

Inhibitory Effects of Mediterranean Sponge Extracts and Metabolites on Larval Settlement of the Barnacle *Balanus amphitrite*

Claire Hellio,^{1*} Maria Tsoukatou,² Jean-Philippe Maréchal,¹ Nick Aldred,¹ Claude Beaupoil,³ Anthony S. Clare,¹ Constantinos Vagias,² Vassilios Roussis²

¹Newcastle University, School of Marine Science and Technology, Newcastle upon Tyne, NE1 7RU, UK

²Division of Pharmacognosy and Chemistry of Natural Products, University of Athens, School of Pharmacy, Panepistimiopolis Zografou, Athens 157 71, Greece

³Station Marine du Muséum National d'Histoire Naturelle, USM 405, BP 225, 29182 Concarneau Cedex, France

Received: 13 December 2003 / Accepted: 14 July 2004 / Online publication: 30 June 2005

Abstract

One of the most promising alternative technologies to antifouling paints based on heavy metals is the development of coatings whose active ingredients are compounds naturally occurring in marine organisms. This approach is based on the problem of epibiosis faced by all marine organisms and the fact that a great number of them cope with it successfully. The present study investigated the antifouling activity of a series of extracts and secondary metabolites from the epibiont-free Mediterranean sponges *Ircinia oros*, *I. spinosula*, *Cacospongia scalaris*, *Dysidea* sp., and *Hippospongia communis*. Antifouling efficacy was evaluated by the settlement inhibition of laboratory-reared *Balanus amphitrite* Darwin cyprids. The most promising activity was exhibited by the metabolites 2-[24-acetoxy]-octaprenyl-1-4-hydroquinone (**8a**), dihydrofurospingin II (**10**), and the alcoholic extract of *Dysidea* sp.

Key words: antifouling activity — *Balanus amphitrite* — *Cacospongia scalaris* — *Dysidea* sp. — *Hippospongia communis* — *Ircinia oros* — *Ircinia spinosula* — settlement inhibition — toxicity.

Introduction

For industries operating in the marine environment, the biofouling of man-made structures has huge

economic implications (Armstrong et al., 2000) and is one of the most important problems that marine technology is currently facing (Hattori and Shizuri, 1996; Rittschof, 2000). Fouling on the hulls of ships increases frictional drag, with a corresponding decrease in speed and maneuverability, and a subsequent increase in both fuel consumption and pollution (Willemensen, 1994).

Hull fouling is an age-old problem. Historically, wooden sailing vessels by, have been protected against fouling by toxic copper or lead sheathing and comparatively primitive coatings containing toxic ingredients such as arsenic and mercury (Omae, 2003). Since the early 1960s, tributyltin (TBT) has been used widely in marine paint formulations. Though undeniably efficient at preventing macrofouling, TBT caused adverse environmental effects (Gibbs 1993; Iwata et al., 1995; Ohira et al., 1996; Ponasik et al., 1998; Sidharthan et al., 2002; Bennett et al., 2003; Cheung et al., 2003; McAllister and Kime, 2003). Tin-based paints have been linked to the pollution of food webs, creating increased concern for human consumers (Peterson et al., 1993; Coehlo et al., 2002). The imminent prohibition of TBT-based coatings (Yebra et al., 2004) means that there is a need to develop new environmentally compatible alternatives that would be equally efficient against severe fouling organisms such as barnacles, blue mussels, bryozoans, and algae (Dahlstrom et al., 2000).

In the marine environment, where all surfaces are constantly exposed to the threat of surface colonization, many sessile organisms remain relatively clean and control epibiont growth using effective antifouling mechanisms. In addition to physical de-

*Present address: Laboratoire MFS, Faculté des Sciences et Techniques, Université du Sud-Toulon Var, BP 20132, 83957 La Garde Cedex, France
Correspondence to: Vassilios Roussis; E-mail: roussis@pharm.uoa.gr

fense mechanisms comprising structural elements made of lignin, CaCO_3 , silica, etc., sessile organisms such as sponges, soft corals, and seaweeds are known to elaborate chemical defense mechanisms against predation and epibiont growth. Many sponges have been shown to synthesize toxic metabolites to prevent predation, and because of this, frequently other organisms attach sponges to themselves for protection (Pawlik et al., 1995).

One promising alternative to heavy metal-based paints for antifouling is the utilization of bioactive ingredients produced by marine organisms. These metabolites already exist in the marine environment, and as natural products they would be target-oriented and biodegradable (Holmström and Kjelleberg, 1994; Clare, 1996, 1998; Burgess et al., 2003; Fusetani, 2004).

In recent years many studies have assessed the potential activity of extracts from sessile marine organisms in antifouling assays (Armstrong et al., 2000; de Nys and Steinberg, 2002a, 2002b). Understanding the antifouling mechanisms of these marine organisms may provide valuable information for new approaches to fouling control.

The use of natural marine products that are capable of inhibiting one or several stages of fouling on ships and other submerged structures may provide an acceptable solution from the aspect of environmental impact (Clare, 1996, 1998). A significant number of marine metabolites exhibiting antifouling activity have already been reported, including terpenoids, steroids, saponins, fatty acid-related compounds, bromotyrosine derivatives, and heterocyclic compounds (Clare, 1996, 1998; Fusetani, 2004; Rittschof, 2000).

