Electronic Supplementary Information (ESI)

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

Keiichi Yamada*[a], Makoto Kodaira[a], Shun-suke Shinoda[a], Keiko Komagoe[b], Hiroyuki Oku[a], Ryoichi Katakai[a], Takashi Katsu[b] and Ichiro Matsuo[a]

[a]Department of Chemistry and Chemical Biology, Gunma University, 1-5-1, Tenjin-cho, Kiryu, Gunma 376-8515, Japan

[b]Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 1-1-1, Tsushima-naka, Kita, Okayama 700-8530, Japan

Experimental procedures

General

Boc-amino acids were purchaised from Peptide Institute, Inc. (Osaka, Japan) Reagents for peptide synthesis were obtainied from Novabiochem (Darmstadt, Germany) and Peptide Institute (Osaka, Japan). Polymyxin B nonapeptide (PMBN) hydrochloride was purchaised from Sigma-Aldrich (St. Louis, MO, USA) Other organic reagents were of commerically avairable grade. All solvents were distilled over CaH_2 or Na before use. TLC was performed on Silicagel $60F_{254}$ aluminum plate (Merck, Germany). The compounds were vidualized with UV light (254nm) and ninhydrin. Column chromatography was performed on Silicagel 60N for flash chromatography (Kanto Chemical, Co. Ltd., Tokyo, Japan, spherical, neutral, 40-60 μ m 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 appratus and were uncorrected. Optical rotations were measured on JASCO (Tokyo, Japan) DIP-1000 digital polarimeter using 10cm-path quartz cell at 20°C. ^{1}H NMR spectra were measured on JEOL (Tokyo, Japan) lambda-500 spectrometer or alpha-300 spectrometers in $CDCl_3$ or DMSO-d_6 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%), R_f 0.15 (1-BuOH:AcOH:H_2O=2:1:1 (v/v/v)), $[\alpha]_D^{20}$ +5.8 (c1.0 in 1M HCl aq.). ^{1}H NMR (300 MHz, D_2O, 25°C) : δ 8.64 (s, 1H), 8.56 (s, 1H), 8.17 (d, 1H, J = 8.1Hz), 7.70 (t, 1H, $J_{1,2}$ = 5.6 Hz, $J_{1,3}$ = 12.4 Hz), 5.06 (d, 1H, J = 4.3 Hz), 3.96 (br, 1H).

Boc-Leu-Δ^Z3Pal-Pro-OMe (8)

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

Boc-Ala-Leu-Δ^Z3Pal-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-Δ^Z3Pal-Pro-OMe·2HCl (678 mg, quantitative)

To a stirred solution of H-Leu-Δ^Z3Pal-Pro-OMe·2HCl (678 mg, 1.47 mmol) and NMM (0.317 ml, 2.88 mmol) in CH_2Cl_3 (10 ml) were added Boc-Ala-OH (225 mg, 1.19 mmol), HOBt·H_2O (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 $NaHCO_3$ (50 ml×3), water (50 ml×2) and brine (100 ml). After the organic layer was dried over anhydrous Na_2SO_4, the solvent was evaporated off to afford a crude. SiO_2 chromatographic purification ($CHCl_3$:MeOH= 9:1(v/v) as a eluent) afforded **9** as a white solid. (708 mg, 88%), *Rf* 0.33 ($CHCl_3$:MeOH=9:1(v/v)); m.p. 99-102ºC; $[\alpha]_D^{20}$ +45 (c0.1 in MeOH); ESI-MS: *m/z*=582.4 ($[M+Na]^+$), $C_{28}H_{41}N_5O_7Na$ requires 582.3

Boc-Orn(For)-Leu-Δ^Z3Pal-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-Δ^Z3Pal-Pro-OMe·2HCl 1.53 g, quantitative)

