:) Young leaves have unique compounds not found in mature leaves. In addition to having higher concentrations of chemical defense metabolites, young tropical leaves frequently have different secondary metabolites than mature leaves (Kursar *et al.* 1999). For example, our ICBG work with alkaloids, an important class of medicinal compounds, showed a greater occurrence of unique compounds in young leaves (Kursar *et al.* 1999). We contrasted the number of alkaloids present in young and mature leaves of the same plant using TLC and Dragendorff's reagent. Ten out of 18 species had alkaloids that were found in the young leaves and absent in mature leaves, while only three species had alkaloids unique to mature leaves (Figure 5). Among the 24 alkaloids that were unique to either young or mature leaves, 71% occurred only in young leaves and 29% occurred only in mature leaves (data not shown). HPLC analyses of the major peaks from alkaloidal extracts of young and mature leaves of 23 species showed 60 peaks unique to young leaves and 40 unique to mature leaves (Kursar *et al.* unpublished).

Young leaves have also provided our ICBG with most of our bioactive compounds. We have pursued isolation of compounds from 15 species that were active in our bioassays. In two cases the active compounds were present in both young and mature leaves, but in 13 species the compound was absent or at such low concentrations in mature leaves that detection of activity and purification were only possible from young leaves. In November 2002 we obtained a provisional patent for compounds extracted from young leaves that are active against leishmaniasis. Five of our seven Submitted In Preparation Submitted Thus, we suggest that much of the chemical diversity lies in young leaves, and that this promising source remains largely untapped.

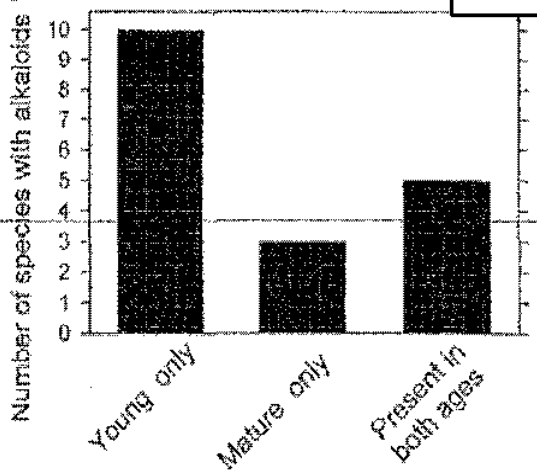


Figure 5. Alkaloid analysis of young and mature leaves from the same individual for 18 species of Panamanian plants. The number of species in which one or more alkaloids were found only in young or only in mature leaves, and the number of species in which all alkaloids were found in both aged leaves are indicated. The presence of alkaloids was determined by TLC of alkaloidal extracts and detected with Dragendorff's reagent. Data from Kursar *et al.* 1999.

d) Biological activity is higher in extracts from fresh as compared to dried leaves. In this project, we have been extracting fresh samples to ensure a higher quality of extracts and greater repeatability. To test the hypothesis that fresh samples have higher bioactivity, we collected a leaf sample and immediately extracted half of it following methods described below. The other half of the sample was subject to a mild air-drying treatment (4 days in the shade) to mimic typical collection techniques. Dried leaves were then extracted using identical methods as for the fresh leaves. This comparison was made for both young and mature leaf samples from over 100 Panamanian species in assays with *Artemia salina* and *Bacillus subtilis*. In general, extracts of fresh leaves had higher activities, with the difference being particularly dramatic for young leaves (Figure 6).

The comparison of particular interest to us is between our approach, with fresh young leaves, and more conventional approaches, with dried mature leaves (Figure 6). For both bioassays, our approach yielded significantly greater activity. In the case of *Bacillus*, fresh young leaves were 2.7 times more active than dried mature leaves. Results for *Artemia* were similar. Therefore, by combining our ecological approach with rapid extraction of fresh leaves, we are substantially increasing our ability to find active compounds.

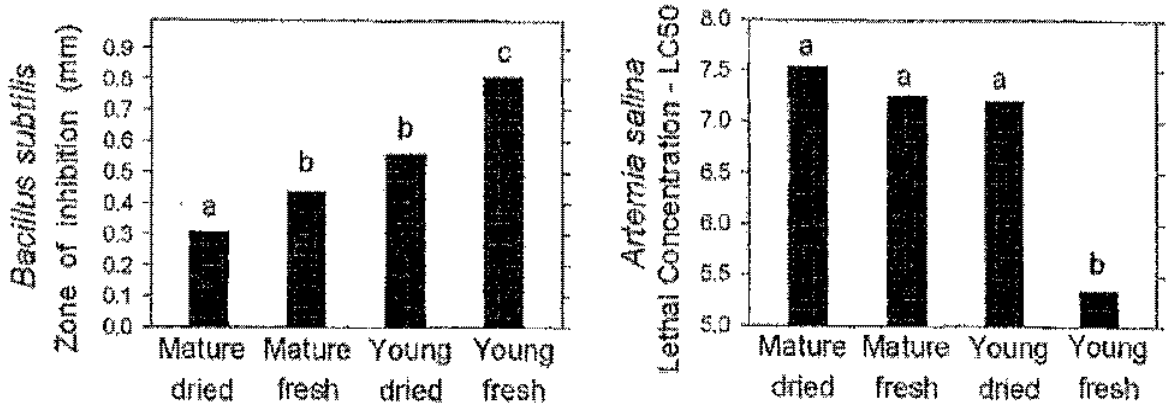


Figure 6: A. Inhibition of *Bacillus subtilis* and *Artemia salina* by extracts from fresh and air-dried samples of young and mature leaves of 104 species. For *Bacillus*, filter paper disks containing extracts were placed on a bacterial lawn and zones of growth inhibition measured. A larger zone of inhibition indicates greater toxicity. For *Artemia*, extracts were dissolved in media in 96-well plates. A lower LC_{50} (mg dry leaf/mL) indicates greater toxicity. Values with different letters are significantly different at $p<0.01$ by both ANOVA and paired t-tests. (Data from Kursar *et al.* 1999).

C.1.2. Collection Sites

As mentioned previously, Panama has an exceptionally rich flora due to strong climatic gradients, geological diversity and its unique position as a corridor between Central and South America. This puts researchers in the enviable position of being able to sample extraordinarily high diversity over small spatial scales (*i.e.* high beta diversity). For example, the beta diversity of plants in Panama is extremely high compared to forests in Ecuador and Peru (Condit *et al.* 2002), with major floristic variation being found in only 60 km across the Isthmus of Panama (Pyke *et al.* 2001).

To date we have made collections at 8 main sites that represent a diversity of habitats and floristic elements (Figure 7). These include several National Parks, Nature Monuments, Protected Forests and Forest Reserves as well an International Park and World Heritage Site. Most of the flowering plant diversity (84%) is in the humid and wet lowland rainforests (D'Arcy 1987), so it was here that much of our initial collecting efforts were targeted. Now that we have established an efficient collection system, and have developed techniques for initiating extractions while still in the field (see Methods, D.3), we have started to sample more distant sites. The lowland sites are distributed from eastern to western Panama to ensure collections of both Central and South American species, as well as from the Atlantic to the Pacific coasts to sample forests across a rainfall gradient. Several sites are in dry forests, one of the most endangered habitats in Central America. In addition, we have collected in four highland sites, two in Western Panama and two sites in Central Panama that are particularly threatened, Cerro Campana and Cerro Azul.

C.1.3. Collections and Recollections

The Panamanian flora is one of the best known tropical floras in the world (D'Arcy 1987, Croat 1978, Gentry 1990, 1993b) so we have had few problems with identification. All plant identifications are made by our excellent team of field botanists (Rafael Aizprua, Blanca Arauz, Nayda Flores) in consultation with Professor Mireya Correa, Director of the herbaria at both the University of Panama and the Smithsonian (see Letter of Support). The botanists have collected 156 families, 629 genera and 1189 species from October 1998 to October 2002. This includes 32 species of non-flowering plants. For 315 species they collected both young and mature leaves. In addition, 105 species with promising activity have been recollected in larger quantities. For each collection, they record the exact location with GPS, photograph the plants, collect voucher specimens, and note

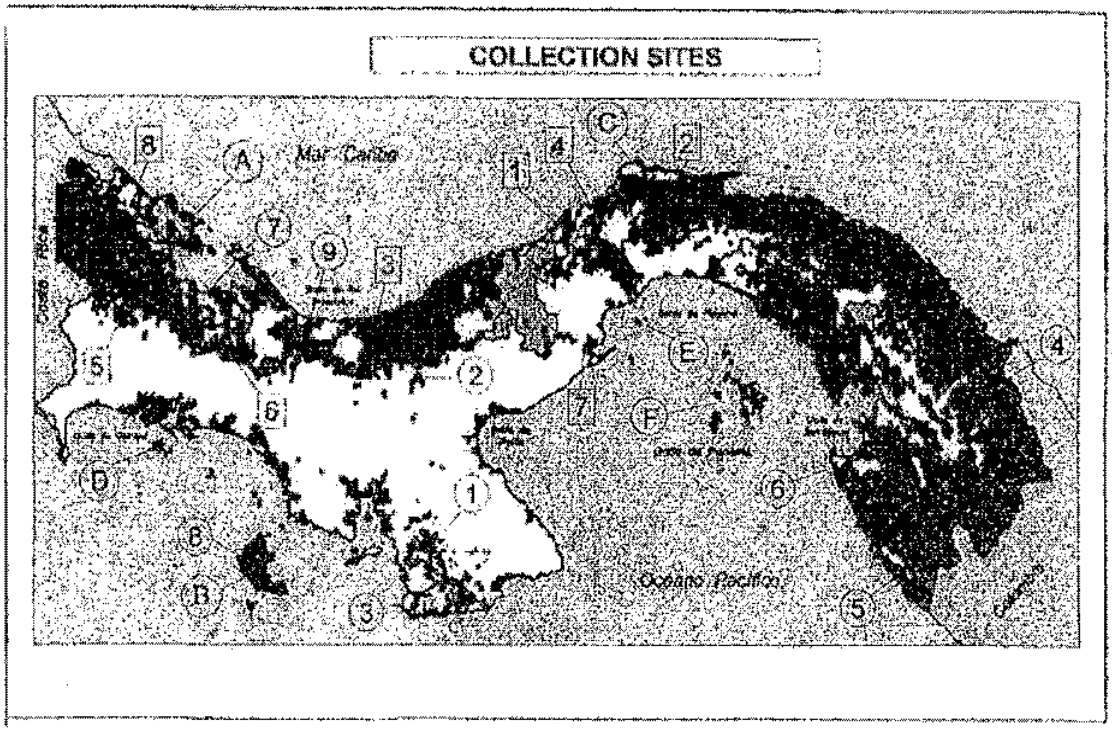


Figure 7. Map of the Republic of Panama indicating forested areas (in gray) and sites where collections have been made (squares). Additional sites where we propose to collect are also marked (circles). Marine sites are indicated by circled letters. A summary of the site characteristics and the number of species collected to date at each are listed below. More detailed site descriptions are in Appendix 8.

Sites collected to date (squares):

1. Barro Colorado Island Nature Monument: <100 m, semi-deciduous forest, 492 species
2. Chagres National Park and Forest Reserve: 1000 m, wet forest, 249 species
3. Omar Torrijos H. National Park: 800-1300 m, premontane to montane forest, 89 species
4. Soberanía National Park: 20-200 m, moist forest, 129 species
5. Volcan Barú National Park: 1800-3474 m, premontane to montane forest, 37 species
6. Fortuna Hydrographic Reserve: 1000-1500 m, montane rainforest, 28 species
7. Altos de Campana National Park: 400-850 m, montane forest; 275 species,
8. La Amistad International Park: 3000 m, premontane to montaine rainforest, cloud forest, paramo; 45 species

Future collection sites (circles):

1. El Montuoso Forest Reserve (Las Minas): 800 m, premontane
2. Los Pozos de Galobre Nature Monument (Santa Fé): 800 m, moist lowland-premontane forest.
3. Cerro Hoya National Park: 0-1500 m, montane to premontane rainforest
4. El Canglón Forest Reserve: 200 m, lowland moist forest
5. Darién National Park, Biosphere & World Heritage Site: 0-1845 m, evergreen wet forest
6. Chepigana Forest Reserve (Garachiné): 200 m, dry forest
7. Pato Seco Protected Forest: 200-2000 m, very wet forest
8. Coiba National Park: 0-416 m, moist tropical forest, very moist premontane forest.
9. Santa Fe National Park: 800 m, moist lowland-premontane forest

Marine Collections sites (circles):

A. Isla Bastimentos Marine National Park; B. Coiba National Park; C. Portobelo National Park; D. Gulf of Chiriquí National Marine Park; E. Taboga and Urabá Wildlife Refuge; F. Pearl Islands

Map Source: (ANAM) http://www.anam.gob.pa/proyectosficha/cboscosa_1992.jpg

pertinent ecological data (see Appendix 7). In the field, our botanists are generally able to identify plants to family and genus, with the vouchers being used to confirm species identities in the herbarium. Often, these vouchers lack reproductive structures, so plants are marked and monitored so that flowers and fruits can be collected and a definitive identification made. Permanent vouchers are stored in herbaria at both the University of Panama and the Smithsonian Tropical Research Institute in Panama.

To minimize the ecological impact of plant collections, we initially collect 20 g fresh weight of leaves. Leaves are harvested by hand and stored in ziploc bags in a cooler during transportation to the laboratory for extraction (see below). Once a species has shown promising bioactivity, we make a larger collection (approximately 500 g) for bioassay-guided fractionation. A significant challenge in any drug discovery program is the ability to quickly recollect samples with promising activity and to ensure that the recollections retain the activity of the original sample. Our program in Panama has several advantages that ensure both rapid and reliable recollections. Firstly, AP1 is based in Panama. Secondly, field sites throughout Panama are relatively accessible, so return collection trips can be made efficiently. Thirdly, because our extraction procedure uses fresh samples and all processing follows strict laboratory protocols, differences in extraction are unlikely to cause loss of activity. And finally, because our botanists are trained at vegetative identification and do not depend on short, infrequent flowering events, accurate recollections can be made at any time of year. Although there is some seasonality to the production of young leaves, leaf production in the understory is much more continuous.

To date, AP1 has been asked to recollect 105 species that showed promising activity in the bioassays. All of these were successfully recollected, most within a few weeks of the request. In some particularly urgent requests, recollections were made the next day. Over 82% of the species we recollected maintained similar activity to the originals. Thus, our recollection success has been particularly high.

C.1.4. Extraction Procedures

We have made three types of extracts: organic extracts for the cancer, tropical disease and HIV bioassays in Panama, protein extracts for the agricultural bioassays in Panama, and detannified, fractionated samples for testing by Novartis in their high-throughput anticancer screens. All of the samples that have been collected to date were immediately extracted and stored at –70°C until needed for bioassays. Detailed methods of our extraction procedures are described below in Section D.4.

C.2. Data Management

The ICBG project has developed an information system specifically tailored to our needs for maintaining records of plant collections, transfers of samples and bioassay and chemical results. Our goal was to make the system easy to use and to have the data readily available to all members of the Panama ICBG. We have developed a relational database that stores the information and also permits the members of the project to introduce new records, update or modify records, and export and import the bioassay results.
The information system is divided in two: a local database located in the AP1 laboratory and a password-protected database accessible via the Internet. The local, AP1 database is written in Microsoft's Visual FoxPro 6.0 and is the main repository of all the data. It maintains records of plant collections, transfers of samples, chemical information and bioassay results. The internet-accessible database is in PHP4 and uses MySQL as a database. It is updated weekly from the AP1 database, making it possible for all members of the Panama ICBG to obtain the species names and other collection information associated with each sample number, download bioassay results, and look up bioassay results of the other Associate Programs. For security reasons, critical information on chemical structures of active compounds will only reside in the AP1 database, but any member of the Associate Programs can consult this information by a) visiting AP1 to use the computer at the Smithsonian, or b) requesting that the website administrator send this information as an attachment to an email. Currently the Associate Programs send their results to AP1 data managers in Excel format via email, or via the internet and these are loaded into the centralized database. Future improvements of the applications and databases are explained in more detail below in Section D.11. We also have an informative public webpage for

the project, with general information about the ICBG-Panama project, the scope and goals of the program, plant species collected and information on collection sites (http://www.icbgpanama.org/).

C.3. Training and Outreach

C.3.1 Training: A major goal of our ICBG is to provide training to Panamanians. In the last four years, AP1 has provided on-the-job training, student internships and volunteer opportunities for 13 biologists and 9 chemists. Seven students completed or are currently conducting their undergraduate thesis research using AP1 resources and guidance, and four have received Master's degrees in Panama. An additional two students have gone abroad for a Master's program, and a third was just accepted into a Ph.D. program in Spain. In addition, one Canadian is finishing her research for a Master's degree from McGill and one US volunteer was just accepted into a Ph.D. program in the US. We have given many other students from Panama and abroad the opportunity to go on botanical collecting trips. We also encourage the full-time AP1 chemists and botanists to study English and to pursue educational opportunities such as short specialty courses or international scientific meetings. We provide release time and in some cases funds.

C.3.2 Outreach: A key component of our ICBG is to provide information about biodiversity to the public, and we have found that giving presentations is an extremely effective and inexpensive means of accomplishing this (see AP 4). Perhaps one of the most successful outreach projects that members of AP1 have initiated is to give talks to elementary and high schools. We have given 13 of these in the last year, and they are extremely well received. Not only do the talks convey information about the value and uses of biodiversity, but they also provide students with role models for continuing in biology. The botanists have also given numerous impromptu and formal talks to park guards and officials at the National Authority of the Environment (ANAM), and one gives weekly nature tours for Panamanian visitors to Barro Colorado Island. In the last year, AP1 members gave 3 talks at international meetings, and 4 talks to citizens, park employees and naturalist guides. Drs. Coley and Kursar also regularly give talks to the scientific community and the general public in the US, Panama and other countries. Additional outreach activities have occurred in all of the Associate Programs.

C.3.3 Use of biological inventories: Our inventory efforts are expanding knowledge of the distribution of plants, a service for both botanists and conservationists. Although the flora of Panama is extremely well known for a tropical flora, many of the parks and protected areas have never been inventoried. The lack of information is particularly marked for the sites in eastern and western Panama. However, even for well known areas we have been able to make contributions. For example, the Barro Colorado Island Nature Monument administered by the Smithsonian, is arguably the best studied tropical forest in the world. Yet two new botanical records were reported by our AP1 collectors. In addition, they were able to collect three species, previously reported from Panama, but for which no voucher specimens existed in Panama. Our fertile voucher collections are now deposited at STRI and the National Herbarium at the University of Panama. One apparently new species of *Symplocos* (Symplocaceae) was collected and has been sent to taxonomic experts for verification.

C.3.4 Strengthening infrastructure: We have also provided technical assistance to the National Authority of the Environment (ANAM), the Panamanian authority responsible for protected areas, on many occasions. Our database containing information on botanical collections is available to them through the online database and application on the Internet. For example, they used our inventories in the recently funded project entitled "Panama Atlantic Mesoamerican Biological Corridor" (CBMAP; http://www.biodiv.org/events/cbmap.asp), a $9 million implementation of the goals of the Convention on Biological Diversity, funded by Private Source. The botanists have also served on several ANAM committees to identify rare and endangered species (Listados de especies en Peligro para la Flora de Panamá, 2002). They have helped design 4 km of educational nature trails in Torrijos National Park and have identified and marked plants along the trail. And finally, our database manager Lorna Sanchez, in consultation with Dr. Todd Capson, has created a computerized system for ANAM so they can approve and track permits. These include permits for scientists as well as commercial logging operations. ANAM has been very pleased with the database and, in November 2002, held a press conference to inaugurate the system. ANAM has indicated that the permit database places them in the forefront of developing nations and should greatly reduce permit abuse and hence illegal or overzealous logging. This database system is described in more detail in AP4.

We are also helping a grass-roots effort to promote conservation. Peace Corps volunteers and the community of Santa Rosa, which borders Soberania National Park, are trying to develop ecotourism such that the local communities can benefit from protected areas. We were asked to help them establish nature trails and provide interpretive information on the ecology and natural history of the area.

C.4. Preliminary Results from Endophytic Fungi

The fungi have been called a "hyperdiverse" group of organisms, and perhaps the most diverse group within the fungi is the endophytic fungi (Hawksworth 1991, Arnold *et al.* 2000, 2001). Endophytes are ubiquitous, may number well over a million species, and probably represent several independent evolutionary lines including symbiotic, pathogenic and saprophytic ancestors (Verhoeff 1974, Hawksworth 1991, Petrini 1986).

One striking attribute of endophytic fungi is the prevalence of chemically mediated interactions with their host plants, other endophytes, herbivores and pathogens (Clay 1990, 1992, Breen 1994, Saikkonen *et al.* 1998). Additionally endophytes have been noted as a proven source of novel secondary metabolites (Gusman and Vanhaelen 2000, Guo *et al.* 2000, Wilson *et al.* 2000, Brady *et al.* 2000, 2001, Schulz *et al.* 2002).

Tropical endophytes may be a particularly promising group to screen for bioactivity because they are more diverse (Arnold *et al.* 2000, 2001), less studied, and more likely to contain potent secondary metabolites than temperate species. In addition, since endophytic fungi are polyphyletic (Verhoef 1974, Petrini 1986), having arisen from many different ancestral taxa, there should be a diversity of chemical classes expressed within the group. Thus, we suggest that the enormous unexplored diversity of tropical endophytes may provide many novel leads.

We initiated collections of endophytic fungi to be tested in screens with our original industrial partner, Monsanto. However, when they merged with Pharmacia-UpJohn, the natural products division was terminated, and we stopped our collection efforts. During the year we were making collections, we isolated endophytes from young, mature and senescent leaves of over 200 plant species. From surface sterilized leaf pieces (1x2 mm) we cultured 1250 pure isolates on 2% malt extract agar and classified 367 species based on morphological traits (see Bills 1996, Arnold *et al.* 2000 for methods). We found that over 95% of the leaves and 74% of the leaf segments were infected. We also found extraordinary diversity compared to temperate systems (Arnold *et al.* 2000, see also Lodge *et al.* 1996, Bayman *et al.* 1997, Arnold *et al.* 2001). Information on each isolate was entered into a data base to determine morphospecies and included traits such as colony color, texture, margin, shape, growth rates and presence of aerial mycelium or reproductive structures. Classification based on morphology showed high congruence with molecular classifications based on DNA sequence data of ITS-1, ITS-2 and the 5.8s gene (Arnold 2002). For long-term storage of fungal strains, we transfer agar blocks containing mycelia excised from the growing margin of pure cultures to sterile plastic cryovials with distilled water (Burdsall and Dorworth1994, Qiangqiang *et al.* 1998).

C.5. Preliminary Results from Marine Plant Collections

The Gerwick research group at Oregon State University has embarked on an unsupported collaboration with the Panama ICBG over the past year with the goal of contributing a new marine component to the ICBG in its anticipated renewal period. This group has focused in recent years on the extraordinary rich chemistry of the marine cyanobacterium *Lyngbya majuscula*, and includes such notable findings as that of curacin A, a novel and potent antitubulin agent (Hamel *et al.* 1995, Blokhin *et al.* 1995), from a Curaçao collection, the novel molluscicidal agent barbamide (LC_{50} 10.0 μg/mL against the freshwater snail *Biomphalaria glabrata*, Orjala and Gerwick 1996), and antillatoxin, a new voltage-gated sodium channel activator (Orjala *et al.* 1995; Li *et al.* 2001). Another *L. majuscula* strain, collected from Hector Bay Jamaica, and now cultured at OSU, yielded hectochlorin (Marquez *et al.* 2002), a potent antifungal agent which causes hyperpolymerization of actin and had greatest potency in the colon, melanoma, ovarian and renal panels in the *in vitro* 60-cell line screen at the National Cancer Institute (overall average GI_{50} = 5.1 μM). Interestingly, hectochlorin was also isolated from a red *L. majuscula* strain collected from Bocas del Toro, Panama (Marquez *et al.* 2002).

Preliminary efforts to collect marine algae in Panama have been rewarded with promising results. Excursions to assess algal diversity and biomass around Coiba Island, Bocas del Toro and the Portobelo region

(Figure 7) have revealed an exciting diversity of species (we have photographed twelve different cyanobacterial specimens on one snorkeling excursion in Bocas del Toro), with biomasses well in excess of that required for large (2 L) collections of several marine cyanobacteria. Several small algal collections obtained in September and October 2002 have been worked up, and the majority of the crude extracts show activity in preliminary tropical disease and brine shrimp bioassays. Three pure compounds that were previously known, as well as two novel peptides that are under continuing investigation, have been isolated from one of the *L. majuscula* strains collected at Bocas del Toro (see details in AP3, Section C.2.1.).

C.5.1. Collections and Cultures

Collections of marine algae have been made at two sites in the Caribbean (Figure 7). In September 2002, a total of sixteen algal samples were collected from four different locations near Portobelo using SCUBA and snorkeling (Isla Drake, Hidden Cove, Playa Francaise, Three Sisters). Of these, three were macroalgae collected in quantities large enough for chemical investigation (1-2 L); four small scale collections of cyanobacteria were made for chemical investigation (250 mL) and samples for culture were taken from three of these; six cyanobacterial specimens were collected only as samples for culture at Oregon State University. In October 2002, four collections of algae were obtained from three different sites around Bocas del Toro: one filamentous red macroalga (500 mL), and three of cyanobacteria (300 mL) identified as different strains or species of the genus *Lyngbya*. A culture specimen of one of the latter samples, a red *Lyngbya majuscula*, was also obtained, and is now growing well in Oregon, thus validating our ability to successfully transport living cultures from Panama to Oregon.

C.5.2. Extractions and Prefractionations

The largest of the cyanobacterial collections (approx 0.5 L wet mass, 85.5 g dry mass) made in the Caribbean Sea near Portobelo (Figure 7) was extracted using the scheme outlined for marine cyanobacteria (see Section D.9, see Appendix 9). The organic extract (4.1 g) of this presently unidentified microalgal sample has been 'prefractionated' according to our standard protocol for marine algae also described in Section D.9 (see Appendix 9). The three other, smaller, cyanobacterial collections made from Portobelo have also been extracted using our standard extraction protocol. Of the samples collected around Bocas del Toro in October 2002, the largest one, a red specimen of *Lyngbya majuscula* (approx 1L wet mass, 56.5 g dry mass), has been extracted and fractionated according to our standard pre-fractionation protocol (see Appendix 9).

C.5.3. Bioassays

A total of five cyanobacterial crude extracts, four from Portobelo and one from Bocas del Toro, have been tested in the standard brine shrimp toxicity assay (Meyer *et al.* 1982). Of these, four were active at a concentration of 100 ppm and two were active at 10 ppm. These more potent extracts were fractionated and the first tier fractions retested in the assay. One, a Portobelo *Lyngbya majuscula*, yielded three brine shrimp toxic fractions (at 10 ppm), while six fractions from the second, a Bocas del Toro *L. majuscula*, were also active (at 10 ppm). Fractions from these two collections have been additionally fractionated according to the protocols and procedures outlined in AP3 to yield five pure compounds and several semi-pure fractions to date. All of these samples, together with the crude extracts have been submitted to AP2 for testing against malaria, leishmaniasis and Chagas' disease. To date, only the crude extracts have been tested; one was active in the malaria assay with an $ID_{50} = 5$ ppm.

D. RESEARCH DESIGN AND METHODS

D.1. Organizational Overview of the Panama ICBG

The Panama ICBG consists of four Associate Programs as illustrated in Figure 8 and described in detail in the Group Plan. Here we summarize the organization and the flow of samples and information among the different Associate Programs (AP).

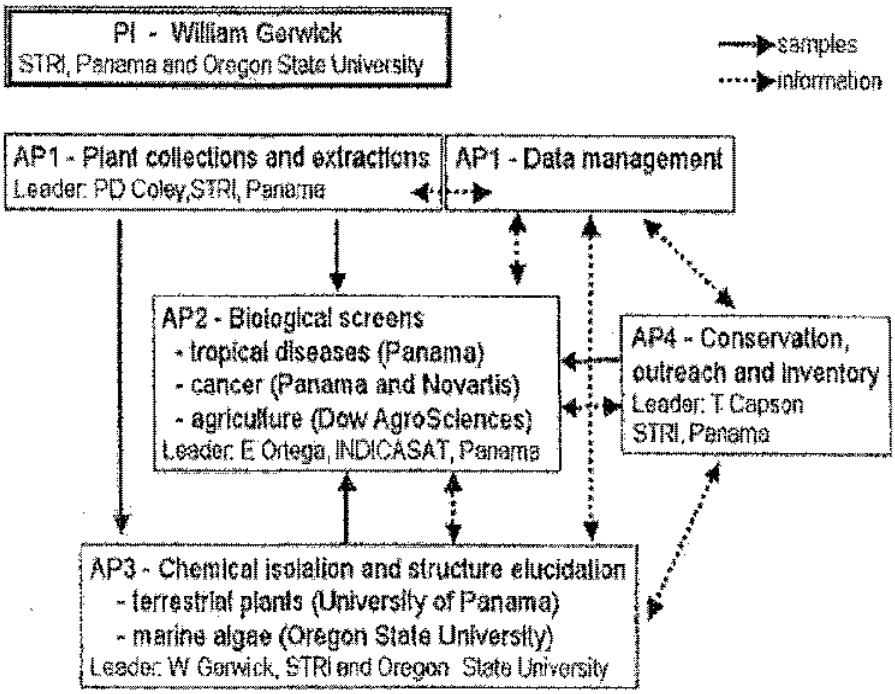


Figure 8: Diagram showing the Associate Program Interactions and Interrelationships in this ICBG Proposal, with sample and data transmission indicated

The proposed research program for AP1 builds on our previous successful work at STRI with terrestrial plant collections and adds a new marine component based on the expertise of Dr. Gerwick's group at Oregon State University. The majority of the AP1 activities will still be based in Panama, and the culture and extraction of marine macroalgae and cyanobacteria will be accomplished in the facilities at Oregon State University. A schematic of the specific aims and flow of samples and information between different components of AP1 is presented in Figure 9.

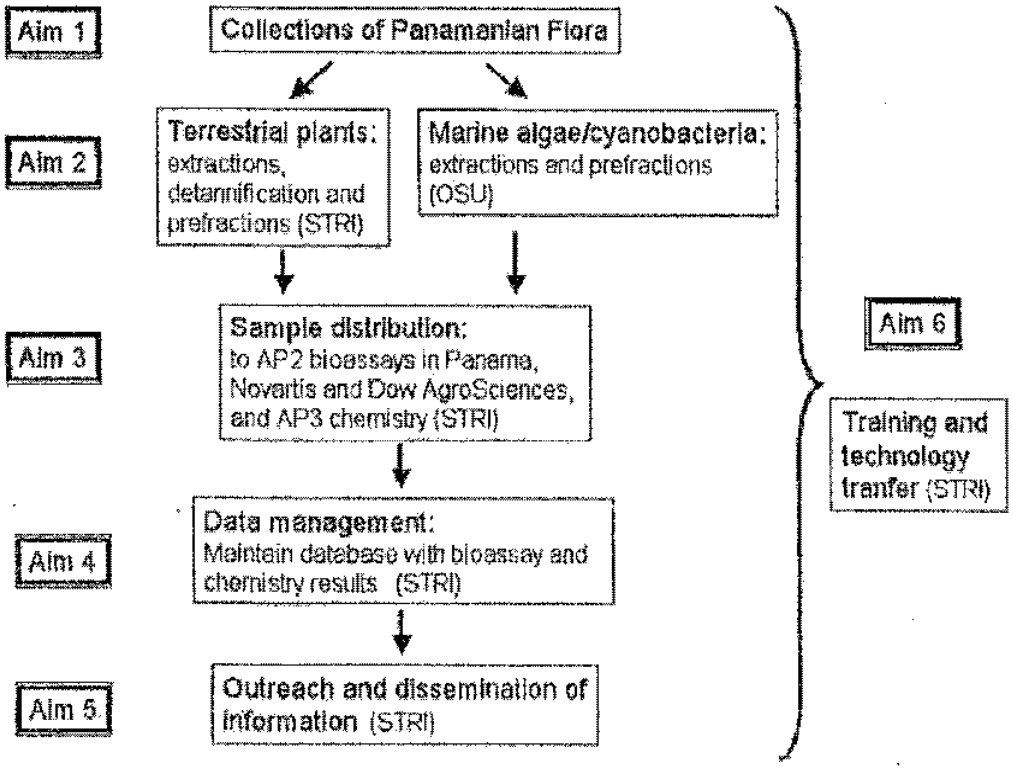


Figure 9: Flow chart of Associate Program 1 activities as they relate to the aims of this project.

D.2. Associate Program 1 Personnel

The interrelationships of the key personnel in Associate Program 1 are shown in Figure 10. Dr. Phyllis Coley is the AP leader and is the PI of the current ICBG. She and Dr. Thomas Kursar have worked on the ecology and evolution of anti-herbivore defenses in tropical plants for over 20 years. Together they designed the collection strategy for terrestrial plants and will continue to supervise this component. They will spend 3-4 months annually in Panama, as they have for the past 20 years. Dr. Maria Heller is based at STRI and currently supervises the collection, extraction and prefractionation of terrestrial plants. She will continue in this capacity in consultations with Drs. Kursar and Coley. Dr. Kerry McPhail is a natural products chemist and a Research Assistant Professor at Oregon State University. She will be in charge of the marine plant collections and extractions and will make three trips to Panama per year. The three field botanists, Mr. Aizprua, Ms. Flores and Ms. Arauz, are all extremely well trained and have worked on the project for several years. Ms. Lakey and Ms. Rios carry out extractions and prefractionations in the STRI laboratory. Ms. Sanchez is the data manager (see letter of support). Professor Mireya Correa is director of the National Herbarium and the herbarium at the Smithsonian Tropical Research Institute. She will confirm all identifications of active plants (see letter of support). Dr. Valerie Paul (Head Scientist at the Smithsonian Marine Station in Fort Pierce, Florida) will help collect and will confirm all macroalgal identifications (see letter of support). In addition, we anticipate involving numerous students and volunteers in the AP1 activities.

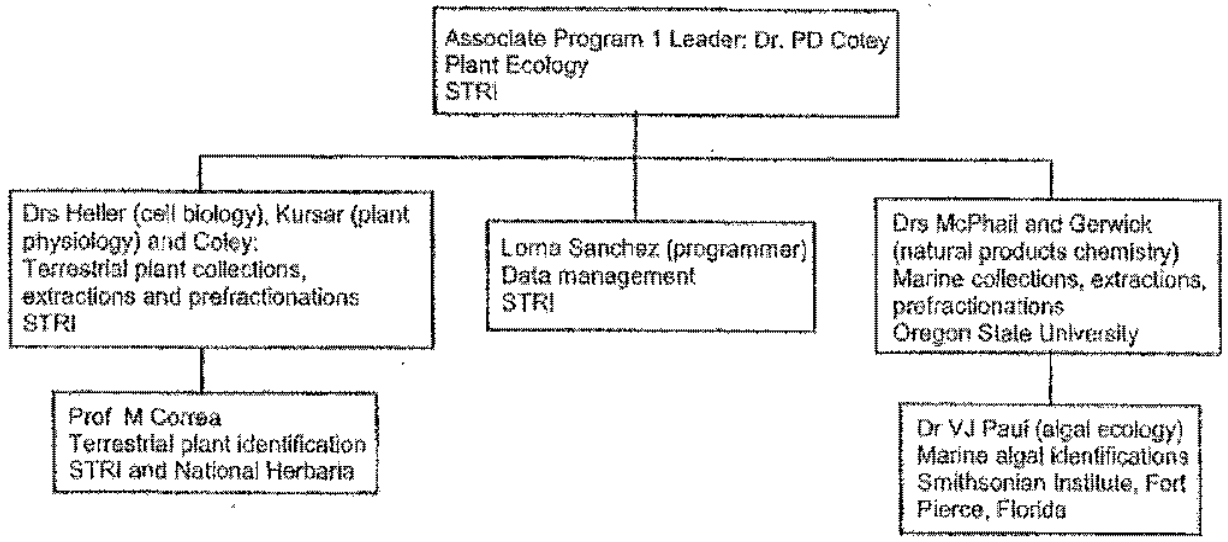


Figure 10: Organizational chart of key personnel in Associate Program 1 and their research areas and affiliations

D.3. Terrestrial plant collections (specific aim #1)

Our approach (described above) of focusing on young leaves and making fresh extractions has proved both feasible and highly effective. Thus, we propose to continue this approach. To date we have collected approximately 12% of the Panamanian flora, and have concentrated our collections on central Panama. Much remains to be explored, and significant contributions will be made to the understanding of the flora of these less-studied National Parks. Based on the past year, we anticipate that 50% of the botanists' collection efforts will be on new collections, and 50% will be recollecting promising leads. We expect to make new collections for 200-300 species annually.

Most of the new collections will be made in more remote locations in western and eastern Panama (Figure 7). Although we have made a few collecting trips to sites in Western Panama, several areas, such as Fortuna, Amistad, Baru and Torrijos need continued effort to adequately sample their unique floras. We also propose to add more collection sites because we find new species at every site, and because ICBG collecting efforts also promote protection of these areas. We have successfully accessed these more remote sites by 4-wheel drive vehicle and occasionally additional transport in dugout canoes. Collections in western Panama present no security risks. However, for the last several years, Colombian guerilla activity in eastern Panama has made parts of the Darién too dangerous for collecting. There is no indication that this situation will change. Dr. Thomas Kursar has considerable experience working in the Darién, and has only suggested sites (Figure 7) in areas that have always been safe and that he and his colleagues continue to visit regularly. Nonetheless, we can access much of the biodiversity of Darien from these sites.

For sites in Central Panama that are within a few hours driving distance from the laboratory, we place leaves in coolers, return to the extraction laboratory in Panama City and extract immediately (see following section). For more remote locations, initial processing of leaves occurs on site: after collecting, leaves are homogenized in a Waring blender in 100% ethanol, or if there is no electricity, chopped finely with scissors. The ethanolic homogenate is then stored in an insulated cooler (H&R Industries, Beecher, IL) at -30 to -40°C on dry ice for up to 3 days before returning to the laboratory where the extractions are completed. This system has worked very effectively. In the field we use ethanol as a solvent instead of methanol because of health safety issues should the ethanol be stolen. Ethanol is widely used for the preparation of crude plant extracts (Ghisalberti 1993, Cragg *et al.* 1993) and can solubilize compounds having a wide range of polarities. We have found no difference in yield, activity or TLC patterns when extracting samples with methanol or ethanol.

When bioassay results and literature surveys suggest promising species, recollections are made from both young and mature leaves. If young leaves of certain species are difficult to obtain, and activity or TLC patterns are similar in mature leaves, we will pursue bioassay-guided fractionation with mature leaves. We followed this procedure for *Mryospermum fructescens*, which had activity against *Trypanosoma cruzi* (Torres *et al.* 2003).

Voucher specimens will continue to be deposited in the herbaria at STRI and the University of Panama.

D.3.1. Endophytic Fungi (specific aim #1)

We will provide DowAgrosciences with endophytic fungal cultures following the procedures we developed over the last few years to collect and isolate morphospecies (Arnold *et al.* 2000). Collections of endophytic fungi will be made at the same time as our plant collections. This has the advantage of being cost-effective in terms of travel and plant identification and is only possible because we are collecting fresh material. We will culture endophytes from surface-sterilized leaf pieces on malt extract agar and ship pure cultures in cryovials with distilled water (see Section C.4.). We will send approximately 500-1000 isolates per year which will take less than 50% effort of one of the AP1 technicians.

D.4. Extraction and Prefractionation Procedures for Terrestrial Plant Collections (specific aim #2)

Organic extracts are prepared from 20 g of fresh leaves or from the ethanolic homogenates made in the field. Samples are homogenized in cold 100% methanol (20 g fresh weight of leaves per 180 mL) in a Waring blender for 30 s. This is followed by 2 min of homogenization at maximum speed with a PT-3000 Polytron (Brinkmann Instruments) at 5°C. Studies in our laboratory have demonstrated that Polytron treatment provides rapid, complete extraction of metabolites at 5°C, even with very tough leaves (Torti *et al.* 1995). After homogenization, the sample is filtered, washed with ethyl acetate, dried under vacuum at room temperature, and stored at -70°C. On average, fresh leaves weighing 20 g yield 1.4 g of extract. Having had extensive experience working with both dried and fresh leaves, we have found that the yields from fresh leaves are higher than those from dried leaves. This protocol avoids drying and grinding the sample to a powder, long extractions, or any other treatments that could lead to degradation of compounds. Glass vials with 3 mg of extract are prepared for testing in the Panama-based bioassays. Each vial receives a unique barcode label that facilitates tracking samples and minimizes potential errors in the use of that sample for bioassays. A subsample of 150 of the crude organic extracts will be detannified and prefractionated before sending to Novartis for testing in their anticancer bioassays and to Dow AgroSciences for testing in their agricultural bioassays.

Detannification: Extracts are tested for tannins by spotting on a silica TLC plate and staining with vanillin/sulfuric acid. If tannins are present, 500-800 mg of extract is dissolved in 5 mL of methanol and passed through a syringe with 15 g of polyamide (Tan *et al.* 1991, Wall *et al.* 1996). The weight of polyamide is adjusted according to the observed tannin level. The extract is eluted with 300-500 mL of methanol. The methanol is removed *in vacuo*, the extract weighed, tested for tannins and detannification is repeated as needed. The yield is about 300 to 500 mg. Polyamide is discarded after one use.

Prefractionation: Prefractionation of plant extracts is accomplished by reversed-phase vacuum-liquid chromatography on a 100 ml vacuum filter funnel following an application developed in the PI's laboratory (similar to that in Appendix 9). After dry-packing a coarse porosity vacuum filter funnel half full with C_{18} silica gel under vacuum, the resulting column is conditioned with methanol (approx. 6 bed volumes). The column is then equilibrated with a starting eluent of 1:1 methanol-water (6 bed vol). The sample (about 300 mg) is dissolved in 1:1 methanol/water and loaded on the column. If insoluble in methanol/water, the sample is dissolved in 100% methanol, C_{18} is added, the methanol removed *in vacuo* and the sample is loaded on the column as a powder. The column is eluted with 6 bed volumes each of six different mobile phases: 1:1 methanol-water, 7:3 methanol-water, 9:1 methanol-water, 19:1 methanol-water, 100 % methanol and 100 % dichloromethane.

Transfer to AP2 Panama-based Assays: 3 mg of each fraction are dissolved in methanol and aliquots are placed into bar-code-labeled glass vials and dried *in vacuo* for transfer to AP2 for bioassays.

Transfer to Novartis and Dow AgroSciences: We propose to send Novartis and Dow AgroSciences (DAS) extracts and prefractions from 150-200 terrestrial plant species and 50-75 marine macroalgae and cyanobacteria

(1,500-2,000 materials in total) per year. Crude extracts chosen for prefractionation will be those that are active in one or more of the Panama-based whole cell anticancer assays of AP2, or from species for which there was no, or little, published information on the species or genus according to the SciFinder database. Fractions for transfer to Novartis (2.5 mg) and DAS (5 mg) are dissolved in 0.5 mL of methanol, aliquoted into a 96-well (Regular and Deep Well, respectively) plate and dried *in vacuo* (MPTR 12-210 rotor and Speed Vac, Savant Instruments). We propose to send extracts from 150 species (900 fractions) per year.

Purification of samples active in Novartis' assays: Purifications for Novartis must be done quickly because Novartis usually keeps an individual assay on-line for less than 6 months. Thus, some actives materials will be further purified in AP1. Upon receiving prefraction activity data from Novartis, candidates for bioassay-guided purification are chosen, samples are recollected (30–100 g of extract), separated again into 6 crude fractions on C_{18} (the methods described above are adjusted for a large sample), the original and new fractions are compared by TLC, and the active fractions are chromatographed into pure or semi-pure fractions on C_{18} HPLC. When only a subset of the prefractions from reversed-phase vacuum-LC are active in Novartis' assays (suggesting that C_{18} is an effective separation method), we have had good success in purifying active compounds using RP-HPLC (C_{18}). In AP1 we have both semi-preparative and preparative capabilities (Waters radial compression with Radial-Pak segments; Waters 2996 HPLC with photodiode array detector and fraction collector). All of the purifications in AP1 will be carried out using the guidance of, and regular consultation with, the ICBG natural products chemists, adjusting the eluting solvent and other run conditions to optimize separation of the abundant compounds (as detected on TLC). Pure or semi-pure fractions will be returned to Novartis for further assay. This approach requires only two rounds of fractionation and assay, and we have used it with success during this past year. In the last few months, a total of 33 plant species (147 fractions) were detannified and prefractionated and tested in Novartis' bioassays for anticancer activity. We have purified 2 active compounds and have 5 more that are almost pure. One is a potent Sirt2 inhibitor (Sirt2 is an NAD-dependent histone deacetylase). The structures of purified, active compounds will be assigned by AP3 chemists.

D.5. Ecologically-guided Macroalgal and Cyanobacterial Collections (specific aim #1)

We intend to consult with Dr. Valerie Paul, a world-renowned marine chemical ecologist, to optimize our collections of chemically-defended algal species. Marine macroalgae are generally opportunistic species and the degeneration of hard corals in coral reefs facilitates establishment of macroalgae as the dominant life form (Littler and Littler 2000). Indeed, since the massive die-off of *Diadema* sea urchins in 1983 in the Caribbean, there has been noticeably increased algal growth and overgrowth on coral reefs (Edmunds and Carpenter 2001). Thus, much of the shallow reef habitat is characterized by a range of macroalgal species. Uncalcified macroalgal species are targeted for collection on the premise that the lack of a physical defense could indicate a chemical defense mechanism in organisms that are not grazed excessively by the myriad predators in this environment (Hay 1996).

In the shallow subtidal zone of protected bays, sea grasses on a sandy bottom provide a sheltered habitat for cyanobacteria. In addition, cyanobacteria are ubiquitous on coral reefs and with their ability to fix atmospheric nitrogen, are primarily responsible for the high productivity of reefs (Littler and Littler 2000). These microalgae can occur as trellised plumes (*e.g. Lyngbya*), as distinct gelatinous puffballs (*e.g. Schizothrix*) or even gelatinous mats on the sandy bottom (*e.g. Phormidium*), typically in locations with high light intensity and temperature, and low wave action. Such conditions can facilitate algal blooming where copious 'swathes' of algae are evident at a particular location. Again, these algae are conspicuously free of associated predators.

The inherently changeable nature of the marine environment dictates that we make as large collections as possible (up to 2 L volume) on first encountering noteworthy species. In our experience, the distribution of marine cyanobacteria is highly unpredictable, varying both in accord with the season, the year of collection, and exposure of the site. Therefore, we plan to obtain enough material from the initial collection to purify a sufficient amount of any active pure compound for structure elucidation and preliminary bioactivity testing. In the case of the cyanobacterial collections, the potential need for recollection can be overcome by the successful culturing of the organism (see Section D.8). Fortunately, the relative accessibility of Panama greatly enhances the likelihood of making successful recollections, should this be necessary.

Samples are collected in fine mesh bags or ziplock plastic bags by hand using SCUBA or snorkeling. An underwater photograph of each species collected is taken for identification purposes using a SONY digital camera (DSC-P1) in a "MARINE-PAK" underwater housing. Upon completion of the collection activity, the samples are processed immediately as follows. A few filaments of interesting cyanobacterial specimens are taken for culture (see Section D.8). Material for chemical investigation is added to 70% ethanol or isopropanol in the appropriate size Nalgene bottle (250 mL, 500 mL, 1 L or 2 L). While packing an alga into a bottle, small amounts of alcohol are continuously added and the bottle is shaken to ensure that the sample becomes completely saturated, without having excess alcohol. At the same time, two voucher specimens are prepared in 70% ethanol in sealed Whirlpak® bags for submission to the herbaria at the University of Panama and STRI. The algal samples are stored at ambient temperature until our return to Oregon, at which time they are deposited at -20 °C to await extraction and pre-fractionation.

D.6. Marine Collection Sites (specific aim #1)

We plan to collect in both the Caribbean and Pacific in order to sample a broad range of algal diversity. Within the Pacific, we intend to target sites in both the warmer Gulf of Chiriqui and the strikingly colder upwellings in the Gulf of Panama. All collections will be made within National Park boundaries, concentrating on the locations listed below. Examples of collection and export permits negotiated through ANAM are presented in Appendix 6.

Bocas del Toro, Western Caribbean: Situated on Panama's northwestern coast, this region comprises a patchwork of islands and shallow water reefs which are mostly at depths of less than 10 m, although some extend to 20 m. This extensive lagoon habitat is dominated by mangroves, grassbeds and sand. Pristine mangrove habitat, which we found to harbor a rarely encountered and extraordinary proliferation of marine cyanobacteria, is extremely abundant. The Smithsonian Tropical Research Institute (STRI) is in the process of completing an extensive marine biological research facility in Bocas del Toro (www.stri.org) where we have been, and will continue to be, based during our collection efforts in this region. Bocas del Toro is also the site of an extensive marine national park known as Bastimentos Island, which comprises 132 square km, of which 115 square km are marine. The park protects the largest extension of Caribbean mangroves in the country as well as extensive coral reefs (Navarro 1998). In the Northeastern section of the park are the Zapatillos cays which comprise coral reefs extending over 500 ha.

Portobelo, Central Caribbean: A recent Reefkeeper report (Panama ReefMonitor Update 2001) presented the reefs off the north coast of Panama around Portobelo as being surprisingly healthy, with a high percentage of hard coral cover. Our brief survey of the shallow subtidal in this area revealed a high diversity of algae, in particular cyanobacteria. While the biomass of cyanobacteria observed was relatively low, this could be seasonal; nevertheless, composite collections over a period of days are certain to yield sufficient biomass for chemical extractions.

East of Portobelo is an area known as San Blas, which is under the jurisdiction of the Kuna Indians (Clifton *et al.* 1997). Due to the potential complexity of negotiating with the Kuna Indians, we do not intend to collect samples in this area of the Caribbean.

Coiba Island, Gulf of Chiriqui: Situated 30 miles off the southwest corner of Panama in the Gulf of Chiriqui (Pacific Ocean), Coiba is a 493 km^2 island which was declared a national park in 1991. The marine environment around Coiba is world-renowned for its diverse, pristine reefs, and the island with its surrounding marine reserve is currently under review as a World Heritage Site (as part of AP4). The relatively stable and warm temperatures in the Gulf of Chiriqui are manifest in differences in coral reef growth and community structure (Glynn and Maté 1997). Generally, coral reefs are more abundant, larger and have a greater vertical buildup and a higher species diversity in non-upwelling western Panama than in upwelling central/eastern Panama. The best developed reefs in the Gulf of Chiriqui have vertical buildups of 10-12 meters and maximum ages of 5,600 years (Glynn and Maté 1997). The Pacific shores of Panama have a diverse coral fauna, representing 91% of the genera of the eastern Pacific reef-building species, many of which are found in the Gulf of Chiriqui (Glynn and Maté 1997). Since Coiba is some distance from the mainland, it is largely free of sedimentation resulting from mainland deforestation and the clean rocky substrate is home to a rich complement

of Pacific marine organisms not found closer inshore. As such it is anticipated to be a unique collection site for marine algae and cyanobacteria.

The Gulf of Panama: Reef development in the Gulf of Panama occurs almost exclusively on the Pearl islands and Isla Iguana. The largest aggregation of coral reefs is located around the Pearl Islands, a group of 53 rock islands, islets and shoals situated 70 km south of Panama City. In this area, the maximum reef depth is 3-4 m below water. Carpets of filamentous algae are known to cover large areas of the reef flat on the 11.7 ha expanse of Contadora Island. The largest reef of the Pearl Islands is Saboga Island (14.3 ha), which is a shallow reef dominated by a few massive reef species where reef fishes are often observed feeding on turf algae. Iguana Island, 5 km east of the Azuero Peninsula is the site of the largest reef in the Gulf of Panama (16 ha) and was declared a wildlife refuge in 1981. We have yet to explore firsthand the promising algal biodiversity of these Gulf of Panama sites.

