Quorum Sensing Hot Paper

International Edition: DOI: 10.1002/anie.201508052
German Edition: DOI: 10.1002/ange.201508052

Fimbrolide Natural Products Disrupt Bioluminescence of *Vibrio* By Targeting Autoinducer Biosynthesis and Luciferase Activity

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Abstract: *Vibrio is a model organism for the study of quorum sensing (QS) signaling and is used to identify QS-interfering drugs. Naturally occurring fimbrolides are important tool compounds known to affect QS in various organisms; however, their cellular targets have so far remained elusive. Here we identify the irreversible fimbrolide targets in the proteome of living V. harveyi and V. campbellii via quantitative mass spectrometry utilizing customized probes. Among the major hits are two protein targets with essential roles in Vibrio QS and bioluminescence. LuxS, responsible for autoinducer 2 biosynthesis, and LuxE, a subunit of the luciferase complex, were both covalently modified at their active-site cysteines leading to inhibition of activity. The identification of LuxE unifies previous reports suggesting inhibition of bioluminescence downstream of the signaling cascade and thus contributes to a better mechanistic understanding of these QS tool compounds.*

Quorum sensing (QS) is an important bacterial communication strategy that coordinates density-dependent gene expression.[1] QS was recognized for the luminescent Gram-negative marine bacteria *Vibrio fischeri* and *Vibrio harveyi*, which became archetypes for the study of bacterial signaling.[2] *V. harveyi* produces three distinct classes of autoinducer (AI) molecules, the species-specific HAI-1 produced by the protein LuxM, the interspecies molecule AI-2 produced by LuxS, and the *Vibrio* genus specific CAI-1 produced by CqsA.[3] The secreted molecules are sensed by a growing population via the AI-specific membrane receptors LuxN, LuxQ (in interplay with LuxP), and CqsS, respectively. Once a threshold concentration is exceeded, a signaling cascade promoted by LuxU and LuxO regulates the production of LuxR, an important transcriptional activator for the luminescence *luxCDABE* operon (Figure 1 A).[4]

Since *Vibrio* species utilize three diverse signaling channels that can be readily measured via luminescence detection, they are important models for understanding QS.[1b] An important tool for these studies are fimbrolides, natural products obtained from the marine algae *Delisea pulchra* which are potent QS disruptors.[5] These halogenated furanones not only inhibit *Vibrio* QS but are also effective against several other bacteria including clinically relevant pathogens (Figure 1 B).[6] Although fimbrolides and other brominated furanones have been extensively used in several studies, so far comprehensive proteomic analysis of the full complement of bacterial targets is lacking. Instead, individual QS-related proteins were tested for brominated furanone binding and inhibition.[7] Fimbrolides exhibit an exocyclic vinyl bromide that can trap nucleophilic residues. Recombinant LuxS was shown to be covalently modified by compound **F1** at a non-catalytic cysteine residue which led to inhibition of enzymatic activity.[7a] However, studies showed that LuxS cannot be the sole target since *V. harveyi* with only one active QS system or with a constitutive luminescent phenotype was attenuated in bioluminescence upon compound treatment.[4a,7b] Thus a target downstream of the different receptors was postulated that consolidates all signaling channels and collectively turns on luminescence. One such target is the master transcriptional regulator LuxR (Figure 1 A).[1b,7c] However, the results are controversial. While some studies indicate halogenated furanone binding and competitive displacement of natural QS ligands,[7c,8] other studies do not observe specific binding and rather suggest for example, degradation of LuxR upon compound addition.[7b,9] Since fimbrolides have been applied as gold standards in the elucidation of these pathways, a full understanding of their mode of action and cellular targets is required and will have implications for related systems.[1b]

Here we identify the cellular binding partners of fimbrolides in *V. harveyi* NBRC 15634 and *V. campbellii* ATCC BAA-1116 (formerly assigned as *V. harveyi* ATCC BAA-1116).[10] Using activity-based protein profiling (ABPP)[11] with quantitative mass spectrometry we indeed confirmed LuxS as a target. However, several additional previously unrecognized proteins were identified as specific binders.

