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Optimized Protocol for Synthesis of Cyclic Gramicidin S: Starting Amino Acid Is Key to High Yield

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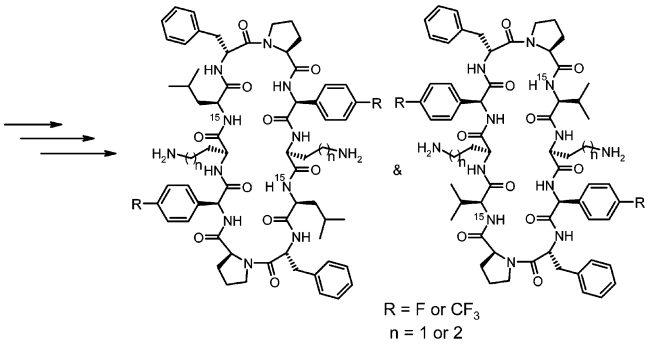

A simple and highly efficient Fmoc solid-phase protocol for synthesizing the antimicrobial decapeptide gramicidin S and various labeled analogues is presented. When preparing the linear precursor peptides (**1a**−**e**), a systematic permutation of the starting amino acid within the cyclic sequence gave different yields between 51% and 93%. Also the subsequent step of cyclization gave widely diverging yields between 26% and 74%, depending again on the starting amino acid. The ease of cyclization was found to correlate with the tendency of the respective linear precursor peptide to assume a preorganized conformation, as observed by circular dichroism. The overall yield is thus critically dependent on the starting amino acid and can be raised from 20% to 70% using D-Phe. The choice of coupling agent and its counterion was found to play only a marginal role. Irrespective of being able to assume a preorganized conformation, none of the linear precursor peptides exhibited any antimicrobial or hemolytic activity. Using the optimized protocol, which involves only simple Fmoc-couplings and requires no intermittent purification steps, several gramicidin S analogues (**3**−**8**) containing ^{19}F-labeled phenylglycine derivatives and/or ^{15}N-labeled amino acids were synthesized for solid-state NMR structure analysis.

Introduction

Many different types of organisms use antimicrobial peptides as a first line of defense against bacterial infection. These cationic peptides are usually amphiphilic and capable of rapidly killing a wide range of bacteria presumably by permeabilizing their cell membranes.[1] A well-studied representative is the decapeptide gramicidin S (GS), which is produced nonribosomally by *Bacillus brevis*.[2−4] It has a symmetric cyclic backbone, consisting of two short antiparallel β-strands connected by two β-turns [*cyclo*-(D-Phe-Pro-Val-Orn-Leu)$_2$] (Figure 1). The backbone conformation is comparatively rigid, as it is stabilized by four intramolecular hydrogen bonds. With its polar side chains (Orn) on one face and the hydrophobic side chains (Val and Leu) on the other, GS is known to bind favorably to lipid bilayers. Even 60 years after its initial discovery, GS continues to attract the interest of chemists and biologists in an attempt to understand its mode of action in membranes.[5,6] Although several NMR,[4,7−10] CD,[11,12] spectroscopic, and biophysical investigations[9,13−15] have been concerned with the peptide structure in solution or in lipid bilayers, it is still not clear how GS interacts with biological membranes. We have recently

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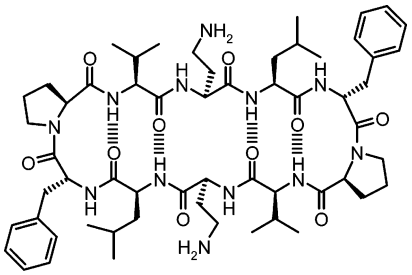

FIGURE 1. Structure of gramicidin S.

investigated the alignment of GS embedded in lipid bilayers, using solid-state ^{19}F and ^{15}N NMR spectroscopy under quasi-native conditions.[16−18] At low concentration GS lies flat on the bilayer surface, whereas at high concentration it can flip upright to self-assemble presumably as an oligomeric pore.[17,19] These studies required large amounts of several analogues of GS labeled with different NMR isotopes. We also note that the commercial supply of GS ceased several years ago; hence it appeared worthwhile to optimize the chemical synthesis and to understand the factors influencing the yield of this natural compound.

