Biological screening of a diverse set of AI-2 analogues in *Vibrio harveyi* suggests that receptors which are involved in synergistic agonism of AI-2 and analogues are promiscuous†

Jacqueline A. I. Smith,[a] Jingxin Wang,[a] Sao-Mai Nguyen-Mau,[b] Vincent Lee[b] and Herman O. Sintim*[a]

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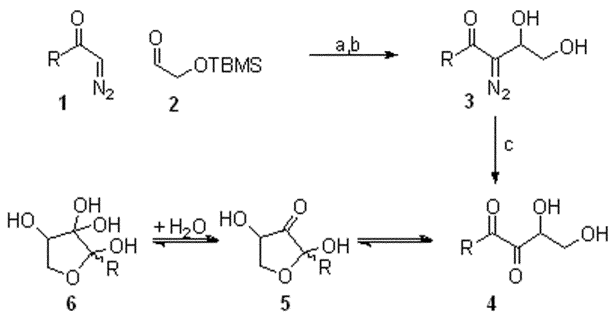
C1-alkyl AI-2 analogues do not induce bioluminescence in *V. harveyi* on their own but enhance the bioluminescence induced by AI-2 in a synergistic fashion. A new facile synthesis of AI-2 facilitates the synthesis of a diverse set of AI-2 analogues and biological screening suggests that receptors that are involved in the synergistic bioluminescence production in *V. harveyi* are promiscuous.

Many bacteria use chemical signals to regulate the expression of important genes as a function of cell density. This phenomenon, known as quorum sensing (QS), is a cell-to-cell communication system that allows bacteria to assess their local population density *via* the secretion and detection of small, diffusible signal molecules called autoinducers and regulate gene expression when a critical population density is reached.[1] QS regulates the expression of virulence factors,[2] and other factors that are important for bacterial colonization of higher organisms,[3] susceptibility to antibiotics,[4] the formation of biofilms[5,6] and the growth rates of some bacteria.[7] Therefore molecules that can inhibit proteins or other macromolecules that are involved in the quorum-sensing process could in principle be used to fight bacterial infection.[8–11]

The LuxS enzyme catalyzes the formation of (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD), the precursor of AI-2, and it has been identified in over 55 bacterial strains.[12] Because AI-2 is produced and sensed by many bacterial strains, it has been termed "the universal autoinducer".[12] It therefore follows that small molecules that antagonize the actions of AI-2 might have broad spectrum antibiotic effects. Six C1-linear alkyl analogues of AI-2 have been shown to be non-toxic to human cells and they exhibit different biological activities in *V. harveyi* and *S. typhimurium*; synergistic agonists for bioluminescence in *V. harveyi* and antagonists for β-galactosidase activity in *S. typhimurium*.[13] Access to C1 branched and cyclic alkyl chain AI-2 analogues will allow a more comprehensive study of how both the size and shape of the C1 alkyl group of AI-2 analogues affect their biological profile.

Despite the structural simplicity of AI-2, its chemical synthesis is not trivial.[14–17] DPD (the precursor of AI-2) is only stable at dilute concentrations and it has been shown that at higher concentrations, DPD dimerizes into an inactive triacetal compound.[14] The "instability" of DPD therefore places a constraint on the synthetic strategies that can be used to access this molecule.[18,19] Crucially, the last step of the synthesis of DPD has to be near quantitative and it is vital to employ reagents that are easy to remove without the use of standard chromatographic separation techniques. For the reported synthesis of C1-linear alkyl chain analogues of AI-2, one of the key steps involved a S_N2 reaction with primary iodides.[13] Difficulties in adapting reported AI-2 syntheses to make C1-branched and cyclic alkyl chain AI-2 analogues are envisioned (nucleophilic substitution reactions at 2° and 3° alkyl halide centers are difficult to accomplish). Herein we report a new and more facile synthesis of AI-2 that has allowed access to the "difficult-to-make" C1-*tert*-butyl, C1-isopropyl and cyclic alkyl chain AI-2 analogues (see Scheme 1).

For our strategy towards AI-2, the products obtained from the condensation of diazocarbonyl **1** and aldehyde **2** in the presence of DBU were not purified but taken to the next step whereby the silyl group was deprotected with tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) to give diol **3** in good yields after silica column chromatography. Diazodiol **3** existed predominantly as an open chain and not as a lactol (as would have been expected for a compound containing



Scheme 1 A two-pot synthesis of AI-2 and analogues; (a) cat. DBU, MeCN; (b) TBAF (1–3 eq), THF; (c) dimethyl dioxirane; acetone. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene.

