Marine Biotechnology (2019) 21:743–752
https://doi.org/10.1007/s10126-019-09920-y

ORIGINAL ARTICLE

The Sponge-Associated Fungus *Eurotium chevalieri* MUT 2316 and its Bioactive Molecules: Potential Applications in the Field of Antifouling

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Received: 29 May 2019 / Accepted: 20 August 2019 / Published online: 7 September 2019

Abstract

The need for new environmentally friendly antifouling and the observation that many marine organisms have developed strategies to keep their surface free of epibionts has stimulated the search for marine natural compounds with antifouling activities. Sponges and in particular fungi associated with them represent one of the most appropriate sources of defence molecules and could represent a promising biomass for the supply of new antifouling compounds. The objective of this work was therefore to evaluate the antifouling potency of 7 compounds isolated from the sponge derived fungus *Eurotium chevalieri* MUT 2316. The assessment of their activity targeted the inhibition of the adhesion and/or growth of selected marine bacteria (5) and microalgae (5), as well as the inhibition of the mussel's byssus thread formation (tyrosinase activity). The 7 compounds showed bioactivity, with various levels of selectivity for species. Cyclo-L-Trp-L-Ala was the most promising active compound, and led to the inhibition, at very low concentrations (0.001 μg ml^{-1} in 61.5% of cases), of adhesion and growth of all the microalgae, of selected bacteria, and towards the inhibition of tyrosinase. Promising results were also obtained for echinulin, neoechinulin A, dihydroauroglaucin and flavoglaucin, respectively, leading to inhibition of adhesion and/or growth of 9, 7, 8 and 8 microfouling species at various concentrations.

Keywords Antifouling · Bacteria · Bioprospection · Marine fungi · Microalgae · Tyrosinase

Introduction

Marine biofouling is defined as the adhesion and growth of microorganisms, algae and invertebrates on biotic and abiotic surfaces immersed in seawater. Biofouling can seriously affect the worldwide economy and environmental integrity (Yebra et al. 2004; Amara et al. 2018). For example, its development on man-made surfaces such as on ship's hulls can increase the frictional drag leading to an augmentation of fuel consumption with a concomitant rise of carbon dioxide and sulphur dioxide emissions (Cronin et al. 1999; Champ 2000; Eckman et al. 2001; Phillippi et al. 2001; Amara et al. 2018). Biofouling is also linked to the increase in frictional drags (leading to a reduction in ship speed), the acceleration of the biocorrosion phenomenon and increased costs of hull repair and maintenance, representing an additional $150 billion per year for the maritime industry (Maréchal and Hellio 2009; Hellio et al. 2015). Biofouling is a key factor in the spreading of invasive species that might be transported by ships, and thus threatening indigenous aquatic life forms (Martins et al. 2018).

Existing antifouling techniques are used to inhibit or reduce surface colonization by biofoulers, but none are fully satisfactory from an environmental point of view. Tributyltin (TBT) was largely employed as antifouling agents until it has been banned in 2008 due to unacceptable toxicity on non-target species and bioaccumulation in the food chain (Alzieu 2000; Satheesh et al. 2016; Le Norcy et al. 2017; Amara et al. 2018). Up to date, the main alternatives to TBT are paints based on copper derivatives and organic molecules (or booster

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biocides) (Le Norcy et al. 2017; Amara et al. 2018). However, these formulations are not entirely environmentally satisfactory: for instance, a copper concentration that exceeds 3 ppb affects various life stages of marine organisms (Le Norcy et al. 2017); as for other new commercial formulations, for example Sea-Nine, Diuron and Irgarol 1051 are bioaccumulated in non-target benthic communities and affect the food web chain (Le Norcy et al. 2017). New antifouling solutions, both active on target organisms (biofoulers) and environmentally friendly, are urgently required.

The observation that many marine organisms have developed strategies to keep their surface free from epibionts has stimulated the search for natural compounds with antifouling activities. This is based on chemical ecology studies and interactions between epibionts and basibionts. Understanding which compounds are used by basibionts to deter epibionts could lead to new development in marine biotechnology. Such compounds can represent an environmentally friendly alternative to biocides. They can act in several ways, for instance by altering the protein expression, blocking the neurotransmission or inducing oxidative stress in fouling organisms (Qian et al. 2013; Kang et al. 2016; Chen and Qian 2017). Alternatively, they can block the attachment site of the bacteria and algae (avoiding the biofilm formation), inhibit the production/release of adhesive compounds by the biofoulers (Qian et al. 2013; Chen and Qian 2017). As their activity is targeted towards foulers only, they are less likely to cause side effects on other organisms.

