# Chemical Defense of Mediterranean Sponges *Aplysina cavernicola* and *Aplysina aerophoba*

Carsten Thoms[a], Matthias Wolff[b], K. Padmakumar[c], Rainer Ebel[a], and Peter Proksch[a,*]

[a] Institut für Pharmazeutische Biologie, Universität Düsseldorf, Universitätsstraße 1, Geb. 26.23, D-40225 Düsseldorf, Germany. Fax: +49-211-81-11923. E-mail: proksch@uni-duesseldorf.de
[b] Zentrum für Marine Tropenökologie, Fahrenheitstr. 6, D-28359 Bremen, Germany
[c] Department of Aquatic Biology and Fisheries, Kerala University Campus, Kariavattom, Trivandrum-695581, India

* Author for correspondence and reprint requests

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The Mediterranean sponges *Aplysina aerophoba* and *A. cavernicola* accumulate brominated isoxazoline alkaloids including aplysinamisin-1 (**1**), aerophobin-2 (**2**), isofistularin-3 (**3**) or aerothionin (**4**) at concentrations up to 10% of their respective dry weights. In laboratory feeding experiments employing the polyphagous Mediterranean fish *Blennius sphinx* crude extracts of both *Aplysina* sponges were incorporated into artificial fish food at their physiological concentrations (based on volume) and offered to *B. sphinx* in choice feeding experiments against untreated control food. In addition to the *Aplysina* sponges, extracts from nine other frequently occurring Mediterranean sponges were likewise included into the experiments. Both *Aplysina* species elicited strong feeding deterrence compared to the other sponges tested. Bioassay-guided fractionation of *A. cavernicola* yielded the isoxazoline alkaloids aerothionin (**4**) and aplysinamisin-1 (**1**) as well as the 3,4-dihydroxyquinoline-2-carboxylic acid (**8**) as major deterrent constituents when tested at their physiological concentrations as present in sponges. Aeroplysinin-1 (**5**) and dienone (**6**), however, which are formed in *A. aerophoba and A. cavernicola* from isoxazoline precursors through bioconversion reactions upon tissue injury showed no or only little deterrent activity. Fractionation of a crude extract of *A. aerophoba* yielded aerophobin-2 (**2**) and isofistularin-3 (**3**) as major deterrent constituents against *B. sphinx*. We propose that the isoxazoline alkaloids **1**–**4** of Mediterranean *Aplysina* sponges as well as the 3,4-dihydroxyquinoline-2-carboxylic acid (**8**) (in the case of *A. cavernicola*) act as defensive metabolites against *B. sphinx* and possibly also against other predators while the antibiotically active bioconversion products aeroplysinin-1 (**5**) and dienone (**6**) may protect sponges from invasion of bacterial pathogens.

*Key words:* Chemical Defense, Fish Feeding Assay, *Aplysina* Sponges

## Introduction

Marine sponges accumulate a large variety of structurally highly diverse secondary metabolites many of which are deterrent towards potential predators such as fishes (*e.g.* Pawlik *et al*., 1995; Schupp *et al*., 1999; Becerro *et al*., 2003), possess anti-fouling activity (*e.g.* Thompson *et al*., 1985; Martín and Uriz, 1993; Becerro *et al*., 1994) and/or suppress the growth of other competing invertebrates (*e.g.* Sullivan *et al*., 1983; Porter and Targett, 1988; Turon *et al*., 1996). Among these various ecological roles, deterrence of potential predators appears to be most significant (*e.g.* Braekman and Daloze, 1986; Proksch, 1999; McClintock and Baker, 2001 and references cited therein). Taking into consideration that sponges often live in ecosystems such as coral reefs that are characterized by an exceptionally high feeding pressure (Carpenter, 1986) and that they furthermore lack effective morphological defense mechanisms (Chanas and Pawlik, 1996), it is obvious that the frequent occurrence of deterrent and/or toxic metabolites in their tissues can be interpreted as chemical defense protecting these sessile and vulnerable invertebrates from potential predators.

