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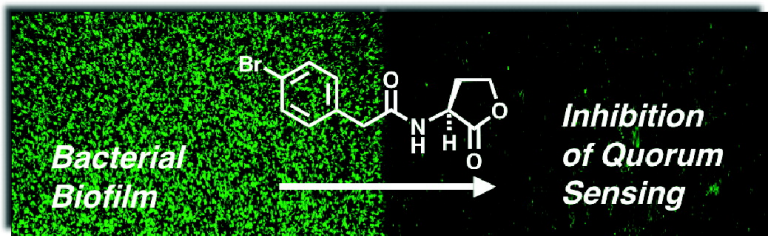

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Small Molecule Inhibitors of Bacterial Quorum Sensing and Biofilm Formation

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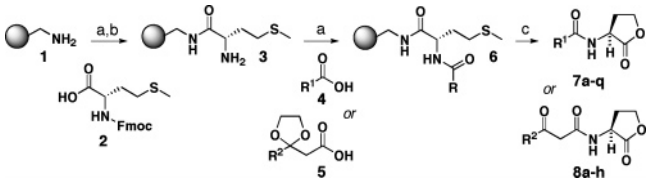
Bacteria use autoinducer ligands to monitor their population densities in a phenomenon called quorum sensing.[1] At high cell densities, bacteria use this chemical signaling process to switch from a nomadic existence to that of multicellular community. This lifestyle switch is significant; quorum sensing can be used to turn on virulence pathways[2] and induce the formation of drug-impervious communities called biofilms that are the basis of myriad chronic infections.[3] Over 80% of bacterial infections in humans involve the formation of biofilms. For example, lung infection by *Pseudomonas aeruginosa* in the biofilm state is the primary cause of morbidity in cystic fibrosis patients.[2] As such, the development of small molecules that attenuate bacterial quorum sensing has become an area of intense research.[4] Here, we report a highly efficient synthetic route to new ligands that modulate quorum sensing. Application of this route in focused library synthesis has delivered a set of potent quorum sensing antagonists, two of which strongly inhibit biofilm formation in *P. aeruginosa*.

We have focused our initial research on quorum sensing pathways in Gram-negative bacteria, as these signaling networks are used by numerous human pathogens, including *P. aeruginosa*. Gram-negative bacteria use *N*-acyl L-homoserine lactone (AHL) signal molecules to activate quorum sensing.[5] At a threshold AHL concentration (and related cell density), the AHL ligand will bind its cognate receptor, a LuxR-type protein, and activate the transcription of target genes involved in group behavior. An attractive strategy for quorum sensing control is to use non-native AHL derivatives that block natural AHL signals. The pace of AHL analogue discovery has been slow, however. Most synthetic routes to AHLs afford the products in low yields and purities, and the biological activity of these compounds has been evaluated primarily on an ad hoc basis.[6] New synthetic approaches are required for the generation of AHL analogues and the *systematic* evaluation of the effects of AHL ligand structure on quorum sensing.

To meet these challenges, we developed a solid-phase synthetic route to both natural and non-natural AHLs (Scheme 1). We chose solid-phase methods because they routinely give improved product purity relative to solution-phase methods and enable combinatorial library construction.[7] To date, the use of combinatorial methods to systemically evaluate AHL analogues remains essentially unexplored.[4c] To further expedite both solid-phase and library synthesis, we incorporated microwave (MW)-assisted reactions throughout the route.[8] Our four-step synthetic approach entails first loading amino polystyrene resin (**1**) with *N*-Fmoc-L-methionine (**2**) using a MW-assisted carbodiimide coupling (DIC).[9] Next, thermal Fmoc group removal followed by a second MW-assisted DIC coupling with various carboxylic acids (**4**) or protected β-keto acids (**5**)[10] generated acylated resin **6**. Finally, we utilized the classical reaction of cyanogen bromide (CNBr) with L-methionine in a MW-assisted, tandem cyclization cleavage step to release AHLs **7** and **8** from the solid support.[11,12]

