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Chemical warfare in the sea: The search for antibiotics from Red Sea corals and sponges*

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Abstract: Marine sponges and corals are widely recognized as rich sources of novel bioactive natural products. These organisms are frequently colonized by bacteria. Some of these bacteria can be pathogenic or serve as beneficial symbionts. Therefore, these organisms need to regulate the bacteria they encounter and resist microbial pathogens. One method is by chemical defense. Antimicrobial assays performed with extracts of 23 Red Sea corals and sponges against bacteria isolated from their natural environment revealed considerable variability in antimicrobial activity. Soft corals exhibited appreciable activity, sponges showed variability, and stony corals had little or no activity. Among the soft corals, *Xenia macrospiculata* exhibited the highest activity. Bioassay-directed fractionation of the extract indicated that the activity was due to a range of compounds, one of which was isolated and identified as the diterpene desoxyhavannahine. Among the sponges, *Amphimedon chloros* exhibited strong activity. Bioassay-directed fractionation resulted in the isolation of the pyridinium alkaloid antibiotics, the halitoxins and amphitoxins. These compounds showed selective activity against specific bacteria, rather than being broad-spectrum. They were highly active against seawater bacteria, whereas bacteria associated with the sponge were resistant. This selective toxicity may be important in enabling certain bacteria to live in close association with their sponge host while it maintains a chemical defense against microbial pathogenesis. The halitoxin-resistant bacteria were identified by 16S rRNA gene analysis as *Alphaproteobacteria*, closely related to other *Alphaproteobacteria* isolated from various marine sponges. The study of microbial communities associated with sponges and corals has important implications for the production of symbiont-derived bioactive compounds and for the use of corals and sponges as source material for microbial diversity in screening programs for natural products.

Keywords: marine natural products; antimicrobial activity; sponges; corals; Red Sea; terpenes; alkaloids; halitoxins.

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CORAL/SPONGE–MICROBE INTERACTIONS AND ANTIMICROBIAL DEFENSE

Bacteria and other microorganisms are ubiquitous in the marine environment. They are taxonomically diverse, biologically active, and colonize all marine habitats, from the deep oceans to the shallowest estuaries [1], as well as coral reefs [2]. Living benthic marine organisms such as corals and sponges are frequently colonized by bacteria [2–4]. Moreover, the surface of living corals is covered by mucus [5]. This mucus layer is colonized by bacteria, allowing for the establishment of a bacterial community that can be characteristic of a particular coral species [3,4,6–8]. Some of these bacteria can be pathogenic and may initiate diseases such as black band disease [9], white plague type II [10,11], and tissue necrosis [12,13]. On the other hand, bacteria could serve as beneficial symbionts or as benign associates. For example, Gil-Turnes et al. [14] showed that bacteria on the surface of externally held eggs of the shrimp *Palaeman macrodactylus* produce a metabolite that inhibits fungal infections that are lethal to the eggs. Since microorganisms are ubiquitous in the marine environment and especially on the surface of benthic invertebrates such as corals and sponges, these organisms need the ability to regulate the bacteria they encounter and to resist microbial colonization and the invasion of potential pathogens, in order to prevent possible detrimental effects. One method of combating microbial attack is by chemical defense.

Corals are able to deter unwanted bacteria by several means, such as the self-cleaning of mucus from their surface [6]. Another potential method is the maintenance of antimicrobial chemical defenses targeted at pathogens or other potentially deleterious microorganisms.

Sponges and corals offer a rich source of unique and diverse natural products (see [15] for a recent review). Many of these compounds have potent pharmacological activities, including anti-tumor, -fungal, -viral, and -bacterial properties, some of which are currently in preclinical or clinical trials [16]. However, natural products were also found to play important biological and ecological roles for the producing organisms such as defense against predators, competition for space, prevention of fouling, roles in reproduction, and antimicrobial activity (see reviews [17–21]).

