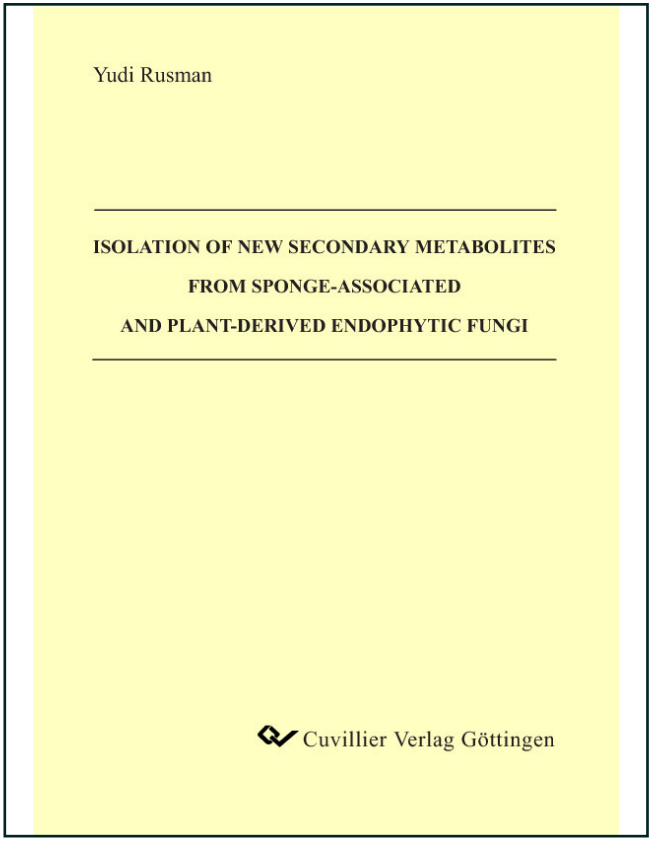
Isolation of New Secondary Metabolites from Sponge-associated and Plant-derived Endophytic Fungi



1 INTRODUCTION

1.1 Natural Products Drug Discovery and Its Current Status

Natural products are chemical compounds derived from living organisms, such as plants, animals, insects, and microorganisms. Since they are highly diverse and often provide highly specific biological activity, natural products have been the basis of human diseases treatment and a major source of new drugs. Many successful drugs in the market today were originally synthesized to mimic the action of molecules found in nature (Feher and Schmidt, 2003).

Natural product derived drugs are usually secondary metabolites and their derivatives. And today they must be pure and highly characterized compounds. Secondary metabolites are those products (chemical compounds) of metabolism that are not essential for normal growth, development or reproduction of organism. They may serve as (Demain, 2000):

- competitive weapons used against other bacteria, fungi, amoebae, plants, insects, and large animals
- metal transporting agents
- agents of symbiosis between microbes and plants, nematodes, insects, and higher animals
- sexual hormones
- differentiation effectors

1.1.3 Traditional Medicines and Natural Products in the Past

The use of natural products, mainly as plant preparations, as medicinal agents has begun since the ancient time. The usefulness of plants to treating diseases was recorded in the Emperor Shennung´s classic herbals (the earliest recorded Chinese herbal medicine, 2700 BC) and Eber's papyrus in Egypt (1550 BC). Ayurveda, the term used for the traditional medicinal system in India, is being used for more than three thousand years, and the earliest Vedic texts, dating from 1500 – 1200 BC were especially concerned with aging, various afflictions and the prescription of cures involving prayers and herbal medicines.

Later, the Greek physician Galen (AD 129 – 200) devised the first pharmacopoeia describing the appearance, properties and use of many plants of his time (Patwardhan et al., 2004).

Today, most knowledge of traditional medicines and therapies has been lost in the daily life, since it was only sporadically recorded in epics and folklore. This fact is not only found in mostly developed countries, but also some developing countries where the indigenous population has been marginalized. Although some traditional medicines and therapies have still survived and are being applied, their percentage is very small compared to modern medications (Patwardhan et al., 2004).

