Sequestration of Dietary Alkaloids by the Spongivorous Marine Mollusc *Tylodina perversa*

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Specimens of the spongivorous Mediterranean opisthobranch *Tylodina perversa* that had been collected while feeding on *Aplysina aerophoba* were shown to sequester the brominated isoxazoline alkaloids of their prey. Alkaloids were stored in the hepatopancreas, mantle tissues, and egg masses in an organ-specific manner. Surprisingly, the known sponge alkaloid aerothionin which is found only in *A. cavernicola* but not in *A. aerophoba* was also among the metabolites identified in wild caught specimens of *T. perversa* as well as in opisthobranchs with a documented feeding history on *A. aerophoba*. Mollusc derived aerothionin is postulated to be derived from a previous feeding encounter with *A. cavernicola* as *T. perversa* was found to freely feed on both *Aplysina* sponges in aquarium bioassays. The possible ecological significance of alkaloid sequestration by *T. perversa* is still unknown.

Key words: Sponge-Opisthobranch Interactions, Marine Natural Products, Chemical Ecology

Introduction

Sponges of the genus *Aplysina* (syn. *Verongia*) harbor a wealth of structurally unique brominated isoxazoline alkaloids (e.g. 1–4, Fig. 1) that are thought to be biogenetically derived from 3,5-dibromotyrosine (Tymiak and Rinehart, 1981). Whereas the spirocyclohexadienylisoxazoline moiety of most brominated alkaloids isolated from *Aplysina* sponges is structurally identical the respective compounds differ by the nature of their amine substituents linked to the carbonyl group adjacent to the isoxazoline ring (Fig. 1). Several isoxazoline alkaloids from *Aplysina* sponges including aerothionin (4), aerophobin-2 (2) or isofistularin-3 (3) (Fig. 1) have recently been shown to act as strong feeding deterrents against marine fishes such as *Blennius sphinx* (Thoms, 2000) and are thought to play a crucial role in the chemical defense of the sponges against predatory fish (Thoms et al., in preparation).

The Mediterranean sea hosts two *Aplysina* species: the sulphurous yellow *A. aerophoba* is a conspicuous sponge that lives exposed on hard bottom substrata. It can be found at water depths as low as 1 m and is among the most common sponges occurring in the Mediterranean sea (Riedl, 1983; Pansini, 1997). The sibling species *A. cavernicola* is pale yellow in appearance and dwells in under-water caves or at depths of up to 40 m and even below. Just like other *Aplysina* sponges – e.g. those occurring in the Caribbean sea (Pawlik et al., 1995) – *A. aerophoba* and *A. cavernicola* are rarely attacked by predatory fish presumably due to the presence of the typical brominated *Aplysina* alkaloids. Nevertheless, the opisthobranch mollusc *Tylodina perversa* is frequently found feeding on *A. aerophoba* and is considered to be a specialized predator on this sponge (Riedl, 1983) and perhaps also on *A. cavernicola* even though up to now it has not been reported from the latter from nature. Morphologically defenseless opisthobranchs that are found in the oceans in a dazzling variety of shapes and colors are known to feed apparently unharmed on chemically defended sponges and other marine invertebrates thereby accumulating the chemical weaponry of their prey which in turn is utilized for the chemical defense of the molluscs e.g. against fishes (Faulkner and Ghiselin, 1983; Paul and van Alstyne, 1988; Pawlik, 1988). *T. perversa* is no exception in this regard since it is likewise morphologically defenseless (the rudimentary shell provides no efficient defense) and has been shown to sequester brominated alkaloids from its...
sponge prey (Teeyapant et al., 1993; Ebel et al., 1999).

The majority of reports on the sequestration of prey-derived secondary metabolites by opisthobranch molluscs originates from analysis of specimens caught in the wild with a poorly documented feeding history (e.g. Faulkner and Ghiselin, 1983; Pawlik et al., 1988). The full host range of prey species amenable to respective opisthobranch species, the organ-specific qualitative and quantitative distribution of sequestered dietary secondary metabolites in the molluscs vs. those in their prey as well as the metabolic fate of sequestered metabolites are often unknown. Knowledge of the different factors that influence accumulation and retention of putative defensive natural products, however, is crucial for an assessment of the ecological consequences of natural product sequestration by opisthobranch molluscs. In this study we report on an investigation of the feeding preference of *T. perversa* as judged by choice feeding experiments and on the organ specific accumulation of dietary alkaloids during long term feeding of *T. perversa* on the sponges *A. aerophoba* and *A. cavernicola* under controlled conditions in sea water tanks. Furthermore we provide evidence based on electron microscopic studies that the sequestration of sponge metabolites by *T. perversa* is not paralleled by an accumulation of sponge-associated bacteria that are harbored in high density in both *A. aerophoba* and *A. cavernicola* (Friedrich et al., 1999; Friedrich et al., 2001; Hentschel et al., 2001; Hentschel et al., 2002; Thoms et al., 2003).

