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Quorum sensing inhibitors from *Leucetta chagosensis* Dendy, 1863

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Abstract :

Sponges are a rich source for investigation of bioactive small molecules. They have been mostly investigated for the search of new pharmacological models or therapeutic agents for the treatment of human diseases. Micro-organisms can also represent a virulent pathogen for marine invertebrates such as sponges, which need to protect themselves against these microbes. Sponges' self defence mechanisms involving dialogue molecules thus represent a pertinent research track for potent anti-infective and anti-biofilm activities such as quorum sensing inhibitors (QSIs). The investigation of the QSI crude extract of *Leucetta chagosensis* Dendy, 1863 led to the isolation of three new alkaloids, isonaamine D, di-isonaamidine A and leucettamine D, along with the known isonaamine A and isonaamidine A. Isonaamidine A and isonaamine D were identified as inhibitors of the three quorum sensing pathways of *Vibrio harveyi* (CAI-1, AI-2 and *harveyi* auto inducer), but isonaamidine A displayed the strongest activity on AI-2 biosensor. Both compounds are new examples of natural QSIs of *V. harveyi*. These results outline the importance of these secondary metabolites for their producing organisms themselves in their natural environment, as well as the potential of the marine resource for aquaculture needs.

Significance and Impact of the Study

A new type of quorum sensing inhibitors was isolated from the sponge *Leucetta chagosensis*. One of them inhibits strongly the AI-2 channel of *Vibrio harveyi*, a marine pathogen of special importance in aquaculture. The activity of five different related compounds, including three new natural products discovered there, was investigated leading to structure-activity relationships which are useful for the design of new quorum sensing inhibitors to control marine infectious pathogens.

Keywords : biofouling, *Leucetta chagosensis*, leucettamine, naamidine, naamine, quorum sensing, quorum sensing inhibitors, sponges, *Vibrio harveyi*

Introduction

The survey of French Polynesia sponges was undertaken with the dual aim of filling the gap of knowledge in this group (Van Soest *et al.* 2012; Hall *et al.* 2013), but also to search for new natural resources as bioactive natural products for aquaculture, a field of special interest for this Pacific island country. Facing the depletion of edible fish stock (over-fishing, ciguatera and cyanobacteria poisoning spreading), marine aquaculture is a promising alternative source of proteins. It experiences an intensification of production systems at a rate of about 6% (Bostock *et al.* 2010; FAO 2012). New species of aquaculture interest are also developed. In French Polynesia, aquaculture development is focusing on pearl oysters (*Pinctada margaritifera*), blue shrimps (*Litopenaeus stylirostris*), and also orbicular batfish (*Platax orbicularis*) farming.

Vibrio species are very common in all marine environments from the ocean to estuaries, as well as in coastal sediments. They occur either as planktonic bacteria or as biofilms, and may be also associated with host organisms. *V. harveyi* has been identified as a serious pathogen in French Polynesia. This bacterium is known to induce severe economic losses in many vertebrate and invertebrate aquacultured species (Austin *et al.* 2006; Schikorski *et al.* 2013). It is responsible for the septicaemia of the cultured orbicular batfish in Tahiti. It may also induce mortality of the pearl oyster by experimental infection and has been found associated to pearl rejection rate and pearl biomineralization abnormalities (Cochennec *et al.* 2006; Ogimura *et al.* 2012). The different *Vibrio spp*. share some quorum sensing (QS) pathways (Tang and Zhang 2014), which control different phenotypic expressions such as bioluminescence (Bassler 1993), extracellular products such as metalloprotease, cystein protease, haemolysin and phospholipase (Liu and Lee 1999; Soto-Rodriguez *et al.* 1999; Mok *et al.* 2003); type III secretion (Henke and Bassler 2004); siderophore (Lilley and Bassler 2000) and biofilm formation (Natrah *et al.* 2011).

