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Specialized Metabolite Mediated Predation Defense in the Marine Actinobacterium *Salinispora*

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Abstract

The obligate marine actinobacterial genus *Salinispora* has become a model organism for natural product discovery, yet little is known about the ecological functions of the compounds produced by this taxon. The aims of this study were to assess the effects of live cultures and culture extracts from two *Salinispora* species on invertebrate predators. In choice-based feeding experiments using the bacterivorous nematode *Caenorhabditis elegans*, live cultures of both *Salinispora* species were less preferred than *E. coli*. When given a choice between the two species, *C. elegans* preferred *S. areniolca* over *S. tropica*. Culture extracts from *S. tropica* deterred

C. elegans while those from *S. arenicola* did not, suggesting that compounds produced by *S. tropica* may account for the feeding deterrence. Bioactivity guided isolation linked compounds in the lomaiviticin series to the deterrent activity. Additional assays using the marine polychaete *Ophryotrocha siberti* and marine nematodes further support the deterrent activity of *S. tropica* against potential predators. These results provide evidence that *Salinispora* natural products may function as a defense against predation and that the strategies of predation defense differ between closely related species.

Importance

Bacteria inhabiting marine sediments are subject to predation by bacterivorous eukaryotes. Here we test the hypothesis that sediment-derived bacteria in the genus *Salinispora* produce biologically active natural products that function as a defense against predation. The results reveal that cultures and culture extracts of *S. tropica* deter feeding by *Caenorhabditis elegans* and negatively affect the habitat preference of a marine annelid (*Ophryotrocha siberti*). These activities were linked to the lomaiviticins, a series of cytotoxic compounds produced by *S. tropica*. Microbial natural products that function as a defense against predation represent a poorly understood trait that can influence community structure in marine sediments.

Introduction

Marine bacteria play fundamental roles in nutrient cycling, primary production, bioremediation, and other ecosystem processes (1-3). These roles are particularly pronounced in ocean sediments where bacterial abundances can exceed 10^9 cells per cm^3 (4). Numerous marine organisms have evolved to prey on this relatively abundant resource including viruses,

protozoans, nematodes, and annelids (5-8). In response, marine bacteria have evolved various strategies to avoid predation (9, 10) including morphological adaptations, such as increasing cell size (11), reduced cell surface hydrophobicity (12), and the formation of colonies or biofilms that are relatively impervious to eukaryotic predators (13, 14). The production of natural products that deter predation represents another defense strategy (15). Microbial natural products have been linked to predation defense in terrestrial systems (16-19) and include the production of potent antinematodal compounds by soil Actinobacteria in the genus *Streptomyces* (20, 21). Yet, there are few examples in which the natural products produced by marine bacteria have been linked to predation defense. One notable exception is violacein, which is produced by *Pseudomonas luteoviolacea*, inhibits protist feeding at sub-millimolar concentrations, and can cause nanoflagellate death following the ingestion of 1-2 bacterial cells (22). Mat and bloom-forming cyanobacteria are also known to produce natural products that deter predation, however in these cases the effects were observed on herbivores that feed on the macroscopic growth forms of cyanobacteria (23-25).

The relatively high abundance of bacteria in marine sediments coupled with their spatially structured communities and the diversity of potential predators provide a strong ecological rationale for the production of natural products that function as feeding deterrents. The marine actinobacterial genus *Salinispora* (order Micromonosporales, family Micromonosporaceae) represents a useful candidate to test for predation defense in that it is readily cultured, broadly distributed in marine sediments, and a rich source of biologically active natural products (26). *Salinispora* currently comprises nine species (27) with over 170 different natural product biosynthetic gene clusters (BGCs) identified to date (28). Of these, several are fixed at the species

level (29) suggesting they represent ecotype-defining traits (30). These include BGCs encoding the cytotoxic compounds lomaiviticin and salinisporamide A, which are fixed in *S. tropica*, and the kinase inhibitor staurosporine, which is found in all *S. arenicola* strains examined to date. Recently it was shown that *S. arenicola* and *S. tropica* maintain different competitive strategies, with *S. arenicola* employing interference competition and *S. tropica* exploiting relatively fast growth (31). Evidence that these two closely related species have diverged ecologically provides a mechanism to support their co-occurrence in marine sediments.

Bacterivorous nematodes are common in marine sediments (32) and thus represent a potential target of bacterial predation defense. Although *C. elegans* does not occur in the marine environment, it is highly chemotactic (33) and has been exploited to study nematode responses to toxins and pathogens (34). *C. elegans* exhibits preference for the types of bacteria on which it feeds, suggesting that individuals will migrate among bacteria in search of an optimal food source (35). With their relatively short growth cycle, ease of culturing, and highly developed chemotaxis (33), *C. elegans* is an ideal organism to use in feeding assays. Similarly, various groups of sediment inhabiting marine annelids feed on bacteria (36), thus making them potential targets for microbial predation defense. Despite the ecological rationale, we could find little evidence that natural products produced by marine bacteria have been tested for this functional role.

This study addressed the hypothesis that *Salinispora* natural products function as feeding deterrents targeting bacterivorous eukaryotes. The results of live culture assays revealed that *C. elegans* prefers *S. arenicola* over *S. tropica*. Organic extracts from *S. tropica* deterred *C. elegans* feeding, suggesting the presence of a chemical defense, with the activity subsequently linked to known cytotoxic compounds in the lomaiviticin class. *Salinispora tropica* extracts similarly

deterred a marine annelid and were lethal to marine nematodes providing further support that *S. tropica* natural products function as feeding deterrents.

