An Adamantyl Amino Acid Containing Gramicidin S Analogue with Broad Spectrum Antibacterial Activity and Reduced Hemolytic Activity

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An Adamantyl Amino Acid Containing Gramicidin S Analogue with Broad Spectrum Antibacterial Activity and Reduced Hemolytic Activity

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Abstract: The cyclic cationic antimicrobial peptide gramicidin S (GS) is an effective topical antibacterial agent that is toxic for human red blood cells (hemolysis). Herein, we present a series of amphiphilic derivatives of GS with either two or four positive charges and characteristics ranging between very polar and very hydrophobic. Screening of this series of peptide derivatives identified a compound that combines effective antibacterial activity with virtually no toxicity within the same concentration range. This peptide acts against both Gram-negative and Gram-positive bacteria, including several MRSA strains, and represents an interesting lead for the development of a broadly applicable antibiotic.

Keywords: amino acids · antibiotics · gramicidin · methicillin-resistant *Staphylococcus aureus* (MRSA) · peptides

Introduction

The discoveries of the antibiotic penicillin and sulfonamide at the beginning of the 20th century, and of their subsequent chemical derivatives, marked the birth of modern medicine.[1] The high toxicity that these early antibiotics exert towards bacterial cells, while at the same time being relatively nontoxic to human cells, explains their huge impact on

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human health. Both penicillin (bacterial cell-wall biosynthesis) and sulfonamide (folate biosynthesis) target physiological processes that are essential to bacterial growth but absent in man. Guided by these early examples, research into new antibiotic agents in the following decades aimed to target physiological processes unique to microorganisms.[2] This strategy has obvious advantages with respect to human toxicity; however, almost without exception, the currently used antibiotics provide pathogens with the opportunity to become resistant.[3] As a result, human health is threatened by a growing number of drug-resistant bacterial strains.[4] Outbreaks of the "hospital-bacterium" methicillin-resistant *Staphylococcus aureus* (MRSA) globally claim the lives of patients who often did not even suffer from infection at the time of hospital admission.[5] Vancomycin, the antibiotic of choice in the treatment of people with MRSA infections, has become less effective because of the emergence of vancomycin-resistant bacteria, and it is clear that restrained application of the currently available antibiotics will not solve the resistance problem.[6] Alternative strategies need to be pursued to arrive at antibiotics that are effective against drug-resistant strains and less prone to elicit new resistance.[7]

An interesting lead in this respect is the cyclic amphiphilic peptide gramicidin S (GS, *cyclo*-(D-Phe-Pro-Val-Orn-Leu)$_2$ (Orn: ornithine), **1**, Scheme 1).[8] GS belongs to the cationic antimicrobial peptides, a large compound family employed by various organisms as a defense mechanism against foreign invaders. GS effectively kills Gram-positive bacteria but is less active against Gram-negative bacteria.

The peptide does not target a specific bacterial gene product but acts by disrupting the bacterial cell membrane, which leads to leakage and cell death. The drawback with GS is the occurrence of toxic effects, which are primarily caused by its indiscriminate disruption of both bacterial and mammalian lipid bilayers.

GS is therefore only topically applied, for instance, in the treatment of ear infections.[9] Thus, so far, no GS-resistant pathogenic strains have emerged, even though the compound has been used for several decades in the clinic. GS is C2 symmetric and adopts a cyclic β-hairpin conformation, in which the two D-phenylalanine–proline sequences have β-turn conformations and the C=O and NH groups of the valine and leucine residues of opposing β strands form intramolecular H-bonds.[10] The basic side chains of the ornithine residues in this structure are on the opposite side of the molecule to the hydrophobic side chains of the valine and leucine residues, which provides the molecule with its amphiphilic nature. The ability of GS to disrupt membranes is attributed to this amphiphilic structure.

