New cationic amphiphilic compounds as potential antibacterial agents

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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	DAA_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]

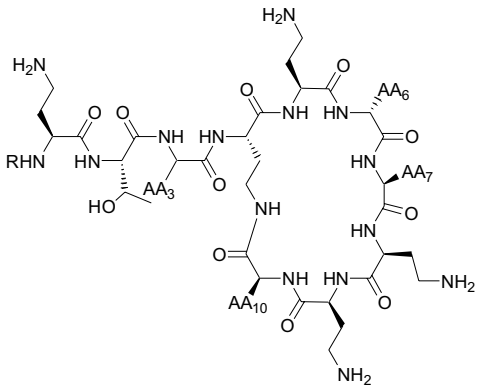


FIGURE 1 | General structures of polymyxins and subdivision of polymyxin B. [A] Additionally, polymyxin F has been reported (ref. 9); [B] Chiralities at C3 and C6 were not established.

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]

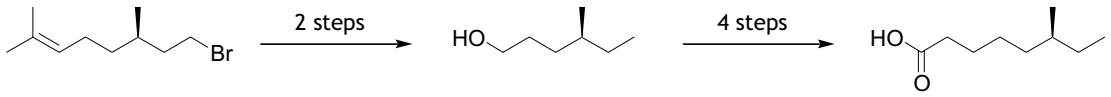
It is also well established that PMB1 decreases the effects of sepsis by binding and neutralizing LPS that is released in the course of Gram-negative infections.[14] These properties make PMB1 a good candidate as antibiotic for therapeutic purposes. However, its nephrotoxicity to humans has thus far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

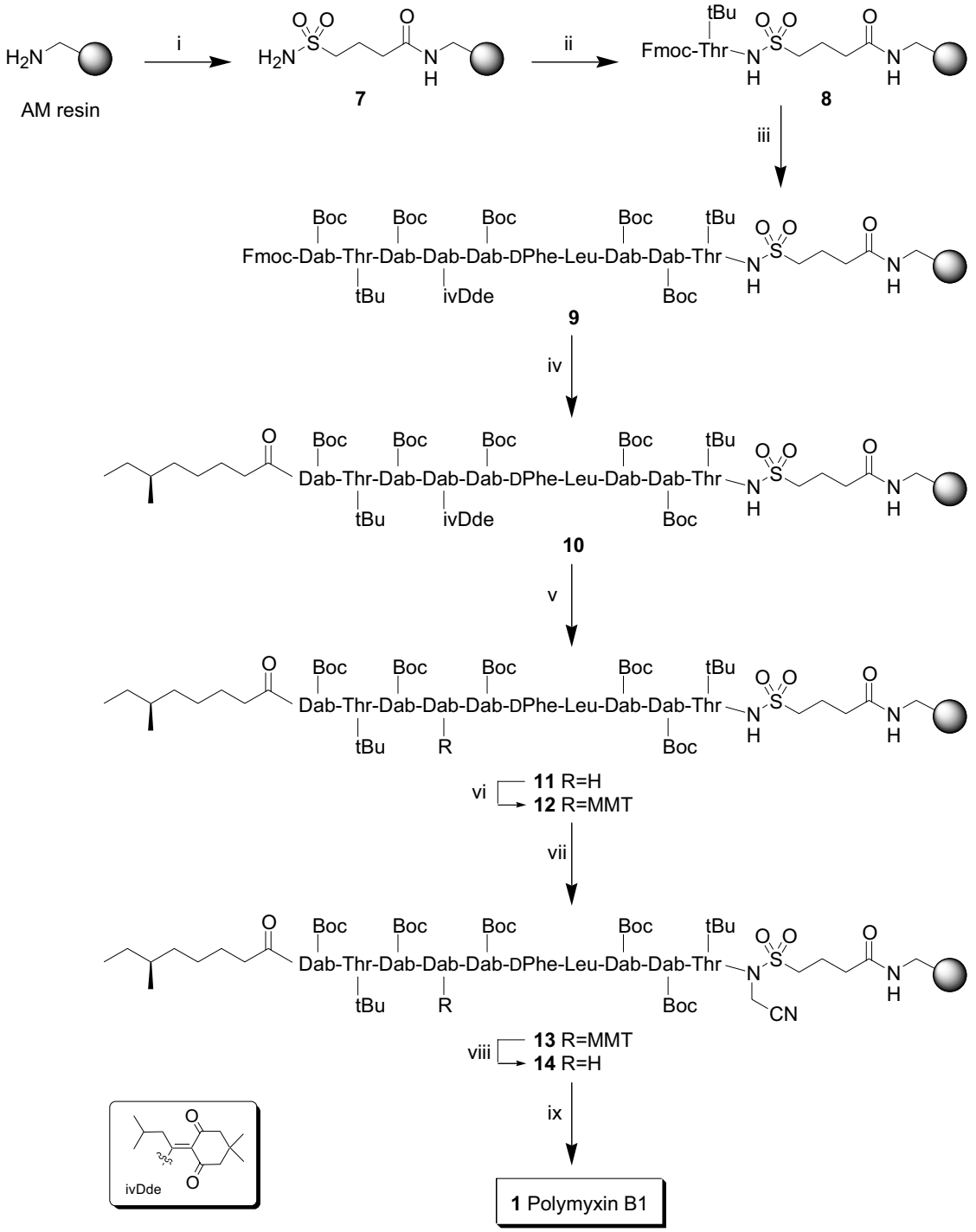
and 18eq., respectively. Sequential elongation of **8**, resulting in immobilized peptide **9**, was effected by BOP/HOBt/DiPEA-mediated condensation with suitably protected Fmoc amino acids.[24] With respect to residue Dab4, efforts were dedicated towards the incorporation of this Dab residue bearing 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**).

