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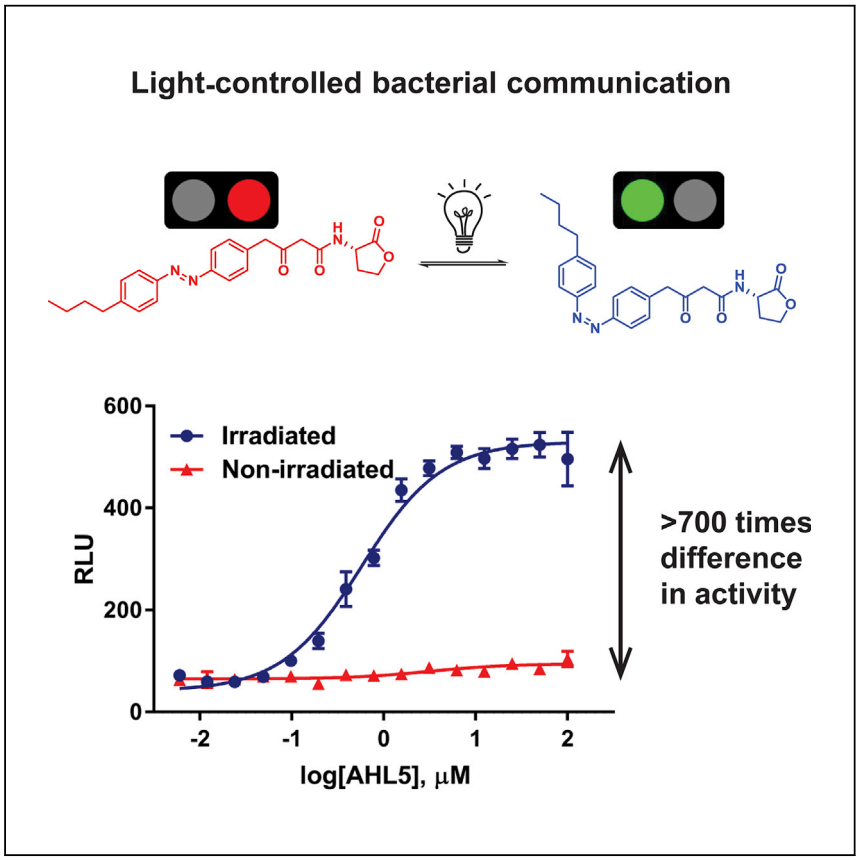
Easily Accessible, Highly Potent, Photocontrolled Modulators of Bacterial Communication



By applying the photopharmacological approach, bacterial communication can be controlled with light through the application of photoswitchable modulators. Interestingly, one of the lead photoswitchable modulators of bacterial communication, presented herein, shows a remarkable (>700-fold) difference in activity between the non-irradiated and irradiated states. The photoresponsive quorum-sensing modulators can be used to control toxin production in *Pseudomonas aeruginosa* and have a promising outlook as next-generation research tools.

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HIGHLIGHTS

Convenient, two-step synthesis of photoswitchable quorum-sensing modulators

>700-fold difference in activity between the two photoisomers

Reversible photoswitching from inhibitor to activator

Light control of toxin production in *Pseudomonas aeruginosa*

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SUMMARY

External control of bacterial communication—quorum sensing—allows for the regulation of a multitude of biological processes. Herein, we describe the development of a new synthetic methodology, as well as the characterization, photoisomerization, and biological evaluation of a privileged series of novel photoswitchable quorum-sensing agonists and antagonists. The presented method allows for the rapid and convenient synthesis of previously unknown photoswitchable agonists with up to 70% quorum-sensing induction and inhibitors reaching up to 40% inhibition, which significantly extends the level of photocontrol over bacterial communication achieved before. Remarkably, for the lead photoswitchable agonist, a >700-fold difference in activity was observed between the irradiated and non-irradiated forms, which allows for antagonism-to-agonism switching upon exposure to light, showing levels of control unprecedented in photopharmacology. Finally, utilizing this system, we were able to regulate toxin production in *Pseudomonas aeruginosa* with light.