To a stirred solution of H-Leu-Δ^Z3Pal-Pro-OMe·2HCl (1.53 g, 3.32 mmol) and NMM (0.740

ml, 6.73 mmol) in CH_2Cl_2 (20 ml) were added Boc-Orn(For)-OH (1.13 g, 4.34 mmol), HOBt·H_2O (45.0 mg, 0.333 mmol) and EDC·HCl (762 mg, 3.99 mmol) at 0ºC. 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 $NaHCO_3$ (50 ml×3), water (50 ml×2) and brine (100 ml). After the organic layer was dried over anhydrous Na_2SO_4, the solvent was evaporated off to afford a crude. SiO_2 chromatographic purification ($CHCl_3$:MeOH=9:1(v/v) as a eluent) afforded **10** as a white solid. (1.89 g, 89 %), *Rf* 0.33 ($CHCl_3$:MeOH=9:1(v/v)); m.p. 104-106ºC (lit.[18] 102-103ºC); $[\alpha]_D^{20}$ +81 (c 0.1, MeOH) (lit.[18] +85.4 (c0.1 in MeOH)); ESI-MS: *m/z*=631.6 ($[M+H]^+$), $C_{31}H_{47}N_6O_8$ requires 631.4

Boc-Val-Ala-Leu-Δ^Z3Pal-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-Δ*Z*3Pal-Pro-OMe·2HCl as a white solid. (639 mg, 95 %)

To a stirred solution of H-Ala-Leu-Δ^Z3Pal-Pro-OMe·2HCl (639 mg, 1.20 mmol) in CH_2Cl_2 (20 ml) were added NMM (0.265 ml, 1.90 mmol), Boc-Val-OH (313 mg, 1.52 mmol), HOBt·H_2O (17 mg, 0.13 mmol) and EDC·HCl (277 mg, 1.52 mmol) at 0ºC. The solution was stirred for 1 h at 0ºC 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 $NaHCO_3$ (50 ml×3), water (50 ml×2) and brine (100 ml). After the organic layer was dried over anhydrous Na_2SO_4, the solvent was evaporated off to afford a crude. SiO_2 chromatographic purification ($CHCl_3$:MeOH=95:5 (v/v)) followed by crystalization from AcOEt-hexane afforded **11** as a white solid. (704 mg, 89 %), *Rf* 0.30 ($CHCl_3$:MeOH=9:1(v/v)); m.p. 124-125ºC; $[\alpha]_D^{20}$ +77 (c 0.1 in MeOH); ESI-MS: *m/z*=659.9 ($[M+H]^+$), $C_{33}H_{51}N_6O_8$ requires 659.4

Boc-Val-Orn(For)-Leu-Δ^Z3Pal-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-Δ^Z3Pal-Pro-OMe·2TFA as a yellowish oil. (1.14 g, 99%)

To a stirred solution of H-Orn(For)-Leu-Δ^Z3Pal-Pro-OMe·2TFA (1.14 g, 1.52 mmol) in CH_2Cl_2 (20 ml) were added NMM (0.326 ml, 2.96 mmol), Boc-Val-OH (390 mg, 1.78 mmol), HOBt·H_2O (20 mg, 0.15 mmol) and EDC·HCl (341 mg, 1.78 mmol) at 0ºC. The solution was stirred for 1 h at 0ºC 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 $NaHCO_3$ (50 ml×3), water (50 ml×2) and brine(100 ml). After the organic layer was dried over anhydrous Na_2SO_4, the solvent was evaporated off to afford a crude. Recrystallization from hexane afforded **12** as a white solid. (781 mg, 73 %), *Rf* 0.36 ($CHCl_3$:MeOH=9:1(v/v)); m.p.

116-117ºC (lit.[18] 117-118ºC); $[\alpha]_D^{20}$ +76 (c 0.1 in MeOH) (lit.[18] +76.3 (c 0.1 in MeOH)); ESI-MS: *m/z*=730.6 ([M+H]+), $C_{36}H_{56}N_7O_9$ requires 730.5

Boc-Val-Orn(For)-Leu-Δ^Z3Pal-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 ºC. The solution was neutralized by addition of 1M aqueous HCl and was concentrated in vacuo. The residue was mixed with CH_3CN and the insoluble material was filtered out. The filtrate was concentrated in vacuo and the residue was crystalized from addition of $CHCl_3$-hexane to afford **13** (154 mg, 78%) as a white solid. *Rf* 0.20 ($CHCl_3$:MeOH:AcOH=25:5:1 (v/v/v)); m.p. 175-176ºC; $[\alpha]_D^{20}$ +17 (c 0.1 in MeOH); ESI-MS: *m/z*=716.4 ($[M+H]^+$), $C_{35}H_{54}N_7O_9$ requires 716.4

