Peer-reviewed version available at *Mar. Drugs* **2020**, *18*, 127; doi:10.3390/md18020127

Article

Co-existence of quorum sensing and quorum sensing inhibitory compounds in marine sponge *Sarcotragus spinosulus*

Kumar Saurav[1,2,3], Nicola Borbone[2], Ilia Burgsdorf[1], Roberta Teta[2], Alessia Caso[2], Rinat Bar-Shalom[1], Germana Esposito[2], Maya Britstein[1], Laura Steindler[1] and Valeria Costantino[2*].

[1]Department of Marine Biology, Leon H. Charney School of Marine Sciences, University of Haifa, Mt. Carmel 31905, Haifa, Israel. sauravverma17@gmail.com (K.S); burgsdorf84@gmail.com (I.B); rbar-shal@univ.haifa.ac.il (R.B.S); mayabritstein@gmail.com (M.B); lsteindler@univ.haifa.ac.il (L.S)
[2]The Blue Chemistry Lab, Dipartimento di Farmacia, Università degli Studi di Napoli Federico II, Via D. Montesano 49, 80131, Napoli, Italy. sauravverma17@gmail.com (K.S); nicola.borbone@unina.it (N.B); roberta.teta@unina.it (R.T); alessia.caso@unina.it (A.C); germana.esposito@unina.it (G.E); valeria.costantino@unina.it (V.C)
[3]Laboratory of Algal Biotechnology-Centre Algatech, Institute of Microbiology of the Czech Academy of Sciences, Opatovický mlýn, Novohradská 237, 379 81, Třeboň, Czech Republic. sauravverma17@gmail.com (K.S)
*Correspondence: valeria.costantino@unina.it; Tel.: +39-081-678-504

Abstract: Marine sponges, a well documented prolific source of natural products, harbors numerous microbial communities believed to possess *N*-acyl homoserine lactones (AHLs) mediated Quorum sensing (QS) as one of the mechanisms of interaction. Bacteria and eukaryotic organisms are known to produce molecules that can interfere with QS signaling, thus affecting microbial genetic regulation and function. In the present study, we established the potential for production of both QS signal molecules as well as QS interfering molecules (QSI) in the same sponge species *Sarcotragus spinosulus*. A total of eighteen saturated acyl chain AHLs were identified along with six putative unsaturated acyl chain AHLs. Bioassay guided purification led to the isolation of two brominated metabolites with QS-interfering activity. The structures of these compounds were elucidated by comparative spectral analysis of ^{1}HNMR and HR-MS data and was identified as 3-Br-*N*-methyltyramine (**1**) and 5,6-dibromo-*N,N*-dimethyltryptamine (**2**). The QSI activity of compounds **1** and **2** were evaluated using reporter gene assays for long- and short-chain signals (*E. coli* pSB1075 and *E. coli* pSB401) and was confirmed by measuring dose dependent inhibition of proteolytic activity and pyocyanin production in *P. aeruginosa* PAO1. The obtained results showed the co-existence of QS and QSI in *S. spinosulus*, a complex network which may mediate the orchestrated function of the microbiome within the sponge holobiont.

Keywords: sponge; quorum sensing; quorum sensing inhibition, *N*-acyl homoserine lactone; *Sarcotrag*us *spinosulus*; 3-Br-*N*-methyltyramine; 5,6-dibromo-*N,N*-dimethyltryptamine

1. Introduction

Overuse of antibiotics is one of the factors involved in the emergence of drug resistant pathogens. The discovery of alternative novel strategy to tackle the infections is required to solve this emergent problem. The understanding of how inter-cellular microbial communication is involved in bacterial pathogenesis has revealed potential for alternative strategies to treat bacterial-mediated diseases

[1,2]. A crucial aspect for the establishment and maintenance of a microbial population is expected to require a tool, based on cell density which triggers the genetic regulation, coordinating the physiologies of the different cell types through cell–cell communication [3] known as quorum sensing (QS). In many cases, the responses elicited by QS signals contribute directly to pathogenesis through the synchronized production of virulence determinants, such as toxins and proteases [4]. It has been theorized that, if the signal communication that coordinates these pathogenic behaviors was blocked by means of quorum sensing inhibition (QSI), bacteria would lose their ability to mount an organized assault on the host and thus their ability to form organized community structures with antibiotic resistance would be compromised [5]. QSI compounds inactivate QS by different quenching mechanisms including enzymatic inactivation of the signal molecule [6,7], inhibition of signal biosynthesis [8] and inhibition of signal detection [9,10].

Sponges (phylum Porifera), likely among the oldest multicellular animals (Metazoa) [11,12], are an important component of aquatic benthic communities, and through their immense filter-feeding capacities, have significant consequences upon coastal food webs and biogeochemical cycles [13,14]. Their arsenal of chemicals has been investigated in terms of chemical ecology [e.g., 15], drug discovery [16], and for biotechnological purpose [e.g., 17]. Growing evidence suggests that microbial symbionts are the main producer of several documented sponge-derived bioactive compounds rather than the host itself [18-21]. Within the densely colonized sponge, there is an ample opportunity for intra-species, inter-species and inter-kingdom chemical signaling [22-24]. QS was shown to be essential for the successful establishment of symbiotic and pathogenic relationships with eukaryotic hosts [25]. The presence of *N*-acyl homoserine lactones (AHLs) mediated QS in sponge extracts, sponge- bacterial isolates and a metagenomics-derived genome of a sponge symbiont [e.g., 23,24,26,27,28] are well documented. Few studies have searched for QSI compounds in sponges and their isolates, mainly in a biotechnological context, as such molecules could provide alternatives for antimicrobials, and presence of QSI molecules was reported [29-33]. Whilst AHLs have a common structure, QSI compounds are way more variable: from being structurally similar to AHLs [34,35] to cyclic peptides [36-38], alkaloids [31,39], lactones [23,40] and diterpenes [41-43]. In our recent work, a novel lactone, named plakofuranolactone, which showed a strong QSI activity at submicromolar concentration, has been discovered from the extract of the Indonesian sponge *Plakortis* cf. *lita* [40].

Recently, we showed that some sponge species have constant presence of AHLs, while other species show high variability both in presence and type of AHLs. This variability was observed in the case of *Sarcotragus* sp., where 3 out of 18 specimens tested harbored AHLs, and based on chromatographic analysis, the AHL profiles differed among these specimens [28]. Such variability does not surprise, being easily explained by differences in microbial communities among different specimens of the same species, by QS regulation of AHL production, or by presence of QSIs in part of the specimens.

In this paper, we wish to report data obtained by testing for the latter hypothesis. We have searched data showing the presence of AHLs as well as AHL based quorum sensing-interfering molecules in the sponge species *Sarcotargus spinosulus* (previously identified as *Sarcotragus* sp.,). Moreover, in this study we highlight that cell-separation is a useful tool to enrich the microbial fractions and lead to the identification of additional AHLs, often missed in direct analysis of the extract. Furthermore, bioassay guided purification led to the isolation of two brominated metabolites: 3-Br-*N*-methyltyramine (**1**) and 5,6-dibromo-*N*,*N*-dimethyltryptamine (**2**), possessing QS-interfering activity.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 December 2019 doi:10.20944/preprints201912.0184.v1

Peer-reviewed version available at Mar. Drugs 2020, 18, 127; doi:10.3390/md18020127

2. Results and Discussion

2.1. Taxonomic identification of sponge

Five samples of *S. spinosulus* were collected along the Mediterranean coast of Sdot Yam, Israel during summer 2016. Sponge specimens used in this study (voucher no: 454, 455, 456, 457 and 460) displayed a high degree of intraspecific mitochondrial COX1 gene conservation with 99-100% identity (E-value = 0.0) to the sequence published for *Sarcotragus spinosulus* (accession number HE591460). Maximum likelihood phylogenies also showed clustering of the sequences from the here analyzed five specimens, within the representatives of the *S. spinosulus* species (**Figure 1**). *S. spinosulus* is a massive southern species recorded from the Atlantic coasts.