We chose to use the luminescence of *V. harveyi* as a test model for our work. Luminescence is controlled by three pathways of QS activation in this species: each of them is controlled by an auto-inducer (Figure 1): *harveyi* auto inducer-1 (HAI-1), *cholerae* auto inducer 1 (CAI-1) and auto inducer 2 (AI-2). The receptors of these autoinducers are respectively produced by LuxN for the HAI-1, *cholerae* quorum sensing sensor kinase/phosphatase (CqsS) for the CAI-1, and LuxP (which activates the histidine kinase receptor LuxQ) for the AI-2. These three pathways activate by a common signal transduction cascade the key protein LuxU and further steps to the final activation of LuxR. The gene *luxR* encodes the master transcription factor of quorum sensing-controlled behaviors such as the expression of the luciferase operon leading to the expression of luminescence.

Figure 1.

Small molecules are a good source of QSIs but still poorly investigated, especially in the marine environment (Dobretsov *et al.* 2011; Villa *et al.* 2013). The major goal of our research is to discover new natural QSIs with potential aquaculture applications, but also to understand the possible role and mode of action of the secondary metabolites in their host sponges such as chemical defence against pathogenic bacteria or the control of biofouling (Dobretsov *et al.* 2009). Natural bioactive compounds also display a special importance for further development of bio-inspired QSI (Müller *et al.* 2013), or marine aquaculture drugs. We describe here the results of our investigation of the bioactive extract of *Leucetta chagosensis* selected here as a promising source of QSI.

Results and Discussion

General screening

A set of 53 sponges were collected in Tuamotu and 11 in Leeward Islands. Organic extracts of these organisms were screened for the inhibition of luminescence of *V. harveyi* on two bioassays, the first one using disk diffusion on agar plates, and the second one kinetic measurement of bacterial growth and luminescence on 96 wells plates. The *V. harveyi* strain used here has its all three active QS pathways: if one pathway is inhibited by a compound, the two other pathways relay for complete luminescence recovery. The luminescence inhibition is thus only measurable during a short time (dark room, results read on a digital slide) on agar media. In liquid media, the kinetic of luminescence is belated. Forty-four extracts upon 64 were detected as QSIs on the disk diffusion bioassay on agar plates and displayed also activity in liquid media. The liquid media bioassay was shown to be more sensitive with more responses either as antibiotic (7), luminescence inhibition (11) or both (12). Some extracts clearly inhibited the bacterial growth but not luminescence. It was not possible to quantitatively correlate antibacterial activity with quantitative luminescence inhibition. The large number of positive responses led us to discriminate luminescence inhibitor extracts, either antibiotic or not, on *V. harveyi* BB120 wild strain and its three derived mutants kindly provided by Dr Bassler. Each of them only express one upon three pathways controlling luminescence expression via three auto-inducers: JAF 375 (CAI-1 activated), JMH 597 (AI-2 activated), JMH 612 (HAI-1 activated) (Bassler *et al.* 1997; Henke and Bassler, 2004).

Chemical investigation of the sponge *Leucetta chagosensis*

The organic extracts of *Leucetta chagosensis* displayed QS inhibition on a dose depending on wild strain BB120 and the mutant JMH 597, and was thus chosen for further studies. This sponge is widespread in French Polynesia, and was proven to be a rich source of new imidazole alkaloids (Hassan *et al*. 2004) that may present new QSI structures. Extracts of different batches of *Leucetta chagosensis* collected in Leewards and Tuamotu Islands (animal material in supplementary information) were submitted to high performance liquid chromatography (HPLC) analysis in order to compare their chemical signature. The HPLC profiles, obtained using diode array detector, evaporative light scattering detector and mass spectrometry detection, showed small differences between both groups of islands. The chemical profile of Society Islands samples containing a mixture of isonaamine A **1** (Molina *et al.* 1999) and isonaamidine A **2** (Nakamura *et al.* 2003), also linked to a stronger QS inhibition on our bioassays, were chosen for further investigation (Figure 2).

Figure 2.

