Chemical Defense and Antifouling Activity of Three Mediterranean Sponges of the Genus *Ircinia*

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The defense roles and the antifouling activity of the organic extracts and the major metabolites of the sponges *Ircinia oros, I. variabilis* and *I. spinosula* were investigated. The antifeedant activity was tested in experimental aquaria on the generalist predator fish *Thalassoma pav*o as well as in coastal ecosystems rich in fishes. Some of the major metabolites exhibited high levels of antifeedant activity. The antifouling activity was tested in laboratory assays, against representatives of the major groups of fouling organisms (marine bacteria, marine fungi, diatoms, macroalgae and mussels). All extracts showed promising levels of activity. As was expected, no single extract was active in all tests and some fractions that were effective against one organism showed little or no activity against the others. The high but variable level of antifouling activity in combination with the absence of toxicity (tested on the development of oyster and sea urchin larvae) shows the potential of these metabolites to become ingredients in environmentally friendly antifouling preparations.

Introduction

Sponges are important members of the marine ecosystem. Because they are sessile and soft-bodied, they appear to be physically vulnerable. The lack of predation on sponges is thought to result from the elaboration of physical (Hartman, 1981) and chemical defenses (Bakus and Green, 1974).

Sponges have for long been the focus of attention, concerning their chemical defense mechanisms (Takur and Anil, 2000). Many studies showed that they are rich in terpenoids and steroids, which function in antipredation, competition for space and control of epibiont overgrowth (Bakus and Schulte, 1992 ; Takur and Anil, 2000).

A number of studies have shown that organic compounds extracted from a variety of non-motile marine taxa, including sponges, inhibit fouling in the laboratory or in the field (Wahl, 1989). It has been stated (Fusetani, 1991) that these organisms secrete chemicals that prevent larvae of other marine organisms from settling and growing on them.

The control of biofouling is of particular concern in modern marine engineering and shipping operations and one of the most important problems marine biotechnology is currently facing (Hattori and Shizuri, 1996). Generally, fouling development can be prevented by means of antifouling paints containing one or more toxic compounds such as organotin derivatives in a paint matrix (Vallee-Rehel *et al*., 1998). However, environmental and human health problems are associated with these metal complexes (Gibbs, 1993; Gibbs *et al*., 1987; Gibbs *et al.,* 1988; Martin *et al*., 1981; Peterson *et al*., 1993; Voulvoulis *et al*., 1999).

The biochemical mechanisms that sponges have developed as a chemical defence for growth inhibition of epiphytic micro and macro organisms might comprise a potential alternative for the prevention of biofouling. Marine sessile invertebrates and algae provide acceptable substrates to fouling organisms, yet many of these are remarkably free of fouling organisms (Ware, 1984; Gerhart *et al*., 1988; Wahl, 1989; Uriz *et al*., 1992; Hellio *et al*., 2000a, 2000b).

The sponges of the genus *Ircinia* found in swallow Mediterranean marine ecosystems are most of the times free of epibionts and have at the same time proven to be rich sources of interesting metabolites, such as linear or cyclic polyprenylhy-

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droquinones (Mihopoulos *et al.*, 1999) and sesterterpenes (Cimino *et al.*, 1972) many of which contain furan and tetronic acid functional groups. Some *Ircinia* metabolites have exhibited activity on phospholipase A_2 with subsequent analgesic and anti-inflammatory effects (De Pasquale *et al.*, 1991; Gil *et al.*, 1993). Additionally, several constituents have been reported to show brine shrimp toxicity (De Rosa *et al.*, 1994) and fish lethality (Fusetani *et al.*, 1984).

In the course of our investigations towards the isolation of bioactive metabolites from marine organisms and the elucidation of their ecological roles (Agalias *et al.*, 2000) we examined the antifeedant activity and the antifouling efficacy of three *Ircinia* species collected from the Aegean sea.

Materials and Methods

Sponge materials

I. oros was collected by SCUBA diving from cave walls (5–15 m) at the island of Melos, Cyclades. *I. spinosula* and *I. variabilis* were hand picked by scuba divers (5–15 m) at the island of Fleves, Saronicos gulf. The freshly collected organisms were initially freeze dried and then exhaustively extracted at room temperature with CH_2Cl_2/MeOH (2/1, v/v) mixtures. The residues were subjected to vacuum chromatography using silica gel and a step-wised gradient solvent system ranging from 100% cyclohexane to 100% ethyl acetate. The active fractions were further purified either by vacuum/column chromatography and/or HPLC until pure metabolites were isolated. Structural elucidation of natural products was based on their spectral data (NMR, MS, IR, UV) and comparison with literature values.

Chemical defense experiments

The preliminary palatability of the sponges was evaluated by laboratory assays on the generalist predator fish *Thalassoma pavo. Ircinia* extracts and pure metabolites were incorporated in food preparations following the methods described by Pawlik (Pawlik *et al.*, 1995; Chanas and Pawlik, 1995). Groups of fish were randomly chosen during feeding assays and offered either a treated or control food pellet, followed by the other choice. If the second pellet was treated and rejected by the fish, another control pellet was offered to determine whether the fish had ceased feeding; groups of fish that would not eat control pellets were not used in assays. Control and treated pellets were given one at a time to 10 different fish, kept separately. A food pellet was considered rejected if the fish accepted the pellet into the mouth cavity and then spat it out. The pellet was considered eaten if swallowed by the fish. Tank assays were repeated several times in order to establish a significant number of experiments.

