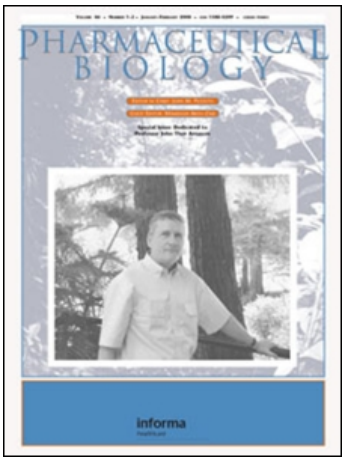

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COSTA RICAN INTERNATIONAL COOPERATIVE BIODIVERSITY GROUP: USING INSECTS AND OTHER ARTHROPODS IN BIODIVERSITY PROSPECTING

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ABSTRACT

This paper describes the Costa Rican International Collaborative Biodiversity Group (ICBG), which was designed to introduce insects and other arthropods as a source of pharmaceutical compounds, and to generate knowledge and economic resources for biodiversity conservation. The National Biodiversity Institute (INBio) and the Area de Conservación Guanacaste (ACG), collected, inventoried and processed insect samples directly from the ACG in northwestern Costa Rica, and developed infrastructure to screen and characterize compounds against microbes and tropical diseases at INBio and the University of Costa Rica (UCR). Cornell University supplied its expertise in chemistry and administration. Bristol-Myers Squibb (BMS) passed samples through part of its screening batteries in six major therapeutic areas. The field team at ACG collected samples, produced vouchers, identified, and obtained natural history information for 1800 insect samples from more than 20 orders of arthropods and 250 species of food plants. Lepidoptera was the most frequently collected (47%), followed by Coleoptera (15%) and Hymenoptera (12%). The adult instar was the most frequent insect stage processed. About 75% of the extracted samples were sent to different screening sites. Analysis of extracts at BMS yielded no ongoing compounds of interest. Several active samples in antibacterial and antimalaria screens at INBio and UCR have entered into bioassay-guided fractionation and structure elucidation. While the chemical characterization of all active samples is still in process, most of the active compounds studied so far are related to unsaturated fatty acids. A very active dehydrochalcone was detected in a host plant after first being detected in a sample of caterpillars that had been feeding on that plant. Costa Rica ICBG information reinforced the National Biodiversity Inventory. During the course of the project, 16 Costa Rican researchers at both professional and paraprofessional levels received training in the field and in laboratories of the collaborators.

Keywords: Bioprospecting, insects, drug discovery, biodiversity conservation, biological inventories, biologically-active extracts, economic development.

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INTRODUCTION

Chemical prospecting and biodiversity prospecting (bioprospecting for short) are recent terms for the search in nature for interesting compounds and molecules and the organisms in which they are contained. Modern bioprospecting attempts to meld two socio-economic goals: (1) conservation of wildland biodiver-

sity through its sustainable use, and (2) the scientific and socioeconomic development of source countries and local communities, with the technical and traditional act of searching for drug leads (Sittenfeld, 1996). Such an attempt faces major challenges in the areas of legal and regulatory frameworks, identification and conservation of biodiversity, distribution of benefits, technology transfer and business development (Sittenfeld, 1996; Tamayo et al., 1997; Sittenfeld & Lovejoy, 1998).

The screening of samples from the wild has always been a prominent activity in ancient and modern pharmaceutical industries. Almost half of the best-selling pharmaceuticals are directly extracted from nature or have active components for which natural products have provided the lead compound, the majority of them obtained from microbial sources (Demain, 1998). The incorporation of automated selection and assay screens in concert with the development of robust molecular biology techniques and information systems has allowed the bioprospecting process to rapidly analyze a large number of samples obtained from plants, microbes, insects, mollusks, etc. However, the frequency of discovering target molecules per sample is low. On another front, societal problems associated with bioprospecting are many (Reid et al., 1993). In addition, a new pharmaceutical agent may require 15 years to bring to market and cost more than $360 million per product in research and development (Thayer, 1998). These barriers severely limit possibilities for many developing countries to fully conduct bioprospecting and subsequent drug development on their own, rendering cooperative agreements with industries in developed nations imperative (Sittenfeld, 1996). In this regard, the US-sponsored International Cooperative Biodiversity Groups (ICBG) Program has been a key exercise in educating bioprospectors in different countries about the complexities and possibilities of modern bioprospecting for drug discovery. Here we reflect on our activities and present some results from the ICBG project entitled "Chemical Prospecting in a Costa Rican Conservation Area," a project that was unique in bioprospecting among insects and other arthropods.

PROJECT DESCRIPTION

A partnership composed of Costa Rica's National Biodiversity Institute (INBio), Cornell University (CU), the Area de Conservación Guanacaste (ACG), the University of Costa Rica (UCR) and the Bristol-Myers Squibb (BMS), began on October 1, 1993, as a five-year bioprospecting project of insects and other arthropods. The project followed the ICBG guidelines for drug discovery, conservation, and economic development. Our emphasis was on exploration of a high diversity of higher taxa and species of correctly identified, vouchered, and re-sourceable adult insects, immature insects, and related biological material (e.g., nests, frass, cocoons) sampled directly from the ACG in northwestern Costa Rica. Samples were collected by a group of national and local bioprospectors and paraprofessionals (parabioprospectors) resident in this rural area and trained explicitly for this purpose. Samples were then processed by the INBio laboratories in Santo Domingo, Heredia, Costa Rica, and subsequently sent to Cornell, BMS, and UCR for initial screening and, potentially, subsequent research and development.

Insects (for practical reasons insects and arthropods are treated here as synonyms) are well known to utilize a wide variety of secondary metabolites as defensive agents, venoms, construction materials, pheromones, and internal regulators. However, they have received much less attention than have plants, microbes, or marine organisms as potential sources of useful pharmaceutical agents. Four major goals were defined for this ICBG:

(a) introduce insects and other arthropods into the modern drug discovery process,
(b) train researchers at both professional and paraprofessional levels in i) tropical ecology, systematics, chemical ecology and insect chemistry, ii) how to conduct the process of sample selection and processing in the field, and iii) how to conduct the laboratory counterparts – while simultaneously increasing in-country bioprospecting capacity as a contribution to the scientific and technological development of Costa Rica,
(c) generate useful knowledge for the conservation of biodiversity in the ACG through regional inventory of species and natural history, and
(d) contribute to national conservation efforts and local economic development.