Three new products isonaamine D **3**, di-isonaamidine A zinc complex **4**, and leucettamine D **5** were also isolated by reverse phase HPLC and characterized. Compound **3** was obtained as a red amorphous solid. The high resolution electrospray mass ionization spectra (HRESIMS) analysis indicated a molecular peak at m/z 310.1563 $[M+H]^+$ ($C_{18}H_{20}N_3O_2$, calculated: 310.1556). The proton nuclear magnetic resonance (^{1}H NMR) spectrum showed its similarity to isonaamine A **1**, but with an additional methoxyl group signal at δ_H 3.79 correlating to carbon C-11 (δ_C 159.7). Further two

dimensional (2-D) NMR analysis including HMBC analysis allowed the positioning of this methoxyl on C-11 (Scheme Fig 2). Structure **3** was thus assigned to isonaamine D.

Compound **4** was isolated as a brown amorphous solid. The HRESIMS showed a characteristic ion cluster at m/z 873.2109 $[M+H]^+$ indicating the presence of one zinc atom in the molecule. The ^1H NMR spectrum showed two diastereotopic systems for the two benzylic protons (H-7, 5.29/5.42 and H-14, 3.21/3.51) confirmed the complexation of two molecules according to the literature [27]. The HRESIMS showed a fragment ion peak at m/z 406.1500 ($C_{21}H_{20}N_5O_4$; calculated: 406.1515) suggesting the presence of isonaamidine A. According to the COSY and HMBC correlations (Figure 2), the structure of compound **4** appeared to be a Zn^{2+} complex of two molecules of isonaamidine A. This was confirmed by the dimerization of the natural isonaamidine A isolated here, in the presence of $ZnCl_2$: the ^1H NMR spectrum of the natural product **4** was identical to the semi-synthetic product.

The HRESIMS analysis of leucettamine D **5** exhibited a peak at 310.1190 $[M+H]^+$ ($C_{17}H_{16}N_3O_3$, calculated 310.1192). The ^1H NMR spectrum contained four doublets consistent with aromatic protons of two phenolic substituents (δ_H 7.82 and 7.13, J = 8,5 Hz; 6.78 and 6.74, J = 8,5 Hz), one olefinic proton at δ_H 6.61 (s) and one benzylic proton at δ_H 4.75 (s). The ^{13}C NMR spectrum indicated the presence of a carbonyl at δ_C 172.1. The molecular skeleton was determined by COSY and HMBC experiments (Figure 2). The position of the carbonyl was established by the correlation of H-7 and H-14 with C-5. The position of the olefinic proton was determined by the correlation of H-14 with C-5 and C-20.

Evaluation of anti-quorum sensing activity of isolated compounds

Compounds **1-5** were tested on the wild *V. harveyi* strain BB120 and the three double mutants (Table 1). None of these five pure molecules affected bacterial growth at concentration tested as high as 50 µg ml^{-1}. Isonaamine A **1** exhibited no effect on QS of all strains, while its *O*-methyl derivative, isonaamine D **3**, displayed a mild but dose-dependent activity on JAF 375 and JMH 612. In contrast, isonaamidine A **2** exhibited a significant inhibition of the AI-2 QS pathway (strain JMH 597), with an $IC_{50\ lum}$ = 1 µg ml^{-1}, but was inactive as a Zn^{2+} dimeric complex. Leucettamine D did not present any significant activity on any strain. Whatever the compounds and bacterial strains tested, a Spearman's rank correlation test indicated no statistical effect of the concentration of tested compounds on bacterial growth, with Pvalue >0.05. Mean and standard deviation of OD_{595} obtained at the five concentrations of isonaamine D and at the end of the incubation time were of 0.094 ± 0.014, 0.074 ± 0.010, 0.122 ± 0.007 and 0.095 ± 0.042 with BB120, JAF 375, JMH 597 and JMH 612 strains respectively. Concerning isonaamidine A, mean and standard deviation of OD_{595} were of 0.123 ± 0.023, 0.072 ± 0.014, 0.128 ± 0.029 and 0.09 ± 0.058 respectively.

Table 1.