D.7. Taxonomy of Algal Collections (specific aim #1)

Identification of macroalgae will be made primarily with the aid of a highly comprehensive guide to the marine algae of the Caribbean provided by Littler and Littler (2000), which provides a superb resource for identifying marine plants from a composite of morphology (outstanding color photographs), anatomical features (line drawings), and clearly understood taxonomic descriptions. Littler and Littler have also produced a general guide to the Southern Pacific flora (2003). Most recently, an extensive Panama survey undertaken as part of a Ph.D. project by Wysor (2002) reported a total of 308 Caribbean taxa, of which 178 appeared to be new records, and 180 Pacific taxa, of which 87 were new for Panama. Thus, including previous records, over 450 taxa have been documented to date. Determinations of the 1500 specimens collected from both coasts of Panama in this study are incomplete to date. Wysor *et al.* (2000) also report that a literature survey revealed that macroalgal diversity in Panama is actually higher than in most other countries of either the Caribbean or the eastern Pacific. When necessary for less easily identified specimens, macroalgal voucher samples will be sent to Dr. Valerie Paul (Smithsonian Marine Station in Fort Pierce, Florida) for her expert identification. Identification of Cyanophyceae genera will be carried out by Dr. McPhail and the Oregon graduate student, with advice from the Gerwick laboratory algal culture technician, and is based on the botanical taxonomies of Geitler (1932) and Desikachary (1959). Rippka's (1988) cyanobacterial taxonomy work is used for comparative descriptions. In addition to Dr. Paul's support (see letter in Appendix 2), we anticipate the continued assistance of several other colleagues in the field of macro- and microalgal taxonomy to help identify problematic specimens (Richard Castenholz, University of Oregon; Gail Hanson, Oregon State University; David Ballantine, University of Puerto Rico, Mayaguez).

D.8. Isolation and Culture of Marine Cyanobacteria (specific aim #1)

Our collection techniques to date have been highly successful in returning to the laboratory a broad assortment of viable material in easily handled small containers. We anticipate using this same successful methodology on this project (Gerwick *et al.* 1994). Our collection containers are sterilized 30 mL Nalgene jars into which are placed small portions of algal material in native seawater. Key parameters in maintaining viable cultures in the field until return to the laboratory are temperature (ambient tropical), light level (low), oxygenation (high), and turbulence (minimal). Once the samples are back in the laboratory, the cultures are surveyed to establish the nature of the initially dominant algae and allowed to acclimate to the culture room conditions (28 °C, 16 h day/8 h dark). We have found it best to let new isolates remain undisturbed for 2-4 weeks following return. After this initial screening and acclimation period, cyanobacteria are physically isolated and put into one of several selected media: CMM (Gerwick *et al.* 1988), BG11SW and F/2 (Castenholz, 1988), ES (Starr and Zeikus 1993), employing multi-well tray technology and agar plates using Noble Agar. Isolations for unialgal cultures are done according to standard literature procedures (Waterbury 1988).

Initial scale-up is a 3 L volume, grown for about four weeks or until a cell mass dense enough for extraction purposes is achieved. Usually, about 3.5 g of freeze-dried cell material is obtained from 3 L of culture volume (average weight of 140 x 3 L scale-up cultures). Cultures are grown as static cultures

supplemented with moderate aeration. Harvest of cyanobacterial filaments is most effectively accomplished by filtration using a custom-modified Whatman filter apparatus and then the cellular material is freeze-dried.

D.9. Extraction and Prefractionation Procedures for Marine Collections (specific aim #2)

Extracts are prepared from macroalgae and cyanobacteria according to the schemes outlined in Appendix 9. In each case, after allowing the algal sample to defrost, a voucher specimen (stored in a Whirlpak® bag at –20 °C) is prepared for the Gerwick research group voucher repository and excess preservation alcohol is filtered off the sample. Macroalgal collections are homogenized in an industrial Waring blender before being immersed in 2:1 dichloromethane-methanol. After standing for 15 minutes, the extraction solvent is filtered off and fresh solvent is added to the algal material and heated gently for 0.5 hour, cooled, and filtered. Addition of fresh solvent, gentle heating and filtration is repeated until the decanted extraction solvent is pale to colorless. The combined filtrates are concentrated *in vacuo* and the resulting aqueous residue is extracted using dichloromethane, filtered to remove particulates, and concentrated *in vacuo*. A portion of this crude extract (100 mg) is kept aside in a 1:1 ethanol-isooctane stock solution (10 mg/mL) as a supply for the bioassays described in AP2.

Cyanobacteria are chopped by hand if necessary, or simply immersed directly in the 2:1 dichloromethane-methanol solvent mix. The same procedure as outlined above for the macroalgae is carried out with the following additional steps: once the alga has been extracted exhaustively in dichloromethane-methanol, it is soaked in 3:1 methanol-water overnight. This aqueous extract is then filtered off, reduced *in vacuo* and twice partitioned between *sec*-butanol and water. The water layer is discarded, while the combined *sec*-butanol layers are concentrated to yield an "aqueous extract." As with the macroalgal extractions, a portion of the cyanobacterial extracts (50-100 mg) are kept aside as stock solutions for bioactivity testing in AP2.

Organic extracts of both macroalgae and cyanobacteria are pre-fractionated by normal-phase vacuum-liquid chromatography as represented in the Appendix 9. A coarse porosity vacuum filter funnel is packed with TLC grade silica gel under vacuum, and conditioned and equilibrated with hexanes. The vacuum is released and then applied intermittently as the sample is loaded into the column, after which a stepped gradient of hexanes through ethyl acetate and methanol is used to elute the sample in 9 distinct fractions. Each resulting fraction is made up in 1:1 ethanol-isooctane (10 mg/mL) as a stock solution, from which the required amounts can be withdrawn for submission to AP2 for bioactivity testing together with the crude extract.

Pre-fractionation of aqueous cyanobacterial extracts is accomplished by reversed-phase vacuum-liquid chromatography (Appendix 9). After dry-packing a coarse porosity vacuum filter funnel with C_{18} silica gel under vacuum, the resulting column is conditioned with methanol (approx. 5 bed volumes). The column is then equilibrated with a starting eluent of 1:1 methanol-water (approx. 3 bed volumes) before the extract sample is loaded as described above for the organic extracts. Four distinct fractions are eluted (1:1 methanol-water, 7:3 methanol-water, 100 % methanol and 100 % dichloromethane) and prepared as bioassay stock solutions (10 mg/mL) in 100 % ethanol. All macroalgal and cyanobacterial crude extracts and pre-fractions to be submitted for initial bioactivity testing in AP2 (Tropical Diseases, Cancer cells, Novartis Mechanism-Based screening, Dow AgroSciences Agricultural screening) will be sent to Panama in 10 mg quantity in screw-top glass vials, and all distributions to AP2 biology groups will occur through the AP1 laboratory.

D.10. Permits for Collection and Sample Transfer (specific aim #3)

As described in the overview, the legal agreement (signed April 8, 1999) between STRI and the National Authority of the Environment (ANAM) allows collecting for the ICBG project and outlines benefit sharing. We set up the agreement to require additional permits from ANAM for each collecting and sample transfer. Thus, before we begin collecting in a particular area, we send ANAM a list of species we expect to collect. This is reviewed and approved, usually within 2 weeks and is good for one year (see Appendix 6). Recollections that are accomplished within a year of the original permit to collect do not require additional permits. We do not collect endangered or threatened species, nor species for which we feel it would be too destructive (such as most orchids). After extraction, we obtain additional permits to transfer samples from STRI to other institutions

for bioassays (Appendix 6). This includes AP2 bioassays at INDICASAT in Panama, Novartis, Dow AgroSciences and any additional collaborators.

Marine collections within National Parks are also under the jurisdiction of ANAM and are covered by the STRI/ANAM agreement. We easily obtained collection and transfer permits for the collecting and subsequent transfer of samples to Oregon State University accomplished in September and October 2002. We anticipate that marine areas within National Parks should provide an excellent diversity of algae and cyanobacteria. However, if additional collections outside of parks seem desirable, we will enter into Agreements with the Panamanian Maritime Authority (AMP).

The permitting process has become very streamlined and has engendered much good will with ANAM. We felt that it was better to have a permit process rather than ask for blanket approval for all collections in the STRI/ANAM agreement. This increases the transparency of our work and allows Panama to maintain more control. Because the majority of the ICBG research is done in Panama by Panamanians, and because we have made every effort to be open, the project is held in high regard (see AP 4 for more details). Nonetheless, bioprospecting projects with involvement of foreigners must be proactive in order to avoid any perception that we might be unfairly exploiting Panamanian biodiversity. The permitting process is just one of the ways we maintain open and positive relations with the Panamanian government.

D.11. Data Management (specific aim #4)

AP1 will continue to manage the relational database (described in C.2.) that we developed for tracking information on collections, bioassay and chemical results for both marine and terrestrial collections. As described in a previous section (see C.2. "Data Management"), the goal of our information system is to maximize accuracy and ease of use, and to facilitate rapid access to results by all members of the Panama ICBG. To accomplish this, the information system has both a database located in the AP1 laboratory at STRI, and an internet-accessible, password-protected database. The AP1 database is the main repository of all the data, a warehouse that stores records of plant collections, transfers of samples, chemical information and bioassay results. The internet-accessible database is updated weekly from the AP1 database and members of our ICBG can transfer data or browse collection information or bioassay results from all of the AP's. For security reasons, critical information on chemical structures of active compounds will only reside in the AP1 database, but any member of the AP's can consult this information by a) visiting AP1 to use the AP1 computer at the Smithsonian, or b) requesting that the website administrator send this information as attachment to an email.

Status of current database: The database included information on collections for terrestrial plants (e.g. taxonomic identifications, collection locales, collectors, dates and GPS coordinates, ecological information, see Appendix 7). All collections by AP1 are given a unique barcode identification, and bar code labels are placed on all samples leaving the laboratory, as well as the herbarium voucher specimens. Information on collections is entered into the database by the botanists. Examples of several pages, all with pull-down menus, are also presented in Appendix 7. Pull-down menus help to avoid inconsistent data entry or spelling mistakes. The database also contains an up-to-date pull-down menu with extensive taxonomic information from the Missouri Botanical Garden's species list. We will continue to confirm all the current taxonomic names as well as synonyms by using the Missouri Botanical Gardens Neotropical Web-based Flora (http://mobot.mobot.org/W3T/Search/vast.html). This web site provides access to the Missouri Botanical Garden's VAST (VAScular Tropicos) nomenclatural database, including plant names, authority, genus and family, synonyms of the current name, references and recent nomenclatural changes. The information at the VAST website on Panama's flora is complete, including new species and range extensions, as Missouri Botanical Gardens has conducted the floristic inventory of Panama and Professor Correa, head of the University of Panama and Smithsonian herbaria, works closely with Missouri.

In addition, the database currently contains information on extracts, distribution of samples to different laboratories, results from our bioassays in Panama, results of bioassay-guided fractionation, and structures of active compounds. Each laboratory has its own barcode reader and software. Results from the bioassays in AP2 are recorded directly from the 96-well plate readers into an Excel file where all identifying information has been obtained from the barcode. The Excel format is the same for all AP's, so the information can easily be imported

directly into the centralized database in AP1. These files can either be sent by email or via the Internet site. Recollections of promising leads are also given unique barcodes, and once retested in bioassays, transferred to AP3 for bioassay-guided fractionation. Again, all pertinent chemical information is stored in the centralized AP1 database. An example of bioassay results are presented in Appendix 7.

The current information system works very well, nevertheless, our needs are constantly evolving and we will be adding a new marine component. In addition, we would like to expand the database and application to be a more complete information tool. We would like to provide more extensive information and, by adding more effective search engines, facilitate sophisticated queries of the database.

Enhancement of the database: In addition to the information already in the current database, we plan to integrate extraction protocols, images of collections, improved management of results from our bioassays in Panama and the US, pertinent biomedical and chemical data from literature searches (*e.g.* SciFinder), structures of active compounds, and a summary of the current status of each sample under investigation at Novartis. We are also adding general information about the collection sites, as well as about the laboratories, methods and bioassay protocols.

Another significant change in the database will be the addition of modules to manage the marine collections. The marine module will be tailored to the specific information associated with these collections and will include the underwater photos. However, with the experience obtained in our management of terrestrial samples, we expect these changes to be relatively straightforward.

Search engines: Because of the large quantity of data generated by the ICBG, we will need search engines that can not only retrieve simple information, but can also uncover relationships among the data. For example, we would like to more easily explore correlations between the bioassay results and ecological or taxonomic information on the active plants, or between chemical similarities of actives in different bioassays.

Additional improvements: We plan on improving the public webpage so that it includes an inventory of all of the species that the project has collected. We would also like to expand the visual and text information about the ecological characteristics of the collection sites.

AP1 will also be responsible for transferring the necessary information in compatible formats to NIH so that they can maintain an overview of results from all the ICBG projects. This information must be compatible with NAPIS, the data management program used by NIH. We will work to streamline this process.

D.12. Outreach and Dissemination of Information (specific aim #5)

A major goal of the ICBG will continue to be outreach and the dissemination of basic scientific results as well as information on the value of and threats to biodiversity. Outreach, along with conservation, is also the primary focus of AP4. However, AP1 botanists and chemists have regularly given talks, workshops and advice at schools, government agencies and public fora. In the last year, AP1 members gave over 20 formal presentations. We propose to continue these presentations, as they have been extremely well received and provide our AP1 team with experience in public speaking. These talks not only convey information about research on Panamanian diversity, but also provide school children with male and female role models of Panamanians in science. Typically, biology degrees are only viewed as training to teach biology, a rather poorly paid profession. However, we have been informed that our presentations have inspired students to continue their studies of biology because of an expanded vision of what might be possible with a biology degree.

We also regularly give formal and informal presentations to park guards, naturalist tour guides and local communities. When in the field, our botanists feel like ambassadors for biodiversity, and always take time to explain what they are doing to interested citizens.

Both the expertise of our botanists and the collections database have provided useful information to the National Authority of the Environment (ANAM). As mentioned before, ANAM has access to all our collection information on line, and we have helped them to become familiar with use of the database. In order for the government to make reasonable decisions about management and conservation, they must have information on species distributions. Furthermore, many international funding organizations such as GEF-World Bank and USAID, require this type of information. We will cooperate with ANAM to help them to be as competitive as possible in their attempts to obtain funding for large-scale conservation initiatives such as the Mesoamerican

Biological Corridor Project (see also AP4). The species lists of collections that we are beginning to make in the more remote parts of Panama will be particularly helpful, as these areas have been much less studied. Almost nothing is known about the algae or cyanobacteria, so this new component will be extremely interesting. We will continue to provide ANAM and any other interested conservation or educational organizations access to our database and update their training when necessary. Because these data will be posted on the web, they may also benefit the large number of scientists working in Panama.

D.13. Training and Technology Transfer (specific aim #6)

A major goal of our ICBG is to provide Panama with the technology and training necessary to conduct drug discovery research, as this is potentially an ecologically gentle and sustainable use of biodiversity. Unfortunately, the probability of having a drug make it to market is extremely low, so it is unlikely that developing nations will receive royalties from uses of their biodiversity. However, large sums are invested in research and development of drugs, for example $30.5 billion for US companies in 2001 (PhRMA Annual Survey 2001). The research and development costs of each novel drug is controversial, but estimates range from $150 to $800 million per approved drug (Grabowski and Vernon 1994, Love 1999, Agnew 2000, ten Kate and Laird 1999, DiMasi et al 2002, Public Citizen 2002). Worldwide pharmaceutical R & D is estimated at 27-43 billion dollars per year (Agnew 2000), with about one third of that spent on research that could be carried out in developing countries such as extraction, synthesis, bioassay and toxicity testing (ten Kate and Laird 1999). If a fraction of these investments could be made in developing nations, then biodiversity-rich countries would receive immediate and guaranteed benefits from the non-destructive use of their natural resources. Furthermore, by conducting as much of the drug discovery research as possible in underdeveloped nations, the potential exists for providing more jobs than alternative uses of biodiversity such as logging or cattle ranching. In addition, bioprospecting is compatible with other non-destructive uses of biodiversity, such as ecotourism and ecosystem services.

Thus, our goal is to design an ICBG project that will provide the training, infrastructure and technology such that Panamanians can conduct a significant proportion of the research in Panama. We have forged a practical link between drug discovery and conservation while also providing benefits to the host country. This approach addresses the widely perceived and pressing need that society recognizes the true economic value of the conservation of biodiversity.

With these goals in mind, all paid API positions have been filled by citizens and permanent residents of Panama. Non-Panamanians have participated only on a volunteer basis. We will continue to provide jobs and unique training opportunities for learning chemistry and taxonomic identification of terrestrial and marine plants. In addition to the laboratory head, there will be positions for three full-time botanists responsible for terrestrial plant collections and endophytes as well as two chemists responsible for extractions. Marine collections will occur during several intensive trips each year, and will include students from the University of Panama as well as local field assistants. We encourage the API employees to view this as a stepping stone to better educational or job options, so we expect there to be turnover.

In addition to these employment opportunities, we will continue to provide opportunities for students to conduct their theses and practicums or simply to gain experience by working with our API team. Because research options are few at the University of Panama, this has opened many opportunities for students. For them, participation in marine or terrestrial collecting trips, or working in the chemistry extraction laboratory, provide wonderful learning opportunities. We anticipate that two to four students per year will take advantage of this. By generating an interest in the biology and chemical ecology of plants, we hope some students will continue with advanced degrees. Dr. Gerwick is open to the possibility of having students interested in marine natural products chemistry and biochemistry pursue degrees in his laboratory at Oregon State University.

E. HUMAN SUBJECTS
None

F. VERTEBRATE ANIMALS
None

G. LITERATURE CITED

Agnew B. 2000. When pharma merges, R & D is the dowry. *Science* 287: 1952-1953.

ANAM (National Authority of the Environment) web report: http://www.anam.gob.pa/parques/coibapn.htm

Arnold AE, Maynard Z, Gilbert GS, Coley PD and TA Kursar. 2000. Are tropical fungal endophytes hyperdiverse? *Ecology Letters* 3: 267-274

Arnold AE. 2002. Morphospecies as functional taxonomic units: A critical assessment for studies of endophyte biodiversity. In: Neotropical fungal endophytes: diversity and ecology. Ph.D. dissertation, University of Arizona.

Arnold AE, Maynard Z and GS Gilbert. 2001. Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. *Mycological Research* 105: 1502-1507.

Balandrin MF, Kinghorn, AD and NR Farnsworth. 1993. Plant-derived natural products in drug discovery and development: an overview. In: *Human Medicinal Agents from Plants*. Kinghorn AD, Balandrin MF (Eds.), American Chemical Society (ACS Symposium Series 534) Washington D.C. pp. 2-12.

Ballou TG, Dodge R, Hess S and A Knap. 1989. ASTM Special Technical Publication, 1018 (Oil Dispersants: New Ecol. Approaches): 229-256.

Barthlott W, Lauer W and A Placke. 1996. Global distribution of species diversity in vascular plants: Towards a world map of phytodiversity. *Erdkunde* 50: 317-327.

Bayman PL, Lebron RL, Tremblay, and DJ Lodge. 1997. Variation in endophytic fungi from roots and leaves of *Lepanthes* (Orchidaceae). *New Phytologist* 135: 143-149.

Bills GF. 1996. Isolation and analysis of endophytic fungal communities from woody plants. In: *Endophytic Fungi in Grasses and Woody Plants*. Redlin SC, Carris LM (Eds). APS Press, St. Paul, MN, pp. 31-65.

Blokhin AV, Yoo H-D, Geralds RS, Nagle DG, Gerwick, WH and E Hamel. 1995. Characterization of the interaction of the marine cyanobacterial natural product curacin A with the colchicine site of tubulin and initial structure-activity studies with analogs. *Mol. Pharm.* 48: 523-531.

Submitted

Brady SF, Wagenaar MM, Singh MP, Janso JE and J Clardy. 2000. The cytosporones, new octaketide antibiotics isolated from an endophytic fungus. *Org. Lett.* 2: 4043-4046.

Brady SF, Bondi SM, and J Clardy 2001. The guanacastepenes: A highly diverse family of secondary metabolites produced by an endophytic fungus. *J. Amer. Chem. Soc.* 123: 9900-9901.

Breen JP. 1994. *Acremonium* endophyte interactions with enhanced plant resistance to insects. *Annual Review of Entomology* 39: 401-423.

Burdsall HH and E Dorworth. 1994. Preserving cultures of wood-decaying Basidiomycotina using sterile distilled water in cryovials. *Mycologia* 86: 275-280.

Burke L. 2001. Reefs at Risk in the Caribbean. In: *Priorities for Caribbean Coral Reef Research: Results from an International Workshop of the National Center for Caribbean Coral Reef Research (NCORE)*. (October 3-5).

Castenholz RW. 1988. Culturing methods for cyanobacteria. *Methods Enzymol.* 167: 68-95.

Cervino J, Goreau TJ, Nagelkerken I, Smith GW and R Hayes. 2001. Yellow band and dark spot syndromes in Caribbean corals: distribution, rate of spread, cytology, and effects on abundance and division rate of zooxanthellae. *Hydrobiologia* 460: 53-63.

Submitted

Clay K. 1990. Fungal endophytes of grasses. *Annual Review of Ecology and Systematics* 21: 275-297.
Clay K. 1992. Fungal endophytes of plants: biological and chemical diversity. *Natural Toxins* 1: 147-149.
Clifton KE, Kim K and JL Wulff. 1997. A field guide to Caribbean Panama with an emphasis on western San Blas, Panama. *8th International Coral Reef Symposium*, pp. 167-184. Smithsonian Tropical Research Institute, Balboa, Republic of Panama.
Coley PD. 1983. Herbivory and defensive characteristics of tree species in a lowland tropical forest. *Ecological Monographs*. 53: 209-233.
Coley PD and TM Aide. 1991. A comparison of herbivory and plant defenses in temperate and tropical broad-leaved forests. In: *Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions*. Price PW, Lewinsohn TM, Fernandes GW Benson WW (Eds). John Wiley & Sons, N.Y. pp. 25-49.
Coley PD and JA Barone. 1996. Herbivory and plant defenses in tropical forests. *Annual Review of Ecology & Systematics* 27: 305-335.
Coley PD and TA Kursar. 1996. Anti-herbivore defenses of young tropical leaves: physiological constraints and ecological tradeoffs. In: *Tropical Forest Plant Ecophysiology*. Smith AP, Mulkey SS, Chazdon RL (Eds). Chapman and Hall, N.Y. pp 305-336.

Submitted

Condit R, Pitman N, Leigh Jr EG, Chave J, Terborgh J, Foster RB, Núñez P, Aguilar VS, Valencia R, Villa G, Muller-Landau HC, Losos E and SP Hubbell. 2002. Beta-diversity in tropical forest trees. *Science* 295: 666-669.
Conservation International 2003. http://www.biodiversityhotspots.org/xp/Hotspots
Cragg GM, Boyd MR, Cardellina II JH, Grever MR, Schepartz SA, Snader KM and M Suffness. 1993. Role of plants in the national cancer institute drug discovery and development program. In: *Human Medicinal Agents from Plants*. Kinghorn AD, Balandrin MF (Eds). American Chemical Society (ACS Symposium Series 534) Washington D.C. pp. 80-95.
Crankshaw DR and JH Langenheim. 1981. Variation in terpenes and phenolics through leaf development in *Hymenaea* and its possible significance to herbivory. *Biochem. Syst. and Ecol.* 9: 115-24.
Croat TB. 1978. *Flora of Barro Colorado Island*. Stanford University Press, Stanford, CA. 943 p.
D'Arcy WC. 1987. *Flora of Panama Checklist and Index*. Missouri Botanical Garden, St. Louis, MO.
Desikachary TV. 1959. *Cyanophyta*. Indian Council of Agricultural Research, New Delhi, 686 pp.
DiMasi, J.A. Ronald W. Hansen and Henry G. Grabowski. 2002. The price of innovation: new estimates of drug development costs. Tufts University Center for the Study of Drug Development, Boston, Massachusetts.
Duflos A, Kruczynski A, Barret J-M. 2002. Novel aspects of natural and modified Vinca alkaloids. *Current Medicinal Chemistry: Anti-Cancer Agents* 2: 55-70.
Edmunds PJ and RC Carpenter. 2001. Recovery of *Diadema antillarum* reduces macroalgal cover and increases abundance of juvenile corals on a Caribbean reef. *Proc. Natl. Acad. Sci.* U.S.A. 98: 5067-5071.
Ehrlich PR and PH Raven. 1964. Butterflies and plants: a study in plant coevolution. *Evolution* 18:586-608.
Fuller RW, Cardellina JH, Kato Y, Brinen LS, Clardy J, Snader KM and MR Boyd. 1992. A pentahalogenated monoterpene from the red alga *Portiera hornemannii* produces a novel cytotoxicity profile against a diverse panel of human tumor cell lines. *J. Med. Chem.* 35: 3007-3011.
Geitler L. 1932. Cyanophyceae. In Rabenhorst L (Ed.), Kryptogamenflora von Deutschland, *Osterreich und der Schweiz*, 14.
Gentry AH. 1990. *Four Neotropical Rainforests*. Yale University Press: New Haven.

Gentry AH. 1993a. Tropical forest biodiversity and the potential for new medicinal plants. pp 13-24 In: *Human Medicinal Agents from Plants*. AD Kinghorn and MF Balandrin (Eds). ACS Symposium Series 534, American Chemical Society, Washington, D.C.

Gentry AH. 1993b. A Field Guide to the Families and Genera of Woody Plants of Northwest South America (Colombia, Ecuador, Peru) with Supplementary Notes on Herbaceous Taxa. Conservation International, Washington, D.C.

Gerwick WH, Mrozek C, Moghaddam MF and SK Agarwal. 1988. Novel cytotoxic peptides from the tropical marine cyanobacterium *Hormothamnion enteromorphoides* Grunow. 1. Discovery, isolation and initial chemical and biological characterization of the hormothamnins from wild and cultured material. *Experientia*. 45: 115-121.

Gerwick WH, Roberts MA, Proteau PJ and J-L Chen 1994. Screening cultured marine microalgae for anticancer-type activity. *J. Applied Phycology*. 6: 143-149.

Gerwick WH, Tan LT and N Sitachitta. 2001. Nitrogen-containing metabolites from marine cyanobacteria. In: *Alkaloids: Chemistry and Biology*. Cordell GA (Ed). Academic Press: N.Y. Vol. 57. pp. 75-184.

Ghisalberti EL. 1993. Detection and isolation of bioactive natural products. In: *Bioactive Natural Products. Detection, Isolation and Structural Determination*. Colegate SM, Molyneux RJ (Eds). CRC Press, Boca Raton, Florida. pp. 9-58.

Glynn PW and JL Maté. 1997. Field guide to the Pacific coral reefs of Panama. *8th International Coral Reef Symposium*, pp. 145-166. Smithsonian Tropical Research Institute, Balboa, Republic of Panama.

Goreau TJ, Hayes RL and T McClanahan. 2000. Conservation of coral reefs after the 1998 global bleaching event. *Conservation Biology* 14: 1-18.

Grabowski HG and JM Vernon. 1994. Returns to R&D on New Drug Introductions in the 1980s. *Journal of Health Economics* 13: 383-406.

Guo B, Dai JR, Ng S, Huang Y, Leong C, Ong W, and BK Carte. 2000. Cytonic acids A and B: novel tridepside inhibitors of hCMV protease from the endophytic fungus *Cytonaema* species. *J. Nat. Prod.* 63: 602-604.

Gusman J and M Vanhaelen. 2000. Endophytic fungi: An underexploited source of biologically active secondary metabolites. *Recent Research Developments in Phytochemistry* 4: 187-206.

Guzmán HM, Jackson JBC and E Weil. 1991. Short-term ecological consequences of a major oil spill on Panamanian subtidal reef corals. *Coral Reefs* 10: 1-12.

Hamel E, Blokhin AV, Nagle DG, Yoo H-Y and WH Gerwick. 1995. Limitations in the use of tubulin polymerization assays as a screen for the identification of new antimitotic agents: The potent marine natural product curacin A as an example. *Drug Development Research* 34: 110-120.

Hawksworth DL. 1991. The fungal dimension of biodiversity: magnitude, significance and conservation. *Mycological Research* 95: 641-655.

Hay ME. 1996. Marine chemical ecology: what's known and what's next? *J. Exp. Mar. Biol. Ecol.* 200: 103-134.

Submitted

Submitted

INRENARE. 1992. Cobertura Boscosa 1992. INRENARE (National Institute of Renewable Natural Resources), Panamá.

Ishibashi M, Moore RE, Patterson GML, Xu C and J Clardy. 1986. Scytophycins, cytotoxic and antimytotic agents from the cyanophyte *Scytonema pseudohofmanni*. *J. Org. Chem.* 51: 5300-5306.

Karelva P. 1999. Coevolutionary arms races - is victory possible? *Proc Nat Acad USA* 96: 8-10.

Kathuria S and KN Ganeshaiah. 2002. Tectonic activity shapes the spatial patchiness in the distribution of global biological diversity. *Current Science* 83: 76-81.

Kursar TA, Coley PD, Capson TL, Gupta MP, Harrison LA, Ortega-Barría E and DA Windsor. 1999. Ecologically guided bioprospecting in Panama. *Journal of Pharmaceutical Biology* 37 (supplement): 114-126.

In Press

Langenheim JH, Macedo CA, Ross MK and WH Stubblebine. 1986. Leaf development in the tropical leguminous tree *Copaifera* in relation to microlepidopteran herbivory. *Biochem. Syst. Ecol.* 14: 51-59.

Leigh Jr. EG 1999. *Tropical forest ecology: A view from Barro Colorado Island.* Oxford Press, N.Y.

Levin DA. 1976. Alkaloid-bearing plants: An ecogeographic perspective. *American Naturalist* 110: 261-284.

Levin DA and BM York. 1978. The toxicity of plant alkaloids: An ecogeographic perspective. *Biochem. Syst. Ecol.* 6: 61-76.

Li WI, Berman FW, Okino T, Yokokawa F, Shioiri T, Gerwick WH, and Murray TF. 2001. Antillatoxin is a novel marine cyanobacterial toxin that potently activates voltage-gated sodium channels. *Proc. Natl. Acad. Sci. USA.* 98: 7599-7604.

Littler DS and MM Littler. 2000. *Carribean Reef Plants.* Offshore Graphics, Inc. Washington, D.C. 542 p.

Littler DS and MM Littler. 2003. *South Pacific Reef Plants.* Offshore Graphics, Inc. Washington, D.C. 331 p.

Lodge DJ, Fisher PJ and BC Sutton. 1996. Endophytic fungi of *Manilkara bidentata* leaves in Puerto Rico. *Mycologia* 88: 733-738.

Love J. 1999. Who pays what in drug development. *Nature* 397: 202.

Lowman MD and JD Box. 1983. Variation in leaf toughness and phenolic content among five species of Australian rain forest trees. *Aust. J. Ecol.* 8: 17-25.

Lucas PW, Turner IM, Dominy NJ and N Yamashita. 2000. Mechanical defences to herbivory. *Annal Botany* 86: 913-920.

Marquez BL, Watts KS, Yokochi A, Roberts MA, Verdier-Pinard P, Jiminez JI, Harnel E, Scheuer PJ and WH Gerwick. 2002. Structure and absolute stereochemistry of hectochlorin, a potent stimulator of actin assembly. *J. Nat. Prod.* 65: 866-871.

Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE and JL McLaughlin. 1982. Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Medica* 45: 31-34.

Submitted

Myers N, Mittermeier RA, Mittermeier CG, Fonseca GAB and J Kent. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403: 853-858.

Navarro JC. 1998. Panama, National Parks. Ediciones Balboa S.A. Panama City, Panama. 221 p.

Nogle LM, Gerwick WH. 2003. Wewakazole, a novel cyclic dodecapeptide from a Papua New Guinea *Lyngbya majuscula*. *Organic Letters* 5: 3-6.

Orjala J and WH Gerwick. 1996. Barbamide, a chlorinated metabolite with molluscicidal activity from the Caribbean cyanobacterium *Lyngbya majuscula*. *J. Nat. Prod.* 59: 427-430.

Orjala J, Nagle DG, Hsu V and WH Gerwick. 1995. Antillatoxin, an exceptionally ichthyotoxic cyclic lipopeptide from the tropical cyanobacterium *Lyngbya majuscula*. *J. Amer. Chem. Soc.* 117: 8281-8282.

Panama ReefMonitor Update http://www.reefkeeper.org/CRM/DownloadSite/PANAMA/Panama.html RMU Portobelo's Coral Reefs: Healthy and Holding - October 2001

Patterson GM and S Carmeli. 1992. Biological effects of tolytoxin (6-hydroxy-7-O-methyl-scytophycin b), a potent bioactive metabolite from cyanobacteria. *Arch. Microbiol.* 157: 406-410.

Petrini O. 1986. Taxonomy of endophytic fungi of aerial plant tissues. In: *Microbiology of the Phyllosphere.* Fokkema NJ, van den Heuval J (Eds). Cambridge University Press. pp. 175-187

PhRMA Annual Survey 2001. Pharmaceutical R & D Spending. Pharmaceutical Research and Manufacturers of America.

Price PW, Lewinsohn TM, Fernandes GW and WW Benson (Eds). 1991. *Plant-Animal Interactions.* John Wiley and Sons, NY. 639 p.

Public Citizen July 2001. "Rx R&D Myths: The Case Against The Drug Industry's R&D Scare Card. http://www.citizen.org/congress/reform/drug_industry/articles.cfm?ID=6514

Pyke CR, Condit R, Aguilar S and S Lao. 2001. Floristic composition across a climatic gradient in a Neotropical lowland forest. *Journal of Vegetation Science* 12: 553-566.

Qiangqiang Z, Jiajun W and L Li. 1998. Storage of fungi using sterile distilled water or lyophilization: Comparison after 12 years. *Mycoses* 41: 255-257.

Rausher MD. 2001. Co-evolution and plant resistance to natural enemies. *Nature* 411: 857-864.

Rippka R. Recognition and identification of cyanobacteria. 1988. *Methods Enzymol.* 167: 28-67.

Rosenthal GA and MR Berenbaum. 1991. *Herbivores. Their interactions with secondary plant metabolites.* Second Edition. Academic Press, NY.

Saikkonen K, Faeth SH, Helander M and TJ Sullivan. 1998. Fungal Endophytes: A Continuum of Interactions with Host Plants. *Annual Review of Ecology and Systematics* 29: 319-343.

Schiff PB, Fant J and SB Horwitz. 1979. Promotion of microtubule assembly *in vitro* by Taxol. *Nature* 277: 665-667.

Schulz B, Boyle C, Draeger S, Roemmert A and K Krohn. 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycological Research* 106: 996-1004.

Starr RC and JA Zeikus. 1993. UTEX-The culture collection of algae at the University of Texas at Austin. *J. Phycol.* 29: 1-105.

Tan GT, Pezzuto JM and AD Kinghorn. 1991. Evaluation of natural products as inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. *J Nat. Prod.* 54: 143-154.

Ten Kate, K and SA Laird. 1999. The Commercial Use of Biodiversity. Access to Genetic Resources and Benefit-Sharing. Earthscan Publications Ltd., London. 398 p.

Thompson, JN. 1999. The evolution of species interactions. *Science* 284: 2116-2118.

Submitted

Tolli SD, Dearing MD and TA Kursar. 1995. Extraction of phenolic compounds from fresh leaves: a comparison of methods. *J. Chem. Ecol.* 21: 117-125.

Tosi J. 1971. Zonas de vida. Republica de Panama FO:SF/PAN 6, *Informe Tecnico* 2. FAO, Roma. 123 p.

Trimurtulu G, Ohtani I, Patterson GML, Moore RE, Corbett TH, Valeriote FA and L Demchik. 1994. Total structures of cryptophycins, potent antitumor depsipeptides from the blue-green alga *Nostoc* sp. Strain GSV224. *J. Am. Chem. Soc.* 116: 4729-4737.

Vaishampayan U, Glode M, Du W, Kraft A, Hudes G, Wright J and M Hussain. 2000. Phase II study of dolastatin-10 in patients with hormone-refractory metastatic prostate adenocarcinoma. *Clin. Cancer. Res.* 6: 4205-4208.

Verhoeff, K. 1974. Latent infections by fungi. *Annual Review of Phytopathology* 12: 99-110.

Wall ME, Wani MC, Brown DM, Fullas F, Olwald JB, Josephson FF, Thornton NM, Pezzuto JM, Beecher CWW, Farnsworth NR, Cordell GA and AD Kinghorn. 1996. Effect of tannins on screening of plants extracts for enzyme inhibitory activity and techniques for their removal. *Phytomedicine* 3: 281-285.

Wani MC, Taylor HL, Wall ME, Coggon P and AT McPhail. 1971. Plant antitumor agents. VI. Isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J. Amer. Chem. Soc.* 93: 2325-2327.

Waterbury JB and JM Willey. 1988. Isolation and growth of marine planktonic cyanobacteria. *Methods Enzymol.* 167: 100-105.

Submitted

Wilson KE, Tsou NN, Guan Z, Ruby CL, Pelaez F, Gorrochategui J, Vicente F, and HR Onishi. 2000 Isolation and structure elucidation of coleophomones A and B, novel inhibitors of bacterial cell wall transglycosylase. *Tetrahedron Lett.* 41: 8705-8709.

World Bank. 2001 http://www.worldbank.org/data/wdi2001/pdfs/tab3_4.pdf.

Wysor B, Kooistra WHCF and S Fredericq. 2000. Marine Macroalgal Diversity of the Republic of Panama. Poster presentation at the Annual Meeting of the Phycological Society of American, San Diego, CA, 15-19 July 2000 http://morayeel.louisiana.edu/SeaweedsLab/Wysor/PSA_2000/poster.html#anchor2837646

Wysor BS. 2002. Biodiversity and biogeography of marine green algae of the Republic of Panama. Univ. of Louisiana, Lafayette, LA, USA. Avail. UMI, Order No. DA3045165. 291 p. From: Diss. Abstr. Int., B 2002 63: 1115.

Yao D and Y Zhang. 1986. Neurotoxicity and utilization of kainic acid. *Shengli Kexue Jinzhan*. 17: 55-57.

Zunino F, Dallavalle S, Laccabue D, Beretta G, Merlini L and G Pratesi. 2002. Current status and perspectives in the development of camptothecins. *Current Pharmaceutical Design* 8: 2505-2520.

H. CONSORTIUM/CONTRACTUAL ARRANGEMENTS

None

I. LETTERS OF SUPPORT (Consultants/Collaborators)

Dr. Mireya Correa (Director of the Herbaria at STRI and the University of Panama) has agreed to confirm all plant identifications (see letter of support, Appendix 2).

Dr. Valerie Paul (Head Scientist at the Smithsonian Marine Station in Fort Pierce, Florida) has worked for over 20 years on the chemical interactions of tropical macroalgae. She will help with the marine collection program and with taxonomic identifications. She will also host student interns (see letter of support, Appendix 2).

Ms. Lorna Sánchez will continue to help with data management (see letter of support, Appendix 2).

ASSOCIATE PROGRAM 2

ASSESSMENT OF BIOACTIVITY AGAINST PARASITES, CANCER AND AGRICULTURAL PESTS

Associate Program Leader: Eduardo Ortega-Barría

Research and Development
Institute of Advanced Scientific Investigations and High Technology Services
Secretariat for Science and Technology, Panama

International Cooperative Biodiversity Groups
"Bioassay and Ecology Directed Drug Discovery in Panama"
Dr. William Gerwick, Group Leader

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. DO NOT EXCEED THE SPACE PROVIDED.

The overall goal of Associate Program 2 is the isolation and development of new remedies for the treatment of neoplastic and tropical parasitic diseases, and the discovery of compounds for the control of agricultural pests. Biological materials will include terrestrial plants, marine algae and marine cyanobacteria from AP1 and selected marine corals from AP4. We will use novel colorimetric and fluorimetric assays developed in our laboratory to test samples for the presence of compounds with activity against parasites that cause American trypanosomiasis, leishmaniasis and malaria. For the discovery of compounds with activity against cancer, we will use cell-based assays [NCI-H460 (lung), SF268 (CNS), and MCF7 (breast)] in our laboratory and mechanism-based assays developed by the Novartis Institute for Biomedical Research (NIBR). The tumor cell lines will allow the detection of a wide variety of cytotoxic agents and are an ideal complement to the more specific mechanism-based assays of the NIBR. The cell lines will be used for bioassay-guided fractionation in Panama and to select extracts to be prefractionated for the NIBR. Dow AgroSciences (DAS) will evaluate the potential agrochemical utility of plant and marine samples from Panama by screening for insecticidal, fungicidal, and herbicidal activity. DAS will pursue secondary testing of all extracts in addition to isolation and structure elucidation of active compounds. An additional objective of this Associate Program is to enhance the capacity of local research institutions, train scientists and develop long-term relationships between Panamanian and US academic, pharmaceutical and agricultural companies that will secure the base for a sustainable Panama-based program for the discovery and commercialization of new drugs and products for agriculture.

PERFORMANCE SITE(S) *(organization, city, state)*
Institute of Advanced Scientific Investigations and High Technology Services Secretariat for Science and Technology, Panama City, Republic of Panama

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
ORTEGA-BARRÍA, Eduardo	Institute of Advanced Scientific Investigations and High Technology Services Secretariat for Science and Technology (Panama)	P.I. Associate Program 2; in charge of antimalarial and anti-Chagas' disease bioassays
ROMERO, Luz I	Institute of Advanced Scientific Investigations and High Technology Services Secretariat for Science and Technology (Panama)	In charge of leishmaniasis and anticancer bioassays

ASSOCIATE PROGRAM 2
ASSESSMENT OF BIOACTIVITY AGAINST PARASITES, CANCER AND AGRICULTURAL PESTS 212

A. SPECIFIC AIMS

The **overall goal** of Associate Program 2 is to conduct medicinally relevant assays for the discovery of treatments for neoplastic and tropical parasitic diseases and compounds for the control of agricultural pests. An additional objective is to enhance the capacity of local scientific infrastructure, to provide research opportunities for training students and to develop long-term relationships between Panamanian and US academic, pharmaceutical and agricultural companies that will help secure the base of a permanent and productive Panama-based program for the discovery of new medicines and products for agriculture.

The **Specific Aims** of Associate Program 2 are to:

1. **To analyze biological samples for medicinally relevant activity against the protozoan parasites responsible for leishmaniasis, American trypanosomiasis (Chagas' disease) and malaria.**
   Crude extracts from 150-200 species of Panamanian terrestrial plants and 50-75 species of Panamanian marine algae and cyanobacteria will be tested for activity in tropical disease assays. The assays will be used to direct the bioassay-guided fractionation that will take place in Associate Program 3. Bioactive molecules that show exceptional promise will be further evaluated in order to study their mechanism of action, potency, stability, and cytotoxic activity.

2. **To evaluate biological samples for anticancer activity using tumor cell lines in Panama and mechanism-based assays at the Novartis Institute for Biomedical Research.**
   We will carry out bioassays in our laboratories with three cancer cell lines [NCI-H460 (lung), SF268 (CNS), and MCF7 (breast)] to detect cytotoxic activity and to direct bioassay-guided fractionation in Associate Program 3. In addition, prefractionated extracts from terrestrial and marine plants (1,500-2,000 materials) will be sent to the Novartis Institute for Biomedical Research for mechanism-based assays.

3. **To evaluate the agrochemical utility of plant and marine samples in collaboration with Dow AgroSciences.**
   We will send crude extracts and prefractions (1,500-2,000 materials) for testing with DowAgroSciences. DAS will screen those samples for insecticidal, fungicidal, and herbicidal activity and carry out the isolation and structure elucidation of the compounds of interest.

4. **To develop the host-country capacity for a permanent Panama-based drug discovery program.**
   We will reinforce the bioassay component of a long-term and sustainable Panama-based drug discovery program by transferring technology and training students in the techniques of tissue-culture, the cultivation of parasites and the evaluation of data.

B. BACKGROUND AND SIGNIFICANCE

Natural products have been and continue to be one of the most important sources of pharmaceutical agents in use today. For example, it has been estimated that 37% of total pharmaceutical sales, and 45% of today's bestselling drugs, are from natural products and natural products-derived molecules (Frormann and Jas 2002). Another analysis reported that 8 of the top 25 top selling drugs worldwide derive from microorganisms (Chicarelli-Robinson et al. 1997). Approximately 60% of the agents in clinical trial for the treatment of cancer owe their origin to natural products (Cragg and Newman 2000). For tropical parasitic diseases, there exists a pressing need to discover novel treatments and to develop techniques that will facilitate the participation of scientists from the developing nations where those diseases have the greatest impact (Gelb and Hol 2002, Sachs 2002) and this Associate Program has attempted to address those needs for the parasites responsible for leishmaniasis, American trypanosomiasis (Chagas' disease) and malaria. In the field of agriculture, natural

products have a long and important history related to crop protection. They have been used as crude extracts, purified components and as templates for the design of new synthetic compounds.

B.1. The Impact of Tropical Parasitic Diseases

The burden imposed on the developing world by tropical diseases continues to generate an enormous price in human suffering and contributes to poverty and underdevelopment (Sachs 2002). Collectively, malaria, Chagas' disease and leishmaniasis affect 3 *billion* people, most of whom survive on less than $2 a day (Gelb and Hol 2002). For most parasitic diseases, drugs remain the mainstay of control (Marshall 2000, Modabber 2002). However, for many of these diseases the current repertoire of pharmaceutical agents is inadequate, a problem which is exacerbated by the emergence of drug resistance (Gelb and Hol 2002). While most pharmaceutical companies have concentrated on finding remedies for the afflictions of industrialized countries, *e.g.*, cancer, heart disease, and chronic pain, no new classes of antiparasitic drugs have been developed since the 1960s, and few are in the pipeline (WHO 2000). Of the 1223 new drugs that entered the market between 1975 and 1996, only 11 were directed against the parasites that affect millions in Africa and the Americas (TDR news 2000). With the exception of AIDS, research and development for the world's greatest causes of premature death (pneumonia, diarrhoeal diseases, tuberculosis and malaria) amounted to less than $500 million, the cost it takes to bring one drug to market. Since it takes an estimated 10-20 years to develop a drug, it is critical that research and development be accelerated and that the pool of researchers be expanded (WHO 2000, Sachs 2002). Beyond the mortality inflicted upon residents of developing countries, outbreaks of parasitic diseases are likely to affect rich countries as well, since drug resistant diseases can spread via immigration and travel (Kirchhoff 1993). Despite the traditional lack of interest on the part of the pharmaceutical industry in the development of novel chemotherapeutic agents for parasitic diseases, recent initiatives have been described that are designed to bring affordable and effective antiparasitic drugs to the market (Gelb and Hol 2002).

B.1.1. The *Leishmaniases*

The leishmaniases comprise a group of infections caused by a number of species of the obligate intracellular parasite *Leishmania* (Desjeux 1996). Infections are acquired through the bite of female sandflies of the genera *Phlebotomus* and *Lutzomyia*. Leishmanial infections occur in nature in a wide range of vertebrate hosts but are particularly common in canids, rodents, and primates, including humans (WHO 1990). Clinical syndromes including asymptomatic infections, self-healing cutaneous or disfiguring mucocutaneous lesions, and severe disseminated disease with fatal outcomes (WHO 1990). An estimated 12 million people are infected worldwide, and 350 million live in endemic areas and are at risk of acquiring leishmaniasis (Desjeux 1996). There are no effective means of prevention as vector and reservoir control are of limited value (WHO 1990).

The clinical manifestations of leishmaniasis are caused by the amastigote form of the parasite, which is an obligate intracellular parasite of host-cell macrophages. Sandflies feeding on infected hosts ingest cells containing amastigotes which rapidly transform into promastigotes. These are extracellular forms of the parasite which multiply rapidly in the lumen of the sandfly gut. Following inoculation into the host skin by an infected sandfly, promastigotes rapidly attach and enter cells of the mononuclear phagocyte system, where they transform into the amastigote form (Figure 1, WHO 1990). In the New World, leishmaniasis is widespread from southern Texas to Northern Argentina and the Caribbean Islands (Weigle and Saravia 1996, Maloney *et al.* 2002). Most cases occur in rural areas where the parasite, the host, and the vector coexist. While leishmaniasis can be treated with pentavalent antimonials, resistance to the treatment is emerging (Croft and Yardley 2002). The efficacy of the antimony treatment is variable (57-100% cure rate), requires long courses of therapy and causes significant liver and cardiac toxicity in up to 20% of patients (Croft and Yardley 2002). Other treatments for leishmaniasis such as amphotericin-B and pentamidine are associated with multiple adverse side effects such as bone marrow suppression, renal toxicity and glucose metabolism disturbances (Berman 1997, Myerhoff 1999). While miltefosine (hexadecylphosphocholine) has shown promise as a treatment of Indian visceral

leishmaniasis (Jha *et al.* 1999) the author of a recent review article reported "there remains a pressing need for new antileishmanials" (Croft and Yardley 2002).

B.1.2. American Trypanosomiasis (Chagas' Disease)

Trypanosoma cruzi is the etiologic agent of Chagas' disease, a chronic multisystemic disease that affects millions of people in temperate, subtropical, and tropical regions in the Americas and West Indies, killing 10-20% of the people it infects (Gelb and Hol 2002). Infection is zoonotic, transmitted to humans by bloodsucking insects of the family Reduviidae. Approximately 90 million people in Latin America are at risk of infection, and 16 to 18 million are actually infected. The overall incidence of the disease has been estimated at 200,000 cases a year, and over 70,000 individuals die annually as a consequence of the infection (WHO 1991, Dusanic 1991). While human infection is rare in areas north of Mexico, a serosurvey conducted in 1989 to determine the prevalence of *T. cruzi* infection among Central American immigrants in the Washington DC area found 5% of them to be infected (Kirchhoff *et al.* 1987), suggesting that 50,000 to 100,000 US immigrants may be living with the disease (Grant *et al.* 1989). Fulminant infections resulting from blood transfusion in the United States and Canada have been reported (Kirchhoff *et al.* 1993).

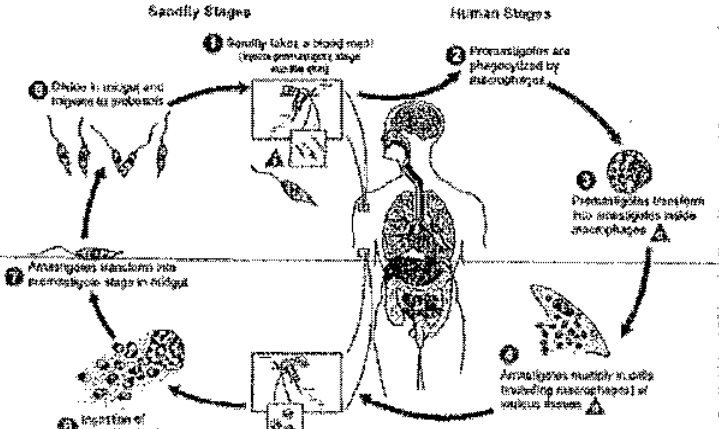


Figure 1. *Leishmania* life cycle (http://www.dpd.cdc.gov/DPDx/).

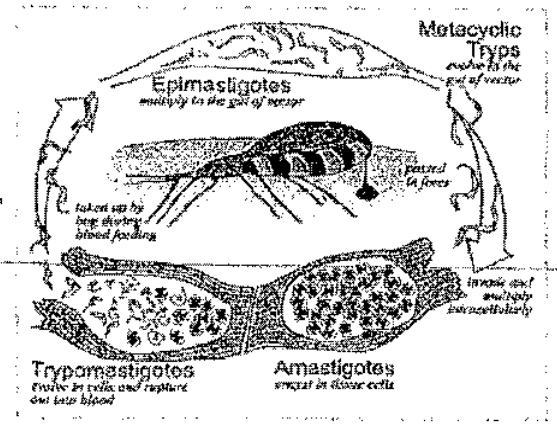


Figure 2. The life cycle of *T. cruzi.*

The developmental cycle of *T. cruzi* includes three morphologic forms: amastigotes, epimastigotes, and trypomastigotes (Figure 2). The amastigote is the dividing intracellular form found in the tissues of the mammalian host. The trypomastigote is found in the tissue and bloodstream of infected mammals and is responsible for the spread of infection from cell to cell and is also the form that is transmitted to the insect vector. The epimastigote is the form that multiplies in the digestive tract of the triatome vector. The epimastigote can also be cultured axenically, *i.e.*, in the absence of its host cell. While the insect feeds, the trypomastigote is excreted in its feces and urine, and then enters the body through an abrasion on the skin, an intact mucous membrane such as the conjunctiva, or the wound caused by the bite.

