New cationic amphiphilic compounds as potential antibacterial agents

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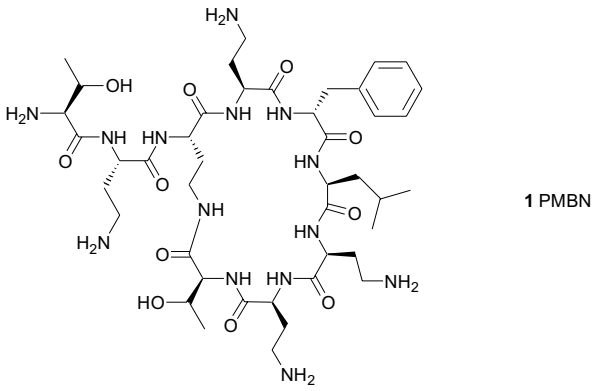
4.1 | Introduction

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]



1 PMBN

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]

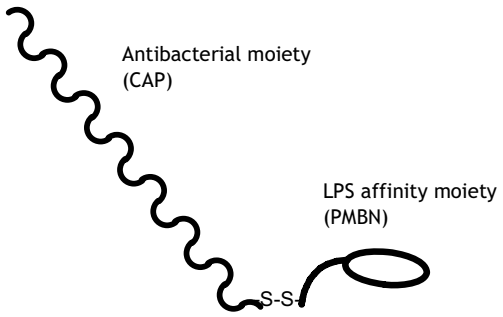
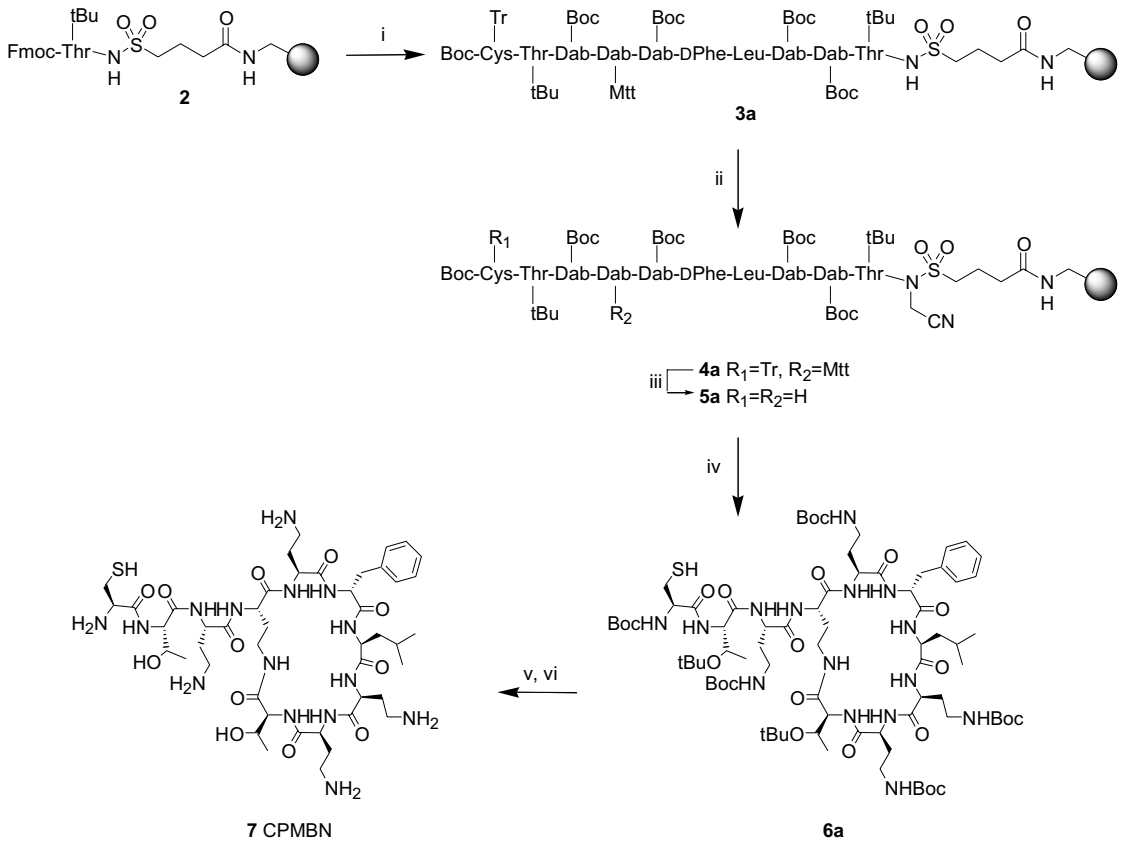


FIGURE 2 | Schematic representation of disulfide conjugates of CAPs with PMBN. The disulfide bridge is located at the C-terminus of the CAP.

