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ACTIVATED CHEMICAL DEFENSE IN *Aplysina* SPONGES REVISITED

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Abstract—Sponges of the genus *Aplysina* accumulate brominated isoxazoline alkaloids in concentrations that sometimes exceed 10% of their dry weight. We previously reported a decrease in concentrations of these compounds and a concomitant increase in concentrations of the monocyclic nitrogenous compounds aeroplysinin-1 and dienone in *Aplysina aerophoba* following injury of the sponge tissue. Further investigations indicated a wound-induced enzymatic cleavage of the former compounds into the latter, and demonstrated that these reactions also occur in other *Aplysina* sponges. A recent study on Caribbean *Aplysina* species, however, introduced doubt regarding the presence of a wound-induced bioconversion in sponges of this genus. This discrepancy motivated us to reinvestigate carefully the fate of brominated alkaloids in *A. aerophoba* and in other *Aplysina* sponges following mechanical injury. As a result of this study we conclude that (1) tissue damage induces a bioconversion of isoxazoline alkaloids into aeroplysinin-1 and dienone in *Aplysina* sponges, (2) this reaction is likely catalyzed by enzymes, and (3) it may be ecologically relevant as the bioconversion products possibly protect the wounded sponge tissue from invasion of bacterial pathogens.

Key Words—Wound-induced bioconversion, chemical defense, biotransformation, brominated alkaloids, enzymatic cleavage, marine sponge.

INTRODUCTION

Sessile organisms are dependent on protective mechanisms other than flight or active defense. Depending on the predictability of the influencing stress factors

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they are exposed to, they follow different strategies. In cases of predictable or constant stress factors, constitutive defense mechanisms (e.g., shells and stings or the constitutive accumulation of protective metabolites) have usually evolved (Hay and Fenical, 1988). However, if the disturbing impacts display a high spatial or temporal variability, facultative defense mechanisms have often developed instead (Adler and Harvell, 1990; Harvell, 1990). The latter can be divided into two different forms: induced defenses and activated defenses.

"Induced defenses" such as induced defense metabolite biosynthesis (Havel, 1986; Roda and Baldwin, 2003) or induced morphological changes (Steneck and Adey, 1976; Lewis et al., 1987; Lurling, 2003) are slow. Inducing factors include predation or attack by harmful microorganisms. After the initial attack, the priorities of resource allocation in the attacked organism may be shifted from growth to defense. Sometimes it takes weeks until a protective effect occurs (Hammerstrom et al., 1998; Taylor et al., 2002). Thus, this type of mechanism can not be expected to provide immediate protection, but rather prepares the harmed organism for future attacks.

A faster response to stress factors is the attack-induced transformation of inactive precursor compounds stored in the tissue of the attacked organism, thereby yielding transformation products that exhibit a pronounced defensive activity. Paul and van Alstyne (1992) described this phenomenon as "activated defense" to distinguish it from the slower "induced defense." Such mechanisms are fast (often occurring within seconds after wounding) in order to facilitate immediate protection against the affecting organism. These reactions are usually catalyzed by enzymes that convert inactive storage compounds into defensive products (Paul and van Alstyne, 1992; Hickel et al., 1996).

In the terrestrial environment, activated defense reactions involving enzymatic transformations of inactive precursors are frequently found in plants. Examples include cyanogenic glycosides that are transformed into highly toxic HCN (Conn, 1979; Wajant and Effenberger, 1996; Gleadow and Woodrow, 2002), or as in Brassicaceae and related plants, glucosinolates that are cleaved to form thiocyanates, isothiocyanate, or isonitriles (Stoewsand, 1995; Fahey et al., 2001). In all reported examples, the respective precursors are physically separated by compartmentalization from the biotransformation enzymes. Upon mechanical damage, the compartments are destroyed, which facilitates the formation of the protective substances as the enzymes and substrates make contact.

In the marine environment, examples of activated chemical defense mechanisms are also found. In green algae, *Halimeda*, tissue damage leads to the transformation of the diterpene halimedatetraacetate to the aldehyde halimedatrial, which is a more potent toxin compared to the precursor (Paul and van Alstyne, 1992). The green alga *Caulerpa taxifolia* accumulates caulerpenyne. After wounding of the algal tissue, it is deacetylated forming a

group of aldehydes, mainly oxytoxins 1 and 2 (Jung and Pohnert, 2001). Presumably, the transformation products are more potent defensive compounds than the precursor. It has been suggested that the rapid biotransformation of caulerpenyne is catalyzed by an esterase (Jung et al., 2002). A third example is the cleavage of dimethylsulfoniopropionate (DMSP) to acrylic acid and dimethylsulfide (DMS) by the enzyme DMSP lyase that was recently observed in macroalgae (van Alstyne et al., 2001; van Alstyne and Houser, 2003). This reaction was previously described in unicellular phytoplankton (Wolfe and Steinke, 1996; Wolfe et al., 1997).

Numerous marine invertebrates face ecological situations that are similar to those in marine or terrestrial plants. Ascidians, sponges, corals, and bryozoans are sessile and unable to protect themselves actively by flight or attack. They have evolved defense mechanisms similar to those of plants. Marine sponges have been particularly well investigated with regard to putative defense metabolites. Within the last 30 yr, over 4000 secondary metabolites have been isolated and described, many of which are biologically active (MarinLit, 2003). In various cases, these metabolites protect invertebrates against predators, competitors for settlement space, or against microbial pathogens (Green, 1977; Bakus et al., 1986; Becerro et al., 1997; McClintock et al., 1997; Thacker et al., 1998; Engel et al., 2002).

Induced chemical defense reactions have also been reported for sponges. Recent examples are *Suberites domuncula* and *Agelas conifera*. In *S. domuncula*, the level of two lyso-PAF (platelet-activating factor) congeners is increased upon exposure to an endotoxin derived from the outer cell wall of gram-negative bacteria (Müller et al. 2004). Lyso-PAF and analogues possess strong antimicrobial activity (Steel et al., 2002) and inhibit the growth of phytopathogenic fungi (Tanaka et al., 1997). In *A. conifera*, the concentrations of the antimicrobial and feeding-deterrent compounds sceptrin and oroidin increase 3- to 4-fold within 0.5–6 d following wounding of the sponge tissue (Richelle-Maurer et al., 2003).

An example for an activated chemical defense mechanism in sponges has been reported for *Aplysina aerophoba*. This sponge, like other *Aplysina* species, accumulates brominated isoxazoline alkaloids in concentrations of up to 10% dry weight (Albrizio et al., 1994; Aiello et al., 1995; Ciminiello et al., 1994a,b, 1995, 1996a,b, 1997, 1999, 2000; Thoms et al., 2003a). X-ray microanalysis revealed that these brominated metabolites are mainly stored in specialized sponge cells called spherulous cells (Turon et al., 2000). Teeyapant and Proksch (1993) reported the cleavage of these isoxazoline alkaloids in tissue of *A. aerophoba* when freeze-dried sponge material was extracted with water or with aqueous MeOH. During this reaction, the monocyclic nitrogenous compounds aeroplysinin-1 (**5**) and dienone (**6**) were formed (Figure 1). By extraction of freeze-dried tissue with 100% MeOH, however, only putative isoxazoline