Two natural brominated furanones commonly used in the elucidation of QS pathways, fimbrolide **F1** and fimbrolide **F2**, were selected for probe synthesis. The natural products were prepared according to published procedures.[12] For in situ target discovery, the scaffolds were additionally equipped with an alkyne moiety at the butyl side chain by means of two synthetic strategies (Scheme 1 and Scheme S1 in the Supporting Information). The alkyne moiety enables bioorthogonal ligation via click chemistry to functionalized azide tags carrying a fluorescent label or biotin for affinity enrichment. The common bromide-substituted precursors for the two reactions, **3** and **4**, were prepared via ring-opening of 3-(2-oxopropyl)tetrahydro-2*H*-pyran-2-one (**2**) with HBr to 5-bromo-2-(2-oxopropyl)pentanoic acid, subsequent bromina-

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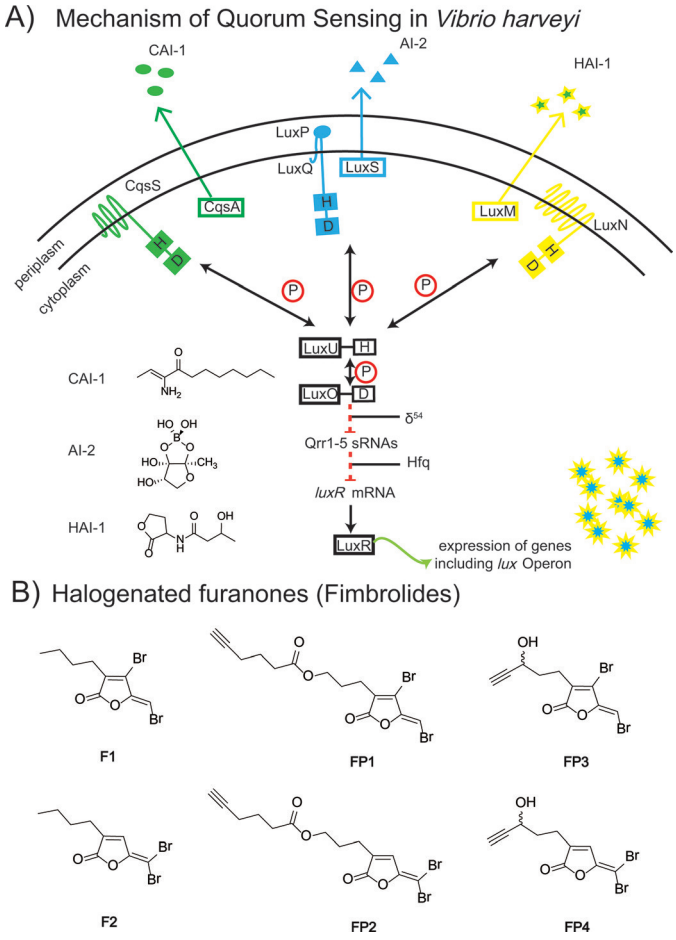


Figure 1. A) Three QS autoinducers are synthesized by LuxM (HAI-1), LuxS (AI-2), and CqsA (CAI-1) and sensed by LuxN (HAI-1), LuxP/LuxQ (AI-2), and CqsS (CAI-1) receptors. At low concentrations of autoinducers, the receptors autophosphorylate and transfer their phosphoryl groups to LuxU which in turn phosphorylates LuxO. LuxO~P activates the expression of Qrr1-5 sRNA which, together with the chaperone Hfq, destabilizes *luxR* mRNA. At high concentrations of autoinducers, the autophosphorylation of the receptors is inhibited and the phosphate is drained from the cascade, resulting in the expression of transcriptional regulator LuxR and thereby activating the *lux* operon and bioluminescence. P in red circle denotes phosphotransfer; H (histidine) and D (aspartate) denote the phosphorylation site. B) Structures of halogenated furanones (fimbrolides) used in this study.

tion, and cyclization. Nucleophilic substitution of the aliphatic side chain bromide with 5-hexynoic acid led to the ester probes **FP1** and **FP2** (Scheme S1). In addition, conversion of the bromo substituent to an acetoxy moiety with KOAc or AgOAc followed by hydrolysis to a hydroxyl group facilitated the oxidation to aldehydes **7** and **8** via Dess–Martin reagent. Compounds **7** and **8** were subsequently reacted with ethynylmagnesium bromide to yield probes **FP3** and **FP4** (Scheme 1).

