The Bromotyrosine Derivative Ianthelline Isolated from the Arctic Marine Sponge *Stryphnus fortis* Inhibits Marine Micro- and Macrobiofouling

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Abstract

The inhibition of marine biofouling by the bromotyrosine derivative ianthelline, isolated from the Arctic marine sponge *Stryphnus fortis*, is described. All major stages of the fouling process are investigated. The effect of ianthelline on adhesion and growth of marine bacteria and microalgae is tested to investigate its influence on the initial microfouling process comparing with the known marine antifoulant barettin as a reference. Macrofouling is studied via barnacle (*Balanus improvisus*) settlement assays and blue mussel (*Mytilus edulis*) phenoloxidase inhibition. Ianthelline is shown to inhibit both marine micro- and macrofoulers with a pronounced effect on marine bacteria (minimum inhibitory concentration (MIC) values 0.1–10 μg/mL) and barnacle larval settlement (IC_{50}=3.0 μg/mL). Moderate effects are recorded on *M. edulis* (IC_{50}=45.2 μg/mL) and microalgae, where growth is more affected than surface adhesion. The effect of ianthelline is also investigated against human pathogenic bacteria. Ianthelline displayed low micromolar MIC values against several bacterial strains, both Gram positive and Gram negative, down to 2.5 μg/mL. In summary, the effect of ianthelline on 20 different representative marine antifouling organisms and seven human pathogenic bacterial strains is presented.

Introduction

Rapid colonisation and successive overgrowth of epibiotic organisms on a surface immersed in water is a process known as biofouling. It is a fast and complex sequence of events which is initiated by surface adhesion of organic molecules, followed by the rapid settlement of microfoulers (marine bacteria, protozoans and microalgae) and, subsequently, macrofoulers (macroalgae and invertebrates) (Qian et al. 2007; Yebra et al. 2004). Biofouling is a natural process, yet it is highly undesired for a wide range of marine economic sectors such as shipping, aquaculture and the offshore petroleum industry. The growth on associated man-made structures leads to substantial economic losses in these sectors mainly due to increased fuel usage, maintenance costs and process parameter interference (Yebra et al. 2004; Fitridge et al. 2012). Employment of antifouling (AF) paints has been the most successful strategy to prevent biofouling on such structures (Almeida et al. 2007). The most efficient AF compounds such as organotin and copper oxide have been shown to come with several serious environmental drawbacks (Okoro et al. 2011; Kotake 2012; Nakanishi 2008) due to the collateral damage inflicted on the marine environment and non-target species. The high toxicity and induction of imposex, i.e. the development of male characteristics by females, amongst marine molluscs (Ellis and Pattisina 1990; Nakanishi 2008) resulted in a total ban on the use of organotin-based compounds in the marine environment which was implemented in 2008.

Not only man-made structures submerged in water but also sessile marine organisms such as sponges and tunicates serve as surfaces for biofouling and are under a constant threat of being overgrown by epibionts. Despite this, many sessile marine organisms appear to have developed strategies to combat epibiosis and maintain a clean exterior surface, free of settling species (Muller et al. 2013). This observation has motivated the search formarine compounds with AF activities from their arsenal of secondary metabolites (Tsukamoto et al. 1996a; Fusetani 2004, 2011; Hellio et al. 2005). Marine sponges in particular are a rich source of bioactive secondary metabolites, some with documented AF activity (Sipkema et al. 2005; Stowe et al. 2011; Gerwick and Moore 2012). Sponges are primitive sessile filter-feeders that lack a specialised immune system and the ability to remove themselves from predators and settling organisms. To prevail, they have developed physical and chemical defence strategies to protect themselves against over-growth by epibionts (Muller et al. 2013). Sponges commonly host a range of microbial symbionts that may produce compounds protecting their host. Sponges therefore represent a particularly rich marine source for discovering novel compounds (Gerwick and Fenner 2013; Gerwick and Moore 2012; Ortlepp et al. 2007).

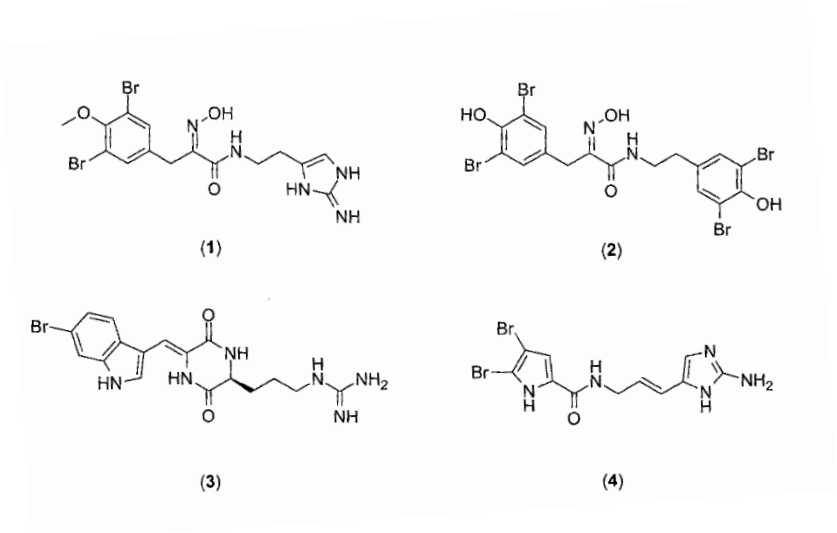
One group of marine compounds described in the literature that display particular potential as antifoulants are bromotyrosine-derived sponge metabolites (Ortlepp et al. 2007). Several such compounds have been studied, and their inhibition of balanide larval settlement has been described (Ortlepp et al. 2007). Similar brominated marine compounds such as barettin and oroidin (Fig. 1) have also been extensively investigated as potential leads for additives in marine paints as “green” settlement deterrents (Sjogren et al. 2004b; Yamada et al. 1997). Both barretin and oroidon and their synthetic derivatives have been incorporated in paint formulations and shown to prevent biofouling in field studies (Melander et al. 2009; Sjogren et al. 2004a). Synthetic analogues of bromotyrosines isolated from sponges have further been shown to inhibit phenoloxidase frombluemussels at low micromolar concentrations (Bayer et al. 2011).

