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2-Aminobenzimidazole Derivatives Strongly Inhibit and Disperse *Pseudomonas aeruginosa* Biofilms**

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Keywords

2-aminobenzimidazole; bacteria; biofilm inhibition; *Pseudomonas aeruginosa*; quorum sensing; small molecule modulator

Bacteria can grow into surface-associated communities termed biofilms that are pervasive virtually everywhere on earth.[1] This mode of growth poses a significant obstacle to the successful treatment of infectious disease, with an estimated 80% of human infections in the biofilm state.[2] Biofilms are particularly problematic to clear due to their encasement in a protective and impermeable extracellular matrix,[3] which render biofilm-associated bacteria resistant to both host immune responses and standard antibiotic agents. Indeed, treatment with ~10-1000-fold higher doses of antibiotic is often required for biofilm clearance relative to planktonic bacteria.[2] Biofilm growth by the Gram-negative bacterium *Pseudomonas aeruginosa* has attracted particular attention, as biofilms of this pathogen are the origin of the fatal chronic lung infections in most cystic fibrosis patients.[4] *P. aeruginosa* biofilm infections also plague burn victims, AIDS patients, and are endemic on the medical implants and devices universal in healthcare today.[5] As such, the development of new methods to attenuate bacterial biofilm growth is of significant importance and represents a major research area.[6] Small molecules capable of inhibiting the growth of or removing (*i.e.*, dispersing) preformed biofilms would be extremely useful to combat bacterial infection and in a range of other applications in industry, agriculture, and the environment.[7] Molecules of this class, however, remain rare.[5-8] Here, we report our discovery of a chemical approach for the inhibition and dispersion of *P. aeruginosa* biofilms based on 2-aminobenzimidazoles.

Biofilm growth only occurs after a critical bacterial cell density is achieved, and in many bacteria is under the direct control of the cell-cell signaling pathway termed quorum sensing (QS).[6-7, 9] Notably, *P. aeruginosa* mutants lacking a functional QS system are unable to grow into mature biofilms and are largely avirulent.[10] Our group and others have previously shown that non-native analogs of natural *N*-acylated L-homoserine lactone (AHL) QS signals can strongly modulate QS in Gram-negative bacteria,[11] and several of

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these AHLs also attenuate biofilm growth in *P. aeruginosa*.[8d] One challenge to the application of AHLs as biofilm or QS inhibitors, however, is the hydrolytic instability of the lactone head group.[12] Hydrolyzed AHLs are biologically inactive, and therefore additional measures (*e.g.*, multiple dosing, controlled delivery, etc.) are required for sustained activity of AHLs.[13] Furthermore, and of particular relevance to biofilms, our early AHL-derived biofilm inhibitors failed to disperse preformed biofilms, similar to most antibiotics.[8d] In this context, we currently seek to identify alternative, hydrolytically stable molecular scaffolds for QS and/or biofilm modulation with enhanced activities.

A few natural products have been shown to inhibit bacterial QS or biofilm growth. The halogenated furanones from the macroalga *Delisea pulchra* have seen the most intensive study in this regard.[8b] Three other notable examples include bromoageliferin, 3-indolylacetonitrile, and resveratrol (Figure 1). Bromoageliferin displays anti-biofilm activity in the Gram-negative bacterium *Rhodospirillum salexigens*, and recent elegant studies by Melander and co-workers have revealed simplified analogs of this marine natural product with anti-biofilm activities (most notably, 2-aminoimidazole (2-AI) derivatives in Gram-negative bacteria[8a] and 5-amido or 5-imido 2-aminobenzimidazole (2-ABI) derivatives in Gram-positive bacteria).[14] The plant auxin 3-indolylacetonitrile was found to inhibit the formation of *P. aeruginosa* biofilms *via* a QS-dependent mechanism,[15] and the phytoalexin resveratrol[16] and related stilbene derivatives[17] have recently been shown to inhibit the LuxR-type QS receptors in Gram-negative bacteria. We reasoned that combining structural attributes of these three compounds into a simple molecular scaffold could reveal molecules with heightened anti-biofilm and/or QS properties.

