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The Biological Promise of Microbial Endophytes and Their Natural Products

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Abstract: 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 slightly pathogenic. Because of what appears to be their contribution to the host plant, the endophytes may produce a plethora of substances that may have potential use to modern medicine, agriculture and industry. Novel antibiotics, antimycotics, immunosuppressants, anticancer compounds are only a few examples of what has been found after the isolation, culture and purification and characterization of some choice endophytes in the recent past. The potential prospects of finding new drugs that may be effective candidates for treating newly developing diseases in humans, plants and animals is great. The valleys and mountain sides of Pakistan that harbor and support native populations of plants offer a treasure trove of biologically interesting endophytic microorganisms and they need to be isolated and studied. In many parts of the world, those areas that are relatively untouched such as national biological reserves, parks, or wildlands are likely to be the best sources of promising endophytes.

Key words: Fungi, microbes, bacteria, pathogens, antibiotics

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 threading viruses, the recurring problems with disease in persons with organ transplants and the tremendous increase in the incidence of fungal infections in the world's population each only underscores our inadequacy to cope with these medical problems. Added to this are enormous difficulties in raising enough food on certain areas 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 and potential sources of novel natural products for exploitation in medicine, agriculture and industry. It is worthy to note, that of the nearly 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes. Only a handful of these plants have ever been completely studied relative to their endophytic biology. Consequently, the opportunity to find new and interesting endophytic microorganisms among myriads of plants in different settings and ecosystems is great. The intent of this review is to provide insights into the presence of endophytes 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, methods and examples of a plethora of endophytes have been *bioprospected* over the course of many years with some emphasis on specific examples of discoveries made in the authors' laboratory. A brief discussion is also presented indicating the possibilities for finding endophytes within the borders of Pakistan.

Needs for new medicines and agrochemical agents: 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. This search is driven by the development of resistance in infectious microorganisms (e.g. *Staphylococcus*, *Mycobacterium*, *Streptococcus*) to existing compounds and by the menacing presence of naturally resistant organisms. The ingress to the human population of new diseases such as AIDS 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 so do new therapies 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 by opportunistic pathogens, such as *Aspergillus* spp., *Cryptococcus* spp.

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and *Candida* spp. that normally are not major problems in the human population. In addition, more drugs are needed to efficiently treat parasitic protozoan and nematode infections such as malaria, leshmaniasis, trypanomiasis and filariasis. Malaria alone claims more lives each year than AIDS and TB[1] 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[2]. 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[3]. These products have been exploited for human use for thousands of years and plants have been the chief source of compounds used for medicine. Even today the largest users of traditional medicines are the Chinese with over 5000 plants and plant products in their pharmacopoeia[4]. In fact, the world's best known and most universally used medicinal is aspirin (salicylic acid) which has it natural origins from the glycoside salicin which is found in many species of the plant genera *Salix* and *Populus*. 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 has identified certain plants to provide relief of symptoms varying from head colds to massive wounds and intestinal ailments[5]. 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[6]. More recently, the Benedictine monks (800 AD) began to apply *Papever somniferum* as an anesthetic and pain reliever as the Greeks had done before them[7]. 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 to usher in the antibiotic era via the discovery of penicillin from the fungus-*Penicillium notatum*. Since then, people 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 practical because of the development of novel and sophisticated screening processes in both medicine and agriculture. These processes use individual organisms, cells, enzymes and site directed techniques, many times in 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 the case that natural product based compounds have had an immense impact on modern medicine since about 40% of prescription drugs are based on them. Furthermore, 49% of the new chemical products registered by the FDA are natural products or derivatives thereof[8]. Excluding biologics, between 1989 and 1995, 60% of approved drugs and pre-new drug application candidates were of natural origin[7]. From 1983-1994, over 60% of all approved and pre-NDA stage cancer drugs were of natural origin as were 78% of all newly approved antibacterial agents[9]. In fact, the world's first billion dollar anticancer drug taxol is a natural product derived from the yew tree[10]. Many other examples abound that illustrate the value and importance of natural products in modern civilizations.

