Journal of Medicinal Chemistry

Perspective

Cite This: J. Med. Chem. 2019, 62, 7603−7617

pubs.acs.org/jmc

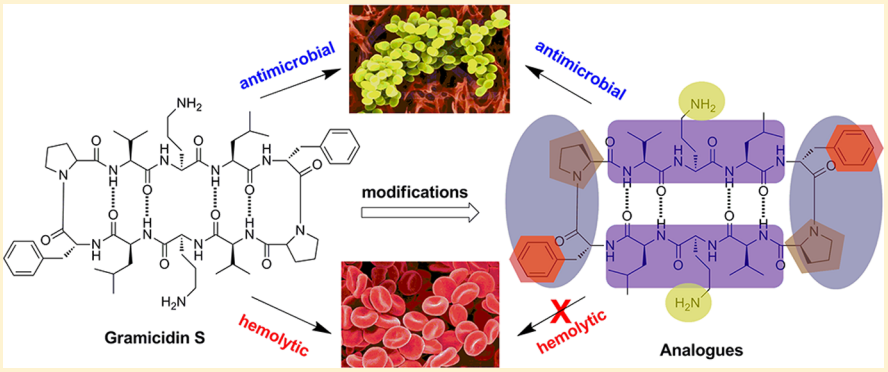
Recent Advances in the Exploration of Therapeutic Analogues of Gramicidin S, an Old but Still Potent Antimicrobial Peptide

Qinkun Guan,[†,∥] Shuhui Huang,[†,§,∥] Yi Jin,[†] Rémy Campagne,[‡] Valérie Alezra,*[,‡] and Yang Wan*[,†,‡]

[†]National Pharmaceutical Engineering Center for Solid Preparation in Chinese Herbal Medicine, Jiangxi University of Traditional Chinese Medicine, Nanchang 330006, P. R. China

[‡]Faculté des Sciences d'Orsay, Université Paris-Sud, Laboratoire de Méthodologie, Synthèse et Molécules Thérapeutiques, ICMMO, UMR 8182, CNRS, Université Paris-Saclay, Bât 410, 91405 Orsay, France

[§]Jiangxi Maternal and Child Hospital, Nanchang 330006, P. R. China



ABSTRACT: Gramicidin S (GS), one of the oldest commercially used peptide antibiotics, is known for its robust antibacterial activity against both Gram-positive and Gram-negative bacterial strains. Although it was discovered well over 70 years ago, its clinical potential was limited to topical applications because of its high hemolytic activity. To overcome this side effect, significant efforts have been invested in the chase for GS analogues with high therapeutic index (e.g., high antimicrobial activity and low hemolytic activity) in the past decades. In this Perspective, the structural properties and biological profiles (including the recently discovered activities) of representative GS analogues designed by different approaches are described and analyzed. We also present how the general structure−activity relationships were established and how they could help in the design of more efficient GS analogues.

INTRODUCTION

The antimicrobial peptide (AMP) gramicidin S (GS), isolated from *Bacillus brevis* in the Soviet Union in 1942,[1] is active against both Gram-positive and Gram-negative bacteria.[2] A number of attractive features have made GS and its analogues powerful antibiotic candidates for clinical use. However, because of its severe toxicity toward human red blood cells,[2] its therapeutic value has been strictly limited to topical application, where it is still in use today, for instance against external ear infections,[3] throat infections,[4] and root canal infections.[5] Since no bacterial resistance was observed after such a long time of use and in the context of the global rise of bacterial drug resistance with very few newly available antibiotics, pharmaceutical scientists have turned their attention to reinvestigate this old and topically used antibiotic peptide.

Since its discovery, significant efforts have been invested in the chase for GS analogues possessing high activity and low cytotoxicity. In this Perspective, a full picture of GS analogues will not be presented. Alternatively, more stress will be placed on those described after 1990 for several reasons. First, although hundreds of analogues have been studied with the goal of better understanding their structure−activity relationships and extend the activity of this peptide (see the reviews by Izumiya et al.,[6] Ovchinnikov and Ivanov,[7] and Pal et al.[8]), regrettably, no significant breakthroughs occurred in the first 50 years. Second, although the antimicrobial activity was studied in almost every work, the hemolytic activity was evaluated in only a few cases before then. Third, in 1996 the Hodges group for the first time described the ability of GS to kill Gram-negative bacteria in a liquid-based assay instead of the previously used solid-based assay.[2] The latter method had severely underestimated the therapeutic potential of this peptide and its analogues against Gram-negative bacteria and fungi. Nevertheless, some structural requirements accounting for the antibacterial activity were concluded from these studies, including β-sheet content, global hydrophobicity, and the presence of cationic groups.[6,7]

Received: January 25, 2019
Published: April 2, 2019

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603−7617

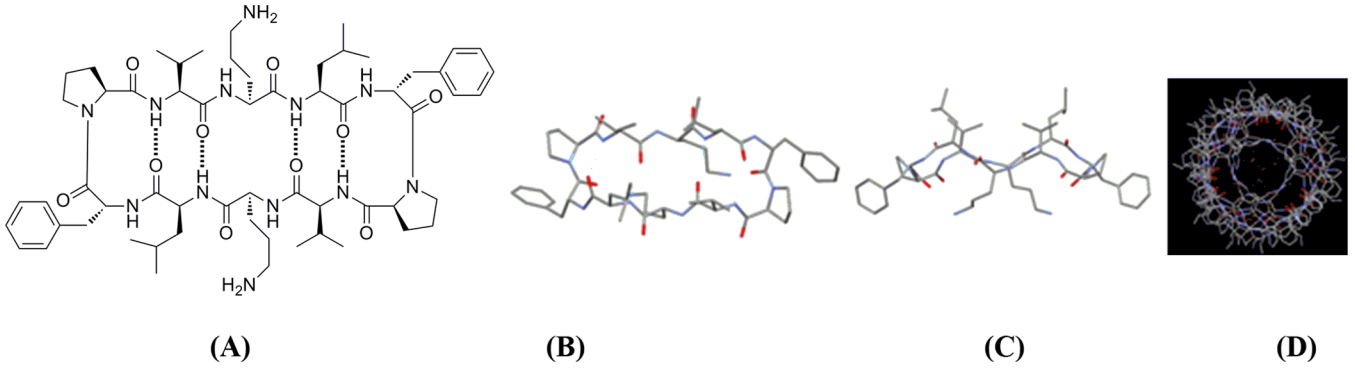


Figure 1. (A) Molecular structure of GS. (B) Top view of GS showing that the β-sheet structure is stabilized by four intramolecular H-bonds. (C) Side view demonstrating that the amphipathic feature of GS is induced by the opposite orientation of hydrophilic and hydrophobic side chains. (D) Molecular packing of GS along the pore axis.[10]

In this Perspective, we first discuss briefly the mechanism of action of GS, although it is not fully understood yet. Then a detailed introduction to recently developed GS analogues along with their structure−activity relationship studies will be given. In addition, some newly discovered therapeutic properties of GS and its analogues will be mentioned.

STRUCTURAL PROPERTIES AND PROPOSED MECHANISM OF ACTION

Structurally, GS is a cyclic decapeptide with the primary sequence *cyclo*-(VOL^{D}FP)$_2$ (where O stands for ornithine), which adopts a rigid C_2-symmetric β-hairpin structure constrained by four intramolecular H-bonds (Figure 1). The hydrophobic residues Val and Leu of both strands are placed on the opposite face from the two Orn cationic side chains, thus inducing strong amphiphilicity.[9,10] During several decades, GS has been the subject of extensive structure−activity relationship studies, in which the peptide sequence has been systematically substituted by a variety of natural and non-natural amino acids.[11−14] It has become clear that the antimicrobial activity of GS is a result of a combination of several physicochemical parameters: cationicity (often related to basicity), hydrophobicity, amphiphilicity, and hydrophobic aromaticity. Amphiphilicity results from segregated hydrophobic and hydrophilic domains.[15] For cyclic peptides, amphiphilicity is largely determined by the strength of the β-sheet character. Therefore, for two cyclic peptides with the same cationic residues, the one with higher β-sheet character will have a stronger amphiphilicity. For cationic AMPs, membrane-targeting specificity mainly relies on the intrinsic difference in cell membrane compositions.[16,17] Prokaryotic membranes usually contain a considerable proportion of negatively charged acidic phospholipids, whereas eukaryotic membranes are exclusively composed of zwitterionic phospholipids. In line with the majority of cationic AMPs, the positively charged residues of GS account for the initial binding to the prokaryotic cell membrane while the hydrophobic domain aids the further insertion into the lipid bilayer.[16] Nevertheless, as an amphiphilic peptide with strong global hydrophobicity, GS is also active against eukaryotic cell membranes as a result of hydrophobic interactions.

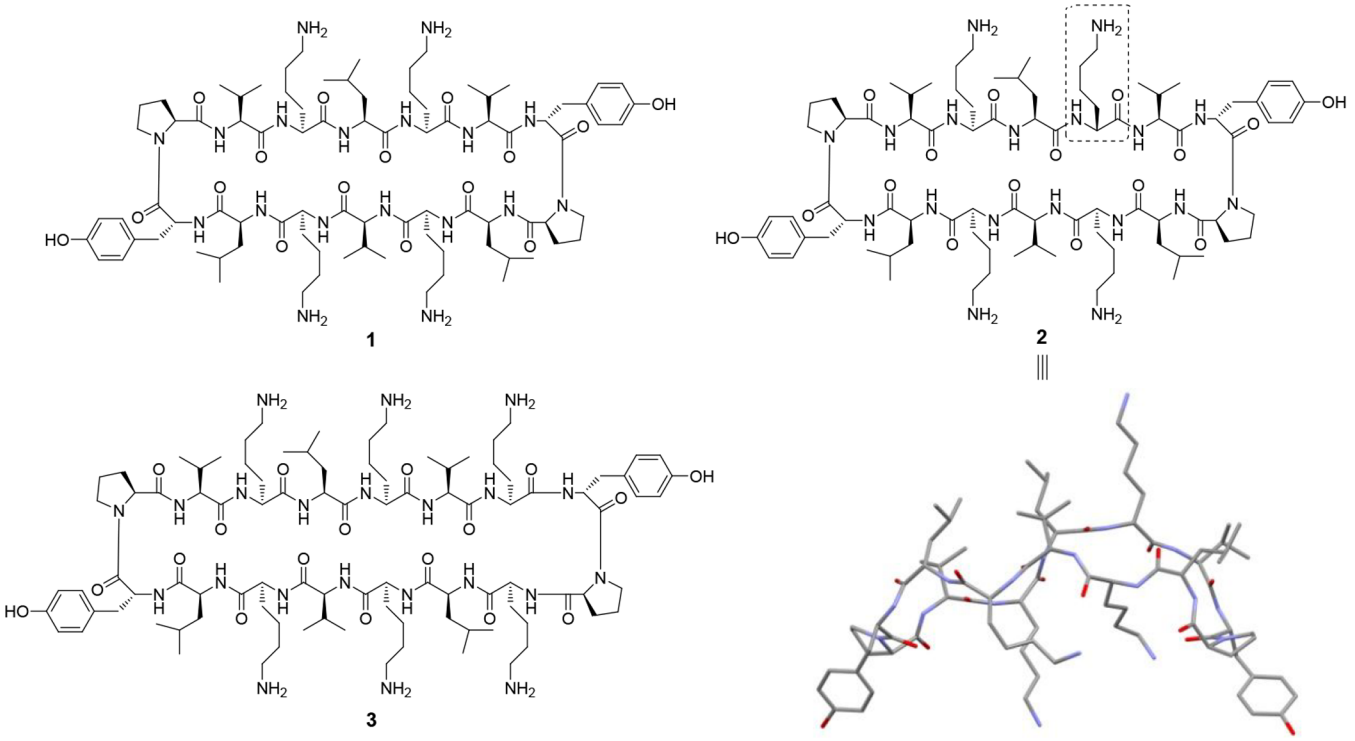
A long-held belief is that GS kills bacteria mainly, if not only, through disruption of the cytoplasmic membrane since the enantiomer of GS exerts essentially the same activity as GS. However, there is a heavy dispute about whether it forms discrete pores or destroys the membrane in a detergent-like manner. The early studies supported the former opinion, which is also the prevailing model of action for the majority of AMPs.[18,19] In contrast, more recent studies have revealed that rather than the formation of well-defined ion-channels, GS induces transient defects to cause cytoplasmic release.[20−22] It is noteworthy that the peptide−membrane interaction behaviors are also associated with the structures of GS and its analogues, their self-association in solution, and the composition of the membrane.[23−25] Moreover, apart from the effect of membrane potential and fluidity, delocalization of the peripheral membrane proteins was also observed through in vivo studies.[22] One can speculate that the multifaceted antimicrobial mechanism encompassed by this peptide may largely explain why it is still a potent peptide antibiotic after more than 70 years of use and why bacterial resistance is barely observed. With this in mind, different strategies have been attempted to reduce the hemolytic side effect of GS while retaining its antibacterial activity: the ring size has been changed, the β-turn regions have been functionalized or replaced, the β-strands have been modified, and light-controllable "smart" analogues have been designed.