INTRODUCTION

Bacterial communication plays a vital role in the regulation of symbiotic processes and pathogenesis of infections.[1–5] The communication between bacteria is mainly based on quorum sensing (QS), a process in which bacteria produce and excrete QS autoinducers that are responsible for the upregulation of gene expression to control cellular organization, virulence, and biofilm formation, among others.[3,5] External control over the activity of QS autoinducers would allow the up- or down-regulation of gene expression in bacteria, enabling remote regulation of, for example, biofilm formation, which is becoming a major threat in the treatment of bacterial infections, with serious implications in surgery.[6–9] Moreover, complementary to the field of optogenetics, genetic engineering combined with the external control of QS induction potentially allows the regulation of a plethora of functions by controlling the activity of the QS operon with light.[10,11]

Light has proven to be an excellent stimulus for the remote control of biological systems.[12,13] In this context, the emerging field of photopharmacology aims at the design and synthesis of bioactive molecules, whose activity can be altered with light.[14–17] Photopharmacology relies on molecular photoswitches to control the structure of bioactive molecules in space and time. Incorporation of photoswitches into the pharmacophore renders the product reversibly photoresponsive. Applying the photopharmacological approach, a variety of biological targets and tools ranging from ion channels,[18–20] glutamate receptors,[21,22] and G-protein-coupled receptors[23] to antibiotics[24–26] and

The Bigger Picture

Photopharmacology is an emerging approach aimed at the regulation of biological function with light. Herein, the application of molecular photoswitches allows for the reversible switching between two distinct structural states of bioactive compounds. Bacterial communication (quorum sensing) is an interesting target for photopharmacology, from the perspective of both clinical and basic research, because of its implications for pathogenicity of bacteria and complex biological mechanism of action. By the novel synthesis and application of photoswitchable modulators, we were able to reversibly control bacterial communication with light. Remarkably, one of our lead compounds allows the control of bacterial communication with very high selectivity, switching from a quorum-sensing inhibitor to a quorum-sensing activator upon irradiation with light, which was further exemplified by the control of quorum-sensing-regulated toxin production in *Pseudomonas aeruginosa*.

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anti-tumor drugs[27–32] have been controlled. In the context of QS, earlier work by our group focused on the design of photoswitchable QS autoinducers, which led to the photocontrol of QS-related gene expression and pyocyanin production.[33] This proof of concept, together with an abundance of synthetic QS ligands developed,[34–36] has paved the way for the further advancement toward more potent and selective autoinducers that can be controlled with light.

Two of the major native autoinducer motifs in *Pseudomonas aeruginosa*, *N*-3-(oxododecanoyl)-L-homoserine lactone (OdDHL) and *N*-butyryl-L-homoserine lactone (BHL), are depicted in Figure 1.[37] These autoinducers both consist of a homoserine lactone "head group" and a hydrophobic alkyl chain. Interestingly, large differences in activity have been observed upon minor alterations of the structure, and the specificity of autoinduction is highly dependent on the bacterial strain, allowing for the selective addressing of virulent strains over the beneficial ones.[8,34] For example, in *P. aeruginosa*, the 3-oxo motif seems to be inherent to the ability to induce QS, while the BHL also shows agonistic properties albeit less pronounced.

Successful photomodulation of highly potent QS autoinducers requires the synthetic access to 3-oxo homoserine lactones. However, the photoswitchable derivatives of 3-oxo homoserine lactone cannot be made via conventional synthetic methodology. While the original synthesis of the native 3-oxo derivative is performed using a low-yielding derivatization of Meldrum's acid that poses problems with both stability (decarboxylation) and purification when non-alkyl substrates are used,[38,39] efforts by the Blackwell group led to the development of an elegant, microwave-assisted synthetic sequence.[40] However, simple dianion formation and nucleophilic substitution, in our hands, did not give satisfying results either; especially the reported protection-deprotection strategy proved troublesome with our azobenzene-based substrates, while we anticipated that elevated temperatures with microwave irradiation potentially would pose problems with the photoswitch scaffold. In developing a new, efficient synthetic pathway toward aryl-substituted 3-oxo homoserine lactones, we were inspired by earlier reports from the groups of Buchwald on the cross-coupling of esters and ketones to aryl chlorides[41] and Skrydstrup on carbonylative vinylogous couplings with dioxinone,[42] which provided evidence that an unprecedented cross-coupling reaction between chlorobenzene and dioxinone could potentially allow the facile synthesis of a protected 3-oxo precursor. Our initial investigations focused on the development and optimization of such a cross-coupling reaction to synthesize aryl-β-keto-amides.

