New cationic amphiphilic compounds as potential antibacterial agents

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1 | Gram-negative Bacterial Sepsis

With the discovery in the 1930s of natural and synthetic compounds that were able to kill pathogenic bacteria, man appeared to leave their natural ancient enemies behind. Thanks to these antibiotics, mortality rates resulting from common diseases indeed steeply declined. However, bacterial resistance grew against the early classes of antibiotics through a combination of careless application and high rates of mutation.[1] Nowadays, with increasing bacterial resistance to conventional antibiotics being an accepted problem, the on-going search for new antibiotics is an important subject worldwide[2] as witnessed by the countless reports on modification of existing antibiotics[3] and the search for antibiotics with new modes of action.[4,5]

Bacterial infections can in principle be cured by removal of the causative agent. In most cases, treatment with the correct antibiotic or a balanced cocktail of drugs will result in countering of the pathogen. In some cases however, *e.g.* if bacterial infection has turned into bacterial infestation (sepsis, or blood poisoning), or if the patient is already immuno-compromised, antibiotics can no longer be of effective assistance to the immune systems in their protective task. Moreover, treatment of Gram-negative (G^-) bacterial infections with established antibiotics might cause aggravation of a patient's condition rather than improving it by release of immunogenic membrane components.[6] If septic patients are not treated carefully, their condition can result in septic shock, an inflammatory syndrome resulting from loss of the homeostasis maintained by the body. Although there is no general definition of this syndrome, microvascular occlusion and vascular instability lead *via* effects of fever, coagulopathy, vasodilatation and capillary leak to multiple organ failure and, eventually, death.[7] The recent estimation of 750,000 annual cases of septic shock in IC (intensive care) units in the USA accompanied by mortality rates of ~30-50%[8] shows that bacterial sepsis and septic shock remain conditions that are difficult to treat.

This introduction presents a global overview of the present day status of established antibiotics and research approaches towards new classes of antibacterial compounds. Focusing on approaches to treat G^- bacterial infections, a biological background of G^- bacterial infections is given, and the potential of the class of cationic antimicrobial peptides (CAPs) will be discussed in greater detail.

2 | Endotoxin and Sepsis

The toxicity of the group of molecules referred to as 'toxins' arises from disruption of cellular processes *e.g.* by binding nucleic acids, inhibiting enzymes or by having modulating effects on the immune response. *Exotoxins* are substances that are secreted by bacteria including anthrax toxic complex, diphtheria toxin, tetanus toxin, botulinum toxin, cholera toxin and heat-labile enterotoxin.[9,10,11] In contrast, *endotoxins* are not secreted but are antigens of a specific bacterium, mostly as integrated part of the membrane.[12]

2.1 | Lipopolysaccharide (LPS)

In G^- bacteria, the term *endotoxin* refers to a unique membrane-associated molecular structure, which is collectively called lipopolysaccharide (LPS). LPS alone can induce all of the characteristic features of septic shock in humans.[13]

Differing from Gram-positive (G^+) bacteria, in which the cell's contents are protected by a single cytoplasmic membrane and a peptidoglycan layer, G^- species contain an extra membrane outside of their peptidoglycan. This characteristic outer membrane consists of phospholipid bilayer, of which the outside possesses an overall anionic character (see Figure 1). The abundant, negatively charged LPS is equally distributed over the outer membrane, with Mg^{2+} ions coordinating to the phosphate groups that connect the LPS moieties near their hydrophobic anchors.[14] LPS contains a few typical segments (Figure 1). The *O*-antigen substructure of LPS, pointing outwards into the extracellular space, is a repeating branched polysaccharide mostly composed of glucose (Glc) and galactose (Gal) units. In this region, the largest structural variation among G^- species is found. Approaching the membrane, the core oligosaccharide structure of LPS is divided into two parts. The outer cores consists mainly of Gal, Glc and occasionally, heptose residues. The inner core typically contains residues of unusual 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid (Kdo) and L-*glycero*-D-*manno*-heptose (Hep). Carbohydrate variations in the core contribute to the general complex heterogeneity of LPS from a single species and presence or absence of modifications is profoundly dependent on the growh conditions of the bacterium.

The base structure of the inner core is decorated with additional carbohydrate residues in non-stoichometric fashion, and with phosphate, pyrophosphorylethanolamine (PPEtN) or phosphorylcholine to a varying degree.

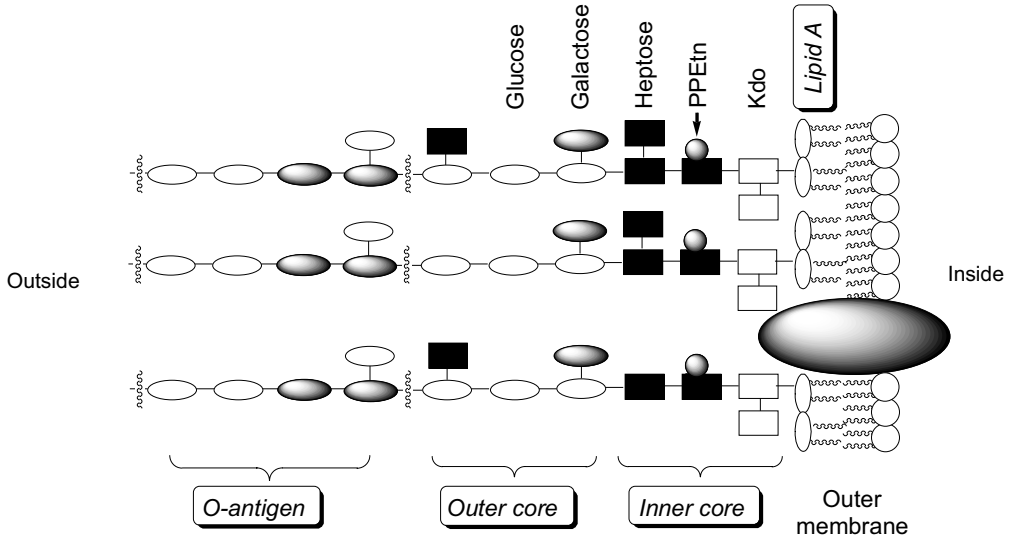


FIGURE 1 | Schematic representation of the structure of *Escherichia coli* K12 LPS, consisting of the *O*-antigen, outer and inner cores, and Lipid A. The oval transmembrane structure represents an outer membrane protein. The overall negative charge is caused by phosphate groups in the inner core and Lipid A.

2.2 | Lipid A

Lipid A (Figure 2) is the most conserved substructure of LPS in G^- bacteria and anchors the core structure of LPS to the membrane. Lipid A, the actual part of LPS responsible for its toxic effects, consists of a glucosamine dimer that is *O*-phosphorylated at the 1 (α) and 4′ positions; the inner core extends from the 6′ primary hydroxyl function connecting to the first Kdo moiety. Lipid A is polyacylated with β-hydroxyalkanoyl chains, providing hydrophobic anchors. Variations in the Lipid A structure from Figure 2 (*e.g.* acyl chain composition, lack of phosphates, different saccharides) can be found in *Rhizobium*, *Aquiflex, Rhodobacter, Campylobacter, Helicobacter* and *Yersinia* species.[15,19b] Different acyl substitution patterns yield overall different shapes, which are at the basis of different signalling pathways (see § 3.1) and toxic effects of LPSs.[16] Synthetic Lipid A analogues lacking a disaccharide motif display potent Lipid A-like activity, assuming a major role for the phosphate and lipid parts in activity;[17] however, 1-*O*-dephosphoryl Lipid A has been reported to be devoid of toxicity.[18] The structure, biosynthesis and diversification of Lipid A/LPS and their separate components have been the subject of a number of reviews.[19]

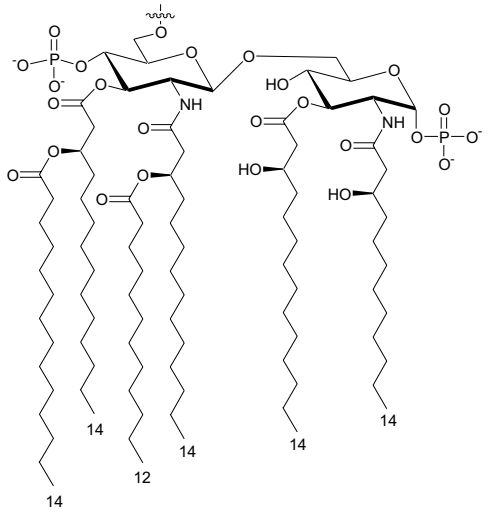


FIGURE 2 | Structure of Lipid A from *E. coli* K12. Numbers denote the number of carbon atoms in each chain.

2.3 | Biological effects of LPS

At the onset of G^- bacterial infection, LPS is bound by LPS-binding protein (LBP), facilitating complex formation with the CD14 receptor. This way, the endotoxin is recognized as pathogen-associated molecular pattern (PAMP)[20] by Toll-like receptor (TLR) 4[21] present on macrophages, neutrophils, monocytes, dendritic cells and endothelial cells in mammals.[22] Atypical (modified) LPSs were found to interact with TLR2 rather than TLR4.[23] TLRs 2 and 4 are two of the 11 human TLRs known to date that are capable of identifying highly conserved PAMPs and mediate the correct immune response upon activation.[24] Originally thought to involve one single TLR per PAMP, it is becoming evident that TLRs might collaborate with each other and with other innate immune receptors for recognition of a specific pathogen, leading to cumulative effects for a response towards this pathogen.[25]

Interaction of LPS with TLR4 triggers the biosynthesis of various immune inflammatory mediators, most notably tumor necrosis factor α (TNF-α),[26] interleukin 1β (IL-1β),[27] IL-6,[28] and IL-8.[29] Besides this, the production of co-stimulatory compounds that are required for the adaptive immune response, is activated.[30] Furthermore, LPS causes upregulation of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin[31] that are involved in recruitment of leukocytes towards inflamed endothelium.[32] The human body normally carefully controls the systemic concentrations of the mediators that regulate the immune response. However, if systemic

concentrations reach too high levels, the homeostasis maintained by the body is disturbed, resulting in septic shock.

