Novel Natural Products from Endophytic Fungi of Egyptian Medicinal Plants - Chemical and Biological Characterization

Neue Naturstoffe aus endophytischen Pilzen ägyptischer Arzneipflanzen - chemische und biologische Charakterisierung

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Erklärung

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation mit dem Titel „Neue Naturstoffe aus endophytischen Pilzen ägyptischer Arzneipflanzen - chemische und biologische Charakterisierung“ selbst angefertigt habe. Außer den angegebenen Quellen und Hilfsmitteln wurden keine weiteren verwendet. Diese Dissertation wurde weder in gleicher noch in abgewandelter Form in einem anderen Prüfungsverfahren vorgelegt. Weiterhin erkläre ich, dass ich früher weder akademische Grade erworben habe, noch dies versucht habe.

Düsseldorf, den 10.05.2007

Amal Hassan

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Zusammenfassung

Endophytische Pilze produzieren Naturstoffe mit einer Vielfalt an chemischen Strukturen, die für spezifische medizinische oder agrochemische Anwendungen von großem Interesse sein könnten. Viele dieser Sekundärstoffe weisen biologische Aktivitäten in pharmakologisch relevanten Assaysystemen auf, die sie zu potentiellen Leitstrukturen für die Entwicklung neuer Arzneistoffe machen.

Ziel dieser Arbeit war die Isolierung von Sekundärstoffen aus Endophyten terrestrischer Pflanzen, gefolgt von Strukturaufklärung und Untersuchung ihres pharmakologischen Potentials. Vier endophytische Pilze, nämlich *Alternaria* sp., *Ampelomyces* sp., *Stemphylium botryosum* und *Chaetomium* sp., gewonnen aus ägyptischen Arzneipflanzen, wurden als Naturstoffquellen ausgewählt und über einen Zeitraum von drei bis vier Wochen in Standkulturen in Wickerham-Flüssigmedium sowie in Reis-Festmedium angezogen. Die aus der folgenden Extraktion erhaltenen Fraktionen wurden zur Isolierung der Naturstoffe weiteren chromatographischen Trennmethoden unterzogen.

Zur Strukturaufklärung wurden moderne analytische Verfahren wie die Massenspektrometrie (MS) und die Kernresonanzspektroskopie (NMR) eingesetzt. Zusätzlich wurden für einige optisch aktive Verbindungen chirale Derivatisierungsreaktionen angewendet, um deren absolute Konfiguration zu ermitteln. Schließlich wurden die erhaltenen Substanzen verschiedenen Biotests unterzogen, um ihre antimikrobiellen, antifungalen und cytotoxischen Eigenschaften sowie die Wirkung als Inhibitoren verschiedener Proteinkinasen sowie der Biofilmbildung von *Staphylococcus epidermidis* zu ermitteln.

1. *Alternaria* sp.

Drei neue Alternariolderivate wurden aus *Alternaria* sp., isoliert aus *Polygonum senegalense*, gewonnen. Des weiteren wurden aus diesem Pilz vier neue Verbindungen, nämlich Desmethylaltenusin, 4`-Epialtenuene, Alterlacton und Alternariasäure, isoliert. Die Alternariolderivate sowie einige strukturverwandte Verbindungen wiesen sowohl ausgeprägte zytotoxische Eigenschaften im Test mit der Zellinie L5178Y (murines T-Zell Lymphom) als auch inhibitorische Aktivität gegenüber Proteinkinasen auf.

2. *Ampelomyces* sp.

Ampelomyces sp. ist ein Isolat aus *Urospermum picroides*. Aus diesem Pilz wurden sechs neue Verbindungen isoliert, darunter ein neues Pyron, zwei neue Isocoumarine, zwei

neue sulfatierte Anthrachinone und ein neues Hexahydroanthronol. In den Biotests zeigten Desmethyldiaportinol, Altersolanol A und Methylalaternin zytotoxische Aktivität gegenüber L5178Y-Zellen. Des weiteren zeigten Altersolanol A und Methylalaternin inhibitorische Aktivität gegenüber der Biofilmbildung von *S. epidermidis*.

3. *Stemphylium botryosum*

Aus *Chenopodium album* wurde der Pilz *Stemphylium botryosum* isoliert. Daraus konnten Curvularinderivate isoliert werden, die ausgeprägte zytotoxische Eigenschaften im Test mit der Zellinie L5178Y zeigten.

4. *Chaetomium* sp.

Schließlich wurde der Pilz *Chaetomium* sp., gewonnen aus *Otanthus maritimus*, untersucht. Ein neues Tetrahydrofuranderivat sowie zwei bekannte Cochliodinolderivate und Orsellinsäure wurden aus Extrakten dieses Pilzes gewonnen. Die Cochliodinolderivate wiesen inhibitorische Aktivität gegenüber Proteinkinasen auf; weiterhin zeigten Cochliodinol und Orsellinsäure ausgeprägte zytotoxische Eigenschaften gegenüber der Zellinie L5178Y.

Insgesamt wurden in dieser Arbeit zweiundvierzig Verbindungen isoliert, von denen vierzehn neue Naturstoffe darstellen. Sowohl die neuen als auch die bekannten Substanzen wurden in Hinsicht auf bioaktiven Eigenschaften in verschiedenen Biotests untersucht.

Die Extrake der jeweiligen Wirtspflanzen wurden mit Hilfe von LC/MS gezielt auf die isolierten Naturstoffe aus den endophytischen Pilzen hin untersucht. Keiner der isolierten Sekundärstoffe des endophytischen Pilzes *Chaetomium* sp. war in den Fraktionen von *O. maritimus* zu detektieren. Dagegen konnten Komponenten der übrigen Pilzextrakte eindeutig in Fraktionen der jeweiligen Wirtspflanzen *P. senegalense*, *U. picroides* and *C. album* nachgewiesen werden.

Table of Contents

1. Introduction

Fungi play pivotal ecological roles in virtually all ecosystems. Saprotrophic fungi are important in the cycling of nutrients, especially the carbon that is sequestered in wood and other plant tissues. Pathogenic and parasitic fungi attack effectively all groups of organisms, including bacteria, plants, other fungi, and animals, including humans. Other fungi function as mutualistic symbionts, including mycangial associates of insects, mycorrhizae, lichens, and endophytes. Through these symbioses, fungi have enabled a diversity of other organisms to exploit novel habitats and resources. Indeed, the establishment of mycorrhizal associations may be a key factor that enabled plants to make the transition from aquatic to terrestrial habitats (Lutzoni *et al.*, 2004).

1.1. Endophytes

Mycologists have come to use the term endophyte for fungi that inhabit living, internal tissues of plants without causing visible disease symptoms. The term refers only to fungi at the moment of detection without regard for the actual status of the interaction. Endophytic fungi living asymptomatically within plant tissues have been found in virtually all plant species (Saikkonen *et al.*, 1998; Bacon and White, 2000). The definition thus includes a wide range of fungi, from fungal plant pathogens and saprophytes that have extended latency periods before disease or external signs of infection appear, to obligate mutualists. Accordingly, the distinction between classical plant fungal pathogens and mutualists is not clear and interactions between fungi and host plant are often variable (Saikkonen *et al.*, 1998).

It is hypothesized that there are no neutral interactions, but rather that endophyte-host interactions involve a balance of antagonisms. There is always at least a certain degree of virulence on the part of the fungus enabling infection, whereas defense of the plant host limits development of fungal invaders and disease (Schulz and Boyle, 2005). Many endophytes are closely related to pathogenic fungi, and presumably evolved from them via an extension of latency periods and a reduction of virulence (White *et al.*, 1993). It is also hypothesized that endophytes, in contrast to known pathogens, generally have far greater phenotypic plasticity and thus more options than pathogens including infection, local but also extensive colonization, latency, virulence, pathogenicity, or saprophytism (Schulz and Boyle, 2005).

1.2. Endophyte-host plant interaction

The interactions between host plants and endophytes in natural populations and communities are poorly understood. The endophyte-host plant symbioses represent a broad continuum of interactions, from pathogenic to mutualistic, even within the lifespan of an individual microorganism and its host plant (Freeman and Rodriguez, 1993; Saikkonen *et al.*, 1998; Schulz *et al.*, 2002). Studies showed that endophytes are more likely to be mutualistic when reproducing vertically (systemic) by growing into seeds, and more antagonistic to the host when transmitted horizontally (nonsystemic) via spores (Schardl *et al.*, 1991; Saikkonen *et al.*, 1998). It is possible to imagine that some of these endophytic microbes may have devised genetic systems allowing for the transfer of information between themselves and the higher plant and *vice versa* (Stierle *et al.*, 1993; Strobel, 2002a). Obviously, this would permit a more rapid and reliable mechanism of the endophyte to deal with environmental conditions and perhaps allow for more compatibility with the plant host leading to symbiosis (Strobel, 2002a).

Endophytic fungi are thought to interact mutualistically with their host plants mainly by increasing host resistance to herbivores and have been termed ‘‘acquired plant defenses’’ (Carroll, 1988; Clay, 1988; Schulz *et al.*, 1999; Faeth and Fagan, 2002). Indeed, agronomic grass species infected with systemic endophytes show striking toxic and noxious effects on vertebrate and invertebrate herbivores and pathogens, purportedly resulting from production of multiple alkaloids by endophytes (Siegel and Bush, 1996). Loline alkaloids, saturated 1-aminopyrrolizidines with an oxygen bridge, were exclusively found in endophyte-infected grasses, such as *Festuca* sp. infected with *Neotyphodium* sp. Recently, it was demonstrated that *N. uncinatum*, the common endophyte of *F. pratensis*, had the full biosynthetic capacity for some of the most common loline alkaloids (Blankenship *et al.*, 2001). Lolines are potent broad-spectrum insecticides, acting both as metabolic toxins and feeding deterrents depending on the specific insect species. Unlike ergot and indole diterpene alkaloids, these loline derivatives are much less toxic to mammals (Casabuono and Pomilio, 1997). Similarly, endophytes of woody plants may provide a defensive role for the host plant because they produce a wide array of mycotoxins and enzymes that can inhibit the growth of microbes and invertebrate herbivores (Saikkonen *et al.*, 1998; Tan and Zou, 2001).

Endophytes may also increase host fitness and competitive abilities, by increasing nutrient uptake, germination success, resistance to drought and water stress, resistance to seed predators, tolerance to heavy metal presence, tolerance to high salinity, and growth rate by evolving biochemical pathways to produce plant growth hormones. For instance, the growth

promoting phytohormone indole-3-acetic acid (IAA) was isolated from cultures of the fungal endophytes *Acremonium coenophialum*, *Aureobasidium pullulans*, *Epicoccum purpurascens* and *Colletotrichum* sp. Together with IAA and indole-3-acetonitrile, cytokinins were also shown to be produced by an endophytic strain of *Hypoxylon serpens* (Tan and Zou, 2001). An imaginable role of endophytes is furthermore to initiate the biological degradation of the dead or dying host plant that begins the critical processes of nutrient recycling (Tan and Zou, 2001; Strobel, 2002a; Zhang *et al.*, 2006).

In return, plants provide spatial structure, protection from desiccation, nutrients, photosynthate and, in the case of vertical-transmission, dissemination to the next generation of hosts (Clay, 1988; Wolock-Madej and Clay, 1991; Knoch *et al.*, 1993; Saikkonen *et al.*, 1998; Faeth and Fagan, 2002; Rudgers *et al.*, 2004). It is also possible that the plant may provide compounds critical for the completion of the life cycle of the endophyte or essential for its growth or self-defense (Metz *et al.*, 2000; Strobel, 2002a). However, in cases in which herbivores facilitate spore or hyphal dispersal, nonsystemic endophyte interactions with their host plants should fall near the antagonistic end of the interaction spectrum (Saikkonen *et al.*, 1998).

Recent studies suggested that plant and endophyte genotypic combinations together with environmental conditions are an important source of variation in endophyte-plant interactions (Faeth and Fagan, 2002). It would seem that many factors changing in the host as related to the season, age, environment and location may influence the biology of the endophyte (Strobel and Daisy, 2003).

1.3. Microbial biodiversity

Fungi make up one of the major clades of life. It had been estimated that approximately 1.5 million fungal species are present on earth of which only about 7% have been described so far (Hawksworth, 1991). Almost all vascular plant species examined to date were found to harbor endophytes, thus they are presumably ubiquitous in the plant kingdom (Tan and Zou, 2001). Because numerous new endophytic species may exist in plants, it follows that endophytic microorganisms are important components of microbial biodiversity (Clay, 1992). Ultimately, biological diversity implies chemical diversity because of the constant chemical innovation that exists in ecosystems where the evolutionary race to survive is the most active (Strobel and Daisy, 2003). Currently, it is hypothesized that ecology has a major impact on the profiles of natural products in filamentous fungi. Temperature, precipitation, humidity, length of season and other climatic factors affect the distribution of

fungi. Moreover, diverse habitats, as tropical forests, the deep sea, sites of extreme temperature, salinity or pH, often provide a source of novel microorganisms with the potential for novel metabolic pathways and compounds (Larsen *et al.*, 2005; Ebel, 2006). However, temperate ecosystems, especially damp temperate regions, such as those of northern Europe, eastern North America and North Africa are also rich in fungal diversity. They are generally taken to have the "standard" fungus flora, *i.e.* the one first and best known (Bisby, 1943). Even cold regions can be rich in fungal diversity as a number of these species have recently been investigated and found to produce several bioactive metabolites (Larsen *et al.*, 2005).

One of the most easily genetically transformable fungal species that has been studied to date is *Pestalotiopsis microspora*. The fungus was found to be capable of adding telomeric repeats to foreign DNA, a phenomenon unusual among fungi (Li *et al.*, 1996). This finding may have important implications in its biology since it explains at least one mechanism by which new DNA can be captured by this organism and eventually expressed and replicated. Such a mechanism may explain how the enormous biochemical variation may have arisen in *Pestalotiopsis microspora* (Li *et al.*, 1996). It is also a start in understanding how this fungus adapts itself to the environment of the plant hosts and it suggests that the uptake of plant DNA into the fungal genome may occur. In addition, the telomeric repeats have sequences very similar to human telomeres, which points to the possibility that *P. microspora* could conceivably serve as a means to construct artificial human chromosomes (Strobel, 2002a).

1.4. Plant selection for isolation of endophytes

Endophytes, by definition, live in close association with living plant tissues. In order to acquire endophytes, host plant species should be selected that may be of interest because of their unique biology, age, endemism, ethnobotanical history, or environmental setting. It seems that endemic plants growing in moist, warm climates or in areas of great biodiversity are among the first choices for study. It would appear that microbial competition in such an area would be fierce given the abundance of both water and plants. As such, the number and diversity of natural products produced by microbes surviving in such an area would be high. Moreover, plants growing in harsh or extremely moist environments are sometimes prone to attack by extremely pathogenic fungi and thus special defense mechanisms are necessary for survival. Such disease defenses may be offered by the endophyte normally associated with the plant (Strobel, 2002a, 2002b; Strobel and Daisy, 2003).

1.5. The potential of natural products in drug discovery

Natural products are produced by all organisms but are mostly known from plants, including algae, and microorganisms including fungi and prokaryotes. Most of these organisms coexist in ecosystems and interact with each other in various ways in which often chemistry plays a major role. It has been proposed that most secondary metabolites serve the producing organisms by improving their survival fitness (Williams *et al.*, 1989). On the contrary to primary metabolites that are common in all living cells and are involved in the formation of biomass and generation of energy, secondary metabolites are often only produced by one or few species. Many are biologically active, and some of them have been used by man for thousands of years as traditional medicines and as natural poisons (Larsen *et al.*, 2005).

From a pharmaceutical point of view, there is a growing need for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity, and have a minor environmental impact. In fact, around 60% of the new drugs registered during the period 1981-2002 by the FDA as anticancer, antimigraine and anti-hypertensive agents were either natural products or based on them (Newman *et al.*, 2003). Moreover, a significant number of the top 35 worldwide selling drugs in the years 2000-2003 were natural product-derived compounds (Butler, 2004). Natural products have been the traditional pathfinder compounds, offering an untold diversity of chemical structures unparalleled by even the largest combinatorial databases. In addition, natural products often serve as lead structures whose activity can be enhanced by manipulation through combinatorial and synthetic chemistry (Strobel and Daisy, 2003). Since there are still many unexplored resources in nature, the potential for finding new organisms and thereby new metabolic pathways is also enormous.

1.6. The potential of fungal natural products in drug discovery

It was not until Alexander Fleming discovered **penicillin G** from *Penicillium notatum* almost 80 years ago (1928) that fungal microorganisms suddenly became a hunting ground for novel drug leads (Strobel and Daisy, 2003; Larsen *et al.*, 2005). Hence many pharmaceutical companies were motivated to start sampling and screening large collections of fungal strains especially for antibiotics (Butler, 2004). Microorganisms represented a promising rich source of novel natural product leads having the advantage of feasible production of large quantities with reasonable cost, by large scale cultivation and fermentation of the source organisms. About 20 years later several other antibacterial agents such as **cephalosporin C** had been

discovered (Newton and Abraham, 1955). Furthermore, **griseofulvin** was one of the first antifungal natural products found in filamentous fungi (Grove *et al.*, 1952). Recently, **echinocandin B** and pneumocandin B, isolated from *Aspergillus rugulovalvus* and *Glarea lozoyensis*, respectively, were the lead compounds and templates for the semisynthetic antifungal drugs anidulafungin (Eraxis®) and caspofungin (Cancidas®) (Butler, 2004). In addition, by the promising new screening strategy for antibiotics, aiming at inhibition of biofilm formation by Gram-negative bacteria, the quorum sensing inhibitory activity of two well known fungal mycotoxins, patulin and penicillic acid, isolated from *Aspergillus* and *Penicillium* sp., was described (Rasmussen *et al.*, 2005).

Furthermore, a new era in immunopharmacology began with the discovery of **cyclosporine**, isolated from *Tolypocladium inflatum*, in 1971. It was the first immunosuppressive drug that allowed selective immunoregulation of T cells without excessive toxicity and was used as immunosuppressant during organ transplantations (Borel and Kis, 1991; Butler, 2004). It is now widely exploited in organ and tissue transplant surgery, to prevent rejection following bone marrow, kidney, liver and heart transplants. It has revolutionized organ transplant surgery, substantially increasing survival rates in transplant patients (Dewick, 2006). Another strongly immunosuppressive fungal metabolite that is used for organ transplantations and for treatment of autoimmune diseases is **mycophenolic acid** (Cellcept®, Myfortic®) (Bentley, 2000). This compound was produced by *Penicillium*, *Aspergillus*, *Byssochlamys* and *Septoria* species (Larsen *et al.*, 2005).

Another group of fungal derived drugs are the antilipidemic statin compounds. Statins are the most potent cholesterol-lowering agents available. They are either fermentation-derived for instance mevastatin and **lovastatin** (Mevacor®), from *Penicillium citrinum* and *Aspergillus terreus*, respectively, or synthetic analogue compounds such as the major selling synthetic statins (lipitor®, crestor® and livalo®). Statins lower cholesterol by reversible competitive inhibition of the rate-limiting enzyme HMG-CoA reductase in the mevalonate pathway of cholesterol biosynthesis, thus reducing total and low-density lipoprotein cholesterol levels. As high blood cholesterol levels contribute to the incidence of coronary heart disease, statins are of potential value in treating high-risk coronary patient (Butler, 2004; Dewick, 2006). Two lipid-regulating drugs of this class, atorvastatin (lipitor®) and simvastatin (Zocor®), feature prominently in the top ten drugs by cost reflecting the widespread implementation of clinical guidelines and recommendations relating to coronary heart disease.

Traditionally, microorganisms were isolated from soil samples and explored for pharmacologically active natural products which might prove to be suitable for specific

medicinal or agrochemical applications. Extremely unusual and valuable organic substances were sometimes produced by these organisms. Nowadays, investigations of soil fungi showed a reduced hit-rate of novel compounds. Thus, in the search for new sources of therapeutic agents, marine microorganisms and endophytic fungi associated with plants were found to be a vast untapped reservoir of metabolic diversity producing a wide array of new biologically active secondary metabolites.

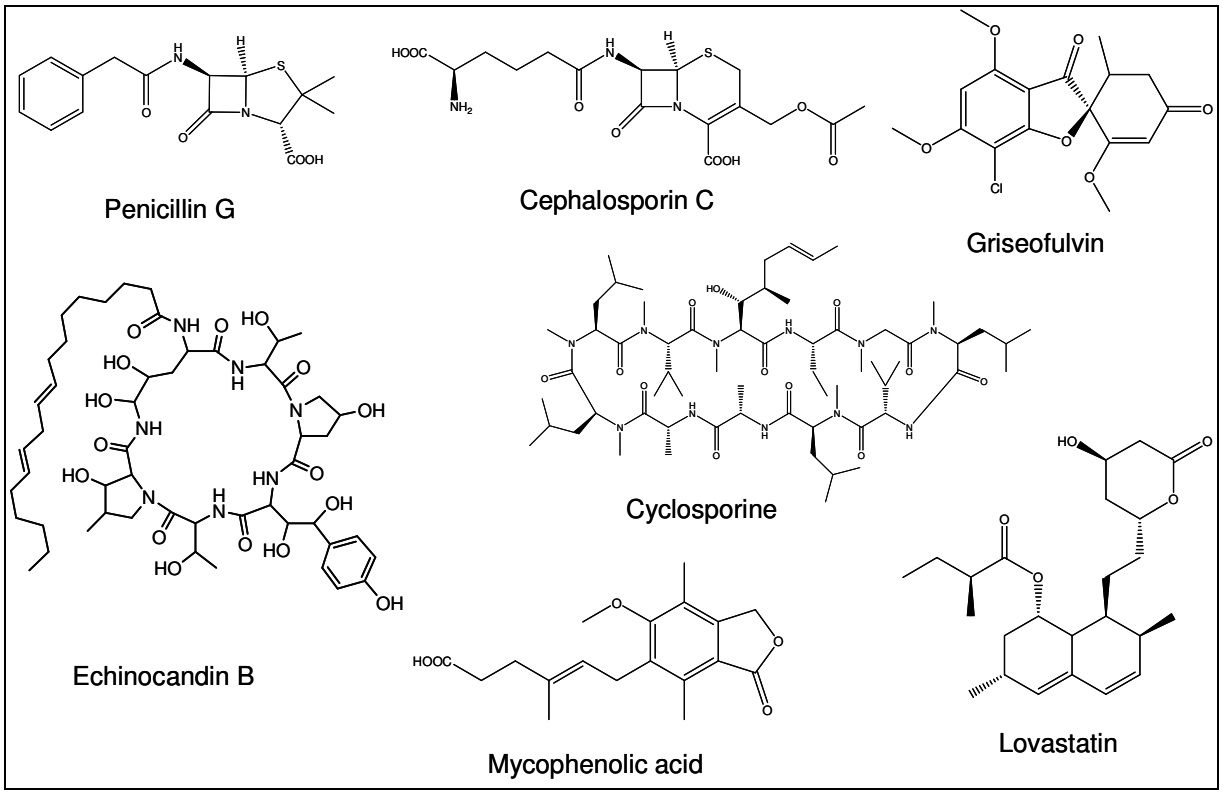


Figure 1.1: Fungal natural products as drugs or drug lead compounds.

1.7. Endophytic fungi as a source of bioactive natural products

There is growing evidence that bioactive substances produced by microbial endophytes may not only be involved in the host-endophyte relationship, but may also ultimately have applicability in medicine, agriculture and industry (Strobel, 2002a). Additionally, it is of great relevance in this context that the number of secondary metabolites produced by fungal endophytes is larger than that of any other endophytic microorganism class (Zhang *et al.*, 2006). Indeed, endophytic fungi are a very promising source of novel biologically active compounds, and have proven to yield a considerable hit-rate of novel compounds when screening larger strain numbers for biological activities (Schulz *et al.*, 2002). This may be the case because endophytes may have developed close biological

associations with and inside their hosts, leading to the production of a high number and diversity of classes of biological derived molecules with a range of biological activities. In fact, a recent comprehensive study has indicated that 51% of biologically active substances isolated from endophytic fungi were previously unknown (Stierle *et al.*, 1999; Strobel, 2002b; Weber *et al.*, 2004; Shen *et al.*, 2006). In the following part examples including novel bioactive secondary metabolites from endophytic fungi are listed according to their indications. So far, only a small percentage of these metabolites have been carried forward as natural product drugs, nevertheless they represent interesting structures which indicate the great chemical diversity and pharmaceutical potential of endophytic fungi as sources for novel drug lead compounds.

1.7.1. Secondary metabolites from endophytes as antibiotics

Even though more than 30 000 diseases are clinically described today less than one-third of these can be treated symptomatically and even a fewer can be cured. The increasing occurrence of multiresistant pathogenic strains has limited the effect of traditional antimicrobial treatment. Hence, there is an urgent need for new therapeutic agents with infectious disease control (Strobel and Daisy, 2003; Larsen *et al.*, 2005).

Guanacastepenes, exemplified by **guanacastepene A**, represent highly diverse diterpenoids produced by an unidentified endophytic fungus isolated from *Daphnopsis americana* tree. They exhibited pronounced antibiotic activity against drug-resistant strains of *Staphylococcus aureus* and *Enterococcus faecium* (Brady *et al.*, 2001). **Chaetoglobosin A** and **rhizotonic acid**, from endophytic *Chaetomium globosum*, in *Maytenus hookeri*, and *Rhizoctonia* sp., in *Cynodon dactylon*, respectively, were reported to be active against the gastric ulcer involved bacterium *Helicobacter pylori* (Tikoo *et al.*, 2000; Ma *et al.*, 2004). Moreover, **altersetin** purified from an endophytic *Alternaria* sp. displayed potent activity against pathogenic Gram-positive bacteria (Hellwig *et al.*, 2002).

1.7.2. Secondary metabolites from endophytes as antimycotic agents

Fungal infections are becoming an increasingly difficult problem as a result of the AIDS epidemic and the increased numbers of patients with organ transplants whose immune systems are weakened. Thus, new antimycotics are needed to combat these problems (Strobel, 2002a). A unique peptide antimycotic, termed **cryptocandin A**, was isolated and characterized from *Cryptosporiopsis quercina*, endophytic in *Tripterigeum wilfordii*, a medicinal plant belonging to the family Celastraceae that is native to Eurasia (Strobel *et al.*,

1999). It is currently being considered by several companies for use against a number of fungi causing diseases of skin and nails (Strobel, 2002a). Other fungal metabolites with promising antifungal activity are **ambuic acid**, described recently from several isolates of *P. microspora* found in many of the world's rainforests (Li *et al*., 2001), as well as **jesterone** and hydroxyjesterone from *Pestalotiopsis jesteri*, a newly described species of *Pestalotiopsis* (Li and Strobel, 2001). Furthermore, a new pentaketide antifungal agent, CR377, was isolated from the culture broth of an endophytic *Fusarium* sp., from the plant *Selaginella pallescens* collected in Costa Rica, and showed potent activity against *Candida albicans* in agar diffusion assays performed on fungal lawns (Brady and Clardy, 2000).

1.7.3. Secondary metabolites from endophytes as antiviral agents

The emergence of resistance and multi-resistance against available drugs, the side effects and high cost of current therapies as well as the HIV/AIDS epidemic and AIDS-associated opportunistic infections, such as cytomegalovirus and polyomavirus, made the development of novel antiviral drugs a central priority.

Cytonic acids A and **B** were reported as human cytomegalovirus protease inhibitors from the culture of the endophytic fungus *Cytonaema* sp. isolated from *Quercus* sp. (Guo *et al.*, 2000). In addition, the novel quinone-related metabolites, **xanthoviridicatins E** and **F**, produced by an endophytic *Penicillium chrysogenum* colonizing an unidentified plant, inhibited the cleavage reaction of HIV-1 integrase (Singh *et al.*, 2003).

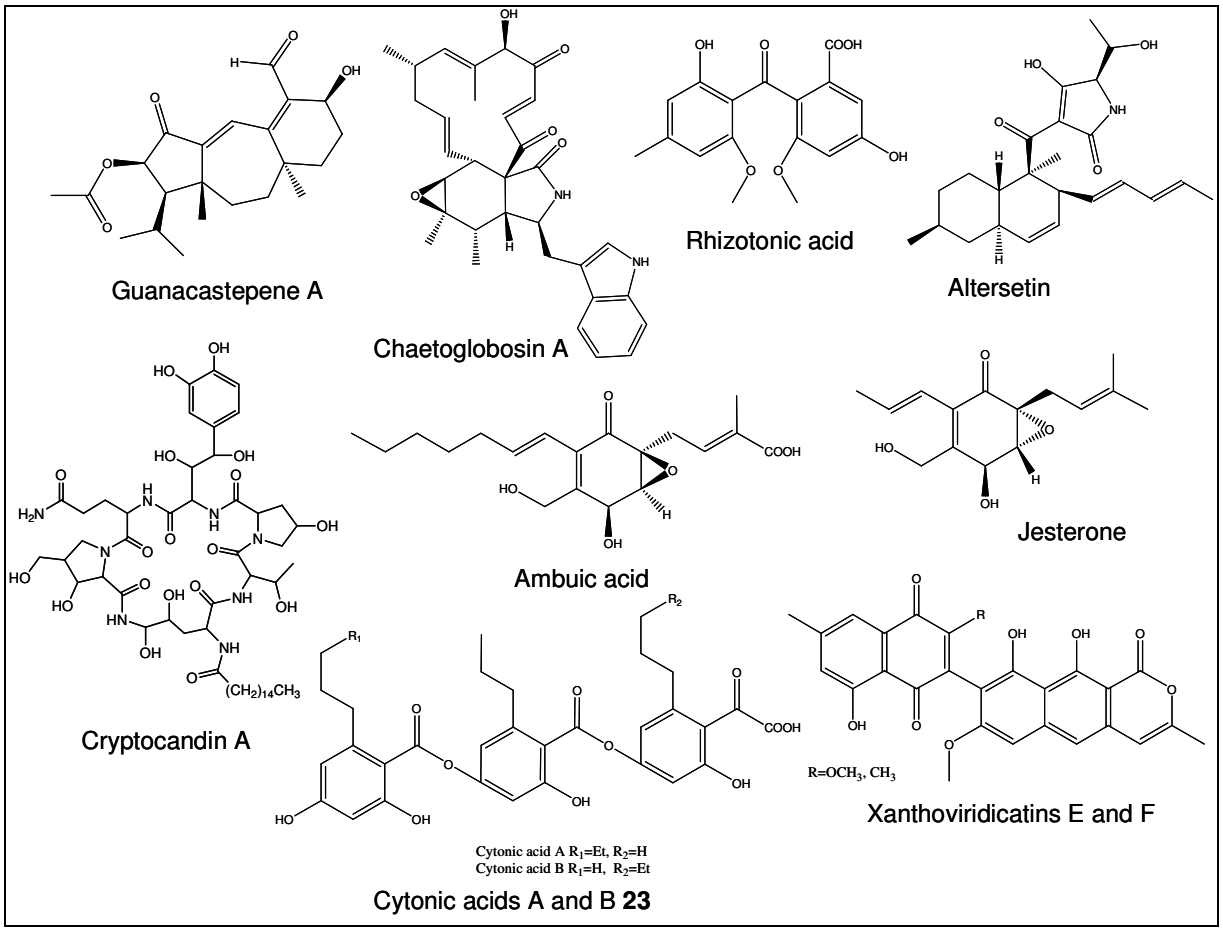


Figure 1.2: Fungal natural products with antimicrobial activity.

1.7.4. Secondary metabolites from endophytes as anticancer agents

The discovery of the **paclitaxel** (taxol®) producing endophytic fungus *Taxomyces andreanae* from *Taxus brevifolia* (Strobel *et al.*, 1993; Stierle and Strobel, 1995) evoked the interest in endophytes as potential new sources for therapeutic agents. 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. Taxol is the world's first billion dollar anticancer drug and is used to treat a number of other human tissue proliferating diseases as well (Strobel, 2002a). The mode of action of paclitaxel is to preclude tubulin molecules from depolymerizing during the processes of cell division (Schiff and Horowitz, 1980). In fact, tubulin molecules in taxol-sensitive plant pathogenic fungi were found to be affected in the same manner as human cancer cells, which indicated that taxol, in nature, may provide a defensive role for the yew tree (*Taxus* sp.) from which it originates (Young *et al.*, 1992). Similarly, paclitaxel has been reported to induce a reversible polymerization of plant tubulin into microtubules, albeit weakly when compared to that of mammalian tubulin (Morejohn and

Fosket, 1984; Bokros *et al.*, 1993). Further examination of the endophytes of *T. wallichiana* yielded *Pestalotiopsis microspora* which was found to produce taxol as well.

By the finding that many other endophytic fungi such as *P. microspora* (Strobel *et al.*, 1996) and *Periconia* sp. (Li *et al.*, 1998), residing in plants other than *Taxus* species were also producing taxol, it appeared that fungi more commonly produce taxol than higher plants, and the distribution of those fungi is worldwide and not confined to endophytes of yews. Thus, it may be that taxol had its origin in certain fungi and ultimately, if there is lateral gene transfer, it may have been in the direction of the microbe to the higher plant. Unfortunately, taxol production upon fermentation by all endophytes investigated so far is only in the range of submicrograms to micrograms per liter. Considerable efforts are being made to determine the feasibility of producing taxol by fermentation, in much the same way as penicillin, which would effectively reduce its market price (Strobel, 2002a; Strobel and Daisy, 2003).

Moreover, the cytotoxic plant alkaloid, camptothecin, originally described from *Camptotheca acuminate* and *Nothapodytes foetida*, and undergoing clinical trials since 1992 as anticancer drug, was identified in cultures of *Entrophospora infrequens* endophytic in *Nothapodytes foetida* (Amna *et al.*, 2006). Another anticancer drug, which has been given in chemotherapy treatment for some types of cancer including leukemia, lymphoma, breast and lung cancer for many years, is the indole derivative **vincristine**. This drug, available under the trade names Oncovin®, Vincasar®, and Vincrex®, was originally obtained from *Catharanthus roseus*. Very recently, a Chinese group reported preliminary evidence that vincristine might be produced by *Fusarium oxysporum* endophytic in the same plant (Zhang *et al.*, 2006).

On the other hand, endophytic fungi were found to produce interesting bioactive metabolites not related to the natural products produced by their host plants. For example, **chaetomellic acids A** and B, isolated from the culture of an endophytic *Chaetomella acutisea*, were found to be specific inhibitors of farnesyl-protein transferase (Lingham *et al.*, 1993; Ishii *et al.*, 2000). Inhibitors of this enzyme prevent posttranslational modification of Ras proteins, which serve as central connectors between signals generated at the plasma membrane and nuclear effectors, thus disrupting the Ras signaling pathway as well as Ras-dependent proliferative activity in cancerous and precancerous lesions (Kelloff *et al.*, 1997). A similar activity was observed for the new metabolites preussomerin N_1, palmarumycin CP_{4a}, and palmarumycin CP_5 produced by an endophytic *Coniothyrium* sp. (Tan and Zou, 2001). Moreover, **microcarpalide**, a microfilament disrupting agent with weak cytotoxicity to

mammalian cells, was characterized from fermentation broths of an unidentified endophytic fungus (Ratnayake *et al.*, 2001).

A further example is the relatively large group of alkaloids known as cytochalasins. Many of these compounds, possessing antitumor and antibiotic activities, were found in endophytic fungi, but because of their cellular toxicity they have not been developed into pharmaceuticals (Wagenaar *et al.*, 2000). Chaetoglobosins are fungal metabolites belonging to the family of cytochalasins. Some chaetoglobosins have been isolated recently from endophytic *Chaetomium globosum* and were shown to exhibit cytotoxic activities against the human nasopharyngeal epidermoid tumour KB cell line (Vesely *et al.*, 1995; Zhang *et al.*, 2006).

1.7.5. Secondary metabolites from endophytes with further interesting pharmacological activities

As mentioned above, 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-dependent diabetes (Strobel and Daisy, 2003). Interestingly, compounds showing immunosuppressive activity were also obtained from endophytic fungi, for example **subglutinols A** and B, which are noncytotoxic diterpene pyrones produced by *Fusarium subglutinans*, an endophyte of *Triptergium wilfordii*. In the mixed lymphocyte reaction assay the subglutinols were roughly as potent as cyclosporine (Lee *et al.*, 1995b).

L-783,281, is a quinine produced by the plant associated fungus *Pseudomassaria* sp. This compound was found to lower blood glucose level in diabetic mice. Thus, the compound mimics the action of the polypeptide hormone insulin, and unlike insulin, it was not destroyed by enzymes in the digestive tract and may be given orally (*Chem. Eng. News*, 2000).

Pestacin and isopestacin, were separated from *Pestalotiopsis microspora* associated with *Terminalia morobensis*. The compounds were able to scavenge superoxide and hydroxyl free radicals in solution. The antioxidant activity of pestacin is at least one order of magnitude higher than that of trolox, a vitamin E derivative (Harper *et al.*, 2003). Two cerebrosides with xanthine oxidase inhibitory activity were identified from an endophytic *Fusarium* sp. (Shu *et al.*, 2004). Aurasperone A, from *Aspergillus niger*, an endophytic fungus obtained from *Cynodon dactylon*, is also a xanthine oxidase inhibitor (Song *et al.*, 2004).

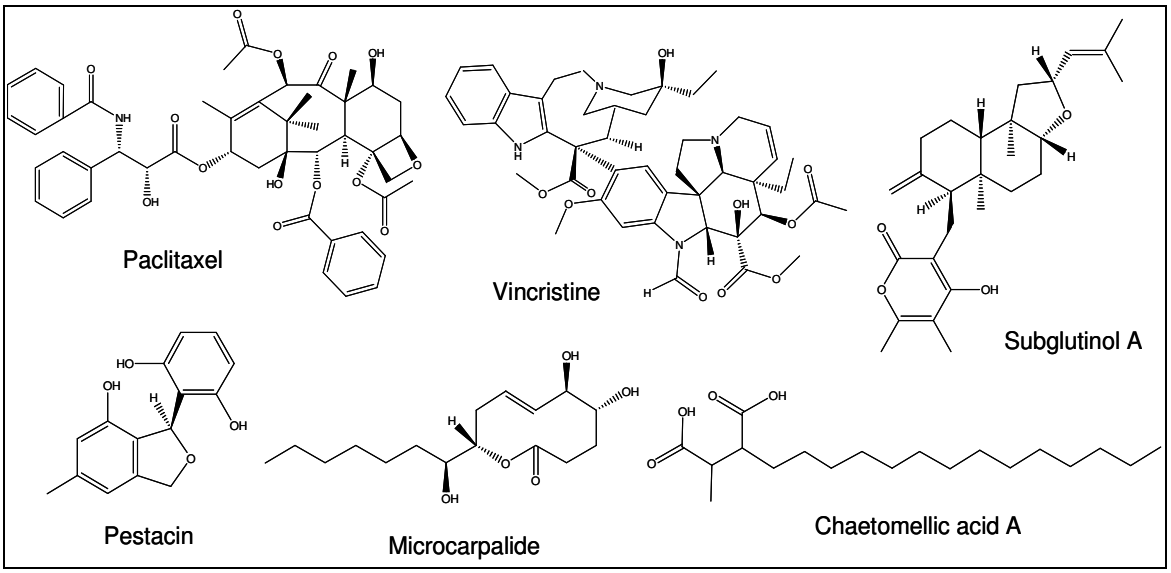


Figure 1.3: Fungal natural products with anticancer, immunosuppressive and antioxidant activities.

1.8. The potential of microbial natural products in agriculture

As the world becomes wary of ecological damage provoked by extensive use of synthetic insecticides, natural product research continues for the discovery of powerful, selective, and safe alternatives (Strobel and Daisy, 2003). Many synthetic agricultural agents have been and currently are being targeted for removal from the market, because of profound harmful effects on human health and environment. Thus, perhaps endophytic fungi could serve as a reservoir of untapped biologically based compounds that may present alternative ways to control farm pests and pathogens (Demain, 2000; Strobel, 2002a). One interesting finding consisted in the discovery of **peramine**, which was toxic to insects without any harmful impact on mammals. This secondary metabolite was characterized in cultures of *Neotyphodium coenophialum*, *N. lolli*, *Epichloë festucae* and *E. typhina* associated with tall fescue, ryegrass and other grasses (Dew *et al.*, 1990). **Nodulisporic acids** were isolated from a *Nodulisporium* sp. endophytic in *Bontia daphnoides*. They were found to exhibit potent insecticidal properties against the larvae of the blowfly (Demain, 2000). Another endophytic fungus, *Muscodor vitigenus* isolated from *Paullina paullinioides*, was found to yield naphthalene as its major product. **Heptelidic acid** and hydroheptelidic acid, from *Phyllosticta* sp. an endophytic fungus of *Abies balsamea*, have been shown to be toxic to spruce bud worm (*Choristoneura fumiferana*) larvae (Calhoun *et al.*, 1992).

Furthermore, several fungal metabolites were inhibitory to the growth of selected crop phytopathogenic fungi. One example is the unique tetramic acid, known as **cryptocin**, which was produced by *Cryptosporiopsis quercina* endophytic in the medicinal plant *Tripterigeum*

wilfordii. It showed potent activity against *Pyricularia oryzae*, causal agent of rice blast, one of the most important plant diseases on earth, and is currently being examined as a natural chemical control agent for rice blast (Li *et al.*, 2000). Some of the first reported sesquiterpenes produced by fungal endophytes were **chokols A-**G. They were isolated from an endophytic *Epichloë typhina*, from *Phleum pretense*, and were found to be fungitoxic to the leaf spot disease pathogen *Cladosporium phlei* (Koshino *et al.*, 1989).

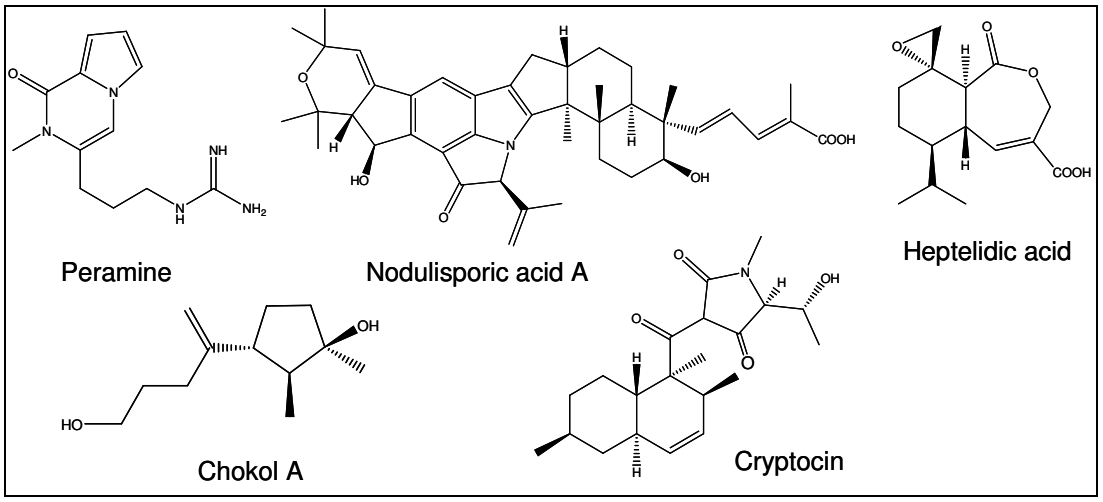


Figure 1.4: Fungal natural products with agricultural potential.

1.9. Aim and scopes of the study

Being poorly investigated, endophytes are obviously a rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential. The aim of this study was the purification of endophytic fungal strains from Egyptian medicinal plants, the isolation, characterization and structure elucidation of biologically active secondary metabolites from the extracts of these endophytic fungal strains, and the preliminary evaluation of their pharmaceutical potential. Four endophytic fungi, *Alternaria* sp., *Ampelomyces* sp., *Stemphylium botryosum* and *Chaetomium* sp., were subjected as biological sources of the study.

In order to isolate the secondary metabolites, the fungi were grown in static liquid Wickerham medium as well as solid rice medium at room temperature. The cultures were allowed to grow for 3-4 weeks, followed by harvesting and subsequent extraction with organic solvents. The obtained raw extracts were then fractionated and separated using various chromatographic techniques and their fractions were analysed by HPLC-DAD for their purity and ESI-LC/MS for their molecular weight and fragmentation patterns. The pure compounds were submitted to state-of-the-art one- and two-dimensional NMR techniques for structure elucidation. In addition, selected compounds were derivatized in order to determine their absolute stereochemistry.

Furthermore, fractions and pure compounds were subjected to selected bioassays to determine their pharmaceutical potential. Thus, antimicrobial activity was studied using the agar diffusion assay as well as the biofilm test, whereas cytotoxicity was studied *in vitro* using mouse lymphoma (L5178Y) cell line. Moreover, fractions and pure compounds were also tested for their protein kinase inhibitory activity. The latter three assays were conducted in cooperation with Prof. U. Hentschel, Würzburg, Prof. W. E. G. Müller, Mainz, and ProQinase, Freiburg, respectively.

Finally, extracts were prepared from the corresponding host plants and fractionated, and the obtained fractions were analyzed by HPLC and LC/MS for the presence of the identified fungal metabolites. The samples were then reanalyzed parallel to the pure substances and retention times as well as MS/MS spectra were compared.

2. Materials and Methods

2.1. Materials

2.1.1. Biological materials

2.1.1.1. Plant material

Plant samples were collected from different areas in Alexandria, Egypt. Voucher specimens were identified by Prof. Dr. Amin El-Sayed Ali, Department of Crops, Faculty of Agriculture, Alexandria University, and Prof. Dr. Rafiq El-Gharib Mahmoud, Department of Botany, Faculty of Science, Alexandria University. Small stem, leaf and flower pieces were cut from the plants and placed in plastic bags after any excess moisture was removed. Every attempt was made to store the materials at 4° C until isolation procedures could be instituted.

2.1.1.2. Pure fungal strains isolated from the collected plants

Table 2.1 shows a list of the endophytic fungal strains isolated from different organs of the collected plant samples and their corresponding botanical sources.

Table 2.1: Pure fungal strains and their botanical sources

Fungal code	Plant part	Source
I7L1 I7L2	leaf	*Chenopodium album* (Amaranthaceae)
II2L1 II2L2 II2L3 II2L4	leaf	*Polygonum senegalense* (Polygonaceae)
II3F1 II3F2 II3F3 II3F4 II3F5 II3F6	flower	*Solanum nigrum* (Solanaceae)
II3S	stem	
III3S2 III3S3 III3S4	stem	*Plantago major* (Plantaginaceae)
III3L1 III3L2	leaf	

Fungal code	Plant part	Source
IV16L	leaf	*Euphorbia helioscopia* (Euphorbiaceae)
V2L V2S1 V2S2	leaf stem	*Otanthus maritimus* (Asteraceae)
VI1F1 VI1F2 VI1F3 VI1F4 VI1S VI1L	flower Stem leaf	*Urospermum picroides* (Asteraceae)
VI2F1 VI2F2 VI2S1 VI2S2 VI2S3 VI2S4 VI2S5	flower stem	*Aegialophila cretica* (Asteraceae)

2.1.2. Media

2.1.2.1. Composition of malt agar (MA) medium

MA medium was used for short term storage of fungal cultures or fresh seeding for preparation of liquid cultures.

Agar-agar	15.0 g
Malt extract	15.0 g
Distilled water	to 1000 mL
pH	7.4 - 7.8 (adjusted with NaOH/HCl)

For the isolation of endophytic fungi from plant tissues chloramphenicol or streptomycin (0.2 or 0.1 g, respectively) were added to the medium to suppress bacterial growth.

2.1.2.2. Composition of Wickerham medium for liquid cultures

Yeast extract	3.0 g
Malt extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Distilled water	to 1000 mL
pH	7.2 - 7.4 (adjusted with NaOH/HCl)

2.1.2.3. Composition of rice medium for solid cultures

Rice	100 g
Distilled water	100 mL

Water was added to the rice and kept overnight before autoclaving.

2.1.2.4. Composition of Luria Bertani (LB) medium

This medium was used to conduct antibacterial assays.

Peptone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Distilled water	To 1000 mL
pH	7.0 (adjusted with NaOH/HCl)

To prepare the agar plates, 15.0 g agar were added to 1 L broth media.

2.1.2.5. Composition of yeast medium

This medium was used to perform bioassays using *Saccharomyces cerevisiae*.

Peptone	5.0 g
Yeast extract	3.0 g
Malt extract	3.0 g
Glucose	10.0 g
Distilled water	To 1000 mL

To prepare the agar plates, 15.0 g agar were added to 1 L broth media.

2.1.2.6. Composition of fungal medium for bioassay

Mannitose	50.0 g
Saccharose	50.0 g
Succinic acid	5.4 g
Yeast extract	3.0 g
KH_2PO_4	0.1 g
$MgSO_4$	0.3 g
$FeSO_4$	10.0 mg
$ZnSO_4$	10.0 mg
Distilled water	To 1000 mL

pH	5.4 (adjusted with NaOH/HCl)

2.1.2.7. Composition of potato dextrose agar (PDA) medium for bioassay

Potato infusion (see below)	1000 mL
Dextrose	20.0 g
Agar	15.0 g

Potato infusion: The potatoes (200 g) were first washed and cut into small pieces, then boiled in 1000 mL distilled water for 1 hour and filtered to get the potato infusion.

2.1.2.8. Composition of trypticase soy broth (TSB)

Peptone from casein	17.0 g
Peptone from soymeal	3.0 g
Glucose	2.5 g
NaCl	5.0 g
K_2HPO_4	2.5 g
Distilled water	To 1000 mL
pH	7.3 (adjusted with NaOH/HCl)

2.1.3. Chemicals

2.1.3.1. General laboratory chemicals

Anisaldehyde (4-methoxybenzaldehyde)	Merck
(-)-2-Butanol	Merck
Dimethylsulfoxide	Merck
Formaldehyde	Merck
L-(+)-Ascorbic acid	Merck
Hydrochloric acid	Merck
Potassium hydroxide	Merck
Pyridine	Merck
Concentrated sulphuric acid	Merck
Trifloroacetic acid (TFA)	Merck
Concentrated ammonia solution	Fluka

Acetic anhydride	Merck
Ortho-phosphoric acid 85% (p.a.)	Merck
Sodium hydrogen carbonate	Sigma
Trifluroacetic acid (TFA)	Merck

2.1.3.2. Chemicals for culture media

Agar-agar	Galke
Chloramphenicol	Sigma
Glucose	Caelo
Malt extract	Merck
NaCl	Merck
Peptone	BD
Streptomycin	Sigma
Yeast extract	Sigma

2.1.3.3. Chemicals for agarose gel electrophoresis

Agarose	Serva
TBE-buffer	Merck
Ethidium bromide	Serva
Standards	NEB

2.1.4. Chromatography

2.1.4.1. Stationary phases

Pre-coated TLC plates, Silica Gel 60 F_{254}, layer thickness 0.2 mm	Merck
Silica Gel 60, 0.04 - 0.063 mm mesh size	Merck
Pre-coated TLC plates , RP-18, F_{254} S, layer thickness 0.25 mm	Merck
RP-18, 0.04 - 0.063 mm mesh size	Merck
Sephadex LH 20, 0.25 - 0.1 mm mesh size	Merck
Diaion HP20	Supelco

2.1.4.2. Spray reagents

The reagents were stored in amber-colored bottles and kept refrigerated until use. TLC was used to monitor the identity of each of the fractions and the qualitative purity of the isolated compounds. It was also utilized to optimize the solvent system that would be applied for column chromatography.

Anisaldehyde/H_2SO_4 Spray Reagent

Methanol	85 mL
Glacial acetic acid	10 mL
Conc. H_2SO_4	5 mL (added slowly)
Anisaldehyde	0.5 mL

Vanillin/H_2SO_4 Spray Reagent

Methanol	85 mL
Conc. H_2SO_4	15 mL (added slowly)
Vanillin	1 g

2.1.5. Solvents

2.1.5.1. General solvents

Acetone, acetonitrile, dichloromethane, ethanol, ethyl acetate, n-hexane and methanol were used. The solvents were purchased from the Institute of Chemistry, University of Duesseldorf. They were distilled before using and special grades were used for spectroscopic measurements.

2.1.5.2. Solvents for HPLC

Acetonitrile	LiChroSolv HPLC grade (Merck)
Methanol	LiChroSolv HPLC grade (Merck)
Nanopure water	distilled and heavy metals free water obtained by passing distilled water through nano- and ion-exchange filter cells (Barnstead, France)

2.1.5.3. Solvents for optical rotation

Chloroform	Spectral grade (Sigma)
Methanol	Spectral grade (Sigma)
Water	Spectral grade (Fluka)

2.1.5.4. Solvents for NMR

Acetone-d_6	Uvasol, Merck
Chloroform-d	Uvasol, Merck
DMF-d_7	Uvasol, Merck
DMSO-d_6	Uvasol, Merck
Methanol-d_4	Uvasol, Merck
Pyridine-d_5	Uvasol, Merck

2.2. Methods

2.2.1. Purification of fungal strains

Plant materials were cut into small pieces, washed with sterilized demineralized water, then thoroughly surface treated with 70% ethanol for 1-2 minutes and ultimately air dried under a laminar flow hood. This is done in order to eliminate surface contaminating microbes. With a sterile scalpel, outer tissues were removed from the plant samples and the inner tissues were carefully dissected under sterile conditions and placed onto malt agar plates containing antibiotic (see section 2.1.2.1). After 3-4 weeks of incubation at room temperature, hyphal tips of the fungi were removed and transferred to fresh malt agar medium. Plates are prepared in duplicates to eliminate the possibility of contamination. Pure strains were isolated by repeated inoculation (see Figure 2.1).

2.2.2. Cultivation of pure fungal strains

2.2.2.1. Cultivation for short term storage

Fungi were grown on malt agar medium under room temperature for several days. When fungal hyphae almost cover the surface of the MA plate, cultures were stored at 4º C for a maximum period of 6 months, and then re-inoculated onto fresh MA media.

2.2.2.2. Cultivation for screening and isolation of secondary metabolites

Mass growth of pure fungi for screening as well as isolation and identification of secondary metabolites was carried out by transferring fresh fungal cultures into Erlenmeyer flasks (1L each) containing 300 mL of Wickerham medium for liquid cultures or 100 g rice for solid cultures. The cultures were then incubated at room temperature (no shaking) for 21 and 30 days, respectively. Large scale cultivation was carried out using 30 and 10 1L Erlenmeyer flasks for liquid and solid rice cultures, respectively.

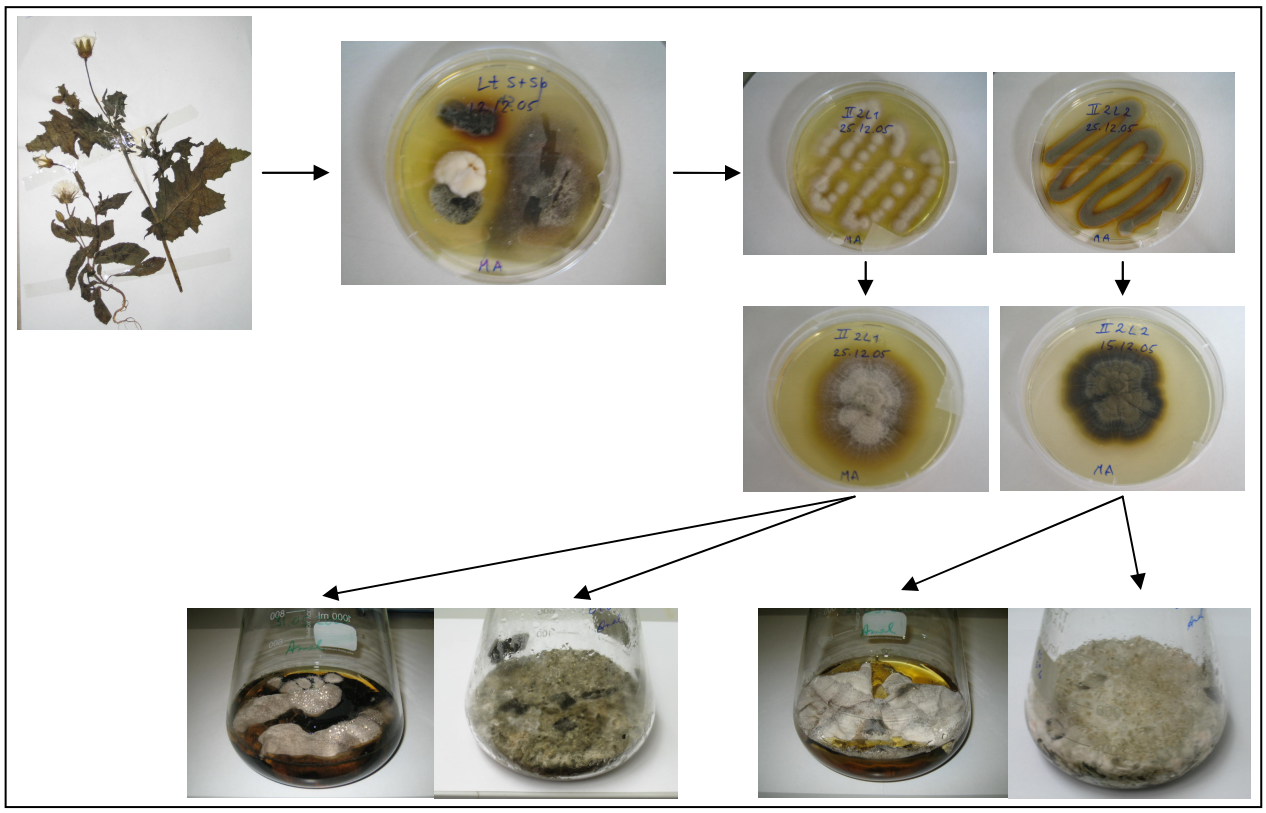


Figure 2.1: Isolation, purification and cultivation of fungal strains

2.2.3. Extraction of fungal cultures and host plant material

2.2.3.1. Extraction of fungal liquid cultures

2.2.3.1.1. Total extraction of culture media and mycelia

250 mL EtOAc were added to each Erlenmeyer flask containing 300 mL culture medium and left overnight to stop cell growth. Culture media and mycelia were then extracted in the Ultraturrax for 10 min for cell destruction, followed by vacuum filtration using Buchner. The mycelium residue was discarded while culture filtrates were collected and

extracted with EtOAc and *n*-BuOH till exhaustion. The combined EtOAc phases were washed with distilled water and then taken to dryness. The dry residue was then partitioned between *n*-hexane and 90% MeOH. The extraction scheme is described in Figure 2.2.

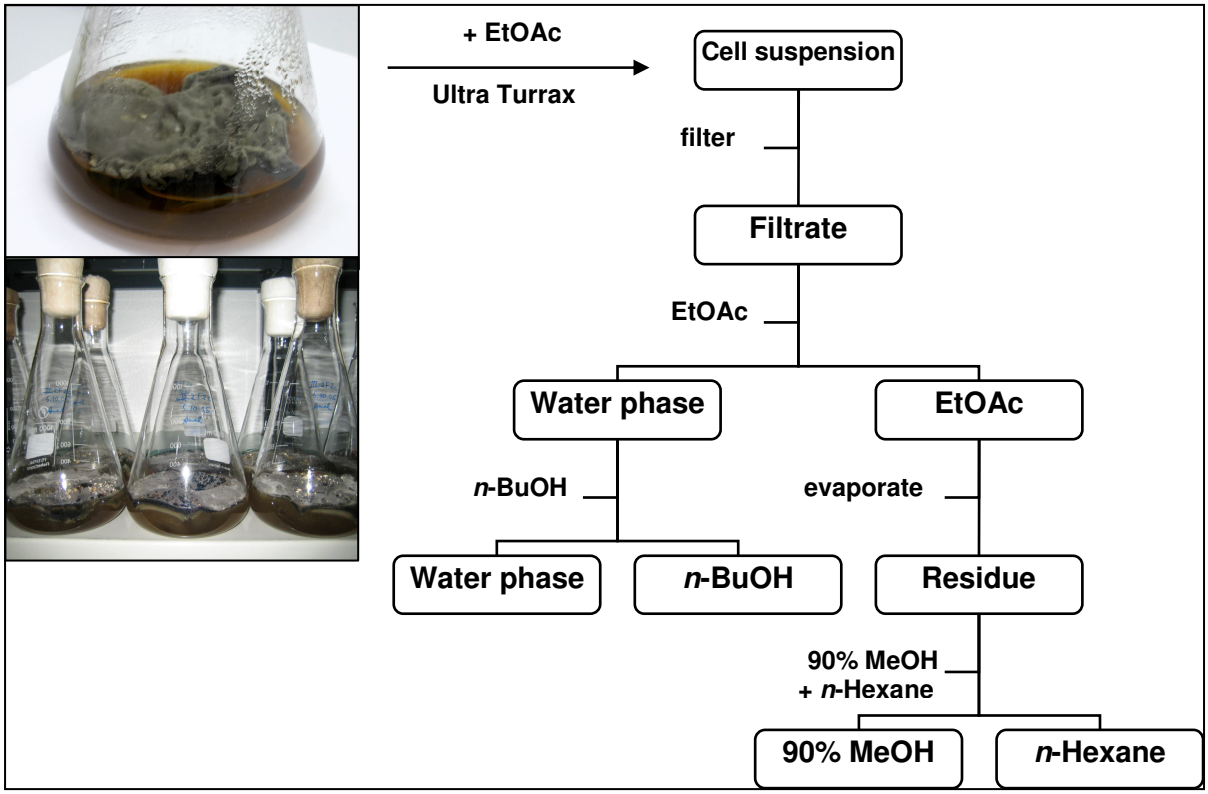


Figure 2.2: Total extraction of culture media and mycelia

2.2.3.1.2. Separate extraction of culture media and mycelia

Fungal mycelia were seperated from culture media and left in MeOH overnight. Using Ultraturrax cells were destructed and extracted for 10 min, followed by filtration and repeated extraction till exhaustion. The culture media were extracted in the same manner as described above in 2.2.3.1.2 to obtain the EtOAc extract. The extraction scheme is described in Figure 2.3.

