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Inhibition of marine biofouling by bacterial quorum sensing inhibitors

Sergey Dobretsov[1,*], **Max Teplitski**[2], **Mirko Bayer**[3], **Sarath Gunasekera**[4], **Peter Proksch**[3], and **Valerie J Paul**[4]

[1]Department of Marine Science and Fisheries, College of Agricultural and Marine Sciences, Sultan Qaboos University, Sultanate of Oman

[2]Soil and Water Science Department, University of Florida-IFAS, Gainesville, USA

[3]Institut für Pharmazeutische Biologie und Biotechnologie, Düsseldorf, Germany

[4]Smithsonian Marine Station, Fort Pierce, FL, USA

Abstract

Seventy eight natural products from chemical libraries containing compounds from marine organisms (sponges, algae, fungi, tunicates and cyanobacteria) and terrestrial plants, were screened for the inhibition of bacterial quorum sensing (QS) using a reporter strain *Chromobacterium violaceum* CV017. About half of the natural products did not show any QS inhibition. Twenty four percent of the tested compounds inhibited QS of the reporter without causing toxicity. The QS inhibitory activities of the most potent and abundant compounds were further investigated using the LuxR-based reporter *E. coli* pSB401 and the LasR-based reporter *E. coli* pSB1075. Midpacamide and tenuazonic acid were toxic to the tested reporters. QS-dependent luminescence of the LasR-based reporter, which is normally induced by N-3-oxo-dodecanoyl-L-homoserine lactone, was reduced by demethoxy encecalin and hymenialdisin at concentrations 46.6 μM and 15μM, respectively. Hymenialdisin, demethoxy encecalin, microcolins A and B and kojic acid inhibited responses of the LuxR-based reporter induced by N-3-oxo-hexanoyl-L-homoserine lactone at concentrations 40.2 μM, 2.2 μM, 1.5 μM, 15 μM and 36 μM, respectively. The ability to prevent microfouling by one of the compounds screened in this study (kojic acid; final concentrations 330 μM and 1 mM) was tested in a controlled mesocosm experiment. Kojic acid inhibited formation of microbial communities on glass slides, decreasing the densities of bacteria and diatoms in comparison with the control lacking kojic acid. The study suggests that natural products with QS inhibitory properties can be used for controlling biofouling communities.

Keywords

quorum sensing; inhibitors; antifouling; natural products; biofilms

Introduction

In the marine environment, all natural and artificial substrata are quickly colonized by marine micro- and macro-organisms in a process that is known as “biofouling”. Micro- and macro-foulers can cause severe industrial problems by increasing drag force, promoting metal corrosion and reducing heat transfer efficiency of heat exchangers (Yebra et al. 2006;

[*]Corresponding author: Dr. Sergey Dobretsov, Department of Marine Science and Fisheries, College of Agricultural and Marine Sciences, Sultan Qaboos University, PO Box 34 Al Khoud 123, Sultanate Oman, Tel.: (968) 24143657, Fax: (968) 24413418, sergey@squ.edu.om, sergey_dobretsov@yahoo.com.

Schultz et al. 2011). Biocides that are used to control biofouling are not effective against some bacterial and diatom species (Cassé and Swain 2006; Molino et al. 2009a,b), which colonize entire surfaces coated with antifouling paints and affect their performance. Therefore, development of new ways to regulate densities of microbes in and on antifouling coatings is urgently required.

Interference with bacterial quorum sensing (QS) has been proposed as one potential approach for controlling biofouling (Dobretsov et al. 2009; Choudhary and Schmidt-Dannert 2010; Qian et al. 2010; Xiong and Liu 2010). QS is a population density-dependent gene regulatory mechanism, which relies on the production and perception of threshold concentrations of low molecular weight signal molecules that activate transcriptional regulators (Antunes et al. 2010). In Gram negative bacteria, QS is affected by N-acyl homoserine lactones (AHLs). These QS molecules are typically produced by the AHL synthethases (homologues of the LuxI) and actively or passively redistributed in the environment (Waters and Bassler 2005; Dickschat 2010). When the intracellular concentration of AHLs reaches a certain threshold level, the AHL molecules bind to the LuxR-type receptor, and this leads to the formation of active dimers allowing interactions with QS-dependent promoter sequences (Boyer and Wisniewski-Dye 2009). Once active complexes within promoter sequences are established, transcription of QS genes responsible for luminescence, biofilm formation, virulence and other relevant processes is effected (Waters and Bassler 2005; Irie and Parsek 2008).

Because bacterial QS is central to the interactions of bacteria with their eukaryotic hosts, it is not surprising that many multicellular organisms evolved different mechanisms to interfere with bacterial QS (reviewed by Dobretsov et al. 2009; Goecke et al. 2010). One of the well-studied examples of organisms producing QS inhibitors is the red alga *Delisea pulchra* (Manefield et al. 1999), which produces a suite of halogenated furanones that reduce bacterial adhesion to algal surfaces and inhibit bacterial swarming (Maximilien et al. 1998). Several recent studies demonstrated that extracts of Great Barrier Reef marine invertebrates (Skindersoe et al. 2008b) and cyanobacteria from Florida waters (Dobretsov et al. 2010) are similarly capable of inhibiting bacterial QS.

It has been proposed that QS inhibitors can be used for antimicrobial protection in aquaculture (Defoirdt et al. 2004; Dobretsov et al. 2009). In the laboratory, it has been shown that synthetic furanones inhibited development of microbial biofilms (Dobretsov et al. 2007). In short field and laboratory experiments, furanones produced by *D. pulchra* strongly inhibited attachment of marine bacteria on rocks and seaweeds (Maximilien et al. 1998).

In this study, we screened 78 natural products from marine invertebrates (mostly sponges), terrestrial plants, fungi and cyanobacteria for the inhibition of bacterial QS reporters. The activities of the most potent and abundant QS inhibitors, such as demethoxy encecalin, midpacamide, tenuazonic acid, hymenialdisin, microcolins A and B and kojic acid, were further investigated using different reporter strains. The AF performance of kojic acid was tested in a mesocosm experiment. The main aims of the study were to investigate: 1) the effects of natural products on QS pigment production in *Chromobacterium violaceum* CV017; 2) the activity of demethoxy encecalin, midpacamide, tenuazonic acid, hymenialdisin, microcolins A and B and kojic acid using the LuxR-based and the LasR-based reporters; 3) AF performance of kojic acid in a mesocosm experiment.

Material and methods

Compounds used in this study

All natural products analyzed in this study were previously isolated by the research groups of P. Proksch and V. Paul (Table 1). These compounds had been isolated from sponges, tunicates, fungi, plants and cyanobacteria and represent major groups of natural products. All isolated compounds were fully characterized structurally by mass spectrometry as well as by one and two dimensional NMR spectroscopy (^{1}H, ^{13}C, COSY, HMBC). All compounds were dissolved in methanol (Fisher Scientific, USA) yielding a stock solution (0.2 mg ml^{-1}).

QS inhibition bioassays

A reporter strain *Chromobacterium violaceum* CV017 was used for screening for QS inhibitors. This biosensor strain produces N-hexanoyl homoserine lactone, which induces production of the purple pigment violacein via the AHL receptor CviR (Chernin et al. 1998). Methanol solutions of the compounds were added into wells of microtiter plates (Nunc, Denmark), solvents were evaporated and extracts were re-dissolved in 3μl of dimethyl sulfoxide (DMSO). DMSO in empty cells was used as a control. Experiments were conducted according to Dobretsov et al. (2010). Briefly, bacterial cells from overnight culture of CV017 were centrifuged and washed with sterile distilled water. Five ml of soft LB agar (Difco) were mixed with 500μl of washed culture of CV017, and 100μl of this mixture were applied to each well. The plates were incubated overnight at 30 °C. A reduction in violacein production was compared to the control treatments visually. The bioassays were repeated three times and the mean minimum inhibitory concentration (MIC) in μM was calculated.

