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ORIGINAL ARTICLE

Terpenes from the Red Alga *Sphaerococcus coronopifolius* Inhibit the Settlement of Barnacles

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Abstract In this study, we screened eight terpenes isolated from the organic extract of *Sphaerococcus coronopifolius* for their antifouling activity in order to find possible new sources of non-toxic or less toxic bioactive antifoulants. The anti-settlement activity (EC_{50}) and the degree of toxicity (LC_{50}) of *S. coronopifolius* metabolites was evaluated using larvae of the cirriped crustacean *Amphibalanus (Balanus) amphitrite* (cyprids and nauplii) as model organism. For five of eight tested metabolites EC_{50} was lower than 5 mg/L. The most promising results were observed for bromosphaerol (**3**), which expressed an EC_{50} value of 0.23 mg/L, in combination with low toxicity levels (LC_{50}>100 mg/L). The therapeutic ratio—an index used to estimate whether settlement inhibition is due to toxicity or other mechanisms—is also calculated and discussed.

Keywords Antifouling · Marine natural products · Barnacle · *Sphaerococcus coronopifolius*

Introduction

Marine fouling affects a wide range of human activities in the aquatic environment and, especially in shipping, it is associated with economic loss due to speed reduction and higher costs both for fuel and for hull maintenance. Technological problems related to biofouling and methods for protecting surfaces have been widely discussed in the past few years (Omae 2003; Yebra et al. 2004; Chambers et al. 2006). The majority of vessels are protected by antifouling paints containing biocides (Almeida et al. 2007); the key property of a good antifouling biocide with respect to the environment is to be effective in preventing fouling, without causing persisting adverse environmental effects. The effects and behaviour of biocides used in antifouling paints have been extensively studied and the related data are available (see reviews: Thomas 2001; Thomas and Brooks 2010). Currently, a wide range of chemicals are used as antifouling biocides, governed by different regulations, depending on the legislation in each country. Copper, copper pyrithione, zinc pyrithione, TPBT, diuron, SeaNine 211, Irgarol 1051, chlorothalonil, cuprous thyocianate, Ziram, Zineb, naphthenic acid copper salts, tolyfluanid, Econea and capsaicin are just a few of the antifoulants in use. Nowadays, marine antifouling technology is at a crossroads: in September 2008, IMO banned the use of self-polishing tributyltin coatings, while there is an increasing opposition to the use of copper.

Silicone-based fouling release technologies are providing an alternative solution, however they are relatively expensive and can be easily damaged (Swain et al. 2007).

Preventing the settlement of fouling organisms in a non-toxic manner is the ideal solution; it is therefore necessary to investigate new avenues, which may be inspired by a biomimetic approach, studying structures and functions of biological systems as models for the design of antifouling solutions. Much effort has been directed to identify natural chemistries that may act as antifoulants (Pawlik 1992; Abarzua et al. 1999; Rittschof 2001; de Nys and Steinberg 2002; Burgess et al. 2003; Ralston and Swain 2009). In the

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marine environment, a number of organisms are equipped with natural physical or chemical defence mechanisms against fouling (Engel et al. 2002; Hellio et al. 2002; Paul and Puglisi 2004). Several marine metabolites show significant levels of antibacterial, antimacrofouling, antifungal, and antiprotozoan properties, and have good potentials to be developed as antifouling paints (Hellio et al. 2000; Da Gama et al. 2002; Steinberg and de Nys 2002; Ali et al. 2002; Kubanek et al. 2003; Faimali et al. 2003; Fusetani 2004; Hellio et al. 2005).

There are many reports on marine organisms that show their ability to resist epibiosis (Fusetani 2004), including several species of algae that contain a diverse spectrum of chemical entities with antifouling activity. Algae seem to be chemically protected by surface-bound or continuously released water-soluble compounds that can deter invertebrate larvae from settling. Algal metabolites can affect the development and grazing behaviour of some settling organisms, pointing to the existence of chemical antifouling mechanisms. Isolation of these bioactive secondary macroalgae metabolites might lead to the development of new eco-friendly antifouling paints.

