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6-BROMOINDOLE-3-CARBALDEHYDE, FROM AN *Acinetobacter* SP. BACTERIUM ASSOCIATED WITH THE ASCIDIAN *Stomozoa murrayi*

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Abstract—The ascidian *Stomozoa murrayi* from Caribbean coral reefs is not overgrown by macroepibionts. Chemical extraction of this organism gave 6-bromoindole-3-carbaldehyde (**1**) and its debromo analog (**2**). These two compounds, previously obtained from several marine organisms, were also extracted from an *Acinetobacter* sp. bacterium isolated from the surface of *S. murrayi*. Compound **1** exhibits in vitro settlement inhibition of barnacle larvae, moderate antibacterial properties, but not antialgal or fish-feeding deterrent activities. Its potential role in contributing to the reduced fouling in *S. murrayi* is proposed.

Key Words—6-Bromoindole-3-carbaldehyde, *Stomozoa murrayi*, marine bacteria, *Acinetobacter*, antifouling activity, chemical defense.

INTRODUCTION

In recent years it has been repeatedly suggested that associated microorganisms could be the true producers of biologically active secondary metabolites obtained from marine benthic invertebrates (e.g., Ireland et al., 1988; Banaigs et al., 1993). Yet there are few data supporting this hypothesis and the fact is still largely a matter of speculation (Fenical, 1993; Kang et al., 1996).

Recent work has provided convincing evidence for the production by microorganisms of natural products originally attributed to their hosts. In a very elegant work, Unson et al. (1994) demonstrated that a brominated diphenyl

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[1]In memory of Hélène Débard, who died during the preparation of this manuscript.

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ether is produced by *Oscillatoria spongeliae*, a cyanobacterial symbiont of the sponge *Dysidea herbacea*.

Oclarit et al. (1994) isolated an anti-bacillus peptide from the sponge *Hyatella* sp. and showed that this product is in fact synthesized by an associated *Vibrio* sp. Bewley et al. (1996) provided another good example of bioactive metabolites from the sponge, *Theonella swinhoei*, produced by microbial symbionts.

The ecological role of microbial metabolites in the marine environment is not yet well understood. Some marine bacteria inhibit marine yeasts (Buck et al., 1963; Burkholder, 1973), while other marine bacteria protect crustacean embryos against fungal infections (Gil-Turnes et al., 1989; Gil-Turnes and Fenical, 1992). Microbial films are known to promote the settlement of many benthic organisms (Pawlik, 1993) or, in contrast, to inhibit larval settlement of barnacles (Maki et al., 1988; Holmström et al., 1992; Rittschof et al., 1992). Kon-Ya et al. (1995) showed that a bacterium of the genus *Alteromonas*, associated with the sponge *Halichondria okadai*, produces ubiquinones that inhibit the settlement of *Balanus amphitrite* larvae.

Among bromoindole derivatives, the metabolite 2,5,6-tribromo-1-methylgramine, isolated by Kon-Ya et al. (1994a) from the bryozoan *Zoobotryon pellucidum*, and other compounds of the same family (Kon-Ya et al., 1994b) have shown potent anti-fouling activity against barnacle larvae.

The present work was undertaken to determine if secondary metabolites present in the Caribbean ascidian *Stomozoa murrayi* (Kott, 1957) could be found in associated bacteria isolated from the surface of the ascidian. If such a hypothesis could be confirmed, then comments about bioactivity of these compounds would be an important part of profiling the compounds, allowing speculation on their ecological role as antifouling agents.

METHODS AND MATERIALS

Collection and Extraction of the Ascidian. In the summer of 1992, 250 g (fresh weight) of *S. murrayi* were collected by scuba diving (3–5 m) near the marine research station of the Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México (ICML-UNAM), at Puerto Morelos, 86°52′W, 20°50′N (Q. Roo, Mexico), on the east coast of the Yucatan Peninsula. This black massive colonial ascidian was identified by Claude Monniot and Françoise Monniot, from the Muséum National d'Histoire Naturelle (Paris), as *Stomozoa murrayi*, a species originally described by Kott (1957) from the Red Sea, and later found in Brazil (Millar 1977) and in New Caledonia (Monniot, 1988). This is the first observation of the species in the Caribbean Sea.