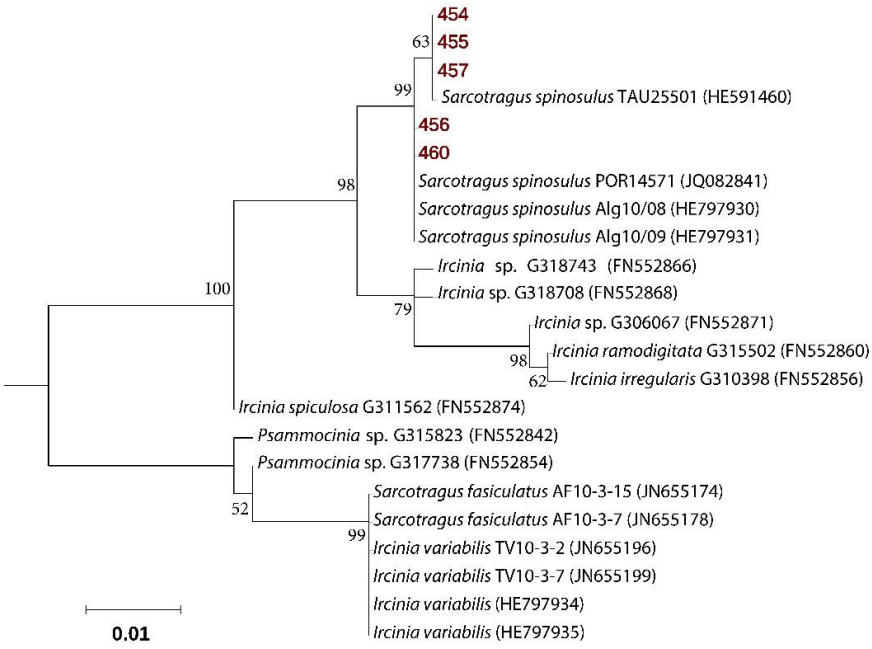

Figure 1. Molecular phylogenetic analysis based on cytochrome oxidase gene, subunit 1 sequences**.** The Maximum Likelihood tree is shown, with sequences repossess in this study highlighted in bold and red. Bootstrap values derive from 1000 replications and are shown at branch nodes. Values above 50% are shown.

2.2. Identification of AHLs in *Sarcotragus spinosulus* crude extracts

Out of the five sponge specimens analyzed, four showed the presence of AHLs in the crude extract using two AHL-biosensors, *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NT1 (pZLR4) . Subsequently, the presence of AHLs in the *S. spinosulus* crude extract was identified by comparison of HRMS/MS fragmentation patterns of individual peaks and confirmed the presence of nine AHL molecules (C6-AHL, C8-AHL, OC10-AHL, C12-AHL, C14-AHL, OHC14-AHL, OHC16-AHL, OC16-AHL and OHC18-AHL) along with the potential presence of two unsaturated AHLs (OHC6:1-AHL and C10:1-AHL) (**Table 1**).

Table 1. Chromatographic and mass spectrometric data of all the observed *N*-acyl homoserine lactones (AHLs) identified by LC-HRMS/MS in the crude extract of *S. spinosulus* (specimens: 454, 455, 456, 457 and 460).

AHLs	M+H (exp)	Rt	454	455	456	457	460
OH-C6:1-AHL	214.1073	1.73		x			
C6-AHL	200.1280	11.86				x	
C8-AHL	228.1593	15.39	x				
C10:1-AHL	254.1749	15.89		x		x	x

OXO-C10-AHL	270.1697	17.46			x	
C12-AHL	284.2217	24.18				x
C14-AHL	312.2527	27.63				x
OH-C14-AHL	328.2479	23.58			x	
OH-C16-AHL	356.2794	25.18			x	
OH-C18-AHL	384.3102	30.25				x
OXO-C16-AHL	354.2635	25.22			x	

The detection of these signal molecules often hindered by several drawbacks, mainly the large sample complexity resulting in additive or multiplicative effects of the compounds present in the extract. Taken in consideration that AHLs are produced by bacteria and not by eukaryotes, we tested whether a cell fractionation procedure that enriches the microbial fraction in the sponge, performed prior to chemical extraction, can improve our detection of AHLs in sponges. The first fraction obtained during microbial enrichment (SCS-A), obtained as filtrate debris from 125 µm sieve filtration of homogenized sponge sample, was expected to contain mainly sponge cells (SCS-A C, SCS-A C:M and SCS-A M). As expected, these sponge cells enriched fractions did not show any presence of AHLs by LC-MS/MS. The next five fractions (SCS-B-F) obtained from cells separation, were expected to be enriched in microbial cells, and led to the identification of a wide variety of AHLs, (C6-AHL, C8-AHL, OH-C14-AHL, OH-C16-AHL, OH-C18-AHL together with two unsaturated AHLs, OH-C6:1-AHL, C10:1-AHL) (**Table 2**) from the eleven AHLs that were detected in sponge extracts (**Table 1**). Additionally, seven AHLs (OHC8-AHL, OHC10-AHL, OHC12-AHL, OXO-C12-AHL and OXO-C14-AHL, together with two unsaturated AHLs, C8:1-AHL and OHC14:1-AHL), which were not identified by direct sponge tissue extraction, were identified in the cell fractions (**Table 2**).

Table 2. Chromatographic and mass spectrometric data of all the observed *N*-acyl homoserine lactones (AHLs) identified by LC-HRMS/MS in the microbial enrichment cell separation fractions of *S. spinosulus*.

AHLs	M+H (exp)	Rt	SCS-B	SCS-C	SCS-D	SCS-E	SCS-F
C10:1-AHL	**254.1749**	**15.89**	**x**	**x**	**x**		
C6-AHL	**200.1280**	**11.86**		**x**			
C8:1-AHL	226.1437	11.16	x	x			
C8-AHL	**228.1593**	**15.39**		**x**			
OXO-C14-AHL	326.2320	24.96	x	x			
OH-C10-AHL	272.1853	16.17		x			
OH-C12-AHL	300.2168	20.08		x			
OH-C14:1-AHL	**326.2321**	**23.86**				**x**	
OH-C14-AHL	328.2479	23.58		x			
OH-C16-AHL	**356.2794**	**25.18**			**x**		
OH-C18-AHL	**384.3102**	**30.25**			**x**		
OH-C6:1-AHL	**214.1073**	**1.73**		**x**			
OH-C8-AHL	244.1544	10.46		x			x
OXO-C12-AHL	298.0009	21.44		x			

Note: AHLs that were only identified by cell fractionation prior to extraction are in bold. Fractions (SCS-B to F) were obtained from microbial enrichment cell separation fractions of *S. spinosulus*.

Furthermore, we also employed reverse phase flash chromatography to create numerous fractions in order to reduce sample complexity, improve the efficiency of detection as well as to increase the titer of minor constituents. This led to identification of six new long chain AHLs (OXO-C10-AHL, C18-AHL, OXO-C18-AHL, C19-AHL and OXO-C19-AHL, C18:1-AHL) apart from OHC18 and OHC14:1, which were previously identified in sponge extract and microbial enrichment methods respectively (**Table 3**). Extraction from enriched bacterial cell fractions was shown to provide additional AHLs that could not be detected when chemical analysis was performed directly on sponge crude extracts, suggesting that the previous reports on AHLs from sponges are underestimating the variety of signals found in these invertebrates. Thus, the utilization of different extraction methods on both sponge tissue and enriched microbial fraction, enabled a more exhaustive description of the wide variety of AHLs that can be found in *S. spinosulus*, identifying a wide range of AHLs including unusual long acyl chain AHLs (Table 3).