Marine sponges are sessile organisms that lack a specialized immune system and have developed chemical defence to protect their surface against epibiosis (Bayer et al. 2011; Hanssen et al. 2014). Bromotyrosine-derived compounds are among the most potent antifouling isolated from marine sponges (Hanssen et al. 2014); for instance, barretin, isolated from the sponge *Geodia barrette*, inhibits fouling adhesion in field studies (Hedner et al. 2008) and synthetic derivatives of bastadin produced by the sponge *Ianthella basta* inhibit microfouling and macrofouling adhesion in laboratory experiments (Le Norcy et al. 2017). Interestingly, marine sponges harbour a rich fungal diversity, but so far, the role of fungi is not completely understood (Suryanarayanan 2012; Bovio et al. 2018).

The use of microbial metabolites as antifouling has several key advantages, avoiding the supply problem of several animals from the environment, when the cultivation in aquaria or the chemical synthesis is not feasible (Hanssen et al. 2014; Wang et al. 2017). In the literature, several microorganisms have been described as promising sources for the formulation of new antifouling paints. Among fungi, *Aspergillus* spp. (anamorph of *Eurotium* spp.) are the most fruitful producers of antifouling compounds (Wang et al. 2017).

In view of the urgent need to find new and environmentally friendly antifouling, and the potential of fungi associated with sponges, 7 molecules isolated from the sponge derived fungus *Eurotium chevalieri* MUT 2316 were evaluated for their antifouling properties. Bacteria and microalgae (microfoulers) representative for the first step of development of the biofilm were chosen as target. In addition, to evaluate the capability of the compounds to inhibit the "macrofoulers" (typical of the last phase of development of the biofouling), an enzymatic assay was performed to evaluate the inhibition of the byssus formation in mussels.

Material and Methods

Fungal Isolation and Molecules Purification

Eurotium chevalieri MUT 2316 was isolated from the Atlantic sponge *Grantia compressa* by lihomogenization of the sponge tissues, as previously described (Bovio et al. 2019; Bovio 2019). *E. chevalieri* MUT 2316 was identified using a polyphasic approach, combining morphological observation of the colony and reproductive structure with molecular data (Bovio et al. 2019; Bovio 2019).

In order to isolate and purify the metabolites produced by *E. chevalieri* MUT 2316, the fungus was inoculated into 200 petri dishes plates containing Potato Dextrose Agar-PDA (potato extract 4 g, dextrose 20 g, agar 15 g, up to 1 L dH_2O) plus 3% NaCl and 20—250-mL Erlenmeyer flasks containing Potato Dextrose Broth - PDB (potato extract 4 g, dextrose 20 g, up to 1 L dH_2O) plus 3% NaCl. *E. chevalieri* MUT 2316 yielded 7 pure compounds from solid cultures and three pure compounds from liquid cultures. The extraction procedures and the chemical structures were previously described by Bovio et al. (2019). Following the previous study on *E. chevalieri* MUT 2316, the bioactivity of 7 compounds (those obtained with a higher yield), namely echinulin (**1**–9.6 mg), neoechinulin A (**2**–7.2 mg), physcion (**3**–9.0 mg), dihydroauroglaucin (**4**–12.0 mg), flavoglaucin (**5**–28.0 mg), cinnalutein (**6**–1.8 mg) and cyclo-L-Trp-L-Ala (**7**–2.8 mg) (Fig. 1), was further investigated to assess their antifouling activity.

The stock solutions for the bioassays were prepared by dissolving the 7 molecules in 100% dimethyl sulfoxide (DMSO) at a final concentration of 10 mg mL^{-1} for (**1**, **2**, **4**, **5**, **6**, **7**) and at 1 mg mL^{-1} for **(3)**, due to the solubility limit of the compound in DMSO.

Marine Antibacterial Assays

The 7 compounds were tested for their potential to inhibit the adhesion and/or growth of 5 bacterial species representative of fouling species in the estuarine and marine environment (Chambers et al. 2011) and obtained from the ATCC: *Halomonas aquamarina* (ATCC 14400), *Polaribacter irgensii* (ATCC 700398), *Roseobacter littoralis* (ATCC 49566), *Vibrio*

