

Chemical defenses in Sacoglossan Opisthobranchs: Taxonomic trends and evolutive implications*

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SUMMARY: Sacoglossan sea slugs (Mollusca: Opisthobranchia) are one of the few groups of specialist herbivores in the marine environment. Sacoglossans feed suctorially on the cell sap of macroalgae, from which they “steal” chloroplasts (kleptoplasty) and deterrent substances (kleptochemistry), retaining intracellularly both host plastids and chemicals. The ingested chloroplasts continue to photosynthesize for periods ranging from a few hours or days up to 3 months in some species. Shelled, more primitive sacoglossans feed only on the siphonalean green algal genus *Caulerpa*, and they do not have functional kleptoplasty. The diet of Sacoglossans has radiated out from this ancestral food. Among the shell-less Plakobranchidae (=Elysiidae), the more primitive species feed on other siphonales (families Derbesiaceae, Caulerpaeaceae, Bryopsidaceae and Codiaceae) and fix carbon, while the more “advanced” species within the Plakobranchidae and Limapontioidea have a more broad dietary range. Most of these “advanced” species are unable to fix carbon because the chloroplasts of their food algae are mechanically disrupted during ingestion. Mesoherbivores are likely to be eaten if they live on palatable seaweeds, their cryptic coloration and form not always keeping them safe from predators. Sacoglossans prefer to live on and eat chemically defended seaweeds, and they use ingested algal chemicals as deterrents of potential predators. The most ancestral shelled sacoglossans (Oxynoidae) and some Plakobranchidae such as *Elysia translucens*, *Thuridilla hopei* and *Bosellia mimetica* have developed a diet-derived chemical defense mechanism. Oxynoids and *Thuridilla hopei* are able to biomodify the algal metabolites. However, the Plakobranchidae *Elysia timida* and *E. viridis*, together with Limapontioidea species, are characterized by their ability to *de novo* synthesize polypropionate metabolites. A whole analysis of kleptoplasty and chemical defenses in Sacoglossans may offer a better understanding of the ecology and evolution of these specialized opisthobranchs. In this paper we summarize some of the latest findings, related mainly to Mediterranean species, and offer a plausible evolutionary scenario based on the biological and chemical trends we can distinguish in them.

Key words: Sacoglossa, chemical defense, evolution.

RESUMEN: DEFENSAS QUÍMICAS EN LOS OPISTHOBANQUIOS SACOGLÓSOS: TENDENCIAS TAXONÓMICAS E IMPLICACIONES EVOLUTIVAS. Los limacos marinos del orden Sacoglossos (Moluscos: Opisthobranquios) son uno de los pocos grupos de herbívoros especializados en el ambiente marino. Los Sacoglossos se alimentan suctorialmente del jugo celular de microalgas, de las que “roban” cloroplastos (cleptoplastia) y sustancias disuasorias (cleptoquímica), y conservan intracelularmente tanto los plástidos como las sustancias químicas del patrón. Los cloroplastos ingeridos continúan fotosintetizando por períodos que van desde unas pocas horas o días hasta 3 meses en algunas especies. Los sacoglossos testáceos, más primitivos, se alimentan sólo de algas del género de sifonales *Caulerpa*, y no tienen cleptoplastia funcional. La dieta de los Sacoglossos ha radiado desde este alimento ancestral. Entre los Placobránquidos (=Elysidos), sin concha, las especies más primitivas se alimentan de otras algas sifonales (familias Derbesiáceas, Caulerpáceas, Briopsidáceas y Codiáceas) y fijan carbono, mientras que las especies más “avanzadas” pertenecientes a Placobránquidos y Limapontioideos poseen una panoplia alimentaria más amplia. La mayoría de estas especies “avanzadas” son incapaces de fijar carbono porque los cloroplastos de sus algas alimento se rompen mecánicamente durante la ingestión. Los mesoherbívoros están expuestos a ser comidos si viven sobre algas marinas comestibles, pues su coloración y forma crípticas no siempre los mantienen a salvo de los depredadores. Los Sacoglossos prefieren vivir sobre algas defendidas químicamente y comerlas, y utilizan sustancias químicas ingeridas como disuasores de depredadores potenciales. Los sacoglossos testáceos más primitivos (Oxinoidos) y algunos placobránquidos tales como *Elysia translucens*, *Thuridilla hopei* y *Bosellia mimetica*, han desarrollado un mecanismo defensivo derivado de la dieta. Los Oxinoidos y *Thuridilla hopei* pueden biomodificar los metabolitos algales. Sin embargo, los placobránquidos

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Elysia timida y *E. viridis*, al igual que las especies de Limapontoideos, se caracterizan por su capacidad de sintetizar *de novo* metabolitos tales como polipropionatos. Un análisis detallado de la cleptoplastia y las defensas químicas en los Sacoglossos puede proporcionar una mejor comprensión de la ecología y evolución de estos opisthobranchios especializados. En este artículo se resumen algunos de los últimos descubrimientos, relacionados principalmente con especies mediterráneas, y se ofrece una hipótesis evolutiva plausible sobre la base de las tendencias biológicas y químicas que podemos distinguir en ellas.

Palabras clave: Sacoglossos, defensa química, evolución.

If you are looking for alternatives to Darwinism, don't waste your time on opisthobranchs.

G. Cimino and M.T. Ghiselin (2001).

Whatever the immediate future of the research on the consortia in which sacoglossans and other opisthobranchs are involved, it will presumably provide as many surprises and as much satisfaction as in the last few years.

J.D. Ros and A. Marín (1991).

It has seemed to me convenient to introduce the term "kleptodefenses" to describe the defensive systems of any type which an opisthobranch obtains borrowed from another animal, from a plant or, even, from inert materials in the environment.

J.D. Ros (1976).

INTRODUCTION

Most marine herbivores (fishes, sea urchins, amphipods, crabs, polychaetes and gastropods) are extreme feeding generalists. In contrast to marine herbivores, terrestrial insect herbivores have numerous feeding modes, such as sap sucking, leaf mining, and galling, that are largely unknown among marine herbivores (Hay and Steinberg, 1992). Seaweeds differ from terrestrial plants by the lack of phloem and xylem transport systems. The only group of seaweeds that have extensive nonseptate tubes of cytoplasm are the siphonous green seaweeds, or Siphonales. Sacoglossans (Mollusca: Opisthobranchia) are one of the few groups of specialized herbivores in the marine environment. These sea slugs feed suctorially on the cell sap of siphonous green algae and of some macroalgae with high cellular cytoplasm volume. Sacoglossans "steal" chloroplasts (kleptoplasty) and deterrent substances (kleptochimistry) from the food algae and retain both host plastids and chemicals intracellularly. The ingested chloroplasts continue to photosynthesize for periods ranging from a few hours

or days up to 3 months in some species (Marín and Ros, 1989). Sacoglossans feed suctorially by piercing the algal cell wall with a specialized stylet-like tooth (Clark, 1992). The radula of sacoglossans is highly modified to sap sucking, with a continuous single row of teeth. There are two basic shapes of teeth in the Sacoglossa: the sabot-shaped teeth found in the genera *Stiliger*, *Ercolania*, *Limapontia* and *Alderia*, and the blade-shaped teeth which occur in all other genera (Fig. 1, top). The particular use of these teeth also varies from one genus to another, the mollusk effectively either piercing, cutting or sawing the algal cell wall depending on its thickness and absence or presence of trabecular reinforcements (Fig. 1, bottom). Adaptive dietary radiation among sacoglossan families shows a correlation between radular teeth

