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Novel Natural Products From Rainforest Endophytes

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Summary

Endophytic microorganisms are found in virtually every higher 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 survival value to the plant. Ultimately, these compounds, once isolated and characterized, may also have potential for use in modern medicine. 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 potential of finding new drugs that may be effective candidates for treating newly developing diseases in humans is great.

Key Words: Munumbicins; kakadumycin; taxol; volatile antibiotics; streptomycetes; anticancer agents; immunosuppressants.

1. 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 new 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. Environmental degradation, loss of biodiversity, and spoilage of land and water also add to problems facing humanity, and each of these in turn can have health-related consequences.

Endophytes, microorganisms that reside in the tissues of living plants, are relatively unstudied as potential sources of novel natural products for exploitation in medicine. However, some of the most extensive and comprehensive work on natural products produced by endophytes has been done on the *Neotyphodium* sp. found on grasses *(1)*. Alkaloids synthesized by this fungus in its grass hosts have been implicated in fescue toxicosis in rangeland animals *(1)*. The chemistry and biology of this and other grass endophytes are reviewed elsewhere *(2)*. Unfortunately, because this work is so comprehensive, one may be led to the conclusion that endophytes produce toxic compounds only in their respective hosts and hold no promise for any medicinal applications whatsoever *(2)*. It turns out that this is simply not the case. As endophytes are examined

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from a plethora of sources, an overwhelming number have been found to produce natural products with promising potential for medicinal applications.

Of the approx 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. Only a handful of these plants (grass species) have ever been completely studied relative to their endophytic biology *(2)*. Consequently, the opportunity to find new and interesting endophytic microorganisms among myriads of plants in different settings and ecosystems is very great. The intent of this review is to provide insights into their occurrence in nature, the products that they make, and indicate how some of these organisms are beginning to show some potential for human use. The majority of the report discusses rationale for study, methods used, and examples of a number of endophytes isolated and studied in the authors' laboratories 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.

2. Needs for New Natural Products

There is a general call for new antibiotics, and for chemotherapeutic agents that are highly effective and possess low toxicity. 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 acquired immunodeficiency syndrome (AIDS), Ebola, and severe acute respiratory syndrome (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*, which 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 exception of AIDS and tuberculosis (TB) *(3)*. However, the enteric diseases claim the most lives each year of any disease complex, and unfortunately, the victims are mostly children *(3)*.

Novel natural products and the organisms that make them offer opportunities for innovation in drug discovery. Exciting possibilities exist for those who are willing to venture into the wild and unexplored territories of the world to experience the thrill of engaging in the discovery of endophytes, their biology, and potential usefulness.

3. 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 *(4)*. Shutz notes that certain microbial metabolites seem to be characteristic of certain biotopes, both on an environmental as well as organismal level *(5)*. 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 *(5)*. 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 exploitation of their medical benefits.

In addition, it also is extremely helpful for the investigator interested in exploiting endophytes to have access to, or have some expertise in, microbial taxonomy, and this includes modern molecular techniques involving sequence analyses of 16S and 18S 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 usually depended on the perspective from which the endophytes were being isolated and subsequently examined *(6)*. 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" *(2)*. 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 *(7)*. It turns out that the vast majority of plants have not been studied for their endophytes. Thus, enormous opportunities exist for the recovery of novel fungal forms, including genera, biotypes, as well as 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 approx 100,000 have been described *(8)*. 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 *(9)*. 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 novel natural products associated with them. This fact alone helps eliminate the problems of dereplication in compound discovery.

4. Rationale for Plant Selection

It is important to understand the methods and rationale used seem 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 *(10)*.

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 are 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 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 *(11)*. These environmental insults created many portals through which common phytopathogenic oomycetes could enter the plant. Still, the plant population appeared to be healthy, possibly owing to protection by 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 both as 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 (**Fig. 1**) *(11)*. 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

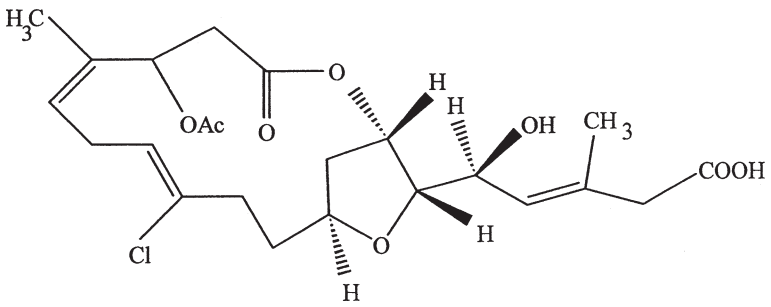

Fig. 1. Oocydin A, a chlorinated macrocyclic lactone isolated and characterized from a strain of *Serratia marcescens*, obtained from *Rhyncholacis penicillata* (stereochemistry is not known).

fungi such as *Pythium* spp. and *Phytophthora* spp. Oocydin A also has activity against a number of rapidly dividing cancer cell lines *(11)*.

