Natural Products from Endophytic Microorganisms[⊥]

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Endophytic microorganisms are to be found in virtually every plant on earth. These organisms reside in the living tissues of the host plant and do so in a variety of relationships ranging from symbiotic to pathogenic. Endophytes may contribute to their host plant by producing a plethora of substances that provide protection and ultimately survival value to the plant. Ultimately, these compounds, once isolated and characterized, may also have potential for use in modern medicine, agriculture, and industry. Novel antibiotics, antimycotics, immunosuppressants, and anticancer compounds are only a few examples of what has been found after the isolation and culturing of individual endophytes followed by purification and characterization of some of their natural products. The prospects of finding new drugs that may be effective candidates for treating newly developing diseases in humans, plants, and animals are great. Other applications in industry and agriculture may also be discovered among the novel products produced by endophytic microbes.

Preamble

This issue of the *Journal of Natural Products* is being dedicated to the memory/work of Dr. Monroe Wall and to the work of Manuskh C. Wani, and so too is this article. Certainly, the greatest scientific contribution coming from the natural products laboratory of these gentlemen, over a lifetime of work in natural products chemistry, was the isolation and characterization of taxol, now known as paclitaxel (Taxol).[1] This seminal contribution has affected and improved the lives of untold numbers of patients in the world as they receive treatment for cancer remediation, especially breast cancer. In addition, from a natural products perspective, this work has served as a model for others to strive to discover other natural products that may have clinical applications. The story of taxol is a classic one that exemplifies all aspects of how a natural product is developed for medicinal purposes. It took the span of one generation for taxol eventually to be a medical wonder drug and the world's first billion-dollar anticancer compound.[2] It started with discovery of the novel natural product taxol by Wall and Wani and co-workers, followed by a 10-year hiatus, then classical mode of action studies by Horwitz and her group that eventually led to animal and clinical trials and finally to a product in the hands of the medical profession.[3] The entire process leading from drug discovery to a final product was not easy, as it had many detractors as well as champions.

I, Gary Strobel, first met both Drs. Wall and Wani at a bioprospecting conference that was held in Caracas, Venezuela, in January 1998. Even at a late age and with physical infirmities, Dr. Wall was still an active chemist, and so too was Dr. Wani. I was told that this conference would be the last major international meeting of Dr. Wall. This, in itself was inspiring, since many elect to retire whenever they can and leave science. In his case, Dr. Wall stayed the course and remained productive almost up to his dying day. After the conference, my wife Suzan and I headed for the tepui lands of the Venzuelan−Guyana border to collect some plant stems for the eventual isolation of endophytic fungi and bacteria. My approach to isolate and grow endophytes for novel and bioactive natural products had resulted, in part, from the lives and legends of Drs. Wall and Wani, who had been pioneers in finding an extremely important and useful anticancer compound. As it is with so many people in science, Dr. Wall probably never knew how his contributions served to inspire and influence others. Certainly, the work of Drs. Wall and Wani has inspired me and the people in my laboratory to continue to search for and find bioactive molecules from unusual places such as the endophytes in plants. Finally, it appears that an important lesson needs to be learned from their approach in natural products chemistry. We need to understand and support the concept that, despite a growing interest in synthetic products, bioactive natural products can have an enormous impact on human health and are still out there waiting to be found and developed.

Introduction

The need for new and useful compounds to provide assistance and relief in all aspects of the human condition is ever-growing. Drug resistance in bacteria, the appearance of life-threatening viruses, the recurrent problems of diseases in persons with organ transplants, and the tremendous increase in the incidence of fungal infections in the world's population all underscore our inadequacy to cope with these medical problems. Added to this are enormous difficulties in raising enough food in certain regions of the earth to support local human populations. Environmental degradation, loss of biodiversity, and spoilage of land and water also add to problems facing mankind.

Endophytes, microorganisms that reside in the tissues of living plants, are relatively unstudied as potential sources of novel natural products for exploitation in medicine, agriculture, and industry. Of the approximately 300 000 higher plant species that exist on the earth, each individual plant, of the billions that exist here, is host to one or more endophytes (Strobel, G. A., unpublished data). Only a handful of these plants (grass species) have ever been completely studied relative to their endophytic biology. Consequently, the opportunity to find new and inter-

[⊥] Dedicated to the late Dr. Monroe E. Wall and to Dr. Manuskh C.Wani of Research Triangle Institute for their pioneering work on bioactive natural products.

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esting endophytic microorganisms among myriads of plants in different settings and ecosystems is very great. The intent of this review is to provide insights into the occurrence in nature, the products that they make, and how some of these organisms are beginning to show some potential for human use. The majority of the report discusses the rationale for study, methods used, and examples of a plethora of endophytes isolated and studied in the authors' laboratory over the course of many years. This review, however, also includes some specific examples that illustrate the work of others in this emerging field of bioprospecting the microbes of the world's rainforests.

The Promise of Bioactive Compounds from Endophytes

Needs for New Natural Products. There is a general call for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity, and will have a minor environmental impact, respectively. This search is driven by the development of resistance in infectious microorganisms (e.g., *Staphylococcus*, *Mycobacterium*, *Streptococcus*) to existing drugs and by the menacing presence of naturally resistant organisms. The ingress to the human population of new disease-causing agents such as AIDS, Ebola, and SARS requires the discovery and development of new drugs to combat them. Not only do diseases such as AIDS require drugs that target them specifically, but new therapies are needed for treating ancillary infections which are a consequence of a weakened immune system. Furthermore, others who are immunocompromised (e.g., cancer and organ transplant patients) are at risk of infection by opportunistic pathogens, such as *Aspergillus*, *Cryptococcus*, and *Candida*, that normally are not major problems in the human population. In addition, more drugs are needed to efficiently treat parasitic protozoan and nematodal infections such as malaria, leishmaniasis, trypanomiasis, and filariasis. Malaria, by itself, is more effective in claiming lives each year than any other single infectious agent with the exceptions of AIDS and TB.[4] However, the enteric diseases claim the most lives each year of any other disease complex, and unfortunately, the victims are mostly children.[4]

Finally, because of safety and environmental problems, many synthetic agricultural agents have been and currently are being targeted for removal from the market, which creates a need to find alternative ways to control farm pests and pathogens.[5] Novel natural products and the organisms that make them offer opportunities for innovation in drug and agrochemical discovery. Exciting possibilities exist for those who are willing to venture into the wild and unexplored territories of the world to experience the excitement and thrill of engaging in the discovery of endophytes, their biology, and potential usefulness.

Natural Products and Traditional Approaches in Medicine. Natural products are naturally derived metabolites and/or byproducts from microorganisms, plants, or animals.[6] These products have been exploited for human use for thousands of years, and plants have been the chief source of compounds used for medicine. Today the largest users of traditional medicines are the Chinese, with over 5000 plants and plant products in their pharmacopeia.[7] In fact, the world's best known and most universally used medicinal agent is aspirin, which is related to salicin, having its origins in the plant genera *Salix* spp. and *Populus* spp. Examples abound of natural product use, especially in small native populations in a myriad of remote locations on earth. For instance, certain tribal groups in the Amazon basin, the highland peoples of Papua New Guinea, and the Aborigines of Australia each have identified certain plants to provide relief of symptoms varying from head colds to massive wounds and intestinal ailments.[8] History also shows that now extinct civilizations had also discovered the benefits of medicinal plants. In fact, nearly 3000 years ago, the Mayans used fungi grown on roasted green corn to treat intestinal ailments.[9] More recently, the Benedictine monks (800 A.D.), following the example of the ancient Greeks, began to apply *Papaver somniferum* as an anesthetic and pain reliever.[10] Many people, in past times, realized that leaf, root, and stem concoctions had the potential to help them. These plant products, in general, enhanced the quality of life, reduced pain and suffering, and provided relief, even though an understanding of the chemical nature of bioactive compounds in these complex mixtures and how they functioned remained a mystery.

