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FULL PAPER

β-Cyclodextrin encapsulation of synthetic AHLs: drug delivery implications and quorum-quenching exploits.

Eric W. Ziegler,[a] Alan B. Brown,[a] Nasri Nesnas,[a] Christopher D. Chouinard,[a] Anil K. Mehta,[b] and Andrew G. Palmer,[a][c]*

We dedicate this manuscript to Dr. J. Clayton Baum, an advisor, mentor, colleague, coauthor, scientist (in the noblest sense of the word), and also — perhaps most importantly — a friend to all at Florida Institute of Technology. We dedicate this work to the effect that Dr. Baum had on our lives and those of his students while serving as a professor and researcher of physical chemistry throughout his tenure at Florida Tech. We dedicate this work to his thoughtfulness and his careful curation of experiments that EWZ performed as a student and TA under his direction that are foundational to the work presented here. Lastly, we dedicate this manuscript to the memories of and lessons learned from Dr. Baum that we will cherish and hold with us for the many years to come. Dr. Baum, *ad astra per scientiam*!

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Abstract: Many bacteria, such as *Pseudomonas aeruginosa*, regulate phenotypic switching in a population density-dependent manner through a phenomenon known as quorum sensing (QS). For Gram-negative bacteria, QS relies on the synthesis, transmission, and perception of low molecular weight signal molecules that are predominantly N-acyl-L-homoserine lactones (AHLs). Efforts to disrupt AHL-mediated QS have largely focused on the development of synthetic AHL analogues (SAHLAs) that are structurally similar to native AHLs. However, like AHLs, these molecules tend to be hydrophobic and are poorly soluble under aqueous conditions. Water-soluble macrocycles, such as cyclodextrins (CDs), that encapsulate hydrophobic guests, have long been used by both agricultural and pharmaceutical industries to overcome solubility issues associated with hydrophobic compounds of interest. Conveniently, CDs have also demonstrated anti-AHL-mediated QS effects. Here, using fluorescence spectroscopy, nuclear magnetic resonance spectrometry, and mass spectrometry, we evaluate the affinity of SAHLAs, as well as their hydrolysis products, for β-CD inclusion. We also evaluated the ability of these complexes to inhibit wildtype *P. aeruginosa* virulence in a *Caenorhabditis elegans* host infection study, for the first time. Our efforts confirm the potential of β-CDs for the improved delivery of SAHLAs at the host:microbial interface, expanding the utility of this approach as a strategy for probing and controlling QS.

Introduction

Since its discovery in 1970, population density-dependent gene expression—typically referred to as quorum sensing (QS)— has been widely observed among bacteria and is frequently associated with phenotypes that, if expressed by a lone bacterium, would be ineffective.[1,2] Common examples of these phenotypes include biofilm and virulence factor production by *Pseudomonas aeruginosa*, an opportunistic pathogen known for its adverse effects on patients suffering with cystic fibrosis or nosocomial (*i.e.*, healthcare-related) infections. When it was observed that pathogenic bacteria in which the underlying QS molecular circuitry was disrupted exhibited less virulence, QS inhibition became an attractive target for anti-infective and anti-virulence strategies, necessitated by the increasing frequency of multidrug resistant bacterial infections and dwindling arsenal of viable antibiotics with which to treat them.[3,4]

QS relies on the synthesis, transmission, and perception of low molecular weight signal molecules generically classified as autoinducers (AIs). Presently, at least a half-dozen different QS strategies have been identified, each differentiable by the molecular structure of the AI employed, their cognate receptor, and the mechanism of signal transduction within the cell.[5] In Gram-negative bacteria, such as *P. aeruginosa*, the predominant AIs are *N*-acyl-L-homoserine lactones (AHLs, Figure 1).[6]

These compounds are composed of a γ-butyrolactone `head group' that participates in highly conserved molecular recognition interactions (*i.e.*, hydrogen bonding) with cognate receptors

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(LuxR-type proteins), and an acyl tail that encodes species and/or signal specificity through exclusive interactions with the cognate receptor facilitated by the chain length and oxidation state at the β-position.[7,8] AHL-mediated QS inhibition, to date, has largely focused on using small molecule ligands capable of emulating the molecular recognition interactions between native AHLs and their receptor proteins (*i.e.*, receptor antagonism). Example classes of AHL receptor antagonists include halogenated furanones,[9,10] anthropogenic AHLs,[11–16] and homocysteine analogues (*i.e.*, thiolactones).[17]

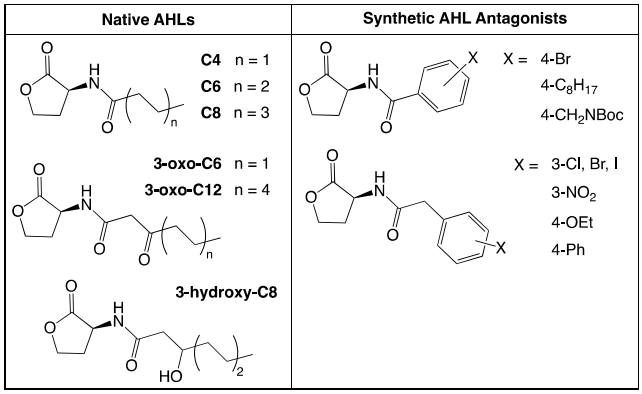

Figure 1. Chemical structures of bioactive, native *N*-acyl-L-homoserine lactones and synthetic antagonists that demonstrate bioactivity against the QscR-mediated QS in *P. aeruginosa*.

Anthropogenic AHLs, sometimes known as synthetic AHL analogues (SAHLAs), have been remarkably successful at modulating QS.[11,18–22] Blackwell and coworkers, since reporting a rapid solid-phase supported method for the synthesis of AHLs and SAHLAs, have made significant contributions to this class of QS modulators by: (a) generating an extensive synthetic AHL library of variable lactone stereochemistry and acyl tail composition, (b) screening that library for bioactivity against well-characterized AHL-mediated QS circuits, and (c) evaluating the effects of SAHLAs on prospective host organisms. Such screens have identified many effective receptor antagonists (for examples, see Figure 2), many of which possess native lactone stereochemistry and acyl tails containing aromatic groups that are highly hydrophobic.[13–16,23,24] Though effective antagonists, these compounds, like native AHLs, are often poorly soluble under aqueous conditions as a consequence of their hydrophobic tails and, as such, frequently must be introduced into biological assays using carrier solvents such as dimethyl sulfoxide (DMSO). DMSO is commonly used in medical formulations; however, it is known to alter membrane permeability, complicating assay results, and, physiologically, can induce adverse effects in patients (*e.g.*, nausea and vomiting.[25]

Improved delivery systems for SAHLAs could significantly contribute to efforts to advance QS regulation as a viable anti-virulence strategy. We propose that, in place of DMSO as a carrier solvent to facilitate solubilization, the hydrophobicity of these antagonists could be exploited to form inclusion complexes with macrocyclic hosts, such as cyclodextrins (CDs, Figure 3).

