SUPPLEMENTARY INFORMATION

Multivalent Attenuation of Quorum Sensing in Staphylococcus aureus

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1. GENERAL.

All chemicals were of reagent grade quality, purchased commercially from Sigma, Aldrich, Fluka, or Acros and used without further purification, unless described else. Solvents were purchased from ABCR or BioLab and used without any further treatment with the exception of THF which was distilled from sodium, and dichloromethane and DMF which were purified via an MBRAUN SPS solvent purification system before use. Flash chromatography was performed using Merck 40-63 µm silica gel. Solvent ratios for the purification of compounds by flash chromatography are reported as percent volume (v/v). Gas chromatography mass spectrometry data was obtained using an Agilent 6850 GC equipped with an Agilent 5973 MS detector working under standard conditions, using an Agilent HP5-MS column. Analytical HPLC analysis was performed using a Surveyor Plus HPLC System (Thermo Scientific) with a Luna C18.5 um (150 x 4.6 mm) column at a flow rate of 1 mL/min. Preparative HPLC was performed using a Sapphire 600 instrument (ECOM) with a Luna C18 column, 10 µm (250 x 21.20 mm), at a flow rate of 20 mL/min. All runs used linear gradients of 0.1% aqueous TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B). Compounds were identified by UV detection with dual wavelengths (230 nm, 260 nm). MS-ESI analyses were performed on a LCQ Fleet mass spectrometer (Thermo Scientific) with an ESI source. Spectra were collected in the positive ion mode and analyzed by Xcalibur software (Thermo Scientific). NMR spectra were recorded on Bruker DPX400 or DMX500 instruments; spectra were calibrated on residual solvent signal. GPC analyses were obtained using an Agilent 1200 HPLC equipped with two Agilent PLgel 5 µm Mixed-C columns and a Phenomenex Phenogel 5u 103A column. Wyatt's miniDAWN TriStar laser light-scattering system, ViscoStarTM viscometer, and Optilab[®] rEX refractometer were used as detectors. Astra 5.3.12.3 was used for calibration and calculation of the polymers polydispersities and molecular weights. Microwave heating was realized by a CEM Discover® instrument.

Microscopy was performed using a Nikon Digital Sight DS-U1 cooled CCD camera mounted on an Nikon inverted fluorescence microscope (Nikon Eclipse TE2000-S, Badhoevedorp, The Netherlands) equipped with a Plan Fluor 100× oil NA1.3 objective (Nikon). Fluorescence excitation was performed using a 100 W mercury lamp in combination with an Endow GFP BP filter set (Chroma Technology, Rockingham, VT; exciter HQ470/40 nm, dichroic mirror Q4951p, emitter HQ525/50). Images were obtained and processed using the microscope domain program NIS-Elements Basic Research (Nikon Instruments, Melville, NY).

2. Synthetic procedures



N-(3,6,9-Trioxadecyl)phthalimide

Phthalimide (1.77 g, 12 mmol) and PPh₃ (3.15 g, 12 mmol) were dried under vacuum and dissolved in 50 mL of distilled THF. Triethyleneglycol monomethylether (1.6 mL, 10 mmol) was added to the mixture, which was stirred for 30 min. Diisopropyl azodicarboxylate (2.36 mL, 12 mmol) was added dropwise and the mixture was stirred for 12 h at rt. The reaction was then quenched by adding 25 mL of EtOH and swirled for 15 min. After removal of solvent by rotary evaporation, 10 mL of 1:1 EtOAc:hexane was added and the mixture was stirred for 1 min at rt and 1 h at 40 °C, and then filtered. The filtrate was concentrated by rotary evaporation and the residue was purified by column chromatography (4:1 hexane:EtOAc) to obtain 2.58 g (82%) of a colorless oil. 1H NMR (CDCl3) δ 3.38 (s, 3H), 3.57 (m, 2H), 3.65 (m, 10H), 7.49 (m, 2H), 7.67 (m, 2H), in agreement with literature data.¹

3,6,9-Trioxadecylamine (MEA)

