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ORIGINAL PAPER

Chemical defenses of tunicates of the genus *Aplidium* from the Weddell Sea (Antarctica)

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Abstract Predation and competition are important factors structuring Antarctic benthic communities and are expected to promote the production of chemical defenses. Tunicates are subject to little predation, and this is often attributed to chemical compounds, although their defensive activity has been poorly demonstrated against sympatric predators. In fact, these animals, particularly the genus *Aplidium*, are rich sources of bioactive metabolites. In this study, we report the natural products, distribution and ecological activity of two *Aplidium* ascidian species from the Weddell Sea (Antarctica). In our investigation, organic extracts obtained from external and internal tissues of specimens of *A. falklandicum* demonstrated to contain deterrent agents that caused repellency against the Antarctic omnivorous predator, the sea star *Odontaster validus*. Chemical analysis performed with Antarctic colonial ascidians *Aplidium meridianum* and *Aplidium falklandicum* allowed the purification of a group of known bioactive indole alkaloids, meridianins A-G. These isolated compounds proved to be responsible for the deterrent activity.

Keywords Chemical defense · Antarctic tunicates · Indole alkaloids · Deterrent activity · *Aplidium* species · *Odontaster validus*

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Introduction

Antarctic benthos is characterized by stable environmental conditions and abundant faunal communities, which are considered to be structured mainly by biological factors (Gutt and Starmans 1998; Arntz et al. 2005). However, perturbations are quite common in shallow areas where ice disturbance can be an important factor (Gutt 2000). Antarctic invertebrate communities are affected by intense predation by other macroinvertebrates (such as sea stars) rather than fish, in contrast to what is common in other geographic areas (Dayton et al. 1974; Dearborn 1977; Bakus et al. 1986; McClintock et al. 1994). These circumstances may favor the evolution of chemical defenses. In fact, bioactivity detected in sessile Antarctic marine organisms has been shown to be very abundant, commensurable with temperate, and perhaps even tropical marine environments (McClintock 1989; Baker et al. 1993; Amsler et al. 2000; McClintock and Baker 2001; Avila 2006; Lebar et al. 2007; Avila et al. 2008; Peters et al. 2009). In spite of this, the

Southern Ocean remains understudied, and only 1.7% of all marine natural products reported so far come from Antarctic organisms (Marin Lit Database).

Many common benthic Ascidians (Chordata, Tunicata, Ascidiacea) lack strong structural elements, such as spicules or a tough tunic, as physical defenses against predators; however, they are relatively free from predation by generalists (Millar 1971; Goodbody and Gibson 1974; López-Legentil et al. 2006). This suggests that chemicals may be responsible for protecting them. In fact, a combination of factors including low caloric content, low digestibility and the presence of chemical defenses, such as high vanadium concentrations, low pH in tunic tissues and, especially, natural products, may be responsible for repellence against predators (Carliste 1968; Stoecker 1980b, a; Pisut and Pawlik 2002; Paul et al. 2008; Koplovitz et al. 2009). Tunicates, especially colonial species, appear to be protected against epibiosis as well, since fouling is rarely observed on them (Tatián et al. 1998; Davis et al. 2002). In general, ascidians are considered rich sources of bioactive natural products (Marchant et al. 1991; Faulkner 2000; Blunt et al. 2009), which may deter invertebrate and fish predators, as well as inhibit the growth of microorganisms (Tarjuelo et al. 2002; McClintock et al. 2004; López-Legentil et al. 2006).

Tunicates have the potential to yield novel compounds of ecological, chemical and also biomedical interest (Davis and Bremner 1999; Blunt et al. 2009; Paul et al. 2008). In particular, the cosmopolitan genus *Aplidium* is renowned for the variability of its metabolites (Zubía et al. 2005). A large variety of alkaloids have been isolated from this group (Arabshahi and Schmitz 1988; Zubía et al. 2005), such as piperidins, tetracyclic alkaloids and indoles, which display potent bioactivities (Table 1). However, even though a wide range of natural products has been isolated from tunicates, little is known about the ecological roles of most of these metabolites and their allocation within ascidian tissues (Paul et al. 1990, 2008; McClintock et al. 1991; Vervoort et al. 1998; Pisut and Pawlik 2002; Avila et al. 2008). According to the Optimal Defense Theory, defensive compounds should be located in areas that are most vital for survival and fitness (Rhoades 1979). In the case of predation by sea stars, for example, we would expect to find chemical defenses in the external body parts.

Ascidians are conspicuous members of Antarctic marine benthic communities (Gutt and Starmans 1998; Gili et al. 2000; Arntz et al. 2005, 2006). However, only seven species of Antarctic tunicates have been studied for their natural products so far (McClintock et al. 1992; Paul et al. 2008; Lebar et al. 2007; Avila et al. 2008) and fourteen species for their chemical ecology (Koplovitz et al. 2009). Among these, we emphasize the potent cytotoxic properties described for aplicyanins from *Aplidium cyaneum* (Reyes et al. 2008), meridianins from *Aplidium meridianum* (Gompel et al. 2004) and palmerolide from *Synoicum adareanum* (Diyabalanage et al. 2006), with both ecological and biomedical potential. Antarctic colonial ascidians, and more precisely those belonging to the genus *Aplidium*, are indeed a little-studied group of animals that are often observed apparently free from obvious macrofouling and predation (personal observations by the authors). For these reasons, they are expected to possess ecologically active compounds, as described for other congeners from other latitudes (Avila et al. 2008; Blunt et al. 2009).