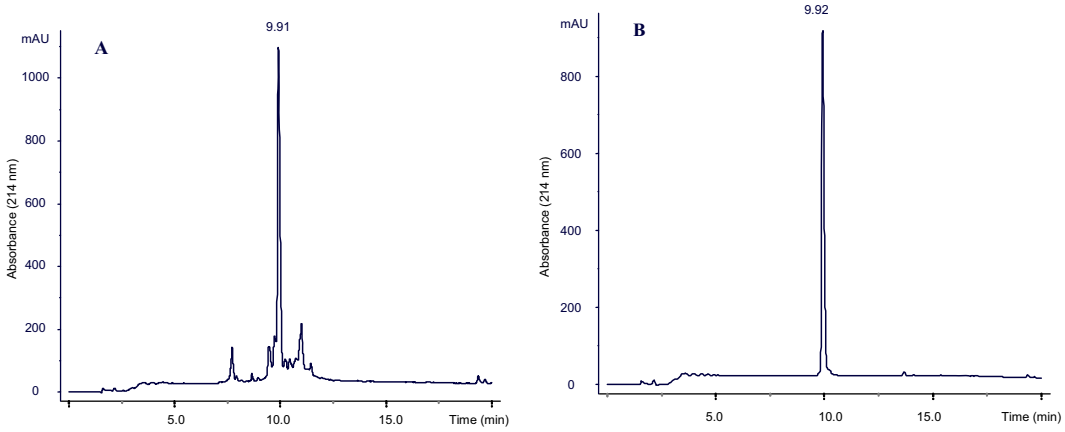


FIGURE 2 | HPLC traces of PMB1 (**1**). (**A**) crude after cyclization and deprotection; (**B**) after HPLC purification.

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**).

