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Chemical ecology and bioactivity of triterpene glycosides from the sea cucumber *Psolus patagonicus* (Dendrochirotida: Psolidae)

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Information on the chemical ecology and bioactivity of chemical compounds present in the sea cucumber Psolus patagonicus *is provided through an interdisciplinary approach. The specimens studied were collected from two different and very distant sampling localities: Bridges Islands (Beagle Channel, Ushuaia, 54° 48′57″ S 66° 25′00″ W) at depths of 4 to 10 m by SCUBA diving, and from the scallop beds of* Zigochlamys patagonica *(39° 27′10″ S 55° 56′76″ W), at depths of 110 m and 115 m (43° 47′84″ S 59° 56′80″ W) by means of non-selective dredge in the South Atlantic Ocean.*

The secondary metabolites were isolated from complete adults by a combination of chromatographic methods and purified by high-performance liquid chromatography. They were characterized as triterpene glycosides through extensive spectroscopic analyses (nuclear magnetic resonance and fast atom bombardment mass spectroscopy) and chemical methods. A purified fraction containing Patagonicoside A as the main triterpene showed a high level of mortality against the brine shrimp Artemia salina *and revealed different antifungal activity of Patagonicoside A and its desulphated glycoside (ds-Patagonicoside A) against the fungi* Cladosporium fulvum, Fusarium oxysporum *and* Monilia *sp., compared with a potent commercial antifungal product.*

Keywords: *Psolus patagonicus*, holothurins, chemical ecology, bioactivity

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INTRODUCTION

The present study is an interdisciplinary contribution of marine biologists and chemists working on natural products derived from mollusc and echinoderm species in the southern Atlantic (Fontana *et al.*, 1998; Gavagnin *et al.*, 1999; Murray *et al.*, 2001; Chludil *et al.*, 2002; Muniain *et al.*, 2005, 2007a,b; Maier *et al.*, 2007).

The saponins derived from sea cucumbers, which are triterpene glycosides commonly known as holothurins, have drawn attention because of their wide spectrum of biological effects and antifungal, cytotoxic, virucidal and haemolytic activities (Shimada, 1969; Kalinin *et al.*, 1996; Stonik *et al.*, 1999; Maier *et al.*, 2001; Chludil *et al.*, 2003; Maier & Murray, 2006).

Previous studies in the genus *Psolus* (Holothuroidea: Dendrochirotida: Psolidae) have been conducted only for the species *P. fabricii* (Dueben & Koren, 1846) and *P. eximius* Saveljeva (Kalinin *et al.*, 1984, 1985, 1989, 1997).

The sea cucumber *Psolus patagonicus* Ekman, 1925 has been scarcely studied since its earliest description and subsequent taxonomic revisions (Ekman, 1925; Deichmann, 1947; Pawson, 1964, 1969). The triterpene saponin Patagonicoside A was first isolated by our team work from complete specimens of *P. patagonicus* collected on the intertidal rocks in Ensenada Bay (Tierra del Fuego), exhibiting an important antifungal activity when tested against *Cladosporium cucumerinum* (Murray *et al.*, 2001; Muniain *et al.*, 2005). In recent preliminary studies we found that this compound and its desulphated glycoside (ds-Patagonicoside A) also have antitumour activity against tumour cell lines (Careaga *et al.*, 2007).

This research is part of a project on bioprospection of marine invertebrates of applied interest; it is a preliminary contribution to the knowledge of chemical–ecological relations occurring in these sea cucumbers, whose biological and ecological aspects are poorly understood. The present work examines chemical aspects of adult specimens of *Psolus patagonicus* collected from two different and distant localities, and compares results with previous data from studies of specimens from intertidal rocks. Antifungal activity was tested against a broad spectrum of fungi and cytotoxicity assays conducted in *Artemia salina*.