Fresh ascidians were preserved in ethanol for two weeks. The ascidians were mixed in a Waring blender, filtered, and the ethanol evaporated. The remaining aqueous extract (700 ml) was partitioned with heptane (3 × 500 ml), ether (3 × 500 ml), dichloromethane (3 × 500 ml) and butanol (3 × 350 ml). The weight of all combined extracts was 3.9 g, and the dry weight of the ascidian residue was 40 g. The extracts were frozen and transported to France. Work-up was done immediately.

Isolation and Extraction of Bacteria from the Ascidian. Four bacterial isolates were obtained by cutting very thin layers from different parts of an ascidian colony (freshly collected) with a sterile razor blade. These layers were gently rinsed with sterile seawater and then added to 10 ml sterile seawater and shaken vigorously. Serial log dilutions were prepared (two replicates). From the third dilution, 200 μl was inoculated in sterile Petri dishes containing Bacto Marine Agar 2216 (Difco). Dishes were incubated for five days at 28°C in darkness. From the two replicates, we obtained two strains from the surface layer of the ascidian, namely SM-S1 and SM-S2. These strains were transferred in separate sterile Petri dishes and subcultured to ensure the purity of the strains. Two other bacterial strains were obtained from the same specimen, one from the inner layer of the tunic (strain SM-INT), and another one from an individual zooid (strain SM-Z). The pure strains were stored in tubes containing Bacto Marine Agar 2216 (Difco) and kept at 4°C. They were transported to France at environmental temperature, placed in a refrigerator and subcultured monthly.

The culture medium was prepared with natural filtered seawater and diluted with distilled water (75:25, v/v). Then, 5 g bactopeptone, 0.5 g NH_4Cl, and 0.1 g $FePO_4$ were dissolved in 1 liter of the diluted seawater. The four strains were grown separately in 0.5 liter of this medium (pH 6.8) for 10 days in darkness at 28°C. No significant pH variation was observed when the growth process was stopped.

Culture broths were extracted with ethyl acetate. The extracts were evaporated to dryness and partitioned between water (500 ml) and heptane (2 × 500 ml), followed by ether (2 × 500 ml). Ether fractions were submitted to GC-MS analysis. Sterile medium (500 ml) was extracted as a reference.

The strain SM-S1 was grown again for two days at 28°C in 50 ml of culture medium (see above), after which 5 ml of the 2-day-old culture was transferred to a 2-liter fermentor containing 1 liter of culture medium and growth continued at 28°C in darkness for two days with 350 rpm agitation and 2 liter/min aeration. The ether extract (5 mg) from 1 liter of bacterial broth of the strain SM-S1 (*Acinetobacter* sp.), was submitted to GC-MS analysis under the conditions described below.

Identification of Metabolites. Gas chromatographic separation was performed with an Unicam 610 gas chromatograph coupled to an Unicam System 2 Benchtop quadrupole mass spectrometer. A 30-m × 0.25-mm SPB 1701

(Supelco) column was used. The oven was programmed at 3°C/min from 160°C to 250°C (He carrier gas, 1 bar). Ether extract from bacterial culture of the strain SM-S1 was evaporated to dryness, and the resulting residue was dissolved in CH_2Cl_2 at 50 mg/ml. One microliter of the dichloromethane solution was injected in splitless mode at 150°C. For the MS determinations the ionization voltage was 70 eV, the pressure of the detector was 10^{-4} torr, and the mass spectral scan range was set to 50–500 *m/z*.

The ether-soluble material (520 mg) from 40 g (evaluated dry weight) of the ascidian was submitted to silica gel chromatography (Si 60). The 9:1 CH_2Cl_2–MeOH fraction (60 mg) was purified by HPLC on a C_8 reverse-phase column, with 8:2 MeOH–H_2O as solvent mixture, and it yielded 3 mg (0.007% of the ascidian dry weight, i.e., 75 μg/g dry tissue) of pure 6-bromoindole-3-carbaldehyde (**1**) and 1.5 mg (0.0035% = 37.5 μg/g dry tissue) of pure indole-3-carbaldehyde (**2**). The structures were determined by NMR spectral data (Wratten et al., 1977; Dellar et al., 1981; Rasmussen et al., 1993).

