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DIVERSITY AND BIOACTIVITY OF MARINE SPONGES OF THE CARIBBEAN: THE TURKS AND CAICOS ISLANDS

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ABSTRACT
Sponges are among the most abundant groups of benthic marine invertebrates and are also known to produce a high diversity of bioactive compounds. As part of a research programme focusing on the discovery of marine natural products with therapeutic potential, we have studied 85 shallow and deep water habitats of the Turks and Caicos Islands. Approximately 500 samples of sponges were photodocumented, collected, taxonomically identified, and biologically and chemically assayed. All classes of the Phylum Porifera and all orders of the Class Demospongiae were represented, although their relative abundance and diversity varied with depth. Nine percent of all sponges tested were found active in one or more enzyme assays used to identify compounds with potential as antitumor or immunoregulatory drugs.

Keywords: bioactivity, biodiversity, deep water, Caribbean, natural products, Porifera, sponges, submersible, Turks and Caicos Islands

INTRODUCTION

Sponges are a significant component of tropical marine benthic communities. They are a source of nutrition for fish, turtles, echinoderms, and molluscs, and they provide refuge for a diversity of micro- and macro-organisms. The Caribbean shallow-water sponge fauna is among the better known of the world's sponge fauna (de Laubenfels, 1936; Hechtel, 1965, 1969; Wiedenmayer, 1977; Van Soest, 1978, 1980, 1984; Zea, 1983; Pulitzer-Finali, 1986). Our knowledge of deep water sponges of the Caribbean is scant, and is restricted to the work of Van Soest and Stentoft (1988) and the earlier works of de Laubenfels (1934) and Schmidt (1879, 1880).

In recent years, major initiatives have been established by academic, governmental and industrial research groups to discover biologically active compounds from marine organisms. This quest for new drugs focuses primarily on sponges, since they contain the widest diversity of biologically-active compounds of any marine phylum (Ireland *et al.*, 1993).

As part of an on-going program in the discovery of marine natural products with therapeutic potential, we have conducted a series of expeditions in the Caribbean region. A significant component of this research has been the documentation and identification of marine flora and fauna from both shallow and deep water environments. In addition, we are actively pursuing alternative methods for sustainable use of marine resources through microbial fermentation, marine invertebrate cell culture (Pomponi and Willoughby, 1994; Pomponi *et al.*, in press), and aquaculture for production of bioactive compounds.

In November 1994, we surveyed, documented, and collected marine invertebrates and algae from the Turks and Caicos Islands. Our preliminary results on the taxonomy, chemistry, and bioactivity of the sponges are described herein.

MATERIALS AND METHODS

Collections

Samples were collected in shallow water (0-45 m) by wading, snorkeling, and scuba diving; and in deep water (45-914 m) with the *Johnson-Sea-Link*

I (JSL) manned submersible and the Research Vessel *Edwin Link*. The JSL is equipped with a multifunctional manipulator arm which enables the collection of specimens by either a claw, suction tube, or scoop. Samples can generally be obtained intact, except for extremely fragile or brittle sponges. The JSL is also equipped with color video and 35-mm cameras for photodocumentation, and a data recorder to log temperature, salinity, and depth.

Environmental Impact

Collections by submersible and scuba were highly selective and were of minimal impact to the environment or species populations. Only one or a few individuals of each species were collected at each site with little or no disturbance to the habitat.

Site and Sample Documentation

For purposes of sample tracking, each sample was given a unique identifying number. Collection site descriptions, including latitude, longitude, habitat, depth, temperature, current, and weather conditions, along with sample descriptions, including morphology, color, abundance, taxonomy, and photographic reference for each sample were recorded in a fieldbook. All data were transcribed on board the ship into a proprietary database (Microsoft ACCESS).

Photography

All deep water (submersible) samples and some shallow water (scuba) samples were photographed *in situ*. Deep water samples were also videotaped *in-situ*. Each sample was also photographed in the ship's laboratory. Original 35-mm slide photographs and videotapes are archived at Harbor Branch Oceanographic Institution (HBOI). Duplicate copies of photographs and videotapes were also submitted to representatives of the Turks and Caicos Islands, Department of Environment and Coastal Resources, Grand Turk Island.