MATERIALS AND METHODS

Collection of specimens

In October 1999, January 2000 and April 2003, a total of 183 specimens were collected from beds of the scallop *Zigochlamys patagonica* (King & Broderip, 1832) at a depth of 110 and

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115 m in the South Atlantic Ocean (39° 27′10S 55° 56′7″W and 43° 47′84″ S 59° 56′80″W) by means of a non-selective dredge of 2.5 m mouth opening and 3 m in length (Lasta & Bremec, 1997). Individuals of *Psolus* were removed manually from each of the scallop valves and frozen immediately on the ship (Coll. S. Campodónico and M. Lasta). They were transported to the Instituto Nacional de Investigaciones Pesqueras (INIDEP) and then to the Department of Organic Chemistry of the National University of Buenos Aires (UBA) for chemical analysis without breaking the cold chain. Taxonomic and biological studies were conducted on 12 individuals at the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' (MACN). In February 2007, 45 specimens were collected from rocky substrates of the Bridges Islands (Beagle Channel, Ushuaia, 54° 48′57″S 66° 25′00″W) at a depth of 4 to 10 m, by SCUBA diving (Coll. C. Muniain and H. Monsalve). Forty specimens were frozen immediately for further chemical analysis (UBA) and 5 specimens for taxonomic analysis (MACN). The voucher material is deposited in MACN-in: 34776 (4 specimens, 0–10 m) and MACN-in: 34777 (2 specimens, 110 m).

GENERAL PROCEDURE

^{1}H and ^{13}C NMR spectra were recorded in CD_3OD on a Bruker AM 500 spectrometer. The fast atom bombardment mass spectroscopy (negative ion mode) was obtained on a VG-ZAB mass spectrometer, on a glycerol matrix. Preparative high-performance liquid chromatography (HPLC) was carried out on a SP liquid chromatograph equipped with a Spectra Series P100 solvent delivery system, a Rheodyne manual injector and a refractive index detector, using a Bondclone 10 μ column (30 cm × 7.8 mm i.d.). Thin layer chromatography (TCL) was performed on precoated Si gel F254 (*n*-BuOH-HOAc-H_2O (12:3:5)) and C_{18} reversed-phase plates (60% MeOH-H_2O) and detected by spraying with *p*-anisaldehyde (5% EtOH).

EXTRACTION AND ISOLATION OF TRITERPENE GLYCOSIDES

Sea cucumbers (45 g, 183 specimens; 10 g, 40 specimens) frozen prior to storage were homogenized in ethanol and centrifuged. After evaporation of the ethanol, the residue was partitioned between MeOH-H_2O (90:10) and cyclohexane. The methanolic extract was evaporated and subjected to vacuum dry column chromatography on Davisil C-18 reversed-phase using MeOH-H_2O mixtures. The fraction eluted with 80% MeOH contained Patagonicoside A (1) as well as minor triterpene glycosides. Fractions eluted with 60%, 50% and 40% MeOH contained Patagonicoside A as the main triterpene glycoside. These fractions were combined and purified by reversed-phase HPLC to obtain the pure glycoside 1 as the main component. Patagonicoside A (1) was desulphated as described elsewhere (Murray *et al.*, 2001) and the residue was subjected to reversed-phase HPLC to give the pure desulphated glycoside, ds-Patagonicoside A (2) (Figure 1).

ANTIFUNGAL ASSAY

Geometric dilutions were obtained from freshly prepared stock solutions of Patagonicoside A (1), ds-Patagonicoside A (2) and reference compound, the commercial fungicide Benomyl (B) at concentrations of 1–10 mg ml^{-1} in an appropriate solvent. A volume of 10 μl of each solution was applied on TLC plates using graduated capillaries. The plates were then sprayed with a suspension of the fungi *Cladosporium fulvum*, *Fusarium oxysporum* and *Monilia* sp. in a nutritive medium and incubated in a glass box with a moist atmosphere for 2–3 d. Samplings were replicated three times; clear inhibition zones appeared against a dark grey background.

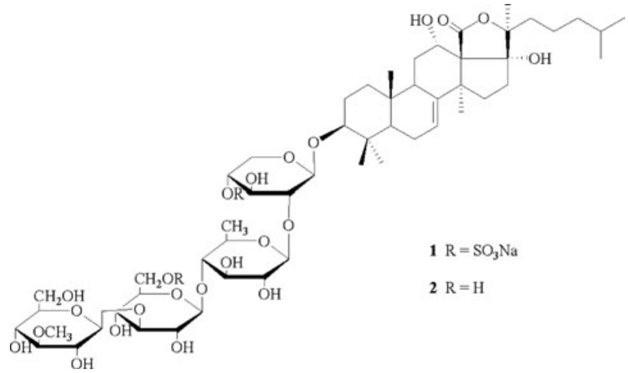

Fig. 1. Structure of the main component, Patagonicoside A (1), and its desulphated derivative ds-Patagonicoside A (2).