**Materials and Methods**

Live specimens of the gastropod *T. perversa* were collected along with their prey sponge *Aplysina aerophoba* in April 2002 off the coast of Banyuls-sur-Mer, France. Specimens of the sponge *Aplysina cavernicola* were collected off the coast of Marseille, France. Three gastropods (group 1) were immediately dissected into mantle, hepatopancreas, alimentary duct, and reproductive organs after collection. Six individuals were kept in a sea water tank containing specimens of *A. aerophoba* and were allowed to feed *ad libitum*. After two weeks feeding on *A. aerophoba* under controlled conditions three gastropods were transferred to a tank containing specimens of the related sponge species *A. cavernicola* (group 3) and maintained there for two weeks (until the first signs of decay at the prey sponge were observed) while the three remaining individuals were kept on *A. aerophoba* for another three weeks, yielding a total of five weeks (group 2). After these periods all gastropods were dissected as described above.

Egg masses that had been produced by molluscs of groups 2 and 3 during captivity and egg masses that had been removed from freshly collected gastropods while feeding on *A. aerophoba* in the wild were lyophilized for subsequent extraction and HPLC analysis. Fresh egg ribbons were also preserved for electron microscopical analysis immediately after collection as described below.

Samples of the sponges (*A. aerophoba* and *A. cavernicola*) used for the feeding experiments were taken at the beginning and in the course of the feeding experiments and analyzed by HPLC.
For HPLC analysis lyophilized samples of mantle, hepatopancreas, and egg ribbons of the gastropods as well as lyophilized samples of the *Aplysina* sponges were ground, extracted with methanol and subsequently injected into 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 μm) (Knauer, Germany). Compounds were identified by their online UV spectra and by direct comparison with previously isolated standards (Ebel et al., 1997). Each compound in the samples analyzed was quantified using calibration curves obtained for the respective isolated natural products.

Electron microscopy was performed employing tissue samples of mantle, hepatopancreas and reproductive organs of gastropods of group 1 as well as with freshly collected egg masses. The samples were cut into small slices with an ethanol-sterilized scalpel, rinsed three times in sterile sea water, fixed in 2.5% glutaraldehyde, and stored at 4°C. They were then cut into smaller pieces of several mm³ in size, rinsed three times for 10 min in 1 × PBS and fixed overnight in 2% osmium tetroxide. After two additional rinses with 1 × PBS, the pieces were dehydrated in an ethanol series (30%, 50%, 70%, 100%) and incubated 3 × 30 min in 1 × propylene oxide. Following overnight incubation in 1:1 (v/v) propylene oxide/Epon 812 (Serva) the samples were embedded in Epon 812 at a temperature of 60°C. The embedded samples were subsequently sectioned with an ultramicrotome (OM-U3, C. Reichert, Austria) and examined by transmission electron microscopy (Zeiss EM 10, Zeiss, Germany).

Feeding experiments with *T. perversa* employing *Aplysina* and *Axinella* sponges were performed in six separate water tanks (size: 20 × 20 × 10 cm), containing one specimen each of the sponges *Aplysina aerophoba*, *A. cavernicola* and either *Axinella damicornis* or *A. polypoides* (all of them approximately 10 cm in diameter) and one individual of *T. perversa*. All the sponge species used for the experiment had a similar yellow color. The gastropod was placed at a starting point at about 15 cm equal distance from the three sponges. As soon as it had selected one of the respective sponges the result was protocollated. For repetition experiments the positions of the sponges in the tank were altered randomly.

**Results and Discussion**

HPLC separation of extracts of the two sponges *A. aerophoba* and *A. cavernicola* revealed the same characteristic profiles of natural products as previously described for both species (Teeyapant et al., 2003).