Taxonomic Reference Samples

Museum voucher specimens were subsampled from most samples and preserved in 70% ethanol. Some duplicate specimens were not vouchered. Museum specimens are stored in the Division of Biomedical Marine Research (DBMR) Reference Museum at HBOI. One duplicate set of museum specimens was submitted to representatives of the Turks and Caicos Islands, Department of Environment and Coastal Resources, Grand Turk Island.

Taxonomy

Sponges were identified in the field by evaluation of morphological characters, including microscopic analysis of spicules and/or fibres. Spicules were prepared using routine methods (e.g., Hechtel, 1965; Van Soest, 1980).

Chemical Extraction

Sub-samples (5-10 g) were extracted by pulverizing with a Virtis grinder in 100% ethanol for subsequent biological and chemical evaluation. The remainder of each sample was stored at -20°C in a portable freezer on board the ship and transfered to DBMR's freezers at HBOI.

Chromatographic Analyses

Most deep water samples were subsampled for subsequent analysis by thin-layer chromatography (TLC). Extracts of the organisms were prepared by crushing a 0.5 cm^3 piece of the organism in ethyl acetate-methanol (3:1). The extracts were chromatographed on silica gel plates using three different solvent systems to determine the relative polarity of the natural products present in the extracts. Nonpolar metabolites were detected using ethyl acetate-heptane (1:1); intermediate polarity compounds were detected using ethyl acetate-methanol (19:1); polar metabolites were detected using n-propanol-ethyl acetate-water (7:2:1). Plates were visualized by spraying the developed plates with a solution of 2% vanillin in sulfuric acid followed by heating. As with any such system, extracts which produce trace amounts of highly bioactive compounds were not detected by this technique.

Bioassays

cdc25A. cdc25A was prepared as a recombinant protein expressed in *Escherichia coli*. The phosphatase activity of the enzyme was assayed in a final volume of 200 µl in 96-well plates by the method of Baratte et. al. (1992). Each well contained 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, 15 mM DTT, 50 mM p-nitrophenylphosphate (pNPP) up to 10 µg recombinant protein and either test samples or controls. Plates were incubated at 37°C for 90 minutes and the liberated p-nitrophenol determined by the change in absorbance at 405 nm. The assay (U.S. Patent No. 5,294,538

assigned to Mitotix, Inc., Cambridge, MA) is used by HBOI under license from Mitotix.

Calcineurin. Calmodulin was prepared from bovine brain according to the method of Wallace *et al.* (1983). The calmodulin was either used directly or coupled to sepharose-4B to form the calmodulin-sepharose affinity column necessary for the isolation of calcineurin. Calcineurin was prepared from bovine brain by the method of Tallant et al. (1983), concentrated, aliquoted and stored at -80°C. Calcineurin activity was assayed in 96-well microtiter plates in a final volume of 50 µl. Each well contained 50 mM Tris-HCl pH 7.5, 0.5 mM $MnCl_2$, 0.05 mM $CaCl_2$, 1 mM DTT, 50 mM pNPP, 0.3 µg calcineurin, a five-fold excess of calmodulin, and either test samples or control compounds. Plates were incubated at 30°C for 60 min. Liberated p-nitrophenol was determined by the change in absorbance at 405 nm.

CD45. CD45 was prepared as a membrane fraction from Jurkat cells (clone E6-1). Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and L-glutamine, lysed in hypotonic lysis buffer (25 mM Tris-HCl, 25 mM sucrose, 0.1 mM EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM phenylmethane sulfonyl fluoride and 10 µg/ml leupeptin, pH 7.5) and centrifuged at 500 x g for 5 minutes. The supernatant was then centrifuged at 100,000 x g for 1 hr. The pellet, containing CD45, was resuspended in assay buffer (100 mM sodium acetate, 1 mM EDTA, pH 6.0) and stored at -80°C. The assay was based on that described by Imoto et al. (1993) and was performed in a total volume of 50 µl. The CD45 membrane suspension (1 µg protein/well) was incubated in assay buffer at 37°C with 2.5 mM o-phosphotyrosine as substrate. Test samples were included as required. The reaction was terminated by the addition of 5% $HClO_4$ (150 µl). Liberated inorganic phosphate was measured by the change in absorbance at 620 nm following the addition of 50 µl of color reagent (6N H_2SO_4, 1 mg/ml malachite green, 2.5% ammonium molybdate and 0.2% Tween 20).