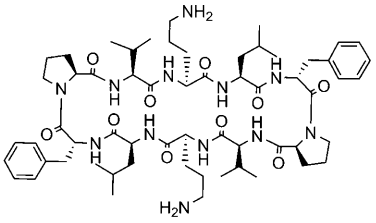
For several decades, GS has been the subject of extensive structure–activity relationship studies, in which amino acids throughout the molecule were systematically replaced with a variety of natural and unnatural residues.[11] It became clear that amino acid substitutions that disrupt the amphiphilic nature or structure of the cyclic hairpin are detrimental with respect to the biological activity. In addition, the biological activity is highly dependent on the hydrophobic nature of the amino acids replacing the valine and leucine residues.[12] Despite these and other specific design criteria, several interesting synthetic derivatives emerged from these studies. For instance, cyclic decapeptide derivatives were reported that contained four ornithine residues, possessed antibacterial activity, and retained structural characteristics similar to those of GS.[13] The latter molecules, however, were only evaluated for their antibacterial activity or used as structural probes, and the relationship between hydrophobicity, number of positive charges, and toxicity was not reported. In our research in this area, we observed a general trend that GS derivatives with reduced hemolytic properties are also less antimicrobial, and we only found molecules with a marginally improved biological profile.[14]

Herein, we report the discovery of a compound that deviates from this trend. This compound (**10**, Scheme 1) has a markedly improved biological profile compared to that of GS, in that it is highly toxic to bacterial strains, including several MRSA strains, but is much less toxic to human cells.

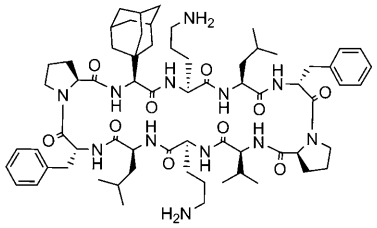
Results and Discussion

Design and synthesis of GS derivatives: The aim of the research described here is to correlate the amphiphilic characteristics of GS-based cyclic decapeptides with both hemolytic and antibacterial activity. Amino acid substitutions in each strand were introduced to obtain a series of amphiphilic derivatives with two or four positive charges and characteristics ranging between very polar and very hydrophobic.[15] To this end, we selected (*S*)-*tert*-butylglycine, (*S*)-cyclohexylglycine, and (*S*)-adamantylglycine as hydrophobic valine analogues and (*S*)-*tert*-butylalanine, (*S*)-cyclohexylalanine, and (*S*)-adamantylalanine as the corresponding leucine analogues, and we assembled GS derivatives in which one or both of these amino acids are introduced at the Val/Leu positions (Scheme 1). Furthermore, two highly charged derivatives that contain four ornithine residues (**9** and **10**) were prepared. Thus, in the nonsymmetric derivative **2**, one valine residue is replaced by an adamantylglycine residue; compound **3** is the corresponding C2-symmetric analogue. Similarly, derivatives **4** and **5** contain one and two adamantylalanine residues, respectively. Compound **6** contains four adamantyl amino acid residues, and compounds **7** and **8** can be viewed as its less hydrophobic counterparts. Compound **9** is an "inverted" GS derivative,[13a] in which the hydrophobic and hydrophilic residues were positionally exchanged. Compound **10** is the adamantane version of **9**.

Previous studies have shown that GS and its derivatives can be readily synthesized by solution-phase cyclization of the corresponding linear precursor peptides with protected ornithine side chains.[16] For this purpose, the linear precursors were assembled by following a solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) strategy. The efficiency of the cyclization reaction of ester-activated linear GS precursors is known to depend on their ability to preorganize into a β-

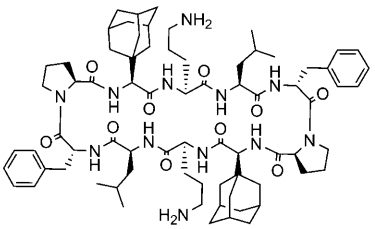


1



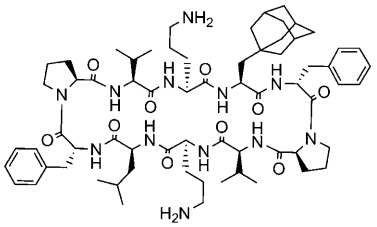
cyclo(DPhe-Pro-Val-Orn-Leu-DPhe-Pro-AdaGly-Orn-Leu)

