0014-4754/94/050510-07$1.50 + 0.20

Transfer of secondary metabolites from the sponges *Dysidea fragilis* and *Pleraplysilla spinifera* to the mantle dermal formations (MDFs) of the nudibranch *Hypserlodoris webbi*

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Received 1 September 1993; accepted 21 December 1993

Abstract. The opisthobranch mollusc *Hypselodoris webbi* is able to select, among its potential preys, sponges chemically rich in furanosesquiterpenoids. The sequestered secondary metabolites act as defensive allomones against predators and are accumulated in some dorsal glands (MDFs). This transfer from sponges to MDFs has been proven by maintaining *H. webbi* together with some selected sponges in an aquarium for a prolonged period.

Key words. Nudibranch; *Hypselodoris*; sponge; *Dysidea*; *Pleraplysilla*; *Microciona*; furanosesquiterpenoids.

Opisthobranch molluscs are uniquely suited to an investigation of the ecology of benthic marine invertebrates. Many of them are unprotected by a shell, but, in spite of this apparent vulnerability, they are rarely victims of predators. Many studies[1-4] have supported a key role of chemical defense operating among opisthobranchs. The origin of the defensive allomones is most frequently linked to the diet (sponges, tunicates, other molluscs, etc.) and sometimes the animals are capable of de novo synthesis[5].

Recent studies of *Hypselodoris* species[6,7] from Italian and Spanish coasts have led to the discovery of many fish-deterrent furanosesquiterpenoids that either are expelled as mucous secretion or are accumulated in small (≈2 mm diameter) spherical glands, so called mantle dermal formations (MDFs)[8], that are strategically located near the gills and rhinophores of the mollusc (fig. 1)[9]. Structural similarity of these terpenoids with typical sponge metabolites strongly supported a prey-predator relationship between *Dysidea* sponges and *Hypselodoris* molluscs. However, the nudibranchs were rarely observed feeding on their prey. In order to prove the transfer of sponge metabolites to *Hypselodoris* spp., we now report controlled laboratory studies of *H. webbi*. This very conspicuous (8 cm) mollusc is brightly coloured (fig. 1) and in spite of its widespread presence along the Mediterranean coasts, it has never been observed grazing upon sponges.

The first chemical study[10] of *H. webbi* (=*Glossodoris valenciennesi*) led to the structural characterization of an ichthyodeterrent furanosesquiterpenoid, longifolin (compound **1**, cf. Scheme), previously known as a minor component of the sponge *Pleraplysilla spinifera*[11]. Subsequently, longifolin (**1**) was found[6] to be the major metabolite of a Dictyoceratid sponge, *Dysidea fragilis*[12].

Combined chemical and histological studies[8] detected large amounts of (**1**) in the spherical MDFs. More recently[6], the discovery in *H. webbi* of other furanosesquiterpenoids, *iso*-tavacfuran (**2**) and nakafuran-9 (**3**), suggested that most likely the diet of *H. webbi* is not selective for a single species but comprises a group of sponges, as e.g. *Dysidea* or related species, which display a metabolite pattern characterized by furanosesquiterpenoids. All Mediterranean *Hypselodoris* spp.[6,7,10], with the exception of *Hypselodoris orsini*[13], seem to possess the same dietary preference toward furanosesquiterpene-rich sponges. In this paper, we report further research on populations of *H. webbi* from two distinct Mediterranean areas (SE Spain and SW Italy). Both populations of molluscs were carefully observed in aquaria which revealed their relationship to their sponge preys: 1) Feeding by *H. webbi* on sponges containing furanosesquiterpenoids has been proven. 2) Ecologically relevant experiments have demonstrated that the sponge metabolites are rapidly transferred into the MDFs of the mollusc.