The present study is part of our continuing research focusing on the discovery of natural marine products that prevent marine biofouling. Barnacles were selected as test organisms because they are one of the most significant forms of animal fouling (Koryakova and Korn, 1993). In the course of our investigations on marine natural product antifoulants (Tsoukatou et al., 2002), we recently demonstrated that sponge extracts can exhibit antifouling activities toward marine bacteria, diatoms, macroalgae, and mussels. In the present study we report on the effectiveness of a series of extracts and metabolites from Mediterranean sponges against the settlement of the cosmopolitan barnacle *Balanus amphitrite*.

Materials and Methods

General Experimental Procedures. ^1H and ^{13}C NMR spectra were recorded using Bruker AC 200 and DRX 400 spectrometers. High-resolution FAB mass spectra

data were recorded on a JEOL AX505HA Mass Selective Detector and were provided by the University of Notre Dame, Department of Chemistry and Biochemistry. Low-resolution EI mass spectra data were recorded on a HP 5973 Mass Selective Detector. Optical rotations were measured using a PerkinElmer model 341 polarimeter and a 10-cm cell. UV spectra were determined in spectroscopic grade C_6H_{14} on a Shimadzu UV-160A spectrophotometer. IR spectra were obtained using a Paragon 500 PerkinElmer spectrophotometer. Column chromatography was performed with a Kieselgel 60 (Merck). High-performance liquid chromatography was conducted using Hewlett Packard (Agilent) 1100, Pharmacia LKB 2248, and EEC LC-1240 systems equipped with refractive index detectors, Spherisorb S10W and Supelcosil SPLC-Si CC1593 (25 cm – 10 mm, 5 μm) columns. Thin-layer chromatography was performed with Kieselgel 60 F₂₅₄ (Merck aluminum support plates).

Collection, Extraction, and Isolation. The black massive sponge *Cacospongia scalaris* was hand-collected by SCUBA diving from depths of 25 to 30 m at Astakos Gulf, Etoloakarnania, Greece (April 1999). Collected organisms were kept frozen (-20°C) until processed. The sponge *Ircinia oros* was collected by SCUBA diving from cave walls (5–15 m) at the island of Melos, Cyclades (July 1998). *Ircinia spinosula* was hand-picked by SCUBA divers (10–20 m) at the island of Fleves, Saronikos Gulf (May 1998). *Dysidea* sp. was collected by snorkelling at the island of Aegina, Saronikos Gulf (August 2001). *Hippospongia communis* was collected by free diving at the island of Patroklos, Saronikos Gulf (10–15 m) (August 2000).

The frozen organisms were initially freeze-dried and then exhaustively extracted at room temperature with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (2:1, v/v) mixtures. The combined extracts were concentrated under vacuum, and the residues were fractionated by vacuum silica gel column chromatography using hexane-EtOAc mixtures of increasing polarity. The active fractions were further purified by flash column chromatography or HPLC until pure metabolites ircinin I and II (**1**, **2**), ircinin I acetate (**1a**), ircinin II acetate (**2a**) (Cimino et al., 1972a); fasciculatin (**9**) (Cafieri et al., 1972); hydroquinone A (**7**), hydroquinone C (**8**), hydroquinone A acetate (**7a**), hydroquinone-C acetate (**8a**) (Mihopoulos et al., 1999); furodysinin (**3**), 7-deacetoxyolepupane (**4**), euryfuran (**11**) (Grode and Cardellina, 1984; Garson et al., 1992; Hochlowski et al., 1982); dihydrofurospingin II (**10**) (Cimino et al., 1972b); spongi-12-en-16-one (**5**) and 11 β -acetoxyspongi-12-en-16-one (**6**) (Cimino et al., 1974) were isolated.

Structural elucidation of the natural products was based on interpretation of their spectral data (NMR, MS, IR, and UV) and comparison with published values.

Preparation of Extracts. After collection, samples were rinsed with sterile seawater to remove associated debris. The surface microflora was removed by washing the sponge samples for 10 minutes with ethanol (30%). The clean material was freeze dried and chopped into small pieces.

Aqueous Extract (A). A 200-g sample of dried sponge was extracted, with stirring, for 2 hours in distilled water (50 g/L) at 4°C. After centrifugation and filtration, the aqueous supernatant was lyophilized.

Ethanollic (B) and Dichloromethane (C) Extract. A 200-g sample of dried sponge was extracted, with stirring, in 300 ml of 95% ethanol. After centrifugation the residual tissue was reextracted 5 times by the same procedure. The alcoholic extracts were combined and concentrated, under vacuum, at low temperature (<40°C). Distilled water (100 ml) was then-added to the organic residue before partitioning against methylene chloride (4 – 100 ml). The aqueous phases were collected, lyophilized, redissolved in absolute ethanol (100 ml), filtered, and concentrated under vacuum at low temperature (ethanollic extract).

The dichloromethane phases were collected, dehydrated with CaCl_2 , filtered, and concentrated under vacuum (dichloromethane extract). All extracts were stored at -20°C until used.

Antifouling Assays. The effect of sponge extracts and metabolites on the settlement of barnacles was tested using cyprids of *Balanus amphitrite*.

Adults were maintained at 22°C and fed on a daily diet of *Anemia* sp. nauplii (7 nauplii/ml) (Hellio et al., 2004). Release and culture of barnacle nauplii were performed as previously described (Hellio et al., 2004). After 4 days, when most of the larvae had metamorphosed to the cyprid stage, cyprids were collected by filtration and aged for 3 days at 6°C, before being used in the settlement experiments (Rittschof et al., 1992).

