New cationic amphiphilic compounds as potential antibacterial agents

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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.

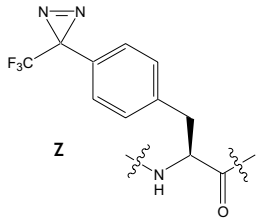
Optimization of CAPs regarding secondary structure, amphiphilicity, toxicity, selectivity and stability for the discovery of lead structures is an on-going process driven by the demand for new antibiotics. The research area of CAPs, further expanded by research into CAP-inspired amphiphilic structures, 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.

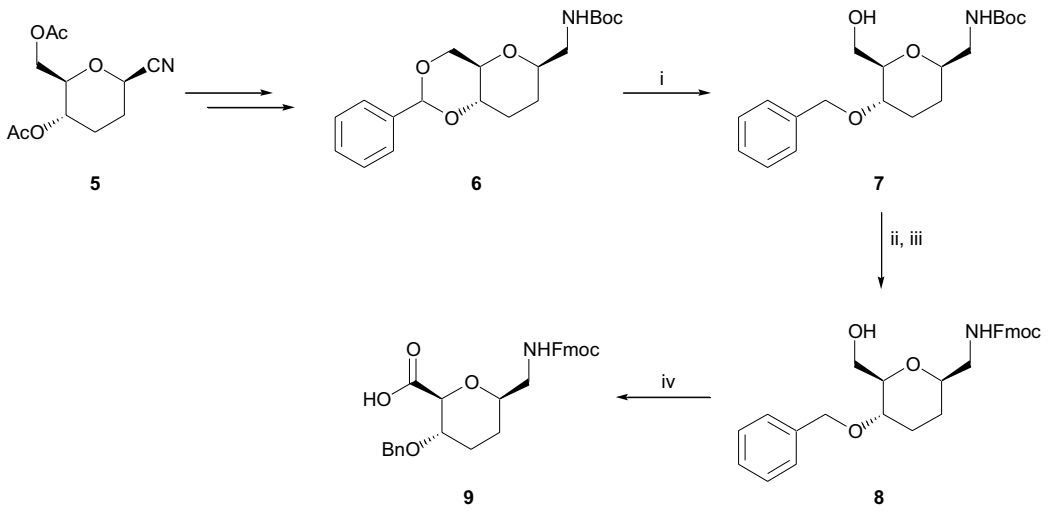
	Sequence[C]	% left[A]	MIC[B] (μM)
DRC	GKPRP Y SPRP TSHPR PI RV	3	6.3
1	**βAla**-KPRP Y **T**PRP TSHPR PI RV	87	3.1
2	GKPRP **SAA**-SPRP TSHPR PI RV	<1	12.5
3	GKPRP Y SPRP TSHPR PI^{Me}**R**V	n/d	50
4	GKPRP **Z** SPRP TSHPR PI RV	n/d	n/d



Z

[A] After 8h in 25% human serum; [B] against *E. coli* ATCC 11775; [C] SAA= sugar amino acid, ^{Me}R=N^{α}-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.

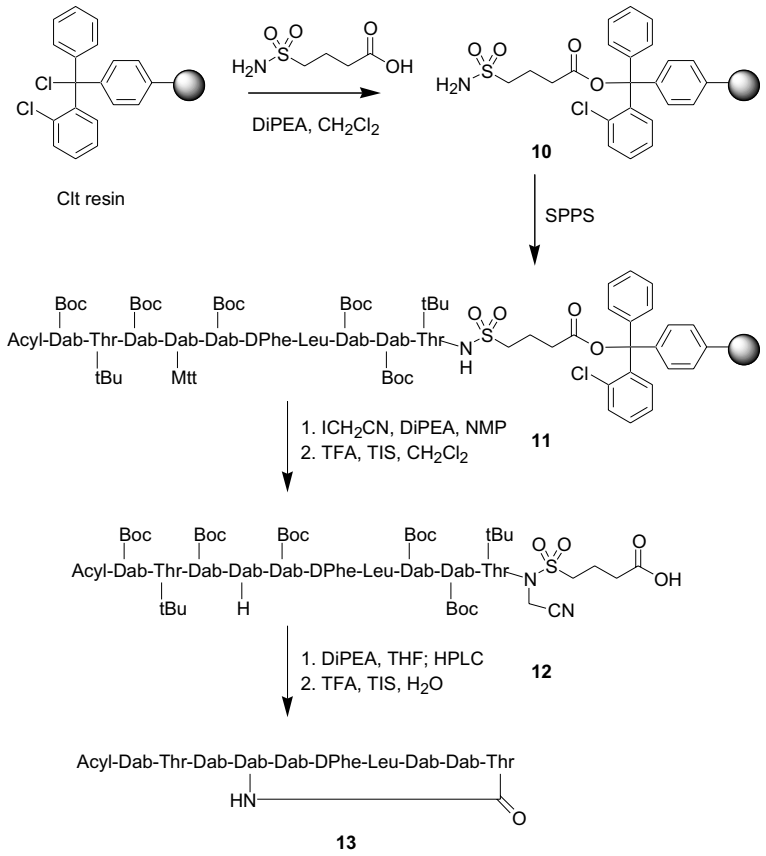
After stabilization of the major Tyr6/Ser7 cleavage site, attention should be directed towards modification of one of the other two minor cleavage sites. To stabilize the Ile17/Arg18 minor cleavage site, substitution with natural and unnatural amino acids can be performed similar to the Ty6/Ser7 dipeptide 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.