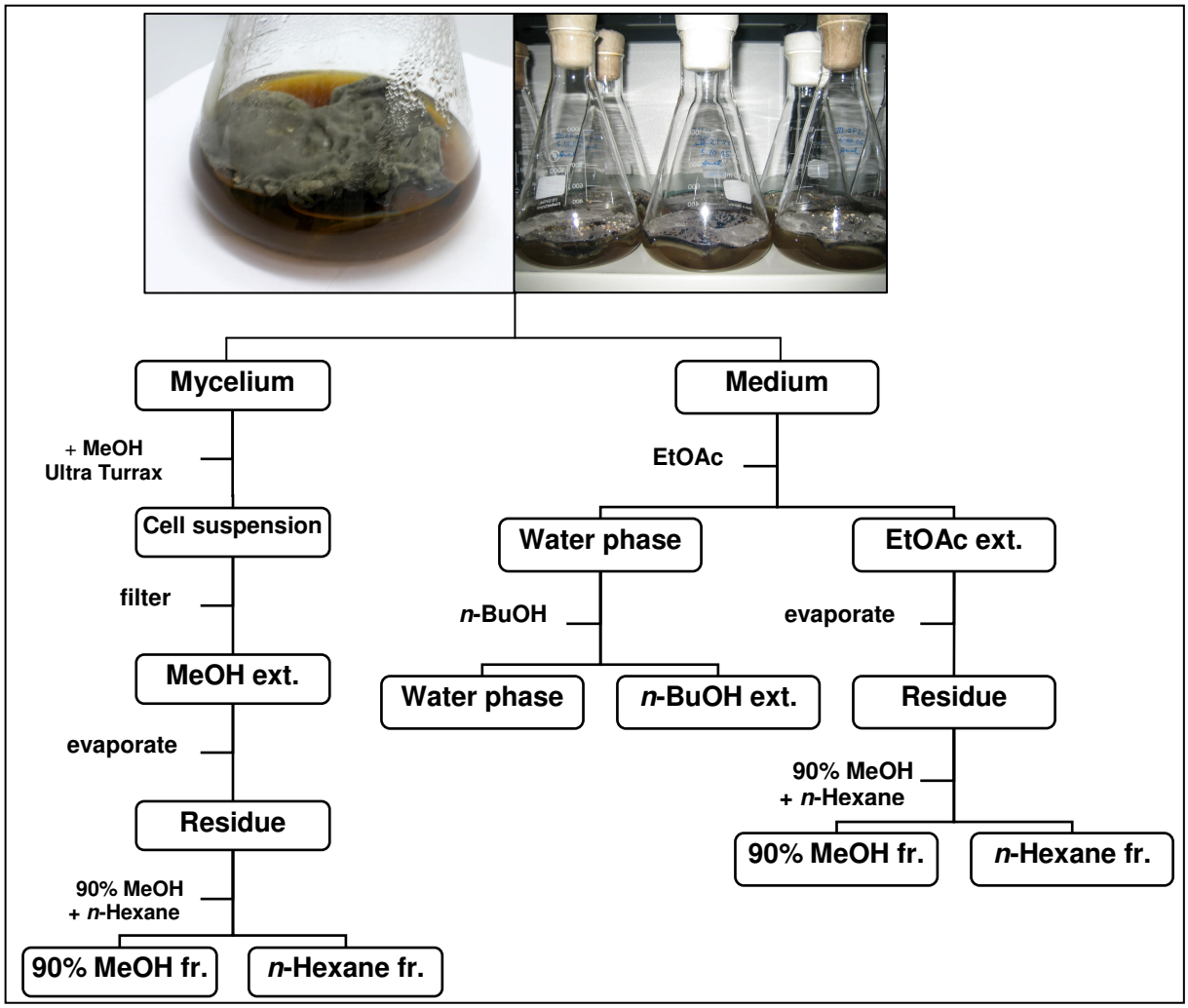


Figure 2.3: Separate extraction of culture media and mycelia

2.2.3.2. Extraction of solid rice cultures

250 mL EtOAc were added to the cultures and left overnight. Culture media were then cut in pieces to allow complete extraction and left for 3–5 days. Then filtration was done followed by repeated extraction with EtOAc and MeOH till exhaustion. The combined EtOAc phases were washed with distilled water and then taken to dryness. The dry residues obtained from EtOAc and MeOH extracts were partitioned between *n*-hexane and 90% MeOH. The extraction scheme is described in Figure 2.4.

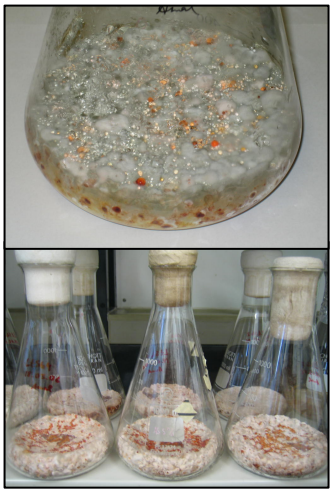


Figure 2.4: Extraction of solid rice media

2.2.3.3. Extraction and fractionation of the plant material

The plant samples were frozen at - 80° C. The freeze dried samples were extracted with 90% MeOH overnight with shaking and the resulting extracts were dried. The dried residues were subjected to partitionation between *n*-hexane and 90% MeOH. The 90% MeOH soluble fractions was fractionated over Diaion HP-20 using H_2O:MeOH and MeOH:acetone gradient elution and the obtained fractions were analysed by HPLC and LC/MS.

2.2.3.4. Solvent-solvent extraction

Solvent-solvent extraction is a widely employed technique to separate organic compounds from a mixture. It involves the separation of compounds into two immiscible solvents. Since the technique is based upon an unequal distribution of solutes between two solvents with different polarities, the solutes will be more soluble in one solvent compared to the other. The distribution of a component A between two phases can be expressed by the distribution coefficient (K):

$$K = \frac{[A]_{\text{top phase}}}{[A]_{\text{lower phase}}}$$

where, [A] is the concentration of solute A.

The following general principles should be considered in choosing the solvents:

- the solvents involved in the extraction must be immiscible
- the solvents must not react with the components that will be separated
- the solvents should be easily removed by evaporation after the process

In this study, solvent-solvent extraction was the first step in the separation process. It was meant to “clean” the ethyl acetate extract from salts and other undesirable polar constituents

by water-ethyl acetate extraction. Subsequently, the methanol-*n*-hexane extraction was applied to remove fatty acids and other undesirable non polar components.

2.2.4. Identification of fungal strains and their taxonomy

2.2.4.1. Fungal identification

Fungal strains were identified using a molecular biological protocol by DNA amplification and sequencing of the internal transcribed spacer (ITS) region. This was carried out by Ine Dewi Indriani and Arnulf Diesel at the Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf.

DNA isolation

Fungal DNA isolation and purification was performed using DNeasy® Plant Mini Kit (QIAgen). The lyophilized fungal mycelia were pulverized and disrupted with the help of glass beads. Then cell lysis was carried out by addition of lysis Buffer AP-1 and RNAse-A solution followed by incubation of the mixture at 65º C. The remaining detergent, protein and polysaccharide were precipitated by addition of Buffer AP-2 to the lysate. The lysate was then applied to the Qiashredder™ Mini Spin Column and centrifuged to remove the cell debris and other remaining precipitates. The lysate was then transferred to a new tube.

An adequate volume of ethanolic Buffer AP3/E was added to the lysate and the mixture was then applied to DNeasy Mini Spin Column. After centrifugation, the filtrate was discarded. The column was washed by addition of ethanolic Buffer AW followed by centrifugation. Another portion of Buffer AW was added to the column and centrifuged at maximum speed to dry the membrane in the column from residual ethanol.

Fungal DNA, which is incorporated into the membrane, was eluted by addition of Buffer AE directly to the membrane in the DNeasy column. The column was then incubated at room temperature for 5 minutes and then centrifuged to collect the filtrate, which was the fungal DNA dissolved in Buffer AE.

DNA amplification

The isolated DNA was then amplified by Polymerase Chain Reaction (PCR). The PCR was carried out using HotStarTaq Master Mix Kit (QIAgen). The Master Mix contains HotStarTaq®DNA Polymerase, PCR buffer (with $MgCl_2$) and dNTPs.

ITS 1 (with base sequences TCCGTAGGTGAACCTGCGG) and ITS 4 (with base sequences TCCTCCGCTTATTGATATGC) (Invitrogen), as primers, were mixed with HotstarTaq Master Mix Kit and DNA template. Thus, each PCR reaction mixture contained 5-10 ng of genomic DNA, 1 µM each of the primers ITS 1 and ITS 4, and 1 U of Hot start Taq-Polymerase (Invitrogen) in a total volume of 50 µL. The mixture was then applied to the thermal cycler (BioRad) using the programmed PCR cycle as outlined below:

- Initial activation step in 95º C for 15 minutes to activate HotStarTaq®DNA Polymerase
- Cycling steps which were repeated 35 times:

  Denaturing: 1 minute at 95º C, annealing: 1 minute at 56º C, extension: 1 minute at 72º C
- Final extension for 10 minutes in 72º C

Purification of PCR products and DNA sequencing

The PCR product was purified using 2% Agarose-Gel-Electrophoresis at 75 V for 60 minutes in TBE buffer. The agarose gel was then stained using 1% ethidium bromide. A 500 bp stained DNA fragment was then excised from the agarose gel. The next step of PCR product purification was performed using Perfectprep® Gel Cleanup Kit (Eppendorf). The binding buffer was mixed to the PCR product and incubated at 50º C for 10 minutes in an eppendorf thermomixer at 1000 rpm. The mixture was mixed with a volume of isopropanol and then centrifuged. The filtrate was discarded and the column was washed with wash buffer twice followed by centrifugation.

Amplified fungal DNA (PCR product), which was incorporated into the column, was eluted by addition of elution buffer or molecular biology grade water to the centre of the column. The column was then centrifuged to collect the filtrate, which was the fungal DNA dissolved in elution buffer. The amplified fungal DNA was then submitted for sequencing by a commercial service and the base sequence was compared with publicly available databases such as GenBank with the help of Blast-Algorithmus.

2.2.4.2. Taxonomy

***Alternaria* sp.**

The fungus *Alternaria* sp. was isolated from fresh leaves of wildly growing *Polygonum senegalense* (Polygonaceae) (see Figure 2.5). The plant was collected in April 2004 from Alexandria, Egypt.

Taxonomy

Phylum	Ascomycota;
Subphylum	Pezizomycotina;
Class	Dothideomycetes;
Order	Pleosporales;
Family	Pleosporaceae;
Genus	*Alternaria*;
Species	*Alternaria* sp.

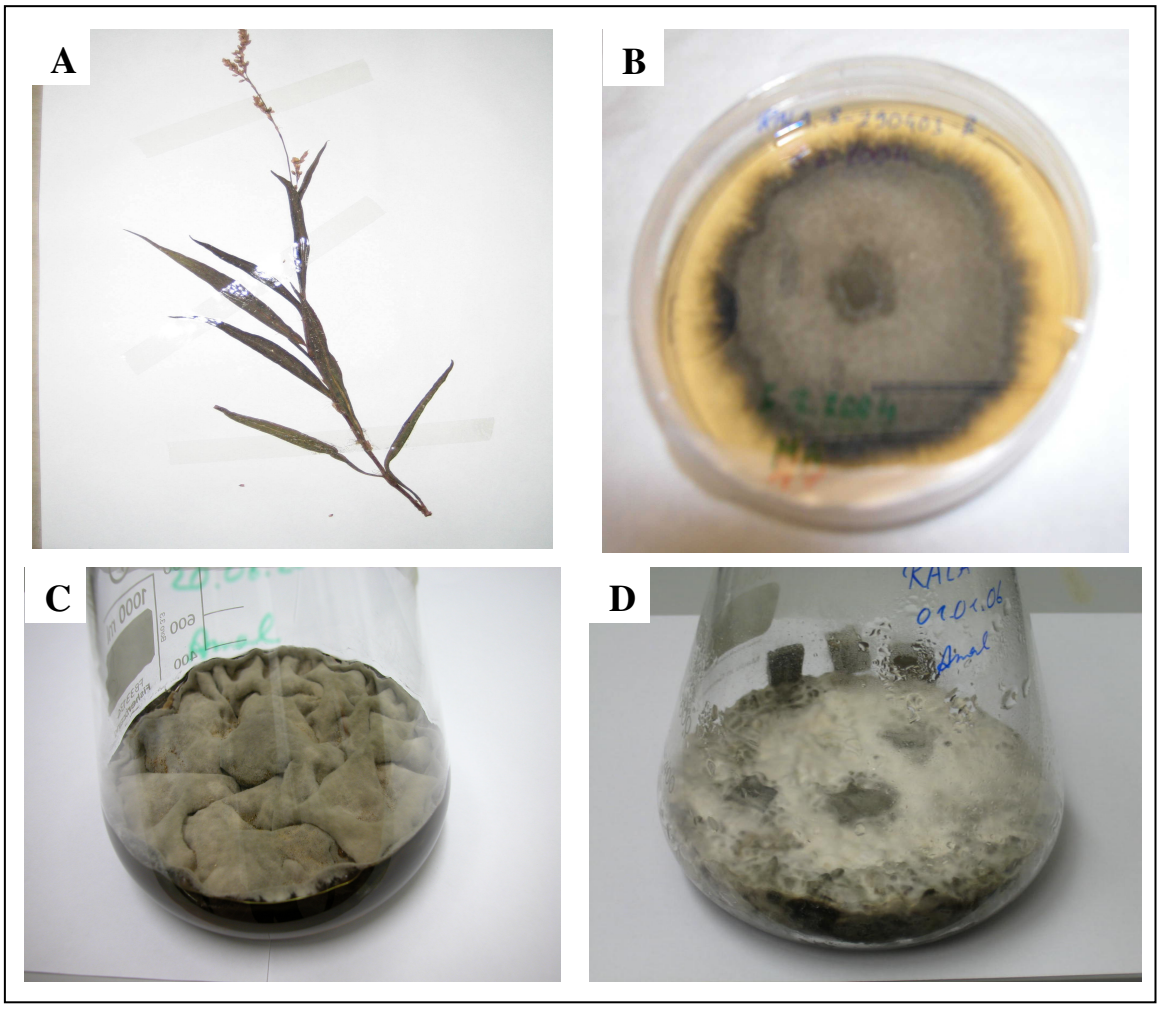


Figure 2.5: *Alternaria* sp. (**A**: *Polygonum senegalense*. **B**: Pure strain on malt agar plate. **C**: Liquid culture in Wickerham medium. **D**: Rice culture).

***Ampelomyces* sp.**

The fungus *Ampelomyces* sp. was isolated from fresh flowers of wildly growing *Urospermum picroides* (Asteraceae) (see Figure 2.6). The plant was collected in April 2004 from Alexandria, Egypt.

Taxonomy

Phylum	Ascomycota;
Subphylum	Pezizomycotina;
Class	Dothideomycetes;
Order	Pleosporales;
Family	Leptosphaeriaceae;
Genus	*Ampelomyces*;
Species	*Ampelomyces* sp.

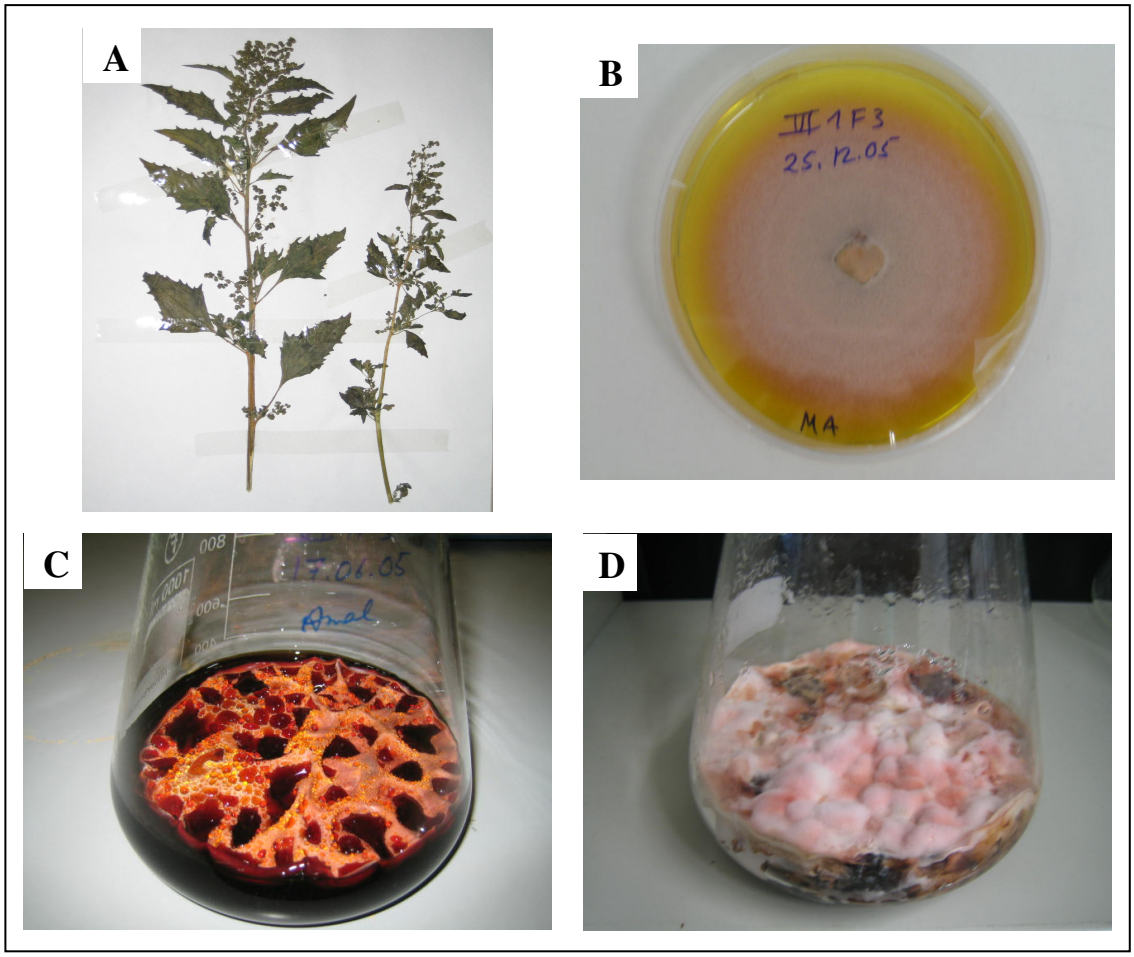


Figure 2.6: *Ampelomyces* sp. (**A**: *Urospermum picroides*. **B**: Pure strain on malt agar plate. **C**: Liquid culture in Wickerham medium. **D**: Rice culture).

Stemphylium botryosum

The fungus *Stemphylium botryosum* was isolated from fresh leaves of wildly growing *Chenopodium album* (Amaranthaceae) (see Figure 2.7). The plant was collected in April 2004 from Alexandria, Egypt.

Taxonomy

Phylum	Ascomycota;
Subphylum	Pezizomycotina;
Class	Dothideomycetes;
Order	Pleosporales;
Family	Pleosporaceae;
Genus	*Stemphylium*;
Species	*Stemphylium botryosum*.

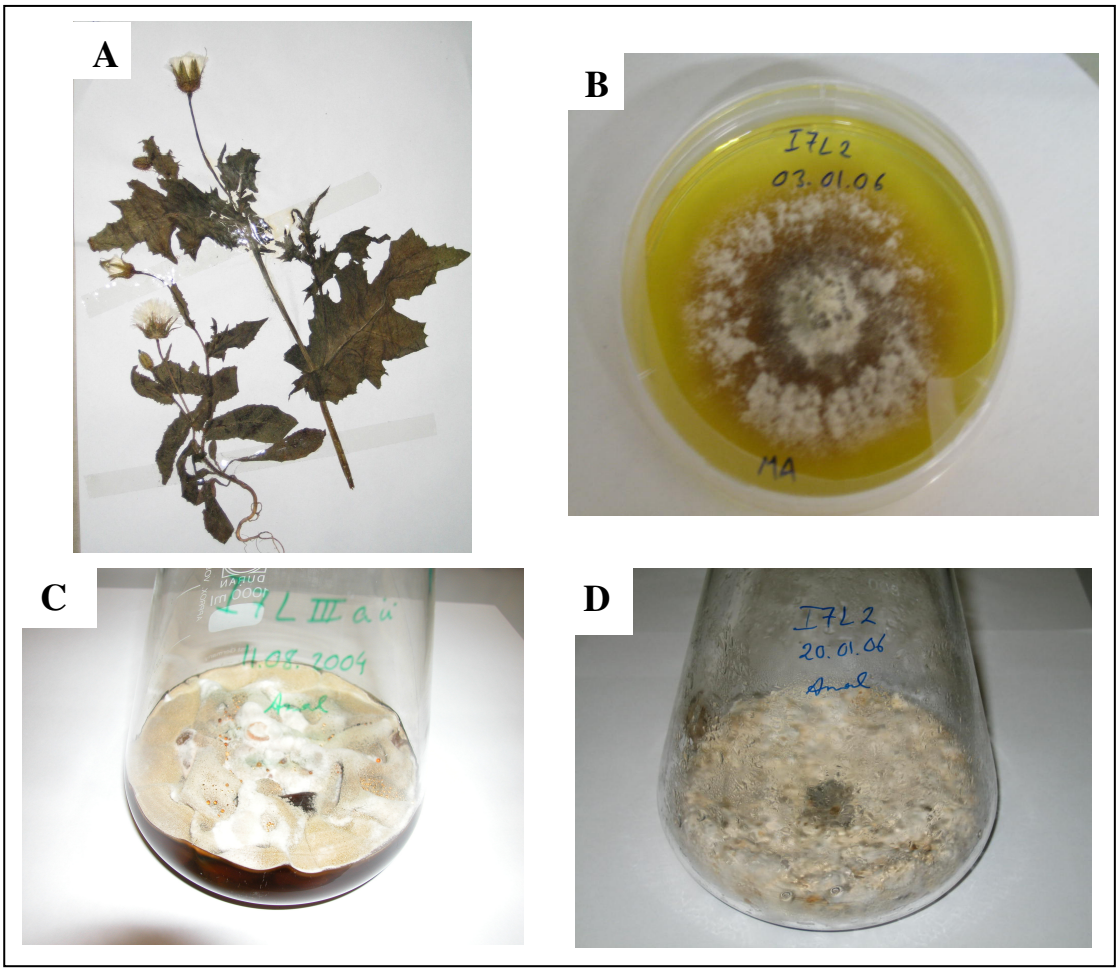


Figure 2.7: *Stemphylium botryosum*. (**A**: *Chenopodium album*. **B**: Pure strain on malt agar plate. **C**: Liquid culture in Wickerham medium. **D**: Rice culture).

***Chaetomium* sp.**

The fungus *Chaetomium* sp. was isolated from fresh stems of wildly growing *Otanthus maritimus* (Asteraceae) (see Figure 2.8). The plant was collected in April 2004 from Alexandria, Egypt.

Taxonomy

Phylum	Ascomycota;
Subphylum	Pezizomycotina;
Class	Sordariomycetes;
Order	Sordariales;
Family	Chaetomiaceae;
Genus	*Chaetomium*;
Species	*Chaetomium* sp.

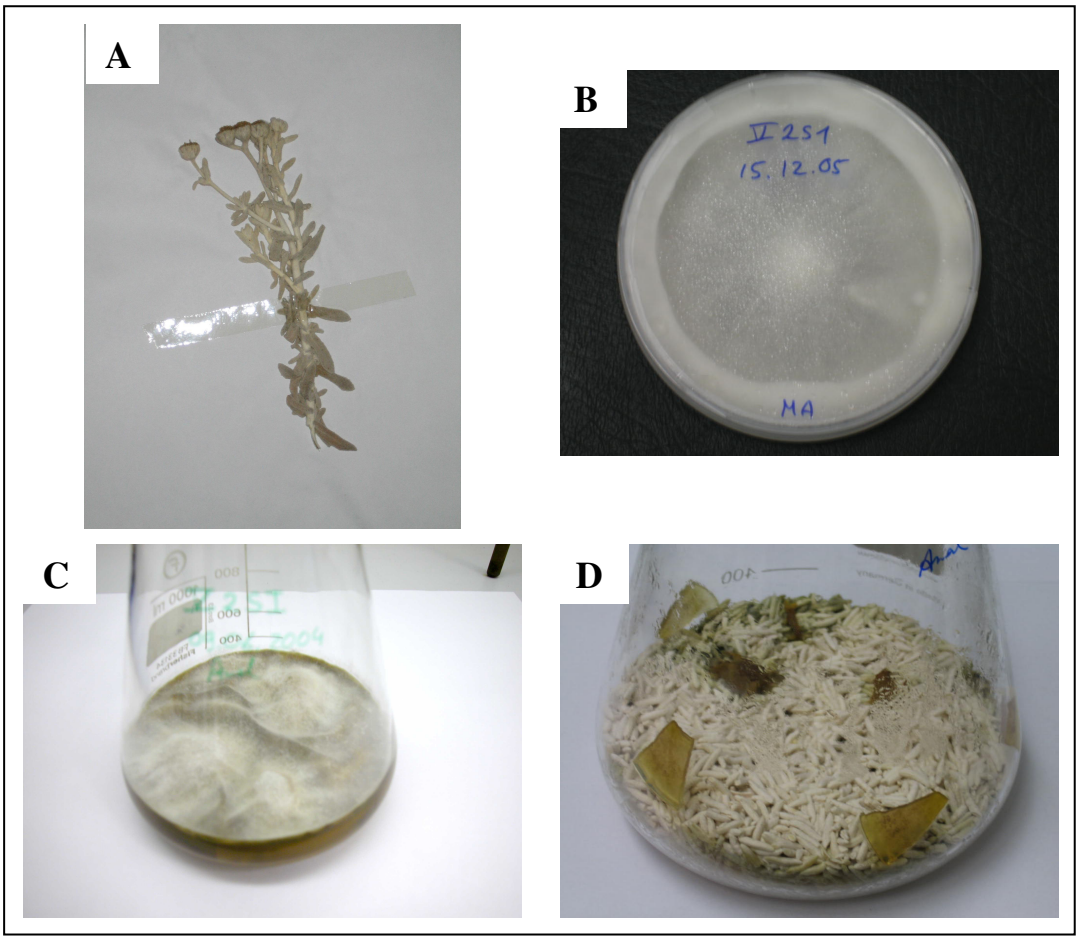
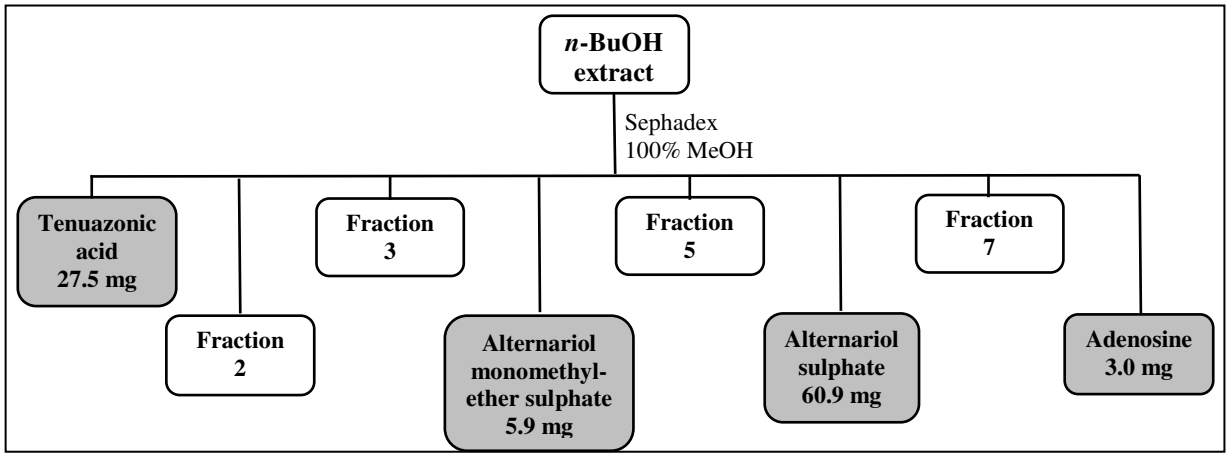


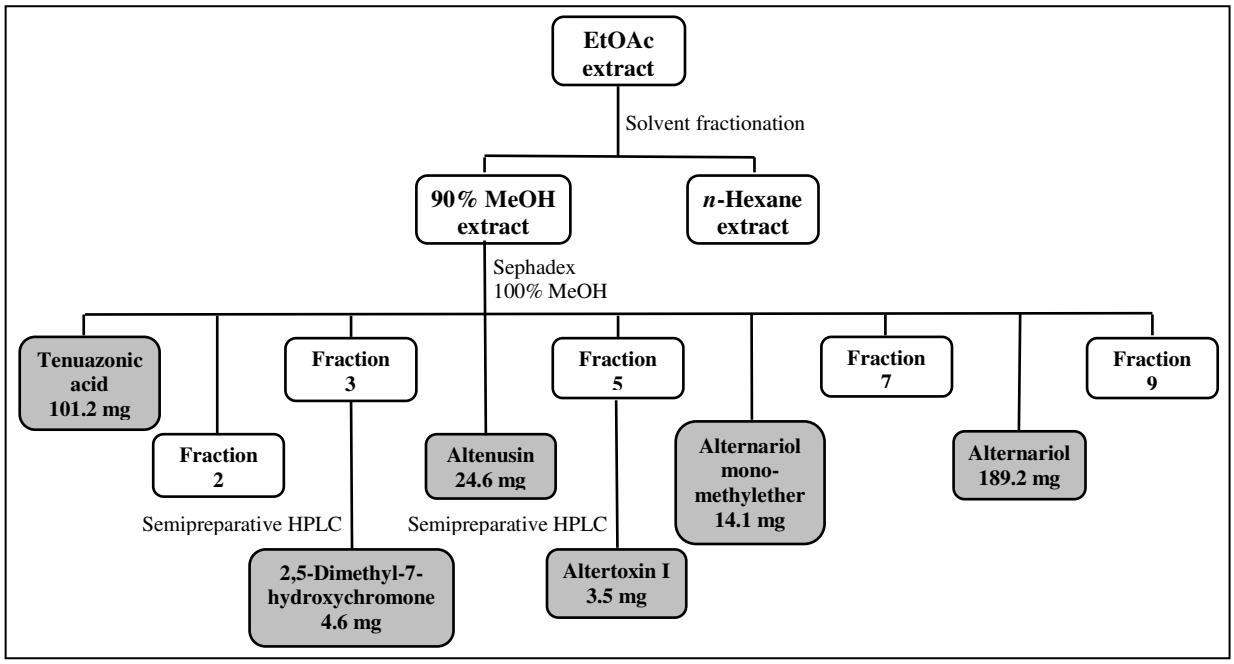
Figure 2.8: *Chaetomium* sp. (**A**: *Otanthus maritimus*. **B**: Pure strain on malt agar plate. **C**: Liquid culture in Wickerham medium. **D**: Rice culture).

2.2.5. Isolation and purification of secondary metabolites

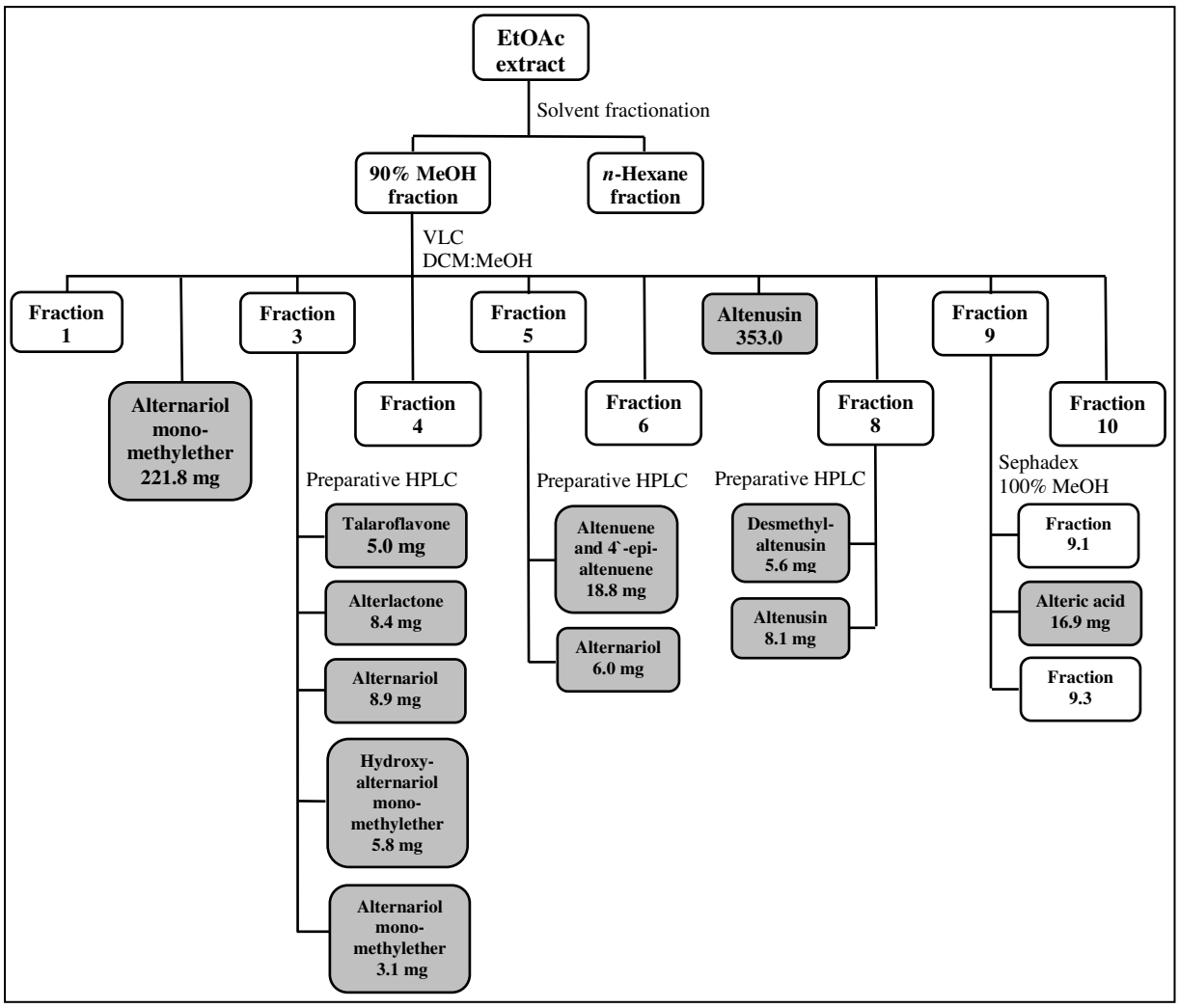
2.2.5.1. Isolation of the secondary metabolites from *Alternaria* sp.

2.2.5.1.1. Secondary metabolites isolated from liquid cultures of *Alternaria* sp.



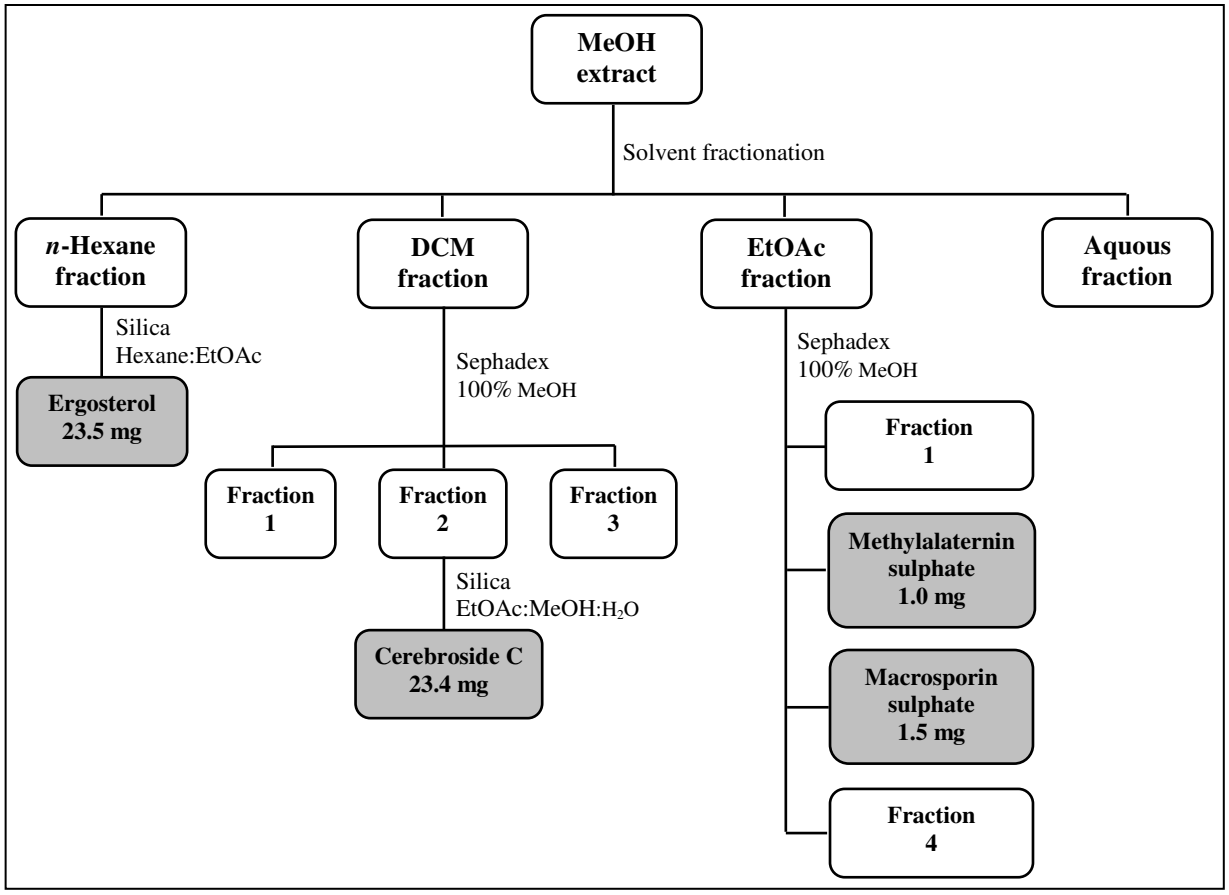


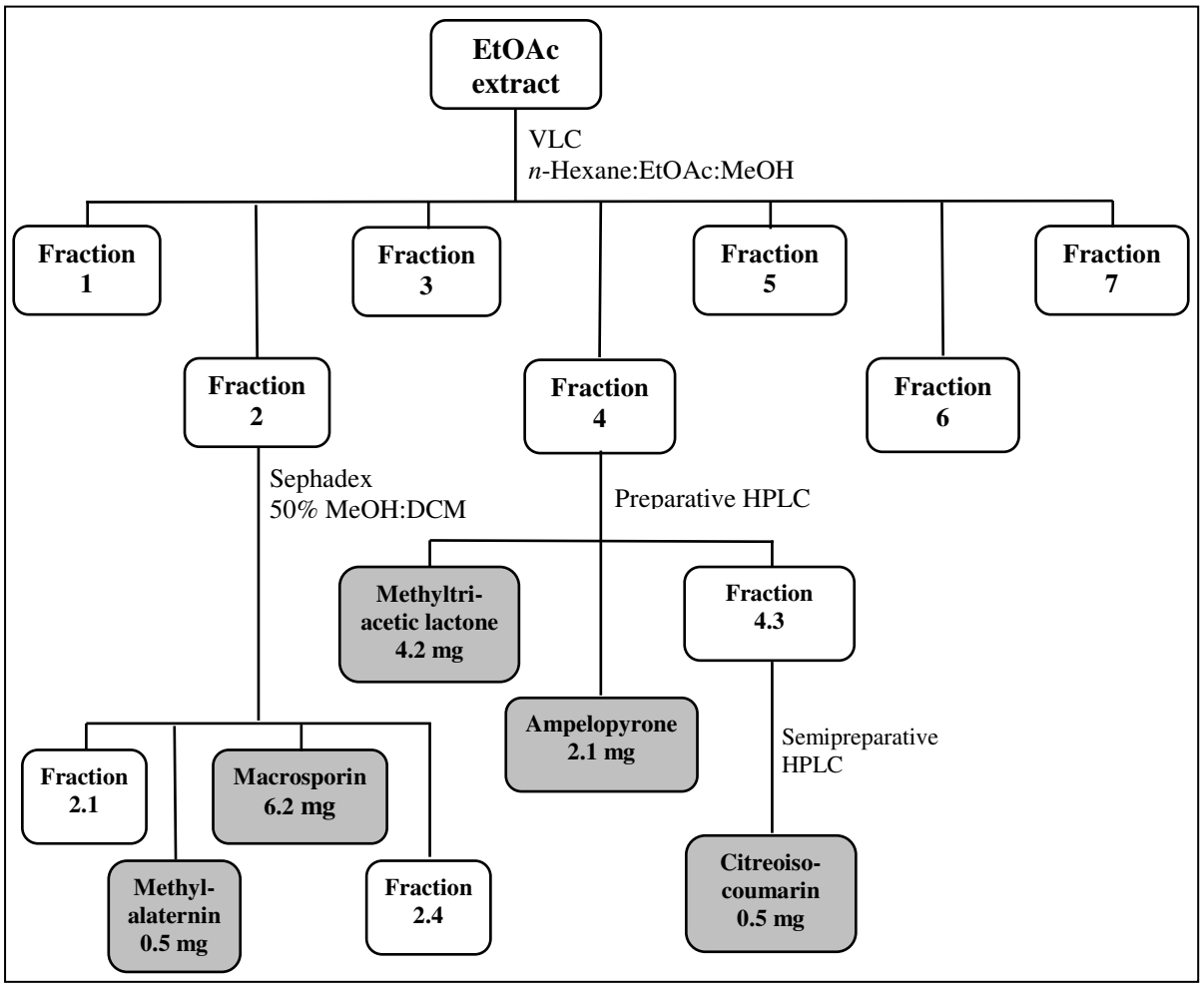
2.2.5.1.2. Secondary metabolites isolated from rice cultures of *Alternaria* sp.



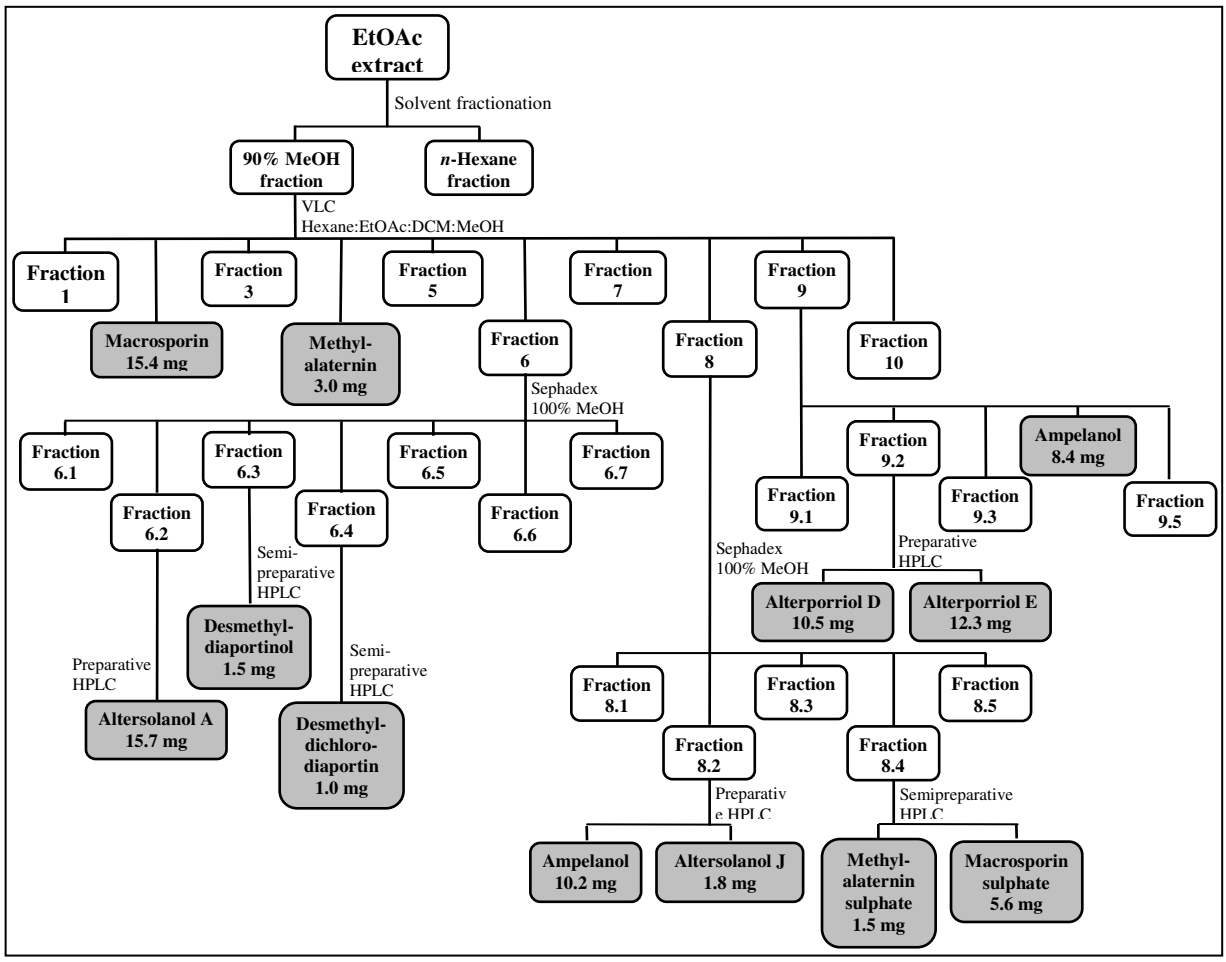
2.2.5.2. Isolation of the secondary metabolites from *Ampelomyces* sp.

2.2.5.2.1. Secondary metabolites isolated from liquid cultures of *Ampelomyces* sp.



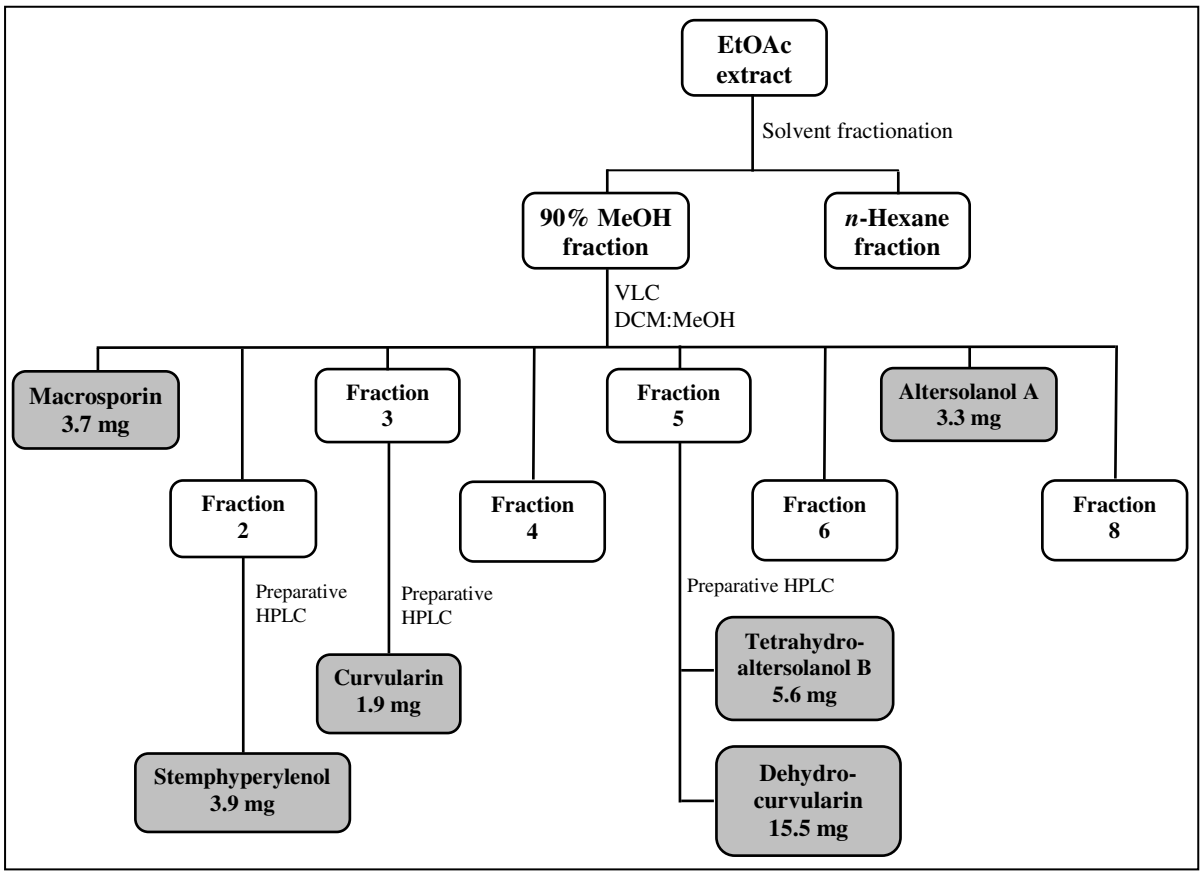

EtOAc extract
VLC
n-Hexane:EtOAc:MeOH
Fraction 1
Fraction 3
Fraction 5
Fraction 7
Fraction 2
Fraction 4
Fraction 6
Sephadex
50% MeOH:DCM
Preparative HPLC
Methyltri-acetic lactone 4.2 mg
Fraction 4.3
Ampelopyrone 2.1 mg
Semipreparative HPLC
Fraction 2.1
Macrosporin 6.2 mg
Methyl-alaternin 0.5 mg
Fraction 2.4
Citreoiso-coumarin 0.5 mg

2.2.5.2.2. Secondary metabolites isolated from rice cultures of *Ampelomyces* sp.



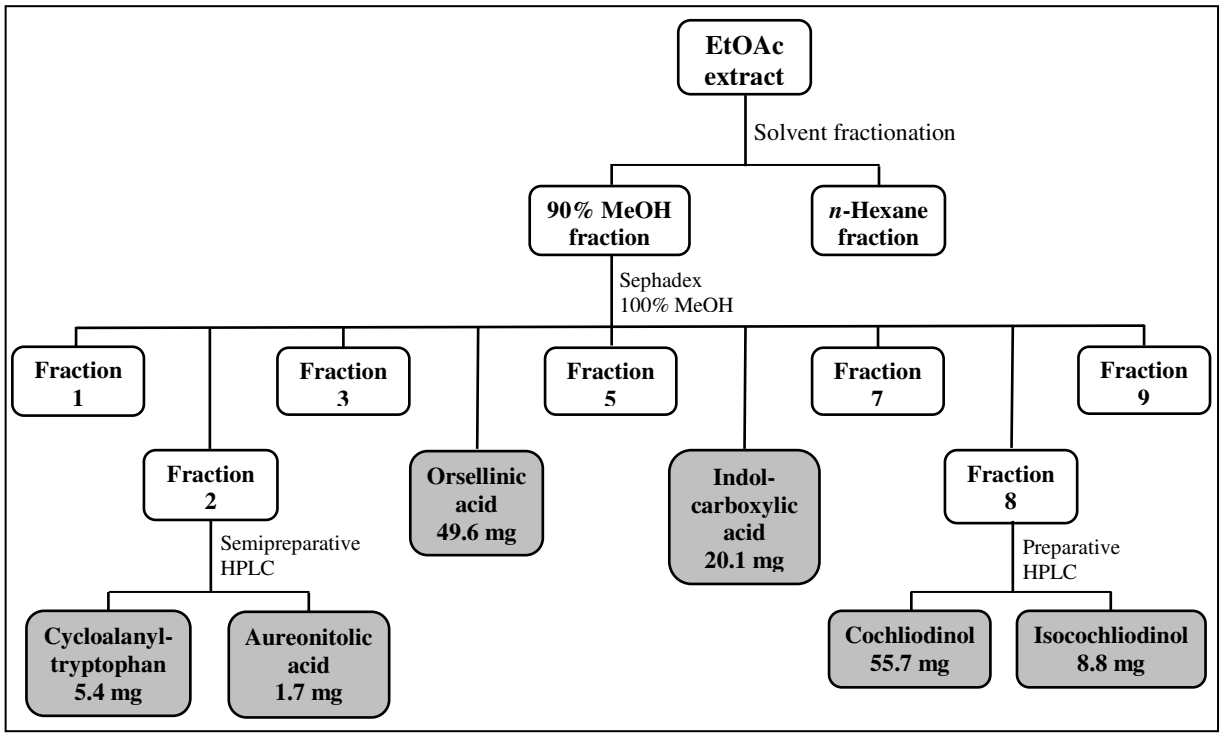
2.2.5.3. Isolation of the secondary metabolites from *Stemphylium botryosum*

Secondary metabolites isolated from rice cultures of *Stemphylium botryosum*



2.2.5.4. Isolation of the secondary metabolites from *Chaetomium* sp.

Secondary metabolites isolated from liquid cultures of *Chaetomium* sp.



2.2.5.5. Chromatographic methods

2.2.5.5.1. Thin layer chromatography (TLC)

Chromatography refers to any separation method in which the components are distributed between stationary phase and mobile phase. The separation occurs because sample components have different affinities for the stationary and mobile phases and therefore move at different rates along the TLC plates and the column. TLC was performed on pre-coated TLC plates with silica gel 60 F_{254} (layer thickness 0.2 mm, E. Merck**,** Darmstadt, Germany) with the following eluents:

For polar compounds	EtOAc:MeOH:H_2O (30:5:4, 30:6:5 and 30:7:6)
For semi-polar compounds	DCM:MeOH (95:5, 90:10, 85:15, 80:20 and 70:30)
	DCM:MeOH:EtOAc (90:10:5 and 80:20:10)
For non-polar compounds	*n*-Hexane:EtOAc (95:5, 90:10, 85:15, 80:20 and 70:30)
	n-Hexane:MeOH (95:5 and 90:10)

TLC on reversed phase RP18 F_{254} (layer thickness 0.25 mm, Merck, Darmstadt, Germany) was used for polar substances and using the different solvent systems of MeOH:H_2O (90:10, 80:20, 70:30 and 60:40). The band separation on TLC was detected under UV lamp at 254 and 366 nm, followed by spraying the TLC plates with anisaldehyde/H_2SO_4 or vaniline/H_2SO_4 reagent and subsequent heating at 110 °C.

2.2.5.5.2. Vacuum liquid chromatography (VLC)

Vacuum liquid chromatography is a useful method as an initial isolation procedure for large amounts of sample. The apparatus consists of a 500 cm sintered glass filter funnel with an inner diameter of 12 cm. Silica gel 60 was packed to a hard cake at a height of 5-10 cm under applied vacuum. The sample used was adsorbed onto a small amount of silica gel using volatile solvents. The resulting sample mixture was then packed onto the top of the column. Using step gradient elution with non-polar solvent (e.g. *n*-Hexane or DCM) and increasing amounts of polar solvent (e.g. EtOAc or MeOH) successive fractions were collected. The flow was produced by vacuum and the column was allowed to run dry after each fraction collected.

2.2.5.5.3. Column chromatography

Fractions derived from VLC were subjected to repeated separation through column chromatography using appropriate stationary and mobile phase solvent systems previously determined by TLC. The following separation systems were used:

I. Normal phase chromatography using a polar stationary phase, typically silica gel, in conjunction with a non-polar mobile phase (e.g. *n*-Hexane, DCM) with gradually increasing amounts of a polar solvent (e.g. EtOAc or MeOH). Thus hydrophobic compounds elute more quickly than do hydrophilic compounds.

II. Reversed phase (RP) chromatography using a non polar stationary phase and a polar mobile phase (e.g. H_2O, MeOH). The stationary phase consists of silica packed with n-alkyl chains covalently bound. For instance, C-8 signifies an octanyl chain and C-18 an octadecyl ligand in the matrix. The more hydrophobic the matrix on each ligand, the

greater the tendency of the column to retain hydrophobic moieties. Thus hydrophilic compounds elute more quickly than do hydrophobic compounds. Elution was performed using H_2O with gradually increasing amounts of MeOH.

III. Size exclusion chromatography involves separations based on molecular size of compounds being analyzed. The stationary phase consists of porous beads (Sephadex LH-20). The larger compounds will be excluded from the interior of the bead and thus will elute first. The smaller compounds will be allowed to enter the beads and elute according to their ability to exit from the small sized pores they were internalized through. Elution was performed using MeOH or MeOH:DCM (1:1).

IV. Ion exclusion chromatography uses ion exchange resin beds (Diaion HP-20) that act as a charged solid separation medium. The components of the processed sample have different electrical affinities to this medium and are, as a result, differently retained by the resins due to these different affinities. Therefore, by elution, these components can be recovered separately at the outlet of the resins bed. Elution was performed using H_2O with gradually increasing amounts of MeOH and acetone.

2.2.5.5.4. Flash chromatography

Flash chromatography is a preparative column chromatography based on optimized prepacked columns and an air pressure driven eluent at a high flow rate. It is a simple and quick technique widely used to separate a variety of organic compounds. Normally, the columns are dry Silica Gel 60 GF_{254} pre-packed, of 18 cm height, vertically clamped and assembled in the system. The column is filled and saturated with the desired mobile phase just prior to sample loading. Samples are dissolved in a small volume of the initial solvent used and the resulting mixture was then packed onto the top of the column using special syringe. The mobile phase (isocratic or gradient elution) is then pumped through the column with the help of air pressure resulting in sample separation. This technique is considered as a low to medium pressure technique and is applied to samples from few milligrams to some gram of sample.

2.2.5.5.5. Preparative high pressure liquid chromatography (HPLC)

This process was used for isolation and purification of compounds from fractions previously separated using column chromatographic separation. The most appropriate solvent systems were determined before running the HPLC separation. The mobile phase combination was MeOH or acetonitrile and nanopure H_2O with or without 0.01 % TFA or 0.1

% formic acid, pumped in gradient or isocratic manner depending on the compounds retention time. Each injection consisted of 20-80 mg of the fraction dissolved in 400 mL of the solvent system. The solvent system was pumped through the column at a rate of 20 mL/min. The eluted peaks were detected by the online UV detector and collected separately in Erlenmeyer flasks.

Preparative HPLC system specifications are described as follows:

Pump	Varian, PrepStar 218
Detector	Varian, ProStar 320 UV-Vis detector
HPLC Program	Varian Star (V. 6)
Column	Varian Dynamax (250 × 4.6 mm, ID and 250 × 21.4 mm, ID), pre-packed with Microsorb 60-8 C18, with integrated pre-column.

2.2.5.5.6. Semi-preparative high pressure liquid chromatography (HPLC)

This process was used for purification of compounds from fractions previously separated using column chromatographic separation. The most appropriate solvent system was determined before running the HPLC separation. The mobile phase combination was MeOH and nanopure H_2O with or without 0.01 % TFA or 0.1 % formic acid, pumped in gradient or isocratic manner depending on the compounds retention time. Each injection consisted of 1-3 mg of the fraction dissolved in 1 mL of the solvent system. The solvent system was pumped through the column at a rate of 5 mL/min. The eluted peaks were detected by the online UV detector and collected separately in Erlenmeyer flasks. The separation column (125 × 4 mm, ID) was pre-filled with Eurospher C18 (Knauer, Berlin, Germany).

Semi-preparative HPLC system specifications are described as follows:

Pump	Merck Hitachi L-7100
Detector	Merck Hitachi UV detector L-7400
Column	Knauer (300 × 8 mm, ID), prepacked with Eurosphere 100-10 C18, with integrated pre-column.

2.2.5.5.7. Analytical high pressure liquid chromatography (HPLC)

Analytical HPLC was used to identify the distribution of peaks either from extracts or fractions, as well as to evaluate the purity of isolated compounds. The solvent gradient used started with MeOH:nanopure H_2O (10:90), adjusted to pH 2 with phosphoric acid, and reached to 100 % MeOH in 35 minutes. The autosampler injected 20 µL sample. All peaks were detected by UV-VIS photodiode array detector. In some cases, special programs were used. HPLC instrument consists of the pump, the detector, the injector, the separation column and the reservoir of mobile phase. The separation column (125 × 2 mm, ID) was pre-filled with Eurospher-100 C18 (5 µm), with integrated pre-column (Knauer, Berlin, Germany).

LC/UV system specifications are described as follows:

Pump	Dionex P580A LPG
Detector	Dionex Photodiode Array Detector UVD 340S
Column thermostat	STH 585
Autosampler	ASI-100T
HPLC Program	Chromeleon (V. 6.3)
Column	Knauer (125 × 4 mm, ID), pre-packed with Eurosphere 100-5 C18, with integrated pre-column.

2.2.6. Structure elucidation of the isolated secondary metabolites

2.2.6.1. Mass spectrometry (MS)

Mass spectrometers use the difference in mass-to-charge ratio (*m/z*) of ionized molecules to separate them from each other. Mass spectrometry is therefore useful for quantification of atoms or molecules and also for determination of chemical and structural information of molecules. A mass spectrometer consists of an ion source, ion detector and mass-selective analyzer. The output of mass spectrometers shows a plot of relative intensity vs. the mass-to-charge ratio (*m/z*).

2.2.6.1.1. Electrospray ionization mass spectrometry (ESI-MS)

A mass spectrometer is an analytical instrument used to determine the molecular weight of a compound. Basically, mass spectrometers are divided into three parts; ionization source, analyzer and detector, which should be maintained under high vacuum conditions in order to maintain the ions travel through the instrument without any hindrance from air molecules. Once a sample was injected into the ionization source, the molecules are ionized.

The ions were then passed and extracted into the analyzer. In the analyzer, the ions were separated according to their mass (*m*) to charge (*z*) ratio (*m/z*). Once the separated ions flow into the detector, the signals are transmitted to the data system where the mass spectrum is recorded.