Following on the ban on organotin compounds, marine natural products have received an increased focus and have even been heralded as the way forward towards environmentally friendly AF solutions (Bhadury and Wright 2004; Marechal and Hellio 2009; Qian et al. 2010; de Nys and Steinberg 2002). Searches for such compounds within the phylum Porifera are ongoing and provide sound basis that an environmentally friendly compound may be isolated from marine sponges (Tsukamoto et al. 1996a; Santos Acevedo et al. 2013). Being of natural origin does not guarantee that the marine AF compounds are non-toxic, but they come with the advantage of having been tuned by evolution for their task. The ideal “green” AF compound should be deterring rather than toxic to the target species (Dobretsov et al. 2006; Marechal and Hellio 2009; Chambers et al. 2006).

In this study, we report the AF activities of ianthelline (1) (displayed in Fig. 1 together with structurally related marine AF compounds), a bromotyrosine derivative isolated from the Arctic sponge *Stryphnus fortis* (Hanssen

et al. 2012). The structure of 1 was determined in 1986 (Litaudon and Guyot 1986), and its synthesis was recently described by Shearman et al. (2010).

Fig. 1 Structure of ianthelline (*1*) and structurally and marinerelated compounds that have been evaluated as AF compounds: 5,5'-dibromohemibastadin-1 (*2*), barettin (*3*) and oroidin (*4*)



Compound 1 was originally isolated from the Caribbean sponge *Ianthella ardis* and has been shown to be a major secondary metabolite in several other warm water sponges (Litaudon and Guyot 1986; Ciminiello et al. 1995). It is a bioactive compound, and the antitumoral activity of 1 was published in 2012 by Hanssen et al. (2012). The initial isolation paper briefly reported antibacterial and antifungal bioactivities against one bacterial and one fungal strain (Litaudon and Guyot 1986). Apart from those studies, little is known about the potential bioactivities of 1, and its ecological role is not understood. A fractionated extract from the Caribbean sponge *Aiolochroia crassa* containing 1 has been reported to inhibit the attachment of marine bacteria to agar blocks (Kelly et al. 2003, 2005). The pooled fractionated extracts prepared in that particular study was studied at natural volumetric concentrations. Thus, the amount, purity, and bioactive concentration of 1 were unknown. The attachment inhibition of *Vibrio harveyi* was examined, and growth values ranging from 14 to 61 %, compared to growth control, in five different fractions rich in 1 was reported (Kelly et al. 2005).

The optimisation of synthetic bromotyrosine derivatives for phenoloxidase inhibition revealed a pharmacophore that is highly analogous to the substructures displayed by 1, suggesting additional bioactivities for 1 (Bayer et al. 2011). Compound 1 contains an oxime moiety, postulated by Proksch and coworkers, to be essential for AF activity, and it represents an interesting compound for further studies towards AF solutions (Ortlepp et al. 2007).

The present study is thus an important continuation of the previously limited studies of the bioactivity and potential ecological role of 1 and also represents an assessment of the exploitability of 1 for biotechnological and medical purposes. Herein, we describe how 1 is isolated from an Arctic source and tested in a wide array of assays (Svenson 2013). In the marine environment, biofouling is undertaken by a great diversity of species, and marine AF compounds should therefore ideally possess a broad-spectrum activity to contend with the

vast amount of epibionts. The effect of 1 on the growth and settlement inhibition of ten marine bacterial and eight microalgal strains relevant to the formation of the biofouling film as well as studies on *Balanus improvisus* barnacle larvae settlement inhibition is described. A potential effect on bivalves is also studied using the *Mytilus edulis* phenoloxidase assay. Finally, the effect of 1 on a test panel of seven human pathogenic bacterial strains is investigated to evaluate the inhibitory effect on medically important strains. Comparisons with relevant structurally related, marine secondary metabolites are included and discussed.

Materials and Methods

Organism and Purification of 1 and 3

The specimen of *S. fortis* was collected off the coast of Spitsbergen (79° 33' N, 8° 53' E) in September 2007. Extraction, purification and structure determination of 1 was carried out as previously described (Hanssen et al. 2012).

For comparison, 3 was isolated from *Geodia barretti*, collected off the coast of Tromsø, Norway, in 2009. The compound was extracted according to previous methods and purified from a desalted 40 % RP-SPE extract (Tadesse et al. 2008) using a Waters SunFire Prep C18 HPLC column (10×250 mm, 5 µm particle size). A linear gradient from 15 to 30% acetonitrile in ultra-pure water containing 0.1 % TFA (*v/v*) was applied at 4 mL/min over 50 min to elute 3. The compound was identified by high-resolution mass spectrometry (HR-MS and HR-MS/MS) using a LTQ Orbitrap XL Hybrid Fourier Transform mass spectrometer (Thermo Fischer Scientific, MA, USA) and by comparison with previously published data on 3 (Lidgren and Bohlin 1986; Solter et al. 2002).

