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Synthesis and application of an N-acylated L-homoserine lactone derivatized affinity matrix for the isolation of quorum sensing signal receptors

Thanit Praneenararat, Teresa M. J. Beary, Anthony S. Breitbach, Helen E. Blackwell *

Department of Chemistry, University of Wisconsin–Madison, 1101 University Avenue, Madison, WI 53706-1322, USA

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ABSTRACT

The design and synthesis of an agarose resin functionalized with a Gram-negative quorum sensing (QS) signaling molecule analogue is described. The modified resin was utilized in affinity pull-down assays to successfully isolate QscR, a LuxR-type QS receptor from *Pseudomonas aeruginosa*. This resin may facilitate the identification of novel QS signal receptors using affinity chromatography techniques.

Bacteria can regulate specific phenotypes as a function of their cell density by sensing the concentration of small molecule chemical signals. This small molecule signaling process is known as quorum sensing (QS), and has attracted considerable interest from the microbiology, chemical biology, and drug discovery communities.[1–5] Gram-negative bacteria use N-acylated L-homoserine lactones (AHLs, Fig. 1A) as their primary QS signals,[6–8] which are produced by synthase proteins (LuxI-type proteins) and are perceived by cytoplasmic transcription factors (LuxR-type proteins).[9] In general, AHLs are cell permeable and their concentration increases with increasing cell density. Once a threshold AHL concentration is achieved, productive AHL:LuxR-type receptor binding occurs. These complexes typically dimerize, associate with DNA, and initiate the transcription of the genes critical for density-dependent phenotypes. Among the diverse phenotypes regulated by this process are biofilm formation in pathogens like *Pseudomonas aeruginosa*,[10,11] bioluminescence in the symbiont *Vibrio fischeri*,[12,13] and root nodulation in mutualists like the *Rhizobia* spp.[14] Due to the impact of these QS-related phenotypes in fields from healthcare to agriculture, there is considerable interest in developing new approaches for modulating and evaluating the chemical dialogue among bacteria.[15,16] Chemical approaches to intercept bacterial QS, whether by blocking AHL:LuxR-type receptor binding or sequestering/degrading AHL signals, have become prominent techniques in the field.[17–21]

Chemical tools for evaluating the biochemical interactions between AHLs and LuxR-type proteins, as well as for the identification of novel AHL receptors, would be useful for a broad range of experiments.[16,22,23] LuxR-type proteins have proven challenging to characterize using standard biochemical and structural techniques (largely due to their instability in the absence of AHL ligands), and this has limited our understanding of how they perceive both native and nonnative AHL signals.[9,24] In addition, increasing evidence suggests that AHLs from one bacterial species can induce phenotypic changes in neighboring species, including other Gram-negative bacteria[25] and potentially even their eukaryotic hosts.[26] This implies that bacteria may have more extensive networks of communication pathways, both at the inter-species and the inter-kingdom levels. Therefore, identifying new receptors for AHLs may lead to a deeper understanding of these phenomena.

Affinity chromatography could provide solutions for several of the challenges described above.[27] In this methodology, small molecules of interest are chemically modified and covalently bound to an insoluble solid support, typically agarose or sepharose resin. These modified resins are then incubated with samples containing

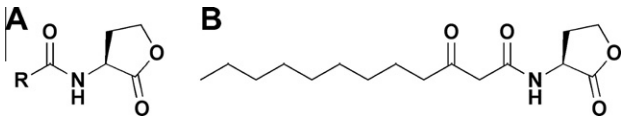

Figure 1. (A) Generic structure of an N-acylated L-homoserine lactone (AHL); (B) *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL), a primary QS signal in *P. aeruginosa*.

* Corresponding author. Tel.: +1 608 262 1503; fax: +1 608 262 4534.
E-mail address: blackwell@chem.wisc.edu (H.E. Blackwell).

