Natural Products from Marine Invertebrates: The Harbor Branch Oceanographic Institution Experience

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Abstract The Division of Biomedical Marine Research (DBMR) at Harbor Branch Oceanographic Institution, Inc. has been involved in the search for potential new medicines from marine orgasnisms for nearly 20 years. Deep-water marine invertebrates and the microorganisms associated with them have been the focus of this group resulting in the discovery of a number of novel compounds with therapeutic potential. Discoveries have been made in-house as well as in collaboration with academic groups, biotechnology companies and the pharmaceutical industry. The most significant discovery to date has been the polyketide, discodermolide. This potent anticancer agent is currently undergoing development by Novartis Pharmaceutical Corporation. As the supply issue is a major challenge in the development of many marine-derived compounds, the DBMR has several ongoing projects to address this issue. The DBMR will continue its discovery efforts through collection of invertebrates and microorganisms from deep-water environments as well as by establishing new collaborations with academic and industrial groups. The success of these efforts will further demonstrate the value of the marine environment as a source of bioactive natural products with therapeutic potential.

Keywords: *Submersibles, Discodermolide, Anticancer, Antifungal, Anti-inflammatory, Microorganism, Supply, Invertebrate cell culture*

The marine environment continues to provide a wealth of organisms from which to discover structurally unique bioactive secondary metabolites. Environmental pressures, including competition for space, light, and resources as well as extreme environments such as those found at depth, have driven the evolution of chemical diversity in a wide variety of benthic invertebrates and algae. Over the last decade, greater than 10,000 novel natural products have been described from the marine environment (Marinlit 2001). One compound Zixonitidine®, a potent analgesic derived from the cone snail, *Conus magus* has been approved for clinical use and at least five additional compounds derived from marine invertebrates are in clinical trials. Additional compounds are in the pipeline.

From its earliest inception in 1984 as an affiliate of the Biotechnology company SeaPharm through its current status as the Division of Biomedical Marine Research (DBMR), scientists at Harbor Branch Oceanographic Institution, Inc. (HBOI), have been involved in the discovery of potential new medicines from marine organisms. Use of the unique collection capabilities of the Johnson-Sea-Link (JSL) and Clelia manned submersibles allow DBMR scientists access to habitats and organisms not available by methods such as scuba, dredging or trawling.

The JSL submersibles, designed, built and operated by HBOI have the capability to dive to a maximum depth of 3000 fsw. Specially designed, interchangeable work platforms allow for either benthic or mid-water collections. Three tools on the manipulator arm allow for the retrieval of a variety of specimens: a standard claw hand can collect larger less fragile specimens; the clam scoop tool, a hydraulically operated cylinder, can encompass specimens and use the sides to cut through the specimen leaving it intact, and the vacuum tool allows for the collection of thin encrusting and smaller organisms. All samples are placed into either a large basket at the front of the submersible or into Plexiglas® containers mounted on a rotating platform where they can be maintained in sea-water for work on deck. In addition to collection of