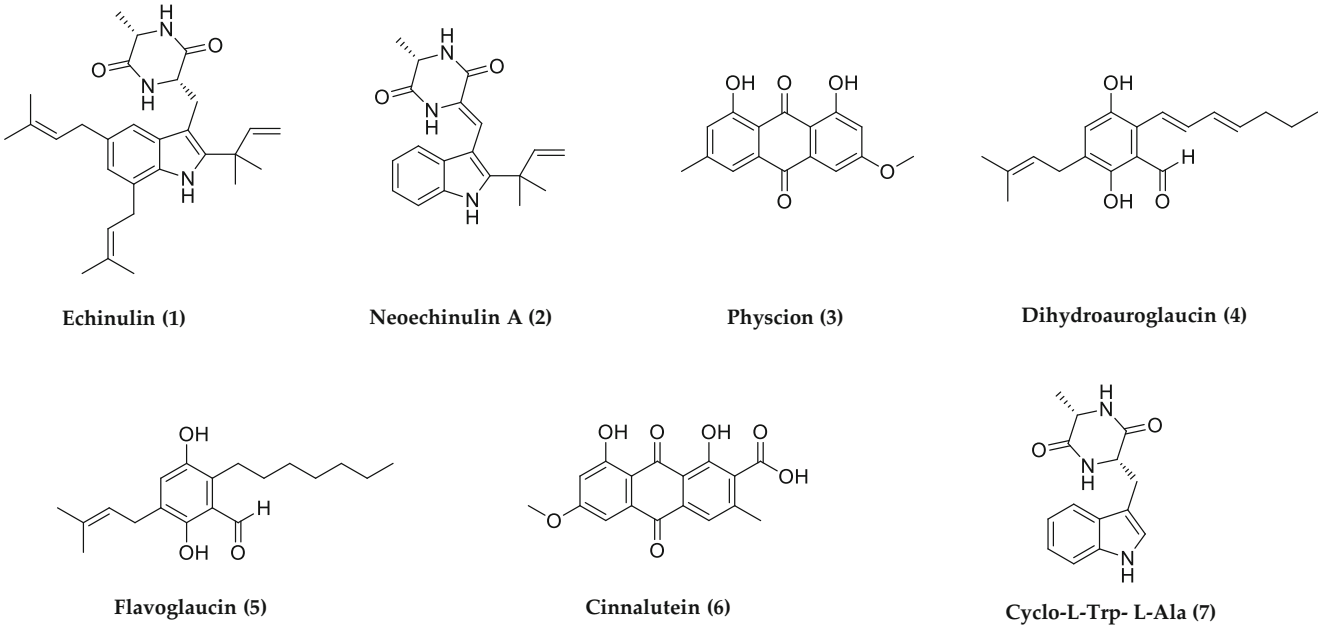

Fig. 1 Compounds isolated from *E. chevalieri* MUT 2316

aesturianus (ATCC 35048), *Pseudoalteromonas citrea* (ATCC 29720). Prior to experiments, bacteria were pre-grown in sterile Marine Bacterial Medium (MBM) (0.5% peptone (Neutralized Bacteriological Peptone, Oxoid Ltd., Basingstoke, UK) in sterile filtered (Whatman 1001–270, pore size 11 μm) natural seawater, at 20 °C.

Bacterial strains adhesion and growth were determined according to the methods of Thabard et al. (2011). Bacterial suspensions (100 μL aliquots, 2×10^8 colony-forming units/mL) were aseptically added to the microplate wells containing the compounds to assay (0.001 to 100 μg ml $^{-1}$), and the plates were incubated for 72 h at 20 °C. Controls were performed with DMSO, making the same dilutions set up to test the molecules, with 1% as the maximum concentration of the solvent; the bacterial strains and the non-inoculated media represented two further controls. The test was replicated 6 times. Bacterial growth was monitored spectroscopically (Tecan Infinite M200) at 630 nm (Trepos et al. 2015) and the low observable effect concentration (LOEC), defined as the lowest test concentration with an average response that is significantly different from the control (Amara et al. 2018), was calculated, based on the OD values at 630 nm. Therefore, the ANOVA was performed to compare the bacterial growth inhibition of the compounds with the control ($p < 0.05$) using IBM SPSS Statistics software. Before the statistical analysis, the controls with the DMSO, that unlikely inhibited the bacterial growth, were subtracted to the results produced by the molecules. Moreover, the potential interference due to the coloured molecules was taken into account by measuring the OD at 630 nm immediately after the inoculum.

In order to evaluate the potential activity of compounds towards adhesion of bacteria, the microplates used to evaluate the bacterial growth inhibition were emptied and rinsed with 100 μL of seawater to remove the non-attached cells. Once dry, the bacterial biofilm was stained with 0.3% aqueous crystal violet, then after 30 min, the plates were rinsed with water and let dry overnight. The OD was then measured at 595 nm (Trepos et al. 2015) to evaluate the LOEC, performing the above-mentioned statistical analysis and taking into account the possible influence of the DMSO.

Marine Anti-microalgal Assays

The 7 molecules were tested for their capability to inhibit the adhesion and/or growth of 5 microalgal species known to be involved in surface colonization (Trepos et al. 2015): *Cylindrotheca closterium* (AC 170), *Exanthemachrysis gayraliae* (AC 15), *Halamphora coffeaeformis* (AC 713), *Porphyridium purpureum* (AC 122), *Phaeodactylum tricornutum* (AC 171). All the strains were obtained from the Algobank culture collection (France). Prior to experiments, microalgae were grown in sterile F/2 medium (Guillard and Ryther 1962) for 7 days at room temperature, exposed to natural daylight irradiance.