This solid-phase route to AHLs is the first to provide access to the ca. 15 known natural AHLs from Gram-negative bacteria and to structural analogues thereof. Further, the route delivers compounds in sufficient purity and quantity for biological testing. To evaluate the scope and generality of our method, we synthesized the majority of the natural AHLs (**7a**−**f**, **8a**−**e**) in good yields and excellent purities in <60 min total reaction times (Table 1). Natural AHLs **8b** (OOHL) and **8d** (ODHL) from *Agrobacterium tumefaciens* and *P. aeruginosa*, respectively, were used as critical control molecules in our later quorum sensing antagonism screens (see below). The high purities of the AHL products overall and the short reaction times used in our approach underscore the value of MW-assisted solid-phase chemistry for AHL synthesis.[8a]

Scheme 1. Microwave-Assisted Solid-Phase Synthetic Route to Natural and Unnatural AHLs[a]



[a] Conditions: a = DIC, HOBT, $CHCl_3$/DMF, MW 50 °C (2 × 10 min); b = DMF, MW 150 °C, 7 min; c = CNBr, TFA, $CHCl_3/H_2O$, MW 60 °C, 30 min.

Table 1. Naturally Occurring AHLs Synthesized via Scheme 1

compound	R^1 or R^2	organism	purity [%][a,b]	yield [%][c]
7a	C_3H_7	*P. aeruginosa*	98	65
7b	C_5H_{11}	*R. leguminosarum*	98	64
7c	C_7H_{15}	*Y. pseudotuberculosis*	98	76
7d	C_9H_{19}	*B. pseudomallei*	97	80
7e	$C_{11}H_{23}$	*S. meliloti*	98	70
7f	$C_{13}H_{27}$	*R. capsulatus*	97	64
8a	C_3H_7	*V. fischeri*	>93	63
8b:OOHL[d]	C_5H_{11}	*A. tumefaciens*	>93	65
8c	C_7H_{15}	*V. anguillarum*	>93	76
8d:ODHL[e]	C_9H_{19}	*P. aeruginosa*	>93	62
8e	$C_{11}H_{23}$	*S. meliloti*	>93	62

[a] Purities of **7a**−**f** determined by integration of GC spectra. [b] Purities of **8a**−**e** determined by ^{1}H NMR. [c] Isolated yields. [d] *N*-3-Oxooctanoyl L-homoserine lactone. [e] *N*-3-Oxododecanoyl L-homoserine lactone.

We next applied our solid-phase route to the parallel synthesis of a small test library of non-natural AHLs (**7g**−**q**, **8f**−**h**). The acyl substituents and stereochemistry of the AHL products were chosen to probe broadly the sterics and functionality present in the AHL binding site of LuxR-type proteins, as revealed in a recent X-ray structure of TraR from *A. tumefaciens*.[13] Our synthetic route again proved robust and delivered the non-native AHLs in good yields (ca. 70%) and high purities (>93%) (Table S-1, Supporting Information).

The AHL library was screened in two well-characterized bacterial reporter strains for antagonism of quorum sensing: *P. aeruginosa* PAO-JP2(p*lasI*-LVAgfp)[14] and *A. tumefaciens* WCF47(pCF372).[6c] Examination of these strains was valuable for two reasons: (1) the direct clinical relevance of *P. aeruginosa*, and (2) the extensive body of biochemical and structural data for TraR in *A. tumefa-*

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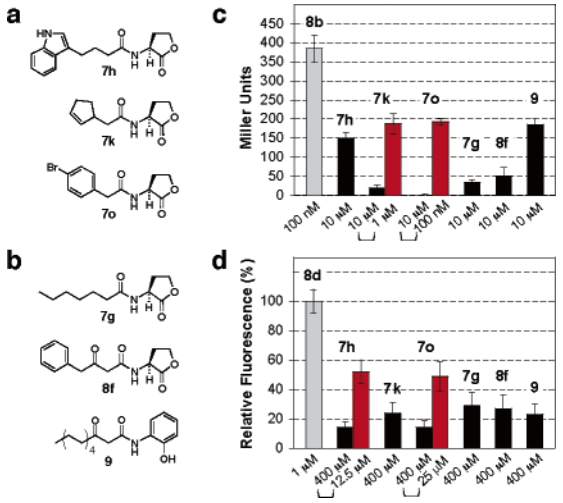