ARTEMIA SALINA BIOASSAY

Geometric dilutions of the combined vacuum dry C18 column chromatography fractions containing the triterpene glycosides from *Psolus patagonicus* were freshly prepared from a 10 mg/mL stock solution in distilled water. Aliquots of this solution (0.5 mL) were added to vials containing 10 shrimp/vial in marine water (3.8% (wt/vol) marine salts in distilled water), and the volume was adjusted to 5 ml/vial. The percentage of larval mortality was determined after exposure to the triterpene glycosides mixture at 25°C for 24 h and was calculated with data from three independent experiments by using the standard procedure of probit analysis (Meyer *et al.*, 1982).

RESULTS

Psolus patagonicus: bioactivity and chemical ecology

CHEMICAL ANALYSIS

Most of the triterpenoid oligoglycosides isolated from sea cucumbers so far belong to two main series: glycosides based on a 3β-hydroxyholost-9(11)-ene aglycon and those containing a 3β-hydroxyholost-7-ene skeleton (Chludil *et al.*, 2003). Patagonicoside A (1) (molecular formula: $C_{54}H_{86}O_{29}S_2Na_2$) is the first example of an holothurin with a 3β,12α,17α -trihydroxy-holost-7-ene type aglycon and a tetrasaccharide linear chain with the most common 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-Xyl structure (Figure 1). It is the main saponin in *P. patagonicus* but other minor glycosides are also present in the mixture of triterpene oligoglycosides, as determined by 1H NMR of fraction eluted with 80% MeOH and by thin layer chromatographic analysis of Patagonicoside A (1), its desulphated derivative (2) and minor holothurins obtained from partially purified HPLC fractions of the oligoglicoside mixture (Figure 2).

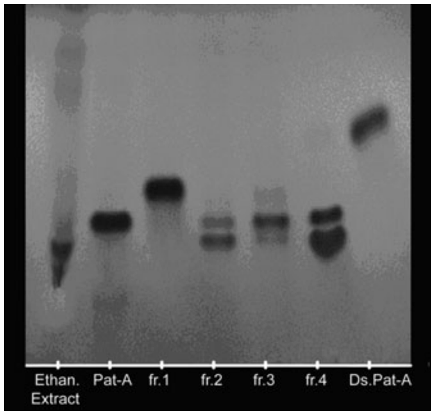

Fig. 2. Silica gel thin layer chromatography of 1, 2 and minority partially purified triterpene glycosides (Fr. 1–4) isolated from *Psolus patagonicus*, solvent: *n*-BuOH:HOAc:H_2O (12:3:5).

Patagonicoside A showed no absorption in the ultraviolet region. The saponin concentration estimated for a *P. patagonicus* individual is 2.4 mg/ind.

ANTIFUNGAL AND CYTOTOXIC ACTIVITIES

The main compound in *P. patagonicus*, Patagonicoside A (1), and its semisynthetic desulphated analogue 2, were evaluated for antifungal activity against three phytopathogenic fungi *Cladosporium fulvum*, *Fusarium oxysporum* and *Monilia* sp. through a bioautographic technique (Homans & Fuchs, 1970). Benomyl, a commercially available fungicide that is selectively toxic to microorganisms and invertebrates, was used as a reference compound. Comparing the antifungal activity against the three fungi, Patagonicoside A (1) was found to be more active than the desulphated analogue 2 against *Cladosporium fulvum* and *Monilia* sp., showing an antifungal activity comparable to Benomyl against *Monilia* sp. On the other hand, 1 was slightly more active than 2 against *Fusarium oxysporum* (Figure 3).

The cytotoxicity of the purified triterpene glycoside mixture of *P. patagonicus* was evaluated using a bioassay to determine brine shrimp (*Artemia salina*) larval mortality (Meyer *et al.*, 1982), showing a significant toxicity in this assay (LC_{50} 54.3 ppm).

CHEMICAL ECOLOGY

The specimens of *Psolus patagonicus* were oval shaped and dorso-ventrally flattened; the external live coloration was intense pink in specimens from intertidal rocks and rocky substrates of the Bridges Islands, whereas specimens collected at 115 m in depth were pale cream to white. All living specimens were photographed during samplings (Figure 4A–D). The length of living specimens ranged from 5 to 40 mm, with an average of approximately 20 mm, those collected from depth reaching the largest sizes. They are well adapted to attach to these hard substrates, both to rocky walls and as part of the epibiont fauna of *Z. patagonicus* (Figure 4A & B).