However, chemical defense of marine sponges through accumulation of bioactive natural products is not only found in the tropics where feeding pressure by fishes is extreme but also in temperate marine habitats and even under the Antarctic ice cover (McClintock, 1987; Uriz *et al*., 1996; Becerro *et al*., 2003). Sponges of the order Verongida [which includes the genus *Aplysina*

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(syn. *Verongia*)], for example, are rich in structurally unusual brominated isoxazoline alkaloids which are thought to be biogenetically derived from dibromotyrosine (Gopichand and Schmitz, 1979; Tymiak and Rinehart, 1981; Carney and Rinehart, 1995). *Aplysina* species occur in the Mediterranean Sea but also in the Atlantic Ocean (*e.g.* around the Canary Isles) and in the Caribbean Sea (Pawlik *et al*., 1995) and usually contribute to the dominating sponges present in the respective habitat. The alkaloid patterns of the two Mediterranean species *A. aerophoba* and *A. cavernicola* share similarities including the presence of aerophobin-2 (**2**) and of aplysinamisin-1 (**1**) (which is not always present in *A. aerophoba*) (Teeyapant *et al*., 1993; Ciminello *et al*., 1997; Thoms *et al*., 2003a, b). The assemblages of secondary metabolites in both *Aplysina* sponges, however, differ mainly with regard to aerothionin (**4**) which occurs only in *A. cavernicola* and with regard to their yellowish pigments. The yellow color of *A. aerophoba* is due to the chemically highly labile pigment uranidine (**7**) which readily undergoes polymerization when exposed to air (Cimino *et al*., 1984) whereas *A. cavernicola* accumulates 3,4-dihydroxyquinoline-2-carboxylic acid (**8**) which is far more stable than uranidine (**7**).

Both species of Mediterranean *Aplysina* sponges feature an unusual wound-induced bioconversion of brominated isoxazoline alkaloids which gives rise to the nitrile aeroplysinin-1 (**5**) which is in turn converted to dienone (**6**) (Teeyapant and Proksch, 1993; Ebel *et al*., 1997). The bioconversion products aeroplysinin-1 (**5**) and dienone (**6**) were previously shown to be biologically strongly active against marine bacteria and algae whereas their isoxazoline precursors were not (Teeyapant *et al*., 1993; Weiss *et al*., 1996). So far, however, no detailed studies on the fish deterrent properties of isolated natural products present in *A. aerophoba* and *A. cavernicola* have been conducted. Since both *Aplysina* species are among the most common sponges found in the Mediterranean Sea (especially *A. aerophoba*) and are apparently not preyed upon by fishes in their natural habitat we initiated a detailed study on the fish deterrent properties of the crude extracts and of the major secondary metabolites of *A. aerophoba* and *A. cavernicola* using the polyphagous Mediterranean fish *Blennius sphinx* as a model organism.

## Material and Methods

### *Collection of sponge material and preparation of crude extracts*

Specimens of ten different sponge species including *A. cavernicola* were collected by SCUBA diving off the coast of Elba in the Mediterranean Sea in April 2000. All samples were kept submerged in zip-lock bags filled with seawater during transportation to the laboratory. Before preserving them in EtOH, weight and volume (measured by displacement of water) of all collected sponges were determined. Following determination of their volumes all sponge samples were minced with a blender and subsequently extracted at room temperature exhaustively in MeOH and $CH_2Cl_2$. EtOH, MeOH and $CH_2Cl_2$ extracts were combined to give crude extracts which in the following are referred to as extracts from EtOH preserved sponges. The collected biomass of *A. cavernicola* was divided into two parts. One part was extracted as explained above whereas the second part of the tissue was lyophilized, ground with a mortar and extracted exhaustively with MeOH and $CH_2Cl_2$. Specimens of the sponge *Aplysina aerophoba* that had been collected at Rovinj, Croatia were likewise lyophilized and extracted. The MeOH and $CH_2Cl_2$ extracts were subsequently combined to give crude extracts which in the following are referred to as extracts from lyophilized sponges.

### *Fractionation of the crude extracts from A. aerophoba and A. cavernicola*

The crude extracts of lyophilized *A. aerophoba* or *A. cavernicola* were taken to dryness and partitioned between EtOAc and $H_2O$. 3,4-Dihydroxyquinoline-2-carboxylic acid (**8**) was mainly detected in the aqueous phase resulting from partitioning of the *A. cavernicola* extract. It was isolated and purified by repeated gel permeation chromatography over LH-20 Sephadex (Amersham Pharmacia Biotech, Freiburg, Germany) using MeOH as a solvent. Compound identification was based on comparison of NMR spectra with previously described data (Fattorusso *et al*., 1971; this study describes 3,4-dihydroxyquinoline-2-carboxylic acid as derived from the sponge *A. aerophoba* instead of from *A. cavernicola*; later the collected sponge proved to be *A. cavernicola*).

