Sequestration and Cyanobacterial Diet Preferences in the Opisthobranch Molluscs *Dolabrifera nicaraguana* and *Stylocheilus rickettsi*

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A multidisciplinary approach was used to assess chemical ecological dietary interactions between marine organisms as a tool to isolate novel ecologically relevant compounds with biotechnological potential. First, laboratory-based feeding preference assays of the sea hare *Dolabrifera nicaraguana* (previously known as *D. dolabrifera*), an anaspidean mollusc, were conducted by simultaneously offering six food options collected from nearby tidal pools in the Coiba National Park in the Tropical Eastern Pacific of Panama. An evaluation of preferred dietary repertoire revealed *D. nicaraguana* significantly preferred cf. *Lyngbya* sp. over the cyanobacterium *Symploca* sp., green alga *Chaetomorpha* sp., and red alga *Spyridia* sp. A no-choice feeding assay using cf. *Lyngbya* sp. or green alga *Cladophora* sp. supported this finding. Secondly, we conducted bioactivity-guided fractionation using the preferred food source of *D. nicaraguana*, the ‘hair-like’ cf. *Lyngbya* sp. from which we also isolated and elucidated two new depsipeptide compounds, veraguamide M (1) and veraguamide N (2). Veraguamides M (1) and N (2) showed *in vitro* activity toward the malaria-causing parasite *Plasmodium falciparum* with GI₅₀ values of 4.2 and 4.3 µM, respectively, and therapeutic windows of 7.0–8.0 (based on moderate cytotoxicities to mammalian Vero cells with GI₅₀ values of 29.3 and 34.1 µM, respectively). Veraguamide N (2) was also active against *Leishmania donovani*, the causative agent of visceral leishmaniasis, with a GI₅₀ value of 6.9 µM. We then evaluated sequestration of these new compounds by *D. nicaraguana* used in the feeding assays and found trace amounts of the dietary sequestered compounds. Finally, we evaluated sequestration of these new compounds by the sea hare *Stylocheilus rickettsi* (previously known as *S. striatus*) that were grazing on the cf. *Lyngbya* sp. used in the feeding assays and found both to be sequestered.
This study is the first example whereby compounds with significant activity against tropical parasites have been found in both the sea hare *S. rickettsi* and its cyanobacterial food source. These results suggest that chemical ecological studies involving sea hares and cyanobacteria continue to provide a diverse source of bioactive compounds with biotechnological potential.

**INTRODUCTION**

Marine cyanobacteria have significant biotechnological potential including as food, fuel, fertilizers, and in mariculture (Thajuddin and Subramanian, 2005; Nunnery et al., 2010). Cyanobacteria are renowned for the wealth of secondary metabolites they produce (Burja et al., 2001; Blunt et al., 2012), many of which have been studied for their potential as pharmaceutical leads, cosmetics, vitamins, enzymes, or for pollution abatement (Thajuddin and Subramanian, 2005; Rotter et al., 2021). In nature, cyanobacteria produce a diverse combination of metabolites, some highly toxic, to maximize survival in competitive habitats where there are a range of grazers (Nagle and Paul, 1999). Many of these cyanobacterial secondary metabolites act as feeding deterrents to generalist grazers, such as crabs, fish, and sea urchins (Pennings et al., 1997; Nagle and Paul, 1998; Capper et al., 2006b), which allows cyanobacteria to avoid predation (Nagle and Paul, 1999; Capper et al., 2016). Fish and other potential grazers often have individualized responses to cyanobacterial secondary metabolites, wherein a compound that deters one herbivore may not deter another (Pennings et al., 1997).

The polyphyletic genus formally known as Lyngbya is renowned for its large number of secondary metabolites with biotechnological potential (Thajuddin and Subramanian, 2005; Rotter et al., 2021). *Lyngbya majuscula*, in particular has been shown to produce a wide array of bioactive secondary metabolites (Liu and Rein, 2010). However, the generic determination in many of these studies was based on morphology with the cyanobacterial consortium formally classified as *Lyngbya majuscula* forming filamentous mats or tangled masses up to 50 cm long (Littler and Littler, 2000). Although *Lyngbya* is known for the array of secondary metabolites it produces, this is likely due to an underestimation of cyanobacterial consortium biodiversity (Engene et al., 2011). With advances in cyanobacterial phylogenetics using the 16S rRNA gene, this morphologically similar cyanobacterial consortium was revealed to contain multiple evolutionarily distinct groups (Engene et al., 2013a). Phylogenetic analysis has revealed the cryptic diversity of *Lyngbya*, resulting in several new genera, including *Moorena* (formerly *Moorea*), *Okeania*, *Dapis*, and *Neolyngbya*, among others (Engene et al., 2012, 2013a,b, 2018; Caires et al., 2018; Tronholm and Engene, 2019). Because of the complexity in identification for this benthic cyanobacterial consortium, in this manuscript we will refer to *Lyngbya majuscula* as cf. *Lyngbya* sp.

Cyanobacterial secondary metabolites comprise a highly diverse range of compounds (Engene et al., 2013a). The bulk of these marine natural products have been isolated from *Okeania* spp. and *Moorena producens* (Engene et al., 2013a,b; Tronholm and Engene, 2019). In addition, several Panamanian compounds from *Okeania, Moorena*, and *Dapis* genera have shown activity against the tropical parasites *Plasmodium falciparum* and *Leishmania donovani*, the causative agents of malaria and leishmaniasis, respectively (Supplementary Tables 1, 2; Linnington et al., 2007; McPhail et al., 2007; Gutierrez et al., 2010; Sanchez et al., 2010; Balunas et al., 2012).

These diverse cyanobacterial secondary metabolites can stimulate feeding by specialized herbivores, such as sea hares (Anaspidea: Opisthobranchia) (Pennings et al., 1993; Nagle and Paul, 1998; Arthur et al., 2009), while providing a safe haven by lowering encounter rates with reef predators that are deterred by the metabolites (Cruz-Rivera and Paul, 2006). Many sea hare species have the capacity to sequester these dietary metabolites and store them in their digestive glands, rather than in external organs, ink or eggs (Pennings and Paul, 1993; Capper et al., 2005). As the predator has to consume the sea hare before being exposed to the toxins (de Nys et al., 1996), it has been suggested that concentrating sequestered compounds in the digestive gland might: (1) aid in detoxifying a diet rich in secondary metabolites (Pennings et al., 1999); (2) aid in the storage of toxic compounds too metabolically expensive to break down (Pennings and Paul, 1993; Capper and Paul, 2008); or (3) act as a site where chemical modification occurs to the molecule before sending through the blood to the ink gland for further modification and use in chemical defense (Kamio et al., 2010). Originally it was thought that these secondary metabolites were produced *de novo* by sea hares (Kato and Scheuer, 1974; Faulkner, 1984; Pettit et al., 1987), although it is now widely accepted that these compounds are of dietary origin (Stallard and Faulkner, 1974; Paul and Pennings, 1991; Rogers et al., 1995; Capper et al., 2005).