RESULTS AND DISCUSSION

A model reaction of chlorobenzene (1 equiv) and dioxinone (1.1 equiv) with catalytic ^{t}BuXPhos-Pd-G1 and 3 equiv LiHMDS in toluene under ambient conditions for 30 min yielded the desired coupling product **1** in a remarkably high yield without the need for laborious purification (Figure 2C). To our delight, the reaction of *p*-chloroazobenzene (1 equiv) with dioxinone (1 equiv) under similar conditions also proceeded with a >95% isolated yield after 30 min reaction time without the need for chromatographic purification. Next, the synthesized intermediate was reacted with homoserine lactone in the presence of a base at elevated temperatures (110°C) to yield the desired photoswitchable 3-oxo homoserine lactone AHL1. Full conversion was obtained after 3 h with only minor impurities, as observed from ^{1}H-NMR spectroscopy. Purification by flash chromatography yielded the pure product in a satisfying yield. The versatility of this two-step method to prepare highly valuable aryl-3-oxo homoserine lactones was further demonstrated by the

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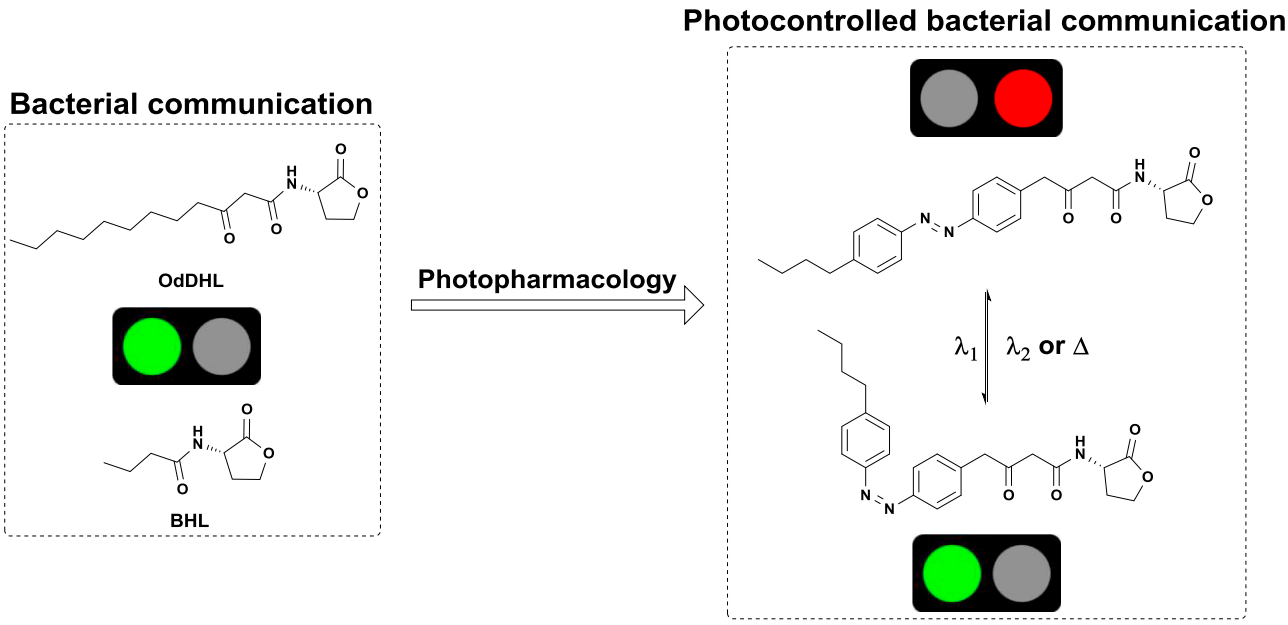

Figure 1. Concept of Photocontrolled Modulators of Bacterial Communication Starting from Native QS Autoinducers toward a Photoswitchable Agonist and Antagonist Toolbox

straightforward synthesis of a library of 16 photoresponsive effectors of bacterial communication (Figure 2D). A diverse set of both azobenzenes and different anilines or aminolactone could be used in a versatile two-step synthetic sequence without the need for specific reaction optimization.