Synthesis of **1**. Bromination of **2** in acetic acid (Da Settimo et al., 1967) gave a mixture of the starting product, with the 5- and 6-brominated homologs. Pure **1** was obtained by repeated HPLC separation on a Lichrosorb Si 60 250-10 column (Merck) with 60:70:70:1 $CHCl_3$–EtOAc–heptane–EtOH.

GC/MS Control. To avoid false positive results, as recently reported for tetrodotoxin production by bacteria (Matsumura, 1995), the sterile culture medium (500 ml) was also examined as background with the same extractive and analytical conditions. Compounds **1** and **2** were not detected even as traces in the culture medium extracts, attesting to the bacterial production of both metabolites.

Biological Activities. Antimicrobial activities of **1** and **2** were assayed by the disc diffusion method against eight marine bacteria: *Deleya marina* (CIP: 748-T), *Alteromonas haloplanktis* (CIP: 103197-T), *Vibrio campbellii* (CIP: 751-T), *Bacillus marinus* (CIP: 103308-T), and SM-S1, SM-S2, SM-INT, and SM-Z (Table 2). Except for *B. marinus*, which is Gram-positive, and the strain SM-S2 (Gram variable), all strains are Gram-negative.

It was necessary to apply 60 μg of each compound to a sterile paper disc (6 mm, Filtres Durieux) to obtain reliable growth inhibition; after solvent evaporation, the discs were placed on a Marine Agar 2216 (Difco) test dish (three discs per dish) containing a bacterial culture. Growth inhibition was measured after 48 hr of incubation at 30°C. Two replicates containing three discs each were performed for each strain, and the results were presented as a simple average of the data.

The antialgal activity of **1** and **2** was tested against the diatom *Nitzschia acicularis* with a similar method of bacterial plating assay. Sterile paper discs were impregnated with 60 μg of each compound. In our experimental conditions,

10 days at 18°C with a 12-hr photoperiod were necessary before measuring zones of inhibition.

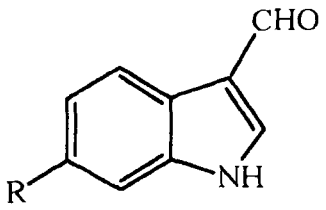
Settlement inhibition was tested in vitro on *Balanus amphitrite* larvae, following the techniques of Rittschof et al. (1984, 1985, 1986, 1992). Compounds **1** and **2** were tested at concentrations ranging from 100 μg to 0.01 μg/ml. Test solutions were prepared by dissolving 1 mg of compound in 30 μl dimethyl sulfoxide (DMSO) and diluting to appropriate concentrations in filtered seawater. Maximal concentration of DMSO in the tests was 0.3%. The tests were carried out in four replicates: 20–30 larvae (4-day-old cyprid larvae) were pipetted into polystyrene cell wells (Corning, 35 mm) containing 2 ml of test situation, filtered seawater control, or 0.3% DMSO in seawater. Test dishes were incubated for 24 hr at 26–28°C with a 15L:9D photoperiod. Tests were stopped by the addition of two drops of 10% formaldehyde, and the numbers of attached and free larvae were counted under a dissecting microscope. The frequency of permanent attachment (expressed as percentage settlements) in treatments was compared to attachment in the control by William's adjusted G test for independence. Results were rejected if the replicates differed significantly or if the settlement in the controls was less than 30%.

Fishes used in the feed-deterrent assay were *Serranus cabrilla*. Compounds **1** and **2** were dissolved in ethanol at 1 mg/ml and 10 mg/ml. For both concentrations, 1 ml of the solution was added to 24 ml of nonsolidified agar (conserved at 40°C) and poured in Petri dishes containing small pieces (0.5 g) of thawed shrimp. After solidification, pieces of agar were cut in pellets of 1 g containing 0.5 g of agar and0.5 g of shrimp. From one extreme to the other, final test concentrations per pellet for both compounds were 250 μg for the highest one and 25 μg for the lowest one. Fishes were fed with four pellets per day. They were conditioned to feeding on control pellets (containing EtOH only) for seven days prior to experimentation.

