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Zebra Mussel Antifouling Activity of the Marine Natural Product Aaptamine and Analogs

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Abstract

Several aaptamine derivatives were selected as potential zebra mussel (*Dreissena polymorpha*) antifoulants because of the noteworthy absence of fouling observed on *Aaptos* sponges. Sponges of the genus *Aaptos* collected in Manado, Indonesia consistently produce aaptamine-type alkaloids. To date, aaptamine and its derivatives have not been carefully evaluated for their antifoulant properties. Structure–activity relationship studies were conducted using several aaptamine derivatives in a zebra mussel antifouling assay. From these data, three analogs have shown significant antifouling activity against zebra mussel attachment. Aaptamine, isoaaptamine, and the demethylated aaptamine compounds used in the zebra mussel assay produced EC_{50} values of 24.2, 11.6, and 18.6 μM, respectively. In addition, neither aaptamine nor isoaaptamine produced a phytotoxic response (as high as 300 μM) toward a nontarget organism, *Lemna pausicostata*, in a 7-day exposure. The use of these aaptamine derivatives from *Aaptos* sp. as potential environmentally benign antifouling alternatives to metal-based paints and preservatives is significant, not only as a possible control of fouling organisms, but also to highlight the ecological importance of these and similar biochemical defenses.

Keywords

Aaptamine; alkaloid; antifouling; biofouling; marine natural product; zebra mussel (*Dreissena polymorpha*)

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Introduction

The search for an effective and benign means to control biofouling accumulation on ship hulls and other artificial surfaces in aquatic environments has reached a level of crisis (Sundberg et al., 1998). Since the early 1970s, primarily organometallic compounds, including toxic metals such as tributyltin (TBT) and tributyltin oxide (TBTO), have been used as antifoulants (AF) (Sundberg et al., 1998; Fusetani, 2004), owing to the lack of alternative replacements. As of the late 1980s, organotin (OT) AF paints have been banned for use on marine vessels in several countries because of elevated OT concentrations in sediments and tissues of organisms found within mooring areas of marinas, harbors, and bays. The gradual discontinued application of OT-based AFs has led to the increased use of organic booster biocides and copper-based coatings (Sundberg et al., 1998). Copper and organic booster biocides blended with copolymers have been used as alternatives to OT AFs for more than a decade (Omae, 2003). Recent data suggest that the copper and organic booster biocide AF coatings currently in use are very cost-effective, but their degradation products are more stable than other biocides and may still pose a potential risk for accumulation in the environment (Omae, 2003; Fusetani, 2004). Copper concentrations have been found in marine sediments (Brady et al., 1994) and soft tissues of mussels (*Mytilus californianus*) that are often used as bio-indicators of marine pollution (Stephenson and Leonard, 1994), suggesting that copper-based coatings, like the OT compounds, pose an unacceptable risk to the marine environment (Sundberg et al., 1998). With these recent findings, the search for natural AF products that are cost-effective and environmentally benign alternatives to the currently utilized AF paints and preservatives has received increasing attention (Diers et al., 2004). New biocides are required to be environmentally safe and to have potent broad-spectrum biocidal activity, shorter half-life in the environment, and higher cost-effectiveness than the currently used biocides (Omae, 2003). Environmentally benign natural products with anesthetic, repellent, and settlement inhibition properties, without being biocidal to nontarget organisms, are desirable as potential AF compounds (Omae, 2003). We have focused our efforts to obtain natural AF compounds from marine organisms with apparent biochemical defense mechanisms against fouling based on our observations in the field. From our previous research with the bromotyrosines (Diers et al., 2004), we learned that they are potent AFs but are not cost effective synthetic targets. This report on the aaptamines represents a more reasonable synthetic target for AF products.

As of December 31, 2003, the EPA added an additional organometallic compound to the list of banned AF products, by canceling the utilization of all wood preservatives containing chromated copper arsenate (CCA), emphasizing the importance of searching for natural benign alternatives. Sponges of the genus *Aaptos* are typically unfouled by macro-organisms and possess distinct morphological and biochemical defense characteristics. The most notable biochemical characteristic is the consistent biosynthesis, in relatively large yields, of aaptamine derivatives by sponges of this genus. The aaptamines are reported to be active in antiviral (Coutinho et al., 2002; Gochfeld et al., 2003) and anticancer (Fedoreev et al., 1988; Longley et al., 1993; Shen et al., 1999) assays, and they also have strong in vitro radical scavenging properties (Takamatsu et al., 2003). They have been shown to block α-

drenoceptor action (Ohizumi et al., 1984) as well as inhibit β-1,3-glucanase (Sova and Fedoreev, 1990), and monoamine oxidase (Ioffina et al., 1990).

