Structural Activity Relationship Studies of Zebra Mussel Antifouling and Antimicrobial Agents from Verongid Sponges

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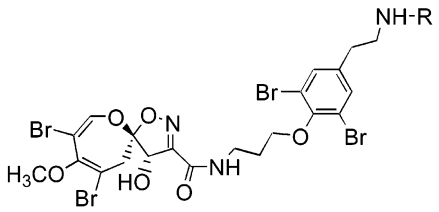
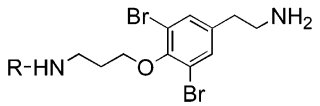
Several dibromotyramine derivatives including moloka'iamine were selected as potential zebra mussel (*Dreissena polymorpha*) antifoulants due to the noteworthy absence of fouling observed on sponges of the order Verongida. Sponges of the order Verongida consistently produce these types of bromotyrosine-derived secondary metabolites. Previously reported antifouling data for the barnacle *Balanus amphitrite* ($EC_{50} = 12.2\ \mu M$) support the results reported here that the compound moloka'iamine may be a potential zebra mussel antifoulant compound ($EC_{50} = 10.4\ \mu M$). The absence of phytotoxic activity of the compound moloka'iamine toward *Lemna pausicostata* and, most importantly, the compound's significant selectivity against macrofouling organisms such as zebra mussels suggest the potential utility of this compound as a naturally derived antifoulant lead.

The search for natural antifouling products that are environmentally benign alternatives to the currently utilized metal-based antifouling paints and preservatives has received increasing attention. We have focused our efforts on marine organisms with apparent biochemical defense mechanisms against fouling based on our observations in the field. As of December 31, 2003, the Environmental Protection Agency (EPA) canceled the utilization of all wood preservatives containing chromated copper arsenate (CCA), emphasizing the importance to search for natural benign alternatives. Sponges of the order Verongida are typically unfouled by macroorganisms and possess distinct morphological and biochemical defense characteristics.[1] The most notable biochemical characteristic of these sponges is the consistent biosynthesis of dibromotyramine derivatives. Many active antibiotic and antifouling compounds identified to date are bromotyrosine derivatives from these Verongid species.[2,3] Moloka'iamine (**5**) is selective against many cell lines and is cytotoxic against P388 leukemia cells with an IC_{50} value of 6.0 μM,[4,5] A-549 lung cells with an IC_{50} value of 28.4 μM,[6] HT-29 colon cells with an IC_{50} value of 14.2 μM,[6] and CV-1 kidney cells with an IC_{50} value of 14.2 μM.[6] However, **5** does not show antifungal or antibacterial activity against *Candida albicans*, *Penicillium chrysogenum*, *Mortierella ramanniana*, *Pseudomonas nautica* (IAM 12929), *Alteromonas macleodii* (IAM 12920), *Vibrio alginolyticus* (ATCC 17749), *Flavobacterium marinotypicum* (ATCC 12960), or *Bacillus marinus* (ATCC 29841) at 10 μg/disk.[4] The selective activity against HIV-1 with an EC_{50} of 52.2 μM without cytotoxicity against human blood mononuclear cells (PBM) shows that **5** also has modest antiviral activity.[7] The dibromotyramine derivative **5** has shown to be a promising antifoulant candidate with significant selective activity against several cancer cell lines and is the focus of this report.

In addition to the antifouling properties of **5**, some of the highly active bromotyrosine derivatives known to date include psammaplysin A (**1**) from the verongid sponge *Psammaplysilla purpurea*.[8] Together with **1**, the bromotyrosine-derived compounds including ceratinamide A (**2**), ceratinamide B (**3**), ceratinamine (**4**), and **5** have been reported from the sponge *Pseudocertina purpurea* collected near Japan.[4,6]



1 R = H
2 R = CHO
3 R = $CO(CH_2)_{11}CH(CH_3)_2$



4 R = COCN
5 R = H

In addition to **5** (Figure 1), several related marine natural products were selected for evaluation in the zebra mussel (*Dreissena polymorpha*) reattachment assay, including fistularin-3 (**6**), hemifistularin-3 (**7**), aerophobin-2 (**8**), 3,5-dibromo-4-*O*-methyl-*N,N,N*-trimethylammonium tyrosine (**9**), 3,5-dibromo-4-*O*-methyl-*N,N,N*-trimethylammonium tyramine (**10**), and dibromotyramine (**12**). These

