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COMMUNICATION

Bromophenazine Derivatives with Potent Inhibition, Dispersion and Eradication Activities against *Staphylococcus aureus* Biofilms

Cite this: DOI: 10.1039/xoxxoooooox

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

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Bacterial biofilms are surface-attached communities of bacteria that are: 1.) highly prevalent in human infections, and 2.) resistant to conventional antibiotic treatments and host immune responses. It has only been in the last ~20 years that bacterial biofilms have been identified as a critical biomedical hurdle in infectious disease and human health. *Staphylococcus aureus* is a leading cause of nosocomial and community-acquired infections and is notorious for its ability to form drug-resistant biofilms. Despite the need for antibacterial agents that target *S. aureus* biofilms, few chemical scaffolds are known that are capable of inhibiting, dispersing or eradicating their biofilms. Here, we report the discovery of bromophenazine derivatives that display antibiofilm activities as either potent biofilm inhibitors (IC_{50} values 0.55-10.3 μM) or dispersal agents (EC_{50} values 1.4-29.3 μM) and biofilm eradicators (MBEC values 100-200 μM) against *S. aureus* strains, including a methicillin-resistant *Staphylococcus aureus* clinical isolate. These discoveries could lead to the development of new treatment options that target drug-resistant, biofilm-associated *S. aureus* infections.

Staphylococcus aureus is a gram-positive human pathogen that is notorious for its role as a leading cause of both nosocomial and community-acquired bacterial infections worldwide.[1,2] During infection, *S. aureus* forms surface-attached bacterial communities, known as biofilms, that are intrinsically resistant to conventional antibiotic treatments and host immune responses.[2,3] As a result of the innate antibiotic resistance displayed by these surface-attached bacterial communities, *S. aureus* biofilms are essentially impossible to clear or eradicate in the clinic.[3]

Unfortunately, our current arsenal of antibiotics does not hit bacterial targets critical to biofilm formation or maintenance. Despite the unmet biomedical challenge posed by biofilm-associated bacterial infections, many pharmaceutical companies have eliminated their antibacterial discovery programs.[4-6] Future antibacterial agents will require the ability to modulate biofilm processes, such as quorum sensing, biofilm formation and maintenance[7] or eradicate established biofilms.[8,9]

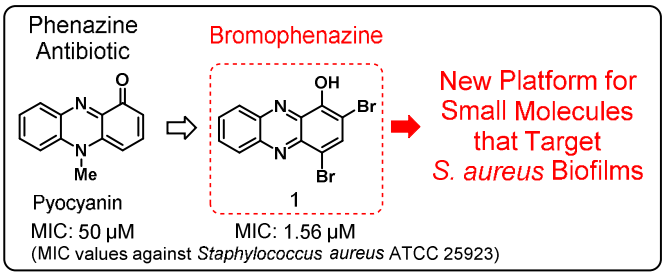

Figure 1. Bromophenazine **1** is a potent antibacterial agent and potentially a new platform to target *S. aureus* biofilms.

S. aureus biofilm-related diseases are highly prevalent in osteomyelitis, indwelling medical device infection, periodontitis and peri-implantitis, chronic wound infection, chronic rhinosinusitis,

