Tropical Bryology 6: 193-202, 1992

Systematic screening of bryophytes for antitumor agents

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Abstract. References are made to cytotoxic and/or antitumor compounds that have been isolated - ansamitocin P-3 from *Claopodium crispifolium* (Hook.) Ren. & Card. and *Anomodon attenuatus* Hueb., or an associated actinomycete, and ohioensins and pallidisetums from *Polytrichum* spp. Several hundred collections, which have been obtained from temperate regions of North America during 1990 and 1991, are currently being screened in new bioassays; active sesquiterpene lactones have been recently isolated from species of *Porella*. The methodologies of collecting and screening bryophyte samples are discussed with consideration to costs based on expected number of samples that might be collected in a day, the diversity in the collections as related to phytogeography and vegetation types, and the bryophyte cover that is vanishing in many forest regions of the United States. The difficulties in obtaining large collections for isolation of active agents are also discussed by example-recollection of *Claopodium crispifolium*.

Introduction

Plants, animals, fungi and microbes possess a wide variety of chemical defensive and/or offensive mechanisms as part of their survival strategy; thus, it is not too surprising to learn that nearly half of all medical prescriptions contain ingredients derived from natural sources of which 20-25% are of plant origin (Marderosian & Liberti 1988; Simpson & Conncer-Ogorzaly 1986). Taxol, isolated from the stembark of a conifer, *Taxus brevifolia*, has recently been recognized as a useful compound for treating ovarian and mammarian cancers (Kingston 1991; Rowinsky *et al*. 1990). However, it is estimated that less than 3% of the plants or animals have been thoroughly evaluated by pharmaceutical industries, and there has only been a preliminary screening of bryophytes for biological activities (Spjut *et al*. 1986).

Although a great potential exists for discovering new drugs from natural product chemistry, the methods in drug screening and evaluation are perhaps still in their infancy. The screening of natural products usually involves stepwise methodical procedures of (1) detecting active chemical substances in crude extracts from a diversity of species collections, (2) isolating chemical agents from samples through fractionation guided by activity in bioassay systems, and (3) chemical and pharmacological evaluation of isolated active compounds employing correlative (accessory) bioassays that have predictability in drug development; the term screening refers to any one step or any combination of steps, collectively. Discoveries of new drugs seem rare, but pharmacological evaluation has often been based on administration of active compounds directly into the blood stream, with usually toxic effects to many organs. On the other hand, if the drug potential of active compounds can be evaluated for using more specific forms of drug delivery, such as binding active agents with carrier proteins or cells that function like antibodies, then the number of new drug entities may increase.

The discovery of novel active compounds from natural product sources and their advancement to clinical evaluation also depends on: (a) collection strategy, (b) the sensitivity of the bioassays employed in preliminary screens (step 1 above), (c) the predictiveness of clinical activity in accessory bioassays, and (d) the flexibility to modify bioassay systems, extraction methods and collection methodology as feedback is obtained from identification and pharmacological evaluation of active compounds.

It should be recognized that our screening methodology in isolating compounds via bioassay-activity-directed fractionation of extracted bryophyte samples differs from that of many investigators who have first isolated compounds before testing them for biological activity (*cf*. Asakawa 1990). Additionally, a high incidence of biological activity in screening crude extracts of bryophyte species (*e.g*., McCleary & Walkington 1960; van Hoof *et al*. 1981) without reference to the active compounds or to bioassays with a track record of discovering novel active compounds, may prove less significant if activity is largely due to the presence of an ubiquitous compound.

Because relatively few active compounds have been isolated by activity-directed fractionation of bryophyte samples, we will review the progress that has been made in the screening of bryophytes and also discuss the factors that need to be considered in collecting bryophytes for biological screening programs. But first we will introduce and define terminology generally employed in the procurement and screening of plant samples for biological activity.