Marine Antibacterial Assays

Tenmarine bacterial strains from the bacterial collection of the University of Portsmouth School of Biological Sciences were used in the current study (Table 1).

Table 1 Marine bacterial strains used in the present study

Marine bacteria	ATCC1
Halomonas aquamarina	14400
Polaribacter irgensii	700398
Pseudoalteromonas elyakovii	700519
Roseobacter litoralis	49566
Shewanella putrefaciens	8071
Vibrio aestuarianus	35048
Vibrio carchariae	35084
Vibrio harveyi	700106
Vibrio natriegens	14058
Vibrio proteolyticus	53559

[1]American tissue culture code

These selected strains are representative of fouling species in both estuarine and marine environments (Chambers et al. 2011). They were grown at 26 °C in a marine medium, composed of 0.5 % peptone (neutralised bacteriological peptone, Oxoid LTD) in filtered (Whatman 1,001-270, pore size 11 µm) natural seawater from the Solent (UK). Compounds 1 and 3 were dissolved in 100 % methanol (general purpose grade, Fisher Chemical) and transferred to clear polystyrene 96-well plates (Fisher Scientific), dried under vacuum and sterilised under UV illumination. Wells were prepared using concentrations of 0.01, 0.1, 1 and 10 µg/mL, and each concentration was replicated six times (Bressy et al. 2010). Both growth and adhesion inhibition were studied.

- Growth inhibition experiments

One hundred microlitres of bacterial culture (2×10^8 colony forming units (CFU) mL^{-1}) was added to each well under aseptic conditions and incubated for 48 h at 26 °C. The minimum inhibitory concentration (MIC) was defined as the minimum concentration resulting in no change in optical density at 630 nm after incubation for 48 h (Thabard et al. 2011).

- Adhesion inhibition experiments

Microplates were prepared and inoculated as stated above. After a 48-h incubation, wells were emptied and rinsed once with 100 µL of sterile seawater to remove the non-attached cells and air-dried at room temperature. The remaining bacterial biofilm was stained with 100 µL of 0.3 % (*v/v*) aqueous crystal violet, and the optical density (OD) was measured at 595 nm (Sonak and Bhosle 1995).

Media was used as blank, and MIC was defined as the lowest concentration that produced a reduction in adhesion.

Microalgal Assay

Eight pure, but non-axenic, marine microalgae (obtained from Algobank, Caen, France) were used in this study (Table 2).

Table 2 Microalgae used in the present study

Microalgae	Algobank code
Cylindrotheca closterium	AC 170
Exanthemachrysis gayraliae	AC 15
Halamphora coffeaeformis	AC 713
Pleurochrysis roscoffensis	AC 32
Porphyridium purpureum	AC 122
Hymenomonas coronata	AC 115
Rhodosorus marinus	AC 119
Pleurochrysis carterae	AC 1

All the included strains are involved in surface colonisation and can lead to increased rates of biocorrosion (Jellali et al. 2013). Each algal strain was grown for 5 days prior to use at 20 °C in F/2 medium. After 5 days, microalgal concentration was assessed via analysis of the chlorophyll *a* content (Chambers et al. 2011), and dilutions of the stock culture were made accordingly to generate stock solutions of each microalgae containing 0.1 mg/L chlorophyll a. One hundred microlitres of the stock solutions were transferred to the wells of black 96-well plates prepared with ranging concentrations of 1 and 3 as described above. Both adhesion and growth inhibition were studied.

- Growth inhibition experiments

The inoculated plates were grown for 5 days under constant light exposure (140 µmol m^{-2} s^{-1}) at 20 °C. After the incubation, the microplates were centrifuged at 4,100 rpm for 10 min at 4 °C using a Beckman Coulter Allegra 25R centrifuge and subsequently emptied. One hundred microlitres of 100% methanol was added to each well to liberate chlorophyll *a*. The pigment concentration was quantified employing the fluorimetric method (Chambers et al. 2011). MIC values were calculated as explained for bacteria (based on OD measurements).

- Adhesion inhibition experiments

Black microplates were prepared and inoculated as stated above. Media was used as blank. After 5 days of incubation, the medium was gently removed using a multichannel pipette to eliminate all the non-attached cells. One hundred microlitres of 100 % methanol was then added to each well to liberate chlorophyll *a*. The pigment concentration was quantified employing the fluorimetric method explained above.

Balanus Cyprid Settlement

Cyprid larvae of *B. improvisus* were reared in a laboratory cultivating system at Tjärnö Marine Biological Laboratory, as described by Berntsson et al. (2000). The settlement assays with *B. improvisus* cyprids were conducted in non-treated polystyrene Petri dishes (Ø 48mm, Nunc #150340) containing 10 mL filtered (0.2 µm) seawater. Compound 1 was dissolved in DMSO and then serially diluted with DMSO to give the desired concentration series of which 10 µL was added to each test dish. A total of 18–22 newly moulted cyprids were added to each test dish which were incubated at room temperature (20–25 °C) for 5 days. At the end of the experiment, the number of metamorphosed juvenile barnacles, as well as live and dead cyprids, was assessed under a dissectionmicroscope. Each concentration of 1 was replicated four times (n=4), and dishes with 10 µL DMSO served as controls. The concentration of 1 leading to 50%inhibition of the settlement compared to the control was reported as the IC_{50} value.