The cosmopolitan red alga *Sphaerococcus coronopifolius* is an unusually prolific source of secondary metabolites, mainly diterpenoids. So far, only a small number of reports have been published on the isolation and characterization of bioactive compounds from *S. coronopifolius*, and even more restricted information is available on their biological activity. Cafieri et al. (1982a and 1988) isolated the diterpenes sphaeopyrane and 12 *S*-hydroxybromosphaerol from *S. coronopifolius*; Etahiri et al. (2001) isolated two new bromoditerpenes that showed antibacterial activity against Gram positive bacteria. Recently, Smyrniotopoulos et al. (2010) tested the cytotoxicity of nine brominated diterpenes against human lung cancer cell lines, and the antibacterial activity of six bromoditerpenes against multidrug-resistant and methicillin-resistant *Staphylococcus aureus* strains.

In this work, we report on the evaluation of eight metabolites from *S. coronopifolius* as to the settlement and larvae mortality of the cirriped crustacean *Amphibalanus amphitrite*. The therapeutic ratio (TR), which indicates whether settlement inhibition is due to the toxicity of the compounds or related to other mechanisms, is also discussed (Rittschof et al. 1994; Vitalina et al. 1997; Clare et al. 1999; Faimali et al. 2005; Fig. 1).

Materials and Methods

Extraction and Isolation of Metabolites

S. coronopifolius was collected by SCUBA diving in Palaiokastritsa bay in the West coast of Corfu Island, Greece, at a depth of 10–15 m, in May 2002. A specimen is kept at the Herbarium of the Laboratory of Pharmacognosy and Chemistry of Natural Products, University of Athens (ATPH/MO/201).

S. coronopifolius was initially freeze-dried (291.4 g dry weight) and then exhaustively extracted with mixtures of CH_2Cl_2/MeOH (3/1 *v*/*v*) at room temperature. The combined extracts were concentrated to give a dark green residue (8.20 g), which was later subjected to vacuum column chromatography on silica gel, using a 10% step gradient of cyclohexane-EtOAc elution sequence. Fraction Ia (10% EtOAc in cyclohexane; 753.8 mg) was fractionated on silica gel isocraticly with 100% cyclohexane, to yield pure compound **1** (449.8 mg). Fraction IIa (20% EtOAc in cyclohexane; 4.01 g) was subjected to gravity column chromatography, using a 2%, initially, to 10% step gradient of cyclohexane-EtOAc. Part (50 mg) of the VIIb fraction (8% EtOAc in cyclohexane; 2.85 g) was subjected to normal phase HPLC chromatography, using 5% EtOAc in cyclohexane as a mobile phase to yield pure compounds **2** (18.7 mg) and **3** (21.0 mg). The CH_3CN soluble portion (173.4 mg) of fraction XIb (50% EtOAc in cyclohexane) (199.6 mg) was subjected to reversed-phase HPLC chromatography, using CH_3CN as mobile phase to yield pure compounds **5** (10.9 mg) and **6** (15.3 mg). The CH_3CN soluble portion (306.7 mg) of fraction IVa (60% EtOAc in cyclohexane; 337.8 mg) was subjected to reversed-phase HPLC chromatography, using 100% CH_3CN as a mobile phase. Peak XIc (retention time 12.52 min; 86.3 mg) was subjected again to reversed-phase HPLC chromatography, using CH_3CN as mobile phase to yield pure compound **4** (54.4 mg). The CH_3CN soluble part (323.4 mg) of fraction Va (70% EtOAc in cyclohexane) (419.8 mg) was subjected to reversed-phase HPLC chromatography, using CH_3CN as a mobile phase to yield pure compound **7** (42.1 mg). Peak XIId (retention time 11.6 min; 48.1 mg) with HPLC normal phase purification, using 70% $CHCl_3$ in *n*-hexane as mobile phase, yielded pure compound **8** (2.0 mg).

Compounds **1**–**8** were identified by comparison of their spectroscopic data (including NMR, MS, IR, UV) with previously reported literature values.

Settlement Inhibition Assays

Cypris larvae were obtained from laboratory cultures of the crustacean cirriped *A. amphitrite* brood stock. Twenty to thirty adult barnacles were reared in 800 mL aerated beakers containing filtered natural sea water (FNSW) at 20±1°C, with a 16 h:8 h light:dark (L:D) cycle. They were fed every 2 days with nauplii of *Artemia salina* sp. (100 mL, 20–35 larvae mL^{-1}), and *Tetraselmis suecica* (100 mL, 2×10^5 cells·mL^{-1}). Twenty beakers containing