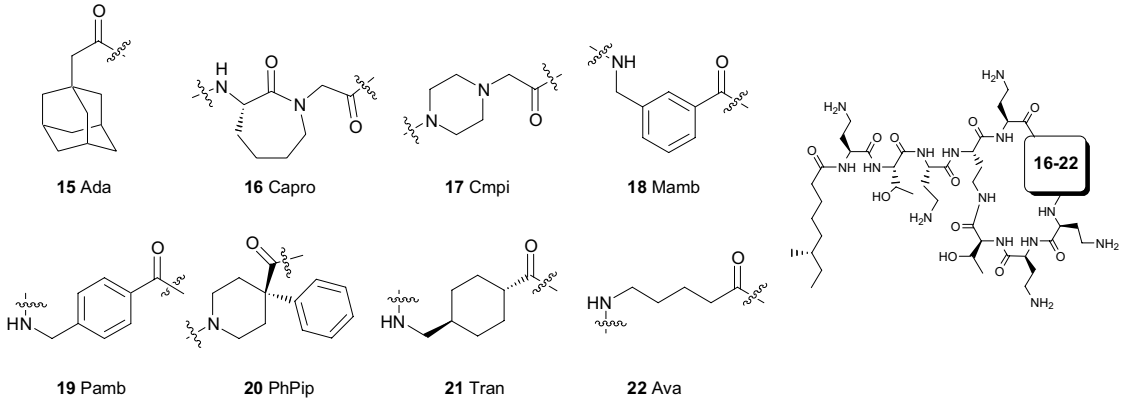


FIGURE 3 | Acyl chain (**15**) and dipeptide mimics (**16**-**22**) used for substitution of the original DPhe6-Leu7 dipeptide in PMB1 (rightmost structure).

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.

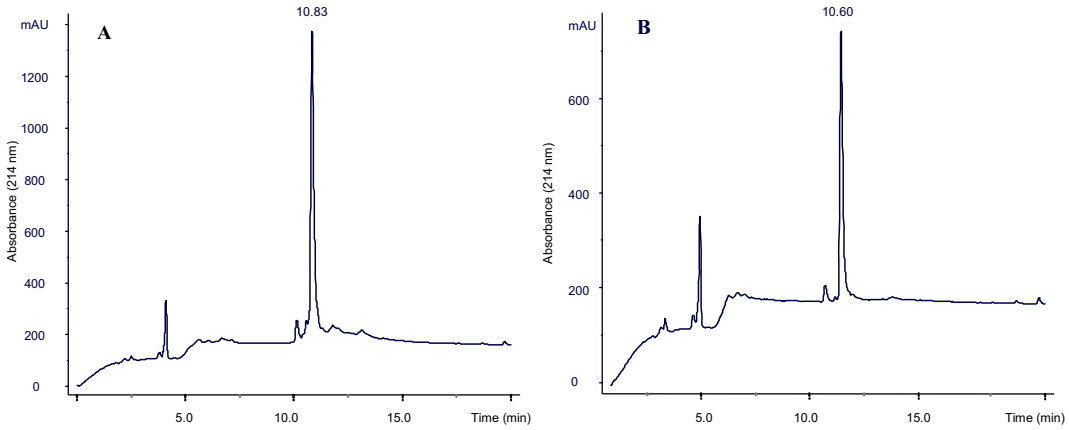


FIGURE 4 | Representative HPLC traces of crude PMB1 analogues; **A** Mamb-PMB1 **29**; **B** Ava-PMB1 **33**.

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.

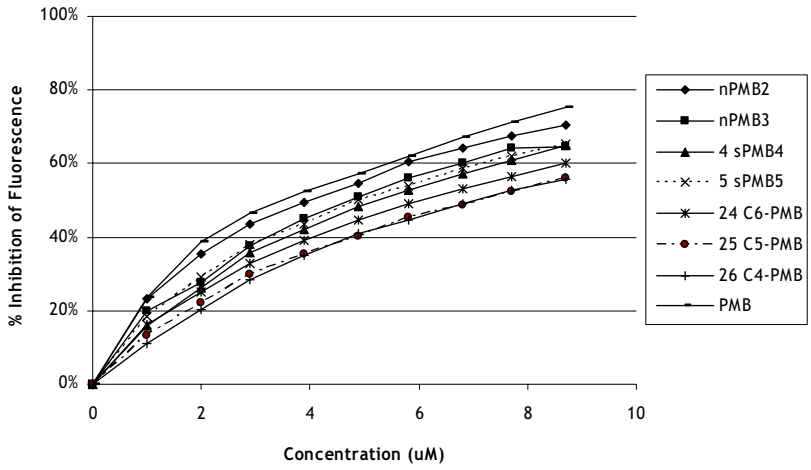


FIGURE 5 | LPS affinity assay of PMB analogues differing in acyl chain length. n - natural (*i.e.* isolated from a commercial (natural) sample), s - synthetic. Concentration in μM.