CDs are commonly composed of six (α-CD), seven (β-CD), or eight (γ-CD) glucopyranosyl subunits bound by $\alpha_{1\rightarrow4}$ linkages. As a result, CDs adopt a toroid geometry such that the alcohol groups line the narrow (1°) and wide (2°) brims while the inner cavity is lined by hydrogens, imbuing it with hydrophobic character. This highly organized architecture makes the macrocycle both water-soluble and capable of hosting poorly soluble, hydrophobic species; the latter of which is driven by the hydrophobic effect. Indeed, many of the molecules used for acyl chain substitutions in SAHLAs are known to associate with β-CD. Notably, β-CD are biocompatible, do not elicit the adverse side effects experienced by patients exposed to DMSO, and have, therefore, been utilized in both agricultural and pharmaceutical formulations to enhance solubility, stability, and bioavailability of high-value hydrophobic molecules, such as chemotherapeutic agents.[26]

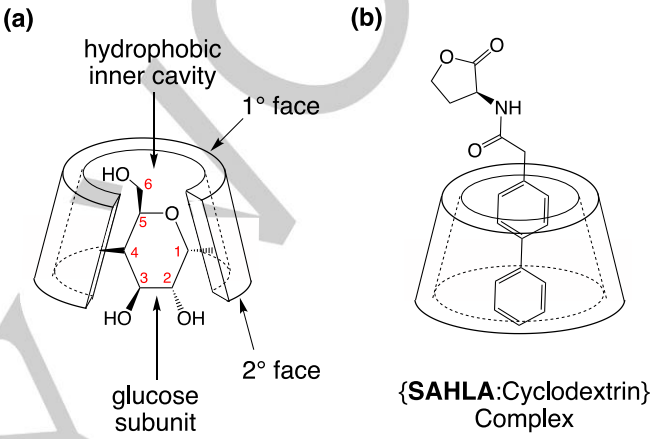

Figure 2. (**a**) Cyclodextrin geometry and (**b**) a possible orientation of a {CD:SAHLA} complex with hydrophobic tail docking within CD cavity.

Apart from their potential utility in solubilizing poorly soluble SAHLAs, which would presumably increase SAHLA bioactivity (via improved delivery), α- and β-CDs (along with their derivatives) — by themselves — have demonstrated efficacy as QS inhibitors through the formation of inclusion complexes with naturally occurring AHLs. In effect, inclusion complex formation reduces the concentration of free AHLs in the bacterial environment.[27–32] These preliminary studies underscore both the potential of this approach, as well as the need for further biological and chemical studies to clearly determine the true benefits.

Biologically, these prior studies have focused on the use of QS reporter lines, which are unable to synthesize AHLs on their own,[27–29] and often behave differently than the wildtype strains from which they are derived.[18] Furthermore, these assays are typically performed in idealized culture conditions in which no host is present, further simplifying the assay but also distancing it from biological relevance. Evaluating the effectiveness of these complexes under native conditions (*i.e.*, during a host-infection assay) is crucial to the successful development of the SAHLA:CD approach as an anti-virulence strategy.

Chemically speaking, previous efforts to obtain binding constants for {CD:AHL} complexes have also encountered certain limitations. For example, binding constants have been determined and/or estimated in either unbuffered or biologically irrelevant aqueous environments (*e.g.*, D_2O,[27] distilled water,[28] and carbonate buffer[29]). Many of these studies have been conducted under conditions where AHLs hydrolyze rapidly (pH 9.5[29]), giving rise to binding constants that likely represent statistical mixtures of AHLs and their hydrolysis products; with considerable error.[28]

Finally, these studies are often performed at relatively high AHL concentrations, which is problematic because AHLs are known to form micelles[33] and the formation of these supramolecular architectures has been suggested as a source of deviation of expected {AHL:receptor} complex formation (as monitored by fluorescence quenching) in at least one binding study.[34] The formation of micelles is particularly problematic in binding assays, most notably those involving indicator displacement methods, [29] because their presence introduces another hydrophobic effect driven association phenomenon (*i.e.*, indicator incorporation into the micelle), which may result in observed spectral changes consistent with displacement from the CD cavity by analyte but not necessarily indicative of it. Indicator displacement assays rely on two competing equilibria; the introduction of additional competing equilibria further complicates assay results and their interpretation.

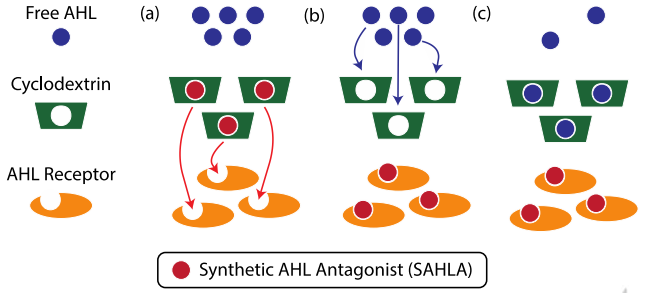

Figure 3. Cartoon of proposed SAHLA-CD synergistic action where (**a**) SAHLA:CD are deployed, SAHLA is released, and SAHLA binds to AHL receptors; (**b**) free native AHL is captured by the now-vacant CD cavity, which leads to (**c**) fewer free AHLs in the chemical environment.

Because of the inherent anti-virulence capabilities exhibited by CDs and their ability to solubilize poorly soluble compounds, we hypothesized that the co-application of these two species could actually work in concert to elicit additive if not synergistic effects. As illustrated in Figure 3, SAHLAs could be solubilized by, and delivered to, the bacterial environment by a CD. Once CD-encapsulated SAHLAs were released, they would compete with native AHLs for receptor binding, while the now free CDs could encapsulate native AHLs, further impeding QS-activation.

In the present study, we have evaluated the association of three representative SAHLAs (**1**-**3**, Figure 4) with β-CD in biologically relevant phosphate buffered saline (pH 7.3), where AHLs hydrolyze significantly slower than at pH 9.5.[35–38] We selected β-CDs for the present study as they are the most economical of the three CDs and more importantly, posses the appropriate dimensions to provide a 'snug' fit for the aromatic moieties of SAHLAs **1-3**.

These ligands were selected both for their physical properties as well as biological utility as QS antagonists. As the acyl tail of all three of these previously synthesized compounds possess fluorophores, it was possible to directly obtain binding constants for complex formation. Inclusion complexes were further characterized by nuclear magnetic resonance (NMR) and mass spectrometries. To demonstrate why the conditions — especially pH — for AHL binding constant determination and/or estimation is highly relevant, we also determined the binding constants of two SAHLA hydrolysis products, noting differences in association behavior.

Lastly, and building on these inclusion complex studies, we confirm, for the first time, the utility of {SAHLA:CD} inclusion complexes for improved anti-virulence under native (*i.e.*, host infection) conditions. Specifically, we establish increased host survival during infection assays of *Caenorhabditis elegans* by wildtype *P. aeruginosa* PAO1. We discuss our findings with regards to their potential benefits to QS regulation and anti-virulence.