Nevertheless, the wisdoms of ancient medicines have often been the basis of modern drug discovery. Thus, some ideas of the currently accepted modern medicine is based on the traditional medicines and therapies. In addition, due to the high cost of modern medications research and production, most developing countries, especially subtropical and tropical countries with a higher biodiversity of plants, still rely on traditional medicine. Today the largest users of traditional medicines are the Chinese, with over 5000 plants and plant products in their Pharmacopeia (Strobel et al., 2000).

1.1.4 Natural Products Today

Natural products are the most successful source of drug leads and continue to provide greater structural diversity than standard combinatorial chemistry, and so they offer major opportunities for finding novel molecules. In the modern area of drug discovery, they will continue to be important as targets for production by biotechnological approaches, a source of lead compounds of novel chemical structures, and as the active ingredients of useful treatments derived from traditional systems of medicine (Harvey, 1993).

The discovery of pure natural compounds as active principles was first described at the beginning of the 19th century. Morphine, produced and commercialised by E. Merck for the first time in 1826, was one among the first isolated active compounds (Newman et al., 2000). Today, a vast range of drugs, which represent the cornerstones of modern pharmaceutical care, are either natural products themselves or have been derived from

them. Currently, there are more than 183.000 known natural products, and new structure are being published at a rate about 10.000 per annum (Dictionary of Natural Products, 2004).

Some analytical and separation techniques have been developed in order to reduce the time recquired for isolation and characterization of natural compounds. High performance liquid chromatography (HPLC) has been the most reliable tool for the separation of complex mixtures of small molecules. More recently, the advent of electrospray (ESI) and atmospheric pressure chemical ionization (APCI) have provided mass spectrometry (MS) interfaces which are applicable to the analysis of a wide range of molecules and are compatible with liquid chromatography (LC) (Elsewijk and Irth, 2003). The high field NMR (originally 200 MHz and then up through 600 – 800 MHz) allows the complete structure elucidation in amount of compounds less than 1 mg.

The rapid identification of already known natural products is an important strategy to screen novel bioactive compounds from natural resources. LC-MS, LC-UV or combination of the two have become a widely used tool for the dereplication of natural products. Data from UV and MS alone will rarely provide sufficient information to distinguish between the known isomers. Even when this is achieved, possible novel isomers have also to be considered. Recently, the use of LC-NMR at this stage has been advocated (Bradshaw et al., 2001).

It is estimated that about one third of currently marketed drugs are related to natural products (Elsewijk and Irth, 2003). The percentage of natural product and natural product derived drugs in the top 35 worldwide ethical drug sales for 2000, 2001 and 2002 are shown in figure 1.1. Their relative percentage of all marketed drugs was 40 % in 2000 and remained approximately constant at 24 % in 2001 and 26 % in 2002 (Butler, 2004).

Most natural product and natural product derived compounds introduced between 1981 – 2002 were compounds with antibacterial and cytotoxic potential, while only one was used as an antiviral agent (see figure 1.2). It thus can be concluded that natural products still serve a major role as anticancer and antibacterial agents.