Definitions

A **natural product** is an entire organism, or any part of it, preserved, modified, extracted or combined with other substances; this includes whole microbes, whole animals, whole plants, organs, plant parts, DNA, RNA or isolated chemical substances. Naturally occurring minerals may also be included (Marderosian & Liberti 1988).

A **biologically active substance** is one that has demonstrated significant experimental results to: (a) increasing the life span of an organism or its cultured cells; or (b) inhibiting cell growth or preventing diseases, or (c) destroying peptides, DNA or RNA, cells or organisms that are naturally harmful to other organisms. These active substances may be crude samples or crude extracts (mixtures of chemicals) or pure compounds; the associated taxonomic names in a hierarchical classification from which the active substances are derived are also defined as active (*e.g*., the active compound taxol from an active species, *Taxus brevifolia* Nuttall; active genus, *Taxus*; active family, Taxaceae).

Protocols for the NCI antitumor screening are according to Geran *et al*. (1972) and Alley *et al*. (1988). The assays that have been employed for screening bryophytes were briefly described in Spjut *et al*. (1986).

A **collection** refers to one or more samples of an organism identified by a collector's name(s) and date of collection, classified by a species name and documented by a voucher specimen. Bryophytes are usually collected as whole samples and not subdivided into parts, in contrast to vascular plants (*e.g*., root, stembark, twig, leaf of *Taxus brevifolia*).

The samples collected for the initial (preliminary) screening are referred to as **general samples**; those obtained for chemical isolation and pharmacological evaluation of the active agents are termed **recollections**. The procurement of each of these two types of samples require different strategies in planning and execution, although both kinds are often obtained during a single field trip. The strategies for general samples and recollections are later discussed separately in this paper. Because activity in bryophytes may be due to associated microorganisms, **accessory samples**, or additional small samples, might be obtained of an active species for reconfirmation of activity.

Patents based on naturally occurring active substances must not only include references to the unique chemical structure(s), discovered biological activities, isolation and purification techniques, but also references to the source materials -scientific name(s) of the organism(s) and the scientific **voucher specimen(s)** as deposited in an internationally recognized institution (*cf*. Index Herbariorum, Holmgren *et al*. 1990). Scientific names are subject to change as new information is obtained, while the voucher specimen is a permanent reference point. For example, taxol was first isolated from stembark samples of *Taxus brevifolia*, the only species of yew generally thought to occur in the Pacific Northwest, which a USDA patent (Christen *et al*. 1991) was filed on obtaining taxol from tissue cultures of this species; however, Spjut (in prep.) recognizes the possibility of several varieties or species of *Taxus* in the Pacific Northwest. Although, the patent (Christen *et al*. 1991) stated that any species of *Taxus* will produce the desired results, this statement is unfounded.

Review of Antitumor Screening of Bryophytes in 3PS (P-388), KB and ASK

The screening of bryophytes in the National Cancer Institute's (NCI) antitumor screening program was reviewed by Spjut *et. al*. (1986). Bryophytes were gathered in amounts of 250-2000 g, extracted and tested against P-388 lymphocytic luekemia (*in vivo*), Cell Culture (9KB) and astrocytoma (9ASK) cell lines. The test results showed correlations with systematic bryophyte groups, particularly in the Thuidiaceae, Mniaceae and Neckeraceae. These results were considered encouraging because the 9KB and P-388 bioassays have lead to isolation of a diversity of novel active compounds from samples of vascular plant species (Hartwell 1976; Suffness & Douros 1979) and taxol, first isolated during the 1960's from *Taxus brevifolia*, was discovered by this screening methodology, which had also included other bioassays (Hartwell 1976).

