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Resolving host-guest interactions between pillararenes and homoserine lactones to restrain bacterial quorum sensing

Luotonen et al. report on a series of pillararenes, “host” molecules that can capture bacterial signal compounds in a molecular pocket to disrupt communication mechanisms involved in disease. Linking chemical and biological analyses reflected a strong link between homoserine lactone capture and blocking bacterial behaviors involved in disease.
Resolving host-guest interactions between pillararenes and homoserine lactones to restrain bacterial quorum sensing

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SUMMARY
Using supramolecular host molecules to hinder bacterial quorum sensing (QS) is a potential approach in circumventing antimicrobial resistance (AMR). The emergent family of pillararenes offers promising candidates for binding homoserine lactones (HSLs), signaling molecules used by gram-negative species, including WHO critical-priority antibiotic-resistant bacteria. Here, we compare seven cationic pillararenes against four HSLs, from (supra)molecular interactions to biological assays. Complexation, characterized by dye displacement assay and NMR spectroscopy, complemented by all-atom molecular dynamics (MD) simulations, was compared to effects in biological systems, studied using a bacterial HSL reporter system as well as biofilm and pyocyanin assays as models of QS-mediated virulence. HSL binding improves approximately 10-fold versus previous reports with a hydroxyl-functionalized pillararene, and a deeper-cavity host with marked preference for the longest-tailed HSL is identified. Successful HSL capture is directly reflected as impaired biofilm formation and pyocyanin production and improved healing in open wound in vivo models.

INTRODUCTION
Antimicrobial resistance (AMR) has been identified as an alarming and growing threat to modern health care. Repercussions of increasing resistance include direct effects like increased mortality and longer illness, loss of protection during invasive medical procedures, as well as broader economic losses. With the antibiotic pipeline steadily dwindling for the past 40 years—faced with economic, regulatory, and scientific barriers—the need to explore alternatives is apparent and further research called for to decrease dependence on classic antibacterial agents.

Among different strategies to mitigate AMR, macrocyclic host molecules have been put forth as potential molecular suppressors of bacterial quorum sensing (QS) systems. They operate through binding small signaling molecules that constitute chemical communication circuits and steer single microbes to act in the interests of the collective. A multitude of signal molecules exist for different species, often forming multi-component and dynamic control systems. These control circuits can evolve dynamically in response to, for example, growth phase, nutrient availability, and physical environment, typically with nanomolar-to-micromolar concentrations.
of signal molecule. The inactivation of these bacterial intercellular signals presents an approach involving a decreased likelihood of developing resistance through lack of direct antibacterial effect.

The multitude of signaling molecules (called autoinducers) used by different bacteria provides a variety of targets to disrupt. Macroyclic hosts may be able to match this variety with their characteristic ability to form noncovalent complexes with a variety of guest molecules through a multitude of intermolecular interactions. The affinity, specificity, and responsiveness of these host-guest complexes can be varied through careful molecular design. Earlier work has been carried out with, for example, the established macrocycle families of cyclodextrins and cucurbiturils.

Pillararenes are a relatively recent addition to the wide host of various macrocycle families. They are characterized by highly symmetrical pillar-like structures with a polygonal core of para-methylene linked aromatic rings that may sport different assortments of substituents that define the “rims” of the macrocycle cavity on both sides of the core cavity. Water-soluble cationic pillararenes also often show a lack of damage or toxicity to host model cells. A screening of 19 assorted macrocycles highlights a trimethylammonium-functionalized pillararene as a particularly promising macrocycle for QS disruption by the binding of N-acyl homoserine lactones (HSLs), small signaling molecules commonly used by gram-negative bacteria. Screening results indicated a lack of antimicrobial activity and evasion of most AMR mechanisms for many compounds.

Systematic studies on binding HSL signal molecules by different pillararenes are limited, and works with other macrocycles have only covered tail lengths of >C8 once. In the present context, the C12-tailed N-(3-oxo-dodecanoyl)-L-homoserine lactone (hereafter C12-HSL) may be considered particularly important, as part of the signaling array of Pseudomonas aeruginosa, which is categorized as a World Health Organization priority pathogen. These partially overlapping gaps are addressed in this work; an array of seven pillararene macrocycles were studied as host molecules for binding four HSL signaling molecules with acyl tail lengths of C4–C14. The effects of different host structures were studied at different levels of complexity, using dye displacement assays and NMR spectroscopy, complemented by all-atom molecular dynamics (MD) simulations to resolve molecular origins of the response and characterization in biological systems with a bacterial HSL reporter system, as well as virulence indicator studies consisting of biofilm and pyocyanin assays.