Boc-Val-Orn(For)-Leu-Δ^Z3Pal-Pro-Val-Ala-Leu-Δ^Z3Pal-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-Δ^Z3Pal-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 CH_2Cl_2 (10 ml) were added NMM (0.060 ml, 0.55 mmol), **14** (212 mg, 0.296 mmol), HOBt·H_2O (4 mg, 0.03 mmol) and EDC HCl (57 mg,

0.30 mmol). The solution was stirred for 1 h at 0ºC 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_2SO_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. 145-148ºC; $[\alpha]_D^{20}$ +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-Δ^Z3Pal-Pro-Val-Ala-Leu-Δ^Z3Pal-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 0ºC. The solution was stirred for 48 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 CH_3CN. 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. 174-179ºC; $[\alpha]_D^{20}$ -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)$^{2'}$,Δ^Z3Pal$^{4,4'}$]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-Δ^Z3Pal-Pro-Val-Ala-Leu-Δ^Z3Pal-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 ºC. 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 H_2O. The resulting solid was collected and purified by Low-pressure liquid chromatography (C_{18} column, 20-80% CH_3CN containing 0.1%TFA, linear gradient). Lyophilization afforded **17** as a white solid. (43 mg, 37%). *Rf* 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,Δ^Z3Pal$^{4,4'}$]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) , *Rf* 0.18 (1-BuOH:AcOH: H_2O=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^Z3Pal-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 ºC. 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 $CHCl_3$-hexane to afford **18** (154 mg, 78%) as a white solid. *Rf* 0.28 (($CHCl_3$:MeOH:AcOH=25:25:1 (v/v/v); m.p. 188-192ºC; $[\alpha]_D^{20}$ -84 (c 0.1 in MeOH)

Boc-(Val-Ala-Leu-Δ^Z3Pal-Pro)$_2$-OMe (19)

To a stirred solution of **13** (267 mg, 0.42 mmol) in CH_2Cl_2 (10 ml) were added NMM (0.093 ml, 0.84 mmol), **18** (329 mg, 1.2 mmol), HOBt·H_2O (6 mg, 0.03 mmol) and EDC HCl (106 mg, 0.55 mmol). The solution was stirred for 1 h at 0 ºC 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 $NaHCO_3$ (50 ml×3), water (50 ml×2) and brine (100 ml). After the organic layer was dried over anhydrous Na_2SO_4, the solvent was evaporated off to afford a crude. Purification by gel filtration chromatography (MeOH) followed by crystalization

from $CHCl_3$-hexane afforded **19** as a white solid. (458 mg, 91 %), *Rf* 0.86 ($CHCl_3$:MeOH: AcOH=25:25:1 (v/v/v)); m.p. 145-147ºC; $[\alpha]_D^{20}$ +33 (c 0.1 in MeOH); ESI-MS: *m/z*=1186.8 ($[M+H]^+$), $C_{60}H_{89}N_{12}O_{13}$ requires 1185.66

Boc-(Val-Ala-Leu-Δ^Z3Pal-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 CH_3CN. 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 ($CHCl_3$:MeOH:AcOH=25:5:1 (v/v/v)); m.p. 169-174ºC; $[\alpha]_D^{20}$ -79 (c 0.1 in MeOH); ESI-MS: *m/z*=1171.9 ($[M+H]^+$), $C_{59}H_{87}N_{12}O_{13}$ requires 1171.64

[Ala$^{2,2'}$, Δ^Z3Pal$^{4,4'}$]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-Δ^Z3Pal-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 ºC. 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 H_2O. The resulting solid was collected and purified by Low-pressure liquid chromatography (C_{18} column, 20-80% CH_3CN containing 0.1%TFA, linear gradient). Lyophilization afforded **3** as a white solid. (41 mg, 26%). *Rf* 0.42 ($CHCl_3$:MeOH:AcOH=25:5:1 (v/v/v)); ESI-MS: *m/z*=1053.8 ($[M+H]^+$), $C_{54}H_{77}N_{12}O_{10}$ 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