The EtOAc phases resulting from partitioning of the crude extracts of lyophilized *A. aerophoba* and *A. cavernicola* were also taken to dryness and

further partitioned between MeOH/$H_2O$ and cyclohexane followed by a separation of the MeOH/$H_2O$ layers by column chromatography on Sephadex LH-20 using MeOH as a solvent. Further purification was achieved by repeated column chromatography on silica gel using various mixtures of $CH_2Cl_2$ and 2-propanol as solvent systems. Fractions were monitored by TLC on pre-coated silica gel plates (Merck, Darmstadt, Germany) employing the same solvent systems and by HPLC analysis using an HPLC system coupled to a photodiode-array detector (Dionex, Germany). Routine detection was at 254 nm. The separation column (125 × 4 mm i.d.) was prefilled with Eurosphere C-18 (5 $\mu$m) (Knauer, Germany). A solvent system consisting of 0.02% phosphoric acid at pH 2 and MeOH at a gradient increasing linearly from 10% MeOH to 100% within 35 min was used for compound separation. Identification of compounds isolated from both *Aplysina* sponges was based on their online UV spectra, on HPLC/ESIMS data and on direct comparison with previously isolated standards (Ebel *et al*., 1997). All compounds were quantified by HPLC using calibration curves obtained for the respective isolated natural products.

For isolation of aeroplysinin-1 (**5**) and dienone (**6**) fresh sponge biomass (containing $H_2O$) that had been immersed into EtOH immediately after collection was used. Compounds were isolated, identified and quantified as described above for the isoxazoline alkaloids isolated from lyophilized *A. cavernicola* material.

### *Fish feeding bioassays*

For feeding experiments with crude sponge extracts and/or fractions derived thereof the respective amounts (in mg) obtained following extraction of 10 ml of EtOH preserved sponge tissue were incorporated into the same volume of artificial fish food. For assays involving isolated pure metabolites of *A. cavernicola* and *A. aerophoba* quantification was performed by HPLC as described above. Aerothionin (**4**), aplysinamisin-1 (**1**) and isofistularin-3 (**3**) (precursors of the injury-induced bioconversion) as well as compound **8** were quantified in lyophilized sponge tissue. For quantification of the bioconversion products aeroplysinin-1 (**5**) and dienone (**6**) EtOH preserved material of fresh *A. cavernicola* was used. Artificial fish food was treated with physiological concentrations of secondary sponge metabolites as determined in 10 ml EtOH preserved sponge material. In addition to experiments with crude extracts, fractions and pure metabolites, feeding assays with mixtures of pure sponge compounds [(3,4-dihydroxyquinoline-2-carboxylic acid + aerothionin) and (3,4-dihydroxyquinoline-2-carboxylic acid + dienone)] were also performed. In these latter bioassays each compound tested was incorporated into fish food at its respective physiological concentration as quantified in 10 ml of sponge tissue.

The fish feeding assays performed in this study were modeled based on a method previously described by Hay *et al*. (1998). This method had originally been designed for feeding experiments with sea urchins but was later successfully adapted by Schupp *et al*. (1999) for fish feeding assays. All metabolites used for feeding experiments were dissolved in MeOH and incorporated into 1.053 g of ground commercial fish food granules (Tetra-Marin Granulat, TetraWerke, Melle, Germany). This mixture was then added to 9 ml of 2% molten agar at 48 °C to give an overall volume of fish food of 10 ml. Thus the concentrations of extracts, fractions and pure compounds used in the bioassays matched the natural concentrations as found in the sponges. Aeroplysinin-1 (**5**) was incorporated into the artificial fish food at the molar concentration as quantified for dienone (**6**) in the EtOH preserved *A. cavernicola* material. Control food was likewise prepared but food granules were mixed only with MeOH without sponge metabolites. All freshly prepared food mixtures (with or without sponge metabolites) were then poured into two rectangular molds (2 mm × 25 mm × 250 mm) placed on a window screen mesh. One of the molds was filled with treated, the other one with control food. When the agar had cooled completely, it became firmly attached to the window screen. By cutting it perpendicular to the food pieces, six equivalent food strips were obtained, each of them containing one rectangle with treated food and one control food piece (each covering an area of 10 × 15 window screen squares) (Fig. 1).