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 because 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 *(12)*. 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 to 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.) *(11,12)*. In 1993, a novel taxol-producing fungus, *Taxomyces andreanae*, from the yew *Taxus brevifolia*, was isolated and characterized *(13)*.

5. Endophytes and Biodiversity

Of the myriad of ecosystems on earth, those having the greatest general biodiversity 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 *(10)*. In addition, each of the 20–25 areas identified as supporting the world's greatest biodiversity support unusually high levels of plant endemism *(10)*. 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 *(14)*.

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 *(15)*. 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. This observation suggests the importance of the host plant as well as the ecosystem in influencing the general metabolism of endophytic microbes.

6. Endophytes and Phytochemistry

Tan and Zou believe the reason why 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 *(6)*. This is a concept that was originally proposed as a mechanism to explain why *T. andreanae* may be producing taxol *(16)*. 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 is a vastly under-investigated and exciting field *(17,18)*. 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 their relationship to various host plant species. Although 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 one can 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 *(19)*. 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, related to the season, 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.

7. Collection, Isolation, and Preservation 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 *(20,21)*.

In the laboratory, the surfaces of plant materials are thoroughly 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 *(20)*. 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 *(21)*. 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 subjected to fermentation, extraction, and the bioactive compounds 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.

8. 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 in endophytes.

8.1. 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*. Basi-

cally, 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 kind of compounds that are produced. 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 *(22)*. 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 *(22)*. This compound contains a number of peculiar hydoxylated amino acids and a novel amino acid, 3-hydroxy-4-hydroxy methyl proline (**Fig. 2**). The bioactive compound is related to known antimycotics—the echinocandins and the pneumocandins *(23)*. 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. 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 fungi causing diseases of the skin and nails.

Cryptocin, a unique tetramic acid, is also produced by *C. quercina* (discussed previously) (**Fig. 3**)*(24)*. 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 *(24)*. The compound was generally ineffective against a general array of human pathogenic fungi. Nevertheless, with minimum inhibitory concentrations against *P. oryzae* at 0.39 µg/mL, this compound is being examined as a natural chemical control agent for rice blast and is being used as a platform for the synthesis of other antifungal compounds.

As mentioned earlier, *P. microspora* is a common rainforest endophyte *(17–20)*. 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 (**Fig. 4**) *(25)*. This compound as well as another endophyte product, terrein, have been used as models to develop new solid-state nuclear magnetic resonance (NMR) tensor methods to assist in the characterization of the molecular stereochemistry of organic molecules.

A strain of *P. microspora* was also isolated from the endangered tree *Torreya taxifolia* and produced several compounds having antifungal activity, including pestaloside, an aromatic β-glucoside (**Fig. 5**), and two pyrones—pestalopyrone and hydroxypestalopyrone *(26)*. These products also possess phytotoxic properties. Other