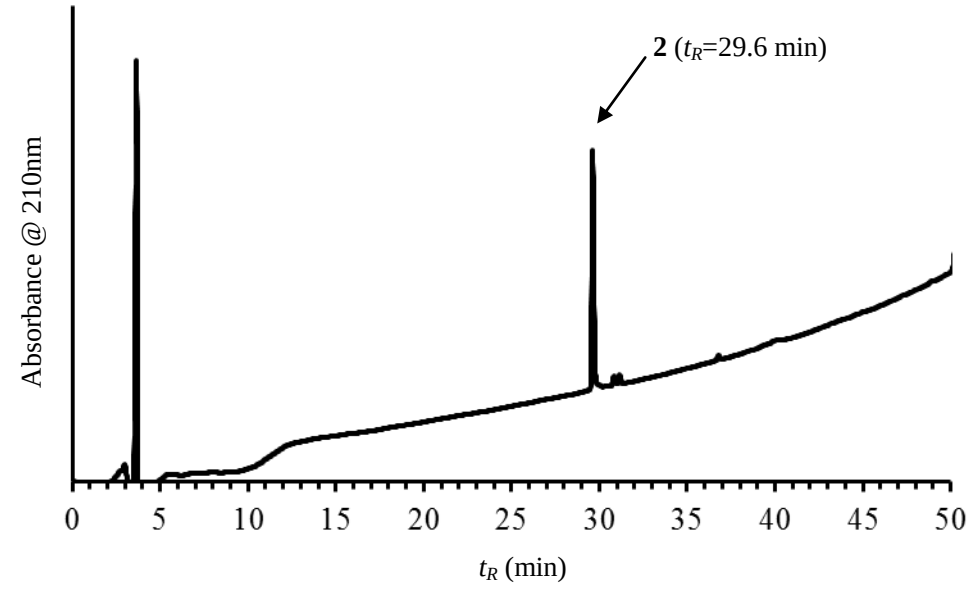
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)/NaOH, pH 7.4), and then suspended in this buffer at a final concnetration of 1% hematocrit. After incubation with peptides at 37 ºC, hemolysis was estimated by measuring the absorbance at 540 nm. Melittin (10 μM) was used to determine the 100% level of hemolysis.[12c,19b]

K^+ efflux and cell viability measurement

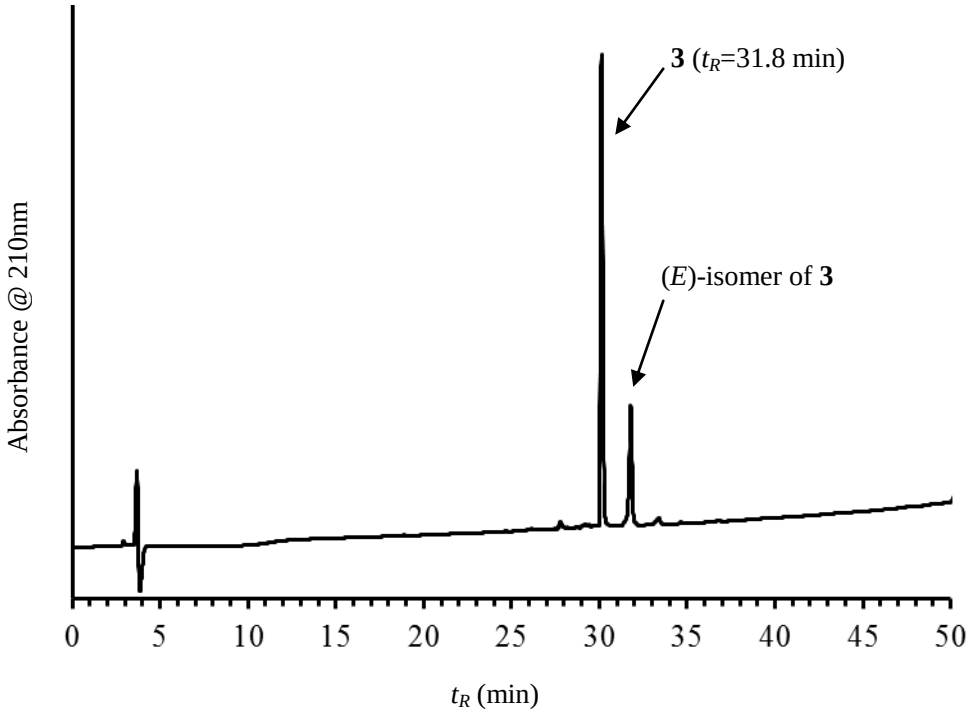
S. aureus FDA 209P and *E. coli* K12 strain W3110 were grown as reported previously. Washed *S. aureus* and *E. coli* cells were suspended in 100mM choline chloride and 50mM 4-morpholine-propanesulfonic acid (Mops)/2-amino-2-hydroxy-methylpropane-1,3-diol (Tris) (pH7.2) at 2×10^9 cells/ml. Cells were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

