran plot for the mean structure | 90 | 90 | 90 | 100 | 100 |
| % residues in the most favorable and additionally allowed region | 10 | 10 | 10 | 0 | 0 |
| % residues in the generously allowed region | 0 | 0 | 0 | 0 | 0 |

FIGURE 6. LPS-bound structure of the designed peptides. A–D, superposition of backbone atoms (N, Cα, C') of the 20 lowest energy conformers, the average conformer, and the electrostatic surface of YII12WF (A), YII12WW (B), YII12WY (C), and YII12AA (D) peptides determined in complex with LPS. This image was produced using the program MOLMOL.

FIGURE 7. Boomerang structure of the influenza hemagglutinin fusion peptide and the designed peptide YII12WF. Shown is the superposition of the structure of fusion domain of hemagglutinin determined in DPC micelles and the LPS-bound structure of YII12WF peptide. The 20-residue viral fusion domain adopts a boomerang-like structure, represented by the two segments of helices, held by a long range (i to i+5) packing between two aromatic residues Phe9 and Trp14. Replacements of the aromatic residues with aliphatic ones showed a dramatic loss of fusogenic activity of the peptide. The LPS-bound conformation of the 12-residue designed peptide studied here shows a close topological similarity with the fusion domain structure. The image was produced using the program MOLMOL.

An LPS Binding Structured Motif Defined by GG8WF Peptide—Encouraged by the observation of a strong tendency of aromatic/aromatic packing between residues 4 and 9, we determined the LPS-bound structure of the short 8-residue peptide GG8WF (Table 1). GG8WF also yields a large number of Tr-NOE cross-peaks while bound to LPS (Fig. 8A). The indole N/H9280Ho of Trp2 shows NOE contact with C/H9252Hs of Phe7 (Fig. 8A, left panel). The C/H9252Hs of Phe7 also shows NOEs with the C6H and C2H ring protons of Trp2 (Fig. 8, right panel). Interestingly, more long range NOEs were detected between C/H9251H protons of residue Gly8 with ring protons of residue Trp2 (Fig. 8A). An ensemble of structures of GG8WF was obtained from Tr-NOE-driven distance constraints including 12 long range NOEs (Fig. 8B and Table 3). The well defined structure shows packing between Trp5/Phe7 rings with the positively charged residues segregated out at the top (Fig. 8C). The Phenyl ring of Phe7 appeared to stack over the indole ring of Trp2 (Fig. 8C).

This structural scaffold derived for the GG8WF peptide in LPS may be termed as a “structured LPS binding motif.” In principle, this independently folded sequence motif could be introduced into other peptides or proteins to develop endotoxin-neutralizing molecules or LPS targeting novel proteins (see “Discussion”).
YI12WF, YI12WY, and YI12WW, and also for the short GG8WF peptide, in LPS. Additions of LPS into peptide solutions caused a progressive blue shift of the emission maxima of Trp fluorescence, saturating at an LPS concentration of ~40 μM (data not shown). The marked blue shift of the Trp fluorescence indicated incorporation of the peptides into LPS micelles (Table 4), indicating that the Trp residue is well buried in LPS but largely exposed in the DPC micelles. Further, to determine insertion of the Trp residue into the LPS bilayer, fluorescence-quenching studies were carried out by two spin-labeled lipids, 5-DSA and 16-DSA. Trp fluorescence intensity was quenched for all four peptides either by the shallow quencher 5-DSA, containing spin label at position 5, or by the deep quencher 16-DSA, containing spin label at position 16. The extent of quenching was used to determine the depth of the insertion into LPS bilayers (see “Experimental Procedures”). The depths of penetration of the Trp residue for the four peptides in their LPS-bound states are shown in Table 4 and found to be located around ~7 Å from the center of LPS bilayer. These depth values indicate that the peptides are rather deeply inserted into LPS bilayer. It is noteworthy that the octapeptide GG8WF also demonstrated a similar depth of insertion (7.18 Å), emission maxima, and $K_{SV}$ values in LPS (Table 4), as compared with the 12-residue active peptides. These data indicate an independent ability of the GG8WF peptide to be localized into the same environment of LPS.

