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# Mechanistic analysis of aliphatic $\beta$ -lactones in *Vibrio harveyi* reveals a quorum sensing independent mode of action†

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***N*-Acylhomoserine lactones are autoinducers of quorum sensing (QS) in Gram-negative bacteria. We exploit here the role of structurally related  $\beta$ -lactones in the inhibition of *Vibrio harveyi* bioluminescence and identify a derivative with nanomolar potency. Surprisingly, QS was not affected and combined proteomic/biochemical studies revealed insights into the cellular mode of action.**

Quorum sensing (QS) represents a common bacterial communication strategy controlling diverse cellular mechanisms including biofilm formation,<sup>1–3</sup> virulence<sup>4,5</sup> and bioluminescence.<sup>6</sup> A growing population of bacteria secretes small autoinducer molecules (AIs) that are sensed by cytoplasmic or transmembrane receptors. Upon exceeding the threshold concentration of AIs an intracellular signaling cascade activates the transcription of QS-regulated genes.<sup>7</sup> Bioluminescent strains such as *Vibrio harveyi* ATCC BAA-1116, recently reclassified as *Vibrio campbellii* ATCC BAA-1116,<sup>8</sup> served as a model organism in the mechanistic analysis of QS<sup>9,10</sup> as well as for inhibitor screens.<sup>11–13</sup> *V. harveyi* recognizes three different classes of AIs including species-specific HAI-1, the interspecies molecule AI-2 and *Vibrio* genus specific CAI-1. The binding of these AIs to their cognate receptors LuxN, LuxQ/P and CqsS induces a phosphorelay involving LuxU and LuxO to regulate the production of the QS master regulator LuxR. LuxR in turn induces transcription of genes required for bioluminescence, biofilm formation, or exoproteolytic activity, and represses the expression of the type III secretion system or siderophores.<sup>6</sup> The inhibition of QS represents an attractive strategy for combating bacterial infections by attenuating virulence and biofilm formation.<sup>4,5,14,15</sup> Several targets were exploited in the past that play crucial roles as key QS regulators, such as the QS-receptor itself or the master regulator LuxR.<sup>16</sup>

Interestingly, most compounds active in QS inhibition exploit certain structural motifs derived from the *N*-acylhomoserine lactone (AHL) scaffold.<sup>7</sup> Here, among other features, the length of the acyl chain as well as the homoserine lactone core was identified as essential to promote QS. Interestingly, structurally related fimbrolide natural products are potent irreversible inhibitors of QS and bioluminescence by binding to LuxS, an enzyme required for AI-2 biosynthesis, as well as LuxE, an enzyme required for luciferase activity.<sup>17</sup> Similarly, the structural composition of natural product  $\beta$ -lactones<sup>18</sup> resembles some features of AHL, raising the question of whether a direct interference with QS pathways is also possible.

We investigate here the effects of diverse  $\beta$ -lactones on *V. harveyi* bioluminescence and identify a  $\beta$ -lactone equipped with a long aliphatic chain that even exceeds the potency of fimbrolides. Target identification *via* activity based protein profiling (ABPP) revealed an outer membrane protein, enzymes involved in fatty acid biosynthesis as well as a cyclic-di-GMP synthase as specific binders. Proteomic studies showed that  $\beta$ -lactones affect pathways relevant for fatty acid synthesis as well as electron transfer required for bioluminescence.

To unravel if the electrophilic  $\beta$ -lactone scaffold could substitute the larger and unreactive  $\gamma$ -lactone core of AHLs, we tested a panel of previously established  $\beta$ -lactones in bioluminescence assays (Fig. S1, ESI†).<sup>19</sup> For benchmarking of their potencies, we included fimbrolides as the gold standard QS and bioluminescence inhibitors.<sup>17</sup> All the compounds (50  $\mu$ M final concentration) were added to the growing culture of *Vibrio harveyi* NBRC 15634, used here for initial screens, and bioluminescence was monitored in a plate reader. Out of the lactones tested, **R1** decorated with a nonenyl-chain at the 2-position and a methyl group at the 3-position revealed a strong reduction of luminescence by 90%, comparable to those of fimbrolides that ranged from 75 to 97% inhibition (Fig. S2, ESI†). A closer inspection of the  $\beta$ -lactone structures revealed that although some of these compounds exhibited an identical nonenyl-substituent at the 2-position, larger decorations at the 3-position such as decenyl (**T1**), pentynyl (**D3**) or phenylethyl (**U1**) significantly decreased the potency down to 20%.

