# New cationic amphiphilic compounds as potential antibacterial agents

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# New Cationic Amphiphilic Compounds as Potential Antibacterial Agents

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in memoriam prof. dr. Jacques H. van Boom

If we knew what it was we were doing, it would not be called research, would it?
*- Albert Einstein (1879-1955)*

*Voor Ilse*

# Table of Contents

# List of Abbreviations

| | | |
|---|---|---|
| a | | C-terminal amide |
| AA | | amino acid residue[1] |
| Abu | | γ-aminobutyric acid |
| Ac | | acetyl |
| ACPC | | *trans*-2-aminocyclopentane carboxylic acid |
| Ada | | 1-adamantaneacetyl |
| ADP | | adenosine 5′-diphosphate |
| Ala | (A) | alanine |
| AM | | aminomethyl |
| anh. | | anhydrous |
| aq. | | aqueous |
| Ar | | aromatic |
| Arg | (R) | arginine |
| Asn | (N) | asparagine |
| Asp | (D) | aspartic acid |
| AP | | alkaline phosphatase |
| ATCC | | American type culture collection |
| Ava | | δ-aminovaleric acid[2] |
| βAla | | β-alanine |
| BF2 | | buforin II |
| BHI | | brain/heart infusion |
| bm | | broad multiplet |
| Bn | | benzyl |
| Boc | | *tert*.-butoxycarbonyl |
| BOP | | (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate |
| Bu | | butyl |
| *c (prefix)* | | cyclo |
| $C_x$ | | *n*-alkyl chain containing *x* carbon atoms |
| CAP | | cationic antimicrobial peptide |
| Capro | | (*S*)-3-amino-1-carboxymethyl caprolactame |
| CD | | circular dichroism |
| CFU | | colony-forming units |
| Clt | | (2-chlorotriphenyl)methyl |
| CMP | | cytidine 5′-monophosphate |
| Cmpi | | *N*-carboxymethylpiperazine |
| COSY | | correlated spectroscopy |
| CPC | | cetylpyridinium chloride |
| CPMBN | | Cys-polymyxin B nonapeptide |
| CTAB | | cetyltrimethylammonium bromide |
| Cys | (C) | cysteine |
| Dab | (X) | α,γ-diaminobutyric acid[2] |
| dansyl | | 5-dimethylamino-1-naphthalenesulfonyl |
| DAST | | diethylaminosulfurtrifluoride |
| DCC | | *N,N′*-dicyclohexylcarbodiimide |
| DCE | | 1,2-dichloroethane |
| Dde | | 1-(4,4-dimethyl-2,6-dioxo-cyclohex-1-ylidene)ethyl |
| Dhb | (U) | α-aminodehydrobutyric acid |
| DIAD | | diisoproyl azodicarboxylate |
| DiBAl-H | | diisobutylaluminum hydride |
| DIC | | *N,N′*-diisopropylcarbodiimide |
| DiPEA | | *N,N*-diisopropylethylamine |
| DMAP | | 4-dimethylaminopyridine |
| DMF | | *N,N*-dimethylformamide |
| DMSO | | dimethylsulfoxide |
| DNA | | deoxyribonucleic acid |
| DOSPER | | 1,3-dioleyloxy-2-(6-carboxy-spermidyl)propylamide |
| DPPA | | diphenylphosphoryl azide |
| DPX | | dansylated polymyxin B |
| DRC | | (S7T)-drosocin |
| DTT | | dithiothreitol |
| EDC | | *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide |
| EDTA | | ethylenediaminetetraacetate |
| ESI | | electrospray interface |
| Et | | ethyl |
| eq. | | equivalent(s) |
| *E*-gel | | gel containing ethylene glycol |
| F *(prefix)* | | fluorous |
| FA *or* fa | | fatty acyl |
| FDA | | (United States) Federal Drug Administration |
| FITC | | fluoresceinyl isothiocyanate |
| Fmoc | | 9-fluorenylmethoxycarbonyl |
| Gal | | galactose |
| $G^-$ | | Gram-negative |
| $G^+$ | | Gram-positive |
| Glc | | glucose |
| Gln | (Q) | glutamine |
| Glu | (E) | glutamic acid |
| Gly | (G) | glycine |
| GRAVY | | grand average of hydropathy |

[1] Where applicable, amino acid residues are of the L-configuration unless otherwise stated.

[2] Due to shortage of unique one-letter codes, Ava is denoted with X in Chapter 4 and Tmd(Phe) with Z in Chapter 7.

| | | |
|---|---|---|
| *G*-gel | | gel containing glycerol |
| HATU | | *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate |
| hBD | | human β-defensin |
| HCTU | | *O*-(6-chlorobenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate |
| Hep | | L-*glycero*-D-*manno*-heptose |
| HEPES | | *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) |
| His | (H) | histidine |
| HMDS | | hexamethyldisilazane |
| HOAt | | 1-hydroxy-7-azabenzotriazole |
| HOBt | | 1-hydroxybenzotriazole |
| HPLC | | high-performance liquid chromatography |
| HRMS | | high-resolution mass spectrometry |
| HSer | ($^{\beta}$S) | $\beta^3$-homoserine |
| HTyr | ($^{\beta}$Y) | $\beta^3$-homotyrosine |
| IBX | | triacetoxyiodobenzoic acid |
| IL | | interleukin |
| Ile | (I) | isoleucine |
| IM | | inner (cytoplasmic) membrane |
| IR | | infrared |
| ISB | | iso-sensitest broth |
| ISO | | International Organization for Standardization |
| ivDde | | 1-(4,4-dimethyl-2,6-dioxocyclo-hex-1-ylidene)-3-methylbutyl |
| Kdo | | 3-deoxy-D-*manno*-oct-2-ulosonic acid |
| KFF | | KFF peptide $(KFF)_3K$ |
| LB | | Luria-Bertani (broth) |
| LBP | | LPS-binding protein |
| LC | | liquid chromatography |
| LCMS | | liquid chromatography/mass spectrometry |
| Leu | (L) | leucine |
| LPS | | lipopolysaccharide |
| Lys | (K) | lysine |
| OM | | outer membrane |
| Orn | (O) | ornithine |
| Mamb | | *m*-(aminomethyl)benzoic acid |
| MALDI | | matrix-assisted laser desorption/ionisation |
| Me | | methyl |
| $^{Me}$Arg | ($^{Me}$R) | $N^{\alpha}$-methylarginine |
| $^{Me}$Ser | ($^{Me}$S) | *N*-methylserine |
| Met | (M) | methionine |
| MHC | | minimal hemolytic concentration |
| MIC | | minimal inhibitory concentration, *i.e.* the lowest concentration at which no bacterial growth can be detected by spectroscopic analysis after incubation for a specified time, compared with a positive control (Triton X-100). |
| MIM | | *N*-methyl-*N'*-alkyl imidazolium |
| MMT | | (4-methoxytriphenyl)methyl |
| MOA | | *(S)*-6-methyloctanoyl, -oic acid |
| Mpa | | 3-maleimidopropionyl |
| MPD | | *N*-methyl-*N*-alkyl-pyrrolidinium |
| MS | | mass spectrometry |
| MS/MS | | tandem mass spectrometry |
| Msc | | methylsulfonylethoxycarbonyl |
| Mtt | | (4-methyltriphenyl)methyl |
| MW | | microwave (oven) |
| n *(prefix)* | | natural |
| *n*- | | normal (linear) |
| n/a | | not applicable |
| n/d | | not determined |
| NBE | | nutrient broth E |
| NCL | | native chemical ligation |
| nG | | not glycosylated |
| NMP | | *N*-methylpyrrolidone |
| NMR | | nuclear magnetic resonance |
| NOE | | nuclear Overhauser effect |
| NOESY | | nuclear Overhauser effect spectroscopy |
| Orn | (O) | ornithine |
| Pamb | | *p*-(aminomethyl)benzoic acid |
| PAMP | | pathogen-associated molecular pattern |
| PBS | | phosphate-buffered saline |
| PE | | petroleum ether 40/60 |
| PEG | | polyethyleneglycol |
| Ph | | phenyl |
| Phe | (F) | phenylalanine |
| PhPip | | 4-phenyl-4-carboxymethyl-piperidine |
| PMB | | polymyxin B |
| PMBN | | polymyxin B nonapeptide |
| PNT | | pentamidine (derivative) |
| Pro | (P) | proline |
| PyBOP | | (benzotriazol-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate |

| | | |
|---|---|---|
| PyBroP | | bromotripyrrolidino-phosphonium hexafluorophosphate |
| QAC | | quaternary ammonium compound |
| RBC | | red blood cell |
| Ref. | | reference |
| $R_f$ | | retardation factor |
| RNA | | ribonucleic acid |
| ROESY | | rotating frame NOESY |
| RP | | reversed phase |
| Rt | | retention time |
| RT | | room temperature |
| RTD | | rhesus θ-defensin |
| s *(prefix)* | | synthetic |
| SAA | | sugar amino acid |
| Sar | | sarcosine |
| sat. | | saturated |
| SCL | | 3-carboxypropanesulfonamide |
| Ser | (S) | serine |
| SIC | | streptococcal inhibitor of complement |
| SPE | | solid-phase extraction |
| SPPS | | solid-phase peptide synthesis |
| SPy | | 2-pyridylsulfenyl |
| SS | | disulfide linkage |
| StBu | | *tert.*butylsulfenyl |
| Su | | succinimidyl |
| tBu | | *tert.*butyl |
| TCA | | trichloroacetic acid |
| TCEP | | tris(carboxyethyl)phoshine |
| TES | | triethylsilane |
| TFA | | trifluoroacetic acid |
| TFE | | 2,2,2-trifluoroethanol |
| THF | | tetrahydrofuran |
| THP | | tetrahydropyran |
| Thr | (T) | threonine |
| TIC | | total ion count |
| TIS | | triisopropylsilane |
| Tmd | | 3-(trifluoromethyl)-3*H*-diazirin-3-yl |
| TMS | | trimethylsilyl |
| TNBS | | 2,4,6-trinitrobenzenesulfonic acid |
| TNF | | tumor necrosis factor |
| TLC | | thin-layer chromatography |
| TLR | | Toll-like receptor |
| TOCSY | | total correlation spectroscopy |
| TOF | | time-of-flight |
| Tr | | triphenylmethyl |
| Tran | | tranexamic acid |
| Trp | (W) | tryptophane |
| TTC | | tritrpticin |
| Tyr | (Y) | tyrosine |
| UDP | | uridine 5′-diphosphate |
| UV | | ultraviolet |
| Val | (V) | valine |
| *W*-gel | | gel containing water |
| Z | | benzyloxycarbonyl[2] |

# General Introduction

## 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.

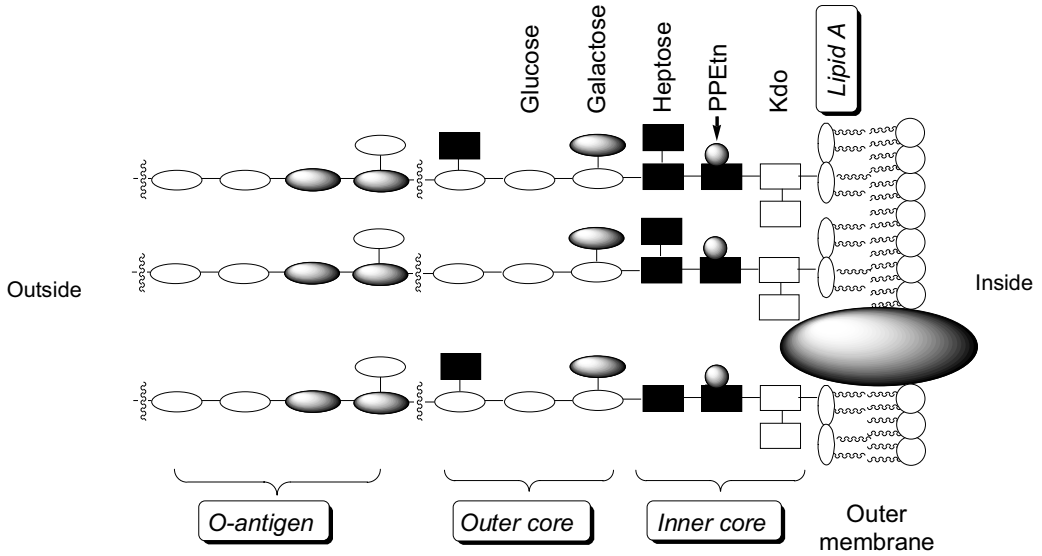



**FIGURE 1** | Schematic representation of the structure of *Escherichia coli* K12 LPS, consisting of the *O*-antigen, outer and inner cores, and Lipid A. The oval transmembrane structure represents an outer membrane protein. The overall negative charge is caused by phosphate groups in the inner core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

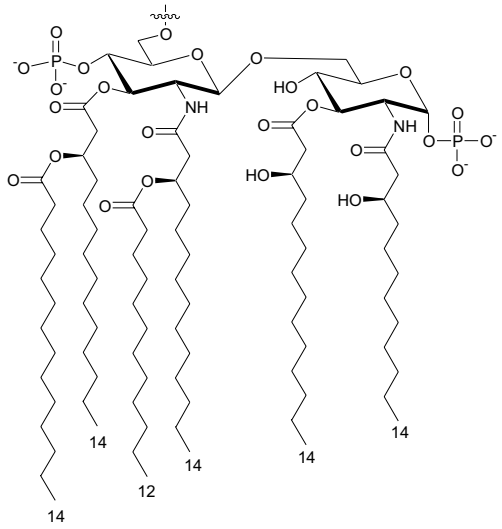


FIGURE 2 | Structure of Lipid A from *E. coli* K12. Numbers denote the number of carbon atoms in each chain.

## 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]

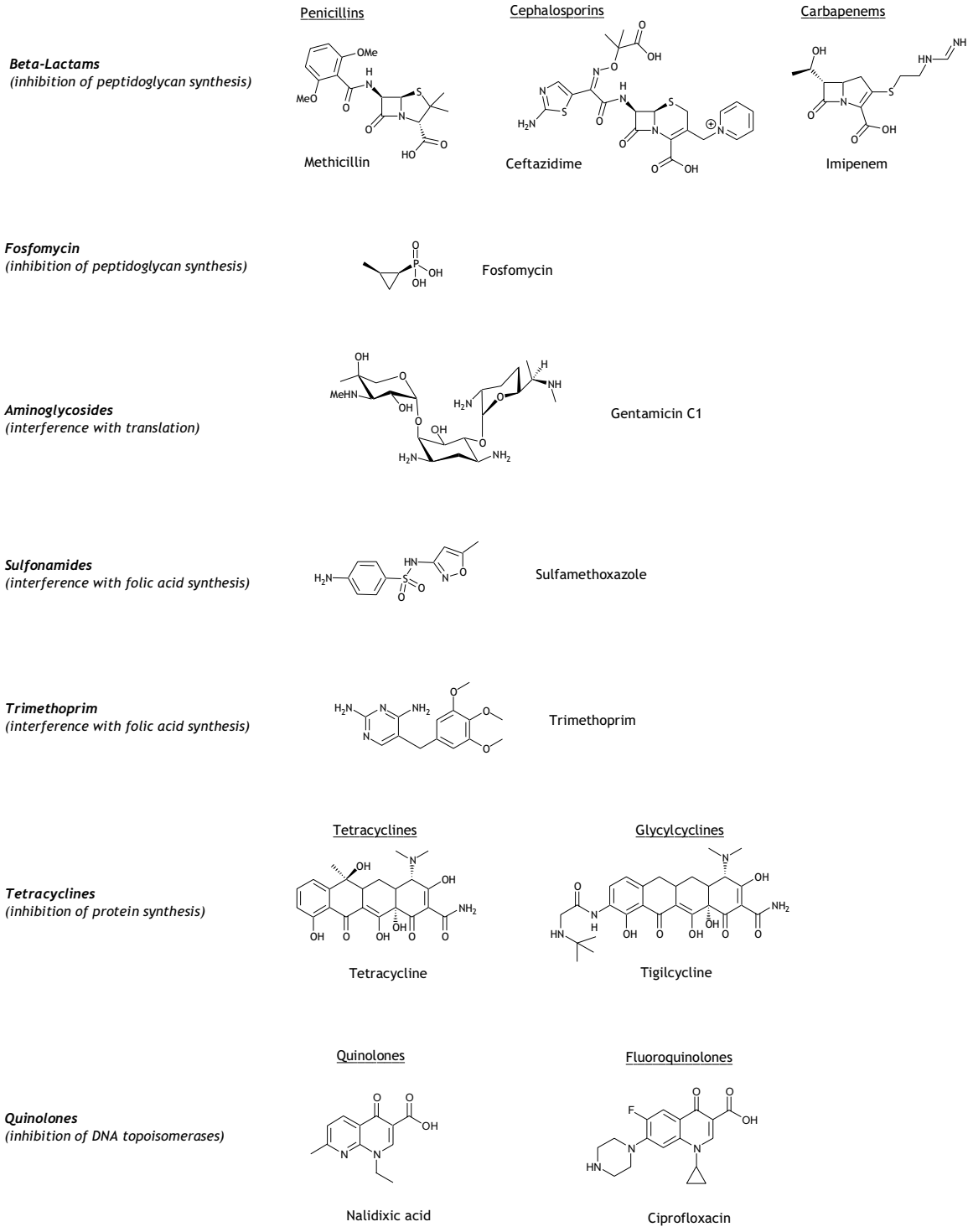



**FIGURE 3** | Structures of representative examples of commonly used classes of antibiotics.

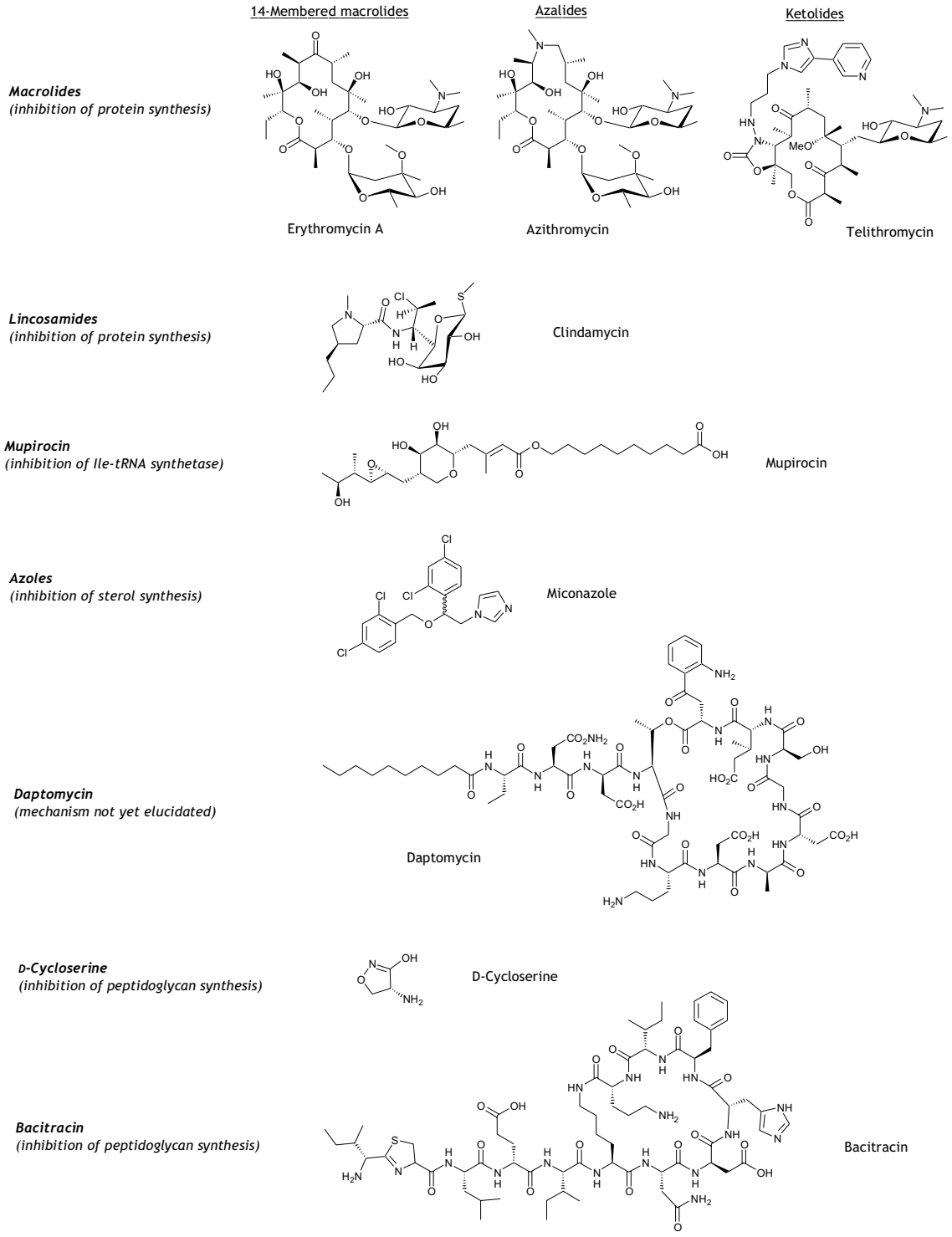



**FIGURE 3 (continued)** | Structures of representative examples of commonly used classes of antibiotics.

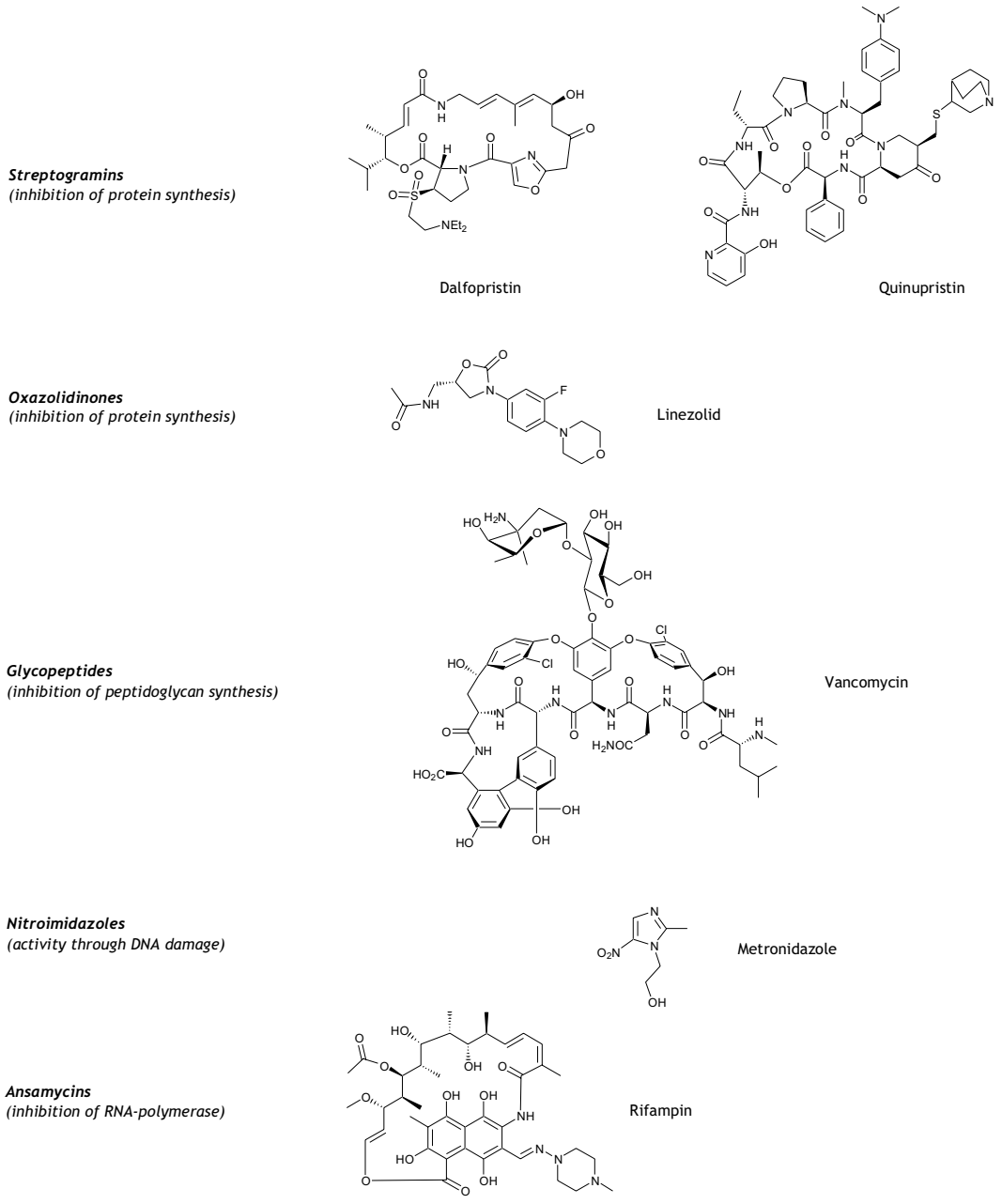



**FIGURE 3 (continued) |** Structures of representative examples of commonly used classes of antibiotics.

## 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.

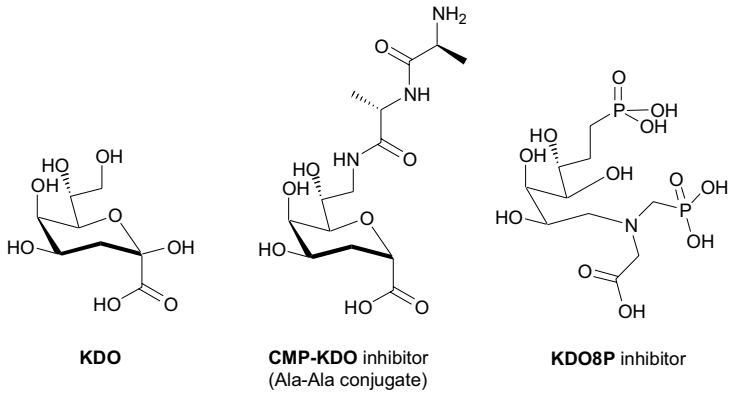



**FIGURE 4** | Kdo analogues as inhibitors of the LPS biosynthesis pathway.

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.

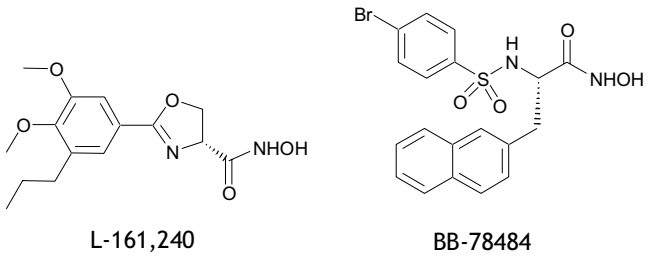



FIGURE 5 | Inhibitors of LpxC, a deacetylase in the LPS biosynthesis pathway.

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]

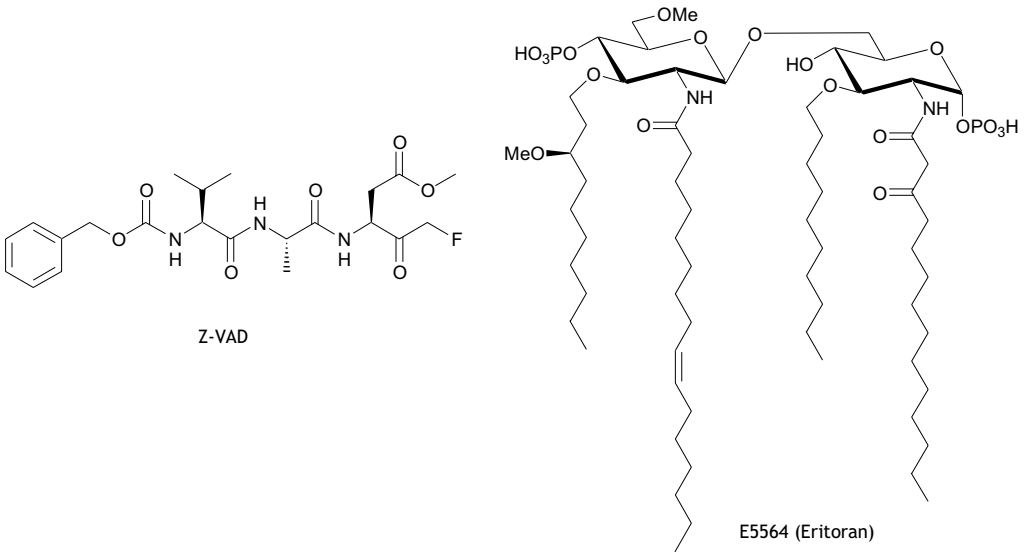



FIGURE 6 | Structure of the caspase inhibitor Z-VAD and E5564, a compound displaying LPS antagonism.

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.

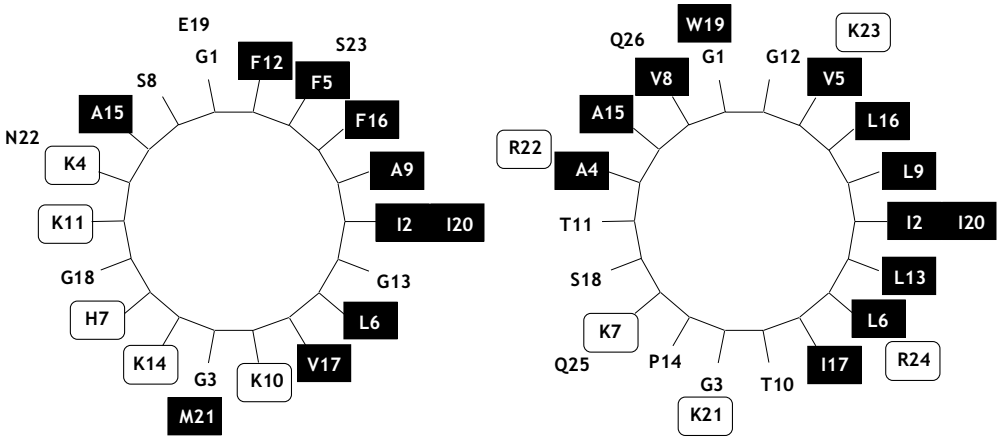
**FIGURE 7** | 3D structures based on NMR models showing the diversity of CAPs, in solution (**A**) or in membrane mimetic conditions (**B-D**).[202b] **A.** human ß-defensin 2 (hBD-2), a triple-stranded ß-sheet with 3 Cys-Cys bridges; **B.** magainin 2, α-helix; **C.** ß-turn/loop structure of bovine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

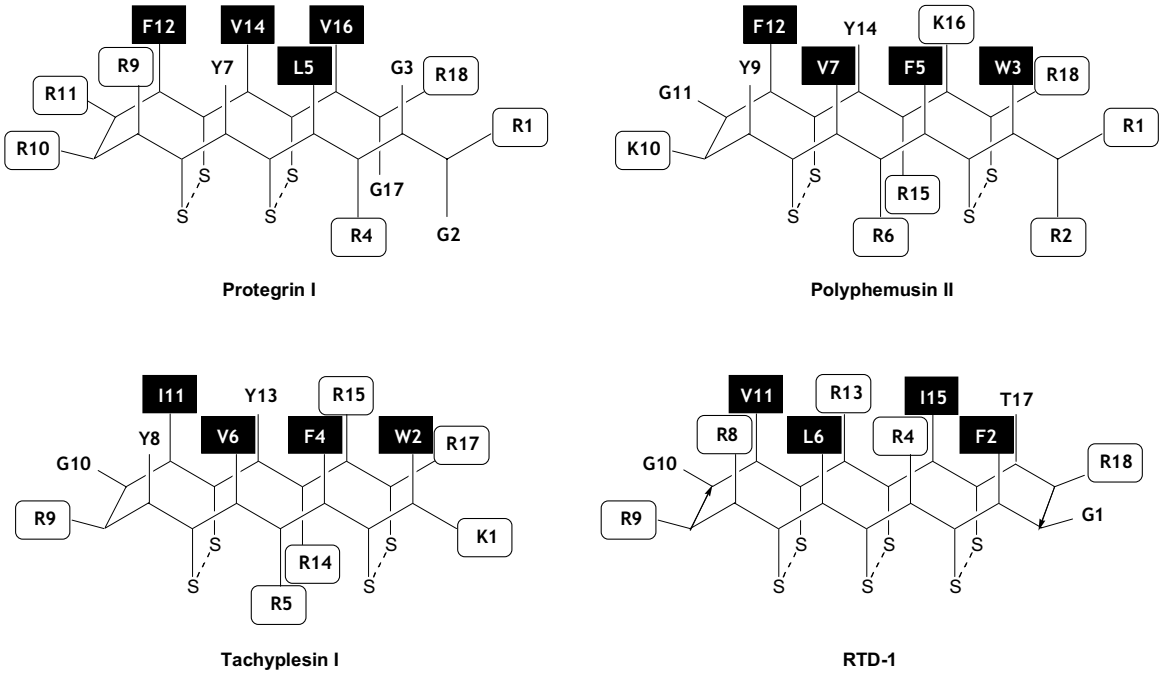



FIGURE 9 | Amphiphilic ß-sheet structures showing hydrophobic (■) and cationic (□) regions for protegrin I, polyphemusin II and tachyplesin I. Note that there is no obvious separation between sides in rhesus θ-defensin RTD-1.

### *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).

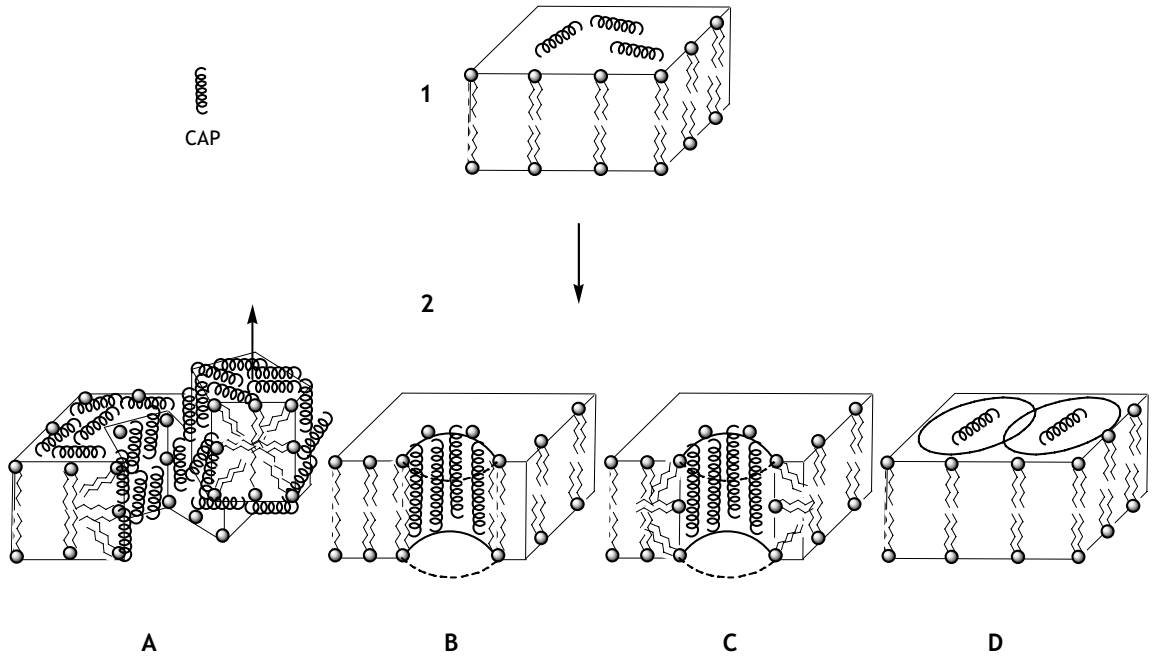



**FIGURE 10** | After initial binding of the CAPs to the membrane by virtue of electrostatic interactions (**1**), four possible models (**2**) have been suggested leading to death of the bacterium; Carpet model (**A**), Barrel/Stave model (**B**), Wormhole model (**C**) and In-Plane Diffusion model (**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 $\beta^3$-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]

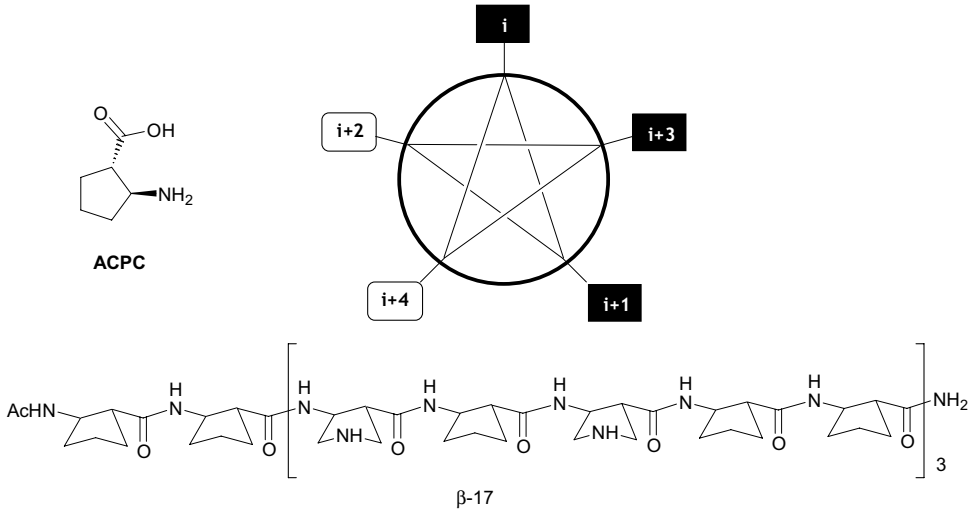



FIGURE 11 | ACPC constrained residue, helical wheel representation indicating ~5 residues per turn in amphiphilic antimicrobial ß-peptide ß-17. View along the helical axis. ■ - hydrophobic residue; □ - cationic residue.

### *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]

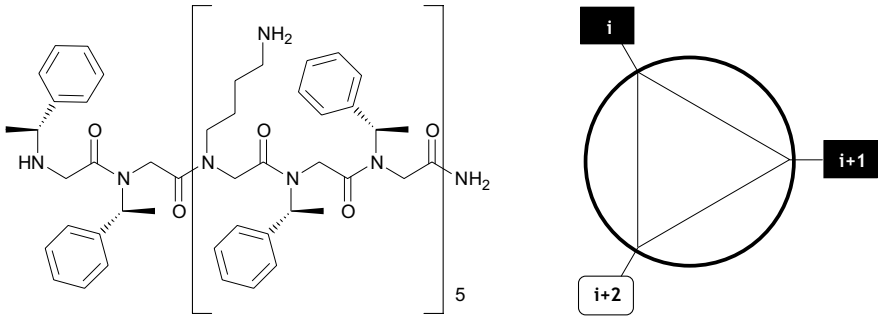



FIGURE 12 | Antimicrobial peptoid and helical wheel representation indicating ~3 residues per turn. View along the helical axis. ■ - hydrophobic residue; □ - cationic residue.

## 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.

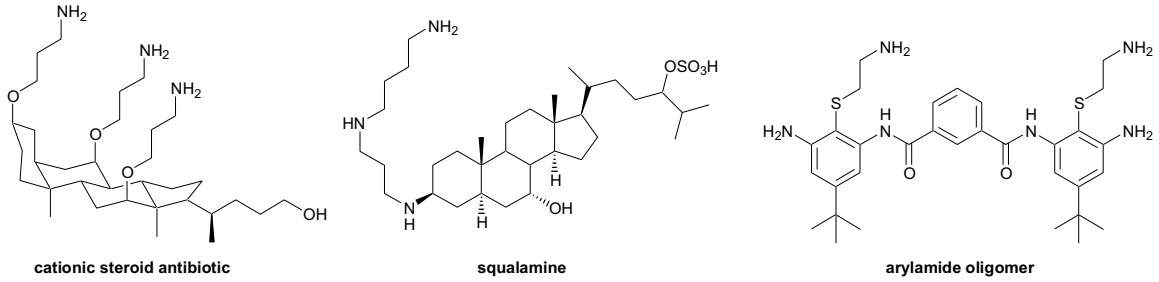



FIGURE 13 | Amphiphilic compounds displaying antibacterial activity.

