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Selected chemo-ecological studies of marine opisthobranchs from Indian coasts[#]

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Abstract

The report summarizes the chemical and ecological studies of benthic marine invertebrates from the southern coast of the Indian peninsula. In particular, the paper is centred on the chemical investigations of opisthobranch molluscs (*Volvatella* sp., *Jorunna funebris*, *Elysia* sp., *Glossodoris atromarginata*, *Chromodoris mandapamensis*, *Hypselodoris kanga* and *Haminoea cymbalum*) and the associated preys, mainly sponges. It also describes the biological activity of the isolated compounds and the ecological role with special attention to their defensive and chemo-taxonomical significance.

Keywords: Chemical ecology, opisthobranchs, molluscs, secondary metabolites, Indo-Pacific Ocean.

1. Introduction

Secondary metabolites can play important role as chemical messengers in marine and terrestrial environments. A variety of sessile and slow-moving invertebrates is organised in marine communities where, in analogy with terrestrial habitats, the interactions are mediated by chemical compounds.[1] Thousands of biologically active natural products have been identified from these organisms and, in the last few years, marine benthos have been the subject of many chemical and ecological studies.[2] Accordingly, over the years, our knowledge of the chemical basis of ecological processes has increased considerably.

Opisthobranch molluscs represent an ideal model for studying chemo-ecological interactions.[3–5] Despite an apparent vulnerability, these invertebrates defend themselves by sophisticated mechanisms that involve both behavioural strategies and use of chemical compounds.[1,3,4] A large number of secondary metabolites, usually with interesting biological properties, has been isolated from opisthobranchs.[3–5] Most of these products show a wide chemical variety and have usually derived from the organisms preyed on by the opisthobranchs. Accordingly, the structure elucidation of these products has often been involved in proving prey–predator relationships even in the absence of direct field observation. Otherwise, the molluscs are rarely able

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to biotransform or biosynthesize *de novo* their own chemicals. In these cases, the chemical characteristics of the secondary metabolites have sometimes been used as chemo-taxonomical parameters.

Despite a large amount of work reported on the chemistry of marine organisms from the Indo-Pacific Ocean, little is known about the opisthobranchs from the Indian sea coast. This paper concentrates on some select examples of recent chemical studies of opisthobranchs from the southern regions of India.[6–8] In particular, we review the major results of a plurennial joint project between the National Institute of Oceanography (NIO, India) and the Istituto per la Chimica di Molecole di Interesse Biologico (ICMIB, Italy). Although the primary aim is to describe the chemistry of Indian opistobranch's secondary metabolites, we also summarize the ecological (prey–predator relationships, defence activity) and chemo-taxonomical aspects, as well as the potential applications related to the chemical studies of these organisms.[9]

2. Opisthobranch biology and chemo-ecology outline

Chemical ecology examines the role of naturally occurring compounds in inter- or intraspecific interactions.[1] Undoubtedly, interest in marine chemical ecology developed as knowledge of the numerous and diverse natural products found in marine organisms increased. Research in this field has spread geometrically and geographically, and, consequently, evidence supporting the role of secondary metabolites as chemical mediators has been accumulating rapidly.[2–5]

Opisthobranchs [Mollusca: Gastropoda] are divided into nine orders according to Rudman[10] (Fig. 1). These invertebrates are diffused worldwide and inhabit many ecological habitats from tropical to polar regions. Opisthobranchs are either herbivorous (feeding on algae) or carnivorous (feeding mainly on sponges, tunicates, soft corals or other molluscs).[11] Only the genera of the order Cephalaspidea displays both the feeding habits. A peculiar aspect of the evolutionary history of the opisthobranch is the progressive reduction of the shell which in the order Nudibranchia is completely absent. The evolutionary aspects related to the gradual loss of mechanical defence provided by the shell and the acquisition of chemical defence among the main orders of opisthobranchs have recently been discussed.[11,12]

Opisthobranch molluscs are, in fact, known to possess a great variety of chemicals. Most of them are associated with specific ecological function including defence, inter- and intraspecific

Phylum	MOLLUSCA
Class	GASTROPODA
OPISTHOBRANCHIA	
Order	**Cephalaspidea**
Order	Acochlidea
Order	Rhodopemorpha
Order	**Sacoglossa**
Order	Anaspidea
Order	Notaspidea
Order	Thecosomata
Order	Gymnosomata
Order	**Nudibranchia**

FIG. 1. Taxonomy of opisthobranchs according to Rudman.[10]

communication. The chemistry and ecology of most of these substances have been deeply investigated and a compendium of this research activity can be found in several reviews.[1–5, 13, 14] The studies we report in this paper are mainly related to nudibranch (*Glossodoris atromarginata*, *Jorunna funebris*, *Hypselodoris kanga*, *Chromodoris mandapamensis*), sacoglossan (*Volvatella* sp., *Elysia* sp.) and cephalaspidean (*Haminoea cymbalum*) molluscs.