Settlement assays utilized 24-well microplates (Iwaki). Compounds were introduced to the wells using methanol as carrier solvent. After evaporation of the solvent (at room temperature), 10 to 15 cyprids were added to the wells that contained the extracts and metabolites in 2 ml of seawater. The concentrations tested were 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0

and 100.0 µg/ml. Each concentration along with the seawater control was replicated 6 times. Test plates were wrapped in aluminum foil and incubated at 28°C. The results were recorded after 24 hours of incubation. Each larva was examined under a dissecting microscope, and its physical condition was recorded. Cyprids that did not move when probed with a metal seeker were regarded as dead (Lau and Qian, 2000; Rittschof et al., 1992). Permanently attached and metamorphosed individuals were counted as settled. All others were counted as swimmers.

Data treatment. The survival curves and the LC_{50} values were determined by applying the log-logistic model to the data (Kooijman, 1981):

$$y = \frac{100}{1 + \exp\left(\frac{\ln(c) - \alpha}{\beta}\right)},$$

where y is the percentage of survival; c is the concentration; α is $\ln(\text{LC}_{50})$; β is slope coefficient; and LC_{50} is the lethal concentration at which 50% of mortality is observed.

In another data treatment, among the surviving individuals, only the swimming larvae were considered. Empirically it was found that the percentage q , of settlers, decreased proportionally to the concentration, following the simple exponential law: $q = q_0 \times \exp(-a \times c)$ where q_0 is the mean percentage of settlers, recorded for $c = 0$; since $p_0 + q_0 = 1$ and $p + q = 1$, the percentage p of swimming larvae becomes $p = 1 - ((1 \times p_0) \times \exp(-a \times c))$.

In both cases data were treated using a nonlinear regression program from SGPLUS software (Uniware).

Percentage settlement values were arcsine-transformed prior to statistical analyses. As Bartlett's test (Minitab) detected departures from homogeneity of variances in some of the data, all were analyzed nonparametrically (Instat) with the Kruskal-Wallis test and, as required, Dunns post hoc test to compare treatment means. The level of significance was set at $P < 0.05$.

Results

Extracts from freeze dried sponges were prepared using $\text{CH}_2\text{Cl}_2/\text{MeOH}$, and the resulting residue was subsequently subjected to chromatographic fractionation to yield a series of metabolites. The structural elucidation of the metabolites (Figure 1) was based on spectral analyses and comparison with published data. *Ircinia oros* yielded a mixture of ircinin I and II (**1**, **2**) (Cimino et al., 1972a) and fasciculatin (**9**) (Cafieri et al., 1972) as the main constituents.

Table 1. Settlement Inhibition Activity Against *B. amphitrite* cyprids

Group I (Active and toxic metabolites/extracts)	<i>LC</i> ₅₀ (ppm)		<i>EC</i> (ppm)
Ircinin I & II	4.7		5.0
Ircinin I & II acetates	4.9		5.0
Furodysinin	18.1		5.0
<i>Ircinia oros</i> CH ₂ Cl ₂ extract	21.7		50.0
7-Deacetoxyolepupane	106.2		100.0
<i>Dysidea</i> sp. CH ₂ Cl ₂ extract	52.5		65.9
Group II (Non-active and nontoxic metabolites/extracts)	% Survival	% Settlement	Concentration (µg/ml)
Spongi-12-en-16-one	100	60.3	100
Hydroquinone A	100	59.4	100
Hydroquinone C	100	57.3	100
Fasciculatin	100	58.0	100
<i>Dysidea</i> sp. aqueous extract	100	59.1	100
11β-Acetoxy-spongi-12-en-16-one	100	53.5	100
Group III (Active and nontoxic metabolites/extracts)	% Survival	% Settlement	Concentration (µg/ml)
Euryfuran	100	24.7	100
Hydroquinone-A acetate	100	19.9	100
Dihydrofuro-spongin II	100	11.2	100
Hydroquinone-C acetate	100	0.0	10
<i>Dysidea</i> sp. alcohol extract	100	0.0	25

Metabolites/extracts are classified according to their activity on inhibition of settlement. In group I, results are expressed as effective concentration for 0% settlement (*EC*) and concentration inducing 50% lethality (*LC*₅₀). For groups II and III, results are expressed as percentage of survival and of settlement for the reported concentrations.

Chromatographic separations of the *I. spinosula* extract resulted in the isolation of the 2 main metabolites, 2-octaprenyl-1,4-hydroquinone [hydroquinone A] (7) and 2-[24-hydroxy]-octaprenyl-1,4-hydroquinone [hydroquinone C] (8) (Mihopoulos et al., 1999).

From the organic extract of *Dysidea* sp., the metabolites furodysinin (3), 7-deacetoxyolepupane (4) and euryfuran (11) (Grode and Cardellina, 1984; Garson et al., 1992; Hochlowski et al., 1982) were isolated in pure form following a series of chromatographic separations.

The furanosesterterpene dihydrofuro-spongin-II (10) was isolated as the main metabolite of *Cacospongia scalaris* (Cimino et al., 1972b). Finally, the main metabolites yielded by the sponge *Hippospongia communis* were the diterpenes spongi-12-en-16-one (5) and 11β-acetoxy-spongi-12-en-16-one (6) (Cimino et al., 1994). Small quantities of a mixture of ircinin I and II, as well as hydroquinone A and hydroquinone C, were acetylated, and the corresponding derivatives 1a, 2a, 7a, 8a were submitted to the same evaluation protocol to determine their structure-activity relationships.