The first significant *in vivo* antitumor activity in bryophytes was not discovered until 1979, based on the collection of *Claopodium crispifolium* in northwestern California during 1978 (Spjut *et al*. 1986), in contrast to the discovery of taxol from samples (of a conifer) first collected in 1962. Not all recollections of *C. crispifolium* gave consistent test results, suggesting that associated microorganism activity might be a factor (Spjut *et al*. 1988). Indeed, ansamitocin P-3, which has been isolated from a culture broth of *Nocardia* sp. (Sakai *et al*. 1988), was isolated from samples of *C. crispifolium* and *Anomodon attenuatus* (Suwanborirux *et al*. 1990). But maytansinoid compounds have also been isolated from vascular plants, and it remains to be shown whether the presence of these compounds in terrestrial plants are produced directly by the associated actinomycetes or are a chemical by-product of a symbiotic relationship between the actinomycete and the plant. Maytansinoid compounds, which have been subjected to extensive preclinical-pharmacological evaluation, are highly toxic and, therefore, show little promise in cancer chemotherapy.

Activity reported by Spjut *et al*. (1986) in species of the Neckeraceae might also be due to maytan-

sinoid compounds (Sakai *et al*. 1988) and, as a result, one may wonder if antitumor activity in bryophytes is largely due to chemical products associated with actinomycetes. However, Cassady's group has isolated novel polycyclic benzonaphthoxanthenones, ohioensins A, B, C, D and E through fractionation guided by 9KB and 9PS bioassay activity from samples of *Polytrichum ohioense* Ren. & Card. (Cassady *et al*. 1990; Zheng 1990; Zheng *et al*. 1989, 1992a). These compounds, also active in the human tumor cell lines (A-549 lung carcinoma, MCF-7 breast adenocarcinoma and HT-29 colon adenocarcinoma), are clearly moss-derived as the biogenetic pathway "apparently involves *O*-hydroxycinnamate and hyroxylated bibenzyls as intermediates" (Huneck 1983; Suire & Asakawa 1981; Zheng *et al*. 1989).

The possibility of a higher incidence of maytansinoid activity in bryophytes in comparison to other plant groups should not discourage further screening of them. Rather, the screening methodology simply should take this into consideration. False-positive leads have previously been encountered with new screening procedures or in the screening of new groups of plants. For example, tannins in angiosperms were showing activity in approximately 10% of the aqueous extract that were being tested in the WM and SA tumor assays before the extraction procedure was modified to precipitate out the tannins, but tannin-sensitive tumor assays were also later dropped from the screen (Hartwell 1976). Recently, lichen polysaccharides in aqueous extracts have been found to give false-positive leads in the NCI anti-AIDS screen.

The cytotoxic compounds in species of *Polytrichum*, the ohioensins, were isolated from samples collected in lowland hardwood-pine forest communities in Maryland based on an extract (B-819218) from the original sample (*Spjut 4357*) that showed only 9KB activity, whereas P-388 activity was reported from another extract (B-856807) based on a sample collected in a coniferous (spruce-hemlock) bog forest of the White Mountains in New Hampshire (*Spjut 6449*) (Spjut *et al*. 1986). However, Spjut has reidentified the voucher for the New Hampshire sample as *Polytrichum pallidisetum* Funck, a closely related species that has been considered by some as conspecific with *P. ohioense*.

Recollections of *Polytrichum ohioense* have been obtained from many locations in Maryland and from one location in Pennsylvania, and recollections of *P. pallidisetum* have been obtained from New York, Vermont, New Hampshire and Tennessee; ethanolic extracts prepared from all samples were active. Although the activity was originally determined from the 9KB and 3PS assays, fractionation of the larger samples was also guided by activity in the human tumor cell lines A-549, HT-29, MCF-7, RPMI-7951 and U251MG (Alley *et al*. 1988). In addition to the isolation of ohioensins A through E from *P. ohioense*, several other novel compounds, namely 1-O-methylohioensin B, 1-O methyldihydroohioensin B, pallidisetin A and pallidisetin B, were isolated from *P. pallidisetum* (Zheng 1990; Zheng *et al*. 1992b). The pallidisetins, which are active against RPMI-7951 and U251MG cell lines, are novel cinnamoyl bibenzyls that differ from the ohioensins structurally. These two classes of compounds may be derived via different biosynthetic pathways from cinnamic acid and bibenzyls as the common precursors. Thus, there appears to be a biochemical activity distinction between the two species.