RESULTS

The main chemical result was the identification of compounds **1** and **2** (Figure 1) from the ether extract of the ascidian *Stomozoa murrayi*. This was accomplished by means of MS analysis and extensive ^{1}H and ^{13}C NMR studies, particularly normal (DQF-COSY) and reverse (HMQC and HMBC) 2D NMR.

Identification of Bacteria. Identification of the four strains isolated from the ascidian *S. murrayi* was not possible with the usual biochemical tests. Strain SM-S1 is a rod-shaped, nonmotile bacterium, which stains Gram-negative and produces white, spreading colonies. It cannot utilize arabinose, lactose, rhamnose, inositol, sorbitol, *N*-acetylglycosamin, acetate, phenylaccetate, pyruvate,



1 R = Br
2 R = H

FIG. 1. 6-bromoindole-3-carbaldehyde **1** and indole-3-carbaldehyde **2**.

γ-hydroxybutyrate, or malonate. It utilizes glucose, galactose, mannose, fructose, saccharose, maltose, fucose, glycerol, mannitol, lactate, succinate, L-alanine, L-histidine, and L-proline. Preliminary studies based on 16S ribosomal DNA sequences of this strain indicate that it belongs to the genus *Acinetobacter*.

Compounds **1** *and* **2** *from* Acinetobacter *sp.* An unidentified polyhalogenated compound (isotopic cluster at *m/z* 327, 329, 331, 333) was detected at R_t 7:43 min. A second compound was detected at R_t 17:42 min (Figure 2B) and identified as the aldehyde **2** (M^+ 145, $[M-H]^+$ 144, $[M-COH]^+$ 116). A third compound was detected at R_t 28:46 min (Figure 3B) and identified as the monobrominated aldehyde **1** based on molecular ion cluster (M^+ 223–225, $[M-H]^+$ 222–224) and the fragmentation pattern ($[M-COH]^+$ 194–196, $[M-COH-HCN]^+$ 167–169, $[M-H-Br]^+$ 143, $[M-COH-Br]^+$ 115). Figure 2 presents the quite similar mass spectra observed at R_t 17 min for two GC-MS independent analyses of bacterial extract and coinjected indole-3-carbaldehyde **2** from ascidian and commercial origin (90:10 w/w).

The mass spectra of **1** from bacterial and ascidian origin (Figure 3) show the same fragmentation pattern with some variation in the relative abundances (Table 1). Table 1 also presents the relative abundance of **1** isolated from the sponge *Pseudosuberites hyalinus* (Rasmussen et al., 1993), for which the Danish group "cannot exclude the possibility that it originates with some associated microorganisms." Synthetic compound **1** (authenticated by NMR spectra: $^{\delta}CDCl_3$ = 8.20, 7.82, 7.61, 7.44) has an R_t of 28:30 min in the same analytical conditions.

Antimicrobial Activity. The bromo compound **1** inhibited the growth of four of eight strains tested, while the debromo analog **2** does not (Table 2). No autotoxicity was observed for strain SM-S1. Compound **2** had no effect on the growth of the diatom *Nitzschia acicularis*. Compound **1** displayed a very low inhibitory activity (300 times less active than Cu^{2+} salts) on this diatom with a zone of inhibition measured as excess radius of 2.5 mm.

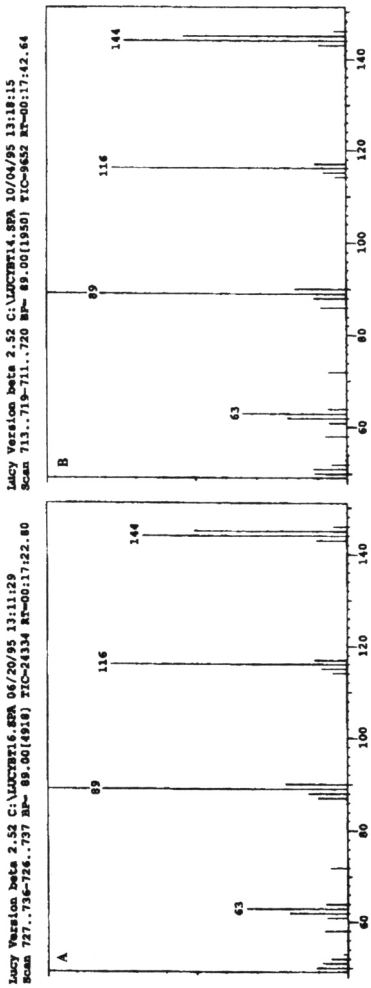


FIG. 2. Mass spectra of indole-3-carbaldehyde **2** from ascidian origin (A) and from bacterial origin (B).