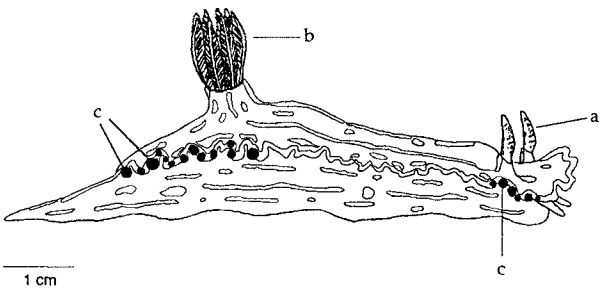
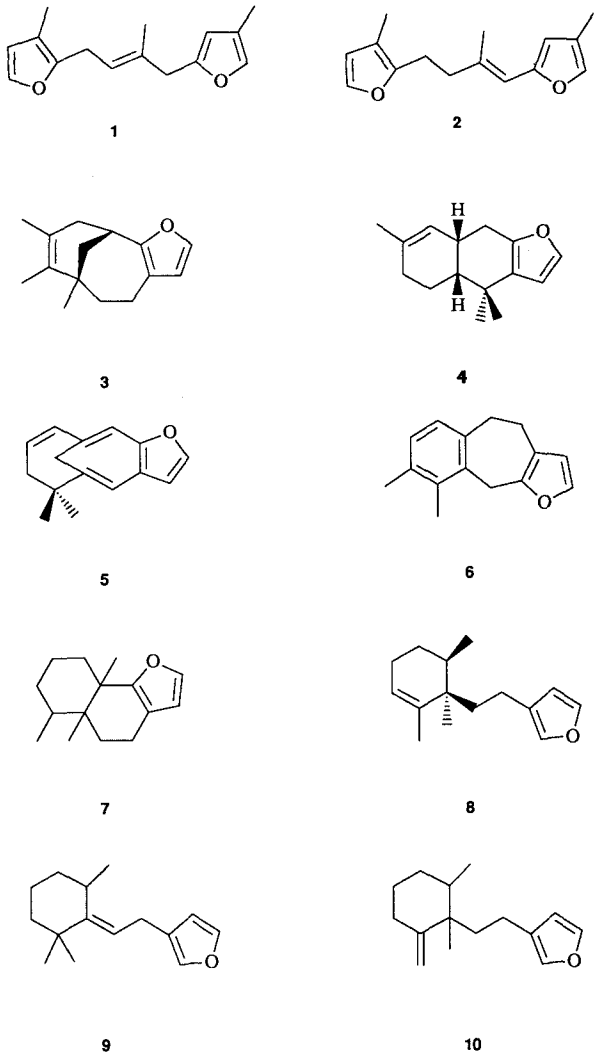


Figure 1. Schematic illustration of *H. webbi* (≈8 cm); a, rhinophores; b, gills; c, MDFs (black balls).

Materials and methods

Instruments. Optical rotations were measured on a JASCO Dip-370 polarimeter and CD curves were performed by JASCO J-710 spectropolarimeter in *n*-hexane



Scheme.

or ethanol. UV spectra were determined on a Varian DMS 90 spectrophotometer. ^{1}H-NMR experiments were recorded at room temperature on an AMX-500 Bruker NMR spectrometer calibrated against the signal of the solvent, C_6D_6 or $CDCl_3$. All mass spectra were taken on an AEI MS 30 instrument. TLC was carried out on Merck precoated analytical Kieselgel 60 F-254 and Merck precoated preparative Kieselgel 60 F254 sprayed with Ehrlich's reagent.

Biological materials. All the biological material was collected by SCUBA from three stations: Alicante (SE Spain), Mar Menor (SE Spain) and Sorrento (SW Italy) (fig. 2).

- *H. webbi*, 9 specimens, Alicante, spring 1992.
- *Dysidea fragilis*, Mar Menor (a hypersaline [39–40%] coastal lagoon), spring 1992.
- *H. webbi*, Sorrento, summer 1992 (3 specimens) and autumn 1992 (4 specimens).
- *Pleraplysilla spinifera*, Sorrento, summer and autumn 1992.
- *Microciona toxystila*, Sorrento, summer 1992.

Extraction of the biological samples and isolation of the metabolites.

