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Evaluation of the ecological function of amphitoxin in the reef-dwelling sponge *Callyspongia (Euplacella) biru* (Haplosclerida: Callyspongiidae) at southwest Sulawesi, Indonesia

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Abstract

The ecological function of the secondary metabolite amphitoxin produced by *Callyspongia (Euplacella) biru* is evaluated by a forced confrontation with the free-living scleractinian coral *Fungia fungites* at different exposure times. Our major goal was to determine whether such a forced confrontation with a spatial competitor would cause a significant change in the concentration of amphitoxin in the sponge tissue, indicating a regulatory mechanism of amphitoxin production. Firstly, the concentrations of amphitoxin of sponge fragments submitted to forced confrontation with a mushroom coral did not differ between the exposure times. Secondly, all sponge fragments, including the controls had a consistently lower amphitoxin concentration during the course of the experiment than the natural variation. Thirdly, the concentration of amphitoxin varied substantially between specimens implying that other ecological factors besides spatial competition regulate the production of bioactive compounds of *C. biru.*

Contents

Introduction

In recent decades, marine invertebrates have attracted increasing interest as sources of bioactive metabolites from pharmaceutical companies and research institutions. Although more than 14,000 distinct natural products have been discovered from marine invertebrates, only few marine-derived compounds have passed pre- and clinical trials (Proksch *et al.,* 2002; Mendola, 2003). In most cases the compound-producing marine organisms were haphazardly collected, without an understanding of the ecological function of these compounds. Recently, the question has arisen whether the function of these compounds could be deduced from field observations in order to collect only specimens of species that are expected to produce metabolites of pharmaceutical importance (Uriz *et al.,* 1992; Proksch *et al.,* 2002). Field observations can also be used to evaluate the biological aspects of the production of bioactive compounds for the feasibility of eventual mass production (Duckworth and Battershill, 2003). For instance, the absence of fouling organisms on soft-bodied invertebrates might indicate that the bioactive compounds in these invertebrates probably have potential anti-bacterial and/or fouling properties (Amade *et al.,* 1987; Kelman *et al.,* 2001). In addition to antifouling properties, the bioactive compounds may also act to deter predators, as shields for UV-protection, and as means for substrate acquisition (Aerts and van Soest, 1997; Assmann *et al.,* 2000; de Voogd *et al.,* 2004). Space is a highly restricted resource for sessile organisms when substrate availability is limited, such as in coral reefs (Dayton, 1971; Beccero *et al.,* 2003). Extensive studies have assessed competitive mechanisms employed by scleractinian corals (Lang, 1973; Lang and Chornesky, 1990), corallimorpharians (Chadwick 1991; Chadwick and Adams, 1991), actiniarians (Bak and Borsboom, 1984), alcyonaceans (Benayahu and

Loya, 1981), zoanthids (Suchanek and Green, 1982), bryozoans (Buss, 1979; Turner and Todd, 1994), and algae (Dai, 1991). Some of these mechanisms include: sweeper tentacles and mesenterial filaments (Bak *et al.,* 1982; van Veghel *et al.,* 1996), overgrowing and smothering (Cope 1982), and exudation of toxic substances (Rinkevich and Loya, 1983; Sammarco *et al.,* 1983). Very few of these studies include sponges (Jackson and Buss, 1979; Buss, 1990). Sponges are a particularly rich source of secondary metabolites with anti-mitotic, cytotoxic, antibacterial, antifungal and/or antiviral properties (Faulkner, 1984; Munro *et al.,* 1999). The ability of sponges to gain and hold space depends mainly on their competitive ability (Aerts and van Soest, 1997). Spatial competition can be visibly determined as overgrowth, necrosis, and bleaching of the interacting neighbour (Aerts, 2000). A few studies have suggested that waterborne biologically active metabolites may deter particular substrate competitors from growing in direct contact with sponges (Turon *et al.,* 1996; Nishiyama and Bakus, 1999), but these chemicals have rarely been isolated or identified (Thompson *et al.,* 1985; Thacker *et al.,* 1998). Aerts and van Soest (1997) remarked that some sponges might be more aggressive towards corals in localities with high coral cover. Highly aggressive corals in the Caribbean are relatively minor reef components (Lang, 1973) while in the Indo-Pacific many of the most aggressive corals dominate (Sheppard, 1979; Dai, 1990). Nevertheless, in the Pacific, Rützler and Muzik (1993) reported spatial dominance and aggression in overgrowing corals of the sponge *Terpios hoshinota* in Japan and Guam.