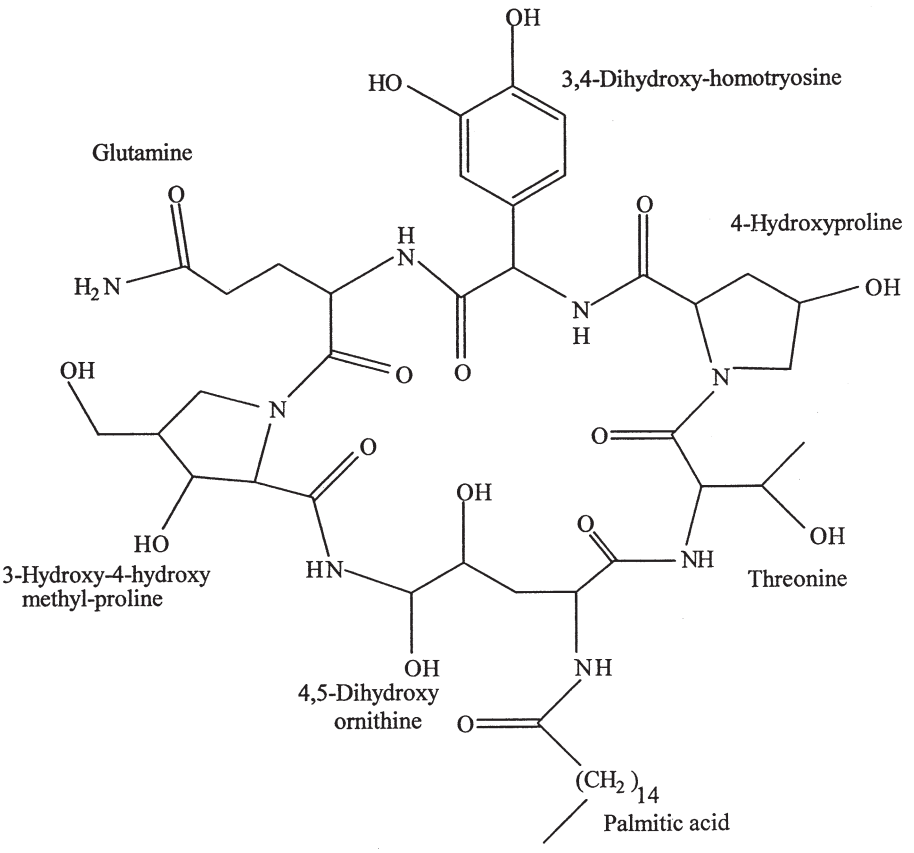

Fig. 2. Cryptocandin A, an antifungal lipopeptide obtained from the endophytic fungus *Cryptosporiopsis* cf. *quercina* (no stereochemistry is intended).

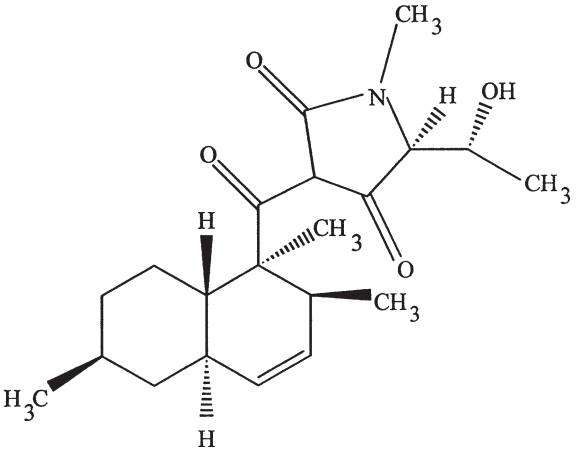

Fig. 3. Cryptocin, a tetramic acid antifungal compound found in *Cryptosporiopsis* cf. *quercina*.

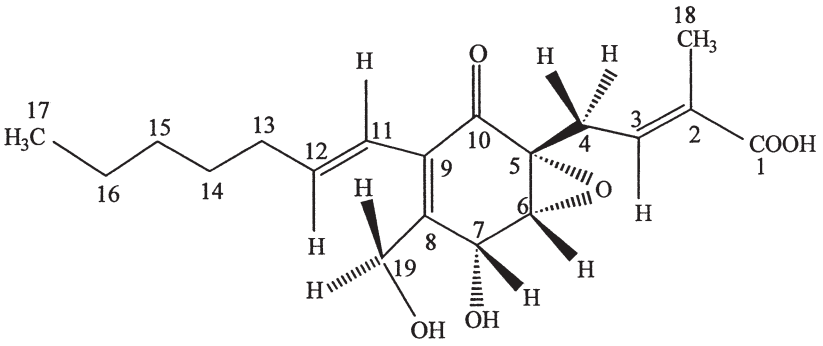

Fig. 4. Ambuic acid, a highly functionalized cyclohexenone produced by a number of isolates of *Pestalotiopsis microspora* found in rainforests around the world. This compound possesses antifungal activity and has been used as a model compound for the development of solid-state nuclear magnetic resonance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

