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Activated Chemical Defense in Marine Sponges—a Case Study on *Aplysinella rhax*

Carsten Thoms · Peter J. Schupp

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Abstract Activated chemical defense, i.e., the rapid conversion of precursor molecules to defensive compounds following tissue damage, has been well documented for terrestrial and marine plants; but evidence for its presence in sessile marine invertebrates remains scarce. We observed a wound-activated conversion of psammaplin A sulfate to psammaplin A in tissue of the tropical sponge *Aplysinella rhax*. The conversion is rapid (requiring only seconds), the turnover rate increases with increasing wounding activity (e.g., ~20% after tissue stabbing vs. ~85% after tissue grinding), and is likely enzyme-catalyzed (no reaction in the absence of water and inhibition of the conversion by heat). Fish feeding assays with the pufferfish *Canthigaster solandri*, an omnivorous sponge predator, revealed an increased antifeeding activity by the conversion product psammaplin A compared to the precursor psammaplin A sulfate. We propose that the wound-activated formation of psammaplin A in *A. rhax* is an activated defense targeted against predator species that are not efficiently repelled by the sponge's constitutive chemical defense. Recent observations of conversion reactions also in other sponge species indicate that more activated defenses may exist in this phylum. Based on the findings of this study, we address the question whether activated defenses may be more common in sponges—and perhaps also in other sessile marine invertebrates—than hitherto believed.

C. Thoms (✉)
Jena School for Microbial Communication,
Friedrich Schiller University Jena,
Jenergasse 8,
07743 Jena, Germany
e-mail: carsten.thoms@gmail.com

P. J. Schupp (✉)
University of Guam Marine Laboratory, UOG Station,
Mangilao, 96923 GU, USA
e-mail: peter.schupp@gmail.com

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Introduction

Activated defenses have been widely reported in terrestrial plants, yet with inconsistent nomenclature. Common synonyms include short-term inducible defense (STID), dynamic defense, and induced direct defense. All these terms describe chemical defense strategies characterized by wound-activated conversions of inactive or less active precursor metabolites to more active forms with pronounced defensive functions (Paul and Van Alstyne 1992; Wittstock and Gershenzon 2002). This strategy permits chemical protection without the need to maintain high levels of potentially autotoxic compounds. Since a major requirement for an efficient activated defense is the rapid formation of active agents—e.g., in time to repel a predator before fatal damage is done—the conversion reactions involved are usually catalyzed by enzyme(s).

In the terrestrial environment, examples of activated defenses include the conversion of cyanogenic glycosides to HCN (see reviews by Wajant and Effenberger 1996; Zagrobelny et al. 2004), the cleavage of glucosinolates in Brassicaceae and related plants to thiocyanates, isothiocyanate, or isonitriles (Stoewsand 1995; Fahey et al. 2001), the conversion of phenolic glycosides in aspen *Populus balsamifera* (Clausen et al. 1989) and of sesquiterpenes in the mushroom *Lactarius vellereus* (Sterner et al. 1985).

In the marine environment, research on activated chemical defenses is just beginning. Advances have recently been summarized in general reviews on marine chemical ecology by Paul and Puglisi (2004) and Pohnert

(2004). So far, most activated defenses have been discovered in algae. Reported examples in macroalgae include the conversion of halimedatetraacetate to the anti-feeding compound halimedatrial in *Halimeda* spp. (Paul and Van Alstyne 1992), and the wound-activated deacetylation of caulerpenyne in *Caulerpa* spp. (Jung and Pohnert 2001; Jung et al. 2002). Phytoplanktonic diatoms convert polyunsaturated fatty acids to α,β,γ,δ-unsaturated aldehydes as an activated anti-predator defense that negatively affects the reproductive success of copepods (Miralto et al. 1999; Pohnert 2000, 2002). Remarkably, a cell damage-activated conversion of dimethylsulfoniopropionate (DMSP) to acrylic acid and dimethylsulfide (DMS) was found in both macro- and microalgae (Wolfe et al. 1997). In both cases, an ecological relevance as an anti-predator defense was proposed (Van Alstyne et al. 2001; Van Alstyne and Houser 2003).

Many sessile marine invertebrates share numerous ecological features and challenges with plants (e.g., modular body organization, high regenerative capabilities, habitat restriction to exposed sites, structural defenses with low efficiency). Thus, it is not surprising that both groups have evolved similar strategies, including chemical defense. In sessile marine invertebrates, examples of activated chemical defenses have also been reported. In *Tridentata marginata*, tissue damage results in an enzymatic formation of a series of dithiocarbamates, tridentatols A–D, that intensify the chemical protection of the hydroid against fish predators (Lindquist 2002). In sponges of the genus *Aplysina*, isoxazoline alkaloids are cleaved to aeroplysinin-1 and dienone following wounding (Teeyapant and Proksch 1993; Weiss et al. 1996; Ebel et al. 1997; Thoms et al. 2006). Puyana et al. 2003, however, have questioned early studies on activated defenses in the genus *Aplysina*. To our knowledge, these two reactions are the only examples of activated defenses that have been reported in sessile marine invertebrates to date. In a recent review on chemical defense in sponges, the problems and challenges encountered when addressing activated defenses in sponges and the limits of conclusions that can be drawn on ecological relevance of these reactions have been discussed (Thoms and Schupp 2007).

The sponge *Aplysinella rhax* (order Verongida, family Aplysinellidae) is closely related to the sponges of the genus *Aplysina* (order Verongida, family Aplysinidae). The dominant secondary metabolites in *A. rhax* (e.g., psammaplins, aplysinellins, bisaprasin) as well as the isoxazoline alkaloids in *Aplysina* spp., are derived from bromotyrosine, and share pronounced structural similarities (Pham et al. 2000; Shin et al. 2000). Psammaplins, in particular psammaplin A, have gained pharmaceutical interest due to their cytotoxic properties (Kim et al. 1999; Jiang et al. 2004; Newman and Cragg 2004). In past studies on *A. rhax*, psammaplin A was described as one of the dominant compounds in the organic extract of the sponge (Pham et al. 2000; Tabudravu et al. 2002).