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).

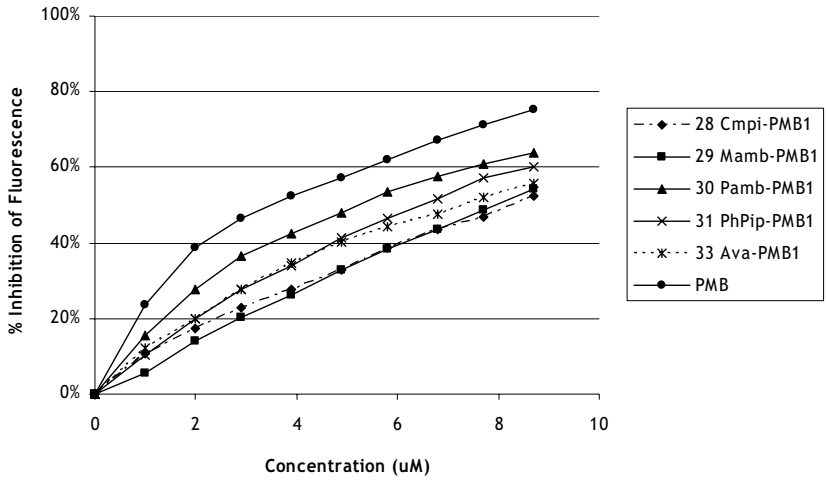


FIGURE 6 | LPS affinity assay of ring-substituted PMB1 analogues and commercial PMB sulfate for comparison. Concentration of polymyxin analogue in μM.