Three Associate Programs worked together to achieve these goals: Associate Program I in Costa Rica consisted of INBio, ACG and UCR; Associate Program II involved members of the chemistry and biology departments at CU; Associate Program III was located at the BMS Research Laboratories in Wallingford, Connecticut.

The INBio Associate Program I was led by Ana Sittenfeld and Giselle Tamayo (both of INBio and UCR),

with consultation from Daniel Janzen and Winifred Hallwachs (University of Pennsylvania), Roger Blanco (ACG), and the bioprospectors in the field. The field team performed the collection of biological materials within the ACG and created a group of in-country personnel. Vanessa Nielssen and Priscilla Hurtado as bioprospector leaders, together with Hazel Mora and Isabel Salas, and parabioprospectors Adrían Guadamuz, Daniel Pérez, Roster Moraga and parataxonomist Roberto Espinoza, conducted the collection of insects and host plants and associated identifications and natural history. These field personnel simultaneously worked in close coordination with, and contributed to, other biodiversity prospecting and biodiversity inventory efforts in the ACG. Insect identification was conducted by project personnel at ACG and taxonomists at INBio, with the collaboration of the international taxasphere. Alberto Jiménez and Allan Jiménez at INBio prepared chemical extracts from biological materials and performed chemical characterization of active compounds. Eugenio Alvarado at UCR worked on developing protocols for extracting small samples of insects. Misael Chinchilla and Olga Guerrero at the Department of Parasitology of UCR performed screening programs for antimalarials and other antiparasites. Jose M. Gutierrez at the Instituto Clodomiro Picado of UCR carried out screens for inhibitors of phospholipases A_2 and Miguel Rojas was in charge of screening programs for antibacterial and antifungal activity at INBio. In addition, Brian Shuster at the Walter Reed Army Institute of Research (WRAIR) coordinated the screening of some extracts for antimalarial activity and the training of researchers from UCR in different "in vitro" assays for *Plasmodium falciparum.* More recently, samples were sent to the National Cancer Institute (NCI), where Gordon Cragg coordinated testing for antitumor agents.

The information from all activities at different sites was organized and stored in a relational database by Maria A. Mora at INBio, using a custom-designed bioprospecting information management system that was evolved from the FileMaker Pro database used in the field for sample documentation. Regional economic development at the ACG occurred through employment and project activities there. The overall contribution to biodiversity conservation in Costa Rica was coordinated by INBio, and the ACG and its associates.

Associate Program II at Cornell, with Jerrold Meinwald as Group Leader, assumed responsibility for the overall coordination of the ICBG´s activities. Jerrold Meinwald, in collaboration with Jon Clardy, Thomas Eisner, Athulla Attygalle, Ulrich Mueller, Frank Schroeder and Melissa Wagenarr, pursued chemical characterization of selected natural products and trained Costa Rican researchers in chemical characterization, chemical ecology, bioassay development and basic field and laboratory disciplines related to bioprospecting.

Associate Program III at Bristol-Myers Squibb Research Laboratories (BMS), was led initially by Terence Doyle and later by Dinesh Vyas and Kim Wright. It received extracts prepared at INBio. BMS carried out screening over a broad range of biological activities, including anticancer, antiviral, cardiovascular, central nervous system (CNS), regulation of the immune system and dermatology.

Why Insects and Other Arthropods?

Tropical arthropods as a whole represent an enormous and extremely diverse group of organisms rich in unexplored organic compounds. More than 360,000 species are estimated for Costa Rica (Gobierno de Costa Rica, 1992). Arthropods are extraordinarily diverse in species and life forms, and are well known for great inter- and intra-taxon diversity of defensive secondary compound, pheromones, developmental and regulatory molecules, digestive compounds, venoms, etc. (similar molecules are responsible for most pharmacologically active material from plants). Furthermore, almost all arthropods carry a complex gut and symbiont microbial biota that also generates a diverse array of organic molecules. A given species of insect usually occurs at a high enough density at some point in its life cycle that the relatively small samples needed for biodiversity prospecting can be collected from a large conserved wildland without fear of creating significant damage to the population.

If insects are so promising, why haven't they been studied more extensively in this context? The delayed incorporation of tropical insects into bioprospecting has been largely due to the difficulty of identifying insects in the field with ease and certainty, uncertainty about obtaining them in sufficiently large samples for chemical extraction and assay, distance of northern research laboratories from tropical diverse sources, and ignorance of insect biology under tropical field circumstances. The latter ignorance traditionally has rendered re-sourcing and replication of samples very difficult. Equally, professional entomologists have been singularly uninvolved in collaboration with the pharmaceutical industry, in striking contrast with the traditions of tropical botanists and botanical gardens.

All of these difficulties are now being addressed by INBio and other institutions in Costa Rica, and by Costa Rica's conservation areas that together make up the National System of Conservation Areas (SINAC). SINAC includes the ACG, where an estimated 3,000-plus arthropod species can now be readily identified in the field and collected with a modicum of natural history understanding, thanks to this ICBG project and a variety of similar ones in the past two decades. This ACG arthropod-based activity complements a moderate national level of taxonomic and natural history understanding of wild tropical insects by Costa Rican and international scientists and parascientists (Janzen, 1996). Finally, the growing and maturing research administration within the ACG and INBio, and a research-friendly set of national laws and regulations, provide the logistic and legal framework for biodiversity inventories and bioprospecting activities.

BIOLOGICAL RESOURCE USE, AND CONSERVATION POLICIES AND REGULATIONS IN COSTA RICA, DURING THE ICBG

Costa Rica has enjoyed a long history of conservation, beginning with the first concerted moves towards protecting resources such as water, deer and oak forests in 1846. In 1945, the first National Park was established and, in 1969, first Forestry Law was established (Ley 4465). Nevertheless, the accelerated growth in area of protected lands, coupled with increasing colonization, deforestation, lack of institutional coordination and declining funding to manage these lands, led to the establishment of SINAC. While SINAC was formally founded in the Biodiversity Law of 1998, it has been evolving in practice throughout the country since the initiation of the ACG in 1986, and was formalized by a Presidential Decree (No. 24652 MIRENEM) in 1995. SINAC is a flexible and dynamic system that conserves about 25% of the national territory of Costa Rica as wildlands aimed at melding conservation, sustainable development, local community awareness, and local participation in conservation management activities. Its eleven Conservation Areas contain Costa Rica's national parks, wildlife refuges, biological reserves, and other government areas established to conserve and use their biodiversity in a non-destructive manner.