The effectiveness of the sponge extracts or metabolites in inhibiting *B. amphitrite* settlement is presented in Table 1. Extracts and metabolites were divided into 3 groups according to their level of activity and degree of toxicity. Group I comprised the mixture of ircinin I and II, the mixture of ircinin I and II acetates, furodysinin, 7-deacetoxyolepupane,

and the CH₂Cl₂ extracts of *Dysidea* sp. and *Ircinia oros*. These substances were highly active in the settlement inhibition assay [higher percentage of swimming larvae and lower percentage of settling larvae], but were also highly toxic.

Group II comprised 11β-acetoxy-spongi-12-en-16-one, spongi-12-en-16-one hydroquinone A, hydroquinone C, fasciculatin, and *Dysidea* sp. aqueous extract. All of the aforementioned substances were nontoxic (100% survival), but were not effective antifoulants. The percentage of swimming larvae did not differ over the concentration range tested.

Group III comprised dihydrofuro-spongin II, euryfuran, hydroquinone-A acetate, hydroquinone-C acetate, and *Dysidea* sp. alcoholic extract. All of these metabolites and extracts were nontoxic, and the percentage of swimming larvae showed a concentration-dependent increase. No mortality was recorded, even at the highest concentrations tested. As shown in Figure 2, settlement in 0.5 µg/ml euryfuran was not significantly different (*P* > 0.005) from the control. At concentrations of 1.0 to 100.0 µg/ml, however, significant differences from the control were observed, especially at 25.0, 50.0 and 100.0 µg/ml (*P* < 0.01), where only 30% settled. Incubation with increasing concentrations of euryfuran (1.0 to 25.0 µg/ml) led to a concomitant decrease in settlers and an increase in swimmers.

Incubating cyprids in 1.0 to 100 µg/ml hydroquinone-A acetate or hydroquinone-C acetate in-

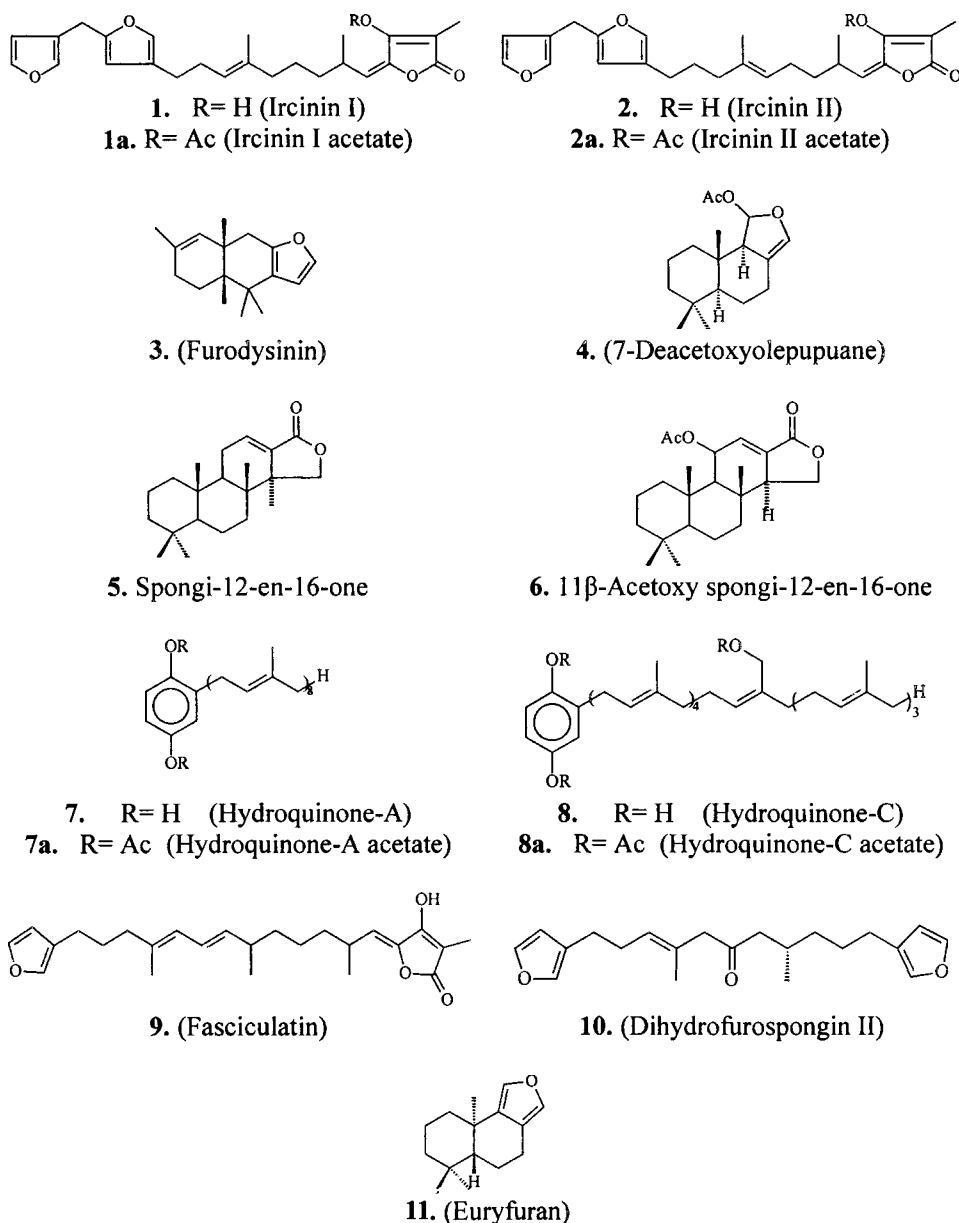


Fig. 1. Structures of the assayed sponge metabolites.

creased significantly the proportion of swimming cyprids with a concomitant decrease in settlers. Hydroquinone-C acetate, when used at 100 $\mu\text{g/ml}$, resulted in 100% swimmers.