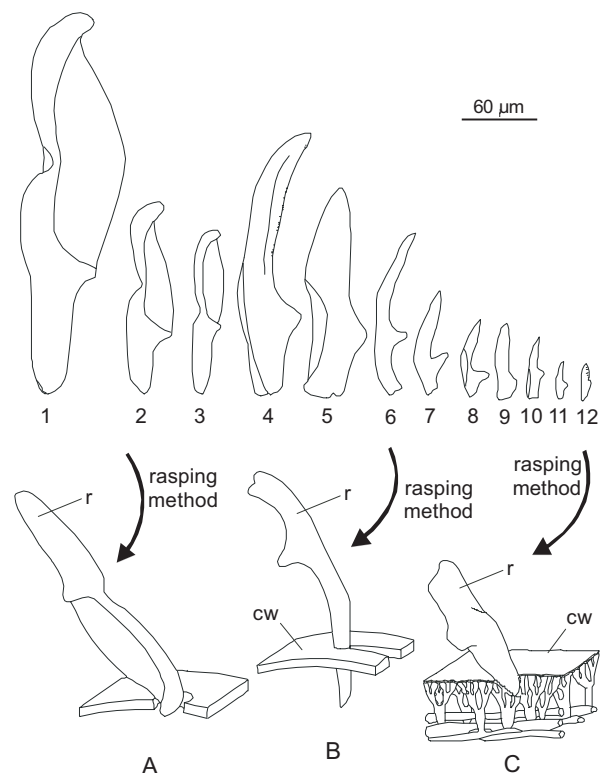


FIG. 1. – The diversity of radular teeth size and feeding methods in the Mediterranean sacoglossans. The diversely-shaped radular teeth actively pierce the algal cell wall of algal species with different cellular volume. 1, *Ercolania coerulea*; 2, *E. funerea*; 3, *Limapontia capitata*; 4, *Elysia viridis*; 5, *Oxynoe olivacea*; 6, *Elysia timida*; 7, *E. translucens*; 8, *E. gordanae*; 9, *Placida dendritica*; 10, *Thuridilla hopei*; 11, *Elysia flava*; 12, *Bosellia mimetica*. A, B, *Elysia*; C, *Bosellia mimetica*. r, radula; cw, algal cell wall.

length and algal cell diameter (Fig. 2). After puncturing the alga, they extract the cell-sap with a muscular pharyngeal pump (Jensen, 1991). The digestive system is adapted to a fluid diet, with a large internal surface area for absorption and a reduced intestine and anus. The digestive gland tends to be highly branched, particularly in those sacoglossans that retain functional chloroplasts from their host plants.

Sacoglossans display a remarkable diversity in shape, ranging from shelled burrowing species (*Ascobulla*) to epifaunal shell-less forms such as *Elysia*, *Ercolania*, and *Caliphylia*. The Sacoglossa are divided into two major classes, shelled sacoglossans (Suborder Oxynoacea) and shell-less sacoglossans (Suborder Plakobranchacea; Williams and Walker, 1999). The taxonomic classification used here is based on the phylogenetic analysis made by Jensen (1997a; Table 1).

In this work we review the factors selecting for host specialisation and the role of secondary metabolites in the evolution of these specialist herbivores, with an emphasis on the chemical ecology of Mediterranean sacoglossans.

TABLE 1. – Classification of the Sacoglossa.

Superfamily and family	Genus
Shelled sacoglossans (Suborder Oxynoacea)	
Family Volvatellidae	<i>Ascobulla</i> <i>Volvatella</i>
Family Juliidae	<i>Julia</i> <i>Berthelinia</i>
Family Oxynoidae	<i>Oxynoe</i> <i>Lobiger</i> <i>Roburnella</i>
Non-shelled sacoglossans (Suborder Plakobranchacea)	
Superfamily Plakobranchioidea (=Plakobranchioidea; =Elysioidae)	
Family Plakobranchidae (=Elysiidae)	<i>Elysia</i> <i>Thuridilla</i> <i>Placobranchus</i> <i>Elysiella</i> <i>Tridachia</i> <i>Tridachiella</i> <i>Pattyclaya</i>
Family Boselliidae	<i>Bosellia</i>
Family Platyhedylidae	<i>Platyhedyle</i> <i>Gascoignella</i>
Superfamily Limapontioidea (=Polybranchioidea; =Stiligerioidea)	
Family Limapontiidae (=Stiligeridae)	<i>Placida</i> <i>Ercolania</i> <i>Stiliger</i> <i>Calliopaea</i> <i>Olea</i> <i>Alderia</i> <i>Alderiopsis</i> <i>Limapontia</i>
Family Hermaeidae	<i>Hermaeopsis</i> <i>Hermaea</i>
Family Polybranchiidae	<i>Aplysiopsis</i> (=Hermaeina) <i>Calliphylia</i> <i>Cyerce</i>

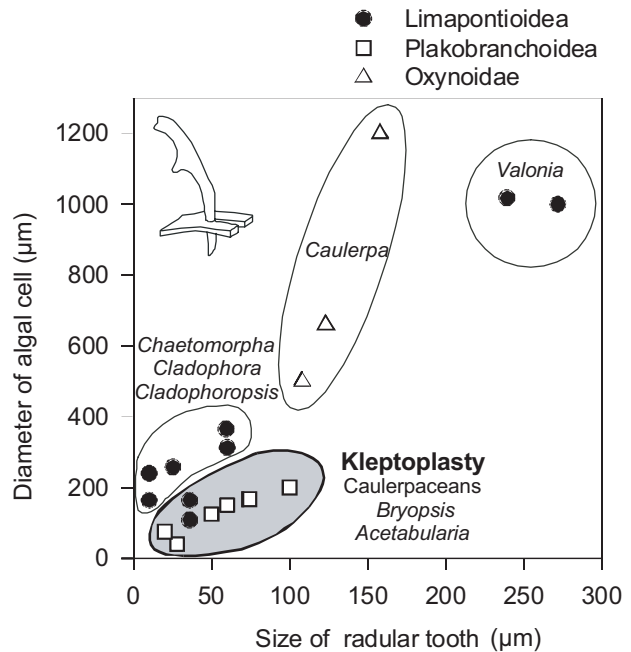


FIG. 2. – Relationship between radular teeth size and diameter of single or coenocytic cells from the algal diet in Mediterranean sacoglossans. Kleptoplasty occurs in the sacoglossans feeding on seaweeds with lower cellular volume.

ORIGIN OF SECONDARY METABOLITES IN SACOGLOSSANS

Most shelled mollusks are protected from predation by a hard shell and do not possess any special secondary metabolites that might function as a chemical defense. However, most opisthobranchs lack the physical protection of a shell yet they compensate for their physical vulnerability with chemical secretions, nematocyst-based defenses or a cryptic coloration (Ros, 1976; Todd, 1981, etc.). The primitive shelled Sacoglossans have both a shell and the ability to produce a chemical exudation. The main source of the chemical defense in opisthobranch mollusks resides in the selective concentration of chemicals contained in their food. The presence of molecules structurally related to typical dietary metabolites in Sacoglossans that feed on algal species from the order Caulerpales could be attributed to the selective accumulation of minor compounds, or to an *in vivo* chemical transformation of major metabolites, both acquired from the diet. The Limapontioidea and some Plakobranchidae species are characterised by their ability to synthesize polypropionate metabolites. Secondary metabolites generated via the propionate route are quite unusual in nature but rather common in marine mollusks, where they have been found in Cephalaspi-

