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Synthesis and biological evaluation of backbone-aminated analogues of gramicidin S

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ABSTRACT

We report the parallel synthesis of gramicidin S derivatives featuring backbone *N*-amino substituents. Analogues were prepared by incorporation of *N*-amino dipeptide subunits on solid support. Nine backbone-aminated macrocycles were evaluated for growth inhibitory activity against ESKAPE pathogens and hemolytic activity against human red blood cells. Diamination of the Orn residues in the β-strand region of gramicidin S was found to enhance broad-spectrum antimicrobial activity without a corresponding increase in hemolytic activity.

Gramicidin S (GS) is a naturally-occurring non-ribosomal peptide first isolated in the early 1940s from *Aneurinibacillus migulanus* (formerly *Bacillus brevis*).[1] GS displays activity against both Gram-positive and Gram-negative bacteria but its poor selectivity over mammalian cells has prevented its systemic use.[2] GS has long been known to increase membrane permeability in bacteria.[3] The emergence of resistance to GS is generally low due to its membranolytic function in addition to its other mechanisms of antimicrobial action.[3–4] As one of the most widely studied antimicrobial peptides, a number of SAR studies have been carried out on GS resulting in several unnatural analogs with improved therapeutic indices.[5]

GS is a C_2-symmetrical cyclic decapeptide that adopts an antiparallel β -sheet-like structure (Fig. 1).[6] Four interstrand hydrogen bonds (between Val and Leu residues) and two type II' β -turns (comprised of D-Phe-Pro) confer substantial conformational stability.[7] Interestingly, cyclization is necessary for bacterial growth inhibition as linear variants are generally inactive.[2a] Various GS backbone modifications have been investigated, including the introduction of dipeptide turn mimics,[8] sugar[9] and tetrahydrofuran[10] amino acid turn mimics, replacement of turn[11] and strand[12] amide bonds with *E* alkene isosteres, and *N*-methylation of backbone amides.[13] For example, Kawai and coworkers synthesized a tetra-*N*-methylated gramicidin derivative and found that the solvent-exposed amide protons were not required for antimicrobial activity, despite substantial perturbation of the native GS conformation.[13a,13c] *N*-Methylation of the hydrogen-bonded Val residues results in significantly decreased antimicrobial activity,[13b] while mono- and di-(*N*-Me)Leu analogues show enhanced activity and selectivity toward bacterial membranes[13d,14]

Given previous studies on backbone modification of GS, we viewed it as a useful template to explore alternative amide substitution strategies. Since *N*-methylation can severely impact the preferred conformation of parent peptides, we sought to explore the effects of *N*-amination on the biological activity of GS. We previously demonstrated that amide *N*-amination of β -strand residues can stabilize β -sheet like conformations due to the cooperative effects shown in Fig. 2 [15] *N*-Amination of solvent-exposed amides in GS may thus yield analogues with unique structural and membranolytic properties. Here, we describe the first solid-phase synthesis of *N*-aminated analogues of GS using a dipeptide fragment incorporation approach. In addition, we assess the activity and selectivity of these derivatives against ESKAPE pathogens – a panel of pathogens that represent leading causes of nosocomial infections and bacterial resistance worldwide. Our studies reveal new *N*-amino peptide (NAP) macrocycles with improved antimicrobial and hemolytic properties relative to GS.

A series of backbone-substituted GS analogues featuring 1–4 amide *N*-amino groups were targeted for synthesis. Although we previously demonstrated that Boc-protected α-hydrazino acid monomers can be utilized in the solid-phase synthesis of NAPs, the deactivated Nα within these building blocks requires on-resin acid chloride condensation for elongation.[16] These conditions often generate insoluble salts that are difficult to separate from the resin, and require multiple rounds of condensation to achieve full conversion. Alternatively, the use of backbone-aminated dipeptide building blocks allows for standard Fmoc-based SPPS and is amenable to automated synthesis. Preparation of the required building blocks for this study is shown in Scheme 1. Compounds **1** and **2** were accessible in 3 steps from the commercially