These results highlight the importance of a lipophilic group such as methoxyl for the isonaamine D **3** activity, and of the uncomplexed conjugated nitrogen core for isonaamidine A **2**. QSI have a special interest for their low potential to provoke resistance of bacterial strains (Tang and Zhang 2014). AI-2 are inter species communication molecules : *luxS* gene, encoding for the protein catalysing the production of AI-2 is widespread amongst either Gram-positive or Gram-negative bacteria, but AI-2

may not be produced for QS purpose since AI-2 receptors are not as widespread as LuxS (Rezzonico and Duffy 2008). LuxPQ-type AI-2 receptor has not been found yet outside Vibrionales. LuxR, another AI-2 receptor, has been found in *Enterobacteriaceae*, *Rhizobiaceae* and *Bacillaceae* but some other receptors remain to be discovered (Pereira *et al.* 2009). N-acyl-homoserine lactone (AHL) may bear different chains such as HAI-1. They are as CAI-1 intra-species communication molecules mostly found in Gram-negative bacteria (Tang and Zhang 2014). Until now, very few marine natural products have been proven to be QSI, and mainly on AHL dependent QS: two flustramine alkaloids from the bryozoan *Flustra foliacea* (IC_{50} = 63 µmol l^{-1}) (Peters *et al.* 2003), hymenialdisin (MIC >0.2 µmol l^{-1}) (Dobretsov *et al.* 2011), manoalide, its monoacetate salt and secomanoalide derivatives (IC_{50} respectively = 0.658 µmol l^{-1}, 1.123 µmol l^{-1}, 1.11 µmol l^{-1}) (Skindersoe *et al.* 2008). Halogenated furanone derivatives from the natural terpene of the red alga *Delisea pulchrea* were shown to interfere with AHL and also AI-2 mediated QS (Hentzer *et al.* 2002). In regards of the activity described for these pure compounds, isonaamidine A displays here an interesting level of QSI activity at the micromolar range (IC_{50} = 2.5 µmol l^{-1}) on the LuxPQ-type AI-2 receptor. Nevertheless, because bioluminescence was inhibited or blocked in our broth medium liquid assay on all three double mutants tested by isonaamine D and isonaamidine A, these results suggest that both molecules act on common QS signal transduction cascade (of the three channels of the QS system), from LuxO to LuxR, rather than on QS signal biosynthesis or detection pathways. Further investigations are needed to infirm or confirm this hypothesis.

In summary, five compounds, two known molecules (isonaamine A and isonaamidine A) and three new natural products (isonaamine D, leucettamine D and di-isonaamidine A) where isolated here. The QSI activity of isonaamidine A is described here for the first time. These first results encourage us to further investigate the potential of marine organisms as a source of QSI compounds and their application towards marine pathogenic targets. Further investigation of the isonaamidine A activity on metalloprotease and phospholipase production will help to investigate active QS channels controlling different bacterial productions. The abundance of this sponge will allow isolation of enough of the bioactive compound for further *in vivo* experiments on aquacultured organisms. The identification of these natural products as QSIs is also interesting for the design of bioinspired active compounds by synthesis or hemisynthesis in this field of activity of great future. On a chemical ecology point of view, it has been shown that *L. chagosensis*' imidazole alkaloids are not toxic to nudibranchs that feed on them (Carroll *et al.* 1993), neither against microalgae (Gross *et al.* 2002), but the role of isonaamidine A here is probably dedicated to the control of biofilm formation through QS control and thus actively participate to the chemical defence of *Leucetta chagosensis* sponges against fouling.

Materials and methods

Extract library

Technical and HPLC grade solvents were obtained from Fisher. Technical CH_2Cl_2 and MeOH were freshly distilled prior extractions.

Sponges were collected in French Polynesia using SCUBA during the sampling cruise aboard the research vessel (R/V) Alis in August 2009 (Leeward Islands) and May 2011 (Tuamotu). Samples were deep frozen immediately aboard Alis and kept at -20 °C until processed. They were then grounded and

lyophilized. Freeze-dried powder (10 g) of each sample was extracted using ASE Dionex extractor at 45 °C, 100 bar, first with water (34 ml), then with CH_2Cl_2 / MeOH: 1 / 1 (2 x 34 ml). The organic extracts were pooled and dried; salts were precipitated in absolute ethanol (EtOH) and CH_2Cl_2. The supernatant was evaporated to give the final extract saved in a library for screenings and preliminary chemical analysis.