The library described here employs the azobenzene motif, an exceptional, versatile class of photoswitches with distinct photochemical properties.[43,44] The structure of azobenzene can be photochemically modulated between the thermally stable *trans*-isomer and the *cis*-isomer. The unstable *cis*-isomer converts thermally back to the *trans*-isomer over time or upon irradiation with another wavelength of light. Utilization of azobenzene derivatives, in a biological context, necessitates photochemical properties such as high photostationary states, appropriate thermal half-lives, and fatigue resistance, i.e., the ability to photoswitch for repetitive cycles without degradation. Photochemical and thermal isomerization studies were performed for all AHLs (see Figure 3; Supplemental Information), exhibiting efficient photostationary states (>95% *trans* before irradiation and between 84% and 97% *cis* under irradiation; see Supplemental Information for details). Moreover, no significant fatigue was observed for any of the AHLs after five cycles of irradiation and half-lives for the *cis*-isomer showed to be between 28 and 100 min, which is within the suitable time range for the biological experiments performed (*vide infra*; see also Figure S7).

The subsequent biological evaluation focused on the Las network of *P. aeruginosa*, which can be quantified by the induction of LasQS as measured by a functional readout of bioluminescence in a QS reporter strain (*E.coli* JM109 pSB1075).[45,46] In this strain, the AHLs potentially bind to the transcriptional activator LasR to form a stable dimer, which can bind to the responsive promoter region of the LasQS system proceeding the *luxCDABE-lasR* promoter fusion reporter genes, resulting in enhanced bioluminescence. Initial studies focused on the potential QS-inhibiting properties by utilizing a competition experiment, in which the induction of the native OdDHL was inhibited with the different AHLs from the herein reported library (see Figure S1).[45,46] Compounds (AHL13–AHL15)