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]

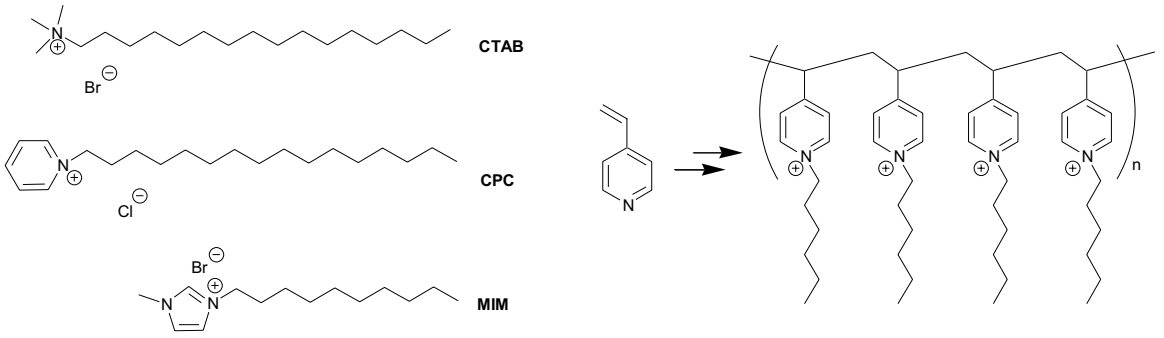



FIGURE 14 | Quaternary ammonium amphiphilic antibacterial compounds (*left*); known cetyltrimethyl-ammonium bromide (CTAB), cetylpyridinium chloride (CPC), and *N*-methyl-*N'*-decyl imidazolium bromide (MIM). *Right:* polymerized alkylated vinylpyridine.

## 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]

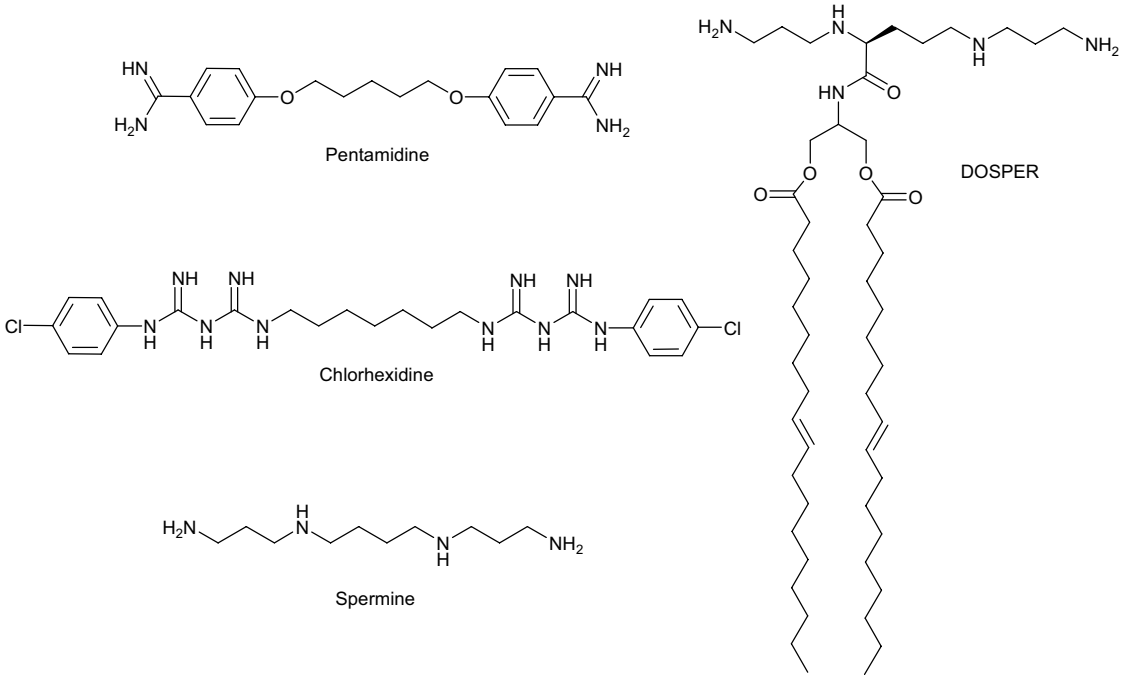



FIGURE 15 | Structures of pentamidine, chlorhexidine, spermine and DOSPER.

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.

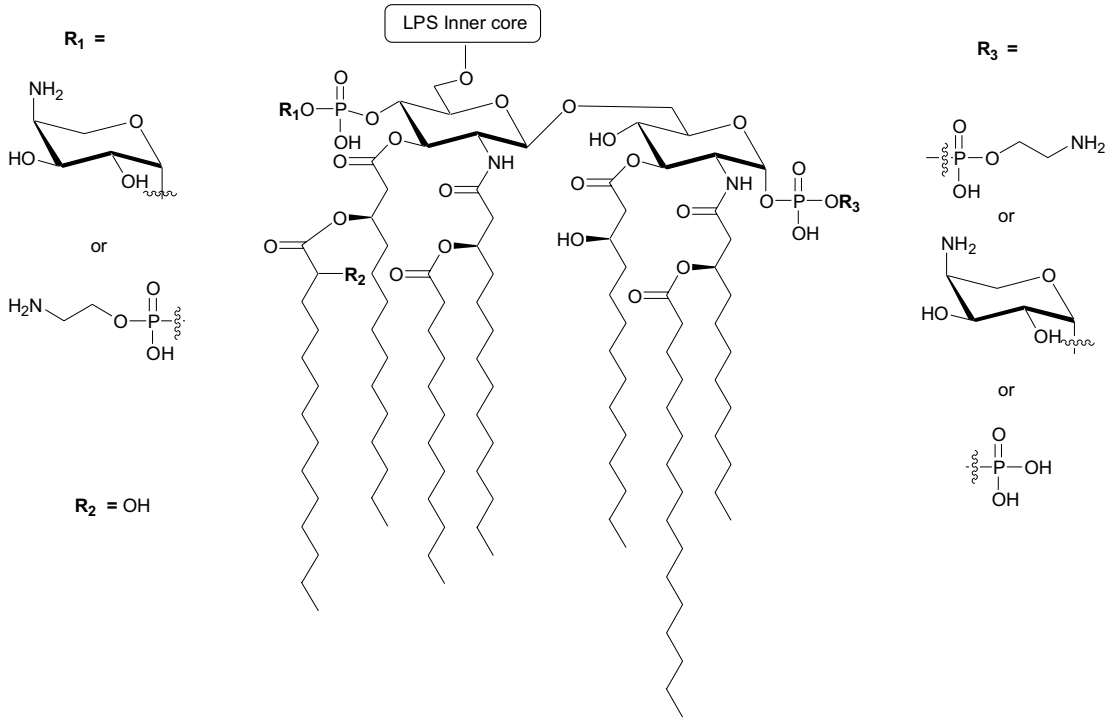



**FIGURE 16** | Covalent modifications observed in *E. coli* and *Salmonella typhimurium* Lipid A, resulting in diminished sensitivity towards CAPs. In unmodified Lipid A, $R_1 = R_2 = R_3 = H$.

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.<br>+ 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.<br>+ 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.<br>- 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.<br>- CAPs composed of proteogenic acids are inherently susceptible towards proteolytic cleavage, requiring studies towards stabilization.<br>- Oral availability of most CAPs is low or zero.<br>- 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.<br>- 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.

## 11 | Notes & References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# CHAPTER 1 | Biological Evaluation of Stabilized Drosocin Analogues

de Visser, P.C.; van Hooft, P.A.V.; de Vries, A.-M.; de Jong, A.; van der Marel, G.A.; Overkleeft, H.S.; Noort, D. Biological evaluation of Tyr6 and Ser7 modified drosocin analogues. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2902

## 1.1 | Introduction

Cationic antimicrobial peptides (CAPs) have found attraction as lead structures in combating bacterial infections,[1] and much research effort has been directed in recent years towards the elucidation of their mode of action.[2,3] From the amassed data it becomes apparent that a majority of CAPs exert their activity through adoption of a defined secondary structure upon contact with anionic bacterial cell membrane components. As a result, either bacterial cell lysis or disturbance of membrane transport events occurs, with bacterial cell death as a result. The fact that a large number of CAPs appear to be indiscriminate to cell type and are often equally effective in killing mammalian cells normally limits their use to topical applications.

Drosocin, a CAP isolated from *Drosophila melanogaster*, is a 19-mer peptide containing three Pro-Arg-Pro repeats and an *O*-glycosylation site at Thr11 (Table 1).[4] The antibacterial activity of drosocin, like other members of the proline-rich CAPs (Table 1), appears not to be based on cell lysis. Rather, drosocin's activity towards bacteria is based on inhibition of a specific intracellular heat-shock protein named DnaK resulting in cell death.[5] This finding explains the observation that *all-D* drosocin, composed of D-amino acids,[6] does not possess any antibacterial activity, in contrast to most *all-D* CAPs.[7] Drosocin, and related proline-rich CAPs, are further distinguished from other CAPs by the lack of toxicity towards human erythrocytes and their apparent bias in activity towards Gram-negative bacteria.[4] These properties combined make drosocin a very attractive lead structure in the search for new and effective antibacterial agents.

**TABLE 1** | Sequences of drosocin and selected related Pro-rich CAP family members.

| CAP | Sequence |
|---|---|
| Drosocin | G - K P R P Y S P R P T* S H P R P I R V |
| Pyrrhocoricin | V D K G S - Y L P R P T* - P P R P I Y N R N |
| Formaecin I | G - R P N P V N N K P T* P H P R - L |

* Glycosylation site

A major hurdle in the development of drosocin-based antibacterial agents is the inherent instability of drosocin towards proteolytic activities present in mammalian sera. In fact, drosocin and its congeners are degraded in sera at such a rate that effective treatment of bacterial infections would require relatively large doses of the peptide.[8] Any strategy that leads to drosocin

analogues with enhanced proteolytic stability without impairing antibacterial activity should therefore be an important step forwards in the development of new antibacterial agents.

## 1.2 | First Generation Drosocin Analogues

### 1.2.1 | Design & Synthesis

The reported finding that the first step in the proteolytic degradation of drosocin in human serum comprises cleavage of the peptide bond[8] between Tyr6 and Ser7 was an incentive to design a series of drosocin (DRC) analogues (**2-12**, Table 2) in which either of the two or both positions at the cleavage site were substituted. This included replacement of Ser7 with Leu (**3**), Thr (**4**, **10**), D-Ser (**5**, **11**), *N*-Me-Ser (**2**) or $\beta^3$-HSer (**6**, **12**; see Figure 1) and replacement of Tyr6 with Phe (**8, 10**), D-Tyr (**7**, **11**) or $\beta^3$-HTyr (**9**, **12**). As a negative control, unrelated peptide **17** was included in the array.[9]

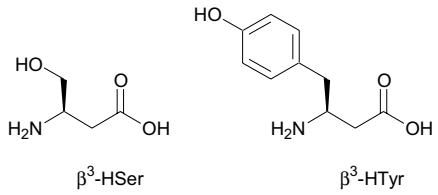


**FIGURE 1** | Structures of $\beta^3$-HSer and $\beta^3$-HTyr used in this study; in this nomenclature, H indicates a homoamino acid with the amine function at the ß-carbon and the sidechain connected to the 3rd backbone carbon atom. Fmoc derivatives of both amino acids are commercially available.

Although non-glycosylated DRC (nG-DRC **1**, Table 2) is about 7 times less active than native drosocin,[4] it was decided to omit the *O*-glycosyl modification at Thr11 to simplify the synthetic procedure. The preparation of the non-glycosylated linear peptides in Table 2 was accomplished using standard Fmoc-based SPPS techniques using the appropriate Fmoc-amino acid building blocks and PyBOP/DiPEA as the activator system. Automated peptide synthesis was employed in all instances except for the Tyr6-*N*-Me-Ser7 stretch in **2** that was introduced manually. In this particular case, the $N^{\alpha}$-methyl amino acid was introduced on the growing peptide chain using PyBOP and successive elongation with tyrosine was achieved using the stronger activator

PyBroP.[10] All peptides were purified to homogeneity by reverse phase HPLC prior to biological assessment.

TABLE 2 | Synthetic peptides used in this study.

| # | Sequence[A] | HRMS [M+H]$^+$ | |
|---|---|---|---|
| | | Calculated | Found |
| **1** | GKPRP **Y** SPRP TSHPR PIRV | 2197.234 | 2197.220 |
| **2** | GKPRP **Y$^{Me}$S**PRP TSHPR PIRV | 2211.250 | 2211.237 |
| **3** | GKPRP **Y L**PRP TSHPR PIRV | 2223.294 | 2223.280 |
| **4** | GKPRP **Y T**PRP TSHPR PIRV | 2211.250 | 2211.241 |
| **5** | GKPRP **Y $^D$S**PRP TSHPR PIRV | 2195.255 | 2195.236 |
| **6** | GKPRP **Y $^ß$S**PRP TSHPR PIRV | 2211.250 | 2211.241 |
| **7** | GKPRP **F** SPRP TSHPR PIRV | 2181.240 | 2181.251 |
| **8** | GKPRP **$^D$Y** SPRP TSHPR PIRV | 2197.234 | 2197.201 |
| **9** | GKPRP **$^ß$Y** SPRP TSHPR PIRV | 2211.250 | 2211.241 |
| **10** | GKPRP **F T**PRP TSHPR PIRV | 2195.255 | 2195.236 |
| **11** | GKPRP **$^D$Y $^D$S**PRP TSHPR PIRV | 2197.234 | 2197.218 |
| **12** | GKPRP **$^ß$Y $^ß$S**PRP TSHPR PIRV | 2225.266 | 2225.197 |
| **17** | NTDGS T DYGI LQINS R | 1753.8[B] | 1753.7[B] |

[A] Modifications are highlighted in bold face, $^{Me}$ = *N*-methylated amino acid $^D$ = D-amino acid, $^ß$ = ß$^3$-homo-amino acid; [B] not HRMS.

## 1.2.2 | Antibacterial Activity

The results of the analysis of the antibacterial activity of DRC analogues **1-12** against *E. coli* ATCC 11775 are summarized in Table 3.[11] None of the prepared peptides possess any hemolytic activity at concentrations up to 300µM. *N*-Methylation of the endopeptidase-sensitive peptide bond (peptide **2**) results in a considerably lower antibacterial activity as compared to the unglycosylated native form (**1**).

In contrast, the DRC analogues containing a single amino acid substitution of Tyr6 or Ser7 (compounds **4-9**) display equal or better antibacterial activity. Analogue **3** however, which contains a Ser7→Leu replacement to resemble the stable Tyr-Leu-Pro-Arg-Pro (YLPRP) pentamer present in the related CAP pyrrhocoricin[9] (Table 1), displayed a loss in activity. The double mutant DRC analogues **11** and **12** were four times less active than reference compound **1**. The positive exception is represented by peptide **10** with a 3.1µM minimal inhibitory concentration.

**TABLE 3** | MIC (minimal inhibitory concentration) values of drosocin analogues and serum stability assay.

| Compound | MIC (μM)[A] | Intact (%)[B] |
|---|---|---|
| **1** (nG-DRC) | 6.3 | 3 |
| **2** | 25 | 11 |
| **3** | 12.5 | n/d |
| **4** | 1.6 | 15 |
| **5** | 3.1 | 1 |
| **6** | 3.1 | 31 |
| **7** | 3.1 | 19 |
| **8** | 6.3 | 16 |
| **9** | 3.1 | 2 |
| **10** | 3.1 | 26 |
| **11** | 25 | 17 |
| **12** | 12.5 | <1 |
| **17** | >100 | n/d |

[A] Against *E. coli* ATCC 11775; [B] Percentage of intact peptide after 8h of digestion in 25% human serum as determined by MALDI-MS peak height of the corresponding peptide; n/d - not determined.

## 1.2.3 | Serum Stability

The next research objective was to evaluate the proteolytic stability of non-glycosylated drosocin (**1**) and analogues **2** and **4-12**. The experiments were performed in 25% pooled human serum to increase peptide recovery and to allow monitoring of the degradation over an 8h period. The relative abundance of the intact peptide was determined by comparison of the MALDI mass spectral peak heights with those at t=0.[8,12]

Based on the results, non-glycosylated drosocin and analogues **1**, **2** and **4-12** can be roughly divided into two categories. The first is represented by the peptides that are almost completely degraded after a digestion period of eight hours (that is, less than 5% intact peptide remains). In this group, analogues **5**, **9**, and **12** accompany reference compound **1.** Peptides **2**, **4**, **6-8, 10** and **11** form the second category and display improved serum stability. Interestingly, all analogues comprising single proteogenic amino acid replacements (**4**, **7**, and **10**) appear to be more stable than parent peptide **1**. On average, 20% of intact peptide was detected after eight hours of digestion in serum, compared to only 3% for **1**. The improved serum stability and antimicrobial activity found for these peptides (**4**, **7** and **10**) illustrate that substitution of one or two amino acids by closely related ones can already be beneficial.

The analogues having substitutions with $\beta^3$- or D-amino acids feature distinct differences in serum stability. For instance, the serum stability was improved ten times by the introduction of $\beta^3$-serine moiety at position 7 (**6**), while the other two $\beta^3$-amino acid containing analogues (**9**, **12**)

demonstrated a proteolytic stability similar to **1**. The opposite result was observed for the D-amino acid containing analogues **5**, **8** and **11**. Here, 16 and 17% of the peptide remained intact after 8h of proteolytic digestion in the case of analogues **8** and **11**, respectively, whereas a single L- to D-serine replacement in **5** did not improve the serum stability at all.

In addition to the percentage of intact peptide after 8h of proteolytic digestion, early degradation products provide useful information about the serum stability of the peptides tested. For this purpose, the degradation fragments formed after two hours of digestion were identified (Table 4), together with their respective intensities.[12]

The most abundant degradation product of non-glycosylated DRC (**1**) after 2h is lacking 6 *N*-terminal residues (*i.e.* cleavage between Tyr6 and Ser7), which is consistent with literature data.[8] This particular cleavage product is considerably less abundant from drosocin analogues **2**-**12** or even fully absent.[13] *N*-methylation (in **2**) yields the lowest increase in stability of the susceptible amide bond. As mentioned previously, the proteolytic stabilities of peptides **5**, **9** and **12** are similar to **1**, but the low serum stability of these analogues is reflected by the high abundances of proteolytic fragments –N1, –N3 and –N5, rather than fragment –N6 which is the most abundant cleavage product in **1**.

**TABLE 4** | Abundances of major degradation products.

| | **Degradation products**[A] | | | | |
|---|---|---|---|---|---|
| **Compound** | -N1 | -N3 | -N5 | **-N6** | -N8 |
| **1** | 2 | 1 | 0 | **5** | 3 |
| **2** | 1 | 1 | 1 | **3** | 0 |
| **4** | 1 | 1 | 1 | **1** | 1 |
| **5** | 5 | 3 | 2 | **1** | 0 |
| **6** | 1 | 1 | 1 | **1** | 1 |
| **7** | 2 | 1 | 1 | **2** | 2 |
| **8** | 1 | 1 | 1 | **0** | 0 |
| **9** | 3 | 2 | 3 | **0** | 0 |
| **10** | 1 | 1 | 1 | **2** | 2 |
| **11** | 2 | 1 | 1 | **1** | 0 |
| **12** | 4 | 3 | 5 | **0** | 1 |

[A] Major degradation products are displayed as missing N-terminal residues after 2h digestion in 25% human serum, *e.g.* -N3 indicates loss of the first three N-terminal residues. The relative MALDI-MS spectral peak heights were measured and divided into 6 classes (not detected (0), 1-25mm (1), 26-50mm (2), 51-75mm (3), 76-100mm (4) and >100mm (5)). Numbers are averages of three experiments.

## 1.3 | Second Generation Drosocin Analogues

Based on the results of the 1st generation DRC analogues, attention was focussed on further stabilization of DRC analogues against proteolytic inactivation. Fragments missing some of the N-terminal residues were encountered in the 1st generation analogues (see Table 4) as a result of aminopeptidase activities. Due to its favorable outcome in the stability/antimicrobial tests and its completely proteogenic composition, compound **4** (Ser7→Thr) was selected as lead structure for further elaboration. On the premise that N-terminal capping of natural peptides may result in enhanced stability towards aminopeptidase activities, the N-terminal Gly residue in **4** was replaced subsequently by sarcosine (Sar, *N*-methylglycine) and the lengthened Gly derivatives β-alanine (βAla), γ-aminobutyric acid (Abu) and δ-aminovaleric acid (Ava).

TABLE 5 | 2nd Generation DRC analogues containing N-terminal modifications.

| Compound | Sequence[A] | HRMS Calculated | HRMS Found |
|---|---|---|---|
| **13** | **Sar** KPRP Y**T**PRP TSHPR PIRV | 2225.266 | 2225.263 |
| **14** | ß**Ala**KPRP Y**T**PRP TSHPR PIRV | 2225.266 | 2225.260 |
| **15** | **Abu** KPRP Y**T**PRP TSHPR PIRV | 2239.281 | 2239.282 |
| **16** | **Ava** KPRP Y**T**PRP TSHPR PIRV | 2253.671 | 2253.308 |

[A] Modifications are highlighted in bold face.

The resulting set of doubly modified DRC analogues **13**-**16** (Table 5) was subjected to identical stability and antimicrobial assays as the 1st generation analogues. Obtained MIC values are summarized in Table 6. Compared to parent peptide **4** (MIC 1.6µM), its derivatives **13**-**16** have on average slightly reduced MIC values, although comparison with DRC (**1**, MIC 6.3µM) shows that these 2nd generation analogues are as potent as the natural non-glycosylated CAP. None of the 2nd generation DRC analogues displayed hemolytic activity up to concentrations of 300µM. N-terminal stability is increased within these N-terminally modified analogues as no –N1, –N3 or –N5 fragments were detected (Table 6). The overall stability is increased dramatically as ~80% of these peptides remains intact after 8h incubation in serum – for analogue **14** an increase of ~5.5-fold compared with peptide **4** and ~30-fold regarding peptide **1** (see also Figure 2 for selected degradation curves).

TABLE 6 | MIC values of 2nd generation DRC analogues and serum stability assay. For comparison, data of lead structure **4** is added.

| | | | Degradation Products after 2h | | | | |
|---|---|---|---|---|---|---|---|
| **Compound** | **MIC (μM)[A]** | **Intact (%)[B]** | -N1 | -N3 | -N5 | **-N6** | -N8 |
| **4** | 1.6 | 15 | 1 | 1 | 1 | **1** | 1 |
| **13** | 6.3 | 77 | 0 | 0 | 0 | **1** | 1 |
| **14** | 3.1 | 87 | 0 | 0 | 0 | **2** | 0 |
| **15** | 6.3 | 76 | 0 | 0 | 0 | **2** | 1 |
| **16** | 6.3 | 84 | 0 | 0 | 0 | **1** | 1 |

[A] Minimal inhibitory concentration against *E. coli* ATCC 11775; [B] Percentage of intact peptide after 8h of digestion in 25% human serum as determined by MALDI-MS peak height of the corresponding peptide.

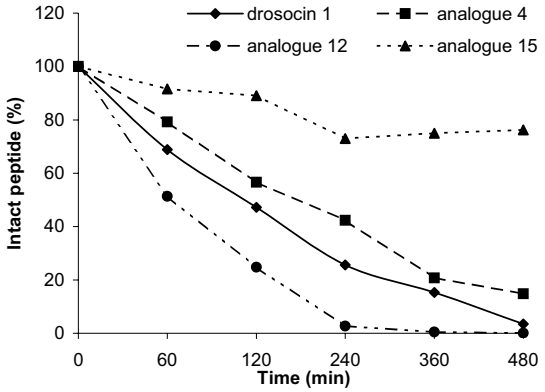


FIGURE 2 | Selected degradation curves (averages of three experiments) of nG-DRC **1** and analogues **4**,**12** and **15**.

## 1.4 | Conclusion

Substitution of selected amino acid residues at sites that are susceptible to proteolytic cleavage results in DRC analogues with enhanced stability, while leaving the antibacterial properties intact. In the 1st generation DRC analogues, both antimicrobial activity and stability were increased with respect to native nG-DRC **1**. Analogue **4** was selected as lead for the design of a 2nd generation of analogues containing a modified N-terminus to overcome unwanted aminopeptidase inactivation. N-terminal modifications on **4** led to the synthesis of a 2nd generation of DRC analogues (**13-16**) with almost 30-fold increase in stability compared to nG-

DRC **1**, while being equally potent. For future studies towards the design of DRC-based drugs, analogues **13-16** can serve as a new lead structures.

## 1.5 | Experimental Section

### 1.5.1 | General

LCMS analyses were performed using a Jasco 900 LC system with detection at 214 and 254nm connected to a Perkin Elmer Q-TOF monoquad mass spectrometer in positive ion mode. Crude peptides were purified on a ÄKTA Explorer HPLC system. In all cases, Alltech Alltima $C_{18}$ analytical (150x4.6mm or 250x4.6mm) and semi-preparative (250x10.0mm) were used. Applied buffer systems – Jasco: A. $H_2O$; B. MeCN; C. 0.1% aq. TFA (always 10% C present in the gradients); ÄKTA: A. 0.1% TFA in 5% MeCN/$H_2O$; B. 0.1% TFA in 80% MeCN/$H_2O$. Eluents were degassed before use and kept under Ar flow. MALDI-MS analyses were performed on a Bruker Biflex III mass spectrometer. Electrospray tandem mass analyses were performed on a Q-TOF mass spectrometer (Micromass) at a cone voltage of 20V.

### 1.5.2 | SPPS

Peptides were synthesized using either an ABI-433A (50µmol scale, Applied Biosystems) synthesizer or Syro 2000 (10µmol scale, MultiSyntech) using customized Fmoc-based protocols. Amino acid residues were used in 5-fold excess, using PyBOP (5eq.)/DiPEA (10eq., ABI-433A) or BOP (5eq.)/DiPEA (10eq., Syro 2000) as activating systems and 20% piperidine/NMP as Fmoc-deprotecting mixture. The first amino acid, Fmoc-Val-OH (5eq.) was manually coupled to TentaGel PHB resin (0.24mmol/g) with DIC (5eq.) and DMAP (0.1eq.) in DMF for 2h. Loading was established by UV determination of the amount of Fmoc-chromophore released after treatment of a sample with 20% piperidine/DMF for 10min. Loading was 0.20mmol/g (85%). *N*-Methyl amino acids (4eq.) were coupled manually with BOP (4eq.)/HOBt (4eq.)/DiPEA(8eq.) in DMF. Amino acids to be reacted with *N*-methyl amino acids were also coupled manually, using the amino acid (5eq.) and preactivation with PyBroP (5eq.) and DiPEA (10eq.) in DMF. The potent activating reagent HATU worked equally efficient in acylations of *N*-methyl amino acids. In case the chloranil test was positive, coupling was repeated. After removal of the N-terminal Fmoc group, crude peptides were treated with TFA/TIS/$H_2O$ (95/2.5/2.5, v/v/v) for cleavage from the resin and removal of protecting groups, and subsequently precipitated in $Et_2O$. Centrifugation and decantation of solvents yielded the crude products. After HPLC purification, the target peptides were obtained: **1** (nG-DRC), yield 42%, purity 96%, HPLC Rt 9.34min; **2** (S7$^{Me}$S), yield 5%, purity 95%, HPLC Rt 9.29min; **3** (S7L), yield 65%, purity 99%, HPLC Rt 9.36min; **4** (S7T), yield 11%, purity 98%, HPLC Rt 9.20min; **5** (S7$^{D}$S), yield 11%, purity 99%, HPLC Rt 9.20min; **6** (S7βS), yield 5%, purity >99%, HPLC Rt 9.18min; **7** (Y6F), yield 12%, purity 99%, HPLC Rt 9.49min; **8** (Y6$^{D}$Y), yield 7%, purity 90%, HPLC Rt 9.32min; **9** (Y6βY), yield 12%, purity 99%, HPLC Rt 9.14min; **10** (Y6F/S7T), yield 11%, purity 99%, HPLC 9.50min; **11** (Y6$^{D}$Y/S7$^{D}$S), yield 13%, purity 99%, HPLC Rt 9.24min; **12** (Y6βY/S7βS), yield 6%, purity 91%, HPLC Rt 9.18min; **13** (G1Sar/S7T), yield 3.1mg (1,41µmol, 14%), purity >99%, HPLC Rt 9.59min; **14** (G1βAla/S7T), yield 2.5mg (1.41µmol, 11%), purity 98%, HPLC Rt 9.56min; **15** (G1Abu/S7T), yield 2.3mg (1.02µmol, 10%), purity 97%, HPLC Rt 9.55min; **16** (G1Ava/S7T), yield 1.6mg (0.72µmol, 7%), purity 98%, HPLC Rt 9.56min.

### 1.5.3 | Antibacterial Assay

Antibacterial assays were performed in sterilized round-bottom 96-well plates with a final volume of 110µL as follows. The bacteria (*E. coli* ATCC 11775) were grown in nutrient broth E (NBE; beef extract 1g/L, yeast extract 2g/L, peptone 5g/L and NaCl 5g/L) and kept at $4^0$C. Lyophilized peptides were dissolved in NBE to give a concentration of 400µM and filtered using 0.22µm filter discs. A 16h culture in NBE was adjusted to

$5\times10^6$ CFU/mL and transferred to the micro titer plate wells (10µL) containing each 100µL of a serial 2-fold dilution (200-0.4µM) of the tested peptide in NBE. Plates were incubated while gently shaking at $37^0$C for 24h. Next, 80µL suspension of each well was transported into a flat-bottom 96-well plate. The absorbance was measured at 600nm using a µQuant micro plate spectrophotometer (Bio-Tek Instruments). All peptides were measured in quadruplo. The peptide with sequence NTDGSTDYGILQINSR was used as negative control.

### 1.5.4 | Hemolysis Assay

The hemolytic activity of the peptides was determined in quadruplo. Human blood was collected in EDTA-tubes and centrifuged to remove the buffy coat. The residual erythrocytes were washed three times in 0.85% saline. Serial dilutions of the peptides in saline were prepared in sterilized round-bottom 96-well plates using 100µL volumes (100-0.2µM). Red blood cells were diluted with saline to 1/25 packed volume of cells and 50µL of the resulting cell suspension was added to each well. Plates were incubated while gently shaking at $37^0$C for 4h. Next, the micro titer plate was quickly centrifuged and 50µL supernatant of each well was transported into a flat-bottom 96-well plate. The absorbance was measured at 405nm using a µQuant micro plate spectrophotometer (Bio-Tek Instruments). The $A_{blank}$ was measured in the absence of additives and 100% hemolysis ($A_{tot}$) in the presence of 1% Triton X-100 in saline. The percentage of hemolysis is determined as $(A_{pep}-A_{blank})/(A_{tot}-A_{blank})\times100$.

### 1.5.5 | Serum Stability Assay

The serum stability studies were carried out in triplicate. Briefly, 10µL of an aqueous peptide solution (0.8mg/mL) was added to 1mL freshly pooled 25% human serum in PBS. The mixtures were thermostated at $37^0$C under gentle stirring. At t=0, 1, 2, 4, 6 and 8h, 100µL of each mixture was taken out and precipitated in 230µL 15% aq. TCA. Samples were stored at $0^0$C for 20min and centrifuged for 5min at $0^0$C. The supernatants (250µL of each) were immediately stored at -80ºC until analyzed by MALDI mass spectrometry. The spectra were recorded in the linear mode, using α-cyanohydroxycinnamic acid in 50% MeCN in 0.1% aqueous TFA. Serum control samples consisted of 100µL of pooled 25% human serum solution in PBS precipitated in 230µL 15% aq. TCA. Peptide reference samples consisted of 10µL of the peptide stock solution diluted in 1mL $H_2O$, of which 100µL was added to 230µL 15% aq. TCA. MALDI peak heights were measured from prints that were generated using standard procedures.[12]

## 1.6 | Notes & References


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12. The relative abundance of these fragments was calculated under the assumption that both the detector sensitivity and MALDI susceptibility was uniform for all fragments. In Table 4, only fragments with molecular weight of 800 or more were taken into consideration
13. The following degradation products were also detected: –C1,N1 for analogue **7** (abundance 1), –C2,N5 for analogues **2**, **6** and **9** (abundance 3, 1 and 1 respectively) and –N10 (abundance 2) for **16**.

# CHAPTER 2 | Safety-Catch Synthesis & Biological Evaluation of Polymyxin B1 and Analogues

Partly published: de Visser, P.C.; Kriek, N.M.A.J.; van Hooft, P.A.V.; Van Schepdael, A.; Filippov, D.V.; van der Marel, G.A.; Overkleeft, H.S.; van Boom, J.H.; Noort, D. Solid-phase synthesis of polymyxin B1 and analogues *via* a safety-catch approach. *J. Peptide Res.* **2003**, *61*, 298

## 2.1 | Introduction

The family of polymyxins is a group of highly potent cationic antimicrobial peptides (CAPs) isolated from *Bacillus polymyxa*.[1] The general structure comprises (see Figure 1) of a cyclic heptapeptide bound to a linear tripeptide, both of which contain a high percentage of the rare amino acid L-α,γ-diaminobutyric acid (Dab). Furthermore, the γ-amino group of Dab4 is linked *via* an amide bond to the C-terminus of residue 10, while its α-amino group is connected to Dab3 of the linear tripeptide. The α-amino group of the N-terminal Dab residue is acylated with a distinctive hydrophobic chain.[2] Of the substantial amount of unique polymyxins known, only polymyxin B is widely used and studied. Within the polymyxin B mixture, polymyxin B1 (PMB1, **1**) is the most abundant component.

**A. Natural polymyxin families (R = acyl chain)**

| Polymyxin[A] | $AA_3$ | D$AA_6$ | $AA_7$ | $AA_{10}$ | Ref. |
|---|---|---|---|---|---|
| A / M | LDab | Leu | Thr | Thr | 1d,3 |
| B | LDab | Phe | Leu | Thr | 1 |
| C / P | LDab | Phe | Thr | Thr | 4 |
| D | DSer | Leu | Thr | Thr | 5 |
| E | LDab | Leu | Leu | Thr | 6 |
| S | DSer | Phe | Leu | Thr | 7 |
| T | LDab | Phe | Leu | Leu | 8 |

**B. Components of polymyxin B**

| Polymyxin | R |
|---|---|
| B1 (**1**) | (*S*)-6-methyloctanoyl |
| Ile7-B1 | (*S*)-6-methyloctanoyl |
| B2 (**2**) | 6-methylheptanoyl |
| B3 (**3**) | $C_8$ |
| B4 (**4**) | $C_7$ |
| B5 (**5**) | $C_9$ |
| B6 (**6**) | 3-hydroxy-6-methyloctanoyl[B] |

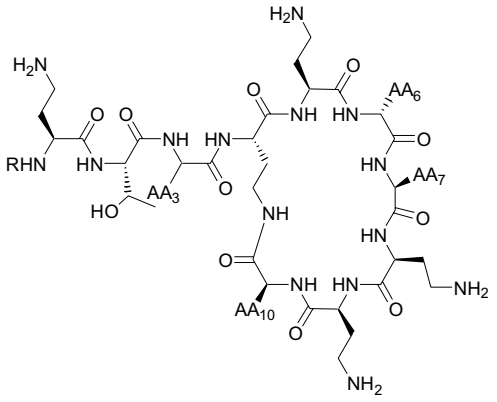



**FIGURE 1** | General structures of polymyxins and subdivision of polymyxin B. [A] Additionally, polymyxin F has been reported (ref. 9); [B] Chiralities at C3 and C6 were not established.

The presence of positively charged residues (*i.e.* the five Dab units), as well as the amphiphilic nature are crucial for PMB1's activity against Gram-negative bacteria. Although the exact mode of action is still a matter of debate, various stages can be distinguished in the membrane

permeabilization process.[10] Initially, binding of **1** to the anionic Lipid A domain[11] leads to disruption of the lipopolysaccharide (LPS) lamellar phase.[12] Next, the hydrophobic tail is inserted into the outer membrane (OM), followed by self-promoted uptake of the remainder of the molecule. After the internalization process, the antibiotic causes disruption of the inner (cytoplasmic) membrane (IM) which eventually leads to cell death.[13]

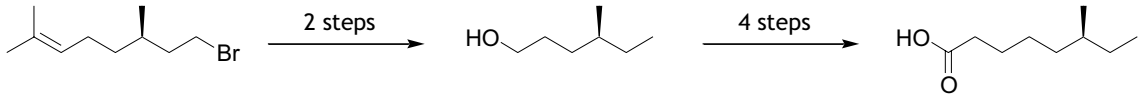
It is also well established that PMB1 decreases the effects of sepsis by binding and neutralizing LPS that is released in the course of Gram-negative infections.[14] These properties make PMB1 a good candidate as antibiotic for therapeutic purposes. However, its nephrotoxicity to humans has thus far limited the clinical use of this antibiotic to topical treatment of infections.[15]

## 2.2 | Design & Synthesis

The first synthesis of PMB1 (**1**) was reported by Vogler and co-workers[16] who constructed the linear peptide by fragment condensation in solution, which was successively cyclized using DCC. Later on, Sharma *et al*.[17] showed for the synthesis of **1** that the linear peptide, obtained *via* SPPS could be cyclized with diphenylphosphoryl azide (DPPA)[18] and DiPEA. Unfortunately, in our hands incomplete DPPA-mediated cyclization led to an inseparable mixture of target compound **1** and its undesired linear counterpart. It was expected that the release of linear fragments could be prevented by executing the cyclization[19] with concomitant cleavage from the solid support.[20] An essential element in this approach is the use of the safety-catch sulfonamide linker, originally developed by Kenner[21] and modified by Backes *et al*.[22] Attractive features of this type of linker are the stability under acidic and nucleophilic conditions and the cleavage by nucleophilic displacement after *N*-alkylation of the sulfonamide moiety. It was reasoned that use of low-substituted resin was desirable in order to prevent any cross coupling during on-resin cyclization.

