

Chapter 14

Genetic Diversity of Microbial Endophytes and Their Biotechnical Applications

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Introduction

The need for new and useful compounds and biological processes to provide assistance and relief in all aspects of the human condition is ever growing. Drug resistance in bacteria, the appearance of life-threatening viruses, and a tremendous increase in the incidence of fungal and drug-resistant bacterial 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. In addition, there is the need for bio-derived fuels to supplant the ever-growing demand for petroleum and petroleum products.

Endophytes, microorganisms that reside in the tissues of living plants, are relatively unstudied and potential sources of novel natural products and processes for exploitation in medicine, agriculture, and industry. The genetic diversity of these organisms seems to parallel that of the diversity of host plants in which they reside. Thus, the areas of the earth having the greatest plant diversity are the wet/damp equatorial regions which have been designated as hot spots (Mittermeier et al. 1999). Frequently, these areas in the developing world and scientists living and working there have an enormous advantage in being close to the source of endophytic genetics and diversity.

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. Less than 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.

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The processes involved in this hunt are relatively straightforward and can be quickly learned. In fact, a few American universities have a cadre of undergraduate students involved in isolating and testing endophytic microbes for their genetic and biological potential (Strobel and Strobel 2007). This approach represents an extremely useful and practical means for students to make first-hand observations and discoveries on endophytes, which enhances their excitement and fascination in doing science.

The intent of this chapter is to provide insights into the presence of endophytes in nature, the products that they make, their genetic potential, and how some of these organisms are beginning to show some potential for human use. The majority of this chapter discusses the rationale, methods, and examples of a plethora of endophytes isolated and studied in the author's lab over the course of many years (Strobel and Daisy 2003). This chapter also includes some specific examples that illustrate the work of others in this emerging field of *bioprospecting* the microbes of the world's rainforests and studying their genetics, biology, and potential applications.

Needs for New Medicines, Agrochemical Agents, and Fuel

There is a general call for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity, and will have 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. In addition, new drugs are needed to efficiently treat parasitic protozoan and nematodal infections such as malaria, leishmaniasis, trypanosomiasis, and filariasis. Malaria alone is more effective in claiming lives each year than any other single infectious agent with the exception of the AIDS virus and *Mycobacterium tuberculosis* (NIH 2001). In addition, 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. Novel natural products and the organisms that make them offer opportunities for innovation in drug and agrochemical discovery. Finally, in the past few years, microbes have been discovered that produce fuel-related compounds upon fermentation (Strobel et al. 2008). Thus, 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. These products have been exploited for human use for thousands of years, and plants have been the chief source of compounds used for medicine. In fact, the world's best known and most universally used medicinal compound is aspirin (salicylic acid) which has its natural origin from the glycoside salicin which is found in many species of the plant genera *Salix* and *Populus*. Many native peoples realized early on 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 (Brewer 2000). In fact, the world's first billion dollar anticancer drug, taxol, is a natural product derived from the yew tree (Wani et al. 1971). Many other examples abound that illustrate the value and importance of natural products in modern civilizations.

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. *There will always be a need for natural products to serve as platforms for new product development!*

Endophytes

Why Study 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 in different parts of the world by armies of collectors. In other words, why do something different (working on endophytic microbes) 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.

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 (Fig. 14.1). Thus, it would appear that these biotypical factors (the nature and location of a host plant) can be important in plant selection since they may govern the novelty and biological activity of the products associated with endophytic microbes.

Bacon and White give an inclusive and widely accepted definition of endophytes—“Microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects” (Bacon and White 2000). 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 and archaeobacteria. 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. It is estimated that there may be as many as one million different fungal species, yet only about 400,000 have been described (Hawksworth and Rossman 1987). As more evidence accumulates, estimates keep rising as to the actual number of fungal species. For instance, Dreyfuss and Chappela (1994) estimate there may be at least one million species of endophytic fungi alone. However, it has been demonstrated through genetic sampling (ITS rDNA sequence information) of over 100 endophytes of Amazonian plants that there were over 10% whose sequence data did not match any known fungi within a respectable level (Smith et al. 2008). This suggests that the one million species estimate may even be low since an ever so diminishingly small sample of the rainforest was studied. 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.

