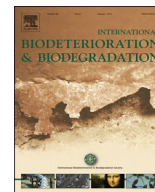


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Effect of secochiliolide acid isolated from the Patagonian shrub *Nardophyllum bryoides* as active component in antifouling paints



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ABSTRACT

Environmental concerns about the use of toxic antifoulants have led to an increased interest in the development of new alternatives. So far, most of the antifouling natural products have been obtained from marine organisms. However, some secondary metabolites from terrestrial plants could be promising antifoulant candidates. The antifouling performance of secochiliolide acid, the main component isolated from *Nardophyllum bryoides* ethanolic extract, was evaluated for inclusion in rosin-based coatings.

Field testing was conducted during the summer months at Mar del Plata harbor, Argentina. The results indicated that secochiliolide acid-based paints completely inhibited the settlement of *Bugula neritina* colonies, *Polydora* sp., *Hydroides elegans*, *Corophium* sp. and solitary ascidians, and also reduced the attachment of some algae as *Enteromorpha intestinalis* and *Ectocarpus* sp. In addition, a lower density and diversity of microfouling species was registered.

These results highlighted the importance of terrestrial plants as a sustainable source of potential environmentally friendly antifoulants.

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1. Introduction

Marine biofouling of ship hulls is an age-old problem, since the surface condition of the hull is of primary importance in the performance of marine vessels. It is well established that biofouling on ships increases the surface roughness of the hull which in turn causes additional frictional resistance, reduces maneuverability and efficiency, increases fuel consumption and decreases top speed (Lewthwaite et al., 1985; Leer-Andersen and Larsson, 2003; Schultz, 2007). These combined factors result in increased fuel costs and a higher frequency of dry-docking with associated economic losses (Schultz et al., 2011). There are also possible ecological

consequences of biofouling due to the inadvertent introduction of invasive foreign species (Minchin and Gollasch, 2003; Floerl et al., 2004, 2005; Lejars et al., 2012). Many submerged structures are consequently protected by biocidal, antifouling coatings in order to minimize the effects due to the colonization of micro and macro-organisms (Evans, 1999).

Since the use of biocides in antifouling paints (in particular organotin) is becoming increasingly restricted, a significant research effort has been focused on the development of environmentally benign technologies to control fouling, of which one of the most promising is the use of non-toxic and potentially biodegradable natural products (Maréchal and Hellio, 2009; Thomas and Brooks, 2010; Blihoghe et al., 2011).

Marine benthic organisms are constantly exposed to colonization by bacterial communities and larvae of fouling organisms. Some of these organisms have developed various strategies to counteract the settlement of fouling organisms, such as the production of antifouling chemicals and/or physical defenses (Tan et al., 2010). To date, several examples of natural products with

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antifouling activity have been isolated from a variety of marine organisms, including marine bacteria, algae, seagrasses, sponges, corals, bryozoans, ascidians, etc. (Clare, 1996; Hellio et al., 2000; Rittschof, 2001; Da Gama et al., 2002; Steinberg and de Nys, 2002; Faimali et al., 2003; Angarano et al., 2007; Tsoukatou et al., 2007; Sjögren et al., 2008; Feng et al., 2009; Raveendran and Limna Mol, 2009; Villa et al., 2010; Qian and Xu, 2012; Nguyen et al., 2013). In fact, many of the natural products that have been discovered from marine organisms on the basis of their pharmacological activity may play an ecological role for their source species in the marine environment, in many cases acting as natural antifoulants. However, the production of these bioactive substances from marine sources on a large scale is a big challenge for the antifouling technology, because, to date, most of these metabolites have been isolated in low yields from delicate and slow-growing marine organisms such as corals, sponges and other invertebrates which cannot be harvested on a commercial scale without environmental harm (Rittschof, 2001). Mariculture of these marine invertebrates is not an easy task, and under different environmental conditions the production of bioactive metabolites may vary substantially. All these issues pose an almost unsolvable sustainability problem for the large-scale production of natural antifoulants of marine origin.