Results

Caenorhabditis elegans *feeding assay*

C. elegans feeding preference was first tested in a choice assay offering *E. coli*, its normal food source, and either of two *Salinispora* species. In these assays, 10 *S. tropica* or nine *S. arenicola* strains (Table S1) were spot inoculated onto agar plates along with *E. coli. C. elegans* eggs were placed equidistant between the *E. coli* and *Salinispora* colonies and the average percentage of *C. elegans* hatchlings associated with either colony type quantified after 24 h. Six replicate plates were prepared for each *Salinispora* species with spatial associations used as a proxy for feeding preference (initial monitoring over 24, 30, and 36 h revealed no major changes over time). The results revealed a significant preference for *E. coli* (Fig. 1) regardless of the *Salinispora* spp. tested (n=9 for *S. tropica*, n=10 for *S. arenicola*, $p<0.05$ for both, Wilcoxon rank sum test), despite one *S. arenicola* strain (CNS-820) being preferred over *E. coli* (Fig. S1). We next asked if there was a difference in *C. elegans* feeding preference between *S. tropica* and *S. arenicola*. These assays were performed in a similar manner except 10 strains from each of the two *Salinispora* species were randomly paired against each other. In each of two trials comparing the *Salinispora* species, significantly more *C. elegans* were associated with *S. arenicola* colonies (Fig. 1) (n=6 per trial, $p<0.05$ for both trials, Wilcoxon rank sum test). While these assays did not directly measure feeding, consumption was often visibly supported by the presence of orange material characteristic of the *Salinispora* carotenoid sioxanthin (37) in the digestive tracts of *C.*

elegans associating with *Salinispora* colonies.

To determine if the difference in *C. elegans* feeding preference between the two *Salinispora* species was chemically mediated, we repeated the feeding assays using organic extracts derived from *Salinispora* cultures. Since *Salinispora* spp. produce antibiotics and other biologically active compounds that could inhibit *E. coli* growth (26), an autoclaved *E. coli* cell paste to which extracts could be added was developed as an alternative food source. In preliminary studies, this cell paste was visibly consumed and shown to maintain *C. elegans* viability over time. *Salinispora* cultures grown on agar plates were extracted with ethyl acetate and the extracts dried, re-solubilized in 50 µl DMSO, and incorporated into the *E. coli* cell paste at a final concentration of 1 mg/mL based on observed yields of ca. 0.5 mg of crude extract per mL of *Salinispora* agar plate culture. When given the choice between an *E. coli* cell paste containing *S. tropica* culture extracts and extracts of the culture media (controls), *C. elegans* preferred the media controls in three of the four strains tested (Fig. S2). On average, the media controls were preferred over the *S. tropica* extracts (Fig. 2), supporting the hypothesis that metabolites produced by *S. tropica* contribute to the reduced preference of *C. elegans* for this species. Conversely, *C. elegans* preferred extracts from *S. arenicola* over the cell paste controls (Fig. 2), with significant differences detected among three of five strains tested (Fig. S2). The difference from the live culture assays, where *E. coli* was preferred over *S. arenicola* (Fig. 1b), could be due to compounds present in the *S. arenicola* colonies that are missed by the extraction process or changes in the nutritional value of the *E. coli* following autoclaving. A similar trend was observed in the *S. tropica* assays, where differences between treatments and controls were greater for live cultures than culture extracts. Physical characteristics of the live colonies could also contribute

129 to these patterns.

130 Efforts were made to identify the compounds responsible for the feeding deterrence
131 exhibited by the *S. tropica* extracts. To generate a larger extract, the three deterrent *S. tropica*
132 strains (Fig. S2) were grown in liquid culture (2L), extracted with XAD resin, and the activity of the
133 extracts confirmed in the *C. elegans* feeding assay. Strain CNY-012 produced the most material
134 and was thus selected for bioassay-guided fractionation. Normal phase flash chromatography
135 resulted in one fraction with nematode deterrent activity (Fr6, 100% MeOH) (Fig. 3A, Fig. S3).
136 Further purification using reversed phase HPLC led to an active fraction that contained
137 lomaiviticin C based comparisons with reported UV and mass spectral data. Further comparison
138 with an authentic standard supported this identification (Fig. 3) along with what appeared to be
139 lomaiviticin A and breakdown products. Both lomaiviticins are known to be cytotoxic, with
140 lomaiviticin A inhibiting numerous cell lines at sub-nanomolar concentrations (38). While
141 lomaiviticin C is the least cytotoxic analogue reported to date (38), an authentic standard
142 nonetheless exhibited *C. elegans* feeding deterrence at 0.05 mg/mL of *E. coli* food paste (Fig. S3).

143 We next quantified lomaiviticin C in the three deterrent *S. tropica* extracts. Based on area
144 under the peak calculations, the extract from the 2L culture of strain CNY-012 contained 8.2
145 (±0.37) µM lomaiviticin C, followed by strains CNS-197 and CNH-898, which contained 5.2 (±0.39)
146 µM and 3.4 (±0.20) µM, respectively (Fig. S4). These concentrations are greater than the cancer
147 cell line IC_{50} cytotoxicity values reported for lomaiviticin C (0.2-0.9 µM) (38), yet below the value
148 that deters *C. elegans* feeding (0.05 mg/mL or 36 µM). Nonetheless, it remains possible that the
149 concentrations surrounding live colonies are considerably greater than those measured in liquid
150 culture, and that more potent analogs present in the extract (e.g., lomaiviticin A) contribute to

the *C. elegans* feeding deterrence, although none could be confidently identified. This may also explain the poor correlation between lomaiviticin C concentration (Fig. S4B) and feeding deterrence (Fig. S2A). Notably, lomaiviticin C was not detected in the extract from strain CNB-440 (Fig. S5), the only *S. tropica* extract that did not deter *C. elegans* feeding (Fig. S2). Identifying additional *S. tropica* strains that fail to produce this compound and generate similar results would provide further support for the role of lomaiviticin C in *C. elegans* feeding deterrence.