- *D. fragilis* (w. wt 13 g) was extracted with acetone. The filtered acetone solution was concentrated and, after dilution with water, extracted with diethyl ether. Evaporation of the solvent under reduced pressure yielded an oily residue that was chromatographed on a silica gel column with petroleum ether as eluant.
- *H. webbi* (2 specimens) of the population from Alicante was frozen and dissected in four parts: mantle dermal formations (MDFs), digestive gland, rest of viscera and rest of mantle. Each section was separately extracted with acetone. After removing the organic solvent, the residues were dissolved in diethyl ether and directly analyzed by TLC (0.25 mm precoated plates; visualization: Ehrlich's reagent; developed with petroleum ether). The extracts of MDFs and digestive gland, which showed the same metabolite pattern, were recombined and fractionated on a SiO_2 column.

Figure 2. Collection stations of nudibranch and sponges.

– *Pleraplysilla spinifera* was extracted as described above to yield oily diethyl ether extracts. The less polar components were purified by preparative SiO_2-TLC (visualization: UV lamp at 256 nm; developed with petroleum ether).
– *H. webbi* (1 specimen) of the population collected during Summer at Sorrento was analyzed like those of *H. webbi* from Alicante. The combined diethyl ether extracts of MDFs and digestive glands were purified by preparative SiO_2-TLC (visualization: UV lamp at 256 nm and Ehrlich's reagent; developed with petroleum ether).
– *H. webbi* (1 specimen) of the population collected during autumn at Sorrento was dissected and directly extracted with diethyl ether. The organic extract was fractionated on a SiO_2 column with petroleum ether.
– *Microciona toxystila* was extracted with acetone. The brown residue obtained by removing the organic solvent was fractionated on SiO_2-gel column by petroleum ether.

Anatomical dissection. Each specimen of *H. webbi* was separately dissected in four parts (mantle dermal formations [MDFs], rest of mantle, digestive gland and rest of viscera) under a Swift microscope (20–40 ×). The MDFs from living specimens were detached in the same way while keeping the animal in a thin layer of salt-water.
Feeding experiments. Before the experiment, some MDFs were detached from 6 specimens of living *H. webbi* from Alicante. The TLC plates (petroleum ether) of these sections showed only compound **1** present in all samples. Thus, the same 6 specimens were kept separately in aquaria with the sponge *D. fragilis* (Mar Menor) for 35 days. During that time some MDFs were periodically detached. After 5 weeks, the animals were frozen and dissected in four parts: MDFs, rest of mantle, digestive gland and rest of viscera. Each section was extracted as described above and the lipid-soluble fraction was chromatographically analyzed.
The MDFs from 2 living specimens of the population of *H. webbi* collected at Sorrento during the summer were preliminarily studied as described above for the nudibranchs from Alicante. The molluscs were then kept in an aquarium with *Pleraplysilla spinifera* for 1 day. After that time, the nudibranchs were sacrificed, frozen and immediately dissected. The diethyl ether extract of the sections, analyzed by TLC, showed a similar pattern of products in digestive gland and MDFs. The combinated extracts of these sections were analyzed on SiO_2-TLC (petroleum ether).
The MDFs of the autumnal population of *H. webbi* (3 specimens) were detached and chromatographically examined as described above. Thus, the molluscs were kept for a week in an aquarium with *D. fragilis* from Mar Menor. After that time, some MDFs of nudibranchs were detached and the remaining parts of *D. fragilis* were removed and substituted by *P. spinifera*. The animals were kept for 5 more days in the aquarium, then they were frozen and dissected. The sections, after extraction with acetone, were characterized by analytical TLC (petroleum ether) and gas-chromatography.
Gas-chromatographic analysis of furanosesquiterpenoids. The ether soluble fractions of MDFs were analyzed by gas-chromatography on a Carlo Erba (Farmitalia Carlo Erba, Milan, Italy) HRGC 5300 instrument equipped with a flame ionization detector. The compounds were separated on a fused silica capillary column (OV1, Farmitalia Carlo Erba, Milan, Italy; 25 m × 0.32 mm). Nitrogen was used as carrier gas. Operating conditions were: detector temperature 280 °C; injector temperature 250 °C. The oven temperature programme was: initial temperature 80 °C followed by a rise to 110 °C at a rate of 5 °C/min; 1 min at 110 °C followed by another rise to 130 °C at a rate of 1 °C/min; finally, the temperature was raised to 210 °C at a rate of 25 °C/min. The samples were dissolved in diethyl ether and injected at a concentration of about 1.0 μg/μl.