For this reason, an additional effort has to be made in the search of natural antifoulants from more sustainable resources such as abundant and easy collectable terrestrial plants. Comparatively little attention has been given to terrestrial plants in the search for natural products which may act as antifoulants, and only a few antifouling compounds have been reported from these sources (Yamashita et al., 1989; Hyodo et al., 1992; Sawant and Wagh, 1994; Sawant et al., 1995; Göransson et al., 2004; Angarano et al., 2007; Pérez et al., 2007; Chen et al., 2008; Zhou et al., 2009; Ovesen et al., 2011). Plant natural products from abundant and widely distributed species represent an attractive and sustainable source of new bioactive compounds. Terrestrial plants produce secondary metabolites that exhibit a variety of biological activities, many of which are of considerable significance to humans (Sánchez et al., 2010). It is worth noting that some plant extracts have shown antifouling activity, e.g. *Quercus dentata* (Yamashita et al., 1989), *Xanthium strumarium* (Harada et al., 1985), *Eucalyptus resinifera* (Hyodo et al., 1992), *Eucalyptus grandis* (Singh et al., 1996), *Eucalyptus rubida* (Yamashita et al., 1986) and *Zingiber officinale* (Etoh et al., 2002) on mussel byssal thread formation, *Acacia pennata* and *Barringtonia acutangula* on some diatoms and invertebrates (Sawant and Wagh, 1997), *Schinopsis* sp. tannin and tannate (Stupak et al., 2003; Pérez et al., 2007; Blustein et al., 2009) and some Chinese herbs on barnacle settlement (Feng et al., 2009). Additionally, some common plants as *Capsicum* sp. (pepper), *Allium* sp. (onion) and *Derris scandens* (hog creeper) restrain the attachment of cirripede larvae and bacteria or inhibit their growth (Sawant et al., 1995; Xu et al., 2005a,b; Lin et al., 2009). This is no surprise, considering the abundance of antibiotic or cytotoxic secondary metabolites in plant extracts that could affect either biofilm formation as the settlement of larvae.

In this context, *Nardophyllum bryoides* (*Chiliotricum* group, Asteraceae), a widely distributed shrub in Argentinean and Chilean Patagonia and the Andes (Jakupovic et al., 1986; Bonifacino, 2005) was selected to evaluate its possible antifouling activity. In previous studies, some metabolites isolated from this species have shown moderate cytotoxic activity against human pancreatic adenocarcinoma cell lines (Sánchez et al., 2010) and strong trypanocidal effect (Silless et al., 2013). Also, the extracts from another species of this genus, *Nardophyllum armatum*, showed antioxidant, antibacterial, antirheumatic and antifungal activity. In addition, digestive,

antitussive and febrifuge properties have been reported for this species (D'Almeida et al., 2007; Barboza et al., 2009; D'Almeida et al., 2011).

The crude ethanolic extract of *N. bryoides* was included in the formulation of a soluble-matrix antifouling paint, which was tested in field trials in Mar del Plata harbor. In this type of paints, water diffusion within the matrix could dissolve any water-soluble components and lead to (i) the diffusion of active species out of the coating (release) and/or (ii) the dissolution of the soluble-matrix paint by the slightly alkaline pH of seawater (erosion). These two mechanisms lead to the release of bioactive species and the renewal of the surface, respectively (Lejars et al., 2012). The release rate of insoluble active molecules from soluble-matrix paints is often controlled by the erosion rate of the immersed coating. The promising results obtained with the paint containing the crude extract of *N. bryoides* led us to also test an enriched fraction of this extract, and finally to the identification of secochilolide acid (**1**), the main component of the extract, as a promising antifouling substance.

2. Material and methods

2.1. General experimental procedures for extraction and isolation

Solvents were distilled for chromatography. NMR spectra were recorded on Bruker AC-200 (200.13 MHz) and Bruker Avance II (500.13 MHz) spectrometers, using the signals of residual non-deuterated solvents as an internal reference. All 2D NMR experiments (COSY, DEPT-HSQC, HMBC, and NOESY) were performed using standard pulse sequences. HRMS were acquired on a Bruker micrOTOF-Q II spectrometer. TLC was carried out on Merck Silicagel 60 F254 plates. TLC plates were sprayed with 2% vanillin in concentrated H₂SO₄. Merck Silicagel (230–400 mesh) was used for column chromatography. Sephadex LH-20 was obtained from Pharmacia Inc.

2.2. Plant material

Specimens of *N. bryoides* were collected at Departamento de Escalante, Province of Chubut (Argentina), in February 2008 (summer). A voucher specimen (HRP6865) was identified by María Elena Arce (Universidad Nacional de la Patagonia San Juan Bosco, Argentina) and was stored at the Herbario Regional Patagónico, Universidad Nacional de la Patagonia San Juan Bosco.