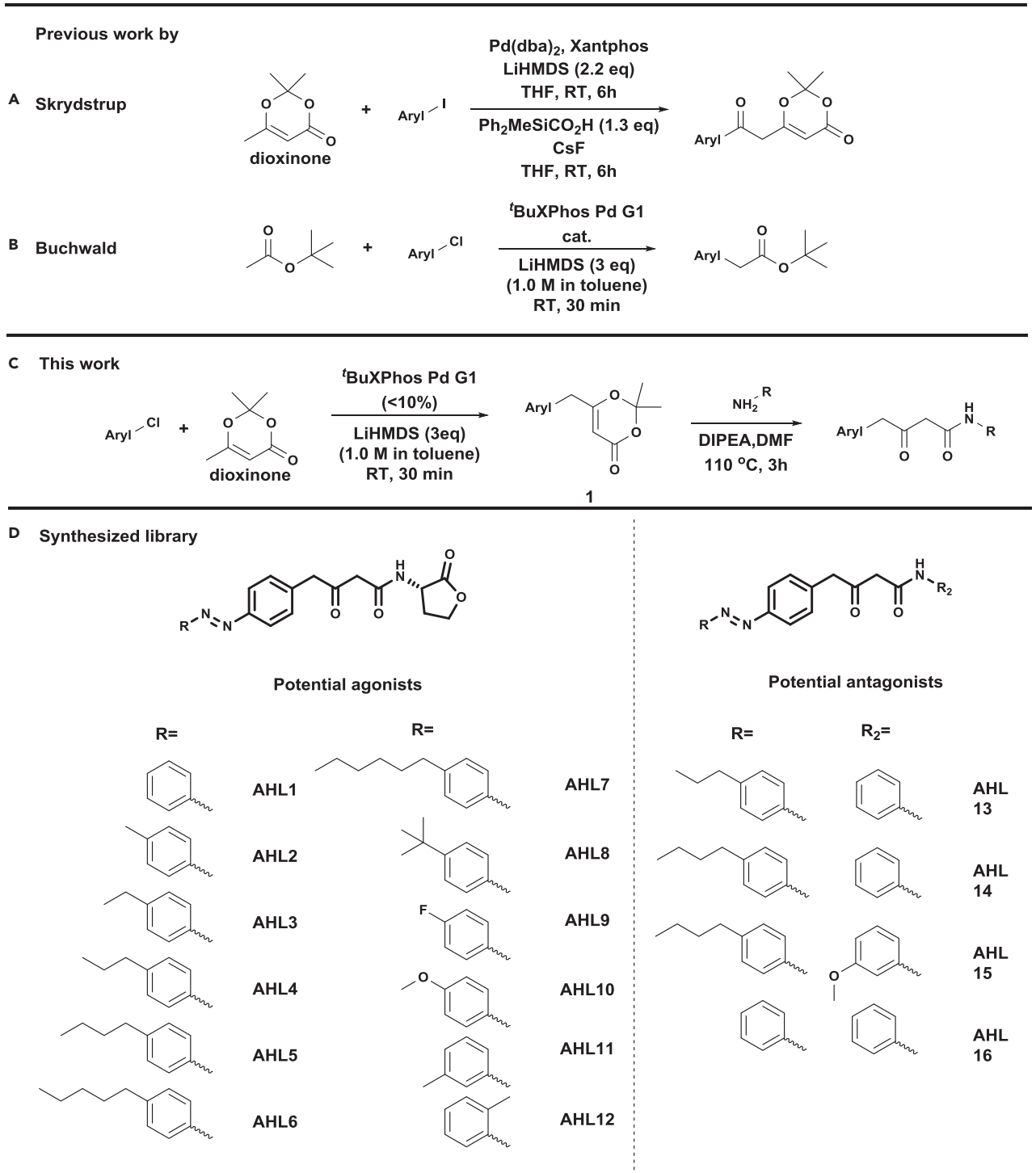

Figure 2. Synthetic Methodology Developed for the Preparation of α- and β-carbonyl Compounds

(A and B) Cross-couplings previously reported by (A) Skrydstrup and co-workers[42] and (B) Biscoe and Buchwald.[41]

(C) Novel methodology for the synthesis of aryl-β-ketoamides in this work.

(D) Synthesized library of potential agonists and antagonists. A diverse set of para-substituted AHLs was prepared for investigation of the structure-activity relationship.

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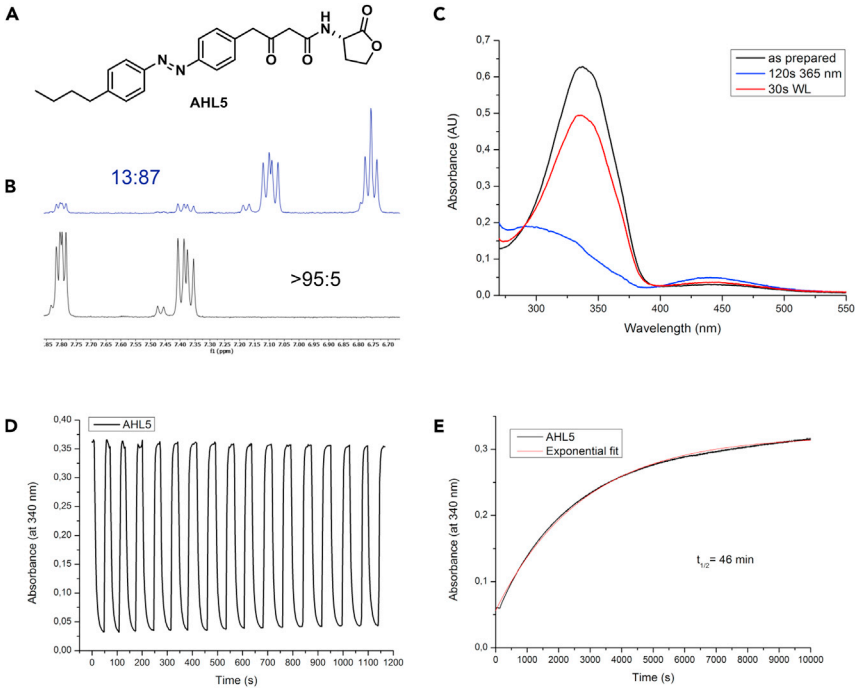