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

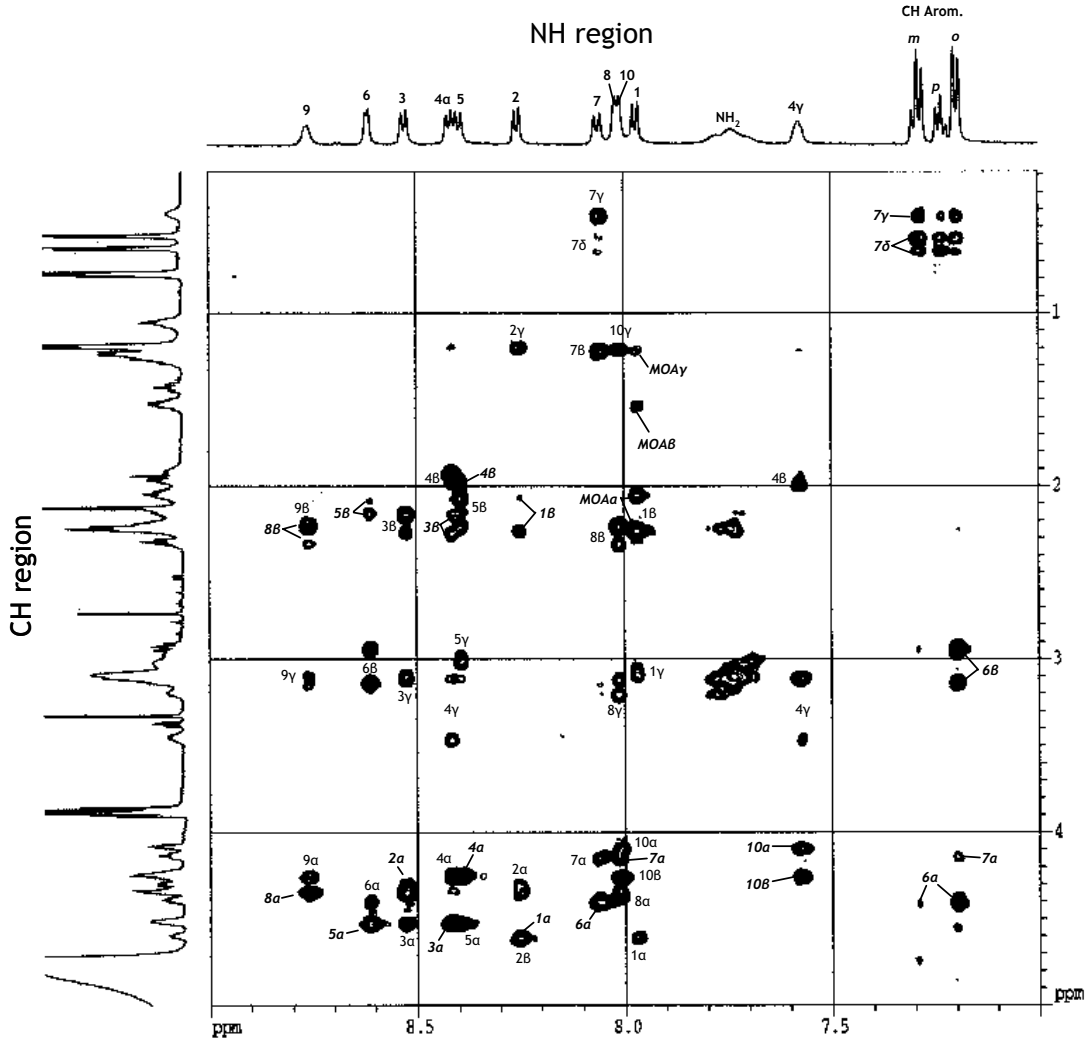


FIGURE 7 | Part of the 600MHz PMB1 (**1**) ROESY spectrum in 43% (v) TFE-d_3/H_2O, 298K, pH 4, mixing time 200msec. *Intraresidual* NOE contacts of amide NH peaks with other atoms are given in regular face; *interresidual* NOEs are denoted in bold/italic face and are connected with lines to the corresponding 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 5β protons (Dab5Hβa and Hβb) and the amide proton of residue 2 (Thr2NH) has intraresidual NOE contacts with Thr2Hγ, Hα and Hβ atoms (denoted in the spectrum as 2γ, 2α and 2β, respectively).