Effects against marine predators

An agar plate assay was designed to test the substrate preference of the annelid *Ophryotrocha siberti* (Dorvilleidae, Eunicida). *Ophryotrocha* is a bactivorous group that will migrate towards areas rich in bacteria and detritus while grazing (36, 39). This assay was used to determine if *O. siberti* avoided substrates containing *Salinispora* cultures or culture extracts relative to media controls. The substrates were A1 media seeded with *Salinispora* cultures or culture extracts and preference was determined by monitoring the position of five *O. siberti* individuals following placement in the center of a petri dish in which one half was comprised of treatment or control substrates. *Ophryotrocha siberti* individuals preferred the media controls to live cultures of either *S. tropica* CNB-440 ($n=5$ $p<0.0005$) or *S. arenicola* CNS-205 ($n=5$, $p<0.05$) (Fig. 4A). Substrates containing extracts from *S. tropica* CNB-440 were significantly less preferred than controls ($n=10$, $p<0.001$), while there was no significant difference between treatment and control for *S. arenicola,* ($n=10$, $p=0.26$) (Fig. 4B). As in the *C. elegans* assays, there was some reduction in the activity observed in extracts relative to live cultures.

We performed similar substrate preference assays using nematodes collected from

sediments at the Smithsonian field station at Carrie Bow Cay, Belize, where *Salinispora* spp. are known to occur (40). Based on 18S rRNA sequence analysis, these nematodes were identified as *Robea hypermnestra* (GenBank accession number MZ787961). In preliminary trials, the nematodes died within several hours of contacting agar containing *Salinispora* culture extracts. We then chose to monitor nematode survivorship on treated and control agar plates over a time course of 6 hours using extracts from *S. tropica* CNB-440 and *S. arenicola* CNS-205, which were available at the time. When subjected to agar substrates containing 1 mg/mL final concentration of crude extract from *S. tropica* strain CNB-440, marine nematode survivorship was reduced to 0% within 6 hours (Fig. 5). In contrast, the extract from *S. arenicola* CNS-205 and the media controls had little effect, with the nematodes surviving for several days.

Discussion

It is well documented that bacteria inhabiting marine sediments produce biologically active natural products (41, 42). These compounds have largely been explored for their biomedical potential, leaving major gaps in our understanding of their ecological functions. This study assessed the effects of two sediment inhabiting *Salinispora* species on the feeding and habitat preference of bacterivorous eukaryotes. The assays tested both live cultures and culture extracts to assess the role of natural products in defense against predation. The results differed for the two species, with *S. tropica* exhibiting higher levels of predator deterrence. This was somewhat surprising given previous evidence that *S. tropica* invests in growth while *S. arenicola* invests in the production of antibacterial compounds (31). While the faster growth rate reported for *S. tropica* was evident in these assays, the results suggest that the natural products produced by the two species serve distinct ecological functions, thus providing further support for their

ecological differentiation. Collectively, it appears that the slower growing species *S. arenicola* is better adapted to compete against bacteria while chemical defenses in the faster growing species *S. tropica* preferentially target eukaryotic predation. While these concepts require further testing, the results indicate that the ecological functions of compounds produced by sediment inhabiting *Salinispora* species can be complex and include defense against predatory eukaryotes.

While it is well understood that marine invertebrates and seaweeds maintain chemical defenses against predation (43, 44), much less is known about this function for bacterial natural products, likely due in part to the limited availability of appropriate bioassays. In response, we developed methods to test the effects of bacterial cultures and culture extracts on *C. elegans* feeding preference. Bioassay-guided fractionation led the isolation of compounds in the lomaivitacin series as the likely candidates for the deterrent activity detected from *S. tropica*. While not marine, *C. elegans* was selected based on past use in feeding preference studies (45) after attempts to develop a similar assay with marine nematodes failed. Despite difficulties working with marine nematodes, it was possible to show that extracts from *S. tropica* were toxic to field collected animals while extracts at similar concentrations from *S. arenicola* were not. Similarly, it was possible to develop a substrate preference assay using the marine annelid *O. siberti* and demonstrate that extracts from *S. tropica* were less preferred than controls while extracts from *S. arenicola* had no significant effect. Since the *S. tropica* strain available for testing (CNB-440) did not produce detectable lomaiviticns, it is likely that other compounds are responsible for the activity.

Several additional issues regarding the ecological relevance of the *C. elegans* results warrant discussion. First, the test concentrations used here were based on laboratory cultures,

which may not reflect natural concentrations. Similarly, it is unknown if the lomaiviticins are produced *in situ* as was previously shown for the *Salinispora* metabolite staurosporine (40). If they are produced, determining ecologically relevant test concentrations for laboratory-based bioassays remains a major challenging given uncertainties over diffusion gradients and the spatial scales at which compounds are encountered by potential predators. A targeted analysis for lomaiviticins in marine sediments known to harbor *S. tropica* would be a useful first step towards assessing *in situ* production.

It was not surprising that lomaiviticins were implicated in the feeding deterrence associated with *S. tropica*. These compounds are potent cytotoxins, causing double stranded DNA breaks at nanomolar to subnanomolar concentrations (46). The lomaiviticin BGC is present in all *S. tropica* strains sequenced to date, whereas it is absent from all *S. arenicola* strains (28). The fixation of this BGC in *S. tropica* indicates that the encoded compounds serve an important ecological role. The results presented here suggest that role may include defense against predation. Inactivation of the *lom* BGC will be an important next step to link lomaiviticins to predation defense and rule out the possibility that other compounds may also be involved. The relatively low activity of the *S. arenicola* cultures and culture extracts was surprising given that all of the strains tested possessed the BGC for the cytotoxic kinase inhibitor staurosporine, which the species is well known to produce (29). Thus, if staurosporine was produced, it did not reach levels that deter *C. elegans* feeding. More work will be needed to determine the potential role of this compound as a defense against predation.