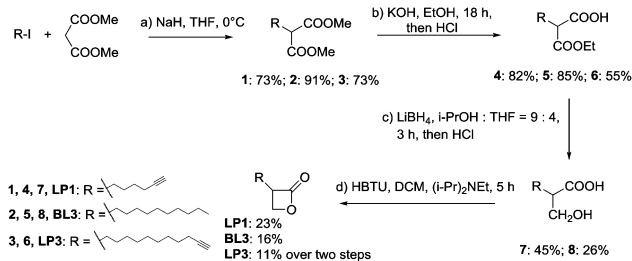
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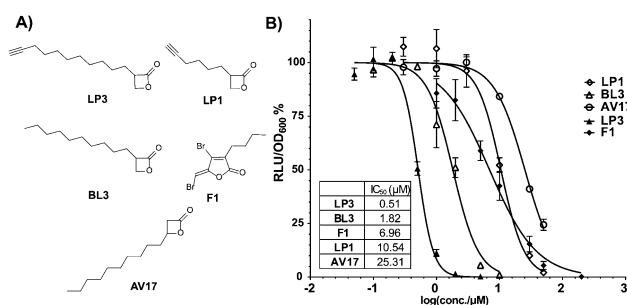


**Scheme 1** Synthesis of  $\beta$ -lactones. (a) NaH, THF, 0 °C, then add R-I; (b) KOH, EtOH, rt, 18 h, then add HCl; (c) LiBH<sub>4</sub>, i-PrOH:THF = 9:4, 3 h, then add HCl; (d) HBTU, DCM, (i-Pr)<sub>2</sub>NEt, 5 h. THF = tetrahydrofuran, DCM = dichloromethane, HBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.

Thus small substituents at the 2-position seem to be a key structural aspect of bioluminescence inhibition by  $\beta$ -lactones.

Based on the **R1** substitution pattern, we expanded the investigation of structural analogs to exploit the inhibitory effect on bioluminescence of various long-chain aliphatic substituents at either 2- or 3-position. Three additional derivatives were synthesized (**LP1**, **BL3** and **LP3**) and the collection was further complemented by the use of one previously established lactone (**AV17**).<sup>19</sup> The derivatives were prepared by the alkylation of dimethyl malonate with alkyl iodide, followed by partial hydrolysis of the diester, reduction of the remaining ester to a hydroxy group with LiBH<sub>4</sub> and cyclization by HBTU (Scheme 1 and Fig. 1A).

For the determination of bioluminescence IC<sub>50</sub> values, we switched to *V. harveyi* BAA-1116 as a commonly applied reference strain. As a general trend an increase of chain length at the 2-position dramatically enhanced the potency, while a substitution at the 3-position was largely inactive (Fig. 1B). Importantly, compounds **BL3** and **LP3** equipped with the longest aliphatic chains of this series, decyl and undecynyl, exhibited the best potencies with IC<sub>50</sub>s of 1.8 and 0.5  $\mu$ M, respectively. **LP3** as the most potent derivative exhibits a terminal alkyne, and was subsequently utilized for target identification *via* ABPP. For the identification of optimal labeling conditions, intact *V. harveyi* cells were incubated with various concentrations of **LP3**. Subsequently, the cells were lysed, separated in soluble and insoluble

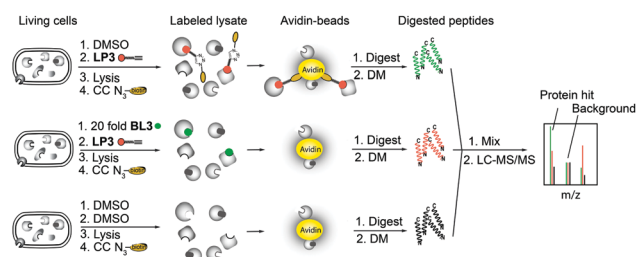


**Fig. 1** (A) Structures of compounds used in *V. harveyi* bioluminescence assay. (B) Residual bioluminescence production of *V. harveyi* after 30 min incubation with the compounds. Relative luminescence units (RLU) are normalized to cell density (OD<sub>600</sub>) and to the DMSO control. **F1** is used as the benchmark. The data were based on three biological experiments with technical triplicates.

fractions and clicked to rhodamine azide under copper catalysis to append a fluorescent dye for target visualization.<sup>20</sup> SDS-PAGE analysis of the labeled proteomes and subsequent fluorescence scanning revealed a concentration dependent labeling of distinct proteins (Fig. S3, ESI<sup>†</sup>). A concentration of 10  $\mu$ M **LP3** showed the optimal signal intensity and was selected for competition experiments. Here the cells were treated with various excesses of **BL3** (no alkyne tag) and subsequently incubated with the **LP3** probe. Interestingly, several protein bands in soluble and insoluble fractions vanished suggesting specific binding of both molecules into the same active sites (Fig. S3, ESI<sup>†</sup>).