Inhibition of Phenoloxidase from the Blue Mussel *M. edulis*

Phenoloxidase was isolated from the byssus gland of the blue mussel (*M. edulis*) as previously described (Hellio et al. 2000; Bayer et al. 2011). Solutions of 1 mg/mL *M. edulis* phenoloxidase, 0.4 mM $_{L}$-dihydroxyphenylalanine (DOPA), and 1 (2‑2,000 µg/mL) were prepared. One millilitre of the LDOPA solution, 10 µL 1 (or 10 µL of water in the case of the control samples), and 10 µL *M. edulis* phenoloxidase (added last) were mixed in a test tube. Phosphate buffered saline (pH 6.8) was added to the solution tomake a total volume of 2 mL. The solution was placed in a cuvette, and the change in absorbance at 475 nm was measured after 30 min using a UV-VIS spectrophotometer. The blank control used was the sample without the enzyme solution. The assay was run in triplicate, and the concentration of 1 leading to 50% inhibition of the phenoloxidase activity result was reported as the IC_{50} value.

Terrestrial Antibacterial Assay

The MIC of 1 was determined against seven human pathogenic bacterial strains presented in Table 3.

Table 3 Human pathogenic bacterial strains used in the current study

Bacterial strain	ATCC
Gram positive	
Staphylococcus aureus	25923
MRSA	33591
Staphylococcus epidermidis	35984
Streptococcus agalactiae	12386
Enterococcus faecalis	29212
Gram negative	
Escherichia coli	25922
Pseudomonas aeruginosa	27853

Enterococcus faecalis and *Streptococcus agalactiae* were grown in brain-heart infusion broth (BHI; Oxoid, Hampshire, England). *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Escherichia coli* and *Pseudomonas aeruginosa* were grown in Mueller Hinton Broth (MH; Merck, Darmstadt, Germany), and Staphylococcus epidermidis in tryptic soy broth (TS; Merck, Darmstadt, Germany). Both growth and biofilm inhibition studies were performed.

‑ Growth inhibition experiments

Suspended bacteria in log phase grown at 37 °C in growth medium were added to 96-well microtiter plates resulting in 1,500‑15,000 CFU/mL. A serial dilution of 1 was subsequently added and left to inoculate for 24 h before growth inhibition was observed with a Victor multilabel counter at 600 nm. Growth medium diluted with water (1:1) was used as negative control, and bacteria suspension diluted with water (1:1) was used as positive control. Gentamicin at ranging concentrations from 0.015 to 16 µg/mL was used as positive assay

control. The MIC was defined as the minimum concentration resulting in no change in optical density after incubation for 24 h at 37 °C. Compound 1 was tested at concentrations ranging from 2.5 to 160 µg/mL.

- Inhibition of biofilm formation

S. epidermidis was used to assess the effect of 1 on biofilm formation. An overnight culture of *S. epidermidis* grown in TS was diluted with fresh TS containing 1 % glucose (1:100). Aliquots of 50 µL were transferred to a 96-well microtiter plate, and 50 µL of 1, dissolved in water at ranging concentrations, was added. After overnight incubation at 37 °C, the bacterial suspension was carefully discarded and the wells washed with water. The plate was dried and the biofilm fixed by incubation for 1 h at 55 °C before the surface attached cells were stained with 100 µL of 0.1 % crystal violet for 5 min. The crystal violet solution was removed and the plate once more washed with water and dried at 55 °C for 1 h. After adding 70 µL of 70 % ethanol, the plate was incubated at room temperature for 10 min. Biofilm formation was observed by visual inspection of the plates. The MIC was defined as the lowest concentration where no biofilm formation was visible. A *S. epidermidis* suspension, diluted with 50 µL of water, was used as a positive control, and 50 µL *Staphylococcus haemolyticus* suspension with 50 µL of water was employed as a negative control. A mixture of 50 µL water and 50 µL TS was used as assay control.

Results and Discussion

Biofouling is a complex chemical and biological process, and no single bioassay can be used to accurately imitate it. Several different assays and organisms are hence needed to evaluate the AF potential of a compound (Briand 2009; Dahms and Hellio 2009). The progression from adsorption of organic molecules to a marine surface to the growth of macroepibionts such as macroalgae, crustaceans and mussels also involves microorganisms such as bacteria and microalgae. It is therefore unrealistic to assume that a single non-toxic AF compound will display activity against all organisms associated with it. In fact, limited access to comprehensive screens against all the different stages of the AF process may lead to the discovery of promising compounds that are later shown to be inactive in complementary studies against other types of foulers (Qian et al. 2010). In the current study of 1, we have included bioassays targeting the main groups of marine organisms involved in the different stages of the biofouling process. The well-established AF brominated diketopiperazine barettin (3), isolated from *G. barretti*, was included for comparison and as a reference in selected assays.