Aplidium is a common genus among Antarctic tunicates, and it is represented by approximately 40 Antarctic and/or Subantarctic species (Varela 2007). *Aplidium falklandicum* Millar, 1960 is a common Antarctic ascidian that forms typically intense lemon-yellow colonies when alive (Tatián 1999). *A. meridianum* (Sluiter 1906) has a variable coloration, often forming gray colonies when alive (Varela 2007).

Table 1 Alkaloid compounds isolated from *Aplidium* species

Aplidium spp.	Compound	Type of Alkaloid	Geographical area	Activity	Reference
A. conicum	Conicamin	Indole alkaloid	Mediterranean	Histamine antagonist	Aiello et al. (2003)
A. cyaneum	Aplicyanins A-F	Indole alkaloid	Weddell Sea, Antarctica	Cytotoxic/antitumoral	Reyes et al. (2008)
A. haouarianum	Haouamines A, B	Alkaloid	Tarifa Island, Spain	Cytotoxic/antitumoral	Garrido et al. (2003)
A. meridianum	Meridianins A-G	Indole alkaloid	South Georgia I., Antarctica	Cytotoxic/antitumoral	Hernández Franco et al. (1998), Gompel et al. (2004), Seldes et al. (2007)
A. pantherinum	Pantherinine	Tetracyclic alkaloid	Australia	Cytotoxic	Kim et al. (1993)
A. tabascum	Lepadins F, G, H	Decahydroquinoline	Great Barrier Reef, Australia	Antiplasmodial, antitrypanosomal	Davis et al. (2002)
A. uouo	Uouamines A, B	Piperidin	Maui, Hawaii	–	McCoy and Faulkner (2001)
Aplidium sp	Aplidites A-G	Macrocyclic alkaloid	Australia	–	Murray et al. (1995)
Aplidium sp1 and *sp2*	3 compounds	Iodinated alkaloid	Australia	Cytotoxic	Carroll et al. (1993)

Both these produce short-lived lecithotrophic larvae throughout the year (Sahade et al. 2003; Tatián et al. 2005) and have a typical Antarctic-Subantarctic distribution (Ramos-Esplá et al. 2005; Primo and Vázquez 2007).

The aim of this study was to establish the presence and location of defensive natural products in Antarctic tunicates of the genus *Aplidium* collected from the Weddell Sea. This geographically remote area was totally unexplored with respect to the chemical ecology of tunicates, until recently. A first analysis of *Aplidium cyaneum* from this area revealed very interesting new metabolites: the aplicyanins (Reyes et al. 2008). Here, we report results for another two Antarctic *Aplidium* species: *A. falklandicum* and *A. meridianum*. Crude extracts were tested for ecological activity against a sympatric generalist predator, the Antarctic sea star *Odontaster validus*. The isolation of some compounds provided the opportunity to evaluate their repellent properties and their antimicrobial activity in laboratory assays against cosmopolitan bacteria and yeasts.

Methods and materials

Collection of samples

Antarctic tunicates of the species *Aplidium falklandicum* and *A. meridianum* were collected in the Eastern Weddell Sea between 280 m and 340 m depth during the ANT XXI/2 cruise of R/V Polarstern (AWI, Bremerhaven, Germany), from November 2003 to January 2004, using Bottom and Agassiz Trawls. Individuals of each species from a single collection site were grouped together as a single sample for experimental analyses (Table 2). A part of each sample was conserved, and pictures of living animals were taken on board for further taxonomical identification at the University of Alicante (Spain). The remaining material was frozen at −20°C and transported to the laboratory in Spain. Later, each sample was dissected into two parts: the tunic or external part and the internal part (visceral tissues), except for samples #4 and #5, which were separately processed as a whole. In total, therefore, eight samples, each consisting of several colonies (see Table 2), were used for chemical analysis (#1int, #1ext, #2int, #2ext, #3int, #3ext, #4 and #5). Sample #5 was processed differently in order to obtain the fraction containing all the meridianins together, for testing the deterrent activity of this mixture, without separating the different compounds.

Organic extractions

Each sample was separately extracted with acetone and sequentially partitioned into diethyl ether and butanol fractions (except for #4 which was processed with different chemical techniques as reported below). Each step was repeated three times, except for butanol which was only done once, and the solvents were then evaporated under reduced pressure, resulting in dry extracts later used for both bioassays and chemical analysis (Table 3). Sample #4 was extracted with hexane, dichloromethane and butanol and was exclusively used for analyzing its chemistry. In addition, a voucher of each sample was extracted with dichloromethane, methanol and water, and the dichloromethane fractions were further used for detailed chemical relative quantification analysis. The detailed description of the extraction procedure has been reported elsewhere (Avila et al. 2000; Iken et al. 2002). Butanolic extracts and water residues were kept for further analysis on compounds with different polarities and are not reported here.

