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# Synthesis and biological evaluation of gramicidin S dimers†

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The design and synthesis of analogues of the cyclic β-sheet peptide gramicidin S (GS), having additional functionalities in their turn regions, is reported. The monomeric GS analogues were transformed into dimers and their activities towards biological membranes, through antimicriobial and hemolytic assays, were evaluated. Finally, conductivity measurements have been performed to elucidate ion channel forming properties.

## Introduction

Cationic antimicrobial peptides (CAPs), a class of structurally diverse peptide-based compounds, are ubiquitously present in nature and are often important compounds of innate immune systems.[1] CAPs exert their biological activity by disturbing the integrity of the cell membrane of pathogenic organisms, and are thought to do so through direct interaction with the lipid bilayer.[2] From the thought that, in most cases, there is no specific subcellular protein target involved in CAP-mediated cell lysis, it follows that the occurrence of pathogens with an acquired CAP-resistance may be unlikely. These considerations, to a large extent, explain current interest in CAPs and research efforts are focussed both on the elucidation of the molecular mechanisms that are at the basis of CAP-mediated membrane disturbance, and on the development of CAPs towards therapeutic agents.

Gramicidin A (GA) is a CAP that has attracted considerable attention over the years.[3] This pentadecapeptide exerts its antibacterial properties by adopting a β-helical secondary structure that associates into a dimer, thereby forming an active ion channel that traverses lipid bilayers. Schreiber and coworkers elegantly demonstrated that covalently linked GA monomers result in unimolecular channels spanning the membrane.[4] More recently, unimolecular membrane-spanning ion channels that have preference for specific ions were obtained by linking two GA monomers through tetrahydrofuran (THF)-based dipeptide isosters.[5] The potential of synthetic dimers is further underscored by the requirement that accumulation of CAPs onto the lipid bilayer precedes pore formation. In this way, synthetic dimers can induce a shift in the dissociation/association equilibrium, resulting in favouring of the conducting over the non-conducting states of ion-channels. This enables the study of the molecular architecture of ion channels and creates an understanding of the dynamics involved. For example, Woolley *et al.* showed that tethered alamethicin monomers selectively stabilize specific conductance states,[6] while Murata and coworkers have prepared bioactive dimers of the polyene antibiotic amphotericin B that enabled the study of pore assemblage.[7]

As part of an ongoing research program,[8] we have developed a practical strategy towards the synthesis of the antimicrobial peptide gramicidin S (GS)[9] and analogues thereof. GS is a cyclic decapeptide of the primary sequence cyclo-(D-Phe-Pro-Val-Orn-Leu)$_2$ that adopts a stable $C_2$-symmetric β-sheet structure.[10] The valine, ornithine and leucine residues of the two opposing β-strands are positioned on alternate sides of the pleated sheet and are flanked by two type II′ β-turns consisting of a D-Phe-Pro dipeptide sequence, thus creating an amphipathic structure that is thought to be at the basis of the cell-permeabilization activity of GS. Through the synthesis of novel GS analogues, we ultimately aim to gain insight into the mechanism of its lytic effects, induce membrane specificity in order to curb its undesirable erythrocytic toxicity and to see whether defined channels can be resolved. In line with these studies, we here present our results in the design, synthesis and biological evaluation of a set of dimeric GS analogues.

## Results and discussion

The synthesis of GS dimer **6** was accomplished as follows (Scheme 1). Standard Fmoc-based solid-phase synthesis using commercially available building blocks and the readily accessible Fmoc-2*S*,4*R*-azidoproline (Azp) and starting from 4-(4-hydroxymethyl-3-methoxyphenoxy)butanoic acid (HMPB) functionalized 4-methylbenzhydrylamine (MBHA) resin **1** that had been esterified with Fmoc-protected leucine,[8a,11] was followed by mild acidolytic cleavage from the solid support and ensuing solution-phase cyclization,[8b,c] to provide Boc-protected cyclic peptide **2** (86%). Reduction of the azide moiety under Staudinger conditions gave free amine **3**, that was treated with succinic anhydride and subsequently condensed with pentafluorophenol (Pfp) under the agency of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) to furnish **4** in 80% over three steps. The activated ester in the monomeric GS analogue was next reacted with a slight excess of **3**, to produce GS dimer **5**. Acidolysis of the Boc-protection groups still present on the Orn-residues followed by reversed-phase HPLC purification, yielded the succinyl-tethered dimer **6** in 69%, the identity of which was confirmed by LC/MS analysis.