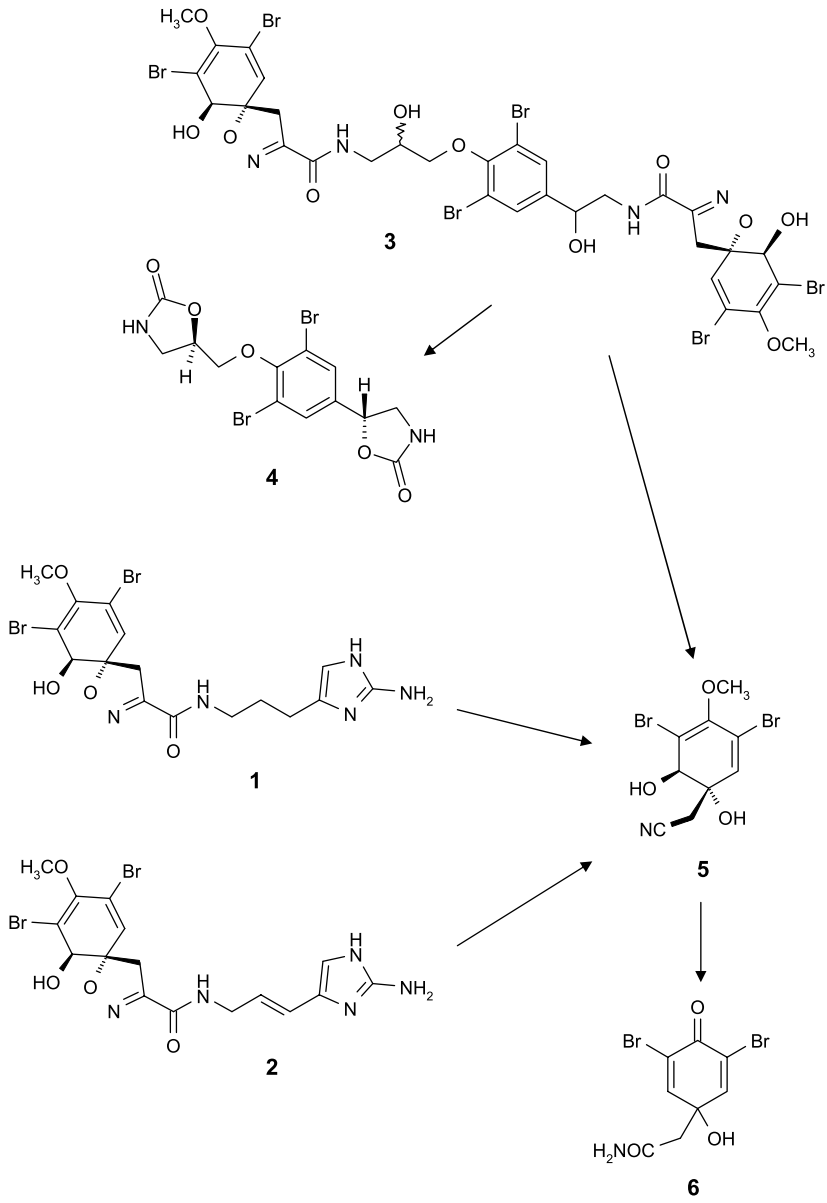

FIG. 1. Wound-induced bioconversion of the brominated isoxazoline alkaloids aerophobin-2 (**1**), aplysinamisin-1 (**2**), and isofistularin-3 (**3**) to aeroplysinin-1 (**5**) and the dienone (**6**) in tissue of *Aplysina aerophoba*. When isofistularin-3 (**3**) is used as a substrate for the reaction, the bisoxazolidinone derivative (**4**) is recovered as a further product.

precursors, such as isofistularin-3 (**3**) or aerophobin-2 (**1**), were recovered. Subsequently, Ebel et al. (1997) reported that the bioconversion of the isoxazoline precursors into aeroplysinin-1 (**5**) and dienone (**6**) also takes place when fresh sponge tissue is crushed mechanically in the presence of seawater. Additional experiments with cell-free extracts from *A. aerophoba* and from six other *Aplysina* species collected in the Caribbean and in the Mediterranean Sea revealed that all *Aplysina* species had the capability to cleave isoxazoline alkaloids, thereby generating aeroplysinin-1 (**5**) (Ebel et al., 1997). This led to the conclusion that the observed cleavage of the isoxazoline precursors is catalyzed by enzymes present in *Aplysina* sponges. It was concluded that the cleavage reactions consist of at least two different steps: in a first step, degradation of the isoxazoline alkaloids gives rise to the β-hydroxynitrile aeroplysinin-1 (**5**), while in a second, this intermediate is converted into the dienone (**6**) via enolether hydrolysis and partial hydrolysis of the nitrile group (Teeyapant and Proksch, 1993; Goldenstein et al., 2000).

Bioactivity studies revealed that bioconversion of the isoxazoline alkaloids in *Aplysina* sponges is paralleled by an increase in antibiotic and cytotoxic activity (Teeyapant et al., 1993; Weiss et al., 1996). In contrast, feeding deterrent activity of the bioconversion products against the Mediterranean fish species *Blennius sphinx* is significantly weaker than observed for their isoxazoline precursors (Thoms et al., 2004).

Recently, Puyana et al. (2003) investigated the Caribbean sponges *A. insularis* and *A. archeri* with regard to a possible biotransformation of isoxazoline alkaloids following mechanical injury, which was simulated by stabbing the sponges with a scalpel. In this study, the authors came to the conclusion, that a conversion of the isoxazoline alkaloids into aeroplysinin-1 (**5**) and dienone (**6**) as reported for *A. aerophoba* was not apparent in the Caribbean *Aplysina* species, and they assumed that past observations of biotransformation reactions within the genus *Aplysina* were the results of either differential tissue extraction efficiency, hydrolysis from insoluble precursors, or a heterogeneous distribution of metabolites in sponge tissue. These contradictory results motivated us to reinvestigate the fate of brominated alkaloids in *Aplysina* sponges following mechanical injury.

METHODS AND MATERIALS

Sponges of the genus *Aplysina* (Demospongiae, Verongiida, Aplysinidae) frequently occur in the Mediterranean as well as in the Caribbean Sea. From both geographical regions, two representatives each were obtained. Specimens of the sponge *A. aerophoba* were collected by scuba diving and snorkeling at

depths between 3 and 10 m at Banyuls-sur-mer, France, in April 2002 and at Rovinj, Croatia, in August 2003. *A. cavernicola* was collected in May 2001 at Elba, Italy, whereas *A. fistularis* and *A. archeri* were from previous collections in the Bahamas in 1995. The Mediterranean sponge *Crambe crambe* (Demospongiae, Poecilosclerida, Myxillidae) was collected off the coast of Rovinj, Croatia, in August 2003. All sponges were carefully detached from the substratum with a knife to avoid tissue damage.

During sampling, specimens were kept submerged in ziplock bags filled with seawater for transport to the laboratory. All experiments with fresh, living sponge tissue were performed with *A. aerophoba* collected at Rovinj, Croatia. These specimens were directly used. For experiments involving lyophilized tissue, specimens were flash frozen and kept at temperatures below −80°C until freeze-drying and homogenization of the lyophilized tissue.

Wounding Intensity Series. Six tissue slices, each comprising a volume of 8 ml (thickness, 1 cm) were cut from one *A. aerophoba* individual with a scalpel. After the respective treatments, all samples were placed in 15-ml cryo tubes and flash frozen in liquid nitrogen.

Sample "t_0" was flash frozen after it was excised from the living sponge. Sample "b" was repeatedly stabbed with a scalpel over its entire surface for a total of 15 sec. After an additional period of 4 min and 45 sec, during which no further treatment was applied, the sample was flash frozen. Sample "c" was ground in a mortar for 15 sec instead. After an additional 4 min and 45 sec (no further treatment), it was flash frozen. Sample "d" was ground for a total of 5 min and immediately flash frozen. Sample "e" was treated like sample "d" with the exception that 1.5 ml EtOH were immediately added to the fresh tissue prior to grinding.

The same series of treatments was repeated four times, each time with samples that had been cut from a different *A. aerophoba* individual. Additional slices of two *A. aerophoba* individuals (samples "a") were placed in a mortar without further treatment for 5 min followed by flash freezing.

Prior to HPLC analysis, all samples were lyophilized, ground to a fine powder, and exhaustively extracted with MeOH. Extracts were then injected into an HPLC system coupled to a photodiode array detector (Dionex, Germany). Routine detection was at 254 and 280 nm. The separation column (125 × 4 mm i.d.) was prefilled with Eurosphere C-18 (5 μm) (Knauer, Germany). Compounds were identified by their online UV spectra and by direct comparison with previously isolated standards (Ebel et al., 1997). Identification of compounds in extracts was further verified by LC-MS analysis using a diode array equipped HPLC system (Agilent 1100 Series; Agilent Technologies, Waldbronn, Germany) coupled to an electrospray ionization (ESI) Mass Spectrometer (LC-Q Deca; Finnigan, Bremen, Germany). Separation was achieved using a Knauer Eurospher C-18 column (5 μm, 2 mm diam × 250 mm long).

