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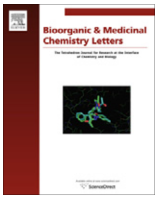

On resin amino acid side chain attachment strategy for the head to tail synthesis of new glutamine containing gramicidin-S analogs and their antimicrobial activity

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ABSTRACT

The alarming increase in infections caused by multiple drug resistant bacteria including methicillin-resistant *Staphylococcus aureus* has prompted a desperate search for new antimicrobials. Augmenting the discoveries of completely new scaffolds with antimicrobial activity are efforts aimed at modifying existing molecules to optimize activity or reduce toxicity. We report herein the parallel solid-phase synthesis of analogues of the cationic antimicrobial peptide gramicidin S (GS) using amino acid side chain attachment strategy. The ornithine (Orn) residues were replaced by glutamine (Gln) and the aromatic D-phenylalanine (Phe) were replaced by different aromatic D-amino acids. Additional Gln containing GS analogues with all the possible combinations of the hydrophobic amino acids valine and leucine were also synthesized. In this work we also report the antibacterial activity of these analogs against several clinically-important drug-resistant Gram-positive and Gram-negative pathogens.

The cyclic decapeptide Gramicidin S (GS) [cyclo-(Val-Orn-Leu-D-Phe-Pro)$_2$] secreted by *Bacillus brevis* is a very stable amphiphilic antiparallel β-sheet structure with a polar and a non-polar surface. It is a powerful antimicrobial agent with potent cidal action on a wide variety of Gram-negative and Gram-positive bacteria as well as on several pathogenic fungi.[1] Numerous reviewers on GS were reported and a large number of studies have been conducted on the relationship between the chemical structure and conformation of GS analogs and their antimicrobial activity.[2] Four major properties were studied, the basic character of Orn residues, the hydrophobic character of the Leu and the Val residues,[2a] the stereochemistry and the aromaticity of D-Phe residues and, the role of proline residues. It was shown that the basic character of the two Orn residues was essential for the antimicrobial activity of GS.[2a] The replacement of one or both Orn with various neutral or acidic amino acids resulted in a major to complete loss of activity. The replacement of both residues with strongly basic Arg or Lys residues maintained the antimicrobial effectiveness.[2d] The hydrophobic character of the Val and Leu proved to be important as well for the antimicrobial activity. The replacement of theses amino acid residues with amino acids, having smaller hydrophobic groups such as Ala or Gly, led to substantial decrease and/or complete loss of activity.[2a] The D-Phe-Pro sequence is very important for the maintenance of the β-sheet structure participating in the two type II′-β-turns.[3] The replacement of the D-Phe with the L-Phe or any other L-amino acid, resulted on destabilization of the β-sheet conformation and consequently to loss of antimicrobial activity.

Herein, we report our work on the parallel synthesis and antimicrobial activity of (GS) analogs where we introduced combined modifications to the hydrophilic, and hydrophobic sides of the GS backbone. The ornithine residues were replaced by glutamine and the aromatic D-Phe residues were replaced with different aromatic D-amino acids (Scheme 1).

The solid phase peptide synthesis (SPPS)[4] of the glutamine containing GS analogs, were performed using amino acid side chain attachment strategy. The anchoring of peptides on resin through the side chain functionalities has been widely employed for the generation of variety cyclic peptides.[5] Starting from MBHA-resin, Fmoc-Glu-O^{t}Bu was attached to the solid support in the presence of DIC and HOBt. The MBHA resin was chosen for its compatibility for both Fmoc and Boc chemistries.[6,7] Following Fmoc deprotection, and subsequent coupling of the amino acids using standard stepwise Fmoc chemistry, the N-terminal amine was deprotected and the *tert*-butyl group on the C$^\alpha$ of glutamic acid was cleaved with a solution of TFA in DCM. The resin-bound linear peptide was treated with HBTU to generate the corresponding cyclic compounds (Scheme 1). The desired glutamine containing GS analogs

