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A Practical Synthesis of Gramicidin S and Sugar Amino Acid Containing Analogues

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A practical gram-scale and high-yielding synthesis of the antimicrobial peptide gramicidin S is presented. An Fmoc-based solid-phase peptide synthesis protocol is employed for the generation of the linear decapeptide precursor, which is cyclized in solution to afford the target compound. The versatility of our method is demonstrated by the construction of eight gramicidin S analogues (**15a**−**h**) having nonproteinogenic sugar amino acid residues (**4**−**7**) incorporated in the turn regions.

Introduction

Gramicidin S (GS) is a naturally occurring antimicrobial peptide that upon accretion on lipid bilayers inflicts a loss of barrier function of cellular membranes. Despite the capacity to lyse microbial cells, the therapeutic value of GS has been restricted to topological applications, since GS also exhibits strong toxicity against human erythrocytes.[1] Due to its potent cidal action toward pathogenic bacteria and fungi, GS and analogues thereof have been extensively studied to elucidate the structure−function relationships. Although the exact mode of action has not yet been resolved, the membrane disruptive properties of GS appear to be contingent on its amphipathic structure. GS (Scheme 1) has the primary sequence *cyclo*-(^{D}Phe-Pro-Val-Orn-Leu)$_2$ and adopts a C_2-symmetric β-sheet structure that is stabilized by four interstrand hydrogen bonds between the Leu and Val residues. The ^{D}Phe-Pro dipeptide sequences hold the $i + 1$ and $i + 2$ position in two type II′ β-turns that further contribute to the stabilization of the pleated sheet structure. In this configuration, the hydrophobic (i.e. Val, Leu) and hydrophilic (i.e. Orn) residues of the two antiparallel β-strands are positioned on opposite sides of the molecule.[2]

An efficient synthetic route toward GS that allows for sequence modifications or incorporation of nonproteinogenic residues is a prerequisite for the tuning of biological properties and to shed further light on the structure−activity relationships. Over the years, several strategies toward the synthesis of GS and its analogues have appeared in the literature.[1a] The earliest accounts involve solution phase synthesis of fragments of the linear decapeptide, followed by connection of these to give the linear decapeptide, which is subsequently cyclized under highly dilute conditions. From these studies it became apparent that specific pentameric sequences have the ability to preorganize into β-hairpin structures that subsequently undergo cyclodimerization. This biomimetic synthesis method was in later years exploited by others, and us, for the construction of GS analogues containing nonnatural amino acids.[3]

After the advent of solid-phase peptide synthesis (SPPS), several protocols have been successfully applied to generate GS and analogues thereof. Early examples involve the assembly of linear, side-chain protected decapeptide precursors by using the Merrifield resin in combination with Boc-chemistry followed by cleavage from the solid support, cyclization, deprotection, and purification.[4] A modification of this procedure was developed by Wishart et al. and entails the use of the

[#] With deep sadness we inform the reader that our colleague, Jacques van Boom, died on July 31th, at the age of 67.

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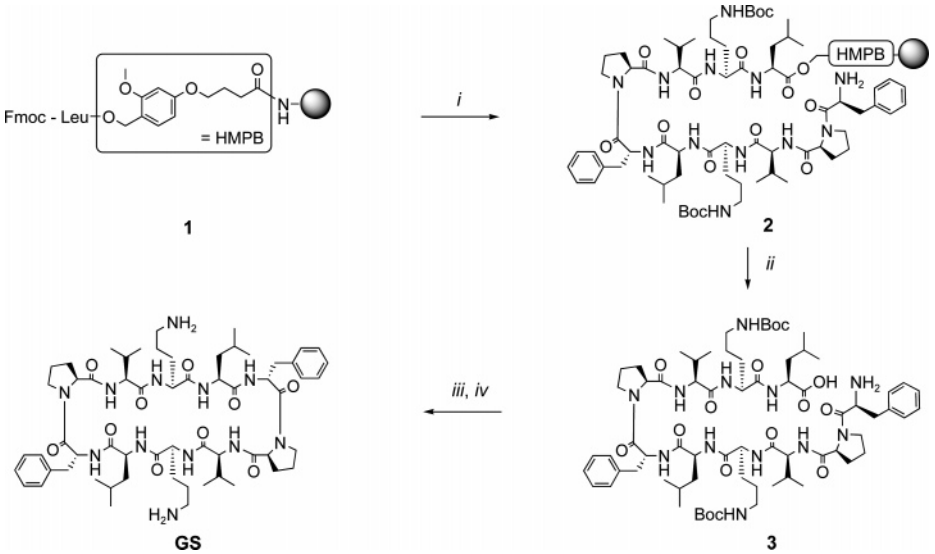
SCHEME 1[a]



[a] Reagents and conditions: (i) Repetitive deprotection; piperidine/NMP (1/4 v/v), condensation; Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-^{D}Phe-OH or Fmoc-Leu-OH (3 equiv), BOP (3 equiv), HOBt (3 equiv), DiPEA (3.6 equiv), NMP; (ii) TFA/DCM (1/99 v/v) 4 × 10 min; (iii) PyBOP (5 equiv), HOBt (5 equiv), DiPEA (15 equiv), DMF, 16 h, 81%; (iv) TFA/DCM (1/1 v/v), 30 min, quant.

4-hydroxymethylphenylacetamidomethyl (PAM) resin in combination with Boc-chemistry.[5] After acidolytic release of the peptide from the resin with concomitant removal of the Z-protection groups from the Orn residues and HPLC purification of the linear peptide, cyclization affords GS and analogues in good yield. Recent developments of novel resin-anchoring methods allow the preparation of GS and analogues, in either protected or unprotected form through exclusive solid-phase chemistry. Specifically, on-resin cyclization[6] and cyclization−cleavage protocols by employing the oxime linker[7] and the safety catch linker[8] have proven effective in the synthesis of GS-like peptides.[9]

Upon perusal of the different strategies, we reasoned that modification of those that are based on solid-phase synthesis of the linear decapeptide and ensuing solution-phase cyclization, with a novel resin and linker system, could unite a mild Fmoc-based SPPS strategy with some advantageous features such as high yields, variable scale, straightforward purification, and the possibility to limit or abolish intermediate HPLC purification steps. We here report the validity of this approach in the efficient synthesis of gramicidin S on a gram-scale, employing the 4-methylbenzhydrylamine (MBHA) resin in combination with the 4-(4-hydroxymethyl-3-methoxyphenoxy)butanoic acid (HMPB) linker system. The versatility of our approach is demonstrated in the construction of GS analogues in which either a single or both reverse turn dipeptide sequences have been replaced with selected sugar amino acids (SAAs).[10] In a recent contribution from our laboratory we revealed that the incorporation of carbohydrate-derived peptidomimetics into the turn region of GS could have a profound effect on the turn as well as the overall structure of the β-sheet of the peptide.[11] Structural and functional data, including antimicrobial and hemolytic activity, of the novel GS analogues are presented.

Results and Discussion

The synthesis of GS commences (Scheme 1) with the installation of the acid-labile HMPB-linker on MBHA-functionalized polystyrene. Subsequent esterification with Fmoc-Leu-OH using *N*,*N*′-diisopropylcarbodiimide (DIC) and a catalytic amount of 4-(dimethylamino)-pyridine (DMAP) furnished loaded resin **1** (0.50 mmol/g).[3c] Further elongation of the peptide was effected by standard SPPS (1.1 mmol scale), employing 20% piperidine in NMP for the liberation of the α-amine functionality followed by condensation of the appropriate Fmoc-protected amino acid building block (3 equiv) with Castro's reagent[12] (3 equiv), *N*-hydroxybenzotriazole (HOBt, 3 equiv), and DiPEA (3.6 equiv). The immobilized decapeptide **2** was subsequently released from the resin

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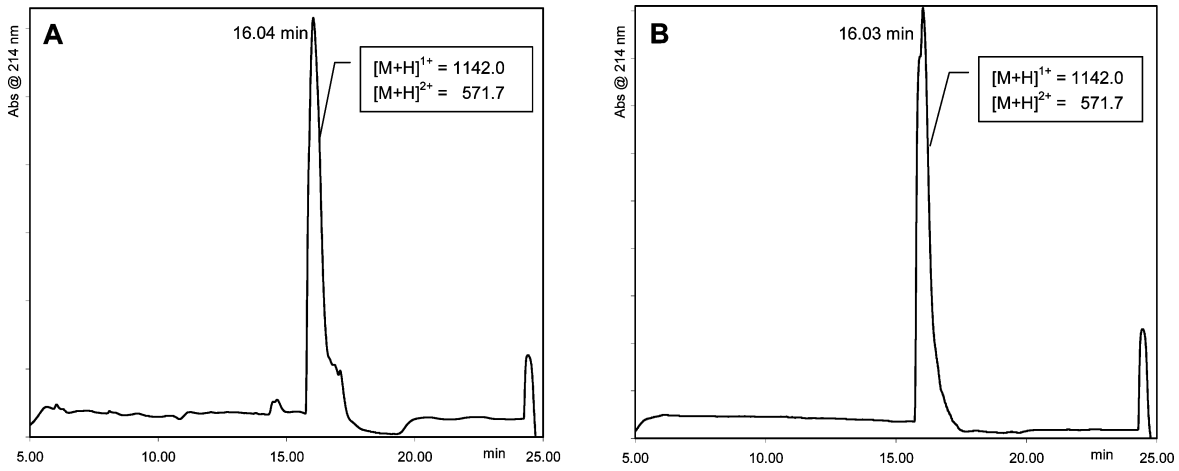
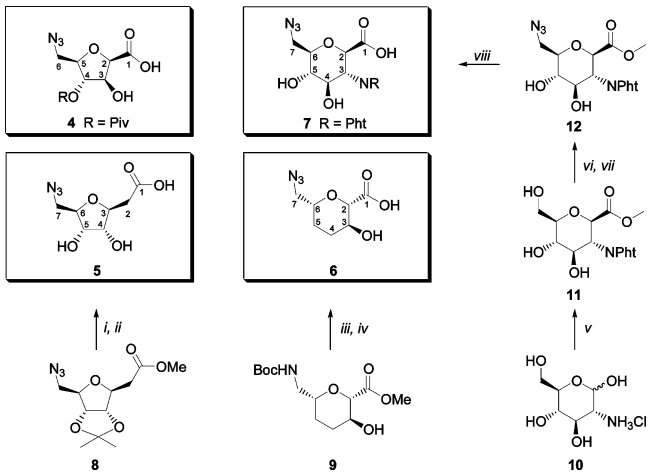