Table 3. Chromatographic and mass spectrometric data of all the observed *N*-acyl homoserine lactones (AHLs) identified by LC-HRMS/MS in the AHL-enriched fraction of sponge by reverse phase flash chromatography fraction of *S. spinosulus*. L stands for "low amount".

AHLs	M+H (exp)	rt	S1	S2	S3	S4	S5	S6
OXO-C10-AHL	270.1697	17.44						x
OH-C14:1-AHL	326.2321	23.86						x
OXO-C16-AHL	354.2635	25.22	L	x	x	L	L	
C18-AHL	368.3148	34.01	L	x	x	L	L	
OXO-C18-AHL	382.2946	31.26	L	x	x	L	L	
OH-C18-AHL	384.3103	29.95	L	x	x	L	L	
C18:1-AHL	366.2998	31.45	L	x	x	L	L	
C19-AHL	382.3312	32.83	L	x	x	L	L	
OXO-C19-AHL	396.3104	30.20	L	x	x	L	L	

The presence of these long chain AHLs in marine sponges may relate to their better stability at the high pH typical of seawater [24,44]. We report here the presence of putative C19-AHL and OXO-C19-AHL in the AHL-enriched fraction of sponge, that, to the best of our knowledge have never been reported from sponges. At one instance, the presence of unsaturated C19-AHL has been reported from a marine *Rhodobacteraceae* strain MOLA 401 [45]. As no standard were available, clues on the structure of the new compounds have been provided by HR-MS and HR-MS/MS spectra (**Figure S1**). In the extracted ion chromatogram generated at *m/z* 102.0550, the peak at t_R = 32.83 min showed a $[M+H]^+$ pseudomolecular ion at *m/z* 382.3312, which was indicative of the molecular formula $C_{23}H_{44}NO_3^+$. In the HR-MS/MS spectrum the typical fragmentation pattern of AHLs were recognized, with the homoserine lactone product ion at *m/z* 102.0550 and the acyl chain at *m/z* 281.2836, corresponding to $C_{19}H_{37}O^+$ ion. The presence of OXO-C19-AHL at *m/z* 396.3104 corresponding to the molecular formula $C_{23}H_{41}NO_4$ was also disclosed; the coherent retention time shorter than C19 and the acyl chain fragment ion at *m/z* 295.2632 ($C_{19}H_{35}O_2^+$) confirmed the hypothesis on its identity [24] .

2.3. Bioassay-guided isolation and Structural Elucidation of 1 and 2

The obtained crude extracts were combined and fractionated using reversed-phase flash column chromatography, eluting with a solvent system of 0 to 100% H_2O/CH_3CN and then with 100% of MeOH, to afford sixteen fractions (FrQ1–FrQ16). Each fraction was evaluated for potential QSI

activity (see section 4.4). Two fractions (Q1 and Q4) showing potential QSI activity were further purified to obtain active molecule. Fraction Q1 was further separated by semi-preparative reversed phase column chromatography, eluting with 10% of CH_3CN, to obtain compound **1** (1.3 mg). Compound **2** (2.1 mg) was obtained by purification of fraction Q4 using Sephadex LH20 resulting in fifteen fractions followed by semi-preparative reversed phase HPLC, eluting with a solvent system of 21% CH_3CN. The chemical structure of the two active molecules **1** and **2** was assessed by ^1H NMR, as well as by HR-ESI MS. In particular, the positive ion mode HR-ESIMS of **1** and **2** displayed M+2 isotopic pseudomolecular peaks in the ratio of 1:1 and 1:2:1, respectively, accounting for the presence of one or two bromine atoms and for the molecular formula $C_{10}H_{15}BrNO^+$ and $C_{12}H_{15}Br_2N_2^+$. Taken together, MS evidence and the comparison of the ^1H-NMR data (**Figure S2**) with compounds **2** in ref. [48] and **3** in ref. [49] allowed us to elucidate the structure of **1** and **2** as 3-Br-*N*-methyltyramine and 5,6-dibromo-*N*,*N*-dimethyltryptamine respectively (**Figure 2**).

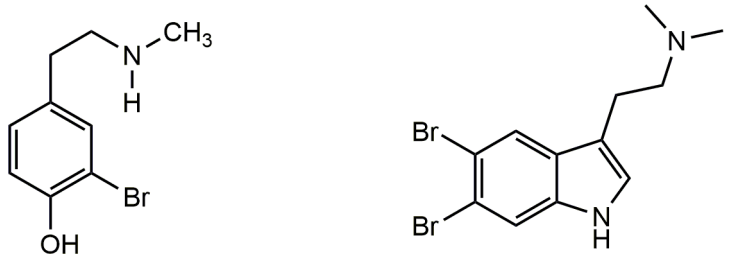

Figure 2. Chemical structure of compounds **1** and **2**.

2.4. Determination of Non-Inhibitory Concentration (NIC)

Compounds **1** and **2** were preliminary evaluated for determination of their non-inhibitory concentration (NIC) against pSB401, pSB1075 and PAO1, the strains used for testing QSI activity. Determination of NIC is important to rule out the growth inhibition artifacts. The growth-inhibitory activities of compound **1** and **2** and of the positive control, penicillic acid (PA), were tested at concentrations between 0.25 μM and 280 μM. Compound **1** and **2** showed inhibitory activity against pSB401, pSB1075 and PAO1 only at the highest concentration, i.e. 560 μM. No growth inhibition compared with the negative control (solvent only) was observed between 0.25 μM and 280 μM. Therefore, this concentration range was used for further evaluation of QSI activity.

2.5. Dose Dependent Quantification of Bioluminescence for QSI Assay

The normalized bioluminescence results for reporter strains treated with our test compounds (**1** and **2**) and activated by incubation (4 hours) with their respective cognate signal molecule are presented in **Figure 3**. A decrease in the bioluminescence in the presence of the test compound **1** and **2** or the control compound PA was interpreted as QSI activity.

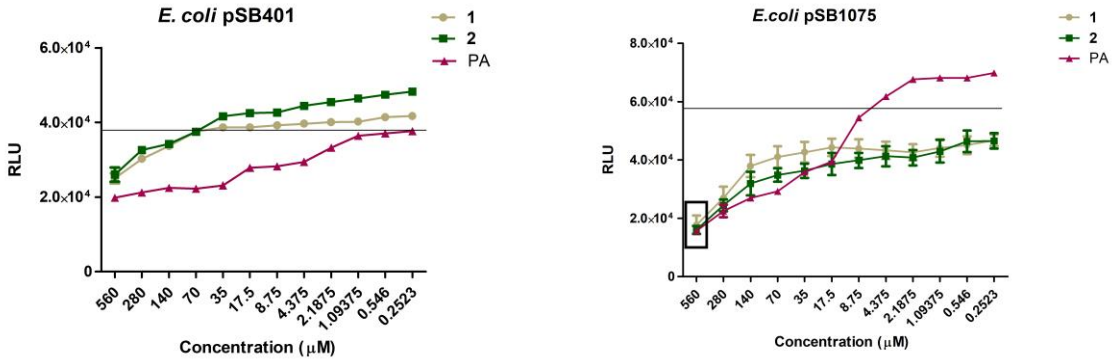

Figure 3. Dose dependent effect of **1**, **2** and penicillic acid (PA) on QS dependent bioluminescence of: **A**) The LuxR-based reporter *E. coli* pSB401 induced by OXO-C6-AHL; B) The LasR-based reporter *E. coli* pSB1075 induced by OXO-C12-AHL. Data is expressed as SD of mean (n = 2). The bioluminescence for the negative control is shown by line.

2.6. Inhibition of Production of the virulence factors Pyocyanin and Protease

To study the ability of **1** and **2** to down-regulate QS-regulated virulence factors of *P. aeruginosa* PAO1, the levels of two extracellular virulence factors were measured in the presence of the compounds. Virulence factors examined included total protease activity, which is directly controlled by the LasI/R system and pyocyanin production, which is mainly controlled by RhlI/R system. PAO1, a wild type opportunistic pathogen strain, was used for these experiments. The activity of protease and pyocyanin production was shown to be inhibited strongly by compound **1** and **2** in a dose-dependent manner (**Figure 4**).

