Use of ecological criteria in designing plant collection strategies for drug discovery

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Tropical forests are one of the most diverse and endangered habitats on earth. They have also been portrayed as a source of future pharmaceuticals, yet finding useful compounds can be both scientifically and politically challenging. Increasingly, over the past decade, the potential value of medicinal compounds derived from plants, microorganisms, and animals has been proposed as a tangible benefit of biodiversity, and therefore a basis for promoting its preservation. Ecological theories of plant defense can increase the probability of discovering compounds with activity in bioassays against human disease targets. In addition, conducting research in tropical countries with local scientists provides immediate and lasting benefits for the sustainable use of biodiversity. This new approach to drug discovery has been effective in identifying bioactive leads. It is both an important step towards understanding the medicinal value of biodiversity, and a practical way to link drug discovery with conservation.

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The conversion of natural habitats for human use represents the primary driving force in the loss of biological diversity; 40–50% of the earth's land surface is already degraded by humans and the fate of remaining biodiversity will probably be determined in the next few decades (Vitousek *et al.* 1997; Sala *et al.* 2000; Tilman *et al.* 2001). Given this massive, rapid assault, multiple approaches with immediate impacts are necessary to protect species-rich habitats (Pimm 2001). One approach, often called "use it or lose it", attempts to identify a habitat's economic value so that protecting biodiversity provides greater benefits than alternative uses (Janzen 1997; Janzen 1999; Balvanera *et al.* 2001). For example, many ecosystem services have been shown to have greater value than logging or agriculture (Daily *et al.* 2000; Beattie and Ehrlich 2001; Cork 2002; Daily and Ellison 2002; Balmford *et al.* 2003; Rosenzweig 2003). Bioprospecting, the search for chemicals or genes with medicinal or agricultural applications, is another sustainable and ecologically gentle use of biodiversity that can promote conservation.

In a nutshell:

- Drug discovery is a non-destructive use of biodiversity that creates incentives to conserve wildlands.
- The typical approach with royalties as the only incentive is insufficient, as the probability of marketing a drug is extremely low.
- Conducting a portion of drug discovery in biodiversity-rich nations guarantees immediate benefits even if royalties never materialize, and promotes conservation.
- The use of ecological theory on plant chemical defenses shows that young leaves are an excellent source of compounds active against human disease targets.

The Convention on Biological Diversity, presented in 1992 at the Earth Summit in Rio de Janeiro, promotes the use and conservation of biological diversity, and requires the fair and equitable distribution of benefits. While bioprospecting is widely cited as a way to give value to biodiversity, its effectiveness has been slowed by the difficulty of providing immediate benefits from the use of genetic resources. In fact, bioprospecting in biodiversity-rich countries is still in its infancy. In 1998, we initiated a project called "Ecologically Guided Bioprospecting in Panama", with the aims of discovering novel agents that promote human health in a manner consonant with the benefit-sharing provisions of the Convention on Biological Diversity, and spurring biodiversity-rich countries to initiate their own conservation measures. The ongoing project (www.icbgpanama.org) is an International Cooperative Biodiversity Group (ICBG) funded by the National Institutes of Health, the National Science Foundation, and the US Department of Agriculture (Kursar *et al.* 1999).

Natural products have been a rich source of therapeutic agents, many of which come from higher plants (Kinghorn and Balandrin 1993; Balick *et al.* 1996; Grifo and Rosenthal 1997). In some areas of medicine, espe-

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cially cancer, plants continue to provide novel drug treatments such as taxol and camptothecan (Shu 1998; Cragg and Newman 1999; Mann 2003). Even so, tropical plants are under-investigated as sources of new medicines. Conventionally, pharmaceutical companies have contracted with botanists who make random collections of dried plant tissue for testing in proprietary bioassays. Less commonly, ethnobotanical information has been used to guide collections (Kingston *et al.* 1999; Lewis *et al.* 1999; Schuster *et al.* 1999; Cox 2001). A third approach, still in its infancy, uses ecological information to discover useful natural products (Reid *et al.* 1993; Beattie and Ehrlich 2001). For example, we spent 4 years collecting leaves at numerous sites in Panama to determined levels of activity in bioassays against cancer, HIV, and tropical diseases. Here we will discuss the inclusion of ecological theories about plant defense in the design of collection strategies for bioprospecting.