Feeding experiments were performed in an aquarium (size: 170 cm × 80 cm × 40 cm) using 70 individuals of the polyphagous Mediterranean fish *Blennius sphinx*. Food strips containing one treated and one control food rectangle were placed at random positions on the bottom of the aquarium. Six parallel experiments (with one sponge extract or fraction, respectively) or less

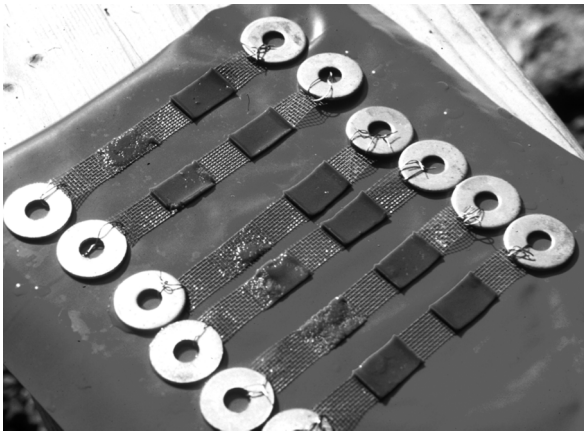

Fig. 1. Food strips containing agar based control food pieces and food pieces treated with sponge metabolites. The picture was taken after preliminary feeding assays employing different sponge extracts.

were usually carried out per day. The food strips were checked at regular time intervals and were removed when approximately a third part of the total food mass had been consumed. As each fish bite made up only for a minute amount of agar based food, few trial bites at strongly feeding deterrent food were almost not visible. The removal of one third of food treated with an edible extract or of control food corresponded to approximately 100 fish bites. Individual experiments lasted between one and two hours. To determine the amount of control and treated food eaten, the number of empty squares in the window screen was counted (Hay *et al*., 1998). The results obtained from several replicate experiments were pooled and analyzed with a paired *t*-test.

### *Determination of nitrogen and carbon content in sponge tissues and in the artificial fish food granules*

Three pieces of each sponge species were lyophilized and ground with a mortar. About 0.3 g of each sample were then measured with a Inductively Coupled Plasma Emission Spectroscope (Jobin Yvon/HORIBA Ltd.). Carbon and nitrogen content in the sponge tissues were determined as percentages of their dry weights in order to facilitate a comparison with the nutrient values of the commercial fish food granules used for the feeding experiments.

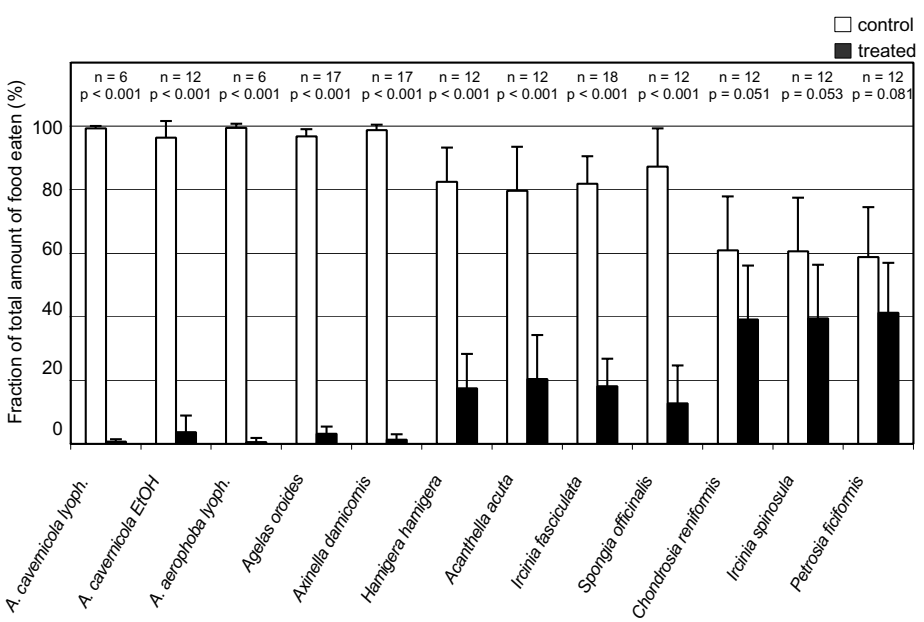


Fig. 2. Feeding experiments with crude extracts from 11 Mediterranean sponge species towards *Blennius sphinx*. Crude extract from *A. cavernicola* was derived from lyophilized (*lyoph.*) and from EtOH preserved (*EtOH*) fresh sponge tissue. Crude extract from *A. aerophoba* was derived only from lyophilized, all other sponges only from EtOH preserved material.

