Small Molecule Potentiation of Gram-positive Selective Antibiotics Against *Acinetobacter baumannii*

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Bacterial strains, media, antibiotics and reagents

Acinetobacter baumannii clinical isolate 5075 was obtained from Dr. Colin Manoil at The University of Washington. Colonies were grown on solid LB agar. Cation-adjusted Mueller-Hinton Broth (CAMHB) (catalog number 212322) and Mueller-Hinton Broth (MHB) (catalog number 211443) were purchased from BD Diagnostics. LB broth was purchased from Fisher Scientific (catalog number BP9722-2). Vancomycin (catalog number V2002) was purchased from Sigma-Aldrich. Erythromycin (catalog number 45673) was purchased from Fluka. Clarithromycin (catalog number C2220) and azithromycin (catalog number A2076) were purchased from TCI. Colistin sulfate salt (catalog number C4461) was purchased from Sigma. Baclight Bacterial Viability Kit (catalog number L7012) was purchased from Invitrogen. Carbonyl cyanide m-chlorophenyl hydrazone (catalog number C2759) was purchased from Sigma. BisBenzimide H33342 trihydrochloride (H33342) (catalog number B2261) was purchased from Sigma. Pentamidine isethionate salt (catalog number P0547) was purchased from Sigma. All assays were completed in duplicate and were repeated at least two separate times. Synthesis of library compounds has been previously reported [1-10] and compounds were dissolved as their HCl salts in molecular biology grade DMSO as 100 mM stock solutions and stored at -20°C. Vancomycin was dissolved in sterile water while erythromycin, clarithromycin, and azithromycin were dissolved in molecular biology grade DMSO.

Broth microdilution method for MIC determination

Duplicate cultures (6 h) in CAMHB were subcultured to 5 x 10^5 CFU/mL in CAMHB. Aliquots (1 mL) were placed in culture tubes, and compound was added from 100 mM stock samples in DMSO, such that the compound concentration equaled the highest concentration tested (200 μM). Samples were then aliquoted (200 μL) into the first row of wells of a 96-well plate, with all remaining wells being filled with 100 μL of initial bacterial subculture. Row one wells were mixed five times before 100 μL was transferred to the following row (row two). Row two was then mixed five times, and 100 μL was transferred to row three. This process provided a serial dilution of the compound and was continued until the final row had been mixed. Plates were covered with Glad Press n' Seal and were incubated under stationary conditions at 37 °C for 16 hours. After 16 hours, plates were examined visually recording the lowest dilution point with no visual growth as the compounds MIC.

Broth microdilution method for antibiotic resensitization

Duplicate cultures (6 h) in CAMHB were subcultured to 5 x 10^5 CFU/mL in CAMHB. Aliquots (4 mL) were placed in culture tubes, and compound was added from 100 mM stock samples in DMSO. One milliliter of the resulting solution was aliquoted into a separate culture tube and was dosed with antibiotic so that the resulting concentration was the highest concentration to be tested. Bacteria treated with antibiotic alone were used as a control. Row one of a 96-well plate was filled with 200 μL of the antibiotic/ compound solution, and the remaining rows were filled with 100 μL per well of the remaining 4 mL bacterial subculture containing compound. The wells in row one were

mixed five times before 100 µL was transferred to the following row (row two). Row two was then mixed five times, and 100 µL was transferred to row three. This process was repeated until the second to last row had been reached. The last row would have only compound and serve as a negative control. The antibiotic only treated bacteria was plated by aliquoting 200 µL of the treated bacteria into row one and filling the remaining rows with untreated bacteria from the original subculture. The rows were mixed in the same way as described above. Plates were covered with Glad Press n' Seal and were incubated under stationary conditions at 37 °C for 16 hours. After 16 hours, plates were examined visually recording the lowest dilution point with no visual growth as the MIC. Fold reductions were determined by comparison of the compound treated wells with the antibiotic only control well.

Biolog

A cell suspension of AB5075 was grown to 85% turbidity and was diluted 200-fold into a solution of 1X IF-10, 1X Dye mix A and 20 µM compound **1** in DMSO or DMSO alone as a control. Biolog plates were inoculated with these suspensions and grown and tetrazolium reduction kinetics assayed according to standard procedures on a Biolog reader.