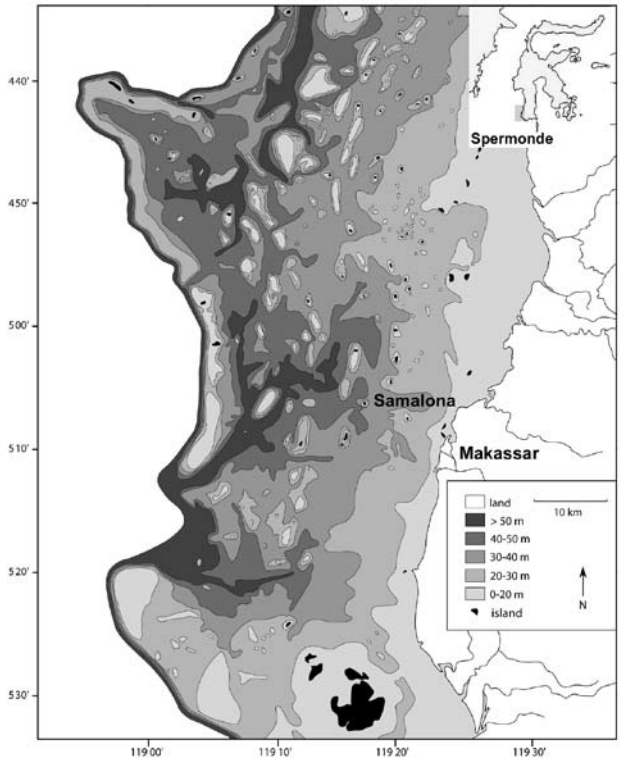

Fig. 1. Bathymetric map of the Spermonde Archipelago. Inset showing the location of the Spermonde Archipelago in relation to the island of Sulawesi.

Sponges of the genus *Callyspongia* (Callyspongiidae: Haplosclerida) are prominent and distinct components of Indonesian shallow-water coral reefs (van Soest 1989, 1990; de Voogd *et al.,* 2004). Approximately 45 species have been described from Indonesia, and many more await description (van Soest, 1990; de Voogd and van Soest, 2002; de Voogd, 2004). Interestingly, a group of secondary metabolites, 3-alkylpiperidine alkaloids, have been recorded in all five families of the Haplosclerida, including Callyspongiidae, and are considered as a chemical marker for the order (Andersen *et al.,* 1996; van Soest and Braekman, 1999). Pharmaceutical properties of these compounds include antifungal (Nicholas and Molinksi, 2000; Albrizio *et al.,* 1995), antimicrobial (Schmitz *et al.,* 1978; Kelman *et al.,* 2001) and anticancer activities (Pettit *et al.,* 1992). Albrizio *et al.* (1995) isolated the 3-alkylpiperidine alkaloid, amphitoxin from the Caribbean *Amphimedon compressa* Duchassaing and Michelotti, 1864 (Niphatidae) and recently this compound was isolated from the Indonesian sponges *Amphimedon paraviridis* Fromont, 1993 and several *Callyspongia* spp., including *Callyspongia (Euplacella) biru* de Voogd, 2004 (unpublished 'Symbiosponge' data).

Ecological studies on sponges producing this compound revealed deterrent properties towards several sponge predators and it was suggested that amphitoxin acts as an anti-feeding agent. (Albrizio *et al.,* 1995; Pawlik *et al.,* 1995; Burns *et al.,* 2003). The selective toxicity of amphitoxin produced by the Red Sea sponge *Amphimedon viridis* Duchassaing and Michelotti, 1864 against different bacterial strains may keep this sponge free from microbial pathogenesis (Kelman *et al.,* 2001). However, de Voogd *et al.* (2004) proposed that amphitoxin might play a role in competition for space with scleractinian corals.

Fig. 2. A fragment of *Callyspongia biru* confronted with *Fungia fungites* at Samalona Island.

