Identification of Potential Secondary Metabolites in Extracted Cyanobacteria and Algae

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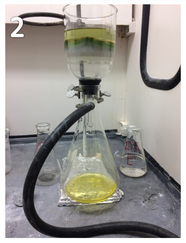
Abstract

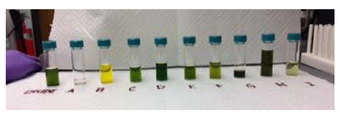
Marine organisms are known to possess architecturally-diverse biologically-active natural products that continue to inspire the development of pharmaceutical drugs. A majority of marine species, especially microbial ones, remain unexamined up until today. But the discovery of these marine life forms has been significantly productive and progressive as new methods of natural product research become available and new species with complex metabolite structures and new compounds with potent biological properties are uncovered. In this study, we aimed to extract secondary metabolites from an assemblage of green algae *Halimeda sp.* from Palmyra Atoll and an unknown species of cyanobacteria from Saipan Island. In order to identify the compounds of our samples, we utilized standard extraction protocol with a 2:1 CH_2Cl_2 and MeOH mixture as our extraction solvent, and VLC (Velocity Liquid Chromatography) to produce fractions of varying polarity and proportions of hexane, ethyl acetate, and methanol. We then ran 0.5 mg of our fractionated extracts in LC-MS/MS (Liquid Chromatography-Tandem Mass spectrometry), and the data produced was used in GNPS (Global Natural Products Social) to conduct molecular networking, a method of dereplicating molecules. This study opens the door to new discoveries as we uncover potent biological compounds from an unknown species of *Halimeda* and Cyanobacteria.

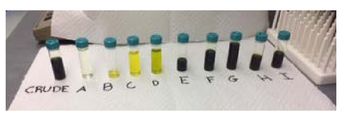
Extraction, Isolation, and LCMS analysis



1) The cyanobacteria ---which we suspect to be part of the Nostocaceae family because of its unbranched filamentous and heterocystous structure--- and the *Halimeda sp.* were covered in CH_2Cl_2/MeOH (2:1, v/v) mixture and filtered through the cheese cloth using a Buchner funnel. The crude extracts were later placed in the RotoVap, where the extraction solvent was evaporated under low pressure. This method was repeated 5 times for Cyanobacteria, and 4 times for *Halimeda.* The first extract of both samples was identified as an aqueous solution (inorganic phase) and were separated from the organic phase for back extraction. In back extraction, we mixed the aqueous solution with CH_2Cl_2 to extract the compounds from the water. The extract from the inorganic phase was later combined with the organic phase.

2) In VLC or Velocity Liquid Chromatography, we used silica gel as the stationary phase and the step-wise gradient of solvents from 100% Hexane to 100% Methanol as the mobile phase. Varying proportions of hexanes, EtOAc, and MeOH were made for each fraction, and were eluted from the column to produce fraction of different colors.



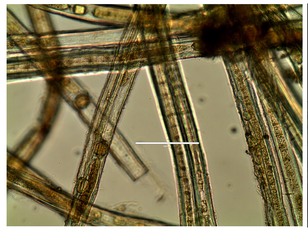


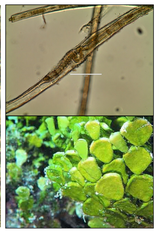
Fractionated crude extracts after VLC: Figure 1. Cyanobacteria (on the left) Figure 2. *Halimeda sp*. (on the right)

Fractions	Composition	Per 150 ml	Per 75 ml
A	100% Hexane	150 Hexane	75 Hexane
B	10% EtOAc: 90% Hex	15 EtOAc: 135 Hex	8 EtOAc: 68 Hex
C	20% EtOAc: 80% Hex	30 EtOAc: 120 Hex	15 EtOAc: 60 Hex
D	40% EtOAc: 60% Hex	60 EtOAc: 90 Hex	30 EtOAc: 45 Hex
E	60% EtOAc: 40% Hex	90 EtOAc: 60 Hex	45 EtOAc: 30 Hex
F	80% EtOAc: 20% Hex	120 EtOAc: 30 Hex	60 EtOAc: 15 Hex
G	100% EtOAc	150 EtOAc	75 EtOAc
H	25% MeOH: 75% EtOAc	38 MeOH: 113 EtOAc	19 MeOH: 57 EtOAc
I	100% MeOH	150 MeOH	75 MeOH

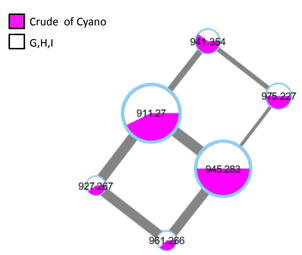
3) The fractionated crude extracts were then evaporated in the RotoVap in a round-bottom flask, transferred into a weighted vial, and were placed in the RotoVap again. In preparation for LC-MS/MS, we added calculated amounts of MeOH in each vial and pipetted 0.5 mg of our fraction into LC-MS vials. The LC-MS profiles were uploaded in GNPS, which analyzed our MS/MS spectra and compared them with other publically available data.

