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Efficient Synthesis and Evaluation of Quorum-Sensing Modulators Using Small Molecule Macroarrays

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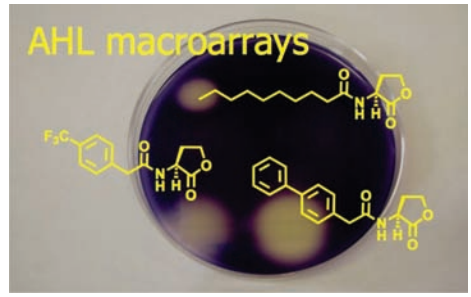
ABSTRACT



A method for the synthesis of small molecule macroarrays of *N*-acylated L-homoserine lactones (AHLs) is reported. A focused library of AHLs was constructed, and the macroarray platform was found to be compatible with both solution and agar-overlay assays using quorum-sensing (QS) reporter strains. Several QS antagonists were discovered and serve to showcase the macroarray as a straightforward technique for QS research.

Bacteria can assess their local population densities and environment using a cell−cell signaling process called quorum sensing (QS).[1] This phenomenon allows bacteria to coordinate group behavior and is widespread in bacteria. Interest in QS is intense and growing due to the critical role of this signaling process in pathogenic and symbiotic host/bacteria relationships. Since its discovery, QS has sparked the attention of chemists due to its reliance on a chemical "language" of small molecule and peptide signals, or autoinducers, and their cognate signal receptors.[2] Indeed, this reliance provides unique opportunities for chemists to manipulate and study QS at the molecular level.

In general, bacterial cell density correlates with autoinducer concentration. Once a threshold concentration is reached, productive autoinducer−receptor binding occurs, and this binding event controls the expression of myriad genes involved in bacterial group behaviors. Pertinent QS phenotypes include virulence factor production, biofilm formation, and swarming in pathogens, and bioluminescence and root nodulation in symbionts. Methods to block or promote QS would allow for the attenuation of these diverse phenotypes, and considerable recent research has focused on the design of non-native molecules that can intercept QS signaling pathways.[3] Much of these efforts have been directed at QS in Gram-negative bacteria,[4] and our laboratory has reported several families of non-native autoinducer mimics capable

(1) For reviews of QS, see: (a) Camilli, A.; Bassler, B. L. *Science* **2006**, *311*, 1113–1116. (b) Fuqua, C.; Parsek, M. R.; Greenberg, E. P. *Annu. Rev. Genet.* **2001**, *35*, 439–468.

(2) Eberhard, A.; Burlingame, A. L.; Eberhard, C.; Kenyon, G. L.; Nealson, K. H.; Oppenheimer, N. J. *Biochemistry* **1981**, *20*, 2444–2449.

(3) (a) Bjarnsholt, T.; Givskov, M. *Phil. Trans. R. Soc. B* **2007**, *362*, 1213–1222. (b) George, E. A.; Muir, T. W. *ChemBioChem* **2007**, *8*, 847–855.

(4) For recent reviews, see: (a) Geske, G. D.; O'Neill, J. C.; Blackwell, H. E. *Chem. Soc. Rev.* **2008**, *37*, 1432–1447. (b) Welch, M.; Mikkelsen, H.; Swatton, J. E.; Smith, D.; Thomas, G. L.; Glansdorp, F. G.; Spring, D. R. *Mol. Biosyst.* **2005**, *1*, 196–202.

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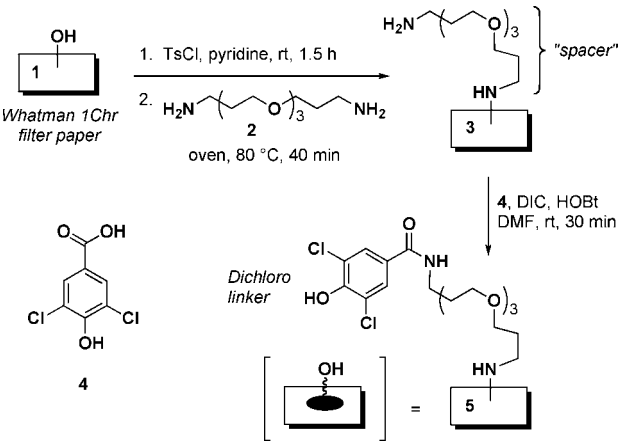
of antagonizing and agonizing QS in these organisms.[5] Potent QS modulators still remain scarce, however. The continued growth of this area demands the development of efficient design, synthesis, and screening strategies for the identification of new QS modulators. Here, we report the application of the small molecule macroarray platform to the synthesis and screening of non-native *N*-acylated L-homoserine lactones (AHLs) for QS modulators. A macroarray of AHLs was constructed using an efficient cyclization-cleavage strategy and demonstrated to be compatible with on- and off-support QS reporter gene assays. This work provides an expedient new method for QS probe discovery.