Zebra mussels (*Dreissena polymorpha*) are sedentary and epibiotic organisms that are well established biofoulers and were selected as the model organism for our reattachment assays using marine natural products including the aaptamine derivatives reported here (**1** to **5**) (Diers et al., 2004). Zebra mussels (ZMs) are relatively easy to maintain, and although ZMs are a freshwater species, they possess a comparable method of attachment to those of many marine hard-fouling organisms, such as the green mussel (*Perna viridis*) and the barnacles. They attach to almost any hard (organic or inorganic) substrate by a byssus or tuft of byssal threads using an essentially permanent "epoxy-like" polyphenolic glue protein (Morton, 1969; Mackie, 1991; Rzepecki et al., 1991; Rzepecki and Waite, 1993) that strongly resists detachment by wave action and currents (Cope et al., 1997). In bivalve development, the byssus is first used to anchor larvae during metamorphosis, and is retained into adulthood by many marine taxa, including ZMs (Rzepecki and Waite, 1993). Large clusters of ZMs, called druses, may develop, forming very dense structures. In fact, ZMs have the ability to "cement" large rock structures to one another by using their unique method of attachment and colonial nature. This type of attachment makes ZMs a very serious nuisance biofouler to utilities and industry, costing billions of dollars in repair, abatement, and remediation from artificial aquatic structures in which they have attached, including cooling water intakes and ship hulls (Taylor and Zheng, 1997). The intent of our AF work is to evaluate marine natural compounds from unfouled invertebrates and select only the compounds that inhibit the permanent fastening of these biofoulers to artificial structures.

In addition to ZM assays, several compounds from the aaptamine class of marine natural products were tested for toxicity against a nontarget aquatic organism (duckweed). Phytotoxicity assays were conducted using **1** to **3** and the sensitive duckweed (*Lemna pausicostata*) assay. The aaptamines represent a promising class of compounds, and to date, are undescribed AF candidates and the focus of this report.

Materials and Methods

The sponge sample used in this study was collected from reef slopes at a depth of −20 m, from Manado and Derawan Island, Indonesia. In life the sponge forms a dense, tough, tuber-like mass, with a relatively smooth surface that may be pitted and feels like sandpaper to the touch. The live sponge is dark orange with a deep yellow interior that can be oxidized to a deep dark gold color, rendering the preservative a fluorescent dark gold. The skeleton consists of large strongyloxeas in three size categories, disposed radially in the ectosome and throughout the choanosome. The sponge was identified as *Aaptos nigra* Lévi, 1961 (Demospongiae: Hadromerida: Suberitidae), first described from Vietnam. A voucher specimen has been deposited in the Natural History Museum, London (BMNH 2005.2.16.1).

The isolation of aaptamine (**3**) and isoaaptamine (**1**) along with the semisynthetic production of the aaptamine derivatives, including 8,9-demethylaaptamine (**2**), 1-*N*-methylaaptamine (**4**), and 4-*N*-methylaaptamine (**5**) shown in Table 1 are detailed in Bowling's masters thesis

(2005). Confirmation of the structures and purity of each compound were achieved by nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) analysis.

Zebra Mussel Antifouling Assay

Zebra Mussel Source, Care and Handling

All ZMs were maintained using the protocols described by Diers et al. (2004). ZMs were obtained from the Mohawk River, NY, kept in 10-gallon aquaria in a torpor-state prior to the assays for no more than 2 months, with aerated reconstituted water (0.5 mM NaCl, 0.4 mM $CaCl_2$, 0.2 mM $MgSO_4$, 0.2 mM $NaHCO_3$, 0.05 mM KCl) (Dietz et al., 1997; Diers et al., 2004), at 7°C in the laboratory prior to testing. ZMs 5 to 10 mm in length were carefully separated from polyvinyl chloride (PVC) pipe strips and placed into a 500-ml glass container with aerated reconstituted water and slowly acclimated to test conditions (15 to 18°C) over 48 h. Time intervals and experimental conditions were determined during methods development.