Results
Chemical characterization of the secondary metabolites. All metabolites were identified by comparison of their spectroscopic and chromatographic properties with those of authentic samples. Each compound isolated from MDFs during the experiment in aquaria was identified by both ^{1}H-NMR data and SiO_2-TLC (petroleum ether), $SiO_2/AgNO_3$ (8%)-TLC (*n*-hexane/benzene 6:4), and gas-chromatographic comparison with pure reference substances.
Longifolin (**1**) was identified by ^{1}H-NMR spectrum in C_6D_6 (ref. 6).
^{1}H-NMR spectrum (C_6D_6), UV and MS data of (−)-flurodysinin (**4**) were identical to those reported in literature[7,14]. The absolute stereochemistry was stated by polarimetric measurement $\{[\alpha]_D = -78$ (c = 0.4, $CHCl_3)\}$, and confirmed by the comparison of the CD maximum (≅220 nm) in *n*-hexane with that of an authentic sample previously isolated from other source.
Spiniferin-1 (**5**) and spiniferin-2 (**6**) were identified by ^{1}H-NMR spectrum in C_6D_6 and $CDCl_3$ (refs 11, 16).

Secondary metabolites contained in the examined animals before (I) and after (II) transfer experiments

Organism	Locality	n[a]	g[b]	Metabolite patterns I	II
H. webbi	Alicante	9		**1**	**1 + 4**
	Sorrento	3		**1, 7 – 10**	**1, 7 – 10 + 5, 6**
	Sorrento	4		**1**	**1 + 4 + 5, 6**
D. fragilis	Mar Menor		8.5	**4**	
P. spinifera	Sorrento		2.0	**5, 6**	
	Sorrento		2.4	**5, 6**	
M. toxystila	Sorrento		1.6	**7 – 10**	

[a]number of specimens; [b]dry weight in g.

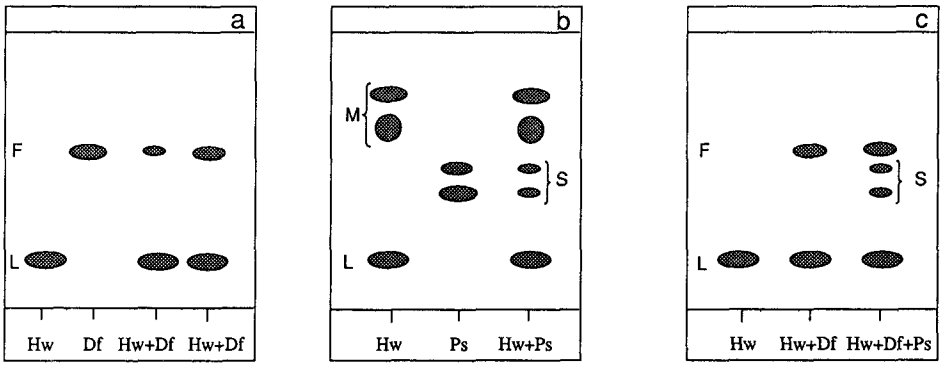


Figure 3. TLC (light petroleum) of the extract of:
a H. webbi from Alicante (Hw), *D. fragilis* from Mar Menor (Df), detached MDFs of *H. webbi* (Alicante) kept upon *D. fragilis* for 10 days (Hw + Df) and 35 days (Hw + Df);
b H. webbi from Sorrento (1st collection) (Hw), *P. spinfera* (Ps), detached MDFs of *H. webbi* kept upon *P. spinifera* for 1 day (Hw + Ps);
c H. webbi from Sorrento (2nd collection) (Hw), detached MDFs of *H. webbi* kept for 7 days in aquarium upon *D. fragilis* (Hw + Df), detached MDFs of *H. webbi* starved for 7 days with *D. fragilis* (Mar Menor) and 5 days upon *P. spinifera* (Hw + Df + Ps).
F = (−)-furodysinin; L = longifolin; M = microcionins; S = spiniferins.

Microcionins 1–4 (**7**–**10**) were identified by comparison of their ^{1}H-NMR (C_6D_6) data with those reported in literature[17].