In order to evaluate the algal growth and adhesion inhibition, the 7 compounds were diluted in F/2 medium and transferred to black 96-multiwell plates to reach the same final concentrations tested for the antibacterial activity. The same controls than for the antibacterial test were carried out (with DMSO, with the algae and the medium alone). All assays were run in 6 replicates. Microalgal stock solutions were

prepared using the chlorophyll analysis methodology described by Chambers et al. (2011). The pre-treated microplate wells were treated with 100 μL of the algal stock solutions (0.1 mg chlorophyll a/mL). The plates were then incubated for 2 weeks at room temperature, exposed to the daylight irradiance. Growth was determined by analysis of liberated chlorophyll a after centrifugation and methanol addition. Chlorophyll a was quantified fluorometrically (Tecan Infinite M200, excitation 485 nm, emission 645 nm). The LOEC was then calculated ($p < 0.05$); the possible influence of the coloured molecules and of the DMSO was taken into account as mentioned above.

Microalgal adhesion was determined in an analogous manner where the non-attached algal cells were removed prior to methanol addition (100 μL), releasing chlorophyll a from the remaining algal biofilms. The LOEC was then calculated ($p < 0.05$) after the evaluation of the possible influence of the coloured molecules and the DMSO.

Bioassays Against Macrofoulers

The inhibition of the mussels' settlement was evaluated through an enzymatic assay based on tyrosinase that plays an essential role in mussel byssus production. Mushroom tyrosinase (EC 1.14.18.1-Sigma-Aldrich) was used for this test (Hellio et al. 2015).

The 7 compounds were diluted in 0.5 mM potassium phosphate buffer pH 6.5 and transferred to clear polystyrene 96-multiwell plates (Fisher Scientific), to reach the same final concentrations tested for antibacterial and anti-microalgal assays. Then, 180 μL of a mixture composed of potassium phosphate buffer, L-tyrosine (1 mM) and dH_2O was added to each well, equilibrated at 25 °C. After addition of tyrosinase (1000 U mL^{-1}, the optical density was measured at 410 nm (Tecan Infinite M200) after 800-s incubation (Duckworth and Coleman 1970). The results were expressed as inhibition of tyrosinase activity compared with the control without the enzyme. A positive control was carried out with kojic acid, known as a tyrosinase inhibitor (Le Norcy et al. 2017), using the same concentrations as the tested molecules. The test was performed in triplicate and the LOEC was then calculated ($p < 0.05$).

Results and Discussion

Antifouling Activities

In the present work, the activity of 7 molecules, produced by the marine fungus *E. chevalieri* MUT 2316, were assessed against pioneer bacteria and microalgal species, that subsequently promote the surfaces colonization by macrofoulers (i.e. mussels, barnacles, ascidians, macroalgae) (Hanssen et al. 2014). The inhibition of adhesion and growth of marine bacteria and microalgae, as well as the inhibition of tyrosinase, was evaluated for all the compounds isolated from *E. chevalieri* MUT 2316 (**1–7**).

In order to study compounds in concentration ranges consistent with industrial exploitation of new antifouling formulations (as defined by the United States Office of Naval Research: < 25 μg mL^{-1}) (Trepos et al. 2014), emphasis will be placed on those compounds with the lowest significant LOEC values.

All the tested molecules, although differently, showed antifouling activity, as summarized in Fig. 2. Among the most promising compounds, cyclo-L-Trp-L-Ala (**7**) displayed activity in 61.5% of the assays at the lowest concentration (0.001 μg mL^{-1}) as reported in Table 1. It is interesting to note that cyclo-L-Trp- L-Ala (**7**) has inhibited the growth and adhesion of all the algal species, including the two diatoms *C. closterium* and *H. coffeaeformis*, that are key microfouling species, since they form permanent slimy layers on marine surfaces (Trepos et al. 2014). In addition, cyclo-L-Trp-L-Ala (**7**) could potentially inhibit the macrofouling, thus limiting the byssus formation in mussels at a concentration lower (0.01 μg mL^{-1}) than the PC kojic acid (Table 2). The antibacterial activity, although reported only against two and one species, for the growth and adhesion inhibition, respectively (Table 1), was never reported before in terrestrial bacteria (Tian et al. 2013; Bovio et al. 2019; Bovio 2019). Flavoglaucin (**5**) showed also a strong and broad antifouling activity, inhibiting all the microalgae tested (except for *C. closterium*); interestingly, flavoglaucin (**5**) was significantly able to inhibit the adhesion of *P. purpureum* and *P. tricornutum* at two and 4 orders magnitude lower, compared with the growth inhibition of the same strains. This makes of flavoglaucin (**5**) a highly desired antifouling compound towards microalgae, with limited biocide activity and high adhesion inhibition. Moreover, flavoglaucin (**5**) inhibited growth and adhesion of 4 and one bacterial species, respectively, and it was the only compound capable to prevent the growth of *R. litoralis* (Table 1). The antibacterial activity of this compound was previously confirmed against different terrestrial bacteria (Gao et al. 2012; Liang et al. 2018).