Zebra Mussel Antifouling Assay

Reattachment assays were performed as described by Diers et al. (2004) with several analogs of the aaptamine class of natural products, conducted in a temperature-controlled chamber (15 to 18°C), in 180-ml PYREX® dishes (70 × 50 mm) with 120 ml of reconstituted water. ZMs were carefully separated from the 500-ml glass container and placed on their sides in 180-ml testing dishes, 10 ZMs/compound/concentration, and allowed to reattach over a 48-h period. The aaptamine analogs **1** to **5** were tested in triplicate. All analogs tested were assayed (methanol 30 mM stock) in parallel with solvent controls. Concentrations of 0.0, 0.3, 1.0, 6.0, 10.0, 15.0, 20.0, and 30.0 μM were used for aaptamine analogs **1** to **5**. Mussels not attached after 48-h were placed into clean dishes with fresh reconstituted water for an additional 48-h postexposure. Any ZMs not attached after the final 48-h postexposure were considered poisoned by the test compound.

Statistical Analyses

The percentage of ZMs not attached versus concentration [x] μM for compounds **1**, **2**, and **3** were plotted using the statistical software package Graphpad Prism, and can be found in Figure 2. EC_{50} and LC_{50} values were calculated and data compared between treatment groups using the Newman-Keuls *post-hoc* test with $P < 0.05$ considered statistically significant (Figure 2).

Phytotoxicity Assay

Duckweed (*Lemna pausicostata*) Source, Care, and Handling

Duckweed (*Lemna pausicostata*) were cultured and maintained according to the protocols described by Michael et al. (2004) with the exception that a modified Hoagland's no. 2 basal salt mixture (Sigma H2395) (1.6 g/liter) was used with added iron (1 ml of 1000× FeEDTA solution to 1 liter of Hoagland media). The iron solution (1000×) contained 18.355 g/liter of Fe-EDTA. The pH of the medium was adjusted to 5.5 with 1 N NaOH, and then the reaction mixture was filter sterilized using a 0.2-μm filter and stored in sterile 1-liter bottles. All

duckweed used for this study originated from a single *L. pausicostata* colony (an aggregate of one mother and two daughter fronds) to ensure genetic uniformity, grown in approximately 100 ml of Hoagland's media in sterile baby food jars with vented lids in a Percival Scientific CU-36L5 incubator under continuous light conditions at 26°C.

Duckweed Phytotoxicity Assay

Both screening and replicate assays were conducted using the protocols described by Michael et al. (2004), in sterile, nonpyrogenic polystyrene six-well plates (CoStar 3506, Corning, Inc.). Each well contained 4950 μl of the Hoagland media plus 50 μl of water and the desired compound dissolved in acetone or dimethyl sulfoxide (DMSO), with appropriate solvent controls run in parallel (at a concentration of 100×). The final concentration of the solvent was therefore 1% by volume. Solutions were prepared in 50-ml centrifuge tubes with 14,850 μl of Hoagland solution and then 150 μl of the 100× concentration of the compound was added, vortex-mixed, and aliquots of 5000 μl were pipetted into three wells. A graphic template of the six-well plates was used to randomly separate the triplicates into the plates. The template sheet was used to set up the test template using LemnaTec software.

Each well was inoculated with two three-frond plants of the same age (4 to 5 days old) and approximate size. All active compounds in the ZM assay were selected for analysis in the phytotoxicity assay. Serial dilutions were made with a 30 mM stock for compounds **2** and **3** and 50 mM for compound **1** using acetone or DMSO as the solvent vehicle, with nominal concentrations of 0.1, 0.3, 1.0, 3.0, 10.0, 30.0, 100, and 300 μM for compounds **1**, **2**, and **3** and 500 μM for compound **1**. Plant growth was typically measured at days 0, 2, 3, 4, and 7. The LemnaTec results were saved as HTML files and recorded as frond number, total frond area, and specific color class present. Only total frond area is represented in this study. These changes indicate any chlorotic or necrotic effects.

Statistical Analysis

Statistical analysis was conducted using the protocols described by Michael et al. (2004). HTML data were used to set up spreadsheets that calculated the frond area change over time. Triplicate means were plotted along with the standard deviation using the statistical software program SigmaPlot. Results were plotted as duckweed growth as a percent increase in frond area during the treatment period versus log concentration (μM) and are shown in Figure 3.

Results

Zebra Mussel Assay

General activity of the aaptamine compounds used in this study toward ZM attachment at single high doses is shown in Figure 1. Aaptamine analogs **1**, **2**, and **3** (Figure 2) showed significant antifouling activity toward ZMs with minor toxicity, which produced EC_{50} values of 11.6, 24.2, and 18.6 μM, respectively. Compounds **4** and **5** (Figure 1) were not active in the ZM assay and will not be evaluated further as lead candidates. Compounds **1** to **3** and the active synthetic aaptamine compounds not shown will be evaluated in AF field studies.

