Evaluation of the antifouling properties of 3-alkylpyridine compounds

Daniela Blihoghe a*, Emiliano Manzo a, Alexandre Villela a, Adele Cutignano a, Gianluca Picariello b, Marco Faimali c* and Angelo Fontana a

aCNR – Istituto di Chimica Biomolecolare (ICB), Via Campi Flegrei 34, 80078 Pozzuoli (NA), Italy; bCNR – Istituto di Scienze dell’Alimentazione (ISA), Via Roma 64, 83100 Avellino, Italy; cCNR – Istituto di Scienze Marine (ISMAR), Via de Marini 6 – IV P, 16149 Genova, Italy

(Received 15 September 2010; final version received 19 November 2010)

One of the most promising alternative technologies to antifouling (AF) biocides based on toxic heavy metals lies in the development of natural eco-friendly biocides. The present study evaluates the AF potential of structurally different compounds containing a 3-alkylpyridine moiety. The products, namely poly 3-alkylpyridinium salts, saraine, and haminols, were either extracted or derived from natural sources (the sponges Haliclona sp. and Reniera sarai and the mollusc Haminoea fusari), or obtained by chemical synthesis. All the molecules tested showed generally good anti-settlement activity against larvae of the barnacle Amphibalanus (= Balanus) amphitrite (EC50 values between 0.19 and 3.61 mg l⁻¹) and low toxicity (LC 50 values ranging from 2.04 to over 100 mg ml⁻¹) with non-target organisms. For the first time, the AF potential of a synthetic monomeric 3-alkylpyridine was demonstrated, suggesting that chemical synthesis is a realistic way to produce large amounts of these compounds for future research and development of environmentally-friendly AF biocides.

Keywords: poly-alkylpyridins; haminols; saraine-1; anti-settlement activity; Amphibalanus (= Balanus) amphitrite; Tigriopus fulvus

Introduction

Biofouling affects all man-made surfaces immersed or in contact with fresh or marine water, such as ships’ hulls, oil-rigs, mariculture cages, pipelines, heat exchangers, and seawater intakes in general. Many submerged structures are consequently protected by biocidal antifouling (AF) coatings in order to minimize the effects due to the colonization of micro- and macro-organisms (Evans 1999). Recently, environmental issues caused by metal-based AF coatings (in particular organotins) and booster biocides have led to an increased interest in developing non-toxic alternatives. Since marine organisms are able to protect their body surface by means that do not damage the environment (Omae 2003), natural substances with AF properties have received considerable attention in environmentally-friendly AF technologies (Wahl 1989; Abarzua and Jakubowski 1995; Clare 1996; Abarzua et al. 1999; Fusetani 2004; Qian et al. 2010). To date several marine metabolites have shown significant AF activity and potential as natural biocides for AF paints (Hellio et al. 2000; Da Gama et al. 2002; Steinberg and de Nys 2002; Faimali et al. 2003a; Angarano et al. 2007, 2009; Tsokatou et al. 2007; Sjögren et al. 2008; Feng et al. 2009; Zhou et al. 2009a,b; Villa et al. 2010). However, it must be acknowledged that, although substantial progress has been made in identifying novel AF candidates, further endeavors are still needed to explore the potential of molecules for marine applications. Issues related to accessing large amounts of these compounds for evaluating their toxicity and environmental impact have already caused considerable delays.

In a recent review, Qian et al. (2010) discussed the perspectives in the field of natural AF biocides and recommended the selection of non-toxic candidates based on a LC50/EC50 ratio > 50 and an EC50 < 5 μg ml⁻¹ against both hard and soft foulers. Compounds which are more toxic should also be considered if they rapidly degrade in the environment. Furthermore, the development of a natural compound into a commercial product also necessitates compliance with environment safety issues (Thomas and Brooks 2010). This requires long and costly studies to fulfill the obligations of national and international regulations, such as the European Biocidal Products Directive (98/8/EC) and CLP Regulation (EC) 1272/2008, or to allow registration for use in North America (by the US EPA and Health Canada), Australia and New Zealand (Thomas and Brooks 2010).

*Corresponding authors. Email: dblihoghe@icb.cnr.it; mfaimali@ismar.cnr.it
Published online 16 December 2010

ISSN 0892-7014 print/ISSN 1029-2454 online
© 2011 Taylor & Francis
DOI: 10.1080/08927014.2010.542587
http://www.informaworld.com
Compounds containing one or more 3-alkylpyridine or 3-alklypyridinium (hereafter 3-AP) units form a family of biosynthetically-related metabolites found in marine molluscs of the order Cephalaspidea (Spinella et al. 1993a,b; Cutignano et al. 2003, 2004; Fontana 2006) and in sponges of the order Haplosclerida (Sepcíc and Turk 2006; Turk et al. 2008). Some members of the family, namely polymeric alklypyridinium salts (hereafter poly 3-APS) with molecular weights (MW) of 5520 and 18,900 Da (29 and 99-100 3-octlypyridinium units) (Sepcíc and Turk 1997b), showed promising AF activity due to inhibition of settlement of macrofouling organisms at a concentration (EC50 = 0.27 µg ml⁻¹) comparable with currently used biocides, such as copper pyrithione (EC50 = 0.01 µg ml⁻¹) or zinc pyrithione (EC50 = 0.02 µg ml⁻¹), but which exhibited lower toxicity against non-target organisms (Faimali et al. 2003a).