Three clinical stages are recognized in Chagas' disease: a short acute stage, a long-lasting chronic stage, and a long, clinically asymptomatic indeterminate stage (WHO 1991). There is no evidence that individuals in the chronic or indeterminate phases of the disease benefit from drug therapy, therefore, treatment is prescribed only for patients in the short acute phase of Chagas' disease. Treatment with nifurtimox (a nitrofuran derivative) and benznidazole (a nitroimidazole) results in adverse side effects, including weakness, anorexia, nausea, and vomiting in most patients (Viotti *et al.* 1994). Continued therapy is frequently associated with

toxic hepatitis, central and peripheral nervous system symptoms, such as loss of memory, tremor polyneuritis, paresthesia, seizures, and hematologic alterations. Of additional concern, lymphomas developed in 33% of the rabbits treated with nifurtimox and 42% of the rabbits given benznidazole (Viotti *et al.* 1994). Thus, there is an urgent need to develop of new pharmacologic agents against *Trypansoma cruzi* (Gelb and Hol 2002).

B.1.3. Malaria

Malaria is the world's most important parasitic infection, ranging among the major health and developmental challenges for the poor countries of the world (Sachs *et al.* 2002). More than a third of the world's population (about 2 billion people) live in malaria endemic areas, and 1 billion people are estimated to carry parasites at any given time (Guerin *et al.* 2002). Each year there are 300 to 500 million new plasmodium infections and 1 to 3 million deaths from malaria in the developing world (Marsh 1998, Marshall 2000). *Plasmodium falciparum* is the most malignant of the four human malarial parasite species (Wellems 2002).

The quinoline antimalarials and related aryl alcohols owe their origins to quinine, isolated from the bark of the Peruvian tree *Cinchona ledgeriana*, which was first imported into Europe from Peru for antimalarial use in the seventeenth century (Meshnick and Dobson 2001). The quinoline antimalarial, chloroquine, has for decades been the primary chemotherapeutic means of malaria treatment and control, but resistance to chloroquine has developed on a global scale and has been met by a few alternative drugs, all of which were more expensive, encountered resistance problems of their own, or were less safe and more difficult to use than chloroquine itself (Ridley 2002, Wellems 2002). In China, infusions prepared from wormwood (*Artemisia annua*) were used for treating fever over a thousand years ago, a property attributed to artemisin, a sesquiterpene lactone that has been used increasingly over the past two decades to treat malaria (Silachamroon *et al.* 2001, Ridley 2002). No clinically relevant resistance of *P. falciparum* to artemisin as yet has been described, nevertheless toxicity associated with high doses has negatively impacted its use (Ridley 2002). The complexity of the malaria parasite's life cycle has made the development of vaccines problematic and antimalarial drugs are considered the last certain line of defense (Figure 3, Marsh 1998, Marshall 2000).

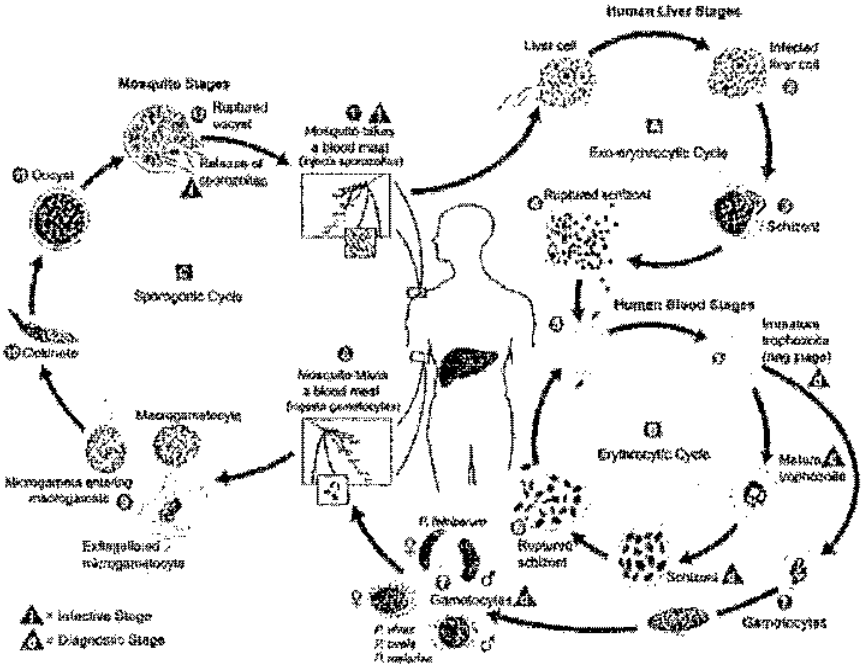


Figure 3. Life cycle of malaria (http://www.dpd.cdc.gov/DPDx/).

B.2. Natural Products as Anticancer Treatments

Approximately 60% of the agents in clinical trial for the treatment of cancer owe their origin to natural products (Cragg and Newman 2000). From a very recent analysis by Newman *et al.* (unpublished), which updates their earlier tally of the origins of clinically useful agents (Cragg *et al.* 2000), 697 agents are currently in use in the clinic. Of these, 30 (4.3%) are unmodified natural products, 155 (22.2%) are modified natural products, 77 (11%) have a natural products "ancestry" and 42 (6%) are "biologicals" (*e.g.* proteins). The remaining 393 (56.4%) are synthetic compounds. Hence, in raw numbers, natural products have played a critical role in the development of 262 (37.5%) of the agents in use today. Approximately 40 chemotherapeutic agents and 20 hormonal agents are used to clinically treat cancer. These compounds used alone or in combination with surgery, radiation, or biomolecules (*e.g.* proteins) have had limited success with only 10% of individuals afflicted with cancer attaining long term survival. Therefore, there exists a need to discover novel potent chemotherapeutic agents. In the past two years, some of the natural products or natural product derivatives entering clinical trials include SGN-10 (from *Escherichia coli*, Smith 2001), flavopiridol (from the Indian tree *Dysoxylum binectariferum*, Wang 2001), rapamycin (from *Streptomyces hygroscopicus*, Elit 2002), irofulven (from the mushroom *Omphalotus illudens*, Baekelandt 2002), FR-901228 (from *Chromobacterium violaceum*, Vigushin 2002), ecteinascidin-743 (from the marine tunicate *Ecteinascidia turbinate* Verschraegen and Glover 2001), and mitomycin C (from *Streptomyces caespitosus*) complexed to hydroxypropyl-β-cyclodextrin (Grosios 2002).

B.2.1. Molecular Target Assays Implemented at Novartis Oncology

Novartis Pharmaceuticals is a world leader in the research and development of products to protect and improve health and well-being. In terms of research and development, Novartis Oncology is a flagship therapeutic area having a pipeline that is the envy of the industry. The recent clinical success of Gleevec, for example, in the treatment of certain leukemia and other cancers has solidified Novartis' role in ushering in a new era of molecular targeted therapeutics (Bakhtiar *et al.* 2002).

The Molecular Target Feasibility Unit (MTFU) of Novartis Oncology has been created to sustain a continuous flow of molecular targeted screens relevant to cancer. These will be used in the current proposed collaboration (Section D.3.2) to discover novel compounds from terrestrial plant and marine algal extracts from Panama that are useful in treating the major types of cancer (colon, lung, breast, prostate, and ovarian). This will be achieved by attacking appropriate targets for cancer drug development including modulation of the activity of oncogenes, tumor suppressor genes, apoptosis, and various signal transduction pathways. Targets that have been addressed by the MTFU of Novartis Oncology in the past calendar year are: Mu B-raf, Flt-3, Mu-Abl, FAK, PKB, P300 HAT, Sirt2, Edg-1 FLIPR, Edg-1/S-1-P Binding, Axl, DNMT3b, CXCR4, PAI, UBCH10, UBC12, UBC9, E2-10,E2-12,E2-17,E2-6, HDAC1, Ephrin 4B Kinase, Ephrin 4B/Eph 2 Binding, USP2, FAS, RET, FGF3-R, ALK, BACE2, PPAR-δ, Hsp90, Bub-1 Kinase.

Some of the more active targets at Novartis include apoptosis screens, kinases, histone acetylases, histone deacetylases, methyltransferases, SMAC/IAP, Hsp90, EDG-1, hypoxia inducible factor/p300 and CXCR-4. Four active kinase programs include protein kinase B, mutant and wild-type Abl, mutant and wild-type β-Raf, and protein dependent kinase -1. Histone acetyltransferases and deacetyltransferases include HAT, HDAC1, SIRT-2, and SIRT-1. There are also programs for DNA methyltransferase 1 and DNA methyltransferase 3b3. The SIRT-2/HDAC-1 system is a recently developed target against which deacetylvismione A showed good activity and is described here, together with two other examples, to provide a background of the criteria of epidemiology, pathophysiology, assayability, and drugability that are considered prior to developing a new target for screening.

B.2.1.1. Sirt-2/HDAC-1 Regulatory Mechanism

Reversible histone acetylation is a major regulatory mechanism that is thought to modulate gene expression by altering the accessibility of transcription factors to DNA. Histone deacetylase (HDA) inhibitors have been shown to result in activation of gene expression and anti-proliferative effects in tumor cells, such as induction of

cell cycle arrest and differentiation in certain transformed cell lines. The collaboration of histones with transcription coactivators and factors in post-translational modification is important in gene regulation. HDA inhibition through histone deacetylation is postulated to produce transcriptional activation by facilitating the accessibility of transcription factors to nucleosomal DNA. The anti-proliferative effects of compounds that inhibit HDA include cell cycle arrest, differentiation or apoptosis of transformed cells (Yoshida *et al.* 1990, Hoshikawa *et al.* 1991, Sugita *et al.* 1992, Hoshikawa *et al.* 1994, Sambucetti *et al.* 1997, Yoshida and Beppu 1998). Furthermore, HDA inhibition leads directly to induction of human p21 cyclin dependent kinase inhibitor (Sambucetti *et al.* 1997), a key mediator of G1 arrest and differentiation, suggesting that p21 induction is involved in mediating certain of the anti-proliferative effects caused by HDA inhibition. A large number of human cancers have low levels of p21 expression possibly due, in part, to loss of p53 activity, a transcriptional activator of p21. Inhibiting HDA may arrest the cell cycle in cells with a defective p53 pathway. This strategy would potentially lead to compounds selectively toxic to cancer cells, since normal tissues generally have lower levels of HDA than tumors. Two HDA enzymes currently being evaluated are HDAC-1 and Sirt-2 using the detection mechanism illustrated in Figure 4.

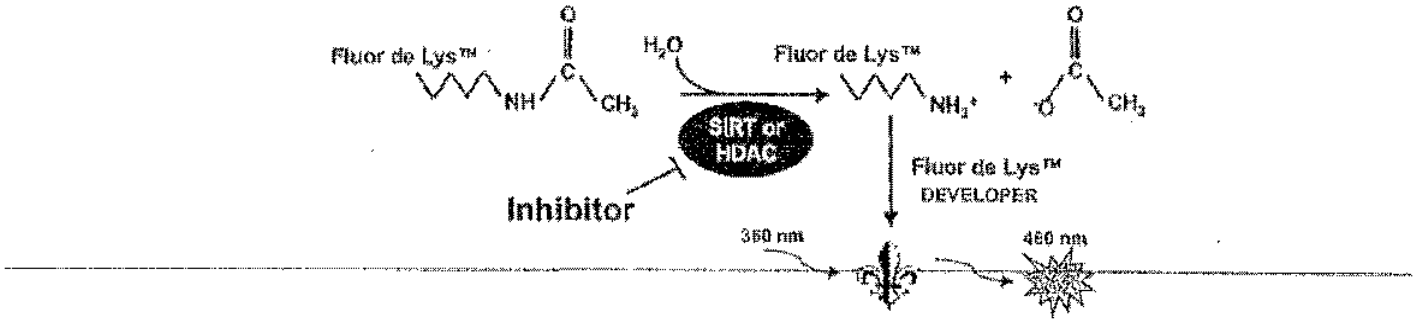


Figure 4: Schematic representation of the Sirt-2/HDAC-1 assay

B.2.1.2. β-Raf Kinase Regulatory Mechanism

The serine/threonine kinase Raf-1 plays a critical role in linking growth factor receptors at the cell membrane with transcription factors. The regulation of Raf-1 activity is complex and involves Ras, as well as several serine/threonine and tyrosine kinases. Through a series of phosphorylation events, extracellular signals are connected through the Raf-1/MAP kinase pathway to transcription factors involved in controlling cell growth, differentiation, and survival (Magnuson *et al.* 1994). This cascade also mediates transformation by many oncogenes (Daum *et al.* 1994). It has been demonstrated that blocking c-Raf-1 protein production by antisense oligonucleotides is sufficient to inhibit both Ras-dependent signal transduction and tumor growth (Monia *et al.* 1996). Therefore, the inhibition of c-Raf-1 kinase activity represents an attractive target for pharmacological intervention and the mechanism for detection of inhibition is presented in Figure 5.

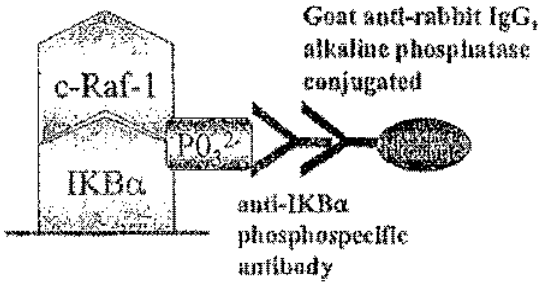


Figure 5. Schematic representation of the c-Raf-1 kinase assay

B.2.1.3. p300/HIF-1 Regulatory Mechanism

New blood vessel formation is essential for tumor growth, invasion, and metastasis. Hypoxia-induced genes such as VEGF play an important role in angiogenesis by acting as potent inducers of vascular permeability, as well as serving as specific endothelial cell mitogens (Semenza 2000). High expression of VEGF was confirmed in tumor tissues, and platelet activation was identified within the tumor vasculature (Verheul *et al.* 1999). The transcriptional activation of hypoxia-induced genes, including VEGF, requires the interaction of HIF with the p300/CBP nuclear coactivators (Kawasaki *et al.* 1998). Antagonists of HIF activity, through disruption of its binding to p300, may prevent neoangiogenesis associated with solid tumors. The principle behind detection of HIF activity antagonists is illustrated in Figure 6.

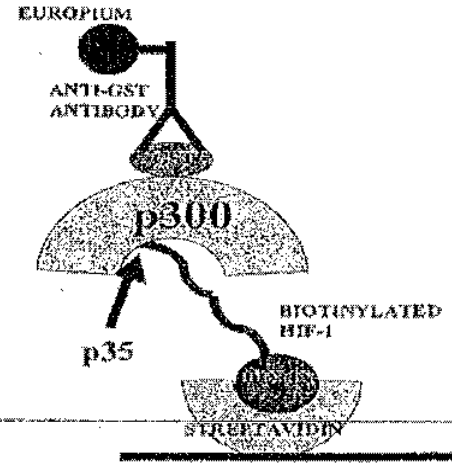


Figure 6. Schematic diagram of the HIF-1/p300 binding assay

B.3. Natural Products as Agrochemicals

Natural products have a long and important history related to crop protection. They have been used as crude extracts, purified components and as templates for the design of new synthetic compounds. The unique structural diversity represented by natural products has been associated with unique modes-of-action (Henkel *et al.* 1999, Duke *et al.* 2000), a key factor in the success of new agrochemicals. The most notable family of commercial products derived from a natural product template is that of the pyrethroids which were developed as synthetic mimics of the pyrethrins. Dried pyrethrum (*Tanacetum cinerariifolium*) flowers have been known for centuries in ancient China and Persia to be effective for insect control (Gullickson 1994). The synthetic pyrethroid analogs are much more active and photostable than the pyrethrins from which they were modeled (Elliot 1996) and represent one of the principal insect control agents used around the world, accounting collectively for over 1 billion dollars in sales annually. Other examples include the discovery and commercialization of the sulcotrione herbicides, modeled after the natural product, leptospermone (Lee *et al.* 1997), and the strobilurin family of fungicides which were developed from the basidomycete natural product, strobilurin A (Sauter *et al.* 1996).

In some cases the biomedical activity of the natural product itself is sufficient to achieve high levels of efficacy under field conditions. Such is the case of spinosad, a new microbially derived insect control agent discovered and developed at Dow AgroSciences. Spinosad is the first commercial representative from this new family of macrolides. A mixture of two closely related factors (spinosyns A and D, Figure 7) are produced by the actinomycete *Saccharopolyspora spinosa* and are present in the final product.

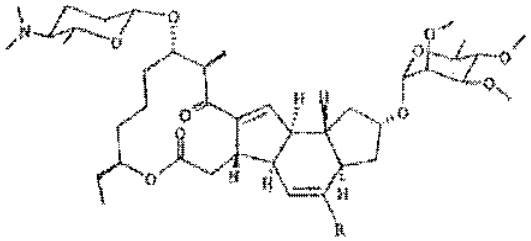


Figure 7. Structures of spinosyn A (R=H) and spinosyn D (R=Me).

Spinosad is an extremely potent insecticide against a number of important lepidopteran pests including tobacco budworm, beet armyworm, European cornborer, and pink bollworm. It is also very selective with respect to predators and certain parasites allowing it to be incorporated into many Integrated Pest Management (IPM) programs. Spinosad has a novel action on nicotinic receptors which has been identified as the primary cause of death. No cross-resistance has been identified and there is no metabolic or other type of resistance known. Tracer*, the spinosad formulation for use in cotton, received U.S. registration in 1997. The expertise of Dow AgroSciences in discovering, developing, and commercializing natural products is illustrated by the recent successful launch of spinosad around the world. Further, there are a number of other novel natural products that have been discovered at Dow AgroSciences with potential agrochemical utility (Fields *et al.* 1996, Gerwick *et al.* 1997, Schmitzer *et al.* 2000, Lewer *et al.* 2002, Lewer *et al.* 2003).

B.3.1. Screening for Novel Agrochemicals by Dow AgroSciences.

Dow AgroSciences (DAS) is the global agrochemical/biotechnology division of The Dow Chemical Co., with headquarters located in Indianapolis, Indiana. Dow AgroSciences discovers, develops, and markets pest management products for the effective control of plant diseases, weeds and insects. The product mix includes insecticides, fungicides, herbicides, soil fumigants, plant growth regulators, and nitrogen stabilizers. Dow AgroSciences employs approximately 5,000 employees globally, with annual sales near 3 billion dollars. The commitment to discovery of new agrochemicals is evidenced by the Research and Development Center which employees over 450 scientists. The Natural Products Discovery group is located within the Research Center and has accountability to deliver leads and products offering new modes-of-action and reduced risk to the applicator, beneficial organisms, and the environment.

A number of proprietary assays have been developed at DAS to identify promising activities against insects, crop diseases, and weeds. The standard testing protocol for crude extracts and natural product mixtures includes a series of screening assays in each therapeutic area, followed by increasingly refined assays to identify those candidates with desirable lead and product attributes. These screening assays include a number of *in vivo* and *in vitro* targets, are adapted to a 96-well microtiter plate format, and can be executed at high throughput with rapid data turn around. Most assays are performed with robot assistance and with automated data acquisition. The precise assays and targets are proprietary but include insect pests representative of the orders, Lepidoptera (caterpillars), Coleoptera (beetles), Homoptera (aphids, scales, hoppers), Diptera (flies), Isoptera (termites), as well as representatives from the class Arachnida (mites). Similarly, assays are in place to identify activity against the major plant diseases including Phycomycetes (late blight), Ascomycetes (blotches), and Basidiomycetes (rust, sheath blight). Phytotoxic (herbicidal) activity is also evaluated in the high throughput 96-well format. Dow AgroSciences routinely evaluates tens of thousands of extracts and fermentation broths each year for promising new activities.

Extracts described in the current proposal will be screened in the above battery of assays and promising activities carefully examined in secondary and advanced levels of testing. Secondary tests typically involve evaluation of the pest in, or on, its crop host and are not conducted in microtiter plate format. Further, a dose range is applied to understand the relative sensitivity of different pests and the level of crop tolerance to the pest control agent. In the case of herbicide testing, extracts are applied directly to one or more of the following target weeds growing in a soil matrix: *Amaranthus retroflexus, Chenopodium album, Lamium amplexicaule,*

Polygonum lapathifolim, Galeopsis tetrahit, Abutilon theophrasti, Ipomoea hederaceae, Pharbitis purpurea, Setaria faberi, Echinochloa crus-galli, and *Sorghum halepense*. Accordingly, it is important that the amount of extract utilized in the initial screening assays be adjusted to achieve a hit rate of 2% or less in a given assay. The level of activity observed in secondary tests is generally sufficient to enable a decision on whether to pursue identification of the active component. Occasionally, other advanced levels of tests may be performed to examine additional performance attributes *e.g.* systemic *vs.* contact activity, curative *vs.* protectant, preemergence *vs.* postemergence. The biology staff at Dow AgroSciences are highly capable of implementing advanced levels of tests to clearly define performance attributes. Those activities that appear promising will be advanced initially to dereplication and subsequently to isolation and identification.

C. PROGRESS REPORT AND PRELIMINARY RESULTS

C.1. Establishment of Research Programs to Find Treatments for Tropical Disease

Over the past four years, scientists working on the tropical disease component of the Panama ICBG have worked with the other ICBG participants to create two new research programs in Panama. Their primary accomplishments include: (i) the creation of two fully-equipped laboratories in Panama, one for tropical disease bioassays and the other for bioassay-guided fractionation, (ii) the training of a group of Panamanian students in the culture of parasites and the techniques of bioassay-guided fractionation, (iii) the establishment for the first time in Panama of cultures of *Plasmodium falciparum*, (iv) the development of a novel DNA-based microfluorimetric bioassay to measure antiplasmodial activity, (v) the development of two novel non-radioactive bioassays for measuring antileishmanial activity, (vi) the implementation of a colorimetric bioassay to measure antitrypanosomal activity, (vii) and the purification and characterization of novel compounds with activity against *Leishmania spp.*, *Plasmodium falciparum*, and *Trypanosoma cruzi*. Presently, the programs of Dr. Ortega-Barría and Dr. Luis Cubilla are widely regarded as among the most productive in the country and have already trained students that are now seeking M.S. and Ph.D. degrees abroad. Dr. Cubilla will be part of Associate Program 3 in the next iteration of the ICBG.

C.1.1. Drug Discovery for Leishmaniasis

C1.1.1. Development and Application of a Novel Promastigote Antileishmanial Assay

The *Leishmania* group is headed by Dr. Luz Romero, who devotes % Effort of her time to the ICBG program. We initially chose to work with the promastigote stage of *Leishmania mexicana* since it is the form of the parasite most easily grown *in vitro* and it can be cultured in well-defined media in the absence of a host cell, reaching high titers (0.5×10^8/mL). A novel colorimetric assay was developed and standardized that provided a quantitative and efficient method for the evaluation of antileishmanial activity in plant extracts and purified compounds Submitted The assay employs the tetrazolium salt, sodium-2,3-bis-[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT). In the presence of the electron-coupling agent, phenazine methasulfate, XTT is reduced by mithocondrial dehydrogenase to a water-soluble product in the culture media supernatant whose concentration can be determined by measuring the optical density at 450 nM in a microplate reader. The XTT method offers several advantages over the conventional colorimetric method that employs 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Unlike the water-insoluble formazan produced upon reduction of MTT, which requires aspiration of the media followed by solubilization with isopropanol prior to measuring the optical density at 570 nm, the formazan product produced upon reduction of XTT is water-soluble, meaning that the optical density can be read directly without aspiration of the media. As a result, the XTT method is simpler and avoids an aspiration step that may result in the inadvertent removal of parasites, an important consideration in time-course experiments. An additional advantage of the XTT method is the shorter period of incubation, a result of the accelerated reduction of the XTT substrate by phenazine methasulfate (Buttke *et al.* 1999).

Optical density values obtained at 450 nM with the XTT method correlated well ($r = 0.965$) with parasite numbers in a growth curve of *L. mexicana* determined by microscopic counting. Similarly, parasite numbers

measured by the XTT method correlated with the values obtained by the conventional MTT method (r=0.961). Likewise, both the XTT- and MTT-based assays showed the same degree of sensitivity in terms of their ability to detect *Leishmania* promastigotes. The minimal number of parasites detected over the control background was 977 promastigotes for both methods. The IC_{50} values obtained for the reference anti-leishmanial drugs, amphotericin-B, hexadecylphosphocholine (Miltefosine) and Ketoconazole were 0.037 μg/mL, 10.2 μg/mL and 0.023 μg/mL, respectively, and were similar to those previously documented (Berman *et al.* 1984, Kuhlencord *et al.* 1992, Golenser 1999).

C.1.1.2. Testing of Plant Extracts and Pure Compounds for Antileishmanial Activity

The XTT-based methodology was employed to screen 1647 crude plant extracts (consisting of original collections, and recollections, of young and mature leaves from the same species) in addition to 609 partially purified fractions and purified compounds. For plant extracts, we considered any extract with IC_{50} values lower than 40 μg/mL as "active". Plants selected for bioassay-guided fractionation were chosen on the basis of consistent bioactivity below 40 μg/mL and the absence of reported bioactivity or known antileishmanial compounds in the SciFinder database. The XTT-based methodology was used to direct the bioassay-guided fractionation of *Guatteria amplifolia* leading to the isolation of the known aporphine alkaloids, xylopine and nornuciferine, while fractionation of *G. dumetorum* yielded aporphine alkaloids, cryptodorine and nornantenine (see AP3 Section C.1.1.1.). The antileishmanial activities of these compounds are shown in Table 1. Due to the promising yet unreported antileishmanial activities of these compounds, an

PATENT PENDING

The isolation of aegeline from *Sarcorhachis naranjoana* (Piperaceae) is described in AP3 (Section C.1.1.1.). While aegeline is a known compound, its antileishmanial properties were not previously recorded (IC_{50} = 12 μM).

Table 1. Antileishmanial activity of purified compounds from *Guatteria amplifolia* and *G. dumetorum*, and cytotoxicity to human foreskin fibroblasts.

Sample	IC_{50}, μg/mL±std dev. (μM)			
	L. mexicana	*L. panamensis*	Macrophages	HFF[b]
Xylopine	1±0.08 (3)	2±0.02 (6)	33±0.05 (112)	34± 0.03(115)
Cryptodorine	1±0.2 (3)	2±0.024 (6)	20±0.01 (64)	18± 0.02(58)
Nornantenine	8±0.1 (24)	5±0.145 (15)	>40	>40
Nornuciferine	4± 0.28 (14)	8±3.2 (28)	>40	>40

[a]Results are expressed as IC_{50} (the concentration of compound that inhibited growth of fifty percent of the parasite or cell line). [b]Human foreskin fibroblasts.

C.1.1.3. Cytotoxicity of Antileishmanial Compounds

To determine if xylopine, cryptodorine, nornantenine and nornuciferine show greater toxicity to the *Leishmania* parasites than to mammalian cell lines, we measured their toxicity towards human foreskin fibroblasts and the murine macrophage cell line, J-774. Fibroblasts are mesenchymal cells commonly found in organs and connective tissue and are isolated in our laboratory from the foreskin of newborns. Macrophages are ubiquitous immune cells in mammalian tissues and are the host cells of the *Leishmania* parasite in infected individuals. As shown in Table 1, xylopine showed approximately 37-fold higher toxicity towards *L. mexicana* than macrophages while the cytotoxicity of nornantenine and nornuciferine was not detectable at the highest concentration tested.

Submitted

In order to assess the potential utility of these four aporphine alkaloids as antileishmanial treatments, STRI recently awarded scientists from our Associate Program $63,000 to test the *in vivo* potential of these alkaloids to cure leishmaniasis in a murine model of the disease. AP3 chemists will work in collaboration with scientists at the Department of Laboratory Animals of the University of Panama.

C.1.1.4. Development and Application of a Novel Amastigote Antileishmanial Assay

The assay described in Section C.1.1.1 utilizes the infective, or promastigote form of the parasite. Of greater clinical relevance is the amastigote form of the parasite which multiplies inside the host macrophages and is responsible of the disease manifestations in humans and which should the target of any novel treatment (Croft and Yardley 2002). A method to convert the *Leishmania* promastigotes into amastigotes by emulating the phagolysosome conditions at low pH (5.5) and temperature (36 °C) has been described (Bates *et al.* 1992). We subsequently found that pH required to induce the promastigote to amastigote transformation was inconsistent with the reduction of the XTT substrate, prompting the search for an alternative method to quantitatively evaluate parasite multiplication. In response, we developed a novel microfluorimetric amastigote assay which employs PicoGreen®, a DNA intercalating agent that forms a fluorescent complex with the parasitic DNA. As the promastigotes are grown axenically (*ie.*, cell-free) there is no potential interference with cellular DNA and growth is measured with a fluorimeter in 96-well plates. The fluorescence values obtained with the microfluorimetric method showed a strong correlation with parasite number when counted manually ($r = 0.97$). This assay was established with *Leishmania panamensis,* which produces both the cutaneous and mucocutaneous clinical forms of the disease (WHO 1990). Since May of 2002, we have used this method for the screening of plant extracts and for bioassay-guided fractionation. A total of 159 crude extracts have been processed, 11 of which (7%) were consistently active and which are considered as candidates for bioassay-guided fractionation.

C.1.2. Drug Discovery for *Trypanosoma cruzi* (Chagas' Disease)

C.1.2.1. Establishment of Colorimetric Assays

Dr. Eduardo Ortega-Barría leads the Chagas' disease group. Ours is one of few laboratories in the world to employ a recombinant strain of *T. cruzi* that express the *Escherichia coli* β-galactosidase gene to test candidate antitrypanosomal compounds. The colorimetric assay was developed by Fred Buckner (Buckner *et al.* 1996) who generously provided the recombinant strain to Dr. Ortega-Barría. Transfected parasites catalyse a colorimetric reaction with chlorophenol red-β-D-galactopyranoside (CPRG) as substrate and the amount of β-galactosidase activity is directly proportional to the number of transfected parasites. The assay is performed in a 96-well plate and is easily and accurately quantitated with a microplate reader.

We initially established a technique to evaluate the extracellular (epimastigote) form of the *T. cruzi* parasite since the growth requirements and conditions of the culture are relatively straightforward. We have since established a bioassay with the intracellular (amastigote) form which we now use routinely to evaluate potential antitrypanosomal compounds. As mentioned in Section B.1.2, while the trypomastigote stage of *T. cruzi* is responsible for spreading the infection from cell to cell, it is short-lived (hours), rapidly transforming into the amastigote form upon entering into the host-cell cytoplasm. It is the amastigote form of the parasite which is the most relevant clinically. The bioassay uses human fibroblasts as host-cell, and to better mimic natural conditions, we allow the trypomastigotes to invade the host cell monolayer overnight prior to exposing the infected cells to the test substance. Controls are performed to accurately determine the effect of the test compound on both the parasite and the host-cell. Plant extracts are initially tested in duplicate at two concentrations, 50 and 10 μg/mL, and extracts that show IC_{50} lower than 50 μg/mL are considered active. Extracts that are active at a single concentration are reconfirmed by testing in duplicate at 50, 10, and 2 μg/mL, and the same concentrations are used for testing fractions from bioassay-guided fractionation. The availability in Panama of assays for both the amastigote and trypomastigote forms of the parasite allows us to explore the specificity of any antitrypanosomal activity and to address the potential mechanism of action of the compounds due to the biochemical and antigenic differences between the developmental stages of *T. cruzi.* (*e.g.* Furuya *et al.* 2000).

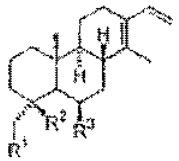
C.1.2.2. Testing of Plant Extracts and Pure Compounds for Antitrypanosomal Activity

A total of 2,181 extracts, (consisting of original collections, young and mature leaves from the same species, and recollections) have been tested for antitrypanosomal activity. In addition, a total of 268 assays

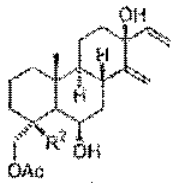
from partially purified fractions as well as purified compounds. The extracellular (epimastigote) method was employed to analyze 896 extracts and 1,285 extracts were studied for activity with the intracellular (amastigote) form of the parasite.

Because the plants initially subjected to purification were selected on the basis of their activity against the extracellular form of the parasite, as purified compounds have become available, they are being evaluated for their effect on the intracellular (amastigote) stage. Although with some differences in the relative values, our results with *Myrospermum frutescens* shows that all the compounds purified using the extracellular method were also active when tested in the intracellular assay (see Table 2).

The bioassays discussed above were used to guide the purification of compounds from *Nectandra lineata, Hedyosmum bonplandianum* and *Myrospermum frutescens*, discussed in greater detail in AP3, Section C.1.1.2. Table 2 shows the results of seven new cassane diterpenoids (**1**-**7**) isolated from *Myrospermum frutescens*. Interestingly, compounds **5** and **7** were more active against the extracellular form of the parasite than the intracellular form, while compounds **1** and **2** were more active against the more clinically relevant intracellular form of the parasite.



	R^1	R^2	R^3
1	OH	CH_3	H
2	OH	CH_3	OH
3	OH	CH_3	=O
4	OAc	CH_3	OAc
5	OAc	CH_3	OH
6	OAc	COOH	H



(**7**)

Table 2. Bioactivities of *Myrospermum* diterpenoids against *T. cruzi*

	Extra-cellular[a]	Intra-cellular[a]	Cytotoxicity[b]
Compound	**IC_{50} (μM)**	**IC_{50} (μM)**	**IC_{50} (μM)**
1	48.6 ± 15.8	17.4 ± 4.1	149
2	56 ± 3.8	16.6 ± 1.5	148
5	11.5 ± 8.4	25.9 ± 1.7	118
7	16.5 ± 1.2	35.8 ± 0.4	124

[a]Results show the IC_{50} value ± *SD* (n = 3). Compounds **3**, **4** and **6** were assayed in the extracellular *T. cruzi* assay and showed IC_{50} values of 36, 59 and 104 μM, respectively. [b]Experiments were performed with human foreskin fibroblasts.

To accurately interpret the results of the intracellular assay, it is necessary to determine whether the measured toxicity of a compound results from the killing of the parasite or from simply killing the host cells. Accordingly, we determined the cytotoxicity of these compounds against mammalian cell lines using primary human foreskin fibroblasts (HFF), the cell line routinely employed to culture the intracellular forms of *T. cruzi* (Chiari *et al.* 1984). In the case of compounds **1** and **2**, the intracellular form of the parasite was approximately nine-fold more sensitive to the compounds than were the HFF cell lines (Table 2). The enhanced toxicity of compounds **1** and **2** towards the intracellular form of the parasite as compared to the HFF cell line is particularly relevant to the development of new drugs for treating Chagas' disease as it involves the more clinically and biologically relevant form of the parasite for which there are currently no effective treatments. Compounds **5** and **7** showed approximately 8- and 10-fold greater effect, respectively, on the extracellular form of the parasite than on the HFF cells.

C.1.3. Drug Discovery for Malaria

C.1.3.1. Development of a Microfluorimetric Technique to Measure Antiplasmodial Activity

The malaria group is led by Dr. Ortega-Barría. Ours is the only laboratory in Panama culturing *Plasmodium falciparum in vitro*. Dennis Kyle (Walter Reed Army Institute for Research) and Phillip Rosenthal (University of

California at San Francisco) provided both materials and training opportunities for personnel. Both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* and have been established.

The standard bioassay for screening potential drugs for antiplasmodial activity is a radioactivity-based method that relies upon the incorporation of [^{3}H]hypoxanthine into the parasite's DNA in order to measure parasitic replication in erythrocytes (Desjardins *et al.* 1979). This method is sensitive and it can be used to screen a large number of compounds, but employs hazardous radioactive materials that require special facilities and procedures. In Panama, the regulations for the importation, use and disposal of radioactive isotopes are not in place, nor do we have the equipment or infrastructure required for their use, therefore it was necessary to seek alternative methods of testing *Plasmodium* susceptibility to potential antimalarial agents. Alternatives to the [^{3}H]hypoxanthine-based methodology included a labor-intensive and time-consuming microscopic method and several colorimetric assays (Makler and Gibbins 1991, Delhaes *et al.* 1999, Makler and Hinrichs 1993, Basco *et al.* 1995). Colorimetric methods, however, are based on enzymatic activity rather than replication of the parasite and may be subject to artifacts caused by pigments present in crude extracts. Alternatively, fluorescence-based techniques benefit from high signal-to-noise ratio available with newly developed fluorochromes, transgenic organisms expressing Green Fluorescent Protein, and the affordability of desk-top flourimeters that use standard 96-well plates. Furthermore, since the erythrocytes in which the parasites are cultivated have no DNA, they do not interfere with the analysis of parasitic DNA. We elected to use PicoGreen® which is an ultrasensitive fluorescent nucleic acid stain for measuring double-stranded DNA (dsDNA) in solution and which enables the detection of quantities as low as 25 pg/mL of dsDNA with a moderately priced spectrofluorometer using fluorescein excitation and emission wavelengths (www.probes.com). The subsequent development of a novel, straightforward, efficient and accurate method for the detection of antimalarial agents based upon a fluorimetric technique marks a significant achievement for the bioassay group of the Panama ICBG. The development of a microfluorimetric method is likely to find wide application, especially in developing nations that contend with logistical problems when using radioactive isotopes. The global need for the establishment of a facility for screening materials for antiplasmodial activity has been evident in samples we have received from Drs. David Kingston (Suriname/Madagascar ICBG) and Abimael Rodriguez (University of Puerto Rico), even though the assay has not yet been published Submitted

To test the sensitivity of the microfluorimetric method as a means of detecting *Plasmodium* DNA in infected erythrocytes, we compared the percentage of infected erythrocytes as determined by microscopic counting with results obtained from the microfluorimetric technique. We used serial double dilutions of infected erythrocyte cultures to prepare Giemsa-stained thin smears and the percentage of parasitemia was then evaluated by light microscopy. Aliquots of the same culture were mixed in a 96-well plate with an equal volume of PicoGreen® cocktail and the amount of fluorescence was quantitated with a fluorescence microplate reader at 485/20nm excitation and 528/20nm emission. As can be seen in Figure 8, there is a direct relationship between the percentage of infected red blood cells and the fluorescence signal between 0.1 and 15% of ring stage infected erythrocytes (r=0.99).

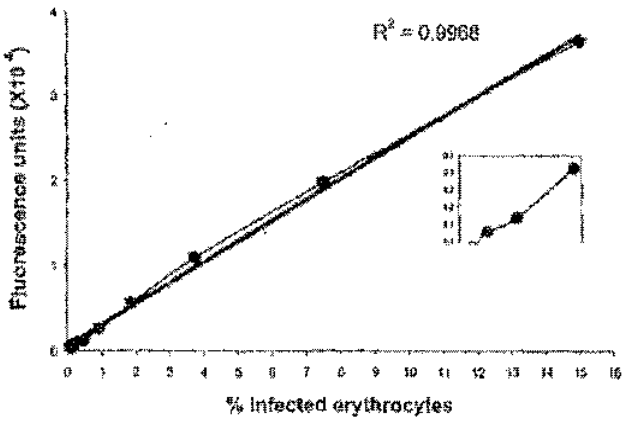


Figure 8. Comparison of the percentage of *Plasmodium falciparum*-infected erythrocytes as determined by microscopic counting with fluorescence intensity obtained from the microfluorimetric technique. The results are from four independently processed samples. The inset shows the relationship below 1% of parasitemia.

The values obtained with microfluorimetric method in dose response experiments with the known antimalarial drugs, chloroquine (IC_{50} of 31 ± 0.7 nM) were comparable to those previously reported by [^{3}H]hypoxanthine incorporation (IC_{50} = 29 ± 9 nM, Makler *et al.* 1993). Comparable results were obtained with mefloquine (Basco *et al.* 1995). When the dose response curves obtained with the radioactivity-based and microfluorimetric methods were compared for measuring the effect of chloroquine on the growth of the chloroquine-resistant W2 clone, similar IC_{50} values were determined by both methods (IC_{50} values of 86.5± 9 and 88.7± 0.72 nM for the radioactivity-based and microfluorimetric methods, respectively). The IC_{50} values determined for chloroquine in these experiments are comparable to the published value of 128±73 nM for the chloroquine-resistant strains (Delhaes *et al.* 1999, Makler *et al.* 1993). We also compared the radioactivity-based, microscopic, and microfluorimetric techniques to detect plant extracts with antiplasmodial activity. We considered as "active" those plant extracts with IC_{50} values <50 µg/mL and used 9 (out of 14) that were known to be active in the radioactivity-based assay. In every case, plants found to be active by the radioactive method were active in the microscopic and microfluorimetric methods.

C.1.3.2. Screening Plants for Antiplasmodial Activity

Species from a total of 889 crude extracts have been assayed, of which 413 were tested during 3 periods of training at the Walter Reed Army Institute for Research and the University of California at San Francisco using the [^{3}H]hypoxanthine method, and 476 were evaluated in Panama by the microfluorimetric technique. A total of 182 (20%) of the crude extracts showed activity below the cut-off point of IC_{50} < 50 µg/mL. Those extracts are currently being tested at 50, 10 and 2 µg/mL in order to confirm their activity. The fluorescent assay has been used to guide the purification of compounds from 3 plants, *Lafoensia punicifolia*, *Trattinickia aspera* and *Coccoloba parimensis*, that showed high levels of activity against *P. falciparum*. A total of 191 solvent partitions, column fractions and pure compounds have also been tested.

C.1.4. Summary of the Impact of the Tropical Disease Research Program in Panama

The development of an antiparasitic disease program in Panama over the past 4 years has had a number of positive effects. The ICBG program has offered an opportunity for Panamanian and American scientists to cooperate towards the common goal of establishing a program for the systematic search of new medicines in the rainforests of Panama, a country in the center of the region with the greatest concentration of terrestrial plant species in the world (Barthlott *et al.* 1996, Myers *et al.* 2000). The majority of the research has been conducted n the host-country, resulting in the creation of two new research laboratories and their respective

research programs. A large group of plant extracts has been analyzed resulting in the isolation of novel anti-parasitic compounds. A provisional patent has been filed for antileishmanial alkaloids and we are in the process of testing the *in vivo* activity of the compounds. Three new bioassays, two for *Leishmania spp.* and one for *Plasmodium falciparum*, were developed and standardized and are used routinely in our drug discovery program. Training opportunities have been created for a large number of students who have had the opportunity to work under a group of highly skilled scientists and learn the principles of good academic research.

C.2. Discovery of Anticancer Medicines

C.2.1. Cancer Cell Lines

In collaboration with the National Cancer Institute (Monks *et al.* 1991), a panel of 3 cancer cell lines was established at the University of Panama in the laboratories of Dr. Mahabir Gupta in the Department of Pharmacy. The cell lines consist of NCI-H460 (lung), SF268 (CNS), and MCF7 (breast). The protocols used to culture cell lines and to measure cytotoxicity are identical to those used by the NCI, with the exception of the use of XTT instead of sulforhodamine B to measure viability (as suggested by Anne Monks, pers. comm). For the testing of crude extracts, after dissolving in DMSO, the extracts are initially tested at a single concentration of 100 µg/mL. Extracts that cause at least 30% cytotoxicity are then tested at five concentrations (1.0, 3.2,10.0, 31.6 and 100 µg/mL), the data from which are used to calculate GI_{50} values. The assays were used to screen 1,152 species at one concentration, from which a total of 2,968 extracts were screened (consisting of young and mature leaves from the same species, recollections and repetitions). From these assays 364 species were active in one or more of the 3 cell lines. A total of 786 samples were tested at five concentrations. Extracts that demonstrate GI_{50} values of 10 µg/mL or less are considered active, revealing 40 species that were active against the MCF-7 line, 37 that were active against the NCI-H460 line and 41 against the SF-268 line. From this group, 8 plants were selected for bioassay-guided purification resulting in the completed structure elucidation of a total of 18 bioactive compounds, as described in detail in AP3 (Section C.1.2.1.), and summarized in the following section.

C.2.1.1. Bioassay-guided Fractionation with the Panama-based 3-Cell Cancer Screens

As elaborated in AP3 (Section C.1.2.1.) and summarized in Table 3 below, both known and novel compounds have been isolated as actives in the 3-cell cancer screens. A novel maquiroside derivative was isolated as the active constituent of *Maquira guianensis* (Moraceae). Three compounds from *Sloanea zuliaensis* (Elaeocarpaceae) showed significant activity: 2-deoxycucurbitacin D and two new cardenolides. The previously uninvestigated species *Prioria copaifera* (Fabaceae) yielded the known compound elliotinoic acid as the active constituent. Elliotinoic acid was first reported from *Pinus elliotii* (Joye and Lawrence 1963), although its anti-cancer activity is unreported. *Adenaria floribunda*, another previously uninvestigated species, yielded 3 active novel compounds. One, a new γ-pyrone, showed good activity, while the activity of the other two compounds was only low to moderate. *Mabea occidentalis* (Euphorbiacea) young leaf extracts showed activity against the SF-268 cell line (GI_{50} = 5.5 µg/mL) and a phenolic compound with low activity has been isolated so far. The extracts of *Vismia macrophylla*, *V. baccifera* and *V. jefensis* were all active against the 3 cancer cell lines. Five active known compounds (Table 3) were isolated from the young leaves of *V. macrophylla:* ferruginin A, ferruginin B and C, vismin and harunganin. *V. baccifera* yielded the three active compounds vismione B, deacetylvismione A and deacetylvismione H. Deacetylvismione A and deacetylvismione H were re-isolated as the active constituents of *V. jefensis*. Deacetylvismione A (8) showed good activity as a Sirt-2 inhibitor in Novartis screens as described below in Section C.2.2.1. While the benzophenone and anthranoid chemistry of the *Vismia* genus is well-documented (Delle Monache 1985), none of the three species, whose chemistry is described in AP3 (Section C.1.2.1.), have been investigated previously.

Table 3: Activity of terrestrial plant metabolites against 3 cancer cell lines.

Source Plant Species	Compound	IC_{50} (ng/mL)			Corresponding Structure numbers in AP3 proposal[a]
		MCF-7	H-460	SF-268	
Maquira guianensis	Maquiroside derivative	33	25	30	60
Sloanea zuliaensis	2-deoxycucurbitacin D	41	-	200	61
	Unnamed Cardenolide A	20	13	21	62
	Unnamed Cardenolide B	110	65	87	63
Prioria copaifera	Elliotinic acid	4,800	3,400	5,600	64
Adenaria floribunda	Unnamed meroterpenoid A	430	290	390	68
	Unnamed meroterpenoid B	1,200	1,100	1,400	69
	Unnamed meroterpenoid C	>10,000	9,300	>10,000	70
Mabea occidentalis	Unnamed polyphenol	4,700	4,100	9,400	71
Vismia macrophylla	Ferruginin A	500	330	490	72
	Ferruginin B	500	500	400	73
	Ferruginin C	480	540	400	74
	Vismin	520	730	570	75
	Harunganin	600	640	600	76
V. baccifera	Vismione B	160	280	250	77
V. jefensis	Deacetylvismione A (8)	180	190	220	78
	Deacetylvismione H	47	67	60	79

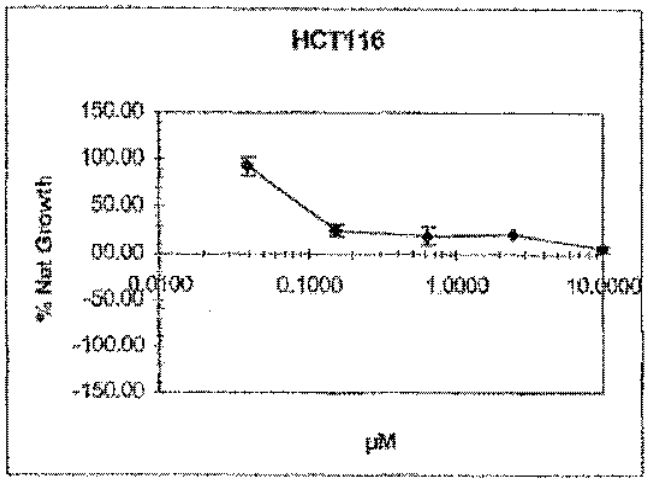
[a] These structure numbers refer to those used in the Associate Program 3 document of this ICBG proposal.

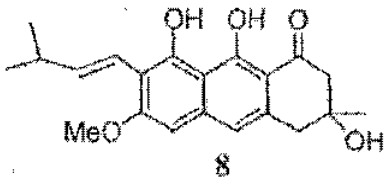
C.2.2. Prefractionation of Plants for Novartis' Screens

In order to provide enriched fractions for screening in Novartis mechanism-based assays, the Panama ICBG collaborated with Novartis chemists to develop prefractionation protocols. A total of 16 crude plant extracts were sent to Novartis in October, 2000 in order to determine the best means of removing tannins and providing enriched fractions for screening. A protocol was developed that involved detannification with polyamide followed by reversed-phase HPLC, and the protocol was used to "prefractionate" an additional 18 plant extracts. Samples were dried under vacuum and the contents transferred with DMSO to 96-well 'Deep-Well' plates which were sent to Novartis for screening. From this group of 18 plants, two species, *Lindackeria laurina* (Flacourtiaceae) and *Allophylus psilospermus* (Sapindaceae), were consistently active in the Sirt2 screen and were targeted for additional purification. Structural elucidation studies of the individual compounds are underway. Subsequently, a second group of 16 samples was prefractionated by reversed-phase vacuum-liquid chromatography using an application developed in the PI Bill Gerwick's laboratory (see AP1 in Section D.9.). Results from those fractions are pending. A total of approximately 1,000 fractions from prefractionated plants have been sent to Novartis for screening.

C.2.2.1. Pure Compounds Sent for Screening at Novartis

To date, a total of 147 pure natural products and partially purified fractions have been provided to Novartis for screening. Deacetylvismione A (8) from *Vismia baccifera and V. jefensis* has been shown to inhibit Sirt2 activity (see Section B.2.1.1.) *in vitro* and was resupplied for additional testing. Novartis scientists subsequently showed that treatment of HCT116 colon cancer cells with 8 inhibits their proliferation (IC_{50} = 113 nM, Figure 9) and induces increased histone and tubulin acetylation. H1299 proliferation is also inhibited (IC_{50} = 312 nM) by 8. Further investigation of this compound is in progress.





8

Figure 9. Inhibition profile of HCT116 cells by compound 8.

C.3. Training and Outreach

Training and education have been a critical component of this Associate Program from the beginning, with the goal of obtaining a critical mass of scientific personnel to maintain a sustainable, independent and productive research agenda. Accordingly, all research activities have been linked to the training of personnel.

C.3.1. Training in Antileishmanial Assays

Another technician, Cornelly Williams, developed the colorimetric method for measuring antileishmanial activity which is discussed in Section C.1.1.1. Submitted The XTT-based methodology was subsequently used to isolate the antileishmanial aporphine alkaloids discussed in Section C.1.1.2. Submitted Submitted Mr. Williams has since finished his Ph.D. studies and is scientific advisor to the Minister of Education in Panama.

With the support of the ICBG program, many other students have benefitted from the infrastructure, equipment, and technology developed in our laboratory. Six biology and 4 medical technology undergraduates have completed their graduation requirements under Dr. Ortega-Barría and Dr. Romero over the last four years. An additional student is pursuing his MS degree in tropical medicine will finish his thesis by March 2003.