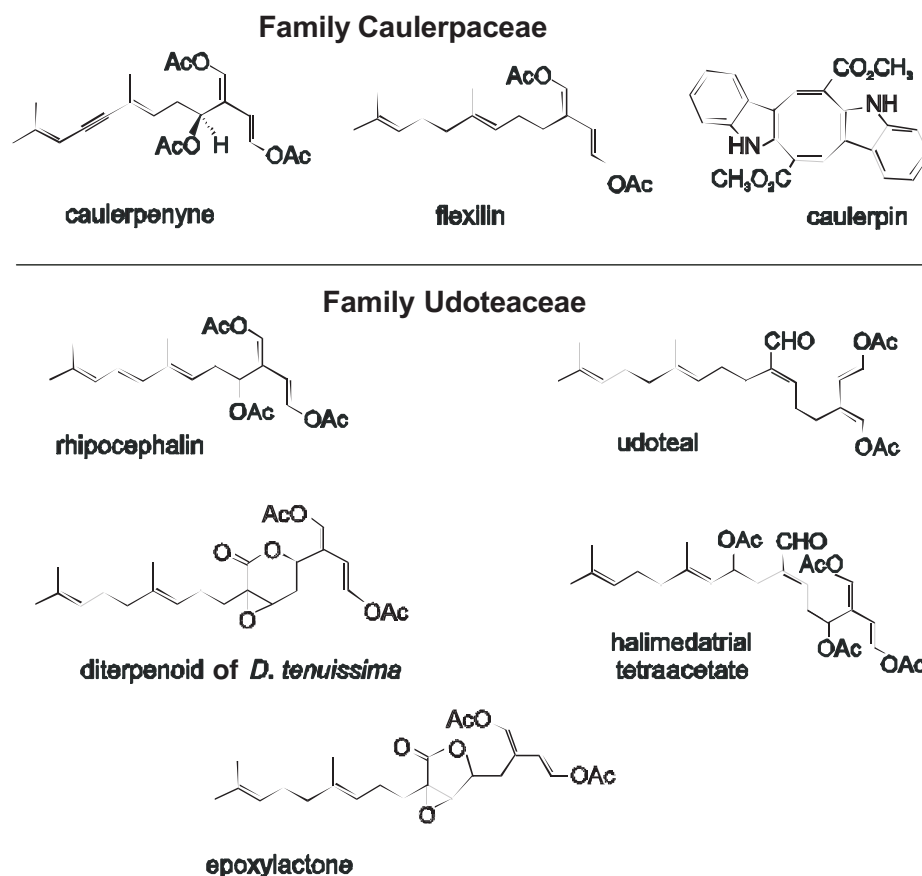


FIG. 3. – Types of secondary metabolites found on coenocytic green algae consumed by Sacoglossans.

deans, Anaspideans, Notaspideans, Pulmonates and Sacoglossans (Faulkner, 1992; Cimino and Sodano, 1993). When disturbed, the elysioids *Elysia timida* and *E. viridis* produce a defensive mucus that contains polypropionate metabolites (Gavagnin *et al.*, 1994b, 1994c). These species are known to feed on algae of the genera *Acetabularia* and *Codium*, which, however, do not contain any polypropionates. Lack of evidence for a food-linked origin of these metabolites lends weight to the hypothesis that Sacoglossans have developed the ability to biosynthesize their defense allomones.

SECONDARY METABOLITES OF CAULERPALEAN ALGAE

Many species of caulerpalean algae grazed by sacoglossans contain secondary metabolites which show biological activity towards microorganisms, invertebrate eggs, sperm, larvae, fishes and mollusks (Paul and Fenical, 1986). Most of the compounds discovered in the algal families Caulerpaceae and Udoteaceae are sesquiterpenoids and diterpenoids,

and are often acyclic. Enol acetates, aldehydes and the terminal bis-enol acetate moiety are common to most of these metabolites, which are functional groups with high biological activity and chemical reactivity. These structural features may be responsible for the wide spectrum of biological activity these metabolites show toward microorganisms, invertebrate eggs, sperm, and larvae, marine fish, and mollusks. Thus, the ancestral sacoglossan would have been able to colonize an algal substratum full of active secondary metabolites which acted as deterrent against generalist herbivores, but not to specialist ones like this early sacoglossan. The first adaptation of the primitive sacoglossan that allowed it to exploit *Caulerpa* might have been to develop a mechanism for the detoxification of the biologically active sesquiterpenoids and diterpenoids. In this way, the ancestral sacoglossan would have gained a free-space without competitors (meso- and macro-herbivores) and potential predators.

Several sesquiterpenoids and diterpenoids have been isolated from the ancestral food of Sacoglossans, the pantropical and subtropical genus *Caulerpa*

(Fig. 3). The Mediterranean *Caulerpa prolifera* contains the acetylenic sesquiterpenoid caulerpenyne, which is toxic and a feeding deterrent against generalist herbivores such as mollusks or sea urchins (Amico *et al.*, 1978; Mc Connell *et al.*, 1982; Paul and Fenical, 1986), although it does not protect the alga against tropical herbivorous fish (Meyer and Paul, 1992). Caulerpenyne has also been found in other common tropical *Caulerpa* species, in both the Caribbean Sea (*C. racemosa*, *C. mexicana*, *C. sertularioides*, *C. paspaloides*, *C. lanuginosa*, *C. cupressoides*, and *C. verticillata*) and the Pacific Ocean (*C. racemosa*, *C. taxifolia*, and *C. cupressoides*). Flexilin is a sesquiterpenoid closely related to caulerpenyne, isolated from the southern Australian species *Caulerpa flexilis*. Both caulerpenyne and flexilin possess the 1,4-diacetoxybuta-1,3-diene (bis-enol acetate) moiety. This functional group is unknown in terrestrial species, but it is common in the majority of green algal metabolites. Although some of the first chemical studies on the genus *Caulerpa* described caulerpicin and caulerpin, their exact biological role has been disputed by several authors. Caulerpin is a bright yellow-orange compound, which has been found in more than half of the investigated *Caulerpa* species (Vest *et al.*, 1983). Although this molecule was originally described as a bioactive metabolite, recent studies suggest that it lacks functionality. Conflicting chemical evidences indicates that caulerpicin does not represent a single pure compound.

Surprisingly, many of the metabolites isolated from the family Udoteaceae have been found to be closely related to, or identical with, those described from *Caulerpa* species (Fig. 3). The sesquiterpenoid rhipocephalin was isolated from the common Caribbean alga *Rhipocephalus phoenix*. Rhipocephalin is closely related to the *Caulerpa prolifera* metabolite, caulerpenyne, but lacks its acetylenic functionality. The sesquiterpenoid flexilin from *C. flexilis* has also been reported in the Caribbean alga *Udotea conglutinata* and in the Pacific species *U. geppii*. Studies of the abundant Mediterranean alga *Udotea petiolata* led to the isolation of udoteal, which has been described in *U. flabellum* from the Caribbean Sea, and *U. argentea* from the Western Pacific. Petiodial has also been reported in *U. petiolata* from the Mediterranean Sea.

Most species from the genus *Halimeda* contain halimedatrial, a cyclopropane-containing trialdehyde (Paul and Fenical, 1984). The major metabolite isolated from the Mediterranean alga *Halimeda tuna* is halimedatrial. The Mediterranean alga *Derbesia*

tenuissima produces an epoxy lactone previously isolated from the cosmopolitan species *Pseudochlorodesmis furcellata*. This compound is structurally related to the epoxy lactone produced by *Udotea argentea*.

It is not clear what chemical features determine the degree of deterrence or toxicity of caulerpalean metabolites. Compounds that differ only slightly in chemical structure can vary greatly in their deterrent effects (Hay *et al.*, 1988). The closely related green algal metabolites udoteal, flexilin, chlorodesmin and caulerpenyne differ in their feeding deterrent effects even though all contain the same major bis-enol acetate functional group (Paul *et al.*, 1988b).

CHEMICAL DEFENSE IN SHELLED SACOGLOSSANS

Shelled sacoglossans (about 50 species, belonging to four families) feed on algae of the genus *Caulerpa* (Clark *et al.*, 1990; Gavagnin *et al.*, 1994a). This specialized diet of *Caulerpa*, the presence of a shell, non-functional chloroplast retention, a chemical defense derived from the diet and burrowing habits, could be considered primitive characters within the Sacoglossa. The most primitive sacoglossan genus, *Ascobulla*, and some species of *Volvatella*, live below the sediment surface. The remaining shelled species are all epialgal but also feed on *Caulerpa*. These primitive members accumulate chemicals from their diet. The transfer of toxic metabolites from caulerpalean algae to grazer sacoglossans was recognized in 1970 by Doty and Aguilar-Santos in *Oxynoe panamensis*, which accumulates caulerpicin and caulerpin from *Caulerpa sertularioides*.