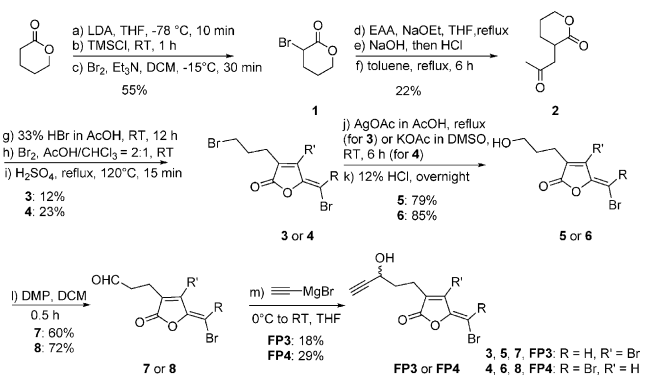
To focus the studies solely on *Vibrio* QS inhibition and not reduction of growth, all molecules were tested for antibacterial activity. In line with previous reports the natural products **F1** and **F2** and also probes **FP1**–**FP3** hardly affected growth in *V. harveyi* NBRC 16534 and *V. campbellii* ATCC BAA-1116 (up to 200 μM tested; Table S1).[8] Only **FP4** had any effect, with an MIC of about 100 μM, and this compound was therefore excluded from further analysis. We tested all the compounds for their effect on bioluminescence in both *Vibrio* strains. **F1** and the corresponding probe molecule **FP3** showed the best IC_{50} values, ranging from 4 to 13 μM, which is in line with previous reports for the natural product (Figure S1).[12] Reduced activity was observed for **F2** and the corresponding ester probe **FP2**. We thus focused all subsequent proteomic target identification studies on the natural product **F1** and its corresponding probe **FP3**.

Incubation of intact *V. harveyi* NBRC 16534 and *V. campbellii* ATCC BAA-1116 with **FP3**, followed by cell lysis, click ligation to rhodamine azide,[13] analysis via SDS-PAGE, and fluorescent scanning revealed several distinct bands in the soluble fraction and less pronounced labeling in the insoluble fraction (Figure 2A, Figure S2). We therefore focused on the soluble proteome and identified a concentration of 50 μM as sufficient for distinct labeling. Fluorescence intensity decreased upon pre-incubation with a 10-fold excess of **F1**, suggesting that these proteins are targets of the natural product.

To obtain a comprehensive overview of all irreversible **FP3** targets shared with the natural product **F1**, three different samples were prepared for gel-free MS analysis: 1) enrichment of **FP3** over DMSO, 2) a competition experiment with a 20-fold excess of **F1** prior to **FP3** addition, 3) a DMSO blank. Enriched proteins of these samples were labeled with light, medium, and heavy isotopes via dimethyl labeling after tryptic digest.[14] Statistical analysis of probe-enriched proteins (compared to DMSO) in *V. harveyi* NBRC 16534 revealed several hits (volcano plot, Figure S3A). Among those hits were LuxS, PhaB, and a cystathionine-β-synthase (CBS) domain protein (inosine monophosphate dehydrogenase related protein(IMPD)). The analysis of the competition study confirmed these hits as binders of the unmodified natural product **F1** (Figure 2B).