To explore this hypothesis, we designed and synthesized a small set of stilbenes containing 2-AI or indole moieties (Scheme 1). The 2-ABI-stilbene derivative **1** was synthesized by initial formation of the stilbene framework *via* the Heck reaction, and then a tin (II) dichloride reduction to form the diamine intermediate. Condensation with cyanogen bromide afforded stilbene **1**. We generated 2-AI-stilbene **2** in good yield *via* the palladium-catalyzed regioselective arylation of imidazo[1,2-*a*]pyrimidine[18] followed by hydrazine-mediated pyrimidine ring cleavage.[19] Indole-stilbenes **4a-b** were synthesized *via* Heck reactions according to published methods.[20]

We next tested the ability of stilbenes **1, 2**, and **4a-b** to inhibit biofilm formation in a wild-type *P. aeruginosa* strain (PAO1) at 500 μM using standard static biofilm growth assays. Biofilms were grown in a modified M9 minimal media in 96-well microtiter plates, and crystal violet staining of the surface-associated biomass was used to quantify biofilm growth at 12 and 24 h (see Supp. Info.) This preliminary screen and subsequent dose-response analyses revealed that **1** and **2** were able to inhibit biofilm growth at 24 h in *P. aeruginosa* by 56% and 48%, respectively, at 100 μM. Neither indole derivative (**4a-b**) showed appreciable anti-biofilm activity.

We selected 2-ABI-stilbene **1** as our most promising lead compound, and sought to identify potential structural motifs within **1** that were responsible for the observed anti-biofilm activity in order to further improve its inhibitory properties. We dissected stilbene **1** into five simple sub-structures (**5-9**, outlined in Figure 2), and tested each of these compounds in analogous *P. aeruginosa* biofilm assays. Removing the amino-group or 2-AI unit, affording **5** and **6**, led to complete loss of inhibitory activity. However, removal of the styrene moiety (to yield 2-ABI **7**) revealed a sub-structure that exhibited greater activity than lead compound **1**, almost completely inhibiting biofilm formation at 24 h (94% inhibition, IC_{50} = 47 μM).[21],[22] The aryl component of 2-ABI (**7**) was essential for anti-biofilm activity, as neither 2-AI (**8**) nor guanidine (**9**) displayed significant inhibitory activity in *P. aeruginosa*.

Intrigued by this result, we synthesized a small library of 2-ABI derivatives to further probe the activity of this compound class in *P. aeruginosa*. Fifteen 2-ABIs (**10-24**) were readily generated in one-step *via* condensation of various functionalized *o*-diaminobenzenes with cyanogen bromide (Table 1).[23] We examined the biofilm inhibitory activities of these compounds in *P. aeruginosa*, and identified several 2-ABI derivatives capable of inhibiting biofilm growth by ~90%, but with improved potencies (*i.e.,* lower IC_{50} values) relative to **7**. An increase in potency was achieved either by halide (**10-13**) or methyl substitutions (**19-21**) on the 2-ABI aryl group. In turn, aryl-substitutions containing hydrogen bond donors or acceptors led to either a total loss (**16**) or significant reductions in biofilm inhibitory activity (Table 1). The most potent biofilm inhibitor identified overall was the 5,6-dimethyl 2-ABI (**21**, IC_{50} = 4.0 μM), which was ~10-fold more active than the parent compound **7**. Amongst the few biofilm inhibitors for which IC_{50} data has been reported,[8a, 8c, 8e, 8g] **21** constitutes one of the most active *P. aeruginosa* biofilm inhibitors known. Dose-response curves and images of crystal violet-stained biofilms in the presence of **7** and **21** are shown in Figure 3A.