The amount of each individual compound in the samples was calculated from the detector response in HPLC-UV and the compound-specific molar extinction coefficients. Based on these data, proportions of the respective brominated alkaloids in the samples were expressed as percentage of the total content of brominated alkaloids (set at 100%) present in 1 g lyophilized sponge tissue.

A control experiment was performed with fresh tissue of the sponge *C. crambe* that had been collected at Rovinj. Three samples of the sponge tissue were spiked with one of the *A. aerophoba* metabolites aeroplysinin-1 (**5**), aerophobin-2 (**1**), or isofistularin-3 (**3**), each at concentrations within the range of their natural abundance in *A. aerophoba* (aeroplysinin-1 as found in damaged sponge tissue). To a fourth *C. crambe* tissue piece, no *A. aerophoba* metabolites were added. Samples were ground for 5 min and subsequently flash frozen. After lyophilization, samples were extracted and analyzed as described above.

Time Course Experiment Fresh tissue slices of *A. aerophoba* of the same volume as described in the experiment above (8 ml) were ground for 15 sec in a mortar. Over a total period of 165 sec, measured from the onset of grinding, aliquots were collected from the ground material and immediately frozen in liquid nitrogen. Following lyophilization, they were extracted and analyzed using HPLC-UV and LC-MS under the conditions described above. All samples comprising one time series resulted from one *A. aerophoba* individual. The four parallel experiments were performed with one individual each.

Experiments with Lyophilized Animal Tissue Experiments were performed with tissue of the two Mediterranean sponges, *A. aerophoba* and *A. cavernicola*, and the Caribbean sponges *A. fistularis* and *A. archeri*. For each species, lyophilized tissue from three different individuals was used. Tissue was ground and homogenized. Subsequently, two portions of 100 mg were sampled. One of these was used for exhaustive extraction with MeOH. The other was mixed with 600 µl seawater, kept therein overnight, and was subsequently lyophilized again. After extraction with MeOH, all samples were analyzed as described above to determine the molar concentrations of the brominated alkaloids.

Additionally, lyophilized and ground tissue of *A. archeri* was spiked with aerophobin-2 (**1**) isolated from *A. aerophoba* in a concentration found naturally in the latter. The mixture was incubated in 600 µl seawater and subsequently lyophilized, extracted, and analyzed as described above.

Control experiments were performed with lyophilized and ground tissue of the marine opisthobranch *Tylodina perversa* and the sponge *C. crambe*. Mantle tissue of *T. perversa* naturally contains *Aplysina* alkaloids (Ebel et al., 1999; Thoms et al., 2003b). The opisthobranch tissue was incubated in seawater, lyophilized again, and subsequently extracted and analyzed as described for the lyophilized *Aplysina* sponge samples. Tissue of the sponge *C. crambe* was treated likewise, but prior to incubation in seawater was mixed with aerophobin-2 (**1**) and isofistularin-3 (**3**) in concentrations as found in *A. aerophoba*.

Data Analyses. Data of the wounding intensity series and the time course experiment were analyzed by randomized block analysis of variance, and multiple comparisons were made with the Tukey–Kramer test for unequal sample sizes (Zar, 1999). Percentage data were arcsine-transformed prior to statistical analyses. In case of missing data in the randomized block design, data were estimated iteratively, and the bias in the group sum of squares (group SS) as well as the total degrees of freedom (*df*) was corrected according to Li (1964) and Glen and Kramer (1958).

For analysis of variations in the relative concentration of bioconversion products in lyophilized *Aplysina* sponge tissue upon treatment with seawater, the percentage data were arcsine-transformed and subsequently analyzed using a two-tailed paired *t*-test (Zar, 1999).

RESULTS

Constitutive Alkaloid Patterns in Intact A. aerophoba Individuals Obtained from Croatia and France. The isoxazoline alkaloids aerophobin-2 (**1**) and isofistularin-3 (**3**) were the dominant compounds in individuals collected at Rovinj, Croatia (Table 1A). Aeroplysinin-1 (**5**) was present only at lower concentrations. Other isoxazoline alkaloids that are known for *A. aerophoba* (e.g., purealidines and aerophobin-1) were detected but not quantified, as they occurred only in negligible concentrations. Dienone (**6**) and aplysinamisin-1 (**2**) were not detected. In contrast, in individuals of *A. aerophoba* collected at the French coast at Banyuls, aplysinamisin-1 (**2**) was dominant, followed by aerophobin-2 (**1**) and isofistularin-3 (**3**) (Table 1B). Neither aeroplysinin-1 (**5**) nor dienone (**6**) was detected in these sponges. The sponge zoochrome uranidine

TABLE 1A. CONTENTS AND RELATIVE CONCENTRATIONS OF BROMINATED ALKALOIDS IN FRESH TISSUE OF *Aplysina aerophoba* COLLECTED AT THE MEDITERRANEAN COAST OF ROVINJ, CROATIA

Alkaloid	Content in 1 g dry weight (μmol)	Content in 1 ml fresh tissue (μmol)	Alkaloid pattern in percentages (%)
Aerophobin-2 (**1**)	134.4 ± 29.6	28.0 ± 6.2	68.9 ± 4.4
Isofistularin-3 (**3**)	48.5 ± 9.0	10.1 ± 1.9	25.0 ± 2.0
Aplysinamisin-1 (**2**)	n.d.	n.d.	0
Aeroplysinin-1 (**5**)	11.7 ± 8.2	2.4 ± 1.7	6.1 ± 4.2
Dienone (**6**)	n.d.	n.d.	0
Total	194.5 ± 37.9	40.5 ± 7.9	

$N = 10$; n.d. = not detected in the sample by HPLC-UV.

TABLE 1B. CONTENTS AND RELATIVE CONCENTRATIONS OF BROMINATED ALKALOIDS IN FRESH TISSUE OF *Aplysina aerophoba* COLLECTED AT THE MEDITERRANEAN COAST OF BANYULS-SUR-MER, FRANCE

Alkaloid	Content in 1 g dry weight (μmol)	Content in 1 ml fresh tissue (μmol)	Alkaloid pattern in percentages (%)
Aerophobin-2 (**1**)	29.2 ± 6.6	6.1 ± 1.4	25.5 ± 3.3
Isofistularin-3 (**3**)	18.5 ± 5.2	3.9 ± 1.1	16.0 ± 2.6
Aplysinamisin-1 (**2**)	67.2 ± 17.9	14.0 ± 3.7	58.4 ± 5.7
Aeroplysinin-1 (**5**)	n.d.	n.d.	0
Dienone (**6**)	n.d.	n.d.	0
Total	114.8 ± 26. 7	23.9 ± 5.6	

$N = 3$; n.d. = not detected in the sample by HPLC-UV.

was abundantly present in all analyzed sponge individuals, but due to its instability upon contact with air it could not be reliably quantified.

The overall concentration of brominated alkaloids was considerably lower in the *A. aerophoba* samples from France (Table 1B) than in the specimens from Croatia (Table 1A). Consistent with previous studies on other *Aplysina* species (Puyana et al., 2003), the absolute amounts of the various alkaloids detected in individuals of *A. aerophoba* (expressed as micromoles per gram dried tissue or as micromoles per ml volume of sponge tissue) varied considerably, whereas the relative proportions (in % compared to the total amount of alkaloids that was set at 100%) of the individual alkaloids turned out to be remarkably consistent for sponge specimens collected in France as well as in Croatia (Table 1A, B).

Wounding Intensity Series. The intensity of artificial damage to tissue taken from freshly collected *A. aerophoba* individuals was gradually increased by applying different methods of tissue destruction (stabbing with scalpel, grinding in mortar, addition of organic solvent plus grinding). This approach revealed an unequivocal correlation between increase in tissue destruction (wounding intensity), gradual disappearance of isoxazoline alkaloids, and concomitant increase in aeroplysinin-1 (**5**) content in injured vs. intact *A. aerophoba* tissue (Figure 2 and Table 2).

The possible effect of air exposure (without accessory wounding) on the metabolite pattern in tissue slices was tested (samples "a" in Figure 2 and Table 2). For this purpose, freshly cut slices of *A. aerophoba* were kept exposed to air in a mortar for 5 min before they were flash frozen. The brominated alkaloid composition in this tissue remained largely unchanged by this treatment, when compared to controls ("t_0" samples) that were flash frozen immediately after having been cut from the living sponge.