Figure 1. (a) Quorum sensing antagonists identified in this study (**7h**, **7k**, and **7o**). (b) Known quorum sensing antagonists (**7g**, **8f**, and **9**).[4c,6b,c] (c) *A. tumefaciens* reporter strain agonism (gray bar: 100 nM **8b**) and antagonism data (red and black bars: against 100 nM **8b**). Miller units report relative β-galactosidase activity. (d) *P. aeruginosa* reporter strain agonism (gray bar: 1 μM **8d**) and antagonism data (red and black bars: against 1 μM **8d**). Error bars reflect at least three experiments.

ciens.[13] Both of these reporter strains lack their native AHL synthases, yet retain active LuxR-type receptors (LasR and TraR proteins, respectively); exogenous ligand is required for receptor activation, which can be measured by fluorescence (green fluorescent protein (GFP) for LasR) or absorbance (via β-galactosidase activity for TraR) measurements.[15]

The antagonism screens revealed a suite of new quorum sensing inhibitors. In these experiments, the strains were treated with non-native AHL in the presence of native AHL ligand (**8b** or **8d**), and a reduction in absorbance or fluorescence signal indicated that the non-native AHL was able to antagonize LuxR-type protein activity. Three compounds (**7h**, **7k**, and **7o**) showed significant activity against TraR in *A. tumefaciens* and were 1−2 orders of magnitude more active than the previously reported LuxR-type protein antagonists examined as controls (**7g**,[6c] **8f**,[6b] and **9**[4c] at 10 μM; Figure 1a−c). Impressively, bromophenyl AHL **7o** displayed 50% inhibition at an equimolar concentration of **8b** (100 nM). Interestingly, the same three ligands were also identified as potent antagonists against LasR in *P. aeruginosa* (Figure 1d). Here, indol AHL **7h** and bromophenyl AHL **7o** were 2-fold as active as the three controls (at 400 μM), with indol AHL **7h** displaying 50% inhibition at a 12.5:1 ratio with native ligand **8d**.[16] Notably, all three ligands contain bulky, hydrophobic acyl groups. This structural similarity, coupled with their cross activity, suggests that the ligands could bind the TraR and LasR receptors in analogous manners; efforts to characterize these interactions are currently underway in our laboratory.

As biofilm formation is largely under the control of LasR in *P. aeruginosa*,[2,3,14] we hypothesized that antagonists **7h** and **7o** could disrupt *P. aeruginosa* biofilm formation. We performed standard static biofilm assays using a *P. aeruginosa* (PAO1(pLVAgfp)) strain that constitutively produces GFP to facilitate visualization.[14] Biofilms were grown in the presence of ligand (50 μM) for 48 h and visualized using scanning laser confocal microscopy (Figure 2). The treated biofilms were significantly less fluorescent relative to the untreated control, which indicates that the treated biofilms have reduced cell densities and are weakly organized.[2,14] These data show that compounds **7h** and **7o** strongly inhibit *P. aeruginosa* biofilm formation. This finding is important; few inhibitors of bacterial biofilm formation are known, yet such compounds should

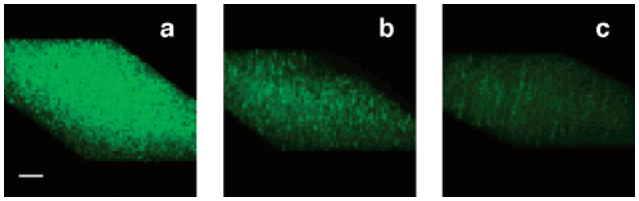

Figure 2. Composite 3-D micrographs of *P. aeruginosa* biofilms grown on glass slides after 48 h in the presence of synthetic ligands (at 50 μM). Scale bar = 50 μm. (a) Untreated. (b) Compound **7h**. (c) Compound **7o**.

have direct clinical impact.[2,3] The discovery of potent inhibitors from a small library highlights the potential utility of focused combinatorial methods for the discovery of additional small molecule modulators of quorum sensing.

In summary, we have developed a robust synthetic route to AHL autoinducers that provides access to both natural and unnatural AHLs in high purity. We have implemented this route to identify a set of non-native AHLs that are among the most potent inhibitors of bacterial quorum sensing reported to date. These molecules represent powerful new tools to elucidate the role of bacterial communication in pathogenesis.

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Supporting Information Available: Full details of solid-phase synthesis, compound characterization, and assay protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) See Supporting Information for full dose response data and IC50 values at 100 nM **8b** in *A. tumefaciens* and 1 μM **8d** in *P. aeruginosa*.

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