A quaternary ammonium-functionalized pillararene with an added hydroxyethyl moiety outperforms any previously reported pillararene by approximately one order of magnitude in binding HSLs. The use of slightly longer substituent chains around the aromatic cavity leads to a strong preference for the C14-tailed HSL over other HSLs, in contrast to other binding pillararenes. A direct relation is observed between binding of HSLs or lack thereof, and the pillararenes’ ability to impair QS-controlled biofilm and pyocyanin production. The findings and employed methods reported here may also become useful beyond our focus on AMR. Many other classes of small molecules used by bacteria for various QS purposes exist that may also provide interesting targets. Problems caused by QS-mediated behaviors are also not limited to healthcare, garnering attention in, for instance, marine transport, food production, and water purification.
RESULTS AND DISCUSSION

Pillararenes and targeted HSLs

A library of functionalized pillararenes were studied as potential host molecules for the binding of HSLs (Figure 1B). The aromatic units of pillararenes form a hydrophobic, π-electron-rich core cavity. Cationic derivatives were chosen; their water solubility (contrary to the poorly water-soluble unfunctionalized pillararenes) and compatibility with bacterial and host model cells greatly improve their relevance in biological settings compared to earlier, less polar derivatives. The studied pillararenes can be thought of as variants of the host with C2-spacered...
trimethylammonium end groups as substituents (Figure 1B, 1), which has been highlighted in an earlier study as having proven activity against HSLs.\textsuperscript{16}

With the reported suitability of the pillar[5]arene cavity diameter\textsuperscript{16} in mind, the focus is on five-membered hosts of the class. The pillararene structure can accommodate different substituent chains that form the rim around the aromatic core cavity. Different aspects that generally affect host-guest binding are explored via the chosen library, including additional modes of intermolecular interaction, different host dimensions, and rim functional groups. The hydrogen bond acceptors present on and near the HSL head invite the use of matching donor groups situated around the pillararenes rims, realized with host 2. The importance of cavity diameter intuitively calls also for studying different cavity depths, leading to the choosing of 3 and 4, characterized by C3 and C6 spacers between the aromatic cores and cationic trimethylammonium or trimethylphosphonium rim groups (in succession with the C2 spacer of 1). In addition to the host dimensions, the length and nature of substituents has been found to influence the dynamic behavior of pillararenes, which can show swinging and flipping of the aromatic panels.\textsuperscript{42-45} While the focus is on pillar[5]arenes, a slightly wider core cavity diameter is also included in the form of pillar[6]arene variant 5 (the diameter is typically reported as approximately 4.6–5 Å for 5-membered forms,\textsuperscript{16,28,30,46} and approximately 6.7 Å for 6-membered forms\textsuperscript{31,47}). Finally, two rim-differentiated\textsuperscript{32} derivatives 6 and 7 were chosen. The short alkyl substituents on one rim can provide more flexible conformations with less charge density around the macrocycle rims compared to the per-substituted compounds. The alkoxy group of 6 is structurally closer to the C2-spacer in 1, 2, 5, and 7, while the subunits of 7 are closer in overall height to the per-substituted 1, 2, and 5. See section 3 of the supplemental information for characterization of the studied pillararenes.

The studied binding targets (Figure 1C) comprised HSLs with diverse structures, which correspond to different gram-negative bacteria and signaling circuits.\textsuperscript{48} N-Butanoyl-L-homoserine lactone (hereafter written as C4-HSL) is the shortest-tailed HSL among the studied four, with four carbon atoms along its tail. C4-HSL is used by many bacteria, including the pathogenic \textit{P. aeruginosa} and \textit{Serratia liquefaciens}.\textsuperscript{49-51} N-(3-oxo-octanoyl)-L-homoserine lactone (hereafter C8-HSL) and C12-HSL are two 3-oxo substituted HSLs with intermediate tail lengths, the latter of which is notably used by \textit{P. aeruginosa}.\textsuperscript{49} The longest carbon chain and a hydroxyl substituent are provided by N-(3-hydroxy-7-cis-tetradecenoyl)-L-homoserine lactone (hereafter C14-HSL).