Using plant defense theory

The evolutionary "arms race" between herbivores and plants has created a huge diversity of plant secondary metabolites (Ehrlich and Raven 1964; Kareiva 1999; Thompson 1999; Rausher 2001), and nowhere in the world are these biotic interactions more intense than in tropical rainforests (Price *et al.* 1991). The high biotic pressure in the tropics has led to higher levels of chemical defense as well as a greater diversity of compounds in tropical species, as compared to temperate ones (Gentry 1993; Coley and Barone 1996; Coley and Kursar 1996). 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 1976; Levin and York 1978). All other classes of compounds that have been surveyed exhibit similar patterns (Coley and Aide 1991; Coley and Barone 1996).

Figure 1. *Young leaves of Panamanian woody plants.*

Despite the many drugs obtained from plants in the past, success rates could be greatly improved by incorporating ecological knowledge. By applying our current understanding of plant defenses and herbivory, we developed a collection strategy aimed at enhancing the discovery of useful pharmaceuticals. To test our ideas, we collected leaves in protected wildlands throughout Panama and prepared extracts from them while still fresh. We evaluated activity in five in vitro bioassays against human disease: three cancer cell lines (breast MCF-7, lung H-460, and central nervous system SF-268); HIV; and three tropical disease cell lines, leishmaniasis (*Leishmania mexicana*), malaria (*Plasmodium falciparum*) and Chagas' disease (*Trypanosoma cruzi*). For the tropical disease bioassays, new methodologies were developed in Panama, as standard methods using radioactive reagents are not possible in developing nations. Extracts were considered highly active if they killed or inhibited growth in the target cells (for methods see Web materials).

Tests of plant defense theory

Activity is greater in young leaves

Most conventional drug discovery programs make collections of mature leaves, roots, or other tissues. We predicted that young, expanding leaves would contain more active secondary metabolites than mature leaves (Figure 1) and thus be more active in bioassays. Mature leaves of tropical plants are tough because of the high lignin and cellulose contents of their thick cell walls (Lucas *et al.* 2000). Although toughness is one of the most effective defenses against herbivores (Coley 1983; Lowman and Box 1983; Coley and Kursar 1996), it has no therapeutic or agricultural potential. In contrast, young leaves cannot toughen until the cells finish expanding, leaving them highly vulnerable to herbivores. In the temperate zone, most young leaves emerge early in the spring while herbivore populations are low. Perhaps because of this, young temperate leaves are not chemically well defended (Coley and Aide 1991).

In the humid tropics, however, herbivory on young leaves is extremely high all year round, accounting for 70% of the lifetime damage to shade-tolerant species (Coley and Aide 1991). This strong biotic pressure has apparently selected for numerous anti-herbivore defenses in young leaves, including investments in secondary metabolites (Coley and Barone 1996; Kursar and Coley 2003). For example, concentrations of terpenes (aromatic hydrocarbons) and alkaloids (a particularly important group of medicinal com-

pounds) are significantly higher in young tropical leaves as compared to mature ones (Crankshaw and Langenheim 1981; Langenheim *et al.* 1986; Kursar *et al.* 1999).

We evaluated the activity in our bioassays of methanolic extracts made from young and mature leaves. Young leaves had greater activity than mature leaves in almost every disease bioassay (Figure 2; Web Table 1). To facilitate comparisons across bioassays with different means and variances, results were normalized so the mean of all extracts tested in a particular bioassay equaled "zero" and the variance equaled "one" (Z-score transformation). In almost all bioassays, the average value for young leaves was greater than zero, indicating above-average activity. In contrast, values for mature leaves were less than zero, indicating below-average activity. This pattern held when bioassay results were compared for young and mature leaves collected from the same plant (Figure 2, top) or for all samples (Figure 2, bottom). In addition, a higher percentage of young leaf samples were highly active and thus merited further investigation for therapeutic potential (Web Figure 1).