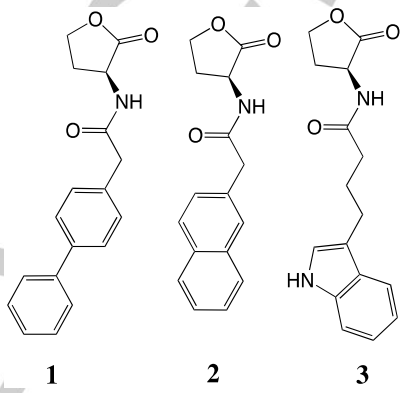

Figure 4. Molecular structures of the three fluorescent synthetic AHLs used in this study to evaluate inclusion complex formation of Blackwell Library constituents with β-CD.

Results and Discussion

SAHLA ASSOCIATION WITH β-CD

Our investigation into the association behavior of synthetic AHLs with β-CD began with **1**, principally because the association of its carboxylic acid precursor (4-biphenylacetic acid, BPAA) with β-CD is well-known through both solubility studies and X-ray crystallography.[39–41] The crystal structure of the {β-CD:BPAA} inclusion complex, solved by Wang and coworkers, indicated that the biphenyl region of BPAA docks within the host's hydrophobic cavity.[41] This SAHLA also has biological relevance as a broad spectrum QS antagonist, capable of inhibiting the TraR[15] (*Agrobacterium tumefaciens*, Ti plasmid replication and conjugation),[42–44] LuxR[15] (*Vibrio fischeri*, bioluminescence),[45,46] and QscR[47] (*P. aeruginosa*, ``anti-virulence'')[48]. Conveniently, biphenyl is also a fluorophore and as fluorescence behavior is highly sensitive to the chemical environment experienced by the fluorophore, we hypothesized that such an approach could provide a relatively facile and rapid way to monitor the association of **1** with β-CD.

As expected, titration of **1** with β-CD altered its fluorescence behavior (Figure 5, *top*), specifically reducing fluorescence intensity, inducing a bathochromic shift (2 nm), and sharpening spectral features. We interpreted the change in fluorescence exhibited by **1** at increasing concentrations of β-CD to indicate inclusion complex formation. Application of the Benesi-Hildebrand equation to the fluorescence at λ_{max} (315 nm) afforded a linear double reciprocal plot that suggested a 1:1 host/guest complexation stoichiometry and an association constant K_{bind} of 3750 ± 160 M^{-1}. Notably, the Benesi-Hildebrand method requires that either host or guest is present in excess and that, as a result,

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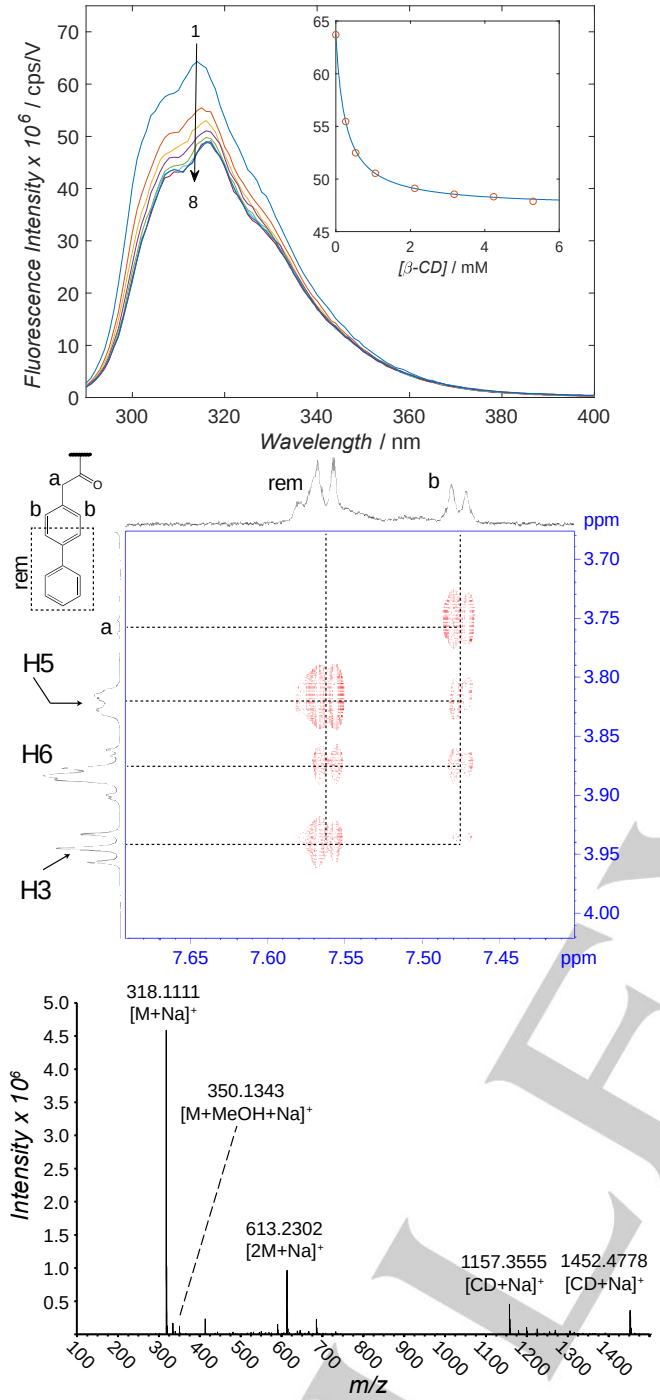

Figure 5. (*top*) Fluorescence titration of **1** (1.8 μM) with increasing concentration of β-CD (0 mM to 5.3 mM; solutions 1 through 8) in phosphate buffered saline (pH 7.3) with (*inset*) fitting to 1:1 binding isotherm determined by non-linear regression analysis at λ_{max} = 315 nm. (*middle*) 2D-ROESY NMR cross-peaks between biphenyl moiety aromatic protons of **1** (0.2 mM) and hydrogens (H3 and H5) internal to the macrocycle's inner cavity ([β-CD] = 2 mM) in D_2O, calibrated against methanol. Cross-peaks between resonances at 7.525 and 3.75 ppm arise from **1** aromatic resonances and acyl α-protons. (*bottom*) Mass spectrum obtained for β-CD:**1** mixture by ESI-MS indicating free **1**, free β-CD, and {β-CD:**1**} as their sodium adducts.

the concentration of the excess reagent is constant throughout the complexation process. Application of non-linear regression (NLR) methods (see Experimental), which do not suffer this assumption, also indicated the data was well-fitted to a 1:1 binding isotherm and afforded a statistically similar association constant of 3850 ± 170 M^{-1}. The agreement between the results was not surprising given that the assumption made by the Benesi-Hildebrand method — in this case: that the host concentration was significantly greater than that of the guest — was satisfied.

It is reasonable to infer that the biphenyl moiety of **1** docks within the macrocycle's hydrophobic cavity, especially given that fluorescence behavior is highly sensitive to changes in chemical environment and the known {β-CD:BPAA} complex crystal structure.