Poly 3-APS have also been reported to exhibit antibacterial activity toward marine bacteria (Chelossi et al. 2006), growth inhibition of marine and freshwater algae (Eleršek et al. 2008), prevention of biofilm formation (Garaventa et al. 2003), inhibition of acetylcholinesterase (AChE) (Sepcíc et al. 1997b, 1998, 1999) and cytotoxic activity (Sepcíc et al. 1997a; Paleari et al. 2006). Interestingly, other members of the 3-AP family do not seem to possess comparable AF properties although they share common chemical traits that arise from the origin of these molecules from related biosynthetic pathways that, in the case of molluscs of the genus Haminoea, are first dependent on polyketide assembly that uses nicotinic acid as the starter unit (Cutignano et al. 2003, 2004).

Here, the AF potential of compounds containing one or more 3-AP moieties (Figure 1) has been assessed by both anti-settlement activity against cyprids and toxicity against nauplii of the barnacle Amphibalanus (=Balanus) amphitrite and non-target (the copepod T. fulvus) organisms. The compounds tested in this study include a mixture of poly 3-APS (1) newly isolated from an Antarctic sponge of the genus Haliclona sp., together with sarain-1 (2) and haminols (3–6), which have been obtained from natural sources (Haminoea fusari) or by chemical synthesis.

**Materials and methods**

**Biological materials**

Haliclona sp. was collected by dredging at a depth of 12–20 m off the Italian Station ‘Mario Zucchelli’ in Terra Nova Bay (Ross Sea, Antarctica) in January 2004. The material was stored in methanol at 4°C until extraction. The sponge was identified by Dr Conxita Avila, University of Barcelona, Spain. R. sarai was collected by scuba diving off Sorrento (Naples, Italy) in May 2006. H. fusari (16 specimens) was collected manually from Lake Fusaro (Naples, Italy) at a depth of 1.5–3.0 m in May 2008.

**Chemical experimental procedures**

**Equipment and reagents**

The mass spectrometry (MS) analyses was carried out by a qTOF micro™ spectrometer (Micromass Ltd, UK) equipped with a ESI source operating on positive-ion mode (capillary 3500 V, sample cone 30 V, extraction cone 2 V) and by a MALDI-TOF Voyager DE-PRO (PerSeptive BioSystems, Framingham, MA, USA) operating in the positive linear ion mode in the range 1000–10,000 Da. However, the m/z range up to 30,000 Da was explored too. Samples (1 µl) were co-crystallized on the target with 1 µl of 2,5-hydroxybenzoic acid (DHB) or α-cyano-4-hydroxycinnamic acid (α-CHCA), both dissolved in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid (10 mg ml⁻¹), used as the matrices. External mass calibration was performed with low and intermediate molecular mass peptide standard kits (PerSeptive BioSystems). NMR spectra were acquired by AMX400 and Avance 600 MHz spectrometers operating at 400 and 600 MHz (Bruker). HPLC purifications were carried out by a Gilson apparatus equipped with Spectra SYSTEM UV 2000 detector (Thermo Finnigan). Chemical reagents for organic synthesis were obtained from Aldrich (Milano, Italy).
Isolation and structure determination of natural 3-AP compounds

Extraction of the Antarctic sponge (25.8 g dry weight) was performed by repeated grinding with methanol followed by sonication (5 min, 50 Watt, 10 KHz). The extract was filtered through paper, which retained residual sponge tissue. After removal of organic solvent at reduced pressure, the concentrated filtrates were recovered in methanol-water 1:1 and partitioned in succession between hexane, diethyl ether and n-butanol. The methanol-soluble part of the butanolic extract was dissolved in water and filtered twice through Centricon-3 (Amicon, Inc.) with 3000 MW cut-off. Retentates contained pure poly 3-APS (1).

Sarain-1 (2, 6 mg) and haminol-2 (3, 1.3 mg) and -4 (4, 1.8 mg) were purified according to the literature (Cimino et al. 1986, 1989; Spinella et al. 1993b; Cutignano et al. 2007).

Synthesis of haminols

Haminol-A (5, 98 mg) and -B (6, 27 mg) were synthesized according to a slightly modified procedure previously reported by Alvarez and de Lera (1998). Intermediate and final compounds were analyzed by NMR and MS (See Supplementary material [Supplementary material is available via a multimedia link on the online article webpage]).

A. amphitrite bioassays

Settlement inhibition

Adult specimens of the barnacle A. amphitrite were collected in Genoa harbor (Italy) in May 2008. Larval rearing and bioassays followed the methods described by Faimali et al. (2005). Cyprid anti-settlement bioassays were generally performed with scalar concentrations of compounds from 0.001 to 100 μg ml⁻¹. Compounds were dissolved in methanol and transferred to experimental plates followed by evaporation. Nauplius stage II larvae were added (n = 15 ± 5) to each well with 2 ml of FNSW (0.22 μm) at 37% salinity. Controls were performed with methanol only, followed by evaporation. The plates were incubated for 48 h at 20 ± 1°C in the dark. The bioassays were performed using three to five replicates. The number of dead larvae was counted under a microscope after 24 and 48 h and the LC50 was determined using Probit software. The therapeutic ratio (TR), defined as LC50/EC50 (Vitalina et al. 1991; Rittschof et al. 1994; Clare et al. 1999) was calculated using mortality and settlement inhibition values. The aim of TR is to determine whether the mechanism of settlement inhibition was based on a toxic effect.