While predation can have a major effect on planktonic bacterial community structure (47), considerably less is known about the effects of predation on bacterial communities in

marine sediments. The results observed here suggest that chemically mediated effects on eukaryote feeding and substrate preference could play a role in structuring sediment microbial communities. These roles could include the creation of spatial barriers that defend producing strains from predatory eukaryotes while providing associational defenses for non-defended community members. Secreted allelochemicals may also help explain the high diversity of microbes detected in marine sediments by providing a mechanism for micro-scale niche segregation. While sediment-derived marine bacteria are well known as a source of natural products, much remains to be learned about their roles in predation defense.

Materials and Methods

Live culture feeding assay

Ten *S. arenicola* and nine *S. tropica* strains with sequenced and annotated genomes were selected to maximize their collective biosynthetic gene cluster diversity (Table S1). *Caenorhabditis elegans* N2 was provided by the Troemel laboratory, UCSD and maintained on nematode growth media (NGM, Fisher scientific) seeded with 300 µL of *E. coli* OP50 (provided by the Troemel lab). Cultures were kept at 20-25°C and transferred to new plates every three days. Axenic *C. elegans* cultures were generated using published protocols (48). Briefly, *C. elegans* cultures were washed with 1 mL DI water after which 700 µL was transferred to a 1.5 mL Eppendorf tube and 300 µL of a 2:1 solution of 5% sodium hypochlorite and 5 M sodium hydroxide added to lyse the adult *C. elegans* and *E. coli*. The slurry was vigorously vortexed every 2 min for 10 min, spun at 1300 x g for 30 sec, and the pellet washed three times with 800 µL of

autoclaved DI water to remove any remaining bleach/sodium hydroxide solution. The washed pellet was mixed with 800 µL of DI water and egg concentration determined using a dissecting scope and diluted with sterile DI water to a final concentration of 8 eggs/µL.

Salinispora strains were cultured in medium A1 (10 g starch, 4 g yeast extract, 2 g peptone, 750 mL 0.2 µm filtered seawater, 250 mL deionized water) for seven days. Twenty-five µL from each strain was then spot inoculated 4 cm apart on a 150 mm x 15 mm petri plate containing 75 mL of medium A1 with 16 g agar/L such that all strains from the same species (either nine or 10) were on one plate. Six replicate plates were generated in this fashion for each species. After 7 days, 25 µL of *E. coli* strain OP50, grown in 50 mL LB broth Miller (Fisher Scientific) for 2 days, was spotted equidistant between the *Salinispora* colonies. After one day of room temperature growth, approx. 75 *C. elegans* eggs were seeded between the *E. coli* and *Salinispora* colonies. The number of live *C. elegans* hatchlings on the *E. coli* and *Salinispora* colonies was counted after 24 h using a dissection scope. The six replicate plates for each species were averaged to determine the preference between *Salinispora* colonies and *E. coli* food sources. Assays to test for preference between the two *Salinispora* species were prepared in a similar manner except 10 *S. tropica* and 10 *S. arenicola* strains were paired and *E. coli* was not offered as a choice. The strains paired were randomized on six replicate plates for the first trial and the process repeated using a new set of strains for the second trial.

Extract feeding assay

One ml of *Salinispora* culture (grown as previously described) was spread onto the surface of 25 individual 100 cm diameter A1 agar plates (1.4% agar) and allowed to grow for 15 days. The

agar plates were then chopped into 1x1 cm blocks, shaken with EtOAc in a 2.5 L culture flask for 2 hours, filtered (qualitative p8 fluted filter paper, Fisher) to remove cell and agar debris, dried on a rotary evaporator, and stored at -4°C. For the feeding assays, 1 mg of extract was dissolved in 50 µL DMSO, transferred to a sterile 10 mL glass culture tube, and combined with 1 mL of autoclaved *E. coli* OP50 that had been cultured in 50 mL LB media for 2 days. This resulted in an extract test concentration of ca. 1 mg/ml. Media controls were similarly prepared and tested at concentrations equivalent to the culture volume required to generate 1 mg of extract. Extract-treated and control *E. coli* cell paste was placed 1 cm apart in 60 mm x 15 mm petri dishes containing 4 mL of solid NGM and ca. 75 *C. elegans* eggs placed between the food sources. On average, 6 plates were tested per trial. The number of *C. elegans* per food source was counted 24 hrs. after *C. elegans* addition.

Bioactivity-guided isolation

Cultures were grown as previously described after which 25 mL was transferred into 4 x 1 L medium A1 in 2.8 culture flasks. After 12 days with shaking at 195 rpm, ca. 5 g of a pre-sterilized, 1:1 mixture of XAD-7 and XAD-16 Amberlite resin (Fisher Scientific) was added to each flask. The cultures grew for another 3 days after which the resin and cell mass was collected using cheese cloth and extracted with 500 mL acetone. The acetone was removed under vacuum and the resulting extracts tested at 1 mg/mL for *C. elegans* feeding deterrence. Active extracts were subjected to normal phase, flash silica column chromatography using the following scheme: 100% Hexanes, 50%:50% EtoAc: Hexanes, 100% EtOAc, 5%:95 MeOH:DCM, 20%:80% MeOH:DCM, and 100% MeOH. The fractions were dried under nitrogen, re-solubilized at concentrations equivalent to their yields per L of culture and tested for feeding deterrence. The