A toxicity assay was performed according to (Dobretsov et al. 2010) in order to test the effect of compounds on the growth of the reporter strain *C. violaceum* CV017. Briefly, solutions of compounds in DMSO were applied onto glass fiber disks (diameter=1 cm) and these disks were placed onto LB agar (Difco, USA) inoculated with *C. violaceum* CV017. Growth inhibition around the disk corresponds to antibacterial activity of the compound against the reporter strains. This experiment was repeated 3 times. These results were expressed as a mean minimal amount of compound in moles that inhibit growth of the reporter. DMSO was used as a control.

In order to further investigate QS inhibitory properties, the most active QS inhibitors were selected. Since some of these inhibitors were isolated in low quantity, only demethoxy encecalin, tenuazonic acid, midpacamide, hymenialdisin and microcolins A and B (Fig. 1, Table 1) were used in this study. Additionally, we tested QS inhibitory properties of kojic acid – a compound that was used for the mesocosm experiment (see below). Several bioassays were performed using *E. coli*-based reporters. Before the bioassays all compounds were re-dissolved in DMSO. Midpacamide was tested at 0.4 – 46 μM, tenuazonic acid was assayed at 0.1– 102 μM, demethoxy encecalin - at 0.04– 6.6μM, hymenialdisin - at 0.007–15μM, microcolins A and B - at 0.015–150 μM and kojic acid - at 0.4–330 μM. Possible toxic effects of compounds on metabolic activity or luminescence of the reporters were tested using a control construct containing a pTIM2442 plasmid in *E. coli* DH5α (Alagely et al. 2011), in which the *lux* cassette is controlled by a constitutive phage lambda promoter. Compounds were tested at the maximal inhibitory concentrations. Midpacamide was tested at 46 μM, tenuazonic acid was tested at 102 μM, demethoxy encecalin was tested at 6.6 μM, hymenialdisin was tested at 15 μM, microcolins A and B - at 150 μM and kojic acid - at 330 μM. This experiment was performed with 8 replicates. For the LasR-based bioassay (*Pseudomonas aeruginosa* LasR/ LasI QS system), we used the LasR-based reporter

(pSB1075) (Winson et al. 1998). It emits light in response to AHLs with long (>C10) acyl side chains. We performed direct and indirect bioassays with non-toxic compounds according to Alagely et al. (2011). In the direct bioassays, the reporter *E. coli* pSB1075 was exposed to the compounds dissolved in DMSO; in indirect bioassays, N-3-oxo-dodecanoyl-L-homeserine lactone (3-oxo-C12-HSL) (final concentration of 2μM) was also added in order to stimulate QS for this reporter. This experiment was performed with 8 replicates. Additionally, we tested the effect of non-toxic compounds on QS in a LuxR-based reporter *E. coli* pSB401 (Winson et al. 1998) that contained the *LuxR* P_{luxI}-*luxCDABE* transcriptional fusion which emits light in response to AHLs with medium (C6–10) acyl side chains. In the direct bioassays, the reporter *E. coli* pSB401 was exposed to the compounds of interest dissolved in DMSO. In indirect bioassay, N-3-oxo-hexanoyl-L-homeserine lactone (3-oxo-C6-HSL) (final concentration of 10 μM) was also added in order to stimulate QS of pSB401. Direct and indirect experiments were performed with 8 replicates each. Since the reporters pSB1075 and pSB401 could give variable counts per second (CPS) for a number of technical and biological reasons (see Alagely et al. 2011), two DMSO controls - positive (reporters with AHLs) and negative (reporters without AHLs) - were included. All concentrations of tested compounds were tested together with the same positive and negative controls and the experiments were replicated 8 times. For all bioassays, compounds were added to the wells of a black microtiter plate and serially diluted. Reporter suspensions (in LB soft agar) were thoroughly mixed with 3% DMSO solutions of compounds. Luminescence and optical density of the reporter suspensions (OD_{595}) were measured every hour using a multimode microtiter plate reader Victor-3 (Perkin Elmer). The data obtained in indirect bioassays are presented as “relative bioluminescence” in order to take into account the population density of the reporters. To calculate relative bioluminescence (RB), we use the following formula:

$$RB=(B_s)/OD_{595}$$

Where B_s is bioluminescence of each sample measured in CPS, and OD_{595} is optical density of the reporter culture measured at 595 nm. The differences between the treatments and the positive control were compared by ANOVA followed by a Dunnet test (Zar 1996).

Mesocosm experiment

Since several grams of QS inhibitor were required for the mesocosm experiment, we selected kojic acid - a QS inhibitor discovered within this study – which is commercially available. Kojic acid was dissolved in unfiltered seawater from the Marina Bandar Rawdha (Muscat, Oman) to give 1 mM and 330 μM final concentrations. Three 3L sterile plastic containers were filled with 1L of the Kojic acid solutions. One sterile microscope slide (size 25 × 75mm) was immersed horizontally in each container. Sterile glass slides placed into 1L of unfiltered seawater from the Marina Bandar Rawdha were used as a control. Each treatment was replicated 3 times and the experiment was conducted 2 times. Each experiment was analyzed separately. Containers with slides were kept under continuous illumination (light intensity 2500 lux) in controlled conditions (temperature =25°C) for 7 days. At the end of experiment, slides were taken out and fouling was fixed with 3% formaldehyde solution in seawater. The slides were stained with the DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI, Fluka Chemie AG, Switzerland) solution (0.5 μg ml^{-1}). The number of bacteria in 10 randomly selected fields of view was counted under an epifluorescence microscope (Axiophot, Zeiss, Germany; magnification 1000x; λ_{Ex} = 359 nm, λ_{Em} = 441 nm). The number of diatoms in 10 randomly selected fields of view was counted under a microscope (Nikon Eclipse, USA; magnification 400x). For counting bacteria and diatoms tables of random x and y coordinates were generated using MS Excel program and these were used to select random field of views. Treatments

were coded; codes were masked prior to the scoring of the treatments. Densities of bacteria and diatoms were log-transformed to normalize the data. The normality assumption was verified with the Shapiro-Wilk test (Zar 1996). Differences in densities of microorganisms between the treatments and the control were compared by ANOVA followed by a Dunnet test (Zar 1996).

Results

QS reporter inhibition bioassays

All tested compounds (Table 1) can be separated into four groups according to their bioactivity. The first group contains compounds that only inhibited QS of *C. violaceum* CV017. The second group includes compounds that inhibited QS of *C. violaceum* CV017 and had some antibiotic properties. The third group represents compounds that only inhibited growth of CV017. The fourth group includes compounds that did not have any bioactive properties in our bioassays. A high proportion (51%) of the natural products did not show any activity. The proportion of compounds that only inhibited QS of *C. violaceum* CV017 without toxicity was the second highest (24%). Twenty percent of compounds inhibited QS of CV017 but had some antibiotic properties. Only a few compounds had only antibacterial activity. Compounds from all tested groups of organisms exhibited some QS inhibitory activity. All major groups of investigated natural products demonstrated some QS inhibitory properties (Table 1).

In the *C. violaceum* CV017 bioassay, QS inhibitory concentrations of tested compounds varied from 3.92 μM to 517 μM (Table 1). Many of the tested natural products had minimum inhibitory concentrations below 100 μM. Demethoxy encecalin from the plant *Baccharis cassinaefolia* was the most effective QS inhibitor in this investigation.