2



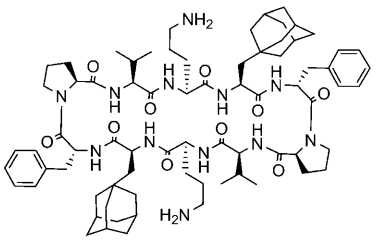
cyclo(DPhe-Pro-AdaGly-Orn-Leu)$_2$

3



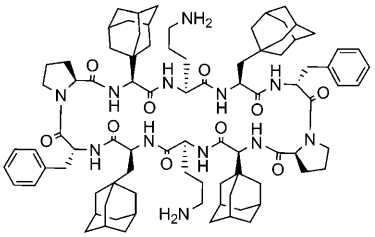
cyclo(DPhe-Pro-Val-Orn-Leu-DPhe-Pro-Val-Orn-AdaAla)

4



cyclo(DPhe-Pro-Val-Orn-AdaAla)$_2$

5



cyclo(DPhe-Pro-AdaGly-Orn-AdaAla)$_2$

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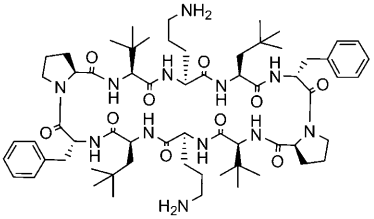
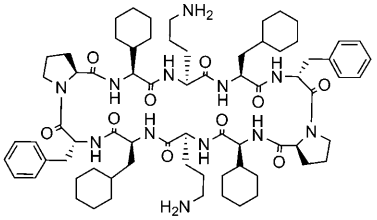


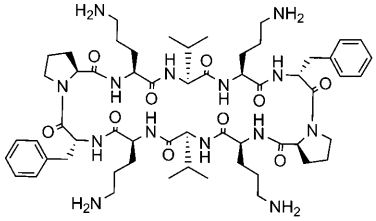
cyclo(DPhe-Pro-*t*BuGly-Orn-*t*BuAla)$_2$

7



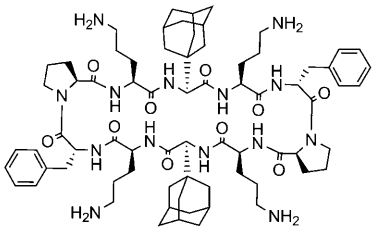
cyclo(DPhe-Pro-Chg-Orn-Cha)$_2$

8



cyclo(DPhe-Pro-Orn-Val-Orn)$_2$

9



cyclo(DPhe-Pro-Orn-AdaGly-Orn)$_2$

10

Scheme 1. Structural formulae of the natural product GS (**1**) and the synthetic derivatives **2**–**10** that are the subject of the present study.

hairpin conformation in solution.[17] This is facilitated by the presence of one β turn in the middle of the linear oligopeptide precursor. Most of the required suitably protected amino acid building blocks are commercially available. Fmoc-protected (*S*)-adamantyl-L-glycine and (*S*)-adamantyl-L-alanine were synthesized as described earlier.[18]

The projected decameric peptides were assembled stepwise on the solid support, with leucine (for peptides **2** and **3**), adamantylalanine (peptides **4**–**6**), *tert*-butylalanine (peptide **7**), cyclohexylalanine (peptide **8**), or ornithine (peptides **9** and **10**) being the first amino acid immobilized on the highly acid-labile resin. Elongation towards the immobilized decapeptides, cleavage from the resin, cyclization under high dilution, full deprotection, and purification of the peptides by using preparative HPLC proceeded uneventfully, and the target peptides were obtained as their trifluoroacetic acid (TFA) salts in high purity (>95%) and good yields (see the Supporting Information).