Sea hares from the *Dolabrifera* genus (Cuvier, 1817) are common opisthobranch molluscs in pantropical waters, especially in the Americas (Rudman, 2003; Valdés et al., 2018). Molecular and morphological research has revealed five taxa within the *Dolabrifera* genus, of which *D. nicaraguana* is endemic to the eastern Pacific (Valdés et al., 2018). *Dolabrifera* spp. defense mechanisms are limited as they do not produce ink (Prince and Johnson, 2006), but do produce a white milky secretion from their mantles when threatened (Ghazali, 2006). They often live under intertidal boulders (Kay, 1979) and avoid predation by emerging to feed during daytime ebbing tide once the tide falls below their tidal pools (Himstead and Wright, 2018). Field observations suggested that *Dolabrifera* spp. feed on diatoms,
microalgae, and algal mats (Miller, 1969; Marshall and Willan, 1999; Cimino and Ghiselin, 2009; Nimbs et al., 2017), whereas Dolabrifera sp. have been reported to feed on cf. Lyngbya sp. and Enteromorpha clathrata when kept in an aquarium with no other food (Prince and Johnson, 2006). However, the preferred diet of Dolabrifera spp. remains unknown.

Metabolites extracted from Dolabrifera spp. have exhibited variable palatability and toxicity. For example, whilst the skin and body walls of Dolabrifera sp. were palatable to the common intertidal hermit crab Pagurus sarmuellis (Takagi et al., 2010), extracts from its mid-gut gland were lethal when injected into mice at a high dose (200 mg/kg) (Waston, 1973). In other studies, egg masses of Dolabrifera sp. have shown antibacterial properties (Benkendorff et al., 2001). A sterol compound (5α,8α-epidioxycholest-6-en-3β-ol) isolated from D. nicaraguana (formerly D. dolabrifera) digestive gland previously demonstrated activity against L. donovani (Clark et al., 2013). A polypropionate metabolite, dolabriferol, isolated from Dolabrifera sp. skin (Ciavatta et al., 1996) and similar compounds, dolabriferol B and C, exhibited inhibitory effects against Mycobacterium tuberculosis (Jiménez-Romero et al., 2012). Given that little is known about Dolabrifera sp. feeding preferences or their ability to sequester dietary metabolites, further study presents an opportunity for marine chemical ecological research and bioactive metabolite investigations.

Sea hares from the Stylocheilus genus (Quoy and Gaimard, 1832) have a circumtropical distribution (Camacho-García et al., 2005). There are ongoing changes to the taxonomy of S. striatus, where phylogenetic testing revealed three allopatric species—S. striatus (Indo-Pacific), S. rickettsi (Eastern Pacific) and S. polyomma (Western Atlantic) (Bazzicalupo et al., 2020). Thus, in this manuscript to refer to a few or all the species we will use Stylocheilus spp. A species designation will be included when it is known based on geographical location of the sea hares. Stylocheilus spp. are specialist grazers of cyanobacteria, preferring cf. Lyngbya sp. over several other cyanobacteria and algae choices (Paul and Pennings, 1991; Capper et al., 2006a; Cruz-Rivera and Paul, 2006). Some cyanobacterial secondary metabolites, such as malyngamide A and B, are preferred by S. striatus while acting as deterents to other grazers (Pennings et al., 1996; Nagle et al., 1998). S. striatus sequesters dietary compounds from cf. Lyngbya sp., storing them in their digestive glands (Kato and Scheuer, 1974; Rose et al., 1978; Gallimore and Scheuer, 2000).

Often these sequestered secondary metabolites show a variety of bioactive properties, such as tumor promotion and anti-proliferation (Kikumori et al., 2012; Youssef et al., 2015; Supplementary Table 3). In some cases, S. striatus transforms these potent compounds in its digestive gland to less toxic forms through acetylation, for example transformation of lyngbyatoxin A to lyngbyatoxin A acetate (Gallimore et al., 2000) and malyngamide B to malyngamide B acetate (Paul and Pennings, 1991; Supplementary Table 3). The ecological connection of Stylocheilus spp. with cf. Lyngbya sp. extends beyond their diet, as chemical cues encourage settlement and development of larvae (Switzer-Dunlap and Hadfield, 1977). Additionally, Stylocheilus spp. have shown feeding attraction to artificial food containing extracts rich in secondary metabolites (Capper et al., 2016). While these cyanobacteria were identified as Lyngbya at the time, S. striatus graze on several of the newly classified cyanobacteria genera, Okeania sp., M. producens, Lyngbya sp., and Dapis sp., altering their feeding preferences based on secondary metabolite type and concentration in their food (Capper et al., 2016). The ecological role, however, of sequestered dietary compounds in Stylocheilus spp. has not yet been fully determined (Bornancin et al., 2017).

In this study, we sought to evaluate the marine chemical ecological interactions of sea hares with their food sources by characterizing dietary metabolites that are sequestered by the sea hares. We also tested these metabolites for anti-parasitic bioactivity, thus linking the ecological interactions with biotechnological applications. Our objectives were to: (1) establish laboratory-based feeding preference of D. nicaraguana; (2) isolate and identify bioactive compounds from the preferred food source cf. Lyngbya sp.; (3) determine sequestration of the compounds from cf. Lyngbya sp. in the sea hares D. nicaraguana and S. rickettsi; and (4) test these sequestered compounds against tropical parasitic diseases in bioassays of relevance to the host country of Panama.

MATERIALS AND METHODS

Study Site

Collections were made in May 2006 during low tide in the littoral zone along an extensive rocky shoreline peninsula between Playa Blanca and Boca Grande, Coiba Island, within Coiba National Park, Veraguas, Panama (07°23′50″ N, 81°39′00″ W) (Figure 1A). The collection site was situated on a large expanse of sedimentary rock weathered to sea-level, extending approximately 0.1 km² from the tip of the peninsula (Figure 1B). At low tide, many tidal pools and exposed boulders provided ample habitat for a variety of organisms on the underside of boulders and in tidal pools.