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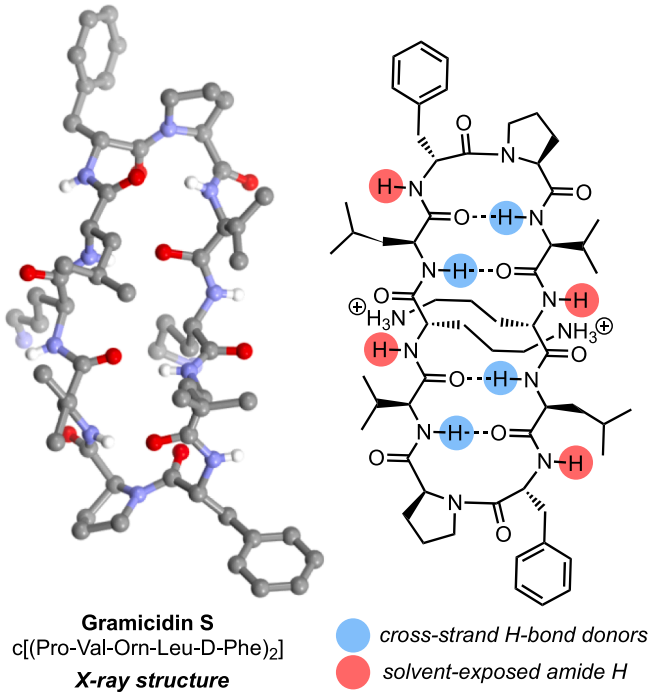


Fig. 1. Structure of gramicidin S.

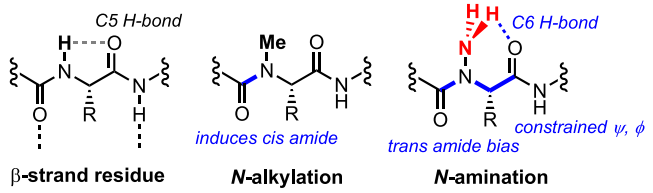
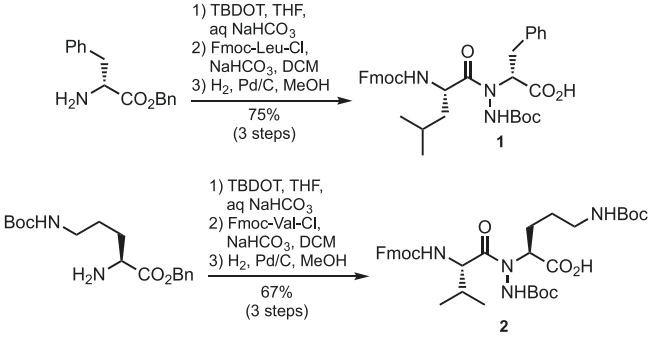


Fig. 2. Conformational effects of peptide backbone substitutions.



Scheme 1. Synthesis of *N*-amino dipeptide building blocks.

available benzyl esters via electrophilic amination with 2-(*t*-butyl)-3,3-(diethyl)-oxaziridine-2,3,3-tricarboxylate (TBDOT), reaction with pre-formed Fmoc-protected amino acid chlorides,[17] and hydrogenolysis.

Since our strategy relies on condensation with a dipeptide fragment, we considered the possibility of C-terminal epimerization upon activation. To test this, we modeled the anticipated solid-phase acylation conditions in solution using isobutylamine as a test nucleophile. As shown in Fig. 3, HCTU-mediated amidation of **(*S,R*)-1** gave **(*S,R*)-3** with > 98:2 d.r. as judged by HPLC. This was confirmed by co-injection of the crude reaction mixture with purified standards of **(*S,R*)-3** and **(*S,S*)-3**, which were well resolved on a normal-phase chiral column. These results confirm that **1** does not undergo appreciable epimerization during condensation. This result may be explained by a

