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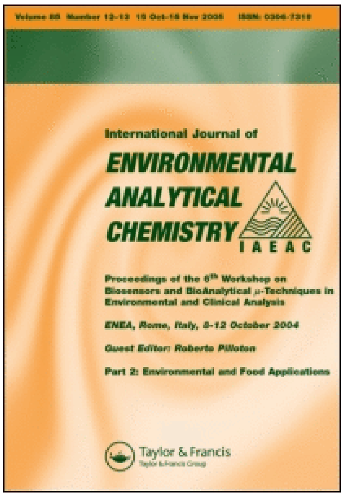

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Naturally occurring organic matter as a chemical trap to scan an ecosystem for natural products

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NATURALLY OCCURRING ORGANIC MATTER AS A CHEMICAL TRAP TO SCAN AN ECOSYSTEM FOR NATURAL PRODUCTS

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A model is proposed that tests an ecosystem for natural products (NPs) using a nonpolar extract of naturally occurring organic matter (NOM), which we demonstrate to be an efficient chemical trap for relatively nonpolar organic molecules. To test the model we collected twenty-six samples of NOM from various locations on the Suwannee River, from its headwaters in the Okeefenokee Swamp to the Gulf of Mexico. We have tentatively identified stearic acid, DDT, chincodine, and a potential precursor to bryostatin. Our data provide evidence that NOM can trap, hold for several decades, concentrate, and transport NP in the environment.

Keywords: Naturally occurring organic matter; Quinine; Chincodine; Bryostatin; Bryozoa

INTRODUCTION

Naturally occurring organic matter (NOM), the product of plant and animal decay, can bind and transport metals through the environment, solubilize nonpolar compounds (e.g., herbicides, pesticides, and petroleum products), fertilize soil, buffer soil and water, impact dissolved oxygen levels in the aqueous phase, etc. [1–3]. A subcomponent of NOM is humic acid (HA). The international standard for HA, distributed by the International Humic Substance Society, is taken from the Suwannee River in Fargo, Georgia [4].

Work in this laboratory with NOM has included using Multiangle Laser Light Scattering (MALLS) to measure the average size and molar mass of HA aggregations and a variety of thermodynamic studies [5–9]. It has been proposed and supported by experimental data that NOM can form micelles and these micelles subsequently solubilize DDT [10]. Specifically, NOMs have a large, nonpolar component that aggregates in

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solution, providing a region that is chemically and physically compatible with larger nonpolar organic species. What should be emphasized is that NOM is an aggregation of many molecules constituting a range of concentrations. Types of structures include aliphatic and aromatic structures [11], multiple-substituted carboxylates, amino acids, peptides and sugars [12], cellulose and lignin fractions [13], and functional groups including thiols, amines, and phenols [14]. A large number of binding studies involving different forms of NOM from different locations globally have also been conducted. These studies include various elemental binding (e.g., Ca^{2+}, Cu^{2+}, lanthanides, actinides, etc.) and the trapping of various organic compounds such as herbicides and pesticides [15–22].

In any ecosystem, from river valleys to deep ocean vents, there exist a large number of living species from bacteria and algae to crustaceans and larger plants, which can potentially contain an NP. Using current methodology, only a few species in a specific ecosystem are identified and tested for their medicinal potential. All species die and leave behind a molecular fingerprint, so we seek a chemical trap that can isolate NPs from dilution or destructive forces in an ecosystem. Because some species may have a very low abundance, or some molecular structures in a specific species may have a very low concentration, it is important that the chemical trap binds, concentrates, and protects the NPs for years or even decades. We show that NOM can be this chemical trap to provide a glimpse into the molecular history of an environment. We believe that aggregates of NOM and fulvic acid are responsible for the transportation of NPs through the environment and that the insoluble humin collects and stores NPs for extended periods of time.

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EXPERIMENTAL

Samples were collected during the first week of August 2000 with details about specific sites provided elsewhere [9]. The Suwannee River, its tributaries, and springs had either low flow or no flow because of a four-year drought when the samples were collected. The following extraction procedure was used for the Suwannee River NOM. Approximately 10 cm^3 of each solid sample was measured, allowed to settle for 48 h, and centrifuged. This removed granular sized limestone and gypsum found throughout the Suwannee River basin. Second, the remaining solid was treated with 0.05 M HNO_3 to dissolve remaining salts and extract the fulvic component of NOM. Third, the sample was washed with 0.05 M KOH to remove the remaining soluble HA component. The remaining solid sample was allowed to dry and subsequently extracted (after 48 h) with a 50/50 methanol–chloroform mixture. The yellow-colored solution was dried with magnesium sulfate. This solution was analyzed by LC-MS, MALDI-MS, and GC-MS for nonpolar species.

