Synthesis of Low-Hemolytic Antimicrobial Dehydropeptides Based on Gramicidin S

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Abstract: The synthesis and biological activity of a novel cyclic β-sheet-type antimicrobial dehydropeptide based on gramicidin S (GS) is described. The GS analogue, containing two (*Z*)-(β-3-pyridyl)-α,β-dehydroalanine (Δ^Z3Pal) residues at the 4 and 4′ positions (**2**), was synthesized by solution-phase methodologies using Boc-Leu-Δ^Z3Pal azlactone. Analogue **2** exhibited high antimicrobial activity against Gram-positive bacteria and had much lower hemolytic activity than wild-type GS and the corresponding (*Z*)-α,β-dehydrophenylalanine (Δ^Z-Phe) analogue (**1**).

The global spread of multidrug-resistant bacteria is a growing threat to human health.[1] Cationic antimicrobial peptides (CAPs[a]) that attack bacterial membranes are promising agents for combating bacterial pathogens;[2] consequently, the modes of action of CAPs, and in particular α-helical CAPs, have been extensively studied by various synthetic and spectroscopic techniques.[3]

Gramicidin S (GS, *cyclo*(Val-Orn-Leu-D-Phe-Pro)$_2$) is a membrane-lytic cyclic peptide antibiotic that acts against both Gram-positive and Gram-negative bacteria.[4,5] NMR[6] and X-ray crystallographic studies[7] established that the main-chain conformation of GS is a stable antiparallel β-sheet conformation. Type II′ β-turn moieties that connect two short β-strands are essential for the bioactive conformation of GS.[5−8] Although the mode of action of GS toward biomembranes is not completely understood, GS is generally believed to perturb lipid packing, resulting in the destruction of the cytoplasmic membrane's integrity and enhancement of its permeability.[8] Unfortunately, the use of GS for therapeutic purposes has been limited to topical application because of its high toxicity to human red blood cells. Therefore, structure−activity relationships of GS and related cyclic peptides have been studied to dissociate its antimicrobial and hemolytic activities and to elucidate the mode of action.[9−18]

The selectivity of CAPs including GS toward biomembrane is governed by a net positive charge of peptides and their amphiphilicity.[19] Polycationic decapeptide analogues of GS, [D-Dap$^{4,4'}$]GS[9] and γ-amino-L-proline-modified GS[13,16] did not induce hemolysis and can highly permeabilize through outer-membrane of Gram-negative bacteria. These peptides, however, are less active against Gram-positive bacteria than wild-type GS, suggesting that polycationic analogues of GS preferentially interact with the outer membrane of Gram-negative bacteria.

Biological activities of GS mutants, with D-Phe residues at the 4 and 4′ positions replaced by other D-amino acids, depend on their conformations. For example, a water-soluble D-Tyr$^{4,4'}$ analogue, [D-Tyr$^{4,4'}$]GS, exhibits a weaker hemolytic activity than wild-type GS by maintaining moderate antimicrobial activity while mutation by D-Asn, D-His, or D-Ser results in loss of both activities because these D-amino acids did not induce β-turn conformation.[5] Very recently, Grotenbreg et al. have reported that aryl substituents in the turn regions of GS and analogues are indispensable for bactericidal action.[20] α,β-Dehydroamino acids (ΔAAs) are hitherto used for β-turn inducers of de novo peptides.[18] Shimohigashi et al. reported that replacement of D-Phe residues in GS with (*Z*)-α,β-dehydrophenylalanine (Δ^ZPhe, Figure 1a) residues stabilizes its β-sheet conformation with maintaining strong antimicrobial activity.[22,23] Recently, we designed a novel ΔAA, (*Z*)-(β-3-pyridyl)-α,β-dehydroalanine (Δ^Z3Pal, Figure 1a).[24] In the present study, we found that replacement of Δ^ZPhe residues in [Δ^Z-Phe$^{4,4'}$]GS (**1**, Figure 1b) with Δ^Z3Pal drastically reduces cytotoxicity to human erythrocyte without loss of antimicrobial activity.