Distribution of the secondary metabolites in sponges and nudibranchs. The secondary metabolites characterized in both molluscs and sponges are listed in the table according to the geographic origin (fig. 2) of the organism. In particular:

– *Disydea fragilis* from Mar Menor. The TLC analysis of the diethyl ether soluble fraction (403 mg) of *D. fragilis* from Mar Menor was mainly characterized by the presence of *ent*-furodysinin[7,14,15] (**4**, Rf 0.6 in petroleum ether), that was isolated (30 mg) by column chromatography.

– *Pleraplysilla spinifera* from Sorrento. Fragments of both samples of *P. spinifera* collected during summer and autumn from Sorrento were extracted to yield, respectively, 9 mg and 15 mg of diethyl ether extract. Spiniferin-1[16] (**5**) and spiniferin-2[11] (**6**) were isolated from both extracts (fig. 3b).

– *Microciona toxystila* from Sorrento. The chromatographic fractionation of the acetone extract (70 mg) of the sponge led to isolate microcionins 1–4 (5 mg; **7**–**10**).

– *Hypselodoris webbi* from Alicante. The population of *H. webbi* (9 specimens) from Alicante was characterized by only longifolin[6,10,11] (**1**). In fact, the analysis of the MDFs and the digestive glands from two frozen specimens yielded **1** (3 mg). Preliminary studies of the detached MDFs from six living molluscs confirmed the compartmentalization of (**1**) in defensive organules. On the other hand, when the same six specimens of *H. webbi* were kept in aquarium together with small pieces of living *D. fragilis* from Mar Menor, the TLC plates and the gas-chromatographic profile of the extracts of the MDFs detached after regular intervals showed the appearance of *ent*-furodysinin (**4**) as well as longifolin (**1**) (fig. 3a). In 5 weeks, a gradual accumulating of (**4**) (fig. 4) was observed. In particular, the GC analysis of defensive organules detached after 4 and 35 days showed a change in ratio of *ent*-furodysinin/longifolin from 2.4:100 to 13:100, respectively (fig. 4).

– *H. webbi collected in summer at Sorrento.* Chemical analysis of both the digestive glands and the MDFs from a frozen specimen showed the presence of (**1**) together with a complex mixture of furanosesquiterpenoids (Rf 0.7 and 0.8 in petroleum ether) identified after comparison with the extract from *Microciona toxystila* as microcionins[17] (**7**–**10**) (fig. 3b). The same chemical pattern was observed in the MDFs detached from two living molluscs. After anatomical dissection, these specimens were kept in an aquarium together with *P. spinifera*, containing spiniferin-1 (**5**) and spiniferin-2 (**6**). The molluscs, kept together with *P. spinifera* for a day, showed the ability to select and to transfer sponge spiniferins in their defensive structures. In fact, the spectroscopic analysis of the sections (totalling 35 mg) from these specimens revealed, exclusively in MDFs and digestive gland, the presence of spiniferin-2 (**6**) together with longifolin (**1**) and microcionins (**7**–**10**). The high unstable spiniferin-1 (**5**) was present in too small an amount to be characterized, but its presence was clearly demonstrated by TLC (fig. 3b) that showed a spot visible under UV lamp at 256 nm and tipically positive to Ehrlich's reagent.

– *H. webbi collected in autumn at Sorrento.* The studies of a dissected specimen and of some detached MDFs of three living animals from the second population of *H. webbi* collected at Sorrento revealed the presence of only longifolin (**1**) (fig. 3c). For a week the molluscs were left in an aquarium together with some small pieces of *D. fragilis* from Mar Menor. After this time, the chemical analysis of the MDFs content led to the identification of (**4**) besides (**1**). Subsequently, the same