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 include compounds that provide social benefits, antihistamines, or ones that can sooth the stomach. It appears that this loss of interest can be attributed to the enormous effort and expense that is required to pick and chose a biological source, then to isolate active natural products, decipher their structures and begin the long road to product development[7]. It is also apparent that combinatorial chemistry and other synthetic chemistries revolving around certain basic chemical structures is now serving as a never ending source of products to feed the screening robots of the drug industry. Professional progress of individuals within

large pharmaceutical companies is primarily based upon numbers of compounds that can be produced and sent to the screening machines. It seems important to realize 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[7]. 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 databases.

Endophytes: 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 indifferent parts of the world by armies of collectors. In other words, why do something different (work with endophytes) 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[12]. Shutz[13] notes that certain microbial metabolites seem to be characteristic of certain biotopes, both on an environmental as well as organismal level. 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 have a totally random approach in the biological 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[13]. 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. 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 these biotypical factors can be important in plant selection since they may govern the novelty and biological activity of the products associated with endophytic microbes.

Since the discovery of endophytes in Darnel in 1904[14], various investigators have defined endophytes in different ways which is usually dependant on the perspective from which the endophytes were being isolated and subsequently examined. Stone *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[15]. 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, but no evidence for them has yet been presented e.g. mycoplasmas, rickettsia and archebacteria. The most frequently isolated endophytes are the fungi. 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, taxa and biotypes. Hawksworth and Rossman estimated there may be as many as 1 million different fungal species, yet only about 100,000 have been described[16]. As more evidence accumulates, estimates keep rising as to the actual number of fungal species. For instance, Dreyfuss and Chappela estimate there may be at least 1 million species of endophytic fungi alone[17]. It seems obvious that endophytes are a rich and reliable source of genetic diversity and novel, undescribed species. Finally, in our experience, novel microbes usually 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 as well as ones making novel bioactive products. 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[18].

A specific rationale for the collection of each plant for endophyte isolation and natural product discovery is

used. Several reasonable hypotheses govern this plant selection strategy and these are as follows:

- Plants from unique environmental settings, especially those with an unusual biology and possessing novel strategies for survival are seriously considered for study.
- 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)
- Plants that are endemic, having an unusual longevity, or that have occupied a certain ancient land mass, such as Gonwanaland, are also more likely to lodge endophytes with active natural products than other plants.
- Plants growing in areas of great biodiversity 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[19]. This 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, a potent antifungal strain of *Serratia marcescens*, was recovered from *R. penicillata* and was shown to produce oocydin A, a novel antioomycetous compound having the properties of a chlorinated macrocyclic lactone (Fig 1.). 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 agriculture use to control the ever threatening presence of oomyceteous fungi such as pythium and phytophthora.

Plants with ethnobotanical history, as mentioned above, also are likely candidates for study since the

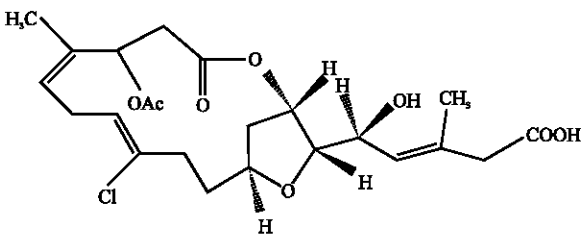

Fig. 1: Oocydin A, a chlorinated macrocyclic lactone from a strain of *Serratia marcescens* isolated from *Rhyncholacis pedicillata*

medical uses to which the plant may have been selected relates 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 long 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 novel endophyte, *Streptomyces* NRRL 30562, that produces wide spectrum novel peptide antibiotics called-munumbicins(discussed below)[20]. It is reasonable to assume that the healing processes, 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.)[21]. In 1993, a novel taxol producing fungus, *Taxomyces andreanae*, from the yew, *Taxus brevifolia* was isolated and characterized[22].

Endophytes and biodiversity: Of the myriad of ecosystems on earth, those having the greatest biodiveristiy seem to be the ones also having endophytes with the greatest number and most biodiversity. 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[18]. In addition, each of the 20-25 areas identified as supporting the world's greatest biodiversity also support unusually high levels of plant endemism[18]. As such, one would expect with high plant endemism there also exists 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 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[23]. Bills *et al.*[11] describe a metabolic distinction between tropical and temperate endophytes through statistical data which compares the number of bioactive natural products isolated from endophytes of tropical regions to the number of those isolated from endophytes of temperate origin. 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 tropical substrata[11]. This observation suggests the importance of the host plant in influencing the general metabolism of endophytic microbes.