Young leaves have unique compounds

We predicted that young leaves, in addition to higher levels of chemical defense, would also have different secondary metabolites than mature leaves. Because young leaves cannot toughen, selection should favor additional chemical defenses specific to this developmental stage (Coley and Kursar 1996). Once leaves stop expanding and toughen, these compounds may no longer be necessary and could be catabolized (metabolically broken down) for other purposes. Many secondary metabolites may thus only be present in young leaves.

In a survey of 18 Panamanian woody species, we contrasted the number of alkaloids present in young and mature leaves of the same plants using thin layer chromatography and Dragendorff's reagent (Kursar *et al.* 1999). Ten out of 18 species had alkaloids that were present only in the young leaves, while only three species had alkaloids unique to mature leaves. Among the 24 alkaloids unique to either young or mature leaves, 71% were found only in young leaves and 29% only in mature leaves. High performance liquid chromatography 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.

To date we have pursued isolation of compounds from 15 species that were highly 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 found in such low concentrations in mature leaves that it was only possible to detect activity and purify it from young leaves (Bonetto *et al.* 2003; Chérigo *et al.* 2003; Hussein *et al.* 2003; Montenegro *et al.* 2003; Mendoza *et al.* 2003). We recently obtained a provisional patent for several alkaloids, isolated only from young leaves, that are active against Leishmania, the parasite that causes leishmaniasis. There is a great deal of chemical diversity in young leaves, and this promising source remains largely untapped (see Web material).

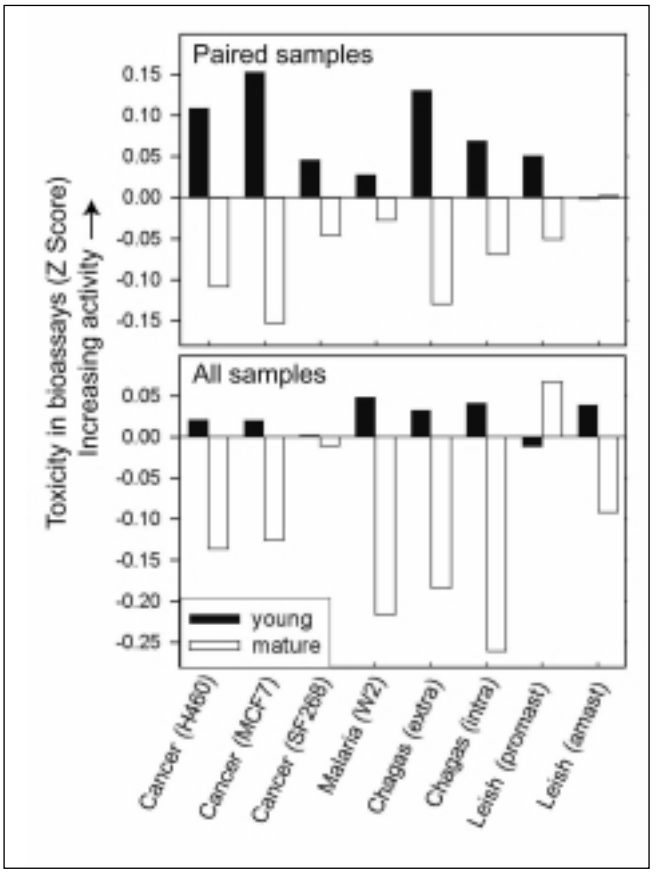

Figure 2. *Activity of extracts from young and mature leaves in bioassays. (top) Paired analysis of species for which we have bioassay data on young and mature leaves from the same plant. (Species were not tested in every bioassay.) We tested 101 species for activity against cancer, 58 for malaria, 76 for Chagas' disease (extracellular form), 18 for Chagas' disease (intracellular form), 40 for leishmaniasis (promastigote cell types), and 84 for leishmaniasis (amastigote cell types). A paired t-test on raw values was significant ($p < 0.05$) for H460, MCF7, and Chagas' (extracellular). Data for HIV are not included as they are scored as active or inactive. (bottom) Analysis of all samples. Extracts from young leaves were significantly more active than those from mature (nonparametric ANOVA, $p < 0.002$). We tested 1077 species against cancer, 443 for malaria, 717 for Chagas' (extracellular), 277 for Chagas' (intracellular), 950 for leishmaniasis (promastigotes), and 158 for leishmaniasis (amastigotes). A two-sided Wilcoxon test on Z-scores of young and mature leaves was significant ($p < 0.05$) for H460, MCF7, malaria, and both forms of Chagas'*