Thus, the safety-catch linker 3-carboxypropanesulfonamide (SCL) was quantitatively coupled to low-loaded polystyrene aminomethyl (AM) resin by using DIC/HOBt to give **7** (see Scheme 2, page 61). Subsequent PyBOP-mediated[22] coupling of Fmoc-Thr(tBu)-OH afforded **8** in low yields (<20%), which is probably caused by the relatively low reactivity of the Thr residue and/or the sulfonamide group. Alternatively, **8** can be obtained *via* coupling of the amino acid fluoride Fmoc-Thr(tBu)-F to **7**.[23] In our hands, the highest yield (64%) was obtained after prolonging the reaction time to 5h, and increasing the excess of Fmoc-Thr(tBu)-F and base to 9

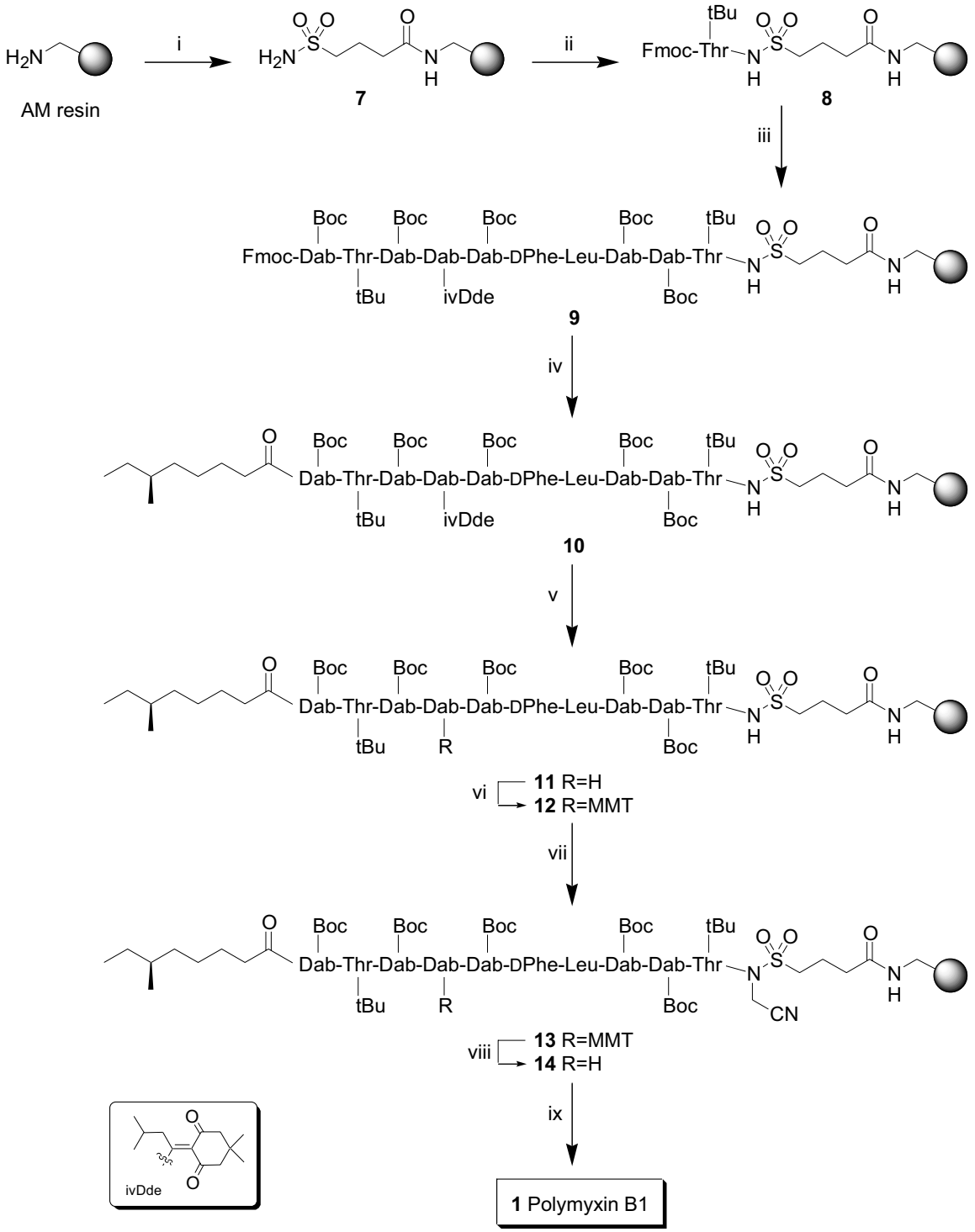
and 18eq., respectively. Sequential elongation of **8**, resulting in immobilized peptide **9**, was effected by BOP/HOBt/DiPEA-mediated condensation with suitably protected Fmoc amino acids.[24] With respect to residue Dab4, efforts were dedicated towards the incorporation of this Dab residue bearing a Mtt functionality, as the mild acidic removal of this group would be fully compatible with our approach. However, the synthesis of Fmoc-Dab(Mtt)-OH gave unsatisfactory low yields.[25] This problem could be avoided by incorporation of an ivDde-protected Dab residue. Compared with the more common Dde group, the ivDde group shows an increased stability towards piperidine treatment and is less prone to migration. The assembly of the linear polymyxin molecule **10** was completed by removal of the Fmoc-group in **9** and subsequent coupling of (*S*)-6-methyloctanoic acid using BOP/HOBt/DiPEA. The octanoic acid derivative was prepared as follows (Scheme 1): reduction of commercially available (*R*)-citronellyl bromide with $LiAlH_4$ was followed by ozonolysis with a reductive work-up ($NaBH_4$) afforded *S*-(+)-4-methylhexanol.[26] Finally, *S*-(+)-4-methylhexanol was converted into (*S*)-6-methyloctanoic acid as described.[26c]




(*R*)-citronellyl bromide (*S*)-4-methylhexanol (*S*)-6-methyloctanoic acid

**SCHEME 1** | Preparation of the chiral fatty acid residue present in polymyxin B1.

In order to secure the final cyclization of the amino group of Dab4 with Thr10 in activated **14**, the ivDde group in **10** was removed by hydrazinolysis (→**11**) and replaced with the MMT group (→**12**). Alkylation of **12** with $ICH_2CN$ afforded activated sulfonamide **13**.[22] Removal of the MMT group from Dab4 in **13** by treatment with TFA/TIS yielded the partially protected and immobilized peptide **14**. Cyclization of **14** with concomitant release of the cyclic peptide from the solid support was effected with DiPEA in THF. Removal of the remaining side-chain protecting groups by acidolysis furnished **1** (Figure 2A).




**SCHEME 2** | Synthesis of polymyxin B1 (**1**). Reagents and conditions: (i) 3-carboxypropanesulfonamide, DIC, HOBt, DMF; (ii) Fmoc-Thr(tBu)-F, DiPEA, $CH_2Cl_2$, 64%; (iii) Fmoc-based SPPS applying 20% piperidine/NMP (Fmoc deprotection), amino acids/BOP/HOBt/DiPEA in DMF/NMP (coupling) and $Ac_2O$/HOBt/DiPEA (capping); (iv) 1. 20% piperidine/DMF; 2. (*S*)-6-methyloctanoic acid, BOP/HOBt/DiPEA, DMF/NMP; (v) 2% $NH_2NH_2{\cdot}H_2O$, DMF; (vi) MMTCl, DiPEA, NMP; (vii) $ICH_2CN$, DiPEA, NMP; (viii) TFA/TIS/$CH_2Cl_2$ 3/5/92 (v/v/v); (ix) 1. DiPEA, THF; 2. TFA/TIS/$H_2O$ 95/2.5/2.5 (v/v/v).

The high purity of the crude product is indicative of the potential of the safety-catch approach towards polymyxins. Semi-preparative HPLC purification afforded pure **1** (Figure 2B). The corresponding linear PMB1 was not present, as indicated by LCMS analysis of the crude product. The synthetic compound co-eluted with PMB1 that was isolated from a commercial sample of polymyxin B.[2] In addition, MS/MS analysis revealed that the fragmentation pattern of **1** was, in every aspect, identical to the one reported recently.[27] Following the same safety-catch procedure as for PMB1, the natural polymyxins PMB3 (**3**), PMB4 (**4**) and PMB5 (**5**) were synthesized for comparison of activity with PMB1 (**1**).

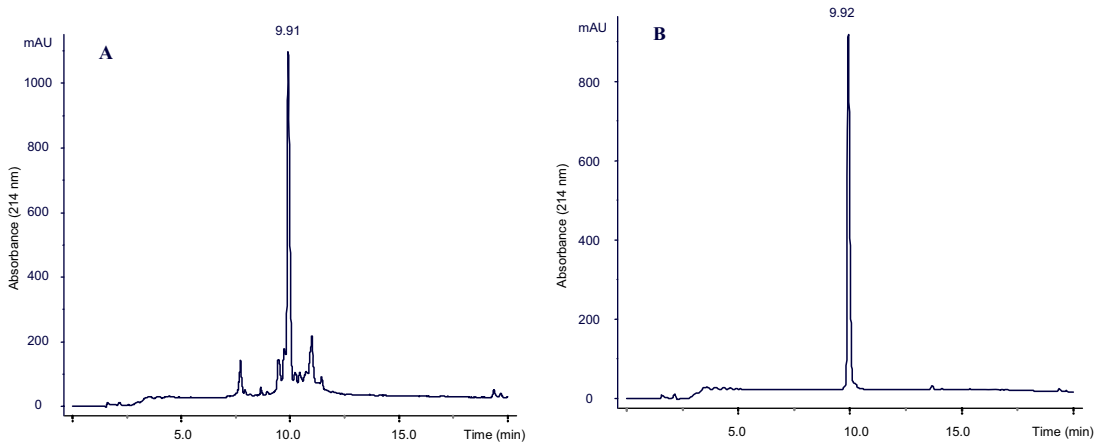



FIGURE 2 | HPLC traces of PMB1 (**1**). (**A**) crude after cyclization and deprotection; (**B**) after HPLC purification.

Having developed a convenient synthetic route towards the natural polymyxins B1 and B3-5, attention was directed towards the design and synthesis of non-toxic analogues. It has been proposed that the acyl chain and hydrophobic DPhe6/Leu7 dipeptide may contribute to the toxicity of PMB1.[28] Based on this concept, both segments were varied independently to give two series of analogues. The first series comprised of those in which the (*S*)-6-methyloctanoic acid moiety was replaced with other acyl chains, including 1-adamantane acetic acid (**15**, Figure 3), synthesized using an identical procedure as for PMB1. In the second series, the dipeptide DPhe6/Leu7 was replaced with unnatural, commercially available Fmoc-protected dipeptide mimics: the β-turn inducing amino acid (*S*)-3-amino-1-carboxymethyl-caprolactame (Capro **16**, Figure 3),[29] the five rigid moieties *N*-carboxymethylpiperazine (Cmpi **17**), *m*-aminomethylbenzoic

acid (Mamb **18**),[30] *p*-aminomethylbenzoic acid (Pamb, **19**), 4-phenylpiperidine-4-carboxylic acid (PhPip **20**) and tranexamic acid (Tran **21**)[31] and finally the flexible δ-aminovaleric acid (Ava **22**).

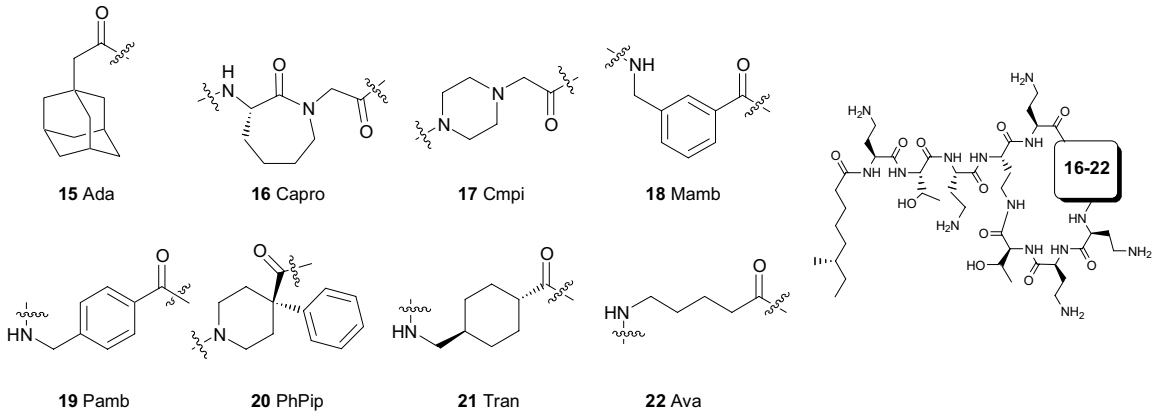



**FIGURE 3** | Acyl chain (**15**) and dipeptide mimics (**16**-**22**) used for substitution of the original DPhe6-Leu7 dipeptide in PMB1 (rightmost structure).

The synthesis of all analogues was performed by slight adaptation of the procedure depicted in Scheme 2: double couplings were applied for the incorporation of the dipeptide mimics **16**, **18**, **19**, and **20**. Application of the cleavage-cyclization strategy afforded analogues **24**-**33** in 37-67% purity based on HPLC analysis of the crude peptides (see Figure 4 for two representative examples). In contrast to a different on-resin cyclization reaction reported recently,[32] these results nicely illustrate that the cleavage-by-cyclization reaction appears to be deprived of structural requirements and is generally applicable.

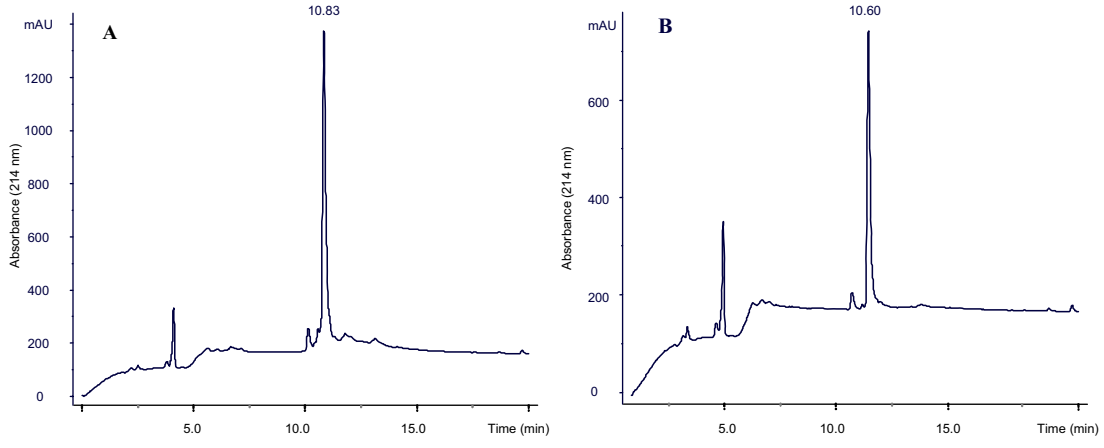



**FIGURE 4** | Representative HPLC traces of crude PMB1 analogues; **A** Mamb-PMB1 **29**; **B** Ava-PMB1 **33**.

## 2.3 | Biological Evaluation

### 2.3.1 | Antibiotic activity

After semi-preparative HPLC purification, natural PMBs and analogues were evaluated for their antibiotic activity against *Escherichia coli* ATCC 11775. As summarized in Table 1, the acyl chain modified derivatives (**23** and **24**) exhibit antimicrobial activities similar to **1**. Contrarily, the shorter pentanoyl and butanoyl derivatives **25** and **26** are considerably less potent than analogues **23** and **24**, implying that stepwise shortening of the acyl chain results in an increase in MIC value. However, analogues containing rigid dipeptide mimics (*i.e.* compounds **27**-**30**) as well as those bearing extended conformation mimicking elements (**31** and **32**) are devoid of any significant antimicrobial activity up to concentrations of 50μg/mL. For PMB1 analogue **33**, containing the flexible δ-aminovaleric acid moiety, a very modest MIC value was determined (500μg/mL).

**TABLE 1** | Antimicrobial data on synthesized natural polymyxins and analogues.

| Polymyxin | R[A] | X-Y[A] | MIC (μg/mL)[B] |
|---|---|---|---|
| **1** | (*S*)-6-methyloctanoyl | DPhe6-Leu7 | 0.3 |
| **3** | $C_8$ | DPhe6-Leu7 | 0.6 |
| **4** | $C_7$ | DPhe6-Leu7 | 0.3 |
| **5** | $C_9$ | DPhe6-Leu7 | 0.3 |
| **23** | Ada **15** | DPhe6-Leu7 | 0.9 |
| **24** | $C_6$ | DPhe6-Leu7 | 0.7 |
| **25** | $C_5$ | DPhe6-Leu7 | 11 |
| **26** | $C_4$ | DPhe6-Leu7 | 23 |
| **PMB1 Analogue** | | | |
| **27** | (*S*)-6-methyloctanoyl | Capro **16** | -[C] |
| **28** | (*S*)-6-methyloctanoyl | Cmpi **17** | -[C] |
| **29** | (*S*)-6-methyloctanoyl | Mamb **18** | -[C] |
| **30** | (*S*)-6-methyloctanoyl | Pamb **19** | -[C] |
| **31** | (*S*)-6-methyloctanoyl | PhPip **20** | -[C] |
| **32** | (*S*)-6-methyloctanoyl | Tran **21** | -[C] |
| **33** | (*S*)-6-methyloctanoyl | Ava **22** | 500 |

[A] See Figure 1 for general polymyxin structure and Figure 3 for structures of **15**-**22**; [B] minimal inhibitory concentration against *E. coli* ATCC 11775; [C] no 100% inhibition detected at concentrations up to 50μg/mL.

### 2.3.2 | LPS Affinity

The affinity for LPS was evaluated using a displacement assay.[33] In this assay, affinity of PMB analogues **4**, **5**, **24**-**26** and **28**-**33** was determined by competitive binding to LPS in the presence of

dansylated polymyxin B (DPX). In short, to an LPS solution in which the LPS was saturated with DPX, aliquots of PMB analogue were added. Displacement of DPX from LPS by the PMB analogues was observed as decrease in fluorescence. In Figure 5, the DPX displacement curves of the polymyxins that differ in the length of the acyl chain are compared. Both 'natural' PMBs 2 and 3, and the synthetic PMBs 4 and 5 have somewhat less affinity for LPS compared to commercial PMB.[34] Shortening of the acyl chain (analogues **24**-**26**) results in a decreased affinity for LPS (Figure 5),[35] an observation that can be correlated with their respective higher MIC values (*i.e.* $C_4$-PMB (**26**) and $C_5$-PMB (**25**) are the least potent). Figure 6 depicts the LPS affinities of polymyxin analogues **28**-**33** carrying ring substituents.

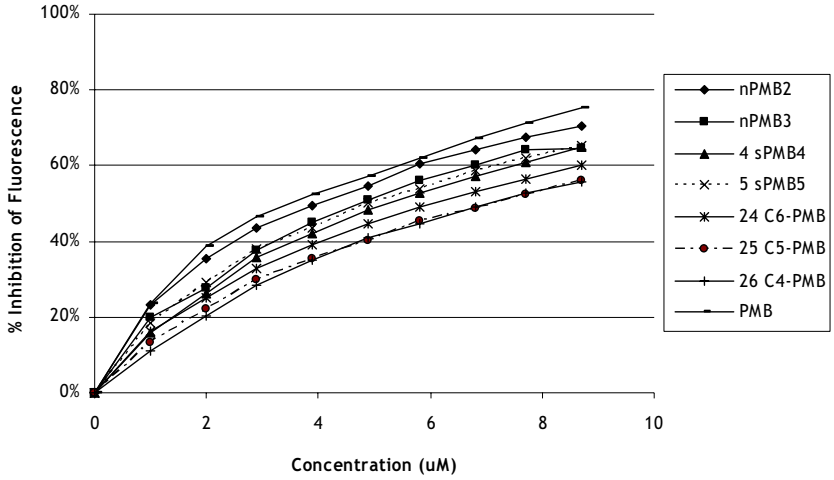



**FIGURE 5** | LPS affinity assay of PMB analogues differing in acyl chain length. n - natural (*i.e.* isolated from a commercial (natural) sample), s - synthetic. Concentration in μM.

Introduction of such ring substituents does not abolish affinity for LPS, but these analogues display lower affinity than PMBs 2-5. However, they are able to displace DPX from LPS to a similar extent as do the PMB analogues with shortened acyl chains (**24**-**26**). The regioisomers Pamb-PMB1 (**30**) and Mamb-PMB1 (**29**) have the highest and lowest affinities, respectively.

Interestingly, Pamb-PMB1 (**30**) and PMBs 3-5 appear to possess similar displacement capacities, although their MIC values are rather different (< 1μg/mL for PMBs 3-5 whereas **30** is not active up to 50μg/mL).

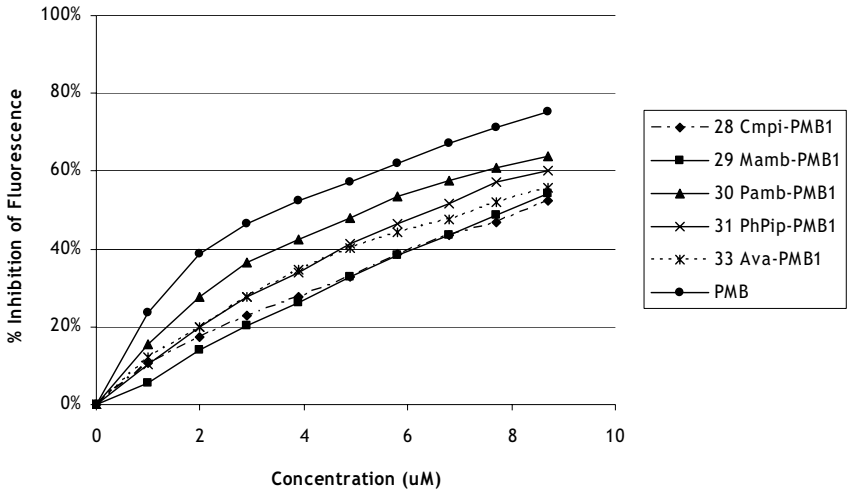



**FIGURE 6** | LPS affinity assay of ring-substituted PMB1 analogues and commercial PMB sulfate for comparison. Concentration of polymyxin analogue in μM.

## 2.4 | Conclusion

A SPPS route towards the cyclic CAP polymyxin B1 and analogues thereof has been presented, based on a safety-catch strategy. The method has the advantage that relatively pure polymyxins are acquired after the final cleavage/cyclization process, obviating extensive purification procedures. Antibacterial assays showed that analogues **23**-**26**, in which the (*S*)-6-methyloctanoyl moiety is replaced with other acyl chains, exhibit distinct antimicrobial activity. Shortening the length of the acyl chain below $C_6$ leads to a significant drop in activity, which appears to correlate with decreased LPS affinities compared to natural polymyxins B,[36] as seen for compounds **24**-**26**. Analogues **27**-**33**, in which the hydrophobic ring segment DPhe6/Leu7 is substituted were basically devoid of antimicrobial activity and for these compounds, display decreased affinity in the displacement assay. Whether this impaired affinity is due to the loss of a hydrophobic site that interacts with the lipid chains of Lipid A or because these analogues are unable to adopt a 3D structure that positions the Dab side chains in proximity of Lipid A's phospate groups for efficient electrostatic interactions, is not clear. There is no evidence that either of these two possible causes of loss of LPS affinity is responsible for the antibacterial inactivity; although the natural PMB2 and B3 and synthetic PMB4 and B5 compared to Pamb-PMB1 **30** display quite similar LPS affinity, their respective MIC values are rather different (<1μg/mL vs. >50μg/mL).

From this, it appears that an intact ring segment is more important for antibacterial activity than the hydrophobic acyl chain (although there is a minimal chain length required for activity in the low µg/mL range).

In general, nature appears to have optimized the ring structure of the polymyxin series of antibiotics to a large extent, as only small variations in the ring section are found in natural compounds (Table 1) and tolerated in synthetic polymyxins[1d,37] and the closely related polymyxin B nonapeptide (PMBN);[38] with a few exceptions, modulation of the hydrophobic segments in polymyxin B1 leads to analogues with slightly decreased LPS affinity, but a significant loss in antibiotic activity.

## 2.5 | Experimental Section

### 2.5.1 | General

Analytical and semi-preparative HPLC was performed on an ÄKTA Explorer chromatography system (Amersham Pharmacia Biotech). The peptides were analyzed using a Zorbax SB $C_{18}$ column (4.6x150mm, 5µ particle size, denoted as column 1 or an Alltech Alltima $C_{18}$ 4.0x250mm, 5µ particle size, column 2). The following buffers were employed: (A) 0.1% TFA in 5% aq. MeCN and (B) 0.1% TFA in 80% aq. MeCN. MALDI-TOF analyses were performed on a Bruker Biflex III mass spectrometer. ESI-MS analyses were performed on a Q-TOF mass spectrometer (Micromass) at a cone voltage of 20V.

### 2.5.2 | SPPS

**Loading of AM resin with SCL and Fmoc-Thr(tBu)-F (8)**
Loading of the AM resin with SCL (3-carboxypropanesulfonamide) and subsequent preparation using DAST and coupling of Fmoc-Thr(tBu)-F was accomplished following the procedure of Ingenito *et al.*[23] with some modifications. Loading of the safety-catch resin (2g, 0.72mmol) was accomplished with DiPEA (2.25mL, 11mmol) and Fmoc-Thr(tBu)-F (2.65g, 7.7mmol) in $CH_2Cl_2$ (12mL) and the reaction time was extended to 5h to give **8** with a loading of 64%.

**SPPS of linear polymyxin B1 (10)**
The acylated decapeptide **10** was synthesized on an ABI 433A (Applied Biosystems) peptide synthesizer. The synthesis was performed on 100µmol scale starting from **8** (0.43g, 0.23mmol/g). Cleavage of Fmoc groups was effected with 20% piperidine/DMF. Single couplings were performed using Fmoc-amino acids (5eq.) in NMP with BOP/HOBt/DiPEA as activator system. Residues 3, 4, and 5 were doubly coupled using 2x4eq. Capping was performed after each coupling step by acetylation ($Ac_2O$/HOBt/DiPEA) in NMP.

**On-resin protective group manipulation (12)**
Immobilized peptide **10** (100µmol) was suspended into a solution of 2% $NH_2NH_2 \cdot H_2O$ in DMF (5mL) and shaken for 3min. The mixture was filtered and the resin rinsed with DMF and $CH_2Cl_2$. This procedure was repeated twice to give resin **11**. Subsequently, the resin was suspended in NMP (1mL), DiPEA (0.1mL, 0.5mmol) and MMTCl (0.11g, 0.35mmol) were added and the mixture was shaken for 4h. The resin was filtered, washed with $CH_2Cl_2$ and MeOH to give **12**.

**Activation of SCL (13)**
A solution of $ICH_2CN$ (0.57mL, 7.9mmol) in NMP (1mL) was passed through a basic alumina plug under the exclusion of light. The resin **12** (100µmol) was suspended in the obtained solution and subsequently DiPEA (0.2mL, 1mmol) was added. The suspension was shaken for 16h under the exclusion of light. The activated resin **13** was filtered and washed with NMP and $CH_2Cl_2$.

**Removal of MTT protecting group (14)**
The activated resin **13** was suspended in TFA/TIS/$CH_2Cl_2$ (3mL, 3/5/92, v/v/v) and shaken for 30min. This procedure was repeated with 10min periods until the filtrate became colourless. After washing with $CH_2Cl_2$, resin **14** was washed with $CH_2Cl_2$ and used immediately in the cyclization reaction.

**Polymyxin B1 (1)**
To a suspension of **14** (100µmol) in freshly distilled THF (3mL), DiPEA (0.17mL, 0.85mmol) was added. The obtained cyclization/cleavage mixture was shaken for 24h at RT. The resin was filtered and washed with $CH_2Cl_2$, MeOH and $CH_2Cl_2$, respectively. The filtrate and washing solutions were concentrated *in vacuo* to afford the protected cyclic peptide. The residue was suspended in a mixture of TFA/TIS/$H_2O$ (5mL, 95/2.5/2.5 v/v/v) and shaken for 2h. The deprotected polymyxin was precipitated in $Et_2O$. The precipitate was centrifuged and the solvent was decanted to give 22.9mg of crude PMB1 (Rt 9.92min, column 1, purity 50%). The crude cyclic peptide was purified using HPLC (linear gradient of 0-100% B in 20min) and lyophilized to furnish pure **1**. Yield after purification: 1.9mg (1.5µmol, 1.5%). ESI-MS: 1203.8 $[M+H]^+$, 602.4 $[M+2H]^{2+}$, 402.3 $[M+3H]^{3+}$. See also Table 2 and Figure 7 (part of the ROESY NMR spectrum).

**Synthesis of PMB1 derivatives (3-5, 23-33)**
Derivatives **3**-**5** and **23**-**33** were synthesized through a similar reaction sequence as was **1**, implementing the modifications in amino acid sequence or acyl chain composition. Analytical data: see Table 2. Exact concentrations of polymyxin solutions were determined by comparison of the LC UV 214nm peak area with that a solution of PMB1 of which the exact content was known. This content was calculated by integration of the phenyl ring proton $^1H$ NMR signals and comparison with the internal reference tetramethylsilane proton signals of known concentration. Part of the ROESY NMR spectrum of **29** is found in Figure 8.

### 2.5.3 | Antibacterial Assay

The bacteria (*E. coli* ATCC 11775) were grown on nutrient agar plates and kept at $4^0C$. Lyophilized peptides were dissolved in Luria-Bertani (LB) broth to give a concentration of 80µM and filtered using 0.22µm filter discs. An overnight culture in LB broth was adjusted to $5x10^6$ CFU/mL and inoculated into the micro titre plate wells containing each 100µL of a serial 2-fold dilution (50-0.1µg/mL) of the tested peptide in LB broth. After incubation for 24h at $37^0C$, absorbance was measured at 600nm using a µQuant micro plate spectrophotometer (Bio-Tek Instruments). The MIC value of Ava-containing polymyxin **33** was determined separately. Hereto, the serial 2-fold dilution assay was adjusted to concentrations ranging from 1000 to 2µg/mL. Analogue **26** was tested in quadruplo; all other peptides were assayed in duplo.

### 2.5.4 | LPS Affinity Assay

Affinity for LPS (Sigma L6761 *S. enteritidis* ATCC 13076 or Sigma L6529 from *E. coli* O55:B5 for **24**-**26**, **28** and **31**; using commercially available PMB no difference between these two LPS preparations was found) was assessed in 2-fold using a competitive displacement assay employing commercially available dansylated polymyxin B. The DPX background fluorescence was determined by addition of an aq. DPX solution (5µL, 100µM) to HEPES buffer (5mM, pH 7.2, 1mL) at 340nm excitation and 495nm emission wavelenghts; this addition was repeated 5-10 times. Saturation of LPS with DPX was determined by measuring fluorescence of a mixture of DPX solution (5µL, 100µM) and LPS solution (3µg/mL in 5mM HEPES buffer pH 7.2, 1mL). Aliquots of 5µL of DPX solution were continuously added to the LPS solution until the fluoresence leveled off and the increase was only a result of the change in background. The amount of DPX to be added to the LPS solution to give 85-90% of saturation (Z-amount) was calculated from these data. For determination of

the amount of displacement of DPX, this Z-amount of DPX solution was added to 1mL LPS solution and equilibrated at RT for 10-15min. Aliquots of synthetic polymyxin analogue (5µL, 100µM in water) were added and the fluorescence measured after 30-60s until the maximum displacement was reached.

**TABLE 2** | Data on synthesized polymyxins.

| # | Modification | Scale (µmol) | Crude (mg) | Rt (min, column) | Crude Purity | Pure (mg, µmol) | Yield (%) | MS |
|---|---|---|---|---|---|---|---|---|
| **1** | PMB1 | 100 | 22.9 | 9.92 (1) | 50% | 1.9, 1.5 | 1.5 | 1203.8[A] |
| **3** | PMB3 | 50 | n/d | 9.87 (1) | 53% | 2.9 | 3.1 | 1188.8[A] |
| **4** | PMB4 | 20 | n/d | 9.85 (1) | 60% | n/d[C] | n/d | 1174.8[A] |
| **5** | PMB5 | 20 | n/d | 9.93 (1) | 62% | n/d[C] | n/d | 1202.7[A] |
| **23** | Ada | 50 | 18.4 | 9.50 (1) | 44% | 2.8, 2.3 | 4.5 | 1239.8[B] |
| **24** | $C_6$ | 50 | 14.1 | 8.42 (1) | 48% | 2.5, 2.2 | 4.3 | 1161.7[B] |
| **25** | $C_5$ | 50 | 17.9 | 8.01 (1) | 46% | 3.3, 2.9 | 5.8 | 1147.7[B] |
| **26** | $C_4$ | 30 | 6.5 | 7.98 (1) | 38% | 1.2, 1.0 | 3.5 | 1133.9[B] |
| **27** | Capro | 45 | n/d | 9.00 (2) | 42% | n/d[C] | n/d | 1112.2[A] |
| **28** | Cmpi | 45 | n/d | 8.72 (2) | 51% | n/d[C] | n/d | 1068.7[A] |
| **29** | Mamb | 100 | 32.7 | 10.83 (2) | 67% | 9.8, 9.1 | 9.1 | 1076.6[B] |
| **30** | Pamb | 40 | 4.7 | 8.43 (1) | 37% | n/d[C] | n/d | 1076.6[B] |
| **31** | PhPip | 40 | 10.9 | 8.45 (1) | 49% | 1.8, 1.6 | 3.9 | 1130.7[B] |
| **32** | Tran | 40 | 3.9 | 8.33 (1) | 49% | n/d[C] | n/d | 1082.6[B] |
| **33** | Ava | 100 | 21.4 | 10.6 (2) | 67% | 2.8, 2.7 | 2.7 | 1042.6[B] |

[A] ESI-MS $[M+H]^+$; [B] MALDI-MS; [C] Synthesis under unoptimized conditions; n/d - not determined

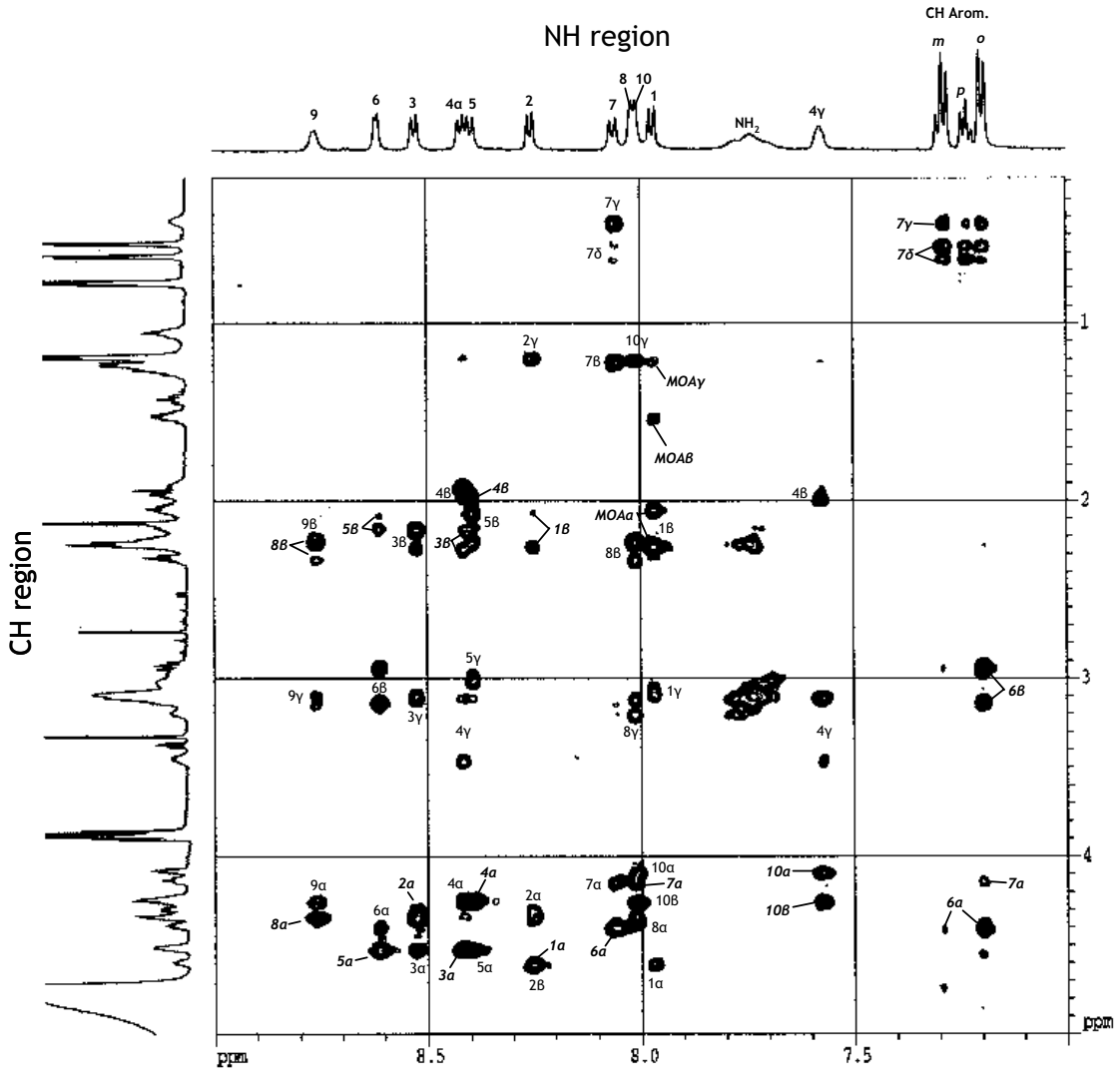



FIGURE 7 | Part of the 600MHz PMB1 (**1**) ROESY spectrum in 43% (v) TFE-$d_3$/$H_2O$, 298K, pH 4, mixing time 200msec. *Intraresidual* NOE contacts of amide NH peaks with other atoms are given in regular face; *interresidual* NOEs are denoted in bold/italic face and are connected with lines to the corresponding signals, residues are depicted by their numbers; MOA is the 6-methyloctanoyl chain. Thus, the amide proton of residue 6 (*i.e.* DPhe6NH) has interresidual NOE contacts with the two 5ß protons (Dab5Hßa and Hßb) and the amide proton of residue 2 (Thr2NH) has intraresidual NOE contacts with Thr2Hγ, Hα and Hß atoms (denoted in the spectrum as 2γ, 2α and 2ß, respectively).

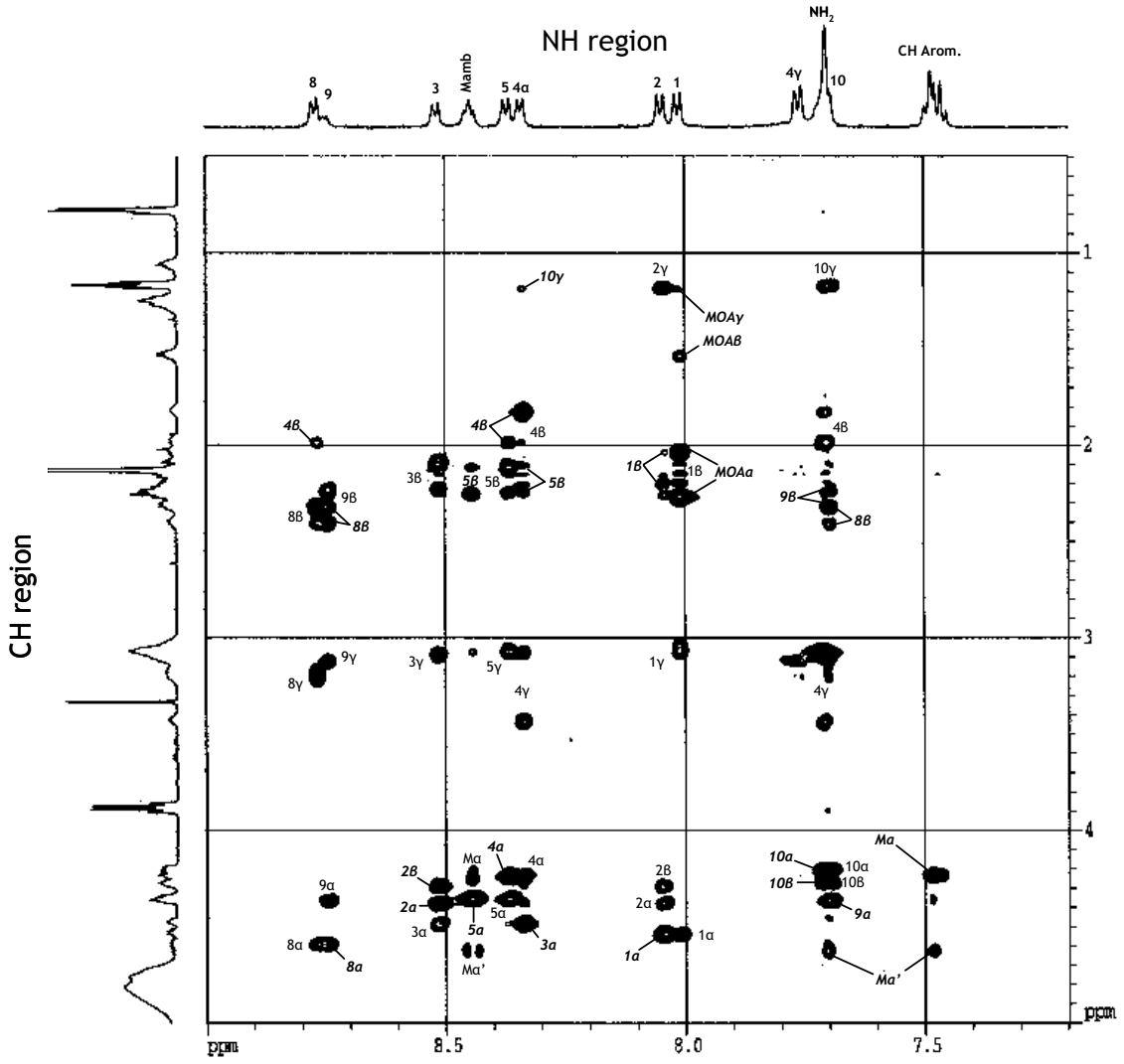



**FIGURE 8** | Part of the 600MHz Mamb-PMB1 (**29**) ROESY spectrum in 43% (v) TFE-$d_3$/$H_2O$, 298K, pH 4, mixing time 200msec. *Intraresidual* NOE contacts of amide NH peaks with other atoms are given in regular face; *interresidual* NOEs are denoted in bold/italic face and are connected with lines to the corresponding signals. Residues are depicted by their numbers; the Mamb moiety replaces both residues 6 and 7 in PMB1 and is denoted with M; MOA is the 6-methyloctanoyl chain. For example, the amide proton of residue 3 (*i.e.* Dab3NH) has interresidual NOE contacts with 2α and 2β protons (Thr2Hα and Hβ) and intraresidual NOE contacts with Dab3Hγ, Hα and the two inequivalent Hβ atoms (denoted in the spectrum as 3γ, 3α and 3β, respectively). The Mamb residue covers residues 6 and 7.