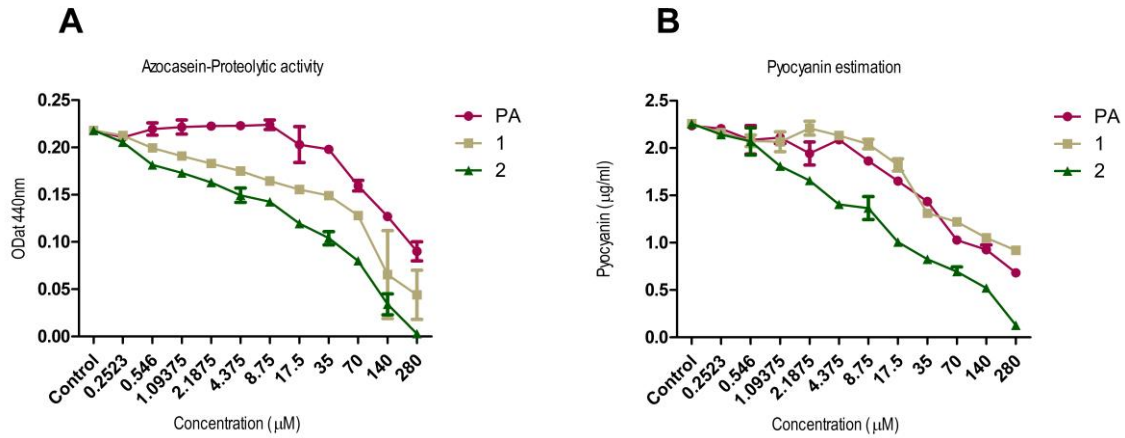

Figure 4. Dose dependent inhibition of proteolytic activity (panel **A**) and pyocyanin production (panel **B**) by **1**, **2** and penicillic acid (PA). *P. aeruginosa* PAO1 grown in the presence of diluting solvent was used as negative control in both experiments. Data is expressed as SD of mean (n = 2).

3-Br-*N*-methyltyramine (**1**), a brominated tryptamine, a natural phenylamine alkaloid found in varieties of plants. Interestingly, **1** was previously isolated from a Mediterranean gorgonian species, *Paramuricea clavata* and was found to have antifouling activity, preventing the adhesion of three bacterial strains, *Pseudoalteromonas* sp. D41 and TC8, and *Paracoccus* sp. 4M6 [46]. Based on the results of this study, we speculate that QSI may be one of the mechanisms laying behind the previously observed antifouling activity of this compound. Compound **2**, (5,6-Dibromo-*N*,*N*-dimethyltryptamine) is a natural indole alkaloid previously isolated from the

marine sponge *Hyrtios* sp. with strong antimicrobial, neurological and antidepressant activity [47,48]. However, we were not able to determine the real producer of these QSI compounds in the holobiont.

3. Experimental Section

3.1. Sponge sampling

Five sponge specimens (voucher no: 454, 455, 456, 457 and 460) were collected along the Mediterranean coast of Sdot Yam, Israel, by scuba diving at 5–12 m depth in compliance with permits n. 2012/38390 and 2013/38920 from the Israel Nature and National Parks Protection Authority. Sponges were identified morphologically following the Systema Porifera classification system [49]. Samples were placed into natural seawater using sterile scalpels and forceps and transported on ice to the laboratory for direct processing.

Each sponge sample was processed as follows. i) Few cm from each specimen was preserved in 90% ethanol as vouchers (deposited in the Marine Microbiology Laboratory, Department Marine Biology, University of Haifa, Israel), ii) part of each specimen was used for cell separation and iii) the rest of each specimen was immediately frozen in liquid nitrogen. The frozen tissues were freeze-dried using a lyophilizer, and the dried tissues were utilized for chemical extractions.

3.2. Taxonomic identification of sponge

Sponges were identified by morphological analysis and by sequencing of their cytochrome coxidase subunit I (COX1) genes. The primers for amplifying the mitochondrial COX1 gene were LCO1490 [50] and COX1-R1 [51]. The conditions of PCR amplifications were: 95 °C for 5 min; 35 cycles of 95 °C for 40 s, 50 °C for 50 s, 72 °C for 90 s; and a final extension at 72 °C for 10 min. The PCR products were purified using the PromegaWizard® SV Gel and PCR Clean-UpSystem. 799 bp-long COX1 amplicons were sequenced at Macrogen Europe (1105 AZ, Amsterdam, The Netherlands) using the LCO1490 primer. For sequence alignment, additional COX1 genes sequences were downloaded from the NCBI nucleotide collection non-redundant database (http://www.ncbi.nlm.nih.gov/). The 519 bp-long final alignment was constructed. Sequences were aligned using PAGAN 0.61 [52]. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model [46] with a discrete Gamma distribution rate variation among sites (+G). Phylogenetic robustness was inferred from 1000 bootstrap replications [40]. Evolutionary analyses were conducted in MEGA7 [53].

3.3. Microbial enrichment by cell separation and its extraction

In addition to direct extraction of sponge tissue, we also tested the potential for AHL detection in the fractions enriched with microbial cells by cell-separation. For cell separation, sponges were first washed in calcium magnesium-free seawater (CMFSW; 25 g NaCl, 0.8 g KCl, 1 g Na_2SO_4, 0.04 g $NaHCO_3$ per 1 L) to remove loosely attached cells. The washed sponge materials were then cut into 1 cm^3 cubes and homogenized for 10–15 seconds using fresh CMFSW. Microbial enrichment by cell separation was then attained using a pre-established protocol by a series of filtration and centrifugation steps as described previously [54]. Briefly, the homogenized samples were filtered through a 125 µm sieve into a sterile centrifuge tube (sample SCS-A) and the filtrate was centrifuged for 15 minutes at 100 × g at 4 °C to remove remaining sponge cells and tissues (sample SCS-B). The supernatant was then centrifuged twice for 15 minutes at 300 × g at 4 °C to remove the diatoms from the sample (sample SCS-C). The supernatant was afterwards filtered through a 11 µm filter using the vacuum filtration unit (sample SCS-D), and the final filtrate were centrifuged for 20 mins at 8,800 × g and 12,000 rpm at 4 °C to pellet microbial cells (samples SCS-E and SCS-F). The first fraction SCS-A,

obtained as filtrate debris from 125 μm sieve filtration of homogenized sponge sample was extracted with $CHCl_3$ (0.5 L × 2) (SCS-A C), $CHCl_3$-MeOH 1:1 (0.5 L × 2) (SCS-A C:M), and MeOH (0.5 L × 2) (SCS-A M). The next five fractions (SCS-B to F) obtained from cell separation were extracted with equal (~0.5 L) volume of butanone. The organic phase was analyzed to check the presence of AHLs and QSI compounds as described below.