The initial microfouling of bacteria and microalgae was studied via both adhesion and growth inhibition assays. It is generally accepted that the microfouling can facilitate the macrofouling (Beech et al. 2005; Qian et al. 2007). Targeting the initial settlement and production of these marine biofilms may thus have beneficial effects for limiting the fouling of macroorganisms. The presence of such marine biofilms on surfaces also increases the rate of biocorrosion (Beech and Sunner 2004). The growth inhibition was studied via standard

serial dilution methods against ten relevant marine bacterial strains. Since biofouling is a process associated with the adhesion of organisms to a surface, the study of bacterial attachment is highly relevant and that was therefore also studied for the same bacterial strains. In addition, eight microalgal strains including the diatoms *Cylindrotheca closterium* and *Halamphora coffeaeformis* were also studied. Diatoms represent particularly relevant strains as they commonly rapidly form resilient slimy layers on marine surfaces (Molino and Wetherbee 2008). The data from the microorganism screening is compiled in Table 4.

Table 4 The effect of 1 and 3 on growth and adhesion of fouling marine bacteria and microalgae

Microorganism	MIC 1 (μg/mL)		MIC 3 (μg/mL)	
	Growth	Adhesion	Growth	Adhesion
Marine bacteria				
Halomonas aquamarina	>10	>10	>10	>10
Polaribacter irgensii	1	>10	>10	>10
Pseudoalteromonas elyakovii	1	>10	>10	>10
Roseobacter litoralis	1	>10	>10	>10
Shewanella putrefaciens	0.1	>10	10	>10
Vibrio aestuarianus	0.1	0.1	0.01	>10
Vibrio carchariae	>10	>10	>10	>10
Vibrio harveyi	10	>10	>10	>10
Vibrio natriegens	>10	0.1	>10	10
Vibrio proteolyticus	10	>10	10	>10
Microalgae				
Cylindrotheca closterium	>10	>10	0.1	0.1
Exanthemachrysis gayraliae	>10	>10	>10	>10
Halamphora coffeaeformis	>10	>10	>10	>10
Pleurochrysis roscoffensis	10	>10	>10	>10
Porphyridium purpureum	>10	>10	0.1	1.0
Hymenomonas coronata	1	n.d.[1]	n.t.[2]	n.t.[2]
Rhodosorus marinus	1	n.d.[1]	n.t.[2]	n.t.[2]
Pleurochrysis carterae	0.1	n.d.[1]	n.t.[2]	n.t.[2]

[1]Not determined due to unfavourable growth conditions for the adhesion study
[2]Not tested

Only concentrations of 1 at or below 10 μg/mL were evaluated for the microorganism studies. This represents a threshold for compounds to be regarded as highly active and of further interest from a biotechnological viewpoint (Hellio et al. 2009). From Table 4, it is clear that 1 has a pronounced effect on the inhibition of marine bacterial growth. All the tested marine bacteria are Gram-negative, and most display MIC values below 10 μg/mL, some as low as 0.1 μg/mL. Only the growth of three bacteria (*Halomonas aquamarina*, *Vibrio carchariae* and *Vibrio natriegens*) of the tested ten strains tested remained unaffected by 1 at 10 μg/mL, illustrating a strong and broad antibacterial activity. In the bacterial adhesion studies, the MIC values were higher. Only the two *Vibrio* species *V. natriegens* and *Vibrio aestuarianus* displayed high sensitivity against 1 with MIC values of 0.1 μg/mL. The high sensitivity of *V. natriegens* in the adhesion assay is of interest as it displayed a MIC >10 μg/mL in the growth assay. *V. natriegens* is a very rapidly growing sulfate-reducing bacterium found on microfouled surfaces (Cheng et al. 2009). Sulfate-reducing bacteria are especially known for their role in biocorrosion (Beech and Sunner 2004), and finding a compound inhibiting the adhesion of *V.*

natriegens would be highly interesting for reducing the biocorrosion process. The oyster industry has grown to be very important for many regions of the world, contributing substantially to social and economic activity in the coastal zones. *V. aestuarianus* is a known pathogen of the commercial pacific oyster *Crassostrea gigas* (Labreuche et al. 2006; De Decker et al. 2011) causing massive mortality outbreaks and a shortage in shellfish. Compound 1 inhibited the growth and adhesion of *V. aestuarianus* at very low concentrations.

In previous studies, 1 has been included in unspecified natural concentrations which are most likely significantly higher than those in the current study. This is based on the amounts of similar brominated secondary metabolites found and analysed in other tropical sponge extracts which frequently contain such compounds (e.g. oroidin, aeroplysinin and sceptrin) in high (0.8-4.9 mg/mL) natural volumetric concentrations (Kelly et al. 2003). *S. fortis* is also rich in 1, and approximately 0.05 % of the organism wet weight is 1, indicating that it is an important secondary metabolite. The antibacterial activity of 1 is similar to the bromotyrosine derivative zamamistatin isolated from the Okinawan sponge Pseudoceratina purpurea by Takada et al. (2001). In comparison to 3, which is both structurally related and a thoroughly studied marine AF compound, 1 displays a higher antibacterial activity. Compound 3 previously isolated from G. barrette was included in the antimicrobial assay and was active against both *Vibrio proteolyticus* and *Shewanella putrefaciens* at 10 µg/mL and highly active (MIC=0.01 µg/mL) against *V. aestuarianus* in analogy to 1. In the bacterial adhesion assay, 3 was only active at 10 µg/mL against *V. natriegens*. No other bacterial strains were affected in the concentration range employed.