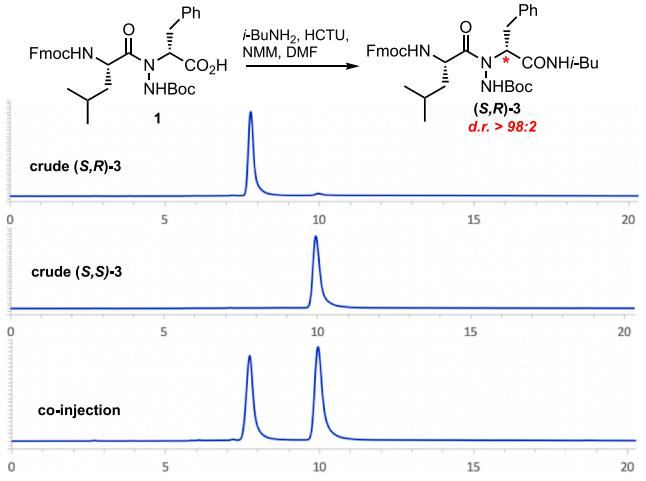
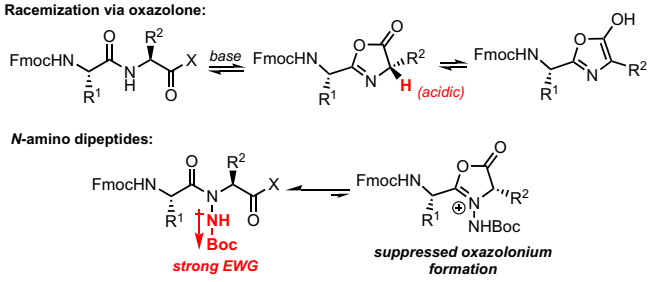


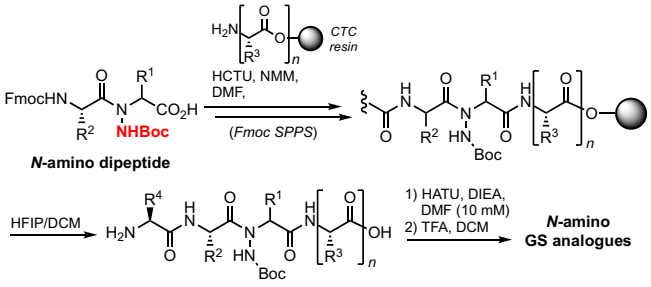
Fig. 3. Assessment of epimerization during dipeptide fragment condensation.



Scheme 2. Mechanism of oxazolone formation and resistance to epimerization.

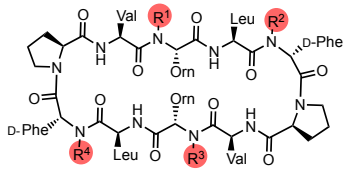
reduced propensity to form the racemization-prone dihydrooxazolonium intermediate. As shown in Scheme 2, active esters of peptide fragments may readily undergo cyclization and aromatization to oxazolones prior to condensation.[18] This pathway is particularly problematic in the case of *N*-alkylated peptide fragments. In contrast, the *N*-NHBoc group acts as an electron withdrawing substituent that disfavors intramolecular cyclization. This feature serves to expand the versatility of *N*-amino dipeptide building blocks for use in SPPS.

Synthesis of the target macrocycles was accomplished using a classical 'tea-bag' approach, wherein resin-filled pouches were separated as needed to introduce diversity elements and recombined for sequence elongation. As shown in Scheme 3, our strategy relied on the introduction of backbone *N*-amino groups via condensation with Fmoc-protected *N*-amino dipeptide building blocks. The substituted peptides were cleaved from the resin with HFIP/DCM to afford Boc-protected linear peptides. Head-to-tail macrocyclization was carried out on the crude material using HATU/DIEA at 10 mM substrate concentration in DMF. The crude macrocyclic peptides were subjected to global Boc deprotection with TFA and purified by RP-HPLC to afford the desired GS analogues.



Scheme 3. Strategy for the synthesis of *N*-amino GS analogues.