Increasing resistance of many bacteria to conventional antibiotics[20,21] has led to a spur of efforts to modify the structure of GS.[22−25] So far there have been no reports of bacterial strains that became tolerant against amphiphilic peptides such as GS.[20,26] That is because any developing resistance would require considerable changes in the bacterial membrane structure rather than simple mutations of some enzyme. As a drawback, however, GS not only acts against bacteria and fungi[14,27,28] but also causes lysis of eukaryotic membranes such as erythrocytes.[8,25,29,30] These hemolytic side effects have limited its pharmaceutical use to topical applications. In an attempt to produce a more selective drug with an improved therapeutic index, i.e., which is more antibiotic and less hemolytic, many different analogues of GS have been synthesized.[8,15,23,29,31−33] In various reports the activity of GS has been correlated with the ring size and overall hydrophobicity of the molecule. Kiricsi et al. investigated three GS-analogues with 10, 12, and 14 residues, of which the 14-mer had the highest therapeutic index.[34] Kondejewski et al. synthesized various GS analogues with ring sizes ranging from 4 to 14 residues, some of which

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showed promise in being less hemolytic.[28] It was furthermore possible to improve the selectivity of a 14-mer against prokaryotic versus eukaryotic membranes by substituting a single L-amino acid for its D-enantiomer.[31,32] These kinds of studies require access to large amounts of GS and its synthetic analogues, preferably via a simple and efficient protocol. Chemical synthesis is the only way of producing a wide variety of GS analogues, especially when certain D-amino acids, sugar derivatives, or other modifications are to be incorporated.[31,32,35,36]

There have been many reports on the synthesis of GS and its analogues.[10,16,37−40] Contrary to the expectation that the small size of this peptide should allow convenient synthesis, the overall yield tends to be quite low. In most cases cyclization of the linear precursor peptide is the most inefficient step.[2,38,41] For example, in an early protocol by Tamaki et al., pentapeptide-active esters were dimerized and cyclized in a biomimetic fashion, which resulted in an overall yield of 38%.[42] Most GS syntheses in the past were carried out either in solution or on a solid phase using t-Boc chemistry. Only some recent reports by Bu et al.[38] and Wu et al.[40] utilize Fmoc chemistry as a more attractive route to synthesize linear GS analogues, though the yields are rarely beyond 25%.[38,40] The method of Bu et al. starts with an Fmoc-Leu preloaded resin and involves an additional step of cyanomethylation. It has the advantage that the δ-amino group of Orn does not need to be selectively protected, as it was argued that a "preorganized" backbone conformation of the linear peptide eliminates the need for Orn protection.[38] The synthetic protocol of Wu et al. utilizes almost the same method, except that the final cyclization is carried out enzymatically, which does not, however, enhance the yield of the final product.[40] Recent Fmoc-based protocols by Grotenbreg et al. offer an attractive route to obtain larger amounts of GS and its sugar-containing analogues.[35,43]

Here, we are interested in a simple, purely chemical synthesis of GS, which involves common coupling reagents, requires a minimal number of purification steps, and affords large amounts of not only the wild-type peptide but also its analogues. We thus decided to optimize the synthesis with regard to several factors that have not yet been addressed in the literature to our knowledge, namely: (i) the starting amino acid of the linear precursor peptide, (ii) a variation of commonly used coupling agents, (iii) the choice of resin, and (iv) the possibility to carry out cyclization directly on the crude linear precursor peptide. Finally, (v) antimicrobial and hemolytic activities of the linear precursor peptides of GS have been addressed in the literature, but there appears to be no conclusion as to whether they are active or not.[27,44] An optimized protocol is thus presented here, based on standard Fmoc solid-phase peptide synthesis (SPPS). It has been used not only to produce wild-type GS in high yield but also a series of ^{19}F- and/or ^{15}N-labeled analogues for solid-state NMR investigations.[17,19] These suitably labeled GS analogues are needed to obtain structural and dynamic information on the membrane-embedded peptide at the molecular level, which may help to develop an improved therapeutic agent. In the present report, the relevant synthetic aspects and key findings will be discussed.

Results and Discussion

Gramicidin S was synthesized according to Scheme 1. First, a series of different linear precursor peptides **1a**−**e** were individually produced by SPPS (steps i and ii) and cleaved from the resin (step iii). The bulk of each crude batch was directly cyclized and deprotected (steps iv and v) without any intermittent purification to give the desired product GS. This product obtained after cyclization showed all characteristic analytical and spectroscopic properties as previously reported.[38−40] In an earlier study, it had been reported that the linear precursor peptides were biologically active only after removal of the protection at the δ-NH_2 groups.[6,44] For that reason we also removed the orthogonal 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) protection from **1a**−**e** to obtain the deprotected linear precursor peptides **2a**−**e** for biological activity tests and CD analysis. Besides producing the wild-type peptide, we also synthesized analogues **3**−**8**, in which specific hydrophobic residues were replaced by 4F-Phg, 4CF_3-Phg, and/or ^{15}N-labeled Leu or Val (Scheme 1). In a few GS analogues the hydrophilic Orn was replaced by Lys, which had been shown not to alter the antimicrobial properties of GS.[27,28] The respective yields of the linear and cyclic peptides, as well as their purities, were quantitatively determined by analytical HPLC as summarized in Table 1. Note that the respective columns report separately the yield of the linear products and the yield of the cyclization step per se. The overall yield of the final product (last column) is then obtained by multiplying the two aforementioned percentages, thus stating the yield of cyclic GS obtained relative to the number of active sites on the resin employed.