Encouraged by these results, we set out to connect turn-modified GS analogues directly through newly introduced amino acid side-chain functionalities. For this purpose, a set of monomeric GS analogues, in which D-Phe- and Pro-residues residing in the same or opposing turn regions are replaced

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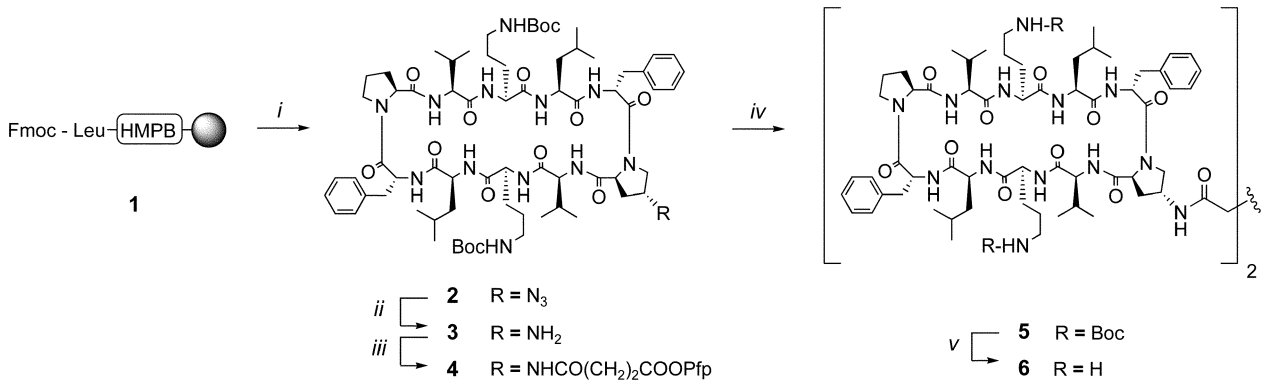



**Scheme 1** Reagents and conditions: (*i*) ref. 8, 86%; (*ii*) $PMe_3$ (1 M in toluene, 8 equiv), 1,4-dioxane/MeCN (1/1 v/v), 6 h; (*iii*) (a) succinic anhydride (4 equiv), TEA (4 equiv), DMF, 16 h. (b) pentafluorophenol (2 equiv), EDC (2 equiv), DCM, 2 h, 80% in 3 steps; (*v*) **3** (1.1 equiv), DiPEA (2 equiv), DMF, 48 h, 92%; (*iv*) TFA/DCM (1/1 v/v), 30 min, 69%.

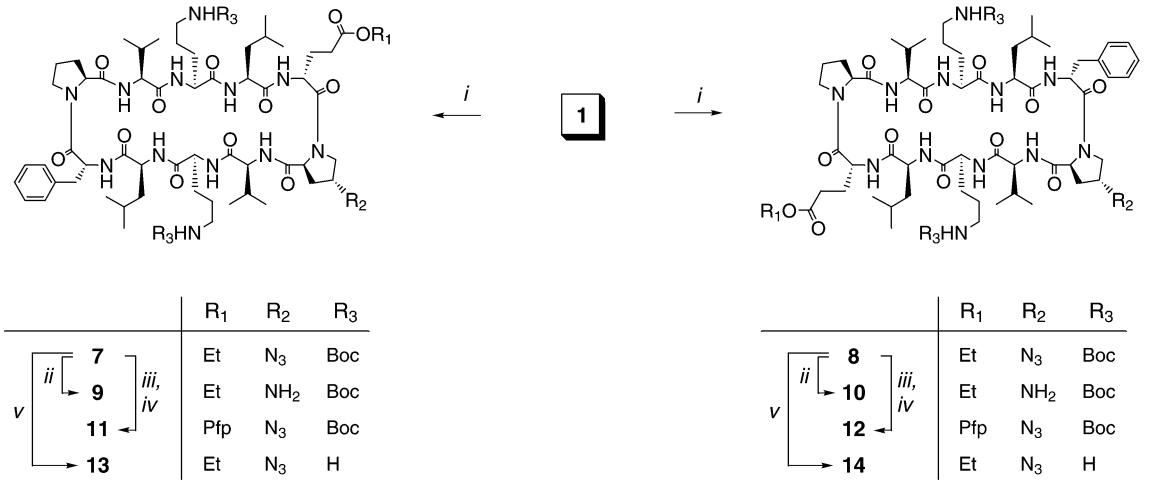
by $^{D}$Glu- and aminoproline (Amp)-residues, respectively, were prepared as follows (Scheme 2). Commencing from solid support **1**, fully protected cyclic peptides **7** and **8** were constructed as described for **2** in 82 and 92% yield, respectively. Staudinger reduction of the azide in **7** and **8** afforded the amines **9** and **10**, respectively. Saponification of the ester moiety in **7** and **8** revealed their carboxylic acid counterparts that were directly esterified with pentafluorophenol employing EDC to furnish **11** and **12**. GS monomers **9**–**12** were used without further purification in the following reaction steps. To facilitate characterization by LC/MS analysis and to evaluate the biological profile of the monomers, small aliquots of **7** and **8** were deprotected and purified by HPLC to produce **13** and **14** in their respective yields of 68 and 89%.