C.3.2. Training in Antiplasmodial Assays

Another technician in our Associate Program, Yolanda Corbett, was given the difficult tasks of establishing the culture of *Plasmodium falciparum* and developing a non-radioactive bioassay to detect antiplasmodial activity. Ms. Corbett spent a total of nine months in the laboratories of Dennis Kyle (Walter Reed) and Phillip Rosenthal (University of California in San Francisco) and eventually established the *Plasmodium falciparum* culture in our laboratory, the only lab in the country with this capacity. Ms. Corbett's experience has since been used to train 3 more technicians in our laboratory in the skills of cultivating the parasite. In collaboration with another technician, Liuris Herrera, the novel microfluorimetric assay discussed in Section C.1.3.1. was established. Yolanda Corbett is now pursuing her Ph.D. studies at the Istituto di Microbiologia, Università Degli Studi di Milano in Italy under Professor Donatella Taramelli

C.3.3. Training in Antitrypanosomal Assays

The antitrypanosomal assay presented in Section C.1.2.1. was adapted by a technician, Venancio Polanco, who first established the assay with the extracellular (epimastigote) stage of the parasite. Mr. Polanco was also responsible for establishing the assay with the intracellular (amastigote) stage of *T. cruzi*. Both

antitrypanosomal assays were used to guide the purification of the novel antitrypanosomal diterpenes from *Myrcevermum frutescens*, as discussed in Section C.1.2.2., work which has been presented for publication Submitted While working in the ICBG program, Mr. Polanco was enrolled in a Masters in Biotechnology program and his MS thesis was associated with his research under Dr. Ortega-Barria. He is now an independent scientist at the Private Source

D. RESEARCH DESIGN AND METHODS

D.1. Organizational Overview of Associate Program 2

Associate Program 2 will be responsible for the screening of terrestrial plant and marine samples from AP's 1, 3 and 4 for antiparasitic, antineoplastic and agricultural activity. For the antiparasitic bioassays and the 3 cancer cell line screens carried out in Panama, we will utilize the high-capacity colorimetric and fluorimetric assays, described in Section D.2. and D.3.1., for which the necessary expertise and infrastructure already exists in Dr. Ortega-Barría's laboratory. Extracts and prefractions will also be screened at Novartis (Section D.3.2) and Dow AgroSciences (Section D.4; see letters of support in Appendix 3). We propose to centralize all of the Panama-based screening activities in our laboratory at the Institute of Advance Scientific Investigations and High Technology Services (INDICASAT) which will allow us to standardize techniques, economize on the necessary equipment and reagents, centralize data-handling and take advantage of the diverse range of tissue culture and parasitology expertise available in our group (see letter of support from Gonzalo Cordoba, National Secretary of INDICASAT, Appendix 1). Plant extracts coming from AP1 are barcoded to minimize errors and enhance efficiency and the exchange of data will be facilitated by our web-based discussed in AP1 (Section D.11., and appendix 7 for example). The whole cell cancer assays will be complemented by the mechanism-based assays of Novartis Oncology. In addition, we will collaborate with DowAgroscience to provide samples for agricultural pests. The overall structure of the proposed Panama ICBG program and interactions between the Associate Programs are summarized in Figure 10, while the key personnel and associated researchers are presented in Figure 11.

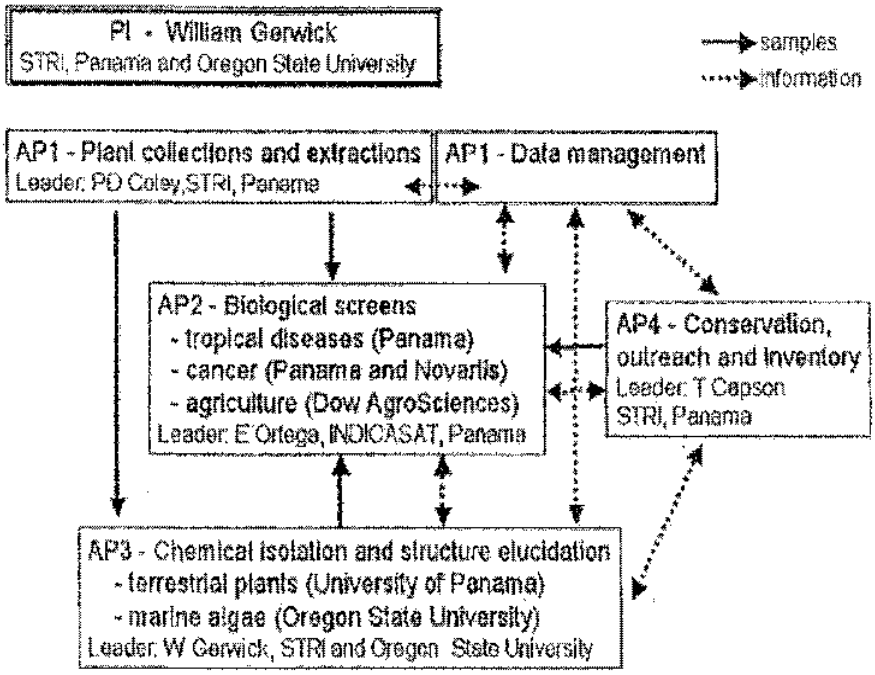


Figure 10: Overview of the ICBG Associate Program interactions and interrelationships in this ICBG proposal including sample and data transmissions.

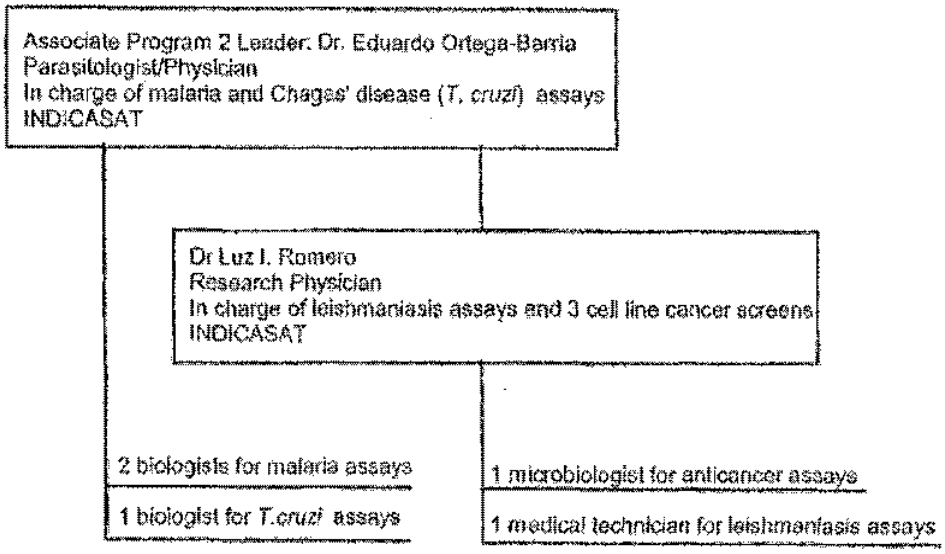


Figure 11: Organizational chart of key personnel in Associate Program 2 and their research areas and affiliations.

D.2. Screening Biological Materials for Activity Against Protozoan Parasites

For each of the bioassays discussed below we will utilize the mammalian host cells in which the parasites survive and multiply, thereby improving the odds that the antiparasitic agents we discover will be of clinical utility. We will use the microfluorimetric and colorimetric techniques that have been developed or adapted to our laboratory that do not require the use of radioactive isotopes, but at the same time are reliable and amenable to the processing of a large number of samples.

D.2.1. Drug Discovery in *Leishmania*

These studies will be the responsibility of Dr. Luz Romero, a dermatologist and expert in leishmaniasis whose group was responsible for developing the methods described in Section C.1. A WHO reference strain of *Leishmania mexicana* MOHM/B2/82/BELZ is used for this bioassay and additional *Leishmania* strains in our laboratory will be used as needed. Assays will be conducted with the amastigote, the stage found in infected human tissues. A method for the axenic growth of amastigotes will be employed in order to assess the effect of test substance without the possible interference of a host cell (Bates *et al.* 1992). We will use the microfluorimetric method described in Section C.1.1.4 to assess parasite viability *in vitro* which employs the fluorochrome PicoGreen®. *Leishmania* parasites will be cultured using established protocols (Morel 1984). Amastigotes will be allowed to grow in the absence and the presence of the test substance for 3 days. At the end of the incubation, the fluorochrome cocktail is added to liberate and label the parasitic DNA. The amount of fluorescence present is detected using a fluorescence microplate reader at 485/20 nm excitation and 528/20 nm emission. Controls include (i) growth of the parasite in the absence of the test substance, (ii) parasite-free controls to determine the fluorescence from the test substance, and (iii) the use of the known antileishmanial substance, amphotericin-B, to evaluate the sensitivity, uniformity and consistency of the parasite response.

We are in the process of developing an additional bioassay to test amastigotes for antileishmanial activity that employs a recently developed transgenic strain of *Leishmania infantum*. This strain constitutively expresses Green Fluorescent Protein (GFP, Kamau *et al.* 2001). The GFP-based methodology has the potential advantage of allowing the measurement of fluorescence without the addition of external fluorochromes such as PicoGreen® and facilitates experiments requiring multiple time points, since measurement does not interfere with parasite development (Gubbels *et al.* 2003). Dr. Adrian Hehl from the Institute of Parasitology at University of Zürich has agreed to provide the transgenic organism (pers comm.).

D.2.2. Drug Discovery for American Trypanosomiasis (Chagas' Disease)

The life cycle of *Trypanosoma cruzi* can be reproduced easily *in vitro* following literature protocols long since established in this laboratory, which allow the evaluation of the susceptibility to test substances of the each developmental stage of the parasite (Buckner et al. 1996). We will continue to utilize the colorimetric assay described in Section C.1.2.1., which employs the recombinant Tulahuen clone C4 of *T. cruzi* expressing bacterial ß-galactosidase (Buckner *et al.* 1996).

We will focus our drug discovery efforts on the amastigote stage, the most clinically relevant form of the parasite, using *T. cruzi*-infected human foreskin fibroblasts (HFF), which models a chronic host cell infection. (Buckner *et al.* 1996). Briefly, 96-well plates are seeded with HFF followed by the addition of ß-Gal-expressing trypomastigotes. After an overnight incubation to allow for parasite invasion, test substances are added. The colorimetric reaction is quantitated with a microplate reader at an optical density of 570 nm. Nifurtimox and amphotericin B, drugs known to have activity against *T. cruzi*, are used as controls to ensure consistency between assays and the susceptibility of the parasite response to test substances. In all cases, aliquots of the *T. cruzi* suspension are subjected to parallel mock treatments with RPMI-1640 alone without test substances. Each expermient also includes dimethyl sulfoxide (DMSO) in the absence of the test substance, and at the same final concentration utilized in the bioassay, in order to assess the effect of the solvent on the experiment. To determine whether the inhibitory effect of bioactive compounds is due to antitrypanosomal activity or is a result of cytoxicity to the host cell, confluent HFF monolayers will be incubated without parasite in the presence and absence of the test substance. Viability of the host-cell will be determined by the reduction of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide thiazolyl blue (MTT) by the mitochondrial dehydrogenase of the fibroblasts as previously published (Kilani *et al.* 2002).

D.2.3. Drug Discovery in *Plasmodium*

We will investigate the antiplasmodial effect of test substances using chloroquine-sensitive (Sierra Leone clone D6 and Tanzania F32) strains and a chloroquine-resistant (Indochina clone W2) strain of *Plasmodium falciparum*. We have established a secure and consistent source of human erythrocytes and serum (Santo Tomas Hospital Blood Bank). The *in vitro* antiplasmodial activities of test substances will be assessed by the microfluorometric assay described in Section C.1.3.1. that measures parasite DNA levels with the PicoGreen® DNA intercalator.

The malarial strains are maintained *in vitro* by a modification of published methods (Trager and Jensen 1976). Sealed culture flasks are filled with RPMI 1640 culture medium supplemented with 10% heat-inactivated human type O+ serum. Cultures are maintained in type O+ human erythrocyte suspensions prepared in citrate-phosphate-dextrose anticoagulant (CPD) as anticoagulant at a hematocrit of 2%. The parasite density is maintained below 2% parasitemia under an atmosphere of a certified gas mixture (5% CO_2, 5% O_2, and 90% N_2) at 37°C. The culture media consist of standard RPMI 1640 supplemented with 25 mM $NaHCO_3$, 2 mM Glutamine and 25 mM *N*-2-hydroxythylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). For each experiment, samples of stock cultures are further diluted in culture medium containing sufficient non-infected type O+ human erythrocytes to yield a final hematocrit of 2% and parasitemia of 1% in preparation for addition to microtitration plates. For some fluorimetric assays the parasites are synchronized using sorbitol as described previously (Lambros *et al.* 1979).

Synchronized ring form cultures (hematocrit 2% and parasitemia 1%) are used to test pure compounds or serial dilutions of test substance in 96-well culture plates. Cultures of *P. falciparum* are placed in a humidified, air-tight container, flushed with the gas mixture described above and incubated at 37°C. After a 48-hour incubation period to allow for parasite growth, an aliquot of culture media is transferred to a new 96-well flat bottom plate. A PicoGreen® fluorochrome cocktail is then added to liberate and label the DNA from parasites present in the erythrocytes and the plates are incubated for 5-30 min in the dark. The amount of fluorescence is measured with a fluorescence microplate reader at 485/20 nm excitation and 528/20 nm emission.

Figure 12 depicts our strategy for lead prioritization. The cut-off for activity has been established as an iC_{50} lower than 50 μg/mL for *P. falciparum* and *T. cruzi*, and 40 μg/mL for *Leishmania spp.* Active extracts are ranked according to their level of antiparasitic activity and extracts that have promising levels of activity are then checked in the SciFinder database. Those active species with little or no literature precedence are considered as candidates for bioassay-guided fractionation. Candidates for purification are selected in meetings between AP1, AP2, AP3 and Central Operations. We will work in close consultation with the PI who will have input on all samples that are selected for bioassay guided fractionation.

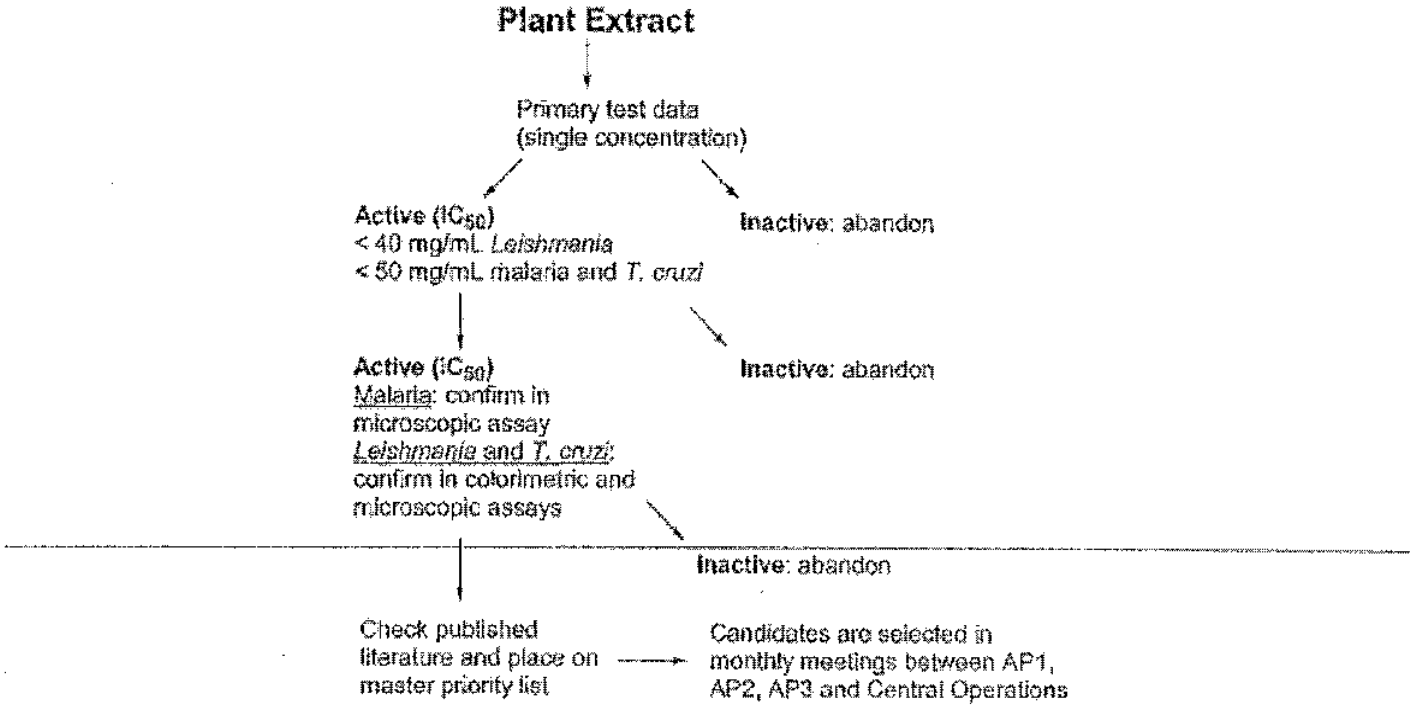


Figure 12. Decision tree for lead prioritization of tropical disease bioactives.

Priorities will be based upon (i) potency, (ii) little or no reported chemistry, (iii) few or no reports in the literature of activity, and ethnobotanical information in the public domain (that indicates that the plant is active). The data will be compiled and compared for each candidate.

D.3. Discovery of Novel Anticancer Agents

D.3.1. Natural Product Screening in Panama-based 3-Cell Cancer Screens

The anticancer bioassay program will be coordinated by Dr. Romero. We will test extracts from terrestrial plants, marine algae and marine cyanobacteria supplied from AP1, AP3 and AP4 in bioassays designed to detect new anticancer agents in our laboratories at INDICASAT (Institute for Scientific Advance Investigations and High Technology Services).

In screening extracts for anticancer activity we will use the MCF7 (breast), NCI-H460 (lung) and SF268 (CNS) cell lines as recommended by the NCI (Gordon Cragg, pers. comm.). The protocols used to culture cell lines and to measure cytotoxicity are identical to those used by the NCI (Monks *et al.* 1991), with the exception of the use of the tetrazolium salt XTT to measure viability instead of sulforhodamine B (Anne Monks, pers. comm.).

D.3.2. Natural Product Screening in Mechanism-based Assays at Novartis

D.3.2.1. Selection of Terrestrial and Marine Samples for Screening

Approximately 1,500-2,000 samples will be screened annually in a suite of molecular target assays similar to that listed in Section C.2.2. through collaboration with Novartis Oncology (see letter of support, Appendix 3). These samples will generally be crude extracts and prefractions from terrestrial plants and marine algae that have already shown interesting activity profiles in the Panama-based *in vitro* 3-cancer cell line cytotoxicity assays. Thus, we anticipate that the prefractions of 150-200 species of plants and 20-40 species of marine algae and cyanobacteria will be screened annually for mechanism-based anticancer properties.

D.3.2.2. Protocols for Screening Samples

Detannified terrestrial plant prefractions, as well as marine macroalgal and cyanobacterial prefractions, will be sent in 96-well microtiter plates to Novartis where they will be assigned a 'BAN' number and registered into Novartis' Molecular Target Feasibility Group computer database. Initial screens can be completed with less than 2.5 mg of extract. The samples are distributed from master plates to daughter plates with the aid of a robot-assisted sample handling system. Materials in daughter plates are dried, resuspended for testing, and evaluated against validated standards. The tests are usually complete within 30 days of reception at Novartis. Actives are noted in an internal tracking database and advanced automatically for secondary testing.

D.3.2.3. Isolation and Structure Elucidation of Mechanism-based Leads

The activities of prefractions showing positive results in the mechanism-based assays will be communicated to AP2 (Dr. Capson will manage information transfer from Novartis) by email, and then entered into the ICBG web-based database in Panama (see database examples in Appendix 7). Subsequent HPLC fractionation of these reduced complexity materials to pure compounds will occur in the ICBG extraction and fractionation laboratories of AP1 at STRI in Panama. Novartis will provide rapid assay of these sub-fractions, usually providing data within one week of sample reception, and these results will be used to direct the isolation process. Pure compounds will be provided to the AP3 chemistry laboratories for structure elucidation. Those deriving from terrestrial plants will be structurally analyzed in the Cubilla laboratories in Panama while those deriving from algae or cyanobacteria will be studied in the Gerwick/McPhail laboratory in Oregon.

D.4. Natural Products as Agrochemicals

DowAgroscience (DAS) will evaluate the potential agrochemical utility of extracts and prefractions from 150-200 terrestrial plant species and 50-75 marine algal and cyanobacterial species from Panama (1,500 – 2,000 materials) by completing primary screening bioassays for insecticidal, fungicidal, and herbicidal activity (see letter of support, Appendix 3). The terrestrial plant samples will have been detannified in the extraction laboratories of AP1 at STRI. DAS will pursue secondary testing of all extracts passing primary screening, and utilize the results of those assays to prioritize samples for subsequent isolation and structure elucidation. To complete preliminary dereplication of extracts passing secondary testing, DAS will use chromatographic profiling linked to LC/MS analysis and commercial natural product database searching. DAS will isolate and identify promising and potentially unique actives emerging from this program internally or in collaboration with the ICBG participants utilizing conventional or SEPBOX™ technology. In effect, DAS will support the isolation efforts of this ICBG by providing rapid bioassay of chromatographic fractions during the isolation process. It will be important to obtain sufficient quantities of lead compounds to pursue advanced levels of testing and development, as warranted by their activity. DAS will initiate synthetic programs, when possible, around lead structures identified by this program, and aggressively file and maintain patents covering composition and utility of compounds discovered in the course of this investigation.

D.4.1. Screening Samples

Screening samples received from ICBG will be registered into Dow AgroSciences' data tracking computer database and extracted with solvent for bioassay. Plant samples evaluated at Dow AgroSciences to date have been processed by extraction with methanol. The resulting solution has been filtered through a polyamide solid phase extraction (SPE) cartridge to remove phenolic compounds, especially tannins. Phenolic compounds from plants have historically caused false or unwanted positives in our *in vitro* screening assays, and also cause weak activity in insect and fungal *in vivo* assays. To eliminate these interferences and allow focus on more potent compounds acting on specific targets, all plant extracts are filtered through polyamide. The extract is dried and distributed to microtiter plates. It is fully acceptable if extracts are prepared at or near the field collection site by a method equivalent to that described above, provided it generates an organic extract free of tannins and that this is clearly documented before we receive the samples.

D.4.2. Sample Distribution

Initial screens in all three therapeutic areas can be completed with 5 mg of extract. The samples are distributed from master plates to daughter plates with the aid of a Hamilton liquid handling system. The daughter plates are dried, resuspended for testing in each biological area, and evaluated against validated standards. The tests are complete in 7-14 days. Actives are noted in an internal tracking database and advanced automatically for secondary testing. The amount of extract required for secondary evaluation varies by discipline, with herbicide testing requiring 10 mg, insecticide testing requiring 40 mg, and fungicide testing requiring 40 mg. All of these samples are dispensed with the aid of a robot-assisted sample handing system and distributed to each therapeutic area for evaluation. Secondary test data is generally available in 10-30 days from receipt of the sample.

D.4.3. Dereplication of Secondary Screen Hits

Preliminary dereplication of actives from secondary testing in each biological area will be completed by chromatography using a standard LC-MS method. Under gradient elution conditions an extract is separated into its components, whose positive and negative ion ESI mass spectra are recorded, and the eluent is collected into a single microtiter plate. A 96-well daughter plate is prepared from this master plate and submitted to the primary screening bioassay. Compounds present in the active regions of the chromatogram are then interrogated using the acquired mass spectral data. Using the taxonomic identity of the organism and the likely mass(es) of the active(s), searching of commercial databases will be completed to rapidly dereplicate most known compounds. Although Dow AgroSciences has access to a number of natural product databases including Antibase and the Berdy database, The Chapman Hall Dictionary of Natural Products will be the primary tool for dereplication of active samples originating from Panama. This database includes over 130,000 entries of diverse origin and is searchable by a combination of biological source and molecular weight (Buckingham and Thompson 1997)

D.4.4. Isolation Chemistry

Extracts demonstrating promising activity that is not associated with known compounds will be communicated to Dr. Gerwick and the Panama ICBG participants. Additional sample will be obtained from the Panama ICBG for isolation and identification (up to 5 g dried extract will be required for fractionation on the SEPBOX™, an automated preparative multi-dimensional HPLC). If isolation is done in collaboration with ICBG participants (Gerwick or Cubilla laboratories), Dow AgroSciences will provide rapid assay of test fractions throughout the isolation and identification process using the primary screening bioassays described above. The results of those assays will promptly be returned via electronic mail.

E. HUMAN SUBJECTS

None.

F. VERTEBRATE ANIMALS

None.

G. LITERATURE CITED

Baekelandt M. 2002. Irofulven. *Current Opinion in Investigational Drugs* 3: 1517-1526.

Bakhtiar R, Lohne J, Ramos L, Khemani L, Hayes M and F Tse. 2002. High-throughput quantification of the anti-leukemia drug STI571 (Gleevec) and its main metabolite (CGP 74588) in human plasma using liquid chromatography-tandem mass spectrometry. *Journal of Chromatography, B: Analytical Technologies in the Biomedical and Life Sciences* 768: 325-340.

Barthlott W, Lauer W and A Placke. 1996. Global distribution of species diversity in vascular plants: Towards a world map of phytodiversity. *Erdkunde* 50: 317-327.

Basco LK, Marquet F, Makler MT and J Le Bras. 1995. *Plasmodium falciparum* and *Plasmodium vivax*: Lactate dehydrogenase activity and its application for *in vitro* drug susceptibility assay. *Exp. Parasitol.* 80: 260-271.

Bates PA, Robertson CD, Tetley L and GH Coombs. 1992. Axenic cultivation and characterization of *Leishmania mexicana* amastigote-like forms. *Parasitology* 105: 193-202.

Berman JD, Holz GG Jr and DH Beach. 1984. Effects of ketoconazole on growth and sterol biosynthesis of *Leishmania mexicana* promastigotes in culture. *Mol. Biochem. Parasitol.* 12: 1-3.

Berman JD. 1997. Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clin. Infect. Dis.* 24: 684-703.

Buckingham J and S Thompson. 1997. The dictionary of natural products and other information sources for natural product scientists. In: *Phytochemical Diversity: a Source of New Industrial Products*. Wrigley S, Hayes M, Thomas R Chrystal E (Eds). The Royal Society of Chemistry, Cambridge, pp. 53-67.

Buckner FS, Verlinde CL, La Flamme AC and WC Van Voorhis. 1996. Efficient technique for screening drugs for activity against *Trypanosoma cruzi* using parasites expressing beta-galactosidase. *Antimicrob. Agents Chemother.* 40: 2592-2597.

Buttke TM, McCubrey JA and TC Owen. 1999. Use of an aqueous soluble tetrazolium/formazan assay to measure viability and proliferation of lymphokine-dependent cell lines. *J. Immunol. Methods* 157: 233-240.

Chiari E and EP Camargo. 1984. In: *Genes and Antigens of parasites. A Laboratory Manual*. Morel CM. (Ed) Fundacão Oswaldo Cruz, Río de Janeiro, Brazil. pp. 23-28.

Chicarelli-Robinson MI, Gibbons S, Mcnicholas C, Robinson N, Moore M, Fauth U and SK Wrigley. 1997. Plants and microbes as complementary sources of chemical diversity for drug discovery. *Special Publication - Royal Society of Chemistry* 200: 30-40.

Submitted

Cragg GM and DJ Newman. 2000. Antineoplastic agents from natural sources: achievements and future directions. *Expert Opinion on Investigational Drugs* 9: 2783-2797.

Cragg GM, Boyd MR, Hallock YF, Newman DJ, Sausville EA and MK Wolpert. 2000. Natural products drug discovery at the National Cancer Institute. Past achievements and new directions for the new millennium. *Special Publication - Royal Society of Chemistry* 257: 22-44.

Croft SL and V Yardley. 2002. Chemotherapy of leishmaniasis. *Current Pharmaceutical Design* 8: 319-342.

Daum G, Eisenmann-Tappe I, Fries HW, Troppmair J and UR Rapp. 1994. The ins and outs of Raf kinases. *Trends Biochem Sci.* 19: 474-480.

Delhaes L, Lazaro JE, Gay F, Thellier M and M Danis. 1999. The microculture tetrazolium assay (MTA): another colorimetric method of testing *Plasmodium falciparum* chemosensitivity. *Ann. Trop. Med. Parasitol.* 93: 31-40.

Desjardins RE, Canfield CJ, Haynes JD and JD Chulay. 1979. Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrob. Agents Chemother.* 16: 710-718.

Desjeux P. 1996. Leishmaniasis, public health aspects and control. *Dermatologic Clinics* 14: 433-450.

Dusanic DG. 1991. *Trypanosoma (Schizotrypanum) cruzi. Parasitic Protozoa.* Academic Press, New York, pp. 137-194.

Duke SO, Dayan FE, Romagni JG and AM Rimando. 2000. Natural products as sources of herbicides: current status and future trends. *Weed Research* 40: 99-111.

Elit L. 2002. Rapamycin. *Current Opinion in Investigational Drugs* 3: 1249-1253.

Elliott M. 1996. Synthetic insecticides related to the natural pyrethrins. In *Crop Protection Agents from Nature: Natural Products and Analogues.* Copping LG (Ed). The Royal Society of Chemistry, Cambridge, pp. 254-300.

Fields SC, Mireles-Lo L, and B Gerwick. Hydroxycornexistin: a new phytotoxin from *Paecilomyces variotii.* 1996. *J. Nat. Prod.* 59: 698-700.

Frormann S and G Jas. 2002. Natural products and combinatorial chemistry - the comeback of nature in drug discovery. *Business Briefing: Future Drug Discovery* 84-90.

Furuya T, Kashuba C, Docampo R and SN Moreno. 2000. A novel phosphatidylinositol-phospholipase C of *Trypanosoma cruzi* that is lipid-modified and activated during trypomastigote to amastigote differentiation. *J. Biol. Chem.* 275: 6428-6438.

Gelb MH and WGJ Hol. 2002. Drugs to combat tropical protozoan parasites. *Science* 297: 343-344.

Gerwick B, Fields SS, Graupner PR, Gray JA, Chapin EC, Cleveland JA and DR Heim. 1997. Pyridazocidin, a new microbial phytotoxin with activity in the Mehler reaction. *Weed Science* 45: 654-657.

Golenser J, Frankenburg S, Ehrenfreund T and AJ Domb. 1999. Efficacious treatment of experimental leishmaniasis with amphotericin B-arabinogalactan water-soluble derivatives. *Antimicrob. Agents Chemother.* 43: 2209-2214.

Grant IH, Gold JWM, Wittner M, Tamowitz HB, Nathan C, Mayer K, O'reilly RJ, and D Armstrong. 1989. Transfusion associated acute Chagas' disease acquired in the U.S.A. *Ann. Intern. Med.* 111: 849-851.

Gresios K. 2002. MitoExtra. *Current Opinion in Investigational Drugs* 3: 648-651.

Guerin PJ, Olliaro P, Nosten F, Druilhe P, Laxminarayan R, Binka F, Kilama WL, Ford N, and NJ White. 2002. Malaria: current status of control, diagnosis, treatment, and a proposed agenda for research and development. *The Lancet Infectious Diseases* 2: 564-573.

Gubbels MJ, Li C and B Striepen. 2003. High-throughput growth assay for *Toxoplasma gondii* using yellow fluorescent protein. *Antimicrob. Agents Chemother.* 47: 309-316.

Gullickson WD. 1994. History of pyrethrum. In Pyrethrum Flowers; Production, Chemistry, Toxicology, and Uses. Casida JE, Quistad BB. (Eds). Oxford University Press, Oxford. pp. 1-32.

Henkel T, Brunne RM, Muller H and F Reichel. 1999. Statistical investigation into the structural complementarity of natural products and synthetic compounds. *Angew. Chem. Int. Ed.* 38: 643-647.

Hoshikawa, Kijima, Yoshida, and Beppu. 1991. Expression of differentiation-related markers in teratocarcinoma cells *via* histone hyperacetylation by trichostatin A. *Agric. Biol. Chem.* 55: 1491-1497.

Hoshikawa, Kwon, Yoshoda, Gorinouchi, and Beppu. 1994. Trichostatin A induces morphological changes and gelsolin expression by inhibiting histone deacetylase in human carcinoma cell lines. *Experimental Cell Research* 214: 189-197.

Jha TK, Sundar S, Thakur CP, Bachmann P, Karbwang J, Fischer C, Voss A and J Berman. 1999. Miltefosine, an oral agent for the treatment of Indian visceral leishmaniasis. *N. Engl. J. Med.* 341: 1795-1800.

Joye NM and RV Lawrence. 1963. Isolation of a new diterpene acid. *J. Org. Chem.* 28: 3274.

Kamau SW, Grimm F and AB Hehl. 2001. Expression of green fluorescent protein as a marker for effects of antileishmanial compounds in vitro. *Antimicrob. Agents Chemother.* 45: 3654-3656.

Kawasaki H, Eckner R, Yao T-P, Taira K, Chiu R, Livingston DM, Yokoyama KK. 1998. Distinct Roles of the Co-Activators p300 and CBP in Retinoic-Acid-Induced F9-Cell Differentiation. *Nature* 393: 284-289.

Kilani RT, Tamimi Y, Karmali S, Mackey J, Hanel EG, Wong KK and RB Moore. 2002. Selective cytotoxicity of gemcitabine in bladder cancer cell lines. *Anticancer Drugs* 13: 557-566.

Kirchhoff LV, Gam BS and FC Gilliam. 1987. American trypanosomiasis (Chagas' disease) in Central American immigrants. *Am. J. Med.* 82: 915-920.

Kirchhoff LV. 1993. American trypanosomiasis (Chagas' disease). A tropical disease now in the United States. *N. Engl. J. Med.* 329: 639-644.

Kuhlencord A, Maniera T, Eibl H and C Unger. 1992. Hexadecylphosphocholine: oral treatment of visceral leishmaniasis in mice. *Antimicrob. Agents Chemother.* 36: 1630-1634.

Lambros C and JP Vanderberg. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol.* 65: 418-420.

Lee DL, Prisbylla MP, Cromartie TH, Dagarin DP, Howard SW, Provan WM, Ellis MK, Fraser T, and LC Mutter. 1997. The discovery and structural requirements of inhibitors of p-hydroxyphenylpyruvate dioxygenase. *Weed Science* 45: 601-609.

Lewer P, Hahn DR, Karr LL, Graupner PR, Gilbert JR, Wordon TV, Yao RC and DW Norton. 2002. Pesticidal macrolides. US Patent 6,455,504 B1.

In Press

Magnuson NS, Beck T, Vahidi H, Hahn H, Sithanandam G and UR Rapp. 1994. The Raf-1 serine/threonine protein kinase. *Semin Cancer Biol.* 5: 247-253.

Makler MT and BL Gibbins. 1991. Laboratory diagnosis of malaria. *Clinics in laboratory medicine* 11: 941-956.

Makler MT and DJ Hinrichs. 1993. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. *Am. J. Tropical. Med. Hyg.* 48: 205-210.

Makler MT, Ries JM, Williams JA, Bancroft JE, Piper RC, Gibbins BL and DJ Hinrichs. 1993. Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *Am. J. Trop. Med. Hyg.* 48: 739-741.

Maloney DM, Maloney JE, Dotson D, Popov VL and RL Sanchez. 2002. Cutaneous leishmaniasis: Texas case diagnosed by electron microscopy. *J. Am. Acad. Dermatol.* 47: 614-616.

Marsh K. 1998. Malaria disaster in Africa. *Lancet* 352: 924-925.

Marshall E. 2000. A renewed assault on an old and deadly foe. *Science* 290: 428-430.

Meshnick S and M Dobson. 2001. In: *Antimalarial chemotherapy. Mechanisms of action, resistance, and new directions in drug discovery* Ed: Rosenthal P, Humana, Totowa, New Jersey, pp. 15-26.

Modabber F. 2002. Eleventh programme report of the UNDP/World Bank/WHO special programme for research and training in tropical diseases, TDR.

Monia BP, Johnston JF, Geiger T, Muller M, and D Fabbro. 1996. Antitumor activity of a phosphorothioate antisense oligodeoxynucleotide targeted against c-raf kinase. *Nat. Med.* 21: 668-675.

Monks A, Scudeiro D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, Gray-Goodrich M, Campbell H, Mayo J and M Boyd. 1991. Feasibility of high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J. Natl. Cancer Inst.* 83: 757-766.

Submitted

Meyerhoff A. 1999. US Food and Drug Administration approval of AmBisome (liposomal amphotericin B) for treatment of visceral leishmaniasis. *Clin. Infect. Dis.* 28: 42-51.

Morel CM. 1984. *Genes and antigens of parasites. A Laboratory Manual.* Fundación. Oswaldo Cruz, Río de Janeiro, Brazil. pp. 67-68.

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB and J Kent. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403: 853-858.

Rosenthal PJ. (Ed). Humana Press. Totowa, N.J. pp. 15-25.

Ridley RG. 2002. Medical need, scientific opportunity and the drive for antimalarial drugs. *Nature* 415: 686–693.

Sachs JD. 2002. A New Global Effort to Control Malaria. *Science* 298: 122-124.

Sambucetti, Trogani, Kwon, Idler, Chamberlin, and Cohen. 1997. Elevated $p21^{waf1}$ levels lead to growth arrest and senescence in human lung carcinoma cells. *Proceedings of the American Association for Cancer Research* 38: 279.

Sauter H, Ammermann E and F Roehl. 1996. Strobilurins-from natural product to a new class of fungicides. *Critical Reviews on Applied Chemistry* 35: 50-81.

Schmitzer PR, Graupner PR, Chapin EL, Fields SC, Gilbert JR, Gray JA, Peacock CL and BC Gerwick. 2000. Ribofuranosyl triazolone: A natural product herbicide with activity on adenylosuccinate synthetase following phosphorylation. *J. Nat. Prod.* 63: 777-781.

Semenza GL. 2000. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J. Appl. Physiol.* 88: 1474-1480.

Silachamroom U, Phumratanaprapin W, Krudsood S, Treeprasertsuk S, Budsaratid V, Pompininworakij K, Wilairatan P and S Looareesuwan. 2001. Frequency of early rising parasitemia in falciparum malaria treated with artemisinin derivatives. *Southeast Asian J. Trop. Med. Public Health* 32: 50-56.

Smith S. 2001. SGN-10. *Current Opinion in Investigational Drugs* 2: 1314-1319.

Sugita, Koizumi, and Yoshida. 1992. Morphological reversion of sis-transformed NIH3T3 cells by trichostatin A. *Cancer Research* 52: 168-172.

TDR news. 2000. New drug targets and leads for kinetoplastid drug discovery. No. 62. June 2000.

Submitted

Trager W and JB Jensen. 1976. Human malaria parasites in continuous culture. *Science* 193: 673-675.

Verheul HMW. *et. al.* High Vascular Endothelial Cell Growth Factor (VEGF) Concentration and Activation of the Coagulation Pathway, including Platelets, in Soft Tissue Sarcomas (STS). 1999. *Proceedings of the American Association for Cancer Research* 40: 227.

Verschraegen C and K Glover. 2001. ET-743. *Current Opinion in Investigational Drugs* 2: 1631-1638.

Vigushin D. 2002. FR-901228 *Current Opinion in Investigational Drugs* 3: 1396-1402.

Viotti R, Vigliano C, Armenti H, and E Segura. 1994. Treatment of chronic Chagas' disease with benznidazole: clinical and serological evolution of patients with long-term follow-up. *American Heart Journal* 127: 151-162.

Wang H-K. 2001. Flavopiridol. *Current Opinion in Investigational Drugs* 2: 1149-1155.

Weigle K and NG Saravia. 1996. Natural history, clinical evolution, and the host-parasite interaction in New World leishmaniasis. *Clin. Dermatol.* 14: 433-450.

Wellems TE. 2002. *Plasmodium* chloroquine resistance and the search for a replacement antimalarial drug *Science* 298: 124-126.

Submitted

World Health Organization (WHO). 1990. Technical Report Series. *Control of the leishmaniases.* Report of a WHO Expert Committee. World Health Organization, Geneva.

World Health Organization (WHO). 1991. *Control of Chagas' disease. Technical Report, Series 811.* Report of a WHO Expert Committee. World Health Organization, Geneva.

World Health Organization (WHO). 2000. *World Health Organization Report on Infectious Diseases.* World Health Organization, Geneva.

Yoshida, Hoshikawa, Koseki, Mori, and Beppu. 1990. Structural specificity for biological activity of trichostatin A, a specific inhibitor of mammalian cell cycle with potent differentiation-inducing activity in Friend leukemia cells. *Antibiotics* 43, 1101-1106.

Yoshida and Beppu. 1998. Reversible arrest of proliferation of rat 3Y1 fibroblasts in both G1 and G2 phases by trichostatin A. *Experimental Cell Research* 177, 122-131.

H. CONSORTIUM/CONTRACTUAL ARRANGEMENTS
None

I. LETTERS OF SUPPORT (Consultants/Collaborators)

Dow AgroSciences (see letter of support, Appendix 3)
Novartis Institute of Biomedical Research (see letter of support, Appendix 3)

ASSOCIATE PROGRAM 3

ISOLATION AND STRUCTURE ELUCIDATION OF PANAMA PLANT BIOACTIVES

Associate Program Leader: William H.Gerwick

Pharmacy Department, Oregon State University, Corvallis, OR, USA
and Smithsonian Tropical Research Institute, Panama

International Cooperative Biodiversity Groups
"Bioassay and Ecology Directed Drug Discovery in Panama"
Dr. William Gerwick, Group Leader

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. DO NOT EXCEED THE SPACE PROVIDED.

Associate Program 3 focuses on the chemical isolation and structure elucidation of bioactive compounds from terrestrial plants, marine algae and cyanobacteria from Panama. Bioassay information from Associate Program 2 will be used to select the most promising extracts for recollection, extraction and bioassay-guided fractionation. Rapid de-replication of known compounds or nuisance substances will use literature databases, taxonomic information, and HPLC profiles. Pure compounds will be analyzed by a contemporary array of analytical techniques, featuring two-dimensional NMR spectroscopy and multidimensional mass spectrometry. A close interaction between the two participating chemistry laboratories, one at the University of Panama and the other at Oregon State University, will provide a mechanism for additional training and capacity building in Panama. Pure and bioactive compounds will be provided in larger scale to the bioassay component of this ICBG for secondary screens, *in vivo* evaluations and mechanism of action studies. Analogs of the most promising lead compounds, both natural as well as semi-synthetic in origin, will also be supplied to Associate Program 2 to develop initial structure-activity relationships. A close interaction with Associate Program 4 will ensure a maximal utilization of these results to foster biodiversity conservation, economic development, and, through our outreach efforts, the public appreciation of the ICBG program and the value of biodiversity. Associate Program 3 participants will contribute to outreach activities through oral and written presentations of the work to both scientific and general populace audiences.

PERFORMANCE SITE(S) *(organization, city, state)*
Oregon State University, Corvallis, Oregon

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
GERWICK, William	Oregon State University	P.I. of Associate Program 3 and Co-P.I. of the Oregon State Univ. Sub-Contract
CUBILLA, Luis	University of Panama	Laboratory Leader
MCPHAIL, Kerry	Oregon State University	P.I. of the Oregon State Univ. Sub-Contract of Associate Program 3
MONTENEGRO, Hector	University of Panama	Natural Products Chemist

A. SPECIFIC AIMS

The overall goals of Associate Program 3 are to provide chemical isolation and structure elucidation support to the drug discovery programs from terrestrial and marine plants as outlined in AP1 and AP2. These latter two AP programs focus on collections of these natural materials, extractions, pre-fractionations, and biomedical evaluations to a battery of targets, including tropical diseases, cancer, and agricultural pests. As such, this AP is a necessary and logical component of this overall ICBG application.

The Specific Aims of this Project are:

1. To use bioassay results from AP2 to select and then direct the chemical isolation of active constituents from pre-fractionated extracts or extracts as appropriate.

Pre-fractionated extracts will be tested for activity in anticancer screens (Novartis, cancer cell lines in Panama) and agrochemical assays (Dow AgroSciences), while crude extracts will be tested in the tropical disease assays and 3 cancer cell lines in Panama, all as part of AP2. Efficient dereplication of known and nuisance compounds from active extracts will be accomplished through the extensive use of electronic scientific literature databases in concert with taxonomic information and HPLC profiles. Active terrestrial and marine plant pre-fractions will be subjected to various chromatographic procedures (SPE, HPLC) to obtain pure compounds, and these will be provided to the appropriate bioassay for further evaluation.

2. To apply state-of-the-art structure elucidation technology, featuring 2D-NMR and various ionization modes of Mass Spectrometry, to rapidly and efficiently determine the structures of new and bioactive compounds.

Chemical structures of new and bioactive compounds will be rapidly and efficiently described utilizing multi-dimensional mass spectrometry, an extended complement of NMR methods (COSY, TOCSY HSQC, HMBC, and NOESY) as well as other standard spectroscopic methods. Absolute stereochemistry will be determined by either spectroscopic methods (CD), NMR methods (Mosher ester), or chemical degradation followed by chiral chromatographic methods (Marfey's).

3. To train students at various levels from the US and Panama in the multidisciplinary science of modern natural products chemistry.

Students both in Panama and in the US will participate in these modern natural products investigations through reciprocal exchanges and participation in graduate programs in Chemistry at the University of Panama and Pharmacy, Chemistry or Biochemistry at Oregon State University. Frequent use of web and email based technologies, in addition to telephone conferencing, will facilitate communication between AP3 and the other AP's.

4. To work with the other AP programs and our industrial partners to advance lead molecules into the drug development process, and to seek their application as research biochemicals.

Newly discovered bioactive molecules will be further evaluated as to mechanism of action, potency, stability, and *in vivo* efficacy both by our various collaborators and within the ICBG. Samples of the natural products as well as derivatives will be supplied to aid and foster the further pharmacological description of lead compounds.

5. To promote our discoveries of valuable bioactive molecules into the economic sector, and to use these discoveries as tools for justifying and directing conservation efforts in Panama.

Scientific publications and presentations along with the provision of new compounds to collaborators will stimulate development efforts. Integration of research findings with the activities and goals of AP4 will foster conservation efforts in Panama by providing the Panamanian population with information related to biological and chemical diversity, and its possible benefits.

B. BACKGROUND

B.1. Introduction

B.1.1. Role of Marine and Terrestrial Natural Products in Human Medicine

Natural products have played an enormous and pivotal role in the development of human medicine, with approximately 45% of all current drugs either being a natural product, natural product derivative, or being inspired by natural products chemistry, and 37% of all pharmaceutical sales being for natural product derived medicines (Frormann and Jas 2002). Terrestrial plant-derived natural products, in particular complex alkaloidal substances, have a long standing history and appreciation in human medicine, in particular in cancer chemotherapy (vinca alkaloids, taxol, camptothecin), pain control (morphine, codeine), as cholinergics and anticholinergics (physostigmine, atropine, scopolamine), anti-infectives (quinine), CNS stimulants (caffeine), and many other drug classes. A relatively large number of these medicinal drugs have been isolated from tropical rainforest plants (Balick *et al.* 1996). Leaves of tropical forest species have both higher levels of chemical defense as well as a greater diversity of compounds than temperate species (Coley and Kursar 1996, Leigh 1999). For example, an extensive survey of the distribution and activity of alkaloids showed that they are more common and more toxic in the tropics (Levin and York 1978). All other classes of compounds that have been surveyed show similar patterns (Coley and Aide 1991). Given that only a small percentage of the tropical flora has been examined (Cox and Balick 1994), investigations of wild tropical plants should produce a wealth of new compounds. In 1995, the value of undiscovered pharmaceuticals in tropical forests was estimated at US $147 billion (Mendelsohn and Balick 1995).

The marine environment is extraordinarily rich in species, especially in tropical environments, but to date has only been examined for its potentially useful natural products chemistry to a cursory extent. Among the major groups of marine organisms known to be rich in secondary metabolite chemistry (*e.g.* sponges, tunicates, soft corals, marine bacteria, bryozoans, and algae), the algae were the first studied (Scheuer 1973), and because of the many novel and surprising natural product structures they yielded, these studies were instrumental in directing more attention to the concept of "Drugs from the Sea". Despite these early successes with algae, few contemporary natural products laboratories target marine plants for drug discovery efforts; instead, more emphasis has been given to sponges, tunicates and marine bacteria in recent years. To date, only very few natural products have been properly evaluated in modern assay-based biochemical or pharmacological screening programs. Moreover, some classes of these organisms, such as the cyanobacteria (blue-green algae), have continued to emerge as extraordinarily rich producers of bioactive natural products (Gerwick *et al.* 2001), and new and surprising molecules of importance to human medicine continue to be discovered at a high rate. In addition to continuing our collections of terrestrial plants, the inclusion of a marine macroalgae and cyanobacterial-based drug discovery component in this ICBG program will add a rich new dimension and provide additional opportunities for training, biodiversity inventory and conservation, and infrastructure development.

B.1.2. Natural Product Anticancer Agents

Isolation and development of new drugs which may aid in the treatment of neoplastic and tropical parasite diseases represents one of the major long term goals of this research proposal. The use of novel pharmacological and cell-based assays to detect new anticancer lead compounds, as well as more traditional yet quite difficult assays to tropical disease parasites, is aimed at the development of new classes of chemotherapies for these diseases, especially those for which there is no truly effective current therapy. Beyond the development of a specific therapeutic lead compound, a significant potential exists to discover from these efforts a new pharmacological probe which may lead to an enhancement of our understanding of the biochemical basis of cancer or tropical diseases. Many natural products have led indirectly to the development of very useful clinical drugs by providing new chemical ideas which have subsequently been explored and further developed by chemical synthesis. This is especially true in the oncology clinic (Cragg and Newman 2000) where there is frequent use of natural product agents (*e.g.* taxol), semi-synthetic compounds (*e.g.* etoposide), or compounds of synthetic origin but which are based on the structure of a natural product (*e.g.* mitoxantrone). However, success in the chemotherapeutic treatment of the complex mosaic of diseases falling

under the general title of "cancer" has generally been disappointing: in the USA alone, 556,500 people are expected to die in 2003 with most succumbing to solid tumors of the lung, stomach, colon, breast, ovarian or prostate as has been the trend in the past (American Cancer Society online report 2003). Unfortunately, until recently the major screening program for new chemotheraeutic agents was *via* mechanism-blind assays directed towards the discovery of new antileukemics or other cancers of a rapidly proliferating nature (Gibbs 2000). It has been argued that this strategy has decreased the likelihood of developing new agents with selectivity to the major solid and slow growing cancers listed above.

Recent increases in knowledge of the biochemical basis of cancer have opened up new opportunities for the design of innovative screening methods (Sikora 2001, Morin 2002). One type of the new generation of assays incorporates understanding about the enzymatic reactions and biochemical pathways that might be involved with the onset of cancer. Examples of these targets include the cyclin-dependent kinases and dual specificity phosphatases (Newman *et al.* 2002), Sirt-2 (Grozinger *et al.* 2001), DNA methyl transferases (Belinsky *et al.* 1996), p21 Cip/Kip proteins (Blagosklonny *et al.* 2002) and inhibitor of apoptosis (IAP) proteins (Deveraux 1999). Programs, including our own, have employed these innovative assays in primary screens to uncover new natural product enzyme inhibitors which are being further evaluated as potential anticancer leads. However, additional understanding at the frontier of biology involves the regulation of cell growth and the genetic and biochemical changes that promote malignancy (Hargnindey 2002). These newly identified targets are highly relevant to the development of rationally-based therapies for the treatment of colorectal, lung, breast, ovarian, and prostate cancers, and many involve inhibition of protein-protein interactions which are critical to unchecked cellular proliferation (Gietema and De Vries 2002). A general proposition to be explored in this research *via* collaboration with Novartis (see letter of support in Appendix 3) is that use of targets such as oncogene products, enzymes involved in signal transduction pathways, and the potential to modulate cell transformation and apoptosis, will enhance our discovery of new classes of terrestrial and marine natural product anticancer leads, and that these leads will be more effective in treating the major lethal solid tumors listed above.