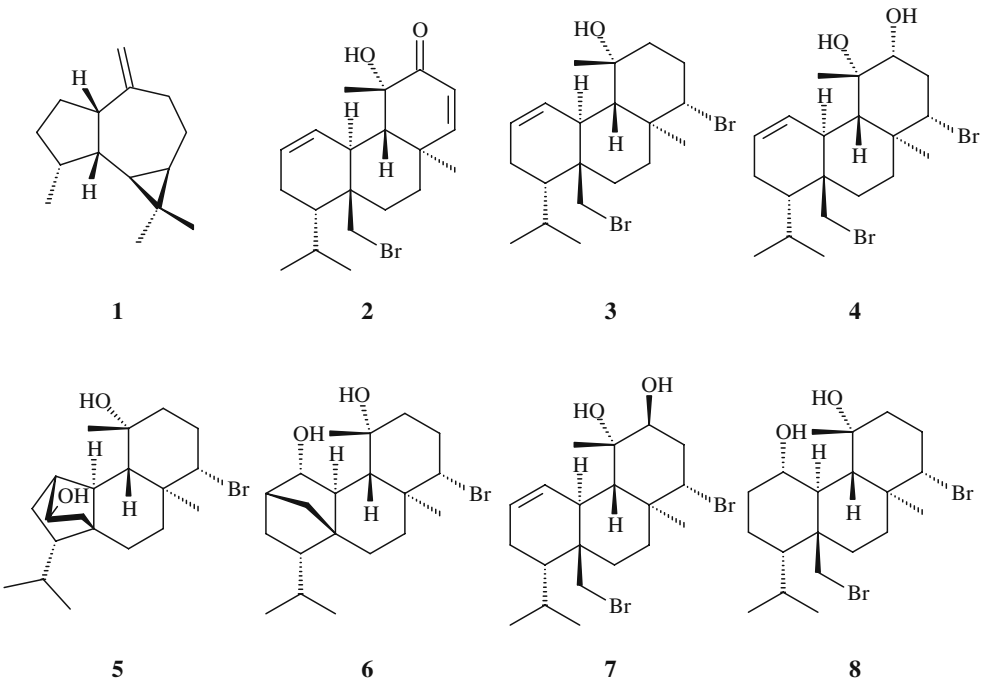

Fig. 1 Structure of *Sphaerococcus coronopifolius* metabolites

adults reared under the above mentioned conditions produced nauplii throughout the year. Nauplii were collected with a 5-mL pipette by positioning the beaker near a light source and reared in 500-mL beakers containing 0.22 μm FNSW gently aerated at 28±1°C with a 16 h:8 h L:D cycle. Nauplii were fed every 48 h with *T. suecica* (5×10^5 cells mL^{-1}) until, after 5–6 days, they reached the cyprid stage.

Newly metamorphosed cyprids were filtered and maintained in filtered (0.22 μm) natural sea water at 6°C for 4 days before being used in settlement assays (Rittschof et al. 1992). Settlement tests were performed by adding 15–20 cyprids (for each replicate) to 24-well polystyrene plates containing 2 mL of bioactive metabolite solution at different concentrations (0; 0.1; 1; 10; 100 mg/L). Four replicates were prepared for each concentration of each tested metabolite and the reported results are the mean values of the four replicates. The 24-well plates were stored for 72 h at 28°C with a 16:8 L:D cycle. After 24, 48 and 72 h, the number of settled, not-settled, and dead larvae was measured under a stereomicroscope. EC_{50} (concentration of metabolite causing 50% settlement inhibition to exposed organisms) was calculated with results obtained after 72 h. Additionally, at the same time, $LC_{50(cypris)}$ was calculated, as the metabolite concentration causing 50% mortality to the exposed organisms.

Naupliar Toxicity Test

Acute environmental toxicity of metabolites was tested by using stage II nauplii of *A. amphitrite*. Nauplii were obtained from adult brood stock as described above, collected and immediately filtered in 0.22 μm FNSW. The toxicity assay was set within 2–4 h from nauplii collection. The test was performed by adding 15 to 25 nauplii II to 24-well polystyrene plates containing 2 mL of bioactive metabolite solution at different concentrations (0; 0.1; 1; 10; 100 mg/L). Four replicates were prepared for each concentration of each tested metabolite and the reported results are the mean values of the four replicates. The plates were stored for 48 h at 20°C with a 16:8 L:D cycle. After 24 and 48 h, the number of dead larvae was observed under a stereomicroscope. $LC_{50(nauplii)}$ was calculated as the concentration of metabolite causing 50% mortality to the exposed organisms after 48 h of contact.