Liquid chromatography mass spectrometry (LC/MS)

High pressure liquid chromatography is a powerful method for the separation of complex mixtures, especially when many of the components may have similar polarities. If a mass spectrum of each component can be recorded as it elutes from the LC column, quick characterization of the components is greatly facilitated. Usually, ESI-MS is interfaced with LC to make an effective on-line LC/MS. HPLC/ESI-MS was carried out using a Finnigan LCQ-DECA mass spectrometer connected to a UV detector. The samples were dissolved in water/MeOH mixtures and injected to HPLC/ESI-MS set-up. For standard MS/MS measurements, a solvent gradient that started with acetonitrile:nanopure H_2O (10:90), adjusted with 0.1 % HCOOH, and reached to 100 % acetonitrile in 35 minutes was used.

LC/UV/MS system specifications are described as follows:

HPLC system	Agilent 1100 series (pump, detector and autosampler)
	Finnigan LC Q-DECA
MS spectrometer	Knauer, (250 × 2 mm, ID), prepacked with Eurosphere 100-5
Column	C18, with integrated pre-column.

2.2.6.1.2. Electron impact mass spectrometry (EI-MS)

Analysis involves vaporizing a compound in an evacuated chamber and then bombarding it with electrons having 25.80 eV (2.4-7.6 MJ/mol) of energy. The high energy electron stream not only ionizes an organic molecule (requiring about 7-10 eV) but also causes extensive fragmentation (the strongest single bonds in organic molecules have strengths of about 4 eV). The advantage is that fragmentation is extensive, giving rise to a pattern of fragment ions which can help to characterize the compound. The disadvantage is the frequent absence of a molecular ion.

Low resolution EI-MS was measured on a Finnigan MAT 8430 mass spectrometer. Measurements were done by Dr. Peter Tommes, Institut für Anorganische and Strukturchemie, Heinrich-Heine Universität, Düsseldorf.

2.2.6.1.3. Fast atom bombardment mass spectrometry (FAB-MS)

This was the first widely accepted method that employs energy sudden ionization. FAB is useful for compounds, especially polar molecules, unresponsive to either EI or CI mass spectrometry. It enables both non-volatile and high molecular weight compounds to be analyzed. In this technique, a sample is dissolved or dispersed in a polar and relatively non-volatile liquid matrix, introduced into the source on a copper probe tip. Then, this matrix is bombarded with a beam of atoms of about 8 Kev. It uses a beam of neutral gas (Ar or Xe atoms) and both positive and negative ion FAB spectra can be obtained.

Low resolution FAB-MS was measured on a Finnigan MAT 8430 mass spectrometer. Measurements were done by Dr. Peter Tommes, Institut für Anorganische and Strukturchemie, Heinrich-Heine Universität, Düsseldorf.

2.2.6.1.4. High resolution mass spectrometry (HR-MS)

High resolution is achieved by passing the ion beam through an electrostatic analyzer before it enters the magnetic sector. In such a double focusing mass spectrometer, ion masses can be measured with an accuracy of about 1 ppm. With measurement of this accuracy, the atomic composition of the molecular ions can be determined.

HRESI-MS was measured on a Micromass Qtof 2 mass spectrometer at Helmholtz Centre for Infection Research, Braunschweig. The time-of-flight analyzer separates ions according to their mass-to-charge ratios (*m/z*) by measuring the time it takes for ions to travel through a field free region known as the flight.

2.2.6.2. Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Some nuclei experience this phenomenon, and others do not, dependent upon whether they possess a property called spin. It is used to study physical, chemical, and biological properties of matter. As a consequence, NMR spectroscopy finds applications in several areas of science. NMR spectroscopy is routinely used by chemists to study chemical structure using simple one dimensional technique. Two dimensional techniques are used to determine the structure of more complicated molecules.

NMR spectra were recorded at 300º K on a Bruker ARX-500 by Dr. Peter Tommes, Institut für Anorganische und Strukturchemie, Heinrich-Heine Universität, Düsseldorf. Some measurements were also performed at the Helmholtz Centre for Infection Research,

Braunschweig, by Dr. Victor Wray using an AVANCE DMX-600 NMR spectrometer. All 1D and 2D spectra were obtained using the standard Bruker software. The samples were dissolved in different solvents, the choice of which was dependent on the solubility of the samples. Residual solvent signals were used as internal standards (reference signal). The observed chemical shift (δ) values were given in ppm and the coupling constants (J) in Hz.

2.2.6.3. Optical activity

Optically active compounds contain at least one chiral centre. Optical activity is a microscopic property of a collection of these molecules that arises from the way they interact with light. Optical rotation was determined on a Perkin-Elmer-241 MC polarimeter. The substance was stored in a 0.5 mL cuvette with 0.1 dm length. The angle of rotation was measured at the wavelength of 546 and 579 nm of a mercury vapour lamp at room temperature (25º C). The specific optical rotation was calculated using the expression:

$$[\alpha]_D^T = \frac{[\alpha]_{579} \times 3.199}{4.199 - \frac{[\alpha]_{579}}{[\alpha]_{546}}}$$

With $[\alpha]_D^T$ = the specific rotation at the wavelength of the sodium D-line, 589 nm, at certain temperature T.

$[\alpha]_{579}$ and $[\alpha]_{546}$ = the optical rotation at wavelengths 579 and 546 nm, respectively, calculated using the formula:

$$[\alpha]_\lambda = \frac{100\,\alpha}{l \times c}$$

Where α = the measured angle of rotation in degrees,

l = the length in dm of the polarimeter tube,

c = the concentration of the substance expressed in g/100 mL.

2.2.6.4. Determination of absolute stereochemistry by Mosher reaction

The reaction was performed according to a modified Mosher ester procedure described by Su *et al.* (Ohtani *et al.*, 1991; Su *et al.*, 2002).

Reaction with (*R*)-(-)-*α*-(trifluoromethyl) phenylacetyl chloride

The compounds (1 mg of each) were transferred into NMR tubes and were dried under vacuum. Deuterated pyridine (0.5 mL) and (*R*)-MTPA chloride were added into the NMR

tube immediately under a N_2 gas stream. The reagent was added in the ratio of 0.14 mM reagent to 0.10 mM of the compound (Dale and Mosher, 1973). The NMR tubes were shaken carefully to mix the samples and MTPA chloride evenly. The reaction NMR tubes were permitted to stand at room temperature and monitored by ^{1}H-NMR until the reaction was found to be complete. ^{1}H-^{1}H COSY was measured to confirm the assignment of the signals.

Reaction with (*S*)-MTPA chloride

Another portion of each compound (1 mg) was transferred into NMR tube. The reaction was performed in the same manner as described before to yield the (*S*)-MTPA ester.

2.2.7. Testing the biological activity

Finding biologically important compounds from endophytic fungi is only achieved if, and when, assay systems have been devised that will allow for successful biologically guided fractionation of the culture extracts.

2.2.7.1. Antimicrobial assay

2.2.7.1.1. Agar diffusion assay

This method was used to detect the capability of a substance to inhibit the growth of microorganisms by measuring the diameter of inhibition zone around a tested compound on agar plate. The agar diffusion assay was performed according to the Bauer-Kirby-Test (Bauer *et al.*, 1966).

Microorganisms

Crude extracts and isolated pure compounds were tested for activity against the following standard strains: Gram-positive bacteria *Bacillus subtilis*,

Gram-negative bacteria *Escherichia coli*,

yeast *Saccharomyces cerevisiae*,

and the fungi *Cladosporium cucumerinum* and *C. herbarum*.

Culture preparation

Prior to testing, bacterial liquid cultures were prepared by subculture of a few colonies (3 to 10) of the organism to be tested in 4 mL semi-liquid medium (containing only 0.01% agar) followed by incubation to allow growth of organisms. The liquid cultures were then

seperately mixed in the Ultraturrax to produce suspensions of moderate cloudiness. The suspension was diluted with sterile saline solution to a density visually equivalent to that of a $BaSO_4$ standards, prepared by adding 0.5 mL of 1% $BaCl_2$ to 99.5 mL of 1% H_2SO_4 (0.36 N). The prepared broth was inoculated onto agar plates using an Eppendorf pipette and homogenously dispersed by means of a sterile spatula.

For the preparation of fungal cultures mycelia of *C. cucumerinum* and *C. herbarum* (after growing the fungi for about one month) were put into a fresh fungal medium (see section 2.1.1.6) and destroyed using Ultraturax. The cell debris (extracted mycellium) was removed by vacuum filtration and the filtrate (medium containing fungal spores) was then used for the next steps of the assay.

Anti-bacterial Assay

For the assay performed using *E. coli* and *B. subtilis*. A 100 – 200 µl of bacterial liquid culture, in an exponential growth phase, was spread on to the surface of Luria Bertani (LB) agar plate (see section 2.1.2.4). Immediately, 10-20 µl of tested samples were loaded onto the disc paper (5 mm diameter, Oxoid Ltd.), to give final disc loading concentrations of 250-500 µg for crude extracts as well as 50-100 µg for pure compounds. The impregnated discs were placed onto the surface of LB plates previously seeded with the selected test organisms, along with discs containing solvent blanks. The culture was then incubated at 37º C for 24-48 hrs (depending on the microbial culture being used). Antimicrobial activity was recorded as the clear zone of inhibition surrounding the disc (diameter measured in mm) and compared to Penicillin G, streptomycin and Gentamycin as positive controls.

The same technique was also applied to the assay using *S. cerevisiae* in a yeast medium (see section 2.1.2.5) and incubation at 27º C for 24 hrs.

Anti-fungal Assay

A 100 ml of fungal spore suspension was spread out onto the surface of potato dextrose agar medium (PDA, see section 2.1.2.7). Immediately, 10-20 µl of tested samples were loaded onto the disc paper (5 mm diameter, Oxoid Ltd.), to give final disc loading concentrations of 250-500 µg for crude extracts as well as 50-100 µg for pure compounds. The impregnated discs were transferred onto the surface of the PDA medium. The fungal cultures were then incubated at room temperature for several days and growth inhibition was measured around the discs. The result was then compared to positive control (nystatin).

2.2.7.1.2. Inhibition of biofilm formation

Biofilm inhibition assays were carried out by PD. Dr. U. Hentschel, Zentrum für Infektionsforschung, Würzburg.

The biofilm formation was determined by a simple adhesion assay in polystyrene microtiter plates. For this purpose, *Staphylococcus epidermidis* cultures were diluted with fresh TSB medium (see section 2.1.2.8) in the ratio of 1:100 (1980 µl medium + 20 µl culture). 200 µl of the prepared suspension were pipetted into each well of a 96-well tissue culture plate (8 time application per strain) and incubated at 37°C for 18 hrs. *S. epidermidis* RP62A (wild type) was used as positive control, and *S. carnosus* TM 300 as negative control. Samples to be tested were added to growing or already formed biofilms. After incubation, the wells were carefully emptied and the plate washed three times with PBS-buffer (phosphate buffered saline), and any remaining biofilm was heat-fixed on a hotplate at *ca* 60° C and stained with crystal violet dye for 5 min, and excess dye was washed off with water. After drying, the optical density of the adhering biofilm was determined by ELISA-Reader at 490 nm. Values lower than 0.120 were considered negative, strains with values between 0.120 and 0.240 were considered as weak adherents and results higher than 0.240 as strong adherents. The limit of 0.120 corresponds to the three-way average value of the negative control.

2.2.7.2. Cytotoxicity test

2.2.7.2.1. Microculture tetrazolium (MTT) assay

Cytotoxicity tests were carried out by Prof. Dr. W. E. G. Müller, Institut für Physiologische Chemie und Pathobiochemie, University of Mainz, Mainz. The cytotoxicity was tested against L5178Y mouse lymphoma cells using the microculture tetrazolium (MTT) assay, and compared to that of untreated controls (Carmichael, DeGraff, Gazdar, Minna, and Mitchell, 1987).

Cell cultures

L5178Y mouse lymphoma cells were grown in Eagle's minimal essential medium supplement with 10% horse serum in roller tube culture. The medium contained 100 units/mL penicillin and 100 µg/mL streptomycin. The cells were maintained in a humified atmosphere at 37° C with 5% CO_2.

MTT colorimetric assay

Of the test samples, stock solutions in ethanol 96% (v/v) were prepared. Exponentially growing cells were harvested, counted and diluted appropriately. Of the cell suspension, 50 µL containing 3750 cells were pipetted into 96-well microtiter plates. Subsequently, 50 µL of a solution of the test samples containing the appropriate concentration was added to each well. The concentration range was 3 and 10 µg/mL. The small amount of ethanol present in the wells did not affect the experiments. The test plates were incubated at 37° C with 5% CO_2 for 72 h. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was prepared at 5 mg/mL in phosphate buffered saline (PBS; 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4) and from this solution, 20 µL was pipetted into each well. The yellow MTT penetrates the healthy living cells and in the presence of mitochondrial dehydrogenases, MTT is transformed to its blue formazan complex. After an incubation period of 3 h 45 min at 37° C in a humidified incubator with 5% CO_2, the medium was centrifuged (15 min, 20 °C, 210 x g) with 200 µL DMSO, the cells were lysed to liberate the formed formazan product. After thorough mixing, the absorbance was measured at 520 nm using a scanning microtiter-well spectrophotometer. The colour intensity is correlated with the number of healthy living cells. Cell survival was calculated using the formula:

$$\text{Survival \%} = 100 \text{ x } \frac{\text{absorbance of treated cells} - \text{absorbance of culture medium}}{\text{absorbance of untreated cells} - \text{absorbance of culture medium}}$$

All experiments were carried out in triplicates and repeated three times. As controls, media with 0.1% EGMME/DMSO were included in the experiments.

2.2.7.2.2. Protein kinase assay

Protein kinase assays were carried out by Dr. Michael Kubbutat (ProQinase GmbH, Freiburg, Germany).

Protein kinase enzymes are integral components of numerous signal transduction pathways involved in the regulation of cell growth, differentiation, and response to changes in the extracellular environtment. Consequently, kinases are major targets for potentially developing novel drugs to treat diseases such as cancer and various inflammatory disorders.

The inhibitory potency of the samples was determined using 24 protein kinases (see Table 2.2). The IC_{50} profile of compounds/fractions showing an inhibitory potency of ≥ 40% with at least one of the 24 kinases at an assay concentration of 1×10^{-06} g/mL was determined. IC_{50} values were measured by testing 10 concentrations of each sample in singlicate (n=1).

Sample preparation

The compounds/fractions were provided as 1×10^{-03} g/mL stock solutions in 100% DMSO (1000 or 500 µL) in micronic boxes. The boxes stored at -20° C. Prior to the assays, 100 µL of the stock solutions was transferred into separate microtiter plates. Subsequently, they were subjected to serial, semi-logarithmic dilution using 100% DMSO as a solvent resulting in 10 different concentrations. 100% DMSO was used as control. Subsequently, 7 × 5 µL of each concentration were aliquoted and diluted with 45 µL H_2O only a few minutes before the transfer into the assay plate to minimize precipitation. The plates were shaken thoroughly and then used for the transfer of 5 µL compound solution into the assay plates.

Recombinant protein kinases

All protein kinases were expressed in Sf9 insect cells as human recombinant GST-fusion proteins or His-tagged proteins by means of the baculovirus expression system. Kinases were purified by affinity chromatography using either GSH-agarose (Sigma) or Ni-NTH-agarose (Qiagen). Purity was checked by SDS-PAGE/silver staining and the identity of each kinase was verified by western blot analysis with kinase specific antibodies or by mass spectrometry.

Protein kinase assay

A proprietary protein kinase assay (^{33}PanQinase® Activity Assay) was used for measuring the kinase activity of the protein kinases. All kinase assays were performed in 96-well FlashPlates™ from Perkin Elmer/NEN (Boston, MA, USA) in a 50 µL reaction volume. The reaction mixture was pipetted in the following order: 20 µL assay buffer, 5 µL ATP solution in H_2O, 5 µL test compound in 10% DMSO and 10 µL substrate/10 µL enzyme solution (premixed). The assay for all enzymes contained 60 mM HEPES-NaOH (pH 7.5), 3 mM $MgCl_2$, 3mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, 50 µg/mL PEG_{20000}, 1 µM [γ-^{33}P]-ATP. The reaction mixtures were incubated at 30° C for 80 minutes and stopped with 50 µL 2% (v/v) H_3PO_4. The plates were aspirated and washed two times with 200 µL of 0.9% (w/v) NaCl or 200 µL H_2O. Incorporation of $^{33}P_i$ was determined with a microplate scintillation counter (Microbeta Trilux, Wallac). All assays were performed with a BeckmanCoulter/Sagian robotic system.

Table 2.2: List of protein kinases and their substrates

Family	Kinase	Substrate	Oncologically relevant mechanism	Disease
Serine/threonine kinases	AKT1/PKB alpha	GCS3(14-27)	Apoptosis	Gastric cancer (Staal, 1987)
	ARK5	Autophos.	Apoptosis	Colorectal cancer (Kusakai *et al.*, 2004)
	Aurora A	tetra(LRRWSLG)	Proliferation	Pancreatic cancers (Li *et al.*, 2003)
	Aurora B	tetra(LRRWSLG)	Proliferation	Breast cancer (Keen and Taylor, 2004)
	CDK2/Cyclin A	Histone H1	Proliferation	Pancreatic cancer (Iseki *et al.*, 1998)
	CDK4/Cyclin D1	Rb-CTF	Proliferation	Breast cancer (Yu *et al.*, 2006)
	CK2-alpha1	p53-CTM	Proliferation	Rhabdomyosarcoma (Izeradjene *et al.*, 2004)
	COT	Autophos.	Proliferation	Breast cancer (Sourvinos, 1999)
	PLK-1	Casein	Proliferation	Prostate cancer (Weichert *et al.*, 2004)
	B-RAF-VE	MEK1-KM	Proliferation	Thyroid cancer (Ouyang *et al.*, 2006)
	SAK	Auotphos.	Proliferation	Colorectal cancer (Macmillan *et al.*, 2001)
Receptor tyrosine kinase	EGFR	$Poly(Glu,Tyr)_{4:1}$	Proliferation	Glioblastoma multiforme (National Cancer Institute, 2005)
	EPHB4	$Poly(Glu,Tyr)_{4:1}$	Angiogenesis	Prostate cancer (Xia *et al.*, 2005)
	ERBB2	$Poly(Glu,Tyr)_{4:1}$	Proliferation	Gastric carcinomas (Lee *et al.*, 2005)
	FLT3	$Poly (Ala,Glu,Lys,tyr)_{6:2:4:1}$	Proliferation	Leukemia (Menezes *et al.*, 2005)
	IGF1-R	$Poly(Glu,Tyr)_{4:1}$	Apoptosis	Breast cancer (Zhang and Yee, 2000)
	INS-R	$Poly (Ala,Glu,Lys,tyr)_{6:2:4:1}$	"counter kinase"	Ovarian cancer (Kalli *et al.*, 2002)
	MET	$Poly (Ala,Glu,Lys,tyr)_{6:2:4:1}$	Metastasis	Lung cancer (Qiao, 2002)

Family	Kinase	Substrate	Oncologically relevant mechanism	Disease
	PDGFR-beta	Poly $(Ala,Glu,Lys,tyr)_{6:2:4:1}$	Proliferation	Prostate cancer (Hofer *et al.*, 2004)
	TIE-2	$Poly(Glu,Tyr)_{4:1}$	Angiogenesis	Rheumatoid arthritis (DeBusk *et al.*, 2003)
	VEGF-R2	$Poly(Glu,Tyr)_{4:1}$	Angiogenesis	Pancreatic cancers (Li *et al.*, 2003)
	VEGF-R3	$Poly(Glu,Tyr)_{4:1}$	Angiogenesis	Breast cancer (Garces *et al.*, 2006)
Soluble tyrosine kinase	FAK	$Poly(Glu,Tyr)_{4:1}$	Metastasis	Breast cancer (Schmitz *et al.*, 2005)
	SRC	$Poly(Glu,Tyr)_{4:1}$	Metastasis	Colon cancer (Dehm *et al.*, 2001)

2.2.8. General laboratory equipments

Autoclave	Varioklav, H&P
Balances	Mettler 200, Mettler AT 250, Mettler PE 1600, Sartorious MC1 AC210S
Centrifuge	Biofuge pico, Heraeus
Cleanbench	HERAsafe, Heraeus
Digital pH meter	420Aplus, Orion
Drying Ovens	Kelvitron t, Heraeus
Fraction collector	Cygnet, ISCO
Freeze dryer	Lyovac GT2, Steris
- 80 °C Freezer	Forma Scientific, 86-Freezer
Hot plate	Camag
Magnetic stirrer	Combi Mag, IKA
Rotary evaporator	Vacuubrand, IKA
Sonicator	Sonorex RK 102, Bandelin
Syringes	Hamilton
Ultra Turrax	T18 basic, IKA
UV Lamp	Camag (254 and 366)
Vacuum centrifuge	SpeedVac SPD 111V, Savant

3. Results

3.1. Compounds isolated from the endophytic fungus *Alternaria* sp.

This endophytic fungal strain of the genus *Alternaria* was isolated from leaves of *Polygonum senegalense* growing in Egypt. The pure fungal strain was cultivated on liquid Wickerham medium and rice solid medium. Interestingly, chemical screening studies indicated a clear difference between *Alternaria* extracts obtained from liquid Wickerham medium and rice cultures. Comparison of the HPLC chromatograms of the EtOAc extracts of both cultures showed a different chemical pattern. While the extract of liquid cultures showed alternariol (**1**) and tenuazonic acid (**14**) as main components, altenusin (**6**) was the major substance detected in the rice culture extract, with no traces of tenuazonic acid (see Figure 3.1A-B). The yield of EtOAc dried extract from rice cultures was much higher than that from liquid cultures with a ratio of 11:1, respectively. Moreover, extracts obtained from liquid and solid cultures were subjected to some preliminary biological screening assays, *i.e.* antibacterial, antifungal, cytotoxicity and protein kinase assays. Interestingly, extracts obtained from rice cultures showed higher cytotoxic and antifungal activity compared to those of liquid cultures, while the latter had higher antibacterial activity (see Table 3.1).

In this part of the investigation results on the natural products produced by *Alternaria* sp. when grown in liquid medium and on solid rice medium are presented.

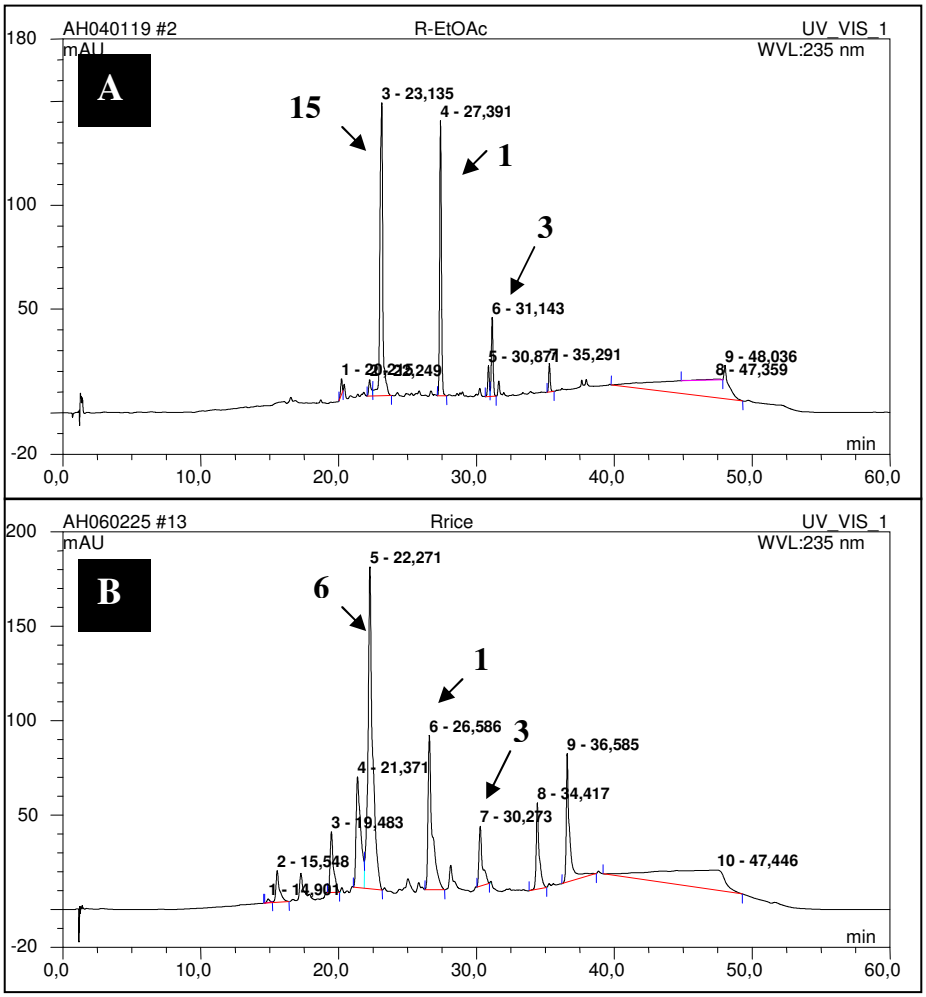


Figure 3.1A-B: EtOAc extracts of *Alternaria* sp. cultures. **A:** HPLC chromatogram of EtOAc extract of liquid cultures (Wickerham medium). **B:** HPLC chromatogram of EtOAc extract of rice cultures. **1:** Alternariol. **3:** Alternariol monomethyl ether. **6:** Altenusin. **15:** Tenuazonic acid.

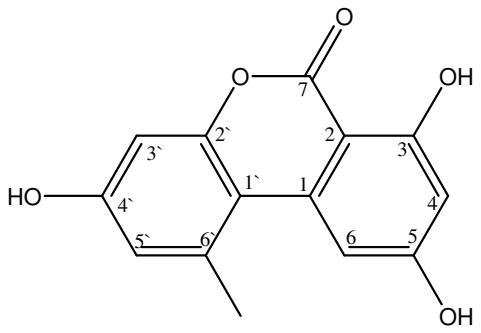
Table 3.1: Biological screening test results for *Alternaria* liquid and rice extracts

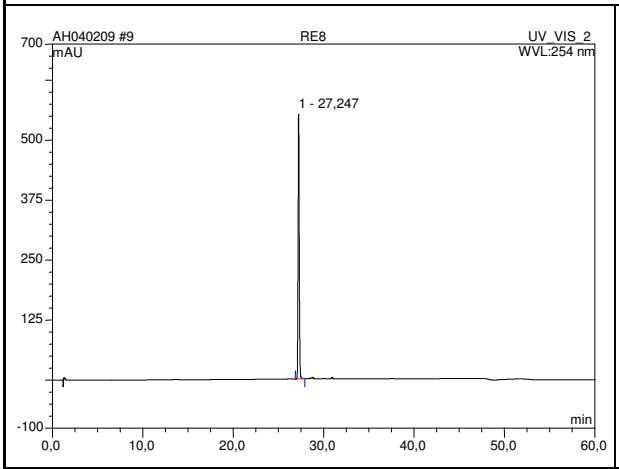
Extracts tested	**L5178Y growth in %** (Conc. 10 µg/mL)	**Protein kinase activity** (Conc. 1 µg/mL)	**Antimicrobial activity** IZ [mm], 0.5 mg		
			BS	SC	CH
Alternaria liquid *n*-BuOH	99.8	Active	7	6	0
Alternaria liquid EtOAc	52.8		13	0	0
Alternaria rice EtOAc	0.1	Active	0	0	9

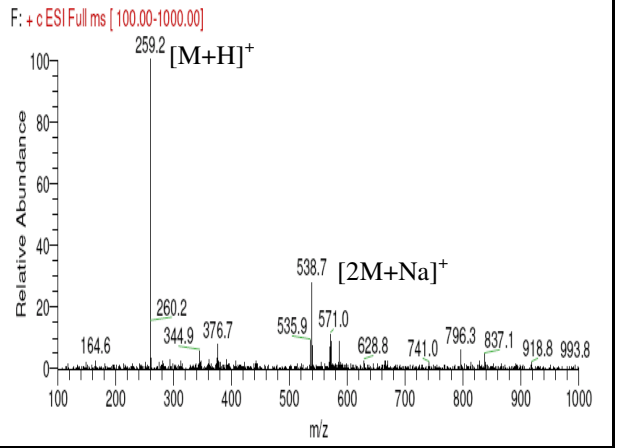
BS: *B. subtilis*, SC: *S. cerevisiae*, CH: *C. herbarum*.

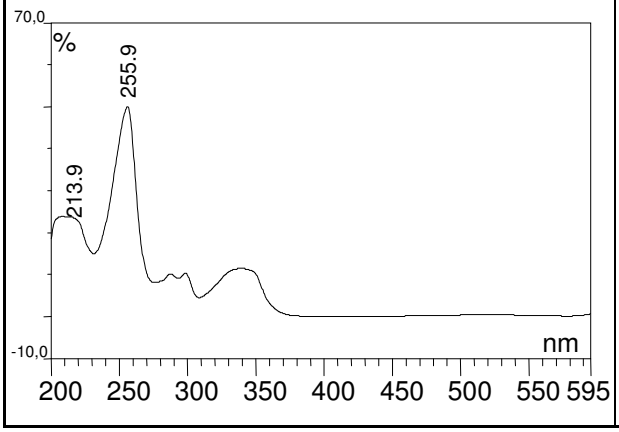
3.1.1. Alternariol (1, known compound)

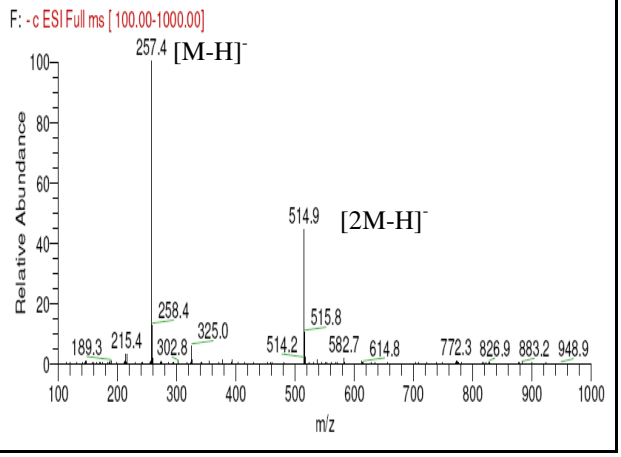
Alternariol	
Synonym(s)	3,7,9-Trihydroxy-1-methyl-6H-dibenzo[b,d]pyran-6-one
Sample code	RE8, RR3.3, RR5.2
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	204.1 mg
Physical Description	reddish white needles
Molecular Formula	$C_{14}H_{10}O_5$
Molecular Weight	258 g/mol
Retention time HPLC	27.3 min (standard gradient)







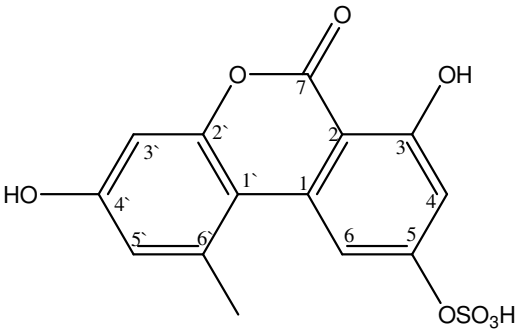


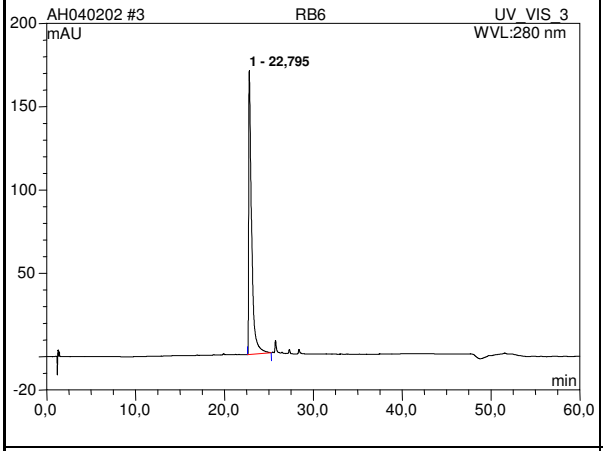


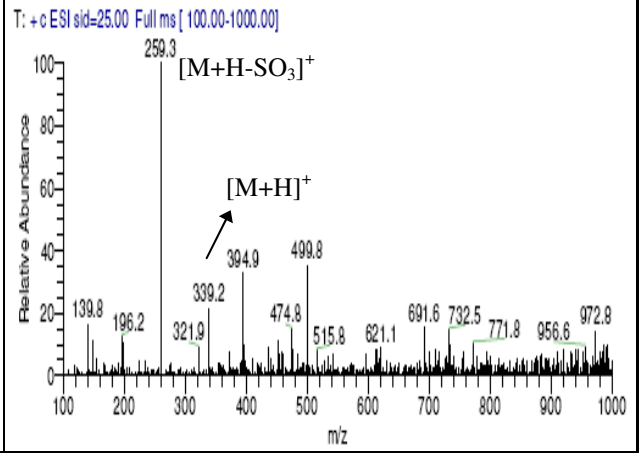
Alternariol (**1**) was isolated from the EtOAc extracts of liquid and rice cultures of *Alternaria* sp. as reddish white needles (204.1 mg). It showed UV absorbances at λ_{max} (MeOH) 206.1, 255.8, 299.8 and 339.7 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 259.2 $[M+H]^+$ (base peak) and *m/z* 257.4 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 258 g/mol. The ^1H and ^{13}C NMR spectra (Table 3.2a and 3.3) indicated the presence of an aromatic methyl group at δ_H 2.71 and δ_C 25.8 as well as four aromatic protons. The coupling constants observed for the aromatic ring protons indicated the presence of two aromatic rings, each bearing a pair of *meta*-coupled protons at δ_H 7.20 (*J*=2.0 Hz), 6.32 (*J*=2.0 Hz), 6.65 (*J*=2.5 Hz) and 6.55 (*J*=2.5 Hz) assigned for H-4, H-6, H-3` and H-5`, respectively. The signal at δ_C 166.8 in the ^{13}C NMR spectrum indicated the presence of a conjugated lactone (C-7). The ^{13}C NMR spectrum revealing the presence of 15 carbon atoms as well as ^1H NMR and mass spectra supported a molecular formula of $C_{15}H_{12}O_5$. Further confirmation was achieved by interpretation of the HMBC spectrum (see Table 3.2a and Figure 3.2) showing correlations of the *meta*-coupled protons, H-4 to C-2, C-3 and C-6 as well as H-6 to C-2, C-4 and C-5, thereby establishing the structure of one aromatic ring. The correlations of the aromatic methyl group to C-(1`-6`) and those observed for H-3` and H-5` confirmed the structure of the second aromatic ring. Furthermore, correlations of the methyl group to C-6 and of H-6 to C-1` proved the point of attachment of both rings. UV, ^1H, ^{13}C NMR and mass spectral data were found to be identical to published data for alternariol (Stinson *et al.*, 1986), previously reported from several *Alternaria* species (Freeman, 1966; Coombe *et al.*, 1970; Bradburn *et al.*, 1994).

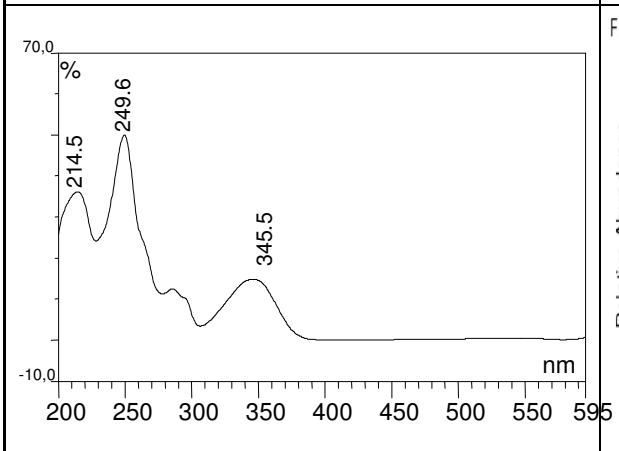
3.1.2. Alternariol-5-*O*-sulphate (2, new compound)

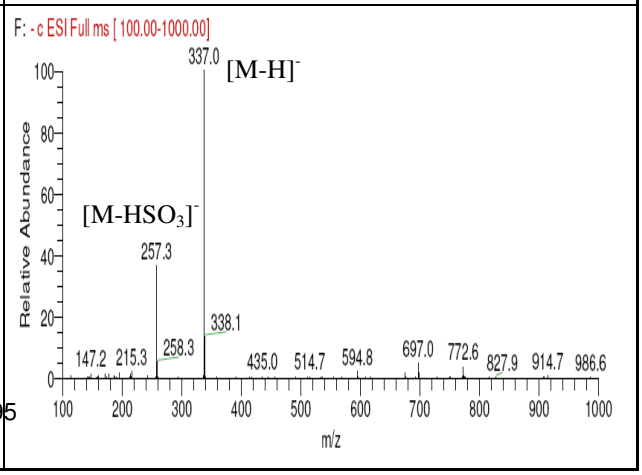
Alternariol-5-*O*-sulphate	
Synonym(s)	3,7,9-Trihydroxy-1-methyl-6H-dibenzo[b,d]pyran-6-one-9-sulphate
Sample code	RB6
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	60.9 mg
Physical Description	reddish white needles
Molecular Formula	$C_{14}H_{10}O_8S$
Molecular Weight	338 g/mol
Retention time HPLC	22.8 min (standard gradient)







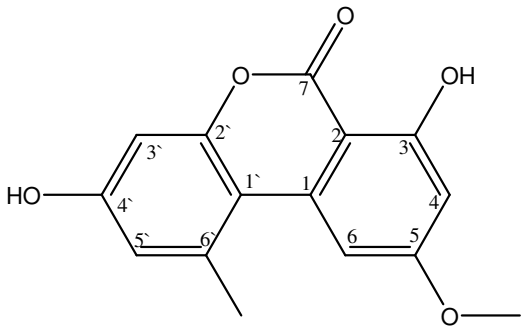


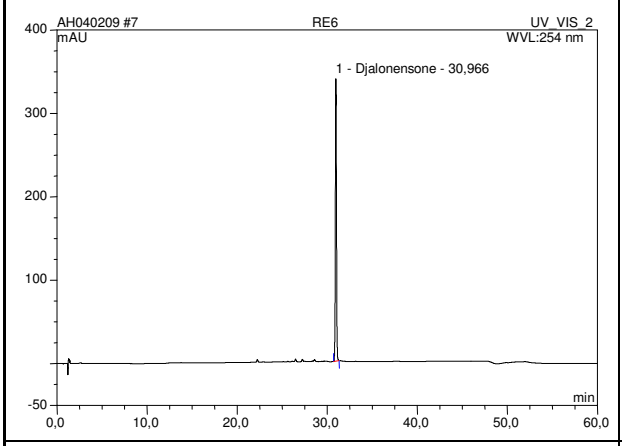


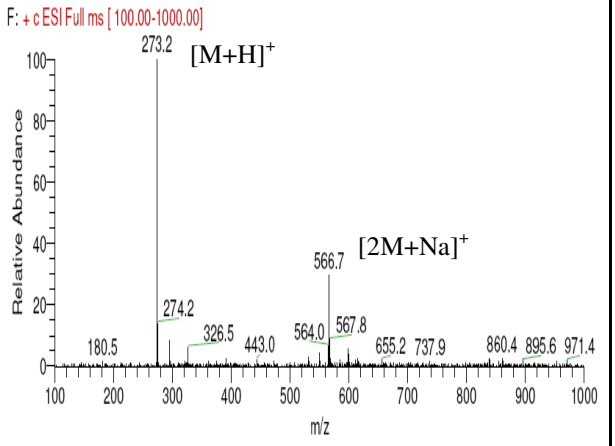
Compound **2** was isolated from the *n*-BuOH extract of liquid cultures of *Alternaria* sp. in the form of reddish white needles (60.9 mg). It displayed UV absorbances at λ_{max} (MeOH) 214.6, 249.7, 287.6 and 345.1 nm, showing high similarity to UV spectra typical for alternariol derivatives. The HRESI-MS exhibited a strong peak at *m/z* 339.0170 $[M+H]^+$ indicating a molecular formula of $C_{14}H_{10}O_8S$ (calculated 339.0174, Δ 0.0004). The ^1H NMR spectrum (see Table 3.2a) showed signals for a methyl group at δ 2.75 and two pairs of *meta*-coupled aromatic protons at δ_H 7.83 (*J*=2.0 Hz), 6.86 (*J*=2.0 Hz), 6.67 (*J*=2.0 Hz) and 6.57 (*J*=2.0 Hz) corresponding to H-6, H-4, H-5` and H-3`, respectively. The dibenzo-α-pyrone structure was confirmed by the NOE effects observed both on H-6 and H-5` upon irradiation of the 6`-methyl group. Interpretation of the HMBC spectrum (see Table 3.2a and Figure 3.2) showed that the correlations observed for 6`-C*H*$_3$ were identical to those observed for **1**. Moreover, correlations of the *meta*-coupled protons, H-4 to C-2, C-3, C-5 and C-6 as well as H-6 to C-2, C-4 and C-5, were similar in both compounds indicating similar structures. However, comparison of ^1H and ^{13}C NMR data (Table 3.2a and 3.3) with those measured for alternariol (**1**) showed good congruence except for the downfield shifts observed for H-4 and H-6, as well as the upfield shift of C-5, of 6.7 ppm, and downfield shifts of C-4 and C-6, of 4.1 and 2.6 ppm, respectively, indicating the presence of a sulphate substitution at C-5 (Ragan, 1978). This deduction was confirmed by the fragment formed by loss of 80 mass units in the mass spectrum of **2** and the hypsochromic shift in the UV spectrum of **2** compared to that of **1**, which is attributed to the electron withdrawing effect of the sulphate group (Plasencia and Mirocha, 1991). The compound was thus identified as the new natural product alternariol-5-*O*-sulphate.

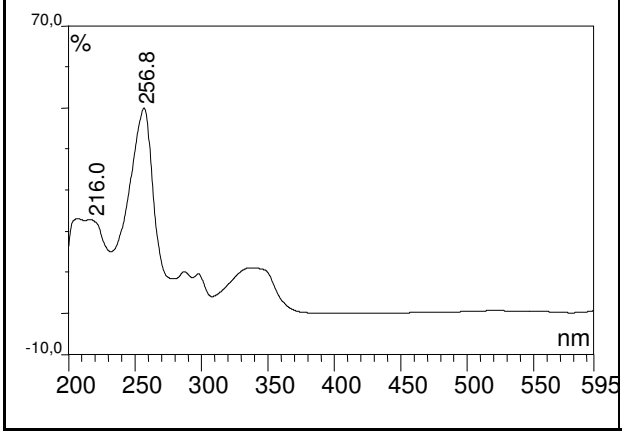
3.1.3. Alternariol-5-*O*-methyl ether (3, known compound)

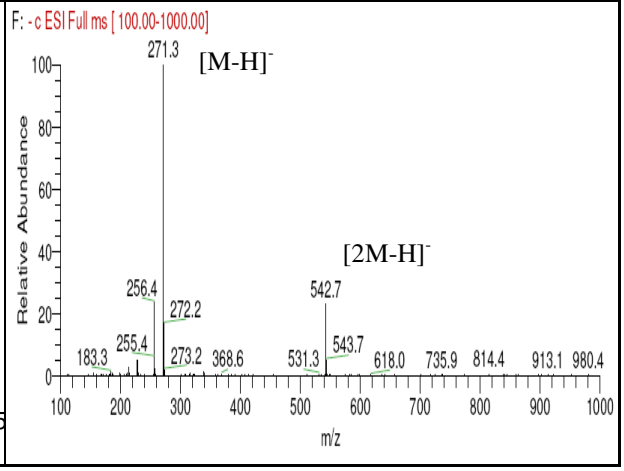
Alternariol-5-*O*-methyl ether	
Synonym(s)	3,7-Dihydroxy-9-methoxy-1-methyl-6H-dibenzo[b,d]pyran-6-one, Djalonensone
Sample code	RE6, RR2, RR3.5
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	239.0 mg
Physical Description	reddish white needles
Molecular Formula	$C_{15}H_{12}O_5$
Molecular Weight	272 g/mol
Retention time HPLC	31.0 min (standard gradient)







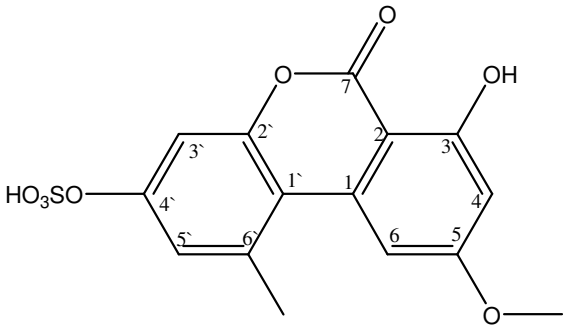


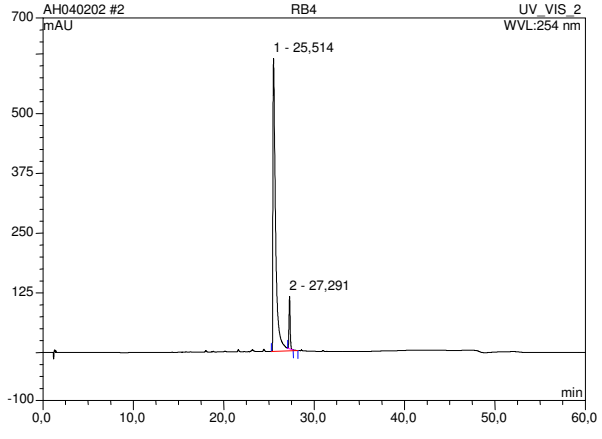


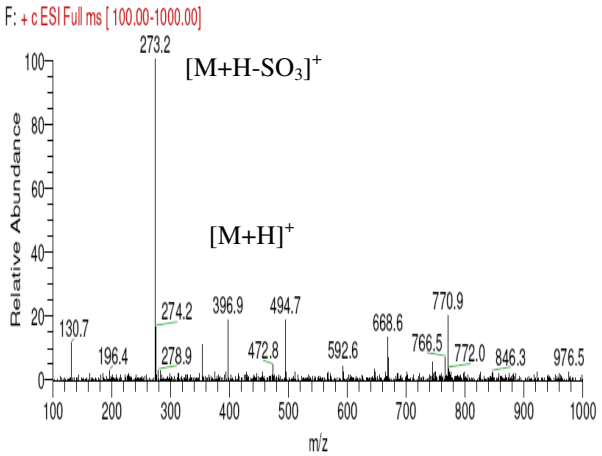
Alternariol-5-*O*-methyl ether (**3**) was isolated from the EtOAc extracts of liquid and rice cultures of *Alternaria* sp. in the form of reddish white needles (239.0 mg). It exhibited UV absorbances at λ_{max} (MeOH) 216.2, 256.9, 287.0 and 338.8 nm, having the typical pattern of alternariol derivatives. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 273.2 $[M+H]^+$ (base peak) and *m/z* 271.3 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 272 g/mol with an increase of 14 mass units compared to alternariol (**1**) and thus supporting a molecular formula of $C_{15}H_{12}O_5$. The ^1H and ^{13}C NMR spectra (see Table 3.2b and 3.3) showed a methoxy group at δ_H 3.91 and δ_C 55.9, an aromatic methyl group at δ_H 2.76 and δ_C 25.3 as well as four aromatic protons at δ_H 7.28 (*J*=1.8 Hz), 6.70 (*J*=2.5 Hz), 6.61 (*J*=2.5 Hz) and 6.54 (*J*=1.8 Hz) assigned for H-6, H-5`, H-3` and H-4, respectively. Interpretation of the HMBC spectrum (see Table 3.2b and Figure 3.3) showing correlations similar to those observed for **1** indicated a close structure resemblance. The structure was confirmed by comparison of UV, ^1H, ^{13}C NMR and mass spectral data with published data for alternariol-5-*O*-methyl ether (Onocha *et al.*, 1995), also known as djalonensone, previously reported from several *Alternaria* species (Freeman, 1966; Coombe *et al.*, 1970; Bradburn *et al.*, 1994) as well as from *Anthocleista djalonensis* (Onocha *et al.*, 1995).

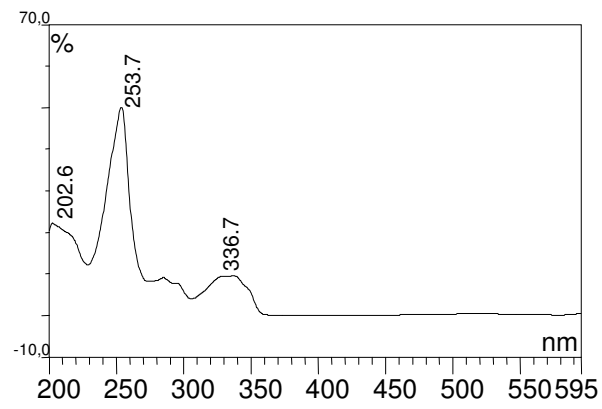
3.1.4. Alternariol-5-*O*-methyl ether-4`-*O*-sulphate (4, new compound)

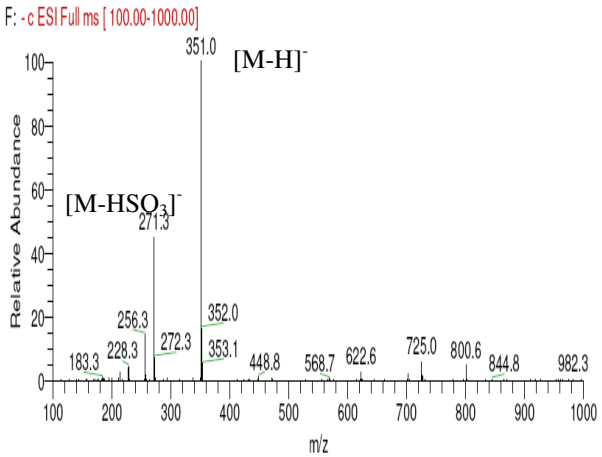
Alternariol-5-*O*-methyl ether-4`-*O*-sulphate	
Synonym(s)	3,7-Dihydroxy-9-methoxy-1-methyl-6H-dibenzo[b,d]pyran-6-one-3-sulphate
Sample code	RB4
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	5.9 mg
Physical Description	reddish white needles
Molecular Formula	$C_{15}H_{12}O_8S$
Molecular Weight	352 g/mol
Retention time HPLC	25.5 min (standard gradient)







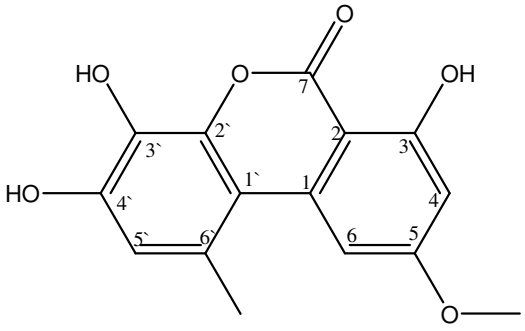


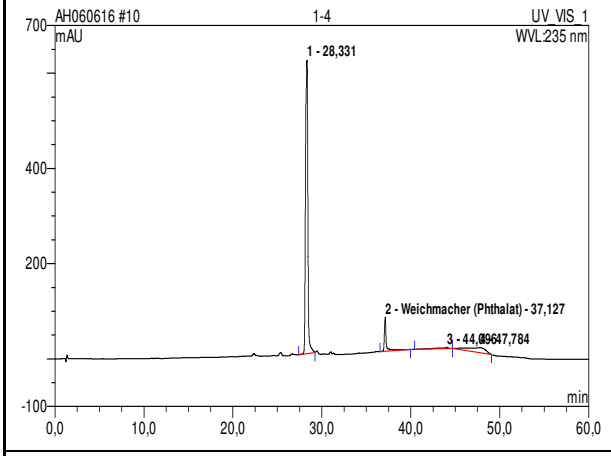


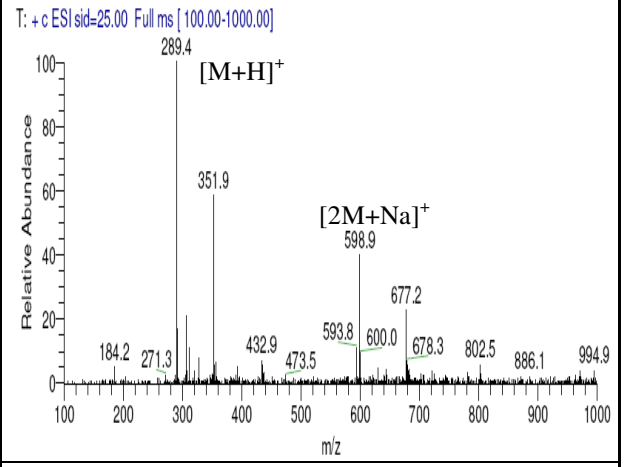
Compound **4** was isolated from the *n*-BuOH extract of liquid cultures of *Alternaria* sp. as reddish white needles (5.9 mg). The UV spectrum showed λ_{max} (MeOH) at 202.5, 253.5, 285.3 and 336.8 nm. The HRESI-MS showed $[M+H]^+$ at *m/z* 353.0320 (calculated 353.0331, Δ 0.0011), indicating a molecular formula of $C_{15}H_{12}O_8S$ and an increase of 14 mass units more than **2**. The compound showed physical and UV spectral features similar to those of **1**, **2** and **3**. The ^{1}H NMR spectrum (see Table 3.2b) showed signals for a methoxy group at δ_H 3.84, an aromatic methyl group at δ_H 2.74 and two pairs of *meta*-coupled aromatic protons at δ_H 7.27 (*J*=2.5 Hz), 7.14 (*J*=2.5 Hz), 7.08 (*J*=2.5 Hz) and 6.52 (*J*=2.5 Hz) corresponding to H-6, H-3`, H-5` and H-4, respectively. Comparison of the ^{1}H and ^{13}C NMR spectra of **4** (see Table 3.2b and 3.3) with those of **2** and **3** suggested a close relationship between their structures. In addition, interpretation of the HMBC spectrum (see Table 3.2b and Figure 3.3) showed that correlations of the *meta*-coupled protons H-4 and H-6 were identical to those observed for **3**. Moreover, correlations of the *meta*-coupled protons, H-3` to C-2`, C-4` and C-5` as well as H-5` to C-1`, C-3`, C-4` and 6`-*C*H$_3$, were similar in both compounds indicating similar structures. However, in spite of close resemblance of $^1$H NMR spectral features of **4** and **3** exceptions were the downfield shifts observed for H-3` and H-5` along with the upfield shift of C-4`, by 5.1 ppm, and downfield shifts of C-3` and C-5`, by 6.4 and 4.6 ppm, respectively, in a similar pattern as in **2** indicating a sulphate group to be attached at C-4` (Ragan, 1978). Presence of the fragment formed by loss of 80 mass units in the mass spectrum and the hypsochromic shift in the UV spectrum of **4** confirmed the structure (Plasencia and Mirocha, 1991). Thus, compound **4** was identified as the new natural product alternariol-5-*O*-methyl ether-4`-*O*-sulphate.

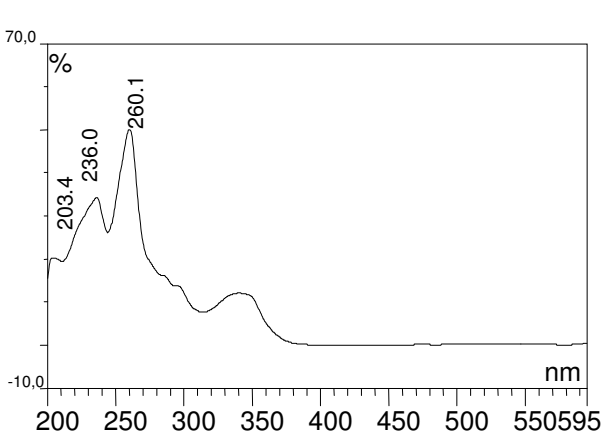
3.1.5. 3`-Hydroxyalternariol-5-*O*-methyl ether (5, new compound)

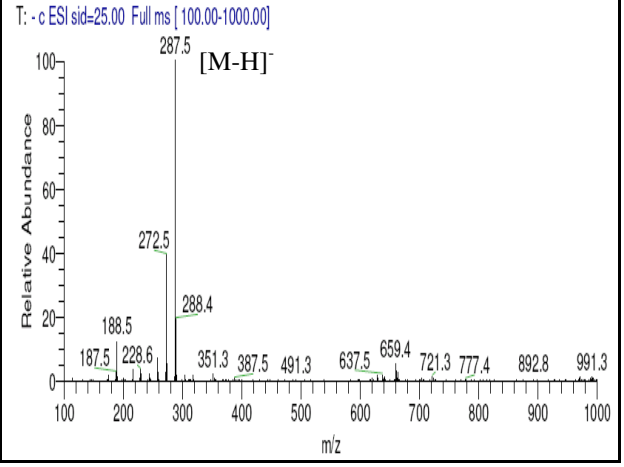
3`-Hydroxyalternariol-5-*O*-methyl ether	
Synonym(s)	3,7,8-Trihydroxy-9-methoxy-1-methyl-6H-dibenzo[b,d]pyran-6-one
Sample code	RR3.4
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	5.8 mg
Physical Description	violet needles
Molecular Formula	$C_{15}H_{12}O_6$
Molecular Weight	288 g/mol
Retention time HPLC	28.3 min (standard gradient)



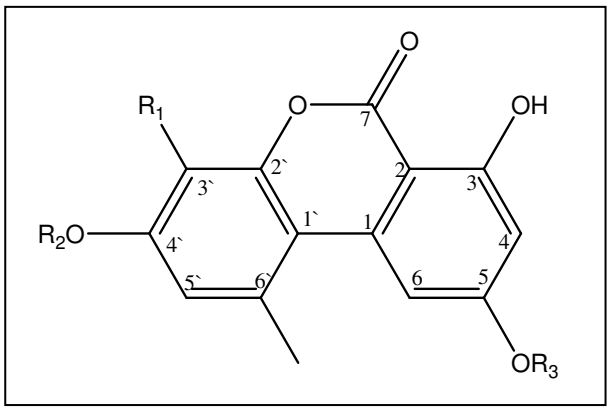








Compound **5** was isolated from the EtOAc extract of rice cultures of *Alternaria* sp.. It was isolated as violet needles (5.8 mg). The UV spectrum showed absorbances at λ_{max} (MeOH) 203.4, 236.0, 260.1 and 340.0 nm, showing high similarity to UV spectra typical for alternariol derivatives **1**, **2**, **3** and **4**. The HRESI-MS showed $[M+H]^+$ at *m/z* 289.0720 (calculated 289.0712, Δ 0.0008), establishing the composition $C_{15}H_{12}O_6$ and indicating an increase of 16 mass units in the molecular weight compared to **3**. UV and NMR spectra of **5** had close similarity to those of **3**. The ^{1}H NMR spectrum (see Table 3.2b) resembled that of **3** except for the absence of one *meta*-coupled pair of aromatic protons and the presence of an aromatic proton singlet at δ_H 6.82 assigned for H-5`. In addition, the ^{1}H and ^{13}C NMR spectra (Table 3.2b and 3.3) showed a methoxy group at δ_H 3.99 and δ_C 56.3, an aromatic methyl group at δ_H 2.74 and δ_C 24.9 and a pair of aromatic protons at δ_H 7.30 (*J*=1.8 Hz) and 6.63 (*J*=1.8 Hz) assigned to H-6 and H-4, respectively. Interpretation of the HMBC spectrum (see Table 3.2b and Figure 3.4) showed that correlations of the *meta*-coupled protons H-4 and H-6 were identical to those observed for **3**. On the other hand, H-5` correlated to C-1`, C-3`, C-4` and 6`-*C*H$_3$ with an upfield shift observed for C-4`and a downfield shift for C-3` compared to the corresponding chemical shifts in compounds **1**-**4**. These findings suggested the presence of an additional hydroxy substitution on the aromatic ring which was placed at C-3`. This was further supported by the upfield shifts of C-2` and C-6` in the ^{13}C NMR spectrum. Thus compound **5** was identified as the new natural product 3`-hydroxyalternariol-5-*O*-methyl ether.



Nr.	Compound	R_1	R_2	R_3
1	Alternariol	H	H	H
2	Alternariol-5-*O*-sulphate	H	H	SO_3H
3	Alternariol-5-*O*-methyl ether	H	H	CH_3
4	Alternariol-5-*O*-methyl ether-4`-*O*-sulphate	H	SO_3H	CH_3
5	3`-Hydroxyalternariol-5-*O*-methyl ether	OH	H	CH_3

Table 3.2a: ^{1}H NMR and HMBC data of compounds **1**-**2** at 500 MHz

Nr.	1		2	
	δ_H (MeOD)	HMBC	δ_H (MeOD)	HMBC
4	6.32,d (2.0)	2,3,6	6.86,d (2.0)	2,3,5,6
6	7.20,d (2.0)	2,4,5,1`	7.83,d (2.0)	2,4,5
3`	6.55,d (2.5)	1`,2`,4`,5`	6.57,d (2.0)	1`,2`,4`,5`
5`	6.65,d (2.5)	CH_3, 1`,3`,4`	6.67,d (2.0)	CH_3, 1`,3`,4`
CH_3	2.71,s	1`,2`,3`,5`,6`,6	2.75,s	1`,2`,3`,5`,6`,6

Table 3.2b: ^{1}H NMR and HMBC data of compounds **3**-**5** at 500 MHz

Nr.	3		3[a]	4		5	
	δ_H (MeOD)	HMBC	δ_H ($CDCl_3$)	δ_H (MeOD)	HMBC	δ_H (DMF-d_7)	HMBC
4	6.54,d (1.8)	2,3,5,6	6.3,d (2.0)	6.52,d (2.5)	2,3,6	6.63, d (1.8)	2,3,5,6
6	7.28,d (1.8)	2,4,5,1`	7.1,d (2.0)	7.27,d (2.5)	2,4,5,1`	7.30, d (1.8)	2,4,5,1`
3`	6.61,d (2.5)	1`,2`,4`,5`	6.5,d (2.0)	7.14,d (2.5)	2`,4`,5`		
5`	6.70,d (2.5)	CH_3, 1`,3`,4`	6.5,d (2.0)	7.08,d (2.5)	CH_3, 1`,3`,4`	6.82, s	CH_3, 1`,3`,4`
CH_3	2.76,s	1`,5`,6`,6	2.62,s	2.74,s	1`,5`,6`,6	2.74, s	1`,2`,3`,5`,6`,6
OCH_3	3.91,s	5	3.79,s	3.84,s	5	3.99, s	5
3-OH						12.03, br s	

a) Onocha *et al.*, 1995.