Compounds capable of not only inhibiting biofilm growth, but also dispersing preformed biofilms, are of particular value for a range of clinical and other applications. We thus tested the ability of compounds **7** and **21** to disperse 24 h-old *P. aeruginosa* biofilms using the crystal violet staining assay (Figure 3B). Biofilms were allowed to develop in the absence of compound for 24 h, after which non-biofilm material was removed by washing with buffer and fresh media with compound was added. Biofilm was quantified after an additional 24 h, and the amount of dispersed biofilm was determined *via* comparison of the amount of biofilm at 48 h in the presence of compound versus the amount of biofilm at 24 h in the absence of compound. We found that 2-ABIs **7** and **21** were capable of strongly dispersing *P. aeruginosa* biofilms (~80%), with half maximal dispersion (DC_{50}) values of 84 μM and 92 μM, respectively (Figure 3B).

Little is known about the actual mechanisms of action of most small molecule biofilm inhibitors. As such, we sought to investigate the mechanism by which the 2-ABI scaffold elicits its biofilm inhibitory and dispersive activity in *P. aeruginosa*. Planktonic growth curve analyses (under conditions identical to biofilm growth) demonstrated that the observed activities were not a result of a bactericidal mechanism. Melander and co-workers have shown that 2-ABI derivatives bearing 5-amido substituents inhibit and disperse biofilms through a zinc-dependent mechanism, albeit in Gram-positive as opposed to Gram-negative bacteria (see above).[14] We screened a wide range of metals, including zinc, in a dose-dependent manner for mitigating effects on the biofilm inhibitory activity of **7** in *P. aeruginosa*, but observed no change in activity.

As introduced above, the role of QS in biofilm formation is well documented,[6-8, 9] and therefore we next evaluated the abilities of **7** and **21** to inhibit QS in *P. aeruginosa*. For this purpose, we prepared two wild-type *P. aeruginosa* QS reporter strains that contain the plasmids p*lasI*-LVA*gfp* and p*rhlI*-LVA*gfp* (see Supp. Info.) These strains report the activity of two intracellular QS receptors in *P. aeruginosa* (LasR and RhlR) by the production of green fluorescent protein (gfp), allowing for LasR and RhlR activities, and thus QS levels, to be quantified by fluorescence. We observed a significant reduction in both LasR and RhlR activities in the presence of **7** and **21** at 1–10× their IC_{50} values for biofilm inhibition (Figures 3C–D). Studies of related 2-ABI derivatives have shown that this class of molecules is bacterial cell permeable, allowing us to surmise that **7** and **21** could act on the Las and Rhl systems intracellularly.[24] Further, we utilized a *P. aeruginosa* strain that constitutively-expresses genomic gfp to demonstrate that **7** and **21** do not simply affect global protein synthesis (see Supp. Info.) Additional experiments are required to elucidate the precise targets of **7** and **21** that result in QS disruption. Nevertheless, these preliminary

findings suggest that biofilm modulation by 2-ABI derivatives could be occurring, at least in part, through interference with the *P. aeruginosa* Las and Rhl QS circuits.

In summary, we have identified 2-ABI derivatives as potent anti-biofilm agents in *P. aeruginosa*. We uncovered this compound class through the study of hybrid compounds derived from the structures of three natural products with known biofilm and QS inhibitory activities, and the subsequent structure-activity analyses of simplified derivatives. This discovery is significant, as several of these 2-ABI derivatives are among the most active *P. aeruginosa* biofilm modulators to be reported. Moreover, these compounds are capable of both inhibiting the growth of and dispersing preformed biofilms. Our results are surprising in light of previous data on related 2-ABI derivatives that indicated they were inactive in *P. aeruginosa*,[14] and support the continued study of this structurally simple, chemically robust compound class in Gram-negative bacteria.[25] Lastly, our studies indicate that 2-ABIs **7** and **21** are also capable of QS inhibition in *P. aeruginosa*, suggesting a possible mechanism for biofilm inhibition. A link between 2-ABI-type anti-biofilm agents and QS, to our knowledge, is previously undocumented. Ongoing work in our laboratory is directed towards the study of additional 2-ABI derivatives, as well as assessing their biofilm inhibitory activities across an expanded set of bacterial species.

Experimental Section

Full details of compound synthesis, compound characterization data, and biological protocols and assay data can be found in the Supporting Information.

