Discovery of a quorum sensing modulator pharmacophore by 3D small-molecule microarray screening†

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Received 22nd June 2010, Accepted 23rd August 2010
DOI: 10.1039/c0ob00300j

The screening of large arrays of drug-like small-molecules was traditionally a time consuming and resource intensive task. New methodology developed within our laboratories provides an attractive low cost, 3D microarray-assisted screening platform that could be used to rapidly assay thousands of compounds. As a proof-of-principle the platform was exploited to screen a number of quorum sensing analogs. Quorum sensing is used by bacterium to initiate and spread infection; in this context its modulation may have significant clinical value. 3D microarray slides were probed with fluorescently labeled ligand-binding domains of the LuxR homolog CarR from *Erwinia carotovora* subsp. *carotovora*. The 3D microarray platform was used to discover the biologically active chloro-pyridine pharmacophore, which was validated using a fluorometric ligand binding assay and ITC. Analogs containing the chloro-pyridine pharmacophore were found to be potent inhibitors of *N*-acyl-homoserine-lactone (AHL) mediated quorum sensing phenotypes in *Serratia* (IC_{50} = ~5 μM) and *Pseudomonas aeruginosa* (IC_{50} = 10–20 μM).

Introduction

Within the fields of medicine and biology small-molecules are valuable tools used to modulate, and hence probe the understanding of a protein's biological function.[1] The discovery of small-molecules with desirable biological properties was traditionally a time and resource intensive approach, requiring the screening of thousands of compounds in well-plate based assays. The microarraying of small-molecules has the potential to provide a faster and cheaper process compared to the more traditional methods. 2D small-molecule microarrays have been used for high throughput protein–binding screening[2] or enzyme assays;[3] however, these screens often lacked sensitivity preventing the identification of weak binders due to surface effects. We have recently shown that 3D small-molecule microarrays display improved loading capacity, signal sensitivity and spot morphology compared with the early 2D approach.[4] As a proof-of-principle we apply our new methodology to efficiently and effectively discover small-molecules that bind to quorum sensing receptors.[5,6]

Bacteria communicate with one another by a process called quorum sensing: a chemical language used to co-ordinate group behavior.[7] Quorum sensing enables bacteria to exploit their environment, this can be a beneficial or negative effect to the surrounding biological system. For example, the Gram negative bacterium *Vibrio fischeri* uses quorum sensing to regulate the production of bioluminescence in order to maintain a symbiotic relationship with certain types of squid, which in turn utilises the light to hunt and mate.[7,8]

Gram negative bacteria such as *V. fischeri* communicate by the production, distribution and detection of a class of small-molecules known as *N*-acyl-homoserine lactones (AHL). In terms of the communication mechanism, 3-oxohexanoyl-L-homoserine-lactone (OHHL, Fig. 1) is free to diffuse in and out of cells and is detected by the LuxR receptor protein. As the localised cell density of *V. fischeri* increases, so does the intracellular concentration of OHHL until a threshold or quorum level is reached. At this point, the LuxR-OHHL complex activates the transcription of certain genes, including those that control bioluminescence.[9] This mode of AHL-mediated quorum sensing is prevalent amongst numberous Gram negative bacteria. For example, *Erwinia carotovora* also uses the signaling pheromone OHHL in addition to hexanoyl-L-homoserine lactone (HHL) to effectively communicate.[10–12] *Pseudomonas aeruginosa* employs both butanoyl-L-homoserine lactone (BHL) and 3-oxododecanoyl-L-homoserine lactone (OdDHL).[13]

P. aeruginosa and *E. carotovora* both employ quorum sensing to initiate and spread infection. *P. aeruginosa* is an opportunistic human pathogen, which uses quorum sensing to infect immune compromised patients such as Cystic Fibrosis (CF) sufferers.[14] In particular, *P. aeruginosa* uses quorum sensing to control the production of biofilms, which protect cell colonies from the host's immune response and antibiotics. This leads to chronic infections, especially in the lungs of a CF patient and ultimately causes premature death. Quorum sensing has therefore emerged as a potential antibacterial target. Several animal models have shown that the spread of infection is reduced when a bacteria's quorum sensing is inhibited, and that the host's immune response is faster when infected with a mutant strain that cannot perform quorum

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† Electronic supplementary information (ESI) available: Full experimental procedures, characterisation data, microarraying details and fluorometric ligand binding assay curves are described. See DOI: 10.1039/c0ob00300j

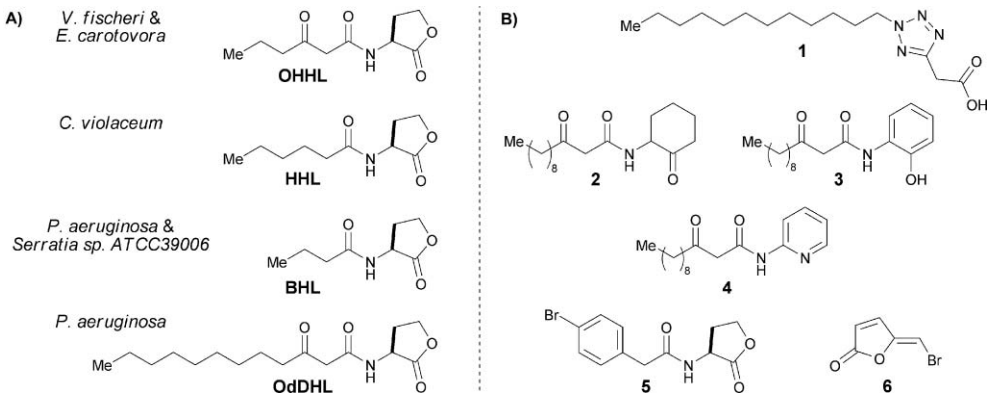


Fig. 1 A) *N*-acyl-homoserine lactones (AHL) used by Gram negative bacteria. B) Inhibitors of quorum sensing in *P. aeruginosa*. OHHL = 3-oxohexanoyl-l-homoserine lactone; HHL = hexanoyl-l-homoserine lactone; BHL = butanoyl-l-homoserine lactone; OdDHL = 3-oxododecanoyl-l-homoserine lactone.

sensing.[15] From a medicinal point of view inhibition of quorum sensing "disarms" bacteria instead of killing them. As a result there is less evolutionary pressure for the bacteria to develop resistance; thus, offering major opportunity to create treatments for multi drug-resistant strains of bacteria.[16,17]

Several quorum sensing blocking strategies exist including: 1) inhibition of the synthase enzyme responsible for the production of the signaling molecule or receptor protein; 2) interference of the chemical signal itself by an antibody;[18] or 3) inhibition of the quorum sensing receptor protein. A number of studies have been published which use the latter approach to inhibit quorum sensing in *P. aeruginosa*. In general, phenotypic based assays have been used to assess the strength of antagonist activity in the presence of the native AHL ligand.

Greenberg and co-workers carried out a well plate screen of 200 000 compounds with the MW1 reporter strain of *P. aeruginosa*.[19] This strain contains the reporter gene for yellow fluorescent protein (yfp). Tetrazole **1** was shown to inhibit quorum sensing with an IC_{50} of 33 μM in the presence of 0.3 μM OdDHL (Fig. 1). Tetrazole **1** was also found to inhibit pyocyanin and elastase in wild type *P. aeruginosa*.[19] Suga and co-workers designed and synthesised a library using a 3,4-dihydropyran resin for screening with a similar reporter strain of *P. aeruginosa.* OdDHL analogs where the lactone head group was substituted, displayed both agonist and antagonistic activity.[20] For example, the substituted cyclohexanone, aniline and pyridine analogues were all inhibitors of LuxR homologue mediated quorum sensing in *P. aeruginosa* (Fig. 1, compounds **2**, **3** & **4** respectively).