Naupliar toxicity

Nauplii II of A. amphitrite were used in both mortality and swimming speed alteration (acute and sub-lethal responses) assays, following the methods described in Faimali et al. (2003a, 2005, 2006). The extract was dissolved in methanol and transferred to experimental plates followed by evaporation. Nauplius stage II larvae were added (n = 15 ± 5) to each well with 2 ml of FNSW (0.22 μm) at 37% salinity. Controls were performed with methanol only, followed by evaporation. The plates were incubated for 48 h at 20 ± 1°C in the dark. The bioassays were performed using three to five replicates. The number of dead larvae was counted under a microscope after 24 and 48 h and the LC50 was determined using Probit software. The therapeutic ratio (TR), defined as LC50/EC50 (Vitalina et al. 1991; Rittschof et al. 1994; Clare et al. 1999) was calculated using mortality and settlement inhibition values. The aim of TR is to determine whether the mechanism of settlement inhibition was based on a toxic effect.

Swimming speed alteration

Changes of swimming speed of A. amphitrite nauplii II were registered by video-graphic techniques (SBR System) to highlight the sub-lethal levels of toxicity in invertebrate larvae (Garaventa et al. 2010). IC50 was determined as the concentration that caused a swimming speed inhibition of 50%, using Probit software.

Tigriopus fulvus mortality assay

T. fulvus specimens collected in coastal micro-environments of the Ligurian Sea have been maintained in a laboratory acclimatized culture for several generations in FNSW at 37 ± 0.5 PSU, 20 ± 0.5°C and fed with algal cells (Tetraselmis suecica) and yeast (Saccharomyces cerevisiae). Toxicity tests were carried out on nauplii according to Pane et al. (2005, 2008).

The assay was performed following the protocol for A. amphitrite nauplii, except for the number of transferred nauplii, which ranged from 8 to 10. Each test was performed using five replicates. The well plates were kept at 20°C, and at 24 and 48 h nauplii were observed under a stereo-microscope to determine the percentage mortality. Living nauplii were easily recognizable as they were swimming, whereas dead nauplii were completely motionless and had an opaque color. LC50 was determined using Probit software.

Inhibition of acetylcholinesterase (AChE) assay

The inhibition of AChE was found to affect A. amphitrite cyprid settlement, with total inhibition of
AChE correlating with total cyprid settlement inhibition (Faimali et al. 2003b). AChE activities were measured using Ellman’s colorimetric method (Ellman et al. 1961). AChE from electric eel, alone or in combination with a test compound, was incubated in the presence of the substrate acetylthiocholine iodide (AcTChI) (29 mg ml\(^{-1}\)) and stained with dithiobis-nitrobenzoic acid (DTNB). The colorimetric reaction was recorded for 10 min at 412 nm in a spectrophotometer (6405 Ultra Violet/Visible, Barloworld Scientific Ltd T/A Jenway, Gransmore Green, UK).

**Statistical methods**

Results are reported as means ± standard error. Data obtained from *A. amphitrite* and *T. fulvus* assays were analyzed by one-way ANOVA, with the concentration of test compound as the fixed factor, followed by post hoc analysis (Dunnett test). The homogeneity of variances was analyzed by the Levene test and normality of data was analyzed by the Kolmogorov–Smirnov test, at a confidence level of 95% prior to the ANOVA test. Statistical calculations were performed with the Statistica\(^\text{®} \) software package, Version 8.

**Results**

**Isolation and characterization of natural compounds 1-4**

The structure poly 3-APS (1) was determined unambiguously by spectroscopic and spectrometric analysis (Figure 2) of pure needle-shaped material obtained by size exclusion chromatography of the MeOH soluble part of the BuOH extract of the Antarctic sponge. The \(^1\)H-NMR spectrum of 1 contained four down-shifted signals typical of a 3-substituted pyridine ring between 8.7 and 7.8 ppm (µg ml\(^{-1}\)), together with the resonances of a saturated alkyl chain (Figure 2A). Within these last signals, those linked to the charged nitrogen atom typically resonated at 4.45 ppm (H\(_2\)-7), whereas the methylene group linked to C-3 of the pyridine ring was observable at 2.75 ppm (H\(_2\)-7'). No methyl groups were present in the spectrum of 1, but integration of the alkyl protons indicated the presence of eight methylenes according to a head-to-tail sequence of unbranched 3-octyl pyridinium units (Davies-Coleman et al. 1993; Sepcić et al. 1997b; Scott et al. 2000). The NMR data were corroborated by ESI qTOF MS/MS analysis that showed three major fragmentation ions at \(m/z\) 190.2, 379.4 and 568.5 assigned to singly charged monomeric (C\(_{13}\)H\(_{20}\)N), dimeric (C\(_{26}\)H\(_{39}\)N\(_2\)) and trimeric (C\(_{39}\)H\(_{58}\)N\(_3\)) structures based on the octyl pyridinium unit (Figure 2C and D). The average size of the molecule was inferred on the basis of MALDI-TOF MS data that showed a main ion cluster around \(m/z\) 3674-3675 using both DHB and x-CHCA matrices (Figure 2B). In MALDI, macromolecular poly-electrolytes almost exclusively produce singly charged ions due to an as yet unknown mechanism (Chang et al. 2007). Sepcić et al. (1997b) hypothesized that the absence of multicharged ions of the alkylpyridinium salts is mostly due to partial neutralization of the positive charges by electrons, whereas Scott et al. (2000) maintained that this is the result of the presence of chloride anions that act as counter-ions under conditions of MS analysis. Following these interpretations of the MALDI data, the poly 3-APS (1) isolated from the Antarctic species of *Haliclona* should consist of an average number of 19 monomeric units according to neutralization by electrons or, alternatively, roughly 16 monomeric units according to the involvement of chloride ions.