However, the fluorescence titration experiment does not explicitly provide structural information regarding the {β-CD:**1**} complex. To this end, we performed 2D-ROESY NMR, which identifies nuclei that are located in close spatial proximity to one another. For a CD host:guest complex, 2D-ROESY would indicate, by the presence of cross peaks, that the internal protons of the CD cavity (*i.e.*, H3 and H5) are in close proximity to the protons on the docking region of the guest. Shown in Figure 5 (*middle*), the aromatic resonances of **1** exhibit cross-peaks with the internal hydrogens of β-CD, indicating that the biphenyl region is responsible for complex formation. This not only confirmed that the fluorophore docked within the CD cavity, but also suggested that, given the similar structure of the {β-CD:BPAA} complex, molecules that possess similar complex-forming regions or *complexophores* demonstrate similar modes of association. Additionally, we observed cross peaks between the aromatic resonances of **1** with CD's H6 resonances, which line the primary rim, suggesting that the biphenyl may dock well within the CD cavity to position these nuclei in close spatial proximity. Notably, these through-space interactions could arise through either the lactone projecting from the primary or secondary faces. To further support the existence of the {β-CD:**1**} complex, we performed ESI mass spectrometry with a methanolic mixture of **1** and β-CD in hopes of observing free **1**, free β-CD, and the {β-CD:**1**} complex as their sodium adducts. All three of these species were readily detected as shown in Figure 5 (*bottom*).

Encouraged by the comparable inclusion complexes of **1** and BPAA with β-CD, we synthesized **2**, a naphthalene substituted SAHLA known to inhibit RhlR[16] (*P. aeruginosa*, host evasion andbiofilm development).[49,50] We hypothesized that **2** would emulate 2-naphthyloxyacetic acid (NOA) in terms of its association with β-CD.[51] The fluorescence of **2**, like NOA, was enhanced in the presence of β-CD (Figure 6, *top*).

The mixture of **2** with β-CD also afforded a slight bathochromic shift (2 nm) of the fluorescence peak at 325 nm and sharpening of spectral features. As with **1**, the linearity of the resulting double reciprocal plot suggested a 1:1 complex stoichiometry. NLR treatment of the fluorescence intensities at λ_{max} (334 nm) also yielded a 1:1 binding isotherm to which the experimental data was well-fitted with an association constant of 395 ± 15 M^{-1}. Similar to **1**, the existence of the {β-CD:**2**} complex and 1:1 stoichiometry was further supported by mass spectrometry (Figure 6, *bottom*)

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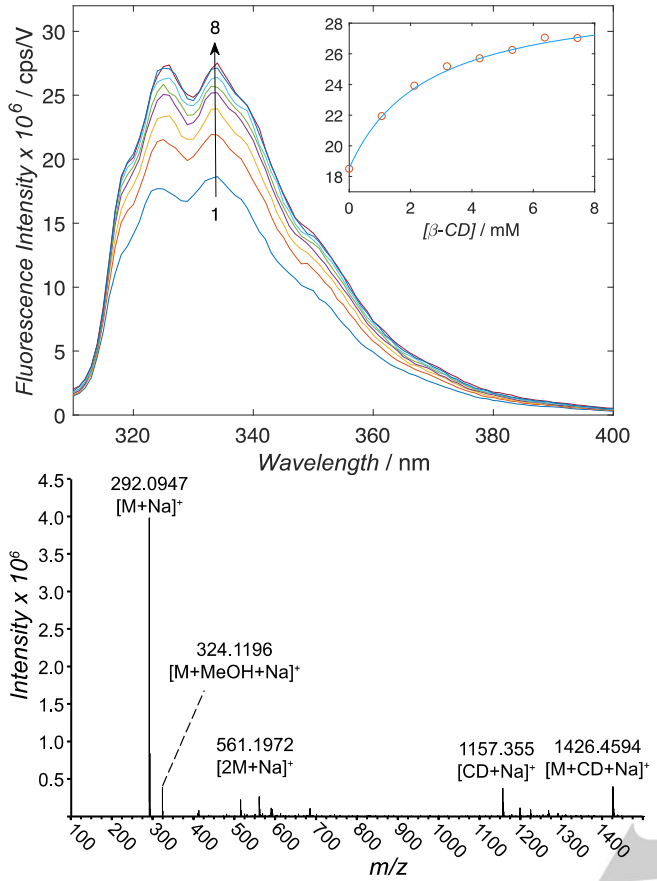

Figure 6. (*top*) Fluorescence titration of **2** (2.9 μM) with increasing concentration of β-CD (0 mM to 7.4 mM; solutions 1 through 8) in phosphate buffered saline (pH 7.3) with (*inset*) fitting to 1:1 binding isotherm determined by NLR analysis at λ_{max} = 333 nm. (*bottom*) ESI-MS spectrum of free **2**, free β-CD, and {β-CD:**2**} complex as sodium adducts.

While the acyl tails of many bioactive synthetic AHLs are primarily all carbon; a number of heterocycles (*e.g.*, furan, indole, oxazole, thiophene) with potential biological activity in bacterial systems have been developed. We, therefore, selected SAHLA **3** as a representative substrate for this class because its acyl tail bears an indole and it is an antagonist for the LuxR receptor that controls bioluminescence in *V. fischeri*.[15] Exposure of **3** to increasing concentrations of β-CD afforded both fluorescence enhancement and a hypsochromic shift (7 nm). This is consistent with the behavior reported for its carboxylic acid precursor, 3-indolebutyric acid, which is known to form an inclusion complex with β-CD.[52] Similar to compounds **1** and **2**, Benesi-Hildebrand treatment of fluorescence intensities at λ_{max} (360 nm) indicated 1:1 host/guest complex stoichiometry and non-linear regression afforded a 1:1 binding isotherm to which our experimental data was well fit (K_{bind} = 374 ± 10 M^{-1}). ESI-MS supported the existence of the {β-CD:**3**} complex with 1:1 stoichiometry.

Taken together, these findings support the potential for CDs to form inclusion complexes with SAHLAs, improving their solubility and, as a result, their potential utility for QS modulation.

ASSOCIATION OF HYDROLYSIS PRODUCTS

AHLs, both native and synthetic, are only active in bacteria if they interact with an AHL receptor through highly conserved molecular recognition interactions (*i.e.*, hydrogen bonding). Such interactions include the lactone carbonyl oxygen, amide carbonyl oxygen, and amide hydrogen that are only possible if the lactone remains intact.[7,8] Under aqueous conditions, however, lactones (and thus AHLs) are prone to abiotic hydrolysis, resulting in loss of biological activity.[53,54] Our group and others have investigated the stability of native AHLs under different acidities/alkalinities.[35–37] The hydrolysis of synthetic AHLs has been reported on by Bertucci *et al.*, specifically how the strength of an intramolecular interaction (*i.e.*, n→π*) influences hydrolysis rate.[38]

Hydrolysis, though, does not chemically alter the complexophore of either native or synthetic AHLs. As such, we reasoned that the AHL hydrolysis products should also form inclusion complexes with β-CD. This would unfortunately allow for the potential formation of unproductive complexes, *i.e.*, the binding and delivery of inactive SAHLAs.