SINAC's mission is saving, knowing, and using biodiversity. This concept provided the groundwork for founding INBio in October 1989 to facilitate the execution of "knowing and using". A private non-profit research institute, INBio operates on the philosophy that unless biodiversity is shown to be economically and intellectually valuable, society is unlikely to continue paying its high maintenance costs and resist the political and economic pressures that lead to its destructive and unsustainable use (Sittenfeld & Villers, 1993). INBio's five administrative units – National Biodiversity Inventory, Bioprospecting, Information Management, Information Dissemination, and Conservation Program – are designed 1) to discover what biodiversity exists in Costa Rica and where it can be found, 2) to facilitate Costa Rica to find sustainable, non-damaging ways to use biodiversity, and 3) to help to conserve wildland biodiversity through this use (*http://www.inbio.ac.cr*).

The ACG is a single conserved wildland of 88,000 terrestrial and 43,000 marine hectares in northwestern Costa Rica, covering an 85 km-long transect from coastal mangroves to dry forest to cloud forest to rain forest (*http://www.acguanacaste.ac.cr*). It may contain as many as 235,000 species of organisms (more than half of which are arthropods) (Janzen, 1996), some 60% of Costa Rica's biodiversity. As a decentralized entity of SINAC, the ACG has a 119-member Costa Rican staff and five biological stations. It is supported by a private $12 million operations endowment, is managed by both its local board of directors and MINAE, and is dedicated to conservation of its biodiversity through restoration, non-damaging use and provision of environmental services to local, national and international society (Janzen, 1998a-d). The ACG is a permanent biological research facility and living biodiversity and ecosystem information repository. It is focused on being both a pilot project for tropical biodiversity development and a major source of scientific understanding of tropical wildlands. The ACG administration views research such as this ICBG project as an intrinsic trait of the ACG and one of the many environmental services that the ACG offers to local, national and international society.

Bioprospecting Legal Framework in Relation to the ICBG

Costa Rica's present national policies and more than 20 laws and regulations determine the framework for biodiversity ownership, access to and use of biological resources. Among them, the Forestry Law 7174 (1990), the National Parks Service Law 4465 (1969), the Law of Creation of the Ministry of Natural Resources and Energy 7152 (1990), the Wild Life Conservation Law 7317 (1992), the Intellectual Property Protection Law 6867 (1983) and the Law for Promotion of Science and

Technology 7169 (1990), contain the key elements of this framework. In April 1998, following the instructions and guidelines of the Biodiversity Convention, Costa Rica approved its Biodiversity Law 7788. These laws contain regulations for permit-based sampling through prior informed consent. They also form the basic policy framework that allows institutional development and collaborations for biodiversity inventories, bioprospecting, and biodiversity conservation and management in state-owned conserved wildlands and private lands.

According to the Wildlife Conservation Law and the Biodiversity Law, specific projects utilizing biological resources found in protected areas are regulated by a system of government permits. Every researcher wishing to gain access to wildland resources must first obtain authorization from the Conservation Area where the research will actually be carried out. Once permission is obtained, the researcher submits a completed research registration form to the General Bureau of Wildlife of MINAE (Ministry of the Environment and Energy), along with approval granted by the Conservation Area hosting the researcher. For the purpose of the Costa Rican ICBG, the INBio Associate Program submitted the permit documents to the General Bureau of Wildlife, and authorization was granted within the framework of the INBio-MINAE collaborative agreement. It is the responsibility of the Conservation Area to provide oversight on collecting sites and processes.

Consortium Contractual Arrangements for the Costa Rican ICBG

There have been prior extensive scientific interactions between participants in this ICBG. On September 24–25, 1992, Jon Clardy, Terrence Doyle, Thomas Eisner, Athulla Attygalle, Ana Sittenfeld, Giselle Tamayo, Daniel Janzen, Winifred Hallwachs and Jerrold Meinwald met at Cornell to plan the creation of this ICBG. This meeting was also attended (in part) by Walter H. Haeussler, at that time President of the Cornell Research Foundation and Director of Patent and Technology Marketing Division, who discussed the protection of intellectual property.

The INBio Associate Program generated sample streams to distinct users: Cornell, UCR, BMS and to "in house" screens for antimicrobials. In the last years of the project, a small number of samples were also delivered to WRAIR and the National Cancer Institute (NCI). Any of these users may develop patentable compounds from ICBG samples. INBio's approach was to have separate agreements with each entity (see below for description). The conditions and shared benefits entered into the same distribution process as INBio has used with other partners (Sittenfeld & Villers, 1993).

For the ICBG, INBio and its partners first outlined the scientific issues (which included capacity building) and workplans. We then broadly defined the business issues, and later placed them in a legal framework (Sittenfeld, 1996). The institutional needs of INBio, and generating income to support protected areas, conservation management activities and local community development, were met through direct contributions as well as royalties. Transfer of processing technologies and a guaranteed future profit sharing were also part of the research negotiations among the Program Associates in this ICBG. Sampling was structured so as to not damage the ACG. Appropriate royalty rates were negotiated based on other industry precedents (e.g., precedents from the biotechnology industry and market perceptions regarding resource supply and demand) (Reid et al., 1993). Pairing institutional representatives and environmental lawyers from Costa Rica with developed-country management consultants and *pro bono* corporate lawyers was highly effective in the development of the contractual arrangements with industrial partners in this ICBG.

INBio-MINAE (formerly MIRENEM)

INBio's overarching agreement with MINAE provides the legal basis for its sampling from the national conservation areas and establishes sharing in financial benefits that result from its collaborative research endeavors. It was signed with MINAE in May 1992 and renewed in 1997. The agreement states that the government's National Park Fund will receive 10% of every industrial research budget and 50% of any realized financial benefits (e.g., royalties, milestone payments or licensing fees) that accrue to INBio as the result of a bioprospecting collaborative research effort. Through returning a share of the financial benefits from bioprospecting directly to Conservation Areas, INBio achieves a part of its core mission. The recently approved Biodiversity Law of 1998 incorporates the profit sharing conditions installed by the INBio-MINAE agreement from 1992.