3 | Countering Infections and Sepsis

3.1 | Classical antibiotic treatment

Classical treatment of infections involves the administration of an appropriate antibiotic. A number of classes of antibiotics are currently in clinical use, including tetracyclines,[33] quinolones,[34] β-lactams,[35] macrolides,[36] aminoglycosides,[37] azoles,[38] oxazolidinones,[39] peptide antibiotics,[40] glycopeptides,[3c] nitroimidazoles,[41] sulfonamides,[42] and ansamycins (Figure 3).[43] Figure 3 also displays fosfomycin,[44] D-cycloserine,[45] trimethoprim[42] and mupirocin,[46] compounds that are the only member in their classes.

Unfortunately, bacteria have adapted to evade antibacterial action by target site residue modification, active efflux, overexpression of degrading proteins or decreased uptake.[49] Serious resistance is encountered in the infamous methicillin-resistant *Staphylococcus aureus* (MRSA).[47] As even the glycopeptide antibiotic vancomycin, an antibiotic of last resort, succumbs to resistance (*Enterococci*),[48] new antibiotics that act through alternative mechanisms are needed. Resistance of potentially pathogenic G^- bacterial serotypes of *Escherichia coli* (commonly involved in urinary and gastrointestinal tract infections) or *Pseudomonas aeruginosa* (infections involving burns and hospital-acquired pneumonia) is a serious matter,[49] especially when considering that these pathogens are less susceptible to conventional antibiotics due to their extra outer membrane.

In the past decades, mostly variations *within* classes (*i.e.* modification of an established scaffold) of antibiotics have been reported,[50] and only a small number of members of completely *new* classes have been approved by the FDA in the past decades. Two of the few are the oxazolidinone linezolid (Zyvox™) and the lipopeptide daptomycin (Cubicin™, Figure 3),[51] and these are indicated against G^+ bacteria only.[52]

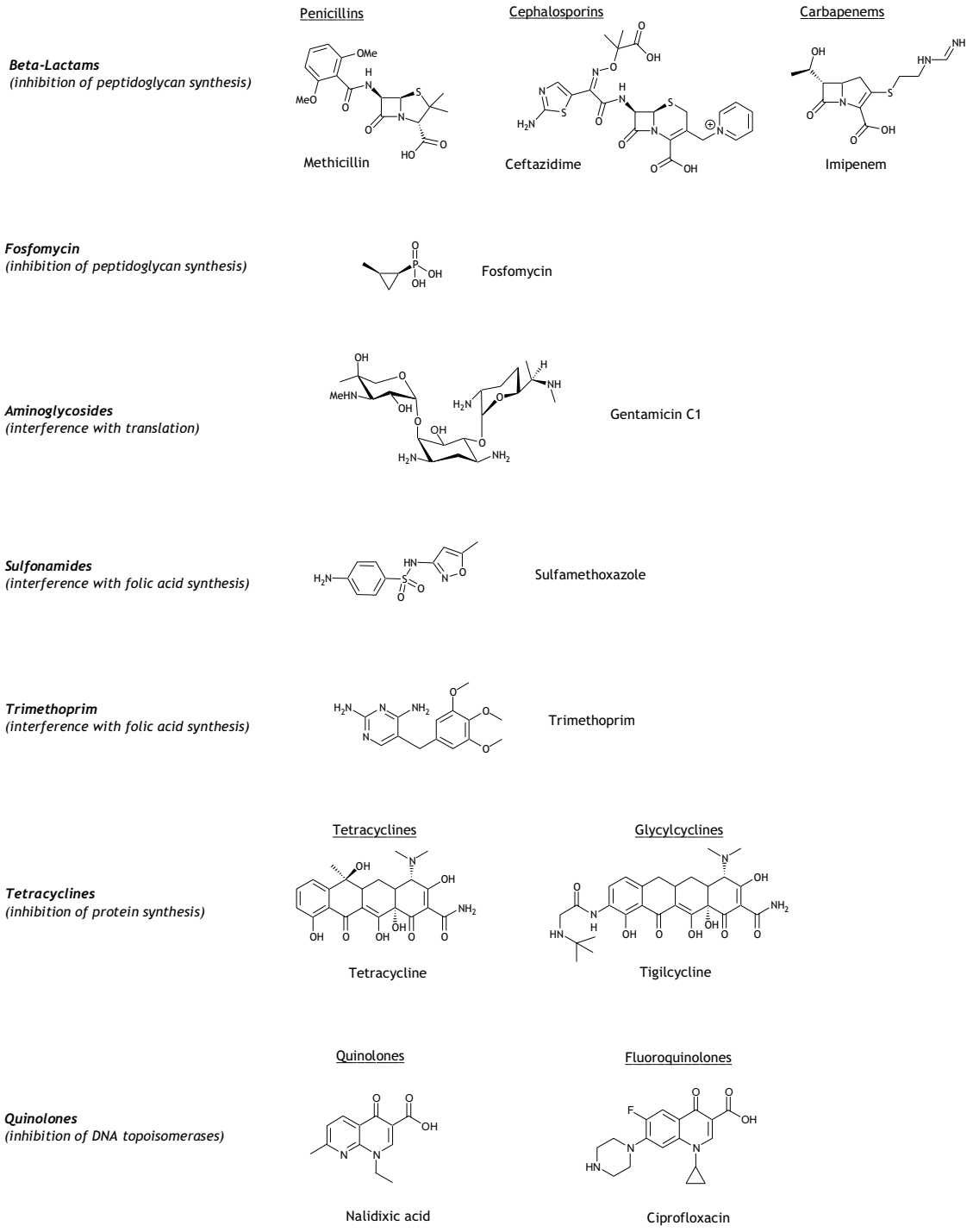


FIGURE 3 | Structures of representative examples of commonly used classes of antibiotics.

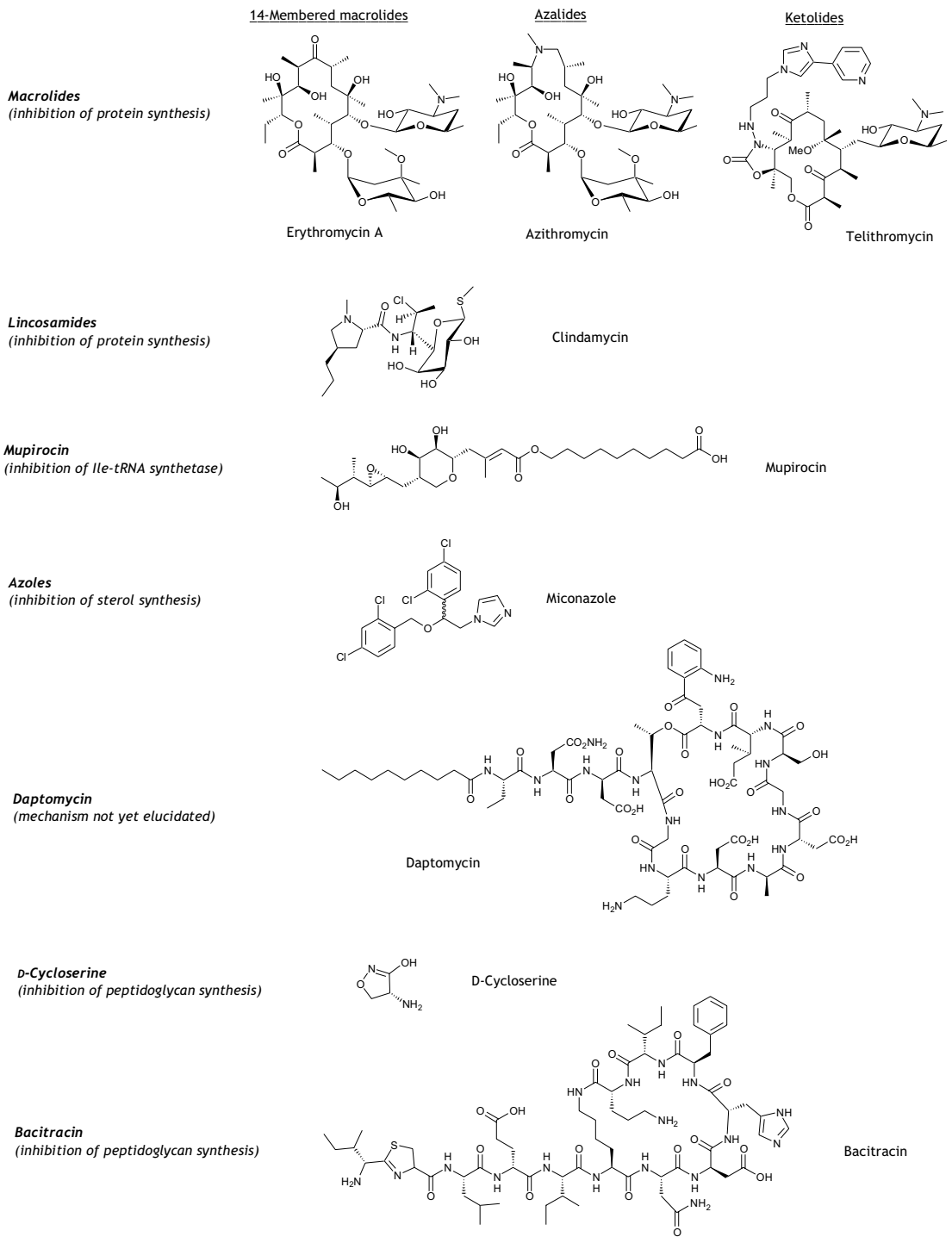
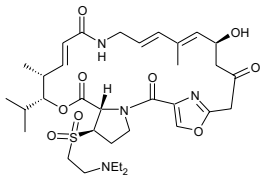
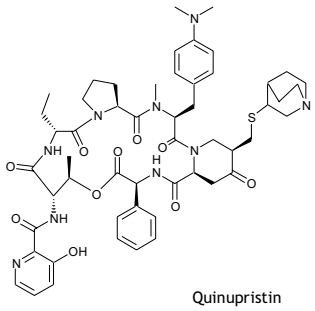


FIGURE 3 (continued) | Structures of representative examples of commonly used classes of antibiotics.