In contrast, our experiments have revealed a dominance of psammaplin A sulfate in intact tissue of *A. rhax*, whereas psammaplin A is present in low quantities, only. However, damage to tissue of *A. rhax* results in a pronounced shift in the chemical profile with psammaplin A increasing considerably. In a series of wounding experiments and feeding assays with generalist reef fish and an omnivorous sponge predator, we investigated a possible ecological role of this reaction as an activated chemical defense. For the experimental design and the interpretation of data, we used the following criteria defined by Paul and Van Alstyne (1992) for activated defenses in macroalgae: (1) a less potent stored secondary metabolite is converted into a more potent one. (2) The process is rapid, requiring only seconds to a few minutes. (3) The conversion is catalyzed by one or few enzymes.

Based on our results and other recent observations of potentially enzyme-catalyzed conversions of secondary metabolites in sponges (e.g., a deacetylation of sesterterpenes in the sponge *Luffariella variabilis* (Ettinger-Epstein et al. 2007) and changes in secondary metabolite profiles following wounding in *Suberea* sp. (Schupp et al., unpublished data)), we question whether, in the past, activated defenses may have been overlooked in the phylum Porifera and possibly also in other sessile invertebrate phyla.

Methods and Materials

Sponge Collection A. rhax De Laubenfels, order Verongida, is commonly found in the West–Central Pacific at depths between 5–30 m. Around Guam, the sponge is most abundant on coral patch reefs in Apra Harbor. We collected *A. rhax* at Western Shoals, Apra Harbor, at depths between 15–20 m. The sponges *Hyrtios altum* and *Stylissa massa* were collected in close vicinity. Replicate samples always were taken from sponge colonies at least 5 m apart to minimize collection of clones. We minimized air contact and damage to fresh *A. rhax* by keeping the specimens submerged in seawater during collection and experiments whenever possible and by transporting them in large wide-mouth plastic bottles. While *A. rhax* is usually not overgrown by algae or other invertebrates, we occasionally observed feeding scars on individuals in the field. However, we never observed predators actually feeding on the sponge, and to our knowledge, no predator has yet been reported in the literature.

Preparation of Extracts and Pure Compounds Extractions and isolation of psammaplin A sulfate (**1**) and psammaplin

A (**2**) (Fig. 1) from *A. rhax* were performed according to previously published methods (Pham et al. 2000; Shin et al. 2000). We simplified the protocol by using fresh intact sponge as source material for (**1**) and fresh sponge material that was ground in a mortar for 1 min as source for (**2**). The extraction procedures for both compounds were similar. Sponge tissue was lyophilized, ground to a fine powder, and extracted exhaustively with methanol. The crude extracts were evaporated in vacuo and partitioned between butanol and water. Repetitive partitioning of the water layer with butanol transferred (**1**) and (**2**) entirely into the butanol fraction (both were no longer detectable in the water layer by HPLC). Since large amounts of salt and precipitating sponge pigment were removed by this technique, the butanol fractions appeared better suited for our bioassays than crude extracts. The butanol fraction of extract from intact sponge (in the following referred to as "Ar intact"; Fig. 1) consisted of ~70% wt. of (**1**) and of ~4% wt. of (**2**). The butanol fraction of ground tissue (in the following referred to as "Ar wounded"; see Fig. 1) consisted of ~20% wt. of (**1**) and of ~50%wt. of (**2**).

To obtain pure (**1**) and (**2**), the butanol fractions were subjected to C_{18} reversed-phase vacuum flash chromatography by using gradient mixtures of methanol and water (elution order: 50, 45, 35% methanol). The 50% fraction yielded almost entirely compound (**1**), whereas almost all (**2**) was found in the 35% fraction. Both fractions were further purified by semipreparative and preparative HPLC on a Waters HPLC system coupled to a refraction index detector (column: Alltech Econosil C_{18}, 250×10 mm; eluents (isocratic): 20% aqueous methanol and 100% methanol).

NMR spectra of the purified compounds were recorded in CD_3OD solutions containing Me_4Si as internal standard on a 500-MHz Unity Inova spectrometer. Samples were run in 3-mm tubes at room temperature. Mass spectra were obtained with an Agilent LCMS-ESI-TOF system. Compounds were identified by comparison of our spectral data with literature data (Pham et al. 2000; Shin et al. 2000; Tabudravu et al. 2002).

Wounding Intensity Series To determine correlations between injury to *A. rhax* tissue and changes in its chemical profile, we caused different intensities of wounding to four pieces of identical volume (5 ml) of one sponge individual. Each piece was kept submerged in 15 ml seawater during the experiment. Piece 1 was not further treated; piece 2 was stabbed 60× with a scalpel; piece 3 was ground for 15 s; and piece 4 was ground for 1 min (both pieces, 3 and 4, in a small mortar to keep them submerged in the 15-ml seawater). After a standardized reaction time of 2 min (including treatment), samples were frozen along with the seawater at −80°C. The protocol was repeated with a total of six sponge individuals.

Forceps Experiment To mimic fish bites, we removed small tissue pieces from fresh *A. rhax* with forceps. Two fragments of 50 ml volume were cut from one individual and kept submerged in 150 ml seawater each. Fifty "bite" pieces (10–100 µl volume) were taken randomly from