3. Biological material and collections

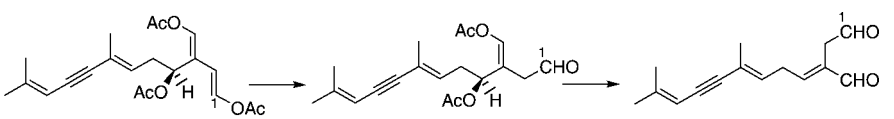
Biological material has been collected during two diving trips along the southern coast of India (Mandapam in Tamil Nadu and Muttom in Kerala) during May 1998 and March 1999. Voucher specimens of the studied organisms are kept at NIO. Taxonomic identification has been carried out by Dr Á. Valdés (Department of Invertebrate Zoology, California Academy of Sciences, USA) and Prof. J. Ortea (Departemento de Biologia, Unversidad de Oviedo, Spain) for opisthobranchs and Dr P. A. Thomas (Centre for Marine Fisheries Research Institute, Vizhinjam, India) for sponges.

4. Sacoglossans

4.1. *Volvatella* sp.

Sacoglossans are herbivore molluscs that feed usually on green algae from which they sequester chloroplast[5] and secondary metabolites. A chemo-ecological survey on sacoglossans has appeared recently in the literature.[3]

The oxynoacean *Volvatella,* Pease 1860 is one of the most primitive members of the order Sacoglossa. Like other molluscs of this order, *Volvatella* lives in close association with algae of the genus *Caulerpa* that represents the main food for the invertebrate.[6] The relation between *Caulerpa* and shelled sacoglossans has been well studied in Mediterranean and Caribbean benthic communities.[15,16] The alga contains caulerpenyne (**1**), a linear sesquiterpene characterized by the presence of the terminal 1,4-diacetoxybutadiene moiety.[17] Caulerpenyne (**1**), the major secondary metabolite of the alga, is a toxic substance with grazing deterrent properties towards herbivorous fish.[18] In analogy with other seaweed products, however, **1** fails to inhibit feeding by specialist herbivores like some sacoglossans.[15, 16] These opisthobranchs are able to eat the *Caulerpa* and even to sequester the toxic component (**1**). Experiments with the Mediterranean sacoglossans have demonstrated that the molluscs are also able to biotransform **1** into more active derivatives, oxytoxin-1 (**2**) and -2 (**3**) by gradual hydrolysis of the enol acetate moieties (Fig. 2). Such a model has been very well studied with the mollusc *Oxynoe olivacea* but it has probably been adapted also by other conchoid sacoglossans from diverse geographical areas.[19] The oxytoxins that are more toxic than **1** in biological assays are regarded as the true defensive allomones of this group of molluscs.



caulerpenyne (**1**) oxytoxin-1 (**2**) oxytoxin-2 (**3**)

FIG. 2. Chemical defensive strategy of the Mediterranean oxynoacean sacoglossans.

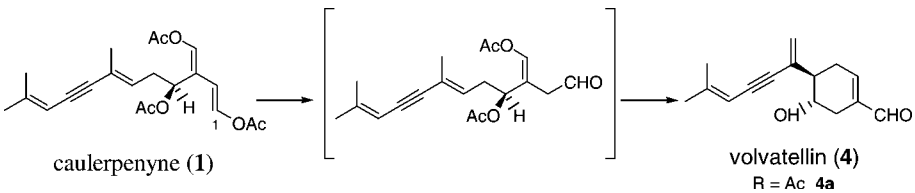


FIG. 3. Hypothetical formation of volvatellin **(4)** from caulerpenyne **(1)**.