Having already synthesized AHLs **1** and **2** — which, like native AHLs, have acyl chains that are carbon-based — and prepared aqueous stock solutions for our binding studies, we allowed these AHLs to undergo hydrolysis under neutral conditions, monitoring progress by TLC (4:1 ethyl acetate/hexanes, vis. on UV active TLC plates). Notably, AHLs migrate off the baseline (R_f ~ 0.4) while their homoserine hydrolysis products do not (R_f ~ 0). AHLs could not be detected after two weeks and we proceeded with binding studies, noting similar changes in fluorescence spectra morphology.

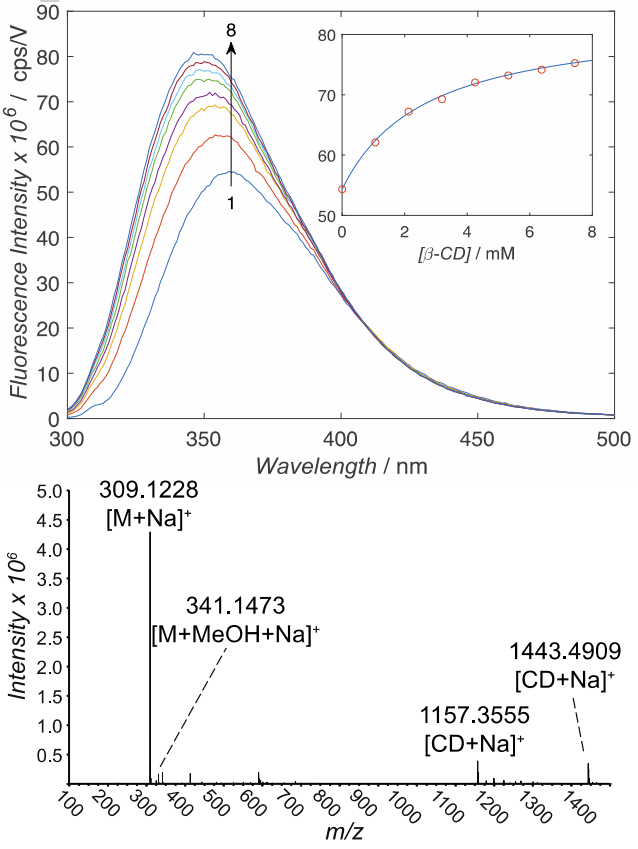

Figure 7. (*top*) Fluorescence titration of **3** (8.7 μM) with increasing concentration of β-CD (0 mM to 7.4 mM; solutions 1 through 8) in phosphate buffered saline (pH 7.3) with (*inset*) fitting to 1:1 binding isotherm determined by NLR analysisat λ_{max} = 360 nm. (*bottom*) ESI-MS spectrum of free **3**, free β-CD, and {β-CD:**3**} complex as sodium adducts.

Summarized in Table 1, the hydrolysis products of **1** and **2**, respectively **1h** and **2h**, demonstrated weaker affinity towards association with β-CD than their lactone precursors, meaning that β-CD preferentially binds active AHLs (and synthetic analogues) over their corresponding inactive, hydrolyzed forms. In terms of SAHLA solubility and delivery, this translates to CDs preferentially forming productive complexes with bioactive molecules rather than unproductive complexes with hydrolysis-inactivated SAHLAs. We assert that this reduction in binding affinity is directly attributable to molecular composition. Explicitly, AHL hydrolysis products bear a formal negative charge (*i.e.*, carboxylate) under the conditions tested and three additional hydrogen bonding sites relative to their respective AHLs. This difference not only affects the polarity of the molecule (as seen by TLC), but also likely increases the hydrolysis products' solvation — particularly about the carboxylate — making it more hydrophilic. Indeed, Dong *et al.* noted that lactone hydrolysis products are in fact more hydrophilic than their respective lactones using HPLC.[55] Additionally, binding constant decrease upon charge adoption is consistent with the observed behavior of many species, including BPAA, as a function of pH. Below its pK_a, BPAA exhibits a significantly higher binding constant with β-CD than above its pKa; upon conversion to its carboxylate, BPAA affinity towards association decreases.[56]

Table 1. Binding Constants (K_{bind}) of SAHLAs and hydrolysis products determined in this study.

Substrate	Binding Constant, K_{bind} (M^{-1}) [a]
1	3850 ± 170
1h	2920 ± 180
2	395 ± 15
2h	337 ± 16
3	374 ± 10

[a] [a]Reported binding constants represent the error-weighted mean obtained from five independent fluorescence titration experiments. Errors reported represent 3 SE (99.7% confidence) about the error-weighted mean.

As an alternative explanation, the hydrolyzed homoserine lactone has more conformational freedom and is therefore more likely to interact with the alcohol groups that line the primary or secondary CD faces. Because there is greater electron density on the homoserine than on its corresponding lactone, electrostatic repulsion between the homoserine and CD alcohols could also decrease the propensity of the hydrolysis products to form inclusion complexes.

As we noted in our report on AHL hydrolysis kinetics,[36] the conditions under which AHL-centric assays are performed – especially pH – are crucial to evaluate whether assays are appropriate and how the results should be interpreted. In that report, we noted the binding constant estimation performed by Morohoshi *et al.*,[29] who used Buvari[57] and coworkers' phenolphthalein displacement assay to determine the binding constant of *N*-hexanoyl-L-homoserine lactone with various CD derivatives, was seemingly inappropriate because, under those conditions (pH 9.5), the AHL readily hydrolyzes ($t_{1/2}$ < 20 min) and the resulting binding constant likely describes a statistical mixture of the AHL and its hydrolysis product. In that report, we predicted that the hydrolysis product would likely have a lower affinity for associating with the macrocycle due to its additional hydrogen bonding sites and formal negative charge. This assertion has been demonstrated here by comparing the binding constants of **1** with **1h** and **2** with **2h**. The effect of hydrolysis on binding affinity is profound and underscores the importance of performing AHL-centric assays under near neutral or acidic conditions, where AHLs are stable.

Additionally, the decreased binding affinity of acyl homoserines as compared to their lactones is a characteristic shared by the AHL lactonase, AiiA, which is deployed by the Gram-positive bacteria *Bacillus thurengiensis* to attenuate AHL-mediated QS. Given CDs can be readily modified, we hypothesize that, in the future, a β-CD based artificial enzyme could be synthesized to accomplish AHL hydrolysis — similar in action to AiiA. Such a catalyst would naturally exploit the desired substrate's propensity for binding over its hydrolysis product to establish a natural turnover cycle.

CD:SAHLA COMBINATION IMPROVES ANTI-VIRULENCE

From these experiments, it is apparent that β-CD can form inclusion complexes with synthetic AHLs and, as a result, can be exploited to better solubilize these molecules. The high propensity of **1** for inclusion is on the same order of magnitude as that of Imatinib ($\log K_{bind}$ = 3.18), a potent and selective inhibitor of protein tyrosine kinase used in the treatment of chronic myelogenous leukemia.[58] The Captisol® formulation of Imatinib uses a β-CD derivative to enhance the compound's solubility 50-fold. On the other hand, as evidenced by the relatively low binding constants that describe the inclusion complexes of **2** and **3** with β-CD, derivatization of the macrocycle (*e.g.*, permethylation) may be necessary to enhance solubility and make β-CD more viable for drug delivery; however, the association constants of **2** and **3** are of the same magnitude as the protonated forms of Imatinib. Perhaps more interestingly, though, the use of β-CD in the context of QS may have even greater effects than simply acting as a delivery vehicle.

