Bonner zoologische Beiträge	Band 55 (2006)	Heft 3/4	Seiten 181–190	Bonn, November 2007

Distribution of homarine in some Opisthobranchia (Gastropoda: Mollusca)*

Sven AFFELD[1),2)], Heike WÄGELE[2),3)], Conxita AVILA[4)], Stefan KEHRAUS[1)] & Gabriele M. KÖNIG[1)]
[1)]Institut für Pharmazeutische Biologie, Universität Bonn, Bonn, Germany
[2]Institut für Evolutionsbiologie und Ökologie, Universität Bonn, Bonn, Germany
[3]Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Bonn, Germany
[4]Dept. of Animal Biology (Invertebrates), Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain

*Paper presented to the 2nd International Workshop on Opisthobranchia, ZFMK, Bonn, Germany, September 20th to 22nd, 2006

Abstract. Homarine, a zwitterionic natural product, is only known from some marine invertebrates, including the opisthobranch *Phestilla lugubris* (Klein 1986 in KARUSO 1987). This natural product is described as having antipredatory and antifouling effects. In the current study we present new results on the distribution of homarine in Opisthobranchia and in a few of their food organisms. Our results indicate that homarine is mainly found in members of the Cladobranchia (with one exception) and is probably derived from cnidarian food sources. Other investigated opisthobranch taxa, which feed on a variety of other marine organisms (algae, Porifera, Bryozoa etc.), lack homarine. An exception is the cephalaspid *Aglaja tricolorata* in which homarine was detected, and foraging of this predatory slug on homarine containing cladobranchs seems likely. It is shown in *Marionia blainvillea* that the characteristic glandular cells in the epidermis of the Dendronotoidea are not adaptations to store homarine.

Keywords. Natural products, Cnidaria, food organisms, Cladobranchia, *Marionia blainvillea*, histology.

1. INTRODUCTION

A wide variety of natural products is known from Opisthobranchia (KARUSO 1987; AVILA 1995; CIMINO et al. 1999; CIMINO et al. 2001; CIMINO & GAVAGNIN 2006; WÄGELE et al. 2006). Some of the opisthobranchs are of particular interest due to their extreme toxicity and the high structural diversity of their secondary metabolite content.

One of these groups is the algae- or cyanobacteria-feeding taxon Anaspidea, which store a high diversity of secondary metabolites. Apart from terpenes, which are the major structural class in Anaspidea, bioactive polyketides and peptides were also found (CAREFOOT 1987; YAMADA & KIGOSHI 1997; CIMINO et al. 2001). One of these peptides, i.e. dolastatin 10, showed potent cytotoxic activity. In clinical studies (phase II), however it had no effect neither against advanced colorectal cancer (SAAD et al. 2002), advanced pancreaticobiliary cancers (KINDLER et al. 2005), nor advanced breast cancer (PEREZ et al. 2005).

The Sacoglossa, which mainly feed on algae of the taxon Chlorophyta, obtain secondary metabolites from their food. One of these compounds, the depsipeptide kahalalide F that *Elysia rufescens* takes up from its food alga *Bryopsis* spec. and algal associated bacteria is currently in a clinical trial (phase II) against hepatocellular carcinoma, non-small cell lung cancer and melanoma (HAMANN 2004). In some cases these sequestered chemicals are biotransformed by the slugs. Thus Mediterranean *Oxynoe oli - vacea, Lobiger serradifalci* and *Ascobulla fragilis*, all of which feeding on *Caulerpa prolifera*, take up the algal sesquiterpenoid caulerpenyne and modify it into oxytoxin-1 and -2, which have an enhanced deterrent effect against fish compared to caulerpenyne (CIMINO et al. 1990; GAVAGNIN et al. 1994a). According to CIMINO & GHISELIN (1998), CIMINO et al. (1999) and MARIN & ROS (2004) highly derived sacoglossans are able to form their defensive compounds *de novo*. E.g. GAVAGNIN et al. (1994b) showed that the polypropionate elysione is produced by *Elysia viridis* itself..

Within the Nudibranchia, the sponge-feeding Doridoidea are probably the group among the Opisthobranchia best examined for the presence of secondary metabolites, most of which are dietary derived terpenes (see e.g. the reviews KARUSO et al. 1987; AVILA 1995; CIMINO et al. 1999; GAVAGNIN & FONTANA 2000; GARSON 2006; WAHIDULLAH et al. 2006; MIYAMOTO 2006). *De novo* biosynthesis, however, has also been shown, e.g., for the sesquiterpenes

polygodial in *Dendrodoris limbata* (CIMINO et al. 1983) and *ent*-pallescensin A in *Doriopsilla areolata* (GAVAGNIN et al. 2001).

In contrast, the nudibranch taxon Cladobranchia has been poorly investigated chemically. Traditionially, Cladobranchia are divided into three taxa, the monophyletic Aeolidoidea and Dendronotoidea, and the paraphyletic Arminoidea (see WÄGELE & WILLAN 2000). Members of the Cladobranchia mainly feed on octocorals and hydrozoans (MCDONALD & NYBAKKEN 1997). Within the Arminoidea few terpenes have been detected. The briarane diterpenoids verecynarmin A to G and the cembranoid preverecynarmin are known from *Armina maculata* and its prey organism, the octocoral *Veretillum cynomorium* (GUERRIERO et al. 1987, 1988, 1990). *Leminda millecra* exhibits four different sesquiterpenoids, millecrone A and B, as well as millecrol A and B (CIMINO et al. 2001). They all are sequestered while feeding on alcyonariids. Further investigation revealed more natural compounds: isofuranodiene, (+)-8-hydroxycalamenene, algoafuran, cubebenone, and a series of seven triprenylquinones and hydroquinones. *Alcyonium fauri* and the gorgonian *Lep - togorgia palma* seem to be the source of at least some of these compounds (MCPHAIL et al. 2001). Origin of janolusimide, extracted from *Janolus cristatus* (SODANO & SPINELLA 1986) seems to be unknown, a *de novo* biosynthesis can not be excluded (CIMINO et al. 2001).

Terpenes are also present in Dendronotoidea. *Tritonia hamneronum* e.g. sequesters the feeding deterrent sesquiterpene julieannafuran from its food, which is the sea fan *Gorgonia ventalina* (CRONIN et al. 1995). Several terpenes (rubifolide, pukalide, cuparane sesquiterpenoides) and other compounds (tochuinyl acetate and its dihydro-derivative, ptilosarcenone) are collected from *Tochuina tetraquetra.* All these products seem to be food derived (CIMINO et al. 2001). Further compounds in other members of Dendronotoidea are summarized by CIMINO et al. (2001): An unknown *Tritonia* sp. revealed a prostanoid, also found in the octocoral *Telesto,* and *Tri - toniella belli* sequestered a chimyl alcohol from the octocoral *Clavularia frankliniana*. *De novo* biosynthesis of terpenes is recorded from one member of the Dendronotoidea: BARSBY et al. (2002) showed through feeding experiments with [1,2-$^{13}C_2$] acetate that *Melibe leonina* produces at least one of its defensive metabolites (2,6-dimethyl-5-heptenal) itself.