**Dye displacement assay of pillararenes against HSLs**

The affinities of the pillararenes toward the HSLs were studied using an UV-visible light (UV-vis) spectroscopy-detected dye displacement assay (a form of indicator displacement assay [IDA]) (Figure 2A). The binding between methyl orange (MO) and macrocycles\textsuperscript{16,52-54} was characterized first, titrating a constant concentration of the dye with increasing amounts of macrocycles (spectra for macrocycles alone and supramolecular titrations are included in section 4 of the supplemental information). This allowed the use of MO for probing the affinities of HSLs toward the macrocycles in displacement assays, with the displacement of MO (a return to uncomplexed spectrum) indicating HSL binding. Free MO shows an absorption maximum at approximately 470 nm, which generally decreases in intensity and widens in the presence of the studied pillararenes (Figures 2B and S16). With 1 and 2, a clear new peak at approximately 395 nm could be identified at high macrocycle/dye ratios (Figure S16). With 3, a similar but less clear formation of a 395-nm
Figure 2. Dye displacement assays of HSL-pillararene pairs, using MO as indicator

(A) Working principle of the dye displacement assay. Binding of MO is detected by a change in absorption. Titration of the indicator with an increasing macrocycle concentration characterizes affinity for the dye (I). Addition of an HSL with affinity for the macrocycle gradually displaces the dye into solution (II).

(B) Absorption at 470 nm for titrations of MO with increasing macrocycle concentration (averages and SDs of triplicates). Log($K_a$) values ($M^{-1}$) indicated for series satisfactorily fitted to 1:1 binding models in the dye displacement assays (average and SD of separately fitted triplicate measurements).

(C) Absorption at 470 nm for displacement assays of HSL-pillararene pairs (each panel corresponds to 1 HSL, respective structures portrayed; averages and SDs of triplicates). Results are displayed for pillararenes satisfactorily fitted to 1:1 binding models for any HSL (1–3) and 1 series that could not be fitted but was further compared against in the biological assays (5). Indicated constants are log($K_a$) ($M^{-1}$), average and SD of separately fitted triplicate measurements.

Dashed lines denote the used MO concentration (2 μM).
peak was seen, leading to two partially overlapping peaks. With 5, 6, and 7, the 470-nm peak widened until reaching a final shape dependent on the host. Increasing amounts of 4 relative to MO produced a particular, back-and-forth evolution of spectra, due to strong non-specific absorption at high concentrations (Figure S16). The results with 4 are discussed further in Note S1. The different absorption spectra of MO with different hosts present is not surprising, and may depend on, for example, different degrees and orientations of immersion into the host cavity.55

Next, solutions of MO and a given host were titrated with increasing HSL concentrations. Should a host have affinity toward HSLs, the displacement of MO and recovery of its “normal” absorption spectrum is expected. With increasing concentrations of HSLs, the low solubility of longer-tailed HSLs in aqueous solutions limits the concentration range available to simple UV-vis characterization. The more hydrophobic guests (with longer carbon tails) proved challenging to characterize due to the limited solubility at high concentration restricting titration completion. The onset of unspecific signal increase due to scattering can be detected as an increase in absorption strongest in the UV region (most prominent under approximately 370 nm) (Figures S17 and S18). With increasing aggregation-related interference, only following the absorption at 470 nm becomes misleading. As such, the spectra were also scrutinized for overall evolution in peak shapes toward that of free MO, to confirm HSL binding.

Among the studied hosts, 1, 2, and 3 showed clear changes in absorption spectra with increasing guest concentrations for the C14-, C12-, and C8-HSLs (Figure 2C). The evolution of the spectra toward that of uncomplexed MO indicates the capture of these HSLs in the concentration ranges studied via IDA. For instance, the beginning state of the 2 + C12-HSL displacement assay (2 μM both of pillararene and MO) shows a peak at approximately 395 nm, with a shoulder at approximately 455 nm, with the shoulder peak growing in height and the initial peak shrinking and slightly shifting as HSL is added, up 100 μM (after which strong non-specific absorption sets in) (Figure S18B).

The onset concentration for the gradual displacement of MO generally follows an expected trend across the different studied HSLs. With macrocycles that bind HSLs, less of the longer-tailed C14- and C12-HSLs is needed to produce a change compared to C8-HSL, with no significant absorption changes detected for C4-HSL (Figure 2C). In other words, longer-tailed HSLs generally have a stronger affinity for the macrocycles. This corresponds with earlier work and descriptions putting forth the hydrophobic effect as a driving force of complexation.16,20

The results from dye displacement experiments involving pillararenes 1–3 were fitted to 1:1 models with competing equilibria of binding MO and HSL to estimate association constants. Various fitting approaches were explored, including different ranges of included wavelengths and use of fixed or freely optimized absorption spectra of individual compounds in the model. First, the quality of different approaches was assessed based on the concordance of absorption spectra assigned to compounds in the model and spectra observed in the indicator titration experiments (i.e., whether peak locations, shape, and relative heights were close to experimental observation). Second, models with spectra corresponding to experimental observations were required to produce viable absorption curves as a function of added HSL that match experimental results. Third, relative magnitudes of obtained association constants between different host-guest pairs need to correspond with how the indicator titration and IDA curves of different pairs are positioned relative
to one another. The absorbances of separate species in the optimized models, choices of fixed absorption spectra, comparisons of fit and experimental data, as well as residuals of the fitting are presented in section 5 of the supplemental information, along with tabulated association constants from separate fits of triplicates.