active fraction was further separated using reversed phase preparative HPLC with the following gradient: 0-95% acetonitrile (0.1% TFA), 100-5% H_2O (0.1% TFA) over 20 minutes with the first 2 minutes discarded. For LC-MS analysis, fractions at 1 mg/mL in MeOH were run through a Phenomenex Kinetex (Torrance, CA, USA) C18 reversed-phase HPLC column (2.6 mm, 100 x 4.6 mm) using an Agilent 1260 LC system (Santa Clara, CA. USA) under the following LC conditions with 0.1% TFA: 1–2 min, 10% MeCN; 2–14 min, 10–100% MeCN; 14-15 min, 100 % MeCN at a flow rate of 0.7 mL/min and analyzed with an Agilent 6530 Accurate-Mass Q-TOF with the divert valve set to waste for the first 2 min. Q-TOF MS settings were as follows: positive ion mode, mass range *m/z* 200–1600, MS scan rate 3/s, MS/MS scan rate 5/s, fixed collision energy 20eV, source: gas temperature 300°C, gas flow 11 L/min, Nebulizer 45 psig, and scan source parameters VCap 3000, fragmentor 100, skimmer1 65, OctopoleRFPeak 750). The MS was auto tuned using Agilent tuning solution in positive mode before each measurement and the data analyzed with the MassHunter software (Agilent). A lomaiviticin C standard was provided by the Seth Herzon.

Ophryotrocha *substrate preference assay*

Dorvilleids identified as *Ophryotrocha siberti* based on morphology were collected from the effluent line of the moon jelly tanks at the Birch Aquarium, SIO. This identification was confirmed based on COI sequence analysis (GenBank accession number MZ820650) performed using standard techniques (49). Voucher specimens are lodged in the Scripps Institution of Oceanography Benthic Invertebrate Collection (A 1152). They were maintained in culture on a frozen spinach diet. Substrate preference assays were performed in 60 mm dia. petri plates containing 4 mL medium A1 (1.2% agar) on one half of the plate and 4 mL of A1 seeded with a *Salinispora* culture on the other half. Five *O. siberti* individuals were added to the center of a

plate, which was then flooded with 1.5 mL of sterile seawater to facilitate movement. Due to a limited supply of *O. siberti*, only one representative *Salinispora* strain was used for each species (CNB-440 for *S. tropica* and CNS-205 for *S. arenicola*). Time-lapse photography was used to document the position of *O. siberti* individuals every 5 min for 1 h and substrate preference recorded as the average percent of individuals per substrate over the 12 time point measurements. Five replicate plates were tested for each trial with randomized plate orientation to negate abiotic factors such as light. The assay was repeated using *Salinispora* extracts (added when agar had cooled to 55°C, final concentration 1 mg/mL) and extract controls generated from 4 mL of medium A1.

Marine nematode assay

Intertidal sediment collected by bucket at the Smithsonian field station, Carrie Bow Cay, Belize in September 2015 was rigorously mixed by hand and the overlying seawater decanted through a 2 mm mesh to collect suspended biota. The samples were transferred to a petri dish and nematodes collected with the aid of a stereomicroscope and identified based on morphology and COI sequence analysis (49). Medium A1 plates containing extracts (1 mg/mL final concentration) of either *S. tropica* (CNB-440) or *S. arenicola* (CNS-205) were prepared along with controls containing extracts of medium A1 at a volumetrically equivalent concentration. Ten nematodes were added to triplicate treatment and control plates and survivorship monitored over six hours based on movement and response to prodding.

Statistical analysis

The non-parametric Wilcoxon rank-sum test (50) was used to evaluate significant

differences between treatments or between treatments and controls. These analyses were conducted in R (51) and graphs generated in Microsoft Excel.

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Figure legends

Figure 1. *C. elegans* feeding assay: live cultures. A) Percent of *C. elegans* individuals associated with live *S. tropica* or *E. coli* colonies (average for nine strains over six independent tests). B) Percent of *C. elegans* individuals associated with live *S. arenicola* or *E. coli* colonies (average for 10 strains over six independent tests). C) Percent of *C. elegans* individuals associated with live *S. tropica* or *S. arenicola* colonies (trial 1, n=6). D) Percent of *C. elegans* individuals associated with live *S. tropica* or *S. arenicola* colonies (trial 2, n=6). Error bars represent standard error. Asterisks denote Wilcoxon rank-sum test p values = 0.00001.

Figure 2. *C. elegans* feeding assay: culture extracts. Percent of *C. elegans* associated with autoclaved *E. coli* paste treated with *S. tropica* or *S. arenicola* culture extracts relative to controls (*E. coli* paste plus ethyl acetate extract of uninoculated culture medium). An ethyl acetate extract

of the uninoculated culture medium (A1) was also compared to a solvent only control. Results are presented out of 100%. Averages shown for four *S. tropica* or five *S. arenicola* strains, each of which was tested in six independent assays. Error bars represent standard error. Asterisks denote Wilcoxon rank-sum test p values <0.05.

Figure 3. Bioassay guided fractionation and compound identification. A) Fractionation scheme (green check indicates significant *C. elegans* feeding deterrence). B) ELSD and 254 nm absorbance spectra for the 100% MeOH fraction. C) UV spectrum for the major peak at 4.41 min matches a lomaiviticin C standard (blue). D) Base peak chromatogram for the major peak in Fr 2 is consistent with lomaiviticin C. E) Structure of lomaiviticin C.

Figure 4. *Ophryotrocha siberti* substrate preference assay. A) Percent of *O. siberti* individuals on A1 media containing live *S. tropica* (CNB-440) or *S. arenicola* (CNS-205) cultures vs. media controls measured at 5 min intervals and averaged over 1 h. B) Percent of *O. siberti* individuals on A1 media containing extracts of *S. tropica* (CNB-440) or *S. arenicola* (CNS-205) vs. extract controls measured at 5 min intervals and averaged over 1 hour. Error bars represent standard error. (n=5, asterisks denote Wilcoxon rank-sum test p values <0.05).