In the Aeolidoidea terpenes are only known from the genus *Phyllodesmium*. *P. longicirrum* accumulates three cembranoid diterpenes, trocheliophorol, thunbergol and epoxythunbergol, from the soft coral *Sarcophyton troche - liophorum* (COLL et al. 1985). *P. guamensis* specifically sequesters the cembranoid 11-beta-acetoxypukalide from its preferred prey, the soft coral *Sinularia maxima* (SLATTERY et al. 1998). Only very few records for other compounds are available. CIAVATTA et al. (1996) discovered two prenylchromanols (1 and 2) and a prenyl-p-hydroxy acid in the skin of *Cratena peregrina.* None of these compounds was detected in the preferred food of *C. peregri - na*, namely the hydrozoan *Eudendrium racemosum*. On the other hand, other metabolites, usually not considered as deterrent to predators have been isolated from *Euden - drium* and its aeolid predators: carotenoids from *Flabel - lina iodinea* (MCBETH 1972), sterols from *Cratena pere - grina*, *Flabellina affinis* and *F. lineolata* (CIMINO et al. 1980), carotenoids and alkaloids from *Phestilla melanobrachia* (OKUDA et al. 1982), sterols and alkaloids from *P. lugubris* (TARGETT 1983; KARUSO 1987). KARUSO (1987, after Klein 1986) reported also the presence of homarine in the latter species.

Members of the Aeolidoidea are known for storing cnidocysts from their prey organisms in special structures located at the cerata tips (see Fig. 1A). Here, a cnidosac is formed as part of the digestive gland, and in the cells of this sac, intact cnidocysts are incorporated and maintained functional. It is assumed that the slugs use these cnidosysts as a major means of defense (e.g. EDMUNDS 1966; GREENWOOD & MARISCAL 1984a, b). This assumption is supported by the studies of FRICK (2003), showing that *Flabelli - na verrucosa* responds to the presence of predators by the variation of nematocyst incorporation.

The incorporation of cnidocysts is regarded as the main key character in the evolution of the Aeolidoidea (WÄGELE 2004). Nevertheless, in some cases secondary metabolites seem to be of importance for aeolids as well, e.g. in the genus *Phyllodesmium* where no incorporation of cnidocysts takes place (SLATTERY et al. 1998). The same holds true for *Phestilla lugubris*, which shows no cnidocysts in their cnidosacs, but homarine was found (KARUSO 1987, after Klein 1986).

Homarine was first isolated in 1933 by HOPPE-SEYLER. Its biological function has been controversially discussed ever since. This product seems to be restricted to marine organisms, and within the Metazoa it is only found in invertebrates (CARR et al. 1996). Therefore it was suspected that its purpose lies in osmoregulation. BEERS (1967) studied the distribution of homarine in Crustacea, Echinodermata and Tunicata and from these results infered that the presence of homarine is "in basic accord with a role in cellular osmotic phenomena". DALL (1971) though could not find any contribution of homarine in osmotic processes in decapod crustaceans. Nevertheless, it is widely accepted that homarine serves as an osmolyte in marine algae. Initial studies have been carried out by DICKSON & KIRST (1986) with the green alga *Platymonas sub -*

cordiformis. SLATTERY et al. (1997) showed that the soft coral *Gersemia antarctica* releases a potent antimicrobial mixture of organic compounds into the surrounding seawater, and homarine is the compound responsible for most of this biological activity. In the tissues of the anthozoans *Actinia equina* and *Calliactis parasitica,* homarine was detected at concentrations of 1.5 and 2.1 mg/g dry weight (MATHIAS et al. 1960). BERKING (1986, 1987) detected homarine in several hydrozoans, where it regulates the colony morphology and prevents the metamorphosis in larvae. Detection of homarine in gastropods is rare. MCCLINTOCK (1994) found the substance in the Antarctic *Marseniopsis mollis* (Caenogastropoda, Marseniidae). It is probably derived from epizoic bryozoans and hydrozoans growing on the ascidian *Cnemidocarpa verrucosa*, the main food of *M. mollis* and serves as a feeding deterrent against the seastar *Odontaster validus*.

Since homarine was found in aeolids without cnidocysts, the question arouse, whether the occurence of homarine with a possible defensive function is more widespread in opisthobranchs.The current study focuses on the distribution of homarine in several opisthobranch taxa, with a main focus on Cladobranchia, since this group is hardly investigated concerning its chemical defensive systems. Furthermore the localisation of this compound within the body of the dendronotoid *Marionia blainvillea* (Fig. 1B) was investigated in detail. The epidermal tissue of the mantle of *M. blainvillea* is densely packed with glands of unknown function (WÄGELE et al. 2006) (Fig. 1C). Therefore a role of these glands in chemical defense was considered likely.

2. MATERIAL AND METHODS

Specimens of the opisthobranchs and of their food were collected worldwide during several field trips. Table 1 lists the investigated specimens, the collection details, and the means of extraction. The animals were fixated either in alcohol (ethanol or methanol) or by deep freezing.

The supernatant alcohol was taken off and used for the preparation of a crude extract, or in the case of the deep frozen samples extraction with methanol was carried out. The solvent was evaporated under reduced pressure with a rotary evaporator in order to obtain a crude extract.

The detection of homarine was carried out via ^{1}H NMR spectroscopy on a Bruker Avance 300 DPX spectrometer, using D_2O as solvent.