Leukocyte Antigen Related Protein Phosphatase (LAR). Recombinant LAR was purchased from New England Biolabs. The assay was performed in 96-well microtiter plates in a final volume of 200 µl in LAR buffer (25 mM imidazole, 50 mM NaCl, 2.5 mM EDTA, 5 mM DTT, pH 5.5) containing 0.1 units of LAR, 100 µg/ml acetylated BSA and 12.5 mM pNPP with or without test samples. Plates were incubated at 30°C for 60 minutes at which point the reaction was terminated by the addition of 10 µl 1N NaOH. Liberated p-nitrophenol was determined by the change in absorbance at 405 nm.

Dipeptidyl Peptidase IV (DPP IV). Recombinant human DPP IV was produced through tran-

Table 1 Biological and chemical activity of sponges by Class and Order.

Class	Order	cdc25A	CAN	LAR	APN	Bioactive # (%)	TLC Active # (%)
Demospongiae							
	Homosclerophorida	1	2			3 (14)	2 (22)
	Astrophorida	1				1 (1)	9 (16)
	Lithistida		2			2 (3)	3 (5)
	Spirophorida					0	0
	Hadromerida					0	0
	Axinellida					0	7 (87)
	Agelasida					0	3 (75)
	Halichondrida	7	3			10 (26)	19 (65)
	Poecilosclerida				4	4 (13)	3 (30)
	Haplosclerida	7	4		4	15 (25)	3 (18)
	Dictyoceratida	2				2 (11)	5 (62)
	Dendroceratida					0	Not tested
	Verongida					0	2 (20)
Hexactinellida						0	1 (12)
Calcarea						0	1 (33)
Total		18	9	2	8	37 (9)	38

sient expression of a human DPP IV plasmid construct (kindly provided by Dr. Laszlo Takacs, AMGEN Corporation, Thousand Oaks, CA) transfected into COS-7 cells by electroporation. The peptidase activity of this enzyme was determined in phosphate buffered saline (PBS) in a final volume of 200 µl in 96-well plates. Each well contained 200 mM gly-pro-pNA as substrate, 10 µl of the recombinant DPP IV and test samples or controls. Liberated p-nitroaniline was determined by the change in absorbance at 405 nm following a 60 min incubation at 37°C.

CD13 or Aminopeptidase N (APN). Aminopeptidase N was assayed using an Enzyme-Linked Immunosorbant (ELISA) capture assay. Briefly, 96-well plates were coated with goat anti-mouse immunoglobulins (Sigma #M4169). Following an overnight incubation the plates were washed three times with PBS-0.1%Triton X-100. The secondary (capture) antibody was then added to the plate (100 µl/well, 3.7 µg/ml; anti-human MY7, Coulter Corporation) and incubated for 3 hours at room temperature. Plates were washed three times with PBS-0.1%Triton X-100. The source of Aminopeptidase N was the KG-1 cell line, 100 µl of an appropriate dilution of the KG-1 cell lysate was added to each well, incubated for 3 hours and then washed a final time. The enzyme assay was performed in PBS in a final volume of 200 µl with 200 µM ala-pNA as substrate and either test samples or controls. Liberated p-nitroaniline was determined by the change in absorbance at 405 nm following an 18 hr incubation at 37°C.

All enzyme assays were performed using either a Tecan 8051 or a Tecan Megaflex automated liquid handling system. Extracts of marine sponges were tested at 5 µg/ml for all except DPP IV and LAR which were tested at 50 µg/ml. The inhibition of enzyme activity found in the presence of extract was compared to that of an uninhibited control. Extracts which inhibited the enzyme >50% were retested in triplicate to confirm activity.