As noted previously, CDs and their derivatives demonstrate efficacy as quorum sensing inhibitors by presumably forming inclusion complexes with active AHL signal molecules, thereby reducing the concentration of free AHLs in the bacterial environment.[27–32] We hypothesized that using a combination treatment of CD and SAHLA, there would by some synergistic effects in that CD would help solubilize the SAHLA and increase its bioavailability and aid in its delivery. Because SAHLAs outcompete native AHLs for receptor binding – they are receptor antagonists – the vacated CDs could uptake the native free AHLs and further impede the QS-circuit (Figure 3). To test this hypothesis, we synthesized **4**, a moderate antagonist of Pseudomonas' LasR-circuit,[15] and evaluated whether it could form an inclusion complex with β-CD by ESI-MS. Notably, SAHLAs **1**-**3** are not significant inhibitors of LasR.[15] To our delight, **4** formed a complex with 1:1 stoichiometry (Figure 8, *top*).

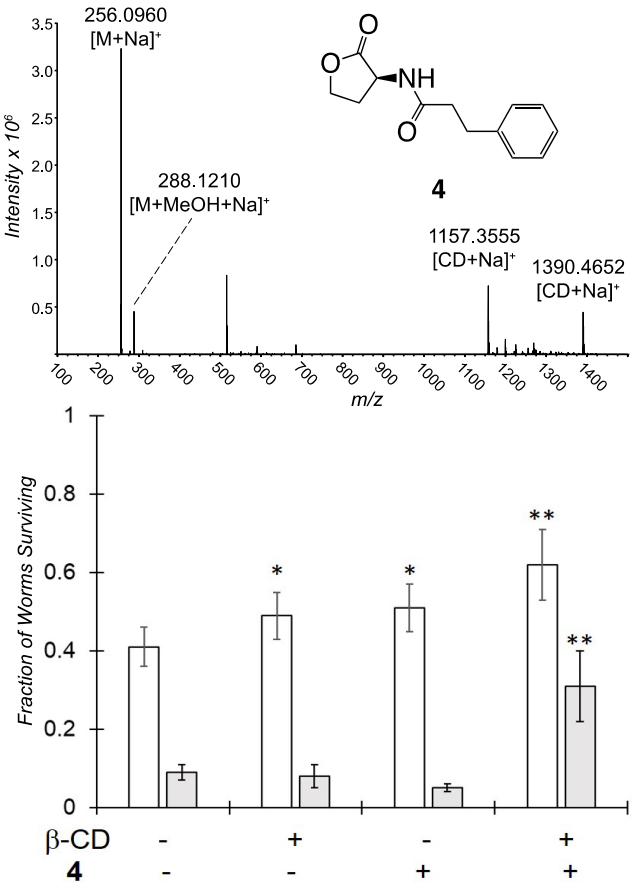

Figure 8. (*top*) Molecular structure of synthetic AHL antagonist **4** that inhibits LasR in *P. aeruginosa* and ESI-MS spectrum indicating **4** forms a 1:1 complex with β-CD. (bottom) Results of *C. elegans* viability assay indicating that when 10 μM **4** and 50 μM β-CD are coapplied, viability at 12 (white bars) and 24 (grey bars) hrs is dramatically increased, implying synergistic effects. Results are the average of 3 trials (N=30 worms/treatment/trial). Error bars represent SE. '*' indicates p-value < 0.05, '**' indicates p-value < 0.01.

Having confirmed the formation of the {β-CD:**4**} complex, we proceeded to test our hypothesis that they could function synergistically to inhibit quorum sensing. Specifically, we evaluated the virulence of wildype *P. aeruginosa* PAO1 with the model eukaryotic host *C. elegans*. Briefly, mature *C. elegans* (*N*=30) were transferred to plates containing *P. aeruginosa* in the presence of β-CD (50 μM) and/or SAHLA **4** (10 μM) and evaluated for viability at 12 and 24 hours under a light microscope. Nematodes were scored for viability by either watching them move across the plate or by applying gentle pressue to the side of the worms using a blunt glass tip to look for reduced motility phenotypes. Shown in Figure 8 (*bottom*), treatment with either β-CD or **4** alone slightly increased *C. elegans* viability at 12 hours; however, in both cases, this improvement was short-lived and did not extend to the 24-hour benchmark. On the other hand, co-application of **4** and β-CD significantly increased *C. elegans* viability beyond 24 hours, suggesting this combination treatment indeed evokes additive, if not synergistic, effects. We note that the addition of either β-CD (50 μM) and/or SAHLA **4** (10 μM) had no effect on *P. aeruginosa* growth in culture (Data not shown) or *C. elegans* viability on pathogen-free plates arguing that this effect comes from the inhibition of QS-mediated virulence factor production rather than any antimicrobial or host effect of these compounds. To our knowledge, this is the first time that a {SAHLA:β-CD} complex has been employed to inhibit virulence in a wildtype pathogen during a host-infection assay.

Conclusion

We have shown that synthetic AHLs, along with their hydrolysis products form inclusion complexes with β-CD using fluorescence spectrophotometry along with NMR and MS spectrometries. We propose that, as a result of that complexation, CDs can be used to better solubilize and deliver SAHLAs to intended target sites. However, as CDs alone demonstrate anti-QS effects, the application of CDs as SAHLA delivery vehicles is a *per se* combination therapy that elicits additive, if not synergistic, effects that we have shown here using a *C. elegans* viability model. Lastly, because there exists some differential in binding affinity of SAHLAs and their hydrolysis products — and, by analogy, native AHLs and their respective hydrolysis products — with β-CD, we assert that that differential could be exploited in the design of an artificial AHL lactonase based on a β-CD scaffold.

Experimental Section

Materials and Instrumentation.

L-homoserine (98%) was obtained from AABlocks and was converted, as needed, to homoserine lactone hydrochloride by reflux with 4M hydrochloric acid and evaporation to dryness. 4-Biphenylacetic acid (98%) was purchased from Acros Organics. 2-Naphthylacetic acid (99%) and indole-3-butyric acid (98%) were purchased from AlfaAesar. 3-Phenylpropanoic acid (95%) was purchased from Enamine, LLC. Triethylamine (99%) was purchased from Acros Organics. *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC-HCl) was purchased from CreoSalus. Dichloromethane was purchased from Fisher Scientific. All reagents were used without further purification.

The identities of products were confirmed using ^{1}H- and ^{13}C-NMR, as well as mass spectroscopy. All NMR spectra were collected using Bruker Avance 400 MHz spectrometers at 22.0 ± 0.5 °C. Mass spectra were collected using an Agilent 6560 IM-QTOF mass spectrometer (MS).

MS samples were prepared by dissolving samples in LC-MS grade methanol (10 μg/mL) and sonicating for 10 minutes. MS samples were then direct infused at 15 μL/min with positive mode electrospray ionization (ESI). The samples were also analyzed after addition of 20 μg/mL β-CD. Noncovalent complexes were identified by accurate mass as primarily the $[M+CD+Na]^{+}$ and $[2M+CD+2Na]^{+2}$ adducts. Collision cross section (CCS) was measured for all complexes.