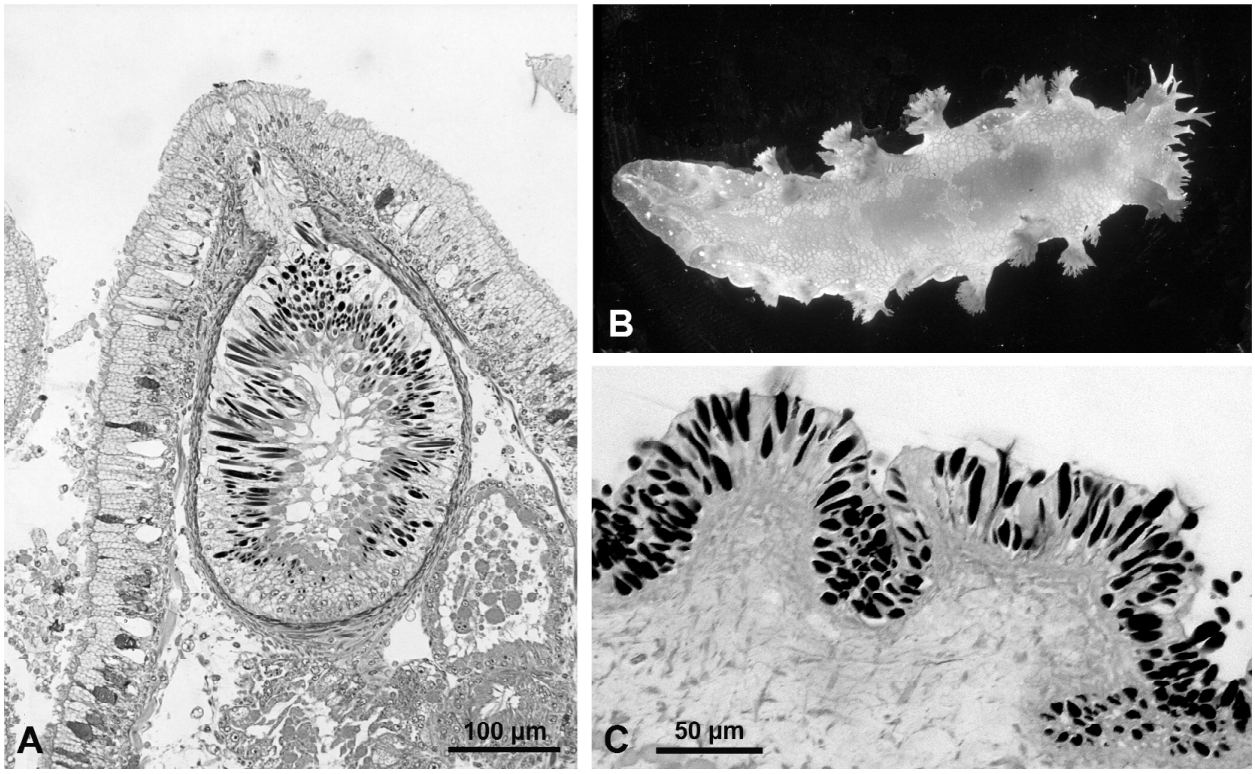

Fig. 1. A. typical cnidosac (here *Aeolidia papillosa*). B. *Marionia blainvillea* C. Histological section of dorsal mantle epithelium of *Marionia blainvillea.* Note the single vacuoles in the cells staining homogenously dark violet.

Table 1. Species investigated for homarine, with information on location, date of collection, extraction type and result. Food organism or substrate is indicated in the last column. These information are taken from MCDONALD & NYBAKKEN (1997) if not stated otherwise. + homarine present; – no signal of homarine present in ^{1}H NMR and total amount of crude extract >100mg; ? insufficient amount of crude extract (<10mg); Column 5 (Extraction): fluid in brackets indicate that only the alcohol for fixation was used for the preparation of a crude extract

Higher ranking Taxon/*Species*	Location	Number of Indiv. Date of collection	Fixation	Extraction	Homarine	Food
		Opisthobranchia				
Aeolidoidea						
Cratena pilata (Gould, 1870)	Blanes, Spain	04.11.1993	Deep frozen	MeOH	+	*Tubularia* spp., and other hydrozoans
Cuthona caerulea (Montagu, 1840)	Banyuls-sur-Mer, France	05.2006	EtOH	(EtOH)	+	diverse hydrozoan species
Cuthona gymnota (Couthouy, 1838)	Blanes, Spain	04.11.1993	Deep frozen	MeOH	+	*Tubularia* spp., and other hydrozoans
Flabellina affinis (Gmelin, 1791)	Banyuls-sur-Mer, France	05.2006	EtOH	(EtOH)	?	*Eudendrium* spp.
Hermissenda crassicornis (Eschscholtz, 1831)	Blanes, Spain	5 Indiv. 24.03.1994	Deep frozen	MeOH	+	Examined specimens found on *Tubularia crocea,* also on diverse hydrozoans
Phestilla lugubris (Bergh, 1870)	Data taken from KARUSO 1987 (after Klein 1986)				+	*Porites* spp.
Phyllodesmium spec.	Lizard Island, Australia	14.07.2006	MeOH	(MeOH)	–	Specialised on Xeniidae. (pers. obs. HW)
Dendronotoidea						
Marionia blainvillea (Risso, 1818)	Blanes, Spain	5 Indiv. 09.2004	Deep frozen	MeOH	+	e.g. *Eunicella singularis*, also on other gorgonians
Marionia blainvillea (Risso, 1818)	Blanes, Spain	10 Indiv. 05.2006	EtOH	(EtOH)	+	
Tritonia striata Haefelfinger, 1963	Blanes, Spain	15 Indiv. 04.–07.2004	Deep frozen	MeOH	?	*Paralcyonium elegans* (see Schmekel & Portmann 1982)
Tritonia manicata Deshayes, 1853	Blanes, Spain	11 Indiv. 03.–08.2004	Deep frozen	MeOH	?	*Clavularia* sp., *Cornularia* spp. (Alcynonacea)
Doridoidea						
Dendrodoris grandiflora Rapp, 1827	Tierra de Malgrat, Spain	05.11.2004	EtOH 96%	(EtOH)	–	Sponges e.g. *Ircinia fasciculata, Clathria toxystila, Spongia officinalis*
Platydoris argo (Linné, 1767)	Giglio, Italy	05.2005	EtOH 70%	(EtOH)	–	Demospongia
Adalaria proxima (Alder & Hancock, 1854)	Bretagne, France	07.1996	EtOH 93%	(EtOH)	–	Bryozoa
Archidoris pseudoargus (Rapp, 1827)	Tierra de Malgrat, Spain	05.11.2004	EtOH 96%	(EtOH)	–	Demospongia
Cephalaspidea						
Scaphander lignarius (Linné, 1758)	Blanes, Spain	5 Indiv. 11.11.2004	EtOH 70%	(EtOH)	–	Predatory e.g. Polychaeta
Aglaja tricolorata Renier, 1807	Giglio, Italy	05.2005	EtOH 70%	(EtOH)	+	Predatory, other Opisthobranchia
Anaspidea						
Aplysia spec.	La Coruña, Spain	08.03.2006	EtOH 96%	(EtOH)	–	Algae
Phyllaplysia spec.	Banyuls-sur-Mer, France	09.1994	EtOH	(EtOH)	–	Algae