INBio-Cornell University

An agreement signed by INBio and Cornell University in 1991 covers all past and future collaborations between INBio and Cornell University. When INBio collaborates with CU in a specific research project in the area of chemistry, and subsequently this project

generates a commercializable, licensable or patentable invention that is either a) constituted of a chemical compound extracted from a biological material supplied by INBio, or b) based on a chemical modification of such a chemical compound, then regardless of legal inventorship, net benefits will be split between INBio and Cornell University as 60:40 in case (a), or 51:49 in case (b). The agreement also covers activities of Cornell and its industrial partners when the source of biological material is INBio.

INBio-University of Costa Rica

INBio has also worked extensively with teams of scientists from the UCR. The two organizations signed a five-year cooperative agreement in March 1991 and renewed it in 1996. This agreement affirms the institutional mutual interest in undertaking joint research activities, such as the development of bioassays and their biochemical follow-up. It allows for the Vice President for Research, together with the Director General of INBio, to determine, on a project-by-project basis, how to share any net financial benefits that are generated from inventions developed as a result of the cooperative research. For the ICBG a specific memorandum of understanding stipulated all conditions for intellectual property protection, benefit distribution, and work plans.

INBio-BMS-Cornell Research Foundation

INBio, Cornell Research Foundation and BMS negotiated a Collaborative Research Agreement in which INBio provided insect samples to BMS for evaluation in its proprietary bioassay screens. The agreement also provided for a direct contribution made by BMS to support part of the administration cost of INBio and SINAC, as stipulated in the INBio-MINAE agreement. BMS further agreed to share with INBio any benefits generated from any future commercializable outcome of this research collaboration. The five-year Collaborative Agreement included a work plan for collecting and processing samples (numbers of samples, conditions for collection and extraction), the terms for sample exclusivity and publication of results, and the training of one INBio-chosen chemist or biologist annually for six weeks at the BMS Natural Products Facility in Wallingford, Connecticut. In addition to training, BMS donated laboratory equipment and other supplies to INBio. Cornell University agreed to pursue chemical characterization and synthesis of those selected natural products that appeared interesting on the bases of ecological leads. During the 10-month negotiation process, INBio supported the negotiation team with a grant from the Rockefeller Foundation and *pro bono* legal support from Hale and Dorr (Boston, MA). Although the BMS-INBio Agreement was for the term of the ICBG, except for benefit sharing clauses into perpetuity, the parties decided to renegotiate the conditions after the first groups of extracts were received by BMS. Draft documents were discussed and an amendment to the main agreement was signed in September 1996.

Other Contractual Arrangements

A letter of intent between INBio and the WRAIR was signed in 1996, with the purpose of facilitating and protecting transference of insect materials for antimalarial screening and fractions from bioassay-guided fractionation. Based on a previous Collaborative Research Agreement signed by INBio and the National Cancer Institute (NCI) in 1993, a material transfer agreement was approved by both parties in 1997. It provided for the antitumor screening of insect samples transferred to NCI by INBio within the context of the Costa Rican ICBG.

FINDINGS, EXPERIENCES AND ACCOMPLISHMENTS

Drug Discovery

Collection Process

Insect and arthropod samples generated most of the material studied in this ICBG. However, some samples derived from endophytic fungi, mollusks and bryophytes were also collected on an opportunistic basis for bioprospecting. The insect collection process carried out by the collecting group (two bioprospectors, three parabioprospectors and one parataxonomist), took place in 21 different collecting sites in the ACG dry forest, rain forest, cloud forest and intergrades among these forest types. During the first three years, collection was primarily at wetter and middle elevations at the Estacion Biologica Cacao, Estacion Biologica Maritza, and Sector Pailas and Sector Santa María on Volcán Rincón de la Vieja (600–1000 m). In 1997, samples were mostly from lowland dry forest in Sector Santa Rosa. In 1998, the collection process emphasized targeted recollection at all sites for samples showing activity on different bioassays. Ease of insect sampling differed within and among the years. The peak times were generally in May-June (soon after the

beginning of the rainy season), July (emergence of the first generation during the rainy season), and October (the peak of the second rainy season generation). In 1997, the driest year since 1983 (which was also a mega-El Niño year), insect densities were the lowest ever recorded in the history of research in the ACG and sampling was exceptionally difficult.

Insects were located by haphazard and directed search, using light, baiting with fruit, dung and other attractants, and returning repeatedly to known host plants or congregating sites. Insects were collected free-ranging in the wild or reared at rearing barns from field-collected females. The bioprospectors avoided or modified traditional insect collecting methods that used solvents as part of mixed species capture, so as to avoid contamination between different species and life stages. Bioprospectors and parabioprospectors collected 1,800 insect samples from the ACG and delivered to INBio laboratories 1,694 insect samples for extraction. Insects were usually collected in the field or in the rearing barns into plastic bags or bottles, and then as soon as possible, frozen at –20°C. Samples for extraction were transported frozen (–20°C) to INBio when the fresh weight of the insect sample reached a minimum of 6 g. An additional 93 samples with less than 2–3 g were collected for micro-extraction procedures. About 6% of the samples were discarded as underweight or of unreliable taxonomy before their delivery to INBio headquarters for extraction. During the first year of collection, 114 samples were collected in the field directly into ethanol and stored at –20°C. However, this method was then used less frequently in view of the inconvenience of walking in the wild with large numbers of jars filled with ethanol, and after different extraction protocols demonstrated that the use of ethanol as a delivery media decreased the yield of the organic extract.

The insect samples included 21 different orders of insects and/or arthropods, 95 families, 288 genera and 642 different species. A single sample contained a single species of insect. However, about half (49%) of the insect samples were members of an array of single species samples separated into two or more conspecific "ecospecies". We used "ecospecies" to mean a sample of a life stage, or sex, and/or stages feeding on different foods, or collected in ecologically different sites. In addition, some samples consisted only of associated material like frass (clean insect feces from the bottom of the rearing container), nests or cocoons. In other words, one species often yielded a number of different ecosamples. *Rothschildia triloba* (Saturniidae), which generated 30 different samples (various instars of caterpillars feeding on different food plants, pupae, males, females, cocoons, eggs) represents an extreme case.