Δ^Z3Pal-containing analogue of GS, [Δ^Z3Pal$^{4,4'}$]GS (**2**), was synthesized by solution-phase method as shown in Scheme 1. Boc-Leu-Δ^Z3Pal azlactone (**9**) was initially synthesized from β-3-pyridyl-DL-serine (**7**) as previously reported.[24] Treatment of **9** with H-Pro-OMe (1.05 equiv) in the presence of a catalytic amount of DMAP (0.05 equiv)[25] afforded Boc-Leu-Δ^Z3Pal-Pro-OMe (**10**) in 96% yield. Addition of excess amounts of H-Pro-OMe (1.5 equiv) gave a mixture of **10** and Boc-D-Leu-Δ^Z3Pal-Pro-OMe (L/D = 51/49 estimated by ^{1}H NMR). Stepwise elongation of Orn(For) and Val residues afforded protected pentapeptide derivative (**12**). Protected decapeptide **15** was derived from **12** by segment condensation. Saponification of **15** followed by acidic deprotection of Boc group gave a linear decapeptide **17**. Cyclization of **17** under a high-dilution condition in DMF afforded [Orn(For)$^{2,2'}$, Δ^Z3Pal$^{4,4'}$]GS (**4**), whose structure was confirmed by ^{1}H NMR and ESI-MS. At first, yield of the cyclic product **4** was extremely low in spite of using potential condensation reagents such as BOP-Cl (trace) or PyBOP (15%). The use of HATU improved the yield up to 85%. Finally, deprotection of formyl groups afforded **2** in good yield. [Δ^Z-Phe$^{4,4'}$]GS (**1**), [Δ^ZPhe,[4] Δ^Z3Pal$^{4'}$]GS (**3**), and a tetrahydro analogue of **2** ([D-3Pal$^{4,4'}$]GS, **6**) were also synthesized in comparison (see Supporting Information). These pyridine-containing analogues were highly soluble in water, while wild-type GS and **1** were hardly soluble. ^{1}H NMR and ESI-MS measurements revealed that **2** contains small amount of Δ^E-3Pal$^{4,4'}$ isomer (*E*/*Z* = 9/91 estimated by HPLC).

As shown in Figure 2, NOESY spectral analysis indicated that the configuration of the β-substituent in Δ3Pal residues is the (*Z*)-form because strong NOE between β-CH(Δ^Z3Pal$^{4,4'}$) and δ-CH$_2$(Pro$^{5,5'}$) was observed. Steric proximity between δ-NH$_3^+$-(Orn$^{2,2'}$) and α-CH(Pro$^{5,5'}$) suggests the stabilization of β-sheet

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[a] Abbreviations: Boc, *tert*-butoxycarbonyl; BOP-Cl, bis(2-oxo-3-oxazolidinyl)phosphinic chloride; CAPs, cationic antimicrobial peptides; CD, circular dichroism; D-Dap, D-α,γ-diaminopropionic acid; Dap(Z), Nγ-benzyloxycarbonyl-D-α,γ-diaminopropionic acid; DIEA, *N*,*N*-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; ESI-MS, electrospray ionization mass spectroscopy; For, formyl; HATU, 1-[bis(dimethylanimo)-methylene]-1*H*-1,2,3-triazolo(4,5-*b*)pyridinium 3-oxide hexafluorophosphate; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HOBt, *N*-hydroxybenzotriazole; NMM, *N*-methylmorpholine; NOESY, nuclear Overhauser and exchange spectroscopy; D-3Pal, (β-3-pyridyl)-D-alanine; PyBOP, benzotriazole-1-yloxypyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.

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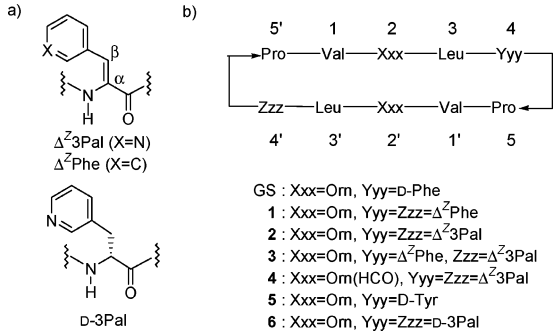
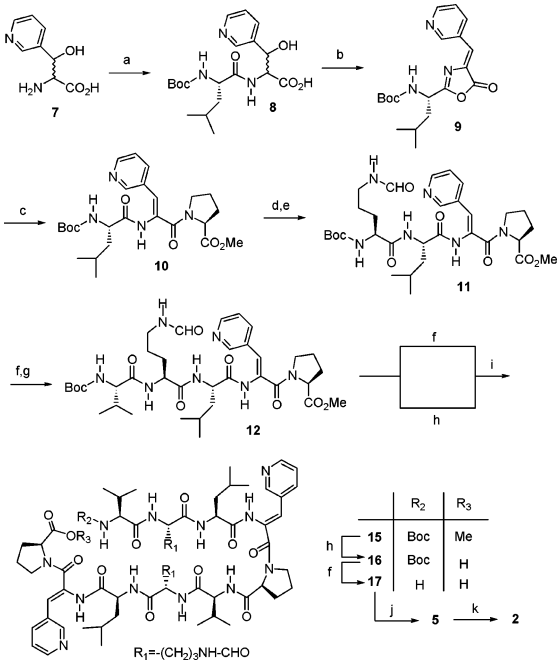