Higher ranking Taxon/*Species*	Location	Number of Indiv. Date of collection	Fixation	Extraction	Homarine	Food
Pleurobranchoidea						
Bathyberthella antarctica Willan & Bertsch, 1987	Antarctica 61°43,30'S 59°12,40'W	27.11.1996	EtOH 70%	(EtOH)	–	unknown
Tylodinoidea						
Umbraculum umbraculum (Lightfoot, 1786)	Meteor Bank ST 486 (N-Atlantic) 29°45,7' N 28°22.9' W	08.09.1998	EtOH 80%	(EtOH)	–	Demospongia
		Other gastropods				
„Prosobranchia"						
Littorina littorea Linné, 1758	Nordstrand, Germany	10 Indiv. 22.08.2006	EtOH 98%	(EtOH)	–	
		Food of the examined Opisthobranchia				
Octocorallia						
Eunicella singularis (Esper, 1791)	Banyuls-sur-Mer, France	05.2006	EtOH 98%	(EtOH)	+	
Xenniidae	Lizard Island, Australia	14.07.2006	MeOH	(MeOH)	–	
Hydrozoa						
Tubularia crocea Agassiz, 1862	Blanes, Spain	04.11.1993	Deep frozen	MeOH	+	

From 1 g of the crude extract of one colony of the gorgonian *Eunicella singularis* 4 mg of homarine were isolated by RP-18 VLC and HPLC. The VLC was carried out using a 2.5 cm glass column filled 10 cm with Silica gel Polygoprep 60-50 C18. Elution was performed using a water/methanol gradient starting with pure water, yielding 13 fractions.

The HPLC was performed on a Shimadzu system equipped with a Merk Hitachi Pump L-7100 and a Shimadzu SPD-M6A Photodiode Array UV-VIS detector, using a Knauer C_{18} Eurospher-100 (5 µm, 250x80 mm) column. A constant flow of 1 mL/sec was applied using Water/Methanol (90:10 V/V) as eluent.

The five deep frozen specimens of *Marionia blainvillea* were dissected before extraction into mantle, foot, digestive gland and the rest of the viscera, in order to examine the distribution of homarine within the body of the animal.

For histological investigation, one specimen each of the dendronotoid *Mariona blainvillea* and the aeolid *Cuthona caerulea* have been preserved in formaldehyde/seawater. After dehydration in alcohol, they were embedded in hydroxyethylmethacrylate for serial sectioning and sections (2.5 µm) were stained with toluidine blue. Investigation was performed under a light microscope.

3. RESULTS

The survey of several members of the Opisthobranchia revealed that the natural compound homarine is present in all examined samples of Cladobranchia, apart from a new species of *Phyllodesmium* from Lizard Island. This compound was also not found in other members of the Opisthobranchia, except for *Aglaja tricolorata.* Results of this survey are listed in Table 1.

The low field shifted ^{1}H NMR signals of homarine δ 7.8 – 8.9 ppm (see Fig. 2) are very characteristic for this compound, and can easily be detected in ^{1}H NMR spectra of crude extracts. In all cases they did not overlap with any other resonance signals. The detection of these resonance signals is a clear indication for the presence of homarine, however only if the concentration of the compound is sufficient, i.e. traces will not be detected.

For specific localisation of the compound different body parts of *M. blainvillea* were extracted separately. ^{1}H NMR spectra of the extracts show that homarine is present throughout the body (see Fig. 3 A to D).

Histological investigation revealed that the epidermis of *M. blainvillea* contains many epithelial glandular cells with one large vacuole filling the whole cell. The vacuole contains a substance that is stained homogeneously dark violett with toluidine blue (Fig. 1C). These glandular cells are evenly distributed over the dorsal and lateral notum.

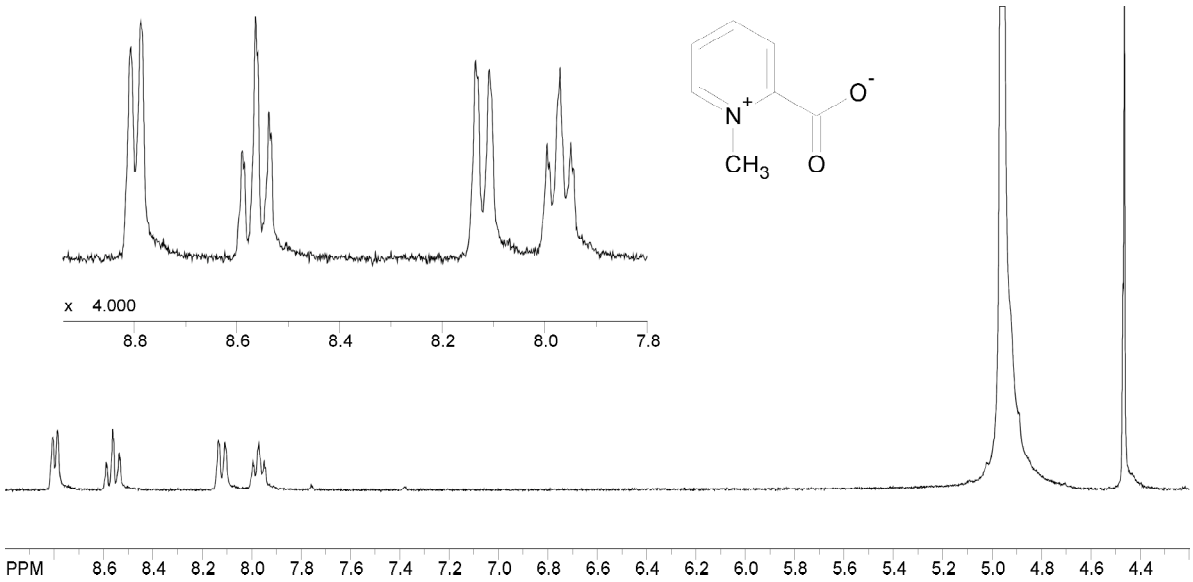

Fig. 2. ^{1}H NMR spectrum of purified homarine in methanol-d_4. 300 MHz, and chemical structure of homarine.

The epithelium of *Cuthona caerulea* showed very few glandular cells, these were usually filled with dark staining granular substances.

4. DISCUSSION

Secondary metabolites clearly seem to play a role in the biotic interactions with potential predators throughout all systematic groups of Opisthobranchia. These chemicals are often taken up from the food while *de novo* synthesis is rare (review see CIMINO et al. 2004).

Only a few field studies on the deterrent effect of the observed compounds have been carried out (THOMPSON 1960; AVILA & PAUL 1997; SLATTERY et al. 1998; JOHNSON & WILLOWS 1999; MARIN et al. 1999; AVILA et al. 2000; BECERRO et al. 2001; IKEN et al. 2002; ROGERS et al. 2002; PENNEY 2004). While even a few studies focus on parasites on opisthobranchs (ARNAUD 1978; HO 1981; CAREFOOT 1987; JENSEN 1987; HUYS 2001; SCHRÖDL 2002, 2003), the effect of secondary metabolites on pathogens is hardly investigated. TEEYAPANT et al. (1993a, b) studied the uptake of secondary metabolites in *Tylodina perversa* (Tylodinoidea) from its food sponge *Aplysina* (*Verongia*) *aerophoba* and the antimicrobial activity of the sponge metabolites. However they only found antimicrobial activity in compounds that were not present in *Tylodina perversa*.