Blackwell and co-workers have screened a number of compounds and discovered that the bromo-phenyl AHL analogue (Fig. 1, compound **5**) has an IC_{50} of 12.5 μM using PAO-JP2 cells harboring the plasB-gfp(ASV) reporter with 1 μM OdDHL.[21,22] Givskov and co-workers have shown that a naturally occurring halogenated furanone (Fig. 1, compound **6**), produced by the algae *Delisea pulchra*, has an IC_{50} of 11.4 μM using the same reporter strain in the presence of 0.1 μM OdDHL.[23]

Herein, we report the first use of a 3D hydrogel small-molecule microarray to discover a novel pharmacophore that binds to LuxR homologues. In our a proof-of-concept study, a total of 95 compounds were synthesised, characterised and printed onto the microarray. Accordingly, the platform has the potential to display and examine 10 000 different small molecules in a miniaturised, low-cost format. The pharmacophore was used to design compounds that inhibited quorum sensing in *Serratia* (IC_{50} = ~5 μM) and *P. aeruginosa* (IC_{50} = 10–20 μM).

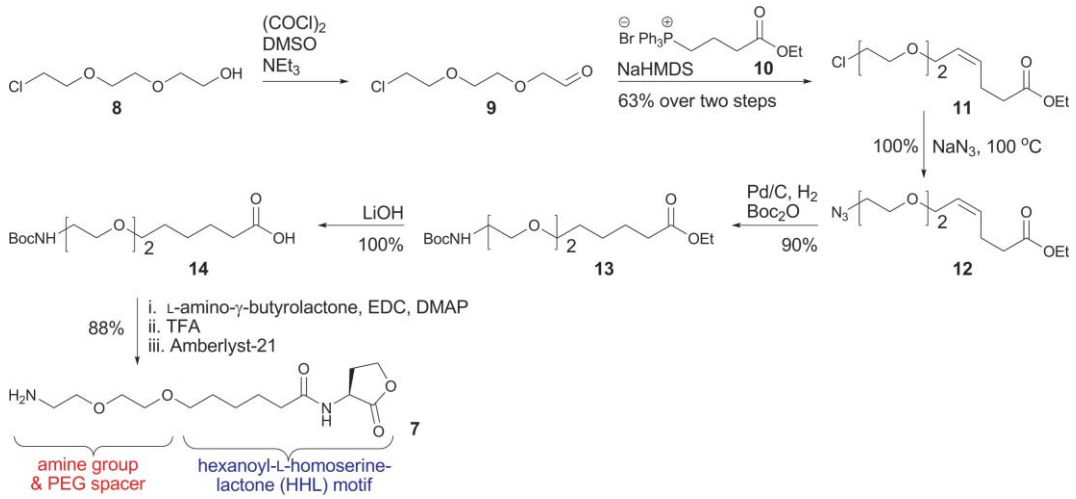
Results and discussion

Rationale

The identification of small-molecule weak binders to targets with potential biological activity and their subsequent development into attractive drug-like hits remains an ongoing challenge. 3D Small-molecule microarrays display improved loading capacity, signal sensitivity and spot morphology compared with comparative 2D slides.[4,24] This reports first highlights the potential of 3D slides to identify relatively weak (10–200 μM) protein-ligand binding interactions. Secondly, the utilisation of these initial leads to discover a novel pharmacophore for quorum sensing inhibition.

LuxR receptor homologues are potential targets for the inhibition of quorum sensing in Gram negative bacteria. Competitive inhibition of LuxR-AHL binding should prevent the transcription of genes associated with virulence. In general, it is notoriously difficult to express unbound LuxR homologues in good soluble yields. However, in a previous report by our group several LuxR homologues (including CarR from *E. carotovora*) have been expressed and isolated.[12,25] *E. carotovora* is a phytopathogenic Gram negative bacterium, which uses OHHL mediated quorum sensing to regulate the production of virulence factors such as *exo*-enzymes and β-lactam antibiotics.[10] CarI, is responsible for the production of OHHL, which is detected by the LuxR homologue, CarR. CarR controls the production of the antibiotic carbapenem. Consequently, CarR was selected as the LuxR homologue protein target because it detects both OHHL and HHL. In addition the LuxR homologue protein can be easily expressed and it contains the ligand binding domain.

The first objective of this research was to synthesise an AHL derivative suitable for immobilisation onto 3D *N*-hydroxy succinimide (NHS) ester microarray slides. The AHL analog **7** (Scheme 1) was selected for the proof-of-principle ligand-CarR binding study on the 3D small-molecule microarray. Subsequently a library based on the chemical scaffold of the AHL analog **7**



Scheme 1 Synthesis of target AHL analog molecule **7**. PEG = polyethyleneglycol, DMSO = dimethoxysulfoxide, NaHMDS = sodium bis(trimethylsilyl)amide, TFA = trifluoroacetic acid, Boc = *tert*-butoxycarbonyl, EDC = 1-(3dimethylaminopropyl)-3-ethylcarbodiimidehydrochloride & DMAP = dimethylaminopyridine.

but with modification of the amide head group was designed and synthesised. This library was then screened against CarR to discover new binding motifs, which were validated using protein binding assays and phenotypic assays.

Synthesis and screening of an AHL attached to a 3D microarray

The AHL analog **7** (Scheme 1) comprises of a hexanonyl lactone ligand attached to a PEG spacer containing a terminal amine group for covalent attachment to NHS ester slides. The PEG spacer was used to avoid surface interactions and to offer accessibility to the CarR ligand binding domains. An alternative AHL ligand containing a biotin tag has previously been described.[26] Compound **7** was successfully synthesised in seven steps (Scheme 1). Swern oxidation of alcohol **8** followed by a Horner–Wadsworth–Emmons reaction of the formed aldehyde **9** with phosphonacetate **10** generated alkene **11** in a 63% yield over two steps. Nucleophilic substitution of chloride **11** proceeded quantitatively upon heating the reaction at 100 °C with sodium azide. The successive one-pot reduction-protection sequence generated compound **13** in an excellent 90% yield. After hydrolysis of ester **13** with LiOH, the formed carboxylic acid **14** was coupled with amino-γ-butyrolactone using EDC and DMAP to produce the amide. Finally, Boc-deprotected using trifluoroacetic acid (TFA) and isolation of the free base generated the target AHL analog **7** in a 50% overall yield for the seven step sequence.

As an initial proof-of-principle, the immobilised AHL analog **7** was monitored for small-molecule protein binding activity with the Cy3 labeled CarR (Fig. 2A). Compound **7** and the control amino-ethoxy-ethanol (**15**) were printed onto a 3D hydrogel slide and probed with Cy3-CarR. The slide was scanned for fluorescence in the Cy3 region. The measured Cy3 fluorescence was corrected for the averaged background Cy3 fluorescence and plotted against the concentration of the AHL analog **7** (Fig. 2B). Compound **7** showed an apparent affinity for CarR, displaying concentration dependency linked with Cy3 fluorescence intensity. In contrast very little fluorescence was observed with the control **15**. A displacement assay was attempted to remove bound CarR, by washing the slide with native HHL and OHHL quorum sensing ligands. The displacement-binding assay revealed that fluorescence fell by approximately 50%. CarR should have had a stronger affinity for solution phase native AHL ligands but the activity of the captured protein on the slide may have been diminished because of denaturation,[25] this might restrict the amount of active protein available to be displaced. Following the successful preliminary investigation, a library of AHL analogues were synthesised for screened against CarR using the same 3D small-molecule microarray approach.

Synthesis and screening of the library

Known inhibitors of quorum sensing in *P. aeruginosa* (Fig. 1) generally all share a common core architecture based on the native AHL ligand, with modification around the homoserine lactone head group. With this in mind, the carboxylic acid intermediate **14** (Scheme 1) was used as a scaffold to generate a library where the amide head group was varied (Scheme 2). The carboxylic acid (**14**) was loaded onto tetrafluorophenol catch-and-release beads (**16**) using diisopropylcarbodiimide (DIC) to generate the solid-supported active ester **17**. The activated ester (**17**) was reacted with 95 different amines (**A1–A95**, see supplementary information for the full list of structures) to yield the Boc-protected amides **LP1–LP95**.[27] This catch-and-release approach was largely successful in producing useful quantities (*ca.* 20–30 mg) of pure (*ca.* >95% by HPLC) AHL analogues in a parallel fashion. However, three sterically hindered anilines failed to yield the required amides. The amines were broadly chosen based upon known quorum sensing inhibitors (Fig. 1). In particular, the library included amines; pyridin-2-amine **A29** (the pyridine moiety was used by Suga and co-workers[20]), cyclic 4-aminocyclo-hexanol **A38** and 2-chloropyridin-4-amine **A86**. The Boc group was deprotected using HCl and the chloride salt was deprotonated to generate the free amine ligands, **L1–L95**.