STRUCTURE−ACTIVITY RELATIONSHIP OF GRAMICIDIN S AND ITS ANALOGUES

Ring Size Variation. Although several analogues of GS with a different ring size were investigated before 1996, the impact of ring size upon β-sheet content, amphiphilicity, hydrophobicity, lipopolysaccharide (LPS, the main component of Gram-negative bacterial outer membrane) binding affinity, and ultimately antimicrobial activity was first examined systemically by the Hodges group.[26−31] In their initial study, a series of cyclic analogues with an even number of residues ranging from 4 to 14 were synthesized and evaluated.[26] Circular dichroism (CD) and aqueous NMR spectroscopy showed that peptides with rings containing 6, 10, and 14 residues exhibited well-defined β-sheet structures, whereas for those with rings containing 8 or 12 residues the β-sheet structure was largely disordered. Interestingly, peptides containing eight or fewer residues were completely inactive against both human and microbial membranes regardless of secondary-structure character, reflecting a minimum requirement for the overall hydrophilicity and hydrophobicity. In terms of outer membrane permeabilization (as determined by *N*-phenyl-1-naphthylamine uptake[26]), peptides containing 10 or more residues all showed a similar capacity to disrupt the *Escherichia coli* membrane as GS itself, whereas the others

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603−7617

Table 1. Structures and Biological Activities of Ring-Extended GS Analogues Developed by the Hodges Group



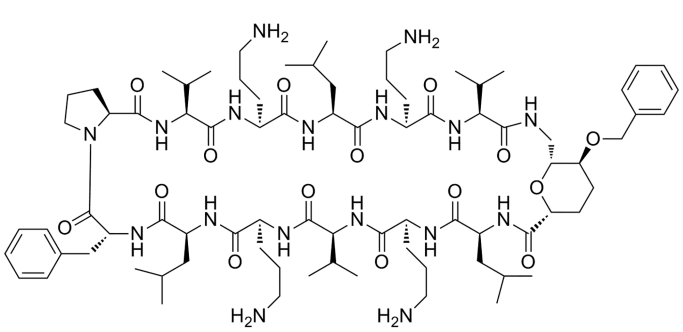
peptide	MIC (TI)[a] *E. coli* UB1005	*P. aeruginosa* H187	*E. faecalis* ATCC 29212	*S. aureus* K147	*C. albicans*[b]	hemolytic activity (μg/mL)[b]	LPS binding activity[c]	β-sheet content[d]
GS[26,e]	6.2[e] (2)	20[e] (0.6)	2.8[e] (4.5)	1.5[e] (8.3)	3.1[e] (4)	12.5[e]	295	+
1[26]	>200 (<0.01)	>200 (<0.01)	2.3 (0.7)	>200 (<0.01)	175 (0.01)	1.5	3	+
2[27,f]	6.2 (32)	25 (8)	1.5 (133)	100 (2)	6.2 (32)	200	93	+
3[28,g]	4 (>50)[g]	64 (>3.1)[g]	4 (>50)[g]	8 (>25)[g]	16[g]	>200[g]	nd[h]	−

[a]Minimal inhibitory concentration (MIC) values are given in μg/mL and unless stated otherwise were measured through a solution-based assay for each study. The therapeutic indexes (TIs) were calculated as 100% × hemolytic activity/antimicrobial activity. [b]Peptide concentration required for 100% lysis of erythrocytes. [c]Peptide concentration to displace 50% of dansyl-polymyxin B from LPS.[26,27,29] [d]Determined by CD spectroscopy, NMR spectroscopy, or crystallographic structure. "+" denotes positive, and "−" denotes negative. [e]Because of differences in experimental conditions and methods of calculation, the determined MICs and hemolytic activities of GS could be slightly different from study to study. [f]^{D}Lys is indicated by the dotted box. [g]The types of tested strains were not stated in detail, so the true MICs may be slightly different. The peptide concentration required for 100% lysis of human erythrocytes was not given either. Instead, they reported that peptide **3** caused 12.7% hemolysis at 100 μg/mL (100% for GS at the same concentration), and we therefore believe that the peptide concentration required for 100% lysis should more than 200 μg/mL.[28] [h]Not determined.

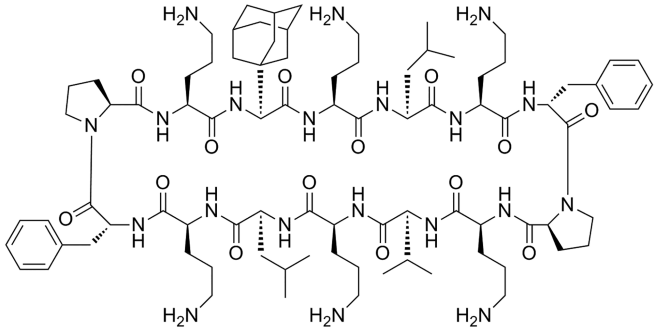
showed a diminished capacity to disrupt membranes. Significantly, the 14-residue peptide *cyclo*-(VKLKV^{D}YPLKVKL^{D}YP) (**1**), with four positive charges and six hydrophobic residues (Table 1), displayed the highest LPS binding affinity but barely any antibacterial activity against both Gram-positive and Gram-negative bacteria. In contrast, it exhibited very strong hemolytic activity. Presumably, the strong affinity between the strong hydrophilic domain of peptide **1** and negatively charged LPS from the Gram-negative bacterial outer membrane or teichoic acid from Gram-positive bacterial peptidoglycans prevent peptide accumulation in the cytoplasmic membrane and further movement into the lipid bilayer. Meanwhile, the high hemolytic activity resulted from the segregated hydrophobic domain composed of three Val and three Leu residues. In this regard, they further synthesized 14 diastereomers of peptide **1**, each containing a single different enantiomeric substitution.[27] Since each diastereomer contained the same hydrophilic and hydrophobic groups, the biological activity was cleverly associated with amphiphilicity, which can be evaluated by the retention time in reversed-phase high-performance liquid chromatography (RP-HPLC) as an empirical method. Impressively, the least amphiphilic peptide, *cyclo*-(VKL^{D}KV^{D}YPLKVKL^{D}YP) (**2**), whose crystallographic structure was lately reported by Knijnenburg et al.;[32] Table 1), had the longest retention time and exhibited 130-fold less hemolytic activity and greatly increased antimicrobial activity compared with peptide **1**. Compared with GS, peptide **2** displayed 16- to 32-fold increased therapeutic indexes (TIs) for Gram-negative bacteria. Obviously, the cationic ^{D}Lys residue positioned in the hydrophobic domain perturbed the peptide insertion in eukaryotic cell membranes while the "less concentrated" hydrophilic domain, with a weaker LPS binding affinity (lower electrostatic interaction), favored the peptide accumulation on prokaryotic cells. In their more recent work, the 16-residue peptide *cyclo*-(VKLKVK^{D}YPKLKVKL^{D}YP) (**3**) (Table 1) displayed comparable antimicrobial activity and much reduced hemolytic activity relative to GS, albeit holding six positive charges and six hydrophilic groups in the β-strand

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603−7617

Table 2. Structures and Biological Activities of Ring-Extended GS Analogues Developed by the Overhand Group



4

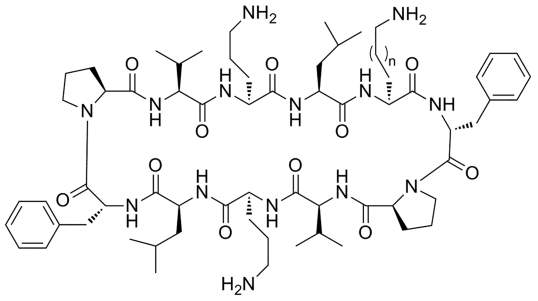


5

	MIC[a] (TI)					
peptide	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*E. faecalis* ATCC 29212	*S. aureus* ATCC 29213	MRSA ATCC 4330	hemolytic activity (μg/mL)
GS[34]	32 (2)	64 (1)	8 (7.8)	4 (15.6)	8 (7.8)	62.5
4[34]	16 (3.9)	8 (7.8)	8 (7.8)	4 (15.6)	nd	62.5
5[35]	8 (15.6)	16 (7.8)	32 (3.9)	16 (7.8)	8 (15.6)	125

[a]In μg/mL.

Table 3. Structures and Biological Activities of Cycloundecapeptides Developed by the Tamaki Group



6a n = 1
6b n = 2

	MIC (μg/mL)					
peptide	*E. coli* NBRC 12734	*P. aeruginosa* NBRC 3080	*B. subtilis* NBRC 3513	*S. aureus* NBRC 12732	*B. megaterium* ATCC 19213	HC_{50} (μg/mL)[a]
GS[38]	25	25	3.13	3.13	3.13	25–35[a]
6a[38]	25	100	6.25	3.13	3.13	90–100[a]
6b[38]	25	100	3.13	3.13	3.13	>100[a]

[a]HC_{50}: peptide concentration required for 50% lysis of erythrocytes. As the metadata were not stated in detail, the HC_{50} values were calculated according to the provided peptide concentration–hemolysis curves.

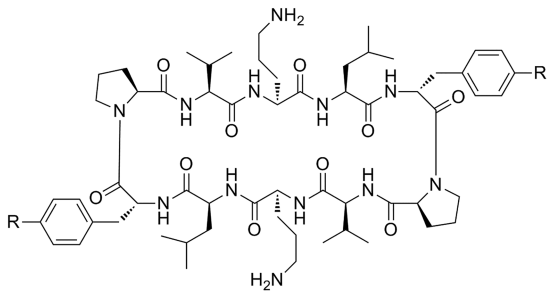
region.[28] This could be largely explained by its disordered β-sheet content, consequently leading to a weakly amphiphilic global structure.