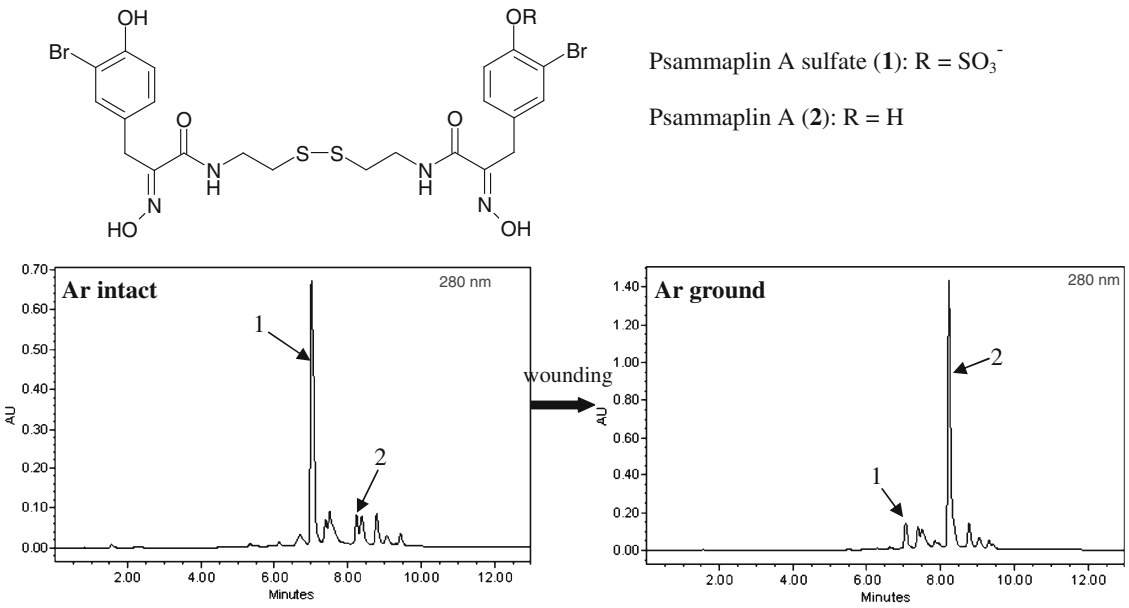

Fig. 1 HPLC chromatograms of the butanol fractions of crude extracts from intact ("Ar intact") and ground ("Ar wounded") *A. rhax* tissue. Tissue wounding resulted in a conversion of psammaplin A sulfate (**1**) to psammaplin A (**2**)

different spots on the sponge surface of one of the pieces and frozen in liquid nitrogen within 2 s. Both 50-ml sponge fragments were frozen at −80°C. The seawater was decanted to remove solid particles and also frozen. The protocol was repeated with a total of four sponge individuals.

Testing for Enzyme-Mediation of the Conversion Reaction Other than grinding of fresh sponge tissue, grinding of lyophilized tissue did not cause any bioconversion, indicating that the conversion enzymes did not function in a water free environment. Two 50-mg subsamples of lyophilized and subsequently ground tissue of *A. rhax* individuals were incubated in 300 µl of distilled water, either at room temperature (25°C) or at 95°C, respectively (2 min incubation time). The suspensions were frozen at −80°C, and the experiment was repeated with tissue powder of three individuals.

Fresh 5-ml pieces of the sponges *A. rhax*, *S. massa*, and *H. altum* were each spiked with 22.8 µmol psammaplin A sulfate dissolved in 75 µl EtOH. Another tissue piece of *A. rhax* was spiked with EtOH only. The tissue pieces were ground vigorously for 4 min and then frozen at −80°C.

Effects of Solvent Exposure In the sponge *Aplysina aerophoba*, reactions similar to those occurring in its activated chemical defense were induced when fresh (wet) tissue contacted organic solvents (Thoms et al. 2006). Preservation of fresh tissue as well as its extraction in organic solvents are common practices in marine chemical ecology. We tested effects of solvent exposure on the metabolite profile in fresh tissue of *A. rhax*.

Five 5-ml pieces of fresh tissue of one *A. rhax* individual were immersed in 10 ml methanol, each. Another piece was frozen immediately at −80°C. In a time series (exposure times 10 min, 30 min, 1 h, 6 h, 24 h), the solvent of each sample was decanted, and the tissue pieces were frozen at −80°C. Solvent samples were dried in a SpeedVac (SPD2010, Thermo Savant). They were re-dissolved in methanol and used to extract the lyophilized and ground sponge pieces as described below. The experiment was repeated with tissue pieces of three sponge individuals.

Chemical Analysis All tissue samples underwent a precisely defined extraction procedure to ensure identical extraction efficiencies. Samples were lyophilized, ground to a fine powder, and homogenized. Fifty milligrams of tissue powder were extracted with 1,500 µl methanol. The samples were vortexed vigorously for 60 s, and after 2 min soaking time, vortexing was repeated for another 30 s. After centrifugation at 13,000 rpm for 2 min, supernatants were analyzed in an HPLC system coupled to a photodiode-array detector (Waters, Milford, Ireland; column: Alltech Rocket Platinum EPS C18, 53×7 mm). Routine detection and quantification of compounds was at 280 nm, but the chromatograms were also inspected with the MaxPlot function of the Waters Empower Pro software that covered wavelengths between 210 and 400 nm.

For quantification, extinction coefficients were determined by repeated analysis of defined amounts of the purified metabolites. Contents in the extracts were calculated based on the detector response at the respective retention times. Seawater samples were lyophilized, redissolved in 50 ml methanol, and an aliquot was analyzed by HPLC as detailed above.

Field Feeding Assay with Generalist Reef Fishes The field assay was conducted based on a method described by Schupp and Paul (1994). In a first set, we tested food treated with psammaplin A sulfate (**1**) against untreated controls. Psammaplin A sulfate (856 mg) dissolved in methanol was added to fish food consisting of 5 g ground catfish pellets, 1.25 g agar, 1.25 g carrageenan, and 80 ml seawater, to match the natural volumetric concentration of (**1**) in *A. rhax* tissue. Controls were prepared by adding the same amount of pure methanol instead. Dissolved extracts and pure compounds always were added to molten food, only after it had cooled down to 60°C in order to avoid compound degeneration due to heat. Mixtures were poured into 1-cm^3 molds containing rubber o-rings. The resulting food cubes were attached with the o-rings and safety pins to ropes. Twenty pairs of ropes, with each rope holding either four treated or four control food cubes, were attached on the reef of Western Shoals, Apra Harbor, Guam. The fish community present was dominated by the species *Abudefduf sexfasciatus*, *A. vaigiensis*, *Amblyglyphidodon curacao*, *Cheilinus fasciatus*, and *Naso vlamingii*. We collected the rope pairs when the fishes had removed half of the cubes. In a second set, we directly compared the repellent activities of psammaplin A sulfate and psammaplin A against generalist reef fish by using the same protocol. In this assay, food treated with natural volumetric concentrations of psammaplin A sulfate was offered together with food treated with an equimolar amount of psammaplin A.