The antisettlement activities of the ethanolic extract of *Dysidea* sp. and hydroquinone-C acetate were similar. All treatments were found to be significantly ($P < 0.05$) different from the control. Incubation of cyprids with increasing concentrations of *Dysidea* sp. ethanolic extract resulted in a progressive increase in the proportion of swimming larvae, reaching nearly 100% at 50 $\mu\text{g/ml}$. At concentrations ranging between 0.5 and 2.5 $\mu\text{g/ml}$ the percentage of swimmers was higher under the influence of *Dysidea* sp. ethanolic extract than with

hydroquinone-C acetate. The opposite was observed at concentrations between 5.0 and 25.0 $\mu\text{g/ml}$.

The settlement rate of *B. amphitrite* cyprids, when exposed to 0.5 $\mu\text{g/ml}$ dihydrofurospongine, was not significantly different ($P > 0.05$) from the control. Significant differences were recorded between the control and all other dihydrofurospongine concentrations ($P < 0.05$), with settlement decreasing as the concentration increased; at 100.0 $\mu\text{g/ml}$, only 11.2 % settlement occurred.

Discussion

Sponges (*Porifera*) are among the oldest groups of multicellular animals (more than 500 million years

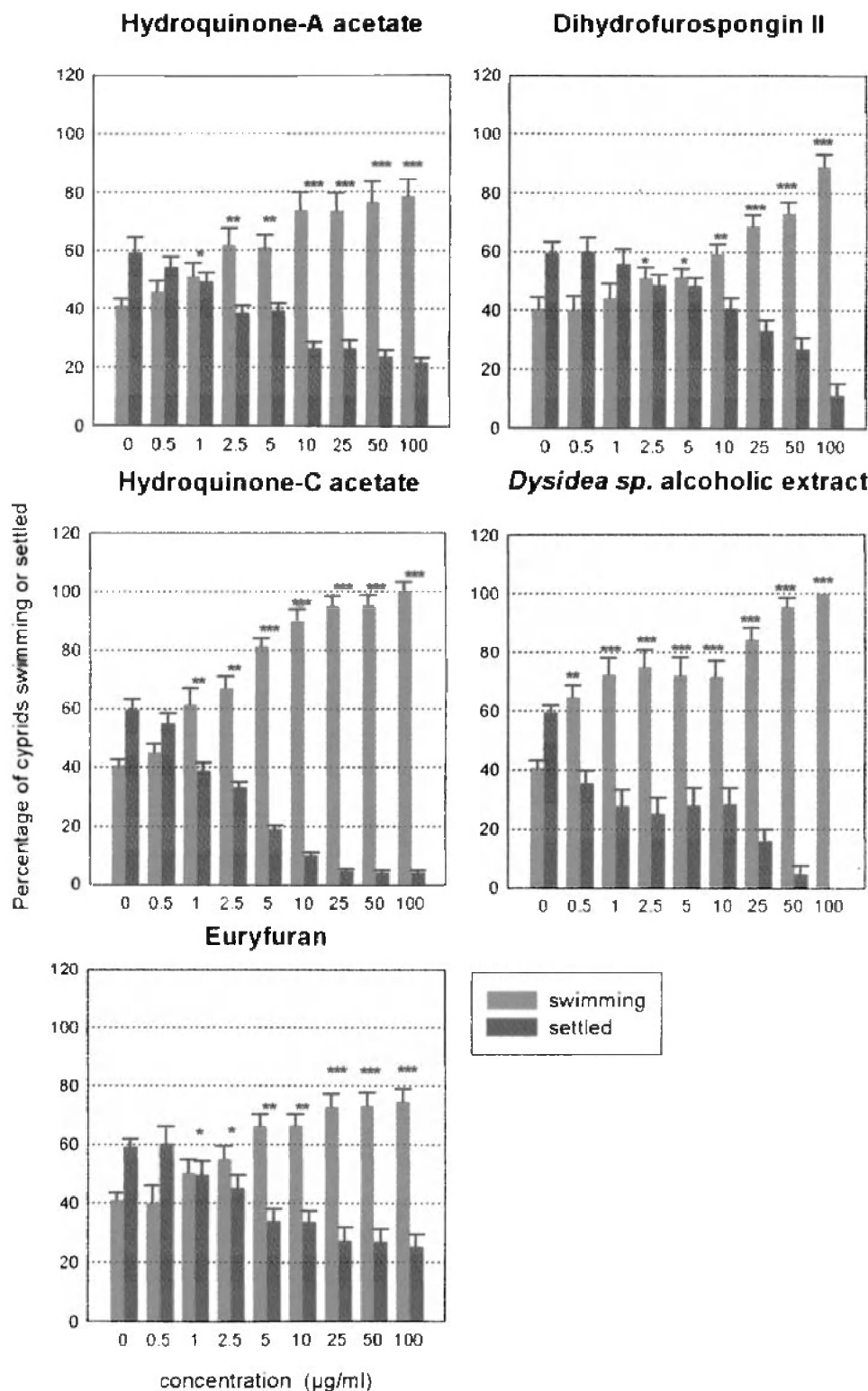


Fig. 2. Effect of the most active nontoxic metabolites/extracts (0 to 100 µg/ml) on *B. amphitrite* cyprid settlement. Results are expressed as percentage settled (\pm SEM) and percentage swimming (\pm SEM). Results significantly different from the control, * P < 0.05; ** P < 0.001; *** P < 0.001.