Using two different resins, we systematically examined the role of the first amino acid to commence the synthesis, which has not been compared before to our knowledge. Most of the previously reported protocols for GS synthesis have used commonly available preloaded resins with Fmoc-protected Leu, Val, or in some cases Pro. We therefore loaded all five Fmoc-amino acids from the GS sequence (Pro, Val, Orn, Leu, and DPhe) onto solid-phase resin, to compare the respective yields of the linear precursor peptides **1a**−**e**. Having synthesized the linear sequences first on Wang resins and later on 2-chlorotrityl resins, we found that the choice of resin makes no significant difference in yield (data not shown). 2-Chlorotrityl resin had

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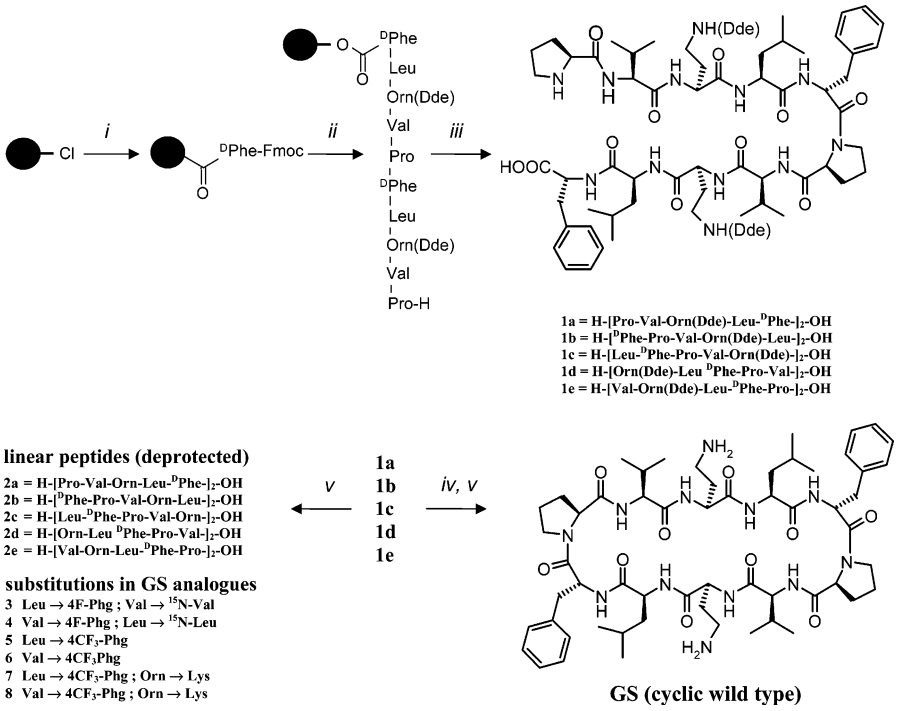
SCHEME 1. Solid-Phase Synthesis of Gramicidin S[a]



[a] Reagents and conditions. (i) Resin loading: starting amino acid (here Fmoc-DPhe-OH), DiPEA, Ch_2Cl_2, 2 h; (ii) standard Fmoc solid-phase peptide synthesis: 9 cycles; deprotection: 22% piperidine in DMF, 30 min; coupling: Fmoc-amino acid (4 equiv), HCTU (3.9 equiv), Cl-HOBt (4 equiv), DiPEA (8 equiv), 2 h; (iii) cleavage: TFA/*i*-Pr_3SiH/H_2O (92.5/5/2.5 v/v), 3.5 h; (iv) cyclization: 1 mg/mL in DCM, PyBOP (3 equiv), HOBt (3 equiv), DiPEA (6 equiv), CH_2Cl_2, 20 h; (v) N_2H_4 (2% in THF), 16 h.