## 2.6 | Notes & References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be due to acetylation during the cyclization step (cross-coupling with activated Ac groups) or to incomplete acetylation, as progress of this reaction cannot be monitored; the sulfonamide group was found to be detectable with the Kaiser test only if present in high concentrations (as in commercial high-loaded SCL-AM resin with loading > 1mmol/g).

25. At the moment of preparation of the manuscript (de Visser, P.C.; Kriek, N.M.A.J.; van Hooft, P.A.V.; Van Schepdael, A.; Filippov, D.V.; van der Marel, G.A.; Overkleeft, H.S.; van Boom, J.H.; Noort, D. *J. Peptide Res.* **2003**, *61*, 298), the building block Fmoc-Dab(Mtt)-OH became commercially available.
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34. The observed displacement percentages appear to be somewhat assay-dependent, as DPX displacement of an identical sample of PMB sulfate in different control assays resulted in different values; for instance, in Chapter 4, PMB sulfate displacement at the highest concentration tested is 91% instead of the 75% determined in the Figure 5 assay. The amount of ~90% has also been reported in ref. 34 and in Loenarz, C.; Jimenez Solomon, M.F.; Tsubery, H.; Fridkin, M. *Scientific Reports of the International Summer Science Institute,* **2001**, *C3*, 29.
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# CHAPTER 3 | Acyl Migration in Polymyxin Synthesis

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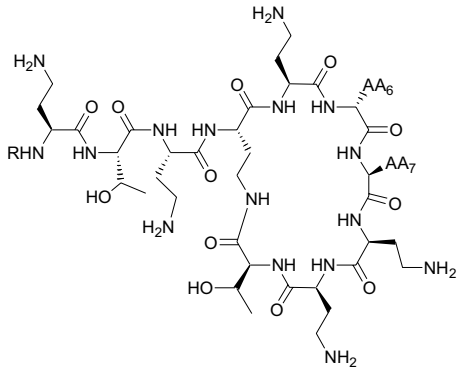
## 3.1 | Introduction

Polymyxins, potent antibiotics of the class of cationic antimicrobial peptides, are receiving increasing attention due to their unique combination of antibacterial and endotoxin-neutralizing properties.[1] Consequently, their chemical properties have been the subject of a number of studies, carried out mostly in the second half of the last century. These studies supply data on the stability of formulations of polymyxins B,[2,3] E,[4,5] and M[6] (see Table 1). Polymyxins B and M were found to be stable in neutral and mildly acidic media, but became inactivate after exposure to basic or strongly acidic conditions.[2a,7,8] This decrease in activity in both basic and acidic solutions were hypothesized to be the result of acyl or aminoacyl migration.[8] Studies on model systems established the possibility of both migrations in *Nα*-(amino)acylated Dab (α,γ-diaminobutyric acid) residues under basic and acidic conditions.[7,8,9]

**TABLE 1** | Composition of polymyxins B, E and M. Different acyl chains (R) can be connected to the Dab1 residue – see Chapter 2. For polymyxin B1, R=*(S)*-6-methyloctanoyl; for polymyxin B3 R=octanoyl.

| Polymyxin | $AA_6$ | $AA_7$ |
|---|---|---|
| B | DPhe | Leu |
| E | DLeu | Leu |
| M | DLeu | Thr |




## 3.2 | Isomerization in Polymyxins

### 3.2.1 | Isolated polymyxin B1

In a recent analytical study by Orwa *et al.* devoted to the stability of single polymyxins isolated from commercial samples (*e.g.* a commercial sample of polymyxin B contains at least 7 compounds in varying amounts, see Chapter 2 Figure 1),[10] formation of degradation products was observed upon exposure to a range of conditions.[11] Interestingly, a number of these products showed different retention time on HPLC but possessed a molecular weight identical to that of

the substrate. For example, when polymyxin B1 (PMB1, Table 1) was subjected to an acid stability test, a side-product was formed with a longer Rt than PMB1 itself, but with identical molecular weight (Figure 1).

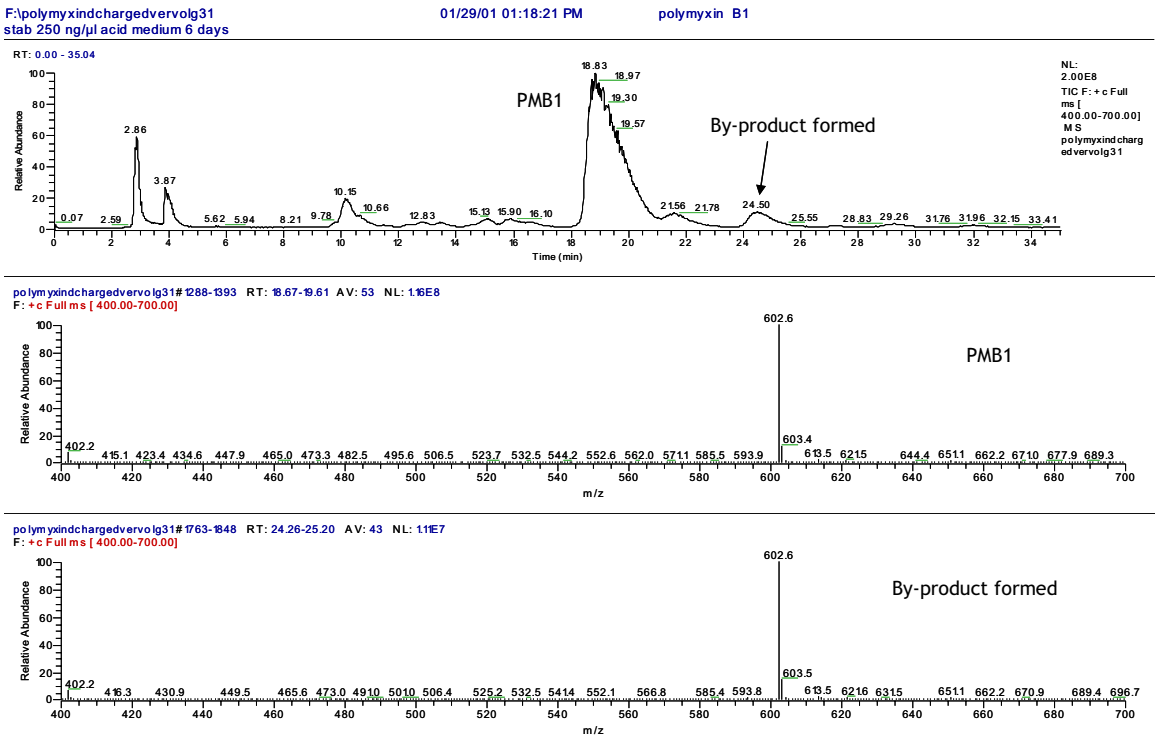



**FIGURE 1** | TIC diagram of isolated PMB1 after acid stability test for 6d at pH 1.4, showing the presence of a side-product, and MS spectra of the two peaks. Peak at 602.6 represents $[M+2H]^{2+}$. Reprinted with permission from A. Van Schepdael, personal communication.

Interestingly, subtle differences were found (see Figure 2) in the MS/MS spectra of both peaks. For example, peaks present in both spectra are different in relative intensity, and a number of peaks are absent in the side-product's spectrum, including 241.0, 414.0, 432.3, 482.4, 863.3 and 963.4. The fatty acyl group (FA, in PMB1 this is *(S)*-6-methyloctanoyl) seems to be involved in all these fragments as in PMB1 these peaks represent FA+Dab1 (241.0), M-(FA+Dab1+Dab5) (863.3) and M-(FA+Dab1) (963.4).[10b-c,12] Peaks 482.4 ($2^+$ ion of 963.4), 432.3 ($2^+$ ion of 863.3) and 414.0 (432.3-$H_2O$) also relate to these fragments. In this study, it was concluded that this isomeric side-product arose from epimerization of PMB1.[11] However, besides the fact that in this study no data

was supplied to justify this theory (no attempts were made to localize the site of isomerization), amino acid epimerization is highly unlikely under acidic conditions as this process proceeds through an anionic species.[13]

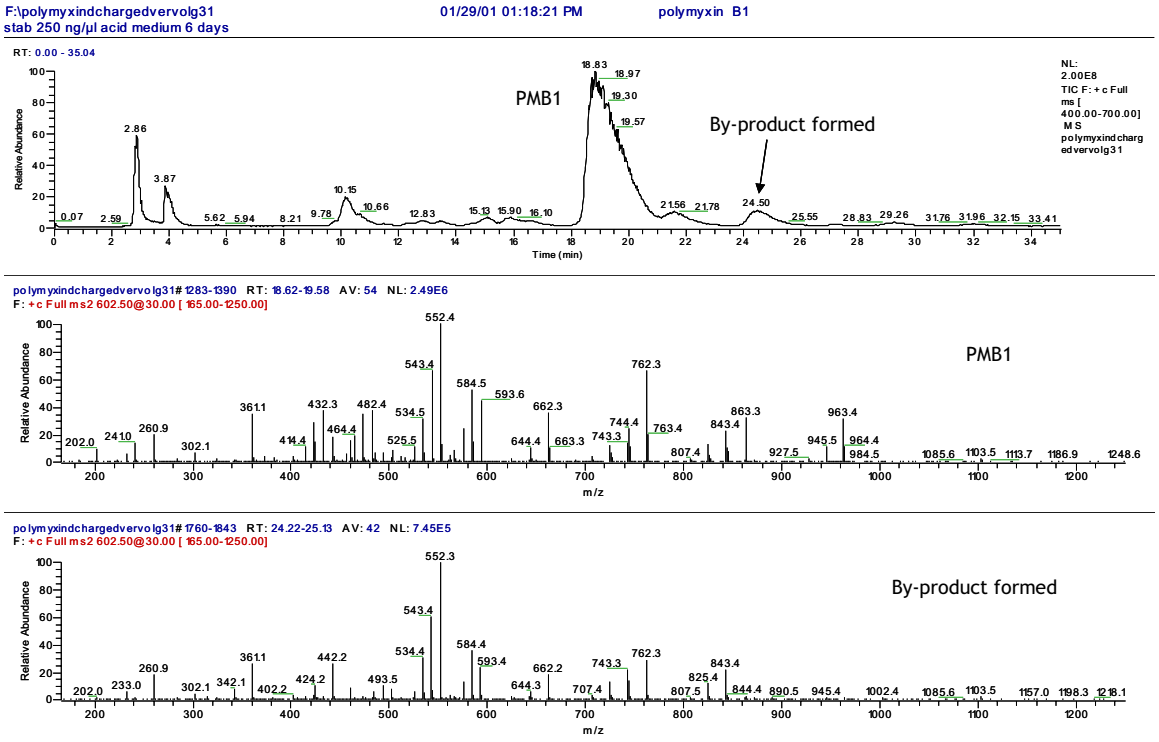



FIGURE 2 | TIC diagram of isolated PMB1 after acid stability test, showing the presence of a side-product, and MS/MS spectra (of the $[M+2H]^{2+}$ ion) of the two peaks. Reprinted with permission from A. Van Schepdael, personal communication.

## 3.2.2 | Synthetic polymyxins

As isolation of substantial amounts of every individual polymyxin from a commercial PMB sample requires an extremely time-consuming purification procedure,[10b] the chemical synthesis of PMB subfamily members was undertaken (Chapter 2). In the course of these studies, formation of a small amount (typically 5-10%) of a side-product with identical molecular weight but different LC retention time was encountered, reminiscent of the isolated PMB1 case (*vide supra*). Remarkably, after HPLC repurification, concentration *in vacuo* to remove solvents and redissolution of *e.g.* synthetic polymyxin B3 (PMB3), LCMS revealed the side-product had formed

again in a similar (5-10%) amount (Figure 3). Peaks in Figure 3 marked with arrows arose from two separate compounds both containing $[M+H]^+$ 1190.0 and $[M+2H]^{2+}$ 595.5 as major ions. Subjection of both peaks present in the mixture to MS/MS analysis displayed differences in abundances of a series of peaks between the spectra of PMB3 and the side-product (Figures 4 and 5).

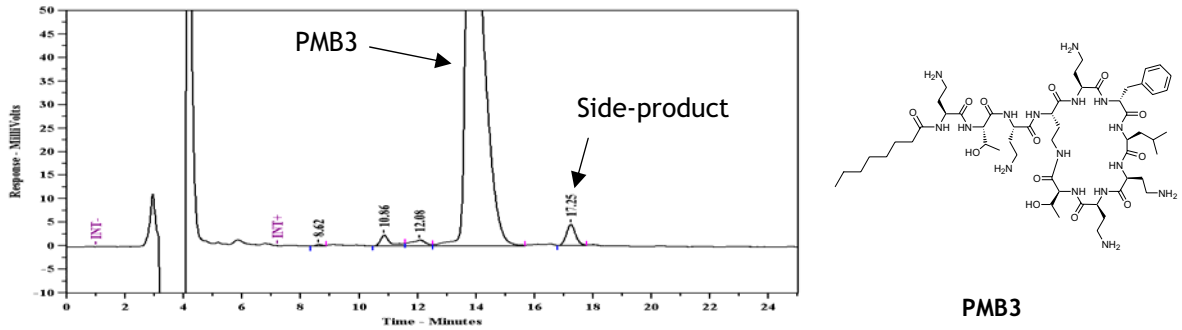



FIGURE 3 | LC diagram of purified PMB3, showing the presence of a side-product with identical molecular weight.

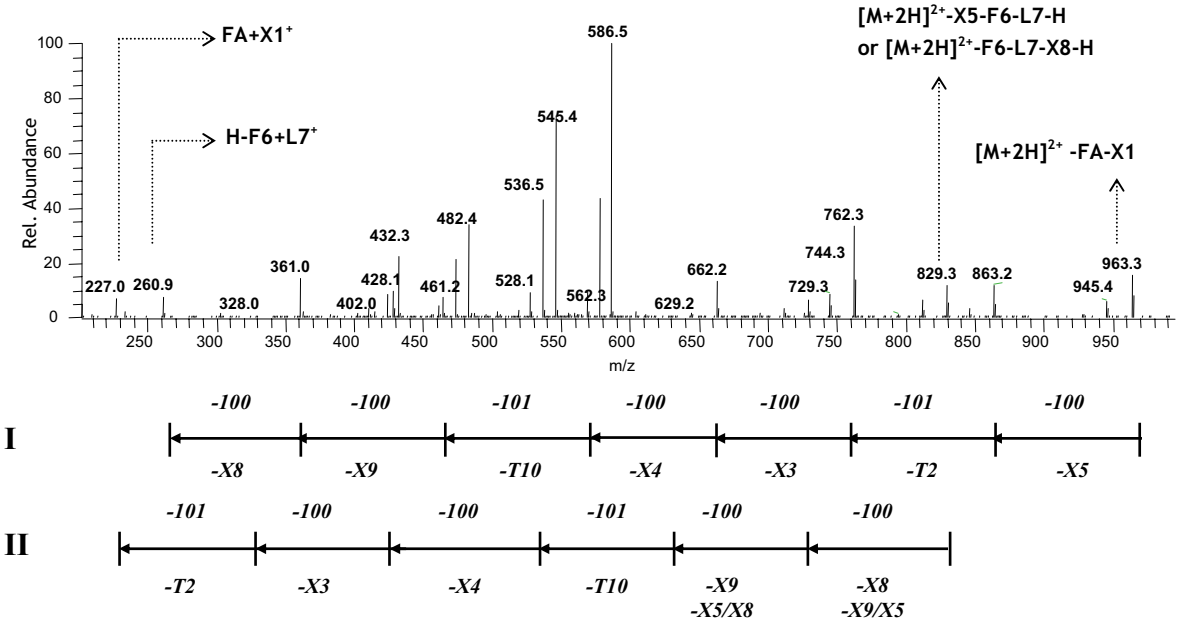



FIGURE 4 | $[M+2H]^{2+}$ MS/MS spectrum acquired for PMB3 and proposed fragmentation routes **I** and **II**.[10b-c,12] The structures of four ions offering information about the FA residue or the ring amino acids DF6 and L7 are shown (FA=fatty acyl (*i.e.* octanoyl), amino acids in one letter code (X=Dab)).

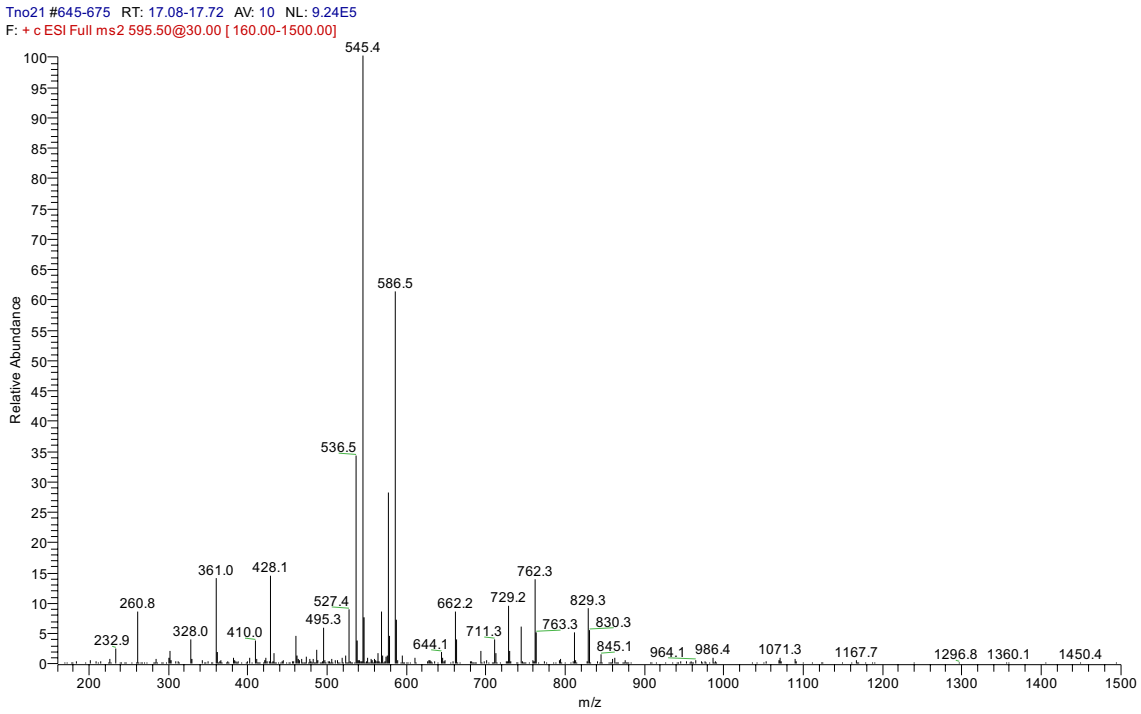



FIGURE 5 | $[M+2H]^{2+}$ MS/MS spectrum obtained from the side-product formed during PMB3 synthesis.

These differences are similar to those seen in the case of PMB1 and the corresponding side-product (*vide supra*); comparable peak disappearance in the side-product's MS/MS relating to fragments containing FA is found in the absence of fragments 227.0, 863.2, 963.3 and the doubly charged ions 432.3 and 482.4 (see Table 2 in the Experimental section). This indicates that a similar isomerization process has taken place in both isolated PMB1 and synthetic PMB3. As acyl migration between Nα and Nγ positions in Dab residues were found possible to occur in comparable systems (*vide supra*) through an energetically favorable 6-membered ring transition state, it was reasoned that acyl migration at Dab1 had taken place rather than epimerization.

## 3.3 | Synthesis & Analysis of the Acyl-Migrated Product

To obtain proof regarding the structure of the observed side-product resulting from Nα→Nγ acyl migration, the acyl-migrated product of PMB3 was synthesized. This regioisomer, the new polymyxin analogue Nγ-PMB3 (Figure 6B), was obtained following the safety-catch procedure

introduced in Chapter 2. Key step in this synthesis is the on-resin cyclization of the linear polymyxin with concomitant cleavage from the resin, which can only occur after activation of the resin by alkylation. This procedure generally yields a crude product of high purity that is not contaminated with its linear counterpart, obviating a tedious purification procedure. The reported procedure was slightly altered, applying Boc-Dab(Fmoc)-OH instead of Fmoc-Dab(Boc)-OH as the last amino acid residue to achieve acylation at the Nγ position of Dab1.

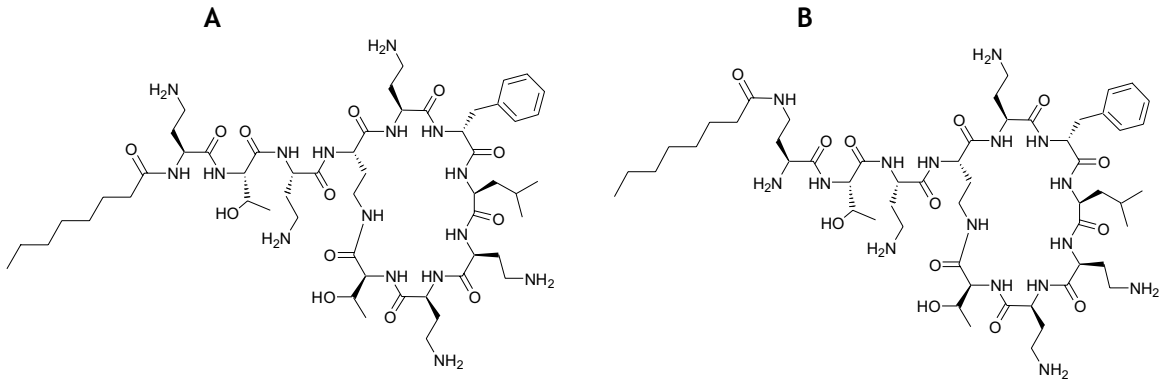



FIGURE 6 | Structures of PMB3 (A) and its regioisomer, Nγ-PMB3 (B).

Upon co-injection of the HPLC-purified, side-product-containing PMB3 mixture (Figure 3) with Nγ-PMB3, the Nγ-PMB3 retention time in LC was found to be identical with the one observed for the side-product (Figure 7). Moreover, comparison of the MS/MS spectra of the PMB3 isomeric side-product with the synthetic Nγ-PMB3 showed that they were identical (Figures 5 & 8).

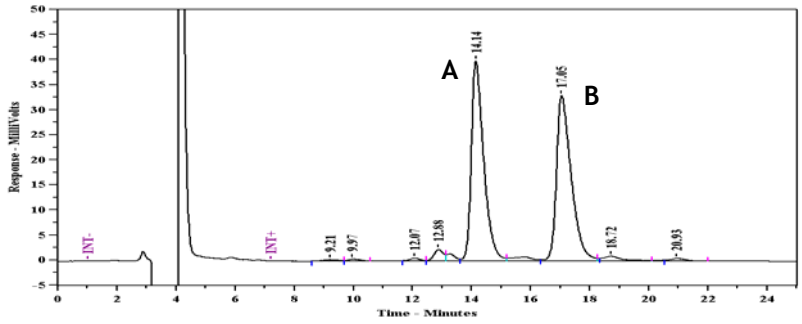



FIGURE 7 | LC chromatogram of crude PMB3 mixture co-injected with Nγ-PMB3. Peak **A**: PMB3, peak **B**: Nγ-PMB3 coincides with the obtained side-product as determined by MS/MS.

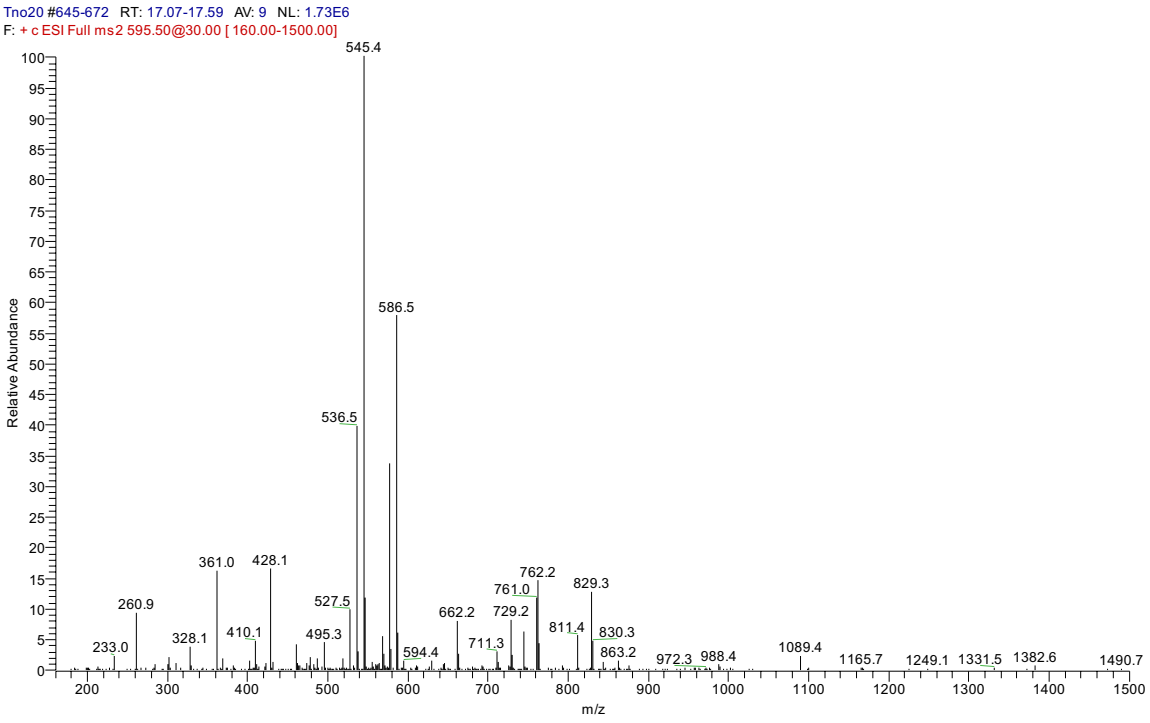



FIGURE 8 | MS/MS spectrum obtained from $[M+2H]^{2+}$ of synthetic Nγ-PMB3.

Based on these data it was concluded that the formed side-product corresponds to the structure in Figure 6B, being the Nγ-regioisomer of the desired polymyxin resulting from N→N′-acyl migration in the Dab1 residue.

## 3.4 | Determination of Favored Isomer

Acyl migration takes place from both *Nα*- and *Nγ*-acylated single Dab residues, in both cases favoring the Nα-product under slightly acidic conditions to yield approx. 60% Nα-product.[7a] To examine their chromatographic behavior, both *Nα*- and *Nγ*-octanoyl-Dab were synthesized by SPPS and treated thereafter in a similar way as was PMB3. In the final product mixture after synthesis of *Nγ*-octanoyl-Dab, only a small amount of this compound was obtained, accompanied by the major product *Nα*-octanoyl-Dab, whereas no *Nγ*-octanoyl-Dab was found in the synthesis

of *Nα*-octanoyl-Dab (by comparison of LCMS chromatograms: both $[M+H]^+$ 244.5, $C_{18}$ RP column, 15→90% MeCN/0.1% aq. TFA in 26min; Nα Rt 8.79min, Nγ Rt 9.15min).

In order to examine the scope of the polymyxin N→N'-acyl migration reported here, a stability study of PMB3 and Nγ-PMB3 was performed as a function of temperature (RT and $60^0$C) and pH (1.4, 4.4 and 7.4). The obtained LCMS/MS chromatograms of these samples showed that acyl migration does not take place at RT. The formation of Nγ-PMB3 from PMB3 at $60^0$C at the different pH values is presented in Figures 9A-C.

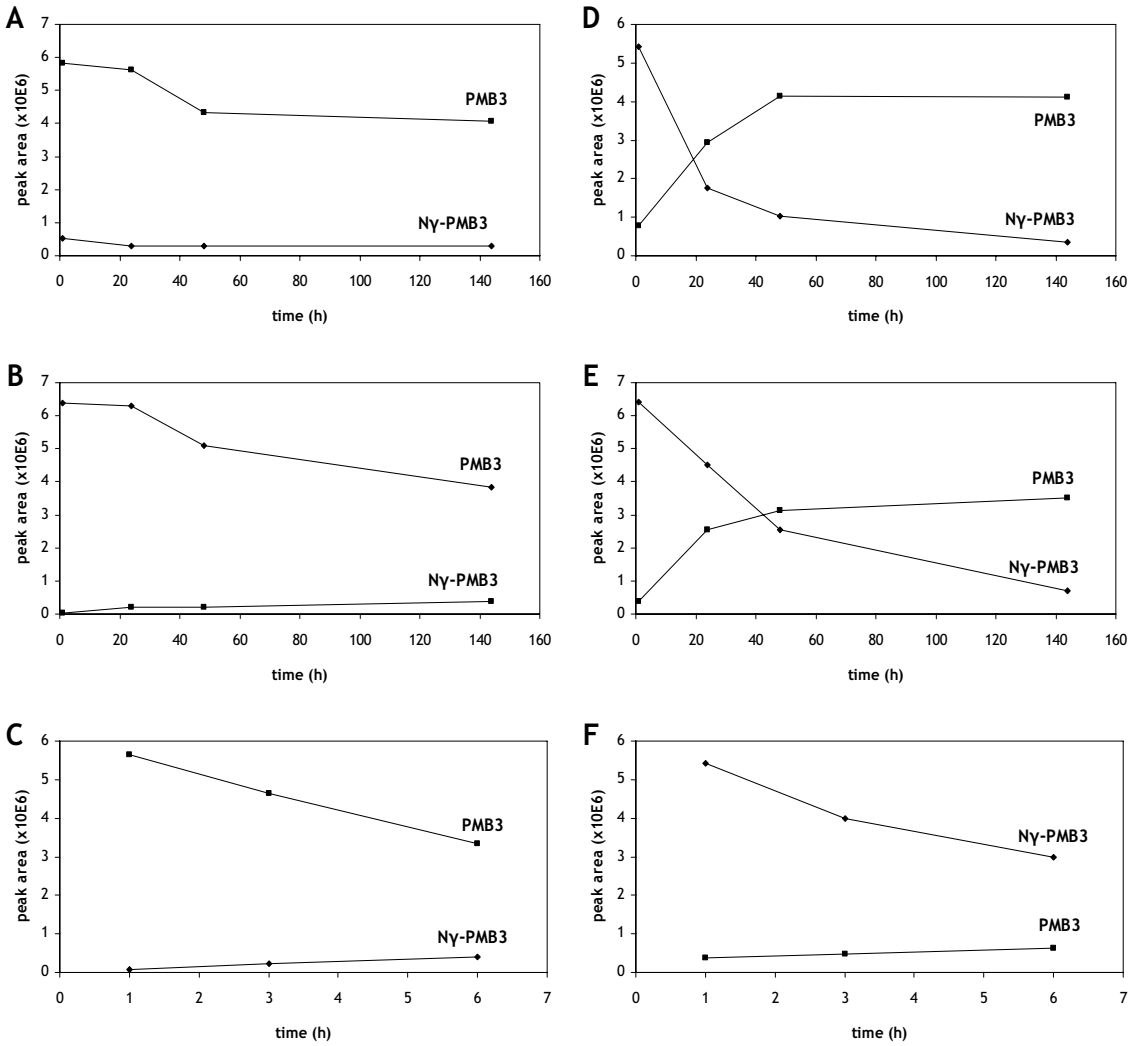



FIGURE 9 | Profiles showing the LC peak areas of PMB3 and Nγ-PMB3 in time at $60^0$C as a function of pH. **A,B,C** starting from PMB3; **D,E,F** starting from Nγ-PMB3. pH 1.4 (**A,D**), 4.4 (**B,E**) and 7.4 (**C,F**).

The Nα→Nγ acyl migration reached a maximum in the medium with pH 1.4 after approx. 48h, by which time ~7% Nγ-PMB3 had formed (Figure 9A). Nγ-PMB3 is formed fastest at neutral pH, which can be explained by the fact that at this pH value the Nγ of Dab1 is more nucleophilic.

The formation of PMB3 out of Nγ-PMB3 (*i.e.* Nγ→Nα migration, Figures 9D-F) occurred most rapidly at low pH and equilibrated after approx. 48h. For pH 1.4, at this point ~20% of the Nγ-PMB3 was left. Conversion of either polymyxin went most rapid at pH 7.4 (Figures 9C and F). It should be noted that the decrease in PMB3 and Nγ-PMB3 levels might be partially ascribed to the formation of other degradation products that were not identified.

## 3.5 | Biological Evaluation

To investigate whether this N→N'-acyl migration has an effect on the potency of the polymyxin as antibiotic, minimal inhibitory concentrations (MIC) values were determined against *Escherichia coli* ATCC 11775 using a 2-fold serial dilution assay (see Chapter 2). PMB3 was found to have a MIC value of 0.6μM, whereas the antibacterial potency of Nγ-PMB3 had decreased to a MIC value of 2.5μM, showing that, at least in PMB3, Dab1 Nα→Nγ acyl migration has a negative effect on antibacterial activity.

## 3.6 | Summary

The structure of a side-product found in the synthesis of polymyxin B3 was elucidated. This side-product, with a mass identical to PMB3 but with different LC retention time, reappeared after purification of PMB3 and resulted from isomerization of the parent compound under acidic conditions. Acyl migration was hypothesized to have taken place at the alkanoylated Dab1 residue. Although the rare amino acid Dab occurs in some other natural products (*e.g.* in the peptide antibiotics friulimicins,[14] syringomycin,[15,16] syringopeptins,[15,17] gavaserin and saltavalin,[18] cepacidine A,[19] pseudomycins,[20] xylocandin,[21] fuscopeptins,[22] and tolaasins[23]), none of these compounds contain an N-terminally alkanoylated Dab residue,[24] providing no precedent for such a hypothesized N→N' acyl shift. LC and MS/MS analyses showed the side-product to be

identical with the synthetic PMB3 regioisomer having the Nα-Dab1 acyl chain migrated to its Nγ position. Analogously, this isomerization most likely also occurred during the acid stability test of isolated PMB1, rather than epimerization as proposed by Orwa *et al*.

## 3.7 | Experimental Section

### 3.7.1 | SPPS

PMB3 was prepared on 50µmol scale as reported previously (see Chapter 2). Nγ-PMB3 was prepared analogously on 50µmol scale, except for the coupling of the last residue to the peptide sequence. This moiety was incorporated using Boc-Dab(Fmoc)-OH instead of Fmoc-Dab(Boc)-OH. After liberating the peptides from the solid support, the compounds were purified using an ÄKTA Explorer™ LC system equipped with an Alltima semi-preparative $C_{18}$ column (250 x 10mm, 5 µm particle size) employing gradients of buffers A (0.1% TFA in 5% (v) MeCN/$H_2O$) and B (0.1% TFA in 80% (v) MeCN/$H_2O$) and simultaneous detection at 214 and 254nm. Collected product fractions were combined and evaporated or lyophilized using a LC1010 vacuum centrifuge (preventing isomerization) to yield PMB3 (1.9mg, 3.2%) and Nγ-PMB3 (2.1mg, 3.5%) respectively. ESI-MS (both compounds): $[M+H]^+$ 1190.0, $[M+2H]^{2+}$ 595.5. LC: PMB3 Rt 14.14min, Nγ-PMB3 Rt 17.05min.

### 3.7.2 | LCMS and MS/MS Analyses

The LC apparatus consisted of a Spectra HPLC system equipped with a YMC-Pack Pro $C_{18}$ column (5µm, 250x2.0mm, Waters), immersed in a water bath at 30°C, employing 0.01M aq. TFA/MeCN (77/23 (v/v)) as buffer. Mass spectra were recorded on a LCQ ion trap mass spectrometer equipped with an ESI interface operated in the positive ion mode at 5kV and capillary temperature of 210°C. Helium was used as collision gas at a pressure of 0.1Pa. MS acquisition over the mass range 500-1500 was performed on-line with UV detection at 215nm. The doubly-charged ions of the products in the sample were isolated mono-isotopically in the ion trap.

### 3.7.3 | Stability Studies

Appropriate amounts of 0.1M aq. $H_2KPO_4$ and 0.1M aq. $HK_2PO_4$ were mixed to pH 7.4. Similarly, buffers of pH 1.4 and pH 4.4 were prepared from 0.1M aq. $H_2KPO_4$ and 0.1M aq. phosphoric acid. For the stability study at different pH values and temperatures, reactions were initiated by diluting stock aqueous solutions of 0.25mg/mL PMB3 and Nγ-PMB3 with equal amounts of buffers pH 1.4, 4.4 or 7.4 to give solutions of 0.125mg/mL. Aliquots in sealed vials were incubated at RT and at 60°C. Vials were removed at regular intervals and immediately frozen at -20°C until they were analyzed as a series. For pH 1.4 and pH 4.4 vials were removed after 1h, 24h, 48h and 144h. Those at pH 7.4 were removed after 1h, 3h and 6h.

### 3.7.4 | Antibacterial Assay

Antibacterial assays against *E. coli* ATCC 11775 were performed in 3-fold as previously described (Chapter 2).

TABLE 2 | Fragments found in MS/MS spectra of PMB3 and its isomer

| Fragment[A] | Origin | PMB3 | isomer |
|---|---|---|---|
| 227.0 | FA+Dab1 | **+** | **-** |
| 232.9 | unknown | + | + |
| 260.9 | DPhe6+Leu7 | + | + |
| 328.0 | FA+Dab1+Thr2 | **-** | **+** |
| 361.0 | DPhe6+Leu7+Dab8 | + | + |
| 410.1 | 428-$H_2O$ | + | + |
| 423 | 2+ ion of 845 | **+** | **-** |
| 428.1 | FA+Dab1+Thr2+Dab3 | + | + |
| 432.3 | 2+ ion of 863 | **+** | **-** |
| 461 | DPhe6+Leu7+Dab8+Dab9 | + | + |
| 464 | 2+ ion of 963-2$H_2O$ | **+** | **-** |
| 473 | 2+ ion of 963-$H_2O$ | **+** | **-** |
| 482.4 | 2+ ion of 963 | **+** | **-** |
| 495.3 | unknown[C] | - | + |
| 527.4 | 2+ ion of 1090 (M-Dab)[B]-2$H_2O$ | - | + |
| 528.1 | FA+Dab1+Thr2+Dab3+Dab4 | + | - |
| 536 | 2+ ion of 1090 (M-Dab)-$H_2O$ | + | + |
| 545.4 | 2+ ion of 1090 (M-Dab) | + | + |
| 569 | 2+ ion of M-3$H_2O$ | + | + |
| 578 | 2+ ion of M-2$H_2O$ | + | + |
| 586.5 | 2+ ion of 1172 (M-$H_2O$) | + | + |
| 662.2 | DPhe6+Leu7+Dab8+Dab9+Thr10+Dab4 | + | + |
| 711.3 | 729-$H_2O$ | + | + |
| 729 | FA+Dab1+Thr2+Dab3+Dab4+Thr10+Dab | + | + |
| 744.3 | 762-$H_2O$ | + | + |
| 762.3 | DPhe6+Leu7+Dab8+Dab9+Thr10+Dab4+Dab3 | + | + |
| 812 | 829-$H_2O$ | + | + |
| 829.3 | M- DPhe6-Leu7-Dab | + | + |
| 845.3 | 863-$H_2O$ | + | + |
| 863 | M-FA-Dab1-Dab5 | + | - |
| 945.4 | 963-$H_2O$ | + | - |
| 963.3 | M-FA-Dab1 | + | - |

These fragments are designated present (+) or absent (-) for PMB3 but are applicable for its isomer on the premise that identical parts in both molecules give identical fragment peaks. [A] Approximate values of peaks that are not denoted in spectra, or in which decimals differ by 0.1 are not given in decimal values; [B] M=1190; [C] might be 2+ ion of 990 (M-2Dab).