Antimicrobial activity has been extensively reported for extracts of various groups of marine organisms, such as sponges [22–29], bryozoans [30], ascidians [31], scleractinian corals [32], scleractinian coral eggs [33], gorgonian octocorals [34–36], and alcyonacean soft corals [37–39].

Several antibiotics have been isolated, such as plakortin [40] and manoalide [41] from marine sponges, and sinulariolide and flexibilide [42] from alcyonacean soft corals. Many of the reports on antimicrobial activity of extracts of marine organisms and the subsequent purified antibiotics isolated from these organisms that were tested against human pathogens as potential novel clinically useful drugs or tested against marine bacteria reveal no obvious ecological relevance to the producing organism. Several recent reports on antimicrobial activity of sponges and corals have examined the activity of secondary metabolites against ecologically relevant bacteria in order to elucidate their function in the chemical mediation of interactions between marine sessile invertebrates and bacteria [27–29,31,36–39,43]. Activity was tested and found mainly in marine sponges and gorgonian octocorals. Little is known, however, on the antimicrobial activity of other corals, especially reef-building scleractinian corals and other soft corals. This is somewhat surprising, considering that the latter organisms are the most dominant and conspicuous members of many reefs.

ANTIMICROBIAL ACTIVITY OF RED SEA CORALS

Scleractinian corals and alcyonacean soft corals are the two most dominant groups of benthic marine organisms inhabiting the coral reefs of the Gulf of Eilat, Red Sea [44]. Therefore, a comparison of the antimicrobial activity of extracts of several of the most dominant stony and soft coral species from the coral reef of Eilat (northern Red Sea) was performed against bacteria isolated from the environment of the corals.

Antimicrobial assays were done with extracts of six dominant Red Sea stony corals and six dominant soft corals against bacterial strains isolated from other corals and from the seawater surrounding the corals. The data revealed considerable variability in antimicrobial activity (Tables 1 and 2). Five out of six (83 %) of the soft coral species inhibited at least 50 % of the test bacteria, while none of the six stony coral species inhibited at least 50 % of the test bacteria. The striking difference in antimicrobial activity between stony and soft corals leads us to conclude that these taxonomically different groups of corals may have developed different means to combat co-occurring microorganisms. While alcyonacean soft corals use chemical defense through the production of antibiotic compounds to combat microbial attack, stony corals seem to rely on other means.

Table 1 Antimicrobial activities of extracts of Red Sea stony corals against various Red Sea marine bacteria. Size of inhibition zones is expressed as: + 0–2 mm; + + 2–4 mm; + + + 4–6 mm; + + + + 6–8 mm; + + + + + 8–12 mm; - No inhibition.

	Test microorganisms										
	Seawater strains			Coral isolates							
Coral species	1	2	3	1	2	3	4	5	6	7	8
Stylophora pistillata	–	+	–	–	–	–	–	–	–	–	–
Favia favus	–	+	–	–	+	–	–	–	–	++	–
Pocillopora damicornis	–	–	–	–	–	–	–	–	–	–	–
Fungia fungites	–	–	+	–	–	–	–	–	–	–	–
Fungia scutaria	–	+	–	–	–	–	–	–	–	–	–
Acropora variabillis	–	+	+	–	–	–	–	–	–	–	–

Table 2 Antimicrobial activities of extracts of Red Sea soft corals against various Red Sea marine bacteria. Size of inhibition zones is expressed as: + 0–2 mm; + + 2–4 mm; + + + 4–6 mm; + + + + 6–8 mm; + + + + + 8–12 mm; – No inhibition.