3.4. Crude extract preparation and preliminary screening for QSI activity

The remaining part of all the five freeze-dried biomass specimens were macerated, and repeatedly extracted with MeOH (0.5 L × 3), MeOH/$CHCl_3$ (0.5 L × 2) and $CHCl_3$ (0.5 L × 2) separately at room temperature. QSI activity was tested using biosensors *Chromobacterium violaceum* CV026 [55] and with an adaptation of the thin layer chromatography (TLC) overlay technique using *Agrobacterium tumefaciens* NTL4 [29,56]. In brief, *A. tumefaciens* NTL4 was cultured in AB broth medium containing 6% (w/v) K_2HPO_4, 2% (w/v) KH_2PO_4, 2% (w/v) NH_4Cl, 0.6% (w/v) $MgSO_4 \cdot 7H_2O$, 0.3% (w/v) KCl, 0.02% (w/v) $CaCl_2$, and 0.005% (w/v) $FeSO_4 \cdot 7H_2O$), supplemented with 30 μg/mL gentamicin and 0.7% (w/v) glucose. Aliquots (~160 μg) of crude extracts were spotted on reverse phase C18-TLC plates (RP-C18 TLC), air-dried, overlaid with AB medium supplemented with 0.7% agar, X-Gal (40 μg/mL), C1-AHL (100 nM) and the biosensor *A. tumefaciens* NTL4, and incubated overnight at 30 °C. In order to improve the detection ability of AHLs in sponge extracts, we combined all the five specimen extracts (7.8 g) and fractionated the combined extract by reversed-phase flash column chromatography (Sigma ODS-A, 60 Å 500/400 mesh), eluting with a solvent system of 0 to 100% H_2O/ACN and then with 100% MeOH, to afford sixteen fractions (FrS1–FrS16). The obtained fractions were then evaluated for the presence of AHLs using LC-HRMS/MS.

3.5. AHLs identification using LC-HRMS/MS analysis

Microbial enriched fraction crude extracts (SCS-A C, SCS-A C:M, and SCS-A M), crude extracts from different sponge specimens (454, 455, 456, 457 and 460), and fractions S1-S16 (obtained by fractionation of combined crude extracts from different sponge specimens) were analyzed for the presence of AHL molecules using high-resolution ESI mass spectrometry experiments (LC-HRMS and LC-HRMS/MS) using a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Spa, Rodano, Italy) coupled to an Agilent model 1100 LC system (Agilent Technologies, Cernusco sul Naviglio, Italy) equipped with a solvent reservoir, an in-line degasser, a binary pump and a refrigerated autosampler. The spectra were recorded by infusion into the ESI source using MeOH as the solvent. A 5 μm Kinetex C18 column (50 × 2.1 mm), maintained at 25 °C, was operated using a gradient elution of H_2O and ACN both with 0.1% formic acid, running at 200 μL/min. The gradient program was as follows: 10% ACN for 3 min, 10%–90% ACN over 30 min, 90% ACN for 3 min. All the mass spectra were recorded in the positive-ion mode. MS parameters were a spray voltage of 5 kV, a capillary temperature of 230 °C, a sheath gas rate of 12 units N_2 (ca. 120 mL/min), and an auxiliary gas rate of 5 units N_2 (ca. 50 mL/min). Data were collected in the Surface Induced Dissociation (SID) mode at 40 eV. 5 μL of a mixture of commercially available synthetic AHLs (C4-AHL, C6-AHL, OXO-C6-AHL, C8-AHL, OXO-C8-AHL, OH-C8-AHL, C10-AHL, OXO-C10-AHL, 3-OH-C10-AHL, C12-AHL, OXO-C12-AHL, OH-C12-AHL, C14-AHL, OXO-C14-AHL, OH-C14-AHL, C16-AHL, OXO-C16-AHL, OH-C16-AHL, C18-AHL, OXO-C18-AHL, OH-C18-AHL) were used (10 μg/mL each); the extracted ion chromatogram was generated at *m/z* 102.0550, corresponding to the characteristic product ion of deacylated homoserine lactone. AHLs in various fractions were identified based on the comparison of their retention time and HRMS/MS spectra with those of the synthetic standards.

3.6. Bioassay guided purification and identification of molecules with QSI activity

The preliminary screening of sponge extracts for QSI activity (as described in the previous section) from all the five specimens displayed moderate activity, hence we collected another set of ten specimens of the same sponge species at the same location. All the specimens were combined, lyophilized (dry wt. ~121 g), macerated, and repeatedly extracted with MeOH (2 L × 3), MeOH/$CHCl_3$ (1.5 L × 2) and $CHCl_3$ (2 L× 2) at room temperature. The obtained crude extracts were combined and fractionated using reversed-phase flash column chromatography (Sigma ODS-A, 60 Å 500/400 mesh), eluting with a solvent system of 0 to 100% H_2O/AcCN and then with 100% of MeOH, to afford sixteen fractions (FrQ1–FrQ16). Fraction Q1 was further separated by semi preparative reversed phase column chromatography (Phenomenax ODS-A, 60 Å, 500/400 mesh), eluting with 10% of CH_3CN, to obtain compound **1** (1.3 mg). Compound **2** (2.1 mg) was obtained by purification of fraction Q4 using Sephadex LH20 resulting in fifteen fractions followed by semi preparative reversed phase HPLC, eluting with a solvent system of 21% CH_3CN. The NMR spectra were acquired on a Varian Unity Inova 700 MHz spectrometer equipped with a triple resonance cryo-probe (Agilent Technologies, Cernusco sul Naviglio, Italy). The chemical shifts were referenced to the residual solvent signal (CD_3OD: δH 3.31, δC 49.01). For an accurate measurement of the coupling constants, the one-dimensional ^1H NMR spectra were transformed at 64-K points (digital resolution: 0.09 Hz). High performance liquid chromatography (HPLC) was performed on a Varian Prostar 210 apparatus equipped with a Varian 350 refractive index detector (Agilent Technologies, Cernusco sul Naviglio, Italy).

Compound (**1**): HRESIMS: t_R = 5.0 min; $[M + H]^+$ m/z 230.0167 and 232.0147 for $C_{10}H_{15}BrNO$, calcd. 230.0181 and 232.0160 (**Figure S2**); ^1H NMR (700 MHz, CD_3OD): δ 7.5 (1H, d, J= 2.0), 7.2 (1H, dd, J=1.8, 8.7), 7.0 (1H, d, J=8.3), 3.8 (3H, s, CH_3-NH), 3.1 (2H, t, J=7.6), 2.8 (t, J=7.6) (**Figure S3**).

Compound (**2**) HRESIMS: t_R = 13.3 min; $[M + H]^+$ m/z 344.9581, 346.9559 and 348.9539 for $C_{12}H_{15}Br_2N_2$; calcd. 344.9602, 346.9582 and 348.9561 (**Figure S2**); ^1H NMR (700 MHz, CD_3OD): 7.9 (1H, s), 7.7 (1H, s), 7.2 (1H, s), 3.2 (2H, br t, J=7.2), 3.1 2H, (br t, J=7.1), 2.8 (6H, s, N-$(CH_3)_2$) (**Figure S3**).

3.7. Determination of Non-Inhibitory concentration (NIC)

The non-inhibitory concentration (NIC) was determined by the broth two-fold micro dilution method (CLSI M100-S20) (CLSI 2000) for compounds **1** and **2** against pSB401, pSB1075 and PAO1, the strains used for testing quorum quenching activity. Briefly, the purified compounds were serially diluted (0.252–560 μM) using methanol in Muller-Hinton broth. The inoculum (approximately 5×10^5 CFU/mL final concentration) was prepared from an overnight culture and was added to each well containing the compound. After incubating 96-well flat-bottomed plates aerobically at 37.8 ºC for 24 h, the optical density (OD) was measured using a spectrophotometer (600 nm) using TriStar Multimode Microplate reader (Berthold Technologies GmbH& Co. KG, Germany) to determine NIC values. Negative controls (culture + methanol) were included. All the experiments were run in triplicates.