Our laboratory has been developing the small-molecule macroarray as a tool for chemical biology research for several years.[6] This technique involves the spatially addressed synthesis of small molecules on planar cellulose supports (0.3 cm^2 spots). Libraries of ~10−1000 can be made routinely in a day, typically on a 10−100 μg/compound scale. The benefits of the small molecule macroarray include ease of manipulation, reduced reagent use, inexpensive support systems, and compatibility with a diverse range of biological assays either performed on or off of the support. We recently demonstrated the use of macroarrays for the discovery of antibacterial small molecules; this was facilitated by straightforward bacterial agar-overlay assays performed directly on the array surface.[7] We sought to broaden the scope of the macroarray approach, and in the current study investigated the feasibility of non-native AHL synthesis *and* screening on the array platform.

AHLs are the primary class of QS signaling molecules used by Gram-negative bacteria, and are sensed by LuxR-type receptors.[8] Non-native analogs of these ligands were among the first synthetic molecules evaluated for QS activity.[3a,4] The majority of these analogs retain the native L-HL headgroup yet contain non-native acyl groups. We previously discovered that phenylacetyl derivatives of this type (PHLs) were extremely potent QS modulators in a range of species.[5] Cinnamyl HLs (CHLs), while less studied, have also been found to have interesting QS activity profiles.[9] We therefore decided to focus largely on PHLs and CHLs in this proof-of-concept study.

In designing our solid-phase synthesis strategy on planar cellulose support, we desired a "traceless" linker that would provide AHLs after cleavage that contained no structural feature originating from the linker. In addition, we needed a cleavage procedure that yielded products with only volatile byproducts in order to permit on-support bacteriological assays. These requirements led us to select a linker compatible with an acid-mediated, tandem lactonization-cleavage reaction to release the AHLs from the support.[10] Careful optimization revealed that a dichlorophenol-type linker facilitated such a cleavage step, and our AHL synthesis route is outlined in detail below (Schemes 1 and 2).

Scheme 1. Planar Support Modification and Linker Installation[a]



[a] TsCl = tosyl chloride; DIC = *N*,*N*′-diisopropylcarbodiimide; HOBt = 1-hydroxybenzotriazole; DMF = *N*,*N*′-dimethylformamide.

Macroarray synthesis commenced with readily available Whatman 1Chr filter paper (**1**; Scheme 1). Grids (1.5 × 1.5 cm) were drawn on paper sheets, and the sheets were subjected to blanket tosylation using previously reported methods.[7,11,12] Blanket displacement of the primary tosyl groups by subjection to neat 4,7,10-trioxa-1,13-tridecanediamine **2** (with gentle heating in a laboratory oven) yielded spacer-functionalized support **3**. This support was synthesized routinely on a multisheet scale and found to be stable at room temperature (rt) for months. Immediately prior to use, the linker unit, 3,5-dichloro-4-hydroxybenzoic acid **4**, was coupled to support **3** in a spatially addressed format (spotting at each grid intersection)[12] using standard carbodiimide conditions (DIC) to give linker-derivatized support **5**.

The first step in AHL synthesis was the spatially addressed esterification of *N*-Fmoc-OTrt-L-homoserine (Hse; **6**) with support **5** using CDI and NMI at rt (spotting repeated 3×; Scheme 2).[13] "Capping" of any unreacted phenols (and residual alcohols or amines) via blanket acetylation yielded support **7**. Thereafter, the loading of Hse (**6**) onto the support was evaluated by UV Fmoc quantification. Test spots of the support were subjected to 4% DBU in DMF (15 min at rt), and UV analysis revealed Hse (**6**) loadings of ~100 nmol

(5) (a) Geske, G. D.; O'Neill, J. C.; Miller, D. M.; Wezeman, R. J.; Mattmann, M. E.; Lin, Q.; Blackwell, H. E. *ChemBioChem* **2008**, *9*, 389–400. (b) Geske, G. D.; O'Neill, J. C.; Miller, D. M.; Mattmann, M. E.; Blackwell, H. E. *J. Am. Chem. Soc.* **2007**, *129*, 13613–13625. (c) Geske, G. D.; O'Neill, J. C.; Blackwell, H. E. *ACS Chem. Biol.* **2007**, *2*, 315–320.