[a] *Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742, USA. E-mail: hsintim@umd.edu*
[b] *Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20742, USA*
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hydroxyl and ketone functionalities in a 1,4-relationship). The ^{13}C NMR showed a carbonyl peak at 192 ppm and there was no peak between 100 and 120 ppm (which would have been indicative of lactolization). Presumably, hyperconjugation between the bonding electrons of the C–N bond and the π* of the carbonyl bond makes the C=O bond less electrophilic for lactolization to occur.

With adequate quantities of diazodiol **3** in hand, after only a one-pot operation, the stage was set to oxidize the diazo functionality into a carbonyl moiety. At this stage, we were cognizant of the fact that the oxidizing reagent or any byproducts of the oxidizing reagent required for the end-game of our synthesis had to be volatile because of the difficulty in purifying DPD using column chromatography, *vide supra*. Therefore, despite the availability of a wide range of oxidizing reagents that have been shown to oxidize the diazo moiety into a carbonyl,[20] only a handful of reagents fitted this requirement. We settled on dioxirane because it is volatile and importantly its byproduct is acetone, which is also volatile.[21] Pleasingly, treatment of diazodiol **3** with an acetone solution of dioxirane afforded DPD **4** and its isomers **5** and **6** in quantitative yield after evaporation of the acetone solvent. The NMR of our synthetic DPD and that of a quinoxaline derivative, which was obtained by reacting the synthetic AI-2 with 1,2-phenyldiamine, matched literature values.[14–17]

We selected bioluminescence production in *V. harveyi* for biological evaluation of our AI-2 analogues because of the rapid readout and reproducibility of this particular assay. Using the *V. harveyi* LuxN$^-$ and LuxS$^-$ strain, MM32, we monitored the bioluminescence of each analogue for up to 8 hours. Our synthetic AI-2 **4a** induced bioluminescence in *V. harveyi* (MM32) at various concentrations (from nanomolar to micromolar).[22]

Our group is interested in deciphering the role of conformation of C1-alkyl analogues of AI-2 on enhancing or inhibiting AI-2-mediated quorum sensing processes. Towards this end, we synthesized a panel of AI-2 analogues with different ring and linear sizes and shapes. Because AI-2 and analogues exist as an equilibrium mixture of compounds, we confirmed the identity of the analogues by converting them into quinoxaline derivatives and fully characterizing these derivatives (see the ESI†). None of the C1-alkyl analogues of AI-2 induced bioluminescence at 2 μM. At AI-2 analogue concentration of 50 μM, only the ethyl analogue **4b** and the cyclopropyl analogue **4g** induced significant bioluminescence, although the bioluminescence intensities from both **4b** and **4g** were at least an order of magnitude less than that from AI-2 after 8 h (see Fig. 1b). The bioluminescence intensities from the rest of the analogues (**4c**, **4d**, **4e**, **4f** and **4h**) were similar to when no analogue was added (*i.e.* background).

In the course of conducting our studies, another group also reported that another diverse group of C1-alkyl analogues of AI-2 enhanced AI-2-induced bioluminescence in *V. harveyi* even though they did not induce bioluminescence on their own (see ref. 13).[23] Four of the compounds in this study (isopropyl **4e**, *tert*-butyl **4f**, cyclopropyl **4g** and cyclohexyl **4h**) are new analogues and were not reported in the recent report.[13] These new analogues were designed to test how shape and size affect AI-2 analogue induced bioluminescence or synergistically-induced bioluminescence. We find that diverse shapes and sizes of the C1-alkyl chain of AI-2 are all able to synergistically induce bioluminescence in *V. harveyi* in the presence of AI-2; receptors that bind to AI-2 analogues in order to promote bioluminescence in *V. harveyi* (in synergism with AI-2) display marked promiscuity of ligand binding.