Purifications and chemical analysis

Diethyl ether extracts were screened by thin layer chromatography (TLC), using Merck Kieselgel plates (20 × 10 cm and 0.25 mm thick), and light petroleum ether/diethyl ether (1:0, 8:2, 1:1, 2:8, 0:1) and chloroform/methanol (8:2) as eluents. The plates were developed with $CeSO_4$. A conspicuous UV–Visible band at R_f 0.63 (chloroform/methanol 9/1) with $CeSO_4$ reaction was observed in all samples. Extracts were further fractioned by molecular exclusion chromatography, using Sephadex LH-20 columns with chloroform/methanol 1:1. ^{1}H-NMR spectroscopic analyses were done to determine pure products or mixtures in the fractions obtained. Fractions composed of a mixture of molecules were further purified with HPLC techniques (Shimadzu with LC-10ADVP pump and SPD-10AVP UV

Table 2 Data of the *Aplidium* samples collected in the Weddell Sea

Species name	Sample code	Number of colonies	Latitude	Longitude	Depth (m)
A. falklandicum	1	5	70° 55.92′ S	010° 32.37′ W	288
A. falklandicum	2	2	70° 56.67′ S	010° 32.05′ W	302.4
A. falklandicum	3	14	70° 52.16′ S	010° 43.69′ W	290.8
A. meridianum	4	13	70° 57.11′ S	010° 33.52′ W	337.2
A. falklandicum	5	1	70° 56.67′ S	010° 32.37′ W	296.4

Table 3 Extracts and weights of the different samples of *Aplidium* spp

Species name	Sample code	WW (g)	DW (g)	EE (mg)	% [N]
A. falklandicum	1 int	13.5	0.6	34.1	7.97
	1 ext	12.3	1.3	74.4	5.64
A. falklandicum	2 int	61.9	0.5	64.2	12.64
	2 ext	84.6	4.0	190.4	4.81
A. falklandicum	3 int	62.8	2.0	38.8	1.97
	3 ext	192.3	5.0	119.0	2.40
A. meridianum	4	331	NA	NA	NA
A. falklandicum	5	9.38	0.4	26.1	6.53

WW wet weight of the sample, *DW* dry weight of the sample, *EE* dry weight of the diethyl ether extract; *% [N]* natural concentration of the ether extract in the sample. % [N] is calculated by dividing the dry weight of the ether extract (EE) by the dry weight of the whole sample (DW). *NA* not available

Table 4 Presence of the different meridianins in the diethyl ether extracts (EE) and dichloromethane extract (DCME) of the two analyzed species of *Aplidium*, *A. falklandicum* and *A. meridianum*

Sample code	Mer A	Mer B	Mer C	Mer D	Mer E	Mer F	Mer G
A. falklandicum							
EE 1 int	+	+	+	−	+	−	−
EE 1 ext	+	+	+	−	+	−	+
EE 2 int	+	+	+	−	+	−	+
EE 2 ext	+	+	+	−	+	+	−
EE 3 int	+	+	+	−	+	+	+
EE 3 ext	+	+	+	−	+	+	−
A. meridianum							
DCME 4	+	+	+	+	+	+	+

Mer, Meridianin; (+), present; (−), absent. Sample codes refer to the sample number, kind of extract and body part (int, internal; ext, external)

Meridianins were detected by ^{1}H NMR (600 MHz). Lowest level of detection was about 1 μM

detector) using a semipreparative column in reverse phase (Supelco Discovery® C_{18}, 25 cm × 46 mm, 5 μm) and water/acetonitrile as solvent.

Spectral analysis of the natural products

The isolated pure compounds were subjected to spectral analysis using both NMR and UV spectroscopy as well as MS spectrometry. The ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker Avance DRX-400, Bruker DRX-600 equipped with in inverse TCI CryoProbe and Bruker DRX-300 spectrometers. The ESIMS and EIMS spectra were obtained on a Micromass Q-TOF Micro™ spectrometer connected to a Waters Alliance 2695 HPLC chromatograph and on a HP-GC 5890 series II spectrometer, respectively. The UV spectra were recorded on an Agilent 8453 spectrophotometer. The spectral data of compounds (Table 4) isolated were compared with the data reported in the literature (Hernández Franco et al. 1998; Gompel et al. 2004; Seldes et al. 2007). NMR spectra of meridianins F and G were also recorded in dimethylsulfoxide (DMSO); the reported δ values are referred to the solvent peaks (2.54 ppm for proton and 40.4 ppm for carbon).

HPLC–MS relative quantification

Relative percentages of each meridianin (A-G) within the total meridianin mixture in the dichloromethane extracts from *Aplidium* samples were quantified in an Orbitrap-MS spectrometer connected to a Thermo Accela-HPLC. Liquid chromatographic separations were performed in a C18 column using a MeOH:water gradient. Meridianins C/D were jointly quantified due to their isomeric nature ($[M+H]^+$ peaks at *m/z* 289.0083), and the same was done for meridianins B/E ($[M+H]^+$ *m/z* 305.0032). In order to quantify ion-counting in the mass spectrometer, the number of ions of 50 μg of flumequine diluted in 1 mL of methanol were used as standard (Table 5).