Time kill curves

AB5075 was grown overnight in CAMHB, and this culture was used to inoculate fresh CAMHB to 5 x 10^5 CFU/mL. Inoculated medium was aliquoted (3 mL) into culture tubes, and compound was added, with untreated inoculated medium serving as the control. Tubes were incubated at 37 °C with shaking. Samples were taken at 2,4,6,8, and 24 hour time points, serially diluting in fresh CAMHB, and plated on LB agar. Plates were incubated at 37 °C overnight in stationary conditions, and the number of colonies were counted. CFU/mL were calculated and graphed.

Galleria mellonella screening

Galleria mellonella larvae (Speedy Worm, Alexandria, MN) were used within 10 days of shipment from the vendor. After reception of worms, larvae were kept in the dark at room temperature for at least 24 h before infection. Larvae weighing between 200 to 300 mg were used in the survival assay. Using a 10 µL glass syringe (Hamilton, Reno, NV) fitted with a 30 G needle (Exel International, St. Petersburg, Fl), a 5 μL of the desired compound and concentration were injected into the last left proleg. For bacterial injections, 50 μL from an overnight culture of AB5075 in Miller LB broth (Fisher Scientific, U.S.) was subcultured into 5 mL Miller LB broth and incubated for an additional 3 h before use. Then, after 2.5 h from the first injection, a second 5 μL injection containing 6 x 10^5 CFU of AB5075 was injected into the second to last left proleg. Injected worms were left at room temperature in the dark while being assessed at 24 h intervals over six days. Larvae were considered dead if they did not respond to physical stimuli. Experiment was repeated 7 times using 10 larvae per experimental group.

Efflux assay with fluorescence read immediately after treatment

AB5075 was cultured 16 hours at 37°C shaking before being subcultured 1:40 in CAMHB. Cultures were then returned to the incubator at 37 °C with shaking for 5 hours. After 5 hours, cultures were centrifuged at 4,000 rpm for 20 minutes and the supernatant was removed. Cell pellets were suspended in 2 mL 1X PBS and subcultured to OD_{600}=0.5 in 1X PBS. Aliquots (1800 μL) were placed into small culture tubes and tubes were dosed appropriately. Compound **1** was added from 100 mM stocks so that the concentration was 30 or 10 μM and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was added so that the concentration was 25 μM within the appropriate tubes. H33342 was added to all tubes so that the concentration was 2.5 μM. 200 μL from the small tubes was pipetted into each well within a column on a 96 well black microtiter plate. Absorbances were read every 10 minutes for 30 minutes at 355 and 460 nm (BioTek Synergy HTX).

Efflux assay with fluorescence read over time

AB5075 was cultured 16 hours at 37 °C with shaking before being subcultured 1:40 in CAMHB. Cultures were divided into 8 mL aliquots and were either treated with compound **1** so that the concentration was 30 μM or left untreated before being returned to the incubator at 37 °C shaking for 5 hours. After 5 hours, 100 μL was removed from each sample, diluted and plated so that CFU/mL could be determined. Cultures were then centrifuged at 4,000 rpm for 20 minutes and the supernatant was removed. Cell pellets were suspended in 2 mL 1X PBS and subcultured to OD_{600}=0.5 in 1X PBS. Aliquots (1800 μL) were placed into small culture tubes and tubes were dosed with H33342 so that the concentration was 2.5 μM. 200 μL from the small tubes was pipetted into each well within a column on a 96 well black microtiter plate. Absorbances were read every 10 minutes for 30 minutes at 355 and 460 nm (BioTek Synergy HTX).

Baclight Assay

The Baclight assay (Invitrogen) was used to determine membrane permeability. AB5075 was grown overnight in CAMHB at 37 °C with shaking. The culture was diluted 1:40 in fresh CAMHB and incubated at 37 °C with shaking for 4 hours. These cultures were centrifuged at 4,000 rpm for 20 minutes at 7 °C and the cell pellet was then washed with 1 mL of sterile water. The cell pellets were then resuspended to 1/10 of the original volume and diluted 1:20 in sterile water or water containing tested compounds at desired concentrations. Suspensions were incubated at 37 °C with shaking for 1 hour and were then centrifuged at 10,000 rpm for 15 minutes. The pellets were washed with sterile water and resuspended in water. A 1:1 mixture of SYTO-9 and propidium iodide was added to each suspension so that the final concentration of each 3 μg/mL. 100 μL of the suspension was added to each well of a 96-well plate and the plate was incubated at room temperature in the dark for 15 minutes. Green fluorescence (SYTO-9) was read at 530nm and red fluorescence (propidium iodide) was read at 645nm. Both fluorescences have an excitation of 485nm (BioTek Synergy HTX). The ratio of green to red fluorescence was expressed as a percentage of the control to determine relative membrane permeability.