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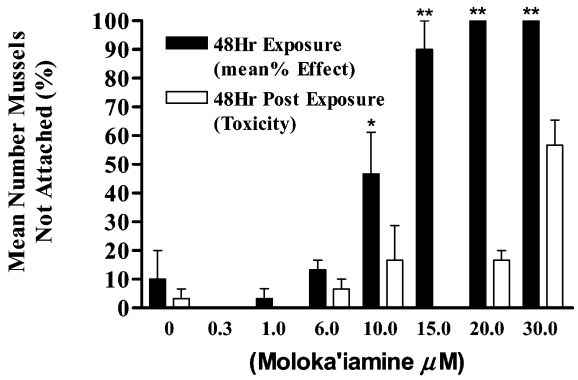
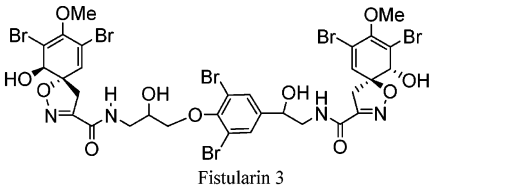


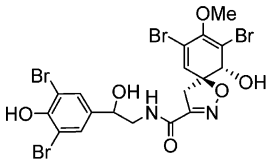
Figure 1. Average number (±1 SE of the mean) of mussels not attached in the 48 h moloka'iamine exposure compared to potential toxicity in the 48 h post-exposure for the three replicates. Methanol was used as the solvent for all compounds and was thus used as the control [0 μM]. There were 10 mussels per concentration per replicate (n = 30). Data were analyzed with the Graphpad Prism software package (Oneway ANOVA, Newman-Keuls *post-hoc* test). Significantly different from the control [0 μM]: *(p < 0.01). **(p < 0.001).

dibromotyramine derivatives were selected to evaluate their structure−activity relationships (SAR).



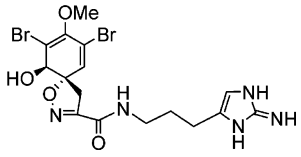
Fistularin 3

6



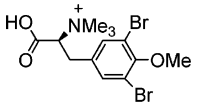
Hemifistularin 3

7



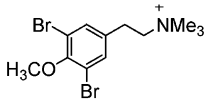
Aerophobin 2

8



3,5-dibromo-4-*O*-methyl-*N,N,N*-trimethylammonium- - tyrosine

9



3,5-dibromo-4-*O*-methyl-*N,N,N*-trimethylammonium -tyramine

10

Compound **5** has been reported to possess a broad spectrum of bioactivity including antiviral,[6] antifouling,[5] and cytotoxic activity.[5,6,9] Antifouling studies of **5**, conducted by Fusetani's lab using *Balanus amphitrite*, proved to be noteworthy, reporting an EC_{50} value of 12.2 μM.[5] The zebra mussel (ZM) reattachment assays reported here yielded a slightly higher antifouling activity, with an EC_{50} value of 10.4 μM (Figure 2) for **5**. Results for the ZM reattachment assay for **5** and relative toxicity at each given concentration are shown in Figure 1. The manner of attachment for these two biofouling species is significantly

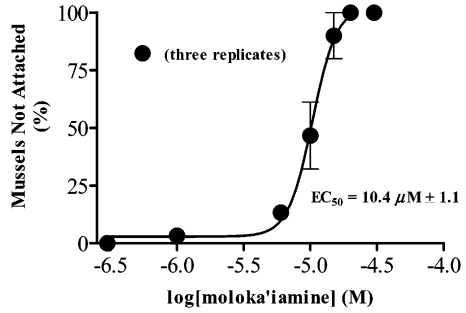
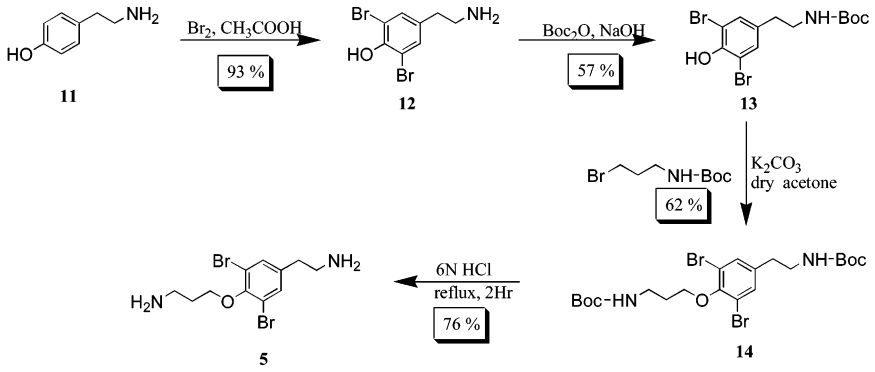


Figure 2. The 48 h reattachment cumulative data from three replicates for moloka'iamine plotted together as one data set. Ten zebra mussels per concentration per replicate (n = 30). Values are means of three replicates ± 1 SE of the mean. Data were analyzed with the Graphpad Prism software package.