Table 3.3: ^{13}C NMR data of compounds **1**-**5** at 125 MHz

Nr.	1	1[a]	2	3	4	5
	δ_C (MeOD)	δ_C (DMSO-d_6)	δ_C (MeOD)[b]	δ_C (MeOD)[b]	δ_C (MeOD)	δ_C (DMF-d_7)
1	139.8	138.1			138.8	139.5
2	99.1	97.4	101.6	99.6	100.5	99.2
3	166.1	164.1		165.8	165.8	165.4
4	101.9	100.9	106.0	99.7	100.9	99.7
5	166.8	165.5	160.1	167.7	168.1	167.3
6	105.4	104.4	108.0	104.5	105.9	104.2
7	166.8	164.7				165.8
1`	110.9	109.0	109.7	110.5	115.1	110.1
2`	154.4	152.6	153.2	154.3	153.6	142.3
3`	102.7	101.6	101.3	102.4	108.8	132.4
4`	159.8	158.4	159.0	159.6	154.5	148.2
5`	118.5	117.6	117.6	118.3	122.9	117.7
6`	140.0	138.3	139.0	139.3	139.5	127.3
CH_3	25.8	25.3	24.6	25.3	25.7	24.9
OCH_3				55.9	56.3	56.3

a) Stinson *et al.*, 1986.
b) Derived from HMBC spectrum.

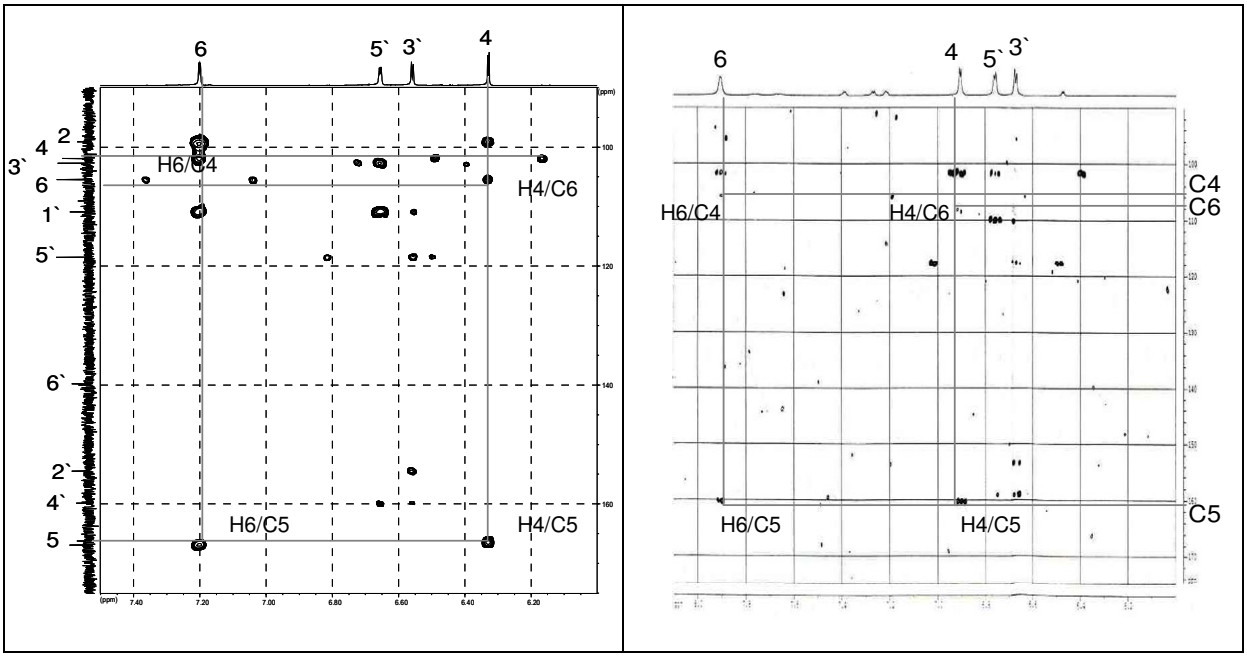


Figure 3.2: HMBC spectra of compounds **1** and **2**.

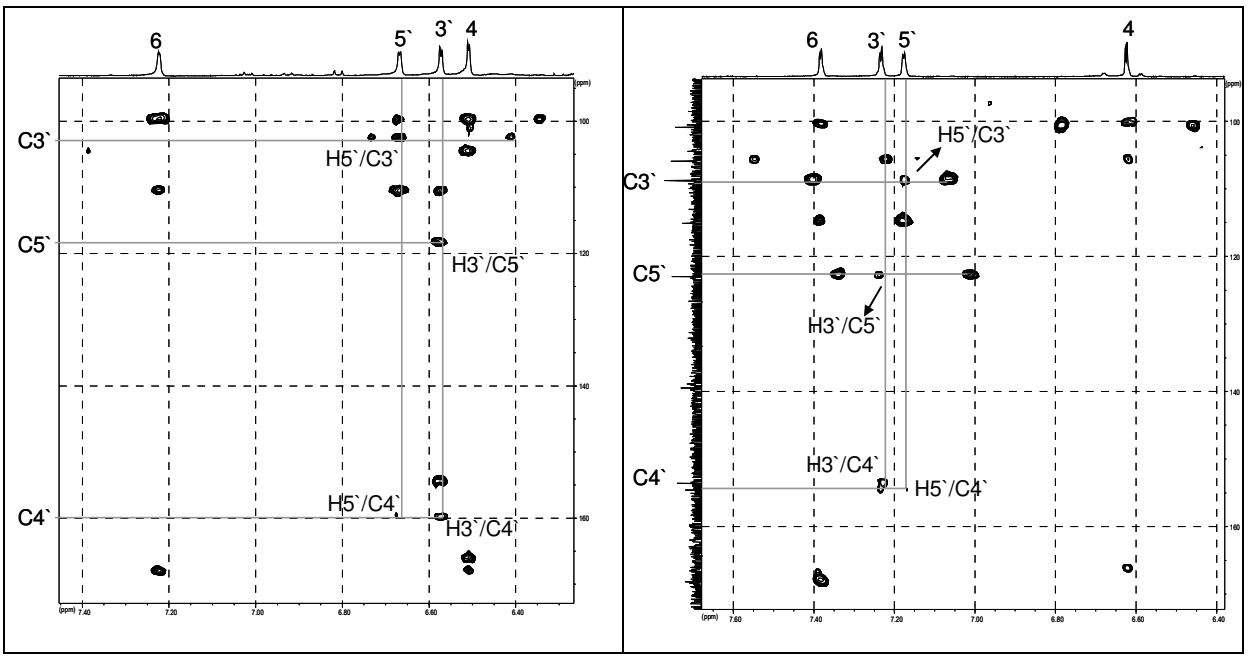


Figure 3.3: HMBC spectra of compounds **3** and **4**.

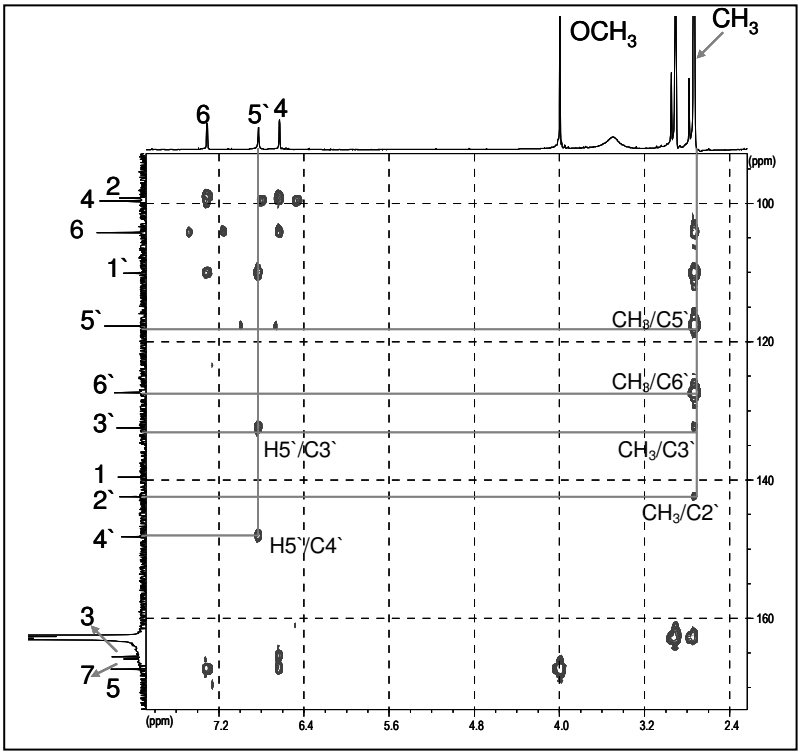
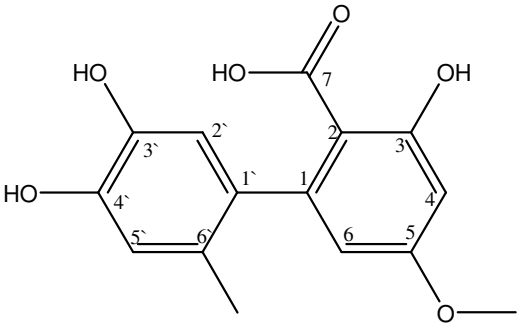
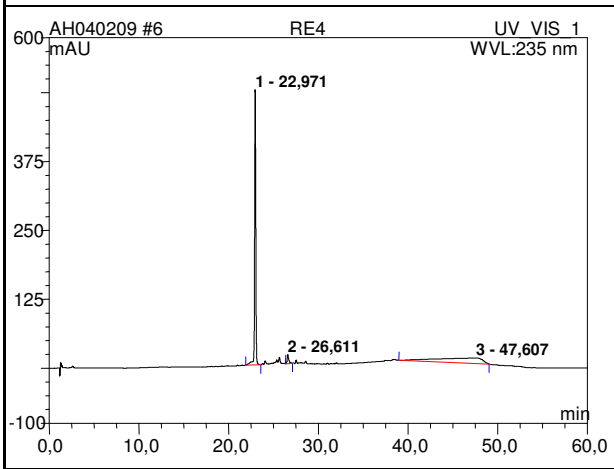


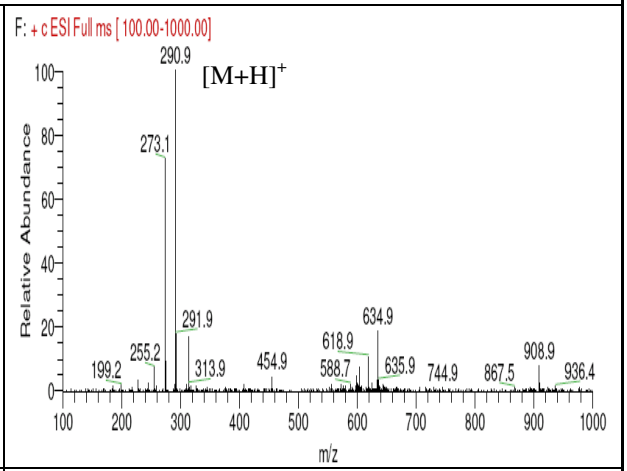
Figure 3.4: HMBC spectra of compound **5**.

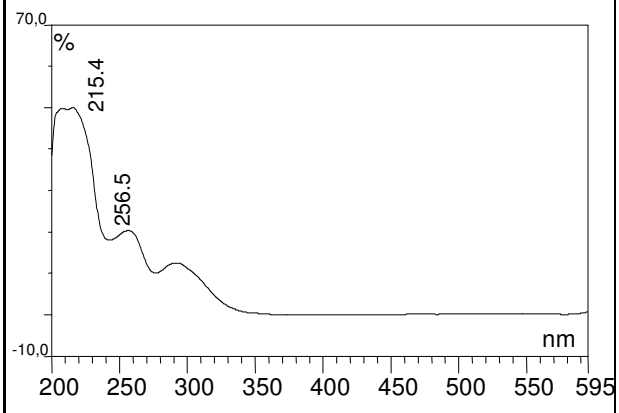
3.1.6. Altenusin (6, known compound)

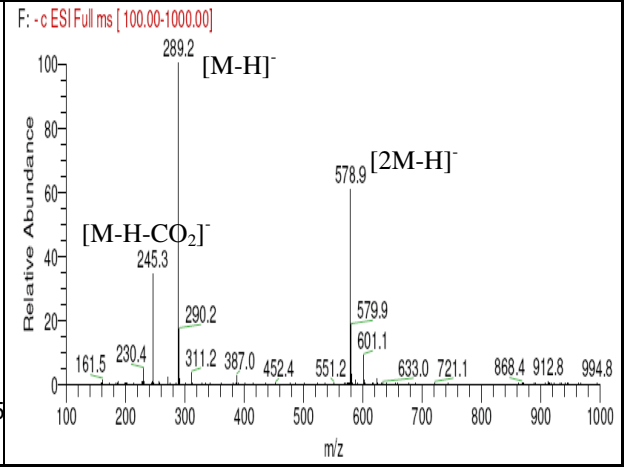
Altenusin	
Synonym(s)	3,4',5'-Trihydroxy-5-methoxy-2'-methyl[1,1'-biphenyl]-2-carboxylic acid
Sample code	RE4, RR7, RR8.2
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	377.6 mg
Physical Description	reddish white prisms
Molecular Formula	$C_{15}H_{14}O_6$
Molecular Weight	290 g/mol
Retention time HPLC	23.0 min (standard gradient)







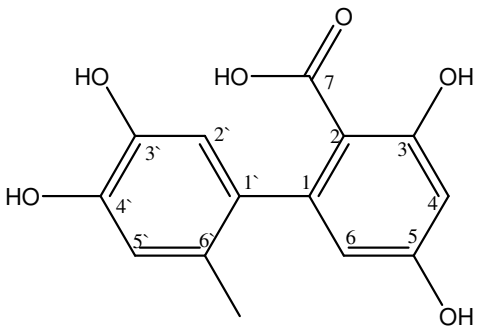


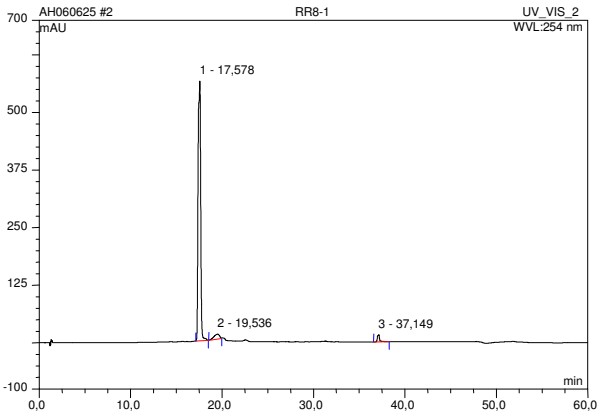


Altenusin (**6**) was isolated from the EtOAc extracts of liquid and rice cultures of *Alternaria* sp. in the form of reddish white prisms (377.6 mg). It exhibited UV absorbances at λ_{max} (MeOH) 202.6, 214.6, 256.9 and 293.0 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 290.9 $[M+H]^+$ (base peak) and *m/z* 289.2 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 290 g/mol. The ^1H NMR spectrum (see Table 3.4) showed two aromatic singlets at δ_H 6.57 and 6.47 assigned to H-5` and H-2`, respectively, as well as *meta*-related hydrogens at δ_H 6.42 and 6.16 (each doublet, J=2.5 Hz) corresponding to H-4 and H-6, respectively. A methoxy signal was seen at δ_H 3.79 and an aromatic methyl at δ_H 1.89. The signal at δ_C 174.3 in the ^{13}C NMR spectrum indicated the presence of a carboxy group (C-7). This was further confirmed by the presence of a fragment at *m/z* 245.3 $[M-CO_2-H]^-$ in the negative ESI-MS. The ^{13}C NMR spectrum (see Table 3.4) revealing the presence of 15 carbon atoms together with ^1H NMR and mass spectra supported a molecular formula of $C_{15}H_{14}O_6$. The structure was established on basis of the HMBC data (see Table 3.4 and Figure 3.5) showing correlations of the *meta*-coupled protons, H-4 to C-2, C-3, C-5 and C-6 as well as H-6 to C-2 and C-4, thereby establishing the structure of one aromatic ring. The methoxy group was found to correlate to C-5 thereby indicating its location there. Furthermore, from the correlations of the aromatic methyl group to C-1`, C-5` and C-6`, H-5` to C-1`, C-3`, C-4` and 6`-CH_3 as well as those observed for H-2` to C-3`, C-4` and C-6` the structure of the second aromatic ring was deduced. The correlation of H-6 to C-1` and that of H-2` to C-1 established the C1-C1` bond attaching both rings. The obtained UV, ^1H, ^{13}C NMR and mass spectral data were found to be identical with published data for altenusin (Nakanishi *et al.*, 1995), previously reported from *Alternaria* (Coombe *et al.*, 1970) and *Penicillium* species (Nakanishi *et al.*, 1995).

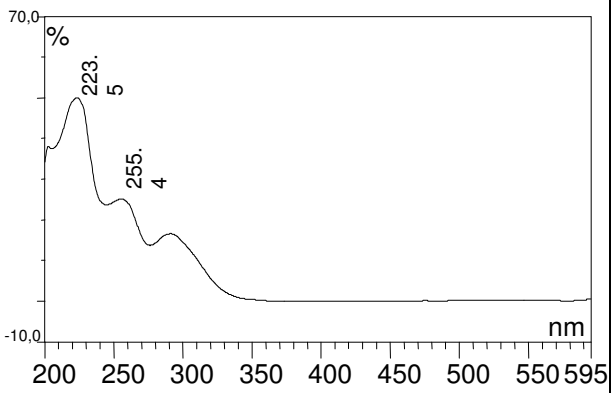
3.1.7. Desmethylaltenusin (7, new compound)

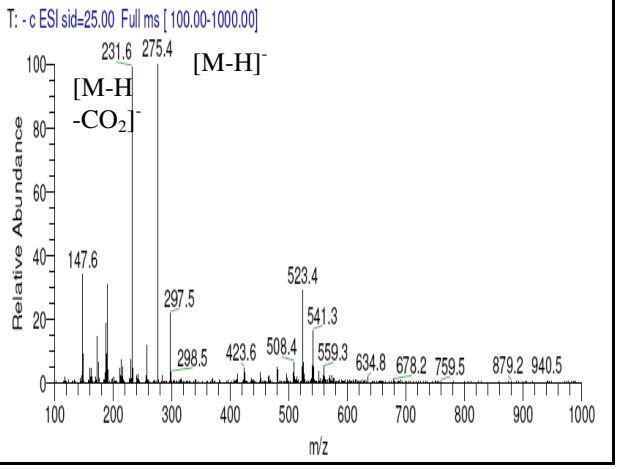
Desmethylaltenusin	
Synonym(s)	3,5,4',5'-Tetrahydroxy-2'-methyl[1,1'-biphenyl]-2-carboxylic acid
Sample code	RR8.1
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	5.6 mg
Physical Description	viscous reddish oil
Molecular Formula	$C_{14}H_{12}O_6$
Molecular Weight	276 g/mol
Retention time HPLC	17.6 min (standard gradient)



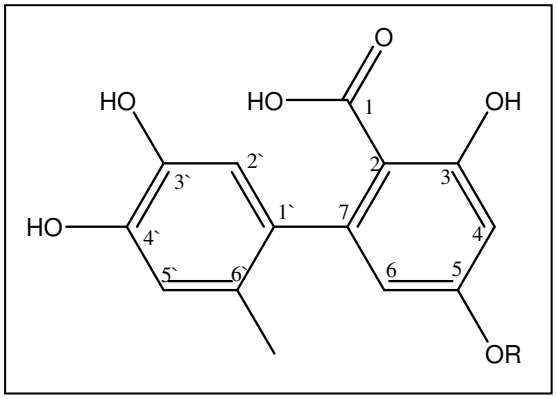


(+)-ESI-MS: no ionization





Compound **7** was isolated from the EtOAc extract of rice cultures of *Alternaria* sp. as viscous reddish oil (5.6 mg). It displayed UV absorbances at λ_{max} (MeOH) 202.6, 223.5, 255.4 and 293.0 nm. The HR-MS exhibited a strong peak at *m/z* 299.0520 $[M+Na]^+$ indicating a molecular formula of $C_{14}H_{12}O_6$ (calculated 299.0531, Δ 0.0011) as well as a loss of 14 mass units compared to altenusin (**6**). Both compounds showed similar UV spectra. Comparing the ^1H and ^{13}C NMR spectra of **7** with those of **6** (see Table 3.4) suggested a close relationship between both structures. The main difference between **7** and **6** was the absence of the methoxy group signal of **6** and the upfield shifts observed for C-4 and C-6, by 1.8 and 3.2 ppm, respectively, as well as for their corresponding *meta*-related hydrogens at δ_H 6.25 and 6.03 (each doublet, *J*=2.2 Hz), respectively. Two aromatic singlets at δ_H 6.55 and 6.46 assigned to H-5` and H-2`, respectively, were also detected in the ^1H NMR spectrum, as well as an aromatic methyl signal at δ_H 1.90. The structure was further confirmed by interpretation of the HMBC spectrum (see Table 3.4 and Figure 3.6) which showed that all observed correlations were corresponding to those observed in the spectrum of **6** except for the lacking methoxy group. In addition, the characteristic fragment at *m/z* 231.6 $[M-CO_2-H]^-$ observed in the negative ESI-MS confirmed the presence of a carboxylic group substituent as in case of altenusin. Thus the compound was confirmed to be desmethylaltenusin, representing a new natural product.



Nr.	Compound	R
6	Altenusin	CH_3
7	Desmethylaltenusin	H

Table 3.4: ^{1}H,^{13}C NMR and HMBC data of compounds **6** and **7** at 500 (^{1}H) and 125 MHz (^{13}C)

Nr.	6		6[a]			7		
	δ_H (MeOD)	HMBC	δ_C (MeOD)	δ_H (DMSO-d_6)	δ_C (DMSO-d_6)	δ_H (MeOD)	HMBC	δ_C (MeOD)
1			148.0		145.0			148.3
2			107.0		108.8			106.5
3			164.9		161.6			163.0
4	6.42, d (2.5)	2,3,5,6	100.5	6.43, d (2.7)	99.6	6.25, d (2.2)	2,3,5,6	102.3
5			165.7		162.0			165.7
6	6.15, d (2.5)	2,4,1`	111.4	6.10, d (2.7)	108.9	6.03, d (2.2)	2,4,1`	112.1
7			174.3		171.6			
1`			135.3		132.4			135.8
2`	6.47, s	3`,4`,6`,1	116.6	6.42, s	115.9	6.46, s	3`,4`,6`,1	116.6
3`			143.2		142.1			143.2
4`			144.9		143.9			144.7
5`	6.57, s	CH_3, 1`,3`,4`	117.3	6.54, s	116.6	6.55, s	CH_3, 1`,3`,4`	117.2
6`			127.3		124.9			127.3
CH_3	1.89, s	1`,5`,6`	19.2	1.86, s	18.8	1.90, s	1`,5`,6`,3`	19.3
OCH_3	3.79, s	5	55.9	3.76, s	55.3			

a) Nakanishi *et al.*, 1995.

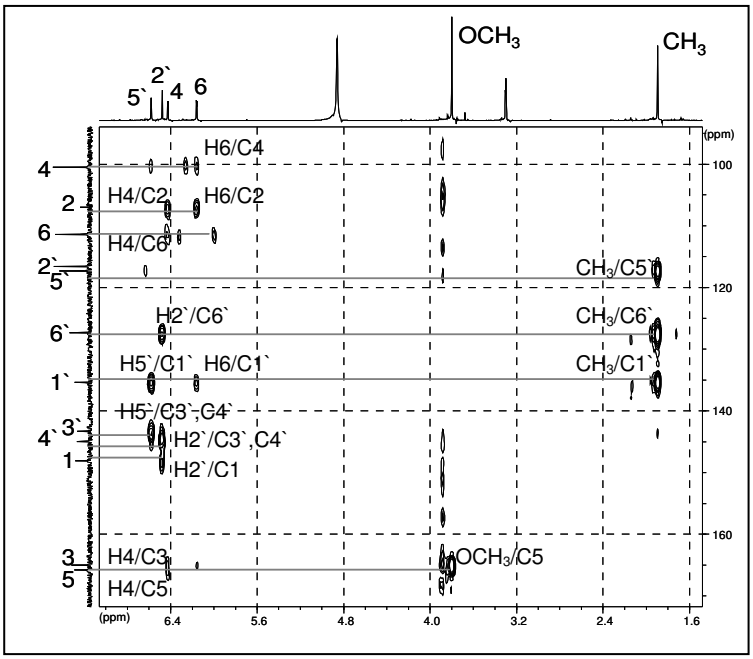


Figure 3.5: HMBC spectra of compound **6**.

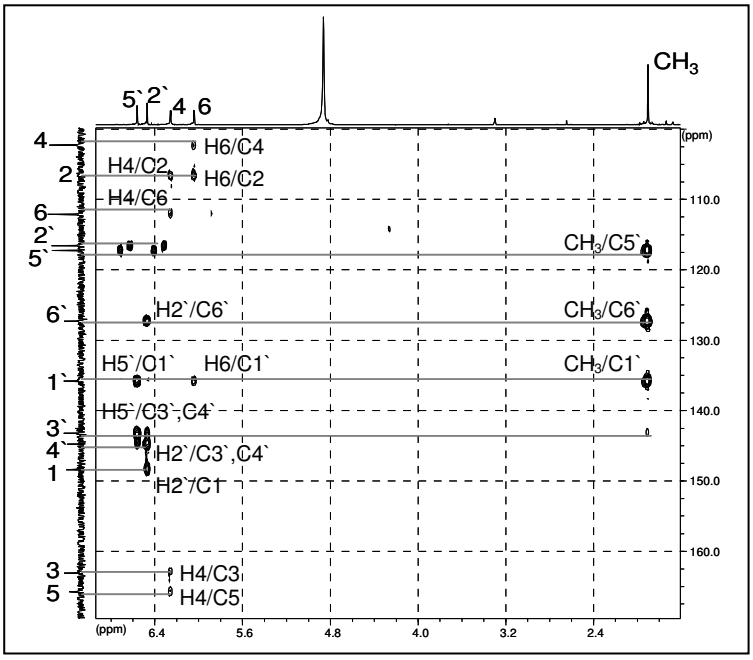
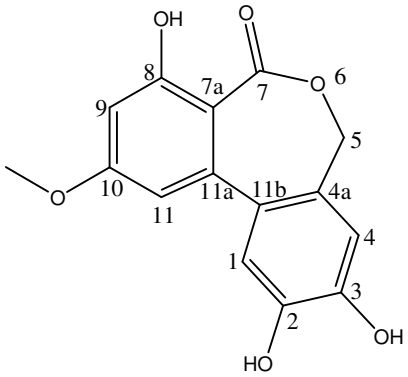
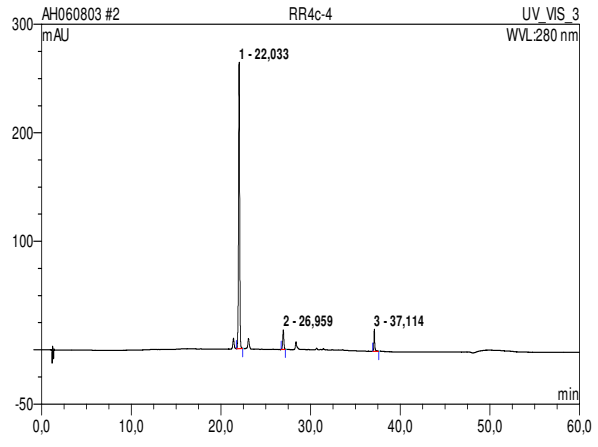


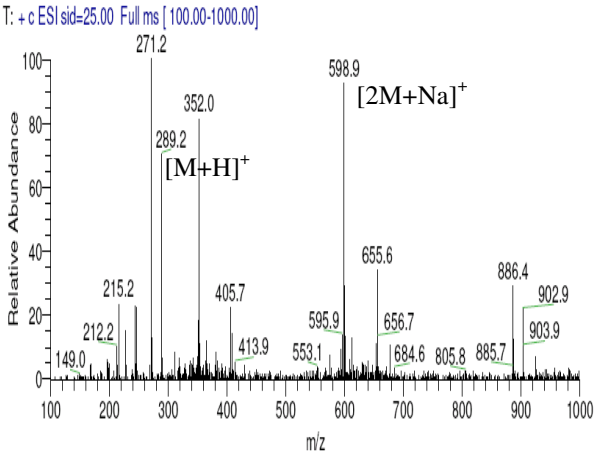
Figure 3.6: HMBC spectra of compound **7**.

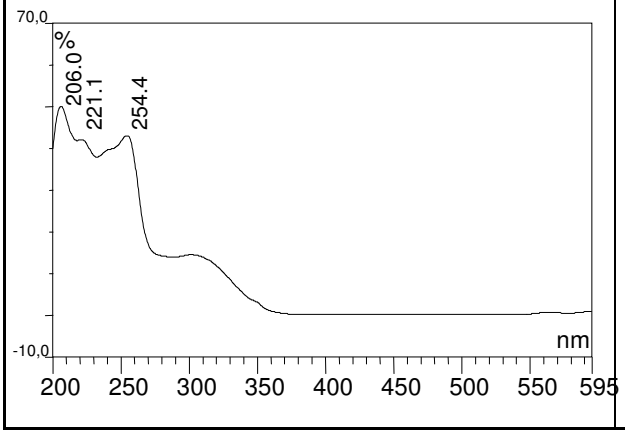
3.1.8. Alterlactone (8, new compound)

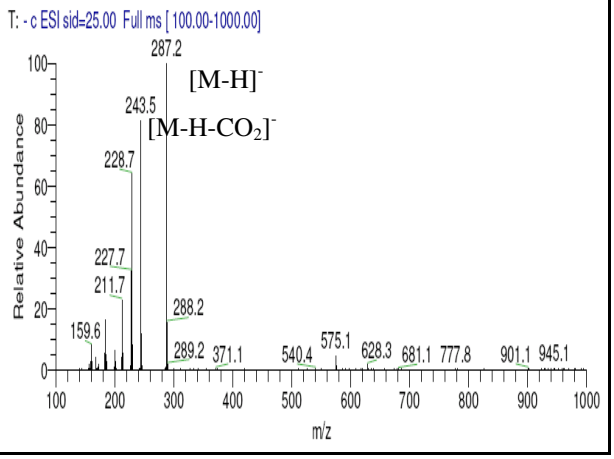
Alterlactone	
Sample code	RR3.2
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	8.4 mg
Physical Description	white flakes
Molecular Formula	$C_{15}H_{12}O_6$
Molecular Weight	288 g/mol
Retention time HPLC	22.0 min (standard gradient)



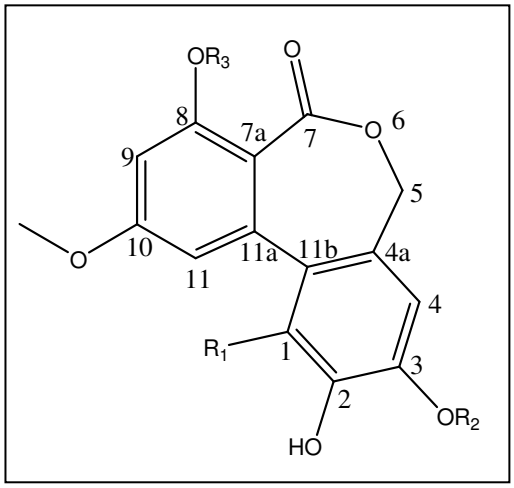








Compound **8** was isolated from the EtOAc extract of rice cultures of *Alternaria* sp. in form of white flakes (8.4 mg). The UV spectrum showed absorbances at λ_{max} (MeOH) 206.0, 221.1 and 254.4 nm. The HRESI-MS gave a $[M+H]^+$ at *m/z* 289.0700 (calculated 289.0712, Δ 0.0012), indicating the molecular formula to be $C_{14}H_{14}O_6$. Its ^{13}C NMR spectrum (see Table 3.5) displayed one methoxy group, twelve aromatic carbons, a carbonyl function and an oxygenated methylene group corresponding to a pair of doublets at δ_H 4.80 and 4.85 (J=11.1 Hz) in the ^1H NMR spectrum. This could be accounted for by assuming that the methylene protons are situated on a ring and their non-equivalence must result from a steric factor of the biphenyl system. Confirmation was achieved by an HMBC ^3J correlation of the methylene protons with the carbonyl carbon (C-7) suggesting that the lactone ring was not formed through connection of the carbonyl carbon to the phenolic hydroxy group as in the previously discussed alternariol derivatives (**1**-**5**), but instead the carbonyl carbon was linked through an ester to the hydroxymethyl group to construct an additional seven-membered lactone ring. The *meta*-coupled hydrogens, observed in the ^1H NMR spectrum at δ_H 6.45 and 6.50 (each doublet, J=2.2 Hz), were assigned to H-9 and H-11, respectively. Both protons showed ROESY correlations (see Table 3.5 and Figure 3.8) to the methoxy group indicating its location at C-10. In addition, the correlations observed for H-9 and H-11 to C-7a in the HMBC spectrum (see Table 3.5 and Figure 3.7), as well as the chelated nature of 8-OH deduced from its appearance at δ_H 10.21, confirmed the attachment of the aromatic ring to the lactone ring at C-7a. Furthermore, the hydroxy substituents were placed in *ortho*-position at C-2 and C-3 on basis of the chemical shift values of the carbon atoms appearing at δ_C 146.6 and 145.9, respectively. The neighboring position of H-1 and H-4, observed at δ_H 7.03 and 6.90 in the ^1H NMR spectrum, respectively, was deduced from their HMBC correlations to C-3 and C-2, respectively. Furthermore, H-4 showed both ROESY and HMBC correlations to the methylene group as well as HMBC correlations to C-4a and C-11b. This together with the ROESY correlation observed between H-1 and H-11 as well as the HMBC correlations of H-11 to C-11b and those of H-1 to C-4a, C-11a and C-11b indicated the attachment of the aromatic rings through the C11a-C11b bond. The structure was further confirmed by comparing NMR data of **8** to those reported for ulocladol (**8a**) (Höller *et al.*, 1999) and graphislactone D (**8b**) (Tanahashi *et al.*, 1997). Both compounds differ from **8** in having an additional hydroxy function at C-1 as well as methoxy groups at C-3 (both) and C-8 (graphislactone D). Thus the compound was identified as the new natural product to which we propose the name alterlactone. It is worth mentioning that this is only the third report for isolation of this carbon skeleton in nature.



Nr.	Compound	R_1	R_2	R_3
8	Alterlactone	H	H	H
8a	Ulocladol	OH	CH_3	H
8b	Graphislactone D	OH	CH_3	CH_3

Table 3.5: ^{1}H,^{13}C NMR, ROESY and HMBC data of compound **8** at 500 (^{1}H) and 125 MHz (^{13}C)

Nr.	8				8b[a]	
	δ_H (DMSO-d_6)	ROESY	HMBC	δ_C (DMSO-d_6)	δ_H (DMSO-d_6)	δ_C (DMSO-d_6)
1	7.03, s	11	3,4a,11a,11b	115.5		143.8
2				146.6		135.7
3				145.9		147.6
4	6.90, s	5	2,3,5,11b,4a	115.5	6.78, s	103.9
4a				140.0		135.1
5	4.80, d (11.3)	4	4,7,11b	67.8	4.74, dd (12.0)	68.3
	4.85, d (11.0)				4.78, dd (12.0)	
7				168.7		166.1
7a				109.5		113.2
8				159.9		158.6
9	6.45, d (2.2)	OCH_3	7a,8,10,11	100.8	6.66, d (2.0)	97.7
10				162.2		160.6
11	6.50, d (2.2)	OCH_3,1	7a,9,10,11b	105.0	6.89, d (2.0)	106.8
11a				126.6		127.3
11b				129.8		118.2
1-OH					9.10, brs[b)]	
2-OH	9.37, brs[b]				8.98, brs[b)]	
3-OH	9.47, brs[b]					
3-OCH_3					3.84, s	56.0
8-OH	10.21, brs					
8-OCH_3					3.81, s	55.3
10-OCH_3	3.81, s	9,11	10	55.4	3.82, s	55.8

a) Tanahashi *et al.*, 1997.

b) Assignements may be interchanged.

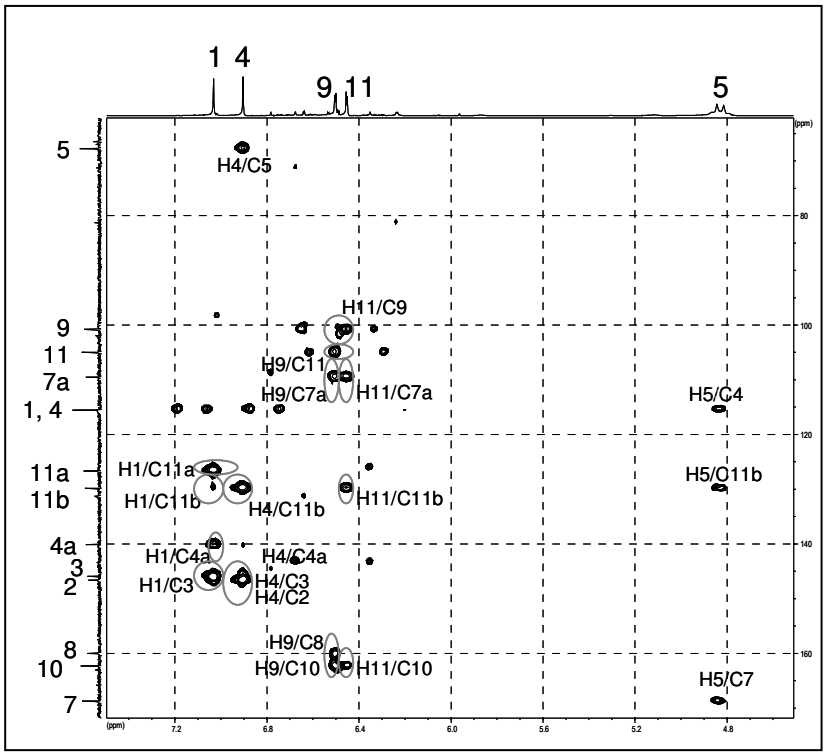


Figure 3.7: HMBC spectrum of compound **8**.

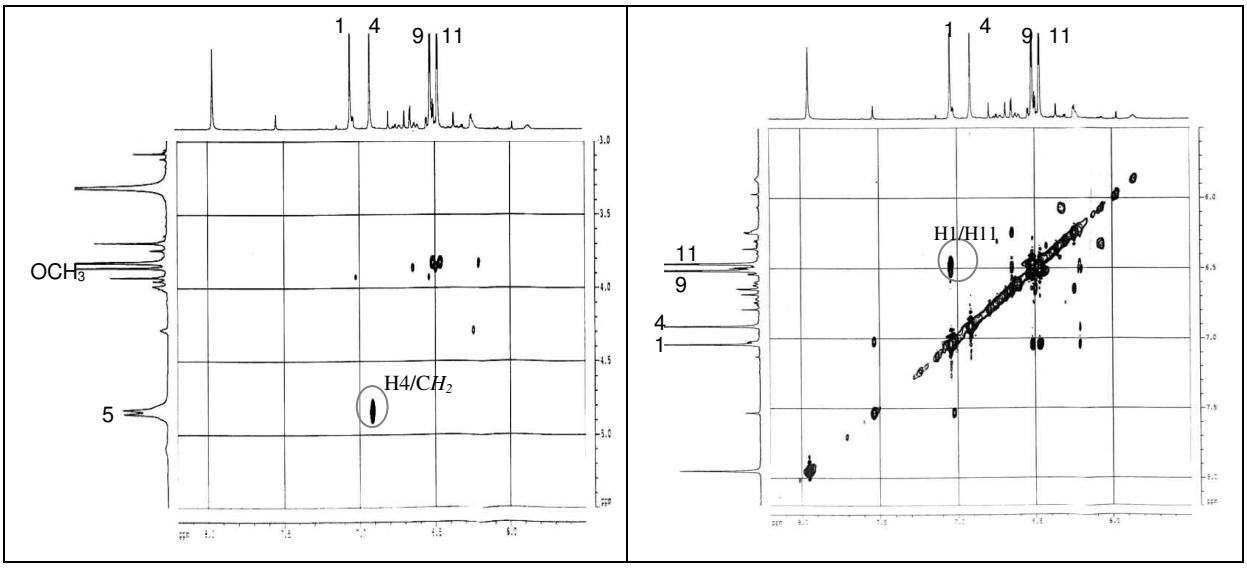
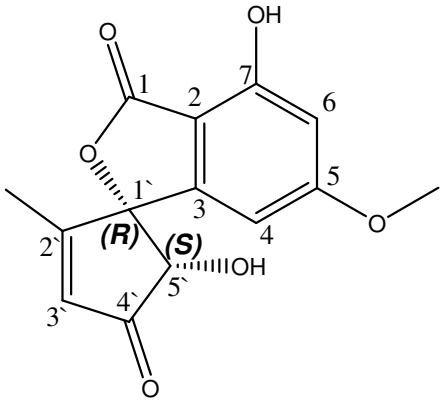
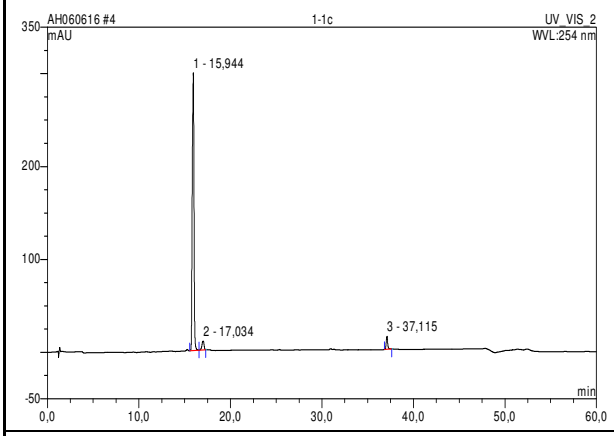


Figure 3.8: ROESY spectrum of compound **8**.

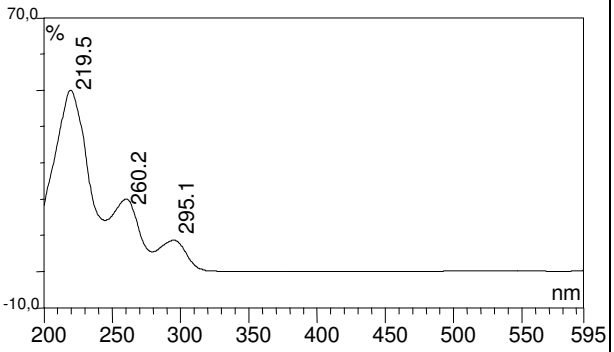
3.1.9. Talaroflavone (9, known compound)

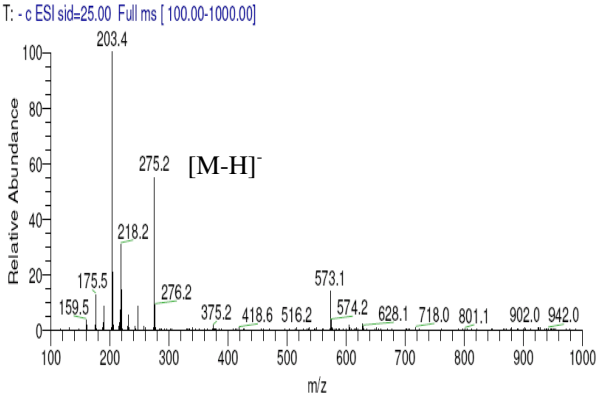
Talaroflavone	
Sample code	RR3.1
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	5.0 mg
Physical Description	white flakes
Molecular Formula	$C_{14}H_{12}O_6$
Molecular Weight	276 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 179 (*c* 0.7, MeOH)
Retention time HPLC	15.9 min (standard gradient)





(+)-ESI-MS: no ionization





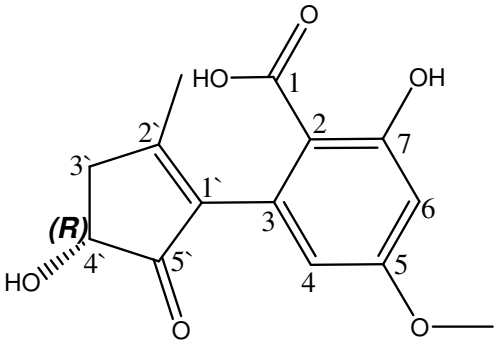
Talaroflavone (**9**) was isolated from the EtOAc extract of rice cultures of *Alternaria* sp. in the form of white flakes (5.0 mg). It displayed UV absorbances at λ_{max} (MeOH) 219.5, 260.2 and 295.1 nm. The negative ESI-MS showed a *pseudo*-molecular ion peak at *m/z* 275.2 $[M-H]^-$ (base peak) indicating a molecular weight of 276 g/mol. ^{1}H NMR and mass spectra supported a molecular formula of $C_{14}H_{12}O_6$. The ^{1}H NMR spectrum exhibited *meta*-coupled protons at δ_H 6.43 (broad singlet) and 6.04 (doublet, J=1.2 Hz) assigned to H-4 and H-6, respectively, as well as a methoxy signal at δ_H 3.79. These ^{1}H NMR data (see Table 3.6) resembled those of the aromatic portion of metabolites **6** and **7**. Furthermore, a carbinolic hydrogen at δ_H 4.73 and a ^{13}C NMR signal at δ_C 79.0 were consistent with the secondary alcohol group at C-5`. Signals in the $^{13}$C NMR (see Table 3.7) spectrum at $\delta_C$ 201.0 (C-4`), 172.0 (C-2`), 130.5 (C-3`) and 13.0 (2`-*C*H$_3$), and $^1$H NMR resonances at $\delta_H$ 1.85 (2`-C*H$_3$*) and 6.34 (H-3`) were consistent with a 3-methylcyclopentenone. This substructure was further confirmed by interpretation of the HMBC spectrum (see Table 3.6) showing correlations of 2`-C*H$_3$* to C-1`, C-2` and C-3`, H-3` to C-1`, C-4` and C-5` as well as H-5` to C-1`and C-4`. The degrees of unsaturation calculated from the molecular formula were found to be 9 [(2C+2-H)/2], where the aromatic ring and the cyclopentenone account for 4 and 3 degrees of unsaturation, respectively. A third ring, joining the aromatic and enone portions, incorporated the quaternary spiro carbon at δ_C 94.0 (C-1`) and the ester carbonyl group (C-1), thus accounting for the remaining two degrees of unsaturation inspired by the molecular formula. This was also supported by the correlation observed for H-4 to C-1` in the HMBC spectrum.

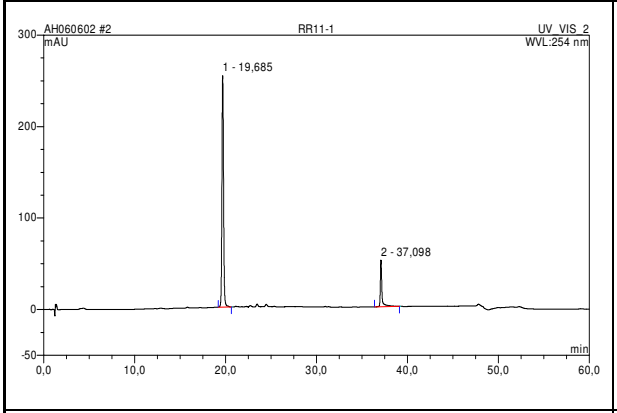
In order to determine the relative configuration of this compound, a NOESY experiment was performed. The 2`-C*H$_3$* group was found to correlate to H-4 and H-3`, additionally, H-5` correlated to H-4 and H-3` (see Table 3.6 and Figure 3.9). Furthermore, for the determination of the absolute configuration of the chiral centre at C-5` the modified Mosher procedure was applied. The observed shift difference between the (*S*)-MTPA ester and its (*R*)-MTPA ester epimer allowed for assigning of the chiral centre at C-5` to have *S*-configuration (see Table 3.6a). Both experiments permitted the assignment of the stereochemistry of talaroflavone to be as shown in **9a**, which is reported for the first time.

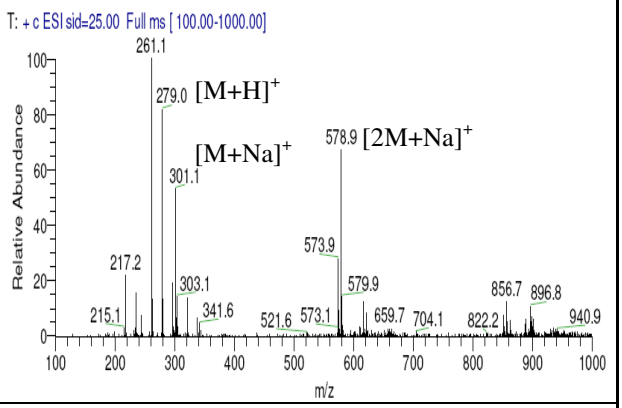
The structure was confirmed by comparison of UV, ^{1}H, ^{13}C NMR and mass spectral data with published data for talaroflavone previously isolated from the soil fungus *Talaromyces flavus* for the first time (Ayer and Racok, 1990a).

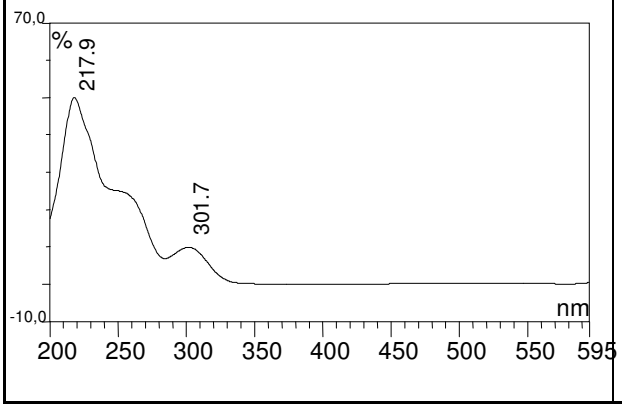
3.1.10. Alternaric acid (10, new compound)

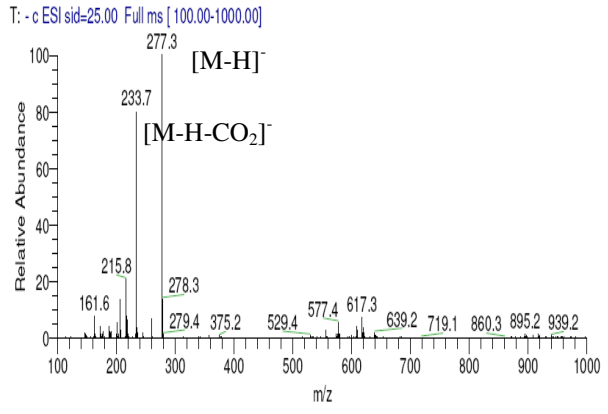
Alternaric acid	
Synonym(s)	2-hydroxy-6-(4-hydroxy-2-methyl-5-oxocyclopent-1-enyl)-4-methoxybenzoic acid
Sample code	RR9.2
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	16.9 mg
Physical Description	viscous yellow oil
Molecular Formula	$C_{14}H_{14}O_6$
Molecular Weight	278 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 75 (*c* 1.0, MeOH)
Retention time HPLC	19.7 min (standard gradient)







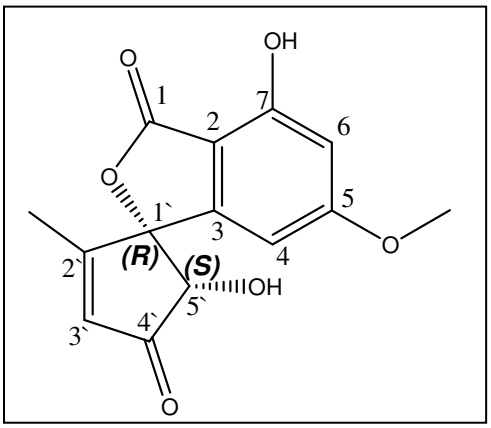




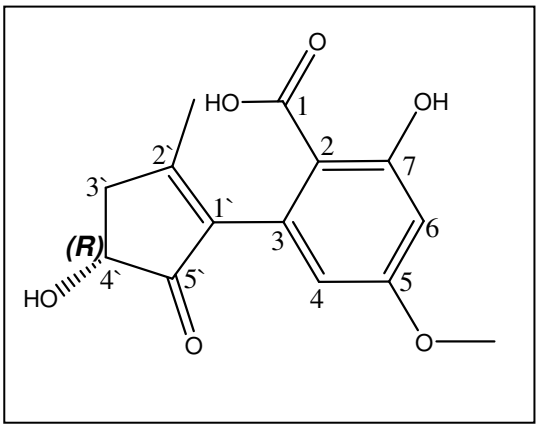
Compound **10** was isolated from the EtOAc extract of rice cultures of *Alternaria* sp. as viscous yellow oil (16.9 mg). It exhibited UV absorbances at λ_{max} (MeOH) 217.9, 260 (sh) and 301.7 nm. The HRESI-MS showed the *pseudo*-molecular ion $[M+Na]^+$ at *m/z* 301.0700 (calculated 301.0688, Δ 0.0012), providing the molecular formula $C_{14}H_{14}O_6$. The ^{1}H NMR spectrum (see Table 3.6) showed *meta*-coupled hydrogens at δ_H 6.40 and 6.07 (each doublet, *J*=2.5 Hz) corresponding to H-6 and H-4, respectively, and a methoxy group at δ_H 3.78, thus resembling ^{1}H NMR data observed for the aromatic portion of metabolites **6**, **7** and **9** (Tables 3.4 and 3.6). Similar to talaroflavone (**9**), a secondary alcohol group was detected, as a carbinolic hydrogen at δ_H 4.38 and a ^{13}C NMR signal at δ_C 73.2, which was found to be part of a saturated spin system by coupling to two methylene hydrogens at δ_H 2.49 (brd, *J*=17.0 Hz) and 3.00 (dd, *J*=6.9, 17.0 Hz). Evidence for this substructure was found in the COSY spectrum (see Table 3.6 and Figure 3.10). A singlet at δ_H 1.99 (3H) in the ^{1}H NMR with a corresponding ^{13}C NMR signal at δ_C 17.8 indicated the presence of a vinyl methyl group. It was located adjacent to the methylene protons on basis of the long range COSY correlations observed for 2`-C*H*$_3$ to 3`-C*H*$_2$ as well as similar correlations appearing in the ROESY spectrum (see Table 3.6, Figure 3.10 and 3.11). The ROESY spectrum showed also strong correlations between the 2`-C*H*$_3$ group and H-4, as well as between the methoxy signal and both H-4 and H-6. Furthermore, interpretation of the HMBC spectrum (see Table 3.6 and Figure 3.12) showed correlations of 2`-C*H*$_3$ to C-1`, C-2` and C-3` as well as H-3` to C-5` confirming the five-membered ring substructure. This was also supported by the correlation observed for H-4 to C-1` in the HMBC spectrum. In addition, the HMBC correlation observed for H-4 to C-1` established the C1`-C3 bond, attaching both rings.

In order to determine the absolute configuration of the metabolite the modified Mosher procedure was applied. The observed shift difference between the (*S*)-MTPA ester and its (*R*)-MTPA ester epimer allowed for the assignment of the chiral centre at C-4` to have *R*-configuration as shown in **10** (see Table 3.6b).

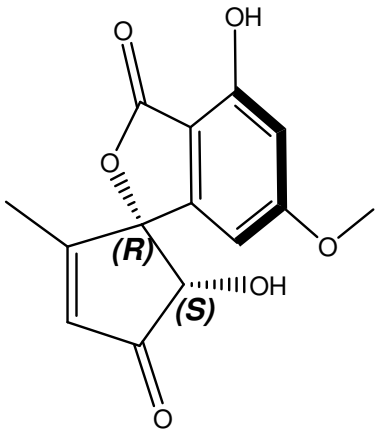
The compound was identified as the new natural product to which we propose the name alternaric acid.



9 Talaroflavone



10 Alteric acid



9a

Table 3.6: ^{1}H NMR, NOESY, COSY, ROESY and HMBC data of compounds **9** and **10** at 500 MHz

Nr.	9				9 [a)]	10			
	δ_H (MeOD)	δ_H (DMSO-d_6)	NOESY	HMBC	δ_H (MeOD)	δ_H (MeOD)	COSY	ROESY	HMBC
1									
2									
3									
4	6.43, brs	6.43, s		2,5,6, 1`	6.44, d (2.0)	6.07, d (2.5)	6	OCH_3,CH_3	2,5,6,1`
5									
6	6.04, d (1.2)	5.90, s		2,4,5,7	6.05, d (2.0)	6.40, d (2.5)	OCH_3,4	OCH_3	2,4,5,7
7									
1`									
2`									
3`	6.34, s	6.38, s		1`,4`,5`	6.34, q (1.5)	A:3.00, dd (6.9, 17.3)	CH_3,3`A,4`	CH_3	1`,2`,4`,5`
						B:2.49, brd (17.0)	CH_3,3`B,4`	CH_3	2`,4`
4`						4.38, dd (2.8, 6.3)	CH_3,3`A,3`B		
5`	4.73, s	4.61, d (6.1)	3`,4	1`,4`	4.74, s				
2`-CH_3	1.85, s	1.74, s	3`,4	1`,2`,3`	1.86, d (1.5)	1.99, s	3`A,3`B,4`		1`,2`,3`,5`
5-OCH_3	3.79, s	3.73, s	4,6	5		3.78, s	6		5
5`-OH		6.08, d (6.1)							
7-OH		11.00, brs							

a) Ayer and Racok, 1990a.

Table 3.6a: Chemical shift difference between the (2`*S*)-MTPA and (2`*R*)-MTPA ester of **9**

Nr.	Chemical shift (δ_H, in C_5D_5N, at 500 MHz)			Δ
	9	**(*S*)-MTPA ester**	**(*R*)-MTPA ester**	**δS - δR**
3`	6.3617	1.7912	1.7279	0.0633
CH_3	1.7953	6.4461	6.4603	- 0.0142

Table 3.6b: Chemical shift difference between the (2`*S*)-MTPA and (2`*R*)-MTPA ester of **10**

Nr.	Chemical shift (δ_H, in C_5D_5N, at 500 MHz)			Δ
	10	**(*S*)-MTPA ester**	**(*R*)-MTPA ester**	**δS - δR**
3`A	3.0503	3.3192	3.3166	0.0026
3`B	2.8074	2.8994	2.7070	0.1924
CH_3	1.9447	1.9352	1.9320	0.0032

Table 3.7: ^{13}C NMR data of compounds **9** and **10** at 125 MHz

Nr.	9		9 [a]	10
	δ_C (DMSO-d_6)	δ_C (MeOD)[d]	δ_C (MeOD)	δ_C (MeOD)
1	166.6		170.2[b)]	164.3
2	104.9	106.2	106.5	109.9
3	149.8		150.8	137.3
4	99.2	100.5	103.5[c]	110.2
5	165.6	168.2	168.5[b]	164.3
6	102.0	102.5	101.0[c]	101.4
7	158.2	159.1	159.9	166.0
1`	91.8	94.0	94.2	142.5
2`	168.7	171.8	171.4[b]	165.2
3`	130.4	130.7	131.4	41.8
4`	200.1	201.5	202.0	73.2
5`	78.2	79.3	79.7	209.0
CH_3	13.0	13.9	13.4	17.8
OCH_3	55.7	56.1	56.5	55.8

a) Ayer and Racok, 1990a.
b), c) Assignements may be interchanged.
d) Derived from HMBC spectrum.

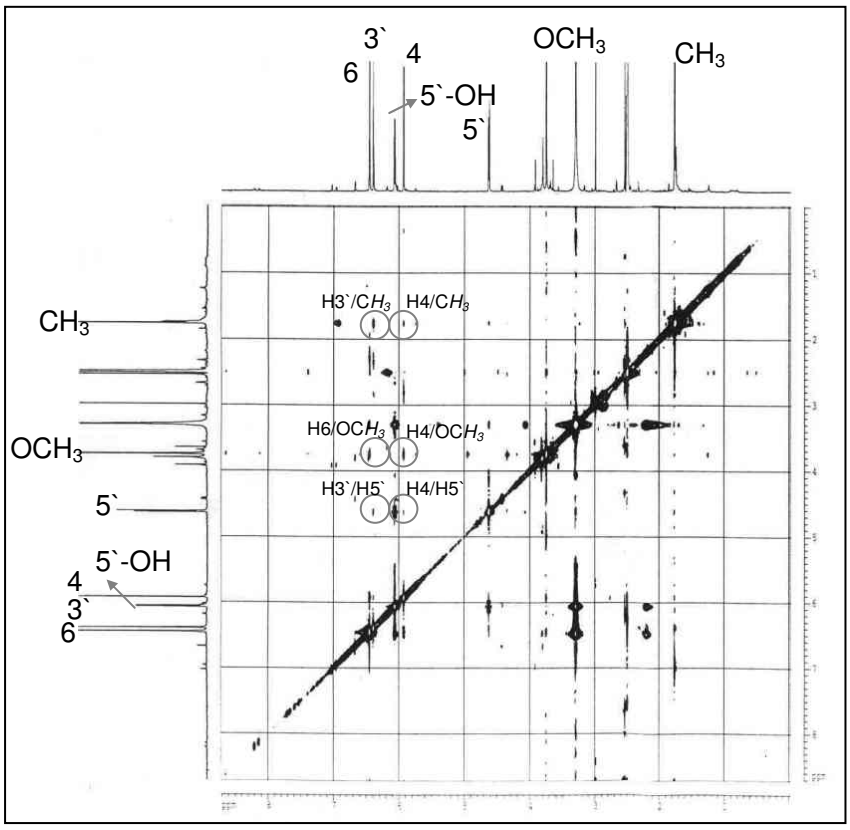


Figure 3.9: NOESY spectrum of compound **9**.