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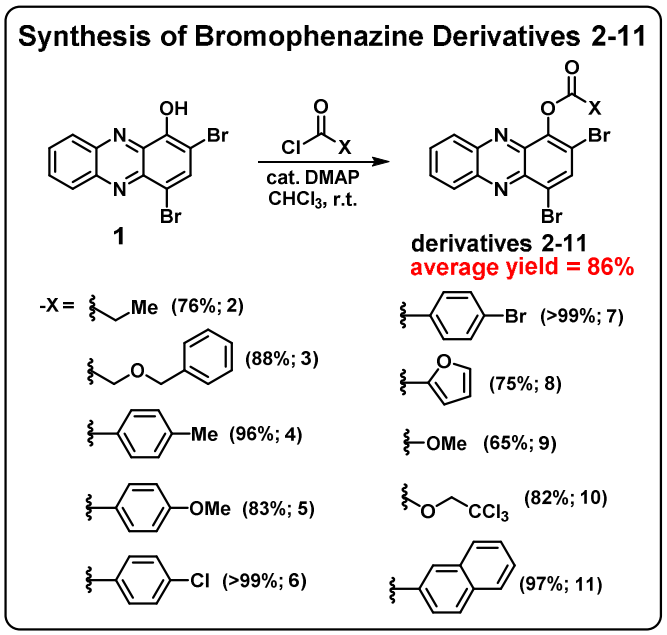
† Electronic Supplementary Information (ESI) available: synthetic procedures, NMR spectra for new compounds synthesized, MIC, biofilm inhibition/dispersion/eradication protocols, dose-response curves and images of MIC/biofilm experiments. See DOI: 10.1039/c000000x/

endocarditis, ocular infection and polymicrobial biofilm infections.[2] Despite the overwhelming need for therapeutic options to treat biofilm-associated *S. aureus* infections, few small molecule scaffolds have been reported that target these biofilms[3] (e.g., ADEP4[10], *cis*-2-decenoic acid[11], 2-aminoimidazoles[12,13], aryl rhodanines[14], quinazolinones[15]). Antibiofilm agents that are clinically effective against *S. aureus* biofilms will have a significant impact on the treatment of drug-resistant, biofilm-associated *S. aureus* infections.

Our group recently identified bromophenazine **1** (Figure 1) as a potent antibacterial agent against *S. aureus* (minimum inhibitory concentration or MIC 1.56 μM) inspired by the redox-active phenazine antibiotic pyocyanin that is produced by *Pseudomonas aeruginosa*.[16] Pyocyanin causes oxidative stress in various cell lines and is associated with toxicity[17]; however, despite these concerns, pyocyanin has promising pharmacological applications.[18] Pyocyanin, in part, enables *P. aeruginosa* to clear cystic fibrosis patients' lungs of established *S. aureus* infection during competitive microbial interactions within the lung.[19] In this study, we were interested in investigating the potential to clear biofilm-associated *S. aureus* infections with bromophenazine small molecules. With the growing demand for effective antibiofilm agents against *S. aureus* biofilms, we were interested in synthesizing and evaluating derivatives of **1** in a series of biofilm inhibition, dispersion and eradication assays against this major pathogen.



Scheme 1. Chemical synthesis of bromophenazine derivatives **2-11**.

Bromophenazine derivatives were synthesized by reacting **1** with commercially available acid chloride or chloroformate starting materials in chloroform with 4-(dimethylamino)pyridine (DMAP) as a catalyst (Scheme 1). These reactions typically required stirring for one hour at room temperature to give an average yield of 86% (range: 65 to >99% yield) for bromophenazines **2-11** following column chromatography purification. Following the synthesis of each bromophenazine derivative (**1-11**), DMSO stock solutions were prepared for biological evaluation in MIC, biofilm inhibition, biofilm dispersion and biofilm eradication experiments against *S. aureus* strains (including ATCC 25923 and MRSA-2, a methicillin-resistant *Staphylococcus aureus* clinical isolate[20]) in 96-well plates.

We began our biological investigations of bromophenazines **1-11** against *S. aureus* by performing two series of assays against *S. aureus* ATCC 25923, which included: 1.) microdilution MIC experiments to evaluate planktonic growth inhibition activity and 2.) biofilm inhibition assays using crystal violet staining to quantify *S. aureus* biofilm formation. Initially, we wanted to have a single assay that would allow us to obtain biofilm inhibition data directly from MIC experiments (i.e., 16-20 hour incubation at 37 °C, ~10^5 $CFUmL^{-1}$, Luria-Bertani medium) against *S. aureus*; however, alternative assay conditions were necessary to have optimal biofilm formation in 96-well plates (i.e., gelatin pre-coated wells, 24 hours at 37 °C, ~$1x10^6$ $CFUmL^{-1}$, Tryptic Soy Broth medium with 0.5% glucose). These assay conditions resulted in a robust *S. aureus* biofilm and served as an excellent model for our investigations.