Dihydroauroglaucin (**4**) showed almost the same target fouling species of flavoglaucin (**5**) (only adhesion inhibition against *R. litoralis* and *P. tricornutum* was not reported). However, dihydroauroglaucin (**4**) was significantly active against bacteria and microalgae at concentrations usually higher than that reported for flavoglaucin (**5**) (Table 1). Dihydroauroglaucin (**4**) was also able to inhibit the tyrosinase (Table 1) even if a too high concentration (100 μg mL^{-1}) to be considered useful for future commercial applications. The same molecule was only recently assessed for the antibacterial activity by Bovio et al. (2019).

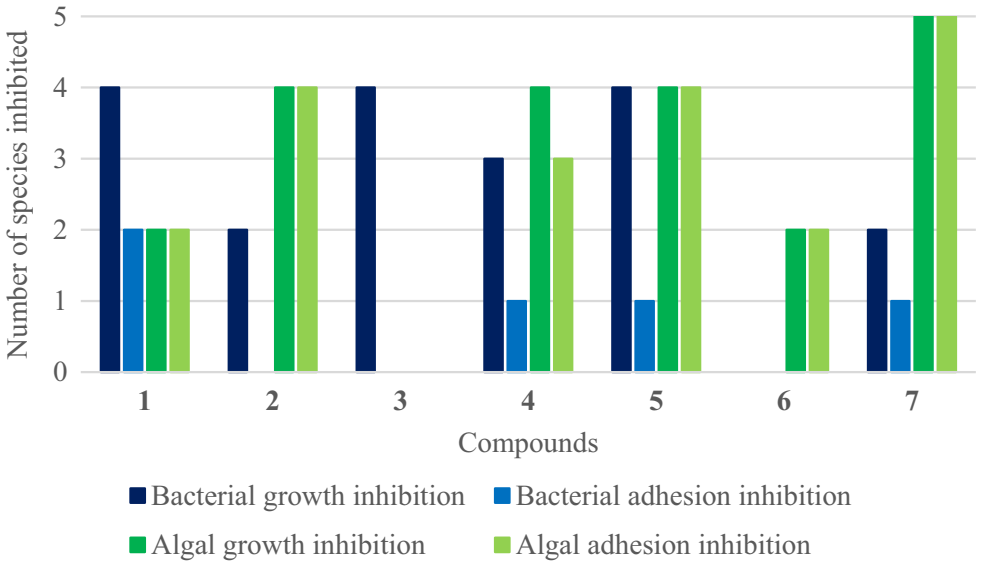

Fig. 2 Number of bacterial and microalgal species inhibited in the growth and adhesion by the 7 tested compounds (**1**–**7**) produced by *E. chevalieri* MUT 2316

Neoechinulin A (**2**) revealed to be more active against microalgae than bacteria (Table 1); indeed, it was able to inhibit growth and adhesion of 4 out of 5 microalgae, including the two diatoms relevant for the biofouling formation, as above mentioned. Regarding bacteria, only two were inhibited at the highest concentration tested (100 μg mL^{-1}). The same compound showed no antibacterial activity in Bovio et al. (2019), while was active against other terrestrial bacteria (Ganihigama et al. 2015; Liang et al. 2018; Zhao et al. 2018). Neoechinulin A (**2**) was previously reported to inhibit the larval settlement of *B. amphitrite* (Sun et al. 2014; Chen et al. 2018). Although it did not display toxicity against the barnacle larvae ($LC_{50} > 50$ μg mL^{-1}), it showed teratogen effects against the embryos of the zebrafish *Danio rerio* (Chen et al. 2018). In this regards, the further use of neoechinulin A (**2**) as antifouling should be carefully evaluated.

Echinulin (**1**) reduced the growth of 4 out of 5 bacteria, and the adhesion of two bacteria, being the only compound significantly limiting the adhesion of *P. citrea* (Table 1). This is the first report of the antibacterial activity of echinulin (**1**) against marine bacteria, while it was inactive against several terrestrial bacteria (Gao et al. 2012; Meng et al. 2015; Pereira et al. 2016; Zin et al. 2017; Bovio et al. 2019); only Liang et al. (2018) reported echinulin (**1**) as active against different bacteria. Echinulin (**1**) limited the adhesion of *R. litoralis*, *E. gayraliae* and *P. tricornutum* without interfering with the growth of the selected bacteria and algae. Probably, among all the possible mechanisms of action of echinulin (**1**), there is the ability to alter the expression of adhesive proteins in the target species or more generally avoiding the release of adhesive compounds in selected bacterial and microalgal species. Certainly, echinulin (**1**) deserves more attention also in light of its capability to inhibit the settlement of *B. amphitrite* at non-toxic concentration for barnacle species and zebrafish (Chen et al. 2018).

Physcion (**3**) and cinnalutein (**6**) showed a specific activity only against one of the two target groups (bacteria or microalgae) assessed in the present work. Pyscion (**3**) inhibited only bacteria (4 out of 5 species) and in particular their growth, not affecting the ability of the selected species to form a biofilm, making of physcion (**3**) not the best candidate for new antifouling formulation. The antibacterial activity of this compound was confirmed also against some human pathogens (Basu et al. 2005; Liang et al. 2018; Bovio et al. 2019).