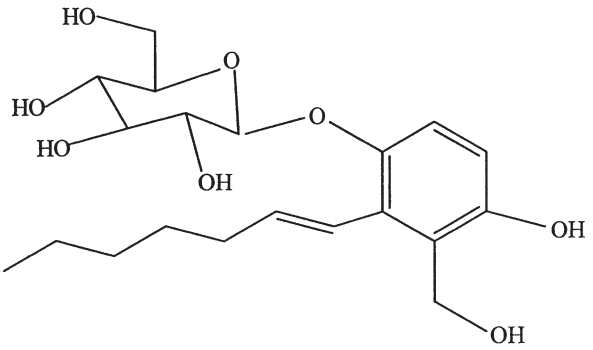

Fig. 5. Pestaloside, a glucosylated aromatic compound with antifungal properties from *Pestalotiopsis microspora*.

newly isolated secondary products obtained from *P. microspora* (endophytic on *Taxus brevifolia*) include two new caryophyllene sesquiterpenes—pestalotiopsins A and B *(27)*. Additional new sesquiterpenes produced by this fungus are 2α-hydroxydimeninol and a highly functionalized humulane *(28,29)*. Variation in the amount and kinds of products found with this fungus depends on both the cultural conditions as well as 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 *(30)*. These compounds are highly functionalized cyclohexenone epoxides. Jesterone, subsequently, has been prepared by organic synthesis with complete retention of biological activity (**Fig. 6**) *(31)*. Jesterone is one of only a few products from endophytic microbes in which total synthesis of a bioactive product has been successfully accomplished.

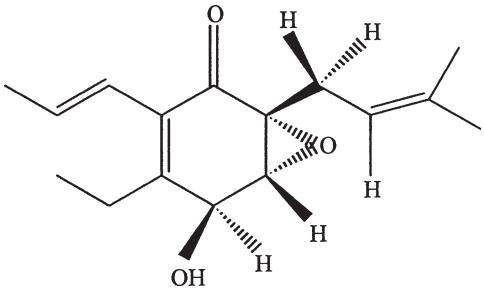

Fig. 6. Jesterone, a cyclohexenone epoxide from *Pestaliotiopsis jesteri*, has antioomycete activity.

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 moderate activity against the yeast *Candida tropicalis (32)*.

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 *C. albicans* in agar diffusion assays *(33)*.

Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus isolated from *Artemisia mongolica*, displays activity against bacteria as well as against the fungus *Helminthsporium sativum (34)*. Another *Colletotrichum* sp., isolated from *Artemisia annua*, produces antimicrobial metabolites 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. Not only did the *Colletotrichum* sp. found in *A. annua* produce metabolites with activity against human pathogenic fungi and bacteria, but also metabolites that were fungistatic to plant pathogenic fungi *(35)*.

8.2. 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 is *Pseudomonas* spp. *Pseudomonas* has 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 (36)*. This bacterium is generally associated with the leaves of many grass species and is located on and within the tissues *(36)*. The ecomycins represent a family of novel lipopeptides and have masses of 1153 and 1181. Besides common amino acids such as alanine, serine, threonine, and glycine, some nonprotein amino acids are incorporated into the structure of the ecomycins, including homoserine and β-hydroxyaspartic acid *(36)*. The ecomycins are active against such human pathogenic fungi as *Cryptococcus neoformans* and *C. albicans*.

The pseudomycins produced by a plant-associated pseudomonad are another group of antifungal peptides *(37,38)*. They are active against a variety of plant and human pathogenic fungi, including *Candida albicans*, *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-hydroxy-tetradecanoate *(38)*. 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 eliminated mammalian toxicity *(39)* The pseudomycins are also effective against a number of ascomycetous fungi, and are being considered for agricultural use for the control of the Black Sigatoka disease in bananas (Strobel, unpublished).

8.3. Endophytic Streptomycetes As Antibiotic Producers

Streptomyces spp. are filamentous bacteria, belonging to the order 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 *(40)*. In fact, just one genus, *Streptomyces*, is the source of 80% of these compounds. The majority of the antibiotic producers are 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 *(41)*. This isolate produces a diketopiperazine that is a weak antibiotic and has been designated "methylalbonoursin" *(41)*.

Using the ethnobotanical approach to plant selection, the snakevine plant, *K. 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 was collected near the Aboriginal Community of Manyallaluk in Northern Territory, Australia, and consistently yielded an endophytic actinomycete designated *Streptomyces* NRRL 30562 *(12)*. 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 these compounds may 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 *(12)*. The munumbicins

are highly functionalized peptides, each containing threonine, aspartic acid (or asparagine), and glutamic acid (or glutamine). Since the peptides are 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, 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). Endophytic actinomycetes are now being tested and considered for use in controlling plant diseases *(42)*.