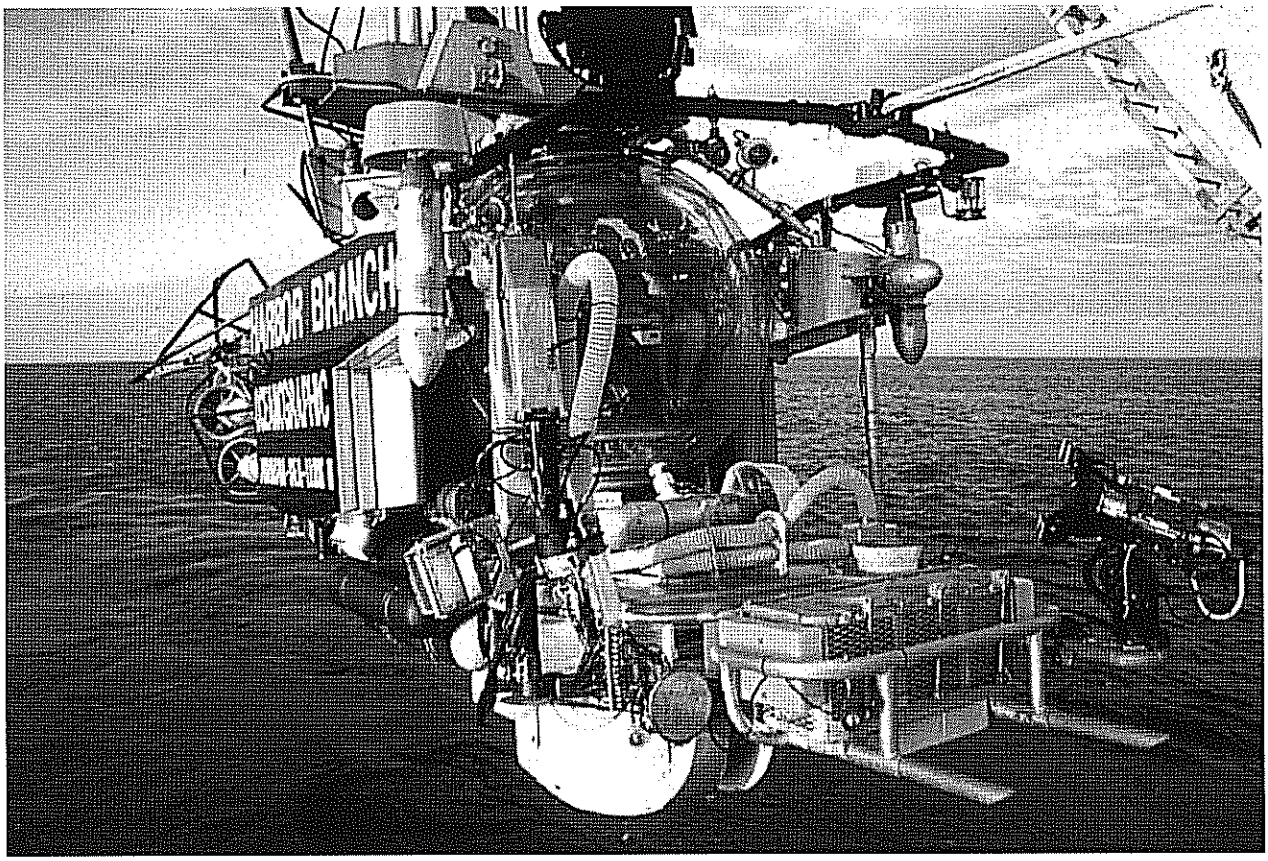


Figure 1.

samples in otherwise inaccessible regions, the submersibles are equipped to document habitat and other in situ collection parameters.

DBMR has conducted over one hundred research expeditions to collect specimens for drug discovery research. The DBMR sample repository contains over 30,000 frozen macro-organism specimens and 16,500 microbial isolates. A proprietary database records collection data including site descriptions (e.g. location, habitat, depth, temperature and conductivity) and biological descriptors (e.g. taxonomy, color, morphology, and observed associations). The database also tracks bioactivity data as the samples are tested. This information facilitates a rapid survey of the collection for identification of additional source organisms, related taxa, habitat and bioactivity data.

The Division also maintains a repository of the pure compounds isolated since the beginning of the program. A database containing the structure, source, depth of collection, bioactivity and patent information is also kept in conjunction with the library. This information can be provided when samples are transferred to collaborators for further evaluation.

The discovery program has evolved through the years. In the early years, all assays were cell based and the disease targets were cancer, anti-infectives and immune modulation. The majority of assays were run in house and included in vivo pre-screens of enriched materials to narrow the hit list. This program successfully identified a number of compounds with strong in vivo activity, but lacked significant information about the molecular target of the agents. In order to address this limitation an emphasis was placed on screening against a panel of molecular targets (enzyme and receptor binding based) many of which are known to be involved in cell cycle regulation. Our current screening regime encompasses both formats. Materials are screened through a panel of multi-drug resistant tumor cell lines and through a cell based reporter assay designed to detect compounds that block the cell cycle at the G2/M checkpoint.

