Electronic Supplementary Information (ESI)

Structure-activity relationships of gramicidin S analogs containing (β-3-pyridyl)-α,β-dehydroalanine residues on membrane permeability

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Experimental procedures

General

Boc-amino acids were purchased from Peptide Institute, Inc. (Osaka, Japan). Reagents for peptide synthesis were obtained from Novabiochem (Darmstadt, Germany) and Peptide Institute (Osaka, Japan). Polymyxin B nonapeptide (PMBN) hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other organic reagents were of commercially available grade. All solvents were distilled over CaH₂ or Na before use. TLC was performed on Silica gel 60F₂₅₄ aluminum plate (Merck, Germany). The compounds were visualized with UV light (254 nm) and ninhydrin. Column chromatography was performed on Silica gel 60N for flash chromatography (Kanto Chemical, Co. Ltd., Tokyo, Japan, spherical, neutral, 40-60 µm particle). Gel filtration chromatography was performed on Sephadex LH-20 (Pharmacia Biotech, Sweden, eluting MeOH). Melting points were measured on a YANAKO (Kyoto, Japan) micromelting points apparatus and were uncorrected. Optical rotations were measured on JASCO (Tokyo, Japan) DIP-1000 digital polarimeter using 10 cm-path quartz cell at 20°C. ¹H NMR spectra were measured on JEOL (Tokyo, Japan) lambda-500 spectrometer or alpha-300 spectrometers in CDCl₃ or DMSO-d₆ as solvents. Tetramethylsilane (TMS) was used as an internal reference. NMR data were processed by using JEOL Alice2 for Windows software. ESI-MS spectra (Positive ion mode) were measured by using API-2000 spectrometer (Applied Biosystems, California, USA). HPLC was performed on JASCO HPLC system. Following
equipments were used; pump: PU-2089plus, UV detector: UV-2075plus, control software: JASCO-BORWIN Chromatography software ver 3.2 (Windows PC).

**H-DL-Ser(3Pyr)-OH (7)**

To a stirred solution of NaOEt, prepared from Na (907mg, 39.0mmol) and EtOH (30ml), was added HCl-H-Gly-OEt (1.47 g, 30.2 mmol) and the mixture was stirred at r.t. After 2h, pyridine-3-carboxaldehyde (3.3 ml, 36.9 mmol) was slowly added to the solution and stirred for 48 h at r.t. The solvent was removed off in vacuo and the residue was dissolved in water (100 ml). The solution was neutralized to pH6 by 4M aqueous HCl and the residue was concentrated in vacuo to afford a crude. The residue was solidified by addition of aqueous EtOH followed by filtration to give H-DL-Ser(3Pyr)-OH as a pale-yellow solid. (1.60 g, 83%), Rf 0.15 (1-BuOH:AcOH:H2O=2:1:1 (v/v/v)), [α]D20 +5.8 (c 1.0 in 1M HCl aq.).

1H NMR (300 MHz, D2O, 25°C) : δ 8.64 (s, 1H), 8.56 (s, 1H), 8.17 (d, 1H, J = 8.1Hz), 7.70 (t, 1H, J1,2 = 5.6 Hz, J1,3 = 12.4 Hz), 5.06 (d, 1H, J = 4.3 Hz), 3.96 (br, 1H).

**Boc-Leu-Δ²3Pal-Pro-OMe (8)**

The titled compound was prepared from 7 by the same method described in our previous report.18

**Boc-Ala-Leu-Δ²3Pal-Pro-OMe (9)**

8 (703 mg, 1.44 mmol) was dissolved in 4M HCl solution in 1,4-dioxane (4 ml). The solution was kept for 3 h at ambient temperature. The solvent was evaporated off and the residue was
solidified by ether. The solid was collected and dried in vacuo. Reprecipitation from ether afforded H-Leu-Δ²3Pal-Pro-OMe·2HCl (678 mg, quantitative)