Instruments

The MALDI is a Reflex produced by Bruker Daltonics. The matrix used was a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in a 50 : 50 solution of acetonitrile : water and 0.1% trifluoroacetic acid. The GC-MS is a Hewlett-Packard 5970 MSD with a 5890 GC. The LC-MS is an Applied Biosystems (ABI) solvent delivery system connected to a PE Sciex API I plus mass spectrometer equipped with an electrospray source.

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RESULTS AND DISCUSSION

Over a period of time, and through the death of many organisms, NOM becomes a window to a molecular history for the surrounding ecosystem. The Suwannee River basin is rich in NOM and has a diverse range of plant and animal life as well as chemical conditions (pH, DO, C content, ORP, etc.) as it travels 300 km from the Okefenokee Swamp to the Gulf of Mexico (USA). In order to test the concept of NOM being a molecular window to the surrounding environment, we outlined four parameters that would have to be demonstrated.

We should identify in the NOM a well-known molecule whose origin is the Suwannee watershed area but that is not found within the limestone riverbed. This would demonstrate NOM's ability to bind and transport an NP to a natural chemical trap. In LC-MS analysis of all nonpolar extracts from the riverbed, NOMs both hexadecanoic acid ($C_{16}H_{32}O_2$) and octadecanoic acid ($C_{18}H_{36}O_2$, stearic acid) were identified [23]. This molecule can be attributed to palmetto trees (*Serenoa repens*, *Sabal minor*, *Rhapidophyllum*) [23], found throughout the Suwannee River basin. These samples were collected at the four-year point of a severe drought, suggesting their transport and accumulation in the river sediment took place several years previously.

The second part of demonstrating that NOM can trap NPs is that they must bind and hold an organic-based molecule for an extended period of time (years to decades) and under harsh conditions (floods, heat, sunlight, etc.). In addition to concentrating a species and preventing it from simply being diluted in the environment, it is also important that the chemical trap minimizes or eliminates degradation by photochemistry, any reactions with oxygen or other species capable of inducing an oxidation or reduction reaction, and consumption by microorganisms. Using MALDI-MS, we analyzed our samples for DDT, which was banned for use in the USA in 1972. If a single site was examined and DDT found, it could be argued that a recent rogue application might be responsible for the molecular fingerprint. We found traces of DDT in NOM over the entire length of the Suwannee River. This is inline with a large-scale soil analysis by the United States Geological Society of the soil of North Florida and South Georgia, which identified DDT [13].

The third piece of data we sought was to identify a known NP that had not yet been identified in this ecosystem. In summary, the molecule cinchonidine was identified in the Suwannee River basin for the first time. It is a derivative of quinine and has the same medicinal value (anti-malarial agent). Both are found in the bark of the Cinchona tree found in Ecuador (Andes Mountains, South America). Using our extraction and detection technique, we identified the molecule trapped in NOM throughout the Suwannee River basin. While this is the first record to identify the molecular species in the southeastern United States, we were able to correlate the find with folklore dating back to the United States Civil War and the locally abundant dogwood tree (*Cornus florida*) [24–29]. Specifically, when quinine was not available due to the naval blockade of southern United States ports during the Civil War (1861–65), the bark of dogwood trees was boiled and the extract ingested to minimize the effects of malaria. After detecting cinchonidine in all samples collected that had significant amounts of NOM, we conducted a separate study of dogwood trees in the Valdosta area. Specifically, we collected root, leaf, bark, and stem samples from six trees in various settings such as on the banks of the Withlachochee River, residential areas, old growth forests, and near slash pine tree stands re-planted for harvest every

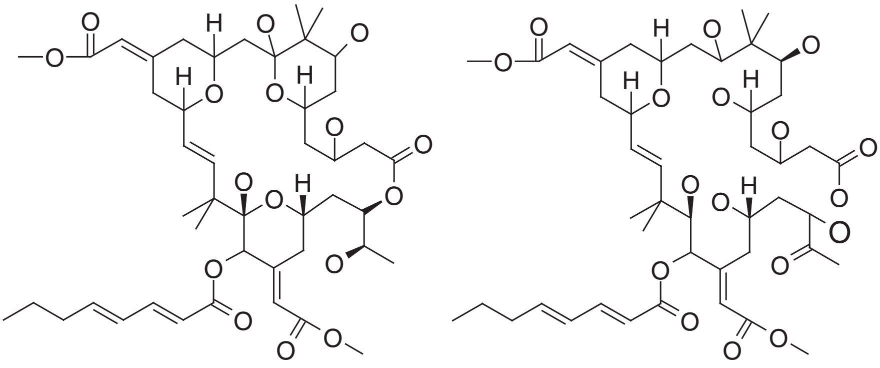

FIGURE 1 One flavor of bryostatin (left) has a molecular weight of 862 amu and has the bryophan ring all bryostatins contain. A potential precursor (right) with an exact mass of 882 amu.