The same set of chemicals, was tested in the field in areas of Saronicos gulf, and the results of these assays were found to follow the pattern of the tank experiments. Control and treated food strips were prepared according to the same protocol and were suspended, in pairs, on 50 cm plastic ropes at a distance ~4–12 cm from one end of the rope (the order was random) and were fastened on rocks at the depth of approximately 15- 25 meters. The ropes were let in the sea until at least 50% of the control strips were consumed. The eaten percentage of the strips was later determined volumetrically.

Antifouling assays

Preparation of the extracts

After collection, samples were rinsed with sterile sea water to remove associated debris. The clean material was subsequently freeze dried.

Extracts: Aqueous, Ethanol, Dichloromethane.

Aqueous extract: 200 g of the dried sponge were added in distilled water (50 g/lt) and suspended by stirring for 2h in an ultrasonic bath at 4^0C. After centrifugation and filtration the aqueous solution was lyophilised.

Ethanol and dichloromethane extracts: A 200 g sample of the dried sponge was extracted in 300 ml of 95% ethanol, for five times. The alcoholic extracts were combined and evaporated under vacuum at low temperature (<40^0C). Distilled water (100 ml) was then added and partitioned with dichloromethane (4 × 100 ml). The aqueous phases were lyophilised, re-suspended in absolute ethanol (100 ml), filtered and concentrated under vacuum (ethanol extract). The organic layers were com-

bined, dried with Na_2SO_4, filtered and concentrated under vacuum (dichloromethane extract).

Inhibition of marine fungi and marine bacteria growth

Ten marine bacterial strains were obtained from the Culture Collection of the University of Quimper (LUMAQ, France). Five Gram-positive bacteria were chosen : B1 and B2 (Bacillus isolated from the surface of *Enteromorpha intestinalis*), B3 and B4 (Bacillus isolated from the surface of *Gigartina sp*) and B5 (Bacillus isolated from the surface of *Laminaria sp.*). Five strains of Gram-negative bacteria were chosen: B6 (Diplococcus isolated from the surface of *Dilsea carnosa*), B8 (Coccus isolated from the surface of *Laminaria sp*), B9 and B10 (Rod isolated from the surface of *Ulva sp.*).

Antibacterial testing of the extracts was performed by the disc diffusion technique in agar plated Petri dishes as previously described in Hellio *et al.*, 2001a. Whatman filter paper discs (6 mm diameter) were initially sterilised at 15 atm pressure for 15 min. A sample consisted of sponge extract/metabolite (30 μg) diluted in DMSO 5% was filtered (Millex-GV unit 0.22 mm Millipore pore size), loaded on each of these discs and was allowed to dry at room temperature for 3 hours. Bacterial cultures were grown in liquid DIFCO 2216 marine broth overnight, and 0.1 ml samples of the culture (106 CFU/ml) were spread over the agar. After incubation for 4 days at 20 °C, the activity was evaluated by measuring the diameter (D, in mm) of the inhibition zones around the discs. Control tests with the solvents were performed for every assay but showed no inhibition of the microbial growth. In addition, the biocide TBTO (bis tributyltin oxide) (10 ppm) was used as positive control to check the sensitivity (Hellio *et al.*, 2000b). All inhibition assays were carried out in triplicate.

Five strains of marine fungi (F1-F5) were obtained from the Culture Collection of the University of Portsmouth (G. Bremer, School of Biological Sciences, UK). F1 and F2 are unidentified marine fungi isolated, respectively, from sand collected in Malaysia and from driftwood collected at the Fleet estuary UK. *Corollospora maritima* (F3), *Lulworthia sp.* (F4) and *Dendryphiella salina* (F5) were isolated from driftwood collected, respectively, from Dinas (Wales, UK), Denmark and Galway. The activity of the sponge extracts and pure metabolites against the marine fungi was evaluated using a modified well-agar diffusion method adapted from Tagg and Mc Given (1971) as previously described (Hellio *et al.*, 2000b). In addition, the biocide TBTO (10 ppm) was used to check the sensitivity (Hellio *et al.*, 2000b).

Determination of the MICs for the bacteria (NCCLS 1993) and fungi (Shadomy *et al.*, 1985) was made by the macrodilution method. The tested extracts' concentrations were: 96, 48, 32, 16, 8 and 4 μg/mL. Micro-organisms (2×108 CFU/ml) were placed in a liquid medium consisted of DIFCO 2216 marine agar, containing the extracts/metabolites for an incubation period of 48h at 20 °C. MIC represents the lowest concentration that inhibits the organism's growth.

Inhibition of microalgae growth

Diatomophyceae strains were obtained from the Algae Culture Collection of the University of Caen and include *Amphora coffeaformis* (AC-2078), *Phaeodactylum tricornutum* (DIA12) and *Cylindrotheca closterium* (DIA6). Cultures were maintained in 100 ml Erlenmeyer flasks under continuous illumination (400 lux white fluorescent lamps) at 18 °C in Guillard's F_2 medium (Guillard and Ryther, 1962). Culture media were autoclaved (120 °C, 20 min.) and inoculated under aseptic conditions. Cultures were checked periodically for bacterial contamination (Hellio and Le Gal, 1998).