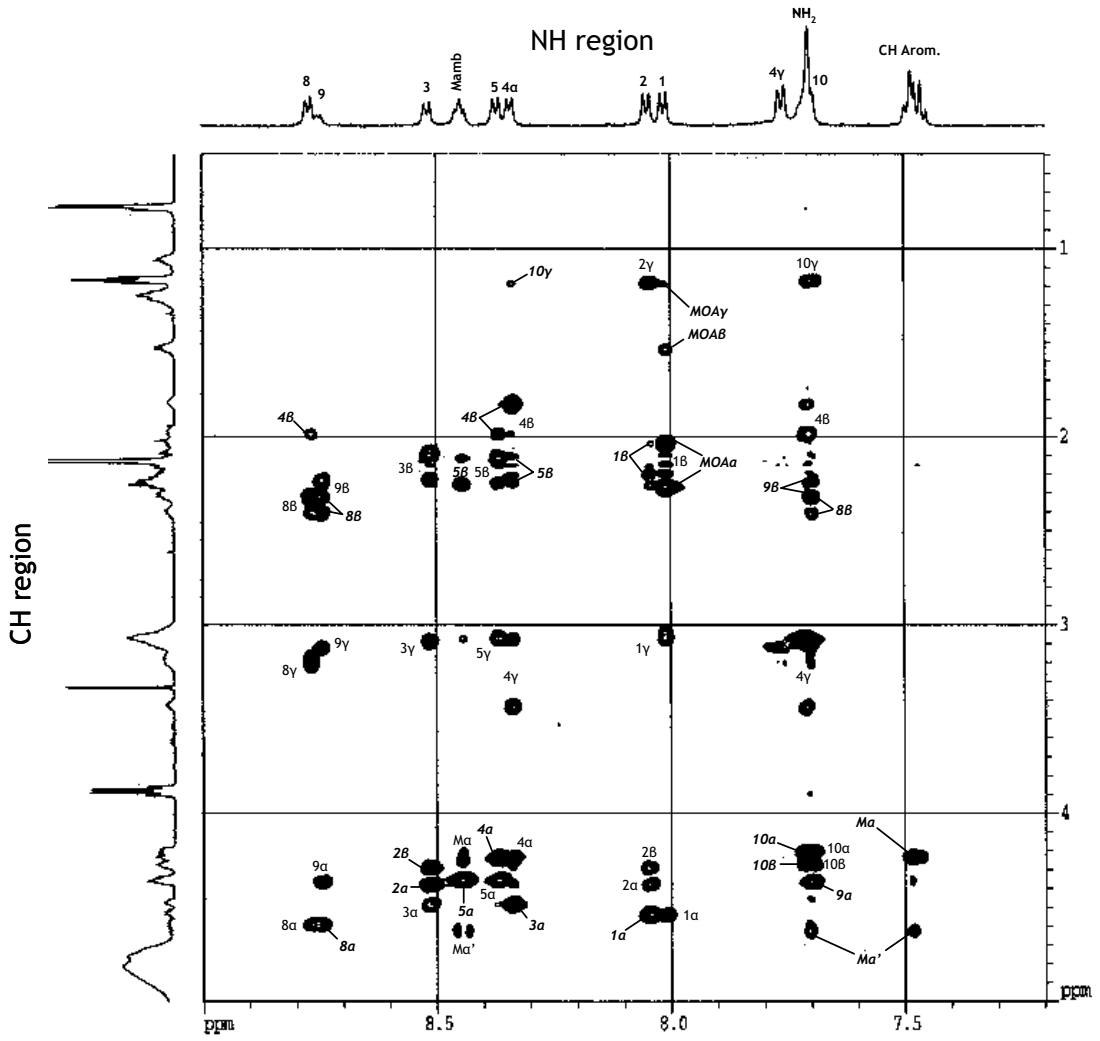


FIGURE 8 | Part of the 600MHz Mamb-PMB1 (**29**) ROESY spectrum in 43% (v) TFE-d_3/H_2O, 298K, pH 4, mixing time 200msec. *Intraresidual* NOE contacts of amide NH peaks with other atoms are given in regular face; *interresidual* NOEs are denoted in bold/italic face and are connected with lines to the 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