Streptogramins
(inhibition of protein synthesis)

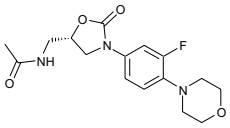


Dalfopristin



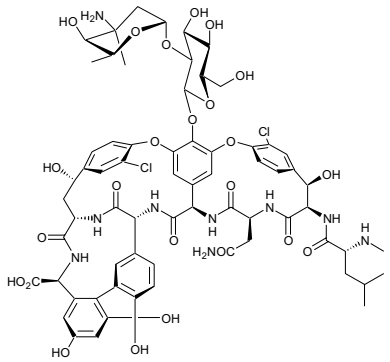
Quinupristin

Oxazolidinones
(inhibition of protein synthesis)



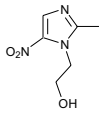
Linezolid

Glycopeptides
(inhibition of peptidoglycan synthesis)



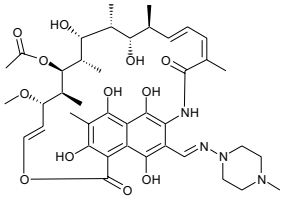
Vancomycin

Nitroimidazoles
(activity through DNA damage)



Metronidazole

Ansamycins
(inhibition of RNA-polymerase)



Rifampin

FIGURE 3 (continued) | Structures of representative examples of commonly used classes of antibiotics.

3.2 | Approaches towards new antibiotics

Research towards new antibiotics acting through other mechanisms than the established arsenal for the treatment of G^- infections has yielded some examples with potential for further investigation. The following examples are illustrative.[53]

The fact that LPS is essential for bacterial growth prompted investigation towards inhibitors of enzymes involved in the biosynthesis of LPS. An inhibitor of the unique enzyme CMP-Kdo synthetase in the Kdo synthesis pathway, 2,8-dideoxy-8-amino-Kdo, showed bacterial growth inhibition in the low μg/mL range. The Ala-Ala conjugate of this compound (Figure 4) was prepared to enhance cellular uptake,[54] but this compound was not therapeutically useful as the dipeptide was hydrolyzed too rapidly.[55] Inhibitors of the enzyme Kdo8P synthetase that catalyzes the condensation of phosphoenolpyruvate with D-arabinose-5-phosphate *en route* to Kdo have been reported (Figure 4).[56] The conserved L-*glycero*-D-*manno*-heptose (Hep) is attached to Kdo, and is not found in mammalian cells. The recent elucidation of the structure of ADP-6-epimerase,[57] an enzyme in the biosynthetic pathway of Hep may inspire the design and synthesis of new antibacterial compounds.

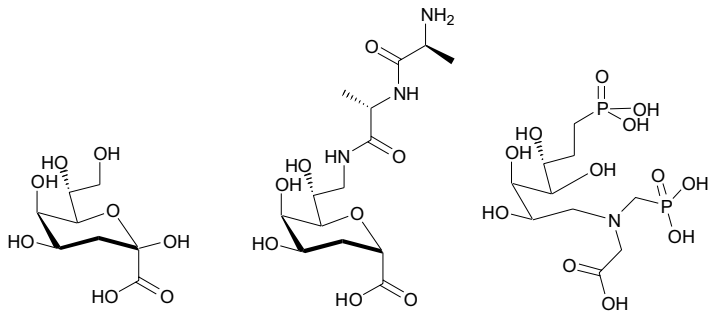


FIGURE 4 | Kdo analogues as inhibitors of the LPS biosynthesis pathway.

Another approach in targeting the biosynthesis of LPS is inhibition of the enzyme LpxC.[58] This enzyme catalyzes the deacetylation of UDP-3-*O*-acyl-GlcNAc, a key step in the synthesis of Lipid A. Indeed, inhibitors are reported based on a hydroxamic acid functionality (*e.g.* L-161,240 and BB-78484, Figure 5).[59]

Removal of the 1-*O*-phosphate from Lipid A is an interesting objective to neutralize G^- bacteria *in situ* as monophosphoryl Lipid A is non-toxic (§ 2). Alkaline phosphatase (AP) from human placenta[60] or calf intestine[61] has proven to be effective in this respect as it improved survival in challenged mice. A possible drawback to this approach is the problem of antigenicity:

treatment with recombinant AP might provoke undesired immunological responses upon application of AP at the next occasion of infection.

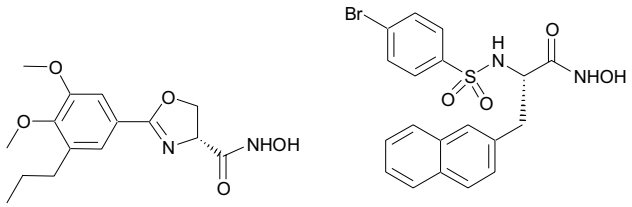


FIGURE 5 | Inhibitors of LpxC, a deacetylase in the LPS biosynthesis pathway.

During bacterial infection, lymphocytes suffer from faster inactivation through apoptosis than in a normal health situation. As this impairs host defenses, preventing the death of these cells might increase the survival of challenged mice. Indeed, mice were successfully treated with the known caspase inhibitor Z-VAD (Figure 6) that inhibits caspase-regulated apoptosis.[62]

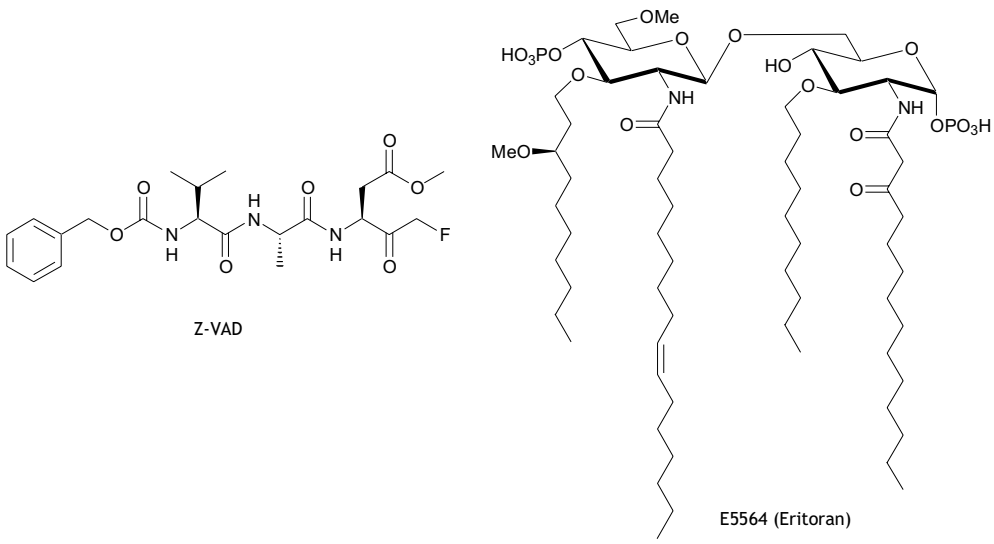


FIGURE 6 | Structure of the caspase inhibitor Z-VAD and E5564, a compound displaying LPS antagonism.

Although a number of the above mentioned research objectives might seem promising, no actual drug has yet arisen from any of these approaches. More progress has been made in the structural

derivatization of Lipid A. This approach has led to the development of the *in vivo* active LPS antagonists E5531 and E5564 (eritoran, Figure 6),[63] the latter showing good results in phase I clinical trials. The structure of eritoran is based on the unusual Lipid A structure of the non-toxic bacterium *Rhodobacter capsulatus* and blocks interaction of LPS with TLR4.[64]

4 | Cationic Antimicrobial Peptides (CAPs)

4.1 | Natural CAPs

Bacteria are an important source of peptide-based antibiotics. In 1947, one of the first peptides that were isolated was polymyxin B, a cyclic, cationic lipopeptide from *Bacillus polymyxa*.[65] From this point on, more bacterial cationic antimicrobial peptides (CAPs) were discovered, all based on peptide structures containing uncommon amino acids. In the 1980s, cecropins[66] and magainins[67] were among the first to be identified in multicellular organisms. Isolated from pig and frog respectively, these CAPs were found to be linear and constructed from proteogenic amino acid residues unlike the bacterial CAPs previously identified. Both cecropins and magainins are specificially active against bacterial cells, in contrast to melittin, the main lytic cationic peptide in bee venom.[68] To date, hundreds of peptides with antibacterial, antifungal, antiviral and/or antiprotozoal activity have been extracted from various organisms, including other mammals[69,70] and amphibians,[71] insects,[72] birds,[73] fish,[74] and shellfish[75] (see Table 2, page 24). The wide-spread presence of CAPs indicates that these peptides may constitute an ancient antibiotic approach. Indeed, one group of antibacterial peptides was determined to stem from a common ancestral precursor around 150 million years old,[76] surviving evolutionary selection.