1, a macrocycle established as having a good affinity toward long-tailed HSLs, produced \( \log(K_A \text{ (M}^{-1})\) values of 5.2, 5.1, and 4.1 for C14-HSL, C12-HSL, and C8-HSL, respectively (\(K_A\) denoting association constant). These reasonably follow the values and trends reported previously.\(^{16}\) Compared with 1, 2 showed improved binding of C14-, C12-, and C8-HSLs, with fitting estimating roughly an order of magnitude higher \(\log(K_A)\) in each case (5.9, 6.2, and 5.1, respectively). The hydroxyethyl groups on the macrocycle rim likely provide hydrogen bond donors that can interact with HSL acceptors (hydroxyl and carbonyl oxygens).

While 3 has a lower affinity for C12- and C8-HSL compared to 1 and 2 (\(\log(K_A)\) 4.3 and 3.2, respectively), the IDA results with C14-HSL show similar affinity with the latter two as the guest carbon tail length increases, with a fitted \(\log(K_A)\) of 5.6. The increased length of the substituent chains of 3 (one carbon more compared to 1 and 2) likely provide a deeper hydrophobic host cavity, which the longer C14-HSL can utilize more fully in terms of hydrophobic binding. The shorter cavities of 1 and 2 can be viewed as being already “saturated” with C12-HSL, where increased length of the guest does not allow for additional solvent molecules to vacate the cavity volume. This marked favoring of longer-tailed HSLs could find uses in preferential binding of signals, for example, specific to strains or messages. Compared to earlier reports covering cyclodextrins and cucurbiturils, a similar trend can be observed in that longer-tailed HSLs produce stronger binding.\(^{12,24,25,27,56}\) Many of these studies have mainly covered shorter HSLs, but association constants with 8-carbon variants have produced \(\log(K_A)\) values of 2.8–5.1, a range that also encompasses our results.

4 did not show measurable changes in the absorption spectra from IDAs, indicating a lack of HSL complexation (Figures S17–S20). This is despite the macrocycle having even longer substituent chains than the rest (six carbons between the ether oxygen and ammonium group), which could at first glance provide an even stronger hydrophobic binding effect. Compared to the other macrocycles, 5 produced a less clear change when complexing MO. In the dye displacement assay, C14- and C12-HSLs produce only a minute change in absorption. Such effects are likely due to a mismatch in cavity size, as has been found in earlier screening of differently sized cavities.\(^{16}\) The water molecules in a larger cavity may be less disadvantaged relative to bulk water, weakening the hydrophobic driving force of complexation.\(^{57}\)

6 and 7 also produced no conclusive evidence of binding HSLs at the measured concentrations. The less shielded cavities (with part of the substituents shorter and less bulky) may contain less constrained and hydrogen bond-deprived water, lessening the benefits of hydrophobic binding.\(^{57}\) Akin to 3 and 4, the flexible substituent chains may also restrict complexation, and another level of motility is introduced by the rotation of aromatic units that would be arrested in a complexed state.

**Structural analysis of host-guest complexes**

1–3 showed clear capture of the studied HSLs, and were therefore chosen for further analysis by NMR. C12-HSL was chosen as the guest due to its relatively strong affinity for the macrocycles (NMR spectra of C12-HSL alone and mixed with hosts are included in section 6 of the supplemental information). Upon the addition of C12-HSL into solutions of 1 and 2 (denoted as guest\(\subset\)host; 5:1 molar ratio, host:guest),
the $^1$H signals from the tail CH$_2$ groups upshifted significantly (close to −2 ppm at most) and split into multiple smaller peaks (Figure 3A). The tail CH$_3$ signals remain close to their original position upon complexation. The shifted CH$_2$ peaks with 2 remain relatively sharp but broaden significantly with 1. With 3, the tail CH$_2$ signal is likely split and upshifted as well, but the peaks are too broad to discern. Further investigation via $^1$H-$^{13}$C heteronuclear single quantum coherence ($^1$H-13C HSQC) and correlated spectroscopy (COSY) experiments did not indicate the tail CH$_2$ peaks being hidden by overlap with larger peaks belonging to 3 (Figure S34B). With 1 and 3, the head signals can be resolved in the same region as the macrocycle peaks via $^1$H-$^{13}$C HSQC (Figure S34) and remain close to their uncomplexed shifts, but were not found with 2 (Figure S35).