QS inhibitory properties of selected compounds (demethoxy encecalin, tenuazonic acid, midpacamide, hymenialdisin, microcolins A and B and kojic acid) (Fig. 1) were further investigated in LasR based and LuxR-based bioassays. Prior to the tests, possible toxic effects of compounds on metabolic activity or luminescence of the reporters were evaluated with the *E. coli* pTIM2442 reporter. This constitutively luminescent reporter demonstrated that both midpacamide and tenuazonic acid significantly (ANOVA, Dunnet test, $p<0.05$) inhibited luminescence of the reporter in the absence of AHLs at the maximal inhibitory concentrations (Fig. 2), suggesting that they were either toxic or inhibited luminescence either directly (i.e. by affecting the luciferase enzyme) or indirectly (by affecting metabolism). This constitutive reporter, when used in conjunction with the toxicity assays, allowed us to eliminate compounds that were both generally toxic and those that inhibited the bioassay. Therefore, compounds that were either toxic or otherwise negatively affected the pTIM2442 reporter were not used for further studies. Demethoxy encecalin, hymenialdisin, microcolins A and B and kojic acid were not toxic and did not interfere with luminescence of *E. coli* pTIM2442. Bioactivity of these compounds was further studied. Demethoxy encecalin and microcolins A and B, hymenialdisin and kojic acid did not affect luminescence of pSB1075 in the direct experiments (data not shown). In the indirect bioassay, both demethoxy encecalin and hymenialdisin at concentrations above 0.36μM and above 0.2μM, correspondingly, significantly reduced QS dependent luminescence of the reporter *E. coli* pSB1075 induced by 3oxo-C12-HSL (Fig. 3). Microcolin A, microcolin B and kojic acid did not significantly (ANOVA, Dunnet test, $p<0.05$) inhibit QS dependent luminescence of the reporter *E. coli* pSB1075 at the tested concentrations. Background relative bioluminescence of pSB1075 without 3oxo-C12-HSL (negative control) was consistently under 2800 CPS, and bioluminescence of this reporter was always below 500 CPS. None of the tested compounds induced luminescence of the reporter *E. coli* pSB401in the direct experiments (data not shown), suggesting that none was capable of stimulating QS

responses in this reporter based on the LuxR system of *Vibrio fischeri.* Both hymenialdisin and demethoxy encecalin at concentrations above 0.06μM and 0.12μM, correspondingly, significantly (ANOVA, Dunnet test, $p<0.05$) reduced QS dependent luminescence of the reporter *E. coli* pSB401 induced by 3-oxo-C6-HSL (Fig. 4A). Kojic acid inhibited QS dependent luminescence of the reporter induced by 3-oxo-C6-HSL only at concentrations above 36μM (Fig. 4A). Microcolins A and B significantly (ANOVA, Dunnet test, $p<0.05$) reduced QS dependent luminescence of the reporter induced by 3-oxo-C6-HSL at concentrations above 1.5μM (Fig. 4B). Background relative bioluminescence of pSB401 without 3-oxo-C6-HSL (negative control) was consistently under 1500 CPS, and bioluminescence of this reporter was always below 114 CPS.

Mesocosm experiment

Kojic acid reduced formation of microbial communities on glass slides (Fig. 5A and B). In both experiments bacterial densities in the presence of 330 μM and 1 mM of kojic acid were significantly reduced (ANOVA, Dunnet test, $p<0.05$) in comparison with ones on the control slides (Fig. 5A). Similarly, significantly lower (ANOVA, Dunnet test, $p<0.05$) densities of diatoms were observed in biofilms developed with kojic acid solutions in two repeated experiments (Fig. 5B).

Discussion

In this study, 78 different natural products from marine organisms (sponges, algae, fungi, tunicates and cyanobacteria) and terrestrial plants were screened for their ability to inhibit bacterial QS. Marine natural products have rarely been screened for QS inhibitory compounds (reviewed by Dobretsov et al. 2009; Ni et al. 2009) in comparison with synthetic compounds (Muh et al. 2006; Soulere et al. 2010). Our results demonstrate that a large proportion of tested compounds (51%) did not interfere with bacterial QS and only 24% of compounds inhibited QS of *C. violaceum* CV017. Inhibition of the *C. violaceum* reporter could be due to the compound's ability to inhibit QS in the reporter (either directly – by blocking AHL perception, or indirectly by affecting, for example, the transcription of the AHL receptor gene or the stability of the QS transcript). Inhibition of the *C. violaceum* reporter could also be due to the direct or indirect inhibition of the synthesis of the tryptophan derivative violacein, a purple pigment which serves as a read-out for this bioassay. Some of the tested natural products inhibited the *C. violaceum* reporter at concentrations above 100 μM. These concentrations are extremely high, and are unlikely to occur in the marine environment (Hmelo and Van Mooy 2009). Therefore, it is likely that these compounds are not truly functioning as QS inhibitors in nature. This fact may explain the relatively high rate of finding QS inhibitors in this study and in other investigations (Skindersoe et al. 2008b).

Some of the *C. violaceum* CV017 QS inhibitors found in this study have antibiotic properties. Due to the different application method, QS inhibitory concentrations of compounds cannot be directly compared to the amount of compounds used for the toxicity bioassay. Further, toxicity of some QS inhibitors was determined by the pTIM2442 reporter. This suggested that indeed some QS inhibitors demonstrate some antibiotic activity. This fact is not novel (Skindersoe et al. 2008a). Previously, 12 antibiotics at sub-lethal concentrations were screened for their QS inhibitory activity (Skindersoe et al. 2008a). The antibiotics azithromycin, ceftazidime, and ciprofloxacin at concentrations of 0.1–11 μM inhibited QS of a LuxR based reporter based on the QS circuit of *Vibrio fischeri.* In a previous study, the cyanobacterial antibiotic malyngolide produced by the cyanobacterium *Lyngbya majuscula* inhibited QS at concentrations ranging from 3.57 μM to 57 μM (Dobretsov et al. 2010). The mechanism of this QS inhibition remains unknown but it was

suggested that antibiotics can change bacterial membrane permeability, thus affecting flux of QS signals (Skindersoe et al. 2008a).

QS activity of some compounds similar to those tested in this study have been investigated earlier. It has been shown that malyngamide C and 8-epi-malyngamide C inhibited luminescence of Las-R based *E. coli* reporter induced with 3-oxo-C12-HSL at concentrations of 10μM -1mM (Kwan et al. 2010), likely by inhibiting transcription of the *lasR* gene. In our study, malyngamides A and B, which are structurally different from malyngamide C, inhibited QS-dependent violacein production by *C. violaceum* CV017 at similar concentrations. Extracts of the plants *Moringa oleifera* and *Acacia nilotica* that contained gallic and ellagic acids had anti-QS potential (Singh et al. 2009a; Singh et al. 2009b). Epigallocatechin gallate (salt of gallic acid) and ellagic acid inhibited Las-R based and Lux-R based QS at concentrations of 15–30μM (Huber et al. 2003). In our study, only gallic but not ellagic acid inhibited QS of *C. violaceum* CV017 at 64.7μM. Different reporters used in both studies likely explain such differences in the results.

