Supporting Information for:

Chloride anion transporters inhibit growth of methicillin-resistant *Staphylococcus aureus* (MRSA) *in vitro*

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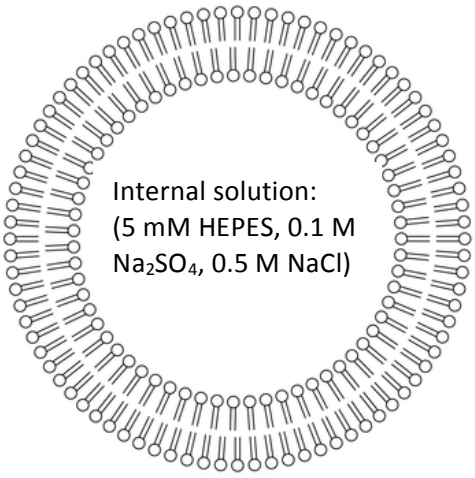
Transport Studies

Chloride concentrations were determined using a Accumet chloride combination ion selective electrode (ISE). The 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in chloroform (25 mg/mL) solution was ordered from Avanti Polar lipids and stored at –20°C.

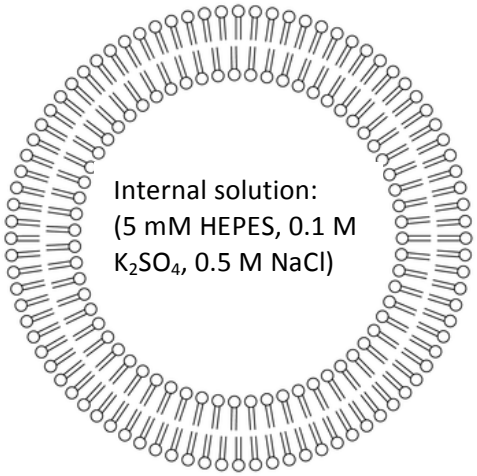
Preparation of NaCl filled POPC vesicles



The solvent of the POPC solution (25 mg / mL in $CHCl_3$) was removed under reduced pressure and then dried under a vacuum for 2 hours. 1 mL HEPES buffer solution (7.2 pH, 5 mM HEPES, 0.1 M Na_2SO_4, 0.5 M NaCl) was added to the POPC film then vortexed for 5 min and mechanically stirred for 30 min. The lipid suspension was subjected to 9 freeze and thaw cycles alternating between a dry ice acetone bath and a room temperature water bath. The solution was allowed to rest for 30 minutes. The solution was then extruded through a 200 nm polycarbonate membrane 39 times. The resulting solution was transferred to dialysis tubing (MWCO: 12,000 – 14,000) dialyzed overnight in distilled water (1 L). The

solution was then diluted to 1 mM POPC with a Na_2SO_4 and HEPES solution (5 mM HEPES, 0.1 M Na_2SO_4). Dynamic light scattering was used determine the size of the vesicles (160 ±10 nm).

Preparation of KCl filled POPC vesicles



External Solution:

(5 mM HEPES, 0.1 M K_2SO_4)

The solvent of the POPC solution (25 mg / mL in $CHCl_3$) was removed under reduced pressure and then dried under a vacuum for 2 hours. 1 mL HEPES buffer solution (7.2 pH, 5 mM HEPES, 0.1 M K_2SO_4, 0.5 M KCl) was added to the POPC film then vortexed for 5 min and mechanically stirred for 30 min. The lipid suspension was subjected to 9 freeze and thaw cycles alternating between a dry ice acetone bath and a room temperature water bath. The solution was allowed to rest for 30 minutes. The solution was then extruded through a 200 nm polycarbonate membrane 39 times. The resulting solution was transferred to dialysis tubing (MWCO: 12,000 – 14,000) dialyzed overnight in distilled water (1 L). The solution was then diluted to 1 mM POPC with a K_2SO_4 and HEPES solution (5 mM HEPES, 0.1 M K_2SO_4). Dynamic light scattering was used determine the size of the vesicles (160 ±10 nm).

Sodium chloride or potassium chloride transport assay: measuring chloride efflux

In a typical experiment, 4 mL of the 1 mM liposome solution was used. A chloride ion selective electrode (ISE) is used to measure chloride ion concentration in the solution external to the POPC vesicles. The ISE was placed into the solution, and readings were taken for 1-2 minutes to ensure a stable response. After the reading from the ISE stabilized, one of the compounds **2**-**15** (0.675% mol compound to lipid concentration) in 9 µL of dimethylformamide (DMF) was added to the liposome solution. The chloride concentration was monitored by the chloride ISE for 9 minutes, after which the liposomes were lysed with a 1:9 Triton X-114 : DMF mixture. The final reading of the ISE was used to calibrate 100% chloride efflux, and the initial reading of the ISE was set to 0% chloride efflux.