A valuable supplement to our in house screening capabilities has been our on-going collaborations with pharmaceutical, biotechnology and academic groups which have provided a broad range of therapeutic areas in which to screen extracts. These areas include cardiovascular, central nervous system, antiviral and inflammation targets. These collaborations have ranged from further evaluation of purified natural products from our

collection to full bioassay-guided purification of natural products from leads identified in targeted screens.

Once a bioacctive extract is identified, the active components are purified using bioassay-guided fractionation. Typical isolation schemes include partitioning, column chromatography, and HPLC. At each step bioactivity is confirmed before proceeding with the isolation. Structures are determined through a combination of spectroscopic methods with a focus on NMR spectroscopy.

The mechanism of action of the active compounds is then investigated. Flow cytometric analysis, confocal microscopy, differential gene expression, and immunochemical chromatography using the natural product bound to matrix are all used to confirm or identify the molecular target of the active compounds.

To date the discovery team at DBMR has been successful in identifying over 200 compounds using bioassay-guided isolation. Of these, several compounds have exceptional bioactivity profiles and are undergoing pre-clinical and advanced pre-clinical evaluation in conjunction with pharmaceutical and biotech companies.

The most significant discovery to date is the polyketide, discodermolide. The compound was originally isolated in 1991 as an immunosuppressive compound from the deep-water sponge *Discodermia dissoluta* (Gunasekera et al, 1990, Longley et al, I and II, 1991). Further investigation indicated that the molecular target of discodermolide is the cytoskeletal protein tubulin and that it induces premature polymerization of tubulin and stabilizes the microtubules, blocking cell proliferation at the G_2/M (metaphase/anaphase) transition. This mechanism is similar to that of the clinically important compound Taxol®. Anti-mitotic activity for discodermolide was confirmed in vitro using cell lines for both breast cancer and Burkitts lymphoma (Ter Haar et al, 1996).

Discodermolide is structurally unrelated to Taxol® and to most of the other tubulin binding marine natural products. Although the mechanism is similar, discodermolide induces irreversible polymerization at much lower concentrations and temperatures and is also more effective than Taxol® against multidrug resistant tumor cell lines. Discodermolide was licensed to Novartis Pharmaceutical Corporation in 1998 and is currently undergoing development as an anticancer agent.

Recently we have identified another tubulin interactive compound. Dictyostatin–1 which was originally isolated in trace quantities from a *Spongia* sp. (Pettit et al, 1994). This compound was isolated in significantly greater yield from a sponge of the Family Corallistidae collected in Jamaica. Dictyostatin 1 stablilizes microtubules at nanomolar concentrations and has been found to be effective against Taxol® resistant lines expressing both PGP and MRP efflux pumps (Isbrucker et al, 2001).

The topsentin class of compounds, isolated from sponges in the genera *Spongosorites, Dragmacidon* and *Hamacantha* sp., are a series of bis-indole alkaloids having potent activity against neurogenic and immunogenic inflammation (Tsujii et al, 1988, Wright et al, 1992, Gunasekera et al, 1994), This activity was discovered in collaboration with Dr. Robert Jacobs at the University of California, Santa Barbara (Wylie et al, 1997). These compounds are being considered for both over the counter and prescription drug use.

