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Antibody Interference with *N*-Acyl Homoserine Lactone-Mediated Bacterial Quorum Sensing

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Cell density-dependent coordination of gene expression in bacteria has been termed "quorum sensing" (QS).[1] *N*-Acyl L-homoserine lactones (AHLs) are produced by over 70 species of Gram-negative bacteria, and structural differences within AHLs occur in the length and oxidation state of the acyl side chain (Figure 1). Upon reaching a critical threshold concentration, AHLs bind to their cognate receptor proteins, triggering the expression of target genes. AHLs have been shown to play an important role in the establishment and course of bacterial infections. One example of a Gram-negative pathogen that employs AHL-based QS to regulate the expression of its pathogenicity factors is *Pseudomonas aeruginosa*. This common environmental microorganism has acquired the ability to take advantage of weaknesses in the host defenses to become an opportunistic pathogen in humans. Most prominent is the role of *P. aeruginosa* in patients suffering from cystic fibrosis (CF). Over the last 15 years, much progress has been made in elucidating the molecular mechanisms underlying *P. aeruginosa* pathogenicity.[2] Two different AHLs, *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C_{12}-AHL) and *N*-butyryl homoserine lactone (C_4-AHL), have been identified as QS signaling molecules in *P. aeruginosa*. Genes regulated by this QS mechanism encode enzymes such as elastases A and B, catalase, and superoxide dismutase as well as exotoxins.[2]

Interference with QS signaling has been suggested as a new approach for anti-infective therapy.[3] In fact, this strategy has yielded interesting results using AHL analogues as QS antagonists in *P. aeruginosa*.[4] Alternatively, we have embarked on a program utilizing antibodies to inhibit AHL-mediated quorum sensing signaling in *P. aeruginosa*. AHL-based QS systems represent an ideal target for antibody-based anti-infective therapy given the highly conserved molecular scaffold and extracellular distribution of AHLs.

Our initial hapten design for the elicitation of anti-AHL antibodies focused on synthesizing a set of molecules highly congruent in structure to AHLs while also possessing a pendant carboxylic acid functionality that would enable carrier proteins BSA or KLH conjugates to be easily accessed. However, we were aware that such molecules might also be prone to hydrolysis (ring-opened products) under conditions required for chemical coupling and also immunization. Thus, to guide our hapten design, the stability of several AHLs under physiological conditions was investigated; we synthesized a number of AHL analogues and their corresponding ring-opened hydrolysis products, both of which contained a 4-methoxyphenyl amide group that allowed for a detailed investigation into the rate of hydrolysis of the AHL analogues using HPLC with UV detection (Figure 2).

Each compound was assayed for its hydrolysis rate in phosphate buffer saline (PBS), pH 7.2, at a concentration of 200 μM at 37 °C. The half-lives of each of the lactones varied from 13.7 to 18.1 h. Interestingly, the oxidation state and chain length did not influence significantly the hydrolytic rate of these molecules.[5] While the expected ring-opened hydrolysis product was produced, a previously undetected side product, a tetramic acid, arising from an intramolecular Claisen-like condensation reaction was also uncovered.[5] On the basis of these results and the insufficient long-term stability of compounds **1**−**4**, our choice was to replace the lactone moiety with a lactam functionality. Thus, the lactam ring would grant more chemical stability toward ring opening/hydrolysis under basic conditions, as well as stability toward the intramolecular Claisen condensation (vide supra).

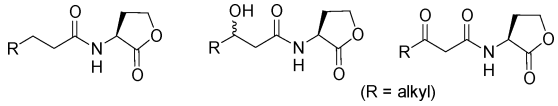

Figure 1. Examples of *N*-acyl homoserine lactones employed by bacteria as quorum sensing molecules.

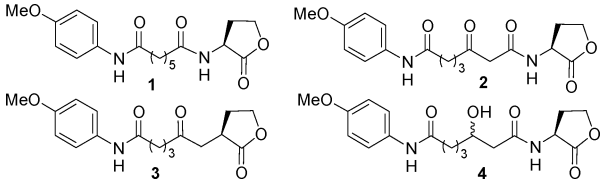

Figure 2. 4-Methoxyphenyl amide AHL analogues.

The amino lactam **6** surrogate was synthesized from commercially available (*S*)-2,4-diaminobutyric acid **5** according to literature precedent.[6] To enhance our chances for the elicitation of antibodies that could differentiate AHL chain length and oxidation state, different linker moieties were designed in recognition of AHLs employed by different bacteria; thus, the linkers of **7**−**9** that mimic the acyl chains of three types of autoinducers were prepared and coupled to lactam **6**, yielding haptens **7**−**9** (Scheme 1). These three haptens were conjugated to carrier proteins KLH and BSA, resulting in 18−23 haptens per carrier protein. Balb/c mice were immunized with the KLH immunoconjugates for hybridoma production using standard protocols. All immunizations resulted in excellent titers (25600−12800), and based on ELISA results, 20 monoclonal antibodies (mAbs) for RS1 (**7**), 21 for RS2 (**8**), and 27 for RS3 (**9**) were selected and purified. The affinities for all mAbs against a series of AHLs (C_4, 3-oxo-C_6, and 3-oxo-C_{12}) as well as the 3-oxo-C_{12}-lactam were determined using competition ELISA (Supporting Information). None of the assayed RS1 antibodies possessed a K_d lower than 100 μM for the 3-oxo-AHLs, nor for the lactam analogue. However, several of the RS2 mAbs generated against the 3-oxo-hapten demonstrated good to excellent affinity (K_d = 150 nM to 5 μM) for 3-oxo-C12-AHL and the lactam analogue and exhibited high specificity as they did not show any recognition of the short chain 3-oxo-AHLs. Finally, the RS3 mAbs exhibited

