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Tuning hydrophobicity of highly cationic tetradecameric Gramicidin S analogues using adamantane amino acids

Annemiek D. Knijnenburg [a], Varsha V. Kapoerchan [a], Emile Spalburg [b], Albert J. de Neeling [b], Roos H. Mars-Groenendijk [c], Daan Noort [c], Gijs A. van der Marel [a], Herman S. Overkleeft [a], Mark Overhand [a,*]

[a] Bio-Organic Synthesis, Leiden Institute of Chemistry, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands
[b] National Institute of Public Health and the Environment, Laboratory for Infectious Diseases PO Box 1, 3720 BA Bilthoven, The Netherlands
[c] TNO Defense, Security and Safety, Business unit Biological and Chemical Protection, Prins Maurits Laboratory, PO Box 45, 2280 AA Rijswijk, The Netherlands

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ABSTRACT

Ring extended Gramicidin S analogues containing adamantane amino acids and six cationic residues were designed and evaluated. Systematic replacement of the hydrophobic residues with adamantane amino acids resulted in a small set of compounds with varying amphipathic character. It was found that the amphipathicity of these compounds is correlated to their biological activity. Several bacterial strains including MRSA strains were shown to be killed by the novel peptides. The most potent antibacterial peptides are tetradecameric GS analogues containing six positives charges and two adamantane moieties.

1. Introduction

Cationic antimicrobial peptides (CAPs) are amphipathic peptides that often contain either an α-helix or a β-sheet as a distinguishing secondary structural element. They are produced by prokaryotes and eukaryotes and are part of the host defence mechanism against invading bacteria. As their mechanism of action is not specifically based on cellular targets, but rather aimed at targeting the cell membrane as a whole, this class of peptides are promising leads for the development of new bactericidal agents. The biological activity of membrane disrupting antimicrobial peptides is frequently based on their common characteristics such as their size, number of positive charges and amphipathicity.[1] As yet, no effective resistance mechanisms are reported against this class of peptides.[2–4]

The antibiotic Gramicidin S (GS, *cyclo*-(PVOL^{D}F)$_2$)[5] is a well-studied member of the CAP family. GS adopts a cyclic β-hairpin structure in solution that is stabilized by four intra-molecular hydrogen bonds. The side-chains of the Val and Leu residues and the side-chains of the Orn residues create a hydrophobic and a hydrophilic face, giving the cyclic β-hairpin structure its amphipathic character.[6,7] GS disrupts bacterial membranes and is able to kill Gram-positive and certain Gram-negative bacteria, however its use is limited only as topical antibiotic because of its haemolytic activity.[8]

Spanning several decades, numerous derivatives based on GS have been investigated with the aim to find new molecules with an improved biological profile. It appears that the amphipathicity of the GS derivative is crucial for its biological activity.[9–12] We recently reported an approach to vary the amphipathicity by the synthesis of 'reversed' GS derivatives,[13,14] as exemplified by the structure of **1** (Fig. 1).[15] In this compound two central hydrophobic adamantane amino acids are flanked by four Orn residues to render the molecule its positive charge. The molecule adopts a cyclic β-hairpin structure dependent on the solvent. Analogue **1** proved to be much less haemolytic as GS, while at the same time being potently active against several bacterial strains, including certain MRSA strains.[15] Another approach we[16] and others[17–22] have pursued involves the synthesis of tetradecameric GS derivatives, such as compound **2**[16] (Fig. 1). This compound also has four positive charges and adopts an extended cyclic β-hairpin structure. In both of these approaches variation of the number and position of the hydrophilic and hydrophobic side-chains to fine-tune the

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; HMPB, 4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid; BHA, benzhydrylamine; NMR, nuclear magnetic resonance; CD, circular dichroism; TOCSY, total correlation spectroscopy; TFE, 2,2,2-trifluoroethanol; MIC, minimal inhibitory concentration; RP-HPLC, reversed phase high performance liquid chromatography; SPPS, solid phase peptide synthesis; NMP, 1-methyl-2-pyrrolidone; DiPEA, *N,N'*-diisopropylethylamine; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium; TFA, 2,2,2-trifluoroacetic acid; DCM, dichloromethane; DMF, *N,N'*-dimethylformamide; HOBt, *N*-hydroxybenzotriazole; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; Fmoc, 9-fluorenylmethyloxycarbonyl.

* Corresponding author. Tel.: +31 715274483; fax: +31 715274307.
E-mail address: overhand@chem.leidenuniv.nl (M. Overhand).

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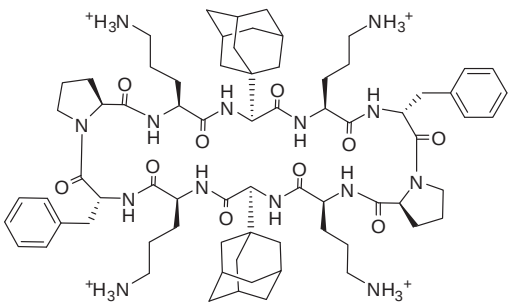
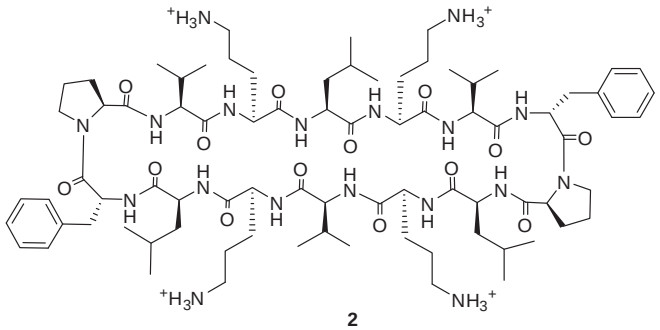


1



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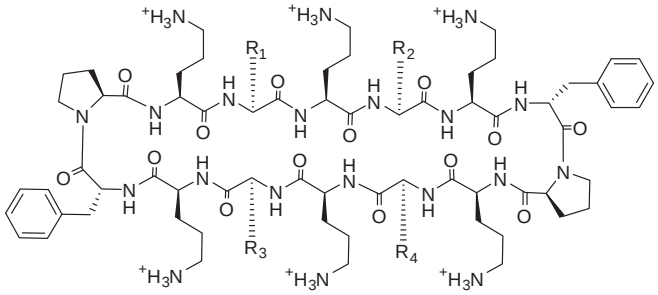