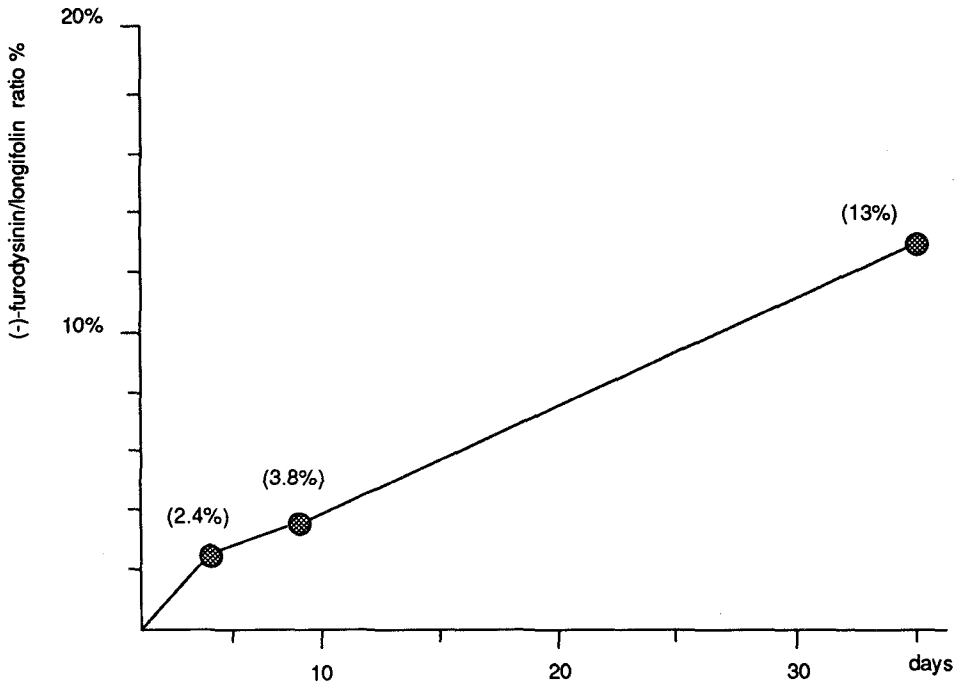


Figure 4. Ratio of (−)-furodysinin (**4**)/longifolin (**1**) from detached MDFs of *H. webbi* (Alicante) kept upon *D. fragilis* (Mar Menor) for 35 days.

specimens were kept with *P. spinifera* for 5 days. After the dissection, compounds (**1**, **4**, **5**, **6**) were found in both MDFs and digestive gland. The presence of the latter products was confirmed by chromatographic comparison with extracts from *D. fragilis* (var. Mar Menor) and *P. spinifera* (fig. 3c).

Discussion

H. webbi (fig. 1) is a dorid nudibranch widespread along the Mediterranean coasts, but, in spite of its wide distribution, no report describes its dietary habits. Only recently, studies of different *Hypselodoris* species[6,7] suggested a general defensive strategy for the molluscs of this genus which would be expected to prey selectively on *Dysidea* (or chemically related) sponges.

We planned to confirm this hypothesis by observing the behaviour of *H. webbi* in the presence of Mediterranean sponges in aquaria. When *Dysidea fragilis* from Mar Menor, a hypersaline coastal lagoon where *H. webbi* was never observed, was placed in the aquarium, the molluscs immediately ate the sponge. To confirm this observation, the metabolite contents of the two organisms were separately analyzed (fig. 3a). The extract of *H. webbi* was mainly characterized, in analogy with our previous studies[6,10], by the presence of longifolin (**1**). Conversely the fractionation of ether extracts of *D. fragilis* (var. Mar Menor) led to (−)-furodysinin (**4**), previously found in a collection of Mediterranean sponges[14] and recently in different populations of *Hypselodoris* from the Cantabrian Sea[7]. Previous studies have led to a hypothesis that *Hypselodoris* spp. are able to transfer defensive allomones into some spherical glands (MDFs) strategically placed near rhinophores and gills[8,9]. In order to prove this transfer we kept *H. webbi* in an aquarium together with *D. fragilis*. Chemical analysis of detached MDFs was the principal means to investigate transfer of the secondary metabolites from the sponge to the defensive organules. Anatomical dissection of a few MDFs did not cause serious damage to the mollusc. Because of this, it was possible to follow the transfer of new metabolites into the MDFs during a long period of observation. Detached MDFs were extracted and chromatographically analyzed before contact with the sponge and after 4, 9 and 35 days of contact. At the beginning, only (**1**) was present, but already after 4 days a second less polar spot, corresponding to (**4**), was observed by SiO_2-TLC. The intensity of the second spot increased during the following days, thus demonstrating that *H. webbi* was able to transfer and accumulate furanosesquiterpene (**4**) from the sponge to MDFs (fig. 3a). Relative amounts of sesquiterpenoids were quantified by gas-chromatography (fig. 4).