Ligands **L1–L95** were printed onto a fresh 3D NHS ester hydrogel slide. A pre-incubation scan revealed that ligand **L77**, which contains a thiazolidinone head group, exhibited

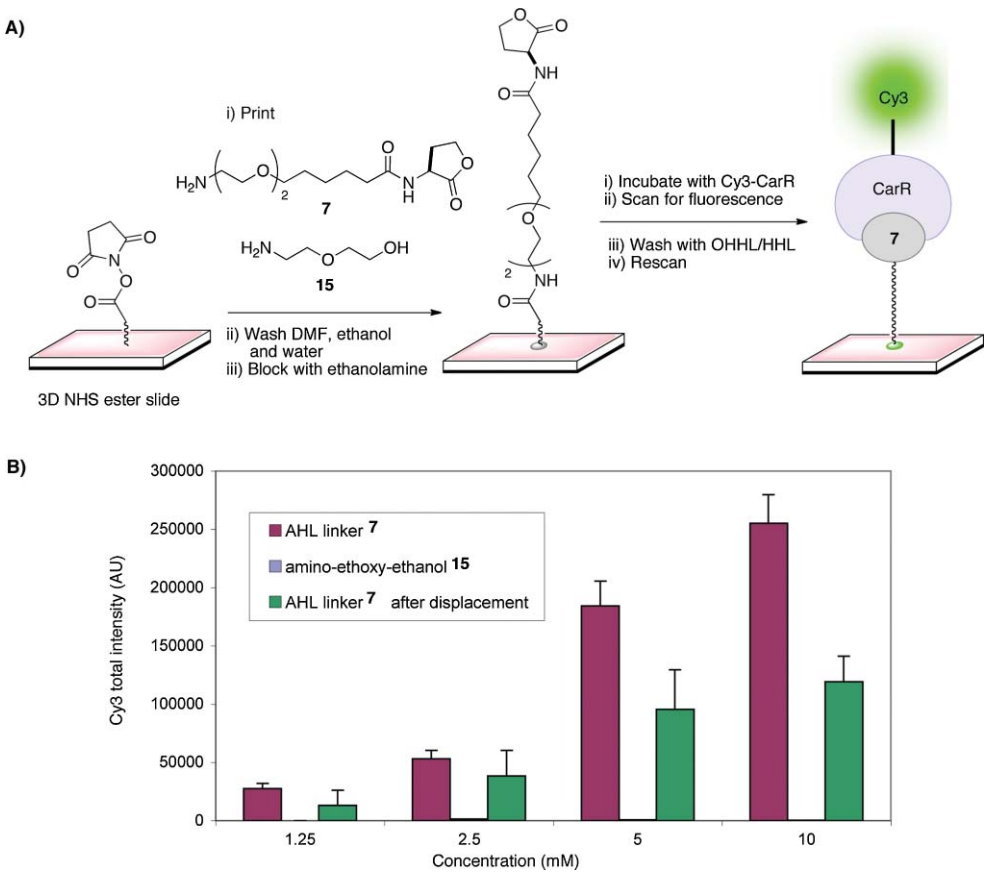
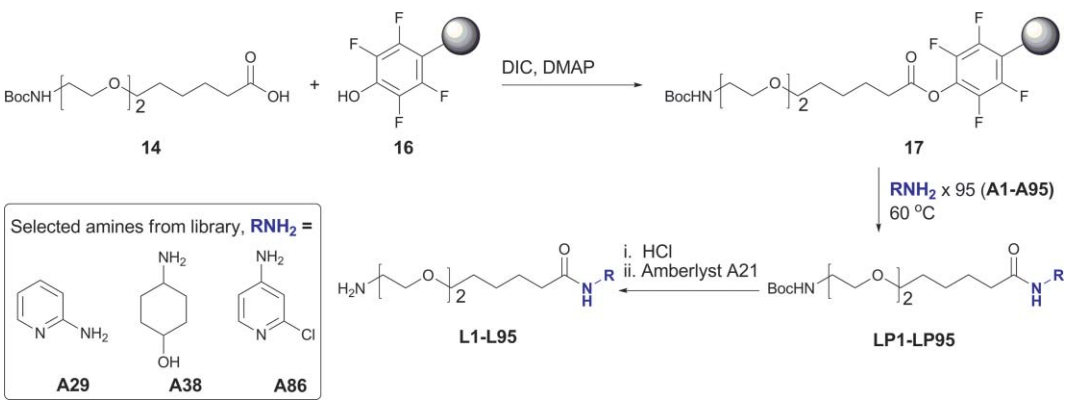


Fig. 2 A) Outline of the small-molecule microarray experiment between the immobilized AHL analog **7** and the protein CarR. The AHL analog **7** and the amino-ethoxy-ethanol control **15** were printed at 10, 5, 2.5 & 1.25 mM concentrations onto a 3D NHS ester slide before washing, blocking and scanning. For the displacement assay, the slide was washed with a solution containing native quorum sensing ligands OHHL and HHL before rescanning. Scanned images can be found in the ESI†. B) The background corrected Cy3 total intensities (based on the average across 8 duplicate spots) were plotted against the concentration of **7** and **15**. Error bars correspond to the standard deviation.



Scheme 2 Synthesis of library using solid phase tetrafluorophenol catch and release beads. The carboxylic acid **14** was loaded onto tetrafluorophenol beads prior to reaction with 95 different amines numbered **A1–A95**. Three amino-benzophenones (**A34**, **A82** & **A83**) did not react with the tetrafluorophenol active ester beads. The library of amides were finally Boc deprotected using HCl and deprotonated to give ligands with reference numbers **L1–L95**.

auto-fluorescence in the Cy3 region and was therefore discounted as a potential binder in the screen. The slide was then probed with Cy3-CarR and Cy5-avidin before scanning for Cy3 and Cy5 fluorescence (see ESI†). Using the Cy5 biotin-avidin interaction as a guide, the average Cy3 fluorescence of each library member was calculated and plotted (Fig. 3A). The pyrrolidine **L15** and chloro-pyridine **L86** were selected as potential hits because they produced the highest average fluorescence and their signal-to-noise ratios were above the lower limit of detection. **L15** and **L86** were also reprinted across a concentration range and probed with Cy3-CarR. The background corrected Cy3 total intensity was plotted against the concentration of **L86** (Fig. 3B) to reveal an apparent concentration dependent fluorescence from 2.5–10 mM **L86**. Ligand **L15** displayed a similar trend but with higher levels of