Psolus patagonicus is a suspension feeder. We collected a higher number of specimens in places with more evident currents and particle movement than in the intertidal. Externally, the sea cucumber appeared ‘hard and protected’, the dorsal surface was covered with imbricating granulated scales, showing distinct oral and anal valves (Figure 4C & D). The middle region, which corresponds to the internal organs, was very small. Ventrally, the soft sole, where the females incubated the eggs and embryos, was transparent and slightly concave and showed several rows of tube feet (podia) that help it stay well attached to the substrate, making its collection difficult.

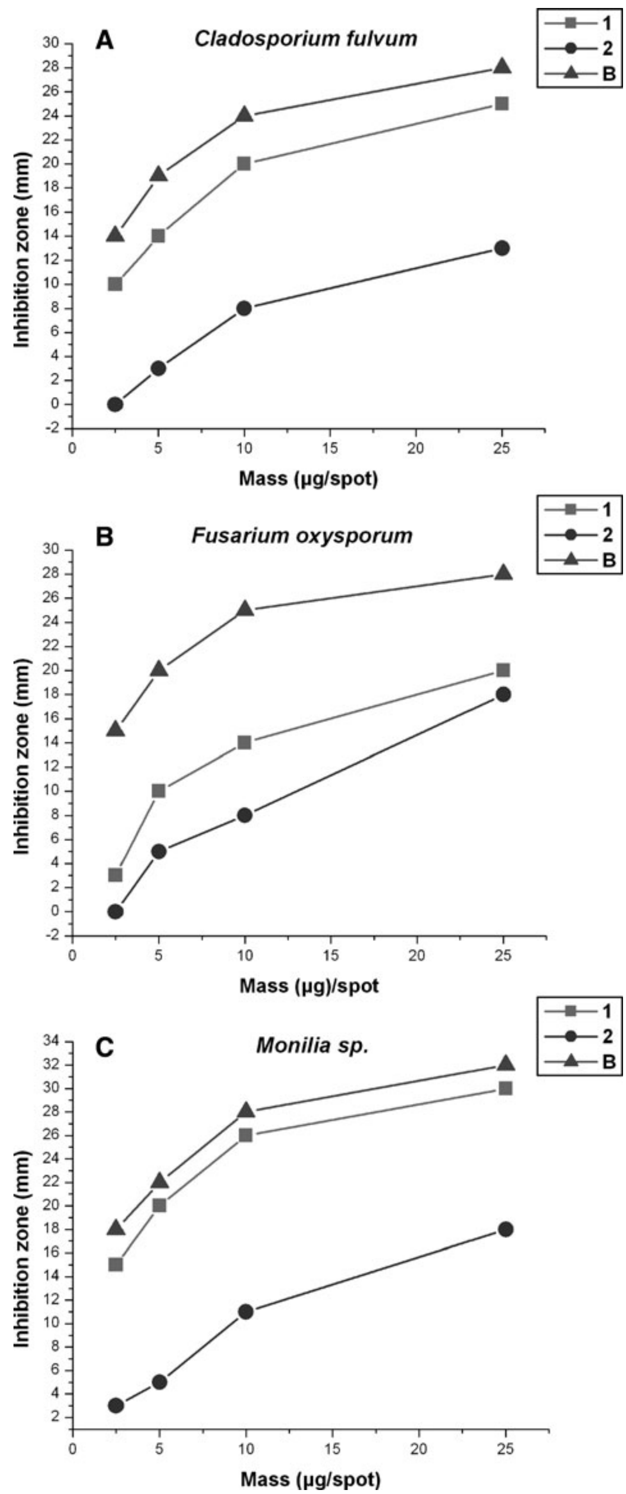

Fig. 3. Response curves for antifungal activity of Patagonicoside A (1), ds-Patagonicoside A (2) and Benomyl (B).