<table>
<thead>
<tr>
<th>Compound</th>
<th><em>T. perversa</em> mantle</th>
<th><em>T. perversa</em> hepatopancreas</th>
<th><em>T. perversa</em> egg masses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>group 1</td>
<td>group 2</td>
<td>group 3</td>
</tr>
<tr>
<td>Aplysaminis-1 (1)</td>
<td>21.2 (±16.8)</td>
<td>3.8 (±2.2)</td>
<td>43 (±1.8)</td>
</tr>
<tr>
<td>Aerophbin-2 (2)</td>
<td>8.2 (±4.9)</td>
<td>6.6 (±3.7)</td>
<td>4.8 (±4.1)</td>
</tr>
<tr>
<td>Isostilatrin-3 (3)</td>
<td>0.0 (±0.0)</td>
<td>0.3 (±0.4)</td>
<td>0.2 (±0.3)</td>
</tr>
<tr>
<td>A. aerophoba (4)</td>
<td>0.0 (±0.0)</td>
<td>2.4 (±2.8)</td>
<td>2.7 (±1.5)</td>
</tr>
<tr>
<td>A. cavernicola pigment (6)</td>
<td>0.0 (±0.0)</td>
<td>18.4 (±14.3)</td>
<td>0.0 (±0.0)</td>
</tr>
</tbody>
</table>

Table I. Concentrations (μmol g⁻¹ dry weight ± standard deviation) of alkaloids in various tissues derived from the opisthobranch *Tylodina perversa* (group 1: collected from the wild; group 2: after five weeks of feeding on *A. aerophoba*; group 3: after two weeks of feeding on *A. aerophoba* followed by another two weeks of feeding on *A. cavernicola*) and in the prey sponges. Numbers of compounds refer to Fig. 1.

* In the course of the aquarium feeding experiment with *A. cavernicola* only one egg ribbon was produced by the gastropods.

** The alkaloid pattern found in *A. cavernicola* tissue was similar to previous findings in earlier studies (e.g. Thoms et al., 2003).
et al., 1993; Ebel et al., 1997; Ebel et al., 1999; Thoms et al., 2003). Aplysinaminis-1 (1), aerophobin-2 (2) and isofistularin-3 (3) were the predominating brominated alkaloids present in A. aerophoba (Table I). No aerothionin (4) was detected in extracts of A. aerophoba which is in congruence with earlier studies (Teeyapant et al., 1993; Ebel et al., 1997; Ebel et al., 1999). In addition, the chemically labile pigment uranidine (5) was also present in A. aerophoba. Since uranidine is easily oxidized to black polymerous compounds it was not attempted to quantify the concentration of the pigment in A. aerophoba unlike the concentrations of the chemically stable brominated alkaloids (Table I). A. cavernicola yielded aerothionin (4) and aplysinaminis-1 (1) as major brominated alkaloids. Isofistularin-3 (3) and aerophobin-2 (2) were present as minor metabolites. In addition to the brominated alkaloids the pigment 3,4-dihydroxyquinoline-2-carboxylic acid (6), which in contrast to uranidine (5) is remarkably stable, was among the predominating secondary compounds identified in A. cavernicola (Table I).

The alkaloid pattern and profile of the hepatopancreas of specimens of T. perversa of group 1 that had been collected from the wild closely resembled that of their prey A. aerophoba. Aplysinaminis-1 (1) was the major alkaloid in the hepatopancreas of T. perversa as well as in A. aerophoba followed by aerophobin-2 (2) and isofistularin-3 (3) which were present in similar concentrations in the hepatopancreas whereas compound 2 dominated over compound 3 in A. aerophoba (Table I). In addition, trace amounts of uranidine (5) were likewise detected in the hepatopancreas thereby confirming that the specimens of T. perversa collected from the wild while present on A. aerophoba had indeed been feeding on this sponge. Interestingly, the mantle tissue of T. perversa from group 1 yielded only alypsinaminis-1 (1) and aerophobin-2 (2) as brominated alkaloids while in contrast to previous studies (Ebel et al., 1999) neither aerothionin (4) nor isofistularin-3 (3) was detected.

Egg masses of T. perversa that had also been collected from the wild yielded a similar pattern of brominated alkaloids as found in mantle tissues of the gastropods with aerophobin-2 (2) followed by alypsinaminis-1 (1) as major secondary constituents. In addition to compounds 1 and 2, however, small amounts of aerothionin (4) were unequivocally identified in the egg masses (Table I).

Electron microscopy of mantle tissue, hepatopancreas, reproductive organs and egg ribbons derived from T. perversa revealed that no bacterial symbionts were present (data not shown) even though both A. aerophoba and A. cavernicola contain large numbers of mostly extracellular bacteria (Friedrich et al., 1999; Friedrich et al., 2001; Hentschel et al., 2001; Hentschel et al., 2002; Thoms et al., 2003). Thus sequestration of sponge alkaloids is apparently not paralleled by uptake of bacteria from the sponge prey.

