VIP Structure Elucidation

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Uncharacterized 4,5-Dihydroxy-2,3-Pentanedione (DPD) Molecules Revealed Through NMR Spectroscopy: Implications for a Greater Signaling Diversity in Bacterial Species**

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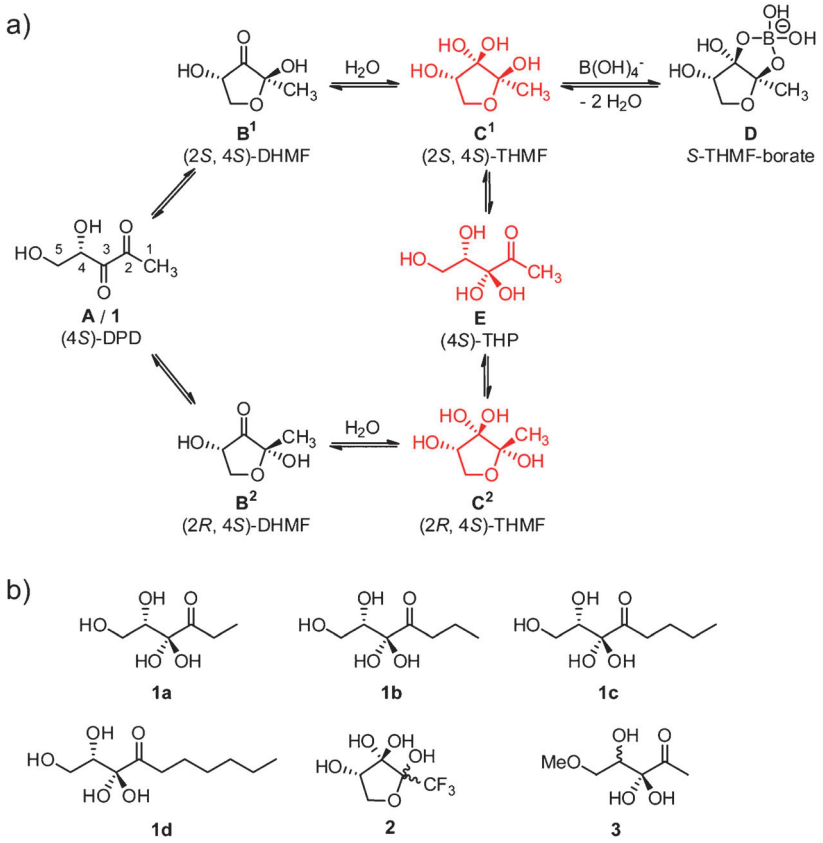
Chemical communication among bacteria, termed quorum sensing (QS), is a phenomenon that has attracted considerable interest over the last three decades. In this process, the exchange of small chemical signals enables bacterial populations to act together as an ensemble, rather than as single organisms.[1] This allows bacteria to achieve functions that are beneficial to an entire population and promotes coexistence with higher organisms. For example, several bacterial species use QS to regulate biofilm formation, which results in an increased survival rate through a higher tolerance to antibiotics.[1] Thus, interference in this communication could lead to improved control of bacterial infections and contamination in healthcare settings. Importantly, this approach presents advantages over traditional antimicrobials because of a presumed diminished selective pressure to develop resistance.[2] Therefore, a sound understanding of this communication system at the molecular level could be vital for new antimicrobial therapeutics.

Autoinducer-2 (AI-2) is an important class of QS signals that are produced by many bacteria and are purported to be interspecies signals. Currently, only two AI-2 chemical signals have been characterized, both of which are derived from 4,5-dihydroxy-2,3-pentanedione (DPD, **1**, Scheme 1a), which is produced by the enzyme LuxS.[3] While DPD seems to be a simple, linear, five-carbon molecule, in aqueous solution DPD exists in a complex equilibrium between linear (**A**) and both cyclic stereoisomers ($\mathbf{B^1}$ and $\mathbf{B^2}$). Indeed, the subsequent hydration of both cyclic forms to give $\mathbf{C^1}$ and $\mathbf{C^2}$ isomers of this highly functionalized molecule is described in almost all reports, and, in fact, X-ray crystallography has shown that $\mathbf{C^2}$ is the isomer that is recognized by the human pathogens *Salmonella typhimurium*, and *Yersinia pestis*, and the plant symbiont *Sinorhizobium meliloti*.[4] In 2002, crystallographic analysis also revealed that the active species in *Vibrio harveyi* is a borate ester (**D**) in complex with LuxP.[5] Adding to the intricacy of DPD is that phosphorylation of its primary alcohol by the kinase LsrK occurs in members of the Enterobacteriaceae family (for example, *S. typhimurium* and *Escherichia coli*) to form the species that directly affects gene expression.[6] Thus, the dense oxygenation of DPD gives an interconversion of several signals from a single precursor.



