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Structure—Activity Relationships of Polymyxin Antibiotics

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1. Polymyxins, a Last-Line Therapy against Gram-Negative "Superbugs"

The world is facing an enormous and growing threat from the emergence of bacteria that are resistant to almost all available antibiotics.[1] Multidrug resistance is a significant public health issue in regard to both Gram-positive and Gram-negative bacteria, but the problem is arguably most grave for the latter class of bacteria. In recent years, virtually no novel drugs targeting multidrug-resistant (MDR[a]) Gram-negative bacteria, in particular *Pseudomonas aeruginosa*, have been developed. As described in the "Bad Bugs, No Drugs" paper published by the Infectious Diseases Society of America (IDSA),[1,2] "as antibiotic discovery stagnates, a public health crisis brews". Therefore, there is an urgent need for new antibiotics, particularly those active against Gram-negative "superbugs", such as *P. aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*.[1–4] It is precisely this mismatch between increasing multidrug resistance and the dry antimicrobial-drug development pipeline, as highlighted in the "Bad Bugs Need Drugs" campaign,[1,2] that led the IDSA to place *P. aeruginosa*, *A. baumannii* and *K. pneumoniae* on a "hit list" of the six top-priority dangerous MDR microorganisms. These pathogens have been identified as requiring the most urgent attention for discovery of novel antibiotics. Meanwhile, the polymyxins are increasingly being used as last-line therapy to treat infections caused by Gram-negative bacteria that are resistant to essentially all other currently available antibiotics.[5–9]

Polymyxins were discovered more than 50 years ago.[10] Polymyxin B (PMB) and colistin (polymyxin E) are secondary metabolite nonribosomal peptides produced by the soil bacterium *Bacillus polymyxa*. PMB and colistin share a common primary sequence, the only difference being at position 6 which is occupied by D-Phe in PMB and D-Leu in colistin (Figure 1). Pharmaceutical grade preparations of PMB and colistin are composed of a mixture of closely related components. PMB_1 **(1)** and PMB_2 **(2)**, the main components of

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[a]Abbreviations: PMB, polymyxin B; LPS, lipopolysaccharide; Dab, L-α-γ-diaminobutyric acid; Dap, 2,3-diaminopropionic acid; DPPA, diphenylphosphorylazide; FA, fatty acyl; MDR, multidrug-resistant; XDR, extremely drug-resistant; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate; Boc, *tert*-butyloxycarbonyl; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl; ivDde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl; Mtt, 4-methyltrityl; 2-ClZ, 2-chlorobenzyloxycarbonyl.

PMB, are *N*-terminally acylated by (*S*)-6-methyloctanoic acid and (*S*)-6-methylheptanoic acid, respectively. Similarly, the two main components of colistin, colistin A **(7)** and colistin B **(8)**, are acylated by (*S*)-6-methyloctanoic acid and (*S*)-6-methylheptanoic acid, respectively. Because the early clinical experience, before the 1970s, with parenteral administration of PMB and colistin (or its nonactive prodrug colistin methanesulfonate11) led to concern over the potential for nephrotoxicity and neurotoxicity, their clinical use waned.[7,9,12–14] Since the mid-1990s, there has been a greatly renewed interest in their clinical use due to prevalent MDR Gram-negative bacteria (especially *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*) and lack of novel antibiotics.[5–9,12,14] Even though there is still a dearth of knowledge on how to use them optimally, polymyxins are mostly being used as last-line antibiotics for otherwise untreatable serious infections. Although the incidence of resistance to polymyxins is currently relatively low, resistance can emerge rapidly *in vitro* in *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*.[6,15,16] More worrying, polymyxin resistance in hospitalized patients has been increasingly reported.[6,15,17] As noted above, there is only one amino acid difference between colistin and PMB and, not surprisingly, cross resistance exists.[7] The emergence of extremely drug-resistant (XDR) *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*, including resistance to polymyxins, is posing the most serious therapeutic challenges.[17–20] In essence, resistance to polymyxins implies a total lack of antibiotics for treatment of life-threatening infections caused by these Gram-negative "superbugs" and highlights the urgency to develop new antibiotics.

In view of the generally good antimicrobial activity of the naturally occurring polymyxins and relatively low prevalence of resistance that exists, it is perhaps not surprising that there has been substantial effort into discovery of polymyxin analogues with improved microbiological, pharmacological, and toxicological profiles. In the early part of this paper, we review the current understanding of the mechanisms of microbiological activity and bacterial resistance. The synthesis of polymyxins is next reviewed, followed by a comprehensive treatise on the structure–activity relationships of the polymyxins and the various analogues that have been investigated. In undertaking this review, a literature search was conducted using PubMed (NLM) with keywords of polymyxin and structure– activity relationships.

2. Mechanisms of Polymyxin Activity and Bacterial Resistance

2.1. Mechanism of Antibacterial Activity of Polymyxins

A key role of the outer membrane (OM) of Gram-negative bacteria is to act as a permeability barrier.[21,22] The initial target of polymyxins is the LPS component of the membrane. An understanding of the mechanism of polymyxin activity therefore requires knowledge of LPS structure. LPS is composed of three domains, a conserved inner core 2-keto-3-deoxyoctonoic acid (Kdo) bound to lipid A and a variable *O*-antigen composed of repeating units of various polysaccharides.[22] The consensus structure of lipid A consists of a β-1′-6-linked D-glucosamine (GlcN) disaccharide that is phosphorylated at the 1- and 4′-positions (Figure 2). Lipid A usually contains six acyl chains (designated A–F in Figure 2). Four acyl chains attached directly to the glucosamine sugars are β-hydroxyacyl chains (usually C12 and C14 in length), while two secondary acyl chains are often attached to the β-hydroxy group. Lipid A acts as a hydrophobic anchor with the tight packing of the fatty acyl chains helping to stabilize the overall OM structure.[22]

Most of the investigations to elucidate mechanisms of action have been conducted using PMB. Polymyxins exert their antimicrobial action by permeabilizing the bacterial OM via direct interaction with the lipid A component of the LPS (Figure 3). The narrow spectrum of activity of polymyxins for Gram-negative bacteria is coincident with their binding selectivity for LPS.[22–24] The amphipathicity of the polymyxins and, possibly, their ability to

form porelike aggregates may be responsible for their OM permeabilizing action.[23–27] The conserved elements in the chemical structure of polymyxins consist of two hydrophobic domains (the *N*-terminal fatty acyl chain and the D-Phe^6-L-Leu^7 segment) separated by segments of polar (Thr) and cationic (L-α-γ-diaminobutyric acid (Dab)) residues. The elucidation of the three-dimensional NMR solution state structure of PMB in complex with LPS revealed the PMB molecule is folded such that the polar and hydrophobic domains form two distinct faces, thereby conferring structural amphipathicity (Figure 4).[28–30] A detailed account of the structural aspects of the polymyxin B–LPS interaction will be given in section 3. The general model for the action of amphipathic antimicrobial peptides involves interaction of the polar face of the peptide with the polar lipid A head groups, while the lipophilic face inserts into the hydrophobic fatty acyl layer of the OM.[23–27] The positive charge of polymyxins allows for accumulation at the anionic bacterial membrane. The electrostatic interaction between the positively charged polymyxin Dab residues and the negatively charged lipid A phosphates is believed to displace divalent cations (Ca^{2+} and Mg^{2+}) that normally function to bridge and stabilize the LPS OM monolayer.[22–27] This initial electrostatic interaction temporarily stabilizes the complex and brings the *N*-terminal fatty acyl chain of the polymyxin molecule into proximity with the OM. Insertion of the fatty acyl chain and D-Phe^6-L-Leu^7 (PMB) or D-Leu^6-L-Leu^7 (colistin) hydrophobic domain acts to weaken the packing of adjacent lipid A fatty acyl chains causing expansion of the OM monolayer. The fact that polymyxin B nonapeptide (PMBN **(193)**, i.e., PMB minus the *N*-terminal fatty acyl chain and Dab^1 residue), lacks antibacterial activity highlights the importance of both the electrostatic and hydrophobic interactions for the mechanism of polymyxin action. Following insertion, polymyxins are purported to transit the OM via a self-promoted uptake mechanism.[23–25,27,29–31] Subsequently, the polymyxin molecule inserts and disrupts the physical integrity of the phospholipid bilayer of the inner membrane via membrane thinning by straddling the interface of the hydrophilic head groups and fatty acyl chains or transient poration.[23–25,27,29–31] This general model, although partly speculative, is consistent with available data in the literature.

An alternative mechanism has been proposed wherein polymyxin mediated contacts between the periplasmic leaflets of the inner and outer membranes. This postulate is based on the observation that PMB bound to anionic phospholipid vesicles is capable of forming vesicle-to-vesicle contacts.[25,32–36] These contacts promote phospholipid exchange between vesicles. In the bacterial membrane, lipid exchange between the inner and outer membrane leaflets would result in the loss of phospholipid compositional specificity, potentially leading to an osmotic imbalance that leads to lytic cell death.

2.2. Mechanisms of Resistance to Polymyxins

As noted above, a critical first step in the action of polymyxins on Gram-negative bacteria is the electrostatic interaction between the positively charged Dab residues of polymyxins and the negatively charged phosphate groups on lipid A.[23,25,37,38] Thus, many bacterial mechanisms of resistance to polymyxins are based on modifications to the lipid A head groups that reduce this initial electrostatic interaction. In *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *K. pneumoniae*, and *P. aeruginosa*, modification of the phosphates of lipid A with positively charged moieties, such as 4-amino-4-deoxy-L-arabinose and/or phosphoethanolamine, reduces the net negative charge of lipid A, thereby increasing resistance to polymyxins (Figure 2).[39–44] In *K. pneumoniae*, the presence of capsule may also be important for polymyxin resistance.[45,46]

In many bacterial species resistance to cationic antimicrobial peptides (CAP) is regulated by a two-component master regulatory system, PhoP–PhoQ. This system is also employed for survival under conditions of low Mg^{2+} that would destabilize the OM because of the decrease in the bridging action of divalent cations between LPS molecules. PhoP– PhoQ

remains repressed in high (mM) Mg^{2+} environments and is activated under conditions of low (micromolar) Mg^{2+}.[39,47] In *S. enterica*, the PhoP–PhoQ two-component system has been well characterized.[40,41,44,47,48] The PhoP– PhoQ acts as a master regulator of *S. enterica* virulence and evasion of CAP killing. PhoQ is an inner membrane sensor kinase that phosphorylates the cognate response regulator PhoP in response to low Mg^{2+} or sublethal concentrations of CAPs. This in turn leads to activation of PmrA–PmrB which confers resistance by activating genes that encode enzymes required for the covalent modification of the phosphate groups on lipid A with 4-amino-4-deoxy-L-arabinose or phosphoethanolamine.[40,41,48] This modification serves to decrease the net negative charge and reduces repulsion between neighboring LPS molecules, thereby strengthening the packing of the OM.[22] CAPs have also been shown to directly activate the PmrA–PmrB system.[49]

3. Polymyxin–LPS Complex

From the foregoing, it is evident that an understanding of the mechanisms of action and resistance of the polymyxins, and of their SAR, requires knowledge of the interaction between polymyxins and LPS. Such knowledge is also central in efforts to develop polymyxin analogues with reduced potential to be affected by the polymyxin resistance mechanisms discussed above. The PMB–LPS binding interaction has been studied by several biophysical techniques including isothermal titration calorimetry (ITC),[50–53] displacement of fluorescent probes,[54–56] surface plasmon resonance (SPR),[57,58] and nuclear magnetic resonance (NMR) spectroscopy.[28–31,59,60]

Thermodynamic analysis of the interaction between PMB and LPS using ITC measurements indicated that binding is entropically driven (i.e., driven by hydrophobic forces) in the gel phase of the hydrocarbon chains of LPS (< 30 °C) but enthalpically driven in the liquid crystalline phase (>35 °C).[50] Furthermore, the ITC binding isotherms indicated the complex is stoichiometric and noncooperative with affinity in the low micromolar range.[50–53] SPR experiments showed a poorer affinity and kinetics of binding for PMBN to LPS compared to PMB, whereas, the binding of a Dab → Lys substituted cyclic decapeptide PMB analogue was comparable to that of PMB.[57,58] These observations were attributed to the amphilic properties of each peptide such that the presence of Lys residues that contain two extra methylene groups in their side chains (compared to a single methylene in the Dab side chain) gives the cyclic decapeptide higher amphilicity than PMBN.[57,58] These data highlight the importance of amphilicity for high affinity binding to LPS.

The interaction between polymyxins and LPS at the molecular level has been well characterized by NMR techniques.[28–31,59,60] This structural information has proved invaluable for the interpretation of available SAR data and toward the development of novel polymyxin compounds against which bacteria cannot easily develop resistance. In the ensuing discussion we will attempt to convey the current consensus regarding the available structural details of the polymyxin–LPS complex.