3: $R_1 = R_4 = CHMe_2$; $R_2 = R_3 = CH_2CHMe_2$
4: $R_1 = R_4 = CHMe_2$; $R_2 = CH_2$adamantyl; $R_3 = CH_2CHMe_2$
5: R_1 = adamantyl; $R_2 = R_3 = CH_2CHMe_2$; $R_4 = CHMe_2$
6: R_1 = adamantyl; $R_2 = CH_2$adamantyl; $R_3 = CH_2CHMe_2$; $R_4 = CHMe_2$
7: $R_1 = R_4$ = adamantyl; $R_2 = R_3 = CH_2CHMe_2$
8: $R_1 = CHMe_2$; $R_2 = CH_2$adamantyl; $R_3 = CH_2CHMe_2$; R_4 = adamantyl
9: $R_1 = R_4 = CHMe_2$; $R_2 = R_3 = CH_2$adamantyl
10: $R_1 = CHMe_2$; $R_2 = R_3 = CH_2$adamantyl; R_4 = adamantyl
11: $R_1 = R_4$ = adamantyl; $R_2 = CH_2$adamantyl; $R_3 = CH_2CHMe_2$
12: $R_1 = R_4$ = adamantyl; $R_2 = R_3 = CH_2$adamantyl

Figure 1. Extended 'reversed' GS analogues containing adamantane amino acids and six cationic residues.

amphipathicity of the GS derivatives plays a key role. We decided to design a series of compounds according to both approaches. For all compounds we used Orn as cationic residue and D-Phe-Pro as two-residue turn element. Starting from extended 'reversed' GS derivative **3**, we systematically replaced either one or both hydrophobic Val and Leu residues by adamantyl-L-glycine[23] and adamantyl-L-alanine[23], respectively (**4**–**12**, Fig. 1) to create a variation in hydrophobicity at the a-polar face of the molecule. We here present the synthesis, structural analysis and biological evaluation of the compounds **3**–**12** and compare the outcome with their parent compounds.

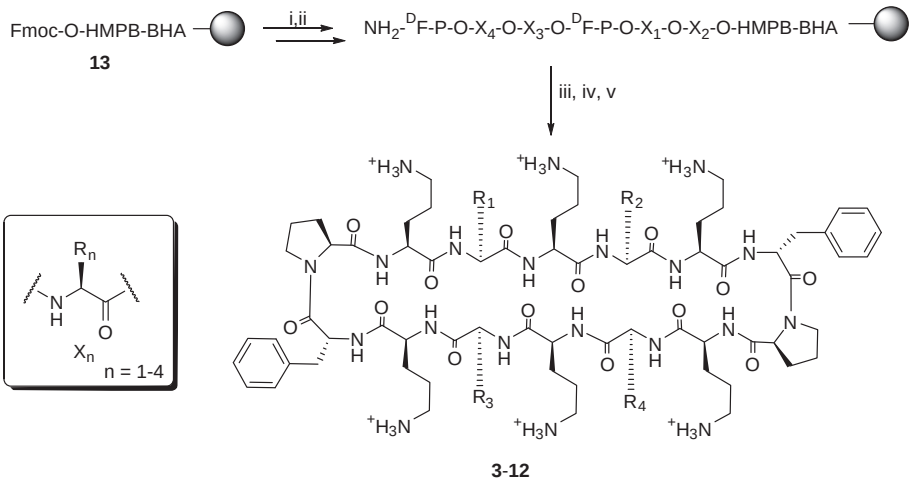
2. Results and discussion

All peptides were prepared using a standard automated stepwise solid phase peptide synthesis protocol and the highly acid sensitive HMPB–BHA resin[24] preloaded with Fmoc protected ornithine **13** (Scheme 1). After mild acidic cleavage from the solid support, the partially protected linear peptides containing six Orn(Boc) residues were cyclised under high dilution conditions and purified by gel filtration to obtain the partially protected cyclised peptides in yields ranging from 45% to 70%. Removal of the Boc protective groups, using strong acidic conditions, was followed by preparative HPLC purification to give the desired cyclic peptides **3**–**12** in 10–35% overall yield (Scheme 1).

The secondary structure of the peptides was evaluated using NMR and CD spectroscopy. NMR spectra were recorded in various solvents, but the 1-D spectra were not that well resolved to determine homonuclear couplings constants. In the 2-D TOCSY spectra of analogues **3–12**, the geminal coupling between the two δ protons of the Pro residues were determined. The chemical shift perturbation value ($\Delta\delta Pro_{\delta d,u}$) of these protons reflects the deformation of the turn propensity of the Pro residue as part of a β-hairpin structure, also called β-sheet content.[25] According to this method all synthesized peptides showed a similar amount of β-sheet content, having a $\Delta\delta$Pro ranging from 0.8 to 0.9 ppm. This finding was corroborated by recording their CD spectra. Peptides **3–12** gave CD curves with negative ellipticities around 210 nm and a slight minimum around 220 nm in a mixture of TFE/H_2O, indicative of a β-sheet/β-hairpin structure (Fig. 2).[26,27] CD spectra recorded in methanol show the same CD curves as for TFE/H_2O (reported in Supplementary data).

The cyclic peptides **3**–**12** were screened for antibacterial activity against several Gram-negative and Gram-positive strains, including six MRSA bacterial strains (Table 1). In addition, their haemolytic properties were investigated (Table 2). For comparison our observed MIC values and haemolytic data of previously reported compounds GS, GS14,[18] **1**,[15] and compound **2**,[16] are also included in Tables 1 and 2. Compounds **3** and **12** showed the lowest antibacterial activity in this series of compounds. Interestingly, several compounds are more active than GS against both Gram-negative and Gram-positive strains. Especially compounds **5**, **7** and **9** containing two adamantane moieties are potently active against the six MRSA strains. Compound **3** is the least haemolytic in this series and compounds **4** and **5** are slightly less haemolytic as compared to the natural product GS (Table 2). The other derivatives are more haemolytic than GS.