The human innate immune system also deploys antimicrobial peptides,[77,78,79,80] most notably the CAP subgroup of defensins,[81] divided in two major classes – the α- and β-defensins (see Table 1).

TABLE 1 | Defensins of the innate immune system.

	kDa	Residues	Cys Pairings	Source
α-defensins	3.5-4.5	29-35	1-6, 2-4, 3-5	Human, rabbit, rat, guinea pig, mouse
ß-defensins	4-6	36-42	1-5, 2-4, 3-6	Human, cow, turkey, chicken, pig, penguin
θ-defensins	2	18	1-4, 2-5, 3-6	rhesus monkey

The 6 known human α-defensins (human neutrophil peptides HNP 1-4 and human defensins HD 5 and 6), are found primarily in neutrophils (HNPs) and intestines (HDs). The human β-defensins (hBD 1-6) are larger and characterized by a different pattern of disulfide bridges (see Table 2 and Figure 7A); they are mainly isolated from epithelia. Members of the α- and β-defensin classes are also encountered in other species. The rhesus monkey θ-defensins are the only cyclic defensins isolated to date.

Besides discrete peptides, naturally occurring (cationic) proteolytic fragments of several proteins were found to exhibit antibacterial activity; *e.g.* from lysozyme,[82] from histone 2A (yielding buforins I and II),[83] and from the N-terminal domain of the *Helicobacter pylori* L1 protein.[84] An α-helical domain in lactoferrin yields lactoferricin,[85] and cathelicidins stem from cathelins.[86] New CAPs are furthermore discovered through screening of protein or DNA sequences for putative amphiphilic stretches, as in the cases of tritrpticin[87] and lactoferrampin.[88]

CAPs come in numerous variations in length, charge, and primary/secondary structures (see Figure 7), but all are amphiphilic.[89] Parameters as hydrophobicity, amphiphilicity, polar angle, charge and conformation govern the activity of a CAP but no general rule exists for predicting activity.

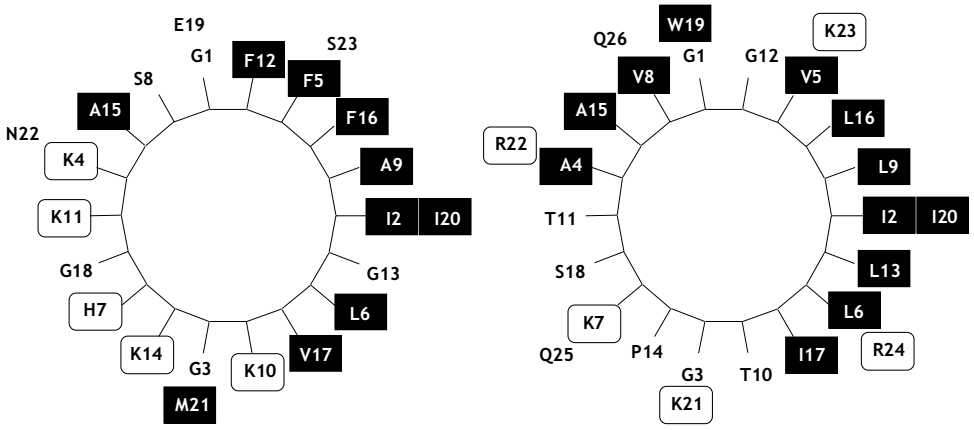
FIGURE 7 | 3D structures based on NMR models showing the diversity of CAPs, in solution (**A**) or in membrane mimetic conditions (**B-D**).[202b] **A.** human ß-defensin 2 (hBD-2), a triple-stranded ß-sheet with 3 Cys-Cys bridges; **B.** magainin 2, α-helix; **C.** ß-turn/loop structure of bovine bactenecin; **D.** Extended structure of indolicidin. Only backbones and SS bridges are shown.

4.2 | Classification of CAPs

Natural CAPs are peptides ranging from ~10 to ~100 amino acids, have an overall net positive charge and are amphiphilic. Some CAPs are classified according to their origin (*e.g.* bacteriocins from bacteria, cathelicidins from cathelins). Reference, however, to their primary/secondary structure, which is fixed or adopted upon interaction with membranes, is more common.[90,91] The following paragraphs discuss the different classes of CAPs.

4.2.1 α-Helical CAPs

Representative members of this class are magainin 2[67] and melittin,[68] both of which adopt an α-helical structure with facial amphiphilicity (see Table 2, Figures 7B and 8) upon interaction with negatively charged membranes. Compared to melittin however, magainin 2 displays far less hemolysis. Although no fundamental rule is available on how residues in the amphiphilic helix influence activity and selectivity, substitution of amino acids on one side of the helix can greatly influence the biological properties.



Magainin 2

GIGKF LHSAK KFGKA FVGEI MNS

Melittin

GIGAV LKVLT TGLPA LISWI KRKRQ Qa

FIGURE 8 | Helical wheel representations of the amphiphilic structures of magainin 2 and melittin. View is along the helical axis. ■ - hydrophobic residue; □ - cationic residue

A number of research groups have applied amino acid substitution[92] to find residues crucial for the selectivity of α-helical CAPs, but the results do not apply for α-helical CAPs other than the one used in the concerning study. Besides this derivatization of natural CAPs, artificial helical peptides have been synthesized displaying antibacterial activity, such as the α-helical KFF peptide $(KFF)_3K$.[93]

4.2.2 β-sheet and looped CAPs

The β-sheet CAPs form the second major class, and can be subdivided into several distinctive subclasses, most notably those with and without intramolecular Cys-Cys disulfide bonds. The cyclic loloatins A-D[94] and tyrocidine A[127] are examples of the group without disulfide bonds. The group of β-sheet/looped CAPs with Cys-Cys bonds comprises peptides ranging from a single S-S bond (bovine 12-peptide) to 3 or more (α- and β-defensins). As for the α-helical CAPs, the spatial distribution of the amino acid side chains in the β-sheet CAPs is crucial for the antibacterial activity, as it governs the amphiphilicity of the CAP (see Table 2, Figures 7C and 9).

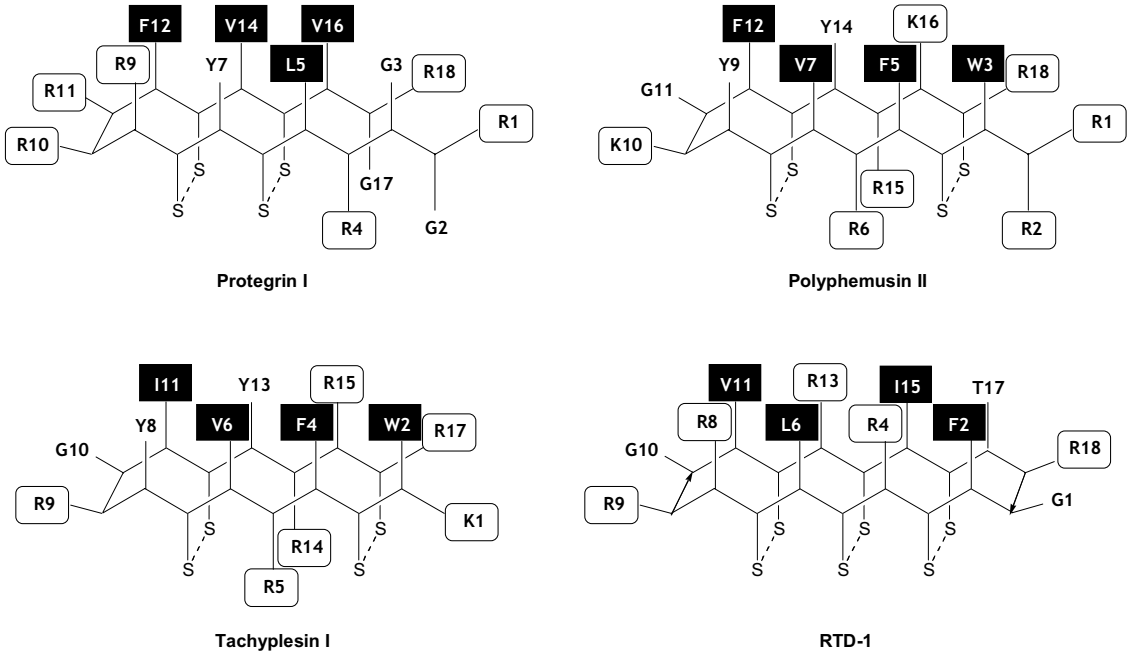


FIGURE 9 | Amphiphilic ß-sheet structures showing hydrophobic (■) and cationic (□) regions for protegrin I, polyphemusin II and tachyplesin I. Note that there is no obvious separation between sides in rhesus θ-defensin RTD-1.