Figure 5. Marine nematode survivorship curves. Percent nematode survival on A1 agar media containing crude extracts (1 mg/ml) from *S. tropica* strain CNB-440 or *S. arenicola* strain CNS-205 compared with a volumetric equivalent of A1 media extract. Error bars represent standard deviation (n=3).

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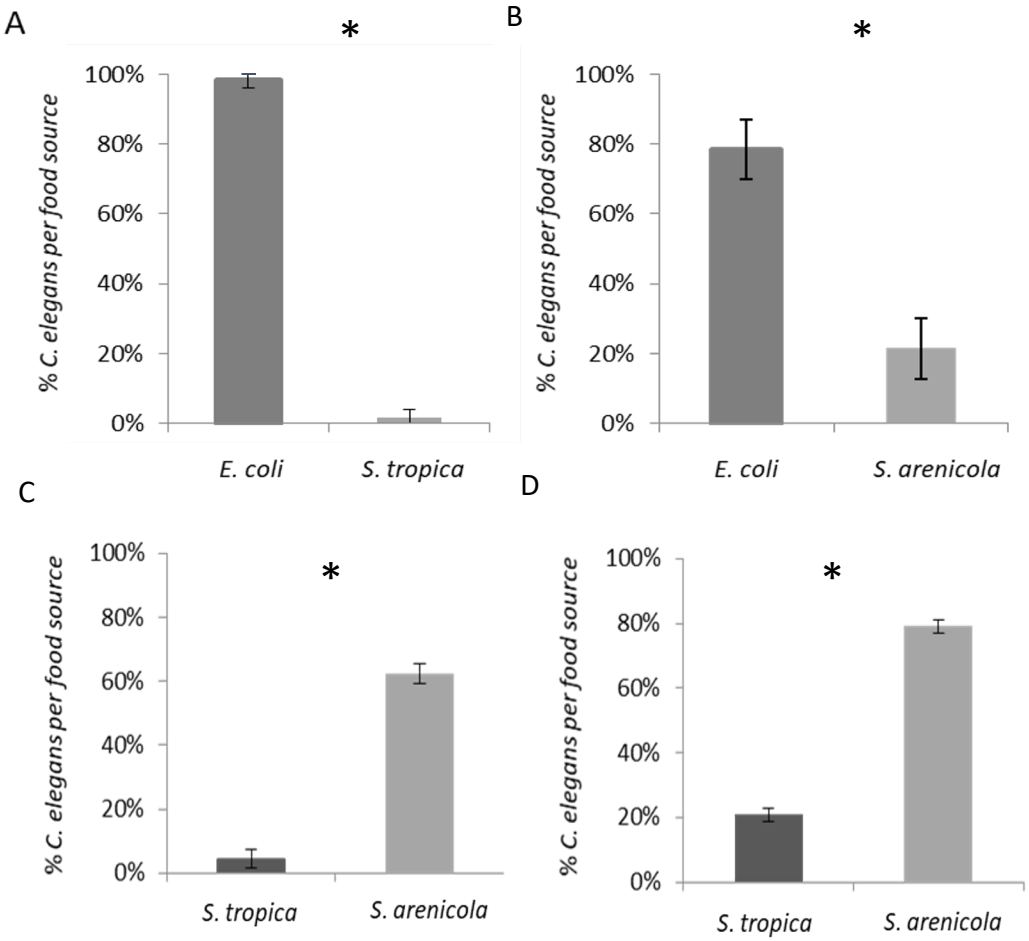

Figure 1. *C. elegans* feeding assay: live cultures. A) Percent of *C. elegans* individuals associated with live *Salinispora* or *E. coli* colonies. Results represent the average for each species (nine *S. tropica* strains, 10 *S. arenicola* strains) over six independent tests. B) Percent of *C. elegans* individuals associated with live *S. tropica* or *S. arenicola* colonies (two trials, n=5 for each trial). Error bars represent standard error. Asterisks denote Wilcoxon rank-sum test p values <0.05.

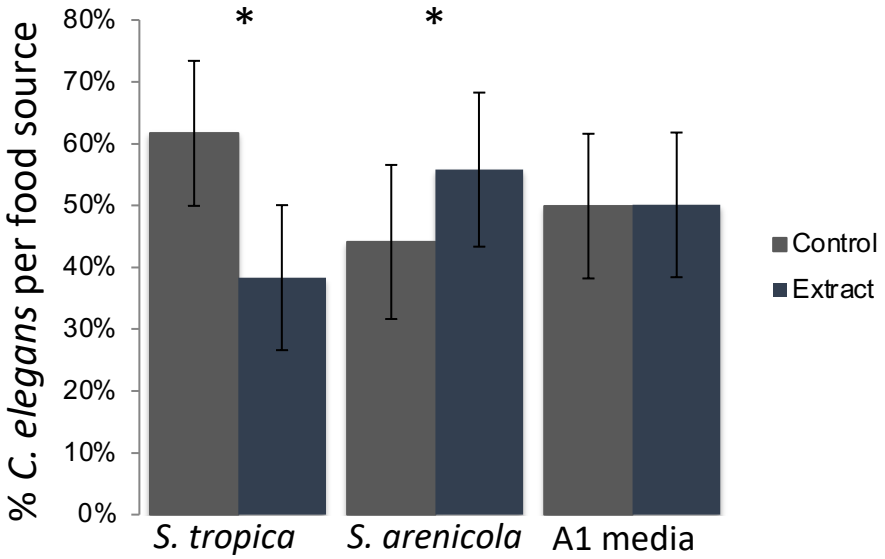

Figure 2. *C. elegans* feeding assay: culture extracts. Percent of *C. elegans* associated with autoclaved *E. coli* paste treated with *S. tropica* or *S. arenicola* culture extracts relative to controls (*E. coli* paste plus extract of culture medium). An extract of the culture medium (A1) was also compared to a solvent only control. Error bars represent standard error (n=6), asterisks denote Wilcoxon rank-sum test p values <0.05.