Incorporation of homarine is shown here for slugs feeding on Hydrozoa as well as on the gorgonian octocoral *E. singularis*. The examined Hydrozoa contained homarine, which is in accord with its role as a morphogen, supposedly in all hydrozoans (BERKING 1986, 1987). The examined gorgonian *E. singularis* also contained homarine as described for other gorgonians where it serves as an antifouling agent (TARGETT et al. 1983). Uptake of homarine can also occur by predation on opisthobranchs containing homarine. According to RUDMAN (1972), members of the genus *Aglaja* are active predators, feeding on vagile prey. Whereas some aglajid species, like *A. cylindrica*, specialize on polychaetes and nemertines, others (e.g. *A. au - reopunctata*) mainly focus on opisthobranchs. It seems likely that *Aglaja tricolorata* obtained the homarine by feeding on other opisthobranchs.

Several of the investigated species belong to the Aeolidoidea with functional cnidocysts incorporated, which are a good means of defense against predatory fishes (FRICK 2003) but certainly useless against crustaceans and probably without effect against seastars. Both latter groups are potential predators on opisthobranchs, though only few thorough investigations on predation have been carried out (review see WÄGELE et al. 2006). The efficiency of homarine as a feeding deterrent is shown e.g. in *Marseniopsis mollis* (MCCLINTOCK 1994). But similar studies have never been performed for opisthobranchs and further investigations are needed to clarify the role of this compound in cladobranchs. The presence of a further defensive system, like homarine, would provide additional protection, e.g. for the cnidocyst containing *Cuthona caerulea.* In species without cnidocysts, e.g. *Marionia blainvillea,* homarine might even represent the sole defensive strategy.

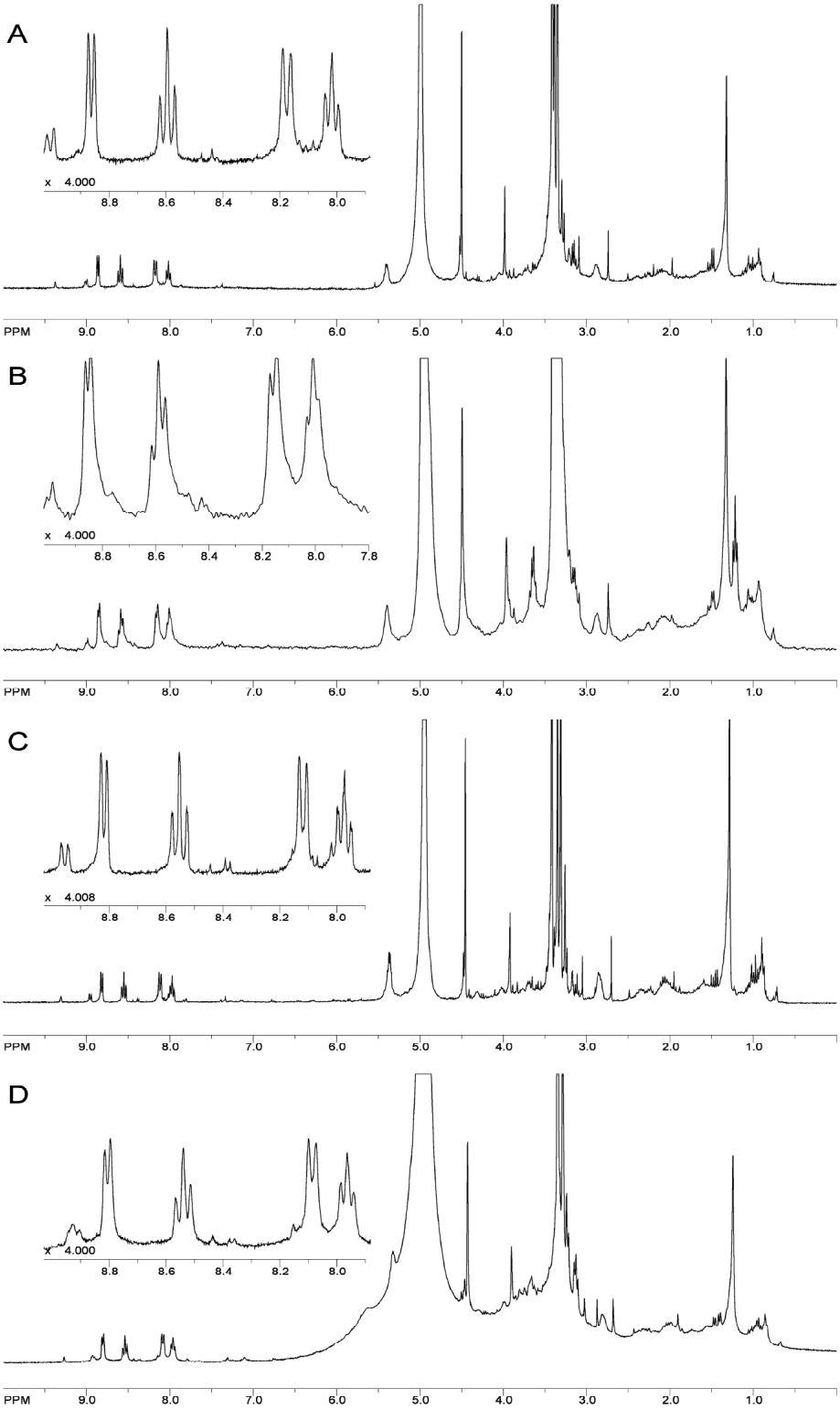

Fig. 3. ^{1}H NMR spectra of crude extracts of dissected *Marionia blainvillea* in methanol-d_4. 300 MHz. A. Mantle, B. Foot, C. Digestive gland, D. Viscera without digestive gland.

Homarine could also serve as an antibacterial agent in the mucus layer of the slugs, independent of its supposed function as a feeding deterrent, since any kind of mucus is a perfect environment for bacteria or fungi. Therefore, organisms with a mucus layer can adopt two ways of keeping their body surface clean. They can either produce big amounts of mucus that wash off continuously or add a substance that inhibits growth of microorganisms. The former strategy is known, e.g. from certain soft corals (DUCKLOW & MITCHELL 1979; DUERDEN 1986), the latter could be the case in the Aeolidoidea and Dendronotoidea.