All the following experiments were carried out in triplicate as previously explained in Hellio *et al.* (2001b). Aliquots (15 ml) of Guillard's F_2 medium were introduced to sterile conical flasks and inoculated with 5×10^5 cells/ml of cultivated microalgae in exponential growth phase. Extracts and/or metabolites were introduced into the flask leading to a final concentration of 30 μg/ml. The flasks were incubated at 18 °C with a 12 hour photoperiod (Gotschalk and Allredge, 1989). Cell growth was estimated daily, for 5 days, by measurement of chlorophyll a (Chl-a) (Sawant and Garg, 1995). In addition, the biocide TBTO (10 ppm) was used to check the sensitivity.

Inhibition of macroalgae growth

Antifouling tests were performed against the macroalgae *Enteromorpha intestinalis*, *Ulva lactuca* and *Sargassum muticum*. Samples were collected in June 1999 in Concarneau Bay, France. After collection, the material was rinsed, in the laboratory, with seawater to remove associated debris and sand grains. The clean algae were then dried by pressing them quickly between paper towels. The algae were then refrigerated for 3–24 hours to maximise the release of the spores (Fletcher, 1989).

For spore and zygote production, fertile blades of algae were selected. No specialised culture medium is necessary for the initial growth of spores. Untreated seawater, from nutrient rich coastal waters, filtrated through Whatman N°1 filter and pasteurised at 72 °C was used. The spores after an incubation at 25 °C for 20 min were collected in a beaker and used for the experiments (Fletcher, 1989). The release and the collections of spores of *Enteromorpha intestinalis* & *Ulva lactuca* and zygotes of *Sargassum muticum* were performed as previously explained in Hellio *et al.,* 2001b.

Petri dishes (35 mm in diameter) were used throughout the experiment as the substrate for settlement of spores (Hattori and Shizuri, 1996). Test samples (1 mg) were dissolved in methanol and spread on the inner surface of the Petri dishes and dried at room temperature. Each Petri dish, containing 5 ml of Guillard's F_2 medium, was inoculated with approximately 3000 spores. Dishes were placed in the dark for 2 hrs to allow even settlement of gametes. TBTO (10 ppm) was used as positive antifouling control.

After incubation for 5 days at 20 °C with 24 hours light (400 lux white fluorescent lamps), the unattached spores were counted on 1 cm^2 areas of each Petri dish using an inverted binocular microscope. The attachment rates were calculated (Hattori and Shizuri, 1996). All experiments were carried out in duplicate.

Inhibition of the blue mussel *Mytilus edulis* settlement

The antifouling activity of the extract towards *M. edulis* was measured spectrophotometrically by recording the activity of the phenoloxidase (Hellio *et al*., 2000a). The purified enzyme was incubated at 25 °C with 10 mM L-DOPA in 50 mM phosphate buffer pH 6.8. The phenoloxidase activity was estimated by monitoring the increase of absorbance at 475 nm. The initial rate of the reaction is proportional to the enzyme concentration. The enzyme activity was calculated from the increment in the absorbance from 30 sec to 1 min after incubation. One unit (U) of enzyme activity was defined as the activity that catalyses the formation of 1 μmol of dopachrome per min under these conditions. Inhibitors were used at concentrations up to 30 μg/mL. In addition, the biocide TBTO (10 ppm) was used as a standard (Hellio *et al.*, 2000a). All inhibition assays were carried out in duplicate.

Toxicity tests on oyster (*Crassostrea gigas*) and sea urchin larva (*Echinus esculentus*)

Toxicity tests were realised as previously described (Hellio *et al*., 2000 b).

Results

Chemical defense experiments

The isolation of the metabolites responsible for the antifeedant activity against the predatory fish was accomplished using a bioassay-guided fractionation scheme. The defense mechanism of the sponges was found to rely on the presence of the major metabolites such as ircinin I and II for *I. oros* and variabilin for *I. variabilis*. The CH_2Cl_2/MeOH extract of *I. spinosula* did not show any activity (Fig. 1). The active factors of *I. oros* and *I. variabilis* were tested in natural concentrations and were inhibiting higher than 80% of the fish feeding (Table I).

Inhibition of marine fungi and bacteria growth

The results of the inhibition of the development of marine fungi and bacteria by sponge extracts/metabolites showed varied antimicrobial activity (Tables II & IV). Although these compounds exhibited a broad spectrum activity as a group, there was a significant variation on the effects of the different extracts or metabolites on the tested microorganisms.

The ethanol fraction of *I. variabilis* inhibited specifically the growth of marine fungi. The dichloromethane fraction of *I. variabilis* lead to inhi-