2.3. Extraction, fractionation and compound identification

Ground aerial parts of fresh plant material (1000 g) were extracted exhaustively with ethanol (3 times, 24 h each) at room temperature (20 ± 1 °C), and the extract was evaporated at reduced pressure to yield a syrupy residue (90 g). This residue was partitioned between MeOH: H₂O (9:1) and cyclohexane. The yield of polar subextract was 5.8 g per 100 g of fresh plant material. The polar subextract (NOP) was chosen for the experiments because, by preliminary chromatographic analysis, it had a higher content of secondary metabolites with the presence of some major components. In contrast, the lipophilic subextract (NOL) showed no major components and, by chromatographic and spectroscopic inspection (NMR), was not interesting from a chemical point of view. The polar subextract (NOP) was concentrated to an aqueous suspension and then partitioned between EtOAc and 10% aqueous NaOH. The basic aqueous phase was then acidified (pH = 3) by addition of 2 M HCl, and extracted three times with EtOAc. This last organic phase was evaporated up to dryness, giving an acidic component-enriched fraction (NOPab). Chromatographic and spectroscopic inspection

(NMR) of NOPab indicated the presence of a major terpenoidal compound and a mixture of flavonoids. NOPab was redissolved in a small volume of MeOH and permeated through a Sephadex LH-20 column (2 × 50 cm) using MeOH as eluant. The fractions that contained the major terpenoidal compound were pooled, and finally purified by vacuum flash chromatography on silicagel, to yield 4.32 g of pure compound **1** (4.8% yield from the crude ethanolic extract, 7.5% from NOP) which was identified by spectroscopic analysis (1D and 2D NMR, MS) as secochiliolide acid, which we previously isolated from the same plant.

Secochiliolide acid (1): oil, ESI⁺HRMS(*m/z*): 347.1858 [M + H]⁺ (Calcd. C₂₀H₂₇O₅⁺: 347.1853; err: 0.500 ppm); 364.2126 [M + NH₄]⁺ (Calcd. C₂₀H₃₀NO₅⁺: 364.2118; err: 0.78 ppm); 369.16732 [M + Na]⁺ (Calcd. C₂₀H₂₆NaO₅⁺: 369.1672; err: -2.3 ppm); 715.3457 [2M + Na]⁺ (Calcd. C₄₀H₅₂NaO₁₀⁺: 715.3453; err: 0.41 ppm). ¹H NMR (500 MHz, CDCl₃) δ 7.47 (br t, *J* = 0.6 Hz, H-16), 7.42 (t, *J* = 1.7 Hz, H-15), 6.41 (dd, *J* = 1.7, 0.6 Hz, H-14), 5.37 (dd, *J* = 10.5, 6.0 Hz, H-12), 2.92 (dd, *J* = 11.6, 3.1 Hz, H-10), 2.46 (dd, *J* = 13.0, 6.0 Hz, H-11a), 2.43 (br t, *J* = 5.6 Hz, H-6a), 2.22 (m, H-2), 2.17 (H-6b), 2.12 (H-1a), 2.04 (H1b), 2.02 (H-11b), 1.73 (d, *J* = 1.0 Hz, H-19), 1.72 (H-7a), 1.72 (H-8), 1.71 (d, *J* = 1.8 Hz, H-18), 1.51 (H-7), 1.33 (d, *J* = 6.7 Hz, H-17). ¹³C NMR (125 MHz, CDCl₃) δ 179.6 (C-3), 177.4 (C-20), 144.0 (C-15), 140.2 (C-16), 128.3 (C-5), 128.1 (C-4), 124.4 (C-13), 108.6 (C-14), 70.7 (C-12), 52.1 (C-9), 45.2 (C-11), 41.6 (C-10), 36.4 (C-8), 32.7 (C-2), 29.6 (C-7), 26.0 (C-1), 21.6 (C-6), 20.9 (C-18), 20.6 (C-19), 16.0 (C-17).

2.4. Soluble matrix paints – general procedure

Soluble matrix antifouling paints were prepared by dissolution of rosin (film forming) and oleic acid (plasticizer) in a xylene/white spirit mixture (1:1) using a high-speed disperser. A laboratory scale ball mill was loaded with this mixture ('vehicle') and pigments (zinc oxide and calcium carbonate), and dispersed for 24 h (Table 1). Then, paints were filtered and fractionated in portions, one of which was used as a negative control and the remaining as treatments. Pigment volume concentration (PVC) was 45% for all paints tested. The average particle's size of the pigment was 0.21 μm for zinc oxide (Oxido Metal S.A) and 1.8 μm for calcium carbonate (Camuati S.A.I.C). For treatments, the polar subextract (NOP), the acidic compounds from NOP (NOPab) and the main compound from NOPab (MC) were incorporated in matrix paints. Finally, paints were dispersed during 1 h.

2.5. Field trials

Sandblasted acrylic tiles (4 × 12 cm) were used for field trials. Paints were applied by brush on tiles previously degreased with toluene. Four coats of paint were applied and allowed to dry for 24 h between each application, resulting in a final dry thickness of 75 ± 5 μm. Coated panels were submerged in a marina in Mar del Plata harbor (Argentina) (38°02'30"S-57°32'00"W). Also, paints without the addition of natural extracts and unpainted acrylic tiles were simultaneously submerged. All tests were performed in triplicate.