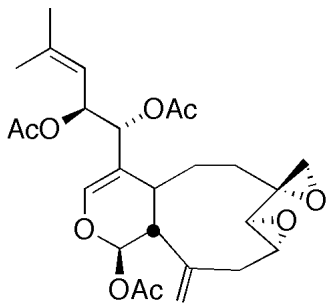
	Test microorganisms										
	Seawater strains			Coral isolates							
Coral species	1	2	3	1	2	3	4	5	6	7	8
Litophyton arboreum	+	++	++	++	+++	+++	+	++++	++++	+++	+
Rythisma f. fulvum[a]	++	+++	++	+++	+++	++	++	+++	+++++	+++	++
Heteroxenia fuscesence	+	+	–	++	++	+	–	+++	++++	++	+
Sarcophyton glaucum	–	++	+	+	+	+	+	++	+++	+	+
Dendronephtia hemprichi	–	++	–	+	–	–	–	+	++	–	–
Xenia macrospiculata	+++	++++	++	+++++	+++++	+++++	++	+++++	+++++	+++++	+++++

[a]Previously known as *Parerythropodium f. fulvum* [65].

The lack of antimicrobial chemical defense in Red Sea stony corals could result from the fact that stony corals may use nonchemical defenses such as mucus production and sloughing against microorganisms [6]. On the other hand, it is also possible that stony corals may produce or release antimicrobial compounds only following induction by certain deleterious microorganisms or upon mechanical stress, as would occur if a coral was bitten by a predator. Geffen and Rosenberg [45] showed that the coral *Pocillopora damicornis* rapidly releases antibacterials following a mechanical stress. Furthermore, in our antimicrobial assays we tested the activity of the coral organic extracts. Nevertheless, water-soluble metabolites of stony corals may possess antimicrobial activity, as was shown by Geffen and Rosenberg [45].

From the active soft coral species examined, *Xenia macrospiculata* exhibited the highest antimicrobial activity (Table 2). Bioassay-directed fractionation of the crude extract of *X. macrospiculata*

indicated that the antimicrobial activity was due to the presence of a range of compounds of different polarities. One of these antibiotic compounds was isolated and identified as desoxyhavannahine (Fig. 1) by NMR spectroscopy (see [39] for details) and comparison with data reported in the literature [46,47]. The estimated volumetric concentration of desoxyhavannahine in tissues of *X. macrospiculata* was ca. 590 μg ml^{-1} of coral tissue (assuming 100 % recovery). This was measured by dividing the yield of the compound by the yield of the crude organic extract, which was measured per volume of tissue. The minimum inhibitory concentration (MIC) of purified desoxyhavannahine was 48 μg ml^{-1} against a marine bacterium, while the MIC of the crude extract of *X. macrospiculata* was 25 μg ml^{-1}. The observed high potency of *X. macrospiculata* (Table 2) led us to choose this coral for further purification. However the MIC of its antibiotic compound desoxyhavannahine was approximately 10-fold lower than its estimated natural concentration. On the other hand, the MIC of the crude extract of *X. macrospiculata* was lower than its antibiotic compound. This may suggest that the extract of this coral contains additional antimicrobial compounds. This conclusion confirms what was also apparent during the fractionation process. However, due to the low concentration of these compounds, they were difficult to purify. Further work is required in order to determine the nature of these compounds, and to show whether these metabolites act in an additive or a synergistic fashion toward potentially harmful bacteria.



Desoxyhavannhine

Fig. 1 Desoxyhavannahine, the antimicrobial compound present in the tissues of the Red Sea soft coral *Xenia macrospiculata*.

Certain symbiotic marine bacteria were shown to be responsible for the production of natural products that were previously thought to be derived from their host [48,49]. It is interesting to note that the soft corals that were examined in the current study were all, except *Dendronephtia hemprichi*, active against the test bacteria. *D. hemprichi* differs from the other five soft corals by the lack of a symbiotic relationship with dinoflagellate zooxanthellae. It therefore will be interesting to investigate the role of symbiotic zooxanthellae, as well as associated bacteria, in the production of natural products, especially metabolites that target co-occurring and potentially harmful microorganisms.