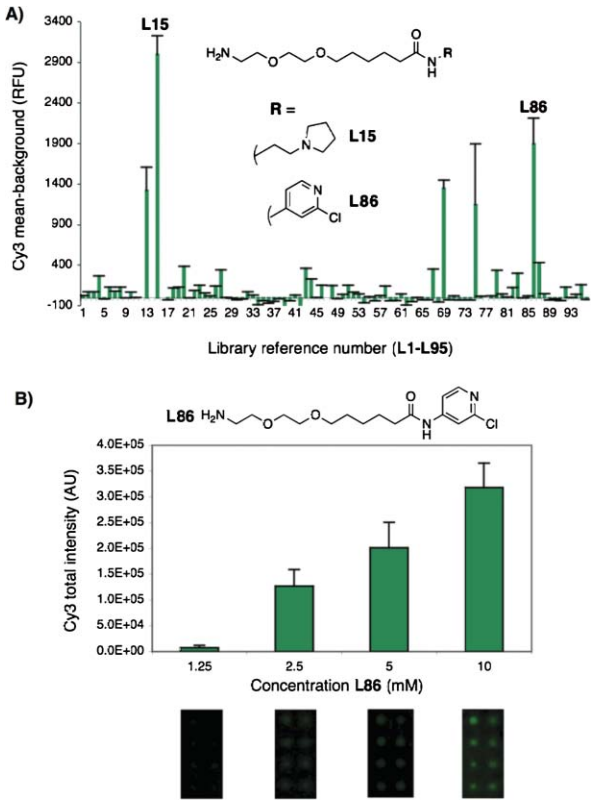


Fig. 3 A) Library-CarR small-molecule microarray screening results of the library reference number *versus* the background corrected Cy3 mean intensity value (based on the average across 4 replicate spots per ligand). Potential binders were selected based on having a signal-to-noise ratio (SNR) higher than 3 (the lower limit for detection), which included **L15** & **L86**. **L77** was removed for clarity due to auto-fluorescence. B) Background corrected Cy3 total intensity (based on the average across 8 replicate spots per concentration) *versus* concentration of **L86** and the corresponding Cy3 images. Error bars for both charts correspond to the standard deviation, RFU = relative fluorescent units, AU = arbitrary units. See ESI† for other scans and plots.

fluorescence (shown in the ESI†). In order to verify these ligand-protein interactions from the microarray screen, fluorometric ligand binding assays and ITC were performed in solution using ligands without the spacer arm.

Hit validation

The crystal structure of TraR (a LuxR homologue belonging to *Agrobacterium tumefaciens*) when bound to its native AHL ligand contains a highly conserved tryptophan residue, which forms a hydrogen bond with the C═O oxygen of the lactone ring.[28] A similar hydrogen bond between LasR and OdDHL is also found in the LasR crystal structure.[29] It is reasonable to predict that a tryptophan residue is involved in the ligand binding of other LuxR homologues such as CarR.[25] The strength of CarR-ligand binding was calculated indirectly by measuring the change in the fluorescent emission spectrum when the tryptophan ligand was titrated into CarR.[12] The pyrrolidine and chloro-pyridine library compounds (**L15** and **L86** respectively) were synthesised without the spacer arm to generate HHL analogues **18** and **19** respectively. Compounds **18** and **19** both showed binding affinity for CarR. Interestingly, the pyrrolidine analogue (**18**) bound with a dissociation constant (K_d) of 181 μM, whilst the chloro-pyridine analogue (**19**) interacted more strongly giving a K_d of 21 μM (Fig. 4). The opposite strength of binding was seen with the microarray screen using the corresponding library compounds, possibly because the binding of **L86** to CarR was impaired on the solid support. Despite the reversal in binding activities between the library screen and the corresponding HHL analogues, it remains that 3D small-molecule microarray has provided a rapid and convenient qualitative method for identifying weak ligand-protein interactions.

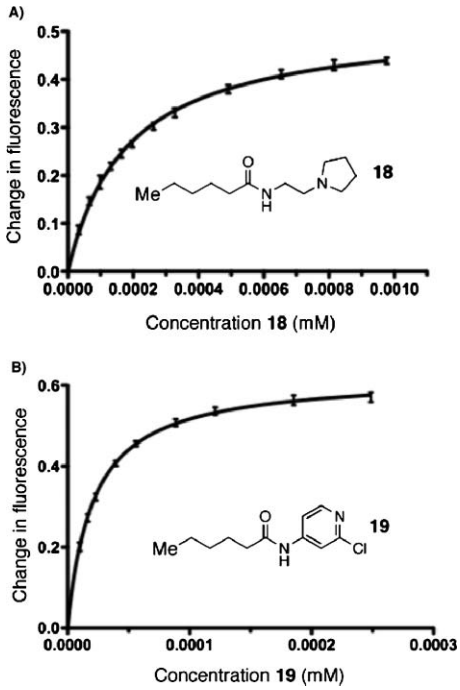


Fig. 4 CarR fluorometric ligand binding assays. The relative change in fluorescence was plotted against concentration using the Graphpad Prism software with a single binding site model. A) CarR-**18** K_d = 181 μM ± 5.0. B) CarR-**19** K_d = 21 μM ± 0.4. Excitation wavelength 292 nm, emission 335 nm & T = 30 °C. Error bars correspond to standard deviation across three replicate experiments. See ESI† for OHHL and **20** binding curves with CarR and EccR.

The 3-oxo chloro-pyridine OHHL analog **20** displayed a dissociation constant of 37 μM; however, the pyrrolidine 3-oxo OHHL analog did not show any binding affinity with CarR. For comparison purposes the native OHHL ligand bound to CarR more tightly with a K_d of 2.0 μM. The interaction between compound **20** and CarR was investigated further using isothermal titration calorimetry (ITC), which measures the heat change associated with the small-molecule: protein binding interaction (Fig. 5). Compound **20** was shown to bind CarR with a dissociation constant of 11.2 μM, *ca.* three fold stronger than the calculated fluorometric value.

The ligand exhibited favourable enthalpic and entropic contributions ([CarR] = 31 μM and [**20**] = 400 μM. T = 30 °C, ΔG = −6.8 kcal mol^{-1}, ΔH = −1.4 kcal mol^{-1}, $-T\Delta S$ = −5.4 kcal mol^{-1}, K_d = 11.2 ± 1.1 μM); however, binding was driven by a larger entropy contribution, probably associated with desolvation of the active site upon binding.

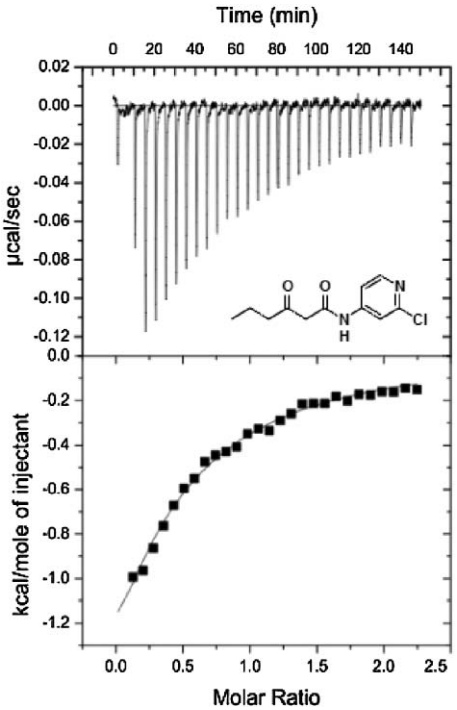


Fig. 5 ITC trace and binding isotherm for the injection of ligand **20** into CarR. [CarR] = 0.031 mM and [**20**] = 0.4 mM. T = 30 °C, ΔG = −6.8 kcal mol^{-1}, ΔH = −1.4 kcal mol^{-1}, $-T\Delta S$ = −5.4 kcal mol^{-1}, K_d = 11.2 ± 1.1 µM. Fitted to single binding site model, stoichiometry fixed to n = 1 based on OHHL binding.

E. carotovora also uses a second LuxR homologue EccR, whose role in quorum sensing is unclear.[10] When tested for ligand binding with EccR, the 3-oxo OHHL analog **20** bound with a K_d of 3.6 µM, which compared favorably with the native OHHL ligand (K_d = 3.9 µM).

Phenotypic assays

Phenotypic assays were performed to study the agonist and antagonist activities of ligands containing the chloro-pyridine group. Despite successful binding affinities with chloro-pyridine HHL ligands **19** and **20** to CarR (and 3-oxo chloro-pyridine OHHL ligand **20** to EccR), no agonist or antagonist activity was observed with *E. carotovra* using either a carbapenem or protease phenotypic assay.[10,30] This lack of activity could be attributed to the inability of the ligands to cross the cell membrane and bind to CarR or EccR intracellularly. However, it was envisaged that the activity of the ligand could be tuned for other Gram negative bacteria such as *Serratia* and *P. aeruginosa*, by adapting the *N*-acyl-chain to match their native AHL ligand(s).