TABLE 1. Characterization of Stepwise Yields in the Synthesis of 1a−e

peptide	starting residue	t_R[a] (min)	purity[b] (%)	linear yield[c] (%)	cyclization yield[d] (%)	overall yield[e] (%)
1a	DPhe	18.1	95	93	74	69
1b	Leu	15.8	68	67	46	31
1c	Orn(Dde)	15.8	70	64	26	17
1d	Val	15.4	89	88	55	48
1e	Pro	17.6	64	51	41	21

[a] HPLC retention time of the peak with correct mass. [b] Ratio of the peak area containing the correct mass over the total area within 5−20 min of the HPLC chromatogram (Supporting Information). [c] Corrected yield of the linear precursor peptide, calculated relative to the amount of resin employed. [d] Yield of the cyclization step per se, i.e., the amount of cyclic GS (purity > 95%) relative to the amount of the linear peptide. [e] Overall yield of GS for the entire synthesis relative to the amount of resin employed, calculated by multiplication of the respective linear yield with the cyclization yield.

been originally selected to prevent diketopiperazine formation when using Pro as the starting residue, but all subsequent syntheses (**3**−**8**) were then also performed on this resin.[45] The efficiency of two different coupling agents was also systematically tested for each different starting amino acid. We compared 2-(1*H*-6-chlorobenzotriazole-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) and 2-(7-azabenzotriazole-1yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TATU). HCTU was employed as it is one of the most economical coupling agents, whereas TATU is often considered as one of the most efficient ones. The yield of **1a**−**e** was always higher when HCTU was used (51−93%, Table 1), as compared to TATU (41−86%) (Supporting Information, Table 1). Hence HCTU is clearly preferred.

The most remarkable observation in Table 1 (and Supporting Information Table 1) is that the yields of **1a**−**e** differ systematically depending on the starting amino acid. With either coupling agent the yields are higher when the sequence is started at DPhe or Val compared to Pro, Orn, or Leu. These observations provide some first guidelines to an optimized GS synthesis. The high yield obtained especially with DPhe probably reflects a favorable situation in which the terminus of the growing sequence is freely accessible throughout all successive coupling steps.

Following cleavage of the linear peptide from the resin, solution-phase cyclization was carried out on the crude lyophilized peptide (without intermittent purification). Depending on the starting amino acid, which now constitutes the free C-terminus of the linear precursor peptide, a considerable variation was observed in the yield of cyclization, ranging from 26% to 74%. The data in Table 1 show that this factor is evidently one of the most critical steps in the synthesis of GS, with DPhe being the best and Orn the worst starting position. It has been generally suggested that certain precursor peptides are able to assume a favorable preorganized conformation which allows for efficient cyclization.[38−40]

Having identified DPhe as the best starting amino acid, we next varied the coupling agents and investigated the effect of their counterions. Among the three major classes of coupling agents, namely, symmetric anhydrides, OBt esters, and acid fluorides, we only investigated OBt esters. Symmetric anhydrides require 2 equiv of the protected amino acids, which makes the synthesis less economical when expensive isotope labels have to be incorporated. The use of acid fluorides was avoided, as most of these require low temperatures not only for their

(45) In an alternative procedure Pro was preferred at the C-terminus to avoid racemization during the synthesis, but in the present study no evidence for racemization of proline was found (ref 31).

TABLE 2. Yield of 1a with Different Coupling Agents

peptide	coupling agent	purity[a] (%)	yield[b] (%)
1a	HCTU	95	93
1a	TCTU	88	86
1a	HBTU	88	86
1a	TBTU	93	83
1a	PyBOP	86	63

[a] Ratio of the peak area containing the correct mass to the total area within 5−20 min of the HPLC chromatogram (Supporting Information). [b] Corrected yield.

formation but also for their subsequent coupling.[46] Since the idea was to develop a simple, efficient, and economical synthesis, we compared the OBt esters of HCTU, 2-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TCTU), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), along with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) as coupling agents. The advantage of OBt active esters is that they are efficient, stable, less prone to racemization, and suitable for automated peptide synthesis. The use of 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), which is known to be a highly efficient coupling agent, was omitted for economic reasons and in view of the fact that the yields of **1a**−**e** with TATU (Supporting Information, Table 1) were never any better than with HCTU. We thus synthesized compound **1a** using HCTU, TCTU, HBTU, TBTU, and PyBOP, and the yields are summarized in Table 2. The yield of **1a** prepared via HCTU was higher than that of the non-chlorinated analogue HBTU. It was also higher with HCTU compared to TCTU, which differ only in their counterions PF_6^- and BF_4^-, respectively. Similarly, the yield of **1a** with HBTU was slightly higher than that of TBTU. The counterion PF_6^- thus seems to give slightly better yields than BF_4^- with regard to the linear peptide precursor. However, this effect is not very significant compared to the more dramatic influence of the starting amino acid.