## Results

### *Intensity of feeding deterrence of crude extracts from eleven Mediterranean sponge species*

The results of the feeding experiments with crude extracts of 11 Mediterranean sponges are shown in Fig. 2. Nine of the analyzed extracts proved to be significantly repellent against the fishes. While food treated with crude extracts from *Aplysina cavernicola*, *A. aerophoba*, *Agelas oroides*, and *Axinella damicornis* remained almost untouched by *B. sphinx*, food containing extracts of *Hamigera hamigera*, *Acanthella acuta*, *Ircinia fasciculata*, and *Spongia officinalis* was consumed more often but still significantly less than the untreated control food (Fig. 2). There was no clear difference in feeding deterrence between the extract from lyophilized *A. cavernicola* and the one obtained by extraction of fresh sponge tissue. Artificial fish food treated with extracts from *Chondrosia reniformis*, *Ircinia spinosula*, and *Petrosia ficiformis* on the other hand had no significant deterrent effects on the fishes.

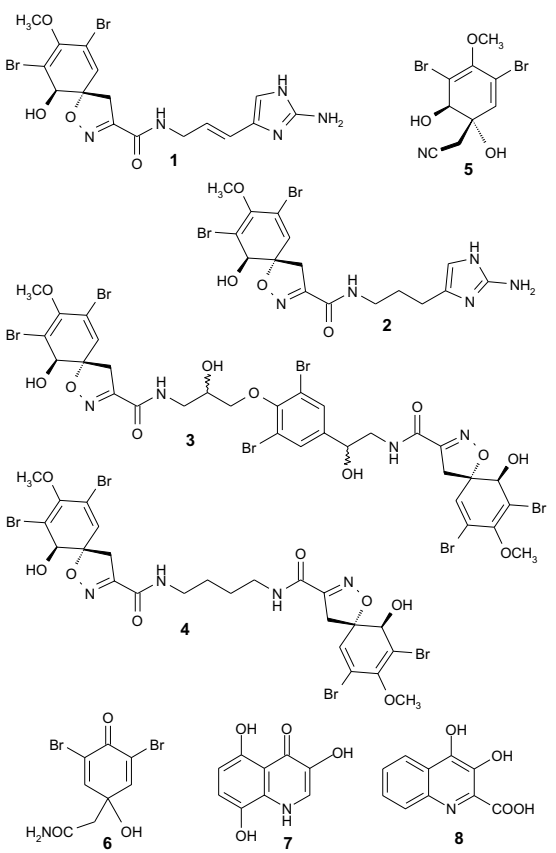


Fig. 3. Structures of *Aplysina* alkaloids: aplysinamisin-1 (**1**), aerophobin-2 (**2**), isofistularin-3 (**3**), aerothionin (**4**), aeroplysinin-1 (**5**), dienone (**6**), uranidine (**7**), and 3,4-dihydroxyquinoline-2-carboxylic acid (**8**).

### *Intensity of feeding deterrence of pure metabolites from A. cavernicola and A. aerophoba*

Bioassay-guided fractionation of the crude extract of lyophilized tissue of *A. cavernicola* using the above described feeding bioassay with *B. sphinx* resulted in the isolation of aplysinamisin-1 (**1**), aerophobin-2 (**2**), aerothionin (**4**) and of 3,4-dihydroxyquinoline-2-carboxylic acid (**8**) as deterrent secondary metabolites (Fig. 3). All of these sponge metabolites when tested at their natural concentrations proved to be highly deterrent to *B. sphinx* with very few fish bites observed at the artificial food pellets (Fig. 4). When the major brominated isoxazoline alkaloid aerothionin (**4**) and 3,4-dihydroxyquinoline-2-carboxylic acid (**8**) were jointly incorporated into the artificial food pellets at their respective natural concentrations an increase of feeding deterrence of this natural mixture towards *B. sphinx* over the individual respective metabolites was observed (Fig. 4).