Study Organisms

All cyanobacterial and algal samples were found growing in tidal pools located nearby the boulders where sea hares were collected. Three cyanobacterial samples, fixed to the sandy bottom, rocks or bedrock, were collected including a cf. Lyngbya sp. cyanobacterium in a mat assemblage, a cf. Lyngbya sp. cyanobacterium with a hair-like morphology, and a Symploca sp. (Figures 2A–C, respectively). Three algal species were collected including a red alga, Spyridia sp. and two green algae, Chaetomorpha sp. and Cladophora sp. (Figures 2D–F, respectively). A 4.5 L bag of each species was collected and then frozen at −20°C for chemical analyses. Voucher specimens were preserved in EtOH:seawater (70:30) and maintained at −20°C for identification. Additional samples of cyanobacteria and algae were maintained until required for feeding assays in aerated aquaria in fresh seawater with 12:12 h light:dark at ambient temperature at the Liquid Jungle Lab, Canales de Tierra, Pacific coast of Veraguas or at Naos Marine and Molecular Laboratories at the Smithsonian Tropical Research Institute, Panama City.
Nine *S. rickettsi* (Figure 3A) were collected in the field grazing on cf. *Lyngbya* sp. with the hair-like morphology (Figure 2B) and were allowed to continue to graze in the laboratory in aquaria (ambient temperature, 12:12 h light:dark) for 5 days, fasted for 24 h (to allow gut evacuation), euthanized in freezing seawater and stored at $-20^\circ$C until tissue analysis was performed. Feeding studies were not conducted on these collections because of the extensive literature on the feeding preferences and metabolite sequestration of *S. rickettsi* (formerly *S. striatus*) (Kato and Scheuer, 1974; Rose et al., 1978; Paul and Pennings, 1991; Gallimore and Scheuer, 2000; Capper et al., 2006a; Cruz-Rivera and Paul, 2006).

Eighteen *D. nicaraguana* (Figure 3B) were collected in the same area as the food choices from the underside of uneven boulders in the intertidal zone. Three of these *D. nicaraguana* were used without laboratory feeding to assess their exposure to the food choices in the wild. They were fasted, euthanized, and stored as described above. Two additional animals died in the laboratory and were kept as vouchers. The remaining 13 animals were maintained in aquaria as above and were used in both multiple choice and no-choice feeding assays.

**Multiple Choice Assay**

*Dolabrifera nicaraguana* (*n* = 12, average initial weight 2.6 ± 0.4 g) were placed individually in separate 2 L aquaria in fresh seawater. Each sea hare was provided a choice between five food types offered simultaneously: “mat-like” and “hair-like” morphologies of cf. *Lyngbya* sp. (average initial wet weight 809 ± 174 mg and 506 ± 117 mg, respectively); *Symploca* sp. (average initial wet weight 407 ± 109 mg); the red alga, *Spyridia* sp. (average initial wet weight 151 ± 32 mg); and the green alga, *Chaetomorpha* sp. (average initial wet weight 448 ± 121 mg). Food items were blotted, weighed, and placed at the bottom of 2 L aquaria with fresh seawater. There were twelve treatment (herbivore) aquaria and twelve control (no-herbivore) aquaria. The no-herbivore control aquaria were used to assess changes
where \( T_i \) and \( T_f \) are the initial and final weights of the treatments, and \( C_i \) and \( C_f \) are the initial and final weights of the controls (Cronin and Hay, 1996). The Friedman test was used to detect significant differences in consumption of the different food types by \( D. \) nicaraguana (Erickson et al., 2006; Capper and Paul, 2008). A Nemenyi post hoc test was used to examine all possible pairwise combinations for significant differences between groups \((P < 0.05)\) (Conover, 1998).

**No Choice Assays**

To confirm food preferences, a no choice assay, using the same \( D. \) nicaraguana from the multiple-choice feeding assays above, was initiated after animals were fasted for 36 h. The no choice assays consisted of two \( D. \) nicaraguana feeding groups, including the cyanobacterium cf. \( Lyngbya \) sp. with “hair-like” morphology \((n = 6, \text{average initial wet weight } 70 \pm 22 \text{ mg})\) or the green alga \( Cladophora \) sp. \((n = 6, \text{average initial wet weight } 41 \pm 8 \text{ mg})\). The green alga \( Cladophora \) sp. was used instead of the green alga \( Chaetomorpha \) sp. because we had already established in the multiple-choice assay that the \( D. \) nicaraguana did not preferentially feed on \( Chaetomorpha \) sp. Food items were blotted dry, weighed, and divided into treatment and no-herbivore control group aquaria, as described above. The cf. \( Lyngbya \) sp. fed \( D. \) nicaraguana had an average initial animal weight of \( 2.1 \pm 0.4 \text{ g} \) while the \( Cladophora \) sp. fed \( D. \) nicaraguana had an average initial animal weight of \( 2.3 \pm 0.3 \text{ g} \). A control group was included to assess changes in cyanobacterial \((n = 6, \text{average initial wet weight } 65 \pm 34 \text{ mg})\) and algal mass \((n = 6, \text{average initial wet weight } 40 \pm 12 \text{ mg})\) throughout the experiment in the absence of herbivores (Peterson and Renaud, 1989). After 60 h, food items were removed, blotted and weights taken as described above. Post-assay, sea hares were fasted for 24 h to allow gut evacuation, euthanized in freezing seawater, and stored at \(-20^\circ \text{C}\). The proportion of food consumed was calculated using equation 1. A Mann–Whitney \( U \) test was used to determine whether there were significant differences between algal and cyanobacterial fed groups.

**General Experimental Chemical Analysis**

Low-resolution mass spectra (MS) were obtained in MeOH and analyzed by direct injection on a JEOL LCMate mass spectrometer (Tokyo, Japan). Nuclear magnetic resonance (NMR) spectra were collected using a JEOL Eclipse 400 MHz spectrometer (United Kingdom). MS/MS analyses were conducted using a Finnigan LTQ MS (Thermo-Electron Corporation). High performance liquid chromatography (HPLC) was carried out in reverse phase using a Prontosil-120 C18 analytical column (4.6 mm × 250 mm, Bischoff Chromatography, Leonberg, Germany) and a Merck Hitachi HPLC (Tokyo, Japan) containing dual pumps (L-7100) and a diode array detector (L-7455) monitoring at 210 nm.