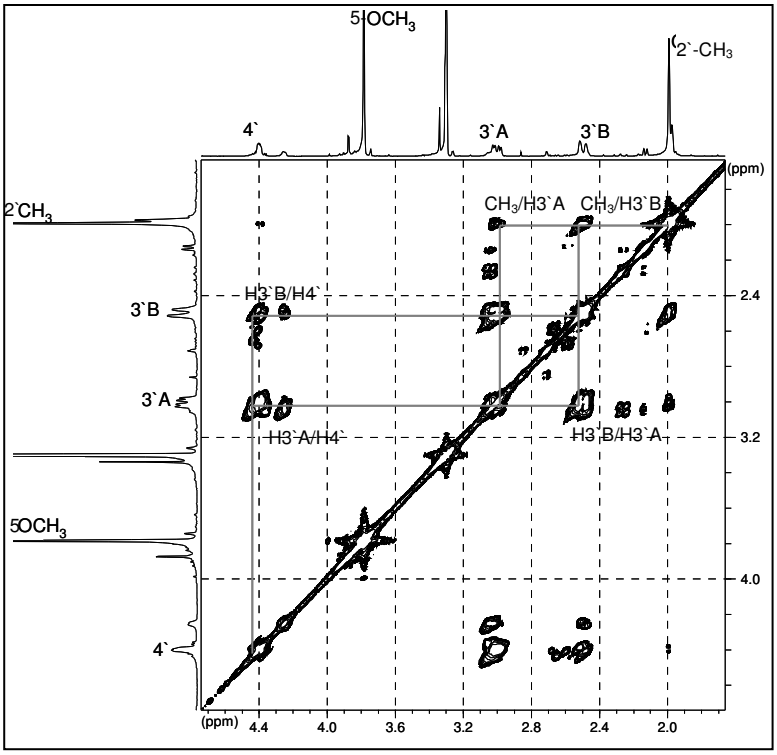


Figure 3.10: COSY spectrum of compound **10**.

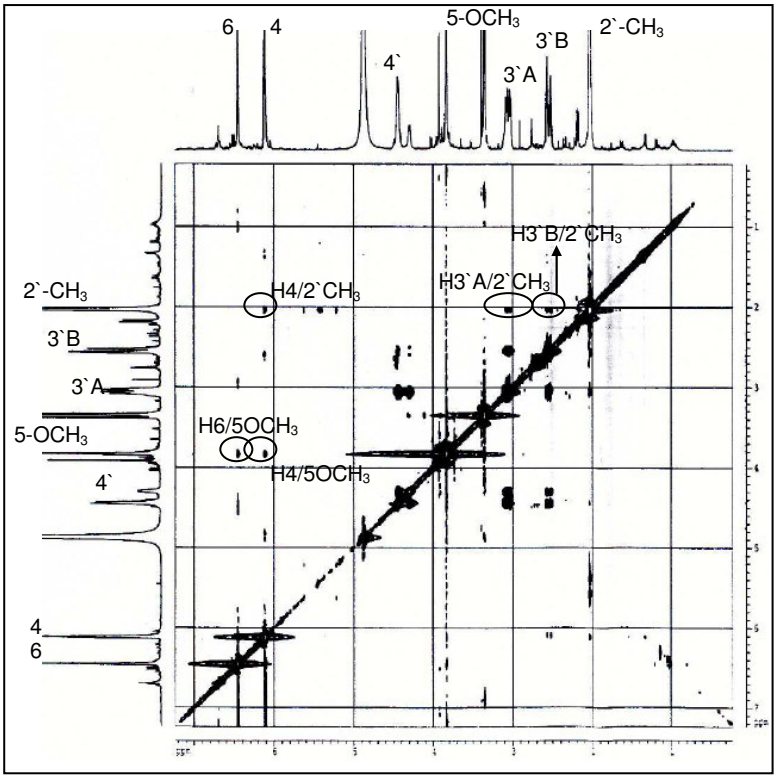


Figure 3.11: ROESY spectrum of compound **10**.

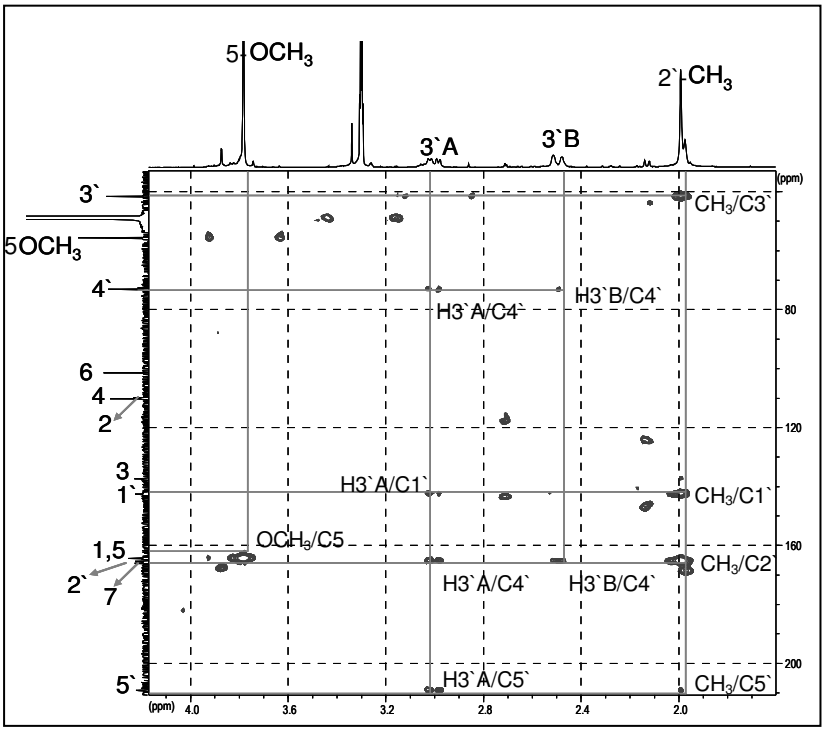
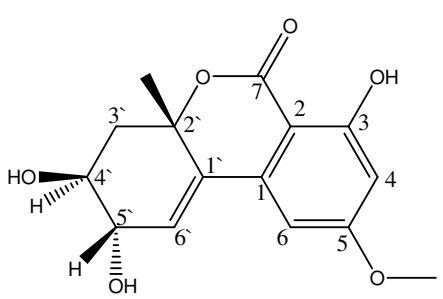


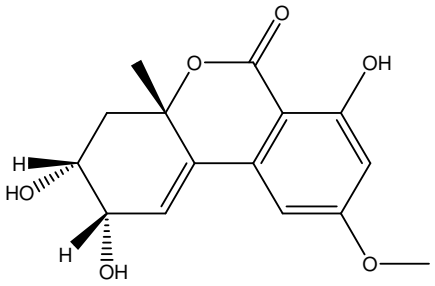
Figure 3.12: HMBC spectrum of compound **10**.

3.1.11. Altenuene and 4`-epialtenuene (11, known, and 12, new compound)

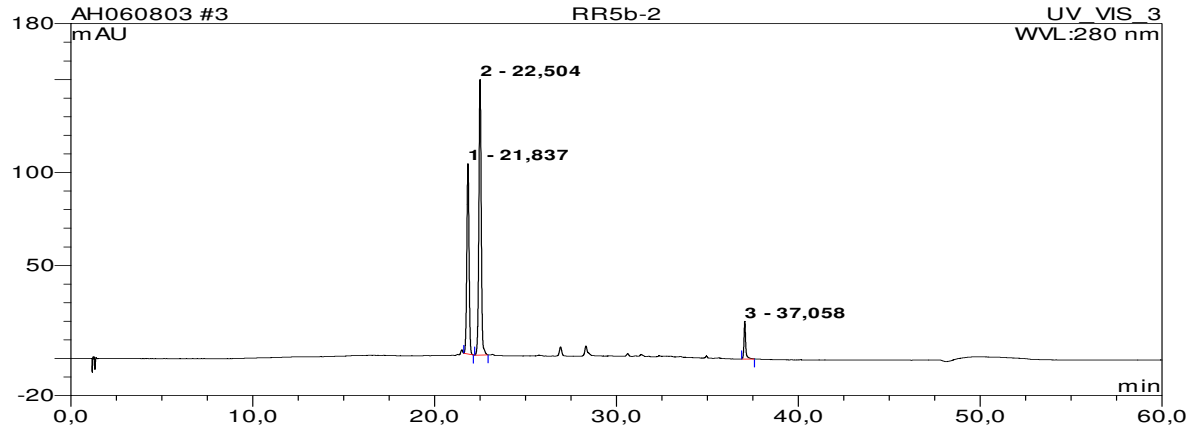
Altenuene and 4`-epialtenuene	
Synonym(s)	2,3,4,4a-Tetrahydro-2,3,7-trihydroxy-9-methoxy-4a-methyl-6H-dibenzo[b,d]pyran-6-one
Sample code	RR5.1
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	18.8 mg
Physical Description	Viscous yellow oil
Molecular Formula	$C_{15}H_{16}O_6$
Molecular Weight	292 g/mol
Optical Rotation $[\alpha]_D^{20}$	racemic
Retention time HPLC	21.8 (4`-epialtenuene), 22.5 min (altenuene) (standard gradient)

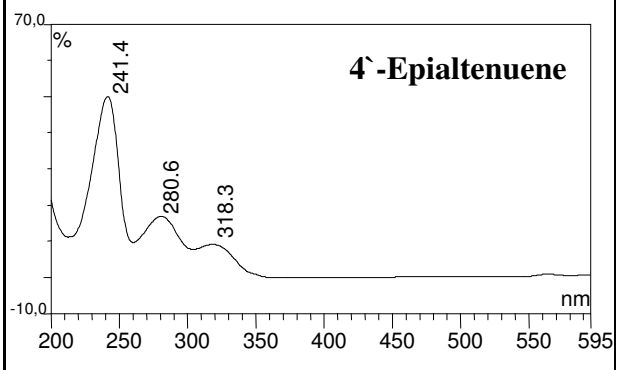


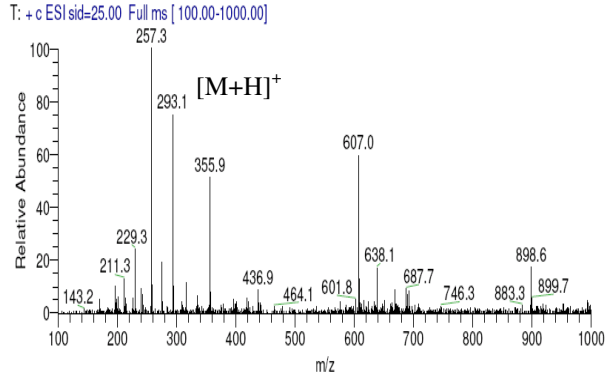
Altenuene

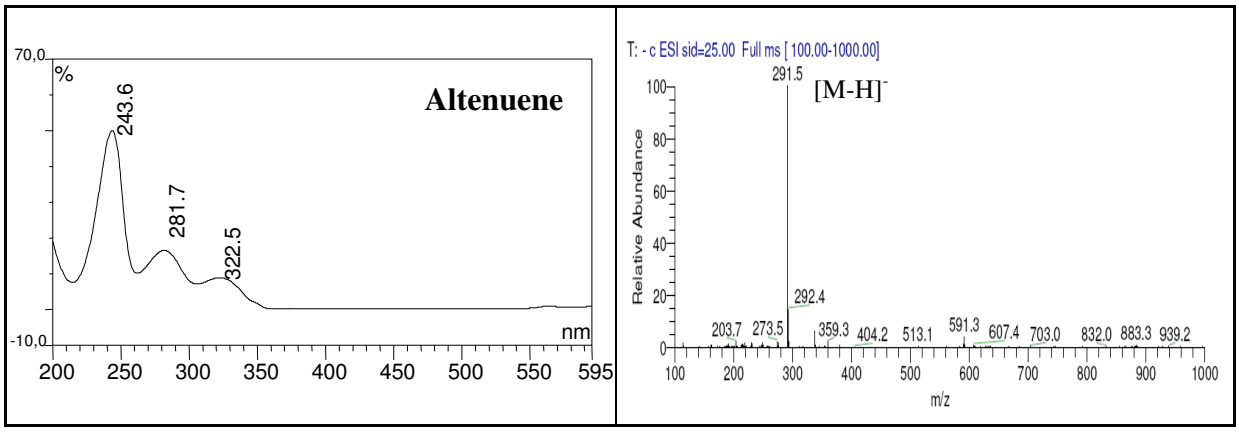


4`-Epialtenuene



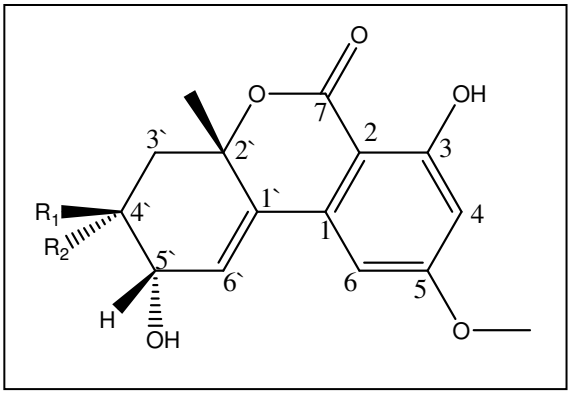




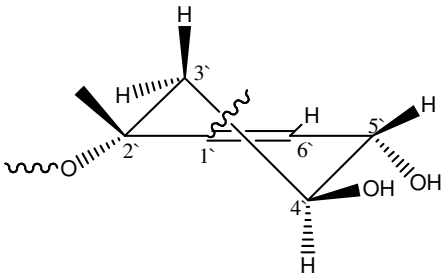


Compounds **11** and **12** were obtained as an inseperable mixture from the EtOAc extract of rice cultures of *Alternaria* sp. in the form of a viscous yellow oil (18.8 mg). The UV spectrum of compound **11** showed λ_{max} (MeOH) at 243.6, 281.7 and 322.5 nm. Compound **12**, on the other hand, showed UV absorbances at λ_{max} (MeOH) 241.4, 280.6 and 318.3 nm. Their HRESI-MS showed $[M+H]^+$ at *m/z* 293.1020 (calculated 293.1025, Δ 0.0005), indicating the molecular formula to be $C_{15}H_{16}O_6$. The major compound in the fraction was identified as altenuene (**11**) by comparison of UV, ^1H, ^{13}C NMR and mass spectral data with published data (Bradburn *et al.*, 1994). The ^1H NMR spectrum showed a pair of *meta*-coupled protons (δ_H 6.64, H-6, and δ_H 6.45, H-4) and a methoxy group at δ_H 3.86, thus reminiscent of the aromatic portion of metabolites **6-9** (see Tables 3.4, 3.5, 3.6 and 3.8). In addition, the diastereotopic methylene protons CH_2-3 appeared as the AB part of an ABX-type system with δ_H 1.96 (dd, *J*=14.5 and 9.1 Hz) and δ_H 2.40 (dd, *J*=14.5 and 3.7 Hz), consistent with axially and equatorially situated protons, respectively, and with a near antiperiplanar relationship between H-3`ax and H-4`ax (δ_H 3.77) in a half-chair conformation (**11a**). Moreover, a coupling constant of 2.8 Hz between H-6` ($\delta_H$ 6.21) and H-5` (δ_H 4.06) was in agreement with a pseudoequatorial orientation of the C-5` hydroxy group (Bradburn *et al.*, 1994). These data were in accordance with reported X-ray crystallographic data for altenuene (McPhail *et al.*, 1973) as well as an in depth analysis of its stereochemistry by Bradburn *et al.* (Bradburn *et al.*, 1994). The structure was corroborated by interpretation of the HMBC spectrum showing correlations of CH_3-2` to C1`-C4`, H-4` to C-2` and C-6`, H-5` to C-1` and C-3` as well as H-6` to C-2`, C-4` and C-1 (see Tables 3.8 and Figure 3.13). Moreover, further evidence was detected in the ROESY spectrum showing correlations between the 2`-methyl group, the C$H_2$-3` methylene protons (δ_H 2.40 and 1.96) and H-5` (δ_H 4.06) (see Tables 3.8 and Figure 3.14). Thus, the structure was confirmed to be that of the known compound altenuene, previously isolated from *Alternaria* species (McPhail *et al.*, 1973; Bradburn *et al.*, 1994).

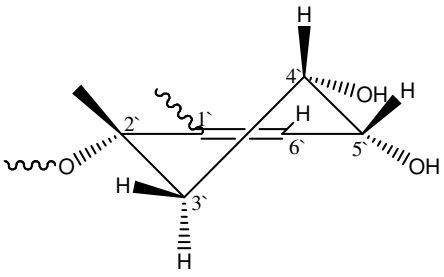
In contrast, the ^{1}H NMR spectrum of **12** showed similar ^{1}H NMR data for the aromatic portion of the compound, but significantly different resonances and coupling patterns for the C*H*$_2$-3` methylene protons at $\delta_H$ 2.15 (br t, *J*=12.3 Hz) and $\delta_H$ 2.25 (dd, *J*=11.9 and 3.7 Hz), which could be explained with this methylene group situated adjacent to an equatorial 4`-hydroxy group. This, taken together with strong ROESY correlations between the 2`-methyl group, one of the methylene protons ($\delta_H$ 2.25), H-4` (δ_H 3.73), and H-5` ($\delta_H$ 4.20) (see Figure 3.14), indicated the adoption of the alternative half-chair conformation and the placement of the 4`-hydroxy group in the equatorial position. Thus, the relative stereochemistry shown in **12a** was assigned to **12**, which identifies the compound as the previously unreported 4`-epialtenuene. All remaining NMR spectral data were in accord with this conclusion (see Tables 3.8 and 3.9, and Figure 3.13). Assignment of all signals belonging to **12** was easily possible because of the lower amount of **12** present in the mixture with **11** (1:2). It was not possible to determine the absolute stereochemistry as the compounds were obtained as inseparable mixture. Moreover, the compounds were found to be optically inactive, probably due to their racemic nature as reported in literature (McPhail *et al.*, 1973).



Nr.	Compound	R_1	R_2
11	Altenuene	OH	H
12	4`-Epialtenuene	H	OH



11a



12a

Table 3.8: ^{1}H NMR and HMBC data of compounds **11** and **12** at 500 MHz

Nr.	11				11[a]	12			
	δ_H (MeOD)	COSY	ROESY	HMBC	δ_H ($CDCl_3$-DMSO-d_6)	δ_H (MeOD)	COSY	ROESY	HMBC
1									
2									
3									
4	6.45, d (2.2)	6		2,3,5,6	6.41, d (2.3)	6.46, d (2.2)	6		2,3,5,6
5									
6	6.64, d (2.2)	4	6`	4,5,1`	6.53, d (2.3)	6.63, d (2.2)	4	6`	4,5,1`
7									
1´									
2´									
3´α	2.40, dd (14.5, 3.7)	3`$\beta$,4`α	2`CH_3,5´β	2`$CH_3$,1`,2`,4`,5`	2.54, dd (14.9, 4.2)	2.15, brt (12.3)	3`$\beta$,4`α	5´β	2`$CH_3$,2`,4`,5`
3`β	1.96, dd (14.5, 9.1)	3`$\alpha$,4`α	2`CH_3,5´β	2`$CH_3$,1`,2`,4`,5`	1.84, dd (14.9, 11.0)	2.25, dd (11.9, 3.7)	3`$\alpha$,4`α	2`CH_3	2`$CH_3$,1`,2`,4`,5`
4´α	3.77, ddd (9.1, 5.6, 3.7)	3`$\alpha$,$\beta$,5`β	6`	2`,5`,6`	3.77, m				
4´β						3.73, ddd (12.3, 8.2, 3.7)	3`$\alpha$,$\beta$,5`β	2`$CH_3$,6`	
5´β	4.06, dd (5.6, 2.8)	4`$\alpha$,6`	2`$CH_3$,3`α,β	1`,3`,4`,6`	4.11, d (4.6)	4.20, dd (8.2, 2.5)	4`$\alpha$,6`	2`$CH_3$,3`α	1`,4`,6`
6´	6.21, d (2.8)	3`α,β	4´α,6	2`,4`,1	6.41, d (1.7)	6.17, d (2.5)	3`α	4´β,6	2`,4`,1
2`-CH_3	1.49, s		3`α,β,5´β	1`,2`,3`,4`	1.49, s	1.54, s		3`β,4´β,5´β	1`,2`,3`
5-OCH_3	3.86,s			5	3.86,s	3.85, s			5

a) Bradburn *et al.*, 1994.

Table 3.9: ^{13}C NMR data of compounds **11** and **12** at 125 MHz

Nr.	11	11[a]	12
	δ_C (MeOD)	δ_C ($CDCl_3$-DMSO-d_6)	δ_C (MeOD)
1	140.7	139.1	139.0
2	101.5		101.2
3	165.2	165.7	165.4
4	101.7	100.4	102.0
5	167.8	168.7[b]	167.9
6	103.7	102.4	103.7
7	170.3	165.7[b]	169.6
1`	134.7	130.9[c]	134.2
2`	82.4	81.0	83.5
3`	40.8	40.2	44.5
4`	70.6	69.6	72.0
5`	72.2	72.4	74.2
6`	131.4	132.5[c]	130.4
2`-CH_3	28.0	27.8	26.6
5-OCH_3	56.3	55.5	56.3

a) Bradburn *et al.*, 1994.

b), c) Assignements may be interchanged.

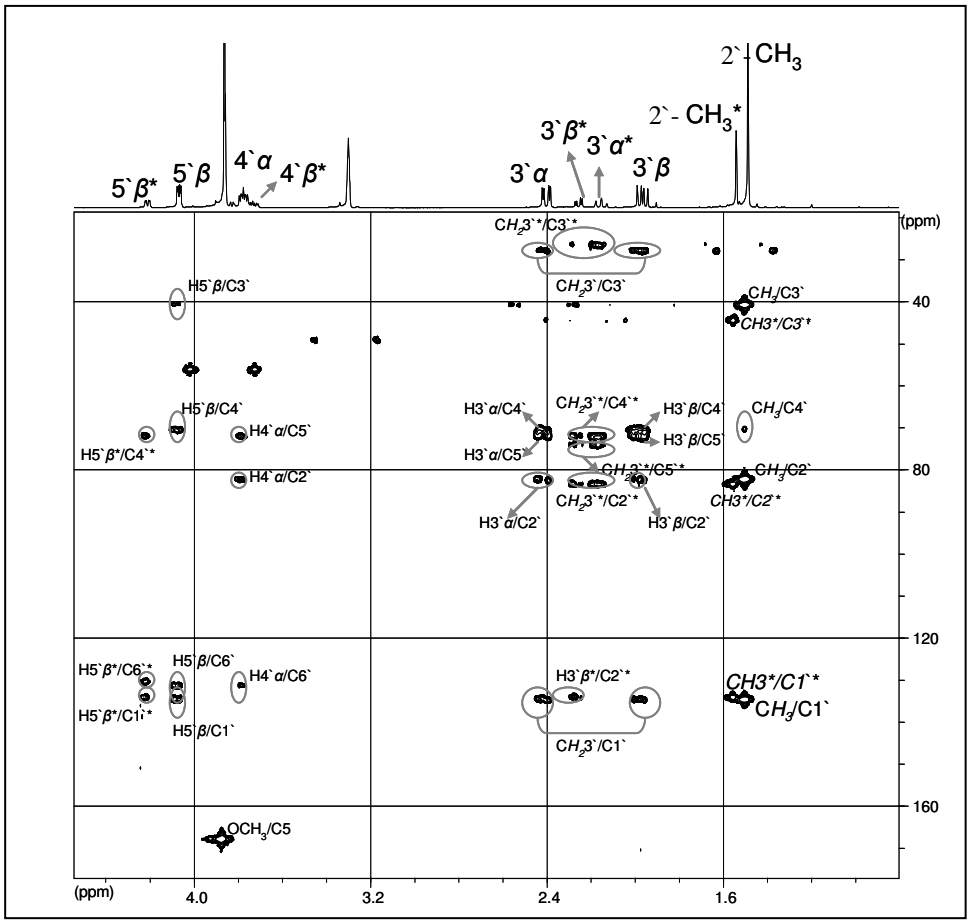


Figure 3.13: HMBC spectrum of compounds **11** and **12**(*) (obtained as inseperable mixture).

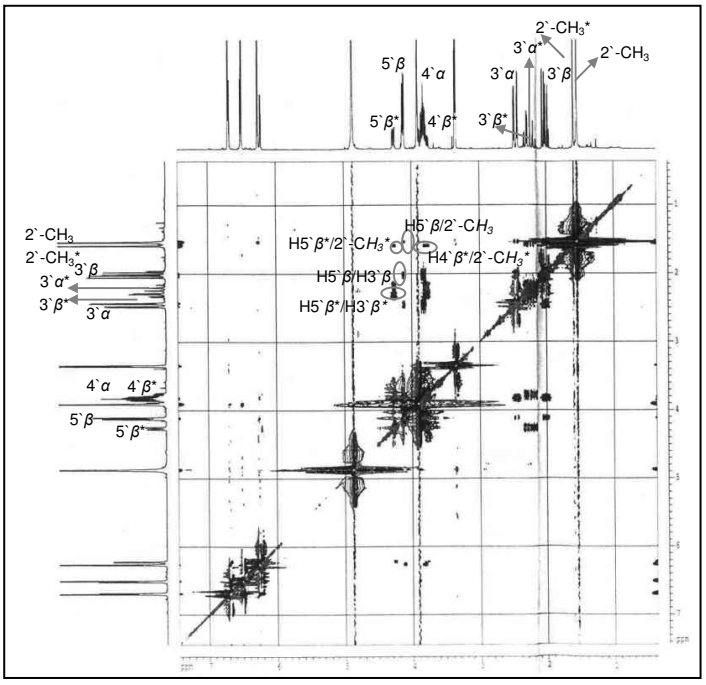
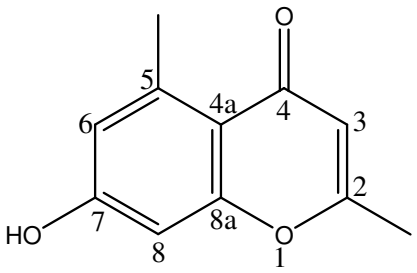
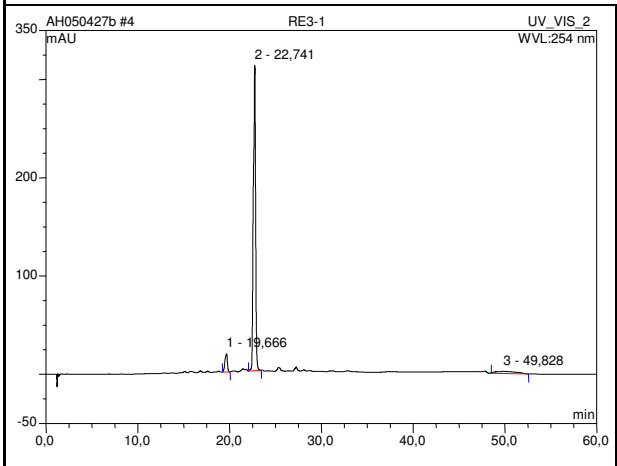


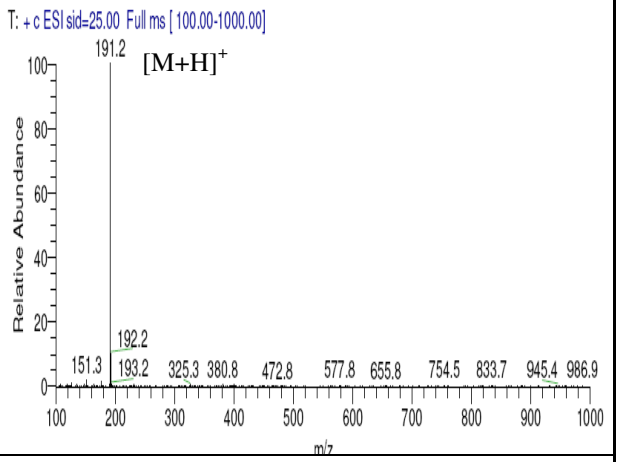
Figure 3.14: ROESY spectrum of compounds **11** and **12**(*) (obtained as inseperable mixture).

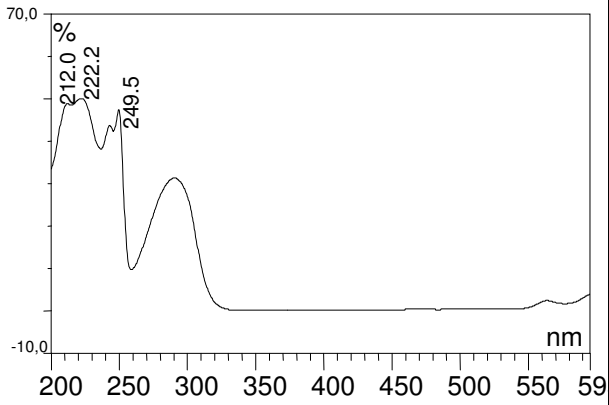
3.1.13. 2,5-Dimethyl-7-hydroxychromone (13, known compound)

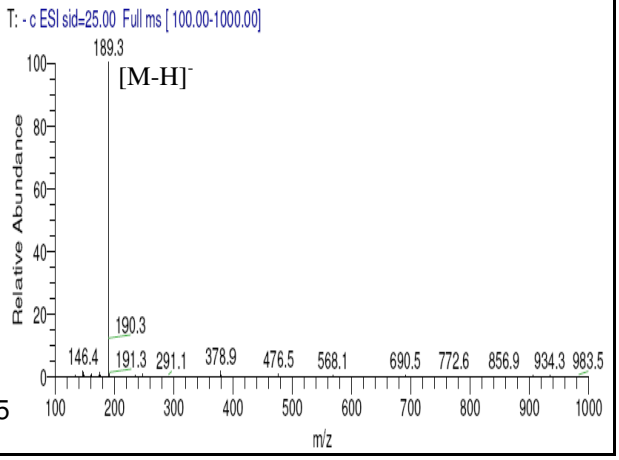
2,5-Dimethyl-7-hydroxychromone	
Synonym(s)	7-Hydroxy-2,5-dimethyl-4*H*-1-benzopyran-4-one
Sample code	RE3.1
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	4.6 mg
Physical Description	Viscous yellow oil
Molecular Formula	$C_{11}H_{10}O_3$
Molecular Weight	190 g/mol
Retention time HPLC	22.7 min (standard gradient)



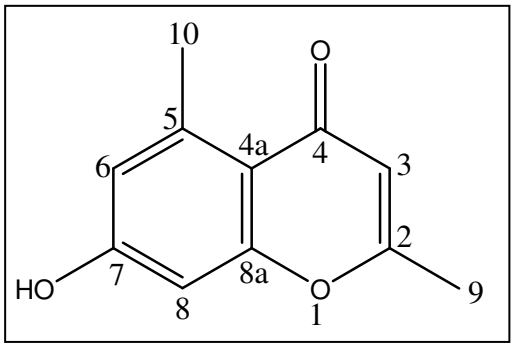








2,5-Dimethyl-7-hydroxychromone (**13**) was isolated from the EtOAc extract of liquid cultures of *Alternaria* sp. as viscous yellow oil (4.6 mg). It displayed UV absorbances at λ_{max} (MeOH) 212.0, 222.2, 243.5, 249.5 and 291.0 nm indicating a chromone skeleton. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 191.2 $[M+H]^+$ (base peak) and *m/z* 189.3 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 190 g/mol. The ^1H NMR (see Table 3.10) indicated the presence of two aromatic methyl groups at δ_H 2.25 and 2.63, a vinyl proton at δ_H 5.96, and a pair of aromatic *meta*-coupled protons at δ_H 6.59 and 6.62 (each d, *J*=2.2 Hz). The upfield chemical shift of the vinyl proton appearing at δ_H 5.96 indicated it to reside at C-3 and thus in the α-position of an α,β-unsaturated carbonyl substructure (Kashiwada *et al.*, 1984; Kimura *et al.*, 1992). The location of the methyl group at C-5 was confirmed by the HMBC correlations of its protons to C-5, C-6 and C-4a (see Table 3.10), while both H-6 and H-8 correlated to C-7 and C-4a, thus establishing the substitution pattern of the aromatic ring. The structure was further confirmed by comparison of UV, ^1H, ^{13}C NMR and mass spectra with published data for 2,5-dimethyl-7-hydroxychromone (Kashiwada *et al.*, 1984). This is the first report of this natural product from an endophytic fungal source. However, it was previously isolated from the soil fungus *Talaromyces flavus* (Ayer and Racok, 1990a) and its positional isomer, altechromone A, was obtained from *Alternaria* sp. (Kimura *et al.*, 1992).



13 2,5-Dimethyl-7-hydroxychromone

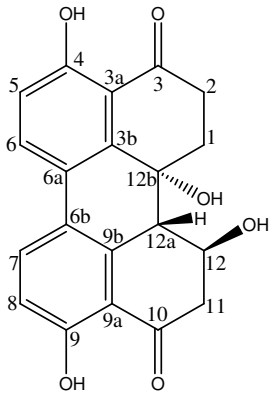
Table 3.10: ^{1}H, ^{13}C NMR and HMBC data of compound **13** at 500 (^{1}H) and 125 MHz (^{13}C)

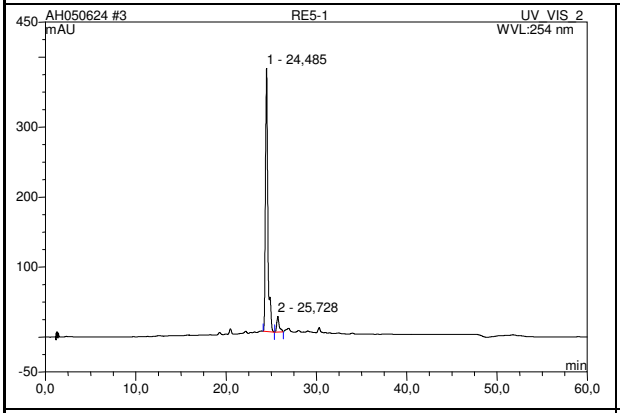
Nr.	**13**			**13**[a]	
	δ_H (DMSO-d_6)	HMBC	δ_C (DMSO-d_6)	δ_H (DMSO-d_6)	δ_C (DMSO-d_6)
2			163.8		163.9
3	5.96, s	2,4a,9	110.7	5.96, s	116.4
4			178.2		178.4
4a			114.2		114.1
5			141.4		141.5
6	6.59, d (2.2)	4a,7,8,10	116.4	6.60, s	110.5
7			160.9		160.6[b]
8	6.62, d (2.2)	4a,6,7,8a	100.5	6.60, s	100.4
8a			159.1		159.1[b]
2-CH_3	2.25, s	2,3	19.3	2.28, s	19.2
5-CH_3	2.63, s	4a,5,6	22.4	2.66, s	22.3
7-OH	10.58, brs				

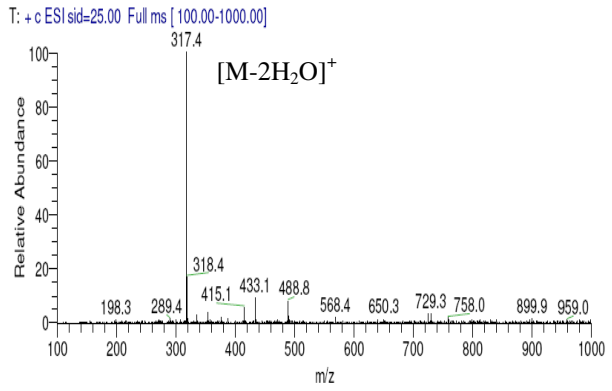
a) Kashiwada, Nonaka and Nishioka, 1984.
b) Assignements may be interchanged.

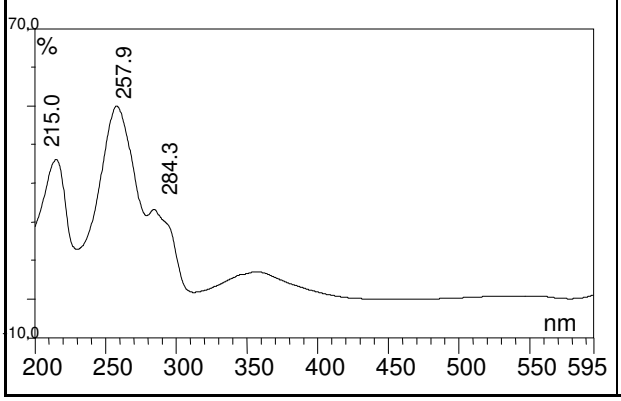
3.1.14. Altertoxin I (14, known compound)

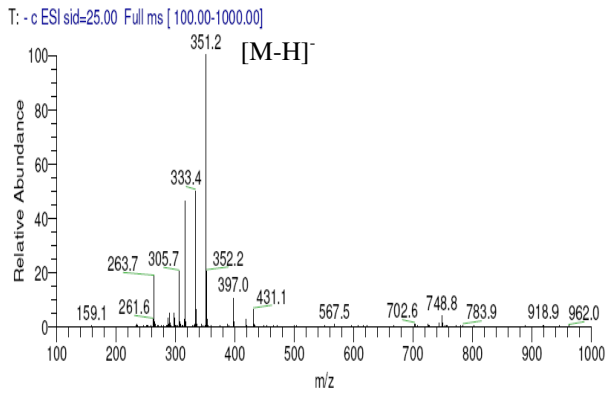
Altertoxin I	
Synonym(s)	Hexahydro-1,4,9,12a-tetrahydroxyperylene-3,10-quinone 1,2,7,8,12b-pentahydro-l,4,6b,l0-tetrahydroxyperylene-3,9-dione
Sample code	RE5.1
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	3.5 mg
Physical Description	Reddish brown powder
Molecular Formula	$C_{20}H_{16}O_6$
Molecular Weight	352 g/mol
Optical Rotation $[\alpha]_D^{26}$	+ 380 (*c* 0.2, acetone)
Retention time HPLC	24.5 min (standard gradient)





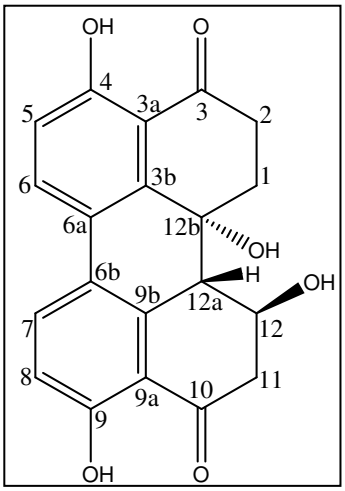






Altertoxin I (**14**) was isolated from the EtOAc extract of liquid cultures of *Alternaria* sp. as reddish brown powder (3.5 mg). It displayed UV absorbances at λ_{max} (MeOH) 215.0, 257.9, 284.3 and 358.0 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 317.3 $[M\text{-}2H_2O]^+$ (base peak) and *m/z* 351.2 $[M\text{-}H]^-$ (base peak), respectively, indicating a molecular weight of 352 g/mol. The ^{13}C NMR spectrum (see Table 3.11) contained signals due to twenty carbon atoms, which together with ^1H NMR and mass spectra indicated a molecular formula of $C_{20}H_{16}O_6$. The ^1H resonances were assigned to two AX spin systems of *ortho*-coupled aromatic protons, one appearing at δ_H 8.05 and 7.03 (H-6 and H-5, respectively), and the other one at δ_H 7.99 and 6.93 (H-7 and H-8, respectively), and to two chelated phenolic hydroxy groups (δ_H 12.72 and 12.31, 4- and 9-OH, respectively). In addition, $C(1)H_2$-$C(2)H_2$ and $C(11)H_2$-C(12)HOH-C(12a)H fragments were observed and assembled on basis of the COSY spectrum (see Table 3.11). The aromatic ^{13}C resonances were attributed to two tetra-substituted aromatic rings and two carbonyl carbon atoms, while the remaining signals were assigned to three methylene and two methine sp^3 hybridized carbons, one of which is oxygen-bearing. The chelated phenolic hydroxy groups were located at C-4 and C-9 on basis of the observed HMBC correlations of H-5, H-6 and 4-OH to C-4 as well as those of H-7, H-8 and 9-OH to C-9 (see Table 3.11). Consequently, the carbonyl groups were located at C-3 and C-10. Furthermore, HMBC correlations were observed for 4-OH to C-3a, H-5 to C-3a and C-6a, H-6 to C-4 and C-3b, CH_2-1 to C-3, C-3b and C-12b besides CH_2-2 to C-3 and C-12b, thereby establishing the structure of the northern part of the molecule. The southern part exhibited similar HMBC correlations for the aromatic portion, in addition to the correlations observed for H-8 to C-9a, CH_2-11 to C-9a and C-10 as well as H-12a to C-6b and C-9a. The attachment of both parts of the molecule was deduced from the HMBC correlations of H-6 to C-6b, H-7 to C-6a along with those of H-12a to C-1 and C-3b suggesting the structure of altertoxin I (**14**) with the phenyl rings being conjugated.

The relative stereochemistry was derived from detailed examination of the proton spectrum (see Table 3.11). The large value of $J_{11ax\text{-}12}$ (11.1 Hz) and $J_{12\text{-}12a}$ (8.1) indicated that H-12 is *trans* to H-11ax and H-12a, and that all of these hydrogens are axially positioned. Accordingly, the hydroxy group 12-OH was placed in an equatorial position. The structure was further confirmed by comparing UV, ^1H, ^{13}C NMR, mass spectral data and $[\alpha]_D$ value with published data for altertoxin I, previously reported from several *Alternaria* species (Okuno *et al.*, 1983; Stack *et al.*, 1986; Hradil *et al.*, 1989).



14 Altertoxin I

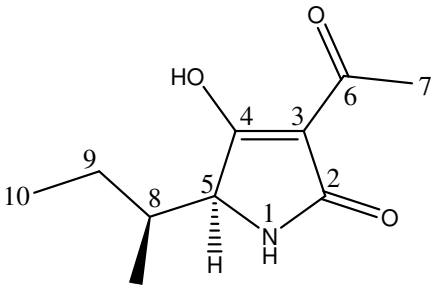
Table 3.11: ^{1}H, ^{13}C NMR, COSY and HMBC data of compound **14** at 500 (^{1}H) and 125 MHz (^{13}C)

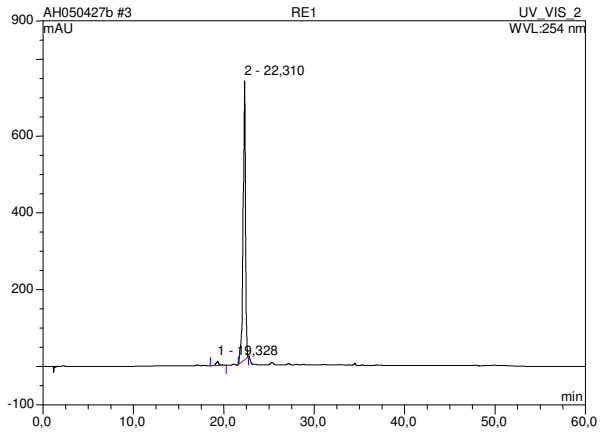
Nr.	**14**			**14**[a]		
	δ_H (DMSO-d_6)	COSY	HMBC	δ_C (DMSO-d_6)	δ_H (DMSO-d_6)	δ_C (DMSO-d_6)
1ax	2.29, dt (14.0, 4.1)	1eq,2ax,2eq		34.7	2.30, dt (13.0, 3.0)	34.8
1eq	2.96, m	1ax,2ax,2eq	2,3,3b,12a,12b		3.0, m	
2ax	3.07, ddd (17.3, 14.0, 4.5)	1ax,1eq,2eq	1,3	33.4	3.0, m	33.5
2eq	2.57, ddd (17.3, 4.1, 2.5)	1ax,1eq,2ax	3,12b		2.59, dt (15.0, 3.0)	
3				206.0		206.0
3a				113.7		113.8
3b				140.6		138.4
4				160.9		161.0
5	7.03, d (8.8)	6	3a,3b,4,6a	117.8	7.1, d (8.8)	117.8
6	8.05, d (8.8)	5	3a,3b,4,6b	132.8	8.1, d (8.8)	132.9
6a				123.4		123.5
6b				124.7		124.8
7	7.99, d (8.8)	8	6a,9,9a,9b	132.5	8.0, d (8.8)	132.5
8	6.93, d (8.8)	7,12a	6b,9,9a,9b	115.5	6.9, d (8.8)	115.5
9				160.4		160.4
9a				116.5		116.5
9b				138.3		140.7
10				204.1		204.2
11ax	2.97, dd (15.4, 11.1)	11eq,12	9a,10,12,12a	47.4	3.0, m	47.5
11eq	2.85, dd (15.4, 4.4)	11ax,12	9a,10,12,12a		2.86, m	
12	4.52, m	11ax,11eq,12a,12-OH		64.6	4.5, m	64.7
12-OH	5.37, brs	12		51.3	2.86, m	51.4
12a	2.94, d (8.1)	8,12	1,3b,6b,7,9,9a,9b,11,12,12b	67.9		68.0
12b						
12b-OH	5.27, s		3b,12a			
4-OH	12.72, s		3a,4,5,6		12.4	
9-OH	12.31, s		7,8,9,9a		12.7	

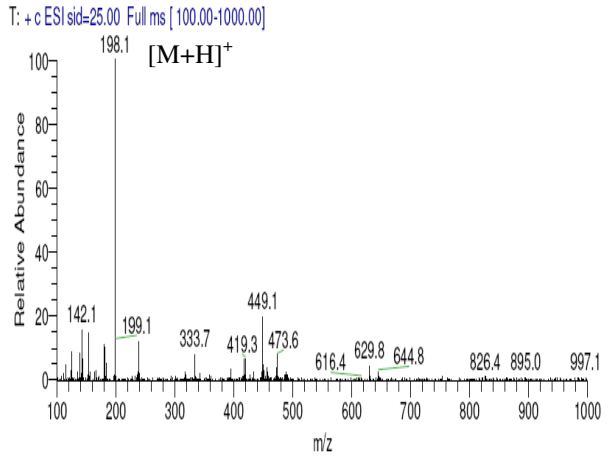
a) Stack *et al.*, 1986.

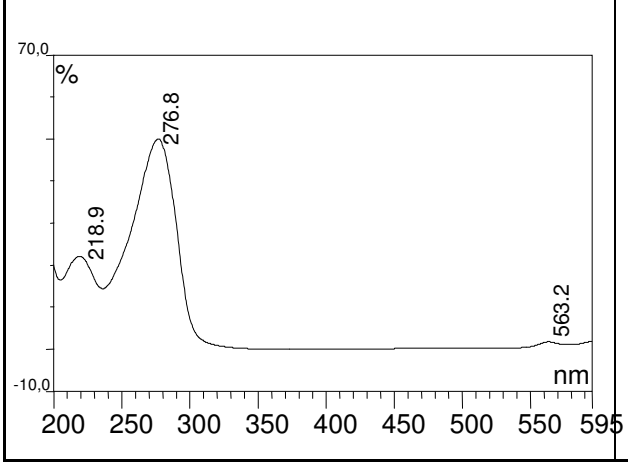
3.1.15. Tenuazonic acid (15, known compound)

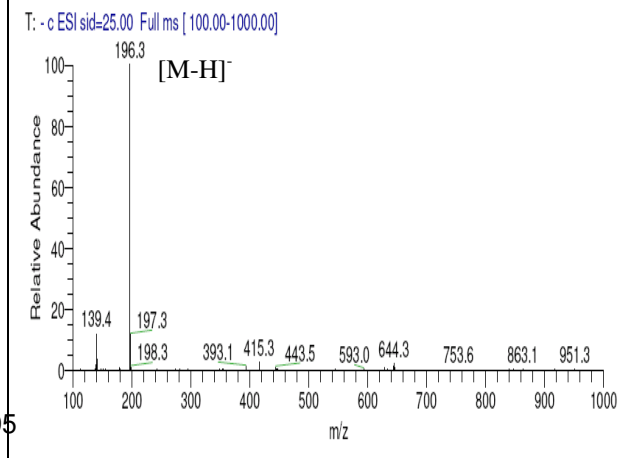
Tenuazonic acid	
Synonym(s)	3-Acetyl-1,5-dihydro-4-hydroxy-5-(1-methylpropyl)-2H-pyrrol-2-one
Sample code	RB1, RE1
Biological source	*Alternaria* sp. (from *Polygonum senegalense*)
Sample amount	128.7 mg
Physical Description	Viscous brown oil
Molecular Formula	$C_{10}H_{15}O_3N$
Molecular Weight	197 g/mol
Optical Rotation $[\alpha]_D^{20}$	- 134 (*c* 0.2, $CHCl_3$)
Retention time HPLC	22.3 min (standard gradient)



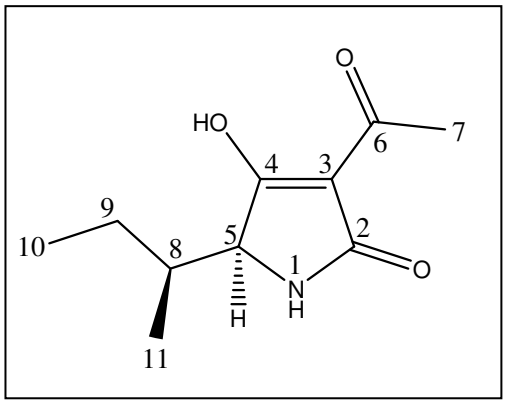








Tenuazonic acid (**15**) was obtained as yellowish brown viscous oil (101.2 mg). It displayed UV absorbances at λ_{max} (MeOH) 218.9 and 276.8 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 198.1 $[M+H]^+$ (base peak) and *m/z* 196.3 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 197 g/mol and thus the presence of an odd number of N-atoms in the structure. The ^{13}C NMR spectrum revealing 10 carbon atoms, together with ^1H NMR and mass spectral data supported a molecular formula of $C_{10}H_{15}O_3N$. The ^1H and ^{13}C NMR spectra (see Table 3.12) indicated three methyl groups, more specifically a methyl ketone group at δ_H 2.33 (s) and δ_C 19.8, a methyl group at δ_H 0.80 (t, *J*=7.2 Hz) and δ_C 11.7, which based on the multiplicity was assigned as adjacent to a methylene group, and a methyl group at δ_H 0.90 (d, *J*=6.9 Hz) and δ_C 15.4 located besides a methine group. In the COSY spectrum the latter signals correlated forming one common spin system of a 2-butyl side chain. Using a substructure and molecular weight based search the structure of tenuazonic acid was found to match the obtained data. The structure was further confirmed by interpretation of the HMBC spectrum (see Table 3.12) in addition to comparison of UV, ^1H, ^{13}C NMR, mass spectral data and $[\alpha]_D$ value with published data for tenuazonic acid (Nolte *et al.*, 1980). This compound was previously isolated from several *Alternaria* (Rosett *et al.*, 1957; Meronuck *et al.*, 1972) and *Aspergillus* species (Kaczka *et al.*, 1964) as well as from *Piricularia oryzae* (Umetsu *et al.*, 1972) and *Phoma sorghina* (Steyn and Rabie, 1976).



15 Tenuazonic acid

Table 3.12: ^{1}H, ^{13}C NMR and HMBC data of compound **15** at 500 (^{1}H) and 125 MHz (^{13}C)

Nr.	**15**					**15**[a]	
	δ_H ($CDCl_3$)	δ_H (DMSO-d_6)	COSY	HMBC	δ_C (DMSO-d_6)[b]	δ_H ($CDCl_3$)	δ_C ($CDCl_3$)
2					174.6		175.6
3					102.2		102.5
4					195.6		195.5
5	3.77, d (3.4)	3.77, d (2.8)	8	2,4,8,9,11	65.9	3.75	67.4
6					184.7		184.0
7	2.43, s	2.33, s		3,6	19.8	2.43	19.4
8	1.94, m	1.78, m	5,9A,9B,11	5,10	36.4	1.92	37.0
9A	1.35, m	1.24, m	8,9B,10	5,8,10,11	23.2	1.30	23.6
9B	1.23, m	1.09, m	8,9A,10	5,8,10,11			
10	0.87, t (7.2)	0.80, t (7.2)	9A,9B	8,9	11.7	0.89	11.7
11	0.99, d (6.9)	0.90, d (6.9)	8	5,8,9	15.4	1.00	15.8
NH	7.06, s	8.74, brs				6.22	
OH		11.10,brs					

a) Nolte, Steyn, and Wessels, 1980.
b) Derived from HMBC spectrum.

3.1.16. Bioactivity test results for compounds isolated from the endophytic fungus *Alternaria* sp.

The isolated compounds were subjected to bioassays aimed to determine their cytotoxicity and their protein kinase inhibitory profiles. The results are shown in Tables 3.13 and 3.14.

Table 3.13: Cytotoxicity assay results for the compounds isolated from *Alternaria* sp. liquid and rice extracts

Nr.	Compound tested	L5178Y growth in %* (Conc. 10 µg/mL)	EC_{50}* (µg/mL)	EC_{50} (µmol/L)
1	Alternariol	3.7	1.7	6.6
2	Alternariol-5-*O*-sulphate	0.9	4.5	13.3
3	Alternariol-5-*O*-methyl ether	1.8	7.8	28.7
4	Alternariol-5-*O*-methyl ether-4`-*O*-sulphate	89.0		
5	3`-Hydroxyalternariol-5-*O*-methyl ether	47.1		
6	Altenusin	1.2	6.8	23.4
7	Desmethylaltenusin	0.8	6.2	22.5
8	Talaroflavone	92.9		
9	Alternaric acid	101.2		
10	Alterlactone	11.8		
11 and 12	Altenuene and 4`-epialtenuene	94.0		
13	Altertoxin I	77.3		
14	2,5-Dimethyl-7-hydroxychromone	95.0		
15	Tenuazonic acid	57.9		

* Data provided by Prof. W. E. G. Müller, Mainz.

All alternariol derivatives as well as alterlactone proved to be highly active against L5178Y cell line except for 3`-hydroxyalternariol-5-*O*-methyl ether and alternariol-5-*O*-methyl ether-4`-*O*-sulphate which showed moderate and very weak cytotoxic activity, respectively. Altenusin and its desmethyl derivative were also highly active in the assay. Tenuazonic acid was moderately active as well, while altertoxin I showed weak activity.

Table 3.14: Protein kinase assay results for the compounds isolated from *Alternaria* sp. liquid and rice extracts

Compound tested (Conc. 1µg/mL)	Activity on various protein kinases based on IC_{50} [g/mL]*																											
	EGF-R	EPHB4	ERBB2	FAK	IGF1-R	SRC	VEGF-R2	VEGF-R3	AKT1	ARK5	Aurora-A	Aurora-B	PAK4	PDK1	CDK2/CycA	CDK4/CycD1	CK2-alpha1	FLT3	INS-R	MET	PDGFR-beta	PLK1	SAK	TIE2	COT	B-RAF-VE
Ellagic acid	A	A	M	A	H	A	A	A	M	A	A	A	0	0	M	A	M	A	A	A	A	A	H	A	M	A
Alternariol	M	0	M	M	M	M	A	M	0	A	A	A	0	0	M	M	M	A	M	M	A	M	A	M	M	M
Alternariol-5-*O*-sulphate	M	M	M	M	M	M	A	M	0	A	A	A	0	0	A	M	M	A	M	M	M	M	A	M	M	A
Alternariol-5-*O*-methyl ether	M	M	M	M	M	M	M	A	M	M	M	M	0	0	M	M	0	M	M	0	M	M	M	M	M	M
Alternariol-5-*O*-methyl ether-4`-*O*- sulphate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3`-Hydroxyalternariol-5-*O*-methyl ether	M	M	M	M	A	M	A	A	M	A	M	M	0	0	M	M	0	A	M	M	M	M	A	M	M	M
Altenusin	M	M	0	0	M	M	M	0	0	M	M	M	0	0	0	M	0	M	M	M	M	M	M	M	M	M
Desmethylaltenusin	M	M	0	0	M	M	M	M	0	M	M	M	0	0	0	M	0	M	M	M	M	0	M	M	M	M
Alterlactone	M	0	0	0	M	M	M	M	0	M	M	M	0	0	0	0	0	M	M	M	0	0	M	0	0	M
Talaroflavone	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alteric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Altenuene and 4`-epialtenuene	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	M	0	0	0	0	0	0	0	0
Altertoxin I	M	M	0	M	M	M	M	M	0	M	M	A	0	0	0	M	0	A	M	M	M	0	M	M	M	M
2,5-Dimethyl-7-hydroxychromone	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tenuazonic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

H: highly active, A: active, M: moderately active, 0: not active
* Data provided by ProQinase, Freiburg.

Results of protein kinase assay showed a similar activity profile as the cytotoxicity assay results. All alternariol derivatives as well as alterlactone were active on various protein kinases, while alternariol-5-*O*-methyl ether-4`-*O*-sulphate proved to be an exception with being inactive for all tested protein kinases. As a reference ellagic acid was used, since it represents a highly active substance with structural similarity to alternariol. Furthermore, altenusin, its desmethyl derivative and altertoxin I were also active in the assay.

3.2. Compounds isolated from the endophytic fungus *Ampelomyces* sp.

This endophytic fungal strain of the genus *Ampelomyces* was isolated from flowers of *Urospermum picroides* growing in Egypt. The pure fungal strain was cultivated on liquid Wickerham medium and solid rice medium. Chemical screening indicated a clear difference between *Ampelomyces* extracts obtained from liquid Wickerham medium and rice cultures. HPLC chromatograms of the EtOAc extract of the fungus grown on solid rice medium showed altersolanol A (**25**) and ampelanol (**26**) as main components. When grown on liquid medium, the major substance detected in the extract was macrosporin (**21**) with no traces of **25** or **26** (see Figure 3.15A-B). Similar to *Alternaria* extracts, the yield of rice cultures was much higher than that of liquid cultures with a weight ratio of 18:1 of dried extracts, respectively. Antibacterial, antifungal, cytotoxicity and protein kinase assay results showed that extracts obtained from rice cultures were much more active in the preliminary biological screening tests compared to the liquid culture extracts (see Table 3.15).

In this part of the investigation results on the natural products produced by *Ampelomyces* sp. when grown in liquid medium and on solid rice medium are presented.

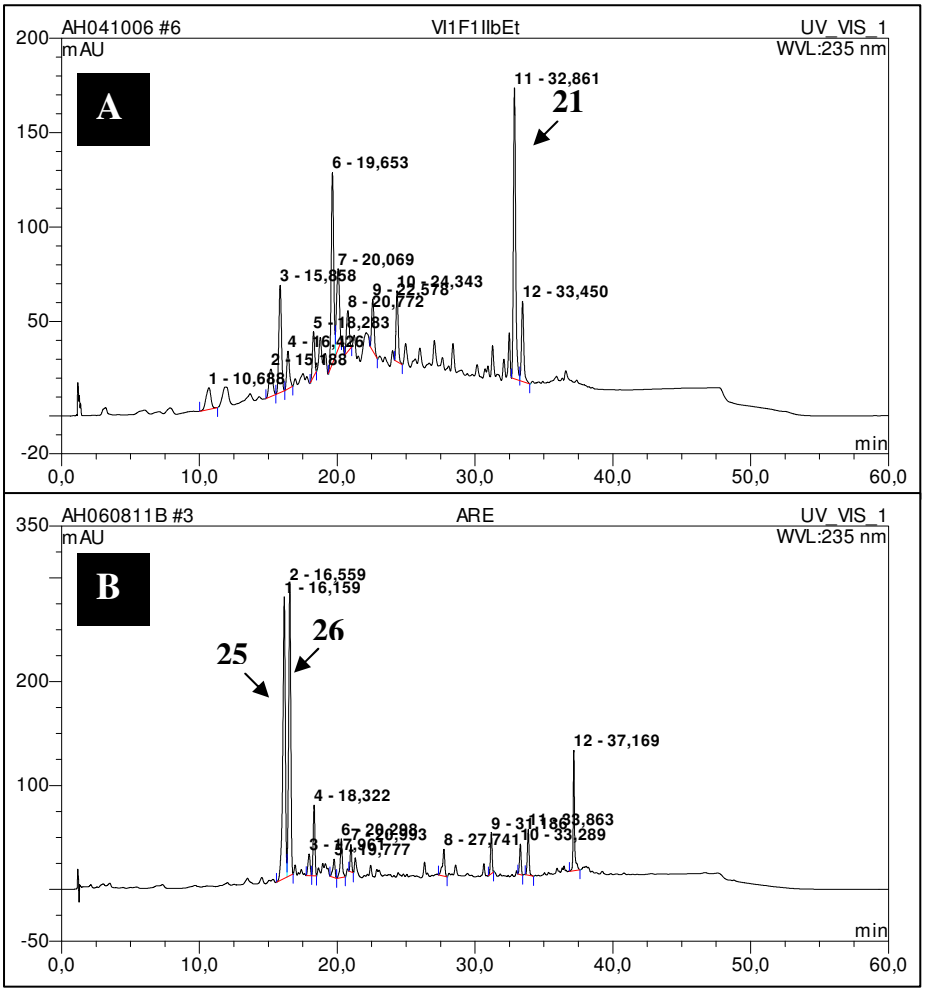


Figure 3.15A-B: EtOAc extracts of *Ampelomyces* sp. cultures. **A:** HPLC chromatogram of EtOAc extract of liquid cultures (Wickerham medium). **B:** HPLC chromatogram of EtOAc extract of rice cultures. **21:** Macrosporin. **25:** Altersolanol A. **26:** Ampelanol.