Statistical Analysis

Settlement inhibition (EC_{50}) at 72 h and mortality (LC_{50}) values at 48 h (for nauplii) and 72 h (for cyprids) were calculated using trimmed Spearman–Karber analysis (Finney 1978). Therapeutic ratio is defined as LC_{50}/EC_{50}. This index was calculated using mortality values measured for larvae at naupliar stage (TR_N) and for larvae at cypris stage (TR_C).

Results

Results of settlement inhibition and cyprid mortality tests for all eight compounds are shown in Fig. 2. The 72 h values of $EC_{50(settl)}$ and $LC_{50(cypris)}$ are summarised in Table 1. For five of these metabolites (**2**, **3**, **5**, **6** and **7**),

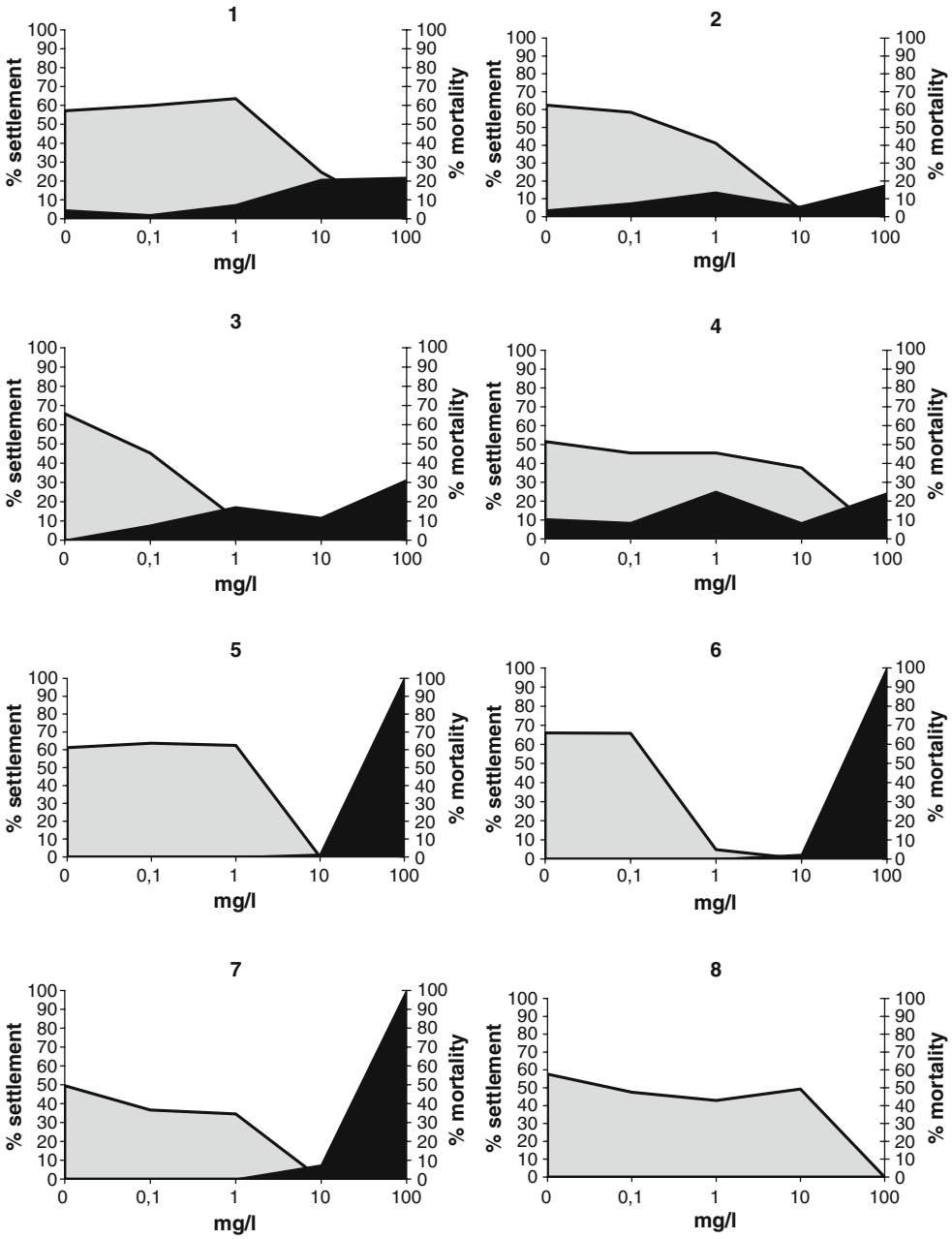

Fig. 2 Results of settlement (*grey area*) and mortality (*black area*) test with cypris larvae of *A. amphitrite* after 72 h of contact (M±ES; n=4)