In the Mediterranean Sea, the endemic sacoglossans *Oxynoe olivacea*, *Lobiger serradifalci* and *Ascobulla* (= *Cylindrobulla*) *fragilis* feed selectively on different parts of the alga *Caulerpa prolifera* (Gavagnin *et al.*, 1994a). Many species of sacoglossans that feed on caulerpalean algae have shapes that closely match those of the algal food. *Oxynoe olivacea* and *Lobiger serradifalci* display homochromy, whereby the pigments contained in the mantle give them an identical colour to that of the alga *Caulerpa prolifera*. The infaunal *A. fragilis* lives below the sediment surface where it feeds upon the runners of *C. prolifera*. This primitive sacoglossan maintains the mechanical defensive role of the shell fully functional, and when the animal is dis-

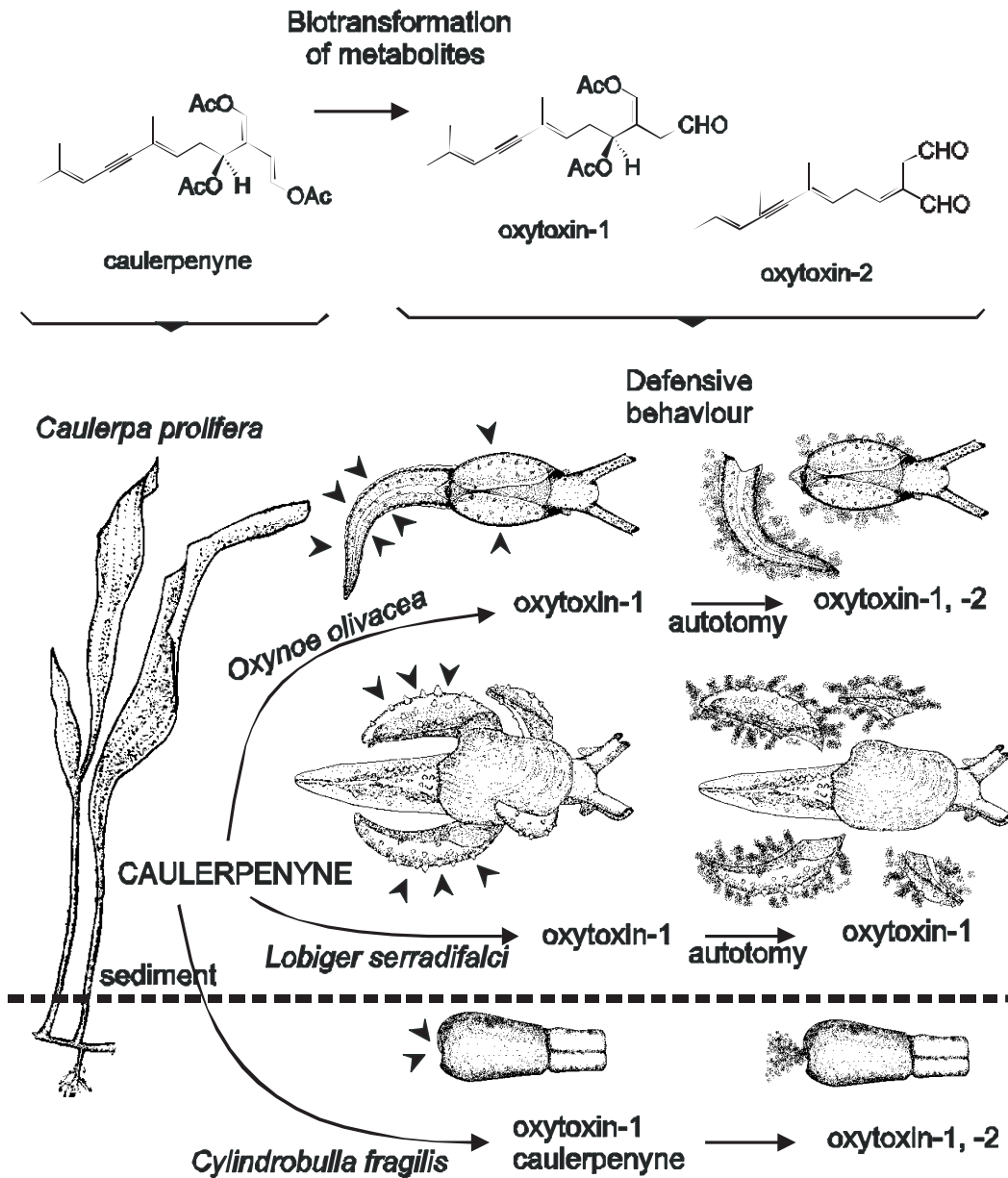


FIG. 4. – Biotransformation of caulerpenyne in the shelled sacoglossans *Oxynoe olivacea*, *Lobiger serradifalci* and *Ascobulla fragilis* from the Mediterranean Sea, associated to the green alga *Caulerpa prolifera*.

turbed its body can be retracted completely into the cylindrical shell. However, when alarmed the mollusk ejects an opalescent white defensive fluid. When disturbed, species of the genus *Lobiger* cast off their parapodia in a process known as autotomy, while species of the genus *Oxynoe* shed their tail since their shell has become reduced in size, allowing the head to be retracted but not the tail or parapodia. The host plant *Caulerpa prolifera* contains the sesquiterpenoid caulerpenyne as the main component. *O. olivacea* and *A. fragilis* are able to modify caulerpenyne into more toxic compounds, oxytoxin-1 and oxytoxin-2, characterized by the pres-

ence of aldehyde groups (Cimino *et al.*, 1990). Caulerpenyne is converted into oxytoxin-1 and oxytoxin-2, which are located in the mucus and external tegument of *A. fragilis* and *O. olivacea*. However, *Ascobulla fragilis* retains unmodified caulerpenyne in the digestive gland as a primitive character. *L. serradifalci* only contained oxytoxin-1 in the parapodial lobes and in the defensive mucus but neither caulerpenyne nor oxytoxin-2 were found. Feeding assays with caulerpenyne demonstrated that this metabolite did not act as a deterrent towards the marine fishes *Thalassoma pavo*, *Chromis chromis* and *Serranellus hepatus* (Gavagnin *et al.*, 1994a,

TABLE 2. – Algal diet, kleptoplasty, defensive behaviour, larval development and secondary metabolites with their biological role in Mediterranean sacoglossans.

	SHELLED	SHELL-LESS			
Family	Volvatellidae Oxynoidae	Plakobranchidae (=Elysiidae) Boselliidae			
Genus	<i>Ascobulla</i> <i>Oxynoe</i> <i>Lobiger</i>	<i>Thuridilla</i>	<i>Elysia</i> <i>Bosellia</i>	<i>Elysia</i>	<i>Tridachia</i>
Species	<i>A. fragilis</i> <i>O. olivacea</i> <i>L. serradifalci</i>	<i>T. hopei</i>	<i>E. translucens</i> <i>B. mimetica</i>	<i>E. viridis</i> <i>E. timida</i>	<i>T. crispata</i>
Diet	<i>Caulerpa prolifera</i>	<i>Derbesia</i>	<i>Udotea, Halimeda</i>	<i>Codium,</i> <i>Acetabularia</i>	<i>Halimeda, Bryopsis,</i> <i>Penicillus</i>
Kleptoplasty	Non-retention	Long-term functional (> 7 days)			
Defensive behaviour	Autotomize body parts, crypsis	conspicuous colour?	Non-autotomy, crypsis; <i>E. timida</i> : conspicuous colour?		
Larval development	Planktotrophic strategy	Lecitotrophic	Planktotrophic strategy; <i>E. timida</i> (direct, lecitotrophic and planktotrophic)		
Secondary metabolites	Sesquiterpenoid, oxytoxin Dietary origin		Diterpenoids: Dietary origin	Polypropionates: Biosynthesized <i>de novo</i>	
Biological role	Fish deterrent: defensive white secretion		Fish deterrent: mucus secretion		
	SHELL-LESS				
Family	Polybranchiidae		Limapontiidae (=Stiligeridae)		
Genus	<i>Calliphylla</i>	<i>Cyerce</i>	<i>Ercolania</i>	<i>Placida</i>	<i>Limapontia</i>
Species	<i>C. mediterranea</i>	<i>C. cristalina</i>	<i>E. funerea</i> <i>E. coerulea</i>	<i>P. dendritica</i>	<i>L. capitata</i>
Diet	<i>Bryopsis</i>	<i>Chlorodesmis?</i>	<i>Chaetomorpha,</i> <i>Valonia</i>	<i>Codium,</i> <i>Bryopsis</i>	<i>Cladophora,</i> <i>Chaetomorpha</i>
Kleptoplasty	Functional retention	Short-term, non-functional retention			
Defensive behaviour	Autotomy of cerata. Defensive mucus. Crypsis.				Non-autotomic mechanism. Crypsis
Larval development	Planktotrophic strategy				
Secondary metabolites	Polypropionates: Biosynthesized <i>de novo</i>				No?
Biological role	Fish deterrent: mucus secretion. Tissue regeneration				Palatable

1994b). These three caulerpivorous sacoglossans are thus able to modify the main algal metabolite into the more toxic metabolites oxytoxin-1 and -2 (Fig. 4). The defensive allomone oxytoxin-1 has been found to be toxic and to act as a feeding deterrent to natural fish predators. Due to the high chemical reactivity of oxytoxin-2, it was not possible to test the pure compound in identical conditions to caulerpene and oxytoxin-1, but the feeding assays realized directly with the mucus secreted by *O. olivacea*, containing a mixture of oxytoxin-1 and -2, resulted highly deterrent to Mediterranean carnivorous fish.

