Structure−Function Studies of Polymyxin B Nonapeptide: Implications to Sensitization of Gram-Negative Bacteria[#]

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Polymyxin B nonapeptide (PMBN), a cationic cyclic peptide derived by enzymatic processing from the naturally occurring peptide polymyxin B, is able to increase the permeability of the outer membrane of Gram-negative bacteria toward hydrophobic antibiotics probably by binding to the bacterial lipopolysaccharide (LPS). We have synthesized 11 cyclic analogues of PMBN and evaluated their activities compared to that of PMBN. The synthetic peptides were much less potent than PMBN in their capacity to sensitize *Escherichia coli* and *Klebsiella pneumoniae* toward novobiocin and to displace dansyl-PMBN from *Escherichia coli* LPS. Moreover, unlike PMBN, none of the analogues were able to inhibit the growth of *Pseudomonas aeruginosa*. The structural−functional features of PMBN were characterized and identified with regard to the ring size, the distance between positive charges and peptide backbone, the chirality of the DPhe-Leu domain, and the nature of the charged groups. Apparently, the structure of PMBN is highly specific for efficient perturbation of the outer membrane of Gram-negative bacteria as well as for LPS binding. The present study further increases our understanding of the complex PMBN−LPS and may, potentially, enable the design of compounds having enhanced permeabilization potency of the Gram-negative outer membrane.

Introduction

The accelerated emergence of pathogenic bacteria exhibiting a multiple pattern of resistance toward conventional antibiotics is a major threat, especially in severe infections such as septicemia where mortality is high.[1] The development of novel, versatile antibiotic arsenal is thus most urgent. Optimal new antimicrobial agents should withstand enzymatic modification and/or degradation and be targeted toward a bacterial component which is readily accessible and not likely to undergo mutations.

The bacterial endotoxin lipopolysaccharide (LPS) is the major antigen of the outer membrane (OM) of Gram-negative bacteria. It is composed of three major parts, one of which is lipid A, a highly conserved hydrophobic domain of LPS, in Gram-negative bacteria.[2] LPS is essential for survival of the bacteria through establishing of an effective permeability barrier. Gram-negative bacteria lacking lipid A have not yet been isolated, and the mutation is thought to be lethal.[2] LPS is a predominant inducer of sepsis.[3] Uncontrolled perturbation of immune cells by LPS followed by cytokine-mediated damage to blood vessels and decrease in vascular resistance frequently lead to collapse of organs and death.[4] Neutralization of the devastating effects of LPS is one of the main targets in combating endotoxicosis.[5] In the past few years various therapeutic strategies and novel agents for the treatment of LPS-mediated septic shock have been evaluated, including anti-LPS antibodies, LPS-neutralizing proteins (BPI and LBP), lipid A antagonist (lipid X), polymyxin B (PMB), and polymyxin B-related synthetic peptides.[5−7] Lipid A is a target for cationic proteins and peptides since it contains negatively charged phosphorylated disaccharides linked, via ester bonds, to long fatty acid chains which are intercalated in the bacterial outer membrane.[8]

PMB is a naturally occurring cationic cyclic decapeptide isolated from *Bacillus polymyxa*.[9,10] PMB is highly bactericidal to Gram-negative bacteria and considered one of the most efficient cell-permeabilizing compounds.[11] This capacity is due to its high-affinity binding to lipid A.[12] However, the therapeutic applications of PMB are very limited because of its relatively high toxicity.[13,14] Polymyxin B nonapeptide (PMBN) is a cyclic peptide obtained from PMB by proteolytic removal of its terminal amino acyl residue (Scheme 1).[15] PMBN is an extremely poor antimicrobial compound,[16] but it is still capable of binding, like its parent compound, to LPS, rendering Gram-negative bacteria susceptible to various hydrophobic antibiotics.[17] This ability of PMBN to bind to bacteria with relatively high affinity and to permeabilize their outer membrane is often referred to as "sensitizing activity" and points at a novel therapeutic direction.[18] This approach is strengthened by the markedly reduced toxicity of the PMBN derivative as compared with its parental PMB molecule.[11] In addition, an enzyme capable of degrading the cyclic PMBN peptide has not yet been reported.

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[#] Abbreviations: Agb, 2-amino-4-guanidinobutyric acid; CD, circular dichroism; CFU, colon-forming unit; Dab, 2,4-diaminobutyric acid; Dap, 2,3-diaminopropionic acid; DIEA, diisopropylethylamine; MIC, minimal inhibitory concentration; NMM, 4-methylmorpholine; LPS, lipopolysaccharide; PMB, polymyxin B; PMBN, polymyxin B nonapeptide; PyBOP, (benzotriazol-1-yloxy)trispyrolidinophosphonium hexafluorophosphate; TES, triethylsilane; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