To a stirred solution of H-Leu-Δ²3Pal-Pro-OMe·2HCl (678 mg, 1.47 mmol) and NMM (0.317 ml, 2.88 mmol) in CH₂Cl₂ (10 ml) were added Boc-Ala-OH (225 mg, 1.19 mmol), HOBt·H₂O (19 mg, 0.14 mmol) and EDC·HCl (332 mg, 1.19 mmol) at 0ºC. The solution was stirred for 4 h at 0ºC. The solvent was removed off in vacuo and the residue was mixed with ethyl acetate (200 ml). The organic layer was washed with saturated aqueous NaHCO₃ (50 ml×3), water (50 ml×2) and brine (100 ml). After the organic layer was dried over anhydrous Na₂SO₄, the solvent was evaporated off to afford a crude. SiO₂ chromatographic purification (CHCl₃:MeOH= 9:1(v/v) as a eluent) afforded 9 as a white solid. (708 mg, 88%), Rf 0.33 (CHCl₃:MeOH=9:1(v/v)); m.p. 99-102ºC; [α]D²⁰ +45 (c0.1 in MeOH); ESI-MS: m/z=582.4 ([M+Na]+), C₂₈H₄₁N₅O₇Na requires 582.3

Boc-Orn(For)-Leu-Δ²3Pal-Pro-OMe (10)

8 (1.63 g, 3.32 mmol) was dissolved in 4M HCl solution in 1,4-dioxane (9 ml). The solution was kept for 2 h at ambient temperature. The solvent was evaporated off and the residue was solidified by ether. The solid was collected and dried in vacuo. Reprecipitation from ether afforded H-Leu-Δ²3Pal-Pro-OMe·2HCl 1.53 g, quantitative)

To a stirred solution of H-Leu-Δ²3Pal-Pro-OMe·2HCl (1.53 g, 3.32 mmol) and NMM (0.740
ml, 6.73 mmol) in CH₂Cl₂ (20 ml) were added Boc-Orn(For)-OH (1.13 g, 4.34 mmol), HOBt·H₂O (45.0 mg, 0.333 mmol) and EDC·HCl (762 mg, 3.99 mmol) at 0°C. The solution was stirred for 1 h at 0 ºC and 36 h at ambient temperature. The solvent was removed off in vacuo and the residue was mixed with ethyl acetate (200ml). The organic layer was washed with saturated aqueous NaHCO₃ (50 ml×3), water (50 ml×2) and brine (100 ml). After the organic layer was dried over anhydrous Na₂SO₄, the solvent was evaporated off to afford a crude. SiO₂ chromatographic purification (CHCl₃:MeOH=9:1(v/v) as a eluent) afforded 10 as a white solid. (1.89 g, 89 %), Rf 0.33 (CHCl₃:MeOH=9:1(v/v)); m.p. 104-106 ºC (lit.18 102-103 ºC); [α]D²⁰ +81 (c 0.1, MeOH) (lit.¹⁸ +85.4 (c0.1 in MeOH)); ESI-MS: m/z=631.6 ([M+H]+), C₃₁H₄₇N₆O₈ requires 631.4

**Boc-Val-Ala-Leu-Δ³3Pal-Pro-OMe (11)**

9 (708 mg, 1.27 mmol) was dissolved in 4M HCl in 1,4-dioxane (3 ml). The solution was kept for 2 h at ambient temperature. The solvent was evaporated off and dried in vacuo to afford H-Ala-Leu-Δ³3Pal-Pro-OMe·2HCl as a white solid. (639 mg, 95 %)