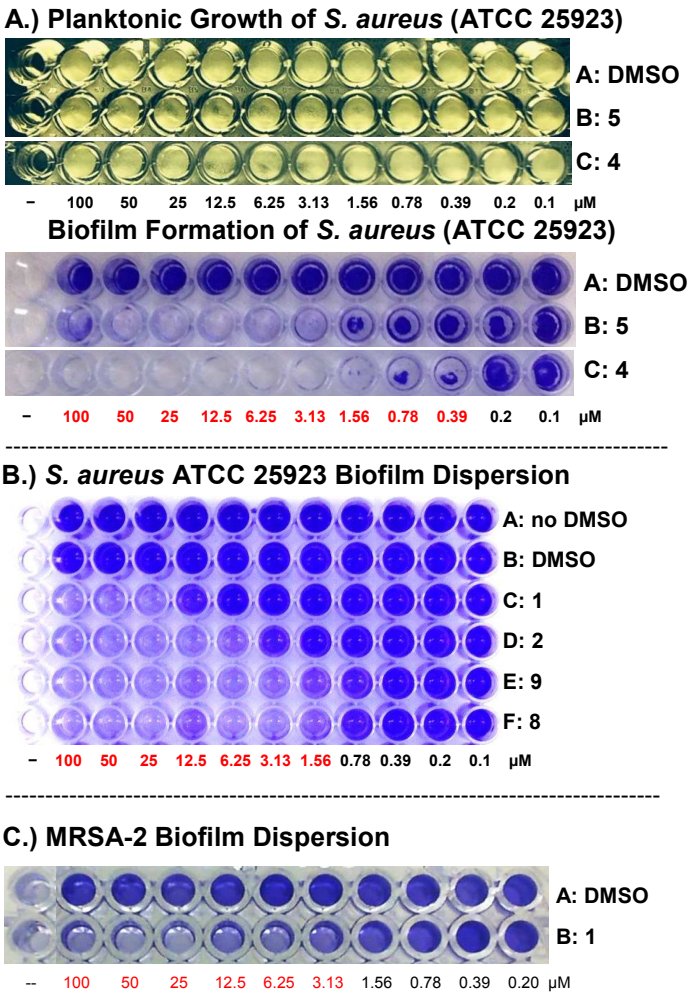


Figure 2. A.) Planktonic growth and biofilm inhibition assay with **4** and **5**.; B.) Biofilm dispersion assays with bromophenazines **1**, **2**, **8**, and 9 against *S. aureus*.; C.) Bromophenazine **1** potently disperses established MRSA-2 biofilms (EC_{50} = 3.53 μM).

In general, compounds that inhibit biofilm formation through non-growth inhibiting mechanisms are considered true biofilm inhibitors whereas compounds that inhibit bacterial growth while inhibiting biofilm formation are considered to demonstrate an antibacterial phenotype.[20] Biofilm-inhibiting small molecules prevent bacterial biofilm formation without placing a selective pressure on bacteria to develop resistance.[3] Bromophenazine derivatives **4**, **5**, **6** and **7** demonstrated potent "biofilm inhibition" activity against *S. aureus* without demonstrating planktonic growth inhibition (Figure 2A. for biofilm inhibitors **4** and **5**; Table 1).

The four bromophenazine derivatives (i.e., **4-7**) that demonstrated potent *S. aureus* biofilm inhibition possess a 4-substituted phenyl ester moiety. These derivatives demonstrated sub-micromolar biofilm inhibition activity (IC_{50} values in biofilm inhibition assays) in our initial single replicate screen against *S. aureus* ATCC 25923. Final IC_{50} values for **4-7** were determined by three independent biofilm inhibition experiments and biofilm inhibitor potency ranged between 0.55-10.3 µM (Table 1; Supporting Information). Bromophenazine **4** demonstrated the most potent biofilm inhibition activity against *S. aureus* ATCC 25923 with an IC_{50} value of 0.55 µM (compared to an MIC >100 µM) to give an MIC:IC_{50} value ratio of >181 (Figure 2A.). Bromophenazine **4** is one of the most potent biofilm inhibitors to be reported against *S. aureus* (IC_{50} = 550 nM).[12]