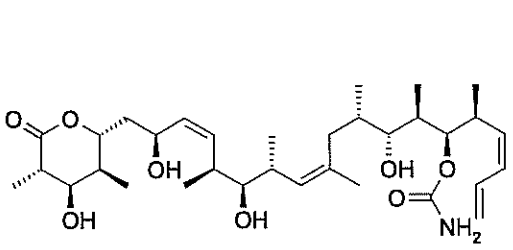
The lasonolides are a series of cytotoxic macrolides isolated from the sponge *Forcepia* sp. (Horton et al, 1994). *Forcepia* typically occurs in deep-water habitats, but can also be found in rare instances in shallow water. Lasonolide A has been screened by the NCI in the 60 cancer cell line panel. Analysis of the results using the COMPARE algorithm suggest that this compound acts by a novel mechanism and has been selected for further investigation. In projects funded by the Florida Sea Grant College Program, work is on going at DBMR to identify the molecular target using both immunochemical methods and differential gene expression. This sponge has also been successfully transplanted from deep water to shallow water and is the subject of an in-the-sea aquaculture study.

A multi-year STTR grant from NIAID allowed a collaborative research program to operate with MycoLogics Inc. (Denver, CO). MycoLogics runs proprietary screens for the identification of antifungal agents which affect the fungal cell wall. During the course of the research 3,500 extracts of marine invertebrates were tested for antifungal activity. Thirty-six of these were found to show sufficient bioactivity and chemical novelty to be studied further. Isolation and characterization of the active components has identified both known and novel compounds. Among the novel compounds are plakinic acid F, epiplakinic acid F and plakortolide F (Chen et al, 2000).

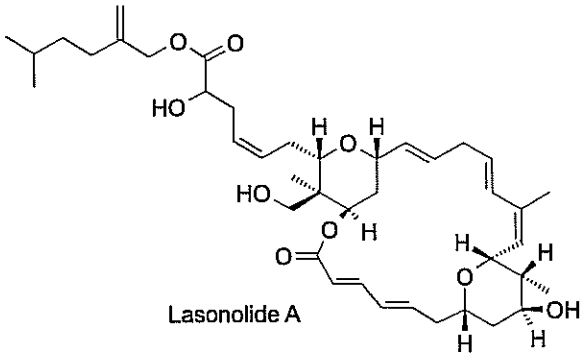
The manzamines are another series of sponge-derived alkaloids having both immunogenic and neurogenic anti-inflammatory activity. The neurogenic activity was demonstrated *in vitro* using the rat brain microglial model developed by Dr. Alejandro Mayer, Midwestern University, Downers Grove, IL (Mayer, A.M.S. et al, 1999). These compounds potently inhibit activated microglia superoxide anion and thromboxane generation with concomitant very low cytotoxicity, as measured by lactate dehydrogenase release (Mayer et al, Inflammation Res. 50(3): S207, 2001). Microglia are thought to play a key role in neuroinflammatory

Table 1. *DBMR compounds with therapeutic potential*

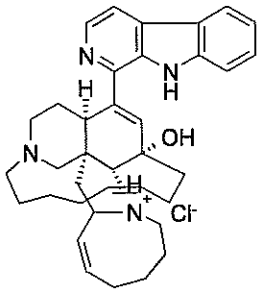
COMPOUND	*SOURCE*	DEPTH (M)	THERAPEUTIC AREA
Discodermolide	*Discodermia dissoluta*	160	anticancer
Dictyostatin	*Corallistes*	442	anticancer
Topsentin	*Spongosorites sp.*	174	anti-inflammatory
Lasonolide	*Forcepia*	19	anticancer
Plakortolide F	*Plakinistrella*	25	antifungal
Manzamine A	*Haliclona sp.*	10	anti-inflammatory



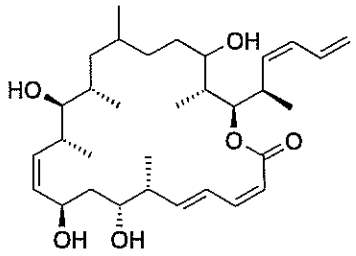
Discodermolide



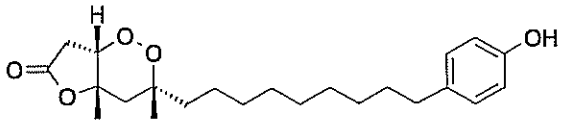
Lasonolide A



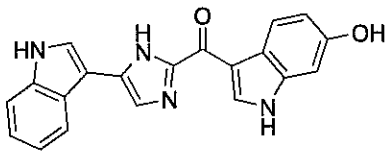
Manzamine A



Dictyostatin-1



Plakortolide F



Topsentin

processes and have been linked to neurodegenerative diseases such as Alzheimer's disease.