Feeding-deterrent experiments

Individuals of the Antarctic omnivorous predator, the sea star *Odontaster validus,* were collected in the South Shetland Islands (Livingston and Deception) on board of B/O

Table 5 Relative percentages among meridianins in dichloromethane extracts from the five different collections of Antarctic *Aplidium* tunicates

Meridianins	Sample #1 (*A. falklandicum*)	Sample #2 (*A. falklandicum*)	Sample #3 (*A. falklandicum*)	Sample #4 (*A. meridianum*)	Sample #5 (*A. falklandicum*)
A	17.8	5.7	18.7	13.7	19.1
G	3.6	2.2	3.3	1.3	2.8
C/D	34.8	26.8	35.5	21.6	35.4
B/E	40.2	62.6	39.1	61.1	38.5
F	3.6	2.6	3.3	2.3	4.2

Meridianins C/D and B/E were jointly quantified in pairs due to their isomeric nature. As explained in the text, for some samples (#1, #2, #3 and probably #5) the percentage values of meridianins C/D are only attributable to meridianin C

Hespérides in January 2006 for feeding-repellence assays. They were kept alive with fresh sea water for the experiments and placed back at the sea at the same location after testing. The experiments took place at the Spanish Base "Gabriel de Castilla" in Deception Island, Antarctica. Dry diethyl ether extracts from the samples #1int, #1ext, #2int, #2ext, #3int, #3ext (*A. falklandicum)* were transported frozen from Spain to the Base "Gabriel de Castilla", where they were diluted in diethyl ether and coated into shrimp pieces, which were then presented to the sea stars. The methodology has been already explained with detail elsewhere (Avila et al. 2000; Iken et al. 2002). Each test consisted of 10 containers filled with 2.5 l of sea water with one sea star and one piece of coated shrimp per container. Shrimp coating was either extract or just the solvent in the control tests. Extracts were applied at their natural tissue concentrations in the assays (Table 3). Dry weight was selected for calculating natural concentrations according to sea star extraoral feeding habits. The extract or the solvent were totally impregnated into the shrimp cube in the coating process, since the size of the cubes was sufficiently small ($5 \times 5 \times 5$ mm) and their dry mass was 13.09 ± 3.43 mg. Solvent was evaporated under flow hood before starting the test. Feeding repellence for the shrimp coatings was evaluated after 24 h exposure, by counting the number of shrimp eaten for each test (Avila et al. 2000; Iken et al. 2002). The remaining shrimp pieces (not eaten) were frozen and later extracted and checked on a TLC, for ensuring the presence of the extracts or compounds on the shrimp after 24 h, which was always the case. Statistical analyses were carried out for each experiment respect to the control run simultaneously using Fisher's exact tests (Sokal and Rohlf 1995).

In a further Antarctic expedition at the Spanish base "Gabriel de Castilla" during the austral summer of 2008–2009, several mixtures of the isolated meridianins were assayed at their natural concentrations in palatability tests following the procedure previously explained and using methanol and diethyl ether as solvents. The meridianin mixtures selected were those abundant enough to do the tests at natural concentrations, and these were samples: #1int, #2int, #2ext and #5 (Table 6). This time the specimens of *O. validus* for testing were collected by scuba diving down to 15 m depth at Whalers Bay (Deception Island) on December 2008. The sea stars were treated as reported above for previous assays.

Antibacterial and antifungal tests

These assays were intended to assess general antibiotic properties of the isolated compounds. Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacterial colonies and yeasts (*Candida albicans*) were cultured in LB medium (Luria–Bertani broth) for one night under agitation at 37°C. Cultures were then diluted at 1:1000 volume in LB medium ($=10^8$ cfu/ml), and 1 ml of each solution was mixed homogeneously with agar and placed onto Petri dishes. Each Petri dish was divided into $n + 1$ regions, being "n" the number of substances to be tested, corresponding to the 6 meridianins tested, plus one region for the positive control with antibiotic activity and one for the negative control. Positive controls were chloramphenicol (10 µg) for Gram-positive and Gram-negative bacteria, and fluconazol (20 µg) for yeasts, while negative controls consisted of solvent only, in this case, methanol. Paper disks (Ø 5/6 mm) soaked with 20 µl (equivalent to 100 µg) of the corresponding testing pure products (meridianins A, B, C, E, F, G) previously dissolved in methanol at 5 mg/ml, or control disks, were placed in the middle of each testing region in the Petri dishes. After 18–24 h at 37°C, inhibition halos were measured to determine antibiotic activity. When the diameter of the inhibition zones was larger than 11 mm Ø, it was considered active.

Table 6 Data and weights of different samples and fractions of *Aplidium falklandicum* and their meridianin mixtures tested in repellency assays

Species name	Sample code	Meridianin Mix	WMer (mg)	% [N]
A. falklandicum	1 int	A, B, C, E	6.3	0.97
A. falklandicum	2 int	A, C, E, G	0.4	0.073
	2 int	A, B	0.8	0.146
A. falklandicum	2 ext	A, C, E, F	22.03	0.545
	2 ext	A, B, C	9.42	0.233
	2 ext	B, C	4.95	0.122
A. falklandicum	5	A-G	9.7	2.425

Meridianin mix, meridianins contained in the mixed fraction; WMer, dry weight of the meridianin mix; % [N], natural concentration of the meridianin mixture in the sample. % [N] is calculated by dividing the dry weight of the ether extract (EE) by the dry weight of the whole sample (DW); EE and DW for each sample are already provided in Table 3. For sample #5, the precise meridianins present in the mixture (A-G) are not known

Results

Morphological characterization of colonies, zooid individuals and larvae allowed the identification of samples #1, #2, #3 and #5 as *A. falklandicum* and sample #4 as *A. meridianum*.