Phytotoxicity Assay

Isoaaptamine (**1**) and aaptamine (**3**) (Figure 3A and B) were not phytotoxic to duckweed up to 300 μM (above practical use), whereas **2** (Figure 3C) inhibited growth after 7 days of exposure with IC_{50} values of approximately 200 and 20 μM respectively. Growth inhibition appeared to be more pronounced with time, as the IC_{50} values for **2** were higher after 6 days of exposure than after 7 days. This could be due to bioaccumulation of the compounds.

Discussion

Among the least explored of the planet's chemically defended organisms are the invertebrates, algae, and microbial communities (Pennaka et al., 2004). Marine-derived natural products obtained from these chemically defended organisms show considerable promise as a source for environmentally benign AF compounds. According to our findings, the aaptamine analogs have significant potential to be utilized in practical AF applications. These previously undescribed and novel AF compounds from abundant and unfouled sponges of the genus *Aaptos* are reasonable synthetic targets and may be of microbial origin owing to their identification from samples of unrelated genera (Calcul et al., 2003). The ability to produce compounds from *Aaptos* sponges through microbial fermentation could enhance the cost-effectiveness of these AF compounds for practical use. Overall, the natural products **1** and **3**, and the semi-synthetic product **2** showed the most significant AF activity with minor toxicity and near zero phytotoxicity at concentrations below 100 μM.

Our preliminary structure–activity relationship (SAR) results from the ZM study for this class of aaptamine-related compounds show that the methyl substitution patterns at the heteroatoms of compounds **1** to **5** significantly affect toxicity and antifouling activity. Compounds **1** and **2** are the most active AF compounds and either have R_1 or both R_1 and R_2 hydroxyl positions free (Table 1). On the other hand, **1** shows no toxicity when compared to **2** and **3**, and is clearly due to the location of the methyl at R_3 (Table 1). The importance of having the free hydroxyls for AF activity and the *N*-methyl groups at R_3 and R_4 (Table 1) to reduce toxicity is confirmed by the decreased activity of **1** and the inactivity of compounds **4** and **5**.

Future studies of the aaptamine class of compounds will focus on additional AF paint formulation, optimizing cost-effective synthesis of the bioactive pharmacophore, and the search for a viable microbial source that may produce these types of AF compounds. The use of natural and semisynthetic aaptamine derivatives similar to those from sponges of the genus *Aaptos* as environmentally benign AF alternatives to metal-based paints and preservatives is important, not only as a potential control for biofouling organisms, but may also explain the ecological importance of these and similar biochemical defenses.

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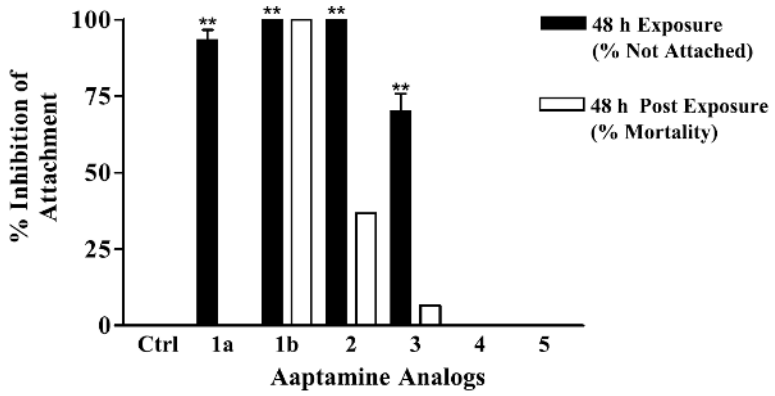


Fig. 1.

Efficacy of the aaptamine analogs at preventing attachment during the 48-h exposure, and mortality in the 48-h postexposure. For the 48-h exposure zebra mussels were exposed to MeOH (Ctrl) or aaptamine analogs (1b at 60 μM and all others at 30 μM). The mean percent inhibition of three replicates ($n = 10$ zebra mussels per replicate) is shown. Mussels that were not attached in the initial 48-h exposure were transferred to clean water and mortality determined by the inability of attachment 48 h later (postexposure). Compounds **4** and **5** (Table 1) showed no effects in the assay. Data were analyzed using one-way ANOVA, Newman-Keuls *post-hoc* test, where $*P < 0.01$ and $**P < 0.001$ were considered significantly different from control (Ctrl).