Scheme 1. a) The equilibrium of autoinducer-2 DPD as typically described in the literature (black color) and the equilibrium identified in this study (red color). DHMF = dihydroxytetrahydrofuran, THMF = tetrahydroxytetrahydrofuran.

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Numerous syntheses of DPD have been described in the last decade, after the structural elucidation of AI-2.[7] Forthcoming from these syntheses have been analogues that reveal how drastic changes in biological activity occur even with minor scaffold alterations such as C1-alkyl analogues **1a–d** (Scheme 1b).[8] Notably, several of these studies have focused on the ring dynamics of DPD and the corresponding requirements for signaling activity. For example, CF_3-DPD (**2**) has been reported to exist exclusively in the cyclic form.[9] However, detailed structural analyses of the equilibrium and hydration states of natural DPD are lacking. Thus, only limited quantitative information has been described for each species of DPD, a problem that is exacerbated by the fact that mass spectrometry techniques are unable to distinguish between molecules of the same molecular weight.[10] Accordingly, we sought to characterize the important but confounding equilibrium and hydration states of DPD by using a variety of NMR spectroscopic techniques. We examined these interactions over a wide range of pH values that we reasoned would provide insights into the stability of DPD as well as the unique isomers that are present at physiological pH. Finally, we prepared and analyzed several key analogues, which enabled the logical deduction of previously unrecognized DPD species.

Previous NMR spectroscopic analysis has shown that the ratio of the linear to both cyclic forms is 1:4 under strongly acidic conditions.[7b] To provide insight into the equilibrium of DPD at physiological pH, we buffered an aqueous solution of **1** to pH 7 (1M NaD_2PO_4/Na_2DPO_4). Based on the previously assigned ^{1}H NMR spectrum under acidic conditions, we anticipated a straightforward assignment of the signals, however, we detected major changes in both the number and intensity of the methyl group signals ($\delta = 1.3$–2.4 ppm) as well as the signals of the DPD core (C4 and C5/$\delta = 3.5$–4.4 ppm), which mainly results in overlapping signals. These findings became even more convoluted as we detected another signal change after 24 h, which we ascribed to be the final stage of the equilibrium (Figure S1 in the Supporting Information). As a result of the complexity of the spectra, we were neither able to clearly assign signals to any species, nor to integrate single signals, but we did detect a decrease in intensity of the original signals. As such, it is evident that the equilibrium of DPD is pH-dependent and that there are species that were previously unaccounted for in equilibrium at physiological pH.

To obtain a more detailed picture of this shift in equilibrium, we titrated DPD with NaOD in D_2O (0.1M) to follow the shifts in equilibrium in a stepwise manner. The most significant changes came between pH 4 and 5, where several new signals appeared in the region of the signals from the methyl protons. Importantly, the final ^{1}H NMR spectrum of DPD at pH 7 under these unbuffered conditions is exactly the same as the spectrum in aqueous phosphate solution after 24 h incubation. Furthermore, we titrated a solution of DPD to pH 10 to investigate the stability and the changes under basic conditions. Interestingly, the new methyl signals at around $\delta = 1.3$ ppm are even more dominant under basic conditions, and the three major signals that are present under acidic conditions disappear completely. We next acidified this solution to pH 1 and obtained the same ^{1}H NMR spectrum as our starting solution (Figure S2 in the Supporting Information). Thus, the changes are reversible, which precludes polymerization or decomposition of DPD during the titration, and indicates an unexpected stability of this highly oxygenated molecule over a broad range of pH values. These results highlight the chemical stability of DPD, which is in complete contrast to other, well-documented QS molecules, such as acylhomoserine lactones (AHLs) and autoinducing peptides (AIPs), which have inherent susceptibility to hydrolytic degradation.[11] The stability/instability of QS signaling molecules has been probed previously[11a] and suggests alternate biochemical roles for DPD and its equilibrium species.