Determination of retention time under controlled conditions on RP-HPLC can be used as a measure of peptide hydrophobicity.[28,29] However, it is reported[30,31] that interactions of reversed-phase matrices with hydrophobic binding domains of the peptide are also influenced by the secondary structure of a peptide. To ensure that all the peptides exhibit a comparable secondary structure on the reversed-phase column, the observed retention times of peptides **3**–**12** were correlated with their hydrophobic moment.[32] The hydrophobic moment is a theoretical value of a peptide sequence indicating its amphipathic character. The calculation is based on the hydrophobicity of the constituting amino acids and secondary structure of the peptide. The retention times correlate well ($r = 1.0$, $R^2 = 0.99$, see Supplementary data) with the hydrophobic moments of peptides **3**–**12** (Table 2). As anticipated, compound **3** is the most polar peptide in this series and compound **12** containing four adamantane moieties the most hydrophobic. Compound **3** is not haemolytic but also not antibacterial active, whereas compound **12** is highly haemolytic without major bactericidal activity. The properties of these molecules are in agreement with data on other



Scheme 1. Synthesis of GS analogues **3**–**12** using automated SPPS. Reagents and conditions: (i) deprotection: 20% pip/NMP; (ii) coupling: standard Fmoc-AA-OH (5 equiv), HATU (90%), DiPEA, NMP, 30 min or Fmoc-Adamantyl-L-glycine, Fmoc-Adamantyl-L-alanine (1.5 equiv), HATU (90%), DiPEA, NMP, 30 min; (iii) cleavage: 1% TFA, DCM; (iv) cyclisation: PyBOP, HOBt, DiPEA, DMF, 16 h; (v) 95% TFA, 2.5% TIS, 2.5% H_2O.

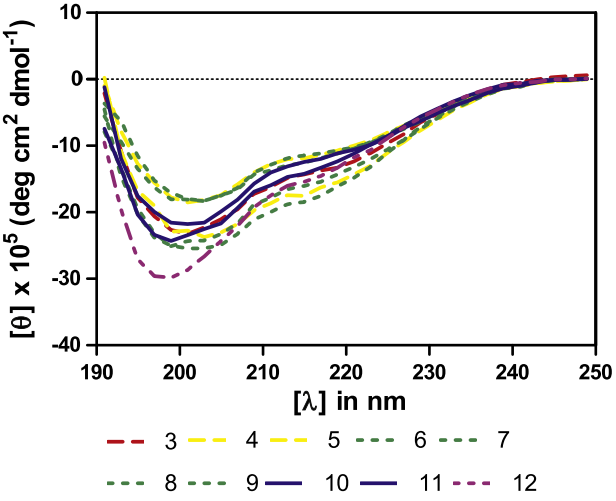


Figure 2. CD-spectra of **3**–**12** analogs containing six basic residues. CD spectra recorded in 0.1 mM in 50% TFE/0.01 M NaOAc (pH 5.3).

hydrophilic and hydrophobic peptides.[22] The amphipathic characteristics of compounds **3** and **12** thus form the outer limits within this series. Compounds **4**–**7** are the most antibacterial active peptides of this series having amphiphatic characteristics corresponding to a hydrophobic moment around 10 μ (Table 2), however, these peptides are also very haemolytic.

3. Conclusion

We here presented our investigation of a series of novel antimicrobial peptides (**3**–**12**) in which two design approaches, 'reversed' GS derivatives[13–15] and tetradecameric GS derivatives[16–22], were combined. These analogues of the natural product Gramicidin S contain fourteen amino acid residues of which six are cationic and in which the Val and Leu residues are systematically replaced by adamantane amino acids. We found that the elongated reversed GS analogues (**3**–**12**) adopt cyclic β-hairpin structures similar to the tetradecameric derivatives reported previously. Having established that the compound series exhibit similar secondary structures, we conclude that differences in biological activity are mainly based on their varied amphipathic character.

A general trend in the correlation between amphipathicity of the molecules and the haemolytic activity was observed. That is, the most hydrophobic molecule **12** displayed the highest haemolytic activity, whereas the least hydrophobic molecule **3** had negligible haemolytic activity. These findings are in agreement with other hydrophilic and hydrophobic peptides.[22] An optimum in antimicrobial activity was observed for compound series **3**–**12**. The outer limits of the peptide series, **3** and **12**, showed low antimicrobial activity. The relation of antimicrobial activity is complex due to the diversity in membrane structures of microorganisms.[33–35] A possible explanation for the observed trend in antimicrobial activity might be the subtle equilibrium between amphipathicity and overall hydrophobicity. Probably compound **3** is too hydrophilic to have any affinity with the lipid bilayers (erythrocytes membrane or bacterial membrane) whereas compound **12** is too hydrophobic and might bind strongly to bacterial cell wall elements.[35] Peptides **4** and **5** showed less haemolytic activity compared to GS and still retained a similar antimicrobial activity, leading to a better therapeutic profile compared to GS, compound **2**[16] and GS14[18] (Table 2). It must be said that our previously reported 'reversed' adamantane compound **1**[15] outperforms all these peptides, by being potently antimicrobial and having low haemolytic activity. The peptides containing two adamantane amino acids **6**–**9** displayed the highest antimicrobial activity of all peptides tested and also displayed very good antimicrobial activity against MRSA strains. Unfortunately these molecules appeared to be highly haemolytic as well, and further modifications need to be pursued in order to discover bactericidal GS derivatives for in vivo applications to treat systemic bacterial infections.