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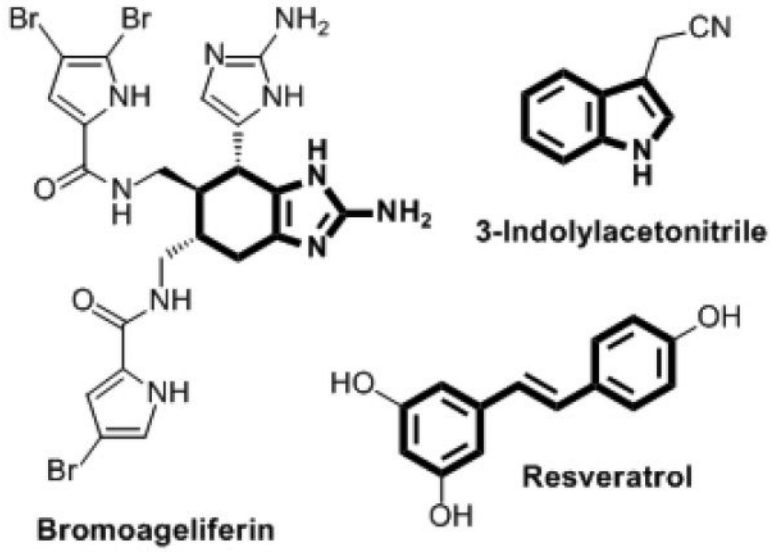
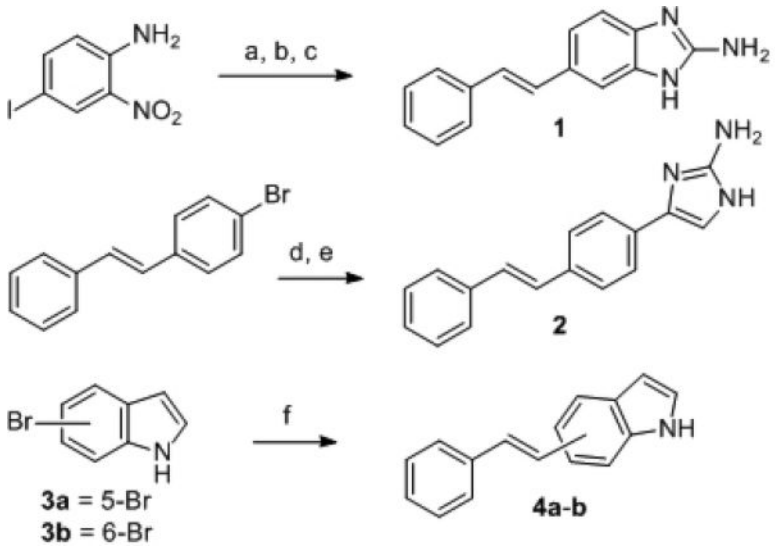


Figure 1.
Selected natural products with anti-biofilm or QS activities. Substructures of interest to this work are shown in bold.



Scheme 1.

Reaction conditions for the synthesis of stilbene derivatives: (a) Styrene, $Pd(OAc)_2$, CH_3CN, DIPEA, 80 °C, 76%; (b) $SnCl_2{\cdot}2H_2O$, EtOAc, 80 °C; (c) CNBr, MeOH:H_2O 1:1, 50 °C, 91% (over two steps); (d) Imidazo[1,2-*a*]pyrimidine hydrobromide, $Pd(OAc)_2$, PPh_3, Cs_2CO_3, 1,4-dioxane, 100 °C, 82%; (e) 20% N_2H_4/EtOH, 105 °C, 84%; (f) Styrene, $Pd(OAc)_2$, P(*o*-tolyl)$_3$, NEt_3, 100 °C, 73-76%. DIPEA = *N,N*-diisopropylethylamine.