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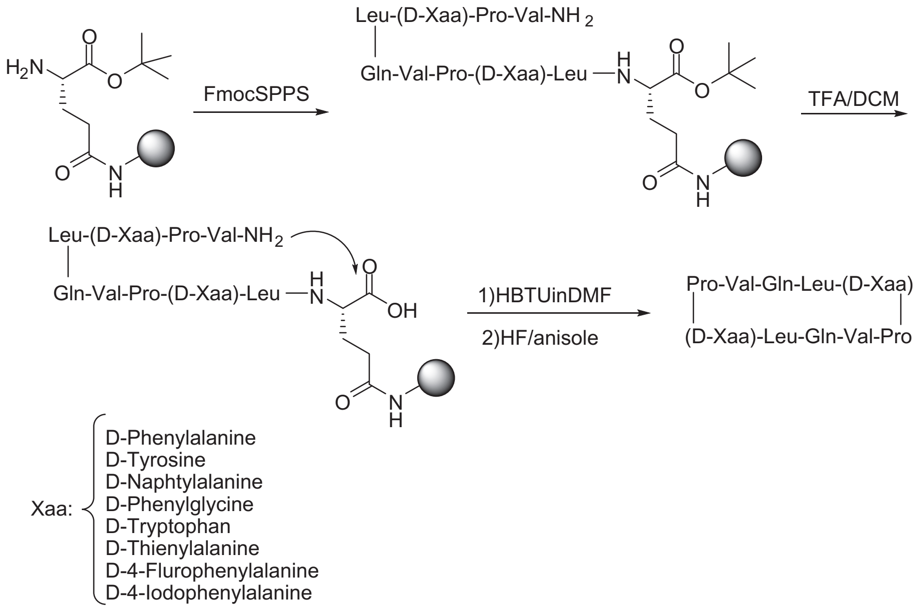


Scheme 1.

were obtained following cleavage of the solid support with HF/anisole in good yields and high purity.

In addition to the Gln-GS analog, we substituted the D-phenylalanine with a variety of D-aromatic amino acids. Thus, seven aromatic amino acids displaying different physicochemical properties were selected (Scheme 1). All the compounds were obtained in good purity and high yields (Table 1).

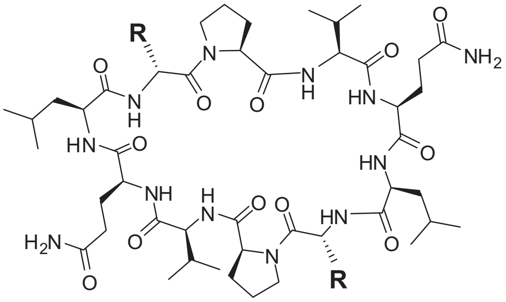
Additional Gln-GS were synthesized in which we performed all the combinations of the two hydrophobic amino acids valine and leucine (Scheme 2). Thus, ten Gln-GS analogs were prepared and tested while maintaining the aromatic D-Phe (Table 2).

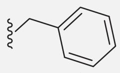
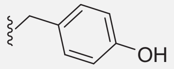
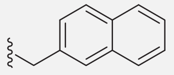
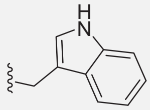
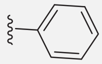
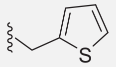
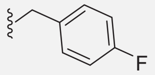
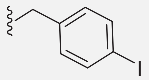
Methods (for biological assays): Susceptibility studies to estimate the minimum inhibitory concentration (MIC) of each analog were done by the microbroth dilution method and included *Streptococcus pyogenes* strain 5448,[8] *Streptococcus agalactiae* strain COH1,[9] *Enterococcus faecalis* (strain ATCC 51299), methicillin-resistant *Staphylococcus aureus* (MRSA) strain ATCC 33591), *Escherichia coli* (strain ATCC 25922), and *Pseudomonas aeruginosa* (strain ATCC 27853). All susceptibility testing was done in Todd–Hewitt broth (THB, Hardy Diagnostics, Santa Maria, CA). Briefly, compounds in 10% dimethylformamide (DMF) were diluted in THB such that the final DMF concentration in the assay was 1% or less. The compounds were tested for evidence of antibacterial activity at 1, 10, and 100 μg/ml. For these experiments an overnight culture of each bacterial strain was diluted in THB and grown to mid-logarithmic phase ($OD_{600} \sim 0.4$) and then added to the diluted compounds at 5×10^5 colony-forming units/ml in a final volume of 100 μl in a 96-well microtiter test plate (Costar, Corning, NY). The test plate was covered and incubated at 37 °C for 16–20 h in a stationary incubator (for the *Streptococcus* strains) or in a shaking incubator (for *Staphylococcus, Enterococcus, E. coli, and P. aeruginosa*). The approximate minimum inhibitory concentration (MIC) was then determined to be the lowest concentration that inhibited detectable bacterial growth at OD_{600}.