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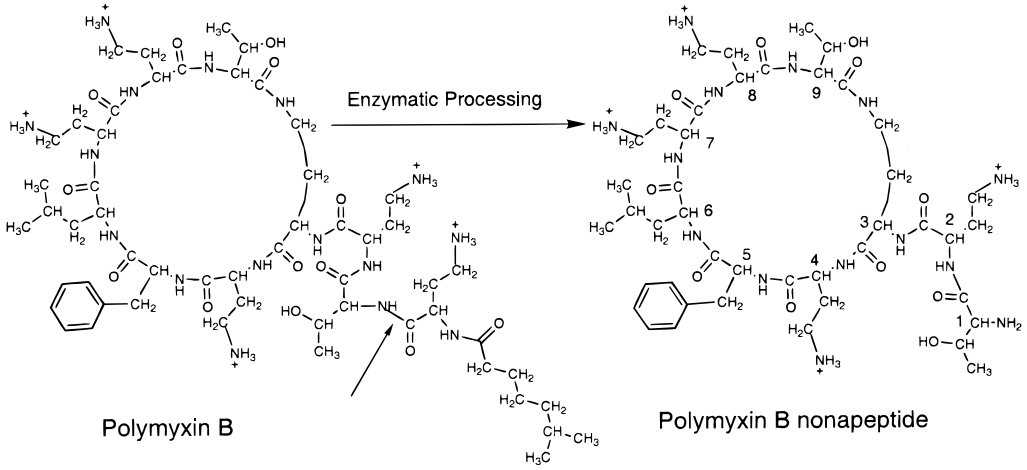
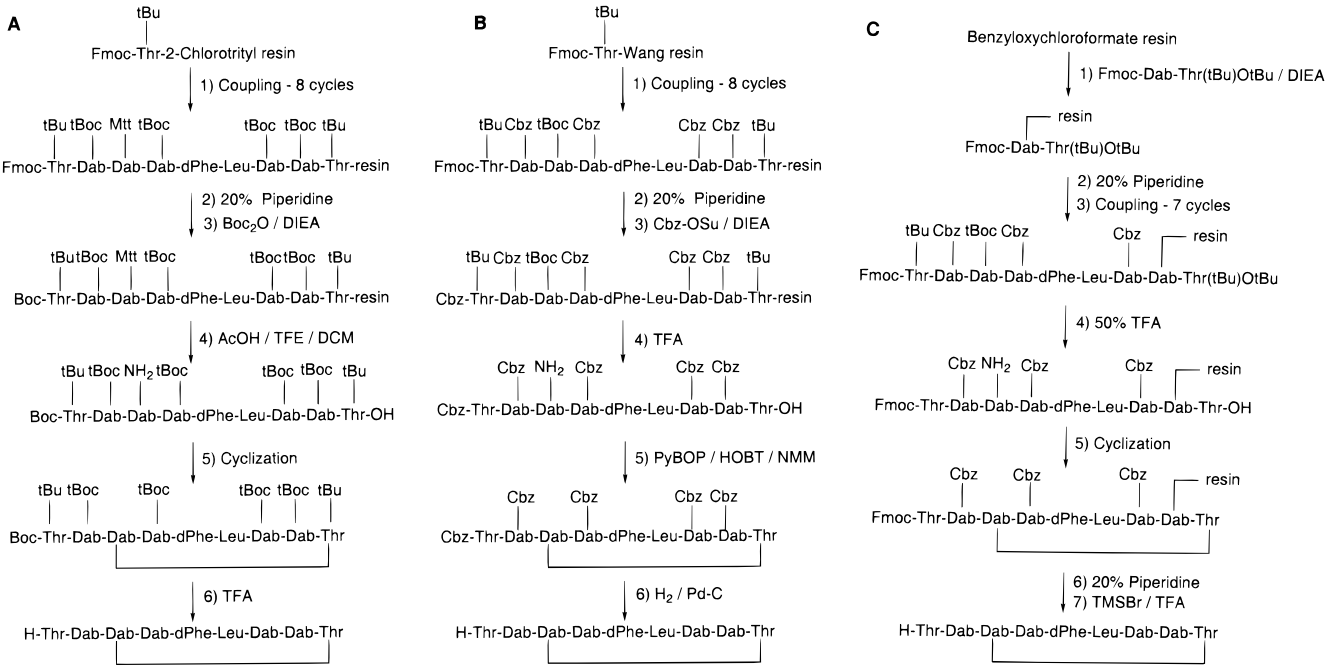
Scheme 1. Generation of PMBN from PMB by Enzymatic Processing



Scheme 2. Synthetic Routes to sPMBN



The binding of PMBN to LPS on bacterial surface and its membrane permeabilization activity (i.e., sensitization) are thought to be consecutive and inseparable. These capacities of the peptide can serve as a starting point to novel anti-Gram-negative drugs. In the present investigation we have performed structure−function studies of PMBN pertaining to its antibacterial activity. Our study indicates that the structural parameters required for LPS binding and membrane permeabilization are rather conserved in the PMBN parent molecule.

Results

Peptides Synthesis. Three approaches were evaluated for the synthesis of PMBN: two involved linear chain assembly on a solid support following by solution cyclization (Scheme 2A,B), whereas the third was based on cyclization on the solid support (Scheme 2C). The 9-fluorenylmethoxycarbonyl (Fmoc) group was used, as a rule, for α-amino protection. γ-Amino groups of Dab^3 were protected by either 4-methyltrityl (Mtt) (Scheme 2A) or *tert*-butyloxycarbonyl (tBoc) (Scheme 2B). The C-terminal carboxylic function of Thr was protected by either the 2-chlorotrityl or the Wang polymeric carriers (Scheme 2A,B, respectively). In route A, following simultaneous cleavage of the linear peptide from the resin and removal of the Mtt protection under mild acidic conditions, cyclization was attempted in high dilution (1 mg/mL) using four different reagents: (benzotriazol-1-yloxy)trispyrolidinophosphonium hexafluorophosphate (PyBOP), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *o*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (all with HOBT in the presence of NMM as a base), and *N*,*N*′-dicyclohexylcarbodiimide/*N*-hydroxybenzotriazole (DCC/HOBT). The reactions were monitored using analytical HPLC (see Experimental Section). The best results, as judged by yield and purity, were obtained with PyBOP. Final deprotection afforded the expected sPMBN, characterized by the expected mass and amino acid content (Table 1) and by HPLC identity with pPMBN obtained via proteolysis of PMB. However, a major byproduct, presumably a result