Absorbance spectra were collected using a Jasco V-650 Spectrophotometer calibrated against a blank of phosphate buffered saline (PBS, pH 7.3). Fluorescence measurements were performed using a Jobin Yvon Horiba Fluoromax-3 fluorimeter and were corrected for the spectral response of the instrument.

Melting points were observed visually using a MelTemp-II apparatus.

AHL Synthesis.

Following the procedure of Ruysbergh[59] *et al.* for synthesizing native AHLs with slight modification, compounds **1**, **2**, and **4** were prepared by first dissolving freshly synthesized homoserine lactone hydrochloride (275 mg, 2 mmol) in deionized water (15 mL) and treating the resulting solution with the corresponding carboxylic acid (2 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (380 mg, 2 mmol, 1 equiv.), and

triethylamine (300 mL, 2.15 mmol, 1 equiv.). The reaction mixture was stirred vigorously overnight at room temperature, diluted with deionized water (15 mL), and extracted with ethyl acetate (3 x 50 mL). The combined organic phases were washed successively with saturated sodium bicarbonate and brine solutions (30 mL each), dried over anhydrous sodium sulfate, and concentrated *in vacuo* by rotary evaporation. Flash silica chromatography (4:1 ethyl acetate/hexanes, visualized by UV and ninhydrin staining) afforded the desired synthetic AHL in moderate yield as a white solid.

Compound **3** was prepared by charging a round-bottomed flask in an ice-water bath with freshly synthesized homoserine lactone hydrochloride (275 mg, 2 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (380 mg, 2 mmol, 1 equiv.), and 3-indolebutyric acid (410 mg, 2 mmol, 1 equiv.). To these solids were added dichloromethane (15 mL) and triethylamine (580 mL, 4 mmol, 2 equiv.). The reaction mixture was warmed to room temperature and vigorously stirred overnight, then diluted with ethyl acetate (30 mL) and extracted with deionized water (30 mL). The aqueous phase was further extracted with ethyl acetate (2 x 30 mL). The combined organic layers were washed successively with saturated sodium bicarbonate and brine solutions (30 mL each), dried over anhydrous sodium sulfate, and concentrated *in vacuo* by rotary evaporation. Flash chromatography (4:1 ethyl acetate/hexanes, visualized by UV and ninhydrin staining) afforded **3** as a white solid.

N-(4-biphenyl)acetyl-L-homoserine lactone (**1**):
^{1}H-NMR (400 MHz; $CDCl_3$, δ): 7.62-7.56 (4H), 7.48-7.42 (2H), 7.39-7.32 (3H), 6.05 (1H, d, J = 3.6 Hz), 4.54 (1H, ddd, J = 11.6, 8.7, 6.0 Hz), 4.44 (1H, ddd, J = 9.0, 9.0, 0.9 Hz), 4.26 (1H, ddd, J = 11.2, 9.3, 6.0 Hz), 3.67 (2H, s), 2.81 (1H, dddd, J = 12.7, 8.4, 5.9, 1.1 Hz), 2.12 (1H, dddd, J = 12.4, 11.5, 11.5, 8.9 Hz); ^{13}C-NMR (100 MHz; $CDCl_3$, δ): 175.22, 171.62, 140.66, 140.61, 133.11, 129.98, 128.96, 127.96, 127.59, 127.19, 66.15, 49.54, 43.02, 30.43; Melting Point: 173-175 °C; Mass (m/z, [M+Na]+): (*theor*.) 318.11061, (*found*) 318.1115; Yield: 65%.

N-(2-naphthalene)acetyl-L-homoserine lactone (**2**):
^{1}H-NMR (400 MHz; $CDCl_3$, δ): 7.87 (3H), 7.74 (1H, s), 7.53-7.46 (2H, 'dd'), 7.39 (1H, dd, J = 8.4, 1.7 Hz), 6.00 (1H, d, NH, J = 4.2 Hz), 4.51 (1H, ddd, J = 11.6, 8.6, 6.1 Hz), 4.41 (1H, ddd, J = 9.2, 8.9, 0.6 Hz), 4.23 (1H, ddd, J = 11.1, 9.3, 6.0 Hz), 3.79 (2H, s), 2.76 (1H, dddd, J = 12.7, 8.5, 5.9, 1.2 Hz), 2.08 (1H, dddd, J = 12.3, 11.4, 11.4, 9.0 Hz); ^{13}C-NMR (100 MHz; $CDCl_3$, δ): 175.13, 171.62, 133.71, 132.78, 131.64, 129.13, 128.52, 127.91, 127.86, 127.27, 126.67, 126.35, 66.11, 49.49, 43.62, 30.34; Melting Point: 193-195 °C; Mass (m/z, [M+Na]+): (*theor*.) 292.0956, (*found*) 292.09496; Yield: 39%.

N-(3-indole)butyryl-L-homoserine lactone (**3**):
^{1}H-NMR (400 MHz; MeOD-*d4*, δ): 7.53 (1H, d, J = 7.8 Hz), 7.31 (1H, d, J = 8.1 Hz), 7.07 (1H, "t"), 7.03 (1H, s), 6.98 (1H, "t") 4.58 (1H, dd, J = 10.8, 9.3 Hz), 4.41 (1H, td, J = 9.0, 1.8 Hz), 4.27 (1H, ddd, J = 10.4, 9.1, 6.6 Hz), 2.8 (2H, t, J = 7.4 Hz), 2.51 (1H, dddd, J = 12.3, 9.0, 6.7, 2.1 Hz), 2.30 (2H,t, J = 7.5 Hz), 2.23 (1H, dddd, J = 12.1, 10.7, 10.7, 9.3 Hz), 2.02 (2H, p, J = 7.4 Hz). ^{13}C-NMR (100 MHz; MeOD-*d4*, δ):177.63, 176.45, 138.39, 128.94, 123.22, 119.56, 119.56, 115.75, 112.28, 67.30, 50.02, 36.52, 29.76, 27.65, 25.66. Melting Point: 63-65 °C; Mass (m/z, [M+Na]+: (*theor*.) 309.12151, (*found*) 309.1221; Yield: 50%.

N-(3-phenylpropanoyl)-L-homoserine lactone (**4**):
^{1}H-NMR (400 MHz; $CDCl_3$, δ): 7.32-7.25 (2H), 7.24-7.16 (3H), 6.07 (1H, d, NH, J = 4.2 Hz), 4.53 (1H, ddd, J = 11.5, 8.5, 6.1 Hz), 4.43 (1H, t, J = 8.9 Hz), 4.26 (1H, ddd, J = 11.1, 9.4, 5.9 Hz), 2.97 (2H, t, AB**X**$_2$, J_{AX} = J_{BX} = 7.7 Hz), 2.78 (1H, dddd, J = 12.6, 8.3, 5.9, 1.0 Hz), 2.58 (1H, **A**BX$_2$, J_{AB} = 14.9 Hz, J_{AX2} = 7.6), 2.53 (1H, A**B**X$_2$, J_{AB} = 14.9 Hz, J_{BX2} = 8.0 Hz), 2.03 (1H, dddd, J = 11.8, 11.8, 11.8, 9.1 Hz); ^{13}C-NMR (100 MHz; $CDCl_3$, δ): 175.60, 172.87, 140.64, 128.78, 128.55, 126.58, 66.27, 49.42, 38.06, 31.62, 30.64; Melting Point:146-148 °C; Mass (m/z, [M+Na]+): (*theor*.) 256.09496, (*found*) 256.0959; Yield: 44 %.