The highly shielded CH$_2$ signals likely arise from the complexation of the HSL tail within the pillararene cavity, where aromatic ring current effects oppose the external magnetic field above and below the ring’s plane. The sharper peaks of the shifted carbon tail in the C12-HSL·2 complex point to slower changes in the chemical environment experienced by the tail groups. This suggests a more rigid complex, where the guest is held more stiffly in a specific conformation in the host cavity. This likely arises from hydrogen bonding between the OH donors of 2 and the HSL hydrogen bond acceptor groups (=O, −O−, and −NH−).

Rotating frame nuclear Overhauser effect spectroscopy (ROESY) experiments were carried out to look for spatial correlations between macrocycles and the C12-HSL guest. C12-HSL·1–3 correlations were found between the tail CH$_3$ group and pillararene hydrogens near the core cavities (Figure 3B). The sharper CH$_2$ peaks of C12-HSL·2 also show correlations with the aromatic hydrogens. The results show that the HSL tail and host cores are in close proximity to one another (ROESY correlations are generally found with distances of under 5 Å, with the clearest signals typically for 1.75–3.5 Å).

For C12-HSL·2, COSY and ROESY correlations between tail groups could be detected (Figures 3D and 3E). Two chains of 8 or 9 neighboring hydrogens were identified, annotated as a, b, ... and a', b', ..., with no ROESY or COSY correlations between the two sets of signals (Figure 3F). The COSY and ROESY spectra therefore suggest the presence of two slightly different complexed states that are resolvable on the NMR timescale, with the HSL guest experiencing slightly different chemical environments between the two. A clear trend for both series of neighboring hydrogens arises, with a maximal shielding effect experienced four connections away (i.e., at the fifth carbon) of the tail from the end (Figure 3G). As such, this carbon experiences on average the strongest shielding, nearest to the center line of the core cavity where the effects of the five aromatic rings are strongest.

Deshielding of hydrogens closer to the head could also be observed with an approximately 1-ppm peak shift for the hydrogens of the seventh or eighth carbon from the tail CH$_3$ group, depending on the correlation chain (Figure 3G). A possible explanation can be found in hydrogen bonding between the carbonyl groups of the HSL and hydroxyls of the host; with electron density pulled away from the acceptor carbonyl, the nearby CH$_2$ groups experience deshielding. This explanation implies a difference in hydrogen-bonding motifs between the two chains of peak correlations. A splitting of the Ar−O−CH$_2$ signal was detected, giving rise to a new peak at 4.4 ppm (Figure S35). This is likely due to contrasting chemical environments of the “inside” and “outside” protons upon complexation with an alkyl tail; the split produces a more shielded peak, and integrals correspond with 14% of these hydrogens.
Figure 3. Characterization of host-guest complexes by NMR and MD simulations
(A) $^1$H-NMR spectra of C12-HSL tail region, alone and mixed with 1–3. Peak assignments are given for unbound C12-HSL, and the HSL tail CH$_3$ is indicated in all spectra.
(B) ROESY spectra of C12-HSL mixed with 1–3, focused on the HSL tail CH$_3$ (peak A) and pillararene cavity signals (peaks A–C). Dashed lines illustrate the detected correlations between hydrogens of the pillararenes and HSL.
(C) MD simulations data of host molecules 1, 2, 4 showing an x-y plane projected two-dimensional probability distribution of substituent chain heavy atoms. The distributions are normalized by the number of data points for host 4 to enable comparison of cavity accessibility between the different hosts.
being more shielded (in theory a 10% value would be expected, with up to every fifth host being complexed and half of the protons splitting off in each complex).

MD simulations of the host molecules were also carried out to investigate the dynamic structure of host molecules with short and long substituent chain lengths (1, 2, 4) to elucidate structural factors that may promote or restrict binding. In addition to the expected cavity diameter and hydrophobicity, the results from dye displacement point to additional criteria that affect complexation (1 and 2: Figure 2C; 4: Figures S17–S19). The MD simulations results present different overall arrangements and structural dynamics adopted by the substituent chains of the pillar[5]arene hosts. The MD results uncover mechanisms that may explain differences in binding when tuning substituent chain lengths and end groups.