**Compound 1**

White fibers (0.4% of sponge dry weight). \(^1\)H-NMR (400 MHz, D\(_2\)O): \(\delta\) 8.62 (1H, br s, H-2), 8.58 (1H, br d, \(J = 5.8\) Hz, H-6), 8.29 (1H, d, \(J = 7.5\) Hz, H-4), 7.85 (1H, m, H-5), 4.45 (2H, t, \(J = 6.5\) Hz, H-7), 2.75 (2H, t, \(J = 6.5\) Hz, H-7'), 1.90 (2H, m, H-8), 1.60 (2H, m, H-8'), 1.20 (8H, m, H-9, 9', 10 and 10'); ESI-MS \(m/z\) 568.5 (C\(_{39}\)H\(_{58}\)N\(_3\)); ESI+ MS/MS spectrum on the fragment at 568.5.

![Figure 2. Structure characterization of poly 3-APS (1) isolated from the Antarctic sponge Haliclona sp. (A) \(^1\)H-NMR spectrum in D\(_2\)O; (B) MALDI-TOF MS spectrum with 2,5-hydroxybenzoyc acid (DHB) as the matrix; (C) ESI- MS spectrum; (D) ESI+ MS/MS spectrum on the fragment at 568.5.](image-url)
in the literature (Cimino et al. 1989; Spinella et al. 1993b; Cutignano et al. 2007).

**Synthesis of haminol-A (5) and -B (6)**

Haminol-A (5) and -B (6) were obtained by chemical synthesis based on the stereocontrolled palladium-catalyzed cross-couplings of 1-alkenyl boronic acids (Figure 3). Unlike the original method proposed by Alvarez and de Lera (1998), the reaction yield was significantly improved by use of the alkylborane II (see Supplementary material for synthetic details [Supplementary material is available via a multimedia link on the online article webpage]). The structure and purity of both compounds were determined by spectroscopic methods.

*Synthetic haminol-A (5; 32% overall yield)*

\[
R_f (\text{Hexane/ethyl acetate 6:4}) = 0.32; [\alpha]_D^{25} = +4.9 (c = 0.2 \text{ in CHCl}_3); ^1H-\text{NMR (400 MHz, CDCl}_3): \delta 1.23 (3H, d, J = 6.1 \text{ Hz, H}_3 \text{-}1), 1.60 (2H, quintet, J = 7.3 \text{ Hz, H}_2 \text{-}9), 2.22-2.11 (3H, m, H-3a, H-2-8), 2.31-2.22 (3H, m, H-3b, H-2-10), 3.83 (1H, m, H-2), 5.56 (1H, dt, J = 14.6, 7.0 Hz, H-4), 5.65 (1H, dt, J = 14.6, 7.0 Hz, H-7), 6.06 (1H, dd, J = 14.6, 10.3 Hz, H-5), 6.11 (1H, dd, J = 14.6, 10.3 Hz, H-6), 6.28 (1H, dt, J = 15.7, 6.8 Hz, H-11), 6.38 (1H, d, J = 15.7 Hz, H-12), 7.26 (1H, m, H-5'), 7.66 (1H, d, J = 7.8 Hz, H-4'), 8.45 (1H, d, J = 2.3 Hz, H-6'), 8.58 (1H, br s, H-2'). ESIMS: m/z 280.1686 (C_{17}H_{23}NO + Na^+).

*Synthetic haminol-B (6; 30% overall yield)*

\[
[\alpha]_D^{25} = -21.2 (c = 0.1 \text{ in CHCl}_3); ^1H-\text{NMR (600 MHz, CDCl}_3): \delta 1.23 (3H, d, J = 6.2 \text{ Hz, H}_3 \text{-}1), 1.60 (2H, quintet, J = 7.2 \text{ Hz, H}_2 \text{-}9), 2.02 (3H, s, COCH}_3), 2.21-2.10 (3H, m, H-3a, H-2-8), 2.41-2.22 (3H, m, H-3b, H-2-10), 4.91 (1H, m, H-2), 5.55 (1H, dt, J = 14.5, 7.0 Hz, H-4), 5.64 (1H, dt, J = 14.5, 7.0 Hz, H-7), 6.09-6.02 (2H, m, H-5, H-6), 6.35 (1H, m, H-11), 6.39 (1H, d, J = 16.0 Hz, H-12), 7.22 (1H, dd, J = 7.7, 4.6 Hz, H-5'), 7.66 (1H, d, J = 7.7 Hz, H-4'). ESIMS: m/z 322.1790 (C_{19}H_{25}NO_2 + Na^+).

Figure 3. Synthetic scheme for obtaining haminol-A (5) and haminol-B (6). Compound I was obtained according to Alvarez and de Lera (1998). Compound II was prepared in situ from (2S) pent-4-yn-2-ol and catecholborane (see Supplementary material [Supplementary material is available via a multimedia link on the online article webpage]).

**Settlement inhibition**

The percentage inhibition of settlement of *A. amphitrite* cypris larvae at different concentrations of compounds 1-6 are provided in Figure 4 and the EC_{50} values in Table 1. Poly 3-APS (1) significantly inhibited...
larval settlement at a concentration slightly above 0.1 μg ml⁻¹ (EC₅₀ 0.19 μg ml⁻¹). Saraine-1 (2) and haminol-2 (3) were also very active in inhibiting larval settlement at the same order of magnitude (EC₂₀ of 0.53 and 0.28 μg ml⁻¹, respectively), whereas haminol-4 (4) showed less potency (EC₂₀ of 2.81 μg ml⁻¹). Both natural haminols (3 and 4) totally inhibited settlement at 10 μg ml⁻¹, and were as potent as haminol-A (5) obtained by chemical synthesis. This last compound (EC₂₀ 2.22 μg ml⁻¹) was more active than the corresponding acetyl derivative haminol-B (6), which showed a significant settlement inhibition starting at concentrations of 5 μg ml⁻¹ (EC₂₀ of 3.6 μg ml⁻¹) and total inhibition only at 50 μg ml⁻¹.