Checkerboard assay

CAMHB was inoculated with *A. baumannii* (5 x 10^5 CFU/mL) and 100 mL aliquots were distributed to all wells of a 96-well plate except for well 1a. Inoculated CAMHB (200 mL) containing a selected compound (at a concentration for 2x the highest concentration being tested) was added to well 1a, and 100 mL of the same sample was added to wells 2a-12a. Column A cells were mixed 6-8 times, and then 100 mL was withdrawn and transferred to column B. This process was repeated up to column G (column H was not mixed to determine the MIC of the antibiotic alone). Inoculated media (100 mL) containing antibiotic at 2x the highest concentration being tested was placed in wells A1-H1 and serially diluted, all the way until row 11 (row 12 was not mixed to determine the MIC of the compound alone). The plates were covered and sealed with Glad Press'n Seal, and incubated under stationary conditions at 37 °C for 16 h. After 16 h the MIC values of both compound and antibiotic were recorded, as well as combination. The ΣFIC values were calculated as follows: ΣFIC = FIC (compound) + FIC (antibiotic), where FIC (compound) is the MIC of the compound in the combination/MIC of the compound alone and FIC (antibiotic) is the MIC of the antibiotic in the combination/MIC of the antibiotic. The combination is considered synergistic when the ΣFIC is ≤0.5, indifferent when the ΣFIC is between 0.5 and 2, and antagonistic when the ΣFIC is ≥2.

LPX mutant generation

LPX deficient strains were generated following the procedure published by Moffatt.[11] In brief, Mueller Hinton II agar plates were made containing 10 μg/mL colistin. AB5075 was cultured for eight hours before 125 μL from the culture was pipetted onto the agar plate containing colistin. Glass beads were used to spread the bacteria. The plates were incubated for 48 hours at 37 °C. After 48 hours, a colony was cultured in CAMHB containing 10 μg/mL colistin and assays were performed.

Mass spectrometry analysis of lipid A

AB5075 cultures (6h) were subcultured to 5 x 10^5 CFU/mL in eight 25 mL cultures of CAMHB. Four of these cultures were treated with the appropriate compound so that the concentration was 30 μM. After treatment the samples were returned to a 37 °C shaker for 16 hours. After 16 hours, the samples were centrifuged at 0 °C and 4000 x *g* for 20 minutes. The supernatant was then removed and samples were stored at -80 °C. Lipid A was extracted from cell pellets using an ammonium hydroxide-isobutyric acid-based procedure as previously described [12]. Briefly, cell pellets were resuspended in 400 μl of 1M ammonium hydroxide and 70% isobutyric acid (5:3 [vol:vol]). Samples were incubated at 100 °C for 1 hour and subsequently centrifuged at 8,000 x *g* for 10 minutes. Supernatants were transferred to endotoxin-free water (1:1 [vol:vol]), snap-frozen on dry ice, and lyophilized overnight. The resultant dried material was washed twice with 1 ml methanol. Lipid A was extracted in 100 μl of a mixture of chloroform, methanol, and water (3:1:0.25 [vol/vol/vol]). 1 μl of the extraction was spotted on a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) stainless steel plate followed by 1 μl

of 10 mg/ml norharmane matrix in chloroform-methanol (2:1 [vol/vol]) (Sigma-Aldrich, St. Louis, MO). All samples were analyzed on a Bruker Microflex mass spectrometer (Bruker Daltonics, Billerica, MA) in the negative-ion mode with reflectron mode. Mass calibration was acheived using an electrospray tuning mix (Agilent, Palo Alto, CA). All spectral data were analyzed with Bruker Daltonics FlexAnalysis software. The resulting spectra were used to estimate the lipid A structures present in each strain based on their predicted structures and molecular weights. Structural diversity of lipid A within a single bacterial membrane is well-described [13; 14].