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 occurs in evolutionary time[14]. This is a concept that was originally proposed as a mechanism to explain why *T. andreanae* may be producing taxol[24]. 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 preserve the world's ever diminishing biodiversity. Furthermore, it is recognized that a microbial source of a valued product may be easier and more economical to produce effectively 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. Thus, more background information on a given plant species and its micro organismal 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, Pestalotiopsis, Xylaria*), 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 of the endophytes will produce a highly biologically active compound in culture[25]. A great deal of uncertainty also exists between what an endophyte produces in culture and what it may produce in nature. It does seem apparent 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 form 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. These interactions are probably all 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 and isolation 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[19,22,25-36].

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. 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 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. 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, 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.

Endophytic microbial products as antibiotics: Antibiotics are defined as low molecular weight organic natural products made by microorganisms that are active at low concentration against other microorganisms[2]. Often, endophytes are a source of these antibiotics. Natural products from endophytic microbes 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.

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[37]. On Petri plates, *C. quercina* demonstrated excellent antifungal activity against some important human fungal pathogens-*Candida albicans* and *Trichophyton* spp. A unique peptide antimycotic, termed cryptocandin, was isolated and characterized from *C. quercina*[37]. 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 the known antimycotics, the echinocandins and the pneumocandins[38]. As is generally true not one but several bioactive and related compounds are produced by a 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 fungi causing diseases of skin and nails.

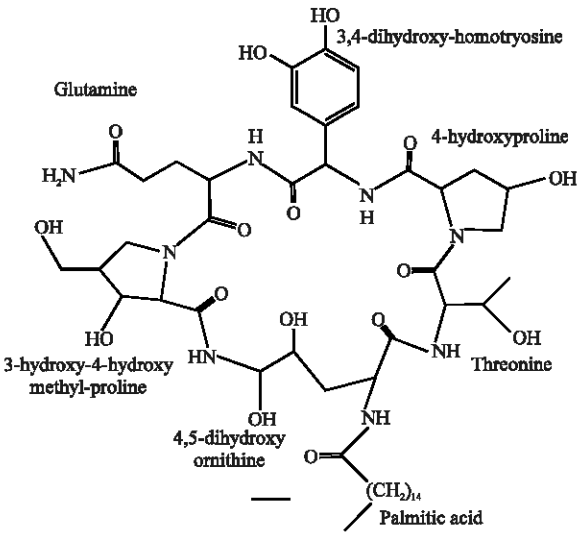

Fig. 2: Cryptocandin A, a peptide antifungal compound obtained from the endophytic fungus *Cryptosporiopsis quercina*

Cryptocin, a unique tetramic acid, is also produced by *C. quercina*[39] (Fig. 3). This unusual compound possesses potent activity against *Pyricularia oryzae* as well as a number of other plant pathogenic fungi[39]. 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^{-1}, this compound is being examined as a natural chemical control agent for rice blast and is being used as a base model to synthesize other antifungal compounds.

The ecomycins are produced by *Pseudomonas viridiflava*[40]. *P. viridiflava* is a member of a group of plant associated fluorescent bacteria. It is generally associated with the leaves of many grass species and is located on and within the tissues[40]. 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 unusual amino acids are also involved in the structure of the ecomycins including homoserine and ß-hydroxyaspartic acid. The ecomycins are active against such human pathogenic fungi as *Cryptococcus neoformans* and *Candida albicans.*

Another group of antifungal compounds is the pseudomycins produced by a plant associated pseudomonad[41,42]. The pseudomycins, represent a family of lipopeptides that are active against a variety of plant and human pathogenic fungi. Some of the notable target organisms include *Candida albicans*, *Cryptococcus neoformans* and a variety of plant pathogenic fungi