As a result of the overlapping signals in the ^{1}H NMR spectra, we next turned to ^{13}C NMR spectroscopy with isotopically labeled DPD derivatives $^{13}CH_3$-DPD (**4**)[7b] as well as the 1,2-bis-^{13}C-labeled compound **5** (Figure 1). As anticipated, both labeled derivatives have the same pH-dependent behavior as natural DPD in terms of ^{1}H NMR spectra (Figure 1a; see also Figure S3 in the Supporting Information). However, the presence of the ^{13}C isotope

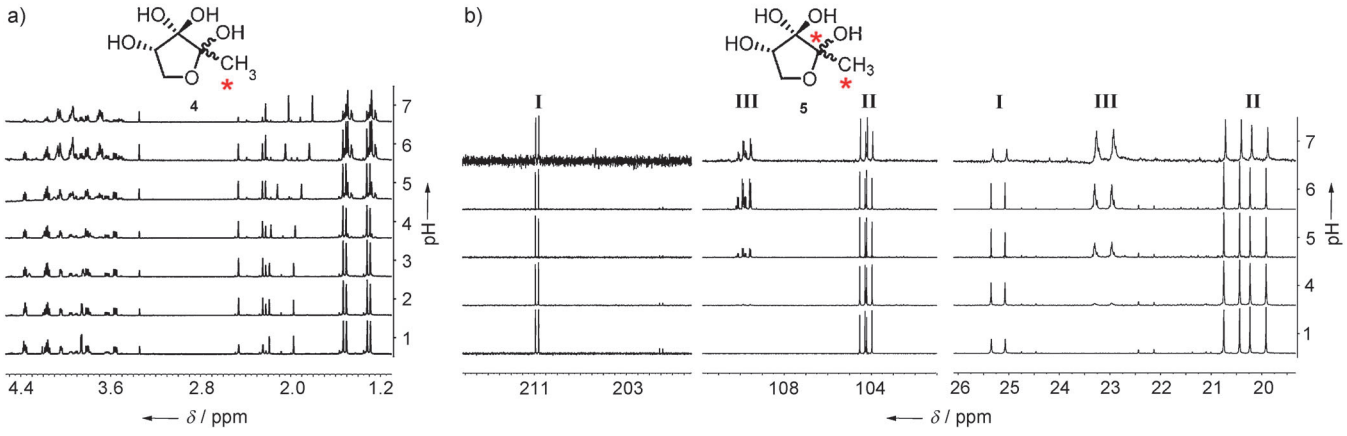

Figure 1. a) Sections of ^{1}H NMR spectra illustrating the pH-dependency of structural equilibrium in ^{13}C-DPD (**4**). b) Sections of ^{13}C NMR spectra for the base titration of bis-^{13}C-DPD (**5**) (Full spectrum in Figure S3 in the Supporting Information). ^{13}C atoms are indicated by *.

resulted in altered splitting patterns for each methyl group in the ^{1}H NMR spectra ($^1J_{CH} = 127.9$–129.1 Hz and $^2J_{CH} = 5.0$–6.1 Hz). Nevertheless, the inclusion of these two isotopically labeled compounds allowed us to focus on two carbon atoms in the ^{13}C NMR spectrum, C1 and C2, which undergo significant changes (see above) based on hydration or cyclization. Analysis of the labeled compounds indeed revealed that the signals in the ^{13}C NMR spectrum are of better resolution than the proton signals, and we detected three major ($\delta = 20.0$, 20.6, and 25.2 ppm) and four minor signals for the methyl carbons at acidic pH values. Basic titration resulted in a decrease in signal intensity of the major signals and the appearance of new signals, which are clearly separated from the original signals, at $\delta = 23.1$ ppm. However, these new signals overlap with each other, which indicates that the new species are all of similar structures. From the ^{13}C NMR spectrum of the monolabeled compound **4** we can surmise that eight or nine different DPD species are in equilibrium at physiological pH (Figure S4 in the Supporting Information). To quantify each labeled signal, we determined the relaxation time for the ^{13}C nuclei, a technique that is rarely used in literature for small molecules, but is made possible with the multiple ^{13}C nuclei. Thus, the overlapping signals at $\delta = 23.1$ ppm were quantified to be 1.5–2 times more abundant at pH 7 than the three originally identified major signals ($\delta = 20.0$, 20.5, and 25.2 ppm). Surprisingly, quantification revealed that the ratio of the original signals is constant over the whole pH range, with an excess of 4.3–4.6 times the amount of both cyclic species relative to the linear species. This clearly shows that the abundance of the original species is reduced with higher pH, but that the linear/cyclic equilibrium is not influenced by the new species at physiological pH.