Table 1
Base paint composition.

Components	% V/V
Zinc oxide	16.2
Calcium carbonate	10.8
Rosin	27.0
Oleic acid	6.0
Xylene/white spirit (1:1)	40.0

Abundance percentages for each species of fouling settled on panels were estimated with a grid after 45 days exposure in the sea (January/February). Settlement of micro and macrofouling organisms was estimated as percentage cover on each plate using a dot-grid estimate method (Foster et al., 1991). In this latitude, the fouling community is characterized by a marked seasonal pattern, with heavy species recruitment during summer months (December–March). For this reason, experiments were carried out in two consecutive years, starting with the study of the activity of the polar extract in the first year, and of fractions and the major component during the second year. Thus, the treatments tested during the first year were: unpainted control panels (T1), painted control panels (paint without antifouling compounds) (T2) and painted panels with 1% Wt NOP (T3). For the second year it were evaluated the following treatments: unpainted control panels (T4), painted control panels (paint without antifouling compounds) (T5), painted panels with 1% Wt NOPab (T6) and painted panels with 0.03% Wt MC (T7).

2.6. Statistical analysis

All statistical analyses were performed with SPSS software. The normality assumption was verified with the Shapiro–Wilk's test (Shapiro and Wilk, 1965) and variance homogeneity with Levene's test. The differences between treatments and control were determined by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Differences were considered to be significant at *p* < 0.05.

3. Results

The main compound (MC) found in NOPab was the diterpene secochiliolide acid (Fig. 1). Several flavonoids were detected in NOPab as minor components, while some previously identified pentacyclic triterpenes remained in the minor neutral residual fraction of NOP, obtained during the original basic extraction.

After an exposure period in Mar el Plata harbor, paints containing NOP, NOPab and MC showed strong antifouling effect on conspicuous species of the fouling community (Figs. 2 and 3).

During the first year of experiments the macrofouling community on both unpainted control and negative control panels (T1 and T2) was dominated by colonial ascidians (*Botryllus* spp.), bryozoans (mainly *Bugula neritina* and *Bowerbankia* sp.), mud-builder tubes *Corophium* sp. and algae (*Enteromorpha intestinalis* and *Ectocarpus* sp.). ANOVA test revealed that there were significant differences between treated and control panels (*p* < 0.05). Paints containing NOP (T3) caused a reduction in the amount of fouling and also in species diversity. These paints completely inhibited the settlement of *B. neritina* and tube building species as *Spirorbis* sp. and *Polydora* sp. and reduced significantly the attachment of *E. intestinalis*, *Botryllus* sp. and *Corophium* sp. (Fig. 4). On the other hand, microfouling was also affected by the extract, particularly the settlement of *Melosira* sp. and *Grammatophora* sp. In contrast, no differences between treated and control panels was observed in the settlement of *Nitzschia longissima* (Fig. 5).

During the second year of experiments NOPab-paints and MC-paints were simultaneously studied, and after immersion time in the sea, both soluble matrix paints exhibited low fouling attachment. Statistical analysis showed significant differences between treatments and controls (T4 and T5) (*p* < 0.005) although there were no differences between NOPab-paints (T6) and MC-paints (T7) (Fig. 2).

The antifouling activity of T6 and T7 was potent against the main fouling organisms. *B. neritina* colonies, *Polydora* sp., *Hydroides*