The Overhand group also contributed to the exploration of highly potent ring-extended GS analogues.[32−37] Both peptides **4** and **5** (Table 2) were designed on the basis of the aforementioned 14-residue structural framework (peptide **1**). Interestingly, unlike peptide **1**, both of them exhibited 2- to 8-fold higher TIs against a wide range of strains (including methicillin-resistant *Staphylococcus aureus*, MRSA) compared with GS. For peptide **4**, the improved antimicrobial activity and slightly reduced hemolytic activity could be attributed to the slight distortion of the cyclic β-hairpin structure, resulting from the mimicking of the β-turn by a *cis*-monobenzyloxypyranoid sugar amino acid (also see β-Turn Region Modifications). Consequently, it showed less amphiphilicity and improved TIs.[34] In terms of the β-strand, for "inverted" peptide **5** (in which the proportions of hydrophilic and hydrophobic residues are reversed; also see β-Strand Region Modifications), which contains six positive charges and four hydrophobic residues (including one adamantane moiety), the reduced hemolytic activity could largely be explained by the increased number of positive charges and decreased global hydrophobicity. However, the highly hydrophobic adamantane moiety effectively compensates for the loss of hydrophobic groups and promotes penetration of the peptide into the prokaryotic lipid bilayer.[35]

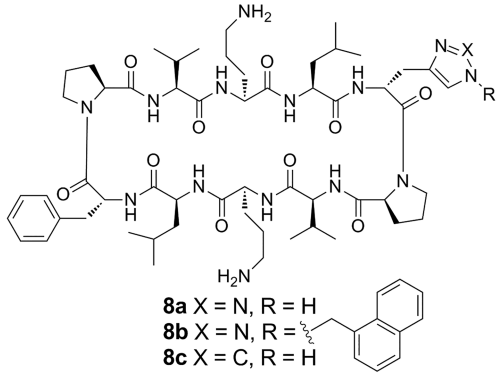
The Tamaki group developed a number of cycloundecapeptides by inserting one additional α-amino acid.[38,39] The best peptides were derived from the incorporation of one cationic residue between the Leu and ^{D}Phe residues (Table 3).[38] Both peptides contained an additional basic amino residue, either Orn (peptide **6a**) or Lys (peptide **6b**), and exerted largely decreased hemolytic activities while having antibacterial

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603−7617

Table 4. Structures and Biological Activities of ^{D}Phe-Residue-Modified GS Analogues Developed by the Overhand Group



7a R = -OH
7b R = -OBn
7c R = -NHCOtBu

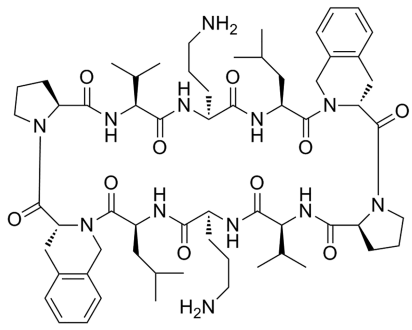


8a X = N, R = H
8b X = N, R =
8c X = C, R = H

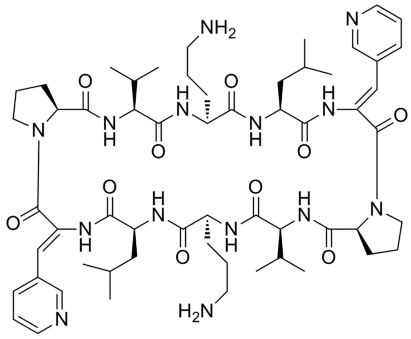
peptide	MIC (μg/mL) *E. coli* ATCC 25922	*P. aeruginosa* ATCC 19582	*E. faecalis* 1131	*S. aureus* 7323	HC_{50} (μg/mL)[a]
GS[43]	32	8	8	8	5–15[a]
7a[45]	64	nd	32[b]	32[c]	nd
7b[45]	64	nd	8[b]	8[c]	nd
7c[46]	32	64	16	8	20–30[a]
8a[43]	32	>64	64	16	45–55[a]
8b[43]	64	>64	4	4	5–10[a]
8c[43]	8	16	16	4	90–110[a]

[a]HC_{50}: peptide concentration required for 50% lysis of erythrocytes. As the metadata were not stated in detail, the HC_{50} values were calculated according to the provided peptide concentration–hemolysis curves. [b]*E. faecalis* ATCC 29212 was tested. [c]*S. aureus* ATCC 29213 was tested.

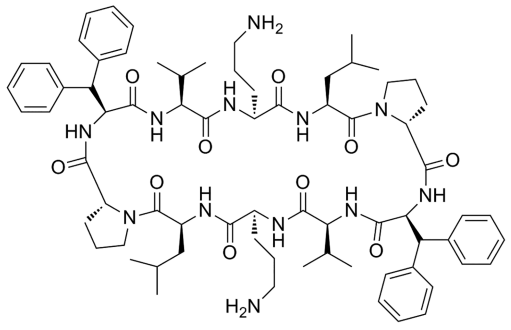
Table 5. Structures and Biological Activities of GS Analogues with a Modified β-Turn Region



9



10



11

peptide	MIC_{50} (TI)[a] *S. aureus* CECT 240	*L. monocytogennes* CECT 4032	*A. baumanni* ATCC 19606	*A. baumanni* 19606R	*E. coli* K12 strain W3110	HC_{50} (μg/mL)
GS[12]	7.9 (2.7)	6.7 (3.1)	10.1 (2.1)	13.1 (1.6)	–	21.1
9[12]	4.9 (>10.2)	8.3 (>6)	>40 (nd)	>40 (nd)	–	>50
10[48]	8[b]	–	–	–	32	>100
11[49]	3.8 (8.9)	3.4 (10.0)	20.3 (1.7)	23.9 (1.4)	–	34

[a]MIC_{50} (in μg/mL) is the peptide concentration required for 50% inhibition of bacterial growth. The TI values (in parentheses) were calculated as HC_{50}/MIC_{50}. [b]*S. aureus* 209P.

activities comparable to that of GS. Extensive NMR studies indicated a novel turn structure around the X-^{D}Phe-Pro sequence with a cis ^{D}Phe–Pro peptide bond. Presumably, the reduced human cell cytotoxicity could be attributed to the existence of extra positive charges and the slightly modified β-hairpin structure.

To summarize, a number of studies have demonstrated that it is possible to modulate the structure and activity of GS analogues by extending their ring size.[27,28] Nevertheless, a simple enlargement of the GS cyclic backbone, with segregated and enlarged hydrophilic and hydrophobic domains, is not desirable for a rational design because a large hydrophobic domain often leads to strong hemolysis while a large hydrophilic domain may result in strong LPS binding affinity and thereby impede the peptide penetration.[26] Therefore, the principle of finding a subtle balance between amphiphilicity and overall hydrophobicity must be respected.

β-Turn Region Modifications. Compared with the well-explored ring-size variation approach, the strategy of β-turn region modifications was investigated only recently but seems

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603–7617

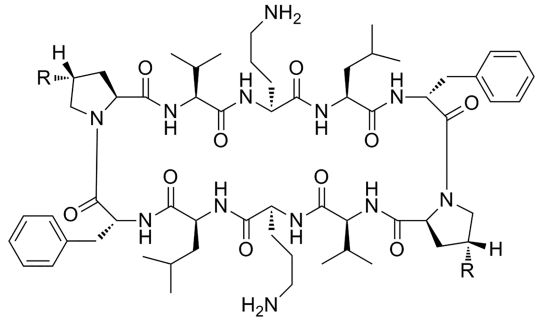
interesting as well. Extensive studies have shown that seemingly subtle changes in the β-turn region could have a dramatic impact on the antimicrobial activity, suggesting that this region may be a hot spot for modulating GS structure and activity. Interestingly, the effect of these modifications on the antimicrobial activity is often not correlated with the cytotoxicity.[12,40−44] Two general methods have been adopted for β-turn region modifications: one involves the modification of ^{D}Phe and/or Pro residues, while the other involves replacement of the entire ^{D}Phe-Pro sequence with dipeptide surrogates.

A. α-Amino Acid Modifications. The Overhand group reported a series of GS analogues in which ^{D}Phe residues were replaced by other natural or unnatural α-amino acid residues (Table 4) .[43,45,46] In accordance with the results of the Hodges group,[2] replacing ^{D}Phe residues with ^{D}Tyr residues led to the loss of almost all antimicrobial activity (peptide **7a**). Impressively, after benzylation of the hydroxyl group or introduction of a hydrophobic moiety,[45,46] the antimicrobial activities of peptides **7b** and **7c**, respectively, were dramatically improved up to that of GS itself, strongly suggesting the need for hydrophobic aromaticity in the β-turn region. Similar findings were obtained in another study from them[43] in which the structure and biological activity of GS analogues were investigated after replacement of one ^{D}Phe residue by substituted and unsubstituted azoles. The unsubstituted triazole peptide **8a** was less potent than naphthylmethyl-substituted **8b**, emphasizing again the importance of hydrophobic aromaticity in this region. Nevertheless, the general trend for the triazole peptide series is that the toxicity toward human erythrocytes is directly correlated to the antimicrobial activity. Given the trend observed for the triazole peptide series, the histidine peptide **8c** exhibited rather promising cytotoxicity specificity. Compared with wild GS, this peptide exhibited much lower hemolytic activity and slightly superior antimicrobial activity against both Gram-positive and Gram-negative bacteria (particularly *E. coli*). The authors claimed that the increased TIs of histidine peptide **8c** could be partly explained by the higher basicity of the imidazole ring ($pK_a \approx 7.0$) compared with the 1*H*-triazole ring of peptide **8a** ($pK_a \approx 1.2$).[43]

Cativiela et al. synthesized and evaluated a panel of analogues with both ^{D}Phe residues replaced, with the aim to understand how the size and conformational mobility of the aromatic moiety affect the structure and biological activity of GS (Table 5).[12] The most potent peptide, **9**, incorporating D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (D-Tic), showed a modest antibacterial activity but a significant increase in therapeutic index. After extensive conformational analysis, the increased biological profile of peptide **9** could be attributed to the relatively close proximity between the D-Tic aromatic ring and the Orn δ-amino group due to the constrained aromatic bicycle. A plausible explanation for the benefits of this orientation is the existence of cation−π interactions between the aromatic ring of D-Tic and the δ-NH_3^+ of the Orn side chain favoring penetration of the peptide into the cell membrane. A similar study was made by Yamada et al., who synthesized a series of dehydropeptides with preserved β-sheet character.[47,48] The most promising peptide, **10** (Table 5), combines an antimicrobial activity comparable to that of wild-type GS with a considerably reduced hemolytic activity. However, an in-depth discussion about the orientation of aromatic rings is absent. In another study, Cativiela et al. designed a series of GS analogues with an inverted β-turn sequence in which the relative chirality (D or L) at each position was preserved.[49] Among all of these analogues, peptide **11** was more active against Gram-positive bacteria and slightly less cytotoxic than GS (Table 5). These studies demonstrated that modest hydrophobicity and adequate orientation at the β-turn region are important to maintain antimicrobial activity, provided that a robust global β-sheet character is preserved.

In general, hydrophobic AMPs show low affinity toward the outer membrane of Gram-negative bacteria. That is why GS exhibited relatively lower activity against Gram-negative bacteria compared with Gram-positive ones.[50] Inspired by Polymyxin B, a cyclic antibiotic peptide with five positive charges that is strongly active against Gram-negative bacteria, Kawai et al. designed a series of polycationic peptides with aminated Pro residues (Table 6).[51] Although peptide **12a** had limited antimicrobial activity against both Gram-positive and Gram-negative bacteria, it exhibited outstanding outer membrane permeability (as determined by hydrophobic tetraphenylphosphonium ion uptake[51]), which was reminiscent of the 14-residue peptide **1**. Intriguingly, after further functionalization with a Phe or Lys residue, peptides **12b** and **12c**, respectively, both displayed low cytotoxicity and increased antimicrobial activity (Table 6).[40] Homologous findings also appeared in a study reported by Tamaki et al.[52] These studies showed that a moderate introduction of basic groups on Pro may effectively lower the hemolytic activity while maintaining the antimicrobial activity.