Structural analysis of GS derivatives: The cyclic decapeptides were analyzed by various 1D and 2D NMR techniques at 298 K in CD_3OH to evaluate the effect of the amino acid

replacements on the cyclic-hairpin secondary structure (Figure 1). The four signals in the amide region of the ^{1}H spectra of peptides **3** and **5–10** indicated that the molecules

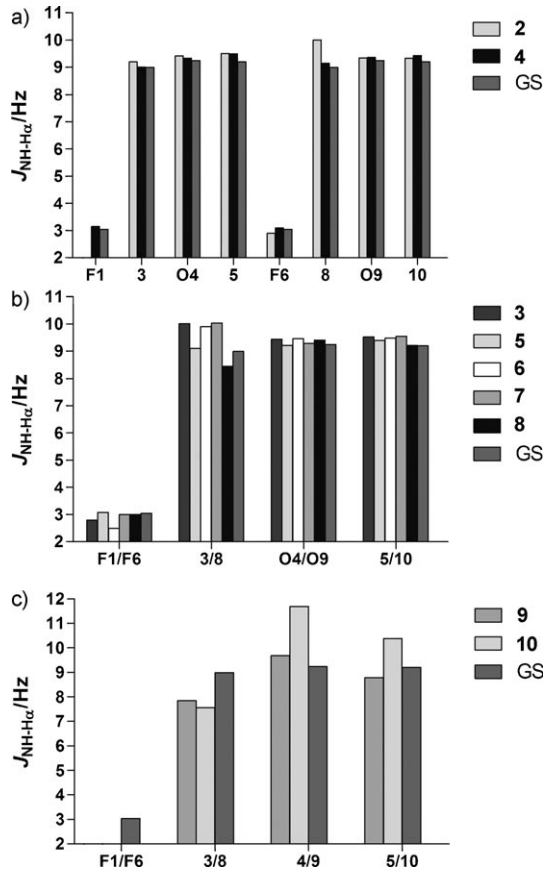


Figure 1. Comparison of the $J_{NH-H\alpha}$ values of GS and its derivatives. Residue numbers are given on the *x* axis; F: Phe; O: Orn. a) Asymmetric peptides **2** and **4**. For **2**, no detectable $J_{NH-H\alpha}$ value was observed for F1. b) Symmetric peptides **3** and **5–8**. c) Inverted symmetric peptides **9** and **10**. No detectable $J_{NH-H\alpha}$ value was observed for either of the phenylalanine residues in both analogues.

possess a C2-symmetric secondary structure, as anticipated. Asymmetric peptides **2** and **4** have eight signals in the amide region. The $J_{NH-H\alpha}$ values of the ornithine, adamantylglycine, adamantylalanine, leucine, and valine residues are all between 8 and 12 Hz (Figure 1), which is a strong indication of a β-strand conformation.[19] The $J_{NH-H\alpha}$ values of the D-phenylalanine residues were in the range of 2–4 Hz, which is typical for an amino acid residue that partakes in a β turn. The NOE signals between the backbone-amide NH protons were similar to the corresponding NOE crosspeaks of GS. Thus, the NMR data of all peptides indicate a rigid cyclic β-hairpin secondary structure in methanol, as is observed for the parent compound GS. For analogues **3** and **7**, crystals suitable for X-ray analysis were obtained. In the crystal, both compounds adopt an amphiphilic cyclic β-hairpin structure (Figure 2), in complete agreement with the NMR data of these compounds in solution and closely resembling the X-ray crystal structure of GS.[10a,b]

We found that the cyclic β-hairpin conformation of peptide **10** (and also **9**) is dependent on the solvent. This is a surprising result because the cyclic β-hairpin conformations of this type of decapeptide normally give rigid structures.[20] This remarkable finding is illustrated in Figure 3, in which the CD spectra of GS and compound **10** at the same concentration in methanol and water are compared. The CD spectrum of GS, both in methanol and water, corresponds to its known cyclic β-hairpin secondary structure. Peptide **10** does not adopt a stable conformation in water, whereas a strong CD effect is observed in methanol to indicate the presence of a well-defined secondary structure (Figure 3).