**Chemical Analysis of Sea Hares**

Sea hare \( D. \) nicaraguana \((n = 3)\) not used in the feeding assays, \( D. \) nicaraguana \((n = 6)\) used in the multiple choice assay and in the cf. \( Lyngbya \) sp. no-choice feeding assay,
and *D. nicaraguana* (*n* = 6) used in the multiple choice assay and in the green alga *Cladophora* sp. no-choice feeding assay were weighed and dissected, separating digestive gland, skin (including parapodia, foot, and head), mucus gland (also referred to as albumen gland), and grouping the remaining internal organs (Figure 3C and Supplementary Table 4). *S. rickettsi* (*n* = 9) were weighed and dissected, separating digestive gland, skin, and grouping the remaining internal organs (Supplementary Figure 1 and Supplementary Table 4). To obtain sufficient material for chemical analysis, tissue samples were grouped, with one or two individuals per replicate, as shown in Supplementary Table 4. Tissue samples were then lyophilized, weighed, and pulverized prior to dissolving in 1:1 EtOAc:MeOH for 20 min, and extracting twice over 48 h. Samples were rinsed in 1:1 EtOAc:MeOH, filtered under vacuum, and dried via rotary evaporation. The excrement collected from *D. nicaraguana* and *S. striatus* during their 24 h fast, an egg mass from *S. striatus*, and mucus from *D. nicaraguana* were also dried and extracted as described above.

**Chemical Analysis of cf. *Lyngbya* sp.**

The cf. *Lyngbya* sp. cyanobacterium with “hair-like” morphology was lyophilized, ground, and 35 g of powdered sample was extracted by soaking in 1:1 EtOAc:MeOH and decanting three times over 72 h. The EtOAc:MeOH extract (given the code 9401) was collected and dried under rotary evaporation, producing 1.68 g of extract. The cf. *Lyngbya* sp. residue was then extracted by soaking in 1:1 EtOH:H₂O and decanting twice over 48 h. The EtOH:H₂O extract (given the code 9402) was collected, dried under rotary evaporation and then lyophilized to remove any remaining water, producing 0.4 g of extract. Both extracts were analyzed by MS, with the EtOAc:MeOH extract (9401) exhibiting mass peaks of m/z 887.1, 717.5, 703.5, 537.4, 313.3, 279.2, 245.2, 227.2 and 213.2. The EtOH:H₂O extract (9402) had two non-solvent peaks m/z 717.5 and 285.2.

The EtOAc:MeOH extract was fractionated using a Supelco Discovery DSC-18 reverse phase (RP) solid phase extraction (SPE) cartridge, with a bed weight of 10 g and 60 mL tube capacity. The cf. *Lyngbya* sp. cyanobacterium was first equilibrated in 1:1 MeOH:EtOAc followed by 150 ml each of 1:1, 3:2, 4:1, and 5:1 MeOH:EtOAc followed by 500 mg of the EtOAc:MeOH (9401) extract onto the cartridge, the sample was sequentially eluted under manually applied pressure with 150 ml each of 1:1, 3:2, 7:3, 4:1, and 5:1 MeOH:EtO₂:O₂ followed by 150 ml each of 100% MeOH, 100% EtOAc, and 100% acetone. The resulting eight fractions (coded 9401A through 9401H) were dried and assessed for bioactivity.

**Bioassay-Guided Fractionation of cf. *Lyngbya* sp.**

To rapidly assess a range of biological activity, *in vitro* bioassays already available through the Panama International Cooperative Biodiversity Group (ICBG) were employed as previously described (Moreno et al., 2011; Pavlik et al., 2013) to detect biological activity using *P. falciparum* (malaria), *L. donovani* (leishmaniasis), *Trypanosoma cruzi* (Chagas’ disease), MCF-7 breast cancer cells, and Vero mammalian cells (used to estimate overall cytotoxicity). Samples were initially screened at 10 µg/mL, with active samples then being further tested to determine the concentration to inhibit 50% growth (GI₅₀). Selectivity indices were calculated by dividing the GI₅₀ value for Vero cell cytotoxicity by the GI₅₀ value of the same sample toward the disease (e.g., *P. falciparum*, *L. donovani*, *T. cruzi*, MCF-7 cells).

Fraction 9401D, eluted with 4:1 MeOH:H₂O, was found to exhibit activity against *P. falciparum* in the malaria assay (GI₅₀ of 1.0 µg/mL) and was thus used for compound isolation via RP-HPLC. After dissolving in MeOH and filtering at 0.45 µm (Altech 17 mm PTFE syringe filters), compound isolation was accomplished using an isocratic system of 55% acetonitrile (CH₃CN) and 45% H₂O, with a flow rate of 1 ml/min to yield veraguamides M (1) and N (2), eluting at 23 and 31 min, respectively.

**Compound Identification**

MS/MS was carried out on compounds 1 and 2 using a Finnigan LTQ MS (Thermo-Electron Corporation) using Tune Plus software version 1.0, as previously described (Mevers et al., 2011). Spectral files were converted to mzXML files (publicly accessible at http://gnps.ucsd.edu under MassIVE accession no. MSV000080055) and analyzed using previously described algorithms (Liu et al., 2009; Ng et al., 2009; Mohimani et al., 2011). Compounds 1 and 2 were analyzed by 1H and 13C NMR, recorded in methylene chloride-d₆ (CDCl₆), Cambridge Isotope Laboratories, Inc., Andover, MA, United States (Supplementary Figures 2–5).

**Veraguamide M (1):** amorphous solid; [α]d²⁰ = –49.5 (c 0.5, CHCl₃); 1H NMR (400 MHz, CDCl₃) δ 6.25 (d, *J* = 8.6 Hz, 1H), 4.88 (d, *J* = 7.7 Hz, 1H), 4.77 (p, *J* = 4.8 Hz, 2H), 4.69 (dd, *J* = 8.5, 5.3 Hz, 1H), 4.05 (d, *J* = 10.2 Hz, 1H), 3.97 (d, *J* = 10.4 Hz, 1H), 3.76 (dt, *J* = 9.4, 6.7 Hz, 1H), 3.55 (dt, *J* = 9.4, 6.9 Hz, 1H), 3.40 (s, 2H), 2.93 (d, *J* = 11.2 Hz, 6H), 2.35–2.15 (m, 3H), 2.15–1.88 (m, 3H), 1.81–1.62 (m, 1H), 1.67–1.35 (m, 4H), 1.32–1.08 (m, 4H), 1.07 (d, *J* = 6.5 Hz, 3H), 1.07–0.79 (m, 20H); 13C NMR (100 MHz, CDCl₃) δ 173.3, 172.3, 172.2, 171.0, 169.8, 165.9, 83.8, 76.9, 76.4, 68.6, 66.3, 64.0, 57.3, 52.6, 50.5, 47.3, 42.1, 38.8, 35.7, 35.2, 29.6, 28.4, 28.3, 28.1, 26.0, 25.5, 25.1, 24.6, 23.7, 19.9, 19.7, 18.1, 16.4, 15.7, 14.1, 13.9, 11.4, 13.3, 10.7; ESIMS/MS m/z 689.44, 590.36, 524.35, 490.36, 470.31, 452.28, 411.33, 339.23, 297.23, 228.19. HREIMS [M + H]⁺ m/z 717.4797 (calcd for C₃₉H₆₅N₄O₈, 717.4802).