One of the early structural studies of PMB and PMBN utilized 2D NMR methods in combination with simulated annealing computational calculations to characterize their structure and dynamics in aqueous trifluoroethanol (TFE).[28,31] Circular dichroism experiments indicated PMB adopts a comparable structure in aqueous TFE and when bound to phospholipid vesicles, indicating TFE does indeed act as a membrane-mimetic solvent. In the free state, the overall structural dynamics of both PMB and PMBN were quite similar.[28,31] The peptides exhibited a differential mobility over the heptapeptide ring, with the more rigid residues acting as pivot points. The data reveal that in both molecules, residues 6, 7, and 10 maintain a stable conformation over a range of TFE concentrations, whereas the

remainder of the molecule is more flexible.[28,31] Simulated annealing calculations suggest that the rigidity about residues 6, 7, and 10 constrains both sides of the ring, thereby limiting flexibility to variations in ring pucker. The linear peptide segment of both molecules displays a greater mobility than the heptapeptide ring. Faster motions are also seen for the side chains than the heptapeptide ring.[28,31] Burch et al.[28,31] purport the increased mobility of the side chains allows the cationic Dab residues to more efficiently dock onto the anionic phosphoester groups on the lipid A component of LPS. This flexibility would also allow the PMB molecule to bind to both mono- and divalent phosphoester ligands. InPMB, the *N*-terminal fatty acyl chain displays a rapid, independent motion from the heptapeptide core. The authors suggest this flexibility facilitates passage of the PMB molecule across the OM layer and into the periplasmic space and accounts for some of the hydrophobic forces driving LPS binding, as shown by thermodynamic measurements.[50–53] Interestingly, all of the internal motions were faster for PMBN compared to PMB, suggesting that despite its independent motion, the *N*-terminal fatty acyl chain somehow restricts the mobility of the heptapeptide ring.[28,31] Burch et al.[28,31] further speculated that the topological flexibility in the higher-order structures is important for accommodating motions that may be necessary to allow for binding to the dynamic environment of the bacterial OM. Nevertheless, since PMBN also displays the same flexibility as PMB, conformational flexibility per se may not be essential for antimicrobial activity.

Subsequently, Pristovsek and Kidric29,[30] successfully characterized the solution-state NMR structure of PMB in aqueous solution, both in the free and in the LPS-bound state. Similar to the conformation observed for the free PMBN structure, the free PMB is in a fast exchanging conformational regime with a preference toward a type II′ β-turn from residues 5 to 8 and a γ-turn about residue 10. Unlike the PMBN–LPS complex where both motifs are preserved, these structures were not evident in the LPS-bound conformation of PMB. Transferred nuclear Overhauser effect (trNOE) NMR techniques were employed to determine the conformation of PMB when bound to LPS. On the basis of the NMR structure of PMB when bound to LPS, a molecular model of the PMB–LPS complex was constructed (Figures 4 and 5A).[29,30] The modeling process took into account the electrostatic interactions between the Dab side chains and lipid A phosphates and maximized the reduction of solvent exposed hydrophobic area on both molecules. The model implies the complex is stabilized by a combination of electrostatic and hydrophobic interactions. The bacterial lytic activity of polymyxins is believed to correlate with their amphiphilic character. Coincidently, in the LPS-bound state the PMB backbone adopts an envelope-like fold separating the polar/charged residues from the hydrophobic components giving the structure amphilicity (Figure 6A). The model indicates that the *N*-terminal fatty acyl chain and D-Phe^{6}-L-Leu^{7} hydrophobic motif penetrate into hydrocarbon portion of the lipid A layer whereas the polar Thr^{2}, Dab^{3}, and Thr^{10} side chains are oriented to point into the hydrophilic environment formed by the Kdo units of the inner core oligosaccharide of LPS. Similar to other cyclic peptides of nonribosomal origin, the backbone conformation of PMB appears to be maintained by an ordered series of intramolecular hydrogen bonds (Figure 6B). Amide protons that are least affected by changes in temperature are most likely shielded from the solvent by participation in hydrogen bonding.[29] The temperature dependence of the amide proton chemical shifts indicated that two of the heptapeptide amide protons from residues Dab^{4,8} participate in intramolecular hydrogen bonding to stabilize the ring conformation. In addition to maintaining the optimal distance between the positive charges, the envelope-like fold of the backbone also forces the two phosphoester binding sites to one face of the PMB molecule. The heptapeptide ring of PMB covers the GlcN disaccharide unit core of lipid A, forming a “charge clamp” such that the positive charges on Dab^{1} and Dab^{5} allow bonding with the negatively charged 4′-phosphate group of lipid A, and Dab^{8} and Dab^{9} similarly bond with the 1-phosphate. The complex is further stabilized by hydrophobic contacts: the *N*-terminal fatty acyl chain of PMB interacts with fatty acyl

chains A and B of lipid A, and the side chains of D-Phe^6 interacts with the A and C fatty acyl chains on lipid A and Leu^7 with C and F (Figures 2 and 5A). In three-dimensional space the Dab^1 and Dab^5 side chains are adjacent to the *N*-terminal fatty acyl chain of PMB, and as such may facilitate its insertion into the lipid A fatty acyl layer of the OM. More recently, this model was consolidated by another NMR study which utilized chemical shift mapping data and intermolecular NOEs to provide additional constraints to model the PMB-LPS complex in the presence of dodecylphosphocholine micelles (Figure 5B).[59] The Mares et al.[59] model was largely consistent with the model of Pristovsek and Kidric[29,30] with the only major difference being polar contacts to the Kdo-C unit of the inner oligosaccharide and the polar Thr^{10} residue of PMB.

As mentioned above, covalent modification of the lipid A phosphoester groups with positively charged, amine-containing moieties such as amino-4-deoxy-L-arabinose or phosphoethanolamine is a mechanism bacteria employ for attaining resistance to cationic antimicrobial peptides such as polymyxins.[41,43,44,48] The aforementioned model implies that an amino-4-deoxy-L-arabinose modification on the lipid A 1′ and 4′-phosphate groups would destabilize the complex by blocking electrostatic interactions with the positively charged amino groups on the side chains of the Dab residues.[41,43,44,48]

The structure of polymyxin M (23) (PMM; syn. mattacin syn. polymyxin A) bound to LPS has also been characterized using trNOE NMR techniques.[60] Similar to PMB, the backbone of the LPS-bound PMM molecule displays a chairlike conformation wherein the side chains of Dab5‚8 point in the opposite direction from those of Leu6 and Thr^7, giving the structure amphilicity (Figure 5C). Similar to PMB, the temperature dependence of the amide proton chemical shifts indicated the amide protons of Dab5‚^{8} are most likely involved in intramolecular hydrogen bonding to stabilize the ring conformation. However, on closer inspection it is clear the backbone conformation of PMM is different from that determined for PMB. In the PMB structure the envelope-like fold of the backbone is so dramatic that the polar and hydrophobic residues in the ring are well separated, whereas in PMM, the backbone is less contorted (i.e., PMM displays a chairlike as opposed to the envelope-like backbone conformation of PMB) giving a less dramatic separation of the polar and hydrophobic side chains. These conformational differences are focused in the region of the hydrophobic motif within the heptapeptide ring and are most likely a result of the amino acid differences in this segment between the two molecules, PMB (D-Phe^6-L-Leu7) and PMM (D-Leu^6-L-Thr7), whereas the rest of the two molecules are identical. The recent chemical shift mapping NMR study by Mares et al.[59] suggested that PMM binds LPS in a similar manner to PMB, with subtle differences at the interaction interface involving the region of the inner oligosaccharide core of LPS. In the PMM–LPS complex, polar interactions between the Dab residues and the GlcN-B and Kdo-C,D carbohydrate units dominate, whereas in the PMB–LPS complex these polar interactions are not prominent. Since PMM displays comparable antimicrobial activity to PMB,[59] the additional polar contacts between the polar side chains of PMM and the Kdo units of the inner core of LPS may help compensate for the loss of binding energy between the hydrophobic domains due to the Leu^7 → Thr substitution.

Bhattacharjya et al.[61] examined the structure of PMBN in aqueous solution both free and in the LPS bound state using trNOE NMR and molecular dynamics techniques. Both the free and LPS bound structures of PMBN are characterized by a type II′ β-turn centered around D-Phe^6-L-Leu7 and an inverse γ-turn at Thr10. The linear dipeptide segment (Thr2-Dab3) and the side chains of the residues forming the heptapeptide ring displayed a reduced mobility in the LPS bound state. The proposed model indicates a different complex topology from that of the PMB–LPS complex (Figure 5D). Although the PMBN molecule straddles the GlcN disaccharide of lipid A similar to PMB, the PMBN molecule sits on top of the lipid A

molecule and does not appear to make the hydrophobic contacts seen in the PMB complex. This model can be used to contrast the OM permeabilizing and antibacterial activities of PMB, to PMBN which possesses OM permeabilizing activity only.[62] In an analogous manner to PMB, PMBN binds lipid A by displacing the divalent cations that bridge adjacent head groups, thereby partially disrupting the membrane packing and creating gaps in the polar surface that allow hydrophobic antibiotics to penetrate into the fatty acyl layer.[22] However, unlike PMB, PMBN lacks the *N*-terminal fatty acyl chain and therefore cannot penetrate the OM; i.e., PMBN lacks the ability to promote its own uptake which is a requirement for antibacterial activity.

The conformation of a lysine substituted PMB analogue in dimethyl sulfoxide has been determined by 2D NMR techniques and molecular dynamics simulations.[63] The backbone of the analogue adopts a rectangular shape stabilized by intramolecular hydrogen bonds. Similar to PMB, the overall structural fold separates the polar and hydrophobic domains, giving the structure amphilicity.

Collectively, the binding and structural data support a two-stage mechanism of the interaction between PMB and LPS, wherein the first stage electrostatic interactions between the positive charges of PMB and the negative LPS head groups stabilize the complex. In the second stage, hydrophobic interactions between the hydrophobic domains of each molecule facilitate penetration of PMB into the OM. The NMR models of the PMB–LPS complex clearly demonstrate that the backbone of PMB adopts an envelope-like fold that separates the polar and hydrophobic domains of the molecule, giving the structure amphilicity that is essential for its antimicrobial action.[29,30,59,60] Most importantly, the model demonstrates a mode of binding that would disrupt the supramolecular structure of the LPS packing in the bacterial OM, ultimately resulting in cell death.

4. Complete Synthesis of Polymyxins

The polymyxin structure possesses elements atypical of peptides in general, such as the N^{α} fatty acyl chain and multiple nonproteogenic L-Dab amino acids, one of which (Dab4) participates in an intramolecular cyclic lactam with the carboxyl terminal L-Thr[10], an element not readily amenable to solid-phase synthesis. The challenge of synthesis has been no small hindrance in the development of polymyxin research.

Total synthesis of a polymyxin was first achieved by Vogler et al.[64–68] using a solution-phase segment condensation approach. However, the efficient generation of analogues has ultimately depended upon the development of solid-phase synthetic methods and specifically the development of orthogonal protection strategies around the L-Dab residues. Such protection provides a mechanism to yield a partially protected linear precursor amenable to cyclization (Figure 7). This has been somewhat challenging, as protected L-Dab residues are not so readily accessible, but in recent years examples such as Fmoc-L-Dab *tert*-Bbutyloxycarbonyl (Boc),[69] Fmoc-L-Dab 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde),[70] Fmoc-L-Dab 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl (ivDde),[71] Fmoc-L-Dab 4-methyltrityl (Mtt),[72] and Fmoc-L-Dab 2-chlorobenzyloxycarbonyl (2-ClZ)[73] have been described.

In the first reported solid-phase method, Sharma et al.70 employed standard Fmoc-based methods but utilizing Dde protection for Dab[7] and synthesis on Sasrin resin to yield the key partially protected precursor peptide after treatment with 2% hydrazine and cleavage with 1% trifluoroacetic acid (TFA). The cyclization was effected with diphenylphosphorylazide (DPPA), and the cyclic peptide was then fully deprotected with 95% TFA.

Similarly, Tsubery et al.72 prepared the polymyxin nonapeptide using Mtt protection for Dab4 and synthesis on 2-chlorotritylresin, yielding the partially protected peptide under mild acidic conditions. Cyclization was best achieved with (benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate) (PyBOP) and again full deprotection with TFA. In the same report, Tsubery et al.72 investigated the use of Fmoc methods for synthesis on Wang resin with Boc protection for the Dab4 but utilizing the TFA-stable 2-(ClZ) protecting group for the other Dab residues. In this way, cleavage with TFA yielded the partially protected peptide. Cyclization was performed again using PyBOP, and final deprotection was achieved with catalytic hydrogenation.

Most recently, Sakura et al.73 and Vaara et al.[74] extended this method for synthesis including the use of OBzl protecting groups for the Thr residues. In this way, cleavage with TFA yielded the partially protected peptide. Cyclization was performed again using DPPA or PyBOP, and final deprotection was achieved with hydrogen fluoride (HF) or hydrogenation. These methods have allowed for the synthesis of approximately 20 peptides in each of these studies.

Total solid phase synthesis of polymyxin peptides via onresin cyclization is rendered a challenge because of the need for an accessible C-terminus and the absence of more obvious side chain linkage points (e.g., Asn or Gln residues). However, the use of a “safety catch” methodology was described by de Visser et al.[71] Standard Fmoc synthesis was performed on Kenner’s safety catch sulfonamide resin with Dab4 protected as an ivDde derivative for chain construction and then changed to the Mmt group for linker activation. Treatment with diisopropylethylamine effected intramolecular cyclization releasing the protected peptide which was then fully deprotected with TFA. Tsuberry72 also reported an on-resin approach using a carbamate linkage through Dab9 side chain but found it less efficient than the solution based methods.

Given the challenge of synthesis, the replacement of the lactam bridge by something more synthetically facile is a logical progression, although the consequences on the cyclic peptide conformation of such changes have the potential to diminish activity. Synthetic analogues where a cystine disulfide replaces the Dab-Thr linkage have been reported by Clausell et al.[35,36] **(211–213)** and Rustici et al.[75] **(153–155)**. The synthesis under these circumstances becomes relatively trivial with a linear sequence prepared and then oxidized under standard conditions.