Most of the active species reported by Spjut *et al*. (1986) were collected during 1986-1989 in small quantities (accessory samples) for reconfirmation of activity, and those that reconfirmed were recollected in large quantities for fractionation and further screening. This work is being conducted by one of our groups at Virginia Polytechnic Institute & State University. Some sixty bryophytes were collected and screened against the 9KB and 3PS assays. Twelve extracts showed T/C >125 in the 3PS assay. Fractionation of these extracts has not to date yielded any pure active compounds; however, either activity was lost on fractionation or recollections failed to confirm the original results. It may be that activity is due in part to associated organisms. Moreover, many of the active species reported by Spjut *et al*. (1986) were only marginally active. The relatively

low percentage of reconfirmation in this particular case should not discourage further screening of bryophytes.

Screening of bryophytes in other bioassays

Several hundred new general samples have been obtained since 1990 for antitumor screening in new bioassays at Virginia Polytechnic Institute & State University. These bioassays employ genetically engineered yeast strains to detect extracts that show DNA-damaging activity in various ways. Activity is determined as an IC_{12} value, which is defined as the dose (in µg/ml) necessary to produce a 12 mm zone of inhibition in a repair-deficient yeast strain. For useful selective activity, the extract must be at least threefold less active (*i.e*. have an IC_{12} at least three times larger) in a repair-deficient yeast strain. An extract is considered marginally active in its initial screening if it has an IC_{12} value between 4000 and 8000, and moderately or highly active if it has an IC_{12} value less than 4000; in each case appropriate selectivity is required.

Based on these criteria, out of some 200 bryophytes collected, a total of 22 (11%) had IC_{12} values less than 4000 in at least one extract, while an additional 27 had IC_{12} values in the 4000-8000 range. Four bryophytes (25) yielded extracts with excellent IC_{12} values of less than 1000.

Regrettably, many of the active species collected have failed to reconfirm activity on recollection. One bryophyte, from which methanol extracts were prepared, gave an IC_{12} value of 110 in the initial small collection but IC_{12} value of greater than 16,000 on recollection, even though recollection was from the same locality as the original sample. Another bryophyte with an IC_{12} value of 427 in one extract was also inactive on recollection. On the other hand, activity can sometimes increase on recollections; one species with an initial IC_{12} value of 4315, gave a much better IC_{12} value of 635 on recollection. In yet other cases, activity is essentially constant between the initial collection and the recollections. A sequiterpene lactone has been isolated from a species of *Porella*.

The failure of a significant number of bryophytes to confirm activity on recollection may indicate that the chemical constituents of these plants are more sensitive to variation in the season, soil type, etc. than what we have generally experienced in the screening of vascular plants. Additionally, the presence of associated microorganisms that may affect the production of active agents in a bryophyte may be subject to the same ecological variables and, in those cases where activity was highly significant, it may be worthwhile to culture the microorganisms.

Systematic collection of general samples

The collection strategy employed by field biologists may be a systematic one in which species are collected as encountered in selected geographic areas, with consideration to phytogeographic and taxonomic relationships as additional species are screened (Spjut 1985), in contrast to selecting species of plants according to their uses in folk medicine, or on the basis of chemosystematic data. The objective is to screen as much taxonomic diversity as possible for the least cost. The factors that need to be assessed in estimating procurement costs are discussed first. Most bryophytes have been collected in North America because procurement and identification costs for these samples are less expensive.

Second, the selection of a geographic area will be exemplified by considering the phytogeography and bryodiversity in tropical Africa. Consideration is given not only to the phytogeographical elements within political boundaries, but also to previous collections that might have been obtained for screening and to the existence of the least-disturbed vegetation types. Additionally, bryophytes are mostly collectable in the upland areas, whereas in the lowland areas they are generally less abundant and often found in the forest canopy.