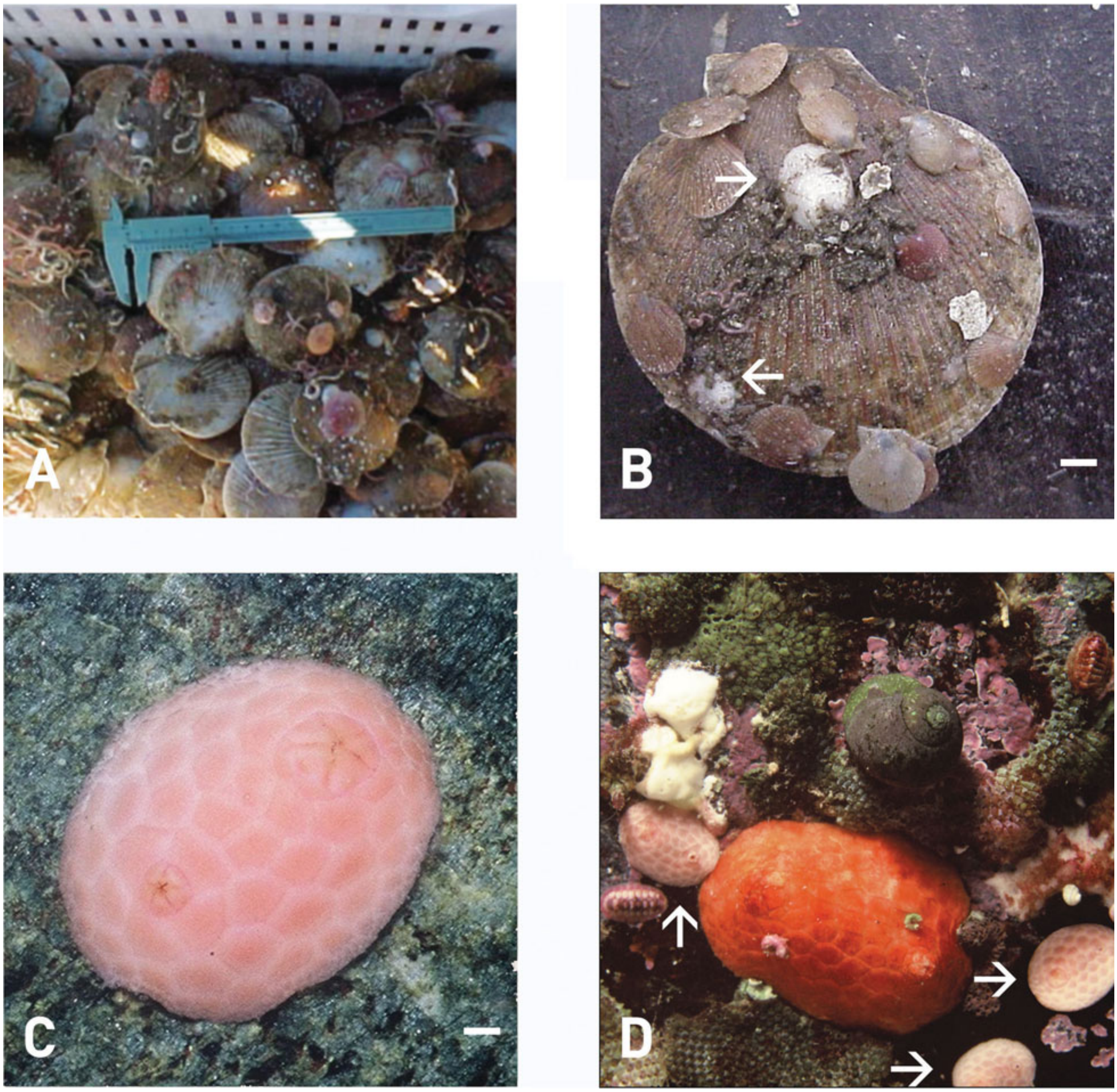

Fig. 4. (A) Samples of *Zygochlamys patagonica* from scallop beds from 110 m; (B) detail of epibionts of *Psolus patagonicus* on the shell. Scale bar: 1 cm. Photographs: S. Campodónico; (C) living specimen of *P. patagonicus* from the intertidal rock of Bahía Ensenada (Tierra del Fuego). Scale bar: 0.25 mm. Photograph: C. Muniain; and (D) specimens of *P. patagonicus* (arrow), around one of *Psolus* cf. *squamatus* (Koren, 1844). (Photograph: H. Monsalve).

Observations with scanning electron microscopy revealed the presence of calcareous ossicles on the body, and each tube foot had conspicuous end plate and submerged rounded plates in all the thin epidermis surfaces. The specimens studied showed a clean aspect, without evidence of fouling by invertebrates or algae, and up to the present, no predator of this species is known.

DISCUSSION

Echinoderms are an important resource with biotechnological potential and have been used for medicinal, agrochemical and feeding purposes since ancient times, especially in the Orient (China and Japan), as described in recent revisions on the topic (Kelly, 2005; Petzelt, 2005; Yokota, 2005).

Basically, 'saponins' are typical bioactive compounds of organisms belonging to the classes Asteroidea and Holothuroidea. The saponins derived from the asteroids are steroidal glycosides, whereas the holothurian saponins are based on triterpenoid aglycones.