It was not until Pasteur discovered that fermentation is caused by living cells that people seriously began to investigate microbes as a source for bioactive natural products. Then, scientific serendipity and the power of observation provided the impetus to Fleming and Chain to usher in the antibiotic era via the discovery and development of penicillin from the fungus *Penicillium notatum*. Since then, scientists have been engaged in the discovery and application of microbial metabolites with activity against both plant and human pathogens. Furthermore, the discovery of a plethora of microbes for applications that span a broad spectrum of utility in medicine (e.g., anticancer and immunosuppressant functions), agriculture, and industry is now extremely practical because of the development of novel and sophisticated relevant screening processes. These processes use individual organisms, cells, enzymes, and site-directed techniques, as well as automated arrays, resulting in the rapid detection of promising leads for product development.

Even with untold centuries of human experience behind us and a movement into a modern era of chemistry and automation, it is still evident that natural product-based compounds have had an immense impact on modern medicine. For instance, about 40% of prescription drugs are based on them. Furthermore, well over 50% of the new chemical products registered by the FDA as anticancer agents, antimigraine agents, and antihypertensive agents were natural products or derivatives thereof in the timeframe of 1981−2002.[11] Excluding biologics, between 1989 and1995, 60% of approved drugs and pre-new drug application candidates were of natural origin.[10] From 1983 to 1994, over 60% of all approved and pre-NDA stage cancer drugs were of natural origin, as were 78% of all newly approved antibacterial agents.[12] Many other examples abound that illustrate the value and importance of natural products from plants and microorganisms in modern civilizations, and paclitaxel (Taxol) is the most recent example of an important natural product that has made an enormous impact on medicine.[1,13]

Recently, however, natural product research efforts have lost popularity in many major drug companies and, in some cases, have been replaced entirely by combinatorial chemistry, which is the automated synthesis of structurally related small molecules.[11] In addition, many drug companies have developed interests in making products that have a larger potential profit base than antiinfectious drugs. These synthetic compounds are ones that may provide social benefits, relieve allergenic responses, reduce the pain of arthritis, or sooth the stomach. It appears that this loss

of interest in natural products not only is an economically driven decision but can be attributed to the enormous effort and expense that is required to pick and choose a biological source, then to isolate active natural products, decipher their structures, and begin the long road to product development.[10] It is also apparent that combinatorial chemistry and other synthetic chemistry methodology revolving around certain basic chemical structures are now serving as a never-ending source of products to feed the screening robots of the drug industry. Within many large pharmaceutical companies, progress of professionals is based primarily upon numbers of compounds that can be produced and sent to the screening machines. This tends to work against the numerous steps needed even to find one compound in natural product discovery. It seems important to emphasize that the primary purpose of combinatorial chemistry should be to complement and assist the efforts of natural product drug discovery and development, not to supersede it.[10] For this reason a few larger companies still retain an interest in natural products chemistry. The natural product often serves as a lead molecule whose activity can be enhanced by manipulation through combinatorial and synthetic chemistry. Natural products have been the traditional pathfinder compounds with an untold diversity of chemical structures unparalleled by even the largest combinatorial libraries.

Endophytic Microbes. It may also be true that a reduction in interest in natural products for use in drug development has happened as a result of people growing weary of dealing with the traditional sources of bioactive compounds, including plants of the temperate zones and microbes from a plethora of soil samples gathered in different parts of the world by armies of collectors. In other words, why continue to do the same thing when robots, combinatorial chemistry, and molecular biology have arrived on the scene? Furthermore, the logic and rationale for time and effort spent on drug discovery using a target-site-directed approach has been overwhelming.

While combinatorial synthesis produces compounds at random, secondary metabolites, defined as low molecular weight compounds not required for growth in pure culture, are produced as an adaptation for specific functions in nature.[14] Shutz notes that certain microbial metabolites seem to be characteristic of certain biotopes, both on an environmental and an organismal level.[15] Accordingly, it appears that the search for novel secondary metabolites should center on organisms that inhabit unique biotopes. Thus, it behooves the investigator to carefully study and select the biological source before proceeding, rather than to take a totally random approach in selecting the source material. Careful study also indicates that organisms and their biotopes that are subjected to constant metabolic and environmental interactions should produce even more secondary metabolites.[15] Endophytes are microbes that inhabit such biotopes, namely, higher plants, which is why they are currently considered as a wellspring of novel secondary metabolites offering the potential for medical, agricultural, and/or industrial exploitation. In addition, it also is extremely helpful for the investigator to either have access to or have some expertise in microbial taxonomy, and this includes modern molecular techniques involving sequence analyses of 16S and 18 S rDNA. Currently, endophytes are viewed as an outstanding source of bioactive natural products because there are so many of them occupying literally millions of unique biological niches (higher plants) growing in so many unusual environments. Thus, it would appear that a myriad of biotypical factors associated with plants can be important in the selection of a plant for study. It may be the case that these factors may govern which microbes are present in the plant as well as the biological activity of the products associated with these organisms.

Since the discovery of endophytes in Darnel, Germany, in 1904, various investigators have defined endophytes in different ways, which is usually dependent on the perspective from which the endophytes were being isolated and subsequently examined.[16] Bacon et al. give an inclusive and widely accepted definition of endophytes: "microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects".[17] While the symptomless nature of endophyte occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggests they can also be aggressive saprophytes or opportunistic pathogens. Both fungi and bacteria are the most common microbes existing as endophytes. It would seem that other microbial forms most certainly exist in plants as endophytes such as mycoplasmas, rickettsia, and archebacteria; however, no evidence for them has yet been presented. The most frequently isolated endophytes are the fungi. It turns out that the vast majority of plants have not been studied for their endophytes. However, the best studied endophyte (plant/microbe) system to date seems to be of the grass species/*Neotyphodium* sp. relationship. A host of biologically active compounds including toxic alkaloids have been comprehensively reviewed elsewhere.[17] However, enormous opportunities exist for the recovery of novel fungal forms, including genera, biotypes, and species in the myriad of plants yet to be studied. Hawksworth and Rossman estimated there may be as many as 1 million different fungal species, yet only about 100 000 have been described.[18] As more evidence accumulates, estimates keep rising as to the actual number of fungal species. For instance, Dreyfuss and Chapela estimate there may be at least 1 million species of endophytic fungi alone.[19] It seems obvious that endophytes are a rich and reliable source of genetic diversity and may represent previously undescribed species. Finally, in our experience, novel microbes (as defined at the morphological and or molecular levels) often have associated with them novel natural products. This fact alone helps eliminate the problems of dereplication in compound discovery.

Rationale for Plant Selection. It is important to understand the methods and rationale used to provide the best opportunities to isolate novel endophytic microorganisms at the genus, species, or biotype level. Thus, since the number of plant species in the world is so great, creative and imaginative strategies must be used to quickly narrow the search for endophytes displaying bioactivity.[20]

A specific rationale for the collection of each plant for endophyte isolation and natural product discovery is used. Several hypotheses govern this plant selection strategy, and these are as follows:

1. Plants from unique environmental settings, especially those with an unusual biology and possessing novel strategies for survival, are seriously considered for study.

2. Plants that have an ethnobotanical history (use by indigenous peoples) that is related to the specific uses or applications of interest are selected for study. These plants are chosen either by direct contact with local peoples or via local literature. Ultimately, it may be learned that the healing powers of the botanical source, in fact, may have

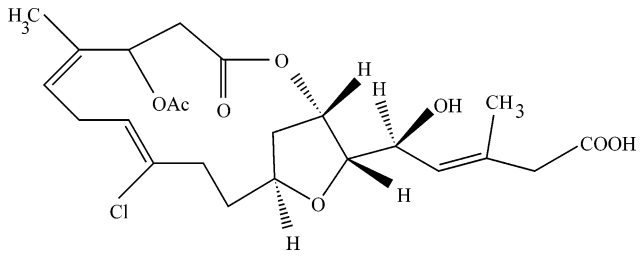

Figure 1. Oocydin A, a chlorinated macrocyclic lactone isolated and characterized from a strain of *Serratia marcescens*, obtained from *Rhyncholacis penicillata*. (Stereochemistry is not completely known.)

nothing to do with the natural products of the plant, but of the endophyte (inhabiting the plant).