**Veraguamide N (2):** amorphous solid; [α]H NMR (400 MHz, CD₂Cl₂) δ 6.22 (t, *J* = 10.5 Hz, 1H), 4.86 (dd, *J* = 13.9, 7.2 Hz, 1H), 4.80–4.73 (m, 2H), 4.71–4.64 (m, 2H), 4.07–3.85 (m, 2H), 3.80–3.67 (m, 1H), 3.59–3.48 (m, 1H), 3.41 (s, 2H), 2.98–2.87 (m, 7H), 2.29–2.10 (m, 2H), 2.04–1.89 (m, 2H), 1.66–1.59 (m, 2H), 1.44 (s, 2H), 1.22 (dd, *J* = 14.0, 8.4 Hz, 4H), 1.12–0.77 (m, 26H); 13C NMR (100 MHz, CD₂Cl₂) δ 173.3, 171.0, 170.8, 169.9, 165.7, 83.9, 77.3, 77.0, 76.5, 76.8, 66.3, 64.0, 57.2, 52.6, 47.2, 42.0, 38.9, 35.7, 35.2, 29.6, 29.4, 28.7, 28.2, 28.0, 25.9, 25.61, 25.57, 25.2, 23.7, 19.92, 19.87, 19.7, 18.2, 18.1, 17.8, 16.4, 15.7, 14.1, 11.4, 11.3, 10.8; HREIMS [M + H]⁺ m/z 703.4641 (calcd for C₃₈H₆₃N₄O₈, 703.4646).
Sequestration Evaluation

Extracts of sea hare tissues, egg mass, and excrement were analyzed via MS to determine the presence of compounds 1 and 2. In addition, $^1$H-NMR of extracts of the sea hare digestive gland and skin tissues were obtained in CDCl$_3$ to compare with those of the pure compounds 1 and 2. HPLC was used to evaluate compound sequestration using an isocratic system of 68% CH$_3$CN and 32% H$_2$O at 1 mL/min. Compounds 1 and 2 eluted at 13 and 15 min, respectively, and were compared with chromatograms of the digestive glands and skin extracts from the feeding assay D. nicaraguana, control D. nicaraguana, and S. rickettsi.

RESULTS

Dietary Preference

In the multiple choice feeding assay, D. nicaraguana individuals were found to exhibit significant differences in preferences for the cyanobacterial and algal food types (Friedman test, $P < 0.001$). The Nemenyi post hoc test revealed no significant difference in preference between the two cf. Lyngbya species but found significant differences in consumption of the “hair-like” assemblage cf. Lyngbya sp. and the other food choices, including red alga Spyridia sp., cyanobacterium Symploca sp., and green alga Chaetomorpha sp. ($P < 0.05$, unadjusted; Figure 4A). Following the multiple choice assay, a no choice assay was performed using the “hair-like” cyanobacterium cf. Lyngbya sp. and the green algae Cladophora sp. In isolation, D. nicaraguana consumed more “hair-like” cf. Lyngbya sp. than it did the green alga Cladophora sp. (Figure 4B, Mann–Whitney U test, $P = 0.008$).

Compound Isolation and Identification From “Hair-Like” cf. Lyngbya sp.

Given that there were significant preferences for the “hair-like” cf. Lyngbya sp. by D. nicaraguana, and that S. rickettsi, known to sequester cf. Lyngbya sp. secondary metabolites, was found grazing on the “hair-like” cf. Lyngbya sp., the “hair-like” collection of cf. Lyngbya sp. was used for further activity-guided isolation. Two extracts were obtained (EtOAc:MeOH and EtOH:H$_2$O) and analyzed by MS with the EtOAc:MeOH extract prioritized for further compound isolation. Fractions were tested for activity and analyzed by MS with the EtOAc:MeOH extract prioritized before those of the pure compounds 1 and 2. HPLC was used to evaluate compound sequestration using an isocratic system of 68% CH$_3$CN and 32% H$_2$O at 1 mL/min. Compounds 1 and 2 eluted at 13 and 15 min, respectively, and were compared with chromatograms of the digestive glands and skin extracts from the feeding assay D. nicaraguana, control D. nicaraguana, and S. rickettsi.

Using HRESIMS, [M + H]$^+$ peaks consistent with molecular formulas of C$_{38}$H$_{56}$N$_4$O$_8$ and C$_{38}$H$_{52}$N$_4$O$_8$, were obtained for compounds 1 and 2, respectively. Literature comparisons revealed several possible compounds with these molecular formulae from cyanobacteria (Nakao et al., 1998; Mevers et al., 2011; Salvador et al., 2011) and $^1$H and $^{13}$C NMR revealed substantially overlapping signals (Supplementary Figures 2–5). Therefore, MS/MS data was obtained for compound 1 and analyzed using software designed for sequencing cyclic peptides (Liu et al., 2009; Ng et al., 2009; Mohimani et al., 2011) and previously used with compounds from the same family [e.g., veraguamide E (3) (Mevers et al., 2011; Salvador et al., 2011)]. Using both manual and computational comparisons of the MS/MS fragmentation patterns, the locations of structural modifications were determined, as compared with veraguamide A (Supplementary Figures 7, 8). Compound 1 was found to have two rearrangements as compared with veraguamide E (3) including substitution of an N-Me-Ile in place of an N-Me-Val in the first residue clockwise from the HMoya, as well as an N-Me-Val in place of the N-Me-Ile for the fourth residue clockwise from the HMoya, thus resulting in designation as a new compound given the trivial name veraguamide M. Stereochemical assignment was not possible with the limited quantities of 1, although the sign and magnitude of the optical rotation is consistent with that reported for veraguamide E (3) (Salvador et al., 2011), making it likely that the absolute configuration of veraguamide M (1) is identical to that reported.

![Figure 4](https://example.com/figure4.png)

**FIGURE 4** | Control adjusted mean (±SE) food consumption (mg, blotted wet weight) of algal or cyanobacterial food options by D. nicaraguana: **A** Multiple choice feeding assay ($n = 12$; Friedman Test, $P < 0.001$). Means with different letters are significantly different ($P < 0.05$) as per an unadjusted Nemenyi post hoc test. cf. Lyngbya sp. (1) refers to “hair-like” morphology and cf. Lyngbya sp. (2) refers to “mat-like” morphology; **B** No choice feeding assay (Mann–Whitney U test, $P = 0.008$) with either Cladophora sp. ($n = 6$) or cf. Lyngbya sp. ($n = 6$).
for compound 3. Similar analyses were performed for compound 2, resulting in the identification of another new compound named veraguamide N. Veraguamide N (2) was found to have one less methyl group, resulting in a 2-hydroxyisovaleric acid (Hiva) group in place of the 2-hydroxy-2-methylpentanoic acid (Hmpa) group found in the third residue clockwise from the HMoYa group of compound 1 (Figure 5).