old) (Muller, 1995), and more than 10,000 species have been described (Zhang et al., 2003). Of all marine organisms investigated, sponges have been proven to biosynthesize the highest number of structurally diverse natural products (Blunt et al.,

2004). A significant number of sponge metabolites exhibit a wide array of bioactivities including anti-cancer, antiviral, and antifungal (Mayer and Hamann, 2004). Sponges, like many other marine invertebrates, host complex microbial communities

capable of synthesizing a variety of microbial-type bioactive metabolites that can impart biological activity to the corresponding extracts (Faulkner et al., 1993; Bewley et al., 1996).

Although most sponges are sessile, soft-bodied, and appear to be physically vulnerable, the lack of predation on some of them indicates advanced elaboration of physical (Hartman, 1981) and chemical defenses (Bakus and Green, 1974). Sponges are rich in terpenoids and steroids, which can function in antipredation, competition for space, and the control of epibiont overgrowth (Becerro et al., 2003; Burns et al., 2003; Kelly et al., 2003; Puyana et al., 2003; Sera et al., 2003; Thakur et al., 2003; Tsoukatou et al., 2003). Fusetani (1991) has proposed that these organisms secrete chemicals that prevent larvae of other marine organisms from settling and growing on them.

In this study we have focused on the ability of sponge extracts and metabolites to inhibit the settlement of *B. amphitrite* cypris larvae. Antifouling activity against settlement of *B. amphitrite* has been reported previously for metabolites found in 2 species of the genus *Axinyssa* (Hirota et al., 1998), from *Pseudoceratina purpurea* (Tsukamoto et al., 1996a, 1996b), from *Acanthella cavernosa* (Hirota et al., 1996; Okino et al., 1995) and from *Stylotella aurantium* (Kato et al., 1995).

The most effective nontoxic antifouling extracts and compounds were obtained from *Dysidea* sp., *I. spinolusa*, and *C. scalaris*. There was no obvious structural correlation between the metabolites that exhibited similar levels of settlement inhibition. The presence of a furan ring, however, seemed important to activity because all metabolites possessing this moiety and all extracts containing furanoterpenes showed strong settlement inhibition. The toxicity that was observed for some of these metabolites is likely to result from other structural functionalities. Interestingly, furan and also lactone rings are a common feature of marine natural product antifoulants (Clare, 1996, 1998). The size of the molecules did not seem to influence the activity, as there was no clear correlation among the tested chemical classes (sesquiterpenes, diterpenes, sesterterpenes, C21-degraded sesterterpenes, and prenylated hydroquinones). Moreover, the presence or absence of the free hydroxyl in ircinin I and II skeletons did not affect either the settlement-inhibitory activity or the toxicity of the metabolites. However, the acetylation of hydroquinones A and C reduced barnacle settlement dramatically without toxicity.

In conclusion, a number of the tested metabolites of epibiont-free sponges had antisettlement

activity when assayed against barnacle, *B. amphitrite*, cyprids. Group III metabolites and extracts, in particular hydroquinone-C acetate and the *Dysidea* sp. alcohol extract, were noteworthy for reducing settlement at nontoxic concentrations. It is of interest to determine, from the perspectives of both fundamental science (antiepibiosis) and applied science (antifouling), whether the group III extracts and metabolites have broad-spectrum efficacy against epibiotic and fouling species.

Acknowledgments

We thank the National Fellowship Foundation for provision of a fellowship to one of us (M.T.), and the General Secretariat for Research and Technology and the University of Athens for financial support of the Greek research team. The British team was supported by an award from the U.S. Office of Naval Research (N00014-02-1-0311). We also thank Dr. Dan Rittschof, Dr. Nikos Mihopoulos, Mr. Dennis Abatis, Mr. Lefteris Marinos, and Mrs. Sheelagh Henry for their assistance on different aspects of the present investigation.

References

1. Armstrong E, Boyd K, Burgess J (2000) Prevention of marine biofouling using natural compounds from marine organisms. *Biotechnol Annu Rev* 6, 221–241
2. Bakus GJ, Green G (1974) Toxicity in sponges and holothurians: a geographic pattern. *Science* 185, 951–953
3. Becerro MA, Thacker RW, Turon X, Uriz MJ, Paul VJ (2003) Biogeography of sponge chemical ecology: comparisons of tropical and temperate defences. *Oecologia* 135, 91–101
4. Bennett SC, Irwin SWB, Fitzpatrick SM (2003) Tributyltin and copper effects on encystment and *in vitro* encystment of *Parorchis acanthus* larvae. *J Helminthol* 77, 291–296
5. Bewley CA, Holland NA, Faulkner DJ (1996) Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts. *Experientia* 52, 71–75
6. Blunt JW, Copp BR, Munro MGH, Northcote TP, Princep MR (2004) Marine natural products. *Nat Prod Rep* 21, 1–49
7. Burgess JG, Boyd KG, Armstrong E, Jiang Z, Yan L, Berggren M, May U, Pisacane T, Granmo A, Adams DR (2003) The development of a marine natural product-based antifouling paint. *Biofouling* 19S, 197–205
8. Burns E, Ifrach I, Carmeli S, Pawlik JR, Ilan M (2003) Comparison of anti-predatory defences of Red Sea and Caribbean sponges, I: chemical defense. *Mar Ecol Prog Ser* 252, 105–114