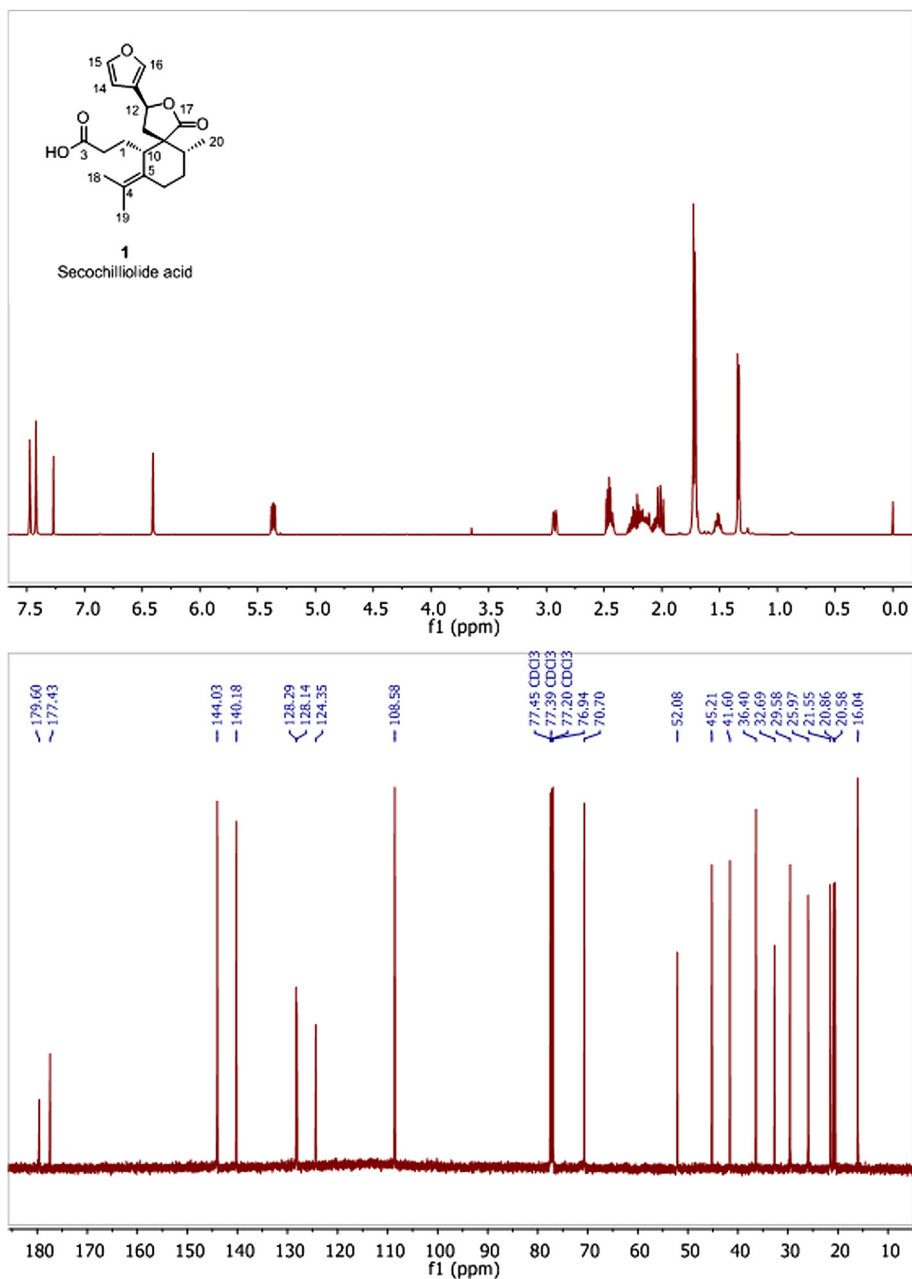


Fig. 1. Secochillilide acid structure, ¹H NMR (CDCl₃, 500 MHz) and ¹³C NMR (CDCl₃, 125 MHz) spectra.

elegans, *Corophium* sp., while solitary ascidians were completely inhibited by these paints. Also, the settlement of *E. intestinalis* and *Ectocarpus* sp. algae was diminished (Fig. 6).

Both treatments, T6 and T7 had successful performances, albeit some differences in two directions were observed. On the panels coated with NOPab, *Ectocarpus* sp. was more common on plates coated with the secochillilide acid-containing paint and, conversely, colonial ascidians were observed on T7 panels, but were virtually absent on T6 panels (Fig. 6).

Microfouling settlement was characterized by a low density and diversity of organisms. T6 and T7 inhibited several common diatoms as *Nitzschia* sp., *Melosira* sp., *Pleurosigma* sp. and *Thalassiotrix* sp. and significantly reduced *Navicula* sp. abundance. However, *N. longissima* was not affected by these formulations (Fig. 7).

4. Discussion

Problems associated with tin and copper antifouling compounds have highlighted the need to develop new environmentally-friendly antifouling coatings. A wide range of chemicals have been used as antifouling biocides, displaying very different physico-chemical properties and therefore differing environmental fates, behavior and effects. In order to be considered a strong candidate for use as an antifouling agent, the water solubility of a bioactive compound should be low enough to ensure that it will be released from paints at a constant rate. Low water-solubility also ensures a low diffusion rate along the water column, which allows the establishment of a stable, relatively higher concentration of the biocide near the protected surface. It is no surprise then, that most of the wealth of natural antifouling substances produced by