We subsequently compared these results with *V. campbellii* ATCC BAA-1116, a strain that exhibits an intrinsically stronger bioluminescence. Here, the comparison of enrichment and competition revealed, among a few other hits including IMPD and LuxS, the fatty acid synthetase LuxE as an additional target protein (Figure 2C and S3B).[15] Interestingly, LuxE was not detected at all in *V. harveyi* NBRC 16534 enrichment experiments (the sequence identity of LuxE in the two strains is 98%), suggesting an expression level below the detection limit. Indeed, a full proteome analysis of both strains showed a 10-fold higher LuxCDABE expression in *V. campbellii* ATCC BAA-1116 compared to that in *V. harveyi* NBRC 16534 which also likely explains the observed differences in their bioluminescence activities (Figure S4). In contrast, the expression levels of IMPD and LuxS remained almost constant. Overall we did not obtain any significant enrichment or competition for the previously discussed target LuxR, although it is expressed to the same extent in both strains, indicating that it is not an irreversible binder of fimbrolides.

The identified targets were grouped in three categories based on available functional data. Category 1 comprises proteins with unknown function such as the 15.6 kDa CBS



Scheme 1. a) LDA, THF, −78 °C, 10 min; b) TMSCl, RT, 1 h; c) Br_2, Et_3N, DCM, −15 °C, 30 min, 55% over 3 steps; d) EAA, NaOEt, THF, reflux; e) aqueous NaOH, overnight, then HCl; f) toluene, reflux, 6 h, 22% over 3 steps; g) 33% HBr in AcOH, RT, 12 h; h) Br_2, AcOH/$CHCl_3$ = 2:1, RT; i) H_2SO_4, reflux, 120 °C, 15 min, 12% for **3** and 23% for **4** over 3 steps; j) AgOAc in AcOH, reflux, 6 h (for **3**) or KOAc in DMSO, RT, 6 h (for **4**); k) 12% HCl, overnight, 79% for **5** and 85% for **6** over 2 steps; l) DMP, DCM, 0.5 hour, 60% for **7** and 72% for **8**; m) ethynylmagnesium bromide in THF, 0 °C to RT, 18% for **FP3** and 29% for **FP4**. LDA = lithium diisopropylamide, THF = tetrahydrofuran, TMSCl = trimethylsilyl chloride, EAA = ethyl acetoacetate, DMSO = dimethyl sulfoxide, DMP = Dess–Martin periodinane, DCM = dichloromethane.