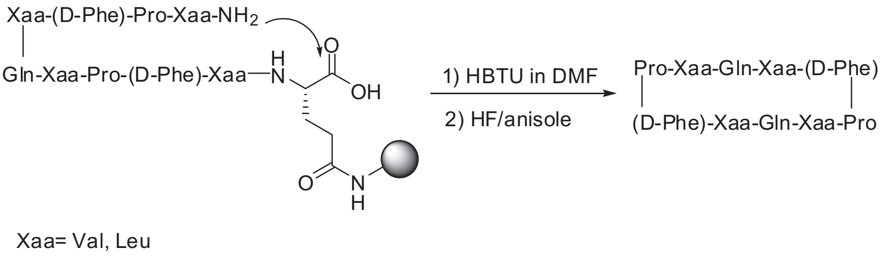
The compounds were screened for their ability to induce red blood cell lysis as a measure of cytotoxicity. Briefly, 1 ml of defibrinated sheep blood (catalog #SB-15, Hardy Diagnostics, Santa Maria, CA) was washed in Dulbecco's phosphate-buffered saline (DPBS, Mediatech, Manassas, VA) to remove lysed red cells. The

Table 1

The products were run on a Vydac column (C18, 3 μm, 100 Å, 3 × 250 mm), gradients 5–95% solvent B (0.1% TFA in ACN) in 7 min. The purity was estimated on analytical traces at $\lambda = 214$ nm



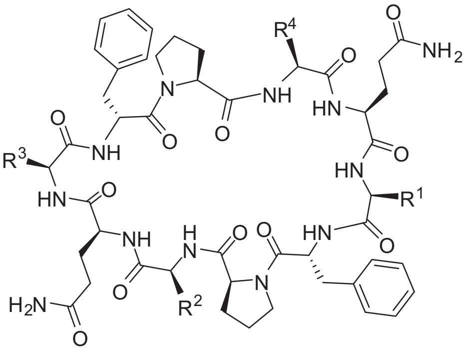
Entry	Xaa	R	Purity (%)
GS-15	D-Phenylamine		86
GS-17	D-Tyrosine		75
GS-17	D-Naphtylalanine		83
GS-18	D-Tryptophan		82
GS-19	D-Phenylglycine		84
GS-20	D-Thienylalanine		87
GS-21	D-4-Flurophenylalanime		88
GS-22	D-4-Lodophenylalanine		86



Scheme 2.

Table 2

The products were run on a Vydac column (C18, 3 µm, 100 Å, 3 × 250 mm), gradients 5–95% solvent B (0.1% TFA in ACN) in 7 min. The purity was estimated on analytical traces at λ = 214 nm



Entry	Xaa$_1$	Xaa$_2$	Xaa$_3$	Xaa$_4$	Purity (%)
GS-1	Val	Val	Val	Val	83
GS-11	Val	Val	Val	Leu	85
GS-2	Val	Val	Leu	Val	87
GS-3	Val	Leu	Val	Val	82
GS-9	Val	Val	Leu	Leu	88
GS-12	Val	Leu	Leu	Val	84
GS-8	Leu	Val	Leu	Val	86
GS-6	Val	Leu	Leu	Leu	85
GS-7	Leu	Leu	Leu	Leu	86
GS-5	Leu	Leu	Leu	Leu	82

washed cells were resuspended to 2% in DPBS. Compounds that were diluted to 2× the test concentration in DPBS in a volume of 100 µl were added to wells of a V-shaped microtiter test plate (Costar, Corning, NY). The washed red cells (100 µl) were then added to the compound dilutions and mixed gently. The plate was covered and incubated at 37 °C for 1 h, after which the remaining intact red cells were removed by centrifugation, and 100 µl of the supernatant was removed for assay of released hemoglobin at OD$_{450}$. Lysis in the presence of the compounds was expressed as a percentage of the maximal lysis obtained by addition of Triton X-100 (0.5%).