Tropical rainforest plants have evolved an unsurpassed chemical arsenal of secondary metabolites against attack by a diverse and abundant set of herbivores and pathogens (Coley and Barone 1996) because no other terrestrial biome on earth has more intense biotic interactions than those in tropical rainforests (Price *et al.* 1991). Considering the high number of clinically useful anticancer and other drugs (Farnsworth and Soejarto 1991) isolated from the small proportion of tropical rainforest plants investigated, there is potentially a large reservoir of metabolites to be tested for their anticancer activity in tropical rainforest biomes. Similarly, the great species diversity available in the marine environment combined with the uniqueness of its competitive environment makes marine organisms a rich source of structurally diverse and bioactive natural products. Sessile marine organisms are particularly rich in this regard as they depend solely on either physical or chemical features in order to escape predation. The group of sessile marine organisms that we have focused on as a source of new potential anticancer agents, the macroalgae and cyanobacteria, are rich in diverse structural classes of natural products, many of which have toxic and other medicinally-relevant properties.

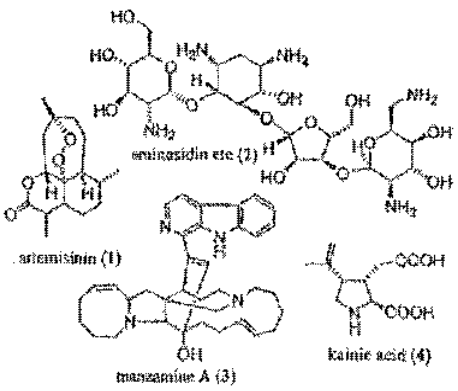
B.1.3. Natural Products in Tropical Disease Medicine

Natural products have played an absolutely crucial role in the development of our rather limited arsenal of agents for treating parasitic diseases. In the antiprotozoal area, terrestrial plants have yielded a number of useful agents, including quinine and its ensuing derivative structures (chloroquine and mefloquine), artemisinin (1), and emetine (Phillipson and Wright 1991). While amphotericin B from *Streptomyces* spp. plays a critical role in the treatment of amoebic meningoencephalitis, artemisinin has recently found some utility in this area (Gupta *et al.* 1995). Derived from different *Streptomyces* spp., the aminocyclitol-aminoglycoside antibiotics, aminosidine, paromomycin, hydroxymycin and monomycin are chemically identical (2). Although, originally developed as anti-bacterial agents they also have significant antiprotozoal activity, against *Leishmania*, *Entamoeba* and *Cryptosporidium* species (Croft and Yardley 2002).

From the marine environment, a number of compounds with antiprotozoal activity have been reported (Crews and Hunter 1993), with some of the most promising being isonitrile-containing terpenoids from sponges of the family Halichondrida (Wright *et al.* 2001), manzamine-type alkaloids [e.g. manzamine A (3), Ang *et al.*

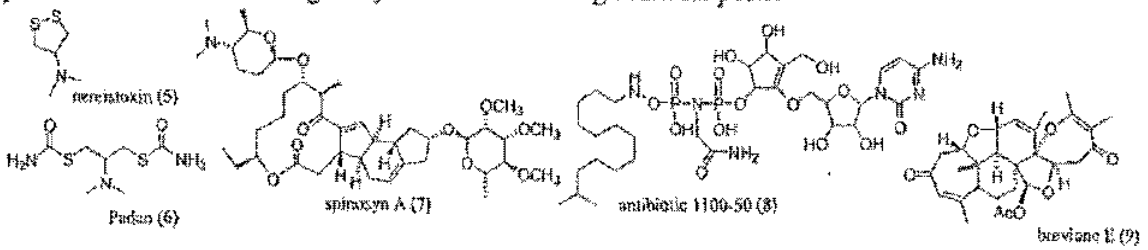
2000] from various sponges including *Xestospongia* sp., and bengamide E from *Jaspis* sp. (Adamczeski *et al.* 1989).

Other types of tropical diseases, such as those caused by helminthes have also benefited enormously from natural products-derived medicines, which include avermectin B_1 (marketed as abamectin) from *Streptomyces avermitilis* and its semisynthetic analog ivermectin (Fisher 1990). From the marine environment, the red algal (*Digenia simplex*) amino acid 'kainic acid' (**4**) is an example of a clinically useful antiparasitic agent. Additionally, kainic acid is an extremely useful tool in neurobiology, and has defined an entire class of receptors known as 'kainate receptors' (McGeer *et al.* 1978). Screening terrestrial and marine natural products for additional chemotypes with antiparasitic properties should be productive in defining new molecules for the treatment of a variety of human diseases, especially those prevalent in impoverished countries. However, the economics of discovering and bringing to market new drugs in this area, one which primarily affects third world populations, are such that large pharmaceutical companies are not able to directly participate in this search. Hence, it is left to other organizations to conduct these types of screening programs, such as the US military, a very few US and European academic laboratories, and tropical disease hospitals and institutions actually located in third world tropical countries.



B.1.4. Natural Products as Agrochemicals

Natural products have also played an important role in the discovery and development of new agrochemicals, both historically as well as more contemporaneously. An older discovery supporting this premise is that of nereistoxin from the Japanese bait worm *Lumbriconereis heteropoda*. Following up on reports from fisherman that insects landing near the worms were rapidly incapacitated and subsequently died, early marine natural products researchers isolated an unusual sulfur containing heterocycle, nereistoxin (**5**, Hashimoto and Okaichi 1960). Synthetic modification of this structure resulted in the simplified derivative **6**, sold under the name Padan, to treat insect infestations of rice fields (Hashimoto *et al.* 1973). A more recent success story of a natural product finding utility in agriculture is represented by the spinosyns (Kirst *et al.* 1991), a novel family of fermentation-derived natural products that exhibit potent insecticidal activities. A naturally-occurring mixture of spinosyn A (**7**) and spinosyn D (Sparks 2001) is sold as Spinosad or Tracer for their utility in crop protective applications. Potential applications of this unique chemical family of macrolides have also been investigated in the field of animal health (Kirst *et al.* 2002). Fueled by these successes, the search for novel natural products with diverse agrochemical utilities has intensified; antibiotic 1100-50 (**8**) which is active to nematodes and helminthes (Takatsu *et al.* 2002), and (+)-brevione E (**9**), active as a herbicidal agent (Macias *et al.* 2000), are examples. Just as in pharmaceutical drug development, natural products play a crucial role in identifying new and unpredictable chemotypes that show useful agrochemical properties, and as such, identify new "lead compounds" as well as new targets by which to inhibit agricultural pests.



B.2. Terrestrial Plants of Panama

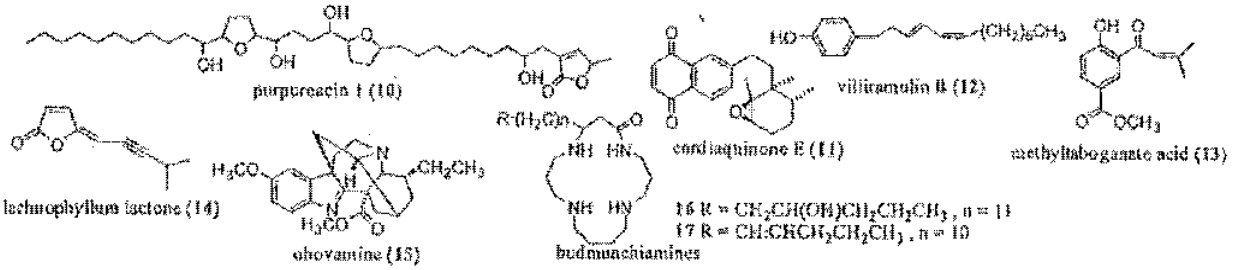
B.2.1. Biodiversity of Terrestrial Plants in Panama

Panama is an excellent site in which to undertake a drug discovery program because of its unsurpassed floristic biodiversity which encompasses habitats of mangrove jungle, lowland forests, floodland forests and

foothill forests. Panama is considered a biodiversity hotspot since it lies at the center of the region with the greatest concentration of terrestrial plant species in the world (>5,000 species/10,000 km^2, Myers *et al.* 2000). In part, this is because Panama links two continents and has large gradients in rainfall and altitude caused by a mountainous backbone. Panama's unique geographic position means that it is home to species from both Central and South America and it is estimated that there are over 13 life zones containing between 8,500 and 9,000 species of flowering plants, 14% of which are endemic (Tosi 1971, D'Arcy 1987, Correa, pers. com.). Furthermore, this terrestrial diversity occurs within a relatively small and accessible area, making collections and recollections feasible.

B.2.2. Chemistry and Medicinally-Relevant Activity of Panama Terrestrial Plants

Although Panama has some of the greatest wealth of vegetative species in the world, the chemical composition of these plants has been studied relatively little and most of these investigations have been carried out by non-Panamanian researchers. However, a number of bioactive pure compounds have been reported from Panamanian plant species. The pronounced activity of leaves of *Annona purpurea* (Annonaceae) against larvae of the *Aedes aegypti* was attributable to 6 acetogenins, of which purpureacin 1 (**10**) and 2 were novel (Cepleanu *et al.* 1993). These compounds also showed antifungal activity against *Candida albicans*. Three new naphthoquinones from the roots of *Cordia linnaei* (Boraginaceae), including cordiaquinone E (**11**), were active against yellow fever mosquito larvae and *Candida albicans* (Ioset *et al.* 1998). The Panamanian shrub *Piper villiramulum* (Piperaceae; Galinis and Wiemer 1993) yielded two new phenol derivatives, one of which was repellent to leafcutter ants (villiramulin B, **12**). Prenylated benzoic acid derivatives, including the new methyltaboganate acid (**13**), were suggested to provide an effective natural defense against leafcutter ants in the leaves of *P. tabogum* after they were found to be repellent at or below their apparent natural concentrations (Roussis *et al.* 1990). Six prenylated benzoic acid derivatives and three chalcones were isolated from the leaves of *P. dilatum*, and four of the benzoic acid derivatives displayed antifungal properties against *Cladosporium cucmerinum* (Terreaux 1998). The antifungal leaf extract of *Baccharis pedunculata* (Asteraceae) yielded a lachnophyllum lactone (**14**), a prenylated coumarin and a 3-methyl ether flavone as the active principles, and an inactive flavone (Rahalison 1995). The acetylenic lactone (**14**) also showed very high toxicity (2 μg/mL) against human keratinocytes. A number of mostly known alkaloids have been isolated from Panamanian plants. The stem bark of *Stemmadenia obovata* afforded ten known alkaloids and the new ibogaine-type alkaloid, obovamine (**15**, Madinaveitia *et al.* 1996). Inhibition of the malarial enzyme plasmepsin II by methanol extracts of the leaves and stem bark of *Albizia adinocephala* (Leguminosae) led to the bioassay-guided isolation of two new spermine alkaloids, budmunchiamines L4 (**16**) and L5 (**17**, Ovenden *et al.* 2002). *Cephaelis inecacuanhaemetic* was shown to be the botanical source of Panamanian ipecac, an emetic syrup widely used in the treatment of poisonings (Hatfield *et al.* 1981).



B.2.3. Biomedical Properties of Panama Terrestrial Plant Extracts

A multitude of Panamanian plant crude extracts have been tested in diverse biomedical assays carried out by the Panama-based research group of Dr. Gupta. In their early studies on medicinal plants from Panama, extracts from 8 species were screened for biomedical activity and toxicity in rats (Eposito-Avella *et al.* 1985). While the extract of *W. glomerata* increased motor activity, all other extracts decreased motor activity. Six plants increased micturition, while four were analgesic. Thirty-four crude extracts of Panamanian plants representing 4 species of Celastraceae and 5 species of Lamiaceoase were subsequently screened for

antimicrobial activity against 13 bacterial strains and 2 yeasts, and cytotoxicity against HeLa and Hep-2 cell lines (Gonzalez *et al.* 1994). Fifty-three percent of the plant extracts had significant antimicrobial activity against at least 2 of the test organisms, while 26.5% possessed IC_{50} < 100 µg/mL against one cell line). Assays for brine shrimp toxicity, tumor inhibition, DNA-intercalation and clonogenic cytotoxicity were carried out on crude extracts from 20 plants used in traditional medicine (Gupta *et al.* 1996). The methanol extracts of 8 plants were active in the DNA-intercalation assay; 16 of the 20 methanol extracts were active in potato disk tumor inhibition assay; only 3 extracts were cytotoxic in the clonogenic assay, while 13 of the 20 extracts tested were toxic to brine shrimp (LC_{50}< 100 ppm). The correlation of brine shrimp activity with DNA intercalation and tumor inhibition supports the use of this rapid and economical bioassay in activity-guided fractionation of plant extracts (Meyer *et al.* 1982). Based on their traditional uses in the treatment of hypertension, cardiovascular, mental and feeding disorders, 19 Panamanian plants were selected, producing 149 extracts that were screened using radioligand-receptor-binding assays (Caballero-George 2001). Several organic extracts inhibited [3H]-AT II binding (angiotensin II AT1 receptor) by more than 50%. Two extracts were potent inhibitors of the [3H] NPY binding (neuropeptide Y Y1 receptor). Extracts from 4 different species showed high inhibition of the [3H] BQ-123 binding (endothelin-1 ET(A) receptor) in preliminary screening. A more recent focus in the Gupta research group has been the testing of Panamanian medicinal plants for anti-HIV activity. Extracts of 39 plants were tested for the inhibition of HIV-induced cytopathic effects in cultured cells, HIV-reverse transcriptase (RT) and HIV-protease (PR) enzymes (Matsuse *et al.* 1999).

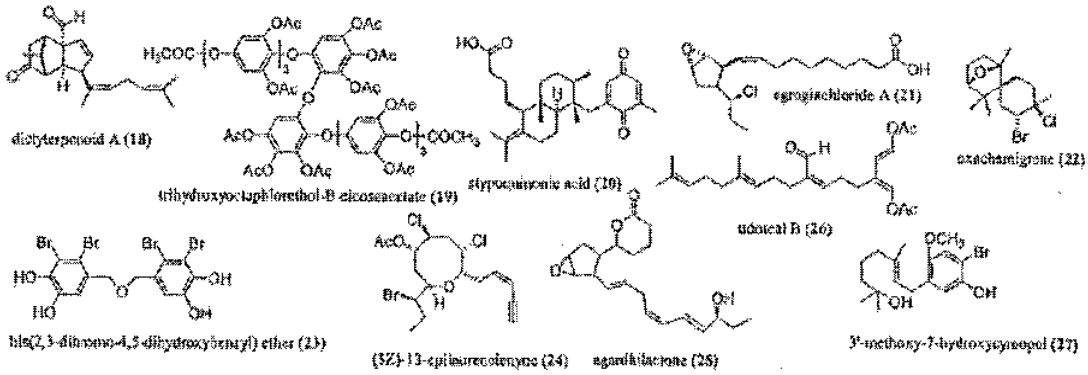
B.3. Macrophytic Algae of Panama

B.3.1. Biodiversity of Macrophytic Algae in Panama

Because the taxonomic standings of macrophytic algae are diverse at the Phylum level, there is inherently even greater genetic diversity among algae than in terrestrial plants (Littler and Littler 2000). The Southern Caribbean Sea (Nicaragua, Costa Rica, Panama and Columbia) is host to a rich diversity of marine algae that is critical to coral reef structure, health, and biogeochemical cycles. Unfortunately, in the past three decades only a single marine floral survey of Panama has been published (Earle 1972), although a handful of studies have focused on particular genera or species (Hay and Gaines 1984, Hay and Norris 1984, Kilar and Norris, 1988). However, Littler and Littler's (2000) recent comprehensive and detailed photographic work on Caribbean Reef Plants is of tremendous aid in taxonomic assignments of both macro- and microalgae. Also, recently completed doctoral research by Wysor (2002, University of Louisiana) describes an extensive survey of the macroalgal biodiversity in Panama along both coasts, and reports an increase of 125% in the total estimated macroalgal diversity of Panama. Including previous records, over 450 taxa have been documented to date. A survey of the literature also revealed that macroalgal diversity in Panama is actually higher than in most other countries of either the Caribbean or the eastern Pacific (Wysor 2000). Littler and Littler have also produced a general guide to the Southern Pacific flora (2003). The Pacific and Bay of Panama zones of Panama's Southern and Western shores remain less well characterized for their algal diversity and habitats than the Caribbean, and this represents one of the outstanding opportunities for discovery and marine biodiversity inventory in this proposed ICBG program.

B.3.2. Chemistry of Macrophytic Algae

Genetic diversity normally equates with diversity in the types of pathways that are used to construct secondary metabolites, and hence, the inherent high diversity among macroalgae results in highly diverse molecular architectures. Marine macroalgae, the first group of marine organisms to be widely investigated for unique constituents (Scheuer 1973), have yielded the greatest number of structures in the literature which are of diverse structural type (Tringali 1997). The major trends in the brown algae include sesquiterpenes and diterpenes (*e.g.* **18**, Suzuki *et al.* 2002), phloroglucinol derivatives (**19**, Glombitza and Schmidt 1999), mixed biogenesis terpene-quinones (*e.g.* **20**, Wessels *et al.* 1999), and oxylipins (**21**, Todd *et al.* 1993). In the red algae, halogenated terpenes of various sizes (**22**, Brito *et al.* 2002), bromophenols (**23**, Kurihara *et al.* 1999), acetylene-containing fatty acids (**24**, Iliopoulou *et al.* 2002) and oxylipins (**25**, Graber *et al.* 1996) represent the major trends. The green algae have been found to contain a number of unusual sesquiterpenoids and diterpenoids (**26**, Iliopoulou *et al.* 2000) and halogenated phenols (*e.g.* **27**, Dorta *et al.* 2002).



However, relatively few macroalgal metabolites have been reported as possessing useful biomedical activities, primarily, we believe, as a consequence of their being studied early in the development of the 'marine natural products' field and pre-dating the current research focus on biologically active natural products. Early efforts into marine natural products chemistry were largely undertaken by organic chemists trained in synthetic organic chemistry. For them, the interest in algal natural products was focused on novel structural motifs. Consequently, the macrophytic algae are known to be abundant producers of structurally interesting natural products, but of perceived low biochemical interest. In all likelihood, it *is* simply the limited range of bioassay-based evaluations that gives rise to this erroneous perception.

B.3.3. Medicinally-Relevant Properties of Macrophytic Algal Natural Products

Supported by our recent results with several different macroalgae from elsewhere in the world, we believe that there are a substantial number of marine macroalgal metabolites previously reported as biomedically inactive or active only in limited arenas (*e.g.* fish toxicity) which will show significant activity in the proposed biochemical and cell-based assays. More specifically, it seems particularly compelling that the many and diverse terpenes produced by macrophytic marine algae will show important activities in the modulation of signal transduction cascades. An example that partially validates this hypothesis comes from the discovery that *d*-limonene is a selective inhibitor of prenylation of 21-26 kDa proteins, including p21ras (Crowell *et al.* 1994). *d*-Limonene and related terpenes are currently in various phases of pre-clinical and clinical trials (Phillips *et al.* 1995, Matthys *et al.* 2000). The discovery of halomon from *Portieria hornemannii*, a simple halogenated monoterpene, as a significant and selective cytotoxin with *in vivo* antitumor effects (Fuller *et al.* 1992), represents another important lead compound from algae in which the class of natural product was discovered years earlier, but not recognized initially for its potentially useful medicinal properties (Konig and Wright 1993). Recently, we have discovered that halomon owes at least a part of its biomedical properties to its ability to inhibit DNA methyltransferases. Unsaturated and halogenated sesquiterpenes of the chamigrene class, common in *Laurencia* spp., have been identified as possessing an interesting profile of cytotoxicity in the NCI-DCT (National Cancer Institute - Division of Cancer Treatment) *in vitro* 60-cell line panel (Kirk Gustafson, pers. comm.). In general, recognition of the subtle yet fundamental mechanisms by which marine algal metabolites, exert their pharmacological activity will be insightful to the cellular process under study and to the development of these compounds into useful drugs.

Only one study of limited scope has focused on the medicinally relevant activity of extracts of marine algae from Panama (Gupta *et al.* 1991). In this work, seven macroalgae from the Pacific coast were collected, methanol extracts formed and tested for antibiotic activity to six different human pathogens (2 Gram +, 2 Gram -, and 2 fungi); five of these showed activity to the Gram positive bacteria (*Caulerpa racemosa, Halimeda opuntia, Gelidiela acerosa, Laurencia papillosa* and *Acanthophora spicifera*). No follow-up isolation of active materials has been reported to date.

B.4. Marine Cyanobacteria of Panama

B.4.1. Biodiversity of Marine Cyanobacteria in Panama

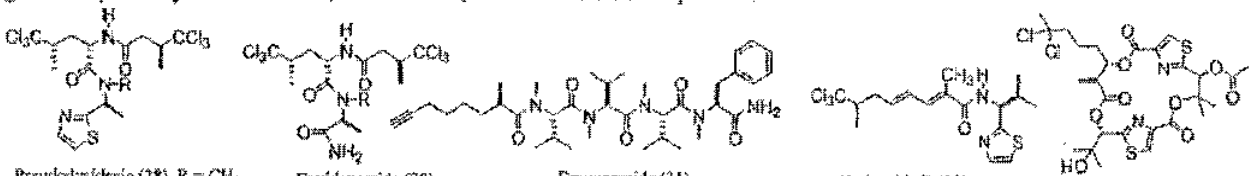
The warm water habitats in the Caribbean and Gulf of Chiriqui sections of Panama are ideal for the growth of diverse marine cyanobacteria, which we have observed on preliminary collection trips. Again, specific collection records and biodiversity inventories of marine cyanobacteria in this region are rather poor, with the recent volume of the Littlers' on Caribbean Reef Plants being one of only a very few sources reporting species from this area. On the other hand, the superb photographic documentation in this book, as well as line drawings of anatomical features, is a tremendous aid in species identification. In the words of the authors of this scholarly work, their "...goal was to create a 'user-friendly' identification guide to approximately 565 macrophytic marine plants of the Caribbean ..." and "...provides a dependable source to identify most tropical western Atlantic marine plants, without the liability of shipping materials to overcommitted specialists for determinations." A total of 37 species of marine cyanobacteria are covered in detail in this treatise (Littler and Littler 2000). Most recently, these authors have also produced a similar guide to the South Pacific marine flora (Littler and Littler 2003).

B.4.2. Chemistry of Marine Cyanobacteria

The natural products chemistry of marine cyanobacteria is highly diverse, and directly reflects the extraordinary biosynthetic capacities of these organisms (Gerwick *et al.* 2001). The major theme in cyanobacterial chemistry is the production of polypeptides modified with various lipid components to make diverse classes of lipopeptides. The types of lipid components found in these metabolites include fatty acids, polyketides, and ketide-extended amino acids. The greatest number of marine cyanobacterial metabolites described to date come from a single species, *Lyngbya majuscula*, from which over 200 different compounds are known. The molecular weight range of these *L. majuscula* metabolites has an even distribution between 250 and 600 daltons (8-10 metabolites per 50 amu division), with only a few compounds of either smaller and larger size. All *L. majuscula* metabolites contain carbon, hydrogen and oxygen; 58% also possess nitrogen, 17% chlorine, 6% sulfur (all chlorine- and sulfur-containing metabolites also contain nitrogen), and 4% possess bromine. While both unbranched and highly branched (up to 27 methine, quaternary carbon, or tertiary amide branches in a single molecule!) metabolites are produced, the majority range from 2 to 8 branch points per molecule. A small number of *L. majuscula* metabolites are acyclic; most have between 10% and 60% of their non-hydrogen/non-halogen atoms involved in ring structures. Dissection of the biogenetic subunits making up these metabolites indicates a range of 1 to 5 units of a different origin per molecule, with 3 being most common. About 25% of the biogenetic subunits of these metabolites are polyketides or fatty acids, another 25% are amino acids, and 25% are methyl groups likely deriving from SAM. The remaining 25% of the biogenetic subunits derive from terpenes and sugars, or are of an uncertain origin. The amino acids present are dominated by the aliphatic (34% leu, ile, val, pro, ala) and aromatic (32% trp, phe, tyr) residues, with the remainder being polar residues (gly, ser, thr, cys, and hypro) and modified amino acids. In general, the types of oxygen-derived functional groups include alcohols, epoxides, ethers, ketones, esters, and carboxylic acids, while nitrogen-containing functional groups include amides, amines, thiazoles and thiazolines. It can be concluded that the roughly 200 metabolites currently known from *Lyngbya majuscula* form an extraordinarily diverse set of natural product structures (Gerwick *et al.* 2001).

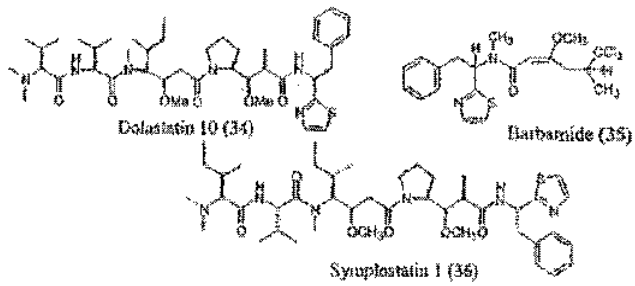
To the best of our knowledge, there are only two reports of chemistry from Panamanian cyanobacterial species. A red strain of *L. majuscula* collected from Bocas del Toro yielded trichlorinated metabolites (Jiminez and Scheuer 2001) similar to those described from the *Dysidea* sponge-associated symbiotic cyanobacterium, *Oscillatoria spongeliae* (Lee and Molinski 1992). Three new trichlorinated metabolites, pseudodysidenin (**28**), dysidenamide (**29**) and nordysidenin (**30**), and a terminal acetylene-containing peptide, dragonamide (**31**), were isolated. Pseudodysidenin (**28**) was cytotoxic to several cancer cell lines with IC_{50}'s > 1 μg/mL. Although less well characterized, herbamide B (**32**), also previously obtained from *Dysidea herbacea* (Clark and Crews 1995), was reported as a metabolite of this Panamanian *L. majuscula* as well. The same *L. majuscula* collection from Bocas del Toro also yielded hectochlorin (**33**), a metabolite which we had recently isolated from an *L. majuscula* strain (now cultured in house) collected from Hector Bay, Jamaica (Marquez *et al.* 2002). In this combined publication we reported that hectochlorin causes hyperpolymerization of actin and was found to have

an average GI_{50} = 5.1 μM in the assay panel of 60 cancer cell lines at the National Cancer Institute, with greatest potency in the colon, melanoma, ovarian and renal panels.

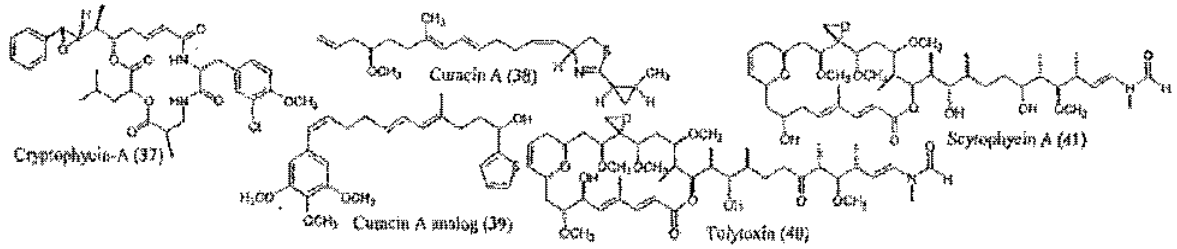


B.4.3. Medicinally-Relevant Properties of Marine Cyanobacterial Natural Products

Arguably, cyanobacteria have been the richest aquatic or marine source of new clinical candidates and lead compounds for the treatment of cancer. Clearly, the flagship of this effort has been the discovery of dolastatin 10 (**34**) as a vinca site antitubulin agent (Bai *et al.* 1990), which is currently in Phase II clinical trials (Margolin *et al.* 2001). Dolastatin 10 is one of a series of ketide-extended polypeptides (Poncet 1999, Luesch *et al.* 2002) originally isolated in very small yields from the herbivorous mollusk, *Dolabella auricularia*. It is now clear from the isolation of close structural homologs, barbamide (**35**) from *Lyngbya majuscula* (Orjala and Gerwick 1996) and the symplostatins (*e.g.* **36**) from *Symploca hydnoides* (Harrigan *et al.* 1998), and even more recently, from the direct isolation of dolastatin 10 from a *Symploca* species (Luesch *et al.* 2001), that the dolastatins derive from marine cyanobacteria.



Cryptophycin A (**37**) is an intriguing vinca site antitubulin agent (Trimurtulu *et al.* 1994) which has shown superb *in vivo* properties, when administered by intravenous routes, and has recently completed Phase I clinical trials. Currently, less toxic analogs are being considered for additional preclinical and clinical evaluation (Shih and Teicher 2001, Sessa *et al.* 2002). From some of our previous work, a Curaçao collection of *L. majuscula* yielded a novel antitubulin agent, curacin A (**38**) which binds at the colchicine binding site on tubulin (Hamel *et al.* 1995, Blohkin *et al.* 1995). Curacin A has shown significant *in vivo* antitumor effects in human xenografts (T/C 21% at 6.7 mg/kg IP daily x 5, NCI H522 non-small cell lung carcinoma; 2/6 animal deaths) as well as an interesting profile in hollow fiber assays (NCI Total Score = 8 with a Cell Kill = 4). However, significant problems with the instability and poor water solubility of curacin A have hindered its development as an anticancer lead compound. Wipf *et al.* (2002) have been applying combinatorial synthesis methods to produce more stable and efficacious analogs (*e.g.* **39**). Tolytoxin (**40**, Carmeli *et al.* 1990) and the scytophycins (**41**, Ishibashi *et al.* 1996) are related complex polyketide metabolites with potent microfilament disrupting properties (Smith *et al.* 1993, Patterson *et al.* 1993).



An interesting trend in the natural products of cyanobacteria is their frequent production of metabolites which interfere with microtubule assembly, as illustrated above. At first consideration, it is difficult to understand how these organisms are able to withstand the toxic effects of their nanomolar level potency inhibitors which in several cases are produced in very large quantities (> 10% of extracted lipids). However, this adaptation of cyanobacteria appears more reasonable if one considers that these organisms lack microtubules, and hence are unaffected by the toxic effects of these antitubulin agents.

As cultivatable classes of organisms, the problem inherent to natural products chemistry of having too little of a compound for full biomedical evaluation, is in principle overcome. Further, it should be pointed out that there is little disturbance to the natural ecology of a habitat from the collection of these microalgae for culture-based activities. With a cultured organism which produces pharmaceutically useful natural products, an exciting array of experimentation aimed at enhancement of drug availability and drug activity is possible, including optimization of drug production, exploration of the physiology of drug production, biosynthetic studies, directed biosynthesis, mutasynthesis and genetic manipulations. We are currently engaged in these types of studies with the collection of *Lyngbya majuscula* which yielded curacin A.

B.5. Advances in Natural Products Technology

B.5.1. Advances in Chromatography

To a large extent, the modern approach to chromatographic isolation of natural products has been influenced by the demand created by industrial high through-put bioassay capabilities (Hook *et al.* 1997). This demand has generated a paradigm shift away from more traditional multi-tiered, exhaustive chromatographic schemes. Modern natural products chemistry investigations routinely involve a bioassay-guided approach, with the bioactivity testing often being carried out by pharmaceutical companies running large-scale operations with a high turn-over of assays. Thus, in both the Panama and Oregon laboratories, "prefractionation" of crude extracts, which allows simultaneous submission of the crude material and its fractions for testing, has been found to provide a mechanism by which the natural product isolation process may be integrated with industrial high through-put screening. In other Oregon projects, we have observed that prefractionation resulting in testing of samples of reduced complexity significantly increases (15-fold) our "hit rates" of bioactive fractions.

A reduction in the number of separations required to obtain pure compounds from crude extracts has been achievable on ever more finely engineered stationary phases and has several advantages. Fewer chromatographic steps may drastically reduce losses of often limited, even irreplaceable sample materials, especially where large-scale column chromatography steps are kept to a minimum. Sample viability is also better preserved by avoiding repeated exposure of the material to potentially acidic column conditions, which rapidly degrade labile components. The isolation of small quantities of difficult to detect, minor components from crude extracts is facilitated by the use of photo diode array or evaporative light scattering detectors such as those available in both the Cubilla and Gerwick laboratories.

B.5.2. Advances in Structure Elucidation Methods

In the last three decades, developments in nuclear magnetic resonance (NMR) spectroscopy and mass (MS) spectrometry, the primary analytical methods in modern natural products research, have been unparalleled (Keifer 2000, Bahktiar and Nelson 2000, respectively). Significant advances have been made in improved hardware, new experimental techniques (new pulse sequence tools) and also more powerful data processing and analysis software.

Perhaps the most prominent advance in mass spectrometry applications has come with the advent of Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) and multi-step tandem mass spectrometry. MALDI-TOF has become an essential tool for the investigation of large biomolecules (Juhasz *et al.* 2002) and its potential uses range from rapid microbial identification (Lay 2002), detection of virulence and antibiotic resistance markers and identification of possible vaccine components (Van de Water *et al.* 1997), to structure determination of large peptides and polyketides such as those we have isolated from cyanobacteria (Cohen and Gusev 2002). Notably, tandem mass spectrometry has been used successfully to sequence cyclic peptides because of the capability to execute several sequential ionizations of a precursor ion and its resulting fragments (Ngoka and Gross 1999). Thus amino acid residues are sequentially removed, one at each stage of the ionization caused by collisionally activated decomposition (CAD) from the C-terminus of the peptide, until a dipeptide ion is produced. A MALDI-TOF instrument has recently been assembled at Oregon State University and this will provide us with enhanced facilities to aid in the efficient structure assignment of metabolites isolated from cyanobacteria collected in Panama, especially where NMR data are complicated by symmetry present in a molecule, or by unresolvable chemical shift overlap.

Compatible with the chromatographic capabilities to detect and isolate smaller amounts of compounds, developments in NMR spectroscopy, an inherently insensitive technique, have made possible the structure elucidation of sub-milligram quantities of higher molecular weight natural products. Besides the increased field strengths of larger and larger superconducting magnets, recent developments in probe technology have contributed enormously to pushing the limits on the amount of sample needed for comprehensive NMR data acquisition and therefore structure elucidation. Cryogenic probe technology relies on the reduced operating temperature of the coil and pre-amplifier which lowers the noise temperature of the coil and pre-amplifier and improves the efficiency of the coil (Kotsubo and Nast 1996). It has improved signal/noise (S/N) ratios by up to a factor of four. However, an even more recent, far less expensive, improvement in probe technology has been the development of solenoidal microcoil probes which have been shown to permit the acquisition of 2D NMR data on samples at concentrations of less than 100 nM (*e.g.* 75 μg for a compound of MW 500 Da) in just a few hours (Subramanian *et al.* 1999). The small size of the solenoidal coils as well as the inherent increase in sensitivity over saddle coil design results in data acquisition times being reduced by over an order of magnitude. A microcoil probe has been purchased for the DRX 600 spectrometer at Oregon State University which is anticipated to greatly increase our efficiency in the structure elucidation of small amounts of relatively large molecules, such as we have routinely isolated from cyanobacteria (Gerwick *et al.* 2001).

Major improvements in data analysis tools have also contributed enormously to efficient data processing and structure elucidation of natural products. The compilation of data for known compounds into databases that are used in software structure elucidation programs such as that marketed by ACD/Labs, which has recently been acquired at Oregon State University, allows for a drastic reduction in the time spent on data processing. This software accelerates not only the de-replication process, which is inherent in natural products chemistry, but also the elucidation of novel structures. Another tool we use to assist in the structure elucidation of complex natural products is NMRSIM, an accessory program in the Bruker XWINNMR package, which is useful for simulation of predicted coupling constant information. Also contributing to efficient NMR data acquisition and processing is the general move away from inherently insecure Unix platforms on expensive Silicon Graphics (SGI) machines to Linux platforms on ordinary personal computers. Following a campus-wide trend at Oregon State University, the Gerwick research laboratories have recently acquired a site license for the Bruker XWINNMR package for Linux to facilitate remote data processing. Such data stations are critical for detailed, unambiguous data analysis which, in our experience, is most efficiently carried out while using the XWINNMR software, rather than working exclusively with printouts. In light of this, networking of the Bruker Avance 300 spectrometer (which uses a Windows platform) at the STRI headquarters in Panama City, and purchase of further site licenses for XWINNMR for Windows are planned to enable data stations for NMR data processing to be installed at the University of Panama.

B.6. Conclusions to Background

There is considerable justification for the screening of terrestrial plants and marine cyanobacteria and macroalgae for new anticancer, antiparasitic and agricultural lead compounds. Previous studies have shown these groups of organisms to possess many structurally-novel natural products, several of which display promising medicinally relevant activities. However, the full potential of these organisms remains to be explored, especially so through application of a broader range of modern biochemical and cell-based biomedical assays. The proposed research will provide critical insights into the prevalence of potential anticancer, antiparasitic and agricultural lead compounds in terrestrial plants and marine cyanobacteria and macroalgae from Panama.

C. PROGRESS REPORT AND PRELIMINARY RESULTS

C.1. Progress Report for Terrestrial Plants

Under the existing Panama ICBG structure, the research groups of Dr. Luis Cubilla and Dr. Mahabir Gupta, both based at the University of Panama, have focused on the bioassay-guided isolation and characterization of novel, bioactive natural products from Panamanian terrestrial plants. In this section, we summarize the results from the terrestrial plant chemical investigations carried out in this ICBG for the last 4 years. Plant materials

were collected according to ecological principles which indicate that fresh, young leaves are richest in natural products, and extracted, as described in AP1. The plant extracts selected for further purification were chosen for their consistent, significant bioactivity in Panama-based tropical disease, anticancer and anti-HIV bioassays described in AP2, as well as for the absence of any reported bioactivity or known compounds, as determined by Napralert and SciFinder database searches (see AP2, Figure 12). In October 1998, Panama ICBG scientists purchased all of the equipment, remodeled laboratories and trained the personnel necessary to establish bioassays for malaria (*Plasmodium falciparum)*, Chagas' disease (*Trypanosoma cruzi*) and leishmaniasis (*Leishmania mexicana*). None of the bioassays had been run previously in Panama and in most cases required the development of novel methodologies, as described in AP2 (Section C.1.). The cell-based assays using 3 tumor cell lines [H-460 (lung), SF-268 (central nervous system) and MCF7 (breast)] from the NCI were run by Dr. Gupta's laboratory, while anti-HIV assays were run in the Department of Microbiology at the University of Panama. These assays have been successfully used to guide the isolation of the bioactive metabolites presented here. Thus, the government of Panama is embracing drug discovery as a sustainable and promising use of their extensive biodiversity and has provided positive support throughout the Panama ICBG project (see letter of support from Ricardo Rivera, National Authority of the Environment).

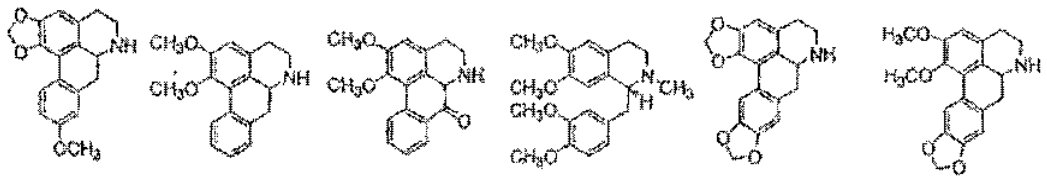
C.1.1. Progress Report for the Cubilla Laboratory

The Cubilla research group collaboration with the Panama ICBG began in January 2000, with a goal to pursue the fractionation of crude extracts active in the tropical disease (leishmaniasis, Chagas' disease and malaria) bioassays run in Panama by Dr. Ortega-Barrià's laboratory. As Dr. Cubilla did not have a laboratory prior to joining the ICBG, the first 6 months were dedicated to preparation of the laboratories and office space and the acquisition of equipment and materials. Initially, the group comprised one research assistant and two students, but the laboratory has since grown to include 3 research assistants and numerous students. During this time, an HPLC system with Photodiode Array (PDA) detector, a Medium Pressure Liquid Chromatography (MPLC) system and a melting point apparatus were acquired. The capacity to carry out separations by Flash Liquid Chromatography (FLC), Vacuum Liquid Chromatography (VLC), Preparative Thin Layer Chromatography (PTLC) and Column Liquid Chromatography (CLC) has also been acquired. Preliminary fractionations of extracts from 31 plant species have been carried out, including 20 extracts from young leaves and 28 extracts from mature leaves. Projects on extracts from 6 of these species are completed and work on a further 10 species is in progress, with 25 pure compounds isolated and at least partially characterized to date. Furthermore, efforts have been initiated to purify gram quantities of alkaloids for *in vivo* testing in AP2. This exciting expansion in the Panama ICBG follows an award of $65,000 from STRI for the development of *in vivo* mouse bioassays for cutaneous and visceral leishmaniasis in Dr. Ortega-Barria's laboratory (see AP2, Section C.1.1.3.). To obtain pure compounds, liquid-liquid partitioning (as is shown in the partition scheme, Appendix 9) of the crude extracts was followed by VLC, CLC, PTLC, FCLC or HPLC at preparative and semi-preparative scale. In cases where the presence of alkaloids was detected, an acid-base extraction of the crude extract or fraction was carried out.

C.1.1.1. Compounds with Activity against Leishmaniasis

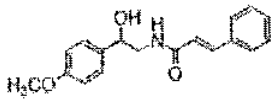
Some of our most relevant results are the isolation of several known aporphine alkaloids from *Guatteria amplifolia* and *G. dumetorum* (Annonaceae) which possessed potent activity in the antileishmaniasis assay. The results summarized below, led us to obtain a [redacted] PATENT PENDING [redacted] and are discussed further in AP2 (Section C.1.1.2.). Three collections of young leaves of these two *Guatteria* species were made on the Barro Colorado Natural Monument (2000-2001), which is the largest island located in Gatún Lake (the main water reservoir for the Panama Canal). Although extracts from *G. amplifolia* were previously reported to have activity against *Leishmania*, the chemical constituents were not isolated and identified (Weniger *et al.* 2001). Two previous studies of *G. amplifolia* led to the isolation of aporphine and oxoaporphine alkaloids (Weniger *et al.* 2000, Lopez *et al.* 1993), some of which had antimalarial properties (Weniger *et al.* 2000). There are no reported chemical investigations of *G. dumetorum*, a species known only to Costa Rica and Panama (Croat 1978). Therefore, after finding that the

crude extracts of *G. amplifolia* and *G. dumetorum* were active in assays against *Leishmania mexicana*, these two species were targeted for further investigation. Bioassay-guided fractionation of the *G. amplifolia* extract led to the isolation of the known alkaloids xylopine (**42**), nornuciferine (**43**), lysicamine (**44**), and laudanosine (**45**), while the *G. dumetorum* extract yielded alkaloids cryptodorine (**46**) and normantenine (**47**). The molecular structures of all of these compounds were determined from 1D and in some cases 2D (COSY, HSQC, HMBC and NOESY) NMR data and confirmed by IR and mass spectroscopy. Compounds **42**, **43**, **46** and **47** demonstrated significant activity against *L. mexicana* and *L. panamensis* (IC_{50} = 7, 13, 24 and 14 μM, respectively) and consequently, cytotoxicity testing against two mammalian cell lines was conducted with compounds **42-44**, **46** and **47** to explore their potential as treatments for leishmaniasis. Xylopine (**42**) and cryptodorine (**46**) showed 33-fold and 65-fold higher toxicity, respectively, towards *L. mexicana* than towards macrophages, the regular host cell of the *Leishmania* parasite. Activities of 172 μM and 21 μM, respectively, for lysicamine (**44**) and nornuciferine (**47**) compare favorably with the reported activities of 135-292 μM for the quinoline alkaloids isolated as antileishmaniasis agents from *Galipea longiflora* (Akedengue *et al.* 1999).



Xylopine (42) Nornuciferine (43) Lysicamine (44) Laudanosine (45) Cryptodorine (46) Normantenine (47)

Crude extracts of young and mature leaves from *Sarcorhachis naranjoana* (Piperaceae) collected in the National Park Altos de Campana (January-April 2002) showed activities of 35 μg/mL and 29 μg/mL respectively against *Leishmania* promastigotes. In this case, silica gel TLC analysis of the young and mature leaf extracts revealed no apparent difference between them. Bioassay-guided fractionation of the hexane soluble portion of the mature leaf extract chosen for further investigation led to the isolation of aegeline (**48**), which was the active constituent (3.5 μg/mL, 12 μM). Although this alkaloid has been isolated previously from *Aegle marmelos* (Patra *et al.* 1979, Patra *et al.* 1981, Sharma *et al.* 1981 and Govindachari and Premila 1983), *Fagara hyemalis* (Patra *et al.* 1981) and *Zanthoxylum ocumarense* (Della Casa de Marcano *et al.* 1972), no medicinally relevant activity for this compound has been reported to our knowledge.

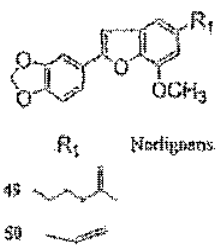


Aegeline (48)

Recently, we have isolated an unidentified alkaloid from a *Chomelia barbellata* (Rubiaceae), also collected in the National Park Altos de Campana (August 2002). Although the activity of this compound is still unknown, the young leaves of this species showed an activity of 17μg/mL against *Leishmania*. The structure elucidation of two alkaloids from *Tachigali versicolor* (Fabaceae), a species collected on the Barro Colorado Natural Monument during August and October 2002, is also underway. Both young and mature leaves showed significant leishmaniasis activity (18 and 11 μg/mL, respectively).

C.1.1.2. Compounds with Activity against Chagas' Disease (American Trypanosomiasis)

The methanol extract of young leaves of *Nectandra lineata* (Lauraceae) collected on the Barro Colorado Natural Monument (2001) displayed an IC_{50} ranging from 4-33 μg/mL in the Chagas' disease bioassay. Bioassay-guided fractionation led to the isolation of two norlignans (**49** and **50**), the structures of which were determined by standard NMR techniques, IR and EI mass spectroscopy. The norlignans have various reported activities, including antibacterial and antifungal (Mendonca *et al.* 2000) as well as *in vitro* vascular activities (Palazzino *et al.* 2000), and are also inhibitors of hyaluronidase (Jeong *et al.* 1999). Norlignan **50** was originally isolated from *Anaxagorea clavata* (Annonaceae) several years ago (Puentes De Diaz 1996). However, to our knowledge, its biomedical activity has never been assessed and thus its activity (IC_{50} = 18.6 μg/mL) against *T. cruzi* is novel. Compound **49**



R_1 Norlignans

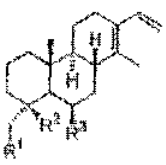
49

50

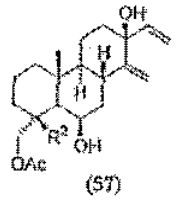
exhibited a lower bioactivity (IC_{50} = 40 μg/mL), which could be due to problems in rendering it completely soluble under the conditions of our bioassay.

Myrospermum frutescens (Fabaceae) was collected on the Barro Colorado Nature Monument in January 2001. Extracts of both young and mature leaves demonstrated significant and consistent activity against the extracellular form of *T. cruzi* (IC_{50} = 36 and 22 μg/mL, respectively). Because previous chemical investigations of *M. frutescens* were limited to the isolation of oils from its seeds (Vieira *et al.* 2002, Da Costa *et al.* 1996), we selected these extracts for further investigation. Bioassay-guided fractionation of the methanol soluble portion of the extract led to the isolation of seven new cassane diterpenoids (**51-57**) and one new cleistanthane (**58**). The molecular structures of these diterpenes were determined primarily by NMR techniques (including 1D selective ^{1}H decoupling, 1D NOE difference and 2D NOESY experiments), and confirmed by IR and mass spectroscopy.

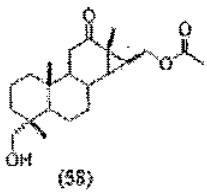
Compounds **51-57** were tested for activity in both the intra- and extra-cellular versions of the *T. cruzi* assay, and the results are presented in full in AP2. Compounds **55** and **57** were most active against the extracellular form of the parasite (16 and 11 μM, respectively), while compounds **51** and **52** were active against the more clinically relevant intracellular form of the parasite (17 and 16 μM, respectively). The majority of diterpenes reported to have activity against *T. cruzi* possess kaurane skeletons (Alves *et al.* 1995, Ragasa *et al.* 2002, Lyder *et al.* 1998). Compounds with the cassane skeletons, such as those described here, have been isolated from the genus *Caesalpinia* and are mostly furanoditerpenoids (Popoff *et al.* 1960, Gunstone *et al.* 1972, Jiang *et al.* 2001).



	R^1	R^2	R^3
51	OH	CH_3	H
52	OH	CH_3	OH
53	OH	CH_3	=O
54	OAc	CH_3	OAc
55	OAc	CH_3	OH
56	OAc	COOH	H

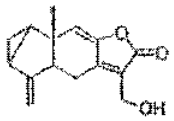


(57)



(58)

Two other plant extracts, from *Hedyosmum bonplandianum* and *Hampea appendiculata*, were selected for fractionation on the basis of their activity against the intracellular form of the parasite. In February 2002, *Hedyosmum bonplandianum* (Chloranthaceae) was collected in the National Park Altos de Campana. Both young and mature leaf extracts, which had similar TLC profiles, showed activities of 6 μg/mL in the Chagas' disease assay. The sesquiterpene lactone onoseriolide (**59**) was isolated as the major active constituent (363 mg) of the young leaf methanol fraction (IC_{50} <6 μg/mL), and its molecular structure was confirmed from NMR, IR and mass spectroscopy. Onoseriolide, isolated initially from *Onoseris albicans* (Bohlmann *et al.* 1980) and subsequently from *Hedyosmum brasiliense* (Trentin *et al.* 1999), is known to produce graded antinociception against acetic acid writhing and capsaicin-induced licking in mice, but no antitrypanosomal activity is reported. Bioassay-guided fractionation of crude extracts of young and mature leaves of *Hampea appendiculata*, (Fabaceae) collected from March to April 2002 in the National Park Chagres, has thus far led to the isolation of two compounds, whose structure elucidation is ongoing.



Onseriolide (59)

C.1.1.3. Compounds with Activity in the Malaria Assay

We are currently pursuing the isolation and characterization of metabolites from *Trattinickia aspera*, young and mature leaf extracts of which demonstrated significant activity in the malaria bioassay (14 and 18 μg/mL, respectively). Similarly, young and mature leaves of *Lafoensia punicifolia* demonstrated significant antimalarial activity (6 μg/mL) and these extracts are also being fractionated further. Both species were collected on the Barro Colorado Natural Monument in September 2001 and April 2002, respectively.

C.1.1.4. Training and Outreach

During the three years in which the Cubilla group has been associated with the current ICBG, 6 chemistry students have finished their undergraduate theses using the ICBG resources and 4 students have obtained training internships. Two research assistants have obtained scholarships and are currently pursuing advanced

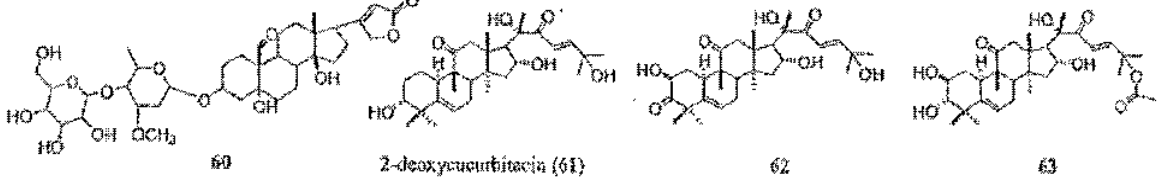
degrees abroad (Mexico and Spain). We are currently trying to secure similar opportunities for 2 other research assistants. Because Dr. Cubilla is the most active researcher in the Department of Chemistry, other professors regularly bring their classes to his laboratory for demonstrations of different chromatographic techniques. Recently we have participated in a scientific workshop organized by the Faculty of Natural Sciences and Technology of the University of Panama, where the process of separation and isolation of terrestrial plant natural products was demonstrated to 150 college level students, with an emphasis on the relationship between natural products and the conservation of the tropical forests. The members of AP3 have presented at conferences to chemistry professionals and Dr. Cubilla has lectured at high school and university levels. Dr. Cubilla's work has also been featured in several articles in the major Panamanian newspaper, *La Prensa* (see AP4, Section C.1).