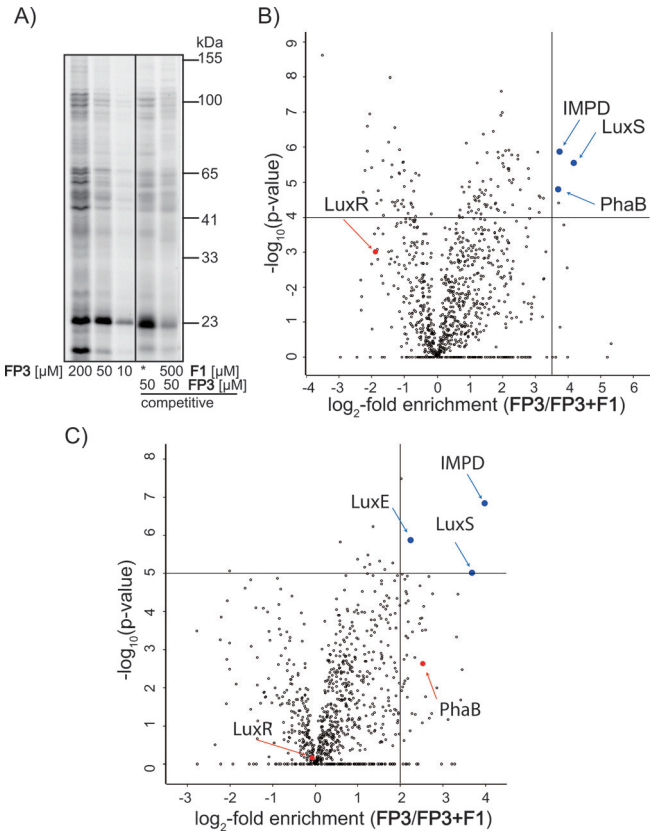


Figure 2. A) Fluorescent SDS-gel of in situ labeled *V. harveyi* with **FP3** (various concentrations) and competitive in situ labeling of 50 µM **FP3** versus a 10-fold excess of **F1** (soluble fraction). *denotes addition of DMSO. B) Volcano plot of gel-free competitive ABPP experiment in *V. harveyi* treated with 50 µM **FP3** vs. a 20-fold excess of **F1** (**FP3** + **F1**). Blue dots depict selected targets that are competed by **F1** (criteria: $\log_2$-fold enrichment ≥ 3.5 and $-\log_{10}(p\text{-value}) \geq 4$) and enriched by **FP3** vs. DMSO (see Figure S3A). C) Volcano plot of gel-free competitive ABPP experiment in *V. campbellii* treated with 50 µM **FP3** vs. a 20-fold excess of **F1** (**FP3** + **F1**). Blue dots depict selected targets competed by **F1** (criteria: $\log_2$-fold enrichment ≥ 2 and $-\log_{10}(p\text{-value}) \geq 5$) and enriched by **FP3** vs. DMSO (see Figure S3B). Results in (B) and (C) are derived from three biological replicates with technical duplicates and $-\log_{10}(p\text{-value})$ were calculated using two sided one sample Student's t-test. Proteins discussed in the text are shown in red. A full list of targets above the cut-off criteria can be found in Table S2.

domain protein IMPD. Proteins with putative roles in QS such as PhaB, an enzyme involved in polyhydroxybutyrate (PHB) biosynthesis, were classified as category 2, while category 3 proteins such as LuxS and LuxE exhibit a confirmed role in QS/bioluminescence pathways. All target proteins were recombinantly expressed in *E. coli* and all bound to **FP3** in this context, as demonstrated via fluorescent SDS-PAGE (Figure 3A). As the major focus of this study is the fimbrolide mode of action in QS and bioluminescence-related pathways, we concentrated all downstream target validation on category 2 and 3 proteins.

PhaB catalyzes the reduction of acetoacetyl-CoA to hydroxybutyryl-CoA, a reaction inhibited by **F1** (Figure S5). Interestingly, a link between QS and PhaB was established previously due to its putative role as an important energy repository for bioluminescence.[16] To clarify its function in this pathway, we generated a *phaB* deletion strain; however, the growth and intensity of bioluminescence remained unchanged (Figure S5B,C). Therefore, category 2 protein PhaB can be excluded as a QS/bioluminescence-associated fimbrolide target.

Validation of category 3 proteins started with LuxS, an enzyme with a confirmed role in QS via AI-2 biosynthesis. In line with a previous report,[7a] fimbrolide **F1** inhibited LuxS activity (Figure S5D). However, in contrast to this report, we determined catalytic Cys83 residue not Cys128 as the site of ligand attachment by MS/MS sequencing (Figure 3B and Figure S6).[17]

Although essential for QS, LuxS inhibition by fimbrolides cannot account for the observed reduction of bioluminescence in strains with a *luxS* or *luxO* deletion (Figure S1).[7b] Therefore, proteins downstream of LuxO in the luminescence pathway such as LuxR and the LuxCDABE luciferase complex are candidate targets. Interestingly, LuxE, a major hit of our MS experiments is part of this downstream luciferase complex. LuxE activates myristoic acid as fatty-acyl-AMP intermediate that is subsequently transferred to LuxC for further processing. We determined Cys362 as the fimbrolide binding site via MS/MS sequencing (Figure S7). This cysteine is essential for the catalytic mechanism suggesting that, as in case of LuxS, the fimbrolide not only binds but also inhibits activity.[15] We next set out to validate this hypothesis. However, since proteins in the LuxCDABE complex directly cooperate, it is difficult to study the function of isolated enzymes. We therefore transformed *E. coli* cells with a plasmid encoding *luxCDABE* from *Photorhabdus luminescens* (LuxE sequence identity 62%). The luminescence system from this strain is advantageous as it does not