THE CHEMICAL DEFENSE OF PLAKOBRANCHOIDEA

The Plakobranchioidea (=Elysioidae) consist of parapodium-bearing taxa. The species belonging to the genus *Elysia* have a narrow foot and a pair of lateral, leaf-like parapodia, often green in color, in which the sequestered algal chloroplasts are retained. Many species are generally found in association with green algae but some species include other algae and even seagrasses in their diet (Table 2). The Mediterranean sacoglossans *Elysia translucens* and *Bosellia mimetica* contain the diterpenoids

udoteal and halimedatrial tetraacetate derived without a chemical biotransformation from the algae they eat (Gavagnin *et al.*, 1994b). These diterpenoids are characterized by a protected 1,4 conjugated dialdehyde system and they exhibit cytotoxic, antimicrobial and feeding-deterrent activity (Paul and Fenical, 1987). The sacoglossan *Elysia translucens* feeds on the caulerpal *Udotea petiolata*, and kleptoplasty confers on it the same green colour. Comparative chemical analysis of *Elysia translucens* and the host plant *U. petiolata* shows that the sacoglossan accumulates large quantities of udoteal (Paul *et al.*, 1982), the main metabolite of the alga, in defensive glands of the parapodia. The diterpenoid halimedatrial tetraacetate is produced by the caulerpalean *Halimeda tuna*. *Bosellia mimetica* feeds on *H. tuna*, and its flat shape and green parapodia (due to chloroplast retention) camouflage it well on the alga. TLC analysis of *Bosellia mimetica* and of its prey *H. tuna* pointed to strong similarity between the two metabolic patterns, the major secondary metabolite of the former being halimedatrial tetraacetate, which was previously identified in the host alga *H. tuna*.

Many sacoglossans are not cryptic on their algal food or usual substrate and make no attempt to conceal themselves, which may suggest that they show aposematic or warning coloration. However, there are no experimental justification for this and the most strikingly colored sacoglossans are cryptic due to disruptive coloration which breaks up the animal's shape. The Mediterranean plakobranchid *Thuridilla hopei* shows a brightly colored pattern in the laboratory with a dark green or black mantle crossed with blue, red and yellow lines. *T. hopei* is an endemic species from the Mediterranean Sea, which is found in association with the caulerpal *Derbesia tenuissima*, whose principal secondary metabolite is a diterpenoid also isolated from the cosmopolitan alga *Pseudochlorodesmis furcellata* (Paul *et al.*, 1988a). The same algal metabolite was found in *T. hopei*, together with other three related diterpenoids, thuridillin-A, -B, and -C (Gavagnin *et al.*, 1993). The chemical analogy of the three thuridillins with the primary algal metabolite suggests that the sacoglossan feeds on *D. tenuissima* and modifies its dietary metabolites with the aim either of reducing their toxicity or of transforming them into more active compounds (Fig. 5).

Polypropionates are rare in nature, but rather common in marine mollusks, where they have been found in Cephalaspideans, Pulmonates and

Sacoglossans. Studies of the sacoglossan species *Elysia* (= *Tridachiella*) *diomedea* and *Elysia* (= *Tridachia*) *crispata* from the Caribbean Sea resulted in the identification of a novel group of propionate-derived γ -pyrones (Ksebete and Schmitz, 1985; Ireland and Faulkner, 1981; Faulkner, 1992). The presence of polypropionates has also been described in the genus *Elysia*. Biosynthetic experiments with labelled sodium [$1-^{14}\text{C}$] propionate confirm that the allomones of the Mediterranean species *Elysia viridis* and *E. timida* are biosynthesized *de novo* by the mollusks. The ether extract of *E. viridis* was found to contain the polypropionate elysione, previously reported in the literature (Dawe and Wright, 1986). This polypropionate is the main component of the defensive mucus secretion of *E. viridis*. The comparative analysis of the algal food *Codium vermilara* and *Elysia viridis* demonstrated that the polypropionates have a *de novo* origin. In the biosynthetic experiments carried out with *E. viridis*, sodium [$1-^{14}\text{C}$] propionate was injected into the mollusks. After 24-72 h, the ^{14}C activity increased in the fractions containing elysione (Fig. 5).

DEFENSE MECHANISMS IN LIMAPONTIOIDEA

The superfamily Limapontioidea (=Polybranchioidea; =Stiligerioidea) contains the cerata-bearing sacoglossans. A comparative study of Mediterranean Limapontioidea revealed a correlation between the occurrence of defensive mechanisms and the morphoanatomical or biochemical features of the cerata of these species (Table 2). Defensive autotomy is a defense strategy largely used by opisthobranch mollusks as the last resource against predation (Stasek, 1967; Ros, 1976). This behaviour has adaptive significance in the prey organism when the autotomized structure: (a) is not essential for the continued existence of the prey itself; (b) is the most frequently attacked part of the prey, and (c) preferably contains the most powerful deterrent substance (Todd, 1981). Thus, detachment of the tail, or parapodia, in the shelled genera *Oxynoe* and *Lobiger*, or the cerata in some species of Limapontioidea, can be very effective in deterring predators, especially if accompanied by the emission of large amounts of toxic mucus from the autotomized appendages.

The Mediterranean species *Cyerce cristallina* is a conspicuous sacoglossan that resembles an eolid opisthobranch because its slender body is flanked by