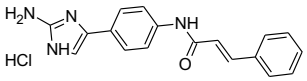
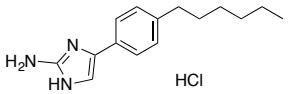
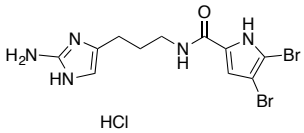
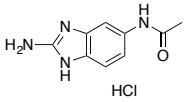
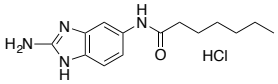
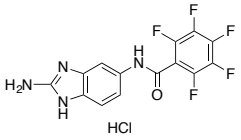
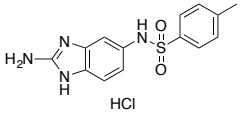
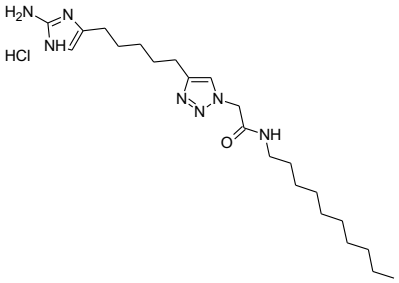
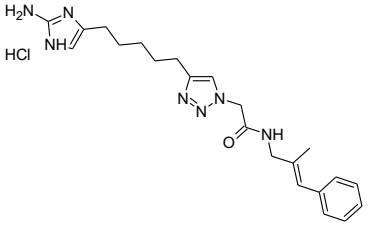
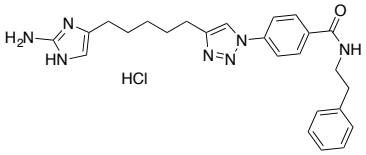
Lipid A analysis by gel electrophoresis

Cultures (16 h) were subcultured to 5 x 10^5 CFU/mL in two 100 mL cultures of CAMHB. These subcultures were further separated into four 10 mL untreated and treated samples that would be incubated for four hours and four 10 mL untreated and treated samples that would be incubated for 16 hours. All treated samples were treated with the compound of interest so that the resulting concentration was 30 μM. After treatment the samples were returned to a 37 °C shaker for their desired treatment time. After the designated treatment time, all eight samples were centrifuged at 4,000 rpm for 20 minutes. Samples were then extracted by the hot phenol method 15 and both the organic fraction and the aqueous fractions were dialyzed and combined prior to lyophilization. Samples were then prepared in Novex LDS sample buffer to 10 μg/mL. Each experimental lane of the Tricine gel was loaded with 20 μg of material and then gel was run using a 10-20% gradient using discontinuous buffering. The 20 μg load was based on a dry weight of lyophilized, extracted product from the hot phenol method. Preparation contained *both* the organic and aqueous fractions.[16] Samples were reconstituted in Novex LDS sample buffer (Thermo) to 10μg/μL and lanes loaded with 20μg. The gel was stained according to Tsai and Frasch silver stain method 17.

Table S.1- Antibiotic resistance profile of AB5075.

Antibiotic	**Class**	**MIC (μg/mL)**
Meropenem		32
Pipericillin		>1024
Ampicillin	β-Lactam	>1024
Ceftazidime		>1024
Cefotaxime		>1024
Ciprofloxacin	Fluoroquinolone	64
Streptomycin		>1024
Gentamicin		>1024
Kanamycin		>1024
Tobramycin	Aminoglycoside	256
Amikacin		256
Neomycin		128
Rifampicin	Rifamycin	2
Tigecycline	Glycylcycline	1
Erythromycin		32
Azithromycin	Macrolide	64
Clarithromycin		32
Linezolid	Oxazolidinone	256
Clindamycin	Lincosamide	128

Table S.2- Screen of a diverse selection of nitrogen dense marine alkaloid scaffolds for potentiation of erythromycin

Compound	Structure	MIC (μM)	Concentration tested (μM)	Erythromycin MIC (μg/mL)
-	-	-	-	32
S.1		100	30	4
S.2		100	30	16
S.3		>200	60	32
S.4		>200	60	32
S.5		>200	60	16
S.6		>200	60	16
S.7		>200	60	32
S.8		>200	60	8
S.9		>200	60	32
S.10		>200	60	32

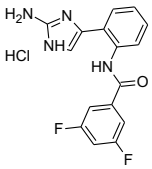
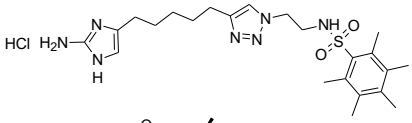
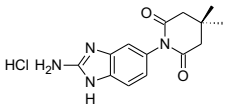
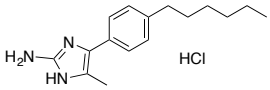
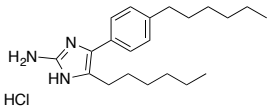
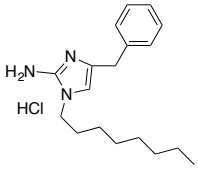
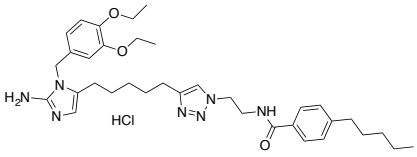
S.11		>200	60	32
S.12		>200	60	32
S.13		>200	60	32
S.14		>200	60	32
S.15		100	30	16
S.16		>200	60	32
S.17		>200	60	32
S.18		100	30	32

Table S.3- Activity of lead compounds at 30 μM with a range of macrolide antibiotics against AB5075.