**Naupliar toxicity**

At the range of concentrations 0.1–50 μg ml⁻¹, poly 3-APS (1) and haminol-B (6) showed low toxicity towards larvae of both A. amphitrite and T. fulvus (Figure 4). The calculated LC₅₀ for poly 3-APS (1) was above 100 μg ml⁻¹ and the therapeutic ratio (Tᵣ = LC₅₀/EC₅₀) was above 500 (Table 1).

Saraine-1 (2), haminol-4 (4), and haminol-A (5) showed similar LC₅₀ values (5.5, 5.5 and 8.2 μg ml⁻¹, respectively). Saraine-1 (2) showed a promising therapeutic ratio > 10, whereas haminol-4 (5) showed a lower Tᵣ value (2) but at the same time a lower toxicity to Tigriopus larvae (LC₂₀ 12.9 μg ml⁻¹).

Unfortunately, the small amount of haminol-2 (3) did not allow an evaluation of toxicity to non-target species to be performed. The mortality of A. amphitrite cyprids was recorded during the anti-settlement tests, which showed a LC₅₀ value of 15.5 μg ml⁻¹, and a calculated Tᵣ of 55.5.

Synthetic haminol-B (6) showed low toxicity towards A. amphitrite nauplii II; a concentration of 50 μg ml⁻¹ significantly increased larval mortality, but the calculated LC₅₀ was > 50 μg ml⁻¹, and the Tᵣ value was ~14.

The effect on the swimming speed of A. amphitrite nauplii was measured for poly 3-APS (1), saraine-1 (2), and synthetic haminol-A (5) and -B (6) at concentrations ranging from 0.1 to 50 μg ml⁻¹ (Supplementary material [Supplementary material is available via a multimedia link on the online article webpage]). At concentrations as high as 50 μg ml⁻¹, poly 3-APS (1) and synthetic haminol-B (6) produced a significant decrease in swimming speed, but inhibited motility (IC₅₀ > 50 and 24.2 μg ml⁻¹, respectively; Table 1). At a concentration of 10 μg ml⁻¹, no movement was registered, in accordance with the results from the barnacle naupliar toxicity assays, in which most of the larvae died.

**AChE inhibition**

The effect of Haliclona sp. poly 3-APS (1) on AChE from the electric eel was tested at concentrations between 0.01 and 10 μg ml⁻¹ (Figure 5). Poly 3-APS significantly inhibited AChE activity starting at a concentration of 0.1 μg ml⁻¹, and total (100%) inhibition was registered at 10 μg ml⁻¹.

**Discussion**

Fouling is a complex and dynamic ecological process, involving micro- and macroorganisms from different kingdoms that continuously interact as they colonize surfaces. Barnacles are among the most troublesome macrofouling marine organisms. These arthropods can comprise up to 28% of the macrofouling community (Thomason et al. 1998) and, for this reason, are

| Source/compound | A. amphitrite | T. fulvus |
|-----------------|---------------|-----------|
|                 | EC₂₀         | LC₅₀      | Tᵣ      | IC₅₀ | LC₅₀ |
| Haliclona sp. poly-3APS (1) | 0.19 (0.12–0.29) | >100 | >526 | >50 | >100 |
| R. sarai saraine-1 (2) | 0.53 (0.47–0.59) | 5.52 (4.79–6.36) | 10.41 | 3.4 (2.67–4.33) | 2.04 (1.92–2.17) |
| H. fusari haminol-2 (3) | 0.28 (0.14–0.49) | 15.53 (10.90–22.12) | 55.45 | – | – |
| H. fusari haminol-4 (4) | 2.81 (2.52–3.13) | 5.49 (4.27–7.05) | 1.95 | – | – |
| Synthetic haminol-A (5) | 2.22 (n.c.) | 8.18 (7.18–9.31) | 3.68 | 2.66 (2.36–2.99) | 9.87 (n.c.) |
| Synthetic haminol-B (6) | 3.61 (2.90–4.49) | >50 | >13.85 | 24.23 (21.29–27.58) | – |

EC₂₀, LC₅₀ and IC₅₀ values are expressed as means and confidence interval (95%) in μg ml⁻¹; n.c. represents a non-calculable confidence interval. Tᵣ (therapeutic ratio) is the ratio LC₅₀ nauplii/EC₅₀ cyprids for A. amphitrite assays.

*Data calculated with LC₅₀ cyprids measured in settlement assay.
Figure 5. AChE inhibition in the presence of Haliclona poly-AP (1). Data are reported as means of AChE inhibition percentage ($n = 5$). Haliclona poly-AP inhibited AChE starting from a concentration of 0.1 $\mu$g ml$^{-1}$, with total inhibition observed at 10 $\mu$g ml$^{-1}$.

commonly used as model organisms in anti-macrofouling laboratory bioassays (Briand 2009). In this study, the effect of natural compounds on settlement of larvae of the barnacle $A$. amphitrite was measured. In order to evaluate the general impact of these compounds on the marine environment, acute toxicity and sub-lethal effects were determined on nauplii of the barnacle as well as on a non-target organism, the copepod $T$. fulvus, that belongs to the second largest meiofaunal group in marine sediment (Figure 4). Zooplankton plays an important role in the food web by linking the primary producers with higher trophic levels. For this reason, copepods have been suggested as key bio-indicators for qualitative as well as quantitative analysis of marine and freshwater environments (Green et al. 1996; Miliou et al. 2000; Ferdous and Muktadir 2009). In particular, members of the genus Tigriopus are regarded as suitable model species for acute toxicity assessment of marine pollutants (Lee et al. 2007, 2008; Raisuddin et al. 2007; Pane et al. 2008; Wang and Wang 2010). The genus offers ideal organisms for evaluating the toxicity of sediments, for example of harbors (Pane et al. 2008), where leaching from AF paints is likely to concentrate biocides in high amounts.