Results (for biological assays): The GS analogs were screened for evidence of inhibitory activity against several clinically-important Gram-positive and -negative pathogens by broth microdilution. Although the parent compound, GS, inhibited the growth of several Gram-positive pathogens including MRSA, *S. pyogenes*, *S. agalactiae*, and *E. faecalis* and the Gram-negative pathogen *E. coli* at 10 µg/ml, very little activity was observed for the GS analogs. Only *S. pyogenes* was very marginally sensitive to twelve of the eighteen GS analogs (Table 3), since inhibition of detectable bacterial growth was observed only at 100 µg/ml and not at 10 µg/ml. Several of the D-aromatic amino acid-substituted analogs (17, 19, 21, and 22) exhibited low activity against *S. pyogenes*. Furthermore, the presence of at least one Leu residue in the Gln-GS analogs (Scheme 2) may contribute to the activity observed for some of these analogs, as compound **1**, which lacks the Leu residue, exhibited no activity.

The ability of the GS analogs to induce red blood cell lysis was examined as an indicator of potential cytotoxicity. The analogs (fi-

Table 3

Analog activity against Gram-positive and Gram-negative pathogens

Compound ID	*S. pyogenes*	*S. agalactiae*	*MRSA*	*E. faecalis*	*E. coli*	*P. aeruginosa*
GS-1	0	0	0	0	0	0
GS-2	*	0	0	0	0	0
GS-3	0	0	0	0	0	0
GS-5	*	0	0	0	0	0
GS-6	*	*	0	0	0	0
GS-7	*	0	0	0	0	0
GS-8	*	0	0	0	0	0
GS-9	*	0	0	0	0	0
GS-11	*	0	0	0	0	0
GS-12	*	0	0	0	0	0
GS-16	0	0	0	0	0	0
GS-17	*	0	0	0	0	0
GS-18	0	0	0	0	0	0
GS-19	*	0	0	0	0	0
GS-20	0	0	0	0	0	0
GS-21	*	0	0	0	0	0
GS-22	*	0	0	0	0	0
GS-23	0	0	0	0	0	0
GS-24	***	***	***	***	*	0

0 = No activity (MIC >100 µg/ml).

* Low activity (MIC greater than 10 but less than 100 µg/ml).

*** Significant activity (MIC less than or equal to 10 µg/ml).

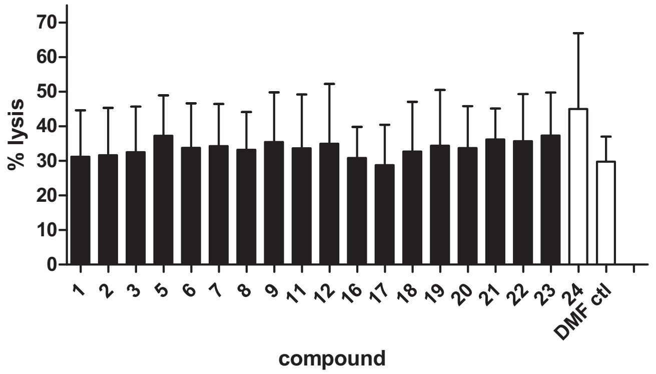


Figure 1. Ability of GS compounds to induce red blood cell lysis. GS compounds were diluted in PBS and added to the wells of a 96-well plate. Washed sheep red blood cells were added to each well, and the plates were incubated for 1 h at 37 °C. Released hemoglobin, indicative of lysis, was measured in the supernatant at A_{450} nm and expressed as a percentage of the lysis induced by 0.5% Triton X-100. Data are shown as the mean ± standard deviation from two independent experiments.

nal concentration = 100 μg/ml) were incubated with washed red blood cells for 1 h at 37 °C. The intact red cells were pelleted, and the supernatants were then examined for the presence of released hemoglobin as an indicator of lysis. Although the vehicle control (1% dimethylformamide) induced some lysis, the percent red cell lysis was slightly increased for red cells treated with the parent compound (#24) at 100 μg/ml compared to the vehicle control as expected (Fig. 1), but this was not statistically significant. In addition, several GS analogs (e.g., #5 and 23) also caused a very slight increase in red cell lysis over the vehicle control, while other analogs (e.g., #17) did not demonstrate this, although these trends were also not statistically significant. These data indicate that these compounds likely retain much of the cytotoxicity apparent in the parent GS compound.

We presented the parallel solid-phase synthesis and the antibacterial activity of glutamine containing gramicidin-S analogues. The replacement of ornithine (Orn) with glutamine and the aromatic D-phenylalanine with different aromatic D-amino acids resulted in decrease of antimicrobial activity. The proposed highly yielding approach for the solid phase synthesis of cyclic peptides can be useful for the combinatorial synthesis of large numbers of cyclic peptides.

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