Figure 1. (a) Δ^Z3Pal, Δ^ZPhe, and D-3Pal and (b) GS analogues in this study.

Scheme 1. Solution-Phase Total Synthesis of **2**[a]



[a] Reagent and conditions: (a) Boc-Leu-OSu, 10% aqueous $NaHCO_3$/1,2-dimethoxyethane = 1:1 (v/v), 0 °C → room temp, 24 h; (b) Ac_2O, AcONa, 3 h; (c)HCl·H-Pro-OMe, DMAP (5 mol%), NMM, $CHCl_3$, 0 °C → room temp, 24 h; (d) 4 M HCl in dioxane, 0 °C, 2 h; (e) Boc-Orn(For)-OH, EDC·HCl, HOBt, NMM, $CHCl_3$, 0 °C → room temp, 24 h; (f) TFA, 0 °C, 2 h; (g) Boc-Val-OH EDC·HCl, HOBt, NMM, $CHCl_3$, 0 °C → room temp, 24 h; (h) 1 M aqueous NaOH, 50% aqueous MeOH, 0 °C → room temp, 2 h; (i) EDC·HCl, HOBt, NMM, $CHCl_3$, 0 °C → room temp, 24 h; (j) HATU, DIEA, DMF (final concentration, 1 mM), 0 °C → room temp, 2 days; (k) 10% HCl in MeOH, room temp, 2 days.

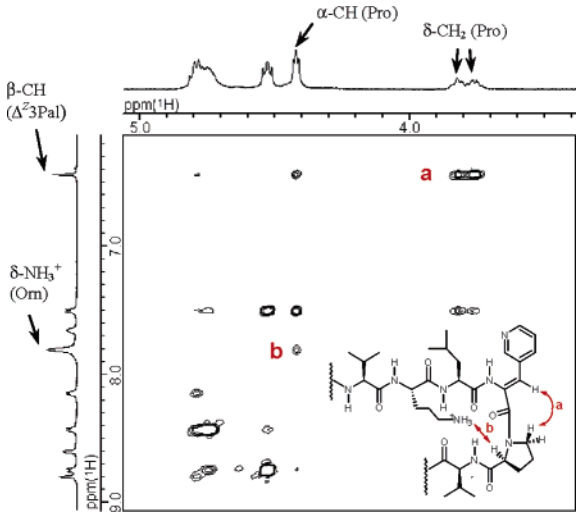


Figure 2. Selective NOESY spectrum of **2** (20 °C, DMSO-d_6, 8.95 mM, τ_m = 1000 ms).

Table 1. Amide Proton Temperature Shift Coefficients ($\Delta\delta/\Delta T$, ppb/K) and Coupling Constants ($^3J_{NH-CH}$, Hz) of GS and Its Analogues in DMSO-d_6[a]

peptides	Val$^{1,1'}$	Orn$^{2,2'}$	Leu$^{3,3'}$	Xaa$^{4,4'}$
GS	−1.8 (-[c])	−4.8 (9.1)	−2.8 (8.8)	−7.2 (3.0)
1	−1.1 (-[c])	−5.6 (8.9)	−2.6 (8.6)	−5.3 (-[d])
2	−0.9 (10.0)	−5.6 (9.5)	−2.6 (8.5)	−4.6 (-[d])
3[b]	*c*	−4.8 (9.1)	−2.6 (9.5)	−5.3 (-[d])
	c	−4.8 (9.1)	−2.8 (9.0)	−4.6 (-[d])
6	−2.3 (-[c])	−4.2 (9.5)	−2.7 (8.5)	−5.7 (4.0)

[a] $^3J_{NH-CH}$ values are in the parentheses and are measured at 20 °C. [b] Upper: Val^1-Δ^ZPhe^4. Lower: Val$^{1'}$-Δ^Z3Pal$^{4'}$. [c] Overlapped with aromatic protons. [d] Singlet.