5. SAR of Polymyxins

This review attempts to provide a comprehensive treatise on the current state of development of polymyxin analogues. Medicinal chemistry strategies for improving the antibacterial activity and toxicity of polymyxins have included modifications of the length and size of the *N*-terminal fatty acyl chain moiety (section 5.1), the Dab side chains and amino acid substitutions (section 5.2), the D-Phe^6-L-Leu^7 hydrophobic motif (section 5.3), the size of the cyclic peptide ring (section 5.4), and the length of the *N*-terminal linear tripeptide segment (section 5.5). Other strategies have involved conjugation (section 5.6), linearization (section 5.7), and generation of mimetic compounds (section 5.8). Hereon in we shall review each of these targeted modifications in detail, and where activity data are available, structure–activity correlations will be made with reference to the three-dimensional models of the PMB–LPS complex.[29,30] This section makes extensive reference to the chemical structures documented in the Supporting Information, and as such, it is intended to be read

Supporting Information Available: Chemical structures of polymyxin analogues, some MICs, and some LPS binding affinity data. This material is available free of charge via the Internet at http://pubs.acs.org.

in tandem with the body of the text. In addition the supplementary section documents minimum inhibitory concentrations (MICs) and LPS binding affinity data where available.

5.1. *N*-Terminal Fatty Acyl Analogues

Functionally the *N*-terminal fatty acyl chain is purported to penetrate the OM and disrupt the packing of the lipid A fatty acyl chains.[29,30] Isothermal titration calorimetry studies have shown that the hydrophobic contribution from the *N*-terminal fatty acyl chain is the predominant energetic driving force for PMB–LPS complexation.[50–53] Therefore, the *N*-terminal fatty acyl chain is crucial for the antimicrobial activity of polymyxins.

The importance of the *N*-terminal fatty acyl segment for the antimicrobial properties of polymyxins first became evident when polymyxin nonapeptides were characterized.[62] Polymyxin nonapeptides are composed of the cyclic heptapeptide and the *N*-terminal Thr^2-Dab^3 dipeptide components. PMBN **(193)** and colistin nonapeptide are generated by proteolytic release of the *N*-terminal fatty acyl-Dab^1 segment with either ficin or papain proteases.[62] Although PMBN and colistin nonapeptide lack any direct antimicrobial activity, they retain the ability to bind LPS with high specificity and efficiently perturb the OM to sensitize Gram-negative bacteria to hydrophobic antibiotics that are normally impermeable to the OM.[62] More importantly, PMBN and colistin nonapeptide display a significantly reduced toxicity profile compared to their parent compounds (LD_{50} of 43 mg/kg for PMBN vs 9 mg/kg for PMB in mice).[76] Thus, the toxicity of polymyxins can partly be attributed to the *N*-terminal fatty acyl segment.[62,74,77] Accordingly, a number of medicinal chemistry programs have focused on generating N^{α} analogues with the goal of developing polymyxins with improved toxicity profiles. A number of different synthetic routes were employed across these reports and will be reviewed briefly preceding each synopsis.

Across the naturally observed PMB peptide components the type of fatty acyl chain linked to the *N*-terminal Dab residue is restricted to (*S*)-6-methyloctanoyl (PMB_1) **(1)**, 6-methylheptanoyl (PMB_2) **(2)**, octanoyl (PMB_3) **(3)**, heptanoyl (PMB_4) **(4)**, nonanoyl (PMB_5) **(5)**, and 3-hydroxy-6-methyloctanoyl (PMB_6) **(6)**.[62,78–82] The naturally occurring polymyxins also display variation of the methyl substitution at the 6 and 7 positions of the fatty acyl chain.[62,78–81] The confirmed synthesis of the naturally occurring 6- and 7-methyloctanoyl isomers of colistin **(7, 17)** indicated that the one carbon migration of the methyl group does not substantially affect antimicrobial activity.[71] The naturally occurring octapeptin subclass of polymyxin peptides are *N*-terminally substituted with long β-hydroxy fatty acyl moieties, including the straight chain β-hydroxydecanoyl and the branched chain 3-hydroxy-8-methylnonanoyl or 3-hydroxy-8-methyldecanoyl **(24–36)**.[83–92]

Early studies of the N^{α} fatty acyl SAR of PMB and colistin component peptides isolated from cultures of producer strains were not sufficiently comprehensive to provide any meaningful SAR data.[93] This is in large part due to the difficulties associated with separation of such closely related structures with the available technology at the time. In more recent times, a few researchers have reported successful separation techniques; however, these studies did not include any microbiological data for the isolated components.[78–81] Subsequently, the N^{α} SAR of the PMB component peptides was confirmed using a solid-phase synthesis approach to generate $PMB_{1–6}$ **(1–6)**.[70,82] Interestingly, the *N*-terminal variation across the naturally occurring $PMB_{1–6}$ structures did not markedly affect antimicrobial activity (MICs against *E. coli* of 0.5–1.0 μM). In contrast, the LPS binding assay results indicated that the more hydrophobic *N*-terminal substituents such as nonanoyl (PMB_5) possess a greater LPS binding affinity compared to the octanoyl (PMB_3) and heptanoyl (PMB_4) substituents.[82,94,95]

Chihara et al.[96,97] were the first to attempt a SAR characterization of the N^{α} fatty acyl segment **(37–58)**. This early work employed colistin nonapeptide as starting material; N^{α} acylation was performed in a buffered aqueous solvent without any protection of the Dab N^{γ}-amino side chain groups.[96,97] The activities of *N*-terminal fatty acyl derivatives containing C9–C14 unbranched fatty acyl chains showed that the longer fatty acyl derivatives were more active against polymyxin-resistant strains and Gram-positive bacteria, whereas the C10 and C12 derivatives were most effective against the polymyxin-susceptible strains.

The most comprehensive N^{α} SAR data have been delivered by Sakura and colleagues.[82,94,95] The group utilized a semisynthetic approach across these studies, wherein purified PMB or colistin were chemically converted to nonapeptides by treatment with *S*-ethyl trifluorothioacetate and used as starting materials. The key step in this successful semisynthetic approach is the preparation of N^{γ}-protected nonapeptide by trifluoroacetylation (Tfa) or with the trichloroethoxycarbonyl (Troc) protecting group, followed by chemical cleavage with methanesulfonic acid to remove N^{α}-alkanoyl-N^{γ}-[Tfa/Troc]-Dab^{1}-OH, yielding the tetrakis-(N^{γ}-protected)nonapeptide.

Following on from the early work of Chihara,[96,97] a complementary study from Sakura et al.[95] also examined the effect of N^{α} fatty acyl chain length on antimicrobial activity **(59–63)**. The study employed the aforementioned semisynthetic approach. All products were HPLC purified, and their identity was confirmed by MS analysis. It was found that *N*-terminal fatty acyl chains intermediate in length (octanoyl) **(63)** are optimal, whereas longer (myristoyl, C14) **(61, 62)** and smaller acetyl-PMBN **(59, 60)** analogues displayed reduced antimicrobial activity compared to PMB. Interestingly, the acetyl-PMBN analogues displayed poor antimicrobial activity against *E. coli*, and *S. enterica* serovar Typhimurium, however, retained potent activity specifically against *P. aeruginosa*. It is noteworthy that the potent activity against *P.aeruginosa* displayed by the compounds **(59, 60)**[95] is not consistent with the observed correlation between the length of the N^{α} fatty acyl chain and antimicrobial activity reported elsewhere.[96,97] This observation can be potentially construed in terms of the differences in the lipid A fatty acyl chain composition across these bacteria which in turn confers different OM properties.[95,98]

In their more recent work Sakura et al.[94] described a comprehensive series of N^{α} analogues were derived by acylation of the tetrakis (N^{γ}-Troc)-PMB or colistin nonapeptides with various hydrophobic acids with aliphatic or hydrophobic ring structures **(64–79)**. The N^{α} analogues were tested for LPS binding affinity and antimicrobial activity against *E. coli*, *S. enterica* serovar Typhimurium, and *P. aeruginosa*. It was found that cyclohexylbutanoyl-**(69, 71)**, 4-biphenylacetyl- **(65, 67)**, and 1-adamantaneacetyl-**(64, 66)** N^{α} analogues displayed comparable activities to the parent compounds (PMB and colistin), with improved LPS binding affinity. Generally, the entire series displayed comparable activity to PMB against *P. aeruginosa*, whereas marked differences in activity against *E. coli* and *S. enterica* serovar Typhimurium were evident across the series.[94]

Clausell et al.[36] reported a PMB analogue that incorporates a pyrene group N^{α} substituent. The compound was employed for examining membrane association and intermembrane contact formation, and no microbiological data were reported. The same compound was reported by Sakura et al.[94] **(72, 74)** as part of their comprehensive N^{α} SAR study; the analogue displayed a reduced antimicrobial activity compared to PMB against *E. coli* and *S. enterica* serovar Typhimurium, and comparable activity to PMB was observed against *P. aeruginosa*.

The Pfizer group[99] explored the effect of increasing the length of the *N*-terminal fatty acyl chain on antimicrobial activity of PMBN analogues **(80–82)**. In this study, PMBN prepared from commercial PMB by papain treatment was employed as the starting material. The Dab N^{γ} amino side chain groups were blocked with a protecting group. The N^{α}-amino was not selectively protected prior to reaction with the protecting group, as it was purported that the Dab N^{γ}-amino groups, being more electron-rich and less sterically hindered, would preferentially react with the protecting group 1-(Boc-oxyimino)-2-phenylacetonitrile (Boc-ON). The reaction was performed over a short period (<20 min at 23 °C) and quenched to avoid complete reaction to the penta-Boc byproduct. N^{α} analogues with unbranched ocatnoyl (C8) fatty acyl chains **(80)** displayed significantly enhanced antimicrobial activity (up to 8-fold based on MICs) compared to PMB (both commercial and synthetically sourced). The nonanoyl (C9) **(81)** and decanoyl (C10) **(82)** fatty acyl N^{α} derivatives showed decreased activity. The findings of this study suggest that N^{α} fatty acyl chains > C8 in length and branching such as the 6-methyl moiety in PMB act to decrease antimicrobial activity, potentially by sterically hindering OM insertion of the fatty acyl moiety. It is interesting to note that in comparison, only marginal differences in activity were evident between the (*S*)-6-methyloctanoyl- (PMB_1) **(1)** and unbranched octanoyl- (PMB_3) **(3)** N^{α} native PMB peptides.[82] However, considering that the Pfizer compounds are PMBN analogues, this discrepancy simply emphasizes the important role of Dab[1] for regulating antimicrobial activity. Other reports have also shown longer N^{α}-terminal fatty acyl substituents such as decanoyl reduce antimicrobial activity compared to the corresponding octanoyl substituted analogues.[74] A similar trend was reported for a series of octapeptins where a decrease in activity was observed with compounds that possess N^{α} fatty acyl chains longer than C8.[100]

There are noticeable discrepancies in the literature with reported N^{α} analogue SAR data. The Sakura[82] and Pfizer groups[99] reported octanoyl- and myristonyl-PMB analogues with potent antimicrobial activity, whereas the same compounds were reported 30 years ago by Chihara et al.[96,97] to be 20 times less active. This discrepancy is most likely due to contamination of the products reported by Chihara et al. [96,97] with N^{γ} acylated nonapeptides, as the acetylation reaction was conducted without protection of the Dab N^{γ}-amino side chain groups. In comparison, the more recent efforts from Sakura et al.[82] and Pfizer[99] employed protecting groups for the Dab N^{γ} side chains prior to N^{α} acetylation. Moreover, the products were purified by HPLC prior to testing for antimicrobial activity. Thus, SAR data obtained from earlier reports should be viewed with caution unless clear evidence of the purity and identity of the compounds is documented.

de Visser et al.[71] synthesized a series of N^{α} PMB analogues with short (<C7) unbranched fatty acyl chains. This study employed a solid-phase synthetic approach based on cleavage-by-cyclization using a safety-catch sulfonamide linker resin.[71] The hexanoyl (C6) **(91)** and 1-adamantane **(83)** N^{α} analogues displayed potent antimicrobial activity (albeit less active than PMB), whereas the pentanoyl (C5) **(92)** and butanoyl (C4) **(90)** fatty acyl derivatives were 10- to 20-fold less active than PMB. It is noted that, unless the MIC of PMB was measured in each study as a control, care is required when comparing the MICs across different series of analogues.

Tsubery et al.[101] adopted a total synthetic approach by employing a combination of solid-phase linear chain elongation (γ-fluorenylmethoxycarbonyl (Fmoc) strategy) and subsequent cyclization after release. Side chain Dab N^{γ}-amino protecting groups were *tert*-butyloxycarbonyl (tBoc) and benzyloxycarbonyl (Cbz). Two pairs of N^{α} PMBN and PMB decapeptide analogues were synthesized **(93–96)**. In one pair the *N*-terminus of PMBN was substituted with $[Ala]_3$ and $[Ala]_6$ oligoalanyl **(93, 95)**. Because of the long hydrophobic chain, $[Ala]_6$-PMBN **(95)** was expected to possess potent antimicrobial activity; however, both oligoalanyl N^{α} analogues did not significantly improve activity compared to the control

compound PMBN. This could potentially be attributed to the extensive methyl side chain branch points along the oligoalanyl structure. The *N*-terminus of the other pair was substituted with the hydrophobic Fmoc group **(94, 96)**. The hydrophobic Fmoc N^{α} substitution significantly enhanced in vitro antimicrobial activity and reduced toxicity in a mouse acute toxicity model. This indicates that the hydrophobicity of the N^{α} substituent influences both antimicrobial activity and acute toxicity. Compared to the Fmoc-PMBN **(94)**, the Fmoc-PMB decapeptide **(96)** was significantly more active against *P. aeruginosa* and *K. pneumoniae* while comparable against *E. coli*.