Table 1. Peptide Analysis

no.	peptide	MS found (calcd)	t_R (min)[a]	amino acid analysis[b]
1	pPMBN	962.5613 (962.5661, $C_{43}H_{74}N_{14}O_{11}$)	25.0	T (2) 1.91; F (1) 0.98; L (1) 1; X (5) 4.98
2	sPMBN	962.5637 (962.5661, $C_{43}H_{74}N_{14}O_{11}$)	25.0	T (2) 1.87; F (1) 0.95; L (1) 1; X (5) 4.9
3	[$Lys^{2,3,4,7,8}$]PMBN	1103.7248 (1103.7305, $C_{53}H_{94}N_{14}O_{11}$)	23.5	T (2) 1.85; F (1) 0.98; L (1) 1; K (5) 5.1
4	[$Orn^{2,3,4,7,8}$]PMBN	1033.6538 (1033.6522, $C_{48}H_{84}N_{14}O_{11}$)	22.3	T (2) 1.81; F (1) 0.91; L (1) 1; O (5) 4.7
5	[$Dap^{2,3,4,7,8}$]PMBN	893.4931(893.4957, $C_{38}H_{64}N_{14}O_{11}$)	23.5	T (2) 1.85; F (1) 0.9; L (1) 1; B (5) 4.6
6	[Lys^{3}]PMBN	991.6041 (991.6052, $C_{45}H_{78}N_{14}O_{11}$)	24.7	T (2) 1.75; F (1) 0.9; L (1) 1; X (4) 3.75; K (1) 0.98
7	[$Lys^{2,4,7,8}$]PMBN	1074.6491 (1074.6914, $C_{51}H_{90}N_{14}O_{11}$)	24.8	T (2) 1.84; F (1) 0.9; L (1) 1; X (1) 0.9; K (4) 3.8
8	[*cyclo*-Dab^{4},Thr^{9}]PMBN	962.7011 (962.5661, $C_{43}H_{74}N_{14}O_{11}$)	24.1	T (2) 1.91; F (1) 0.95; L (1) 1; X (5) 4.75
9	[*cyclo*-Dab^{2},Thr^{9}]PMBN	962.6402 (962.5661, $C_{43}H_{74}N_{14}O_{11}$)	24.9	T (2) 1.9; F (1) 0.95; L (1) 1; X (5) 4.8
10	[$Lys^{2,4}$]PMBN	1018.6754 (1018.6288, $C_{47}H_{82}N_{14}O_{11}$)	25.1	T (2) 1.8; F (1) 0.9; L (1) 1; X (3) 2.9; K (2) 1.9
11	[$Lys^{7,8}$]PMBN	1018.7112 (1018.6288, $C_{47}H_{82}N_{14}O_{11}$)	24.0	T (2) 1.8; F (1) 0.9; L (1) 1; X (3) 2.9; K (2) 1.9
12	[$lPhe^{5}$]PMBN	962.5788 (962.5661, $C_{43}H_{74}N_{14}O_{11}$)	25.0	T (2) 1.86; F (1) 0.95; L (1) 1; X (5) 4.8
13	[$Agb^{2,4,7,8}$]PMBN	1130.6498 (1130.6533, $C_{47}H_{82}N_{22}O_{11}$)	26.1	T (2) 1.88; F (1) 0.92; L (1) 1; X (1) 0.95

[a] t_R, retention time on analytical RP-18 HPLC column (see Experimental Section). [b] Calculated values are in parentheses. X = Dab, T = Thr, F = D- or LPhe, K = Lys, B = Dap, O = Orn.

Table 2. Chemical Structures of PMBN and PMBN Analogues

				length of alkyl chain of charged aa[a]				
				position of aa[b]				
no.	peptide	ring size (atoms)	peptide structure	2	3	4	7	8
1	pPMBN	23	T-X-*cyclo*[X–X-DF-L-X-X-T]	2	**2**	2	2	2
2	sPMBN	23	T-X-*cyclo*[X-X-DF-L-X-X-T]	2	**2**	2	2	2
3	[$Lys^{2,3,4,7,8}$]PMBN	25	T-K-*cyclo*[K-K-DF-L-K-K-T]	4	**4**	4	4	4
4	[$Orn^{2,3,4,7,8}$]PMBN	24	T-O-*cyclo*[O-O-DF-L-O-O-T]	3	**3**	3	3	3
5	[$Dap^{2,3,4,7,8}$]PMBN	22	T-B-*cyclo*[B-B-DF-L-B-B-T]	1	**1**	1	1	1
6	[Lys^{3}]PMBN	25	T-X-*cyclo*[K-X-DF-L-X-X-T]	2	**4**	2	2	2
7	[$Lys^{2,4,7,8}$]PMBN	23	T-K-*cyclo*[X-K-DF-L-K-K-T]	4	**2**	4	4	4
8	[*cyclo*-Dab^{4},Thr^{9}]PMBN	20	T-X-X-*cyclo* [X-DF-L-X-X-T]	2	2	**2**	2	2
9	[*cyclo*-Dab^{2},Thr^{9}]PMBN	26	T- *cyclo*[X-X-X-DF-L-X-X-T]	**2**	2	2	2	2
10	[$Lys^{2,4}$]PMBN	23	T-K-*cyclo*[X-K-DF-L-X-X-T]	4	**2**	4	2	2
11	[$Lys^{7,8}$]PMBN	23	T-X-*cyclo*[X-X-DF-L-K-K-T]	2	**2**	2	4	4
12	[$lPhe^{5}$]PMBN	23	T-X-*cyclo*[X-X-F-L-X-X-T]	2	**2**	2	2	2
13	[$Agb^{2,4,7,8}$]PMBN	23	T-Z-*cyclo*[X-Z-DF-L-Z-Z-T]	4	**2**	4	4	4
14	PMB	23	6-methylheptanoyl/octanoyl-X-T-X-*cyclo*[X-X-DF-L-X-X-T]	2	**2**	2	2	2

[a] Bold numbers represent amino acids that participate in the formation of the peptide ring. [b] Numbers are according to Scheme 1. X = Dab, T = Thr, DF = DPhe, K = Lys, B = Dap, O = Orn, Z = Agb.

of β-elimination reaction of the C-terminal Thr(tBu) residue (i.e., α-amino crotonic acid derivative),[21] accompanied the desired product. An alternative route (Scheme 2B) involved cleavage from Wang resin of a partially protected linear peptide, i.e., possessing free γ-amino Dab^{3} and α-carboxyl Thr^{9} residues, followed by cyclization using PyBOP/HOBT/NMM as reagents and base (see Experimental Section). This procedure was accomplished in less than 1 h. Complete peptide deprotection was achieved by catalytic hydrogenation. The single product obtained was purified to homogeneity by HPLC and characterized by amino acid analysis, mass spectrometry, ^{1}H NMR, and its identity with pPMBN (HPLC). All 11 synthetic peptides (**2**–**13**, Tables 1 and 2) evaluated in this study were prepared according to route B.