In our study, the Indian specimens of *Volvatella* sp. were observed grazing upon *Caulerpa* sp.[6] The field observation that *Volvatella* feeds on the alga was biochemically supported by the presence of caulerpenyne (**1**) in both the organisms. Otherwise, the extracts of *Volvatella* contained also an unknown component that was later characterized as the aldehyde (**4**).[6] The structure of the product, called volvatellin from the name of the opisthobranch, is closely related to caulerpenyne (**1**). The relative stereochemistry of **4** was proposed on the basis of selective decoupling experiments on both the natural compound (**4**) and its acetyl derivative (**4a**). GC-MS analysis also provides evidence of the presence of **4** in the mucus of the molested animals.[6] This finding is in agreement with anatomical studies that suggest the storage of defensive allomones in the mantle cavity linked to the siphonal spout at rear of the shell.[6] In analogy with oxytoxins (**1** and **2**), volvatellin (**4**) may derive from **1** by an enzymatic process, such as that reported in Fig. 3.

Otherwise, Pietra and coworkers have recently reported the spontaneous transformation of **2** into the cyclic skeleton of **4** under acidic condition.[20] This finding rises the question about the origin of volvatellin (**4**) in the molluscs, since neither of the hypotheses, biotransformation or spontaneous conversion, can be ruled out. The ecological properties of **4** were not tested for the inherent instability of the molecule, but a defensive role was proposed on the basis of analogy with other sacoglossans.[6]

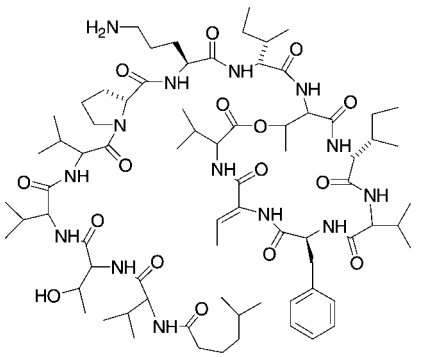
4.2. *Elysia* sp.

Among the sacoglossans, *Elysia rufescens* and more recently *Elysia ornata* are known to accumulate toxic cyclic depsipeptides from their food source, the green alga *Bryopsis*.[21–23] These compounds, called kahalides, show very promising biological activity, including antiviral, antimalarial and, first of all, antitumor properties. In particular, kahalide F[21] (**5**) is in preclinical trials against lung and colon cancers. An Indian collection of *Elysia* sp. yielded a mixture of kahalides from which kahalide A (**6**)[23] was obtained after chromatographic purification. The same compound has also been found in the *Bryopsis* alga associated with the opisthobranch.

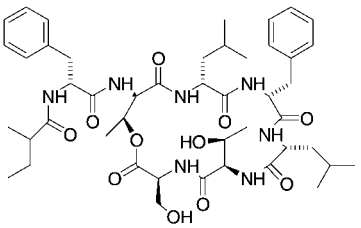
5. Nudibranchs

5.1. *Jorunna funebris*

J. funebris, Kelaar 1858 is a white nudibranch belonging to the family Kentrodorididae.[10] In a previous study of Pacific specimens of this mollusc, Scheuer and coworkers had characterized a series of isoquinoline alkaloids, e.g. *N*-formyl-1,2-dihydroreneirol acetate **(7)**, probably derived

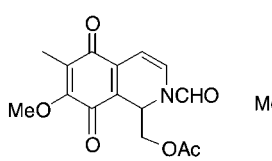


Kahalide F (**5**)

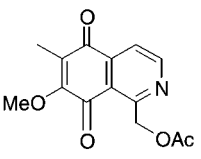


kahalide A (**6**)

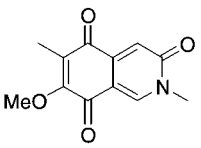
from sponges of the genus *Xestospongia* that was regarded as a likely prey of the nudibranch.[24] Isoquinoline alkaloids are rather common in marine sponges [for example, renierol (**8**)[25] and mimosamycin (**9**)[26]] where these compounds are thought to act as antimicrobial agents. The Indian specimens of *J. funebris* were collected in two different campaigns together with a blue sponge, later identified as *Oceanapia* sp.[7] The extract of the nudibranch was characterized by a high content of a very unstable product, jorumycin (**10**), of which the isolation and characterization were however challenging.[7] The spectroscopic data of **10** suggested a dimeric structure related to a very promising group of antitumor and antimicrobial metabolites isolated from bacteria (saframycins, e.g. **11**),[27–29] tunicates (ecteinascidins, e.g. **12**)[30,31] and sponges (renieramycins, e.g. **13**).[32–34] Jorumycin (**10**) was indeed cytotoxic against several tumor cell lines [HT29 (human colon carcinoma), A549 (human lung carcinoma), Mel28 (human melanoma), P388 (mouse lymphoma)] at very low concentration (IC_{50}–1.5 ng/ml).[7] In justification of the instability of **8**, its