An extract prepared from EtOH preserved fresh *A. cavernicola* proved to be almost deterrent towards *B. sphinx* as the one prepared from lyophilized sponge tissue (Fig. 2) even though HPLC analysis revealed both extracts to be very different with regard to their major secondary metabolites. Whereas the extract from lyophilized *A. cavernicola* featured aerothionin (**4**), aplysinamisin-1 (**1**), aerophobin-2 (**2**) and **8** as major UV-absorbing compounds the extract prepared from EtOH preserved fresh sponge tissue was characterized by two major peaks which included **8** and dienone (**6**). Origin of the latter through bioconversion of isoxazoline precursors in aqueous media containing organic solvents has been demonstrated before (Ebel *et al*., 1997). A known intermediate of this bioconversion of isoxazoline alkaloids is the nitrile aeroplysinin-1 (**5**) (Fig. 3) (Ebel *et al*., 1997). When the bioconversion products aeroplysinin-1 (**5**) and dienone (**6**) were incorporated into artificial fish food (each tested at the concentration of the latter compound as elucidated through HPLC analysis of the extract prepared from EtOH preserved *A. cavernicola*) aeroplysinin-1 (**5**) proved to be weakly (compared for example to **3** or **4**) but nev-

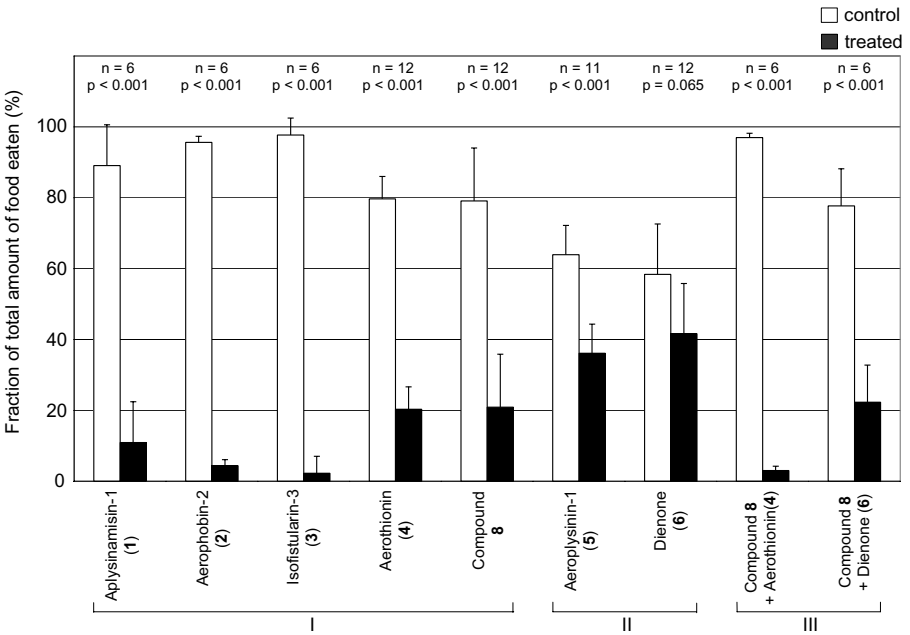


Fig. 4. Feeding experiments with isolated metabolites from *Aplysina* sponges towards *Blennius sphinx*.
(I) Metabolites isolated from lyophilized sponge tissue: Aerothionin (**4**), aplysinamisin-1 (**1**), and 3,4-dihydroxyquinoline-2-carboxylic acid (**8**) were tested in concentrations as determined in *A. cavernicola*, isofistularin-3 (**3**), and aerophobin-2 (**2**) as determined in *A. aerophoba* tissue.
(II) Metabolites isolated from EtOH preserved tissue: Dienone (**6**) was tested in concentrations as determined in *A. cavernicola*. Aeroplysinin-1 (**5**) was tested at the same molar concentration as dienone (**6**).
(III) Mixtures of 3,4-dihydroxyquinoline-2-carboxylic acid (**8**) with other pure metabolites isolated from *A. cavernicola* tissue.

ertheless significantly deterrent towards *B. sphinx* whereas dienone (**6**) had no feeding deterrent effect at the chosen concentration (Fig. 4). When dienone (**6**) and compound **8** isolated from *A. cavernicola* were jointly added to artificial fish food and presented to *B. sphinx* the intensity of feeding deterrence was comparable to that of **8** alone (Fig. 4). Thus there was no significant additive effect caused by the presence of dienone (**6**), while in feeding experiments with compound **8** plus aerothionin (**4**) an enhanced deterrent effect was observed (Fig. 4).

The extract of lyophilized tissue of *A. aerophoba* which had likewise proven to be highly deterrent towards *B. sphinx* contained isofistularin-3 (**3**) and aerophobin-2 (**2**) as major brominated isoxazoline alkaloids as indicated by HPLC analysis (Fig. 3). In addition traces of the chemically unstable pigment uranidine (**7**) which readily polymerizes when exposed to oxygen were detected. Due to the instability of uranidine which prevented accurate quantification in the sponge tissue this compound was not included into the fish feeding bioassays. Isofistularin-3 (**3**) and aerophobin-2 (**2**), however, just like the brominated isoxazoline alkaloids isolated from *A. cavernicola* (**1** and **4**) proved to be highly deterrent towards *B. sphinx* when added to artificial fish food at its natural concentration as present in lyophilized sponge tissue (Fig. 4).