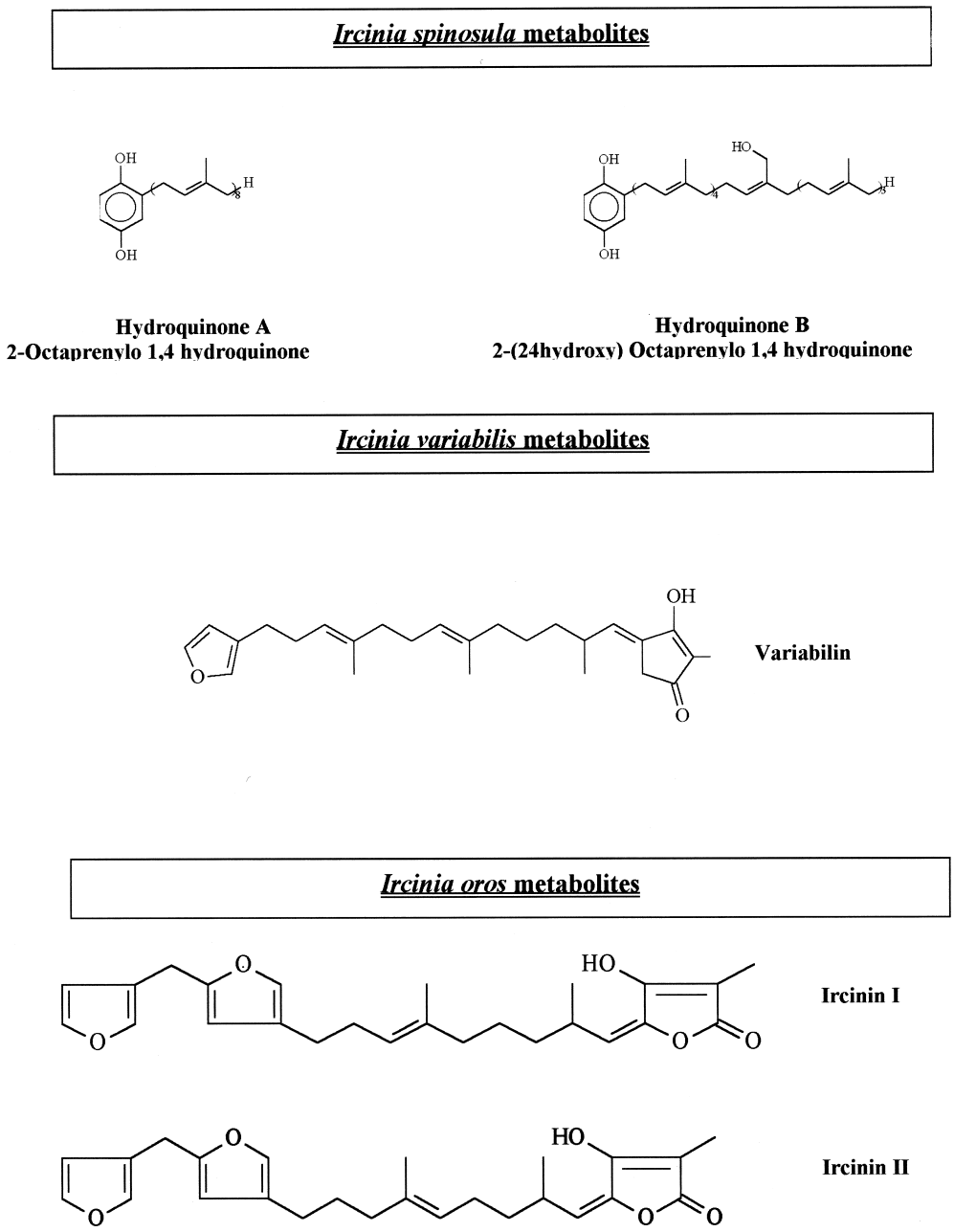

Fig. 1. Major metabolites of the three investigated *Ircinia* species.

bition of marine fungi and marine Gram positive bacteria. The ethanol fraction of *I. spinosula* inhibited the development of Gram positive and negative marine bacteria.

The dichloromethane fraction of *I. spinosula* and hydroquinone A inhibited all studied marine strains and in cases at levels as high as the TBTO.

Table I. Palatability assessment of the three CH_2Cl_2/MeOH *Ircinia* extracts and the major metabolites.

Sample	Aquaria assays (eaten/total pellets)	Number of repetitions	Field assays % Consumption of strips
Control	10 out of 10	7	100
Extract (*I. oros*)	0 out of 10	7	0
Extract (*I. spinosula*)	10 out of 10	7	100
Extract (*I. variabilis*)	3 out of 10	7	15
Mixture of Ircinin I & II	0 out of 10	5	0
Variabilin	1 out of 10	5	5
Hydroquinone A	10 out of 10	5	100
Hydroquinone B	10 out of 10	5	100

Neither the extracts of *I. oros* nor the mixture of ircinin I and II showed any inhibition on the growth of marine fungi and bacteria.

The MICs values are shown in Tables III and V. The most active metabolite towards marine fungi and marine bacteria is hydroquinone A which exhibited the lowest value of MIC. The level of activity for some marine fungi and bacteria are comparable to the activity obtained with TBTO.

Inhibition of microalgae growth

The results of the inhibition on the development of diatoms by the addition of 30 μg/ml of sponge extracts/metabolites are presented in Table VI. The highest level of inhibitory activity was obtained with the ethanol extract of *I. oros* which is comparable to that obtained with TBTO. Some inhibitory effects were also exerted by the aqueous extract of *I. variabilis* and the ethanol extract of *I. spinosula*. None of the pure metabolites exhibited significant activity.

Inhibition of macroalgae growth

The inhibition on the attachment rates of macroalgae spores and zygotes is presented in Table VII. Most active were the dichloromethane extracts of *I. oros* and *I. spinosula*. The mixture of metabolites ircinin I and II and hydroquinone A showed high level of inhibition.

Table II. Antifungal activity of the extracts/metabolites (30 μg).

	F1	F2	F3	F4	F5
TBTO 10 ppm	+++	+++	+++	+++	+++
I. variabilis					
Aqueous extract	–	–	–	–	–
Ethanol extract	+	++	+++	++	++
Dichloromethane extract	+	++	++	++	++
Variabilin	–	–	–	–	–
I. oros					
Aqueous extract	–	–	–	–	–
Ethanol extract	–	–	–	–	–
Dichloromethane extract	–	–	–	–	–
Mixture of Ircinin I & II	–	–	–	–	–
I. spinosula					
Aqueous extract	–	–	–	–	–
Ethanol extract	–	–	–	–	–
Dichloromethane extract	++	+++	+	++	++
Hydroquinone A	+++	+++	+	++	+++
Hydroquinone B	+	+	–	–	–

–: no zone of inhibition; +: 1–5 mm zone of inhibition; ++: 6–9 mm zone of inhibition, +++: more than 10 mm zone of inhibition.