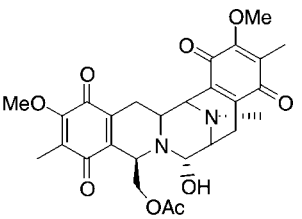
N-formyl-1,2-dihydrorenierol acetate (**7**)



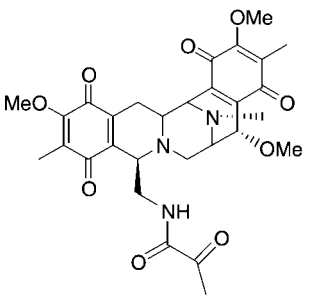
renierol (**8**)
acetyl renierol (**8a**)



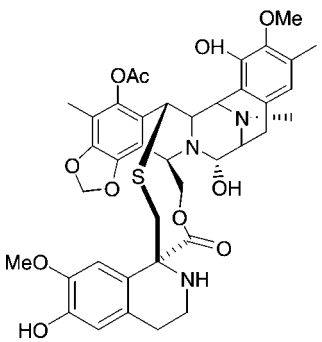
mimosamycin (**9**)



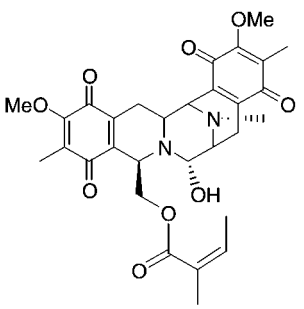
jorumycin (**10**)



saframycin C (**11**)



ecteinascidin 743 (**12**)



renieramycin E (**13**)

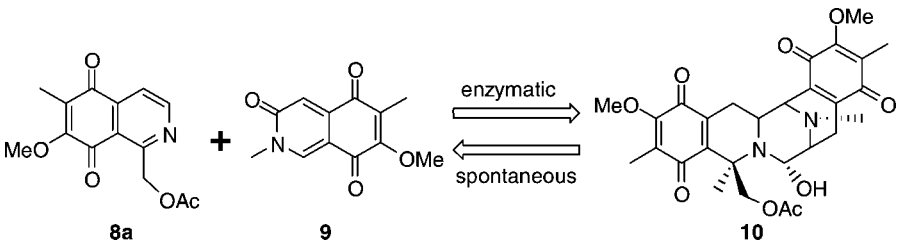


FIG. 4. Hypothetical relationship between monomeric isoquinolines (**8a** and **9**) and jorumycin (**10**).

structure contained two easily reducible quinone rings and a very reactive carbinolamine moiety (C-21). The solution conformation of jorumycin (**10**), that was obtained by restrained molecular dynamic, also resembled the crystal structures of ecteinascidins[31] and saframycins.[28] The molecule assumes a rather constrained L-shape with the two quinone rings that appear orthogonally flipped.[7] This conformation exposes the carbinolamine group towards the attack of nucleophiles and may be essential for the activity of **10**, as already proposed for the cytotoxic ecteinascidins and saframycins.

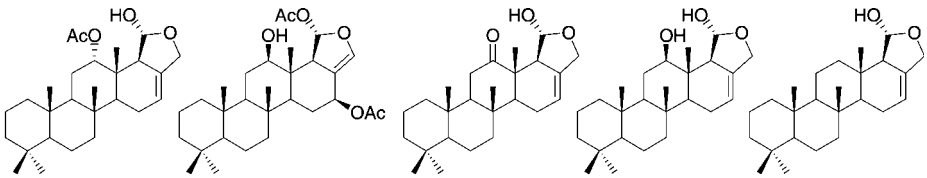
The extracts of *J. funebris* also contained acetyl renierol (**8a**) as a minor component. This compound probably derived from the sponge where the nudibranch had been collected upon, since **8a**, together with mimosamycin (**9**), was also isolated from the *Oceanapia* extracts. If this fact confirmed the prey–predator relationship between mollusc and sponge, it did not clarify the origin of jorumycin (**10**), since this alkaloid was totally absent in the sponge extracts. Nevertheless, other researchers have pointed[33] out that isoquinolinones (for example, **8** and **9**) may derive from oxidative cleavage of dimeric structures, such as **10**, and therefore one may suggest that the occurrence of **8a** and **9** in the sponge may be indicative of the presence of the unstable jorumycin (**10**). On the other hand, chance is that the nudibranch may dimerize the sponge isoquinolines **8a** and **9** to produce jorumycin (**10**). Both possibilities (Fig. 4) are open at this time.