Greater activity in shade-tolerant species

Plant defense theory predicts that the mature leaves of slow-growing, shade-tolerant species should have better chemical and physical protection than mature leaves of

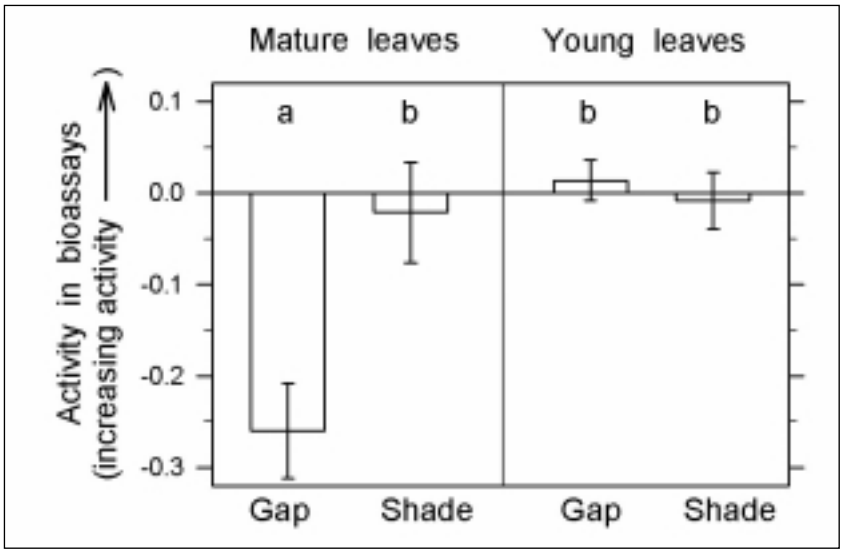

Figure 3. *Activity of young and mature leaves from shade-tolerant and gap-specialist species (± SE). Values with different letters are significantly different at $p < 0.05$ (Duncan Multiple Range Test, ANOVA). A higher value for the Z-score indicates greater activity averaged across all bioassays. There were 3612 activity results, resulting from testing different plant species and leaf ages in multiple bioassays.*

fast-growing species found in the high-light conditions of tree-fall gaps (Coley *et al.* 1985). Because replacing lost leaves entails a greater cost in low-resource environments, shade-tolerant species have well defended leaves that suffer little herbivory (Grime 1979; Coley 1983; Coley 1988; Reich *et al.* 1999; Wright *et al.* 2002). Extracts from the mature leaves of shade-tolerant species should therefore have higher activity than extracts from gap specialists. This prediction was confirmed by our bioassay results (Figure 3). The extremely low rates of herbivory on mature shade-tolerant leaves (Marquis and Braker 1994; Coley and Barone 1996) can therefore be attributed to greater investment in both physical and chemical defenses.

The activity in young leaves from gap specialists and shade-tolerant species did not differ (Figure 3). All young leaves were highly active, and similar in activity to mature leaves of shade-tolerant species. The mature leaves of gap species were the least active. These results are consistent with theory and field data on herbivory (Coley 1983; Coley *et al.* 1985; Kursar and Coley 2003; Web Table 2).

Plant growth form weakly correlated with activity

Plant growth rates, leaf age, and leaf lifespans should be good predictors of investment in secondary metabolites, but growth form per se should not. However, others have argued that particular forms may be a rich source of potential drugs. For example, it has been predicted that epiphytes (Bennett 1992) and lianas (Hegarty *et al.* 1991) should be especially good sources of active compounds. Our data suggest that neither were particularly active (Figure 4). Extracts from shrubs and trees were the most active. Palms were the poorest source of active extracts, undoubtedly due to the extremely fibrous nature of both young and mature leaves. There was a significant effect of growth form on activity (Web Table 1). However, when palms were excluded from the analysis, growth form was only marginally significant ($p = 0.06$), suggesting that growth form effects were dominated by the low activity of palms.