*Chloride/Nitrate transport assay (compound **5**)*

A POPC vesicle solution was prepared in the manner described above, except that the concentration of NaCl in the internal solution of the vesicles was 1.0 M. In the transport experiment, 4 mL of the 1 mM liposome solution was used. A chloride ISE was used to measure chloride ion concentration in the solution external to the POPC vesicles. The ISE was placed into the solution, and readings were taken for 2 minutes to ensure a stable response. After the reading from the ISE stabilized, a $NaNO_3$ solution (5 M) was added to the liposome solution so that the final concentration of $NaNO_3$ was 250 mM. Readings from the ISE were taken for 2 minutes to insure that there was no change in chloride concentration after the addition of $NaNO_3$. Compound **5** (0.675% mol compound to lipid concentration) in 9 μL of DMF was added to the liposome solution, and the chloride concentration was monitored with the chloride ISE for 9 minutes, after which the liposomes were lysed with a 9:1 DMF : Triton X-114 mixture. The final reading of the ISE was used to calibrate 100% chloride efflux, and the initial reading of the ISE was set to 0% chloride efflux.

*Chloride/Bicarbonate transport assay (compound **5**)*

A POPC vesicle solution was prepared in the manner described above, except that the concentration of NaCl in the internal solution of the vesicles was 1M. In the transport experiment, 4 mL of the 1 mM liposome solution was used. A chloride ISE was used to measure chloride ion concentration in the solution external to the POPC vesicles. The ISE was placed into the solution, and readings were taken for 2 minutes to ensure a stable response. After the reading from the ISE stabilized, a $NaHCO_3$ solution (1 M) was added to the liposome solution so that the final concentration of $NaHCO_3$ was 100 mM. Readings from the ISE were taken for 2 minutes to insure that there was no change in chloride concentration after the addition of $NaHCO_3$. Compound **5** (0.675% mol compound to lipid concentration) in 9 μL of DMF was added to the liposome solution, and the chloride concentration was monitored with the chloride ISE for 9 minutes, after which the liposomes were lysed with a 9:1 DMF : Triton X-114 mixture. The final reading of the ISE was used to calibrate 100% chloride efflux, and the initial reading of the ISE was set to 0% chloride efflux.

Transport assays with (thiocarlide, trichlorocarbanalide)

Sodium chloride or potassium chloride transport assay

A POPC vesicle solution was prepared in the manner described above, except that the internal solution of the vesicles is 0.5 M NaCl, 20 mM Phosphate buffer, and 0.1 M Na_2SO_4. A chloride ion selective electrode (ISE) is used to measure chloride ion concentration in the solution external to the POPC vesicles. The ISE was placed into the solution, and readings were taken for 1-2 minutes to ensure a stable response. After the reading from the ISE stabilized, one of the compounds thiocarlide or trichlorocarbanalide (0.675% mol compound to lipid concentration) in 9 μL of dimethyl sulfoxide (DMSO) was added to the liposome solution. The chloride concentration was monitored by the chloride ISE for 9 minutes, after which the liposomes were lysed with a 1:9 Triton X-114 : DMF mixture. The final reading

of the ISE was used to calibrate 100% chloride efflux, and the initial reading of the ISE was set to 0% chloride efflux.

Chloride/nitrate transport assay

A POPC vesicle solution was prepared in the manner described above, except that the internal solution of the vesicles is 0.5 M NaCl, 20 mM Phosphate buffer, and 0.1 M Na_2SO_4. In the transport experiment, 4 mL of the 1 mM liposome solution was used. A chloride ISE was used to measure chloride ion concentration in the solution external to the POPC vesicles. The ISE was placed into the solution, and readings were taken for 2 minutes to ensure a stable response. After the reading from the ISE stabilized, a $NaNO_3$ solution (5 M) was added to the liposome solution so that the final concentration of $NaNO_3$ was 250 mM. Readings from the ISE were taken for 2 minutes to insure that there was no change in chloride concentration after the addition of $NaNO_3$. Compound **5** (0.675% mol compound to lipid concentration) in 9 µL of DMSO was added to the liposome solution, and the chloride concentration was monitored with the chloride ISE for 9 minutes, after which the liposomes were lysed with a 9:1 DMF : Triton X-114 mixture. The final reading of the ISE was used to calibrate 100% chloride efflux, and the initial reading of the ISE was set to 0% chloride efflux.