Because the inhibition of the *Chromobacterium violaceum* reporter is likely to identify a number of bioactive substances, not all of which are inhibitory to the QS regulatory cascades, the activity of the compounds was tested using semi-synthetic LuxR and LasR reporters based on *E. coli*. These reporters contain AHL receptor genes on a multi-copy plasmid (Winson et al. 1998; Alagely et al. 2011). Unfortunately, we were unable to investigate the effect of all promising QS inhibitors in this experiment because most of the natural products tested in this study were available at low quantities. Midpacamide and tenuazonic acid were toxic to the reporters at the tested concentrations. Demethoxy encecalin and hymenialdisin interfered with induced luminescence of LasR and LuxR reporters, while kojic acid and microcolin A and B only interfered with LuxR reporters. Inhibitory concentrations of hymenialdisin, demethoxy encecalin, kojic acid and microcolins A and B were comparable with ones of natural furanones (Maximilien et al. 1998; Martinelli et al. 2004), ellagic acid (Huber et al. 2003), malyngolide (Dobretsov et al. 2010) and manoalide (Skindersoe et al. 2008b).

Usually, QS inhibitors have been tested in the laboratory against monocultures of pathogens (see review by Dobretsov et al. 2009) or environmental isolates (Maximilien et al. 1998). Only a few studies investigated the effect of quorum sensing inhibitors, such as furanones, on multispecies of bacteria in the laboratory (Dobretsov et al. 2007) and over 2h in the field (Maximilien et al. 1998). In the latter study, crude extracts from the red alga *Delisea pulchra* and pure furanone-1 and -2 at the concentration 1 $\mu g\ cm^{-2}$ applied to Perspex disks or glass Petridishes inhibited attachment of bacteria to less than 20% of control numbers. In this study, we tested antifouling performance of the QS inhibitor - kojic acid - against environmental microbes. This compound was selected because of its QS inhibitory activity in the experiments and its commercial availability that ensured sufficient quantities of the compound. In a preliminary field experiment (data not shown) kojic acid incorporated into a non-toxic paint matrix at a concentration of 0.5% significantly reduced densities of bacteria and diatoms growing on the paint and decreased macro-fouling over 1 month. Interpretation of these results poses unique technical and scientific challenges that make it difficult to attribute inhibition of micro- and macro-fouling solely to QS inhibitory activity of kojic acid. Therefore, a controlled mesocosm experiment with kojic acid at non-toxic (330 μM) and 3 fold higher concentrations was conducted. In this experiment, which was repeated two times, kojic acid at non-toxic concentrations inhibited 2.5 – 3.2 fold bacterial density and 4.7 – 3.6 fold diatom density in biofilms on glass slides.

How did kojic acid affect micro-fouling in our experiment? It is possible that kojic acid inhibited QS of bacteria and this led to low bacterial attachment/recruitment and biofilm

formation. This was supported by the data showing that kojic acid at tested concentrations inhibited QS of the reporters and was not toxic to the reporters or the diatom *Amphora coffeaeformis* (data not presented). Kojic acid is widely used as a food additive for preventing enzymatic browning, and in cosmetic preparations as a skin-lightening or bleaching agent because of its tyrosinase inhibitory action (Cabanes et al. 1994; Burdock et al. 2001). Unfortunately, direct measurements of AHLs in the biofilms treated and not treated with kojic acid are technically challenging and would not help proven this hypothesis. Previous studies suggested that QS inhibitors, such as furanones, affect microbial composition and densities of certain groups of bacteria (Dobretsov et al. 2007), shifting the composition of microbial communities from being dominated by Gram-negative bacteria to those dominated by Gram-positive species (Maximilien et al. 1998; Kjelleberg et al. 2001). In this case, a decrease in AHL concentrations might reflect changes in microbial composition. Changes in bacterial species composition and chemical compound production could possibly result in changes in diatom communities, as presence of particular bacteria affect recruitment of diatoms (Gawne et al. 1998; Wigglesworth-Cooksey and Cooksey 2005). Alternatively, there is a possibility that kojic acid reduced formation of microbial communities by means other than QS inhibition. For example, kojic acid could have a toxic effect on some marine bacteria and diatoms more sensitive to this acid than the tested reporters. It is possible that kojic acid could inhibit other regulatory cascades that affect biofilm formation. Brominated furanones are known to inhibit multiple regulatory pathways leading to biofilm formation even without interference with QS (Janssens et al. 2008). Overall, results of this experiment demonstrate a high antifouling potential of kojic acid.

In conclusion, results of this study suggest that screening of natural products is a promising way to find novel QS inhibitors. Natural products with QS inhibitory properties can control formation of microbial communities and potentially can be used in the future for antifouling applications.

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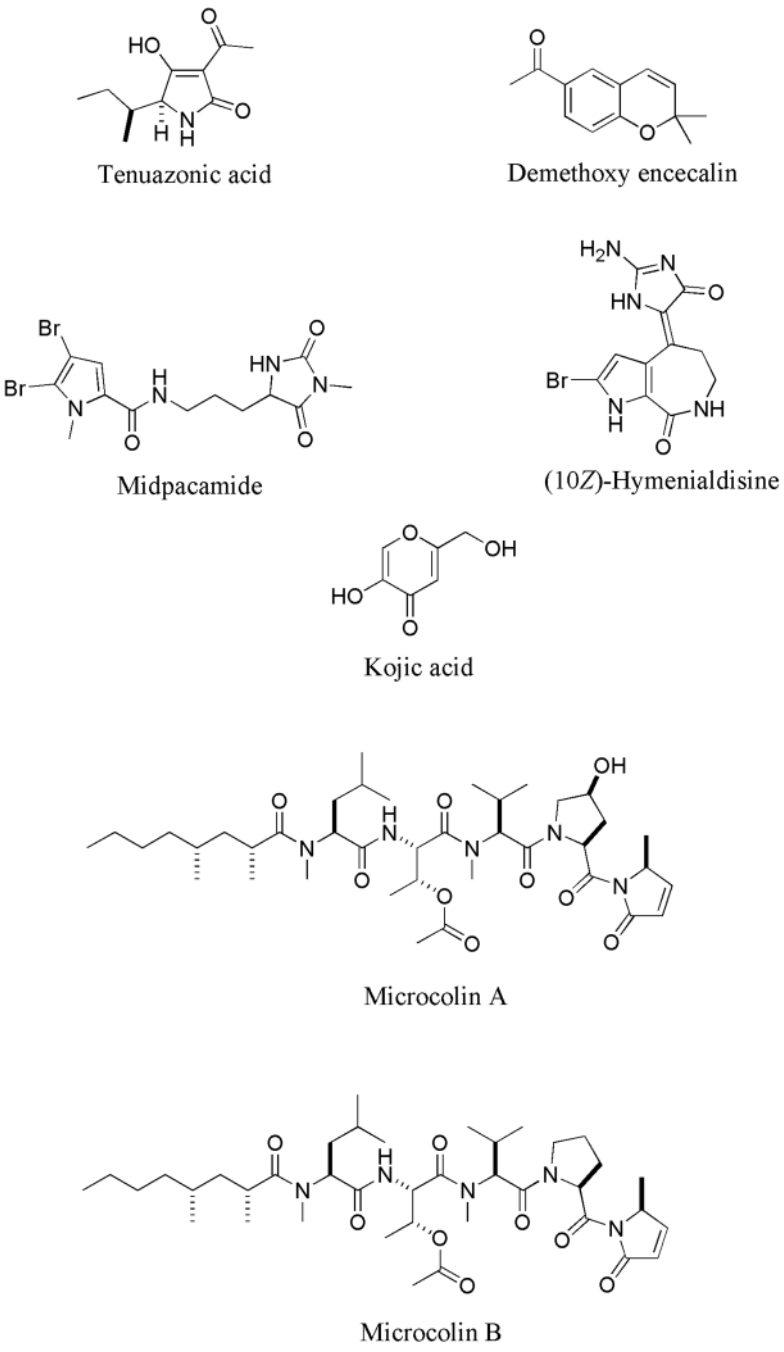


Figure 1.
Structures of tenuazonic acid, demethoxy encecalin, midpacamide, hymenialdisin, microcolins A and B and kojic acid.