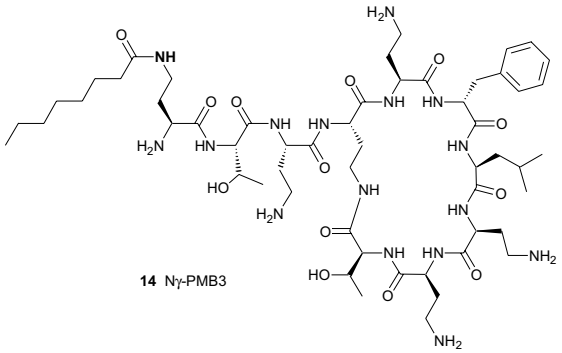


FIGURE 1 | Structure of Nγ-PMB3, the by-product formed from PMB3 by acyl migration.

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.

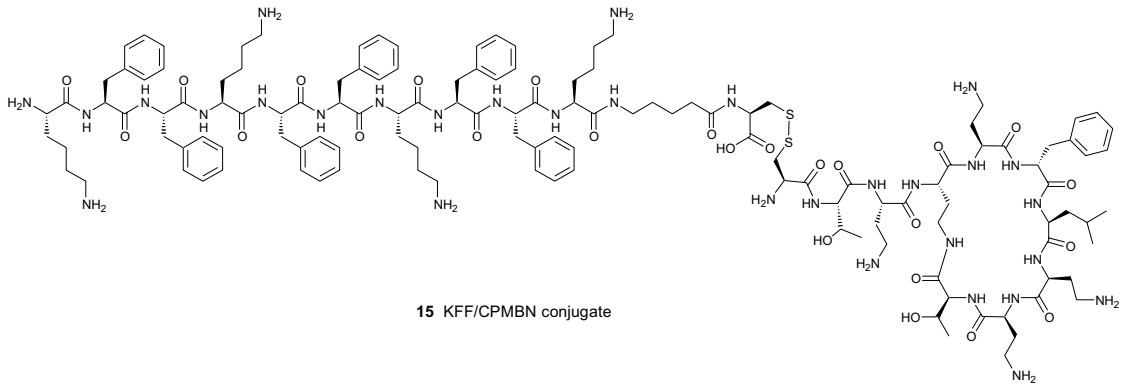
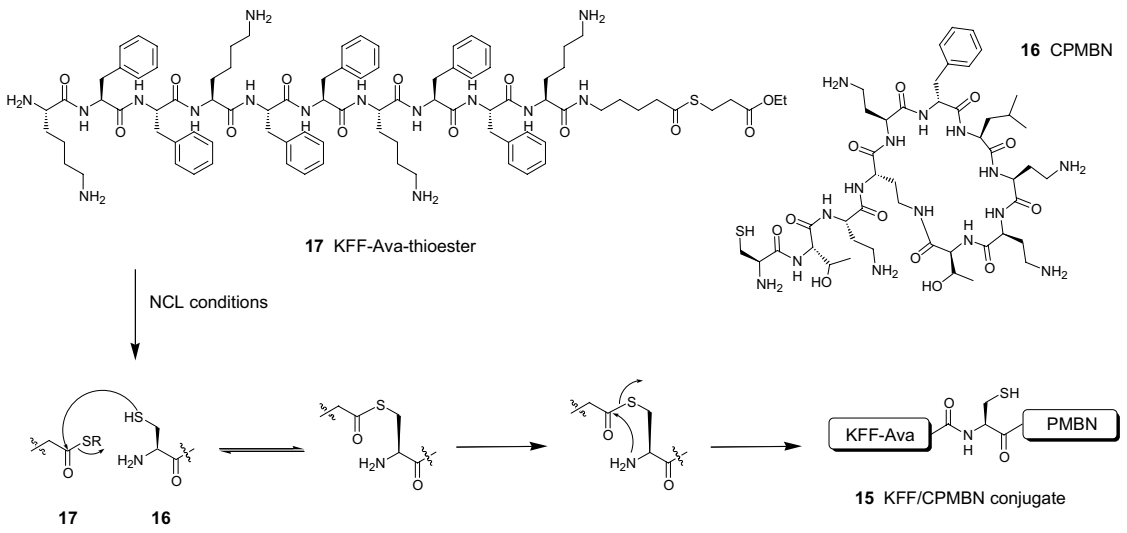


FIGURE 2 | CAP/PMBN conjugate that showed higher affinity for LPS than the control polymyxin.