To a stirred solution of H-Ala-Leu-Δ³3Pal-Pro-OMe·2HCl (639 mg, 1.20 mmol) in CH₂Cl₂ (20 ml) were added NMM (0.265 ml, 1.90 mmol), Boc-Val-OH (313 mg, 1.52 mmol), HOBt·H₂O (17 mg, 0.13 mmol) and EDC·HCl (277 mg, 1.52 mmol) at 0°C. The solution was stirred for 1 h at 0°C and 6 h at ambient temperature. The solvent was removed off in vacuo and the residue was
mixed with ethyl acetate (200 ml). The organic layer was washed with saturated aqueous NaHCO₃ (50 ml×3), water (50 ml×2) and brine (100 ml). After the organic layer was dried over anhydrous Na₂SO₄, the solvent was evaporated off to afford a crude. SiO₂ chromatographic purification (CHCl₃:MeOH=95:5 (v/v)) followed by crystalization from AcOEt-hexane afforded 11 as a white solid. (704 mg, 89 %), Rf 0.30 (CHCl₃:MeOH=9:1(v/v)); m.p. 124-125°C; [α]D²⁰ +77 (c 0.1 in MeOH); ESI-MS: m/z=659.9 ([M+H]+), C₃₃H₅₁N₆O₈ requires 659.4

Boc-Val-Orn(For)-Leu-Δ²3Pal-Pro-OMe (12)

10 (1.02 g, 1.52 mmol) was dissolved in TFA (10 ml). The solution was kept for 2h at ambient temperature. The solvent was evaporated off and dried in vacuo to afford H-Orn(For)-Leu-Δ²3Pal-Pro-OMe·2TFA as a yellowish oil. (1.14 g, 99%)

To a stirred solution of H-Orn(For)-Leu-Δ²3Pal-Pro-OMe·2TFA (1.14 g, 1.52 mmol) in CH₂Cl₂ (20 ml) were added NMM (0.326 ml, 2.96 mmol), Boc-Val-OH (390 mg, 1.78 mmol), HOBT·H₂O (20 mg, 0.15 mmol) and EDC·HCl (341 mg, 1.78 mmol) at 0°C. The solution was stirred for 1 h at 0°C and 8 h at ambient temperature. The solvent was removed off in vacuo and the residue was mixed with ethyl acetate (200 ml). The organic layer was washed with saturated aqueous NaHCO₃ (50 ml×3), water (50 ml×2) and brine(100 ml). After the organic layer was dried over anhydrous Na₂SO₄, the solvent was evaporated off to afford a crude. Recrystallization from hexane afforded 12 as a white solid. (781 mg, 73 %), Rf 0.36 (CHCl₃:MeOH=9:1(v/v)); m.p.

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116-117° C (lit.18 117-118° C); [α]D20 +76 (c 0.1 in MeOH) (lit.18 +76.3 (c 0.1 in MeOH));

ESI-MS: m/z=730.6 ([M+H]+), C36H56N7O9 requires 730.5

**Boc-Val-Orn(For)-Leu-Δ23Pal-Pro-OH (14)**

To a stirred solution of 12 (201 mg, 0.275 mmol) in 50% MeOHaq. (10 ml) was added 1M aqueous NaOH (0.413 ml). The solution was stirred for 19 h at 0 °C. The solution was neutralized by addition of 1M aqueous HCl and was concentrated in vacuo. The residue was mixed with CH3CN and the insoluble material was filtered out. The filtrate was concentrated in vacuo and the residue was crystalized from addition of CHCl3-hexane to afford 13 (154 mg, 78%) as a white solid. Rf 0.20 (CHCl3:MeOH:AcOH=25:5:1 (v/v/v)); m.p. 175-176° C; [α]D20 +17 (c 0.1 in MeOH); ESI-MS: m/z=716.4 ([M+H]+), C35H54N7O9 requires 716.4

**Boc-Val-Orn(For)-Leu-Δ23Pal-Pro-Val-Ala-Leu-Δ23Pal-Pro-OMe (15)**

11 (278 mg, 0.42 mmol) was dissolved in 4M HCl in 1,4-dioxane (1.1 ml) The solution was kept for 4 h at ambient temperature. The solvent was evaporated off and dried in vacuo to afford H-Val-Ala-Leu-Δ23Pal-Pro-OMe·2HCl (13) as a white solid. (267 mg, quantitative), which was used without further purification.