Feeding Assay with a Sponge Predator Feeding experiments were performed with the pufferfish *Canthigaster solandri* based on a laboratory assay described by Schupp et al. (1999). The *A. rhax* extracts "Ar intact" and "Ar wounded" were added in their natural volumetric concentrations to fish food prepared of 2% molten agar and 0.1 g ml^{-1} finely ground catfish food. In a first set, food pieces treated with "Ar intact" and "Ar wounded" were tested separately against controls. Extracts were dissolved in methanol and mixed with the fish food. Control food was prepared similarly, but the same volume of pure methanol was added instead. Treated and control food were poured in

parallel molds backed with fiberglass window screening. When the agar had cooled down, the food was cut into identical strips, each holding a treated and a control food piece (each piece covering 120 squares of the screening). Part of the agar was lyophilized and re-extracted with the same protocol as for extraction of sponge tissue. HPLC comparison with the original extracts revealed no changes in the chemical profile of the extracts after incorporation into the agar.

Food strips were placed in 30-l flow-through tanks and offered to individual pufferfish. Strips were pulled from the tanks, and the number of screening squares where food had been removed was counted when at least 25% of the total food had been eaten. The experiment was terminated after 4 h. In a second set, sponge extracts were compared directly with each other by offering food strips that held two treated food pieces (one with "Ar intact" and the other with "Ar wounded"). Only food strips where no food at all had been removed after 4 h were disregarded in the statistical analysis. A third set of treated food strips was kept in a seawater tank without fish for 4 h. The extract of these strips was compared to extract from strips not exposed to seawater to test for possible diffusion of compounds into the seawater.

Data Analysis Data of the wounding intensity series, the "forceps experiment", and the experiments to test for effects of solvent exposure were analyzed by randomized block analysis of variance. Multiple comparisons were made with the Tukey test (Zar 1999). Levels of significances are given for the treatments. For the field feeding assay, we used Wilcoxon's signed-ranks test for paired comparisons to test for significant differences in the number of cubes eaten. For the feeding assay with the omnivorous sponge predator, we used paired *t*-tests to test for significant differences in the percentage of food squares eaten.

Results

Wounding Intensity Series Mechanical damage to tissue of *A. rhax* resulted in pronounced changes in the concentrations of psammaplin A sulfate (**1**) and psammaplin A (**2**) (Fig. 1). In intact tissue, psammaplin A sulfate (**1**) was present at 52.8±13.4 µmol g^{-1} dry weight (Fig. 2). Upon 1 min grinding, the concentration of (**1**) decreased to 8.2± 3.6 µmol g^{-1}. At the same time, psammaplin A (**2**) concentration increased from 2.1±0.8 to 46.0±8.9 µmol g^{-1} dry weight. Upon gradually increasing the wounding intensity (no wounding<stabbing<grinding for 15 s<grinding for 1 min), (**1**) gradually decreased. This was paralleled by a gradual increase of (**2**) (Fig. 2).

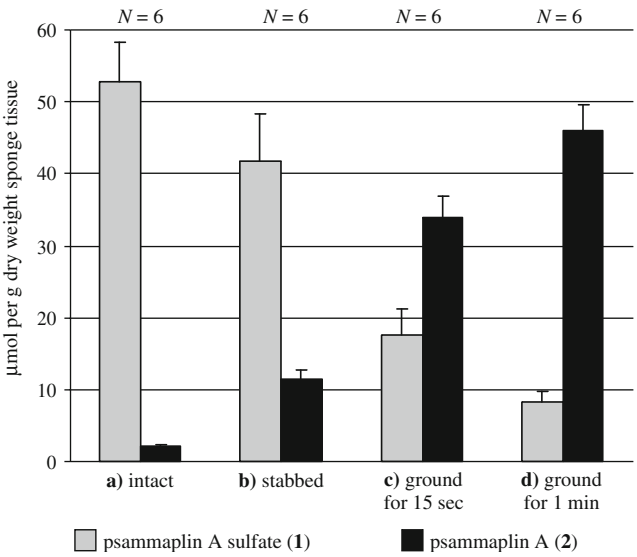

Fig. 2 Changes in psammaplin A sulfate (**1**) and psammaplin A (**2**) concentrations in tissue of *A. rhax* upon different intensities of wounding (overall reaction time was 2 min for all wounding intensities). *Vertical bars* represent SE. Randomized block ANOVA: (**1**) F=36.9, P<0.001; (**2**) F=99.5, P<0.001. Tukey test (significant differences are indicated only): (**1**): $a \neq (c, d)$; $b \neq (c, d)$. (**2**): $a \neq (b, c, d)$; $b \neq (c, d)$; $c \neq d$

Forceps Experiment Tissue pieces that were excised from *A. rhax* with forceps and shock-frozen after 2 s showed a significant decrease in psammaplin A sulfate (**1**) and a concomitant increase in psammaplin A (**2**) when compared to intact sponge tissue (Fig. 3). Similarly, the injured 50-ml sponge fragments (after forceps treatment) showed an increase in psammaplin A concentration, yet to a considerably lesser extent.

Whenever we removed a tissue piece from *A. rhax*, we observed the release of a mucus-like cloud from the wounded sponge area into the ambient seawater. We, therefore, analyzed the seawater and calculated the amounts of (**1**) and (**2**) released per "bite". The amounts, as well as the relative proportions of (**1**) and (**2**) in the seawater came out to be similar to those determined in the removed tissue pieces themselves (Fig. 3).