C.1.2. Highlights of ICBG Research in the Gupta Laboratory

Dr. Mahabir Gupta, the present leader of AP2, heads the Center for Pharmacognistic Research on Panamanian Flora (CIFLORPAN) which has a well-established record in the field of terrestrial plant natural products. As mentioned above, Dr. Gupta's laboratories have been responsible for running the cell-based assays using 3 tumor cell lines [H-460 (lung), SF-268 (central nervous system) and MCF7 (breast)] obtained from the National Cancer Institute. This research group follows up investigations of extracts active in these anticancer assays, and also extracts active in an anti-HIV whole cell assay (noninfectious HIV strain $^{\Delta tat/Rev}$MC99), which is run by Professor Basilio Gómez, a microbiologist from the Department of Microbiology at the University of Panama. Highlights of their recent work are presented below.

C.1.2.1. Compounds with Activity in the Panama-based 3-Cell Cancer Assays

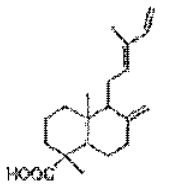
The extracts of young and mature leaves from *Maquira guianensis* (Moraceae) collected in Barro Colorado Natural Monument showed activity in the 3 tumor cell line assays (GI_{50} < 2 μg/mL in each case). Thus, the water liquid-liquid partition fraction from the mature leaf extract was fractionated by DIAION$^{(R)}$ gradient chromatography (water, water-methanol, methanol and ethyl acetate). The maquiroside derivative **60** was isolated as an active constituent (GI_{50} MCF-7: 0.033, H-460: 0.025, SF-268: 0.03 μg/mL) by C_{18} reversed-phase MPLC. Young and mature leaf extracts of *Sloanea zuliaensis* (Elaeocarpaceae) also showed activity in all 3 cancer cell lines and both kinds of leaves were recollected in Barro Colorado Natural Monument during 2001 and 2002. For both extracts, liquid-liquid partitioning produced active methanol partitions which yielded 3 active fractions after chromatography on silica gel. Three compounds were obtained upon further silica gel purification: 2-deoxycucurbitacin D (**61**, GI_{50}, MCF-7: 0.041, SF-268: 0.20 μg/mL) and **62** (GI_{50} MCF-7: 0.02, H-460: 0.013, SF-268 0.021 μg/mL) from both young and mature leaves, and **63** (GI_{50} MCF-7: 0.11, H-460: 0.065, SF-268 0.087 μg/mL) from mature leaves only.



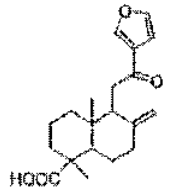
60 2-deoxycucurbitacin (**61**) **62** **63**

Bioassay-guided fractionation of the methanolic extract from young leaves of *Prioria copaifera* (Fabaceae), collected in the Barro Colorado Natural Monument in October 1998, yielded elliotinoic acid (**64**) as the active constituent (GI_{50}, MCF-7 = 4.8 μg/mL, H-460 = 3.4 μg/mL, SF-268 = 5.6 μg/mL). Elliotinoic acid was first reported from *Pinus ellioti* (Joye and Lawrence 1963), although its anticancer activity is unreported. Additionally, two other inactive and ubiquitous labdane diterpenes were isolated from *P. copaifera*: 12-oxolambertianic acid (**65**), originally isolated from *Brickellia glomerata* (Calderon *et al.* 1987), and hardwickiic acid (**66**), reported as an insecticidal agent from *Croton aromaticus* (*e.g.* Bandara *et al.* 1987). To the best of our knowledge, this is the first report on the natural products chemistry of the single *Prioria* species known.

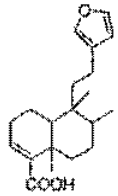
Liquid-liquid partitioning of the total methanolic extract from young leaves of *Adenaria floribunda* yielded active hexanes and methanol partitions. Further chromatographic purification of both partitions yielded 4 new compounds (**67-70**), of which 3 were active. The γ-pyrone **68** showed good activity (GI_{50}, MCF-7: 0.43 μg/mL, H-460: 0.29 μg/mL, SF-268: 0.39 μg/mL), while the activity of compounds **69** (GI_{50}, MCF-7: 1.2 μg/mL, H-460: 1.1 μg/mL, SF-268:1.4 μg/mL) and **70** (GI_{50} MCF-7 :>10 μg/mL, H-460: 9.3 μg/mL, SF-268: >10 μg/mL) was moderate to low. *Adenaria* is another genus for which there is no report of any chemical investigations in the literature.



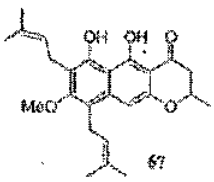
elliotinoic acid (64)



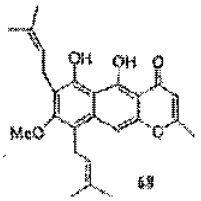
12-oxolambertianic acid (65)



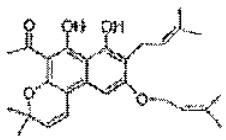
bardwickiic acid (66)



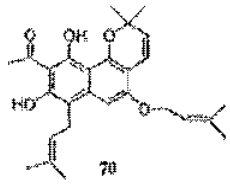
67



68

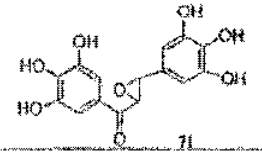


69



70

The extract of young leaves from *Mabea occidentalis* (Euphorbiacea) showed significant activity against the SF-268 cell line (GI_{50} = 5.5 μg/mL). Notably, the mature leaf extract showed no activity against the 3 cell lines. Working on the methanolic extract of the young leaves, we obtained pure white crystals (80 mg), of polyphenolic compound **71**, the structure of which was deduced from NMR data, and which showed moderate activity in all 3 cell lines (GI_{50}, MCF = 4.7 μg/mL, H-460= 4.1 μg/mL, SF-268 = 9.4 μg/mL). Other species of *Mabea* are reported to contain highly inflammatory ingenane diterpenes (as "chechum toxin", Brooks *et al.* 1990) and coumaroylglucosides (Barros *et al.* 1982).

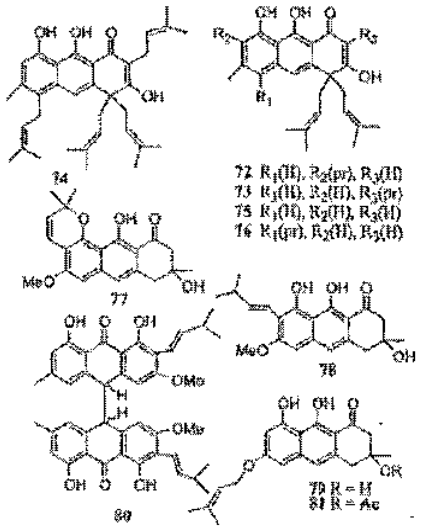


71

Some of our most exciting results have come from *Vismia* species. Upon testing against the 3 cancer cell lines, activity was found in the methanolic extracts of the young leaves of *Vismia macrophylla* (GI_{50} = 2.4, 4.4 and 3.7 μg/mL, respectively), *V. baccifera* (GI_{50} = <1.0, 1.3 and 2.3 μg/mL, respectively), and *V. jefensis* (GI_{50} = 0.5, 0.5 and 0.4 μg/mL, respectively). The fractionation process was carried out using both liquid-liquid partition and column chromatography. Five active compounds (Table 1) were isolated from the young leaves of *V. macrophylla:* ferruginin A (**72**), ferruginin B (**73**) and C (**74**), vismin (**75**) and harunganin (**76**). *V. baccifera* yielded the three active compounds vismione B (**77**), deacetylvismione A (**78**) and deacetylvismione H (**79**, Table 1). A new bianthrone (**80**), which we have named bivismiaquionone, and the known vismiaquinone (Goncalves *et al.* 1981) were also isolated as inactive constituents from the same species. Compounds **78** and **79** were re-isolated as the active constituents of *V. jefensis*. Vismione H (**81**) is reported to be a potent antimalarial agent against *Plasmodium falciparum* (IC_{50} 88 ng/mL, Francois *et al.* 1999). Deacetylvismione A (**78**) was submitted to Novartis and has been found to potently inhibit HCT-116 colon cancer cells (IC_{50} = 113 nM) as a Sirt-2 inhibitor. It also showed activity against H1299 lung carcinoma cells (IC_{50} = 312 nM). These exciting results and the mechanism of inhibition are described in AP2 (Section C.2.2.1). While the benzophenone and anthranoid chemistry of the *Vismia* genus is well-documented (Delle Monache 1985), none of the three species whose chemistry is described here appear in the chemical literature.

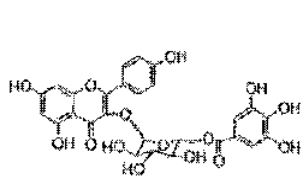
Table 1: Activity of *Vismia* metabolites against 3 cancer cell lines.

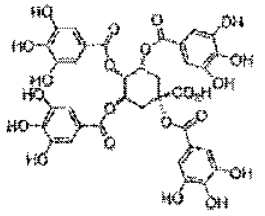
Compound	IC_{50} (ng/mL)		
	MCF-7	H-460	SF-268
Ferruginin A (72)	500	330	490
Ferruginin B (73)	500	500	400
Ferruginin C (74)	480	540	400
Vismin (75)	520	730	570
Haruganin (76)	600	640	600
Vismione B (77)	160	280	250
Deacetylvismione A (78)	180	190	220
Deacetylvismione H (79)	47	67	60



C.1.2.2. Compounds with Activity in the Anti-HIV Assay

The anti-HIV activity demonstrated by the total methanolic extract of the young leaves of *Hiraea reclinata*, prompted us to purify the anti-HIV active constituent(s) of this plant. Leaves were collected from two National Parks in Central Panama (2001). Again, the genus *Hiraea* does not appear in the chemical literature and this species was therefore a prime target for further investigation. Liquid-liquid partitioning and column chromatography of the active crude fraction from the methanol extract of *H. reclinata* afforded the new metabolite kaempferol 3-*O*-(6''-galloyl)-β-D-galactopyranoside (82), as well as known compounds hyperin 6''-gallate, 1,3,4,5-tetragalloylquinic acid (83), vitexin 2''-rhamnoside, orientin 2''-rhamnoside, isovitexin 2''-rhamnoside, and isoorientin 2''-rhamnoside. However, when these metabolites were evaluated for their anti-HIV activity, only **83** showed consistent activity (IC_{50} 8.3 μg/mL, Protection = 55.3%). Similar anti-HIV activity for **83** has been reported previously following the isolation of **83** from a commercial tannic acid (Nishizawa *et al.* 1989).


kaempferol (82)


1,3,4,5-tetra-O-galloylquinic acid (83)

C.2. Preliminary Results for Marine Algae and Cyanobacteria

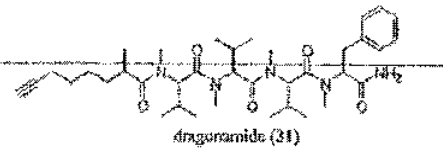
The Gerwick research group at Oregon State University has recently embarked on an unsupported collaboration with the Panama ICBG. Over the last 14 months of active effort to develop a marine component to this already highly successful ICBG project, we have made tangible and productive progress in efforts to 1) demonstrate the biological and chemical richness Panama's marine flora, 2) to show the effectiveness of bi-directional transmission of plant and algal samples for structural chemical studies as well as for biomedical testing, 3) to test and improve bi-directional information transfer between Oregon and Panama, and 4) to establish a positively-focused drug discovery team involving scientists from all of the applicant organizations. Chemical investigations of marine cyanobacteria carried out in the last five months have resulted in the isolation of several new and bioactive compounds, some of which are active in the tropical disease bioassays being conducted in Panama. A relatively large number of compounds isolated by the 2 Panama-based laboratories have been analyzed by detailed NMR studies, mass spectrometry, and polarimetry in the Oregon laboratories, helping to complete the delineation of their chemical structures. In a few cases, the structure elucidation of these terrestrial plant derived natural products has been a collaborative effort, and has resulted in research accomplishments which will be under joint authorship between Oregon and Panama scientists. Thus,

stimulating preliminary interactions between the current ICBG program and Oregon State University bode well for the productive addition of this marine component to the Panama ICBG in its proposed renewal period.

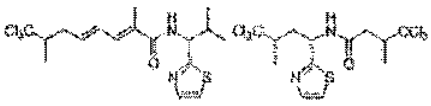
C.2.1. Evaluation of Marine Algal Biodiversity and Preliminary Collections

An initial trip to Panama in January 2002 allowed the P.I. to assess the high biological diversity around the Pacific island of Coiba, which is the principal conservation project of this ICBG application (see AP4). A rich subtropical macroalgal flora was observed with many members of the chemically-rich Dictyotaceae in evidence. Tropical and sub-tropical green and red algae were also present, although less conspicuously. Unidentified marine cyanobacteria were found as tufts and small mats in several shallow water habitats. In September 2002, algal collections made by Oregon participants while using SCUBA and snorkeling near to Portobelo presented an opportunity to assess marine algal biodiversity of this Caribbean site as well. A total of sixteen different algal samples were collected, as described in AP1 (Section C.5.1). Of these samples, three were macroalgae collected in quantities large enough for chemical investigation; four small scale collections of cyanobacteria were made for chemical investigation and samples for culture were taken from three of these; six cyanobacterial specimens were only collected as culture samples. The island of Bocas del Toro was also visited where pristine mangroves proved to be the site of prolific cyanobacterial life and we anticipate large scale collections from this area in the future. Four algal samples from Bocas del Toro (one brown macroalga, and three *Lyngbya* cyanobacterial species) were received in Oregon in November 2002 as a result of preliminary collection efforts made by Panama participants.

Organic extracts of the largest cyanobacterial sample from each site (a brown and a red *L. majuscula*) were generated following the standard AP1 protocol for cyanobacterial extraction (Appendix 9) and were found to be toxic at 100 ppm and 10 ppm in our standard brine shrimp toxicity assay used to guide extract fractionation.



dragonamide (31)



herbamide B (32) dysidenthiazole (34)

AP1 fractionation by normal-phase vacuum-liquid chromatography to give nine 'prefractions' was performed according to the standard pre-fractionation protocol for organic extracts from marine algae (Appendix 9). The most active brine shrimp fractions from each extract were then targeted for further purification using HPLC. Thus, two fractions (80 % EtOAc-hexanes and 100 % EtOAc) from the brown Portobelo *L. majuscula* extract and three fractions (40 % EtOAc-hexanes, 100 % EtOAc and 25 % methanol-EtOAc) from the red Bocas del Toro *L. majuscula* extract were fractionated by HPLC. To date, the red *L. majuscula* has yielded a complex series of intriguing compounds including two unidentified, novel peptides and the known substances dragonamide (**31**), herbamide B (**32**) and dysidenthiazole (**84**). These compounds, and a semi-pure fraction containing similar trichlorinated compounds, have been submitted to AP2 for testing against malaria, leishmaniasis and Chagas' disease.

Five crude extracts are currently undergoing testing in AP2. Thus far, one of these samples, an unidentified *Lyngbya* species, displays promising activity in the antimalarial bioassay, and therefore is likely to be the next target for AP3 bioassay-guided fractionation at Oregon State University.

C.2.2. Collaborative OSU-Panama Analytical Data Acquisition for Current ICBG Projects

Oregon State University has provided and is continuing to provide mass spectroscopic support to the current ICBG grant (28 low resolution and 14 high resolution pure compound samples to date, see Group Plan Figure 4). That these forerunner samples have been easily and efficiently transferred between Panama and Oregon State University portends well for the effectiveness of the Panama-Oregon logistic. We have also acquired optical rotation data on 10 terrestrial plant products for the Cubilla and Gupta research groups since August 2002. Returning MS data in electronic format to our Panamanian colleagues has insured their acquisition of these materials in a timely fashion. Providing this analytical support to the current ICBG, as well as working out the logistics for compound shipment and data return, in advance of our formally participating in this ICBG project, helps to insure an efficient and effective interaction between the collaborating AP's in this anticipated renewal of the program.

In cases where structurally complex, high molecular weight terrestrial natural products are isolated, the 300 MHz field of the spectrometer housed at STRI may be insufficient to provide the spectral resolution necessary to permit structure elucidation. We anticipate that the acquisition of NMR data for these compounds on the DRX 600 spectrometer housed at OSU will provide extended resources for the Panamanian chemists. The enhanced capabilities of the DRX 600 may also facilitate the assignment of stereochemistry in problematic compounds isolated and structurally assigned in Panama.

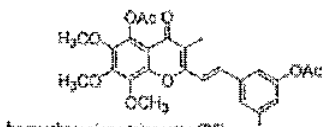
C.2.3. Training Opportunities Through Natural Products Investigations

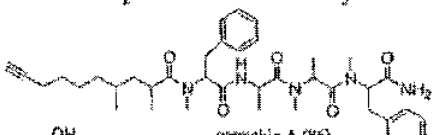
In September 2002, program participants from Panama and Oregon met in Panama. Interactions included training opportunities provided by discussion of the spectroscopic data for novel compounds isolated from terrestrial plants by the Panama participants (Cubilla), and possible modifications to the procedures for producing and screening chromatographic fractions to generate higher 'hit' rates for bioactive compounds. These interactions have generated ongoing dialogue between the Panama and Oregon groups. Additionally, the Bruker Avance 300 NMR spectrometer located at STRI headquarters in Panama was assessed by the Oregon participants with the goal of improving its efficient use by modifications in its operation and data processing (*e.g.* networking it, and acquiring software to permit remote data analysis at the University of Panama). Plans are in place to bring Panama participants to Oregon to gain further experience in NMR data acquisition and processing by using the NMR facilities at Oregon State University while interacting with a cross-section of NMR spectroscopists on the OSU campus.

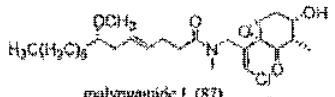
C.2.4. Further Collaborative Interactions between the Gerwick Group and the Current ICBG

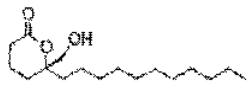
C.2.4.1. Testing Marine Natural Products in Tropical Disease Assays

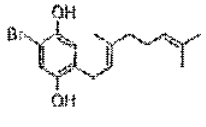
Seven pure marine algal natural products selected to represent a range of bioactive chemotypes from the Gerwick laboratories' pure compound library have been tested in the tropical disease bioassays run by AP2 in Panama. Hormothamnione triacetate (**85**) from the marine cryptophyte *Chrysophaeum taylori*, the lipopeptides carmabin A (**86**) and malyngamide I (**87**) from Caribbean and Okinawan collections of *Lyngbya majuscula* respectively, the lactone tanikolide (**88**) from a Madagascan collection of *L. majuscula*, cymopol (**89**) from the calcareous green alga *Cynopolia barbata*, an unreported indole (**90**) from a species of *Martensia* red alga and the halogenated chamigrane sesquiterpene, obtusol (**91**) from the red alga *Laurencia obtusa* were tested for activity against malaria, Chagas' disease and leishmaniasis (Table 2). Obtusol was by far the most cytotoxic metabolite and was also the most bioactive compound in general, with its most notable activity in the intracellular Chagas' disease assays against HFF and VERO cells. Notably, malyngamide I showed significant activity in both the intracellular and extracellular Chagas' disease assays, being the most active of the seven compounds in the extracellular assay. Cymopol showed the best activity in the antimalarial assay, while tanikolide showed promising activity in the assay against leishmaniasis. Plans for further investigations of the most promising activities shown by these compounds are currently under discussion.

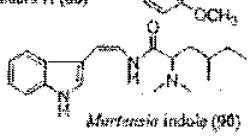












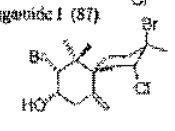


Table 2. Results from the Screening of Oregon Pure Compounds in Panama-based Tropical Disease Bioassays

Compound	Code	Assay (IC_{50} µg/mL)						Cytotoxicity (µg/mL)
		Leishmaniasis		Chagas' disease			Malaria	
				Intracellular in HFF	Intracellular in VERO	Extracellular		
Hormothamnion triacetate (85)	SW-66	> 300	> 300	29.09	17.44	> 50	11	10.51
Carmabin (86)	SW-107	> 300	> 300	21.10	5.08	15.52	> 40	14.37
malyngamide I (87)	SW-86	35	52	6.14	12.90	8.95	11	15.62
Tanikolide (88)	SW-119	3	19	29.78	22.10	24.04	12	14.48
Cynopol (89)	SW-102	124	53	25.37	2.27	10.6	5	21.80
Martensia indole (90)	SW-78	163	263	39.31	42.69	> 50	17	19.44
Obtusol (91)	SW-56	58	32	< 2	< 2	26.39	13	< 0.04

C.2.4.2. Collaboration Planning Meetings

This ICBG team has met several times to establish close working relationships between all of the team members, to plan for the renewal application, and to develop a work plan for the proposed new ICBG program. Two of these meetings were held in Panama City, Panama in January and September 2002, and a third was held in Salt Lake City in June 2002. On-going projects were reviewed and evaluated, and the strengths of the existing program identified. New dimensions of this ICBG were developed through work sessions involving all of the ICBG participants, and goals were established for the anticipated renewal period.

C.2.4.3. Preparation of this ICBG Renewal Application

The construction of this competitive renewal application and research plan has required extensive communication and effort on the part of all associated personnel. The timely completion of this application is testament to the growing sense of cohesion between the new ICBG participants. Communication has been most frequent by email, using a "list-serve" approach such that information is copied to all participants in an automatic fashion. Numerous phone calls have taken place on a regular basis during the course of the preparation and review of this document, including two conference calls involving all key participants. These communications have generated and sustained a momentum of efficient responses between participants in the compilation and exchange of written materials.

D. RESEARCH DESIGN AND METHODS

D.1. Overview of the Research Plan for Associate Program 3

The chart below (Figure 1) summarizes the organization and interactions among the four Associate Programs (AP's) of this Panama ICBG proposal, which are described in detail in the Group Plan. Associate Program 3 will focus on chemical investigations of first tier chromatographic fractions ("prefractions") and extracts generated by AP1, which show activity in the tropical disease, anticancer and agrochemical bioassays of AP2, with a goal to structurally characterize novel, biologically active natural products from terrestrial plants and marine macroalgae and cyanobacteria. Investigations of terrestrial plants and some macroalgae will be carried out at the University of Panama in the Cubilla laboratories, while the purification of marine cyanobacterial and some macroalgal natural products will be accomplished in the Gerwick laboratories at Oregon State University (OSU; Figures 2 and 3). Both research groups have access to comprehensive chromatographic and spectroscopic facilities, and protocols are in place to collaborate in the acquisition and analysis of data, as is necessary. In all cases, AP3 fractionations will be bioassay-guided, with derivative fractions being sent to AP1 laboratories at STRI in Panama for distribution to the appropriate tropical disease or cell-based anticancer assays run in the Ortega laboratories, mechanism-based assays at Novartis (see letter of support, Appendix 3) or the agrochemical assays at Dow AgroSciences (see letter of support, Appendix 3).

Crude prefractions active in the mechanism-based cancer assays at Novartis Institute for Biomedical Research (NIBR) will be subfractionated by AP1 technicians. Only the structure elucidation of pure bioactives from these Novartis samples will be undertaken by AP3 chemists. Panamanian chemists of AP3 will participate in training in general isolation and structural elucidation techniques at OSU, and at least once a year OSU chemists will lead NMR workshops at the University of Panama and STRI in Panama. In line with overall ICBG goals, we will participate in AP4-coordinated activities to give workshops, public presentations, and scientific lectures.

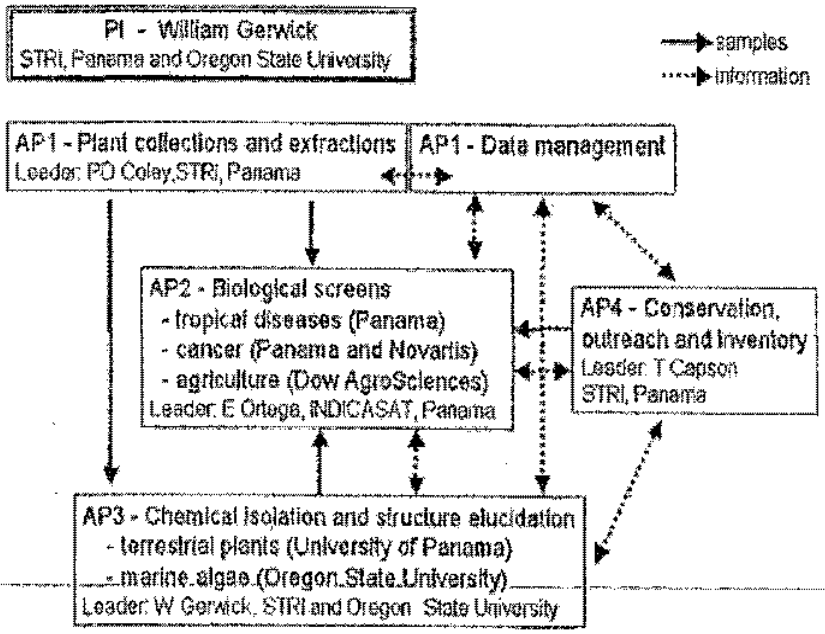


Figure 1: Overview of the Associate Program interactions and interrelationships in this ICBG proposal, including sample and data transmissions.

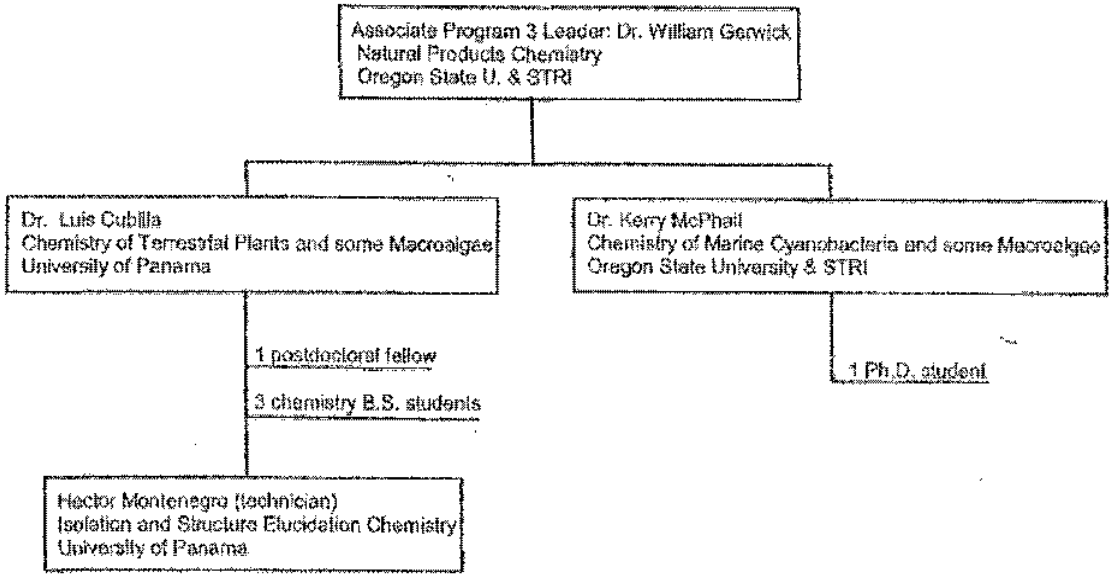


Figure 2: Organizational chart of key personnel in Associate Program 3 and their research areas and affiliations.

D.2. Research Plan to Accomplish Specific Aims

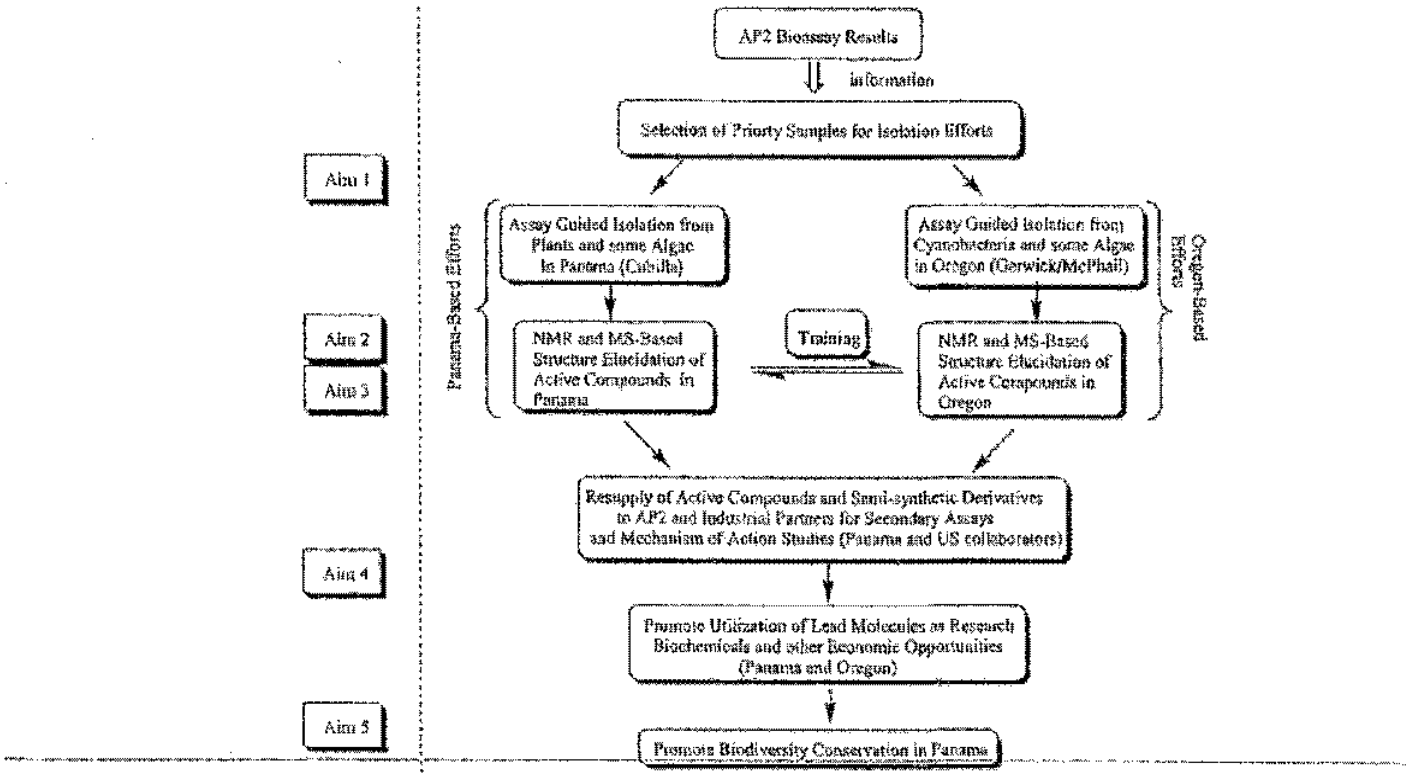


Figure 3: Flow chart of Associate Program 3 activities as they relate to the aims of this project.

D.2.1. Aim 1 - To use bioassay results from AP2 to select and then direct the chemical isolation of active constituents from pre-fractionated extracts or extracts as appropriate

Generally, a larger number of species is collected than can be processed immediately by AP3. In addition, there are many previously described natural products from terrestrial plants, and a significant number from marine macroalgae and *Lyngbya* species of cyanobacteria. Thus, in order to optimize the possibility of obtaining novel and bioactive compounds, candidate species for further fractionation will be selected according to the following criteria (also see AP2, Figure 12). Species with poorly documented chemistry or bioactivity will be given greater priority than those whose natural products chemistry is well known. Other considerations are the specificity of the extract activity to the relevant bioassay, and whether the source organism is endemic to Panama. Bioassays which identify an AP1 prefraction as active will be used to guide subsequent fractionation.

De-replication of known and nuisance substances will occur by three overlapping strategies. Firstly, the taxonomic identities of plants and algae will be used, in conjunction with various literature databases including Napralert, SciFinder, and MarineLit, to identify known constituents of a given plant species. This information will be used in assigning the priority with which an extract is pursued. Moreover, it will be consulted during fractionation so as to identify known or nuisance compounds at the earliest stage possible (*e.g.* by comparison with the ^{1}H NMR of crude fractions). Secondly, a growing library of Diode-Array HPLC information is being generated in Panama for terrestrial plants and in Oregon for algae and cyanobacteria, and these will be utilized to de-replicate substances previously encountered by these respective laboratories. The Oracle® database in the Millennium-32 HPLC software can associate chemical structures with chromatographic peaks, and this can be searched by a computer algorithm in a semi-automatic process. Thirdly, in the Oregon laboratories, a substantial number of previously encountered marine cyanobacterial and algal substances are present as a searchable library on our HP Gas Chromatograph-Mass Spectrometer (GC-MS). Active non-polar fractions of

algal and cyanobacterial extracts will be profiled by GC-MS, and previously encountered compounds identified by the mass spectral pattern matching software. Additionally, mass spectra of peaks in these active fractions will be searched against the commercial library of 54,000 known substances that is provided in the MS software. Lastly, the Oregon laboratories will soon have regular access to a new Liquid-Chromatograph-Mass Spectrometer (LC-MS) and anticipate integrating this into their de-replication strategy. Purification strategies for terrestrial plant fractions are tailored to take account of the complexity of the fractions. While it is desirable to go directly to HPLC using a radial compression column (up to 250 mg per injection) or Medium Pressure Liquid Chromatography (MPLC), for more complex fractions flash chromatography or column chromatography (normal-phase silica gel, C_{18} silica gel, ionic interchange resins or sephadex gel filtration) will be carried out before HPLC. Preparative Thin Layer Chromatography (TLC) and HPLC at preparative or semi-preparative scales are other useful techniques available for the purification of terrestrial plant fractions. Derivative fractions will be sent, in parallel with the parent fraction, to the AP1 extraction and prefractionation laboratory at STRI in Panama, for distribution to the appropriate bioassay laboratories at INDICASAT in Panama, or at Novartis or Dow AgroSciences in the USA. Samples will be submitted to the tropical disease and 3-cell cancer bioassays in glass vials, while 96-well "Regular-Well" plates of samples will be supplied to Novartis and 96-well "Deep-Well" plates of samples will be sent to Dow AgroSciences. The amount of sample will vary according to the size of the fraction. Generally, 2 mg of fractions larger than 10 mg, and 10% of smaller fractions will be sent for bioactivity testing.

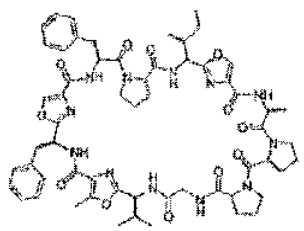
In our experience with marine macroalgae and cyanobacteria, it is often possible to go directly to HPLC with the reduced complexity AP1 prefractions, a sequence which is highly desirable for conserving limited materials. However, we favor reversed-phase HPLC at this stage because there is a greater range of stationary materials available and it is a more efficient separation technique. Because the organic extract first tier "prefractions" result from fractionation on normal-phase silica gel, they require pre-HPLC filtering over C_{18} Solid Phase Extraction (SPE) cartridges. We find it advantageous to collect four to five secondary fractions, eluted with mixtures of water in methanol, at this intervening step. Since we have used prefractionation on silica gel followed by SPE on C_{18} over the past two years, we have developed considerable in-house knowledge of the most effective chromatographic approaches using this general paradigm for fractionation. For example, prefraction D compounds from normal phase VLC eluted with 40% ethyl acetate-hexanes require secondary fractionation on C_{18} SPE and these are well separated by HPLC on C_{12} in acetonitrile-water mixtures, whereas prefractions F (80% ethyl acetate-hexanes) and G (100% ethyl acetate) require no C_{18} SPE and are better approached using HPLC on C_{18} columns in methanol-water mixtures. While samples are first profiled by gradient elution using a Photo Diode Array (PDA) detector to establish optimal separation conditions, multiple related compounds often co-elute under the relatively standard conditions used to achieve best separation of the crude prefraction. To facilitate these separations, we have an array of more specialized analytical columns with, for example, phenyl-hexyl, Lichrosorb diol and amino stationary phases. A D-penicillamine column is also available for chiral analyses of amino acid residues from hydrolyzed peptidic natural products. Marine samples from Oregon will be prepared as described above and will be sent to AP1 laboratories at STRI by FedEx shipment.

D.2.2. Aim 2 - To apply state-of-the-art structure elucidation technology, featuring 2D-NMR and various ionization modes of Mass Spectrometry, to rapidly and efficiently determine the structures of new and bioactive compounds

Terrestrial plant and any macroalgal metabolites isolated at the University of Panama will be effectively characterized using the equipment and infrastructure existing at the University of Panama and INDICASAT (Panama) with the additional support of the facilities at Oregon State University. Metabolites will be routinely characterized by NMR spectroscopy on the Bruker Avance 300 spectrometer located at STRI. Where greater chemical shift resolution is required, compound data will be acquired on the JEOL 400 housed at INDICASAT (available August 2003) or if necessary on the Bruker DRX 600 at Oregon State University. Mass spectral data will also be acquired at either INDICASAT or Oregon State University.

At Oregon State University, new and bioactive marine algal and cyanobacterial natural products will be characterized by ultraviolet and Fourier-transform infrared spectrophotometry, optical rotation and circular

dichroism, mass spectrometry, and high resolution NMR spectroscopy (Bruker Avance DRX 600, DPX 400 and DPX 300). To help facilitate the rapid structure elucidation of newly isolated natural products, we have developed and described several new NMR pulse sequences. For these studies, we utilize our Bruker DRX 600 MHz spectrometer, equipped with triple axis gradients and a waveform generator for selective excitation. Recently, we have encountered numerous examples of structurally-remarkable cyclic peptides from marine cyanobacteria. The structure elucidation of these peptides is made challenging by several considerations, including 1) they are generally of large molecular size (700 to 1500 MW), 2) they commonly possess multiple aliphatic amino acid residues that results in considerable signal degeneracy in the region 0.7 to 2.5 ppm, 3) marine cyanobacterial cyclic peptides frequently possess one or more N-methyl amide groups which results in the molecule existing in solution as a mixture of tertiary amide conformers, 4) they are often isolated in small quantity from marine cyanobacteria (0.2 to 2 mg), 5) they often contain many sites of stereochemistry that are remote from one another, and 6) they frequently contain unusual functional groups or unusual constellations of atoms for which there is little spectroscopic precedent. Therefore, we have needed new tools by which to solve their structures. In addition to application of various published NMR experiments, such as HSQC-TOCSY, 1D DPFGSE NOE, GHMBC, GHNMBC, 1D HMBC and GS-ECOSY, we have created several new experiments. To help to define the contiguous spin-systems of the constitutive amino acids, we have developed the ^{1}H-^{15}N PEP-HSQC-TOCSY experiment (Williamson *et al.* 1999a). In addition, we have described a GHNMBC experiment for looking at the interaction between ^{1}H and their long-range scalar-coupled ^{15}N nuclei, for both structural information as well as investigation into biosynthetic pathways via isotopically-labeled precursors (Williamson *et al.* 1999b). To facilitate the use of heteronuclear coupling constants for determining stereochemical features in new molecules of interest, we have developed both 1D and 2D HSQMBC pulse sequences (Williamson *et al.* 2000a). These experiments allow the accurate measurement of all $^{2,3}J_{CH}$ coupling constants, and facilitate the use of *J*-based configurational analysis (Williamson *et al.* 2002). Recently, we have developed and described a new variant of the HMBC, named the *J*-IMPEACH-MBC (Williamson *et al.* 2001). This experiment allows qualitative determination of long-range heteronuclear coupling constants in the F_1 dimension and retains the ability to make quantitative measurements of this coupling interaction in F_2. Another experiment recently developed by our laboratory for stereochemical investigations is a derivation of the BIRD pulse sequence. This new experiment allows for a clear distinction between *cis* and *trans* olefins even if they contain isochronous proton resonances (Williamson *et al.* 2000b). Of the NMR experiments from the literature, we have found the 1D HMBC experiment to be highly useful in the structure elucidation of complex cyanobacterial depsipeptides and macrolides. This pulse program allows one to target problematic connectivities in a highly selective fashion and observe long-range ^{1}H-^{13}C interactions with great sensitivity. Our success with this experiment is illustrated in the structure elucidation of wewakazole (92), a cyclic depsipeptide from *L. majuscula* (Nogle *et al.* 2003), which appears in a recent Chemical and Engineering News report (C&EN, Jan 13 2003, p35). In this case, long range interactions indicated in the 1D HMBC spectrum enabled unequivocal orientation of a tripeptide fragment within the dodecapeptide macrocycle.



wewakazole (92)

These spectroscopic methods will be used in conjunction with standard natural product degradation, derivative formation, and synthetic schemes aimed at providing unequivocal structural proofs. Finally, X-ray crystallography will be used to determine the structures of crystalline metabolites (or derivatives) which are exceptionally complex. These serives are available in the Departments of Chemistry, and Biochemistry and Biophysics, at Oregon State University, or through collaboration with a number of X-ray crystallographers with whom we have worked in the past.

D.2.3. Aim 3 - To train students at various levels from Panama and the USA in the multidisciplinary science of modern natural products chemistry

One of the most important elements of this ICBG project is the transfer of technology with the goal of contributing to an elevated level of education in Panama. This project expects Bachelor degree students in Panama to carry out laboratory research for up to 2 years and to prepare a dissertation on their research.

Students both in Panama and in the US will also participate in these modern natural products investigations through reciprocal exchanges and participation in graduate programs in Pharmacy, Chemistry or Biochemistry at Oregon State University. In addition, we will continue to provide Panamanian students with the opportunity to conduct their BS thesis research in Dr. Cubilla's laboratory in Panama. His laboratory is the only one in the Departments of Chemistry or Biochemistry wherein research is actively being pursued. Based on experiences of the current ICBG program, we anticipate 2-3 students per year will take advantage of this unique opportunity. Once a year, Dr. Cubilla will visit OSU to gain experience using the NMR facilities there and observe general isolation and structural elucidation techniques at OSU, and at least once a year OSU chemists will participate in workshops at the University of Panama and STRI in Panama. Frequent use of internet and email based technologies, in addition to telephone conferencing, will facilitate continuous communication between members of AP3, as well as between AP3 and the other AP's.

D.2.4. Aim 4 - To work with the other AP programs and our industrial partners to advance lead molecules into the drug development process, and to seek their application as research biochemicals

Newly discovered bioactive molecules will be further evaluated as to mechanism of action, potency, stability, and *in vivo* efficacy by our various collaborators within the ICBG. Key to this development process is the re-isolation of larger quantities of materials as well as the production of simple analogs by semisynthesis. AP3 chemists will re-isolate active natural products as necessary using scaled-up collections or cultures, extractions, and chromatographic procedures. In fact, the large-scale isolation of alkaloids for newly instigated AP2 *in vivo* mouse assays is already in progress in the Cubilla laboratories. While it is a widely held perception that re-supply or scaled-up supply of a natural product can be problematic, to date our laboratories have been highly successful in providing up to multigram quantities of pure natural products to industrial collaborators as well as to vendors of specialty research biochemicals (Molecular Probes – curacin A, debromoaplysiatoxin; Sigma – scytonemin). The combination of accurate collection records, culture samples taken at the time of collection of cyanobacterial species, and the stability of production of secondary metabolites in laboratory cultures of cyanobacteria, has allowed us to be successful in this pursuit several times. For example, following up on results from a mechanism-based assay in another project, we were able to provide 1.5 g of stypoldione (Gerwick and Fenical 1981) as well as 1.5 g of a prodrug analog to our collaborators at Novartis. On another occasion, we provided 2 g of hectochlorin (Marquez *et al.* 2002) to an industrial partner that was exploring the medicinally relevant properties of this molecule. In general, the accurate collection records associated with our terrestrial plant samples allow reliable recollections. Samples of the natural products as well as derivatives will be supplied as necessary to AP2 and our collaborating industrial partners, and will aid and foster the development of these molecules as lead compounds.

D.2.5. Aim 5 - To promote our discoveries of valuable bioactive molecules into the economic sector, and to use these discoveries as tools for justifying and directing conservation efforts in Panama

Scientific publications and presentations, along with the provision of new compounds to collaborators and commercial vendors of research biochemicals, will stimulate development efforts in Panama. Integration of research findings with the activities and goals of AP4 will also foster conservation efforts in Panama. For example, our discovery of a marine cyanobacterium in Curaçao in 1993 which produced a potent anticancer lead compound, curacin A (Gerwick *et al.* 1994), helped to defeat an attempt by a development group to change a protected classification for a sensitive mangrove bay habitat so that they could build a tourist facility. Additionally, in tandem with the pervasive poverty of countries in tropical regions, a general lack of education as to the value and importance of environmental conservation contributes enormously to the destruction of tropical ecosystems. It is our goal that the AP3 participants will annually contribute at least 12 oral presentations to the general public, or in scientific meetings or workshops, in order to inform the Panamanian population about the ICBG project, with an emphasis on the economic and scientific value of chemical, biochemical and biological diversity.

E. HUMAN SUBJECTS
None

F. VERTEBRATE ANIMALS
None

G. LITERATURE CITED

Adamczeski M, Quinoa E and P Crews. 1989. Novel sponge-derived amino acids. 5. Structures, stereochemistry, and synthesis of several new heterocycles. *J. Amer. Chem. Soc.* 111: 647-654.

Akendengue B, Ngou-Milama E, Laurens A and R. Hocquemiller 1999. Recent advances in the fight against leishmaniasis with natural products. *Parasite* 6: 3-8.

Alves TMA, Chaves PG, Santos LMST, Nagem TJ, Murta SMF, Ceravolo IP, Romanha AJ and CL Zani. 1995. A diterpene from *Mikania obtusata* active on *Trypanosoma cruzi*. *Planta Med.* 61: 85-87.

American Chemical Society. Cancer Facts and Figures 2003. http://www.cancer.org/downloads/STT/CAFF2003PWSecured.pdf

Ang KKH, Holmes MJ, Higa T, Hamann MT and UAK Kara. 2000. In vivo antimalarial activity of the β-carboline alkaloid manzamine A. Antimicrobial Agents and Chemotherapy 44: 1645-1649.

Bai R, Pettit GR and E Hamel. 1990. Dolastatin 10, a powerful cytostatic peptide derived from a marine animal. Inhibition of tubulin polymerization mediated through the vinca alkaloid binding domain. *Biochem. Pharm.* 39: 1941-1949.

Bakhtiar R and RW Nelson. 2000. Electrospray ionization and matrix-assisted laser desorption ionization mass spectrometry. Emerging technologies in biomedical sciences. *Biochem. Pharm.* 59: 891-905.

Balick MJ, Elisabetsky E and SA Laird. (Eds). 1996. *Medicinal Resources of the Tropical Forest: Biodiversity and Its Importance to Human Health*. Columbia Univ Press, Irvington-on-Hudson, N. Y. 440 p.

Bandara BMR, Wimalasiri, WR and KANP Bandara. 1987. Isolation and insecticidal activity of (-)-harwickiic acid from *Croton aromaticus*. *Planta medica* 53: 575.

Barros DAD, De Alvarenga MA, Gottlieb OR and HE Gottlieb. 1982. The chemistry of Brazilian Euphorbiaceae. Part 4. Naringenin coumaroylglucosides from *Mabea caudata*. *Phytochemistry* 21: 2107-2109.

Belinsky SA, Nikula KJ, Baylin SB and JP Issa. 1996. Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer. *Proc. Natl. Acad. Sci. USA* 93: 4045-4050.

Blagosklonny MV, Robey R, Sackett DL, Du L, Traganos F, Darzynkiewicz Z, Fojo T and SE Bates. 2002. Histone deacetylase inhibitors all induce p21 but differentially cause tubulin acetylation, mitotic arrest, and cytotoxicity. *Molecular Cancer Therapeutics* 1: 937-941.

Blokhin AV, Yoo H-D, Geralds RS, Nagle DG, Gerwick, WH and E Hamel. 1995. Characterization of the interaction of the marine cyanobacterial natural product curacin A with the colchicine site of tubulin and initial structure-activity studies with analogs. *Mol. Pharm.* 48: 523-531.

Bohlmann F, Zdero C, King RM and H Robinson. 1980. Naturally occurring terpene derivatives. Part 254. Onoseriolide, a new sesquiterpene lactone from *Onoseris albicans*. *Phytochemistry* 19: 689-691.

Brito I, Cueto M, Diaz-Marrero AR, Darias J and A San Martin. 2002. Oxachamigrenes, new halogenated sesquiterpenes from Laurencia obtusa. *J. Nat. Prod.* 65: 946-948.

Brooks G, Evans AT, Markby DP, Harrison ME, Baldwin MA and FJ Evans. 1990. An ingenane diterpene from Belizian *Mabea excelsa*. *Phytochemistry* 29: 1615-1617.

Caballero-George C, Vanderheyden PM, Solis PN, Pieters L, Shahat AA, Gupta MP, Vauquelin G and AJ Vlietinck. 2001. Biological screening of selected medicinal Panamanian plants by radioligand-binding techniques. *Phytomedicine* 8: 59-70.

Calderon JS, Quijano L, Gomez-Garibay F, Moran M and T Rios. 1987. Labdane diterpenes from *Brickellia glomerata*. *Phytochemistry* 26: 2639-2641.

Carmeli S, Moore RE and GML Patterson. 1990. Tolytoxin and new scytophycins from three species of *Scytonema*. *J. Nat. Prod.* 53: 1533-1542.

Cepleanu F, Obtani K, Hamburger M, Gupta MP, Solis P and K. Hostettmann. 1993. Novel acetogenins from

the leaves of *Annona purpurea*. *Helv. Chim. Acta* 76: 1379-1388.
Clark WD and P Crews. 1995. Novel sponge derived amino acids. 17. A novel chlorinated ketide amino acid, herbamide A, from the marine sponge *Dysidea herbacea*. *Tetrahedron Lett.* 36: 1185-1188.
Cohen LH and AI Gusev. 2002. Small molecule analysis by MALDI mass spectrometry. *Analytical and Bioanalytical Chemistry* 373: 571-586.
Coley PD and TM Aide. 1991. A comparison of herbivory and plant defenses in temperate and tropical broad-leaved forests. In: *Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions*. Price PW, Lewinsohn TM, Fernandes GW, Benson WW. (Eds). John Wiley & Sons, N.Y. pp. 25-49.
Coley PD and JA Barone. 1996. Herbivory and plant defenses in tropical forests. *Annual Review of Ecology & Systematics* 27: 305-335.
Coley PD and TA Kursar. 1996. Anti-herbivore defenses of young tropical leaves: physiological constraints and ecological tradeoffs. In: *Tropical Forest Plant Ecophysiology*. Smith AP, Mulkey SS, Chazdon RL. (Eds). Chapman and Hall, N.Y. pp 305-336.
Cox PA and MJ Balick. 1994. The ethnobotanical approach to drug discovery. *Scientific American* 270: 82-87.
Cragg GM and DJ Newman. 2000. Antineoplastic agents from natural sources: achievements and future directions. *Expert Opinion on Investigational Drugs* 9: 2783-2797.
Crews P and LM Hunter. 1993. The search for antiparasitic agents from marine animals. In: *Mar. Biotechnol.* Attaway DH, Zaborsky OR. (Eds). Plenum, New York, N.Y. Vol. 1: 343-389.
Croat TB. 1978. *Flora of Barro Colorado Island*. Standford University Press, Stanford, CA 401 p.
Croft, SL and V Yardley. 2002. Chemotherapy of leishmaniasis. *Current Pharmaceutical Design* 8: 319-342.
Crowell PL, Ren Z, Lin S, Vedejs E and MN Gould. 1994. Structure-activity relationships among monoterpene inhibitors of protein isoprenylation and cell proliferation. *Biochem. Pharmacol.* 47: 1405-1415.