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24. Gavaserin and saltavalin share analogy with known polymyxins regarding amino acid and fatty acid composition. As no chemical structures of the complete peptides were given, it cannot be excluded that an acylated Dab residue is present.

# CHAPTER 4 | Design, Synthesis & Biological Evaluation of PMBN/CAP Conjugates

## 4.1 | Introduction

In addition to its potent antibiotic activity against Gram-negative bacteria, polymyxin B (PMB, see Chapter 2) is able to bind and neutralize endotoxin (or lipopolysaccharide, LPS). This neutralizing capacity may prevent a Gram-negative bacterial infection from reaching the state of sepsis or the aggravated, often lethal form septic shock.[1] Furthermore, PMB is able to sensitize the Gram-negative bacterial outer membrane. In an attempt to impose these favorable features of PMB onto another antibiotic, a conjugate with the ristomycin A aglycon[2] was prepared. The resulting compound was cidal to both Gram-positive and Gram-negative bacteria. However, PMB exerts toxicity and is therefore restricted to topical use.

The polymyxin B nonapeptide (PMBN **1**, Figure 1), a truncated form of PMB without the fatty acyl chain and the Dab1 residue, was reported to have lost both toxicity and antibacterial activity. It still sensitizes Gram-negative bacteria to other drugs[3] and neutralizes LPS (indicating a strong binding to LPS), albeit to a somewhat lower extent than PMB.[4]

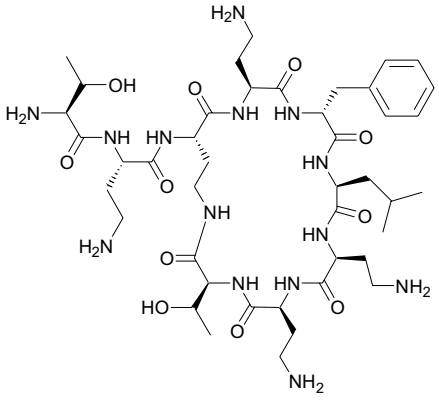



FIGURE 1 | Structure of polymyxin B nonapeptide (PMBN).

It was envisaged that conjugation of PMBN to other members of the cationic antimicrobial peptide (CAP) class of antibiotics would favorably influence their biological activities. The membranolytic CAPs tritrpticin (TTC)[5] and KFF[6] (Table 1), known to be rather unselective in their lytic actions in the sense that they also lyse red blood cells (RBC), might be rendered less hemolytic (more selective) by conjugation to non-hemolytic PMBN. Furthermore, the PMBN

moiety could attribute to bacterial uptake and enhance antimicrobial activity by sensitizing the outer membrane to these CAPs.[7]

**TABLE 1** | Linear CAPs used in this study

| Abbreviation | Sequence[B] | Target |
|---|---|---|
| BF2 | TRSSR AGLQF PVGRV HRLLR K | DNA/RNA |
| DRC[A] | GKPRP YTPRP TSHPR PIRV | DnaK |
| KFF | KFFKF FKFFKa | Membrane |
| TTC | VRRFP WWWPF LRRa | Membrane |

[A] Drosocin analogue (Ser7→Thr)DRC nG. [B] a = C-terminal carboxamide

Linear CAPs with targets in the inside of the bacterium, such as buforin II (BF2, binds to nucleic acids)[8] and drosocin (DRC, targets the bacterial heat shock protein DnaK)[9] would also benefit from conjugation to PMBN. Besides the aforementioned features of sensitization and selectivity, these particular CAPs might experience enhanced uptake through the inner membrane to arrive at the cytosol and thus show enhanced antibacterial activity. Finally, linear CAPs conjugated to PMBN are expected to be endowed with LPS neutralizing activity arising from the polymyxin moiety.

## 4.2 | Design & Synthesis

Conjugates of PMBN with the linear CAPs listed in Table 1 were designed to consist of two separate moieties connected *via* a disulfide linkage (Figure 2). This linkage enables disconnection of the two moieties by reductive activities present in the bacterial cell[10] and was implemented to overcome possible interference of PMBN with the antimicrobial actions of the linear CAPs. This is especially important in the case of conjugates with BF2 and DRC that target internal structures. Construction of this disulfide-linked type of PMBN/CAP conjugates calls for separate syntheses of thiol-functionalized PMBN and linear CAP parts. The thiol function in the linear CAP part is equipped with a 2-pyridylsulfenyl (SPy) leaving group, which enables selective heterodisulfide formation (*vide infra*).[11]

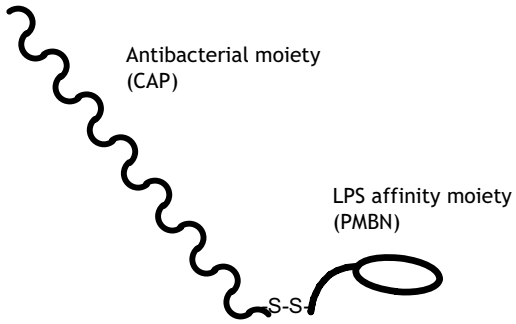
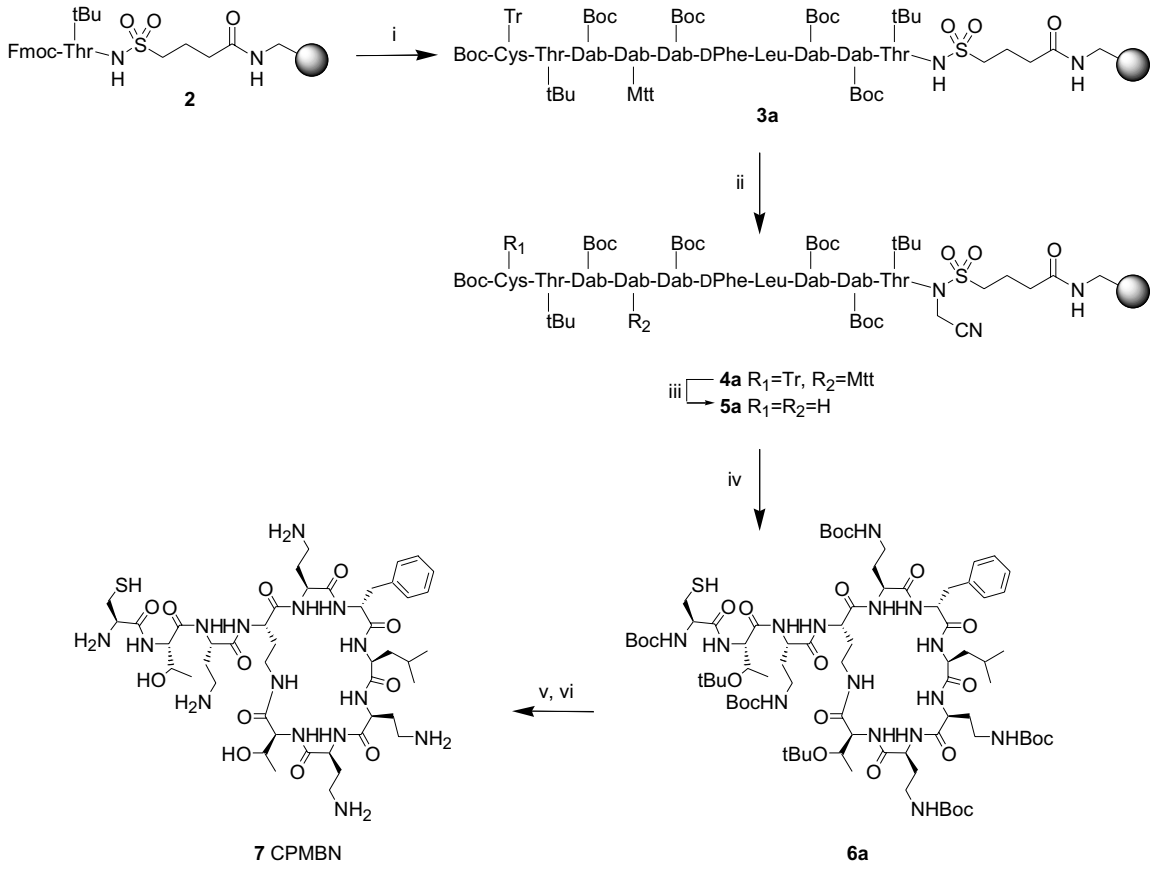



FIGURE 2 | Schematic representation of disulfide conjugates of CAPs with PMBN. The disulfide bridge is located at the C-terminus of the CAP.

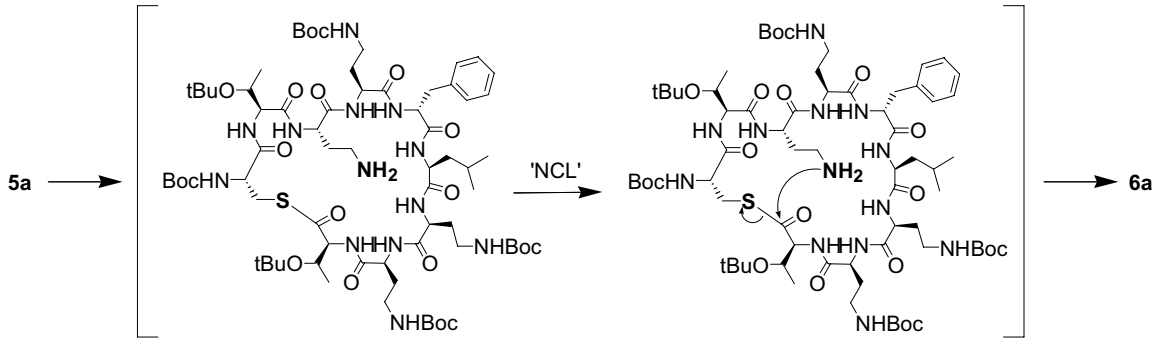
### 4.2.1 | Synthesis of a thiol-functionalized PMBN derivative

An important consideration in the design of a PMBN derivative that can be used in conjugation is the position of the thiol modification; it is known that modification of specific residues in PMB(N) results in loss of LPS affinity.[12] The fact that, from PMB, the fatty acyl (FA)-Dab1 moiety can be removed without abolishing LPS affinity (yielding PMBN), indicates that the PMBN N-terminal Thr residue can serve as attachment point for conjugation. This approach yields defined conjugates (Figure 2).[7,13] Thus, a Cys residue was added to the N-terminal Thr residue to provide a thiol function for participation in disulfide conjugation.

The convenient route towards the synthesis of natural polymyxins reported in Chapter 2 was slightly adapted for the synthesis of this new polymyxin derivative. Starting from Thr-loaded resin **2** (Scheme 1), Boc-Cys(Tr)-OH was coupled as last residue to the linear, protected PMBN to give **3a**. Resin **3a** was then alkylated with $ICH_2CN$/DiPEA (→**4a**) and subsequently treated with acid to liberate the Dab4 side chain. Although the *N*-Mtt group in **4a** was expected to be more acid labile than the *S*-Tr group, it was not possible to detect full orthogonality between the *N*-Mtt and *S*-Tr (Ellman test) groups in test reactions. Therefore, both groups were removed to yield **5a**, in which two nucleophiles can now take part in the subsequent 'cleavage-by-cyclization' reaction: attack of the Dab4 side chain amine on the Thr carbonyl yields the correctly cyclized polymyxin derivative **6a** directly. Nucleophilic attack of the Cys1 side chain yields the internal thioester instead (Scheme 2). If formed, this thioester could undergo conversion through a 'native chemical ligation'-like mechanism (S→N acyl migration) to give the correct product **6a** as well.



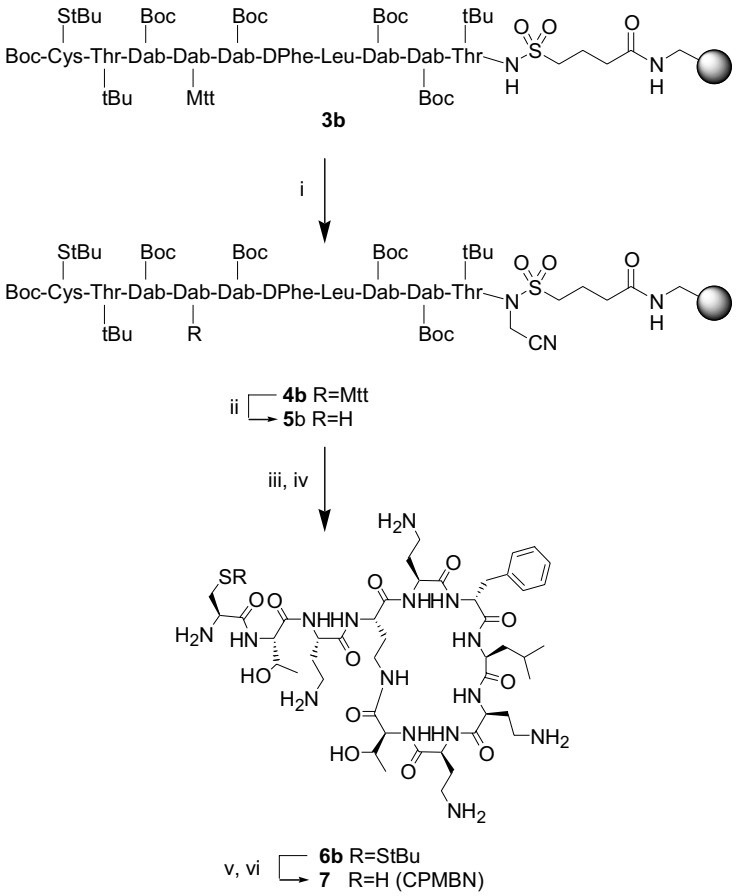

**SCHEME 1** | Synthesis of CPMBN. Reagents & conditions: i. Fmoc-based SPPS (see Chapter 2); ii. $ICH_2CN$, DiPEA, NMP; iii. TFA/TIS/$CH_2Cl_2$ 5/3/92 (v/v/v) iv. DiPEA, THF; v. TFA/TIS/$H_2O$ 95/2.5/2.5 (v/v/v); vi. HPLC purification.




**SCHEME 2** | Formation of **6a** through conversion of an internal thioester formed from **5a** *via* a native chemical ligation (NCL)-like mechanism.

After subjection to cyclization conditions and deprotection, Cys1-modified PMBN (CPMBN) **7** was obtained in a crude cyclization yield of 25%.

Unfortunately, the CPMBN **7** obtained *via* this route was contaminated with a small amount of undesired linear CPMBN, which is likely to have resulted from hydrolysis of unrearranged internal thioester. The formation of this hydrolysis product, together with the fact that both **6a** and **7** are susceptible to the formation of disulfide-linked homodimers during storage, led to adjustement of the synthesis of **7**. The Cys side chain *S*-Tr group was replaced by an *S*-StBu group giving **3b** (Scheme 3). This peptide was carried through a similar reaction sequence as was **3a** in Scheme 1A.




SCHEME 3 | Alternative synthesis of CPMBN **7**. Reagents & conditions: i. $ICH_2CN$, DiPEA, NMP; ii. TFA/TIS/$CH_2Cl_2$ 5/3/92 (v/v/v) iii. DiPEA, THF; iv. TFA/TIS/$H_2O$ 95/2.5/2.5 (v/v/v); v. 0.1M TCEP·HCl, pH 4.5; vi. HPLC purification.

This *S*-StBu strategy had a similar crude cyclization yield as the *S*-Tr strategy, but with the advantage that the Dab4 side chain can be liberated selectively (→**5b**), and the cyclized product **6b** could be stored conveniently without risk of oxidation. The Cys protecting group in **6b** was removed quantitatively, as gauged by LCMS, by the action of tris(carboxyethyl)phosphine (TCEP),[14] after which the compound was purified by HPLC to give fully deprotected **7**.

### 4.2.2 | Synthesis of thiol-functionalized linear CAP moieties

Four linear CAPs differing from each other regarding secondary structure, target and composition were selected for conjugation (see Tables 1 and 2). In short, buforin II (BF2) and a modified drosocin analogue (DRC, Chapter 1) with increased serum stability were selected as CAPs targeting structures inside the bacterial cell. Tritrpticin (TTC) and the synthetic peptide $(KFF)_3K$ (denoted KFF) act through lysis of the bacterial inner membrane. Two of these CAPs (BF2 and KFF) display α-helical structures upon interaction with the bacterial membrane, whereas the structures of DRC and TTC were found to be largely extended. The selected CAPs were equipped with a C-terminal Cys(Tr) residue to provide the thiol function for disulfide formation with CPMBN **7**. Additionally, a δ-aminovaleric acid (Ava) linker was incorporated separating the Cys residue from the CAP to circumvent sterical interference from the linear CAP with the interaction of PMBN with LPS. The linear peptides were synthesized through standard automated Fmoc-based SPPS protocols using HCTU as coupling reagent and cleaved from their resins in the presence of 2,2′-dithiobispyridine to obtain CAPs **8**-**11** with a 2-pyridylsulfenyl (SPy) leaving group attached to the side chain of the Cys residue (Table 2).[11]

**TABLE 2** | Modified peptides

| # | From CAP | Structure[A] | Sequence[B] |
|---|---|---|---|
| **8** | BF2 | α-helical | TRSSR AGLQF PVGRV HRLLR KXC(SPy)a |
| **9** | DRC | extended | GKPRP YTPRP TSHPR PIRVX C(SPy)a |
| **10** | KFF | α-helical | KFFKF FKFFK XC(SPy)a |
| **11** | TTC | extended | VRRFP WWWPF LRRXC(SPy) |

[A] of the unmodified CAPs; [B] X = Ava, SPy = 2-pyridylsulfenyl, a = carboxamide

## 4.2.3 | Conjugation

Having CPMBN **7** and CAPs **8-11** in hand, attention was focussed on their conjugation. Selective asymmetric disulfide formation was accomplished through the use of the SPy leaving groups in a neutral aqueous environment, as monitored by LCMS; once expelled, the SPy group does not take part in disulfide formation due to its inactivity at pH 7. Using an excess of CAP, all CPMBN was found consumed after 16h of reaction (Figure 3). HPLC purification of the mixtures yielded conjugates **12-15** (Table 3).

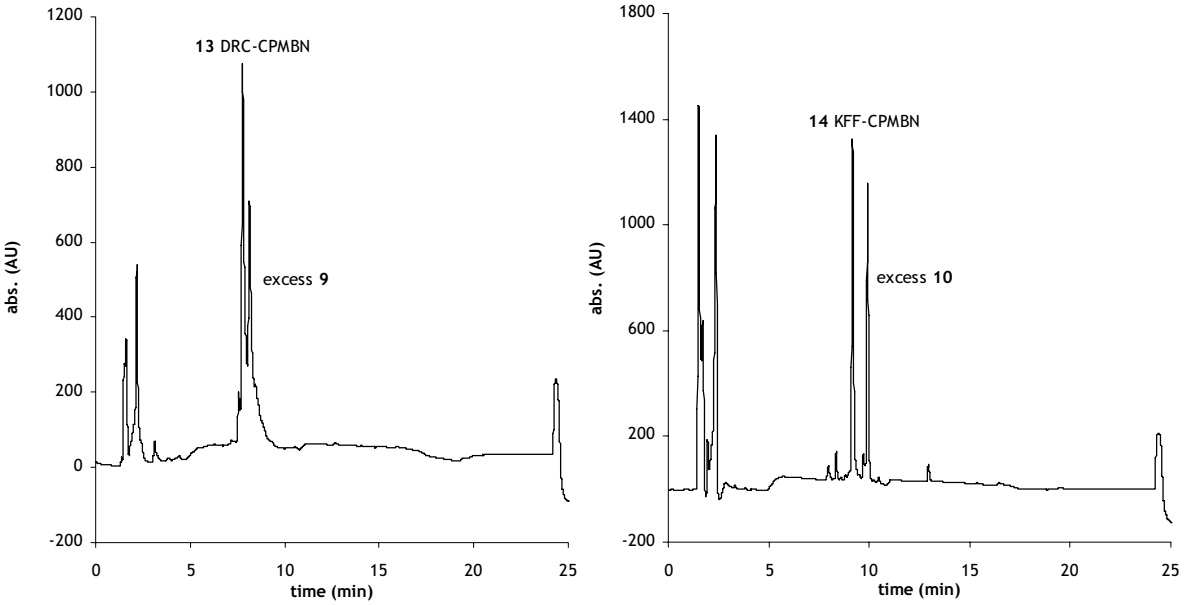



**FIGURE 3** | Representative LC chromatograms (214nm, 10→90% MeCN in 0.1% aq. TFA in 17min) of crude conjugation mixtures showing the formation of **13** (left panel, Rt 7.77min) and **14** (right panel, Rt 9.17min) after 16h reaction; in all cases, all CPMBN **7** was found consumed. Peaks of Rt <5min arise from injection.

**TABLE 3** | Conjugates prepared.

| Compound | Name | Sequence[A] | ESI-MS |
|---|---|---|---|
| **12** | BF2-CPMBN | TRSSR AGLQF PVGRV HRLLR KXC-(SS)-CPMBN | 1851.8 $[M+2H]^{2+}$ |
| **13** | DRC-CPMBN | GKPRP YTPRP TSHPR PIRVX C-(SS)-CPMBN | 1740.2 $[M+2H]^{2+}$ |
| **14** | KFF-CPMBN | KFFKF FKFFK XC-(SS)-CPMBN | 2683.0 $[M+H]^{+}$ |
| **15** | TTC-CPMBN | VRRFP WWWPF LRRXC-(SS)-CPMBN | 1575.5 $[M+2H]^{2+}$ |

[A] X = Ava, SPy = 2-pyridylsulfenyl, -(SS)- indicates disulfide bond, CPMBN is cysteine-modified PMBN

## 4.3 | Biological Evaluation

### 4.3.1 | Antibacterial activity

The biological activities of conjugates **12-15** against *E. coli* ATCC 11775 were assessed. For none of the conjugates, 100% inhibition (*i.e.* MIC value, Figure 4) was reached beneath concentrations of 100µM. Conjugates **12** and **13** were found to be completely devoid of activity while **14** and **15** did kill bacteria. KFF (as in conjugate **14**) alone displays only very modest activity (MIC 300µM); extrapolation of the activity curve of **14** in Figure 4 coincides with a similar MIC value. Attachment of the CPMBN moiety apparently does not interfere with the antibiotic action of KFF; it however slightly impairs the antibacterial activity of TTC (active in the low mM range).[15]

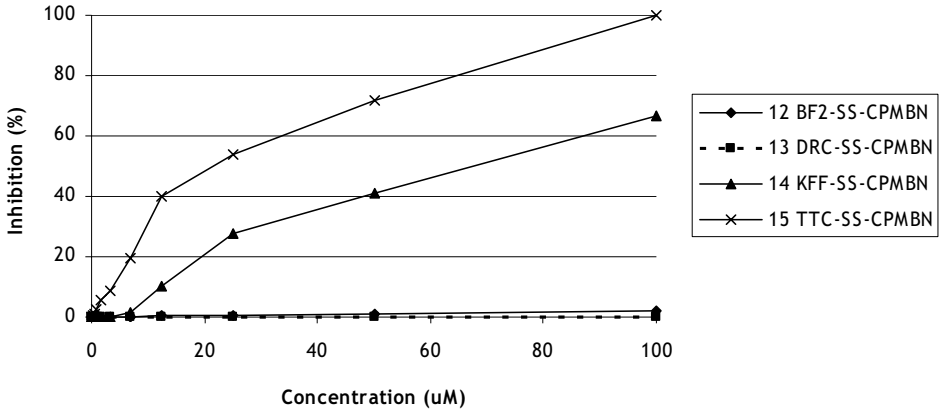



FIGURE 4 | Antimicrobial effects of C-terminal PMBN/CAP conjugates against *E. coli* ATCC 11775.

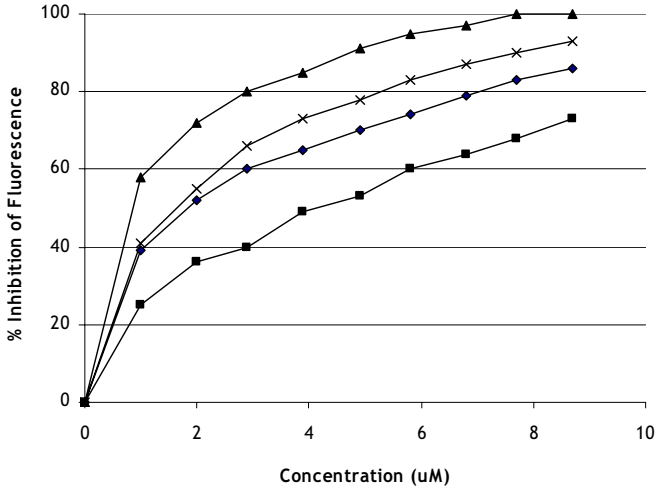
### 4.3.2 | Hemolytic activity

To assess the affinity of conjugates **12-15** for membranes of different composition (*i.e.* selectivity), hemolysis of red blood cells (RBC) was determined. No hemolysis was detected for compounds **12** and **13** up to concentrations of 250µM. This feature appears to be inherited from the three parent compounds DRC, BF2 and PMBN, which are known to lack hemolytic activity. Unfortunately, the antimicrobial conjugates (**14** and **15**) have retained the hemolytic activity of their parent compounds TTC and KFF; at the highest concentration tested (250µM), conjugates **14** and **15** accounted for 65% and 100% hemolysis, respectively.

### 4.3.3 | LPS binding affinity

The affinity for LPS of compounds **12-14** by virtue of their PMBN moieties was assessed in a LPS displacement assay (see Chapter 2).[16,17] In this assay, competitive binding of the conjugates with dansylated polymyxin B (DPX) to LPS is observed as decrease in fluorescence. Using commercially available LPS from *Salmonella enteritidis*, comparison of LPS affinity of these conjugates with that of commercially available PMB sulfate is shown in Figure 5. The linear CAP parts in **12-14** do not abolish LPS binding by the PMBN moiety; all conjugates are able to displace DPX from LPS.

The order of activity in the displacement assay (conjugate of KFF > BF2 > DRC) reflects the 'grand average of hydropathicity' (GRAVY)[18] value ranking of the CAP parts, being KFF(0.120) > BF2(-0.638) > DRC(-1.574). These values imply that KFF has the highest overall hydrophobic character and DRC lowest. KFF-containing conjugate **14** displays the highest LPS affinity of the conjugates assayed, and its affinity even surpasses that of commercial PMB and that of DPX.[19] The enhancing effect of KFF on PMBN's LPS binding might be explained by the fact that the KFF amphiphilic α-helix formed upon contact with LPS creates a large hydrophobic area that allows interactions with the lipid chains of LPS (*cf.* the N-terminal acyl chain in PMB).




| # | Maximal inhibition[A] |
|---|---|
| **12** | 94% |
| **13** | 81% |
| **14** | 100% |
| **PMB** | 100% |

FIGURE 5 | Inhibition of DPX fluorescence by conjugates and PMB. [A] See Experimental section for calculations.

In fact, compound **14** is unique in the sense that there are no literature reports on polymyxin B-based[4,12,20] or other CAP-inspired compounds[20b,21] that exert displacement activity higher than PMB. The percentage of maximal inhibition of DPX LPS binding can be calculated from the reciprocals plot (see Experimental Section, and Figure 5). In agreement with earlier reports, PMB is capable of displacing all DPX; in the same report, PMBN was calculated to be able to displace DPX for a maximum of 77%.[16]

## 4.4 | Conclusion

Disulfide conjugates of PMBN with linear CAPs were prepared to evaluate whether or not the favorable features of PMBN (sensitization/LPS scavenging/selectivity) could be imposed onto the antibiotic part. Through a cleavage-by-cyclization strategy, a Cys-modified derivative of PMBN was obtained. The Cys side chain was used in subsequent conjugation with a number of linear CAPs. Conjugation of PMBN to the membrane-active CAPs KFF and TTC did not abolish the hemolytic activity of the parent linear CAPs, nor did it improve the antibacterial actions of these linear CAPs. Antibacterial activity was absent in PMBN conjugates with BF2 and DRC, both acting on targets inside the bacterium. It can be speculated that the disulfide bond in these conjugates is not reduced inside the bacterium, leading to steric hindrance. Alternatively, the modification of their C-termini might simply be the cause of inactivity: C-terminal derivatization of DRC has not been reported in literature, and the only C-terminally modified BF2 derivative[22] was not evaluated for antimicrobial potency. Finally, the affinity of the conjugates for LPS effected by the PMBN moiety was compared with that of PMB and a fluorescent PMB derivative (DPX) in competition experiments. All conjugates showed affinity for LPS, and the KFF conjugate **14** was found to possess a higher affinity for LPS than PMB and DPX, indicating that KFF contributes to LPS affinity.

## 4.5 | Experimental Section

### 4.5.1 | Synthesis

**Cys1-modified polymyxin B nonapeptide CPMBN (7).**
Compound **6b** was prepared *via* the procedure discussed in Chapter 2, which was slightly modified. In short, Boc-Cys(StBu)-OH was used as last amino acid. After completion of the synthesis of the linear

CPMBN, the resin was alkylated with $ICH_2CN$, and the product was cyclized and cleaved under the agency of DiPEA. All acid-labile protecting groups were removed with 95% TFA. Reductive removal of the Cys(StBu) protecting group was achieved by dissolving compound **6b** under Ar atmosphere in 0.1M aq. TCEP·HCl (brought to pH 4.5 with $Na_3PO_4 \cdot 12H_2O$) and stirring overnight. LCMS showed disappearance of **6b** and formation of **7**; this compound was purified by HPLC (gradients of MeCN in 0.1% aq. TFA), concentrated *in vacuo* and stored at -20$^0$C under Ar.

**Preparation of 2-pyridylsulfenylated CAPs (8-11)**
After completion of the SPPS of the linear peptides (Fmoc-based automated synthesis using HCTU as activator), adding Ava and Cys(Tr) to the C-terminus, the peptides were cleaved from their resins using TFA/TIS/$H_2O$ 95/2.5/2.5 (v/v/v) and 2,2'-dithiobispyridine (20eq.) for 1h. Peptides were precipitated in $Et_2O$, centrifuged, decanted and washed three times with $Et_2O$ to remove the yellow color. Peptides were purified by HPLC and lyophilized. Analytical data: see Table 3.

**Preparation of C-terminal conjugates (12-15)**
Linear cationic peptides containing Cys(SPy) (ca. 1.5-2.5eq.) were dissolved in aq. 1M $NH_4OAc$ to which CPMBN **7** (0.7-1.2µmol) was added. The pH was adjusted to pH 7 with AcOH and the mixture stirred overnight. LCMS analysis of the crude mixtures showed complete consumption of CPMBN in each case. The crude conjugates were purified by semi-preparative HPLC to yield pure compounds **12** (1.6mg, 0.51µmol), **13** (2.9mg, 0.83µmol), **14** (2.1mg, 0.57µmol) and **15** (2.7mg, 1.01µmol). Analytical data: see Table 3.

TABLE 3 | Data on synthetic peptides and conjugates used in this study.

| # | Compound | Rt (min) | ESI-MS | HRMS (calcd.) |
|---|---|---|---|---|
| **6b** | $CPMBN^{StBu}$ | 8.59[A] | 1155.0 $[M+H]^+$ | n/d |
| **7** | CPMBN | 7.99[A] | 1066.4 $[M+H]^+$ | 1065.584 (1065.576) |
| **8** | BF2-Ava-Cys(SPy) | 8.57[A] | 2742.8 $[M+H]^+$ | 2744.517 (2744.507) |
| **9** | DRC-Ava-Cys(SPy) | 8.13[A] | 2525.8 $[M+H]^+$ | 2522.332 (2522.326) |
| **10** | KFF-Ava-Cys(SPy) | 9.92[A] | 1725.2 $[M+H]^+$ | 1723.891 (1723.877) |
| **11** | TTC-Ava-Cys(SPy) | 12.61[A] | 2215.0 $[M+H]^+$ | n/d |
| **12** | BF2-Ava-Cys(SS)-CPMBN | 8.27[B] | 1851.8 $[M+2H]^{2+}$ | 3699.072 (3699.066) |
| **13** | DRC-Ava-Cys-(SS)-CPMBN | 7.77[B] | 1740.2 $[M+2H]^{2+}$ | 3476.873 (3476.872) |
| **14** | KFF-Ava-Cys(SS)-CPMBN | 9.17[B] | 2683.0 $[M+H]^+$ | 2678.401 (2678.393) |
| **15** | TTC-Ava-Cys(SS)-CPMBN | 8.20[C] | 1575.5 $[M+2H]^{2+}$ [D] | 3166.679 (3166.665) |

[A] LC Rt 10→90% MeCN in 0.1% aq. TFA in 20min; detection at 214nm. [B] LC Rt 10→65% MeCN in 0.1% aq. TFA in 9.7min; detection at 214nm. [C] LC Rt 10→65% MeCN in 0.1% aq. TFA in 13.4min; [D] Calculated 1583.8; this is the main peak, originating from fragment M-16, presumably caused by loss of an $NH_2$ group during ionization. LC detection in all cases at 214nm, n/d – not determined.

### 4.5.2 | Antimicrobial Assay

From an overnight culture of ~$10^9$-$10^{10}$ *E. coli* ATCC 11775, a suspension of 5x$10^6$ CFU/mL in iso-sensitest broth (ISB) was prepared. The conjugates were dissolved in ISB to give 0.2mM solutions. Using a 96-well plate, in duplo, all conjugates where dispensed using 2-fold serial dilution down from 100 to 0.21µM. Suspensions were incubated for 18-24h at 37$^0$C while shaking gently. The absorbance at 600nm was measured from which the MIC value was determined.

### 4.5.3 | Hemolysis Assay

Freshly drawn blood samples were centrifuged for 10min after which the erythrocyte pellet was washed with 0.85% saline. The RBC were diluted with saline to 1/25 packed volume of cells. The compounds were

dissolved in a minimal amount of DMSO (max. 30% (v)) and diluted further with saline to give a 0.75mM solution. Two-fold serial dilution of the compounds was applied in triplo against 1% Triton X-100 in saline as positive control. After addition of 50µL RBC solution, the plate was incubated at $37^0$C for 4h, centrifuged (5min at $10^0$C) and 50µL of each well was dispensed into a new plate. The percentage of hemolysis was determined from the absorbance at 405nm.

### 4.5.4 | Displacement Assay

Affinity for LPS (Sigma L6761 *S. enteritidis* ATCC 13076) was assessed in 2-fold using a competitive displacement assay employing commercially available dansylated polymyxin B (DPX). The DPX background fluorescence was determined by addition of an aq. DPX solution (5µL, 100µM) to HEPES buffer (5mM, pH 7.2, 1mL) at 340nm excitation and 495nm emission wavelengths; this addition was repeated 5-10 times. Saturation of LPS with DPX was determined by measuring fluorescence of a mixture of DPX solution (5µL, 100µM) and LPS solution (3µg/mL in 5mM HEPES buffer pH 7.2, 1mL). Aliquots of 5µL of DPX solution were continuously added until the fluorescence levelled off and the increase was a result only of the change in background. The amount of DPX to be added to the LPS solution to give 85-90% of saturation (Z-amount) was calculated from these data. For determination of the amount of displacement of DPX, 2 times the Z-amount of DPX solution was added to 2mL LPS solution and equilibrated at RT for 10-15min. Aliquots of synthetic polymyxin analogue (5µL, 400µM in water) were added and the fluorescence measured after 30-60s until the maximum displacement was reached. The maximum inhibition by a given compound was determined from the extrapolated *y* intercept of a plot of the reciprocal of % fluorescence inhibition as a function of the reciprocal of the compound concentration.[16]

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# CHAPTER 5 | Antimicrobial Gels Based on QACs

## 5.1 | Introduction

Quaternary ammonium compounds (QACs) are readily accessible cationic substances in which the hydrophobicity can be adjusted easily. QACs such as cetylpyridinium chloride or cetyltrimethylammonium bromide (CTAB, Figure 1) are widely used as disinfectives.[1] These compounds exhibit antibacterial activity from their interference with, or destruction of the bacterial cytoplasmic membrane.[2] At low concentrations in water, CTAB forms gels with worm-like micelle structures.[3]

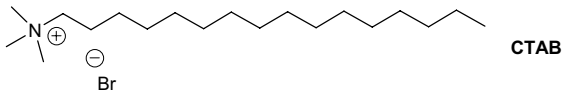


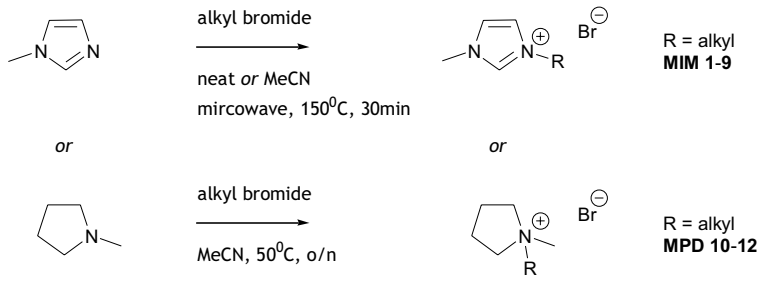

FIGURE 1 | Structure of the QAC cetyltrimethylammonium bromide (CTAB).

Ionic liquids[4] based on quaternary imidazolium salts display amphiphilicity like CTAB. Gelation could be induced in imidazolium-based ionic liquids by addition of organogelators[5] or mesogens (molecules that can exhibit a liquid-crystalline phase).[6] Interestingly, addition of water in low concentrations (~5-40% (wt)) to the ionic liquid *N*-decyl-*N'*-methyl imidazolium bromide was found to induce nearly instantaneous formation of a lyotrophic (*i.e.* concentration-dependent) liquid-crystalline gel phase.[7] The obtained so-called 'ionogels' resisted flow against gravity for an indefinite period of time. As *N*-alkyl-*N'*-methyl imidazolium QACs are expected[8,9] to show antibacterial activity, their encapsulation into gravity-stable gels leads to interesting antibiotic formulations that might be useful for personal (topical) decontamination purposes.[10] This Chapter describes the synthesis and the antibiotic activity of an array of *N*-alkyl-*N'*-methyl imidazolium (MIM) bromides **1**-**9** and **15**-**18** (Table 1) as well as *N*-alkyl-*N*-methyl pyrrolidinium (MPD) bromides **10**-**14**, and of their encapsulation in stable gels obtained by mixing with polar liquids.

## 5.2 | MIM and MPD QACs

### 5.2.1 | Syntheses

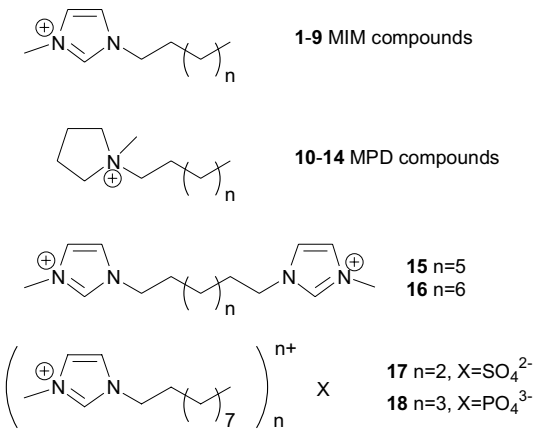
*N*-alkyl-*N'*-methyl imidazolium (MIM) salts were readily obtained through established synthesis procedures, performing the alkylation of *N*-methylimidazole with *n*-alkyl bromides equimolarly, neat or in MeCN,[11] at 150$^0$C for 30min in a microwave oven (Scheme 1). After extraction and vacuum drying at 70$^0$C, compounds **1-9** were obtained in 85-90% yield and were found to be pure apart from some residual $H_2O$. The physical appearances of these MIM salts changed with alkyl chain length. Compounds **1-6** were obtained as ionic liquids whereas **7-9** were solids.