Samples were originally vouchered with field notebook data and identification tags (both handwritten and bar-coded), and with specimen vouchers for permanent reference collections at INBio. A field reference collection was also maintained at the ACG. Field notes and subsequent sample information were then computerized into a FileMaker Pro database and then transferred with the samples to INBio. Initial identification of an insect to be sampled, and checking of the sample purity, occurred in the field at the time of collection. This was based on field guides and/or knowledge of specimens previously identified by INBio, visiting taxonomists, and ACG researchers. In all cases the identifications were also verified through the ACG reference collection, and by curators and other identification services available at INBio. Although all insect samples were separated and taxonomically identified to the species or morphospecies level, reference specimens for all samples have been identified to different levels at this time: all to order level, 95% to family level, 75% to genus level, and 53% to species level. Further identification, and more strict corroboration of identifications, was left to occur when results from a sample indicated that the additional cost and effort was warranted.

Sampling of insects included the very time-consuming process of locating concentrations of insects in the forest. Insects were extremely heterogeneously distributed in time and space, and much of the art of bioprospecting consisted of studying insects in the field until the time and place can be determined where they will appear at one point for mating, feeding, oviposition, etc. At this time and place, an adequate sample (determined to be between 6 and 10 g fresh weight for normal extraction procedures and less than 2 g for micro-extraction procedures) may be collectable in a few minutes. However, several days of effort are often required to obtain a typical sample at the rate of one or several insects at a time. For 1,775 samples (with a fresh weight average of 13 g), the average duration of the collection period was 12 days (only a small fraction of which was actually involved in actual collection of that species). It was normal for many samples to be being collected at any one time. Ten samples of very small insects, required a yet greater effort (18 days on average for a 12 g sample).

Lepidoptera was the most common order collected (47%), followed by Coleoptera (15%) and Hymenoptera (12%). The most frequent insect stage collected

and extracted is the adult (53%), followed by larvae (17%), pupae (12%), frass (10%) and other stages (6%). Other insect-associated material such as nests of social wasps (their nest materials plus immature stages) represented 2% of the samples. Other than caterpillars (Lepidoptera larvae), immature insects are a small percent of the samples because of the difficulty of identifying immature stages with certainty in the field.

The search for insects to be sampled yielded a valuable by-product. It led to a very large number of observations of the natural history of the insects sampled and others. Many of these natural history observations were recorded in the bioprospecting database and serve as an ever-growing body of clues to follow-up sampling and re-supply when "hits" occur, and can guide future ecologically-driven sampling. Results from screening also suggested taxonomic relatives, associated life-stages, and food plants to explore for further sample collection.

Sample documentation was incorporated and organized into a bioprospecting information management system that contained information from all related activities and processes. It began in the field at the ACG and moved to the extraction laboratory for management of samples and data after arrival to INBio, internal processing of samples, extraction protocols, preparing and selecting samples for screening, recollection, bioassay-guided fractionation and compound isolation and characterization. Then samples were sent on to the screening process at different sites: BMS, CU, UCR, WRAIR and the "in-house" screening program at INBio. Owing to the accumulated natural history and taxonomic information all from the same geographic area, and owing to these insects being studied by a variety of other research programs, the species being sampled became well-known to the bioprospectors. This greatly facilitated questions related to resupply and taxonomic purity of the samples, and allowed confirmation that the biological source was not being harmed.

Bioprospectors re-collected samples, repeating the same procedures used for the original sample collection. In the field, bioprospectors marked the resupplies either with a red or yellow code. Red-coded samples corresponded to copies of the original collection (same species, same site, same host plant, etc.), while yellow-coded samples corresponded to ecospecies related to the first collection that produced the active sample in the screening systems. As an example, from 1,062 samples tested for bioactivity, 131 were active on retest and 198 related ecospecies (yellow code) and 21 duplicate recollections (red code) were made in a relatively short time after the first "hit" was obtained. The recollected samples also provided material for further chemical and bioassay characterization.

Sample Extraction and Screening for Biological Activity

At INBio, from 1,694 insect samples delivered for extraction, 1,401 were processed into 2,802 extracts. One insect sample normally generated two extracts. The organic and aqueous extracts followed different extraction protocols (see below). From the total of extracted samples, 1,357 were sent to different screening sites and only 20% of the samples were discarded because of low extraction yields.

Tables 1 and 2 show the information on fresh weight, dry weight (weight of the insect sample after lyophilization), and water content for 863 insect samples obtained from 19 different insect orders. Samples were collected directly into plastic bags, frozen at –20°C, further extracted with dichloromethane/methanol 2:1 (see below for method of choice), and sent to different screening sites. Fresh weight, dry weight and water content varied with the insect order. Samples from Polidesmida, Opiliones and Hymenoptera, were among the orders with the highest average of extractable weight (dry weight), while samples from Lepidoptera, Phasmatodea and Blattodea, showed the highest water content. In general, the weight of an insect sample is about 66.5% water. Larvae contained the highest water content (80.5%) and pupal cuticle the lowest (25.4%).

Three different extraction protocols were tested. The first two protocols used hexane and ethanol/water in different proportions. The use of these two protocols was discontinued after extracting 114 samples because of low extraction yields. The third, and the eventual method of choice, was as follows: two extractions with dichloromethane (DM)/methanol 2:1, followed by ethanol 95% and water. Finally, the DM/methanol extractions were pooled together to form the "organic extract", and the ethanol and water extractions were mixed to generate the hydro-ethanolic extract. This third method gave the best extraction yields, and reduced the minimal requirement of the fresh weight of the insect sample to be collected from 10 g to 6 g (to obtain a minimum of 120 mg of extract). However, this method increased the lipid content in the extracts and lipids may produce false positives (O'Neill & Lewis, 1993). Extraction yields for the organic extract averaged 17.92% while that for the hydroalcoholic extraction averaged 13.07%. Extraction yields organized by insect order and life-stage are presented in Tables 3 and 4, respectively. The averages obtained for the organic and hydroalcoholic extracts depended on the insect

Table 1. Fresh weight, dry weight and water content as percentage (%) of insect[1] samples according to different orders.