The additional ^{13}C label that is incorporated into compound **5** at position C2 is important because multiple ring-closing equilibria are taking place at this position. Furthermore, hydration and dehydration processes could be followed at this position, which again are readily detectable by ^{13}C NMR spectroscopy (Figure 1 b). Importantly, this compound allows direct assignments of each methyl group to the corresponding quaternary signal of the C2 position by using the C–C coupling constants. We identified three major species that we have labeled as species I, II, and III as seen in Figure 1 b (species II consists of both closed species).

The signal at $\delta = 211$ ppm corresponds to a linear species of DPD that contains a carbonyl group and can be assigned to the methyl group of species I at $\delta = 25.2$ ppm with $^1J_{CC} =$ 42 Hz (Figure 1 b). Furthermore, we assigned both cyclic forms of DPD at $\delta = 20.0$ and 20.5 ppm to the quaternary carbon signals at $\delta = 104.0$ and 104.5 ppm with a higher coupling constant ($^1J_{CC} = 47$–48 Hz for species II). The tendency of higher coupling constants for the more rigid cyclic forms relative to the linear form is in accordance with literature values.[12] Yet, the newly observed signals from species III have an even higher coupling constant of $^1J_{CC} =$ 51 Hz. These large values provide a glimpse that other cyclic forms exist; however, these NMR spectra did not serve to fully dissect their exact structure.

We detected only one major signal at $\delta = 211$ ppm that corresponds to the C2 carbonyl group of the linear species and no shift was detected during the titration. Consequently, we can exclude any hydration/dehydration processes that occur at the C3 position (this position is readily hydrated in aqueous solution upon deprotection). These findings are in agreement with NMR studies that describe a high tendency of monohydration of α-diketones in aqueous solutions of up to 70 %;[13] however, we believe that the electron-withdrawing effects of the adjacent alcohol also serve to increase the ratio of monohydration of the diketone in DPD. These findings allow us to assign the signals from species I to (4*S*)-3,3,4,5-tetrahydroxypentan-2-one ((4*S*)-THP, structure **E**, Scheme 1 a). The corresponding species $\mathbf{C^1}$ and $\mathbf{C^2}$ are in equilibrium with **E** and this can be detected in NOESY and ROESY spectra (Figure S5 in the Supporting Information). However, we are not able to fully exclude the linear diketone **A** and both nonhydrated cyclic forms $\mathbf{B^1}$ and $\mathbf{B^2}$ (Scheme 1 a), but we postulate that they are of minor importance in aqueous solutions. This is also in agreement with the calculated equilibrium constant of the hydration of this molecule, which favors hydration, as well as the equilibrium that was suggested in another study.[7b,14]

Our structural analysis of DPD by NMR spectroscopy has opened up an entirely new series of equilibrium structures that were previously not thought to be important. However, we wished to probe the chemical basis of these equilibrating chemical partners further. Therefore, we synthesized two derivatives as model systems for the cyclic and the linear species of DPD (Figure 2). As our model for the cyclized form we examined the CF_3 analogue of DPD (**2**); interestingly, this derivative is the most active agonist in *V. harveyi* that has been described to date and is only present in the cyclic form, hydrated at the C3 position.[9] In our analysis, the equilibrium of **2** is highly pH-dependent (Figure 2 a; see also Figure S6 in the Supporting Information). Thus, the ^{1}H NMR signal changes for the C4 and C5 positions are very similar to the cyclic isomers of natural DPD, and again show complete hydration at C3, whereas the spectra of **2** and DPD parallel each other and reemphasize that the equilibrium of the hydrated species **E** with $\mathbf{C^1}$ and $\mathbf{C^2}$ is important.