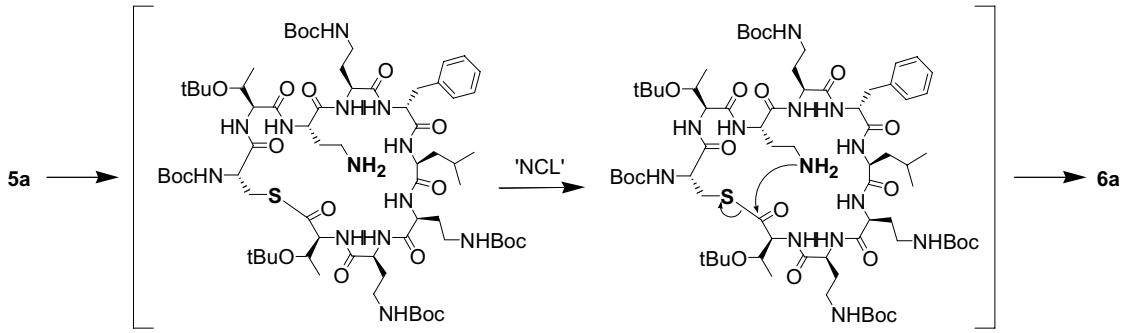
4.2.1 | Synthesis of a thiol-functionalized PMBN derivative

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.



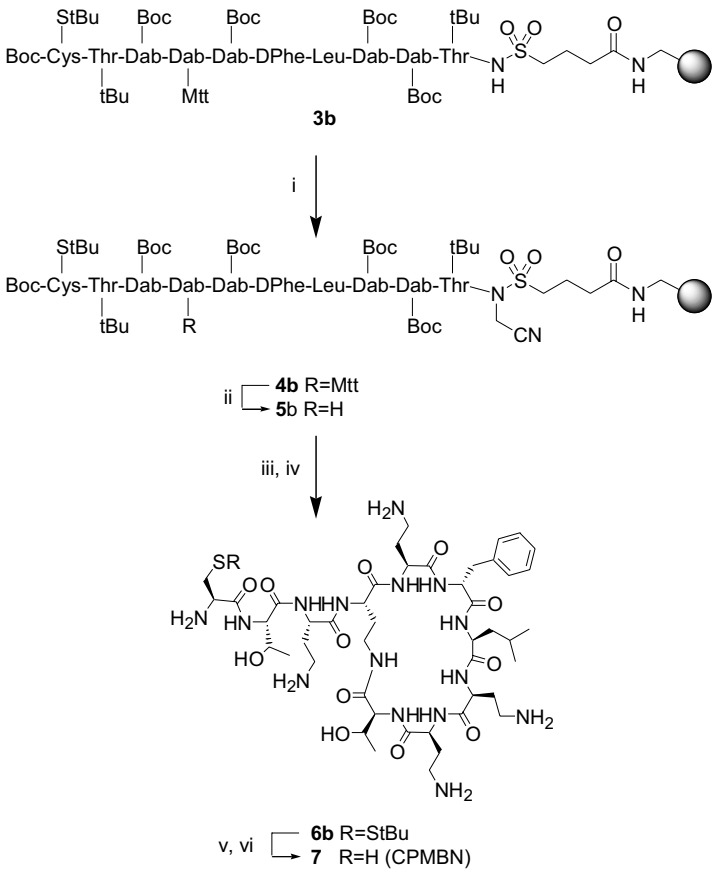
SCHEME 1 | Synthesis of CPMBN. Reagents & conditions: i. Fmoc-based SPPS (see Chapter 2); ii. ICH_2CN, DiPEA, NMP; iii. TFA/TIS/CH_2Cl_2 5/3/92 (v/v/v) iv. DiPEA, THF; v. TFA/TIS/H_2O 95/2.5/2.5 (v/v/v); vi. HPLC purification.



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).