Having set the stage for coupling of the separate monomeric building blocks, equimolar amounts of amine **9** and activated ester **11** were reacted to furnish dimer **15** in 45%, as is depicted in Scheme 3. Similarly, acylation of GS analogue **10** with Pfp-ester **12** gave side-chain linked dimer **16** in 85%. The remaining Boc groups in **15** and **16** were subsequently removed to provide, after HPLC purification, their respective unprotected dimers **17** (66%) and **18** (31%). The azide and ester moieties in dimers **15** and **16** were also quantitatively transformed into the amine and carboxylic acid functionalities present in **19** and **20** and were subsequently deprotected to give dimers **21** (53%) and **22** (53%) as gauged by LC/MS analysis.[12]

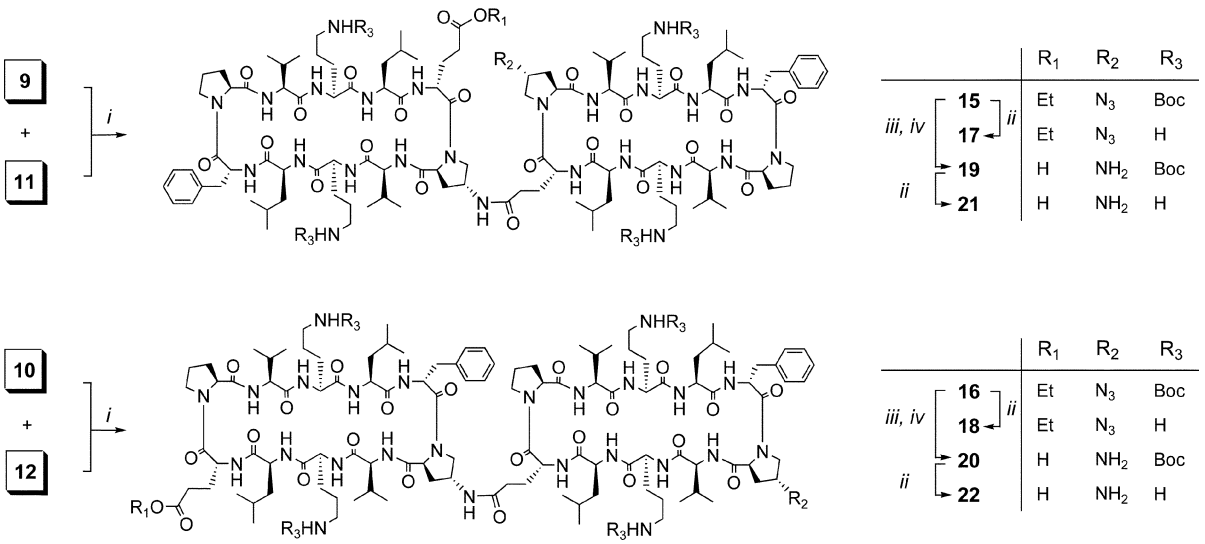
As a final synthetic objective, we investigated whether intramolecular cyclization of **19** and **20** was feasible. Condensation of the carboxylic acid and amine functionalities in **19** could be effected by dropwise addition of the peptide solution to a mixture of benzotriazole-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP), *N*-hydroxybenzotriazole (HOBt) and *N*,*N*-diisopropylethylamine (DiPEA) in DMF. Liberation of the Boc-protection groups yielded the rigid GS dimer **23** as the major product, albeit in 25% yield over two steps. A similar intramolecular cyclization was also performed on peptide **20**. Previous observations of β-sheet alignment in GS,[13a] and the proposed 'cross-β' aggregates by Goodman *et al.*,[13b] were expected to facilitate this cyclization reaction. However, only the reaction conditions described for the cyclization of **19** proved to be marginally effective in yielding product **24** (15%; Scheme 4).[14]

Having the various GS dimers in hand, attention was focussed on the evaluation of their antimicrobial, hemolytic and conductance-increasing properties. The capacity of the GS dimers, and the monomers from which they are assembled, to arrest the proliferation of several Gram-positive and -negative bacterial strains was examined using a standard minimal inhibitory concentration (MIC) test (Table 1).[15] From these results, it can be concluded that the modifications in the reverse turn, an Azp- for a Pro-residue and a $^{D}$Glu- for $^{D}$Phe-residue in monomers **13** and **14**, have no adverse effect on the antimicrobial properties compared to native GS. Conversely, the dimers had lost virtually all activity towards the tested Gram-positive and -negative strains with only dimer **22** displaying limited activity against *Staphylococcus epidermidis*.