Serratia marcescens is a clinically significant pathogen as multi-drug resistance has been observed in this species.[31] *Serratia* species ATCC39006 produces both the antibiotic carbapenem and the red pigment prodigiosin under BHL mediated quorum sensing control.[30] For the phenotypic assays, a mutant *LIS* was used, which cannot produce BHL but can still recognise it. The BHL analog **21**, which contains the chloro-pyridine head-group was synthesised and found to be a potent antagonist of carbapenem production in *Serratia*, (Fig. 6A). Wells contained the mutant *LIS* in addition to an overlay of carbapenem sensitive *E. coli*. Carbapenem production in *LIS* was activated at 0.5 µM BHL (shown in the lower left-hand well of plate i.), which produces a halo where the *E. coli* overlay strain had been killed. The red prodigiosin pigment was also activated at this concentration of BHL. Carbapenem production was inhibited at higher concentrations of **21** in plate i, whilst plate ii showed that carbapenem production was blocked from approximately 5 µM **21**. Below this concentration, a halo is seen corresponding to the release of carbapenem by *LIS* and the death of the *E. coli* reporter strain. Compound **21** is a competitive inhibitor of BHL-LuxR homolgue binding and represents the strongest known antagonist of quorum sensing in *Serratia.*

A goal of this work was to discover antagonists of quorum sensing in *P. aeruginosa*. Since the OdDHL signaling pathway in this organism lies at the top of the quorum sensing hierarchy, we grafted the requisite acyl chain onto the chloro-pyridine head group to generate analogue **22**. This was assessed for its ability to block the transcription of a key quorum sensing-controlled gene, *lasB*, which encodes the secreted virulence factor, elastase B. Strain *MH602* contains a chromosomal insertion (introduced on a mini-Tn5-Gm cassette) carrying a construct composed of (a) the *lasB* promoter (p*lasB*) fused to *gfp*(ASV) and (b) the *lasR* gene under control of the *lac* promoter.[22] Sub-growth-inhibitory concentrations of compound **22** suppressed *gfp* expression in this strain very effectively with an IC_{50} of between 10–20 µM in the presence of 0.66 µM OdDHL (Fig. 6B).

Significantly, analogues **21** and **22** were both identfied as competitive inhibitors of AHL mediated quorum sensing. The chloro-pyridine head group has been successfully recognised as a cross-species pharmacophore for LuxR homologue binding and quorum sensing inhibition. Future work within this field should include structure activity relationships to optimise binding and quorum sensing inhibition.

Conclusions

These results demonstrate a proof-of-principle that 3D small-molecule microarray technology can be used for cost–effective, high throughput compound screening to identify relatively weak protein-ligand interactions (K_d *ca.* 10–200 µM). On analysing the data we discovered a pharmacophore that was subsequently adapted into *Serratia* and *P. aeruginosa* quorum sensing antagonists. In particular the BHL analogue **21** was a potent inhibitor of carbapenem production in *Serratia* (from 5 µM **21** in presence of 0.5 µM BHL) whilst the OdDHL analogue **22** was an inhibitor of LasR mediated quorum sensing in *P. aeruginosa* (IC_{50} = 10–20 µM in the presence of 0.66 µM OdDHL). The 3D small molecule microarray approach is likely to find important application within the fields of medicinal chemistry, chemical biology, molecular recognition and chemical genetics, where the aim is to discover small molecules that bind to macromolecular complexes.

Experimental

Synthetic details of the AHL library and NMR data for the compounds synthesised are found in the ESI† along with microarray data and fluorometric ligand binding assays. CarR and EccR were expressed and purified using the method described in the literature.[12,25] CarR was labeled with bis-NHS-Cy3 dye using the literature procedure.[6]

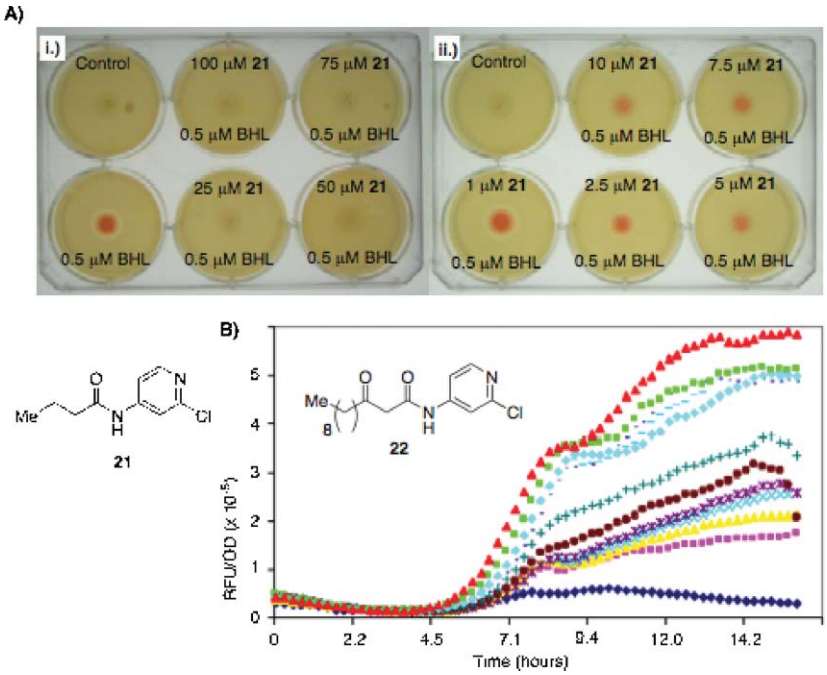


Fig. 6 A) Phenotypic assay of carbapenem production in *Serratia* sp. ATCC39006. Wells contained a lawn of carbapenem sensitive *E. Coli* and the inoculated *Serratia* sp. ATCC39006 mutant called LIS, which is unable to produce BHL and the indicated concentration of its native quorum sensing ligand BHL. Activation of carbapenem production is observed by the generation of a halo in the *E. coli* overlay at 0.5 μM BHL in well 6 of plate i. Carbapenem production was inhibited by the additional presence of the 2-chloro-pyridine BHL analogue **21** from 10–2.5 μM. B) Quorum sensing antagonist (IC_{50}) determination in *P. aeruginosa* by suppression of *gfp* production in the MH602 strain. IC_{50} for **22** = 10–20 μM. Concentrations of **22**: ♦ 166 μM, ▪ 83 μM, ▲ 42 μM, × 21 μM, ✱ 10 μM, ● 5 μM, + 2.6 μM, - 1.3 μM, _ 0.6 μM, ♦ 0.3 μM, ▪ 0.2 μM, ▲ Control.

6-[2-(2-Chloro-ethoxy)-ethoxy]-hex-4-enoic acid ethyl ester (11)