An additional synthetic route (Scheme 2C) was initiated by coupling a dipeptide, i.e., Fmoc-Dab-Thr(tBu)-OtBu, to benzyl chloroformate resin.[22] Following completion of chain assembly, the tBoc and tBu protections of Thr^{1}, Dab^{3}, and Thr^{9} were removed by TFA. Cyclization was then performed on the resin-bound peptide using either PyBOP or HATU as reagents. Treatment with bromotrimethylsilane (TMSBr)/TFA accomplished the final cleavage and deprotection. Cyclization with PyBOP led to the desired, fully identical PMBN product. Cyclization with HATU afforded, in addition, a byproduct identified as a tetramethylguanidinium (Tmg) derivative of Dab.[23] Although the cyclization reaction with PyBOP was successful, the method was rather laborious and not suited for fast and efficient synthesis of PMBN analogues.

The conversion of pPMBN to [$Agb^{2,4,7,8}$]PMBN (**13**) resulted in good yield, using 3,5-dimethylpyrazole-1-carboxamidine nitrate, while the α-amino function of Thr^{1} was not modified.

Rationale of Analogues Design. Eleven PMBN analogues were synthesized (Tables 1 and 2) via route B (Scheme 2B) in an attempt to locate key structural features and amino acid residues essential for membrane disorganizing activity of the parent PMBN molecule. Thus, three analogous peptides, namely, [$Lys^{2,3,4,7,8}$]-PMBN (**3**), [$Orn^{2,3,4,7,8}$]PMBN (**4**), and [$Dap^{2,3,4,7,8}$]PMBN (**5**), differing in the length of the alkyl chains of the charged amino acids and in the size of the peptide ring, were synthesized. The number of the side chain methylene groups varied from 2 in the original PMBN molecule to 4, 3, and 1 in the corresponding analogues. In addition, the ring size of PMBN was modified from 23 atoms in the original molecule to 25, 24, and 22 atoms in the above analogues. [Lys^{3}]PMBN (**6**) and [$Lys^{2,4,7,8}$]PMBN (**7**) were synthesized in an attempt to differentiate between the above two factors, i.e., ring size and length of the alkyl chain of the charged amino acids. Thus, in [Lys^{3}]PMBN (**6**) the ring size was extended to 25 atoms by the addition of two methylene groups. The alkyl chain length of the charged amino acids was changed in [$Lys^{2,4,7,8}$]PMBN (**7**) by replacing

the Dab residues with Lys while the ring size was left unchanged. Further modifications in the size of the PMBN ring were performed in [*cyclo*-Dab^4,Thr^9]PMBN (**8**) by subtracting one charged residue (Dab^3) from the peptide ring while adding a charged residue (Dab^2) in [*cyclo*-Dab^2,Thr^9]PMBN (**9**). Since there are two separated positively charged sites at the PMBN structure, i.e., positions 2,4 and positions 7,8, interspaced by DPhe-Leu, [Lys^{2,4}]PMBN (**10**) and [Lys^{7,8}]PMBN (**11**) were synthesized in order to reveal the significance of each site. Thus, the lengths of the alkyl chains of the charged residues were extended from 2 to 4 methylene groups. The hydrophobic core of PMBN DPhe-Leu was modified by replacing DPhe with LPhe in [LPhe^5]PMBN (**12**). This substitution could possibly affect the amphiphilic nature of the molecule. Finally, the γ-amino side chain function of the Dab residues was modified to yield the corresponding guanidino peptide [Agb^{2,4,7,8}]PMBN (**13**) in order to evaluate the effect of enhanced p*K* value of the positively charged moiety, i.e., 10.79 of Dab and 12.48 of Agb, and the nature of the charged group.

Affinity of PMBN and Analogues to LPS. The interaction of PMB with cell-bound and cell-free forms of LPS can be quantified using the dansyl-PMB binding assay.[20] The method is based on enhanced dansyl fluorescence upon the interaction of LPS with the labeled peptide. Binding experiments with PMBN were performed by modification of the above method. Thus, we have synthesized and used dansyl-PMBN rather than dansyl-PMB. Dansyl-PMBN was synthesized by reacting the free N-terminal α-amino function of the peptide with dansyl chloride prior to its cleavage from the polymeric support. A plot of enhanced fluorescence as a function of dansyl-PMBN added to LPS is illustrated in Figure 1. A Hill plot (insert) of the data suggests a cooperative peptide−LPS association. The molar ratio peptide/LPS as determined from the Hill slope curve (Hill number, *n*) was calculated to be 2.3. According to calculations based on maximal binding curve (not shown) with excess LPS (400 μg/mL), a rather similar ratio of dansyl-PMBN/LPS (1.98) was obtained. The plot intercept ($S_{0.5}$), i.e., binding affinity, was found to be 0.41 μM.

The ability of the peptides pPMBN, sPMBN, and the synthetic analogues to displace dansyl-PMBN (0.55 μM) from cell-free LPS (3 μg/mL) was evaluated. Several representative patterns are illustrated in Figure 2. The calculated values for maximal and 50% displacement, I_{max} and IC_{50}, respectively, are summarized in Table 3.

The analogue [Agb^{2,4,7,8}]PMBN (**13**) was almost as potent as PMBN in displacing dansyl-PMBN from LPS, having IC_{50} of 3.8 and 2.5 μM, respectively. [Lys^3]-PMBN, [*cyclo*-Dab^2,Thr^9]PMBN, and [lPhe^5]PMBN, i.e., peptides **6**, **9**, and **12**, respectively, were somewhat less effective, whereas the other analogues were significantly less active (Table 2). PMBN (**1, 2**) and [Agb^{2,4,7,8}]PMBN (**13**) were able to inhibit 80% of fluorescence, while the other analogues were capable of up to 64% inhibition (Table 3).

Circular Dichroism (CD) Measurements. The CD spectra of PMBN and analogues were measured at 0.1 mM peptide concentrations in 5 mM PB, pH 7.5. pPMBN (**1**) has a maximal negative ellipticity at 200 nm, characteristic of an ensemble of random conformations, and a minor shoulder at 210−222 nm. The CD spectrum of sPMBN (**2**) was, as expected, almost identical to that of pPMBN (**1**). Similar patterns were obtain for the two peptides in trifluoroethanol (TFE)/aqueous buffer mixture (1:1; v/v) or in bulk TFE. The CD spectra of all analogues suggest random conformations.