In the simulations, the short-spacer hosts (1 and 2) show self-complexation (Figures 3C and S38) where one substituent chain bends into the aromatic cavity, while the other substituents point outward, leaving an accessible cavity on one side of the pillararene. The simulations show that self-complexation occurs with a high likelihood; however, the molecular configurations show one side of the cavity remaining open (1–64% and 2–83%). Dual-sided self-complexation was not observed in the simulations timescale (Figure S37). However, 34% and 17% of configurations showed both sides of the cavity obstructed by the substituent chains for host 1 and 2, respectively. The simulations data point to an overall accessibility of the cavity for both these hosts from at least one side of the molecule (Figure 3C), which likely arises from charge repulsion of the cationic substituents. For 1, the NMR results concord with the self-complexation seen in simulations; a smaller peak at 3.81 ppm is split off the 4.00-ppm N–CH₂ peak, sharing the same chemical shift on the carbon axis of ¹H–¹³C HSQC. COSY and ROESY also show a connection between this split-off peak and the O–CH₂ peak. Similarly, a split-off peak of the CH₃ group (main peak at 3.36 ppm) can be seen at 3.18 ppm, which shows no COSY correlations and proximity to the other substituent chain groups in ROESY. Both split-off peaks are more shielded, which agrees with a self-complexation into the aromatic cavity. For 2, similar split-offs can be seen for the CH₃ groups and OH-adjacent CH₂ group, sharing carbon-associated shifts with their main peaks. However, the more complex COSY and ROESY spectra of 2 make discerning additional correlations difficult.

The host 4 has longer alkyl spacers in its substituent groups, which lead to a markedly different arrangement around the core cavity (Figures 3C and S37). The simulations show that the rocking of aromatic panels (a behavior experimentally observed in pillararenes)²⁰ is more pronounced than for 1 and 2 (Figure S38). The long chains move in front of and away from the cavity entrance, collectively maintaining a steric block of substituent chain bundles on both sides of the core cavity (Figure 3C). The continuous obstruction of the cavity by bulky substituent chains rationalizes the lack of detected affinity of the host for HSLs in the dye displacement experiments (Figures S17–S19).
Additionally, the MD simulations reveal significant differences in the structural dynamics of 1, 2, and 4 (Figure S38). The simulations point to a more dynamic structure for 1 compared to 2. 1 shows rocking and flipping of the aromatic rings (Figure S38) that result in several short-lived self-complexed states and two flipping events in the simulations trajectory. On the contrary, rocking of the aromatic rings is not necessary for self-complexation of 2, and there is only one long-lived self-complexed configuration observed in the simulation trajectory. The rigidity of 2 as a host compared to 1 is also seen when complexed with a guest in the NMR studies (sharp shifted signals of the C12-HSL tail; Figure 3A). In addition to the self-complexation timescale, the structural dynamics of pillar[5]arenes are highly influenced by substituent chain lengths. The MD simulations reveal a fast rocking of the aromatic panels in 4, producing structural changes in nanosecond timescales (Figure S38C). The findings point to the high tunability of both dynamics and conformations adopted by pillar[5]arenes using substituent chain lengths and end groups. The MD simulations methodology and more detailed data analysis are discussed further in section 7 of the supplemental information. The simulation results, together with binding assay outcomes, may be used in the optimization of new host structures for improved binding. For instance, the accessibility of 4 may be improved by stabilizing/rigidifying the host via inter-substituent interactions, to harness properties of the deeper cavity.

**GFP reporter, biofilm, and pyocyanin**

To understand the potential ramifications of the interactions between the studied macrocycles and HSLs within a biological context, we utilized an *Escherichia coli*-based reporter system (Figure 4A). In these reporter strains, unique receptor proteins for each HSL bind their cognate ligand, enabling the binding to specific promoters and the production of EGFP in response to added HSLs (C4, C8, C12, or C14). Binding of HSLs by pillararenes inhibits the activation of EGFP expression (Figure 4B). The stronger the binding between the macrocycle and HSL, the less free signaling molecule is present that can activate fluorescence expression in *E. coli*.