A total of seven aromatic alkaloids, meridianins A-G (Fig. 1), which had been previously reported only in *A. meridianum* from the South Atlantic Ocean (Hernández Franco et al. 1998; Seldes et al. 2007), were isolated from distinct individuals of these two Antarctic species. All

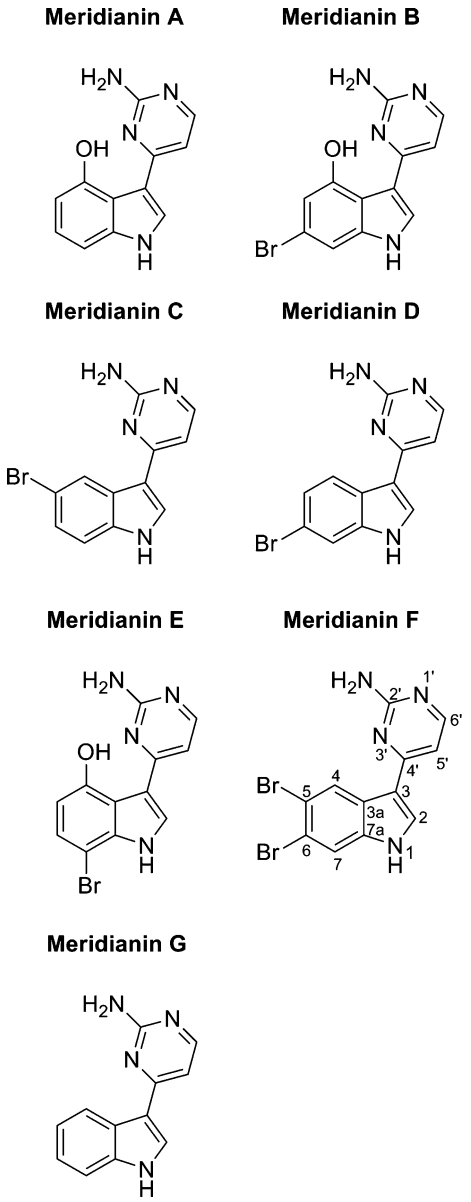

Fig. 1 Chemical structures of the seven indole alkaloids, meridianins A-G, found in one or both of the studied species, *A. falklandicum* and *A. meridianum*

compounds were identified by comparison with their spectral data (^{1}H- and ^{13}C-NMR, UV and MS) with the literature (Hernández Franco et al. 1998; Gompel et al. 2004; Seldes et al. 2007). NMR spectra of meridianins F and G that had been described in methanol (Seldes et al. 2007) were also recorded here in dimethylsulfoxide-d_6 (DMSO-d_6), the same solvent used for the other meridianins (Hernández Franco et al. 1998). All carbon and proton values of meridianins F and G were assigned as reported below.

Meridianin F: ^{1}H-NMR (600 MHz, DMSO-d_6) δ 11.93 (1H, br s, H-1), 8.95 (1H, s, H-4), 8.30 (1H, br s, H-2), 8.12 (1H, d, J = 5.3 Hz, H-6′), 7.83 (1H, s, H-7), 7.00 (1H, d, J = 5.3 Hz, H-5′), 6.53 (s, -NH_2); ^{13}C-NMR (300 MHz, DMSO-D_6) δ 163.4 (s, C-2′), 161.7 (s, C-4′), 157.2 (d, C-6′), 136.7 (s, C-7a), 130.4 (d, C-2), 126.4 (d, C-4), 126.1 (s, C-3a), 116.4 (d, C-7), 116.0 (s, C-5), 114.6 (s, C-6), 113.1 (s, C-3), 105.1 (d, C-5′).

Meridianin G: ^{1}H-NMR (600 MHz, DMSO-d_6) δ 11.93 (1H, br s, H-1), 8.56 (1H, d, J = 7.8 Hz, H-4), 8.17 (1H, d, J = 2.4 Hz, H-2), 8.08 (1H, d, J = 5.3 Hz, H-6′), 7.42 (1H, d, J = 7.9 Hz, H-7), 7.16 (1H, t, J = 6.8 Hz, H-5), 7.10 (1H, t, J = 6.8 Hz, H-6), 7.00 (1H, d, J = 5.3 Hz, H-5′), 6.38 (s, -NH_2); ^{13}C-NMR (300 MHz, DMSO) δ 157.0 (d, C-6′), 137.0 (s, C-3a), 128.2 (d, C-2), 125.2 (s, C-7a), 122.4 (d, C-5), 121.9 (d, C-4), 120.2 (d, C-6), 113.2 (s, C-3), 111.8 (d, C-7), 105.3 (d, C-5′).