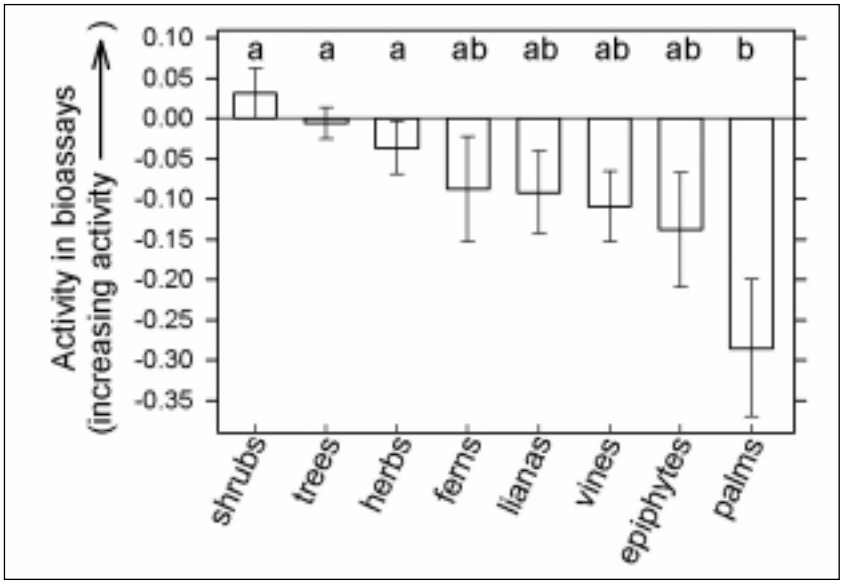

Figure 4. *Activity of extracts from species with different growth forms (± SE). Values with different letters are significantly different at $p < 0.05$ (Duncan Multiple Range Test, ANOVA). A higher value for the Z-score indicates greater activity averaged across all bioassays. Sample sizes (resulting from testing different leaf ages in multiple bioassays) were: 1078 for shrubs, 2738 for trees, 744 for herbs, 158 for ferns, 373 for lianas, 438 for vines, 161 for epiphytes, and 70 for palms.*

■ Phylogenetic patterns of activity

One approach often used in drug discovery is to focus on families known to have unusual chemical structures (eg Euphorbiaceae) or to contain classes of known bioactive compounds such as alkaloids (eg Rubiaceae, Solanaceae, Fabaceae, and Apocynaceae). Although there is some justification for this approach (Barclay and Perdue 1976), our data do not provide strong support. For example, Euphorbiaceae ranked 105 out of 147 families we tested. The four alka-

loid-rich families showed intermediate activity, with ranks ranging from 28 to 90. In fact, the most active families were small and rather poorly studied from a chemical or pharmacological perspective. The families are listed in Web Table 3, in order of decreasing activity in our bioassays. Plant families differed considerably in their activity and there was no interaction between family and bioassay type (Web Table 1), suggesting that the activity level for a given family is similar across many different disease targets.

We also tested the idea that the more recently evolved species would have greater activity, as might be predicted by the chemical "arms race" (Ehrlich and Raven 1964; Kareiva 1999; Thompson 1999; Rausher 2001). This was not strongly supported, as the basal angiosperms (the Magnoliids and Chloranthales) were the most active, followed by the more derived Asterids and Rosids (Figure 5). Monocots were least active, perhaps because parallel leaf venation permits extensive toughening early in leaf development, resulting in less attack by herbivores and less selection for chemical defense. Overall, we found a strong phylogenetic signature, with some clades being clearly more active than others.