1. (a) Benedict, R.G.; Langlykke, A.F. *J. Bacteriol.* **1947**, *54*, 24; (b) Ainsworth, G.C.; Brown, A.M.; Brownlee, G. *Nature* **1947**, *160*, 263; (c) Suzuki, T.; Hayashi, K.; Fujikawa, K.; Tsukamoto, K. *J. Biochem.* **1963**, *54*, 555; (d) Storm, D.R.; Rosenthal, K.S.; Swanson, P.E. *Annu. Rev. Biochem.* **1977**, *46*, 723
2. Orwa, J.A.; Govaerts, C.; Busson, R.; Roets, E.; Van Schepdael, A.; Hoogmartens, J. *J. Chromat. A* **2001**, *912*, 369
3. Martin, N.I.; Hu, H.; Moake, M.M.; Churey, J.J.; Whittal, R.; Worobo, R.W.; Vederas, J.C. *J. Biol. Chem.* **2003**, *278*, 13124
4. Kimura, Y.; Murai, E.; Fujisawa, M.; Tatsuki, T.; Nobue, F. *J. Antibiot. (Tokyo)* **1969**, *22*, 449
5. (a) Studer, R.O.; Lergier, W. *Helv. Chim. Acta* **1970**, *53*, 929; (b) Hayashi, K.; Suketa, Y.; Tsukamoto, K.; Suzuki, T. *Experientia* **1966**, *22*, 354
6. Studer, R.O.; Lergier, W.; Lanz, P.; Böhni, E.; Vogler, K. *Helv. Chim. Acta* **1965**, *148*, 1371
7. Shoji, J.; Kato, T.; Hinoo, H. *J. Antibiot. (Tokyo)* **1977**, *30*, 1035
8. Shoji, J.; Kato, T.; Hinoo, H. *J. Antibiot. (Tokyo)* **1977**, *30*, 1042
9. Parker, W.L.; Rathnum, M.L.; Dean, L.D.; Nimeck, M.W.; Brown, W.E.; Meyers, E. *J. Antibiot. (Tokyo)* **1977**, *30*, 767
10. Li, C.; Budge, L.P.; Driscoll, C.D.; Willardson, B.M.; Allman, G.W.; Savage, P.B. *J. Am..Chem. Soc.* **1999**, *121*, 931
11. (a) Pristovšek, P.; Kidrič, J. *J. Med. Chem.* **1999**, *42*, 4604; (b) Thomas, C.J.; Surolia, A. *FEBS Lett.* **1999**, *445*, 420; (c) Thomas, C.J.; Surolia, N.; Surolia, A. *J. Biol. Chem.* **1999**, *274*, 29624; (d) David, S.A.; Balasubramanian, S.; Mathan, V.I.; Balaram, P. *Biochim. Biophys. Acta* **1992**, *1165*, 147; (e) Bruch, M.D.; Cajal, Y.; Koh, J.T.; Jain, M.K. *J. Am. Chem. Soc.* **1999**, *121*, 11993
12. (a) Brandenburg, K.; Moriyon, I.; Arraiza, M.D.; Lewark-Yvetot, G.; Koch, M.H.J.; Seydel, U. *Thermochim. Acta* **2002**, *382*, 189; (b) Clausell, A.; Pujol, M.; Alsina, M.A.; Cajal, Y. *J. Phys. IV* **2001**, *11*, 227, (c) Clausell, A.; Pujol, M.; Alsina, M.A.; Cajal, Y. *Talanta* **2003**, *60*, 225
13. (a) Vaara, M. *Microbiol. Rev.* **1992**, *56*, 395 ; (b) Daugelavičius, R.; Bakiene, E.; Bamford, D.H. *Antimicrob. Agents Chemother.* **2000**, *44*, 2969; (c) Clausell, A.; Busquets, M.A.; Pujol, M.; Alsina, A.; Cajal, Y. *Biopolymers* **2004**, *E-publication ahead of print*
14. (a) Srimal, S.; Surolia, N.; Balasubramanian, S.; Surolia, A. *Biochem. J.* **1996**, *315*, 679; (b) Mayumi, T.; Takezawa, J.; Takahashi, H.; Kuwayama, N.; Fukuoka, T.; Shimizu, K.; Yamada, K.; Kondo, S.; Aono, K. *Shock* **1999**, *11*, 82; (c) Bannatyne, R.M. *Int. J. Antimicrob. Agents* **2000**, *14*, 165; (d) Cohen, J.; McConnell, J.S. *Eur. J. Clin. Microbiol.* **1986**, *5*, 13
15. (a) Evans, M.E.; Feola, D.J.; Rapp, R.P. *Ann. Pharmacother.* **1999**, *33*, 960; (b) Ouderkirk, J.P.; Nord, J.A.; Turett, G.S.; Kislak, J.W. *Antimicr. Agents Chemother.* **2003**, *47*, 2659
16. (a) Vogler, K.; Studer, R.O.; Lanz, P.; Lergier, W.; Böhni, E. *Experientia* **1964**, *20*, 365; (b) Vogler, K.; Studer, R.O.; Lergier, W.; Böhni, E. *Helv. Chim. Acta.* **1965**, *48*, 1161
17. Sharma, S.K.; Wu, A.D.; Chandramouli, N.; Fotsch, C.; Kardash, G.; Bair, K.W. *J. Peptide Res.* **1999**, *53*, 501
18. Shiori, T.; Nimomiya, K.; Yamada, S. *J. Am. Chem. Soc.* **1972**, *94*, 6203
19. (a) Yang, L.; Morriello, G. *Tetrahedron Lett.* **1999**, *40*, 8197; (b) Tsubery, H.; Ofek, I.; Cohen, S.; Fridkin, M. *J. Med. Chem.* **2000**, *43*, 3085
20. Bourne, G.T.; Golding, S.W.; McGeary, R.P.; Meutermans, W.D.; Jones, A.; Marshall, G.R.; Alewood, P.F.; Smythe, M.L. *J. Org. Chem.* **2001**, *66*, 7706
21. Kenner, G.W.; McDermott, J.R.; Sheppard, R.C. *J. Chem. Soc. Chem. Commun.* **1971**, 636
22. (a) Backes, B.J.; Virgilio, A.A.; Ellman, J.A. *J. Am. Chem. Soc.* **1996**, *118*, 3055; (b) Backes, B.J.; Ellman, J.A. *J. Org. Chem.* **1999**, *64*, 2322
23. Ingenito, R.; Drežnjak, D.; Guffler, S.; Wenschuh, H. *Org. Lett.* **2002**, *4*, 1187
24. Commonly used stronger activators as PyBOP, HCTU (both either with or without HOBt), and HATU (with or without HOAt) could not be applied as these reagents were found to be able to acylate the SCL-loaded resin (**7**) during synthesis, creating incomplete sequences. Capping of unloaded SCL by acetylation (Ac_2O, DMAP) to prevent unwanted acylation and subsequent usage of HCTU was not beneficial with respect to yield and/or purity of the final compounds; this might