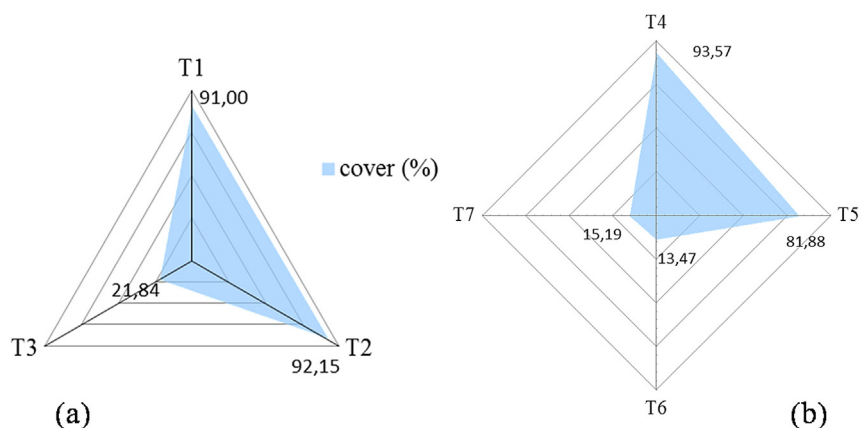


Fig. 2. Total cover percentage on treated and control panels after 45 days exposure in Mar el Plata harbor. (a) first year; T1: unpainted control, T2: control paint, T3: NOP-paint. (b) second year; T4: unpainted control, T5: control paint, T6: NOP-paint, T7: MC-paint.

sponges and other marine invertebrates are compounds with very low water-solubility. For these reasons the search for antifouling natural substances is oriented towards organic extracts of organisms rather than aqueous extracts. The typical isolation process of natural antifouling substances usually begins with the identification of bioactive natural extracts, followed by bioassay-guided fractionation, usually helped by chromatographic and spectroscopic analysis of the fractions until the final isolation and identification of the bioactive compound(s), much the same as in the search for compounds with pharmacological interest.

Based on the previously described problems associated with the sustainability of natural antifoulants of marine origin, the focus of this study was set in the search of a plant extract, or a plant-derived natural product which could be incorporated as active component in an antifouling paint. In this context, recent laboratory experiments employing terrestrial plant extracts detected antimicrobial properties in coconut husk fiber extracts (Viju et al., 2013) and also the production of cyclotides (small proteins) from Rubiaceae and Violaceae with reversible, nontoxic, antisettlement effects on the barnacle *Balanus improvisus* (Göransson et al., 2004; Ovesen et al., 2011).

The focus of this research was a search for a plant extract that could meet some of the following criteria: a) abundant and easy to collect (without ecological harm) natural source, b) chemical

richness, in terms of the presence of major secondary metabolites, and c) if possible, previous bioactivity studies which may be related to a possible antifouling action, such as antibiotic activity or cytotoxicity. Incorporation of such an extract as active component in a marine coating would enable field tests in which the performance of the extract would be simultaneously tested against the whole fouling community of the test site. On the other hand, it will test the potential use as additive of the extract as a whole, without the need of purification steps.

A plant species which met the above mentioned criteria was the Patagonian shrub *Nardophyllum bryoides*. This species grows profusely on the arid Patagonian plateau in Argentina, under harsh conditions (low rain, strong winds, extreme temperature amplitudes), and is relatively easy to collect. The chemical composition of *N. bryoides* has been studied only recently, and the biological and pharmacological activities of its extract and chemical components are just being untapped. This plant produces a large amount of organic extract, which is particularly rich in secochiliolide acid, a very interesting diterpenoid that can be easily isolated in fairly large quantities (2–3 g/kg of fresh aerial parts). This substance has shown moderate cytotoxicity (Sánchez et al., 2010) as well as potent trypanocidal action (Siless et al., 2013). All these factors made the extract of *N. bryoides* an ideal candidate to test for antifouling activity.



Fig. 3. Painted panels after immersion time.

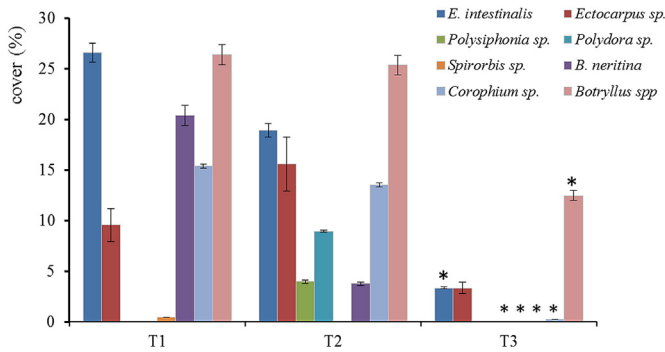


Fig. 4. Macrofouling percentage cover on painted panels vs. controls, 45 days exposure (first year). T1: unpainted control, T2: control paint, T3: NOP-paint. Bars = mean ± SE; *significant differences from controls ($p < 0.05$).