Finally, jorumycin (**10**) was also isolated from the mucus of the mollusc, thus suggesting a defensive role for the alkaloid.

5.2. *Glossodoris atromarginata*

Glossodoris atromarginata, Cuvier 1804 is a relatively common nudibranch, widely distributed in temperate and tropical seas. The mollusc belongs to the family Chromodorididae that includes a large number of brightly coloured animals mainly of the genera *Chromodoris*, *Hypselodoris*, *Glossodoris* and *Cadlina*. The systematic of the family is often a matter of debate and many taxonomic revisions are reported in the literature. Particularly conflicting is the taxonomy of the genus *Glossodoris* that, for long time, has been considered synonymous with *Chromodoris* and *Hypselodoris*.

We have analyzed two different populations of *G. atromarginata* from the Indian areas.[8] The molluscs were collected on their potential preys, the sponge *Hyatella cribriformis* and an unidentified sponge probably belonging to the genus *Spongia*. A series of pentacyclic scalaranes, compounds **14**–**17**, were isolated as main metabolites of the nudibranchs, whereas **14** and **16** were separately isolated from the sponges. The population of *G. atromarginata* that we analyzed



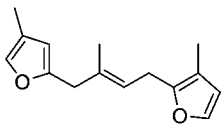
deoxoscalarin (**14**) heteronemin (**15**) **16** **17** **18**

first contained only deoxoscalarin (**14**), previously isolated from sponges of the genus *Cacospongia* and the Mediterranean dorid *Glossodoris* (*Hypselodoris*) *tricolor*.[35,36] The product was equally distributed in the skin and the inner organs of the Indian molluscs. This suggested a dietary origin that was definitely confirmed by the presence of **14** in the extract of the associated sponge *H. cribriformis*. The study of the second collection of *G. atromarginata* was interesting and generated new issues. The extracts of these animals contained the known sesterterpene heteronemin (**15**) together with two new scalaranes **16** and **17**, the structures of which were determined by chemical and spectroscopic methods.[8] In particular, the relative stereochemistry of **17** was confirmed by comparison with the semisynthetic product **18**, that was obtained in agreement with a methodology recently developed.[37] Interestingly, **15–17** were accum- ulated mainly in small, round formations placed along the mantle border of the mollusc. These anatomical structures strongly resembled both shape and function of the so-called mantle dermal formations (MDFs), histologically characterized in Mediterranean *Hypselodoris* nudibranchs.[38] Compound **17** was also found in the encrusting sponge, tentatively identified as *Spongia* sp., collected together with the population of *G. atromarginata*, thus confirming their prey–predator relationship. The origin of **16** is otherwise intriguing. In fact, the co-occurrence of **16** and **17** in the mollusc, together with the finding of only **17** in the sponge, may suggest that *G. atromarginata* can biotransform the sponge compound by oxidation of the hydroxy group at C-12 to produce the keto derivative **16**. A similar attitude has also been proposed for another of dorid mollusc, the Mediterranean *Hypselodoris orsini*, that seems to be able to biotransform scalarane compounds.[36] However, the presence of **16** in *G. atromarginata* may also be due to selective accumulation of this metabolite from other dietary sponges.

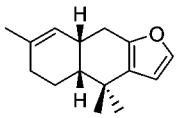
5.3. *Hypselodoris kanga*

The molluscs of genus *Hypselodoris* are among the most brightly colored nudibranchs. These invertebrates have developed a very well-studied strategy of defence based on chemicals stored either in specific organelles of mantle[38] or actively secreted in the mucus. In the past years, we have chemically studied many different species of *Hypselodoris* from diverse geographical regions.[39–41] With the exception of *Hypselodoris orsini*,[36] all nudibranchs of this genus are characterized by the presence of furanosesquiterpenoids (for example, longifolin, **19**) that are sequestered from dietary sponges. This seems to be a specific metabolic marker of these organisms and we have proposed to regard it as a chemo-taxonomical parameter. In order to support such a proposal, we have continued analyzing populations of *Hypselodoris* and associated sponges from all over the world.