N-(3,6,9-Trioxadecyl)phthalimide (675 mg, 2.36 mmol) was added to EtOH (10 mL) and treated with 0.2 mL (3.9 mmol) of hydrazine monohydrate. The mixture was refluxed for 5h, after which it was cooled to rt, treated with 2 mL of concentrated HCl, and refluxed for 1 h. The mixture was cooled to rt and a white solid was removed by filtration. The filtrate was concentrated by rotary evaporation and then treated with 20 mL of water and sufficient NaOH (1N) to bring the pH > 11. The mixture was saturated with NaCl and extracted with CH₂Cl₂ (4 x 20 mL). The extracts were dried over MgSO₄ and concentrated by rotary evaporation to give 310 mg of crude product. 1H NMR analysis showed the presence of diisopropyl hydrazinedicarboxylate, so the crude material was taken into 20 mL of 5% HCl and washed with CH₂Cl₂ (3 x 20 mL). The aqueous layer was basified to pH > 11 and extracted with CH₂Cl₂ (3 x 20 mL), dried over MgSO₄ and concentrated by rotary evaporation to give 150 mg (40%) ¹H NMR (CDCl₃) δ 1.64 (bs, 2H + H2O), 2.87 (bs, 2H), 3.37 (s, 3H), 3.52 (m, 2H), 3.56 (m, 2H), 3.66 (m, 6H), in agreement with the published spectral data.¹



Norbornene acid, exo

Commercially available racemic *exo/endo* norbornene carboxylic acid (25.0 mL, 0.204 mol, 1.0 eq.) was dissolved in aqueous 0.75 M NaHCO₃ (300 mL). A solution of iodine (45.0 g, 0.178 mol, 0.9 eq) and KI (89.0 g, 0.534 mol, 2.6 eq.) in H₂O (250 mL) was added dropwise until a brown color persisted. The aqueous layer was extracted with diethyl ether (Et₂O) (5 x 100 mL) to remove residual iodolactone. An aqueous 10% sodium thiosulfate solution (30 mL) was added to decolorize the aqueous layer. The solution pH was adjusted to 2 using aqueous 1 N H₂SO₄, and a yellow precipitate was

observed. The mixture was extracted with Et2O (4 x 100 mL) while maintaining pH 2. The combined Et2O layers were dried over MgSO4 and concentrated under reduced pressure to afford the *exo* acid product as a white powder (5.83 g, 21.0% yield). ¹H-NMR (400 MHz, CDCl₃): δ 12.70 – 9.96 (br s, 1H), 6.16 (dd, 1H), 6.13 (dd, 1H), 3.12 (br s, 1H), 2.95 (br s, 1H), 2.30 – 2.25 (ddd, 1H), 1.96 (dt,1H), 1.55 (d, 1H), 1.45 – 1.38 (m, 2H); ¹³C-NMR (135 MHz, CDCl₃) δ 182.7, 138.1, 135.7, 46.7, 46.4, 43.2, 41.7.²



exo Norbornene N-hydroxysuccinimidyl ester (1)

The exo norbornene acid, (1.00 g, 7.20 mmol, 1.0 eq.), *N*-hydroxysuccinimide (1.12 g, 9.72 mol, 1.4 eq.), and *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDCI·HCl) (1.79 g, 9.36 mmol, 1.3 eq.) were stirred in CH₂Cl₂ for 15 hours. The reaction mixture was concentrated under reduced pressure, and *N*-hydroxysuccinimidyl (NHS) ester **1** was purified by flash chromatography (2:1 hexanes/ethyl acetate) to afford product as a white powder (1.35 g, 5.76 mmol, 80.0% yield). ¹HNR (400 MHz, CDCl₃) δ 6.21 (dd, *J* = 5.3 Hz, 2.9 Hz, 1H), 6.15 (dd, *J* = 5.7 Hz, 3.4 Hz, 1H), 3.28 (br s, 1H), 3.01 (br s, 1H), 2.85 (s, 4H), 2.52 (ddd, *J* = 10.48 Hz, 4.78 Hz, 1.65 Hz, 1H), 2.06 (dt, *J* = 11.95 Hz, 4.23 Hz, 1H), 1.57 (d, *J* = 9.2 Hz, 1H), 1.51 – 1.44 (m, 2H); ¹³C-NMR (135 MHz, CDCl₃) δ 171.6, 169.2, 138.5, 135.3, 47.1, 46.4, 41.8, 40.3, 31.0, 25.6.²



NHS ester polymer 2.