Collectively, these experiments underscore the important contributions made by the *N*-terminal fatty acyl chain to the binding interactions with LPS and consequent antibacterial activities of polymyxins. A comparison across all N^{α} analogues documented indicates antimicrobial activity appears to correlate with the length and bulkiness of the N^{α} substituent. The optimal fatty acyl chain length appears to be C7–C9 (as per the native peptides), as longer or shorter chain N^{α} analogues display reduced antimicrobial activity. LPS binding affinity appears to correlate with the length of the N^{α} fatty acyl chain.[82,95,99] By reference back to the molecular model of the PMB–LPS complex (Figure 5A),it is likely that hydrophilic, long (>C9 fatty acyl chain), bulky, or extensively branched N^{α} substituents sterically hinder OM insertion. Therefore, the packing of the lipid A fatty acyl chain layer is not sufficiently disrupted to produce antibacterial activity.

5.2. Dab Side Chain Derivatives and Substitutions

Even at the very beginnings of polymyxin research,it was understood that the positive charges of the Dab residues are important for antimicrobial activity.[102] This was first evidenced by the inactive sulfomethyl PMB **(97)**.[102] Sulfomethyl PMB is a derivative of PMB where the free amino groups are blocked by sulfomethylation, yielding a compound that is inactive in vitro.[102] In this section we shall discuss the numerous synthetic or semisynthetic modifications targeted at the Dab positions in an effort to improve microbiological activity and minimize potential for toxicity.

Early attempts to neutralize the untoward effects of the Dab residues mostly involved simple chemistries for derivitization of the Dab N^{γ}-amino side chain groups.[102–107] Most of these studies used commercial preparations of PMB (sulfate) or colistin (sulfate) as the starting material and did not employ protecting groups; as such, the amine coupling reactions were performed in a nonspecific manner. Chemical modifications to the Dab N^{γ}-amino groups through amide linkages or Schiff base formation have included acetylation **(98–102)**,[103,105] deamination **(103)**,[103] formylation **(104–108)**,[103] dinitrophenylation **(110)**,[103,107] guanidation **(113)**,[103] cabamylation **(115–130)**,[107] and reactions with 5-amino-2,4-dinitrofluorobenzene **(111)**,[103] pyridoxal phosphate **(114)**,[103] or dimethylaminonaphthalene-5-sulfonyl (dansyl) **(205)**.[108] Most of these compounds were inactive; with exception, an early report from Srinivasa and Ramachandran[103] described a set of Dab N^{γ}-formylpolymyxin B derivatives **(105–108)** that displayed potent antimicrobial activity (except **108**). Interestingly, triformyl-PMB **(106)** was as active as PMB, and diformyl-PMB **(105)** was 70% more active than PMB.[103] In stark contrast, another early study reported that Dab N^{γ} mono-, di-, and triacyl derivatives **(98, 100, 102)** retained only partial activity, whereas the tetra- and pentaacyl derivatives **(99, 101)** were inactive.[105] Witzke and Heding[107] reported a series of PMB and colistin *N*-benzyl derivatives **(115–130)** prepared from Schiff bases that displayed an increased activity against the Gram-positive test strain, *Staphylococcus aureus* ATCC 6538P. The reduction of cationic character through substitution of Dab side chains with lipophilic groups appears to increase activity against Gram-positive bacteria **(131, 133**, **138, 140, 142**).[106,107]

Weinstein et al.[106] employed tetra-Boc PMB as starting material to prepare a series of analogues selectively modified at the Dab[1] and Dab[9] N^{γ}-amino side chain groups **(131–151)**. Monofunctionalization of PMB at the Dab[1] and Dab[9] positions was achieved by protection of the most basic Dab N^{γ}-amino groups (Dab1 and Dab9) by protonation with strong acids while introducing protecting Boc groups on the remaining Dab N^{γ}-amino groups. PMB monohydrochloride prepared by dissolving PMB in methanol containing acetone, and HCl (1 equiv) was reacted with di-*tert*-butyl dicarbonate to yield tetra-Boc[N^{γ}-Dab1]PMB (15%) and tetra-Boc[N^{γ}-Dab9]PMB (36%). The tetra-Boc products were used to prepare N^{γ}-alkylated and N^{γ}-acylated derivatives. Only derivatives with positively charged or polar side chain substituents displayed better antimicrobial activity than PMB. Notably, the arginyl-Dab[9] **(149)** derivative displayed a significantly improved therapeutic index determined acutely in male CF_1 mice (LD_{50}, 15 mg/kg intravenously)/protective dose (PD_{50}, 0.25 mg/kg subcutaneously) compared to PMB (LD_{50}, 9 mg/kg intravenously/ PD_{50}, 4.5 mg/kg subcutaneously). Across the entire series, the Dab[9] position appeared to be more important for antibacterial activity, as the Dab[9] derivatives were 4-fold less active than the corresponding Dab[1] derivatives. The more lipophilic α-aminoacyl **(131, 133, 138, 140, 142)** derivatives displayed a broader antibacterial spectrum, with potent activity against Gram-positive pathogens. Interestingly, the geometric mean MIC of PMB against the Gram-positive test strain (*S. aureus*) was 2.8 μg/mL, which is surprisingly lower than commonly observed.[7] In light of the other PMB analogues with hydrophobic modifications on the Dab side chains reviewed herein, it appears that the introduction of lipophilic groups acts to broaden the antibacterial spectrum. Although this appears promising, one concern is the potential for hemolytic activity against the host associated with such lipophilic peptides.[109]

The inception of solid-phase peptide synthesis in 1963[110] has allowed chemists to more readily explore amino acid substitutions in bioactive peptides. In an effort to circumvent the potential toxicity, many medicinal chemistry programs have employed solid-phase synthesis to substitute the nonproteogenic Dab residues with neutral or basic proteogenic amino acids.

The earliest report of an all Dab → Lys substituted colistin analogue **(152)** was from Kurihara et al.[111] The microbiological data reported for the compound indicated a comparable antimicrobial activity to native colistin. Rustici et al.[75] synthesized a series of des-N^{α}-acyl-PMB analogues with Lys replacing all of the Dab positions, L-Phe replacing D-Phe, and cyclization via an intramolecular disulfide bridge **(153–156)**. The compounds displayed poor antimicrobial activity, albeit **154–156** possessed appreciable binding affinity for LPS. Once again this study demonstrated that the ability to bind LPS does not necessarily correlate with antimicrobial activity. Acute toxicity testing in mice showed these analogues were less toxic than PMB.

In their patent descriptions, Porro et al.[112–115] described a large series of polymyxin analogues wherein each Dab was substituted with a basic amino acid (Lys, Arg, or His). All of the peptides displayed a high affinity for LPS (even better than PMB) and sensitizing activity toward hydrophobic antibiotics against the Gram-negative bacterial strains tested, namely, *S. Typhimurium*, *Haemophilus influenzae*, and *Vibrio cholerae*.

A recent study from the Sakura group[73] reported a series of PMB alanine scanning analogues aimed at examining the contribution of each amino acid position to antimicrobial activity and LPS binding **(158–167)**. The [Dab[5] → Ala]PMB **(161)** analogue displayed the most significant reduction in activity. In addition, it was found the Dab positions within the heptapeptide ring (Dab5,8,9) were more important for antimicrobial activity than the Dab residues in the linear tripeptide segment (Dab1,3). This can be fully appreciated from the model of the PMB–LPS complex, which shows that the Dab positions in the heptapeptide provide the critical electrostatic contacts that anchor the whole PMB structure to the lipid A

phosphate groups (Figures 4 and 5A). All of the Ala substitution analogues displayed a reduced LPS binding affinity compared to PMB.

In an attempt to reduce the nephrotoxicity associated with the cationic nature of polymyxins, Vaara et al.[74] recently reported a series of polymyxin analogues carrying only three positive charges, all within the heptapeptide ring **(174–192)**. Analogues substituted with Thr, Ser, or aminobutyryl groupat the Dab1,[3] positions within the tripeptide linker all displayed potent antimicrobial activity (≥PMB). Moreover, irrespective of whether they had any direct antimicrobial activity, all of the analogues displayed a potent sensitizing action toward hydrophobic antibiotics. Substitution of the Dab positions in the cyclic heptapeptide with neutral aminobutyric acid residues resulted in a complete loss of antimicrobial activity. The compounds displayed a markedly reduced affinity for isolated rat kidney brush border membranes, which is purported to be indicative of reduced nephrotoxicity.[74]

Tsubery et al.72,[116] described a series of PMBN analogues where the Dab residues were selectively substituted with basic amino acids with longer or shorter side chains compared to Dab (i.e., Lys; ornithine; 2-amino-4-guanidinobutyric acid; 2,3-diaminopropionic) **(194–204)**. The SAR data garnered from this study clearly showed that in addition to the cationic character, the length of the amino acid side chain alkyl arm is also crucial for antimicrobial activity. The optimum side chain length appears to be two methylene groups as per the native Dab residue. PMBN analogues with side chains that varied by four (Lys), three (ornithine), and one (2-amino-4-guanidinobutyric acid) methylene groups in length were all found to display a reduced sensitizing activity. In line with these findings, the model of the PMB–LPS complex (Figures 4 and 5A) indicates that a two methylene side chain length provides the ideal bridging distance between the N^{γ}-amino side chain groups and the lipid A phosphates.

Interestingly, as noted above, a number of studies have indicated that the antimicrobial activities and LPS binding affinities of polymyxins and their analogues are not parallel.[73,94,95] A polymyxin analogue can display potent antimicrobial activity and concomitantly only moderate LPS binding affinity and vice versa.[73,94,95] This is possibly related to the biophysical nature of the assay system employed to measure LPS binding affinity. The most commonly used assay is the *N*-dimethylaminonaphthalene-5-sulfonyl-PMB (dansyl-PMB) **(205, 230)** fluorometric displacement assay. The dansyl moiety is an environmentally sensitive fluorophore that has a low quantum yield in the high dielectric environment of the aqueous buffered assay solution. Whereas in the low dielectric hydrophobic environment formed by the LPS binding surface, it is highly fluorescent. Therefore, the assay measures the enhancement in fluorescence emission of the dansyl moiety upon binding to the hydrophobic LPS molecule. Titration with unlabeled polymyxin results in a quenching of fluorescence by competitive displacement of dansyl-PMB from the LPS binding surface. Dansyl-PMB is usually prepared from commercial preparations of PMB by reaction of the Dab N^{γ}-amino side chain groups with dansyl chloride in solution.108 The reaction products are usually heterogeneous. Our recent unpublished data show mixtures consisting of mono-, di-, tri-, tetra-, and pentalabeled products, which are inactive, yet bind LPS. The original report108 described a dansyl-PMB product that retained potent antimicrobial activity; however, this preparation was not purified, and therefore, the observed antimicrobial activity is probably attributable to unreacted PMB. The ideal approach is to employ a structurally well-defined monodansylated [dansyl-Gly1]PMB **(230)** generated via solid-phase synthesis.[82] The [dansyl-Gly1]PMB retains almost complete antimicrobial activity as PMB; therefore, the assay more faithfully measures competitive binding for the same sites on the LPS molecule.[82]

The critical involvement of the cationic Dab residues in conferring the antimicrobial activity of polymyxins has been well documented.[56,102,117–119] In summary, the key features of the Dab residues that are important for antimicrobial activity and LPS binding affinity include (a) the cationic character of the side chain groups, (b) the two-methylene length of the Dab side chain, and (c) the specific order of the Dab residues within the primary sequence that confers the proper spatial distribution of the positive charges for electrostatic interactions with the phosphates of lipid A. To date, attempts to substitute or derivatize the Dab positions have met with variable success. In general, the Dab residues, particularly within the cyclic heptapeptide, are indispensable for the antimicrobial activity of polymyxins.

5.3. D-Phe^6-L-Leu^7 Hydrophobic Motif Analogues

Polymyxins possess two hydrophobic domains, the N^{α}-terminal fatty acyl moiety and the D-Phe^6-L-Leu^7 segment in the heptapeptide ring. Functionally, the hydrophobic side chains of the D-Phe^6-L-Leu^7 motif are believed to insert into the bacterial OM and stabilize LPS complexation via hydrophobic interactions with the fatty acyl chains of lipid A.[29,30] Molecular dynamics and NMR structural studies indicate that PMB adopts a type II′ β-turn centered around the D-Phe^6-L-Leu^7 motif in the free state.[28–30] A type II′ β-turn is commonly found in many nonribosomal cyclic peptides.[120–122] This structural feature is believed to allow the backbone of the cyclic peptide to adopt the biologically active conformation when binding to its target receptors.[120–122] The position $i + 1$ of the type II′ β-turn is generally populated by a D-amino acid or Gly that serves as the β-turn forming element. Across the naturally occurring polymyxins, position 6 (about which the type II′ β-turn is centered) is commonly populated by D-Phe (PMB) or D-Leu (colistin). In PMB **(1–6)** and colistin **(7, 8, 15–17, 19)** position 7 is occupied by L-Leu. Moreover, [Ileu7] **(12, 18)**, [Thr7] **(20–23)**, [NorVal7] **(10, 13)**, and [Val7] **(11, 14)** peptides have been identified in PMB and colistin minor components.[80–82] By employment of complete synthesis to generate defined products, substitution of Leu^7 → Ileuor Thr in PMB or colistin has been shown not to noticeably affect antimicrobial activity.[82,123]

The contribution of the D-Phe^6-L-Leu^7 segment was first evaluated in PMBN by substitution of D-Phe^6 with D-Trp or D-Tyr **(206–209)** and substitution of the L-Leu^7 position with L-Phe **(206)** or L-Ala **(207)**.[124] PMBN was employed as the control compound such that the contribution of the D-Phe^6-L-Leu^7 motif could be evaluated without the influence of the N^{α} fatty acyl chain.[124] The shortcoming of this approach is that only relative sensitizing activity can be measured, as PMBN lacks any antimicrobial activity. Substitution of either D-Phe^6 or L-Leu^7 with more polar amino acids such as Ala or Tyr significantly reduced OM permeabilizing activity. Replacing the D-Phe^6 with L-Phe resulted in an almost complete loss of OM permeabilizing activity.[124] Together, these results possibly reflect an impairment of the stability of the type II′ β-turn or the inability of the polar residues to insert into the fatty acyl layer formed by lipid A in the bacterial OM. The D-Trp^6 **(209)** analogue displayed marginally reduced permeabilizing activity relative to PMBN, consistent with the conservative nature of these substitutions.