3. Plants that are endemic, having an unusual longevity, or that have occupied a certain ancient land mass, such as Gondwanaland, are also more likely to lodge endophytes with active natural products than other plants.

4. Plants growing in areas of great biodiversity, it follows, also have the prospect of housing endophytes with great biodiversity.

Just as plants from a distinct environmental setting are considered to be a promising source of novel endophytes and their compounds, so too are plants with an unconventional biology. For example, an aquatic plant, *Rhyncholacis penicillata*, was collected from a river system in southwest Venezuela where the harsh aquatic environment subjected the plant to constant beating by virtue of rushing waters, debris, and tumbling rocks and pebbles.[21] These environmental insults created many portals through which common phytopathogenic oomycetes could enter the plant. Still, the plant population appeared to be healthy, possibly due to protection from an endophytic product. This was the environmental biological clue used to pick this plant for a comprehensive study of its endophytes. Eventually, an unusual and potent antifungal strain of *Serratia marcescens*, living as both an epiphyte and an endophyte, was recovered from *R. penicillata*. This bacterium was shown to produce oocydin A, a novel antioomycetous compound, having the properties of a chlorinated macrocyclic lactone (Figure 1).[21] It is conceivable that the production of oocydin A by *S. marcescens* is directly related to the endophyte's relationship with its higher plant host. Currently, oocydin A is being considered for agricultural use to control the ever-threatening presence of oomyceteous fungi such as *Pythium* spp. and *Phytophthora* spp.

Plants with ethnobotanical history, as mentioned above, also are likely candidates for study since the medical uses for which the plant was selected may relate more to its population of endophytes than to the plant biochemistry itself. For example, a sample of the snakevine, *Kennedia nigriscans*, from the Northern Territory of Australia, was selected for study since its sap has traditionally been used as bush medicine for many millenia. In fact, this area was selected for plant sampling since it has been home to the world's longest standing civilization—the Australian Aborigines. The snakevine is harvested, crushed, and heated in an aqueous brew by local Aborigines in southwest Arnhemland to treat cuts, wounds, and infections. As it turned out, the plant contained a streptomycete that possessed unique partial 16S rDNA sequences when compared to those in GenBank. The organism was designated *Streptomyces* NRRL 30562, and it produces broad spectrum novel peptide antibiotics called munumbicins, which are discussed below.[22] It seems likely that some of the healing properties in plants, as discovered by indigenous peoples, might be facilitated by compounds produced by one or more specific plant-associated endophytes as well as the plant products themselves.

In addition, it is worthy of note that some plants generating bioactive natural products have associated endophytes that produce the same natural products. Such is the case with taxol, a highly functionalized diterpenoid and famed anticancer agent that is found in each of the world's yew tree species (*Taxus* spp.).[2] In 1993, a novel taxol-producing fungus, *Taxomyces andreanae*, from the yew *Taxus brevifolia* was isolated and characterized.[23]

Endophytes and Biodiversity. Of the myriad of ecosystems on earth, those having the greatest general biodiversity of life seem to be the ones also having the greatest number and most diverse endophytes. Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth. The most threatened of these spots cover only 1.44% of the land's surface; yet, they harbor over 60% of the world's terrestrial biodiversity.[20] In addition, each of the 20−25 areas identified as supporting the world's greatest biodiversity also supports unusually high levels of plant endemism.[20] As such, one would expect with high plant endemism there also should exist specific endophytes that may have evolved with the endemic plant species. Biological diversity implies chemical diversity because of the constant chemical innovation that is required to survive in ecosystems where the evolutionary race to survive is most active. Tropical rainforests are a remarkable example of this type of environment. Competition is great, resources are limited, and selection pressure is at its peak. This gives rise to a high probability that rainforests are a source of novel molecular structures and biologically active compounds.[24]

Bills et al. describe a metabolic distinction between tropical and temperate endophytes through statistical data that compare the number of bioactive natural products isolated from endophytes of tropical regions to the number of those isolated from endophytes of temperate origin.[13] Not only did they find that tropical endophytes provide more active natural products than temperate endophytes, but they also noted that a significantly higher number of tropical endophytes produced a larger number of active secondary metabolites than did fungi from other substrata, as illustrated by the discovery of cyclosporin A.[25] This observation suggests the importance of the host plant as well as the ecosystem in influencing the general metabolism of endophytic microbes.

Endophytes and Phytochemistry. Tan and Zou believe the reason some endophytes produce certain phytochemicals, originally characteristic of the host, might be related to a genetic recombination of the endophyte with the host that occurred in evolutionary time.[16] This is a concept that was originally proposed as a mechanism to explain why *T. andreanae* may be producing taxol.[26] Thus, if endophytes can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow-growing and possibly rare plants but also help to preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a high value product may be easier and more economical to produce effectively, thereby reducing its market price.

All aspects of the biology and interrelatedness of endophytes with their respective hosts are a vastly underinvestigated and exciting field.[27,28] Thus, more background information on a given plant species and its microorganismal biology would be exceedingly helpful in directing the

search for bioactive products. Presently, no one is quite certain of the role of endophytes in nature and what appears to be their relationship to various host plant species. While some endophytic fungi appear to be ubiquitous (e.g., *Fusarium* spp., *Pestalotiopsis* spp.,and *Xylaria* spp.), one cannot definitively state that endophytes are truly host specific or even systemic within plants any more than assume that their associations are chance encounters. Frequently, many endophytes of the same species are isolated from the same plant, and only one or a few biotypes of a given fungus will produce a highly biologically active compound in culture.[29] A great deal of uncertainty also exists between what an endophyte produces in culture and what it may produce in nature. It does seem possible that the production of certain bioactive compounds by the endophyte in situ may facilitate the domination of its biological niche within the plant or even provide protection to the plant from harmful invading pathogens. Furthermore, little information exists relative to the biochemistry and physiology of the interactions of the endophyte with its host plant. It would seem that many factors changing in the host as related to the season and other factors including age, environment, and location may influence the biology of the endophyte. Indeed, further research at the molecular level must be conducted in the field to study endophyte interactions and ecology. All of these interactions are probably chemically mediated for some purpose in nature. An ecological awareness of the role these organisms play in nature will provide the best clues for targeting particular types of endophytic bioactivity with the greatest potential for bioprospecting.

Collection, Isolation, and Preservation Techniques of Endophytes. After a plant is selected for study, it is identified, and its location is plotted using a global positioning device. Small stem pieces are cut from the plant and placed in sealed plastic bags after excess moisture is removed. Every attempt is made to store the materials at 4 °C until isolation procedures can begin.[30,31]

In the laboratory, plant materials are thoroughly surface treated with 70% ethanol, sometimes flamed, and ultimately they are air-dried under a laminar flow hood. This is done in order to eliminate surface contaminating microbes.[30] Then, with a sterile knife blade, outer tissues are removed from the samples and the inner tissues carefully excised and placed on water agar plates. After several days of incubation, hyphal tips of the fungi are removed and transferred to potato dextrose or other suitable agar. Bacterial forms also emerge from the plant tissues including, on rare occasions, certain *Streptomyces* spp. The endophytes are encouraged to sporulate on specific plant materials and are eventually identified via standard morphological and molecular biological techniques and methods. Eventually, when an endophyte is acquired in pure culture, it is tested for its ability to be grown in shake or still culture using various media and growth conditions.[31] It is also immediately placed in storage under various conditions including 15% glycerol at −70 °C. Ultimately, once appropriate growth conditions are found, the microbe is fermented and extracted and the bioactive compound(s) are isolated and characterized. Virtually all of the common and advanced procedures for product isolation and characterization are utilized in order to acquire the product(s) of interest. Central to the processes of isolation is the establishment of one or more bioassays that will guide the compound purification processes. *One cannot put too much emphasis on this point since the ultimate success of any natural product isolation activity is directly related to the development or selection of appropriate bioassay procedures*. These can involve target organisms, enzymes, tissues, or model chemical systems that relate to the purpose for which the new compound is needed.