To a stirred solution of 13 (212 mg, 0.269 mmol) in CH2Cl2 (10 ml) were added NMM (0.060 ml, 0.55 mmol), 14 (212 mg, 0.296 mmol), HOBt·H2O (4 mg, 0.03 mmol) and EDC HCl (57 mg,
0.30 mmol). The solution was stirred for 1 h at 0°C and 15 h at ambient temperature. The solvent was removed off in vacuo and the residue was mixed with ethyl acetate (200 ml). The organic layer was washed with saturated aqueous NaHCO$_3$ (50 ml×3), water (50 ml×2) and brine (100 ml). After the organic layer was dried over anhydrous Na$_2$SO$_4$, the solvent was evaporated off to afford a crude. Purification by gel filtration chromatography (MeOH) followed by crystalization from CHCl$_3$-hexane afforded 15 as a white solid. (704 mg, 89 %), $Rf$ 0.43 (CHCl$_3$:MeOH:AcOH=25:25:1 (v/v/v)); m.p. 145-148°C; $[\alpha]_D^{20}$ +4 (c 0.1 in MeOH); ESI-MS: $m/z$=1256.9 ([M+H]$^+$), C$_{63}$H$_{94}$N$_{13}$O$_{14}$ requires 1256.7

**Boc-Val-Orn(For)-Leu-$\Delta^Z$3Pal-Pro-Val-Ala-Leu-$\Delta^Z$3Pal-Pro-OH** (16)

To a stirred solution of 15 (203 mg, 0.162mmol) in 50% aqueous MeOH (8 ml) was added 1M aqueous NaOH (0.21 ml) at 0°C. The solution was stirred for 48 h at 0°C. The solvent was removed off in vacuo and the residue was neutralized by addition of 1M aqueous HCl. The residue was dissolved in CH$_3$CN. After the insoluble solid was filtered off, the filtrate was concentrated and lyophilized to afford 16 as a white solid. (244 mg, quantitative), $Rf$ 0.20 (CHCl$_3$:MeOH:AcOH=25:5:1 (v/v/v)); m.p. 174-179°C; $[\alpha]_D^{20}$ -30 (c 0.1 in MeOH); ESI-MS: $m/z$=1242.7 ([M+H]$^+$), C$_{62}$H$_{92}$N$_{13}$O$_{14}$ requires 1242.7
[Ala\(^2\), Orn(For)\(^2\), \(\Delta^Z\)3Pal\(^{4,4'}\)]GS (17)

16 (244 mg, 0.162 mmol) was dissolved in TFA (3 ml). The solution was stand for 3 h at 0° C. The solvent was evaporated off and the residue was solidified by ether. Reprecipitation from ether afforded H-Val-Orn(For)-Leu-\(\Delta^Z\)3Pal-Pro-Val-Ala-Leu-\(\Delta^Z\)3Pal-Pro-OH·3TFA as a white solid (296 mg, quantitative)

A solution of the decapeptide (104 mg, 0.073 mmol) in DMF (20ml) was added dropwise to a stirred solution of HATU (32 mg, 0.117 mmol) and DIEA (0.072 ml, 0.435 mmol) in DMF (80 ml) at 0 °C. The reaction mixture was stirred for 1 h at 0°C and for 24 h at room temperature. The reaction mixture was concentrated in vacuo. The residue was solidified by H\(_2\)O. The resulting solid was collected and purified by Low-pressure liquid chromatography (C\(_{18}\) column, 20-80% CH\(_3\)CN containing 0.1%TFA, linear gradient). Lyophilization afforded 17 as a white solid. (43 mg, 37%). \(R_f\) 0.43 (CHCl\(_3\):MeOH:AcOH=25:5:1 (v/v/v)); ESI-MS: \(m/z=1124.9\) ([M+H]+), C\(_{57}\)H\(_{83}\)N\(_{13}\)O\(_{11}\) requires 1124.6