ANTIMICROBIAL ACTIVITY OF RED SEA SPONGES

The ecological role of sponge secondary metabolites in antimicrobial defense against co-occurring and potentially pathogenic marine bacteria is unclear. Moreover, their role in regulating symbiotic relationships between bacteria and their sponge hosts is also obscure. We tested the activity of crude organic extracts of 11 dominant Red Sea reef sponges against a panel of bacteria isolated from their natural environment. The results showed considerable variability in antimicrobial activity (Table 3). Eight out of eleven (73 %) of the sponge species inhibited at least one bacterial isolate. Among them, *Amphimedon chloros* (previously known as *A. viridis* [50]) exhibited the highest antimicrobial activity.

Table 3 Antimicrobial activity of extracts of 11 dominant Red Sea reef sponges, applied at natural concentrations, against various Red Sea marine bacteria (for more details on these strains see [29]). Size of inhibition zones is expressed as: + 0–2 mm; + + 2–4 mm; + + + 4–6 mm; + + + + 6–8 mm; – No inhibition; nd not determined.

	Test microorganisms							
	Seawater strains			Coral isolates			Sediment strains	
Sponge	1	2	3	1	2	3	1	2
Amphimedon chloros[a]	+++	++++	+	++++	+++	+++	–	+
Callyspongia siphonella	+	–	–	+	–	–	–	–
Callyspongia sp.	+	nd	–	–	–	–	–	–
Petrosia sp.	–	nd	–	–	–	–	–	–
Epipolasis sp.	++	++	–	+	–	–	–	–
Suberites clavatus	+	–	–	++	–	–	++	–
Theonella swinhoei	+	nd	–	–	–	–	–	–
Grayella cyatophora	–	–	–	–	–	–	–	–
Biemna erhenbergi	+	–	–	–	–	–	–	–
Diacarnus erythraenus	–	++	–	+++	–	–	–	–
Negombata magnifica	–	–	–	–	–	–	–	–

[a]Previously known as *Amphimedon viridis* [50].

Bioassay-directed fractionation of the active butanol partition of the crude extract of *A. chloros* resulted in the isolation of an active fraction containing inseparable mixture of two closely related toxin homologues. These metabolites were identified as a mixture of amphitoxins and halitoxins (Fig. 2) by NMR spectroscopy (see [29] for details) and comparison with data reported in the literature [51–53].

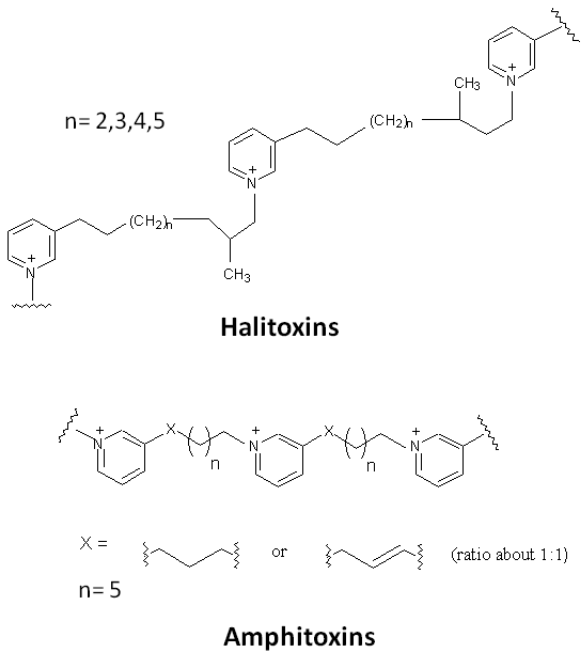


Fig. 2 The halitoxins and amphitoxins, the antimicrobial compounds present in the tissues of the Red Sea sponge *Amphimedon chloros*.