Third, the NCI's intent to develop cooperative agreements with foreign institutions will be mentioned. The long-term cooperation between the host organization and the collectors is criti-

cal. Although the discovery of a new drug might be seen as a humanitarian benefit, especially in regard to diseases like AIDS and cancer, there is potential economic benefit to the host country by the addition of a new agricultural commodity. However, others feel that further compensation in the form of royalities of 1-2% should be paid to societies and/or governing institutions who make their resources available to foreign drug discovery programs, especially if drug discovery is the result of contacts with local medical practitioners.

Costs. Costs are a necessary consideration to proposals and become part of the competitive process for deciding awards; costs are then accounted for in the activities of carrying out a proposal. Scientists also have to report their results if they want to continue their drug discovery efforts in screening bryophytes.

Cost estimates for general samples are based on the expected number of species collections that might be obtained in one day and the number of days spent in the field before diminishing returns on the daily yield becomes apparent. For example, Spjut and Norris (Spjut *et al*. 1986) collected on the average 6 bryophyte samples per day for a period of several weeks in northern California before it became obvious that further collecting would not be productive in northern California.

The costs to collect bryophytes for pharmacological screening are more difficult to estimate than those for collecting samples of higher plants. Bryofloras are less known for most areas and field identification requires applying taxonomic characters not easily visible with a hand lens.

Probably not more than 20% of the bryoflora in an area is available in adequate quantity for screening, but this estimate is being reduced by deterioration of tropical and temperate vegetation due to clear-cutting, burning and atmospheric pollution. During 1986-1990, Spjut (1991) noted the rapid disappearance of bryophyte cover from rocks and soil near streams across North America, and in the northeastern United States many of the drier shaded rocks in forests that were once recalled as being covered by bryophytes and lichens, are now completely bare. As another example, *Thuidium erectum* Duby (synonym *T. delicatulum* (Hedw.) BSG), which was observed in many forest communities of the eastern United States prior to 1986, was rarely seen during field work in 1990 and 1991; the same was also true for lichen species of *Peltigera*. Some taxonomic groups of bryophytes appear to be more tolerant to pollutants (Spjut 1991; species of Polytrichaceae and liverworts).

In many areas, bryophyte species are not easily identified in the field, and laboratory identification can be a costly activity. The bryotaxonomist will not want to provide complete species names until the taxonomic characters of the specimens have been checked under a microscope and compared with authenticated specimens in the herbaria, sometimes requiring assistance from other specialists. On the other hand, the ethnopharmacologist or ethnochemist, the one usually designated as the project leader, is more concerned about getting the samples into the screen. Thus, there may have to be some compromise between the level of taxonomic identification and need for samples in the screen. This situation is more applicable to bryophytes of tropical regions where knowledge of the bryoflora is incomplete. General collections that show activity should be re-examined for identification before recollection.

Prior to 1985, general samples for preliminary antitumor screening by NCI required 1 kg, and up to 100 kg for isolation and identification of the active constituents (Spjut *et al*. 1986, 1988). Bryophyte samples are currently being screened for antitumor activity by Virginia Polytechnic Institute & State University utilizing samples weighing less than 100 g. The lesser amount of material has not changed the overall yield in number of species collected because of apparent diminishing bryophyte growth.

Cost effectiveness depends on the abilities of the field personnel to distinguish species in the field and to recognize which species are likely to be available in sufficient quantity for screening. This requires both training and practice. Furthermore, it is also advantageous to employ a

bryologist who has familiarity with the local geography.