Microalgae forms slimy layers on marine surfaces that are generally challenging to prevent (Molino and Wetherbee 2008), and it was shown that 1 was active against half of the tested microalgal strains. *Hymenomonas coronata*, *Rhodosorus marinus* and *Pleurochrysis carterae* were all sensitive to 1 in solution with MIC values ranging from 0.1 to 1 µg/mL while *Pleurochrysis roscoffensis* displayed a MIC value of 10 µg/mL. The other four strains, including the two diatom species, were unaffected at the tested concentrations. In analogy to the bacterial studies, microalgal adhesion was also evaluated. No effect on algal adhesion was seen for 1. Growth conditions for the three most sensitive species in the growth assay were unfavourable at the time of the adhesion studies, and no data could be obtained for those species. No algal toxicity was seen upon extended exposure times. In contrast, 3 was active against both the growth and adhesion of the diatom *C. closterium* at 0.1 µg/mL and *Porphyridium purpureum* at 0.1 and 1 µg/mL, respectively.

The major macrofoulers, barnacles and mussels, were studied via both enzyme and settlement inhibition assays. Attachment of bivalves was studied by inhibition studies of phenoloxidase isolated from the byssus gland of the common blue mussel, *M. edulis*. Inhibition of the production of byssus threads by mussels has become an established method for evaluating their settlement (Hellio et al. 2000). Other marine invertebrates such as barnacle larvae also depend on phenoloxidase for generating a secure surface attachment. The

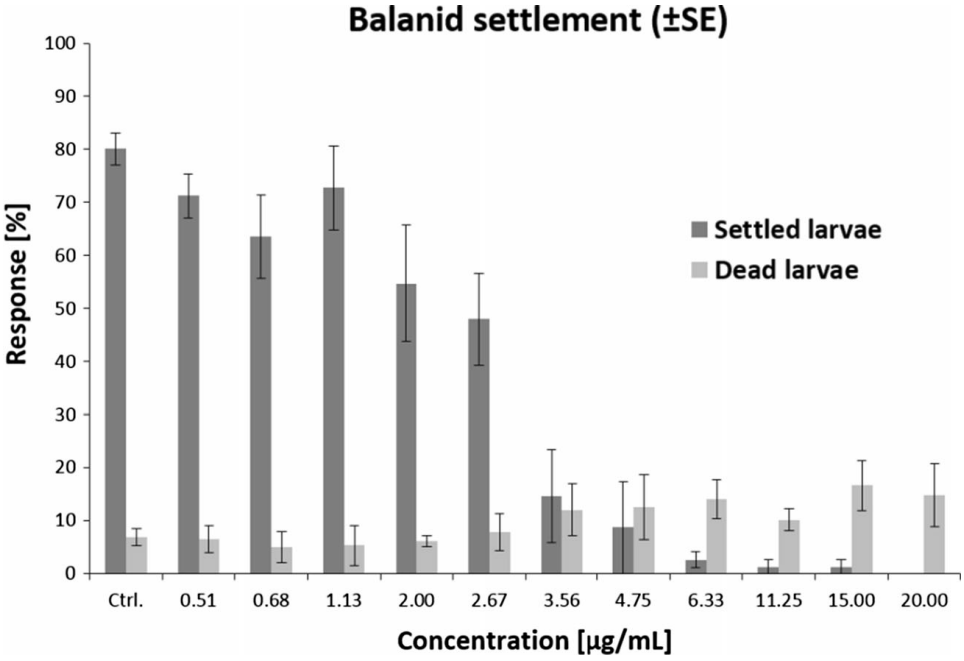
function of phenoloxidase is to oxidise both phenylalanine and tyrosine in protein secretions to quinone derivatives that crosslink to generate a strong biopolymer enabling the organism to anchor to a surface (Bayer et al. 2011). The release of active phenoloxidase is controlled by Ca^{2+} -dependent signalling pathways, and therefore, the intracellular concentration of Ca^{2+} is thus also an interesting target for the development of AF strategies. An inhibitory effect of 1 on phenoloxidase was seen at 1 µg/mL, and the IC_{50} was determined to 45.2 µg/mL. Such an IC_{50} suggests only moderate affinity of 1 for the phenoloxidase despite the α-oxo-oxime functionality previously reported to be beneficial for blue mussel phenoloxidase inhibition. The synthetic 5,5'-dibromohemibastadin-1 (2 in Fig. 1) is a strong phenoloxidase inhibitor with an IC_{50} value of 0.84 µg/mL (Bayer et al. 2011). Compounds 1 and 2 share several structural features such as the dibrominated phenol ring, the overall molecular length and the placement of the α-oxo-oxime functionality. The α-oxooxime functionality has been shown to be involved with complexation of the two copper(II) ions coordinated by six histidine residues in the catalytic centre of the phenoloxidase (Kim and Uyama 2005), and the oxime functionality was crucial for the strong inhibition of synthetic bastadin derivatives (Bayer et al. 2011). The main structural differences between 2 and 1 lie in the methoxylated phenolic hydroxyl in 1 and the cationic iminoimidazole ring. The inhibitory effect of 1 was reduced by nearly 50-fold in comparison to 2, and it is clear that the α-oxo-oxime functionality is not sufficient for a high affinity interaction with the catalytic site of the enzyme given the other structural features of 1. Compound 1 is also positively charged, and charge repulsion within the active site of phenoloxidase is a potential explanation for the lower inhibitory activity of 1. The lack of a phenolic hydroxyl is also believed to additionally impair the enzyme binding as it represents the natural enzyme substrate binding motif (Kim and Uyama 2005).