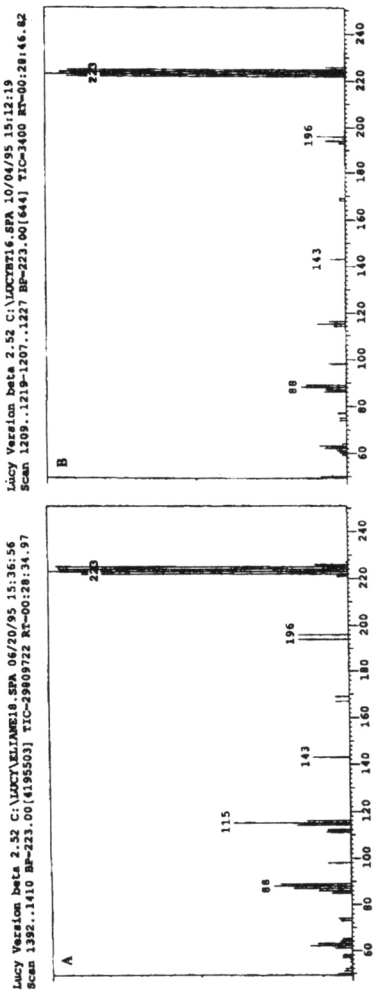


FIG. 3. Mass spectra of 6-bromoindole-3-carbaldehyde **1** from ascidian origin (A) and from bacterial origin (B).

TABLE 1. FRAGMENTATION PATTERN OF 6-BROMOINDOLE-3-CARBALDEHYDE **1** FROM NATURAL AND SYNTHETIC ORIGIN

	$[M]^+$ $[M-H]^+$				$[M-COH]^+$		$[M-COH-HCN]^+$		$[M-H-Br]^+$	$[M-COH-Br]^+$
Origin	225	224	223	222	196	194	169	167	143	115
Stomozoa murrayi	98	97	100	89	17	17	5	6	12	39
Acinetobacter sp.	93	96	100	93	9	7	2	2	2	10
Synthetic **1**	97	100	95	83	17	18	not observed		6	22
Pseudosuberites hyalinus[a]	100	100	100	100	28	28	12	12	24	37

[a]Rasmussen et al. (1993).

TABLE 2. ANTIBACTERIAL ACTIVITY OF **1** AND **2** ON EIGHT MARINE STRAINS[a]

	Strain SM-S1	Strain SM-S2	Strain SM-INT	Strain SM-Z	*Alteromonas haloplanktis*	*Abcillus marinus*	*Deleya marina*	*Vibrio campbellii*
1 (60)	−	++	−	+	−	++	−	++
2 (60)	−	−	−	−	−	−	−	−
C (30)	+++	+++	+++	+++	nt	nt	nt	nt
S (10)	+	++	++	+	nt	nt	nt	nt
P (10)	−	+++	−	−	nt	nt	nt	nt
E (15)	++	+++	++	++	nt	nt	nt	nt

[a]Values are indicated as excess radius. Concentration is indicated in parentheses in μg. +, 4–5 mm radius; ++, 5–10 mm radius, +++, 10–15 mm radius. C = Chloramphenicol, S = Streptomycin, P = Penicillin, and E = Erythromycin. nt = non tested.