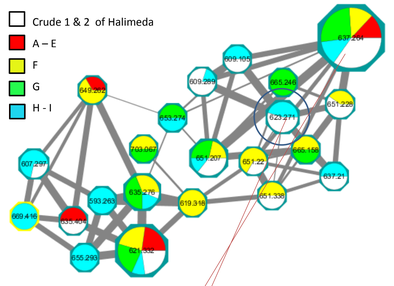
Cyanobacteria and *Halimeda sp.*

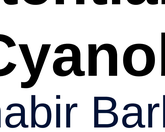




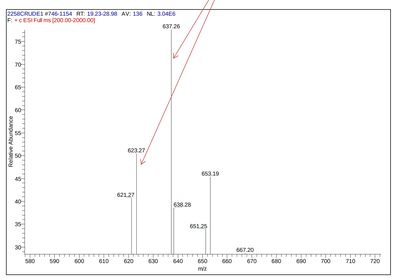
Molecular Networking as a dereplication strategy



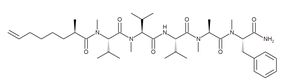




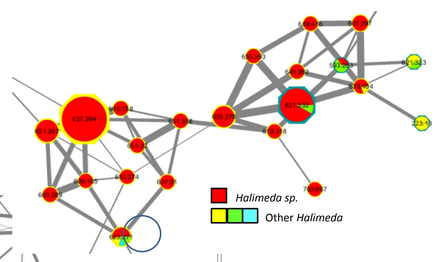
Compound: 2-oxo-3(*R*)-hydroxy-13-*N*-desmethyl-lyngbyatoxin A

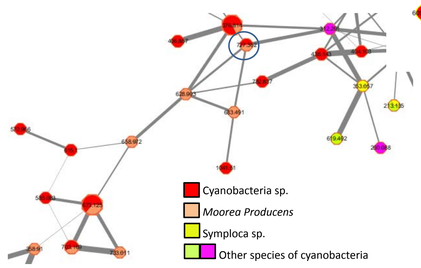


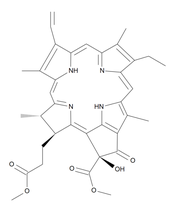
After uploading the MS/MS data to GNPS, we then used its analysis to do molecular networking. **Molecular networking** is an effective dereplication strategy by relying on MS/MS data. A molecular network is generated using "cosine scores," which measures chemical similarity in the MS/MS spectra and can be visualized using Cytoscape. A node, labeled with precursor parent mass, in the molecular network represents a MS/MS spectrum, and its size varies depending on its peak in the MS/MS data. The thickness of the edges, the lines connecting the nodes, illustrates the relatedness between the molecules as defined by the cosine scores. Compounds with chemical similarity tend to form a cluster in a molecular network.



Compound: almiramide c

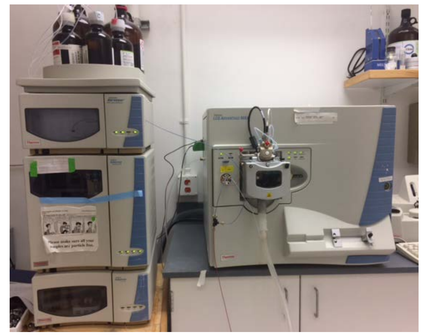






Compound: $C_{36}H_{38}N_4O_6$

Liquid Chromatography-Tandem Mass Spectrometry



Fractions *Halimeda sp.*	Extract in mg/ml	mg/µl	Added MeOH in ml
Crude 1	40.9/4.09	0.5/50	950
Crude 2	56.49/5.649	0.5/50	950
A	12.66/1.266	0.5/50	950
B	0.71/0.7042	0.5/704	296
C	4.13/0.121	0.5/121	879
D	14.62/1.462	0.5/50	950
E	39.15/3.915	0.5/50	950
F	39.91/3.991	0.5/50	950
G	27.54/2.754	0.5/50	950
H	13.98/1.398	0.5/50	950
I	171.44/3.439	0.5/10	990

Liquid Chromatography- Tandem Mass spectrometry is one of the ways for identifying compounds. In this study, we used LC-MS/MS prior to molecular networking, and used the data generated by the mass spectrometers to create our network. We analyzed calculated amounts from each of our samples depending on its concentration. In LC, the components of the samples separate depending on their chemical affinity with the mobile phase (MeOH) and the stationary phase; therefore, molecules will have different retention times. After LC, molecules are sent into MS/MS where the first MS ionize the compounds using low energy. Finally, the second MS executes fragmentations of the ions. MS/MS is more discriminatory than MS alone, and therefore it allowed us to gain more structural information of our compounds.

Fractions Cyanobacteria	Extract in mg/ml	mg/µl	Added MeOH in ml
Crude 1	9.62	0.5/52	948
A	1.40	0.5/357	643
B	4.12	0.5/121	879
C	7.46	0.5/67	933
D	5.75	0.5/87	913
E	6.80	0.5/74	926
F	3.52	0.5/142	858
G	4.22	0.5/118	882
H	20.05	0.5/25	975
I	2.44	0.5/205	795

Discussions and Conclusions

Due to time restrictions, we were unable to conduct a brine shrimp cytotoxicity assay to determine whether or not the secondary metabolites of our samples were biologically active. Exposing the brine shrimp to our samples would have revealed the compounds' potential to be developed into therapeutic drugs; the bioactivity of our compounds could be measured through the survival rate of the brine shrimp. This experiment displays the difficulty of dereplicating compounds by the sole use of precursor or parent masses to search for known molecules in databases such as MarinLit and SciFinder. Nevertheless, molecular networking, using Cytoscape, facilitated the identification of compounds and revealed to us the connections between different natural products and those of our own. For example, we networked our Halimeda to those from different geographic locations and our Cyanobacteria to a variety of species. These networks allow us to visualize the relationships between and identify different samples by comparing it to others similar to it. Further research and testing is required in order to elucidate the biological properties and chemical structure of the compounds discovered in the experiment. Finally, our study exemplifies the challenges that the field of marine natural product biotechnology faces. However, it also illustrates the bright future that this field awaits as it advances in the discovery of marine secondary metabolites that can be applied on human health.

References

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