The initial ethanolic extract was partitioned between MeOH:H₂O (9:1) and cyclohexane to yield lipophylic (NOL) and polar (NOP) subextracts. Based on previous chemical investigation of this species, NOL was discarded since it consisted mainly of fats and waxes, and did not contain bioactive components. NOP was incorporated in soluble-matrix paint and tested in the Mar del Plata harbor during the summer period, with promising results. The initial finding of antifouling activity in the polar subextract of this species, prompted us to try a further fractionation to get insight on the nature of the active substances. Plant extracts are complex mixtures of several classes of compounds, with different bioactivities. For example, some of the components may have antibiotic activity, while others can be cytotoxic. For this reason, the activity of a crude extract as a whole may differ from the activity of the obtained fractions, since in the fractionation process, substances are sorted out based on their chemical or structural properties, such as polarity, hydrophobicity, acid-base character, etc.

Previous knowledge on the chemical composition of NOP dictated that an acid-base extraction would be profitable since the main components were acidic. Besides secochiliolide acid, NOP had other classes of compounds such as flavonoids (acidic) and some minor neutral pentacyclic triterpenoids. For this reason, NOP was extracted with aqueous base to yield a major acidic fraction (NOPab) and a very minor neutral fraction. The initial hypothesis that the main, acidic components of the extract were responsible for the observed bioactivity was rewarded, since NOPab retained the antifouling activity initially observed in NOP. The pentacyclic triterpenoids of NOP, which lack acidic groups, were not extracted

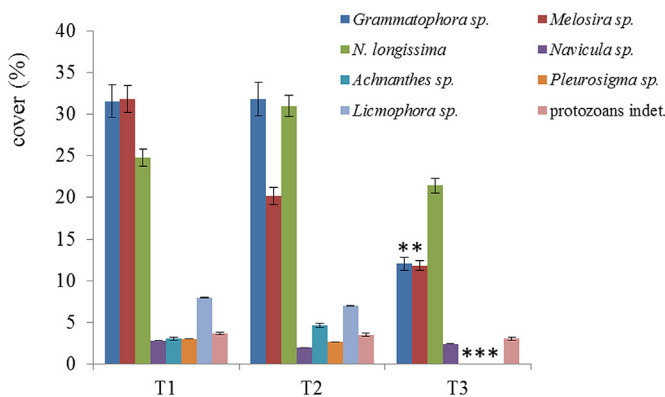


Fig. 5. Microfouling percentage cover on painted panels vs. controls, 45 days exposure (first year). T1: unpainted control, T2: control paint, T3: NOP-paint. Bars = mean ± SE; *significant differences ($p < 0.05$).

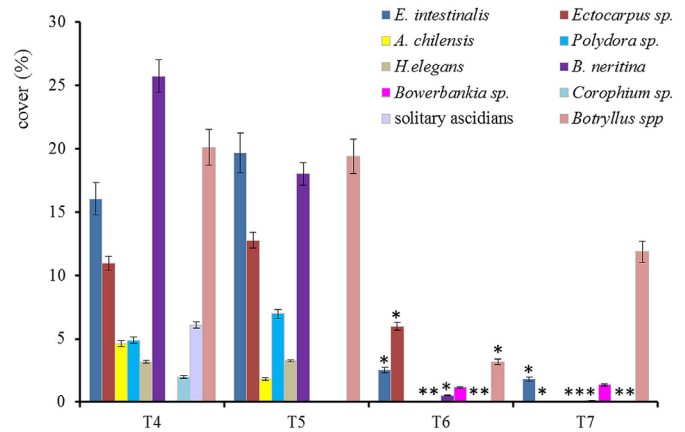


Fig. 6. Macrofouling percentage cover on painted panels vs. controls, 45 days exposure (second year). T4: unpainted control, T5: control paint, T6: NOPab-paint, T7: MC-paint. Bars = mean ± SE; *significant differences ($p < 0.05$).

by the aqueous basic solution and for that reason were not present in NOPab, and obviously played no part in the antifouling activity exhibited by this fraction. Secochiliolide acid (**1**) was purified, incorporated into soluble-matrix paint (MC), and tested in parallel with NOPab paint in the sea. As for the flavonoids present in NOPab, they were not used in the experiments because these compounds are ubiquitous in the plant kingdom, and have well known properties, which include antifouling activity.

In earlier investigations, the antifouling effect of flavonoids on the barnacle *Balanus amphitrite* was confirmed (Zhou et al., 2009), a repellent effect of flavonoids from the bark of *Prunus jamasakura* on the blue mussel *Mytilus edulis* (Takasawa et al., 1990; Yoshioka et al., 1990), and on other fouling organisms (Brango Vanegas, 2011; Gavin and Durako, 2011; Iyapparaj et al., 2012). Our interest was instead focused on secochiliolide acid which is a major and easy-to-purify component of the extract.