Meridianins are structurally characterized by the presence of an indolic nucleus connected to an amino-pyrimidinic moiety through a C-3/C-4′ linkage. With the exception of meridianin A and meridianin G, all meridianins contain one or two bromine atoms in their structure (Fig. 1). Meridianins were detected in the diethyl ether fractions of both the internal and the external parts of *A. falklandicum* (samples #1int, #1ext, #2int, #2ext, #3int, #3ext and #5) and in the dichloromethane extract of specimens of *A. meridianum* (sample #4) (Table 4). The distribution of the diverse meridianins in the samples analyzed was quite homogeneous, especially for meridianins A, B, C and E, which were present in the liposoluble fractions of all samples (Table 4). Meridianin D was detected only in *A. meridianum* (sample #4), while meridianins F and G were found in some samples but not in others (Table 4).

Relative quantification of the secondary metabolites by means of HPLC–MS using an internal standard revealed that meridianins B/E were the major joint compounds, followed by meridianins C/D and meridianin A. As for meridianins F and G, similar values of much smaller range were detected in all samples (Table 5). Meridianins B/E were detected in significantly major quantities in *Aplidium meridianum* (sample #4), and A and C/D presented similarly higher percentages than in other samples.

All the ether extracts from the tunic (external) and the internal parts of the three tested samples (#1, #2, #3) of *A. falklandicum* caused significant (P = 0.01) feeding repellence against the sea star *O. validus* at natural concentrations according to the Fisher's exact test. Control assays conducted using only the solvent, diethyl ether, as shrimp coating, showed a minimum consumption of six pieces of shrimp out of ten. Pieces of shrimp coated with diethyl ether fractions at natural concentrations from the samples tested were never consumed by the sea stars (Fig. 2). Regarding the tests conducted with meridianin mixtures (Table 6), control tests (with methanol or diethyl ether only) were eaten at a ratio of 8 pieces out of ten, while experiments treated with meridianin mixture coatings at natural concentrations showed significant ($P < 0.05$*)

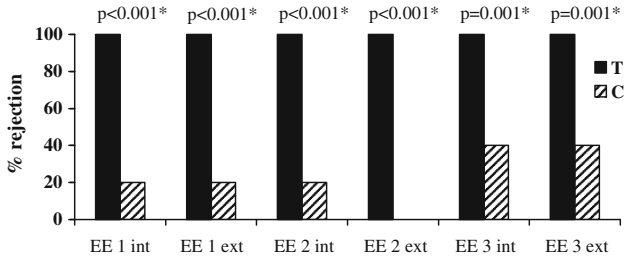

Fig. 2 Results of the repellence experiments with shrimp pieces coated with ether extracts of samples of *A. falklandicum*, using the sea star *Odontaster validus* as a predator. Tests were done using natural concentrations. Controls consisted of coating only the solvent (diethyl ether). Each test was compared to the control run simultaneously. *P**: significant differences from the controls according to Fisher's exact test; T, treatment; C, control

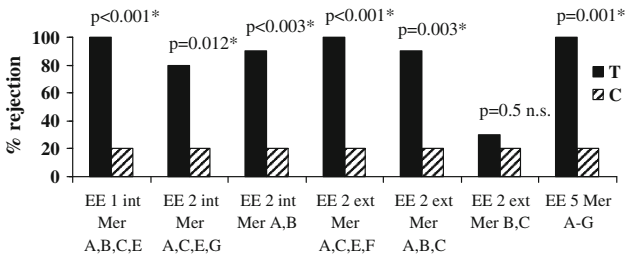

Fig. 3 Results of the repellence experiments with shrimp pieces coated with fractions of meridianin mixtures from samples of *A. falklandicum*, using the sea star *Odontaster validus* as a predator. Tests were done using natural concentrations. Controls consisted of coating only the solvent (diethyl ether). Each test was compared to the control run simultaneously. *P**: significant differences from the controls according to Fisher's exact test; n.s. not significant; T, treatment; C, control. For sample #5, the precise meridianins present in the mixture (A-G) are not known

deterrency against the sea star, with a maximum consumption of 2 out of ten pieces of coated shrimp (except for fraction #2ext mer B, C) (Fig. 3).

None of the isolated meridianins from *Aplidium* species caused growth inhibition on cultures of cosmopolitan yeasts and Gram-negative and Gram-positive bacteria (with the exception of meridianin D which was not tested because there was not enough material). The same was observed in the solvent control. Positive controls (chloramphenicol and fluconazol) significantly inhibited Gram-positive and Gram-negative bacteria and yeasts, respectively. Therefore, no antifungal or antibacterial activity has been detected in meridianins A, B, C, E, F and G in our laboratory assays.