In the present study we evaluate the ecological function of amphitoxin produced by *Callyspongia biru* by a forced confrontation with a free-living scleractinian coral. Our major goal was to determine whether such a forced confrontation with a spatial competitor would cause a significant change in the concentration of amphitoxin in the sponge tissue, indicating a regulatory mechanism of amphitoxin production.

Material and methods

Experimental set-up and research site

This study took place at the northwest side of the fringing reef of Samalona island (05º07.326'S 119º20.410'E) in the Spermonde Archipelago, 7 km off Makassar, SW Sulawesi, Indonesia from September to November 2002 (Fig. 1).

The sponge *Callyspongia (Euplacella) biru* de Voogd, 2004 (Callyspongiidae; Haplosclerida) was selected because its bioactivity and secondary metabolites are known. (Albrizio *et al.,* 1995: unpublished 'Symbiosponge' data www.science.uva.nl/ZMA/Invertebrates/Symbiosponge). Three types of experiments were performed, prior to the current experiment, to evaluate what environmental parameters might induce a change in bioactivity of the sponge crude extract. Sponge fragments were confronted with corals, injured repeatedly to simulate predator bites, and transplanted from deep to shallow water and vice versa to evaluate the impact of UV-radiation. A change in bioactivity was detected with the *Artemia*-toxicity test (for a description of the Artemia-toxicity test see de Voogd, 2005). This test is an effective assay method to detect cytotoxic activity of secondary metabolites from terrestrial and marine organisms (Meyer *et al.,* 1982; Richelle-Maurer *et al.,* 2003), it has not been used to detect changes in bioactivity so far. Nevertheless, a change in bioactivity (mortality of *Artemia* nauplii ≥ 50% exposed to the crude extract of *C. biru* varying between 1 mg/L and 10 mg/L) was revealed in the experimental confrontation of the sponge with a coral (Bavinck, 2001; Becking, 2002); thus this experimental set-up was chosen for quantification of amphitoxin. Twenty branch fragments of approximately 12 cm length of living sponge were collected at a depth of 12 m and attached to a frame with electric cable wire with a protective plastic sheath at the same depth. Frames were made of PVC tubes of a rectangular form (70 × 100 cm) on which nylon fishing net had been tightened. The free-living coral *Fungia fungites* Linnaeus, 1758 (Scleractinia: Fungiidae; see Hoeksema, 1989) is known to be an aggressive competitor for space (Hildemann *et al.* 1975; Thomason and Brown, 1986). After the sponges were allowed to acclimatize

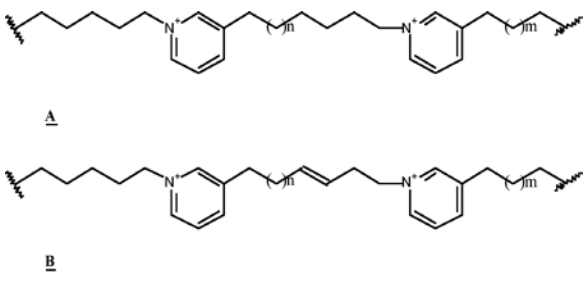

Fig. 3. Chemical structure of isolated amphitoxin A and amphitoxin B.

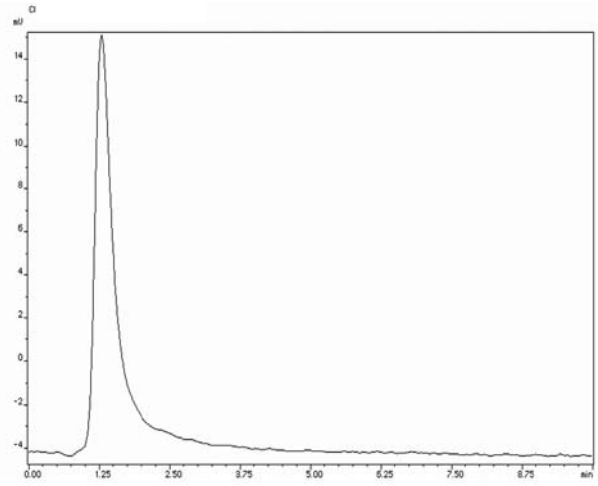

Fig. 4. Callyspongia biru. HPLC chromatogram of MeOH extract. The peak corresponds to Amphitoxin A + B.