Potassium Transport Studies

The efflux of chloride was measured when compounds **2**-**15** were added to KCl filled liposomes (Fig. S1). There was a significant difference between the rate of chloride efflux between the KCl and NaCl filled liposomes (Fig. S2). The difference in transport rates indicates that the counter cation plays a role in anion transport. We suspect that in the case with KCl filled liposomes, we are seeing a mixture of KCl and HCl transport across the liposome membrane.

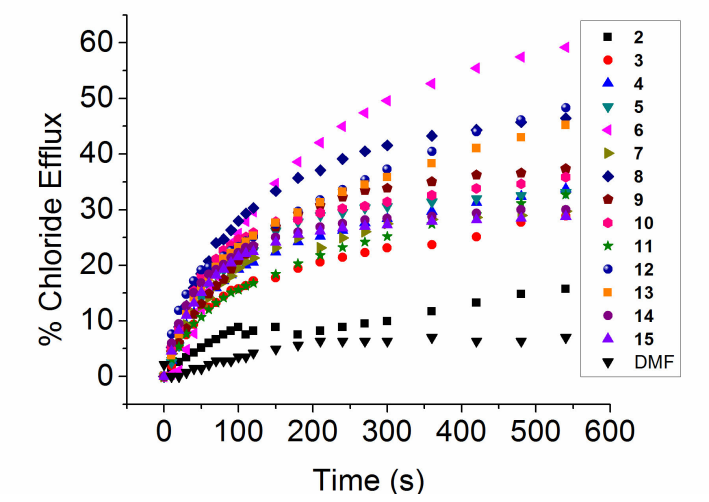


Fig. S1. Chloride efflux was initiated by the addition of **2**-**15** (0.675 mol%) to a solution of POPC vesicles (1 mM POPC, internal solution: 0.5 M KCl, 0.1 M Na_2SO_4, 5 mM HEPES, pH = 7.2, external solution: 0.1 M Na_2SO_4, 5 mM HEPES pH = 7.2)

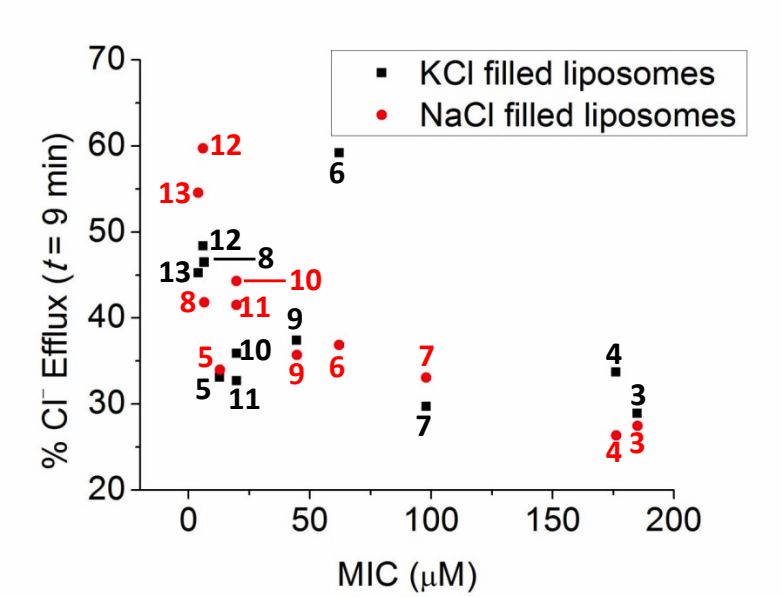


Fig. S2 Plot of chloride efflux promoted by **2**-**13** in liposomal transport models against their effectiveness in reducing the growth of the Mu50 (resistant) strain of *S. aureus*. The black points are from the studies with the KCl filled liposomes (internal solution: 0.5 M KCl, 0.1 M Na_2SO_4, 5 mM HEPES, pH = 7.2), and the red points are from studies with the NaCl filled liposomes (internal solution: 0.5 M KCl, 0.1 M Na_2SO_4, 5 mM HEPES, pH = 7.2).