Compound	**Troleandomycin MIC (μg/mL)**	**Josamycin MIC (μg/mL)**	**Spiramycin MIC (μg/mL)**	**Oleandomycin MIC (μg/mL)**
	>512	128	>512	256
1	32	8	128	64
2	16	4	64	128

Table S.4- Screening of two lead adjuvants at 30 μM with range of aminoglycoside antibiotics against AB5075.

	Gentamicin MIC (μg/mL)	**Kanamycin MIC (μg/mL)**	**Tobramycin MIC (μg/mL)**	**Amikacin MIC (μg/mL)**	**Neomycin MIC (μg/mL)**	**Streptomycin MIC (μg/mL)**
-	>512	>512	256	128	64	>512
1	>512	>512	64	128	16	256
2	>512	>512	64	128	64	512

Table S.5- Screen for potentiation of clarithromycin and vancomycin using compounds **1** and **2**[a] against *A. baumannii* isolates.

***A. baumannii* strain**	**Clarithromycin MIC (µg/mL)**			**Vancomycin MIC (µg/mL)**		
	-	**With compound 1**	**With compound 2**	**-**	**With compound 1**	**With compound 2**
3560	32	0.5	0.5	256	0.5	32
3785	64	0.5	0.5	512	1	128
3806	16	0.25	≤0.125	256	2	32
3927	32	0.5	0.5	256	8	64
4025	64	0.25	0.25	512	2	16
4026	64	0.5	1	512	2	16
4027	64	≤0.25	0.25	512	1	32
4052	32	≤0.25	≤0.25	>512	4	64
4269	32	≤0.25	≤0.25	512	2	64
4448	32	≤0.25	≤0.25	512	1	4
4456	32	0.5	≤0.25	512	16	64
4490	32	0.5	≤0.25	256	1	8
4498	64	≤0.25	≤0.25	512	1	16
4795	32	0.5	0.5	>512	4	4
4857	64	0.5	0.5	-	Toxic	Toxic
4878	32	0.5	1	512	2	1
4957	32	0.5	0.5	512	8	8
4991	32	0.5	0.5	512	32	32
5001	32	0.5	0.5	512	2	1
5197	64	0.5	0.5	512	16	8
5256	32	0.25	0.25	512	2	8
5711	64	0.5	0.5	>512	8	64
8967	16	0.25	0.25	512	4	8

[a]- Both compounds tested at 30 µM for all strains.

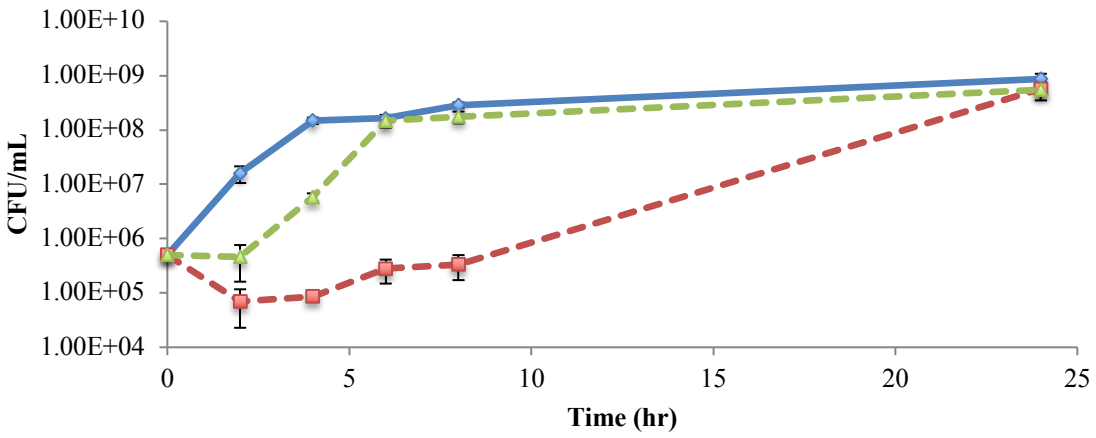


Figure S.1- Growth and survival of AB5075 in the presence of compound **1**. The solid line indicates the growth in the absence of compound **1**, and the dashed line indicates the growth in presence of compound **1**. Blue, control; green, 20 µM compound **1**; red, 30 µM compound **1**.