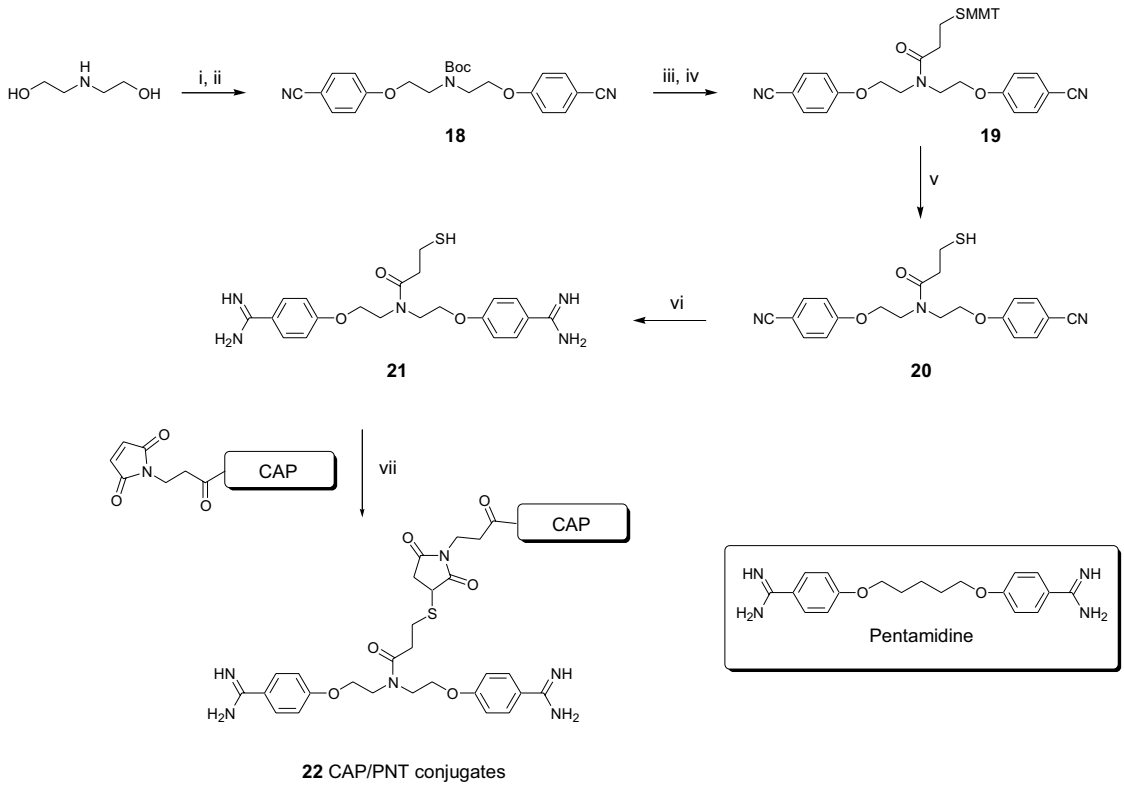
Future prospects of this project include the conjugation of PMBN to the N-terminus of the linear CAP rather than its C-terminus. Derivatization of the N-terminus of drosocin (Chapter 1) appears to be allowed with respect to antibiotic activity; as for BF2, the reported N-terminal biotinyl-BF2 conjugate 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**

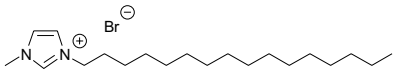
was conjugated in aqueous solution to CAPs which were equipped with a 3-maleimidopropionyl (Mpa) group.[14] After HPLC purification of these mixtures, conjugates **22** of PNT with TTC, DRC and BF2 were obtained. These compounds are to be assayed for bioactivity and LPS affinity.



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)-β-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-

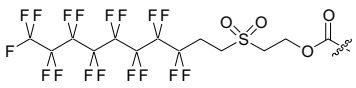
positive *S. aureus*) used. Compound **23** (Figure 3) in a gel containing 35% ethylene glycol as additive showed to be a candidate for further development of an antibacterial gel formulation for decontamination purposes. This particular gel of **23** showed increased stability characteristics, being 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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