Table III. Determination of the minimum inhibitory concentration (MIC) for marine fungi.

	F1	F2	F3	F4	F5
TBTO 10 ppm	8	8	4	8	8
I. variabilis					
Aqueous extract	–	–	–	–	–
Ethanol extract	32	16	8	16	16
Dichloromethane extract	32	16	16	16	16
Variabilin	–	–	–	–	–
I. oros					
Aqueous extract	–	–	–	–	–
Ethanol extract	–	–	96	–	–
Dichloromethane extract	–	–	–	–	96
Mixture of Ircinin I & II	–	–	–	–	–
I. spinosula					
Aqueous extract	–	–	–	–	–
Ethanol extract	96	–	–	–	–
Dichloromethane extract	16	8	32	16	16
Hydroquinone A	8	8	32	16	8
Hydroquinone B	32	32	–	–	–

–: MIC > 96 μg/ml.

Table IV. Antibacterial activity of the extracts/metabolites (30 μg).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
TBTO 10 ppm	+++	+++	+++	+++	+++	++	++	++	+++	+++
I. variabilis										
Aqueous extract	–	–	–	–	–	–	–	–	–	–
Ethanol extract	–	–	–	–	–	–	–	–	–	–
Dichloromethane extract	++	+++	+	+++	+	–	–	–	–	–
Variabilin	–	–	–	–	–	–	–	–	–	–
I. oros										
Aqueous extract	–	–	–	–	–	–	–	–	–	–
Ethanol extract	–	–	–	–	–	–	–	–	–	–
Dichloromethane extract	–	–	–	–	–	–	–	–	–	–
Mixture of Ircinin I & II	–	–	–	–	–	–	–	–	–	–
I. spinosula										
Aqueous extract	–	–	–	–	–	–	–	–	–	–
Ethanol extract	++	++	+++	++	+++	+	+	++	++	+
Dichloromethane extract	++	++	++	+	++	++	+	+	++	+
Hydroquinone A	++	+++	+++	+	+++	+++	++	+	+++	+
Hydroquinone B	+	+	–	–	–	–	–	–	–	–

–: no zone of inhibition; +: 1–5 mm zone of inhibition; ++: 6–9 mm zone of inhibition, +++: more than 10 mm zone of inhibition.

Table V. Determination of the minimum inhibitory concentration (MIC) for marine bacteria.

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
TBTO 10 ppm	8	4	4	8	8	8	4	4	4	8
I. variabilis										
Aqueous extract	–	–	–	–	–	–	–	–	–	–
Ethanol extract	–	–	–	–	–	–	–	–	–	–
Dichloromethane extract	16	8	32	8	32	–	–	–	–	–
Variabilin	–	–	–	–	–	–	–	–	–	–
I. oros										
Aqueous extract	–	–	–	–	–	–	–	–	–	–
Ethanol extract	–	–	–	–	–	–	–	–	–	–
Dichloromethane extract	–	–	–	–	–	–	–	–	–	–
Mixture of Ircinin I & II	–	–	–	–	–	–	–	–	–	–
I. spinosula										
Aqueous extract	–	–	–	–	–	–	–	–	–	–
Ethanol extract	16	16	8	16	8	32	32	16	16	32
Dichloromethane extract	16	16	16	32	16	16	32	32	16	32
Hydroquinone A	16	8	8	32	8	8	16	32	8	32
Hydroquinone B	32	32	–	–	–	96	–	–	–	96

–: MIC > 96 μg/mL.

Inhibition of the blue mussel Mytilus edulis settlement

The results are summarised in Table VIII. Among the 9 tested extracts, only the ethanol extract of *I. oros* exhibited significant inhibition of the phenoloxidase activity (68%). None of the pure metabolites reduced the enzymatic activity.

Toxicity tests on oyster (Crassostrea gigas) and sea urchin larva (Echinus esculentus)

Table VI. Effect of extracts/metabolites (30 μg/mL) on the growth of diatoms.

	A. coffeaformis	*P. tricornutum*	*C. closterium*
TBTO	+++	+++	+++
I. variabilis			
Aqueous extract	+	+	+
Ethanol extract	–	–	–
Dichloromethane extract	+	–	–
Variabilin	–	–	–
I. oros			
Aqueous extract	–	–	–
Ethanol extract	++	++	+++
Dichloromethane extract	–	–	–
Mixture of Ircinin I & II	–	–	–
I. spinosula			
Aqueous extract	–	–	–
Ethanol extract	++	+	+
Dichloromethane extract	–	–	–
Hydroquinone A	–	–	–
Hydroquinone B	–	–	–

–: no inhibition; +: 1–30% of inhibition; ++: 31–59% of inhibition; +++: ≥60% of inhibition. TBTO is used as positive antifouling control.

Table VII. Inhibition on the attachment rate of macroalgae.