Oxalyl chloride (1.4 mL,, 1.1 eq. 0.014 mol) was dissolved in DCM (30 mL) and cooled to −78 °C under a nitrogen atmosphere. DMSO (1.7 mL, 2.0 eq. 0.024 mol) was added followed by the dropwise addition of 2-[2-(2-chloro-ethoxy)-ethoxy]-ethanol **8** (1.72 mL, 1 eq., 0.012 mol) over 15 min. The reaction was stirred for a further 15 min. Triethylamine (8.36 mL, 0.06 mol) was added and the solution warmed to room temperature. The reaction mixture was stirred for a further 12 h at room temperature. The reaction was quenched with water (20 mL), extracted with DCM (3 × 20 mL), washed with brine (15 mL) and dried ($MgSO_4$). The solvent was removed under reduced pressure and the intermediate aldehyde **9** was used in the next step crude. Phosphonacetate **10** (1.7 g, 1 eq., 8.1 mmol), was dissolved in THF (30 mL) and cooled to −78 °C. NaHMDS (9.2 mL, 9.2 mmol of 1 M in THF) was added and stirred for 30 min. The aldehyde **9** (1.55 g, 9.2 mmol) was added dropwise and the reaction was allowed to warm to room temperature and stirred over night. The reaction was quenched with water (20 mL), and extracted with EtOAc (3 × 15 mL). The combined organic layers were dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by flash chromatography to yield the product **11** as a straw coloured oil. (2.0 g, 63% over two steps): R_f 0.48 (SiO_2, 1 : 1 Et_2O : 40–60 petroleum ether); ν_{max}(neat)/cm^{-1} 2868w, 1732 s, 1447w, 1372w, 1298w, 1252w, 1163 m, 1110st; δ_H (400 MHz, $CDCl_3$) 5.65–5.53 (2H, m), 4.16–4.12 (4H, m), 3.75 (2H, t, J = 6.0 Hz), 3.70–3.60 (6H, m), 2.40–2.34 (4H, m), 1.20 (3H, t, J = 7.0 Hz); δ_C (100 MHz, $CDCl_3$) 172.9, 131.2, 127.5, 71.4, 70.8, 69.4, 66.7, 65.8, 42.6, 34.0, 23.1, 14.2; m/z (MH^+) 264 (MH^+); HRMS found 282.1468, $C_{12}H_{25}NO_4Cl$ (MNH_4^+) requires 282.1467.

6-[2-(2-Azido-ethoxy)-ethoxy]-hex-4-enoic acid ethyl ester (12)

11 (400 mg, 1 eq., 1.50 mmol) and sodium azide (110 mg, 1.1 eq., 1.68 mmol) were dissolved in DMF (5 mL) and heated at 100 °C for five hours behind a blast shield. The solution was filtered, diluted with water (10 mL) and extracted with DCM (3 × 15 mL). The organic layers were combined and dried over $MgSO_4$, and the solvent removed under reduced pressure to give the product **12** as a pale yellow oil (430 mg, 100%); ν_{max}(neat)/cm^{-1} 2868w, 2102st, 1729st, 1445w, 1372w, 1345w, 1252 m, 1163 m, 1112st; δ_H (500 MHz, $CDCl_3$) 5.55–5.63 (2H, m), 4.11–4.16 (4H, m) 3.71–3.66 (4H, m), 3.64–3.61 (2H, m), 3.41 (2H, t, J = 5.0 Hz), 2.43–2.36 (4H, m), 1.27 (3H, t, J = 7.0 Hz); δ_C (125 MHz, $CDCl_3$) 172.9, 131.2, 127.6, 70.8, 70.0, 69.5, 66.7, 60.4, 50.7, 34.0, 23.1, 14.2; m/z (ES^+) 272 (MH^+), 289 (MNH_4^+); HRMS found 289.1868, $C_{12}H_{25}N_4O_4$ (MNH_4^+) requires 289.1870.

6-[2-(2-*tert*-Butoxycarbonylamino-ethoxy)-ethoxy]-hexanoic acid ethyl ester (13)

The azido-alkene **12** (100 mg, 1 eq., 0.37 mmol) and carbonic acid di-*tert*-butyl ester (96.3 mg, 1.2 eq., 0.44 mmol) were added to a degassed solution of EtOAc. Pd/C (10% on carbon, 30 mg) was added and the reaction stirred under a H_2 atmosphere for 18 h.[32] The mixture was filtered through celite and concentrated under reduced pressure. The crude product was purifed by column

chromatography (1 : 1 30–40 petroleum ether : Et_2O) to yield the product **13** as a pale yellow oil (116 mg, 90%): R_f 0.12 (SiO_2, 1 : 1 30–40 petroleum ether : Et_2O); ν_{max}(neat)/cm^{-1} 3356w, 2977w, 2935w, 2865w, 1734st, 1713st, 1513 m, 1455w, 1391w, 1365 m, 1248 m, 1116st; δ_H (500 MHz, $CDCl_3$) 5.05 (1H, s), 4.14 (2H, q, J = 7.0 Hz), 3.62–3.60 (2H, m), 3.58–3.54 (4H, m), 3.51–3.50 (2H, m), 3.33–3.32 (2H, m), 2.31 (2H, t, J = 7.5 Hz), 1.68–1.60 (4H, m), 1.45 (9H, s), 1.44–1.38 (2H, m), 1.26 (3H, t, J = 7.0 Hz); δ_C (100 MHz, $CDCl_3$) 173.8, 156.3, 79.4, 70.8, 70.6, 70.5, 70.4, 60.6, 40.7, 31.3, 28.7, 28.6, 25.3, 25.2, 14.6; m/z (ES$^+$) 348 (MH$^+$), HRMS found 348.2382, $C_{17}H_{34}NO_6$ (MH$^+$) requires 348.2381.

6-[2-(2-*tert*-Butoxycarbonylamino-ethoxy)-ethoxy]-hexanoic acid (14)

The Boc protected ester **13** (26 mg, 1 eq., 0.07 mmol) and lithuim hydroxide monohydrate (6 mg, 0.14 mmol) were dissolved in aqueous methanol (2 : 1 MeOH–H_2O, 2 mL) at room temperature. The reaction was monitored by TLC and stirred for 2 h. Water (3 mL) was added to dilute the reaction, which was neutralised with 2 M HCl. The product was extracted using EtOAc (3 × 5 mL) and the combined organic layers dried ($MgSO_4$) and concentrated under reduced pressure to give the product **14** as a colourless oil (23 mg, 100%): ν_{max}(neat)/cm^{-1} 2935w, 2866w, 1706st, 1516 m, 1366 m, 1249 m, 1169st; δ_H (400 MHz, $CDCl_3$) 5.13 (1H, s), 3.57–3.48 (6H, m), 3.43 (2H, t, J = 6.5 Hz), 3.37 (2H, s), 2.30 (2H, t, J = 7.5 Hz), 1.63–1.55 (4H, m), 1.40 (9H, s), 1.43–1.35 (2H, m); δ_C (125 MHz, $CDCl_3$) 176.1, 156.1, 79.2, 71.1, 70.1, 69.9, 40.3, 33.9, 29.1, 28.6, 25.3, 24.4, 20.7; m/z (ES$^+$) 220 (MH$^+$-Boc); HRMS found 320.2067, $C_{15}H_{29}NO_6$ (MH$^+$) requires 320.2068.

6-(2-(2-aminoethoxy)ethoxy)-*N*-(2-oxotetrahydrofuran-3-yl)hexanamide (7) TFA salt

EDC (41 mg, 0.216 mmol) and DMAP (26 mg, 0.216 mmol) were added to a stirred solution of the acid **14** (23 mg, 1 eq., 0.072 mmol) and S-α-amino-γ-butyrolactone.HBr (16 mg, 1.2 eq., 0.086 mmol) in DCM (3 mL). The reaction was stirred over night at room temperature, then diluted with DCM (10 mL) and washed with 1M HCl (3 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to yield a pale yellow oil which was purifed by chromatography to give the Boc protected AHL linker **7** (29 mg, 90%). To a solution of the Boc-protected AHL linker **7** (25 mg, 1 eq., 0.06 mmol) in DCM (2 mL) was added TFA (29 μL, 4 eq., 0.24 mmol). The reaction was stirred for 4 h at room temperature, and monitored using TLC to check the reaction had gone to completion. The solution was concentrated under reduced pressure to give the TFA salt of product 7 as a pale yellow oil (20 mg, 100%): R_f 0.1 (SiO_2, 1 : 9 MeOH–DCM); $[\alpha]_D$ + 18.8 (c = 0.8, $CHCl_3$, T = 25 °C); ν_{max}(neat)/cm^{-1} 2930w, 1774 m, 1674 m, 1544w, 1171st, 1132st; δ_H (400 MHz, $CDCl_3$) 7.70–7.50 (4H, m), 4.56–4.50 (1H, m), 4.44 (1H, t, J = 8.5 Hz), 4.27–4.20 (1H, m), 3.73–3.71 (2H, m), 3.64–3.62 (2H, m), 3.52–3.50(2H, m), 3.45 (2H, t, J = 6.0 Hz), 3.20–3.14 (2H, s), 2.60–2.50 (1H, m), 2.34–2.24 (3H, m), 1.64–1.50 (4H, m), 1.36–1.30 (2H, m); δ_C (125 MHz, $CDCl_3$) 177.4, 175.8, 71.7, 70.7, 70.4, 67.1, 66.9, 49.6, 40.9, 35.6, 29.3, 28.8, 26.0, 25.4; HRMS found 325.1736, $C_{14}H_{26}N_2O_5Na$ (MNa$^+$) requires 325.1739.