Table 6. Structures and Biological Activities of GS Analogues with a Modified β-Turn Region



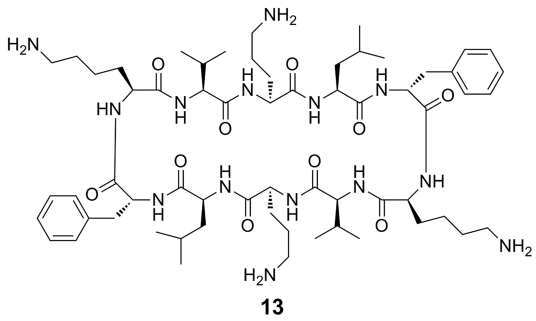
12a R = NH_2
12b R = L-Phe-NH
12c R = L-Lys-NH

peptide	MIC (μg/mL) *E. coli* K12 strain W3110	MIC (μg/mL) *S. aureus* 209P	HC_{50} (μg/mL)
GS[40]	32	4	5−10
12a[51]	64	32	50−100
12b[40]	16	8	20−50
12c[40]	16	8	>100

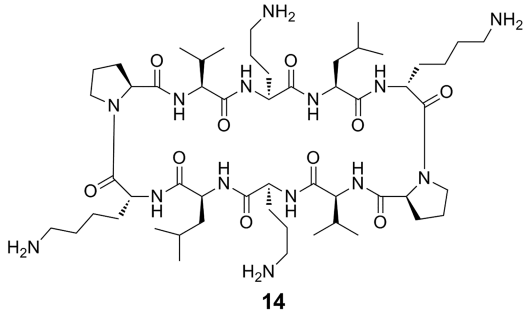
The Tamaki group developed a panel of analogues with the ^{D}Phe or Pro residues substituted by basic residues (Table 7).[44] Like the already-described peptides **12b** and **12c**, peptide **13** with both Pro residues replaced by Lys residues exhibited high antibacterial activity and very low hemolytic activity. Surprisingly, another peptide, **14**, in which both ^{D}Phe residues were substituted by ^{D}Lys residues, also displayed high cytotoxicity specificity. Since there is a long-held belief that

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603−7617

Table 7. Structures and Biological Activities of ^{D}Phe- or Pro-Residue-Substituted GS Analogues Developed by the Tamaki Group



13

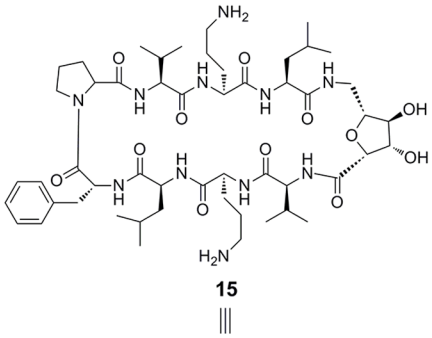


14

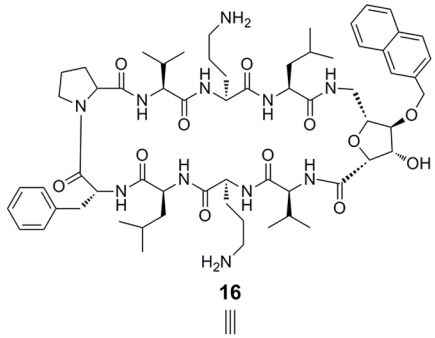
	MIC (μg/mL)					
peptide	*E. coli* NBRC 12734	*P. aeruginosa* NBRC 3080	*B. subtilis* NBRC 3513	*S. aureus* NBRC 12732	*B. megaterium* ATCC 19213	HC_{50} (μg/mL)[a]
GS[44]	25	25	3.13	3.13	3.13	25–35[a]
13[44]	25	50	6.25	3.13	3.13	>200[a]
14[44]	12.5	12.5	6.25	3.13	3.13	150–200[a]

[a]As the metadata were not stated in detail, the HC_{50} values were calculated according to the provided peptide concentration–hemolysis curves.

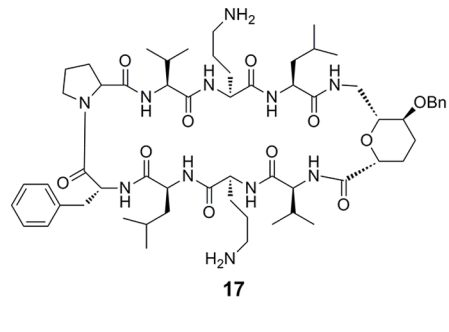
Table 8. Structures and Biological Activities of GS Analogues with a Modified β-Turn Region Developed by the Overhand Group



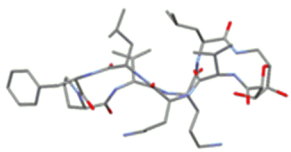
15

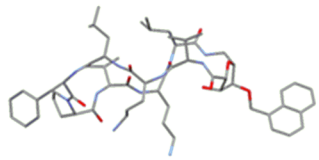


16



17





	MIC (μg/mL)						
peptide	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*E. faecalis* ATCC 29212	*S. aureus* ATCC 29213	*S. epidermidis* ATCC 12228	*B. cereus* ATCC 11778	hemolytic activity (μg/mL)
GS[42]	32	64	8	8	8	8	31.2
15[54]	nd	nd	nd	nd	nd	nd	nd
16[13]	>64	>64	8	4–8	2	4	10–20
17[42]	32	>64	16	16	8	8	500

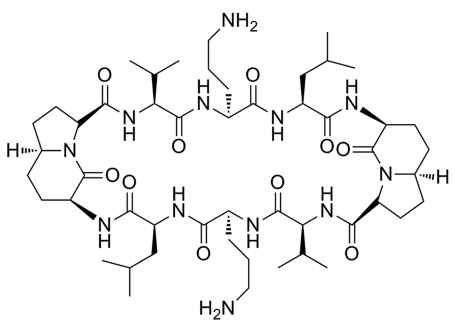
hydrophobic aromaticity is critical to keep the antimicrobial activity,[2,20,45,46] these findings may illustrate a novel possibility to access GS analogues with increased therapeutic index.

B. Replacement with Dipeptide Surrogates. Minor changes solely on ^{D}Phe or Pro residues usually modulate the structural features and biological activities of GS analogues without perturbing the β-sheet character. Alternatively, the β-turn region could be successfully replaced by dipeptide surrogates, provided that the mimics have an aromatic/hydrophobic character and an adequate geometry. In some cases, the antimicrobial and hemolytic activities of GS analogues were shown to be dissociated after the introduction of peptidomimetics.[42,53]

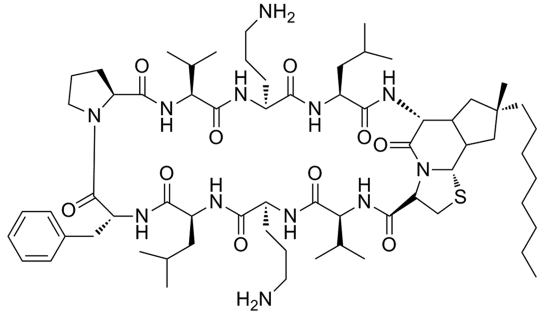
The Overhand group synthesized peptide **15** monosubstituted with a sugar amino acid (SAA) (Table 8), which adopted a bent β-sheet structure similar to that of GS but showed absolutely no activity (antimicrobial nor hemolytic).[54] After arylation of the hydroxyl group, peptide **16** exhibited dramatically improved antimicrobial activity.[13] The results are consistent with the previously mentioned series of ^{D}Phe-modified analogues. We can therefore conclude that the existence of aromaticity in the β-turn regions of GS analogues is essential for biological activity while a perfectly formed β-

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603–7617

Table 9. Structures and Biological Activities of GS Analogues with the β-Turn Region Modified



18

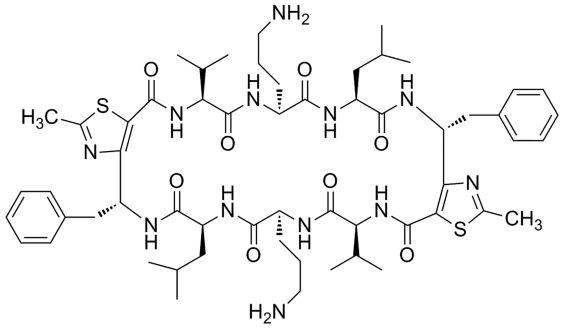


19

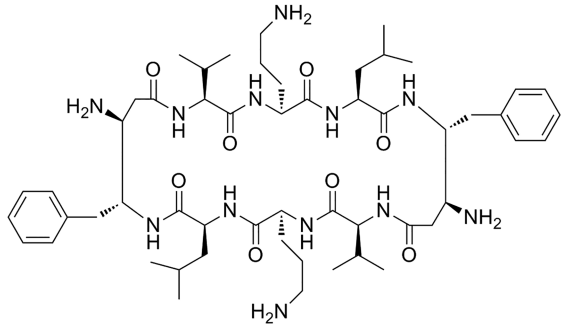
	MIC (TI)[a]					
peptide	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*E. faecalis* ATCC 29212	*S. aureus* ATCC 25923	*S. epidermidis* ATCC 12228	HC_{90} (μg/mL)[b]
GS[53]	32 (0.38)	64 (0.19)	8 (1.50)	4 (3.00)	2 (6.00)	12
18[55]	25[c] (nd)	12.5[d] (nd)	25[e] (nd)	12.5[f] (nd)	12.5[g] (nd)	400[h]
19[53]	64 (0.59)	128 (0.30)	8 (4.75)	4 (9.5)	4 (9.5)	38

[a]The MIC values are in μg/mL. The TI values (in parentheses) were calculated as HC_{90}/MIC. [b]HC_{90} is the peptide concentration required for 90% lysis of erythrocytes. [c]*E. coli* DC2. [d]*P. aeruginosa* H188. [e]*E. faecalis* C625. [f]*S. aureus* K147. [g]*S. epidermidis* C621. [h]The peptide concentration required for 100% lysis of erythrocytes in 4 h (100 μg/mL for GS).

Table 10. Structures and Biological Activities of GS Analogues with the β-Turn Region Replaced



20



21

	MIC (μg/mL)				
peptide	*E. coli* ATCC 8739	*P. aeruginosa* ATCC 9027	*S. aureus* ATCC 6538	*B. subtilis* CIP 52.65	% hemolysis at 100 μg/mL
GS[41]	12.5	50	3.125	1.56	80
20[41]	25	>50	4.68	3.125	14
21[56]	25	50	25	12.5	1

hairpin may not. In another study, they further explored the potential of SAA analogues with varying flexibility and ring size.[42] Compared with mother molecule **16**, the most potent peptide **17** largely maintained its antimicrobial activity while exerting a much lower hemolytic activity.

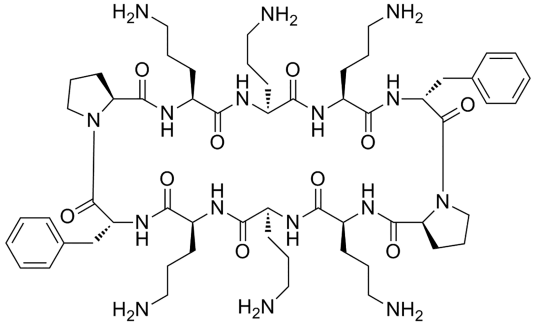
Roy et al. introduced indolizidin-2-one amino acids (I2aa's) possessing 6*S* and 6*R* ring-fusion stereochemistry into GS to explore the influence of the ring-fusion stereochemistry on the biological activity.[55] In terms of antibacterial and antifungal activity, (6*S*)-I2aa peptide **18** presented much higher potency than the (6*R*)-I2aa diastereomer (Table 9). Globally, peptide **18** displayed usually one-half to one-fourth of the antimicrobial activity as well as one-fourth of the hemolytic activity of GS. In another study, Priem et al. synthesized a series of GS analogues with a scaled amphiphilic character by introducing fatty chains with different lengths and topologies (Table 9).[53] In particular, peptide **19** showed similar antibacterial activities and reduced hemolytic activity compared with GS.

Legrand et al.[41] described a GS analogue with the β-turn region monosubstituted by heterocyclic γ-amino acids named ATCs (4-amino(methyl)-1,3-thiazole-5-carboxylic acids) (Table 10). Notably, when incorporated into α-peptides, ATCs have a tendency to form C_9 intramolecular turns. Compared with GS, peptide **20** exhibited lower hemolytic activity while maintaining an interesting antimicrobial activity. Wan et al. described a pair of GS analogues using β,γ-diamino acids as β-turn mimics (e.g., peptide **21** in Table 10).[56] Though the antibacterial activity was partly reduced, the strongly diminished hemolysis allows these analogues to be considered as promising structural frameworks for further studies. For both peptides **20** and **21**, the decrease in hemolytic activity could be partly explained by a higher hydrophilicity.

Investigations of GS analogues with a modified β-turn region have indicated that the hydrophobic aromaticity is critical for maintaining the antimicrobial activity,[13,45] with only a few

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603−7617

Table 11. Structure and Biological Activities of a GS Analogue Containing Six Basic Amino Acid Residues



22

	MIC (μg/mL)					
peptide	*E. coli* NBRC 12734	*P. aeruginosa* NBRC 3080	*B. subtilis* NBRC 3513	*S. aureus* NBRC 12732	*B. megaterium* ATCC 19213	HC_{50} (μg/mL)[a]
GS[59]	25	25	3.13	3.13	3.13	25−35[a]
22[59]	>100	>100	12.5	50	12.5	>200[a]

[a]As the metadata was not stated in detail, the HC_{50} values were calculated according to the provided peptide concentration−hemolysis curves.