	E. intestinalis	*U. lactuca*	*S. muticum*
TBTO	+++	+++	+++
I. variabilis			
Aqueous extract	++	+	+
Ethanol extract	–	–	–
Dichloromethane extract	–	–	–
Variabilin	–	–	–
I. oros			
Aqueous extract	–	–	–
Ethanol extract	+	+	–
Dichloromethane extract	++	++	++
Mixture of Ircinin I & II	+++	+++	++
I. spinosula			
Aqueous extract	–	–	–
Ethanol extract	–	+	+
Dichloromethane extract	++	+++	++
Hydroquinone A	++	+++	+++
Hydroquinone B	+	+	+

– no inhibition; +: 1–30% of inhibition; ++: 31–59% of inhibition; +++: ≥60% of inhibition. TBTO is used as positive antifouling control.

Toxicity tests were performed against non target species of oyster and sea urchins (*Crassostrea gigas* and *Echinus esculentus*) and showed insignificant toxicity levels (5–10% mortality) with concentrations of 500 μg/ml whereas TBTO caused 100% mortality with concentrations of 30 μg/ml.

Discussion

The chemical defense strategy of the three *Ircinia* species was tested in experimental aquaria as well as in the field. The assays were performed on the organic extracts of the sponges, as well as in

Table VIII. Inhibition of the phenoloxidase activity in *M. edulis*.

Compound	Inhibition of the phenoloxidase activity
TBTO 10 ppm	+++
I. variabilis	
Aqueous extract	–
Ethanol extract	–
Dichloromethane extract	–
Variabilin	–
I. oros	
Aqueous extract	–
Ethanol extract	++
Dichloromethane extract	–
Mixture of Ircinin I & II	–
I. spinosula	
Aqueous extract	–
Ethanol extract	+
Dichloromethane extract	–
Hydroquinone A	–
Hydroquinone B	–

–: 0–25% of inhibition; +: 26–50% of inhibition; ++: 51–75% of inhibition; +++: 76 to 100% of inhibition.

the chromatographically separated fractions and pure metabolites.

The CH_2Cl_2/MeOH extract of *I. spinosula*, which contains mainly polyprenylated hydroquinones, was found to be inactive in the field as well as in aquaria experiments. The major metabolites were tested and were found to be inactive as expected.

The CH_2Cl_2/MeOH extract of *I. variabilis* was highly deterrent to fish, both in the tank and in the field assays. Variabilin, the major metabolite of the sponge was found to be responsible for the defense of this sponge. Variabilin was first isolated in 1973 from *I. variabilis* (Faulkner,1973).

The CH_2Cl_2/MeOH extract of *I. oros* was also found to be highly unpalatable. Vacuum flash chromatography of the crude extract of *I. oros* resulted in 16 fractions of increasing polarity. On the basis of TLC analysis, it was determined that some fractions shared the same compounds; these were combined and subjected to aquarium and field assays. From fractions 6–10 ircinin I and II, the major furanosesterterpenes constituents of the species, were isolated as a mixture, assayed and were found to be the deterrent factors of the extract. Ircinin I and II were first isolated from sponges of the genus *Ircinia* (Cimino *et al.*, 1972). Although these furanosesterterpenes have been observed as constituents from diverse sources, their role in predator defense had not previously been demonstrated.

All tested extracts besides the aqueous, significantly inhibited fouling. These extracts can be considered as anti-settlement agents. Significant bioactivity has also been reported from other screening strategies (Huysecom *et al.*, 1985; McCaffrey and Endean, 1985; Thompson *et al.*, 1985; Amade *et al.*, 1987 ; Munro *et al.*, 1989; Uriz *et al.*, 1992; Willemensen, 1994; Sera *et al.*, 1999; Mihopoulos *et al.*, 1999; Takur and Anil, 2000).

The most active extracts (dichloromethane extracts of *I. oros* and *I. spinosula*) gave promising inhibition on the attachment rate of *E. intestinalis*, *U. lactuca* and *S. muticum* at 30 μg/mL. This is a particularly promising result, since algae are often the most difficult organisms to control (Willemensen, 1994) and can be resistant even to tin and copper antifouling paints (Evans, 1990).

The strong antimicrobial activity of the dichloromethane extract of *I. spinosula* (against marine fungi and bacteria) and the ethanol extract of *I. oros* (against the diatoms) can be useful in the prevention of biofilm formation. Biofilms are an important step in the biofouling process (Holmström and Kjelleberg, 1994; Willemensen, 1994) and provide a supporting substrate for the subsequent attachment of other fouling organisms. The reduction of this layer is fundamental for the effective control of biofouling (Hellio *et al.*, 2000b). In addition, marine bacteria and marine fungi are collocated with corrosion products (Little *et al.*, 1999). The extended degree of bacterial (Barkay, 1987), fungal (Duxbury and Bicknell, 1983) and diatom (Jackson, 1991) resistances to heavy metals shows the urgency for new antifouling products.

Mussels are among the major fouling macroorganisms that cause serious problems by settling on man-made surfaces. *Mytilus edulis* attaches to the substrate by means of adhesive plaques connected to a stem of byssus. Theses plaques are thought to be produced by the action of a phenoloxidase (Waite, 1987). The ethanol extract of *I. oros* lead to a high level of inhibition of the enzymatic activity of the phenoloxidase, whereas the ethanol extract of *I. spinosula* moderately inhibits the activity of the enzyme.

Most of the extracts exhibited significant activity against fouling, with strong effect on the three major groups of fouling organisms (micro-organisms, algae and invertebrates). Since every antifouling coating must work against a range (De Nys *et al.*, 1995) of fouling organisms, the above activities are promising. As far as we know this is the first broad spectrum evaluation of the antifouling activities of sponge extracts and metabolites.