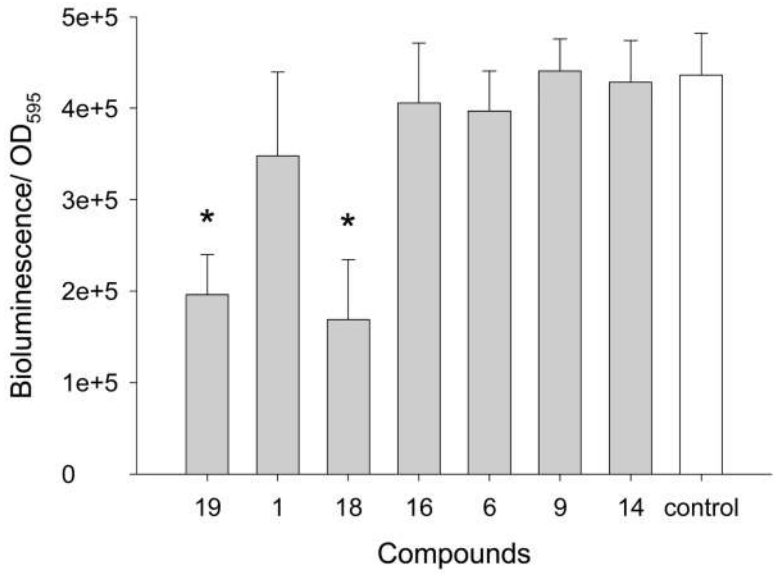


Figure 2.

The effect of tenuazonic acid (#19), demethoxy encecalin (#1), midpacamide (#18), hymenialdisin (#16) and microcolins A (#6) and B (#9) as well as kojic acid (#14) on bioluminescence of *E. coli* DH5α containing a pTIM2442 plasmid. The data are shown as mean + SD relative bioluminescence (bioluminescence/OD_{595}) of the reporter with added compounds in dimethyl sulfoxide (DMSO) (n=8) or without (only DMSO, control, n=8). Toxic compounds highlighted by asterisks have significantly (Dunnet, $p<0.05$) lower relative bioluminescence compared to the control. Midpacamide was tested at 46 μM, tenuazonic acid was tested at 102 μM, demethoxy encecalin was tested at 6.6 μM, hymenialdisin was tested at 15 μM, microcolins A and B - at 150 μM and kojic acid - at 330 μM. Measurements were taken every 1 h but results obtained at 4h are shown.

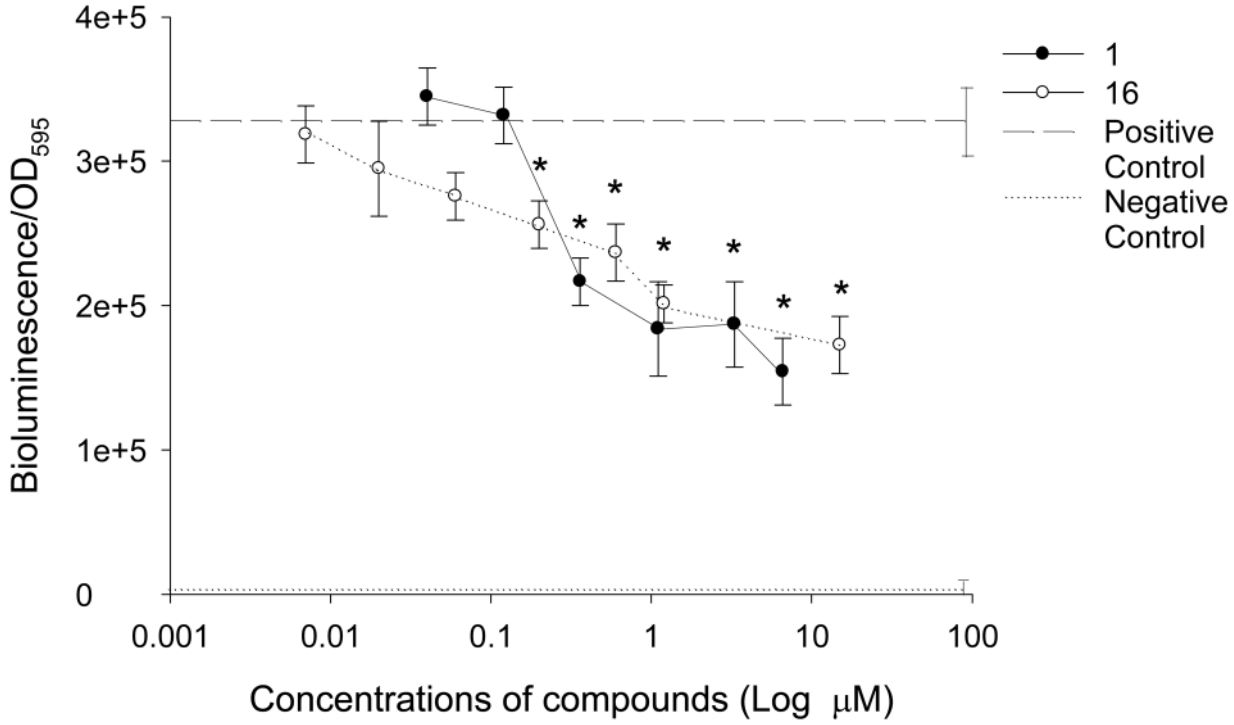


Figure 3.
The effect of demethoxy encecalin (#1) and hymenialdisin (#16) on QS dependent bioluminescence of the LasR-based reporter *E. coli* pSB1075 induced by 3oxo-C12-HSL (final concentration of 2 μM). Data show mean + SD relative bioluminescence (bioluminescence/OD_{595}) of the reporter with added compounds (n= 8). Compound concentrations that significantly (Dunnet, $p<0.05$) inhibited QS of the reporter are marked with asterisks. Dash line - positive control (reporters with AHLs) (n= 8) and dotted line - negative control (reporters without AHLs) (n= 8). All treatments and controls contained dimethyl sulfoxide (DMSO). Measurements were taken every 1 h but results obtained at 4h are shown.