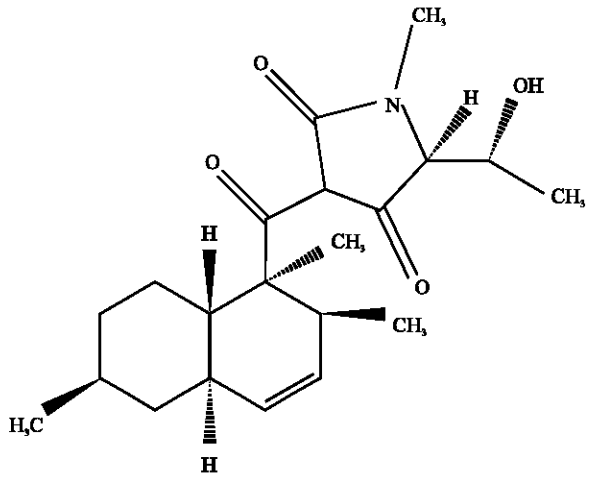

Fig. 3: Cryptocin, a tetramic acid antifungal compound also found in *C. quercina*

including *Ceratocystis ulmi* (the dutch elm disease pathogen) and *Mycosphaerella fijiensis* (the causal agent of Black Sigatoka disease of banana)[41] (Strobel, unpublished). The key conserved part of the pseudomycins is a cyclic nona-peptide. The terminal carboxyl group of L-chlorothreonine closes the macrocyclic ring on the OH group of the N-terminal serine. Variety to this family of compounds is added by virtue of N-acetylation by one of a series of fatty acids including 3, 4-dihydroxydecanoate, or 3-hydroxytetradecanoate and others[42]. The pseudomycins contain several non-traditional amino acids including L-chlorothreonine, L-hydroxy aspartic acid and both D and L-diaminobutryic acid. The molecules are candidates for use in human medicine especially after structural modification has successfully removed mammalian toxicity[43]. Although, the pseudomycins are also effective against a number of ascomycetous fungi, they are also being considered for agricultural use.

As mentioned earlier, *P. microspora*, is a common rainforest endophyte[25,27,36,44,45]. It turns out that enormous biochemical diversity does exist in this endophytic fungus and as such there seems to be many secondary metabolites produced by a myriad of strains of this widely dispersed fungus. 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[25](Fig. 4). In fact, 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[50,51].

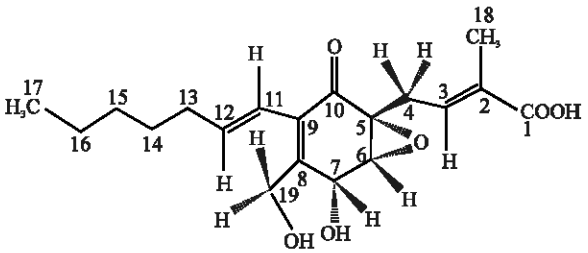

Fig. 4: Ambuic acid, a highly functionalized cyclohexenone produced by a number of isolates of *Pestalotiopsis microspora* found in rainforests around the world. This compound possesses antifungal activity

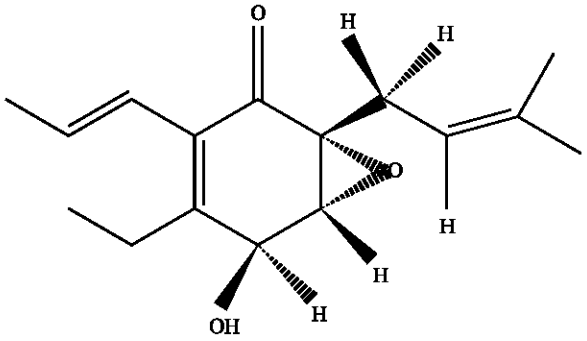

Fig. 5: Jesterone a cyclohexenone epoxide from *P. jesteri* has antioomycete activity

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 and two pyrones-pestalopyrone and hydroxypestalopyrone[48]. 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[49]. Other novel sesquiterpenes produced by this fungus are 2-α-hydroxydimeninol and a highly functionalized humulane[50,51]. Variation in the amount and kinds of products found in this fungus depends on both the cultural conditions of the organism as well as the original plant source from which it was isolated.

A newly described species of pestalotiopsis, namely *Pestalotiopsis jesteri*, from the Sepik river area of Papua New Guinea produces jesterone and hydroxy-jesterone, which exhibit antifungal activity against a variety of plant pathogenic fungi[52]. Jesterone, subsequently, has been prepared by organic synthesis with complete retention of biological activity[53] (Fig. 5).