(6) Blackwell, H. E. *Curr. Opin. Chem. Biol.* **2006**, *10*, 203–212.

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(8) For a review of AHL-LuxR-type QS, see ref 4 and: Lazdunski, A. M.; Ventre, I.; Sturgis, J. N. *Nat. Rev. Microbiol.* **2004**, *2*, 581–592.

(9) Palmer, A. G.; Blackwell, H. E. *Nat. Chem. Biol.* **2008**, *4*, 452–454.

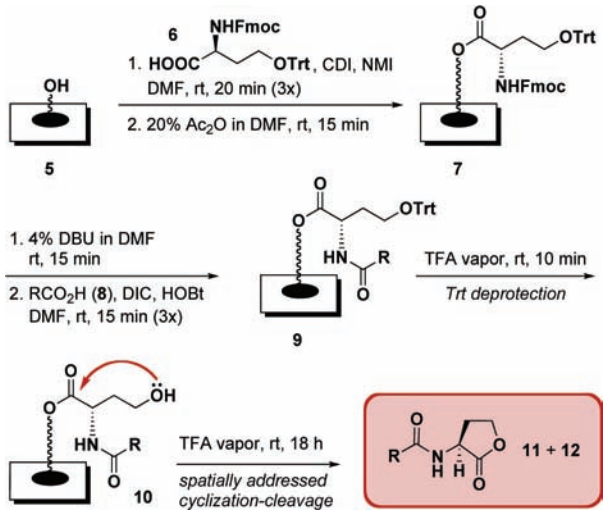
(10) For other examples of cyclization−cleavage routes to γ-lactones, see: (a) Reference 5. (b) LeHetet, C.; David, M.; Carreaux, F.; Carboni, B.; Sauleau, A. *Tetrahedron Lett.* **1997**, *38*, 5153–5156.

(11) (a) Bowman, M. D.; Jeske, R. C.; Blackwell, H. E. *Org. Lett.* **2004**, *6*, 2019–2022. (b) Lin, Q.; O'Neill, J. C.; Blackwell, H. E. *Org. Lett.* **2005**, *7*, 4455–4458.

(12) "Blanket" reactions involve full submersion of the support in reagents. "Spotted" reactions involve delivery of reagents onto support.

(13) This method is modified from the following report. Ay, B.; Volkmer, R.; Boisguerin, P. *Tetrahedron Lett.* **2007**, *48*, 361–364.

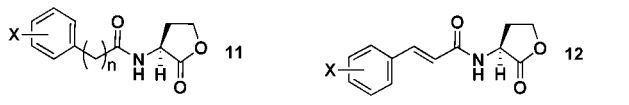
Scheme 2. AHL Synthesis on Planar Support **5**[a]



[a] CDI = 1,1′-carbonyldiimidazole; NMI = *N*-methylimidazole; DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene.

per spot. This loading level was comparable to other small-molecule macroarray syntheses.[6,7,11]

Support **7** was next subjected to blanket Fmoc deprotection with DBU/DMF, preparing it for acylation with various carboxylic acids (**8**; Scheme 2). We chose 26 phenylacetic and cinnamic acids (with one exception) as our acid building blocks, which were readily coupled onto the support in a spatially addressed manner using DIC at rt to generate support **9**. Notably, we selected a subset of acids in this step that would yield several PHLs and one CHL previously studied by our laboratory (Figure 1); this choice was



11a: X = H
11b: X = 4-Br
11c: X = 3-Br
11d: X = 2-Br
11e: X = 4-I
11f: X = 3-I
11g: X = 2-I
11h: X = 4-NO_2
11i: X = 3-NO_2
11j: X = 2-NO_2
11k: X = 4-CF_3
11l: X = 4-Ph
11m: X = 4-Br **

12a: X = H
12b: X = 4-F
12c: X = 3-F
12d: X = 2-F
12e: X = 2,6-F_2
12f: X = F_5
12g: X = 4-Cl
12h: X = 2-Br
12i: X = 4-NO_2
12j: X = 3-NO_2
12k: X = 2-NO_2
12l: X = 4-Br-2-F
12m: X = 4-Cl-3-NO_2

Figure 1. Structures of the PHL (**11**) and CHL (**12**) macroarray products. **For **11**, $n = 1$ except for **11m** ($n = 2$).

advantageous for two reasons: it would (1) allow us to directly compare the macroarray synthesis route to our earlier AHL synthetic route that utilized traditional, polystyrene solid support,[5] and (2) provide built-in controls for our later development of QS bioassays performed on and off of the array surface.