Table S.6- Repotentiation and Baclight data for inactive adjuvant **3** (fold reductions in parenthesis).

MIC (µM)	Conc. Tested (µM)	Vancomycin MIC (µg/mL)	Erythromycin MIC (µg/mL)	Clarithromycin MIC (µg/mL)	Azithromycin MIC (µg/mL)	Membrane Disruption (%)
>200	60	256 (0)	32 (0)	16 (2)	32 (2)	72.9
	30	256 (0)	32 (0)	32 (0)	64 (0)	60.0
	20	256 (0)	32 (0)	32 (0)	64 (0)	54.0
	10	256 (0)	32 (0)	32 (0)	64 (0)	53.7

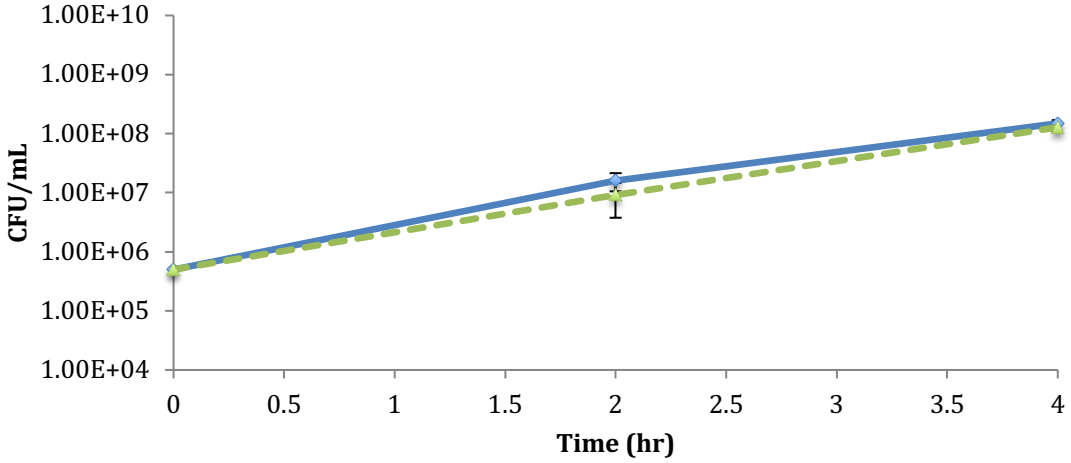


Figure S.2- Growth and survival of AB5075 in the presence of compound **3**. The solid blue line indicates the growth in the absence of compound **3**, and the dashed line indicates the growth in presence of compound **3** at 60µM.

Concentration of compound **1** (μM)									
Colistin MIC (μg/mL)		200	100	50	25	12.5	6.25	3.125	Colistin MIC (μg/mL)
	64								
	32								
	16								
	8								
	4								
	2								
	1								
	0.5								
	0.25								
	0.125								
	0.0625								
	Compound **1** MIC (μM)								

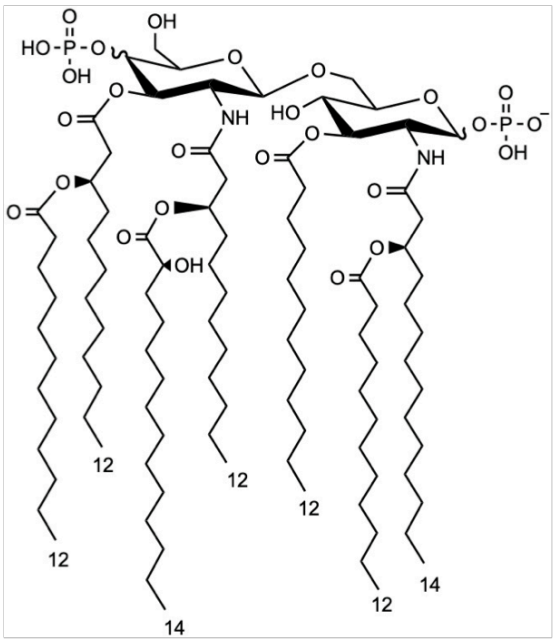
Figure S.3- Heat map from the checkerboard assay used to determine the FIC values from the relationship between colistin and compound **1**. Red cells indicate no bacterial growth while green cells indicate bacterial growth.