The recent work of the Sakura group[73] thoroughly examined the effect substitutions at D-Phe^6-L-Leu^7 on antimicrobial activity and LPS binding. PMB analogues substituted at position 6 with D-Trp **(168)**, D-Ala **(167)**, and L-Phe **(172)** or at position 7 with L-Ala **(162)** and L-Trp **(173)** retained both potent antimicrobial activity and LPS binding. These findings would suggest that neither a loss of hydrophobicity within the D-Phe^6-L-Leu^7 motif nor the D-configuration of the position 6 amino acid was essential for antimicrobial activity. These results are somewhat at odds with the findings of Tsubery et al.[124] and the line of thought that the D-configuration of the position 6 amino acid is necessary for the formation of the type II′ β-turn. However, the findings of Sakura et al.[73] cannot be directly compared to that

of Tsubery et al.,[124] as the latter study examined PMBN analogues. Nevertheless, the discrepancy between the two studies indicates the N^{α} fatty acyl chain is more than capable of compensating for a loss of hydrophobicity within the D-Phe^6-L-Leu7 motif. The [Gly6-Gly7]PMB **(170)** double substitution analogue was completely inactive; this is most likely a result of the complete loss of hydrophobicity or a dramatic conformational change in the heptapeptide ring.[73] The position 7 substitution analogues [L-Ala7]PMB **(163)**, [Gly7]PMB **(171)**, and [L-Trp7]PMB **(173)** displayed marginally reduced antibacterial activity and LPS binding compared to PMB.

Clausell et al.[35] described an analogue that incorporated a permutation where the D-Phe^6-L-Leu^7 motif was disrupted by an intervening Dab residue **(212)**. Although no microbiological data were reported, the analogue was shown to lack selectivity for lipids that exchange through vesicle-to-vesicle contacts and had a significantly lower permeabilizing activity toward artificial vesicle membranes. Another analogue containing D-Phe^6→ D-Trp substitution, comparable to PMB, showed binding to vesicles and formation of vesicle-to-vesicle contacts.

de Visser et al.[71] reported a series of analogues where the D-Phe^6-L-Leu^7 motif was substituted with dipeptide mimics: (a) δ-aminovaleric acid **(84)**, (b) the extended conformation inducer 4-phenyl-4-carboxymethylpiperidine **(85)**, (c) *m*-aminomethylbenzoic acid **(86)**, (d) the β-turn inducing amino acid (*S*)-3-amino-1-carboxymethylcaprolactam **(87)**, (e) *N*-carboxymethylpiperazine **(88)**, and (f) *trans*-4-aminomethylcyclohexane **(89)**. Unfortunately none of these analogues were active against *E. coli*.

In summary, the D-Phe^6-L-Leu^7 segment in the polymyxin heptapeptide ring forms a hydrophobic domain that is highly conserved across the naturally occurring polymyxins and appears to be important. However, it is not indispensable for antibacterial activity and LPS binding.

5.4. Cyclic Peptide Ring Modifications

Many nonribosomal antimicrobial peptides display a cyclized backbone.[120,121,125] Studies with deacylated polymyxins clearly demonstrated that the major structural requirement for direct antimicrobial activity is the cyclic heptapeptide structure. In the polymyxin structure, the N^{γ}-amino side chain of Dab^4 is deacylated by the C-terminal Thr^{10} to form a 23-membered lactam ring. The molecular model of the PMB–LPS complex (Figures 4 and 5A) indicates it is the precise 23-atom size of the native heptapeptide ring that acts as a scaffold for electrostatic and hydrophobic LPS contact points. This has been well accepted, and only a few attempts have been made to examine modifications to the native ring size on antimicrobial activity.[64,72,116]

Vogler et al.[64,66] were the first to describe a PMB analogue with an extended ring structure **(214, 215)** generated by the insertion of an additional Dab residue. The resultant compounds were significantly less active than PMB against *E. coli*.

The Fridkin group[72,76,101,116,124,126] have been very active in the area of polymyxin SAR. Most notably, the group demonstrated that the sensitizing activity of PMBN is stereospecific and highly dependent on the overall structural orientation of the heptapeptide.[126] Compared to the native L-enantiomeric PMBN, the D-enantiomeric PMBN analogue (in which all L-amino acids are replaced by their optical D-isomers) lacks the ability to sensitize Gram-negative bacteria toward hydrophobic antibiotics. Interestingly, both enantiomers displayed comparable LPS binding affinity, again suggesting that LPS binding and OM permeabilizing activity are not coincident. In another study, Fridkin and colleagues described the synthesis of a series of PMBN analogues wherein the ring size was varied from 20 to 26 atoms (**193–**

200).[72,116] All of the ring variants displayed a significantly reduced OM permeabilizing activity compared to PMBN, indicating that the native 23 atom ring provides the optimal structural configuration.

Taken together, these SAR data demonstrate it is the precise combination of topographic chemical features and the 23 atom ring structure that are essential for efficient binding to LPS and subsequent OM permeabilizing activity. Thus, the 23-atom size of the native PMB ring provides the most ideal structural configuration for potent antimicrobial activity.

5.5. Linear Tripeptide Segment Analogues

The heptapeptide cyclic core of the polymyxin molecule is bridged to the fatty acyl chain by a linear tripeptide segment (Dab1-Thr2-Dab3) (Figure 1). Although the isolated fatty acyl–tripeptide moiety (6-methyloctanyl fatty acyl-Dab1-Thr2-Dab3-COOH) lacks both direct antimicrobial activity and OM permeabilizing activity,93 the structure represents a key functional feature of polymyxins. Functionally, this segment contributes two positive charges toward the binding interaction with LPS. Moreover, the molecular model of the PMB–LPS complex indicates hydrogen bonds (a) between the amide nitrogen of Dab3 and the hydroxyl side chain of Thr2 and (b) between the main chain carbonyl of Dab4 and the amide nitrogen of Thr2, which bends the tripeptide toward the heptapeptide core (Figure 6B). This configuration ensures that the amphipathic structure of the molecule remains well organized into the respective hydrophobic and hydrophilic domains.

A number of medicinal chemistry studies have explored the SAR of the linear tripeptide segment by examining the effects of amino acid deletions and/or substitutions.[64,74,76,82,98,127–129] A variety of preparative techniques including enzymatic, semisynthetic, and complete solid-phase synthesis methods have been employed.[64,74,76,82,98,127–129] Both fatty acyl-PMB and des-fatty acyl-PMB analogues have been employed as the parent structure across these studies.

The very first such study was from Vogler et al.[64,66] A pair of ring modified PMB analogues were synthesized which also featured Dab deletions within the tripeptide segment **(214, 215)**. Both analogues displayed significantly lower antimicrobial activity compared to PMB_1. Nevertheless, the reduced activity cannot unequivocally be attributed to the tripeptide segment modifications due to altered cyclic core.

Sakura.et al.[82] described three fatty acyl-PMB analogues with truncated tripeptide segments, octanoyl fatty acyl-PMB nonapeptide (octanoyl FA-PMBN) **(231)**, octanoyl fatty acyl-PMB octapeptide (octanoyl FA-PMBO) **(232)**, and octanoyl fatty acyl-PMB heptapeptide (octanoyl FA-PMBH) **(230)**. Octanoyl FA-PMBN **(231)** displayed comparable activity to PMB, whereas the double deletion analogue octanoyl FA-PMBO **(232)** was 6 times less active against *E. coli*. The complete tripeptide deletion analogue, octanoyl FA-PMBH **(230)**, was devoid of antimicrobial activity. In addition, a corresponding set of des-fatty acyl tripeptide analogues were synthesized.[82] The nona-, octa- **(234)**, and heptapeptide **(235)** des-fatty acyl analogues were devoid of direct antimicrobial activity, whereas the des-fatty acyl decapeptide analogue with the intact tripeptide (des-FA-[Dab1-Thr2-Dab3]-PMB) **(233)** retained antibacterial activity. The activity of these fully synthetic analogues is consistent with the antimicrobial activity reported for corresponding analogues derived enzymatically, as discussed below.[128,129]

The importance of the tripeptide side chain for OM permeabilizing activity was examined in the early work of Vaara,[128,129] which compared the OM permeabilizing activity of PMBN to PMB decapeptide (PMBD) **(233)**, PMB octapeptide (PBMO) **(234)**, and PMB heptapeptide (PMBH) **(235)**. The compounds were prepared by proteolytic digestion of

commercial PMB with polymyxin acylase (yielding PMBD), nagarse (yielding PMBO), or bromelain (yielding PMBH). All products were purified by reversed-phase HPLC. PMBO displayed comparable sensitizing activity to PMBN against *E. coli* and *S. enterica* serovar Typhimurium. In comparison, a 3-fold greater concentration of PMHP **(236)** was required to achieve the same sensitizing effect. PMBD was a slightly more effective OM permeabilizing agent than PMBN.[129] In contrast to PMBN, at high concentrations (30 μg/mL) PMBD displayed direct antimicrobial activity. The report by Kimura et al. is in remarkable contrast to the report by Ito-Kagama and Koyama[127,130] that showed an approximate 100-fold difference in OM permeabilizing activity of colistin nonapeptide and colistin heptapeptide. This discrepancy may be a result of the different preparative and purification procedures employed by the two groups.

Naturally occurring polymyxins with amino acid deletions within the tripeptide segment have been documented.[83–92] The octapeptins are naturally occurring nonribosomal peptides that display the same heptapeptide primary structure as PMB but lack the *N*-terminal tripeptide segment **(24–36)**.[83–92] Instead the heptapeptide core is linked to a single D-Dab or D-Ser residue attached to a long β-hydroxy fatty acyl chain. In a structural perspective, it would appear the loss of the Dab^1-Thr^2 segment (which interacts with the 4′-PO_4 of lipid A in the PMB-LPS complex, cf. Figures 4 and 5A)is compensated by the additional hydrophobic and polar contributions from the longer chain and secondary hydroxyl groups of the fatty acid. Despite their structural similarities at the primary level, the octapeptins and polymyxins display marked differences in their antimicrobial spectrum.[83–92] The octapeptins are active against polymyxin-resistant strains.[64,83–85,89] For example, the MIC of octapeptin EM49β **(24)** for the polymyxin-resistant strain *E. coli* SC9253 was 0.3 μg/mL whereas the MIC of PMB was >200 μg/mL.[64,83–85,89] Moreover, octapeptins are active against Gram-positive bacteria as well.[64,83–85,89,100]

Sakura et al. examined the contribution of each amino acid position in the tripeptide by employing alanine scanning substitutions into synthetic fatty acyl-PMB peptides **(158–166)**.[73] The Thr2 position appears to be more important compared to the Dab1,3 positions, as the FA-[Ala2]PMB **(159)** analogue displayed significantly reduced antimicrobial activity and LPS binding compared to the corresponding positions **1** and Ala substituted analogues. Similarly, a decrease in antimicrobial activity was observed with neutral or hydrophobic amino acid substitutions (Ala or 2-aminobutyric acic (Abu)) **(174, 178, 179, 185)** in the tripeptide segment of octanyl-PMB analogues described by the Vaara group.[74] Across the naturally occurring polymyxins, Thr^2 → Ser polymyxin E_4 **(16)**, Dab^3 → Ser **(19)**, polymyxin D_1 **(20)**, polymyxin D_2 **(21)**, polymyxin S_1 **(22)**, substitutions are observed within the tripeptide linker, whereas the $Dab^{1,3}$ positions are strictly conserved.[131–134] This conservative substitution does not appear to affect antimicrobial activity.[131–134]

The PMB–LPS structural model shows an electrostatic interaction between Dab^1 and the 4′ phosphate of lipid A (Figures 4 and 5A). However, a few studies have indicated that the Dab^1 position is dispensable for the LPS binding and antimicrobial activity of PMB.[73,82,98] [Ala1]PMB **(158)** and [dansyl-Gly1]PMB **(229)** analogues both displayed antimicrobial activity comparable to PMB.[73,82] Moreover, the semisynthetic fatty acyl-[Thr2-Dab3]PMB **(231)** analogue[82,95] displayed comparable activity to PMB, again suggesting the Dab^1 position is dispensable for antimicrobial activity. Tsubery et al.[101] reported a N^{α} Fmoc-PMB decapeptide (Fmoc-PMBD) **(96)** and nonapeptide (Fmoc-PMBN) **(94)** analogue pair. The Fmoc-PMBD displayed an approximately 2-fold greater activity than the Fmoc-PMBN, suggesting Dab1 provides a significant contribution to antimicrobial activity and LPS binding. In one of their recent studies, Sakura and colleagues described a series of des-fatty acyl-PMB analogues substituted at Dab1 with various polar or hydrophobic amino acids (des-FA-[X1]-PMB) **(216–228)**.[98] The des-FA-[Ser, 2,3-

diaminopropionic acid (Dap), Arg, Phe, Trp1]PMB **(218, 221, 222, 226, 227)** analogues displayed comparable antimicrobial activity as the control compound des-FA-[Dab1]PMB **(220)** against most of the test strains. The des-FA-[Lys, Ala, Leu1]PMB **(216, 224, 225)** analogues displayed a reduced antimicrobial activity relative to the control compound **(220)**. Not surprisingly, the introduction of a negatively charged residue generated an inactive compound des-FA-[Glu1]PMB **(223)**, presumably because of electrostatic repulsion with the negatively charged phosphate groups of lipid A. All of the compounds exhibited a reduced affinity for LPS compared to PMB. Collectively, these data would imply that the Dab^1 position plays more of a fine-tuning as opposed to a critical role toward the binding interaction.