Fig.4A

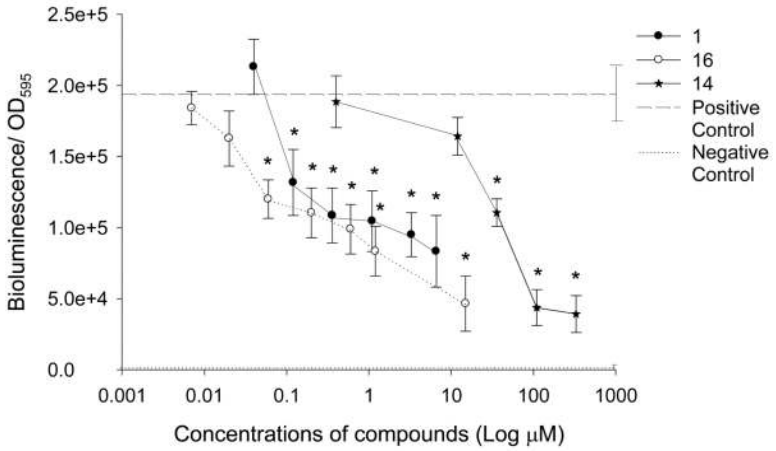


Fig 4B

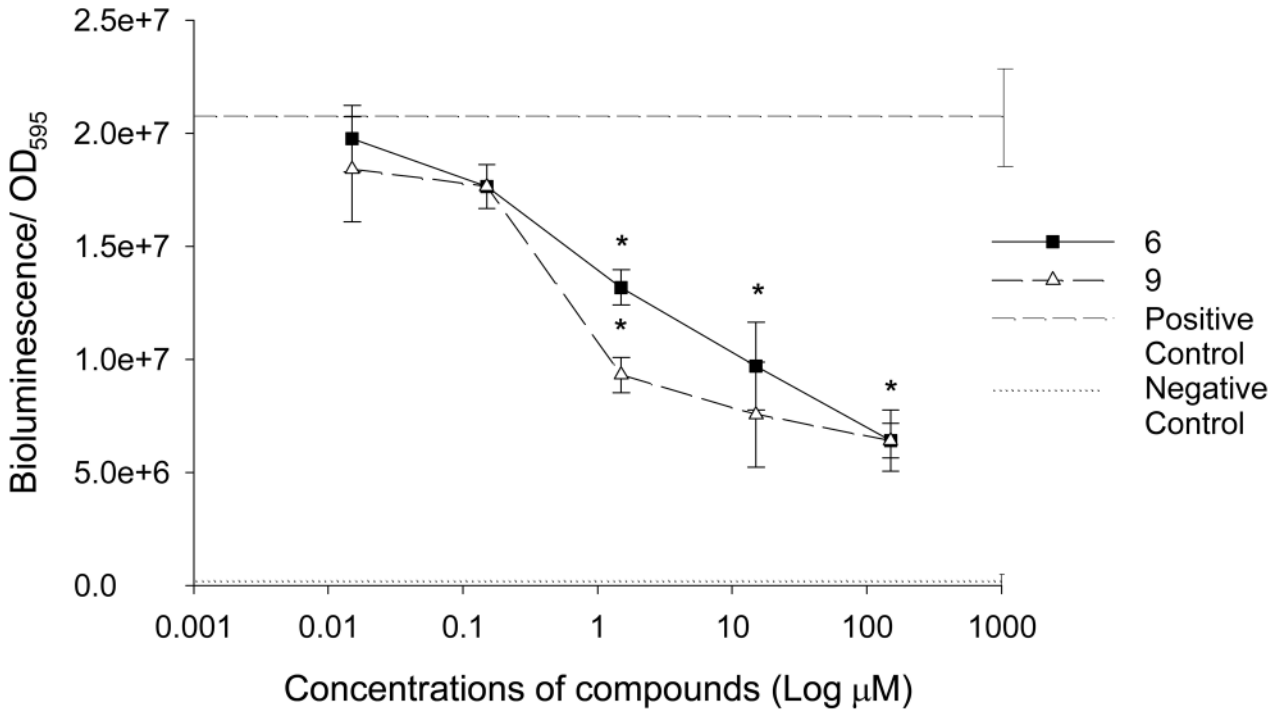
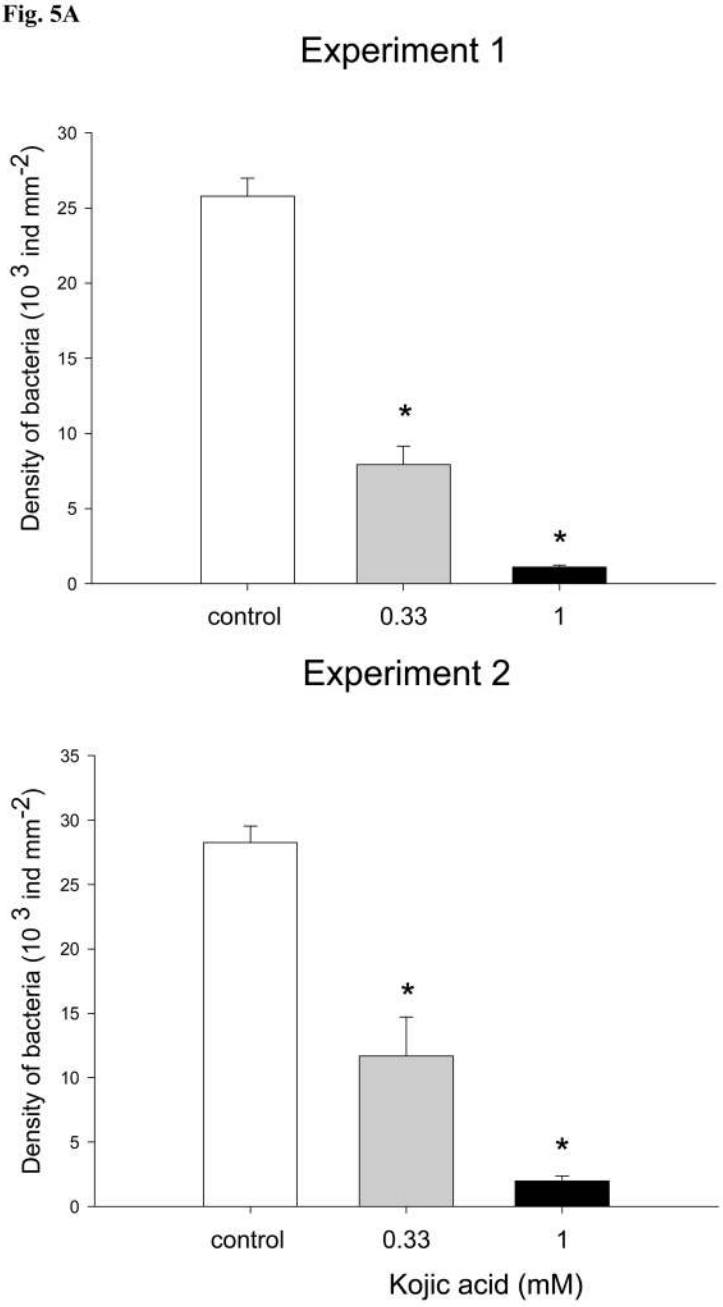


Figure 4.

The effect of **A**: demethoxy encecalin (#1), hymenialdisin (#16) and kojic acid (#14), and **B**: microcolin A (#6) and microcolin B (#9) on QS dependent bioluminescence of the LuxR-based reporter *E. coli* pSB401 induced by 3-oxo-C6-HSL (final concentration of 10 μM). Data show relative mean + SD bioluminescence (bioluminescence/OD_{595}) of the reporter with added compounds (n= 8). Compound concentrations that significantly (Dunnet, $p<0.05$) inhibited QS of the reporter are marked with asterisks. Dashed line -positive control (reporters with AHLs) (n= 8) and dotted line - negative control (reporters without AHLs)

(n= 8). All treatments and controls contained dimethyl sulfoxide (DMSO). Measurements were taken every 1 h but results obtained at 4h are shown.