**SCHEME 1** | Preparation of *N*-alkyl-*N'*-methyl imidazolium (MIM) and *N*-alkyl-*N*-methyl pyrrolidinium (MPD) bromides. For the R (alkyl) groups, see Table 1 below.

**TABLE 1** | Synthesized QACs.[A]

| Compound | | Compound | |
|---|---|---|---|
| **1** | $C_7$MIM Br | **10** | $C_{10}$MPD Br |
| **2** | $C_8$MIM Br | **11** | $C_{11}$MPD Br |
| **3** | $C_9$MIM Br | **12** | $C_{12}$MPD Br |
| **4** | $C_{10}$MIM Br | **13** | $C_{13}$MPD Br |
| **5** | $C_{11}$MIM Br | **14** | $C_{14}$MPD Br |
| **6** | $C_{12}$MIM Br | **15** | $C_9MIM_2$ 2Br |
| **7** | $C_{13}$MIM Br | **16** | $C_{10}MIM_2$ 2Br |
| **8** | $C_{14}$MIM Br | **17** | $(C_{10}MIM)_2$ $SO_4$ |
| **9** | $C_{16}$MIM Br | **18** | $(C_{10}MIM)_3$ $PO_4$ |




[A] MIM = *N*-alkyl-*N'*-methyl imidazolium, MPD = *N*-alkyl-*N*-methyl pyrrolidinium, where '*N*-alkyl' refers to the linear $C_x$ alkyl group.

Non-aromatic *N*-alkyl-*N*-methyl pyrrolidinium (MPD) bromides were prepared through overnight reaction of *N*-methylpyrrolidine and *n*-alkyl bromide in MeCN at $50^0$C (Scheme 1). [12,13] Pure MPD salts **10-14** (Table 1) were obtained by either spontaneous crystallization or after precipitation upon addition of $Et_2O$. Application of the microwave-based synthesis of these bromides gave rise to the formation of unidentified impurities that could not be removed easily. All MPD bromides were obtained as crystalline white solids in yields of 70-95%. The choice of limiting the array of MPD salts to the ones containing $C_{10}$ to $C_{14}$ alkyl chains was based on the data obtained from MIC determinations of analogous MIM compounds (*vide infra*).

Next, dicationic MIM salts **15** and **16** (structure see Table 1), in which the two cationic sites are connected through a hydrophobic stretch were prepared from *N*-methylimidazole and linear α,ω-dibromoalkanes (0.5eq.) applying the microwave approach. Finally, the 'bis-MIM' sulfate **17** and 'tris-MIM' phosphate **18** were prepared by conversion of compound **4** with either $Ag_2SO_4$ or $Ag_3PO_4$[14] and were included to examine the effect of a multivalent anion.

## 5.2.2 | Biological Evaluation

The minimal inhibitory concentration (MIC) values of all MIM compounds were determined against *Escherichia coli* ATCC 11775. Results are found in Table 2 and show an obvious relationship between length of n-alkyl chain and antimicrobial activity. QACs with alkyl chains < 10 carbon atoms did not display any antimicrobial activity up to 200μM. On the other hand, lengthening the alkyl chain >14 atoms did not increase the MIC value, a finding that agrees with earlier reports.[9a]

**TABLE 2** | MIC[A] and MHC[B] values of MIM and MPD QACs.

| # | MIC (μM) | MHC (μM) | # | MIC (μM) | MHC (μM) |
|---|---|---|---|---|---|
| **1** | >200 | n/d | **10** | >1000 | >1000 |
| **2** | >200 | n/d | **11** | 500 | >1000 |
| **3** | >200 | 1000 | **12** | 250 | 500 |
| **4** | 200 | n/d | **13** | 125 | 250 |
| **5** | 100 | 125 | **14** | 62.5 | 125 |
| **6** | 50 | n/d | **15** | >1000 | n/d |
| **7** | 25 | 125 | **16** | 1000 | n/d |
| **8** | 12.5 | n/d | **17** | 250 | n/d |
| **9** | 12.5 | 31 | **18** | 31 | n/d |

[A] Minimal inhibitory concentration against *E. coli* ATCC 11775; [B] Minimal hemolytic concentration; n/d - not determined. It should be noted that for MIM and MPD QACs different bacterial growth media were used (see Experimental section).

Dicationic MIM species **15** and **16** are virtually devoid of antibacterial activity, which can be explained by the fact that a terminal hydrophobic tail is needed to penetrate the membrane, a feature absent in **15** and **16**. Furthermore, no conclusion can be drawn from the MIC values regarding the effect of multivalent anions; compounds **4** (bromide) and **17** (sulfate) have MIC values in the same range whereas **18** (phosphate) is >6 times as active as **4**.[15] Similarly, the MIC values of the MPD bromides were determined against *E. coli* ATCC 11775. As expected, the same trend was observed as with the MIM salts: a longer alkyl chain constitutes higher antimicrobial activity.[16] Additionally, hemolytic indexes were determined of some of these compounds. MIM compounds **3**, **5**, **7** and **9** and the MPD bromides were tested for their potency to lyse erythrocytes and were found to have MHC (minimal hemolytic concentration, see Table 2) values that were close to the MIC values (1.25-5 fold (MIM) or 2-fold (MPD) the MIC values).

## 5.3 | Gel Formation

Having established the antimicrobial potency of the synthesized MIM and MPD bromides, attention was focussed on the gel formation of selected salts by mixing with water and two other polar liquids (ethylene glycol and glycerol).[17]

### 5.3.1 | Water-based gels (*W*-gels)

Following the procedure in which **4** showed phase transition upon mixing with ~5-40% (wt) $H_2O$, water-based gels (*W*-gels) of MIM bromides **4**-**7** were prepared. In case of MIM bromides **4**-**7** (see Table 3) homogeneous gels were obtained after addition of $H_2O$ at percentages ranging from 10-35% (wt) and homogenization by centrifugation. Obtaining homogeneous gels containing the lowest percentages of additive and longest alkyl chains required additional heating and/or sonication. For the MPD bromides (**10**-**14**, Table 3), gels with water (*W*-gels) could also be prepared with percentage of additive ranging from 10-50% (wt). In contrast, commercially available CTAB (**19**) did not form a *W*-gel. Both MIM and MPD bromides appear to follow a trend in which gel formation of compounds containing longer alkyl chains requires a higher percentage of additive. Some of the prepared *W*-gels ($C_{14}$MIM gels **8**) were found unstable (*i.e.* remained no longer in gel phase) when exposed to the air movements in a fume hood (*vide infra*), very likely due to a decrease in % (wt) of $H_2O$. Gels based on ethylene glycol or glycerol, with

boiling points higher than that of water, were therefore expected to be more stable under such conditions.

TABLE 3 | Gels of selected QACs containing water (*W*).

| | | Additive ($H_2O$ wt%) | | | |
|---|---|---|---|---|---|
| **#** | **Composition** | **a. 10%** | **b. 16%** | **c. 25%** | **d. 35%** |
| **4** | $C_{10}$MIM | Clear gel | Clear gel | Clear gel | Clear gel |
| **5** | $C_{11}$MIM | Clear gel | Clear gel | Clear gel | Clear gel |
| **6** | $C_{12}$MIM | Clear gel | Clear gel | Clear gel | Clear gel |
| **7** | $C_{13}$MIM | Solid | Clear gel | Clear gel | Clear gel |
| **8** | $C_{14}$MIM | Solid | Inh. | Clear gel | Clear gel |
| **9** | $C_{16}$MIM | Solid | Solid | Solid | Solid |
| | | **e. 20%** | **f. 30%** | **g. 40%** | **h. 50%** |
| **10** | $C_{10}$MPD | Clear gel[A] | Clear gel | Fluid | Fluid |
| **11** | $C_{11}$MPD | Inh. | Clear gel | Clear gel | Clear gel |
| **12** | $C_{12}$MPD | Inh. | Clear gel | Clear gel | Fluid |
| **13** | $C_{13}$MPD | Solid | Inh. | Clear gel | Clear gel |
| **14** | $C_{14}$MPD | Solid | Inh. | Clear gel | Clear gel |
| **19** | CTAB | Solid | Solid | Solid | Solid |

[A] A gel with 10% additive could also be constructed from this compound (**10a**); Inh. – inhomogeneous.

## 5.3.2 | Ethylene glycol-based gels (*E*-gels)

Compounds **4**, **5**, **10** and **19** could not be transformed into a gel with ethylene glycol (*E*-gel) in a range of 10-40% (wt). Whereas gelation of **9** was not induced with $H_2O$, 35% (wt) ethylene glycol caused gel formation.

TABLE 4 | Physical appearances of QACs upon addition of ethylene glycol (*E*).

| | Additive (ethylene glycol wt%) | | | |
|---|---|---|---|---|
| **Compound** | **i. 10%** | **j. 20%** | **k. 30%** | **l. 40%** |
| **4** $C_{10}$MIM | Fluid | Fluid | Fluid | Fluid |
| **5** $C_{11}$MIM | Fluid | Fluid | Fluid | Fluid |
| **6** $C_{12}$MIM | Inhomogeneous | Clear gel | Fluid | --- |
| **7** $C_{13}$MIM | Inhomogeneous | Inhomogeneous | Clear gel | Clear gel |
| **8** $C_{14}$MIM | Solid | Inhomogeneous | Inhomogeneous | Clear gel |
| **9** $C_{16}$MIM | Solid | Solid | Inhomogeneous | Clear gel[A] |
| **10** $C_{10}$MPD | Fluid | Fluid | Fluid | Fluid |
| **11** $C_{11}$MPD | Clear gel | Clear gel | --- | --- |
| **12** $C_{12}$MPD | Clear gel | Clear gel | Fluid | --- |
| **13** $C_{13}$MPD | Inhomogeneous | Clear gel | Clear gel | --- |
| **14** $C_{14}$MPD | Solid | Inhomogeneous | Clear gel | Fluid |
| **19** CTAB | Solid | Solid | Solid | Solid |

[A] 35% (wt) instead of 40% (wt) of ethylene glycol was used. --- not prepared.

In contrast to some of the $C_{14}$MIM *W*-gels, the *E*-gel **8l** proved to be stable towards air movements in a fume hood. Although the trend observed for *W*-gels regarding % additive/alkyl chain length also applies to *E*-gels, a higher % (wt) of additive was necessary to form homogeneous *E*-gels with increasing alkyl chain length as compared to *W*-gels.

### 5.3.3 | Glycerol-based gels (*G*-gels)

Preparation of MIM and MPD gels containing glycerol (*G*-gels) yielded similar trends as did the *E*-gels; all three **4**, **5** and **10** would not form a gel with 10-40% glycerol (Table 5). In general, higher percentages of glycerol were needed to form gels than was necessary with ethylene glycol. $C_{16}$MIM **9** could not be transformed into a *G*-gel, nor could CTAB **19**.

**TABLE 5** | Physical appearances of QACs upon addition of glycerol (*G*).

| | Additive (glycerol wt%) | | | |
|---|---|---|---|---|
| **Compound** | **m. 10%** | **n. 20%** | **p. 30%** | **q. 40%** |
| **4** $C_{10}$MIM | Fluid | Fluid | Fluid | Fluid |
| **5** $C_{11}$MIM | Clear gel | Fluid | Fluid | --- |
| **6** $C_{12}$MIM | Inhomogeneous | Clear gel | Clear gel | --- |
| **7** $C_{13}$MIM | Inhomogeneous | Inhomogeneous | Clear gel | Clear gel |
| **8** $C_{14}$MIM | Solid | Inhomogeneous | Inhomogeneous | Clear gel |
| **9** $C_{16}$MIM | Solid | Solid | Solid | Solid |
| **10** $C_{10}$MPD | Fluid | Fluid | Fluid | Fluid |
| **11** $C_{11}$MPD | Clear gel | Clear gel | --- | --- |
| **12** $C_{12}$MPD | Inhomogeneous | Clear gel | Clear gel | Fluid |
| **13** $C_{13}$MPD | Inhomogeneous | Inhomogeneous | Clear gel | Fluid |
| **14** $C_{14}$MPD | Solid | Solid | Inhomogeneous | Clear gel |
| **19** CTAB | Solid | Solid | Solid | Solid |

* Inhomogeneity is due to inability to mix QAC and additive thoroughly ('partly gelated'). --- not prepared.

## 5.4 | Gel Stability

Gel stability against a range of external factors determines their potential applicability. Preliminary results of studies towards the resistance of gels against gravity, temperature and water are discussed in the following paragraphs.

### 5.4.1 | Gravity

The *W*-gels **4b**, **5b** and **6b**, as well as **7c** and **8c** were examined for their ability to resist flow against gravity.[18] They were found to be resistant against gravity for at least 5 consecutive days, whereas the other MIM *W*-gels were stable for at least 6h (the longest period examined for these gels). MPD *W*-gels all were gravity-stable (at least 5 days), as were both all MIM and MPD *E*-gels. In the *G*-gel series, only MIM gel **6p** and MPD gel **12n** were found to be gravity- sensitive.

### 5.4.2 | Temperature

When examined for thermal stability, nearly all gels proved to be stable (*i.e.* did not become fluid, Table 6) up to temperatures of 60-65$^0$C for 30min. Exceptions were *W*-gels **4b** and **4c** (already becoming fluid at slightly elevated temperatures within seconds), and **10a** and **11f** that liquefied after heating to 65$^0$C after a few minutes. Increase in stability is observed in gels of **5** and **6** with increasing % (wt) of $H_2O$; the 30% versions are more stable than are their 16% counterparts. The results obtained with *E*-gels show that none of the MIM gels tested are stable except $C_{16}$MIM **9l**, whereas those composed of the corresponding MPD salts easily seem to handle heating to 60-65$^0$C for 30min. *G*-gels all appeared to be stable under the conditions given.

### 5.4.3 | Water resistance

Water resistance is an important aspect regarding applicability. It is undesirable for antimicrobial gels used as protective coatings outdoors to be easily removed *e.g.* at the event of rain. In this light, preliminary studies on the 'water-stability' of the prepared gels show that $C_{16}$MIM gel **9l** is a promising candidate. This particular gel, when dispensed on a vertical glass plate, showed the ability to withstand a one-minute, continuous drop-wise flow of 20mL/min of tap water. Although **9l** was not completely unaffected by this treatment, the water stability observed was not found in the vast majority of the gels reported in this study.

**TABLE 6** | Visual effects upon heating of selected gels.

| Gel | Composition | T ($^0$C)[A] | t (s) | Observation |
|---|---|---|---|---|
| **4b** | $C_{10}$MIM / 16% *W* | 24 | 5 | - |
| **4c** | $C_{10}$MIM / 30% *W* | 24 | 5 | - |
| **5b** | $C_{11}$MIM / 16% *W* | 42 | 90 | +/- |
| **5c** | $C_{11}$MIM / 30% *W* | 65 | 1800[B] | + |
| **6b** | $C_{12}$MIM / 16% *W* | 65 | 600 | +/- |
| **6c** | $C_{12}$MIM / 30% *W* | 65 | 1800 | + |
| **7c** | $C_{13}$MIM / 30% *W* | 65 | 1800 | + |
| **8c** | $C_{14}$MIM / 30% *W* | 65 | 1800 | + |
| **10a** | $C_{10}$MPD / 10% *W* | 65 | 360 | +/- |
| **11f** | $C_{11}$MPD / 30% *W* | 65 | 510 | +/- |
| **12f** | $C_{12}$MPD / 30% *W* | 65 | 1800 | + |
| **13g** | $C_{13}$MPD / 40% *W* | 65 | 1800 | + |
| **14f** | $C_{14}$MPD / 30% *W* | 60 | 1800 | + |
| **6k** | $C_{12}$MIM / 30% *E* | 65 | 360 | +/- |
| **7k** | $C_{13}$MIM / 30% *E* | 65 | 1200 | +/- |
| **8l** | $C_{14}$MIM / 40% *E* | 40 | 30 | - |
| **9l** | $C_{16}$MIM / 35% *E* | 65 | 1800 | + |
| **11i** | $C_{11}$MPD / 10% *E* | 65 | 360 | +/- |
| **12j** | $C_{12}$MPD / 20% *E* | 60 | 1800 | + |
| **13k** | $C_{13}$MPD / 30% *E* | 60 | 1800 | + |
| **6n** | $C_{12}$MIM / 20% *G* | 65 | 1800 | + |
| **7p** | $C_{13}$MIM / 30% *G* | 65 | 1800 | + |
| **8q** | $C_{14}$MIM / 40% *G* | 65 | 1800 | + |
| **11m** | $C_{11}$MPD / 10% *G* | 60 | 1800 | + |
| **12p** | $C_{12}$MPD / 30% *G* | 60 | 1800 | + |
| **13p** | $C_{13}$MPD / 30% *G* | 60 | 1800 | + |
| **14q** | $C_{14}$MPD / 40% *G* | 65 | 1800 | + |

[A] Temperature reached; [B] No apparent changes after prolonged heating to 3600s; + no apparent changes; - becomes fluid; +/- becomes partially fluid at time indicated.

## 5.5 | Biological Evaluation of Gels

Selected gels were assayed for their antimicrobial potency using an ISO standardized film adherence method.[19] In this method, small glass plates are coated with a thin layer of gel. A bacterial suspension containing Gram-negative *Escherichia coli* ATCC 8739 or Gram-positive *Staphylococcus aureus* ATCC 6538P was then added to the coating, covered, and incubated for 24h at 37$^0$C. Surviving colonies were replated and counted. Results are summarized in Tables 7-10 below (key: see Table 10) and show that all *W*-, *E*- and *G*-gels tested killed >99.9% of *E. coli*; a selection of the gels also eradicated Gram-positive *S. aureus* for >99.9%.

TABLE 7 | Antibacterial evaluation of selected MIM *W*-gels.

| # | Composition | *E. coli* ATCC 8739 | | *S. aureus* ATCC 6538P | |
|---|---|---|---|---|---|
| | | $t_0$ (log CFU)[A,B] | $t_{24}$ (log CFU)[A,C] | $t_0$ (log CFU)[A,B] | $t_{24}$ (log CFU)[A,C] |
| | Negative control | 5.11 | 6.49 | 4.87 | 5.06 |
| **4b** | $C_{10}$MIM/16% | 5.11 | <1.0[D] | 4.87 | <1.0[D] |
| **5b** | $C_{11}$MIM/16% | 5.11 | <1.0 | 4.87 | <1.0 |
| **6b** | $C_{12}$MIM/16% | 5.11 | <1.0 | 4.87 | <1.0 |
| **7c** | $C_{13}$MIM/30% | 5.11 | <1.0 | 4.87 | <1.0 |
| **8c** | $C_{14}$MIM/30% | 5.11 | <1.0 | 4.87 | <1.0 |

TABLE 8 | Antibacterial evaluation of selected MPD *W*-gels.

| # | Composition | *E. coli* ATCC 8739 | |
|---|---|---|---|
| | | $t_0$ (log CFU)[A,B] | $t_{24}$ (log CFU)[A,C] |
| | Negative control | 4.54 | 5.91 |
| **10e** | $C_{10}$MPD/20% | 4.54 | <1.0[D] |
| **11e** | $C_{11}$MPD/20% | 4.54 | <1.0 |
| **12g** | $C_{12}$MPD/40% | 4.54 | <1.0 |
| **13h** | $C_{13}$MPD/50% | 4.54 | <1.0 |
| **14h** | $C_{14}$MPD/50% | 4.54 | <1.0 |

TABLE 9 | Antibacterial evaluation of selected MIM & MPD *E*-gels.

| # | Composition | *E. coli* ATCC 8739 | |
|---|---|---|---|
| | | $t_0$ (log CFU)[A,B] | $t_{24}$ (log CFU)[A,C] |
| | Negative control | 4.54 | 5.91 |
| **7l** | $C_{13}$MIM/40% | 4.54 | <1.0[D] |
| **8l** | $C_{14}$MIM/40% | 4.54 | <1.0 |
| **11j** | $C_{11}$MPD/20% | 4.54 | <1.0 |
| **12i** | $C_{12}$MPD/10% | 4.54 | <1.0 |
| **13j** | $C_{13}$MPD/20% | 4.54 | <1.0 |
| **14k** | $C_{14}$MPD/30% | 4.54 | <1.0 |

TABLE 10 | Antibacterial evaluation of selected MIM & MPD *G*-gels.

| # | Composition | *E. coli* ATCC 8739 | |
|---|---|---|---|
| | | $t_0$ (log CFU)[A,B] | $t_{24}$ (log CFU)[A,C] |
| | Negative control | 4.54 | 5.91 |
| **5n** | $C_{11}$MIM/20% | 4.54 | <1.0[D] |
| **6n** | $C_{12}$MIM/20% | 4.54 | <1.0 |
| **7q** | $C_{13}$MIM/40% | 4.54 | <1.0 |
| **8q** | $C_{14}$MIM/40% | 4.54 | <1.0 |
| **11n** | $C_{11}$MPD/20% | 4.54 | <1.0 |
| **12p** | $C_{12}$MPD/30% | 4.54 | <1.0 |
| **13p** | $C_{13}$MPD/30% | 4.54 | <1.0 |

Key for Tables 7-10: [A] Average of triplo measurement; [B] At time t=0; [C] At time t=24h; [D] <1.0 equals >99.9% eradication. CFU - colony forming units.

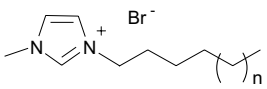
## 5.6 | Conclusion

Alkylated MIM and MPD bromides were synthesized and their antibiotic activity was determined against *E. coli* ATCC 11775. Increased activity was observed along with increasing chain length up to $C_{14}$. The relatively high MIC values, in combination with their MHC values (only a factor ~2 higher than the MIC values) indicate a lack of cell-selectivity and do not allow for systemic use.[2b]

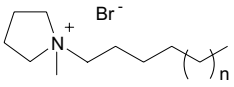
Unlike commercially available CTAB, most (ionic liquid and solid) MIM and MPD bromides could be brought into gel phase by addition of water, ethylene glycol, or glycerol. In general, the length of the alkyl chain appears to impose limitations on gel formation: whereas only an *E*-gel of $C_{16}$MIM bromide **9** could be obtained, CTAB **19** would not form a *W*-, *E*-, or *G*-gel in the range of 10-50% (wt), nor would $C_{18}$MIM (not shown). Preliminary tests to assess the susceptibility of the gels to external factors showed that the majority of the gels resisted flow against gravity and could withstand a temperature of 60-65$^0$C for 30min. Gel **9l** appeared to be also largely stable against running water. A selection of gravity-stable gels were assayed for antibacterial activity against *E. coli* ATCC 8739, and in some cases against *S. aureus* ATCC 6538P. Both these Gram-negative and Gram-positive bacteria were eradicated for >99.9%.

## 5.7 | Experimental Section

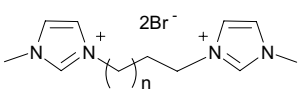
### 5.7.1 | Syntheses




**MIM bromides (1-9).** Typical procedure for the synthesis and analysis of MIM bromides: *n*-alkyl bromide (10mmol) and *N*-methylimidazole (1eq.) were stirred in a microwave oven (Personal Chemistry) at 150$^0$C for 30min with the Absorption leve option set at 'high'. The product was extracted in $Et_2O/H_2O$/MeOH, removing all unreacted reagents, and the solvents were removed through lyophilization. The bromides were obtained as ionic liquids (1-6) whereas 7-9 were solids. Compounds were analyzed by $^1$H NMR, $^{13}$C NMR and LCMS. All compounds were found to be pure except for residual water (~5-20% (wt) after vacuum drying for 24h at 70$^0$C; percentage increases along with alkyl chain). Yields: $C_{10}$MIM 4: 83%, $C_{11}$MIM 5: 90%, $C_{12}$MIM 6: 87%, $C_{13}$MIM 7: 88%, $C_{14}$MIM 8: 87%. $^1$H NMR data was found to be consistent with the data published.[9a] Representative analysis for $C_{14}$MIM Br 8: $^1$H NMR (MeOD): δ 9.01 (s, 1H, H2), 7.67 (t, 1H, H3), 7.59 (t, 1H, H4), 4.23 (t, 2H, H5), 3.95 (s, 3H, H1), 1.90 (m, 2H, H6), 1.34 (bm, 22H, H7-17), 0.90 (t, 3H, H18). $^{13}$C NMR (MeOD): δ 124.8, 123.5 (C3, C4), 50.6 (C5), 36.3 (C1), 32.8, 30.9, 30.5, 30.2, 29.9, 27.0, 23.5 (C6-17), 14.2 (C18). LC (254nm): Rt 18.2min. ESI-MS: 265.2 $[M–Me+H]^+$, 279.3 $[M]^+$.



**MPD bromides (10-14).**Typical procedure for synthesis and analysis of MPD salts: alkyl bromide (15mmol) and *N*-methylpyrrolidine (1eq.) were stirred at 50$^0$C for 16h, yielding a white precipitate in each case. The precipitate was filtered off, washed with $Et_2O$ (3x) removing all unreacted reagents and dried, yielding alkyl MPD bromides as white solids. Yields: $C_{10}$MPD **10**: 85%, $C_{11}$MPD **11**: 92%, $C_{12}$MPD **12**: 92%, $C_{13}$MPD **13**: 96%, $C_{14}$MPD **14**: 70%. All MPD salts were obtained as white solids containing some residual water (~8-20% (wt) after vacuum drying for 24h at 70$^0$C). $C_{13}$MPD contains ~25% (wt) $H_2O$ as exception. Representative analysis for $C_{10}$MPD Br **10**: $^1$H NMR (DMSO-$d_6$): δ 3.52 (m, 4H, H2), 3.38 (m, 2H, H4), 3.02 (s, 3H, H1), 2.09 (bm, 4H, H3), 1.69 (bm, 2H, H5), 1.26 (bm, 14H, H6-12), 0.87 (t, 3H, H13). $^{13}$C NMR (DMSO-$d_6$): δ 63.4 (C2), 63.0 (C4), 47.4 (C1), 31.4, 29.0, 28.8, 28.6, 26.0, 23.0, 22.2, 21.1 (C3, C5-12), 14.0 (C13). ESI-MS: 212.0 $[M\text{-}Me+H^+]^+$, 225.8 $[M^+]^+$, 565.5 $[2M^+ + TFA^-]^+$.



**MIM$_2$ bromides (15, 16).** Typical synthetic procedure: *n*-alkyl-α,ω-di-bromides (3mmol) were treated according to the described microwave procedure using 10mmol of *N*-methylimidazole; MeCN was added to a final volume of 3mL. After repeated of the extraction, residual *N*-methyl imidazole was found to be present by NMR, and compounds were purified by gel filtration using an LH20 column (88x2.8cm) and MeOH as eluent. Yields: $C_9MIM_2$ **15**: 64%, $C_{10}MIM_2$ **16**: 60%. Representative analysis for $C_9MIM_2$·2Br **15**: $^1$H NMR (MeOD): δ 9.23 (s, 2H, 2xH2), 7.82 (t, 2H, 2xH3), 7.74 (t, 2H, 2xH4), 4.35 (t, 4H, 2xH5), 4.05 (s, 6H, 2xH1), 1.95 (m, 4H, 2xH6), 1.37 (bm, 10H, 2xH7, 2xH8, H9). $^{13}$C NMR (MeOD): δ 124.9, 123.6 (C3, C4), 50.7 (C5), 37.0 (C1), 31.0, 29.7, 29.5, 27.0 (C6-C9).

**Metathesis of $C_{10}$MIM (17, 18)**
Bromide **4** (0.5mmol) was dissolved in 1mL MeOH/$H_2O$ 1/1 (v/v), and $Ag_2SO_4$ (**17**) or $Ag_3PO_4$ (**18**), both 1.0eq (taking into account the multivalent anions), were added under the exclusion of light. After stirring for 72h, samples were filtered and solvents were evaporated. After standing for 14d in daylight, newly formed precipitate was filtered off. $^{31}$P NMR ($D_2O$) of **18** showed a single peak. Materials were then subjected to antibacterial assays.

## 5.7.2 | Gel formation

***W*-gels (a-h)**
Gels of MIM compounds **4-8** were prepared by determination of the amount of residual $H_2O$ in a sample by $^1$H NMR (acetone-$d_6$) and then addition of $H_2O$ to obtain a gel with defined weight percentage of $H_2O$ (final concentrations of 10, 16, 25 and 35% (wt)). Gels containing MPD compounds **10-14** were obtained by determination of the $H_2O$ content through $^1$H NMR (DMSO-$d_6$) and subsequent addition of $H_2O$ to obtain the gels (final concentrations of 10, 20, 30 and 40% (wt)).

***E*-gels (i-l) and *G*-gels (m-p)**
Compounds were dried for 16h under vacuum at 70$^0$C. Ethylene glycol (*E*-gels) or glycerol (*G*-gels) was added to samples starting at 10% (wt), and increasing stepwise with 10% (wt) until a homogeneous gel would form (after gently heating/sonication and centrifugation if necessary) to a maximum of 50% (wt).

## 5.7.3 | Antibacterial assay in solution

*E. coli* ATCC 11775 were grown on nutrient agar plates and kept at 4$^0$C. Imidazolium salts were dissolved in Luria-Bertani (LB) and MPD salts in Brain-Heart Infusion (BHI) to give a concentration of 200μM and filtered using 0.22μm filter discs. An overnight culture in LB broth was adjusted to 5x10$^6$ CFU/mL and inoculated into the micro titre plate wells containing each 100μL of a serial two-fold dilution (200μM-down) of the tested compound in LB/BHI broth. After incubation for 24h at 37$^0$C, absorbance was measured at 600nm using a μQuant micro plate spectrophotometer. MIC values of compounds were measured in 3-fold and averaged.

### 5.7.4 | ISO Film adherence assay

Antimicrobial activities of the gels were quantitatively established using the film adherence method using Gram-negative *E. coli* ATCC 8739 and Gram-positive *S. aureus* ATCC 6538P. At time $t_0$, in 3-fold, object glasses were thinly coated in an area of $3cm^2$ with 2–5mg of the antimicrobial gel. Subsequently, bacterial suspension (50µL) containing approx. $1x10^5$ CFU was applied onto the coating and covered with a plastic film. Test samples were incubated for 24h at $37^0C$, after which the number of surviving bacteria was determined (*i.e.* $t_{24}$): bacteria were removed with swabs from the glasses, suspended and plated in tryptic soy agar (TSA). The TSA plates were incubated for 3 days at $37^0C$, after which the number of developing colonies was counted. The number of surviving bacteria were calculated in CFU and the antibacterial activity was calculated using log CFU @ $t_0$ (negative control) – log CFU @ $t_{24}$ (test sample).

### 5.7.5 | Hemolysis Assay

Minimal hemolytic concentration (MHC) values were determined by averaging the results of three measurements, according to the method described in see Chapter 1.

## 5.8 | Notes & References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9. During this research, a number of *n*-alkylated imidazolium salts (*N*-alkyl-*N'*-methyl imidazolium (MIM) salts) were indeed found (a) to exhibit antibiotic activity against a variety of bacteria, fungi and the model nematode *C. elegans* (b): (a) Demberelnyamba, D.; Kim, K.-S.; Choi, S.; Park, S.-Y.; Lee, H.; Kim, C.-J.; Yoo, I.-D. *Bioorg. Med. Chem.* **2004**, *12*, 853; (b) Swatloski, R.P.; Holbrey, J.D.; Memon, S.B.; Caldwell, G.A.; Caldwell, K.A.; Rogers, R.D. *Chem. Commun.* **2004**, 668
10. As are for example, the well-known Betadine-gel (also known as povidone-iodine) as in (a) O'Connor Jr, L.T.; Goldstein, M. *J. Am. Coll. Surg.* **2002**, *194*, 407; (b) Eason, E.; Wells, G.; Gerber, G.; Hemmings, R.; Luskey, G.; Gillett, P.; Martin, M. *BJOG* **2004**, *111*, 695; (c) Ostrander, R.V.; Brage, M.E.; Botte, M.J. *Clin. Orthop. Relat. Res.* **2003**, 246; (d) http://www.vidal.fr/Medicament/betadine-2054.htm or a gel containing polymyxin B and a QAC: Langford, J.H.; Artemi, P.; Benrimoj, S.I. *Ann. Pharmacother.* **1997**, *31*, 559
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15. As a part of the $PO_4^{3-}$ anions will be protonated due to their basicity ($pK_a$ of $HPO_4^{2-}$ 12.32) in solution, compound **18** will not be encountered in this composition. This makes a mono/ multivalent anion comparison rather complicated. Besides, it cannot be excluded that trace amounts of $Ag^+$ (bactericidal in the low µM range) are present in **17** and **18** as the metathesis method used relies on precipitation of AgBr. The minimum achievable level of $Ag^+$ contamination is dictated by the solubility product constant of AgBr in water, $5.2x10^{-13}$ (ref. 14).
16. It should be noted that for MIC determinations for MIM and MPD bromides, different growth media were applied (see Experimental section).
17. Based on their structural similarity: Ivanova, R.; Lindmann, B.; Alexandridis, P. *J. Colloid Interface Sci.* **2002**, *252*, 226
18. A 'gravity-resistant'or 'gravity-stable' gel refers here to the ability of a gel to remain at the same location at the bottom of a test tube if the tube is turned upside down for a defined period of time.
19. Japanese Industrial Standard JIS Z 2801: 2000 (E) Antimicrobial products - Test for antimicrobial activity and efficacy.

# CHAPTER 6 | Fluorous Techniques in Solid-Phase Peptide Synthesis

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## 6.1 | Introduction

The use of fluorous techniques[1] for the separation of reaction mixtures has found wide attraction in synthetic organic disciplines in recent years.[2] An important development comprises the so-called 'light-fluorous' strategy, developed by Curran and co-workers.[1a,3] In this strategy, compounds differing in fluorine content are separated by chromatography using a fluorous stationary phase. For this purpose, an array of fluorinated handles have become available in recent years, including fluorous versions of the Z,[4] Boc,[5] tBu,[6] Bn,[7] THP,[8] acyl-based,[9] silyl-based,[10] and alkoxyethylether[11] protecting groups.

## 6.2 | Fluorous Techniques in SPPS

Although synthetic peptides containing up to 50 residues have become available *via* improved solid-phase synthesis techniques in recent decades, facile HPLC purification of the desired full-length peptide (of n residues) is often hampered by the presence of incomplete sequences (*e.g.* n-1 and n-2 sequences). This is especially true for longer peptides as the chromatographic behavior of full-length and n-1 and n-2 sequences becomes less distinguishable with increasing number of residues, resulting in tedious and lengthy HPLC purification procedures along with unavoidable handling losses. This Chapter describes two approaches for application of fluorous strategies in the purification of synthetic peptides.

### 6.2.1 | Fluorous chromatography

Introduction of long, polyfluorinated alkyl groups increases both hydrophobicity and fluorophilicity of molecules. To verify that the 'light-fluorous' strategy relies on fluorophilic rather than hydrophobic properties of compounds, a test peptide GEPKPAG was equipped with two handles differing from each other only in fluorine content. Thus, in order to examine their influence on the chromatographic behaviour of the peptide, the peptide was equipped with either a lipophilic *n*-undecanoyl ($C_8H_{17}(CH_2)_2COR$) moiety or a fluorinated alkanoyl handle ($C_8F_{17}(CH_2)_2COR$) were introduced.

The three peptides GEPKPAG, **L**-GEPKPAG (lipophilic) and **F**-GEPKPAG (fluorophilic) were synthesized via standard Fmoc-based SPPS procedures and evaluated for their affinity towards a FluoPhase LC column, the stationary phase of which is coated with polyfluorinated alkyl chains. Elution of the three peptides occurred with a gradient of 20→100% TFE in 0.1% aq. TFA in 18min. GEPKPAG, a peptide that is neither lipophilic nor fluorophilic, was expected to have minimal retention on the FluoPhase column; indeed, it elutes in the injection peak (Rt < 2min, not shown). Peptide **L**-GEPKPAG shows a distinct retention time (Rt 7.21min, Figure 1, trace A), indicating there is a significant hydrophobic interaction with the FluoPhase column. Fluorophilic peptide **F**-GEPKPAG however displays a significantly larger retention time than **L**-GEPKPAG (Figure 1, trace B), which is indicative of additional effects and confirms the proposed concepts of 'light-fluorous' chromatography techniques.

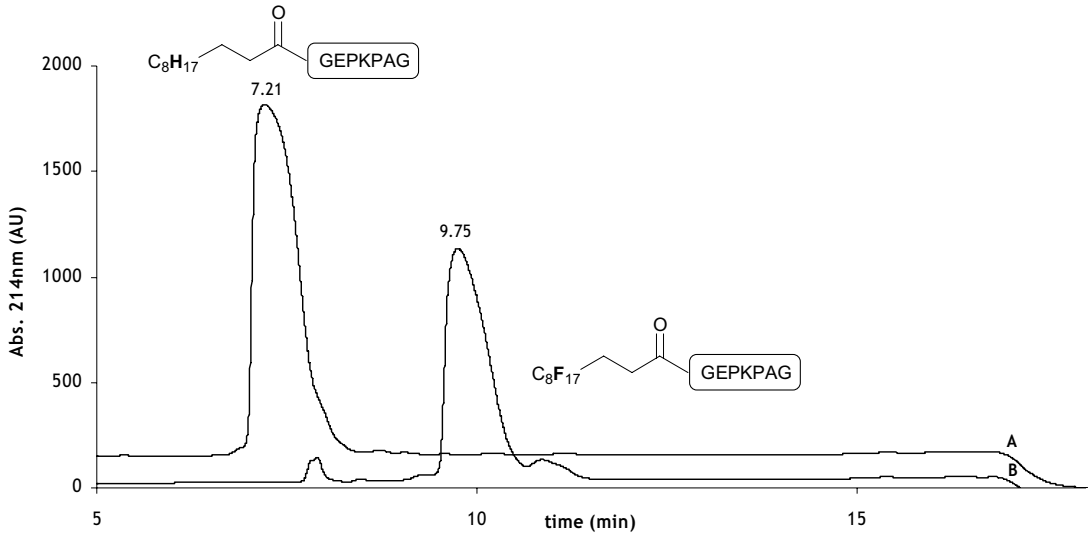
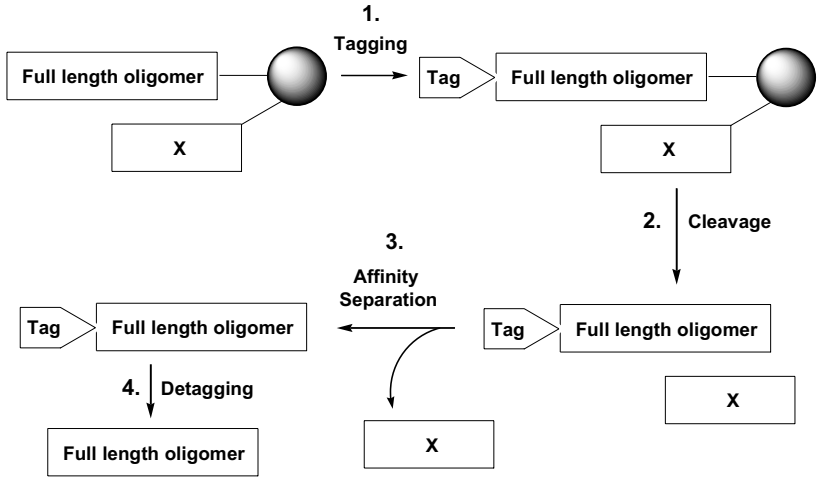



**FIGURE 1** | Chromatograms (5-18min) of peptides **L**-GEPKPAG (trace A) and **F**-GEPKPAG (trace B) on a FluoPhase $C_{18}$ RP column, applying a gradient of 20→100% TFE/$H_2O$ + 0.05% TFA in 13min, with UV detection at 214nm.

## 6.2.2 | Fluorous Strategies in SPPS

The application of fluorous handles promises to be a valuable asset in stepwise solid-phase synthesis procedures. Target molecules, of which the purification from final product mixtures is hampered by the occurrence of closely eluting impurities, can be effectively isolated with the aid of fluorous-based separation.




SCHEME 1 | Schematic representation of tagging strategy. On-resin tagging of the desired product (1) yields a mixture after cleavage (2) from which the desired product can be selectively obtained through affinity separation (3). X = capped incomplete sequences.