The three other bromophenazine "biofilm inhibitors" (derivatives **5-7**) report MIC:IC_{50} value ratios of >10 to >130 in comparison to bromophenazines that demonstrated "antibacterial" activity (i.e., derivatives **1-3**; **8-10**) that report MIC:IC_{50} ratios of 1.0 to 3.8 during these investigations against *S. aureus* (Table 1). We recently reported several antibiotics (i.e., vancoymycin, ciprofloxacin, erythromycin) to have MIC:IC_{50} value ratios between 3.1 and 7.8 against *S. aureus* ATCC 29213 in biofilm inhibition assays to reference "antibacterial" activity.[20] In the same study, our group identified two biofilm inhibitors that had MIC:IC_{50} ratios of >22 against *S. aureus* ATCC 29213.[20] Previously, 2-aminobenzimidazole biofilm inhibitors have been reported with MIC:IC_{50} value ratios that range between 4 to >20.[21] As part of the discussion to differentiate "biofilm inhibition" activity from "antibacterial" activity, a norspermidine analogue was recently reported to possess biofilm inhibition activity that had no observable growth inhibitory activity until concentrations were 40-fold higher than the concentration of this compound's minimum biofilm inhibitory concentration against *B. subtilis*.[22]

Five of the remaining six bromophenazine derivatives (i.e., **1-3**; **8-10**) demonstrated antibacterial activity (MIC 0.78-1.56 µM) while bromophenazine **11** demonstrated neither antibacterial nor biofilm inhibition activity at the concentrations tested. Despite potent antibacterial activities against *S. aureus*, bromophenazines demonstrate weak antibacterial activity against gram-negative bacteria. Against *A. baumannii*, bromophenazine **1** gave an MIC 50 µM (Supporting Information) and an MIC >100 µM against *P. aeruginosa* (PAO1).[16]

We evaluated our bromophenazine derivatives in biofilm dispersion assays to determine if these bromophenazines were capable of dispersing, or clearing, established *S. aureus* biofilms. In biofilm dispersion assays, bacterial biofilms are established in 96-well plates in the absence of test compound. Following the establishment of biofilms, media/planktonic bacteria are removed and test compound is added (in buffer or media) to the established biofilms inside microtiter wells and allowed to incubate. Biofilm dispersion is quantified via crystal violet staining of treated biofilms with the use of a spectrophotometer (OD_{540}).

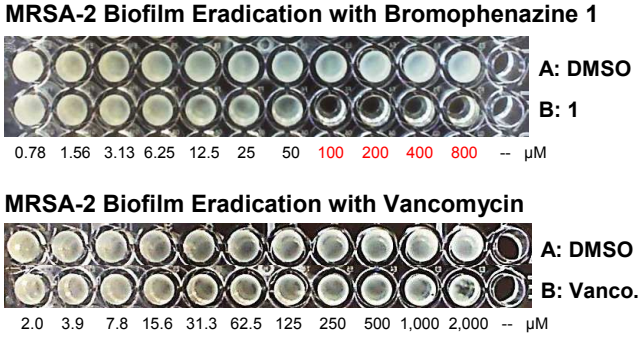


Figure 3. Biofilm eradication of MRSA-2 with bromophenazine **1** (MBEC = 100-200 µM) and vancomycin (MBEC >2,000 µM).