Natural Products from Endophytic Microbes

The following section shows some examples of natural products obtained from endophytic microbes and their potential in the pharmaceutical and agrochemical arenas. Many of the examples are taken from our work, and thus, this review is by no means inclusive of all natural product work on endophytes.

Endophytic Fungal Products as Antibiotics. Fungi are the most commonly isolated endophytic microbes. They usually appear as fine filaments growing from the plant material on the agar surface. Generally, the most commonly isolated fungi are in the group *Fungi Imperfecti* or Deuteromycetes. Basically, they produce asexual spores in or on various fruiting structures. Also, it is quite common to isolate endophytes that are producing no fruiting structures whatsoever such as *Mycelia sterilia*. Quite commonly, endophytes do produce secondary metabolites when placed in culture. However, the temperature, the composition of the medium, and the degree of aeration will affect the amount and kinds of compounds that are produced by an endophytic fungus. Sometimes endophytic fungi produce antibiotics. Natural products from endophytic fungi have been observed to inhibit or kill a wide variety of harmful microorganisms including, but not limited to, phytopathogens, as well as bacteria, fungi, viruses, and protozoans that affect humans and animals. Described below are some examples of bioactive products from endophytic fungi.

Cryptosporiopsis cf. *quercina* is the imperfect stage of *Pezicula cinnamomea*, a fungus commonly associated with hardwood species in Europe. It was isolated as an endophyte from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia.[32] On Petri plates, *C. quercina* demonstrated excellent antifungal activity against some important human fungal pathogens including *Candida albicans* and *Trichophyton* spp. A unique peptide antimycotic, termed cryptocandin, was isolated and characterized from *C. quercina*.[32] This compound contains a number of peculiar hydroxylated amino acids and a novel amino acid, 3-hydroxy-4-hydroxymethylproline (Figure 2). The bioactive compound is related to the known antimycotics, the echinocandins and the pneumocandins.[33] As is generally true, not one but several bioactive and related compounds are produced by an endophytic microbe. Thus, other antifungal agents related to cryptocandin are also produced by *C.* cf. *quercina*. Cryptocandin is also active against a number of plant pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea*. Cryptocandin and its related compounds are currently being considered for use against a number of fungal-causing diseases of the skin and nails.

Cryptocin, a unique tetramic acid, is also produced by *C. quercina* (see above) (Figure 3).[34] This unusual compound possesses potent activity against *Pyricularia oryzae*, the causal organism of one of the worst plant diseases in the world, as well as a number of other plant pathogenic fungi.[34] The compound was generally ineffective against a general array of human pathogenic fungi. Nevertheless, with a minimum inhibitory concentration against *P. oryzae* of 0.39 μg/mL, this compound is being examined as a natural chemical control agent for rice blast and is being used as a model to synthesize other antifungal compounds.

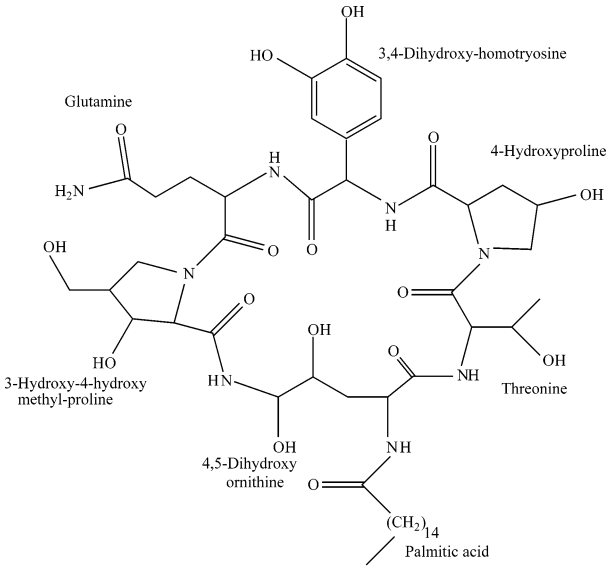

Figure 2. Cryptocandin A, an antifungal lipopeptide obtained from the endophytic fungus *Cryptosporiopsis* cf. *quercina*. (No stereochemistry is intended.)

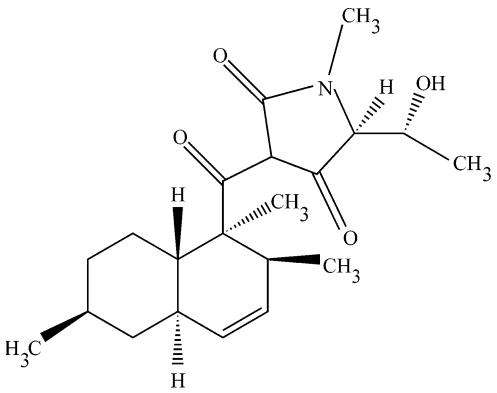

Figure 3. Cryptocin, a tetramic acid antifungal compound found in *Cryptosporiopsis* cf. *quercin*.

As mentioned earlier, *P. microspora* is a common rainforest endophyte.[27−30] It turns out that enormous biochemical diversity does exist in this endophytic fungus, and many secondary metabolites are produced by various strains of this widely dispersed organism. One such secondary metabolite is ambuic acid, an antifungal agent, which has been recently described from several isolates of *P. microspora* found as representative isolates in many of the world's rainforests (Figure 4).[35] This compound as well as another endophyte product, terrein, have been used as models to develop new solid-state NMR tensor methods to assist in the characterization of molecular stereochemistry of organic molecules. The rationale and methods used and developed are described below.

A strain of *P. microspora* was also isolated from the endangered tree *Torreya taxifolia* and produces several compounds having antifungal activity including pestaloside, an aromatic β-glucoside (Figure 5), and two pyrones, pestalopyrone and hydroxypestalopyrone.[36] These products also possess phytotoxic properties. Other newly isolated secondary products obtained from *P. microspora* (endophytic on *Taxus brevifolia*) include two new caryophyllene sesquiterpenes, pestalotiopsins A and B.[37] Additional new sesquiterpenes produced by this fungus are 2α-hydroxydimeninol and a highly functionalized humulane.[38,39] Variation in the amount and kinds of products found in this

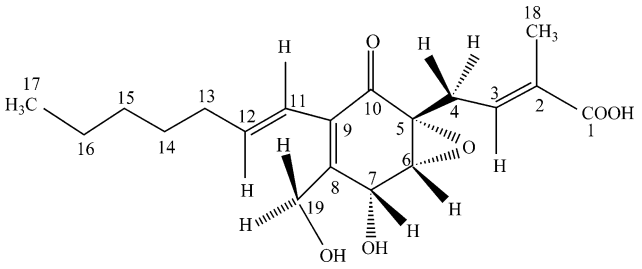

Figure 4. Ambuic acid, a highly functionalized cyclohexenone produced by a number of isolates of *Pestalotiopsis microspora* found in rainforests around the world. This compound possesses antifungal activity and has been used as a model compound for the development of solid-state NMR methods for the structural determination of natural products.

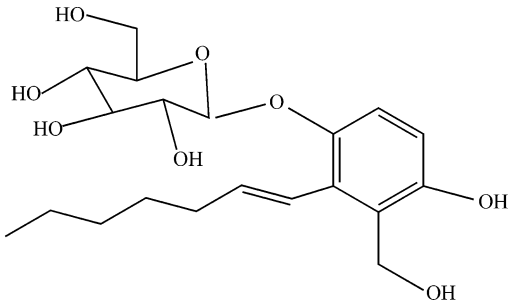

Figure 5. Pestaloside, a glucosylated aromatic compound with antifungal properties from *Pestalotiopsis microspora*.