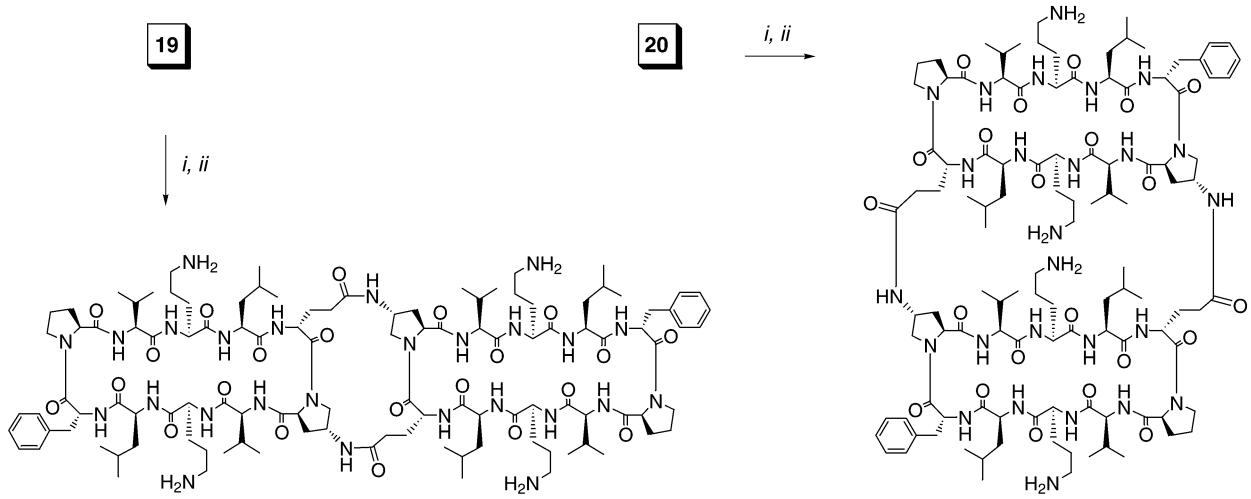
A standard assay was applied to assess the hemolytic activity of the GS analogues towards human erythrocytes using a two-fold dilution series of the appropriate peptide and interpolating




**Scheme 2** Reagents and conditions: (*i*) ref. 8, **7**; 82% and **8**; 92%; (*ii*) $PMe_3$ (1 M in toluene, 8 equiv), 1,4-dioxane/MeCN (1/1 v/v), 6 h; (*iii*) 1 M NaOH, 1,4-dioxane, 4 h, then Amberlite IR-120 ($H^+$); (*iv*) pentafluorophenol (2 equiv), EDC (2 equiv), DCM, 2 h; (*v*) TFA/DCM (1/1 v/v), 30 min, **13**; 68% and **14**; 89%.




**Scheme 3** Reagents and conditions: (*i*) DMF, **15**; 45% and **16**; 85%; (*ii*) TFA/DCM (1/1 v/v), 30 min, **17**; 66%, **18**; 31%, **21**; 53% and **22**; 53%; (*iii*) $PMe_3$ (1 M in toluene, 8 equiv), 1,4-dioxane/MeCN (1/1 v/v), 6 h; (*iv*) 1 M NaOH, 1,4-dioxane, 4 h, then Amberlite IR-120 ($H^+$) **19**; quant. and **20**; quant. (two steps).




**Scheme 4** Reagents and conditions: (*i*) BOP (5 equiv), HOBt (5 equiv), DiPEA (15 equiv), DMF, 16 h. (*ii*) TFA/DCM (1/1 v/v), 30 min, **23**; 25% and **24**; 15% (two steps).

**Table 1** Antimicrobial activity (MIC in μg $ml^{-1}$)[a]

| | *S. aureus*[b] | | *S. epidermidis*[b] | | *E. faecalis*[b] | | *B. cereus*[b] | | *E. coli*[c] | | *P. aeruginosa*[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Peptide | 25W[d] | MT[e] | 25W[d] | MT[e] | 25W[d] | MT[e] | 25W[d] | MT[e] | 25W[d] | MT[e] | 25W[d] | MT[e] |
| **GS** | 8 | 8 | 4 | 4 | n.d. | 8 | 4–8 | 4 | >64 | 32 | >64 | >64 |
| **6** | >64 | >64 | 64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 |
| **13** | 8 | 8 | 4 | 8 | 16 | 8–16 | 8 | 8 | 64 | 64–>64 | >64 | >64 |
| **14** | 8 | 8 | 4 | 8 | 8 | 8–16 | 8 | 8 | >64 | 64 | >64 | >64 |
| **17** | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 |
| **18** | 64–>64 | >64 | 64–>64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 |
| **21** | >64 | 64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 |
| **22** | >64 | >64 | 64 | 32 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 |
| **23** | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 |

[a] Measurements were executed using standard agar two-fold dilution techniques (n.d. = not determined). [b] Gram-positive. [c] Gram-negative [d] 3 ml/25 well plates [e] 100 μl/96 microtiter plates.

between 100% lysis induced by 1% Triton X-100 in saline and a blank measurement. As can be gauged from the results in Table 2, all tested peptides, both GS monomers and GS dimers, cause considerable hemolysis in the 60 to 30 μM range, making them similarly or even more (*i.e.* **17**) toxic as the native peptide. However, most GS dimers in this assay display the propensity to lyse erythrocytes over a broader range of concentrations starting as low as 1.0 to 0.5 μM but often reaching 100% lysis only at 250 to 125 μM.