### *Nitrogen and carbon content in analyzed sponges and artificial fish food*

For an assessment of the nutritional values of the sponge tissues and for comparison with the artificial fish food used in this study nitrogen and carbon contents were measured. The data revealed that the nitrogen fractions in the sponge samples vary from 5.05% of dry weight as was the case for *H. hamigera* to 11.02% as found for *A. oroides*. Carbon contents of the analyzed sponges ranged from 25.30% for *A. damicornis* to 39.42% as found for *I. spinosula*. Nitrogen and

carbon contents of the artificial fish food employed in this study (7.47 and 45.03%, respectively) were almost in the same ranges as found in the sponge specimens.

## Discussion

The remarkable chemical diversity and biological activity of natural products found in marine sponges is often explained in terms of chemical defense of these sessile soft-bodied invertebrates against various biotic stress factors such as predation, allelopathy and biofouling (*e.g.* Braekman and Daloze, 1986; Uriz *et al*., 1991; Proksch, 1999; McClintock and Baker, 2001). Among the different stress factors that affect and influence their fitness and ecological success, predation by fishes is probably the most important.

The nutrient analysis of eleven different sponges from the Mediterranean Sea performed in this study revealed similar carbon and nitrogen contents as in the artificial fish food used for the feeding experiments indicating that these sponges could be a valuable food source to fishes. However, the fact that sizeable tissue damage of sponges due to feeding by fishes is usually rare in their natural environment (Green, 1977) points towards an effective defense of sponges against predatory fishes.

In this study we have focused on the chemical defense of Mediterranean *Aplysina* sponges against fishes. We selected *Blennius sphinx* as a test organism for our feeding bioassays as this small fish (about 5 cm in length) is (a) very abundant at the coastlines of the Mediterranean Sea, (b) polyphagous, feeding on algae as well as on invertebrates in its natural environment by grazing on rock surfaces and (c) inhabits the same ecosystem as the sponge species selected for our study (Riedl, 1983). Thus, we consider *B. sphinx* as a potential predator of Mediterranean sponges. By using a feeding assay similar to that originally described by Hay *et al*. (1998) (placing the experimental fish food at the bottom of the aquarium) we intended to simulate the natural feeding conditions of the test fishes. In order to minimize loss of compounds due to leaching into the surrounding sea water during the feeding experiments we minimized exposure time of the food pieces in the aquarium by using a large number of test fishes to obtain test results within a rather short time of 1–2 h or even less in case of inactive components.

As part of our feeding studies we offered extracts of eleven common sponge species from the Mediterranean Sea to fishes of the species *B. sphinx* which were kept in an aquarium. The results obtained in this experiment (Fig. 2) were remarkably similar to findings recently reported by Becerro *et al*. (2003) who tested the deterrent properties of sponge extracts against fishes under field conditions. In agreement with our results Becerro *et al*. (2003) found *Aplysina aerophoba*, *Agelas oroides* and *Axinella damicornis* to rank amongst those sponges that are well protected against small Mediterranean fishes living close to the substrate. As found in our study, extracts from *Ircinia fasciculata* and *Petrosia ficiformis* were likewise significantly avoided when compared to controls. However, fishes fed on them more often during the experiments as on artificial food containing extracts of the former three sponges. This similarity of the results obtained in our feeding experiments with *B. sphinx* compared to the results obtained by Becerro *et al*. (2003) under field conditions indicates that the data obtained by the laboratory test system with *B. sphinx* has ecological relevance.

Our feeding experiments with *B. sphinx* indicated that both *Aplysina* sponges contain highly deterrent secondary metabolites (Fig. 2). This finding is in agreement with the results of a study by Pawlik *et al*. (1995) who analyzed 71 Caribbean sponges for fish deterrence and reported five *Aplysina* species (not including the species studied in this report) to rank among the chemically best protected sponges encountered.