WÄGELE et al. (2006) noted the presence of homogenously staining violet glands as a characteristic of Dendronotoidea. In their survey on glandular structures in Opisthobranchia, they observed these glands for nine (out of 17 investigated) members of the Dendronotoidea. These glands are absent in any members of the Aeolidoidea and "Arminoidea". We confirmed the presence of these particular glands in *Marionia blainvillea* and their absence in *Cuthona caerulea*. The uniform distribution of homarine in the different body parts of *M. blainvillea* suggests that no bioaccumulation in the glandular epithelium takes place in this case. Homarine is a very small and very polar molecule, therefore it is likely that it is taken up passively in the digestive gland or even in the whole digestive system. Quantitative analyses still should be carried out to support this supposition. Since homarine is also present in *C. caerulea*, we can conclude that these particular epidermal glandular structures of the Dendronotoidea are not a morphological adaptation to store homarine. Their function remains unclear.

Acknowledgments. We thank Sabrina Bleidissel (Wuppertal), Katharina Händeler (Bonn), Yvonne Grzymbowski (Bonn) and Wolfgang Wägele (Bonn) for help in collecting material. This study was partly funded by the German Science Foundation to HW (SPP 1127, "Adaptive radiation – origin of biological diversity": Wa 618/8).

REFERENCES

ARNAUD, F. 1978. A new species of *Ascorhynchus* (Pycnogonida) found parasitic on an opisthobranch mollusc. Zoological Journal of the Linnean Society **63**: 99–104.

AVILA, C. 1995. Natural products of opisthobranch Molluscs: A biological review. Oceanography and Marine Biology: An A nual Review **33**: 487–559.

AVILA, C. & PAUL, V.J. 1997. Chemical ecology of the nudibranch *Glossodoris pallida*: is the location of diet-derived metabolites important for defence? Marine Ecology Progress Series **150**: 171–180.

AVILA, C., IKEN, K., FONTANA, A. & CIMINO, G. 2000. Chemical ecology of the Antarctic nudibranch *Bathydoris hodgsoni* Eliot, 1907: defensive role and origin of its natural products. Journal of Experimental Marine Biology end Ecology **252**: 27–44.

BARSBY, T., LININGTON, R.G. & ANDERSEN, R.J. 2002. De Novo terpenoid biosynthesis by the dentronotid nudibranch *Melibe leonina*. Chemoecology **12** (4): 199–202.

BECERRO, M.A., GOETZ, G., PAUL, V.J. & SCHEUER, P.J. 2001. Chemical defences of the sacoglossan mollusk *Elysia rufescens* and its host alga *Bryopsis* sp. Journal of Chemical Ecology **27**: 2287–2299.

BEERS, J.R. 1967. The species distribution of some naturally-occurring Quaternary Ammonium Compounds. Comparative Biochemistry and Physiology **21**: 11–21

BERKING, S. 1986. Is homarine a morphogen in the marine hydroid *Hydractinia*? Roux's Archive of Developmental Biology **195**: 33–38.

BERKING, S. 1987. Homarine (N-methylpicolinic acid) and trigonelline (N-methylnicotinic acid) appear to be involved in pattern control in a marine hydroid. Development **99**: 211–220.

CAREFOOT, T.H. 1987. *Aplysia:* its biology and ecology. Oceanography and Marine Biology: An Annual Review **25**: 167–284.

CARR, W.E.S., NETHERTON, J.C., GLEESON, R.A. & DERBY, C.D. 1996. Stimulants of feeding behavior in fish: Analyses of Tissues of Diverse Marine Organisms. Biological Bulletin **190**: 149–160

CIAVATTA, M. L., TRIVELLONE, E., VILLANI, G. & CIMINO, G. 1996. Prenylphenols from the skin of the aeolid mollusc *Cratena peregrina*. Gazzetta Chimica Italiana **12**: 707–710.

CIMINO,G., DE ROSA, C., DE STEFANO, C., SODANO, G. 1980. Cholest-4-en-4, 16β, 18, 22R-tetrol-one 16,18-diacetate, a novel polyhydroxylated steroid from the hydroid *Eudendrium* sp. Tetrahedron Letters 21: 3303–3304.

CIMINO, G., DE ROSA, C. DE STEFANO, C., SODANO, G. & VILLANI, G. 1983. Dorid nudibranch elaborates its own chemical defense. Science **219**: 1237–1238.

CIMINO, G., CRISPINO, A., DI MARZO, V., GAVAGNIN, M. & ROS, J.D. 1990. Oxytoxins, bioactive molecules produced by the marine opisthobranch mollusc *Oxynoe olivacea* from a diet-derived precursor. Cellular and Molecular Life Sciences **46**(7): 767–770.

CIMINO, G. & GHISELIN, T.G. 1998. Chemical defense and evolution in the Sacoglossa (Mollusca: Gastropoda: Opisthobranchia). Chemoecology **8**(2): 51–60.

CIMINO, G., FONTANA, A. & GAVAGNIN, M. 1999. Marine opisthobranch molluscs: chemistry and ecology in sacoglossans and dorids. (Review) Current Organic Chemistry **3**: 327–372.

CIMINO, G., CIAVATTA, M. L., FONTANA, A. & GAVAGNIN, M. 2001. Metabolites of marine opisthobranchs: chemistry and biological activity. Pp. 578–637 in: TRINGALI, C. (ed.) Bioactive Compounds from Natural Sources. Taylor & Francis Ltd, London.

CIMINO, G., FONTANA, A., CUTIGNANO, A. & GAVAGNIN, M. 2004. Biosynthesis in opisthobranch molluscs: General outline in the light of recent use of stable isotopes. Phytochemistry Reviews **3**: 285–307.

CIMINO, G. & GAVAGNIN, M. (Eds.) 2006. Molluscs. From Chemo-ecological Study to Biotechnological Application. Series: Progress in Molecular and Subcellular Biology, Vol. 43. Subseries Marine Molecular Biotechnology, Springer.

COLL, J.C., BOWDEN, B.F., TAPIOLAS, D.M., WILLIS, R.H., DIURA, P., STREAMER, M. & TROTT, L. 1985. The terpenoid chemistry of soft corals and its implications. Tetrahedron **41 (6)**: 1085–1092.

CRONIN, G., HAY, M.E., FENICAL, W. & LINDQUIST, N. 1995. Distribution, density, and sequestration of host chemical defenses by the specialist nudibranch *Tritonia hamnerorum* found

at high densities on the sea fan *Gorgonia ventalina*. Marine Ecology Progress Series **119**: 177–189.

DALL, W. 1971. The role of homarine in decapod Crustacea. Comparative Biochemistry and Physiology **39B**: 31–44.

DICKSON, D.M.J. & KIRST, G.O. 1986. The role of b-dimethylsulphoniopropionate, glycine betaine and homarine in the osmoacclimation of *Platymonas subcordiformis*. Planta **167**: 536–543.