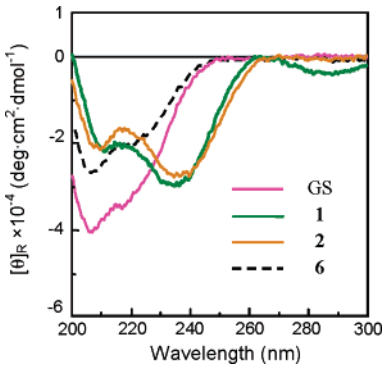


Figure 3. CD spectra of GS, **1**, **2**, and **6** in MeOH (20 °C).

conformation by hydrogen-bonding interaction between δ-NH_3^+-(Orn$^{2,2'}$) and C═O(Δ^Z3Pal$^{4,4'}$) like wild-type GS.[26]

Shimohigashi et al. have previously reported that Δ^ZPhe$^{4,4'}$ analogue **1** adopts a stable antiparallel β-sheet conformation like parent GS by ^{1}H NMR analysis.[22,23] Variable-temperature ^{1}H NMR experiments revealed that four N$^\alpha$Hs of Val$^{1,1'}$ and Leu$^{3,3'}$ in Δ^Z3Pal-containing analogues **2** and **3** were hydrogen-bonded. $^3J_{NH-CH}$ values of Val, Orn, and Leu residues (ranging from 8.6 to 9.5 Hz) correspond to the β-sheet conformation (Table 1). In contrast, the temperature shift coefficient ($\Delta\delta/\Delta T$) and the $^3J_{NH-CH}$ value of α-NH(D-3Pal) in **6** slightly differ from that of α-NH(D-Phe) in wild-type GS, suggesting that the main-chain conformation of **6** is slightly distorted.

CD is also useful for studying conformational changes of GS analogues from parent GS. Figure 3 shows CD spectra of GS and analogues in MeOH. **1** has three negative bands at 210, 236, and 280 nm, while GS has a negative band at 206 nm with a shoulder at 217 nm. The difference is mainly due to the α,β-double bond chromophore of Δ^ZPhe, and the band at 280 nm is characterized as a Δ^ZPhe-Pro β-turn structure.[23] In the case of Δ^Z3Pal$^{4,4'}$ analogue **2**, the spectrum is essentially similar to that of **1**, suggesting that **2** adopts a cyclic β-sheet conformation similar to that of **1** and GS. D-3Pal$^{4,4'}$ analogue **6** exhibited a CD spectrum similar to that of wild-type GS but with much weaker intensity. In an earlier study by Kopple et al., cyclic hexapeptide related to GS, *cyclo*(Orn-D-Phe-Pro)$_2$, exhibits two negative bands centered at 222 and 200 nm in HFIP, which is characteristic of type II′ β-turn conformation.[27] In the case of **6**, the decrement of the ellipticity suggests that the main-chain conformation of **6** was distorted or destabilized compared with the wild-type GS, which is consistent with the result of ^{1}H NMR analysis.

Biological activities of GS analogues were evaluated by a similar method reported previously.[16] Table 2 shows the

Table 2. Antimicrobial Activity of GS Analogues

peptides[a]	MIC (μg/mL) *Staphylococcus aureus* 209P	MIC (μg/mL) *Escherichia coli* K12 strain W3110
GS	4	16
1	4	32
2	8	32
3	4	32
5	4	32
6	64	64

[a] Cells were cultured at 37 °C for 20 h in a Mueller−Hinton broth.