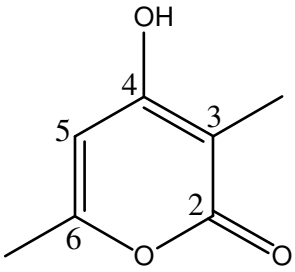
Table 3.15: Biological screening test results for *Ampelomyces* liquid and rice extracts

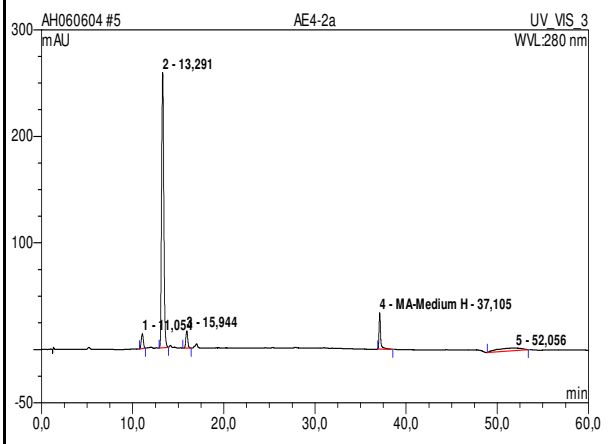
Extracts tested	**L5178Y growth in %** (Conc. 10 µg/mL)	**Protein kinase activity** (Conc. 1 µg/mL)	**Antimicrobial activity** IZ [mm], 0.5 mg		
			BS	SC	CH
Ampelomyces liquid *n*-BuOH	98.3		0	0	0
Ampelomyces liquid EtOAc	108.2		0	0	0
Ampelomyces liquid MeOH	65.9		0	0	0
Ampelomyces rice EtOAc	0.3	Active	9	0	0

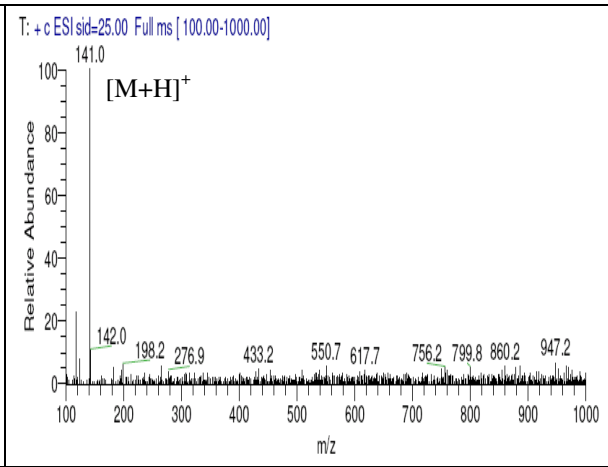
BS: *B. subtilis*, SC: *S. cerevisiae*, CH: *C. herbarum*.

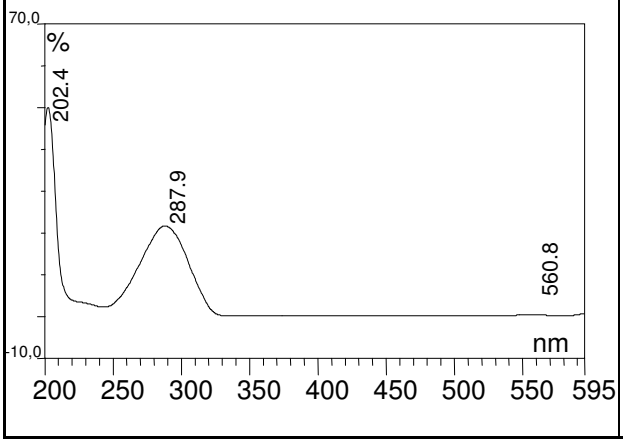
3.2.1. Methyltriacetic lactone (16, known compound)

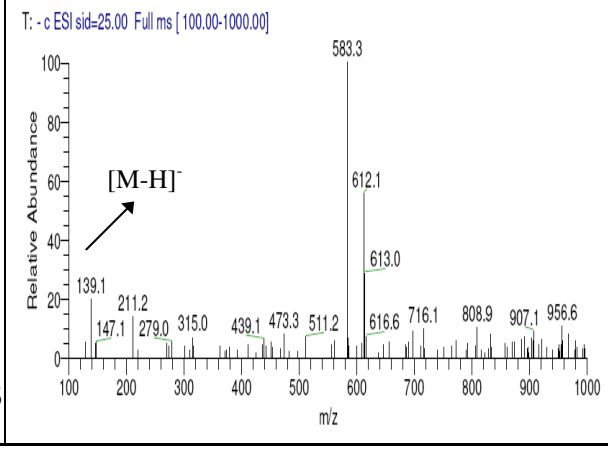
Methyltriacetic lactone	
Synonym(s)	4-Hydroxy-3,6-dimethyl-2H-pyran-2-one
Sample code	AE4.2
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	4.2 mg
Physical Description	viscous yellow oil
Molecular Formula	$C_7H_8O_3$
Molecular Weight	140 g/mol
Retention time HPLC	13.3 min (standard gradient)







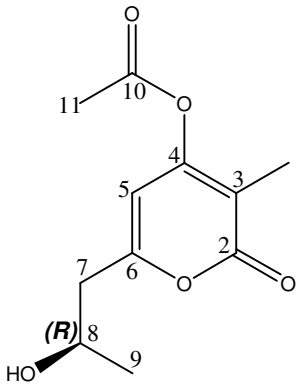


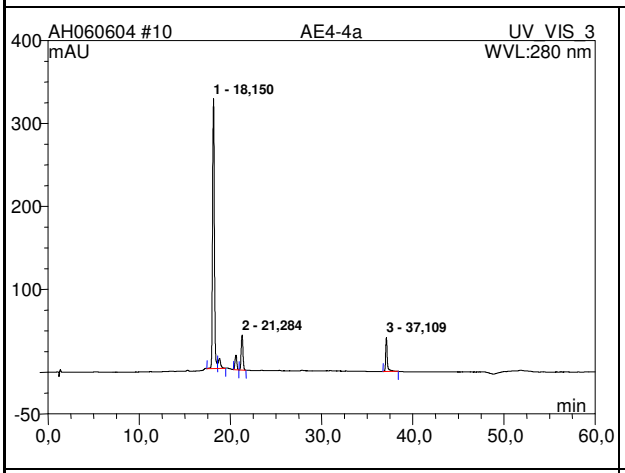


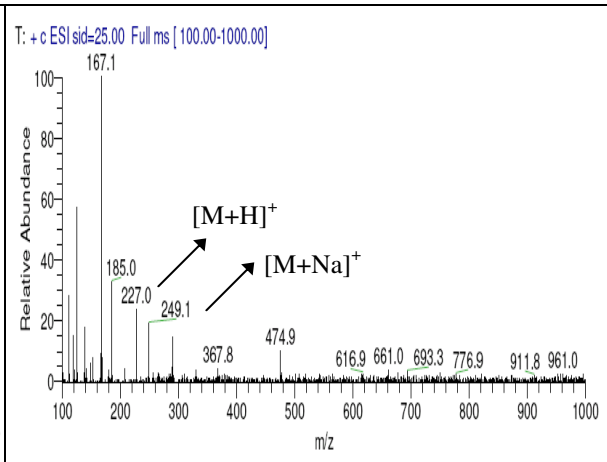
Methyltriacetic lactone (**16**) was isolated from the EtOAc extract of liquid cultures of *Ampelomyces* sp. as viscous yellow oil (4.2 mg). It displayed UV absorbances at λ_{max} (MeOH) 202.4 and 287.9 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 141.0 $[M+H]^+$ (base peak) and *m/z* 139.1 $[M-H]^-$, respectively, indicating a molecular weight of 140 g/mol. The ^1H and ^{13}C NMR spectra (Table 3.16) indicated the presence of two aromatic methyl groups at δ_H 2.08 and δ_C 19.2 (6-*CH*$_3$) as well as δ_H 1.69 and δ_C 8.5 (3-*CH*$_3$), an aromatic proton singlet at δ_H 5.88 assigned to H-5 as well as the corresponding tertiary aromatic carbon at δ_C 101.0. Furthermore, the ^{13}C NMR spectrum showed a quaternary carbon at δ_C 95.0 corresponding to C-3, and two oxygenated quaternary carbons at δ_C 167.3 and 158.6, corresponding to C-4 and C-6, respectively. The signal at δ_C 165.3 indicated the presence of a conjugated lactone (C-2). The ^{13}C NMR spectrum revealing the presence of 7 carbon atoms as well as ^1H NMR and mass spectra supported a molecular formula of $C_7H_8O_3$. The attachment of the methyl groups at C-3 and C-6 was confirmed by the observed HMBC correlation of 3-C*H*$_3$ to C-2, C-3 and C-4, and of 6-C*H*$_3$ to C-5 and C-6 (see Table 3.16 and Figure 3.16). The structure was confirmed by comparison of UV, ^1H, ^{13}C NMR and mass spectral data with published data for methyltriacetic lactone (Fehr *et al.*, 1999), previously isolated from *Penicillium* species (Acker *et al.*, 1966; Savard *et al.*, 1994).

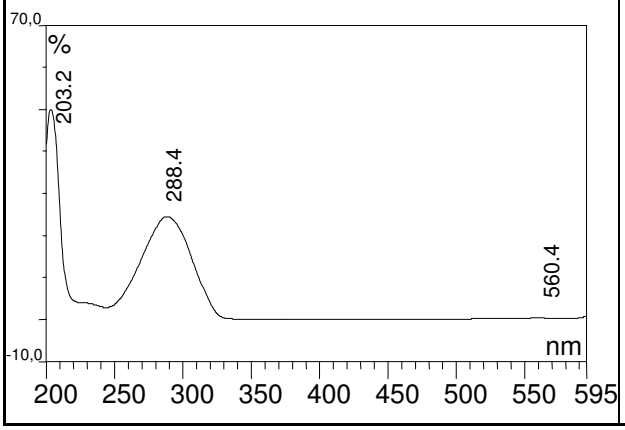
3.2.2. Ampelopyrone (17, new compound)

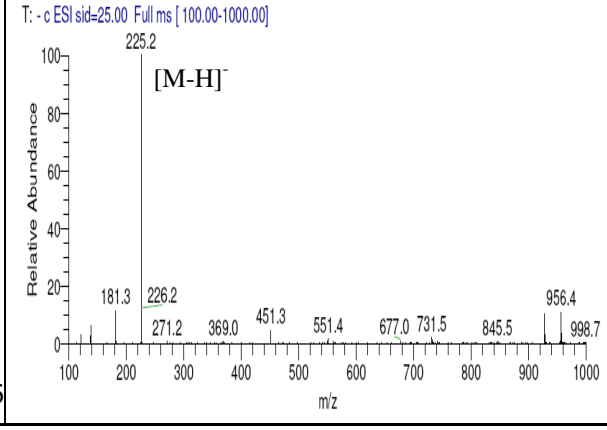
Ampelopyrone	
Synonym(s)	6-(2-hydroxypropyl)-3-methyl-2-oxo-2H-pyran-4-yl acetate
Sample code	AE4.4
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	2.1 mg
Physical Description	viscous yellow oil
Molecular Formula	$C_{11}H_{13}O_5$
Molecular Weight	226 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 67 (*c* 0.2, MeOH)
Retention time HPLC	18.2 min (standard gradient)







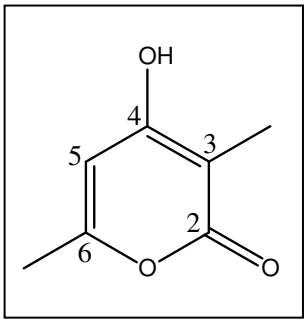




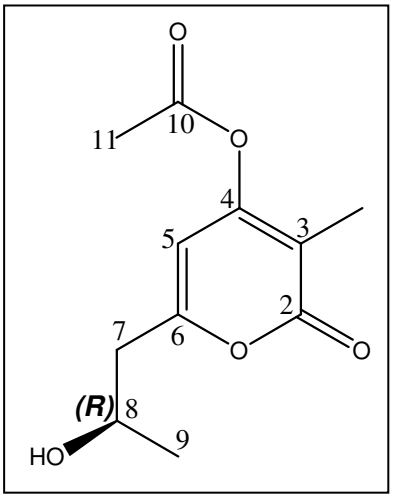
Compound **17** was isolated from the EtOAc extract of liquid cultures of *Ampelomyces* sp. in the form of viscous yellow oil (2.1 mg). It showed UV absorbances at λ_{max} (MeOH) 203.2 and 288.4 nm, showing high similarity to the UV spectrum of methyltriacetic lactone (**16**). The HRESI-MS exhibited a strong peak at *m/z* 227.0910 $[M+H]^+$ indicating a molecular formula of $C_{11}H_{14}O_5$ (calculated 227.0919, Δ 0.0009). The ^1H and ^{13}C NMR spectra (Table 3.16) indicated the presence of three methyl groups, *i.e.* an aromatic methyl group located at the α-position of the carbonyl group of the conjugated lactone at δ_H 1.71 and δ_C 8.4 (3-*CH*$_3$), an acetoxy methyl group at δ_H 1.94 and δ_C 20.8, and a methyl group at δ_H 1.19 (d, *J*=6.3 Hz) and δ_C 19.4 (*CH*$_3$-9). The latter was found to be part of a saturated spin system by coupling to a carbinolic hydrogen at δ_H 5.02 and a ^{13}C NMR signal at δ_C 67.6, indicating a secondary alcohol group, which in turn was adjacent to a methylene group (δ_H 2.65 and δ_C 38.5). Evidence for this substructure was found in the COSY spectrum. The attachment of the side chain to C-6 was established by the HMBC correlation of C*H*$_2$-7 to C-5 (Table 3.16 and Figure 3.17). Similar to methyltriacetic lactone, an aromatic proton singlet at δ_H 5.97 assigned to H-5 as well as the corresponding tertiary aromatic carbon at δ_C 101.2 were detected in the ^1H and ^{13}C NMR spectra. The ^{13}C NMR spectrum showed also a quaternary carbon at δ_C 96.7 corresponding to C-3, and two oxygenated quaternary carbons at δ_C 165.5 and 158.5, corresponding to C-4 and C-6, respectively. The signal at δ_C 164.7 indicated the presence of a conjugated lactone (C-2). The α-pyrone found in **17** was confirmed by the HMBC correlation of 3-C*H*$_3$ to C-2, C-3 and C-4 and of H-5 to C-3 (Table 3.16 and Figure 3.17).

In order to determine the absolute configuration of the metabolite we applied the modified Mosher procedure in an NMR tube. The observed shift differences between the (*S*)-MTPA ester and its (*R*)-MTPA ester epimer led to the assignment of the chiral centre at C-8 of ampelopyrone as shown in **17** (see Table 3.16a).

Thus, **17** was identified as a new natural product for which we suggest the name ampelopyrone.



16 Methyltriacetic lactone



17 Ampelopyrone

Table 3.16: ^{1}H,^{13}C NMR and HMBC data of compounds **16** and **17** at 500 (^{1}H) and 125 MHz (^{13}C)

Nr.	16		16[a]		17			
	δ_H (MeOD)	HMBC	δC (MeOD)	δ_H (DMSO-d_6)	δ_C (DMSO-d_6)	δ_H (DMSO-d_6)	COSY	HMBC	δ_C (DMSO-d_6)
2			165.3		164.8				164.7
3			95.0		96.2				96.7
4			167.3		165.0				165.5
5	5.88,s	6-CH_3,3,4,6	101.0	5.98, s	99.6	5.97, s	7	3,6,7	101.2
6			158.6		159.2				158.5
7						2.65, m	5,8	5,6,8	38.5
8						5.02, m	7,9		67.6
9						1.19, d (6.3)	8	7,8	19.4
10									169.6
11						1.94, s		10	20.8
3-CH_3	1.69, s	2,3,4	8.5	1.74, s	8.2	1.71, s		2,3,4	8.4
6-CH_3	2.08, s	5,6	19.2	2.14	19.1				
8-OH						4.02, brs			

a) Fehr *et al.*, 1999.

Table 3.16a: Chemical shift difference between the (2`*S*)-MTPA and (2`*R*)-MTPA ester of **17**

Nr.	Chemical shift (δ_H, in C_5D_5N, at 500 MHz)			Δ
	17	(*S*)-MTPA ester	(*R*)-MTPA ester	δS - δR
5	6.1884	6.4853	6.4909	- 0.0056
7	2.6693	2.7084	2.7138	- 0.0054
9	1.1900	1.1914	1.1908	0.0006
11	2.2442	1.9920	1.9895	0.0025
3-CH_3	1.9138	1.8155	1.8313	- 0.0158

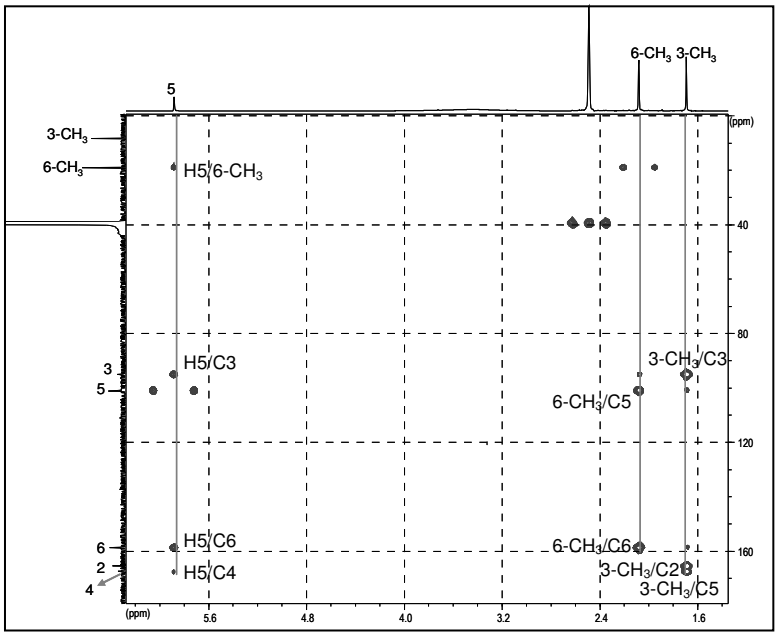


Figure 3.16: HMBC spectrum of compound **16**.

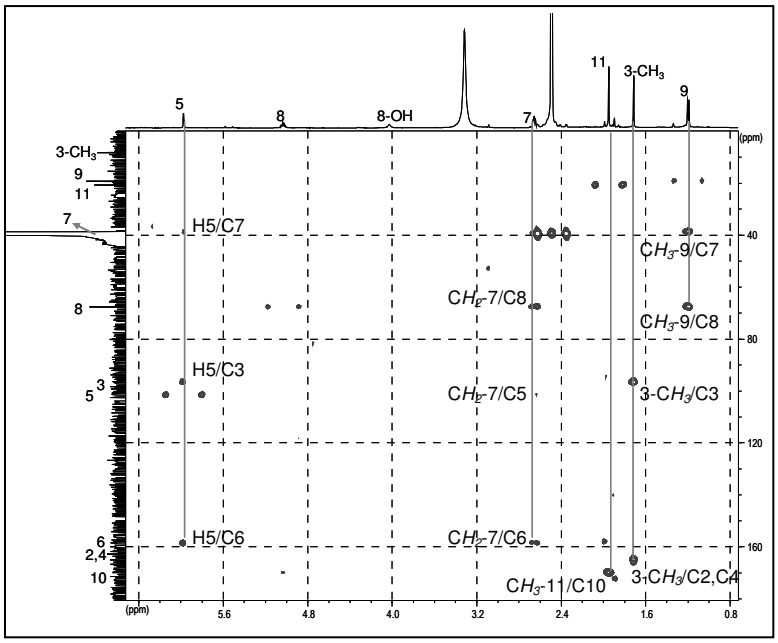
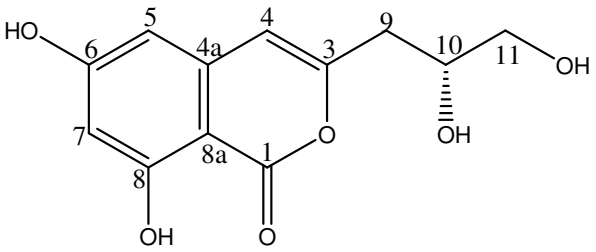
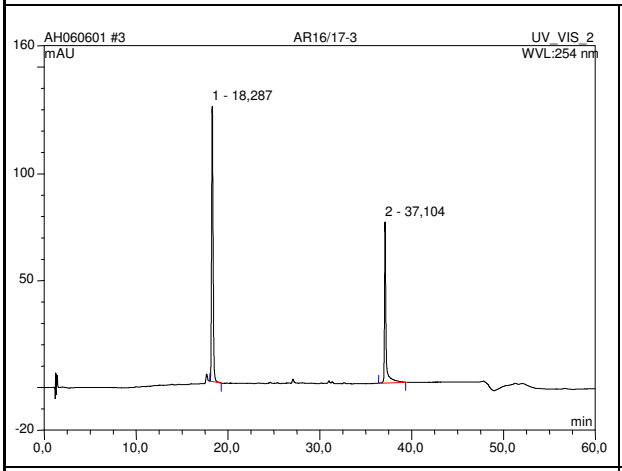


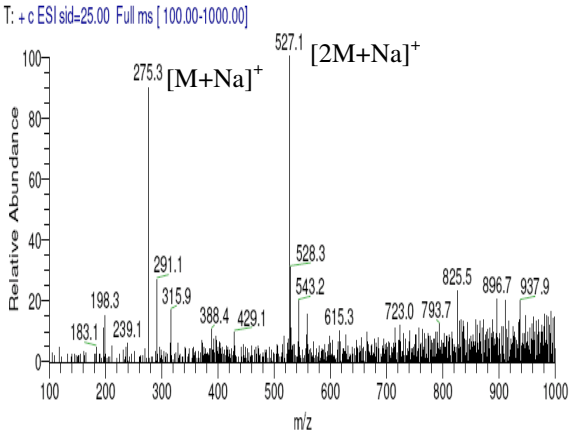
Figure 3.17: HMBC spectrum of compound **17**.

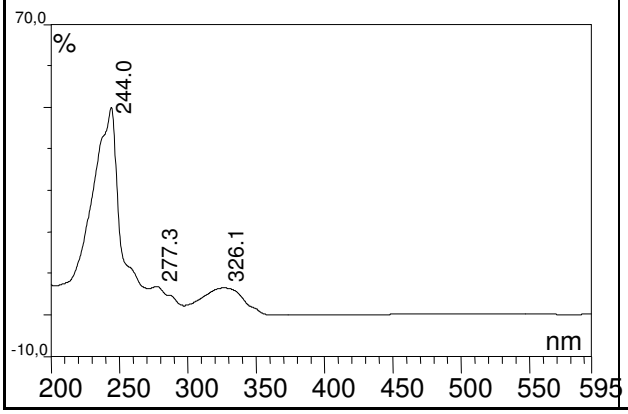
3.2.3. Desmethyldiaportinol (18, new compound)

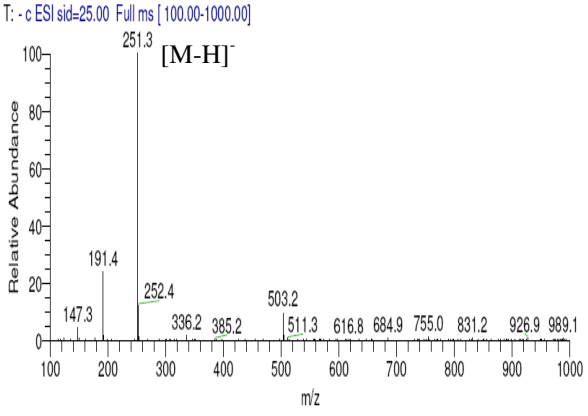
Desmethyldiaportinol	
Synonym(s)	6,8-Dihydroxy-3-(2-hydroxypropyl)-isocoumarin
Sample code	AR6.3.1
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	1.5 mg
Physical Description	viscous yellowish oil
Molecular Formula	$C_{12}H_{12}O_6$
Molecular Weight	252 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 51 (*c* 0.5, MeOH)
Retention time HPLC	18.3 min (standard gradient)







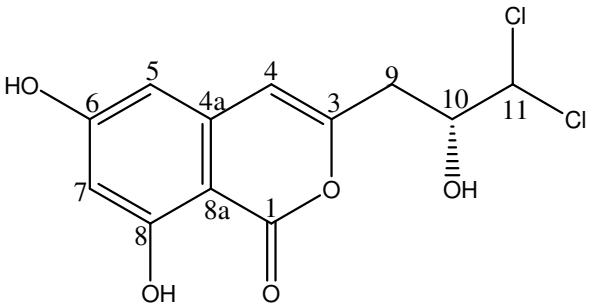


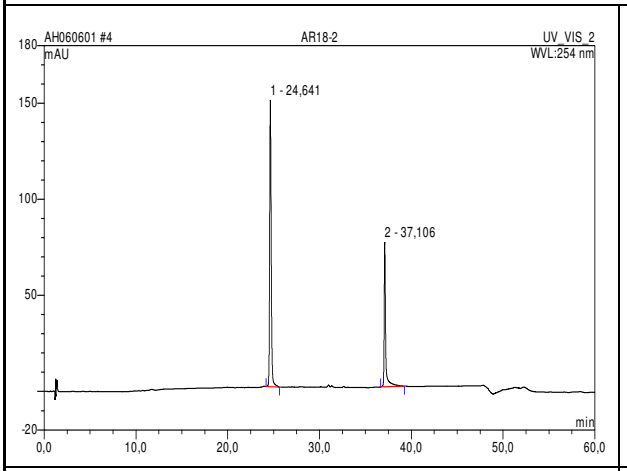


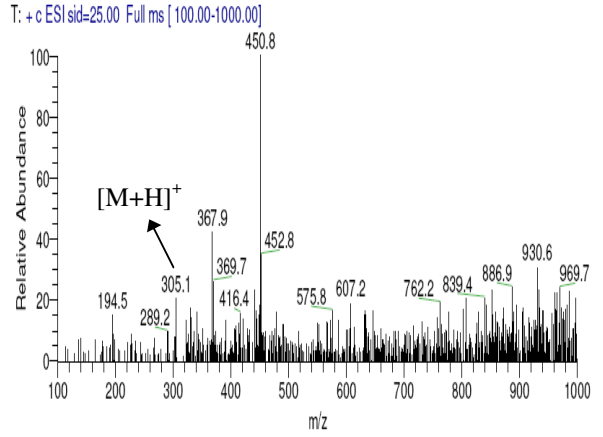
Compound **18** was isolated from the EtOAc extract of rice cultures of *Ampelomyces* sp. as a viscous yellow oil (1.5 mg). It showed UV absorbances at λ_{max} (MeOH) 244.0, 277.3 and 326.1 nm characteristic of isocoumarin derivatives (Larsen and Breinholt, 1999). The HRESI-MS exhibited a prominent peak at *m/z* 275.0540 $[M+Na]^+$ indicating a molecular formula of $C_{12}H_{12}O_6$ (calculated 275.0532, Δ 0.0008). The ^1H NMR spectrum (see Table 3.17) displayed characteristic signals attributable to protons H-4, H-5 and H-7, appearing at δ_H 6.43 (s), 6.42 (d, *J*=2.2 Hz) and 6.37 (d, *J*=2.2 Hz), respectively, in a 3,6,8-trisubstituted isocoumarin ring system. A downfield one-proton singlet signal (δ_H 11.14) indicated the presence of a strongly hydrogen-bonded phenolic proton at C-8. ^1H NMR, COSY and NOE spectra (see Table 3.17) confirmed the substitution pattern and demonstrated the presence of a CH_2CHCH_2 fragment consisting of two methylene protons detected at δ_H 2.76 (dd, *J*=14.5, 3.7 Hz, H-9A) and 2.52 (dd, *J*=14.5, 8.8 Hz, H-9B), a carbinolic hydrogen at δ_H 4.02, indicating a secondary alcohol group, and a hydroxymethyl group at δ_H 3.55 (d, *J*=5.3 Hz) (see Figure 3.18). The attachment of the side chain at C-3 was further confirmed by the NOE correlation of H-4 to C*H*$_2$-9 (Table 3.17). Comparison of UV, ^1H, ^{13}C NMR and mass spectral data with literature data indicated the similarity of **18** to the known diaportinol (**18a**), in which the hydroxy group at C-6 is methoxylated (Larsen and Breinholt, 1999). The relative stereochemistry was derived from the obtained $[\alpha]_D$ value found to have identical sign as that measured for similar structures (Larsen and Breinholt, 1999). Thus, **18** was identified as a new natural product, and was given the name desmethyldiaportinol.

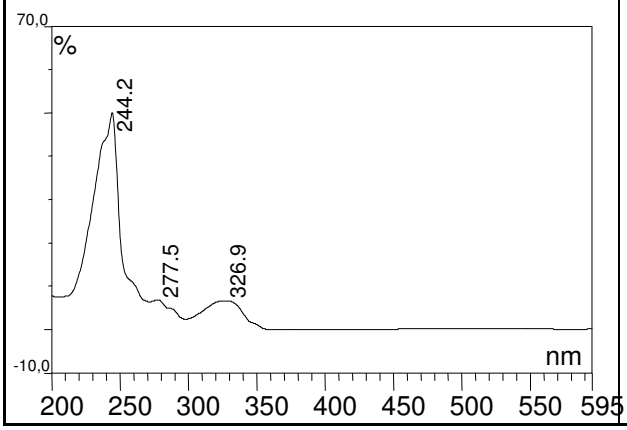
3.2.4. Desmethyldichlorodiaportin (19, new compound)

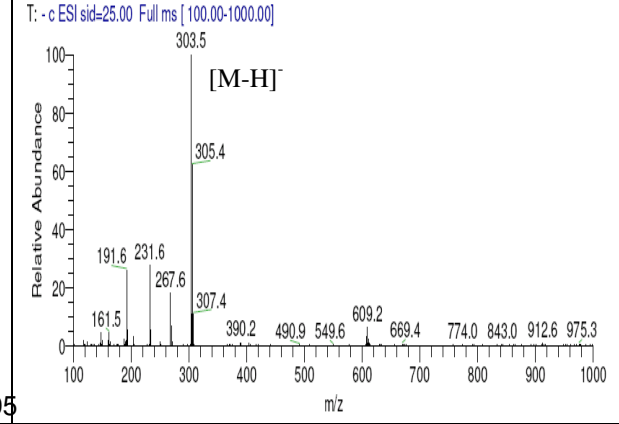
Desmethyldichlorodiaportin	
Synonym(s)	6,8-Dihydroxy-3-(2-hydroxy-3,3-dichloropropyl)-isocoumarin
Sample code	AR6.4.1
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	1.0 mg
Physical Description	viscous yellowish oil
Molecular Formula	$C_{12}H_{10}O_5Cl_2$
Molecular Weight	304 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 19 (*c* 0.3, MeOH)
Retention time HPLC	24.6 min (standard gradient)







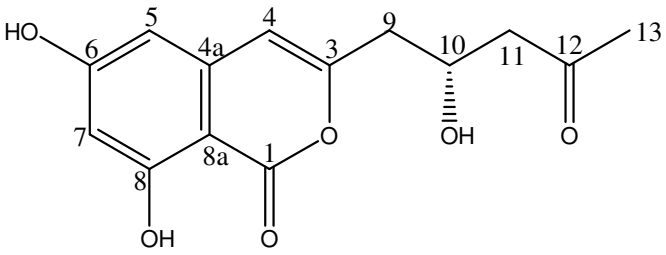


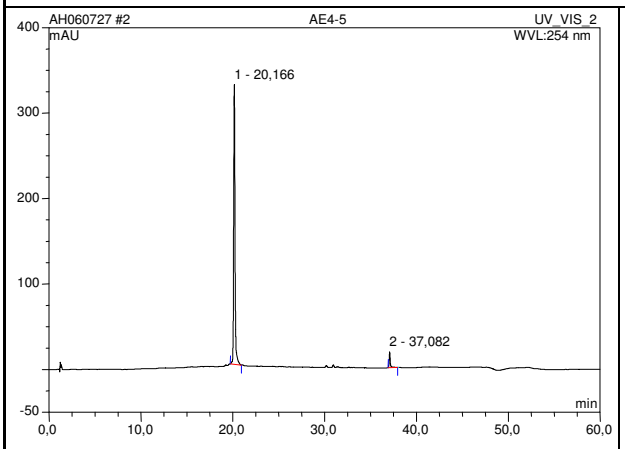


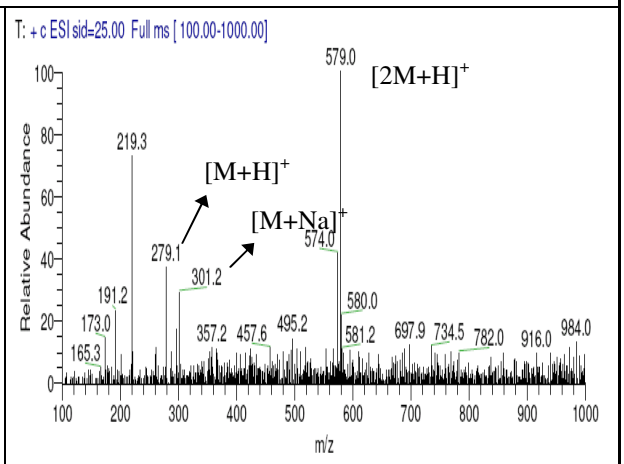
Compound **19** was isolated from the EtOAc extract of rice cultures of *Ampelomyces* sp. in the form of a viscous yellow oil (1.0 mg). It displayed UV absorbances at λ_{max} (MeOH) 244.2, 277.5 and 326.9 nm characteristic of isocoumarin derivatives and very similar to those reported for desmethyldiaportinol (**18**). The HRESI-MS exhibited a prominent peak at *m/z* 326.9800 $[M+Na]^+$ indicating a molecular formula of $C_{12}H_{10}O_5Cl_2$ (calculated 326.9802, Δ 0.0002). The negative ESI-MS showed the presence of molecular ion peaks at *m/z* 303.5, 305.4 and 307.4 $[M-H]^-$, with the distinctive isotope pattern caused by two chloro atoms in the molecule, indicating the dichlorosubstitution in this compound. The ^{1}H NMR spectrum (see Table 3.17) showed the characteristic signals assigned to protons H-4, H-5 and H-7 observed at δ_H 6.50 (s), 6.42 (d, *J*=2.0 Hz) and 6.38 (d, *J*=2.0 Hz), respectively, indicating a 3,6,8-trisubstituted isocoumarin ring system, as in the case of desmethyldiaportinol. ^{1}H NMR, COSY and NOE spectra (see Table 3.17) confirmed the substitution pattern and demonstrated the presence of an analogous $CH_2CH(OH)CH$ as described above, with two methylene protons detected at δ_H 2.98 (dd, *J*=14.5, 3.4 Hz, H-9A) and 2.76 (dd, *J*=14.5, 9.2 Hz, H-9B), an oxygenated carbinolic hydrogen at δ_H 4.39, and a methine group at δ_H 6.20 (d, *J*=3.1 Hz) (see Figure 3.18). Thus, the ^{1}H NMR data were highly similar to those of desmethyldiaportinol (**18**), except for the marked downfield shift of H-11 which indicated dichlorosubstitution at this position (Table 3.17). The attachment of the side chain at C-3 was confirmed by the NOE correlation of H-4 to C*H*$_2$-9 (Table 3.17). Thus, **19** was identified as the 10-deoxy-10,10-dichloro congener of **18** and represents a new natural product for which we suggest the name desmethyldichlorodiaportin.

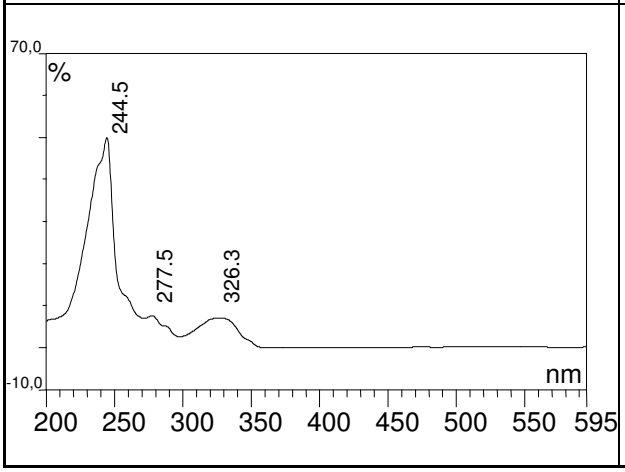
3.2.5. (+)-Citreoisocoumarin (20, known compound)

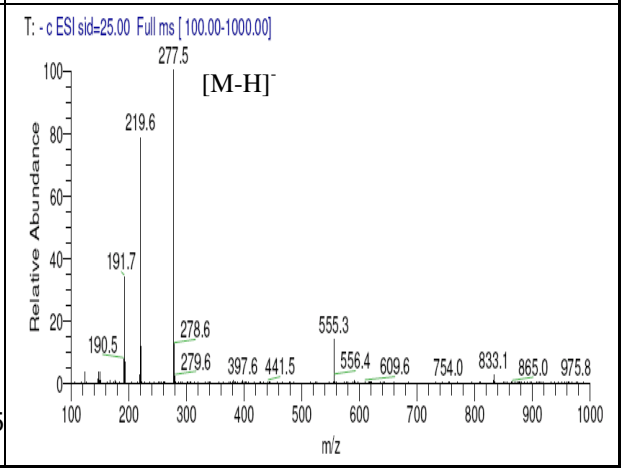
(+)-Citreoisocoumarin	
Synonym(s)	3-(2-Hydroxypentyl-4-ketone)-6,8-dihydroxyisocoumarin
Sample code	AE4.3.1
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	0.5 mg
Physical Description	viscous yellowish oil
Molecular Formula	$C_{14}H_{14}O_6$
Molecular Weight	278 g/mol
Optical Rotation $[\alpha]_D^{20}$	+ 15 (*c* 0.1, MeOH)
Retention time HPLC	20.2 min (standard gradient)



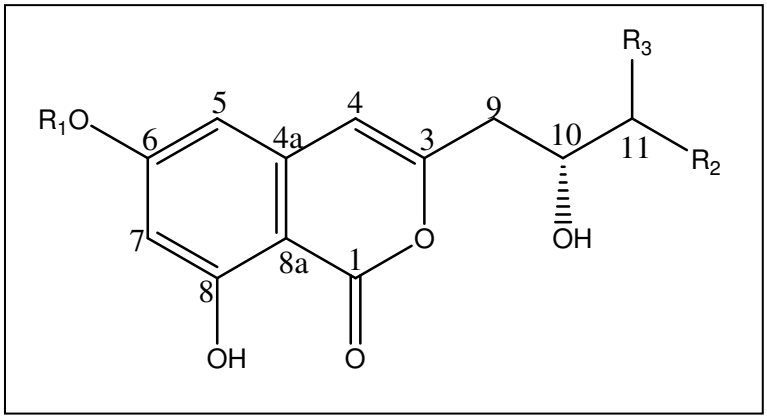








(+)-Citreoisocoumarin (**20**) was isolated from the EtOAc extract of liquid cultures of *Ampelomyces* sp. as viscous yellow oil (0.5 mg). It showed UV absorbances at λ_{max} (MeOH) 244.5, 277.5 and 326.3 nm characteristic of isocoumarin derivatives as described for **18** and **19**. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 279.1 $[M+H]^+$ and *m/z* 277.5 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 278 g/mol. The ^1H NMR spectrum (see Table 3.18) showed the characteristic signals corresponding to protons H-4, H-5 and H-7 observed at δ_H 6.39, 6.36 (d, *J*=2.0 Hz) and 6.3 (d, *J*=2.0 Hz), respectively, indicating a 3,6,8-trisubstituted isocoumarin ring system, similar to previously discussed isocoumarin derivatives **18** and **19** (see Table 3.17). It showed two methylene groups, with the protons resonating at δ_H 2.71 (dd, *J*=16.1, 4.4 Hz, H-11A), 2.65 (dd, *J*=16.1, 7.8 Hz, H-11B), 2.66 (dd, *J*=14.3, 4.4 Hz, H-9A) and 2.57 (dd, *J*=14.3, 8.2 Hz, H-9B), both of which coupled to an oxygenated methine group at δ_H 4.44, indicating a secondary alcohol group. Additionally, a singlet at δ_H 2.13 (3H) was assigned to a methyl group adjacent to a keto function. Thus, **20** was identified as the known (+)citreoisocoumarin, and its UV, ^1H NMR, mass spectral data and $[\alpha]_D$ value were in agreement with published data (Lai *et al.*, 1991; Watanabe *et al.*, 1998). This compound was originally isolated from *Aspergillus* (Watanabe *et al.*, 1998, 1999) and *Penicillium* species (Lai *et al.*, 1991).



Nr.	Compound	R_1	R_2	R_3
18	Desmethyldiaportinol	H	OH	H
18a	Diaportinol	CH_3	OH	H
19	Desmethyldichlorodiaportin	H	Cl	Cl
19a	Dichlorodiaportin	CH_3	Cl	Cl
20	Citreoisocoumarin	H	$COCH_3$	H

Table 3.17: ^{1}H NMR, COSY and NOE data of compounds **18** and **19** at 500 MHz

Nr.	18				18a[a]	19				19a[a]
	δ_H (MeOD)	δ_H (Acetone-d_6)	COSY	NOE	δ_H (Acetone-d_6)	δ_H (MeOD)	δ_H (Acetone-d_6)	COSY	NOE	δ_H (Acetone-d_6)
4	6.38, s	6.43, s	9A	5,9A,9B	6.46, s	6.41, s	6.50, s	9A	5,9B,9A	6.57, s
5	6.29, s	6.42, d (2.2)	7	4	6.53, s	6.30, s	6.42, d (2.0)	7	4	6.53, d (2.2)
7	6.29, s	6.37, d (2.2)	5		6.53, s	6.30, s	6.38, d (2.0)	5		6.47, d (2.2)
9A	2.74, dd (14.6, 4.1)	2.76, dd (14.5, 3.7)	4,9B,10		2.79, dd (14.7, 3.4)	2.96, dd (14.8, 3.1)	2.98, dd (14.5, 3.4)	4,9B,10		3.02, dd (14.7, 3.3)
9B	2.52, dd (14.6, 8.8)	2.52, dd (14.5, 8.8)	9A,10		2.56, dd (14.7, 8.8)	2.72, dd (14.8, 9.4)	2.76, dd (14.5, 9.2)	9A,10		2.80, dd (14.7, 9.2)
10	3.99, m	4.02, m	9A,9B,11		4.04, m	4.29, ddd (9.4, 3.4, 3.1)	4.39, ddd (9.2,3.4, 3.1)	9A,9B,11		4.41, m
11	3.54, d (5.3)	3.55, d (5.3)	10		3.58, d (5.4)	6.02, d (3.4)	6.20, d (3.1)	10	10	6.20, d (3.3)
OCH_3					3.91, s					3.90, s
6-OH										
8-OH		11.14, brs			11.16, s					11.08, s
10-OH										5.25, brs

a) Larsen and Breinholt, 1999.

Table 3.18: [1]H NMR data of compound **20** at 500 MHz

Nr.	(+)-20		(-)-20[a]	(+)-20[b]
	δ_H (MeOD)	δ_H (Acetone-d_6)	δ_H (MeOD)	δ_H (Acetone-d_6)
4	6.33, s	6.39, s	6.33, s	6.53, s
5	6.24, d (2.2)	6.36, d (2.0)	6.39, d (2.2)	6.49, d (2.1)
7	6.23, d (2.2)	6.33, d (2.0)	6.32, d (2.2)	6.45, d (2.1)
9	A 2.66, dd (14.5, 5.0)	A 2.66, dd (14.3, 4.4)	2.67, d (6.3)	A 2.83, dd (14.5, 4.8)
	B 2.58, dd (14.5, 7.5)	B 2.57, dd (14.3, 8.2)		B 2.72, dd (14.5, 8.1)
10	4.43, m	4.44, m	4.47, m	4.55, m
11	2.68, d (5.9)	A 2.71, dd (16.1, 4.4)	2.71, d (6.3)	A 2.86, dd (16.3, 4.7)
		B 2.65, dd (16.1, 7.8)		B 2.80, dd (16.3, 7.8)
13	2.17, s	2.13, s	2.23, s	2.23, s
6-OH				9.70, brs
8-OH				11.22, brs

a) Lai *et al.*, 1991.
b) Watanabe *et al.*, 1998.

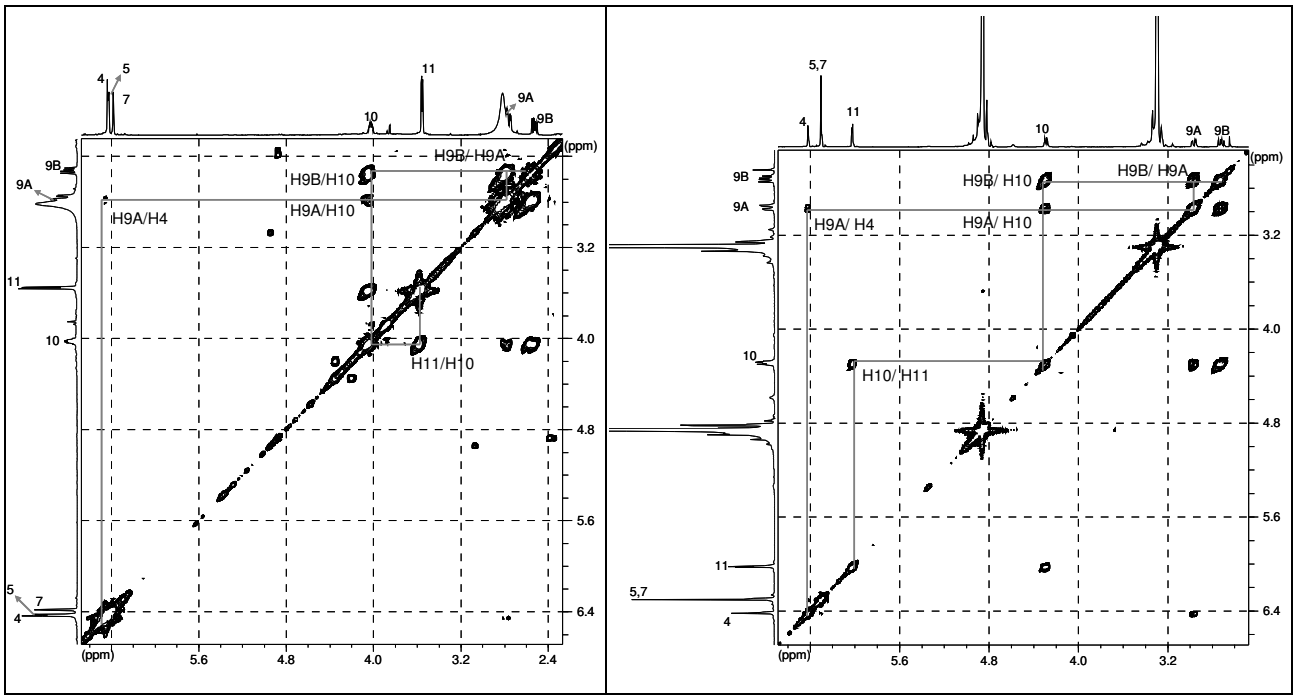
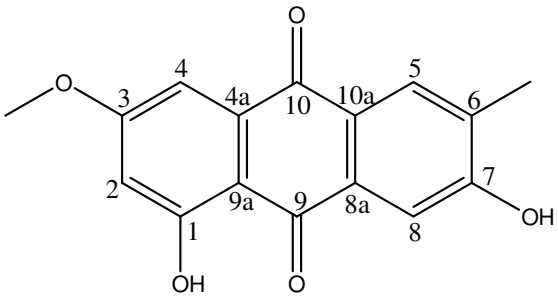
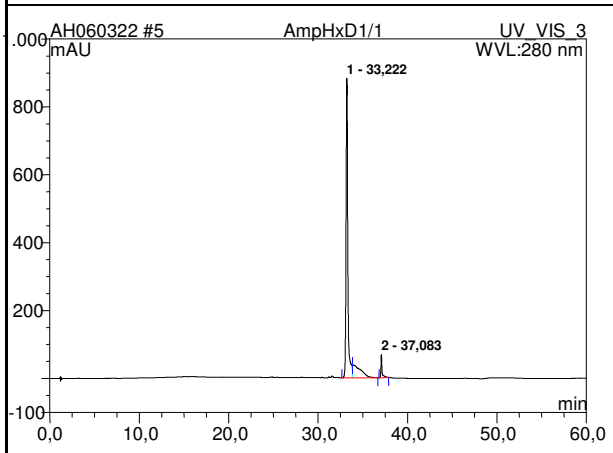


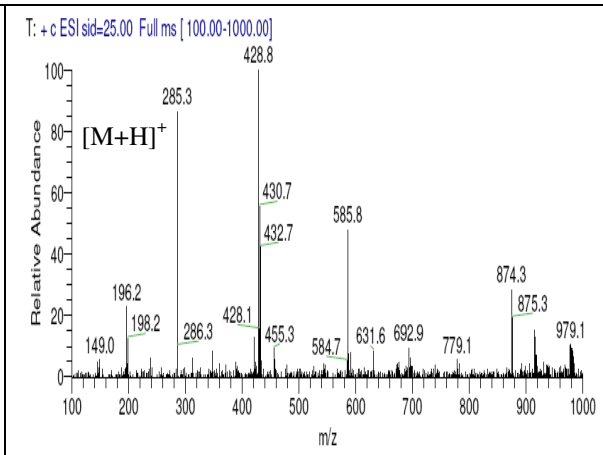
Figure 3.18: COSY spectra of compounds **18** and **19**.

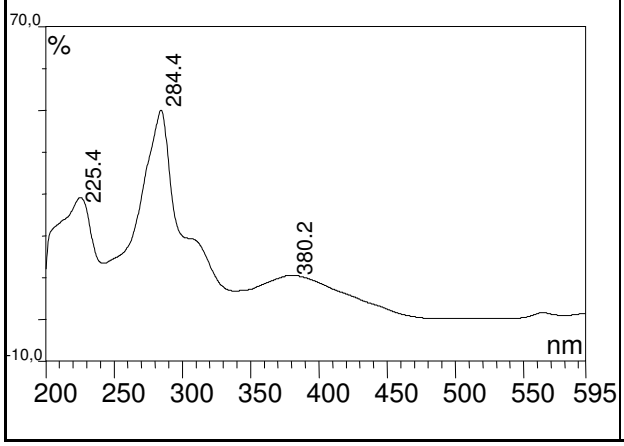
3.2.6. Macrosporin (21, known compound)

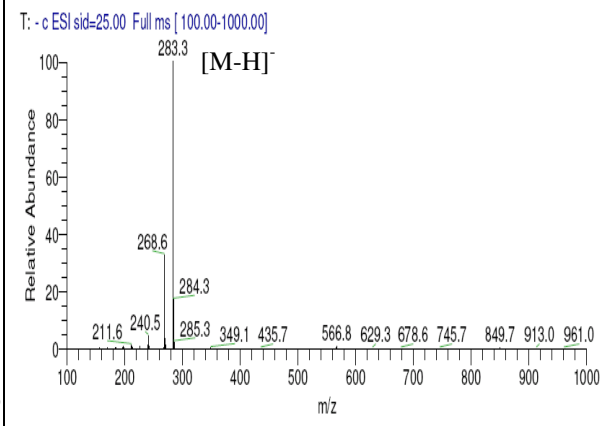
Macrosporin	
Synonym(s)	1,7-Dihydroxy-3-methoxy-6-methylanthraquinone
Sample code	AE2.3, AR2
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	21.6 mg
Physical Description	yellow crystals
Molecular Formula	$C_{16}H_{12}O_5$
Molecular Weight	284 g/mol
Retention time HPLC	33.2 min (standard gradient)







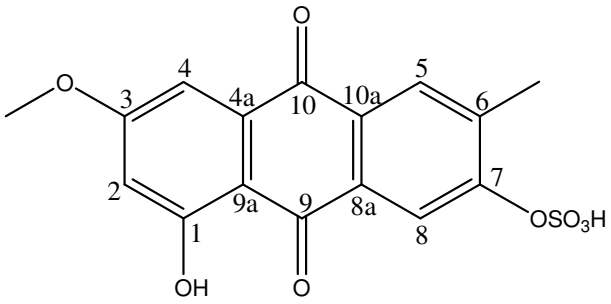


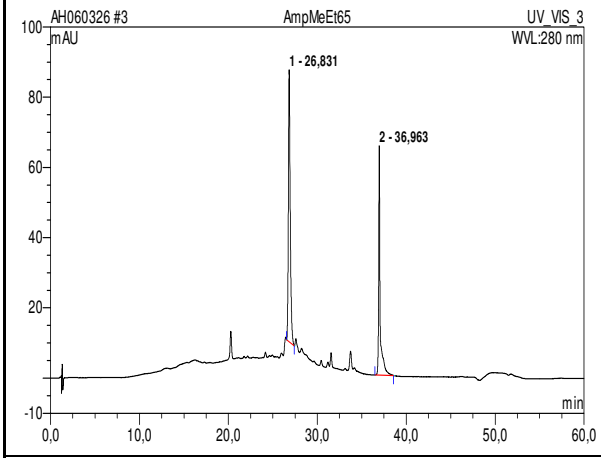


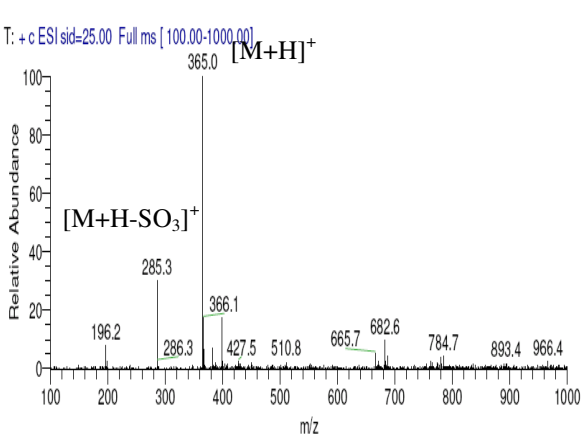
Macrosporin (**21**) was isolated from the EtOAc extract of liquid and rice cultures of *Ampelomyces* sp. as yellow crystals (21.6 mg). It exhibited UV absorbances at λ_{max} (MeOH) 225.4, 284.4 and 380.2 nm suggesting an anthraquinone as the basic structure. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 285.3 $[M+H]^+$ (base peak) and *m/z* 283.6 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 284 g/mol. ^1H and ^{13}C NMR spectra (see Table 3.19) indicated the presence of an aromatic methyl group at δ_H 2.34 and δ_C 16.4, and a methoxy singlet at δ_H 4.00 and δ_C 56.7. The ^{13}C NMR spectrum showed resonances at δ_C 187.6 and 181.2, indicative of a quinone, and three aromatic carbons connected to oxygens at δ_C 167.0, 165.8 and 162.5. The ^1H NMR spectrum revealed the presence of four aromatic protons, two of which were doublets occurring at δ_H 7.19 and 6.80 with a coupling constant of 2.5 Hz, indicating their *meta* disposition in the ring system, corresponding to H-4 and H-2, respectively, while the remaining two aromatic proton singlets at δ_H 7.95 and 7.67, were assigned to the *para*-coupled protons H-5 and H-8, respectively, of the other aromatic ring. The ^{13}C NMR spectrum displayed 16 carbon atoms, and together with ^1H NMR and mass spectral data the molecular formula of $C_{16}H_{12}O_5$ was derived. The compound was thus identified as the known macrosporin, which was confirmed by interpretation of HMBC spectra (see Table 3.19 and Figure 3.19) and comparison of UV, ^1H, ^{13}C NMR and mass spectral data with published data (Suemitsu et al., 1984, 1989). Macrosporin was previously reported from several *Alternaria* species (Stoessl *et al.*, 1983; Lazarovits *et al.*, 1988; Suemitsu *et al.*, 1989) as well as from *Phomopsis juniperovora* (Wheeler and Wheeler, 1975), *Dactylaria lutea* (Becker *et al.*, 1978), *Dichotomophthora lutea* (Hosoe *et al.*, 1990) and *Pleospora* sp. (Ge *et al.*, 2005).

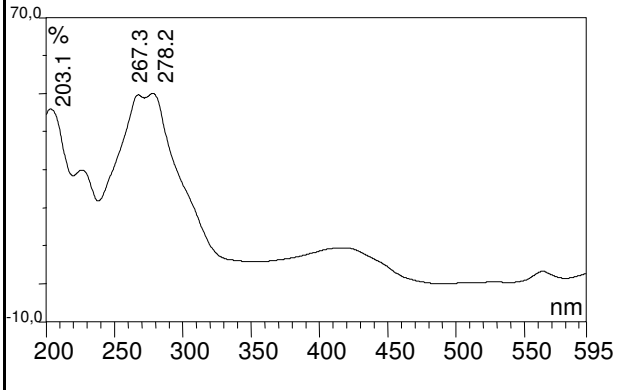
3.2.7. Macrosporin-7-*O*-sulphate (22, new compound)

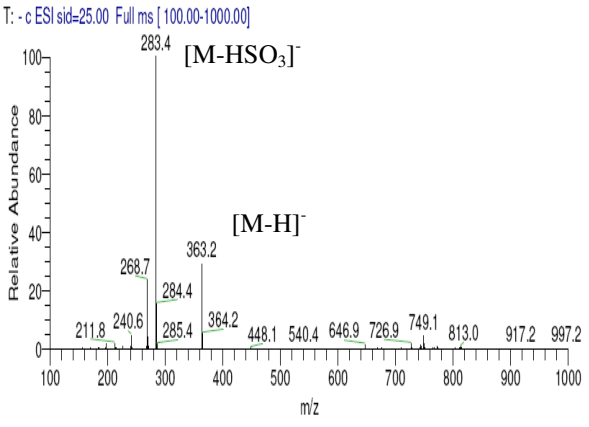
Macrosporin-7-*O*-sulphate	
Synonym(s)	1-Hydroxy-3-methoxy-6-methylanthraquinone-7-sulphate
Sample code	AME3, AR8.4.2
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	7.1 mg
Physical Description	yellow crystals
Molecular Formula	$C_{16}H_{12}O_8S$
Molecular Weight	364 g/mol
Retention time HPLC	26.8 min (standard gradient)



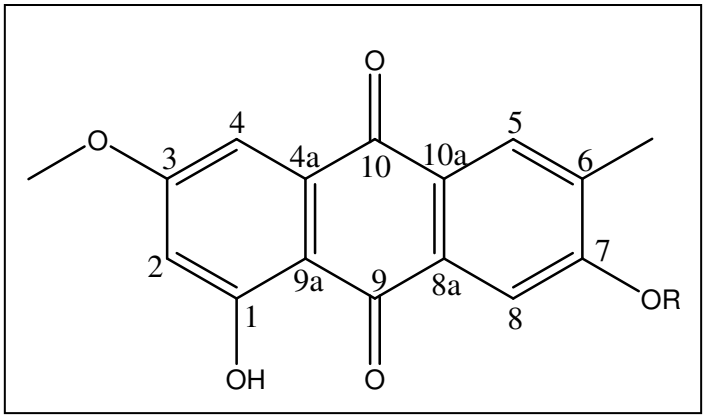








Compound **22** was isolated from the MeOH extract of liquid cultures and EtOAc extract of rice cultures of *Ampelomyces* sp. in the form of yellow crystals (7.1 mg). Its UV spectrum showed λ_{max} (MeOH) at 203.1, 267.3, 278.3 and 420.0 nm. The HRESI-MS exhibited a prominent peak at *m/z* 408.9970 $[M+2Na]^+$ indicating a molecular formula of $C_{16}H_{12}O_8S$ (calculated 408.9969, Δ 0.0001). Comparison of ^1H, ^{13}C NMR and HMBC data (Table 3.19 and Figure 3.19) with those measured for macrosporin (**21**) showed good accordance except for the downfield shifts observed for H-8, as well as the upfield shift of C-7, by 6.5 ppm, and downfield shifts of C-6 and C-8, by 5.0 and 7.2 ppm, respectively, indicating the presence of a sulphate substitution at C-7 (Ragan, 1978). This assumption was corroborated by the fragment formed through loss of 80 mass units in the mass spectra of **22** and the hypsochromic shift in the UV spectrum of **22** compared to that of **21**, which is attributed to the electron withdrawing effect of the sulphate group (Plasencia and Mirocha, 1991). The compound was thus identified as the new natural product macrosporin-7-*O*-sulphate.



Nr.	Compound	R
21	Macrosporin	H
22	Macrosporin sulphate	SO_3H

Table 3.19: ^{1}H, ^{13}C NMR and HMBC data of compounds **21** and **22** at 500 (^{1}H) and 125 MHz (^{13}C)

Nr.	**21**			**21**[a,b]		**22**		
	δ_H (DMF-d_7)	HMBC	δ_C (DMF-d_7)	δ_H (THF-d_8)[a]	δ_C (THF-d_8)[b]	δ_H (MeOD)	HMBC	δ_C (MeOD)
1			165.8		166.4			166.0
2	6.80, d (2.5)	1,3,4,9a	106.0	6.72, d (2.5)	106.1	6.74, d (2.5)	1,3,4,9a	107.0
3			167.0		167.4			167.0
4	7.19, d (2.5)	2,9a,10	107.8	7.30, d (2.5)	108.0	7.27, d (2.5)	2,3,9a,10	108.0
4a			136.0		136.5			
5	7.95, s	CH_3,7,8a,9,10	130.8	8.00, s	131.1	8.06, s	CH_3,7,8a,9,10	131.0
6			133.0		133.0			138.0
7			162.5		162.3			156.0
8	7.67, s	6,7,8a,9,10,10a	111.8	7.54, s	111.6	8.36, s	6,7,8a,9,10,10a	119.0
8a			134.1		134.6			134.0
9			187.6		187.9			187.0
9a			111.1		111.5			111.0
10			181.2		181.2			182.0
10a			126.0		126.9			130.0
CH_3	2.34, s	5,6,7,8	16.4	2.33, s	16.4	2.46, s	5,6,7,8	17.0
OCH_3	4.00, s	3	56.7	3.93, s	56.4	3.92, s	3	56.0

a) Suemitsu et al., 1984.
b) Suemitsu et al., 1989.