FIGURE 1. HPLC trace of GS before (A) and after (B) semipreparative HPLC.

by mild acidic cleavage (1% TFA in DCM). Next, the crude linear peptide **3** was dissolved in DMF and added dropwise, over a period of 60 min, to a vigorously stirred solution of benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 5 equiv), HOBt (5 equiv), and DiPEA (15 equiv) to give a final concentration of 1.3×10^{-3} M of peptide. This mixture was then stirred overnight, concentrated in vacuo, applied to a Sephadex size exclusion column, and eluted with MeOH. The fractions containing Boc-protected GS were pooled and concentrated, yielding a white amorphous solid (1.19 g, 0.89 mmol, 81%). Deprotection of the exocyclic amine functionalities by acidolysis (50% TFA in DCM) followed by LC/MS analysis afforded gramicidin S in 87% purity (Figure 1A). For analytical purposes, a small portion of GS was purified by semipreparative HPLC and subsequently lyophilized. The homogeneity of the product was confirmed by LC/MS analysis (Figure 1B). All spectroscopic and spectrometric data of GS were in full agreement with those reported in the literature.[3c,5,9a,13]

To establish the versatility of the above-presented route in the preparation of GS analogues, we set out to synthesize a set of turn region modified analogues. Accordingly, we selected four SAA building blocks (**4**, **5**, **6**, and **7**, Scheme 2) and applied these for the construction of GS-analogues **15a**−**d**, which have a single type II′ β-turn replaced, and **15e**−**f**, which have both D-Phe-Pro dipeptide sequences substituted (Scheme 3). The synthesis of the furanoid SAA building block **4** has been previously reported by our laboratory.[11] Removal of the isopropylidene protection group in **8**[14] by acidic methanolysis followed by saponification of the methyl ester afforded **5** in 80% yield over the two steps. The partially deoxygenated gluconic amino acid **9**[15] was transformed by acidic deblocking (50% TFA in DCM) of the Boc-protected amine, installation of the azide group by Cu-catalyzed diazo-transfer in a procedure developed by Wong and co-workers,[16] and saponification of the methyl ester, to give **6** in 58% yield over 2 steps. Finally, the

SCHEME 2[a]



[a] Reagents and conditions: (i) 2 M HCl/MeOH (1/3 v/v), 16 h, 82%; (ii) 1 M NaOH/THF (1/1 v/v), 3 h, then Amberlite IR-120 (H^+), 98%; (iii) (a) TFA/DCM (1/1 v/v), 30 min; (b) TfN_3 (2 equiv), K_2CO_3, $CuSO_4$ (cat.), H_2O, MeOH, 16 h, 58%; (iv) 0.2 M LiOH/1,4-dioxane (5/4 v/v), 3 h, then Amberlite IR-120 (H^+), 98%; (v) see ref 17; (vi) TosCl (1.1 equiv), pyridine, 16 h, 73%; (vii) NaN_3 (10 equiv), DMF, 80 °C, 48 h, 85%; (viii) 1 M HCl/AcOH (1/1 v/v), 60 °C, 3 h, quant.

novel β-D-glucosaminopyranosyl template **7** was prepared through adaptation of the synthetic strategy developed by Ichikawa and co-workers.[17] Starting from D-(+)-glucosamine hydrochloride (**10**), the *N*-phthaloyl protected methyl ester **11** was obtained in a straightforward manner in 6 steps. Regioselective tosylation of the primary hydroxyl function proceeded in 73% yield and subsequent nucleophilic displacement with sodium azide

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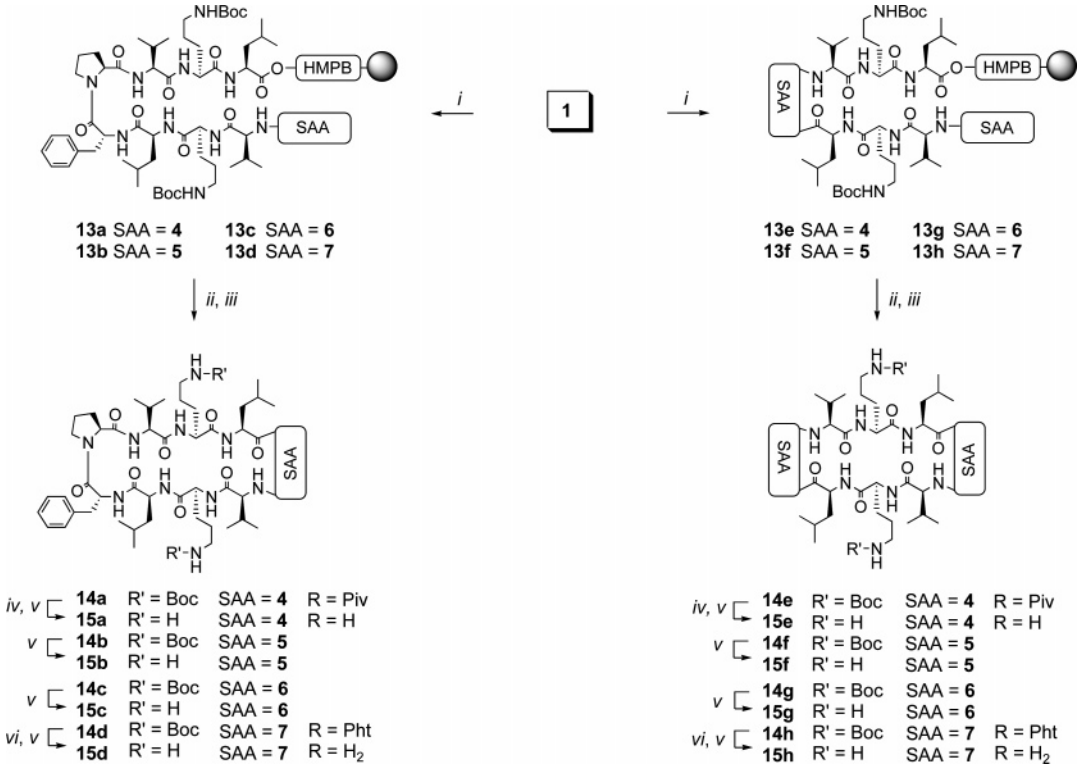
SCHEME 3[a]



[a] Reagents and conditions: (i) Fmoc deprotection: piperidine/NMP (1/4 v/v), azide deprotection: PMe_3 (16 equiv), 1,4-dioxane/H_2O (10/1 v/v); condensation: Fmoc-aa-OH (3 equiv) or SAA **4**, **5**, **6**, and **7** (2 equiv), BOP (3 equiv), HOBt (3 equiv), DiPEA (3.3 equiv), NMP, 90 min; (ii) TFA/DCM (1/99 v/v), 4 × 10 min; (iii) PyBOP (5 equiv), HOBt (5 equiv), DiPEA (15 equiv), DMF, 16 h, **14a**, 96%; **14b**, 63%; **14c**, 85%; **14d**, 78%, **14e**, 36%; **14f**, 43%; **14g**, 72%; **14h**, 78%; (iv) NaOMe (16 equiv), MeOH, 16 h, then Amberlite IR-120 (H^+); (v) TFA/DCM (1/1 v/v) 30 min; (vi) $H_2NNH_2 \cdot H_2O$ (50 equiv), MeOH, 65 °C, 16 h.

afforded the 2,6-dideoxy sugar **12** in 85%. Hydrolysis of the methyl ester under acidic conditions furnished SAA **7** quantitatively.[18]

Having the sugar amino acid building blocks **4**, **5**, **6**, and **7** in hand, attention was focused on their incorporation into GS, as is outlined in Scheme 3. The construction of the first four targets, having a single SAA substitution, comprises the stepwise elongation of the first seven amino acids, starting from Fmoc-protected leucine on a HMPB-MBHA resin **1**, in a similar manner as described for GS. Ensuing condensation with SAAs **4**, **5**, **6**, and **7** gave immobilized nonapeptides **13a**−**d**, respectively. To ensure complete condensation, an excess of 3 equiv of coupling reagents (i.e. BOP, HOBt) and 2 equiv of the SAA building block was employed. Next, the azide functionalities were subjected to Staudinger reduction to liberate the terminal amines. The linear resulting peptides were released from the solid support through mild acidolysis and cyclized following the same procedure as for GS. This led to the isolation of homogeneous, fully protected GS analogues **14a** (96%),[11] **14b** (63%), **14c** (85%), and **14d** (78%), respectively. The assembly of the final four targets, having a sugar amino acid scaffold in both turn regions, commenced with resin **1** that was sequentially elongated with Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, and the appropriate SAAs (i.e. **4**, **5**, **6**, and **7**). Subjection of the thus obtained immobilized peptides to Staudinger reduction resulted in the formation of the terminal amines. Further elongation applying the proper amino acid building blocks gave the anchored linear peptides **13e**−**h**. Abiding by the abovementioned three-step procedure for solid support release, cyclization, and purification, the cyclic peptides **14e**−**h** were obtained in their respective yields of 36%, 43%, 72%, and 78%. The protected GS analogues **14a**−**h** were transformed into their unprotected counterparts by basic methanolysis of the pivaloyl esters (in the case of **14a** and **14e**), hydrazinolysis of the *N*-phthaloyl amide (in the case of **14d** and **14h**), and finally treatment with 50% TFA in DCM. HPLC purification led to homogeneous cyclic peptides **15a**−**h** as gauged by LC/MS analysis.

At this stage, GS analogues **15a**−**h** were subjected to ^{1}H NMR studies and the results were compared with proton NMR data of native GS. The resonance assignment of the assembled GS analogues was undertaken by using a combination of COSY, TOCSY, and ROESY data sets. It was gratifying to establish that GS analogues **15a**−**h** showed large resonance dispersion, allowing for facile and unambiguous assignment of all residues. Perusal of the acquired data subsequently enabled the identification of the presence of secondary structure elements in those peptides. In this respect, it has been postulated that the vicinal spin−spin coupling constants can be indicative of turn and β-sheet structures.[19] For

(18) In an alternative procedure, it was established that saponification of the methyl ester led to a diastereoisomeric mixture of acids.