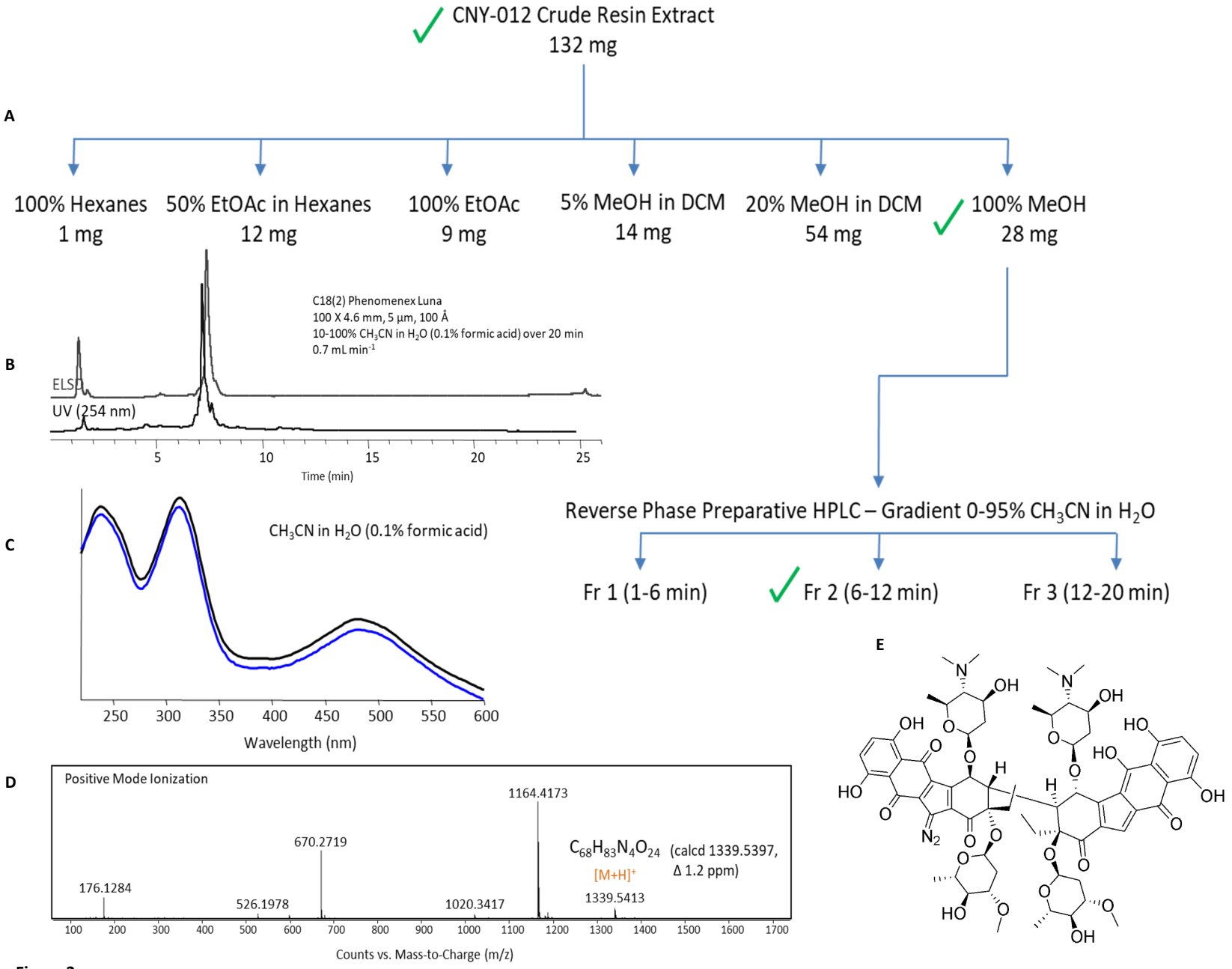
A
CNY-012 Crude Resin Extract
132 mg
100% Hexanes
1 mg
50% EtOAc in Hexanes
12 mg
100% EtOAc
9 mg
5% MeOH in DCM
14 mg
20% MeOH in DCM
54 mg
100% MeOH
28 mg
B
C18(2) Phenomenex Luna
100 X 4.6 mm, 5 µm, 100 Å
10-100% CH3CN in H2O (0.1% formic acid) over 20 min
0.7 mL min-1
ELSD
UV (254 nm)
Time (min)
Reverse Phase Preparative HPLC – Gradient 0-95% CH3CN in H2O
Fr 1 (1-6 min)
Fr 2 (6-12 min)
Fr 3 (12-20 min)
C
CH3CN in H2O (0.1% formic acid)
Wavelength (nm)
D
Positive Mode Ionization
1164.4173
670.2719
176.1284
526.1978
1020.3417
1339.5413
C68H83N4O24 (calcd 1339.5397,
[M+H]+
Δ 1.2 ppm)
Counts vs. Mass-to-Charge (m/z)
E

Figure 3.

Figure 3. Bioassay guided fractionation and compound identification. A) Fractionation scheme (green check indicates significant *C. elegans* feeding deterrence). B) ELSD and 254 nm absorbance spectra for the 100% MeOH fraction. C) UV spectrum for the major peak at 4.41 min matches a lomaiviticin C standard (blue). D) Base peak chromatogram for the major peak in Fr 2 is consistent with lomaiviticin C. E) Structure of lomaiviticin C.