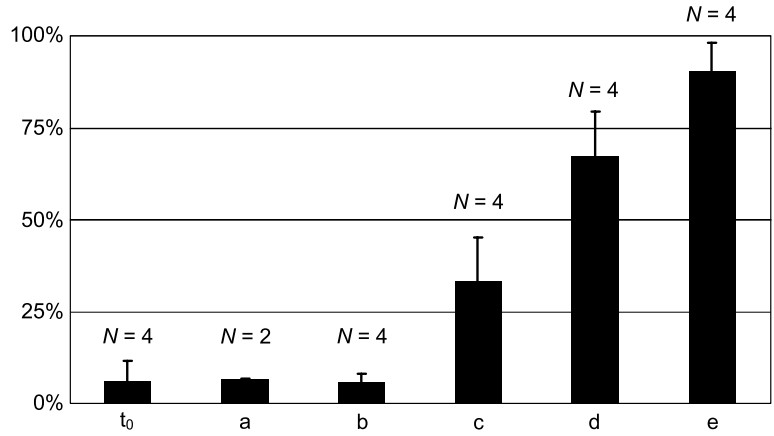

FIG. 2. Variations in the relative concentration of aeroplysinin-1 (**5**) in *A. aerophoba* tissue upon different intensities of wounding. Aeroplysinin-1 proportion is given in percent relative to the total brominated alkaloid content (set at 100%) in the sponge. For explanation of t_0, a, b, c, d, and e, refer to Table 2. Data were arcsine-transformed prior to statistical analysis. Randomized block analysis of variance: $F = 21.13$, $P < 0.001$. Tukey–Kramer multiple comparisons (significant differences are indicated, only): $d \neq t_0$, $d \neq a$, $d \neq b$, $e \neq t_0$, $e \neq a$, $e \neq b$, $e \neq c$.

Stabbing of the sponge tissue with a scalpel over a period of 15 sec followed by subsequent exposure to air for 4 min 45 sec similarly had no apparent effect on the alkaloid pattern (samples *b*). However, the pronounced increase in tissue destruction caused by grinding the tissue in a mortar for 15 sec substantially changed the metabolite composition (samples *c*). By this treatment, a heterogeneous mixture of different-sized tissue pieces was obtained. As a result, aeroplysinin-1 (**5**) abundance in the sponge tissue increased more than 3-fold when compared to samples "a" or "b" (Table 2), whereas the concentrations of aerophobin-2 (**1**) and isofistularin-3 (**3**) decreased markedly.

When the grinding time of the sponge tissue was extended to 5 min (sample "d"), the tissue was more effectively crushed than observed for sample "c," yielding a rather homogenous mixture of cellular debris. This resulted in a pronounced increase in the abundance of aeroplysinin-1 (**5**) (compared, for example, to sample "c"), which now accounted for almost two-thirds of all brominated alkaloids detected (Figure 2). In sample "e," which had been subjected to grinding for 5 min in the presence of 16% EtOH (v/v), aeroplysinin-1 accounted for almost 90% of all brominated metabolites (Figure 2). In samples "c," "d," and "e," the rise in concentration of aeroplysinin-1 was

TABLE 2. VARIATIONS IN THE ABSOLUTE CONCENTRATIONS OF BROMINATED ALKALOIDS IN CRUDE EXTRACTS OF *Aplysina aerophoba* AFTER DIFFERENT INTENSITIES OF WOUNDING

	t_0 (N = 4)	a (N = 2)	b (N = 4)	c (N = 4)	d (N = 4)	e (N = 4)
Aerophobin-2 (**1**)	136.0 ± 36.4	105.0 ± 14.3	141.8 ± 33.5	53.3 ± 25.9	15.5 ± 3.5	2.7 ± 1.8
Isofistularin-3 (**2**)	47.2 ± 13.1	36.2 ± 3.5	48.8 ± 10.2	25.1 ± 12.6	11.5 ± 2.3	6.1 ± 4.1
Aeroplysinin-1 (**5**)	11.8 ± 9.2	10.2 ± 1.1	11.9 ± 5.4	37.8 ± 14.4	62.3 ± 28.8	107.6 ± 54.4
Dienone (**6**)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	195.0 ± 49.5	151.4 ± 15.4	202.5 ± 46.8	116.1 ± 45.6	89.3 ± 30.2	116.4 ± 51.3

Quantities are expressed in μmol of compound per gram dry weight of sponge tissue ± SD. n.d. = not detected in the sample by HPLC-UV.
t_0 Controls that were immediately flash frozen.
a Controls that were kept exposed to air for 5 min before flash freezing.
b Tissue pieces that were stabbed over 15 sec with a scalpel and were then subsequently left untreated for another 4 min 45 sec before flash freezing.
c Tissue pieces that were ground over 15 sec and were subsequently left untreated for another 4 min 45 sec before flash freezing.
d Tissue pieces that were ground over 5 min.
e Tissue pieces that were ground over 5 min after 16% (v/v) EtOH had been added.
Randomized block analysis of variance: aerophobin-2: $F = 24.68$, $P < 0.001$; isofistularin-3: $F = 15.21$, $P < 0.001$; aeroplysinin-1: $F = 7.98$, $P < 0.001$. Tukey–Kramer multiple comparisons (significant differences are indicated, only): aerophobin-2: $c \neq t_0$; $c \neq b$, $d \neq t_0$, $d \neq a$, $d \neq b$, $e \neq t_0$, $e \neq a$, $e \neq b$; isofistularin-3: $c \neq t_0$; $c \neq b$, $d \neq t_0$, $d \neq b$, $e \neq t_0$, $e \neq b$; aeroplysinin-1: $e \neq t_0$, $e \neq a$, $e \neq b$, $e \neq c$.

paralleled by an equally impressing decrease in the concentrations of isofistularin-3 (**3**) or of aerophobin-2 (**1**) (Table 2). Dienone (**6**) was not detected in any of the samples.

A control experiment was performed with the Mediterranean sponge *C. crambe* that contains no brominated alkaloids. Aeroplysinin-1 (**5**), aerophobin-2 (**1**), and isofistularin-3 (**3**) isolated from *A. aerophoba* were added to fresh tissue of *C. crambe*. The concentrations remained unchanged (recovery rates of 97–98%) after they were ground with *C. crambe* tissue over 5 min (similar treatment to sample "d" in the experiment with *A. aerophoba*; Table 2) (Figure 3). Neither aeroplysinin-1 (**5**) nor dienone (**6**) was detected in the ground samples that had been mixed with aerophobin-2 (**1**) and isofistularin-3 (**3**). In the ground sample that had been mixed with aeroplysinin-1 (**5**), no dienone (**6**) was detected. The metabolite composition of *C. crambe* also remained unaffected by the treatment.

Time Course Experiment. Grinding of fresh *A. aerophoba* tissue for 15 sec and subsequent collection of samples at set time intervals revealed a gradual and time-dependent increase in the relative proportions of aeroplysinin-1 (**5**) from

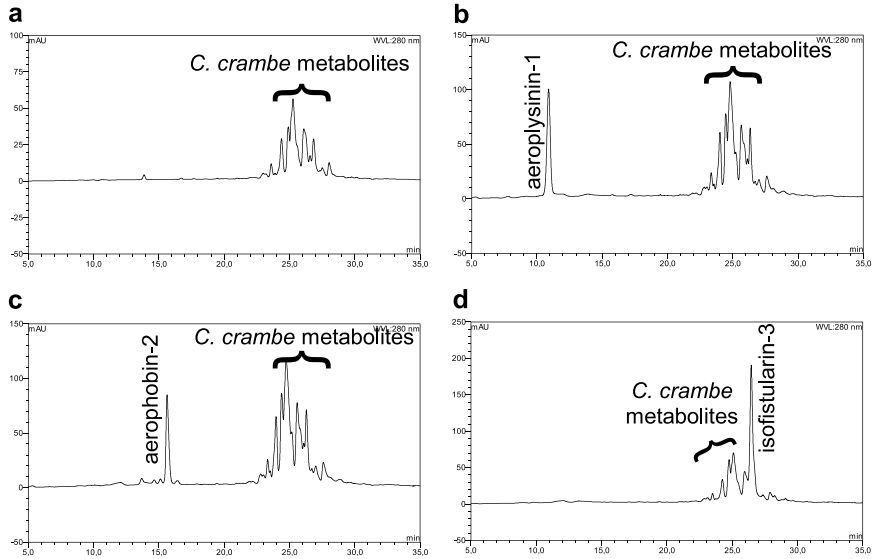

FIG. 3. Comparative HPLC analysis of crude extracts from ground fresh *Crambe crambe* tissue spiked with *A. aerophoba* metabolites (grinding time: 5 min). (a) *C. crambe* tissue without *A. aerophoba* metabolites added. (b) *C. crambe* tissue + aeroplysinin-1 (**5**). (c) *C. crambe* tissue + aerophobin-2 (**1**). (d) *C. crambe* tissue + isofistularin-3 (**3**). Recovery rates for *A. aerophoba* metabolites after grinding: 97–98%.