Determination of antibacterial and hemolytic activity: The antibacterial activities of GS and analogues **2–10** against a variety of Gram-negative and Gram-positive bacteria are shown in Table 1. GS shows, as expected,[21] strong activity against Gram-positive bacteria and, to a lesser extent, Gram-negative bacteria. Also, it is very effective against the different MRSA strains. Compounds **2** and **4** are as active as GS against Gram-positive bacteria and less active against Gram-negative bacteria, but **3** and **5** show less activity against all of the bacteria assayed. Peptide **6** is completely inactive. Peptide **7** shows activity comparable to that of GS against Gram-positive bacteria and performs well against the MRSA strains. It is slightly active against the Gram-negative bacteria. Peptide **8** is less active against all bacteria. The "inverted" compound **9**, with four positive charges, does not display any inhibition of bacterial growth, but the adamantane variant **10** is as active as GS towards Gram-positive bacteria. Furthermore, compared to GS, this peptide shows an increased activity against the panel of Gram-negative bacteria. The most active compounds **7** and **10** (with **9** as a control) were also screened for their ability to kill several MRSA strains. Compounds **7** and **10** are as active in killing the MRSA bacteria as GS.

Next, the ability of peptides **2–10** to lyze human red blood cells was determined. The observed hemolytic activity of GS is in agreement with the data as reported in the literature (Figure 4).[21] Peptides **2** and **4–8** are more hemolytic than GS, as can be judged from the lower concentrations needed to effect 50% hemolysis (Table 2). Peptide **3** did not dissolve well in the buffers used for the assay and therefore no reliable measurements could be performed with this analogue. Peptide **9** is not toxic towards human red blood cells at the tested concentrations. Importantly, the most antibacterial-active peptide, **10**, also shows reduced hemolytic properties relative to those of GS.

Estimation of the amphiphilic characteristics: To evaluate the amphiphilic characteristics of peptides **2–10** relative to those of GS (logP = 2.51),[22] reversed-phase liquid chromatography was used under controlled conditions (Figure 5).[23] By using this empiric method, the cyclic amphiphilic peptides with the shortest retention times are termed hydrophil-

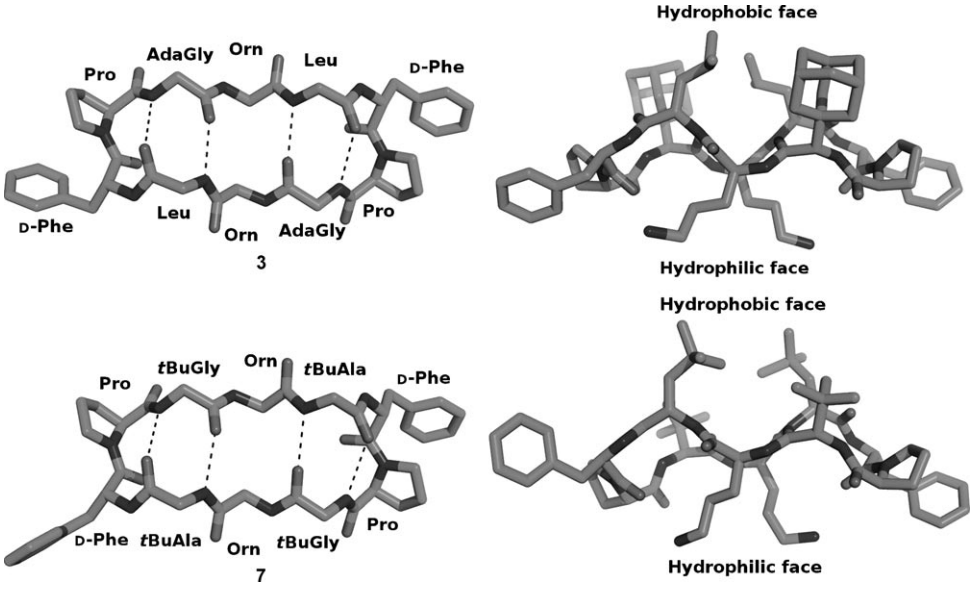


Figure 2. The crystal structures of peptides **3** and **7**. a) Left: top view of **3**. Right: side view of **3**. b) Left: top view of **7**. Right: side view of **7**. In the top views, the side chains are partly omitted for clarity. Dotted lines indicate hydrogen bonds stabilizing the secondary structure. For a color version of the figure, see the Supporting Information.