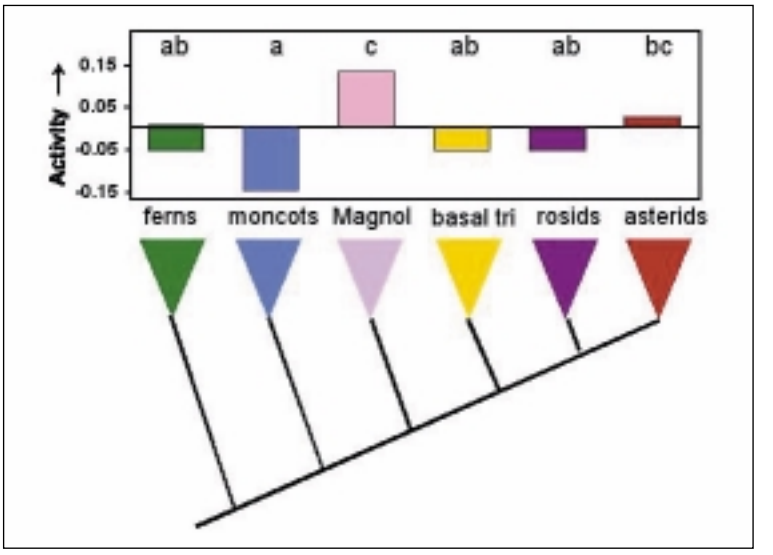

Figure 5. *The activity and phylogenetic relationships among 34 orders of flowering plants. Units of activity are Z-scores averaged across all bioassays. Values with different letters are significantly different at $p < 0.05$ (Duncan Multiple Range Test, ANOVA). The study species were grouped into six clades: (1) ferns; (2) monocots; (3) Magnoliid complex (Magnoliales, Laurales, Piperales) plus Chloranthales; (4) basal tricolpates (Ranunculales, Proteales), Gunnerales, Caryophyllid clade (Dillenales, Caryophyllales), Santalales, and Vitales; (5) rosids (Myrtales, Celestrales, Malpighiales, Oxalidales, Fabales, Rosales, Curcurbitales, Fagales, Malvales and Sapindales); and (6) asterids (Ericales, Gentianales, Lamiales, Solanales, Aquifoliales, Apiales, Asterales and Dipsacales). The nomenclature and phylogenetic reconstruction follows those of Judd et al. (2002) and the Angiosperm Phylogeny Group of the Missouri Botanical Garden (Stevens PF, Version 4, May 2003, www.mobot.org/MOBOT/research/Apweb)*

■ Ecological insight in drug discovery

Application of plant defense theory allowed us to collect plant species and tissues with greater activity in bioassays. The ecological criteria used to collect organisms must be easy to implement in the field and broad enough to generate a reasonable number of samples for bioassays. Ecological approaches should also be based on sound science, so that increases in active extracts justify the extra effort of selecting samples. The ecological criteria outlined in this paper arose from work in Africa, Southeast Asia, and Panama (Coley and Kursar 1996; Kursar and Coley 2003) and should therefore be applicable in tropical forests worldwide.

When research leads to the commercialization of a drug, large quantities of the compound are required. The preferred option is synthesis of the compound, a derivative, or an analog. These options still provide royalties to the source countries, through patents or contract stipulations that ensure equitable benefit sharing. If synthesis is not cost effective, then the plants can be grown in plantations, providing an alternative "cash crop" for the host country. Legal agreements as well as public pressure can prohibit the destructive harvesting of wild plants as a source.

■ Drug discovery as a conservation tool

Drug discovery has been portrayed as a way to combine research to improve human health with the sustainable use of biodiversity (Rosenthal *et al.* 1999). Nevertheless, the effective application of bioprospecting to conservation remains a fundamental, unsolved problem. A key criticism from conservationists and economists is that royalties are the sole source of benefits provided to the host country in most benefit-sharing arrangements. Unfortunately, the probability of a drug making it to the market is extremely low, so developing nations are unlikely to receive any royalties from uses of their biodiversity. The challenge, therefore, is to provide immediate and guaranteed benefits even if royalties are not forthcoming.