4.2.3 CAPs with extended structures

The last major group (Table 2, Figure 7D) comprises the linear CAPs with no propensity to form specific α-helical or β-sheet structures upon interaction with a G^- membrane. A number of members of this subgroup act through lysis of the bacterial membrane, while for others the antibiotic action appears to arise from specific interaction with intracellular bacterial components (*vide infra,* § 4.3.2). The lack of a clear secondary structure appears to be linked to prevalence of certain amino acid residues as found in indolicidin (Trp),[95] tritrpticin (Trp),[96] drosocin (Pro),[97] pyrrhocoricin (Pro),[98] bactenecins (Pro),[99] and histatins (His).[100]

4.2.4 CAPs containing structural modifications

Non-ribosomal synthesis or post-translational modification of CAPs results in compounds with distinct features. Through these processes, CAPs may display incorporation non-proteogenic amino acids, as can be seen in polymyxins,[101] ramoplanins,[102] nisin Z[103] and other bacteriocins, [104] and can contain modifications including glycosylation (*e.g.* drosocin,[97] pyrrhocoricin,[98] mannopeptimycins),[105] fatty acid conjugation (*e.g.* polymyxins,[101] syringomycins,[106] friulimicin),[107] and cyclization to macrolactams (*e.g.* tyrocidins,[127] gramicidin S)[108] or macrolactones (*e.g.* kahalalide F).[109]

TABLE 2 | Examples of natural CAPs sorted by secondary structures.

CAP	Sequence	Origin
α-helical		
Buforin II	TRSSR AGLQF PVGRV HRLLR K	frog
Cecropin A	KWKLF KKIEK VGQNI RDGII KAGPA VAWGQ ATQIA Ka	silk moth
Cecropin P1	SWLSK TAKKL ENSAK KRISE GIAIA IQGGP R	pig
Clavanin A	VFQFL GKIIH HVGNF VHGFS HVFa	tunicate
Crabrolin	FLPLI LRKIV TALa	hornet venom
Dermaseptin 1	ALWKT MLKKL GTMAL HAGKA ALGAA ADTIS QGTQ	frog
Gaegurin 5	FLGAL FKVAS KVLPS VKCAI TKKC	frog
Lactoferrampin	WKLLS KAQEK FGKNK SR	milk protein
Lactoferricin B	FKCRR WQWRM KKLG	milk protein
LL-37	LLGDF FRKSK EKIGK EFKRI VQRIK DFLRN LVPRT ES	human
Magainin 2	GIGKF LHSAK KFGKA FVGEI MNS	frog
Mastoparan B	LKLKS IVSWA KKVLa	hornet venom
Melittin	GIGAV LKVLT TGLPA LISWI KRKRQ Qa	bee venom
Misgurin	RQRVE ELSKF SKKGA AARRR K	fish

Nigrocin 2	GLLSK VLGVG KKVLC GVSGL C	frog
PGLa	GMASK AGAIA GKIAK VALKA La	frog
Piscidin 3	FIHHI HRGIV HAGRS IGRFL TG	fish
Pleurocidin	GWGSF FKKAA HVGKH VGKAA LTHYL	fish
Temporin A	FLPLI GRVLS GILa	frog
Temporin L	FVQWF SKFLG RIL	frog
β-sheet/loop with Cys-Cys bonds		
α-Defensin HNP-1	ACYCR IPACI AGERR YGTCI YQGRL WAFCC	human
β-Defensin hBD-1	DHYNC VSSGG QCLYS ACPIF TKIQG TCYRG KAKCC K	human
θ-Defensin RTD-1	c(GFCRL CRRGV CRCIC TR)	monkey
Androctonin	RSVCR QIKIC RRRGG CYYKC TNRPY	scorpion
Bovine 12-peptide	RLCRI VVIRV CR	cow
Gomesin	ZCRRL CYKQR CVTYC RGR	spider
Protegrin 1	RGGRL CYCRR RFCVC VGGRa	pig
Polyphemusin I	RRWCF RVCYR GFCYR KCRa	crab
Polyphemusin II	RRWCF RVCYK GFCYR KCRa	crab
Tachyplesin I	KWCFR VCYRG ICYRR CRa	crab
β-sheet no Cys-Cys		
Gramicidin S	*c*(VOLfP VOLfP)	bacterium
Loloatin D	*c*(VOLyP WfNDW)	bacterium
Tyrocidine A	*c*(VOLfP FfNQY)	bacterium
Extended structure/rich in certain residues		
Apidaecin 1A	GNNRP VYIPQ PRPPH PRIa	bee
Drosocin	GKPRP YSPRP T*SHPR PIRV	fruit fly
Formaecin I	GRPNP VNNKP T*PHPR L	ant
Histatin 5	DSHAK RHHGY KRKFH EKHSH RGY	human
Indolicidin	ILPWK WPWWP WRRa	cow
PR-39	RRRPR PPYLP RPRPP PFFPP RLPPR IPPGF PPRFP PRFPa	pig
Pyrrhocoricin	VDKGS YLPRP T*PPRP IYNRN	bug
Tritrpticin	VRRFP WWWPF LRR	synthetic
Miscellaneous		
Polymyxin B	fa XTX *c*(XfLXXT)	bacterium
Polymyxin E	fa XTX *c*(XlLXXT)	bacterium
Syringomycin E	fa *c*(SSXXRFUBJ)	bacterium

Amino acids in lowercase are of the D-configuration. *c*=cyclo; fa=fatty acyl; U=Dhb; B=Asp(OH) J=Thr(Cl), * - glycosylation site, X=Dab, a=carboxamide

4.3 | Targets of CAPs

Due to their cationic nature, CAPs generally prefer interactions with anionic membranes and hence display higher activity against G^- bacteria than G^+ species, but exceptions (*e.g.* nisin Z) that preferentially target G^+ bacteria are known. Although the majority of CAPs kill bacteria by destabilizing the cytoplasmic membrane, some peptides rather bind to essential structures inside the bacterial cell.

4.3.1 Targeting the cytoplasmic membrane

Many studies have been devoted to elucidate the interaction of CAPs with bacterial membranes in order to define a general mode-of-action for CAPs that kill through lysis of the bacterial cell.[110,111] By virtue of their positive charges, CAPs substitute the divalent metal ions that neutralize and cluster LPS. This creates local disturbances of the outer membrane's integrity, and enables more CAPs to translocate over the outer membrane, a process called 'self-promoted uptake'.[112] Having bridged the outer membrane, CAPs target the inner membrane by any of the postulated general mechanisms (Figure 10).[113] Although described here for α-helical CAPs, these mechanisms are thought to apply for other subgroups as well.[114]

One mechanism, referred to as the *Carpet* mechanism, is based on the covering of the membrane by CAPs in a carpet-like fashion. Upon reaching a peptide concentration threshold, the membrane becomes unstable and eventually collapses, resulting in permeation and pore formation. Ultimately, the membrane disintegrates in a detergent-like manner (Figure 10A).

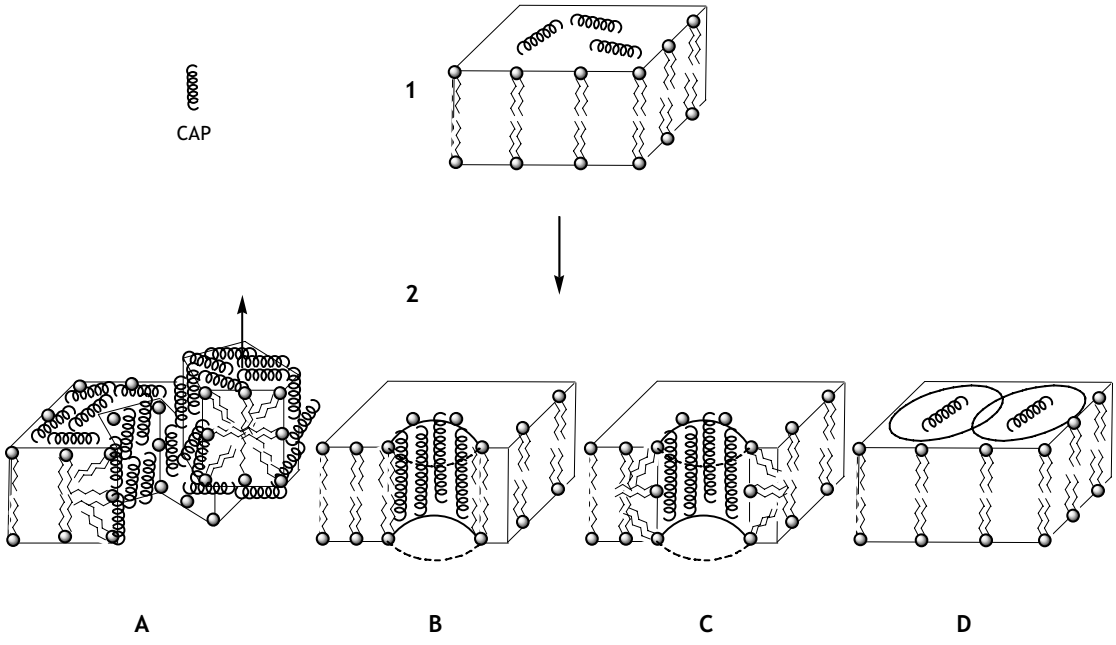


FIGURE 10 | After initial binding of the CAPs to the membrane by virtue of electrostatic interactions (**1**), four possible models (**2**) have been suggested leading to death of the bacterium; Carpet model (**A**), Barrel/Stave model (**B**), Wormhole model (**C**) and In-Plane Diffusion model (**D**).

CAPs exerting activity through this mode of action are considered to be non-cell selective, as carpet-like covering may also occur in the cases of non- or less-anionic membranes. Indeed, these CAPs (*e.g.* melittin) display mostly minimal hemolytic concentration (MHC) values close to their MIC (minimal inhibitory concentration) values.