QSI screenings

NaCl, KCl, $MgSO_4$, $CaCl_2$ used for preparing artificial sea water were purchased by SIGMA; Lennox broth by Life technologies; Tryptone, yeast extract and agar-agar by Difco; Bacto™ Peptone and yeast extract for Zobell liquid media bioassays by Becton Dickinson Co.

Agar media screening: 1 mg of each extract was deposited on a cellulose disk (6 mm diameter) and used for agar diffusion bioassay on Zobell agar plates (trypton: 5 g; yeast extract: 1 g; agar : 17 g l^{-1}, sterilized sea water 1 l). A 24 hours old *V. harveyi* colony was suspended in 5 ml of Zobell broth and plated. Disks were deposited (duplicate for each extract) and the dishes incubated at 27 °C for 15 to 18 hours. The diameters of bacterial growth inhibition were measured under daylight, but the luminescence inhibition diameters were measured in a dark room using a digital camera (1600 ASA, exposure: 30 s, aperture: 2.8, d=15 cm). Bioactive extracts, either antibiotic and / or luminescence inhibitors, were tested twice.

Liquid broth media screening: The strains used for this bioassay are *V. harveyi* BB120 wild type and its derived double mutants (JAF 375, JMH 597 and JMH 612) obtained from Bassler's laboratory (Bassler *et al.* 1997; Henke and Bassler, 2004). Their purity was verified by real-time PCR (Schikorski *et al.* 2013). BB120 strain was shown to exhibit strong virulence in brine shrimp *Artemia franciscana* whereas the use of AI-2 and CAI-1-deficient mutants abolished virulence of *V. harveyi* to brine shrimp (Defoirdt and Sorgeloos 2012). Twenty four hours old bacteria colonies were suspended in 5 ml Lennox broth prepared in artificial sea water (2.3% w / vol NaCl, 20 mmol l^{-1} KCl, 5 mmol l^{-1} MgSO4 and 2 mmol l^{-1} $CaCl_2$ in distilled water) and incubated for 16 hours under stirring at 27 °C. The optical absorbance (OD) of each bacterial culture at wavelengh $\lambda = 595$ nm was adjusted at 0.012 (approximately 10^7 bacteria ml^{-1} after numeration on a Malassez cell) by dilution in Zobell media (0.5% w / vol Bacto™ peptone, 0.1% w / vol yeast extract in artificial sea-water). The compounds were dissolved in EtOH and deposited in the 96 wells plates and dried. Different bacterial suspensions (200 µl) were added in the appropriate wells and incubated at 27 °C in a BMG FLUOstar OPTIMA microplate reader (Labtech), where luminescence is measured every 10 min after 1 min double orbital stirring during 5h 50 min (35 cycles). At the end of the incubation time, the absence of bacterial growth inhibition due to the tested compounds was verified by OD_{595} measurements and a Spearman's rank correlation test to determine the correlation between the concentration of tested compounds and OD_{595} measurements.

Every compound was tested in duplicate at 5 concentrations against each strain, for both luminescence production (relative luminescence units) and antibiotic or bacteriostatic activity (OD_{595}). For each strain, luminescence and absorbance values were obtained after subtracting the background signal obtained with media and compounds sterility tests to the raw data.

Extraction and isolation of the compounds from *Leucetta chagosensis*

Analytical HPLC was carried out using a Waters Sunfire (4.6 × 100 mm, 5 µm) column and was performed on a Waters 1695 Alliance system equipped with a photodiode array detector (Waters 996), an evaporative light scattering detector (Waters 2420), and a mass spectrometer (Waters Micromass ZQ 2000). Preparative HPLC (preparative column: Waters Sunfire C18, 19 × 150 mm, 5 µm) and semi-preparative (column: Waters Sunfire C18 column, 10 × 150 mm, 5 µm); HPLC system (Waters 600, Alliance) coupled with UV detector (Waters 2996 photodiode array detector)