Figure 3. Photochemical Evaluation of AHL5

(A) Molecular structure of AHL5.

(B) Photostationary state and *trans-cis* ratio after thermal adaption of AHL5: thermally adapted (black) and 365 nm irradiated (blue). See Table S1 for all AHLs.

(C) UV-visible spectra showing the photoswitching of AHL5 with an isosbestic point at 385 nm.

(D) Fatigue determination of AHL5; no significant fatigue was observed after 15 rounds of irradiation.

(E) Kinetic evaluation of the half-life of AHL at 30°C (see Figure S7; Supplemental Information for evaluation of the half-life under assay conditions).

All measurements were performed in DMSO at a concentration of 20 μM (or DMSO-d_6 at 2 mM for B).

showed satisfactory inhibitory activity, as expected from earlier structure-activity-relationship (SAR) studies.[47,48] Also, AHL1 showed good inhibitory activity against OdDHL with a minor difference in activity between the irradiated and non-irradiated samples. Inspired by these results, we synthesized and tested AHL16, which unfortunately exhibited similar inhibition with a minor difference in activity. Notably, the 1-oxo-azo, reported before by our group,[33] proved to be the most potent photoswitchable inhibitor in our library with 57% inhibition of QS activity.

Next, we tested the agonizing properties of the synthesized AHLs. Remarkably, only limited enhancement of the activity was observed for the 3-oxo AHL1 when compared with the previously reported 1-oxo-azo derivative,[33] whereas from previous SAR studies,[8,34] a considerable enhancement was expected. However, substitution of the azobenzene core had a major effect on the agonistic properties of the resulting photoswitchable AHLs. At the thermally stable states, both *p*-propyl and *p*-fluoro AHLs (AHL4 and AHL9) proved to be the main agonists of QS in this library with 15%–18% induction (compared with that of the native AHL, OdDHL; Figure 1). However, irradiation (λ = 365 nm for 5 min) of the AHLs and subsequent evaluation as QS agonists revealed a dramatic increase in activity for AHL3–AHL7 and AHL10, whereas the activity of AHL9 showed a notable decrease (see Figure 4). Especially AHL4 and AHL5 stood out in this respect with 71% and 46% QS induction,

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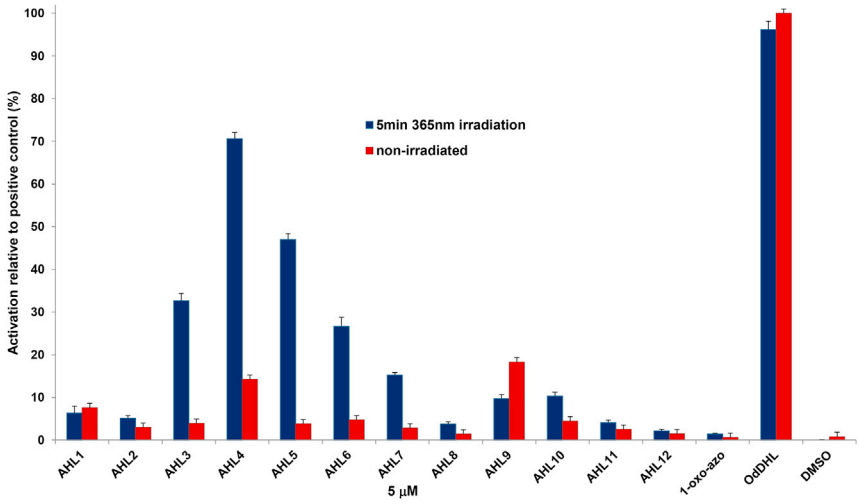