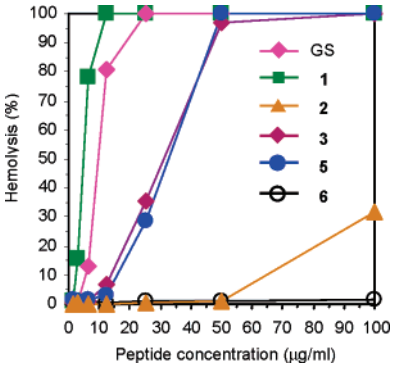


Figure 4. Dose-dependence curves of hemolysis induced by GS analogues.

antibacterial activity determined by the liquid-broth method and that Δ^Z3Pal$^{4,4'}$ analogue **2** exhibited potent antimicrobial activity against Gram-positive *Staphylococcus aureus*, which was comparable to those of wild-type GS and Δ^ZPhe$^{4,4'}$ analogue **1**. The MIC value of the analogue containing Δ^Z3Pal and Δ^ZPhe residues (**3**) was comparable to that of [D-Tyr$^{4,4'}$]GS (**5**). It is noteworthy that hemolytic activity of **2** was drastically reduced in comparison with those of other analogues (Figure 4). These observations suggest that incorporation of Δ^Z3Pal residues leads to diminished cytotoxicity to human erythrocyte without disrupting the bioactive conformation of GS. To clarify the structural importance of Δ^Z3Pal residues in antibacterial action, biological activities of [D-3Pal$^{4,4'}$]GS (**6**) were evaluated. As expected, water-soluble **6** did not induce hemolysis (Figure 4), but it was inactive against *S. aureus* and *E. coli* (Table 2).

Relative hydrophobicity and hydrophilicity of peptides including GS-related peptides could be evaluated by HPLC analysis.[28] Figure 5 shows the correlation among biological activities of GS-based peptides and their retention time. Δ^Z-3Pal analogue **2** had a lower retention time compared with all hemolytic analogues and higher retention time compared with **6**, indicating that **6** is slightly more hydrophilic than **2**. At present, we speculate that the lost of antimicrobial activity of **6** is related to its higher hydrophilicity and conformational distortion mentioned above. Increased hemolytic activity was accompanied by a retardation of elution time. A remarkable difference between MIC and ED_{50} was observed only in the case of two Δ^Z3Pal-containing **2**. As a result, introduction of two Δ^Z3Pal residues into the GS framework provides moderate hydrophilicity without interfering with permeation of **2** across bacterial membranes. Interestingly, Katsu et al. have reported that antimicrobial [Ala$^{2,2'}$,Δ^ZPhe$^{4,4'}$]GS, an analogue of **1** lacking cationic Orn residues, enhances the K^+ efflux from human erythrocyte, resulting in changes in its morphology. On the other hand, inactive [Ala$^{2,2'}$]GS scarcely induces the change.[29] The difference implies that the aromatic and conjugated planes in Δ^ZPhe residues play a crucial role in membrane−peptide interaction and/or its permeation through a phospholipid bilayer.

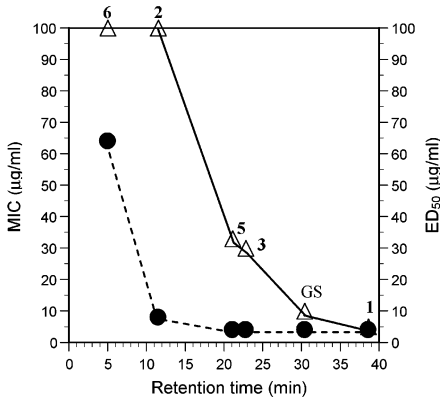


Figure 5. Correlation among biological activities of GS-based cyclic decapeptides and their retention time in HPLC analysis. Closed circles and opened triangles indicate MIC values toward *S. aureus* 209P and ED_{50} values toward human erythrocyte. The following conditions were applied for RP-HPLC analysis: column, YMC-pack ODS R&D (6.0 mm i.d. × 250 mm); flow rate, 1 mL/min; eluent, 40−85% aqueous CH_3CN (containing 0.1%TFA); detection, 270 nm (**1**−**3**), 220 nm (others).

Therefore, it is considered that Δ^Z3Pal analogue **2** possessing similar aromatic ΔAA residues in the β-turn moieties could interact with bacterial membranes like **1** and [Ala$^{2,2'}$,Δ^ZPhe$^{4,4'}$]-GS. At present, however, the mechanisms of action of **2** are not fully understood.

In conclusion, we have discovered a novel antimicrobial cyclic peptide **2** that exhibits high solubility in water and low hemolytic activity. Our findings should be helpful in obtaining new insights to the rational design of novel β-sheet-type CAPs with low hemolytic activity.

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Supporting Information Available: Experimental details, spectroscopic data, and HPLC of GS analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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