Another endophytic *Streptomyces* sp. (NRRL 30566), from a fern-leaved grevillea (*Grevillea pteridifolia*) tree growing in the Northern Territory of Australia, produces novel antibiotics called "kakadumycins," which are related to the echinomycins *(43)*. 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 of 7–10 ng/mL *(43)*. 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 qualities, chromatographic retention times, and biological activities *(53)*.

Echinomycin and kakadymycin A were studied as inhibitors of macromolecular synthesis, with control substances such as ciprofloxacin, rifampin, chloramphenicol, and vancomycin 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 RNA synthesis in *B. subtilis (43)*. Kakadumycin A also inhibited protein synthesis and cell-wall synthesis substantially, but had a lower effect 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)*.

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 named "coronamycins" *(44)*.

8.4. 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*-tridepside isomers by MS and NMR methods *(45)*. It is apparent that the potential for the discovery of compounds having antiviral activity from endophytes is in its infancy. The main limitation to com-

pound discovery to date is probably related to the absence of common antiviral screening systems in most compound-discovery programs.

8.5. Volatile Antibiotics From Endophytes

Muscodor albus is a newly described endophytic fungus obtained from small limbs of *Cinnamomum zeylanicum* (cinnamon tree) *(46)*. This xylariaceaous (non-spore producing) fungus effectively inhibits and kills certain fungi and bacteria by producing a mixture of volatile compounds *(47)*. The majority of these compounds have been identified by gas chromatography (GC)/MS, synthesized or acquired, and then 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 *(47)*. 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, 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 *(48)*. 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. Methyl bromide is not only a hazard to human health, but it has been implicated in causing destruction of the ozone layer. 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 plant parts in storage and while being transported. *Muscodor albus* already has a limited market for the treatment of human wastes. Its gases have both inhibitory and lethal effects on such fecal-inhabiting organisms as *Escherichia coli* and *Vibrio cholera*.

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 obtained twice 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 (49)*. In addition, for the first time, a nonmuscodor species (*Gliocladium* sp.) was discovered as a producer of volatile antibiotics. The volatile components of this organism are totally different from 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. However, the bioactivity of the volatiles of this *Gliocladium* sp. is not as good or comprehensive as those from *Muscodor* spp. *(21,47)*.

8.6. 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 (**Fig. 6**). Taxol (**Fig. 7**), a highly functionalized diterpenoid, is found in each of the world's yew (*Taxus*) species, but was originally isolated from *Taxus brevifolia (50)*. The original targets for this compound were ovarian and breast cancers, but now it is used to treat a number of other human tissue

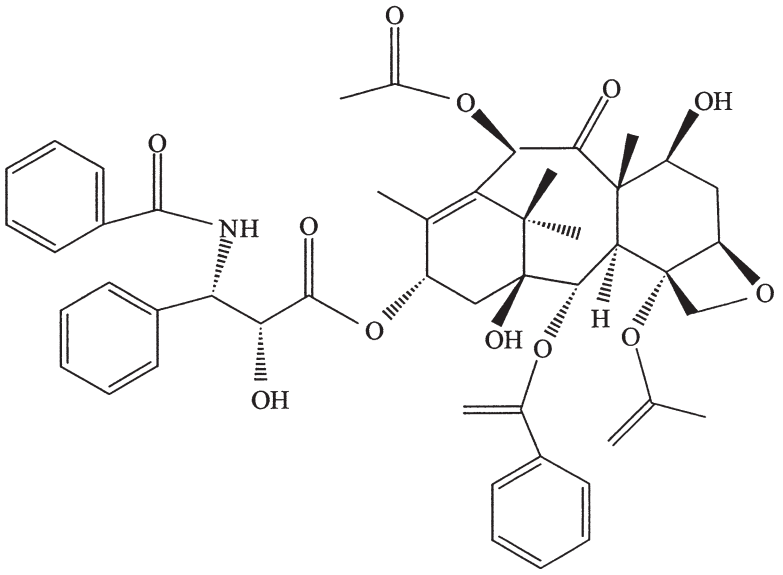

Fig. 7. Taxol, the world's first billion-dollar anticancer drug, is produced by many endophytic fungi. It too, possesses outstanding anti-oomycete activity.