exo Norbornene N-hydroxysuccinimidyl ester (1) (100 mg, 0.425 mmol) was dissolved in 4 mL DCM. Grubbs 3_{rd} generation catalyst (3.9 mg, 0.004 mmol) was added. The reaction was stirred under nitrogen at room temperature for 45 min. The reaction appeared complete by TLC, and an excess of ethyl vinyl ether was added. The reaction mixture was filtered through a small plug of silica gel with DCM as eluent. The solvent was removed under reduced pressure to afford a white solid (96.8 mg). Yield 98%; 1H NMR δ (400 MHz, CDCl₃) 7.3 (m), 5.7-5.2 (m), 3.5-0.90 (br m)²

Polymers 3a-c

AIP-4' peptide (0.7 mg 0.7 μ mol) was dissolved in 2 mL anhydrous DMF in 3 different reaction vials, and different amounts of polymer **2** (17.7, 3.5, 1.8 mg; 70.0, 14.0, 7.0 μ mol) and 2 μ L of TEA were added and the solutions were stirred under nitrogen at room temperature. The reaction was followed via depletion of AIP-4' by LCMS (~20 h), after which an excess of MEA (1.5 eq) was added for another 24h. The reaction

solutions were concentrated under vacuum. The three polymers (with 100, 20, and 10 molar equivalents per monomer unit) were used without further purification.



Polymers 5a-c

AIP-4' peptide (0.7 mg 0.7 μ mol) was dissolved in 2 mL anhydrous DMF in 3 different reaction vials, and polymer **4** (9.8, 2.0, 1.0 mg; 70.0, 14.0, 7.0 μ mol) was added and the solutions were stirred under nitrogen at room temperature. The reaction was followed via depletion of AIP-4' by LCMS (24h), after which an excess of MEA (1.5 eq) was added for another 24h. The reaction solutions were concentrated under vacuum. The three polymers (with 100, 20, and 10 molar equivalents per monomer unit) were used without further purification.



Polymer 6a

AIP-4' peptide (0.7 mg 0.7 μ mol) and *N*-(2-aminoethyl)rhodamine 6G-amide bis(trifluoroacetate (0.2 mg 0.28 μ mol) were dissolved in 2 mL anhydrous DMF. Polymer **4** (2.0 mg) was added and the solutions were stirred under nitrogen at room temperature. The reaction was followed via depletion of AIP-4' and the rhodamine by LCMS (~24h), after which an excess of MEA (1.5 eq) was added for another 24 h. Purification of the polymer was done by dialysis followed by lyophilization.

Polymer 6b

N-(2-Aminoethyl)rhodamine 6G-amide bis(trifluoroacetate (0.2 mg 0.28 μ mol) was dissolved in 2 mL anhydrous DMF. Polymer **4** (2.0 mg) was added and the solutions were stirred under nitrogen at room temperature. The reaction was followed via depletion of AIP-4' and the rhodamine by LCMS (~24 h), after which an excess of MEA (1.5 eq) was added for another 24 h. Purification of the polymer was done by dialysis followed by lyophilization.

AIP-4'³

<u>Preparation of linker – tritylmercaptopropionic acid.</u> 10 mmol (0.87 mL) of 3-mercaptopropionic acid was dissolved in a mixture of 6 mL DCM and 6 mL of acetic acid. Trityl chloride (10mmol, 2.87g) was added to the solution, after which $BF_3 \cdot Et_2O$ (2 mL) was added dropwise as a catalyst, and the color of the reaction turned yellow-green. After the reaction solution has been stirred at rt for 20 min, the solvent was removed *in vacuo*. Water (10 mL) was added to the residue and the white precipitate was collected by filtration and washed with distilled water, acetonitrile and cold ether.

<u>Handling of the resin</u>: MBHA resin (0.2 mmol, 166.7 mg) was placed in a frit syringe. The resin was washed with fresh DMF 3 times, flushed and then washed twice with DCM; the resin was left wet until used in a coupling reaction.

<u>Coupling linker to the resin</u>: trityl-mercaptopropionic acid (4 eq, 0.8 mmol, 276 mg) was placed in a 4 ml vial. HBTU (4.25eq, 0.85mmol, 322.4mg) and DIEA (10 eq, 2 mmol, 330 μ L) was added, followed by the edition of 2 mL DMF.After 2 minutes, the resin was air flushed to remove DCM, and the pre-activated linker was added. 1 mL of DCM was then added and the resin was shaken for 30 min. After this coupling the resin was washed with DMF and DCM.