We synthesized the methylated primary alcohol 5-MeO-DPD (**3**) as our linear model compound, which is unable to engage in any ring closing events. The titration of **3** in the range of pH 1–7 indicates the presence of a single major species over the entire pH range, as shown by ^{1}H NMR spectroscopy (Figure S7 in the Supporting Information). Only one new minor species that makes up less than 5 % of the sample was detected, and the ^{13}C NMR spectrum clearly shows the presence of only one carbonyl moiety (Figure 2 b). This linear structure thus houses a single ketone, which further strengthens our hypothesis that the linear form of DPD is species **E** in aqueous solution (Scheme 1 a). However, seemingly in contradiction, diketone **A** has always been implicated as the major linear species of DPD in solution, largely because the addition of 1,2-phenylenediamine gives the corresponding quinoxaline derivative.[3b,7a,b] To test this, we treated **3** with an aqueous solution of 1,2-phenylenediamine and achieved complete conversion to the corresponding quinoxaline derivative (Figure S7 in the Supporting Information). To reconcile this finding, we postulate that

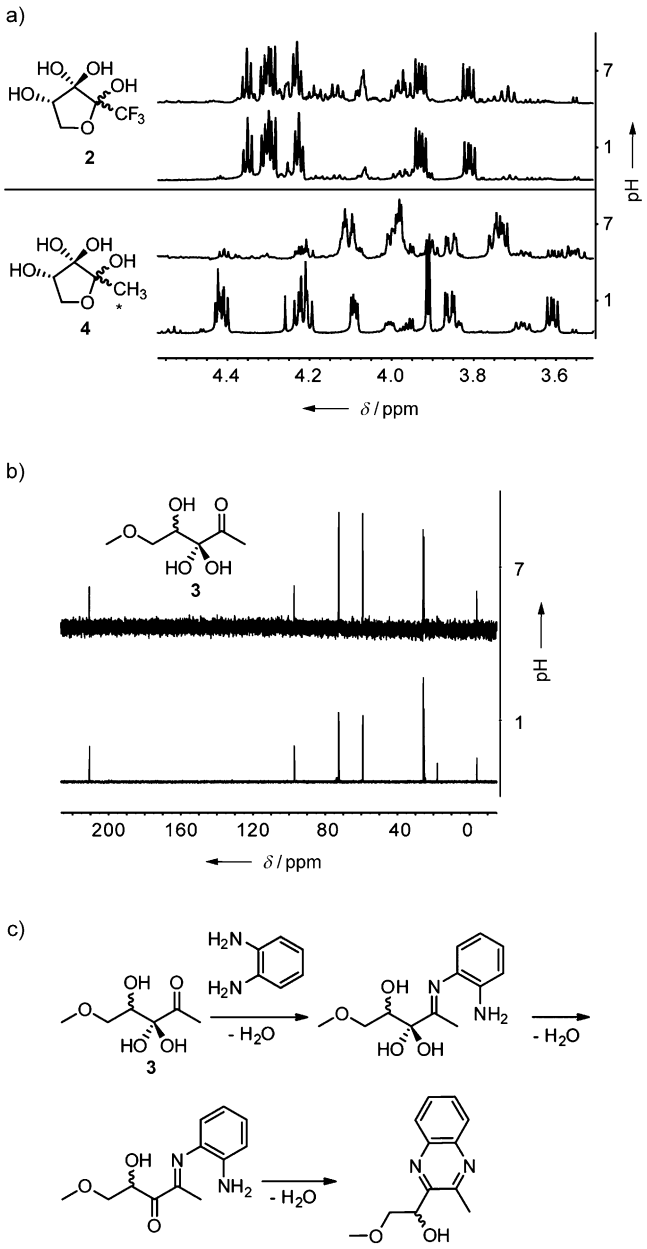

Figure 2. a) Sections of the ^{1}H NMR spectra of model compound **2** versus ^{13}C-DPD (**4**) at pH 1 and pH 7. b) Sections of ^{13}C NMR spectra of model compound **3** at pH 1 and pH 7. c) The proposed mechanism for the reaction of **3** with 1,2-phenylenediamine.

the reaction likely occurs through rapid dehydration of the C3 position after the conversion of the C2 carbonyl group to the corresponding imine, thus obviating the requirement of the α-diketone (Figure 2c).