PATENT PENDING

D'Arcy WC. 1987. Flora of Panama Checklist and Index. Missouri Botanical Garden, St. Louis, MO.
Da Costa FB, Albuquerque S and W Vichnewski. 1996. Diterpenes and synthetic derivatives from *Viguiera asplilicides* with trypanomicidal activity. *Planta Med.* 62: 557-559.
Della Casa de Marcano D, Hasegawa M and A Castaldi. 1972. Neutral compounds and alkaloids of *Zanthoxylum ocumarense*. *Phytochemistry* 11: 1531-1532.
Delle Monache F. 1985. Chemistry and biological activity of the secondary metabolites of Vismieae. *Revista Latinoamericana de Quimica* 16: 5-15.
Deveraux QL and JC Reed. 1999. IAP family proteins-suppressors of apoptosis. *Genes & Development* 13: 239-252.
Dorta E, Darias J, San Martin A and M Cueto. 2002. New prenylated bromoquinols from the green alga *Cymopolia barbata*. *J. Nat. Prod.* 65: 329-333.
Dutcher JD. 1968. The discovery and development of amphotericin B. *Diseases of the Chest* 54 S1: 296-298.
Earle SA. 1972. A review of the marine plants of Panamá. *Bull. Biol. Soc.Wash.* 2: 69-87.
Esposito AM, Brown P, Tejeira I, Buitrago R, Barrios L, Sanchez C, Gupta MP and J Cedeno. 1985. Pharmacological screening of Panamanian medicinal plants. *International Journal of Crude Drug Research* 23: 17-25.
Farnsworth NR and DD Soejarto. 1991. Global importance of medicinal plants. In: *The Conservation of Medicinal Plants*. Akerele O, Heywood V, Synge H. (Eds). Cambridge University Press, Cambridge. pp. 25-52.
Fisher MH. 1990. Recent advances in avermectin research. *Pure Appl. Chem.* 62: 1231-1240.
Francois G, Steenackers T, Assi LA, Steglich W, Lamottke K, Holenz J and G Bringmann. 1999. Vismione H and structurally related anthranoid compounds of natural and synthetic origin as promising drugs against the human malaria parasite *Plasmodium falciparum*: structure-activity relationships. *Parasitology Res.* 85: 582-588.
Fromann S and G Jas. 2002. Natural Products and Combinatorial Chemistry -- The Comeback of Nature in Drug Discovery. *Business Briefing: Future Drug Discovery* 84-90.

http://www.wmrc.com/businessbriefing/pdf/drug2_2002/publication/frormann.pdf

Fuller RW, Cardellina II JH, Kato Y, Brinen LS, Clardy J, Snader K and MR Boyd. 1992. A pentahalogenated monoterpene from the red alga *Portieria hornemanii* produces a novel cytotoxicity profile against a diverse panel of human tumor cell lines. *J. Med. Chem.* 35: 3007-3011.

Galinis DL and DF Wiemer. 1993. Villiramulins A and B: new phenol derivatives from *Piper villiramulum*. *J. Org. Chem.* 27: 7804-7807.

Gerwick WH and W Fenical. 1981. Ichthyotoxic and cytotoxic compounds from the tropical brown alga *Stypopodium zonale* (Lamouroux) Papenfuss. *J. Org. Chem.* 46: 22-27.

Gerwick WH, Proteau PJ, Nagle DG, Hamel E, Blokhin A and DL Slate. 1994. Structure of Curacin A, a Novel Antimitotic, Antiproliferative and Brine Shrimp Toxic Natural Product from the Marine Cyanobacterium *Lyngbya majuscula*. *J. Org. Chem.* 59: 1243-1245.

Gerwick WH, Tan LT, N. Sitachitta. 2001. Nitrogen-containing metabolites from marine cyanobacteria. In: *Alkaloids: Chemistry and Biology*. Cordell, GA (Ed). Academic Press: N.Y. Vol. 57, pp 75-184.

Gibbs JB. 2000. Mechanism-based target identification and drug discovery in cancer research. *Science* 287: 1969-1973.

Gietema JA and EGE De Vries. 2002. Clinical cancer research 2002: New agents and therapies. *Drug Resistance Updates* 5: 192-203.

Glombitza K-W and A Schmidt. 1999. Trihydroxyphloroethols from the brown alga *Carpophyllum angustifolium*. *Phytochemistry* 51: 1095-1100.

Goncalves M de Lourdes S and WB Mors. 1981. Vismiaquinone, a Δ1-isopentenyl substituted anthraquinone from *Vismia reichardtiana*. *Phytochemistry* 20: 1947-50.

Gonvindachari TR and MS Premila. 1983. Some alkaloids from *Aegle marmelos*. *Phytochemistry* 22: 755-757.

Gonzalez AG, Moujir L, Bazzocchi IL, Correa MD and MP Gupta. 1994. Screening of antimicrobial and cytotoxic activities of Panamanian plants. *Phytomedicine* 1: 149-153.

Graber MA, Gerwick WH and DP Cheney. 1996. The isolation and characterization of agardhilactone, a novel oxylipin from the marine red alga Agardhiella subulata. *Tetrahedron Lett.* 37: 4635-4638.

Grozinger CM, Chao ED, Blackwell HE, Moazed D and SL Schreiber. 2001. Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. *J. Biol. Chem.* 276: 38837-38843.

Gunstone FD, Steward SR, Cornelius JA and TW Hammonds. 1972. New tropical seed oils. IV. Component acids of leguminous and other seed oils including useful sources of crepenynic and dehydrocrepenynic acid. *J. Sci. Food Agr.* 23: 53-60.

Gupta S, Ghosh PK, Dutta GP and RA Vishwakarma. 1995. *In vivo* study of artemisinin and its derivatives against primary amoebic meningoencephalitis caused by *Naegleria fowleri*. *Journal of Parasitology* 81: 1012-1013.

Gupta MP, Gomez NE, Santana AI, Solis PN and G Palacios. 1991. Antimicrobial activity of various algae of the Panamanian Atlantic coast. *Rev Med Panama* 16: 64-68.

Gupta MP, Monge A, Karikas GA, Lopez de Cerain A, Solis PN, de Leon E, Trujillo M, Suarez O and F Wilson. 1996. Screening of Panamanian medicinal plants for brine shrimp toxicity, crown gall tumor inhibition, cytotoxicity and DNA intercalation. *International Journal of Pharmacognosy* 34: 19-27.

Hamel E, Blokhin AV, Nagle DG, Yoo H-Y and WH Gerwick. 1995. Limitations in the use of tubulin polymerization assays as a screen for the identification of new antimitotic agents: The potent marine natural product curacin A as an example. *Drug Development Research* 34: 110-120.

Harguindey S. 2002. Integrating fields of cancer research through pivotal mechanisms and synthetic final pathways: a unifying and creative overview. *Medical Hypotheses* 58: 444-452.

Harrigan GG, Luesch H, Yoshida WY, Moore RE, Nagle DG, Paul VJ, Mooberry SL, Corbett TH and FA Valeriote. 1998. Symplostatin 1: A dolastatin 10 analogue from the marine cyanobacterium *Symploca hydnoides*. *J. Nat. Prod.* 61: 1075-1077.

Hashimoto Y and T Okaichi. 1960. Some chemical properties of nereistoxin. *Ann. N.Y. Acad. Sci.* 90: 667-673.

Hashimoto Y, Sakai M and K Konishi. 1973. New insecticide developed from nereistoxin. *Food-Drugs Sea, Proc. [Conf.]* Worthen LR (Ed). Mar. Technol. Soc., Washington, D. C. (Meeting Date 1972), 3: 129-138.

Hatfield GM, Arteaga L, Dwyer JD, Arias TD and MP Gupta. 1981. An investigation of Panamanian ipecac: botanical source and alkaloid analysis. *J. Nat. Prod.* 44: 452-456.

Hay ME and SD Gaines. 1984. Geographic differences in herbivore impact: Do Pacific herbivores prevent Caribbean seaweeds from colonizing via the Panama Canal? *Biotropica* 16: 24-30.

Hay ME and JN Norris. 1984. Seasonal reproduction and abundance of six sympatric species of *Gracilaria* Grev. (Gracilariaceae; Rhodophyta) on a Caribbean subtidal sand plain. *Hydrobiologia.* 116/117: 63-94.

Hook DJ, Pack EJ, Yacobucci JJ and J Guss. 1997. Approaches to automating the dereplication of bioactive natural products-the key step in high throughput screening of bioactive materials from natural sources. *Journal of Biomolecular Screening* 2: 145-152.

Iliopoulou D, Vagias C, Harvala C and V Roussis. 2000. Udoteal B, a new linear diterpenoid from the green alga *Udotea petiolata. Nat. Prod. Lett.* 14: 373-378.

Iliopoulou D, Vagias C, Harvala C and V Roussis. 2002. C15 acetogenins from the red alga Laurencia obtusa. *Phytochemistry* 59: 111-116.

Ioset JR, Marston A, Gupta MP and K Hostettmann. 1998. Antifungal and larvicidal meroterpenoid naphthoquinones and a naphthoxirene from the roots of *Cordia linnaei. Phytochemistry* 47: 729-734.

Ishibashi M, Moore RE, Patterson GML, Xu C and J Clardy. 1986. Scytophycins, cytotoxic and antimycotic agents from the cyanophyte *Scytonema pseudohofmanni. J. Org. Chem.* 51: 5300-5306.

Jeong S-J, Ahn N-H, Kim Y-C, Inagaki M, Miyamoto T and R Higuchi. 1999. Norlignans with hyaluronidase inhibitory activity from *Anemarrhena asphodeloides. Planta Medica* 65: 367-368.

Jiang RW, Ma SC, But PP and TC Mak. 2001. New antiviral cassane furanoditerpenes from *Caesalpinia minax. J. Nat. Prod.* 64: 1266-1272.

Jimenez, JI and PJ Scheuer. 2001. New lipopeptides from the Caribbean cyanobacterium *Lyngbya majuscula. J. Nat. Prod.* 64: 200-203.

Joye NM and RV Lawrence. 1963. Isolation of a new diterpene acid. *J. Org. Chem.* 28: 3274.

Juhasz P, Campbell JM and ML Vestal. 2002. MALDI-TOF/TOF technology for peptide sequencing and protein identification. In: *Mass Spectrometry and Hyphenated Techniques in Neuropeptide Research.* Silberring J, Ekman R (Eds). 375-413.

Keifer PA. 2000. NMR spectroscopy in drug discovery: tools for combinatorial chemistry, natural products, and metabolism research. *Progress in Drug Research* 55: 137-211.

Kilar JA and JN Norris. 1988. Composition, export, and import of drift vegetation on a tropical, plant-dominated, fringing-reef platform (Caribbean Panamá). *Coral Reefs* 7: 93-103.

Kirst HA, Michel KH, Martin JW, Creemer LC, Chio EH, Yao RC, Nakatsukasa WM, Boeck LD, Occolowitz JL *et al.* 1991. A83543A-D, unique fermentation-derived tetracyclic macrolides. *Tetrahedron Lett.* 32: 4839-4842.

Kirst HA, Creemer LC, Naylor SA, Pugh PT, Snyder DE, Winkle JR, Lowe LB, Rothwell JT, Sparks TC and TV Worden. 2002. Evaluation and development of spinosyns to control ectoparasites on cattle and sheep. *Current Topics in Medicinal Chemistry* 2: 675-699.

Konig GM and AD Wright. 1993. Algal secondary metabolites and their pharmaceutical potential. In: *Human Medicinal Agents from Plants. ACS Symposium Series 534.* Kinghorn AD, Balandrin MF (Eds). American Chemical Society, Washington, DC, pp. 276-293.

Kotsubo V and R Nast. 1996. Cryogenic system for a high temperature superconductor NMR probe. *Advances in Cryogenic Engineering* Volume Date 1995, 41(Pt. B): 1857-1864.

Kurihara H, Mitani T, Kawabata J and K Takahashi. Two New Bromophenols from the Red Alga Odonthalia corymbifera. 1999. *J. Nat. Prod.* 62: 882-884.

Lay Jr. JO. 2002. MALDI-TOF mass spectrometry of bacteria. *Mass Spectrometry Reviews* Volume Date 2001, 20: 172-194.

Leigh Jr. EG 1999. *Tropical forest ecology: A view from Barro Colorado Island.* Oxford Press, N.Y.

Levin DA and BM York. 1978. The toxicity of plant alkaloids: An ecogeographic perspective. *Biochem. Syst. Ecol.* 6: 61-76.

Littler DS and MM Littler. 2000. *Carribean Reef Plants.* Offshore Graphics, Inc., Washington, D.C. 542 p.

Littler DS and MM Littler. 2003. *South Pacific Reef Plants.* Offshore Graphics, Inc., Washington, D.C. 331 p.

Lopez JA, Laurito JG, Lin FT, Sharaf M, Wong LK and PL Schiff 1993. Alkaloids of *Guatteria diospyroides. Planta Med.* 59: 191.

Lee GM and TF Molinski. 1992. Herbaceamide, a chlorinated N-acyl amino ester from the marine sponge *Dysidea herbacea. Tetrahedron Lett.* 33: 7671-7674.

Levin DA and BM York. 1978. The toxicity of plant alkaloids: an ecogeographic perspective. *Biochem. Syst. Ecol.* 6: 61-76.

Luesch H, Moore RE, Paul VJ, Mooberry SL and TH Corbett. 2001. Isolation of dolastatin 10 from the marine cyanobacterium *Symploca* species VP642 and total stereochemistry and biological evaluation of its analogue symplostatin 1. *J. Nat. Prod.* 64: 907-910.

Luesch H, Harrigan GG, Goetz G and FD Horgen. 2002. The cyanobacterial origin of potent anticancer agents originally isolated from sea hares. *Curr. Med. Chem.* 9: 1791-1806.

Lyder DL, Peter SR, Tinto WF, Bissada SM, McLean S and WF Reynolds. 1998. Minor Cassane Diterpenoids of *Caesalpinia bonduc. J. Nat. Prod.* 61: 1462-1465.

Macias FA, Varela RM, Simonet AM, Cutler HG, Cutler SJ, Dugan FM and RA Hill. 2000. Novel Bioactive Breviane Spiroditerpenoids from *Penicillium brevicompactum* Dierckx. *J. Org. Chem* 65: 9039-9046.

Madinaveitia A, Reina M, de la Fuente G, Gonzalez AG and E Valencia. 1996. Obovamine, a New Indole Alkaloid from *Stemmadenia obovata. J. Nat. Prod.* 59: 185-189.

Margolin K, Longmate J, Synold TW, Gandara DR, Weber J, Gonzalez R, Johansen MJ, Newman R, Baratta T and JH Doroshow. 2001. Dolastatin-10 in metastatic melanoma: A phase II and pharmacokinetic trial of the California Cancer Consortium. *Investigational New Drugs* 19: 335-340.

Marquez BL, Watts KS, Yokochi A, Roberts MA, Verdier-Pinard P, Jimenez JI, Harnel E, Scheuer PJ and WH Gerwick. 2002. Structure and absolute stereochemistry of hectochlorin, a potent stimulator of actin assembly. *J. Nat. Prod.* 65: 866-871.

Matsuse I T, Lim YA, Hattori M, Correa M and MP Gupta. 1999. A search for anti-viral properties in Panamanian medicinal plants. The effects on HIV and its essential enzymes. *Journal of Ethnopharmacology* 64: 15-22.

Matthys H, De Mey C, Carls C, Rys A, Geib A and T Wittig. 2000. Efficacy and tolerability of myrtol standardized in acute bronchitis: a multi-center, randomised, double-blind, placebo-controlled parallel group clinical trial vs. cefuroxime and ambroxol. *Arzneimittel-Forschung* 50: 700-711.

McGeer PL, McGeer EG and T Hattori. 1978. Kainic acid as a tool in neurobiology. *Kainic Acid Tool Neurobiol.* 123-138.

Mendelsohn R and MJ Balick. 1995. The value of undiscovered pharmaceuticals in tropical forests. *Economic Botany* 49: 223-228

Mendonca Pauletti P, Araujo AR, Young MCM, Giesbrecht AM and V da Silva Bolzani. 2000. Nor-lignans from the leaves of *Styrax ferrugineus* (Styracaceae) with antibacterial and antifungal activity. *Phytochemistry* 55: 597-601.

Meyer BN, Ferrigni NR, Putnam LB, Jacobsen LB, Nichols DE and JL McLaughlin. 1982. *Planta Med.* 45: 31-33.

Morin PJ. 2002. Functional genomics approaches in cancer research. *Current Genomics* 3: 425-436.

Myers N, Mittermeier RA, Mittermeier CG, Fonseca GAB and J Kent. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403: 853-858.

Newman DJ, Cragg GM, Holbeck S and EA Sausville. 2002. Natural products and derivatives as leads to cell cycle pathway targets in cancer chemotherapy. *Current Cancer Drug Targets* 2: 279-308.

Ngoka LCM and ML Gross. 1999. Multistep tandem mass spectrometry for sequencing cyclic peptides in an ion-trap mass spectrometer. *J. Am. Soc. Mass Spectrom.* 10: 732-746.

Nishizawa M, Yamagishi T, Dutschman GE, Parker WB, Bodner AJ, Kilkuskie RE, Cheng YC and KH Lee. 1989. Anti-AIDS agents, 1. Isolation and characterization of four new tetragalloylquinic acids as a new class of HIV reverse transcriptase inhibitors from tannic acid. *J. Nat. Prod.* 52: 762-768.

Nogle LM, Marquez BL and WH Gerwick. 2003. Wewakazole, a Novel Cyclic Dodecapeptide from a Papua New Guinea Lyngbya majuscula. *Organic Letters* 5: 3-6.

Orjala J and WH Gerwick. 1996. Barbamide, a chlorinated metabolite with molluscicidal activity from the Caribbean cyanobacterium *Lyngbya majuscula*. *J. Nat. Prod.* 59: 427-430.

Ovenden SPB, Cao S, Leong C, Flotow H, Gupta MP, Buss AD and MS Butler. 2002. Spermine alkaloids from *Albizia adinocephala* with activity against Plasmodium falciparum plasmepsin II. *Phytochemistry* 60: 175-177.

Palazzino G, Galeffi C, Federico E, Delle Monache F, Francesca Cometa M and M Palmery. 2000. Benzylbenzoate and norlignan glucosides from *Curculigo pilosa*: structural analysis and in vitro vascular activity. *Phytochemistry* 55: 411-417.

Patra A., Mukhopadhyay AK, Ghosh A and AK Mitra. 1979. Carbon-13 NMR spectra of some furocoumarins. *Indian J. Chem.* 17B: 385.

Patra A, Mitra AK, Ghosh A and PK Mukhopadhyay. 1981. Carbon-13 NMR spectra of tembamide, aegeline, and related amides. *Org. Mag. Res.* 16: 65-67.

Patterson GML, Smith CD, Kimura LH, Britton BA and S Carmeli. 1993. Action of tolytoxin on cell morphology, cytoskeletal organization, and actin polymerization. *Cell Motility Cytoskeleton* 24: 39-48.

Phillips LR, Malspeis L and JG Supko. 1995. Pharmacokinetics of active drug metabolites after oral administration of perillyl alcohol, an investigational antineoplastic agent, to the dog. *Drug Metabolism and Disposition* 23: 676-80.

Phillipson JD and CW Wright. 1991. Antiprotozoal agents from plant sources. *Planta Medica* 57(Suppl. 1): S53-S59.

Poncet J. 1999. The dolastatins, a family of promising antineoplastic agents. *Current Pharmaceutical Design* 5: 139-162.

Popoff S, Stino MR and H de Lima. 1960. Chemical study of *Myrospermum frutescens*. *Rev. Soc. Venezalana Quim.* 31: 31-36.

Price PW, Lewinsohn TM, Fernandes GW and WW Benson. (Eds). 1991. *Plant-Animal Interactions*, John Wiley and Sons, NY. 639 p.

Puentes De Díaz AM. 1996. Neolignans from *Anaxagorea clavata*. *Phytochemistry* 44: 345-346.

Ragasa CY, Hofileña JG and JA Rideaout. 2002. New furanoid diterpenes from *Caesalpinia pulcherrima*. *J. Nat. Prod.* 65: 1107-1110.

Rahalison L, Benathan M, Monod M, Frenk E, Gupta MP, Solis PN, Fuzzati N and K Hostettmann. 1995. Antifungal principles of *Baccharis pedunculata*. *Planta Med.* 61: 360-362.

Roussis V, Ampofo SA and DF Wiemer. 1990. A prenylated benzoic acid derivative from the leaves of *Piper taboganum*. *Phytochemistry* 29: 1787-1788.

Scheuer PJ. 1973. *Chemistry of marine natural products*. Academic Press, N.Y. 201 p.

Sessa C, Weigang-Kohler K, Pagani O, Greim G, Mora O, De Pas T, Burgess M, Weimer I and R Johnson. 2002. Phase I and pharmacological studies of the cryptophycin analogue LY355703 administered on a single intermittent or weekly schedule. *European Journal of Cancer* 38: 2388-2396.

Sharma B, Rattan R and P Sharma. 1981. Constituents of *Aegle marmelos*. Part III. Marmeline, an alkaloid, and other components of unripe fruits of *Aegle marmelos*. *Phytochemistry* 20: 2606-2607.

Shih C and BA Teicher. 2001. Cryptophycins: a novel class of potent antimitotic antitumor depsipeptides. *Current Pharmaceutical Design* 7: 1259-1276.

Sikora K. 2001. Cancer drug development in the post-genomic age. Current Science 81: 549-554.

Smith CD, Carmeli S, Moore RE and GML Patterson. 1993. Scytophycins, novel microfilament-depolymerizing agents which circumvent P-glycoprotein-mediated multidrug resistance. *Cancer Res.* 53: 1343-1347.

Sparks TC, Crouse GD and G Durst. 2001. Natural products as insecticides: the biology, biochemistry and quantitative structure-activity relationships of spinosyns and spinosoids. *Pest Management Science* 57: 896-905.

Subramanian R, Sweedler J and AJ Webb. 1999. Rapid two-dimensional inverse detected heteronuclear correlation experiments with <100 nmol samples with solenoidal microcoil NMR probes. *J. Amer. Chem. Soc.* 121: 2333-2334.

Suzuki M, Yamada H and K Kurata. 2002. Dictyterpenoids A and B, two novel diterpenoids with feeding-deterrent activity from the brown alga *Dilophus okamurai*. *J. Nat. Prod.* 65: 121-125.

Takatsu T, Matsumoto T, Takahashi S, Ishikawa M and K Wanibuchi. 2002. Manufacture of antibiotic 1160-50, Streptomyces producing it, and nematocides containing it. *Jpn. Kokai Tokkyo Koho* 12 p.

Terreaux C, Gupta MP and K Hostettmann. 1998. Antifungal benzoic acid derivative from *Piper dilatatum*. *Phytochemistry* 49: 461-464.

Todd JS, Proteau PJ and WH Gerwick. 1993. Egregiachlorides A-C: new chlorinated oxylipins from the marine brown alga *Egregia menziesii*. *Tetrahedron Lett.* 34: 7689-7692.

Tosi J, 1971. *Zonas de vida. Republica de Panamá*. FO:SF/PAN 6, Informe Tecnico 2. FAO, Roma. 123 p.

Trentin AP, Santos ARS, Guedes A, Pizzolati MG, RA Yunes and JB Calixto. 1999. Antinociception caused by the extract of *Hedyosmum brasiliense* and its active principle, the sesquiterpene lactone 13-hydroxy-8,9-dehydroshizukanolide. *Planta Medica* 65: 517-521.

Trimurtulu G, Ohtani I, Patterson GML, Moore RE, Corbett TH, Valeriote FA and L Demchik. 1994. Total structures of cryptophycins, potent antitumor depsipeptides from the blue-green alga *Nostoc* sp. strain GSV 224. *J. Amer. Chem. Soc.* 116: 4729-4737.

Tringali C. 1997. Bioactive metabolites from marine algae: recent results. *Curr. Org. Chem.* 1: 375-394.

Van De Water J, Deininger SO, Macht M, Przybylski M and ME Gershwin. 1997. Detection of molecular determinants and epitope mapping using MALDI-TOF mass spectrometry. *Clinical Immunology and Immunopathology* 85: 229-235.

Vieira HS, Takahashi JA, De Oliveira AB, Chiari E and MAD Boaventura. 2002. Novel derivatives of kaurenoic acid: preparation and evaluation of their trypanocidal activity. *J.Brazilian Chem. Soc.* 13: 151-157.

Weniger B, Aragon R, Deharo E, Bastida J, Codina C, Lobstein A and R Antón. 2000. Antimalarial constituents from *Guatteria amplifolia*. *Pharmazie* 55: 867-868.

Weniger B, Robledo S, Arango GJ, Deharo E, Aragon R, Munoz V, Callapa J, Lobstein A and R Antón. 2001. Antiprotozoal activities of Colombian plants. *Journal of Ethnopharmacology* 78:193-200.

Wessels M, Koenig GM and AD Wright. 1999. A new tyrosine kinase inhibitor from the marine brown alga *Stypopodium zonale*. *J. Nat. Prod.* 62: 927-930.

Williamson RT, Marquez BL and WH Gerwick. 1999a. Use of 1H-15N PEP-HSQC-TOCSY at natural abundance to facilitate the structure elucidation of naturally occurring peptides. *Tetrahedron* 55: 2881-2888.

Williamson RT, Sitachitta N and WH Gerwick. 1999b. Biosynthesis of the marine cyanobacterial metabolite barbamide. 2: Elucidation of the origin of the thiazole ring by application of a new GHNMBC experiment. *Tetrahedron Lett.* 40: 5175-5178.

Williamson RT, Marquez BL, Gerwick WH and KE Kover. 2000a. One- and two-dimensional gradient-selected HSQMBC NMR experiments for the efficient analysis of long-range heteronuclear coupling constants. *Mag. Res. Chem.* 38: 265-273.

Williamson RT, Carney JR and WH Gerwick. 2000b. Application of the BIRD Sandwich for the Rapid and Accurate Determination of 1H-1H NMR Coupling Constants in Higher Order Spin Systems. *J. Nat. Prod.* 63: 876-878.

Williamson RT, Marquez BL, Gerwick WH, Martin GE and VV Krishnamurthy. 2001. J-IMPEACH-MBC: a new concatenated NMR experiment for F1 scalable, J-resolved HMBC. *Mag. Res. Chem.* 39: 127-132.

Williamson RT, Boulanger A, Vulpanovici A, Roberts MA and WH Gerwick. 2002. Structure and absolute stereochemistry of phormidolide, a new toxic metabolite from the marine cyanobacterium *Phormidium* sp. *J. Org. Chem.* 67: 7927-7936.

Wipf P. *et al.* 2002. In news report: A Pharmaceutical Company Would Never Do This. *Chem. Eng. News* November 11: 52.

Wright AD, Wang H, Gurrath M, Koenig GM, Kocak G, Neumann G, Loria P, Foley M and LTilley. 2001. Inhibition of Heme Detoxification Processes Underlies the Antimalarial Activity of Terpene Isonitrile Compounds from Marine Sponges. *J. Med. Chem.* 44: 873-885.

Wysor B, Kooistra WHCF and S Fredericq. 2000. Marine Macroalgal Diversity of the Republic of Panama. Poster presentation at the Annual Meeting of the Phycological Society of America, San Diego, CA, 15-19 July 2000 http://morayeel.louisiana.edu/SeaweedsLab/Wysor/PSA_2000/poster.html#anchor2837646

Wysor BS. 2002. Biodiversity and biogeography of marine green algae of the Republic of Panama. Univ. of Louisiana, Lafayette, LA, USA. Avail. UMI, Order No. DA3045165. 291 p. From: Diss. Abstr. Int., B 2002 63: 1115.

H. CONSORTIUM/CONTRACTUAL ARRANGEMENTS

Sub-contract to Oregon State University

I. LETTERS OF SUPPORT (Consultants/Collaborators)

None

ASSOCIATE PROGRAM 4

CONSERVATION, OUTREACH AND BIODIVERSITY INVENTORY IN PANAMA

Associate Program Leader: Todd L. Capson

Smithsonian Tropical Research Institute, Panama City, Panama

International Cooperative Biodiversity Groups
"Bioassay and Ecology Directed Drug Discovery in Panama"
Dr. William Gerwick, Group Leader

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. DO NOT EXCEED THE SPACE PROVIDED.

Associate Program 4 has three basic objectives: (i) to use the resources of the Panama ICBG to develop programs that contribute to biodiversity conservation in Panama, (ii) to conduct biodiversity inventories in unique marine and terrestrial ecosystems in Panama, and (iii) to provide materials for the drug discovery components of the Panama ICBG. Objective (i) will be achieved by engaging in extensive public outreach and environmental educational activities in Panama, and by working with the State agency for protecting natural resources, the National Authority of the Environment (ANAM). We will work with ANAM to strengthen their capacity to protect Panama's extensive system of national parks through improved database management, to design and implement strategies to protect the Coiba National Park (in collaboration with Conservation International and the Woods Hole Oceanographic Institution) and to place the park on UNESCO's list of World Heritage Sites. Objective (ii) will involve biodiversity inventories in the Coiba National Park. Botanical inventories will be conducted on Coiba Island, whose intact flora is largely unstudied but known to contain endemic species. In the park's marine environment, we will work with STRI marine biologists to conduct an inventory of corals. Cyanobacteria and other algae collected in the Coiba National Park will also be collected and inventoried. Objective (iii) will provide an interface between the biodiversity inventories in the Coiba National Park and the drug discovery program of the ICBG (Associate Programs 1-3). The extracts of plants from Coiba Island that are new to the ICBG will be tested for activity in tropical disease and anticancer assays. Octocorals will be provided to chemists working in collaboration with the Panama ICBG for bioassay-guided fractionation.

PERFORMANCE SITE(S) *(organization, city, state)*
Smithsonian Tropical Research Institute, Panama City, Republic of Panama

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
CAPSON, Todd	Smithsonian Tropical Research Institute	Associate Program 4 leader
GUZMÁN, Hector	Smithsonian Tropical Research Institute	Coral biodiversity inventories
IBÁÑEZ, Alicia	Smithsonian Tropical Research Institute	Plant biodiversity inventories
SÁNCHEZ, Lorna	Smithsonian Tropical Research Institute	Database management

A. SPECIFIC AIMS

The **overall goals** of Associate Program 4 are to (i) use the resources of the Panama ICBG to develop programs that contribute to biodiversity conservation in Panama, (ii) conduct biodiversity inventories in unique marine and terrestrial ecosystems in Panama, and (iii) provide materials for the drug discovery components of the Panama ICBG.

The **Specific Aims** of this Associate Program are:

1. To link the activities of the Panama ICBG in Associate Programs 1, 2 and 3 to biodiversity conservation.
Working in collaboration with the Panamanian government and local NGOs we will engage in a range of public outreach and environmental educational activities in Panama. The programs will emphasize the importance of marine and terrestrial ecosystems in the tropics, the connection between human health and biological diversity and the activities of the Panama ICBG program.

2. To strengthen Panama's National Authority of the Environment.
We will work with the National Authority of the Environment to strengthen their capacity to protect natural resources through improved database management and by contributing to environmental education programs.

3. To design and implement programs to protect Panama's Coiba National Park.
We will work with the National Authority of the Environment, local and international NGOs, the Woods Hole Oceanographic Institution and private individuals in the design and implementation of programs to protect the Coiba National Park.

4. To carry out biodiversity inventories of terrestrial and marine plants and corals in the Coiba National Park.
Terrestrial botanical inventories will be carried out on Coiba Island whose flora is largely unstudied but known to include endemic species. STRI staff scientists to conduct an inventory of corals in the Coiba National Park's marine environment. Cyanobacteria and other algae will also be collected and inventoried.

5. To provide terrestrial and marine plants from the Coiba National Park for drug discovery activities of the Panama ICBG.
Plant collected in the Coiba National Park that are new to the Panama ICBG will be supplied to Associate Programs 1, 2 and 3. Octocorals collected in the Coiba National Park will be subjected to bioassay-guided fractionation by chemists working in collaboration with the Panama ICBG.

6. To work with the National Authority of the Environment to have the Coiba National Park included in UNESCO's list of World Heritage Sites.
Once added to the list of World Heritage Sites, we will use that status to raise awareness of the importance of the Coiba National Park and to help secure the funds necessary for the park's protection.

B. BACKGROUND AND SIGNIFICANCE

A priority of the Panama ICBG over the past 4.5 years has been the design and implementation of initiatives that promote biodiversity conservation in Panama. These activities were not part of any particular Associate Program but arose from an appreciation of two important factors that contribute to the loss of biodiversity in Panama. Firstly, there is a general lack of awareness in Panamanian society of the importance of biological diversity other than as a source of raw materials such as timber. When national development priorities are

established, the strategic value of biological diversity for uses such as drug discovery, and the importance of the goods and services that intact ecosystems provide, are not a significant priority. Secondly, the institution that is responsible for protecting biological diversity in Panama, the National Authority of the Environment (known as "ANAM" for its acronym in Spanish) needs to be strengthened in order to effectively protect Panama's natural resources.

In response to these needs, we have implemented two basic strategies to link the Panama ICBG to biodiversity conservation: (i) We have engaged in consistent public outreach efforts that have taken the form of lectures, newspaper articles, and other activities. As a result of these efforts, the Panama ICBG is well-known and widely perceived as beneficial for the country. Consequently, there is a greater appreciation of the potential benefits to be gained from natural products-based drug discovery. (ii) We have worked closely with ANAM in a collaboration that has ranged from investments in infrastructure to the design of strategies for the protection of national parks.

In order to increase the impact of these programs, we propose to create a separate Associate Program that will expand upon the initiatives described above as well as coordinate biodiversity inventories in the Coiba National Park, the focus of our conservation efforts. The biodiversity inventories will also supply biological materials and information for Associate Program 2 (Bioactivity Testing) and Associate Program 3 (Chemical Isolation and Structural Elucidation).

B.1. Panama's Biological Diversity

B.1.1. Terrestrial Biological Diversity

As described in Associate Program 1, Panama (Figure 1) is at the center of the region with the greatest concentration of terrestrial plant species in the world (>5,000 species per 10,000 km^2) and is considered a "biodiversity hotspot" (Myers *et al.* 2000). It is estimated that there are over 13 life zones containing between 8,500 and 9,000 species of flowering plants, with over 8,400 already identified by taxonomic experts (Tosi 1971, D'Arcy 1987, M. Correa, pers. com.). Endemic species account for 14% of the flora. In addition, Panama's unique geographic position means it is home to species from both Central and South America. For example, Panama is the most northern extent for approximately 4,000 South American species. The Panama forests also serve as important migratory corridors for flora and fauna between Central and South America (Coates and Obando 1996). Today, 104 species of birds and 38 species of mammals are found only in Panama and are thus completely dependent on the country's protected habitats. Of the 900 species of birds that have been recorded in Panama, over 200 are North American migrants that use Panama as a wintertime destination. Destruction of migration destinations may be responsible for the observed decline in North American songbird populations (Robbins *et al.* 1989). As extreme climate fluctuations become more severe, it becomes increasingly important for species to migrate in response to adverse conditions. This role of Panama as a biological corridor is particularly vulnerable because the isthmus is only 100 km wide. In recognition of the key role of this biological corridor, 7 Central American Countries recently pledged to help preserve this forested connection (The Pantera Paseo Agenda, Illueca 1998).

Panama is unique in Central America in having 38.6% of its land still in forest cover with an estimated 29,000 square km of remaining forest cover. Almost a quarter of Panama's land area is protected in the country's 43 parks and reserves. However, deforestation continues at a rapid rate; 1.6% or 519 square km per year. At this rate, Panamanian forests will disappear within approximately 50 years (FAO 2001). Due to its extraordinary yet threatened biodiversity, Panama is now considered a threatened biodiversity hotspot by Conservation International (Myers *et al.* 2000).

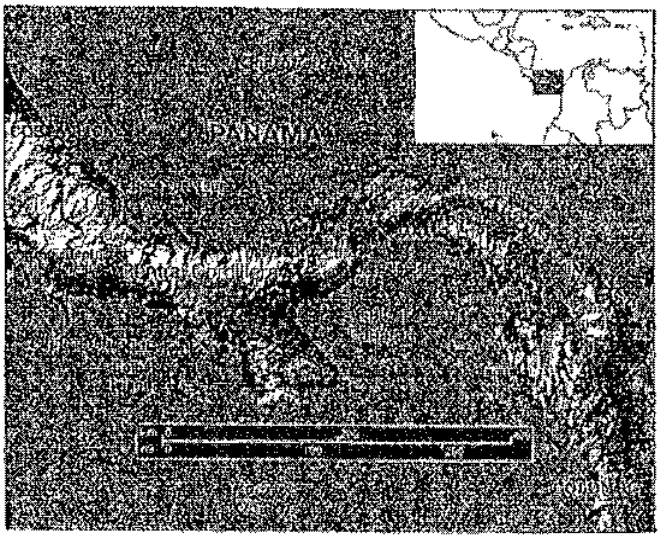


Figure 1. The Republic of Panama. The Coiba National Park is located within the Gulf of Chiriqui and its borders are indicated by solid lines. See Appendix 5 for the color version.

B.1.2. Marine Biological Diversity and the Gulf of Chiriquí

Panama's 2,490 km coastline and the surrounding oceans are home to an extraordinary marine biological diversity (see AP1, Section B.1.; Allen and Robertson 1994, Glynn and Maté 1997). Panama is surrounded by three distinct bodies of water, the Caribbean (to the north), the Gulf of Chiriquí (southwest) and the Gulf of Panama (southeast). The temperature differences between the Gulf of Chiriqui and the Gulf of Panama can be seen in the satellite-derived sea-surface temperature field taken during the dry season of 1995 (Figure 2). The trade winds blow year-round over the Isthmus of Panama and where their flow is unimpeded, such as the Gulf of Panama, the surface flow produces an upwelling of cold nutrient-laden water, resulting in a marked drop in temperature, which dips below 20 °C during upwelling phases (Glynn and Maté 1997). By contrast, the Central Cordillera (shown in Figure 1) blocks the flow of trade winds in western Panama, protecting areas such as the Gulf of Chiriquí.

The relatively stable and warm temperatures in the Gulf of Chiriqui are manifest in differences in coral reef growth and community structure (Glynn and Maté 1997). Generally, coral reefs are more abundant, larger and have higher species diversity in non-upwelling western Panama than in upwelling central/eastern Panama. The best-developed reefs in the Gulf of Chiriquí have vertical buildups of 10-12 meters and maximum ages of 5,600 years. The Pacific shores of Panama have a diverse coral fauna, representing 91% of the genera of the eastern Pacific reef-building species, many of which are found in the Gulf of Chiriquí (Glynn and Maté 1997).

The Gulf of Chiriquí is situated within the eastern tropical Pacific Ocean, in a region known as the Panama Bight, an area consisting of the islands and their surrounding waters in the east central Pacific off the coasts of Ecuador, Colombia, Panama and Costa Rica. The Panama Bight is one of the most biologically productive areas of the eastern tropical Pacific and one of the world's most biologically diverse geographical provinces (Glynn and Maté 1997). The area has a high degree of ecological interconnection and complex oceanographic characteristics, mainly due to the convergence of major marine currents which facilitate the dispersal of marine larvae (*e.g.*, from corals, crustaceans, echinoderms, mollusks and fishes) and affect the migrations, movements and distribution of many species of regional and global significance. The waters and currents which link these islands play an important role as bridges for the species within the region (Glynn and Maté 1997, Wellington 1997).

Due to the global importance of this marine region, at the Summit on Sustainable Development held in Johannesburg, South Africa (September 4, 2002), the initiative "Marine Conservation and Sustainable

Development Corridor: Galapagos, Cocos, Coiba, Gorgona and Malpelo Islands" was launched. The program involves the establishment of a marine conservation corridor that encompasses each of the islands mentioned above and their surrounding waters and involves the governments of Costa Rica, Panama, Ecuador and Colombia in addition to the United Nations Environment Programme, the World Conservation Union and Conservation International. The Coiba National Park is a key link in this initiative, and its protection is significant on both national and international levels.

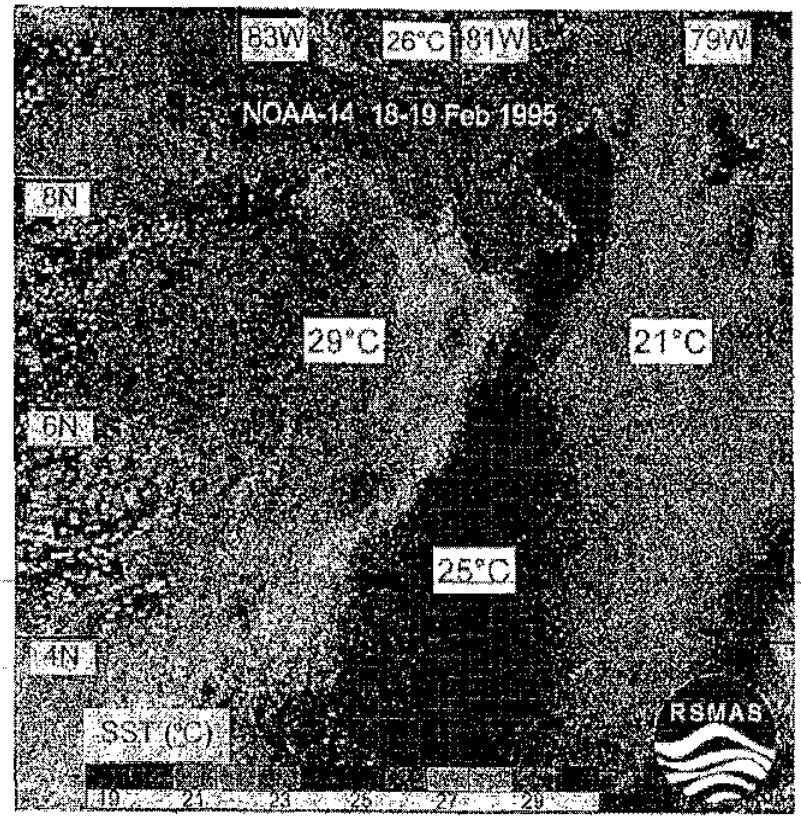


Figure 2. Satellite derived sea-surface temperature (SST) field taken during the dry season of 1995 demonstrating the temperature differences between the Gulf of Chiriqui and the Gulf of Panamá. Temperatures are indicated on the scale at the bottom of the figure (from Glynn and Maté 1997). See Appendix 5 for the color version.

B.2. The Coiba National Park

While there are numerous marine and terrestrial ecosystems in Panama worthy of national and international conservation efforts, the case for the Coiba National Park (Figures 1 and 3) is particularly compelling. Much of the Gulf of Chiriqui's terrestrial and marine biodiversity occurs within the Coiba National Park, of which 2,165 square km are marine and 535 square km are insular, including Coiba Island (503 square km) and 38 smaller islands (Figure 3).

With its largely intact marine and terrestrial ecosystems, the Coiba National Park is one of the greatest remaining natural treasures in Panama. The pristine nature of the Coiba National Park is largely a consequence of Coiba Island's use as a penal colony since 1919 (Castroviejo and Ibáñez 2001). However, the prisoners are being removed from the island and there are now less than 70 inmates, down from a high of over 1,000. By creating the Coiba National Park in 1991, the government of Panama took a key step by providing the legal basis for the area's

protection, and it is now a question of coordinating with ANAM to devise and implement strategies that will protect the park.

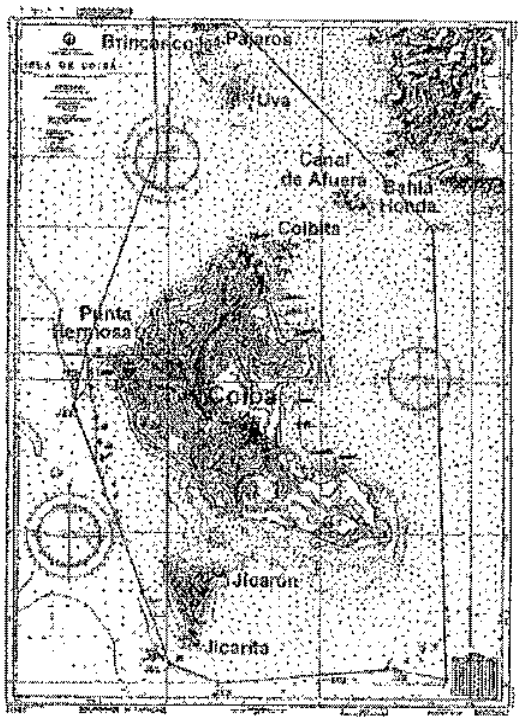


Figure 3. The Coiba National Park. The park boundaries are indicated with solid lines. See Appendix 5 for the color version.

B.2.1. The Coiba National Park's Biological Diversity

Coiba Island has a variety of endemic birds, mammals and plants (Castroviejo 1997, Ibáñez 2001). Coiba Island possesses one of the last relicts of tropical moist forest in the Pacific slope of all Central America (Ibáñez 2001). Coiba Island encompasses 94% of the insular area of the park and is the largest island in the Central American Pacific. Approximately 80% of the forests of Coiba Island are primary and largely untouched (Ibáñez 2001). The following section will focus on those species of relevance to drug discovery.

B.2.1.1. Terrestrial Plant Biodiversity

The flora of Coiba Island has been the subject of preliminary studies only (Velayos *et al.* 1997, Castroviejo and Ibáñez 2001, Ibáñez 2001). These studies suggest that there are around 2,000 species of vascular plants, 758 of which have been identified, and have resulted in the discovery of a new genus, endemic to Coiba, *Desmotes* (family: Rutaceae) and three endemic species (Castroviejo and Ibáñez 2001). The main forest types are tropical moist forest and very moist premontane forests in addition to stands of mangroves, *Prioria copaifera* and *Mora oleifera*. Remarkably, most of the interior of Coiba Island is still unexplored botanically (Ibáñez 2001, M. Correa, pers. comm.). The authors of a recent study reported, "we do not doubt that a more detailed study will yield many more endemic species" (Castroviejo and Ibáñez 2001). Professor Mireya Correa of STRI, a foremost authority on the Panamanian flora, has reached a similar conclusion (pers. comm.).

B.2.1.2. Marine Biodiversity

The reefs found in the Coiba National Park are an extraordinary resource as they contain some of the few remaining eastern Pacific coral reefs in relatively good condition (Cortés 1997). The marine ecosystems of the Coiba National Park are poorly understood and largely unstudied (Juan Maté, Hector Guzmán, pers. com.). During

a recent study of the distribution and status of coral reefs and communities in the Coiba National Park by Dr. Hector Guzmán, a STRI staff scientist and expert on the ecology and population dynamics of coral reefs, important "hot spots" of coral diversity were identified. These areas contain numerous octocoral species, some of which are known to constitute new species (Breedy and Guzmán 2003, discussed in Preliminary Results, Section C.4). These octocorals, together with the hard corals (scleractinians), are considered major reef-building species in the region. The survey indicated that high coral diversity was observed in coral communities rather than coral reefs (H. Guzmán pers. com.).

Eastern Pacific reefs are considered small and sparsely distributed by comparison to their counterparts in the central and western Pacific. However, an exception is seen in the Ensenada Maria reef, which is immediately adjacent to Coiba Island and is the second largest reef in the eastern Pacific covering 160 hectares (1.6 square km, Glynn and Maté 1997). In addition to the Ensenada Maria reef, there are smaller coral reefs at sites throughout the Coiba National Park, including the Islands of Jicarón, Jicarita, Uva, Brincanco and Canal de Afuera (Figure 3). Coral reefs in the Gulf of Chiriquí also occur at mainland sites not associated with islands (Glynn and Maté 1997). In addition to the coral diversity, a reconnaissance trip to several different sites in the Coiba National Park by Dr. Bill Gerwick in February 2002 revealed a significant diversity of cyanobacteria and other algae.

B.3. Threats to the Coiba National Park

While the most immediate threat to the Coiba National Park is from illegal fishing, threats to the terrestrial environment are likely to increase upon removal of the penal colony.

B.3.1. Threats to the Marine Environment

Fishing is one of the human activities associated with coral reef destruction in the Central American region. It causes removal of herbivores which results in algal blooms and the destruction of reefs, over and above the direct physical effect of nets, traps and anchors (Cortés 1997). The need to incorporate local communities and resource users into programs involving marine protected areas is well documented (Barahona and Guzmán 1998, White *et al.* 2002, Christie *et al.* 2002) and in the Coiba National Park, there was substantial anecdotal evidence from ANAM park guards and dive operators that illegal fishing within the park's boundaries is extensive. As defined by ANAM's Management Plan for the Coiba National Park, "illegal fishing" is anything other than fishing with hand lines. The estimated threat to coral reefs within the Coiba National Park is considered medium- to high-risk by the World Resources Institute (Bryant *et al.* 1998).

To assess the nature of the fishing activities accurately, the Panama ICBG coordinated a detailed survey of fishermen from the region by scientists from the Duke University School of the Environment and STRI (Moretti 2002, Submitted). The survey was implemented in 14 towns in the Gulf of Chiriquí (Figure 1) as well as in two large commercial fishing ports. A total of 170 fishermen participated, providing invaluable data for the first systematic analysis of fishing in the Coiba National Park. Among the issues addressed were the extent of illegal fishing within the park boundaries, the techniques employed, the species caught (including unintended bycatch), the productivity of the fishery, and the fishermen's knowledge and attitudes towards the Coiba National Park (Moretti 2002). The survey revealed that illegal fishing within the Coiba National Park is widespread. The survey results are consistent with the observations of recreational dive operators who have seen marked decreases in the number of sharks, billfish, rays, groupers and snappers over the past four to five years, which is the same time that commercial fishing operations increased in the Coiba National Park.

Only 11% of the commercial shrimp fishermen interviewed knew that the Coiba National Park was anything more than Coiba Island, even though half of those interviewed were boat captains. When these same fishermen were shown a map of the Gulf of Chiriquí, all of them indicated that they routinely fish within the park boundaries (Moretti 2002). In addition to shrimp fishing, indiscriminate fishing techniques such as gill nets and long lines are widely employed within the park boundaries. The survey identified a total of six communities that have the greatest impact on the park's marine resources: between 86% and 100% of the fishermen from those areas routinely fish within the Coiba National Park. The same fishermen showed a widespread unawareness of the park's boundaries.

Despite the extent of illegal fishing within the Coiba National Park, the survey indicated that many of the fishermen were supportive of the concept of a national park: fully 67% of the fishermen from the six communities mentioned above responded that protecting the region as a National Park was beneficial (Moretti 2002). Seventy-six percent of those interviewed said that the development of tourism would be beneficial for their community.

B.3.2. Threats to the Terrestrial Environment

The Coiba National Park is part of the province of Veraguas, which is among the most impoverished of Panama's interior provinces (Elton 1997). The pressure from squatting and terrestrial resource extraction is likely to increase once the penal colony has been completely removed.

B.3.2.1. Colonization by Rural Peasants

It is a common practice throughout Panama for peasants to invade unoccupied lands which are then cleared for housing and subsistence activities. Even National Parks within the Panama Canal watershed are subject to squatting [see, for example, *La Prensa*, May 8, 2002, "The footprints left by squatters in Mata Redonda in the National Park, Camino de Cruces].

B.3.2.2. Logging and Sale of Wildlife

Commercial logging also poses a threat since Coiba Island has valuable hardwoods such as *Anacardium excelsum*, *Calophyllum longifolium* and *Manilkara sp.* which have long since disappeared from the Pacific slopes of the nearby provinces of Veraguas and Los Santos. The capture of Scarlet Macaws (*Ara macao*) for commercial sale has resulted in their disappearance from every forest in Panama except in two areas, Coiba Island and the remote Cerro Hoya National Park.

B.4. The Need to Enhance ANAM's Capacity to Protect the Coiba National Park

The fundamental causes of the loss of biodiversity in Panama are complex, but a key component is economic: 40.5% of the population live in general poverty, of which 26.5% live in extreme poverty (UNDP 2002). Under such circumstances, social issues are a far greater priority to the government than those pertaining to conservation of natural resources. Subsequently, institutions such as ANAM are chronically under-funded (Elton 1997). The situation in Panama is similar to many biologically diverse developing countries in terms of poverty, loss of biodiversity, ineffective management of protected areas and insufficient financial resources for the institutions responsible for the protection of those areas (Terborgh 1999). There is an obvious and urgent need to develop models for the protection and management of protected areas that integrate and strengthen host-country institutions. Recognizing the financial constraints upon the governments where those protected areas are found, finding innovative and effective means of using funds and expertise from external sources is crucial (Terborgh 1999).