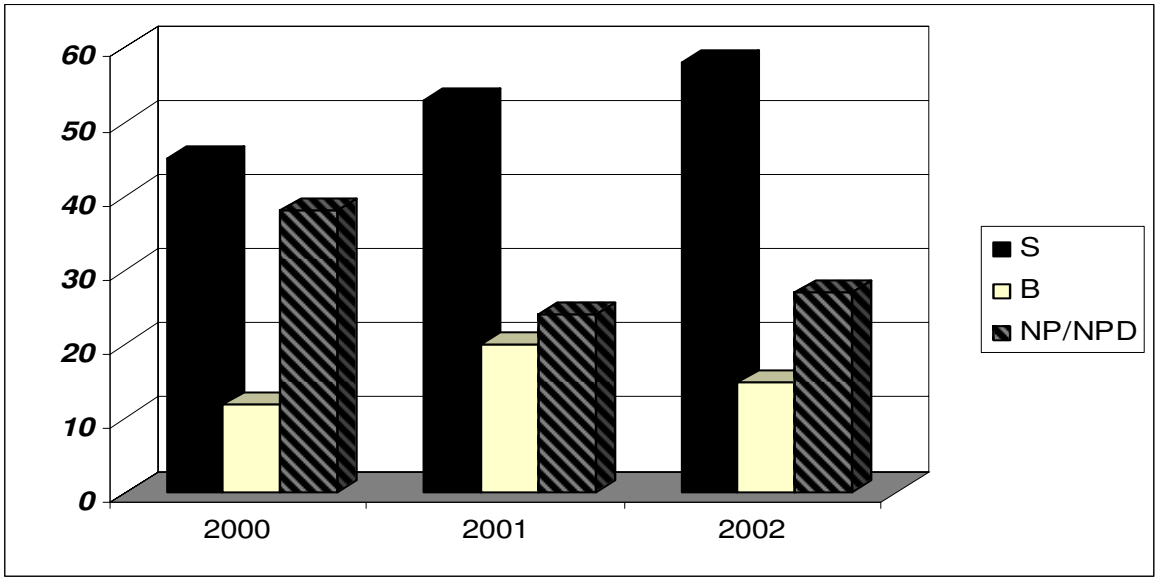


Figure 1.1 Percentage of synthetic (S), biologic (B) and natural product (NP)/natural product-derived (NPD) drugs in the top 35 worldwide ethical drug sales for 2000, 2001, and 2002 (Butler, 2004).

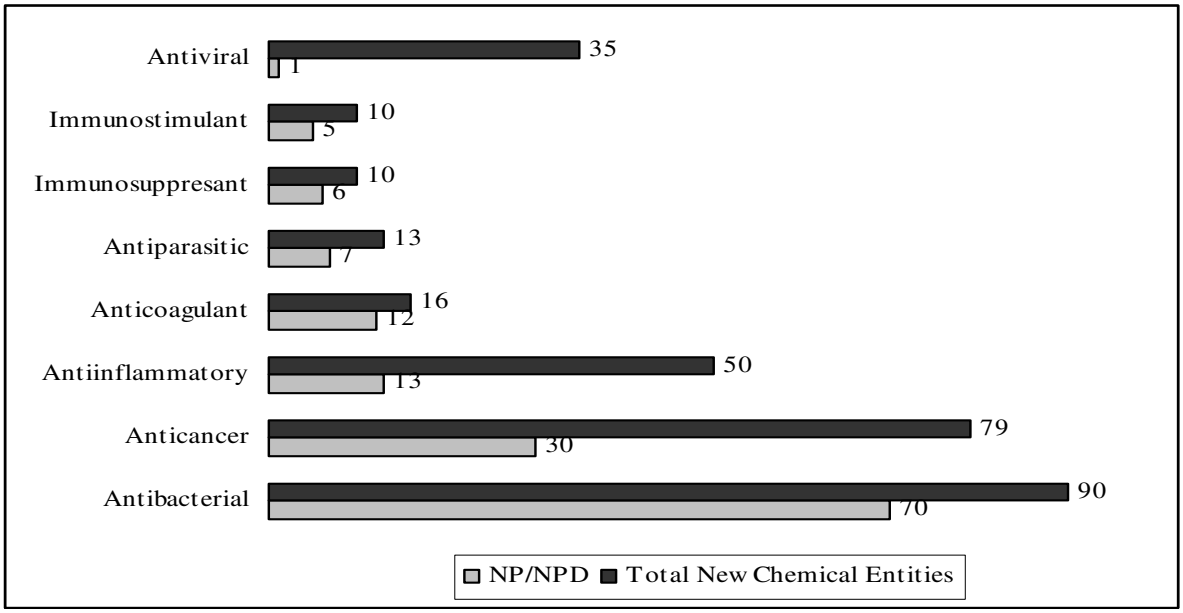


Figure 1.1 Comparison of new natural product (NP) and natural product derived (NPD) compounds to total new chemical entities per selected medical indication in the frame time 1981 – 2002 (Newman et al., 2003).