Since the different linear peptide sequences had such impact on determining the yield of cyclization, we addressed their conformation by circular dichroism (CD). Of the deprotected and purified precursor peptides **2a**−**e** we recorded CD spectrum under different conditions, namely, in aqueous buffer, in 50% trifluoroethanol (TFE), and in 100% TFE. The CD spectrum of cyclic GS in aqueous buffer (not shown) exhibits large negative ellipticities around 206 and 223 nm, as previously reported.[11,25,29] The CD spectra of the five linear precursor peptides **2a**−**e** in aqueous buffer are quite similar to one another but differ distinctively from the cyclic GS spectrum (data not shown). They exhibit a minimum around 198 nm and a substantially reduced ellipticity in the range of 205−225 nm, indicating a largely disordered structure. A more interesting conformational trend is observed in 50% TFE as illustrated in Figure 2. GS shows an overall increase in ellipticity compared to aqueous solution and a more pronounced minimum at 206 nm. While the CD spectra of **2b**, **2c**, and **2d** reveal a mainly disordered structure, **2a** and **2e** exhibit a more ordered conformation.[47]

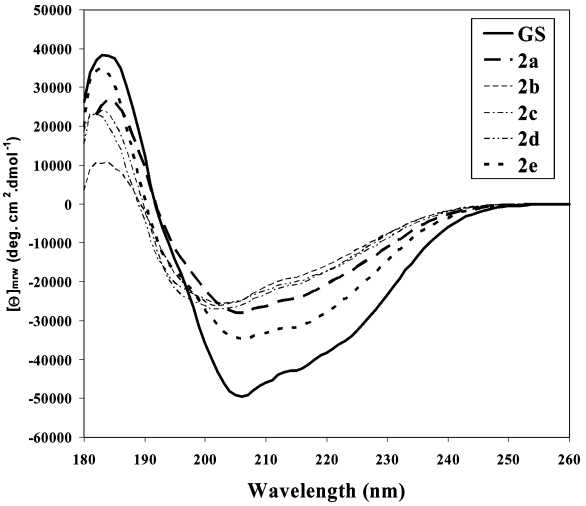


FIGURE 2. Circular dichroism spectra of cyclic gramicidin S and its linear precursor peptides **2a**−**e** in 50% TFE.

Especially the CD line shape of linear precursor peptide **2a**, with DPhe as starting (C-terminal) amino acid, resembles very closely that of cyclic GS. Both exhibit negative bands at 206 nm and around 220 nm and pass through zero at 192 nm. The line shape of **2e**, on the other hand, suggests some secondary structure but does not resemble that of GS so closely. In 100% TFE, finally, the spectrum of cyclic GS is similar to that of **2a**, **2d**, and **2e**, with two distinct negative bands around 206 and 220 nm, while showing subtle differences to **2b** and **2c** especially in the lower wavelength region (data not shown). This series of CD experiments demonstrates that of all linear precursor peptides the one starting with DPhe has the most pronounced tendency to assume a conformation in solution (as seen in 50% TFE) that is very similar to the structure of cyclic GS.

The remarkable pre-organized conformation observed for the precursor peptide **2a** correlates very well with its high yield of cyclization (Table 1). This conformational aspect, which is critical for the synthesis of cyclic peptides, has not been experimentally addressed before, although the concept has been discussed.[38] In practice, preloaded resins with Fmoc-Pro-OH, Fmoc-Leu-OH, or Fmoc-Val-OH have been most commonly used for GS synthesis, as they are readily available and cheap (being a factor of 5 less expensive than Fmoc-DPhe-resin, while Fmoc-Orn(Dde)-resin is not available at all). When starting a synthesis from any of these three preloaded amino acids, it usually leads to an acceptable yield of the respective linear precursor peptide. However, when this peptide is not appropriately preorganized with the N- and C-termini close together, as illustrated in Figure 3, cyclization remains the major yield-limiting step. Starting the synthesis of GS with DPhe appears to be the only route to achive an efficient cyclization. An additional advantage of DPhe that becomes apparent when inspecting Table 1 is the fact that also the yield of synthesizing the linear peptide **1a** is the highest of all. This synergistic effect of DPhe being favorable both in the linear couplings as well as cyclization appears to be a fortuitous coincidence but it leads to a dramatic improvement in the overall yield of GS up to about 70%.

The concept of a preorganized conformation raises the question as to whether the linear peptides exhibit any functional activity. Conflicting observations have been reported on such

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(47) CD measurements were not possible in dichloromethane as the solvent absorbs heavily in the lower wavelength region.