Two different approaches can be distinguished, that is, attachment of an appropriate fluorous protecting group to either the final products (*tagging*, Scheme 1) or to the intermediate unreacted species (*capping*). The success of fluorous capping in solid-phase chemistry was demonstrated by Seeberger *et al*.[10b,c] who were able to separate the desired products from the fluorous impurities after the solid-phase assembly of oligosaccharides.

## 6.3 | Fluorous Tag: Design & Synthesis

Recently, the first example of the use of a fluorous tag (a fluorinated derivative of the Z-protecting group, *i.e.* FZ tag, Figure 2) in the purification of peptides obtained through solid-phase peptide synthesis was disclosed.[4a]

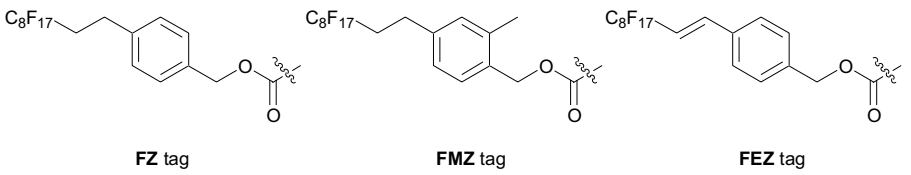



FIGURE 2 | Structures of the designed FZ tag and derivatives for tuning of acid lability.

Although the acid-lability of the FZ tag could be tuned by the introduction of additional substituents (FMZ and FEZ tags), the application of the FZ tag in Fmoc-based SPPS proved to be restricted. It requires the use of amino acids with highly acid-labile side-chain protecting groups and a highly acid-labile linker. It was reasoned that a base-labile fluorous protecting group would be an attractive alternative tag for standard Fmoc-based SPPS with acid-labile side-chain protections and standard linker/resin systems such as Wang and Rink amide resins. To this end, a fluorous version of the Msc (methylsulfonylethoxycarbonyl, Figure 3)[12] protecting group for amines, introduced by Tesser and co-workers nearly 30 years ago,[13] was designed and synthesized. The new fluorous protecting group was named FMsc (**1**), in analogy with FZ.

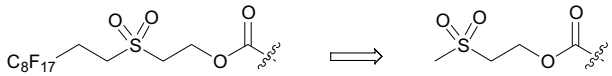
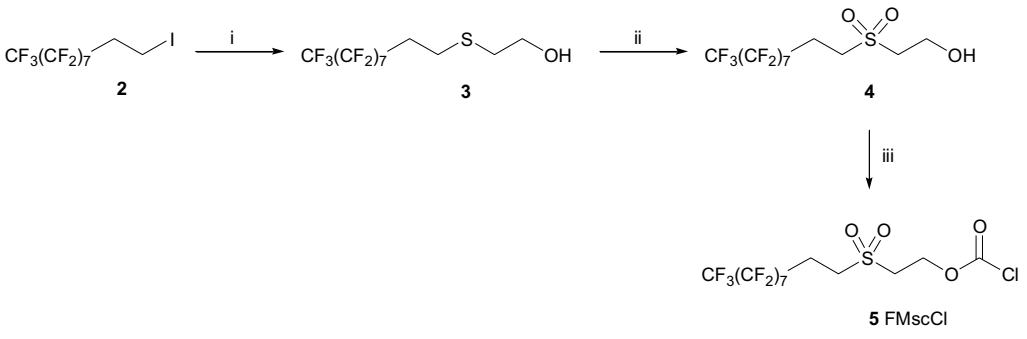



FIGURE 3 | Structures of the Msc protecting group and the derived tag **1**.

The FMscCl tagging reagent **5** was readily synthesised as follows (Scheme 2). In the first step, commercially available [1H,1H,2H,2H]-perfluorodecyl iodide **2** was substituted with 2-mercaptoethanol to give **3**. After oxidation of thioether **3** to the corresponding sulfone **4** (30% AcOOH/AcOH), target FMscCl **5** was obtained by chloroformylation of **4**, in an excellent overall yield of 88%.




SCHEME 2 | *Reagents and conditions:* (i) 2-mercaptoethanol, NaOH, tBuOH, reflux (91%); (ii) AcOOH (30% in AcOH), $H_2O$, 97%; (iii) phosgene (20% in toluene), THF (quant.).

## 6.4 | Application of the FMsc Tag

With reagent **5** in hand, the ease and efficiency of introduction and removal of the FMsc tag **1** was explored. Model peptide **6** (Table 1) was treated with FMscCl **5** (5eq.) and DiPEA (10eq.) in DMF to give the corresponding tagged peptide FMsc-**6**, as corroborated by LCMS (Figure 4, trace A). Treatment of FMsc-**6** with 2% aq. $NH_3$ for 15min furnished starting peptide **6** (Figure 4, trace B).

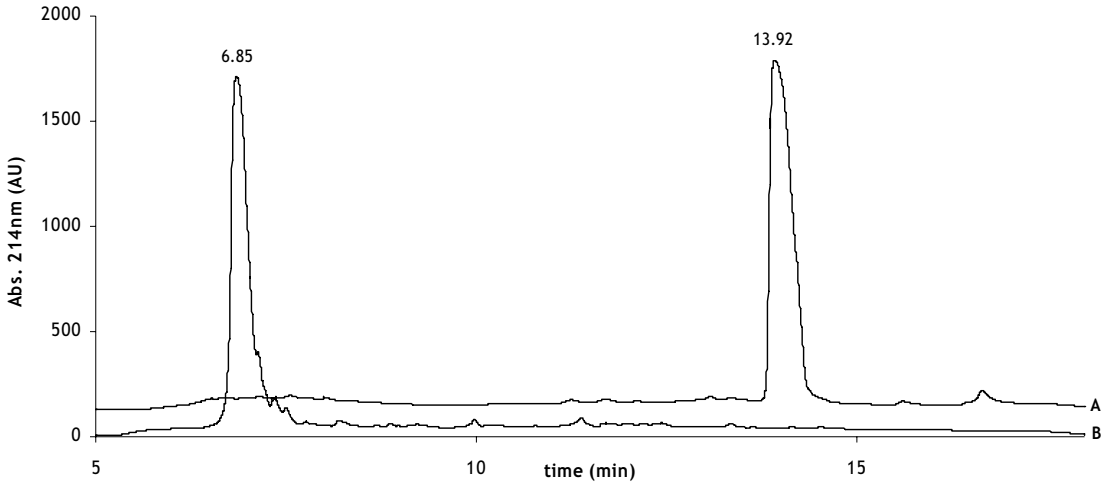



FIGURE 4 | LC chromatograms (5-18min, C18 RP column, 5→90% MeCN/0.1% aq. TFA, detection at 214nm) of peptide GEPKPAG; **A.** after on-resin tagging (FMsc-**6**); **B.** after detagging (**6**).

### 6.4.1 | Fluorous HPLC (FHPLC) purification

On the basis of these results, we turned our attention to the application of the FMsc tag in the purification of peptides **7**-**9** (Table 1) obtained through SPPS. These peptides are difficult to isolate in pure form using reversed-phase HPLC.[14,15] For instance, the assembly of 35-mer **9** by a standard SPPS protocol suffered from incomplete couplings leading to a tedious isolation procedure, as shown by the HPLC pattern of crude **9** (Figure 5E).

TABLE 1 | Synthetic peptides used in this study.

| Peptide | Sequence |
|---|---|
| **6** | GEPKP AGa |
| **7** | GCCSL PPCAL NNPDYa |
| **8** | RQIKI WFQNR RMKWK Ka |
| **9** | LSELD DRADA LQAGF SQFES SAAKL KRKYW WKNLK |

a = C-terminal carboxamide

Peptides **7**-**9** were synthesised using standard Fmoc-based SPPS with either HCTU (**7** and **8**) or BOP/HOBt (**9**) as condensing agents, starting from Rink amide (**7**, **8**) and Wang resins (**9**). In the latter case, the first amino acid was condensed with the resin using DIC and DMAP. Each condensation step was followed by capping ($Ac_2O$, DiPEA, HOBt) of the residual unreacted amines. After completion of the synthesis of the respective oligopeptide sequences and removal of the final Fmoc group, the resin-bound peptides were tagged with FMscCl/DiPEA (5 and 10eq. respectively) in DMF. The peptides were then cleaved from the solid support with concomitant liberation of the side-chain functionalities using TFA/TIS/$H_2O$ (95/2.5/2.5, v/v/v).

At this stage, we turned our attention to the purification of tagged 35-mer **9**. As can be seen (Figure 5F), full length, FMsc-tagged **9** eluted significantly later from a fluorous HPLC (FHPLC) column than non-tagged impurities. Semi-preparative FHPLC with an aq. TFE gradient afforded FMsc-**9** in 99% purity (Figure 5G). Detagging (2% aq. $NH_3$, Figure 5H) finally afforded target peptide **9** in 21% overall yield (based on initial loading of the resin). Tagged peptides **7** (Figure 5) and **8** were purified with FHPLC and detagged with equal efficiency (Table 2).

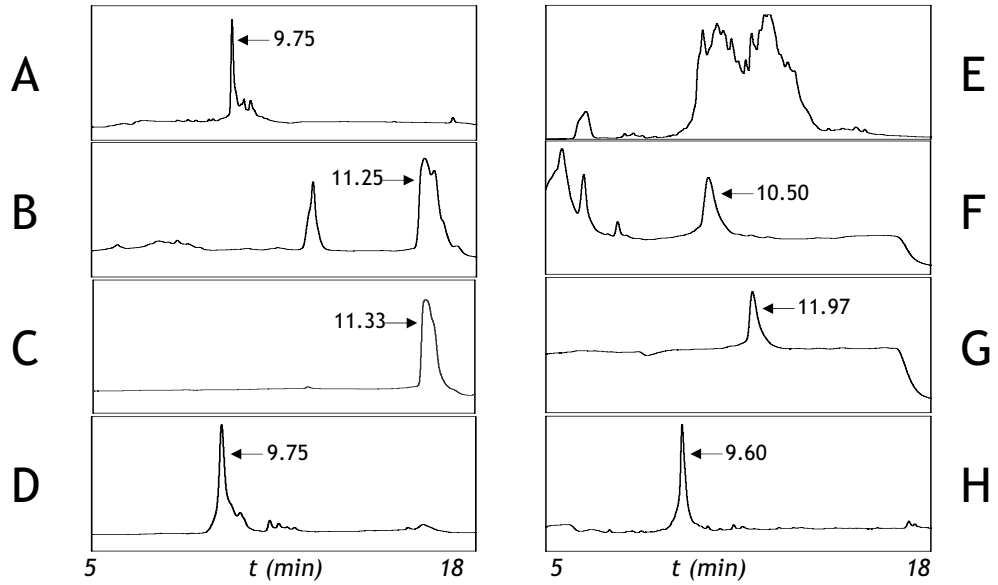



FIGURE 5 | Chromatograms (5-18min except **B** and **C**, 5-12min), UV detection at 214nm) of peptides purified by FHPLC. *Left:* peptide **7**: **A.** Crude **7**; **B**. Crude-FMsc-**7**, the apparent double peak is due to overloading of the column; **C**. FHPLC-purified FMsc-**7**; **D**. Pure **7** after detagging; shoulder is caused by S-S oxidation. *Right:* 35-mer **9**: **E**. Crude **9**; **F**. Crude FMsc-**9**; **G**. FHPLC-purified FMsc-**9**; **H**. Pure **9** after detagging. HPLC methods (see Experimental section): **A**,**D**,**E**,**H** method ***X***; **F**,**G** method ***Y***. **B**,**C** method ***Z***. Peaks with correct mass are indicated with arrows. Shift of retention time is due to different sample composition.

TABLE 2 | Data on detagged peptides purified by FHPLC.

| Peptide | Yield (%)[A] | Purity (%)[D] | ESI-MS | |
|---|---|---|---|---|
| | | | Tagged | Detagged |
| **7** | 37 | 98 | 2252.0 | 1669.0 |
| **8** | 10 | 94 | 2828.8 | 2246.6 |
| **9** | 21 | 99 | 2343.0[B] | 4099.121[C] |

[A] Based on loading of the first amino acid; [B] $[M+2H]^{2+}$; [C] HRMS (calcd. 4099.128); [D] purity determined by integration of LC peaks.

## 6.4.2 | Fluorous SPE (FSPE) purification

In an alternative approach, it was investigated whether FMsc-tagged peptides **7**-**9** could be purified by fluorous silica gel extraction.[16] As an example, crude FMsc-**7** was applied to a fluorous solid-phase extraction (FSPE) cartridge. With the aim of removing non-tagged impurities, the cartridge was eluted first with 0.1% aq. TFA and then with 0.1% TFA in MeOH/$H_2O$ 1/1 (v/v). Subsequent elution of the cartridge with 0.1% TFA/MeOH afforded FMsc-**7** in a significant enhanced purity after detagging (59% yield, 91% purity, Table 3). Although the purity of the final product was somewhat lower than was observed after FHPLC, the ease of execution and potential scale (~50mg crude FMsc-**7** could be applied to the FSPE cartridge in one single run) makes this purification procedure an attractive alternative. In a similar way, FMsc-tagged **8** was purified and detagged (Table 3). In this case, the use of a mixture of 0.1% TFA in TFE/$H_2O$ 1/1 (v/v) proved to be more effective for elution of the fluorous peptide. In contrast, purification of FMsc-**9** on a FSPE cartridge failed, arguably due to the relative low fluorine content (only 7% by weight compared to 14% in **7** and 11% in **8**).

TABLE 3 | Data on detagged peptides purified by FSPE.

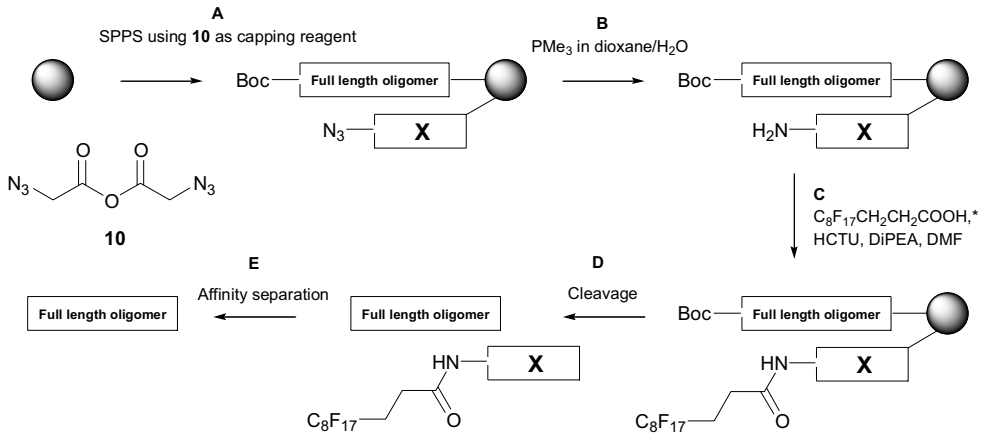
| Peptide | Crude Material (mg) | Detagged Yield (%)[A] | Purity (%)[B] | Eluents[C] |
|---|---|---|---|---|
| **7** | 45.9 | 59 | 91 | MeOH/$H_2O$ |
| **8** | 17.2 | 7 | 72 | TFE/$H_2O$ |

[A] Based on loading of the first amino acid; [B] purity determined through integration of LC peaks;
[C] containing 0.1% TFA.

## 6.5 | Fluorous Capping

The described fluorous methods (FZ, FMsc) based on *tagging* of the desired product, discussed in the previous paragraphs, have the common disadvantage that the fluorous product obtained after purification by either FHPLC or FSPE must be subjected to one final detagging step, which leads to inevitable handling losses. Attachment of a fluorous handle to all incomplete sequences, *i.e.* the use of fluorous *capping* reagents,[10b,c] overcomes this last reaction step. However, substitution of commonly used $Ac_2O$ for capping in SPPS with a fluorous anhydride (*e.g.* [1H,1H,2H,2H]-perfluoroundecanoic acid anhydride) brings about some practical issues: [1H,1H,2H,2H]-perfluoroalkanoic anhydrides with sufficient fluorine content are not commercially available, and the precursor acids are rather expensive for large-scale use. Besides, the reactivity of the fluorous capping reagent is unknown and might well differ from $Ac_2O$.

To circumvent these issues, the following two-step fluorous capping strategy was designed. As outlined in Scheme 3, $Ac_2O$ in standard SPPS procedures (step **A**) has been replaced by azidoacetic acid anhydride (**10**) or azidoacetic acid/HCTU. After completion of the SPPS (**A**), in which the N-terminal residue is incorporated as its *N*α-Boc-protected derivative, the on-resin azides are reduced by phosphine treatment (**B**) and subsequently capped by reaction with a perfluoroalkyl acid or its anhydride (step **C**). As the last two steps are performed after automated synthesis, their progress can be easily followed by TNBS or Kaiser tests.




SCHEME 3 | Outline of the two-step fluorous capping strategy. * or the corresponding anhydride/DiPEA if desired; the azidoacetic acid/HCTU/DiPEA conditions can be applied instead of compound **10** as well.

After cleavage from the resin (**D**), fluorous affinity purification (FHPLC/FSPE, **E**) can be applied to obtain the desired product as the first, non-fluorous, peptide eluting. This way, the fluorous acylation step **C** is applied only once (after completion of the SPPS) as in the tagging approach, rather than after every coupling step as in the original capping approach.

In a preliminary experiment, the peptide GEPKPAG was synthesized following the two-step fluorous capping strategy. During automated Fmoc-based SPPS, 0.85eq. of amino acid was used to ensure incomplete sequences. After each coupling, the resin was treated with azidoacetic acid/HCTU/DiPEA. After completion of the synthesis, treatment with $PMe_3$/1,4-dioxane/$H_2O$ yielded the free amines, as verified by TNBS test. Subsequent acylation with [1H,1H,2H,2H]-perfluoroundecanoic acid/HCTU/DiPEA resulted in fluorous capped incomplete sequences. After cleavage from the resin, the crude material (14.2mg) was purified in a single run using FSPE. The FSPE cartridge, containing 0.7mL fluorous silica, was eluted with 4x5mL 10% aq. TFA, and the desired product was found primarily in the first 5mL fraction, with very low amounts in the other fractions. Importantly, no fluorous incomplete sequences were detected by LCMS in these fractions. The yield of the desired peptide was 28% of the theoretical maximum yield. Although this method does need optimization, it might eventually lead to a simple and time-effective synthesis/purification procedure for peptides.

## 6.6 | Summary

In summary, the development and application of a new fluorous FMsc protecting group **1** was disclosed. Application of FMscCl **5** in Fmoc-based SPPS allows the generation of full-length peptides equipped with a base-labile fluorous tag. These peptides were purified by either FHPLC or FSPE, after which the tag is easily and quantitatively removed. Furthermore, a *two-step fluorous capping* approach towards a capping strategy was discussed along with preliminary results. In conclusion, fluorous strategies can be valuable tools enabling isolation of desired products from complex crude synthetic peptide mixtures.

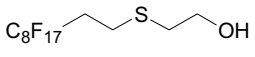
## 6.7 | Experimental Section

### 6.7.1 | HPLC methods

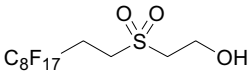
HPLC methods: **X**. Alltech Alltima C18 column (150x4.6mm), 5→90% MeCN/H2O+0.05% TFA in 17min at 1.0mL/min; **Y**. Keystone Scientific Operations FluoPhase WP column (100x4.6mm), 20→100% TFE/$H_2O$ + 0.05% TFA in 18min at 1.5mL/min; **Z**. Identical to method **Y** but gradient elution time is 12min. UV detection in all methods at 214nm.

### 6.7.2 | Syntheses

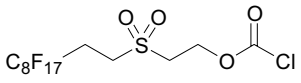
**Acylated GEPKPAG dervatives**
GEPKPAG was synthesized using automated Fmoc-based SPPS procedures using Rink amide resin and HCTU/DiPEA as coupling reagents. After completion of the synthesis, the last Fmoc group of GEPKPAG was removed and the resin was split into three batches (6.5µmol each), two of which were treated with undecanoic acid/DiPEA (5 and 10eq. respectively) or [1H,1H,2H,2H]-perfluoroundecanoic acid/DiPEA (5 and 10eq. respectively) in DMF for 2h. After acidic cleavage of all batches from the resins, three peptides were obtained; GEPKPAG, **F**-GEPKPAG and **L**-GEPKPAG. These peptides were analyzed by FHPLC (method **Y**): GEPKPAG Rt < 2min, **L**-GEPKPAG Rt 7.21min, **F**-GEPKPAG Rt 9.75min.




**([1H,1H,2H,2H]-perfluorodecyl)sulfidylethanol (3).** Under nitrogen atmosphere, NaOH (0.50g, 12.5mmol, 1.5eq.) and 2-mercaptoethanol (1.41mL, 20mmol, 2.45eq.) were refluxed in tBuOH (30mL) for 30min. Next, [1H,1H,2H,2H]-perfluorodecyl iodide **2** (4.71g, 8.15mmol) was added and the mixture was stirred under reflux conditions for an additional 3h. After evaporation of all volatiles, the crude product was subjected to silica gel column purification, yielding 3.88g (7.41mmol, 91%) of pure **3**. TLC (PE/EtOAc 1/1 (v/v)): $R_f$ 0.70. $^1$H NMR ($CDCl_3$): δ 3.75 (s, 1H, OH), 3.75-3.68 (m, 2H, H1), 2.71-2.42 (m, 4H, H2, H3), 2.40-2.09 (m, 2H, H4). $^{13}$C NMR ($CDCl_3$): δ 123.1-104.7 (CF), 59.5 (C1), 24.6 (C2), 32.3, 31.9, 31.4 (C4), 22.4 (C3); $^{19}$F NMR ($CDCl_3$): δ -5.03 ($CF_3$), -38.1, -45.6, -46.7, -47.0, -50.1 ($CF_2$). ESI-MS: 547.1 $[M+Na]^+$.




**([1H,1H,2H,2H]-perfluorodecyl)sulfonylethanol (4).** To a solution of **3** (3.12g, 5.95mmol) in ice-cooled AcOH (1.6mL), 32% AcOOH in AcOH (3.26mL, 1.2eq) and water (1.1mL, 10eq) were added. If gel formation occurred, EtOAc (5mL) was added to dilute the reaction mixture. After 2h, TLC showed total conversion of the starting material and $NaHCO_3$ (s) was carefully added to neutralize the mixture. Next, the mixture was extracted using a large excess of EtOAc (3x) and dried over $MgSO_4$. After filtration and removal of solvents *in vacuo*, the crude product was purified by silica column chromatography. Compound **4** was obtained as a white solid (yield: 5.77mmol, 97%). TLC (EtOAc/PE 1/1 (v/v)): $R_f$ 0.49. $^1$H NMR (MeOD): δ 3.90 (t, 2H, H1, $J_{1,2}$ = 5.1Hz), 3.60-3.33 (m, 2H, H3, $J_{3,4}$ = 8.0Hz), 3.25 (t, 2H, H2), 2.84-2.48 (m, 2H, H4). $^{13}$C NMR (MeOD): δ 56.8 (C1), 47.5 (C2, C3), 25.9, 25.4, 25.0 (C4). $^{19}$F NMR (MeOD): δ -1.17 ($CF_3$), -33.5, -41.6, -42.5, -43.0, -46.1 ($CF_2$). ESI-MS: 579.1 $[M+Na]^+$.




**([1H,1H,2H,2H]-perfluorodecyl)sulfonylethoxycarbonyl chloride (5).** Compound **4** (5.77mmol) was dissolved in freshly distilled THF (50mL). To this solution, phosgene (20% in toluene, 24mL, 9eq.) was added. After 16h, TLC indicated total conversion of the starting material. Phosgene and solvents were carefully removed *in vacuo* in a fumehood. The residue was coevaporated with toluene (2x), yielding 3.56g (5.75mmol, quant.) as a pinkish white solid. TLC (EtOAc/PE 1/1 (v/v)): $R_f$ 0.85. $^1$H NMR ($CDCl_3$): δ 4.82-4.71 (m, 2H, H1), 3.51-3.41 (m, 2H, H2), 3.41-3.26 (m, 2H, H3), 2.86-2.53 (m, 2H, H4). $^{13}$C NMR (acetone-$d_6$): δ 120.3-110.6 (CF), 65.4 (C1), 51.8 (C2), 45.9 (C3), 24.8, 24.4, 24.0 (C4). $^{19}$F NMR ($CDCl_3$): δ -3.5 ($CF_3$), -36.3, -44.6, -45.2, -45.8, -48.8 ($CF_2$). A sample of **5** was solvolyzed in MeOH to give the methyl carbonate, ESI-MS: 637.1 $[M+Na]^+$.

**Loading of Wang Resin**
A flask was silylated using 20% TMSCl in $CHCl_3$. Therein, the Wang resin was coevaporated with DCE, suspended in $CH_2Cl_2$ and treated with the first amino acid (3eq.), DIC (3eq.) and DMAP (0.1eq.) for 2h. The resin was filtered off, rinsed with $CH_2Cl_2$, MeOH and $Et_2O$ and air-dried. Loading of the resin was determined by measuring the UV absorbance at 300nm of released Fmoc-chromophore upon treatment of a resin sample with 20% piperidine/DMF for 10min.

### 6.7.3 | FMsc Tagging Procedure

After SPPS, the free N-terminus of the full-length peptide was tagged by suspending the resin in DMF or NMP (1.5mL per 50µmol on-resin peptide), and adding FMscCl (**5**, 5eq.) and DiPEA (10eq.) and shaking at RT for 1h. The resin was filtered off and washed with DMF or NMP, MeOH and $CH_2Cl_2$. If TNBS or Kaiser test was positive, procedure was repeated.

### 6.7.4 | FSPE Procedure

Crude peptide mixture was dissolved in the smallest amount of $H_2O$ possible, and AcOH was added to aid dissolution if necessary. This mixture was loaded onto a 5mL syringe that contained 0.7-2.0mL fluorous silica material (FTI, Pittsburg, USA) with a filter on top of the silica layer. The cartridge was subsequently eluted with volumes of 0.1% aq. TFA, 0.1% aq. TFA/MeOH 1/1 (v/v) and 0.1% TFA/MeOH and fractions (1-5mL) were collected. If LCMS analysis required the use of TFE, TFE instead of MeOH was used in the FSPE purification of these particular peptides.

## 6.8 | Notes & References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# CHAPTER 7 | Summary & Future Prospects

## 7.1 | Summary & Future Prospects

Given the alarming rate of evolving bacterial resistance against commonly used antibiotics, the demand for new classes of antimicrobials to stay ahead of bacteria is increasing. Cationic antimicrobial peptides (CAPs) are ubiquitous in nature and play key roles in the innate immune systems of virtually all living species. CAPs come in large variations regarding length, primary and secondary structures, but all share amphiphilicity. In fact, this amphiphilicity is the factor that appears to determine the preference of the majority of CAPs for Gram-negative bacteria by allowing initial electrostatic interactions with their anionic outer membranes. The **General Introduction** gives an overview on the current status of common antibacterial drugs and research towards new entities for combating especially Gram-negative bacterial infections, with special attention for the relatively new antibiotic class of CAPs and compounds inspired by CAPs.

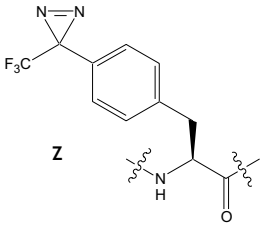
Optimization of CAPs regarding secondary structure, amphiphilicity, toxicity, selectivity and stability for the discovery of lead structures is an on-going process driven by the demand for new antibiotics. The research area of CAPs, further expanded by research into CAP-inspired amphiphilic structures, inspires a large global scientific community and has led to a number of projects in which CAP-based compounds have arrived in different stages of clinical trials.

**Chapter 1** deals with the stability issue of drosocin (DRC), a CAP isolated from *Drosophila melanogaster* (fruit fly). This CAP is a potential candidate for further drug development: it possesses desired characteristics in that it kills Gram-negative bacteria in the low micromolar concentration range, does not bind the human equivalent of its target bacterial protein (*i.e.* it is selective) and is non-toxic to human erythrocytes. Unfortunately, it is broken down by proteases in serum before it can exert its activity. Substitution of amino acid residues 1, 6 and/or 7 led to a series of analogues of which the best compound (**1**, Table 1) showed a ~30-fold increased serum stability. This compound might be further optimized regarding stability and activity to become a new CAP-based drug lead.

Substitutions are by no means limited to the Tyr6/Ser7 dipeptide nor limited to closely related amino acids as is illustrated by compounds **2**-**4** in the text below (see also Table 1).

**TABLE 1** | The most stable DRC analogue (**1**), analogues containing a sugar amino acid (SAA) (**2**) or a $^{Me}$Arg residue (**3**). Derivative **4** enables identification of the binding location in its target protein DnaK. Structure of the amino acid (Tmd)Phe (**Z**) is depicted on the right.

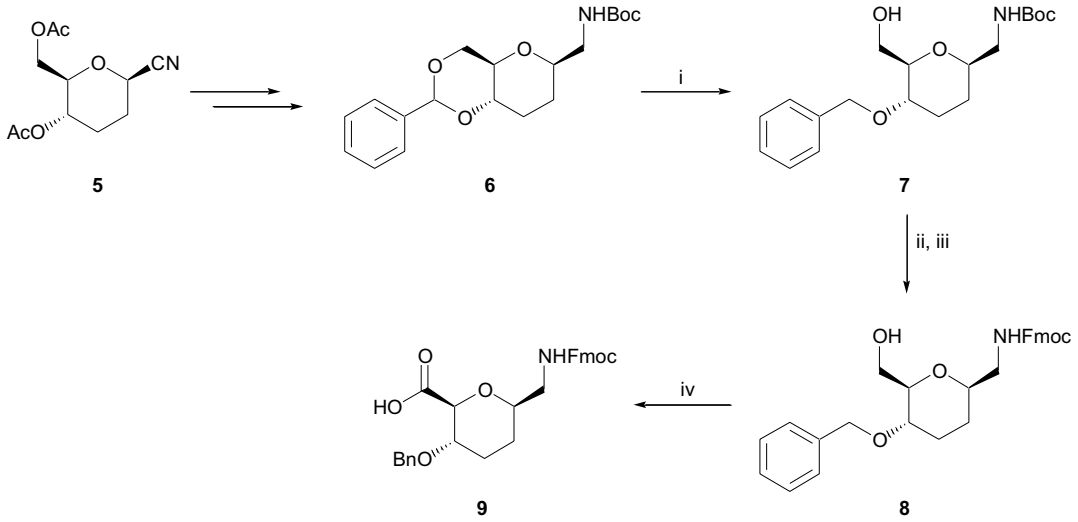
| | Sequence[C] | % left[A] | MIC[B] (μM) |
|---|---|---|---|
| DRC | GKPRP Y SPRP TSHPR PI RV | 3 | 6.3 |
| **1** | **βAla**-KPRP Y **T**PRP TSHPR PI RV | 87 | 3.1 |
| **2** | GKPRP **SAA-**SPRP TSHPR PI RV | <1 | 12.5 |
| **3** | GKPRP Y SPRP TSHPR PI$^{Me}$RV | n/d | 50 |
| **4** | GKPRP **Z** SPRP TSHPR PI RV | n/d | n/d |



**Z**

[A] After 8h in 25% human serum; [B] against *E. coli* ATCC 11775; [C] SAA= sugar amino acid, $^{Me}$R=$N^{\alpha}$-methylargininyl, Z=(Tmd)Phe.

For example, in structure **2**, a sugar amino acid (SAA) residue was designed in an attempt to combine two desired characteristics of such a substitute. First, the unnatural SAA acts as a dipeptide isostere, replacing the labile Tyr6/Ser7 amide bond. Second, these types of SAA structures are known to be capable of inducing a flexible β-turn.[1] The presence of a turn element was observed in proximity of this dipeptide in NMR studies of glycosylated DRC, and this turn element is suspected be involved in interactions with drosocin's target.[2] The Bn protecting group was retained to add hydrophobicity which is present in the original dipeptide. The intermediate Boc-protected building block **6** was synthesized in parallel with a recently published procedure[3] from cyanide **5** (Scheme 1).[4] Selective opening of the benzylidene moiety towards the 4-*O*-benzyl protected compound **7** was realized with DiBAl-H at -40$^0$C.




**SCHEME 1** | Key steps in synthesis of Fmoc-SAA-OH (**9**). Reagents & conditions: i. DiBAl-H, toluene, -40$^0$C (70%) ii. 25% TFA/$CH_2Cl_2$; iii. FmocOSu, dioxane/$H_2O$ (62% over 2 steps); iv. (1) IBX, $CH_2Cl_2$ (2) $NaClO_2$, tBuOH, $H_2O$, 2-methyl-2-butene (60% over 2 steps).

Amine protecting group manipulations (→**8**) and two-step oxidation of the primary alcohol function yielded the Fmoc-derivative **9** which was used in standard automated SPPS protocols to give DRC derivative **2** (HRMS: $[M+H]^+$ 2194.258, calcd. 2194.260). Peptide **2** had a MIC value of 12.5μM, but was completely degraded after 8h in 25% human serum.

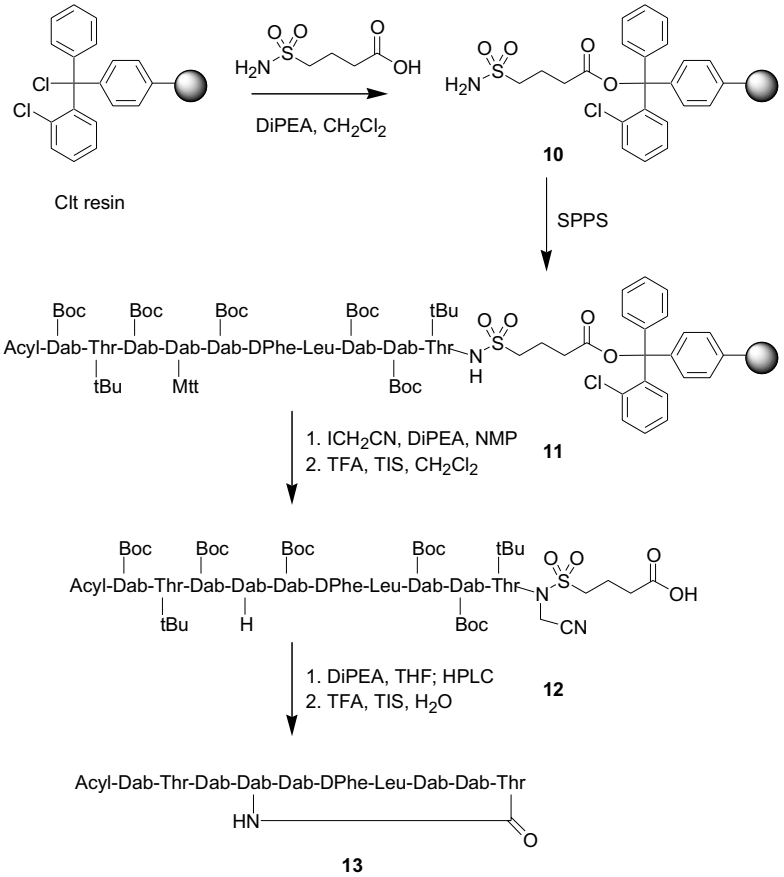
After stabilization of the major Tyr6/Ser7 cleavage site, attention should be directed towards modification of one of the other two minor cleavage sites. To stabilize the Ile17/Arg18 minor cleavage site, substitution with natural and unnatural amino acids can be performed similar to the Ty6/Ser7 dipeptide modifications. To this end, *N*-Me-Arg18 was incorporated (**3**, Table 1); this substitution however impairs antibacterial activity (MIC: 50μM).

DRC finds its target, the bacterial heat shock protein DnaK, inside the Gram-negative bacterial cell. Determination of the exact location of binding to this protein is a step forward in the design of new DRC-based antibiotics. To this end, the Tyr6 residue in DRC could be substituted with a light-activatable alkylating L-4'-[3-(trifluoromethyl)-3H-diazirin-3-yl]-phenylalanine ((Tmd)Phe, **Z**) residue[5] to yield compound **4** (Table 1). After binding to commercially available *Escherichia coli* DnaK, light-activation of the (Tmd)Phe residue in **4** would result in a stable, covalent crosslink between the two compounds; subsequent digestion of the complex with a protease (*e.g.* trypsin) gives small peptide fragments that can be identified by MS to locate the position of the crosslink.[6]

The polymyxin family of CAPs, produced by *Bacillus polymyxa*, are among the most potent anti-Gram-negative bacterial peptides known. As reported strategies towards the synthesis of polymyxin B1 (PMB1), based on cyclization in solution, resulted in inseparable mixtures of linear and cyclized products, a new synthetic approach towards PMB1 was devised. Through the use of the safety-catch approach described in **Chapter 2**, PMB1 was obtained conveniently as this strategy prevents any uncyclized products to be released from the resin after SPPS. A number of PMB1 analogues containing substitutions in the hydrophobic regions were synthesized *via* this route. These compounds, containing analogues with different acyl chains or amino acid substitutions of hydrophobic amino acids in the ring, showed distinct MIC values. Unfortunately, none of the analogues proved to be more potent than the parent compound.

Although cyclized polymyxins can be conveniently obtained using this procedure, the yield of the cyclization step might be optimized (yield for polymyxins B ~10%). An alternative cleavage/cyclization strategy is the approach depicted in Scheme 2, which might lead to higher yields of cyclized material. In this approach, the 4-butyrylsulfonamide safety-catch linker (SCL) is first coupled to 2-chlorotrityl (Clt) resin (→**10**), rather than AM resin. After automated SPPS of

the linear polymyxin (**11**), the SCL is activated by alkylation. Subsequently, the activated resin is treated with treated with 3% TFA to liberate the Mtt group of Dab4 and cleave the peptide, still C-terminally connected to the SCL, off the resin (→**12**). Cyclization (DiPEA/THF) in a highly diluted solution then gives the protected polymyxin. As this cyclization occurs fully in solution, cyclization yields can be expected to be higher than the on-resin cyclization/cleavage strategy in Chapter 2. At this stage, the protected polymyxin should be purified by chromatography if necessary to remove unwanted linear **12** which is still connected to the SCL; these two can be expected, in contrast to the linear and cyclized PMB1 after cyclization in solutions, to differ in chromatographic behaviour. Care should be taken when searching for the optimal purification conditions, as the activated butyryl sulfonamide in **12** might react with the solvents used to give products with near-identical retention times. In any case, cyclization yields are expected to increase. Final acid treatment of the purified cyclized peptide removes all protecting groups (→**13**).




SCHEME 2 | Alternative pathway for synthesis of polymyxins starting from Clt resin. The nature of the Dab1 acyl group determines the polymyxin B subtype. HPLC purification of **12** might be established by gel filtration due to the hydrophobic nature of the compound.

In **Chapter 3**, the identity of a by-product found in the last step of the synthesis of synthetic polymyxin B3 (PMB3) from Chapter 2 was elucidated. This by-product, which was also detected after acid treatment of polymyxin B1 isolated from a commercial sample in a different assay, displayed a different HPLC retention time than did the parent compound; however, its mass spectrum was identical. This isomeric molecule was hypothesized to be the polymyxin with the acyl chain migrated from the Nα to the Nγ position of the Dab1 residue. Following a synthetic route slightly adapted from the one described in Chapter 2, synthetic Nγ-PMB3 (**14**) was obtained (Figure 1). Nγ-PMB3 coeluted with the by-product formed from PMB3, and showed identical MS/MS spectra.