3.8. Dose dependent quantification of Bioluminescence for QSI assay

The well diffusion QSI assay was performed using *C. violaceum* CV026 following a pre-established protocol [55], whereas the bioluminescence based QSI assay using *E. coli* pSB401 and *E. coli* pSB1075 reporters were quantified on a TriStar Multimode Microplate reader (Berthold Technologies GmbH & Co. KG, Germany) following [57]. Plasmid psB401 has a *luxR* gene and the promoter of *luxI* controlling *luxCDABE* which is responsible for luminescence in the presence of exogenous addition

of AHLs with medium (C6-C8) acyl side chain length. Plasmid pSB1075 contains *lasR* gene and cognate *lasI* gene promoter controlling *luxCDABE* expression, and responds well to exogenously provided long chain AHLs (e.g. C12-AHL, OXO-C10-AHL, and OXO-C12-AHL) [57]. Briefly, the stock solutions (10 mM) of compounds 1 and 2 were serially diluted to NIC concentrations (0.252–280 µM) in Luria-Bertani (LB) medium. The inocula of the reporter strains (OD600 0.01, final concentration) were prepared from an overnight culture and added (50 µL) to each well. The inoculum was supplemented with N-(3-oxo-hexanoyl)-l-homoserine lactone (3-oxo-C6-AHL, 1 µM final concentration) and N-(3-oxo-dodecanoyl)-l-homoserine lactone (3-oxo-C12-AHL, 2 µM final concentration) to stimulate QS of pSB401 and pSB1075 biosensors, respectively. The bioluminescence was recorded every 30 min for 7 hours at 30 °C. The production of bioluminescence in the graphs is given as the relative light units (RLU), obtained at 4 h.

3.9. Inhibition of production of virulence factors – pyocyanin and protease

The inhibition of pyocyanin and protease was tested for compound **1** and **2** in a dose dependent manner at NIC concentration using *Pseudomonas aeruginosa* PAO1 as described earlier [40,55,58]. Methanol (solvent in which test compounds were dissolved) and penicillic acid (PA) were used for negative and positive control, respectively, for both the experiments. Briefly, for inhibition of pyocyanin production, an overnight culture of *P. aeruginosa* PAO1 was diluted to an OD600 of 0.2 using LB medium (4.5 mL). The diluted cultures were then supplemented with 250 µL of compounds (**1** and **2**) at the NIC concentration range. After overnight incubation at 37 °C and 200 rpm, 3 mL of chloroform was added to each test tube and mixed vigorously. The organic layer was collected by centrifugation (2,000 g) and transferred to a fresh tube. 1 mL of 0.2 M hydrochloric acid was added to the organic layer and the absorbance was measured at 520 nm. The experiments were performed in triplicate. Pyocyanin concentration (µL/mL) was calculated as:

$$P = (OD \times 17.072) \times 1.5$$

Where, OD is the optical density value obtained at 520 nm, 17.072 is the extinction coefficient to obtain the value in µg/mL, and 1.5 is the dilution factor (3 mL from initial 4.5 mL of chloroform were used) [59].

Proteolytic inhibition activity was performed in a 96-well plate containing the overnight inoculums of PAO1 (approximately $5X10^5$ CFU/mL, final concentration) treated with test compounds (**1** and **2**) and incubated at 37 ºC for 16 h. Once the OD600 reached 0.4, the plate was centrifuged and 30 µL of supernatant was transferred to clean Eppendorf tubes containing 50 µL of 0.8% azocasein. The mixture was further incubated at 37 °C for 12 h. Subsequently, the mixture was incubated at room temperature for 15 min with 240 µL of 10% (w/v) trichloroacetic acid (TCA) followed by centrifugation for 10 min (15,000 g). Finally, 240 µL of 1 M NaOH was added to the mixture and spectroscopic measurement was recorded at OD440.

3.10. Data Deposition

Sequences of the amplified *cox1* of *S. spinosulus* were deposited on NCBI with accession numbers MK350313 - MK350317.

4. Conclusion

In this study, we report the QSI activity of two brominated tryptamines coexisting together with QS molecules (AHLs), which provides an evidence for opposite functions related to cell-cell signaling within the same sponge species *S. spinosulus*. Molecules with QS interfering activity, such as the here identified 3-Br-*N*-methyltyramine (**1**) and 5,6-dibromo-*N*, *N*-dimethyltryptamine (**2**) compounds,

may be involved in a fine-tuned regulation of concentration of AHL signals through concomitant production and interference of QS signals. The real producer of the QSI compounds here elucidated remains to be determined and could be either microbial or eukaryotic. The potential for interaction between QS and QSI molecules within *S. spinosulus* remains to be determined, and future studies will aim at the chemical localization of these molecules within the sponge tissue.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Figure S1: HR-MS and HR-MS/MS spectra of the new compounds C19-HSL and OC19-HSL; a) extracted ion chromatogram from the LC-HRMS analysis of the sponge enriched fraction S2 at m/z 102.0550 (black trace), corresponding to deacylated homoserine lactone, m/z 382.3312 (pink trace), corresponding to C19-HSL and m/z 396.3104 (green trace), corresponding to OC19-HSL; b), c) HR-MS and HR-MS/MS spectra of C19-HSL; d), e) HR-MS and HR-MS/MS spectra of OC19-HSL. Figure S2: HR-MS spectra of compounds 1 and 2. Figure S3: 1HNMR spectra of compounds 1 and 2.

Author's Contributions: All authors have read and agree to the published version of the manuscript. Conceptualization, K.S., N.B., I.B., L.S. and V.C.; methodology, K.S., N.B., R.T., R.B.S., I.B., G.E., A.C. and M.B.; data curation, K.S., I.B., L.S., and V.C.; writing—original draft preparation, K.S., I.B., R.T., N.B., A.C., L.S., and V.C.; writing—review and editing, K.S., I.B., R.T., N.B., A.C., L.S., and V.C.; supervision, L.S. and V.C.; project administration, R.B.S., L.S. and V.C.; funding acquisition, L.S. and V.C.

Funding: This study was supported by the Israel Science Foundation [grant no. 1243/16] titled "Identification of molecular mechanisms underlying sponge-microbiome symbiosis" and the "Programma Sostegno Territoriale alle attività di ricerca (STAR)", Università degli Studi di Napoli Federico II (UNINA) Project entitled 'SeaLeads'. K.S had a post-doctoral fellowship from the Israeli Council for Higher Education (VATAT) and University of Haifa. K.S post-doctoral fellowship was also supported by the Università degli Studi di Napoli Federico II, bilateral agreement Haifa-Naples 2015-2018. This research was also funded by Regione Campania, PO FESR 2014-2020, O.S. 1.2, Project "Campania Oncoterapie" No. B61G18000470007.

Acknowledgement: We would like to thank Dr. Markus Haber and the Staff at the Morris Khan Marine Research Station for support with the diving activities during this study. Sponge samples were collected in compliance with permits 2012/38390 and 2013/38920 from the Israel Nature and National Parks Protection Authority.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rasko, D.A.; Sperandio, V. Anti-virulence strategies to combat bacteria-mediated disease. *Nat Rev Drug Discov* **2010**, *9*, 117-128.
2. LaSarre, B.; Federle, M.J. Exploiting Quorum Sensing To Confuse Bacterial Pathogens. *Microbiology and Molecular Biology Reviews : MMBR* **2013**, *77*, 73-111.
3. López, D.; Vlamakis, H.; Losick, R.; Kolter, R. Paracrine signaling in a bacterium. *Genes Dev* **2009**, *23*, 1631-1638.
4. Rutherford, S.T.; Bassler, B.L. Bacterial Quorum Sensing: Its Role in Virulence and Possibilities for Its Control. *Cold Spring Harbor Perspectives in Medicine* **2012**, *2*.
5. Cegelski, L.; Marshall, G.R.; Eldridge, G.R.; Hultgren, S.J. The biology and future prospects of antivirulence therapies. *Nat Rev Microbiol* **2008**, *6*, 17-27.
6. Dong, Y.H.; Wang, L.H.; Xu, J.L.; Zhang, H.B.; Zhang, X.F.; Zhang, L.H. Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature* **2001**, *411*, 813-817.