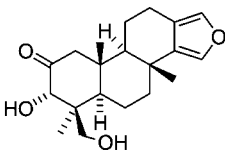
Hypselodoris kanga is a brightly coloured nudibranch widely diffused along the southern coast of the Indian peninsula. It was collected upon an encrusting sponge, later identified as



longifolin (**19**)



furodysinin (**20**)



spongiadiol (**21**)

Dysidea sp. In agreement with our hypothesis, the chemical analysis of both the organisms revealed the presence of large amount of furodysinin (**20**), a furanosesquiterpene previously isolated from nudibranchs and sponges.[3] The co-occurence of the same metabolite in the nudibranch and sponge further supported the prey–predator relationship between these two organisms. Like other *Hypselodoris*, the specimens of *H. kanga* possessed the typical spherical glands of mantle[38] that, after chemical analysis, showed to contain pure **20**.

In conclusion, the Indian collection of *Hypselodoris* confirms the chemical composition and biological information on this genus of nudibranchs. The Indian animals live closely with sponges of the genus *Dysidea*, which are the sources of furanosesquiterpenoids that are accumulated selectively by the mollusc into the MDFs. The defensive role of MDF-contained furanosesquiterpenes and pure furodysinin (**20**) has been rigorously demonstrated in earlier papers.[3]

5.4. *Chromodoris mandapamensis*

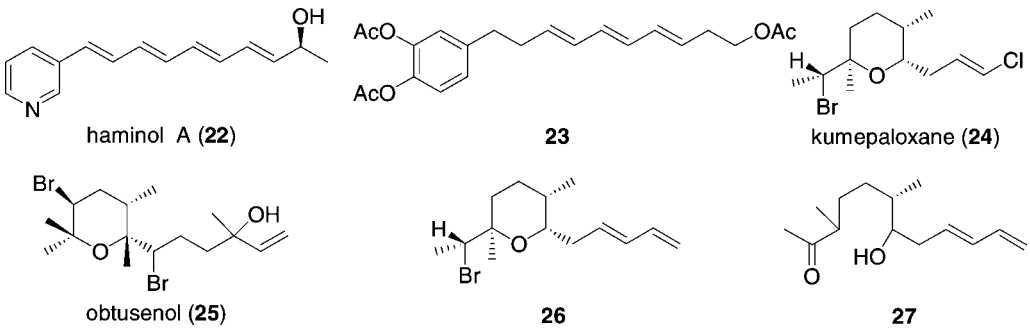
Chemical studies of *Chromodoris mandapamensis*, a new species[42] collected at Mandapam, yielded spongiadiol (**21**),[43] together with a mixture of related spongiane compounds. Compound **21** was preferentially accumulated in ramified, subepidermical mantle glands that were situated along the mantle margin of the mollusc.[42] Spongiadiol (**21**) and other compounds were also isolated from an unidentified sponge collected together with molluscs, thus proving the dietary origin of the terpenoids in *C. mandapamensis*.

6. Cephalaspideans

6.1. *Haminoea cymbalum*

Mollusc of the genus *Haminoea* has been studied regarding the use of aromatic substances, for example, **22** or **23**, as intraspecific mediators.[44–46] These products, that are presumably biosynthesized *de novo* by the molluscs, are released into the slime trail. When another specimen encounters the product it turns away and follows another path. The ecological role (alarm pheromone) of these compounds has been deeply proved by in-field and aquarium assays with diverse opisthobranchs of the genus *Haminoea*.[44–46]

In spite of the results, it has been reported that Guam specimens of *Haminoea cymbalum* (Quoy and Gaimard, 1835) contain only the tetrahydropyranyl kumepaloxane (**24**), a metabolite with deterrent properties toward carnivorous fish.[47] The origin of kumepaloxane is very uncertain, although its structure strongly resembles that of some algae metabolites (for example, obtusenol, **25**).[48] Although *H. cymbalum* is uncommon in Indo-Pacific, a large community of the mollusc (about 200 specimens) was found in shallow waters at Mandapam, Tamil Nadu region. The cephalaspideans were collected together with a dense mucus that appeared to be released by the animals. No evidence of prey–predator association was observed, even if the animals were