In the present work we conducted a chemical study of specimens of *Psolus patagonicus* from two distant and different sampling environments, the Bridges Islands (Beagle Channel, Ushuaia) at a depth of 4 to 10 m, and the epibiont specimens of the scallop *Z. patagonica*, at about 100 m in depth (Figure 4A–D). The results of the chemical analysis indicated identical compounds in both samples (Figure 3), Patagonicoside A being the main one, originally described from specimens of the intertidal zone (Bahía Ensenada, Ushuaia) (Murray *et al.*, 2001; Muniain *et al.*, 2005).

Regarding the bioactivity found, data on structure–activity correlations of saponins isolated from sea cucumbers by the work of our team (Murray *et al.*, 2001; Chludil *et al.*, 2003) and previous studies (Kalinin *et al.*, 1996; Kalinin, 2000) suggest that the aglycone structure and the presence of sulphate groups in the oligosaccharide chain might play an important role in the antifungal and cytotoxic activities of these triterpene glycosides. The presence of a linear tetrasaccharide fragment appears to be a determining structural feature for membranotropic action and antifungal activity. For example, the antifungal disulphated triterpene glycoside, Hemoiedemoside A, isolated from *Hemioedema spectabilis* (Ludwig, 1882) from the southern Atlantic coast, showed remarkable toxicity against *Artemia salina* (LC_{50} 18.7 ppm), whereas the desulphated analogue was weakly active (LC_{50} 424.5 ppm) (Chludil *et al.*, 2002) and Patagonicoside A showed significant toxicity in this assay (LC_{50} 54.3 ppm). Patagonicoside A (1) and Hemoiedemoside A present the same desulphated tetrasaccharide chain and differ in the triterpene aglycone.

Underwater observations and examination of specimens of *P. patagonicus* prior to chemical processing revealed the absence of epibiont organisms, which may indicate antifouling activity provided by the presence of Patagonicoside A on the body wall. Through *in vitro* assays we recently found that both Patagonicoside A(1) and its desulphated analogue (2) inhibit the proliferation of three different types of tumour cells (Careaga *et al.*, 2007). This possible antifouling activity of the compound is currently under investigation by our research group.

The Cuvierian tubules, peculiar organs that play a defensive role, are present only in some species of Aspidochirotida. Cooper (1880) reported that some holothuroids discharged saponin concentrate in Cuvierian tubules. After expulsion and autotomy, tubules are readily regenerated as an efficient defensive mechanism (Hamel & Mercier, 2000; Vandenspiegel *et al.*, 2000; Flammang *et al.*, 2002, 2005). Kerr & Chen (1995) found that the triterpene precursor of saponins in the sea cucumbers, *Holothuria floridea* and *Actinopyga agassize*, have been identified as parkeol, and demonstrated that biosynthesis occurs exclusively in the holothurian Cuvier gland. On the other hand, in holothurian species without Cuvierian tubules, such as the family Psolidae, saponins are distributed in the body wall (Yokota, 2005; present work), and they could be originated in the synthesis by modifying precursors obtained through feeding (Harper *et al.*, 2001).

Up to the present, no likely predators of *P. patagonicus* are known; we only found the reference of Bingham & Braithwaite (1986) stating that the asteroid *Dermasterias imbricate* (Grube) is the only main predator of *Psolus chitonoides* H. L. Clark 1901. In *P. patagonicus*, the presence of a dorsal surface covered with imbricating scales and calcareous ossicles, and the tube feet (podia) involved in the attachment to the substratum through adhesive secretions play an important role in the defence mechanism of this organism. The presence of *P. patagonicus* as an epibiont species associated with the scallop *Zygochlamys patagonicus* has been mentioned for different areas in the Argentine Sea (Bremec & Lasta, 2002). Based on the concentrations of Patagonicoside A located in the body wall, we may preliminarily suggest a defensive role of this compound; further experimental *in situ* studies with fish, crustaceans and sea stars would allow us to test this hypothesis. On the other hand, further investigations on the presence of Patagonicoside A during the reproductive behaviour of incubating eggs and embryos under the sole (Hernández, 1981; Martínez *et al.*, 2006), will permit more precise conclusions regarding the chemical ecology in this species. Previous studies have demonstrated that eggs, larva and juveniles of many holothurian species possess chemical deterrents to predation that persist through early life stages (McEuen, 1984; Iyengar & Harvell, 2001).

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