Providing adequate supplies of marine natural products remains a daunting challenge. There are currently several ongoing projects with the aim of identifying sustainable supplies of sponge-derived metabolites. For the past 13 years the Invertebrate Cell Culture Group has used a model sponge system to develop methods for establishing primary cell cultures and monitoring growth and production of bioactive metabolites. Cryopreservation methods allow for the storage of cell suspensions for subsequent laboratory studies using material from the original sample. In addition, a basal sponge cell culture medium has been formulated and can be supplemented with mitogens and antibiotics for the initiation of primary cultures (Pomponi et al, 1997). Multi-well plate assays have been established to monitor DNA, protein and esterase levels in primary cultures (Willoughby and Pomponi, 2000).

Aquaculture efforts funded by Harbor Branch and Sea Grant have provided base line data for the culture of both sponges and ascidians. Studies have allowed both sponges and ascidians to be grown in aquaculture tank and "in-the-sea" farming environments. Preliminary work has allowed a deep-water sponge to be grown in a shallow water marine environment allowing ready access to samples

without the use of a submersible and thus significantly reducing costs. Aquaculture tank experiments have focussed on defining the diet required by both sponges and tunicates to promote both growth and production of bioactive compounds. A recent appropriation from the Health Resources and Services Administration (HSRA) is being used to upgrade facilities to support a program for the aquaculture of biomedically important species.

The microbiology of sponges is proving to be increasingly significant in our drug discovery program. Many groups, including DBMR, consider the microbial population of the invertebrate to be the original source for the synthesis of many marine natural products. Although the goal of producing a complex sponge metabolite in a heterotrophic microbial culture has yet to be achieved this remains the driving force behind our microbiology program. The program can be divided into two major research areas: identifying the microbes present in the sponge-associated microbial community; and the cultivation of these microbes as a drug discovery resource. Although the proportion of the microbial population that is amenable to culture is very low (<1% of the total microbial population can be brought into culture) we have identified media modifications that have increased this number to as much as 3% for certain sponges (Olson et al, 2002).

Using molecular techniques such as DGGE (denaturing gradient gel electrophoresis) of the cloned 16S rRNA gene from the whole sponge community and sequence analysis of this gene we have been able to begin the elucidation of the uncultured bacterial community of the sponge. This information will be used in planning future isolation work: understanding what is not being cultured may allow specific techniques to be used which will allow another subset to be brought in culture.

The HBOI Marine Microbial Culture Collection currently contains approximately 16,500 cultures of marine-derived microorganisms (heterotrophic bacteria and fungi). Current NSF funding is allowing us to determine the diversity of approximately 20% of the collection using the 16S and 18S rRNA genes as taxonomic markers: RFLP (Restriction Fragment Length Polymorphism) using two restriction endonucleases is used as a preliminary screen to identify unique clones which are then sequenced and compared to closest matches in GenBank. The microbes held within the culture collection are a unique resource for drug discovery. Over 75% of the collection have been derived from sponges collected from diverse geographic locations and depths to 3,500 fsw. Microbes in the collection are routinely fermented and extracts prepared for screening through both in house and collaborator's screens.

One final supply option is to identify, clone and express the genes responsible for biosynthesis of bioactive compounds. This can be done from either isolated cell types or from the whole-organism "metagenome". In collaboration with partners at the University of Minnesota, Oregon State University and Florida Atlantic University, DBMR is currently working on recombinant production of a number of important compounds.