The final steps in AHL macroarray synthesis were the acid-mediated stepwise deprotection of the Trt group of **9**, followed by cyclization−cleavage to give the PHL and CHL products (**11** and **12**; Scheme 2). We sought to perform the latter step using acid vapor, as this would yield the PHLs and CHLs as spatially addressed (yet non-covalently bound) arrays on the support after cleavage and amenable to bacteriological agar overlay assays. Further, our previous studies had shown that such vapor-phase cleavage steps on intact macroarrays are operationally straightforward.[6,7] Significant optimization work revealed that both the deprotection and the cleavage could be performed sequentially using TFA vapor in a vacuum desiccator. A 10 min treatment was sufficient to cleave the Trt group of support **9** with minimal premature lactonization. The support (**10**) was then washed and dried prior to a longer TFA vapor treatment (18 h, rt) to generate AHLs **11** and **12** (Figure 1). Any residual TFA was neutralized by subjecting the array to NH_3 (generated from NH_4OH) and subsequent drying at rt.

The purities of the AHL library members were assessed by punching out spots, elution, and either GC−MS or HPLC analysis. Compound purity was excellent (≥93%), reflecting the advantage of the cyclization−cleavage strategy. To analyze the isolated yields of AHLs, 4-bromo PHL (**11b**) was selected as a representative compound. Comparison of the quantity of **11b** cleaved from the macroarray to a calibration curve generated from an authentic sample of **11b** indicated 40 nmol (∼10 μg) of product was formed per spot (∼40% yield based on Hse Fmoc analysis; see the Supporting Information). Qualitative analysis of the GC and HPLC data suggested that this conversion level could be approximated for all 26 library members. The cleavage step was determined to be the primary reaction reducing the overall product yield. However, longer reaction times, or resubjection of the array to cleavage conditions, failed to give higher yields of product.[14] In comparison to our previous solid-phase synthetic route to AHLs (incorporating a CNBr-mediated lactonization-cleavage),[5] the macroarray route gave comparable if not higher product purities yet reduced yields (by ∼10−20%). The absence of toxic CNBr in the current route, however, coupled with the ease of planar support manipulation versus beads, is advantageous. Importantly, the quantities of AHLs isolated from the macroarray were more than sufficient for biological assays (see below).

For quality control, we first examined the biological activities of a subset of the cleaved PHLs (**11e**, **11i**, **11l** and **11m)** in solution-phase bacterial reporter gene assays. Stock solutions (∼0.5 mM) were prepared in DMSO, and examined in a reporter strain of the marine symbiont *Vibrio fischeri* (ES114 (Δ-*luxI*)) that only luminesces in the presence of QS activators.[5c] Antagonistic QS activities (versus the native AHL signal for *V. fischeri*, *N*-3-oxo-hexanoyl HL (OHHL)) were assessed at ∼5 μM and compared to authentic samples[5] of each PHL at 5 μM. The activities of the macroarray-derived PHLs were comparable (±5%) to that of authentic PHL samples (see Figure S-4, Supporting Information), indicating both that our estimation of macroarray compound yields was reasonable and that the samples were chemically identical. Further, these results demonstrate the compatibility

(14) For comparison, solution-phase cleavage of spots (50% TFA in CH_2Cl_2 for 18 h) gave a 31% isolated yield of **11h.**

of the macroarray products with solution-phase reporter gene assays.[15] Encouraged by these results, we examined the activity of the entire structurally novel CHL library (**12**) in analogous QS antagonism assays, and identified several potent QS antagonists (most notably, F_5-CHL **12f** and 4-Br, 2-F-CHL **12l**; Figure 2). These two CHLs were capable of