Testing for Enzyme-Mediation of the Conversion Reaction Following a 2 min incubation of lyophilized and subsequently ground tissue of *A. rhax* in water at room temperature (~25°C), the concentration of psammaplin A sulfate (**1**) decreased from 67.8±2.5 to 1.9±1.6 µmol g^{-1} dry weight sponge tissue. The concentration of psammaplin A (**2**) increased from 2.1±0.4 to 31.1±6.2 µmol g^{-1} dry weight. Upon treatment of another subsample of the same material with hot water (~95°C), instead, the compound concentrations changed to a considerably lesser extent: after a 2-min incubation, psammaplin A sulfate (**1**) amounted for 27.9±7.4 and psammaplin A (**2**) for 4.1±1.1 µmol g^{-1} dry

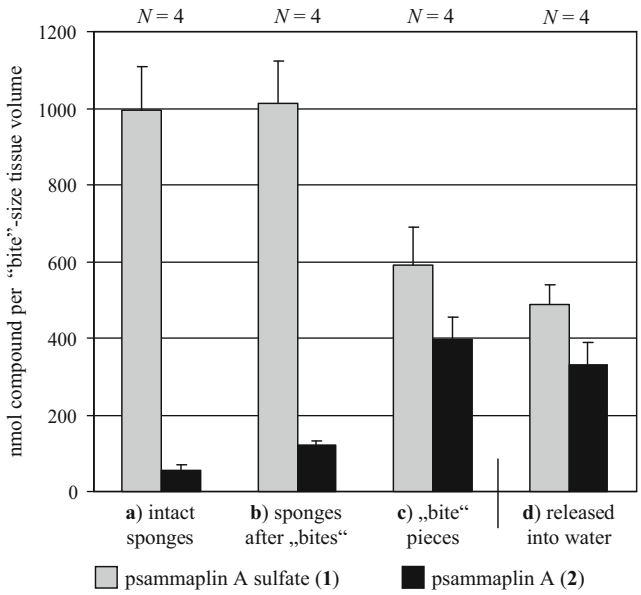

Fig. 3 "Forceps experiment": Contents of psammaplin A sulfate (**1**) and psammaplin A (**2**) in sponge tissue and seawater after mimicked fish bites. "Bite" pieces were shock-frozen within 2 s after removal from the sponges. Concentrations in the sponge tissue samples (*a–c*) are calculated per average "bite" volume (~76 µl). Amounts of compounds released into the water (*d*) are calculated per "bite" caused to the sponge tissue. *Vertical bars* represent SE. Randomized block ANOVA: (**1**) F=12.7, P<0.0025; (**2**) F=15.3, P<0.001. Tukey test (significant differences are indicated, only): (**1**): $a \neq (c, d)$; $b \neq (c, d)$. (**2**): $a \neq (c, d)$; $b \neq (c, d)$

weight. The discrepancy between the total concentrations of psammaplins before and after addition of water (i.e., the apparent loss of compounds) may be explained by the formation of non-UV-detectable side products.

Grinding with additional EtOH-dissolved psammaplin A sulfate (**1**) together with fresh *A. rhax* resulted in an increase of the psammaplin A (**2**) concentration in the sponge tissue to 75.1 µmol g^{-1} dry weight (compared to 50.3 µmol g^{-1} dry weight in sponge tissue ground with EtOH, only). Apparently, artificially added (**1**) was converted to (**2**) when ground together with fresh *A. rhax*. In contrast, no (**2**) was formed when (**1**) was ground together with fresh tissue of the sponges *H. altum* and *S. massa*.

Isolated (**1**) proved stable for at least 6 days when stored at room temperature in seawater or methanol, respectively.

Effects of Solvent Exposure Similar to mechanical damage, solvent exposure had an activating effect on the conversion of psammaplin A sulfate (**1**) to psammaplin A (**2**) in fresh *A. rhax* tissue (Table 1). The concentration of (**1**) dropped quickly—yet much more slowly than upon mechanical damage—within 10 min of exposure, and remained largely constant over the next 24 h. At the same time, (**2**) considerably increased in concentration—likewise mostly within the first 10 min of exposure.

Table 1 Concentration of psammaplin A sulfate (**1**) and psammaplin A (**2**) following methanol exposure of fresh *Aplysinella rhax* tissue

Exposure time	Psammaplin A sulfate (1) µmol g^{-1} dry weight±SE	Psammaplin A (2) µmol g^{-1} dry
a, no exposure	79.7±2.0	6.6±0.6
b, 10 min	28.9±2.8	51.4±1.5
c, 30 min	23.8±3.8	69.4±9.9
d, 1 h	21.2±1.5	76.0±4.7
e, 6 h	25.1±7.9	68.9±7.4
f, 24 h	16.9±1.6	76.1±5.4

N=3 for all time points. Randomized block ANOVA: (**1**) F=48.5, P< 0.001; (**2**) F=41.9, P<0.001. Tukey test (significant differences are indicated, only): (**1**) $a \neq$(b, *c*, *d*, *e*, *f*) and (**2**) $a \neq (b, c, d, e, f)$; $b \neq (d, f)$.

Field Feeding Assay with Generalist Reef Fishes Psammaplin A sulfate (**1**) was strongly deterrent against feeding by generalist reef fish. While control food was readily eaten, food treated with (**1**) was scarcely removed (Fig. 4a). When pairs of ropes, both containing treated food [one with (**1**) and the other with (**2**)] were offered to the fish, they only occasionally took bites; repetitive feeding was rarely observed. Over time, similar amounts of both types of food were removed (Fig. 4b). This indicates a strong repellent effect also by (**2**), but it precludes conclusions on differences in anti-feeding intensity between (**1**) and (**2**), since the occasional choices by the fishes in this assay have to be considered random.

Feeding Assay with a Sponge Predator Both "Ar intact" (butanol fraction of crude extract from intact *A. rhax* tissue) and "Ar wounded" (butanol fraction of crude extract from ground *A. rhax* tissue) had significant repellent effects in

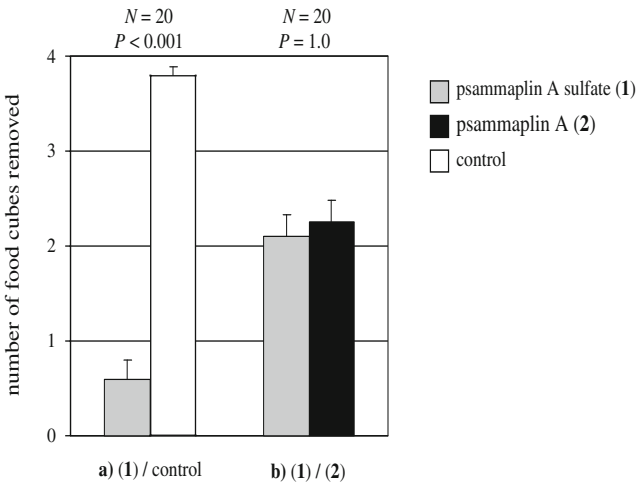

Fig. 4 Results of two field feeding assays testing (*a*) food treated with psammaplin A sulfate against untreated controls and (*b*) food treated with psammaplin A sulfate against food treated with psammaplin A. In each assay, 20 pairs of ropes (each rope holding four food pieces) were offered to generalist reef fish. *Vertical bars* represent SE

choice feeding assays with *C. solandri* when they were tested separately against controls (Fig. 5a and b). When offered in a direct choice without control food, "Ar wounded" proved to be a significantly stronger deterrent than "Ar intact" (Fig. 5c).