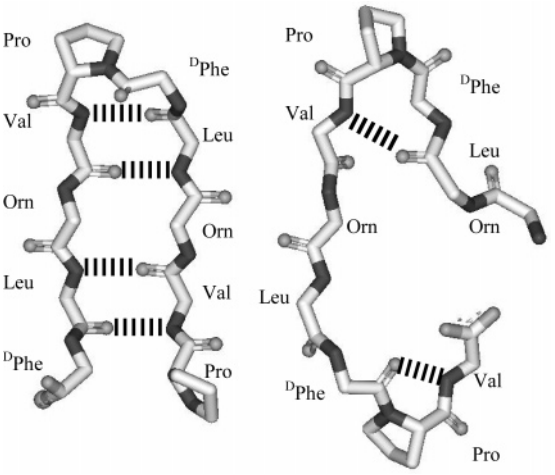

FIGURE 3. Molecular models illustrating possible conformations and hydrogen bonds of **1a** (left) and **1d** (right).

linear GS analogues in the literature.[27,44] We thus determined systematically the antimicrobial and hemolytic activities of the deprotected linear precursor peptides **2a**−**e** (Supporting Information, Table 2). The antimicrobial activities were judged by their minimum inhibitory concentration (MIC) against a Gram-positive (*Bacillus subtilis* ATCC 6633) and a Gram-negative strain (*Escherichia coli* DH5α). Gram-positive bacteria are generally more sensitive to GS than Gram-negative, giving MICs of 0.7 and 11 μM, respectively. For the linear precursor peptides **2a**−**e**, bacterial growth continued up to 80 μM, regardless of the type of bacterial strain. Likewise, GS caused 50% hemolysis of human erythrocytes at 12 μM, but none of the linear precursor peptides showed any activity up to a concentration of 36 μM. This indicates that GS needs to be cyclic for its activity. This finding is reminiscent of the antimicrobial β-hairpin peptide protegrin-1, which is cyclized by two intramolecular disulfide bridges. It requires these links for its biological activity, as the linear analogues were shown to be inactive.[48] Likewise the antiparallel β-strands of tachyplesin are held together by two disulfide bridges, and the linear derivatives are inactive.[49,50] Only when a preorganization of linear tachyplesin analogue was noncovalently induced by aromatic stacking, could the activity of some of these derivatives be recovered.[50]

In summary, we have shown that for the synthesis of backbone-cyclized peptides such as GS, the starting amino acid is very important and can severely influence the overall yield. This is a convolution of two independent factors: first, the yield obtained in coupling the linear precursor peptide, and second, the efficiency of cyclization. In the case of GS, both factors happen to potentiate each other, giving an optimal yield in both steps when starting with DPhe. The first factor seems to be a fortuitous coincidence related to the accessibility of the free N-terminus of the growing peptide chain throughout successive coupling steps. The second factor, related to cyclization, appears to correlate with a preorganized conformation of the linear precursor peptide as observed by CD. The optimum choice of DPhe as a starting amino acid is rationalized by the formation of a hairpin structure of the linear precursor peptide **1a**. This sequence carries the characteristic β-turn motif DPhe-Pro in its center, which may act as a nucleation site for the formation of a preorganized hairpin, as observed in the folding process of larger proteins.[51] Placing the N-and C-termini of **1a** into close apposition would allow the maximum number of four intramolecular hydrogen bonds to form, as illustrated in Figure 3. For any other linear precursor peptide starting with a different amino acid, the entropic cost of forming two β-turns and tying in two loose ends during cyclization is presumably much higher. Many other backbone cyclized peptide antibiotics, such as tyrocidines, streptocidins and loloatins, also possess a hairpin structure with two β-turns consisting of proline together with a D-amino acid. We may speculate that the relative position of this β-turn within the linear sequence is likely to be a relevant factor in determining the yield of cyclization of these other peptides.

In terms of chemical coupling efficiency, we may summarize that all OBt esters give acceptable yields, but HCTU seems to be the best coupling agent. The choice of counterion or resin was not as significant as the starting amino acid. The overall yield could be raised from about 20% to 70% for the final cyclic peptide, which is a significant improvement over previously reported protocols for GS. The optimized method employs reagents that are economical (HCTU) and usually available in most peptide laboratories. The use of Fmoc chemistry should make the synthesis of gramicidin S, its analogues, and presumably also other cyclic peptides much easier and more efficient. The entire protocol requires only a single HPLC purification step at the very end, thus minimizing any loss of intermediates.

With a view to solid-state NMR applications, we have used the optimized protocol to prepare several selectively isotope-labeled GS analogues **3**−**8**, containing a ^{15}N-labeled amino acid (Leu or Val) and a suitably substituted flurophenylglycine side chain (4F-Phg or 4CF_3-Phg). By incorporating a labeled ^{15}N-amino acid into the peptide bond or when a highly sensitive ^{19}F-reporter is rigidly attached to the GS backbone, it is possible to determine the detailed structure and dynamics of GS in its biologically relevant membrane-bound state.[17,19] This information may then be used to develop an analogue that is more selective toward bacterial membranes.