Discussion

Meridianins from Antarctic colonial ascidians of the genus *Aplidium* were shown to provide protection from predation by a sympatric macroinvertebrate. The common Antarctic sea star, *O. validus*, is a voracious omnivorous predator that feeds on a wide range of prey, even on conspecifics (Dearborn 1977; McClintock 1994). Previous trials and experiments, as well as controls in the present study, show how specimens of this sea star avidly consume pieces of shrimp in laboratory assays (Avila et al. 2000; Iken et al. 2002). However, when individuals of *O. validus* are presented with pieces of shrimp treated with coatings of ether extracts from inner or outer parts of specimens of *A. falklandicum* at natural concentrations, no consumption was detected (Fig. 2). In some cases, the sea stars were even observed to move quickly away from the shrimp piece (personal observations). Natural concentrations were calculated according to the total dry mass, since sea stars usually extrude their stomach out against their prey. Sea stars pre-digest their food externally by enzymatic processes, rather that biting and performing internal digestion, as is usual in other predators such as fish. Therefore, one or more deterrent compounds must be present in the lipophilic fractions from *A. falklandicum*, causing the unpalatability to the coated shrimp pieces, and rejection from the sea stars. The meridianins (Fig. 1) isolated from diethyl ether extracts of *A. flaklandicum* (Table 6) were shown to be the agents responsible for the repellent activity, since shrimp pieces coated with several meridianin mixtures proved to be significantly unpalatable to the sea stars (Fig. 3). This deterrent property cannot be attributed to a specific meridianin but to the mixture of these alkaloids. The only fraction not active in the assays was fraction #2ext mer B, C (Fig. 3), and this could be due to a problem during the coating procedure resulting in not enough extract being coated onto the shrimp pieces in that particular experiment.

The total meridianin mixture represents an important proportion within the total dry mass of the lipophilic fraction of the animal, e.g., 37.2% taking as an example sample #5 (Table 6). Considering the fact that these molecules are secondary metabolites, they must play an important role for the tunicate's integrity to appear in such high concentrations. Even though scarce, there are a few studies on Antarctic tunicates containing anti-predatory defenses in the literature: the colonial ascidian *Distaplia cylindrica* (McClintock et al. 2004) and the solitary ascidian *Cnemidocarpa verrucosa* (McClintock et al. 1991; McClintock and Baker 1997), as well as a recent study of fourteen species of tunicates (Koplovitz et al. 2009), although the repellence has not been traced to any particular metabolites. In this last study, however, Koplovitz et al. (2009) did not detect activity in extracts of an unidentified *Aplidium* sp. near Palmer Station (Antarctic Peninsula). In other geographical areas, however, some compounds have been described as providing ascidians with antipredator chemical defense (Paul 1992; Blunt et al. 2009). For instance, tambjamines (Paul et al. 1990), 15′ didemnin B and nordidemnin B, and patellamide C (Paul 1992) have icthyodeterrent

properties. Also, tambjamines and ecteinascidin alkaloids, extracted from two different tunicates, confer chemical protection to their larvae (Young and Bingham 1987). Furthermore, defensive mechanisms often act at different levels, such as fouling avoidance or space competition (Stoecker 1980a; Becerro et al. 1997; Davis and Bremner 1999; López-Legentil et al. 2006). Similarly, the alkaloid eudistomins isolated from a colonial tunicate (*Eudistoma olivaceum*) exhibit potent antiviral, antimicrobial and antifouling properties (Davis et al. 2002). Our tests with meridianins, however, did not show apparent antimicrobial activity against cosmopolitan bacteria or yeasts in laboratory assays. Further experiments using sympatric marine bacteria or fouling organisms should be conducted in order to evaluate other possible defensive activities with ecological relevance for these compounds.

It was suggested that tunicates use both physical (spicules, tunic toughness) (Lambert and Lambert 1987) and chemical (natural products, acidity, heavy metals, vanadium) strategies to defend themselves (Stoecker 1980b, a; Parry 1984; Pawlik 1993; Davis et al. 2002; Tarjuelo et al. 2002; López-Legentil et al. 2006; Koplovitz et al. 2009). Nonetheless, assays performed using silicious (Pawlik et al. 1995; Chanas and Pawlik 1995, 1996) or calcareous spicules and sclerites (Lindquist and Hay 1996; Pawlik et al. 1995; Puglisi et al. 2002) failed to demonstrate rejection by fish, suggesting that natural products are the primary means of defense against predators, even if occasionally combined with other defensive systems (Stoecker 1980a; Pisut and Pawlik 2002). According to the Theory of Optimal Defense (Rhoades 1979), an ascidian would be expected to store organic or inorganic chemical defenses in body regions that maximize fitness, considering its potential predators' habits. In the case of Antarctic ecosystems with sea stars as frequent predators, protection would be most useful in outer regions. Chemical defenses are thus expected to accumulate in the tunic for adult protection. However, if defending larval stages leads to a higher survival of the species, then internal body tissues, such as the gonads, are likely to be protected as well (Rhoades 1979; Young and Bingham 1987; Lindquist et al. 1992; Lindquist and Hay 1996; Pisut and Pawlik 2002). Tunics are commonly less attractive to predators since they have very little nutritive value, whereas visceral mass and gonads contain the bulk of usable protein and lipid (McClintock et al. 1991). This could explain why a number of ascidians have undefended tunics (Pisut and Pawlik 2002). Defenses stored in the gonads would not seem to protect solitary adult ascidians, since a predator would need to open the tunic to encounter such localized defenses, resulting in the death of the tunicate. In contrast, for clonal ascidians, a predator could attack and kill a single (or a few) zooid(s) and then be deterred from further feeding without killing the whole colony (Stoecker 1980b). For this reason, allocation of defensive metabolites may not be as necessary in colonial ascidians as it is in solitary ascidians. From our study, we conclude that the colonial tunicate *A. falklandicum* contains chemical defenses which are not concentrated in specific tissues. Meridianins are distributed throughout the inner parts, as well as in the tunic, and provide protection from sympatric predators, such as the sea star *O. validus* (Tables 4; Figs. 2, 3). Isolated meridianins from both *A. falklandicum* and *A. meridianum* were shown to repel the sea star. This is the first example of characterized secondary metabolites from Antarctic tunicates with ecological activity.