In our assays the extract obtained from lyophilized *A. cavernicola* was comparable with regard to deterrency to the extract that had been prepared by immersing fresh sponge into EtOH (Fig. 2) even though both extracts clearly differ with regard to their major secondary metabolites. Whereas the former is characterized by the presence of several brominated isoxazoline alkaloids such as aplysinamisin-1 (**1**), aerophobin-2 (**2**) and aerothionin (**4**) as well as copious amounts of **8** the latter features compound **8** and the dienone (**6**) as major secondary metabolites with isoxazoline alkaloids present only in trace amounts. Ebel *et al*. (1997) had shown previously that isoxazoline alkaloids can be converted *in vitro* into the nitrile aeroplysinin-1 (**5**) which in turn gives rise to dienone (**6**) using a cell free extract obtained from *A. aerophoba or A. cavernicola*. Furthermore it

had been suggested that similar reactions occur also *in situ* upon wounding of sponge tissue (Ebel *et al*., 1997) or when fresh sponges are extracted in presence of water (Teeyapant and Proksch, 1993). Compound **8**, which is clearly no isoxazoline alkaloid, remains apparently unaffected by these conversions and is detected in extracts *of A. cavernicola* regardless of the method chosen for extraction. In this context, it should be noted that Puyana *et al*. (2003) found no evidence for a similar conversion of isoxazoline alkaloids in the Caribbean sponges *Aplysina insularis* and *A. archeri*.

Through bioassay guided fractionation of an extract obtained from lyophilized *A. cavernicola* aerothionin (**4**) and aplysinamisin-1 (**1**) as well as 3,4-dihydroxyquinoline-2-carboxylic acid (**8**) were found to be the major deterrent secondary metabolites. Each compound when tested at its physiological concentration as present in the sponges significantly deterred feeding by *B. sphinx* (Fig. 4). When pieces of artificial fish food were treated with a mixture of compound **8** and aerothionin (**4**) an additive or synergistic effect with regard to feeding deterrence was found (Fig. 4).

When the extract prepared from fresh *A. cavernicola* that had been immersed into EtOH was subjected to a bioassay-guided fractionation only compound **8** was identified as defensive metabolite (Fig. 4). The dienone (**6**) on the other hand had no significant fish deterrent activity (Fig. 4).

Adding dienone (**6**) together with compound **8** to artificial food at their respective physiological concentrations the feeding deterrent effect of the treated fish food increased only to a small extent when compared to fish food that had been treated only with compound **8** (Fig. 4). In contrast to the dienone (**6**), which apparently had no significant feeding deterrent activity, aeroplysinin-1 (**5**) when tested at the concentration of the dienone (**6**) caused a weak (compared for example to **3** or **4**) but nevertheless statistically significant feeding inhibition of *B. sphinx* (Fig. 4).

Isofistularin-3 (**3**) and aerophobin-2 (**2**) are among the major isoxazoline alkaloids present in the sibling sponge species *A. aerophoba*. When the latter alkaloids were incorporated into artificial fish food at the concentrations as found in *A. aerophoba* and offered to *B. sphinx* both isofistularin-3 (**3**) and aerophobin-2 (**2**) provoked pronounced antifeedant activity (Fig. 4). Direct comparison of the antifeedant activity of the various isoxazoline alkaloids **1–4** from *A. aerophoba* and *A. cavernicola* towards *B. sphinx* with regard to possible structure-activity relationships, however, is not possible, since the respective compounds were not tested at equimolar but rather at their physiological concentrations as detected in both *Aplysina* sponges.

Based on the results obtained in our study we propose that the brominated isoxazoline alkaloids of *A. aerophoba* and *A. cavernicola* as well as compound **8** that is present only in *A. cavernicola* act as constitutive defense compounds against potential fish predators. Aeroplysinin-1 (**5**) and dienone (**6**), on the other hand, are clearly inferior in comparison with regard to inhibiting feeding of *B. sphinx*. Nevertheless they are characterized by pronounced antibiotic properties against a multitude of terrestrial as well as marine bacteria (Teeyapant and Proksch, 1993; Teeyapant *et al*., 1993; Weiss *et al*., 1996; Debitus *et al*., 1998). Interestingly, isoxazoline alkaloids analyzed in these studies (*e.g.* **1–4**) are devoid of antibacterial activity when tested against the same range of bacteria (Teeyapant *et al*., 1993; Weiss *et al*., 1996). Thus, one may speculate that aeroplysinin-1 (**5**) and dienone (**6**) protect the sponges primarily from bacterial invasion (*e.g.* following wounding) whereas their isoxazoline precursors serve as chemical defense metabolites against fishes such as *B. sphinx*.

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