The Sakura group also described a series of N-terminal tripeptide PMB analogues **(217, 219, 228)**.98 The des-FA-[Dab-Dab-Dab1]PMB **(228)** and des-FA-[Arg-Arg-Arg1] PMB **(219)** analogues showed potent antimicrobial activity against *P. aeruginosa* but were poorly active against *E. coli* and *S. enterica*. Moreover, both compounds displayed comparable LPS binding affinity to PMB. The des-FA-[Ala-Ala-Ala1]PMB **(217)** analogue was inactive across all three test strains and displayed poor LPS binding. The authors' purport that the selective antimicrobial activity of these compounds against *P. aeruginosa* can be attributed to structural differences between the lipid A of *P. aeruginosa* compared to that of *E. coli* and *S. enterica*. The lipid A of *E. coli* and *S. enteric* are similar, both display four C_{14} and two C_{12} fatty acyl chains. In comparison, the lipid A of *P. aeruginosa* possesses four C_{12} and two C_{10} fatty acyl chains. Consequently, the OM of *P. aeruginosa* is less tightly packed because of the reduced hydrophobic interactions between the lipid A fatty acyl chains. Thus, given the presence of a strong ionic bonding potential between the polymyxin analogue and LPS, the hydrophobic interaction from the side chains of $_D$-Phe^6-$_L$-Leu^7 is sufficient to cause disordering of the OM that leads to cell death.

Tsuebery et al.[76] described the synthesis of PMB and colistin tripeptide analogues where a short chemotactic peptide consisting of [Met-Leu-Phe] was linked to Thr^2 **(236–239)**. Although the analogues lacked any direct antibacterial activity, they retained the ability to sensitize the OM to hydrophobic antibiotics and displayed binding to LPS. Upon binding to the bacterial OM, the analogues acted as potent opsonins to promote bacterial destruction by phagocytic cells.[76] This novel design strategy was primarily aimed at exploiting the PMB structure as a delivery vector to target chemotactic peptides to the bacterial cell surface.

In summary, the available SAR data relating to the tripeptide segment demonstrate that it represents an integral functional feature of the polymyxin structure. Two main principles can be drawn from the SAR data: (a) the tripeptide segment can only be truncated by one amino acid position from the *N*-terminus with a negligible loss of antimicrobial activity; (b) only conservative amino acid substitutions appear to be tolerated.

5.6. Linear Polymyxin Analogues

To date a number of attempts have been made to generate linear polymyxins **(156, 240–243)**. Again, these efforts have been underpinned by the need to improve the toxicity profile while maintaining potent antimicrobial activity.[75,77,111] Across most reports it was found the loss of the cyclic structure completely abrogates antimicrobial and OM permeabilizing activity; this indicates that the high degree of organization imposed by the rigid cyclic heptapeptide (Figures 4 and 5A) is required to maintain the active conformation.

5.7. Conjugated Polymyxin Analogues

Griffin et al.[135] described a large number of multivalent compounds consisting of 2–10 PMB molecules covalently attached to one or more linker structures **(244)**. The compounds

were intended as neutralizing agents against the septic effects of LPS and as prophylactics for Gram-negative bacterial infections.[135] Detailed microbiological or toxicological data for these compounds were not documented; however, it is likely that they would not improve upon the nephrotoxicity of PMB.

5.8. Polymyxin Mimics

Many groups have abandoned making direct modifications to the polymyxin scaffold while attempting to mimic the physicochemical properties of polymyxins. Frecer et al.[136] reported a series of cyclic amphipathic peptides **(245)** consisting of alternating cationic (Lys) and nonpolar (Val or Phe) residues, loosely based on the amphipathic properties of the PMB structure. The compounds displayed potent antimicrobial activity against *E. coli*, *S. enterica* serovar Typhimurium, *P. aeruginosa*, *K. pneumoniae*, and *Shigella sonnei*, and a high affinity for LPS.

Other notable efforts that produced highly active compounds include the spermine derivatives **(246)** reported by David and colleagues[137] and the synthetic amine and guanidine functionalized cholic acid-derived mimics **(247)** reported by Savage and colleagues.[138,139]

6. Polymyxin Pharmacophore

As reviewed in preceding sections, the current understanding of polymyxin SAR is that both electrostatic and hydrophobic interactions with the lipid A component of LPS are essential for antimicrobial activity. Here, we have conducted pharmacophore modeling based upon the collective two-dimensional polymyxin SAR data in the literature combined with the three-dimensional model of the PMB–LPS complex. A pharmacophore describes the essential, steric, electronic, and function-determining points necessary for an optimal interaction with the pharmacological target.[140] Pharmacophore generation was performed with the Catalyst HypoGen algorithm as implemented in Accelyrs Discovery Studio, version 2.1.[140]

The polymyxin pharmacophore indicates that the positive charge of the $Dab^{1,3,5,8,9}$ side chains represent key features (Figure 8, red location spheres). Hydrophobic properties are key features in the regions of the N^{α} fatty acyl chain and positions 6 and 7 in the cyclic heptapeptide ring (Figure 8, cyan location spheres). Amino acid positions 2–5 and 8–10 display a hydrogen bond donor capacity (Figure 8, purple vector arrows). This also explains the ability of the hydroxyl side chain of Thr to functionally substitute at Dab side chain positions.[74]

The pharmacophore model clearly demonstrates that the polymyxin molecule can be divided into a set of polar and hydrophobic domains, namely, the polar Dab and Thr residue segments (Figure 4), and the hydrophobic N^{α} fatty acyl chain and $_{D}$-Phe^{6}-$_{L}$-Leu^{7} motif. The model further emphasizes the integral scaffolding function of the linear tripeptide and the cyclic heptapeptide for maintaining the optimal distance between each domain, giving the structure its amphilicity, a property that is essential for antimicrobial activity.[29,30]

7. Toxicological Properties of the Polymyxins

The currently available polymyxins (polymyxin E (colistin) and polymyxin B) have the potential to cause toxicity, the most concerning being nephrotoxicity and neurotoxicity which serve to be dose limiting.[9,19] Toxicity was not evaluated in the majority of previous studies of new polymyxin derivatives. NAB7061 **(174)** and NAB739 **(184)** contain three primary amine groups, and their affinities to isolated rat kidney brush-border membrane

were substantially lower (i.e., ~15–20%) than that of polymyxin B.74 This suggests that they might be less nephrotoxic and better tolerated in vivo than polymyxin B. The urinary recovery of both NAB compounds was substantially higher than those of colistin.141 For des-FA-[Trp1]-PMB **(221)** and des-FA-[Dap1]-PMB **(222)**, their acute toxicity, measured by administration into a mouse tail vein, was much lower than polymyxin B (LD_{50} values of 19.0 and 23.5 μM/kg, respectively, versus 4.8 μM/ kg).98 Tsubery et al.[101] examined the acute toxicity of a series of PMBN derivatives by injecting them (15 mg/kg) into the tail veins of mice. Fmoc-PMBN **(94)** was not toxic at this dose, while PMB and Fmoc-PMB decapeptide **(96)** showed very similar toxicity. It was concluded that hydrophobic aromatic substitutions of PMBN may significantly reduce their toxicity.[101] In general, there is very little information on the relationship between structure and toxicity (including nephrotoxicity). Clearly, because of the potential for toxicity to be dose limiting, there is need to consider for polymyxin derivatives the balance between antibacterial activity and toxicity, although as noted above this has not occurred for many derivatives reviewed herein.

8. Conclusions

Naturally occurring polymyxins are cationic antimicrobial peptides with a narrow spectrum of activity mainly against Gram-negative bacteria. Multiple chemical and structural properties of the polymyxin molecule are responsible for LPS binding and antibacterial activity, including (a) the amphipathic and cationic distribution of charges across the primary sequence, (b) the size of the lactam ring, and (c) the N^{α} fatty acyl chain. Interest in polymyxins has increased dramatically in recent times because of the emergence of bacterial strains multiresistant to other clinically available antibiotics. In an attempt to ameliorate potential toxicity and improve the antimicrobial activity, a large number of polymyxin analogues have been generated across several medicinal chemistry programs. Medicinal chemistry strategies for improving the activity profile of polymyxins included targeted modifications of the Dab side chains, modifications to the cyclic peptide ring, and N^{α} fatty acyl chain derivatives. SARs that can be drawn from the antimicrobial activity of analogues synthesized to date suggest that many of the aforementioned modifications generate inactive compounds. The most common modifications reported in the literature involve alterations that negate the positive charges of the Dab side chains, often resulting in a complete loss of antimicrobial activity. Expansion or reduction of the cyclic ring also results in a substantial loss of antibacterial activity. Modifications to the N^{α} fatty acyl chain indicate that the optimal chain length is between seven and nine carbons. Collectively, the SAR data clearly indicate that the unique three-dimensional architecture of polymyxins is required for both LPS binding and antimicrobial activity. In addition to the medicinal chemistry approaches reviewed above, the recent cloning of the PMB biosynthetic gene cluster has opened up the prospect of generating polymyxin analogues by a recombinant approach to re-engineer the biosynthetic enzymes by module swapping.[142]

In future it is hoped that the collective knowledge from the SAR inferred from reported polymyxin analogues will be utilized for the design of synthetic lead compounds that display significantly greater antimicrobial activity against multidrug-resistant Gram-negative bacteria.

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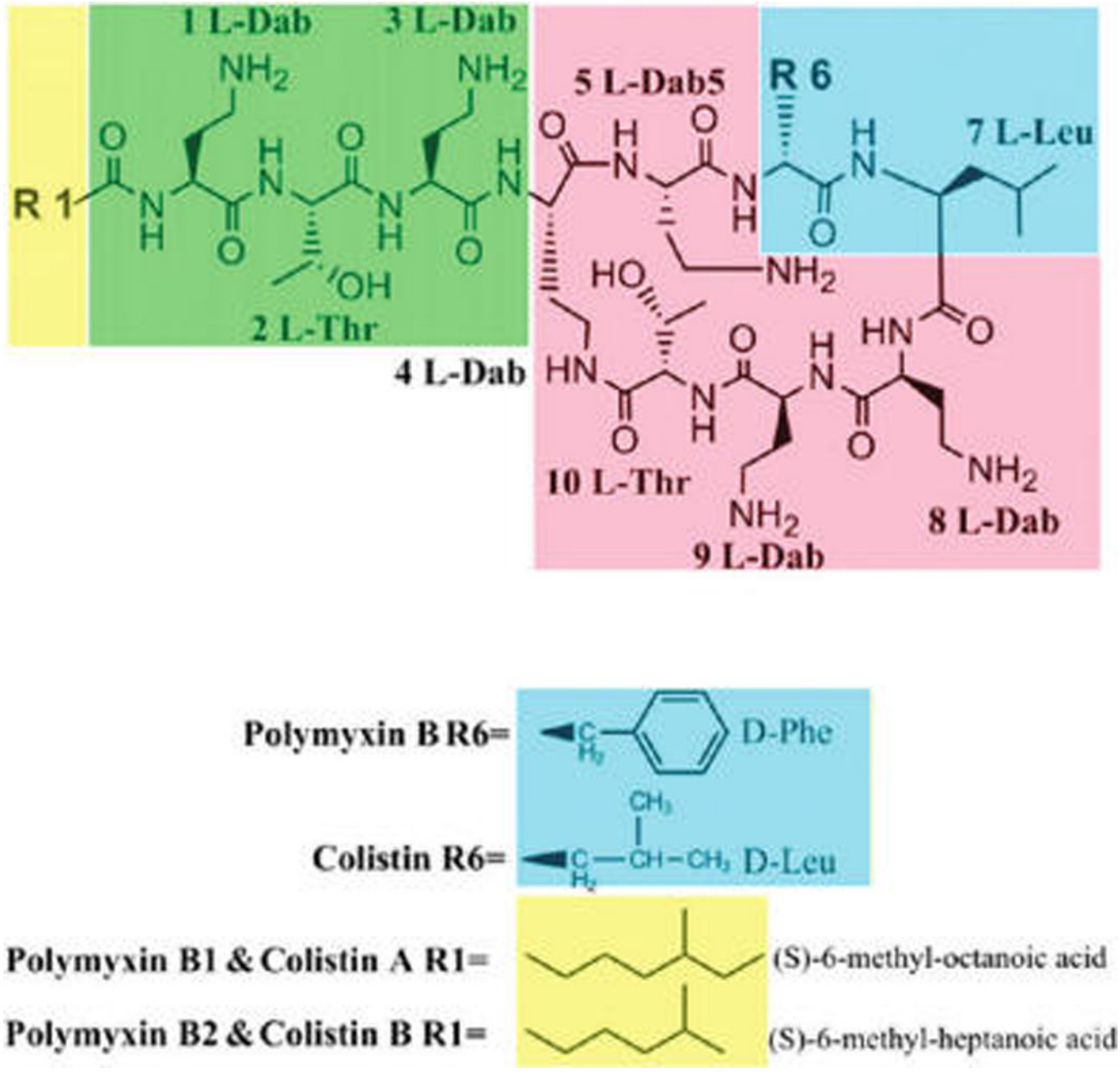

Figure 1.

Chemical structures of polymyxin B and colistin. The functional segments of polymyxins are colored as follows: yellow, N^{a} fatty acyl chain; green, linear tripeptide segment; red, the polar residues of the heptapeptide; blue, the hydrophobic motif within the heptapeptide ring. The amino acids positions are numbered in accordance to references in the text.