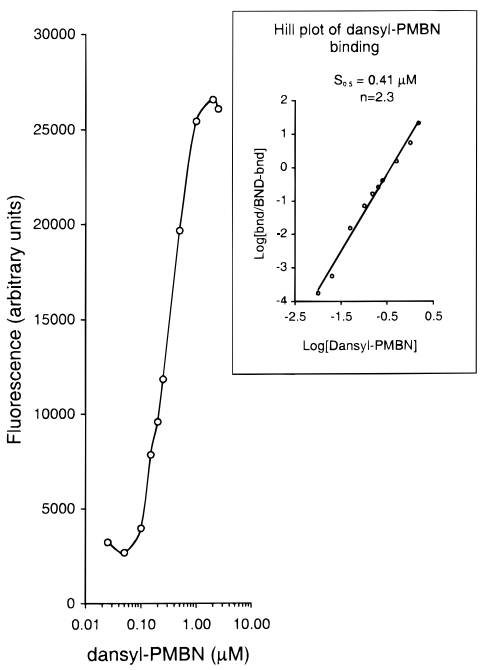


Figure 1. Binding curve of dansyl-PMBN to *E. coli* LPS. Increasing concentrations of dansyl-PMBN were added to *E. coli* LPS solution (3 μg/mL). The augmented fluorescence was measured 5 min after each addition at excitation and emission wavelengths of 340 and 485 nm, respectively. Hill plot of dansyl-PMBN binding (insert) was generated as described (Experimental Section), where BND and bnd are the fluorescence determined with 400 and 3 μg/mL LPS, respectively, for the same dansyl-PMBN concentrations.

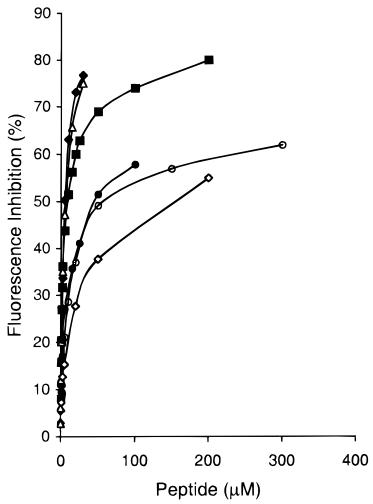


Figure 2. Displacement of dansyl-PMBN bound to LPS by PMBN and PMBN analogues. Increasing concentrations of PMBN peptides [pPMBN (◆), sPMBN (△), [Agb^{2,4,7,8}]PMBN (■), [Dap^{2,3,4,7,8}]PMBN (◇), [Lys^{7,8}]PMBN (○), and [lPhe^5]-PMBN (●) were added to *E. coli* LPS solution (3 μg/mL) bound to dansyl-PMBN (0.55 μM). The fluorescence inhibition was measured 5 min after each addition at excitation and emission wavelengths of 340 and 485 nm, respectively.

Antimicrobial and Sensitizing Activity. The PMBN analogues were tested in vitro for their antimi-

Table 3. Sensitizing Activity and LPS Binding of PMBN Analogues[a]

		relative sensitization activity (%)[a]			
no.	peptide	*E. coli*	*K. pneumoniae*	IC_{50} (μM)[b]	I_{max} (%)[c]
1	pPMBN	100	100	2.5	80
2	sPMBN	96	93	3	80
3	[$Lys^{2,3,4,7,8}$]PMBN	4	3	35	60
4	[$Orn^{2,3,4,7,8}$]PMBN	2	6	25	62
5	[$Dap^{2,3,4,7,8}$]PMBN	11	5	30	64
6	[Lys^{3}]PMBN	17	18	8	60
7	[$Lys^{2,4,7,8}$]PMBN	5	3	30	45
8	[*cyclo*-Dab^{4},Thr^{9}]PMBN	4	5	15	45
9	[*cyclo*-Dab^{2},Thr^{9}]PMBN	7	5	8	60
10	[$Lys^{2,4}$]PMBN	9	13	14	64
11	[$Lys^{7,8}$]PMBN	40	26	12	62
12	[lPhe^{5}]PMBN	5	9	8	64
13	[$Agb^{2,4,7,8}$]PMBN	25	25	3.8	80
14	PMB			0.5	100

[a] Relative sensitizing activity was determined as a percent of pPMBN (50 μg/mL) employed as a control. The MIC values of novobiocin for *E. coli* and *K. pneumoniae* were 125 and 250 μg/mL, respectively, in the absence of peptides. pPMBN at 50 μg/mL reduced the MIC to 1 and 4 μg/mL novobiocin, representing a sensitization activity of 125 and 62 μg/mL for *E. coli* and *K. pneumoniae*, respectively. The numbers are the average values derived from 4–8 experiments. [b] The concentration required to displace 50% of dansyl-PMBN (0.55 μM) from maximal specific binding to *E. coli* LPS. [c] Maximal displacement of dansyl-PMBN.

crobial activity toward clinical isolates of *E. coli*, *K. pneumoniae*, and *P. aeruginosa*. As expected, PMBN and its analogues lacked antimicrobial activity against the first two bacterial species (i.e., MIC ≥ 1000 μg/mL). However, the MIC of pPMBN (**1**) and sPMBN (**2**) for *P. aeruginosa* was 8 μg/mL. The susceptibility of *P. aeruginosa* to PMBN was previously reported and could seemingly be attributed to the relative high number of negative charges on the *P. aeruginosa* outer membrane.[12] A further study focused on the peptides ability to disorganize the outer membrane of Gram-negative bacteria. This activity was evaluated in terms of the ability to reduce the MIC values of novobiocin toward *E. coli* and *K. pneumoniae* as a function of increased drug penetration into the bacteria. Both sPMBN and pPMBN exhibited considerable activity by reducing the MIC of novobiocin by a factor of 2 orders of magnitude toward *E. coli* (from 125 down to 1 μg/mL) and a rather similar activity against *K. pneumoniae* (from 250 down to 4 μg/mL). However, PMBN analogues exhibited significantly reduced activity at identical concentrations (Table 3).