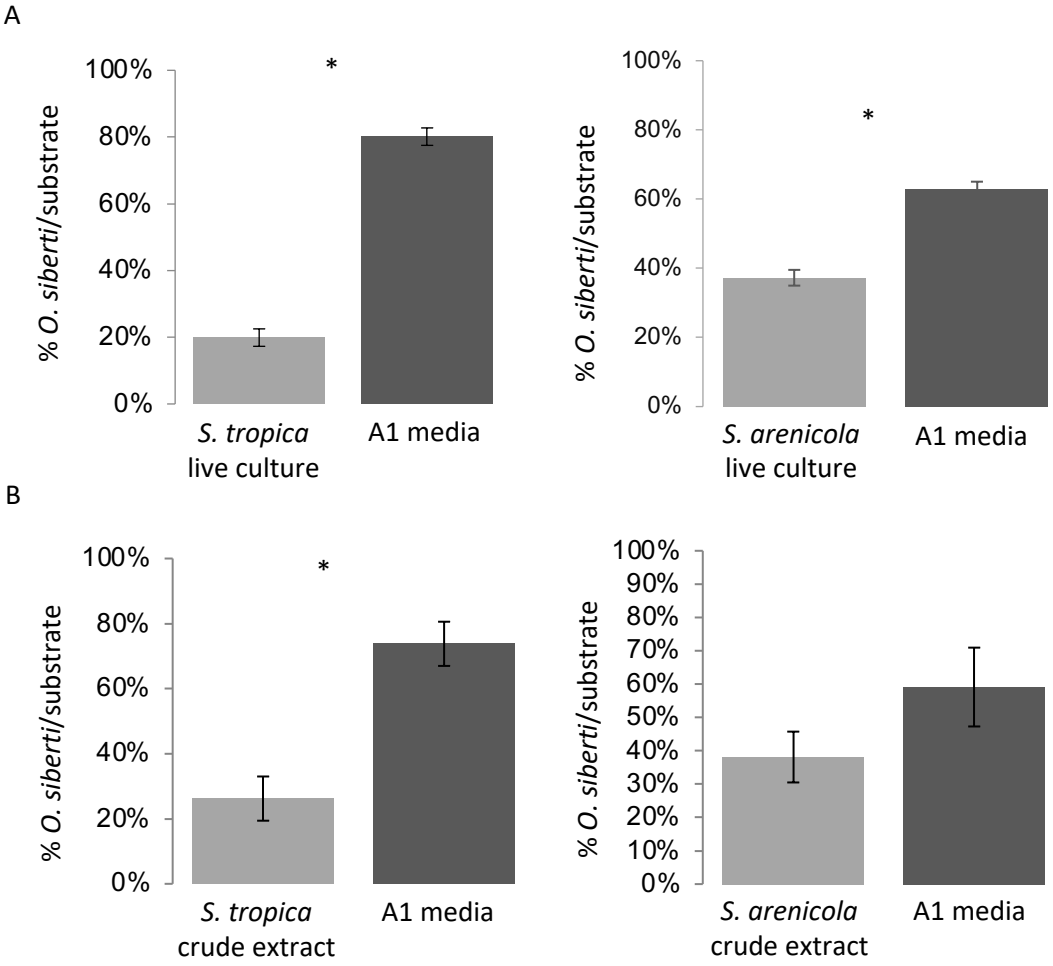

Figure 4. *Ophryotrocha siberti* substrate preference assay. A) Percent of *O. siberti* individuals on A1 media containing *Salinispora* cultures vs. media controls measured at 5 min intervals and averaged over 1 h. B) Percent of *O. siberti* individuals on A1 media containing *Salinispora* extracts vs. extract controls measured at 5 min intervals and averaged over 1 hour. Error bars represent standard error (n=5, asterisks denote Wilcoxon rank-sum test p values <0.05.

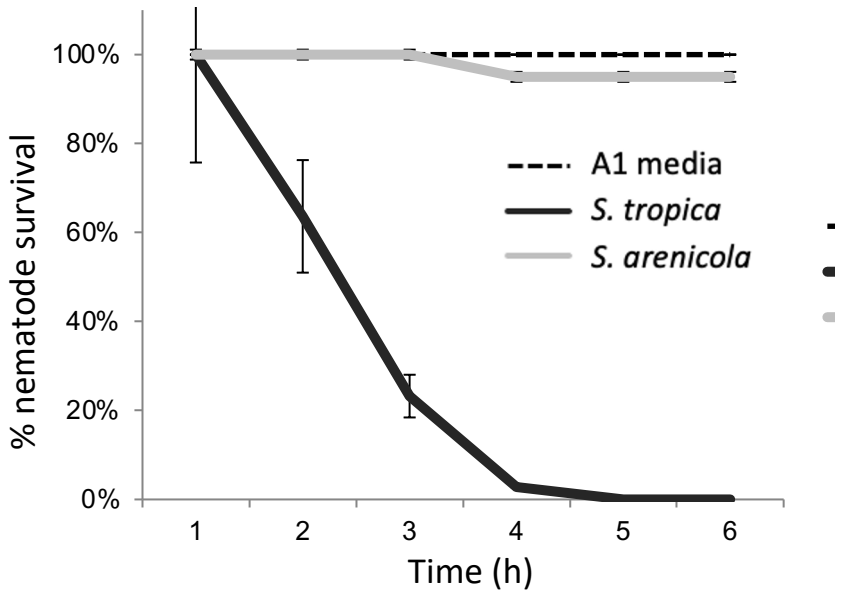

Figure 5. Nematode survivorship curves. Percent nematode survival on A1 agar media containing crude extracts (1 mg/ml) from *S. tropica* strain CNB-440 or *S. arenicola* strain CNS-205 compared with a volumetric equivalent of A1 media extract. Error bars represent standard deviation (n=3).