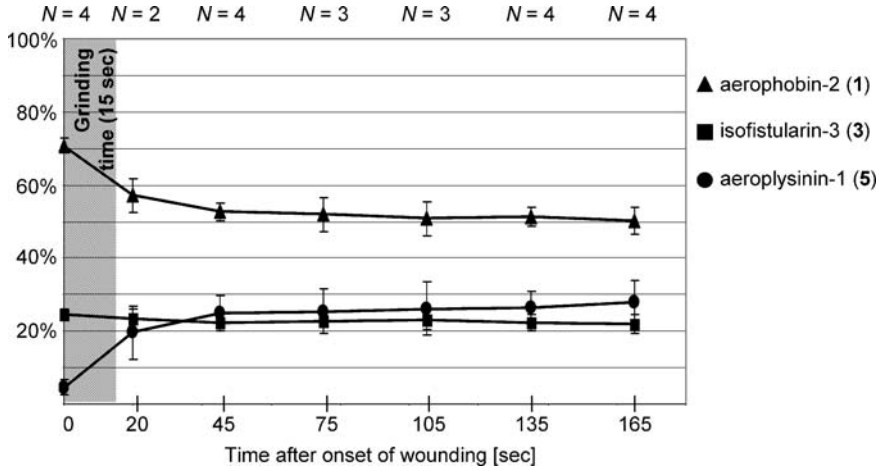

FIG. 4. Variations in the relative concentrations of aerophobin-2 (**1**), isofistularin-3 (**3**), and aeroplysinin-1 (**5**) in *A. aerophoba* tissue after 15 sec of grinding over a 165-sec time course. Proportions of the respective alkaloids are given in percent relative to the total brominated alkaloid content (set at 100%) in the sponge. Data were arcsine-transformed prior to statistical analysis. Randomized block analysis of variance: aeroplysinin-1: $F = 10.75$, $P < 0.001$; aerophobin-2 and isofistularin-3: no statistically significant differences. Tukey–Kramer multiple comparisons for aeroplysinin-1 (significant differences are indicated only): $0 \neq 20$ sec, $0 \neq 45$ sec, $0 \neq 75$ sec, $0 \neq 105$ sec, $0 \neq 135$ sec, $0 \neq 165$ sec.

4.5 ± 2.0% in undamaged controls (t_0) to 25.0 ± 4.8% in samples taken after 45 sec (Figure 4). Thereafter, the aeroplyinin-1 concentrations remained largely unchanged until the observation was terminated after 165 sec.

Experiments with Lyophilized Animal Tissue. In lyophilized tissue of intact *Aplysina* sponges from the Mediterranean Sea (*A. aerophoba* and *A. cavernicola*) and from the Caribbean Sea (*A. fistularis* and *A. archeri*), brominated isoxazoline alkaloids consistently dominated over aeroplysinin-1 (**5**) or dienone (**6**) (Tables 1A, B, and 3) even though the alkaloid concentrations showed considerable inter- and intraspecific variation. Following incubation of lyophilized tissue in seawater, the concentrations declined in a pronounced manner, whereas the concentrations of both aeroplysinin-1 (**5**) and the dienone (**6**) increased sharply (Table 3, Figure 5). This phenomenon was consistent for all analyzed samples.

A second experiment was performed with lyophilized tissue of *A. archeri*. This sponge contains large amounts of various isoxazoline alkaloids (Table 3). Aerophobin-2 (**1**), however, which is a major alkaloid in *A. aerophoba*, was not detected in the specimens of *A. archeri* available for this study. Powdered

TABLE 3. CHANGES IN THE BROMINATED ALKALOID CONTENT IN GROUND LYOPHILIZED TISSUE OF DIFFERENT *Aplysina* SPECIES UPON OVERNIGHT INCUBATION IN SEAWATER

Sponge species	Individual no.	Before seawater treatment			After seawater treatment		
		Isoxazol.	Aeroplys-1	Dienone	Isoxazol.	Aeroplys-1	Dienone
A. aerophoba	1	184.7	26.7	n.d.	43.1	130.4	19.9
	2	232.7	6.4	n.d.	44.3	149.8	18.6
	3	214.9	7.0	0.9	72.8	1.7	43.4
A. cavernicola	1	284.7	12.3	n.d.	22.6	10.8	173.7
	2	223.3	9.4	n.d.	11.9	6.1	122.7
	3	115.1	4.0	6.9	2.4	1.8	76.5
A. fistularis	1	95.3	0.0	0.7	20.4	n.d.	13.2
	2	67.0	0.3	2.2	6.9	0.4	23.4
	3	83.4	0.0	1.4	20.5	n.d.	10.7
A. archeri	1	327.1	0.0	0.8	36.9	2.2	257.1
	2	173.8	11.7	n.d.	90.0	121.8	11.3
	3	196.7	5.7	n.d.	6.9	233.8	71.9

Quantities are expressed in μmol of compound per gram dry weight of sponge tissue.
Isoxazol. = pooled content of all detected isoxazoline alkaloids in the tissue; aeroplys-1 = aeroplyinin-1 (**5**) content; n.d. = not detected in the sample by HPLC-UV.

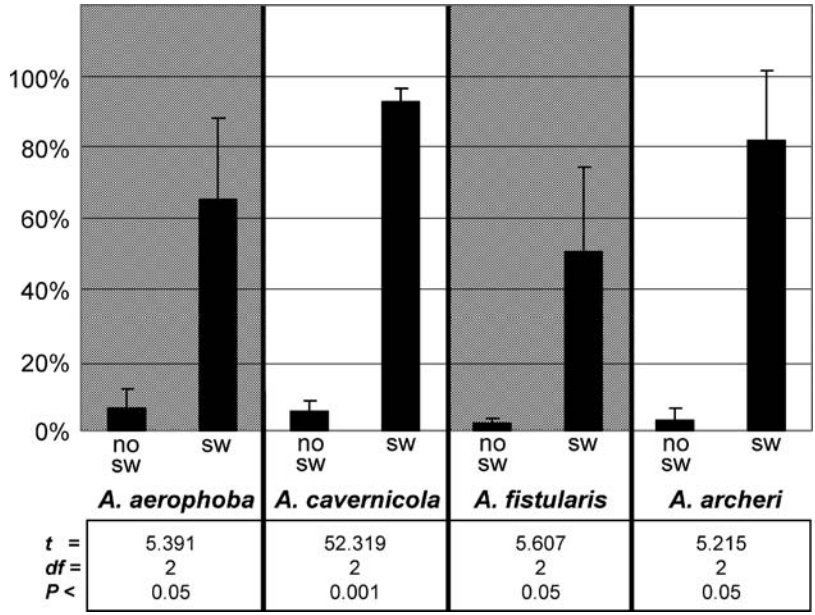

FIG. 5. Variations in the relative concentration of the postulated bioconversion products in ground lyophilized tissue of various *Aplysina* species upon treatment with seawater. The concentrations of bioconversion products aeroplysinin-1 (**5**) and dienone (**6**) are pooled. Their proportion is given in percent relative to the total brominated alkaloid content (set at 100%) in the respective sponge. Data were arcsine-transformed prior to statistical analysis. no sw = before treatment with seawater; sw = after treatment with seawater.