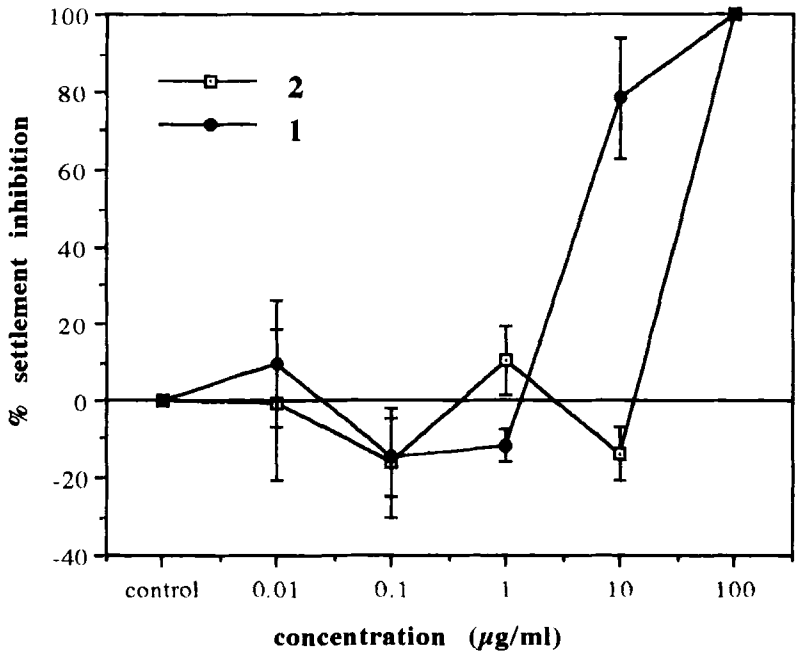


FIG. 4. Inhibition of settlement of *Balanus amphitrite* larvae by 6-bromoindole-3-carbaldehyde **1** and indole-3-carbaldehyde **2**. Control includes seawater and DMSO controls. Each point is the mean of four replicates ± SD.

Inhibition of Settlement. Both compounds completely inhibited settlement of cyprid larvae of *Balanus amphitrite* at the highest concentration tested (100 μg/ml) (Figure 4). The most active metabolite **1** inhibited settlement of larvae by 80% at 10 μg/ml. Settlement inhibition by compound **2** showed a sharp increase at 100 μg/ml, with no significant effect at lower concentrations. EC_{50} values of compounds **1** and **2** were calculated to be 5 μg/ml and 28 μg/ml, respectively. In similar experiments, the EC_{50} value of $CuSO_4$ was found to be 0.85 μg/ml.

Feeding Deterrency Activity. We have not observed any natural predation on *Stomozoa murrayi* by fishes, but when we cut the tunic, damselfishes greedily ate the exposed zooids and spat out tunic fragments. Although tested at concentrations corresponding to between 2 and 20 times stronger than the natural concentrations in the ascidian, no repellent effect of **1** and **2** has been observed. It is worth noting that fishes in this experiment are Mediterranean *Serranus cabrilla* and do not belong to the environment of the Caribbean ascidian.

DISCUSSION

As in the case of other marine benthic invertebrates, the ascidian *S. murrayi* is subjected to a continuous pressure of settlement by larvae and spores, but neither our field observations nor earlier descriptions give any evidence of fouling on this species. Thus, the existence of an antifouling defense is expected. The biological activity of compound **1** isolated from the ascidian supports this hypothesis. The present of compounds **1** and **2** in the ascidian and in a bacterium from its surface may indicate a symbiotic relationship. Compound **1** together with the debromo analog **2** previously was described from at least one bacterium and different taxonomic groups of invertebrates (Table 3).

Four bacterial strains from the ascidian *S. murrayi* have been isolated and cultured. Only one strain produced compounds **1** and **2**. Compound **1** displays low antibacterial activity (Table 2). Moreover, this compound is not present in large quantity in the ascidian (75 μg/g dry tissue, and 12 μg/g wet tissue). For the first time, these indolic compounds are shown to be produced by a bacterium associated with an ascidian.

The activity of **1** on strain SM-S2 is surprising because this bacterium grows at the ascidian surface along with strain SM-S1 that produces **1**. When grown together, the two bacteria are present in Petri dishes after seven days and in a fermentor after three days. Possibilities that could explain this observation are that strains SM-S1 and SM-S2 in the ascidian are separated by many layers of cell-tissue; the production of **1** in culture by SM-S1 is low; and the concentration of **1** in the test is higher than in the environment.