for a period of four weeks, 16 individuals of the coral were collected and placed on the frames at a distance of 0.5-1 cm to an equal number of sponge fragments. In a similar manner to the sponges, the corals were tied to the frame with cable wire to keep the neighbouring coral and sponge in place (Fig. 2). Four sponge fragments were used as controls, and these were not confronted with the coral. Two of these fragments were collected at the beginning of the experiment and the remaining two fragments were left on the frame without coral confrontation, until their collection at the end of the experiment. Of the 16 sponge fragments confronted with the corals, two fragments were collected at eight different time intervals; T1 = ½ hour, T2 = 1 hour, T3 = 4 hours, T4 = 24 hours, and T5 = 72 hours, T6 = 8 days, T7 = 19 days and T8 = 28 days. For the natural variation, three sponge fragments of approximately 12 cm in length were collected from different depths at different locations at the reef surrounding the island of Samalona.

Extraction and isolation

The sponge fragments were measured (length in cm; growth in thickness was not observed) and extracted in ± 50 ml pure methanol for 24 hours at room temperature immediately upon collection. Preliminary investigation with this sponge showed that the methanolic extract was found to be active and contained the 3-alkylpiperidine alkaloids. We therefore only used pure methanol for extraction. The sponges were cut into smaller pieces and were washed twice in succession with the solvent to ensure an exhaustive extraction. All the filtrates were combined, and placed in a cooled evaporator. The crude extract was collected and weighed after approximately 24 hours of evaporation and stored at 7°C. Detection of the 3-alkylpiperidine alkaloids was determined using Thin Layer Chromatography.

Thin Layer Chromatography

Although 3-alkylpiperidine alkaloids have previously been isolated from *C. biru*, the presence of these alkaloids was verified for several sponge fragments prior to HPLC-quantification. A capillary tube was held in the methanolic sponge extract and carefully spotted five times onto 0.25 mm Si-gel coated sheets (5 × 2 cm). The plates were eluted with a solvent mixture containing water / acetonitrile (7:3) + 0.1% trifluoroacetic acid. After the completion of the chromatography, the dried plates were sprayed with a solution of Dragendorff reagent. 3-alkylpiperidine alkaloids can be detected as one of the major compounds present in the extract and appears as a bright orange spot.

Quantification of amphitoxin

Analysis of amphitoxin was performed by high-performance liquid chromatography (HPLC) with a

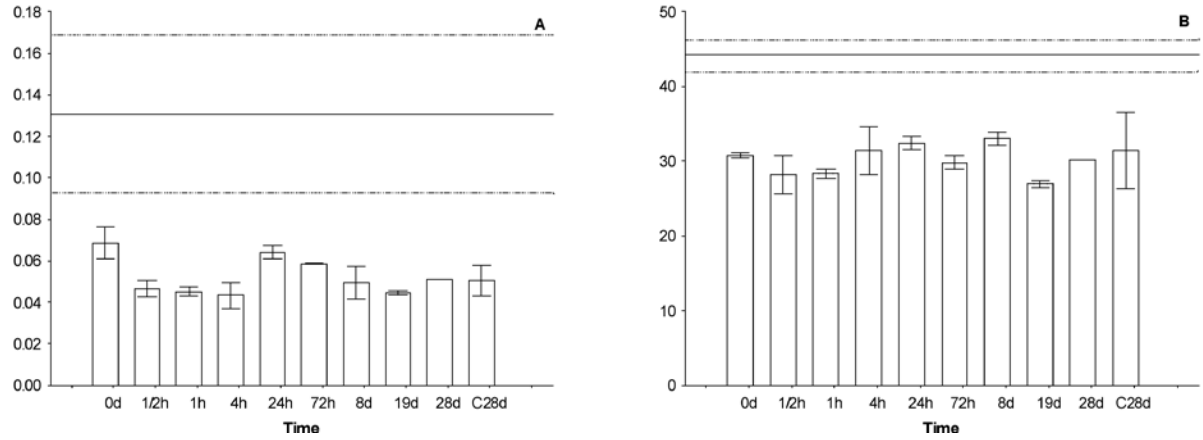

Fig. 5.(A) Mean % concentration of amphitoxin / dry weight of sponge mass (bars and ± 1SE). (*B)* Mean % concentration of crude extract /dry weight of sponge mass over time (in h=hours and d=days) and in control sponges at the start and the end of the experimental period (0d and C28d). Mean natural concentrations are indicated by a line ± (dotted line 1SE).