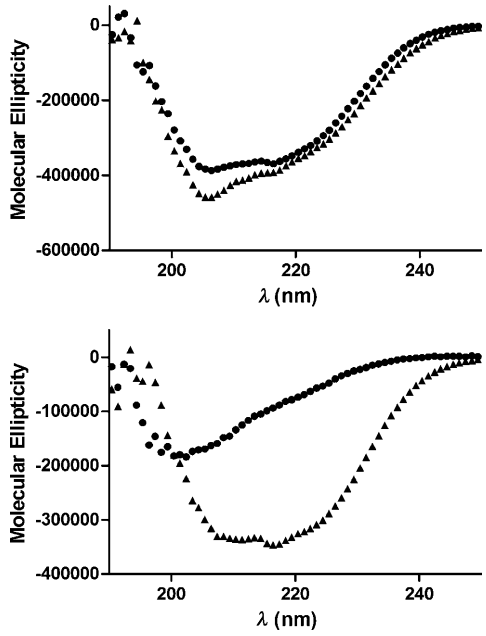


Figure 3. CD spectra of compound **10** (bottom) and GS (top), measured in water (●) and methanol (▲).

ic and those with the longest retention times are termed hydrophobic. In this series, peptides that are more hydrophobic than GS generally combine reduced antibacterial activity with increased hemolytic activity (Figure 5). Peptide **6** is an extreme example of this phenomenon; it very potently lyzes red blood cells but is inactive against bacteria. At the other extreme is the "inverted" GS derivative **9**,[13a] the most hydrophilic compound (with four positively charged amine groups) in this series, which is completely inactive against both bacterial and mammalian cells (Figure 5). Compound **10** (the adamantane version of **9**) is also somewhat less hydrophobic than GS. Compound **10** has an improved biological profile. It is as active as GS against Gram-positive bacteria, including four MRSA strains, and more active against certain Gram-negative species. Remarkably, compound **10** is much less hemolytic than GS: it is not hemolytic in the low micromolar range, the concentration at which it potently kills bacteria. This corresponds to results published earlier, in which inverted analogues of the compound GS10 (*cyclo*-(D-Tyr-Pro-Val-Lys-Leu)$_2$) also displayed decreased retention times and reduced hemolytic activity.[11l] From Table 1, it follows that a concentration of 16 μg mL^{-1} of compound **10** effectively kills all of the screened bacteria (except for P. mirabilis). This corresponds to 9 μM, a concentration at which compound **10** is not hemolytic (see Figure 4).

Conclusion

We prepared a series of hydrophobic and hydrophilic GS analogues with two or four positive charges. The structural characteristics were evaluated by using NMR spectroscopy, X-ray crystallography, and CD spectroscopy. There appears to be an optimal distribution between charge and hydrophobicity in this series of peptides with respect to their biological profile. This finding is corroborated by results obtained with related cyclic peptides.[11n,24] In addition, the compact and hydrophobic nature of the incorporated adamantane moiety may be an important factor in the interaction of these GS analogues with biomembranes. Peptide **10** emerges as the most promising compound because of its ability to distinguish, at a specific concentration, between bacteria and mammalian cells. Peptide **10** is also very active against four MRSA strains. It has been previously shown that a commercially available wound-care product containing gramicidin and polymyxin B is fairly effective against selected MRSA strains.[25] However, there are multiple members of the gramicidin family and it is not clear which one is used in this formulation or which component is actually killing the MRSA strains. Interestingly, the modified amphiphilic profile of peptide **10** not only affects its antibacterial and hemolytic activity but possibly its structural characteristics as

Table 1. Antimicrobial properties of synthesized GS and analogues **2–10** against Gram-positive, Gram-negative, and MRSA bacterial strains. The minimal inhibitory concentration (MIC) values are given in $\mu g\,mL^{-1}$ and were measured after 24 h of incubation.[a]