lyophilized tissue of *A. archeri* was spiked with aerophobin-2 (**1**) and subsequently incubated in seawater. Extraction and chromatographic analysis of the sample revealed that the added aerophobin-2, as well as the native isoxazoline alkaloids originally present in *A. archeri*, completely disappeared, whereas dienone (**6**) showed a considerable increase when compared to a parallel sample that had been lyophilized and extracted without seawater (Figure 6). In contrast, concentrations of the isoxazoline alkaloids aerophobin-2 (**1**) and isofistularin-3 (**3**) added to lyophilized and ground tissue of the sponge *C. crambe* remained unchanged, and neither aeroplysinin-1 (**5**) nor dienone (**6**) occurred after the tissue had been incubated in seawater [resulting HPLC chromatograms were similar to those shown for the experiments with fresh tissue of *C. crambe* (Figure 3) and, therefore, are not shown separately]. Similarly, in mantle tissue of the marine opisthobranch *T. perversa*, the

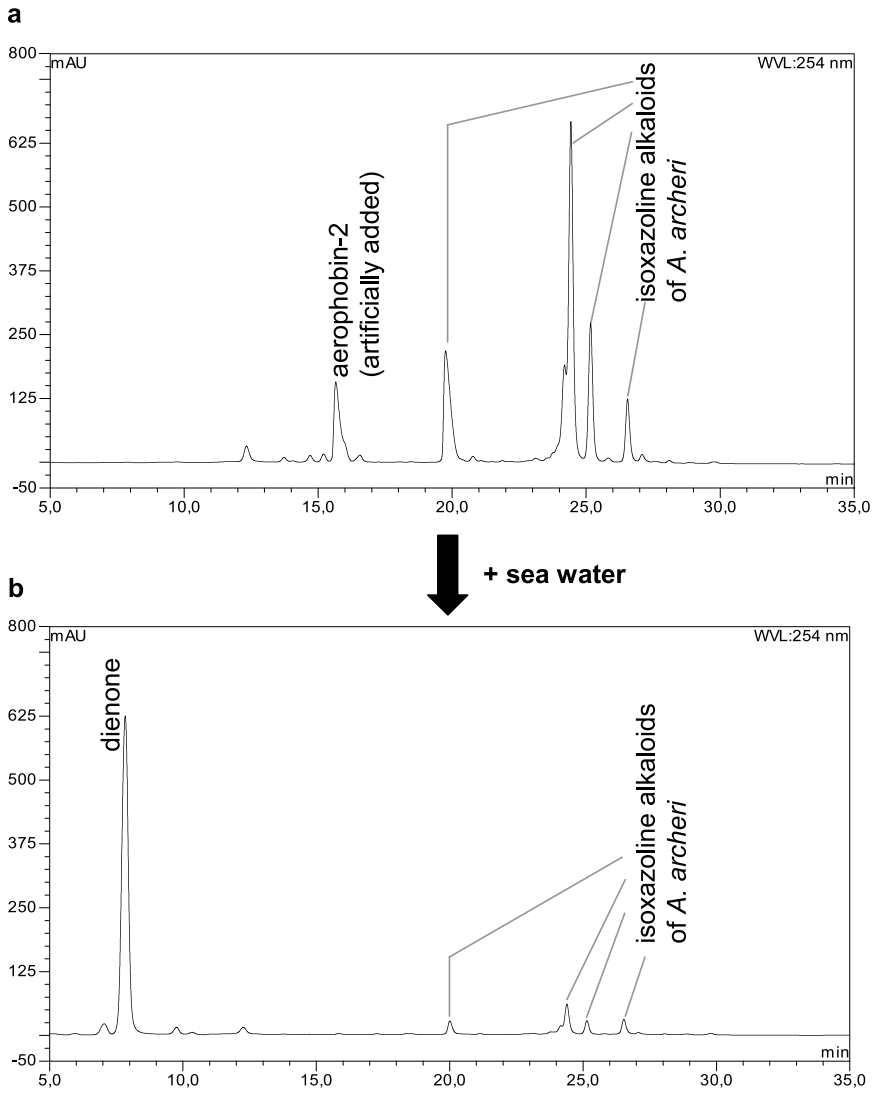

FIG. 6. Comparative HPLC analysis of ground lyophilized tissue of *Aplysina archeri* spiked with aerophobin-2 (**1**) before (a) and after (b) treatment with seawater. The signal intensity axis in both chromatograms is uniformly scaled to facilitate direct comparison.

concentrations of naturally occurring isoxazoline alkaloids remained unchanged, and neither aeroplysinin-1 nor dienone was detected after incubation of the lyophilized and ground material in seawater (data not shown).

DISCUSSION

Comparison of *A. aerophoba* individuals collected at Banyuls-sur-mer with those collected at Rovinj revealed considerable qualitative and quantitative differences in alkaloid patterns (Table 1A, B). The isoxazoline alkaloid aplysinamisin-1 (**2**) that consistently dominated the alkaloid pattern of the sponges from the French Mediterranean coast was not detected in sponges from Croatia. In sponges collected at Rovinj, we detected minor amounts of aeroplysinin-1 (**5**), which, in turn, were not found in individuals from Banyuls (Table 1A, B).

Within 10 specimens of *A. aerophoba* collected along a transect spanning several kilometers at Rovinj, the qualitative composition of the alkaloid patterns turned out to be remarkably similar (Table 1A). However, the absolute contents of brominated alkaloid showed a pronounced variability. Even different tissue samples within a single individual had considerable differences in total brominated alkaloid content. Pronounced variability of secondary metabolite concentrations is a commonly observed phenomenon in Porifera (e.g., Becerro et al., 1995; Betancourt-Lozano et al., 1998; Page et al., 2005), and in regard to the distinct natural variability of the alkaloid pattern in sponges of the genus *Aplysina*, our observations are in accordance with those of Puyana et al. (2003).

This natural variability in *Aplysina* sponges complicates investigations on artificially induced changes in the alkaloid profile, as large standard deviations resulting from replicates with erratic values are likely to obscure possible changes. For our experiments on the injury-induced bioconversion, we, therefore, selected the sponges from Rovinj that showed similarity in relative compositions of their alkaloids. By comparison before and after an experimental treatment (next to the comparison of the absolute values), we circumvented the problem of the vast standard deviations arising when absolute values are compared. We also complimented the experiments involving fresh tissue of *A. aerophoba* with experiments employing lyophilized sponge material. We had determined in our earlier studies (Teeyapant and Proksch, 1993) that the latter has the advantage of being amenable to grinding and homogenization prior to experimental treatment without inducing changes in the alkaloid pattern. Hereby, identical starting material is provided for all samples of an experiment, and even absolute values of the metabolite contents can be compared.

We caused injuries of different intensities to fresh, living tissue of *A. aerophoba* individuals (Table 2, Figure 2). In all samples, the reaction time, i.e.,

the period of time between the onset of the wounding and the flash freezing of the sample, was kept constant at 5 min. Thus, the only variable parameter was the intensity of wounding. In tissue samples that were only damaged to the degree necessary for removal of the sample from the whole sponge (samples "a"), the isoxazoline alkaloid (**1** and **3**) proportion relative to the total brominated alkaloid content amounted to 93.2 ± 0.1% (Figure 2). Alkaloid proportions in these samples were almost identical to samples that were immediately flash frozen after being cut from the sponge (samples "t_0"). Also, wounding by stabbing with a scalpel (samples "b") did not cause any observable changes in the alkaloid patterns. However, grinding the tissue over 15 sec lowered the isoxazoline alkaloid (**1**+**3**) proportion to 66.9 ± 11.9%, whereas proportions of aeroplysinin-1 (**5**) increased (samples "c"). By grinding for 5 min, the sponge tissue was more finely homogenized, causing a decrease in the isoxazoline alkaloid proportion to 32.7 ± 12.0% (samples "d"). Addition of EtOH before 5 min grinding resulted in an isoxazoline alkaloid proportion of merely 9.6 ± 7.5% (samples "e"). The absolute content of the alkaloids (**1** + **3**) consequently decreased from an original value of 183.3 ± 49 μmol g^{-1} dry weight in the samples "t_0" to 8.8 ± 5.0 μmol g^{-1} in samples "e" (Table 2). At the same time, the aeroplysinin-1 (**5**) content increased from 11.8 ± 9.2 to 107.6 ± 54.4 μmol g^{-1} dry weight sponge tissue. Thus, this experiment revealed a clear relationship between intensity of injury, decline of isoxazoline alkaloids (**1** + **3**), and rise of aeroplysinin-1 (**5**).