We established *S. aureus* biofilms in 96-well plates using conditions similar to biofilm inhibition assays (Supporting Information). Following the initial *S. aureus* biofilm establishment, bromophenazine derivatives **1-11** were added to 96-well plates in 2-fold serial dilutions with established *S. aureus* biofilms in either: 1.) phosphate buffered saline (PBS) with room temperature incubation for 24 hours with established *S. aureus* ATCC 25923 biofilms or 2.) media with 37 °C incubation for 24 hours with MRSA-2 biofilms. Following the final incubation of established *S. aureus* biofilms with our bromophenazine derivatives, crystal violet was used to stain and quantify remaining biofilms to determine biofilm dispersal activity of bromophenazines **1-11** as effective concentrations from our test concentrations (EC_{50} values; Table 1).

We identified four bromophenazine derivatives (e.g., **1**, **2**, **8** and **9**; Figure 2B.; Table 1) capable of dispersing established *S. aureus* ATCC 25923 biofilms. The potency of these four biofilm dispersion-active bromophenazines ranged in EC_{50} values between 1.4 and 29.3 µM while the three most potent dispersal agents gave EC_{50} values of 1.4 µM, 2.6 µM and 2.9 µM for derivatives **8**, **2** and **9** respectively. Despite several potent antibacterial or biofilm inhibitors, many bromophenazine derivatives were found to be completely inactive in head-to-head dispersion assays against established *S. aureus* ATCC 25923 biofilms (EC_{50} >100 µM). Bromophenazine **1** effectively dispersed MRSA-2 biofilms and reported an EC_{50} value of 3.53 µM (Figure 2C.).

Since the most potent antibacterial bromophenazines demonstrated a tendency to be potent biofilm dispersal compounds, we wanted to evaluate bromophenazines **1**, **2**, **8**, **9** and **10** in biofilm eradication assays[8,9,23] against MRSA-2 to see if our active compounds were demolishing MRSA biofilms. Biofilm eradication assays are essentially biofilm dispersion assays with the addition of a final treatment with fresh media (and incubation at 37 °C for 24 hours) instead of crystal violet staining. This final incubation allows viable cells within the biofilm to grow. At the end of this final incubation in biofilm eradication assays, microtiter wells void of turbidity represent eradicated biofilms and the lowest concentration at which no visible growth is observed is referred to as the minimum biofilm eradication concentration (MBEC). Potent biofilm-eradicating small molecules are extremely rare.[8,9] For these

experiments, we selected MRSA-2 as our model since it is a multidrug-resistant, biofilm-forming clinical isolate of *S. aureus*.[18] Against MRSA-2, bromophenazine **1** reported an MIC value of 1.56 μM and an MBEC of 100-200 (Figure 3) while **2**, **8**, **9** and **10** gave MBEC values between 62.5 and 250 μM. Only bromophenazine **8** was found to be more potent (MBEC 62.5-100 μM) than parent compound **1**. Bromophenazine **8** was found to be equipotent to known biofilm eradicating agent[9] QAC 10 in head-to-head eradication assays against MRSA-2 biofilms.

Table 1. Antibiofilm activity of bromophenazine derivatives **1-11** against *Staphylococcus aureus* (ATCC 25923[a] and MRSA-2[b]).

Compound	[a]Growth Inhibition MIC (μM)	[a]Biofilm Inhibition IC_{50} (μM)	[a]Biofilm Dispersion EC_{50} (μM)	[b]Biofilm Eradication MBEC
1	1.56[a,b]	0.41	29.3	100-200*
2	1.56	0.92	2.6	125
3	1.56	0.76	>100	--
4	>100	0.55	>100	--
5	>100	10.3	>100	--
6	>100	0.77	>100	--
7	>100	1.13	>100	--
8	0.78	0.76	1.4	62.5-100
9	1.56	0.77	2.9	250
10	0.78	0.76	>100	125
11	>100	>100	>100	--
vancomycin	--	--	--	>2,000**
QAC 10	--	--	--	62.5-125

Note: [a] *S. aureus* ATCC 25923; [b] MRSA-2. IC_{50} values reported for a single replicate screen except for **4-7** which are reported from 3 independent biofilm inhibition experiments following the initial screen. EC_{50} (biofilm dispersion) and MBEC (biofilm eradication) values are reported from 2 to 4 independent experiments. *Bromophenazine **1** gave an EC_{50} value of 3.53 μM in MRSA-2 dispersion assays (not reported here). **MRSA-2 is "sensitive" to vancomycin (MIC 0.78 μM) as a growth inhibitor.[20]