Compound	Gram+ *S. aureus*	Gram+ *S. epidermidis*	Gram+ *E. faecalis*	Gram+ *B. cereus*	Gram− *E. coli*	Gram− *P. aeruginosa*	Gram− *P. mirabilis*	Gram− *E. cloacae*	Gram− *K. pneumoniae*	MRSA-NT 1110301981H-T034-PVL+	MRSA-NT N133-T034-PVL−	MRSA-NT N229-T034-PVL−	MRSA-Cluster218 USA300-1110301146 PVL+
GS	8	4	8	8	32	64	>64	64	32	8		16	8
2	8	4	8	8	>64	>64	>64	>64	>64	–	–	–	–
3	16	16	16	16	64	64	>64	>64	>64	–	–	–	–
4	8	4-8	8	4	>64	>64	>64	>64	>64	–	–	–	–
5	>64	16	16	64	>64	>64	>64	>64	>64	–	–	–	–
6	>64	>64	>64	>64	>64	>64	>64	>64	>64	–	–	–	–
7	4	4	4	4	64	32	>64	>64	>64	8	8	8	8
8	16	16	32	16	>64	>64	>64	>64	>64	–	–	–	–
9	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64		>64	>64
10	8	4	8	8	8	16	>64	8	16	8		8	8

[a] Full details and names of the bacterial strains can be found in the Experimental Section. –: not tested against the strain indicated. The error margin in each MIC determination is a factor of two. For instance, when one compound exhibits an MIC value of 8 $\mu g\,mL^{-1}$ and another displays an MIC value of 4 $\mu g\,mL^{-1}$, there is no significant difference in activity.

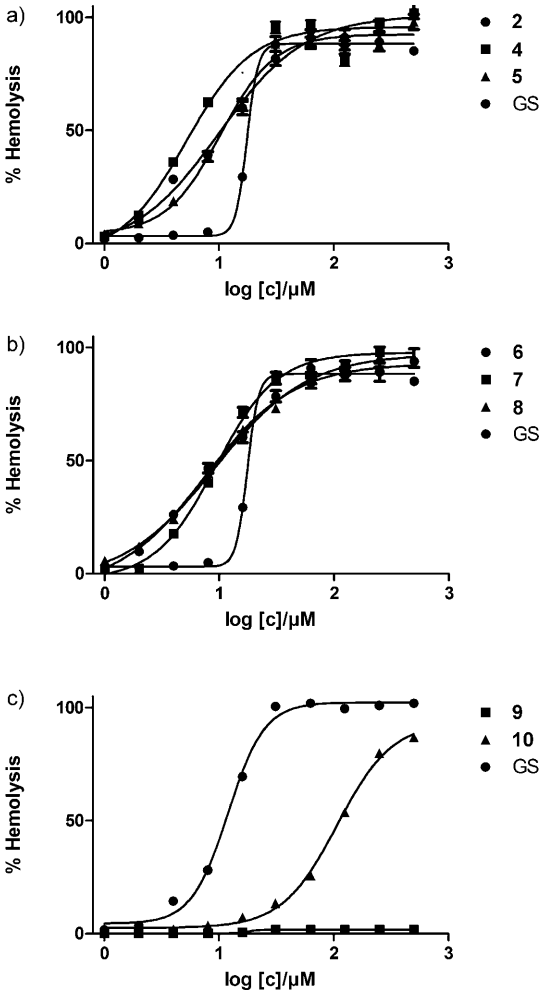


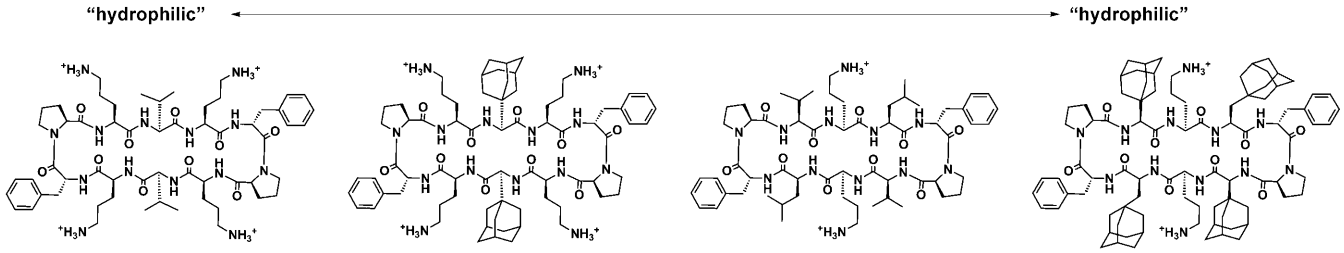
Figure 4. Hemolytic activity of GS analogues **2–10** on human red blood cells, fitted as logarithmic dose-response curves. Experiments were carried out in triplicate. a) Peptides **2–5** and GS. Compound **3** did not dissolve well in the buffers used in the assay and is therefore not included in the graph. b) Peptides **6–8** and GS. c) Inverted GS analogues **9** and **10** and GS.