Figure S1. HPLC chromatograms of the GS analogues.

[Ala^{2},Δ^{Z}3Pal$^{4,4'}$]GS (**2**)



[Ala$^{2,2'}$,Δ^{Z}3Pal$^{4,4'}$]GS (**3**)



Column : YMC-pack ODS-AM (250×4.6 mm)

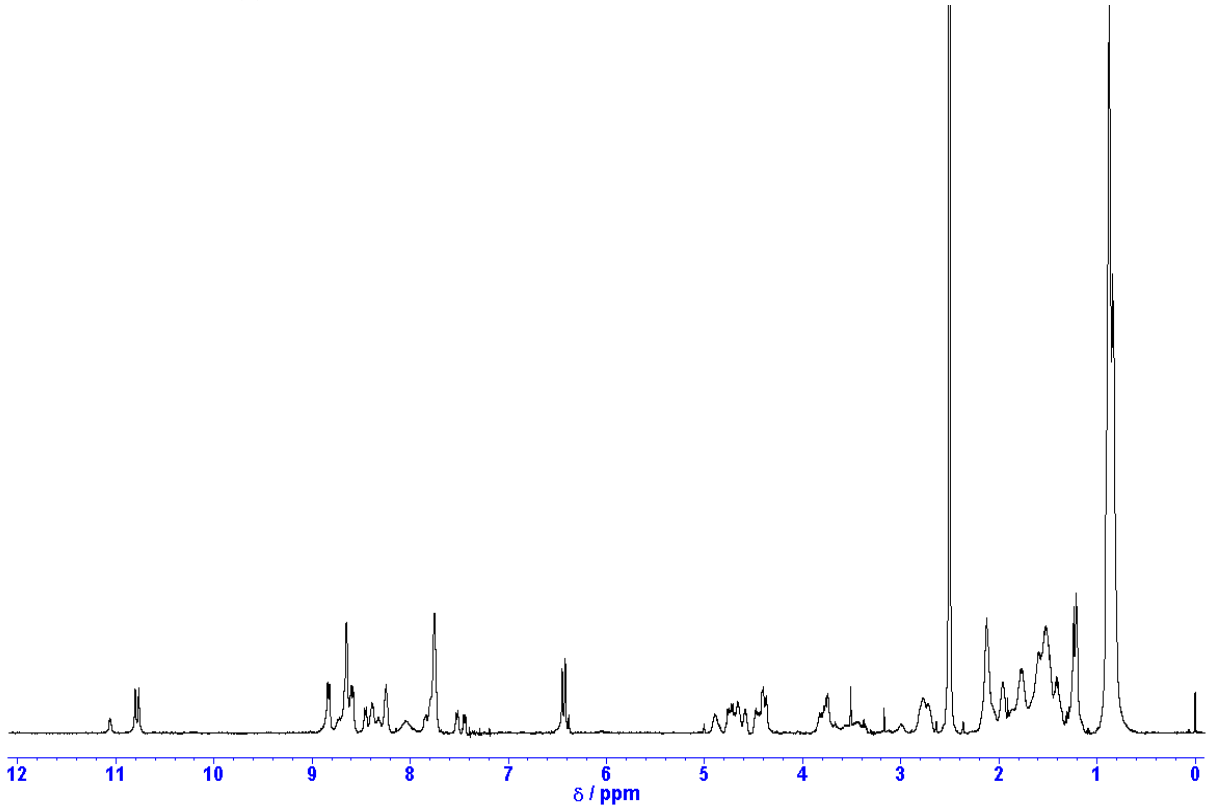
Eluent: 30-80% MeCNaq. (Containing 0.1%TFA)