RESULTS AND DISCUSSION

Collection Sites

The cruise track and general location of dive sites are shown in Figure 1. A total of 85 dive sites (39 deep submersible sites, 46 shallow water sites) were sampled. Most of the scuba collections focused on the deep fore reef slopes and walls from depths of 20 to 45 m. At many of these sites, the top of the wall was between 15 and 30 m. The wall was transected at some sites by sand chutes and tunnels which allowed sediment from the shelf to flow onto the deep slope. In shallow water, various mangrove, *Thalassia* seagrass or algal beds were sampled at sites at Providenciales Island, North Caicos Island, West Caicos Island, South Caicos Island, Salt Cay and Grand Turk Island. Only a few shallow water reefs were visited. No sites were surveyed on the shallow flats of the Turks and Caicos Banks.

Deep water habitats from depths of 305 to 915 m were primarily rock and sand-mud slopes. Rock slopes (50-80°) consisted of smooth rock pavement or low ledges. Some were covered with thin sand or mud veneer whereas others were clean of sediment. Sand slopes (40-50°) consisted of coarse sand or shell hash to muddy-sand sediment. Some sand slopes had scattered boulders, and at one site off South Caicos, 3-15 m tall boulders were encountered. A steep or near-vertical wall was generally found from 75 m to approximately 150 m and then graded into a sediment and/or rubble slope. The base of the deep fore reef slope or escarpment generally occurred between 150 and 180 m. From 150 to 300 m, the abundance of samples decreased dramatically (Figure 2).

Physical Parameters

Surface water temperature averaged between 27.5 and 28.5° C and was fairly constant down to the thermocline, which ranged from 50 to 100 m. From the thermocline to 200 m, temperatures dropped approximately 10° C, from 28.5° C to 18° C, on the average. From 200 m to the maximum depth, the temperature gradually declined; the minimum temperature recorded was 7.02° C at 911 m. Surface salinity averaged 36.0 to 36.5 ppt, and increased to approximately 36.5 to 37.0 ppt near the thermocline. Below 200 m, the salinity gradually decreased to a minimum of 35.06 ppt at 911 m. Bottom currents recorded with the JSL submersible generally ranged from 0 to 0.1-0.2 knots, and occasionally increased to 0.3 knots.

Taxonomy and Biodiversity

Of the 667 samples of benthic macroinvertebrates and algae collected, the sponges comprised 73% (n=489 samples). The Class Demospongiae dominated both shallow and deep water environments, with only 5 Calcarea and 12 Hexactinellida collected. All orders of the Demospongiae were represented (Figure 3), although the most abundant groups were the

Astrophorida (17%), Lithistida (15%), and Haplosclerida (13%). The relative abundance of the groups varied with depth (Figure 3). The Hadromerida, Axinellida, Poecilosclerida, and Haplosclerida were most abundant at depths shallower than 150 m, while the Lithistida, Astrophorida, Halichondrida, and the Class Hexactinellida were most abundant at depths greater than 150 m. To date, 47 families, 91 genera and 67 species of sponges have been identified.

Chemical Screening - Thin Layer Chromatography

Deep water sponges collected by submersible were examined by thin layer chromatography to assess which organisms were the most productive in terms of natural products chemistry. In our experience, this has often correlated well with bioactivity (Table 1). Samples were scored in terms of their overall productivity of natural products. Those which produce either large amounts of one or two compounds as well as those which produce many different compounds were given the highest ranking. Sixty-one sponges were found to be of special interest (Table 1). They include specimens of the Homosclerophorida (*Plakortis*), Astrophorida (*Asteropus, Erylus, Geodia, Stellettinopsis, Poecillastra, Pachataxa*), Lithistida (*Racodiscula, Corallistes, Vetulina*), Axinellida (*Auletta, Phakellia*), Agelasida (*Agelas*), Halichondrida (*Axinyssa, Epipolasis,*