Regarding cinnalutein (**6**), it was active only towards microalgae by inhibiting growth and/or adhesion of three species. Bovio et al. (2019) reported a selective activity of cinnalutein (**6**) against few bacterial strains, probably highlighting a specific mechanism of action for this compound (**6**).

Overall, all the compounds except cinnalutein (**6**) displayed activity against the Gram-negative bacteria tested, that due to the outer membrane represent a more difficult target compared with the Gram-positive (Trepos et al. 2015). Noteworthy, all the compounds were active against *V. aestuarianus*; this species has been found worldwide and is recognized as a virulent pathogen of oysters, leading to high mortality on both adult and juvenile (Travers et al. 2017). Therefore, the fungal molecules tested in the present study could be suitable both as antifouling and for the protection of organisms in aquaculture against pathogenic strains.

Moreover, to put these results in an industrial and applicative context, the minimum inhibitory concentration (comparable to the LOEC) of the biocide TBT oxide and copper sulphate, against the growth of a range of bacteria, was observed

Table 1 Low observable effect concentration (LOEC μg/mL) values of compounds (**1**–**7**) against the growth (Gr) and adhesion (Ad) of marine bacteria and microalgae representatives of fouling organisms. Positive values are italicized

Molecules	Echinulin (1)		Neoechinulin A (2)		Physcion (3)		Dihydroauroglaucin (4)		Flavoglaucin (5)		Cinnalutein (6)		Cyclo-L-Trp- L-Ala (7)		Sea-Nine[a]	
	Gr	Ad	Gr	Ad	Gr	Ad	Gr	Ad	Gr	Ad	Gr	Ad	Gr	Ad	Gr	Ad
Marine bacteria																
H. aquamarina	*100*	> 100	> 100	> 100	*0.01*	> 100	> 100	> 100	> 100	> 100	> 100	> 100	*0.001*	> 100	*0.1*	*< 0.01*
P. irgensii	*1*	> 100	*100*	> 100	*1*	> 100	*0.01*	> 100	*1*	> 100	> 100	> 100	> 100	*0.001*	*1*	*0.1*
P. citrea	*0.01*	*0.001*	> 100	> 100	*0.01*	> 100	*0.01*	> 100	*1*	> 100	> 100	> 100	> 100	> 100	N/A	N/A
R. litoralis	> 100	*0.1*	> 100	> 100	> 100	> 100	> 100	*0.001*	*0.01*	*0.1*	> 100	> 100	> 100	> 100	*1*	*< 0.01*
V. aestuarianus	*100*	> 100	*100*	> 100	*10*	> 100	*0.001*	> 100	*0.001*	> 100	> 100	> 100	*0.001*	> 100	*< 0.01*	*1*
Marine microalgae																
C. closterium	> 100	> 100	*0.001*	*1*	> 100	> 100	> 100	> 100	> 100	> 100	> 100	*0.001*	*0.001*	*0.001*	*< 0.01*	*< 0.01*
E. gayraliae	> 100	*0.01*	> 100	*0.01*	> 100	> 100	*1*	*1*	*0.01*	*0.01*	> 100	> 100	*0.001*	*0.001*	*< 0.01*	*< 0.01*
H. coffeaeformis	*0.1*	> 100	*0.01*	*1*	> 100	> 100	*10*	*1*	*1*	*1*	*0.01*	*1*	*0.001*	*1*	*< 0.01*	*< 0.01*
P. tricornutum	> 100	*0.01*	*1*	*0.001*	> 100	> 100	*1*	> 100	*10*	*0.001*	*1*	> 100	*100*	*1*	N/A	N/A
P. purpureum	*0.001*	> 100	*100*	> 100	> 100	> 100	*1*	*0.1*	*0.1*	*0.001*	> 100	> 100	*10*	*100*	*< 0.01*	*< 0.01*

N/A not available

[a] Trepos et al. (2015)

Table 2 Low observable effect concentration (LOEC μg/mL) values of compounds (**1**–**7**) in the tyrosinase assay. Positive values are italicized

Molecules	Tyrosinase assays
Echinulin (**1**)	> 100
Neoechinulin A (**2**)	> 100
Physcion (**3**)	> 100
Dihydroauroglaucin (**4**)	*100*
Flavoglaucin (**5**)	> 100
Cinnalutein (**6**)	>100
Cyclo-L-Trp- L-Ala (**7**)	*0.01*
Kojic acid	*1*

to be 6 μg mL^{-1} (Hellio et al. 2001). In 73.7% of cases, the molecules assessed here were able to inhibit the bacterial growth at concentrations lower than 1 μg mL^{-1}.