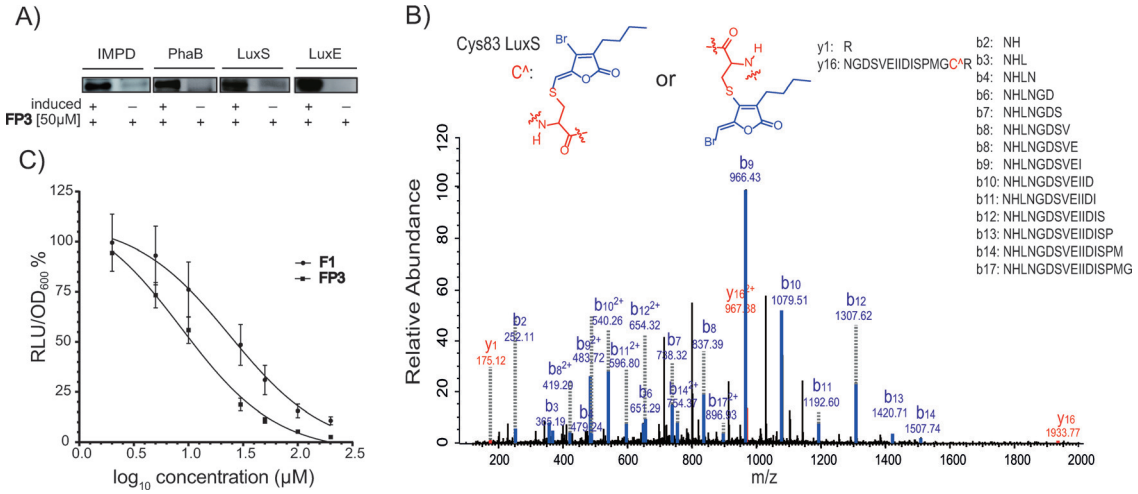


Figure 3. A) Analytical in situ labeling of recombinant IMPD, PhaB, LuxS, LuxE expressed in *E. coli* with 50 μM **FP3**; "induced" denotes induction of protein overexpression. B) MS/MS sequencing shows the binding of **F1** to Cys83 of LuxS. C) Bioluminescence of luminescent *E. coli* DH5α pBluelux containing *luxCDABE* operon under the control of *lacZ* promoter was inhibited by **F1** and **FP3**. RLU (relative luminescence units) were normalized to cell density (OD_{600}) and then to DMSO control. The data is derived from three biological experiments with technical triplicates and error bars indicate standard deviation from the means.

require a QS signaling cascade in order to produce light.[18] Recombinant *P. luminescens* LuxE was labeled by the probe (Figure S8). Moreover, addition of **F1** and **FP3** dose-dependently reduced bioluminescence with an IC_{50} of 24 μM and 9 μM respectively, supporting that LuxE of the luciferase complex is an additional target of fimbrolides (Figure 3C).

In conclusion, our study confirms LuxS, LuxE, PhaB, and the uncharacterized IMPD protein as fimbrolide targets. Catalytic cysteines of LuxS and LuxE were identified as binding sites. The lack of binding to LuxR in our proteomic experiments suggests that this transcriptional regulator is at least not an irreversible target of fimbrolides. LuxR came into focus as putative fimbrolide target to consolidate results that showed an inhibition of bioluminescence downstream of LuxO; however, the binding to LuxE as reported here provides an alternative explanation. Overall, it is intriguing to note that one natural product targets different proteins of a single pathway at divergent steps to achieve one phenotype.

Acknowledgements

This work was funded by the Deutsche Forschungsgemeinschaft FOR1406 and the ERC starting grant (250924-antibacterials) (to S.A.S), and Exc114/2 and Ju270/13-1 (to K.J.). W.Z. acknowledges support from the China Scholarship Council. We thank Prof. Tom Coenye (Ghent University) for the generous donation of luminescent *Escherichia coli* DH5α with a pBluelux plasmid, encoding *luxCDABE*, Priv.-Doz. Dr. Ralf Heermann for providing *Photorhabdus* strains, Elena Kunold for help with MS analysis, and Vadim Korotkov and Megan Wright for critical reading of the manuscript.

Keywords: bioluminescence · fimbrolides · proteomics · quorum sensing

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 1187–1191
Angew. Chem. **2016**, *128*, 1203–1207

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Received: August 27, 2015
Published online: November 26, 2015