Concentration of compound **2** (μM)									
Colistin MIC (μg/mL)		200	100	50	25	12.5	6.25	3.125	Colistin MIC (μg/mL)
	64								
	32								
	16								
	8								
	4								
	2								
	1								
	0.5								
	0.25								
	0.125								
	0.0625								
	Compound **2** MIC (μM)								

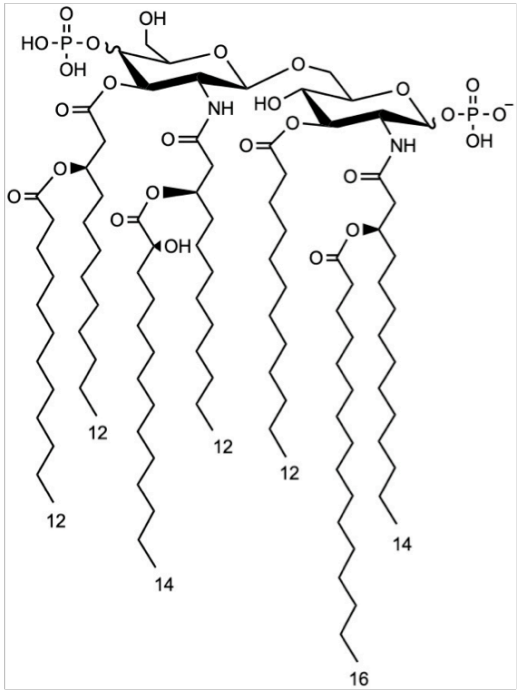
Figure S.4- Heat map from the checkerboard assay used to determine the FIC values from the relationship between colistin and compound **2**. Red cells indicate no bacterial growth while green cells indicate bacterial growth.

Table S.7- Potentiation of vancomycin and the macrolides with compound **1** against LPS deficient AB5075.

	Concentration tested (µM)	**Vancomycin MIC (µg/mL)**	**Erythromycin MIC (µg/mL)**	**Clarithromycin MIC (µg/mL)**	**Azithromycin MIC (µg/mL)**
		0.0625	0.03125	0.0039	0.0625
1	20	0.0625	0.03125	0.0039	0.0625



WT lipid A



Palmitoylated lipid A

Figure S.5-Lipid A structures for wild type and palmitoylated *A. baumannii.*

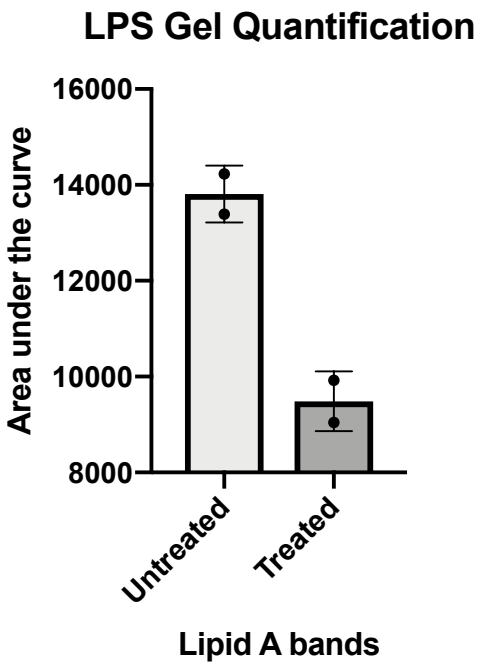


Figure S.6-Quantification of lipid A bands from treated (compound **1**) and untreated samples using ImageJ software. Lipid A bands from treated and untreated lanes were selected and the area under the curve was analyzed. The graph is a result of both replicates. [Note – there is a significant difference between treated and untreated; P=0.0192;