DUCKLOW, H.W. & MITCHELL, R. 1979. Bacterial populations and adaptations in the mucus layers on living corals. Limnology and Oceanography **24 (4)**: 715–725

DUERDEN, J.E. 1906. The role of mucus in corals. Quarterly Journal of Microscopical Science **49**: 591–614.

EDMUNDS, M. 1966. Protective mechanisms in the Eolidacea (Mollusca Nudibranchia). Journal of the Linnean Society, Zoology **46**: 27–71.

FRICK, K. 2003. Response in nematocyst uptake by the Nudibranch *Flabellina verrucosa* to the presence of various predators in the Southern Gulf of Maine. Biological Bulletin 205: 367–376.

GARSON, M.J. 2006. Marine Mollusks from Australia and New Zealand: Chemical and ecological studies. Pp. 159–174 in: CIMINO, G. & GAVAGNIN, M. (eds.) Molluscs from Chemo-ecological Study to Biotechnological Application. Springer.

GAVAGNIN, M., MARIN, A., CASTELLUCCIO, F., VILLANI,G. & CIMINO,G. 1994a. Defensive relationships between *Caulerpa prolifera* and its shelled sacoglossan predators. Journal of Experimental Marine Biology and Ecology **175:** 197–210.

GAVAGNIN, M., MARIN, A., MOLLO, E., CRISPINO, A., VILLANI, G. & CIMINO, G. 1994b. Secondary metabolites from Mediterranean Elysioidea: origin and biological role. Comparative Biochemistry and Physiology **108** B: 107–115.

GAVAGNIN, M. & FONTANA, A. 2000. Diterpenes from Marine Opisthobranch Molluscs. Current Organic Chemistry **4**: 1201–1248.

GAVAGNIN, M., MOLLO, E., CASTELLUCCIO, F., GHISELIN, M. T., CALADO, G. & CIMINO, G. 2001. Can mollusks biosynthesize typical sponge metabolites? The case of the nudibranch *Doriopsilla areolata*. Tetrahedron **57**: 8913–8916.

GREENWOOD, P. G. AND MARISCAL, R. N. 1984a. The utilization of cnidarian nematocysts by aeolid nudibranchs: nematocyst maintenance and release in *Spurilla*. Tissue Cell **16**: 719–730.

GREENWOOD, P. G. AND MARISCAL, R. N. 1984b. Immature nematocyst incorporation by the aeolid nudibranch *Spurilla neapolitana.* Marine Biology **80**: 35–38.

GUERRIERO, A., D'AMBROSIO, M. & PIETRA, F. 1987. Verecynarmin A, a novel briarane diterpenoid isolated from both the Mediterranean nudibranch mollusc *Armina maculata* and its prey, the pennatulacean octocoral *Veretillum cynomorium*. Helvetica Chimica Acta **70**: 984–991.

GUERRIERO, A., D'AMBROSIO, M. & PIETRA, F. 1988. Slowly interconverting conformers of the briarane diterpenoids verecynarmin B, C, and D, isolated from the nudibranch mollusc *Armina maculata* and the pennatulacean octocoral *Veretillum cynomorium* of East Pyrenean waters. Helvetica Chimica Acta **71**: 472–485.

GUERRIERO, A., D'AMBROSIO, M. & PIETRA, F. 1990. Isolation of the cembranoid preverecynarmin alongside some briaranes, the verecynarmins, from both the nudibranch mollusc *Armina maculata* and the octocoral *Veretillum cynomorium* of the East Pyrenean Mediterrandean Sea. Helvetica Chimica Acta **73**: 277–283.

HAMANN, M.T. 2004. Technology evaluation: Kahalalide F, PharmaMar. Current Opinion in Molecular Therapeutics **6 (6)**: 657–665.

HO, J.S. 1981. *Ismaila occulta*, a new species of poecilostomatoid copepod parasitic in a dendronotid nudibranch from California. Journal of Crustacean Biology **1**: 130–136.

HOPPE-SEYLER 1933. Zur Isolierung von Homarin. Hoppe Seyler's Zeitschrift für physiologische Chemie **22**: 105

HUYS, R. 2001. Splanchnotrophid systematics: a case of polyphyly and taxonomic myopia. Journal of Crustacean Biology **21**: 551–561.

IKEN, K., AVILA, C., FONTANA, A. & GAVAGNIN, M. 2002. Chemical ecology and origin of defensive compounds in the Antarctic nudibranch *Austrodoris kerguelensis* (Opisthobranchia: Gastropoda). Marine Biology **141**: 101–109.

JENSEN, K. R. 1987. *Ismaila monstrosa* Bergh (Copepoda: Splanchnotrophidae) found parasitizing in *Ercolania funerea* (Costa) (Gastropoda: Ascoglossa). Ophelia **28**: 75–84.

JOHNSON, P.M. & WILLOWS, A.O.D. 1999. Defence in sea hares (Gastropoda, Opisthobranchia, Anaspidea): multiple layers of protection from egg to adult. Marine and Freshwater Behaviour and Physiology **32**: 147–180.

KARUSO, P. 1987. Chemical Ecology of the Nudibranchs. Bioorganic Marine Chemistry **1**: 31–60.

KINDLER, H.L., TOTHY, P.K., WOLFF, R., MCCORMACK, R.A., ABBRUZZESE, J.L., MANI, S., WADE-OLIVER, K.T. & VOKES, E.E. 2005. Phase II trials of dolastatin-10 in advanced pancreaticobiliary cancers. Investigational New Drugs **23 (5)**: 489–493.

MARIN, A. & ROS, J. 2004. Chemical defenses in Sacoglossan Opisthobranchs: Taxonomic trends and evolutive implications. Scientia Marina **68** (Supplement 1): 227–241.

MARIN, A., ALVAREZ, L.A., CIMINO, G., & SPINELLA, A. 1999. Chemical defence in cephalaspidean gastropods: Origin, anatomical location and ecological roles. Journal of Molluscan Studies **65**: 121–131.

MATHIAS, A.P., ROSS, D.M. & SCHACHTER, M. 1960. The distribution of 5-hydroxytryptamine, tetra-methylammonium, homarine, and other substances in sea anemones. The Journal of Physiology **151**: 296–311.

MCBETH, J.W. 1972. Carotenoids from nudibranchs II. The partial characterization of hopkinsiaxanthin. Comperative Biochemistry and Physiology **41B**: 69–77.

MCCLINTOCK, J.B., BAKER, B.J., HAMANN, M.T., YOSHIDA, W., SLATTERY, M., HEINE, J.N., BRYAN, P.J., JAYTILAKE, G.S., MOON, B.H. 1994. Homarine as a feeding deterrent in common shallow water antarctic lamellarian gastropod *Marseniopsis mollis*: A rare example of chemical defense in a marine prosobranch. Journal of Chemical Ecology **20 (10)**: 2539–2549.