Waters 600E system controller equipped with a Waters 717 plus autosampler and a Waters 848 Tunable Absorbance Detector. Crude extracts from the sponge fragments were dissolved in a mixture (70:30) of water and acetonitrile + 0.1% trifluoroacetic acid and 10 µl was injected by auto-sampling into an HPLC-system. The column used was a Lichrospher RP18 (125 by 2.0 mm with a 3 µm pore size) and the mobile phase consisted of acetonitrile (70%) and nanopure water (30%) with trifluoroacetic acid (0.1%) at a flow rate of 1 ml/min. Amphitoxin was detected at its UV-maximum of 266 nm. The quantitative analysis was based on peak area calibration using earlier purified amphitoxin (Fig. 3). We compared our data with spectral data reported in the literature (Albrizio *et al.,* 1995). To calculate the percent yield of amphitoxin, we calculated the mass amphitoxin divided by the mass of dried sponge, multiplied by 100. The percentage yield of the total crude extract was calculated as the mass of the extract divided by the mass of dried sponge, multiplied by 100.

Results

The concentrations of amphitoxin / dry weight of sponge fragments submitted to forced confrontation with a coral did not differ between the time intervals. All sponge fragments, including the controls (0d and C28d), attached to the PVC-frame had a consistently lower mean amphitoxin concentration per dry weight than the natural variation (Fig. 5A). The percentage amphitoxin varied from 0.04-0.07% of the dry weight of the fragments, whereas the percentage for the natural variation varied from 0.06-0.20% of the dry weight. Also, the mean percentage crude extracts / dry weights were consistently lower than that of the natural variation. All sponge fragments, including the natural variation, exhibited a large interindividual variability. The mean crude extract per dry weight of the natural variation varied between 42-48%, whereas this was only 26-37% for the fragments of the experimental set-up (Fig. 5B). The sponge fragments were measured after transplantation and after collection, and all sponge fragments showed at least 10% growth in a period of at least four weeks. White (bleached) spots in the area adjacent to the sponges were observed in some of the individuals of *Fungia fungites* after several days.

Discussion

In the present study we evaluated the ecological function of the major secondary metabolite, amphitoxin, produced by *Callyspongia (Euplacella) biru* by confronting this species with the space competitor *Fungia fungites.* Importantly, all sponge specimens analysed with HPLC in this study were found to contain amphitoxin. Amphitoxin is known to be

mainly responsible for the bioactivity of the crude extract of the Indonesian sponge *C. biru* (Dubut, 2000). The mean percentage amphitoxin concentration per dry weight was consistently lower for all sponge fragments, including the control fragments, during the course of the experiment than the amphitoxin concentration of the natural variation. In a similar experiment to ours, the Caribbean sponge *Agelas conifera* Schmidt, 1870 was experimentally confronted with the scleractinian coral *Madracis* sp., and no significant change in bioactive compound concentration was observed. In the same study, inflicted sponge damage induced a fourfold rise in concentration, and yielded detectable amounts of the *Agelas* alkaloids in the surrounding seawater, suggesting allelopathic activity (Richelle-Maurer *et al.,* 2003). Although a detection analysis of the chemicals in the surrounding seawater was not performed in the present study, the lower concentrations of amphitoxin in the experimental sponge fragments might imply allelopathic activity. Several studies (Porter and Targett, 1988; Nishiyma and Bakus, 1999; Engel and Pawlik, 2000) suggested that water borne allelochemicals might deter substrate competitors from growing in direct contact with sponges. Sullivan *et al.* (1983) isolated secondary metabolites excreted within mucus from an excavating sponge, which inhibits coral growth. Turon *et al.* (1996) suggested that the sponge *Crambe crambe* Schmidt, 1862 releases waterborne chemicals and may cause tissue necrosis to adjacent organisms at small scales.