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.
26. (a) Saplay, K.M.; Sahni, R.; Damodaran, N.P.; Dev, S. *Tetrahedron* **1980**, *36*, 1455; (b) Odinokov, V.N.; Ishimuratov, G.Y.; Kharisov, R.Y.; Serebryakov, É.P.; Tolstikov, G.A. *J. Org. Chem. USSR (Engl. Trans.)* **1992**, *28*, 1286; (c) Kriek, N.M.A.J. Thesis Leiden University, The Netherlands 2002
27. Govaerts, C.; Orwa, J.; Van Schepdael, A.; Roets, E.; Hoogmartens, J. *J. Peptide Sci.* **2002**, *8*, 45
28. (a) Tsubery, H.; Ofek, I.; Cohen, S.; Fridkin, M. *Peptides* **2001**, *22*, 1675; (b) Hancock, R.E.W.; Chapple, D.S. *Antimicrob. Agents Chemother.* **1999**, *43*, 1317
29. (a) Liskamp, R.M.J. *Recl. Trav. Chim. Pays-Bas* **1994**, *113*, 1; (b) Cristau, M.; Devin, C.; Oiry, C.; Chaloin, O.; Amblard, M.; Bernad, N.; Heitz, A.; Fehrentz, J.-A. *J. Med. Chem.* **2000**, *43*, 2356
30. Jackson, S.; DeGrado, W.F.; Dwivedi, A.; Parthasarathy, A.; Higley, A.; Krywko, J.; Rockwell, A.; Markwalder, J.; Wells, G.; Wexler, R.; Mousa, S.; Harlow, R. *J. Am. Chem. Soc.* **1994**, *116*, 3220
31. Snyder, K.R.; Murray, T.F.; DeLander, G.E.; Aldrich, J.V. *J. Med. Chem.* **1993**, *36*, 1100
32. Bu, X.; Wu, X.; Xie, G.; Guo, Z. *Org. Lett.* **2002**, *4*, 2893
33. Moore, R.A.; Bates, N.C.; Hancock, R.E. *Antimicrob. Agents Chemother.* **1986**, *29*, 496
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.
35. During preparation of this Chapter, the following publication appeared on LPS affinities of natural polymyxins B. Sakura, N.; Itoh, T.; Uchida, Y.; Ohki, K.; Okimura, K.; Chiba, K.; Sato, Y.; Sawanishi, H. *Bull. Chem. Soc. Jpn.* **2004**, *77*, 1915
36 . It should be noted that the DPX displacement assay employs LPS *in solution*; this is physically different from the *membrane-bound* LPS encountered in the antibacterial assay, for which the affinity might be different.
37. (a) Weinstein, J.; Afonso, A.; Moss Jr, E.; Miller, G.H. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3391; (b) Kline, T.; Holub, D.; Therrien, J.; Leung, T.; Ryckman, D. *J. Peptide. Res.* **2000**, *57*, 175; (c) Srinivasa, B.R.; Ramachandran, L.K. *Ind. J. Biochem. Biophys.* **1978**, *14*, 54; (d) Salem, E.-E.M.; El-Gammal, A.A. *Pharmazie* **1980**, *35*, 761
38. (a) Tsubery, H.; Ofek, I.; Cohen, S.; Fridkin, M. *J. Med. Chem.* **2000**, *43*, 3085; (b) Tsubery, H.; Ofek, I.; Cohen, S.; Eisenstein, M.; Fridkin, M. *Mol. Pharmacol.* **2002**, *62*, 1036

CHAPTER 3 | Acyl Migration in Polymyxin Synthesis

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