Whereas the preceding studies were focused on the equilibrium of the natural DPD signals, we also sought to understand the equilibria of DPD analogues **1a–d** and their capability in modulating QS. Overall, these compounds exhibit variable biological activity in *V. harveyi* and *S. typhimurium* reporter assays. In *V. harveyi*, **1a–d** act only as synergistic activators of QS in the presence of DPD.[8] However, in *S. typhimurium* the biological activity varies based on the simple addition of methylene groups: ethyl-DPD (**1a**) is a weak agonist, whereas propyl-DPD (**1b**), butyl-DPD (**1c**), and hexyl-DPD (**1d**) are potent antagonists.[8a,b]

In stark contrast to DPD, which has a ratio of the two cyclic forms to the single linear form of 4.3:1, the ratios of cyclic to linear isomers of the alkyl-DPD analogues were about 1:1 at pH 1.5 (Figure 3). Only **1a** has a ratio slightly above 1:1, whereas it is less than 1:1 for **1b–d** that have the longer alkyl chains. This drastic change in equilibrium is surprising given the only weakly electron-donating capability of the additional methylene groups. However, even these minor changes appear sufficient to alter the electrophilicity of the C2 carbonyl group.

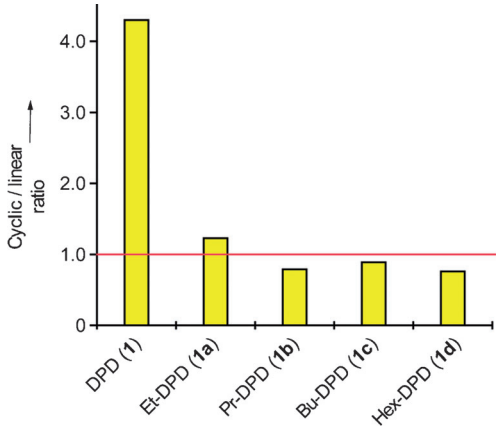

Figure 3. ^{1}H NMR quantification of the cyclic/linear ratio of DPD (**1**) and the C1-alkyl-DPD analogues **1a–d** at approximately pH 1.5. The values for both cyclic species $\mathbf{C^1}$ and $\mathbf{C^2}$ combined over species **E** are shown.

We adjusted the pH of these solutions to pH 7 and detected the appearance of new signals in a manner similar to those detected for DPD (Figure S8 in the Supporting Information). Because of the complexity of the spectra, we were unable to rigorously characterize the equilibria of **1a–d** at pH 7. However, as we measured a constant value of 4.3:1 for the ratio of the equilibrium of species $\mathbf{C^1}$ and $\mathbf{C^2}$ combined over species **E** for DPD, we posit that the corresponding ratios of the C1-alkyl derivatives **1a–d** at pH 1.5 (Figure 3) are also similar over the whole investigated pH range.

According to our NMR data for DPD, we postulate that the lower abundance of the cyclic forms may explain the lack of signaling activity of the DPD analogues in *V. harveyi*, as a boron complexed cyclic isomer of DPD must bind to the receptor protein LuxP to initiate the QS cascade inside the cell.[5a] In *S. typhimurium*, on the other hand, activity does not depend on the signal from cyclic DPD or other such species binding to its receptor, and the linear forms of these analogues may enter the cell where they can then activate or antagonize gene expression.[8b]

In summary, a detailed NMR analysis has provided us with a better understanding of the structural diversity of DPD at physiological pH. Our results allow us to conclude that the linear species of DPD is present as the monohydrate **E** ((4*S*)-

THP), which is in equilibrium with both hydrated forms $\mathbf{C}^1$ and $\mathbf{C}^2$ at pH 7. The species previously postulated to be major components of the DPD equilibrium, **A**, $\mathbf{B}^1$, and $\mathbf{B}^2$, were not observed in this study and are at best only minor species. Furthermore, the presence of the multitude of closed DPD isomers at physiological pH, coupled with the ability of DPD to complex anions, as with $[B(OH)_4]^-$, highlights the complexity of AI-2 based signaling, in that any of these isomers could potentially mediate bacterial communication. This study suggests that the lexicon of AI-2 communication may be significantly larger than appreciated.

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[1] a) J. W. Costerton, P. S. Stewart, E. P. Greenberg, *Science* **1999**, *284*, 1318 – 1322; b) W.-L. Ng, B. L. Bassler, *Annu. Rev. Genet.* **2009**, *43*, 197 – 222; c) C. A. Lowery, T. J. Dickerson, K. D. Janda, *Chem. Soc. Rev.* **2008**, *37*, 1337 – 1346; d) W. R. J. D. Galloway, J. T. Hodgkinson, S. D. Bowden, M. Welch, D. R. Spring, *Chem. Rev.* **2011**, *111*, 28 – 67.