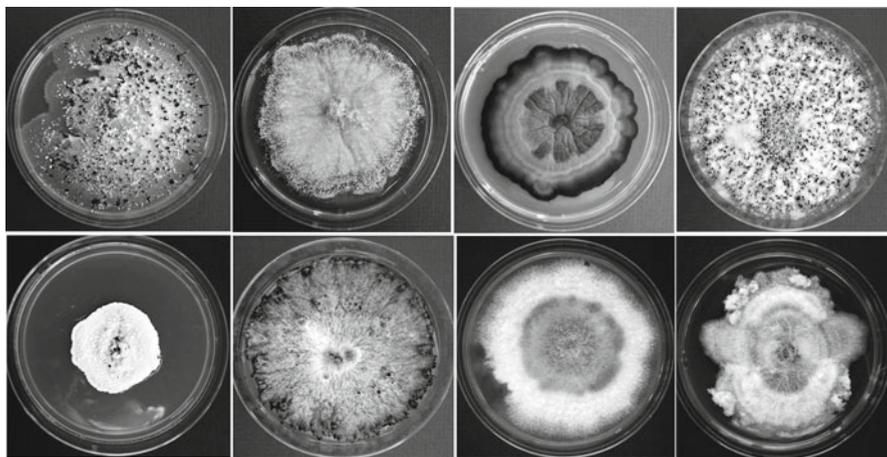


Fig. 14.1 Some endophytic fungi isolated from various tree species in Madagascar. The genetic diversity of these fungi is truly astonishing. The products made by endophytic microbes have promise in many aspects of human endeavor

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.

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:

- (a) Plants from unique environmental settings, especially those with an unusual biology and possessing novel strategies for survival.
- (b) Plants that have an ethnobotanical history (used by indigenous peoples) that are related to the specific uses or applications of interest are selected for study. Ultimately, it may be the healing powers of the botanical source, in fact, that may have nothing to do with the natural products of the plant, but of the endophyte (inhabiting the plant).
- (c) 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.
- (d) Plants growing in areas of great biodiversity also have the prospect of housing endophytes with great biodiversity.

Plants with ethnobotanical history, as mentioned above, also are likely candidates for study since the medical uses to which the plant may have been selected

relate more to its population of endophytes than to the plant biochemistry itself. 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 by the plant 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.) (Suffness 1995); a novel taxol-producing fungus, *Taxomyces andreanae*, from the yew, *Taxus brevifolia*, was isolated and characterized (Stierle et al. 1993).

Endophytes and Biodiversity

Of the myriad of ecosystems on earth, those having the greatest biodiversity also seem to be the ones also having endophytes with the greatest number and the most diverse microorganisms. Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth. As such, scientists in countries in and bordering these regions on earth have ready access to this enormous diversity. 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 (Mittermeier et al. 1999). As such, one would expect that areas having high plant endemism also possess specific endophytes that may have evolved with the endemic plant species.

Ultimately, biological diversity implies chemical diversity because of the constant chemical innovation that exists 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 (Redell and Gordon 2000). There is 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 (Bills et al. 2002). 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. This observation suggests the importance of the host plant in influencing the general metabolism of endophytic microbes.

Collection, Isolation, and Storage 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.

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 . One of the most effective methods for storage of these organisms is by growing them on sterile barley seed and then placing the infested seed directly at -70°C .

Growing Endophytes

Most commonly, standard commercially available media can be used to culture endophytes with potato dextrose broth being one of the most frequently used media. Other media include oatmeal, lima bean, or other infusions of various plant seeds, stems, or roots. This is not to exclude the prospects of actually making an infusion of the host plant itself for use as a cocktail for endophyte fermentation. Ideally, if working on natural product isolation, a medium that is well defined is desirable to use since media substances that would otherwise interfere with the purification processes are not present.

Ultimately, once appropriate growth conditions are found, the microbe is fermented, extracted, 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, human sniff tests, or model chemical systems that relate to the purpose for which the new compound is needed. Once a set of endophytes is obtained from natural sources, all avenues of product discovery should be open, and this includes a target for organisms performing novel and interesting activities such as the manufacture of fuel-like substances or volatile antibiotics.