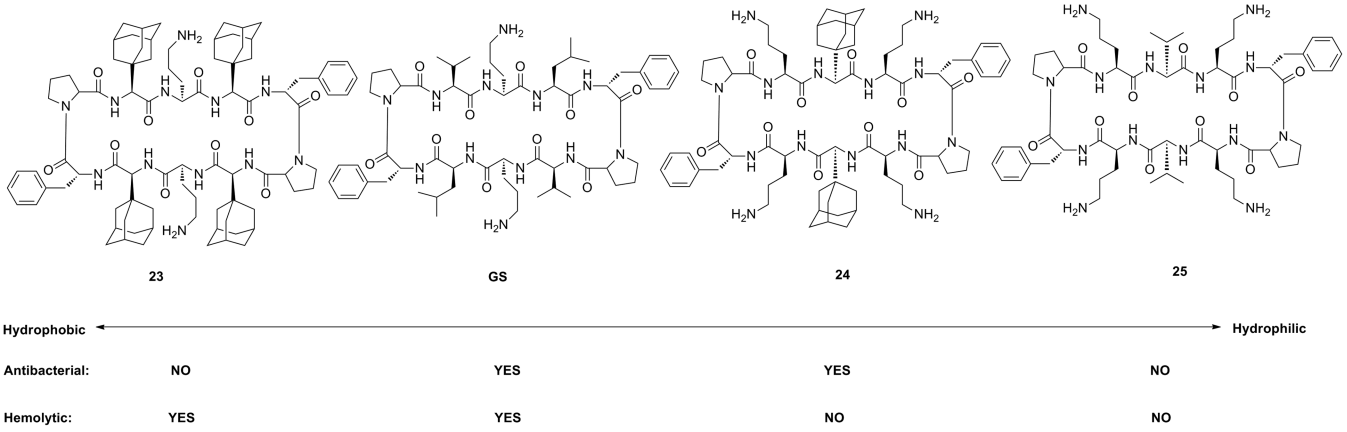


Figure 2. Upper panel: GS and GS analogues ranked from least to most hydrophobic (as determined by LC−MS retention time). Lower panel: overview of the antibacterial and hemolytic activities of GS and analogues with respect to the amphiphilic characteristics.

Table 12. Biological Activities of Some GS Analogues

	MIC (μg/mL)					
peptide	*E. coli* ATCC25922	*P. aeruginosa* ATCC 27853	*E. faecalis* ATCC 29212	*S. aureus* ATCC 29213	MRSA-NT 1110301981H-T034-PVL+	HC_{50} (μg/mL)
GS[60]	32	64	8	8	8	17.5
23[60]	>64	>64	>64	>64	nd	7.55
24[60]	8	16	8	8	8	107
25[60]	>64	>64	>64	>64	>64	n.o.[a]

[a]n.o.: not observed.

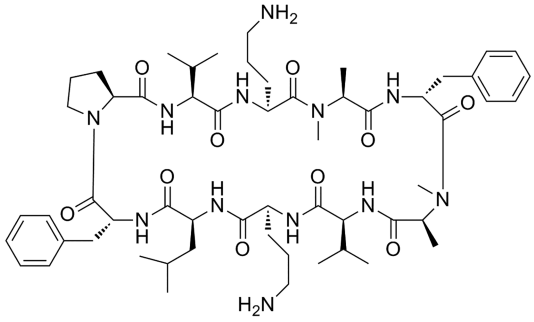
exceptions.[44] Impressively, extra amino groups on Pro often resulted in decreased hemolytic activity while largely maintaining the antimicrobial activity, leading to a popular modification strategy.[40] In addition to the analogues presented here, there are some other similar GS analogues, including some very recently reported examples.[57,58] Nevertheless, either no biological profile improvement was observed or their design is similar to the peptides already described, and therefore, they will not be presented in detail here.

***β*-Strand Region Modifications.** Compared with the widely investigated impacts of ring size and *β*-turn region, modifications of the *β*-strand region received little attention after the 1990s.[59−63] Nevertheless, extending the molecular ring size or modifying the *β*-turn region bears the risk of disrupting the *β*-hairpin structure and suppressing the amphiphilicity. In contrast, replacement of amino acids of the *β*-strand region usually does not perturb the *β*-hairpin content, provided that the relative chirality (D or L) at each position is preserved.

The Tamaki group reported a pair of GS analogues with no hydrophobic groups.[59] Peptide **22** containing six basic amino acid residues (Table 11) shared the same *β*-hairpin structure with GS. Although the hemolytic activity was totally lost, peptide **22** exhibited limited antibacterial activity against

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603−7617

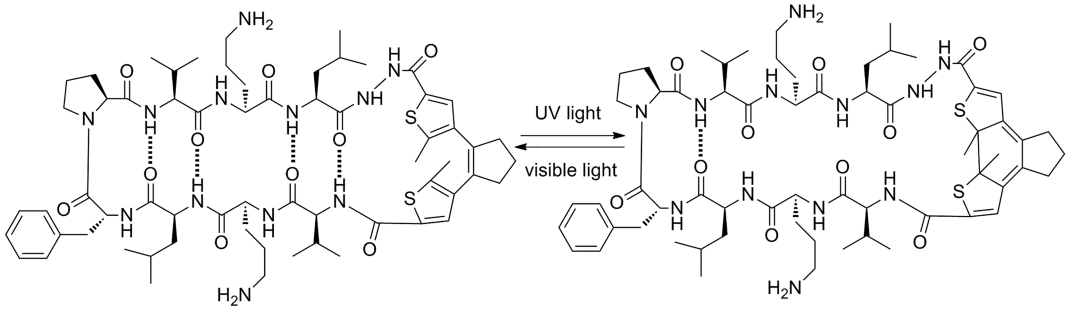
Table 13. Structure and Biological Activities of N-Methylated GS Analogue 26 Reported by Li et al.



26

peptide	MIC (μg/mL)						HC_{50} (μg/mL)
	E. coli	*K. pneumonia*	*Salmonella*	MDRSA	VRSA	MRSE	
GS[63]	28	3.6	28	1.8	3.6	1.8	14.3
26[63]	28	7	28	7	3.5	1.8	69.2

Table 14. Structures and Biological Activities of Photoswitchable GS Analogues Developed by Babii et al.



27a open (more active) **27b** closed (less active)

peptide	MIC (μg/mL)			HC_{50} (μg/mL)
	S. aureus	*S. epidermidis*	*S. xylosus*	
GS[65]	2	2	1	12
27a[65]	4	4	4	6
27b[65]	16	32	32	58

Gram-positive bacteria and was absolutely inactive against Gram-negative bacteria, highlighting the importance of hydrophobicity in the β-strand regions.

By adopting the "inverted" β-strand approach,[64] Kapoerchan et al. synthesized a series of hydrophobic and hydrophilic GS analogues with two or four positive charges (Figure 2).[37,60] Since all of the peptides shared the same β-hairpin content, the hydrophobicities of the analogues were evaluated using the RP-HPLC retention times as an empirical method. Impressively, the moderately hydrophobic peptide **24** with four positive charges and two adamantyl amino acid residues displayed antibacterial activity comparable to that of GS against several bacteria (Table 12). Meanwhile, the hemolytic activity of peptide **24** was significantly suppressed, highlighting the critical role of an optimal amphiphilicity for cytotoxicity specificity. Moreover, a number of bacterial pathogens trending to develop multidrug resistance (MDR) were treated with peptide **24**. Impressively, peptide **24** exhibited globally lower MIC values than GS.[36]

In order to dissociate the antibacterial and hemolytic activities, Li et al. reported a number of GS analogues with low β-sheet content obtained by N-methylation.[63] Compared with native GS, peptide **26** containing *N*-methylleucine in the β-strand region and *N*-methylalanine in the β-turn region exhibited a 4-fold improvement in selectivity index (Table 13). Although the internal H-bond originally involving the Leu residue was not present, the other H-bonds were preserved under solution conditions. Hence, the dramatically improved biological profile was the result of a minor change in the β-hairpin content.

"Smart" GS Analogues. Recently, Babii et al. designed and synthesized several "smart" GS analogues relying on a photodynamic technique (Table 14).[65,66] More precisely, a reversibly photoisomerizable amino acid analogue based on a diarylethylene scaffold was incorporated into the β-turn region of GS. The molecular conformation could thereby be controlled by irradiation with either visible or UV light. Therefore, peptide **27a** is able to form a stable β-hairpin structure (indicated by H-bonds), whereas peptide **27b** is not. Since the β-hairpin structure is correlated with the amphiphilicity, a critical parameter responsible for the antimicrobial activity, the biological activity of these GS analogues could be switched by light. Similarly, by incorporating a meta-/para-substituted azobenzene photoswitch, Yeoh et

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603−7617

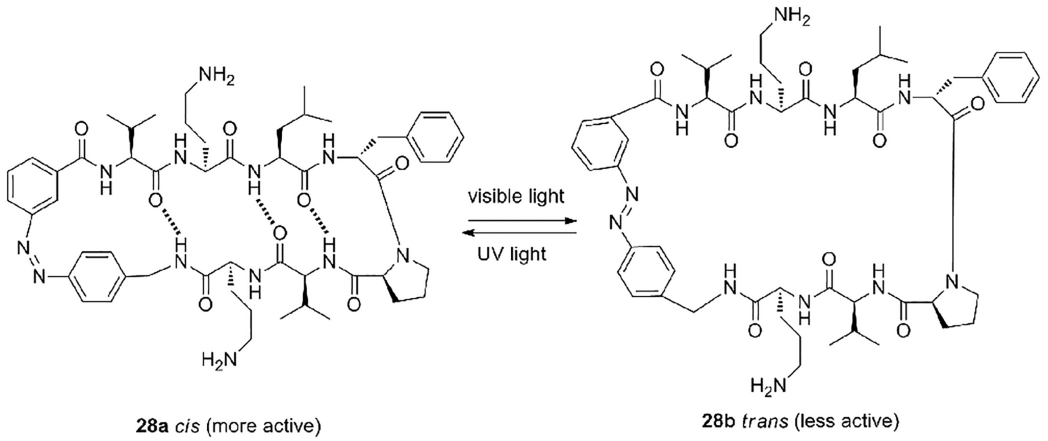


Figure 3. Photoswitchable GS analogues developed by Yeoh et al.

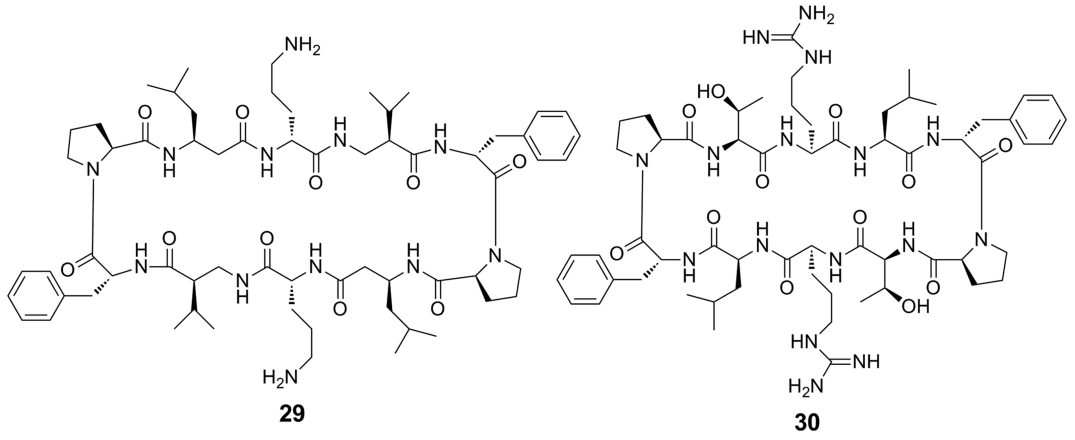


Figure 4. GS analogues with anti-Aβ amyloid activity.