[2] L. Cegelski, G. R. Marshall, G. R. Eldridge, S. J. Hultgren, *Nat. Rev. Microbiol.* **2008**, *6*, 17 – 27.

[3] a) M. G. Surette, M. B. Miller, B. L. Bassler, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 1639 – 1644; b) J. G. Zhu, X. B. Hu, E. Dizin, D. H. Pei, *J. Am. Chem. Soc.* **2003**, *125*, 13379 – 13381.

[4] a) C. S. Pereira, J. R. McAuley, M. E. Taga, K. B. Xavier, S. T. Miller, *Mol. Microbiol.* **2008**, *70*, 1223 – 1235; b) S. T. Miller, K. B. Xavier, S. R. Campagna, M. E. Taga, M. F. Semmelhack, B. L. Bassler, F. M. Hughson, *Mol. Cell* **2004**, *15*, 677 – 687; c) J. S. Kavanaugh, L. Gakhar, A. R. Horswill, *Acta Crystallogr. Sect. F* **2011**, *67*, 1501 – 1505.

[5] a) X. Chen, S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B. L. Bassler, F. M. Hughson, *Nature* **2002**, *415*, 545 – 549; b) X. Xue et al., *Angew. Chem.* **2011**, *123*, 10026 – 10030; *Angew. Chem. Int. Ed.* **2011**, *50*, 9852 – 9856.

[6] K. B. Xavier, B. L. Bassler, *J. Bacteriol.* **2005**, *187*, 238 – 248.

[7] a) M. M. Meijler, L. G. Hom, G. F. Kaufmann, K. M. McKenzie, C. Sun, J. A. Moss, M. Matsushita, K. D. Janda, *Angew. Chem.* **2004**, *116*, 2158 – 2160; *Angew. Chem. Int. Ed.* **2004**, *43*, 2106 – 2108; b) M. F. Semmelhack, S. R. Campagna, M. J. Federle, B. L. Bassler, *Org. Lett.* **2005**, *7*, 569 – 572; c) S. C. J. De Keersmaecker et al., *J. Biol. Chem.* **2005**, *280*, 19563 – 19568.

[8] a) C. A. Lowery, J. Park, G. F. Kaufmann, K. D. Janda, *J. Am. Chem. Soc.* **2008**, *130*, 9200 – 9201; b) V. Roy, J. A. I. Smith, J. Wang, J. E. Stewart, W. E. Bentley, H. O. Sintim, *J. Am. Chem. Soc.* **2010**, *132*, 11141 – 11150; c) H. Ganin, X. Tang, M. M. Meijler, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3941 – 3944.

[9] M. Frezza, D. Balestrino, L. Soulere, S. Reverchon, Y. Queneau, C. Forestier, A. Doutheau, *Eur. J. Org. Chem.* **2006**, 4731 – 4736.

[10] a) S. R. Campagna, J. R. Gooding, A. L. May, *Anal. Chem.* **2009**, *81*, 6374 – 6381; b) J. R. Gooding, A. L. May, K. R. Hilliard, S. R. Campagna, *Biochemistry* **2010**, *49*, 5621 – 5623.

[11] a) G. F. Kaufmann et al., *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 309 – 314; b) A. R. Horswill, P. Stoodley, P. S. Stewart, M. R. Parsek, *Anal. Bioanal. Chem.* **2007**, *387*, 371 – 380; c) J. Park et al., *Chem. Biol.* **2007**, *14*, 1119 – 1127.

[12] a) B. Bose et al., *J. Am. Chem. Soc.* **1998**, *120*, 11158 – 11173; b) B. Bose-Basu, T. Klepach, G. Bondo, P. B. Bondo, W. Zhang, I. Carmichael, A. S. Serianni, *J. Org. Chem.* **2007**, *72*, 7511 – 7522.

[13] a) E. Lissi, E. Abuin, C. Espinoza, M. A. Rubio, *Langmuir* **1994**, *10*, 1071 – 1074; b) J. P. Segretario, N. Sleszynski, R. E. Partch, P. Zuman, V. Horak, *J. Org. Chem.* **1986**, *51*, 5393 – 5396.

[14] K. Tsuchikama, C. A. Lowery, K. D. Janda, *J. Org. Chem.* **2011**, *76*, 6981 – 6989.