### Secondary Structures and LPS Interactions by FTIR Studies

- The assignment of the bands in the amide I region was performed as reported (56). The spectra in the amide I region of the different peptides examined are shown in Fig. 9A. In all cases, except in the peptide GG8WF, there is a strong component at ~1630 cm$^{-1}$ indicative of β-structure (57). When these structures are anti-parallel, it is accompanied by a much less intense band at ~1690 cm$^{-1}$, but in this case, it may be obscured by the strong band at 1670 cm$^{-1}$ corresponding to turns. By contrast, for the peptide GG8WF, the spectrum is consistent with turns (band at 1670 cm$^{-1}$) and also some flexible structures (broad band centered at ~1640 cm$^{-1}$).
The effect of the peptides on the phosphate of LPS was examined by looking at the antisymmetric phosphate bands in the region 1200–1260 cm\(^{-1}\) where the band is split in at least two vibrational bands with different hydration states, with the band at a higher wave number corresponding to representing lower hydration (57). In this case, two main bands were observed in LPS at both low and high levels of hydration, at 1237 and 1210 cm\(^{-1}\) (Fig. 9, B and C). In agreement with the above, when the sample was fully hydrated, the band at 1237 cm\(^{-1}\) (lower hydration) became attenuated (in Fig. 9, B and C, compare spectrum LPS, see arrow). After the addition of the peptides, another band appears at 1200 cm\(^{-1}\), as reported previously for the interaction of other peptides with LPS (22, 57). At both low and high hydration, the bands from the antisymmetric phosphate of LPS disappear, indicating immobilization of the phosphate groups (22). Taken together, these results demonstrate that all of the 12-residue peptides, active or inactive, form \(\beta\)-type structures in LPS with a turn component. These results are in complete corroboration with the LPS-bound NMR structures of the peptides whereby the central positively charged residues form the turn or loop and the N and C termini hydrophobic residues are in \(\beta\)-strand conformations.

**DISCUSSION**

Due to the prime importance as an endotoxic agent, LPS is a target for the development of antiseptic drugs (27, 59, 60). LPS also serves as a potential barrier toward antimicrobials at the outer membrane of Gram-negative bacteria (3, 4, 61). In addition, LPS has been found to be highly necessary for the correct folding of the outer membrane proteins (5–7). Antimicrobial and endotoxic functionalities of LPS had recently generated considerable interest in investigating naturally occurring antimicrobial peptide–LPS interactions (18–23, 62–66). Helical amphipathic cationic peptides, consisting of amino acids Leu and Lys, were designed to understand interactions with LPS (22, 40). Highly \(\beta\)-sheet peptides (33-residue) were designed based on \(\alpha\)-chemokines and human neutrophil bactericidal protein (BPI) for LPS neutralization and antimicrobial activities (67, 68).

It is noteworthy that the design of YI12 peptides, as studied here, does not follow the usual amphipathic sequence pattern for helices or \(\beta\)-sheets as described earlier (22, 40, 67, 68). Instead, we assume that the centrally located four positively charged residues will help in initiating a binding process via electrostatic interactions with LPS head groups; this may allow the hydrophobic sequences at the N and C termini to integrate into the acyl chains of LPS, stabilizing defined \(\beta\)-type structures (34). The structural organization of these peptides may essentially act as outer membrane protein mimics (see below). Here, we are able to demonstrate critical structural features that are essential for the LPS neutralization and antimicrobial activities of these peptides. We established that a long range aromatic-aromatic packing (\(i\) to \(i+5\)) between residues located at positions 4 and 9 in the 12-residue sequences is highly important for the antiendotoxic and also antimicrobial activities of the designed sequences. In the LAL assay, the active peptides,

| Table 4 |
|---|
| Fluorescence emission maxima, Stern-Volmer quenching constants (\(K_{SV}\)), and depth of insertion of the designed peptides in the zwitterionic DPC micelles and LPS bilayer |
| Peptides | Buffer | DPC | LPS | Distance of Trp from the center of the LPS bilayer |
|---|---|---|---|---|
| YI12WF | 357 | 31.2 | 355 | 14.2 | 345 | 5.2 | 7.12 |
| YI12WW | 357 | 53.2 | 353 | 16.9 | 346 | 9.0 | 7.01 |
| YI12WY | 357 | 42.8 | 355 | 12.6 | 342 | 6.9 | 7.09 |
| GG 8WF | 355 | 25.4 | 352 | 13.3 | 344 | 7.1 | 7.18 |