We also evaluated vancomycin against MRSA-2 since it is considered to be the drug of last resort against MRSA infections. Vancomycin gave an MIC of 0.78 μM against MRSA-2 and an MIC of 0.39-0.78 against *S. aureus* ATCC 29213, therefore MRSA-2 is considered to be "sensitive" to vancomycin.[20] When tested against MRSA-2 in biofilm eradication assays, vancomycin reported an MBEC of >2,000 μM (inactive at all concentrations). The MRSA-2 biofilms are >2,564-fold more resistant against vancomycin when compared to their planktonic counterparts (i.e., MBEC:MIC ratio). Bromophenazine **1** reported an MBEC:MIC ratio of 64-128.

We also tested the stability of several ester derivatives towards hydrolysis by subjecting these compounds to conditions mimicking our biofilm assays. All bromophenazines tested were found to be completely stable to hydrolysis (Supporting Information).

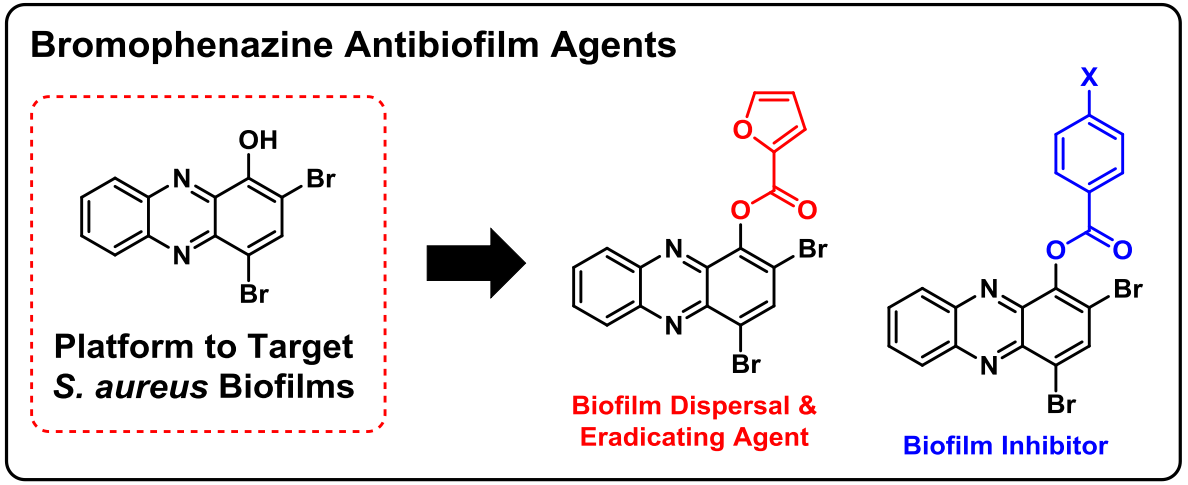
In conclusion, we have discovered several bromophenazine derivatives that are potent inhibitors, dispersal agents and eradicators against *S. aureus* biofilms, including a MRSA clinical isolate. These bromophenazines are inspired by pyocyanin, thus future investigations to determine the therapeutic index of these compounds are critical. Bromophenazine **1** is a promising small molecule platform to develop agents that target *S. aureus* biofilms. These findings could lead to breakthroughs in the treatment of drug-resistant, biofilm-associated *S. aureus* infections worldwide.

This work was supported by a Research Opportunity Seed Fund Award and the College of Pharmacy at the University of Florida. We would like to thank Christian Melander for useful comments during the course of these studies. A.T.G. has been awarded a UF Graduate School Fellowship.