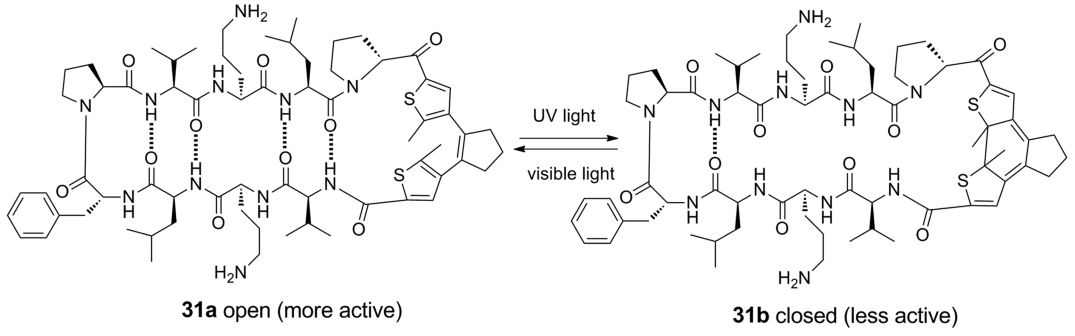


Figure 5. GS analogues with antitumor activity.

al. synthesized a series of photoswitchable AMPs based on GS (Figure 3).[67] Although not comparable with GS (MIC = 2 μg/mL against *S. aureus*), the cis-enriched peptide **28a** (64 μg/mL) exhibited a 4-fold increased antibacterial activity over the trans-enriched peptide **28b** (256 μg/mL).

RECENTLY EXPLORED NEW THERAPEUTIC PROPERTIES

Since it has been defined as an AMP, most research has been focused on the antimicrobial activity of GS. Nevertheless, GS also displays some other therapeutic properties, such as anti-Aβ amyloid and antitumor activities, which have been only recently studied.

Luo et al. showed that native GS significantly inhibits Aβ amyloid formation in vitro at a low concentration (20 μM) and exhibited the ability to dissolve amyloids that had formed in the absence of the inhibitor.[68] After tuning the hydrophobic and hydrophilic residues in the β-strand region and the hydrophobic side chain of the ^{D}Phe residues in the β-turns, they were able to identify α/β-mixed peptide **29** (Figure 4) with a potency 4 times higher than that of wild-type GS. In silico docking suggested that both GS and peptide **29** bind to the channel of the Aβ fibril, which is surrounded by hydrophobic and hydrophilic residues.

In contrast to the widely studied hydrophobic segment, Bu et al. demonstrated that the flexible N-terminus of Aβ fibril is a

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603−7617

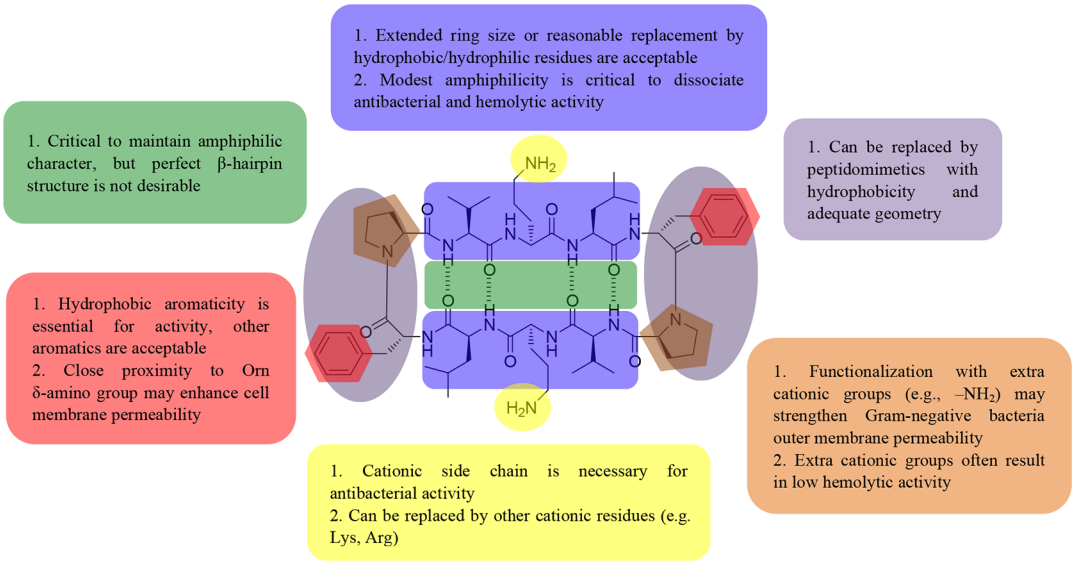


Figure 6. Main concepts of GS modifications and structure–activity relationship.

novel target.[69] In the proposed model of action, GS and analogues were considered as a "lasso" to host the flexible N-terminus. Significantly, the designed GS analogues are able to stabilize Aβ_{42} as a monomer from both the aggregation and disassembly processes under such a model of action. After modulating the structural properties, they obtained peptide **30** with dissociated hemolytic and oligomerization-preventing activities (Figure 4).[70]

Besides the high cytotoxicity toward prokaryotic and eukaryotic cells, wild-type GS has been reported to be able to inhibit tumor growth in vivo.[71] More recently, Babii et al.[72] synthesized GS-based peptide **31b** incorporating a diarylethylene photoswitch (Figure 5). When applied to an animal tumor, this peptide could be administered in the less toxic form and then activated only locally inside the solid tumor. In vivo studies demonstrated that peptide **31b** in combination with light is able to eliminate a tumor after a few weeks.

CONCLUSIONS AND PERSPECTIVE

Different approaches have been adopted with the ultimate goal of obtaining highly active and less toxic therapeutic candidates. With lessons taken from those modification strategies, we have summarized the structure–activity relationship of GS as shown in Figure 6. In most cases, the β-hairpin structure is essential to separate the hydrophobic and hydrophilic domains. However, an ideally formed β-hairpin structure, in the case of GS analogues, is not always desirable. In contrast, a slightly disordered secondary structure often leads to an optimal amphiphilicity and a better therapeutic index.[13,34,38,63] Several studies have demonstrated that a proper GS analogue should contain a good balance between positive charges and hydrophobic groups and have a well-controlled amphiphilicity.[27,35] Moreover, hydrophobic aromaticity (including both the size and spatial positioning of the aromatic ring) in the β-turn regions is crucial and should also be respected.[12,13] As also stated by Hodges et al, although the difference between the compositions of prokaryotic and eukaryotic membranes may not be large enough to obtain complete specificity of GS analogues, the therapeutic index could be optimized to provide the greatest discrimination possible.[27] Impressively, the photopharmacological approach allows antimicrobial activity to be controlled through photochemical control of a photoswitch. It is worth noting that clinical application of such "smart" antibiotics is of great interest to minimize the hemolytic side effects on noninfected tissues and to slow the rise of bacterial resistance. The application of photodynamic techniques thereby provides new paths in the design and synthesis of GS-based antibiotics. Beyond the strategies described above, there are also some other independently designed interesting examples, for instance, nonamphiphilic peptides[73] and GS dimers.[74] With or without a good biological profile, each designed GS analogue helped us to better understand this potent AMP and to make the next analogue a better one.

Currently, the extensive rise of bacterial resistance to classical antibiotics has become a major global health concern that has spurred significant efforts in the development of novel anti-infection agents. Significantly, although cyclic β-sheet peptide antibiotics like gramicidin S and tyrocidines have been clinically applied for more than half a century, there is virtually no resistance to them, which has made these old peptide antibiotics promising for new applications. Notwithstanding the fact that there is no clinically applicable antibiotic related to GS to date, we have been excited to witness the appearance of some druggable candidates along with the technical advance of structural analysis and mechanism studies. We hope that this Perspective will encourage and benefit continuous investment in the revival of this old but still potent AMP.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: valerie.alezra@u-psud.fr.
*E-mail: wy15506@gmail.com.

ORCID

Valérie Alezra: 0000-0003-3734-0052
Yang Wan: 0000-0003-3842-5074

Author Contributions

‖Q.G. and S.H. contributed equally.

Notes

The authors declare no competing financial interest.

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603–7617

Biographies

Qinkun Guan obtained his B.S. degree in Pharmacy in 2017 from Jiangxi University of Traditional Chinese Medicine in China. He is currently doing his Master's research in Medicinal Chemistry under the supervision of Dr. Yang Wan and Professor Yi Jin at Jiangxi University of Traditional Chinese Medicine. His research focuses on the design and synthesis of high-therapeutic-value gramicidin S analogues.

Shuhui Huang received her Master's degree at Nanchang University Medical College in 2007. Currently she is working at Jiangxi Maternal and Child Hospital. Her research focuses on the discovery of effective antibiotics.

Yi Jin received his Ph.D. degree in 1996 from Jiaosai University School of Medicine in Japan. After two years of postdoctoral training on TTS technology in Japan, he set up his own lab at Zhejiang University School of Medicine. Since 2014 he has been a full-time professor at Jiangxi University of Traditional Chinese Medicine. His research focuses on the development of hybrid nanoparticles for drug delivery and bioimaging.

Rémy Campagne received his Ph.D. degree from Université Paris-Saclay–Université Paris-Sud in 2017 after graduating from the Ecole Normale Supérieure de Cachan. His research interests have included the total synthesis of therapeutic molecules and the design of dipeptide mimics. Although currently working in the field of computer engineering, he is still involved in the field of medicinal chemistry.

Valérie Alezra received her Ph.D. degree in 2000 from the Faculty of Pharmacy of Université Paris Descartes. After one year of postdoctoral studies at the University of Geneva with Prof. E. P. Kündig in organometallic chemistry, she was appointed as an Assistant Professor at Ecole Normale Superieure de Lyon in 2001 in bioorganic chemistry. She moved to ICMMO at Université Paris-Sud in 2003, where she developed new methods for asymmetric synthesis of non-natural amino acids. Her research also focuses on the development of new therapeutic peptides.

Yang Wan received his Ph.D. degree from Université Paris-Saclay–Université Paris-Sud in 2017, and he started his independent career at Jiangxi University of Traditional Chinese Medicine in 2018. His scientific interest is mainly focused on the development of small peptidic and peptidomimetic molecules with therapeutic potential for antibacterial and antiamyloid activities.

■ ACKNOWLEDGMENTS

This work was supported by the Health Commission of Jiangxi Province Foundation (Grant 20195647) and the Major Program of Jiangxi Province Foundation (Grant S2017ZDYFE0080).

■ ABBREVIATIONS USED

Aβ, amyloid β protein; AMP, antimicrobial peptide; ATC, 4-amino(methyl)-1,3-thiazole-5-carboxylic acid; CD, circular dichroism; GS, gramicidin S; I2aa, indolizidin-2-one amino acid; Leu, leucine; LPS, lipopolysaccharide; Lys, lysine; MDR, multidrug resistance; MIC, minimal inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; NMR, nuclear magnetic resonance; Orn, ornithine; Phe, phenylalanine; Pro, proline; RP-HPLC, reversed-phase high-performance liquid chromatography; SAA, sugar amino acid; TI, therapeutic index; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Tyr, tyrosine; Val, valine