A solution becomes apparent upon recognizing that the research and development pyramid underlying the successful development of a drug is based upon many basic but essential discoveries, a tiny fraction of which result in a product. Many of these inventions originate from discoveries that were initially made in academia or by small companies. Also, large sums are invested in research and development of drugs. Worldwide pharmaceutical investment in research and development is estimated at $27–43 billion per year (Agnew 2000); about one third of that is spent on research that could be carried out in developing countries, including extraction, synthesis, in vitro bioas-

Figure 6. *(left) Nayda Flores, ICBG project's botanist, collects leaves in protected forests in Panama. (right) Dr Luz Romero and assistant Yolanda Corbett conduct a bioassay to determine activity of leaf extracts against Leishmania mexicana in Panama.*

says, and activity and efficacy testing in vertebrate models (ten Kate and Laird 1999). In addition to pharmaceutical companies, governments and non-profit organizations also provide substantial support for research in biodiversity and drug discovery. If a part of these huge investments by industry, governments of developed nations, and NGOs could be redirected toward bioprospecting research in the source country, then biodiversity-rich countries would receive immediate and guaranteed benefits from the non-destructive use of their natural resources. If only a fraction of the drug-discovery research were conducted in developing nations, this would provide many educational and job opportunities. For example, in our NIH-funded project, substantial results have been obtained with an investment of only $500 000 per year in Panama, a country of 3 million people (Figure 6). We are using this base to forge new collaborations with pharmaceutical companies and to attract additional international funding. A similar outcome could be derived in many of the biodiversity-rich nations of the world with an investment that would not be prohibitive.

For bioprospecting to have a positive impact on conservation, immediate benefits must first be realized by the host country. While they must be included in legal agreements, royalties are unlikely to materialize. However, one does not need to find a drug in order to link bioprospecting to conservation. By conducting all of the research in Panama, we circumvent the issue of uncertain royalties and provide immediate and lasting benefits in the form of training, employment, technology transfer, and infrastructure development (Capson *et al.* 1996; Kursar *et al.* 1999). These benefits provide an important but generally neglected mechanism for demonstrating the value of intact forests to Panamanians. Furthermore, the entire research effort is funded by foreign monies, and requires no investment by the country's government. In principle, very similar benefits are provided by the National Biodiversity Institute in Costa Rica, the National Commission on Biodiversity Use and Knowledge of Mexico, the Brazilian Program of Molecular Ecology for the Sustainable Use of Amazon Biodiversity, the Ibero-American Program of Science and Technology Development, and a number of bioprospecting companies in Brazil such as Extracta, Chamma da Amazonia, and Crodamazon. The actual contributions that such efforts may make towards conservation are enmeshed in politics, and evaluating impacts on conservation is beyond the scope of this paper. Nevertheless, the broad awareness of, and support for, their missions suggest that these in-country bioprospecting projects generate considerable incentive for conservation.

The first step towards effectively linking bioprospecting and conservation is to ensure the host country receives immediate benefits from the use of its biodiversity. In Panama we have tried to use bioprospecting as a conservation tool by providing tangible benefits, and after only 5 years, our efforts have been acknowledged by the government, the national university, the public, and the press. A second key step is to influence policy and public perception towards conservation. Several of our team members encouraged the government to recognize the value of Panama's unique biodiversity and to initiate the Institute

of Advanced Scientific Investigations and High Technology, a research institute with a substantial bioprospecting component. The National Authority of the Environment (ANAM) has called upon us with increasing frequency to provide technical assistance, and our inventories of plants and insects have been helpful in establishing management plans for protected areas. As part of the ICBG project, we also recently played an instrumental role in helping ANAM apply for UNESCO World Heritage Site status for Coiba National Park and surrounding areas (270 125 ha in all), thereby increasing the probability of effective protection for this unique marine and terrestrial habitat.

Thus, we are using several approaches to link bioprospecting, sustainable use, and economic development with the preservation of Panama's biodiversity. Although bioprospecting can only be one of many simultaneous efforts to promote conservation, it is compatible with other non-destructive uses of biodiversity, such as ecotourism and ecosystem services.

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