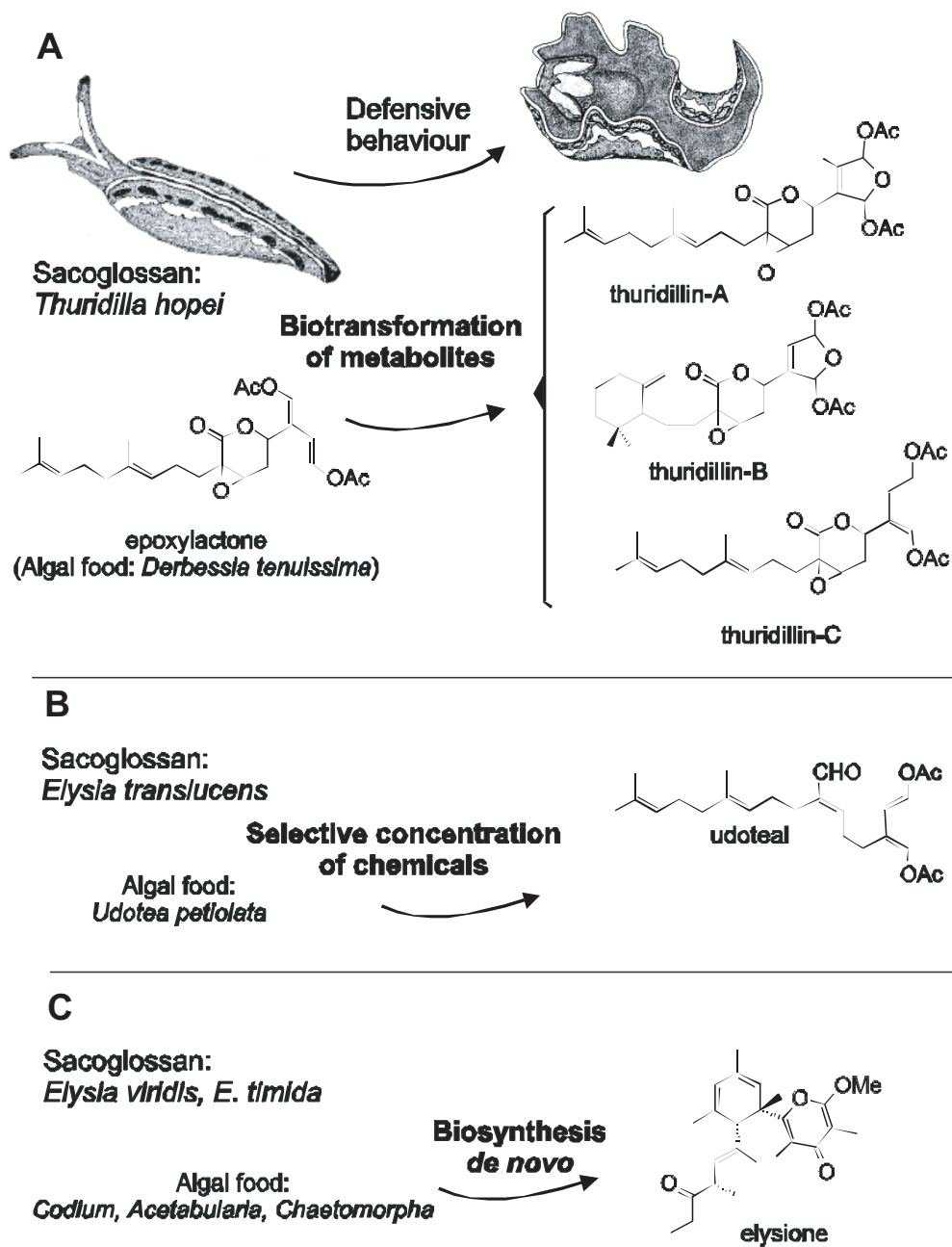


FIG. 5. – Origin of secondary metabolites in Plakobranchidae (=Elysiidae): A, biotransformation of active compounds sequestered from the food in defensive allomones; B, selective accumulation of algal metabolites; C, biosynthesis *de novo* of their own chemical defenses.

voluminous cerata. Each cerata is flattened and observed to be covered by red bands on a white background. When the animal is attacked by predators, the cerata are detached from the mantle and exhibit prolonged contractions while secreting large amounts of toxic mucus (Di Marzo *et al.*, 1991; Vardaro *et al.*, 1991). After the autotomy, the cerata are regenerated within 7-10 days. The internal structure of the cerata exhibit peculiar adaptations to defensive autotomy. Electron microscopy has shown that the flattened cerata do not contain branches of the

digestive gland as is the case for other cerata-bearing sacoglossans and, therefore, the origin of the white and red colour of the appendages cannot be attributed to rhodoplast retention. The major part of the volume of the appendages is filled by cells with an enormous central vacuole, with no haemocoelic spaces. The cerata contain both transverse and longitudinal muscles, which are responsible for its prolonged contractions, and sphincter-like muscle fibres located at the base of each cerata, which cause the appendage autotomy.

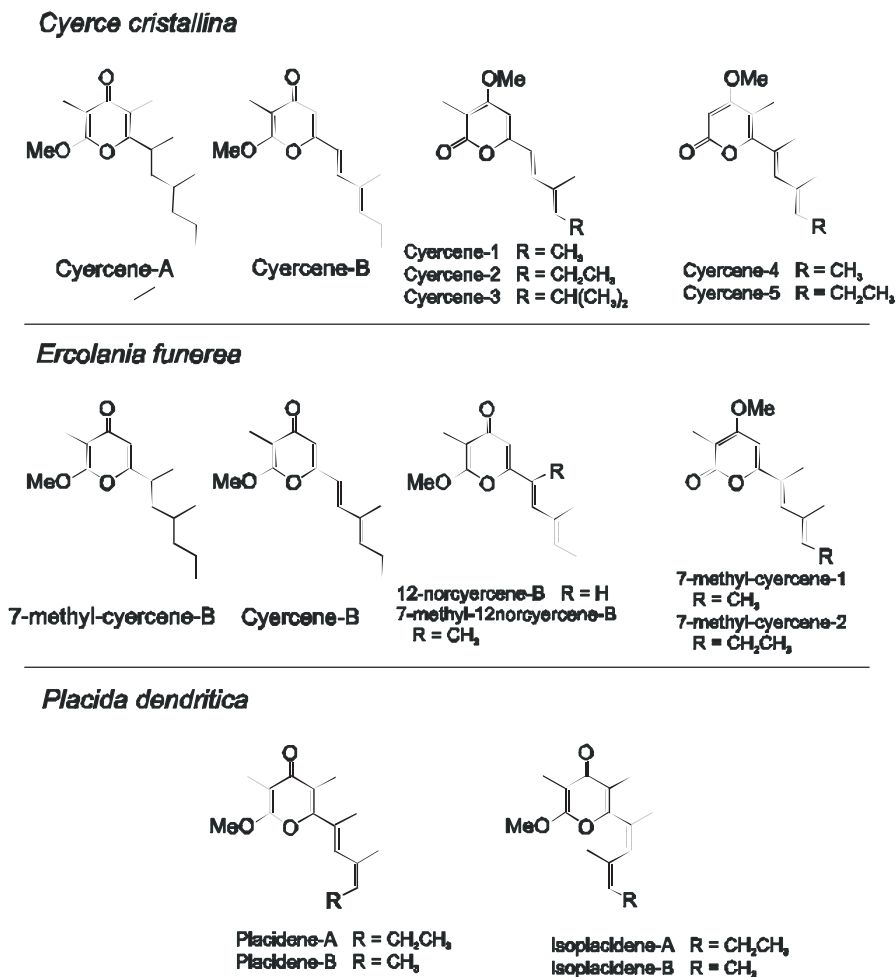


FIG. 6. – Chemical structure of the polypropionate pyrones isolated from the limapontioidea *Cyerce cristallina*, *Ercolania funerea* and *Placida dendritica*.

The body of the limapontioid *Ercolania funerea* is translucent, and has several latero-dorsal rows of cerata. Each cerata is rounded and white spotted, with green digestive gland branches appearing through the skin. The ceratal green colour is due to the presence of intact chloroplasts in the digestive cells sequestered from the chlorophycean *Chaetomorpha linum*. Among the ciliated cells, the epithelium contains unicellular glands, which are probably responsible for the secretion of slime. Both longitudinal and transversal muscular fibers, inserted in broad haemocoelic spaces, are also found between the epithelium and the digestive gland, and, as in *Cyerce cristallina*, this musculature is better developed at the base of each cerata, where it forms sphincter-like structures which, when the mollusk is disturbed, contract strongly, thus cutting off the cerata.

In *Placida dendritica* both the albumen gland and the digestive gland protrude into the cerata. The

ceratal digestive gland branches contain functional chloroplasts identical to those of the algal food *Bryopsis plumosa*. The cerata are covered with a ciliated epithelium which is intercalated with unicellular defensive glands. The base of the cerata is solid with no special muscular system. The cerata of *Calliophylla mediterranea* are leaf-shaped and transparent, so that the green branches of the digestive gland can be discerned. These branches show intact chloroplasts sequestered from the algal food, *B. plumosa*. The albumen gland does not protrude into the cerata. Other histological features include the presence of unicellular glands that are not located in the proximity of the epithelium and the absence of sphincter-like muscles at the base of the cerata. When molested, *C. cristallina* and *E. funerea* produce a defensive mucous secretion and can easily shed their appendages. However, *P. dendritica* and *C. mediterranea* normally do not autotomize the

cerata if they are disturbed. For a comparative analysis of the rate of regeneration among species, the cerata were removed by means of dissecting forceps from specimens of *P. dendritica* and *C. mediterranea*. After loss of the cerata, spontaneous or aided with dissecting forceps, the fastest regeneration was observed in *C. cristallina* and *E. funerea* (8 to 10 days), and the slowest in *P. dendritica* (15 days). *C. mediterranea* showed an incipient regeneration, but it was too slow to be measured (> 30 days). Histological sections of regenerated cerata of *C. cristallina* and *E. funerea* exhibited the same appendicular structure and the same cell types as the fully developed cerata.