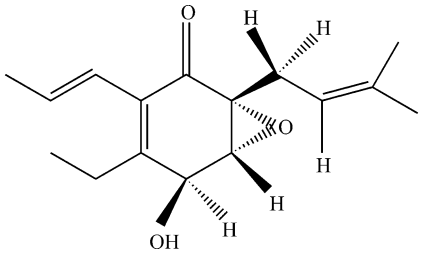

Figure 6. Jesterone, a cyclohexenone epoxide from *Pestaliotiopsis jesteri* with antioomycete activity.

fungus depends on both the cultural conditions and the original plant source from which it was isolated.

Pestalotiopsis jesteri is a newly described endophytic fungal species from the Sepik river area of Papua New Guinea, and it produces jesterone and hydroxyjesterone, which exhibit antifungal activity against a variety of plant pathogenic fungi.[40] These compounds are highly functionalized cyclohexenone epoxides. Jesterone, subsequently, has been prepared by organic synthesis with complete retention of biological activity (Figure 6).[41] Jesterone is one of only a few products from endophytic microbes in which total synthesis of a bioactive product has been successfully accomplished.

Phomopsichalasin, a metabolite from an endophytic *Phomopsis* sp., represents the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring. This metabolite exhibits antibacterial activity in disk diffusion assays (at a concentration of 4 μg/disk) against *Bacillus subtilis*, *Salmonella gallinarum*, and *Staphylococcus aureus*. It also displays a moderate activity against the yeast *Candida tropicalis*.[42]

An endophytic *Fusarium* sp. from the plant, *Selaginella pallescens*, collected in the Guanacaste Conservation Area of Costa Rica, was screened for antifungal activity. A new pentaketide antifungal agent, CR377, was isolated from the culture broth of the fungus and showed potent activity against *Candida albicans* in agar diffusion assays.[43]

Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus isolated from *Artemisia mongolica*, displays antibacterial activity against bacteria as well as against the fungus *Helminthsporium sativum*.[44] Another *Colletotrichum* sp., isolated from *Artemisia annua*, produces bioactive metabolites that showed antimicrobial activity as well. *A. annua* is a traditional Chinese herb that is well recognized for its synthesis of artemisinin (an antimalarial drug) and its ability to inhabit many geographically different areas. The *Colletotrichum* sp. found in *A. annua* produced not only metabolites with activity against human pathogenic fungi and bacteria but also metabolites that were fungistatic to plant pathogenic fungi.[45]

Endophytic Bacterial Products as Antibiotics. There are only a limited number of bacterial species known to be associated with plants, and one of the most common genera encountered is *Pseudomonas* spp. *Pseudomonas* spp. have representative biotypes and species that are epiphytic, endophytic, and pathogenic. They have been reported from every continent including the Antarctic. Some of these species produce phytotoxic compounds as well as antibiotics. The ecomycins are produced by *Pseudomonas viridiflava*.[46] This bacterium is generally associated with the leaves of many grass species and is located on and within the tissues.[46] The ecomycins represent a family of novel lipopeptides and have masses of 1153 and 1181 Da. Besides common amino acids such as alanine, serine, threonine, and glycine, some unusual amino acids are incorporated into the structure of the ecomycins, including homoserine and β-hydroxyaspartic acid.[46] The ecomycins are active against such human pathogenic fungi as *Cryptococcus neoformans* and *Candida albicans*.

The pseudomycins produced by a plant-associated pseudomonad are another group of antifungal peptides.[47,48] The pseudomycins are active against a variety of plant and human pathogenic fungi including *Candida albicans* and *Cryptococcus neoformans* and a variety of plant pathogenic fungi including *Ceratocystis ulmi* (the dutch elm disease pathogen) and *Mycosphaerella fijiensis* (causal agent of Black Sigatoka disease in bananas). The pseudomycins are cyclic depsipeptides formed by acylation of the OH group of the N-terminal serine with the terminal carboxyl group of L-chlorothreonine. Variety in this family of compounds is imparted via N-acetylation by one of a series of fatty acids including 3,4-dihydroxydecanoate, 3-hydroxytetradecanoate, and others.[48] The pseudomycins contain several nontraditional amino acids including L-chlorothreonine, L-hydroxyaspartic acid, and both D- and L-diaminobutryic acid. The molecules are candidates for use in human medicine especially after structural modification by chemical synthesis has successfully removed mammalian toxicity.[49] The pseudomycins are also effective against a number of ascomycetous fungi, and are also being considered for agricultural use for the control of the Black Sigatoka disease in bananas (Strobel, G. A., unpublished data).

Endophytic Streptomycetes as Antibiotic Producers. *Streptomyces* spp. are filamentous bacteria, belonging to the group Actinomycetales, that live in widely diverse ecological settings. Generally, this group is Gram positive, has a high G+C content, and does not have an organized nucleus. To date, actinomycetes have been the world's greatest source of natural antibiotics.[50] In fact, just one genus, *Streptomyces* spp., is the source of 80% of these compounds. The majority of the antibiotic producers are known from soil sources, and until recently it was not realized that these organisms can exist as endophytes. One of the first endophytic *Streptomyces* spp. isolated was that from *Lolium perenne*, a grass species.[51] This isolate produces a diketopiperazine that is only weakly antibiotic and has been designated methylalbonoursin.[51]

Using the ethnobotanical approach to plant selection, the snakevine plant, *Kennedia nigriscans*, was chosen as a possible source of endophytic microbes, because of its long held traditional use by Australian Aborigines to treat cuts and open wounds resulting in reduced infection and rapid healing. This plant, collected near the Aboriginal Community of Manyallaluk in Northern Territory, Australia, consistently produced an endophytic actinomycete designated *Streptomyces* NRRL 30562.[22] The organism was not found in several tree species supporting the vine, suggesting a host selective or specific association of the endophyte with a specific plant genus. This streptomycete produces a family of extremely potent peptide antibiotics, and it may be the case that these compounds not only protect the plant from fungal and bacterial infections but also have unknowingly served the Aborigines as a source of bush medicine.

The antibiotics produced by *Streptomyces* NRRL 30562, called munumbicins, possess widely differing biological activities, depending on the target organism. In general, the munumbicins demonstrate activity against Gram-positive bacteria such as *Bacillus anthracis* and multidrug-resistant *Mycobacterium tuberculosis* as well as a number of other drug-resistant bacteria. However, the most impressive biological activity of any of the munumbicins is that of munumbicin D against the malarial parasite *Plasmodium falciparum*, having an IC_{50} of 4.5 ± 0.07 ng/mL.[22] The munumbicins are highly functionalized peptides each containing threonine, aspartic acid (or asparagine), and glutamic acid (or glutamine). Since the peptides are colored yellowish orange, they also contain one or more chromophoric groups whose structures have not been determined. Their masses range from 1269 to 1326 Da. The isolation of this endophytic streptomycete represents an important finding in providing one of the first examples of plants serving as reservoirs of actinomycetes. More than 40 of these endophytic streptomycetes, now in hand in our laboratory, possess antibiotic activity (Castillo, U., Strobel, G. A., unpublished data). In fact, endophytic actinomycetes are now being tested and considered for use in controlling plant diseases.[52]