Diffusion of compounds from the food strips into the seawater was minimal in the course of the experiment as revealed by HPLC comparison of extracts from strips kept in the seawater tanks (without fish) with food strips not exposed to water. Compound concentrations were >90% compared to the original concentrations after 4 h of seawater exposure.

Discussion

Whereas numerous activated chemical defenses have been documented in terrestrial plants and marine algae (see reviews by Zagrobelny et al. 2004 for terrestrial and Pohnert 2004 for marine-activated defenses), in sessile marine invertebrates, chemical defense is considered largely static. Our study revealed that psammaplin A, a natural product that has been considered constitutive in the marine sponge *A. rhax* (e.g., Shin et al. 2000; Tabudravu et al. 2002), is, in fact, a cleavage product of psammaplin A sulfate. This cleavage reaction is triggered by wounding of the sponge tissue (Figs. 1 and 2).

The conversion of psammaplin A sulfate to psammaplin A occurs within a few seconds following damage of *A. rhax* tissue, as revealed by the "forceps experiment" (Fig. 3). Simulated predator "bites" caused a more than six-fold increase in psammaplin A in the excised tissue compared to undamaged tissue. Psammaplin A was also formed in the remaining damaged sponge, yet to a much lesser extent. Apparently, the conversion reaction was restricted to the wounded tissue area and did not disperse through the sponge. Analogous localized reactions in plants are usually induced by disintegration of compartments that facilitates contact between precursors and enzymes previously stored in separate compartments (Matile 1984; Wittstock and Gershenzon 2002). Storage of secondary metabolites in compartments also has been observed in sponges (Turon et al. 2000; Richelle-Maurer et al. 2003). *Aplysina* sponges, which are closely related to *A. rhax*, contain isoxazoline alkaloids, compounds structurally and biogenetically similar to psammaplin A sulfate (Ciminiello et al. 1994, 1996). These compounds are stored in so-called spherulous cells (Thompson et al. 1983; Turon et al. 2000). By electron microscopy, we found this cell type also to be highly abundant in tissue of *A. rhax* (Thoms et al., unpublished). This suggests that the mechanism that results in the formation of psammaplin A in *A. rhax* is analogous to those that are typically involved in plant activated defenses (i.e., the disruption of compartments to trigger enzymatic compound conversion). We found several further indications that the conversion reaction in *A. rhax* is, indeed, enzyme-mediated. First, the rapidness of the conversion of psammaplin A sulfate, a compound that proved highly stable when isolated from the sponge tissue, indicates enzyme-catalysis. Second, the factor that mediates the conversion appears to be specific to *A. rhax* tissue, since no psammaplin A was formed when psammaplin A sulfate was ground together with tissue of other sponge species. This indicates that *A. rhax* possesses a specific enzyme that catalyzes the cleavage of the sulfate ester group in psammaplin A sulfate. Third, the conversion reaction occurs only in the presence of water. No conversion occurred when lyophilized tissue of *A. rhax* was ground. If, however, water was added to the lyophilized and ground sponge material, psammaplin A was formed at high concentrations. Finally, the conversion reaction was inhibited by heat. In case of a non-enzymatic reaction, the turnover rate should increase with higher reaction temperature. In contrast, the yield of psammaplin A was considerably lower when lyophilized *A. rhax* tissue was treated with hot water compared to water at room temperature. This indicates heat denaturation of the enzyme(s) that catalyzes the cleavage of psammaplin A sulfate in *A. rhax*. In fact, arylsulfatases, enzymes catalyzing reactions such as the cleavage of psammaplin A sulfate in *A. rhax* (i.e., the hydrolysis of a phenolic sulfate ester group) have been described from most organisms, including sponges and marine microorganisms (Mraz and Jatzkewitz 1974; Byun et al. 2004).

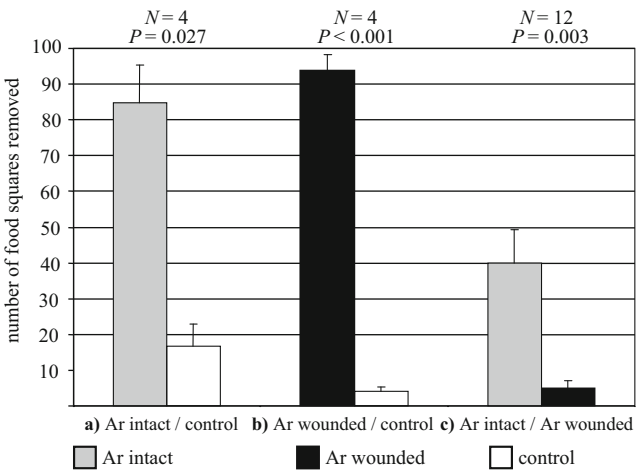

Fig. 5 Results of three choice feeding experiments with the sponge predator *C. solandri*. Fish food was treated with the butanol fractions of extracts from intact ("Ar intact") and ground ("Ar wounded") *A. rhax* tissue. The two fractions were tested separately against controls (*a*+*b*) and in a direct comparison against each other (*c*). *Vertical bars* represent SE