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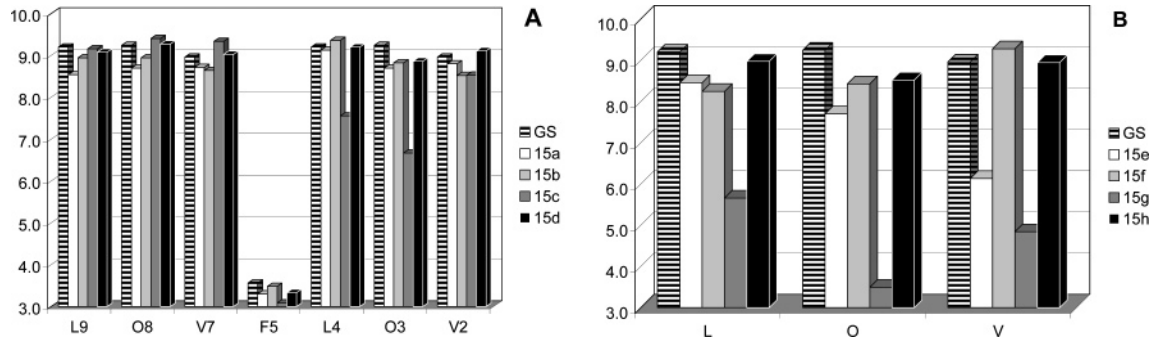

FIGURE 2. Coupling constants ($^3J_{HN\alpha}$) found in GS analogues **15a**−**d** (A) and **15e**−**h** (B) in hertz.

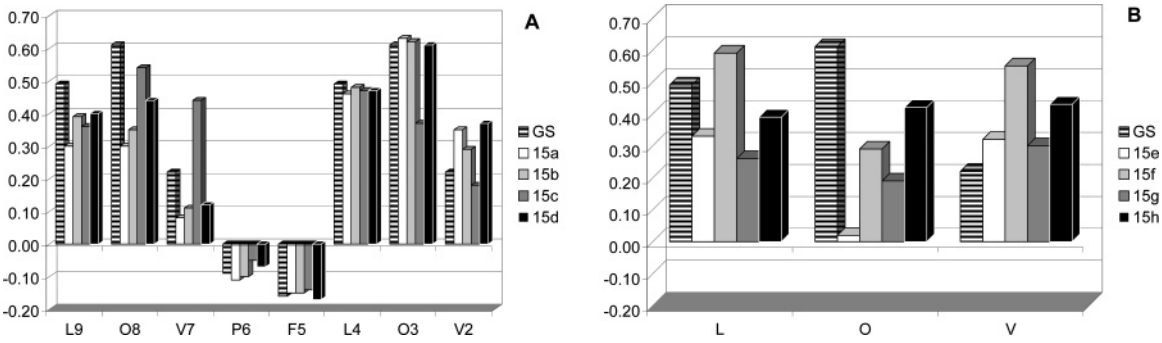

FIGURE 3. Chemical shift perturbation ($\Delta\delta H_\alpha$ = observed δH_α − random coil δH_α) found in GS analogues **15a**−**d** (A) and **15e**−**h** (B).[3c,13,21]

example, in native GS, the $^3J_{HN\alpha}$ values of the Val, Orn, and Leu vary between 8.5 and 9.0 Hz and correspond to those found in β-sheet structures. Furthermore, the $^3J_{HN\alpha}$ of the ^{D}Phe residues are typically small (<4 Hz) as they occupy a position in the turn regions.[3c] Therefore, the vicinal coupling constants found in peptides **15a**−**d** (Figure 2A) that are largely idiosyncratic to GS strongly suggest that these analogues adopt a conformation closely related to that assumed by the native peptide. Only a small deviation of the coupling constants toward random coil values was observed in a single β-strand of GS analogue **15c**. Consequently, as we demonstrated earlier for **15a**,[11] these single SAA residues do not appear to interfere with β-sheet formation. Rather, they induce a turn conformation that can be locally distorted. For GS analogues **15e-h**, the spectra collapse into a unique set of resonances of four amino acid residues (i.e. Val, Orn, Leu, and the appropriate SAA) that signify C_2-symmetric peptides reminiscent of native GS. The furanoid ϵ-SAA **5** and pyranoid δ-SAA **7** in peptides **15f** and **15h**, respectively, display $^3J_{HN\alpha}$ values charactaristic of a β-sheet structure (Figure 2B). However, in peptides **15e** and **15g**, featuring furanoid δ-SAA **4** and pyranoid δ-SAA **6**, spectral line broadening was observed and the vicinal spin−spin coupling constants are considerably smaller compared to GS, representing a lesser degree of β-sheet formation.

An alternative ^{1}H NMR spectral analysis focuses on the position of the NMR lines of the individual amino acids. Wishart and co-workers have defined the perturbation of chemical shift as the difference between the measured chemical shift for the H_α of an amino acid and the H_α chemical shift value of the same residue reported for a random coil peptide.[20] When three or more successive residues have $\Delta\delta H_\alpha > 0.1$ ppm, it can be assumed that the peptide exists in an extended β-strand conformation. In the case of GS analogues **15a**−**d**, the secondary chemical shifts follow a similar trend compared to the native peptide (Figure 3A). The large values found in the Leu-Orn-Val tripeptide sequences confirm that both are involved in β-strand formation, whereas the negative values of the Pro and ^{D}Phe residues imply the presence of a turn motif, further validating a β-sheet conformation for peptides **15a**−**d**. The chemical shift perturbation of the Leu, Orn, and Val residues found in GS analogues **15e**−**h** (Figure 3B) show the largest values for peptides **15f** and **15h**. Positive values for peptides **15e** and **15g** were also observed although these proved to be considerably smaller, corroborating the data found in the $^3J_{HN\alpha}$ values.

The potential of peptides **15a**−**h** as antibacterial agents was assessed by employing a standard minimal inhibitory concentration (MIC) assay against four Gram-positive and two Gram-negative bacterial strains.[3c] The results of these tests demonstrate that GS analogues **15a**−**d** have substantially lost activity against the Gram-positive strains (Table 1). Generally, the *S. epidermidis* strain is still the most sensitive toward lysis by these antimicrobial peptides. GS analogue **15c** proved to be the most active in this series. Peptides **15e**−**h** show a complete loss of activity against all bacterial strains in this assay.

The hemolytic activity toward human erythrocytes of **15a**−**h** was examined by a standard 2-fold dilution assay

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TABLE 1. Antimicrobial Activity (MIC in μg/mL)[e]

	S. aureus[a]		*S. epidermidis*[a]		*S. epidermidis*[a]		*B. cereus*[a]		*E. coli*[b]		*P. aeruginosa*[b]	
peptide	25W[c]	MT[d]	25W[c]	MT[d]	25W[c]	MT[d]	25W[c]	MT[d]	25W[c]	MT[d]	25W[c]	MT[d]
GS	4	4	2	2	8	8	2	4	64−>64	>64	>64	>64
15a	64	64	8−16	16−32	>64	>64	16	16−32	>64	>64	>64	>64
15b	>64	>64	32−64	32−64	>64	>64	32	64−>64	>64	>64	>64	>64
15c	32	64	16	16	>64	>64	>64	16−32	>64	>64	>64	>64
15d	64	>64	16	16	>64	>64	16−32	64	>64	>64	>64	>64
15e	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
15f	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
15g	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
15h	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64

[a] Gram-positive. [b] Gram-negative. [c] 3 mL/25 well plates. [d] 100 μL/96 microtiter plates. [e] Measurements were executed using standard agar 2-fold dilution techniques.

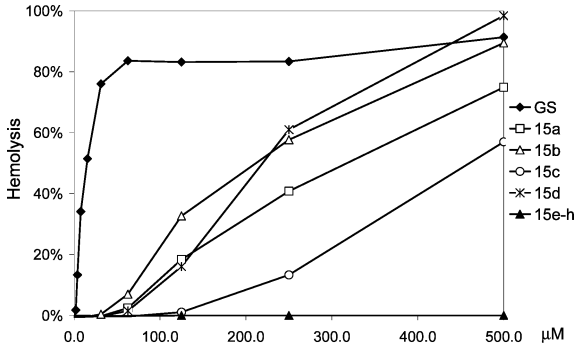

FIGURE 4. Hemolytic activity of GS analogues **15a**−**h**.

of the appropriate peptide, interpolating between a blank measurement and 100% lysis induced by 1% Triton X-100 in saline. As can be seen in Figure 4, peptides **15a**−**d** displayed a reduced toxicity profile, showing appreciable lysis only around 500 μM, as compared to 32 μM for native GS. Furthermore, peptides **15e**−**h** lost all toxicity toward human erythrocytes. Since these results correlate with the abovementioned antimicrobial activity and the same trend for antimicrobial activity and hemolytic activity was observed, it can be concluded that the therapeutic value of the peptides presented in these studies is limited.

In summery, we have presented a highly efficient synthesis strategy toward gramicidin S. The strategy proved to be sufficiently versatile for the incorporation of nonproteinogenic sugar amino acids **4**, **5**, **6**, or **7**, furnishing eight GS analogues **15a**−**h** in moderate to good yields, and necessitating only a single HPLC purification step. Earlier analogous methods rely on the HPLC purification of both the linear precursor and cyclic product, posing limits to the scale of the synthesis. Moreover, the harsh reaction conditions for release of GS peptides from the resin employed in most common methods are often not compatible with conventional protecting groups. The ^{1}H NMR characterization of the GS analogues **15a**−**h** revealed that these peptides prevalently adopt a β-sheet secondary structure. Assaying their biological profile showed a deleterious effect on the antimicrobial activity with a similar decrease in toxicity toward human erythrocytes.