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 mainly exhibits antibacterial activity in disk diffusion assays (at a concentration of 4 μg/disk) against *Bacillus subtilis* (12 mm zone of inhibition), *Salmonella gallinarum* (11 mm zone of inhibition) and *Staphylococcus aureus* (8mm zone of inhibition). It also displays a moderate activity against the yeast *Candida tropicalis* (8 mm zone of inhibition)[54].

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 performed on fungal lawns[55].

Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus in *Artemisia mongolica*, displays antimicrobial activity against bacteria as well as against the fungus *Helminthsporium sativum*[56]. Another *Colletotrichum* sp., isolated from *Artemisia annua*, produces bioactive metabolites that showed varied 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. 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[57].

In addition to plants such as *A. annua* producing antimalarial compounds, some endophytes have shown powerful activity against protozoal diseases as well. Wide-spectrum antibiotics are produced by *Streptomyces* NRRL 30562, an endophyte in *Kennedia nigriscans*[20]. These antibiotics, 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 the IC_{50} of 4.5±0.07 ng mL^{-1}[20]. 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. Their masses are ranging from 1269 to 1326 daltons. The isolation of an endophytic streptomycete-Streptomyces NRRL 30562, represents an important clue in providing one of the first examples of plants serving as reservoirs of actinomycetes which are the world's primary source of antibiotics. However, virtually all of them, in the past, used for modern antibiotic production had been isolated from soils. Now, of the more than twenty of these are on hand as endophytes, many possess antibiotic activity (Strobel, unpublished). In fact, endophytic actinomycetes are now being tested and seriously considered for use in controlling plant diseases[58].

Another endophytic Streptomycete (NRRL 30566), from a fern-leaved grevillea (*Grevillea pteridifolia*) tree growing in the Northern Territory of Australia, produce, in culture, novel antibiotics called kakadumycins[59]. Each of these antibiotics contains, by virtue of their amino acid compositions; alanine, serine and an unknown amino acid. Kakadumycin A has wide spectrum antibiotic activity similar to munumbicin D, especially against Gram-positive bacteria and it generally displays better bioactivity than echinomycin. For instance, against *Bacillus anthracis* strains, kakadumycin A has MIC's of 0.2-0.3 μg mL^{-1} in contrast to echinomycin at 1.0-1.2 μg mL^{-1}. Both echinomycin and kakadumycin A have impressive activity against *Plasmodium falciparum* with $LD_{50's}$ in the range of 7-10 ng mL^{-1}[59]. Kakadumycin A and echinomycin are related by virtue of their very similar chemistries (amino acid content and quinoaxoline rings), but differ slightly with respect to their elemental compositions, aspects of their spectral qualities and biological activities[59]. This is yet another example of an endophytic actinomycete having promising antibiotic properties.

Antiviral compounds: Another fascinating use of antibiotic 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 the solid-state fermentation of the endophytic fungus *Cytonaema* sp., Their structures as *p*-tridepside isomers were elucidated by MS and NMR methods[60]. It is apparent that the potential for the discovery of compounds, from endophytes, having antiviral activity is in its infancy. The fact, however, that some compounds have been found is promising. The main limitation is compound discovery is probably related to absence of appropriate 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)[61]. This xylariaceaous (non-spore producing) fungus

effectively inhibits and kills certain other fungi and bacteria by producing a mixture of volatile compounds[62]. The majority of these compounds have been identified by gas chromatography/mass spectrometry, synthesized or acquired and then ultimately made into an artificial mixture. This mixture mimicked the antibiotic effects of the volatile compounds produced by the fungus[62]. Each of the five classes of volatile compounds produced by the fungus had some inhibitory effect 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 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.

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*[63]. In addition, for the first time, a non-muscodor species was discovered as a volatile antibiotic producer *Gliocladium* sp. The volatile components of this organism are totally different that either *M. albus* or *M. roseus*. In fact, the most abundant volatile inhibitor is annulene, formerly used as a rocket fuel and discovered for the first time as a natural product in an endophytic fungus[64]. The bioactivity of the volatiles, however of *Gliocladium* sp is not as good or comprehensive as the *Muscodor* spp.[64].