**Evaluation of Sequestration**

The animal tissues were analyzed under the same conditions as the compounds, veraguamides M (1) and N (2), and demonstrated that there was strong evidence of dietary compound sequestration by *S. rickettsi*, and possible evidence of compound sequestration in our study (Table 1). Separate HPLC and MS analyses of the digestive gland of the *D. nicaraguana* fed the “hair-like” cf. *Lyngbya* sp. and of the tissues of *D. nicaraguana* not used in feeding assays exhibited low abundance signals indicative of possible evidence of the presence of veraguamide M (1) (Table 1). Veraguamide N (2) was not detected in *D. nicaraguana* tissues (Table 1). Other *D. nicaraguana* tissue samples, including skin, other internal organs, mucus gland, and mucus did not show evidence of compound sequestration, as assessed by MS and HPLC.

**Economically and Medicinally Relevant Compounds**

Veraguamide M (1) showed moderate activity against *P. falciparum* with a GI₅₀ value of 4.2 μM and moderate cytotoxicity to mammalian Vero cells with a GI₅₀ value of 29.3 μM (Table 2), resulting in a selectivity index of 7.0. Veraguamide N (2) showed moderate activity against *P. falciparum* with a GI₅₀ value of 4.3 μM and *L. donovani* with a GI₅₀ value of 6.9 μM, moderate cytotoxicity to mammalian Vero cells with a GI₅₀ value of 34.1 μM (Table 2), resulting in selectivity indices of 7.9 and 5.0 for antimalarial and anti-leishmanial activity, respectively.

Since both sequestered metabolites showed activity toward *P. falciparum*, sea hare tissues were also screened for bioactivity. *D. nicaraguana* tissue samples, including the digestive gland, skin and other internal organs, from animals used in the feeding assays and control animals were shown to be inactive (Table 1). In contrast, an extract from the *S. rickettsi* digestive gland showed similar antimalarial activity as the cyanobacterial compounds, veraguamides M (1) and N (2), with a GI₅₀ value of 3.0 μg/mL (Table 2). The *S. rickettsi* digestive gland extract was also cytotoxic to mammalian Vero cells with a GI₅₀ of 10 μg/mL, consistent with the detection of the two veraguamides by MS and NMR (Table 1) in the digestive gland extract. Extracts from the *S. rickettsi* skin, other internal organs, and excrement were shown to be inactive (Table 1).

**DISCUSSION**

**Feeding Assays and Preferences**

Multiple choice feeding assay results revealed *D. nicaraguana* significantly preferred both cf. *Lyngbya* sp. morphologies “hair-like” and “mat” assemblage more than the cyanobacterium *Symplaca* sp., the green alga *Chaetomorpha* sp., or the red alga *Spyridia* sp. In no-choice assays, the “hair-like” cf. *Lyngbya* sp. cyanobacterium was also significantly preferred to the green alga *Cladophora* sp. Whilst Prince and Johnson (2006) observed *D. nicaraguana* consuming cf. *Lyngbya* sp. in a no-choice feeding experiment, they also observed it consuming...
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TABLE 1 | Sea hare sequestration of veraguamide M (1) and veraguamide N (2).

| Sea hare | Body part                     | Replicate #<sup>a</sup> | Veraguamide M (1) | Veraguamide N (2) | % inhibition<sup>b</sup> (at 10 µg/ml) |
|----------|-------------------------------|--------------------------|-------------------|-------------------|--------------------------------------|
|          |                               |                          | MS   | NMR | HPLC | MS   | NMR | HPLC |                          |
| D. nicaraguana used in the feeding assays with cf. Lyngbya sp. | Digestive Gland           | 1–3<sup>c</sup>         | P    | –   | P    | –    | –   | –    |                          |
|          | Skin                          | 1                        | –    | –   | –    | –    | –   | –    |                          |
|          | Skin                          | 2                        | –    | –   | –    | –    | –   | –    |                          |
|          | Skin                          | 3                        | –    | –   | –    | –    | –   | –    |                          |
|          | Other Internal Organs         | 3                        | –    | –   | –    | –    | –   | –    |                          |
|          | Mucus Gland                   | 2                        | –    | –   | –    | –    | –    | –    |                          |
|          | Mucus<sup>d</sup>            |                          | –    | –   | –    | –    | –    | –    |                          |
|          | Excrement<sup>d</sup>        |                          | –    | –   | –    | –    | –    | –    |                          |
| D. nicaraguana (control) | Digestive Gland           | 8–10<sup>c</sup>         | –    | –   | –    | –    | –    | –    |                          |
|          | Skin                          | 8                        | –    | –   | –    | –    | –    | –    |                          |
|          | Skin                          | 9                        | –    | –   | –    | –    | –    | –    |                          |
|          | Other Internal Organs         | 8                        | –    | –   | –    | –    | –    | –    |                          |
|          | Mucus Gland                   | 8                        | –    | –   | –    | –    | –    | –    |                          |
| Stylocheilus rickettsi collected from and solely fed cf. Lyngbya sp. | Digestive Gland           | 1                          | S    | S   | S    | S    | S   | S    | 92                          |
|          | Digestive Gland              | 2                        | S    | S   | S    | S    | S   | S    |                          |
|          | Digestive Gland              | 3                        | S    | S   | S    | S    | S   | S    |                          |
|          | Skin                          | 1                        | S    | –   | S    | S    | –    | –    |                          |
|          | Skin                          | 2                        | S    | S   | P    | S    | S   | –    |                          |
|          | Skin                          | 3                        | S    | S   | P    | S    | S   | –    |                          |
|          | Other Internal Organs         | 2                        | S    | –   | S    | –    | –    | –    |                          |
|          | Excrement<sup>d</sup>        | 2                        | –    | –   | –    | –    | –    | –    |                          |
|          | Egg Mass<sup>d</sup>         |                          | –    | –   | –    | –    | –    | –    |                          |

<sup>a</sup> Description of replicates provided in Supplementary Table 4.
<sup>b</sup> Against Plasmodium falciparum.
<sup>c</sup> Replicates combined prior to analysis to obtain sufficient material.
<sup>d</sup> Sample collected from tank and so cannot be assigned to an individual replicate.
S, strong evidence; P, possible evidence (HPLC and/or MS peaks present but low abundance); –, no evidence; (blank), not tested.