Experimental Section

General. Reactions were monitored by TLC analysis, using DC-fertigfolien (Schleicher & Schuell, F1500, LS254) with detection by spraying with 20% H_2SO_4 in EtOH, $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25 g/L), and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) in 10% sulfuric acid or by spraying with a solution of ninhydrin (3 g/L) in EtOH/AcOH (20/1 v/v), followed by charring at ~150 °C. Column chromatography was performed on Fluka silicagel (0.04−0.063 mm) and size exclusion chromatography on Sephadex LH-20. For LC/MS analysis, an HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical C_{18} column (4.6 mm D × 250 mm L, 5 μm particle size) in combination with buffers (A) H_2O, (B) MeCN, and (C) 0.5% aq TFA and coupled to a mass instrument with a custom-made Electronspray Interface (ESI) was used. For reversed-phase HPLC purification of the peptides, an automated HPLC system supplied with a semipreperative C_{18} column (10.0 mmD × 250 mmL, 5μ particle size) was used. The applied buffers were (A) H_2O, (B) MeCN, and (C) 1.0% aq TFA.

General Procedure for Peptide Synthesis. (a) Stepwise elongation: Resin **1** (2.2 g, 0.5 mmol/g, 1.1 mmol) was submitted to nine cycles of Fmoc solid-phase synthesis with use of commercially available building blocks in the order Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-^{D}Phe-OH, Fmoc-Leu-OH, Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, and Fmoc-^{D}Phe-OH: (a) deprotection with piperidine in NMP (1/4 v/v, 30 mL, 15 min); (b) washing with NMP (30 mL, 3 × 3 min); (c) coupling of the appropriate Fmoc amino acid (3 equiv, 3.3 mmol) in the presence of BOP (3 equiv, 1.46 g, 3.3 mmol), HOBt (3 equiv, 446 mg, 3.3 mmol), and DiPEA (3.6 equiv, 664 μL, 3.9 mmol) which was preactivated for 2 min in NMP (30 mL) and shaken for 90 min; and (d) washing with NMP (30 mL, 3 × 3 min). Couplings were monitored for completion by the Kaiser test[22] and Val-residues were standardly immobilized by applying a double coupling procedure.

(b) Cleavage from the resin: The *N*-terminal amine was liberated with piperidine in NMP (1/4 v/v, 30 mL, 15 min) followed by washing with NMP (30 mL, 3 × 3 min) and DCM (30 mL, 3 × 3 min). Afterward, peptide **2** was treated with TFA/DCM (1/99, v/v, 40 mL, 4 × 10 min). The fractions were collected and coevaporated with toluene (250 mL) three times to give crude linear peptide **3** that was directly cyclized without further purification.

(c) Cyclization: Crude **3** was taken up in DMF (55 mL) and slowly added to a solution of PyBOP (5 equiv, 2.86 g, 5.5 mmol), HOBt (5 equiv, 743 mg, 5.5 mmol), and DiPEA (15 equiv, 2.87 mL, 16.5 mmol) in DMF (800 mL) over the period of an hour and subsequently allowed to stir for 16 h. The mixture was concentrated and directly applied to a Sephadex LH-20 column (50.0 mm D × 1500 mm L) that was eluted with MeOH to yield Boc-protected GS as a white amorphous solid (1.19 g, 0.89 mmol, 81%).

(21) To enhance solubility, CD_3OH was used as solvent. The δH_α values of the amino acid residues in GS are not significantly affected when using methanol instead of water as the solvent system.

(22) Kaiser, E.; Colescott, R. L.; Bossering, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.

(d) Deprotection: The cyclic peptide (124 mg, 92 μmol) was dissolved in DCM (5 mL) and the solution cooled to 0 °C. TFA (5 mL) was added slowly and the mixture was stirred for 30 min, after which all volatiles were removed in vacuo. The crude product was analyzed by LC/MS (R_t 16.03 min, linear gradient 50→90% B in 26 min; *m/z* 1142.0 $[M + H]^+$, 571.7 $[M + H]^{2+}$) and purified by RP-HPLC (linear gradient of 3.5 CV; 40→75% B; R_t 3.2 CV) and the combined fractions lyophilized to produce GS (82 mg, 72 μmol, 78%) as a white amorphous powder. ^{1}H NMR (400 MHz, CD_3OH): δ 8.90 (d, 1H, NH ^{D}Phe, $J_{NH,H\alpha}$ = 3.6 Hz), 8.80 (d, 1H, NH Leu, $J_{NH,H\alpha}$ = 9.2 Hz), 8.70 (d, 1H, NH_α Orn, $J_{NH,H\alpha}$ = 9.2 Hz), 7.73 (d, 1H, NH Val, $J_{NH,H\alpha}$ = 9.0 Hz), 7.33−7.24 (m, 5H, H_{ar} ^{D}Phe), 4.97 (m, 1H, H_α Orn), 4.66 (m, 1H, H_α Leu), 4.50 (m, 1H, H_α ^{D}Phe), 4.35 (m, 1H, H_α Pro), 4.17 (m, 1H, H_α Val), 3.73 (m, 1H, $H_{\delta d}$ Pro), 3.10 (m, 1H, $H_{\beta d}$ ^{D}Phe), 3.05 (m, 1H, $H_{\delta d}$ Orn), 2.96 (m, 1H, $H_{\beta u}$ ^{D}Phe), 2.91 (m, 1H, $H_{\delta u}$ Orn), 2.48 (m, 1H, $H_{\delta u}$ Pro), 2.26 (m, 1H, H_β Val), 2.05 (m, 1H, $H_{\beta d}$ Orn), 2.00 (m, 1H, $H_{\beta d}$ Pro), 1.79 (m, 2H, H_γ Orn), 1.71 (m, 1H, $H_{\gamma d}$ Pro), 1.67 (m, 1H, $H_{\beta u}$ Pro), 1.64 (m, 1H, $H_{\beta d}$, Orn), 1.59 (m, 1H, $H_{\gamma u}$ Pro), 1.55 (m, 1H, $H_{\beta d}$ Leu), 1.50 (m, 1H, $H_{\gamma d}$ Leu), 1.41 (m, 1H, $H_{\beta u}$ Leu), 0.96 (m, 3H, $H_{\gamma d}$ Val), 0.87 (m, 6H, H_γ Leu), 0.86 (m, 3H, $H_{\gamma u}$ Val). ATR-IR (thin film): 3262.0, 3073.0, 2956.6, 2931.9, 2872.7, 1734.2, 1674.8, 1634.2, 1534.4, 1451.5, 1201.0, 1185.1, 1136.4, 834.9, 798.8, 748.8, 722.1, 702.2 cm^{-1}. HRMS: calcd for $C_{60}H_{92}N_{12}O_{10}H^+$ 1141.71321, found 1141.71362 and $C_{60}H_{92}N_{12}O_{10}H^{2+}$ 571.36025, found 571.35986.

3,6-Anhydro-7-azido-2,7-dideoxy-D-*allo*-heptonic Acid (5). Isopropylidene protected methyl ester **8** (5.03 g, 18.56 mmol) was dissolved in MeOH (75 mL) and 2 M aq HCl (25 mL) was added, after which the solution was stirred overnight. The mixture was neutralized in 1 M aq NaOH (50 mL), partially concentrated, and extracted with EtOAc three times. The organics were dried ($MgSO_4$), filtered, and concentrated. Silica gel column chromatography (50→100% EtOAc in light PE) gave the free diol as a clear oil (3.52 g, 15.25 mmol, 82%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.15 (ddd, 1H, H_3, $J_{3,2a} = J_{3,2b}$ = 6.5 Hz, $J_{3,4}$ = 6.3 Hz), 4.06 (dd, 1H, H_5, $J_{5,6}$ = 5.4 Hz, $J_{5,4}$= 6.3 Hz), 4.00 (dd, 1H, H_6, $J_{6,7}$ = 4.3 Hz, $J_{6,5}$ = 5.4 Hz), 3.95 (dd, 1H, H_4, $J_{4,5} = J_{4,3}$ = 6.3 Hz), 3.57 (dd, 1H, H_{7a}, $J_{7a,6}$ = 3.4 Hz, $J_{7a,7b}$ = 13.3 Hz), 3.31 (dd, 1H, H_{7b}, $J_{7b,6}$ = 4.3 Hz, $J_{7b,7a}$ = 13.3 Hz), 2.77 (dd, 1H, H_{2a}, $J_{2a,3}$ = 6.5 Hz, $J_{2a,2b}$ = 16.3 Hz), 2.69 (dd, 1H, H_{2b}, $J_{2b,3}$ = 6.5 Hz, $J_{2b,2a}$ = 16.3 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.3 (C=O), 82.8 (C_6), 79.1 (C_3), 74.6 (C_4), 72.1 (C_5), 52.1 (C_7), 52.0 (OMe), 37.9 (C_2). ATR-IR (thin film): 3396.4, 2956.2, 2098.4, 1728.1, 1438.8, 1400.2, 1274.9, 1172.6, 1097.4, 1037.6, 987.5, 910.3, 850.5, 829.3, 731.0 cm^{-1}. $[\alpha]^{23}_D$ +80.4 (*c* 1.0, CH_2Cl_2). MS (ESI): *m/z* 232.1 $[M + H]^+$, 253.8 $[M + Na]^+$, 463.0 $[2M + H]^+$.