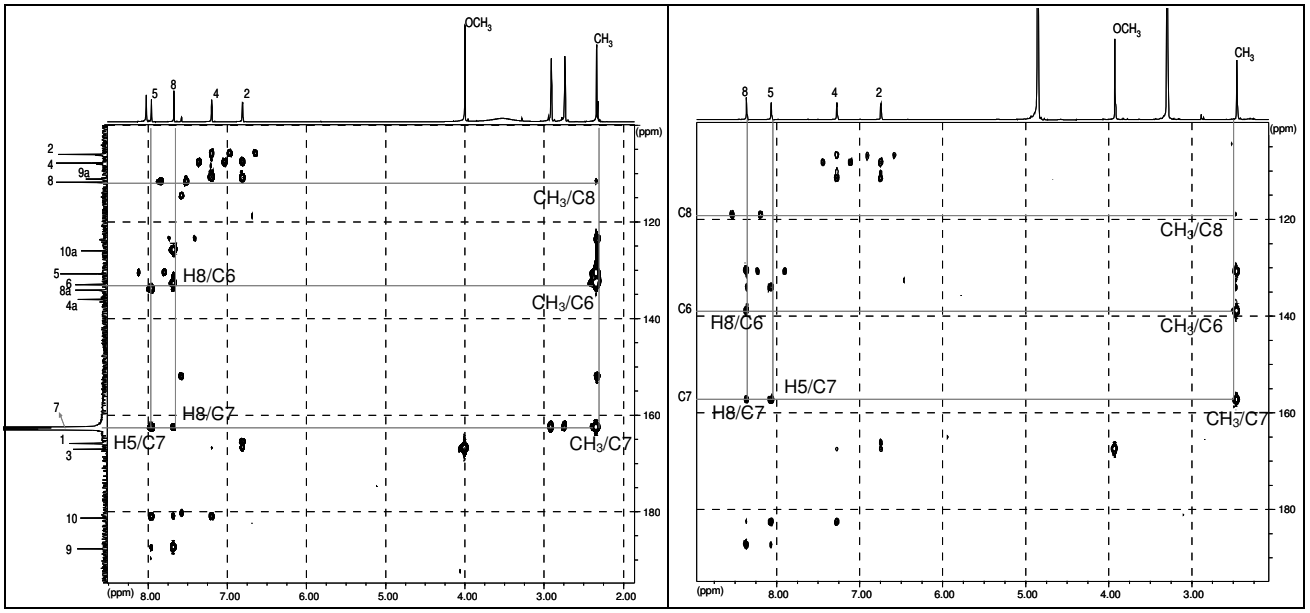
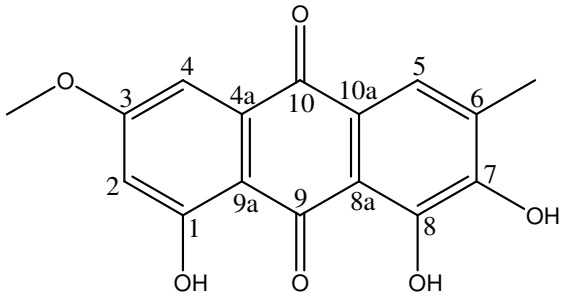
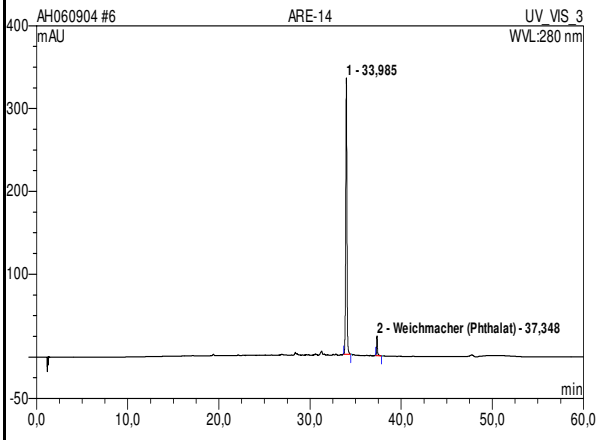


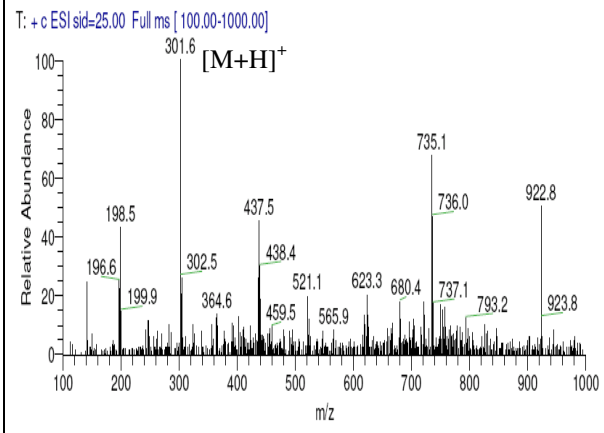
Figure 3.18: HMBC spectra of compounds **21** and **22**.

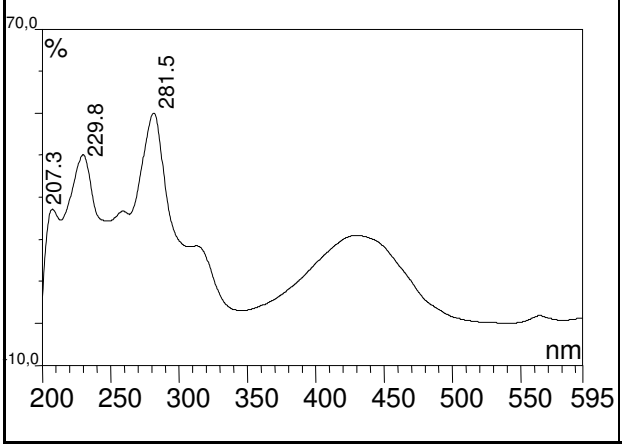
3.2.8. 3-*O*-Methylalaternin (23, known compound)

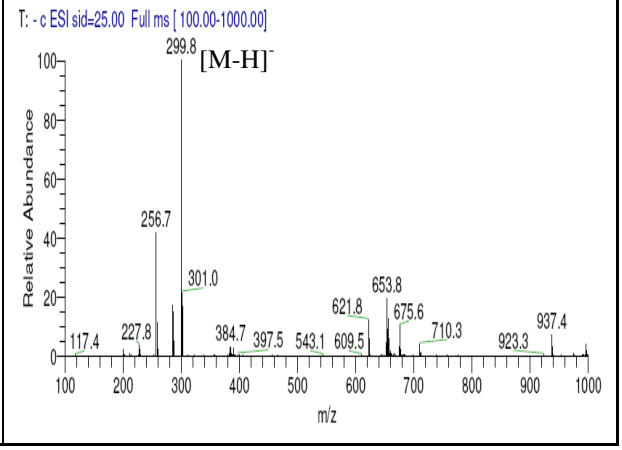
3-*O*-Methylalaternin	
Synonym(s)	1,2,8-Trihydroxy-6-methoxy-3-methylanthraquinone
Sample code	AE2.2, AR4
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	3.5 mg
Physical Description	orange crystals
Molecular Formula	$C_{16}H_{12}O_6$
Molecular Weight	300 g/mol
Retention time HPLC	34.0 min (standard gradient)







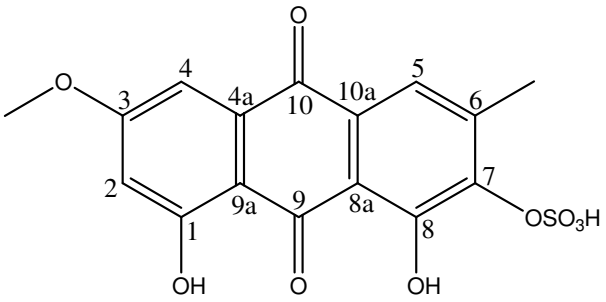


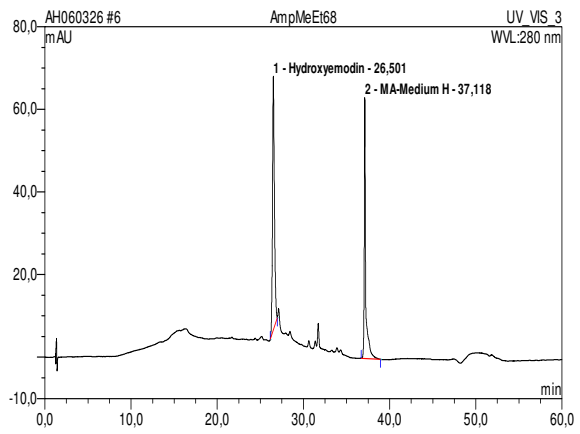


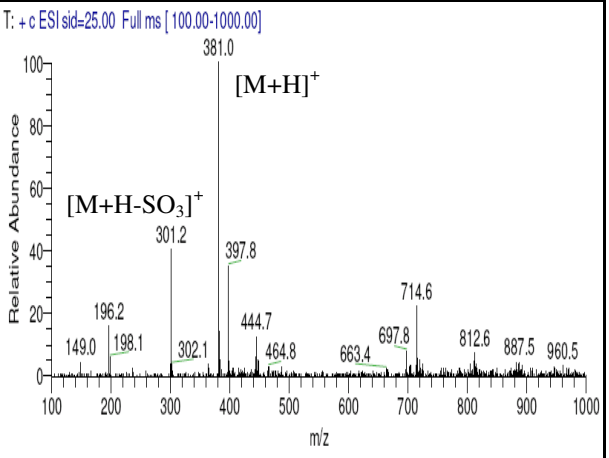
3-*O*-Methylalaternin (**23**) was isolated from the EtOAc extract of liquid and rice cultures of *Ampelomyces* sp. as orange crystals (3.5 mg). It showed UV absorbances at λ_{max} (MeOH) 207.3, 229.8, 281.5 and 435.0 nm suggesting an anthraquinone as the basic structure and showing high similarity to UV spectra of macrosporin (**21**). Positive and negative ESI-MS showed molecular ion peaks at *m/z* 301.6 $[M+H]^+$ (base peak) and *m/z* 299.8 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 300 g/mol, and further indicating an increase of 16 mass units compared to **21** which established the composition $C_{16}H_{12}O_6$. UV and NMR spectra of **23** had close similarity to those of **21**. The ^{1}H NMR spectrum (see Table 3.20) resembled that of **21** except for the absence of the *para*-coupled pair of aromatic protons and the presence of an aromatic proton singlet at δ_H 7.62 assigned to H-5. The *meta*-coupled pair appeared as doublets occurring at δ_H 7.24 and 6.84 with a coupling constant of 2.0 Hz, corresponding to H-4 and H-2, respectively. The ^{13}C resonances at δ_C 187.6 and 180.1 indicated a quinone structure, and both ^{1}H NMR and ^{13}C NMR data (see Table 3.20) indicated the presence of an aromatic methyl group at δ_H 2.34 and δ_C 16.4, and a methoxy singlet at δ_H 4.01 and δ_C 56.7. Proton signals were assigned to their corresponding carbons by HMBC (see Table 3.20 and Figure 3.19). The structure was confirmed by comparison of UV, ^{1}H , ^{13}C NMR and mass spectral data with published data for alaternin (**23a**) (Lee *et al.*, 1998). 3-*O*-Methylalaternin was previously reported from *Alternaria* species (Stoessl, 1969b; Stoessl *et al.*, 1983).

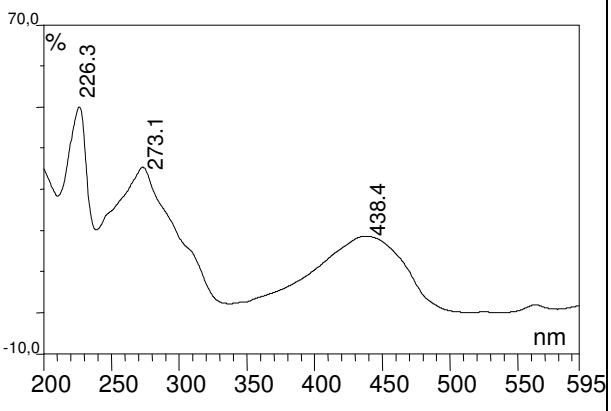
3.2.9. 3-*O*-Methylalaternin-7-*O*-sulphate (24, new compound)

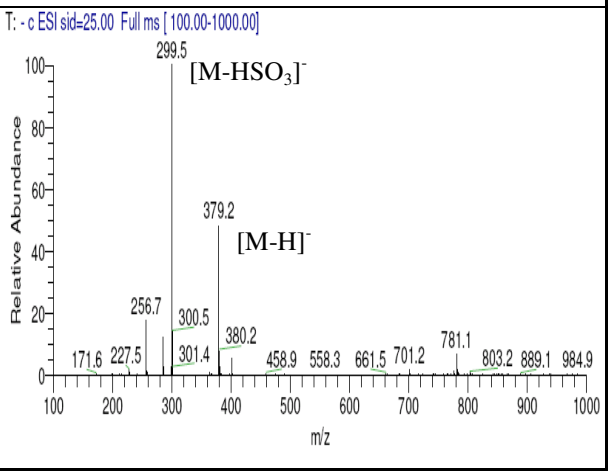
3-*O*-Methylalaternin-7-*O*-sulphate	
Synonym(s)	1,8-Dihydroxy-6-methoxy-3-methylanthraquinone-2-sulphate
Sample code	AME3, AR8.4.1
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	2.5 mg
Physical Description	orange crystals
Molecular Formula	$C_{16}H_{12}O_9S$
Molecular Weight	380 g/mol
Retention time HPLC	26.5 min (standard gradient)



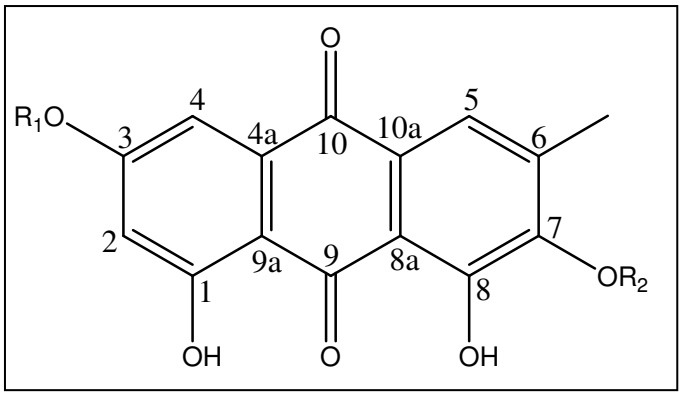








Compound **24** was isolated from the MeOH extract of liquid cultures as well as EtOAc extract of rice cultures of *Ampelomyces* sp. in the form of orange crystals (2.5 mg). It had UV absorbances at λ_{max} (MeOH) 226.3, 273.1 and 438.4 nm. The HRESI-MS exhibited a prominent peak at *m/z* 424.9910 $[M+2Na]^+$, consistent with a molecular formula of $C_{16}H_{12}O_9S$ (calculated 424.9919, Δ 0.0009). The ^1H NMR spectrum (see Table 3.20) showed signals for a methyl group at δ_H 2.52, a methoxy group at δ_H 3.94, a pair of *meta*-coupled aromatic protons at δ_H 7.31 and 6.77 (J=2.5 Hz), and an aromatic singlet at δ_H 7.65, corresponding to H-4, H-2 and H-5, respectively. The aromatic methyl group showed HMBC correlations to carbons at δ_C 146.0, 143.5 and 122.5, assigned to C-7, C-6, and C-5, respectively. Similar to macrosporin sulphate (**22**), comparison of the chemical shifts observed for C-7 and C-6 to those recorded for the respective carbon atoms in methylalaternin (**23**) (see Table 3.20 and Figure 3.19) clearly revealed a prominent upfield shift for C-7 and a downfield shift of C-6, thus indicating the presence of a sulphate substitution at C-7 (Ragan, 1978). Presence of the sulphate substituent was confirmed by the fragment formed by loss of 80 mass units in the mass spectrum of **24** and the hypsochromic shift in the UV spectrum of **24** compared to that of **23**, which is attributed to the electron withdrawing effect of the sulphate group (Plasencia and Mirocha, 1991). The compound was thus identified as 3-*O*-methylalaternin-7-*O*-sulphate. This is the first example of the isolation of **24** as a natural product.



Nr.	Compound	R_1	R_2
23	Methylalaternin	CH_3	H
23a	Alaternin	H	H
24	Methylalaternin sulphate	CH_3	SO_3H

Table 3.20: ^{1}H, ^{13}C NMR and HMBC data of compounds **23** and **24** at 500 (^{1}H) and 125 MHz (^{13}C)

Nr.	23			23a[a]		24		
	δ_H (DMF-d_7)	HMBC	δ_C (DMF-d_7)	δ_H (DMSO-d_6)	δ_C (DMSO-d_6)	δ_H (MeOD)	HMBC	δ_C (MeOD)[b]
1			165.7		164.4			
2	6.84, d (2.0)	1,3,4,9a	106.1	6.72, d (2.5)	107.2	6.77, d (2.5)		
3			167.5		165.6			167.0
4	7.24, d (2.0)	2,9a,10	107.9	7.30, d (2.5)	108.5	7.31, d (2.5)		
4a			136.9		131.3			
5	7.62, s	CH_3,7,8a,10	123.3	7.47, s	122.9	7.65, s		122.5
6			132.0		135.6			143.5
7			152.5		150.1			146.0
8					149.2			
8a			114.9		113.9			
9			187.6		190.1			
9a			110.0		109.0			
10			180.1		179.9			
10a			124.0		122.9			
CH_3	2.34, s	5,6,7	16.4		16.2	2.52, s	5,6,7	
OCH_3	4.01, s	3	56.7			3.94, s	3	

a) Lee *et al.*, 1998.
b) Derived from HMBC spectrum.

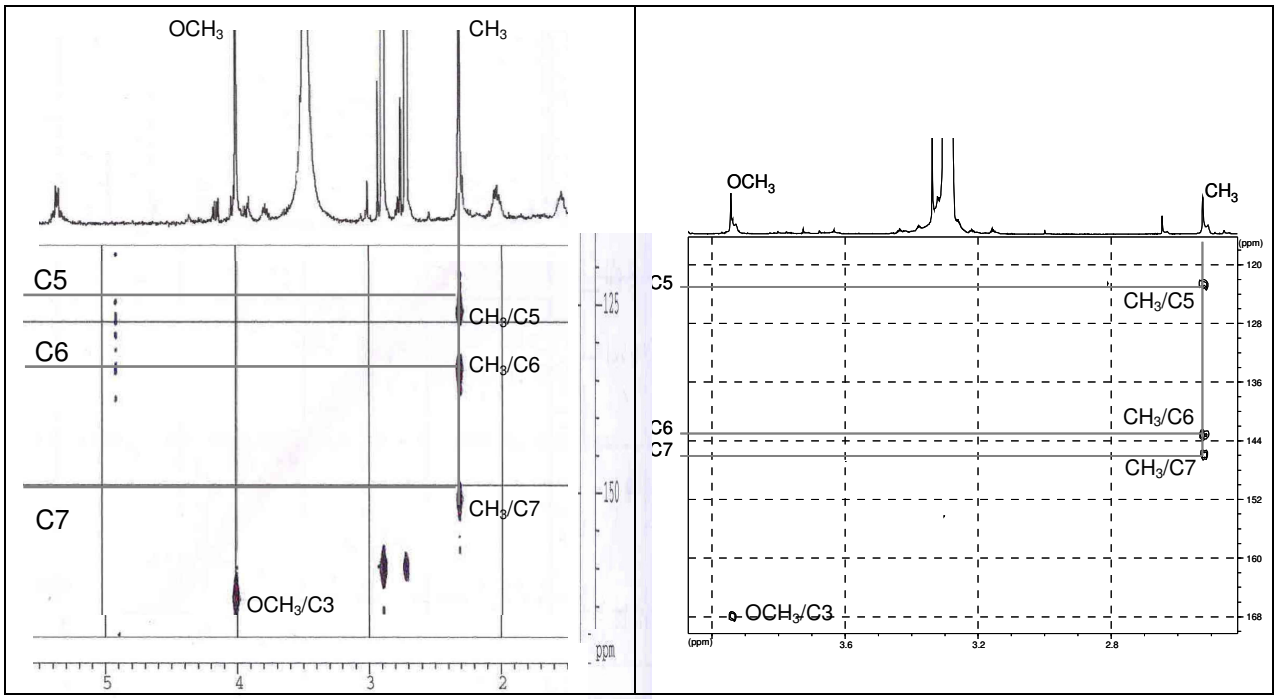
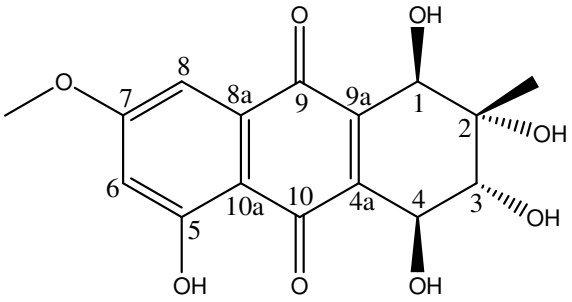
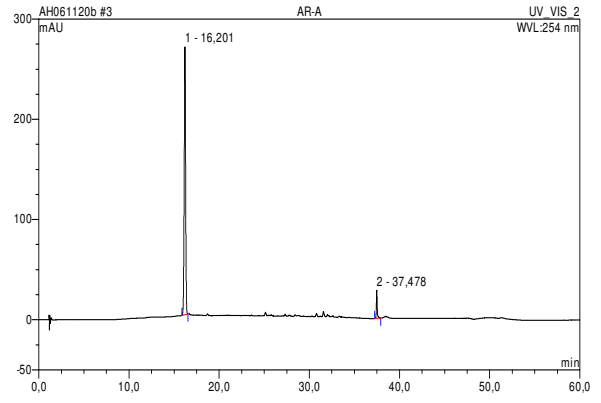


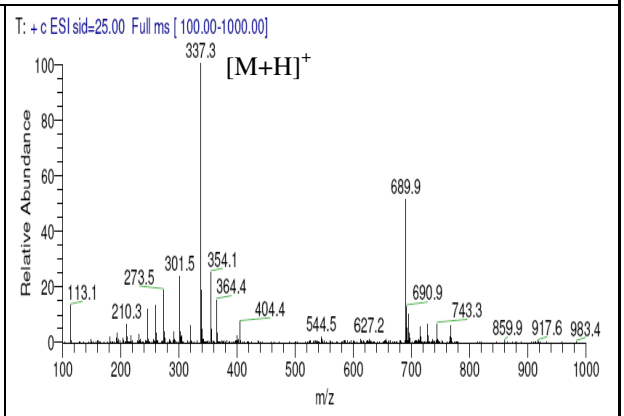
Figure 3.19: HMBC spectra of compounds **23** and **24**.

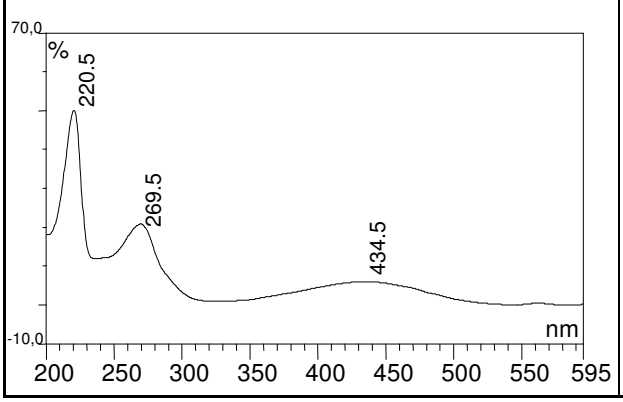
3.2.10. Altersolanol A (25, known compound)

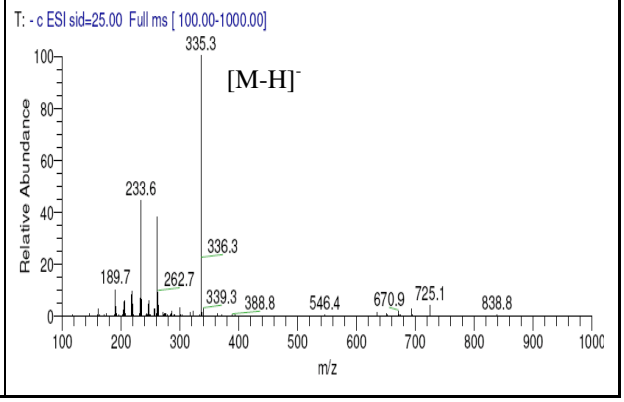
Altersolanol A	
Synonym(s)	7-Methoxy-2-methyl-1β,2α,3α,4β,5-pentahydroxy-1,3,4-trihydroanthraquinone
Sample code	AR6.2.1
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	15.7 mg
Physical Description	orange yellow crystals
Molecular Formula	$C_{16}H_{16}O_8$
Molecular Weight	336 g/mol
Optical Rotation $[\alpha]_D^{22}$	- 149 (*c* 1.2 mg/mL, EtOH)
Retention time HPLC	16.2 min (standard gradient)







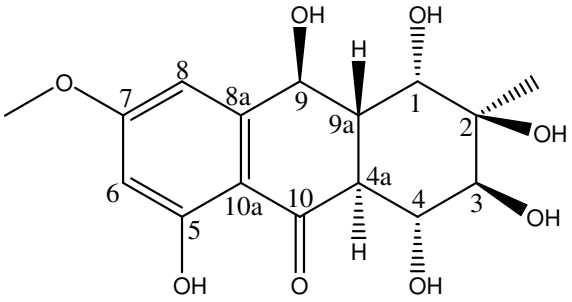


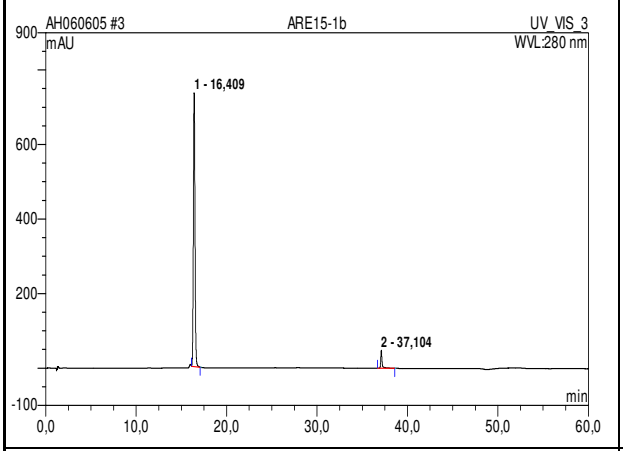


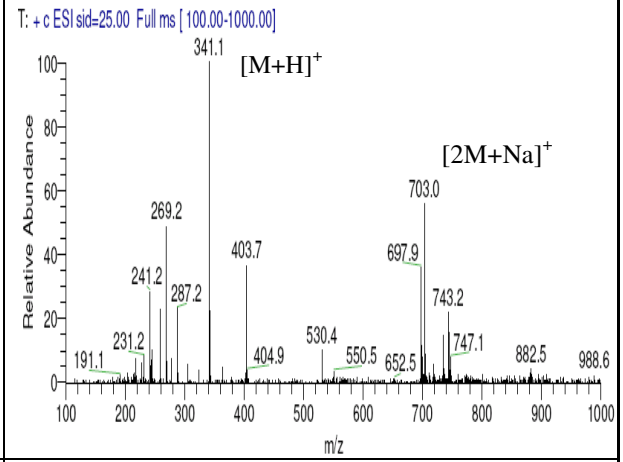
Altersolanol A (**25**) was isolated from the EtOAc extract of rice cultures of *Ampelomyces* sp. as orange yellow crystals (15.7 mg). It showed UV absorbances at λ_{max} (MeOH) 220.5, 269.5 and 434.5 nm suggesting a quinone as the basic structure. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 337.3 $[M+H]^+$ (base peak) and *m/z* 335.3 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 336 g/mol, while inspection of the NMR data suggested a molecular formula of $C_{16}H_{16}O_8$. The ^1H NMR spectrum (see Table 3.21) displayed signals for four alcoholic hydroxyl groups, three doublets at δ_H 5.67, 5.03 and 4.88 (*J*=5.9, 5.3, and 6.3 Hz, respectively) and one singlet at δ_H 4.46, assigned for 1-OH, 4-OH, and 3-OH, respectively. In addition, the spectrum contained a broad singlet (chelated phenol) at δ_H 12.11 (5-OH). These signals were not observed in the ^1H NMR spectrum measured in MeOD, confirming their facile exchange. Furthermore, a singlet was detected at δ_H 1.23 corresponding to an aliphatic methyl group (2-CH_3), together with three carbinolic protons at δ_H 4.47 (H-4), 4.31 (H-1) and 3.63 (H-3). The pair of *meta*-coupled aromatic protons at δ_H 7.00 (H-8) and 6.81 (H-6) and the aromatic methoxy group (δ_H 3.89) were comparable to the respective signals of metabolites **21**-**24** (see Tables 3.19 and 3.20). The nature of the non-aromatic carbocycle was evident from the COSY spectrum (see Table 3.21), establishing the planar structure of **25** as identical to altersolanol A (**25a**) (Stoessl, 1969a). Moreover, correlations of 2-CH_3 with H-3, 1-OH, and 4-OH in the ROESY spectrum indicated that also the relative stereochemistry corresponded to that of altersolanol A (**25a**), which was also in agreement with the observed coupling constants. This assignment was further corroborated by the very similar experimental UV, ^1H, ^{13}C NMR, mass spectral data and $[\alpha]_D$ value obtained for **25a** in comparison to published data for altersolanol A (Yagi *et al.*, 1993; Okamura *et al.*, 1993, 1996). Altersolanol A was previously isolated from several *Alternaria* species (Stoessl *et al.*, 1983; Lazarovits *et al.*, 1988; Yagi *et al.*, 1993; Okamura *et al.*, 1993, 1996).

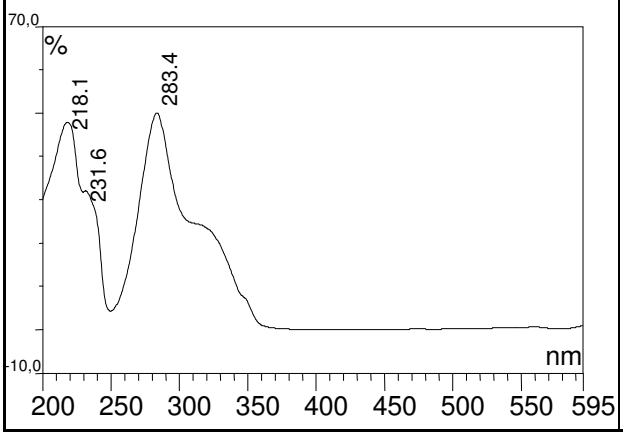
3.2.11. Ampelanol (26, new compound)

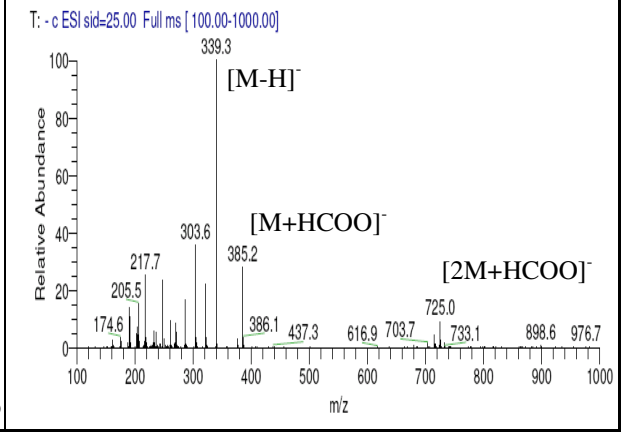
Ampelanol	
Synonym(s)	7-Methoxy-2-methyl-1β,2α,3α,4β,5,9α-hexahydroxy-1,3,4,4a,9,9a-hexahydroanthracen-10-one
Sample code	AR8.2.1, AR9.4
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	18.6 mg
Physical Description	white crystals
Molecular Formula	$C_{16}H_{20}O_8$
Molecular Weight	340 g/mol
Optical Rotation $[\alpha]_D^{20}$	- 64 (*c* 0.5 mg/mL, MeOH)
Retention time HPLC	16.4 min (standard gradient)







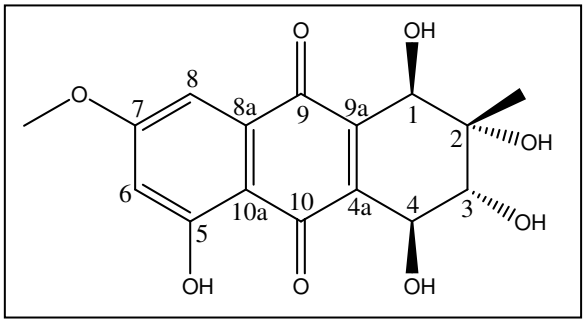




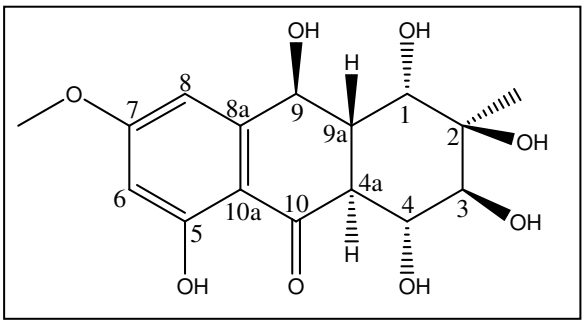
Compound **26** was isolated from the EtOAc extract of rice cultures of *Ampelomyces* sp. in the form of white crystals (18.6 mg). It had UV absorbances at λ_{max} (MeOH) 218.1, 231.6, 283.4 and 318.0 nm. The HRESI-MS exhibited a prominent peak at *m/z* 341.1230 $[M+H]^+$ indicating a molecular formula of $C_{16}H_{20}O_8$ (calculated 341.1236, Δ 0.0006) as well as an increase of four mass units compared to altersolanol A (**25**). The ^{1}H NMR spectrum (see Table 3.21) contained five exchangeable alcoholic hydroxyl groups, two doublets at δ_H 5.49 and 5.06, a broad singlet at δ_H 4.41 and two singlets at δ_H 4.46 and 4.21, assigned to 9-OH, 1-OH, 3-OH, 4-OH, and 2-OH, respectively. In addition, a singlet for a chelated phenol appeared at δ_H 12.57 which was likewise exchangeable and was attributed to 5-OH. A singlet corresponding to an aliphatic methyl group was detected at δ_H 1.20 (2-C*H*$_3$), while four carbinolic protons resonated at δ_H 4.67 (H-9), 3.82 (H-4), 3.76 (H-1), and 3.36 (H-3). Similar to compounds **21**-**25**, the *meta*-coupled H-8 and H-6 appeared at δ_H 6.72 and 6.35, while the aromatic methoxy group was detected at δ_H 3.83. In the COSY spectrum, the less shielded aryl proton H-8 exhibited a long range correlation to a *peri*-proton (H-9), which in turn coupled to both the hydroxy signal at δ_H 5.49 (9-OH) and the ring junction proton at δ_H 2.30 (H-9a). These results indicated that the quinone carbonyl at C-9 in **25** had been reduced to a hydroxy group at the respective position in **26**, while the double bond between C-9a and C-4a in **25** likewise was reduced in **26**, also in accord with the increase in the molecular weight of 4 amu compared to altersolanol A and the absence of color for this compound. The complete aliphatic spin system comprising H-9, H-9a, H-1, H-4a, H-4, and H-3, together with the corresponding hydroxy functions, was clearly discernible in the COSY spectrum (see Figure 3.20). Furthermore, in the HMBC spectrum (see Figure 3.22) the correlations attributed to the two protons at the ring junction, *i.e.* H-9a (to C-4a and C-9) and H-4a (to C-4, C-9, C-9a, and C-10), as well as the correlation of H-8 to C-9 fully supported the assignment of the planar structure as depicted.

The relative stereochemistry was deduced from the coupling constants in the ^{1}H NMR spectrum as well as correlations in the ROESY spectrum (see Table 3.21). The large values of $J_{3\text{-}4}$ (9.4 Hz), $J_{4\text{-}4a}$ (9.4 Hz), $J_{4a\text{-}9a}$ (13.2 Hz) and $J_{9\text{-}9a}$ (10.5 Hz) could only be explained by a series of mutual diaxial relationships and thus proved that all of these hydrogens were axially positioned, while correspondingly, the 2.2 Hz coupling between H-9a and H-1 indicated an equatorial position for the latter. Correlations of H-9 to H-1 and H-4a, 2-CH_3 to both H-1 and H-3, as well as H-4a to H-3 and 4-OH in the ROESY spectrum, indicated their position at the β-face of the molecule. On the other hand, correlations of H4 to 2-OH and 3-OH, 9-OH to both H-1 and H-9a, and H-9a to 2-OH indicated their α-orientation (see Table 3.21). These

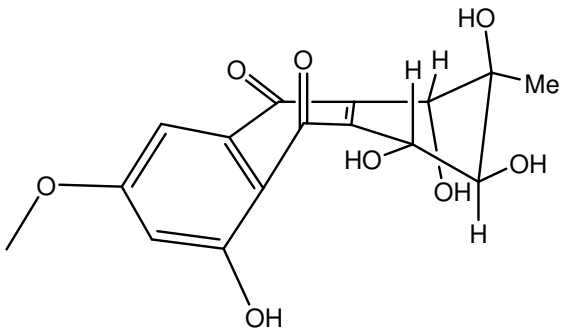
data indicated the adoption of chair conformation for the aliphatic carbocycle and allowed to deducing the relative stereochemistry as shown in **26a**. The structure was further confirmed by comparing NMR data of **26** to those reported for altersolanol A (**25**) (Yagi *et al.*, 1993; Okamura *et al.*, 1993, 1996) and tetrahydroaltersolanol B (Stoessl and Stothers, 1983) which differ from **26** in lacking the 1- and 4-OH groups. Thus, **26** was identified as a new natural product for which we propose the name ampelanol.



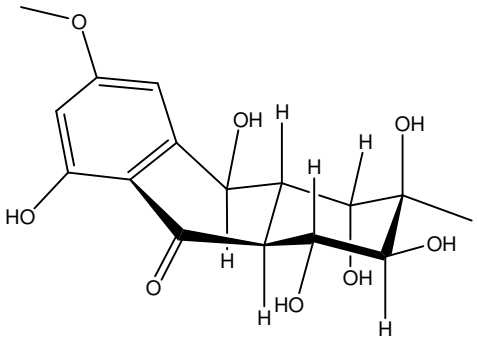
25 Altersolanol A



26 Ampelanol



25a



26a

Table 3.21: ^{1}H NMR, COSY, ROESY and HMBC data of compounds **25** and **26** at 500 MHz

Nr.	25			25[a]	26			
	δ_H (DMSO-d_6)	ROESY	HMBC	δ_H (DMSO-d_6)	δ_H (DMSO-d_6)	COSY	ROESY	HMBC
1	4.31, d (3.7)	CH_3	CH_3,2,3,4a,9,9a	4.38, d (4.0)	3.76, br s	9a,1OH	CH_3,9,9a,9OH	
1-OH	5.67, d (5.9)	CH_3,3	1,2,9a	5.30, s	5.06, d (4.7)	1	CH_3,9	
2-OH	4.46, s		1,3	4.48, br s	4.21, s	CH_3	CH_3,4,9a	1
3	3.63, dd (5.6, 6.3)	CH_3,1OH, 4OH	CH_3,1,4	3.64, m (7.0)	3.36, d (9.4)	4,3OH	CH_3,4a	4
3-OH	4.88, d (6.3)		2,3,4	5.00, d (7.0)	4.41, br s	3	4	
4	4.47, m		3,4a,9a	4.54, m (7.0)	3.82, m	3,4a,4OH	2OH,3OH	3,10
4-OH	5.03, d (5.3)	CH_3,3		5.71, d (7.0)	4.46, s	4	4a	3
4a					2.63, dd (13.2, 9.4)	4	3,9,4OH	4,9,9a,10
5-OH	12.11, br s			12.15, s	12.57, s		6	5,6,10a
6	6.81, br s	OCH_3	5,7,8,10a	6.72, d (2.0)	6.35, d (1.8)	8	OCH_3,5OH	5,7,8,10a
8	7.00, br s	OCH_3	6,7,9,10a	6.93, d (2.0)	6.72, d (1.8)	6,9	OCH_3,9,9OH	6,7,9,10a
9					4.67, dd (10.5, 6.2)	8,9a,9OH	1,4a,8,1OH	
9-OH					5.49, d (6.2)	9	1,8,9a	
9a					2.30, ddd (13.2, 10.5, 2.2)	1,4a,9	1,2OH,9OH	4a,9
CH_3	1.23, s	1,3,1OH,4OH	1,2,3	1.24, s	1.20, s	2OH	1,3,1OH,2OH	1,2,3
OCH_3	3.89, s	6,8	7	3.90, s	3.83, s		6,8	7

a) Yagi *et al.*, 1993.

Table 3.22: ^{13}C NMR data of compounds **25** and **26** at 125 MHz

Nr.	25	25[a]	26
	δ_C (DMSO-d_6)	δ_C (DMSO-d_6)	δ_C (DMSO-d_6)
1	68.5	68.3	71.2
2	72.9	72.7	72.9
3	73.8	73.6	74.0
4	68.5	68.3	70.7
4a	144.5	144.2	48.1
5	163.2	162.9	164.2
6	105.9	105.6	99.0
7	165.4	165.1	166.0
8	106.6	106.4	104.6
8a	133.3	132.9	152.0
9	183.7	183.3	66.4
9a	142.1	141.8	44.5
10	188.5	188.1	206.1
10a	109.5	109.2	109.1
CH_3	22.3	22.2	23.8
OCH_3	56.2	56.1	55.6

a) Yagi *et al.*, 1993.

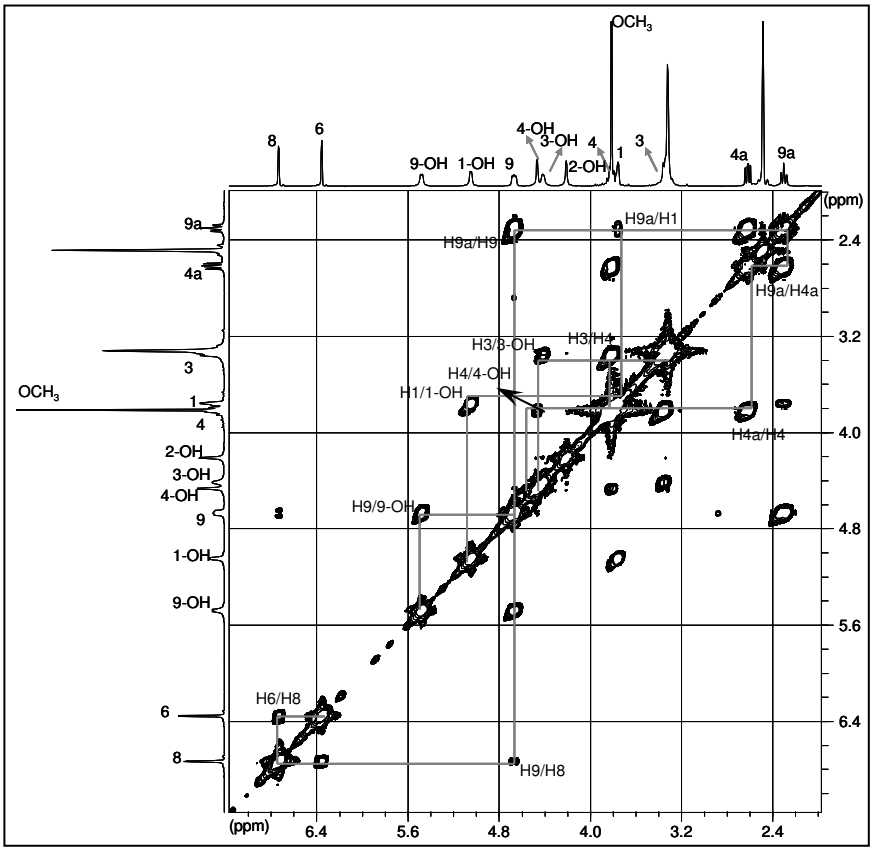


Figure 3.20: COSY spectrum of compound **26**.

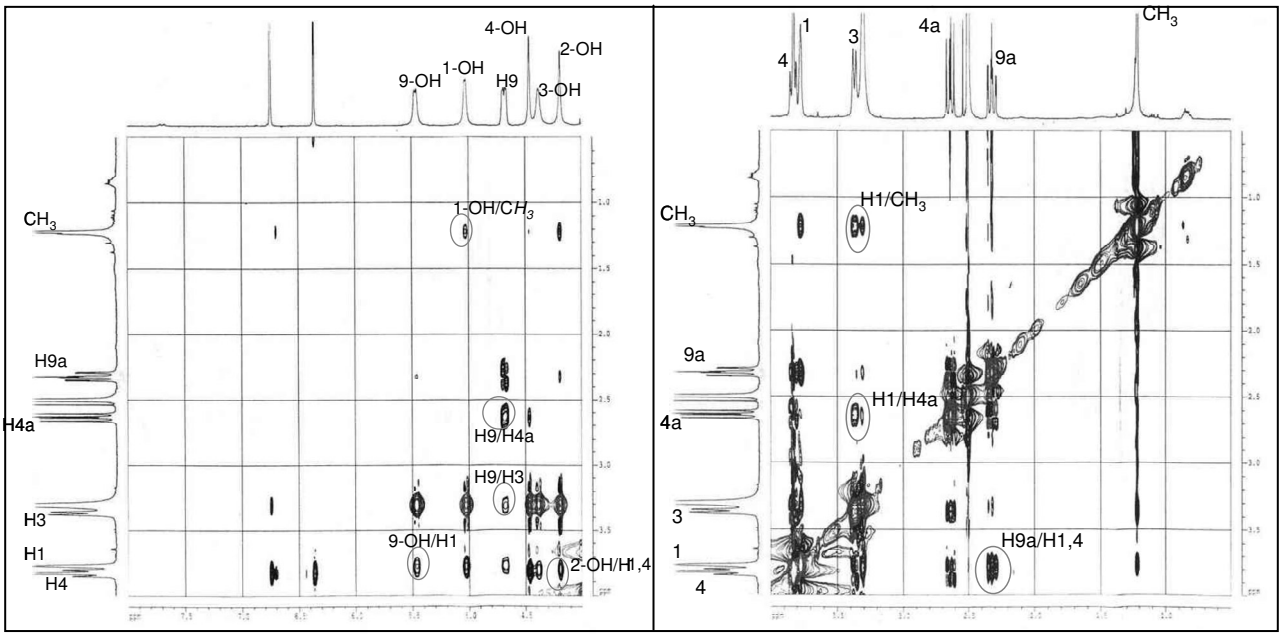


Figure 3.21A: ROESY spectrum of compound **26**.

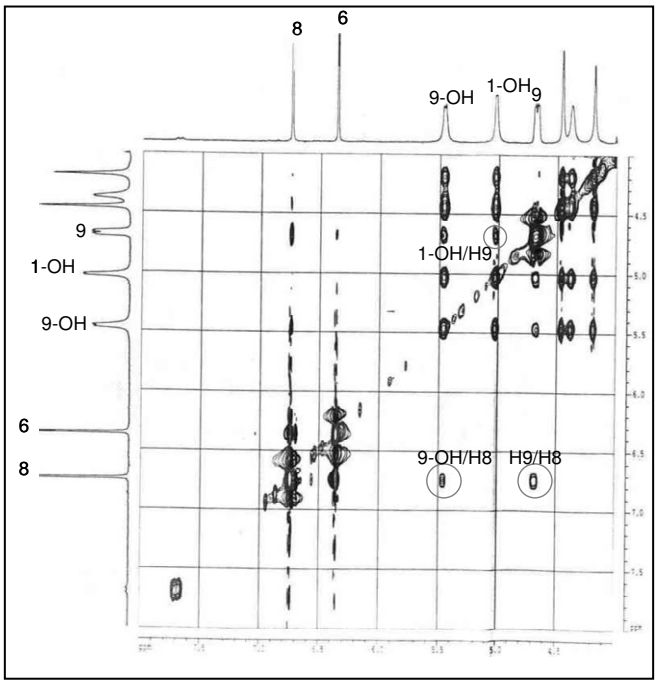


Figure 3.21A: ROESY spectrum of compound **26**.

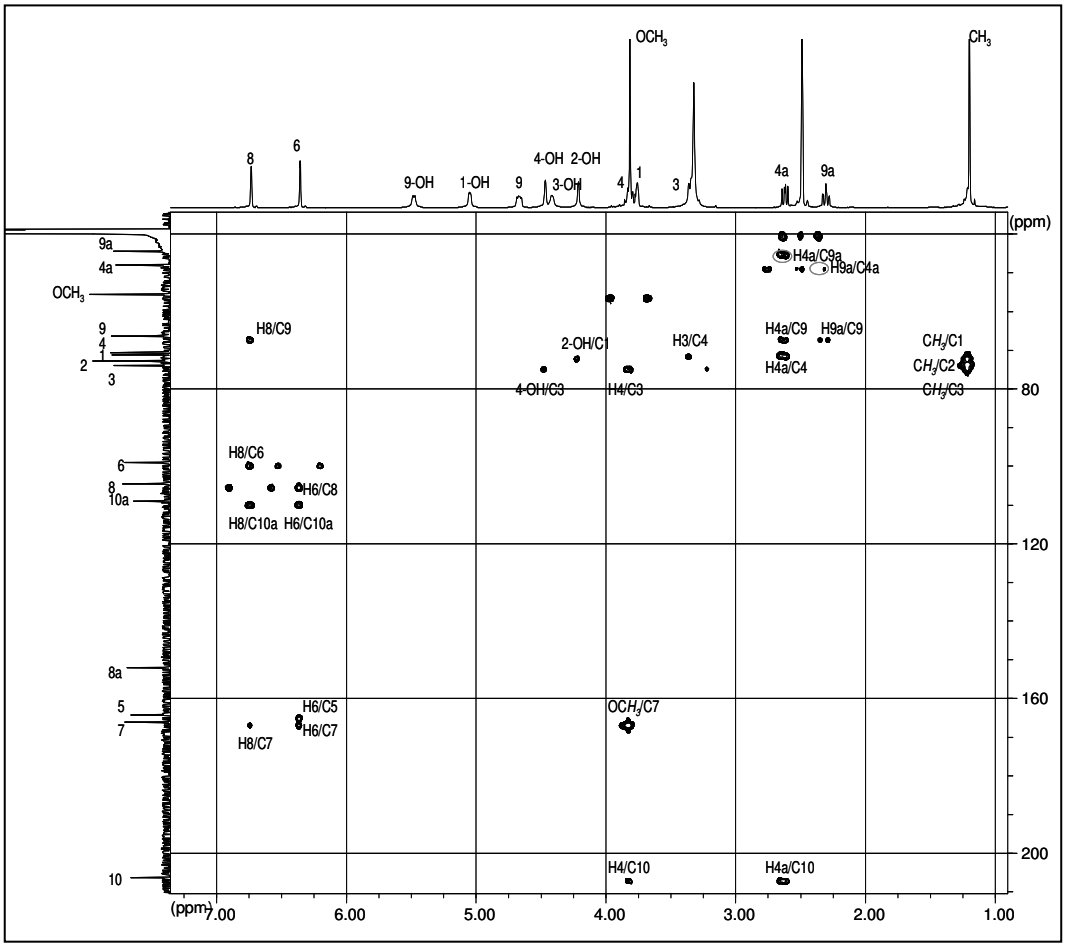
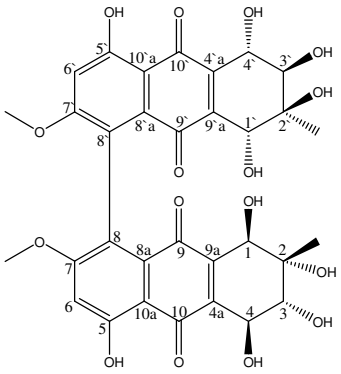


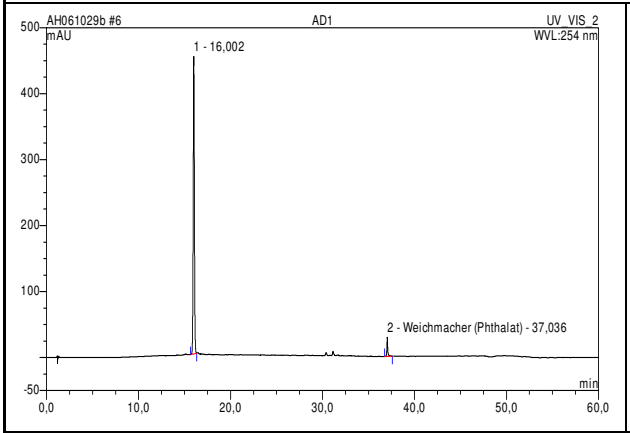
Figure 3.22: HMBC spectrum of compound **26**.

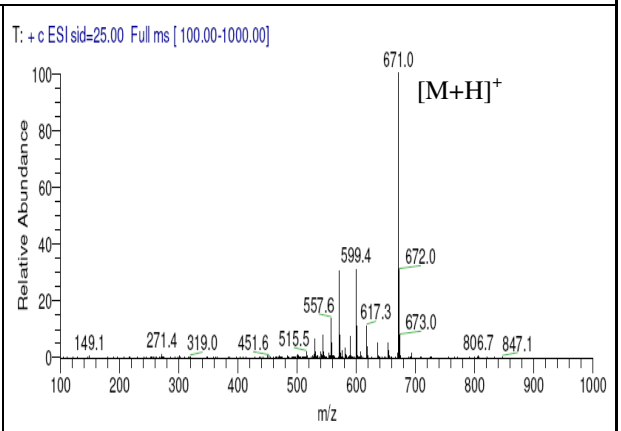
3.2.12. Alterporriol D (27, known compound)

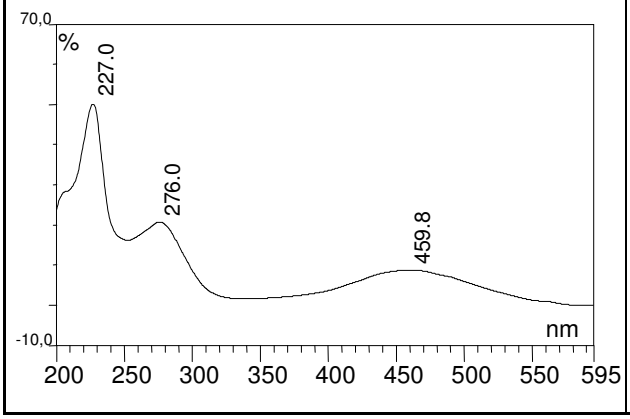
Alterporriol D	
Synonym(s)	7,7`-Dimethoxy-2,2`-dimethyl-1β,2α,3α,4β,5,1`$\alpha$,2`β,3`$\beta$,4`α,5`-decahydroxy-1,1`,3,3`,4,4`-hexahydro-8,8`-bianthraquinone
Sample code	AR9.2.1
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	10.5 mg
Physical Description	red crystals
Molecular Formula	$C_{32}H_{30}O_{16}$
Molecular Weight	670 g/mol
Optical Rotation $[\alpha]_D^{20}$	- 795 (*c* 0.01, EtOH)
Retention time HPLC	16.0 min (standard gradient)

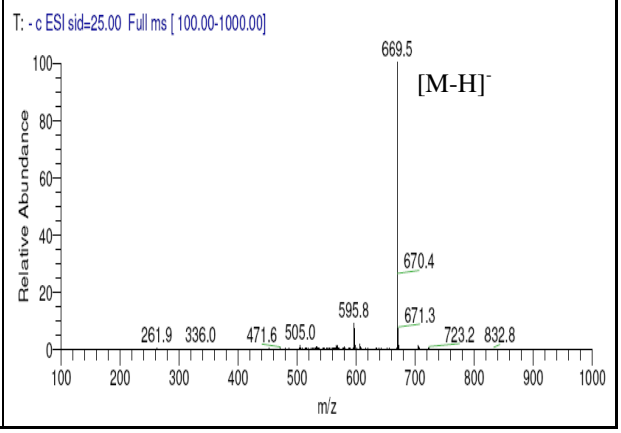


(atropisomer of **28**)





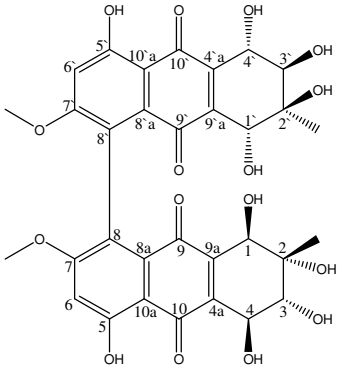




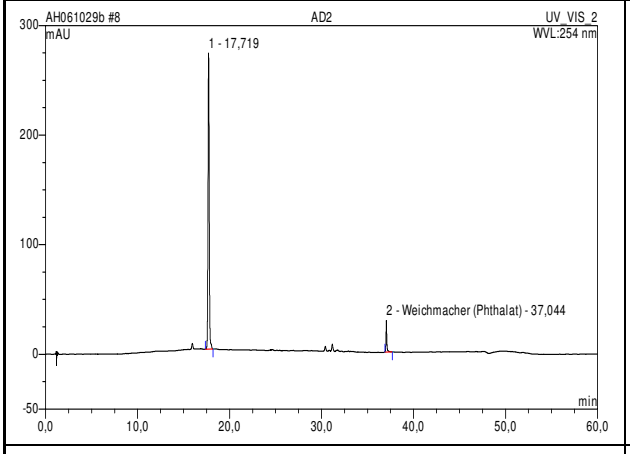
Alterporriol D (**27**) was isolated from the EtOAc extract of rice cultures of *Ampelomyces* sp. as red crystals (10.5 mg). It showed UV absorbances at λ_{max} (MeOH) 227.0, 276.0 and 459.8 nm, thereby almost superimposable and closely resembling those of altersolanol A (**25**). Positive and negative ESI-MS showed molecular ion peaks at *m/z* 671.0 $[M+H]^+$ (base peak) and *m/z* 669.5 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 670 g/mol. The ^1H NMR spectral data of alterporriol D and altersolanol A resembled each other in many aspects except for the absence of the *meta*-coupled pair of aromatic protons found for the latter, which was replaced by a single aromatic proton singlet at δ_H 6.90 in the spectrum of **27**, assigned for H-6,6` (see Table 3.21 and 3.23). Likewise, the ^{13}C NMR spectra of both compounds showed high similarity with the exception of the downfield shift of the signal corresponding to C-8 and the slight upfield shift of C-8a in the spectrum of **27** compared to that of **25** (see Table 3.22 and 3.24). Similar NMR and UV spectra as well as the molecular weight being twice that of altersolanol A (336 g/mol) with the loss of two hydrogens suggested that **27** was a known compound, representing a symmetrical dimer of **25**, formed through phenolic oxidation at C-8. This assignment was corroborated by interpretation of COSY, ROESY and HMBC spectra which were reminiscent to a high degree to those acquired for altersolanol A (see Table 3.21 and 3.23, and Figure 3.23). Further confirmation was achieved by comparison of UV, ^1H, ^{13}C NMR, mass spectral data and $[\alpha]_D$ with published data for alterporriol D, previously reported from *Alternaria solani* and *A. porri* (Lazarovits *et al.*, 1988; Suemitsu *et al.*, 1989).