<u>Capping of unreacted sites</u>: capping was performed by reacting the resin with an Ac_2O : DIEA solution (1:1, 30 equiv.). A solution of Ac_2O (6 mmol, 567 µL), DIEA (6 mmol, 992 µL) and 1 mL DMF was added to the air flushed resin. After 15 min. shaking, the resin was washed twice with DMF and twice with DCM.

<u>Cleavage of trityl group</u>: The resin was air flushed and incubated for with a mixture of triisopropylsilane (750 μ L), water (750 μ L) and TFA (28.5 mL) for 15 min., after which the resin was washed with DCM (twice) and DMF (twice).

<u>Coupling of amino acids</u>: amino acids were pre-activated for 5 min. with a pre-activation solution^{*} (0.2 mmol amino acid per 2 mL of pre-activation solution). After activation, 1 mL of DCM was added to the solution and the mixture was added to the resin. The resin was then shaken for 30 min, flushed and washed with DMF (twice) and DCM (twice). The Boc protecting groups were removed with 2 flow washes of TFA, followed by a wash with DMF (twice) and DCM (twice).

*Pre-activation solution for each amino acid (0.2 mmol scale): 0.85 mmol HBTU, 2 mmol DIEA, 2 mL DMF

** cys and ser are pre-activated for 2 min only to prevent racemization.

<u>Cleavage of peptide</u>: after the coupling of the last amino acid, the Boc group was removed and the resin was dried under nitrogen flow. 250 mg of resin was placed in a 50 mL round bottom flask with a stirring bar and 750 μ L of methyl sulfide/ethanedithiol (2:1) was added. The flask was chilled on an ice bath and 5 mL of TFA was added, ans the suspension was stirred for 5 min. 500 μ L of TFMSA was added dropwise at 0 ^oC with vigorous stirring. The reaction was stirred for 1.5 hours, after which TFA was evaporated quickly under an air flow. The resulting solution was filtered through a clean frit syringe, into a centrifuge tube, and an 8 fold volume of cold ether was added dropwise. The solution was centrifuged and the liquid was decanted. The resulting white precipitate was dissolved in a water/acetic acid (1:1) solution and purified by HPLC. Cyclization of AIP:

The linear peptides were dissolved in a mixture of 0.1 M sodium phosphate buffer (pH 7.0) and acetonitrile (1:1). After 24 hours, the solvent was removed by lyophilization, followed by HPLC purification.

Figure SI - 1: MS-ESI for AIP-4':



AIP-4'- norbornene:

AIP-4' (11 mg, 1.1 μ mol) and norbornene N-hydroxysuccinimidyl ester (1) (5 mg, 2.2 μ mol) were dissolved in anhydrous DMF (1.5 mL). Dry triethylamine (3 μ L) was added to this solution and the reaction mixture was stirred under nitrogen at room temperature for 24 h. The resulting peptide was purified by HPLC and analyzed by LC/MS. Lyophilization afforded a white powder, in 85% yield: MS-ESI m/z, 1133.2 [M+Na]⁺

Figure SI - 2: MS-ESI for AIP-4'- norbornene:



3. Biological Evaluations

3.1 Bacterial strains and growth conditions:

Assays were performed with *S. aureus* wild-type laboratory strain and RN9371 strain (containing an agr P3:blaZ fusion plasmid). Cells were grown in CYGP medium at 37°C overnight. Cultures on GL plates were routinely used as inocula and for plating. The antibiotics erythromycin (10 μ g/mL) and tetracycline (5 μ g/mL) were added to the *S. aureus* RN 9371 CYGP medium and GL plates.

3.2 Activator assays: 4,5

Cells were grown in CYGP medium at 37°C. After 6-10 h the culture was diluted to $OD_{600nm} = 0.25$ by CYGP medium. A mixture of 40 µL cells, 5 µL AIP-4' / poly-AIP-4' solution (20 µM in DMSO) and 55 µL CYGP medium was incubated at 37°C with shaking for 90 min. β -lactamase activity was measured by the nitrocefin method. 50 µL culture samples were transferred to a 96 well microtiter plate that contained 10 µL sodium azide solution (10 mM in sodium phosphate buffer, pH 5.8) and 5 µL nitrocefin solution (0.5 mM in MeOH) diluted with CYGP medium to a final volume of 100 µL. The microtiter reader was set to read at ϵ_{490nm} - ϵ_{650nm} every 5-10 min for 3 h. In the control wells CYGP medium was added instead of AIP-4' solution.