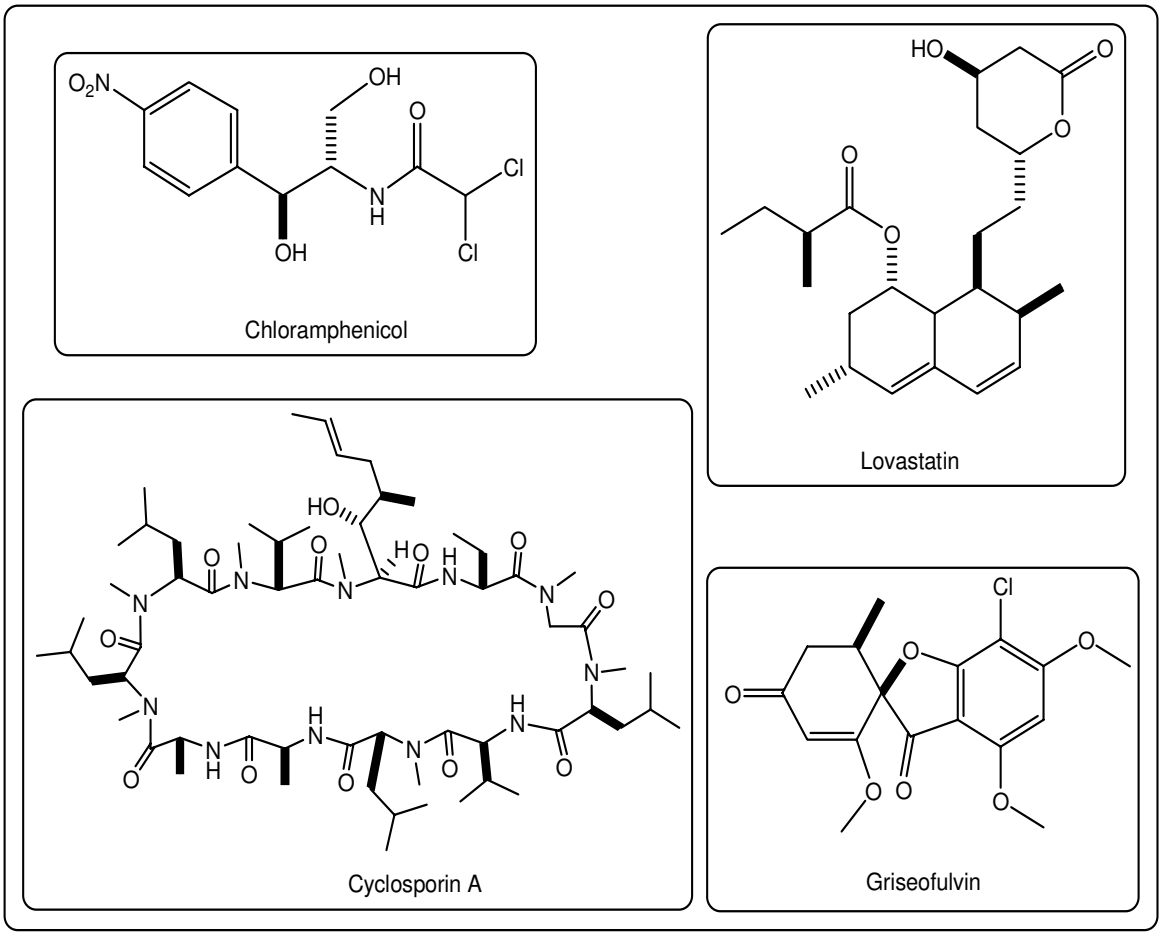
In the frame time 1998 – 2004 at least 21 natural product and natural product derived drugs have been launched onto the market in USA, Europe, and Japan. The 21 drugs can be classified as 3 natural products, 10 semi synthetic natural products, and 8 natural product-derived drugs. Although the number was low, there are still many natural product-derived

compounds in Phase III or registration that may be launched in 2005 and 2006 (Butler, 2005)