A second mechanism, the so-called *Barrel-Stave* mechanism is used to explain the mechanism of most CAPs that display high cell-selectivity. In this model, CAPs do not cover the bacterial membrane, but, after binding to the membrane, assemble to form supramolecular structures in the membrane (hydrophilic pores, Figure 10B). Recruitment of additional peptides increases the pore size, causes efflux of cell components and eventually leads to cell death. As the complexation process is dependent on the composition of the membrane, the CAPs following this concept (*e.g.* magainin 2) are generally non-toxic to erythrocytes. In the *Barrel-Stave* model, the cationic charges are located in energetically unfavorable close proximity. Therefore, this model has been slightly adjusted to give the *Wormhole* model,[113] in which these charges are neutralized by negatively charged phospholipid head groups from the membrane (Figure 10C).

Another model, the *In-Plane Diffusion* model, explains the activity of CAPs that were found to have their α-helical axes aligned (in-plane) with the membrane rather than a transmembrane fashion as predicted by the *Barrel/Stave* mechanism.[115] According to this model, overlap of long-range disturbances in the membrane induced upon in-plane binding of CAPs causes local, transient openings in the inner membrane (Figure 10D).

4.3.2 Targeting internal structures

A small number of peptides within the CAP class do not act by destruction of bacterial membranes, but meet their ultimate targets inside. Bac7(1-35) is able to interfere with bacterial components other than the membrane,[116] and the bactericidal effects of apidaecin involve interactions with molecular targets inside *E. coli*.[117] Well-documented are the cases of the Pro-rich insect CAPs drosocin and pyrrhocoricin. These peptide antibiotics were found to bind specifically to the *E. coli* heat-shock protein DnaK, inhibiting its cellular functions.[118] Most interestingly, the human homologue of this bacterial protein (Hsp60) is not affected by either one. The absence of cytotoxicity for these peptides makes them interesting candidates for drug development. Internal targets are by no means limited to extended-structured CAPs as is demonstrated by the α-helical CAPs buforin II and lactoferricin B, that were found to respectively bind to nucleic acids and to inhibit the synthesis of macromolecules in both *Escherichia coli* and *Bacillus subtilis*.[119,120]

5 | Beyond Natural CAPs

Besides amino acid substitution in natural CAPs for structure/activity studies,[121] many reports deal with the design of new CAPs and derivatives that are inspired by their amphiphilic nature, a number of which is highlighted in the following paragraphs.

5.1 | Peptides & Peptidomimetics

5.1.1 Synthetic cationic antimicrobial α-peptides

Compounds inspired by CAP helices[122] such as the KFF peptide,[93] stabilized β-sheet structures based on protegrins[123,124] and the LPS binding region in LALF (*Limulus* anti-lipopolysaccharide factor) have been designed, displaying natural CAP-like biological activities.[125] Even small, *de novo* designed extended-structured CAPs composed of 6 amino acids can exert antimicrobial activity.[126] Furthermore, a combinatorial approach towards cyclic decapeptides yielded derivatives that were more potent than the natural CAP tyrocidine A.[127]

5.1.2 Hybrids

Several CAPs contain areas with different functionalities. Pyrrhocoricin contains a putative pharmacophore and an intracellular delivery domain,[128] as does drosocin. Mixing these putative domains resulted in peptides with strongly reduced activities.[129] However, hybrids of membrane active CAPs, cecropin/melittin[130] and cecropin/magainin,[131] were found to have the characteristics of both CAPs. Dimers of a magainin analogue[132] and magainin 2 cross-linked to PGLa[133] showed distinct biological profiles with respect to the monomers. A conjugate of a dermaseptin derivative with an RNA III-inhibiting peptide (for the prevention of biofilm formation) was able to interfere in *Staphylococcus*-associated infections.[134]

5.1.3 Conjugates with lipophilic groups

Inspired by the architecture of natural antibacterial lipopeptaibols[135] and polymyxins, the effects of fatty acid conjugation to CAPs have been reported. In polymyxin B, the acyl moiety is

considered to be important for activity as deacylated polymyxin B shows significant loss in antimicrobial potency.[136] Indeed, acylated derivatives of a synthetic D,L-peptide,[137] SC4,[138] cathepsin G(117-136),[139] lactoferrin-derived peptides,[140] a cecropin/melittin hybrid[141] and magainin[142] displayed improved activity and/or altered selectivity.

5.1.4 D-Amino acid incorporation

Incorporation of enantiomeric amino acids influences 3D structure and stability, activity, toxicity or selectivity. Substitution of L-amino acid residues in melittin,[143] pardaxin[144] and synthetic peptides[145] with their D-counterparts leads to analogues of these CAPs with improved selectivity and slightly influenced antibacterial activity. A synthetic α-helical peptide containing only DLys and DLeu residues (an *all-D* peptide) was significantly more stable against trypsin treatment than the corresponding *all-L* analogue.[146] Furthermore, only the *all-D* peptide could cure mice from infection with *Pseudomonas aeruginosa* and gentamicin-resistant *Acinetobacter baumanii*, underlining the importance of CAP stability in serum, which is greatly improved upon introduction of enantiomeric amino acid residues. However, the *all-D* strategy is limited to membrane-active CAPs; enantiomeric analogues of pyrrhocoricin and drosocin showed no antibacterial activity because of their stereospecific interaction with target proteins inside bacterial cells.[119]

5.1.5 β-Peptides

Peptides completely composed of β-amino acids (β-peptides) were found to be able to form helices.[147] Following the concept of amphiphilic helices present in α-peptidic CAPs, the groups of Seebach[148] and DeGrado[149] reported antibacterial activity of their amphiphilic β^3-peptides. Using constrained *trans*-2-aminocyclopentane carboxylic acid (ACPC)-based monomers for optimal induction of a helical structure,[150] β-peptide β-17 (Figure 11)[151] was constructed. This peptide possessed antibacterial activity comparable to that of magainin 2 amide and melittin, but its hemolytic activity was considerably lower. β-Peptides have been shown to be stable towards a number of proteases.[152]

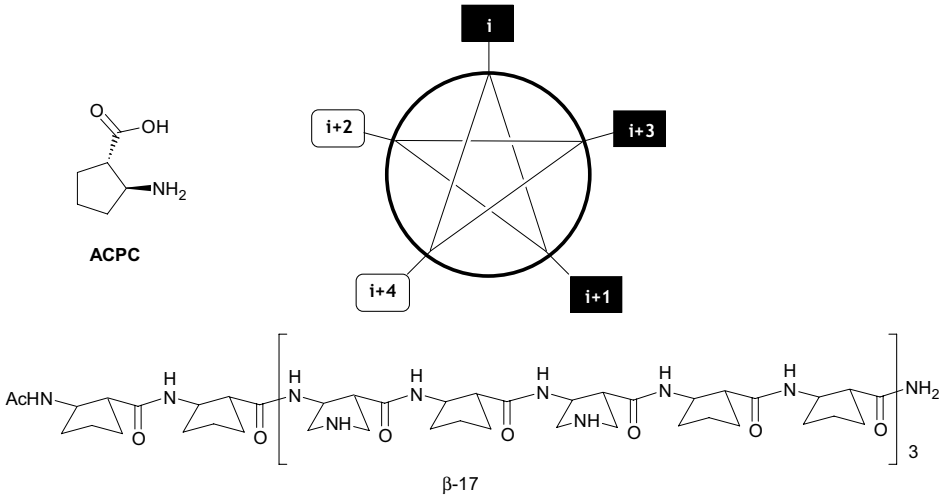


FIGURE 11 | ACPC constrained residue, helical wheel representation indicating ~5 residues per turn in amphiphilic antimicrobial ß-peptide ß-17. View along the helical axis. ■ - hydrophobic residue; □ - cationic residue.

5.1.6 Peptoids

Attachment of the side chains of amino acids to the nitrogen atom rather than the Cα atom yields a class of peptide derivatives known as *peptoids* (Figure 12). Chiral peptoids have been constructed that form amphiphilic helices and show antibacterial activity.[153] Through combinatorial chemistry, tripeptoids have been constructed that display antimicrobial activity.[154]

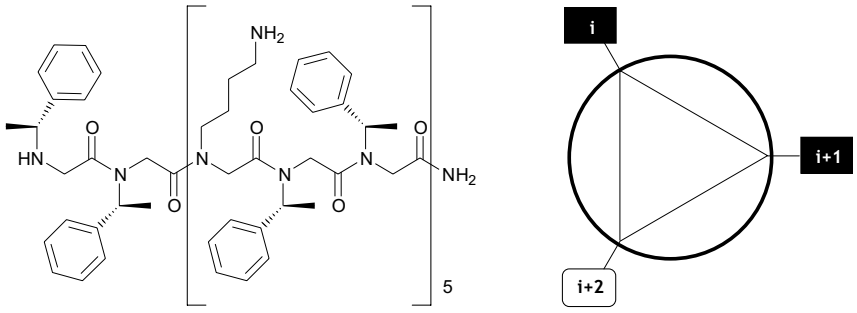


FIGURE 12 | Antimicrobial peptoid and helical wheel representation indicating ~3 residues per turn. View along the helical axis. ■ - hydrophobic residue; □ - cationic residue.

5.2 | Amphiphilic scaffolds

Amphiphilic scaffolds mimicking the separation of cationic and hydrophobic sides in CAPs have been synthesized and evaluated for biological activity. For example, the cholic acid scaffold was applied (Figure 13) in the preparation of amphiphiles.[155] The synthesized cationic steroid-derived compounds displayed activity comparable to some natural CAPs.