7. Dong, Y.H.; Xu, J.L.; Li, X.Z.; Zhang, L.H. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc Natl Acad Sci U S A* **2000**, *97*, 3526-3531.
8. Hoang, T.T.; Schweizer, H.P. Characterization of *Pseudomonas aeruginosa* enoyl-acyl carrier protein reductase (FabI): a target for the antimicrobial triclosan and its role in acylated homoserine lactone synthesis. *J Bacteriol* **1999**, *181*, 5489-5497.
9. Ding, X.; Yin, B.; Qian, L.; Zeng, Z.; Yang, Z.; Li, H.; Lu, Y.; Zhou, S. Screening for novel quorum-sensing inhibitors to interfere with the formation of *Pseudomonas aeruginosa* biofilm. *J Med Microbiol* **2011**, *60*, 1827-1834.
10. Yang, L.; Rybtke, M.T.; Jakobsen, T.H.; Hentzer, M.; Bjarnsholt, T.; Givskov, M.; Tolker-Nielsen, T. Computer-aided identification of recognized drugs as *Pseudomonas aeruginosa* quorum-sensing inhibitors. *Antimicrob Agents Chemother* **2009**, *53*, 2432-2443.
11. Gold, D.A.; Grabenstatter, J.; de Mendoza, A.; Riesgo, A.; Ruiz-Trillo, I.; Summons, R.E. Sterol and genomic analyses validate the sponge biomarker hypothesis. *Proceedings of the National Academy of Sciences* **2016**, *113*, 2684-2689.
12. Littlewood, D.T.J. Animal Evolution: Last Word on Sponges-First? *Current Biology* **2017**, *27*, R259-R261.
13. Maldonado, M.; Ribes, M.; van Duyl, F.C. Nutrient fluxes through sponges: biology, budgets, and ecological implications. *Adv Mar Biol* **2012**, *62*, 113-182.
14. Bell, J.J. The functional roles of marine sponges. *Estuarine, Coastal and Shelf Science* **2008**, *79*, 341-353.
15. Pawlik, J.R. The Chemical Ecology of Sponges on Caribbean Reefs: Natural Products Shape Natural Systems. *BioScience* **2011**, *61*, 888-898.
16. Carroll, A.R.; Copp, B.R.; Davis, R.A.; Keyzers, R.A.; Prinsep, M.R. Marine natural products. *Natural Product Reports* **2019**, *36*, 122-173.
17. Martins, M.L.; Pinto, U.M.; Riedel, K.; Vanetti, M.C.; Mantovani, H.C.; de Araujo, E.F. Lack of AHL-based quorum sensing in *Pseudomonas fluorescens* isolated from milk. *Braz J Microbiol* **2014**, *45*, 1039-1046.
18. Esteves, A.I.S.; Hardoim, C.C.P.; Xavier, J.R.; Gonçalves, J.M.S.; Costa, R. Molecular richness and biotechnological potential of bacteria cultured from Irciniidae sponges in the north-east Atlantic. *FEMS Microbiology Ecology* **2013**, *85*, 519-536.
19. Hentschel, U.; Piel, J.; Degnan, S.M.; Taylor, M.W. Genomic insights into the marine sponge microbiome. *Nat Rev Micro* **2012**, *10*, 641-654.
20. Piel, J.; Hui, D.; Wen, G.; Butzke, D.; Platzer, M.; Fusetani, N.; Matsunaga, S. Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge *Theonella swinhoei*. *Proc Natl Acad Sci U S A* **2004**, *101*, 16222-16227.
21. Wilson, M.C.; Mori, T.; Ruckert, C.; Uria, A.R.; Helf, M.J.; Takada, K.; Gernert, C.; Steffens, U.A.; Heycke, N.; Schmitt, S., *et al.* An environmental bacterial taxon with a large and distinct metabolic repertoire. *Nature* **2014**, *506*, 58-62.
22. Zan, J.; Fuqua, C.; Hill, R.T. Diversity and functional analysis of luxS genes in *Vibrios* from marine sponges *Mycale laxissima* and *Ircinia strobilina*. *The ISME Journal* **2011**, *5*, 1505-1516.
23. Esposito, G.; Teta, R.; Della Sala, G.; Pawlik, J.R.; Mangoni, A.; Costantino, V. Isolation of Smenopyrone, a Bis-gamma-Pyrone Polypropionate from the Caribbean Sponge *Smenospongia aurea*. *Mar Drugs* **2018**, *16*.

24. Saurav, K.; Burgsdorf, I.; Teta, R.; Esposito, G.; Bar-Shalom, R.; Costantino, V.; Steindler, L. Isolation of Marine Paracoccus sp. Ss63 from the Sponge *Sarcotragus sp.* and Characterization of its Quorum-Sensing Chemical-Signaling Molecules by LC-MS/MS Analysis. *Israel Journal of Chemistry* **2016**, *56*, 330-340.

25. González, J.E.; Keshavan, N.D. Messing with Bacterial Quorum Sensing. *Microbiology and Molecular Biology Reviews* **2006**, *70*, 859-875.

26. Gardères, J.; Taupin, L.; Saïdin, J.; Dufour, A.; Le Pennec, G. N-acyl homoserine lactone production by bacteria within the sponge *Suberites domuncula* (Olivi, 1792) (Porifera, Demospongiae). *Mar Biol* **2012**, *159*, 1685-1692.

27. Mohamed, N.M.; Cicirelli, E.M.; Kan, J.; Chen, F.; Fuqua, C.; Hill, R.T. Diversity and quorum-sensing signal production of Proteobacteria associated with marine sponges. *Environ Microbiol* **2008**, *10*, 75-86.

28. Britstein, M.; Saurav, K.; Teta, R.; Sala, G.D.; Bar-Shalom, R.; Stoppelli, N.; Zoccarato, L.; Costantino, V.; Steindler, L. Identification and chemical characterization of *N*-acyl-homoserine lactone quorum sensing signals across sponge species and time. *FEMS Microbiol Ecol* **2018**, *94*.

29. Saurav, K.; Bar-Shalom, R.; Haber, M.; Burgsdorf, I.; Oliviero, G.; Costantino, V.; Morgenstern, D.; Steindler, L. In Search of Alternative Antibiotic Drugs: Quorum-Quenching Activity in Sponges and their Bacterial Isolates. *Front Microbiol* **2016**, *7*, 416.

30. Skindersoe, M.E.; Ettinger-Epstein, P.; Rasmussen, T.B.; Bjarnsholt, T.; de Nys, R.; Givskov, M. Quorum Sensing Antagonism from Marine Organisms. *Marine Biotechnology* **2008**, *10*, 56-63.

31. Mai, T.; Tintillier, F.; Lucasson, A.; Moriou, C.; Bonno, E.; Petek, S.; Magre, K.; Al Mourabit, A.; Saulnier, D.; Debitus, C. Quorum sensing inhibitors from *Leucetta chagosensis* Dendy, 1863. *Lett Appl Microbiol* **2015**, *61*, 311-317.

32. Dobretsov, S.; Teplitski, M.; Bayer, M.; Gunasekera, S.; Proksch, P.; Paul, V.J. Inhibition of marine biofouling by bacterial quorum sensing inhibitors. *Biofouling* **2011**, *27*, 893-905.

33. Ong, J.F.M.; Goh, H.C.; Lim, S.C.; Pang, L.M.; Chin, J.S.F.; Tan, K.S.; Liang, Z.-X.; Yang, L.; Glukhov, E.; Gerwick, W.H., *et al.* Integrated Genomic and Metabolomic Approach to the Discovery of Potential Anti-Quorum Sensing Natural Products from Microbes Associated with Marine Samples from Singapore. *Marine Drugs* **2019**, *17*, 72.

34. Teplitski, M.; Robinson, J.B.; Bauer, W.D. Plants secrete substances that mimic bacterial *N*-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. *Mol Plant Microbe Interact* **2000**, *13*, 637-648.

35. Manefield, M.; de Nys, R.; Kumar, N.; Read, R.; Givskov, M.; Steinberg, P.; Kjelleberg, S. Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. *Microbiology* **1999**, *145 (Pt 2)*, 283-291.