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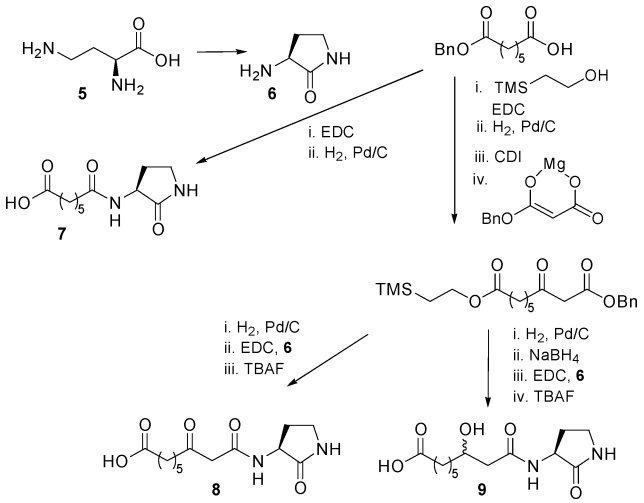
Scheme 1. Synthesis of Lactam-Containing Haptens



low to moderate affinity ($K_d = 10-50\ \mu M$) to the short-chain 3-oxo-AHLs and virtually no recognition of 3-oxo-C12-AHL nor the lactam.

In total, the overall immune response seen for haptens **7**−**9** might be construed as only moderately effective. However, we note that this is one of the few demonstrations of the generation of tight binding antibodies to structures of basically aliphatic composition with no aromaticity, charge, and few hydrogen bond donor/acceptor opportunities.[7] All of these interactions are normally considered critical for the generation of antibodies with high affinity and specificity.

To investigate potential disruption of bacterial cell−cell communication, five RS2 mAbs and one unrelated control mAb were selected and evaluated for their ability to inhibit 3-oxo-C_{12}-AHL-mediated QS signaling in a green fluorescent protein (GFP) reporter assay using the wild-type *P. aeruginosa* PAO strain and the double knockout PAO-JM2 (Δ*rhlI*, Δ*lasI*) strain. 3-oxo-C12-AHL (either endogenously (PAO) produced or exogenously (PAO-JM2) added) is required for reporter gene transcription activation, which in turn can be measured as the level of GFP fluorescence.

The mAb RS2-1G9 exhibited excellent inhibition of QS signaling in both *P. aeruginosa* strains, while all other tested mAbs show little or no inhibition (Figure 3A). Excitingly, RS2-1G9 is the first reported antibody to show an inhibitory effect on QS signaling in both wild-type and mutant *P. aeruginosa* PAO cells. Furthermore, in our evaluation of mAb RS2-1G9 for its ability to prevent virulence factor expression, we observed a clear inhibitory effect in both PAO and PAO-JM2 cultures on the production of pyocyanin, a QS-controlled virulence factor in *P. aeruginosa* (Figure 3B).[4]

In summary, we have demonstrated the validity of our hapten design strategy to obtain specific mAbs to AHLs. One mAb, RS2-1G9, possesses the ability to inhibit 3-oxo-C_{12}-AHL-based QS signaling. This antibody may be envisioned as a tool for future investigations into 3-oxo-C_{12}-AHL-based QS, which may aid in gaining new insights into the pathogenesis of *P. aeruginosa* and may ultimately lead to the development of new strategies to combat bacterial diseases.

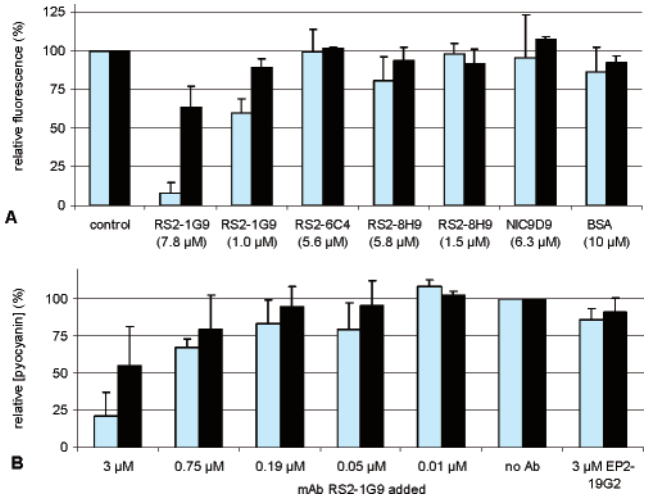


Figure 3. Inhibition of 3-oxo-C12-AHL-mediated QS signaling by RS2 mAbs and control mAb in *P. aeruginosa* PAO (wild-type, filled columns) and in PAO-JM2 (open columns) in a GFP reporter assay (A). Inhibition of pyocyanin production in *P. aeruginosa* PAO (wild-type, filled columns) and in PAO-JM2 (open columns) by mAb RS2−1G9 (B). Anti-nicotine and anti-stilbene mAbs (NIC9D9 and EP2-19G2) and BSA were used as additional controls.

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Supporting Information Available: Experimental procedures for the syntheses of compounds **1**−**9** and bioassays. This material is available free of charge via the Internet at http://pubs.acs.org.

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