Binding Constant Determination.

Methanolic stock solutions of AHLs **1**, **2**, and **3** were prepared and subsequently diluted with PBS (pH 7.3) to 90 μM, 145 μM, and 435 μM, respectively ([MeOH] = 2% v/v). An aqueous β-CD stock (10.6 mM) was prepared by dissolving the macrocycle in PBS. For binding constant determination studies, 100 μL aliquots of aqueous AHL stock solution were transferred into 5 mL volumetric flasks charged with varying amounts of β-CD stock solution and filled to the mark with PBS ([MeOH] < 0.04% v/v). Solutions were inverted and left to equilibrate for 15 minutes prior to performing fluorescence measurements.

For **1**, the excitation and emission wavelengths were selected to be 254 nm and 315 nm. For **2**, the excitation and emission wavelengths were selected to be 277 nm and 334 nm. For **3**, the excitation and emission wavelengths were selected to be 280 nm and 360 nm. All spectra were collected with increments of 1 nm, integration times of 0.05 sec, and bandwidths (both emission and excitation) of 3 nm.

Association constants were initially determined by application of the Benesi-Hildebrand equation (Equation 1), which suggested a 1:1 host/guest complex stoichiometry by a linear double-reciprocal plot. Here, I indicates the intensity of a given solution, I_0 is the intensity of the solution without CD present, [CD] denotes the total concentration of CD present in solution, $\Delta\phi_{complex}$ denotes the change between a fluorescence quantum efficiency factor of the free and bound fluorophore, and K_{bind} denotes the binding constant.

$$\frac{1}{I - I_0} = \frac{1}{[CD]\Delta\phi_{complex}K_{bind}} + \frac{1}{\Delta\phi_{complex}} \quad (1)$$

However, to overcome the limitations posed and circumvent assumptions made by the Benesi-Hildebrand method, we fitted the data using non-linear regression (NLR).

Briefly, at low enough concentrations (*i.e.*, A(λ)<0.05), the fluorescence (*F*) of a solution can be thought of as the sum of the individual components in that solution multiplied by that component's "fluorescence efficiency factor," ϕ, which is proportional to its fluorescence quantum yield. Assuming the macrocycle's fluorescence is trivial, the fluorescence can be written as a function of total guest concentration (G_0, known) and the concentration of the host:guest complex ([HG], unknowable given an unknown association constant).

$$F = [G]\phi_G + [HG]\phi_{HG} \quad (2)$$

$$F = (G_0 - [HG])\phi_G + [HG]\phi_{HG} \quad (3)$$

The concentration of the host:guest complex can rarely be known experimentally; however, it can be written as a solution to the quadratic equation resulting from the definition of the 1:1 host:guest association constant K_{bind}, where [H] and [G] are the free host and guest concentrations, respectively.

$$K_{bind} = \frac{[HG]}{[H][G]} \quad (4)$$

$$K_{bind} = \frac{[HG]}{(H_0 - [HG])(G_0 - [HG])}$$

$$[HG]^2 - \left(H_0 + G_0 + \frac{1}{K_{bind}}\right)[HG] + H_0G_0 = 0$$

$$[HG] = \frac{1}{2}\left(\left(H_0 + G_0 + \frac{1}{K_{bind}}\right) - \sqrt{\left(H_0 + G_0 + \frac{1}{K_{bind}}\right)^2 - 4H_0G_0}\right) \quad (5)$$

Fitting the fluorescence data using Equations 3 and 5, optimized using the Simplex algorithm on PSI-PLOT software, afforded the association

constants and fluorescence efficiency factor of the host:guest complex.[60] The fluorescence efficiency factor of the guest was determined by dividing the fluorescence of the null/guest only solution by analyte concentration. Initial guesses for the association constants were the association constants determined by the Benesi-Hildebrand method (using regression in Microsoft Excel) while guesses for the host:guest complex fluorescence quantum efficiency factor were input as the fluorescence of the highest β-CD concentration solution divided by the analyte concentration.

Binding constant determination studies were per-formed with five replicates. The values reported reflect the weighted mean of those results and the error indicates three SE (99.7% confidence interval) about the reported average.

2D-ROESY NMR Experiments.

Synthetic AHL **1** was dissolved in CD_3OD and combined with β-CD in D_2O. For all samples, CD_3OD was kept at 2%. Spectra were recorded on Brucker AVANCE III 800 MHz spectrometer at a transmitter frequency of 799.9 MHz with a Bruker TCI H-C/N-D 5mm cryo probe at 12 °C. ^{1}H 1D spectra were taken before and after 2D spectra to determine if any hydrolysis of AHL occurred. Spectra were calibrated against the methanol residual solvent peak in D_2O.[61] EASY-ROESY[62] experiments were collected with a 200 ms ROESY mixing time, a spectral width of 10.02172 ppm, acquisition time of 255 ms, 90° pulse width of 8.75 μs, 64 scans and a relaxation delay of 2.5 s.

C. elegans viability assay.

Worms were synchronized by hypochlorite treatment to ensure only eggs survived. Eggs were hatched overnight and transferred to plates of nematode growth media (NGM) until they reached the L4 stage. Infection plates were prepared by taking an overnight culture of *P. aeruginosa* PAO1 grown in Lysogeny Broth (LB) at 37 °C, diluting it 100-fold into fresh LB, and transferring 100 μL to the top of nematode-free plates of NGM media. SAHLA and CD plates were prepared by adding these compounds directly to molten NGM as the plates were poured from master stocks to yield the indicated concentrations. The transferred LB media was allowed to air dry 30 minutes in a BSL2 hood. L4 stage nematodes (*N*=30) were then transferred to infection plates with or without SAHLA (10 μM **4** or 50 μM β-CD. Samples were visualized using a dissecting steromicroscope (Meiji Techno) Nematode viability was measured at the indicated time points by observing movement across the plate or by mechanoresponse (movement) in response to gentle prodding from a blunt glass tip .[63,64]

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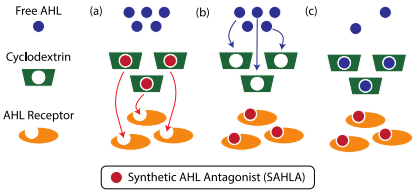

Synthetic AHL analogues (SAHLAs) have demonstrated efficacy as quorum sensing inhibitors by receptor antagonism. However, like native AHLs, these compounds are poorly soluble. To improve their solubility, we investigate the association of three representative SAHLAs, along with their hydrolysis products, with β-cyclodextrin by spectrofluorimetry and evaluate co-application of CDs with SAHLAs using a *C. elegans* infection model.