All the compounds tested in this study showed promising AF activity ($EC_{50}$ values ranging from 0.19 and 3.61 $\mu$g ml$^{-1}$) and generally low toxicity ($LC_{50}$ between 2.04 and over 100 $\mu$g ml$^{-1}$) (Table 1). Poly 3-APS (1) isolated from an Antarctic sponge totally blocked acetylcholinesterase (AChE) activity (Houssen et al. 2007). Poly 3-APS (1) is an example of harbors (Pane et al. 2008), and CuSO$_4$ ($EC_{50}$ of 0.3 $\mu$g ml$^{-1}$) (Faimali et al. 2003a). By analogy with other studies, the inhibition of cyprid settlement was reversible (data not shown). The activity of poly-APS on cyprid settlement has been explained in terms of the surfactant properties of these molecules (Malovrh et al. 1999) that can prevent adhesion or induce lysis of the fouling organisms, as well as in terms of interference with the metabolism of acetylcholine. AChE activity has been detected in the cement gland and in the sensory setae of the antennules of cyprids. Total inhibition of AChE activity produces total inhibition of larval settlement, suggesting that the neurotransmitter modulates settlement and adhesion of cypris larvae (Faimali et al. 2003a). Poly 3-APS generally modulate AChE activity (Turk et al. 2007). Recently, synthetic analogues of large 3-AP-based polymers with 12- and 8-membered chains have shown potent reversible non-competitive inhibition of AChE activity (Houssen et al. 2010). In line with these results, poly 3-APS (1) isolated from the Antarctic sponge totally blocked acetylcholine stimulation (Figure 5) at a range of concentrations (around 10 $\mu$g ml$^{-1}$) that also produced significant inhibition of larval settlement (12% of average settlement; Figure 4). This evident parallelism corroborates the hypothesis of a non-toxic anti-settlement activity based on alteration of the cholinergic signal system.

The degree of polymerization is a key factor for the biological activities of poly 3-APS compounds (Sepčić and Turk 2006) and, for this reason, several synthetic strategies have explored laboratory preparation of these polymers, generally with the intention of obtaining macromolecules with MW $> 3000$ Da. Nevertheless, the 3-AP family embraces more than 70 structurally different metabolites, including a large number of compounds with low MW (below 1000 Da) (Fontana 2006). Although many of these compounds have promising biological activities that vary with the chemical traits of the molecules, their AF properties have been rarely investigated (for a review see Turk et al. 2008). The characterization of poly APS (1) from
the Antarctic sponge prompted the evaluation of the effects on barnacle settlement of two classes of molecules, sarains and haminols, which have been the subject of numerous chemo-ecological studies in the authors’ laboratory. Sarains are dimeric 3-AP compounds that have been exclusively isolated from the Mediterranean sponge *R. sarai* (Cimino et al. 1986, 1989). Haminols are based on a single AP unit and serve as chemical mediators in intra-specific interactions within mollusces of the genus *Haminoea* (Cimino et al. 1991; Spinella et al. 1993a, b; Marin et al. 1999). Interestingly, AF properties have not been reported for either class of compound, although specimens of *R. sarai* and *H. fusarii* are not fouled in nature.

Saraine-1 (2) showed good results for anti-settlement activity towards *A. amphitrite* cyprids (EC50 0.53 μg ml^-1^) and excellent non-toxic properties (TR 10.41), although it was less effective than poly 3-APS (1). Haminol-2 (3) and -4 (6) also showed good AF activity towards *A. amphitrite*. Haminol-4 (4) possessed high specificity for barnacle larvae, which were killed at a dosage half of that required for the non-target organism *T. fulvus* (LC50 5.5 μg ml^-1^ vs 12.9 μg ml^-1^). Due to the small amount of haminol–2 (3) in *Haminoea*, a rigorous evaluation of toxicity could not be performed, even though a non-toxic mechanism (TR > 55.5) is suggested on the basis of the low mortality of *A. amphitrite* cyprids during the anti-settlement assay.

In comparison with the alkylpyridinium polymers, low MW 3-AP compounds, such as the haminols (3 and 4), possess much simpler structures than those that have been the subject of several laboratory syntheses. In view of the potential application of this class of compounds as natural biocides, the synthetic studies offer the possibility of achieving large quantities of these molecules. Thus, haminol-A (5) and haminol-B (6) were prepared and tested according to the synthetic strategy proposed by Alvarez and de Lera (1998). Both compounds were obtained in high yields (above 30%) through a convergent synthesis based on the independent preparation of two structurally-simple syrtos (Figure 3). Compounds 5 and 6, previously isolated from *Haminoea orteai* (Spinella et al. 1993b), are structural analogs of haminol-4 (4) having the presence of an additional double bond and substitution of the acetyl function with a hydroxy group (Figure 1). These structural changes did not affect the activity against macro-foulers since both synthetic compounds showed inhibition of larval settlement in line with those reported for the natural products (Table 1). The absence of the acetyl group in product 5 led to an apparent increase of toxicity (LC50 ~ 8 μg ml^-1^) but the results with haminol-B (6) confirmed the non-toxic mechanism of action (TR > 13.85) observed with natural product 4, which showed a median lethal concentration (LC50) on *A. amphitrite* larvae above the highest tested concentration (50 μg ml^-1^) and a median sub-lethal concentration (IC50) of 24.2 μg ml^-1^. Except for the anti-microfouling activity reported for untenines, nitroalkyl pyridine members isolated from the Okinawan marine sponge *Callyspongia* sp. (Wang et al. 1996), haminols (3–6) are the first monomeric 3-AP compounds that have shown AF properties.