C. cristallina, *E. funerea* and *P. dendritica* produce α - and γ -pyrone polypropionates while *C. mediterranea* does not contain polypropionates (Vardaro *et al.*, 1991, 1992a, 1992b; Di Marzo *et al.*, 1991). These secondary metabolites have structures that differ only in the degree of methylation and the geometry of the double bonds of the side chain and are only distributed in the cerata and defensive slime of these species (Fig. 6). Experiments to evaluate the role of polypropionates in cerata regeneration indicated that the most active compounds were the ones with an all-trans-alkyl chain, that were found specifically in the cerata of the quickly regenerating species (*C. cristallina* and *E. funerea*), while the γ -pyrones with inverted geometry of double bonds isolated from *P. dendritica* were either not active or very weakly active in cerata regeneration. The defensive mucus produced by *C. cristallina*, *E. funerea* and *P. dendritica* were toxic to the fish *Gambusia affinis*, while the slime of *C. mediterranea* was harmless. Therefore, polypropionate metabolites from *C. cristallina* and *E. funerea* may function to prevent predation and as inductors of cerata regeneration.

The defensive mechanisms of these four Limapontioidea species show the evolutionary plasticity of Sacoglossans in exploiting different algal resources. *Calyphylla mediterranea* feeds on algae pertaining to the genus *Bryopsis*, all of which are coenocytic species with a morphology close to the primitive food *Caulerpa*. This sacoglossan retains functional chloroplasts but does not exhibit autotomy nor chemical deterrence. Apparently, chloroplast-provided camouflage is the only means of defense in this species. Cryptic behaviour is also used by *Placida dendritica* and *Ercolania funerea*. The ceratiform *P. dendritica* grazes on the coenocytic thallii of *Bryopsis* and *Codium* algae. The defense of this species is based on the exudation of large vol-

umes of pyrone-containing slime, which generates a defensive "cloud" around the mollusks. The defensive secretion is accompanied by defensive behavior when the animal is molested, and the cerata are erected and waved frantically. The small sacoglossan *Ercolania funerea* retains non-functional chloroplasts from the cladophoral *Chaetomorpha linum*. *E. funerea* combines several defensive strategies found in Limapontioidea species: cryptic coloration, autotomy, ceratal regeneration and chemical deterrence (Vardaro *et al.*, 1992a). The cerata-bearing *Cyerce cristallina* exhibits the most evolved defensive strategy. The autotomy of the cerata, which in this species do not contain any prolongation of inner organs and can be compared to large reservoirs containing toxic polypropionates, has been highly improved. *C. cristallina* detaches its cerata when attacked by predators; the detached cerata exude a large volume of deterrent mucus and contract visibly, thus diverting the predator from the animal body to the contracting cerata.

EVOLUTION OF KLEPTOCHEMISTRY AND KLEPTOPLASTY IN SACOGLOSSANS

The evolution of Sacoglossans has led to trophic specialisation, which is a characteristic of species of mature ecosystems with high species diversity. The species diversity of the Mediterranean Sea is impoverished relative to the Caribbean Sea or to the tropical Indo-Pacific Ocean. There are fossil evidences that Sacoglossans could be one of the "oldest European inhabitants". The first record of a sacoglossan fossil belongs to the "bivalve" *Berthelinia* (Juliidae) from the Eocene of the Paris Basin (Keen and Smith, 1961). Valdés and Lozouet (2000) have described the fossil record of four shelled species in a exceptionally well-preserved collection of Tertiary from the Aquitaine Basin, France. The Tertiary European sacoglossans include three species of the family Juliidae, *Julia girondica*, *Squamulina* sp. and *Berthelinia* sp., and one of the family Volvatellidae, *Volvatella faviae*. The presence of these fossils indicate the probable existence of algae of the genus *Caulerpa* in this shallow sea due to the close trophic relationship between shelled sacoglossans and this genus. Most species of *Caulerpa* are uncalcified and do not leave direct remains of their presence. Definitive separation of the Indo-Pacific and Atlantic species occurred during the closure of the main East-West connection, during the Oligocene-

Miocene transition. The geographical range of the genus *Volvatella* is today restricted to the tropical Indo-Pacific and Caribbean, being absent from Europe and West Africa (Valdés and Lozouet, 2000). This discontinuous geographical range is all that remain of a wider distribution through the Tethys Sea. The extinction of this high diversity of shelled sacoglossan species in Europe might have occurred during the first major cooling period in the mid Miocene (Lozouet *et al.*, 1994). From this point of view, there is a general tendency to think of the Mediterranean sacoglossans only as a relictual fauna or even as a group of invasive species from tropical areas. However, we believe the Mediterranean sacoglossans are a particularly instructive example of how trophic specialisation in ancestral sacoglossans from the Tethys Sea have lead to similar defensive and chloroplast exploitation strategies in different geographical areas.

For Sacoglossans, one may think of the following evolutionary history. First of all, we must place ourselves in the paleo-scenario of the tropical sea that was the Tethys. In this time, the shallow, well light and warm waters favored the growth of uncalcified algal meadows, made up from caulerpacean species chemically defended against generalist herbivores. The ancestral sacoglossan must have developed a diet-derived chemical defense mechanism prior to the shrinking or loss of the shell. However, it must first have overcome the secondary metabolites contained on the ancestral food, algae of the genus *Caulerpa*, to deter generalist herbivores. The shelled sacoglossans developed a mechanism to sort sesquiterpenoid and diterpenoid metabolites from *Caulerpa* into those that could be catabolized and those that were noxious or toxic. The sesquiterpenoids or diterpenoids were then transferred to defensive glands of the mantle, from which they could be excreted in response to an attack. The burrowing species of Volvatellidae, including the genera *Volvatella* and *Ascobulla*, could be considered an evolutive link between the infaunal cephalaspideans and the most advanced sacoglossans (Clark *et al.*, 1990). Only when the mechanism of chemical exudation in defensive glands was fully operative could the ancestral burrowing sacoglossan exploit the aboveground, erect parts of *Caulerpa*. *Oxynoe olivacea* and *Lobiger serradifalci* living and thriving on *Caulerpa prolifera* are modern examples.

Jensen (1997b) suggests that the original host could be other than *Caulerpa* because she found *Cylindrobulla phuketi* feeding upon algae of the

genus *Halimeda*. However, if we compare the parallel evolution in the Indo-Pacific, Atlantic and Mediterranean areas, the initial association with *Caulerpa* seems to be the more probable starting point. We do not found arguments enough to change the traditional scenario for this alternative hypothesis. Fossils of the udoteacean alga *Halimeda* date back to the Cretaceous (Hillis-Colinvaux, 1980). Fossils of calcareous algae of the order Dasycladales are usually associated to the Tethyan transgression. The possible paleoecological scenario having changed, the majority of uncalcified caulerpaceans became extinct due to interspecific competition, feeding by herbivores or climatic changes. The present-day tropical seaweeds on coral reefs are heavily grazed by generalist herbivorous species (fish, sea urchins and gastropods), which thus can seize from 60 to 100 % of total production (Hay and Steinberg, 1992). Several studies have shown that increasing the mineral content of food plants by adding powdered calcite and/or aragonite can strongly deter feeding by some herbivores. In *Halimeda*, the CaCO_3 crystals in the thallii fuse to form a larger skeleton which has an additional negative effect on feeding by herbivorous fishes and sea urchins (Pennings and Svedberg, 1993). Many actual calcified caulerpacean algae are also chemically defended. The selection should favour seaweeds with structural and/or chemical defenses active against numerous types of generalist herbivores. The evolutionary adjustment to generalist herbivores probably exerted a parallel selective pressure for the evolution of sacoglossans. Here the metaphor of the Red Queen to describe biotic evolution (Van Valen, 1973) is of application: the consistent change is necessary, not to increase adaptiveness, but merely to maintain it.