Fig. 5A



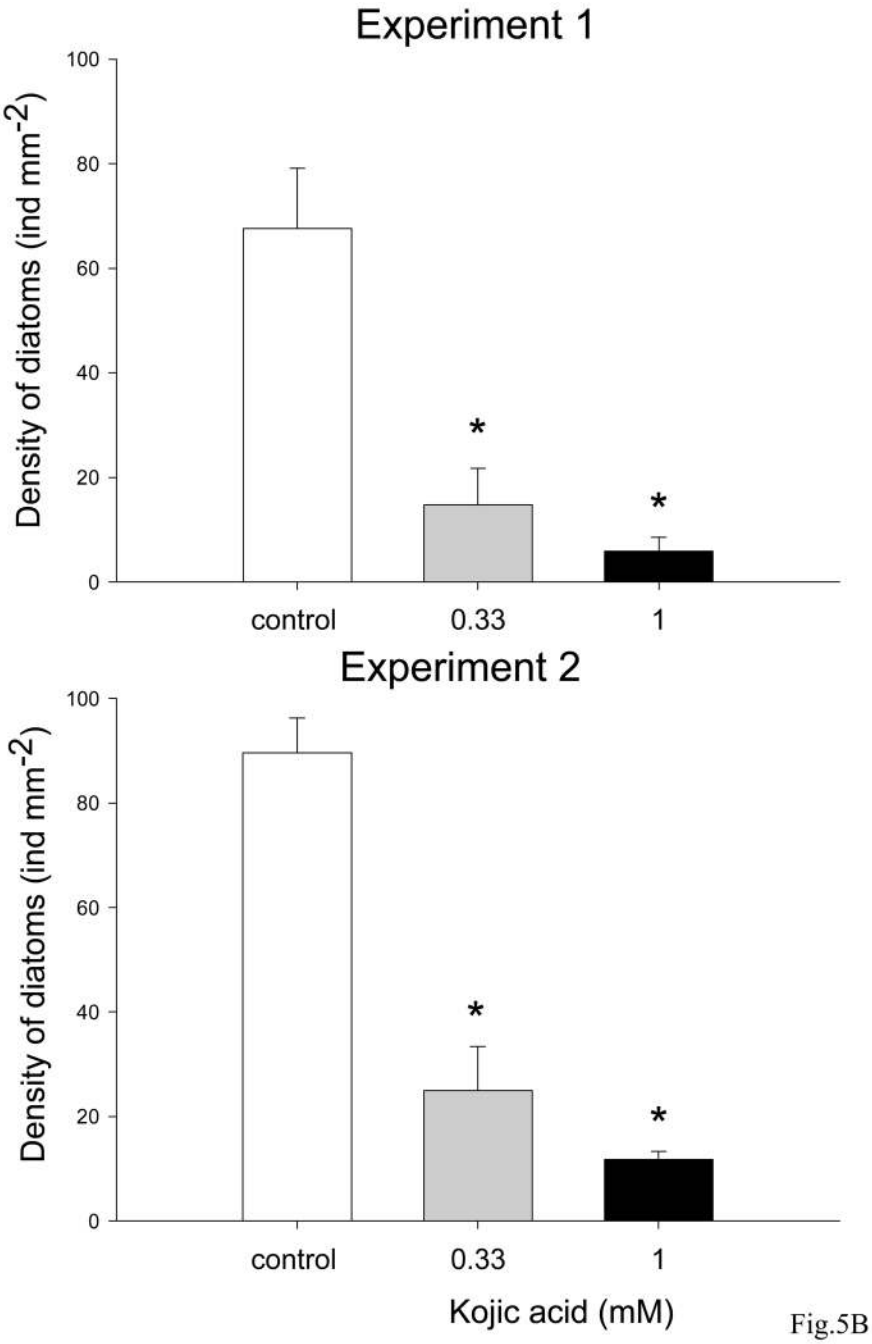


Figure 5.

Mean densities of **A:** bacteria (ind mm^2) and **B:** diatoms (ind mm^2) on glass slides exposed to 330 μM and 1 mM kojic acid solutions prepared with unfiltered seawater from the Marina Bandar Rawdha (Muscat, Oman). Three glass slides were incubated in containers filled with unfiltered seawater for 7 days to allow development of biofouling. Sterile glass slides placed into unfiltered seawater without addition of kojic acid were used as a control. Bars are the means of 3 replicates + SD. Data that are significantly different from the control (ANOVA, Dunnet test: $p<0.05$) are indicated by an asterisk above the bars. Data are from 2 independent experiments.

Table 1

Origin of tested natural products and their effect on quorum sensing of *Chromobacteruium violaceum* CV017 and its growth. Quorum sensing inhibition reported as mean of 3 replicates ± SD minimum inhibitory concentrations–MIC (μM). Toxicity effect of compounds is presented as a minimal amount of natural product necessary to inhibit growth of the reporter strain. Compounds are sorted according to their bioactivity. The first group contains compounds that only inhibited QS of *C. violaceum* CV017. The second group includes compounds that inhibited QS of *C. violaceum* CV017 but have some toxicity. The third group represents compounds that inhibited growth of CV017. The fourth group includes compounds that did not have any bioactive properties.

Abbreviation	Name	Origin		Groups of compounds	Reference	QS (μM)	Growth (mole × 10^{-6})
		Species	Group				
First group of compounds							
1	Demethoxy encecalin	*Baccharis cassinaefolia*	Plant	Benzopyran	Proksch and Rodriguez 1982	3.92 ± 1.11	-
2	Orientin	*Polygonum orientale*	Plant	Flavonoid	Weber 2007	4.46 ± 1.3	-
3	Kuanoniamin D	*Cystodytes sp.*	Tunicate	Alkaloid	Eder et al. 1998	5.55 ± 3.11	-
4	Malyngamide A	*Lyngbya majuscula*	Cyanobacteria	Amide	Cardellina et al.1979	7.46 ± 2.11	-
5	Ageliferin	*Agelas conifera*	Sponge	Alkaloid	Hertiani et al. 2010	11.29 ± 1.93	-
6	Microcolin A	*Lyngbya sp.*	Cyanobacteria	Peptide	Koehn et al. 1992	15.23 ± 2.75	-
7	Mauritamide B	*Agelas nakamurai*	Sponge	Alkaloid	Hertiani et al. 2010	36.76 ± 4.47	-
8	Pinoresinol	*Pinus ssp.*	Plant	Lignan	Weber 2007	41.85 ± 6.13	-
9	Microcolin B	*Lyngbya sp.*	Cyanobacteria	Peptide	Koehn et al. 1992	43.21 ± 3.14	-
10	Gallic acid	*Plants*	Plant	Phenol	Bayer 2009	64.66 ± 12.27	-
11	Glucobrassicin	*Brassica napus var. napus*	Plant	Alkaloid	Weber 2007	133.71 ± 16.39	-
12	Meleagrin	*Penicillium chrysogenum*	Fungus	Alkaloid	Rusman 2006	138.42 ± 22.18	-
13	Alterporriol E	*Alternaria porri*	Fungus	Polyketide	Aly et al. 2008	298.26 ± 57.30	-
14	Kojic acid	*Aspergillus spp*	Fungus	Pyranone	Indriani 2008	239.25 ± 8.92	-
15	4-(4,5-dibromo-1- methyl-1H-pyrrole- 2-carboxamido) butanoic acid	*Agelas sp.*	Sponge	Alkaloid	2010	271.73 ± 9.13	-
16	Hymenialdisin	*Hymeniacidon aldis*	Sponge	Alkaloid	Supriyono et al. 1995	308.52 ± 8.19	-
17	Dulcitol	*Spatoglossum sp.*	Plant	Sugar	Queiroz et al. 2008	380.02 ± 56.23	-
18	Midpacamide	*Agelas mauritiana*	Sponge	Alkaloid	Hertiani et al. 2010	458.61 ± 34.11	-
19	Tenuazonic acid	*Alternaria tenuis*	Fungus	Alkaloid	Hassan 2007	517.03 ± 39.71	-