The origin of the concentration-dependent synergistic enhancement (by **4b**, **4c**, **4d**, **4e**, **4f** and **4g**) of AI-2-induced bioluminescence remains unknown. The analogues might act on a different target protein and the action of the second target sensitizes the LuxP/LuxQ system that is involved in *V. harveyi* bioluminescence or (b) the analogues may activate a protein which is further downstream of the LuxP/LuxQ AI-2 signaling pathway. It is also plausible that the enhancement of AI-2-induced bioluminescence by C1-alkyl analogues of AI-2 is due to one AI-2 molecule binding to one active site in the LuxP/LuxQ dimer whereas the analogue binds to the other active site; resulting in a desymmetrized complex that is more active at eliciting bioluminescence than the complex that contains two identical AI-2 or AI-2 analogue molecules; Hughson *et al.* have previously shown that two AI-2 molecules bind to the LuxP/LuxQ dimer, in an asymmetric manner, to elicit bioluminescence in *V. harveyi*.[24]

In conclusion, we have provided a straight-forward synthesis of the universal autoinducer, AI-2. This development has facilitated the synthesis of a panel of seven C1-alkyl analogues of AI-2, four of which are novel. The analogues

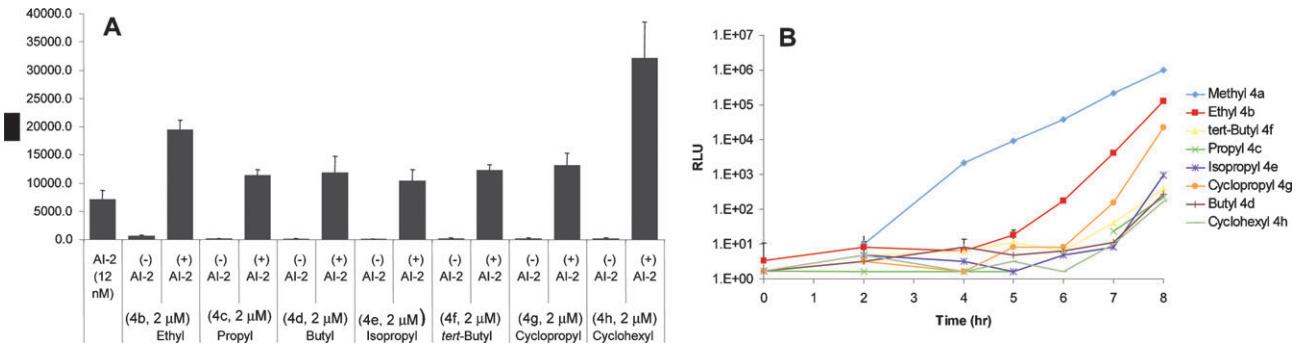

Fig. 1 (a) Bioluminescence induction in *V. harveyi* MM32 (at 8 h) by the addition of a DPD mixture containing analogue (2 μM), AI-2 (12 nM) and boric acid (100 μM); synergistic agonism. Fold-activations are as follows: ethyl (4.3), propyl (2.6), butyl (2.7), isopropyl (2.4), *tert*-butyl (2.9), cyclopropyl (3.1), cyclohexyl (9.1). The analogues (2 μM) did not induce bioluminescence on their own. (b) Bioluminescence induction in *V. harveyi* MM32 by the addition of 50 μM of each analogue and boric acid (100 μM).

were screened for bioluminescence induction in *V. harveyi* and they enhanced AI-2-mediated bioluminescence, although they did not induce bioluminescence in *V. harveyi* on their own. Interestingly, a clear trend does not emerge as regards to the size or shape of an AI-2 analogue and its fold enhancement of AI-2-mediated bioluminescence in *V. harveyi*. This suggests that the receptors that mediate the AI-2 and analogue synergistic agonism may be promiscuous. Our new and expeditious synthesis of AI-2 analogues could be used to prepare an expanded set of AI-2 analogues for further biological testing, such as investigating promiscuity or lack-thereof in proteins that signal AI-2 binding into other quorum sensing processes.

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23 Although synergistic agonism assays of AI-2 analogues have been previously done without added boric acid (see ref. 13), we added boric acid (100 μM) to our assay. We thank an anonymous reviewer who pointed out that when boric acid is not added to the culture media for the bioluminescence assay, the analogues might scavenge for adventitious borate; thereby affecting the results of different assays that contained different amounts of adventitious borate. We nonetheless confirm Janda's report that AI-2 analogues cause fold enhancement of AI-2 induced bioluminescence in *V. harveyi*, although that study did not add boric acid to the media for the bioluminescence assay.
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