16–22 years. Although not consistently, we did find the same molecular fingerprint for cinchonidine in these trees. We were not able to correlate the fingerprint with a specific part of the tree or a specific location, and this will be left to a separate study. The relatively low quantities we detected coupled with lack of consistency between different trees would account for dogwood trees not being actively pursued as a medicinal source when compared to quinine.

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The fourth parameter outlined in our research, to use the extracts from NOM to open up a new research area, centered on finding either a molecule from an extinct species or a precursor for a molecule of known medicinal value in the NOM. Before starting this work we compiled a list of freshwater and saltwater organisms that gave rise to Bryostatin (Fig. 1), isolated from the marine organism bryozoan, *Bugula neritina*. While there are over 5000 species of bryozoa found in fresh and salt water supplies globally, some saltwater species have been found to contain very low quantities of bryostatin. Bryozoa are millimeter-sized filter feeders that live in colonies. We hope to identify a water-soluble molecular species (see Fig. 1) ubiquitous in the marine environment that can be simply rearranged to form bryostatin. There have been more than 30 derivatives of the molecule bryostatin isolated and several have been shown to have strong anti-cancer properties [30–31].

In viewing MALDI-MS spectra of the MeOH/chloroform extract of our Suwannee River NOM samples, a group of peaks in the 840–900 amu range were constant and relatively intense (Fig. 2), which is intriguing because bryostatins weigh in the 800–910 amu range. With our extraction and separation procedure we would typically have a large number of mass spectral peaks in the low molecular mass range ($<$ 500 amu), but as molar mass increases, the number of molecular species decreased, and with the exception of the spectral features in the 800–900 amu region, there were no features consistently present. As Figs. 2(a) and (b) illustrate, in two different samples taken on the Suwannee River from different locations we routinely identified peaks at 882 and 877, and occasionally a peak at 887 amu.

CONCLUSION

In this work we positively identified DDT, stearic acid, and cinchonidine by LC-MS, and identified potential precursors to bryostatin by MALDI-MS. While the search

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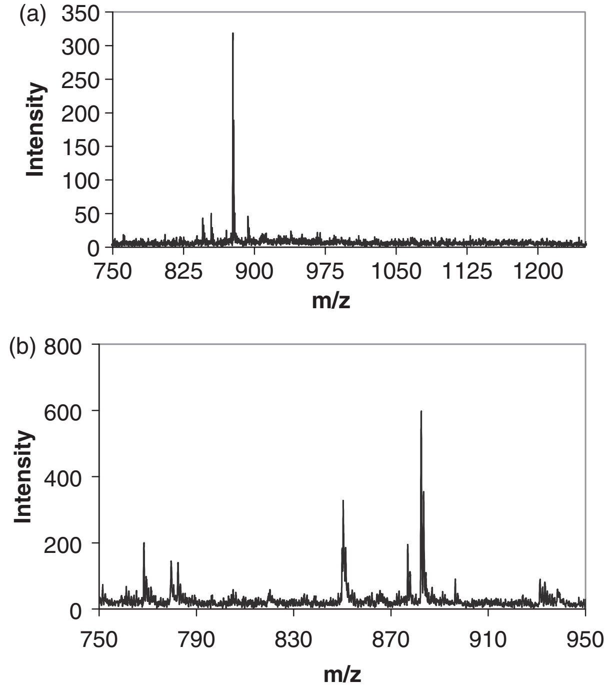

FIGURE 2 MALDI-MS of methanol/chloroform extracts of Suwannee River NOM. (a) Shows a typical scan of a different river sediment sample where the spectral features in the upper 800 amu range are evident; (b) Illustrates mass spectral features at 882, 877, and 854 amu.

for NPs centered on NOMs from a river basin, a range of other applications exist. For example, it is difficult to collect a large number of individual organisms from the ocean's depths, but a core containing NOM can be collected, extracted, and unique molecular structures separately identified. A range of other complex ecosystems, current or past, from tropical estuary systems to dried lakebeds, could be tested via NOM extractions. Many NPs, such as bryostatin with its bryophan ring, have molecular structures that synthetic chemists never knew existed. There may also be a number of potential NPs that are from organisms that no longer exist in an ecosystem (i.e. they are extinct, mutated, etc.); for example: molecular derivatives that have been structurally transformed chemically (i.e. by redox, pH or DO shifts, etc.), biologically (i.e. transformed by microbes, etc.), and physically (UV light, temperature changes, etc.), and therefore would not be found when analyzing a specific organism.

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