TABLE 2 | Biological activity (GI<sub>50</sub> values) for veraguamide M (1), veraguamide N (2), and the digestive gland extract of Stylocheilus rickettsi fed exclusively cf. Lyngbya sp.

| Activity | P. falciparum | L. donovani | T. cruzi | MCF-7 | Vero cells |
|----------|---------------|-------------|----------|-------|-----------|
|          | µg/L | µM  | µg/L | µM  | µg/L | µM  | µg/L | µM  | µg/L | µM  | µg/L | µM  |
| Veraguamide M (1) | 3.0 | 4.2 | >10 | 10 | >10 | 10 | 21 | 10 | 29.3 |
| Veraguamide N (2) | 3.0 | 4.3 | 4.8 | 6.9 | >10 | >10 | 24 | 10 | 34.1 |
| Stylocheilus rickettsi digestive gland extract | 3.0 | 4.0 | >10 | >10 | >10 | <10 | 10 | 10 |

the green alga Enteromorpha clathrata. As no collections were made of E. clathrata, this alga could not be used in feeding preference assays reported herein. As a generalist grazer, it is likely that D. nicaraguana may capitalize on variety of food types, including diatoms, algal mats, and bacterial biofilms (Miller, 1969; Marshall and Willan, 1999; Rudman, 2003;
Cimino and Ghiselin, 2009; Nimbs et al., 2017), as part of wider dietary repertoire. These grazing habits could be beneficial to *D. nicaraguana*, allowing it to survive varying environmental conditions and food availability. In a previous study in Panama City, at Punta Culebra on Naos island, scores of *D. nicaraguana* were reported to emerge to forage during the daytime once the tide fell below their tidal pools (Himstead and Wright, 2018). In our study, *D. nicaraguana* were also collected during this tidal period, although we did not find any individuals on the food treatment types used in our feeding assays, but rather nearby on the undersides of boulders. While *D. nicaraguana* preferred cf. *Lyngbya* sp. in our laboratory-based feeding assays, further research is required to assess the diet of *D. nicaraguana* in the wild.

**Compound Sequestration**

Although there was possible evidence for sequestration of veraguamide M (1) in the digestive gland of *D. nicaraguana*, the low abundance signals found in the HPLC and MS analyses were not definitive. Additionally, *D. nicaraguana* tissue samples were biologically inactive, further suggesting minimal sequestration of veraguamides M (1) or N (2). It is possible that if the *D. nicaraguana* feeding assays ran for longer periods of time (e.g., 10 to 20 days (Pennings and Paul, 1993; Capper et al., 2005)) that these compounds may have bioaccumulated and demonstrated stronger evidence for sequestration. Additionally, detection of sequestered secondary metabolites may also depend on the ability of the organism to store or detoxify these compounds into less toxic metabolites (Capper et al., 2005). While some sea hares can sequester secondary metabolites with no apparent harm (Paul and Pennings, 1991), *D. nicaraguana* may not be able to tolerate these secondary metabolites and may, therefore, metabolize them into less harmful compounds.

In contrast, it is well known that *Stylocheilus* spp. sequester dietary-derived compounds from *Lyngbya* spp. and store them in their digestive glands (Pennings and Paul, 1993). Herein, *S. rickettsi* was found to sequester and store the cf. *Lyngbya* sp. compounds veraguamide M (1) and N (2), mainly in its digestive gland, but also in skin, other internal organs, and excrement. However, only the digestive gland of *S. rickettsi* exhibited biological activity, demonstrating the accumulation of sequestered compounds in the digestive gland. Moreover, the digestive gland extract showed similar antimalarial activity as the cyanobacterial compounds, while it was more cytotoxic toward mammalian Vero cells than were either of the sequestered compounds, suggesting there may be additional cytotoxic compounds stored in the *S. rickettsi* digestive gland. This is plausible given that *Stylocheilus* species process diverse secondary metabolites from their food (Paul and Pennings, 1991; Pennings and Paul, 1993; de Nys et al., 1996; Pennings et al., 1996; Capper et al., 2005) and concentrate the bulk of sequestered compounds in their digestive gland (Pennings and Paul, 1993). *S. rickettsi* provided an excellent opportunity to compare sequestration to an animal whose capability of sequestering was uncertain, in this case *D. nicaraguana*. In addition, this is the first study to demonstrate these phenomena in S. rickettsi, a species located in the Eastern Pacific. Additional studies incorporating the isolated compounds into artificial diets for sea hare feeding experiments are needed to fully assess consumption and subsequent compound sequestration by the sea hares.

**Cyanobacteria and Stylocheilus Ecological Interactions**

Ongoing phylogenetic assessments of marine benthic cyanobacteria have revealed that these cyanobacteria are much more diverse than previously thought (Engene et al., 2011). Although cf. *Lyngbya* sp. and related species may be morphologically similar, their genetic diversity has allowed them to produce effective and biodiverse chemical defenses (Engene et al., 2013a). *Stylocheilus* are known to graze on several of these new genera (Capper et al., 2016), including cyanobacteria recently reclassified using molecular sequencing as *Okeania* sp., *Moorena producens*, *Lyngbya* sp., and *Dapis* sp. (Engene et al., 2013a,b, 2018; Tronholm and Engene, 2019). *Stylocheilus* are also known to exhibit an altered feeding preference based on secondary metabolite type (Capper and Paul, 2008; Capper et al., 2016). Thus, it may be that *Stylocheilus* species select their food based on morphology of cyanobacteria and the secondary metabolites they produce.

*Stylocheilus* species are located in tropical oceans and have been geographically separated for at least the last three million years, with the closure of the Isthmus of Panama (Bacon et al., 2015; O’Dea et al., 2016), diverging into three allopatric species found in the Indo-Pacific, Western Atlantic, and Eastern Pacific (Bazzicalupo et al., 2020). Although these geographical barriers likely resulted in *Stylocheilus* speciation, multiple species from this genus are known to evade predation by preferring metabolite rich *Lyngbya* spp. cyanobacterial assemblages, thus benefiting from the structural diversity of the defensive compounds found in these diverse cyanobacterial species. Results of this study are consistent with previous findings and confirm the food preference of *S. rickettsi* for these filamentous cyanobacteria, consistent with other species of *Stylocheilus* (Pennings and Paul, 1993; Pennings et al., 1996; Capper and Paul, 2008).