While phenoloxidase inhibition is a common representative indicator of a compound's ability to reduce the settling abilities for a range of invertebrates (Bayer et al. 2011), studies on *B. improvisus* larvae were also performed to assess the potential effects 1 may specifically have on barnacles. Based on the moderate phenoloxidase inhibition, a similar moderate deterring effect on Balanus settlement was anticipated. The most commonly studied barnacle in AF studies is *Balanus amphitrite* which is found in tropical waters. The sponge *S. fortis* (Fig. 2), from which 1 was isolated, is a cold water species which is why we in this study chose to target B. improvisus, which is also found in cold water. From a chemical ecology perspective, it has been established that when working on marine natural products, it is more judicious to sample organisms and also test them primarily on fouling species from the same area (Marechal and Hellio 2011). *B. improvisus* is commonly found in the shallow littoral zone attached to stones, algae or man-made constructions (Barnes and Barnes 1962). It is a notorious fouler on smooth artificial substrates such as ship hulls, which has enabled its rapid spread amongst continents during the last century. The species has a planktotrophic larval development consisting of six naupliar stages and one non-feeding cyprid stage, the latter being responsible for finding and attaching to a suitable substratum. The processes of attachment and metamorphosis of the cyprid larva into an adult barnacle are collectively referred to as settlement. The cyprids are capable of exploring surfaces actively, and the site of settlement is determined using physical and biochemical properties

of the substratum (Berntsson et al. 2000). The effect of 1 on *B. improvisus* cyprid larvae is presented below in Fig. 3.

Fig. 2 A specimen of S. fortis from which 1 was isolated for the present study. Scale bar represents 2 cm

Fig. 3 Effect of 1 on the settlement of *B. improvisus* cyprid larvae as presented as percentages of settled (*dark grey columns*) and dead cyprids (*light grey columns*) and given as means ±SE (*n*=4). IC_{50} was determined to 3.0 µg/mL. 0.1 % DMSO (*v/v*) in FSW was used as negative control



As evident from the settlement and metamorphosis inhibition data, 1 represents a potent AF compound against *B. improvisus* cyprid larvae. An IC_{50} of 3.0 µg/mL indicates a high deterring effect and makes 1 significantly more active against balanide settlement thanmost reported bromotyrosinederived sponge metabolites and their synthetic analogues (Ortlepp et al. 2007; Tsukamoto et al. 1996a). Compound 1 is four times as active as 2, and no apparent coupling between the moderate phenoloxidase inhibition and Balanus settlement is seen for 1, as has been previously reported for the synthetic hemibastadins (Bayer et al. 2011). Compound 3 displays an IC_{50} around 1 µg/mL (Sjogren et al. 2004b) while the structurally related dibromopyrrole oroidin (4 in Fig. 1) exhibits an IC_{50} of 19 µg/mL against the settlement and metamorphosis of *B. amphitrite* cyprids (Tsukamoto et al. 1996b). Compound 1 displays activity similar to Ceratinamide B but is one order of magnitude less active than Ceratinamide A, a highly potent AF bromotyrosine derivate with low

toxicity isolated from the marine sponge *P. purpurea* (Tsukamoto et al. 1996a). Even at the highest concentration tested (20 μg/mL), 1 exhibited low toxicity. Furthermore, an increase in larval time in the water column caused by settlement inhibition will inevitably result in higher larval mortality, which may partly explain the tendency for a higher cyprid mortality at the highest test concentrations of 1 (Fig. 3).

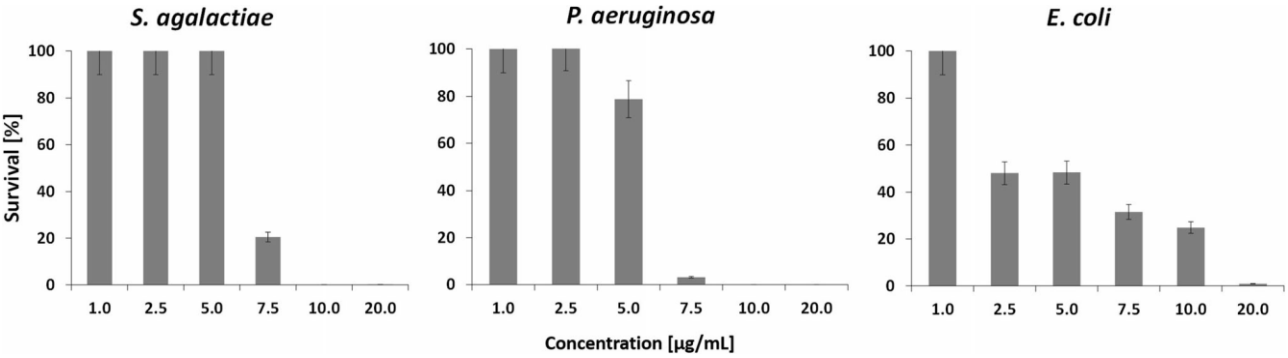
When the isolation of 1 was initially reported by Litaudon and Guyot in 1986, an activity against *S. aureus* using a disc diffusion assay (15 mm growth inhibition radius using a disc loaded with 50 μg of 1) was included (Litaudon and Guyot 1986). Based on that initial data, a screening against human pathogenic bacterial strains was also performed to provide a comprehensive overview of the antibacterial effects of 1. Both strains resistant to conventional antibiotics and biofilm forming bacteria were included in the test panel of seven bacterial strains. Serial dilution experiments were performed to assess the antibacterial activity of 1 against human pathogenic bacterial strains, and the data is compiled in Table 5.