4. Experimental section

Peptides were synthesized on an Applied Biosystems 433A Peptide Synthesizer. LC/MS analyses (detection simultaneously at 214 and 254 nm) were performed on a LCQ Adventage Max (Thermo Finnigan) equipped with a Gemini C18 column (250 mmL × 4.6 mmD, 5 μ particle size, Phenomenex). The applied buffers were A: H_2O, B: MeCN and C: 1.0% aq TFA. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in H_2O/MeCN; 50/50: v/v and 0.1% formic acid) on a mass sp-ectrometer Thermo Finnigan LTQ Orbitrap equipped with an electrospray ion source in positive mode (source voltage 3.5 kV,

Table 1
Antimicrobial activity (MIC, mg/L) of GS, GS14 and GS analogues **1**–**12**

Analogues[a]	*S. aureus*[b] ATCC 29213	*S. epider midis*[b] ATCC 12228	*E. faecalis*[b] ATCC 29212	*B. cereus*[b] ATCC 11778	*P. aeruginosa*[c] ATCC 27853	*E. coli*[c] ATCC 25922	MRSA[b] 1110301146	MRSA[b] 1110301981	MRSA[b] N229	MRSA[b] N133	MRSA[b] ATCC 49775	MRSA[b] ATCC 43300
GS	32	8	8	8	64	32	16	16	8	16	8	8
GS14[18]	>64	64	32	32	>64	>64	–	–	–	–	–	–
1	8	4	8	8	16	8	8	8	8	8	–	–
2	16	8	8	4	64	32	–	–	–	–	–	–
3	64	8	>64	32	32	64	>64	64	64	>64	64	>64
4	16	8	32	8	16	16	16	8	16	8	8	8
5	16	4	32	8	16	8	16	8	8	8	8	8
6	8	4	8	1	16	8	16	16	16	16	16	16
7	8	8	8	2	16	16	8	8	8	8	8	8
8	8	4	16	4	16	16	16	16	16	32	16	16
9	8	8	8	8	32	8	8	8	8	8	8	8
10	16	4	8	4	32	16	32	16	16	16	16	16
11	16	8	8	4	32	16	8	8	8	8	16	16
12	64	32	32	2	64	64	32	32	32	32	32	32

[a] Molecular weight GS: 1369.49; GS14: 2126.23; **1**: 1783.79; **2**: 2038.12; **3**: 2282.17; **4**,**5**: 2374.31; **6**–**9**: 2466.45; **10**,**11**: 2558.59; **12**: 2650.72. GS14 is an 14-meric analogue of GS with ^{D}Tyr and Lys instead of ^{D}Phe and Orn (Ref. 18).
[b] Gram-positive bacteria, MIC in mg/L. For detailed experimental set up: see Section 4.
[c] Gram-negative bacteria, MIC mg/L.

Table 2
Correlation of physical and biological properties of peptide **3**–**12**

Analogue	Sequence[a]	Observed retention time[b]	Hydrophobic moment (μ)[c]	Haemolytic activity[d]	Antimicrobial activity[e] Gram positive bacteria	Antimicrobial activity[f] Gram negative bacteria	Therapeutic index[g] *S. aureus*	Therapeutic index[h] *E. coli*
GS	*cyclo*-(PVOL^{D}F)$_2$	8.38	–	62.5	+	+/–	2.0	2.0
GS14[18]	*cyclo*-(PVKLKV^{D}YPLKVKL^{D}Y)	7.26	–	3.9	+/–	–	0.03	0.03
1	*cyclo*-(POX$_{gly}$O^{D}F)$_2$	6.39	–	500	++	+	62.5	62.5
2	*cyclo*-(PVOLOV^{D}FPLOVOL^{D}F)	8.00	–	3.9	++	+/–	0.2	0.1
3	*cyclo*-(POVOLO^{D}F)$_2$	4.02	7.3	>500	–	+/–	11.7	11.7
4	*cyclo*-POVOX$_{ala}$O^{D}FPOVOLO^{D}F	4.58	8.5	125	+	++	7.8	7.8
5	*cyclo*-POX$_{gly}$OLO^{D}FPOVOLO^{D}F	4.69	8.6	125	++	++	7.8	15.6
6	*cyclo*-POX$_{gly}$OX$_{ala}$O^{D}FPOVOLO^{D}F	5.09	9.8	31.3	++	++	3.9	3.9
7	*cyclo*-(POX$_{gly}$OLO^{D}F)$_2$	5.13	9.9	31.3	++	++	3.9	2.0
8	*cyclo*-POVOX$_{ala}$O^{D}FPOX$_{gly}$OLO^{D}F	5.16	9.9	31.3	++	++	3.9	2.0
9	*cyclo*-(POVOX$_{ala}$O^{D}F)$_2$	5.23	10.0	31.3	++	+	3.9	3.9
10	*cyclo*-POVOX$_{ala}$O^{D}FPOX$_{gly}$OX$_{ala}$O^{D}F	5.63	11.1	15.6	++	+	1.0	1.0
11	*cyclo*-POX$_{gly}$OX$_{ala}$O^{D}FPOX$_{gly}$OLO^{D}F	5.69	11.2	15.6	++	+	1.0	1.0
12	*cyclo*-(POX$_{gly}$OX$_{ala}$O^{D}F)$_2$	6.12	12.5	7.8	+/–	+/–	0.1	0.12

[a] Linear sequences of cyclic peptides. One-letter amino acid code is used; amino acids with superscripted D represent D-amino acids. X$_{gly}$ = Adamantyl-L-glycine; X$_{ala}$ = Adamantyl-L-alanine.
[b] Observed retention time on RP-HPLC at 25 °C (for more information see Section 4).
[c] The hydrophobic moment is only calculated for the homologues series **3**–**12**. Calculated hydrophobic moment (μ) with values for Leu, Val, Orn, Pro, and Phe as 9.7, 4.1, −9.0, −0.2 and 10, respectively. The values for X$_{gly}$ and X$_{ala}$ were calculated as 24.1 and 28.7 (see Section 4).
[d] Peptide concentration (in μM) required for 100% lysis of erythrocytes. Haemolytic curves reported in Supporting information.
[e] General trend observed for Gram positive antimicrobial activity with most MIC (mg/L) values in the range of: – = 64–>64 mg/L; +/– = 32–64 mg/L; + = 8–32 mg/L, ++ = 1–16 mg/L.
[f] General trend observed for Gram negative antimicrobial activity with most MIC (mg/L) values in the range of: – = 64–>64 mg/L; +/– = 32–64 mg/L; + = 8–32 mg/L, ++ = 1–16 mg/L.
[g] Therapeutic index of *S. aureus* = haemolytic activity at 100% lysis/MIC. For calculation of the therapeutic index, values of 128 mg/L were used for MIC values of >64 mg/L, and values of 750 were used for haemolytic activity values of >500 μM.[18]
[h] Therapeutic index of *E. coli* = haemolytic activity at 100% lysis/MIC. For calculation of the therapeutic index, values of 128 mg/L were used for MIC values of >64 mg/L, and values of 750 were used for haemolytic activity values of >500 μM.[18]