Ascidians produce many nitrogen-containing metabolites, almost all derived from amino acids (Davidson 1993). In our study, meridianins were found in *A. falklandicum* and *A. meridianum*. The presence of meridianins in *A. falklandicum* is reported here for the first time. Previously, meridianins had been reported in *A. meridianum* from South Georgia Islands (Hernández Franco et al. 1998; Gompel et al. 2004; Seldes et al. 2007). To date, a total of seven of these metabolites have been described: meridanins A, B, C, D, E, F and G, and they frequently appear together as a mixture of indolic alkaloids. Meridianins F and G are two of the least common compounds in the mixture (Hernández Franco et al. 1998). The carbon and proton values of meridianins F and G in DMSO were assigned in our study for the first time. These molecules are composed of a brominated and/or hydroxylated indole nucleus with a 2-aminopyrimidine substituent at C3.

Meridianin composition in external and internal lipophilic extracts of *A. falklandicum* varied slightly among samples and compared to *A. meridianum*. Specimens of *A. meridianum* (sample #4) contained all seven meridianins (A-G). This is not surprising, since this was the organism from which these metabolites were originally described. All the samples analyzed, of both species, contained meridianins A, B, C and E. Meridianin D was only found in *A. meridianum* and meridianins F and G had a peculiar distribution, appearing in some samples but not in others (Table 4). Differences in meridianin composition could be due to interspecific variability, and thus, the different species might have characteristic meridianin profiles. Also the variability could have a geographic component, since animals living in separate habitats, under different ecological and/or environmental conditions, may produce different compounds. Another hypothesis is the presence of diverse symbiotic organisms in the samples that produce different metabolites. However, a more plausible explanation for the absence of meridianins F and G in some of the samples is the small amounts in which they appear; this makes detection difficult. This is probably the cause of their apparent absence in some extracts analyzed in the past (Seldes et al.

2007). However, the absence of meridianin D in *A. falklandicum* samples must have a different explanation, since it is not considered a minor metabolite in the indolic mixture. Meridianin D could be a characteristic metabolite of the species *A. meridianum*, although more data are needed to support this hypothesis.

From the relative chemical quantification, we can confirm that B/E are the most common meridianins followed by C/D and then by A. Meridianins F and G are clearly minor compounds in the mixture and appear at constant ratios in all samples. Similar ratios are also observed for meridianins C/D, except for a slight increase in sample #4 with respect to the rest. Meridianins B/E and A show more variable relative percentages (Table 5). Meridianins B/E and C/D constitute two isomeric couples which appear jointly in the chromatographic peaks, and so the contribution of each isomer to the ratio of the isomeric pair is impossible to calculate using this method. Nonetheless, for samples #1, #2 and #3 (*A. falklandicum*), the relative ratio recorded for the couple C/D is all due to meridianin C, since meridianin D was never detected. For sample #5, although it also corresponds to *A. falklandicum*, we cannot draw any conclusion, since we did not demonstrate the absence of meridianin D. The increased ratio of the couple C/D detected in *A. meridianum* (sample #4) could be due to meridianin D.

It is also noteworthy that the species studied here are currently subject to intensive taxonomic studies, due to their high intraspecific variability, which is very common in colonial ascidians (Tatián 1999; Varela 2007). In fact, it has been suggested that *A. meridianum* and *A. falklandicum* might be synonymous species and could be considered as two morphotypes of the same species. However, more detailed morphogenetic studies are needed to confirm this (Varela 2007). In that case, *A. meridianum* could be a morphotype containing meridianin D while *A. falklandicum* lacks it. Finding meridianins in both species is also remarkable for this reason; however, these metabolites have also been reported in collections of the related tunicate *Synoicum* sp. from Palmer Station, Antarctica (Lebar et al. 2007; Ankisetty and Baker, unpublished). Further studies are needed to explain this variability.

Indole alkaloids, frequently isolated from tunicates and sponges, are important potential antitumoral natural products (Davidson 1993). Some of them display interesting ecological defensive activities, as demonstrated here for the meridianins. Moreover, meridianins have been reported to exhibit protein kinase inhibitory properties, as well as a moderate cytotoxicity toward human tumor cell lines. Meridianins B and E are the most potent inhibitors (Hernández Franco et al. 1998; Gompel et al. 2004; Seldes et al. 2007). The interesting bioactivities found in meridianins make these compounds a promising scaffold for pharmacological anticancer research (Gompel et al. 2004). The biological activity of organic products extracted from marine organisms has generated considerable pharmacological interest, but the ecological roles of most of these metabolites remain unclear and much experimental research is still needed (Fenical 2007; Avila et al. 2008; Taboada et al. 2010). To date, more than 18,000 compounds have been reported from marine sources; however, only about 300 marine natural products originate from organisms collected in Antarctic habitats (MarineLit Database; Munro and Blunt 2009; Lebar et al. 2007; Avila et al. 2008). Thus, cold-water marine habitats represent a source of natural products that has yet to be fully explored.

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