**Diversity and Distribution of the Veraguamides**

Veraguamides are encompassed within the kulolide superfamily of related cyclodepsipeptides produced by marine benthic cyanobacteria (Boudreau et al., 2012). In 2011, veraguamides A to L were published in parallel efforts (Meyers et al., 2011; Salvador et al., 2011) (two representative structures shown in Figure 5). Veraguamides A-G were isolated from *Symplaca cf. hydroides* from reef habitats in Guam (Salvador et al., 2011) and veraguamides A-C and H-L were isolated from *Okeania* sp. PAC-17-FEB-10-2 from an intertidal area offshore of a small island within Coiba National Park, Panama (Meyers et al., 2011). Interestingly, veraguamides A to C were isolated from two different cyanobacterial genera, as well as from geographically isolated locations in the Indo-Pacific and Eastern Pacific. It is possible that the cyanobacterial source of veraguamides M (1) and N (2), which we refer to as cf. *Lyngbya* sp., may be *Okeania* sp., since the morphologically similar cyanobacteria
were both collected in Coiba National Park in Panama and contain similar compounds. However, it was not possible to carry out phylogenetic testing on the voucher specimens collected for the present study.

In 1998, two related depsipeptides within the kulolide superfamily, kulomo’opunalide-1 (5) and -2 (6), were isolated from the marine gastropod Philinopsis speciosa, collected in the intertidal area offshore of O’ahu, Hawai’i (Nakao et al., 1998). P. speciosa is a generalist carnivore, predaing on opisthobranch molluscs, including S. striatus (Nakao et al., 1998; Zamora-Silva and Malaquias, 2016). In feeding experiments, Nakao et al. (1998) demonstrated that P. speciosa fed on S. striatus, and they were able to isolate kulolide-1, a related depsipeptide, from both the predator and its prey. In addition, they noted that S. striatus feeds on cf. Lyngbya sp., although isolation and/or identification was not performed from the cyanobacterial food source (Nakao et al., 1998). However, inclusive of this current study, there are now three distinct reports of isolation of veraguamides from at least two genera of marine cyanobacteria (Meyers et al., 2011; Salvador et al., 2011). Thus, the dietary source of the veraguamide-type compounds, kulomo’opunalide-1 (5) and -2 (6) and kulolide-1, were likely from marine cyanobacteria, sequestered by S. striatus and then accumulated by its predator P. speciosa. Additionally, P. speciosa had higher concentrations than S. striatus, suggesting that P. speciosa bioaccumulated this dietary-derived compound (Nakao et al., 1998).

### Ecologically and Medicinally Relevant Compounds

Numerous compounds isolated from cyanobacteria have been shown to exhibit strong antimalarial properties (Supplementary Table 1; Fennell et al., 2003; Linington et al., 2007; McPhail et al., 2007; Barbaras et al., 2008; Gutierrez et al., 2010; Shao et al., 2015). Several cyanobacterial compounds, such as nostocarboline (Barbaras et al., 2008), dolastatin-10 (Fennell et al., 2003), and dragomabin (McPhail et al., 2007) have selectivity indexes two to three orders of magnitude greater than veraguamides M (1) and N (2). There are only a few known cyanobacterial compounds, however, with anti-leishmanial properties against L. donovani (Barbaras et al., 2008; Balunas et al., 2010, 2012; Sanchez et al., 2010). Herein, veraguamide N (2) was shown to exhibit modest anti-leishmanial properties (Supplementary Table 2). In addition, some of the previously isolated veraguamides showed moderate to weak cytotoxicity against cancer cell lines, although none of the previous veraguamide isolates were assessed for anti-leishmanial or antimalarial properties (Meyers et al., 2011).

Marine chemical ecology studies have important applications in the field of natural products based drug discovery (Simmons et al., 2005; Matthew et al., 2007; Wan et al., 2021). There are several examples of potential anticancer agents, sourced from marine cyanobacteria, which have been shown to be sequestered by the sea hare S. striatus (Luesch et al., 2002) including malyngamidine A (Paul and Pennings, 1991), lyngbyatoxin A (Cardellina et al., 1979; Capper et al., 2005) and aplysiatoxin (Kato and Scheuer, 1974; Supplementary Table 3). The findings presented in this study provide further examples of dietary-derived cf. Lyngbya sp. compounds, veraguamide M (1) and N (2), sequestered by S. rickettsi and possibly by D. nicaraguana, which are also active against the clinically important diseases malaria and leishmaniasis.

Both sequestered compounds are active toward protozoan parasites, with this bioactivity evident in the digestive gland of the S. rickettsi. However, it remains untested whether dietary-acquired secondary metabolites provide defense against protozoan parasites in molluscs. Marine parasites, however, affect all trophic levels from individual to ecosystems, regulating host abundance, modifying traits, and can indirectly affect species interactions and community structure (Coen and Bishop, 2015). Molluscs have a complex response to parasites, including anatomic barriers, immune cell response, and physiological elements such as the complement system including proteins mainly synthesized in the liver (Al-Khalafah and Al-Nasser, 2019).

### CONCLUSION

In laboratory-based feeding assays and using food sources collected from nearby tidal pools, D. nicaraguana was offered a variety of cyanobacteria and algae food options. Out of all the food options offered, D. nicaraguana significantly preferred cf. Lyngbya sp. Trace amounts of bioactive compounds isolated from cf. Lyngbya sp., were found in the Dolabrifera tissues. This study suggests that Dolabrifera, like fellow sea hare Stylocheilus, may have the capacity to sequester and store dietary-derived compounds with biotechnological potential, albeit in much lower abundance. Further feeding studies with the isolated compounds incorporated into artificial diets are needed to fully assess sequestration by D. nicaraguana.

Moreover, this study is the first example whereby compounds with significant activity against parasites responsible for tropical diseases have been found in both sea hares and their cyanobacterial food source. This study provides additional evidence that chemical ecological studies of sea hares and their cyanobacterial food sources not only provide insight into trophic relationships in marine invertebrates and their food sources but may also facilitate the search for compounds with important biological activities and biotechnological potential.

### DATA AVAILABILITY STATEMENT

The LC-MS/MS datasets presented in this study can be found at http://gnps.ucsd.edu in the MassIVE repository (accession: MSV00080055).

### AUTHOR CONTRIBUTIONS

KEC collected the field samples, ran the feeding assays and carried out statistical analysis under the advice of AC and VJP. KEC processed samples under the advice of AC, VJP, and MJB. KEC, W-TL, AMF, PCD, and MJB ran analytical analyses.
on the cyanobacterial samples. AA, GDT, and LH carried out the bioassays. W-TL and MJB elucidated the cyanobacterial compounds. KEC was supervised by TJ, TLC, and MJB. KEC and MJB wrote the manuscript. All authors assisted in editing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.766282/full#supplementary-material
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Conflict of Interest: PCD has stock in Sirenas and Galileo and is a scientific advisor to Cybele and scientific advisor and co-founder of Ometa and Enveda.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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