1.2 Fungi as Natural Products Sources

Fungi are heterotrophic eukaryotes that lack chlorophyll. Thus they must absorb all recquired nutrients from external sources, but since they are independent of light, they can also inhabit damp and dark places. As true eukaryotes, they have membrane bound organels such as nuclei, mitochondria, endoplasmic reticulum, etc. Fungi are characterized by a distinctive, filamentous, multinucleate vegetative structure known as the mycelium. It is composed of hyphae, a branching system of tubular structures which contain protoplasm and continually extend by apical growth and lateral branching (Ainsworth and Sussman, 1965).

Fungi are remarkable organisms that produce a wide range of secondary metabolites. In many cases, the benefit these compounds confer on the organism is unknown. However, interest in these compounds is considerable, as many natural products are of medical, industrial, or agricultural importance (Calvo et al., 2002). The exploration of fungal bioactive secondary metabolites was initiated by the discovery of penicillin in 1928 by Alexander Fleming, further re-isolation and clinical studies by Chain, Florey and co-workers in early 1940s, and its subsequent commercialization in a synthetic form (Butler, 2004). About twenty years after the discovery of penicillin, several other antimicrobial agents such as chloramphenicol (Long and Troutman, 1949) and griseofulvin (Grove et al., 1952) had been discovered from fungi. Furthermore, cyclosporine A (Traber et al., 1982 and Traber et al., 1987), and lovastatin (Endo, 1979) are fungal metabolites used as immunosuppressants during organ transplantation and antihyperlipidaemic agents, respectively.



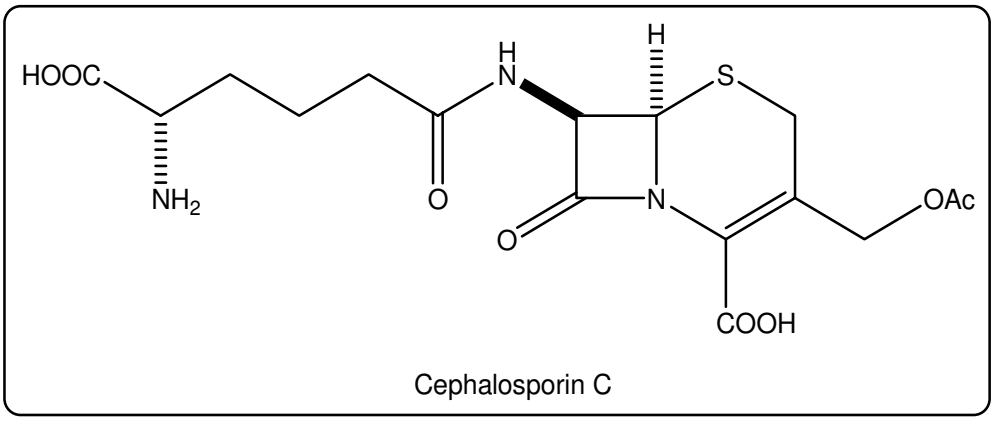
Some secondary metabolites can stimulate spore formation and inhibit or stimulate germination. Since formation of secondary metabolites and spores are regulated by similar factors, this phenomenon can ensure secondary metabolites production during sporulation. Thus, the secondary metabolites can slow down germination until a less competitive environment and more favourable conditions for growth exist, protect the dormant or initiated spore from consumption by amoebae or cleanse the immediate environment of competing microorganisms during germination (Demain, 2000).