Experimental Section

Solid-Phase Peptide Synthesis. All linear precursor peptides **1a**−**e** were synthesized manually, using standard Fmoc solid-phase protocols.[52] Resin loading was performed as described by the manufacturers' protocol, and the loading was determined to be 0.63−0.87 mmol/g. The synthesis was performed on a 100 μmol scale and was scaled up to 0.5 mmol. Deprotection was carried out with piperidine (22% in DMF, 30 min). Peptide elongation was carried out sequentially using commercially available Fmoc-amino acids. The appropriate Fmoc-amino acid (4 equiv) was preactivated with 6Cl-HOBt (4 equiv) and HCTU (3.9 equiv) in the presence of the base DiPEA (8 equiv) in 2 mL of *N*-methyl pyrrolidone (NMP). The resulting solution was preactivated for 2 min, followed by coupling for 2 h. NMP or DMF were used as solvents. The Fmoc-deprotection and coupling efficiencies were monitored by performing a Kaiser test.[53] The linear decapeptides were cleaved

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from the resin using a cocktail of TFA/*i*-Pr_3SiH/H_2O = 92.5:5:2.5. Typically 25 mL of cleavage cocktail was used per gram of resin. A higher percentage of *i*-Pr_3SiH (5%) was found to be necessary compared to the usual 1−2% reported in the literature, to minimize the formation of peptide fragments with undesired mass. The cleavage was performed at room temperature for 3−5 h with occasional shaking. The resin was filtered off and washed twice with 5 mL of pure TFA. The volume of combined filtrates was reduced under a gentle stream of nitrogen. The product was precipitated with cold diethyl ether. The solid product was taken up in 1:1 acetonitrile/water and lyophilized. The crude product was characterized with mass spectrometry, and the purity of the product was determined using analytical reverse-phase HPLC. Yields were determined by integrating the peak area and determining the ratio of this area to the total area over 5−20 min of the HPLC chromatogram (Supporting Information)

Cyclization. Cyclization was performed using PyBOP as previously reported.[24,54] The linear Dde-protected decapeptide (0.5 mg/mL) was dissolved in degassed dichloromethane (DCM) in a 1-L round-bottom flask. Solid PyBOP (3 equiv) and a solution of HOBt (3 equiv) in degassed DMF (0.5 mL) were added to the flask. The mixture was stirred to dissolve. DiPEA (6 equiv) was then added to initiate the reaction. The reaction was monitored by HPLC (Supporting Information) and allowed to proceed overnight under argon atmosphere. Completion of cyclization was confirmed by performing mass spectra on an aliquot of the reaction mixture. After removing the solvent DCM by rotary evaporation, the resulting oil was taken up in THF and treated directly with 2% hydrazine solution in THF (v/v) to remove the Dde-protection group on the δ-amino groups of Orn. The progress of the reaction was monitored for about 3 h at room temperature, followed by stirring overnight. Dde-deprotection was monitored using analytical HPLC (Supporting Information). The solution was rotary-evaporated to give a yellowish oil, which was dissolved in acetonitrile/water (1:1) and purified directly by preparative HPLC. Mass spectrometry of this sample provided evidence of proper cyclization and of complete removal of the Dde-protection group.

H-Pro-Val-Orn(Dde)-Leu-^{D}Phe-Pro-Val-Orn(Dde)-Leu-^{D}Phe-OH (1a). All linear analogues were synthesized by standard Fmoc-SPPS protocols. Typically, in a reaction vessel fitted with a filter and stopper, Fmoc-^{D}Phe-2-chlorotrityl resin (100 μmol) was swollen for 45 min in 5 mL of degassed DMF under continuous nitrogen flow. After DMF was drained, 4 mL of 22% piperidine was added and the solution allowed to drain through the resin bed over a period of 5 min. A second aliquot of 5 mL of 22% piperidine was then added, and deprotection was allowed to proceed for another 30 min under N_2 atmosphere. The piperidine solution was drained under positive pressure of nitrogen, and the resin was washed with NMP (10 mL × 2). The presence of free NH_2 groups was checked with a Kaiser test.[53] Next, Fmoc-Leu-OH (4 equiv) was dissolved with 6Cl-HOBt (4 equiv) and HCTU (3.9 equiv) in 2 mL degassed DMF in the presence of DiPEA (8 equiv). The mixture was vortexed for 2 min and added to the reaction vessel, and the coupling was allowed to proceed at room temperature under N_2 atmosphere for 2 h. The resin was then washed with NMP (10 mL × 2), and the completion of coupling was checked by a Kaiser test. Deprotection-coupling cycles were repeated to obtain the complete decameric sequence. Final Fmoc-deprotection and cleavage from the resin afforded the linear peptide **1a**. MALDI-TOF: *m*/*z* 1487.3 $[M + H]^+$, calcd mass 1486.88. ^{1}H NMR (400 MHz, DMSO-d_6, 25 °C): δ 13.34−13.19 (m, 2H), 9.35 (s, 1H), 8.60−8.37 (m, 3H), 8.34−8.28 (d, J = 8.6 Hz, 1H), 8.22−8.0 (m, 1H), 8.0−7.9 (m, 2H), 7.28−7.10 (m, 10H), 4.49−4.14 (m, 10H), 3.25−2.65 (m, 10H), 2.47−2.42 (m, 6H), 2.29−2.21 (m, 10H), 2.04−1.04 (m, 30H), 0.96−0.63 (m, 42H).