Endophyte Identification with Microscopy and Molecular Genetics

Identifying Endophytes with Microscopic and Other Techniques

The pathway in the production of a product of one's research on endophytes is to know the identity of the organism under investigation. This activity is central to any publication or patent that is desired from the scientific effort. Initially, after isolation and obtaining some indication of the activity of the organism, work is on microbe identity. This is initiated observing the cultural characteristics of the microbe including form, color, pattern development, and the appearance of fruiting bodies on various media. Experience of the investigator can be extremely helpful in making initial guesses on microbe identity. Then microscopic observations are made on spores, mycelia, and other fruiting structures. The best images are generally those obtained from scanning electron microscopy (SEM) which provides unique and important details of the endophyte (Fig. 14.2). Even more interesting maybe the images obtained by environmental SEM (ESEM) which reveals the actual unaltered structural features of the organism since no fixatives are used in this process.

Molecular Genetics of Endophytes

While getting microscopic data on an organism is important, it is also valuable to acquire genetic data. In fact, some scientific journals and patent offices are now requiring such information before things can proceed. This is because in more recent times, the advent of the enormous reservoir of genetic information in GenBank has allowed for immediate molecular comparisons of any unknown organism with molecular genetic data on deposit, and this is true for endophytic microbes. The most common gene sequences entered for most organisms at the present time are selected ribosomal encoded regions of the organismal DNA. In particular, the ITS regions are conserved genetically, and as such the sequences obtained are most likely to be unique to the organism being studied. The procedures for acquiring these genetic data are very straightforward and generally involve the use of already commercially available kits and local, regional, national, or commercially available sequencing facilities. An example of a statement on the molecular identification of a fungal endophyte is shown below:

Once limited genomic data are obtained and comparisons are made with GenBank entries, the results of the genetic analyses are compared with the findings of the light and SEM observations. As for the genetic analytical data, it is generally the case that greater than a 95% level of nucleotide matching is needed to consider an organism close or identical to an already established genus. However, less than 95% identity is enough different to call in taxonomic uncertainty. Ideally, both the visual data and the genomic data sets will be in agreement. However, as frequently occurs with