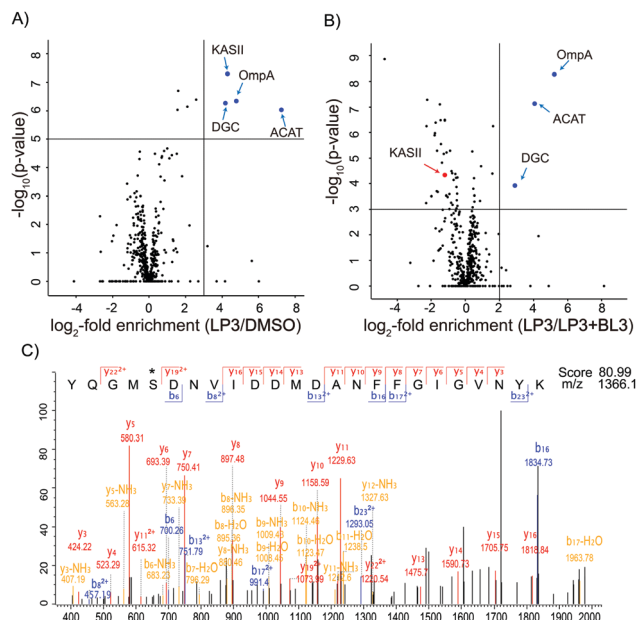
As fluorescence labeling does not directly reveal protein identities, we utilized a quantitative proteomic labeling strategy *via* direct mass-spectrometric target identification. In brief, intact cells were treated with the probe, lysed, clicked to biotin azide and labeled proteins were enriched on avidin beads (Fig. 2).<sup>21</sup> To account for unspecific avidin background binders, an identical experiment was carried out with DMSO instead of the probe in parallel. Avidin bound proteins were released by tryptic digestion and the resulting peptides were appended to different isotopes *via* dimethyl labeling.<sup>22</sup> The samples were pooled and analyzed *via* LC-MS/MS to determine isotope ratios. These were ranked in the corresponding volcano plots according to their enrichment and significance (proteins with an enrichment ratio of 8 and *p*-value < 0.00001 for insoluble samples and a ratio of 4 with *p*-value < 0.003 for soluble proteins were regarded as hits). Interestingly, insoluble and soluble proteome fractions showed distinct target proteins including a 3-oxoacyl-[acyl-carrier-protein] synthase (KASII), the outer membrane protein A (OmpA) and an acetyl-CoA-acetyltransferase (ACAT). Surprisingly, a putative diguanylate cyclase with a GGDEF-domain (DGC) was not detected in the soluble fraction but emerged as the best target from the insoluble samples (Fig. 3A and Fig. S4A, ESI<sup>†</sup>). To confirm that these proteins are specific  $\beta$ -lactone targets, we performed a competition experiment with a 20-fold excess of **BL3** and subsequent probe labeling in parallel (Fig. 2, 3B and Fig. S4B, ESI<sup>†</sup>). Interestingly, with the exception of KASII, all other targets were efficiently outcompeted. A recombinant expression of the remaining three proteins in *Escherichia coli* confirmed their specific interaction with **LP3** upon fluorescence labeling of SDS-gels (Fig. S5, ESI<sup>†</sup>).

A closer inspection of the three proteins revealed that, with the exception of ACAT, the functional roles of DGC and



**Fig. 2** Principle of gel-free activity based protein profiling (ABPP) employing isotope labeling of amino acids. CC denotes click chemistry and DM denotes stable isotope dimethyl labeling.





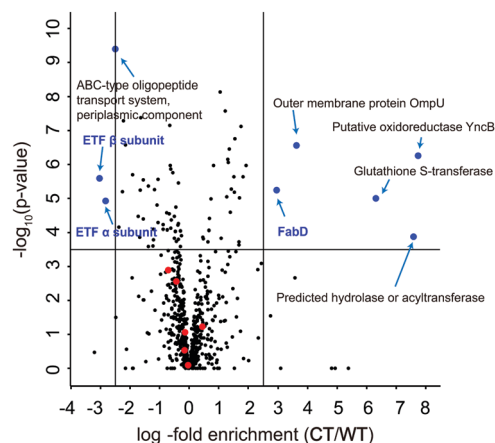


Fig. 4 Volcano plot of whole proteome comparison between 25  $\mu$ M BL3 treated *V. harveyi* ATCC BAA-1116 (CT) and non-treated samples (WT). The blue dots depict selected targets that are significantly up-regulated or down-regulated (criteria:  $\log_2$ -fold enrichment  $\geq 2.5$  or  $\leq -2.5$  and  $-\log_{10}(p\text{-value}) \geq 3.5$ ). Red dots represent proteins expressed from *luxCDABEG* operon. The targets discussed in the text are highlighted in blue. The data were derived from three biological replicates with technical triplicates and  $-\log_{10}(p\text{-value})$  were calculated using a two-sided one sample Student's *t*-test based on Z scores with Perseus.

bioluminescence inhibitor, a detailed mode of action analysis suggested no direct link to QS. Instead the compound likely interfered with the fatty acid metabolism, which is largely attributed to its long aliphatic chain. None of the identified LP3 targets were directly linked to bioluminescence; however, the down-regulation of ETF  $\alpha$  and  $\beta$  subunits pointed towards luciferase activity, which needs to be further evaluated in future studies, *e.g.* by the use of photoprobes to analyze reversible binding target proteins. In conclusion, although AHLs and long chain aliphatic  $\beta$ -lactones share similar structural motifs the identity of the lactone core scaffold (4 vs. 5 membered ring) remains a crucial switch between QS regulation and QS-independent inhibition of bioluminescence.

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