Endophytes making anticancer agents: Taxol and some of its derivatives represent the first major group of anticancer agents that are produced by endophytes (Fig 6.). Taxol, a highly functionalized diterpenoid, is found in each of the world's yew (*Taxus*) species[65]. The mode of action of taxol is to preclude tubulin molecules from depolymerizing during the processes of cell division[66]. This compound is the world's first billion dollar anticancer drug. It is used to treat a number of other human tissue proliferating diseases as well. The presence of taxol in yew species prompted study of their endophytes. By the early 1990's, however, no endophytic fungi had been isolated from any of the world's representative yew species. After several years of effort,

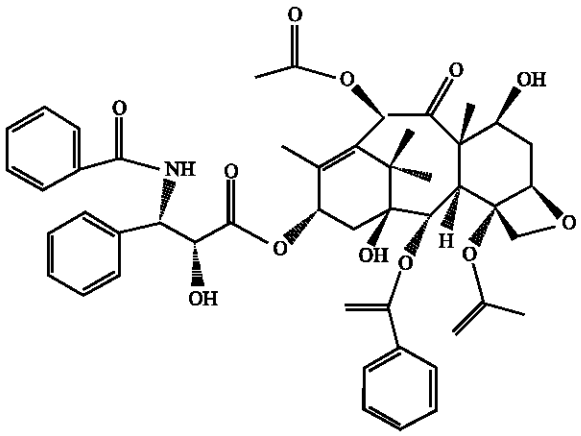

Fig. 6: Taxol, the world's first billion dollar anticancer drug is produced by many endophytic fungi. It too, possesses outstanding antioomycete activity

a novel taxol producing endophytic fungus, *Taxomyces andreanae,* was discovered in *Taxus brevifolia*[22]. 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 $(M+Na^+)$ and fungal taxol had an identical mass spectrum to authentic taxol[24]. Then, ^{14}C labelling studies irrefutably showed the presence of fungal derived taxol in the culture medium[24]. This early work set the stage for a more comprehensive examination of the ability of other *Taxus* species and other plants to yield endophytes producing taxol.

Some of the most commonly found endophytes of the world's yews are *Pestalotiopsis* sp.[26]. One of the most commonly isolated endophytic species is *Pestalotiopsis microspora*[25-28]. An examination of the endophytes of *Taxus wallichiana* yielded *P. microspora* and a preliminary monoclonal antibody test indicated that it might produce taxol[25]. 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[25]. Furthermore, several other *P. microspora* isolates were obtained from bald cypress in South Carolina and also shown to produce taxol[26]. 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 an examination of 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[29]. Also, quite surprisingly, a rubiaceous plant-*Maguireothamnus speciosus*, yielded a novel fungus *Seimatoantlerium tepuiense* that produces taxol. This endemic plant grows on the tops of the tepuis in the Venzuelan-Guyana in S.W. Venezuela[19]. Furthermore, fungal taxol production has also been noted in *Periconia* sp.[30] and *Seimatoantlerium nepalense*, another novel endophytic fungal species[31]. Simply, it appears that the distribution of those fungi making taxol is worldwide and 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.[67]. 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[66]. Thus, bona fide endophytes may be producing taxol to protect their respective host plant from degradation and disease caused by these pathogens.

As time has passed, many investigators in different parts of the world have made observations on taxol production by endophytes, including the discovery of taxol production by *Tubercularia* sp. isolated from Southern Chinese yew (*Taxus mairei*) in the Fujian province of Southeastern China[32]. At least three endophytes of *Taxus wallichiana* produce taxol including *Sporormia minima* and *Trichothecium* sp. were found in Nepal[33]. Using high performance liquid chromatography and electrospray mass spectroscopy, taxol has been discovered in *Corylus avellana* cv. Gasaway[34]. Several fungal endophytes of this plant (filbert) produce taxol in culture[34]. It is important to note, however, that taxol production by all endophytes in culture is in the range of sub-microgram to microgram 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[30]. Efforts are being made to determine the feasibility of making microbial taxol a commercial possibility.