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Agalias A., Mihopoulos N., Tsoukatou M., Marinos L., Vagias C., Harvala C., and Roussis V. (2000), New prostaglandins from the chemically defended soft coral *Plexaura nina.* Z. Naturforsch. **55C**, 425–430.

Amade P., Charroin C., Baby C. and Vacelet J. (1987), Antimicrobial activities of marine sponges from the Mediterranean Sea. Mar. Biol. **94**, 271–275.

Bakus G. J. and Green G. (1974), Toxicity in sponges and holothurians : a geographic pattern. Science. 185, 951–953.

Bakus G. J. and Schulte A. (1992), Predation deterrence in marine sponges: laboratory versus field studies. Bull. Mar. Sci. **50**(1), 205–211.

Barkay T. (1987), Adaptation of aquatic microbial communities to Hg stress. Appl. Environ. Microb. **53**, 2725–2732.

Chanas B. and Pawlik J. R. (1995), Defenses of Caribbean sponges against predatory fish: II. Spicules, tissue toughness, and nutrinional quality. Mar. Ecol. Prog. Ser. **127**, 195–211.

Cimino G., De Stefano S., Fattoruso E. and Minale L. (1972), Ircinin-1 and –2 linear sesterterpenes from the marine sponge *Ircinia oros.* Tetrahedron **28**, 333–341.

De Nys R., Steinberg P., Willemensen P., Dworjanyn S., Gabelish C. and King R. (1995), Broad spectrum effects of secondary metabolites from the red alga *Delisea pulchra* in antifouling assays. Biofouling. **8**, 295–271.

De Pasquale R., Circosta C., Occhiuto F., De Rosa S.and De Stefano S. (1991), Pharmacological studies on terpenoids from marine sponges analgesic and muscle relaxant effects. Phytotherapy Research. **5**, 49–53.

De Rosa S., De Giulio A. and Iodice C. (1994), Biological effects of prenylated hydroquinones: structure-activity relationship studies in antimicrobial, brine shrimp and fish lethality assays. J. Nat. Prod. **57**, 1711–1716.

Duxbury T. and Bicknell B. (1983), Metal-tolerant bacterial populations from natural and metal-polluted soils. Soil Biochem. **15**, 243–250.

Evans L. (1990), Underwater foul play. Biol. Sci. Rev. **2**, 35–38.

Faulkner J. (1973), Variabilin, an antibiotic from the sponge *Ircinia variabilis.* Tetrahedron. Lett. **39**, 3821–3822.

Fletcher R. (1989), A bioassay technique using the marine fouling green alga *Enteromorpha*. Inter. Biodeter. Biodegrad. **25**, 407–422.

Fusetani N. (1991), The Fusetani biofouling project. Biofouling. **12**, 2–8.

Fusetani N., Kato Y., Matsunaga S. and Hashimoto K. (1984), Two new furanosesterterpenes, inhibitors of cell division of the fertilized starfish eggs, from the marine sponge *Cacospongia scalaris.* Tetrahedron Lett. **25**, 4941–4942.

Gerhart D., Rittschof D., and Mayo S. (1988), Chemical ecology and the search for marine antifoulants: studies of predator-prey symbiosis. J. Chem. Ecol. **14**, 1903–1915.

Gibbs P. (1993), A male genital deffect in the dog-welk, *Nucella lapilus* (Neogasteropoda), favouring survival in TBT-polluted area. J. Mar. Biol. Assoc. U.K. **73**, 667–678.

Gibbs P., Bryan G., Pascoe P. and Burt G. (1987), The use of the dog-whelk, *Nucella lapilus*, as an indicator of tributyltin (TBT) contamination. J. Mar. Biol. Assoc U. K. **67**, 407–423.

Gibbs P., Pascoe P. and Burt G. (1988), Sex changes in the female dog-whelk, *Nucella lapilus*, induced by tributyltin from antifouling paints. J. Mar. Biol. Assoc U. K. **68**, 715–731.

Gil B., Sanz M., Terencio M., De Giulio A., De Rosa S., Teeyapant R., Woerdenbag H., Kreis P., Hacker J., Wray V., Witte L. and Proksch P. (1993), Antibiotic and cytotoxic activity of brominated compounds from the marine sponge *Verongia aerophoba.* Z. Naturforsch. **48C**, 939–945.

Gotschalk C. and Alldredge A. (1989), Enhanced primary production and nutrient regeneration within aggregated marine diatoms. Mar. Biol. **103**, 119–129.

Guillard R. and Ryther J. (1962), Studies of marine planktonic diatoms. I. *Cyclotella nana* (Hustedt), and *Detonula confervacea* (Cleve). Can. J. Microbiol. **8**, 229–239.