[Ala\(^2\), \(\Delta^Z\)3Pal\(^{4,4'}\)]GS·HCl (2)

17 (13.9 mg, 0.012 mmol) was dissolved in 10% methanolic HCl. (5 ml) The solution was gently stirred for 2 days at room temperature. The solvent was evaporated off and the residue was lyophilized to afford 2 as a white solid (14.2 mg, quantitative), \(R_f\) 0.18 (1-BuOH:AcOH: H\(_2\)O=2:2:1(v/v/v)); m.p. 254-256 °C (decomp.); ESI-MS: \(m/z=1096.0\) ([M+H]+), C\(_{56}\)H\(_{82}\)N\(_{13}\)O\(_{10}\)
requires 1096.6

**Boc-Val-Ala-Leu-Δ^3Pal-Pro-OH (18)**

To a stirred solution of **11** (330 mg, 0.50 mmol) in 50% MeOHaq. (10 ml) was added 1M aqueous NaOH (0.751 ml). The solution was stirred for 11 h at 0 °C. The solution was neutralized by addition of 1M aqueous HCl and was concentrated in vacuo. The residue was mixed with CH3CN and the insoluble material was filtered out. The filtrate was concentrated in vacuo and the residue was crystalized from addition of CHCl₃-hexane to afford **18** (154 mg, 78%) as a white solid. Rf 0.28 ((CHCl₃:MeOH:AcOH=25:25:1 (v/v/v); m.p. 188-192°C; [α]D²₀ -84 (c 0.1 in MeOH)

**Boc-(Val-Ala-Leu-Δ^3Pal-Pro)₂-OMe (19)**

To a stirred solution of **13** (267 mg, 0.42 mmol) in CH₂Cl₂ (10 ml) were added NMM (0.093 ml, 0.84 mmol), **18** (329 mg, 1.2 mmol), HOBt·H₂O (6 mg, 0.03 mmol) and EDC HCl (106 mg, 0.55 mmol). The solution was stirred for 1 h at 0 °C and 23 h at ambient temperature. The solvent was removed off in vacuo and the residue was mixed with ethyl acetate (200 ml). The organic layer was washed with saturated aqueous NaHCO₃ (50 ml×3), water (50 ml×2) and brine (100 ml). After the organic layer was dried over anhydrous Na₂SO₄, the solvent was evaporated off to afford a crude. Purification by gel filtration chromatography (MeOH) followed by crystalization
from CHCl₃-hexane afforded 19 as a white solid. (458 mg, 91 %), Rf 0.86 (CHCl₃:MeOH:AcOH=25:25:1 (v/v/v)); m.p. 145-147ºC; [α]D²⁰ +33 (c 0.1 in MeOH); ESI-MS: m/z=1186.8 ([M+H]⁺), C₆₀H₈₉N₁₂O₁₃ requires 1185.66

Boc-(Val-Ala-Leu-Δ₂₃Pal-Pro)₂-OH (20)

To a stirred solution of 19 (131 mg, 0.11mmol) in 50% aqueous MeOH (8 ml) was added 1M aqueous NaOH (0.131 ml) at 0ºC. The solution was stirred for 30 h at 0ºC. The solvent was removed off _in vacuo_ and the residue was neutralized by addition of 1M aqueous HCl. The residue was dissolved in CH₃CN. After the insoluble solid was filtered off, the filtrate was concentrated and lyophilized to afford 20 as a white solid. (109 mg, 84%), Rf 0.45 (CHCl₃:MeOH:AcOH=25:5:1 (v/v/v)); m.p. 169-174ºC; [α]D²⁰ -79 (c 0.1 in MeOH); ESI-MS: m/z=1171.9 ([M+H]⁺), C₅₉H₈₇N₁₂O₁₃ requires 1171.64

[Ala²⁻, Δ₂₃ Pal⁴⁻]GS (3)