Table 1
GS analogues synthesized by SPPS.



peptide	R^1	R^2	R^3	R^4	yield
GS	H	H	H	H	53
4	NH_2	H	H	H	38
5	H	NH_2	H	H	32
6	NH_2	NH_2	H	H	36
7	NH_2	H	NH_2	H	33
8	NH_2	H	H	NH_2	28
9	H	NH_2	H	NH_2	13
10	NH_2	NH_2	NH_2	H	23
11	NH_2	NH_2	H	NH_2	14
12	NH_2	NH_2	NH_2	NH_2	13

Table 1 depicts the nine GS analogues synthesized for this study, along with their isolated yields following cleavage from the resin, macrocyclization, Boc-deprotection, and purification by RP-HPLC. This series includes all possible combinations of mono-, di-, and tri-aminated variants substituted at the non-H-bonded backbone amides. All macrocycles were characterized by analytical HPLC, HRMS and, NMR.

We tested all analogues for their ability to inhibit the growth of ESKAPE pathogens using a microbroth dilution assay. A minimum inhibitory concentration (MIC) was obtained for each macrocycle and compared against the parent compound. As shown in Table 2, several backbone-aminated analogues exhibited similar or enhanced activity against both Gram-positive and Gram-negative bacterial strains relative to GS. In general, *N*-amination resulted in increased activity against *S. aureus* and *P. aeruginosa*, and decreased activity against *K. pneumoniae*. The effect of backbone substitution was highly dependent on the modified residue. Analogues **4** and **7**, which are mono- and di-aminated at the β -strand region Orn residues, showed improved activity relative

Table 2
Antibacterial activity of **4–12** against ESKAPE pathogens.[a].

	MIC (μM)					
peptide	*Ef(+)*	*Sa(+)*	*Kp(-)*	*Ab(-)*	*Pa(-)*	*Ec(-)*
GS	3	3	40	6.5	20	35
4	3	2	40	6.5	13	30
5	5	4	45	6	20	40
6	3	2	45	6.5	15	15
7	2	2	45	4	10	20
8	3	2	45	6.5	15	30
9	4	4	>50	12.5	35	>50
10	2	2	>50	6	12	>50
11	3	3	50	6.5	20	35
12	2	2	>50	5	15	>50

Ef(+) = Enterococcus faecium 1450
Sa(+) = Staphylococcus aureus 635
Kp(-) = Klebsiella pneumoniae 1433
Ab(-) = Acinetobacter baumanni 5075
Pa(-) = Pseudomonas aeruginosa 1419
Ec(-) = Eneterobacter cloacae 1454

[a]Enhancement or reduction in growth inhibition relative to GS is highlighted (green and red, respectively).

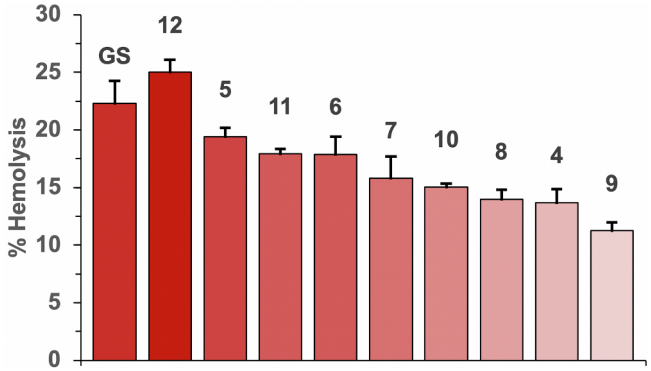


Fig. 4. Hemolytic activity of **4–12** toward hRBCs at 50 μM.

to GS. Compound **7** exhibited the lowest overall MIC values among the backbone-aminated variants. In contrast, D-aPhe analogues **5** and **9** were notably less effective at inhibiting bacterial growth. Macrocycles harboring both Orn and D-Phe *N*-amino groups confirmed this trend, with broad spectrum inhibitory activity that roughly correlated with the ratio of Orn/D-Phe amination (see **6**, **8**, **10**, and **11**). Tetra-aminated macrocycle **12** retained much of the activity of the more potent di-aOrn analogue **7**.