The macrocycles 4, 6, and 7 showed significant bacteria growth inhibition (\(I/I_0\) optical density >20%; Figure S39). Since alterations in growth rate impact the accuracy of the reporter system, these hosts were excluded (Figure S39). While quaternary ammonium compounds have generally garnered attention for antimicrobial uses, the differences in growth inhibition point to additional structural aspects that affect the macrocycles’ suitability for HSL capture without direct antimicrobial effect. The strongest dose-dependent effects were observed with 1 and 2 (Figure 4C). The strength of the interaction for both 1 and 2 correlated well with the length of the branched carbon chain of the HSLs, where the strongest inhibition was observed with the longest-tailed C14-HSL and the weakest inhibition with C4-HSL. This result is in line with the previous observations in dye displacement assays and NMR. 3 and 5 did not exhibit notable dose-dependent changes on the reporter signal intensities. The findings for 3 diverge from the dye displacement assays, which showed interactions particularly with the longest-tailed C14-HSL. 3 had a small yet observable impact on the bacterial growth rate, which could influence the difference between the dye displacement assay and the reporter screen results. Host 5 has an enlarged cavity size as compared to 1–3 (6.7 Å\(^3\)) compared to approximately 4.6–5 Å\(^3\). Given the importance of the inner cavity size on the strength of non-covalent interactions, the lack of changes was expected and in line with the dye displacement results.

Notably, some host HSL combinations resulted in full inhibition of the reporter systems (1 with C14-HSL and 2 with C12- and C14-HSL). This suggests that these hosts
could act as potent signaling disrupters, since the HSL concentrations utilized in this screen exceed natural production levels. HSL signaling serves as an important mechanism employed by numerous bacterial species across various physiological processes. To better understand the influence of our hosts on microbial environments, our focus shifted toward studying their effects within the context of *P. aeruginosa*, which is a key and clinically important pathogen that is reliant on
HSL signaling. *P. aeruginosa* is an opportunistic pathogen known for its versatility in causing a wide spectrum of infections across various hosts. From respiratory tract infections in people with compromised immunity to severe bloodstream infections in hospitalized patients, this bacterium is implicated in diverse pathological conditions. Its ability to adapt to different environments and resist antibiotics makes it particularly challenging to manage clinically.63

*P. aeruginosa* employs two principal HSL-type signaling molecules, namely C4-HSL and C12-HSL, alongside two additional recognized signaling pathways.17 These systems operate in a hierarchical manner, with the C12-HSL and its receptor protein LasR positioned at the top of the cascade. This intricate signaling cascade is instrumental in enhancing the likelihood of a successful infection by directly orchestrating the expression of virulence factors. These factors include the production of offensive virulence factors such as exotoxins like pyocyanin, elastases, and heightened motility, aimed at facilitating host invasion and tissue damage. The signaling cascade also plays a crucial role in mediating defensive virulence factors by regulating the formation of bacterial biofilms, thereby aiding in resisting antibiotic therapies and host immune responses.64

To demonstrate the potential implications of host-guest interactions between macrocycles and HSLs in disrupting bacterial virulence, we first examined the impact of the hosts on offensive virulence factors by looking at pyocyanin production (Figure 4D). The addition of 1, 2, and 3 led to a concentration-dependent decrease in pyocyanin levels. Whereas we did not observe the concentration-dependent effects of 3 in the reporter screening system, the effect of 3 on pyocyanin levels is comparable to that of 1 and 2. Somewhat surprisingly, 2 showed stronger binding of HSLs compared to 1, but showed a weaker antipyocyanin effect between the two. The QS circuitry of *P. aeruginosa* is also known to involve 2-heptyl-3-hydroxy-4-quinolone (PQS) and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde autoinducers.17 The C7-tailed PQS may also be able to interact with the pillararene cavity to a certain degree, and it may provide an additional connection to the pyocyanin expression.65 Overall, 1–3 show effective decreases in pyocyanin levels, as opposed to 5. This corresponds with the binding ability of HSLs shown by the 1–3 in dye displacement assays, compared to the lack of binding by 5.

Next, we turned to bacterial biofilm levels, the defensive virulence factors that help resist bacterial clearance. Concentration dependencies similar to binding affinities found in dye displacement assays were observed with the impact of 1–3 on biofilm levels. 1 and 2, which bound C8- and C12-HSL more strongly than 3 and C14-HSL equally well, showed better antibiofilm activity between the three (Figure 4F). In both virulence factor assays, no impact could be detected with 5. This matches with the lack of binding observed in dye displacement assays and the *E. coli* reporter system. The lack of antivirulence effects with 5 is intriguing when contrasted with the increasing attention garnered by quaternary ammonium compounds for antibiofilm and antibacterial purposes.61 The difference against the otherwise similar but smaller in cavity 1 points to the importance of a specific host diameter. While too direct comparisons should not be drawn, similar effects have been noted with gram-positive bacteria and cationic pillararenes; simple tetramethylammonium or pillararene “monomers” did not offer results comparable to the antibiofilm effects of the corresponding macrocyclic hosts.33,34

The concentration-dependent effects of both 1 and 2 on bacterial biofilms in *P. aeruginosa* reference strain PAO1 strongly correlate with the effects of 1 and 2
in the *E. coli* reporter screen for C12-HSL, whereas no correlation was observed with the C4-HSL (Figure S40). This further suggests that in *P. aeruginosa*, which utilizes both of these signaling systems, the disruption of virulence is solely mediated through interaction with the C12-HSL. This signaling system sits at the top of its interconnected signaling cascade, making it a very effective target for disruption strategies.