A time course experiment conducted for an aliquot of sample "c" that ran over a total of 165 sec (15 sec grinding followed by an additional exposure of the treated tissue for 150 sec) demonstrated the time-dependent increase in aeroplysinin-1 (**5**) (Figure 4). The injury-induced reactions take place within less than 1 min after wounding.

The capability of converting isoxazoline alkaloids to aeroplysinin-1 (**5**) and to the dienone (**6**), which possibly arises from the former by enolether hydrolysis and by partial hydrolysis of the nitrile group thereby giving rise to the amide function, can also be demonstrated for other *Aplysina* sponges such as *A. cavernicola*, *A. fistularis*, and *A. archeri*. From the latter three, only freeze-dried material was available. To obtain comparable results for the different sponges tested, *A. aerophoba* tissue was also lyophilized. Lyophilized and ground material of each *Aplysina* sponge analyzed was divided into two aliquots. One cohort was extracted with MeOH, the other aliquot was incubated in seawater, again lyophilized and subsequently extracted with MeOH. HPLC analysis revealed clear differences in alkaloid profiles between the two treatments (Table 3). Isoxazoline alkaloids dominated in all sponges that had been directly extracted after lyophilization, whereas samples that had been incubated in seawater showed decreased levels and dramatic increases in amounts of aeroplysinin-1 (**5**) and/or dienone (**6**). Exogenously added

aerophobin-2 (**1**), which was not among the brominated isoxazoline alkaloids detected in *A. archeri*, was similarly metabolized by lyophilized powder of this sponge when incubated in seawater (Figure 6). The possibility that the cleavage of the isoxazoline alkaloids is mediated by seawater alone can be excluded as it does not occur in the absence of *Aplysina* sponge tissue (or crude protein extract of these sponges) as demonstrated in our controls with tissue of *C. crambe* and *T. perversa* (Figure 3).

In contrast to compounds such as aerophobin-1 (**1**), alkaloid precursors consisting of two isoxazoline moieties (e.g., isofistularin-3 (**3**) in *A. aerophoba* and aerothionin in *A. cavernicola* and in the Caribbean *Aplysina* species) give rise to two molecules of aeroplysinin-1 upon enzymatic cleavage. This may explain the observation that the total molar concentrations of alkaloids (isoxazoline precursors + generated bioconversion products) in samples treated with seawater in some cases exceed the molar concentrations of precursors in the untreated samples (Table 3). The fact that the dienone (**6**) was also observed for *A. aerophoba* (Table 3) in experiments using lyophilized sponge tissue, whereas it had not been detected for the same sponge in the wounding intensity experiment (Table 2), can probably be explained by the different incubation times used in the experiments (overnight vs. 5 min in case of living tissue).

All experiments unequivocally suggest a wound-induced conversion of brominated isoxazoline alkaloids to aeroplysinin-1 (**5**), which may react further yielding the dienone (**6**). These results are in line with our earlier studies (Teeyapant and Proksch, 1993; Ebel et al., 1997), but are in contrast to the observations and conclusions made by Puyana et al. (2003) in their recent study. Puyana et al. (2003) suggest that our reports on the bioconversion phenomenon were the result of either (1) differential tissue extraction efficiency, (2) formation of aeroplysinin-1 (**5**) and/or dienone (**6**) upon contact of the fresh, wet sponge tissue with organic solvents as artifacts from otherwise insoluble precursors (other than the isoxazoline alkaloids), or (3) heterogeneous distribution of the putative bioconversion products in sponges. Based on these new results and on those from previous studies (Teeyapant and Proksch, 1993; Ebel et al., 1997), we believe that the arguments raised by Puyana et al. (2003) are insufficient to explain the observed phenomenon. We comment on them point by point:

(1) *Differential tissue extraction efficiency*: Upon artificial wounding of living tissue of *A. aerophoba*, an obvious correlation between wounding intensity, decrease in brominated isoxazoline alkaloids (**1** and **3**), and a concomitant rise of aeroplysinin-1 (**5**) became obvious (Table 2, Figure 2). Different extraction efficiencies as an explanation for this phenomenon are unlikely as all tissue samples had been treated the same way (5 min of exposure followed by flash freezing, lyophilization, and extraction). The only

parameter in which the samples differed was the degree of tissue damage inflicted. While in the aforementioned experiment wounding intensity varied, this parameter was identical for all samples of the time course experiment (Figure 4). Nevertheless, we observed a steady increase the aeroplysinin-1 (**5**) concentration paralleled by a decrease in isoxazoline alkaloids that was positively correlated with the time between wounding and flash freezing of the damaged tissue.

(2) *Formation of aeroplysinin-1* (**5**) *and/or dienone* (**6**) *upon contact of the fresh, wet sponge tissue with organic solvents as artifacts from otherwise insoluble precursors:* Aeroplysinin-1 (**5**) is indeed accumulated when living tissue of *A. aerophoba* contacts organic solvents as shown, for example, for sample "e" (Table 2), where living tissue of *A. aerophoba* was treated with diluted EtOH. Except for sample "e," however, all other samples of living *A. aerophoba* tissue analyzed in this study had been lyophilized prior to exposure to organic solvents. Nevertheless, in all of these samples, aeroplysinin-1 (**5**) occurred at high concentrations when the respective tissue had been damaged in a substantial manner prior to extraction (Table 2 and Figure 4). At the same time, isoxazoline alkaloid concentrations decreased significantly. Thus, the formation of artifacts upon organic solvent exposition of wet sponge tissue can be ruled out as a plausible explanation for the observed changes in the alkaloid pattern.

(3) *Heterogeneous distribution of the putative bioconversion products in sponge tissue:* As described in Table 1A and B, alkaloid patterns as well as alkaloid concentrations in different specimens of *A. aerophoba* are subject to intraspecific and even intraindividual variation. Within a given sponge population, these differences are, however, mostly quantitative and not qualitative. For example, all specimens of *A. aerophoba* collected in Rovinj (Croatia) exhibited remarkably homogenous alkaloid profiles and differed only in regard to total alkaloid concentrations (expressed as μmol/g dried tissue or as μmol/ml volume of sponge tissue). The observed changes (reproducible decrease in isoxazoline alkaloids paralleled by a concomitant increase in aeroplysinin-1) we encountered during our experiments, therefore, can not be ascribed to lack of homogeneity of the sponge samples used.

While experiments with lyophilized sponge tissue provide only limited information for processes taking place under *in situ* conditions in living tissue, these experiments have the advantage of providing homogenous material in regard to alkaloid patterns and concentrations. Therefore, differences in alkaloid concentrations of control samples vs. treated samples can not be attributed to nonhomogeneous sponge tissue in experiments using lyophilized material (Table 3, Figure 5 and 6).

The only plausible explanation for the observed changes in the alkaloid patterns of *Aplysina* species is the effect of wounding itself, which results in a breakdown of cellular compartmentalization. We assume that the spherulous cells containing the isoxazoline alkaloids are broken down by mechanical damage. This results in the activation of the bioconversion of isoxazoline alkaloids yielding aeroplysinin-1 (**5**), which may further react to the dienone (**6**). This process would be similar to other known wound-induced bioconversion reactions (e.g., cleavage of cyanogenic glycosides or glucosinolates) in the plant and animal kingdom (Conn, 1979; Paul and van Alstyne, 1992; Wajant and Effenberger, 1996).