Table 5 Antibacterial activity of 1 against human pathogenic bacterial strains

Bacterial strain	MIC (μg/mL)
Gram positive	
Staphylococcus aureus	2.5
MRSA	20
Staphylococcus epidermidis	50
Streptococcus agalactiae	7.5
Enterococcus faecalis	22.5
Gram negative	
Escherichia coli	7.5
Pseudomonas aeruginosa	7.5
Biofilm inhibition	
Staphylococcus epidermidis	30

The Gram-positive bacteria displayed sensitivities towards 1 ranging from 2.5 to 50 μg/mL while the two Gram-negative strains were both highly sensitive with MIC values of 7.5 μg/mL. *S. aureus* displayed the highest sensitivity towards 1 at 2.5 μg/mL, and the methicillin-resistant strain was slightly more tolerant. These activities are similar to those reported for synoxazolidinone A which is a closely structurally related marine compound isolated from the Arctic ascidian Synoicum pulmonaria (Tadesse et al. 2010). In this molecule, the central oxime is replaced with an oxazolidinone core. Previous cytotoxicity studies indicate that 1 does not act via cellular membrane disruption against mammalian cells (Hanssen et al. 2012). Instead, the kill kinetics suggests intracellular targets as no biological effect was seen until cells were incubated with 1 for extended periods and at higher concentrations. Even though the antibacterialmode of action of 1 was not studied in detail, it appears from the data that the antibacterial effect is primarily induced at the bacterial membrane interface. The rapid killing, once a threshold concentration on
1 has been reached, is typical for a compound acting by a nonspecific membrane depolarisation mechanism (Shai 1999) and is exemplified for selected strains in Fig. 4.

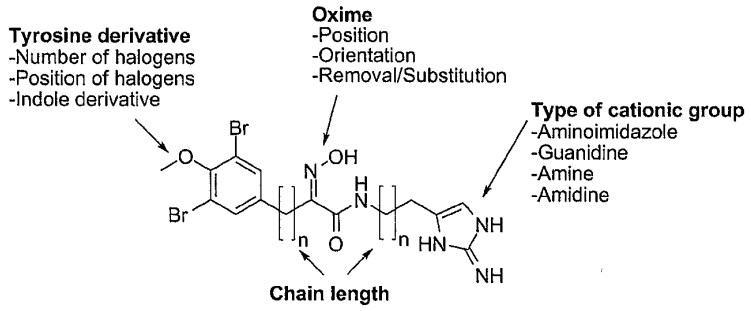
Fig. 4 Effects of 1 on selected bacterial strains illustrating the threshold concentrations needed for activity, particularly against Gram-positive bacteria



The lipid composition of the bacterial cell membrane differs significantly from the neutral mammalian cell membrane (Lohner et al. 2008), and a different mode of action on the anionic bacterial cell membrane is suspected. Cationic amphiphilic antimicrobial peptidic derivatives, which 1 can be regarded as, are generally active at the bacterial membrane interfaces via several mechanisms (Melo et al. 2009), and a pronounced antibacterial effect can be seen even for small diand tripeptidomimetics (Flaten et al. 2011). The inhibition of *S. epidermidis* biofilm formation at 30 µg/mL is seen at a lower concentration than the MIC for growth inhibition and may suggest an additional mode of action for 1.

Given the many promising bioactivities of 1 and the relative ease of preparing it, and similar analogues, synthetically (Shearman et al. 2010; Ortlepp et al. 2007) it is realistic to assume that libraries of analogues of 1 is within reach. Compound 1 can be dissected into smaller building blocks allowing for alternative molecular assemblies where the key functional groups such as the tyrosine moiety, the central oxime and the cationic iminoimidazole can be substituted with analogous chemistries. Several different structural motifs displayed by 1 can be thus be targeted to generate diverse libraries of compounds that would aid in future structure activity relationship studies (SAR) as depicted in Fig. 5.

Fig. 5 Synthetic targets for structure activity relationship studies for establishing the AF pharmacophore of 1



It is expected that such analogues will be active in several assays and also structurally simplified in comparison with one. This is certainly a prerequisite if a large-scale marine use is considered. The structurally related two

has undergone extensive SAR studies, and those libraries of synthetic analogues have been shown to display several medically relevant bioactivities (Richards et al. 2008, 2009) and are being studied further.

The studied extract of *S. fortis* contains a range of secondary metabolites (unpublished data), but 1 is by far the most abundant compound constituting 0.05 % of the organism's wet weight. This strongly indicates that 1 serves important ecological functions such as inhibition of the recruitment of potential epibionts and competitors, maybe in synergy with other compounds. A tissue specific production or localisation of 1 to exposed areas of the organism may further aid to increase the local concentration to levels higher than those evaluated in the present study to effectively prevent settlement to generate a broad resistance against competing marine species.

Conclusion

Brominated secondary metabolites are common in the marine environment, and the sponge-derived tyrosine derivatives are particularly interesting due to their diverse bioactivities and their potential application areas. In the present study, 1 was shown to be a powerful micro- and macro-AF compound, mainly against marine bacteria and the settlement of barnacles. A lower activity is seen against microalgae, and a moderate bioactivity is reported for blue mussel phenoloxidase inhibition. In addition, 1 is active against all tested human pathogenic bacterial strains included in the current study, both Gram positive and Gram negative. Based on the structural similarities with barettin, oroidin and the synoxazolidinones, it is anticipated that ianthelline also exhibits other biological activities yet to be investigated.

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