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Extra amino group-containing gramicidin S analogs possessing outer membrane-permeabilizing activity

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Novel (2*S*,4*R*)- and (2*S*,4*S*)-4-aminoproline residue-containing analogs of the cyclic decapeptide antibiotic gramicidin S were synthesized, which exhibited marked permeabilizing activity on the outer membrane of gram-negative bacteria.

Because of the increasing resistance of bacteria to conventional antibiotics,[1] peptide antibiotics which attack the bacterial membrane, leading to membrane permeation, are attracting attention as promising antibacterial agents against multidrug-resistant pathogens.[2] Gramicidin S (GS) is a cyclic decapeptide with the primary structure *cyclo*-(-Val-Orn-Leu-D-Phe-Pro-)$_2$, which is one of the peptide antibiotics that have been most extensively studied by the synthetic approach.[3] Although the mode of action is not completely established in detail, GS is generally considered to perturb lipid packing, resulting in destruction of the integrity and enhancement of the permeability of the lipid bilayer of the cytoplasmic membrane.[4] The stable amphiphilic pleated β-sheet structure of GS with two cationic Orn side chains (Fig. 1) is considered essential for its strong antibacterial activity against gram-positive bacteria. In contrast to the strong antibacterial activity against gram-positive bacteria, GS is inactive or only moderately active against gram-negative bacteria.†[3,5]

Polymyxin B, which is also an amphiphilic, membrane-active cyclic peptide antibiotic, is strongly active against gram-negative bacteria and its pentacationic nature is considered to be responsible for the antibacterial activity.[6] It has been shown that polycationic compounds with much simpler structures efficiently permeabilize the outer membrane of gram-negative bacteria.[7] Therefore, we decided to synthesize the analogs of GS which possess additional amino groups, expecting the polycationic analogs to be active against both gram-positive and gram-negative bacteria.

We selected Pro residues as the sites to which additional amino groups are introduced. The amino group of Boc-Pro(4*R*-NH_2)-OH and Boc-Pro(4*S*-NH_2)-OH,[8] prepared from 4*R*-hydroxyproline, was protected by the Tfa group. Each of the diastereomeric protected Pro analogs, Boc-Pro(4*R*/*S*-TfaNH)-OH,‡ was incorporated into solid-phase peptide synthesis using Kaiser's oxime resin[9] to give Boc-(-[D-Phe-Pro(4*R*/*S*-TfaNH)-Val-Orn(Cbz)-Leu]-)$_2$-oxime resin. Removal of the Boc group followed by cyclisation-cleavage with AcOH–Et_3N,[9] afforded the protected GS analogs [Orn(Cbz)$^{2,2'}$,Pro(4*R*/*S*-TfaNH)$^{5,5'}$]GS in *ca*. 80% yield.§ Complete deprotection furnished novel GS analogs possessing extra amino groups at the Pro residues, namely [Pro(4*R*-NH_2)$^{5,5'}$]GS·4HCl (**1a**) and [Pro(4*S*-NH_2)$^{5,5'}$]GS·4HCl (**1b**).

The stereochemistry of the analogs and derivatives of GS can be studied by their CD spectra and a negative maximum around 207 nm with a shoulder around 220 nm are known to be good criteria for the pleated β-sheet-type structure of GS.[3] As shown in Fig. 2 the CD spectra of the synthetic analogs **1a** and **1b** measured in MeOH were similar to that of GS, indicating that these analogs also adopt β-sheet conformations. However, their weaker intensity suggested that the conformation was distorted or destabilized compared with the parent GS. In particular, the molecular ellipticity of **1b**, which possesses extra amino groups directed towards the hydrophobic side of the amphiphilic β-sheet plane, was less than a half of that of GS.

The conformational stability of the GS analogs was also studied by ^{1}H NMR spectroscopy. It is known that, upon the addition of D_2O to the DMSO-d_6 solutions of GS, the signals for the NHs of the Val and Leu residues remain unchanged, indicating the resistance of the intramolecularly hydrogen-bonded NHs to H–D exchange. Thus D_2O was added to the DMSO-d_6 solutions of the GS analogs and the intensity changes of the NH signals were followed. In the case of protected precursors [Orn(Cbz)$^{2,2'}$,Pro(4*R*/*S*-TfaNH)$^{5,5'}$]GS, the intramolecularly hydrogen-bonded NH of Leu remained essentially unchanged 3 days after the addition of D_2O, indicating that their β-sheet conformations are highly stable. On the other hand, the Leu–NH of **1a** decreased to less than half of the original intensity and that of **1b** to a very weak residual peak after one day. Thus, consistent with the CD spectral data, the relative stability of the β-sheet structures were shown to be GS > **1a** > **1b**.