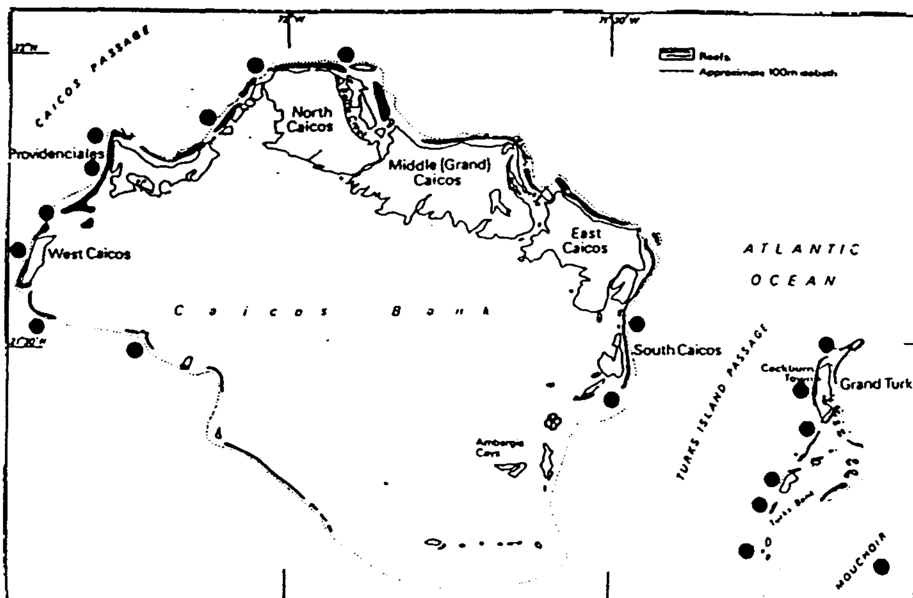


Figure 1. Map of the Turks and Caicos Islands with dots indicating location of study areas (from Wells, 1988).

Myrmekioderma, Spongosorites), Poecilosclerida (*Phlyctaenopora*), Haplosclerida (*Amphimedon, Strongylophora*), Dictyoceratida (*Ircinia, Sarcotragus, Smenospongia*), and Verongida (*Pseudoceratina*). The Orders Axinellida, Agelasida, Halichondrida, and Dictyoceratida showed especially interesting chemical patterns. In these orders, over 50% of all the specimens analyzed were rich in natural products chemistry (Table 1). Lithistid sponges have been reported to be the source of many interesting bioactive compounds, however, in this study only 5% of the lithistid sponges showed interesting TLC patterns. This low hit rate in our chemical screen is probably due to the stony nature of these specimens and corresponding low concentration of organic material in the small samples that were analyzed.

Biological Activity

Our primary screening programme focuses on two major therapeutic areas: cancer and immunoregulation. There are two general approaches to identifying extracts that are of interest: (1) testing for compounds which affect

Figure 2. Distribution of sponges with depth. Note marked decrease in sponge populations from 150 to 300 m.

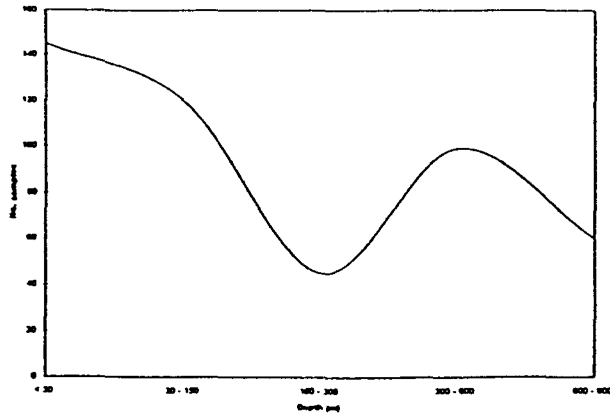
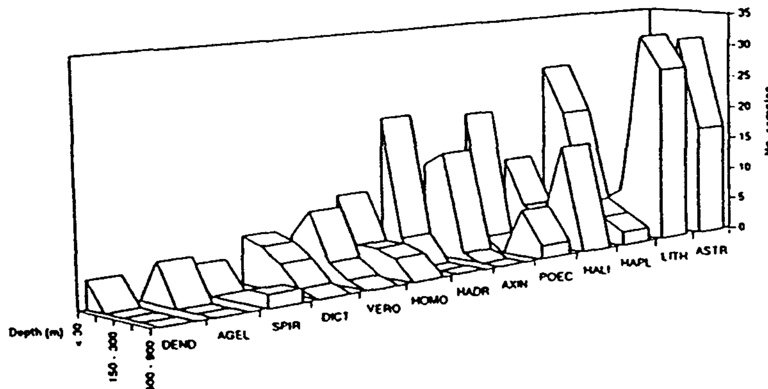