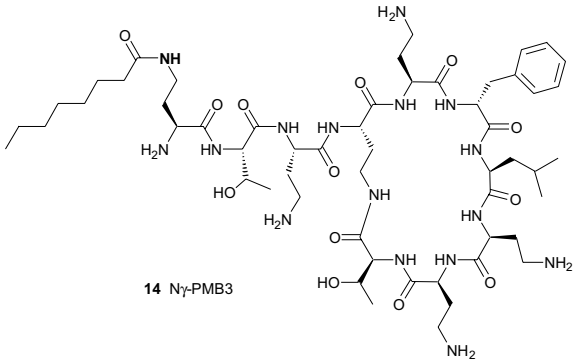
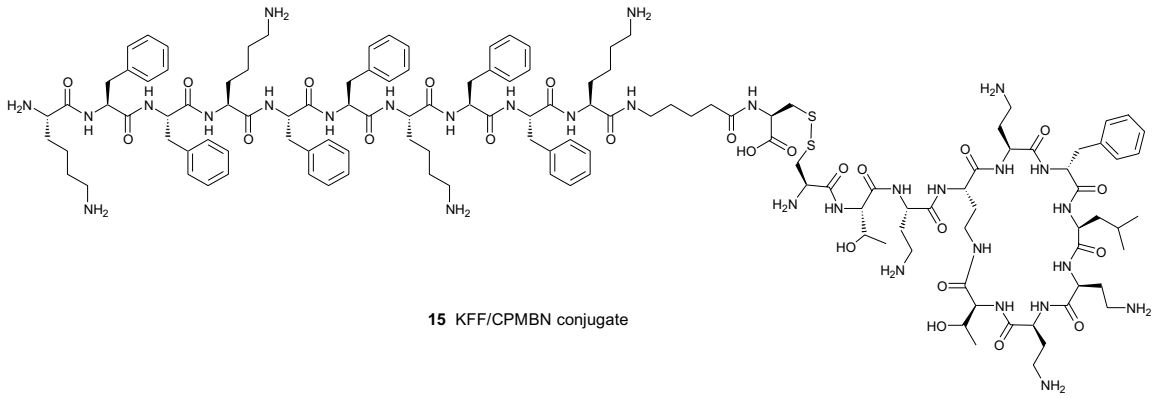



FIGURE 1 | Structure of Nγ-PMB3, the by-product formed from PMB3 by acyl migration.

**Chapter 4** describes an approach to create conjugates of linear CAPs with the polymyxin B nonapeptide (PMBN). The two parts are incorporated for specific purposes. Whereas the CAP part was expected to exert the antibacterial activity, the PMBN moiety was incorporated for a three-fold task. First, it imposes selectivity upon the conjugate towards Gram-negative bacteria due to its selectivity for anionic membranes. Second, it sensitizes the outer membrane for enhanced uptake of the conjugate, and third, after antibacterial action had taken place, it is able to bind and neutralize LPS that is released from the killed bacteria. Compared to the untruncated polymyxin B (PMB) itself, PMBN has lost antibacterial activity but also its toxicity. Four conjugates were prepared that were linked by disulfide bonds (*e.g.* **15**, Figure 2) to enable separation of the two moieties once inside the bacteria. To this end, the polymyxin synthesis of Chapter 2 was adjusted to incorporate a Cys residue to create CPMBN (**16**, Scheme 3).

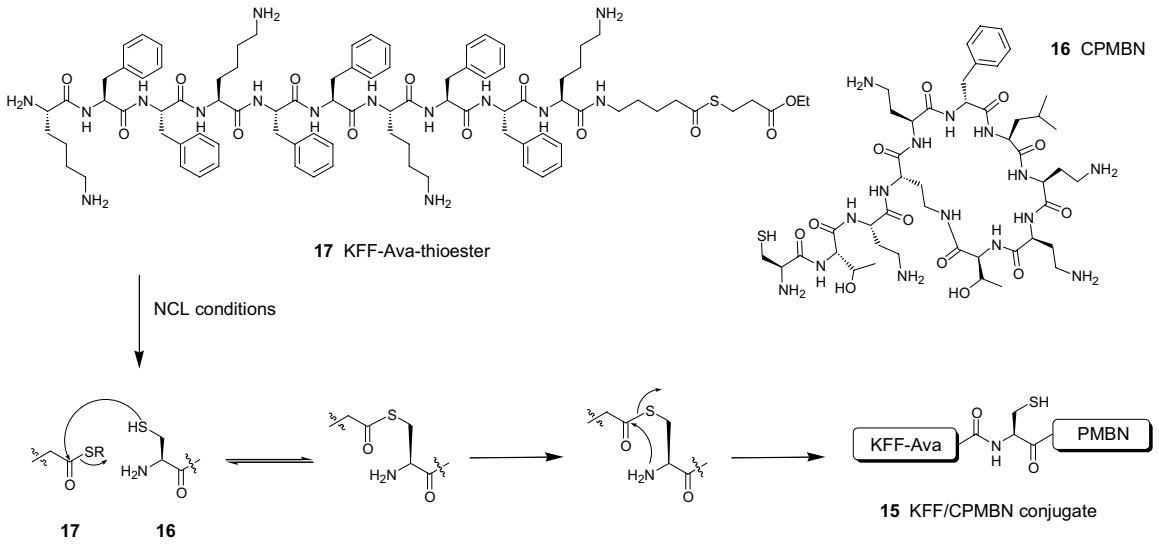
The conjugates with membrane-active CAPs (tritrpticin and KFF) were found to possess antibacterial and hemolytic activity; one of them (KFF/PMBN **15**, Figure 2) showed higher affinity for LPS than did the control polymyxin. Conjugates with peptides targeting internal structures (buforin II, drosocin) were devoid of antibacterial and hemolytic activity.



**15** KFF/CPMBN conjugate

**FIGURE 2** | CAP/PMBN conjugate that showed higher affinity for LPS than the control polymyxin.

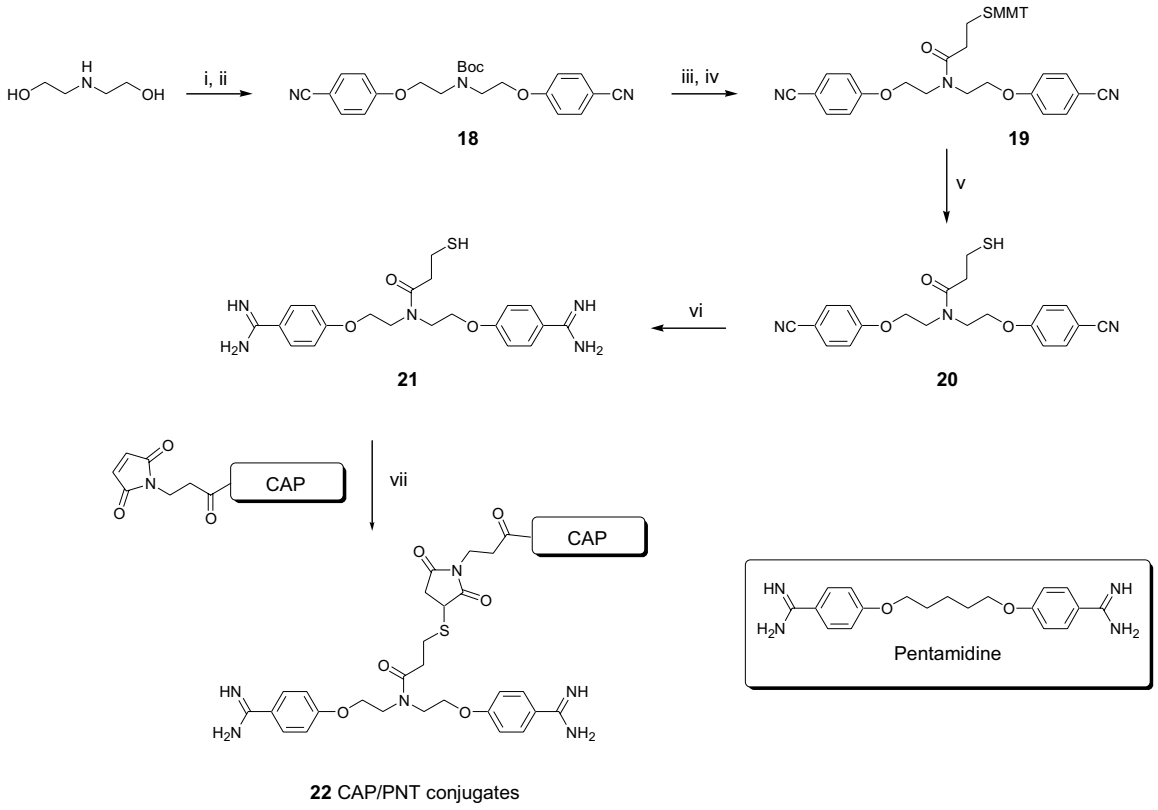
Future prospects of this project include the conjugation of PMBN to the N-terminus of the linear CAP rather than its C-terminus. Derivatization of the N-terminus of drosocin (Chapter 1) appears to be allowed with respect to antibiotic activity; as for BF2, the reported N-terminal biotinyl-BF2 conjugate was not subjected to antimicrobial tests,[7] whereas inhomogeneous (*i.a.* N-terminal) FITC-labeling of BF2 did not interfere with antimicrobial action.[8] Thus, the N-terminal conjugates CPMBN-SS-Cys-Ava-BF2 and CPMBN-SS-Cys-Ava-DRC were prepared *via* similar chemistry as the C-terminal conjugates, and will be evaluated for their biological properties. Furthermore, PMBN/CAP conjugates can be constructed through different methods. For instance, conjugation by native chemical ligation (NCL, Scheme 3) employs the earlier synthesized compound CPMBN. In a preliminary experiment, compound **15** was constructed through NCL from **16** and **17**.




SCHEME 3 | Preparation of a KFF/CPMBN conjugate through NCL.

Instead of conjugating PMBN to CAPs, the LPS-affinity moiety could well be coupled to other antibiotics such as β-lactam or macrolide derivatives to generate 'dual-action' antibiotics of which the antibiotic activity might be enhanced due to the sensitizing effect of the present PMBN. The approach of conjugating LPS-affinity moieties to CAPs can also be extended to other LPS-affinity moieties. Pentamidine,[9] a drug mainly used to treat African trypanosomiasis (sleeping sickness) was elaborated to give a compound that could be used in thiol/maleimide conjugation to CAPs (Scheme 4). Pentamidine derivatives (PNT) are structurally and synthetically less complicated than CPMBN; moreover, LPS affinity of pentamidine and congeners was found to be higher than that of polymyxin B.[10] The unoptimized synthetic route commenced with diethanolamine which was *N*-protected[11] with Boc and converted into compound **18** by double Mitsunobu reaction with 4-cyanophenol. After removal of the Boc protecting group, a linker containing an MMT-protected thiol moiety was introduced by coupling of *S*-(4-methoxytrityl)-β-mercaptopropionic acid with the aid of EDC (→**19**). Of the many conditions[12] tested for conversion of test-compound benzonitrile to their amidines (*e.g.* LiHMDS or KHMDS followed by aq. HCl, CuCl followed by $NH_3$/NaOH/$H_2O$, MeAl(Cl)$NH_2$ followed by $H_2O$, or Ac-Cys-OH followed by $NH_3/H^+$),[13] the classical Pinner conversion (HCl/EtOH followed by $NH_3$/EtOH) gave best results. Removal of the MMT group (→**20**) proved necessary prior to the Pinner conversion of the nitriles into the intermediate imidate salts, which are subsequently ammonolyzed with saturated $NH_3$/EtOH, yielding the diamidine **21** (MS: 429.4 $[M+H]^+$, 857.4 $[2M+H]^+$). Following HPLC purification, **21**

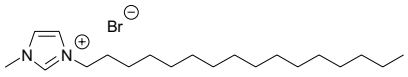
was conjugated in aqueous solution to CAPs which were equipped with a 3-maleimidopropionyl (Mpa) group.[14] After HPLC purification of these mixtures, conjugates **22** of PNT with TTC, DRC and BF2 were obtained. These compounds are to be assayed for bioactivity and LPS affinity.




**SCHEME 4** | Synthesis of CAP/PNT conjugates. Reagents & conditions: i. $Boc_2O$, THF, $0^0C$ (94%); ii. $PPh_3$, DIAD, 4-cyanophenol, THF, $0^0C$ (31%); iii. TFA/TES/$CH_2Cl_2$ (8/1/11 v/v/v) (95%); iv. S-(4-methoxytrityl)-ß-mercaptopropionic acid, EDC, sat. aq. $NaHCO_3$, DMF (99%); v. TFA/TIS/$CH_2Cl_2$ (8/5/87 v/v/v) (98%); vi. (1) dry HCl (g), EtOH/$CH_2Cl_2$ (4/1 v/v), $0^0C$ to RT (2) sat. $NH_3$/EtOH, microwave, $85^0C$ (3) HPLC purification (28% over these 3 steps); vii. (1) CAP with N-terminal Mpa group, $H_2O$/MeCN, phosphate buffer, pH 7 (2) HPLC purification.

Hydrophobicity and cationicity, governing the antibacterial activity of CAPs, are also present in smaller structures, among which are the quaternary ammonium compounds (QACs). In **Chapter 5**, a number of QACs were synthesized, based on either *N*-alkyl-*N'*-methyl imidazolium (MIM) or *N*-alkyl-*N*-methyl pyrrolidinium (MPD) cations. Gel formulations of a selection of these compounds were prepared using either water, ethylene glycol or glycerol as additive. All gels tested showed effective eradication (>99.9%) of the bacteria (Gram-negative *E. coli* or Gram-

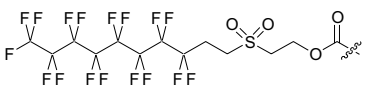
positive *S. aureus*) used. Compound **23** (Figure 3) in a gel containing 35% ethylene glycol as additive showed to be a candidate for further development of an antibacterial gel formulation for decontamination purposes. This particular gel of **23** showed increased stability characteristics, being largely stable against a one-minute, 20mL/min dropwise continuous flow of water when applied to a vertical surface, unlike the vast majority of the gels tested. The favorability of QACs as antibacterial compounds has also been proven by Klibanov *et al*. who prepared *i.a.* quaternized poly(vinylpyridine)[15] that showed antibacterial effects similar to the gels reported here.




**23** $C_{16}$MIM

FIGURE 3 | Structure of **23**, which, in a gel of 35% (wt) ethylene glycol, showed highest water stability.

As crude mixtures of synthetic peptides generated through SPPS generally contain impurities, HPLC purification is necessary to obtain these peptides in pure form. In some cases, HPLC purification can be a tedious and time-consuming procedure if truncated sequences display rather similar chromatographic behaviour as the desired product. In **Chapter 6**, two approaches to the use of fluorous techniques in the purification of synthetic peptides are presented. The first one is based on purification by tagging the desired full-length product during SPPS and subsequent chromatographic purification of the crude peptide mixture using fluorous HPLC or fluorous SPE. Non-tagged incomplete sequences elute before the (fluorous) desired product does. To this end, a novel, base-labile amine protecting group based on the Msc group (FMsc **24**, Figure 4) was synthesized.




**24** FMsc protecting group

FIGURE 4 | Structure of Msc-derived fluorous protecting group for use in fluorous chromatography of synthetic peptides (FMsc **24**).

The second method describes a 'two-step fluorous capping' approach, in which all non-desired impurities are equipped with a fluorous handle as the last step in SPPS sequence. The preliminary results of this approach still need optimization but omit a final detagging step (as in the tagging strategy) and allow more convenient fluorous purification as the desired product elutes first.

The fluorous purification techniques described in this Chapter were successfully applied to a number of synthetic peptides; optimization of the two-step fluorous capping approach might lead to a simple, cost and time-effective procedure able to compete with conventional HPLC purification.

## 7.2 | Notes & References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# General Materials & Methods

## Chemicals

Chemicals used in the reactions described in this thesis were obtained from the following commercial sources and used as received, unless otherwise stated (see last section of this paragraph). Chemicals were stored at temperatures indicated by the supplier and under inert atmospheres and dry conditions where necessary.

**Acros**: 30% AcOOH/AcOH, 1-adamantaneacetic acid, 29% ammonium hydroxide, $Boc_2O$, 1-bromodecane, 1-bromododecane, 1-bromoheptane, 1-bromohexadecane, 1-bromooctadecane, 1-bromooctane, 1-bromoundecane, butyric acid, chloranil, DAST, DIAD, 1,10-dibromodecane, 1,9-dibromononane, DIC, 2,2'-dithiobispyridine, DMAP, ethylene glycol, glycerol, heptanoic acid, hexanoic acid, HFIP, hydrazine hydrate, $ICH_2CN$, $LiAlH_4$, 2-mercaptoethanol, 1-methylimidazole, MMTCl, $NaBH_4$, nonanoic acid, octanoic acid, sodium thiophenolate, TFE, TMSCl, valeric acid. **Aldrich**: $Ag_2SO_4$, $Ag_3PO_4$, 1-bromononane, 1-bromotridecane, EDC.HCl, ethyl-3-mercaptopropionate, 20% phosgene/toluene. **Applied Biosystems**: HATU. **Bachem**: Fmoc-*N*-Me-Arg(Mtr)-OH, Fmoc-*N*-Me-Ser(tBu)-OH, 3-maleimidopropionic acid. **Baker**: $Na_2PO_4.12H_2O$ (z.A.). **BDH**: cetyltrimethylammonium bromide. **Biosolve**: MeCN (HPLC-S gradient grade), DiPEA, DMF, NMP, piperidine, TFA (peptide synthesis grade), $Ac_2O$, $CHCl_3$, $CH_2Cl_2$, 1,4-dioxane, DMSO, $Et_2O$, pyridine, tBuOH, THF (AR), MeOH (absolute HPLC), DCE (DNA synthesis grade). **Boom**: NaOH. **Bruker Daltonik:** α-cyanohydroxycinnamic acid (recrystalized). **Bufa**: $NaHCO_3$, $MgSO_4$. **Cambridge Isotopes**: acetone-$d_6$, $CDCl_3$, aq. DCl in $D_2O$, DMSO-$d_6$, $D_2O$, MeOD, TFE-$d_3$. **Fluka**: Boc-Cys(StBu)-OH, (*R*)-citronellyl bromide, DTT, Fmoc-βHSer(tBu)-OH, Fmoc-βHTyr(tBu)-OH), Fmoc-Cys(StBu)-OH, Fmoc-Mamb-OH, Fmoc-Pamb-OH, MS 3Å, MS 4Å, 1M $PMe_3$/toluene, PyBroP, TCEP·HCl, TentaGel PHB (0.24mmol/g), TentaGel S $NH_2$ (0.26mmol/g), TIS, 1% TNBS/DMF. **FTI**: fluorous silica gel, [1H,1H,2H,2H]-perfluorodecyl iodide, [1H,1H,2H,2H]-perfluoroundecanoic acid. **ICN**: basic alumina. **Iris Biotech**: HCTU. **Merck**: AcOH (glacial), $NH_4Oac$, silica gel. **Molecular Probes**: DPX. **NeoSystem**: Boc-Dab(Fmoc)-OH, Fmoc-Abu-OH, Fmoc-Ava-OH, Fmoc-Capro-OH, Fmoc-Cmpi-OH, Fmoc-Dab(Boc)-OH, Fmoc-DPhe-OH, Fmoc-Sar-OH, Fmoc-Tran-OH, HBTU. **Nova Biochem**: Boc-Cys(Tr)-OH, 3-carboxypropanesulfonamide, Fmoc-βAla-OH, Fmoc-Dab(ivDde)-OH, Fmoc-Dab(Mtt)-OH, Fmoc-DSer(tBu)-OH, Fmoc-DTyr(tBu)-OH, Rink amide resin (0.64mmol/g), Wang resin (0.96mmol/g). **Perseptive Biosystems**: HOAt. **Riedel-de Haën**: EtOAc, PE 40/60 (puriss.), toluene (purum). **Senn Chemicals**: AM resin (0.36mmol/g), BOP, Fmoc-Abu-OH, Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Asn(Tr)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Tr)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ava-OH, Fmoc-Cys(Tr)-OH, Fmoc-Gly-OH, Fmoc-His(Tr)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Sar-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH, FmocOSu, HOBt, PyBOP

$H_2O$ was purified using a Millpore MilliQ purification instrument. MeOH was stored on MS 3Å, $CH_2Cl_2$ and DMF on MS 4Å. THF was distilled prior to use from $LiAlH_4$ (5g/L). Toluene, PE and EtOAc used in sgcc and work-up procedures were distilled before use.

## Analytical Techniques

**TLC** – Reactions were followed by TLC on silica gel (Schleider & Schull F 1500 LS 254) or HPTLC sheets (Merck, silica gel 60, F 254), with detection by UV absorption (254nm) where applicable followed by charring at $150^0$C after spraying with ninhydrin (3g/L) in EtOH/AcOH (100/3 v/v), 20% $H_2SO_4$ in EtOH (25g/L), $NH_4Mo_7O_{24}{\cdot}4H_2O$ (25g/L) and $NH_4Ce(SO_4)_4{\cdot}2H_2O$ (10g/L) in 10% aq. $H_2SO_4$, or 2% $KMnO_4$ in 1% aq. $K_2CO_3$.

**NMR** – $^1$H NMR, $^{13}$C NMR, $^{19}$F NMR and $^{31}$P NMR were recorded on Bruker AC200 (200MHz), Bruker DPX-400 (400MHz) and/or Bruker DMX-600 (600MHz). H-H COSY, C-H COSY, TOCSY, ROESY and NOESY spectra were recorded on a Bruker DMX-600 (600MHz). Chemical shifts are in ppm relative to tetramethylsilane as internal standard at 0.0ppm ($^1$H NMR) or relative to shifts of deuterated solvents ($^{13}$C NMR) according to literature values ($CDCl_3$ middle resonance at 77.0ppm)

**HPLC** – RP-HPLC analyses and purifications were performed on a ÄKTA Explorer HPLC system. Alltech Alltima $C_{18}$ analytical (250x4.6mm) and semi-preparative (250x10.0mm) columns were used. Applied buffer systems: maximal gradient of 5→80% MeCN in 0.1% aq. TFA. Eluents were degassed before use with helium.

**(LC)MS** – Analyses were performed using a Jasco 900 LC system with simultaneous detection at 214 and 254nm connected to a Perkin Elmer SCIEX API 165 Q-TOF monoquad ESI mass spectrometer in positive ion mode. The applied LC buffer system runs with a maximal gradient of 10→90% MeCN in 0.1% aq. TFA (eluents were degassed with helium) over an Alltima Alltech analytical $C_{18}$ column (150x4.6mm) at 1mL/min. LCMS samples were dissolved in mixtures containing any of AcOH, $H_2O$, MeCN, tBuOH, TFA, TFE, MeOH and HFIP, and for MS any of MeCN, MeOH, DMSO, DMF or $H_2O$, where applicable.

**HRMS** – HRMS spectra were recorded on an API QSTAR Pulsar (Applied Biosystems) or TSQ Quantum (Thermo Finnigan) fitted with an acurate mass option, interpolating between PEG peaks.

**MALDI-MS** – MALDI-MS analyses were performed on a Bruker Biflex III reflectron TOF mass spectrometer, equipped with delayed extraction and with a UV ionization laser ($N_2$, 337nm). For the analyses in Chapter 1, 5µL amounts of peptide samples were mixed with 5µL matrix solution (sat. α-cyanohydroxycinnamic acid with 0.1% TFA in MeCN/$H_2O$ 1/1 (v/v)). Of this matrix mixture, 0.5µL was brought on the target plate.

**Determination of loading of resin with first amino acid** – The loading of resin with the first amino acid was determined by photospectrometric detection. To this end, a sample of resin 2-5mg was treated for 10min 20% piperidine/DMF (1mL), then the total volume was adjusted to 10mL by addition of MeOH, and UV-absorption was measured at 300nm in a 1cm cuvet (Perkin Elmer DMS 200 spectrophotometer). The loading was caluclated using the expression below derived from Lambert-Beer's Law where L is the loading in mmol/g, $A_{300}$ the absorbance at 300nm, vol the initial volume of the sample (10mL) and wt is the weight of the resin sample in mg.

$L=(A_{300} \times vol)/(7.8 \times wt)$

**Kaiser test**[1] – For identification of free primary amines, the Kaiser test was performed: to 2-3mg of resin, 20µL of the three solutions were added (5g ninhydrin in 100mL EtOH; 80g PhOH in 20mL EtOH; 2mL 0.001M KCN in 98mL pyridine) and the mixture heated to 120$^0$C for 5min. A blue/purple resin colour indicates a positive test.

**TNBS analysis**[2] – As alternative to the Kaiser test, the TNBS test for identification of primary amines was applied. To this end, 20µL of 1% DiPEA/DMF and 20µL of 1% TNBS/DMF were added to samples of 2-3mg of resin. A red/orange resin colour after standing for 5min at RT indicates a positive test.

**Chloranil analysis**[3] – For identification of secondary amines, the chloranil test was applied: to a 2-3mg sample of resin, 20µL of 1% chloranil/DMF and 20µL of 1% DiPEA/DMF were added and the sample allowed to react for 5min at RT. Coloured resin indicates a positive test.

## Solid-Phase Peptide Synthesis

Peptides were prepared by using automated Fmoc-based SPPS custom protocols using any of the following synthesizers; ABI433A (Applied Biosystems), Syro 2000 (MultiSyntech) or 336 System 3 Peptide Synthesizer (CS Bio). Standard cycles (of which the duration differed for the three synthesizers, but followed customized programs) for acylating preloaded Wang or Rink amide resins commenced with washing of the resin with NMP (and swelling with $CH_2Cl_2$ in the first cycle), followed by a Fmoc deprotection step applying 20% piperidine/NMP. In general. acylation was effected by using 3-6eq. of Fmoc-amino acid/DiPEA (6-12eq.), with PyBOP (Syro 2000, in NMP with LiCl) or HCTU (ABI433A and CS Bio) in NMP or NMP/DMF 1/1 (v/v). Less often used activators include BOP, HBTU and HATU. After coupling, the resin was washed with NMP and a capping step comprising either $Ac_2O$/NMP, $Ac_2O$/DiPEA/NMP or $Ac_2O$/DiPEA/HOBt/NMP was applied. SPPS were performed with $N_2$ as pressurizing gas and resulted in on-resin peptides with either the last Fmoc removed (ABI433A, Syro 2000) or maintained (CS Bio). Synthesis scales ranged from 10-100µmol.

1. Kaiser, E.; Colescott, R.L.; Bossinger, C.D.; Cook, P.I.; *Anal. Biochem.* **1970**, *34*, 595
2. Hancock, W.S.; Battersby, J.E. *Anal. Biochem.* **1976**, *71*, 261
3. Vojkovsky, J. *Peptide Res.* **1995**, *8*, 236

# Samenvatting

**New Cationic Amphiphilic Compounds**
**as Potential Antibacterial Agents**

Bacteriële resistentie tegen antibiotica is een wereldwijd, steeds groter wordend probleem. Door foutief en overgebruik van bestaande antibiotica is het aantal typen bacteriën dat niet meer te bestrijden valt met deze middelen sterk gestegen. In het bijzonder voor Gram-negatieve bacteriën is resistentie een niet te verwaarlozen bedreiging; naast het feit dat dit soort bacteriën moeilijk te bestrijden is door het vrij impermeabele extra celmembraan, zijn patiënten die te maken hebben met een verergerde Gram-negatieve bacteriële infectie (bloedvergiftiging of sepsis) niet atijd gebaat bij de huidige antibiotica. Immers, nadat de bacterie gedood is door het antibioticum, komen membraanfragmenten vrij in de bloedbaan, die elders (weer) immunologische reacties kunnen veroorzaken waardoor de toestand van de patiënt verslechtert. De kans op overleven bij een dergelijke septische shock (de verergerde toestand van sepsis) is slechts 50-70%.

Het onderwerp van het in dit proefschrift beschreven onderzoek is de ontwikkeling van nieuwe verbindingen met antibacteriële activiteit, specifiek gericht tegen Gram-negatieve bacteriën. Deze verbindingen zijn afgeleid van kationische antimicrobiële peptides (CAPs), een klasse van antibiotica die volgens een alternatief mechanisme werkt. Hoewel in het midden van de vorige eeuw ontdekt, kreeg deze klasse van antibiotica pas de laatste paar decennia meer aandacht vanwege de unieke eigenschappen. De meeste CAPs zijn selectief voor Gram-negatieve bacteriën vanwege hun kationisch karakter, waardoor elektrostatische interacties kunnen optreden met anionische bacteriële membraancomponenten. Bovendien zijn de meeste CAPs door hun natuurlijke samenstelling niet toxisch voor het menselijk lichaam; het menselijk lichaam maakt zelf ook gebruik van CAPs (bijvoorbeeld α- en β-defensines) in het aangeboren immuunsysteem.

De **Algemene Introductie** van dit proefschrift geeft een overzicht van de huidige status van antibiotica en lopend onderzoek naar nieuwe antibiotica gericht tegen Gram-negatieve bacteriën. De pathogenese van Gram-negatieve sepsis en septische shock worden verklaard aan de hand van de samenstelling van het buitenmembraan van Gram-negatieve bacteriën. In deze

Introductie wordt ook de CAP klasse van antibiotica besproken en een kort overzicht gegeven van onderzoek naar CAPs en door CAPs geïnspireerde verbindingen.

**Hoofdstuk 1** beschrijft de synthese van een aantal analoga van het CAP drosocine. Dit CAP, geïsoleerd uit de fruitvlieg, mag op grond van een aantal gewenste eigenschappen als *lead compound* voor de ontwikkeling van nieuwe antibiotica worden beschouwd; het is volledig selectief voor Gram-negatieve bacteriën waarin het een *heat shock protein* inactiveert. Het bindt niet aan de menselijke variant van dit eiwit, is niet hemolytisch en niet toxisch. Het wordt echter afgebroken in menselijk serum voordat het zijn werk heeft kunnen doen. Door substituties van aminozuren aan weerszijden van de amide binding die in serum verbroken wordt, alsmede van het N-terminale aminozuur, werd een drosocine derivaat verkregen dat ~30x stabieler is dan drosocine zelf, maar met eenzelfde antibiotische activiteit.

De CAP subfamilie van polymyxines is onderwerp van **Hoofdstuk 2**. In dit hoofdstuk wordt een nieuwe syntheseroute voor deze cyclische lipopeptides gepresenteerd, die gebaseerd is op het gelijktijdig vormen van het cyclische peptide en afsplitsing van de hars waarop het peptide opgebouwd is. Deze synthese procedure vergemakkelijkt de zuivering van het eindprodukt aanzienlijk omdat niet-gecycliseerde peptides achterblijven op de vaste drager. Via deze syntheseroute werden een aantal polymyxine B1 analoga bereid met substituties in een van beide hydrofobe regionen, omdat de toxiciteit van polymyxines aan deze regionen wordt toegedicht. De verkregen analoga vertoonden echter lagere tot geen antibacteriële activiteit en verminderde affiniteit voor lipopolysaccharide (LPS), de anionische membraancomponent van Gram-negatieve bacteriën.

**Hoofdstuk 3** laat zien hoe de structuur van een bijprodukt opgehelderd is dat gevormd werd tijdens de laatste zuiveringsstap van de polymyxines beschreven in Hoofdstuk 2. Dit bijprodukt bleek hetzelfde molekuulgewicht te hebben als de betreffende polymyxine, maar een andere LC retentietijd en MS/MS fragmentatiepatroon. De hypothese dat het hier gaat om een isomeer waarin de acyl groep van de Nα van Dab1 verhuisd is naar de Nγ positie van hetzelfde aminozuur werd bevestigd door deze isomeer te synthetiseren en te karakteriseren.

**Hoofdstuk 4** behandelt het ontwerp en de synthese van antibiotica conjugaten gebaseerd op CAPs. In deze conjugaten zijn twee aparte verbindingen geconjugeerd door middel van een disulfide brug. Het betreft conjugaten van de CAPs tritrpticine, buforine II, het synthetische KFF peptide en een drosocine derivaat uit Hoofdstuk 1, met het polymyxine B nonapeptide (PMBN). Dit nonapeptide is een kleinere variant van polymyxine B dat niet alleen de antibiotische werking verloren heeft, maar tegelijkertijd ook de toxiciteit. Het nonapeptide heeft echter nog wel affiniteit voor LPS, en kan in deze conjugaten optreden als *sensitizer*; plaatselijke verstoring van

het buitenmembraan (*sensitizing*) zorgt ervoor dat het gehele conjugaat gemakkelijker het buitenmembraan kan passeren. Slechts de conjugaten met membraanactieve CAPs zijn antibacterieel actief; het KFF/PMBN conjugaat bleek in de tests zelfs een hogere affiniteit voor LPS te hebben dan de controle polymyxines.

Quaternaire ammonium verbindingen (QACs) behoren tot de eenvoudigste structuren die, net als CAPs, hydrofoob en kationisch zijn. In **Hoofdstuk 5** wordt de synthese en de antibacteriële activiteit besproken van een aantal op imidazool- en pyrrolidine-gebaseerde QACs. De meeste van deze verbindingen konden in gelfase gebracht worden door toevoeging van water, ethyleen glycol of glycerol. Deze gels bleken alle zeer effectief in antibiotische tests tegen *Escherichia coli*. Van de gel bestaande uit *N*-hexadecyl-*N*'-methylimidazolium bromide met 35% (gew.) ethyleen glycol bleek, in tegenstelling tot bijna alle andere gels, slechts een klein gedeelte weg te spoelen in een waterstroom. Deze positieve eigenschappen kunnen leiden tot de ontwikkeling van antibacteriële gels voor (preventieve) ontsmettingstoepassingen.

Het onderwerp van **Hoofdstuk 6** is peptide-gerelateerd en betreft de toepassing van polygefluoreerde groepen in de chemische synthese van peptiden. Door een polygefluoreerde (*fluorous*) groep te koppelen selectief aan een synthetisch peptide op de vaste drager, bleek dit gewenste peptide eenvoudig te zuiveren uit een mengsel met ongewenste incomplete verbindingen door middel van *fluorous* vloeistofchromatografie of *fluorous* vastestofextractie. Deze groep, FMsc genaamd, werd speciaal ontwikkeld voor dit doel. Voorts beschrijft dit Hoofdstuk ook een *fluorous* strategie waarin juist alle ongewenste verbindingen van een polygefluoreerde groep werden voorzien, zodat het gewenste peptide meteen verkregen kan worden zonder dat eerst de FMsc groep verwijderd hoeft te worden.

Tot slot wordt in **Hoofdstuk 7** een samenvatting gegeven van de voorgaande Hoofdstukken en worden een aantal toekomstperspectieven behandeld, waaronder de synthese van pentamidine/CAP conjugaten, als uitbreiding op Hoofdstuk 4. Pentamidine, een geneesmiddel voor de behandeling van de Afrikaanse slaapziekte, blijkt een grotere affiniteit voor LPS te hebben dan het polymyxine B nonapeptide, en kan de laatstgenoemde vervangen in de constructie van verbindingen die zowel een antibacterieel als een LPS-affiniteitsgedeelte hebben.

# List of Publications

**Chemical and enzymatic synthesis of DNA fragments containing 5-(β-D-glucopyranosyloxy-methyl)-2'-deoxycytidine – a modified nucleoside in T4 phage DNA**
de Kort, M.; de Visser, P.C.; Kurzeck, J.; Meeuwenoord, N.J.; van der Marel, G.A.; Rüger, W.; van Boom, J.H. *Eur. J. Org. Chem.* **2001**, 2075

**Solid-phase synthesis of new saphenamycin analogues with antimicrobial activity**
Laursen, J.B.; de Visser, P.C.; Nielsen, H.K.; Nielsen, J. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 171

**Solid-phase synthesis of polymyxin B1 and analogues *via* a safety-catch approach**
de Visser, P.C.; Kriek, N.M.A.J.; van Hooft, P.A.V.; Van Schepdael, A.; Filippov, D.V.; van der Marel, G.A.; Overkleeft, H.S.; van Boom, J.H.; Noort ,D. *J. Pept. Res.* **2003**, *61*, 298

**A novel, base-labile fluorous amine protecting group: synthesis and use as a tag in the purification of synthetic peptides**
de Visser, P.C.; van Helden, M.; Filippov, D.V.; van der Marel, G.A.; Drijfhout, J.W.; van Boom, J.H.; Noort, D.; Overkleeft, H.S. *Tetrahedron Lett.* **2003**, *44*, 9013

**Liquid chromatography-mass spectrometry study towards the pH and temperature-induced *N*-acyl migration in polymyxin B**
de Visser, P.C.; Govaerts, C.; van Hooft, P.A.V.; Overkleeft, H.S.; Van Schepdael, A.; Hoogmartens, J. *J. Chromat. A.* **2004**, *1058*, 183

**Improved Antibiotics**
van Hooft, P.A.V.; Noort, D.; de Visser, P.C.; Overkleeft, H.S.; van Boom, J.H. *European Patent* 04078089.2 (2004)

**Biological evaluation of Tyr6 and Ser7 modified drosocin analogues**
de Visser, P.C.; van Hooft, P.A.V.; de Vries, A.-M.; de Jong, A.; van der Marel, G.A.; Overkleeft, H.S.; Noort, D. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2902

# Curriculum Vitae

Peter Christian de Visser was born in Delft, The Netherlands, on the 19th of March, 1977. After the completion of his secondary education at the Christelijk Lyceum Delft in July 1995, he started his academic studies in Chemistry at Leiden University, Leiden, The Netherlands, in September of the same year. From February 1998 to May 1999, undergraduate research was conducted at the BIOSYN group (Leiden University) of the late prof. dr. Jacques H. van Boom, under supervision of dr. Martin de Kort. The M.Sc. thesis describes the synthesis of modified nucleoside building blocks for studies towards the phenomenon of DNA glycosylation. This work was presented on a poster during the PAC Symposium in Amsterdam (1999). From August to December 1999, he performed research at the Danske Tekniske Universitet (DTU), Kgs. Lyngby, Denmark under the guidance of prof. dr. John Nielsen and dr. Knud J. Jensen. The project entailed the solid-phase synthesis of derivatives of the phenazine antibiotic saphenamycin. During the last years of his Chemistry studies, as Leiden University correspondent, a number of articles were published in the Dutch former weekly national magazine Chemisch Weekblad. In June 2000, the doctorandus (drs., equivalent to M.Sc.) title was obtained. From July of that year, he was employed at Nalco Europe BV, Leiden, where he conducted research in the wastewater treatment laboratory, until the start of his Ph.D. studies in November 2000. These were conducted at the BIOSYN group under the supervision of prof. dr. Jacques H. van Boom, prof. dr. Herman S. Overkleeft and prof. dr. Gijsbert A. van der Marel, in close cooperation with dr. Daan Noort and dr. Peter A.V. van Hooft of TNO Defense, Security & Safety, Rijswijk ZH, The Netherlands. In September 2004, he participated in the 3rd International/28th European Peptide Symposium (Prague, Czech Republic). Parts of the research described in this dissertation have been presented at annual meetings of the NWO (Netherlands Organisation for Scientific Research) Organic Chemisty section in Lunteren, The Netherlands (2002, 2003, 2004, poster presentations) and by oral presentations at the 9th Dutch annual meeting for peptide chemists (Utrecht, April 2002), the BIOMAC colloquium (Leiden, 2002) and the Katholieke Universiteit Leuven (Leuven, Belgium, August 2002). At the end of the Ph.D. research period (November 2004), he became temporarily affiliated with ProSensa BV, Leiden, as research scientist, in which period he finished writing this thesis. At present, he is employed by ProSensa BV on a project in cooperation with prof. dr. Herman de Boer (Leiden University) and prof. dr. Jaap T. van Dissel (Leiden University Medical Center) regarding design, synthesis and evaluation of new antibiotics for which he was granted a subsidy by the Dutch government (June 2005).

# Nawoord

Het onderzoek beschreven in dit proefschrift is het resultaat van samenwerking met velerlei mensen –in en buiten de laboratoria- die ik hier dan ook graag wil bedanken. Allereerst mijn ouders; zij stonden aan de basis van dit proefschrift door mij de mogelijkheid te bieden een academische opleiding te volgen.

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De afgelopen jaren heeft onder andere het Delta RA-team gezorgd voor sportieve activiteiten buiten de chemie. Voor buiten-chemische activiteiten moet ik ook zeker de (ex-) bandleden van Shavin'KojaK niet vergeten! De energieke repetities en optredens waren meestal precies wat ik nodig had. Rest mij nog Linda, goede vrienden en familie te bedanken voor hun steun en interesse ('hoe staat het met je fruitvliegen?') en natuurlijk Ilse: in tegenstelling tot de vaak voorkomende onvoorspelbaarheid van de chemie kon en kan ik blindelings uit gaan van jouw steun, interesse, begrip en liefde – eenvoudig onmisbaar.