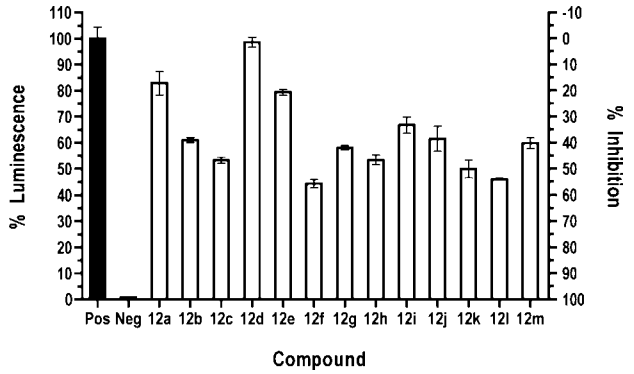


Figure 2. *V. fischeri* QS antagonism solution-phase assay data for CHLs **12**. Assay performed at 1:1 against OHHL; concentration of all compounds ~5 μM. Pos = positive control, OHHL at 5 μM. Neg = negative control, 1% DMSO.

inhibiting QS responses in *V. fischeri* by 50% at 1:1 against OHHL and represent new leads for QS probe development.

We next examined the feasibility of performing QS reporter gene assays on intact, cleaved macroarrays of **11** and **12** in an agar overlay format and focused again on antagonism-type assays. The soil bacterium *Chromobacterium violaceum* (CV026) was selected for these studies, as this organism produces a brilliant violet pigment (violacein) upon QS activation, providing an excellent visual reporter.[16] In this reporter strain, QS inhibitors cause a loss of pigmentation in the presence of its native ligand, *N*-hexanoyl HL (HHL). A molten agar suspension of *C. violaceum* and HHL (1 μM) was added to Petri dishes, the cleaved arrays were submerged in the agar, and the dishes were incubated until white zones were apparent (16−20 h). *N*-Decanoyl HL (DHL), a known inhibitor of *C. violaceum* QS,[16] was also synthesized on the arrays for use as an internal control.

The overlay assay procedure proved successful on the macroarrays and revealed two new QS antagonists in *C. violaceum* (4-CF_3 PHL **11k** and 4-Ph PHL **11l**; Figure 3). PHL **11k** has previously been shown to be a cross-species QS inhibitor,[5] and its activity in *C. violaceum* further supports this activity trend. However, 4-Ph PHL **11l** has exhibited only limited and muted activity in other strains[5]

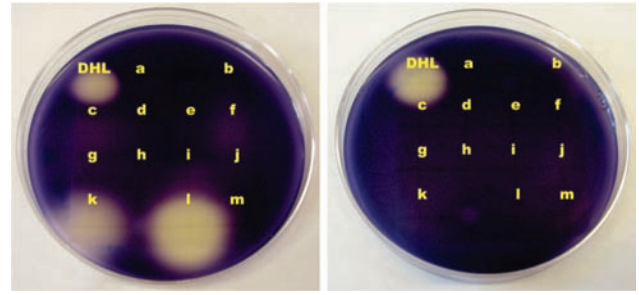


Figure 3. *C. violaceum* QS antagonism overlay assays (against HHL at 1 μM) performed on cleaved PHL (**11a-m**, left) and CHL (**12a-m**, right) macroarrays. DHL = decanoyl HL control; blank areas were punched out and used for loading calculations.

and appeared to be a comparable inhibitor to the control DHL in this overlay assay. Interestingly, in contrast to the *V. fischeri* assay above, none of the CHLs **12** were active in the overlay assay. To better quantitate the activities of **11k**, **11l**, and DHL in *C. violaceum*, we determined their IC_{50} values using a solution-phase violacein production assay (see the Supporting Information).[17] These values indicated that DHL and **11l** had similar inhibitory activities, and **11k** was ~10-fold less active (IC_{50} = 4.12, 4.98, and 40.5 nM, respectively (vs 400 nM HHL)), a trend approximated by visual inspection of the overlay assay data (Figure 3).

In summary, we have developed an efficient synthesis of AHLs on the small molecule macroarray platform and generated a focused library of AHLs in high purities. The macroarray was found to be compatible with both on- and off-support QS assays, and several new QS antagonists were identified in *V. fischeri* (**12f** and **12l)** and *C. violaceum* (**11k** and **11l)**. We anticipate that these methods are compatible with the synthesis and screening of AHL macroarrays of expanded structural diversity and size, and such studies are ongoing in our laboratory.

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Supporting Information Available: Full experimental procedures and characterization data, structures of carboxylic acids **8**, and additional biological assay data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Sufficient compound was generated for ~30 assays per spot.

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