sheath gas flow 10, capillary temperature 523 K) with resolution R = 60,000 at m/z 400 (mass range m/z = 150-2000) and dioctylphthalate (m/z = 391.28428) as lock mass. HPLC purifications were performed on a Gilson GX-281 automated HPLC system, equipped with a preparative Gemini C18 column (150 mmL × 21.20 mmD, 5 μ, particle size). The applied buffers were: A: 0.2% aq TFA, B: MeCN. CD and haemolytic curves were analyzed with GRAPHPAD PRISM version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. 1H-, ^{13}C-, Standard DGF-COSY (512c × 2084c) and TOCSY (400c × 2048c) NMR spectra were recorded on a Bruker DMX 600 equipped with a pulsed field gradient accessory and a cryo-probe. Chemical shifts are given in ppm (δ) relative to CD_3OH (3.33) ppm.

4.1. General peptide synthesis

(a) *Peptide chain elongation:* Preloaded resin (HMPB–BHA, 100-200 mesh) with Fmoc-Orn(Boc) (133 mg, 0.75 mmol/g, 0.1 mmol) was subjected to 13 cycles of automated Fmoc solid-phase synthesis using the Fmoc based solid phase peptide synthesis protocols. Commercially available Fmoc protected amino acids were coupled with 90% HATU with respect to 5 equiv of amino acid in 30 min.

The Fmoc adamantyl-L-glycine and Fmoc adamantyl-L-alanine were coupled with 90% HATU coupling reagent with respect to 1.5 equiv amino acid in 30 min.

(b) *Cleavage from resin*: The peptides were released from the resin by mild acidic cleavage (4 × 10 min, 10 mL 1% TFA in DCM). The fractions were collected and coevaporated with toluene (3 × 50 mL) to give the crude linear peptide which was immediately cyclised without further purification.

(c) *Cyclisation*: The crude partially protected peptide in DMF (20 mL) was dropwise added over 16 h to a solution of HOBt (5 equiv), pyBOP (5 equiv) and DiPEA (15 equiv) in DMF (160 mL). The solvent was removed under diminished pressure and the residue applied to a Sephadex™ LH-20 size exclusion column (50.0 mmD × 1500 mmL) and eluted with MeOH. The volatiles were removed under diminished pressure and the protected peptides were analyzed by LC/MS and HRMS.

(d) *Deprotection*: The Boc-protection groups of the peptides were removed by addition of TFA/TIS/H_2O mixture (10 mL, 95/2.5/2.5) and subsequently the peptide was purified by preparative RP-HPLC.

4.1.1. *cyclo*-(POVOLO^{D}F)$_2$ (3)

Prepared and cyclised according to the general procedure. Purified protected yield: 103 mg, 46.8 μmol; 47%. HRMS (ESI) *m/z* 1099.67703 $[M+H]^{2+}$, calcd 1099.67616 for $C_{110}H_{180}N_{20}O_{26}$; Removal of the Boc group, purification by preparative RP-HPLC (linear gradient of 46–76%, 3 CV) and lyophilisation of the combined pure fractions furnished peptide **3** (18.62 mg, 8.16 μmol, 8%); LCMS R_f 4.02 min, linear gradient 10→90% B in 13.5 min; *m/z* = 1599.4 $[M+H]^+$; HRMS (ESI) *m/z* 799.51914 $[M+H]^{2+}$, calcd 799.51887 for $C_{80}H_{132}N_{20}O_{14}$; ^{1}H NMR (600 MHz, CD_3OH) δ 9.07 (br s, 1H), 8.64 (m, 3H), 7.91 (m, 5H), 7.74 (m, 2H), 7.46–7.10 (m, 12H), 4.68–4.16 (m, 7H), 3.67 (s, 1H), 3.56 (s, 2H), 3.18–2.89 (m, 18H), 2.68 (m, 2H), 2.25–1.48 (m, 51H), 1.45–1.21 (m, 4H), 1.10–0.68 (m, 24H). ^{13}C NMR (151 MHz, CD_3OH) δ 181.60, 181.51, 173.61, 173.58, 173.00, 172.97, 162.99, 162.77, 136.92, 130.34, 129.65, 128.46, 101.28, 61.33, 40.27, 37.80, 30.00, 29.68, 25.62, 25.10, 23.62, 19.68.

4.1.2. *cyclo*-POVOX$_{ala}$O^{D}FPOVOLO^{D}F (4)

Prepared and cyclised according to the general procedure. Purified protected yield: 147 mg, 64.17 μmol; 64%. HRMS (ESI) *m/z* 1145.70846 $[M+H]^{2+}$, calcd 1145.70746 for $C_{117}H_{188}N_{20}O_{26}$ Removal of the Boc group, purification by preparative RP-HPLC (linear gradient of 46–76%, 3 CV) and lyophilisation of the combined pure fractions furnished peptide **4** (40, 62 mg, 24.03 μmol, 24%); LCMS R_f 4.58 min, linear gradient 10→90% B in 13.5 min; *m/z* = 1691.6 $[M+H]^+$; HRMS (ESI) *m/z* 845.55070 $[M+H]^{2+}$, calcd 845.55017 for $C_{87}H_{140}N_{20}O_{14}$; ^{1}H NMR (600 MHz, CD_3OH) δ 9.05 (br s, 1H), 8.68 (m, 2H), 8.53 (br s, 2H), 7.91 (m, 5H), 7.75 (br s, 1H), 7.39–7.20 (m, 12H), 4.44 (m, 5H), 3.67 (s, 1H), 3.55 (s, 2H), 3.18–2.85 (m, 18H), 2.15–1.35 (m, 72H), 1.30 (s, 1H), 1.04–0.74 (m, 18H). ^{13}C NMR (151 MHz, CD_3OH) δ 173.51, 172.53, 162.99, 162.76, 130.33, 129.65, 128.46, 68.02, 61.35, 43.29, 40.26, 40.11, 37.78, 33.67, 30.02, 29.89, 25.11, 19.58.