20 (203 mg, 0.172 mmol) was dissolved in TFA (3 ml). The solution was stand for 3 h at 0° C. The solvent was evaporated off and the residue was solidified by ether. Reprecipitation from ether afforded H-(Val-Ala-Leu-Δ₂₃Pal-Pro)₂-OH·2TFA as a white solid (225 mg, 92%)

A solution of the decapeptide (215 mg, 0.15 mmol) in DMF (5ml) was added dropwise to a stirred solution of HATU (65 mg, 0.17 mmol) and DIEA (0.156 ml, 0.91 mmol) in DMF (205 ml) at 0 °C. The reaction mixture was stirred for 1 h at 0ºC and for 24 h at room temperature. The
reaction mixture was concentrated in vacuo. The residue was solidified by H2O. The resulting solid was collected and purified by Low-pressure liquid chromatography (C18 column, 20-80% CH3CN containing 0.1% TFA, linear gradient). Lyophilization afforded 3 as a white solid. (41 mg, 26%). Rf 0.42 (CHCl3:MeOH:AcOH=25:5:1 (v/v/v)); ESI-MS: m/z=1053.8 ([M+H]+), C54H77N12O10 requires 1053.58.

Antimicrobial activity

Staphylococcus aureus FDA 209P and Escherichia coli K12 strain W3110 were used as Gram-positive and Gram-negative bacteria, respectively. Wild-type GS was initially dissolved in EtOH followed by dilution with water. We have checked that no bacterial killing happened under this experimental condition. Other peptides were dissolved in water to prepare stock solutions. Minimum inhibitory concentrations (MIC) were determined by the liquid microdilution method, using serially diluted (two-fold) peptides. Cells (1×10^4) were cultured at 37ºC for 20 h in 100 μl of Mueller-Hinton broth containing peptide in 96-well microtiter plates. The minimum inhibitory concentration was determined as the lowest concentration of peptide in which cells were unable to grow.

Hemolytic activity

Human erythrocytes were washed twice with buffer (150 mM NaCl and 10 mM
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)/NaOH, pH 7.4), and then suspended in this buffer at a final concentration of 1% hematocrit. After incubation with peptides at 37 °C, hemolysis was estimated by measuring the absorbance at 540 nm. Melittin (10 μM) was used to determine the 100% level of hemolysis.\textsuperscript{12c,19b}

*K+ efflux and cell viability measurement*

*Staphylococcus aureus* FDA 209P and *Escherichia coli* K12 strain W3110 were grown as reported previously. Washed *S. aureus* and *E. coli* cells were suspended in 100mM choline chloride and 50mM 4-morpholinepropanesulfonic acid (Mops)/2-amino-2-hydroxy-methylpropane-1,3-diol (Tris) (pH7.2) at 2×10^9 cells/ml. Cells were incubated with a peptide at 37 °C for 30 min. The amount of K\textsuperscript{+} efflux was measured with a K\textsuperscript{+}-selective electrode.\textsuperscript{19,23} The total amount of K\textsuperscript{+} was determined by disrupting cells with melittin (10 μM) or polymyxin B (200 μg/ml).\textsuperscript{23b} The viability of bacterial cells was determined by counting colonies.\textsuperscript{19,23b}
Figure S1. HPLC chromatograms of the GS analogues.

[Ala²,Δ²Z3Pal⁴,⁴']GS (2)

Column: YMC-pack ODS-AM (250×4.6 mm)
Eluent: 30-80% MeCNaq. (Containing 0.1% TFA)
Flow rate: 1 mL/min
Temperature: 30 ºC
Figure S2. $^1$H NMR spectra of the GS analogues. (500MHz, DMSO-$d_6$, 30 °C)

[Ala$^{2, \Delta 3}$Pal$^{4,4'}$]GS (2)

[Ala$^{2,2'}$Pal$^{4,4'}$]GS (3)
Figure S3. CD spectra of the GS analogues. (0.1mm-path quartz cell, MeOH, 25 °C, 1mg/mL)