The methyl ester (350 mg, 1.52 mmol) was next dissolved in THF (4 mL) and 1 M aq NaOH (2 mL) was added. After being stirred for 3 h, the mixture was neutralized with Amberlite IR-120 (H^+), filtered, and concentrated. Purification by silica column chromatography (0→2% AcOH in EtOAc) gave **5** as a clear oil (323 mg, 1.49 mmol, 98%). ^{1}H NMR (400 MHz, CD_3OD): δ 4.16 (ddd, 1H, H_3, $J_{3,2a}$ = 4.9 Hz, $J_{3,4}$ = 5.3 Hz, $J_{3,2b}$ = 8.4 Hz), 3.96 (m, 2H, H_5, H_6), 3.84 (dd, 1H, H_4, $J_{4,3}$ = 5.3 Hz, $J_{4,5}$ = 5.4 Hz), 3.51 (dd, 1H, H_{7a}, $J_{7a,6}$ = 3.1 Hz, $J_{7a,7b}$ = 13.2 Hz), 3.29 (dd, 1H, H_{7b}, $J_{7b,6}$ = 4.4 Hz, $J_{7b,7a}$ = 13.2 Hz), 2.67 (dd, 1H, H_{2a}, $J_{2a,3}$ = 4.9 Hz, $J_{2a,2b}$ = 15.7 Hz), 2.50 (dd, 1H, H_{2b}, $J_{2b,3}$ = 8.4 Hz, $J_{2b,2a}$ = 15.7 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 174.6 (C=O), 83.9 (C_6), 81.2 (C_3), 75.7 (C_4), 73.0 (C_5), 53.5 (C_7), 39.4 (C_2). ATR-IR (thin film): 3434.6, 2927.7, 2100.3, 1706.9, 1406.0, 1272.9, 1180.4, 1097.4, 1033.8, 977.8, 912.3, 827.4, 748.3 cm^{-1}. $[\alpha]^{23}_D$ +54.4 (*c* 1.0, MeOH). MS (ESI): *m/z* 217.9 $[M + H]^+$, 241.0 $[M + Na]^+$, 435.1 $[2M + H]^+$, 457.1 $[2M + Na]^+$. HRMS: calcd for $C_7H_{11}N_3O_5H$ 218.07715, found 218.07724.

2,6-Anhydro-7-azido-3-hydroxy-4,5,7-trideoxy-l-*ribo*-heptonic Acid (6). Methyl ester **9** (289 mg, 1.00 mmol) was dissolved in DCM (5 mL) and treated with triisopropylsilane (1.3 mmol, 266 μL) and TFA (5 mL). After being stirred for 30 min, the solvents were removed in vacuo. The crude was coevaporated with toluene (5× 5 mL) after which a solution of K_2CO_3 (1.5 equiv, 207 mg, 1.5 mmol) and $CuSO_4$ (3 mg, cat.) in H_2O (3.3 mL) was added, followed by MeOH (5 mL) and a freshly prepared solution of TfN_3 (2 equiv) in DCM. The reaction mixture was homogenized with additional MeOH and stirred overnight. The organics were removed by evaporation and the product was purified by silica column chromatography to produce the azide in 58% over 2 steps (0.58 mmol, 125 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.83 (s, 3H, CH_3), 3.77 (s, 1H, H_2), 3.61−3.59 (m, 1H, H_6), 3.48 (s, 1H, H_3), 3.39 and 3.24 (2 × dd, 2H, H_{7ab}, J = 6.1, 6.8, J = 3.8, 9.2 Hz), 1.79−1.68 and 1.62−1.43 (m, 4H, H_{4ab} and H_{5ab}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.4 (C_1), 79.8 (C_2), 76.9 (C_6), 67.2 (C_3), 54.3 (C_7), 52.6 (CH_3), 30.6 (C_4), 27.2 (C_5). ATR-IR (thin film): 2096.5, 1733.9, 1438.8, 1290.3, 1209.3, 1089.7, 1047.3 cm^{-1}. $[\alpha]^{20}_D$ +22.5 (*c* 0.24, $CHCl_3$). MS (ESI): *m/z* 237.9 $[M + Na]^+$. HRMS: calcd for $C_8H_{13}N_3O_4NH_4^+$ 233.12443, found 233.12435.

Subsequently, a solution of the azide (50 mg, 0.23 mmol) in 1,4-dioxane/H_2O (1/1, v/v, 4 mL) was cooled to 0 °C and treated with 1 M aq LiOH (1.0 equiv, 0.23 mL) and the reaction mixture was allowed to warm to room temperature. After being stirred for 1 h, the reaction mixture was neutralized with Amberlite IR-120 (H^+), filtered, and concentrated. The product was purified by silica column chromatography (0→15% MeOH in DCM) furnishing the title compound **6** quantitatively (46 mg, 0.23 mmol). ^{1}H NMR (400 MHz, MeOD): δ 3.64 (m, 1H, H_6), 3.57 (m, 2H, H_2 and H_4), 3.46 (dd, 2H, H_{7ab} J = 4.0, 7.6 Hz), 2.12 (m, 1H, H_{4a}), 1.73 (m, 1H, H_{5a}), 1.54−1.43 (m, 2H, $H_{4b,5b}$). ^{13}C NMR (100 MHz, MeOD): δ 179.4 (C_1), 82.1 (C_2) , 77.6 (C_6), 69.2 (C_3), 55.8 (C_7), 32.3 (C_4), 28.5 (C_5). ATR-IR (thin film): 2100.3, 1589.2, 1431.1, 1292.2, 1085.8, 1045.3 cm^{-1}. $[\alpha]^{20}_D$ −3.8 (*c* 0.16, MeOH). MS (ESI): *m/z* 201.9 $[M + H]^+$.

6-Azido-2,6-dideoxy-2-phthalimido-β-D-glucopyranosyl Formic Acid (7). Methyl ester **12** (120 mg, 0.32 mmol) was dissolved in glacial acetic acid (4 mL) and 1 M aq HCl (4 mL) was added. The reaction mixture was heated to 60 °C and stirred for 3 h until TLC analysis revealed complete consumption of starting material. All solvents were removed by repeated evaporation with toluene, to quantitatively furnish carboxylic acid **7** (115 mg, 0.32 mmol) as off-white foam. ^{1}H NMR (400 MHz, CD_3OD): δ 7.87−7.80 (m, 4H, Phth), 4.94 (br s, 3H, 3× OH), 4.73 (d, 1H, H_2, $J_{2,3}$ = 10.6 Hz), 4.36 (dd, 1H, H_4, $J_{4,3}$ = 10.6 Hz, $J_{4,5}$= 9.0 Hz), 4.21 (dd, 1H, H_3, $J_{3,4}$ = 10.6 Hz, $J_{3,2}$ = 10.6 Hz), 3.66−3.57 (m, 2H, H_6 and H_{7a}), 3.55 (dd, 1H, H_{7b}, $J_{7b,6}$ = 6.5 Hz, $J_{7b,7a}$ = 13.1 Hz), 3.41 (dd, 1H, H_5, $J_{5,4} = J_{5,6}$ = 9.0 Hz).^{13}C NMR (100 MHz, CD_3OD): δ 171.6 (COOMe, C=O Phth), 135.5 (CH Phth), 131.4 (C_q Phth), 124.2 (CH Phth), 80.6 (C_6), 74.7 (C_2), 73.0 (C_4, C_5), 55.7 (C_3), 52.6 (C_7). ATR-IR (thin film): 3348.2, 2102.3, 1772.5, 1701.1, 1386.7, 1234.4, 1112.9, 1058.8, 1010.6, 966.3, 873.7, 719.4 cm^{-1}. $[\alpha]^{23}_D$ +17.6 (*c* 1.00, $CHCl_3$). HRMS: calcd for $C_{15}H_{14}N_4O_7$-NH_4 380.1206, found 380.1213.

Methyl 6-Azido-2,6-dideoxy-2-phthalimido-β-D-glucopyranosyl Formate (12). Triol **11** (3.26 g, 9.27 mmol) was dried by repeated coevaporation with pyridine and redissolved in pyridine (50 mL). The solution was stirred at 0 °C and *p*-toluenesulfonyl chloride (1.95 g, 10.2 mmol) was added, after which the mixture was stirred overnight at room temperature. Then, the reaction mixture was concentrated in vacuo and partitioned between water and EtOAc. The organic layer was washed successively with saturated aqueous $NaHCO_3$, saturated aqueous $CuSO_4$, and brine after which it was dried ($MgSO_4$) and evaporated. The crude product was purified by silica column chromatography (40→70% EtOAc in toluene) to afford the tosylate (3.42 g, 6.76 mmol, 73%) as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.79−7.67 (m, 6H, Tos, Phth), 7.32 (d, 2H, Tos), 4.65 (d, 1H, H_2, $J_{2,3}$ = 10.6 Hz), 4.42 (dd, 1H, H_4, $J_{4,3}$ = 10.6 Hz, $J_{4,5}$ = 9.0 Hz), 4.34 (dd, 1H, H_{7a}, $J_{7a,6}$ = 2.0 Hz, $J_{7a,7b}$ = 11.2 Hz), 4.29 (dd, 1H, H_{7b}, $J_{7b,6}$ = 5.4 Hz, $J_{7b,7a}$ = 11.2 Hz), 4.21 (dd, 1H, H_3, $J_{3,4}$ = 10.6 Hz, $J_{3,2}$ = 10.6 Hz), 3.63 (m, 1H, H_6), 3.51 (s, 3H, OCH_3), 3.49 (m, 1H, H_5),

2.42 (s, 3H, CH_3 Tos). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.1 (COOMe, C=O Phth), 144.9 (C_q Tos), 134.2 (CH Phth), 132.4 (C_q Tos), 131.3 (C_q Phth), 129.8, 128.1 (CH Phth), 77.2 (C_6), 73.4 (C_2), 71.6 (C_4), 70.6 (C_5), 68.9 (C_7), 53.5 (C_3), 52.5 (OMe), 21.5 (CH_3 Tos). ATR-IR (thin film): 3456.5, 2923.9, 1774.4, 1708.8, 1386.7, 1359.7, 1190.0, 1174.6, 1118.6, 1095.5, 966.3, 813.9, 719.4 cm^{-1}. $[\alpha]^{23}_D$ +20.4 (*c* 1.0, $CHCl_3$). MS (ESI): *m/z* 506.0 $[M + H]^+$, 528.3 $[M + Na]^+$. HRMS: calcd for $C_{23}H_{23}NO_{10}SNH_4$ 523.1386, found 523.1396.