References

1. Bunders, C. A., Richards, J. J., & Melander, C. (2010). Identification of aryl 2-aminoimidazoles as biofilm inhibitors in Gram-negative bacteria. *Bioorg Med Chem Lett, 20*(12), 3797-3800. doi:S0960-894X(10)00512-3 [pii] 10.1016/j.bmcl.2010.04.042
2. Worthington, R. J., Bunders, C. A., Reed, C. S., & Melander, C. (2012). Small molecule suppression of carbapenem resistance in NDM-1 producing Klebsiella pneumoniae. *ACS Med Chem Lett, 3*(5), 357-361. doi:10.1021/ml200290p
3. Brackett, C. M., Melander, R. J., An, I. H., Krishnamurthy, A., Thompson, R. J., Cavanagh, J., & Melander, C. (2014). Small-molecule suppression of beta-lactam resistance in multidrug-resistant gram-negative pathogens. *J Med Chem, 57*(17), 7450-7458. doi:10.1021/jm501050e
4. Rogers, S. A., Huigens, R. W., 3rd, & Melander, C. (2009). A 2-aminobenzimidazole that inhibits and disperses gram-positive biofilms through a zinc-dependent mechanism. *J Am Chem Soc, 131*(29), 9868-9869. doi:10.1021/ja9024676
5. Huigens, R. W., 3rd, Reyes, S., Reed, C. S., Bunders, C., Rogers, S. A., Steinhauer, A. T., & Melander, C. (2010). The chemical synthesis and antibiotic activity of a diverse library of 2-aminobenzimidazole small molecules against MRSA and multidrug-resistant A. baumannii. *Bioorg Med Chem, 18*(2), 663-674. doi:10.1016/j.bmc.2009.12.003
6. Richards, J. J., Ballard, T. E., Huigens, R. W., 3rd, & Melander, C. (2008). Synthesis and screening of an oroidin library against Pseudomonas aeruginosa biofilms. *Chembiochem, 9*(8), 1267-1279. doi:10.1002/cbic.200700774
7. Reed, C. S., Huigens, R. W., 3rd, Rogers, S. A., & Melander, C. (2010). Modulating the development of E. coli biofilms with 2-aminoimidazoles. *Bioorg Med Chem Lett, 20*(21), 6310-6312. doi:10.1016/j.bmcl.2010.08.075
8. Liu, C., Worthington, R. J., Melander, C., & Wu, H. (2011). A new small molecule specifically inhibits the cariogenic bacterium Streptococcus mutans in multispecies biofilms. *Antimicrob Agents Chemother, 55*(6), 2679-2687. doi:10.1128/AAC.01496-10
9. Rogers, S. A., Bero, J. D., & Melander, C. (2010). Chemical synthesis and biological screening of 2-aminoimidazole-based bacterial and fungal antibiofilm agents. *Chembiochem, 11*(3), 396-410. doi:10.1002/cbic.200900617
10. Harris, T. L., Worthington, R. J., & Melander, C. (2012). Potent Small-Molecule Suppression of Oxacillin Resistance in Methicillin-Resistant Staphylococcus aureus. *Angew Chem Int Ed Engl, 51*(45), 11254-11257. doi:10.1002/anie.201206911
11. Moffatt, J. H., Harper, M., Harrison, P., Hale, J. D., Vinogradov, E., Seemann, T., . . . Boyce, J. D. (2010). Colistin resistance in Acinetobacter baumannii is mediated by complete loss of lipopolysaccharide production. *Antimicrob Agents Chemother, 54*(12), 4971-4977. doi:10.1128/AAC.00834-10
12. El Hamidi, A., Tirsoaga, A., Novikov, A., Hussein, A., & Caroff, M. (2005). Microextraction of bacterial lipid A: easy and rapid method for mass

spectrometric characterization. *J Lipid Res, 46*(8), 1773-1778. doi:10.1194/jlr.D500014-JLR200
13. Shaffer, S. A., Harvey, M. D., Goodlett, D. R., & Ernst, R. K. (2007). Structural heterogeneity and environmentally regulated remodeling of Francisella tularensis subspecies novicida lipid A characterized by tandem mass spectrometry. *J Am Soc Mass Spectrom, 18*(6), 1080-1092. doi:10.1016/j.jasms.2007.03.008
14. Dixon, D. R., & Darveau, R. P. (2005). Lipopolysaccharide heterogeneity: innate host responses to bacterial modification of lipid a structure. *J Dent Res, 84*(7), 584-595. doi:10.1177/154405910508400702
15. Haseley, S. R., Holst, O., & Brade, H. (1997). Structural studies of the O-antigenic polysaccharide of the lipopolysaccharide from Acinetobacter (DNA group 11) strain 94 containing 3-amino-3,6-dideoxy-D-galactose substituted by the previously unknown amide-linked L-2-acetoxypropionic acid or L-2-hydroxypropionic acid. *Eur J Biochem, 247*(3), 815-819.
16. Haseley, S. R., Holst, O., & Brade, H. (1998). Structural studies of the O-antigen isolated from the phenol-soluble lipopolysaccharide of Acinetobacter baumannii (DNA group 2) strain 9. *Eur J Biochem, 251*(1-2), 189-194.
17. Tsai, C. M., & Frasch, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem, 119*(1), 115-119.