General amide coupling using hexanoyl chloride

To a stirred solution of the amine (1.2 eq.) and Na_2CO_3 (2.3 eq.) dissolved in water (2.5 mL mmol^{-1} of amine), was added hexanoyl chloride (1 eq.) dissolved in DCM (2.5 mL/0.8mmol of acid chloride). The reaction was stirred vigorously at room temperature for 12 h. The two-phase mixture was separated and the aqueous layer extracted twice with DCM. The organic layers were combined, washed with 10% aqueous Na_2CO_3 and saturated aqueous NaCl. The organic layer was dried over $MgSO_4$, filtered, concentrated under reduced pressure and purified.

Hexanoic acid (2-pyrolidin-1-yl-ethyl)-amide (18)

By following general method above and using amine **A15**, product **18** was isolated as a pale oil (61% yield); R_f 0.08 (SiO_2; EtOAc); ν_{max}(neat)/cm^{-1} 2956 m, 2929 m, 2872w, 2794w, 1697 s, 1460w, 1377 m, 1177 m, 1128m; δ_H (400 MHz, $CDCl_3$) 7.0 (1H, s), 3.37 (2H, s), 3.35–3.40 (2H, m), 2.59 (2H, t, J = 6.5 Hz), 2.49–2.51 (4H, m), 1.76–1.84 (4H, m), 1.61–1.65 (2H, m), 1.28–1.32 (4H, m), 0.88 (3H, t, J = 6.5 Hz]; δ_C (100 MHz, $CDCl_3$) 176.4, 54.8, 54.4, 43.4, 37.9, 31.3, 24.6, 23.5, 22.5, 13.9; HRMS found 216.1965, $C_{12}H_{25}N_2O$ (M$^+$) requires 213.1967.

Hexanoic acid (2-chloro-pyridin-4-yl)-amide (19)

By following general method above and using amine **A86**, product **19** was isolated as a pale yellow oil; R_f 0.1 (SiO_2; 1 : 1; 30–40 petroleum ether : Et_2O); ν_{max}(neat)/cm^{-1} 2958w, 2931w, 1684w, 1584 s, 1506 m, 1222 m, 1468w, 1383 m, 1264w; δ_H (400 MHz, $CDCl_3$) 8.30 (1H, br s) 8.20 (1H, d, J = 6.0 Hz), 7.69 (1H, d, J = 2.0 Hz), 7.38–7.40 (1H, m), 2.34–2.40 (2H, m), 1.66–1.72 (2H, m), 1.29–1.34 (4H, m), 0.86–0.90 (3H, m); δ_C (100 MHz, $CDCl_3$) 172.7, 152.3, 149.8, 147.6, 113.4, 112.4, 37.6, 31.3, 24.9, 22.3, 13.8; m/z (APCI$^+$) 227 (MH$^+$, 100%); HRMS found 227.0946, $C_{11}H_{16}N_2OCl$ (MH$^+$) requires 227.0946.

3-Oxo-hexanoic acid (2-chloro-pyridin-4-yl)-amide (20)

The carboxylic acid starting material: 2-(2-propyl-1,3-dioxolan-2-yl)acetic acid was made according to a standard literature procedure.[33] Thionyl chloride (1.6 eq., 1.4 mmol) was added to a stirred solution of the carboxylic acid (1 eq., 0.9 mmol) in DCM (5 mL per mmol of acid) at 0 °C. 1 drop of DMF was added and the reaction was stirred for 30 min at 0 °C. The reaction was warmed to room temperature and concentrated under reduced pressure to give the acid chloride intermediate. The residue was redissolved in DCM (5 mL). Na_2CO_3 (2.8 mmol) and amine **A86** (1.4 mmol) were dissolved in water (5 mL per mmol of acid) and added to the acid chloride solution. The reaction mixture was stirred for 12 h at room temperature. The reaction was extracted with DCM (3×) and the combined organic layers were washed with aqueous Na_2CO_3 (2×) and brine (2×) before drying over $MgSO_4$ and concentrating under reduced pressure. The crude product was purified by flash chromatography to give the protected product. For the deprotection, TFA (2 eq.) was added to a solution of the protected product (1 eq.) in DCM. The reaction was stirred for one hour before quenching with Na_2CO_3 solution (10%). The product was extracted with DCM (×3) and the combined organic layers were washed with brine (×2) and dried ($MgSO_4$). The solvent

was removed under reduced pressure and the residue purified by flash chromatography to give the final product **20** as a viscous yellow oil (49.5 mg, 53%); R_f 0.55 (SiO_2, EtOAc); ν_{max}(neat)/cm^{-1} 3311br, 2965w, 1718 m, 1698 m, 1579st, 1506st, 1380st, 1317st, 1266st, 838m; δ_H (400 MHz, $CDCl_3$) 9.70 (1H, s), 8.26 (1H, d, J = 5.5 Hz), 7.66 (1H, d, J = 2 Hz), 7.25 (1H, dd, J = 5.5, 2.0 Hz), 3.58 (2H, s), 2.54 (2H, t, J = 7.5 Hz), 1.70–1.62 (2H, m), 0.95 (3H, t, J = 7.5 Hz); δ_C (100 MHz, $CDCl_3$) 207.7, 164.2, 152.2, 150.3, 146.9, 113.9, 112.8, 48.2, 46.1, 16.8, 13.5; m/z (ES$^+$) 241.1 (MH$^+$), HRMS found 241.0756, $C_{11}H_{14}ClN_2O_2$ (MH$^+$) requires 241.0744.

(2-Chloro-pyridin-4-yl)-butyramide (21)

To a stirred solution of 2-chloro-pyridin-4-ylamine **A86** (256 mg, 1.2 eq., 2.0 mmol) and Na_2CO_3 (400 mg, 2.3 eq. 3.77 mmol) in water (5 mL) was added butyryl chloride (200 µL, 1 eq., 1.66 mmol) dissolved in DCM (5mL).[17] The reaction was stirred vigorously at room temperature for 12 h. The two-phase mixture was separated and the aqueous layer extracted twice with DCM. The organic layers were combined, washed with 10% aqueous Na_2CO_3 and saturated aqueous NaCl. The organic layer was dried over $MgSO_4$, filtered, concentrated under reduced pressure. Product was purified by flash chromatography to give **21** as a yellow oil (251 mg, 78%): R_f 0.55 (SiO_2, 3 : 1 EtOAc : 40–60 petroleum ether); ν_{max}(neat)/cm^{-1} 3253br, 2966 m, 1684 m, 1579st, 1504st, 1380st, 1264st, 1078st, 837st; δ_H (400 MHz, $CDCl_3$) 8.22 (1H, d, J = 5.5 Hz), 8.03 (1H, br s), 7.67 (1H, s), 7.38–7.36 (1H, m), 2.37 (2H, t, J = 7.5 Hz), 1.71–1.69 (2H, m), 0.99 (3H, t, J = 7.5 Hz); δ_C (100 MHz, $CDCl_3$) 172.5, 152.4, 150.3, 147.6, 113.4, 112.4, 39.5, 18.7, 13.6; m/z (ES$^+$) 199.1 (MH$^+$), HRMS found 199.0632, $C_9H_{13}N_2O$ (MH$^+$) requires 199.0639.