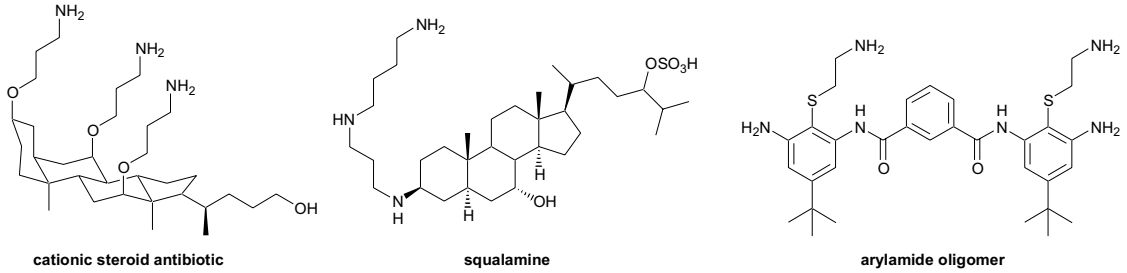


FIGURE 13 | Amphiphilic compounds displaying antibacterial activity.

It should be noted that natural steroid compounds such as squalamine[156] (Figure 13) and derivatives[157] display antibacterial activity as well. Amphiphilic compounds based on the *ter*-cyclopentane scaffold[158] and indane-based compounds[159] also exerted antibiotic activity. The group of DeGrado synthesized biologically active, facially amphiphilic arylamide oligomers (Figure 13).[160] Amphiphilicity also inspired the work on cyclic D,L-α-peptides that were able to form tubular structures by self-assembly to permeate membranes and kill both G^- and G^+ bacteria.[161]

5.3 | Structural minimization

Based on the two activity-determining parameters of CAPs (cationicity and hydrophobicity), biologically active structures far less complicated than those of CAPs can be synthesized. Amphiphilic molecules composed of no more than a few non-proteogenic, bulky amino acid residues already display antibacterial activity against both G^- and G^+ bacteria as well as hemolysis.[162] Extending this simplification further, the bioactive ammonium compounds are

among the smallest possible structures displaying both cationicity and hydrophobicity (Figure 14). For instance, amphiphilic coatings based on alkylated poly(vinylpyridine) applied to surfaces kill airborne bacteria upon contact.[163,164] However, the trade-off for structural simplification is a loss in selectivity: whereas CAPs can be highly selective in their actions, most quaternary ammonium compounds lyse bacterial cells and mammalian erythrocytes alike.[165]

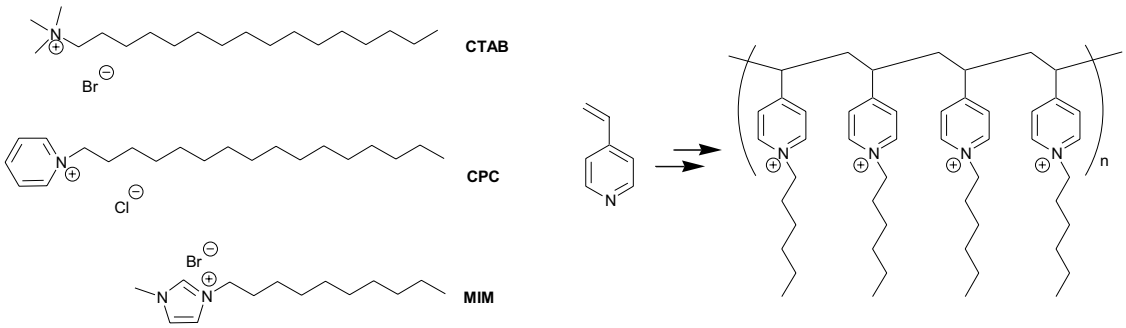


FIGURE 14 | Quaternary ammonium amphiphilic antibacterial compounds (*left*); known cetyltrimethyl-ammonium bromide (CTAB), cetylpyridinium chloride (CPC), and *N*-methyl-*N'*-decyl imidazolium bromide (MIM). *Right:* polymerized alkylated vinylpyridine.

6 | Neutralization of LPS

A number of natural CAPs are capable of strong binding to and neutralizing LPS.[166] Unfortunately, the usage of the CAPs tested (*e.g.* melittin and polymyxin B) is limited to topical systems as they display undesired characteristics (hemolysis or nephrotoxicity, respectively). Based on these results, structural studies towards LPS-binding optimization of synthetic peptides have been reported.[167] A recombinant N-terminal sequence of BPI ($rBPI_{23}$), an LPS binding protein,[168] fused to the human immunoglobulin IgG abolished the physiological response to LPS challenge in human volunteers.[169] Other CAPs were also reported to interfere with the LPS/LBP complexation process.[170] A successful approach that preserves the favorable LPS-neutralizing properties of polymyxin B, but circumvents toxicity issues, is the application of hemoperfusion.

In this approach, blood from septic patients is cleared from LPS extracorporally by using a cartridge containing immobilized polymyxin B.[171]

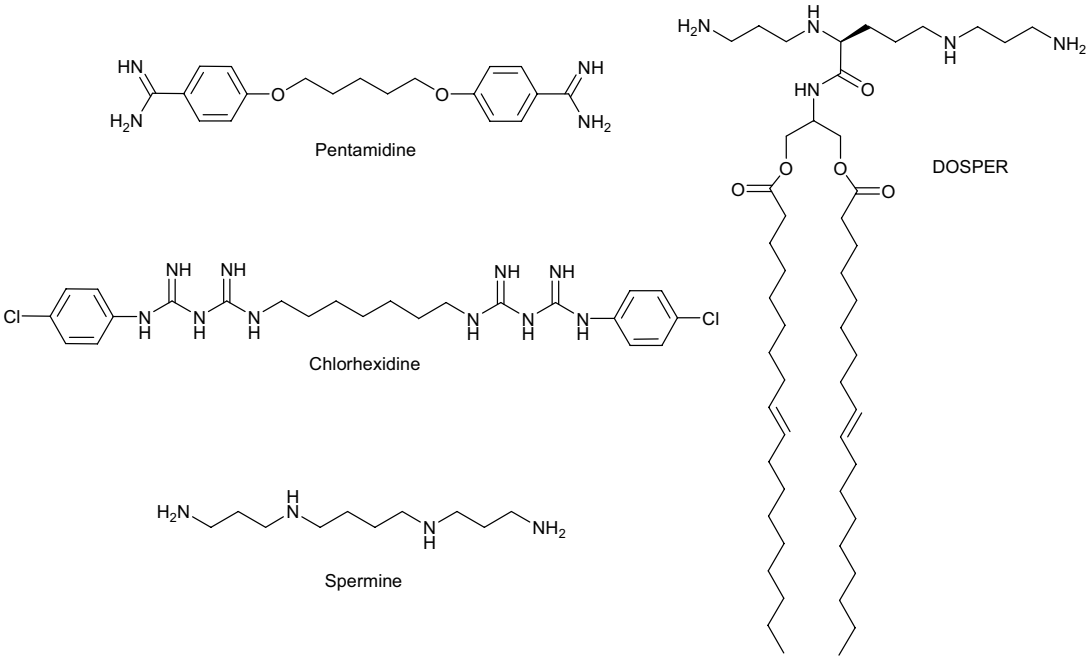


FIGURE 15 | Structures of pentamidine, chlorhexidine, spermine and DOSPER.

Research towards existing natural or synthetic structures that are able to scavenge LPS has attracted interest in recent years.[172] The geometry of the five cationic Dab residues in polymyxin B inspired research towards small molecules in which appropriately spaced cationic groups are present. Established antibiotics as pentamidine,[173] pentamidine congeners,[174] and chlorhexidine[175] (Figure 15) were found to exhibit Lipid A affinity. The affinity of pentamidine was found to be 3-fold higher than that of polymyxin B. The appropriate intercation distance for simultaneous recognition of both phosphate groups in Lipid A was also observed in the polyamine spermine.[174] Lipophilic spermine derivatives[176] were shown to have a neutralizing effect on endotoxin as did lipopolyamines such as DOSPER (used in nucleic acid transfection studies, Figure 15).[177] Although DOSPER alone could not prevent mortality in challenged mice, survival increased upon its co-administration with the β-lactam antibiotic ceftazidime compared to ceftazidime alone.[178]

7 | Clinical & Commercial Application of CAPs

Colimycin (the methosulfate derivative of polymyxin E) appears to be well-tolerated and is successfully used in an aerosol formulation.[179] The mixture of polymyxin B, gramicidin S and bacitracin is a highly active topical preparation.[180] Polymyxin B is also present as topical agent in ophthalmologic formulations,[181] along with bacitracin, which can be found in cosmetics.[182] Nisin Z, active against G^+ bacteria, is currently used as a food additive and is referred to as E 234.[183]

The magainin derivative MSI-78 (pexiganan) was rejected by an FDA panel in phase III clinical trials against both polymicrobic diabetic foot ulcers and impetigo.[5] Nisin has succesfully undergone phase I trials against *Helicobacter pylori* stomach ulcers.[180] Iseganan (IB-367, a protegrin derivative) is currently in phase III trials for treatment of oral mucositis.[184] BPI[185] and its recombinant fragment ($rBPI_{23}$) linked to IgG, were reported to be in clinical trials.[186] A topical 1% gel preparation of omiganan (MBI-226, a 12-residue indolicidin analogue) is currently in phase III clinical trials for the prevention of catheter-related bloodstream infections.[187]

8 | Evolution of Resistance?