■ REFERENCES

(1) Gause, G. G.; Brazhnikova, M. G. Gramicidin S and Its Use in the Treatment of Infected Wounds. *Nature* **1944**, *154*, 703.
(2) Kondejewski, L. H.; Farmer, S. W.; Wishart, D. S.; Hancock, R. E. W.; Hodges, R. S. Gramicidin S Is Active against Both Gram-Positive and Gram-Negative Bacteria. *Int. J. Pept. Protein Res.* **1996**, *47* (6), 460–466.
(3) Wadsten, C.-J.; Bertilsson, C. A.; Sieradzki, H.; Edström, S. A Randomized Clinical Trial of Two Topical Preparations (Framycitin/Gramicidin and Oxytetracycline/ Hydrocortisone with Polymyxin B) in the Treatment of External Otitis. *Arch. Oto-Rhino-Laryngol.* **1985**, *242* (2), 135–139.
(4) Smirnov, S.; Belashov, A.; Demin, O. Optimization of Antimicrobial Drug Gramicidin S Dosing Regime Using Biosimulations. *Eur. J. Pharm. Sci.* **2009**, *36* (1), 105–109.
(5) Berditsch, M.; Lux, H.; Babii, O.; Afonin, S.; Ulrich, A. S. Therapeutic Potential of Gramicidin S in the Treatment of Root Canal Infections. *Pharmaceuticals* **2016**, *9* (3), 56.
(6) Izumiya, N.; Kato, T.; Aoyagi, H.; Waki, M.; Kondo, M. *Synthetic Aspects of Biologically Active Cyclic Peptides: Gramicidin S and Tyrocidines*; Halsted Press: New York, 1979.
(7) Ovchinnikov, Y. A.; Ivanov, V. T. The Cyclic Peptides: Structure, Conformation, and Function. In *The Proteins, Volume V*, 3rd ed.; Neurath, H., Hill, R. L., Eds.; Academic Press: New York, 1982; Chapter 3, pp 310–640.
(8) Pal, S.; Ghosh, U.; Ampapathi, R. S.; Chakraborty, T. K. Recent Studies on Gramicidin S Analog Structure and Antimicrobial Activity. *Top. Heterocycl. Chem.* **2015**, *49*, 159–202.
(9) Hull, S. E.; Karlsson, R.; Main, P.; Woolfson, M. M.; Dodson, E. J. The Crystal Structure of a Hydrated Gramicidin S–Urea Complex. *Nature* **1978**, *275* (5677), 206–207.
(10) Llamas-Saiz, A. L.; Grotenbreg, G. M.; Overhand, M.; van Raaij, M. J. Double-Stranded Helical Twisted β-Sheet Channels in Crystals of Gramicidin S Grown in the Presence of Trifluoroacetic and Hydrochloric Acids. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2007**, *63* (3), 401–407.
(11) Lee, D. L.; Hodges, R. S. Structure–Activity Relationships of de Novo Designed Cyclic Antimicrobial Peptides Based on Gramicidin S. *Biopolymers* **2003**, *71* (1), 28–48.
(12) Solanas, C.; de la Torre, B. G.; Fernández-Reyes, M.; Santiveri, C. M.; Jiménez, M. Á.; Rivas, L.; Jiménez, A. I.; Andreu, D.; Cativiela, C. Therapeutic Index of Gramicidin S Is Strongly Modulated by D-Phenylalanine Analogues at the β-Turn. *J. Med. Chem.* **2009**, *52* (3), 664–674.
(13) Grotenbreg, G. M.; Buizert, A. E. M.; Llamas-Saiz, A. L.; Spalburg, E.; van Hooft, P. A. V.; de Neeling, A. J.; Noort, D.; van Raaij, M. J.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. β-Turn Modified Gramicidin S Analogues Containing Arylated Sugar Amino Acids Display Antimicrobial and Hemolytic Activity Comparable to the Natural Product. *J. Am. Chem. Soc.* **2006**, *128* (23), 7559–7565.
(14) Jelokhani-Niaraki, M.; Kondejewski, L. H.; Farmer, S. W.; Hancock, R. E. W.; Kay, C. M.; Hodges, R. S. Diastereoisomeric Analogues of Gramicidin S: Structure, Biological Activity and Interaction with Lipid Bilayers. *Biochem. J.* **2000**, *349* (3), 747–755.
(15) Eisenberg, D.; Weiss, R. M.; Terwilliger, T. C.; Wilcox, W. Hydrophobic Moments and Protein Structure. *Faraday Symp. Chem. Soc.* **1982**, *17* (0), 109–120.
(16) Yeaman, M. R.; Yount, N. Y. Mechanisms of Antimicrobial Peptide Action and Resistance. *Pharmacol. Rev.* **2003**, *55* (1), 27–55.
(17) Zasloff, M. Antimicrobial Peptides of Multicellular Organisms. *Nature* **2002**, *415* (6870), 389–395.
(18) Katsu, T.; Imamura, T.; Komagoe, K.; Masuda, K.; Mizushima, T. Simultaneous Measurements of K+ and Calcein Release from Liposomes and the Determination of Pore Size Formed in a Membrane. *Anal. Sci.* **2007**, *23* (5), 517–522.
(19) Afonin, S.; Dürr, U. H. N.; Wadhwani, P.; Salgado, J.; Ulrich, A. S. Solid State NMR Structure Analysis of the Antimicrobial Peptide Gramicidin S in Lipid Membranes: Concentration-Dependent Re-

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603–7617

alignment and Self-Assembly as a β-Barrel. *Top. Curr. Chem.* **2008**, *273*, 139−154.
(20) Jelokhani-Niaraki, M.; Hodges, R. S.; Meissner, J. E.; Hassenstein, U. E.; Wheaton, L. Interaction of Gramicidin S and Its Aromatic Amino-Acid Analog with Phospholipid Membranes. *Biophys. J.* **2008**, *95* (7), 3306−3321.
(21) Ashrafuzzaman, M.; Andersen, O. S.; McElhaney, R. N. The Antimicrobial Peptide Gramicidin S Permeabilizes Phospholipid Bilayer Membranes without Forming Discrete Ion Channels. *Biochim. Biophys. Acta, Biomembr.* **2008**, *1778* (12), 2814−2822.
(22) Wenzel, M.; Rautenbach, M.; Vosloo, J. A.; Siersma, T.; Aisenbrey, C. H. M.; Zaitseva, E.; Laubscher, W. E.; van Rensburg, W.; Behrends, J. C.; Bechinger, B.; Hamoen, L. W. The Multifaceted Antibacterial Mechanisms of the Pioneering Peptide Antibiotics Tyrocidine and Gramicidin S. *mBio* **2018**, *9* (5), No. e00802-18.
(23) Jelokhani-Niaraki, M.; Prenner, E. J.; Kay, C. M.; McElhaney, R. N.; Hodges, R. S. Conformation and Interaction of the Cyclic Cationic Antimicrobial Peptides in Lipid Bilayers: Cyclic Antimicrobial Peptides in Lipid Bilayers. *J. Pept. Res.* **2002**, *60* (1), 23−36.
(24) Jelokhani-Niaraki, M.; Prenner, E. J.; Kay, C. M.; McElhaney, R. N.; Hodges, R. S.; Kondejewski, L. H. Conformation and Other Biophysical Properties of Cyclic Antimicrobial Peptides in Aqueous Solutions. *J. Pept. Res.* **2001**, *58* (4), 293−306.
(25) Prenner, E. J.; Lewis, R. N. A. H.; McElhaney, R. N. The Interaction of the Antimicrobial Peptide Gramicidin S with Lipid Bilayer Model and Biological Membranes. *Biochim. Biophys. Acta, Biomembr.* **1999**, *1462* (1−2), 201−221.
(26) Kondejewski, L. H.; Farmer, S. W.; Wishart, D. S.; Kay, C. M.; Hancock, R. E. W.; Hodges, R. S. Modulation of Structure and Antibacterial and Hemolytic Activity by Ring Size in Cyclic Gramicidin S Analogs. *J. Biol. Chem.* **1996**, *271* (41), 25261−25268.
(27) Kondejewski, L. H.; Jelokhani-Niaraki, M.; Farmer, S. W.; Lix, B.; Kay, C. M.; Sykes, B. D.; Hancock, R. E. W.; Hodges, R. S. Dissociation of Antimicrobial and Hemolytic Activities in Cyclic Peptide Diastereomers by Systematic Alterations in Amphipathicity. *J. Biol. Chem.* **1999**, *274* (19), 13181−13192.
(28) Jelokhani-Niaraki, M.; Kondejewski, L. H.; Wheaton, L. C.; Hodges, R. S. Effect of Ring Size on Conformation and Biological Activity of Cyclic Cationic Antimicrobial Peptides. *J. Med. Chem.* **2009**, *52* (7), 2090−2097.
(29) Kondejewski, L. H.; Lee, D. L.; Jelokhani-Niaraki, M.; Farmer, S. W.; Hancock, R. E. W.; Hodges, R. S. Optimization of Microbial Specificity in Cyclic Peptides by Modulation of Hydrophobicity within a Defined Structural Framework. *J. Biol. Chem.* **2002**, *277* (1), 67−74.
(30) McInnes, C.; Kondejewski, L. H.; Hodges, R. S.; Sykes, B. D. Development of the Structural Basis for Antimicrobial and Hemolytic Activities of Peptides Based on Gramicidin S and Design of Novel Analogs Using NMR Spectroscopy. *J. Biol. Chem.* **2000**, *275* (19), 14287−14294.
(31) Lee, D. L.; Powers, J.-P. S.; Pflegerl, K.; Vasil, M. L.; Hancock, R. E. W.; Hodges, R. S. Effects of Single D-Amino Acid Substitutions on Disruption of β-Sheet Structure and Hydrophobicity in Cyclic 14-Residue Antimicrobial Peptide Analogs Related to Gramicidin S. *J. Pept. Res.* **2004**, *63* (2), 69−84.
(32) Knijnenburg, A. D.; Kapoerchan, V. V.; Grotenbreg, G. M.; Spalburg, E.; de Neeling, A. J.; Mars-Groenendijk, R. H.; Noort, D.; Otero, J. M.; Llamas-Saiz, A. L.; van Raaij, M. J.; Ravensbergen, B.; Nibbering, P. H.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. Synthesis and Evaluation of Strand and Turn Modified Ring-Extended Gramicidin S Derivatives. *Bioorg. Med. Chem.* **2011**, *19* (11), 3402−3409.
(33) Knijnenburg, A. D.; Spalburg, E.; de Neeling, A. J.; Mars-Groenendijk, R. H.; Noort, D.; Grotenbreg, G. M.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. Ring-Extended Derivatives of Gramicidin S with Furanoid Sugar Amino Acids in the Turn Region Have Enhanced Antimicrobial Activity. *ChemMedChem* **2009**, *4* (12), 1976−1979.
(34) Knijnenburg, A. D.; Spalburg, E.; de Neeling, A. J.; Mars-Groenendijk, R. H.; Noort, D.; Grotenbreg, G. M.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. Ring-Extended Gramicidin S Analogs Containing Cis δ-Sugar Amino Acid Turn Mimetics with Varying Ring Size. *Helv. Chim. Acta* **2012**, *95* (12), 2544−2561.
(35) Knijnenburg, A. D.; Kapoerchan, V. V.; Spalburg, E.; de Neeling, A. J.; Mars-Groenendijk, R. H.; Noort, D.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. Tuning Hydrophobicity of Highly Cationic Tetradecameric Gramicidin S Analogues Using Adamantane Amino Acids. *Bioorg. Med. Chem.* **2010**, *18* (23), 8403−8409.
(36) Swierstra, J.; Kapoerchan, V.; Knijnenburg, A.; van Belkum, A.; Overhand, M. Structure, Toxicity and Antibiotic Activity of Gramicidin S and Derivatives. *Eur. J. Clin. Microbiol. Infect. Dis.* **2016**, *35*, 763−769.
(37) Overhand, M.; Kapoerchan, V. V.; Van der Marel, G. A.; Overkleeft, H. S.; De Neeling, A. J.; Noort, D.; Knijnenburg, A. D. Preparation of Antimicrobial Cyclic Peptides. WO 2010/113042 A1, 2010.
(38) Tamaki, M.; Sasaki, I.; Kokuno, M.; Shindo, M.; Kimura, M.; Uchida, Y. Antimicrobially Active Cycloundecapeptides Related to Gramicidin S Having a Novel Turn Structure with Cisd-Phe-Pro Peptide Bond. *Org. Biomol. Chem.* **2010**, *8* (8), 1791.
(39) Tamaki, M.; Takanashi, K.; Harada, T.; Fujinuma, K.; Shindo, M.; Kimura, M.; Uchida, Y. Novel Cycloundecapeptides Related to Gramicidin S with Both High Antibiotic Activity and Low Hemolytic Activity. *Chem. Pharm. Bull.* **2011**, *59* (12), 1481−1484.
(40) Kawai, M.; Yamamura, H.; Tanaka, R.; Umemoto, H.; Ohmizo, C.; Higuchi, S.; Katsu, T. Proline Residue-Modified Polycationic Analogs of Gramicidin S with High Antibacterial Activity against Both Gram-Positive and Gram-Negative Bacteria and Low Hemolytic Activity*: Polycationic Gramicidin S Analogs. *J. Pept. Res.* **2005**, *65* (1), 98−104.
(41) Legrand, B.; Mathieu, L.; Lebrun, A.; Andriamanarivo, S.; Lisowski, V.; Masurier, N.; Zirah, S.; Kang, Y. K.; Martinez, J.; Maillard, L. T. Thiazole-Based γ-Building Blocks as Reverse-Turn Mimetic to Design a Gramicidin S Analogue: Conformational and Biological Evaluation. *Chem. - Eur. J.* **2014**, *20* (22), 6713−6720.
(42) Knijnenburg, A. D.; Tuin, A. W.; Spalburg, E.; de Neeling, A. J.; Mars-Groenendijk, R. H.; Noort, D.; Otero, J. M.; Llamas-Saiz, A. L.; van Raaij, M. J.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. Exploring the Conformational and Biological Versatility of β-Turn-Modified Gramicidin S by Using Sugar Amino Acid Homologues That Vary in Ring Size. *Chem. - Eur. J.* **2011**, *17* (14), 3995−4004.
(43) van der Knaap, M.; Lageveen, L. T.; Busscher, H. J.; Mars-Groenendijk, R.; Noort, D.; Otero, J. M.; Llamas-Saiz, A. L.; van Raaij, M. J.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. Evaluation of Readily Accessible Azoles as Mimics of the Aromatic Ring of D-Phenylalanine in the Turn Region of Gramicidin S. *ChemMedChem* **2011**, *6* (5), 840−847.
(44) Tamaki, M.; Harada, T.; Fujinuma, K.; Takanashi, K.; Shindo, M.; Kimura, M.; Uchida, Y. Polycationic Gramicidin S Analogues with Both High Antibiotic Activity and Very Low Hemolytic Activity. *Chem. Pharm. Bull.* **2012**, *60* (9), 1134−1138.
(45) Grotenbreg, G. M.; Spalburg, E.; de Neeling, A. J.; van der Marel, G. A.; Overkleeft, H. S.; van Boom, J. H.; Overhand, M. Synthesis and Biological Evaluation of Novel Turn-Modified Gramicidin S Analogues. *Bioorg. Med. Chem.* **2003**, *11* (13), 2835−2841.
(46) van der Knaap, M.; Engels, E.; Busscher, H. J.; Otero, J. M.; Llamas-Saiz, A. L.; van Raaij, M. J.; Mars-Groenendijk, R. H.; Noort, D.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. Synthesis and Biological Evaluation of Asymmetric Gramicidin S Analogues Containing Modified D-Phenylalanine Residues. *Bioorg. Med. Chem.* **2009**, *17* (17), 6318−6328.
(47) Yamada, K.; Kodaira, M.; Shinoda, S.; Komagoe, K.; Oku, H.; Katakai, R.; Katsu, T.; Matsuo, I. Structure−Activity Relationships of Gramicidin S Analogs Containing (β-3-Pyridyl)-α,β-Dehydroalanine Residues on Membrane Permeability. *MedChemComm* **2011**, *2* (7), 644−649.