Diversity and Phytogeography. To exemplify the phytogeographic approach, one might look at the bryoflora of Africa. A good representation of the African bryoflora might be obtained by first collecting in Kenya from the following localities as characterized by their vegetational/floristic types: (1) Mount Londiani, a seasonally dry, sclerophyll type forest, primarily found in the montane regions of eastern Africa from Ethiopia to Malawi; (2) Mount Kenya, typical of the Afromontane rain forest; (3) the Kikuyu Escarpment, seasonally dry forest with many deciduous trees and understory shrubs, somewhat spotty in distribution, extending into southern Africa; and (4) the Kakamega, an upland humid rain forest with affinities to west African lowland rain forests. Further collections in East Africa might be obtained from the Usambara Mountains of Tanzania where one may expect to find many species not previously collected in Kenya because of affinities to the bryoflora of Madagascar (Pócs 1975; Rodgers & Homewood 1982), which is in contrast to the bryoflora of the southern highlands of Tanzania that would yield fewer new species because the bryoflora there has stronger affinities to species that would have been collected on Mount Kenya. Similar examples could be described for Latin America, perhaps beginning with Mexico taking into consideration its phytogeography from data in Bartram (1949) and Delgadillo (1971, 1979).

Prior to 1982, many taxa of vascular plants were precluded from antitumor screening because of known active compounds, *e.g.*, cucurbitacins (9KB activity) in species of Cucurbitaceae, cardenolides (9KB activity) in *Asclepias* (Asclepiadaceae), quassinoids (9KB and P-388 activity) in species of Simaroubaceae (Spjut 1985). Grasses were generally not collected because they rarely showed activity. The screening of bryophytes has never progressed to the point of precluding taxa from collection, although one might want to limit the number of species of *Sphagnum* in the screen because of the numerous species in a genus that has a cosmopolitan distribution; Spjut *et al*. (1986) listed samples from ten or more species that were screened but none were active.

In screening programs, there is often emphasis on numerical quotas without regard to the taxonomic diversity being represented in the samples. Spjut (1985) proposed an identification manual that would make it possible for collectors to readily identify many common tropical vascular plant species. The same author has also considered developing a field key to common bryophytes of North America; however, he is discouraged by the rapid decline in bryophyte cover in many regions; thus, such a key might only have temporary value.

Cooperative agreements between collectors and host countries. NCI, in developing agreements with foreign government institutions, expresses their intent to have a percentage of the monetary profits from sales of new drugs be returned to the country that was the originating source of the natural product. NCI also will allow foreign institution the first opportunity to develop the cultivation of the source for the drug. World Botanical Associates proposes to follow the NCI guidelines with modification to have the monetary returns be divided among those institutions who collaborate based on the geographical distribution of a species since species are not strictly confined to political boundaries.

Recollections

In biological screening of bryophytes, reconfirmation of bioassay activity should take into consideration various associated microorganisms (Spjut *et al*. 1988). Indeed, a bryophyte sample might be viewed as a whole community of organisms dominated by a particular species of moss, liverwort or hornwort. Therefore, an extracted sample from a bryophyte species that shows activity should be evaluated using new (accessory) samples of the same species of a moss from different locations before large-scale collections are obtained for isolation of the active compounds.

Recollections are often obtained by a trial and error process of learning the ideal conditions under which a species grows. For vascular plants, locality data on herbarium specimens and publications on vegetational composition of species often provide good leads to specific locations where a species occurs in abundance; however, bryotaxonomists in conducting floristic field projects rarely provide information on the abundance or luxuriance of growth for their collections. As already indicated, bryophyte identification is based on microscopic characters, and bryotaxonomists who collect for floristic reasons will focus more on differences in microhabitats to be certain of obtaining a good representation of the flora rather than spend time in the field being concerned about the identification and abundance of a species. On the other hand, one who is trained to collect for biological screening programs will have developed the ability to automatically keep a mental record on abundance of species, which is particularly helpful when estimates for the cost of recollections are requested. Nonetheless, locality data on labels of bryophyte herbarium specimens may narrow the geographical range of a search by the apparent concentration of collections within an area.