Another endophytic *Streptomyces* sp. (NRRL 30566), from a fern-leaved grevillea (*Grevillea pteridifolia*) tree growing in the Northern Territory of Australia, produces, in culture, novel antibiotics called kakadumycins that are related to the echinomycins.[53] Each of these antibiotics contains alanine, serine, and an unknown amino acid. Kakadumycin A has wide spectrum antibiotic activity similar to that of munumbicin D, especially against Gram-positive bacteria, and it generally displays better bioactivity than echinomycin. For instance, against *B. anthracis* strains, kakadumycin A has MICs of 0.2−0.3 μg/mL, in contrast to echinomycin at 1.0−1.2 μg/mL. Both echinomycin and kakadumycin A have impressive activity against *P. falciparum*, with LD_{50}'s in the range 7−10 ng/mL.[53] Kakadumycin A and echinomycin are related by virtue of their very similar structures (amino acid content and quinoxoline rings), but differ slightly with respect to their elemental compositions, aspects of their spectral parameters, chromatographic retention times, and biological activities.[53]

Echinomycin and kakadymycin A were studied as macromolecular synthesis inhibitors with control substances such as ciprofloxacin, rifampin, chloramphenicol, and van-

comycin used as standards, with well-established modes of action. Tests were done for DNA, RNA, protein, and cell wall synthesis inhibition activities, respectively. Kakadumycin A significantly inhibited the RNA synthesis rate in *B. subtilis*.[53] Kakadumycin A also inhibited protein synthesis and cell wall synthesis substantially, but the effect was lower on DNA synthesis. Kakadumycin A shares a very similar inhibitory profile with echinomycin in four macromolecular synthesis assays. Kakadumycin A preferentially inhibits RNA synthesis and may have the same mode of action as echinomycin, which inhibits RNA synthesis by binding to a DNA template.[53] This is yet another example of an endophytic actinomycete having promising antibiotic properties.

More recently, endophytic streptomycetes have been discovered in an area of the world claimed to be one of the most biologically diverse—the upper Amazon of Peru. The inner tissues of the Follow Me Vine, *Monstera* sp., commonly yielded a verticillated streptomycete with outstanding inhibitory activities against pythiaceous fungi as well as the malarial parasite *Plasmodium falciparum*. The bioactive component is a mixture of lipopeptides and named coronamycin.[54]

Antiviral Compounds. Another fascinating use of products from endophytic fungi is the inhibition of viruses. Two novel human cytomegalovirus (hCMV) protease inhibitors, cytonic acids A and B, have been isolated from solid-state fermentation of the endophytic fungus *Cytonaema* sp. Their structures were elucidated as *p*-tridepsides isomers by MS and NMR methods.[55] It is apparent that the potential for the discovery of compounds having antiviral activity from endophytes is in its infancy. The fact, however, that some compounds have been found already is promising. The main limitation to compound discovery to date is probably related to the absence of common antiviral screening systems in most compound discovery programs.

Volatile Antibiotics from Endophytes. *Muscodor albus* is a newly described endophytic fungus obtained from small limbs of *Cinnamomum zeylanicum* (cinnamon tree).[56] This xylariaceaous (non-spore producing) fungus effectively inhibits and kills certain other fungi and bacteria by producing a mixture of volatile compounds.[57] The majority of these compounds have been identified by GC/MS, synthesized or acquired, and then ultimately formulated into an artificial mixture. This mixture not only mimicked the antibiotic effects of the volatile compounds produced by the fungus but also was used to confirm the identity of the majority of the volatiles emitted by this organism.[57] Each of the five classes of volatile compounds produced by the fungus had some microbial effects against the test fungi and bacteria, but none was lethal. However, collectively they acted synergistically to cause death in a broad range of plant and human pathogenic fungi and bacteria. The most effective class of inhibitory compounds was the esters, of which isoamyl acetate was the most biologically active. The composition of the medium on which *M. albus* grows dramatically influences the kind of volatile compounds that are produced.[58] The ecological implications and potential practical benefits of the "mycofumigation" effects of *M. albus* are very promising given the fact that soil fumigation utilizing methyl bromide will soon be illegal in the United States. The potential use of mycofumigation to treat soil, seeds, and plants may soon be a reality. The artificial mixture of volatile compounds may also have usefulness in treating seeds, fruits, and other plant parts in storage and while being transported.

Using *M. albus* as a screening tool, it has now been possible to isolate other endophytic fungi producing volatile antibiotics. The newly described *M. roseus* was twice obtained from tree species growing in the Northern Territory of Australia. This fungus is just as effective in causing inhibition and death of test microbes in the laboratory as *M. albus*.[59] In addition, for the first time, a non-muscodor species (*Gliocladium* sp.) was discovered as a volatile antibiotic producer. The volatile components of this organism are totally different than those of either *M. albus* or *M. roseus*. In fact, the most abundant volatile inhibitor is [8]-annulene, formerly used as a rocket fuel and discovered for the first time as a natural product.[31] However, the bioactivity of the volatiles of this *Gliocladium* sp. is not as good or comprehensive as that of the *Muscodor* spp.[31]

Endophytic Fungal Products as Anticancer Agents. Taxol and some of its derivatives represent the first major group of anticancer agents that are produced by endophytes (Figure 6). Taxol, a highly functionalized diterpenoid, is found in each of the world's yew (*Taxus*) species, but was originally isolated from *Taxus brevifolia*.[1,2] The original target diseases for this compound were ovarian and breast cancers, but now it is used to treat a number of other human tissue-proliferating diseases as well. The presence of taxol in yew species prompted the study of their endophytes. By the early 1990s, however, no endophytic fungi had been isolated from any of the world's representative yew species. After several years of effort, a novel taxol-producing endophytic fungus, *Taxomyces andreanae*, was discovered in *Taxus brevifolia*.[23] The most critical line of evidence for the presence of taxol in the culture fluids of this fungus was the electrospray mass spectrum of the putative taxol isolated from *T. andreanae*. In electrospray mass spectroscopy, taxol usually gives two peaks, one at *m/z* 854 which is $M + H^+$ and the other at *m/z* 876 which is $M + Na^+$. Fungal taxol had a mass spectrum identical to that of authentic taxol.[26] Then, ^{14}C labeling studies showed the presence of fungal-derived taxol in the culture medium.[26] This early work set the stage for a more comprehensive examination of the ability of other *Taxus* species and many other plants to yield endophytes producing taxol.

Some of the most commonly found endophytes of the world's yews and many other plants are *Pestalotiopsis* spp.[27−30] One of the most frequently isolated endophytic species is *Pestalotiopsis microspora*.[27] An examination of the endophytes of *Taxus wallichiana* yielded *P. microspora*, and a preliminary monoclonal antibody test indicated that it might produce taxol.[30] After preparative TLC, a compound was isolated and shown by spectroscopic techniques to be taxol. Labeled (^{14}C) taxol was produced by this organism from several ^{14}C precursors that had been administered to it.[30] Furthermore, other *P. microspora* isolates were obtained from a bald cypress tree in South Carolina and also were shown to produce taxol.[29] This was the first indication that endophytes residing in plants other than *Taxus* spp. were producing taxol. Therefore, a specific search was conducted for taxol-producing endophytes on continents not being known for any indigenous *Taxus* spp. This included investigating the prospects that taxol-producing endophytes exist in South America and Australia. From the extremely rare and previously thought to be extinct Wollemi Pine (*Wollemia nobilis*), *Pestalotiopsis guepini* was isolated, which was shown to produce taxol.[60] Also, quite surprisingly, a rubiaceous plant, *Maguireothamnus speciosus*, yielded a novel fungus, *Seimatoantlerium*

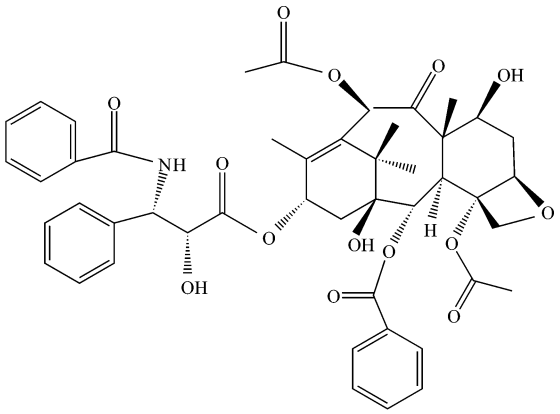

Figure 7. Taxol, the world's first billion-dollar anticancer drug produced by many endophytic fungi. It, too, possesses outstanding antioomycete activity.

tepuiense, that produces taxol. This endemic plant grows on the top of the tepuis in the Venzuelan−Guyana border in southwest Venezuela.[61] Furthermore, fungal taxol production has also been noted in *Periconia* sp.[62] and *Seimatoantlerium nepalense*, another novel endophytic fungal species.[63] Simply, it appears that the distribution of those fungi making taxol is worldwide and is not confined to endophytes of yews. The ecological and physiological explanation for the wide distribution of fungi making taxol seems to be related to the fact that taxol is a fungicide, and the most sensitive organisms to it are plant pathogens such as *Pythium* spp. and *Phytophthora* spp.[64] These pythiaceous organisms are some of the world's most important plant pathogens and are strong competitors with endophytic fungi for niches within plants. In fact, their sensitivity to taxol is based on their interaction with tubulin in a manner identical to that in rapidly dividing human cancer cells.[3,64] Thus, bona fide endophytes may be producing taxol and related taxanes to protect their respective host plant from degradation and disease caused by these pathogens.