GS (Cyclic Wild Type). Cyclization and Dde-deprotection of linear precursor peptides **1a**−**e** were performed as described above to give the final product gramicidin S. MALDI-TOF: *m*/*z* 1141.1 $[M + H]^+$, calcd mass 1140.7. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.13−8.06 (m, 2H), 8.72 (d, J = 9.3 Hz, 2H), 8.35 (d, J = 9.1 Hz, 2H), 7.92−7.79 (m, 6H), 7.35−7.20 (m, 12H), 7.19 (s, 1H), 6.98 (s, 1H), 4.82−4.72 (q, 2H), 4.62−4.54 (q, 2H), 4.44−4.26 (m, 6H), 3.66−3.54 (m, 2H), 3.03−2.69 (m, 10H), 2.13−1.92 (m, 4H), 1.84−1.20 (m, 24H), 0.90−0.71 (m, 24H).

Hemolysis Assay. Hemolytic activity was examined by a serial 2-fold dilution assay.[55] Citrate phosphate dextrose-stabilized blood bags (erythrocyte suspensions of healthy donors) were obtained from the blood bank of the municipal hospital (Karlsruhe, Germany). Erythrocytes were washed twice with 9-fold excess of Tris (172 mM, pH 7.6, 0 °C) (1000 g, 10 min, 4 °C) and kept on ice between. After the second wash the erythrocytes were transferred from the sediment to a fresh tube with the same precooled buffer to be diluted to about 5% (v/v) hematocrit, giving the stock cell suspension, which was kept on ice. For each peptide two individual shifted serial 2-fold dilutions in Tris (pH 7.6 at 37 °C)/DMSO (9/1 v/v) were prepared. The stock cell suspension was further diluted to 0.25% (v/v). After preincubating for 5 min, 200 μL of the resulting erythrocyte dilution was transferred to each tube of the corresponding peptide serial dilution (final concentration 0.125% (v/v)). For each dilution series, zero hemolysis was obtained by adding the erythrocytes to Tris (pH 7.6 at 37 °C)/DMSO (9/1 v/v) and measuring the background lysis in the absence of peptide. For 100% hemolysis, 0.1% of Triton X-100 in the same buffer was added. Incubation was performed at 37 °C for 20 min with gentle shaking. The tubes were centrifuged (20 000*g*, 5 min) to pellet the cells and debris, and the absorbance at 540 nm was recorded against Tris buffer. The percentage lysis was then calculated relative to 0% lysis with buffer and 100% lysis by Triton X-100.

Antimicrobial Activity. Antimicrobial activity was studied by a standard minimal inhibitory concentration (MIC) assay, carried out with Gram-positive *Bacillus subtilis* ATCC 6633 and Gram-negative *Escherichia coli* DH5α. Bacteria were grown in Luria-Bertani medium at 37 °C and 230 rpm overnight, and diluted in 1% trypticase soy broth (TSB). Microtiter plates (96 wells of 100 μL) were filled with 50 μL of 1% TSB, and serial 2-fold dilutions of peptides were arranged in quadruple. The two final rows of each plate remained without peptide, so that the penultimate data point served as the positive control (no peptide) and the final one as the negative control (not inoculated). Fifty microliters of bacterial suspension was added to the wells (except for the final row of each plate) to give a final concentration of 10^6 colony forming units per milliliter. The plates were incubated at 37 °C for 20 h, and the MIC was determined visually on the basis of turbidity as the lowest concentration inhibiting bacterial growth.

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Supporting Information Available: Table characterizing the linear GS precursor peptides using TATU; ^{1}H NMR of **1a**−**e**, *cyclo*-**1a** (GS), **3**, **4**; HPLC traces of **1a**−**e**, **1a** with various coupling agents, cyclization and Dde-deprotection of **3**; mass spectra of **1a**−**e**, *cyclo*-**1a** (GS), **2a**−**e**, **3**−**8**; and table summarizing the antimicrobial and hemolytic activities of **2a,b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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