The amphitoxins differ from the halitoxins in having an additional carbon–carbon double bond in the alkyl chain linking the pyridinium rings. The estimated volumetric concentration of the amphitoxins/halitoxins mixture in tissues of *A. chloros* was ca. 6.2 mg ml^{-1} of sponge tissue (assuming 100 % recovery). Again, this was measured by dividing the yield of the metabolites by the yield of the crude organic extract, which was measured per volume of tissue.

The MICs of purified amphitoxins/halitoxins mixture and crude extracts of *A. chloros*, as well as selected commercial antibiotics against *A. chloros*-associated and seawater bacteria are presented in Table 4. Five of the six *A. chloros*-associated bacteria were not inhibited by 250 μg ml^{-1} of purified amphitoxins/halitoxins mixture and crude extracts of *A. chloros*. Strain AV-2 was inhibited at an MIC of 125 μg ml^{-1} of purified amphitoxins/halitoxins mixture and 63 μg ml^{-1} of crude extracts of *A. chloros*. In contrast, purified amphitoxins/halitoxins mixture and crude extracts of *A. chloros* inhibited the growth of eight out of eight seawater bacteria in MICs ranging from 4 to 250 μg ml^{-1}. The eight seawater bacteria consisted of equal numbers of Gram-negative and -positive strains. The differences in MICs of the amphitoxins/halitoxins mixture against *A. chloros*-associated bacteria and seawater bacteria were significant ($p < 0.05$). The selected commercial antibiotics exhibited variable MICs against *A. chloros*-associated and seawater bacteria (Table 4).

Table 4 Minimum inhibitory concentrations (μg ml^{-1}) of amphitoxins/halitoxins mixture, crude extract of *Amphimedon chloros* and selected commercial antibiotics against growth of bacterial isolates from *A. chloros* and surrounding seawater. Poly–B = polymixin B, Pen–G = penicillin–G, Chl = chloramphenicol, Nal = nalidixic acid, Strep = streptomycin, Tet = tetracycline, nd = not determined.

Source	Isolate	Crude *A. chloros*	Amphitoxins/ halitoxins	Poly–B	Pen–G	Chl	Nal	Strep	Tet
A. chloros	AV–1	>250	>250	>250	1	2	16	31	2
	AV–2	63	125	>250	63	2	16	31	8
	AV–3	>250	>250	>250	1	2	16	63	2
	AV–4	>250	>250	>250	1	1	31	31	2
	AV–5	>250	>250	>250	>250	2	16	31	8
	AV–6	>250	>250	>250	1	2	16	31	4
Seawater	RSW–2	31	31	1	1	4	>125	8	2
	RSW–3	31	31	250	1	8	>125	16	8
	RSW–16	31	31	1	1	31	63	16	8
	RSW–13	31	31	8	1	4	>125	16	63
	RSW–17	125	250	2	1	4	1	16	8
	RSW–18	250	250	4	63	4	16	16	8
	RSW–14	125	8	4	4	8	nd	63	nd
	RSW–1	31	4	125	8	31	nd	63	nd

Previous studies have shown that the halitoxins exhibit general cytotoxicity and were toxic to mice and sea urchin eggs, as well as possessing hemolytic and neurotoxic activity [53]. The amphitoxins were shown to deter the feeding of a generalist predatory fish in laboratory experiments [52]. However, our study with the halitoxins and amphitoxins from a Red Sea sponge showed selective activity to specific bacteria rather than being broad-spectrum. The amphitoxins/halitoxins mixture isolated from *A. chloros* was highly active against eight strains of bacteria isolated from the seawater surrounding these sponges, whereas six strains associated with the sponge were resistant to these compounds. This selective toxicity may be important in enabling certain bacteria to live in close association with their sponge host while it maintains a chemical defense against microbial pathogenesis.