The hepatopancreas, mantle tissues as well as egg masses of specimens of T. perversa from group 2 that had been feeding on A. aerophoba under controlled conditions for a total of five weeks all contained small but unequivocally detectable amounts of aerothionin (4). The largest concentration of aerothionin (2.4 µmol g⁻¹ dry wt.) was detected in the mantle, whereas the concentrations of compound 4 in the hepatopancreas and in the egg masses amounted only to 0.7 µmol g⁻¹ dry wt., respectively (Table I). With the exception of aerothionin (4) the patterns of brominated isoaxazoline alkaloids (1–3) found in the hepatopancreas, in the mantle tissues and in the egg masses of the gastropods from group 2 were similar to those of T. perversa from group 1 (Table I). This chemical similarity was most pronounced in case of the egg masses produced by gastropods of groups 1 and 2 that both contained aerophobin-2 (2) as major brominated alkaloid at almost identical concentrations (11.9 and 11.7 µmol g⁻¹ dry wt., respectively). The alkaloid pattern of gastropods from group 3 which had been feeding for two weeks on A. cavernicola (after an initial three weeks feeding period on A. aerophoba) closely resembled that of their last prey. This was again most striking for the hepatopancreas which yielded large concentrations of the A. cavernicola pigment (6) (45.6 µmol g⁻¹ dry wt.) (Table I). Aerothionin (4) (17.1 µmol g⁻¹ dry wt.) and alypsinaminis-1 (1) (16.9 µmol g⁻¹ dry wt.) were present in almost the same concentrations whereas aerophobin-2 (2) and isofistularin-3 (3) constituted only minor secondary metabolites. The mantle tissues featured the pigment (6) as major nitrogenous constituent (18.4 µmol g⁻¹ dry wt.) followed by comparable concentra-
tions of aerophobin-2 (2) (4.8 µmol g\(^{-1}\) dry wt.) and aplysinamin-1 (1) (4.3 µmol g\(^{-1}\) dry wt.). Egg masses that had been produced by *T. perversa* while feeding on *A. cavernicola* contained again aerophobin-2 (2) (16.1 µmol g\(^{-1}\) dry wt.) as major alkaloid followed by the pigment (6) (11.5 µmol g\(^{-1}\) dry wt.), aplysinamin-1 (1) (6.3 µmol g\(^{-1}\) dry wt.) and aerothionin (4) (0.4 µmol g\(^{-1}\) dry wt.).

It is particularly interesting to note that not only the pattern but also the concentrations of brominated alkaloids present in all egg masses of *T. perversa* analyzed in this study were remarkably similar irrespective of the large quantitative differences of alkaloids found in *A. aerophoba* and *A. cavernicola* (Table I). Aerophobin-2 (2) constituted the major brominated alkaloid in all analyzed egg masses irrespective of the fact that aplysinamin-1 (1) (in both *Aplysina* species) and aerothionin (4) (in *A. cavernicola*) clearly predominate over 2 in the sponges. The more lipophilic (based on comparison of reversed phase HPLC retention times) alkaloids isofistularin-3 (3) and aerothionin (4) were only present in small concentrations in the egg masses even though they are conspicuous secondary metabolites in both *Aplysina* sponges. The chemical comparison of egg masses and to a lesser degree also of mantle tissue of *T. perversa* with both *Aplysina* sponges reveals that sequestration of alkaloids by the gastropods not merely mirrors the alkaloid profiles as present in *Aplysina* sponges but appears to favor certain compounds such as aerophobin-2 (2). The reasons for the obvious differences in alkaloid profiles found in *Aplysina* sponges and e.g. egg masses of *T. perversa* are unknown.

Previous studies on the presence of dietary alkaloids in specimens of *T. perversa* that had been either collected in the wild while feeding on *A. aerophoba* (Teeyapant et al., 1993) or that had been allowed to feed on this sponge species for one week under controlled conditions (Ebel et al., 1999) correspondingly reported on the occurrence of considerable amounts of aerothionin (4) in addition to the typical alkaloids from *A. aerophoba* (1–3). The presence of aerothionin (4) in the opisthobranchs has been confirmed in this study (see results on alkaloid distribution in *T. perversa* from group 2 feeding on *A. aerophoba* under controlled conditions), but its origin still remains unclear. In general, four different hypotheses can be raised:

- Hypothesis 1: the presence of aerothionin traces back to a previous feeding encounter with *A. cavernicola*; 2: aerothionin is occasionally accumulated also by *A. aerophoba*; 3: aerothionin in *T. perversa* results from biotransformation of *A. aerophoba* alkaloids; 4: *de novo* biosynthesis of aerothionin by the gastropods. Hypothesis 1 should involve feeding of *T. perversa* on *A. cavernicola* which up to now, however, has not been reported from nature. Therefore a feeding experiment was conducted in this study involving specimens of *T. perversa* that were kept in tanks together with *A. aerophoba*, *A. cavernicola* and the sponges *Axinella damicornis* and *Axinella polypoides*. When given the choice between the different sponges the gastropods clearly preferred the *Aplysina* species (Fig. 2). Both *A. aerophoba* and *A. cavernicola* proved to be similarly attractive to *T. perversa* based on the comparable numbers of observation of the gastropods on the sponges. This experiment as well as the observed feeding of *T. perversa* on *A. cavernicola* over a period of two weeks (group 3) unequivocally demonstrate that the gastropods freely feed on both Mediterranean *Aplysina* sponges. The fact that unlike *A. aerophoba* no specimens of *A. cavernicola* were observed at the site of collection of *T. perversa* at Banyuls-sur-Mer is not necessarily a valid argument against hypothesis 1 but probably reflects differences in the ecology of both species.
Whereas *A. aerophoba* grows exposed and can be found even at low water depths (around 1 m) *A. cavernicola* generally lives hidden in caves or at greater depths and is thus harder to find than the conspicuous *A. aerophoba*.

Hypothesis 2 postulates at least occasional occurrence of aerothionin in *A. aerophoba*. Whereas this possibility can certainly not generally be ruled out, it must nevertheless be clearly pointed out that all previous chemical studies on *A. aerophoba* and *T. perversa* that involved specimens from the Canary islands (Teeyapant *et al*., 1993), the Mediterranean coast of Spain (Ebel *et al*., 1999) and France (Ebel *et al*., 1997) as well as from Croatia (Teeyapant, 1994) unequivocally failed to detect aerothionin in the sponges. Hypothesis 3 which explains the origin of aerothionin through biotransformation of sequestered *A. aerophoba* alkaloids occurring in the gastropods would not only involve cleavage of sponge alkaloids such as 1 – 3 at the amide bond(s) but also re-assembly of the two spirocyclohexadienylisoxazoline moieties obtained upon cleavage and one putrescine unit thereby giving rise to aerothionin. Compared to hypotheses 1 and 2 hypotheses 3 and 4 appear highly speculative with no experimental data available in support. Based on the presently available data hypothesis 1 (previous feeding history of the gastropods on *A. cavernicola*) is in our view the most likely explanation for the origin of aerothionin in *T. perversa*, although this would involve a switch of host sponges by the gastropods.

However, if we assume that the presence of aerothionin in specimens of *T. perversa* from group 2 dates back from previous feeding on *A. cavernicola*, retention of this alkaloid (4) for clearly longer than five weeks and selective transfer into the egg masses have to be postulated. In this context it is of interest to note that the anthraquinone paretin which had been sequestered by specimens of the terrestrial lichen-feeding snail *Balea perversa* could be detected in the snails for at least up to four weeks after transferring *B. perversa* to a paretin-free diet (Hesbacher *et al*., 1995).

It is tempting to speculate on the possible ecological roles of sequestered sponge alkaloids in *T. perversa* especially as it has been repeatedly shown that sponge-derived natural products are utilized by sponge-feeding gastropods such as nudibranchs for their own chemical defense against predators such as fishes (Cimino *et al*., 1993, Avila, 1995; Avila and Paul, 1997; Dumdei *et al*., 1997; Becerro *et al*., 1998). Recently it has been shown that brominated alkaloids (1–4) as well as the *A. cavernicola* pigment (6) act as feeding deterrents against the Mediterranean fish *Blennius sphinx* (Thoms, 2000). However, the concentrations at which compounds 1–4 and 6 were tested and at which they proved to be effective followed those that are present in *A. aerophoba* and *A. cavernicola* (see Table I). Since the alkaloid concentrations in the mantle tissues and egg masses of *T. perversa* that are most vulnerable to an attack by predators are considerably lower than found in *Aplysina* sponges it is difficult to simply transfer the results obtained in the previous study (Thoms, 2000) to our present investigation on *T. perversa*. The interesting question regarding the possible ecological advantage of alkaloid sequestration for *T. perversa* can therefore presently not be answered but will have to await further studies.

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