A second population of *H. webbi* was collected at Sorrento. The analysis of the secondary metabolites present in the mucous secretion and in the dissected parts of a single specimen suggested a predation upon *Microciona toxystila* and *Dysidea fragilis*. In fact, the metabolic pattern was characterized by the series of microcionins (**7**–**10**) previously isolated from *M. toxystila*[17] and by longifolin (**1**) recently found in *D. fragilis*[6]. Even though *M. toxystila* (Poecilosclerida) does not belong

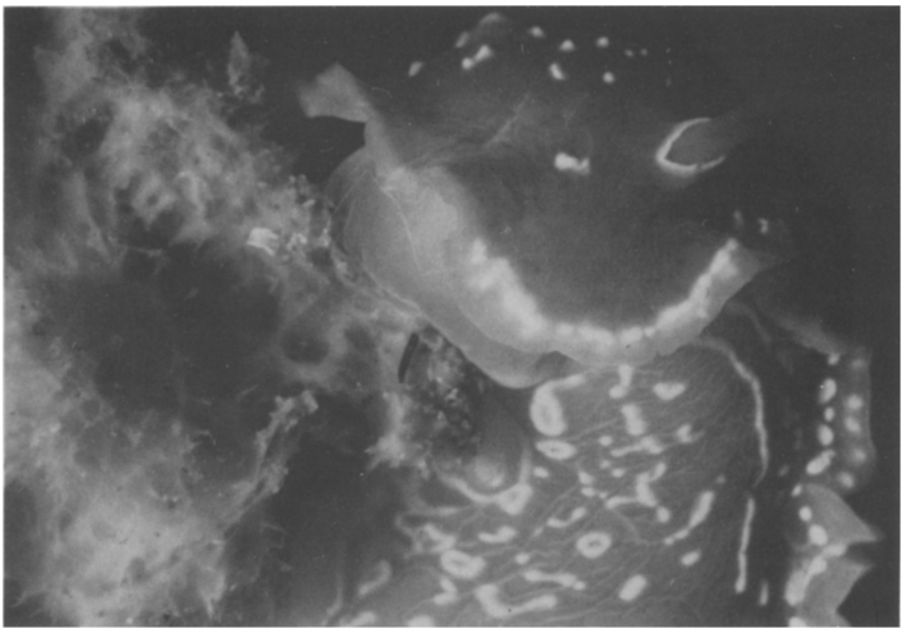


Figure 5. Photograph of *H. webbi* while it is sucking the soft tissues of *D. fragilis* (var. Mar Menor). The white spots on the right border of the mollusc are the mantle dermal formations (MDFs).

to the Dictyoceratida order[12], it, like *D. fragilis*, contains large amounts of furanosesquiterpenoids. These data were also confirmed by the analysis of some MDFs detached from living *H. webbi*, which displayed the metabolic pattern shown in figure 3b. These nudibranchs were kept in an aquarium with another Mediterranean sponge, *Pleraplysilla spinifera* (Dendroceratida) that contains the highly unstable spiniferin-1[16] (**5**) and spiniferin-2 (**6**)[11]. The molluscs immediately ate the sponge. After one day, by the same procedure as described above, spiniferin-1 (**5**) and spiniferin-2 (**6**) were detected in both MDFs and digestive glands (fig. 3b).

The food preference of *H. webbi* for three sponges (table), all containing furanosesquiterpenoids, but belonging to three different orders[12], suggests a need for careful taxonomical re-analysis of these sponges.

As the metabolite pattern of *D. fragilis* from Mar Menor was clearly different from that of *P. spinifera* from Sorrento, we performed a third experiment with the population of *H. webbi* collected in autumn 1992, chemically characterized by longifolin (**1**) (fig. 3c). The molluscs were placed in an aquarium with first *D. fragilis* (var. Mar Menor) and then with *P. spinifera* from Sorrento. Both sponges were rapidly devoured and the chromatographic studies of the MDF extracts confirmed the transfer of the furanosesquiterpenoids (fig. 3c).