3-Oxo-dodecanoic acid (2-chloro-pyridin-4-yl)-amide (22)

The carboxylic acid starting material: 2-(2-nonyl-1,3-dioxolan-2-yl)acetic acid was made according to a standard literature procedure.[33] DCC (1.5 eq.) and DMAP (1 eq.) were added to a solution of the carboxylic acid (1 eq., 160 mg, 0.62 mmol) dissolved in DCM (5 mL). After 10 min, amine **A86** (1 eq.) was added and the reaction was stirred for 18 h before filtering and washing with 1M HCl. The residue was dried and purified by column chromatography to give the protected product. For the deprotection, TFA (2 eq.) was added to a solution of the protected product (1 eq.) in DCM. The reaction was stirred for one hour before quenching with Na_2CO_3 solution (10%). The product was extracted with DCM and the combined organic layers were washed with brine and dried ($MgSO_4$). The solvent was removed under reduced pressure and the residue purified by flash chromatography to give the final product **22** as a pale yellow oil (119 mg, 49%); R_f 0.14 (SiO_2; 1 : 2 30–40 petroleum ether : Et_2O); ν_{max}(neat)/cm^{-1} 1710 m, 1694 m, 1586 s, 1513 m, 1387 m, 1324 m, 1261w, 1175w, 1079m; δ_H (400 MHz, $CDCl_3$) 9.77 (1H, br s), 8.25 (1H, br d, J = 4.5 Hz), 7.67 (1H, s), 7.37 (1H, d, J = 4.5 Hz), 3.56 (2H, s), 2.57 (2H, t, J = 7.5 Hz), 1.58–1.63 (2H, m), 1.25–1.29 (12H, m), 0.87 (3H, t, J = 6.5 Hz); δ_C (100 MHz, $CDCl_3$) 207.7, 173.0, 164.4, 150.2, 146.6, 113.9, 112.8, 48.5, 44.2, 31.8, 29.3, 29.3, 29.2, 28.9, 23.3, 22.6, 14.1; m/z (ES$^+$) 325, 327 (MH$^+$); HRMS found 325.1671, $C_{17}H_{26}N_2O_2Cl$ [MH$^+$] requires 325.1683.

Microarraying

Printing was undertaken on three separate occasions, using a new slide each time. i) The AHL analog **7** and aminoethoxy-ethanol control **15** were printed at 10, 5, 2.5 and 1.25 mM concentrations in DMF (8 replicates per concentration). ii) The library was printed at 10 mM, whilst biotin-amine was printed at 0.25 mM (4 duplicate spots per compound). iii) **L15** and **L86** were reprinted at 10, 5, 2.5 and 1.25 mM concentrations (8 replicates per concentration). *General procedure*: Compounds were printed onto NHS ester 3D hydrogel slides[24] using a Genetix QArray-lite microarrayer. Slides were left overnight at 65% humidity before washing for one hour in DMF, ethanol and water. Unreacted NHS ester groups were blocked using 50 mM ethanolamine in 25 mM PBS buffer pH 8.5 for 30 min.

Probing

General procedure. 200 µL Cy3-CarR (0.075 mg mL^{-1}) was pipetted onto PC200 cover wells from Sigma and slides were incubated by inverting the slide over the cover well. For the library slide, 50 µL of Cy5-Avidin (0.01 mg mL^{-1}) was also added to the incubation solution. Slides were incubated for two hours in the dark at room temperature. Following incubation slides were transferred to a slide washer rack (Genetix Ltd.) and immersed vertically in 600 mL of Tris Tween buffer (0.5% Tween-20, 50 mM Tris, 0.1 M NaCl, pH 7.4). The beaker was covered in foil and the buffer was stirred for 15 min. The buffer was replaced and washed for 2 × 15 mins with Tris buffer (50 mM Tris, 0.1 M NaCl, pH 7.4). The slide rack was removed from the final buffer and all slides were rinsed thoroughly with distilled water to remove any salts from the slide surface. The slide rack was transferred to a dry beaker, and the slides were dried under a stream of nitrogen.

Displacement assay. Following incubation and scanning, the slide printed with the AHL analog **7** was placed in a shallow plastic container which contained 5 mM HHL and OHHL dissolved in 30 mL of Tris buffer (50 mM Tris, 0.1 M NaCl, pH 7.4). Slides were covered and were shaken at 300 rpm on a Heidolph Vibramax 100 for 1 h at room temperature. Slides were transferred to a slide rack and washed in Tris buffer (50 mM Tris, 0.1 M NaCl, pH 7.4) for 10 min before rinsing with water and drying under nitrogen. Slides were scanned at the original scanned PMT settings and the Cy3 fluorescence was plotted for comparison purposes against concentration in Fig. 2B.

Scanning

All slides were scanned using a Genetix QScan scanner in the Cy3 region at 50% PMT with no focus adjustment. Additionally the library slide was scanned in the Cy5 region at 50% PMT in order to use the biotin-avidin interaction as a control for lining up the data tracking overlay of the library. Because the Cy3 background was high using Cy3-CarR, the palette feature was used for better visualisation of spots. Note, this does not alter the underlying fluorescence data and the analysis results are unaffected. i) For the AHL analog slide, the background corrected Cy3 total intensity was plotted against the concentration in Fig. 2. ii) For the library slide, the library reference code (**L1–L95**) was plotted against the background corrected Cy3 mean intensity in Fig. 3A (see

ESI† for the scan itself). The amino-ethoxy-ethanol control and the biotin-amine values were plotted in the 96 and 97 position respectively. The library reference code was also plotted against the background corrected Cy5 total intensity (shown in the ESI†). iii) Scanned sections and background corrected Cy3 total intensity *versus* concentration of **L86** was plotted in Fig. 3B, whilst the equivalent plot and images for **L15** are found in the ESI†.

Fluorometric ligand binding assays[12]

Each experiment was repeated three times to give an average. Graphpad Prism software was used, using a single binding site model fitted with non-linear regression to give returned K_d values. See Fig. 4 for binding assays of ligands **18** & **19** and ESI† for others.

Serratia Carbapenem Assay

Carbapenem production in *Serratia* sp. ATCC39006 was measured using a mutant called LIS and a ESS super-sensitive *E. coli* as a reporter strain.[30] The *Serratia* mutant LIS carries a mini-Tn*5* Sm/Sp, SpR transposon insertion in the *smaI* gene and is unable to generate its own BHL. As a consequence, it no longer makes the vivid red pigment, prodigiosin, the biosynthesis of which is normally under quorum sensing control. For agonist assays, the compounds to be tested were dispensed into the wells of a 6-well cell culture plate (Nunc) and mixed with LB-agar (1.5% w/v agar). For antagonist assays, the same procedure was followed except that the agar contained the indicated concentration of BHL. The agar was allowed to set and, after briefly drying in a warm oven to remove excess moisture from the surface of the agar in the wells, the plates were overlain with a layer of 0.5% LB-agar seeded with a 1/500 dilution of an overnight culture of ESS. The soft agar layer was allowed to solidify before spotting on 2 µl droplets of a stationary-phase LIS culture. The plates were allowed to develop overnight and then photographed. Production of carbapenem gives rise to a clear zone (halo) in the overlay shown in Fig. 6A.

Pseudomonas aeruginosa - GFP Assay

To investigate the efficiency of a putative inhibitor on *P. aeruginosa* of quorum sensing, the quorum sensing reporter strain MH602 was applied as follows: 150 ml ABT medium supplemented with 0.5% cas-amino acids were added to wells in a microtiter dish (Black Isoplate®, Perkin Elmer). A 2-fold serial dilution row of the pure compound was made, leaving the last well for reference (concentration of the tested agent = zero). Finally 150 ml 50x diluted overnight culture of MH602 (OD450 of diluted culture = 0.1) was added to the wells. The Gfp(ASV)-expression (measured as fluorescence; excitation and emission wave length 485 and 535 nm, respectively) was measured every 15 min during the following 18 h by the use of the multilabel plate reader Wallac 1420 VICTOR2 (Perkin Elmer). The OD450 was also determined as a measure for growth. During the assay the temperature was held constantly at 34 °C. See Fig. 6B.

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