Bacterial Studies

Bacterial Assay – Broth dilution method

The bacterial strains used for the assays were *S. aureus* UAMS1 (Table S1), *S. aureus* HP1173 (Table S2), *S. aureus* Mu50 (Table S3), *P. aeruginosa* PA01 (Table S4), and *P. aeruginosa* PA14 (Table S5). MICs were determined by the broth microdilution method recommended by the Clinical and Laboratory Standards Institute[1] in brain-heart infusion broth (BHI). The MIC was defined as the lowest concentration of an antibacterial agent that inhibited visible growth after incubation

In general, bacteria from a freezer stock were plated on BHI agar plates. The bacteria were grown overnight in an incubator at 37 °C. Three separate colonies were transferred into BHI media, and were grown overnight in an incubator (37°C, 250 rpm), and then diluted to an optical density at 600 nm (OD_{600}) of 0.05 in BHI media the next day. The bacteria diluent was incubated for 1-2 h to an OD_{600} between 0.2 to 0.8. The bacteria were diluted in BHI to OD_{600} of 0.0001 (approximately 10^5 cells / mL). The bacterial solution (99 μL / well, approximately 10^4 cell / well) was transferred to 96 well plates (Sarstedt, 82.1581.001, also called "assay plate").

Serial dilution of testing compounds were prepared on 96well microtiter plate (Nunc #297245, called as "dilution plate") using a multichannel pipet. In detail, 50 μL of 100 % DMSO was first filled into columns 2 through 12. Next, 100 μL of compounds dissolved in 100 % DMSO at 10 mg/μL were plated on column 1 and then 50 μL was diluted to next column containing 50 μL of 100 % DMSO to make 2 fold dilution.

Sequential 2 fold dilution was continued until column 11. Column 12 was filled with 100 μL of 100 % DMSO as a negative control (Fig. S3). Wells on column 12 with only DMSO will serve as a negative control, while wells with piperacillin will serve as a positive control. Compounds (**15** and **16**) were diluted in 50 % DMSO in aqueous solution due to solubility issues. Addition of 1 μL of compound dilution series from a dilution plate to an assay plate in which 99 μL of bacterial solution were plated was conducted using a Janus automated workstation (Perkin Elmer, Waltham, MA). This resulted in 100 fold dilutions of compounds with final concentration of 1% DMSO in the assay mixture. Three replicated assay plates per compound dilution were prepared.

After addition of the compounds, the assay plates were incubated overnight (16 hours) at 37°C. Compound's activity was first determined by the turbidity of wells when they were inspected visually next day. Cloudy wells indicated bacterial growth, and clear wells indicated bacterial inhibition. The most active compounds **5**, **8**, **10**, **12, 16**, **17**, and **18** were further validated.