Finally, we have conducted studies concerning the pore-forming properties of GS, succinyl-tethered GS dimer **6** and rigid GS dimer **23** (Fig. 1). Events of this type are commonly

**Table 2** Hemolytic activity (% hemolysis)[a]

| Peptide | Peptide concentration (μM) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 250.0 | 125.0 | 62.5 | 31.3 | 15.6 | 7.8 | 3.9 | 2.0 | 1.0 | 0.5 | 0.2 |
| **GS** | | | 100 ± 2 | 80 ± 3 | 50 ± 1 | 24 ± 0 | 2 ± 2 | 0 ± 0 | 0 ± 1 | 0 ± 0 | n.d. |
| **6** | 100 ± 5 | 79 ± 9 | 50 ± 3 | 62 ± 5 | 65 ± 3 | 49 ± 6 | 32 ± 5 | 8 ± 4 | 8 ± 1 | 3 ± 3 | n.d. |
| **13** | 80 ± 5 | 90 ± 3 | 92 ± 3 | 52 ± 7 | 21 ± 3 | 3 ± 1 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | n.d. |
| **14** | | 100 ± 5 | 66 ± 1 | 35 ± 7 | 25 ± 2 | 8 ± 0 | 0 ± 0 | 0 ± 1 | 0 ± 0 | 0 ± 0 | n.d. |
| **17** | | | | 100 ± 1 | 93 ± 3 | 90 ± 31 | 77 ± 2 | 29 ± 3 | 14 ± 3 | 6 ± 2 | 0 ± 0 |
| **18** | | 100 ± 2 | 87 ± 1 | 70 ± 9 | 62 ± 7 | 66 ± 2 | 44 ± 3 | 18 ± 3 | 6 ± 1 | 0 ± 0 | n.d. |
| **21** | 100 ± 0 | 98 ± 2 | 87 ± 7 | 76 ± 2 | 52 ± 2 | 26 ± 0 | 18 ± 2 | 10 ± 1 | 3 ± 4 | 0 ± 1 | 0 ± 0 |
| **22** | | 100 ± 1 | 78 ± 3 | 52 ± 6 | 41 ± 5 | 23 ± 2 | 14 ± 1 | 2 ± 0 | 1 ± 0 | 0 ± 1 | n.d. |
| **23** | 96 ± 1 | 99 ± 4 | 82 ± 4 | 64 ± 4 | 51 ± 3 | 39 ± 4 | 26 ± 1 | 11 ± 1 | 1 ± 4 | 0 ± 0 | n.d. |

[a] Measurements were executed using standard two-fold dilution techniques. (n.d. = not determined).

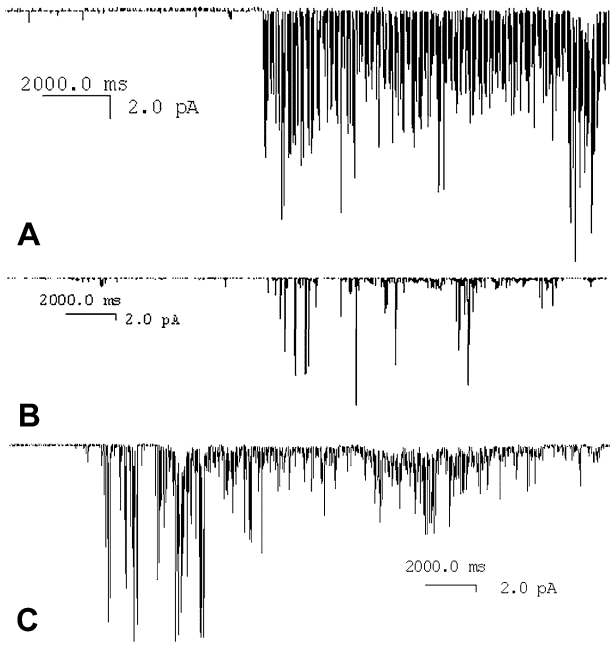



**Fig. 1** Conductivity traces for GS (A), dimer **6** (B) and dimer **23** (C) performed with 1 M KCl in a DPhPC/DPhPGlycerol 4 : 1 membrane.

described as bursts and depending on the concentration, there is a minimal voltage required for these bursts. Below $10^{-6}$ molar, no effects were observed for GS and dimer **6**, but at $10^{-6}$ molar they induced rapid changes in conductances. Dimer **23** was slightly more active, displaying conductance-increasing effects at $10^{-7}$ molar concentrations. Although all three compounds showed conductance-increasing properties,[16] no series of discrete single channels could be resolved.