**In vivo wound infection model**

Next, we continued to validate the most potent hosts, 1 and 2, in a more clinically relevant setting: open skin wounds in PAO1 infected mice over a 3-day period (Figure 5A). The addition of differing concentrations of 1 and 2 (1, 5, and 30 mg/kg) had no observable impact on the body weight of mice throughout the 3-day infection (Figure S41). However, the addition of 1 and 2 resulted in a reduction in total
bacterial burden after 3 days, measured in colony-forming units (CFU) (Figures 5B and 5C). For 1, all concentrations resulted in a significant reduction in bacterial burden, with the highest reduction observed in the highest dose of 30 mg/kg. For 2, although a reduction was observed in all concentrations, significance was observed only in the highest dose of 30 mg/kg. The total reduction in bacterial burden in the highest dose is comparable between 1 and 2. The wound closure over time with different treatments was also monitored. After the 3-day treatment, with the highest dose of 30 mg/kg, we observed a notable improvement in wound healing for both 1 and 2, where 60% and 40% of the wounds were closed, compared to none of the wounds in the untreated control group. Taken together, these results suggest that disruption of signaling using host compounds is potentially an effective strategy to disrupt bacterial virulence. Furthermore, it suggests that bacterial virulence plays an important role during bacterial infections and that strategies that disrupt bacterial virulence could provide relief in clinically relevant settings.

To summarize, a series of cationic pillararenes were studied as potential macrocyclic hosts for the binding of bacterial HSLs in the context of disrupting QS as an alternative to classic antibacterial agents. The binding of HSLs and its effects was studied in systems of increasing complexity, from fundamental supramolecular assays to microbial system studies and in vivo proof-of-concept validation in an open wound infection model. All-atom MD simulations were employed to investigate the configurations and structural dynamics of pillararene macrocycles. The simulations revealed that the pillararenes have at a molecular level a complex free energy landscape that results in differences in molecular arrangements and accessibility of the cavity HSLs when varying substituent chain length and end group. The binding of HSLs was detected for two new hosts with 8–14 carbon-tailed HSLs; a hydroxyethyl-equipped host showed improved binding over the previously studied trimethylammonium-functionalized pillar[5]arene across these HSLs, while a long-spacer host molecule showed marked preference for the longest-tailed guest (C14). The binding and affinities between host and HSL combinations correlated strongly with the effects on pyocyanin and bacterial biofilm levels in P. aeruginosa, processes that are under the direct control of HSL signaling. For hosts that showed low affinity toward all HSLs, the binding of HSLs or lack thereof was directly reflected in their ability to disrupt pyocyanin production and biofilm formation. Finally, we show that two lead variants effectively lower bacterial burden and inflammation in open wounds infected with P. aeruginosa. The findings and methods used may find applications in designing macrocycles to bind other QS autoinducers, including other HSLs and separate families of signal molecules. Additional characterization of host-autoinducer binding and the concurrent mapping of QS systems and their interplay may allow for a more specific understanding and targeting of bacterial control systems. Possibilities in alternative motifs include hydrogen bond donors other than hydroxyl groups, as well as modulating structural dynamics via bulkier groups or intramolecular interactions between subunits. The disruption of bacterial QS may also find uses other than the conceptualized circumventing of AMR, where, for example, biofilm- or toxin-related problems arise.

EXPERIMENTAL PROCEDURES

Full experimental and computational procedures can be found in the supplemental information (Sections 2 and 7 of supplemental information, respectively).

Resource availability

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Materials availability
All reagents used in this study, their sources, and experimental details can be found in the supplemental information.

Data and code availability
Data associated with the simulations are available at https://doi.org/10.23729/8c190fc-98cc-4f68-96c3-57bf0fabe3ed. All experimental data supporting the findings of this study are available within this article and the supplemental information files.

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AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
C.J. and E.O. are co-founders of Arivin Therapeutics Oy. Arivin Therapeutics Oy has filed patent applications FI20185841A1, FI20205369A1, and FI20205368A1 on the effects of macrocycles on gram-negative pathogens, with C.J., E.O., and M.B.L. listed as co-inventors.

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