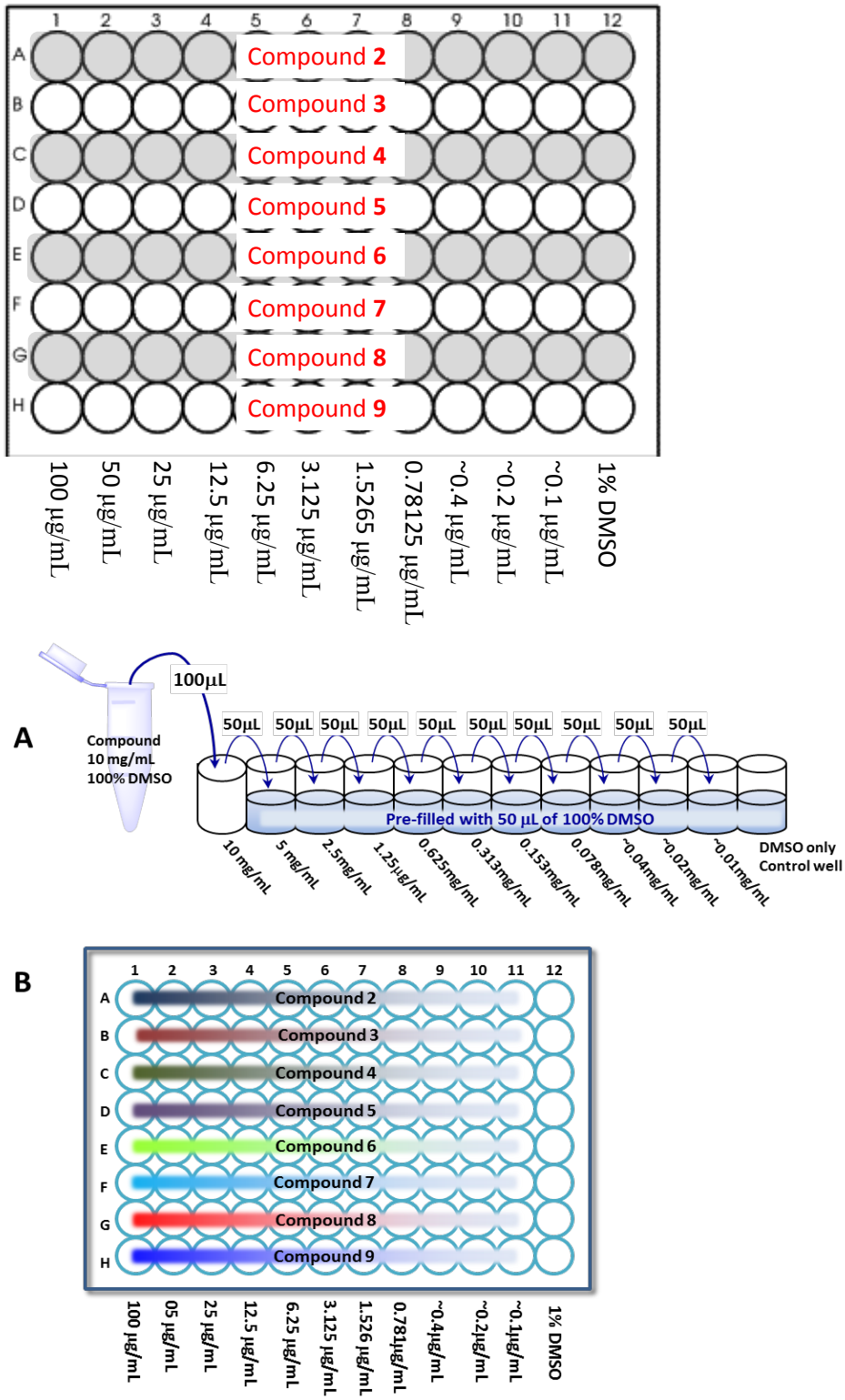


Fig. S3 Schematic illustration of compound dilution procedure (A) and layout of a typical 96 well plate (B) used in the bacterial assay

Compound	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	Average
2	none	none	none								none
3	50	50	50								50
4	100	100	50								83.3
5	12.5	6.25	12.5					12.5	12.5	12.5	11.46
6	25	25	50								33.3
7	50	50	50								50
8	1.53	3.13	3.13					3.13	3.13	3.13	1.86
9	25	25	25								25
10	12.5	12.5	12.5					12.5	12.5	12.5	12.5
11	12.5	12.5	50								25
12	6.25	6.25	6.25					6.25	6.25	6.25	6.25
13	6.25	6.25	25								12.5
14	none	none	none								none
15	none	none	none								none
16	1.53	1.53	1.53	0.39	0.39	0.39	1.53	1.53	1.53	1.53	1.188
17	1.53	0.78	1.53	1.53	1.53	0.78	0.78	0.78	0.78	1.53	1.155
18	3.13	3.13	3.13	1.53	1.53	1.53	3.13	3.13	3.13	3.13	2.65
DMSO	none	none	none	none	none	none	none	none	none	none	none
Piperacillin				6.25	6.25	12.5	12.5	12.5	12.5	12.5	10.71

Table S1. MIC (μg/mL) results from each individual antibacterial assay against *S. aureus* UAMS1. The term none indicates that no detectible bacterial inhibition was observed. The blank spaces indicate that no trials were performed. The terms "trial" and "none" refer to an independent experiment and an MIC value >100 μg/mL.

Compound	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	Average
2	none	none	none								none
3	50	50	50								50
4	50	50	100								66.67
5	12.5	12.5	6.25					12.5	12.5	12.5	11.45
6	25	25	25								25
7	50	50	50								50
8	3.13	3.13	3.13					3.13	3.13	3.13	3.13
9	50	50	50								50
10	12.5	12.5	12.5					25	25	12.5	16.67
11	12.5	12.5	12.5								12.5
12	6.25	6.25	6.25					12.5	12.5	12.5	9.375
13	6.25	6.25	6.25								6.25
14	none	none	none								none
15	none	none	none								none
16	1.53	1.53	3.13	0.39	0.78	0.78	0.78	1.53	1.53	3.13	1.51
17	1.53	0.78	0.78	0.39	0.39	0.39	0.39	1.53	1.53	0.78	0.85
18	3.13	3.13	3.13	1.53	1.53	1.53	1.53	3.13	3.13	3.13	2.49
DMSO	none	none	none	none	none	none	none	none	none	none	none
Piperacillin				none	none	none	none	100	none	none	>100

Table S2. MIC (μg/mL) results from each individual antibacterial assay against *S. aureus* HP1173. The term none indicates that no detectible bacterial inhibition was observed. The blank spaces indicate that no trials were performed.