Other investigators have also made observations on taxol production by endophytes, including the discovery of taxol production by *Tubercularia* sp. isolated from the Chinese yew (*Taxus mairei*) in the Fujian province of southeastern mainland China.[65] At least three endophytes of *Taxus wallichiana* produce taxol including *Sporormia minima* and *Trichothecium* sp.[66] Using HPLC and ESIMS, taxol has been discovered in *Corylus avellana* cv. Gasaway.[67] Several fungal endophytes of this plant (filbert) produce taxol in culture.[67] It is important to note, however, that taxol production by all endophytes in culture is in the range of sub-micrograms to micrograms per liter. Also, commonly, the fungi will attenuate taxol production in culture, with some possibility for recovery, if certain activator compounds are added to the medium.[62] Efforts are being made to determine the feasibility of making microbial taxol a commercial possibility. The greatest prospect of making microbial taxol a commercial reality may be the discovery of endophytes that make large quantities of one or more taxanes that could then be used as platforms for the organic synthesis of taxol or one of its anticancer relatives.

Torreyanic acid, a selectively cytotoxic quinone dimer and potential anticancer agent, was isolated from a *P. microspora* strain (Figure 8). This strain was originally obtained as an endophyte associated with the endangered tree *Torreya taxifolia* (Florida torreya) as mentioned above.[68] Torreyanic acid was tested in several cancer cell lines, and it demonstrated 5−10 times more potent cytotoxicity in those lines that are sensitive to protein kinase C agonists and causes cell death by apoptosis. Recently, torreyanic acid has been successfully synthesized by application of a biomimetic oxidation/dimerization cascade.[69]

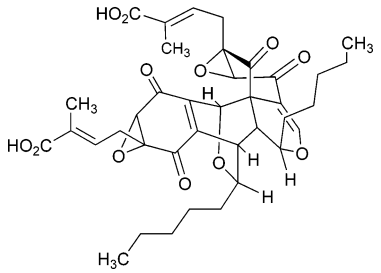

Figure 8. Torreyanic acid, an anticancer compound, from *Pestalotiopsis microspora*.

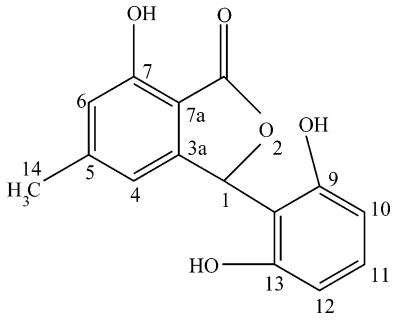

Figure 9. Isopestacin, an antioxidant produced by an endophytic *Pestalotiopsis microspora* strain, isolated from *Terminalia morobensis* growing on the north coast of Papua New Guinea.

Alkaloids are also commonly found in endophytic fungi. Such fungal genera as xylaria, phoma, hypoxylon, and chalara are representative producers of a relatively large group of substances known as the cytochalasins, of which over 20 are now known.[70] Many of these compounds possess antitumor and antibiotic activities, but because of their cellular toxicity, they have not been developed into pharmaceuticals. Three novel cytochalasins have recently been reported from *Rhinocladiella* sp. as an endophyte on *Tripterygium wilfordii*. These compounds have antitumor activity and have been identified as 22-oxa-[12]-cytochalasins.[70] Thus, it is not uncommon to find one or more cytochalasins in endophytic fungi, and this provides an example of the fact that redundancy in discovery does occur, making dereplication an issue even for these underinvestigated sources.

Products from Endophytes as Antioxidants. Two compounds, pestacin and isopestacin, have been obtained from culture fluids of *Pestalotiopsis microspora*, an endophyte isolated from a combretaceaous plant, *Terminalia morobensis*, growing in the Sepik River drainage system of Papua New Guinea.[71,72] Both pestacin and isopestacin display antimicrobial as well as antioxidant activity. Isopestacin was attributed with antioxidant activity based on its structural similarity to the flavonoids (Figure 9). Electron spin resonance spectroscopy measurements confirmed this antioxidant activity; the compound is able to scavenge superoxide and hydroxyl free radicals in solution.[71] Pestacin was later described from the same culture fluid, occurring naturally as a racemic mixture and also possessing potent antioxidant activity (Figure 10).[72] The proposed antioxidant activity of pestacin arises primarily via cleavage of an unusually reactive C−H bond and, to a lesser extent, through O−H abstraction.[72] The antioxidant activity of pestacin is at least 1 order of magnitude more potent than that of trolox, a vitamin E derivative.[72]

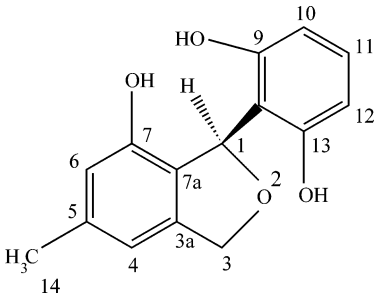

Figure 10. Pestacin, produced by *Pestalotiopsis microspora*. It, too, is an antioxidant.

Products of Endophytes with Insecticidal Activities. Bioinsecticides are only a small part of the insecticide field, but their market is increasing.[5] Several endophytes are known to have anti-insect properties. Nodulisporic acids, novel indole diterpenes that exhibit potent insecticidal properties against the larvae of the blowfly, work by activating insect glutamate-gated chloride channels. The first nodulisporic acids were isolated from an endophyte, a *Nodulisporium* sp., from the plant *Bontia daphnoides*. This discovery has since resulted in an intensive search for additional *Nodulisporium* spp. or other producers of more potent nodulisporic acid analogues.[13] Insect toxins have also been isolated from an unidentified endophytic fungus from wintergreen (*Gaultheria procumbens*). The two new compounds, 5-hydroxy-2-(1′-hydroxy-5′-methyl-4′-hexenyl)benzofuran and 5-hydroxy-2-(1′-oxo-5′-methyl-4′-hexenyl)benzofuran, both show toxicity to spruce budworm, and the latter is also toxic to the larvae of spruce budworm.[73] Another endophytic fungus, *Muscodor vitigenus*, isolated from a liana (*Paullina paullinioides*) yields naphthalene as its major product. Naphthalene, the active ingredient in common mothballs, is a widely exploited insect repellant. *M. vitigenus* shows promising preliminary results as an insect deterrent and has exhibited potent insect repellency against the wheat stem sawfly (*Cephus cinctus*).[74,75] As the world becomes wary of ecological damage done by synthetic insecticides, endophytic research continues for the discovery of powerful, selective, and safe alternatives.