We next determined the ability of GS analogues to lyse human red blood cells (hRBCs). Hemolysis assays carried out a 50 μM concentration revealed that GS leads to 22% toxicity toward hRBCs (Fig. 4). Despite its highly substituted backbone, analogue **12** exhibited slightly higher hemolytic activity relative to GS. The most potent inhibitor of ESKAPE pathogen growth, compound **7**, displayed reduced hemolytic activity (~16%). Although efficacy against bacterial growth did not strictly correlate with hRBC toxicity, the least hemolytic analogue **9** similarly displayed diminished antimicrobial activity.[15]

RP-HPLC retention times of GS derivatives can provide a qualitative measure of amphipathicity, especially when comparing stereo- or regioisomeric analogues.[19] Within their respective groups, mono-, di-, and tri-aminated GS analogues would be expected to have the same intrinsic hydrophobicities. However, disruption of the amphipathic nature of GS typically results in lower RP-HPLC retention times. By this measure, amphipathicity was found to correlate directly with amination of the Orn residues and inversely with D-Phe amination (see Supporting Information). This is presumably due to a disruption of type II' β -turn geometry upon substitution at D-Phe residues. Circular dichroism (CD) spectroscopy further supported the disruptive effect of multiple D-aPhe substitutions on native structure. As shown in Fig. 5, compound **9** featuring two D-aPhe residues deviates significantly from the CD signature of GS, particularly in the β -turn region of the spectrum (200–210 nm). The di-aOrn analogue **7** showed only minor changes relative to the parent structure with native-like minima in both the β

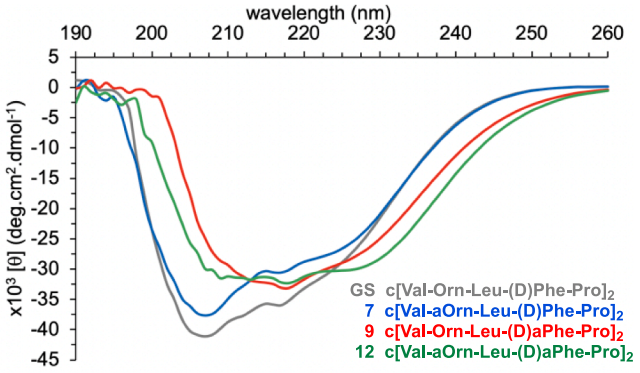


Fig. 5. CD spectra of selected *N*-aminated GS analogues.

-sheet and turn regions. The structure of tetra-aminated analogue **12** was qualitatively most like that of **9**, again demonstrating the disruptive effect of amination at both D-Phe residues.

Structural and biological data from this analogue set reveals that *N*-amination of Orn within GS is beneficial for antimicrobial activity while also reducing toxicity toward hRBCs. This is despite an overall enhancement in amphipathicity that is typically also associated with increased hemolysis. It is tempting to speculate that the *N*-amino group in the β -strand region of the macrocycle is better able to shield polar surface area through the previously observed intraresidue H-bond. The transient nature of this interaction could also serve to decouple antimicrobial activity from hRBC toxicity, although the extent of and reasons for this divergence would require additional studies.

In summary, we have probed the effect of backbone *N*-amination on the biological activity of gramicidin S analogues. As part of this study, we demonstrated the synthesis of NAP macrocycles via incorporation of substituted dipeptide building blocks on solid-support. These *N*-aminated building blocks are resistant to racemization and allow for efficient assembly of mono- and poly-*N*-aminated linear peptides. Among the nine GS analogues evaluated, a macrocycle featuring *N*-amination at both Orn residues (**7**) exhibited enhanced amphipathicity and antimicrobial activity against ESKAPE pathogens, and reduced hemolytic activity relative to GS. This represents the first evaluation of NAPs as antimicrobial agents and provides novel GS analogues with improved therapeutic indices. Backbone aminated macrocyclic peptides such as **7** may thus serve as useful lead structures in design of optimized disruptors of bacterial cell membranes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127283.

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