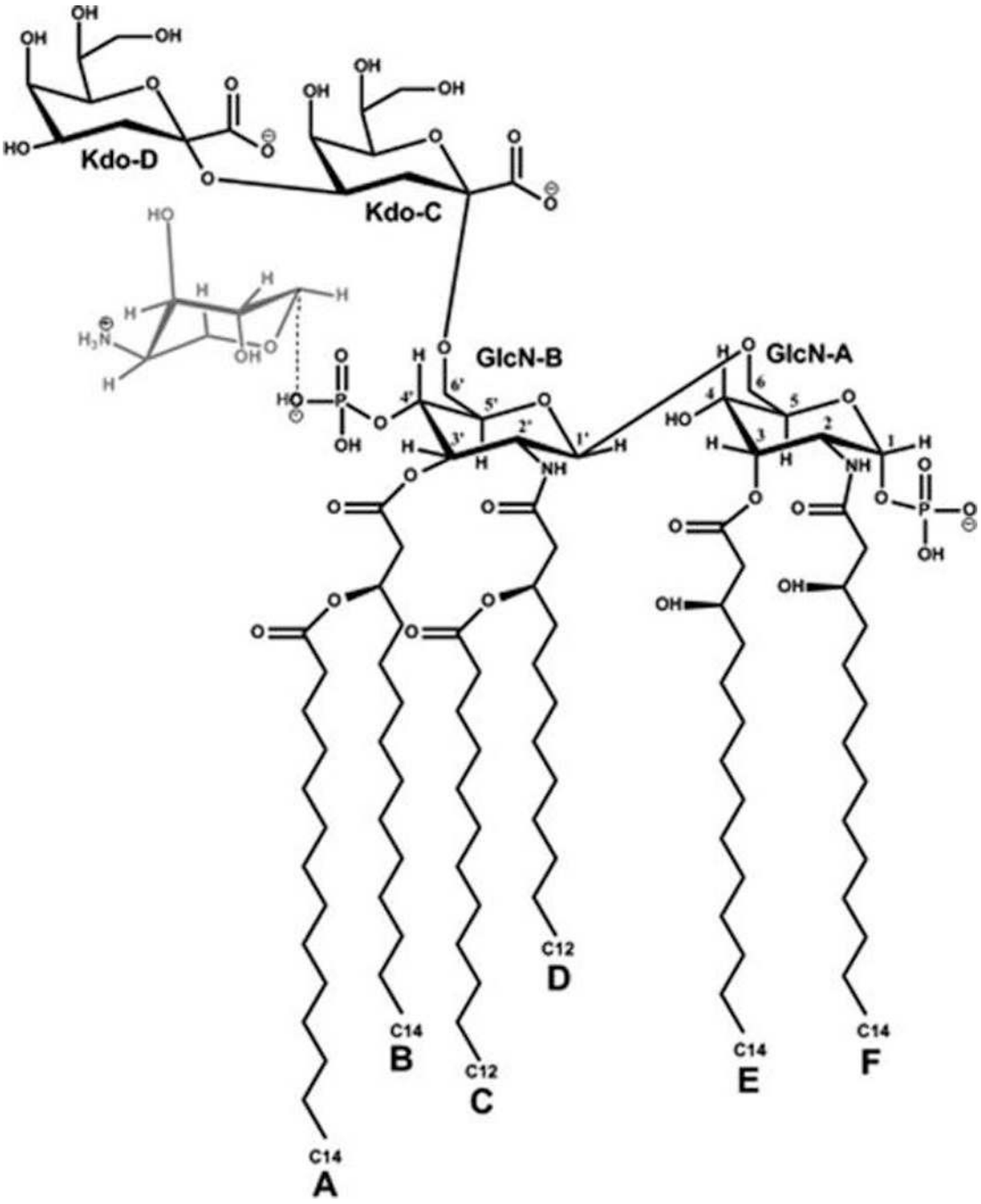

Figure 2.
Chemical structure of *E. coli* lipid A. The amino-4-deoxy-L-arabinose modification of the phosphate groups observed in polymyxin-resistant strains is shown in gray.

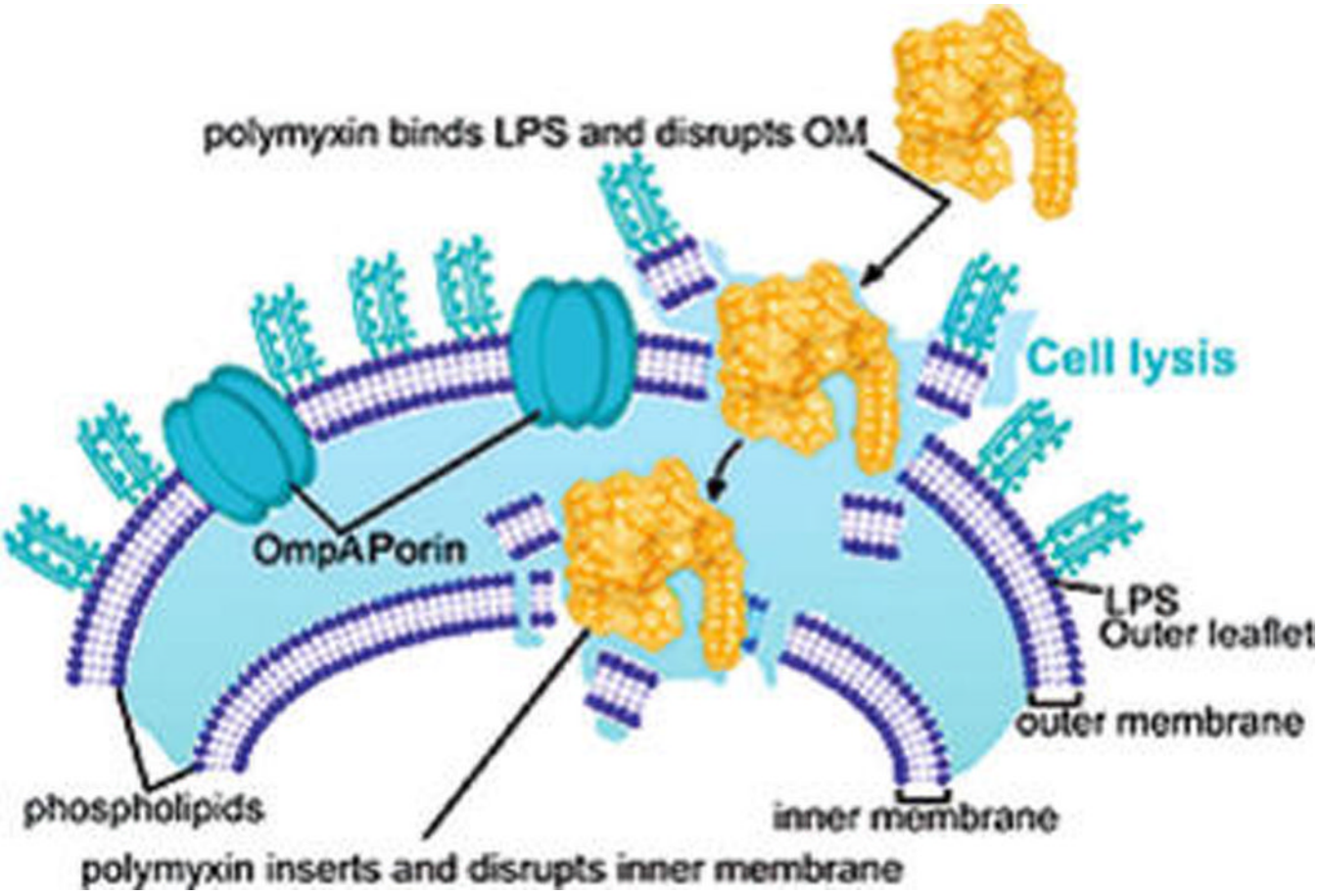

Figure 3.
Diagram depicting putative antimicrobial action of PMB on Gram-negative bacterial outer membrane.

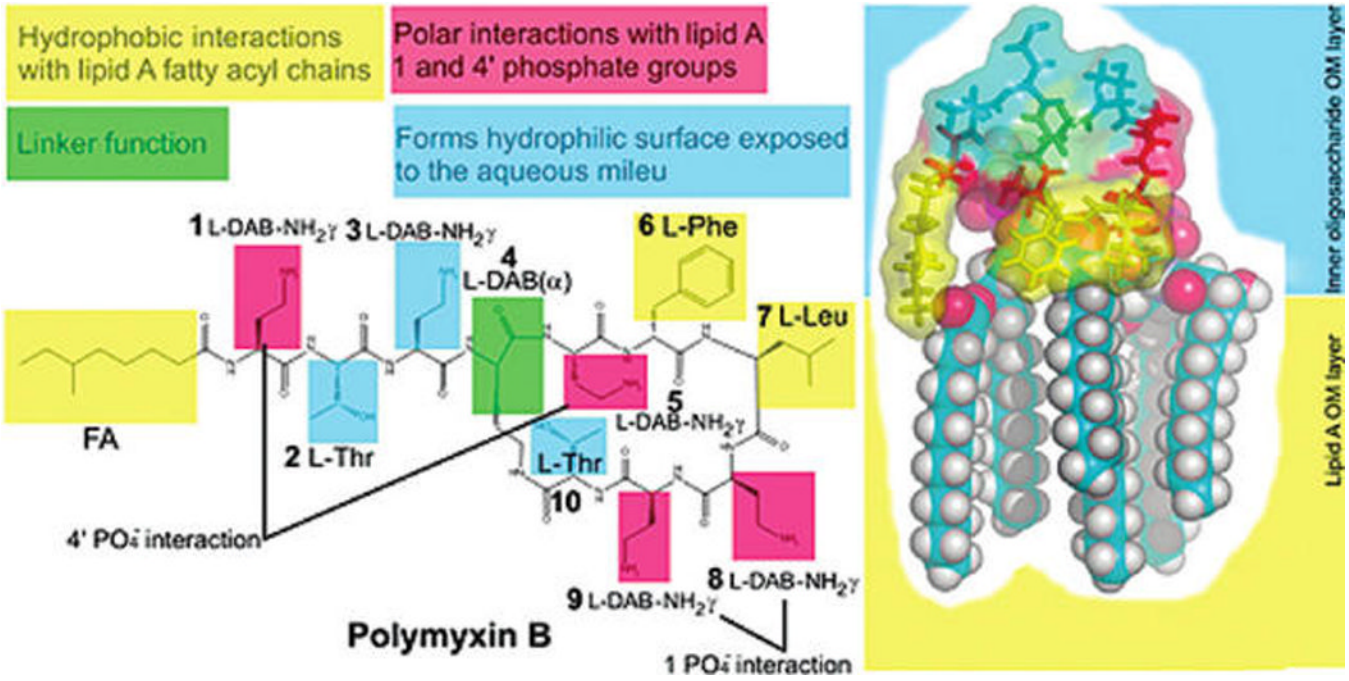

Figure 4.

A color coded schematic diagram summarizing the key contacts involved in complex formation between PMB and the lipid A component of LPS. FA = *N*-terminal fatty acyl chain.

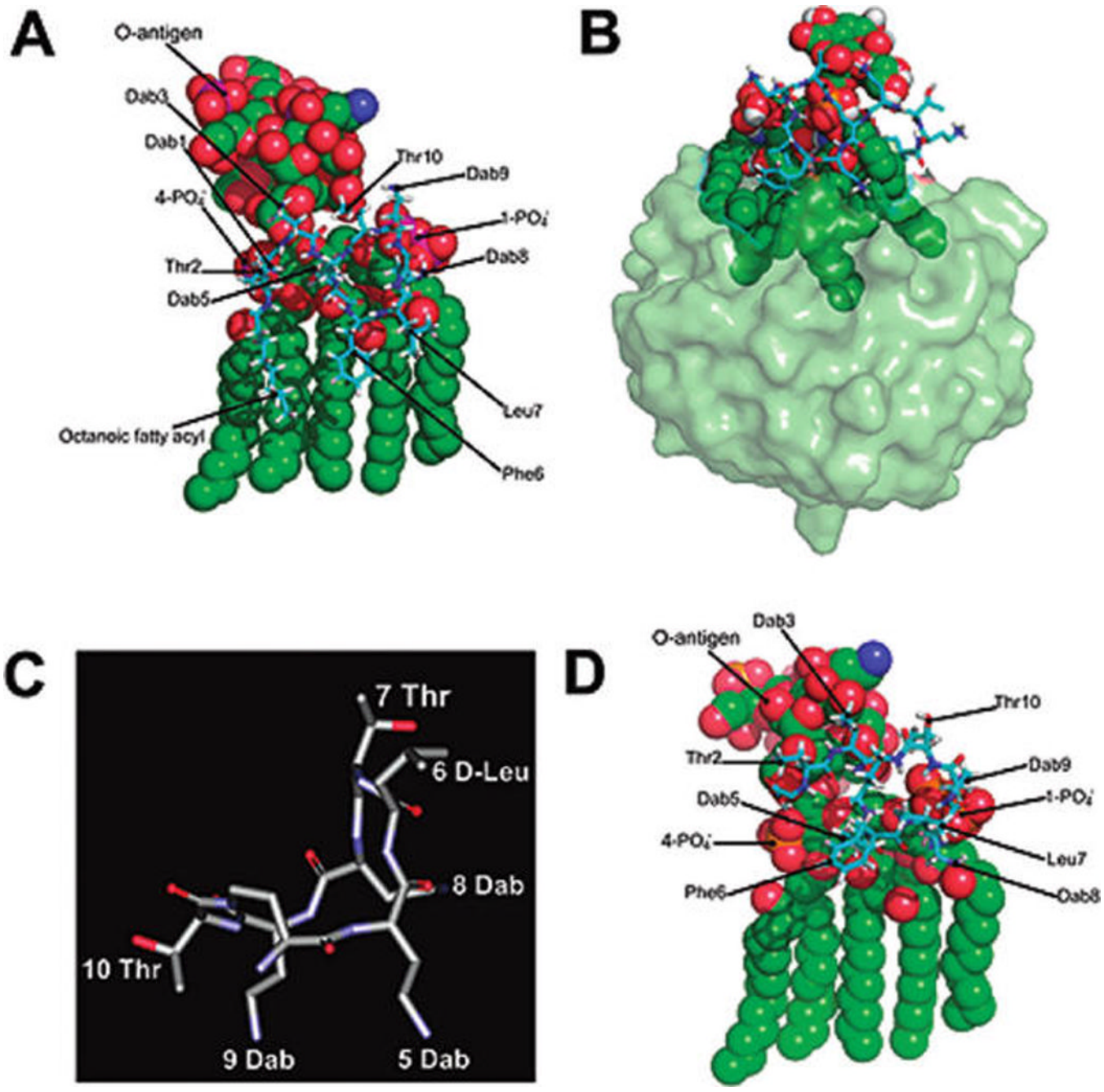

Figure 5.