Table 1 72 h—$EC_{50(settl)}$, 72 h—$LC_{50(cypris)}$ derived respectively from settlement inhibition test and mortality test with cypris larvae and 48 h—$LC_{50(nauplii)}$ from mortality test with nauplii of *Amphibalanus amphitrite* (with 95% confidence limits)

Compounds	72 h—$EC_{50(settl)}$ mg/L (CL 95%) Cypris larvae settlement'	72 h—$LC_{50(cypris)}$ mg/L (CL 95%) Cypris larvae mortality	48 h—$LC_{50(nauplii)}$ mg/L (CL 95%) Naupliar mortality
1	7.94 (6.34–9.95)	>100	8.32 (6.63–10.44)
2	1.53 (1.17–2.00)	>100	0.32 (0.28–0.45)
3	0.23 (0.17–0.30)	>100	3.63 (3.05–4.33)
4	15.97 (11.94–22.10)	>100	>100
5	3.10 (2.98–3.23)	30.20 (28.31–32.21)	2.82 (2.55–3.12)
6	0.38 (0.33–0.43)	30.20 (28.31–32.21)	0.39 (0.34–0.44)
7	1.68 (1.09–2.59)	26.92 (23.93–30.27)	0.33 (0.31–0.35)
8	21.29 (13.66–33.18)	> 100	8.46 (6.56–10.91)

$EC_{50(settl)}$ was lower than 5 mg/L. Very high activity was observed for metabolites **3** and **6,** which showed $EC_{50(settl)}$ values lower than 0.5 mg/L. Concerning cyprids mortality, five compounds (**1**, **2**, **3**, **4** and **8**) showed $LC_{50(cypris)}$ values higher than the maximum tested concentration (100 mg/L), while the remaining three compounds showed toxicity values in the range of 30 mg/L. A naupliar toxicity assay was performed and results for all compounds are shown in Fig. 3. It is evident that naupliar response to tested molecules is very different from cyprids; indeed, naupliar mortality occurs at lower concentrations than for cyprids, and all $LC_{50\ (nauplii)}$ values referring to nauplii (see Table 1) are lower than 10 mg/L (except for **4**). In Table 2, we report the Therapeutic Ratio values, calculated both for naupliar (TR_N) and cyprids (TR_C). High TR values, indicate low toxicity of the tested compound. With regard to TR_N values, only **3** showed a quite high value (15.78), while for TR_C three metabolites (**2**, **3**, **6**) had values higher than 50.

Discussion

Isolated metabolites, tested in pure form and showing antifouling activity, belong to the chemical classes of fatty