Bryologists, of course, are able to recognize certain species in the field, often in part by taxonomic characters that can be seen with a hand lens, and by other characters that are often not mentioned in the keys. For instance, species of *Polytrichum* and related genera are often recognized at a glance by the miniature conifer-like habit, *i.e*., erect stiff wiry stems bearing spirally arranged needlelike leaves. Depending on the knowledge of the bryoflora, field identification to species is often possible; in North America, *Polytrichum piliferum* Hedw. may be identified by its preference for open sunny siliceous rocks and by examination of the leaf for the presence of a whitish-hyaline awn and incurved leaf margins that cover the lamellae.

Recollection of *Claopodium crispifolium*. The recollections of up to 50 kg of *Claopodium crispifolium* (Spjut *et al*. 1988) were largely obtained by a trial and error process in field observations to narrow the search to the most preferred habitat type for the species. Prior to conducting field work, Spjut compiled data on ecology and geographical distribution from various sources that included communication with Daniel Norris, labels on herbarium specimens at US and from Lawton (1971). *C. crispifolium* appeared to have been frequently collected on logs or on bark at the base of big-leaf maple (*Acer macrophyllum* Pursh) in the redwood forest and mixed-evergreen forest regions of northern California to Washington. Norris also indicated that *C. bolanderi* Best might be mistaken for *C. crispifolium*, but as he further indicated this species usually occurs on rock at higher elevations [in California (Spjut 1971) and in Oregon, but not in Washington]. While this preliminary information was generally accurate, it was still inadequate for identifying sites with unusually large quantities of *C. crispifolium* that were needed for screening. As a result of conducting transect-surveys of the coast ranges in California and Oregon, *C. crispifolium* was most frequently observed within a narrow longitudinal zone, which seems to correspond to the most inland occurrence of fog. This led to focusing the search within this zone with further refinement to elevation, in California generally near 250 m altitude, and in Oregon from 150-225 m altitude. The best growth of *C. crispifolium* was then observed in riparian forests of old growth maple or mixed Douglas fir and maple. In addition to occurring on bark or logs, this species was collected as a dominant moss on large rocks (Spjut *et al*. 1988).

Approximately 7 days were spent conducting a survey of the northern Coast Ranges of California and Oregon before reasonable collection sites for *C. crispifolium* were found. Once the preferred ecological parameters were learned, good quantities of *C. crispifolium* could be located within a days time.

Subsequent recollections of other bryophyte species have required less investment of time for learning ecological parameters, but environmental pollution is throwing a ringer into the learning curve because bryophytes are simply dying out in many places. Currently, up to three days are allowed for recollection of a

bryophyte species. Recollections that require more time are considered too expensive, unless the screening results are highly active or significant progress has already been made on isolating the active compounds.

Environomental impact on recollecting *Claopodium crispifolium*. Samples of *C. crispifolium* were collected in habitats where this species was unusally abundant, thus, the removal of this moss from various rocks and trees is expected to have little effect on the surivival of the species and its general distribution; nonetheless, the structure of the remaining bryophyte-fern community has been changed and one may be concerned about the redevelopment of the community on the surface of rocks or trees that were exposed as a result of collecting *C. crispifoliun*. The recollections sites of *C. crispifolium* in California (Spjut *et al*. 1988) were revisited by Spjut four years later and he found these to be mostly bare; however, Norris (pers. comm.) has since revisited these sites and indicated that species of *Encalypta* have filled in the bare spots. It is generally recognized that the composition of bryophyte cover on a particular habitat will change, especially in secondary growth forests, but there is little published information on changes in bryophyte composition in seral communities.

Acknowledgments We thank Daniel Norris, Professor Emeritus, Oregon State University, for his review and comments on the manuscript and also for identification checks of many of the moss collections; David Wagner, Curator and Professor, University of Oregon, for checking the identifications of liverwort collections; and David K. Ho, Research Associate, Ohio State University (now at NCI), for information from Dean Cassady's group on the isolation of active compounds from samples of *Polytrichum pallidisetum*.

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