The results indicated that inhibition of organism settlement by *N. bryoides* polar subextract (NOP) was due mainly to secochiliolide acid; the present studies add a new potential use to this compound as an antifoulant because it showed inhibition settlement on most conspicuous fouling species at Mar del Plata harbor. Secochiliolide acid demonstrated strong antifouling activity on most of the micro and macrofouling species, except for *Botryllus sp.* However, NOPab coating did inhibit the attachment of this colonial ascidian suggesting that other substances, such as the flavonoids present in this

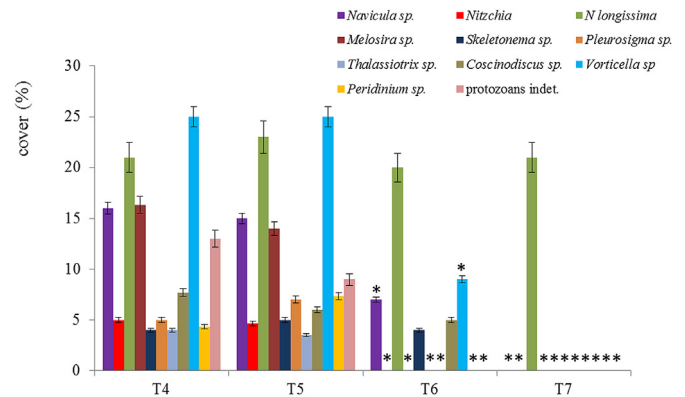


Fig. 7. Microfouling percentage cover on painted panels vs. controls, 45 days exposure (second year). T4: unpainted control, T5: control paint, T6: NOPab-paint, T7: MC-paint. Bars = mean ± SE; *significant differences from controls ($p < 0.05$).

acidic fraction may be responsible of the antifouling effect on this particular species. Also, possible interactions and synergism among different compounds should be considered, taking into account that NOPab is still a mixture of different substances, although less complex than the original subextract.

Secochilolide acid is a diterpenoid that contains an unusual core bearing furan and spiro-lactone fragments. Interestingly, these fragments are common features in some antifouling marine natural products which have been detected in algae, fungi, sponges, and octocorals (Keifer et al., 1986; Clare, 1996, 1998; Clare et al., 1999; Hellio et al., 2005; Shao et al., 2011). For example, furanones inhibit bacterial colonization and biofilm formation through interference with quorum sensing (Steinberg et al., 1997; Lowery et al., 2009). Also, the presence of a furan group deters the settlement and growth of macrofouling organisms and, indirectly affects invertebrate larval attachment by modifying bacterial biofilm density and/or composition (de Nys et al., 2006; Dobretsov et al., 2007; Xu et al., 2010; Li et al., 2013). Furan and lactone rings are also frequent structural motifs in natural products isolated from terrestrial plants and actinomycetes, with properties that may be related to a possible antifouling activity. For example, methyl angolensate, a compound isolated from the Indian plant *Soymida febrifuga*, has the same structural motifs as secochilolide acid, and displayed antibacterial, antifungal and insect antifeedant properties (Samir et al., 2001; Chiruvella et al., 2007). Also, benzofurans, furanocoumarins, furoflavones, furanochalcones and furanoterpenes, all of which share with compound **1** a furan ring moiety, and can be isolated from terrestrial plants, have shown a broad range of biological activities such as antibacterial and antifungal properties (Sardari et al., 2000; DellaGreca et al., 2001; Waridel et al., 2003; Widelski et al., 2009; Alam and Lee, 2011; Kamal et al., 2011; Tran et al., 2012; Casero et al., 2013). In particular, imperatorin, a furanocoumarin, showed high inhibitory activity against larval settlement of *Balanus albicostatus* (Wang et al., 2013). Besides, it is well known that many macrocyclic lactones (macrolides) are commercially current antibiotics. Recently, rosin-based coatings loaded with a macrocyclic lactone (ivermectin) were found to be effective in preventing colonization by barnacles (Pinori et al., 2011).

Consequently, it is possible that the presence of furan and/or lactone moieties in secochilolide acid may be responsible of the displayed antifouling activity. Further experiments will be needed, and suitable derivatives will have to be tested in order to establish the structure-activity relationship of this compound, as well as a determination of the active concentration and other performance and safety parameters. The present work represents the first report of antifouling activity of the organic extract of a Patagonian plant, and an addition of another interesting bioactivity to this fascinating substance. The present results show that, taking into account the availability of the plant source, ease and good yield of purification, and the displayed bioactivity in field trials, secochilolide acid may be a good natural and sustainable candidate for the replacement of toxic compounds in antifouling paints.

Further experimental investigations will be needed to estimate whether secochilolide acid affects non target species and how this substance is degraded in the environment. In addition, assessments of the time for retention of the antifouling properties of secochilolide-based paints will probably have to be made before they can be adopted for its use as efficacious antifouling paints.

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