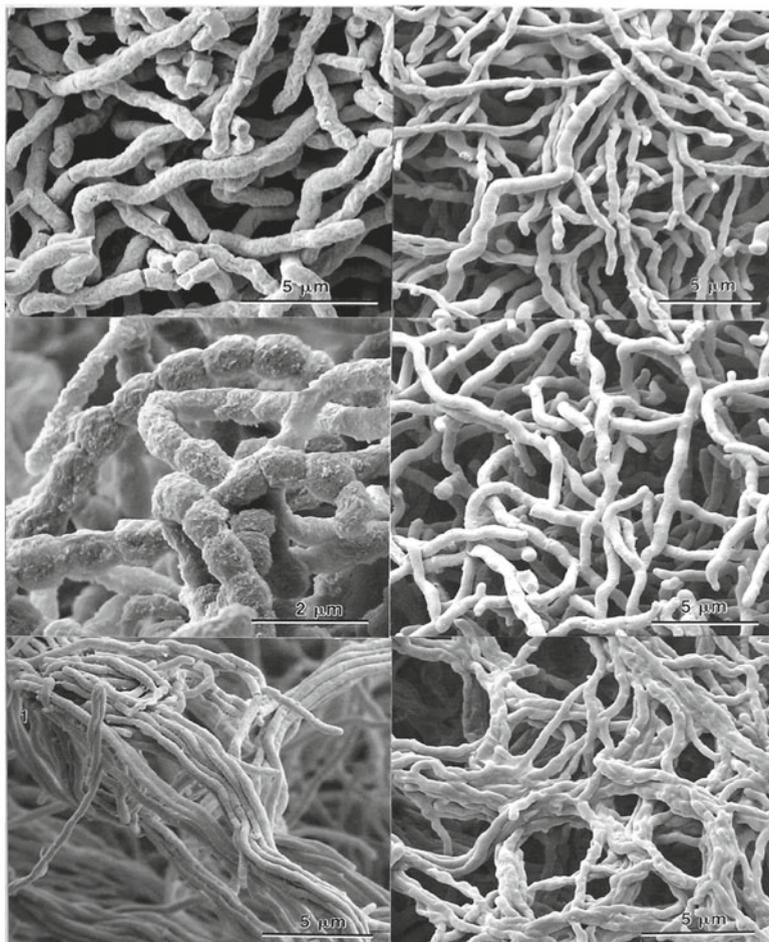


Fig. 14.2 Scanning electron micrographs of various *Streptomyces* spp. from Patagonian plants (a, upper left) C-1 (from *Nothofagus pumilio*) showing early spore formation, (b, upper right) C-2 (from *Chilotrimum diffusum*) showing early spore formation and young hyphal cells, (c, center left) C-3 (from *Nothofagus betuloides*) showing distinct segmentation of hyphal cells into spores, (d, center right) C-4 (from *Misodendrum punctulatum*) showing young hyphae, (e, lower left) C-5 (from *Nothofagus betuloides*) showing ropy strands of hyphae, (lower right) C-6 (from *Nothofagus betuloides*) showing ropy net-like hyphae. Compare C-2 with the images in C-4

endophytes, all of the data are unique, and thus one has the opportunity to name a new fungal genus and/or species or both. An example of how this is done is provided in (Mitchell et al. 2010).

Total genomic sequencing has been done on many organisms with a bacterium being the first followed by completion of the complete human genome. To date, only a few endophytes have been totally sequenced and annotated. Probably the best work is that of Strobel's group at Yale who have completely sequenced and

annotated *Ascocoryne sarcoides*, an endophyte that makes fuel-related compounds (Strobel et al. 2011, unpublished). Such efforts are costly, time consuming, and require a working group. In the future, fungal genomics will be done more efficiently, and the results will undoubtedly show the way toward manipulation of microbial products and processes.

Natural Products from Endophytic Microbes

The following section shows a few examples of natural products obtained from endophytic microbes and their potential in the pharmaceutical and agrochemical arenas.

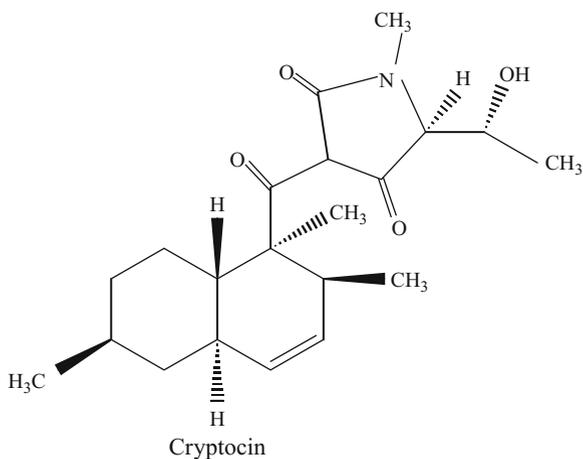
Endophytic Microbial Products as Antibiotics

Antibiotics are defined as low-molecular-weight organic natural products made by microorganisms that are active at low concentration against other microorganisms (Demain 1981). 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 disease-causing agents 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 *Tripterygeum wilfordii*, a medicinal plant native to Eurasia (Strobel et al. 1999). 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* (Strobel et al. 1999). This compound contains a number of peculiar hydroxylated amino acids and a novel amino acid—3-hydroxy-4-hydroxy methyl proline. The bioactive compound is related to the known antimycotics, the echinocandins and the pneumocandins (Walsh 1992). 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.

Cryptocin, a unique tetramic acid, is also produced by *C. quercina* (see above) (Li et al. 2000) (Fig. 14.1). This unusual compound possesses potent activity against *Pyricularia oryzae* as well as a number of other plant pathogenic fungi (Li et al. 2000). The compound was generally ineffective against a general array of human pathogenic fungi. Nevertheless, with minimum inhibitory concentrations against

Fig. 14.3 The structure of cryptocin as determined by x-ray crystallography. It is an antifungal agent produced by *Cryptosporiopsis cf. quercina*