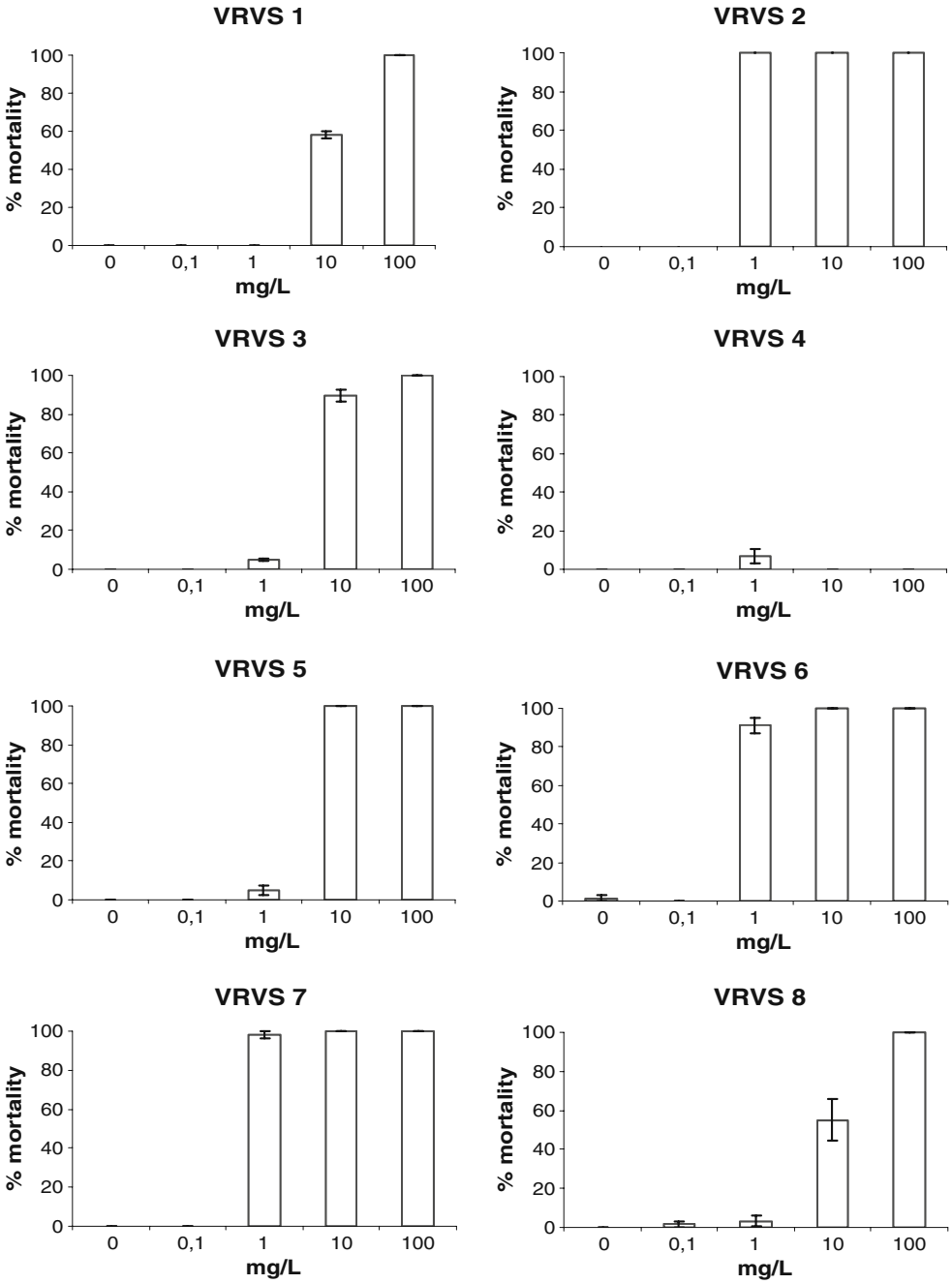

Fig. 3 Results of acute toxicity test with nauplii of *A. amphitrite* (M±ES; *n*=4)

Table 2 Therapeutic ratio for all eight tested compounds calculated both with $LC_{50\ (nauplii)}$ values from naupliar toxicity test (TR_N) and with $LC_{50(cypris)}$ from cyprids toxicity assay (TR_C)

Compounds	TR_N ($LC_{50\ (nauplii)}$/ $EC_{50(settl)}$)	TR_C ($LC_{50(cypris)}$/ $EC_{50(settl)}$)
1	1.05	12.59
2	0.21	65.3
3	15.78	434.78
4	6.26	6.26
5	0.91	9.74
6	1.02	79.47
7	0.19	16.02
8	0.39	4.69

acids, lipopeptides, amides, alkaloids, terpenoids, lactones, pyrroles, and steroids. Seaweeds produce a wealth of bioactive metabolites (Tringali 1997), which, among other ecological roles, are responsible for the protection against other settling organisms. Chemical investigations of the benthic macroalga *Delisea pulchra* resulted in the isolation and identification of halogenated furanones, which, when tested against the barnacle *A. amphitrite* (Steinberg et al. 1998) and the macroalga *Ulva lactuca* (Maximilien et al. 1998), showed significant inhibitory effects against both fouling organisms in both field and laboratory experiments (de Nys and Steinberg 2002). Phlorotannins, isolated from the Australian brown algae *Ecklonia radiata*, have also shown to be effective against the settlement and growth of the green alga *Ulva* sp. (Jennings and Steinberg 1997). Non-polar secondary metabolites—dictyol E and pachydictyol A—isolated from the brown alga *Dictyota menstrualis*, were found capable of inhibiting the settlement of *Bugula neretina* (Schmitt et al. 1995). Extracts of the marine alga *Sargassum muticum* were found to inhibit the development of fouling organisms in a non-toxic manner (Hellio et al. 2000), whereas the dichloromethane extract of the same alga was found effective against germination of *U. lactuca* (Bazes et al. 2009). Elatol and deschloroelatol, isolated from the red alga *Laurencia rigida*, were capable of inhibiting larval settlement of both *A. amphitrite* and *B. neretina* (de Nys et al. 1996; Konig and Wright 1997). Two halogenated monoterpenes, exhibiting deterrent effects against *A. amphitrite*, have been isolated from the Tasmanian red alga *Plocamium costatum*, (Konig et al. 1999). Furthermore, Lau and Qian (1997) reported that phlorotannins from *Sargassum tenerrimum* inhibited the metamorphosis of the polychaete *Hydroides elegans*. Larval settlement of *A. amphitrite* was found to be inhibited by crude extracts of the brown alga *Bifurcaria bifurcata* (Marèchal et al. 2004).