36. Keshavan, N.D.; Chowdhary, P.K.; Haines, D.C.; Gonzalez, J.E. L-Canavanine made by Medicago sativa interferes with quorum sensing in *Sinorhizobium meliloti*. *J Bacteriol* **2005**, *187*, 8427-8436.

37. Igarashi, Y.; Yamamoto, K.; Fukuda, T.; Shojima, A.; Nakayama, J.; Carro, L.; Trujillo, M.E. Arthroamide, a Cyclic Depsipeptide with Quorum Sensing Inhibitory Activity from *Arthrobacter sp*. *Journal of Natural Products* **2015**, *78*, 2827-2831.

38. Abed, R.M.; Dobretsov, S.; Al-Fori, M.; Gunasekera, S.P.; Sudesh, K.; Paul, V.J. Quorum-sensing inhibitory compounds from extremophilic microorganisms isolated from a hypersaline cyanobacterial mat. *J Ind Microbiol Biotechnol* **2013**, *40*, 759-772.

39. Gross, H.; Goeger, D.E.; Hills, P.; Mooberry, S.L.; Ballantine, D.L.; Murray, T.F.; Valeriote, F.A.; Gerwick, W.H. Lophocladines, Bioactive Alkaloids from the Red Alga *Lophocladia sp. Journal of Natural Products* **2006**, *69*, 640-644.

40. Costantino, V.; Della Sala, G.; Saurav, K.; Teta, R.; Bar-Shalom, R.; Mangoni, A.; Steindler, L. Plakofuranolactone as a Quorum Quenching Agent from the Indonesian Sponge *Plakortis cf. lita*. *Mar Drugs* **2017**, *15*.

41. Tello, E.; Castellanos, L.; Arévalo-Ferro, C.; Duque, C. Disruption in Quorum-Sensing Systems and Bacterial Biofilm Inhibition by Cembranoid Diterpenes Isolated from the Octocoral *Eunicea knighti*. *Journal of Natural Products* **2012**, *75*, 1637-1642.

42. Gilabert, M.; Ramos, A.N.; Schiavone, M.M.; Arena, M.E.; Bardón, A. Bioactive Sesqui- and Diterpenoids from the Argentine Liverwort *Porella chilensis*. *Journal of Natural Products* **2011**, *74*, 574-579.

43. Kawamura, A.; Iacovidou, M.; Hirokawa, E.; Soll, C.E.; Trujillo, M. 17-Hydroxycyclooctatin, a Fused 5−8−5 Ring Diterpene, from *Streptomyces sp*. MTE4a. *Journal of Natural Products* **2011**, *74*, 492-495.

44. Bojko, B.; Onat, B.; Boyaci, E.; Psillakis, E.; Dailianis, T.; Pawliszyn, J. Application of in situ Solid-Phase Microextraction on Mediterranean Sponges for Untargeted Exometabolome Screening and Environmental Monitoring. *Frontiers in Marine Science* **2019**, *6*.

45. Doberva, M.; Stien, D.; Sorres, J.; Hue, N.; Sanchez-Ferandin, S.; Eparvier, V.; Ferandin, Y.; Lebaron, P.; Lami, R. Large Diversity and Original Structures of Acyl-Homoserine Lactones in Strain MOLA 401, a Marine Rhodobacteraceae Bacterium. *Frontiers in Microbiology* **2017**, *8*.

46. Pénez, N.; Culioli, G.; Pérez, T.; Briand, J.-F.; Thomas, O.P.; Blache, Y. Antifouling Properties of Simple Indole and Purine Alkaloids from the Mediterranean Gorgonian *Paramuricea clavata*. *Journal of Natural Products* **2011**, *74*, 2304-2308.

47. Mollica, A.; Locatelli, M.; Stefanucci, A.; Pinnen, F. Synthesis and bioactivity of secondary metabolites from marine sponges containing dibrominated indolic systems. *Molecules* **2012**, *17*, 6083-6099.

48. Longeon, A.; Copp, B.R.; Quevrain, E.; Roue, M.; Kientz, B.; Cresteil, T.; Petek, S.; Debitus, C.; Bourguet-Kondracki, M.L. Bioactive indole derivatives from the South Pacific marine sponges *Rhopaloeides odorabile* and Hyrtios sp. *Mar Drugs* **2011**, *9*, 879-888.

49. J.N.A. Hooper; Soest, R.W.M.v. Systema Porifera, a guide to the classification of the sponges (in 2 volumes). *eds J. N. A. Hooper, R. W. M. Van Soest, and P. Willenz (New York, NY: Springer).* **2002**, 1706.

50. Doberva, M.; Stien, D.; Sorres, J.; Hue, N.; Sanchez-Ferandin, S.; Eparvier, V.; Ferandin, Y.; Lebaron, P.; Lami, R. Large Diversity and Original Structures of Acyl-Homoserine Lactones in Strain MOLA 401, a Marine Rhodobacteraceae Bacterium. *Front Microbiol* **2017**, *8*, 1152.

51. Wagner-Dobler, I.; Thiel, V.; Eberl, L.; Allgaier, M.; Bodor, A.; Meyer, S.; Ebner, S.; Hennig, A.; Pukall, R.; Schulz, S. Discovery of complex mixtures of novel long-chain quorum sensing signals in free-living and host-associated marine alphaproteobacteria. *Chembiochem* **2005**, *6*, 2195-2206.

52. Zan, J.; Cicirelli, E.M.; Mohamed, N.M.; Sibhatu, H.; Kroll, S.; Choi, O.; Uhlson, C.L.; Wysoczynski, C.L.; Murphy, R.C.; Churchill, M.E., *et al.* A complex LuxR-LuxI type quorum sensing network in a roseobacterial marine sponge symbiont activates flagellar motility and inhibits biofilm formation. *Mol Microbiol* **2012**, *85*, 916-933.

53. Sperry, J. Concise syntheses of 5,6-dibromotryptamine and 5,6-dibromo-N,N-dimethyltryptamine en route to the antibiotic alternatamide D. *Tetrahedron Letters* **2011**, *52*, 4042-4044.

54. Burgsdorf, I.; Slaby, B.M.; Handley, K.M.; Haber, M.; Blom, J.; Marshall, C.W.; Gilbert, J.A.; Hentschel, U.; Steindler, L. Lifestyle evolution in cyanobacterial symbionts of sponges. *MBio* **2015**, *6*, e00391-00315.

55. McClean, K.H.; Winson, M.K.; Fish, L.; Taylor, A.; Chhabra, S.R.; Camara, M.; Daykin, M.; Lamb, J.H.; Swift, S.; Bycroft, B.W., *et al.* Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* **1997**, *143 (Pt 12)*, 3703-3711.

56. Hardoim, C.C.; Costa, R. Temporal dynamics of prokaryotic communities in the marine sponge *Sarcotragus spinosulus*. *Mol Ecol* **2014**, *23*, 3097-3112.

57. Winson, M.K.; Swift, S.; Fish, L.; Throup, J.P.; Jorgensen, F.; Chhabra, S.R.; Bycroft, B.W.; Williams, P.; Stewart, G.S. Construction and analysis of luxCDABE-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol Lett* **1998**, *163*, 185-192.

58. Pejin, B.; Talevska, A.; Ciric, A.; Glamoclija, J.; Nikolic, M.; Talevski, T.; Sokovic, M. Anti-quorum sensing activity of selected sponge extracts: a case study of *Pseudomonas aeruginosa*. *Nat Prod Res* **2014**, *28*, 2330-2333.

59. El-Fouly, M.Z.; Sharaf, A.M.; Shahin, A.A.M.; El-Bialy, H.A.; Omara, A.M.A. Biosynthesis of pyocyanin pigment by *Pseudomonas aeruginosa*. *Journal of Radiation Research and Applied Sciences* **2015**, *8*, 36-48.