Figure 3. Distribution of Demospongiae with depth. DEND, Dendroceratida; AGEL, Agelasida; SPIR, Spirophorida; DICT, Dictyoceratida; VERO, Verongida; HOMO, Homosclerophorida; HADR, Hadromerida; AXIN, Axinellida; POEC, Poecilosclerida; HALI, Halichondrida; LITH, Lithistida; ASTR, Astrophorida.



cells growing *in vitro* or (2) testing for inhibitors of certain enzyme and receptor targets. We use a combination of these approaches, however, in which we emphasize the use of enzyme and receptor targets in our primary screening programme followed by *in vitro* evaluation of pure compounds. Our primary tests identify extracts that affect proteins which are known to be important for the proliferation of cancer cells or the activation of immune cells. Our secondary tests study the effects which the pure compounds isolated from the extracts have on living cells.

cdc25A. cdc25A is a tyrosine phosphatase which activates the G_2/M transition of the cell cycle. Inhibitors of this enzyme may be able to block the cell cycle at this transition point and produce cell cycle arrest (Baratte et al., 1992). The compounds discovered will have antimitotic activity and may be useful in the treatment of cancer.

Calcineurin. Calcineurin is a serine/threonine phosphatase which is critical to the signal transduction pathway in mammalian cells. Based upon the activity of immunosuppressive agents such as cyclosporin and FK506, which affect the activity of calcineurin (Fruman et al., 1992), we expect that inhibitors of this enzyme will have immunosuppressive activity.

CD45. CD45 is a family of cell surface receptors expressed as the major surface component of lymphoid and myeloid cells. The cytoplasmic portion of the molecule has protein

tyrosine phosphatase activity. CD45 is a critical component of the signal transduction pathway which leads to T-cell activation (Trowbridge, 1991). Inhibitors of CD45 are therefore expected to have immunosuppressive activity.

LAR. The leukocyte antigen related protein tyrosine phosphatase (LAR) is a member of the CD45 family. Some cells which have transformed to a cancerous state express higher levels of LAR (Zhai et al., 1993). Although the precise role of this enzyme in cancer has not been defined, specific inhibitors of this enzyme may have potential as anti-cancer agents.

DPP IV: Dipeptidyl peptidase IV (DPP IV) is a serine protease that is involved in the induction of interleukin 2 (IL-2) and interferon by T-lymphocytes (Schonn et al., 1989). It is expected that inhibitors of DPP IV will have immunosuppressive activity.

CD13 or Aminopeptidase N (APN): APN is a membrane bound aminopeptidase which is over-expressed in a number of human tumors. Inhibition of the activity of this enzyme inhibits growth of cell lines which overexpress this enzyme (Ino et al., 1994). Inhibitors of APN may have use as antitumor agents. Extracts of thirty-seven sponges (9% of all sponges tested) were active (Table 1). Only those samples that were tested twice and produced over 50% inhibition each time were considered active. Bioactivity was restricted to 7 of the 13 orders of Demospongiae. The greatest level of activity was found in the Halichondrida and Haplosclerida (26% and 25% of all samples tested in their respective orders). The cdc25A assay had the highest level of bioactivity (4.2% of all sponges tested), followed by calcineurin (2.1%), aminopeptidase N (1.9%), and LAR (0.5%). None of the samples tested showed inhibition of CD45 or DPP IV.

DISCUSSION

The results of this preliminary study of the biodiversity and bioactivity of sponges from the Turks and Caicos Islands support the hypotheses that sponges are a dominant component of both shallow and deep water environments in the Caribbean, and that they produce secondary metabolites with a relatively high level of bioactivity. Our knowledge of the structure of deep water sponge communities is scant and limited to the general observations made in the older literature about hexactinellid and lithistid sponges. More recently, Van Soest and Stentoft (1988) recorded differences in sponge abundance and diversity, but these were based upon two dredge-transects off Barbados. Based on the 39 submersible transects around the Turks and Caicos Islands, we provide further evidence for distinct differences in sponge species richness and composition with depth. We emphasize that this study is preliminary, however, the detailed site data we have collected, as well as more in-depth taxonomic analyses will facilitate assessment of sponge community structure with depth, habitat, and locality.

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