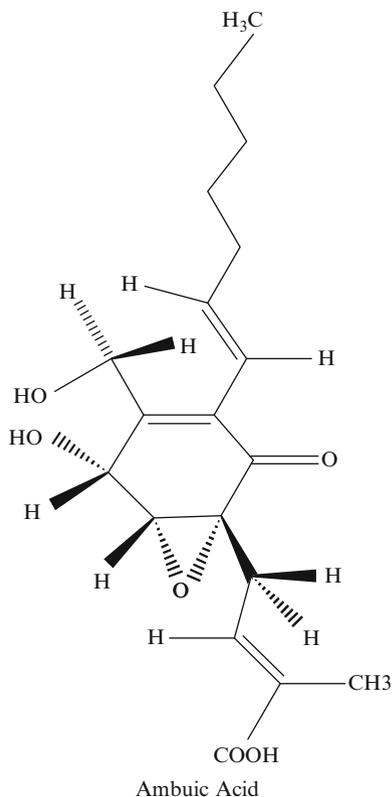
P. oryzae at 0.39 $\mu\text{g/mL}$, this compound is being examined as a natural chemical control agent for rice blast and is being used as a base model to synthesize other antifungal compounds. Its structure was deduced by x-ray crystallography which should be an ultimate goal of any natural product research effort since the structural result is probably not refutable (Fig. 14.3).

As mentioned earlier, *Pestalotiopsis microspora* is a common rainforest endophyte (Strobel 2002). It turns out that enormous genetic diversity exists in this endophytic fungus, and as such there seems to be many secondary metabolites produced by a myriad of strains of this widely dispersed and common rainforest 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 (Li et al. 2001) (Fig. 14.2). In fact, this compound has been used as a model to develop new solid-state NMR tensor methods to assist in the characterization of molecular stereochemistry of organic molecules (Harper et al. 2001). Recently, and quite surprisingly, ambuic acid has been shown as an effective agent in precluding the quorum sensing response in certain bacteria, and a whole new field of microbiology has been born (Nakayama et al. 2009) (Fig. 14.4).

Other Products and Processes from Endophytes

Over the past 15 years, there has been a concerted worldwide effort to find, describe, and study the secondary products of endophytes. Many of these organisms have come from both temperate and tropical rainforests. The products range from antibiotics, antioxidants, antiviral agents, anticancer agents, to antibacterial agents and now—mycodiesel. In addition, there is an interesting fungal group making volatile

Fig. 14.4 The structure of ambuic acid from *P. microspora*. Its structure was deduced by NMR and mass spectroscopy. The compound is an effective inhibitor of the quorum sensing processes in bacteria



biologically active products—the Muscodors. These products and processes are included in a number of review articles (Strobel 2002; Strobel 2006; Verma et al. 2009). It is to be noted that isolation and characterization of natural products does require some expensive sophisticated equipment and people who know and understand how to gather and interpret data sets in order to make meaningful conclusions. In order to do this, the investigator needs to make alliances with those who have these skills. Ultimately, publication or the patent process will mean that there are multiple contributors. This is evident on the majority of papers in this field of study.

Conclusions

The enormous field of endophyte biology and genetics is a relatively untapped resource for finding novel compounds that may be useful to humankind. On top of this is the prospect that those in countries (hot spots) with these genetic resources do

have immediate access to this genetic potential. Because the endophyte does not cause any harm to its plant host, there arises a plethora of questions about the relationship of the microbe to its host. There are also numerous questions about the environmental, genetic, and host factors controlling the relationship of host and endophyte. For the most part, investigators have concentrated on isolating and identifying the various endophytes of a few of the world's higher plants. Yet, the same questions can be asked of lower plant types including aquatic and marine plants, ferns, and mosses. It appears that this field is open for all to inquire and make important observations and discoveries.

This chapter briefly discussed some of the approaches needed to isolate and characterize an endophyte. Underlying natural products made by these organisms is the larger prospect of examining the entire genome of the endophyte and learning how important products are made and if improvements in production can be achieved. To this end, this field is in its infancy since so little has been performed to date, but the prospects of finding new products and processes seem unlimited. Fungal genomics is an important part of endophyte isolation and discovery, and with time, complete endophyte genomes will provide a better understanding of the biology of these organisms.

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