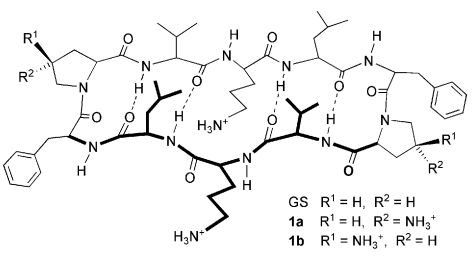

Fig. 1 Antiparallel β-sheet conformation of GS and the analogs **1a** and **1b** containing extra amino groups.

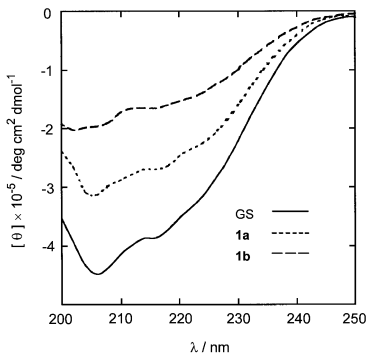

Fig. 2 CD spectra of GS and the synthetic analogs **1a** and **1b** in MeOH.

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The antibacterial activity of these synthetic GS analogs against gram-positive and gram-negative bacteria was examined in Mueller–Hinton broth using *Staphylococcus aureus* and *Escherichia coli*, respectively, and the results are summarized in Table 1.† Contrary to our expectation, the tetracationic analogs **1a** and **1b** exhibited lower activity against gram-negative bacteria. Furthermore, their activity against gram-positive bacteria was much less than that of the parent GS. The unexpectedly weak activity against gram-positive bacteria might be related to the destabilization of the β-sheet structure, which was believed to be essential for the interaction of the antibiotic with the membrane of the sensitive bacteria.[3,10] Therefore, the action of the synthetic analogs **1a** and **1b** on bacterial membranes was studied.

Table 1 The antibacterial activity (minimum inhibitory concentration in μg mL^{-1})[a] of GS and the synthetic analogs **1a** and **1b**

Bacteria	**1a**	**1b**	GS	**1b** + GS [b]
Staphylococcus aureus 209P	32	64	4	
Escherichia coli K12 strain W3110	64	64	32	4 + 4

[a] Cells were cultured at 37 °C for 20 h in Mueller–Hinton broth. [b] 1:1 mixture.

In general, the outer membrane of gram-negative bacteria prevents the penetration of hydrophobic antibiotics into cells, which explains the low activity of GS against gram-negative bacteria.† Polycationic antibiotics such as polymyxin B are considered to bind to the outer membrane, leading to its disorganization and permeabilization. The action of GS and its analogs **1a** and **1b** on the outer membrane of *E. coli* cells and on the cytoplasmic membranes of *E. coli* and *S. aureus* were examined using ion-selective electrodes.[7] Permeabilization of the outer and cytoplasmic membranes was monitored by the enhanced uptake of the hydrophobic tetraphenylphosphonium ion (TPP^+) and the leakage of K^+ ions from the cells, respectively, and the results are shown in Fig. 3. As expected, the tetracationic analogs **1a** and **1b** markedly increased the outer membrane permeability compared with the parent dicationic GS (**1b** > **1a** > GS), as shown in Fig. 3a. However, the effects of these analogs on the cytoplasmic membranes were exactly the opposite to that observed for the outer membranes as shown in Fig. 3b, *i.e.*, the synthetic analogs were much less active than GS.

In spite of the high permeabilizing activity of the analog **1b** on the outer membrane, it possessed poor antibacterial activity against gram-negative bacteria, which is consistent with the low antibacterial activity of **1b** against gram-positive bacteria lacking outer membrane. Polymyxin B nonapeptide, which is a proteolytic product of polymyxin B lacking the long fatty acid tail, possesses no antibacterial activity while maintaining the high outer membrane-permeabilizing activity.[6] Since such an outer membrane permeabilizer is generally known to sensitize gram-negative bacteria towards hydrophobic antibiotics, the synergistic action of **1b** and GS was studied. The presence of both **1b** and GS (each 4 μg mL^{-1}) completely inhibited the growth of *E. coli*, although, when dosed independently, a much higher concentration of each was required to effect the same activity.