Discussion

We have evaluated here three routes for the synthesis of PMBN. Route B (Scheme 2B) was found to be the method of choice as determined by its convenient chemical manipulations, good yield, and quality of products. The various PMBN analogues described hereby were prepared successfully using this approach. Route C, i.e., on-polymer cyclization (Scheme 2C), seems, however, to have the highest potential as a means for the synthesis of cyclic peptides. Notably, the removal of the side chain tBu protecting group from the C-terminal threonine residue prior the cyclization step (Scheme 2B,C) minimized substantially the formation of the α-crotonic acid-related side product.

The association of PMB with the bacterial LPS, mediated by the lipid A moiety, has been studied rather intensively with regard to affinity, stoichiometry, cooperativity, and molecular features; yet, certain related conflicts were detected.[12,24,25] Based on isothermal calorimetry, 2D ^{1}H NMR, and molecular dynamics studies, it appears that the intermolecular peptide–lipid interactions are attributed to the amphiphilic feature of PMB.[24,26] This interaction is translated into major related hydrophobic peptide–lipid contacts which are properly directed and strengthened by interactions of the positively charged side chains of the 2,4-diaminobutyric acid residues of PMB with the negatively charged phosphate groups of lipid A.

We aimed in the present study to examine the effect of structural alterations – specifically in the peptide ring size, distance of positive charges from the peptidic backbone, and amphiphilic character on the binding of PMBN to LPS and on its synergistic antimicrobial activity, i.e., capacity for bacterial cell permeabilization. Thus, the fluorescence binding-inhibition assay employing dansyl-PMBN and *E. coli* LPS enabled to calculate the number of peptide molecule associated to each LPS molecule. The results are correlated with a previous study with dansyl-PMB showing a ratio of ca. 2:1.[12] The LPS–peptide binding assay also enabled to conclude that none of the PMBN analogues exhibited higher affinity to LPS than PMBN.

The sensitizing activity of the various PMBN analogues is consistent with the LPS binding affinity. In general, reduced affinity (IC_{50} and I_{max}) to LPS, compared to PMBN, reduced the peptide-mediated perturbation activity and abolished the growth-inhibition capacity toward *P. aeruginosa*. The results obtained (Table 3) suggest that the structural parameters of the natural pPMBN peptide, namely, ring size of 23 atoms, a space of 2 methylene groups between the positive charge and the backbone, the hydrophobic order of DPhe-Leu, and the nature of the charged group, are optimal for bacterial sensitization toward hydrophobic antimicrobial agents. However, other modifications of the peptidic ring, including insertion of peptidomimetic elements or suitable enhancement of structural constraints pertaining to the stabilization of the "active conformation" of the peptide, may yield more effective derivatives. These results could also indicate that the assembly of the LPS molecule in the bacterial OM has a specific array and order.

CD studies suggest, however, that PMBN as well as its analogues have a random conformation. An active architecture can, theoretically, be assumed upon as-

sociation of PMBN and PMB, with their LPS target. Recently, the solution structure of LPS-bound PMB was elucidated as an envelope-like fold of the peptide ring and as a β-turn type II′ for the free peptide.[26,27] However, both structures could not be observed by the CD measurements.

Conclusions

The structure of PMBN appears to be highly specific for efficient perturbation of the outer membrane and consequent sensitization of the Gram-negative bacteria toward hydrophobic antibiotics as well as for LPS binding. Moreover, it is not a mere platform for a set of positive charges but rather a molecule with precise and unique topographic features which are essential for complementarity in LPS binding and subsequent perturbation of the Gram-negative outer membrane. In light of this study, the use of PMBN as a scaffold for antibiotic targeting should be considered.

Experimental Section

Synthesis of sPMBN and Analogues 2−12. All protected amino acids, coupling reagents and polymers were obtained from Nova Biochemicals (Laufelfingen, Switzerland) or from Bachem (Bubendorf, Switzerland). Synthesis grade solvents were obtained from Labscan (Dublin, Ireland). Linear peptide chains were assembled by conventional solid-phase synthesis, using an ABIMED AMS-422 automated solid-phase multiple peptide synthesizer (Langenfeld, Germany). The Fmoc strategy was employed throughout the peptide chain assembly[19] following the company's commercial protocols. Synthesis was initiated using Fmoc-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 (Dab), lysine (Lys), ornithine (Orn), and 2,3-diaminopropionic acid (Dap) were *tert*-butyloxycarbonyl (tBoc) and benzyloxycarbonyl (Cbz). Fmoc-Thr(tBu)-OH was employed as the final building unit. Coupling was achieved, as a rule, using two successive reactions with 4 equiv of PyBOP as a coupling reagent and 8 equiv of NMM, all dissolved in DMF. The fully protected peptide-bound resin was treated with piperidine (20% in DMF) for 20 min, then washed (DMF), and the free N-terminus amino moiety was reacted with 4 equiv of Cbz-OSu and 4 equiv of DIEA in DMF for 3 h. The fully protected peptide-bound resin was then treated with TFA/water/TES (95:2.5:2.5, v/v/v) for 1 h at room temperature. The cleavage mixture was cooled to 4 °C, and the partially protected linear peptides were 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 peptide concentration of 1 mM, using PyBOP/HOBT/NMM (4:4:8, equiv) as reagents for 2 h at room temperature (yield, >90% according to analytical HPLC). The reaction mixture was concentrated in high vacuum and the peptidic cyclic product was precipitated by treatment with water. Final deprotection, i.e., removal of Cbz, was achieved by catalytic hydrogenation (Pd/C) in acetic acid/methanol/water (5:4:1, v/v/v).