Flow rate: 1 mL/min

Temperature: 30 °C

Figure S2. ^{1}H NMR spectra of the GS analogues. (500MHz, DMSO-d_6, 30 °C)

[Ala^{2},Δ^{Z}3Pal$^{4,4'}$]GS (**2**)



[Ala$^{2,2'}$,Δ^{Z}3Pal$^{4,4'}$]GS (**3**)

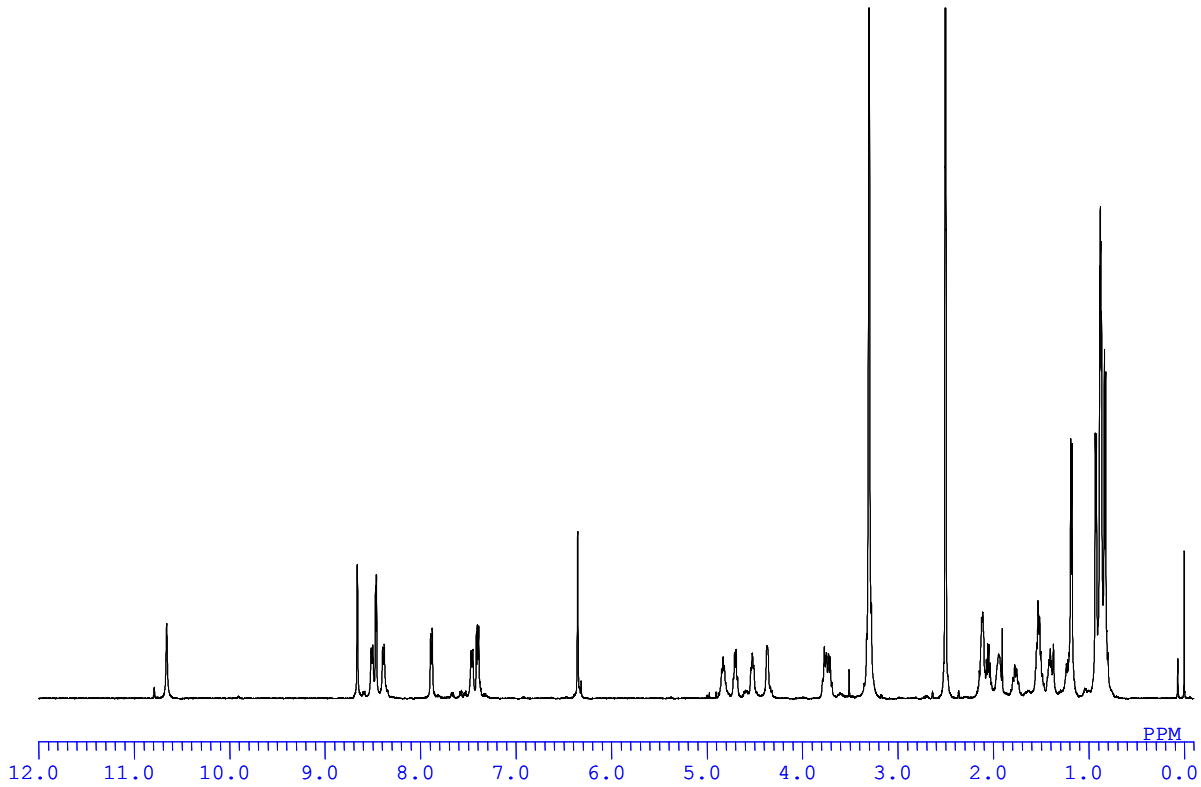


Figure S3. CD spectra of the GS analogues. (0.1mm-path quartz cell, MeOH, 25 °C, 1mg/mL)