Figure 4. Biological Evaluation of Photocontrolled Quorum-Sensing Autoinducers

All compounds were evaluated at 5 μM, with *E. coli* JM109 psB1075 as the reporter strain, after 65 min of incubation. Native autoinducer OdDHL was used as a positive control. Measurements all represent the mean of triplicates with standard deviation. AHLs were used in their thermally adapted form (red, *trans*-isomer) or were pre-irradiated for 5 min with λ = 365 nm light (blue, mainly *cis*-isomer) (see Supplemental Information for details).

respectively. The activity of irradiated AHL4 is closely comparable to the best known synthetic autoinducers reported so far.[8,35] From the small library reported herein, it can be concluded that the alkyl substituent at the *para*-position is crucial to enhance activity upon photoisomerization. As shown in Figure 4 (AHL1–AHL7), elongation of the hydrophobic tail initially increases the activity until it reaches a maximum at the C_3 tail, whereas further elongation up to a C_6 substituent leads to a loss of activity.

Moreover, the selectivity of the *cis*-isomer (over the *trans*-isomer) also significantly changes with the substitution pattern. A profound 12-fold difference in activity at a single concentration (5 μM) between the irradiated and non-irradiated form was observed for AHL5. Subsequent investigation of the dose response of both the irradiated and non-irradiated AHL5 (see Figure 5A) revealed a stunning >700-fold difference in activity between the irradiated (*cis*, EC_{50} = 0.57 μM) and non-irradiated (*trans*, no activity up to 400 μM; see Supplemental Information for details) forms. This difference in activity represents an unprecedented selectivity in photopharmacology, hitherto observed only for irreversible activation of photocaged systems,[49] and additionally illustrates the sensitivity of the LasQS system. Remarkably, the irradiated form showed a threshold at which activation reached the maximum. After 100 μM, the activity significantly decreased without interfering with bacterial growth (see Supplemental Information for details). All the tested compounds showed no antibacterial activity, i.e., the AHLs did not alter the growth of the reporter strain at relevant concentrations (see Supplemental Information for growth curves), proving that the drop of the QS signal is not caused by compound toxicity and cell death.

To emphasize the reversibility of the induction attained with photoswitchable AHL5 and exclude the possibility that the difference in potency between the two forms of AHL5 stems from photodegradation to a more potent compound, we sequentially activated and deactivated this compound with different wavelengths of light. As can be observed from Figure 5B, at least two cycles of activation and deactivation with 365 nm and WL are feasible without the observation of significant fatigue or degradation. Moreover, the lack

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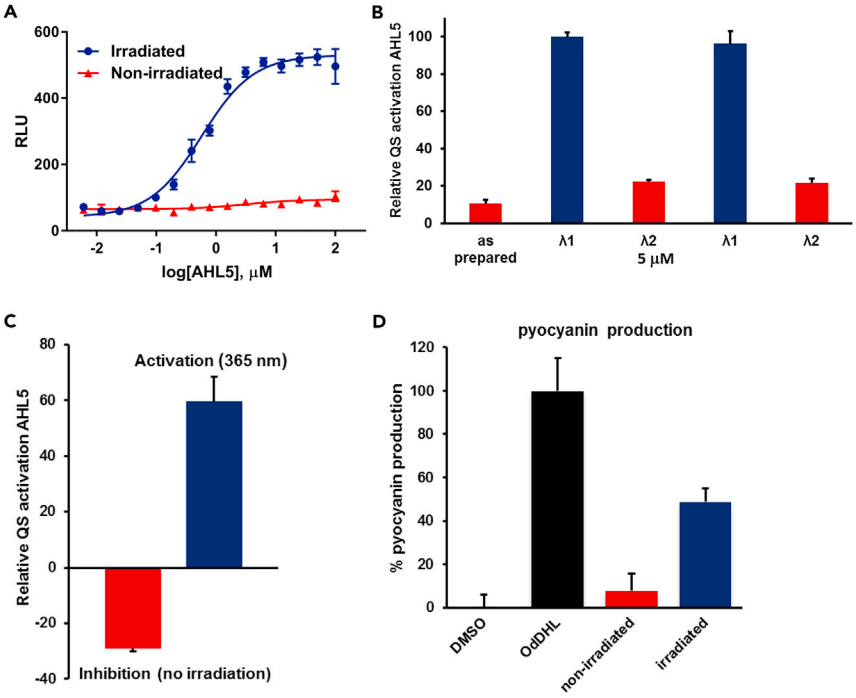