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 (13)*. 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 mass 854, which is $M+H^+$, and the other at 876, which is $M+Na^+$. Fungal taxol had a mass spectrum identical to authentic taxol *(16)*. 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. *(17–20)*. One of the most frequently isolated endophytic species is *Pestalotiopsis microspora (17)*. An examination of the endophytes of *Taxus wallichiana* yielded *P. microspora*, and a preliminary monoclonal antibody test indicated that it might produce taxol *(20)*. 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 *(20)*. Furthermore, several other *P. microspora* isolates that produce taxol were obtained from a bald cypress tree in South Carolina *(19)*. This was the first indication that endophytes, residing in plants other than *Taxus* spp., produce taxol. Therefore, a specific search was conducted for taxol-producing endophytes on continents not known for any indigenous *Taxus* spp., e.g., 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 *(51)*. Also, quite surprisingly, a rubiaceous plant, *Maguireothamnus speciosus*, yielded a novel fungus, *Seimatoantlerium tepuiense*, that produces taxol. This endemic plant grows on the top of the tepuis in the Venzuelan-Guyana border in southwest Venezuela *(52)*. Furthermore, fungal taxol production has also been noted in *Periconia* sp. *(53)* and *Seimatoantlerium nepalense*, another novel endophytic fungal species *(54)*. Simply, it appears that the distribution of taxol-making fungi is worldwide and is not confined to endophytes of yews. The ecological and physiological explanation for fungi making taxol seems to be related to the fact that taxol is a fungicide, and the organisms most sensitive to it are plant pathogens such as *Pythium* spp. and *Phytophthora* spp. *(55)*. 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 an identical manner as in rapidly dividing human cancer cells *(55)*. 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 *(56)*. At least three endophytes of *Taxus wallichiana* produce taxol, including *Spordormia minima* and *Trichothecium* sp. *(57)*. Using HPLC and ESIMS, taxol has been discovered in *Corylus avellana* cv. Gasaway *(58)*. Several fungal endophytes of this plant (filbert) produce taxol in culture *(58)*. 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 *(53)*. Efforts are being made to determine the feasibility of making microbial taxol a commercial possibility, e.g., the discovery of endophytes that make large quantities of one or more taxanes that could then be used as intermediates 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 (**Fig. 8**). This strain was originally obtained as an endophyte associated with the endangered tree *Torreya taxifolia* (Florida torreya) *(59)*. Torreyanic acid was tested in several cancer cell lines, and it demonstrated 5 to 10 times more potent cytotoxicity in lines that are sensitive to protein kinase C agonists; it causes cell death by apoptosis. Recently, torreyanic acid has been successfully synthesized by a biomimetic oxidation/dimerization cascade *(60)*.

Alkaloids are also commonly found in endophytic fungi. Fungal genera such as *Xylaria*, *Phoma*, *Hypoxylon*, and *Chalara* are representative producers of a relatively large group of substances known as the cytochalasins, of which more than 20 are now known. 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 *(61)*. Thus, it is not uncommon to find one or more cytochalasins in endophytic fungi, and this provides an example of

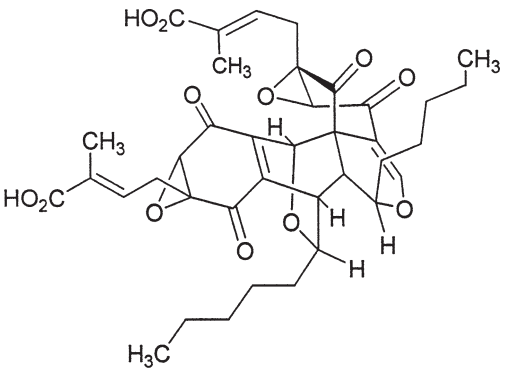

Fig. 8. Torreyanic acid, an anticancer compound, from *Pestalotiopsis microspora*.

the fact that redundancy in discovery does occur, making dereplication an issue even for these under-investigated sources.

8.7. 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 *(62,63)*. 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 (**Fig. 9**). Electron spin resonance spectroscopy confirmed this antioxidant activity; the compound is able to scavenge superoxide and hydroxyl free radicals in solution *(62)*. Pestacin was later described from the same culture fluid, occurring naturally as a racemic mixture and also possessing potent antioxidant activity (**Fig. 10**) *(63)*. 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 *(63)*. The antioxidant activity of pestacin is at least one order of magnitude more potent than that of trolox, a vitamin E derivative *(63)*.