Order	Number of samples[2]	Fresh weight (g)[3]	Dry weight (g)[3,4,]	Water (%)[3]
Lepidoptera	456	15.24	3.93	72.48
Coleoptera	146	17.34	5.21	59.40
Hymenoptera	79	28.03	7.84	55.60
Orthoptera	61	12.76	4.35	65.58
Hemiptera	40	13.55	6.15	58.36
Homoptera	26	7.51	2.93	59.48
Polydesmida	15	51.74	15.47	65.59
Others[5]	14	8.67	3.85	55.59
Blattodea	8	10.88	3.44	65.86
Phasmatodea	7	11.35	3.18	71.58
Opiliones	6	26.32	11.13	54.32
Mantodea	5	10.45	5.38	49.65
Total/average	863	16.81	4.88	66.52

[1]For practical reasons, insect and other related arthropods are treated here as synonyms.
[2]Includes only samples collected into plastic bags, frozen at –20°C, extracted with dichloromethane/methanol 2:1 and sent to different screening sites.
[3]Numbers represent averages.
[4]Weight of the sample after lyophilization.
[5]Includes samples from the following orders: Araneae, Diptera, Amblypigy, Isoptera, Iulida, Neuroptera, Odonata and Thysanoptera.

Table 2. Fresh weight, dry weight and water content as percentage (%) of insect[1] samples, according to different life stages and associated materials.

Life stage and associated material	Number of samples[2]	Fresh weight (g)[3]	Dry weight (g)[3,4,]	Water (%)[3]
Adults	361	17.17	5.87	60.36
Larvae	206	13.57	2.49	80.52
Pupae	124	10.84	2.71	70.36
Frass[1]	120	23.06	7.90	62.22
Various stages [5]	22	45.70	2.59	70.16
Wasp nests	14	16.41	11.69	25.44
Nymphs	13	9.40	3.17	64.89
Eggs	1	24.40	7.81	67.99
Oothecaria	1	14.20	11.84	16.62
Moltings	1	6.49	5.62	13.41
Total/average	863	16.81	4.88	66.52

[1]For practical reasons, insect and other related arthropods are treated here as synonyms. Frass refer to insect feces.
[2]Includes only samples collected into plastic bags, frozen at -20°C, extracted with dichloromethane/methanol 2:1 and sent to different screening sites.
[3]Numbers represent averages.
[4]Weight of the sample after lyophilization.
[5]Includes different life-stages per sample.

orders and life-stages, suggesting different chemical constituents that are related to taxonomy and life-stages. This information should be useful to improve and customize extraction procedures according to the insect order and the life-stages. Research is currently underway on this topic.

In order to increase the number of samples for drug discovery, 93 insect samples of less than 2–3 g entered a micro-extraction process. After testing various protocols, it was found that samples with as little as 79 mg can produce 2–12 mg of extract. This allowed testing extracts for antimicrobial and phospholipase A_2 inhibitory activity. The protocol used two extractions with 15 ml DM:methanol 2:1 overnight, followed by one extraction with 10 ml of DM:methanol 1:1 for 6 h. Two extractions with 15 ml of water overnight and one final extraction with 10 ml of water completed the extraction protocol. By increasing the amount of solvent and extraction time, efficiency was not improved. Also, centrifugation was replaced by filtration where filtration was slow. The average percentage of extractable material with DM:methanol was 9% and for aqueous extracts was 5%.

Screening for Biological Activity

Insect samples were screened at UCR and INBio in Costa Rica for activity against malaria and other parasites (*Leishmania mexicana mexicana*, *Toxoplasma*

Table 3. Distribution of dichloromethane:methanol and hydroethanolic extractions according to different insect[1] orders.

Order	Number of samples[2]	Average % DM/Methanol extraction	Average % Hydroalcoholic extraction[3]
Lepidoptera	511	18.81	14.72
Coleoptera	158	20.85	10.69
Hymenoptera	144	12.39	12.48
Orthoptera	96	13.68	14.65
Hemiptera	50	31.15	8.46
Homoptera	27	21.23	8.98
Polydesmida	16	6.05	9.37
Blattodea	15	13.06	10.83
Phasmatodea	8	11.67	23.81
Mantodea	7	12.55	8.76
Diptera	6	22.29	8.42
Megaloptera	6	3.86	2.97
Opiliones	6	16.37	7.15
Araneae	5	15.63	12.71
Odonata	3	10.96	5.89
Isoptera	1	23.41	13.48
Iulida	1	5.39	7.56
Thysanoptera	1	25.66	14.11
Uropigidae	1	9.59	7.86
Total/average	1062	17.92	13.07

[1]For practical reasons, insect and other related arthropods are treated here as synonyms.
[2]Includes only samples extracted with dichloromethane:metanol 2:1.
[3]First extraction is made with ethanol 95% followed by a water extraction and both extracts are pooled together.

Table 4. Distribution of dichloromethane/methanol and hydroethanolic extractions according to different insect[1] life stages and associated materials.

Insect life stages and associated materials	Number of samples[2]	Average % Dichloromethane/methanol extraction	Average % Hydroethanolic extraction[3]
Adults	490	17.22	10.07
Larvae	212	22.04	20.19
Pupae	146	23.08	14.34
Frass[1]	132	10.69	11.55
Different stages[4]	29	17.54	15.90
Wasp nests	27	6.20	6.99
Nymphs	20	20.11	16.59
Eggs	3	19.42	19.79
Moltings	2	1.40	0.76
Oothecaria	1	11.41	2.39
Total/average	1062	17.93	13.07

[1]For practical reasons, insect and other related arthropods are treated here as synonyms. Frass refer to insect feces.
[2]Includes only samples extracted with dichloromethane:metanol 2:1.
[3]First extraction is made with ethanol 95% followed by a water extraction and both extracts are pooled together.
[4]Includes different life-stages per sample.

gondii and *Trypanosoma cruzi*). We also screened for anti-fungal and antibacterial activity, and for inhibitors of phospholipase A_2. We reconfirmed all active samples. Host plants were collected and tested as alternative sources of interesting molecules that had been first detected in insect extracts. No positive results were obtained when 74 insect extracts were tested for inhibitors of phospholipase A_2. Fractionation and structure elucidation of compounds in positive insect samples at INBio was accomplished by means of typical chromatographic techniques, NMR and other analytical tools.