In summary, we have described the synthesis of GS analogues that have been modified in the β-turn region, with $^{D}$Phe residues being replaced by $^{D}$Glu(OEt) and Pro-residues by Azp-residues. These GS monomers have subsequently been covalently linked either *via* a succinyl-tether or directly through their side-chains to produce several differently functionalized GS dimers. Intramolecular cyclization also produced more conformationally restricted dimers, albeit in moderate yields. As was previously observed with a GS dimer,[17] no bactericidal effect against either Gram-positive or -negative strains could be detected. However, the GS dimers displayed a significant increase in hemolytic activity. Moreover, these compounds proved hemolytic over a broader range of concentrations, which might suggest a different mode of action on the lipid bilayer. Upon studying the conductance-increasing properties of selected GS-dimers, they showed membrane disruptive properties but no discrete channels could be observed.

## Experimental

### 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 $L^{-1}$) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g $L^{-1}$) in 10% sulfuric acid or by spraying with a solution of ninhydrin (3 g $L^{-1}$) in EtOH/AcOH (20/1 v/v), followed by charring at ≈150 °C. Size exclusion chromatography was performed on Sephadex™ LH-20. For LC/MS analysis, a Jasco HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical Alltima $C_{18}$ column (Alltech, 4.6 × 250 mm (id × l), 5 μm particle size) in combination with buffers A: $H_2O$, B: MeCN and C: 0.5% aq. TFA and coupled to a Perkin Elmer Sciex API 165 mass instrument with a custom-made Electronspray Interface (ESI) was used. For reversed-phase HPLC purification of the peptides, a BioCAD 'Vision' automated HPLC system (PerSeptive Biosystems, inc.) supplied with a semi-preperative Alltima $C_{18}$ column (Alltech, 10.0 × 250 mm (id × l), 5 μ particle size) was used. The applied buffers were A: $H_2O$, B: MeCN and C: 1.0% aq. TFA.

**Monomer 2.** From resin **1** (200 mg, 0.1 mmol), the peptide was constructed following a literature procedures[8b,c] to give the title compound **2** (120 mg, 86 μmol) in 86% as a white amorphous solid. To confirm the identity of **2**, an aliquot was dissolved in DCM (1 mL), cooled to 0 °C and TFA (1 mL) was added slowly. After 30 min of stirring, the solvents were removed *in vacuo* and the identity of peptide **2** was established by LC/MS: $R_t$ 13.94 min; linear gradient 10→90% B in 20 min.; $m/z = 1183.0$ $[M + H]^+$, 592.4 $[M + H]^{2+}$.

**Monomer 3.** Azide **2** (26 mg, 19 μmol) was dissolved in 1,4-dioxane (2 mL) and acetonitrile (2 mL), to which trimethylphosphine (0.15 mL, 0.15 mmol, 8 equiv, 1 M in toluene) was added. The mixture was stirred for 3 h, water (0.1 mL) was added and stirring was continued for another 3 h. Next, all solvents were removed *in vacuo* and the crude peptide was thrice coevaporated with dry toluene.

**Monomer 4.** Crude peptide **3** was dissolved in DMF (2 mL) and succinic anhydride (76 mg, 76 μmol, 4 equiv) and triethylamine (76 mg, 76 μmol, 4 equiv) were added. After stirring for 16 h, the solvent was evaporated and the mixture was directly applied to a LH-20 column that was eluted with MeOH. The peptide-containing fractions were pooled, evaporated and redissolved in DCM (2 mL). To the solution were added pentafluorophenol (6.3 mg, 34 μmol, 2 equiv) and EDC (6.5 mg, 34 μmol, 2 equiv) and stirring was continued for 2 h. The mixture was subsequently concentrated and partitioned between 0.1 M HCl and $CHCl_3$. The organic layer was dried ($MgSO_4$), filtered and concentrated, to furnish the activated ester **4** (22 mg, 14 μmol) in 80% over 3 steps as amorphous white solid.

**Dimer 5.** To Pfp-ester **4** (22 mg, 14 μmol) in DMF (0.5 mL), a fresly prepared batch of amine **3** (22 mg, 16 μmol, 1.1 equiv) in DMF (0.5 mL) was added and the resulting mixture was stirred for 48 h. The solvents were removed under reduced pressure and the mixture separated by size-exclusion chromatography using MeOH as eluent. The fractions containing peptide were pooled and evaporated to dryness to produce the title compound **5** (36 mg, 13 μmol, 92%) as amorphous white solid.

**Dimer 6.** The dimer **5** (18 mg, 6.4 μmol) was deprotected as described for monomer **2** to give crude **6**, that was analyzed by LC/MS ($R_t$ 15.26 min; linear gradient 10→90% B in 20 min.; $m/z$ = 2395.8 $[M + H]^+$, 1198.6 $[M + H]^{2+}$, 799.6 $[M + H]^{3+}$) and purified by reversed-phase HPLC (linear gradient of 3.0 CV; 50→60% B; $R_t$ 3.1 CV) to give dimer **6** (10.6 mg, 4.4 μmol, 69%) as a fluffy white solid.