9. Cafieri F, Fattorusso E, Santacroce C, Minale L (1972) Fasciculatin: a novel sesterterpene from the sponge *Ircinia fasciculata*. *Tetrahedron* 28, 579–583
10. Cheung KC, Wong MH, Yung YK (2003) Toxicity assessment of sediments containing tributyltin around Hong Kong harbour. *Toxicol Lett* 137, 121–131
11. Cimino G, de Stefano S, Minale L (1972a) Ircinin-1 and -2, linear sesterterpenes from the marine sponge *Ircinia oros*. *Tetrahedron* 28, 333–341
12. Cimino G, de Stefano S, Minale L, Fattorusso E (1972b) Minor C-21 furanoterpenes from the sponges *Spongia officinalis* and *Hippospongia communis*. *Tetrahedron* 28, 267–273
13. Cimino G, de Rosa D, de Stefano S, Minale L (1974) Isoagatholactone, a diterpene of a new structural type from the sponge *Spongia officinalis*. *Tetrahedron* 30, 645–649
14. Clare AS (1996) Marine natural product antifoulants: status and potential. *Biofouling* 9, 211–229
15. Clare AS (1998) Towards non-toxic antifouling. *J Mar Biotechnol* 6, 3–6
16. Coehlo MR, Bebianno MJ, Langston WJ (2002) Routes of TBT uptake in the clam *Ruditapes decussates*, II: food as a vector of TBT uptake. *Mar Environ Res* 54, 193–207
17. Dahlstrom M, Martensson L, Jonsson P, Amebrant T, Elwing H (2000) Surface active adrenoreceptor compounds prevent the settlement of cyprid larvae of *Balanus improvisus*. *Biofouling* 16, 191–198
18. de Nys R, Steinberg PD (2002a) Antifouling: the sea approach. *Biofutur* 228, 40–42
19. de Nys R, Steinberg PD (2002b) Linking marine biology and biotechnology. *Curr Opin Biotech* 13(3), 244–248
20. Faulkner DJ, He HY, Unson MD, Bewley CA (1993) New metabolites from marine sponges: are symbionts important?. *Gazz Chim Ital* 123, 301–305
21. Fusetani N (1991) The Fusetani biofouling project. *Biofouling* 12, 2–8
22. Fusetani N (2004) Biofouling and antifouling. *Nat Prod Rep* 21, 94–104
23. Garson MJ, Dexter AF, Lambert LK, Liokas V (1992) Isolation of the bioactive terpene 7-deacetoxyolepupane from the temperate marine sponge *Dysidea* sp. *J Nat Prod* 55, 364–367
24. Gibbs P (1993) A male genital defect in the dog-whelk, *Nucella lapillus* (Neogastropoda), favouring survival in TBT-polluted area. *J Mar Biol Assoc U* 73, 667–678
25. Grode SH, Gardellina JH II (1984) Sesquiterpenes from the sponge *Dysidea etheria* and the nudibranch *Hypselodoris zebra*. *J Nat Prod* 47, 76–83
26. Hartman W (1981) Form and distribution of silica in sponges. In: *Silicon and Siliceous Structures in Biological Systems*, Volkani TL, Simpson BE, eds. (New York, NY: Springer, Simpson) pp 454–493
27. Hattori T, Shizuri Y (1996) A screening method for antifouling substances using spores of the fouling macroalga *Ulva conglobata* and new antifouling technology. *Biofouling* 8, 147–160
28. Hellio C, Maréchal JP, Véron B, Bremer G, Clare AS, Le Gal Y (2004) Seasonal variation of antifouling activities of marine algae from the Brittany coast (France). *Mar Biotechnol* 6, 67–82
29. Hirota H, Tomono Y, Fusetani N (1996) Terpenoids with antifouling activity against barnacle larvae from the marine sponge *Acanthella cavernosa*. *Tetrahedron* 52, 2359–2368
30. Hirota H, Okino T, Yoshimura E, Fusetani N (1998) Five new antifouling sesquiterpenes from two marine sponges of the genus *Axinyssa* and the nudibranch *Phyllidia pustulosa*. *Tetrahedron* 54, 13791–13980
31. Hochlowski JE, Walker RP, Ireland C, Faulkner DJ (1982) Metabolites of four nudibranchs of the genus *Hypselodoris*. *J Org Chem* 47, 88–91
32. Holmstrom C, Kjelleberg S (1994) The effect of external biological factors on settlement of marine invertebrate and new antifouling technology. *Biofouling* 8, 147–160
33. Iwata H, Tanabe S, Mizuno T, Tatsukawa R (1995) High accumulation of toxic butylins in marine mammals from Japanese coastal waters. *Environ Sci Technol* 29, 2959–2965
34. Kato T, Shizuri Y, Izumida H, Yokoyama A, Endo M (1995) Styloguanidines, new chitinase inhibitors from the marine sponge *Stylotella aurantium*. *Tetrahedron Lett* 36, 2133–2136
35. Kelly SR, Jensen PR, Henkel TP, Fenical W, Pawlik JR (2003) Effects of Caribbean sponge extracts on bacterial attachment. *Aquat Microb Ecol* 31, 175–182
36. Kooijman S (1981) Parametric analyses of mortality rates in bioassays. *Water Res* 15, 107–119
37. Koryakova M, Korn O (1993) Using barnacle larvae for evaluation of the toxicity of antifouling paint compounds. *Russ J Mar Biol* 19, 212–216
38. Lau S, Qian PY (2000) Inhibitory effect of phenolic compounds and marine bacteria on larval settlement of the barnacle *Balanus amphitrite* Darwin. *Biofouling* 16, 47–58
39. Mayer AMS, Hamann MT (2004) Marine pharmacology in 2000: marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiplatelet, antituberculosis and antiviral activities affecting the cardiovascular, immune and nervous systems and other miscellaneous mechanisms of action. *Mar Biotechnol* 6, 37–52
40. McAllister BG, Kime DE (2003) Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (*Danio rerio*). *Aquat Toxicol* 65, 309–316
41. Mihopoulos N, Vagias C, Chinou I, Roussakis C, Scoullou M, Harvala C, Roussis V (1999) Antibacterial and cytotoxic natural and synthesized hydroquinones from the sponge *Ircinia spinosula*. *Z Naturforsch* 54C, 417–423
42. Muller WE (1995) Molecular phylogeny of metazoa (animals): monophyletic origin. *Naturwissenschaften* 82, 321–329
43. Ohira S, Matsui H, Nitta K (1996) Subchronic study of the metabolism of triphenyltin in hamster. *Vet Hum Toxicol* 38, 206–210