different, both in mechanism of attachment and in chemistry of the primary glue proteins; therefore correlation is not possible. Compounds **6**, **8**, **9**, **10**, and **12** showed no antifouling activity against ZMs. Compound **7** had significant activity in a preliminary study against ZM attachment, and we could test only for a single high point concentration at 30 μM due to lack of adequate sample material. No apparent structure−activity relationships can be provided from the dibromotyramine derivatives used in this study. To determine if compound **5** would be toxic to other aquatic organisms during practical applications, we assayed the compound in the aquatic plant *Lemna paucicostata* phytotoxicity assay, widely used by toxicologists. Compound **5** was not phytotoxic to the duck weed *L. pausicostata* at elevated concentrations (up to 200 μM) used in a 7-day dose−response bioassay.[10]

The total synthesis of **5** was achieved in 1998.[3] We completed the gram-scale synthesis of **5** following the published method[3] with slight modifications, i.e., using di-*tert*-butyl dicarbonate (Boc) as the protecting group for the primary amino groups of dibromotyramine and 3-bromopropylamine. The first published synthesis of **5** involved protecting the amino group of dibromotyramine with Bpoc-OC_6H_4(p-CO_2CH_3). The first step in the synthesis of **5** is the bromination of tyramine (**11**) to obtain 3,5-dibromo-4-hydroxy-β-phenethylamine (**12**).[11] We selected the preferential Boc protection[12−14] of the primary amino function of dibromotyramine (**12**), followed by alkylation of the phenolic hydroxyl with Boc-protected 3-bromopropylamine, and finally removal of the Boc groups with 6 N hydrochloric acid, affording **5**. Marine organisms are a rich source of chemically diverse bioactive substances that include the dibromotyrosine derivatives typically isolated from Verongid species.[15] The EPA's decision to discontinue the use of all wood preservatives containing chromated copper arsenate (CCA) stresses the importance of searching for natural, benign alternatives. The use of natural dibromotyramine metabolites from unfouled Verongid sponges as environmentally benign antifouling alternatives to metal-based paints and preservatives is important, not only as a potential control of fouling organisms, but may also explain the ecological importance of these and similar biochemical defenses. Our data suggest that fistularin-3 (**6**), which is inactive, may act as a pro-antifoulant molecule for the formation of the potent hemifisturlarin-3 (**7**), the active form of the molecule. Structure−activity relationships clearly indicate that the bromotyrosine moiety does not in itself elicit bioactivity, as illustrated in **9**, **10**, and **12**, which do not show antifouling activity. Moloka'iamine (**5**) may act by a different mechanism than the compounds presented here, and further studies are needed to address

Scheme 1



these issues. Moloka'iamine (**5**) has been previously characterized as being active as an antifoulant for use with cyprinids of the barnacle *Balanus amphitrite* (12.2 μM),[4,5] but has not been fully described using epibiotic organisms such as ZMs and other aquatic nuisance biofouling species with similar glue homology used for attachment. The results from this study support the significant potential of dibromotyramine derivatives such as moloka'iamine (**5**) as antifoulant candidates for use with the control of aquatic biofouling organisms such as ZMs. Phytotoxicity results show that moloka'iamine does not pose an obvious environmental hazard to aquatic plants. Future studies focused on additional toxicity testing, and optimizing a cost-effective and simple synthesis of the bioactive pharmacophore may lead to the potential application of moloka'iamine (**5**), hemifistularin-3 (**7**), or related dibromotyramine derivatives as antifouling products.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Bruker Avance DRX-400 spectrometer. Carbon multiplicity was established by a DEPT experiment. Chromatographic separations were carried out on Sephadex LH-20 (Pharmacia) from Sigma (USA). TLC was performed on aluminum sheets (silica gel 60 F_{254}, Merck KGaA, Germany).

3,5-Dibromo-4-hydroxy-β-phenethylamine (12). A solution of **11** (0.68 g) in HOAc (5 mL) was treated portionwise with a solution of Br_2 (0.95 g) in HOAc (5 mL) until the color of excess bromine persisted. When the mixture was cooled in an ice bath, a solid precipitated, which was collected and washed with ether to yield (3,5-dibromo-4-hydroxy-β-phenethylamine, **12**) (1.37 g; 93%): ^{1}H NMR (CD_3OD, 400 MHz) δ 7.44 (2H, s, H-2,6), 2.87 (2H, t, J = 7.5 Hz, H_2-8), 2.72 (2H, t, J = 7.5 Hz, H_2-7); ^{13}C NMR (CD_3OD, 100 MHz) δ 149.2 (C-4), 137.3 (C-1), 132.8 (C-2,6), 116.9 (C-3,5), 31.8 (C-7), 39.3 (C-8); HRESIMS *m/z* 293.9135 $[M + H]^+$ (calcd for $C_8H_{10}Br_2NO$, 293.9124).