The tosylate (3.42 g, 6.76 mmol) was then dissolved in DMF (35 mL) and NaN_3 (4.4 g, 67.6 mmol) was added. The reaction mixture was stirred at 80 °C for 48 h and subsequently concentrated. The residue was diluted with water and extracted twice with EtOAc. The combined organic layers were successively washed with saturated aqueous $NaHCO_3$ and brine, dried ($MgSO_4$), and concentrated. The crude product was applied to a silica gel column (60→80% EtOAc in light PE) to yield azide **12** (2.15 g, 5.72 mmol, 85%) as a white foam. ^1H NMR (400 MHz, $CDCl_3$): δ 7.80−7.72 (m, 4H, Phth), 4.72 (d, 1H, H_2, $J_{2,3}$ = 10.4 Hz), 4.39 (dd, 1H, H_4, $J_{4,3}$ = 10.4 Hz, $J_{4,5}$= 9.2 Hz), 4.25 (dd, 1H, H_3, $J_{3,4}$ = 10.4 Hz, $J_{3,2}$ = 10.4 Hz), 3.59−3.41 (m, 4H, H_5, H_6, H_7), 3.55 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.3, 168.2 (COOMe, C=O Phth), 134.3 (CH Phth), 131.4 (C_q Phth), 123.5 (CH Phth), 78.5 (C_6), 73.4 (C_2), 71.8 (C_4), 71.8 (C_5), 53.6 (C_3), 52.6 (OMe), 51.2 (C_7). ATR-IR (thin film): 3455.0, 2100.3, 1774.4, 1741.0, 1705.0, 1436.9, 1384.8, 1286.4, 1114.8, 1066.6, 1010.6, 964.3, 873.7, 719.4 cm^{-1}. $[\alpha]^{23}_D$ +54.4 (*c* 1.00, $CHCl_3$). MS (ESI): *m/z* 377.2 $[M + H]^+$, 398.9 $[M + Na]^+$. HRMS: calcd for $C_{16}H_{16}N_4O_7NH_4$ 394.1363, found 394.1340.

Assembly of GS Analogues 14a−h. Resin-anchored peptides **13a−d** (100 μmol) were constructed from **1** according to the general SPPS procedure. Reduction of the *N*-terminal azide was accomplished by washing the solid support with 1,4-dioxane (5 mL, 3 × 3 min) and dispersing it in 1,4-dioxane (10 mL), to which trimethylphosphine (16 equiv, 1.6 mL, 1.6 mmol, 1 M in toluene) was added. The resin was shaken for 2 h, water (1 mL) was added, and shaking was continued another 4 h. The resin was washed with 1,4-dioxane (5 mL, 3 × 3 min) and DCM (5 mL, 3 × 3 min). The peptides were released from the resin, cyclized, and purified as described above to yield the protected **14a** (96%), **14b** (63%), **14c** (85%), and **14d** (78%), respectively, as amorphous white solids. The assembly of peptides **13e−h** was performed in a similar manner, to furnish **14e** (36%), **14f** (43%), **14g** (72%), and **14h** (78%), respectively.

Deprotection of 14a−h. The pivaloyl protection groups in **14a** (32 mg, 24 μmol) and **14e** (12 mg, 10 μmol) were removed by dissolving the peptides in MeOH (5 mL), followed by addition of NaOMe (16 equiv, 20 mg, 370 mmol) and stirring overnight. The mixtures were neutralized with Amberlite IR-120 (H^+), filtered, and concentrated and the crudes were used directly in the following Boc-deprotection step. For peptides **14d** (17 mg, 13 μmol) and **14h** (17 mg, 11.4 μmol), the phthaloyl protection groups were removed by dissolving the peptides in MeOH (5 mL), followed by addition of hydrazine-monohydrate (50 equiv, 28 μL, 0.57 mmol). After refluxing for 16 h, the solvents were evaporated and the crude compounds were used without further purification in the following Boc-deprotection. Removal of the Boc protection groups in the aforementioned peptides, as well as **14b** (14 mg, 11.0 μmol), **14c** (14 mg, 11 μmol), **14f** (6 mg, 5.0 μmol), and **14g** (12 mg, 10.3 μmol), was performed according to the general procedure to give **15a** (22.0 mg, 20.8 μmol, 87%), **15b** (8.1 mg, 7.6 μmol, 69%), **15c** (9.8 mg, 9.3 μmol, 85%), **15d** (12.5 mg, 11.5 μmol, 88%), **15e** (9.0 mg, 9.3 μmol, 93%), **15f** (4.2 mg, 4.2 μmol, 84%), **15g** (9.6 mg, 9.9 μmol, 96%), and **15h** (6.9 mg, 6.7 μmol, 59%), respectively, as white amorphous powders.

***cyclo*-[SAA_4−Val-Orn-Leu-^{D}Phe-Pro-Val-Orn-Leu] (15a).** Prepared as described previously.[11]

***cyclo*-[SAA_5-Val-Orn-Leu-^{D}Phe-Pro-Val-Orn-Leu] (15b).** Analyzed by LC/MS (R_t 14.71 min; linear gradient 10→90% B in 20 min; *m/z* 1070.8 $[M + H]^+$, 536.1 $[M + H]^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 40→50% B; R_t 1.9 CV). ^1H NMR (600 MHz, CD_3OH): δ 8.90 (d, 1H, NH ^{D}Phe$_5$, $J_{NH,H\alpha}$ = 3.5 Hz), 8.68 (d, 1H, NH_α Orn$_3$, $J_{NH,H\alpha}$ = 8.1 Hz), 8.62 (d, 1H, NH Leu$_4$, $J_{NH,H\alpha}$ = 9.4 Hz), 8.61 (d, 1H, NH_α Orn$_8$, $J_{NH,H\alpha}$ = 8.9 Hz), 8.56 (d, 1H, NH Leu$_9$, $J_{NH,H\alpha}$ = 8.9 Hz), 8.07 (t, 1H, NH SAA_1, $J_{NH,7}$ = 6.1 Hz), 7.86 (br s, 2H, NH_δ Orn$_{3,8}$), 7.74 (d, 1H, NH Val$_7$, $J_{NH,H\alpha}$ = 8.6 Hz), 7.55 (d, 1H, NH Val$_2$, $J_{NH,H\alpha}$ = 8.5 Hz), 7.38−7.21 (m, 5H, H_{ar}), 4.98 (m, 1H, H_α Orn$_3$), 4.71 (m, 1H, H_α Orn$_8$), 4.65 (m, 1H, H_α Leu$_4$), 4.56 (m, 1H, H_α Leu$_9$), 4.51 (m, 1H, H_α ^{D}Phe$_5$), 4.34 (m, 1H, H_α Pro$_6$), 4.24 (m, 1H, H_α Val$_2$), 4.06 (m, 1H, H_α Val$_7$), 3.95 (m, 2H, $H_{3,6}$ SAA_1), 3.86 (dd, 1H, H_5 SAA_1, $J_{5,4}$ = 5.2 Hz, $J_{5,6}$ = 3.0 Hz), 3.78 (dd, 1H, H_4 SAA_1, $J_{4,5}$ = 5.2 Hz, $J_{4,3}$ = 6.5 Hz), 3.72 (m, 1H, $H_{\delta d}$ Pro$_6$), 3.36 (m, 1H, H_{7d} SAA_1), 3.31 (m, 1H, H_{7u} SAA_1), 3.07 (dd, 1H, $H_{\beta d}$ ^{D}Phe$_5$, $J_{\beta d,\beta u}$ = 12.6 Hz, $J_{\beta d,\alpha}$ = 5.0 Hz), 3.02 (m, 1H, $H_{\delta d}$ Orn$_3$), 2.98 (m, 1H, $H_{\delta d}$ Orn$_8$), 2.96 (m, 3H, $H_{\delta u}$ Orn$_3$, $H_{\delta u}$ Orn$_8$, $H_{\beta u}$ ^{D}Phe$_5$), 2.50 (m, 3H, H_2 SAA_1, $H_{\delta u}$ Pro$_6$), 2.28 (m, 1H, H_β Val$_7$), 1.99 (m, 3H, $H_{\beta d}$ Pro$_6$, $H_{\beta d}$ Orn$_3$, H_β Val$_2$), 1.83 (m, 1H, $H_{\beta d}$ Orn$_8$), 1.74 (m, 2H, H $_\gamma$ Orn$_3$), 1.71 (m, 2H, $H_{\beta u,\ \gamma d}$ Pro$_6$), 1.67 (m, 1H, $H_{\beta u}$ Orn$_3$), 1.66 (m, 2H, H_γ Orn$_8$), 1.64 (m, 3H, $H_{\beta,\ \gamma}$ Leu$_9$), 1.59 (m, 1H, $H_{\gamma u}$ Pro$_6$), 1.56 (m, 2H, $H_{\beta d,\ \gamma}$ Leu$_4$), 1.39 (m, 1H, $H_{\beta u}$ Leu$_4$), 0.95 (m, 3H, $H_{\gamma d}$ Val$_7$), 0.94 (m, 3H, $H_{\gamma d}$ Val$_2$), 0.92 (m, 3H, $H_{\gamma u}$ Val$_2$), 0.90 (m, 6H, H_δ Leu$_4$), 0.88 (m, 3H, $H_{\gamma u}$ Val$_7$), 0.86 (m, 6H, H_δ Leu$_9$). ATR-IR (thin film): 3278.1, 3071.9, 2959.2, 2935.6, 2873.4, 1669.8, 1636.5, 1539.2, 1464.7, 1456.7, 1437.0, 1203.7, 1182.7, 1135.0, 1033.3, 1020.8, 837.1, 800.1, 722.6, 702.5 cm^{-1}. HRMS: calcd for $C_{53}H_{87}N_{11}O_{12}H$ 1079.6608, found 1070.6521.