To test for ecological relevance of the observed wound-activated conversion in *A. rhax* as a chemical defense mechanism, we conducted fish-feeding assays comparing the repellent effects of the precursor and the product. When generalist reef fish in the field were offered a choice between food treated with psammaplin A sulfate and controls, they significantly preferred the controls (Fig. 4a). When offered a choice between food treated with psammaplin A sulfate and food treated with psammaplin A (Fig. 4b), the fish only occasionally took bites. By contrast, untreated food—offered to attract the fishes to our experiment—was readily taken. We found that both psammaplin A sulfate and psammaplin A barely leak from the experimental fish food. Thus, their deterrent properties are presumably only perceived by the fish when bites are taken. During the experiment, tasting of both compounds by one individual fish or repetitive feeding were rarely observed, making the occasional fish bites in this experiment rather random. This precludes conclusions on differences in anti-feeding activity between the two compounds. However, the observation that both types of treated food were largely avoided whereas untreated food was readily taken, suggests that both psammaplin A sulfate and psammaplin A had a pronounced feeding deterrent effect against the generalist reef fish.

Despite this pronounced repellence, we observed feeding scars on *A. rhax* in the field, suggesting that there are predators that are able to overcome the strong constitutive chemical defense of the sponge. We, therefore, performed another feeding experiment—this time with a "non-specialist" omnivorous sponge predator, i.e., a predator that is not adapted to the defense compounds of a particular sponge species, but may be less susceptible to sponge chemical defense compared to common reef fish. We selected the pufferfish *C. solandri* for this assay, since it is known to feed on sessile invertebrates such as sponges (Myers 1991). Moreover, this species is highly abundant on the coral reefs around Guam. Similar to the generalist reef fish in the field experiment, *C. solandri* strongly preferred control food over food treated with the *A. rhax* compounds (Fig. 5a and b). However, when no control but only a choice between the two *A. rhax* compounds was offered, *C. solandri* was significantly more deterred by the bioconversion product psammaplin A (Fig. 5c). Thus, the wound-activated conversion reaction in *A. rhax* resulted in an enhanced chemical protection against the potential predator *C. solandri*.

Taken together, we found a rapid, most likely enzyme-mediated bioconversion in *A. rhax* that was activated by tissue wounding and enhanced protection of the sponge against a known sponge predator. Thus, all criteria for an activated chemical defense as defined by Paul and Van Alstyne (1992) are fulfilled. Apparently, *A. rhax* is sufficiently protected against generalist reef fish by its constitutive chemical defense (i.e., the storage of psammaplin A sulfate). However, if despite this defense *A. rhax* is wounded—for instance by a sponge predator that is less susceptible to defensive chemicals than the average reef fish—the sponge's protection against predators is rapidly boosted by the wound-activated formation of psammaplin A.

When simulating fish grazing by picking tissue pieces from *A. rhax*, we observed the release of mucus-like clouds from the wounded tissue. We quantified the psammaplin A sulfate and psammaplin A concentrations released with these clouds and found that they were similarly high as the concentrations in the removed tissue bits (Fig. 3). The latter had proven sufficient to efficiently repel fishes. The mucus clouds may facilitate a rapid transport of the rather lipophilic defense compounds psammaplin A sulfate and psammaplin A to the olfactory organs of the predator, thus accelerating and intensifying their repellent effect.

To our knowledge, the bioconversion in *A. rhax* is so far only the second reported example of an activated chemical defense in sponges—the third among sessile marine invertebrates. Interestingly, all compounds in the sponges *A. rhax* and *Aplysina* spp., as well as in the hydroid *Tridentata marginata*, were initially considered constitutive (Fattorusso et al. 1970; Lindquist et al. 1996; Pham et al. 2000; Shin et al. 2000). In all cases, it was only later discovered that they are, in fact, products of wound-activated conversions (Teeyapant and Proksch 1993; Lindquist 2002; this study). Recently, a cleavage of acetylated sesterterpenes in the sponge *L. variabilis* was reported to occur when fresh tissue of the sponge is frozen and subsequently thawed (Ettinger-Epstein et al. 2007). Here, again, the cleavage products had originally been considered constitutive (de Silva and Scheuer 1981; Kernan et al. 1987). Ettinger-Eppstein et al. proposed an enzyme-mediated cleavage reaction and speculated about its relevance as an activated defense. We have lately observed pronounced changes in the chemical profiles of yet unidentified deep-water sponges collected off the coast of Guam that occurred upon damage to their tissue (Thoms C. and Schupp P.J., unpublished data). More detailed investigations on these preliminary findings need to be done. Based on all these recent observations, however, we deem it reasonable to address the question whether more activated defenses in sponges—and perhaps also in other sessile marine invertebrate phyla—may have been overlooked in the past. In fact, this would be analogous to the development in research on marine algae. Here, it took almost a decade from the first discovery of activated defenses, until it was realized that this strategy is, in fact, widespread among both micro- and macroalgal species (Pohnert 2004). Even for algae, it was recently proposed that the prevalence

of this strategy may still be underestimated due to a "restricted methodological approach by marine chemical ecologists that focuses on constitutive chemical defense" (Pohnert 2004).

In this context, it is interesting to note that the reaction mechanisms of the bioconversions in *A. rhax* and in the *Aplysina* spp. (the genus that accommodates the only other wound-activated defense reported in sponges as yet) are dissimilar. In the latter, isoxazoline alkaloids are cleaved between the isoxazoline moiety and the carbonyl function common to all these molecules (Fig. 6a; Ebel et al. 1997; Thoms et al. 2006). The cleavage is catalyzed by a yet unknown enzyme and results in the formation of the antimicrobially active compounds aeroplysinin-1 and dienone. In contrast, the activated defense reaction in the hydroid *Tridentata marginata* shares many similarities with the conversion reaction we report in this study. In *T. marginata*, as well, a phenolic sulfate ester is enzymatically hydrolyzed and a fish-feeding-deterrent phenol is formed (Fig. 6b; Lindquist 2002). Interestingly, the precursors of all three reactions (in *Aplysina* spp., *A. rhax*, and *T. marginata*) are structurally similar tyrosine-derivatives (Figs. 1 and 6). This may indicate a predisposition of this class of compounds for being converted in activated defenses. An alternative explanation for the repeated discovery of activated defenses that involve tyrosine-derivatives may be found in the fact that these compounds are accumulated in extraordinarily high concentrations in the sponges and the hydroid (Lindquist et al. 1996; Pham et al. 2000; Thoms et al. 2006). Consequently, conversion reactions in these invertebrates may simply be more conspicuous than analogous processes in other species with lower compound yields and more complex chemical profiles. Moreover, if the conversion reactions involve a high number of different precursors, intermediates, or products, this may additionally conceal activated defense reactions.