By comparing the obtained results with those of the commercial antifouling Sea-Nine (Table 1, data from Trepos et al. 2015), available for 4 out of 5 bacterial and microalgal species here tested, several *E. chevalieri* MUT 2316–derived molecules can be considered highly promising. For instance, echinulin (**1**), neoechinulin A (**2**), flavoglaucin (**5**) and cyclo-L-Trp-L-Ala (**7**) were active at 0.001 μg mL^{-1} against at least one algal species (Table 1), being therefore comparable and probably even more active than the Sea-Nine (MIC < 0.01 μg mL^{-1}) reported by Trepos et al. (2015). As for the antibacterial assays physcion (**3**), dihydroauroglaucin (**4**), flavoglaucin (**5**) and cyclo-L-Trp-L-Ala (**7**) when active showed LOEC values lower or comparable with those reported for the Sea-Nine (Table 1).

Structure-Activity Relationship

Physcion (**3**) and cinnalutein (**6**) are both anthraquinones; this family of compounds although being of one of the most relevant chemical class of polyketides is scarcely reported for the antifouling activity (Wang et al. 2017). Physcion (**3**) was exclusively able to inhibit the growth of bacteria, while cinnalutein (**6**) inhibited the growth/adhesion of few microalgae. Thus demonstrating that the functional groups are key elements in determining the biological activity compared with the core structure in physcion (**3**) and cinnalutein (**6**).

Among the same chemical family of anthraquinones, there are no reports of compounds inhibiting the marine microfoulers, while several reports underline the antibacterial activity of anthraquinones against human pathogens. In addition, different anthraquinones compounds (9,10-anthraquinone-2-sulfonic acid, anthraquinone–disulfonate/polypyrrole composite) inhibited the fouling formation on bioreactors' membranes (Li et al. 2014; Xu et al. 2015). Regarding macrofoulers, Wang et al. (2016) highlighted the bioactivity of 5 out of 8 marine-derived anthraquinones (Averufin, 8-*O*-methylnidurufin, Asperinine A, Asperinine B and Rhodoptilometrin) against the larval settlement of *Balanus amphitrite* at 25.0 μg ml^{-1}. Marine fungal anthraquinones were reported to both inhibit (< 0.0125 μg ml^{-1}) and paralyze *B. amphitrite* larvae (Li et al. 2013). A weak inhibition towards *B. amphitrite* was also observed for several anthraquinones isolated from the marine *Penicillium* sp. (Bao et al. 2013). In our study, only an enzymatic test was performed to evaluate the antifouling activity against macrofoulers where physcion (**3**) and cinnalutein (**6**) did not shown any result, not allowing further comparison with the literature.

Dihydroauroglaucin (**4**) and flavoglaucin (**5**) are phenolic terpenoids which share structural similarities. Dihydroauroglaucin (**4**) differs from flavoglaucin (**5**) by the presence of two double bonds in positions one and three. The high similarity of these compounds is reflected in their analogous biological activities, with as main difference the concentration at which they showed a positive response. Indeed, the lack of double bonds in flavoglaucin (**5**) might guarantee more flexible structure that can easily interact with the target.

Similar to our observation that little variation in the chemical structure does not strongly influence the biological activity of this class of compounds, Tsukamoto et al. (1997) reported a comparable anti-larval activity of (+)-curcuphenol and (+)-curcudiol against *B. amphitrite*. The latter mentioned compound only differs for the presence of a hydroxyl group; both molecules were isolated from the marine sponge *Myrmekioderma dendyi* (Tsukamoto et al. 1997).

As already observed for anthraquinones, the antifouling assays are mainly performed against macrofoulers, for instance two phenolic sesquiterpenoids derivatives of *Aspergillus* sp., isolated from a marine sponge, were able to inhibit the settlement of *B. amphitrite* (Li et al. 2012). However, (*Z*)-5-(hydroxymethyl)-2-(6′-methylhept-2′-en-2′-yl)-phenol was toxic for *B. amphitrite* larvae, thus only (−)5-(hydroxymethyl)-2-(2′,6′,6′-trimethyltetrahydro-2*H*-pyran-2-yl)phenol was a promising antifouling (Li et al. 2012). These two compounds showed also a weak antibacterial activity against non-target fouling species.

The remaining three compounds, echinulin (**1**), neoechinulin A (**2**) and cyclo-L-Trp-L-Ala (**7**), are diketopiperazines. They present different levels of structural complexity, from the simplest structure of (**7**) to the most complex ones of (**2**) and (**1**) to which one and three functional groups are respectively added.

Looking to other diketopiperazines, Cho et al. (2012) isolated from the marine bacterium *Streptomyces praecox* two small diketopiperazine namely (6*S*,3*S*)-6-benzyl-3-methyl-2,5-diketopiperazine and (6*S*,3*S*)-6-isobutyl-3-methyl-2,5-diketopiperazine. Both compounds inhibited the settlement of *Ulva pertusa* zoospores and the growth of the diatom *Navicula annexa* (Cho et al. 2012). Interestingly, in our study, only two compounds were able to inhibit the growth and also

the adhesion of the diatoms, they both are diketopiperazines (neoechinulin A (**2**) and cyclo-L-Trp-L-Ala (**7**)).