4.1.3. *cyclo*-POX$_{gly}$OLO^{D}FPOVOLO^{D}F (5)

Prepared and cyclised according to the general procedure. Purified protected yield: 164 mg, 71.59 μmol; 71%. HRMS (ESI) *m/z* 1145.70824 $[M+H]^{2+}$, calcd 1145.70746 for $C_{117}H_{189}N_{20}O_{26}$; Removal of the Boc group, purification by preparative RP-HPLC (linear gradient of 46–76%, 3 CV) and lyophilisation of the combined pure fractions furnished peptide **5** (32, 58 mg, 19.28 μmol, 19%); LCMS R_f 4.69 min, linear gradient 10→90% B in 13.5 min; *m/z* = 1690.6 $[M+H]^+$; HRMS (ESI) *m/z* 845.55045 $[M+H]^{2+}$, calcd 845.55017 for $C_{87}H_{140}N_{20}O_{14}$; ^{1}H NMR (600 MHz, CD_3OH) δ 9.09 (m, 2H), 8.62 (m, 3H), 7.92 (m, 6H), 7.31 (m, 12H), 4.36 (m, 5H), 3.67 (s, 1H), 3.60 (m, 2H), 3.01 (m, 18H), 2.30–1.28 (m, 73H), 1.03–0.68 (m, 18H). ^{13}C NMR (151 MHz, CD_3OH) δ 173.52, 162.99, 162.76, 130.35, 129.67, 128.49, 68.02, 61.34, 40.26, 39.57, 37.63, 29.98, 29.66, 29.31, 25.10.

4.1.4. *cyclo*-POX$_{gly}$OX$_{ala}$O^{D}FPOVOLO^{D}F (6)

Prepared and cyclised according to the general procedure. Purified protected yield: 129 mg, 54.13 μmol; 54%. HRMS (ESI) *m/z* 1191.73959 $[M+H]^{2+}$, calcd 1191.73876 for $C_{124}H_{197}N_{20}O_{26}$; Removal of the Boc group, purification by preparative RP-HPLC (linear gradient of 46–76%, 3 CV) and lyophilisation of the combined pure fractions furnished peptide **6** (39, 62 mg, 22.23 μmol, 22%); LCMS R_f 5.09 min, linear gradient 10→90% B in 13.5 min; *m/z* = 1783.8 $[M+H]^+$; HRMS (ESI) *m/z* 891.58195 $[M+H]^{2+}$, calcd 891.58147 for $C_{94}H_{148}N_{20}O_{14}$; ^{1}H NMR (600 MHz, CD_3OH) δ 9.00 (br s, 1H), 8.71 (br s, 1H), 8.37 (br s, 2H), 7.93 (br s, 2H), 7.85 (br s, 2H), 7.72 (br s, 2H), 7.43–7.13 (m, 12H), 4.44 (m, 6H), 3.67 (s, 2H), 3.54 (s, 2H), 3.15–2.81 (m, 18H), 2.70 (s, 2H), 2.18–1.43 (m, 88H), 1.39 (m, 1H), 1.30 (s, 1H), 0.89 (m, 12H). ^{13}C NMR (151 MHz, CD_3OH) δ 173.41, 163.00, 137.01, 130.33, 129.64, 128.43, 101.28, 68.02, 61.39, 45.75, 43.28, 40.25, 40.12, 37.77, 33.70, 29.90, 25.13, 19.54.

4.1.5. *cyclo*-(POX$_{gly}$OLO^{D}F)$_2$ (7)

Prepared and cyclised according to the general procedure. Purified protected yield: 138 mg, 57.91 μmol; 57%. HRMS (ESI) *m/z* 1191.73980 $[M+H]^{2+}$, calcd 1191.73876 for $C_{124}H_{196}N_{20}O_{26}$; Removal of the Boc group, purification by preparative RP-HPLC (linear gradient of 46–76%, 3 CV) and lyophilisation of the combined pure fractions furnished peptide **7** (56, 81 mg, 31.87 μmol, 32%); LCMS R_f 5.13 min, linear gradient 10→90% B in 13.5 min; *m/z* = 1783.8 $[M+H]^+$; HRMS (ESI) *m/z* 891.58179 $[M+H]^{2+}$, calcd 891.58147 for $C_{94}H_{148}N_{20}O_{14}$; ^{1}H NMR (600 MHz, CD_3OH) δ 9.06 (s, 2H), 8.78–8.38 (m, 4H), 7.92 (m, 6H), 7.71 (s, 2H), 7.46–7.20 (m, 12H), 4.47 (m, 5H), 3.67 (br s, 4H), 3.58 (m, 2H), 3.20–2.86 (m, 17H), 2.22–1.40 (m, 81H), 1.32 (m, 1H), 1.14–0.64 (m, 12H). ^{13}C NMR (151 MHz, CD_3OH) δ 173.53, 162.71, 137.38, 130.35, 129.65, 128.47, 68.02, 61.28, 43.33, 40.24, 39.65, 37.79, 37.70, 33.82, 30.11, 29.88, 29.69, 25.15.

4.1.6. *cyclo*-POVOX$_{ala}$O^{D}FPOX$_{gly}$OLO^{D}F (8)

Prepared and cyclised according to the general procedure. Purified protected yield: 150 mg, 62.95 μmol; 63%. HRMS (ESI) *m/z* 1191.73974 $[M+H]^{2+}$, calcd 1191.73876 for $C_{124}H_{196}N_{20}O_{26}$; Removal of the Boc group, purification by preparative RP-HPLC (linear gradient of 46–76%, 3 CV) and lyophilisation of the combined pure fractions furnished peptide **8** (49, 03 mg, 27.51 μmol, 28%); LCMS R_f 5.16 min, linear gradient 10→90% B in 13.5 min; *m/z* = 1783.8 $[M+H]^+$; HRMS (ESI) *m/z* 891.58182 $[M+H]^{2+}$, calcd 891.58147 for $C_{94}H_{148}N_{20}O_{14}$; ^{1}H NMR (600 MHz, CD_3OH) δ 9.07 (br s, 1H), 8.67 (br s, 2H), 8.60 (br s, 1H), 7.94 (br s, 5H), 7.87 (br s, 2H), 7.31 (m, 12H), 4.45 (m, 5H), 3.67 (s, 3H), 3.57 (s, 2H), 3.01 (m, 17H), 2.11–1.40 (m, 85H), 1.30 (s, 1H), 0.98–0.79 (m, 12H). ^{13}C NMR (151 MHz, CD_3OH) δ 162.98, 162.75, 130.33, 129.65, 128.47, 68.02, 61.34, 43.30, 40.25, 39.52, 37.79, 37.63, 29.89, 29.68, 25.11.