**FIGURE 9.** Secondary structures and interactions of the peptides with LPS by FTIR. Infrared data for the peptides examined are shown. A, spectra in the amide I region of LPS alone (indicated by LPS) or LPS mixed with the peptides indicated in a low hydration state. Spectra in a high hydration state were similar and are not shown. B and C, spectra in the antisymmetric phosphate region, in low (B) and high (C) hydration states. **rel units**, relative units.
YI12WF, YI12WY, and YI12WW, showed very high LPS neutralization potency (Fig. 1 and Table 1). A much lower LPS neutralization activity of the YI12FF peptide (Fig. 1 and Table 1) indicates a specific role of the Trp residue at this position. Trp has been known to play an important function toward the membrane incorporation of antimicrobial peptides (69). The Tr-NOE-driven structures obtained for the active peptides, YI12WF, YI12WW, and YI12WY, in LPS showed aromatic packing interactions between Trp⁴ and Phe⁹/Tyr⁹/Trp⁹ (Fig. 6). The central positively charged residues assume loop-like structures (Fig. 6). The hydrophobic residues at the N and C termini are extended or β-strands and lay close to the packed aromatic-aromatic cluster. IR studies also demonstrated the presence of turn and β-strand structures of the peptides in LPS (Fig. 9).

The active and inactive peptides bind to LPS with similar affinity (Fig. 2 and Table 2) and acquire folded structure in complex with LPS (Fig. 6). It appears that the aromatic packing may render a specific orientation of the hydrophobic termini, resembling a boomerang. The replacement of the critical aromatic residues either by Ala or by Leu resulted in a non-packed structure, although amphipathic with impaired activities (Figs. 1 and 6). The electrostatic surface of the active peptides clearly shows a positively charged “head” and a well packed elongated non-polar “body” (Fig. 6). We have examined surface charges of a number of outer membrane proteins with known three-dimensional structures. Very interestingly, several outer membrane proteins contain structural regions that show similar surface topology to the designed peptides (Fig. 10). Fig. 10 illustrates a selected region from outer membrane proteins, FhuA (A), OmpA (B), and PhoE (C), showing positively charged patches and non-polar β-sheet regions. This image was produced with the program PyMOL (DeLano Scientific, San Carlos, CA). The Protein Data Bank (PDB) coordinates are as follows: for FhuA, 1QFZ; for OmpA, 1g90; and for PhoE, 1PHO.

The probable mechanism by which active peptides are able to neutralize LPS appeared to correlate with their ability to disaggregate and/or perturb LPS micelles (Figs. 3 and 4). Dynamic light-scattering and FITC fluorescence studies clearly demonstrated that active peptides can dissociate LPS micelles. Dynamic light-scattering experiments revealed that the large aggregated forms of LPS converted into smaller sizes upon binding with active peptides (Fig. 3B). 3¹P NMR resonances of LPS were highly affected in the presence of active peptide YI12WF (Fig. 4). However, the inactive peptide YI12AA did not cause any perturbation to 3¹P resonance of LPS (Fig. 4), although ITC, NMR, and IR studies show that YI12AA peptide interacts and adopts a folded structure in LPS. Therefore, the perturbation of 3¹P resonances of LPS micelles can be used as a specific probe for the LPS-neutralizing and antimicrobial peptides. Recently, we reported that 3¹P resonances of LPS undergo
The hydrophobic arms, at the N and C termini, are expected to serve as a lock to secure the insertion of the peptide into LPS. The hydrophobic arms, at the N and C termini of the most active LPS-neutralizing peptide YI12WF showed a dramatic diminution of LPS neutralization (71). Intrinsic Trp fluorescence emission spectra and quenching of fluorescence intensity by soluble peptides are transmembrane proteins. These proteins are targets for the development of antimicrobial compounds. The current LPS-induced structure-sequence correlation of the designed peptides, presented here, might aid in developing novel proteins that may fold into the outer membrane with β-sheet topology. Designing proteins for the outer membrane could be useful to disrupt transmembrane organization of the therapeutically important pathogenic proteins. Disruption of transmembrane helical interfaces by designed peptides has recently been demonstrated in type I membrane protein integrins (73). In conclusion, we have established, using a set of design peptides, vital structural determinants for LPS neutralizations by β-sheet peptides. The ability of the active and inactive peptides and the short peptide motif to fold into well defined β-type structures in LPS will be a starting point to build up β-sheet-rich native outer membrane proteins.

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