***N*-(*tert*-Butoxycarbonyl)-3,5-dibromo-4-hydroxy-β-phenethylamine (13).** To a stirred colorless solution of **12** (0.88 g) in 1,4-dioxane/H_2O (8:2, 50 mL) at 0 °C was added NaOH (0.16 g). After 5 min, the ice bath was removed and the mixture was allowed to stir at room temperature for 10 min. The reaction mixture was cooled to 0 °C, and Boc_2O (0.76 g) was added. After 30 min, the ice bath was removed, and a slurry formed within 30 min. The reaction mixture was stirred for an additional 2 h at room temperature and monitored by TLC. The reaction mixture was poured into H_2O (200 mL) and extracted with ether (3 × 75 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give **13** (0.67 g) as an off-white solid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.30 (2H, s, H-2,6), 3.29 (2H, t, J = 6.5 Hz, H_2-8), 2.72 (2H, t, J = 6.5 Hz, H_2-7), 1.41 (9H, s, Boc-Me_3).

Alkylation of 13 with *N*-(*tert*-Butoxycarbonyl)bromopropylamine. A solution of **13** (0.59 g), 0.41 g of K_2CO_3, and 0.40 g of *N*-(*tert*-butoxycarbonyl)bromopropylamine in 40 mL of dry acetone was refluxed for 3 h, and the reaction mixture was concentrated in vacuo. H_2O (100 mL) was added to the reaction mixture and extracted with ether (3 × 50 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give **14** (0.51 g) as a gummy colorless solid: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.30 (2H, s, H-2,6), 4.02 (2H, t, J = 7.5 Hz, H_2-9), 3.01 (2H, t, J = 7.5 Hz, H_2-11), 3.29 (2H, t, J = 6.5 Hz, H_2-8), 2.72 (2H, t, J = 6.5 Hz, H_2-7), 2.06 (2H, m, H_2-10), 1.41 (18H, bs, Boc-Me_6).

Moloka'iamine (5). To a solution of **14** (0.41 g) in 20 mL of MeOH was added 6 N HCl (2 mL), and the mixture was refluxed for 2 h at room temperature. The reaction mixture was neutralized with saturated $NaHCO_3$ solution and concentrated in vacuo. The residue was purified over Sephadex LH-20 gel using MeOH as eluent and yielded 0.21 g of **5** as a colorless solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.43 (2H, s, H-2,6), 4.07 (2H, t, J = 7.5 Hz, H_2-9), 3.05 (2H, t, J = 7.5 Hz, H_2-11), 2.86 (2H, t, J = 7.5 Hz, H_2-8), 2.71 (2H, t, J = 7.5 Hz, H_2-7), 2.06 (2H, m, H_2-10); ^{13}C NMR (CD_3OD, 100 MHz) δ 149.2 (C-4), 137.3 (C-1), 132.8 (C-2,6), 116.9 (C-3,5), 70.7 (C-9), 31.8 (C-7), 39.3 (C-8), 36.1 (C-11), 27.8 (C-10); HRESIMS *m/z* 350.9707 $[M + H]^+$ (calcd for $C_{11}H_{17}Br_2N_2O$, 350.9704).

Zebra Mussel Antifouling Assay. The sedentary and epibiotic zebra mussel (*Dreissena polymorpha*) is a well-established biofouling organism and was selected for the reattachment assays using marine natural products including the dibromotyramine derivatives **5**, **6**−**10**, and **12**. Zebra mussels (ZMs) used in the assays were collected from the Mohawk River, Crescent, NY, by researchers from the New York State History Museum Field Research Laboratory and shipped to the University of Mississippi. All mussels were maintained (not fed) in 10-gallon aquaria 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)[16] at 7 °C in the laboratory prior to testing. ZMs 5−10 mm in length were carefully severed from PVC pipe strips with a razor blade at the byssal threads and placed into a 500 mL glass container with aerated reconstituted water and slowly acclimated to testing conditions (15−18 °C). Reattachment assays were conducted in a temperature-controlled chamber (15−18 °C) in 180 mL Pyrex dishes (70 × 50 mm) with 120 mL of reconstituted water. Selected ZMs were carefully placed on their sides in each 180 mL testing dish, 10 mussels/compound/concentration, and allowed to reattach over a 48 h period. All compounds with the exception of **7** (limited sample) were tested in triplicate with solvent controls assayed in parallel. A blunt probe was used to determine ZM attachment. Mussels not attached after 48 h were placed in clean dishes with fresh reconstituted water for an additional 48 post-exposure. Any ZMs not attached after the final 48 h post-exposure were considered poisoned by the test compound.

Note Added after ASAP Publication: Structure **7** was incorrectly drawn in the version posted on November 19, 2004. The correct structure appears in the version posted on November 29, 2004.

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