The evolutionary trend observed in Mediterranean Plakobranchoidea suggests that the next event could have been parapodium-bearing species with kleptoplasty colonizing other caulerpalean algal species with structurally similar chemicals and robust chloroplasts. The change of the shelled sacoglossans from a *Caulerpa*-based diet to other green algae may have involved a very small genetic change in Plakobranchoidea species. Chloroplast retention in the ancestral epialgal sacoglossans probably functioned as nutritional homochromy, in which intact chloroplasts provided a cryptic coloration identical to that of the host alga. There is a convergent evolution between functional plastid retention and the origin of secondary metabolites of Sacoglossans. Siphonolean green algae are known

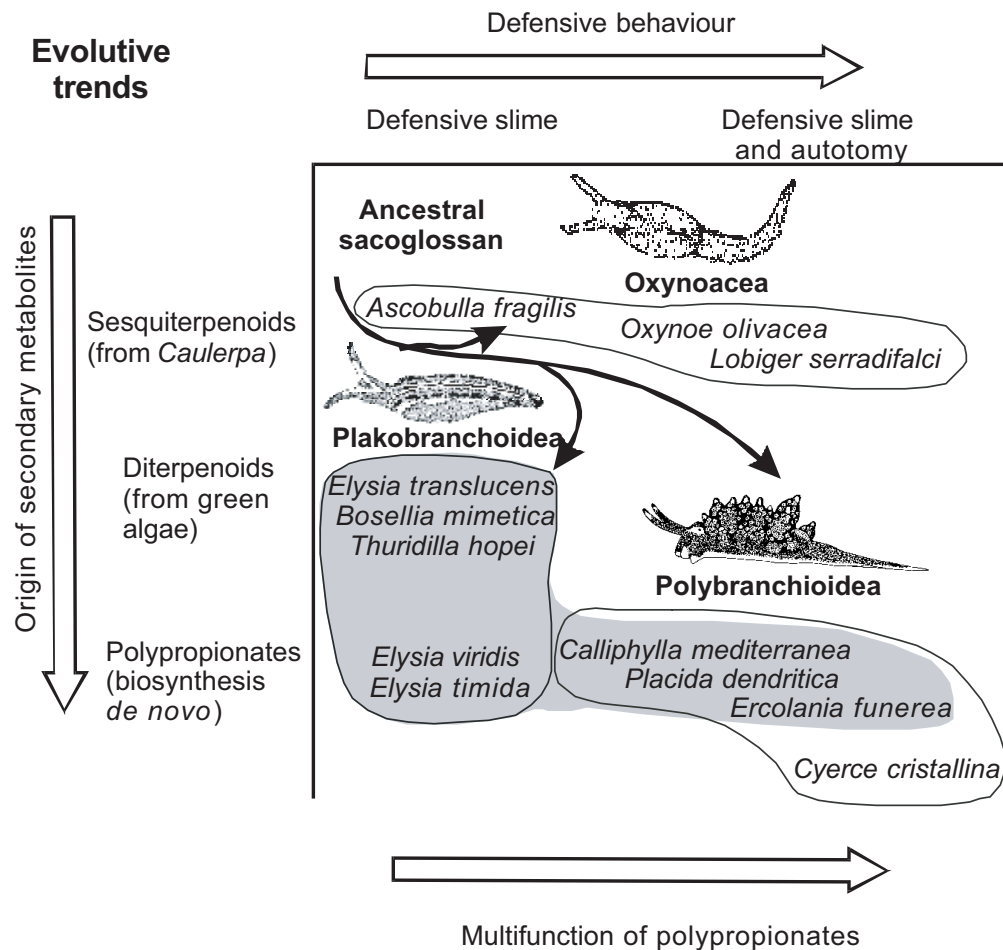


FIG. 7. – Adaptive radiation in Sacoglossans and the evolutionary trends on the origin of secondary metabolites and defensive behaviour. Shaded areas indicates the presence of kleptoplasty in sacoglossan species (only Mediterranean ones are cited).

to contain secondary metabolites, which act as feeding deterrents against generalist herbivores (Norris and Fenical, 1982). Most “primitive” Mediterranean Plakobranchoidea, *Elysia translucens* and *Bosellia mimetica*, accumulate udoteal and halimedatrical from the caulerpalean species *Udotea petiolata* and *Halimeda tuna* respectively, which are metabolites closely related to those described from *Caulerpa* species. *E. translucens* and *B. mimetica* retain intact chloroplasts which provide a coloration virtually identical to the algal background. In this pattern, more advanced taxa feed on other Siphonales, Siphonocladales, Cladophorales and other exotic foods. With the dietary radiation of the Sacoglossans, the chemical defense mechanisms probably adjusted to the habitat characteristics. Some species maintained the ability of kleptoplasty with a chemical defense derived from the diet. The elysioid *Thuridilla hopei* probably modifies algal metabolites to produce a more effective antifeedant chemical and retains functional chloroplasts. The exploita-

tion of algal plastids in shell-less sacoglossans seems associated with the amount of cell sap that the host plant provides to the slug due to the own algal architecture (e.g., uniseriate filamentous, blade fronds), to structural defenses (e.g., calcification), or low host plant density (Fig. 7). An example of the evolutionary pressure that food limitation can exert on sacoglossans to acquire functional chloroplasts is to be found in the relationship between the elysioid *Elysia timida* and the unicellular alga *Acetabularia acetabulum* (Marín and Ros, 1992). *Elysia timida* is an stenophagous herbivore that feeds exclusively on the green alga *Acetabularia acetabulum*. In the Mediterranean Spanish coast, the life cycle of the alga begins in October, when the young single-celled plant has two growing apices, one giving rise to the rhizoidal system that attaches the plant to the substrate, the other growing apex becoming the erect thallus. Starting in January, the cell walls of the thallus undergo progressive calcification; by April the base of the thallus is very highly calcified,

although the apex continues its vegetative growth. The apex does not calcify until June, when the thallus vegetative growth stops and a cap of gametangial radial chambers is formed. The gametangial rays contain numerous cysts which when mature release pyriform biflagellate isogametes. The calcification process is not simultaneous; algae whose thallii are calcified to different extents coexist in the same population at any one time, and different *A. acetabulum* populations also feature an unequal calcification period. *Elysia timida* features a whole series of adaptations for optimum kleptoplast exploitation. Its photosynthetic production is one of the highest amongst the Mediterranean sacoglossans and in the laboratory it can survive for up to three months when starved. All these adaptations are related to the seasonal dynamics of the food used. The young *E. timida* can be found on shallow rocks when the first *A. acetabulum* stalks appear in October. The population density of the sacoglossan species then increases to a maximum in December and a secondary maximum in May; the mollusks disappear in summer. The combination of number of individuals and individual growth produces a maximum sacoglossan biomass out of step with the population maximum. Calcification of the alga cell wall first complicates browsing by *E. timida*, then impedes it altogether. Between January and June, the sacoglossan moves to the non-calcified apices of the algae, where it eats. From June, when all the thallii are totally calcified, the *E. timida* population falls drastically, until it disappears completely the following month. Progressive calcification of the alga not only diminishes the food supply, but also makes it more difficult to find. If we divide the total number of stalks of *A. acetabulum* found in a given month by the number of non-calcified stalks, we obtain a parameter measuring the energetic effort the mollusks must devote to obtain the same amount of food. Even in March, virtually all attempts to find and eat a non-calcified stalk would have a positive result. In April, however, to obtain the same amount of food *E. timida* would have to double efforts, and in May the effort necessary to obtain the same amount of food would be nearly ten times greater than in autumn and winter. The effort would become infinite in summer, when all *Acetabularia* stalks are calcified. This may explain the disappearance of the slug by mid-summer.

The food resource limitation hypothesis exposed previously to try explain the role of kleptoplasty in Sacoglossans must be complemented, however, with

the defensive role which the cryptic coloration that sequestered algal plastids confer to these mesoherbivores. Aposematic coloration patterns have been only described in sacoglossans that feed on seaweeds with patchy distribution or whose small size make useless a cryptic pattern on a variable background. Ghiselin (1966) and Jensen (1996) reject the view of some authors that the shell-less sacoglossans are polyphyletically derived from shelled forms in favour of treating the shell-less forms as a single clade. This hypothesis suggests that Limapontioidea and Plakobranchoidea had a common shell-less ancestor, in which polypropionate metabolites together with chloroplast retention first appeared. But these characters could have derived separately from the common ancestor with a convergent evolution of polypropionate biosynthesis and functional kleptoplasty in each line. The Red Queen effect referred to above lead us to consider that the *de novo* biosynthesis of polypropionates by Sacoglossans is forced by the host-plant specialisation of these mollusks where kleptochimistry is not possible, and is the result of their selective predation.

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