Torreyanic acid, a selectively cytotoxic quinone dimer (anticancer agent), was isolated from a *P. microspora* strain. 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 potency in those lines that are sensitive to protein kinase C agonists and causes cell death by apoptosis. Recently, a complete synthesis of torreyanic acid has been successfully completed using the application of a biomimetic oxidation/dimerization cascade[69].

The 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[35]. 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-cytochalasins[35]. Thus, it is not uncommon to find one or more cytochalasins in endophytic fungi and workers in this field need to be alerted to the fact that redundancy in discovery does occur. Chemical redundancy (dereplication) usually occurs with certain groups of organisms on which previous studies have already established the chemical identity of major biologically active compounds. For instance, as with the cytochalasins, they are commonly associated with the xylariaceaous fungi.

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 of Papua New Guinea[36,38]. Both pestacin and isopestacin display antimicrobial as well as antioxidant activity. Isopestacin was suspected of antioxidant activity based on its structural similarity to the flavanoids (Fig. 7). Electron spin resonance spectroscopy measurements confirmed this antioxidant activity; the compound is able to scavenge superoxide and hydroxyl free radicals in solution[36]. Pestacin was later described from the same culture fluid, occurring naturally as a racemic mixture and also possessing potent antioxidant activity[38](Fig. 8). Proposed antioxidant activity of pestacin arised primarily via cleavage of an unusually reactive C-H bond and to a lesser extent, though O-H abstraction[38]. The antioxidant activity of pestacin is at least one order of magnitude greater than that of trolox, a vitamin E derivative[39].

Products of endophytes with insecticidal activities: Bioinsecticides are only a small part of the insecticide field but their market is increasing[2]. Several endophytes are

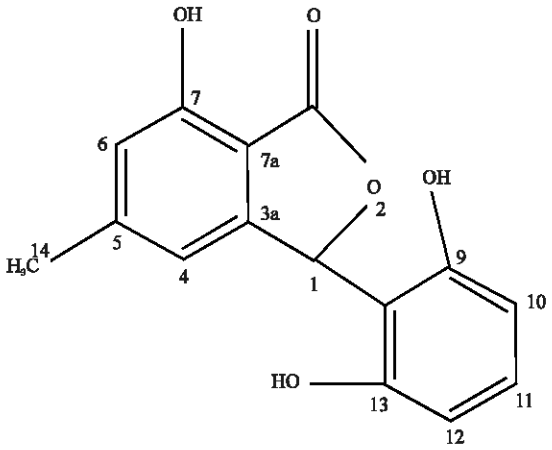

Fig. 7: Isopestacin, an antioxidant produced by an endophytic *P. microspora* strain isolated from a tree growing on the north coast of Papua New Guinea

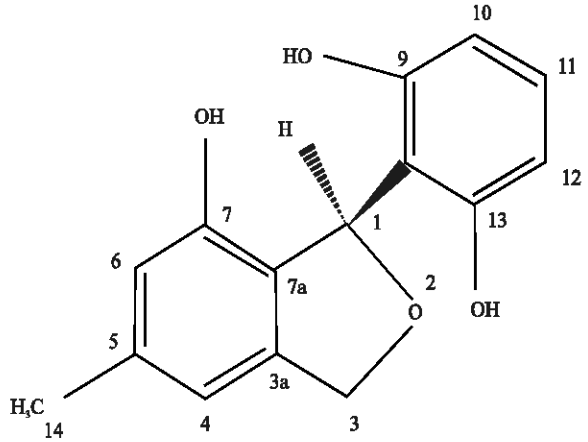

Fig. 8: Pestacin is also produced by the same fungus as in Fig. 7 and it too is an antioxidant

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 compounds were isolated from an endophyte, a *Nodulisporium* sp., from the plant *Bontia daphnoides*. This discovery has since resulted in an intensive search for more *Nodulisporium* spp. or other producers of more potent nodulisporic acid analogues[11]. 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[70]. 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*)[71,72]. 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[73]. This compound acts as an insulin mimetic and, unlike insulin, is not destroyed in the digestive tract and may be given orally. Oral administration of L-783, 281 to two mouse models of diabetes resulted in significant lowering in blood glucose levels. These results may lead to new therapies for diabetes[73].