44. Okino T, Yoshimura E, Hirota H, Fusetani N (1995) Antifouling Kalihinenes from the Marine Sponge *Acanthella cavernosa*. *Tetrahedron Lett* 36, 8637–8640
45. Omae I (2003) Organotin antifouling paints and their alternatives. *Appl Organomet Chem* 17, 81–105
46. Pawlik JR, Chanas B, Toonen RJ, Fenical W (1995) Defences of Caribbean sponges against predatory reef fish. *Mar Ecol Prog Ser* 127, 195–211
47. Peterson S, Batley G, Scammell M (1993) Tetracycline in antifouling paints. *Mar Pollut Bull* 26, 96–100
48. Ponasik J, Conova S, Kinghorn D, Kinney W, Rittschof D, Ganem B (1998) Pseudocreatine, a marine natural product with antifouling activity: synthetic and biological studies. *Tetrahedron* 54, 6977–6986
49. Puyana M, Fenical W, Pawlik JR (2003) Are there activated chemical defenses in sponges of the genus *Aplysina* from the Caribbean? *Mar Ecol Prog Ser* 246, 127–135
50. Rittschof D (2000) Natural product antifoulants: one perspective on the challenges related to coatings developments. *Biofouling* 15, 119–127
51. Rittschof D, Clare AS, Gerhart DJ, Avelin Sr.M, Bonaventura J (1992) Barnacle *in vitro* assays for biologically active substances: toxicity and settlement inhibition assays using mass cultured *Balanus amphitrite* Darwin. *Biofouling* 6, 115–122
52. Sera Y, Adachi K, Fujii K, Shizuri Y (2003) A new antifouling hexapeptide from a Palauan sponge, *Haliclona* sp. *J Nat Prod* 66, 719–721
53. Sidharthan M, Young KS, Woul LH, Soon PK, Shin HW (2002) TBT toxicity on the marine microalga *Nannochloropsis oculata*. *Mar Pollut Bull* 45, 177–180
54. Thakur NL, Hentschel U, Krasko A, Pabel CT, Anil AC, Muller WEG (2003) Antibacterial activity of the sponge *Suberites domuncula* and its primmorphs: potential basis for epibacterial chemical defence. *Aquat Microb Ecol* 31, 77–83
55. Tsoukatou M, Hellio C, Vagias C, Harvala C, Roussis V (2002) Chemical defense and antifouling activity of three Mediterranean sponges of the genus *Ircinia*. *Z Naturforsch* 57C, 161–171
56. Tsoukatou M, Siapi H, Vagias C, Roussis V (2003) New sesterterpene metabolites from the Mediterranean sponge *Cacospongia scalaris*. *J Nat Prod* 66, 444–446
57. Tsukamoto S, Kato H, Hirota H, Fusetani N (1996a) Ceratinamides A and B: new antifouling dibromotyrosine derivatives from the marine sponge *Pseudoceratina purpurea*. *Tetrahedron* 52, 8181–8186
58. Tsukamoto S, Kato H, Hirota H, Fusetani N (1996b) Pseudoceratidine: a new antifouling spermidine derivative from the marine sponge *Pseudoceratina purpurea*. *Tetrahedron Lett* 37, 1439–1440
59. 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* 10, 361–373
60. Yebra DM, Kill S, Dam-Johansen K (2004) Antifouling technology: past, present and future steps towards efficient and environmentally friendly antifouling coatings. *Prog Org Coat* 50, 75–104
61. Zhang W, Xue S, Zhao Q, Zhang X, Li J, Jin M, Yu X, Yuan Q (2003) Biopotential of marine sponges from China oceans: past and future. *Biomol Eng* 20, 413–419