Molecular models of polymyxins in complex with LPS derived from NMR restraints. The LPS molecule is shown in space filling representation, and polymyxins are shown in stick representation: (A) molecular model of the complex between *E. coli* LPS with PMB;[29,30] (B) molecular model of the complex between *E. coli* LPS with PMB in a dodecylphosphocholine micelle shown in surface representation;59 (C) backbone conformation of polymyxin M;[60] (D) molecular model of the complex between *E. coli* LPS with PMBN.[61]

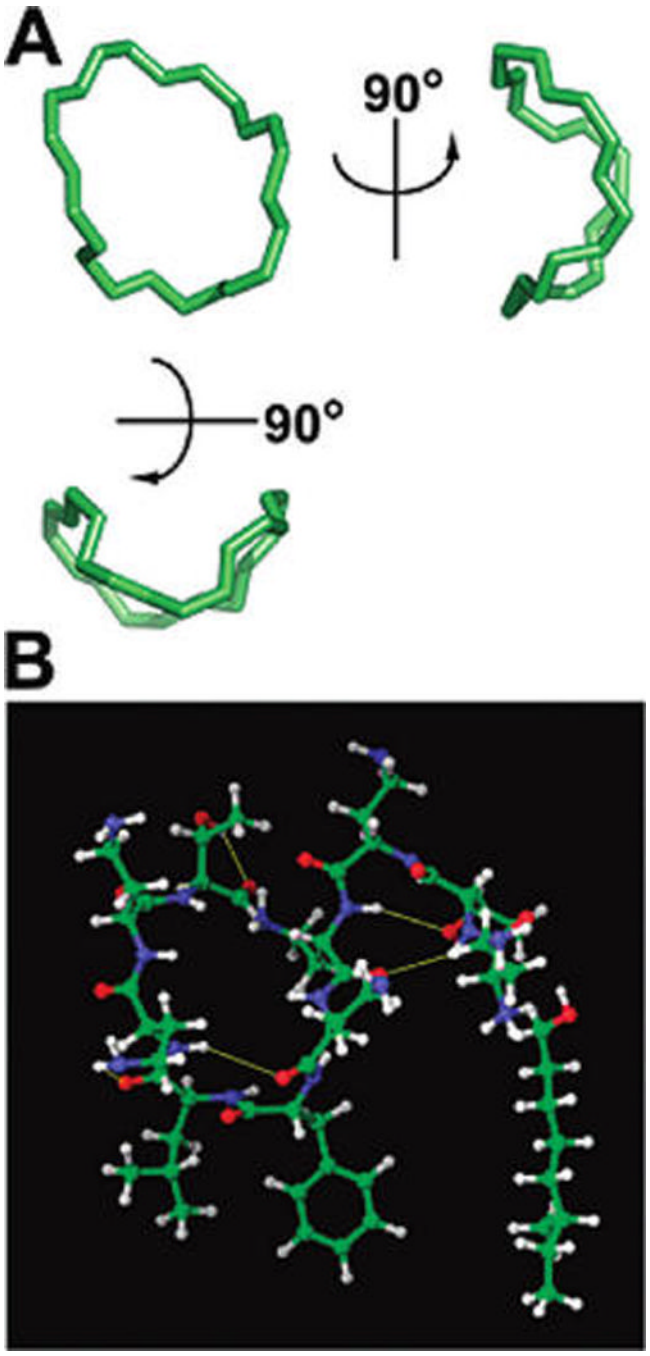

Figure 6.

(A) Conformation of the cyclic heptapeptide backbone of PMB when bound to LPS.[29,30] The structure is shown in two different views from the top left orientation by 90° rotation about the *x*- and *y*-axis. (B) NMR structure of PMB when bound to LPS showing predicted intramolecular hydrogen bonds.

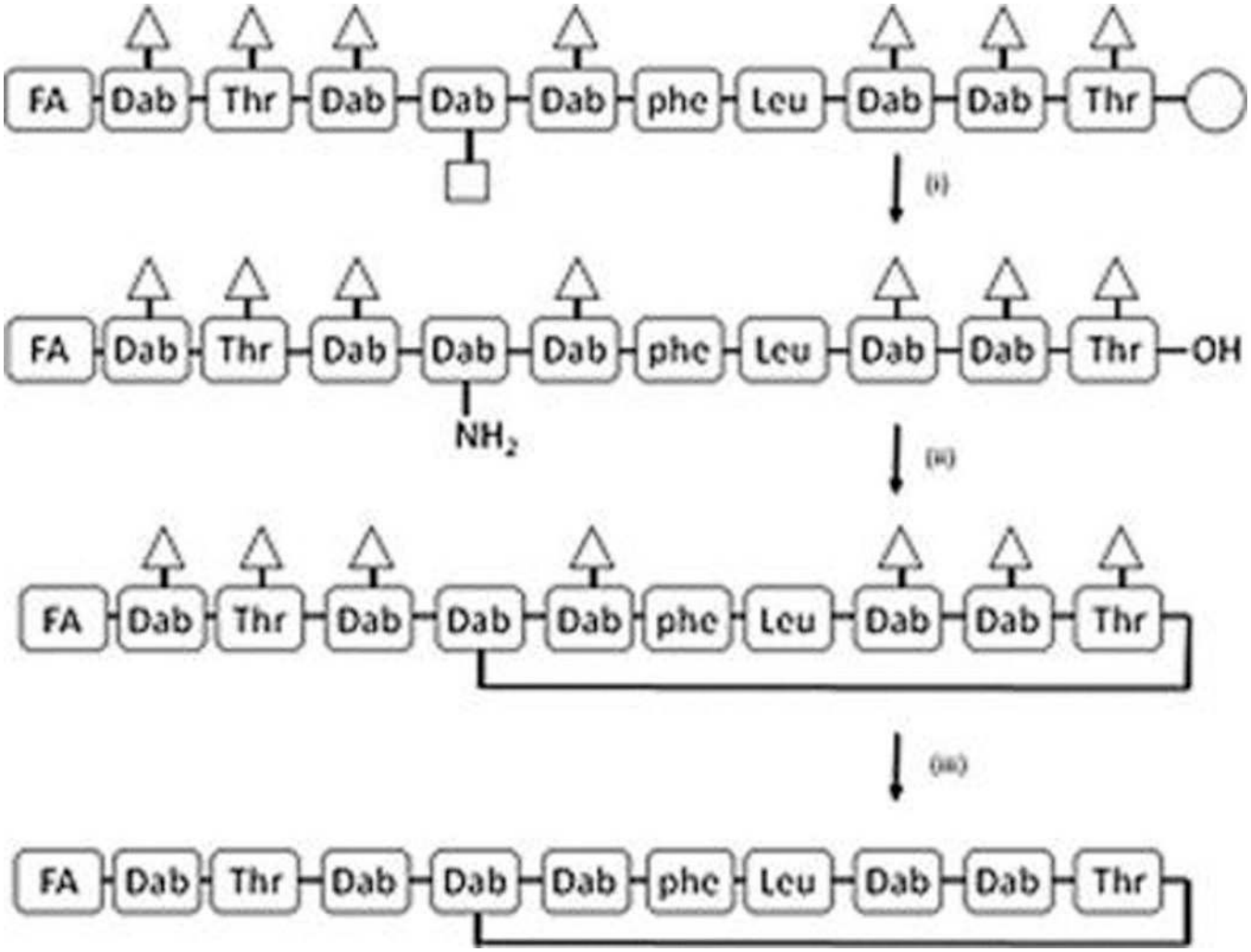

Figure 7.

Generalized schemes for peptide syntheses of polymyxin peptides. From Sharma et al.:[70] ○ = Sasrin resin, □ = Dde protecting group, Δ = Boc/^{t}Bu protection, FA = fatty acid; (i) hydrazine and then 1% TFA; (ii) DPPA; (iii) 95:5 TFA/H_2O. From Tsuberry et al.:[72] ○ = 2-ClTrt resin, □ = Mtt protecting group, Δ = Boc/^{t}Bu protection, FA = fatty acid; (i) 1% TFA; (ii) PyBOP; (iii) 95:5 TFA/H_2O (PMBN only). From Sakura et al.73 and Vaara et al.:74 ○ = Wang resin, □ = Boc protecting group, Δ = Bz/Cbz protection, FA = fatty acid; (i) 95% TFA; (ii) PyBOP or DPPA; (iii) HF or hydrogenation.

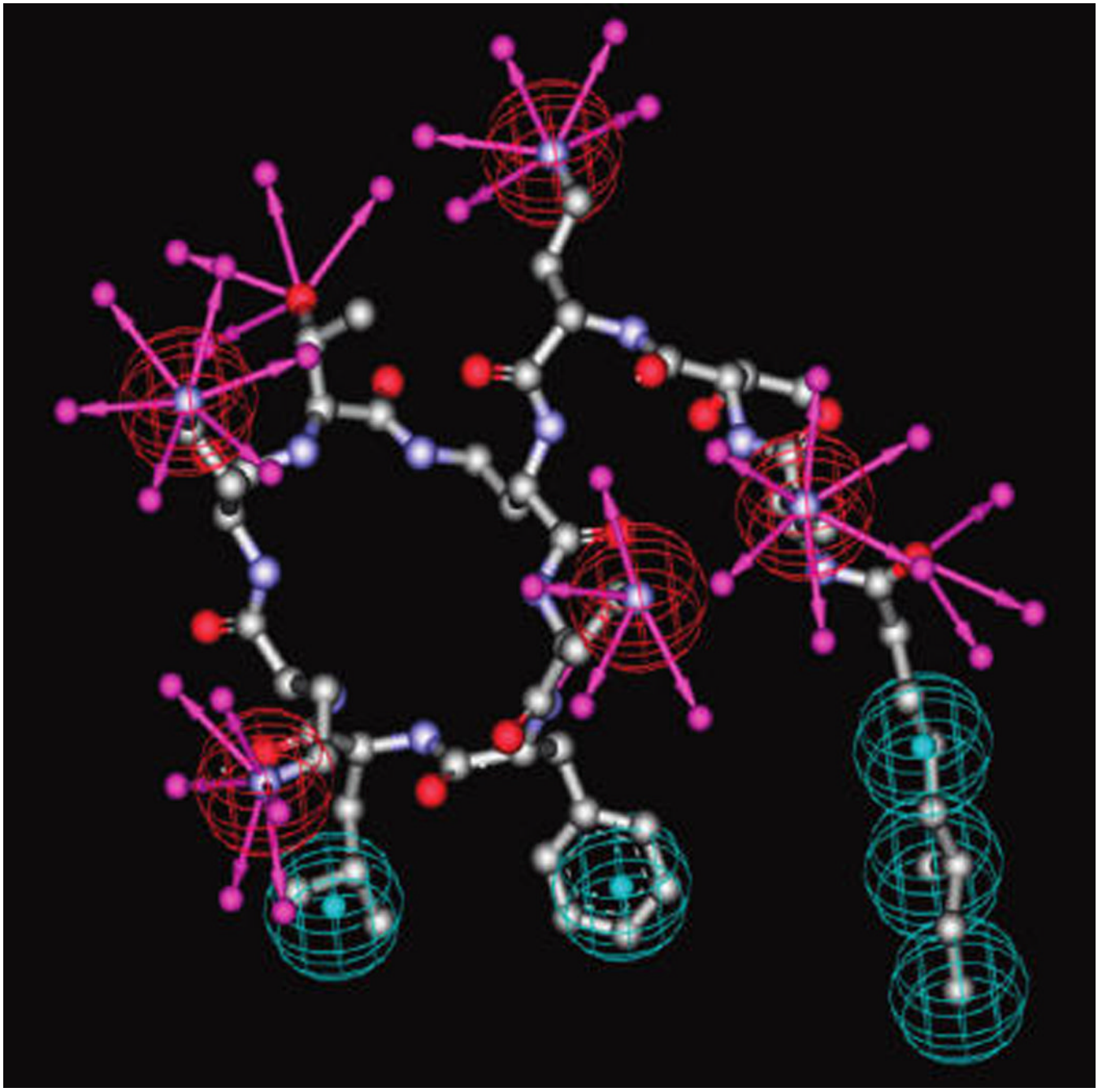

Figure 8.
Polymyxin B pharmacophore model. Red location spheres indicate positive charge property. Hydrophobic property is represented by cyan location spheres. Hydrogen bond donor vectors are shown in purple. The polymyxin backbone is shown in ball and stick representation.