The HBOI drug discovery program has successfully identified and developed a number of novel marine natural products. This success has come through both in house research and collaborations with other academic groups, biotechnology companies and the pharmaceutical industry.

The DBMR will continue to explore the chemical diversity in the marine environment through collection of macro- and micro- organisms from deep-water environments. We will also expand our discovery efforts into new therapeutic areas by establishing new collaborations with academic and industrial groups.

As the supply issue continues to be the major challenge in the development of marine natural products, we will continue to address this issue on many levels: aquaculture of marine invertebrates; isolation and culture of marine invertebrate-derived microorganisms; culture of invertebrate cells; and cloning and expression of biosynthetic pathways. Such approaches, in addition to chemical synthesis of the natural products, will make marine natural products an even more attractive resource for drug discovery programs.

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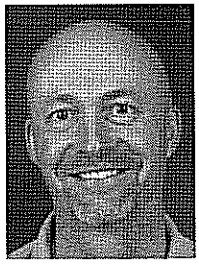
Dr. Susan Sennett has worked in the field of marine natural products for the last 16 years, investigating both ecological and applied roles for bioactive metabolites. As a graduate student at the University of Delaware she evaluated wound induced production of phytoalexins in halophytic plants. As a post-doctoral associate at Harbor Branch Oceanographic Institution she studied the utility of marine natural products as feeding deterrents and chemotaxonomic markers in sponges. Dr. Sennett has been a staff scientist in the Division of Biomedical Marine Research for 11 years, where she has been involved in several aspects of the drug discovery program. Her research focus in the Invertebrate Cell Culture group is directed towards the localization of bioactive natural products in marine invertebrates and their associated microorganisms.



Dr. Peter McCarthy was educated in England and received his Ph.D. in Microbiology from the University of Kent at Canterbury. Following a two year post-doctoral fellowship at Smith Kline Beckman working in the Natural Products Discovery division, Dr. McCarthy joined Harbor Branch Oceanographic Institution in 1985 and was responsible for the establishment of both the fermentation group and the antifungal screening program. He is currently the Head of the Microbiology Program and is responsible for the development of novel isolation methods, the maintenance of the HBOI marine microorganism culture collection, and the fermentation of marine microorganisms to provide extracts for the drug discovery program. Dr. McCarthy has over 35 publications in the scientific literature and is currently the President of the Florida Branch of the American Society for Microbiology.

Dr Amy Wright is the Director of the Division of Biomedical Marine Research at Harbor Branch Oceanographic Institution. Dr. Wright received her Ph.D. in Organic Chemistry from the University of California, Riverside, and has been working in the field of Natural Products Chemistry for over 20 years. She has contributed over forty publications in this area and is an inventor on 15 patents. Dr. Wright's responsibilities at Harbor Branch Oceanographic Institution have included the management of the in-house chemistry discovery program as well as a number of industrial contracts seeking to discover compounds active in the areas of cancer, fungal infections, viral diseases, inflammation, cardiovascular disease and central nervous system disorders.

Dr. Shirley Pomponi is Vice President and Director of Research at Harbor Branch Oceanographic Institution. Dr. Pomponi received her Ph.D. in Biological Oceanography from the University of Miami's Rosenstiel School of Marine and Atmospheric Science (RSMAS). Dr. Pomponi joined Harbor Branch in 1984, and from 1992 to 2002 directed the Division of Biomedical Marine Research in the discovery of novel, marine-derived chemicals with pharmaceutical potential. She has authored or co-authored more than 70 publications in marine biotechnology, biodiversity, cell and molecular biology, systematics and natural products chemistry, and is a co-inventor on several Harbor Branch patents. Dr. Pomponi currently serves on the National Academies of Science Committee on Exploration of the Seas, and is a member of the Scientific Advisory Panel to the U.S. Commission on Ocean Policy.