8.8. 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 *(64)*. 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 of blood glucose levels. These results may lead to new therapies for diabetes.

8.9. 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 (**Fig. 11**) *(65)*. Subglutinol A and

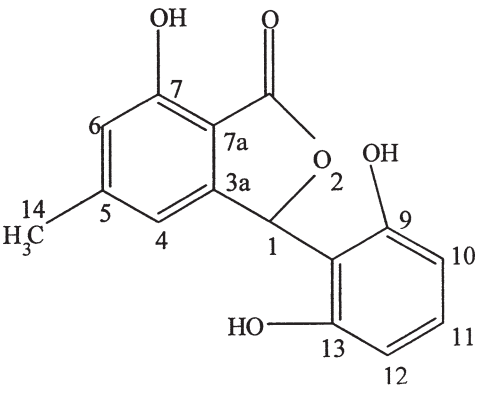

Fig. 9. Isopestacin, an antioxidant produced by an endophytic *Pestalotiopsis microspora* strain, isolated from *Terminalia morobensis* growing on the north coast of Papua New Guinea.

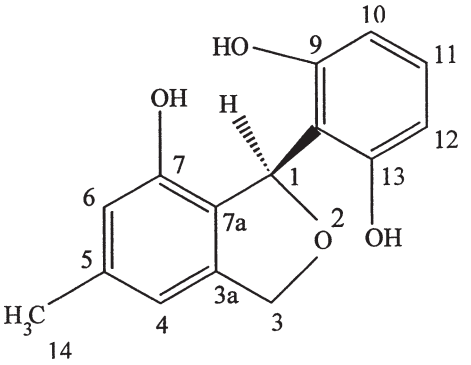

Fig. 10. Pestacin is also produced by *Pestalotiopsis microspora*, and it too is an antioxidant.

B are equipotent in the mixed lymphocyte reaction (MLR) assay and thymocyte proliferation (TP) assay, with an IC_{50} of 0.1 μ*M*. 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 *(65)*.

9. 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 (66)*. Although the complete answer to this question is not at hand, relevant genetic studies have been performed on this organism. *P. microspora* Ne 32 is one of the most easily genetically transformable fungi that have been studied to date. In vivo addition of telomeric repeats to foreign DNA generates extrachromosomal DNAs in this fungus *(66)*. Repeats of the telomeric sequence 5'-TTAGGG-3' were appended to nontelomeric transforming DNA termini. The new

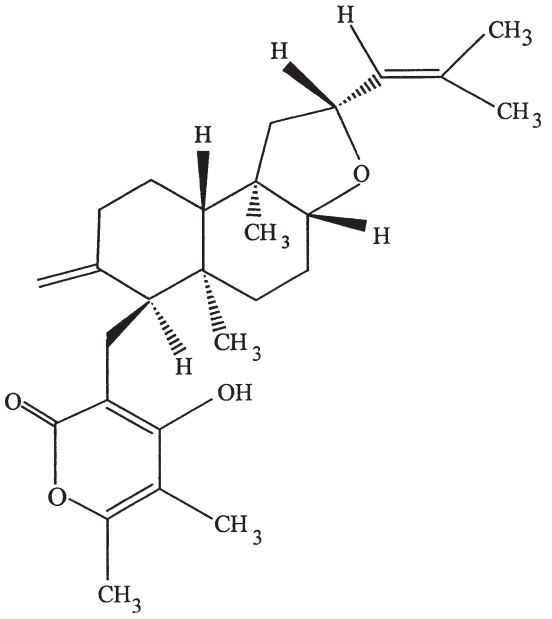

Fig. 11. Subglutinol A, an immunosuppressant, is produced by an endophytic *Fusarium subglutinans* strain.

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, because 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 *(19)*. 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.

10. Conclusion

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 applications. 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. 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 *(10)*. Each year, an area the size of Vermont or greater is lost to clearing, harvesting, fire, agricultural development, mining, or other human-oriented activities *(10)*. Presently, it is estimated that only a small fraction (10–20%) of what were the original rainforests existing 1000–2000 yr ago, are currently present on the earth *(10)*. 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 at the same frequency as the loss of mega-life forms, especially because 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 natural products with medicinal potential. Multistep processes are needed now to secure information and life forms before they are 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.

Acknowledgments

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