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 a rheumatoid arthritis and insulin dependant diabetes. The endophytic fungus, *Fusariu subglutinans*, isolated from *T. wilfordii*, produces the immunosuppressive but noncytotoxic diterpene pyrones subglutinol A and B[74] (Fig. 9). Subglutinol A and B are equipotent in the mixed lymphocyte reaction (MLR) assay and thymocyte proliferation (TP) assay with an IC_{50} of 0.1 μM. In the same assay systems, the famed immunosuppressant drug, Cyclosporin A, is 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[74].

The Microbiology Department at Sandoz Ltd., developed a computer aided evaluation program to screen and evaluate fungi for bioactivity. The program can recognize and eliminate from study common fungi producing known compounds and thereby direct attention to the evaluation of rare samples, which are more likely to produce metabolites with novel bioactivity. This approach resulted in the discovery of the fungus-*Tolypocladium inflatum*, from which cyclosporin, a hugely beneficial immunosuppressant, was isolated[75]. This example perfectly depicts the current aim of many investigators to seek out rare endophytes from interesting and uncommon hosts and environments.

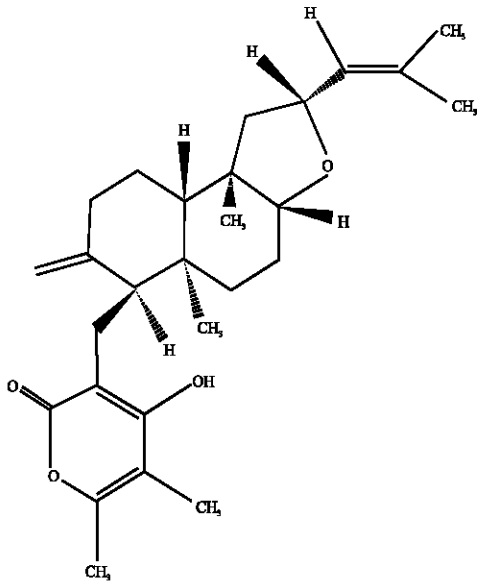

Fig. 9: Subglutinol A, an immunosuppressant, is produced by an endophytic *Fusarium subglutinans* strain.

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*[26]. Although the complete answer to this question is not at hand, some other relevant genetic studies have been done 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[76]. Repeats of the telomeric sequence 5'-TTAGGG-3' were appended to non-telomeric 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[25]. 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.

Endophytes in Pakistan: Certainly, as pointed out above, a search for interesting endophytes begins with some knowledge of the plant source from which they originate. In Pakistan, to our knowledge, little or no work has been done on endophytic microbes. Currently, a checklist of the plants of Pakistan has been produced (http://www.mobot.org/MOBOT/research/pakistan/intro.html). It is to be noted that the great range of altitudes of Pakistan, from sea level to the famous K2 mountain (second highest on earth) provides huge possibilities for very diverse biological communities and a rich flora composing at least 5,700 species. A Flora representing all of Pakistan has not been done, but Flora Iranica by K. H. Reichinger provides a good staring point for the identification of plants in many areas of Pakistan with the exception of the very rich northeastern areas.

The flora of Pakistan has no endemic plant families and three endemic plant genera including *Douepia* in Brassicacae, *Stewartiella* in Apiaceae and *Decalepidanthus* in Boraginaceae. In addition, there are over 200 endemic species or about 4% of Pakistan's flora. These endemics are generally found in the mountain regions of Pakistan. These unique plants, as well as plants used for medicinal purposes and those growing in areas of high rainfall, such as in the monsoonal uplands of the Kashmir are those recommended for study. Generally, those endophytic microorganisms that represent novel taxonomy also possess novel chemistry. Searching unusual plants growing in remote areas is likely to produce some interesting and important results. The plants of Pakistan are waiting to be studied for their endophytes.

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 spend time isolating microfloral components. This may facilitate the product discovery processes.

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 witnessed by the discovery of a wide range of products and microorganisms already that hold an inkling for future prospects 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 and other forested areas 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[18]. 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 40-50 %, of what were the original rainforests existing 1000-2000 years ago, are currently present on the earth. 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 disappears. It can only 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. Multi-step 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 hold enormous promise to impact many areas of human existence. The problem of the loss of biodiversity should be one of concern to the entire world.

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