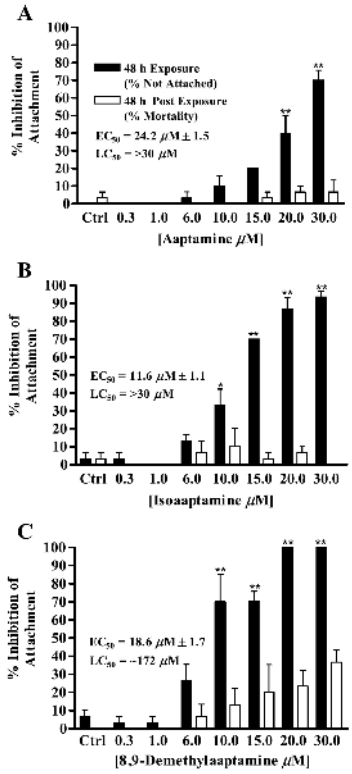


Fig. 2.

Efficacy of select aaptamine analogs to prevent attachment during the 48-h exposure, and mortality in the 48-h postexposure. (**A**) Aaptamine (**3**, Table 1), (**B**) isoaaptamine (**1**, Table 1), and (**C**) 8,9-demethylaaptamine (**2**, Table 1). Zebra mussels were exposed for 48-h to MeOH (Ctrl) or various concentrations of aaptamine analogs to determine inhibition of attachment (black bars). The mean (+ SEM) percent inhibition of three replicates ($n = 10$ zebra mussels per replicate) is shown. Mussels that were not attached in the initial 48-h exposure were transferred to clean water and mortality determined by the inability of attachment 48 h later (white bars). Data were analyzed using one-way ANOVA, Newman-Keuls *post-hoc* test, where $^{*}P < 0.01$ and $^{**}P < 0.001$ were considered significantly different from control (Ctrl).

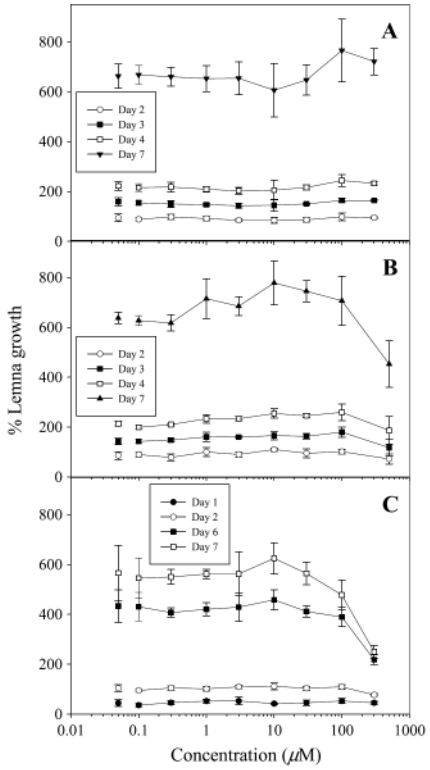
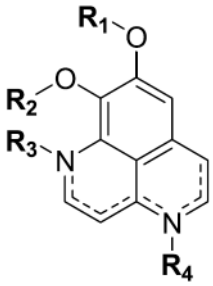


Fig. 3.

Efficacy of select aaptamine analogs on growth of duckweed (*Lemna pausicostata*) expressed as percent increased frond area (*Lemna* growth) after exposure to the compounds. (**A**) Aaptamine (**3**, Table 1), (**B**) isoaaptamine (**1**, Table 1), and (**C**) 8,9-demethylaaptamine (**2**, Table 1). The control values are placed to the left of each graph, unconnected to the dose–response curve. The compounds were dissolved in either acetone (**3**) or DMSO (**1**, **2**) and added to the growth medium. All controls contained 1% acetone or 1% DMSO. The mean percent growth (± SEM) is shown.

Table 1

Structures of Aaptamine Analogs Used in the AF Assays (1–5)



Compound	R_1	R_2	R_3	R_4
(1) Isoaaptamine	CH_3	H	CH_3	—
(2) 8, 9-demethylaaptamine	H	H	H	—
(3) Aaptamine	CH_3	CH_3	H	—
(4) 1-*N*-methylaaptamine	CH_3	CH_3	CH_3	—
(5) 4-*N*-methylaaptamine	CH_3	CH_3	H	CH_3