Four hundred thirty grams of freeze dried sponges were extracted by maceration with aqueous methanol and then with CH_2Cl_2. The solvent was evaporated under vacuum. The residue was dissolved in water and then partitioned between heptane, ethyl acetate and butyl alcohol. The ethyl acetate fraction (1.3 g) was subjected to reverse-phase silica gel column chromatography and eluted with MeOH / H_2O / formic acid by increasing polarity. Further preparative and semi-preparative HPLC allowed the isolation of five pure compounds : isonaamine A **1** (15 mg; 0. 35% w/w), isonaamidine A **2** (33 mg; 0.77%), isonaamine D **3** (6 mg; 0.12%), a homodimer Zn^{2+} complex of isonaamidine A **4** (5 mg; 0.12%) and leucettamine D **5** (3 mg; 0.07%). Spectral data are given in supplementary material.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure 1. Structure of known auto-inducers of QS pathways in *Vibrio harveyi*: 3-OH-C4-HSL (HAI-1), S-THMF-borate (AI-2) and Ea-C8-CAI-1 (CAI-1).

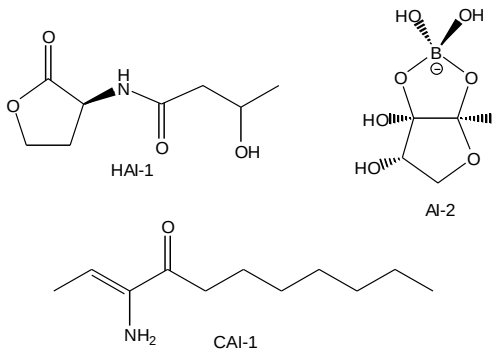


Figure 2. Molecules isolated from *Leucetta chagosensis*; correlation spectroscopy (COSY, bold) and heteronuclear multiple bond correlation (HMBC, arrows) correlations of new compounds **3** and **5.**