Figure 5. Dose-Response Profile and Fatigue Resistance of AHL5

(A) Relative luminescence obtained at different concentrations of AHL5. A dramatic difference in agonizing activity was observed between irradiated and non-irradiated samples.

(B) Repetitive switching cycles without observable fatigue. λ_1, 365 nm for 5 min; λ_2, white light for 1 min; [AHL5], 5 μM in Luria Bertani (LB) broth with <1% DMSO.

(C) Antagonist to agonist switching of AHL5 (60 μM): 0% relative QS activity was induced by the EC_{50} concentration (0.6 nM) of OdDHL, and −100% activation (100% inhibition) is based on a blank with the appropriate DMSO concentration (see Supplemental Information for details; Figure S8).

(D) Pyocyanin production in *Pseudomonas aeruginosa* (PA14 ΔlasI) upon exposure to a negative control (2% DMSO), positive control (20 μM ODdHL), and AHL5 (2 × 50 μM) without (red) and after (blue) irradiation (see Supplemental Information for details).

of agonizing activity, even at increasing concentrations of our non-irradiated AHL5, raised the question whether the *trans*-AHL5 would potentially possess antagonizing properties. To evaluate such behavior, we induced QS activation by EC_{50} concentrations of the native autoinducer (OdDHL). The induction of limited QS activity allows both QS activation and inhibition to be measured. Interestingly, at a concentration of 60 μM, AHL5 could be reversibly switched from a QS inhibitor (30% inhibition) to a QS activator (up to 60% activation) upon irradiation with light. This showcases one of the first examples of the photopharmacological approach with opposing activities (inhibitor versus activator) of the different isomers.[23] Next, we applied AHL5 to *P. aeruginosa* (PA14 Δ*lasI*) to control toxin production (pyocyanin) with light. Pyocyanin production is under direct QS control, making it an interesting target to confirm the applicability of our approach *in vitro*. Utilizing AHL5 in the non-irradiated and irradiated form resulted in a significant difference in pyocyanin levels (7% and 48%, respectively), confirming the ability to control QS-regulated phenotypes with light using the photoswitchable modulators presented here.

In conclusion, we have developed a synthetic methodology to gain access to a library of photoswitchable 3-oxo-derived QS agonists and antagonists via a novel Pd-catalyzed cross-coupling reaction. Up to 71% QS induction was obtained, whereas the best inhibitor showed almost 40% OdDHL inhibition. Moreover, our

lead compound, AHL5, showed privileged properties for further development in photopharmacology because of the excellent difference in activity between the inactive (non-irradiated) and activated (irradiated) states. Strikingly, utilizing reversible photoswitching, a >700-fold difference in activity between the irradiated and non-irradiated forms has been achieved, which could be translated to a significant phenotypic effect, i.e., toxin production in *P. aeruginosa*. Furthermore, the possibility to switch from a QS inhibitor to a QS activator by using light opens up new possibilities in photopharmacology and QS research. This excellent photopharmacological behavior paves the way for future application of these novel photoswitchable QS agonists, and distinct AHLs from the here-reported series are highly promising as next-generation light-controlled research tools.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.chempr.2019.03.005.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.J.H., W.S., A.J.M.D., and B.L.F.; Methodology, M.J.H. and J.I.C.H.; Investigation, M.J.H. and J.I.C.H.; Writing – Original Draft, M.J.H.; Writing – Review & Editing, M.J.H., W.S., A.J.M.D., and B.L.F.; Resources, W.S., A.J.M.D., and B.L.F.; Supervision, W.S., A.J.M.D., and B.L.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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