**Conclusions**

In addition to the well-documented AF activity of poly 3-APS (1), this study showed that 3-AP compounds of lower MW displayed good anti-settlement activity and low toxicity to non-target organisms. In particular, the results with the natural haminol-4 (4) and synthetic haminol-B (6) indicated that the structural motif of these monomeric AP compounds could be a promising model for the development of a non-toxic class of biocides. Except for generic antimicrobial activities (Caprioli et al. 1992; Peltari et al. 2002), haminols do not show any toxicity towards eukaryotic cells or fish at micromolar concentrations. There have been no studies on the stability of these molecules in marine ecosystems, although their function as chemical mediators would suggest a transient persistence (Cimino et al. 1991). Large amounts of haminols are easily produced in a pure form by the simple method of synthesis described in this study. The availability of a diverse array of haminol analogs will lead to improvement of therapeutic ratios of compounds that are potential biocides. Structure-activity studies will improve knowledge of some of the poorly understood mechanisms underlying the development of biofouling.

**Acknowledgements**

DB was supported by a Marie Curie FP6 RTN fellowship from the European Community Program MRTN-CT-2004-512301. The authors thank Dr Maria Grazia Aluigi (University of Genoa, Italy) for help with the inhibition of acetylcholinesterase assay, Dr Guido Villani (CNR-ICB) for *H. fusarii* collection, Mr Antonio Esposito (CNR-ICB) for the work on fractionating and screening the *Haliclona* sp. extract, and Dr Conxita Avila (University of Barcelona, Spain) for identification of the Antarctic sponge. The authors also kindly acknowledge Prof. Micha Ilan (Tel Aviv University) for valuable advice and comments on the manuscript, Ms Naomi Paz (Tel Aviv University) for editorial help, the staff of CNR-ISMAR, especially Dr Veronica Piazza, for the help with barnacle and algal.
rearing, and the staff of CNR-ICB for technical support. Many thanks also to the three anonymous referees for constructive remarks on the manuscript.

References

Abazua S, Jakubowski S. 1995. Biotechnological investigation for the prevention of biofouling.1. Biological and biochemical principles for the prevention of biofouling. Mar Ecol Prog Ser 123:301–312.

Abazua S, Jakubowski S, Eckert S, Fuchs P. 1999. Biotechnological investigation for the prevention of marine biofouling II. Blue-green algae as potential producers of biogenic agents for the growth inhibition of microfouling organisms. Bot Mar 42:459–465.

Alvarez R, de Lera AR. 1998. Synthesis of haminol-A and haminol-B, polyenic alarm pheromones of cephalaspidean molluscs. Tetrahedron: Asymmetry 9:3065–3072.

Angarano MB, McMahon RD, Schetz JA. 2009. Cannabinoids as inhibitors of zebra mussel (Dreissena polymorpha) byssal attachment: a potentially green antifouling technology. Biofouling 25:127–138.

Angarano MB, McMahon RF, Hawkins DL, Schetz JA. 2009. Cannabinoids as inhibitors of zebra mussel (Dreissena polymorpha) byssal attachment: an overview of their diversity. Biofouling 25:297–311.

Caprioli V, Cimino G, De Giulio A, Madaio A, Scognamiglio G, Trivellone E. 1992. Selected biological activities of saraines. Comp Biochem Physiol 103B:293–296.

Chang WC, Huang LC, Wang YS, Peng EP, Chang HC, Hsu NY, Yang WB, Chen CH. 2007. Matrix-assisted laser desorption/ionization (MALDI) mechanism revisited. Anal Chim Acta 582:1–9.

Chelossi E, Mancini I, Sepcic K, Turk T, Faimali M. 2006. Comparative antibacterial activity of polymeric 3-alkylpyridinium salts isolated from the Mediterranean sponge Reniera sarai and their synthetic analogues. Biomol Eng 23:317–323.

Cimino G, De Stefano S, Scognamiglio G, Sodano G, Trivellone E. 1986. Saraines: a new class of alkaloids from the marine sponge Reniera sarai. Bull Soc Chim Belges 95:783.

Cimino G, Passeggio A, Sodano G, Spinella A, Villani G. 1991. Alarm pheromones from the Mediterranean opisthobranch Haminoea navicula. Cell Mol Life Sci 47:61–63.

Cimino G, Puliti R, Scognamiglio G, Spinella A, Trivellone E, Mattia CA, Mazzarella L. 1989. Amazing new alkaloid skeletons from the marine sponge Reniera sarai. Pure Appl Chem 61:535–538.

Clare AS. 1986. Marine natural product antifoulants: status and potential. Biofouling 9:211–229.

Clare AS, Rittschof D, Gerhart DJ, Hooper IR, Bonaventura J. 1999. Antisettlement and narcotic action of analogues of diterpene marine natural product antifoulants from octocorals. Mar Biotechnol 1:427–436.

Cutignano A, Cimino G, Giordano A, d'Ippolito G, Fontana A. 2004. Polypeptide from the Mediterranean mussel Haminoea orbignyana. Tetrahedron Lett 45:2627–2629.
Green AS, Chandler GT, Piegorsch WW. 1996. Life-stage-specific toxicity of sediment-associated chlorpyrifos to a marine, infaunal copepod. Environ Toxicol Chem 15:1182–1188.