The hypothesis that aeroplysinin-1 (**5**) and dienone (**6**) arise from a cleavage of isoxazoline alkaloids is corroborated by a series of further observations: (1) after damaging tissue of *Aplysina* sponges, we never observed an increase in the concentration of the postulated bioconversion products aeroplysinin-1 (**5**) and dienone (**6**) without a concomitant decrease in the concentrations of the isoxazoline alkaloids; (2) the structures of the postulated bioconversion products (**5** + **6**) are directly deducible from the structures of the brominated isoxazoline alkaloids (**1**–**3**) (Figure 1); (3) the bisoxazolidinone derivative (**4**) would be expected as a byproduct of the proposed cleavage of isofistularin-3 (**3**) into aeroplysinin-1 (**5**). When performing *in vitro* assays on the bioconversion of isofistularin-3 using a cell-free extract of *A. aerophoba*, we detected the formation of the bisoxazolidinone derivative (**4**) next to aeroplysinin-1 (**5**) and dienone (**6**) (Ebel et al., 1997).

The fact that Puyana et al. (2003) failed to detect alkaloid conversions is probably attributable to insufficient wounding of the sponges, as a mere stabbing of sponge tissue with a scalpel was insufficient to provoke a measurable conversion of isoxazoline alkaloids in our study as well (Table 2). More severe tissue damage as can be achieved by grinding are necessary (Table 2), as otherwise small changes in the alkaloid composition that will occur only in injured cells are likely to be masked by the unchanged alkaloid profiles remaining in the intact cells that will by far outnumber the former.

Activated chemical defense mechanisms, i.e., rapid conversions of inactive precursor compounds into ecologically active products (reviewed in Havel, 1986; Adler and Harvell, 1990; Paul and Puglisi, 2004), are typically catalyzed by enzymes that are usually separated from their substrates (precursors) by compartmentalization. Upon tissue damage, these compartments are disrupted, facilitating the contact between substrates and the enzymes, which in turn induces the bioconversion. In many cases, the compounds emanating from such reactions arise from cleavage of the precursors. Compartmentalization is also observed in the mesohyl tissue of sponges of the genus *Aplysina*: the brominated isoxazoline alkaloids are mainly stored in specialized cells, the so-

called spherulous cells, in the sponge tissue (Thompson et al., 1983; Turon et al., 2000).

The bioconversion reactions that are observed for living as well as for lyophilized tissue of *Aplysina* species appear to be specific for these sponges, as other marine invertebrates, the sponge *C. crambe* and the opisthobranch *T. perversa*, were unable to convert isoxazoline alkaloids. This is of special interest with regard to *T. perversa*, as this gastropod sequesters isoxazoline alkaloids from its prey *A. aerophoba* (Ebel et al., 1999; Thoms et al., 2003b). In contrast to *C. crambe*, the tissue of this gastropod "naturally" contains brominated isoxazoline alkaloids. However, after grinding and incubating lyophilized tissue of *T. perversa*, we observed no changes in alkaloid profiles or concentrations. Neither aeroplysinin-1 nor dienone was detected, indicating that no biotransformation reactions had occurred.

Earlier experiments with cell-free protein extracts from various sponges revealed that only extracts from the sponges *A. aerophoba*, *A. cavernicola* (both from the Mediterranean Sea), *A. archeri*, *A. cauliformis*, *A. fistularis*, *A. fulva*, and *A. lacunosa* (all from the Caribbean) were able to cleave isoxazoline alkaloids into aeroplysinin-1 and dienone. In cell-free protein extracts from other sponges, *Stelletta globostellata* (order Astrophorida), *Axinella carteri* (order Halichondrida), and *Theonella swinhoei* (order Lithistida) that do not accumulate brominated isoxazoline alkaloids, the concentrations of added isofistularin-3 or aerophobin-2 remained unchanged, and no aeroplysinin-1 or dienone occurred under otherwise identical conditions (Ebel et al., 1997). Alkaloid biotransformation by cell-free protein extracts from *Aplysina* sponges may be inhibited by addition of trichloric acid as well as by boiling (Ebel et al., 1997; Fendert, 2000). Substrate specificity studies that were carried out *in vitro* with naturally occurring isoxazoline alkaloids and with synthetic analogues indicated that the postulated alkaloid splitting enzyme(s) are specific for substrates containing spirocyclohexadienisoxazoline moieties and acyl amide chains (Fendert, 2000; Goldenstein et al., 2000). Unfortunately, attempts to isolate the responsible enzyme(s) from this crude extract have been unsuccessful due to activity loss during purification (Ebel, 1998). Thus, while a final proof for the enzymatic nature of the bioconversion [e.g., the purification and detailed characterization of the responsible enzyme(s)] is still lacking, our observations point toward an involvement of enzymes in the bioconversion reactions.

The question remains whether the alkaloid conversion is ecologically advantageous for *Aplysina* sponges and can be considered an example of an activated chemical defense. Choice feeding experiments with the Mediterranean fish *B. sphinx* revealed that aeroplysinin-1 (**5**), as well as dienone (**6**), possesses considerably lower feeding deterrent properties against test fishes than their precursors (e.g., aerophobin-2 or isofistularin-3) when tested at their natural concentrations (Thoms et al., 2004). Even if in the course of the bioconversion

the feeding deterrent properties intensified, this could hardly be considered an activated protection against predatory fishes. First, the bioconversion rate (45 sec to 1 min; Figure 4 and Ebel et al., 1997) would probably be too slow to deter the predators in time, before serious wounding was caused. Second, the bioconversion products would presumably only arise at the surface of the sponge tissue piece bitten off, as only here does a decompartmentalization of the spherulous cells occur. The arising bioconversion product concentration would probably be too low to have a noteworthy effect on predators. It appears unlikely that the wound-induced bioconversion of alkaloids in *Aplysina* species will increase the fitness of sponges toward fish predators.

The situation is different, however, when a defense against microbial pathogens is considered. In this case, it might possibly be sufficient if the bioconversion products were formed only in injured cells, as penetration of pathogenic microorganisms is likely to occur through wounded tissue. It is difficult to determine the actual metabolite concentration in the thin surface layer covering the wounded tissue, because it is virtually impossible to harvest and analyze only wounded cells without adherent healthy tissue that will mask the results of a chemical analysis due to the "static" alkaloid profile that will remain unchanged. The only reasonable approximation for the concentrations of aeroplysinin-1 (**5**) and the dienone (**6**) that arise locally in wounded surface tissue are the amounts of bioconversion products that are formed in finely macerated sponge tissue. We determined the concentration of aeroplysinin-1 (**5**) in tissue of *A. aerophoba* after 5 min of grinding to amount to 4400 ± 2036 μg ml^{-1} sponge tissue (Table 2). Teeyapant et al. (1993) determined the Minimum Inhibitory Concentrations (MIC) of aeroplysinin-1 and dienone against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* to range between 12.5 and 50 μg ml^{-1} depending on the bacterial species tested. Kelly et al. (2003) were able to show that seawater bacteria attachment to agar blocks treated with aeroplysinin-1 at a concentration of 1000 μg ml^{-1} was reduced to 1.4 ± 0.1% compared to untreated controls. Dienone at a concentration of 250 μg ml^{-1} reduced bacterial attachment to 4.1 ± 0.1%. An amount of 5 μg aeroplysinin-1 showed activity against two out of six marine bacteria species tested in an agar diffusion assay (Weiss et al., 1996). Dienone at the same concentration inhibited the growth of five of six species in this experiment. One hundred μg of aeroplysinin-1 as well as 100 μg dienone were active against all eight marine bacteria species tested. Debitus et al. (1998) found activity for both compounds when they tested 10 μg of them against *S. aureus* and *Vibrio anguillarum* in agar diffusion assays. They proposed dienone (**6**) for use as an antibiotic in mariculture, as their investigation revealed that it rivals chloramphenicol with regard to decreasing mortality of scallop (*Pecten maximus*) larvae in culture. Thus, the concentrations of the bioconversion products arising in ground fresh *A. aerophoba* tissue exceed concentrations that have proven effective against

bacterial invasion, suggesting that the generation of aeroplysinin-1 (**5**) and subsequently of the dienone (**6**) at the site of wounding might protect injured sponge tissue against pathogenic bacteria. Consequently, in the course of the wound-induced bioconversion, the antipredatory isoxazoline alkaloids stored constitutively in tissue of *Aplysina* sponges are cleaved into compounds with pronounced antimicrobial activity. This process seems to correlate with the change in needs of the sponges after their tissue has been wounded and reveals the multiple ecological functions that marine natural products can possess.

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