Table 2. HC_{50} values of GS and peptides **2** and **4–10**.[a]

Compound	HC_{50} [μM]
GS	17.5 ± 1.07
2	10.1 ± 1.11
4	5.20 ± 1.13
5	10.3 ± 1.07
6	7.55 ± 1.10
7	9.30 ± 1.07
8	9.12 ± 1.13
9	n.o.
10	107 ± 1.07

[a] HC_{50}: concentration needed to effect 50% hemolysis; n.o.: not observed.

well, as indicated by the differences in CD spectra obtained when measured in water and methanol (see Figure 3). Whether these structural changes are representative of the peptide conformation in a membrane environment and



	9	10	GS	6
antibacterial:	no	yes	yes	no
hemolytic:	no	no	yes	yes

Figure 5. Upper panel: LC-MS retention times and the number of charges of GS and analogues **2**–**10**, ranked from least to most hydrophobic. The LC-MS spectra were recorded with a gradient of 10→90% MeCN in the presence of 0.1% TFA on a C18 column and with a run duration of 15 min. Lower panel: Overview of the antibacterial and hemolytic activities of compounds **6**, **9**, **10**, and GS with respect to the amphiphilic characteristics obtained from their LC-MS retention times.

whether they correlate to its biological activity remains the subject of further research.

Experimental Section

Antibacterial assays: The following bacterial strains were used: *Staphylococcus aureus* (ATCC 29213), *S. epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) *Proteus mirabilis* (1190901236), *Enterobacter cloacae* (1190900252), *Klebsiella pneumoniae* (1190901525), and the MRSA strains MRSA-NT 1110301981H-T034-PVL+, MRSA-NT N133-T034-PVL−, MRSA-NT N229-T034-PVL− (all cattle-related strains), and MRSA-Cluster218 USA300-1110301146 PVL+ (an MRSA strain well known in the USA). Bacteria were stored at −70 °C and grown at 35 °C on Columbia agar with sheep's blood (Oxoid, Wesel, Germany) suspended in physiological saline until an optical density of 0.1 AU (at 595 nm, 1 cm cuvette) was achieved. The suspension was diluted (100×) with physiological saline, and this inoculum (2 μL) was added to the growth medium (100 μL; cation-adjusted Mueller Hinton II broth, BBL ref. no. 212322, lot no. 7079753) in microtiter plates (96 wells). The peptides GS and **2**–**10** were dissolved in methanol (1 g L^{-1}) and twofold diluted in the broth (64, 32, 16, 8, 4, and 2 mg L^{-1}). The plates were incubated at 35 °C (24 h), and the MIC was determined as the lowest concentration inhibiting bacterial growth.

Hemolytic 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 (**2**–**10** and GS) were dissolved in a 30% dimethylsulfoxide/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, saline solution (100 μL) was dispensed in columns 1–11 of a microtiter plate, and 1% Triton solution (100 μL) was dispensed in column 12. The peptide (100 μL) was added to wells A1–C1 and mixed thoroughly. Aliquots (100 μL) from wells A1–C1 were dispensed into wells A2–C2. This process was repeated until wells A10–C10, and aliquots (100 μL) from wells A10–C10 were then discarded. These steps were repeated for the other peptides. Subsequently, the red blood cell suspension (50 μL) 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, the supernatant of each well (50 μL) was dispensed into a corresponding well. The absorbance at 405 nm was measured, and the percentage of hemolysis was determined.

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