The screening process for compounds against malaria at UCR began in the first year of the program. At first we used an *in vivo* test with a mouse model using *Plasmodium berghei berghei*. This was soon replaced by an *in vitro* system, which was extended to other parasites following the methods described by Chinchilla et al. (1995). For *in vivo* protocols it was necessary to prepare large numbers of mice and

required at least 100 mg of extract per test. Both factors drove us to utilize *in vitro* protocols, which required fewer animals and less extract. In both cases, extract dilutions as well as *P. berghei berghei* inocula were standardized. Screening for other parasites was implemented in the last year of the Costa Rican ICBG. The numbers of samples tested to date are small and research in still in progress. Significant increased survival in the *in vivo* system was obtained for 22 out of 150 extracts. Characterization of bioactive compounds was not possible due to the large amounts of extract required for bioassay-guided fractionation. However, material from host plants was obtained and extracts from two plant species caused a significant increase in survival of infected animals. Bioassay-guided fractionation is currently in process.

The detection of reconfirmed inhibitory activity of *P. berghei berghei* using *in vitro* systems occurred in 42 insect samples out of 756 insect samples tested (we used dilutions equal to or higher than 1:80 of insect extracts and used the inhibition produced by 50 ng/ml of chloroquine as cut-off for a positive result). None of the host plants analyzed were positive in the *in vitro* screening process, but fractions of these plants showed activity in the *in vitro* assays. This suggests problems related to dilution factors. In order to compare the murine malaria models implemented at UCR with the human malaria screening systems (using *P. falciparum*) at WRAIR, 75 extracts were evaluated at both sites. From 75 extracts, 25 were partially active and only one extract showed a similar effect to chloroquine used at 50 ng/ml. Of 32 samples reported inactive by WRAIR, five were reported with some level of activity by UCR, 27 samples (84%) were negative at both UCR and WRAIR. UCR reported activity in 14 of the 21 samples (67%) reported active by WRAIR.

The microbiological facility at INBio tested insect extracts using standard agar diffusion assays (González et al., 1994), *Candida albicans* ATCC 10231, *C. wisconsin* UCR 121, *Escherichia coli* ATCC 9637, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 10031, *Staphylococcus aureus* ATCC 13709, and *Pseudomonas aeruginosa* ATCC 27853. From 649 insect samples, 32 were confirmed active. Following the method of Rahalison et al. (1991), bioautography was performed in all cases and samples are currently under bioassay-guided fractionation. Host plants were collected and analyzed. One plant extract tested positive for *S. aureus*. Bioassay-guided fractionation and structure elucidation resulted in a very active dihydrochalcone. Antimicrobial screening at Cornell was performed on samples from ecochemical leads that produced very polar antibiotics, but were of no further interest.

A total of 198 insect extracts obtained by microextraction protocols were submitted for antimicrobial assays. Three samples tested positive, one for *C. albicans* and two for Gram-negative bacteria. Recollections of active samples in sufficient amounts for bioassay-guided fractionation was not possible. This is a reflection of the difficulty of resupply when a sample is underweight in the first collection.

Over a five-year period, BMS received 1,354 insect extracts from INBio. These were prepared from 677 insect samples. Extracts were examined using a five-tier protocol. At tier one, extracts were tested in all screens running at the time of receipt. Extracts were judged inactive, presumed active or confirmed active. At tier two, a fresh sample of a confirmed active was retested. If active a second time, the extract moved to tier three, dereplication. At this tier, extracts were examined for the presence of known active compounds by both chromatography and activity profile. If known active materials were not detected by either method, a resupply was requested from INBio and tested at tier four. Active resupplies became candidates for purification studies (tier five). During the five-year period, 117 extracts were confirmed active at tier one and 57 proved active on retest at tier two. At tier three, 35 extracts survived dereplication. A resupply of 15 extracts were requested and received at tier four. Of these, seven extracts were subjected to tier five, purification. The remaining eight were dropped for one of two reasons. Either the original selection screen was no longer of interest and abandoned, or known false positive substances were observed.

Insect extracts were tested in 21 high-throughput screens covering six therapeutic areas; infectious disease (5 screens), oncology (5 screens), central nervous system (4 screens), cardiovascular (2 screens), dermatology (1 screen) and immunology (4 screens). A total of 15,253 bioassays were used to evaluate extracts at tiers one through four (giving an average of 11.3 screens per extract) and 173 bioassays were performed during bioassay-guided purification at tier five. Samples were thoroughly tested by BMS in all running high-throughput screens at the time of their receipt. As new high-throughput screens came on-line over the years, old residual extracts were not retested because of solution instability and logistic issues.

Bioassay-guided purification and structure determination of extracts at tier five from seven frass samples

found phaeophorbide A, phaeophorbide B, or other porphyrin type molecules to be the active agents. These substances proved to be false positive leads in secondary assays and frass samples were of no further interest to BMS.

Thirty six percent of all hits at tier one were from Lepidoptera, followed by Hymenoptera with 19%, Orthoptera with 16% and Coleoptera with 14%. According to the order representation (in percentage) of the extracted samples analyzed by BMS, samples from lepidopterans account for the most common order (41%), followed by Hymenoptera (18%), Coleoptera and Orthoptera (13%). The highest number of hits occurred in adults (49%), frass (19%) and larvae (13%).

From 677 insect samples delivered to BMS, positive confirmed initial activity was detected in 117 insect samples (17.3%). This is a high percentage by drug discovery standards. This high rate could be explained at least in part by the presence of compounds interfering with screens and by the presence of a high percentage of related ecospecies – if one ecospecies of a species is active, often the others are as well. Approximately 28% of the samples with confirmed activity were among pools of conspecific ecospecies. However, we still support the exploration of a series of conspecific ecospecies derived from one species. For example, from 13 related samples of *Neoconocephalus triops* (Tettigoniidae), a large green ordinary-looking tettigoniid-grasshopper, separated according to collection site and sex, five were negative, but eight were positive. A similar situation was observed for conspecific *Polybia occidentalis* (Vespidae) samples separated by life-stage and/or associated material; only six out of 14 conspecific ecospecies samples were positive.