Hartman W. (1981), Form and distribution of silica in sponges. In: Silicon and Siliceous Structures in Biolog-

ical Systems (Simpson T. L., Volkani B. E., eds). Springer, Berlin Heidelberg New York, 454–493.
Hattori T. and Shizuri Y. (1996), A screening method for antifouling substances using spores of the fouling macroalga *Ulva conglobata* larvae and new antifouling technology. Biofouling. **8**, 147–160.
Hellio C. and Le Gal Y. (1998), Histidine utilization by the unicellular alga *Dunaliella tertiolecta.* Comp. Biochem. Physiol. **119A**, 753–758.
Hellio C. and Bourgougnon N., Le Gal Y. (2000a), Phenoloxidase (E. C. 1.14.18.1) from *Mytilus edulis* byssus gland: purification, partial characterization and application for screening products with potential antifouling activities. Biofouling. **16**, 235–244.
Hellio C., Bremer G., Pons A. M., Le Gal Y. and Bourgougnon N. (2000b), Inhibition of the development of microorganisms (bacteria and fungi) by extracts of marine algae from Brittany (France). Appl. Microb. Biotech. **54**, 543–549.
Hellio C., De La Broise D., Dufosse L., Le Gal Y. and Bourgougnon N. (2001a), Inhibition of marine bacteria by extracts of macroalgae: potential use for environmentally friendly antifouling paints. Mar. Environ. Res. **52**, 231–247.
Hellio C., Thomas-Guyon H., Culioli G, Piovetti L., Bourgougnon N. and Le Gal Y. (2001b), Marine antifoulants from *Bifurcaria bifurcata* (phaeophyceae, cystoseiraceaea) and other brown macroalgae. Biofouling. **17** (3), in press.
Holmstroem C. and Kjelleberg S. (1994), The effect of external biological factors on settlement of marine invertebrate larvae and new antifouling technology. Biofouling. **8**, 147–160.
Huysecom J., Vyver Van de G., Braekman J. and Daloze D. (1985), Chemical defense in sponges from North Brittany. In : New Perspectives in Sponge Biology, K. Rutzler, ed. 3d Int. Sponge Conf., pp 115–118.
Jackson S. (1991), Microalgae: their status as fouling organisms. Oebalia. **17**, 295–303.
Little B., Ray R., Wagner P., Jones-Meehan J., Lee C. and Mansfeld F. (1999), Spatial relationship between marine bacteria and localized corrosion on polymer steel. Biofouling **13**, 301–321.
Martin M., Osborn K., Billig P. and Glickstein N. (1981), Toxicity of ten metals to *Crassostrea gigas* and *Mytilus edulis* embryos and *Cancer magister* larvae. Mar. Poll. Bull. **12**, 305–308.
McCaffrey E. and Endean R. (1985), Antimicrobial activity of tropical and subtropical sponges. Mar. Biol. **89**, 1–8.
Mihopoulos N., Vagias C., Chinou I., Roussakis C., Scoullos M., Harvala C. and Roussis V. (1999), Antibacterial and cytotoxic natural and synthetisized hydroquinones from sponge *Ircinia spinolusa*. Z. Naturforsch. **54C**, 417–423.
Munro M., Blunt J., Barns G., Battershill C., Lake R. and Perry N. (1989), Biological activity in New Zeland marine organisms. Pure Appl. Chem. **61**, 529–534.
Pawlik J., Chanas B., Toonen R. and Fenical W. (1995), Defenses of Caribbean sponges against predatory reef fish: I. Chemical deterrency. Mar. Ecol. Prog. Ser. **127**, 183–194.
Peterson S., Batley G. and Scammell M. (1993), Tetracycline in Antifouling paints. Mar. Poll. Bull. **26**, 96–100.
Sawant S. and Garg A. (1995), Growth inhibition of fouling bacteria and diatoms by extract of terrestrial plant, *Derris scandens* (Dicotyledonae: Leguminosae). Indian J. Mar. Sci. **24**, 229–230.
Sera Y., Adachi K. and Shizuri Y. (1999), A new epiodoxy sterol as an antifouling substance from a palauan marine sponge, *Lendenfolia chondrodes.* J. Nat. Prod. **62**, 152–154.
Shadomy S., Espinel-Ingroff A. and Cartwright R. (1985), Laboratory studies with antifungal agents : susceptibility tests and bioassays, pp. 991–999. In: Manual of Clinical Microbiology, 4th ed American Society for Microbiology (Lenette E. H., Balows A., Hausler W. J., Shadomy H. J., eds). Washington, DC.
Takur N. and Anil A. (2000), Antibacterial activity of the sponge *Ircinia ramosa* : importance of its surface-associated bacteria. J. Chem. Ecol. **26**, 57–71.
Thompson J., Walker R. and Faulkner D. (1985), Screening and bioassay for biologically-active substances from forty marine sponge species from San Diego, California, USA. Mar. Biol. **88**, 11–21.
Uriz M., Martin D. and Rosell D. (1992), Relationships of biological and taxonomic characteristics to chemically mediated bioactivity in Mediterranean littoral sponges. Mar. Biol. **113**, 287–297.
Vallee-Rehel K., Mariette B., Hoarau P., Guerin P., Langlois V. and Langlois J. (1998), A new approach in the development and testing of antifouling paints without organotin derivatives. J. Coating Technol. **70**, 55–63.
Voulvoulis N., Scrimshaw M. and Lester J. (1999), Alternative antifouling biocides. Appl. Organometal. Chem. **13**, 135–143.
Wahl M. (1989), Marine epibiosis: I. Fouling and antifouling: some basic aspects. Mar. Ecol. Prog. **58**, 175–189.
Waite J. (1987), Nature's underwater adhesive specialist. Chemtech. **13**, 692–697.
Ware G. (1984), The patterns and mechanisms of antifouling in some temperate sponges. PhD dissertation, Duke University, Durham, NC.
Willemensen P. (1994), The screening of sponge extracts for antifouling activity using a bioassay with laboratory-reared cyprid larvae of the barnacle *Balanus amphrite.* Int. Biodeter. Biodegrad. **5**, 361–373.