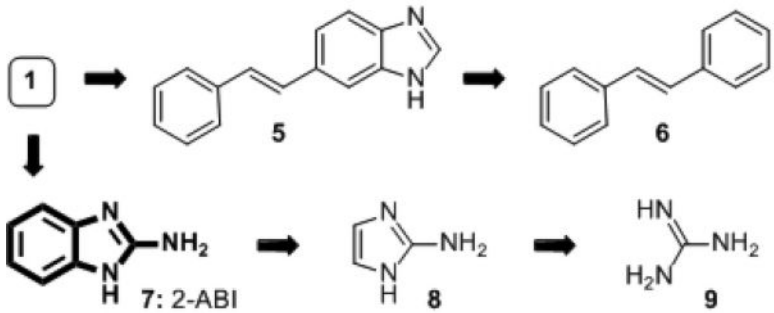


Figure 2.
Compounds studied (**5–9**) to dissect the structural features necessary for the activity of initial lead stilbene **1**.

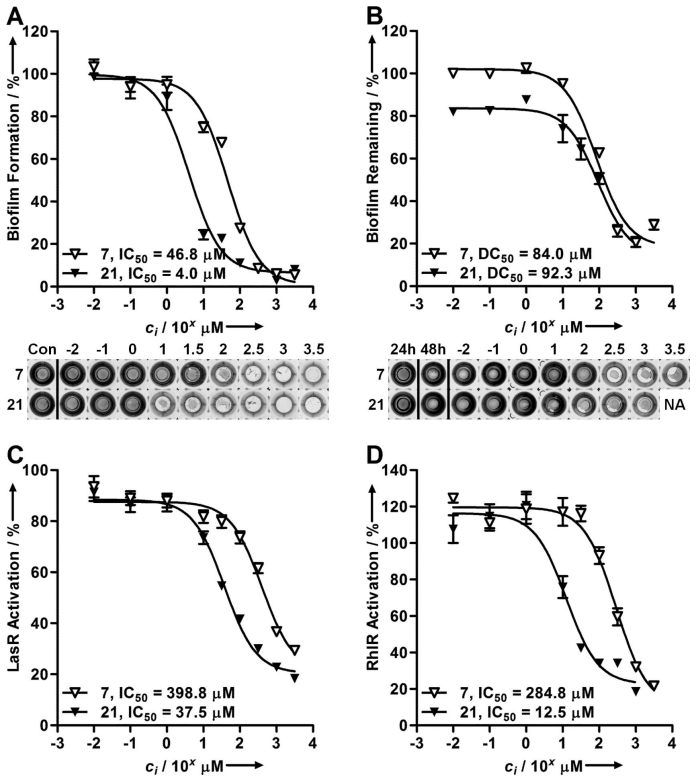
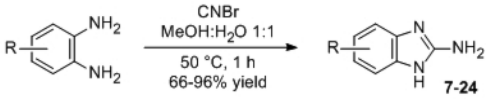


Figure 3.

Dose-response curves and images of crystal violet biofilm inhibitory (A), and dispersion (B) assays for **7** and **21** in *P. aeruginosa* (PAO1). NA = not available. Dose-response curves for **7** and **21** in *P. aeruginosa* PAO1/p*lasI*-LVAgfp (C), and PAO1/p*RhI*-LVAgfp (D) QS reporter strains.

Table 1

P. aeruginosa PAO1 biofilm inhibition data for 2-ABI derivatives.



Compound	R	IC_{50} (μM)[a]
7	H	47
10	5-I	20
11	5-Br	22
12	5-Cl	28
13	5-F	35
14	5-COPh	*ND*
15	5-CO_2Me	140
16	5-CO_2H	*ND*
17	5-CN	180
18	5-NO_2	63
19	4-Me	39
20	5-Me	25
21	5,6-Me	4.0
22	5-OMe	80
23	5-NH_2	*ND*
24	Fused 5,6-Ph[b]	48

[a] IC_{50} values were only obtained for 2-ABI derivatives exhibiting >60% biofilm inhibition after 24 h; *ND* = not determined. See Supp. Info. for 95% confidence intervals for IC_{50} values.

[b] Full name: 2- amino-1H-naphtho[2,3-d]imidazole.