We recently reviewed the various difficulties involved in studies investigating wound-activated reactions in chemical profiles of sponges in regard to both methodological constraints and limitations of conclusions that can be drawn on the ecological relevance of these reactions (Thoms and Schupp 2007). One of our conclusions is that, in order to clarify the true prevalence of activated defenses in sponges (and in other sessile marine invertebrate phyla), studies are required that systematically compare chemical profiles of these organisms before and after tissue wounding at the

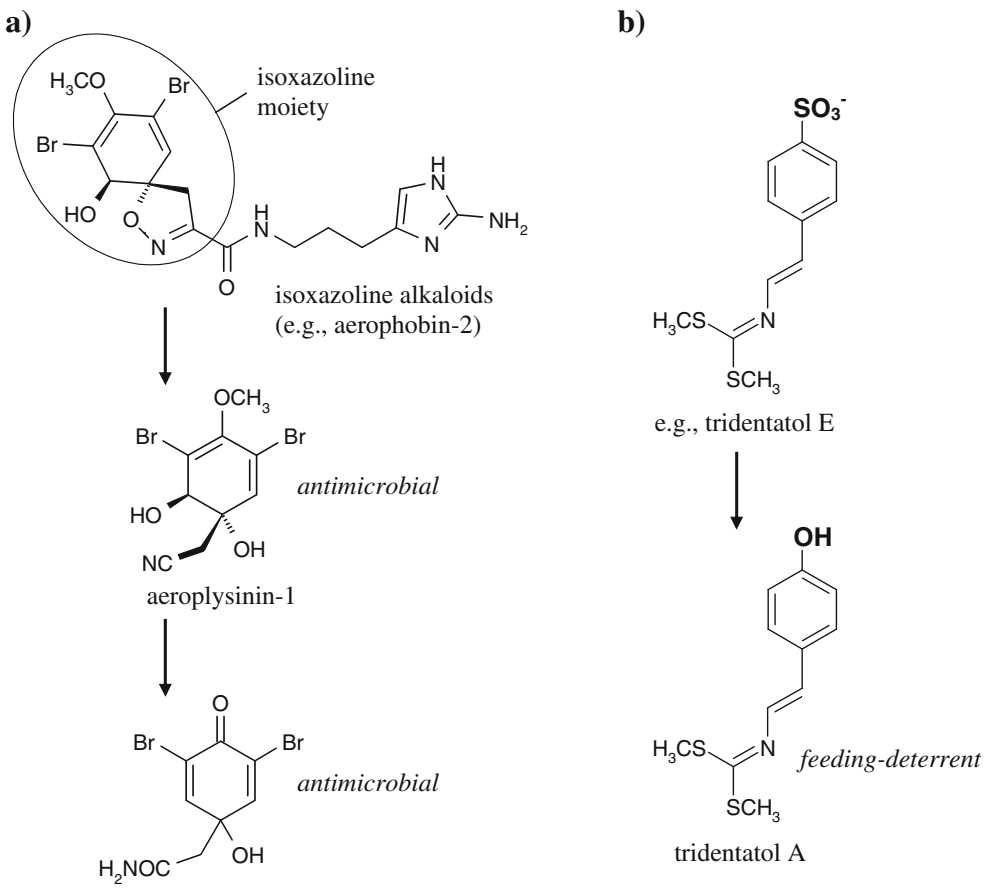

Fig. 6 Activated chemical defenses in sessile marine invertebrates: **a** wound-activated cleavage of the isoxazoline moiety of isoxazoline alkaloids in sponges of the genus *Aplysina* (Thoms et al. 2006). **b** Wound-activated hydrolysis of the sulfate ester groups of tridentatols in the hydroid *Tridentata marginata* (Lindquist 2002)

species level. An important issue that needs to be considered in this context is the methodology for preservation and extraction of invertebrate tissue that is employed in such studies. Preservation and extraction of fresh (wet) tissue in organic solvents are common practices in marine natural product chemistry and ecology. We observed that conversion reactions similar to those activated in *A. rhax* upon tissue wounding also occurred when fresh sponge tissue was stored in methanol. This finding parallels earlier observations in *Aplysina* spp. where storage of fresh sponge tissue in organic solvents elicited the cleavage of isoxazoline alkaloids to an extent similar to that of mechanical injury (Teeyapant and Proksch 1993; Thoms et al. 2006). The activation of enzymatic reactions in invertebrate tissue may be explained by the lacerating effect of organic solvents on biomembranes (Jones 1989; Weber and DeBont 1996). Similar to mechanical damage, this likely results in decompartmentalization and, thus, facilitates contact between enzymes and precursors. If the solvent concentration increases gradually (as occurs if fresh invertebrate tissue is soaked by solvents), enzymes can still sustain their activity and catalyze compound conversions (see Klibanov 2001 and references cited therein).

Another possible trigger for enzyme-mediated reactions in invertebrate tissue after sampling is freezing and thawing for preservation and subsequent extraction (see for example Ettinger-Epstein et al. 2007). Here, the formation of intracellular ice crystals may result in disruption of compartments (Gahan 1981; Hällgren and Öquist 1990). Thawing of the tissue may then re-activate the enzymes. Last but not least, mechanical damage caused during sampling and handling has to be taken into account as a potential elicitor of wound-activated defenses.

Taken together, various factors could potentially have led to the oversight of activated chemical defenses, and, in consequence, to the misinterpretation of products of these defense mechanisms as constitutive chemical profile components. If future systematic investigations on activated defenses in sessile marine invertebrates reveal additional examples of this strategy, this could add another interesting aspect to the chemical ecology of this group of animals.

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