***cyclo*-[SAA_6-Val-Orn-Leu-^{D}Phe-Pro-Val-Orn-Leu] (15c).** Analyzed by LC/MS (R_t 15.79 min; linear gradient 10→90% B in 20 min; *m/z* 1054.8 $[M + H]^+$, 528.2 $[M + H]^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 35→55% B; R_t 2.7 CV). ^1H NMR (600 MHz, CD_3OH): δ 8.99 (d, 1H, NH ^{D}Phe$_5$, $J_{NH,H\alpha}$ = 3.1 Hz), 8.76 (d, 1H, NH_α Orn$_3$, $J_{NH,H\alpha}$ = 6.7 Hz), 8.74 (d, 1H, NH Leu$_4$, $J_{NH,H\alpha}$ = 7.6 Hz), 8.56 (d, 1H, NH Leu$_9$, $J_{NH,H\alpha}$ = 9.2 Hz), 8.51 (d, 1H, NH_α Orn$_8$, $J_{NH,H\alpha}$ = 9.4 Hz), 8.11 (t, 1H, NH SAA_1, $J_{NH,7}$ = 6.3 Hz), 7.87 (br s, 2H, NH_δ Orn$_{3,8}$), 7.69 (d, 1H, NH Val$_2$, $J_{NH,H\alpha}$ = 8.5 Hz), 7.60 (d, 1H, NH Val$_7$, $J_{NH,H\alpha}$ = 9.3 Hz), 7.32−7.23 (m, 5H, H_{ar}), 4.90 (m, 1H, H_α Orn$_8$), 4.73 (m, 1H, H_α Orn$_3$), 4.64 (m, 1H, H_α Leu$_4$), 4.50 (m, 2H, H_α ^{D}Phe$_5$, H_α Leu$_9$), 4.39 (m, 2H, H_α Pro$_6$, H_α Val$_7$), 4.13 (m, 1H, H_α Val$_2$), 3.71 (m, 1H, $H_{\delta d}$ Pro$_6$), 3.52 (m, 1H, H_{7d} SAA_1), 3.44 (2, 2H, $H_{2,3}$ SAA_1), 3.41 (m, 1H, H_6 SAA_1), 3.08 (dd, 1H, $H_{\beta d}$ ^{D}Phe$_5$, $J_{\beta d,\beta u}$ = 12.9 Hz, $J_{\beta d,\alpha}$ = 4.9 Hz), 3.07 (m, 1H, H_{7u} SAA_1), 3.01 (m, 1H, $H_{\delta d}$ Orn$_3$), 2.93 (dd, 1H, $H_{\beta u}$ ^{D}Phe$_5$, $J_{\beta d,\beta u}$ = $J_{\beta d,\alpha}$ = 12.9 Hz), 2.87 (m, 3H, H_δ Orn$_8$, $H_{\delta u}$ Orn$_3$), 2.46 (m, 1H, $H_{\delta u}$ Pro$_6$), 2.23 (m, 1H, H_β Val$_7$), 2.14 (m, 1H, H_β Val$_2$), 2.09 (m, 1H, H_{4d} SAA_1), 2.01 (m, 2H, $H_{\beta d}$ Pro$_6$, $H_{\beta d}$ Orn$_3$), 1.77 (m, 2H, H_β Orn$_3$), 1.71 (m, 1H, H_{5d} SAA_1), 1.62 (m, 5H, $H_{\beta d}$ Leu$_9$, $H_{\beta d}$ Leu$_6$, $H_{\beta d}$ Orn$_8$, $H_{\beta u}$ Pro$_6$, $H_{\gamma u}$ Orn$_3$), 1.52 (m, 7H, $H_{\beta u,\ \gamma d}$ Orn$_8$, H_γ Leu$_4$, H_γ Leu$_9$, H_γ Pro$_6$, H_{4u} SAA_1), 1.42 (m, 4H, $H_{\beta u,}$ Leu$_9$, $H_{\beta u,}$ Leu$_6$, $H_{\gamma u}$ Orn$_8$, H_{4u} SAA_1), 1.42 (m, 1H, $H_{\beta u}$ Leu$_4$), 1.02 (d, 3H, $H_{\gamma d}$ Val$_2$ $J_{\gamma,\beta}$ = 6.7 Hz), 0.97 (d, 3H, $H_{\gamma u}$ Val$_2$ $J_{\gamma,\beta}$ = 6.8 Hz), 0.92 (d, 6H, H_γ Val$_7$), 0.93−0.87 (m, 12H, H_δ Leu$_4$, H_δ Leu$_9$). ATR-IR (thin film): 3267.7, 3061.3, 2957.6, 2933.1, 2870.1, 1675.2, 1639.5, 1538.9, 1456.8, 1203.4, 1182.1, 1133.3, 1060.1, 1033.4, 838.3, 800.0, 749.1, 722.8, 701.7 cm^{-1}. HRMS: calcd for $C_{53}H_{87}N_{11}O_{11}H$ 1054.6659, found 1054.6622.

***cyclo*-[SAA_7-Val-Orn-Leu-^{D}Phe-Pro-Val-Orn-Leu] (15d).** Analyzed by LC/MS (R_t 12.92 min; linear gradient 10→90% B in 20 min; *m/z* 1086.0 $[M + H]^+$, 543.6 $[M + H]^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 30→50% B; R_t 2.8 CV). ^1H NMR (600 MHz, CD_3OH): δ 8.92 (d, 1H, NH ^{D}Phe$_5$, $J_{NH,H\alpha}$ = 3.3 Hz), 8.69 (d, 1H, NH Leu$_4$, $J_{NH,H\alpha}$ = 9.2 Hz), 8.61 (d, 1H, NH_α Orn$_3$, $J_{NH,H\alpha}$ = 8.9 Hz), 8.58 (d, 1H, NH_α Orn$_8$, $J_{NH,H\alpha}$ = 9.3 Hz), 8.47 (d, 1H, NH Leu$_9$, $J_{NH,H\alpha}$ = 9.1 Hz), 8.09 (t, 1H, NH SAA_1, $J_{NH,7}$ = 6.1 Hz), 7.72 (d, 1H, NH Val$_7$, $J_{NH,H\alpha}$ = 9.0 Hz), 7.70 (d, 1H, NH Val$_2$, $J_{NH,H\alpha}$ = 9.1 Hz), 7.38−7.21 (m, 5H, H_{ar}), 4.97 (m, 1H, H_α Orn$_3$), 4.80 (m, 1H, H_α Orn$_8$), 4.64 (m, 1H, H_α Leu$_4$), 4.57 (m, 1H, H_α Leu$_9$), 4.49 (m, 1H, H_α ^{D}Phe$_5$), 4.34 (m, 1H, H_α Pro$_6$), 4.32 (m, 1H, H_α Val$_2$), 4.07 (m,

2H, H_2 SAA_1, H_α Val_7), 3.87 (m, 1H, H_{7d} SAA_1), 3.71 (m, 1H, $H_{\delta d}$ Pro_6), 3.58 (dd, 1H, H_4 SAA_1, $J_{4,5} = J_{4,3} = 9.2$ Hz), 3.49 (m, 1H, H_6 SAA_1), 3.34 (m, 1H, H_{7u} SAA_1), 3.18 (dd, 1H, H_5 SAA_1, $J_{5,4} = J_{5,6} = 9.2$ Hz), 3.08 (dd, 1H, $H_{\beta d}$ ^{D}Phe_5, $J_{\beta d,\beta u} = 12.6$ Hz, $J_{\beta d,\alpha} = 5.0$ Hz), 3.02 (m, 1H, $H_{\delta d}$ Orn_3), 3.00 (m, 2H, H_δ Orn_8), 2.98 (m, 1H, $H_{\delta u}$ Orn_3), 2.93 (m, 1H, $H_{\beta u}$ ^{D}Phe_5), 2.80 (dd, 1H, H_3 SAA_1, $J_{3,2} = 10.2$ Hz, $J_{3,4} = 9.2$ Hz), 2.47 (m, 1H, $H_{\delta u}$ Pro_6), 2.29 (m, 1H, H_β Val_7), 2.17 (m, 1H, H_β Val_2), 1.98 (m, 2H, $H_{\beta d}$ Pro_6, $H_{\beta d}$ Orn_3), 1.75 (m, 2H, $H_{\beta u}$ Orn_3, $H_{\beta d}$ Orn_8), 1.68 (m, 6H, $H_{\beta d}$ Leu_9, $H_{\beta u}$ Pro_6, H_γ Orn_3, H_γ Orn_8), 1.55 (m, 3H, $H_{\beta u}$ Orn_8, $H_{\beta d,\gamma}$ Leu_4), 1.52 (m, 4H, H_γ Pro_6, $H_{\beta u,\gamma}$ Leu_9), 1.42 (m, 1H, $H_{\beta u}$ Leu_4), 0.98 (d, 3H, $H_{\gamma d}$ Val_2 $J_{\gamma,\beta} = 6.8$ Hz), 0.95 (d, 3H, $H_{\gamma d}$ Val_7 $J_{\gamma,\beta} = 6.6$ Hz), 0.93 (d, 3H, $H_{\gamma u}$ Val_2 $J_{\gamma,\beta} = 6.8$ Hz), 0.91−0.86 (m, 15H, $H_{\gamma u}$ Val_7, H_δ Leu_4, H_δ Leu_9). ATR-IR (thin film): 3273.4, 3066.7, 2954.8, 2936.6, 2872.3, 1672.1, 1645.9, 1539.5, 1454.0, 1437.0, 1203.7, 1182.5, 1133.5, 1033.2, 1021.5, 838.7, 799.9, 748.0, 722.9, 702.4 cm^{-1}. HRMS: calcd for $C_{53}H_{88}N_{12}O_{12}H$ 1085.6717, found 1085.6691.

***cyclo*-[SAA_4-Val-Orn-Leu-]$_2$ (15e).** Analyzed by LC/MS (R_t 10.96 min; linear gradient 10→90% B in 20 min; *m/z* 971.8 [M + H]$^+$, 486.6 [M + H]$^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 20→40% B; R_t 2.7 CV). ^{1}H NMR (600 MHz, CD_3OH): δ 8.46 (d, 1H, NH_α Orn, $J_{NH,H\alpha} = 7.7$ Hz), 8.00 (d, 1H, NH Leu, $J_{NH,H\alpha} = 8.5$ Hz), 7.99 (t, 1H, NH SAA, $J_{NH,6} = 8.5$ Hz), 7.89 (d, 1H, NH Val, $J_{NH,H\alpha} = 6.1$ Hz), 4.57 (d, 1H, H_2 SAA, $J_{2,3} = 4.0$ Hz), 4.50 (m, 1H, H_α Leu), 4.38 (m, 1H, H_α Orn), 4.27 (m, 2H, H_α Val, H_3 SAA), 4.02 (m, 1H, H_5 SAA), 3.95 (s, 1H, H_4 SAA), 3.63 (m, 1H, H_{6d} SAA), 3.37 (m, 1H, H_{6u} SAA), 2.94 (m, 2H, H_δ Orn), 2.25 (m, 1H, H_β Val), 1.95 (m, 1H, $H_{\beta d}$ Orn), 1.84 (m, 1H, $H_{\beta u}$ Orn), 1.71 (m, 2H, H_γ Orn), 1.59 (m, 3H, $H_{\beta,\gamma}$ Leu), 0.96 (m, 6H, H_γ Val), 0.89 (m, 6H, H_δ Leu). ATR-IR (thin film): 3279.5, 2961.1, 2933.9, 2875.5, 1648.7, 1528.3, 1435.9, 1202.6, 1182.4, 1135.4, 1042.1, 837.6, 799.8, 722.3 cm^{-1}. HRMS: calcd for $C_{44}H_{78}N_{10}O_{14}H$ 971.5771, found 971.5736.