Abbreviation	Name	Origin		Groups of compounds	Reference	QS (μM)	Growth (mole × 10^{-6})
		Species	Group				
Second group of compounds							
20	Malyngamide B	*Lyngbya majuscula*	Cyanobacteria	Amide	Cardellina et al. 1978	5.89 ± 1.77	17.27 ± 1.26
21	(+)Avarol	*Dysidea avara*	Sponge	Terpene	Putz 2009	6.36 ± 2.78	0.40 ± 0.04
22	Alternariol monomethyl ether	*Alternaria sp.*	Fungus	Polyketide	Aly et al. 2008	7.32 ± 1.25	7.35 ± 1.16
23	Aaptamin	*Aaptos aaptos*	Sponge	Alkaloid	Supriyono 1997	8.76 ± 2.38	0.61 ± 0.02
24	8-OH-manzamien A	*Acanthostrongylo phore ingens*	Sponge	Alkaloid	Edrada 1998	12.41± 1.34	0.50 ± 0.03
25	Lyngbyastatin 3	*Lyngbya majuscula*	Cyanobacteria	Peptide	Williams et al. 2003	12.00 ± 3.41	16.19 ± 1.67
26	Aeroplysinin	*Aeroplysisnin sp.*	Sponge	Alkaloid	Ebel 1997	16.55 ± 5.51	0.31 ± 0.02
27	(−)Dibromophakelline	*Pseudaxinyssa cantharella*	Sponge	Alkaloid	Hertiani 2007	17.99 ± 5.10	0.46 ± 0.05
28	Alterlactone	*Alternaria sp.*	Fungus	Polyketide	Aly et al. 2008	24.31 ± 5.29	1.74 ± 0.13
29	Emodin	*Rhamnus purshiana*	Plant	Polyketide	Debbab 2007	25.90 ± 5.14	0.93 ± 0.04
30	Encecalin	*Eupatorium californica*	Plant	Benzopyran	Proksch and Rodriguez 1982	30.12 ± 6.39	1.29 ± 0.09
31	Agelanesin C	*Agelas sp.*	Sponge	Alkaloid	Hertiani et al.2010	36.30 ± 8.94	0.91 ± 0.01
32	Cyclo Colorenon (1)*	*Porella vernicosa*	Plant	Terpene	Handayani 1998	91.60 ± 16.22	1.38 ± 0.04
33	Aerothionin	*Aplysina aerophoba*	Sponge	Alkaloid	Ebel 1997	244.47 ± 19.71	0.27 ± 0.02
34	(−) Agelasidine D	*Agelas clathrodes*	Sponge	Alkaloid	Hertiani et al.2010	454.90 ± 14.22	0.46 ± 0.06
35	Altersolanol A	*Alternaria solani*	Fungus	Polyketide	Aly et al. 2008	594.71 ± 48.39	0.41 ± 0.04
Third group of compounds							
36	Curacin D	*Lyngbya majuscula*	Cyanobacteria	Lipid	Marquez et al. 1998	-	69.88 ± 3.54
37	Alternariol sulphate	*Alternaria sp.*	Fungus	Polyketide	Aly et al. 2008	-	0.59 ± 0.06
Forth group of compounds							
38	Alteric acid	*Alternaria sp.*	Fungus	Polyketide	Aly et al. 2008	-	-
39	Alternariol	*Alternaria tenuis*	Fungus	Polyketide	Aly et al. 2008	-	-
40	Altenuene 4′-Epialtenuene	*Alternaria sp.*	Fungus	Polyketide	Aly et al. 2008	-	-
41	Altenusin	*Alternaria tenuis*	Fungus	Polyketide	Aly et al. 2008	-	-
42	Alterporriol D	*Alternaria porri*	Fungus	Polyketide	Aly et al. 2008	-	-
43	Ampelanol	*Ampelomyces sp.*	Fungus	Polyketide	Hassan 2007	-	-
44	Aposhaerin A	*Aposphaeria sp.*	Fungus	Polyketide	Hassan 2007	-	-

Abbreviation	Name	Origin		Groups of compounds	Reference	QS (μM)	Growth (mole × 10^{-6})
		Species	Group				
45	Atromentine	*Aglaia odorata*	Fungus	Phenol	Duong 2006	-	-
46	Chaetomin	*Chaetomium cochliodes*	Fungus	Alkaloid	Aly et al. 2008	-	-
47	Cochliodinol	*Chaetomium globosum*	Fungus	Alkaloid	Aly et al. 2008	-	-
48	Citrinin	*Penicillium citrinum*	Fungus	Phenol	Hiort et al. 2004	-	-
49	Cyclo(prolyl-Valyl)*	*Aspergillus flavipes*	Fungus	Peptide	Indriani 2008	-	-
50	Cyclo(L-tyr-L-pro)*	*Alternaria alternata*	Fungus	Peptide	Indriani 2008	-	-
51	Cytochalasin E	*Rosellinia necatrix*	Fungus	Alkaloid	Indriani 2008	-	-
52	Equisetin	*Fusarium equiseti*	Fungus	Alkaloid	Kjer 2010	-	-
53	Ageraton	*Ageratum houstonianum*	Plant	Benzofuran	Kunze 1995	-	-
54	Aglaiol	*Aglaia odorata*	Plant	Terpene	Duong 2006	-	-
55	(+) Curcudiol	*Didiscus flavus*	Plant	Terpene	Hertiani 2007	-	-
56	Piscidinol A	*Phellodendron chinense*	Plant	Terpene	Duong 2006	-	-
57	Septicine	*Tylophora asthmatica*	Plant	Alkaloid	Moustafa 2009	-	-
58	Ellagic acid	*Plants*	Plant	Phenol	Bayer 2009	-	-
59	Agelasine I	*Agelas sp.*	Sponge	Alkaloid	Hertiani et al. 2010	-	-
60	Agelanesin A	*Agelas sp.*	Sponge	Alkaloid	Hertiani et al. 2010	-	-
61	Agelanesin B	*Agelas sp.*	Sponge	Alkaloid	Hertiani et al. 2010	-	-
62	Agelanin A	*Agelas sp.*	Sponge	Alkaloid	Hertiani et al. 2010	-	-
63	Agelanin B	*Agelas sp.*	Sponge	Alkaloid	Hertiani et al. 2010	-	-
64	(+) Agelasidine-C	*Agelas nakamurai*	Sponge	Alkaloid	Hertiani et al. 2010	-	-
65	Agelongine	*Agelas longissima*	Sponge	Alkaloid	Hertiani et al. 2010	-	-
66	(−)-Ageloxime D	*Agelas nakamurai*	Sponge	Alkaloid	Hertiani et al. 2010	-	-
67	Aldisine	*Hymeniacidon aldis*	Sponge	Alkaloid	Hertiani 2007	-	-
68	Aplysamine-2	*Psammaplysilla purpurea*	Sponge	Alkaloid	Hertiani 2007	-	-
69	E/Z-aplysinopsin	*Verongia spengelii*	Sponge	Alkaloid	Hertiani 2007	-	-
70	(+)Avarone	*Dysidea avara*	Sponge	Terpene	Putz 2009		-
71	Bastadin- 4	*Ianthella basta*	Sponge	Alkaloid	Ortlepp et al.2007	-	-
72	2 Bromoaldisine	*Hymeniacidon aldis*	Sponge	Alkaloid	Hertiani 2007	-	-

Abbreviation	Name	Origin		Groups of compounds	Reference	QS (μM)	Growth (mole × 10^{-6})
		Species	Group				
73	4-(4-Bromo-1H- pyrrole-2-carboxamido) butanoic acid	*Agelas nakamurai*	Sponge	Alkaloid	Hertiani 2007	-	-
74	4-Bromopyrrole-2- carboxamide	*Agelas nakamurai*	Sponge	Alkaloid	Hertiani 2007	-	-
75	Dienone dimethoxyketal	*Pseudoceratina purpura*	Sponge	Alkaloid	Fendert 2000	-	-
76	Hymenidin	*Agelas clathrodes*	Sponge	Alkaloid	Supriyono 1997	-	-
77	Mauritamide C	*Agelas nakamurai*	Sponge	Alkaloid	Hertiani et al. 2010	-	-
78	Dragonamide C	*Lyngbya cf. polychroa*	Cyanobacteria	Peptide	Gunasekera et al. 2008	-	-