found in a photophilic habitat. The digestive gland, the mantle and the mucus of the molluscs contained the same metabolite, a brominated tetrahydropyran (**26**), which is very similar to kumepaloxone (**25**).[49] Although **26** has been previously described from the extracts of the sponge *Haliclona* sp.,[50] its origin is not clear. In fact, no other examples of halogenated tetrahydropyrans have been reported from sponges, whereas they have been described from algae extracts.[2] However, no mention of symbionts or epiphytes is reported for the studied specimen of *Haliclona*, and we did not observe any evidence of feeding upon algae for *H. cymbalum*. On the other hand, the mucus and mantle extracts of the Indian opisthobranchs contained linear ketoalcohol **27** as minor component. The structure of this product was determined by NMR and MS techniques. The molecule has an unusual arrangement that can be derived from either norsesquiterpene or acetogenin precursors. Obviously, **27** is closely related to the bromotetrapyrane **26**, but it is not clear if it derives from this latter compound or it is a biosynthetic precursor of the halogenated pyrane. The absence of **27** from the digestive gland of *H. cymbalum* is very interesting. This may suggest that the Indian cephalaspidean is able to biosynthesize *de novo* its own chemicals or biotransform dietary metabolites. Unfortunately, no case is reported in the literature in support of one or the other hypothesis.

Finally, the occurrence of **26** and **27** in the mantle and mucus of *H. cymbalum* suggests a defensive role for both the compounds. In the *absence* of field observation, this view is supported by antifeedant properties towards fish that are observed by similar halogenated compounds including kumepaloxane (**24**).[47]

7. Conclusion

Previous chemical studies of opisthobranch molluscs are relevant from different points of view: 1) the new chemicals have exhibited unique features; 2) chemistry has strongly favoured the construction of an evolutionary scenario; 3) some chemical roles have been clarified in order to understand the presence of opisthobranchs in very different habitats; 4) it has been proposed that many compounds play relevant ecological functions in controlling inter- and intraspecific communication; and 5) the research can select promising pharmacologically active molecules.

Since the opisthobranchs of the Indian coasts have rarely been studied for chemical composition, a systematic research can contribute to the growth of knowledge in this field and can give impetus to economic exploitation of the Indian marine resources. The early results from the project between NIO and ICMIB, started three years ago to investigate the benthic communities from the southern regions of India, are very promising.

In the family Chromodorididae, the three studied species confirm the selective dietary habits. *H. kanga* and the associated *Dysidea* sp. contained furanosesquiterpenes, whereas the specimens of *C. mandapamensis* were featured by polycyclic diterpenoids. The presence of scalarane sesterterpenes in *G. atromarginata* extracts was apparently conflicting with previous results that indicated the presence of furanoditerpenoids in a population of the same mollusc from Red Sea.[51] However, a re-analysis of the Red Sea specimens led to the taxonomic revision of these molluscs as *Glossodoris cincta.* The study of the Indian populations, therefore, supported the general characteristic of this genus of molluscs that, with a few exceptions, is associated with sponges containing scalarane metabolites.[1, 2]

The chemical study of the cephalaspidean *H. cymbalum* confirmed the previous results obtained with the population of the mollusc from Guam.[47] The secondary metabolites, **26** and **27**, are very likely derived from dietary sources. Interestingly, no aromatic substances, such as those isolated from Mediterranean *Haminoea* molluscs,[45–47] have been revealed in the extracts of *H. cymbalum*. Further studies, however, are needed to rule out their presence in the molluscs.

Volvatella sp. was a missing part in the chemical scenario of sacoglossan molluscs, since no other chemical studies have been carried out on this genus before. *Volvatella* chemistry confirms the general capability of oxyneacean molluscs to modify the main metabolite, caulerpenyne (**1**), present in their preferred diet, the algae belonging to the genus *Caulerpa*. Analogously, the chemical aspects of the Indian specimen of *Elysia* sp. are in agreement with the Scheuer's studies that showed the presence of dietary cyclic depsipeptides.

Finally, the most intriguing results were obtained studying the nudibranch *J. funebris*. The pharmacological properties of its main metabolite jorumycin (**10**) are very promising. However, the ecological aspects need further investigation. The origin of **10** is still not clear. The presence of related metabolites in very different marine organisms (sponges, tunicates, molluscs) may suggest their formation in symbiont microorganisms. The concentration of jorumycin in the mucus of the mollusc strongly supports its defensive role.

In conclusion, despite the little number of genera that have been studied, the outcome of this research is extremely interesting. We have described a few new molecules, some of which are involved in ecological interactions and thus have potential technological applications.

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