Antidiabetic Agents from Rainforest Fungi. A nonpeptidal fungal metabolite (L-783,281) was isolated from an endophytic fungus (*Pseudomassaria* sp.) collected from an African rainforest near Kinshasa in the Democratic Republic of the Congo.[76] This compound acts as an insulin mimetic but, unlike insulin, is not destroyed in the digestive tract and may be given orally. Oral administration of L-783,281 in two mouse models of diabetes resulted in significant lowering in blood glucose levels. These results may lead to new therapies for diabetes.[7]

Immunosuppressive Compounds from Endophytes. Immunosuppressive drugs are used today to prevent allograft rejection in transplant patients, and in the future they could be used to treat autoimmune diseases such as rheumatoid arthritis and insulin-dependent diabetes. The endophytic fungus *Fusarium subglutinans*, isolated from *T. wilfordii*, produces the immunosuppressive but noncytotoxic diterpene pyrones subglutinols A and B (Figure 11).[77] Subglutinols A and B are equipotent in the mixed lymphocyte reaction (MLR) and thymocyte proliferation (TP) assays with an IC_{50} of 0.1 μM. In the same assay systems, the famed immunosuppressant drug cyclosporin A, also a fungal metabolite, was roughly as potent in the MLR assay and 10^4 more potent in the TP assay. Still, the lack of toxicity associated with subglutinols A and B suggests that they should be explored in greater detail as potential immunosuppressants.[77]

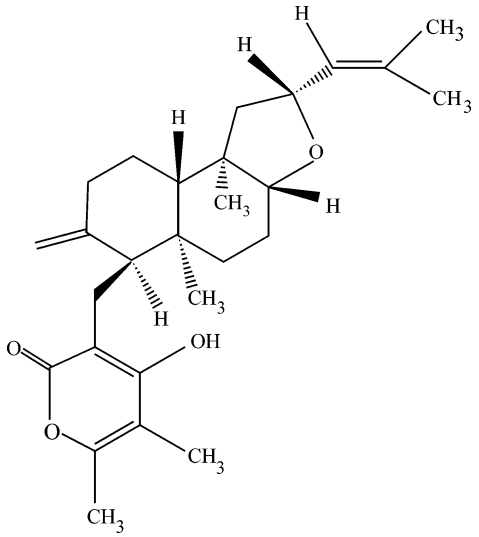

Figure 11. Subglutinol A, an immunosuppressant produced by an endophytic *Fusarium subglutinans* strain.

Natural Products Motivating Analytical Methodology Development. The remarkable structural diversity found in natural products has frequently stimulated the development of new approaches to structural elucidation. This is especially true in NMR, where natural products were among the first examples of successful application of multidimensional methods. In our laboratories, uncertainty in the stereochemistry of certain natural products has motivated the development of new solid-state NMR methods. These techniques were initially intended to provide unambiguous assignments for relative stereochemistry, but the approach has also proven able to establish solid-state molecular conformation and hydrogen-bonding modes.

Characterization of stereochemistry from solid-state NMR data involves a statistical comparison of the three experimental principal shift values per nucleus with corresponding computed values for a variety of model structures.[78,79] In addition, a solid sample of 80−100 mg is required. The natural product terrein provided an initial test compound to which these methods were applied. Terrein is a relatively small molecule with only two possible diastereomers and a fairly rigid skeleton, eliminating the need to consider a large number of conformers. The relative stereochemistry is also known, allowing independent corroboration of NMR predictions.[80] In terrein, the solid-state NMR analysis correctly predicted the relative stereochemistry with greater than 99.5% probability, establishing the feasibility of performing stereochemical analysis in this manner with other natural products.[78]

An extension of this technique was then made to a molecule with unknown stereochemistry. Ambuic acid had been isolated and characterized in our labs, but stereochemical characterization had proven difficult (Figure 4).[35] NOESY NMR interactions were unsuited to differentiating the two diastereomers, as the distance between relevant hydrogens in both structures differed by only 0.06 Å in energy-minimized model structures (positions 5, 6, and 7 in Figure 4). Proton−proton scalar couplings ($^3J_{HH}$) were likewise of little value, as the relevant dihedral angles of the two possible structures differed by only 7°. Repeated attempts to grow crystals also proved futile. However, the solid-state NMR approach selected a best relative stereochemistry at approximately the 82% probability level.[81] The predicted stereochemistry has recently been shown to be

correct by an independent synthesis of both diastereomers of ambuic acid (Porco, J., Li, C., unpublished data).

Surprising Results from Molecular Biological Studies on *Pestalotiopsis microspora*. Of some compelling interest is an explanation as to how the genes for taxol production may have been acquired by *P. microspora*.[82] Although the complete answer to this question is not at hand, some other relevant genetic studies have been performed on this organism. *P. microspora* Ne 32 is one of the most easily genetically transformable fungi that has been studied to date. In vivo addition of telomeric repeats to foreign DNA generates extrachromosomal DNAs in this fungus.[82] Repeats of the telomeric sequence 5′-TTAGGG-3′ were appended to nontelomeric transforming DNA termini. The new DNAs, carrying foreign genes and the telomeric repeats, replicated independently of the chromosome and expressed the information carried by the foreign genes. The addition of telomeric repeats to foreign DNA is unusual among fungi. This finding may have important implications in the biology of *P. microspora* Ne 32 since it explains at least one mechanism as to how new DNA can be captured by this organism and eventually expressed and replicated. Such a mechanism may begin to explain how the enormous biochemical variation may have arisen in this fungus.[29] Also, this initial work represents a framework to aid in the understanding of how this fungus may adapt itself to the environment of its plant hosts and suggests that the uptake of plant DNA into its own genome may occur. In addition, the telomeric repeats have the same sequence as human telomeres, and this points to the possibility that *P. microspora* may serve as a means to make artificial human chromosomes, a totally unexpected result.

Concluding Statements

Endophytes are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial arenas. The mechanisms through which endophytes exist and respond to their surroundings must be better understood in order to be more predictive about which higher plants to seek, study, and employ in isolating microfloral components. This may facilitate the natural product discovery process.

Although work on the utilization of this vast resource of poorly understood microorganisms has just begun, it has already become obvious that an enormous potential for organism, product, and utilitarian discovery in this field holds exciting promise. This is evidenced by the discovery of a wide range of products and microorganisms that present potential as mentioned in this report. *It is important for all involved in this work to realize the importance of acquiring the necessary permits from governmental, local, and other sources to pick and transport plant materials (especially from abroad) from which endophytes are to be eventually isolated. In addition to this aspect of the work is the added activity of producing the necessary agreements and financial sharing arrangements with indigenous peoples or governments in case a product does develop an income stream.*

Certainly, one of the major problems facing the future of endophyte biology and natural product discovery is the rapidly diminishing rainforests, which hold the greatest possible resource for acquiring novel microorganisms and their products. The total land mass of the world that currently supports rainforests is about equal to the area of the United States.[20] Each year, an area the size of Vermont or greater is lost to clearing, harvesting, fire, agricultural development, mining, or other human-oriented activities. Presently, it is estimated that only a small fraction (10−20%) of what were the original rainforests existing 1000−2000 years ago are currently present on Earth.[20] The advent of major negative pressures on them from these human-related activities appears to be eliminating entire mega-life forms at an alarming rate. Few have ever expressed information or opinions about what is happening to the potential loss of microbial diversity as entire plant species disappear. It can only be guessed that this loss is also happening, perhaps with the same frequency as the loss of mega-life forms, especially since certain microorganisms may have developed unique specific symbiotic relationships with their plant hosts. *Thus, when a plant species disappears, so too does its entire suite of associated endophytes and consequently all of the capabilities that they might possess to make important natural products*. Multistep processes are needed now to secure information and life forms before they continue to be lost. Areas of the planet that represent unique places housing biodiversity need immediate preservation. Countries need to establish information bases of their biodiversity and at the same time begin to make national collections of microorganisms that live in these areas. Endophytes are only one example of a life form source that holds enormous promise to impact many aspects of human existence. The problem of the loss of biodiversity should be one of concern to the entire world.

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