The National System of Protected Areas in Panama includes 43 protected areas and encompasses an area of 19,000 square kilometers, approximately 25% of the national territory (Navarro 1998). In the Coiba National Park, there are three park guards on duty at any given time. As the park comprises 2700 square kilometers, each park guard is thus responsible for patrolling 900 square km (pers. comm. from Clemente Nuñez, ANAM's Director of the Coiba National Park).

B.5. Conducting Scientific Research within the Coiba National Park

As described in Section B.2, the prisoners are gradually being removed from Coiba Island, which will eliminate a significant impediment to scientific research. The ANAM facility on Coiba Island includes a simple field station that has served as a base for the research carried out to date (Castroviejo 1997). STRI has lodging facilities on the Island of Coibita (Figure 3), less than a kilometer from the ANAM facilities.

A biological laboratory is currently being constructed by Mr. Jean Pigozzi, a private landowner interested in conservation and in promoting scientific research in the Coiba National Park. The 20,000 square foot facility is located approximately 15 km from Coiba Island on the Island of Canal de Tierra, near the island of Bahía Honda (Figure 3). The laboratory will be ready by March 2003 and will comprise both a chemical laboratory and a

biological laboratory, an herbarium and a small workshop (www.liquidjunglelab.com/facilitiesfinal.html). The biological laboratory on the Island of Canal de Tierra will also include boats and diving facilities and will be at the disposal of the researchers associated with the Panama ICBG (see letter of support from Mr. Jean Pigozzi, Appendix 1). The ANAM facilities on Coiba Island, the STRI lodging facilities on Coibita and the laboratory on the Island of Canal de Tierra will greatly facilitate the collections of marine and terrestrial organisms within the Coiba National Park. STRI and the Woods Hole Oceanographic Institution have signed collaborative agreements with Mr. Pigozzi and will be involved in the administration of the laboratory.

C. PRELIMINARY STUDIES

C.1. Public Outreach

A significant emphasis of the Panama ICBG has been to actively inform governmental institutions, the scientific community, and, in particular, the public at large, about the nature of the program and the connection between biodiversity and human health. The outreach activities emphasize how the country can benefit from drug discovery research through educational opportunities, creation and support of Panama-based research programs, development of local scientific infrastructure, opportunities for economic development and contributions to human health. During the past 4.5 years, ICBG members engaged in a total of 110 outreach activities in Panama in the following fora:

- lectures at elementary and high schools
- presentations to governmental officials
- lectures to non-governmental organizations
- lectures to scientific organizations
- lectures at local universities
- lectures to the public
- radio and television appearances
- newspaper articles

There have been a total of 13 newspaper articles written on the Panama ICBG over the past 4.5 years, 11 of which appeared in *La Prensa*, Panama's most widely respected newspaper. The articles dealt with subjects such as the overall structure of the program, emphasizing the large host-country component (April 30, 1999; April 20, 2000; June 7, 2000; October 22, 2000), an article of Panamanian forests and chemical diversity (April 14, 2002),

PATENT PENDING

training African scientists in antiplasmodial assays (May 21, 2001), three articles on conservation efforts in the Coiba National Park (August 4, 2002), the use of aposematic insects (those with warning coloration) to guide plant collections for drug discovery (October 21, 2002), the research program of Dr. Luis Cubilla, an ICBG participant at the University of Panama (October 28, 2002), the development of a flourimetric technique to detect antiplasmodial compounds (December 23, 2002) and the nomination of the Coiba National Park as a World Heritage Site (January 27, 2003). Most of the lectures were given by the PIs (25), but many were given by the Panamanian participants in the ICBG as well (17). Although the impact of these outreach efforts is not easily measured, they have undoubtedly contributed to a positive perception of the program in Panama (see letter of support from Líder Sucre, Appendix 1). In response to a talk by an ICBG participant on the design of the program and the importance of biodiversity to human health, an ex-Government Minister published an editorial entitled "Save Our Forests" in *La Prensa* (August 20, 2001) in which he proposed an immediate ban on the felling of primary forests and the opening of those forests to drug discovery. In addition to these outreach efforts in Panama, a total of nine lectures have been presented by the current co-PI's in the US, Mexico, Colombia and Australia.

C.1.1. Conservation Workshops

During the previous funding period, the Panama ICBG program held three workshops which were designed to coordinate the efforts of governmental organizations, environmental non-governmental organizations (NGOs) and research institutions involved in biodiversity conservation in Panama. Two of the workshops dealt with the Coiba National Park. The workshops also provided a forum for each group to learn from the experiences of the others, which is significant for the Panamanian NGO community which does not a history of collaboration. The workshops were organized around the following themes:

- The workshop "Conservation and Sustainable Development in Panama: lessons learned and visions for the future" brought together the coordinators from Panama's five largest conservation programs, including Panama-based NGOs, foreign aid institutions, STRI and the representatives from ANAM. This half-day workshop was attended by over 80 people (November 29, 2002).
- Representatives from STRI, ANCON (a Panama-based NGO), The Nature Conservancy-Panama, and private landowners with holdings near Coiba National Park attended a workshop to coordinate activities among the conservation organizations working in the Coiba National Park (March 7, 2002).
- Representatives from STRI, ANCON, the Panamanian Maritime Authority, and ANAM attended a workshop to coordinate activities among the institutions working in the Coiba National Park (September 23, 2002).

C.2. Working with the National Authority of the Environment (ANAM)

There are two basic means by which the Panama ICBG has worked with ANAM. Firstly, permits are obtained from ANAM for all plant and marine collections and for exports of extracts and compounds to laboratories outside of Panama. The contractual framework for these transactions was established through a legal agreement between STRI and ANAM that was signed on the 17th of August of 1999. ANAM officials have expressed their willingness to renew the agreement for the next award cycle (see letter of support from Mr. Ricardo Rivera, Head of Natural Patrimony, Appendix 1). Secondly, we have provided resources and expertise to ANAM to increase the effectiveness of their National System of Protected Areas. Our collaboration with ANAM to help protect the Coiba National Park will be treated separately (Section C.3).

C.2.1. Permits for Terrestrial Plant and Marine Collections and Exports

To date, a total of 40 permits have been sought and obtained from ANAM for collecting plants and marine samples and for the export of extracts and purified compounds. Permits are routinely approved between 1-2 weeks from the date of submission.

C.2.1.1. Collecting Permits

The ICBG carries out plant collections in the National System of Protected Areas for the following reasons: (i) Panama's National System of Protected Areas encompasses 25% of the national territory and is largely representative of the country's terrestrial and marine ecosystems, (ii) collecting in National Parks provides an unambiguous means of working with the Panamanian Government (*i.e.*, ANAM), (iii) by collecting in Protected Areas, we help provide valuable information on the flora, supporting ANAM programs as well as other research and conservation programs. A total of 10 collecting permits have been solicited and granted. Each permit is valid for a period of one year and includes several Protected Areas.

C.2.1.2. Export Permits

A total of 18 permits have been obtained from ANAM in order to export samples from the ICBG to the following institutions: (i) Novartis Pharmaceuticals Corporation, (ii) the National Cancer Institute, (iii) Oregon State University, (iv) the Universidad de Santiago de Compostela in Spain, and (v) the Walter Reed Army Hospital.

C.2.2. Institutional Strengthening and Technical Cooperation

As mentioned in Section B.4 in the context of the Coiba National Park, an important element in the conservation of biodiversity in developing countries is the need to strengthen host-country institutions such as ANAM. The Panama ICBG has worked closely with ANAM both in the field and with their administrative headquarters in Panama City to provide information and resources that has resulted in substantial improvements in ANAM's operations. Contributions to ANAM include, (i) investments in database management, and (ii) providing biodiversity inventory data on plants in National Parks. Both are discussed below.

C.2.2.1. Development of Databases and Permit Management Systems for ANAM

The Panama ICBG recently developed a database that fulfills two roles: (i) it provides a digital interface for preparing research, extractive (*e.g.*, logging) and export permits and (ii) it facilitates data management. The database was created at the request of ANAM's National Director for Natural Patrimony and was built by the Panama ICBG Systems Analyst, Lorna Sánchez. ANAM personnel were involved in every aspect of the design and implementation of the system.

This system has fundamentally changed every transaction mediated by permits, from monitoring timber concessions to scientific research. Previously, permits were filled out on typewriters, an inefficient and error-prone process. The lack of an electronic system for evaluating permits made it difficult to monitor compliance, for example, determining if the species approved for harvest in a timber concession were the same as the species that were eventually harvested. The new system helps ANAM monitor the compliance of permit holders and makes it easier to compare separate permits dealing with the same area or resource. The system allows ANAM to determine easily the status of a given species, for example, to see whether a species is listed on the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) list of endangered species.

A second database was also developed by the Panama ICBG for use by ANAM's Division of Natural Patrimony in which their library of over 800 publications was cataloged, organized and labeled, and the information was subsequently placed on a computerized database. They previously relied on non-computerized methods for organizing their library and documentation on natural resources, such as environmental impact statements, management plans for National Parks and international treaties.

C.2.3. Providing Biodiversity Inventory Data on Terrestrial Plants in National Parks

We created an internet-based database system that allows all ICBG collaborators, including ANAM, to have instant access to information on ICBG plant collections and on the status of samples associated with the Panama ICBG. This database (also discussed in API, Section D.11. "Data management") gives ANAM instant access to information on all of our plant collections including their GPS coordinates, the date of collection and ecological conditions. We coordinated with a major, World Bank-supported ANAM program for sustainable development, known as the Panama Atlantic Mesoamerican Biological Corridor, to provide information on our plant collections in the Omar Torrijos H National Park. This park is a World Biosphere Reserve that includes both Costa Rica and Panama (www.biodiv.org/events/cbmap.asp).

C.3. Design and Implementation of Programs to Protect the Coiba National Park

As described in Section B.2, the Coiba National Park is an extraordinary repository of terrestrial and marine biological diversity as well as a key link in marine biological corridors of regional and international importance. There are a number of circumstances that combine to make the Coiba National Park an attractive setting for the development of novel and effective conservation strategies: (i) its terrestrial ecosystems are largely intact and the home of endemic species, (ii) it is relatively isolated and has no significant resident population, (iii) its borders can be clearly marked and enforced and (iv) its protection has elicited the interest of a range of influential organizations including Panamanian governmental institutions, international non-governmental organizations (*e.g.*, Conservation International, World Conservation Union), international research institutions (STRI, Woods Hole Oceanographic

Institution), wealthy private individuals (*e.g.*, Jean Pigozzi) and the United Nations (exemplified by the Marine Biological Corridor discussed in Section B.1.2).

Accordingly, the Panama ICBG has worked with a number of local and international institutions to develop a strategy that is designed to secure the protection of the Coiba National Park. The elements of this strategy are described in the following sections and include:

1. To have the Coiba National Park designated as a World Heritage Site by the United Nations Educational, Scientific and Cultural Organization (UNESCO).

2. To carry out an environmental education campaign with the local communities about the importance of the Coiba National Park and the potential benefits they may derive when the park's ecosystems are effectively conserved.

3. To coordinate with ANAM, the Woods Hole Oceanographic Institution and Conservation International to install a system of buoys to delimit the park's boundaries and radars to detect the entry of fishing boats within the park's boundaries.

The combined impact of these activities is likely to have a significant impact on the park's protection, most immediately, to reduce fishing within the park boundaries. It has been demonstrated in other marine protected areas that when an area is protected from over-fishing, the adjacent fisheries are enhanced to the benefit of surrounding communities (Sumaila 2000, Roberts *et al.* 2001). Other benefits to those communities are also seen by enhanced activities such as tourism and recreation (Sumaila 2000, White *et al.* 2002). By creating economic opportunities, the combined benefits to local communities are expected to decrease the threats to the Coiba National Park that were described in Section B.3. (White *et al.* 2002).

C.3.1. World Heritage Site Status for the Coiba National Park

From October 2002 to January 2003, the Panama ICBG worked closely with ANAM in order to nominate the Coiba National Park for placement on the list of UNESCO's World Heritage sites. The information for the application was supplied primarily by ICBG personnel who then worked closely with the Division of Natural Patrimony on subsequent drafts. The final application was submitted by ANAM to UNESCO headquarters on the 21st of January, 2003. The inscription of the Coiba National Park to this list will be conducive to the park's protection in several ways:

C.3.1.1. The Convention for World Heritage Sites is a legally binding treaty in which the State Party pledges protection of its properties on the list of World Heritage Sites

Among other pledges, the state is obliged to "...ensure that effective and active measures are taken for the protection, conservation and presentation of the cultural and natural heritage situated on its territory" and "not to take any deliberate measures which might damage directly or indirectly the cultural and natural heritage".

C.3.1.2. The Coiba National Park will benefit from the enhanced national and international profile that will be associated with the nomination

The park still suffers from its image as a distant, inaccessible and largely unseen penal colony, leading to a degree of neglect that has been conducive to illegal fishing within the park's boundaries, resource extraction and illegal squatting.

C.3.1.3. Fund-raising efforts for the park's protection will be facilitated

Institutions and individuals will be more inclined to donate money and resources to the protection of the Coiba National Park when the government takes concrete and conspicuous steps that promote the park's protection. There

are now funds available only to World Heritage Sites, in particular, the newly created $15 million fund from Private Source press release from November 15, 2002, www.conservation.org/xp/CIWEB/newsroom/press_releases/ 111502.xml).

C.3.2. Working with Local Communities that Fish within the Park Boundaries

C.3.2.1. Socioeconomic Survey and Environmental Education in Bahía Honda

One of the six communities whose fishermen most routinely fish in the Coiba National Park is that of Bahía Honda (Figure 3). Located 15 km from Coiba Island, the residents of Bahía Honda have traditionally used the waters of the Coiba National Park for fishing and they will be key players in the development of any realistic strategy to protect the park (Moretti 2002). Their proximity to the Coiba National Park and destitute poverty (UNDP 2002) suggests that the resources of the park such as valuable hardwoods, and marketable wildlife such as Scarlet Macaws, are likely to represent a significant temptation once the prisoners are removed from the island.

To gain a greater understanding of the community of Bahía Honda, from October 2002-June 2003, a visiting Fulbright Scholar working with the Panama ICBG has worked in the community to gain insight into some basic issues such as: (i) What are the greatest socioeconomic issues facing the communities surrounding the Coiba National Park that may impede successful conservation programs?, (ii) How can environmental education and sustainable development programs increase motivation to promote conservation?, and (iii) How can programs and alternative sources of income be developed that are sensitive to the socioeconomic needs of the residents of Bahía Honda, while effectively protecting the Coiba National Park?

A standardized interview is being implemented in each of the 116 households in the community of Bahía Honda. The questionnaire includes basic socioeconomic information such as housing, health and nutrition, culture and religion, and individual attitudes regarding fishing practices, conservation, tourism and alternative sources of income. The survey was designed in collaboration the Panamanian Maritime Authority (which has extensive experience in the implementation of Integrated Coastal Management programs in Panama), the Duke University Nicholas School of the Environment and the Duke University Center for Aging and Human Development (both with experience in the design and implementation of qualitative surveys), and physicians and epidemiologists from Panama's Gorgas Memorial Institute for Health Research. The program in Bahía Honda is being implemented in collaboration with Panamanian non-governmental organizations (NGOs) ANCON (Panama's largest environmental NGO) and ProMar (an NGO dedicated to environmental education regarding marine resources) (see Letters of Support from Líder Sucer and Carlos Bieberach, Appendix 1).

It is expected that the results of this work will not only provide insight into the community of Bahía Honda, but will also facilitate our work with other communities within the Gulf of Chiriquí that fish within the Coiba National Park and who may also pose a threat to the park's natural resources. The extensive coordination with Panama-based organizations will help ensure that the work will have the continuity and consistency necessary for a long-term impact.

C.3.2.2. Environmental Education in Bahía Honda

The survey discussed in the preceding section indicated that the residents of Bahía Honda do not have a clear understanding of the purpose of Coiba National Park nor of the benefits that the park could provide to the community. In response, an environmental education program has been organized for the community by the ICBG and the NGOs ProMar and ANCON and includes an introduction to ecology; environmental contamination and its effects; the purpose and characteristics of national parks in Panama; the importance of Coiba National Park and potential economic benefits that can result from the park's protection (*e.g.*, from an increase in research and tourism activities). A major goal of this education campaign is to develop an appreciation among community members of the potential ecological and personal benefits of conserving the Coiba National Park.

C.3.2.3. Previous Coastal Management Studies

Hector Guzmán, whose studies of the corals of the Coiba National Park were mentioned in Section B.2.1.2, has carried out research in Bocas del Toro in northwestern Panama (Figure 1) and in Cayos Cochinos, Honduras, in order to understand the impact of local communities on nearby marine protected areas, their attitudes towards protected areas and their relationships with park authorities (Barahona and Guzmán 1998, Guzmán and Guevara 1998, Guzmán and Jácome 1998). The results of his research have been used to make recommendations to local authorities regarding the design of marine protected areas and to develop strategies for working with communities. Dr. Guzmán has also contributed to the design of the studies with the community of Bahía Honda, discussed in the preceding section.

C.3.3. Installation of a System of Buoys to Indicate the Boundaries of the Coiba National Park and Radar to Detect the Entry of Boats

The survey discussed in Section B.3 indicated a widespread unawareness of the park's boundaries by local fishermen in addition to the need for enhanced enforcement of fishing activities by ANAM personnel. In response to those needs, a proposal was submitted to Conservation International's Global Conservation Fund (GCF) for a feasibility study that would determine; (i) a cost-effective strategy for placing buoys around the boundaries of the Coiba National Park in order to clearly indicate the park's boundaries and, (ii) to employ a system of radars to detect the entry of boats into the park. The proposal was submitted in collaboration with the Woods Hole Oceanographic Institution, the largest independent oceanographic research institution in the United States and a leader in the design and installation of buoys (see letter of support from Larry Madin, Appendix 1). The feasibility study was approved and work began in August of 2002. A collaborative agreement has been drafted between ANAM, STRI and Conservation International that details the role of each of the institutions. The draft has been approved by STRI and Conservation International and is currently under revision by ANAM.

C.3.3.1. The Use of Buoys to Delimit the Boundaries of the Coiba National Park

Buoys can be used to mark the boundaries of marine protected areas and help reduce illegal fishing (Roberts and Hawkins 2000). As part of the feasibility study supported by the Global Conservation Fund, engineers from the Woods Hole Oceanographic Institution carried out a study of the ocean floor and the ocean depths around the entire boundary of the Coiba National Park (Figure 3) in order to design the appropriate buoys and a strategy for their deployment. Combining these data with the results from the survey of fishermen (Section B.3.1) and the observations of ANAM park guards, a program was designed that involves the placement of buoys around the perimeter of the park. The spacing of the buoys is contingent upon cost and the nature of the fishing activities. Buoys will be spaced closely (1 mile intervals) where small scale fishing activities are greatest and where fishermen are likely to rely upon visual contact with the buoys (northern, eastern and southern boundaries of the park) and at greater intervals where larger commercial boats operate that employ radar (western boundary, Figure 3).

C.3.3.2. The Use of Radar to Detect the Entry of Boats within the Coiba National Park

The extensive marine area of the Coiba National Park (2,165 square km) suggests that routine patrolling by boat to detect illegal fishing is not a cost-effective option. Alternatively, the use of conventional marine radars based on Coiba Island will permit the detection of boats within the park boundaries without patrolling. Radars have been employed in other marine protected areas, such as the Tubbataha Reef National Marine Park in the Philippines, which is also to World Heritage Site (Dobkowski 2001, White *et al.* 2002). Upon detection of a boat by radar, fishermen can be contacted by radio and, if necessary, the boat can then be approached by personnel from ANAM and the National Police. Technicians from a local supplier of marine navigational radars made two trips to the Coiba National Park and have suggested the placement of 10 KW radars at the following locations, (i) the biological station maintained by ANAM (the northern end of Coiba Island), (ii) the island of

Jicarón and, (iii) Punta Hermosa, on the western side of the island (Figure 3). The regions covered by the three radars are subjected to heavy illegal fishing according to both ANAM park guards and fishermen.

Based on the results of the feasibility study, on February 20-23, 2003, the full proposal to employ buoys and radars in the Coiba National Park will be presented to Conservation International's Board of Directors, a reflection of the importance of the program to the organization. The activity will be presented in the context of the Marine Biological Corridor program mentioned previously in which Conservation International is a key participant (Section B.1.2). Additional funds for this program have also been pledged from

Private Source

C.4. Biodiversity Inventory of Corals from the Coiba National Park and Preliminary Bioassay Results

In Section B.2.1.2, the results of preliminary biodiversity inventories of the coral reefs and communities in the Coiba National Park by Dr. Hector Guzmán were described. In the summer of 2002, octocoral and hard corals (scleractinians) were collected from sites throughout the Coiba National Park. While the taxonomy of those species is currently being determined by Dr. Guzmán, at least one of the octocorals, *Pacifigorgia rubinoffi*, is a new species (Breedy and Guzmán 2003). The holotype of *P. rubinoffi* was collected near Brincanco Island (Figure 3). Several other species appear to be new to science as well but they have not yet been named (H. Guzmán pers. com).

As octocorals are known to be sources of biologically active compounds (*e.g.*, Ospina *et al.* 2003), we combined Dr. Guzmán's investigation with the drug discovery activities of the Panama ICBG. Working in collaboration with Dr. Ricardo Riguera, a marine natural products chemist from the Department of Organic Chemistry in Spain's Universidad de Santiago de Compostela, extracts from 13 octocoral species were tested for anti-parasitic activity using the assays described in Associate Program 2. Preliminary tests indicated that extracts from several of the octocoral species have activity against *Plasmodium falciparum* including *P. rubinoffi* (percentage plasmodial viability < 12 % when tested at a single concentration of 50 µg/mL). Species that showed anti-plasmodial activity are currently being evaluated at lower concentrations in order to confirm their activity. Promising candidates will be subjected to bioassay-guided fractionation in the laboratory of Dr. Riguera by Mr. Marcelino Gutiérrez, a chemist formerly associated with the Panama ICBG and currently pursuing doctoral studies with Dr. Riguera.

While the primary focus of the drug discovery activities of the ICBG will involve terrestrial and marine plants (Associate Programs 1 and 3), the isolation of biologically active compounds from octocorals represents a means of adding value to the marine biodiversity inventory work we propose to carry out in the Coiba National Park. It is also a means by which a young Panamanian chemist can pursue drug discovery on organisms from Panama while training in a well recognized natural products laboratory. The collaboration between STRI and Universidad de Santiago de Compostela is carried out under a Memorandum of Understanding between the two institutions signed on May 8th, 2002, and which establishes that any compounds of interest to drug discovery will be subject to the provisions of the legal agreement between STRI and ANAM for the ICBG program. Representatives from STRI and Universidad de Santiago de Compostela are currently developing a comprehensive legal agreement that will fully define the working relationship.

D. RESEARCH DESIGN AND METHODS

This ICBG proposal comprises four Associate Programs which are described in detail in the Group Proposal. The chart in Figure 4 provides an overview of the Associate Program interactions and their leaders.

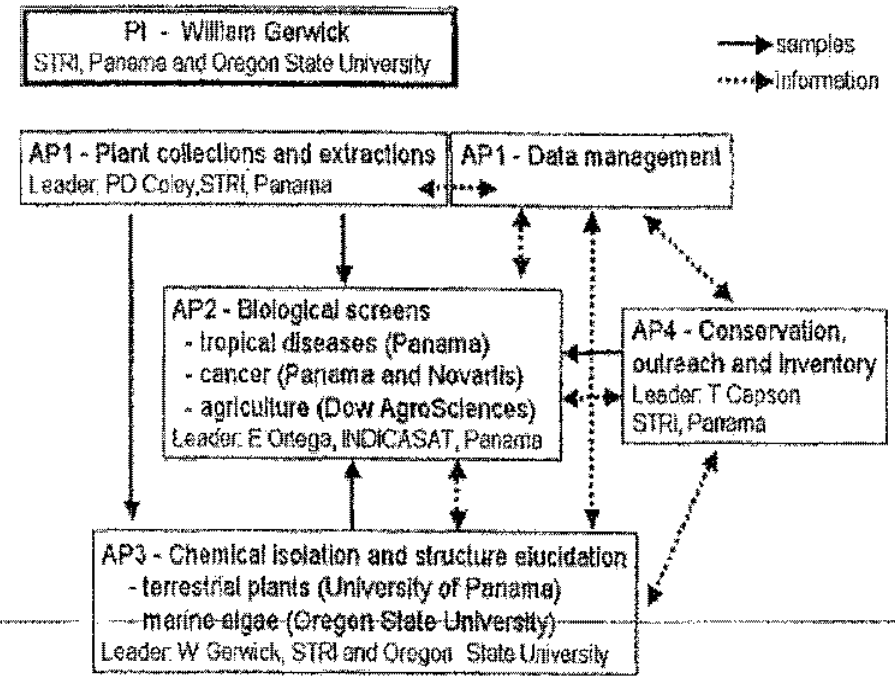


Figure 4: Overview of the Associate Program interactions and interrelationships in this ICBG proposal, including sample and data transmissions.

The conservation initiatives for the protection of Coiba National Park will be spearheaded by Dr. Capson in collaboration with the organizations shown in Figure 5. The other key personnel in Associate Program 4 are Drs. Ibáñez and Guzmán, who are responsible for the AP4 terrestrial and marine biodiversity inventories, respectively.

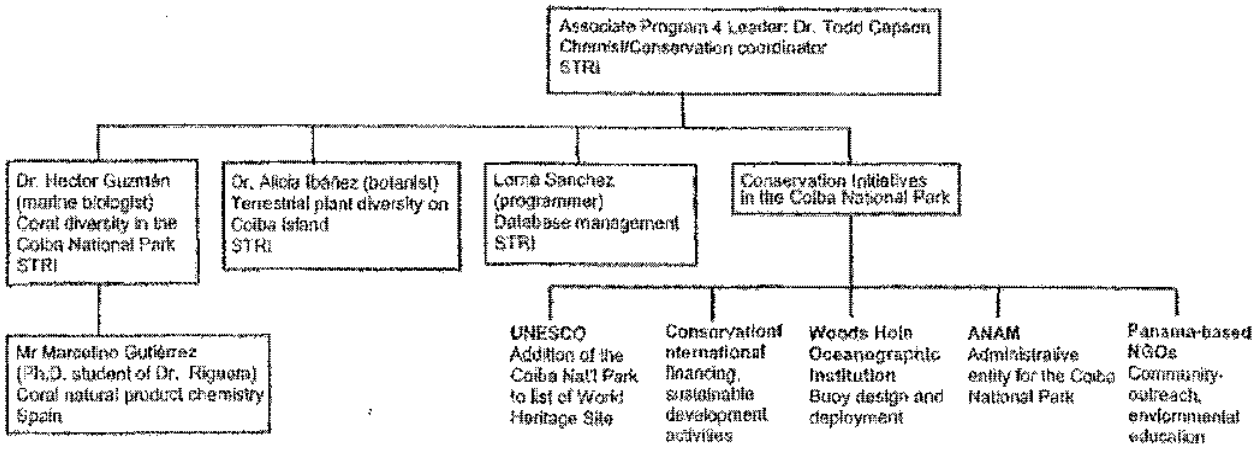


Figure 5: Organizational chart of key personnel/collaborators in Associate Program 4 and their research areas and affiliations

D.1. Public Outreach

D.1.1. Lectures and Workshops

The Panama ICBG will continue to engage in outreach activities that reach all levels of Panamanian society, using the fora described in Section C.1., *e.g.*, lectures at elementary and high schools, universities, to governmental and non-governmental organizations (NGOs) and to community groups. Similar to the preceding 4.5 years, workshops between governmental and NGOs will be held as frequently as necessary in order to ensure coordination and cohesion, in particular, when different groups are working in the same area, such as the Coiba National Park. STRI provides an excellent setting for such workshops. The contacts that have been established during the past 4.5 years, for example, with public schools, with ANAM, with Panama-based NGOs and newspapers such as *La Prensa*, will provide an invaluable network for disseminating results.

D.1.2. New Directions for Public Outreach

In addition to the activities described in the preceding section, we will seek new channels for promoting the importance of biological diversity in Panama. An explicit goal of the public outreach efforts for the next five years will be to make contributions with greater permanence, such as classroom and textbook materials. ANAM has an effective environmental education program and has worked with the Panamanian Ministry of Education in the preparation of a wide variety of materials for schools and local communities. We will coordinate with ANAM in order to incorporate information pertaining to the importance of biological diversity for human health. Materials such as that shown in Figure 6, which shows the use of aposematic insects (insects with defensive coloration) to guide drug discovery will be prepared (see letter of support from Ms. Mabel Morcillo, National Director of Promotion of Environmental Education, ANAM, Appendix 1).

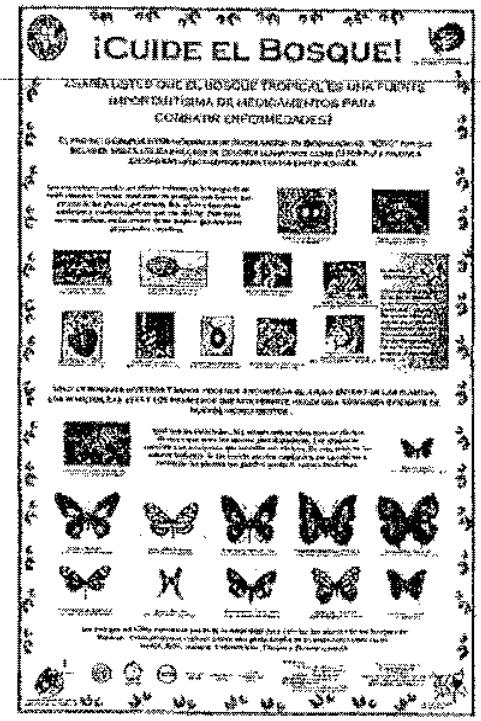


Figure 6. Didactic material prepared by the Panama ICBG for environmental education purposes. The title reads "Protect the forest! Did you know that the forest is an important source of medicines to treat disease?" The poster shows aposematic insects that feed on host plants with toxic secondary metabolites. See Appendix 5 for the color version.

D.2. Working with the National Authority of the Environment (ANAM)

We will continue to work with ANAM over the next five years much as we have in the past. The Office of Natural Patrimony has requested our assistance in the design and implementation of databases similar to the database that was developed for managing permits (Section C.2.2.1) for the Departments of Hydrologic Resources (see letter of support from Ricardo Rivera, Director of Natural Patrimony, Appendix 1). Such measures can be very cost-effective and constitute permanent and substantive contributions to ANAM's operational capacity in addition to creating good-will for the ICBG program.

D.3. Conservation Activities in the Coiba National Park

D.3.1. Installation of a System of Buoys and Radar to prevent Illegal Fishing in the Park

Upon approval of the funds by Private Source work will begin on the installation of the buoys and radars around the Coiba National Park (Section C.3.3), most likely in the fall of 2003. ICBG funds will not be used for these activities other than a portion of the Associate Program leader's time (Todd Capson). For all conservation activities carried out by the Panama ICBG in the Coiba National Park, we will continue to work closely with the Panama-based NGO, ANCON, to ensure that our programs are complementary. ANCON has received funds from the AVINA foundation, an institution that works in partnership with civil society and business leaders in sustainable development initiatives in Latin America (www.avina.net). As described in Section C.3.3.2, with funds from Private Source will hire 6 additional park guards and build the two additional ANAM outposts (Figure 3, see letter of support from Lider Sucre, Executive Director of ANCON, Appendix 1).

D.3.2. Working with Marine Resource Users in the Coiba National Park

The environmental education and community outreach work with the community of Bahía Honda described in Section C.3.2. is in progress. The experience gained in that community will be applied to other communities in the Gulf of Chiriquí that are known to fish within the limits of the Coiba National Park (Section B.3.1). In order to add continuity and consistency to the community outreach work, we will work closely with the Panamanian Maritime Authority and the Panama-based NGOs, ANCON and ProMar with whom we are already coordinating our activities (see letters of support from Lider Sucre, Executive Director of ANCON, and from Carlos Bieberach, President of ProMar, Appendix 1). All of these organizations have extensive experience in environmental education in Panama. The Panamanian Maritime Authority has worked in the Panamanian province of Darién to implement programs of integrated coastal management and their experience will be valuable in the Coiba National Park (see letter of support from Arnulfo Franco, Director of Marine Resources for the Panamanian Maritime Authority, Appendix 1). We will also consult routinely with Dr. Hector Guzmán whose experience with coastal communities in Bocas del Toro (northwestern Panama) and Honduras will be valuable (Barahona and Guzmán 1998, Guzmán and Guevara 1998).

D.3.3. Development of Sustainable Economic Initiatives in the Coiba National Park in Collaboration with Conservation International

As mentioned in Section C.3, examples from other marine protected areas show that the adjacent communities benefit in a number of ways once an area is protected from over-fishing, including enhanced fisheries outside of the protected area and economic opportunities that result from tourism and recreation (Sumaila 2000, White *et al.* 2002). Accordingly, an expected outcome of the conservation activities in the Coiba National Park is the creation of new economic opportunities for the communities surrounding the park and a decrease of the threats to the Coiba National Park that were described in Section B.3.

The Panama ICBG has worked closely with Conservation International in the development of conservation strategies for the Coiba National Park, and will continue to do so for the next funding cycle (see letter of support from Russell Mittermeier, President of Conservation International, Appendix 1). Conservation International's Conservation Enterprise division works worldwide with communities to develop products and to open markets that create economic incentives to conserve their natural resources rather than destroy habitats. Conservation

International has developed conservation enterprises in nearly 20 countries and also has extensive experience in the development of ecotourism programs throughout Latin America.

Combined with the contacts established by the Panama ICBG with the government and other institutions, the expertise of Conservation International will facilitate the development of programs with the communities in the Gulf of Chiriquí that will provide alternatives to resource extraction from the Coiba National Park. Much of the information needed to implement those programs will be provided by the survey work now being carried out in Bahía Honda, which will be extended to the other communities in the Gulf of Chiriquí that are known to have an impact on the Coiba National Park's resources.

D.4. Biodiversity Inventories in the Coiba National Park and Drug Discovery

Aside from the AP leader's salary, the primary use of ICBG funds for this Associate Program will be for the biodiversity inventories in the terrestrial and marine ecosystems of the Coiba National Park. In order to take advantage of available expertise and to provide a logical interface to the drug discovery activities of the ICBG we will focus on terrestrial plants on Coiba Island and octocorals in the park's marine ecosystems.

D.4.1. Terrestrial Plant Biodivesity Inventory

The primary ICBG-sponsored activity for this Associate Program is the botanical inventory for Coiba Island. As mentioned in Section B.2.1.1, the interior section of the island is largely unexplored and is likely yield additional endemic species. We propose to hire Dr. Alicia Ibáñez, a Ethnicity botanist who spent 4 years studying the flora of Coiba Island for her Ph.D. work and is uniquely qualified for this task (Ibáñez 2001, Ibáñez and Castroviejo 2001, see letter of support from Alicia Ibáñez, Appendix 4). Dr. Ibáñez will work closely with other ICBG botanists and STRI botanist, Mireya Correa (Associate Program 1, see letter of support from Mireya Correa, Appendix 2). Dr. Ibáñez will be working with two student interns who will receive training while working as her assistants. Dr. Ibáñez estimates that there is a total of 2000-2500 species on Coiba Island (pers. com.). She estimates that she will collect 1,000 species during year 1 of the study, 500 species during year 2, 250 species during year 3, 150 species during year 4 and 100 species during year 5, as it becomes increasingly difficult to encounter new species. Herbarium vouchers will be stored in herbariums at STRI, the University of Panama and the Royal Botanical Garden in Madrid, Spain.

D.4.2. Marine Coral Biodiversity Inventory

Dr. Guzmán estimates that he will make two trips per year to the Coiba National Park for each of the 5 years of the program. He will utilize the R/V Urracá, STRI's 96-foot research vessel that is fully equipped for making marine collections. There are an estimated 60 octocoral species and 20 hard corals (scleractinians) in the Coiba National Park. When the species are unknown, they will be collected and their taxonomy determined. For both known and unknown coral species within the park, data will be recorded with regard to their abundance, precise location (determined by GPS), distribution, and any other data relevant to the ecology or biology of the species. Work is already underway, resulting in the discovery of *Pacifigorgia rubinoffi* (Breedy and Guzmán 2003, Section C.4). The work will take place largely but not exclusively in the Coiba National Park as there are areas outside of the park, but within the Gulf of Chiriquí, that are known to have a rich diversity of corals and marine invertebrates, e.g., Secas Islands, approximately 45 km northwest of Coiba Island (Glynn and Maté 1997). It is anticipated that all of the corals within the Coiba National Park, and other areas of interest within the Gulf of Chiriquí, will have been identified and mapped during the course of the ICBG program. His taxonomic work will be supported in part by ICBG funds, in particular the travel to museums for the taxonomic work necessary to identify new species (an estimated two trips per year). As an expert in the ecology and population dynamics of coral reefs, and with extensive experience in both the Panamanian Caribbean and Pacific, he is highly qualified for the proposed work (see letter of support from H.Guzmán, Appendix 2). The coral holotypes he collects will be stored in the Museo Zoología, Escuela de Biología, Universidad de Costa Rica (UCR) and paratypes will be deposited in the UCR, the Smithsonian Institution's National Museum of Natural History and at the Museum of Comparative Zoology,

Harvard University, Cambridge, MA. The marine plant biodiversity inventories in the Coiba National Park will include algae and cyanobacteria, both of which are important elements of the drug discovery components of this ICBG (Associate Programs 1 and 3) and a major component of Dr Gerwick's research program. The survey of algae and cyanobacteria will be carried out by Drs. Bill Gerwick, Valerie Paul and Kerry McPhail (Associate Program 1).

D.4.3. Marine Macroalgae and Cyanobacteria Biodiversity Inventory

We anticipate that at least one collection trip per year will be made to Coiba National Park by Oregon State University participants (Drs. McPhail and Gerwick). Two voucher specimens for submission to the STRI and University of Panama herbaria (see AP1, Section D.5) will be prepared from each macroalgal or cyanobacterial sample collected. Collection samples will then be transported to Oregon for extraction and prefractionation (as part of AP1). In Oregon, the algal specimens will be identified with assistance from Dr. Valerie Paul when necessary for more problematic species. Identification of macroalgae will be made primarily with the aid of two comprehensive guides by Littler and Littler, one for the Caribbean flora (2000) and one for South Pacific flora (2003; see AP1 Section D.7). We also intend to use the extensive survey data resulting from a Ph.D. project by Wysor (2002) some of which are presented online (Wysor 2000). These taxonomic records will be added to the biodiversity inventory databases maintained by Associate Program 1.

D.5. Providing Selected Plants and Octocorals for Drug Discovery

D.5.1. Drug Discovery from Terrestrial Plants Collected in the Coiba National Park

When species new to the Panama ICBG are discovered from the botanical inventories described in the preceding section, plants will be stored in ethanol and stored in freezers on Coiba Island or in the biological laboratory on the Island of Canal de Tierra. Samples will then be shipped as necessary to STRI laboratories using boats and terrestrial transportation from ANAM or the laboratory on the Island of Canal de Tierra. Plants will be processed in the ICBG laboratories at STRI (Associate Program 1) and tested for antiparasitic and cytotoxic activity in the bioassays described in Associate Program 2.

D.5.2. Drug Discovery from Octocorals from the Coiba National Park

This project has been initiated through collaboration with a previous student of the University of Panama, Mr. Marcelino Gutiérrez, who is currently pursuing doctoral studies in Spain (described in Section C.4). He will carry out bioassay-guided fractionation in Dr. Riguera's laboratory in Spain. Extracts of these Panamanian corals will be prepared in the STRI laboratories currently in use by the ICBG program, using protocols established by the Riguera laboratory (Fernandez 1996). Extracts will be tested for antiparasitic and cytotoxic activity in the medicinally-relevant assays established in Associate Program 2. All collections and recollections of octocorals will be coincident with Dr. Guzmán's studies and will not require the use of ICBG funds, although support is requested for Dr. Guzman's taxonomic studies.

D.6. Using World Heritage Sites Status for the Coiba National Park to Promote the Protection of the Park and Additional Fund-raising Activities

We will work closely with ANAM and UNESCO in order to place the Coiba National Park on the list of World Heritage Sites a process which takes approximately 1 year from the time of submission (January 24, 2003, pers. com. from Marjaana Kokkonen, Associate Expert, Natural Heritage, UNESCO). As stated in Section C.3.1., the addition of the Coiba National Park to the list of World Heritage Sites will facilitate the search for additional funds which will allow us to increase the impact of the activities supported by the ICBG program. One potential source of funding is the $15 million fund established by the Private Source Private Source Two uses of additional funds are listed below:

(i) To create of a trust fund which will be used to fund the long-term protection of the Coiba National Park. The interest generated from a trust fund would be used to pay for items such as maintenance of buoys and radars and salaries of park guards.

(ii) To increase the scale of both marine and terrestrial biodiversity inventories in the Coiba National Park described in Section D.4. Additional funds would permit more frequent trips to the park and would facilitate the additional involvement of Panamanian scientists and students.

E. HUMAN SUBJECTS
None

F. VERTEBRATE ANIMALS
None

G. LITERATURE CITED

Allen GR and DR Robertson. 1994. *Fishes of the tropical eastern Pacific.* University of Hawaii Press, Honolulu, Hawaii.

Barahona GM and HM Guzmán. 1998. Encuesta socio-ecológica de las poblaciones residentes de la Reserva Biológica Cayos Cochinos, Honduras. *Rev. Biol. Trop.* 46:39-55.

Breedy O and HM Guzmán. 2003. A new species of *Pacifigorgia* (Coelenterata: Octocorallia: Gorgoniidae) from Panamá. *Zootaxa* 128:1-10.

Bryant D, Burke L, McManus J and M Spalding. 1998. *Reefs at Risk: A map-based indicators of threats to the World's coral reefs.* World Resources Institute, Washington D.C.

Castroviejo S. 1997. *Flora y fauna del Parque Nacional de Coiba (Panamá).* Ed. Castroviejo, S. Agencia Española de Cooperación Internacional, Madrid, Spain.

Castroviejo S and A Ibáñez. 2001. Origen y análisis de la diversidad biológica de la isla de Coiba. *Quercus.* Octubre, 29-32.

Christie P, White A and E Deguit. 2002. Starting point or solution? Community-based marine protected areas in the Phillippines. *Journal of Environmental Management* 66: 441-454.

Coates AG and JA Obando. 1996. The geologic evolution of the Central American isthmus. In *Evolution and Environments in Tropical America.* Ed(s) B.C. Jackson, A.F. Budd and A.G. Coates. p 21-56.

Cortés J. 1997. Biology and geology of eastern Pacific coral reefs. *8th International Coral Reef Symposium*, Smithsonian Tropical Research Institute, Balboa, Republic of Panama, pp. 57-64.

D'Arcy WC. 1987. Flora of Panama Checklist and Index. Missouri Botanical Garden, St. Louis, MO.

Dobkowski K, Gallegos Y, Hoffman J and A Mapa. 2001. Fact Sheet on Management of Nine Coastal and Marine Protected Areas. Internship of the University of San Francisco, School of Law.

Elton C. 1997. *Panamá: Evaluación de la Sostenibilidad Nacional.* Centro de Acción Social Panameño. Poligráfica S.A. Panama, Republic of Panama.

Fernandez R, Rodriguez J, Quiñoa E, Riguera R, Muñoz L, Fernandez-Suarez M and C Debitus. 1996. Onchidin B. A new cyclodepsipeptide from the marine mollusc *Onchidium sp. J. Am. Chem. Soc.* 118:11635-11643.

Food and Agricultural Organization (FAO). 2001. *State of the World's Forests.* Food and Agricultural Organization of the United Nations, Rome, Italy.

Guzmán HM and CA Guevara. 1998. Arrecifes coralinos de Bocas del Toro, Panama: I. Distribución, estructura y estado de conservación de los arrecifes continentales de la Laguna de Chiriquí y Bahía Almirante. *Rev. Biol. Trop.* 46: 601-623.

Guzmán HM and G Jácome. 1998. Pesca artesanal en la Reserva Biológica Cayos Cochinos, Honduras. *Rev. Biol. Trop.* 46:151-163.

Glynn PW and JL Maté. 1997. A field guide to the Pacific coral reefs of Panama. *8th International Coral Reef Symposium*, Smithsonian Tropical Research Institute, Balboa, Republic of Panama, pp. 145-166.

Ibáñez A. 2001. *Estudio de la composición florística y ecología del bosque tropical de la isla de Coiba (Panamá).* Dissertation, Facultad de Biología, Department of Botany, University of Salamanca, Spain.

Littler DS and MM Littler. 2000. *Carribean Reef Plants.* Offshore Graphics, Inc. Washington, D.C. 542 p.

Littler DS and MM Littler. 2003. *South Pacific Reef Plants.* Offshore Graphics, Inc. Washington, D.C. 331 p.

Moretti GS. 2002. *Identifying and understanding resource users of Panama's Coiba National Park.* M.S. thesis for the Duke University Nicholas School of the Environment and earth sciences.

Submitted

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB and J Kent. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403: 853-858.

Navarro JC. 1998. *Panama. National Parks.* Ediciones Balboa S.A. Panama City, Panama.

In Press

Robbins CS, Sauer JR, Greenberg RS and S Droege. 1989. Population declines in North American birds that migrate to the Neotropics. *Proc. Natl. Acad. Sci.* 86: 7658-7662.

Roberts CM and JP Hawkins 2000. *Fully protected marine reserves: a guide.* World Wildlife Fund, Washington, D.C.

Roberts CM, Bohnsack JA, Gell F, Hawkins JP and R Goodridge. 2001. Effects of marine reserves on adjacent fisheries. *Science* 294:1920-1923.

Sumaila UR, Guénette S, Alder J and R Chuenpagdee. 2000. Addressing ecosystem effects of fishing using marine areas. *Journal of Marine Science* 57:752-760.

Terborgh J. 1999. *Requiem for Nature.* Island Press, Washington, D.C.

Tosi J. 1971. *Zonas de vida. Republica de Panamá.* FO:SF/PAN 6, Informe Tecnico 2. FAO, Roma. p. 123.

UNDP (United Nations Development Programme). 2002. *Informe Nacional de Desarrollo Humano Panama 2002.* United Nations Development Programme.

Velayos M, Correa M, Galdames C, Castroviejo S and B Araúz. 1997. Primera aproximación al catálogo de la isla de Coiba (Panamá). In: *Flora y fauna del Parque Nacional de Coiba (Panamá).* Ed. Castroviejo S. Agencia Española de Cooperación Internacional. pp. 245-328

Wellington GM. 1997. A field guide to the corals and coral reefs of the Galapagos Islands, Ecuador. 8th *International Coral Reef Symposium*, pp. 145-166. Smithsonian Tropical Research Institute, Balboa, Republic of Panama.

White AT and P Christie. 2000. Conclusions. *Coastal Management* 28:119-122.

White AT, Courtney CA and A Salamanca. 2002. Experience with marine protected area planning and management in the Phillippines. *Coastal Management* 30:1-26.

Wysor B, Kooistra WHCF and S Fredericq. 2000. Marine Macroalgal Diversity of the Republic of Panama. Poster presentation at the Annual Meeting of the Phycological Society of America, San Diego, CA, 15-19 July 2000 http://moravec.louisiana.edu/SeaweedsLab/Wysor/PSA_2000/poster.html#anchor2837646

Wysor BS. 2002. Biodiversity and biogeography of marine green algae of the Republic of Panama. Univ. of Louisiana, Lafayette, LA, USA. Avail. UMI, Order No. DA3045165. 291 p. From: Diss. Abstr. Int., B 2002 63: 1115.

H. CONSORTIUM/CONTRACTUAL ARRANGEMENTS

None

I. LETTERS OF SUPPORT (Consultants/Collaborators)

Mr. Carlos Bieberach, President of ProMar (see letter of support, Appendix 1).

Mr. Arnulfo Franco, Director of Marine Resources for the Panamanian Maritime Authority (see letter of support, Appendix 1).

Mr. Marcelino Gutiérrez, Ph.D. Candidate, Department of Organic Chemistry, Chemistry Faculty, Universidad de Santiago de Compostela, Spain (see letter of support, Appendix 2).

Dr. Hector Guzmán, Staff Scientist, Smithsonian Tropical Research Institute (see letter of support, Appendix 2)

Dr. Ricardo Riguera, Professor, Department of Organic Chemistry, Chemistry Faculty
Universidad de Santiago de Compostela, Spain (see letter of support, Appendix 2)

Private Source

Mr. Ricardo Rivera, Director of Natural Patrimony, ANAM (see letter of support, Appendix 1).

Ms. Lorna Sánchez. Systems Analyst for the Panama ICBG (see letter of support, Appendix 2).

Mr. Líder Sucre, Executive Director, ANCON (see letter of support, Appendix 1).

Ms Mabel Moreillo, Director of Promotion of Environmental Education, National Authority of the Environment, Panama (see letter of support, Appendix 1).

Mr. Russell Mittermeier, President of Conservation International (see letter of support, Appendix 1)

Principal Investigator/Program Director (Last, first, middle): Gerwick, William Henry

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

[X] NEW application. *(This application is being submitted to the PHS for the first time.)*

[ ] SBIR Phase I [ ] SBIR Phase II; SBIR Phase I Grant No. _____ [ ] SBIR Fast Track

[ ] STTR Phase I [ ] STTR Phase II; STTR Phase I Grant No. _____ [ ] STTR Fast Track

[ ] REVISION of application number: _____
(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

[ ] COMPETING CONTINUATION of grant number: _____
(This application is to extend a funded grant beyond its current project period.)

INVENTIONS AND PATENTS
(Competing continuation appl. and Phase II only)

[ ] No [ ] Previously reported

[ ] SUPPLEMENT to grant number: _____
(This application is for additional funds to supplement a currently funded grant.)

[ ] Yes. If "Yes," [ ] Not previously reported

[ ] CHANGE of principal investigator/program director.
Name of former principal investigator/program director: _____

[ ] FOREIGN application or significant foreign component.

1. PROGRAM INCOME *(See Instructions.)*
All applications must indicate whether program income is anticipated during the period(s) for which grant support is request. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)

2. ASSURANCES/CERTIFICATIONS *(See Instructions.)*
The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/certifications are provided in Section III. If unable to certify compliance, where applicable, provide an explanation and place it after this page.

•Human Subjects; •Research Using Human Embryonic Stem Cells• •Research on Transplantation of Human Fetal Tissue •Women and Minority Inclusion Policy •Inclusion of Children Policy• Vertebrate Animals• •Debarment and Suspension; •Drug- Free Workplace *(applicable to new [Type 1] or revised [Type 1] applications only)*; •Lobbying; •Non-Delinquency on Federal Debt; •Research Misconduct; •Civil Rights (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age Discrimination (Form HHS 680 or HHS 690); •Recombinant DNA and Human Gene Transfer Research; •Financial Conflict of Interest (except Phase I SBIR/STTR) •STTR ONLY: Certification of Research Institution Participation.

3. FACILITIES AND ADMINSTRATIVE COSTS (F&A) INDIRECT COSTS. See specific instructions.

[ ] DHHS Agreement dated: _____ [ ] No Facilities And Administrative Costs Requested.

[ ] DHHS Agreement being negotiated with _____ Regional Office.

[X] No DHHS Agreement, but rate established with Office of Naval Research Date 10/01/02

CALCULATION* *(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information.)*

a. Initial budget period:	Amount of base $		x Rate applied		% = F&A costs	$ 78,456
b. 02 year	Amount of base $		x Rate applied		% = F&A costs	$ 79,956
c. 03 year	Amount of base $		x Rate applied		% = F&A costs	$ 79,956
d. 04 year	Amount of base $		x Rate applied		% = F&A costs	$ 79,956
e. 05 year	Amount of base $		x Rate applied		% = F&A costs	$ 79,956
					TOTAL F&A Costs $	398,281

*Check appropriate box(es):

[X] Salary and wages base [ ] Modified total direct cost base [ ] Other base *(Explain)*

[X] Off-site, other special rate, or more than one rate involved *(Explain)*

Explanation *(Attach separate sheet, if necessary.)*: See enclosed

4. SMOKE-FREE WORKPLACE [X] Yes [ ] No *(The response to this question has no impact on the review or funding of this application.)*