1.2.1 Sponge-Associated Fungi

The marine environment became a focus of natural products drug discovery because of its relatively untapped biodiversity compared to terrestrial habitats. Marine plants, animals, and microbes produce secondary metabolites that have a promising potential as new drugs

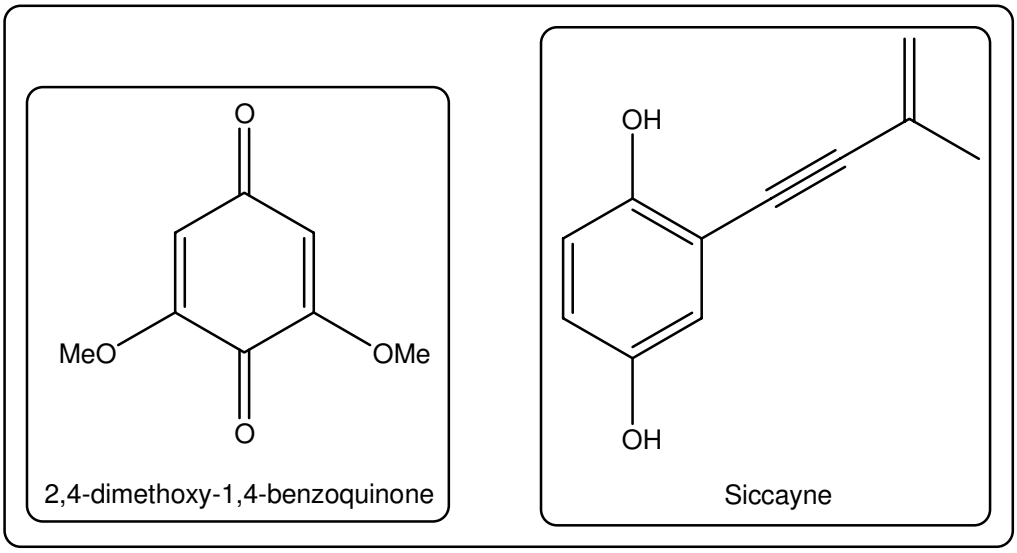
for the treatment of cancer, infectious diseases, and inflammation (Faulkner, 1998). Furthermore, it possesses a unique feature of chemical structures which could not be found in terrestrial metabolites (Larsen et al., 2005). However, the major problem is that most promising pharmacologically active marine natural products can only be isolated in an extremely low yield. In addition, the limited amounts of biomass of most marine organisms, especially invertebrates, in nature usually causes the supply problems of the promising compounds for drug development and sustainable production (Proksch et al., 2003). Thus, most of natural products or its derivatives that are being used as medicines in the market were isolated from terrestrial organisms which can either be fermented (in the case of microorganisms) or be produced by agriculture (in the case of medicinal plants).

The exploration of microorganisms living inside invertebrates is one of the most exciting strategies to solve the pressing supply issue inherent to marine drug discovery. Marine microorganisms including fungi, have shown to be potential sources of pharmacologically active metabolites because of their capability to adapt and survive in the marine environment, and to produce unique secondary metabolites (Bugni and Ireland, 2004). The first chemical investigation of marine-derived fungi was carried out by Giuseppe Brotzu in 1945. He found that a fungus (later identified as *Cephalosporium acremonium*) isolated from seawater near a sewage outlet off the Sardinian coast exhibited pronounced antibacterial activities (Demain and Elander, 1999). Ten years later Newton and Abraham discovered that the β-lactame cephalosporin C was responsible for the antibacterial activity of this fungus (Newton and Abraham , 1955). Cephalosporin C shows structural similarity to the penicillins (which were already marketed at that time), but differs from the latter by the cephalosporane (instead of the penicillane) backbone. Nowadays, semisynthetic cephalosporine derivatives have by far outnumbered medicinally applied penicillines, especially in the clinical use.