Only four bacterial strains have been isolated, but marine bacteria are dif-

TABLE 3. SUMMARY OF NATURALLY OCCURRING 6-BROMOINDOLE-3-CARBALDEHYDE **1** TOGETHER WITH INDOLE-3-CARBALDEHYDE **2**

Species	Taxonomic group	Site	Reference
Pseudomonas sp.	Bacterium	California	Wratten et al. (1977)
Acinetobacter sp.	Bacterium	Southeastern Mexico	This paper
Iotrochota sp.	Sponge	Western Australia	Dellar et al. (1981)
Pleroma menoui	Sponge	Southeastern Noumea	Guella et al. (1989)
Oceanapia bartschi	Sponge	Bahamas	Cafieri et al. (1993)
Pseudosuberites hyalinus	Sponge	Faroe Islands	Rasmussen et al. (1993)
Tubastrea coccinea	Coral	Hawaii	Okuda et al. (1982)
Tubastrea faulkneri	Coral	Northeastern Australia	Rashid et al. (1995)
Pyura sacciformis	Tunicate	Japan	Niwa et al. (1988)
Stomozoa murrayi	Tunicate	Southeastern Mexico	This paper

ficult to isolate and cultivate, because only a small percentage of the viable bacterial cells in marine samples ultimately grow under standard culture conditions (Fenical, 1993). The exact nature of the association between the *Acinetobacter* sp. strain (SM-S1) and the ascidian *S. murrayi* is not yet established, and we do not know if this bacterium is typical to *S. murrayi*.

Marine life is the largest source of naturally occurring organohalogen compounds, and in many marine organisms these compounds may serve in chemical defense, as either feeding deterrents, irritants, or poisons (Gribble, 1992). The question of the true origin of these metabolites has not been resolved. Bromoindole alkaloids isolated from several phyla have been shown, at least in one case, to originate from a dietary source (Fahy et al., 1991). A symbiotic origin is often suggested but rarely proved (Rasmussen et al., 1993). Very few microorganisms express the biochemical potential for introducing a bromo atom in the indole nucleus (Wratten et al., 1977; Norton and Wells, 1982).

When a metabolite is widely distributed, bacteria or cyanobacteria either as true symbionts or as nonobligate associates have been suspected to be responsible for production (Fenical, 1993). We have no information on whether compound **1** obtained from other marine invertebrates is of bacterial origin or whether those organisms are fouled or not. The production of both compounds **1** and **2** by a marine bacterium isolated from the surface of a benthic invertebrate could explain the presence of the same products in phylogenetically unrelated benthic invertebrates. Pietra (1995) hypothesized that the presence of similar natural products in different phyla could stem from endosymbiotic bacterial contribution of genetic information to the host, but this hypothesis remains to be proved.

The information about bacterial epibiosis on ascidians is quite limited (Wahl et al., 1994; Wahl, 1995). Bacterial associations with ascidians have been described (Holmström et al., 1992; Holmström and Kjelleberg, 1993), and reveal that bacteria produce inhibitory substances in the marine environment, even if they are not specifically antibiotic producers.

Marine organisms, such as ascidians, can prevent fouling by the production of secondary metabolites (Davis and Wright, 1990). For the ascidian *S. murrayi*, bromo compound **1** inhibits larval settlement, but compound **2** does not. Compound **1**, although not present at a high level, was present in sufficient quantity to inhibit settlement of barnacles (EC_{50} = 5 μg/ml). This could explain why the ascidian *S. murrayi* is not overgrown by macroepibionts such as *Balanus amphitrite*. We cannot estimate an equivalence between the concentration of the products on the ascidian, in dry weight, to the test in solution, and we think that it is necessary to incorporate the compound in a matrix to evaluate its antifouling properties. Compound **1** displays an activity against barnacle larvae settlement close to the activities of 2,5,6-tribromo-1-methylgramine (EC_{50} = 1 μg/ml) studied by Kon-Ya et al. (1994a) or $CuSO_4$. The fact that compound **1** is also produced by strain SM-S1, probably the true producer, agrees with the concept that larval settlement can be affected in a negative way by bioactive secondary metabolites released by associated bacteria (Avelin et al., 1993).

Wratten et al. (1977) report that products **1** and **2** are from extracellular origin, and our results are in agreement with the suggestion of Maki et al. (1988) that bacterial extracellular products may be the causal agents of larval settlement inhibition. We propose that compound **1** reduces fouling on the surface of the ascidian *S. murrayi*, but it should be tested against naturally occurring epibionts to ascribe to it a defensive role.

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