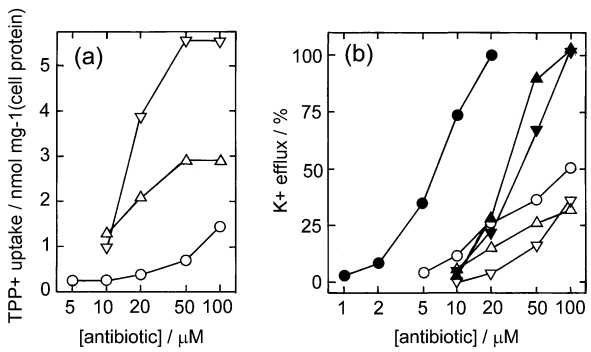

Fig. 3 Action of GS, **1a** and **1b** on bacterial membranes analyzed by ion-selective electrodes. (a) Uptake of TPP^+ for *E. coli* (○ GS, △**1a**, ▽**1b**). (b) Efflux of K^+ for *S. aureus* (● GS, ▲**1a**, ▼**1b**) and for *E. coli* (○ GS, △**1a**, ▽**1b**).

In summary, we have prepared extra amino group-containing analogs of GS, which effectively permeabilized the outer membrane of gram-negative bacteria, although they exhibited poor antibacterial activity against both gram-positive and gram-negative bacteria. The introduction of hydrophilic amino groups possibly caused an unallowable perturbation of the hydrophobic–hydrophilic balance, which is essential for the activity of GS. Therefore, simultaneous introduction of both amino groups and hydrophobic groups to GS seems to be a promising strategy for obtaining analogs possessing broad spectra of antibiotic activity. As preliminary results, a new tetracationic analog possessing D-Phe–NH groups at the 4*R*-position of the Pro residues has exhibited higher activity than GS against *E. coli*, while maintaining high activity against *S. aureus*, and further studies are in progress. Since GS is an important molecular scaffold for the synthesis of functional molecules,[9,11] the unique GS analogs and their orthogonally protected precursors described in this communication should also be useful as β-sheet-based frameworks for the construction of functional molecular systems.

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Notes and references

† Although GS had long been considered to be active against only gram-positive bacteria,[3] Kondejewski *et al*.[5] showed that the antibacterial activity of GS against gram-negative bacteria depends on the assay system. Also, in this communication GS manifested moderate activity against *E. coli*, which is inconsistent with our earlier reports describing GS as being inactive or almost inactive.[12]

‡ Boc-Pro(4*R*-TfaNH)-OH, colourless oil; dicyclohexylammonium salt, mp 144–146 °C. Found: C, 56.77; H, 8.04; N, 8.10%. $C_{12}H_{17}F_3N_2O_5{\cdot}C_{12}H_{23}N$ requires C, 56.79; H, 7.94; N, 8.28%. Boc-Pro(4*S*-TfaNH)-OH, colourless oil; dicyclohexylammonium salt, mp 165–168 °C. Found: C, 56.89; H, 7.94; N, 8.14%. $C_{12}H_{17}F_3N_2O_5{\cdot}C_{12}H_{23}N$ requires C, 56.79; H, 7.94; N, 8.28%.

§ [Orn(Cbz)$^{2,2'}$,Pro(4*R*-TfaNH)$^{5,5'}$]GS, mp 298–300 °C (browns above 280 °C). Found: C, 57.64; H, 6.54; N, 11.76%. $C_{80}H_{104}F_6N_{14}O_{16}{\cdot}2H_2O$ requires C, 57.61; H, 6.53; N, 11.76%. [Orn(Cbz)$^{2,2'}$,Pro(4*S*-TfaNH)$^{5,5'}$]GS; mp 263–266 °C. Found: C, 57.95; H, 6.54; N, 11.71%. $C_{80}H_{104}F_6N_{14}O_{16}{\cdot}H_2O$ requires C, 58.24; H, 6.48; N, 11.89%.

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