Reversed-Phase HPLC and Analyses. The crude synthetic peptides were purified by using a prepacked LichroCart RP-18 column (250 × 10 mm; 7-μm bead size) employing a binary gradient formed from 0.1% TFA in water (solution A) and 0.1% TFA in 75% acetonitrile in water (solution B), eluted at t = 0 min, B = 0%; t = 48 min, B = 60%; t = 60 min, B = 100% at 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) and the following binary gradient: t = 0 min, B = 10%; t = 40 min, B = 60%; t = 50 min, B = 100% at 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 UV absorbance at 220 nm. Following HPLC purification the lyophilized peptides (>97% pure) were analyzed, after exhaustive acid hydrolysis and precolumn reaction with 6-aminoquinolyl *N*-hydroxysuccinimidylcarbamate (AQC), to ascertain amino acid composition (Waters 2690 separations module, Milford, MA). Yields were 35−40%. Molecular weights of compounds were determined by FAB mass spectrometry on a VG-high-resolution magnetic sector (Fisons, U.K.) with PEG or PEG-Me as internal standard or by ESI mass spectrometery on a VG-platform-II electrospray single quadropole mass spectrometer (Micro Mass, U.K.). Proton magnetic resonance spectra were recorded at 25 °C on a Bruker AVANCE 400 spectrometer (400 MHz). J values are given in hertz (Hz).

sPMBN (2): ^{1}H NMR (400 MHz, H_2O:D_2O, 9:1; pH = 2.3) δ 0.69 (d, 3H, J = 5.9), 0.75 (d, 3H, J = 5.87), 1.19 (d, 3H, J = 6.24), 1.30 (d, 3H, J = 6.4), 2.07 (m, 10H), 3.10 (m, 10H), 3.95 (d, 1H, J = 5.0), 4.23 (m, 9H), 7.26 (d, 2H, J = 6.9) 7.36 (m, 4H), 7.76 (t, 1H, J = 5.4), 7.88 (d, 1H, J = 7.6), 8.24 (d, 1H, J = 6.4), 8.69 (d, 1H, J = 4.7), 8.74 (d, 1H, J = 5.8), 8.98 (d, 1H, J = 6).

[Lys^{2,3,4,7,8}]PMBN (3): ^{1}H NMR (400 MHz, H_2O:D_2O, 9:1; pH = 3.1) δ 0.82 (d, 3H, J = 6.5), 0.88 (d, 3H, J = 6.6), 1.23 (d, 6H, J = 6.4), 1.33 (d, 6H, J = 6.43), 1.51 (m, 16H), 1.73 (m, 11H), 1.88 (m, 6H), 2.90 (m, 3H), 3.05 (m, 10H), 3.21 (m, 4H), 3.92 (d, 1H, J = 3.2), 4.16 (m, 3H), 4.26 (m, 6H), 4.36 (m, 4H), 7.32 (d, 3H, J = 6.9), 7.40 (m, 4H), 7.52 (d, 1H, J = 4.81), 7.58 (t, 1H, J = 5.8), 8.13 (d, 1H, J = 6.3), 8.33 (d, 1H, J = 7.54), 8.43 (d, 1H, J = 6.4), 8.63 (d, 2H, J = 6.6), 8.70 (d, 1H, J = 7.5), 8.8 (d, 1H, J = 6.9).

[Dap^{2,3,4,7,8}]PMBN (5): ^{1}H NMR (400 MHz, H_2O:D_2O, 9:1; pH = 2.38) δ 0.87 (d, 3H, J = 6.5), 0.92 (d, 3H, J = 6.6), 1.22 (d, 1H, J = 6.0), 1.38 (d, 3H, J = 6.5), 1.56 (m, 2H), 2.26 (s, 1H), 3.07 (m, 4H), 3.34 (m, 2H), 3.56 (m, 2H), 3.67 (m, 4H), 4.09 (d, 1H, J = 4.6), 4.16 (m, 4H), 4.37 (m, 3H), 4.49 (d, 1H, J = 6.8), 7.30 (d, 3H, J = 7.2), 7.41 (m, 4H), 7.49 (d, 1H, J = 6.1), 7.93 (t, 1H, J = 6.4), 8.32 (d, 1H, J = 6.5), 8.71 (m, 3H), 9.35 (d, 1H, J = 7.3).

[*cyclo*-Dab^2,Thr^9]PMBN (9): ^{1}H NMR (400 MHz, H_2O:D_2O, 9:1; pH = 2.35) δ 0.79 (d, 3H, J = 6.4), 0.86 (d, 3H, J = 6.6), 1.21 (d, 3H, J = 6.5), 1.31 (t, 2H, J = 7.3), 1.36 (d, 3H, J = 6.5), 1.54 (m, 2H), 2.03 (m, 4H), 3.18 (m, 10H), 3.44 (m, 1H), 3.95 (d, 1H, J = 6.1), 7.31 (d, 3H, J = 6.9), 7.40 (m, 4H), 7.95 (d, 1H, J = 7.5), 8.11 (t, 1H, J = 5.6), 8.45 (d, 1H, J = 7.1), 8.54 (d, 1H, J = 6.0) 8.57 (d, 1H, J = 6.7), 8.65 (d, 1H, J = 6.9), 8.68 (d, 1H, J = 6.4), 8.80 (d, 1H, J = 7.2), 8.96 (d, 1H, J = 6.1).

pPMBN (1). pPMBN was prepared by proteolysis of PMB with papain or ficin as described elsewhere[11] (PMB, papain and ficin were purchased from Sigma Chemical Co., St. Louis, MO). Crude product was purified (>98%) by HPLC and characterized as described above (yield 60%): ^{1}H NMR (400 MHz, H_2O:D_2O, 9:1; pH = 2.3) δ 0.70 (d, 3H, J = 5.8), 0.77 (d, 3H, J = 5.8), 1.20 (d, 3H, J = 6.3), 1.32 (d, 3H, J = 6.4), 2.06 (m, 10H), 3.13 (m, 10H), 3.95 (d, 1H, J = 5.5), 4.23 (m, 9H), 7.26 (d, 2H, J = 6.7), 7.36 (m, 4H), 7.78 (t, 1H, J = 5.6, 5.9), 8.24 (d, 1H, J = 6.2), 8.49 (d, 1H, J = 7.8), 8.69 (d, 1H, J = 4.7), 8.74 (d, 1H, J = 5.8), 8.98 (d, 1H, J = 6.1).