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603−7617

(48) Yamada, K.; Shinoda, S.; Oku, H.; Komagoe, K.; Katsu, T.; Katakai, R. Synthesis of Low-Hemolytic Antimicrobial Dehydropeptides Based on Gramicidin S. *J. Med. Chem.* **2006**, *49* (26), 7592−7595.
(49) Solanas, C.; de la Torre, B. G.; Fernández-Reyes, M.; Santiveri, C. M.; Jiménez, M. Á.; Rivas, L.; Jiménez, A. I.; Andreu, D.; Cativiela, C. Sequence Inversion and Phenylalanine Surrogates at the β-Turn Enhance the Antibiotic Activity of Gramicidin S. *J. Med. Chem.* **2010**, *53* (10), 4119−4129.
(50) Katsu, T.; Nakagawa, H.; Yasuda, K. Interaction between Polyamines and Bacterial Outer Membranes as Investigated with Ion-Selective Electrodes. *Antimicrob. Agents Chemother.* **2002**, *46* (4), 1073−1079.
(51) Kawai, M.; Tanaka, R.; Yamamura, H.; Yasuda, K.; Narita, S.; Umemoto, H.; Ando, S.; Katsu, T. Extra Amino Group-Containing Gramicidin S Analogs Possessing Outer Membrane-Permeabilizing Activity. *Chem. Commun.* **2003**, No. 11, 1264−1265.
(52) Tamaki, M.; Fujinuma, K.; Harada, T.; Takanashi, K.; Shindo, M.; Kimura, M.; Uchida, Y. Fatty Acyl-Gramicidin S Derivatives with Both High Antibiotic Activity and Low Hemolytic Activity. *Bioorg. Med. Chem. Lett.* **2012**, *22* (1), 106−109.
(53) Priem, C.; Wuttke, A.; Berditsch, M.; Ulrich, A. S.; Geyer, A. Scaling the Amphiphilic Character and Antimicrobial Activity of Gramicidin S by Dihydroxylation or Ketal Formation. *J. Org. Chem.* **2017**, *82* (23), 12366−12376.
(54) Grotenbreg, G. M.; Timmer, M. S. M.; Llamas-Saiz, A. L.; Verdoes, M.; van der Marel, G. A.; van Raaij, M. J.; Overkleeft, H. S.; Overhand, M. An Unusual Reverse Turn Structure Adopted by a Furanoid Sugar Amino Acid Incorporated in Gramicidin S. *J. Am. Chem. Soc.* **2004**, *126* (11), 3444−3446.
(55) Roy, S.; Lombart, H.-G.; Lubell, W. D.; Hancock, R. E. W.; Farmer, S. W. Exploring Relationships between Mimic Configuration, Peptide Conformation and Biological Activity in Indolizidin-2-One Amino Acid Analogs of Gramicidin S. *J. Pept. Res.* **2002**, *60* (4), 198−214.
(56) Wan, Y.; Stanovych, A.; Gori, D.; Zirah, S.; Kouklovsky, C.; Alezra, V. β,γ-Diamino Acids as Building Blocks for New Analogues of Gramicidin S: Synthesis and Biological Activity. *Eur. J. Med. Chem.* **2018**, *149*, 122−128.
(57) Kotmale, A. S.; Sangtani, E.; Gonnade, R. G.; Sarkar, D.; Burade, S.; Rajamohanan, P. R.; Sanjayan, G. J. Conformational Studies of Ant−Pro Motif-Incorporated Cyclic Peptides: Gramicidin S and Avellanin. *New J. Chem.* **2018**, *42* (2), 1197−1201.
(58) Pal, S.; Singh, G.; Singh, S.; Tripathi, J. K.; Ghosh, J. K.; Sinha, S.; Ampapathi, R. S.; Chakraborty, T. K. Tetrahydrofuran Amino Acid-Containing Gramicidin S Analogues with Improved Biological Profiles. *Org. Biomol. Chem.* **2015**, *13* (24), 6789−6802.
(59) Tamaki, M.; Sasaki, I.; Nakao, Y.; Shindo, M.; Kimura, M.; Uchida, Y. Gramicidin S Analogs Having Six Basic Amino Acid Residues. *J. Antibiot.* **2009**, *62* (10), 597−599.
(60) Kapoerchan, V. V.; Knijnenburg, A. D.; Niamat, M.; Spalburg, E.; de Neeling, A. J.; Nibbering, P. H.; Mars-Groenendijk, R. H.; Noort, D.; Otero, J. M.; Llamas-Saiz, A. L.; van Raaij, M. J.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. An Adamantyl Amino Acid Containing Gramicidin S Analogue with Broad Spectrum Antibacterial Activity and Reduced Hemolytic Activity. *Chem. - Eur. J.* **2010**, *16* (40), 12174−12181.
(61) Kapoerchan, V. V.; Knijnenburg, A. D.; Keizer, P.; Spalburg, E.; de Neeling, A. J.; Mars-Groenendijk, R. H.; Noort, D.; Otero, J. M.; Llamas-Saiz, A. L.; van Raaij, M. J.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. 'Inverted' Analogs of the Antibiotic Gramicidin S with an Improved Biological Profile. *Bioorg. Med. Chem.* **2012**, *20* (20), 6059−6062.
(62) Tamaki, M.; Fujinuma, K.; Harada, T.; Takanashi, K.; Shindo, M.; Kimura, M.; Uchida, Y. Design and Syntheses of Gramicidin S Analogs, Cyclo(-X-Leu-X-D-Phe-Pro-)2 (X = His, Lys, Orn, Dab and Dap). *J. Antibiot.* **2011**, *64* (8), 583−585.
(63) Li, Y.; Bionda, N.; Yongye, A.; Geer, P.; Stawikowski, M.; Cudic, P.; Martinez, K.; Houghten, R. A. Dissociation of Antimicrobial and Hemolytic Activities of Gramicidin S through N-Methylation Modification. *ChemMedChem* **2013**, *8* (11), 1865−1872.
(64) Soejima, Y.; Hashiguchi, A.; Izumiya, N. Syntheses and Antibacterial Activities of Gramicidin S Analogs Containing L-Ornithine in Place of L-Valine. *Biosci., Biotechnol., Biochem.* **1994**, *58* (5), 826−829.
(65) Babii, O.; Afonin, S.; Berditsch, M.; Reisser, S.; Mykhailiuk, P. K.; Kubyshkin, V. S.; Steinbrecher, T.; Ulrich, A. S.; Komarov, I. V. Controlling Biological Activity with Light: Diarylethene- Containing Cyclic Peptidomimetics. *Angew. Chem., Int. Ed.* **2014**, *53* (13), 3392−3395.
(66) Babii, O.; Afonin, S.; Ishchenko, A. Y.; Schober, T.; Negelia, A. O.; Tolstanova, G. M.; Garmanchuk, L. V.; Ostapchenko, L. I.; Komarov, I. V.; Ulrich, A. S. Structure−Activity Relationships of Photoswitchable Diarylethene-Based β-Hairpin Peptides as Membranolytic Antimicrobial and Anticancer Agents. *J. Med. Chem.* **2018**, *61* (23), 10793−10813.
(67) Yeoh, Y. Q.; Yu, J.; Polyak, S. W.; Horsley, J. R.; Abell, A. D. Photopharmacological Control of Cyclic Antimicrobial Peptides. *ChemBioChem* **2018**, *19* (24), 2591−2597.
(68) Luo, J.; Otero, J. M.; Yu, C.-H.; Wärmländer, S. K. T. S.; Gräslund, A.; Overhand, M.; Abrahams, J. P. Inhibiting and Reversing Amyloid-β Peptide (1−40) Fibril Formation with Gramicidin S and Engineered Analogues. *Chem. - Eur. J.* **2013**, *19* (51), 17338−17348.
(69) Wen, G.; Chen, D.; Qin, W.; Zhou, B.; Wang, Y.; Liu, Z.; Du, J.; Zhou, Q.; Quan, J.; Bu, X. Stabilizing Amyloid-β Peptide by the N-Terminus Capture Is Capable of Preventing and Eliminating Amyloid-β Oligomers. *Chem. Commun.* **2017**, *53* (54), 7673−7676.
(70) Chen, D.; Qin, W.; Wen, G.; Shi, B.; Liu, Z.; Wang, Y.; Zhou, Q.; Quan, J.; Zhou, B.; Bu, X. Dissociation of Haemolytic and Oligomer-Preventing Activities of Gramicidin S Derivatives Targeting the Amyloid-β N-Terminus. *Chem. Commun.* **2017**, *53*, 13340−13343.
(71) Okamoto, K.; Tomita, Y.; Yonezawa, H.; Hirohata, T.; Ogura, R.; Izumiya, N. Inhibitory Effect of Gramicidin S on the Growth of Murine Tumor Cells in Vitro and in Vivo. *Oncology* **2004**, *41* (1), 43−48.
(72) Babii, O.; Afonin, S.; Garmanchuk, L. V.; Nikulina, V. V.; Nikolaienko, T. V.; Storozhuk, O. V.; Shelest, D. V.; Dasyukevich, O. I.; Ostapchenko, L. I.; Iurchenko, V.; Zozulya, S.; Ulrich, A. S.; Komarov, I. V. Direct Photocontrol of Peptidomimetics: An Alternative to Oxygen-Dependent Photodynamic Cancer Therapy. *Angew. Chem., Int. Ed.* **2016**, *55* (18), 5493−5496.
(73) Tamaki, M.; Sawa, K.; Kikuchi, S.; Shindo, M.; Uchida, Y. A Novel, Antimicrobially Active Analog of Gramicidin S without Amphiphilic Conformation. *J. Antibiot.* **2006**, *59* (6), 370−372.
(74) Grotenbreg, G. M.; Witte, M. D.; van Hooft, P. A. V.; Spalburg, E.; Reiß, P.; Noort, D.; de Neeling, A. J.; Koert, U.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. Synthesis and Biological Evaluation of Gramicidin S Dimers. *Org. Biomol. Chem.* **2005**, *3* (2), 233−238.

DOI: 10.1021/acs.jmedchem.9b00156
J. Med. Chem. 2019, 62, 7603−7617