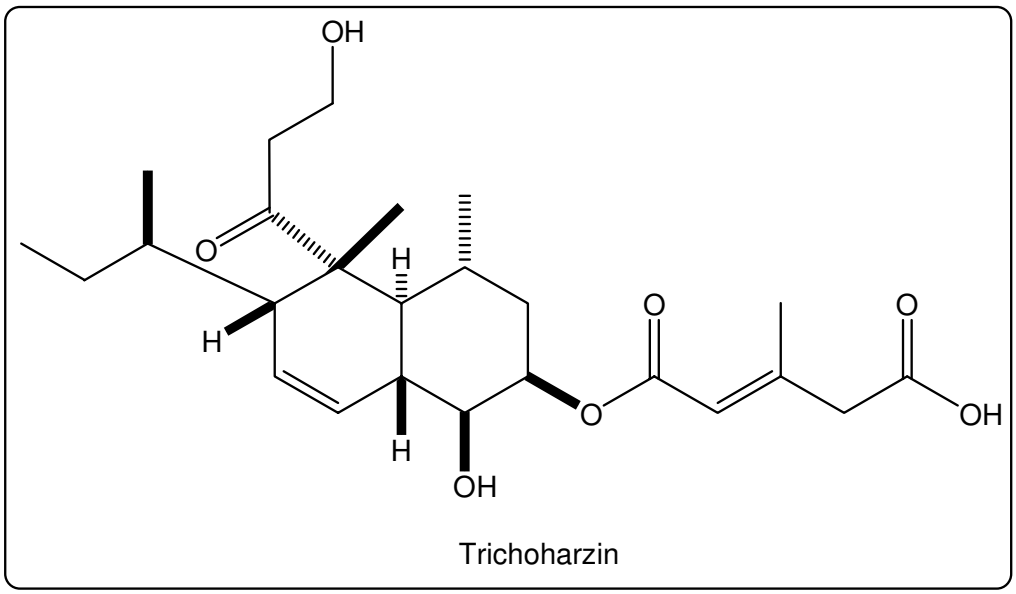


Cephalosporin C

After the discovery of cephalosporin C, the detailed studies of secondary metabolites from marine-derived fungi were still scarce until 1975, when the second secondary metabolite of marine- derived fungi, 2,6-dimethoxy-1,4-benzoquinone, was succesfully identified in a culture of *Dendryphiella salina* (Fukuzumi, et al., 1975). Six years later, siccayne was isolated from *Halocyphina vilosa* and identified as the second antibiotic from marine-derived fungi (Kupta et al., 1981). Starting from 1980'es, marine derived fungi have been systematically studied with regard to their potential to produce bovel bioactive secondary metabolites, yielding up to now more than 300 structure in more than 200 publications (Bugni and Ireland, 2004 ; Ebel, 2006)



In particular, sponge-associated fungi have yielded novel metabolites with potent antibacterial and anticancer activities (Jensen, 2000) which have not been previously reported from terrestrial strains of the same species (Hiort et al., 2004). Trichoharzin, a compound isolated from *Trichoderma harzianum* associated with the sponge *Mycale cecilia*, was the first novel metabolite from sponge-associated fungi (Kobayashi et al., 1993), while gymnastatins A, B and C were the first novel cytotoxic metabolites from sponge-associated fungi (Amagata et al., 1998)



Trichoharzin

1.2.2 Terrestrial Endophytic Fungi

"An endophyte is a bacterial (including actinomycete) or fungal microorganism, which spends the whole or part of its life cycle colonizing inter- and/or intra-cellularly inside the healthy tissues of the host plant" (Tan and Zou, 2001). Since the relationship between the endophyte and its host plant may range from symbiotic to bordering on pathogenic, some secondary metabolites involved in the host-endophyte relationships may also be produced by the endophytes during the colonization. As a direct result of the role of these secondary metabolites in the nature, they may show to have applicability in medicine, agriculture and industry (Strobel, 2002).