Several diketopiperazines isolated from marine microorganisms and invertebrates, including fungi, bacteria and sponges, were able to inhibit *B. amphitrite*, making of this class of compounds highly promising antifouling (Li et al. 2006; Yang et al. 2007; Yang et al. 2016).

Moreover, cyclo-L-Trp-L-Ala (**7**), one of the most promising compounds in this study, presents the same core structure of barretin. This highly promising natural antifouling has been isolated from a sponge. Certainly it is not feasible to collect sponges from the natural environment to extract barrettin, therefore different chemical syntheses have been performed (Bergman 2013; Kelley et al. 2017). However, if further studies will confirm that cyclo-L-Trp-L-Ala (**7**) is a promising antifouling, several techniques can be applied to increase its production. Indeed, compounds isolated from microorganisms do not face the same supply problems as those isolated from animals, plants or algae (Wang et al. 2017). Microorganisms can be cultivated in bioreactors using the most appropriate conditions to enhance the production of specific metabolites. As proved by Bode et al. (2002) with the OSMAC approach (One Strain-Many Compounds), the modification of easily and accessible growing parameters can stimulate the production of target metabolites.

Finally, since the biological activity of the molecules is strongly related with the species used in the bioassays, it is important to contextualize the results obtained in this study, with works that used the same species of bacteria and microalgae. Therefore, looking at the antifouling activity of other natural products (from fungi or sponges), it emerges that molecules produced by the sponge derived fungus *Aspergillus* sp. were active at one of the lowest value reported in literature (0.001 μg mL^{-1}) (Zhou et al. 2014), as in our case. Two bromotyrosine derivatives (ianthelline and barettin) isolated from the marine sponge *Stryphnus fortis* showed MIC values between 0.1 and 10 μg mL^{-1} (Hanssen et al. 2014).

In the future, it would be interesting to better understand the mechanisms behind the inhibition of bacterial and algal growth by compounds produced by *E. chevalieri* MUT 2316. Moreover, it would be fascinating to understand whether fungi in their natural environment and in association with the sponge produce the same compounds as in axenic culture. It has been hypothesized that different degrees of complexity characterize the interactions among sponge holobiont components, including mutualism, commensalism and parasitism (Rodríguez-Marconi et al. 2015). Non-pathogenic microorganisms can positively contribute to sponge metabolism, by increasing the uptake of nitrogen, sulphur and carbon, producing hydrolytic enzymes able to convert complex organic matter in easily accessible nutrients for sponges (Taylor et al. 2007; Debbab et al. 2012; Pita et al. 2018).

The most fascinating theory about the role of fungi in the sponge holobiont is that marine fungi produce a wide range of bioactive molecules acting as a chemical defence against sponges' predators, pathogens and fouling organisms (Debbab et al. 2012). This statement is supported by the high yield of bioactive molecules isolated from sponge's derived fungi (Debbab et al. 2012). So far, metabolites knew to be produced by sponges could be produced by fungi.

In conclusion, the molecules produced by *E. chevalieri* MUT 2316 revealed to be promising antifouling. This was particularly evident for cyclo-L-Trp-L-Ala (**7**) showing antibacterial, anti-microalgal activity and the inhibition of tyrosinase, at comparable or lower concentrations than commercial antifouling formulations. Other compounds, presented a broad-spectrum activity, this was the case of dihydroauroglaucin (**4**) and flavoglaucin (**5**). However, it is unrealistic to think that only a molecule would be able to inhibit all the fouling species, without being a common biocide, active also against non-target species. Based on this study, a formulation composed by echinulin (**1**), dihydroauroglaucin (**4**), flavoglaucin (**5**) and cyclo-L-Trp-L-Ala (**7**) would inhibit the growth and adhesion of all tested bacteria (except the adhesion of *Vibrio aestuarianus*) at concentrations < 0.01 μg mL^{-1}. Similarly, neoechinulin A (**2**), flavoglaucin (**5**) and cyclo-L-Trp-L-Ala (**7**) would be able to inhibit the growth and adhesion of all tested microalgae at concentrations < 1 μg mL^{-1}, observing the requirements of the U.S. Office of Naval Research for new antifouling formulations.

Funding Information This research was supported by the Galileo project [grant number G18-701] and the Vinci project [grant number C2-22] from the Italo-French University. Biodimar team was supported by funding from Biogenouest. M. Mehiri is supported by the EMBRIC project (EU grant No 654008) and the Galileo project from the Italo-French University [grant number 34595SA]. This work was supported by the French government, managed by the French National Research Agency under the project Investissements d'Avenir UCA^{JEDI} (# ANR-15-IDEX-01).

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