MCDONALD, G.R. & NYBAKKEN, J.W. 1997. A list of the worldwide food habits of nudibranchs: http://people.ucsc.edu/~mcduck/nudifood.htm. last access 18-05-2007.

MCPHAIL, K.L., DAVIES-COLEMAN, M.T., STARMER, J. 2001. Sequestered chemistry of the arminacean nudibranch *Leminda millecra* in Algoa Bay, South Africa. Journal of Natural Products **64** (9)*:* 1183–1190

MIYAMOTO, T. 2006. Selected bioactive compounds from Japanese anaspideans and nudibranchs. Pp. 199–214 in: CIMINO, G. & GAVAGNIN, M. (eds.) Molluscs from Chemo–ecological study to biotechnological application. Springer.

OKUDA, R. K., KLEIN, D., KINNEL R., Li M.& SCHEUER P. J. 1982. Marine natural products: the past twenty years and beyond. Pure and Applied Chemistry **54**: 1907–1915.

PENNEY, B.K. 2004. Individual selection and the evolution of chemical defence in nudibranchs: experiments with whole *Cadlina luteomarginata* (Nudibranchia: Doridina). Journal of Molluscan Studies Research Notes **70**: 399–400.

PEREZ, E.A., HILLMAN, D.W., FISHKIN, P.A., KROOK, J.E., TAN, W.W., KURIAKOSE, P.A., ALBERTS, S.R. & DAKHIL, S.R. 2005. Phase II trial of dolastatin–10 in patients with advanced breast cancer. Investigational New Drugs **23 (3)**: 257–261.

ROGERS, C.N., DE NYS, R. & STEINBERG, P.D. 2002. Effects of algal diet on the performance and susceptibility to predation of the sea hare *Aplysia parvula*. Marine Ecology Progress Series **236**: 241–254.

RUDMAN, W. B. 1972. Structure and functioning of the gut in the Bullomorpha (Opisthobranchia). Journal of Natural History 6: 547–560.

SAAD, E.D., KRAUT, E.H., HOFF, P.M., MOORE, D.F. JR, JONES, D., PAZDUR, R. & ABBRUZZESE, J.L. 2002. Phase II study of dolastatin-10 as first-line treatment for advanced colorectal cancer. American Journal of Clinical Oncology **25** (5): 451–453.

SCHRÖDL, M. 2002. Heavy infestation by endoparasitic copepod crustaceans (Poecilostomatoidea: Splanchnotrophidae) in Chilean opisthobranch gastropods, with aspects of splanchnotrophid evolution. Organisms, Diversity and Evolution **2**: 19–26

SCHRÖDL, M. (ed.) 2003. Sea Slugs of Southern South America. Hackenheim: ConchBooks

SLATTERY, M., HAMANN, M.T., MCCLINTOCK, J.B., PERRY, T.L., PUGLISI, M.P., YOSHIDA, W.Y. 1997. Ecological roles for water-borne metabolites from Antarctic soft corals. Marine Ecology Progress Series **161**: 133–144.

SLATTERY, M., AVILA, C., STARMER, J. & PAUL, V.J. 1998. A sequestered soft coral diterpene in the aeolid nudibranch *Phyllodesmium guamensis* Avila, Ballesteros, Slattery, Starmer and Paul. Journal of Experimental Marine Biology and Ecology **226** (1): 33–49.

SODANO, G., & SPINELLA, A. 1986. Janolusimide: a lipophilic tripeptide toxin from the nudibranch mollusc *Janolus cristatus*. Tetrahedron Letters **27**: 2505–2508.

TARGETT, N.M., BISHOP, S.S., MCCONNELL, O.J. & YODER, J.A. 1983. Antifouling agents against the benthic marine diatom, *Navicula salinicola*. Journal of Chemical Ecology **9** (7): 817–829.

TEEYAPANT, R., KREIS, P., WRAY, V., WITTE, L. & PROKSCH, P. 1993a. Brominated secondary compounds from the marine sponge *Verongia aerophoba* and the sponge feeding gastropod *Tylodina perversa*. Zeitschrift für Naturforschung, **48**c: 640–644.

TEEYAPANT, R., WOERDENBAG, H. J., KREIS, P., HACKER, J., WRAY, V., WITTE, L. & PROKSCH, P. 1993b. Antibiotic and cytotoxic activity of brominated compounds from the marine sponge *Verongia aerophoba*. Zeitschrift für Naturforschung, **48**c: 939–944.

THOMPSON, T.E. 1960. Defensive adaptations in opisthobranchs. Journal of the Marine Biological Association of the United Kingdom **39**: 123–134.

WÄGELE, H. & Willan, R. C. 2000. On the phylogeny of the Nudibranchia. Zoological Journal of the Linnean Society **130**: 83–181.

WÄGELE, H. 2004. Potential key characters in Opisthobranchia (Gastropoda, Mollusca) enhancing adaptive radiation. Organisms, Diversity & Evolution **4**: 175–188.

WÄGELE, H., BALLESTEROS, M. & AVILA, C. 2006. Defensive glandular structures in opisthobranch molluscs: from histology to ecology. Oceanography and Marine Biology: An Annual Review **44**: 197–276.

WAHIDULLAH, S., GUO, Y.-W., FAKHR, I.M.I. & MOLLO, E. 2006. Chemical diversity in opisthobranch molluscs from scarcely investigated Indo-Pacific areas. Pp. 175–198 in: CIMINO, G. & GAVAGNIN, M. (eds.) Molluscs from chemo-ecological study to biotechnological application. Springer.

YAMADA, K. & KIGOSHI, H.1997. Bioactive compounds from the sea hares of two genera: *Aplysia* and *Dolabella*. Bulletin of the Chemical Society, Japan **70**: 1479–1489.

Authors' adresses: Sven AFFELD, Institut für Pharmazeutische Biologie, Universität Bonn, Nussallee 6, 53121 Bonn, Germany, E-mail: sven.affeld@uni-bonn.de; Heike WÄGELE (corresponding author) Institut für Evolutionsbiologie und Ökologie, Universität Bonn, An der Immenburg 1, 53121 Bonn, Germany, and Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany, E-mail: hwaegele@evolution.uni-bonn.de; Conxita AVILA, Dept. of Animal Biology (Invertebrates), Facultat de Biologia, Universitat de Barcelona, Av. Diagonal, 645 08028 Barcelona, Spain, E-mail: conxita.avila@ub.edu; Stefan KEHRAUS, Institut für Pharmazeutische Biologie, Universität Bonn, Nussallee 6, 53121 Bonn, Germany; Gabriele KÖNIG, Institut für Pharmazeutische Biologie, Universität Bonn, Nussallee 6, 53121 Bonn, Germany, E-mail: g.koenig@uni-bonn.de.