4.1.7. *cyclo*-(POVOX$_{ala}$O^{D}F)$_2$ (9)

Prepared and cyclised according to the general procedure. Purified protected yield: 161 mg, 67.56 μmol; 68%. HRMS (ESI) *m/z* 1191.73976 $[M+H]^{2+}$, calcd 1191.73876 for $C_{124}H_{196}N_{20}O_{26}$; Removal of the Boc group, purification by preparative RP-HPLC (linear gradient of 46–76%, 3 CV) and lyophilisation of the combined pure fractions furnished peptide **9** (63, 46 mg, 35.61 μmol, 36%); LCMS R_f 5.23 min, linear gradient 10→90% B in 13.5 min; *m/z* = 1783.8 $[M+H]^+$; HRMS (ESI) *m/z* 891.58183 $[M+H]^{2+}$, calcd 891.58147 for $C_{94}H_{148}N_{20}O_{14}$; ^{1}H NMR (600 MHz, CD_3OH) δ 9.08 (br s, 1H), 8.58

(br s, 2H), 8.17 (br s, 1H), 7.92 (m, 4H), 7.62 (br s, 2H), 7.31 (m, 12H), 4.39 (m, 7H), 3.67 (s, 4H), 3.58 (s, 2H), 3.01 (m, 17H), 2.19–1.35 (m, 83H), 1.30 (s, 1H), 0.87 (m, 12H). ^{13}C NMR (151 MHz, CD_3OH) δ 162.89, 162.66, 130.35, 129.64, 128.47, 68.02, 61.62, 61.26, 47.88, 40.26, 39.45, 37.64, 32.07, 29.82, 29.66, 29.26, 25.26, 25.17, 21.26.

4.1.8. *cyclo*-POVOX$_{ala}$O^{D}FPOX$_{gly}$OX$_{ala}$O^{D}F (10)

Prepared and cyclised according to the general procedure. Purified protected yield: 139 mg, 56.16 μmol; 56%. HRMS (ESI) *m/z* 1237.77097 $[M+H]^{2+}$, calcd 1237.77006 for $C_{131}H_{204}N_{20}O_{26}$; Removal of the Boc group, purification by preparative RP-HPLC (linear gradient of 46–76%, 3 CV) and lyophilisation of the combined pure fractions furnished peptide **10** (12, 02 mg, 6.41 μmol, 6%); LCMS R_f 5.63 min, linear gradient 10→90% B in 13.5 min; *m/z* = 1875.4 $[M+H]^+$; HRMS (ESI) *m/z* 937.61366 $[M+H]^{2+}$, calcd 937.61277 for $C_{101}H_{156}N_{20}O_{14}$; ^{1}H NMR (600 MHz, CD_3OH) δ 9.06 (br s, 1H), 8.68 (br s, 1H), 8.36 (br s, 2H), 8.13 (br s, 2H), 7.93 (br s, 2H), 7.70 (br s, 2H), 7.31 (m, 12H), 4.51 (s, 2H), 4.41 (m, 3H), 4.12 (m, 1H), 3.87–3.76 (m, 1H), 3.57 (m, 2H), 3.44 (m, 1H), 3.16–2.78 (m, 18H), 2.69 (m, 1H), 2.37 (s, 1H), 2.21–1.37 (m, 96H), 1.32 (m, 2H), 1.09–0.75 (m, 6H). ^{13}C NMR (151 MHz, CD_3OH) δ 162.99, 130.32, 129.65, 128.46, 68.02, 61.37, 43.33, 40.24, 37.77, 33.80, 30.74, 29.90, 29.71.

4.1.9. *cyclo*-POX$_{gly}$OX$_{ala}$O^{D}FPOX$_{gly}$OLO^{D}F (11)

Prepared and cyclised according to the general procedure. Purified protected yield: 133 mg, 53.73 μmol; 54%. HRMS (ESI) *m/z* 1237.77107 $[M+H]^{2+}$, calcd 1237.77006 for $C_{131}H_{204}N_{20}O_{26}$; Removal of the Boc group, purification by preparative RP-HPLC (linear gradient of 46–76%, 3 CV) and lyophilisation of the combined pure fractions furnished peptide **11** (50, 87 mg, 27.14 μmol, 27%); LCMS R_f 5.69 min, linear gradient 10→90% B in 13.5 min; *m/z* = 1875.4 $[M+H]^+$; HRMS (ESI) *m/z* 937.61316 $[M+H]^{2+}$, calcd 937.61277 for $C_{101}H_{156}N_{20}O_{14}$; ^{1}H NMR (600 MHz, CD_3OH) δ 9.08 (br s, 1H), 8.63 (br s, 2H), 8.20 (m, 1H), 7.95 (br s, 4H), 7.64 (br s, 2H), 7.31 (m, 12H), 4.67–4.07 (m, 8H), 3.67 (s, 3H), 3.60 (m, 2H), 3.00 (m, 17H), 2.72 (s, 1H), 2.14–1.34 (m, 96H), 1.30 (s, 1H), 1.05–0.77 (m, 6H). ^{13}C NMR (151 MHz, CD_3OH) δ 173.51, 162.95, 162.72, 130.35, 129.65, 101.26, 68.02, 44.06, 43.35, 40.25, 37.70, 29.85, 29.67, 25.16, 21.31.