**Monomer 7 and 8.** From resin **1** (500 mg, 0.25 mmol), the peptides were assembled according to literature procedure[8b,c] to furnish cyclic peptides **7** (299 mg, 0.21 mmol, 82%) and **8** (320 mg, 0.23 mmol, 92%) as a white solids.

**Monomer 9 and 10.** Azides **7** (125 mg, 90 μmol) and **8** (125 mg, 90 μmol) were individually treated with $PMe_3$, as described for monomer **3**, to furnish crude amines **9** and **10** as white solids that were directly used in the next reaction step.

**Monomer 11 and 12.** Ethyl esters **7** (125 mg, 90 μmol) and **8** (125 mg, 90 μmol) were individually dissolved in EtOH (5 mL) and 1 M aq. NaOH (0.5 mL) was added. After stirring the mixtures for 2 h, TLC analysis showed completed conversion of starting material and Amberlite IR-120 ($H^+$) was added. The neutral solutions were subsequently concentrated, coevaporated thrice with dry toluene and the crude acids were individually redissolved in DCM. To these were added pentafluorophenol (33 mg, 0.18 mmol, 2 equiv) and EDC (35 mg, 0.18 mmol, 2 equiv) and stirring was continued for 2 h. The mixture was diluted with $CHCl_3$ and extracted with 0.1 M HCl after which the organic layer was dried ($MgSO_4$), filtered and concentrated, to furnish the crude Pfp-esters **11** and **12** that were directly used in the next reaction step.

**Monomer 13.** To a cooled solution of cyclic peptide **7** (25 mg, 18 μmol) in DCM (4 mL) was added TFA (4 mL) and the mixture was stirred for 30 min after which it was evaporated to dryness. The crude product was analyzed by LC/MS ($R_t$ 18.53 min, linear gradient 10→90% B in 30 min; $m/z$ = 1193.1 $[M + H]^+$, 597.0 $[M + H]^{2+}$), purified by RP-HPLC (linear gradient of 3.0 CV; 50→60% B; $R_t$ 2.0 CV) and lyophilized, to produce peptide **13** (14.7 mg, 12 μmol, 68%) as white amorphous powder.

**Monomer 14.** The cyclic peptide **8** (30 mg, 22 μmol) was similarly deprotected as for **13**, to give the crude peptide that was analyzed by LC/MS ($R_t$ 18.48 min; linear gradient 10→90% B in 30 min; $m/z$ = 1193.1 $[M + H]^+$, 597.0 $[M + H]^{2+}$), purified by RP-HPLC (linear gradient of 3.0 CV; 50→60% B; $R_t$ 2.3 CV) and lyophilized, to produce **14** (23.1 mg, 19 μmol, 89%) as white amorphous powder.

**Dimer 15 and 16.** Pfp-ester **11** was dissolved in DMF (2 mL) and a solution of amine **9** in DMF (2 mL) was slowly added. To this mixture, DiPEA (29 μL, 0.18 mmol, 2 equiv) was added and the reaction was stirred 48 h. The solvents were subsequently removed *in vacuo* and the resulting mixture applied to a size-exclusion column that was eluted with MeOH. Pooling of the peptide-containing fractions gave dimer **15** (109 mg, 40 μmol, 45%) as white amorphous solid. Monomer **12** was treated in an equal manner with amine **10** to furnish dimer **16** (207 mg, 76 μmol, 85%) as white amorphous solid.

**Dimer 17 and 18.** Dimers **15** (13 mg, 4.8 μmol) and **16** (40 mg, 14.7 μmol) were treated as described for **13**, to give crude **17** and **18**, respectively, and were analyzed by LC/MS; **17**: $R_t$ 15.97 min; linear gradient 10→90% B in 20 min; $m/z$ = 1157.5 $[M + H]^{2+}$, 771.8 $[M + H]^{3+}$, 597.4 $[M + H]^{4+}$ and **18**: $R_t$ 18.71 min; linear gradient 10→90% B in 30 min; $m/z$ = 1157.6 $[M + H]^{2+}$, 772.0 $[M + H]^{3+}$, 597.4 $[M + H]^{4+}$. Subsequent purification by RP-HPLC of **17**: linear gradient of 3.0 CV; 55→70% B; $R_t$ 2.2 CV and **18**: linear gradient of 3.0 CV; 50→60% B; $R_t$ 2.9 CV, followed by freeze-drying, furnished **17** (7.4 mg, 3.2 μmol, 66%) and **18** (10.4 mg, 4.5 μmol, 31%) as white amorphous powders.