Results from different screening sites and chemical characterization of active components led to either chlorophyll or lipid derivatives. Most of the active samples in the in-house screening at INBio that have been isolated and characterized are related to unsaturated fatty acids. However, the number of samples studied is still small, since analytical methods such as NMR were not available until 1998. More chemistry is presently being performed to better understand the significance of these results. It is significant in this regard that Pitt et al. (1998) have recently reported the antimalarial activity of hydroperoxide derivatives of polyunsaturated fatty acids. Others (Giamerellos-Bourboulis et al., 1998) have found as well that some polyunsaturated fatty acids were responsible for antibacterial activity. These findings demonstrate the increasing interest in lipids. In addition to the lack of analytical tools, the most important practical problem found at INBio, has been the relatively small amount of active crude extract available to pursue bioassay-guided fractionation. This suggests that larger amounts of insect material should be collected when using insects as a source of potential compounds for drug discovery. If the exhibited activity could be demonstrated to be present in the host plant, the isolation and characterization of active components is straightforward, as it was demonstrated in the isolation of a dihydrochalcone from the host plant for *Mimallo ammalia* (Mimallonidae).

Biodiversity Conservation, National Biodiversity Inventory and Economic Development

The Costa Rican ICBG had a major positive impact on biodiversity conservation by being part of the social and government transition from viewing national parks as "untouchable" to being places that are appropriate for non-destructive research, and research that may also create national income. The project has taken advantage of the national inventory being conducted by INBio, and in fact could not have been conducted without this taxonomic background. Taxonomy and natural history collected by parabioprospectors and bioprospectors have also produced important information on the 1800 insect samples and 250 species of food plants collected during the ICBG. The plant background work for the ICBG initiated the construction of a plant database and computer capture with digital photography of the vegetative traits of ACG plants, and began to generate one Species Home Page for each plant. This was expanded during the fifth year of the project, and now continues as an ACG plant inventory supported for two years by other sources of funding. The overall goal is to develop a protocol for all the users of living wild plants, so that they have a simple and straightforward way of identifying the plant, flower, leaf, fruit, seed, etc., in the field at the time that they need to collect a sample or make some observation on its biology. The goal is also to create a team of experienced professional and paraprofessional field biologists who can establish this. This computer-based knowledge system is to be readily applied by the nontaxonomist to complicated taxonomic arrays in the field. It builds on a several-year-old lineage of thought about confronting this problem with insects. As this is written, 433 plant species have been placed on the ACG web site. Five people, three of whom are former bioprospectors and parabioprospectors for the ICBG, conduct the project.

The information generated by the ICBG continues to support and build useful knowledge for conservation. It generates income at the ACG directly through employment, expenses, and payments for research fees, and indirectly by encouraging other investments at the ACG. From the total budget allocated to Costa Rica of $1,650,975, 30.3% or $500,643 was expenditures made directly in the ACG. This contributed directly to local economic development. An additional, $84,400 was generated by the ACG from visitors' expenses that were directly a result of the ICBG, and represents income indirectly generated by the project for the ACG and its neighborhood.

Taxonomy and natural history obtained by bioprospectors continues to support the selection and design of trails for ecotourism. It also supports educational activities for schools, scientists and policy makers, as well as provides raw information for the ACG biological education program. This provides basic biological education to all grade school and high school students in the region (more than 2,500 students per year). In addition, part of the information generated by the ICBG is of easy access to the local and international community through INBio´s and ACG publications in the Internet and through the inventory information that both are making available through their servers (*www.inbio.ac.cr; www.acguanacaste.ac.cr*).

The project generated contributions to conservation management at SINAC (MINAE) by the way of the 10% of the research budget allocated to Costa Rica. MINAE is allocating these funds to the conservation of the Isla del Coco. Funds for this direct contribution to MINAE were obtained in part from a contribution from BMS. Unfortunately, BMS decided in this second portion of the project to discontinue the support to this part of the project. As an alternative to fulfill INBio´s agreement with MINAE, a contribution of equipment was negotiated with MINAE as part of the overall consortium agreement.

Scientific and Technological Development of Costa Rica

During the course of the project two parataxonomists, six parabioprospectors, four bioprospectors, two taxonomists and six chemists and microbiologists from Costa Rica received training in the laboratories of the collaborators, other research centers and on the job. Expertise has been acquired in chemical ecology, tropical ecology, taxonomy, entomology, bioassay-guided fractionation, bioassay development and implementation, chemical separation and characterization, and microbial biotechnology. Multidisciplinary and interdisciplinary groups of scientists (biologists, chemists, computer experts and microbiologists) were established in the field and laboratory while working with a focus on drug discovery. The creation of a Bioprospecting Information Management System and the installation of both the extraction-chemical analysis and microbiology laboratories at INBio, as part of the ICBG, represented key steps in institutional capacity building. This capacity was instrumental for INBio in the process of developing new projects with industrial and academic partners outside the collaborators of the ICBG.

CONCLUSIONS

The Costa Rican ICBG focused on drug discovery with insects. It improved access to wild insect biodiversity for conservation and helped to build in-country capacity. BMS provided part of its screening batteries in six major therapeutic areas and made the results available to the other associate groups. INBio and ACG generated access to biodiversity, developed their own infrastructure to extend the screening and chemical characterization of drugs against tropical diseases, and reinforced the National Biodiversity Inventory. Cornell supplied its know-how in chemistry, microbiology and administration. Scientists, administrators and politicians from other countries that participated in five international workshops at INBio (funded by other sources), visited the collection sites in the ACG, and learned about technical, political and legal issues of the ICBG. The national and international impact of this Cooperative Group continues both in technical and political directions.

The program involved the extensive chemical and ecological training of field experts, termed "bioprospectors" (biodiversity prospectors and parabiodiversity prospectors), who in addition to their regular collection activities, were also trained to look for and record ecological indications of potentially powerful chemical compounds. This ICBG has met many of its expectations in terms of technology transfer, impact on biodiversity conservation, biodiversity inventory, local economic development at ACG, and scientific development of Costa Rica. Although the screening process at BMS yielded no ongoing compound of interest, the INBio Associated Program is currently working on several leads from the antibacterial and antimalarial screens performed in Costa Rica. All participants

expect that this consortium effort, and the knowledge resulting from it, will provide the impetus to introduce compounds from insects into the pharmaceutical market while generating valuable new ecological information that could increase the efficiency and scope of drug screening in the long run.

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