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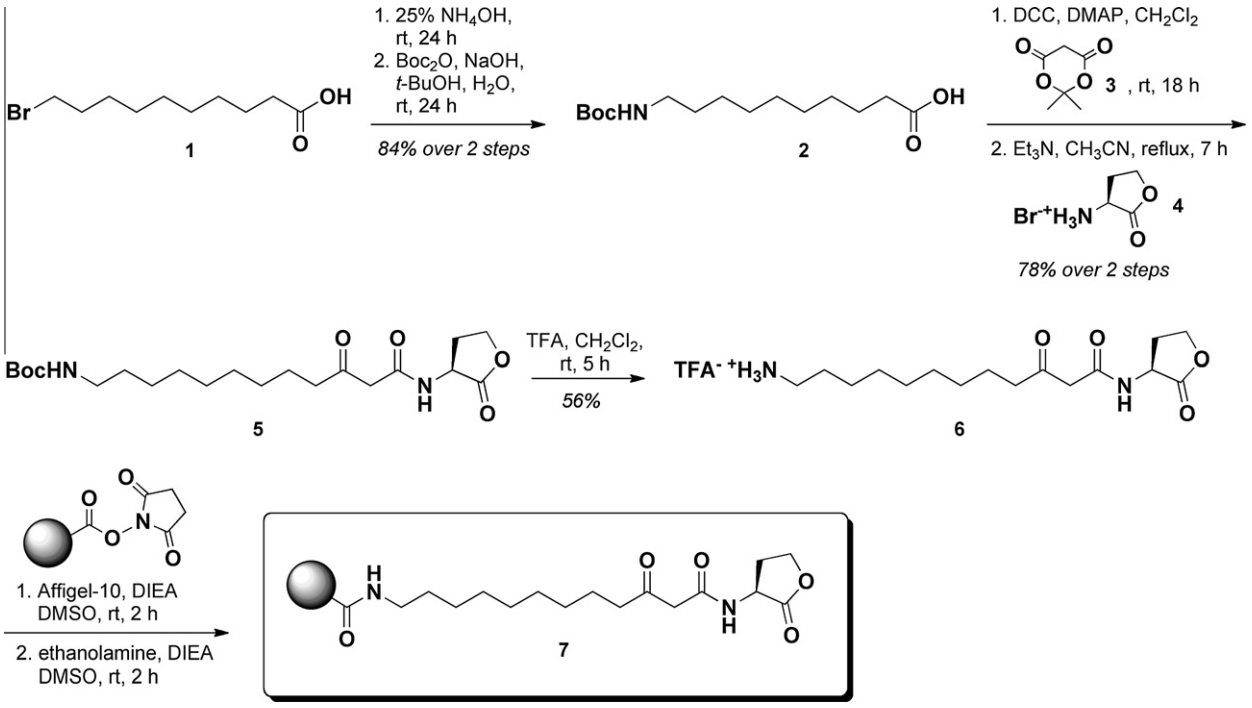
putative biological targets. Theoretically, small molecule moieties on the resin should only bind to their specific protein partners, permitting the latter to be retained throughout resin washing steps. Isolated proteins can then be eluted, analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and identified using mass spectrometry (MS) or other techniques. This strategy has successfully identified several small-molecule target proteins, including histone deacetylase[28] and the FK-506 binding protein (FKBP).[29] Follow-up studies with these molecular targets have contributed to the overall understanding of their associated biological processes.

To date, the design of affinity matrices to assist in the identification of QS-related protein targets has been limited. Certain matrices and related chemical tools are relevant, however, and are introduced briefly here. Spring and co-workers have utilized an azithromycin-modified sepharose resin to identify its biological targets in *P. aeruginosa*.[30] Sub-bacteriocidal doses of azithromycin have been shown to inhibit biofilm formation in *P. aeruginosa*, and because biofilm formation and QS are linked in this pathogen, QS pathways would appear to be a plausible target for azithromycin. Interestingly, analysis of the azithromycin–sepharose resin showed that many of the captured targets were ribosomal proteins, which suggests *P. aeruginosa* biofilm inhibition via azithromycin occurs through the ribosome as opposed to QS pathways. Concurrently, Spring and co-workers have developed an elegant 3D small-molecule microarray system[31] that allows for the printing of AHL derivatives in a spatially addressable format.[32] These arrays consist of reactive polymer gel matrices, and can be readily derivatized with AHL analogues containing nucleophilic groups at their acyl chain termini. The 3D arrays have proven useful for the screening of new AHL analogues for LuxR-type protein binding. In related work, the Meijler group has synthesized an AHL probe equipped with both a photoactive diazirine and 'click' chemistry reactive alkyne.[22] In this proof-of-concept work, they demonstrated that their AHL probe binds to LasR, a prominent LuxR-type protein in *P. aeruginosa*, with only a slight decrease in activity relative to LasR's native AHL, *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL; Fig. 1B). The authors also demonstrated that UV exposure leads to covalent attachment of the AHL probe to LasR through the diazirine moiety. These results suggest that the alkyne moiety could be used to further react the AHL-LasR complex with an affinity resin via click chemistry, and the authors highlight this possibility as a goal for future work. Lastly, motivated by reports of the immunomodulatory effects of OdDHL in humans,[33] Seabra et al. prepared two OdDHL-derivatized resins and applied them for the identification of human receptors for AHLs.[34] The OdDHL ligand was appended to the matrix through either the acyl chain termini or a mid-chain branch point using a piperazine linker. Two proteins were isolated using these matrices, but neither appeared to have immunological relevance.

Inspired by these recent studies, we sought to apply the affinity chromatography technique in our own QS research with the ultimate goal of developing a new tool to aid in the discovery of novel QS targets. To the best of our knowledge, the application of AHL-derivatized affinity resins for the identification of LuxR-type receptors has not been reported to date. Herein, we describe our initial studies toward the design, synthesis, and application of an AHL-derivatized agarose matrix. Affinity pull-down assays using this new AHL-functionalized matrix confirmed the binding of QscR, an 'orphan' LuxR-type protein from *P. aeruginosa*,[35] to the matrix. These results serve to demonstrate the feasibility of utilizing AHL-derivatized affinity matrices for LuxR-type receptor isolation and characterization.

To guide our design of an appropriate ligand for matrix derivatization, we first scrutinized the AHL:receptor binding interactions in the three reported structures of AHL:LuxR-type proteins (TraR, LasR, and SdiA).[36–40] Each of these structures shows a series of conserved hydrogen-bonding interactions between the AHL lactone ring and residues in the binding pocket of the receptor. To prevent disruption of these key interactions, we decided that attachment of an AHL to the resin matrix should occur through the acyl tail as opposed to the lactone head group. Such an attachment strategy was success-



Scheme 1. Synthesis of OdDHL-derivatized resin **7**. Abbreviations: Boc = *tert*-butoxycarbanoyl, DCC = *N*,*N*′-dicyclohexylcarbodiimide, DMAP = 4-(dimethylamino)pyridine; TFA = trifluoroacetic acid; DIEA = diisopropylethylamine.