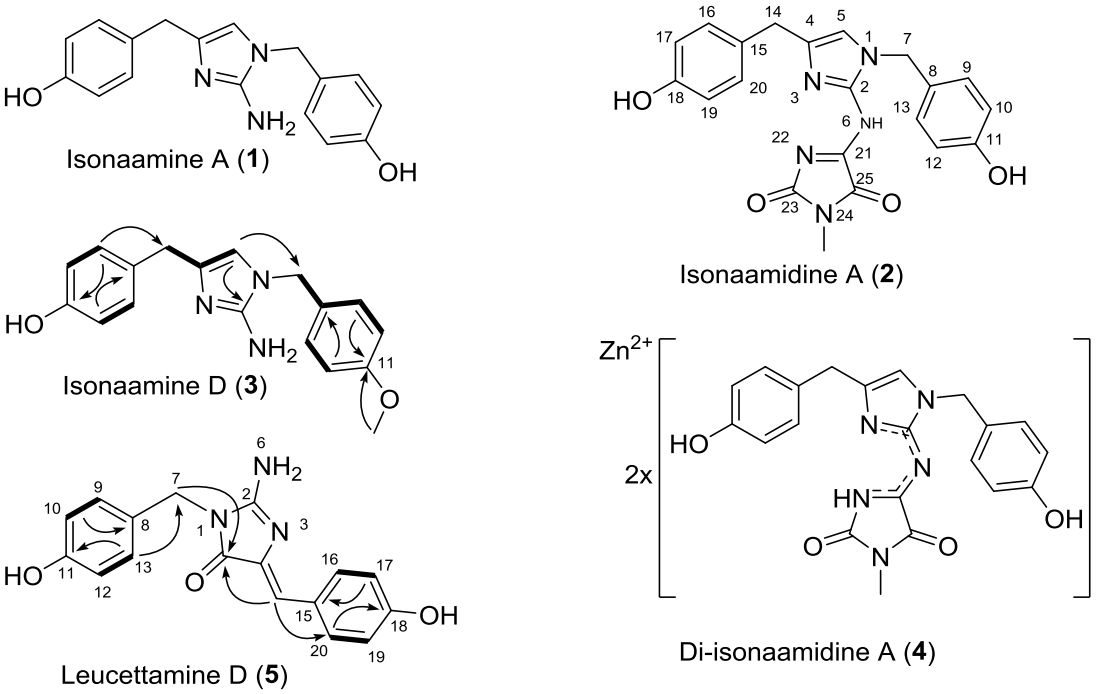
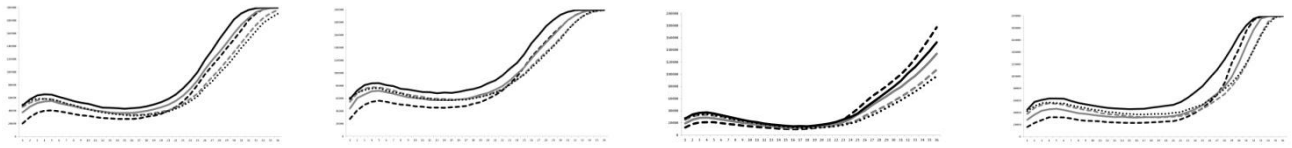
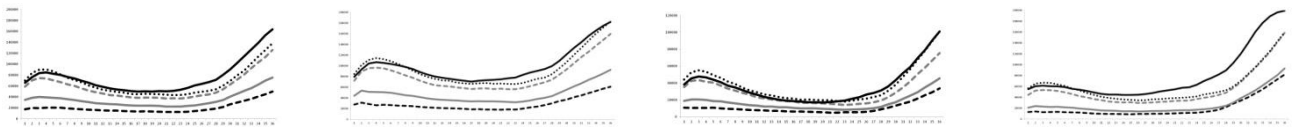
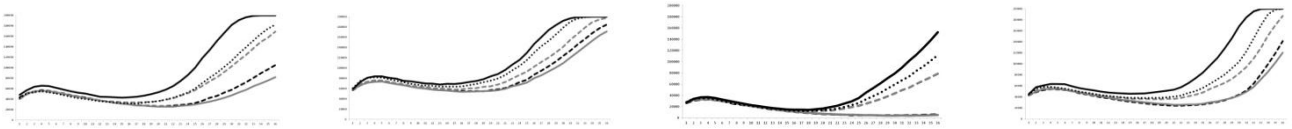
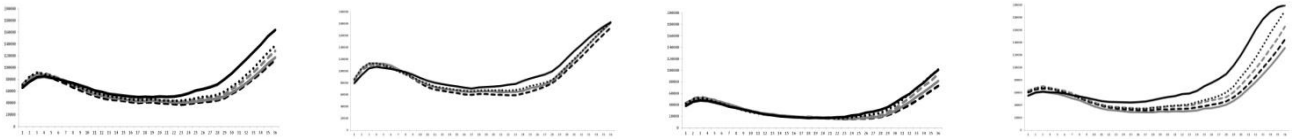
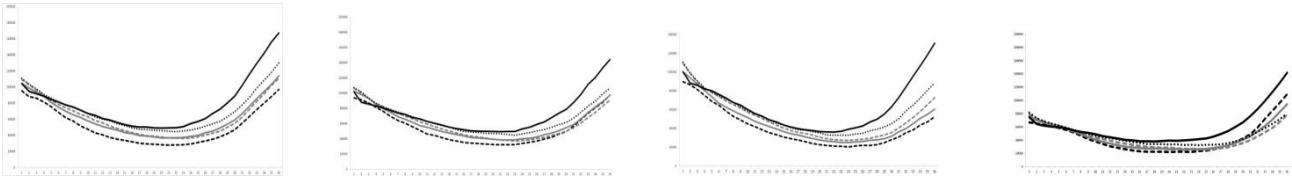


Table 1. Activity curves of purified compounds (Y-axis : relative luminescence units (RLU) ; X-Axis : n cycles) on each strain. Luminescence values were obtained for each strain after subtracting the background signal obtained with media and compounds sterility tests to the raw data.

── : control without product ; - - - : 50 µg ml^{-1} ; ── : 10 µg ml^{-1} ; - - - : 5 µg ml^{-1}; ····· : 1 µg ml^{-1}			
BB120	JAF 375 (CAI-1 +)	JMH 597 (AI-2 +)	JMH 612 (HAI-1 +)
			
Isonaamine A (**1**)			
			
Isonaamine D (**3**)			
			
Isonaamidine A (**2**)			
			
(Di-isonaamidine A) Zn^{2+} (**4**)			
			
Leucettamine D (**5**)			