The behaviour of *H. webbi* in an aquarium is very interesting. The nudibranch does not crawl directly toward the sponge, but it finds its bearing following the flowing water. The nudibranch, once in contact with the sponge, extrudes its proboscis (fig. 5) sucking sponge tissue from spongine fibres. *H. webbi* selectively feeds on soft tissues without injuring the spongine fibers which allow rapid regeneration of the sponge tissue.

In conclusion, this work has proved by rigorous laboratory experiments that *H. webbi*, in aquaria, is a predator of the sponge *D. fragilis* and *P. spinifera* and that the secondary metabolites of the sponge are quickly transferred into the protective mantle organules of the mollusc.

Acknowledgments. The authors are deeply grateful to Prof. P. J. Scheuer (Hawaii University) for critical reading of the manuscript. Thanks are due to Mr. G. Scognamiglio and Miss D. Ricciardi, Mr. R. Turco and Dr. G. Villani for technical assistance. NMR spectra were recorded at the I.C.M.I.B.-NMR Service. Mass spectra were provided by the Servizio di Spettrometria di Massa. Staffs of both services are gratefully acknowledged. This work was supported by EEC project 'Sciences and Technologies Marines Mast II' Contract MAS2-CT91-0004. A. Martin is grateful to the Spanish Government (I + D project AMB92-0617) for financial assistance.

1 Faulkner, D. J., and Ghiselin, M. T., Mar. Ecol. Progr. Ser. *13* (1983) 295.
2 Karuso, P., in: Biorganic Marine Chemistry, vol. 1, p. 31. Ed. P. J. Scheuer. Springer-Verlag, New York-Berlin-Heidelberg 1987.
3 Cimino, G., and Sodano, G., Chem. Scripta, *29* (1989) 389.
4 Faulkner, D. J., in: Ecological Roles of Marine Natural Products, p. 119. Ed. V. J. Paul. Comstock Publishing Associates, Ithaca and London 1992.
5 Cimino, G., and Sodano, G., in: Topics in Current Chemistry, p. 77. Ed. P. J. Scheuer. Springer-Verlag, New York 1993.
6 Avila, C., Cimino, G., Fontana, A., Gavagnin, M., Ortea, J., and Trivellone, E., J. chem. Ecol. *17* (1991) 625.
7 Fontana, A., Avila, C., Martinez, E., Ortea, J., Trivellone, E., and Cimino, G., J. chem. Ecol. *19* (1993) 339.

8 Garcîa-Gômez, J. C., Cimino, G., and Medina, A., Mar. Biol. *106* (1990) 254.
9 Garcîa-Gômez, J. C., Medina, A., and Coveñas, R., Malacologia *32* (1991) 233.
10 Cimino, G., De Rosa, S., De Stefano, S., and Sodano, G., Comp. Biochem. Physiol. *73(B)* (1982) 471.
11 Cimino, G., De Stefano, S., Minale, L., and Trivellone, E., Tetrahedron, Lett., *16* (1975) 3727.
12 Bergquist, P. R., and Wells, R. J., in: Marine Natural Products, vol. 5, p. 1. Ed. P. J. Scheuer. Academic Press, New York-London 1983.
13 Cimino, G., Fontana, A., Giménez, F., Marin, A., Mollo, E., Trivellone, E., and Zubìa, E., Experientia *49* (1993) 582.
14 Guella, G., Mancini, I., Guerriero, A., and Pietra, F., Helv. chim. Acta *68* (1985) 1276.
15 Vaillancourt, V., Agharaimi, M. R., Sundram, U. N., Richou, O., Faulkner, D. J., and Albizati, K. F., J. org. Chem. *56* (1991) 378.
16 Cimino, G., De Steano, S., Minale, L., and Trivellone, E., Experientia *34* (1978) 1425.
17 Cimino, G., De Stefano, S., Guerriero, A., and Minale, L., Tetrahedron Lett. *16* (1975) 3723.