Figure SI - 3 : Activation curve of AIP-4':

Activation curves of AIP-4'. V_{max} is the initial rate of nitrocefin cleavage by the β -lactamase reporter strain, as measured by absorbance ϵ 490nm - ϵ 650nm of the hydrolyzed lactam. X-axis values are nanomolar. EC50 of the native AIP-4 Lit.13-20 nM⁶

Figure SI - 4 : Activation curve of AIP-4'- norbornene:



Activation curve of AIP-4'. V_{max} is the initial rate of nitrocefin cleavage by the β -lactamase reporter strain, as measured by absorbance ϵ 490nm - ϵ 650nm of the hydrolyzed lactam. X-axis values are nanomolar. Lactamase activity drops sharply at AIP-4' concentrations above 5 μ M.





QS activation curves for AIP-4' containing polymers 3a-c.

 Δ [O.D/min] - Vmax is the initial rate of nitrocefin cleavage by the β -lactamase reporter strain, as measured by absorbance ϵ 490nm - ϵ 650nm of the hydrolyzed lactam. Data are presented as mean \pm SD (n=3).







QS activation and inhibition curves for AIP-4' containing polymers 5a-c Δ [O.D/min] - Vmax is the initial rate of nitrocefin cleavage by the β -lactamase reporter strain, as measured by absorbance ϵ 490nm - ϵ 650nm of the hydrolyzed lactam. Data are presented as mean \pm SD (n=3).

3.3 Growth assays:

Cultures of *S. aureus* (OD₆₀₀: 0.15) were incubated for 90 min at 37 °C in the presence of polymers **3a-c** and AIP-4'. OD₆₀₀ values were measured before and after incubation. None of the studied polymers showed growth inhibitory effects at the highest concentrations used in the studies.







Figure SI - 8: Growth assays (polymers 5a-c)

3.4 Sheep RBC Hemolysis Assay:

Bacteria RN4282 (agr+, group 4, OD₆₀₀ nm of 0.1) were cultured at 37 °C with serially diluted sub-inhibitory concentrations of AIP-4', **5a** or **5b** to an OD₆₀₀ of 1.0. Bacterial samples were centrifuged (4,000 × g, 4 °C, 3 min), culture cell densities were measured and cells were centrifuged at 4000 RCF at 4 °C for 3 min. The culture supernatants were removed from the pellet, passed through a 0.2 μ m filter to remove any remaining bacteria. Hemolytic activity in the culture supernatant was determined using defibrinated sheep red blood cells (RBCs) that were obtained from Hy-labs (Park Tamar, Rehovot, Israel). RBCs were washed three times in sterile PBS, by centrifugation for 2 min at 4000 RCF at 4°C followed by resuspension, and then diluted to 2% (v/v) in sterile PBS. 200 μ L of 2% RBC solution was added to 100 μ l of culture supernatant and incubated for 15 min at 37°C with intermittent shaking. The remaining (non-lyzed) blood cells were removed by centrifugation (4,000 × g, room temperature, 3 min) and the hemolytic activity of the supernatant was determined by measuring the optical densities at 405 nm. The control culture supernatant served suspended in CYGP (0% hemolysis) and 1% Triton X-100 (100% hemolysis).





4. GPC- analysis

The samples were dissolved in GPC-grade THF (~10 mg in 1.0 mL) and analyzed by Wyatt triple detector GPC (Mini-Dawn-laser light-scattering, Viscostar-viscometer and Optilab-rEX-refractometer). The molecular weight was calculated using Zimm plot, assuming a dn/dc of 0.185ml/g – typical for PS in THF at 35°C. All samples contained some insoluble particles, which were filtered out after trying to dissolve them by heat and ultra-sound. Higher errors are observed for small polymers (10^2 g/mol) as a consequence of their very low light scattering.



Figure SI - 10: GPC analysis of polymer 2

RESULTS

Peak #1

Peak #1 Polydispersity Mw/Mn 1.136(3%) Mz/Mn 1.311(5%) Molar mass moments (g/mol) Mn 2.398e+4(2%) Mw 2.724e+4(2%) Mz 3.144e+4(4%) rms radius moments (nm) Rn 23.6(18%) Rw 22.0(18%) Rz 20.5(18%) Intrinsic viscosity moments (mL/g) 17.4(5%) ηn 17.5(5%) ηw 17.4(6%) ηz

ηw

ηz

88.6(0.6%) 97.8(0.7%)



Figure SI - 11: GPC analysis of polymer 4

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