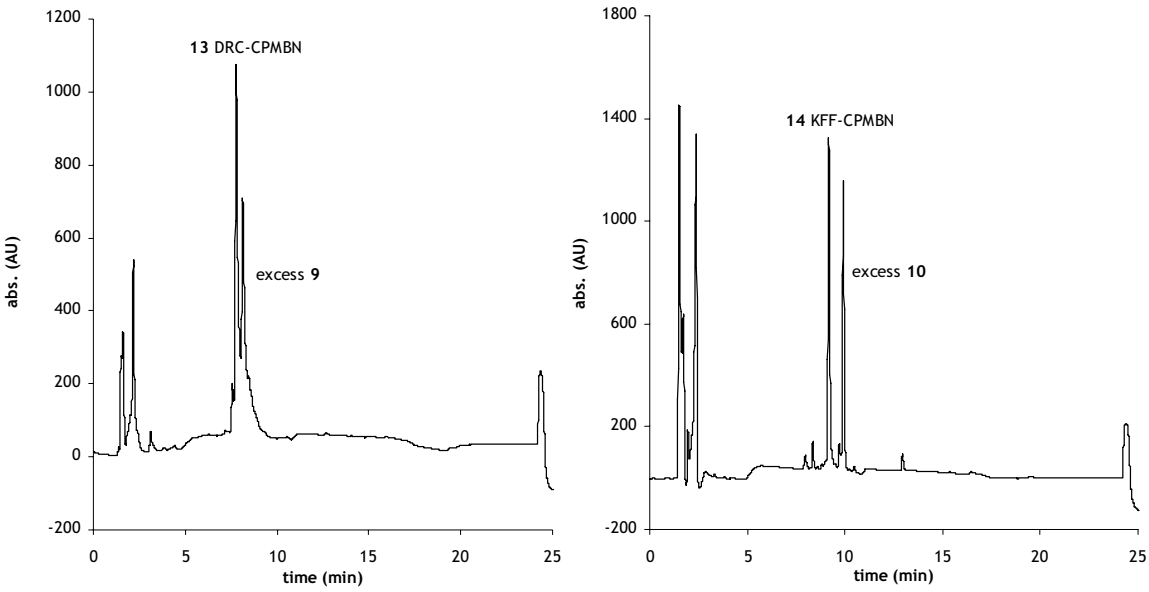


FIGURE 3 | Representative LC chromatograms (214nm, 10→90% MeCN in 0.1% aq. TFA in 17min) of crude conjugation mixtures showing the formation of **13** (left panel, Rt 7.77min) and **14** (right panel, Rt 9.17min) after 16h reaction; in all cases, all CPMBN **7** was found consumed. Peaks of Rt <5min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

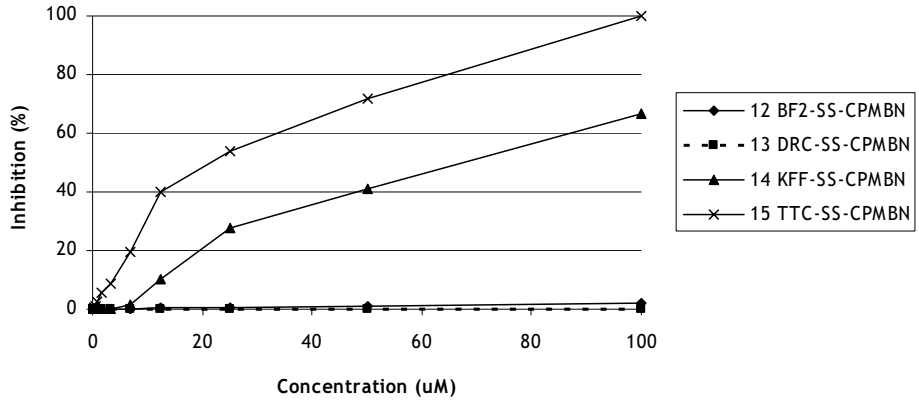


FIGURE 4 | Antimicrobial effects of C-terminal PMBN/CAP conjugates against *E. coli* ATCC 11775.

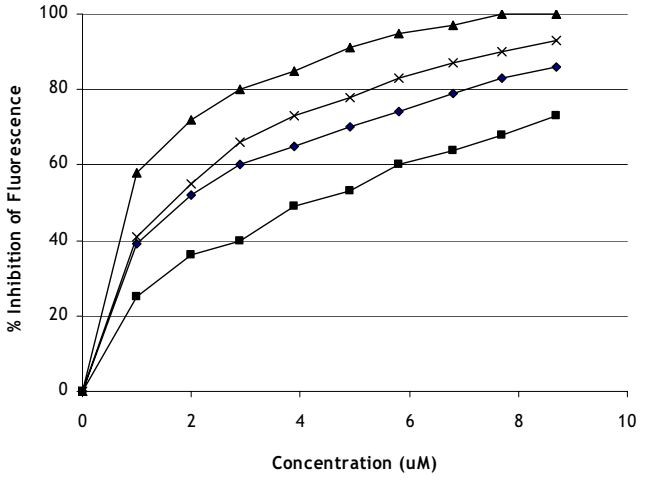
4.3.2 | Hemolytic activity

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 5x10^6 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 37^0C for 4h, centrifuged (5min at 10^0C) 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]

4.6 | Notes & References

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CHAPTER 5 | Antimicrobial Gels Based on QACs