***cyclo*-[SAA_5-Val-Orn-Leu-]$_2$ (15f).** Analyzed by LC/MS (R_t 10.95 min; linear gradient 10→90% B in 20 min; *m/z* 999.8 [M + H]$^+$, 500.7 [M + H]$^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 20→40% B; R_t 2.5 CV). ^{1}H NMR (600 MHz, CD_3OH): δ 8.64 (d, 1H, NH Leu, $J_{NH,H\alpha} = 8.2$ Hz), 8.50 (d, 1H, NH_α Orn, $J_{NH,H\alpha} = 8.4$ Hz), 8.46 (t, 1H, NH SAA, $J_{NH,7} = 4.3$ Hz), 8.04 (d, 1H, NH Val, $J_{NH,H\alpha} = 9.3$ Hz), 4.76 (m, 1H, H_α Leu), 4.65 (m, 1H, H_α Orn), 4.50 (m, 1H, H_α Val), 4.06 (m, 2H, $H_{4,6}$ SAA), 4.01 (m, 1H, H_3 SAA), 3.93 (dd, 1H, H_5 SAA, $J_{5,4} = J_{5,6} = 4.6$ Hz), 3.55 (m, 1H, H_{7d} SAA), 3.32 (m, 1H, H_{7u} SAA), 2.93 (m, 2H, H_δ Orn), 2.63 (dd, 1H, H_{2d} SAA, $J_{2d,3} = 3.3$ Hz, $J_{2d,2u} = 15.3$ Hz), 2.37 (dd, 1H, H_{2u} SAA, $J_{2u,3} = 7.3$ Hz, $J_{2u,2d} = 15.3$ Hz), 2.12 (m, 1H, H_β Val), 1.71 (m, 3H, $H_{\beta,\gamma d}$ Orn), 1.61 (m, 3H, $H_{\gamma u}$ Orn, $H_{\beta d,\gamma}$ Leu), 1.44 (m, 1H, $H_{\beta u}$ Leu), 0.92 (m, 6H, H_γ Val), 0.88 (m, 6H, H_δ Leu). ATR-IR (thin film): 3279.4, 3072.3, 2957.8, 2930.1, 2872.2, 2857,6, 1663.5, 1642.8, 1539.4, 1534.0, 1437.0, 1202.3, 1182.8, 1134.3, 839.3, 800.5, 722.8 cm^{-1}. HRMS: calcd for $C_{46}H_{82}N_{10}O_{14}H$ 999.6084, found 999.6097.

***cyclo*-[SAA_6-Val-Orn-Leu-]$_2$ (15g)**. Analyzed by LC/MS (R_t 12.02 min; linear gradient 10→90% B in 20 min; *m/z* 967.7 [M + H]$^+$, 484.6 [M + H]$^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 25→40% B; R_t 2.8 CV). ^{1}H NMR (600 MHz, CD_3OH): δ 8.49 (d, 1H, NH_α Orn, $J_{NH,H\alpha} = 3.5$ Hz), 8.34 (d, 1H, NH Leu, $J_{NH,H\alpha} = 5.6$ Hz), 8.06 (t, 1H, NH SAA, $J_{NH,6} = 3.2$ Hz), 7.76 (d, 1H, NH Val, $J_{NH,H\alpha} = 4.8$ Hz), 4.55 (m, 1H, H_α Orn), 4.43 (m, 1H, H_α Leu), 4.25 (m, 1H, H_α Val), 3.52 (m, 2H, $H_{2,3}$ SAA), 3.47 (m, 2H, $H_{6,7d}$ SAA), 3.16 (dd, 1H, H_{7u} SAA, $J_{7u,7d} = 8.4$ Hz, $J_{7u,6} = 1.7$ Hz), 2.89 (m, 2H, H_δ Orn), 2.14 (m, 2H, H_β Val, H_{4d} SAA), 1.69 (m, 2H, $H_{\beta d}$ Orn, H_{5d} SAA), 1.61 (m, 4H, $H_{\beta u,\gamma d}$ Orn, $H_{\beta d,\gamma}$ Leu), 1.52 (m, 4H, $H_{\gamma u}$ Orn, $H_{\beta u}$ Leu $H_{5u,4u}$ SAA), 0.99 (m, 6H, H_γ Val), 0.92 (d, 3H, $H_{\delta u}$ Leu $J_{\delta,\gamma} = 6.2$ Hz), 0.89 (d, 3H, $H_{\delta d}$ Leu $J_{\delta,\gamma} = 6.1$ Hz). ATR-IR (thin film): 3285.8, 3070.5, 2957.8, 2932.4, 2872.1, 1652.6, 1533.2, 1468.4, 1437.0, 1202.3, 1180.2, 1130.8, 837.1, 799.7, 722.1 cm^{-1}. HRMS: calcd for $C_{46}H_{82}N_{10}O_{12}H$ 967.6186, found 967.6191.

***cyclo*-[SAA_7-Val-Orn-Leu-]$_2$ (15h).** Analyzed by LC/MS (R_t 9.73 min; linear gradient 10→90% B in 20 min; *m/z* 1029.8 [M + H]$^+$, 515.6 [M + H]$^{2+}$) and purified by RP-HPLC (linear gradient of 3.0 CV; 20→40% B; R_t 2.0 CV). ^{1}H NMR (600 MHz, CD_3OH): δ 8.55 (d, 1H, NH_α Orn, $J_{NH,H\alpha} = 8.5$ Hz), 8.47 (d, 1H, NH Leu, $J_{NH,H\alpha} = 9.0$ Hz), 8.10 (t, 1H, NH SAA, $J_{NH,7} = 6.3$ Hz), 7.76 (d, 1H, NH Val, $J_{NH,H\alpha} = 8.9$ Hz), 4.78 (m, 1H, H_α Orn), 4.56 (m, 1H, H_α Leu), 4.38 (m, 1H, H_α Val), 4.53 (d, 1H, H_2 SAA, $J_{2,3} = 10.6$ Hz), 3.83 (m, 1H, H_{7d} SAA), 3.58 (dd, 1H, H_4 SAA, $J_{4,5} = 9.3$ Hz, $J_{4,3} = 10.0$ Hz), 3.48 (m, 1H, H_6 SAA), 3.39 (m, 1H, H_{7u} SAA), 3.18 (dd, 1H, H_5 SAA, $J_{5,6} = 9.1$ Hz, $J_{5,4} = 9.3$ Hz), 3.02 (m, 1H, $H_{\delta d}$ Orn), 2.98 (m, 1H, $H_{\delta u}$ Orn), 2.79 (dd, 1H, H_3 SAA, $J_{3,4} = 10.0$ Hz, $J_{3,2} = 10.6$ Hz), 2.19 (m, 1H, H_β Val), 1.71 (m, 4H, $H_{\beta,\gamma d}$ Orn, $H_{\beta d}$ Leu), 1.55 (m, 2H, $H_{\gamma u}$ Orn, H_γ Leu), 1.48 (m, 1H, $H_{\beta u}$ Leu), 0.98 (d, 3H, $H_{\gamma d}$ Val $J_{\gamma,\beta} = 6.8$ Hz), 0.95 (d, 3H, $H_{\gamma u}$ Val $J_{\gamma,\beta} = 6.8$ Hz), 0.89 (m, 6H, H_δ Leu). ATR-IR (thin film): 3280.4, 3072.9, 2957.5, 2932.8, 2872.4, 1671.5, 1647.8, 1544.5, 1437.6, 1203.3, 1186.7, 1136.2, 1084.3, 840.9, 800.1, 723.6 cm^{-1}. HRMS: calcd for $C_{46}H_{84}N_{12}O_{14}H$ 1029.6302, found 1029.6280.

Biological Activity. The following bacterial strains were used: *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853). Bacteria were stored at −70 °C and grown at 35 °C on Columbia Agar with sheep blood (Oxoid, Wesel, Germany) overnight and diluted in 0.9% NaCl. Microtiter plates (96 wells of 100 μL) and large plates (25 wells of 3 mL) were filled with Mueller Hinton II Agar (Becton Dickinson, Cockeysvill, USA) containing serial 2-fold dilutions of peptides **15a**−**h**. To the wells were added 3 μL of bacteria, to give a final inoculum of 10^4 colony forming units (CFU) per well. The plates were incubated overnight at 35 °C and the MIC was determined as the lowest concentration inhibiting bacterial growth.

Hemolytic Activity. The hemolytic activity of the peptides was determined in quadruple. Human blood was collected into EDTA tubes and centrifuged to remove the buffy coat. The residual erythrocytes were washed three times in 0.85% saline. Serial 2-fold dilutions of the peptides **15a**−**h** in saline were prepared in sterilized round-bottom 96-well plates (polystyrene, U-bottom, Costar), using 100 μL volumes (500−0.5 μM). Red blood cells were diluted with saline to 1/25 packed volume of cells and 50 μL of the resulting cell suspension was added to each well. Plates were incubated while gently shaking at 37 °C for 4 h. Next, the microtiter plate was quickly centrifuged (1000 g, 5 min) and 50 μL of supernatant of each well was transported into a flat-bottom 96-well plate (Costar). The absorbance was measured at 405 nm with a mQuant microplate spectrophotometer (Bio-Tek Instruments). The A_{blank} was measured in the absence of additives and 100% hemolysis (A_{tot}) in the presence of 1% Triton X-100 in saline. The percentage hemolysis is determined as $(A_{pep} - A_{blank})/(A_{tot} - A_{blank}) \times 100$.

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