Hellio C, Bourgougnon N, Le Gal Y. 2000. Phenoloxidase from Mytilus edulis byssus gland: purification, partial characterization and application for screening products with potential antifouling activities. Biofouling 16:235–244.

Houssen WE, Lu Z, Edrada-Ebel RA, Chatzi C, Tucker SJ, Sepčić K, Turk T, Žovko A, Shen S, Mancini I, et al. 2010. Chemical synthesis and biological activities of 3-alkyl pyridinium polymeric analogues of marine toxins. J Chem Biol 3:113–125.

Lee KW, Raisuddin S, Hwang DS, Park HG, Lee JS. 2007. Acute toxicities of trace metals and common xenobiotics to the marine copepod Tigriopus japonicus: evaluation of its use as a benchmark species for routine ecotoxicology tests in Western Pacific coastal regions. Environ Toxicol 22:532–538.

Lee KW, Raisuddin S, Hwang DS, Park HG, Dahms HU, Ahn IY, Lee JS. 2008. Two-generation toxicity study on the copepod model species Tigriopus japonicus. Chemosphere 72:1359–1365.

Malovrh P, Sepčić K, Turk T, Maček P. 1999. Characterization of haemolytic activity of 3-alkylpyridinium polymers from the marine sponge Reniera sarai. Comp Biochem Physiol 124C:221–226.

Martin A, Alvarez LA, CIMINO G, Spinella A. 1999. Chemical defence in cephalaspidean gastropods: origin, anatomical location and ecological roles. J Molluscan Stud 65:121–131.

Miliou H, Verriopoulos G, Maroulis D, Bouloukos D, Moraitou-Apostolopoulou M. 2000. Influence of life-history adaptations on the fidelity of laboratory bioassays for the impact of heavy metals (Co²⁺ and Cr⁶⁺) on tolerance and population dynamics of Tisbe holothuriae. Mar Pollut Bull 40:352–359.

Onae M. 2003. General aspects of tin-free antifouling paints. Chem Rev 103:3431–3448.

Paleari L, Trombino S, Falugi C, Gallus L, Carlone S, Angelini C, Sepčić K, Turk T, Faimali M, Nokzan DM, et al. 2006. Marine sponge-derived polymeric alkylpyridinium salts as a novel tumor chemotherapeutic target. Int J Oncol 29:1381–1388.

Pane L, Giacco E, Mariotti GL. 2005. Acute and chronic heavy metal bioassay on Tigrigopus fulvus Fischer (Copepoda: Harpacticoida). 13th Symposium PRIMO 13, Pollutant response in marine organisms. Alessandria, Italy. p. 113.

Pane L, Giacco E, Corrà C, Greco G, Mariotti GL, Varisco F, Faimali M. 2008. Ecotoxicological evaluation of harbour sediments using marine organisms from different trophic levels. J Soils Sedim 8:74–79.

Peltari E, Matikainen J, Elo H. 2002. Antimicrobial activity of the marine alkaloids humiloin and pulo’upone and related compounds. Z Naturforsch 57c:548–552.

Qian PY, Xu Y, Fusetani N. 2010. Natural products as antifouling compounds: recent progress and future perspectives. Biofouling 26:223–234.

Raisuddin S, Kwok KWH, Leung KMY, Schlenk D, Lee JS. 2007. The copepod Tigriopus: a promising marine model organism for ecotoxicology and environmental genomics. Aquat Toxicol 83:161–173.
Tsoukatou M, Marechal JP, Hellio C, Novakovic I, Tufegdzie S, Sladic D, Gasic MJ, Clare AS, Vagias C, Roussis V. 2007. Evaluation of the activity of the sponge metabolites avarol and avarone and their synthetic derivatives against fouling micro- and macroorganisms. Molecules 12:1022–1034.

Vitalina S, Avelin S, Rittschof D, Sarojini R, Nagabhushanam R. 1991. Compounds from octocorals that inhibit barnacle settlement: isolation and biological potency. In: Thompson MF, Sorojini R, Nagabhushanam R, editors. Bioactive compounds from marine organisms. New Delhi: Oxford and IBH Publ. Co. p. 331–339.

Villa F, Albanese D, Giussani B, Stewart PS, Daffonchio D, Cappitelli F. 2010. Hindering biofilm formation with zosteric acid. Biofouling 26:739–752.

Wahl M. 1989. Marine epibiosis. 1. Fouling and antifouling – some basic aspects. Mar Ecol Prog Ser 58:175–189.

Wang GYS, Kuramoto M, Daisuke U, Yamada A, Yamaguchi K, Yazawa K. 1996. Three novel antimicrofouling nitroalkyl pyridine alkaloids from the okinawan marine sponge Callyspongia sp. Tetrahedron Lett 37:1813–1816.

Wang M, Wang G. 2010. Oxidative damage effects in the copepod Tigriopus japonicus Mori experimentally exposed to nickel. Ecotoxicology 19:273–284.

Zhou X, Xu Y, Jin C, Qian PY. 2009a. Reversible anti-settlement activity against Amphibalanus (=Balanus) amphitrite, Bugula neritina, and Hydroides elegans by a nontoxic pharmaceutical compound, mizolastine. Biofouling 25:739–747.

Zhou X, Zhang Z, Xu Y, Jin C, He H, Hao X, Qian Pei-Yuan. 2009b. Flavone and isoflavone derivatives of terrestrial plants as larval settlement inhibitors of the barnacle Balanus amphitrite. Biofouling 25:69–76.