S. coronopifolius is known to produce halogenated analogues of diterpenoids (Dembitsky et al. 2002). The sesquiterpene alloaromadendrene (**1**) (De Rosa et al. 1988; Smyrniotopoulos et al. 2010) along with the brominated diterpenes sphaerococcenol A (**2**) (Fenical et al. 1976; Smyrniotopoulos et al. 2008a), bromosphaerol (**3**) (Fattorusso et al. 1976; De Rosa et al. 1988; Smyrniotopoulos et al. 2008a), 12*R*-hydroxybromosphaerol (**4**) (Cafieri et al. 1987; Smyrniotopoulos et al. 2008a), coronopifoliol (**5**) (Cafieri et al. 1985), bromotetrasphaerol (**6**) (Cafieri et al. 1988), 12 *S*-hydroxybromosphaerol (**7**) (Cafieri et al. 1982a; Smyrniotopoulos et al. 2008a) and 1 *S*-hydroxy-1,2-dihydro-bromosphaerol (**8**) (Cafieri et al. 1982b; Smyrniotopoulos et al. 2008b), have been previously isolated from various collections of *S. coronopifolius* collected from different areas of the Mediterranean Sea.

The algal and not microbial biogenetic origin of the above mentioned metabolites is strongly supported by their high percentage in the algal biomass. Specifically, the sesquiterpene alloaromadendrene (**1**) and the bromoditerpenes sphaerococcenol (**2**), bromosphaerol (**3**) and 12*R*-hydroxybromosphaerol (**4**), are major constituents of the alga, accounting for the 28.1% (*w*/*w*) of the organic extract (**1**: 8.1%, **2**: 9.1%, **3**: 10.4%, **4**: 0.5%; Smyrniotopoulos, unpublished data). The coexisting minor diterpenoids (**5**–**8**) are structurally related to the main metabolites indicating a common biosynthetic origin from genanyl-geranylpyrophosphate.

Recently, diterpenoids extracted from the Mediterranean brown alga *Dictyota* sp. have been evaluated as antifouling substances against marine bacterial biofilm (Viano et al. 2009), while Culioli et al. (2008) investigated the antifouling activity against *A. amphitrite* of four meroditerpenoids isolated from the marine brown alga *Halidrys siliquosa*. Meroditerpenoids from the brown alga *Cystoseira baccata* also showed antifouling activity against the growth of microalgae and macroalgal settlement (Mokrini et al. 2008).

Antifouling diterpenes produced by the brown seaweed *Canistrocarpus cervicornis* strongly inhibited the establishment of the mussel *Perna perna* (Bianco et al. 2009) and a diterpene isolated from the gorgonian *Junceella juncea* showed potent antifouling activity against larval settlement of *A. amphitrite* at non-toxic concentrations (Qi et al. 2009).

Complying with the guidelines of the US Navy Program that require an $EC_{50(settl)}$ lower than 25 mg/L for a compound to be considered a promising natural antifoulant, all eight compounds evaluated in this study meet this requirement. The EC_{50} values obtained are comparable to seven of the nine reported by Qi et al. (2006), even if two of the nine diterpenoids isolated from the gorgonian *J. juncea* showed a very high antifouling activity against the barnacle *A. amphitrite* (EC_{50}=0.004 mg/L). Levels of antifouling activity showed in this study can are also similar to those obtained from a marine-derived fungus *Ampelomyces* sp. (Kwong et al. 2006).