Certain tolerant coral species, however, may grow adjacent to sponges, whereas others are rarely or never found in close proximity. Nishiyama and Bakus (1999) demonstrated that three sponges released allelochemicals into the surrounding seawater, and were toxic to several scleractinian coral species. Although they did not quantify the secondary metabolites in the sponge tissue, these results suggest the release of compounds into the seawater. It is possible that the release of allelochemicals into the surrounding seawater of the confronted sponges in the present study might trigger non-confronted proximate individuals to release their toxins as well, and might explain the lower concentrations of amphitoxin in the control sponges. Besides, it is known that certain green algae rapidly transform stored non-active compounds into very reactive toxins upon mechanical wounding (Paul and Van Alstyne, 1992; Jung and Pohnert, 2001). However, it is not known in sponges, whether such enzymatic transformations take place upon wounding or during other stress events. Notwithstanding, the sponges exhibited a large interindividual variation, indicating a regulatory mechanism for the production of amphitoxin. Thacker *et al.* (1998) found no evidence that the production of secondary metabolites was induced by the presence of a spatial competitor, rather they suggested that the constant threat of predators may maintain high concentrations of the compound. They also observed a large variation in the production of secondary metabolites, which according to them might be due to differences in nutrient availability or genetic differences. Thus an increase or decline in the production of secondary metabolites may not be so clearly attributed to the presence of spatial competitors or predators. On the other hand, the ecological role and strength of pure metabolites, in this case amphitoxin, may be quite different from the total crude extract. From unpublished records, it was hypothesized that amphitoxin was responsible for the general toxicity of this particular species (*Artemia*-toxicity-test). Besides, the mean percentage crude extract per dry weight was consistently lower from the manipulated sponge fragments than those of the natural variation. However, it has been suggested that different metabolites present in a species may interact and produce different bioactivities when tested together or separately. Although no other major metabolites have been isolated from this sponge species, other chemical products, even salts, may have a synergetic effect on its bioactivity (Marti *et al.,* 2003).

The optimal defense theory assumes that the production of defensive secondary metabolites is costly and consequently would repress growth and reproduction (Schupp *et al.,* 1999). In the present study, all sponge fragments did grow at least 10% during the experimental period, and upon collection, larvae were observed in the tissue of several sponge fragments. Richelle-Maurer *et al.* (2003) suggested that there is always a minimal production of bioactive compounds possibly at the boundary of efficiency. Thus, it is probable, that wounded individuals invest their limited available energy in healing and growth rather than in the production of secondary metabolites. However, some of the individuals of *F. fungites* were observed with white spots during the course of the experiment confronted with *C. biru* suggesting

exudation of secondary metabolites and competitive dominance of the sponge, although other enviromental stressors may have induced bleaching of the corals (cf. Hoeksema, 1991). Scleractinian corals are in general sedentary animals, however most mushroom corals (Fungiidae) have a free-living phase in their life cycle (Hoeksema, 1989). Mobility may help mushroom corals to disperse and reach favourable habitats, to prevent burial in soft sediments or to function in avoiding competition for space (Hoeksema, 1988, 1993; Chadwick, 1988; Chadwick-Furman and Loya, 1992). Fungiid corals, however, have been shown to competitively dominate non-fungiid scleractinians during interactions in physical contact experiments (Chadwick-Forman and Loya, 1992). The thick mucus secreted by most unattached mushroom corals may have a protective function in aggressive reactions during accidental contact with other sedentary organisms (Hildemann *et al.,* 1975; Chadwick, 1988). Competitive interactions with other sessile invertebrates though, are only known with octocorals, where the interacting organisms appear not to harm each other (Chadwick-Forman and Loya, 1992). Also, observations of natural interactions of *C. biru* with scleractinian corals demonstrated necrosis in more than 90% of overgrowth interactions (de Voogd *et al.,* 2004). In conclusion, although a forced confrontation of the sponge *C. biru* with the scleractinian *F. fungites* showed a consistently lower amphitoxin concentration compared to the natural variation, this may not be so straightforward be attributed to a competitive regulatory mechanism induced by the spatial competitor. Rather, the concentration of amphitoxin varies substantially between specimens implying that other ecological factors besides spatial competition interact to regulate the production of bioactive compounds of *C. biru*. More importantly, in scope of eventual mass production, manipulated sponges might produce consistently lower concentrations of the target bioactive compound than sponges in their natural environment.

Acknowledgements

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