4.1.10. *cyclo*-(POX$_{gly}$OX$_{ala}$O^{D}F)$_2$ (12)

Prepared and cyclised according to the general procedure. Purified protected yield: 169 mg, 65.83 μmol; 66%. HRMS (ESI) *m/z* 1283.80267 $[M+H]^{2+}$, calcd 1283.80136 for $C_{138}H_{212}N_{20}O_{26}$; Removal of the Boc group, purification by preparative RP-HPLC (linear gradient of 46–76%, 3 CV) and lyophilisation of the combined pure fractions furnished peptide **12** (57, 53 mg, 29.25 μmol, 29%); LCMS R_f 6.12 min, linear gradient 10→90% B in 13.5 min; *m/z* = 1967.6 $[M+H]^+$; HRMS (ESI) *m/z* 983.64407 $[M+H]^{2+}$, calcd 983.64420 for $C_{108}H_{164}N_{20}O_{14}$; ^{1}H NMR (600 MHz, CD_3OH) δ 9.41 (br s, 1H), 9.10 (br s, 1H), 8.67 (br s, 2H), 8.22 (br s, 2H), 7.94 (br s, 2H), 7.64 (br s, 2H), 7.42–7.22 (m, 12H), 7.07 (m, 1H), 4.47 (m, 6H), 4.31–3.97 (m, 1H), 3.67 (s, 9H), 3.57 (s, 2H), 3.00 (m, 19H), 2.52–2.22 (m, 1H), 2.21–1.34 (m, 102H), 1.30 (s, 1H). ^{13}C NMR (151 MHz, CD_3OH) δ 162.75, 130.35, 129.65, 68.02, 43.35, 40.24, 37.69, 29.89, 29.70.

4.2. Circular dichroism spectroscopy

CD spectra were recorded at 298 K on a Jasco J-815 spectropolarimeter using 0.1 cm path length quartz cells The CD spectra are averages of four scans, collected at 0.1 nm intervals between 190 and 250 nm with scanning speed 50 nm/min. The peptides were prepared at concentrations 0.1 mM in 50% TFE/0.01 M NaOAc (pH 5.3). Ellipticity is reported as mean residue ellipticity [θ], with approximate errors of ±10% at 220 nm.

4.3. Antimicrobial assays

The following bacterial strains were used: *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and the MRSA strains MRSA-USA300-1110301146 PVL+ (community acquired MRSA), and MRSA-*NT* 1110301981-*spa*/t034-PVL+, MRSA-*NT* N133-*spa*/t034-PVL-, MRSA-*NT* N229-*spa*t/034-PVL- (cattle-related strains), MRSA (ATCC 49775) en MRSA (ATCC 43300). Bacteria were stored at −70 °C and grown at 35 °C on Columbia Agar with sheep blood (Oxoid, Wesel, Germany) suspended in physiological saline until an optical density of 0.1 AU (at 595 nm, 1 cm cuvette). The suspension was diluted (100×) with physiological saline, and 2 μL of this inoculum was added to 50 μL growth medium, Cation Adjusted Mueller Hinton II Broth (BBL Ref. No. 212322), in microtiter plates (96 wells).

All peptides including GS were dissolved in methanol (4 g/L) and diluted in distilled water (1000 mg/L and two-fold diluted in the broth (64, 32, 16, 8, 4 and 1 mg/L). The plates were incubated at 35 °C (24–48 h) and the MIC was determined as the lowest concentration inhibiting bacterial growth.

4.4. Haemolytic assays

Freshly drawn heparinized blood was centrifuged for 10 min at 1000*g* at 10 °C, Subsequently, the erythrocyte pellet was washed three times with 0.85% saline solution and diluted with saline to a 1/25 packed volume of red blood cells. The peptides to be evaluated were dissolved in a 30% DMSO/0.5 mM saline solution to give a 1.5 mM solution of peptide. If a suspension was formed, the suspension was sonicated for a few seconds. A 1% Triton-X solution was prepared. Subsequently, 100 μL of saline solution was dispensed in columns 1–11 of a microtiter plate, and 100 μL of 1% Triton solution was dispensed in column 12. To wells A1–C1, 100 μL of the peptide was added and mixed properly. 100 μL of wells A1–C1 was dispensed into wells A2–C2. This process was repeated until wells A10–C10, followed by discarding 100 μL of wells A10–C10. These steps were repeated for the other peptides. Subequently, 50 μL of the red blood cell solution was added to the wells and the plates were incubated at 37 °C for 4 h. After incubation, the plates were centrifuged at 1000*g* at 10 °C for 4 min. In a new microtiter plate, 50 μL of the supernatant of each well was dispensed into a corresponding well. The absorbance at 405 nm was measured and the percentage of haemolysis was determined.

4.5. Calculation peptide hydrophobic moment

The hydrophobic moment of peptides **3–12** was calculated using the hydrophobic moment relationship of Eisenberg et al.[32] To determine the hydrophobicity scale of the non-proteinogenic amino acids Adamantyl-L-glycine and Adamantyl-L-alanine, the method was used of Tossi et al.[36] The retention time of a collection of Fmoc-N-capped amino acids were determined using HPLC (Val: 7.83 min; Met: 7.76; Phe: 8.34; Ile: 8.32; ^{D}Phe: 8.35; Leu: 8.37; Ala: 6.98; Gly: 6.58; Pro: 7.32; 13.5 min run time with 1 mL/min flow rate, using a Gemini C18 column, the applied buffers were A: H_2O, B: MeCN and C: 1.0% aq TFA). The observed retention times of the Fmoc protected amino acids agreed well with the consensus hydrophobicity scale and showed a good correlation (r = 0.98).[36] The retention times of Fmoc-*N*-Adamantyl-L-glycine and Adamantyl-L-alanine are 10.43 and 11.07 min, respectively. The values for

Adamantyl-L-glycine and Adamantyl-L-alanine were calculated as 24.1 and 28.7. The hydrophobicity scale values used for Leu, Val, Orn, Pro, and DPhe were 9.7, 4.1, −9.0, −0.2 and 10, respectively.[36] To account for the two β-turns in the molecule the hydrophobic moment (μ) was calculated for each half of the molecule (e.g., for **2** OVOLO^{D}YP and OX$_1$OLO^{D}YP) and averaged out. A value of $\delta = 180°$ was used to define the angle of the backbone β-sheet.[20]

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Supplementary data

Supplementary data (HRMS, ^{1}H NMR, CD, haemolytic curves, correlation of retention time and hydrophobic moment are reported in the supporting information) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.09.018.

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