**Dimer 19 and 20.** Peptide **15** (55 mg, 20 μmol) was dissolved in 1,4-dioxane (2 mL) and MeCN (2 mL) and $PMe_3$ (0.16 mL, 0.16 mmol, 8 equiv, 1 M in toluene) was added. The solution was stirred for 3 h, water (0.1 mL) was added and stirring was continued for another 3 h. All solvents were evaporated and the crude peptide was redissolved in EtOH (2 mL) and 1 M aq. NaOH (0.5 mL) was added. After stirring for 2 h, Amberlite IR-120 ($H^+$) was added and the neutral solution was subsequently concentrated and coevaporated thrice with dry toluene to quantitatively provide **19** (53 mg, 20 μmol) as amorphous solid. Dimer **16** (20 mg, 7.5 μmol) treated as described above to quantitatively furnish **20** (20 mg, 7.5 μmol).

**Dimer 21 and 22.** Dimers **19** (15 mg, 5.4 μmol) and **20** (20 mg, 7.5 μmol) were treated as described for **13**, to give crude **21** and **22**, respectively, and were analyzed by LC/MS; **21**: $R_t$ 17.10 min; linear gradient 10→90% B in 30 min; $m/z$ = 1130.5 $[M + H]^{2+}$, 754.0 $[M + H]^{3+}$, 556.7 $[M + H]^{4+}$ and **22**: $R_t$ 16.40 min; linear gradient 10→90% B in 30 min; $m/z$ = 1130.6 $[M + H]^{2+}$, 754.0 $[M + H]^{3+}$, 565.7 $[M + H]^{4+}$. Subsequent purification by RP-HPLC of **21**: linear gradient of 3.0 CV; 40→60% B; $R_t$ 2.7 CV and **22**: linear gradient of 3.0 CV; 40→55% B; $R_t$ 2.1 CV, followed by freeze-drying, furnished **21** (6.5 mg, 2.9 μmol, 53%) and **22** (8.9 mg, 3.9 μmol, 53%) as white amorphous powders.

**Dimer 23.** The crude **19** (45 mg, 20 μmol) was taken up in DMF (3 mL) and slowly added to a solution of BOP (52 mg, 100 μmol, 5 equiv), HOBt (14 mg, 100 μmol, 5 equiv) and DiPEA (50 μL, 300 μmol, 15 equiv) in DMF (8 mL). The reaction was then stirred overnight, concentrated and the resulting mixture applied to a size-exclusion column that was eluted with MeOH, after which the peptide-containing fractions were combined and evaporated to dryness. Deprotection, as descibed for **13**, was followed by LC/MS analysis ($R_t$ 19.10 min, linear gradient 10→90% B in 30 min; $m/z$ = 1121.4 $[M + H]^{2+}$, 747.8 $[M + H]^{3+}$, 561.1 $[M + H]^{4+}$ of the crude product, purification by RP-HPLC (linear gradient of 4.0 CV; 50→65% B; $R_t$ 3.4 CV) and lyophilization, to produce title compound **23** (8.4 mg, 4.5 μmol, 25%) as white amorphous powder over 2 steps.

**Dimer 24.** The crude **20** (20 mg, 7.5 μmol) was treated as described for **23**, to give the crude product (LC/MS analysis: $R_t$ 17.80 min, linear gradient 10→90% B in 30 min; $m/z$ = 1121.8 $[M + H]^{2+}$, 748.4 $[M + H]^{3+}$) that was purified by RP-HPLC (linear gradient of 4.0 CV; 40→65% B; $R_t$ 3.2 CV) and freeze-dried, to furnish in 2 steps the title compound **24** (2.5 mg, 1.1 μmol, 15%) as white amorphous powder.

**Antimicrobial 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. Microtitre plates (96 wells of 100μL) as well as large plates (25 wells of 3 mL) were filled with Mueller Hinton II Agar (Becton Dickinson, Cockeysvill, USA) containing serial two-fold dilutions of the peptides. 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 quadruplo*. 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 two-fold dilutions of the peptides 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 supernatant of each well was transported into a flat-bottom 96-well plate (Costar). The absorbance was measured at 405 nm using 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 $(A_{pep} - A_{blank})/(A_{tot} - A_{blank})100$.

**Conductivity measurements.** Planar lipid membranes were prepared by painting a solution of diphytanoylphosphatidylcholin (DPhPC, Avanti Polar Lipids, Alabaster, AL) in n-decane (25 mg $ml^{-1}$) over the aperture of a polystyrene cuvette with a diameter of 0.15 mm.[18] All experiments were performed at ambient temperature. The used electrolyte solutions at a concentration of 1 M each were unbuffered. Probes were dissolved in methanol and added to the *trans* or *cis* side (containing the measuring electrode) of the cuvette. Current detection and recording was performed with a patch-clamp amplifier Axopatch 200B, a Digidata A/D converter and pClamp6 software (Axon Instruments, Foster City, MA). The acquisition frequency was 5 kHz. The data were filtered with an digital filter at 50 Hz for further analysis.

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