Notes and references

1 J. M. Yarwood, D. J. Bartels, E. M. Volper and E. P. Greenberg, *J. Bacteriol.,* 2004, **186**, 1838.
2 N. K. Archer, M. J. Mazaitis, J. W. Costerton, J. G. Leid, M. E. Powers and M. E. Shirtliff, *Virulence,* 2011, **2**, 445.
3 P. Y. Chung and Y. S. Toh, *Pathog. Dis.,* 2014, **70**, 231.
4 K. Lewis, *Nat. Rev. Drug Discovery,* 2013, **12**, 371.
5 S. J. Projan, *Curr. Opin. Microbiol.*, 2003, **6**, 427.
6 J. F. Barrett, *Curr. Opin. Microbiol.*, 2005, **8**, 498.
7 D. J. Musk and P. J. Hergenrother, *Curr. Med. Chem.,* 2006, **13**, 2163.
8 M. H. Fletcher, M. C. Jennings and W. M. Wuest, *Tetrahedron*, 2014, **70**, 6373.
9 M. C. Jennings, L. Ator, T. J. Paniak, K. P. C. Minbiole, W. M. Wuest, *ChemBioChem,* 2014, **15**, 2211.
10 B. P. Conlon, E. S. Nakayasu, L. E. Fleck, M. D. LaFleur, V. M. Isabella, K. Coleman, S. N. Leonard, R. D. Smith, J. N. Adkins, K. Lewis, *Nature*, 2013, **503**, 365.
11 D. G. Davies and C. N. H. Marques, *J. Bacteriol.*, 2009, **191**, 1393.
12 R. W. Huigens III, S. A. Rogers, A. T. Steinhauer and C. Melander, *Org. Biomol. Chem.,* 2009, **7**, 794.
13 Z. Su, L. Peng, R. J. Worthington and C. Melander, *ChemMedChem*, 2011, **6**, 2243.
14 T. J. Opperman, S. M. Kwasny, J. D. Williams, A. R. Khan, N. P. Peet, D. T. Moir and T. L. Bowlin, *Antimicrob. Agents Chemother.*, 2009, **53**, 4357.
15 Y. Ma, Y. Xu, B. D. Yestrepsky, R. J. Sorenson, M. Chen, S. D. Larsen and H. Sun, *PLoS ONE,* 2012, **7**, e47255. doi:10.1371/journal.pone.0047255
16 N. V. Borrero, F. Bai, C. Perez, B. Q. Duong, J. R. Rocca, S. Jin and R. W. Huigens III, *Org. Biomol. Chem.,* 2014, **12**, 881.
17 H. Ran, D. J. Hassett and G. W. Lau, *Proc. Natl. Acad. Sci.*, 2003, **100**, 14315-14320.
18 P. Priyaja, P. Jayesh, R. Phillip and I. S. Bright Signh, *Cytotechnol.*, 2014. doi: 10.1007/s10616-014-9765-5.
19 Z. A. Machan, T. L. Pitt, W. White, D. Watson, G. W. Taylor, P. J. Cole and R. Wilson, *J. Med. Microbiol.*, 1991, **34**, 213.
20 Y. Abouelhassan, A. T. Garrison, G. M. Burch, W. Wong, V. M. Norwood IV and R. W. Huigens III, *Bioorg. Med. Chem. Lett.*, 2014, **24**, 5076.
21 E. A. Lindsey, C. M. Brackett, T. Mullikin, C. Alcaraz and C. Melander, *ChemMedChem*, 2012, ***3***, 1462.
22 T. Böttcher, I. Kolodkin-Gal, R. Kolter, R. Losick and J. Clardy, *J. Am. Chem. Soc.,* 2013, ***135***, 2927.
23 Biofilm eradication assays adopted from: a.) Y.-J. Eun, M. H. Foss, D. Kiekebusch, D. A. Pauw, W. M. Westler, M. Tanbichler, D. B. Weibel, *J. Am. Chem. Soc.,* 2012, ***134***, 11322.; b.) H Ceri, M. E. Olson, C. Stremick, R. R. Read, D. Morck and A. Buret, *J. Clin. Microbiol.*, 1999, **37**, 1771.

Graphical Abstract



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