[Agb^{2,4,7,8}]PMBN (13). pPMBN (40 mg, 41.5 μmol) was dissolved in water (40 mL) and 3,5-dimethylpyrazole-1-carboxamidine nitrate (125 mg, 0.62 mmol; Sigma) was added. The pH of the solution was adjusted to 10 with sodium carbonate solution (1 M) and the reaction mixture was stirred overnight at room temperature. Progress of guanidation was followed using analytical HPLC. The major product was purified to homogeneity using semipreparative HPLC as described above, characterized by amino acid analysis and mass spectrometry: yield, 82%; ^{1}H NMR (400 MHz, H_2O:D_2O, 9:1; pH = 2.30) δ 0.71 (s, 3H), 0.79 (s, 4H), 1.24 (d, 3H, J = 6.4), 1.31 (t, 2H, J = 7.3), 1.35 (d, 3H, J = 6.4), 1.5 (m, 2H), 2.03 (m, 9H), 3.10 (m, 3H), 3.23 (m, 2H), 3.36 (m, 6H), 3.97 (d,

1H, $J = 5.8$), 4.25 (m, 6H), 4.55 (m, 2H), 6.8 (broad s, 12H), 7.15 (t, 1H, $J = 5.3$), 7.34 (m, 9H), 7.73 (t, 1H, $J = 5.5$), 7.90 (d, 1H, $J = 7.3$), 8.22 (d, 1H, $J = 6.2$), 8.49 (d, 1H, $J = 7.6$), 8.66 (m, 2H), 8.76 (d, 1H, $J = 5.4$), 8.96 (d, 1H, $J = 7.0$).

Dansyl-PMBN. Labeling of PMBN with dansyl was achieved by the following method: 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 the threonine. The resin-bound peptide was then reacted with dansyl chloride (100 μmol, 27 mg; Sigma) and NMM (200 μmol, 22 μL) in DMF for 2 h. The labeled peptide was cleaved off the resin, cyclized, purified by semipreparative HPLC (>98%), and characterized as described above: yield, 40%; *m*/*z* 1197.6 (calcd 1197.2); Thr, 1.1; Dab, 4.9; DPhe, 1; Leu, 1; t_R, 33.54 min.

Determination of Minimal Inhibitory Concentration (MIC). Clinical isolates of *E. coli*, *K. pneumoniae* and *P. aeruginosa*, obtained as described elsewhere, were employed.[18] The Gram-negative bacteria were grown on nutrient agar plates (Difco Laboratories, Detroit, MI) and kept at 4 °C. An overnight culture in isotonic sensitest broth (ISB, Oxoid) was adjusted to 1×10^5 CFU/mL and inoculated into microtiter plate wells containing each 100 μL of a serial 2-fold dilution (1000−0.5 μg/mL) of the tested antibiotics in ISB. 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 4−8 separate tests that varied by no more than one dilution.

Sensitizing Activity. Bacterial suspension (10 μL, 1×10^5 CFU) was inoculated into microtiter plate wells containing 100 μL of a serial 2-fold dilution (1000−0.5 μg/mL) of novobiocin (Sigma) 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 comparing wells with and without test peptides was calculated and designated as sensitizing activity. The relative sensitizing activity of the test peptide was calculated as percent of the sensitizing activity of 50 μg/mL pPMBN.

Binding of Peptides to LPS. The fluorescence of dansyl-PMBN bound to *E. coli* LPS was measured using a MC200 monochromator (SLM Aminco, SLM Instruments, Inc.) set at an excitation wavelength of 340 nm and an emission wavelength of 485 nm.[20] To a quartz cuvette containing LPS solution (2 mL, 3 μg/mL) in *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] buffer (HEPES; 5 mM, pH 7.2) was added 5 or 10 μL of dansyl-PMBN solution (1×10^{-6}−1×10^{-3} M) at 5-min intervals for up to 1 h until a plateau in the fluorescence intensity was reached (i.e., saturation of dansyl-PMBN binding to LPS). The amount of dansyl-PMBN bound to LPS at saturation was calculated as described elsewhere.[12] Briefly, a binding curve of dansyl-PMBN to excess LPS (400 μg/mL) was plotted and referred to as maximal binding (F_{max}). The amount of bound dansyl-PMBN was calculated from: $[\text{dansyl-PMBN}] = (F_{exp}/F_{max}) \times [\text{dansyl-PMBN}_{total}]$, where F_{exp} is the fluorescence obtained with 3 μg/mL LPS.

To determine the binding of a given peptide to LPS, a displacement assay was performed in which 5 or 10 μL (1×10^{-5}−1×10^{-3} M) of the tested peptide at desired concentrations were added at 5-min intervals to a preequilibrated mixture of LPS solution (2 mL, 3 μg/mL, ~2×10^{-7} M) in HEPES buffer (5 mM, pH 7.2) and dansyl-PMBN (0.55 μM). The fluorescence intensity was recorded after each time interval of adding the tested peptide. Each experiment was repeated 2−3 times. The percent inhibition of fluorescence intensity was plotted as a function of the peptide concentration from which the concentration required for maximal (I_{max}) and 50% (IC_{50}) displacement of the dansyl-PMBN from LPS was derived.

Circular Dichroism (CD) Studies. CD spectra were recorded on an Aviv-202 circular dichroism spectrometer (Lakewood, NJ). Duplicate scans over a wavelength range of 190−250 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 (PB), pH 7.2, at a final concentration of 0.1 mM. The CD of pPMBN and sPMBN were evaluated, as well, in a mixture of TFE/aqueous buffer (1:1, v/v) or in bulk TFE. A baseline was recorded and subtracted after each spectrum. Ellipticity is reported as the mean residue ellipticity [Θ] in deg cm^2 dmol^{-1}. $[\Theta] = [\Theta]_{obs} \times (\text{MRW}/10lc)$, where $[\Theta]_{obs}$ is the ellipticity measured in mdeg, MRW is the mean residue molecular weight of the peptide (molecular weight divided by the number of peptide bonds), *c* is the concentration of the sample in mg/mL, and *l* is the optical path length of the cell in cm.

Supporting Information Available: CD spectra of compounds **1**, **2**, **5**, **12**, and **13** are available free of charge via the Internet at http://pubs.acs.org.

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