Investigation of antimicrobial effects of secondary metabolites with disc-diffusion assays using agar media is limited, due to the variable diffusion rates of compounds in agar [54]. The level of activ-

ity that is measured in the disc diffusion assay is dependent on both the rate of diffusion of the extract into the agar and the potency of the extract. Extracts that contain highly active compounds (i.e., more potent), but have physical properties that generate a lower diffusion rate, may appear to have low activity in the assay. This is particularly significant when testing cationic high-molecular-weight antibiotics, such as the halitoxins and amphitoxins. In our study, this problem was overcome by performing MIC assays in liquid media. Moreover, the simulation of natural concentrations on a volumetric basis, as described by Kim [35], assumes that the extracts are equally distributed throughout the volume of the organism being tested. In corals, algae, and some sponges, it is reasonable to assume that higher concentrations of bioactive compounds accumulate on their surfaces, thus providing greater defense against bacterial colonization. However, since sponges pass large volumes of water through their tissues [55], which contain potentially harmful bacteria, as well as encountering them on their external surfaces, it may be advantageous for sponges to distribute their antimicrobials throughout their tissues. Several studies provided evidence for the localization of natural products within the tissues of sponges (e.g., [56,57]). Further investigations on the localization of antibiotics in tissues of marine sponges are therefore warranted. In our antimicrobial studies we used MIC assays performed in liquid media, which ensures a uniform concentration of antibiotic and increases interaction between the antimicrobial agents and the tested microorganisms. In our view, such assay conditions better simulate the conditions experienced by microbes in nature.

It is interesting to note that, as a group, the six strains of *A. chloros*-associated bacteria were resistant to the amphitoxins/halitoxins mixture, as well as to polymixin B (Table 4). The latter is a bactericidal cyclic peptide antibiotic, which acts by binding to the bacterial cytoplasmic membrane. The positively charged peptide ring is thought to bind electrostatically with the anionic phosphate head groups of the membrane, thus affecting the normal organization of the membrane and altering its permeability characteristics [58]. The fact that the amphitoxins and halitoxins are also positively charged and all isolated *A. chloros*-associated bacteria were resistant to them as well as to polymixin B, leads us to suggest that the amphitoxins and halitoxins mimic the mode of action of this class of antibiotics. Furthermore, it leads us to hypothesize that these resistant bacteria probably have special membrane properties that interfere with the antibiotic action. It will be interesting to test the charge on the surface of these microbes and investigate the ability of positively charged antibiotics to bind to these membranes. Halitoxin preparations from the Papua New Guinea sponge *Callyspongia ridleyi* were shown to cause irreversible membrane potential depolarization, collapse in membrane potential, reduction in input resistance, increased Ca^{2+} permeability, and pore-forming action [59,60]. Further studies are required to investigate the mode of resistance of *A. chloros*-associated bacteria to these highly potent antibiotics.

The halitoxin-resistant bacteria associated with the Red Sea sponge *A. chloros* were found to be members of the *Alphaproteobacteria* by 16S rRNA gene analysis [61]. They were found to be closely related to other *Alphaproteobacteria* isolated from various marine sponges. An *Alphaproteobacterium* closely related to the halitoxin-resistant strain AV-1 was isolated previously from the Great Barrier Reef sponge *Rhopaloeides odorabile* [62]. Another *Alphaproteobacterium* closely related to the halitoxin-resistant strain AV-1 was isolated from the Mediterranean sponge *Aplysina aerophoba* [63]. These *Alphaproteobacteria* were recently shown to be widely distributed in sponges and to be vertically transmitted in sponge larvae in one case, indicating a role as important sponge symbiont [64]. The findings that different species of sponges from different oceans harbor closely related *Alphaproteobacteria* and that these *Alphaproteobacteria* may be vertically transmitted suggest that *Alphaproteobacteria* closely related to the halitoxin-resistant strain AV-1 may be important members of the sponge-microbial community. The study of microbial communities associated with sponges and corals has important implications for the production of symbiont-derived bioactive compounds and for the use of corals and sponges as source material for microbial diversity in screening programs for natural products.

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