Some bacteria are able to withstand the antibiotic activity of CAPs, and resistance of G^- bacteria against CAP family members has been documented.[188] For instance, the two-component regulatory protein systems PmrA/PmrB (**p**oly**m**yxin **r**esistance) and PhoP/PhoQ govern resistance towards CAPs in *Pseudomonas aeruginosa.*[189,190] In *P. aeruginosa* and *Salmonella* species, the latter system induces modification of Lipid A moieties in the LPS by covalent addition of 4-amino-4-deoxy-L-arabinose or phosphoethanolamine, decreasing the overall negative charge of the bacterial outer membrane (Figure 16).[19a,191] Likewise, resistance towards defensins and protegrins is enhanced by modification of phosphatidylglycerol with Lys in the cytoplasmic membrane of *Staphylococcus aureus* (G^+), changing net charge.[192] Efflux pumps belong to the arsenal of resistance mechanisms of bacteria,[193] along with PgtE endoprotease/peptidase, whose presence was demonstrated in the outer membrane of *Salmonella* species.[194] This enzyme, its homologue OmpT (*Escherichia coli*)[195] and the porin OmpU (*Vibrio cholerae*),[196] were found to decrease susceptibility towards CAPs.

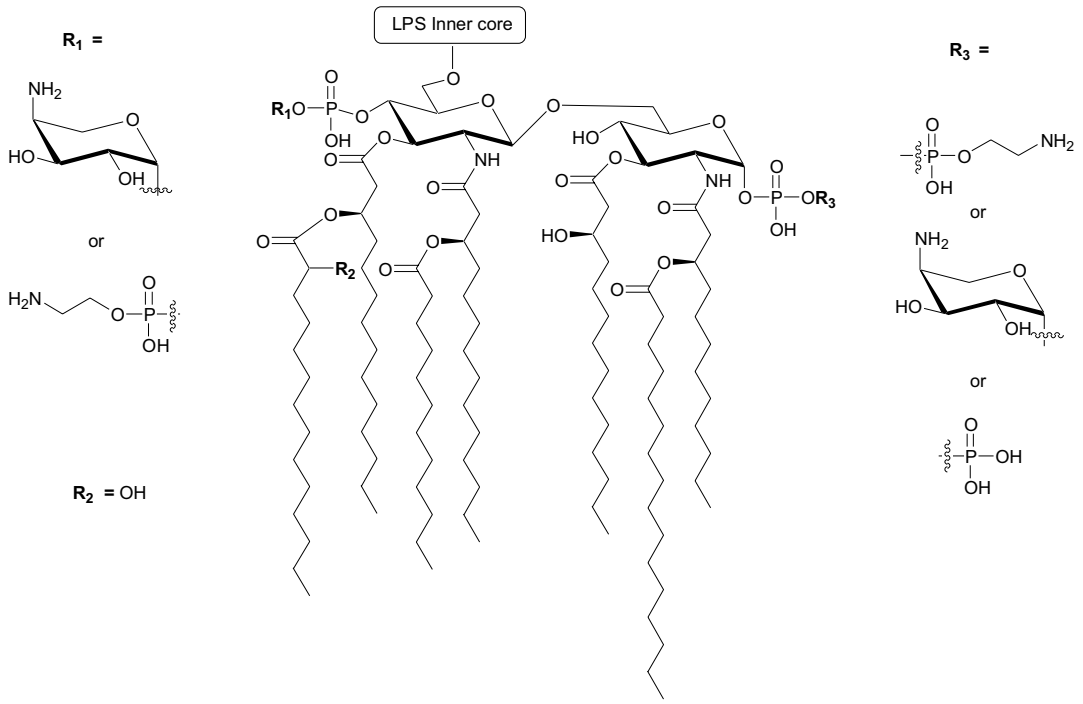


FIGURE 16 | Covalent modifications observed in *E. coli* and *Salmonella typhimurium* Lipid A, resulting in diminished sensitivity towards CAPs. In unmodified Lipid A, $R_1 = R_2 = R_3 = H$.

Some reports suggest that association of the antimicrobial peptide with the bacterial membrane's phospholipids is only a partial process in the overall interaction between the two. Nisin Z[197] and mesentericin Y,[198] both active against G^+ bacteria, were found to lose target cell specificity upon removal of a receptor-binding element in their structures. The corresponding membrane-bound receptors are thought to be produced by bacteria as multidrug-resistant (MDR) proteins.[199] An illustration of this concept is the SIC protein secreted by pathogenic *Streptococcus pyogenes*, which was found to be able to render human α-defensins and LL-37 inactive. The high prevalence of *S. pyogenes* M1 serotype infections is most likely caused by the high level of SIC protein secreted by this particularly serotype.[200]

Finally, it has been stated that introduction of CAPs into clinical use may induce the evolution of bacterial resistance to our own cationic antimicrobial defense proteins and thus severely compromise our natural defenses against infection.[201] However, reports have appeared that claimed zero to marginal evolving bacterial resistance against certain CAPs,[51,202] leaving the possibility for these particular CAPs to become clinically useful antibiotics.

9 | Conclusion

The research towards, and development of, antibiotics with new modes of action are important objectives in attempting to counter the growing bacterial resistance against commonly used antibacterial drugs. Despite all efforts, only a small number of new compounds have been approved for clinical use in the last decade, of which only two have a novel mechanism of action. In particular, (potential) resistance of pathogenic G^- bacteria poses a threat to public health. However, among the newest antibacterials approved there are no compounds indicated against G^- infections. Besides the fact that treatment of G^- pathogens is intrinsically hampered by the presence of an extra membrane, countering a G^- pathogen leads to release of immunogenic endotoxins that may very well aggravate the patient's condition. Members of the class of cationic antimicrobial peptides (CAPs), appear to represent a solution to these issues. The favorable properties of CAPs are summarized in Table 4, together with issues that will need to be addressed in the development of CAPs.

TABLE 4 | Properties of CAPs.

Resistance	+ The minute time scale antibacterial action of membrane-active CAPs does not allow for spontaneous bacterial adaptations. + Mutations in targets of CAPs targeting internal structures are unlikely to yield viable resistant species as these internal structures are mostly essential for bacterial growth. + Resistance against the secondary structure types of CAPs is unlikely as this would yield unviable 'self-resistant' species.
Selectivity	+ Most CAPs (both membrane-active CAPs and CAPs with internal targets) target prokaryotes selectively (in particular G^- bacteria), allowing for directed treatment in mammals. - Many CAPs show hemolytic activity (although at higher concentrations than needed for antibacterial activity).
LPS Neutralization	+ A number of CAPs are able to neutralize LPS and might be able to prevent sepsis during/after treatment of the bacterial infection.
Stability	+ Mammalian CAPs composed of proteogenic amino acids can be metabolized and excreted by the body. - CAPs composed of proteogenic acids are inherently susceptible towards proteolytic cleavage, requiring studies towards stabilization. - Oral availability of most CAPs is low or zero. - CAPs that are proteolytically too stable might exert toxicity.
Toxicity	- Non-ribosomally synthesized bacterial CAPs might exert (organ-specific) toxicity due to the fact that they are rather resistant towards proteolytic breakdown. - CAPs that are less-selective display hemolysis.
Optimization	- As there is no general rule by which the activities of natural or synthetic CAPs can be predicted, optimization of lead structures might be a time-consuming process.

Based on their specific characteristics, a number of CAPs was considered promising for clinical development (see § 7). In order to become lead structures for clinical antibiotic development, CAPs should possess the favorable properties from Table 4 regarding cell-selectivity, activity and stability, ideally combined with the ability to take care of LPS after eradication of the G^- bacterium.[5,203,204]

10 | Outline

Chapter 1 of this thesis deals with the biological evaluation of analogues of the CAP drosocin from the fruit fly *Drosophila melanogaster*. This CAP is fully selective towards G^- bacteria, but is rather unstable in serum. Amino acid substitutions yielded a series of lead analogues that display a far higher stability than the natural CAP while maintaining or slightly increasing the antibacterial activity.

Polymyxin B1 (from *Bacillus polymyxa*) is the subject of **Chapter 2**. This bactericide is among the most potent CAPs known and is used as standard control in various biological assays. Nature appears to have optimized the structure of polymyxins, as no analogues more active than polymyxin B1 have been reported to date. A new synthetic route towards polymyxin B1 is presented and applied in the synthesis of several polymyxin analogues.

During the polymyxin syntheses, a by-product was detected having identical molecular weight but a different retention time on LC. **Chapter 3** deals with the identification of this by-product as a regioisomer of the polymyxins, resulting from an Nα→Nγ acyl migration.

In an approach to circumvent the negative nephrotoxic aspects of polymyxin B1 while preserving its Lipid A affinity, conjugates of non-toxic, deacylated polymyxin B1 (polymyxin B nonapeptide) and other CAPs were designed. The preparation of these conjugates and their biological evaluation are described in **Chapter 4**.

Chapter 5 describes the synthesis of amphiphilic compounds inspired by the cationic and hydrophobic properties of CAPs. Quaternary ammonium compounds (QACs) are among the most easily synthesized compounds displaying antimicrobial activity in solution. Stable gel formulations containing biologically active quaternized *N*-methylimidazolium and *N*-methylpyrrolidinium bromides and water, ethylene glycol or glycerol were prepared and assayed for antimicrobial activity against G^- and G^+ bacteria.

Chapter 6 discloses a peptide-related topic. As the chemical synthesis of peptides is not always straightforward and purification procedures can be tedious, a new approach for synthetic peptide purification is presented. Exploiting specific fluorine-fluorine interactions, purifications using fluorous HPLC or fluorous SPE were performed to solely yield the desired compounds. To enable this, a novel base-labile fluorous amine protecting group was designed and synthesized.

Finally, **Chapter 7** discusses some future prospects regarding the research described in this thesis. Notably, the approach of conjugating a Lipid A-affinity moiety to a CAP is further extended, and the anti-malarial drug pentamidine, displaying a higher affinity for Lipid A than polymyxin B, was derivatized to provide it with a handle for conjugation to CAPs.

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CHAPTER 1 | Biological Evaluation of Stabilized Drosocin Analogues

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