Bromosphaerol (**3**) is the most active among the eight tested metabolites; showing the lowest $EC_{50(settl)}$ value (0.23 mg/L) without toxic effects on the cypris larvae ($LC_{50\ (cypris)}$>100 mg/L). TR_C value of **3**, calculated considering cyprids mortality, is impressive (434.78), whereas its toxicity towards nauplii is relatively low ($LC_{50\ (nauplii)}$= 3.63 mg/L). When compared with all assayed compounds, **3** clearly results to be the most promising natural antifoulant candidate.

Besides **3**, metabolites **2** (sphaerococcenol A) and **6** (bromotetrasphaerol) were significantly active compounds, showing $EC_{50(settl)}$ values of 1.53 and 0.38 mg/L, respectively, and a TR_C (calculated with cyprids mortality) of 65.3 and 79.47. Their toxicity among naupliar stage is however high ($LC_{50(nauplii)}$ values of 0.32 and 0.39 mg/L, respectively).

Settlement inhibition levels of metabolites examined in this work are comparable to some of the most significant ones found in the literature for natural products such as those from *Callyspongia truncata* (EC_{50}=0.24 mg/L) (Tsukamoto et al. 1997), *Phyllidia pustulosa* (EC_{50}=0.17 mg/L) (Hirota et al. 1998), and *Reniera sarai* (EC_{50}=0.27 mg/L) (Faimali et al. 2003).

Traditionally, the TR is calculated by taking into account naupliar mortality (Rittschof et al. 1994; Clare et al. 1999; Faimali et al. 2003). A recent review from Qian et al. (2010) underlined how the TR is commonly used as a yardstick of the potential antifouling activity of a compound. In this review, it is suggested that a compound with a TR>15 can be considered as a non-toxic antifouling compound, even if a much higher TR is recommended when selecting candidate compounds. It is suggested that only small molecules with a TR>50 and an EC_{50}<5 mg/L against both hard and soft foulers should be considered.

Since the aim of TR is to determine whether the mechanism of settlement inhibition is based on toxic effect, it would be more appropriate to measure mortality on the same larval stage on which settlement is evaluated (competent larval stage). On the other hand, LC_{50} on nauplii is a good index of toxicity against non-target organisms, since nauplii can be considered a more representative class of zooplankton.

It is clear (see Table 2) that the proposed approach for therapeutic ratio calculation, using cyprids (TR_C) instead of naupliar mortality (TR_N), yields very different results. For instance, taking TR_N of compound **2** (0.21), we can assert that this compound is characterised by good antifouling properties (EC_{50}=1.53 mg/L), but acts with a toxic mechanism. However, looking at TR_C (65.3) of the same compound, the conclusions are completely different, and this compound becomes a well-performing antifoulant, acting through a non-toxic mechanism. These considerations are also valid for the rest of the tested compounds. Settlement inhibition of compounds **2**, **3** and **6** can be associated to a non-toxic mechanism, since its TR_C is quite high (more than 60).

On the basis of settlement and mortality assay results, the tested compounds can be divided into three groups. Only compound **3** belongs to the first group, since it is able to inhibit settlement of *A. amphitrite* with a non-toxic mechanism and without toxicity to the naupliar stage. Indeed, TR_C of metabolite 3 is 434.78, nearly five times higher than that of **6** and almost 100 times greater than that of **8** (which was the lowest among all the eight tested compounds). Furthermore, its toxicity towards nauplii is quite low, with $LC_{50\ (nauplii)}$ amounting to 3.36 mg/L. The second group includes compounds **2**, 5, **6** and **7,** which showed relatively low $EC_{50(settl)}$ values (<5 mg/L), low toxicity towards cyprids, but were toxic to the naupliar stage. The third group contains metabolites **1**, **4** and **8**, which are characterised by low settlement inhibition (EC50 >5 mg/L) and low toxicity towards cypris larvae and nauplii and, basically, are of no biological interest. It is important to note that this division into three groups would be very different if naupliar toxicity (TR_N) was to be used for therapeutic ratio calculation. In this case, for example, compound **4** would appear as one of the best performing chemicals, even if showing a high $EC_{50(settl)}$ value (15.97 mg/L), while **2** would be considered as the worst compound, due to its high toxicity towards nauplii.

The results obtained in this study highlight a new perspective in the quest for environment-friendly antifouling agents, and encourage further field experiments. Finally, the issue of cultivation is worth mentioning, which is easier for macroalgae, than for other marine organisms, thus giving them a considerable advantage as potential sources of antifouling agents in the future.

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