Compound	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	Average
2	none	none	none								none
3	50	50	50								50
4	50	50	100								66.67
5	6.25	6.25	6.25					6.25	6.25	6.25	6.25
6	25	25	25								25
7	50	50	50								50
8	3.13	3.13	3.13					6.25	6.25	6.25	4.69
9	25	25	25								25
10	12.5	12.5	12.5					12.5	12.5	12.5	12.5
11	12.5	12.5	12.5								12.5
12	3.13	3.13	3.13					6.25	6.25	6.25	4.69
13	3.13	3.13	3.13								3.13
14	none	none	none								none
15	none	none	none								none
16	1.53	1.53	1.53	0.78	0.78	0.78	0.78	1.53	1.53	1.53	1.23
17	0.78	0.78	0.78	1.53	0.78	0.78	1.53	0.78	0.78	0.78	0.93
18	1.53	1.53	1.53	3.13	1.53	1.53	0.78	1.53	3.13	1.53	1.78
DMSO	none	none	none	none	none	none	none	none	none	none	none
Piperacillin				25	25	100	100	50	50	50	57.1

Table S3. MIC (μg/mL) results from each individual antibacterial assay against *S. aureus* Mu50. The term none indicates that no detectible bacterial inhibition was observed. The blank spaces indicate that no trials were performed.

Compound	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Average
2	none	none	none	none	none	none				none
3	none	none	none	none	none	none				none
4	none	none	none	none	none	none				none
5	none	none	none	100	25	12.5	none	none	none	>45.8
6	none	100	100	none	none	none				none
7	none	none	none	none	none	none				none
8	25	25	25	50	50	25	25	25	25	30.56
9	none	none	none	100	none	100				none
10	none	none	none	none	none	none	none	none	none	none
11	none	none	none	none	none	none				none
12	none	none	none	none	none	none	none	none	none	none
13	none	none	none	none	none	none				none
14	none	none	none	none	none	none				none
15	none	none	none	none	none	none				none
16	none	none	none	none	none	none	none	none	none	none
17	none	none	none	none	none	none	none	none	none	none
18	none	none	none	none	none	none	none	none	none	none
DMSO	none	none	none	none	none	none	none	none	none	none

Piperacillin			1.53	1.53	1.53	1.53	1.53	0.78	1.41

Table S4. MIC (μg/mL) results from each individual antibacterial assay against *P. aeruginosa* PA01. The term none indicates that no detectible bacterial inhibition was observed. The blank spaces indicate that no trials were performed.

Compound	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Average
2	none	none	none	none	none	none				none?
3	100	100	100	50	none	none				>87.5
4	none	100	none	25	none	none				>62.5
5	25	25	25	12.5	6.25	12.5	25	25	25	20.14
6	none	none	none	none	none	none				none
7	none	none	none	none	none	none				none
8	none	none	none	none	none	none	100	100	50	>83.3
9	none	none	none	100	100	100				>100
10	none	none	none	none	none	none	none	none	none	none
11	none	none	none	none	none	none				none
12	none	none	none	none	none	none	none	none	none	none
13	none	none	none	none	none	none				none
14	none	none	none	none	none	none				none
15	none	none	none	none	none	none				none
16	none	none	none	none	none	none	none	none	none	none
17	none	none	none	none	none	none	none	none	none	none
18	none	none	none	none	none	none	none	none	none	none
DMSO	none	none	none	none	none	none	none	none	none	none
Piperacillin				6.25	3.13	6.25	3.13	3.13	3.13	4.17

Table S5. MIC (μg/mL) results from each individual antibacterial assay against *P. aeruginosa* PA14. The term none indicates that no detectible bacterial inhibition was observed. The blank spaces indicate that no trials were performed.

1 S. B. Singh, J. D. Polishook, D. L. Zink, O. Genilloud, M. A. Goetz, and F. Vicente, U.S. patent application WO2011/079034A1