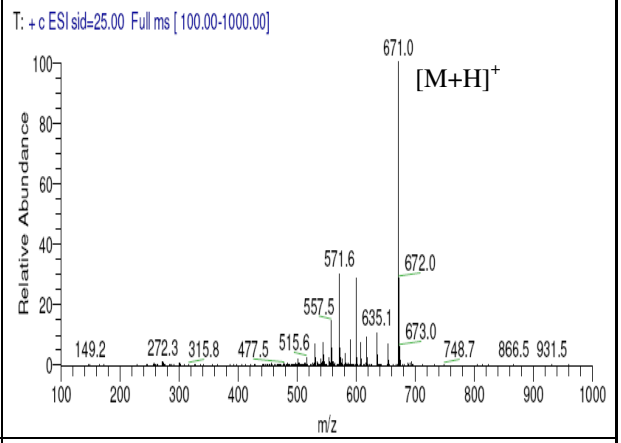
3.2.13. Alterporriol E (28, known compound)

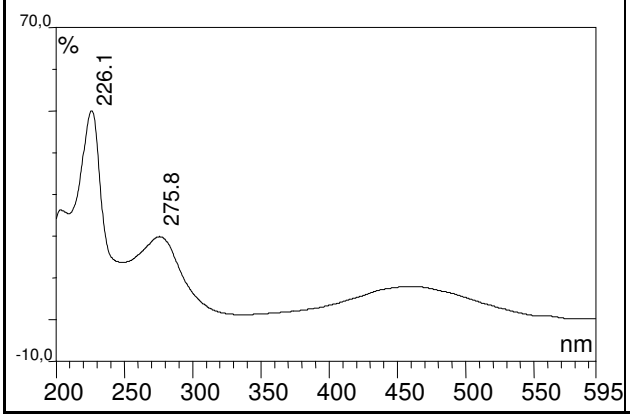
Alterporriol E	
Synonym(s)	7,7`-Dimethoxy-2,2`-dimethyl-1*β*,2*α*,3*α*,4*β*,5,1`*α*,2`*β*,3`*β*,4`*α*,5`-decahydroxy-1,1`,3,3`,4,4`-hexahydro-8,8`-bianthraquinone
Sample code	AR9.2.2
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	12.3 mg
Physical Description	red crystals
Molecular Formula	$C_{32}H_{30}O_{16}$
Molecular Weight	670 g/mol
Optical Rotation $[\alpha]_D^{20}$	- 688 (*c* 0.01, EtOH)
Retention time HPLC	17.7 min (standard gradient)

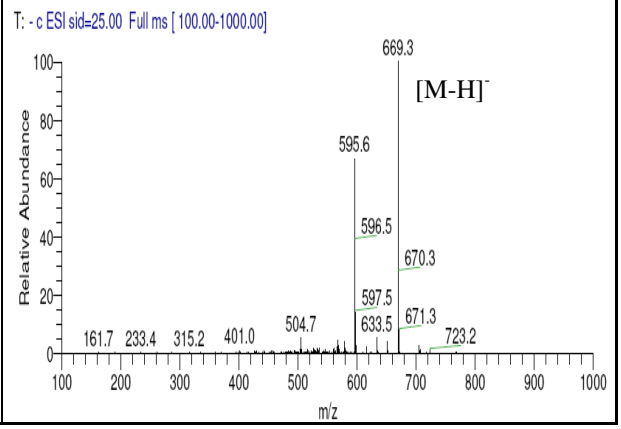


(atropisomer of **27**)

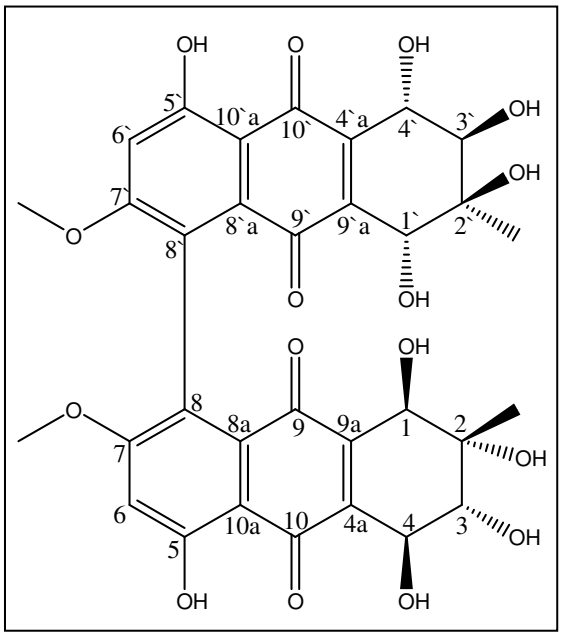








Alterporriol E (**28**) was isolated from the EtOAc extract of rice cultures of *Ampelomyces* sp. as red crystals (12.3 mg). It had UV absorbances at λ_{max} (MeOH) 226.1, 275.8 and 460.0 nm, almost superimposable to those of altersolanol A (**25**) and alterporriol D (**27**). Positive and negative ESI-MS showed molecular ion peaks at *m/z* 671.0 $[M+H]^+$ (base peak) and *m/z* 669.3 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 670 g/mol identical to that of **27**. Moreover, the fragment patterns in the mass spectra of both compounds were similar to each other. The ^{1}H NMR spectral data of alterporriol E were almost identical to those of alterporriol D (**27**) (see Table 3.23). Moreover, by comparing the ^{13}C NMR spectra of both compounds, the agreement between them was within 1 ppm (see Table 3.24). Likewise, COSY, ROESY and HMBC spectra showed basically the same set of correlations as observed for alterporriol D (see Table 3.23 and Figure 3.23). This striking similarity ruled out the possibility of **28** being a diastereomer of **27**, and could neatly be explained with both compounds representing atropisomers. Due to a complete set of *ortho* substituents around the C8-C8` bond, rotation about this single bond is restricted and thus lead to the existence of two separate atropisomers which do not interconvert at room temperature. However, since the compounds do contain additional stereogenic centres with identical configuration, both atropisomers do not behave like mirror images of each other, and can thus be isolated using achiral chromatographic separation techniques. Further confirmation of the structure was achieved by comparison of UV, ^{1}H, ^{13}C NMR, mass spectral data and $[\alpha]_D$ with published data for alterporriol E, previously reported from *Alternaria solani* and *A. porri* (Lazarovits *et al.*, 1988; Suemitsu *et al.*, 1989). Interestingly, both publications also reported the simultaneous occurrence of both atropisomers alterporriol D and alterporriol E in the respective fungal strains.



27 Alterporriol D
28 Alterporriol E (atropisomer of **27**)

Table 3.23: ^{1}H NMR, COSY, ROESY and HMBC data of compounds **27** and **28** at 500 MHz

Nr.	27				27[a]	28				28[a]
	δ_H (DMSO-d_6)	COSY	ROESY	HMBC	δ_H (THF-d_8)	δ_H (DMSO-d_6)	COSY	ROESY	HMBC	δ_H (THF-d_8)
1,1`	4.08, d (6.7)	1OH		CH_3,2,3,4a, 9,9a	4.26	4.05, d (6.6)	1OH		CH_3,2,3,4a, 9,9a	4.26
1,1`OH	5.65, d (6.7)	1		1,2,9a		5.65, d (6.6)	1		1,2,9a	
2,2`OH	4.39, s	CH_3		1,2,3		4.42, s				
3,3`	3.57, d (6.7)	4,3OH		1,4	4.32, d (6.5)	3.54, d (6.6)	4,3OH		1,4	4.35, d (6.4)
3,3`OH	4.83, br s	3				4.86, br s	3			
4,4`	4.45, dd (6.7, 4.4)	3,4OH		3,4a,9a	4.74, d (6.5)	4.45, dd (6.6, 4.7)	3,4OH			4.75, d (6.4)
4,4`OH	5.02, d (4.4)	4		3		5.07, d (4.7)	4			
5,5`OH	12.92, br s					13.07, br s				
6,6`	6.90, s	OCH_3		5,7,8,10,10a	6.77	6.92, s	OCH_3		5,7,8,10a	6.77
2,2`CH_3	1.13, s	2OH		1,2,3	1.33	1.12, s			1,2,3	1.36
7,7`OCH_3	3.66, s	6		7	3.71	3.69, s	6		7	3.67

a) Suemitsu *et al.*, 1989.

Table 3.24: ^{13}C NMR data of compounds **27** and **28** at 125 MHz

Nr.	27	27[a]	28	28[a]
	δ_C (DMSO-d_6)	δ_C (THF-d_8)	δ_C (DMSO-d_6)	δ_C (THF-d_8)
1,1`	68.1	68.2	68.1	68.3
2,2`	72.8	72.8	72.9	72.9
3,3`	73.8	73.7	73.7	73.8
4,4`	68.3	68.4	68.4	68.3
4a,4`a	143.4	144.5	143.3	143.5
5,5`	163.5	163.5	164.0	163.9
6,6`	104.2	104.1	103.7	103.9
7,7`	163.6	163.6	164.6	164.7
8,8`	121.4	121.4	122.4	122.5
8a,8`a	129.8	129.8	128.8	128.9
9,9`	184.1	184.0	183.8	183.8
9a,9`a	142.7	142.7	142.8	142.8
10,10`	188.8	188.8	188.7	188.8
10a,10`a	109.3	109.3	109.2	109.3
2,2`-CH_3	22.2	22.2	22.2	22.2
7,7`-OCH_3	56.7	56.7	56.8	56.8

a) Suemitsu *et al.*, 1989.

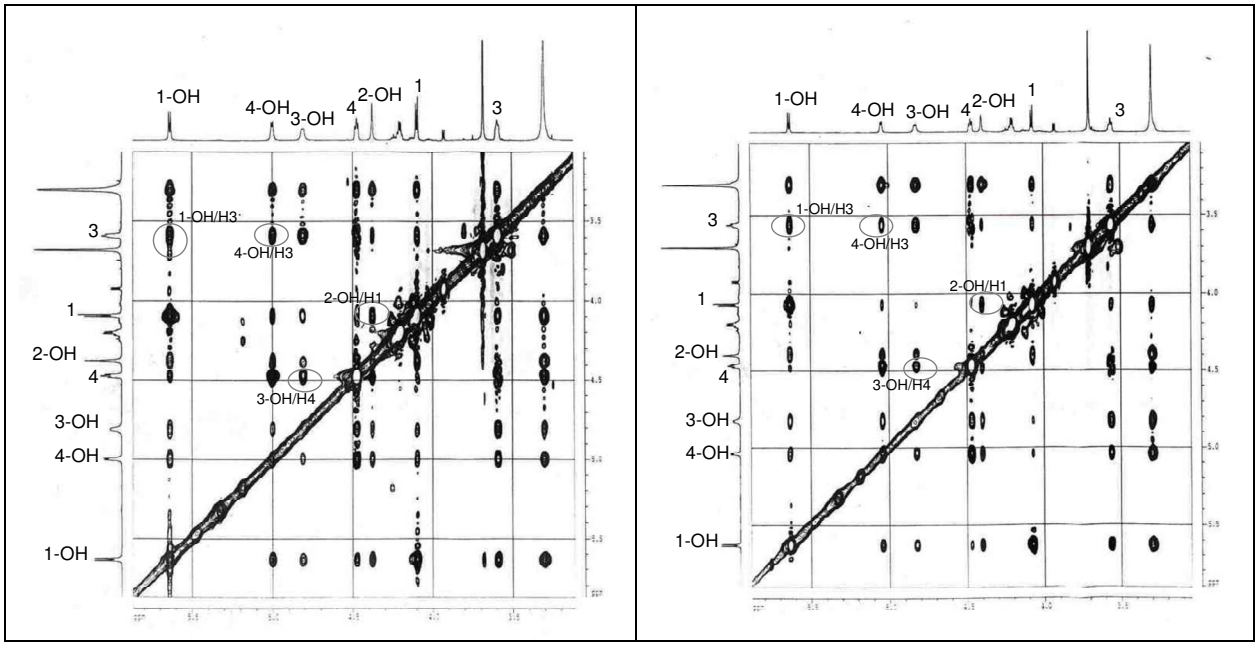
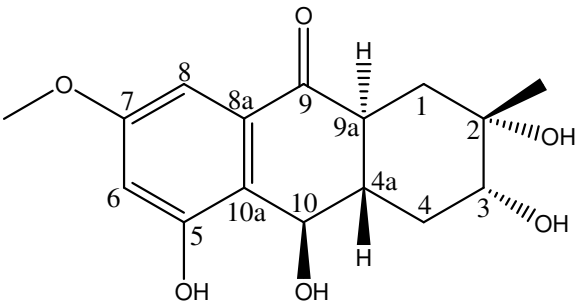
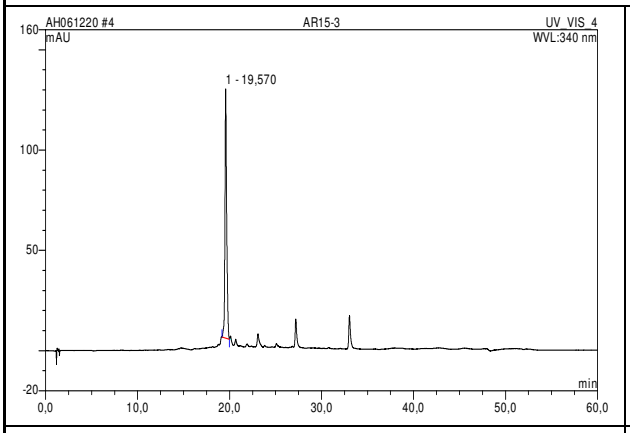


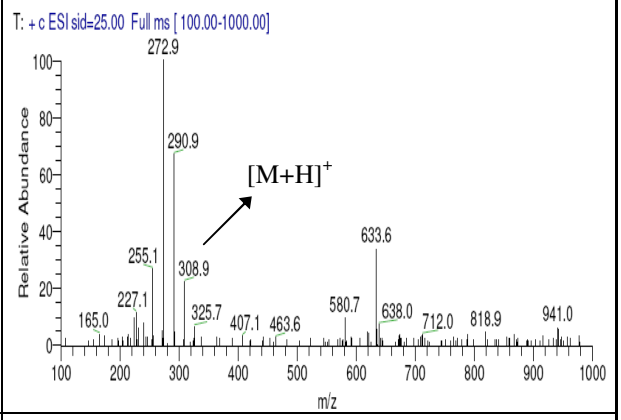
Figure 3.23: ROESY spectra of compounds **27** and **28**.

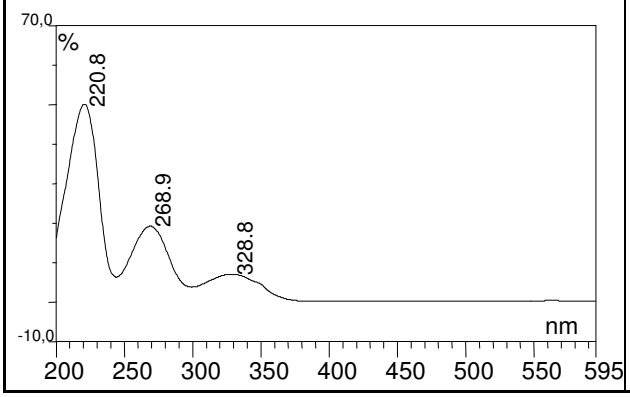
3.2.14. Altersolanol J (29, known compound)

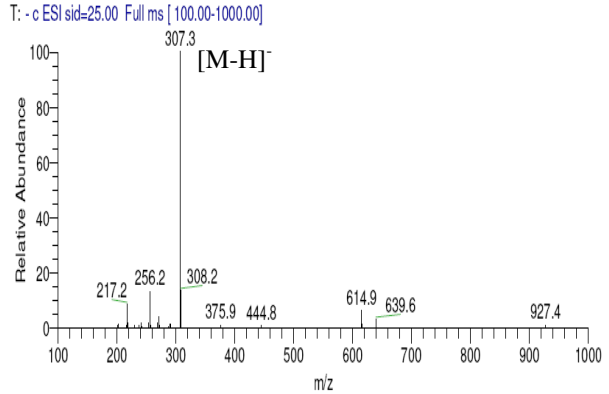
Altersolanol J	
Synonym(s)	7-Methoxy-2-methyl-2*α*,3*α*,5,10*β*-tetrahydroxy-1,3,4,4a,9a,10-hexahydroanthracen-9-one
Sample code	AR8.2.2
Biological source	*Ampelomyces* sp. (from *Urospermum picroides*)
Sample amount	1.8 mg
Physical Description	yellowish white powder
Molecular Formula	$C_{16}H_{20}O_6$
Molecular Weight	308 g/mol
Optical Rotation $[\alpha]_D^{22}$	- 46 (*c* 0.7, MeOH)
Retention time HPLC	19.6 min (standard gradient)





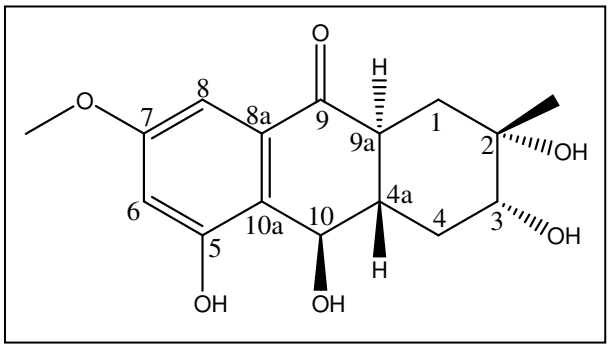






Compound **29** was isolated from the EtOAc extract of rice cultures of *Ampelomyces* sp. in the form of yellowish white powder (1.8 mg). It showed UV absorbances at λ_{max} (MeOH) 218.5, 268.9 and 328.7 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 308.9 $[M+H]^+$ and *m/z* 307.3 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 308 g/mol and a decrease of 32 amu compared to ampelanol (**26**), formally corresponding to the loss of two oxygen atoms. The ^{1}H NMR spectrum (see Table 3.25) showed two alcoholic hydroxyl groups at δ_H 4.37 (3-OH) and 3.92 (2-OHs), while a singlet corresponding to the aliphatic methyl group 2-CH_3 was detected at δ_H 1.16. Additionally, the spectrum contained two carbinolic protons at δ_H 4.76 (H-10) and 3.15 (H-3), a set of *meta*-coupled aromatic protons at δ_H 6.80 (H-8) and 6.57 (H-6), as well as an aromatic methoxy group (δ_H 3.71). The latter features were already familiar from the spectra of **25** and **26** (see Tables 3.21). However, absence of the long range correlation of H-8 to a *peri*-proton, observed in the COSY spectrum of **26**, together with the singlet at δ_H 9.91 corresponding to a non-chelated aromatic hydroxyl group (5-OH) indicated that the quinone carbonyl present at C-10 in **25**, had been reduced to a hydroxyl group. As in the case of ampelanol (**26**), this explained the absence of color for this compound. The complete aliphatic spin system in **29** was assembled in a straight forward manner on the basis of COSY data (see Table 3.25).

The relative stereochemistry was deduced from the coupling constants in the ^{1}H NMR spectrum (see Table 3.25). The large values of $J_{4a\text{-}9a}$ (13.2 Hz) and $J_{4a\text{-}10}$ (9.8 Hz) indicated the diaxial orientation of H-4a to both H-9a and H-10. A 12.1 Hz value for $J_{3\text{-}4ax}$ indicated an axial position for also for H-3. The structure was further confirmed by comparing UV, ^{1}H NMR, mass spectral data and $[\alpha]_D$ value with published data for altersolanol J, previously reported from an undetermined fungicolous hyphomycete resembling *Cladosporium* (Höller *et al.*, 2002).



29 Altersolanol J

Table 3.25: [1]H-NMR and COSY data of compound **29** at 500 MHz

Nr.	**29**		**29**[a]
	δ_H (DMSO-d_6)	COSY	δ_H (DMSO-d_6)
1ax	1.26, dd (13.8, 11.9)	1eq,9a	1.27, dd (14.0, 12.0)
1eq	2.06, dd (13.8 ,3.7)	1ax,9a	2.07, dd (14.0, 3.8)
2-OH	3.92, br s		3.93, s
3	masked by water peak	4ax,4eq,3-OH	3.16, ddd (12.0, 6.6, 4.5)
3-OH	4.37, brs	3	4.38, d (6.6)
4ax	1.56, br ddd (12.1, 12.1, 12.1)	3,4eq,4a	1.57, br ddd (12.0, 12.0, 12.0)
4eq	2.09, ddd (3.7, 4.4, 11.7)	3,4ax,4a	2.10, m
4a	1.82, dddd (13.2, 12.1, 9.8, 4.4)	4ax,4eq,9a,10	1.84, m
5-OH	9.91, s		10.1, s
6	6.57, d (2.2)	8	6.59, d (2.5)
8	6.80, d (2.2)	6	6.83, d (2.5)
9a	masked by solvent peak	1ax,1eq,4a	2.51, ddd (13.0, 12.0, 3.8)
10	4.76, d (9.8)	4a,10-OH	4.78, dd (9.6, 6.6)
10-OH			6.38, d (6.6)
CH_3	1.16, s		1.17, s
OCH_3	3.71, s		3.73, s

a) Höller, Gloer and Wicklow, 2002.

3.2.15. Bioactivity test results for compounds isolated from the endophytic fungus *Ampelomyces* sp.

The isolated compounds were subjected to cytotoxicity and protein kinase bioassays. Some of the isolated pure compounds were also subjected to *Staphylococcus epidermidis* biofilm inhibition assays. The results are shown in Tables 3.26 and 3.27.

Table 3.26: Cytotoxicity and biofilm inhibition test results for the compounds isolated from *Ampelomyces* sp. liquid and rice extracts

Nr.	Compound tested	L5178Y growth in %[a] (Conc. 10 µg/mL)	EC_{50}[a] (µg/mL)	EC_{50} (µmol/L)	Biofilm inhibition in %[b] (Conc. 50 µg/mL)	MIC[b] (µg/mL)
16	Methyltriacetic lactone	100.5				
17	Ampelopyrone	102.6				
18	Desmethyldiaportinol	- 0.4	7.30	1.6		
19	Desmethyl-dichlorodiaportin	41.4				
20	Citreoisocoumarin	99.5				
21	Macrosporin	54.5			na	> 50.0
22	Macrosporin sulphate	74.8			na	> 50.0
23	Methylalaternin	4.9	1.25	4.2	100	12.5
24	Methylalaternin sulphate	93.6			na	> 50.0
25	Altersolanol A	0.0	0.21	0.6	50	> 50.0
26	Ampelanol	69.1			na	> 50.0
27	Alterporriol D	64.8			na	> 50.0
28	Alterporriol E	92.1			na	> 50.0
29	Altersolanol J	35.7			na	> 50.0

na: not active
a) Data provided by Prof. W. E. G. Müller, Mainz.
b) Data provided by Dr. U. Hentschel, Würzburg.

The isocoumarins, desmethyldiaportinol and desmethyldichlorodiaportin, showed high and moderate activity against L5178Y cell line, respectively. Furthermore, the anthraquinone metabolites, macrosporin and 3-*O*-methylalaternin, proved to be moderately and highly active, respectively, while no activity was detected for their sulphated derivatives. Altersolanol A was very active in this bioassay compared to ampelanol, altersolanol J and alterporriol D, which showed moderate activity. The latter was more active than its atropisomer, alterporriol E, which displayed almost no activity against the cancer cell line used in the assay. On the other hand, 3-*O*-methylalaternin inhibited biofilm formation of *S. epidermidis* by 100%, while altersolanol A was moderately active in the assay showing 50% inhibition of biofilm formation.

Table 3.27: Protein kinase assay results for the compounds isolated from *Ampelomyces* sp. liquid and rice extracts

Compound tested (Conc. 1µg/mL)	Activity on various protein kinases based on IC_{50} [g/mL]*																												
	EGF-R	EPHB4	ERBB2	FAK	IGF1-R	SRC	VEGF-R2	VEGF-R3	AKT1	ARK5	Aurora-A	Aurora-B	PAK4	PDK1	CDK2/CycA	CDK4/CycD1	CK2-alpha1	FLT3	INS-R	MET	PDGFR-beta	PLK1	SAK	TIE2	COT	B-RAF-VE			
Methyltriacetic lactone	0	0	0	0	0	0	0	0	0	0	M	0	0	0	0	0	0	0	0	0	0	M	0	0	0	0			
Ampelopyrone	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	A	0	0	0	M	0	0	0	0			
Desmethyldiaportinol	0	0	0	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Desmethyl-dichlorodiaportin	0	0	0	0	0	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	M	0	0	0	0			
Citreoisocoumarin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Macrosporin	M	0	M	0	0	0	M	0	0	0	M	A	0	0	0	0	0	A	M	M	0	0	M	M	M	M			
Macrosporin sulphate	0	0	M	0	0	0	0	0	0	0	0	M	0	0	0	0	0	M	0	0	0	M	0	0	0	0			
Methylalaternin	M	0	0	0	M	M	M	M	0	0	M	M	0	0	0	M	0	M	M	M	0	0	M	M	M	0			
Methylalaternin sulphate	0	0	M	0	0	0	0	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Altersolanol A	M	0	0	M	M	M	M	M	M	0	M	A	0	0	0	A	0	M	0	0	0	M	M	M	0	0			
Ampelanol	0	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Alterporriol D	0	0	0	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Alterporriol E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	M	0	0	0	0	0			
Altersolanol J	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			

S: strongly active, A: active, M: moderately active, 0: not active
* Data provided by ProQinase, Freiburg.

Results of protein kinase assay showed a similar activity profile as the cytotoxicity assay results. Only altersolanol A and 3-*O*-methylalaternin were active on various protein kinases, macrosporin was also active on some protein kinases, while the remaining compounds were either active on very few protein kinases or completely inactive for all tested protein kinases.

3.3. Compounds isolated from the endophytic fungus *Stemphylium botryosum*

The endophytic fungus *Stemphylium botryosum* was isolated from leaves of *Chenopodium album* growing in Egypt. The pure fungal strain was cultivated on liquid Wickerham medium and on rice solid medium. Interestingly, chemical screening studies indicated a clear difference between *Stemphylium botryosum* extracts obtained from liquid (Wickerham) and rice cultures. Comparison of the HPLC chromatograms of the EtOAc extracts of both cultures showed that extracts of liquid cultures had a very complex chemical pattern compared to those obtained from rice cultures. HPLC chromatograms of the EtOAc extract of the fungus grown on solid rice medium showed dehydrocurvularin (**33**) and macrosporin (**21**) as main components. When grown on liquid medium the major substance detected in the extract was stemphyperylenol (**31**) (see Figure 3.25A-B). Moreover, the yield of rice cultures was higher than that of liquid cultures with a ratio of 4:1 of dried extract, respectively. Antibacterial, antifungal, cytotoxicity and protein kinase assay results showed that extracts obtained from rice cultures were much more active in the preliminary biological screening tests compared to the liquid culture extracts (see Table 3.28). Due to the complex chemical pattern, low yield and low activity of extract obtained from liquid cultures, rice culture extracts were chosen for further investigation.

In this part results of investigation of the natural products produced by *Stemphylium botryosum* when grown on solid rice medium are presented.

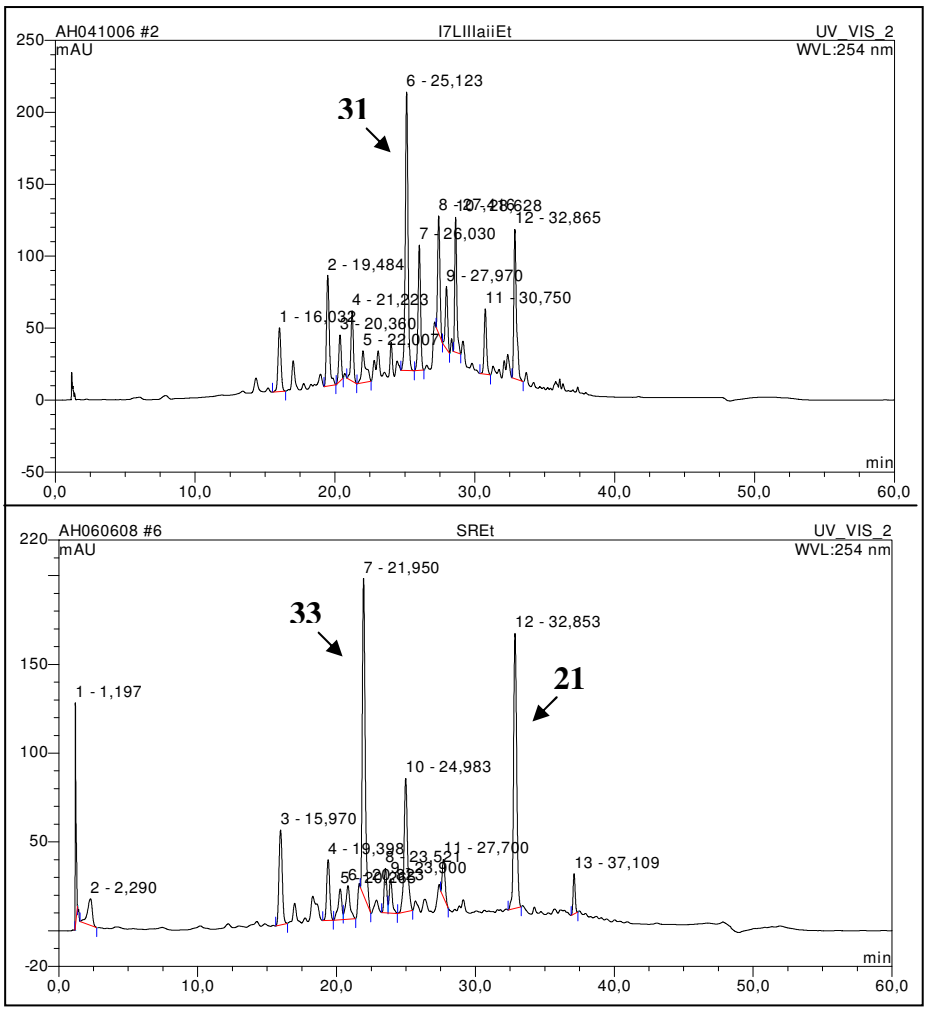


Figure 3.25A-B: EtOAc extracts of *Stemphylium botryosum* cultures. **A:** HPLC chromatogram of EtOAc extract of liquid cultures (Wickerham medium). **B:** HPLC chromatogram of EtOAc extract of rice cultures. **21:** Macrosporin. **31:** Stemphyperylenol. **33:** Dehydrocurvularin.

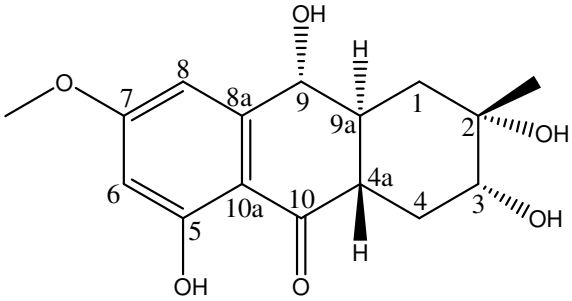
Table 3.28: Biological screening test results for *Stemphylium botryosum* liquid and rice extracts

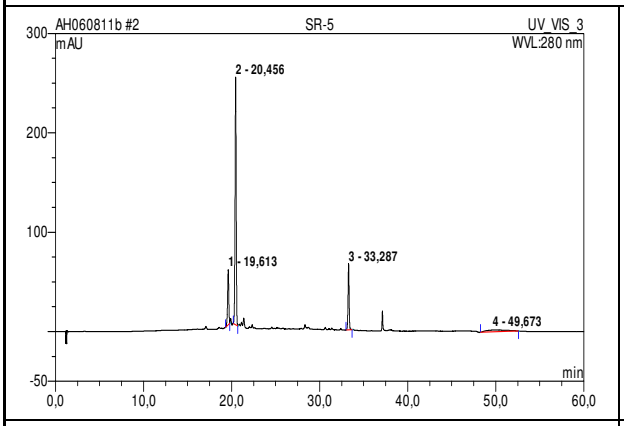
Extracts tested	**L5178Y growth in %** (Conc. 10 µg/mL)	**Protein kinase activity** (Conc. 1 µg/mL)	**Antimicrobial activity** IZ [mm], 0.5 mg		
			BS	SC	CH
Stemphylium liquid *n*-BuOH	96.5		0	0	0
Stemphylium liquid EtOAc	66.8		0	0	0
Stemphylium rice EtOAc	- 2.9	Active	12	0	0

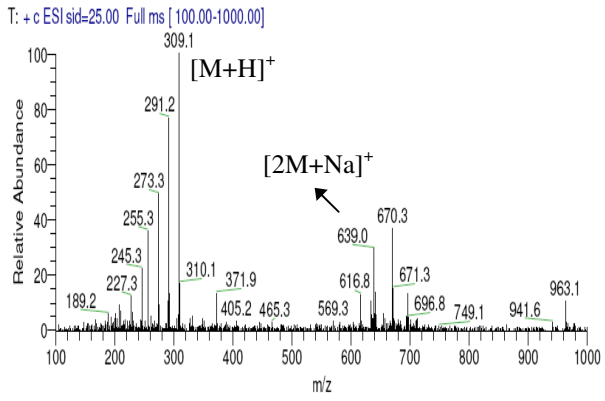
BS: *B. subtilis*, SC: *S. cerevisiae*, CH: *C. herbarum*.

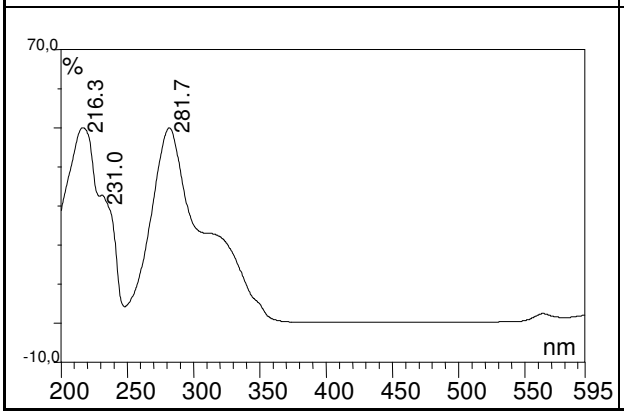
3.3.1. Tetrahydroaltersolanol B (30, known compound)

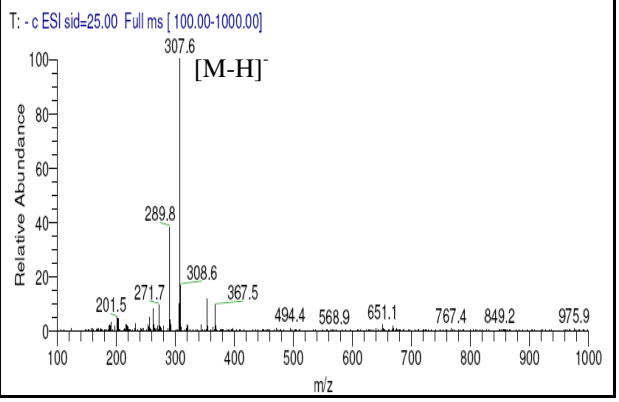
Tetrahydroaltersolanol B	
Synonym(s)	7-Methoxy-2-methyl-2*α*,3*α*,5,9*α*-tetrahydroxy-1,3,4,4a,9,9a-hexahydroanthracen-10-one
Sample code	SR5.1
Biological source	*Stemphylium botryosum* (from *Urospermum picroides*)
Sample amount	5.6 mg
Physical Description	yellowish white powder
Molecular Formula	$C_{16}H_{20}O_6$
Molecular Weight	308 g/mol
Optical Rotation $[\alpha]_D^{25}$	- 27.0º (*c* 0.18, MeOH)
Retention time HPLC	20.5 min (standard gradient)





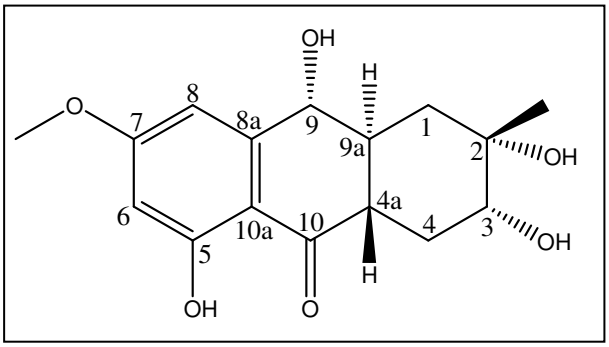






Tetrahydroaltersolanol B (**30**) was isolated from the EtOAc extract of rice cultures of *Stemphylium botryosum* as a yellowish white powder (5.6 mg). It showed UV absorbances at λ_{max} (MeOH) 216.1, 231.0, 281.7 and 320.0 nm, with a similar pattern to the UV spectrum recorded for ampelanol (**26**). Positive and negative ESI-MS showed molecular ion peaks at *m/z* 309.1 $[M+H]^+$ (base peak) and *m/z* 307.6 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 308 g/mol, identical to that of altersolanol J (**29**). The ^{1}H NMR spectrum resembled that of **29** (see Tables 3.25 and 3.29), showing three exchangeable alcoholic hydroxyl groups at δ_H 5.61, 4.43, and 3.79 assigned to 9-OH, 3-OH, and 2-OH, respectively. The singlet corresponding to the aliphatic methyl group (2-C*H*$_3$) was detected at δ_H 1.15. Additionally, two carbinolic protons appearing at δ_H 4.26 (d, *J*=10.8 Hz) and 3.28 (dd, *J*=12.2, 4.2 Hz) were assigned to H-9 and H-3, respectively. The ^{1}H NMR spectrum also showed *meta*-coupled aromatic protons at δ_H 6.68 (H-8) and 6.34 (H-6) as well as an aromatic methoxy group (δ_H 3.80). These were features already familiar from the spectra of **25** and **26** (see Table 3.21). In addition, a chelated phenolic OH was found to resonate at δ_H 12.89 (5-OH). Importantly, the less shielded of the two aryl protons H-8 exhibited a second splitting of 1.2 Hz, indicating a long range coupling with the *peri*-proton H-9, which was confirmed by the corresponding correlation observed in the COSY spectrum. Accordingly, similar to ampelanol (**26**), the quinone carbonyl corresponding to that at C-9 in **25** had been reduced to a hydroxy group, with the consequent loss of color for this compound. As for **26**, H-9 coupled to the proton situated at the junction of the *trans*-decaline-like ring system (H-9a), which in turn coupled to a diastereotopic methylene group (C*H*$_2$-1) as well as to the other ring junction proton (H-4a). The further members of the aliphatic spin system, *i.e.* C*H*$_2$-4 and H-3, were clearly discernible in the COSY spectrum (see Table 3.29).

The relative stereochemistry of **30** with the exception of C-2 could be resolved by analysis of the coupling constants of signals in the high field region (see Table 3.29). The large values of $J_{4a\text{-}9a}$ (11.6 Hz) and $J_{9a\text{-}9}$ (10.8 Hz) indicated their respective diaxial relationship. The value for $J_{3\text{-}4ax}$ (12.2 Hz) furthermore indicated an axial position for H-3. In addition, spectroscopical properties discovered during this study proved virtually identical to UV, ^{1}H NMR, mass spectral data, and the $[\alpha]_D$ value published for tetrahydroaltersolanol B (Stoessl and Stothers, 1983), thus confirming the identity of **30** with this compound. Tetrahydroaltersolanol B had previously been reported from culture filtrates of *Alternaria solani* (Stoessl and Stothers, 1983).



30 Tetrahydroaltersolanol B

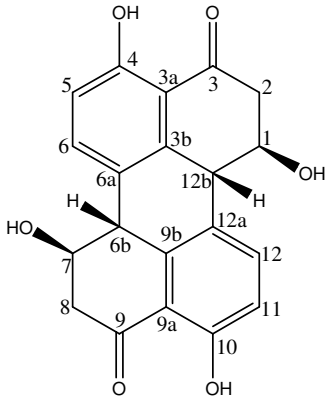
Table 3.29: ^{1}H NMR and COSY data of compound **30** at 500 MHz

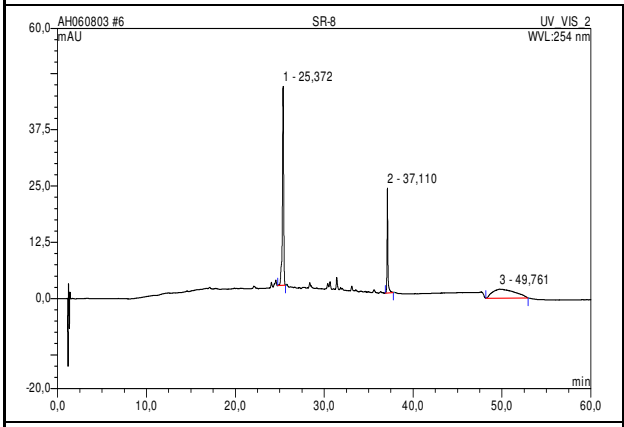
Nr.	**30**		**30**[a]
	δ_H (DMSO-d_6)	COSY	δ_H (DMSO-d_6)
1ax	1.19, dd (13.2, 12.1)	1eq,9a	1.20, dd (13.0, 12.0)
1eq	2.15, dd (13.2 ,3.4)	1ax,9a	2.16, dd (13.0, 3.3)
2-OH	3.79, brs		3.80, s
3	3.28, dd (12.2, 4.2)	4ax,4eq,3-OH	3.30, ddd (10.7, 6.5, 4.6)
3-OH	4.43, brs	3	4.45, d (6.5)
4ax	1.45, q (12.2)	3,4eq,4a	1.46, dt (12.0, 12.0)
4eq	2.11, ddd (12.2, 4.2, 3.7)	3,4ax,4a	2.13, m
4a	2.44, m	4ax,4eq,9a	2.46, dt (12.0, 3.7)
5-OH	12.89, s		12.91, s
6	6.34, d (2.3)	8	6.35, d (2.5)
8	6.68, dd (2.3, 1.2)	6,9	6.70, dd (2.5, 1.1)
9	4.26, br d (10.8)	8,9a,9-OH	4.29, m
9-OH	5.61, br s	9	5.63, d (7.4)
9a	1.94, dddd (12.1, 11.6, 10.8, 3.4)	1ax,1eq,4a,9	1.96, m
CH_3	1.15, s		1.16, s
OCH_3	3.80, s		3.82, s

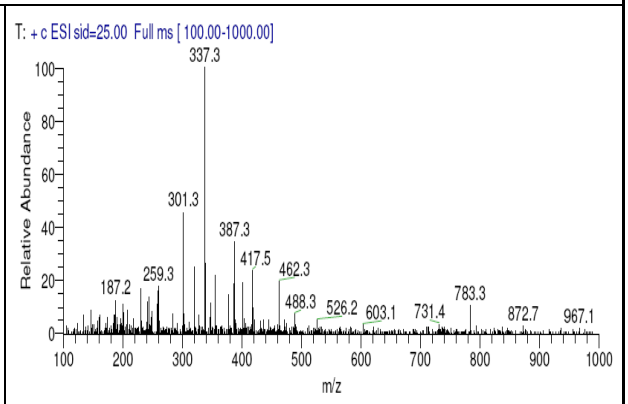
a) Stoessl and Stothers, 1983.

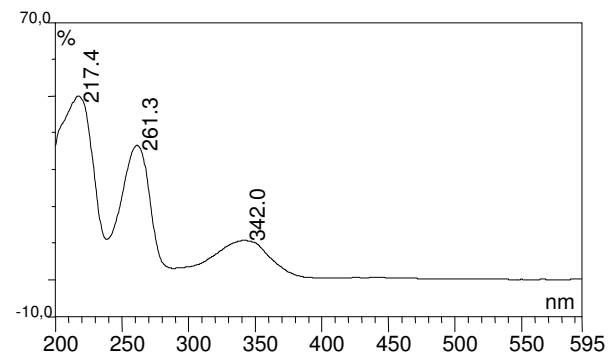
3.3.2. Stemphyperylenol (31, known compound)

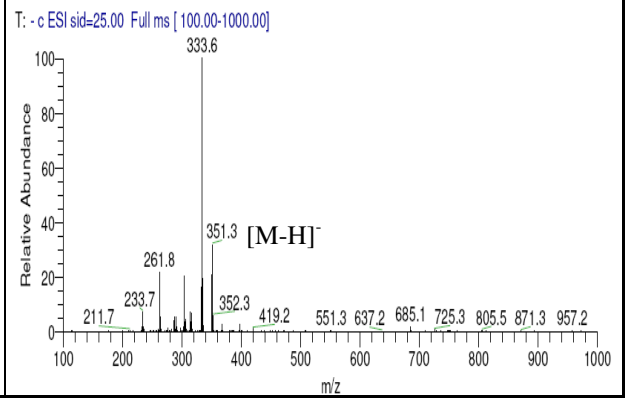
Stemphyperylenol	
Synonym(s)	Hexahydro-1,4,7,10-tetrahydroxyperylene-3,9-quinone
Sample code	SR2.1
Biological source	*Stemphylium botryosum* (from *Urospermum picroides*)
Sample amount	3.9 mg
Physical Description	Reddish brown powder
Molecular Formula	$C_{20}H_{16}O_6$
Molecular Weight	352 g/mol
Optical Rotation $[\alpha]_D^{23}$	+ 411° (*c* 0.2, MeOH)
Retention time HPLC	25.4 min (standard gradient)





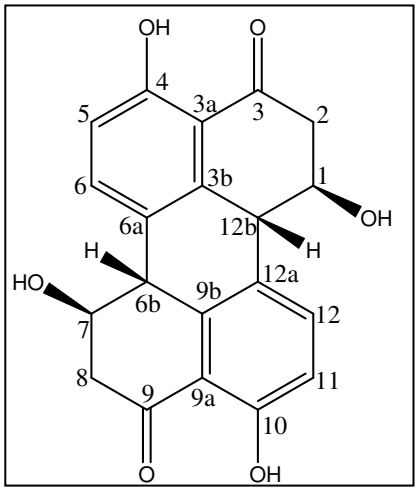






Stemphyperylenol (**31**) was isolated from the EtOAc extract of rice cultures of *Stemphylium botryosum* in the form of a reddish brown powder (3.9 mg). The UV spectrum showed λ_{max} (MeOH) at 217.4, 261.3 and 342.0 nm. Negative ESI-MS showed a molecular ion peak at *m/z* 351.3 $[M-H]^-$ (base peak) indicating a molecular weight of 352 g/mol, identical to that of altertoxin I (**14**). The ^1H and ^{13}C NMR spectra (see Table 3.30), which contained signals due to only eight protons and ten carbon atoms, indicated that the molecule was a symmetrical C_{10} dimer. The ^1H resonances were assigned to an AX spin system of *ortho*-coupled aromatic protons appearing at δ_H 8.14 and 6.81 (H-6/H-12 and H-5/H-11, respectively), to a chelated phenolic hydroxyl group (δ_H 12.09, 4-OH/10-OH), and to a CH_2-CHOH-CH fragment (C*H*$_2$-2, H-1 and H-12b/C*H*$_2$-8, H-7 and H-6b, respectively). The respective substructures were also assembled on basis of the COSY spectrum (see Table 3.30). The ^{13}C resonances were attributed to a tetra-substituted aromatic ring and a carbonyl carbon atom, the remaining signals assigned to one methylene and two methine sp^3 carbons, one of which was oxygen-bearing. From the HMBC spectrum, it was possible to place the chelated phenolic hydroxyl group at C-4, since this carbon showed HMBC correlations to H-5, H-6 and 4-OH. Consequently, the carbonyl group was located at C-3. Furthermore, C*H*$_2$-2/8 displayed correlations with C-3/9, thereby establishing the C2-C3/C8-C9 bonds. The correlations of H-6b/12b to C-6a/12a and of H-6/12 to C-6b/12b indicated the connection of the two halves of the molecule to give the planar structure of **31**. This was further confirmed by the long-range coupling between the aromatic (H-6/12) and benzylic (H-6b/12b) protons observed in the COSY spectrum.

The relative stereochemistry was derived from detailed examination of the coupling constants (see Table 3.30). The large values of $J_{1/7\text{-}2ax/8ax}$ (11.9 Hz) and $J_{1/7\text{-}12b/6b}$ (8.8 Hz) indicated the diaxial (= *trans*) position of H-1/7 with regard to both H-12b/6b and H-2ax/8ax. This suggested that the cyclohexanone rings preferentially adopted a half-chair conformation with the hydroxy groups in an equatorial position and H-6b/H-12b in an axial position. The obtained data were in excellent agreement with UV, ^1H, ^{13}C NMR, mass spectral data and $[\alpha]_D$ value published for stemphyperylenol (Arnone and Nasini, 1986), confirming that **31** and the latter were identical. Stemphyperylenol had previously been described from *Alternaria cassiae* (Hradil *et al.*, 1989) and *Stemphylium botryosum* var. Lactucum (Arnone and Nasini, 1986).



31 Stemphyperylenol

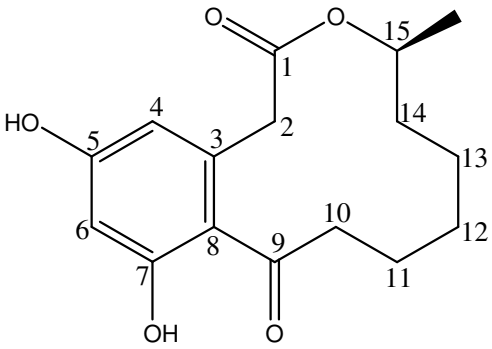
Table 3.30: ^{1}H-, ^{13}C-NMR, COSY and HMBC data of compound **31** at 500 (^{1}H) nd 125 MHz (^{13}C)

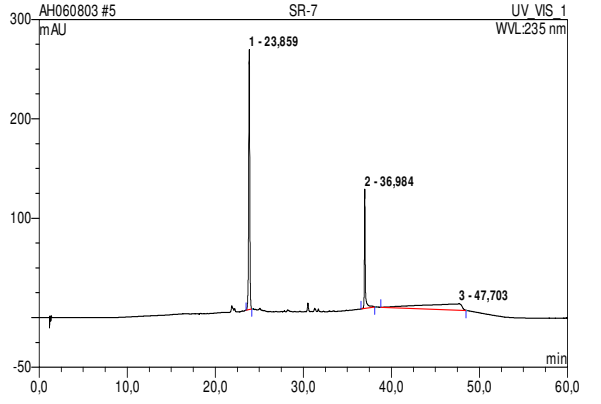
Nr.	31				31[a]	
	δ_H (DMSO-d_6)	COSY	HMBC	δ_C (DMSO-d_6)	δ_H (Acetone-d_6)	δ_C (Acetone-d_6)
1,7	4.59, m	2/8ax,2/8eq,6/12b,1/7-OH		66.5	4.76	68.32
1-,7-OH	5.75, brs	1/7			4.97	
2ax,8ax	3.14, dd (15.4, 11.9)	1/7,2/8eq	1/7,3/9,6/12b	46.8	3.17	47.84
2eq,8eq	2.91, dd (15.4, 4.4)	1/7,2/8ax	1/7,3/9,6/12b		3.07	
3,9				203.5		204.01
3a,9a				114.8		115.92
3b,9b				143.0		143.73
4,10				159.1		161.03
5,11	6.85, d (8.8)	6/12	3/9a,4/10,6/12a	114.4	6.81	115.55
6,12	8.01, d (8.8)	5/11,6/12b	3/9b,4/10,6/12b	134.6	8.14	135.58
6a,12a				129.9		130.90
6b,12b	3.71, d (8.8)	1/7,6/12	1/7,3/9b,6/12a	44.6	3.75	46.05
4-,10-OH	12.02, s		3/9a,4/10,5/11		12.09	

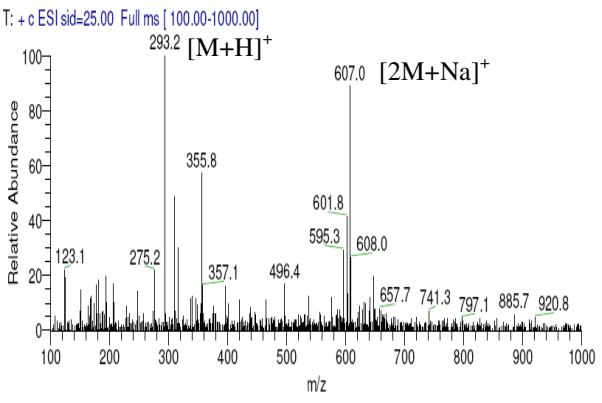
a) Arnone and Nasini, 1986.

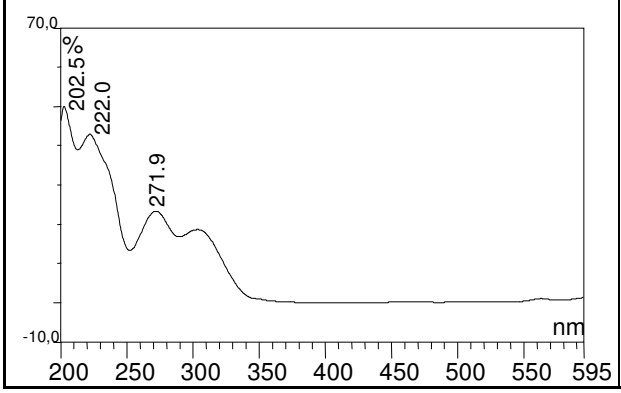
3.3.3. Curvularin (32, known compound)

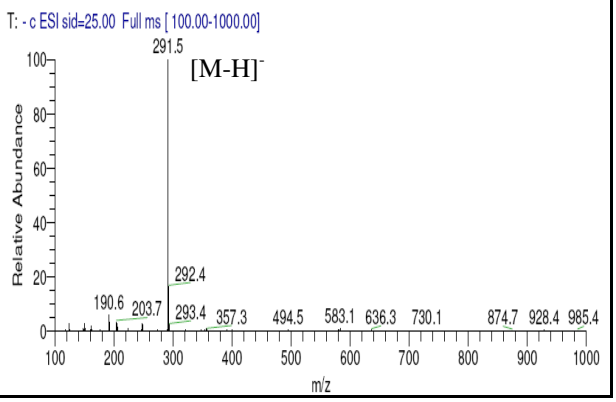
Curvularin	
Synonym(s)	11,13-dihydroxy-4-methyl-4,5,6,7,8,9-hexahydro-1H-benzo[d][1]oxacyclododecine-2,10-dione
Sample code	SR3.1
Biological source	*Stemphylium botryosum* (from *Urospermum picroides*)
Sample amount	1.9 mg
Physical Description	yellow powder
Molecular Formula	$C_{16}H_{20}O_5$
Molecular Weight	292 g/mol
Optical Rotation $[\alpha]_D^{20}$	- 28.5º (*c* 0.4, EtOH)
Retention time HPLC	23.9 min (standard gradient)







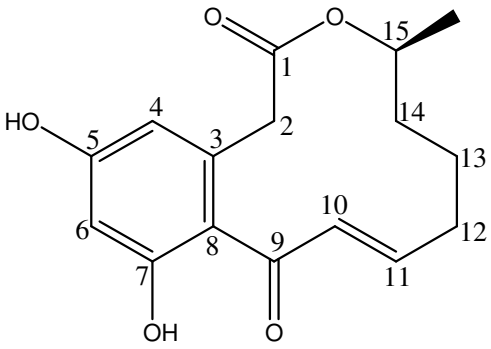


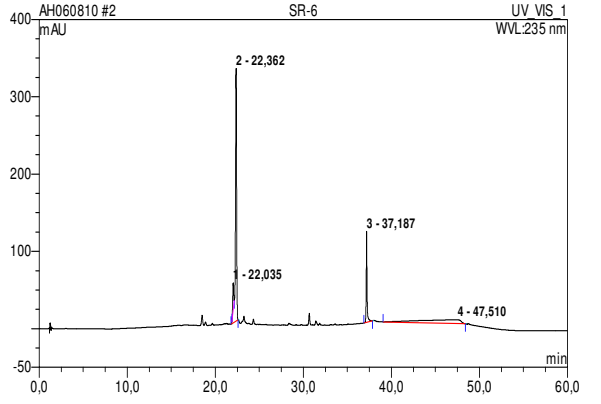


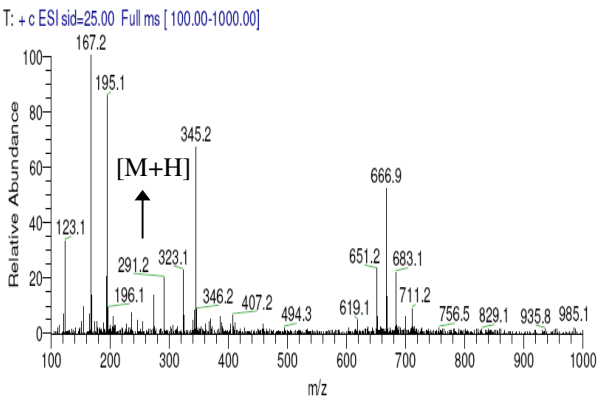
Curvularin (**32**) was isolated from the EtOAc extract of rice cultures of *Stemphylium botryosum* in the form of a yellow powder (1.9 mg). It showed UV absorbances at λ_{max} (MeOH) 202.5, 222.0, 271.9 and 298.9 nm, reminiscent to those of ampelanol (**26**) and tetrahydroaltersolanol B (**30**). Positive and negative ESI-MS displayed molecular ion peaks at *m/z* 293.2 $[M+H]^+$ (base peak) and *m/z* 291.5 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 292 g/mol. The ^1H NMR spectrum (see Table 3.31) showed a pair of *meta*-coupled aromatic protons at δ_H 6.24 (H-6) and 6.21 (H-4), respectively. In addition, the spectrum contained a diastereotopic methylene function resonating at δ_H 3.85 and 3.61 (C*H*$_2$-2) as well as an extended aliphatic spin system which could only be completely assembled with help of the COSY spectrum due to a significant degree of overlapping (see Table 3.31). It consisted of an aliphatic methyl group (δ_H 1.12, 15-C*H*$_3$) adjacent to an oxygenated methine group (δ_H 4.92, H-15), which was further connected to a chain of five methylene groups. Four of them appeared as a complex set of multiplets resonating between 1.26 and 1.74 ppm (C*H*$_2$-11-C*H*$_2$-14), while the downfield chemical shift of the last one (δ_H 3.20 and 2.73, C*H*$_2$-10) indicated its position adjacent to a carbonyl function, which was conjugated to the aromatic ring on basis of the occurrence of the characteristic ion with *m/z* 205 ($C_{11}H_9O_4$) in the mass spectrum, the structure of which is presumably **32a** (Munro *et al.*, 1967). Furthermore, the COSY spectrum also revealed a long range coupling between H-4 and C*H*$_2$-2. In the HMBC spectrum, C*H*$_2$-2 displayed correlations to C-3, C-4, C-8, and the ester carbonyl at δ_C 173.0 (C-1), the oxygen atom of which had to be connected to H-15. Overall, the substructures established so far suggested that **32** was identical to the known curvularin, a macrocyclic lactone rather widespread in fungi, which had previously been reported from *Curvularia* sp. (Birch *et al.*, 1959), *Alternaria* sp. (Robeson and Strobel, 1981, 1985) and *Penicillium* sp. (Lai *et al.*, 1989). This assumption was confirmed by comparison of spectral properties of **32** with data reported in the literature (Munro *et al.*, 1967; Ghisalberti *et al.*, 1993). Based on the almost identical value obtained for the $[\alpha]_D$ it was possible to deduce the absolute configuration at C-15 to be *S* (Ghisalberti *et al.*, 1993).

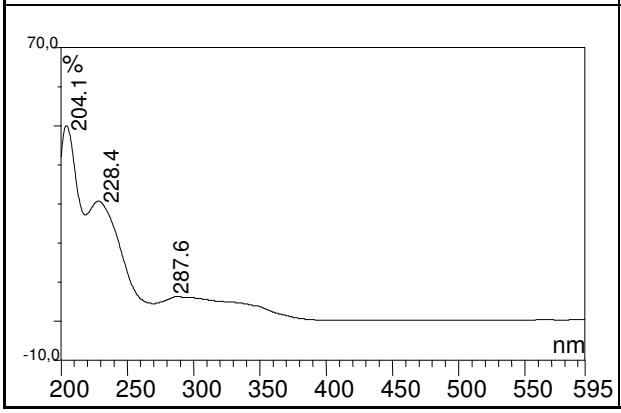
3.3.4. Dehydrocurvularin (33, known compound)

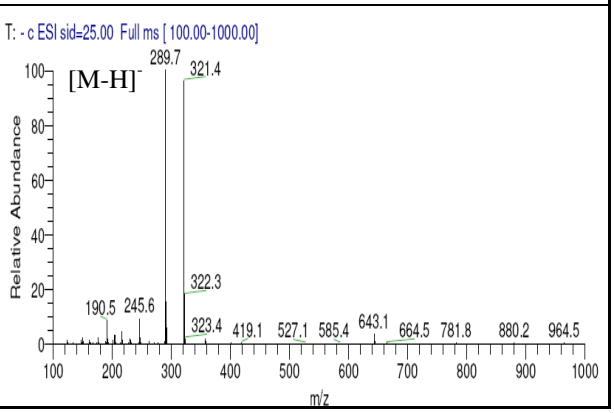
Dehydrocurvularin	
Synonym(s)	11,13-dihydroxy-4-methyl-4,5,6,7-tetrahydro-1H-benzo[d][1]oxacyclododecine-2,10-dione
Sample code	SR5.2
Biological source	*Stemphylium botryosum* (from *Urospermum picroides*)
Sample amount	15.5 mg
Physical Description	brown powder
Molecular Formula	$C_{16}H_{18}O_5$
Molecular Weight	290 g/mol
Optical Rotation $[\alpha]_D^{22}$	- 79.8º (*c* 3.0, EtOH)
Retention time HPLC	22.4 min (standard gradient)



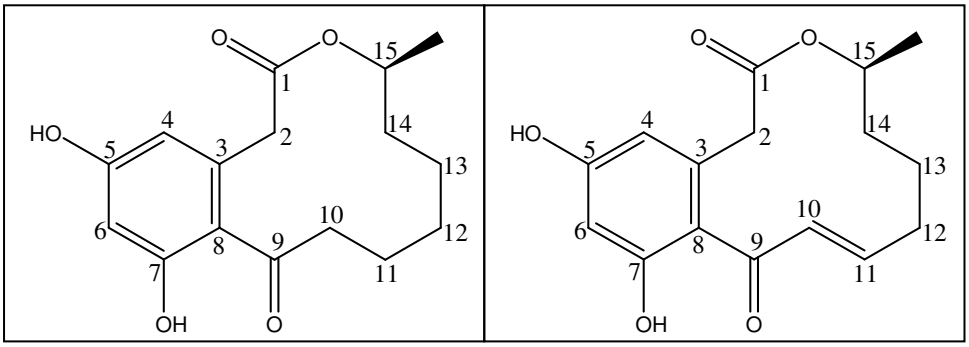






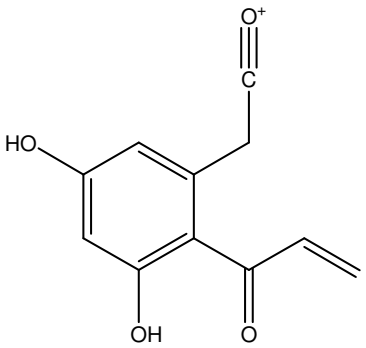


Dehydrocurvularin (**33**) was isolated from the EtOAc extract of rice cultures of *Stemphylium botryosum* in the form of brown powder (15.5 mg). Its UV spectrum showed λ_{max} (MeOH) at 204.1, 228.4 and 287.6 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 291.2 $[M+H]^+$ and *m/z* 289.7 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 290 g/mol, and thus 2 mass units less than that of curvularin (**32**). The ^1H NMR spectrum (see Table 3.31) suggested a close relationship to **32**, except for the appearance of an ABX_2 system, with two olefinic protons forming the AB part (δ_H 6.49, H-10, and δ_H 6.57, H-11), and a coupling constant of 15.4 Hz consistent with a *trans*-configuration at the double bond. These olefinic protons were found to be part of an extended spin system detected in the COSY spectrum, consisting of the adjacent C*H*$_2$-12 (δ_H 2.38, 2.29), and the remaining signals for C*H*$_2$-13, C*H*$_2$-14, H-15, and 15-C*H*$_3$ which showed close similarity to the respective signals observed for curvularin **32**. As in the case of curvularin, presence of the characteristic ion with *m/z* 203 ($C_{11}H_7O_4$, **33a**) in the EI-mass spectrum indicated that the carbonyl function at C-9 was conjugated to the aromatic ring (Munro *et al.*, 1967). Thus, **33** was identified as the known 10-dehydro congener of curvularin. This was corroborated by very similar spectral characteristics both in the HMBC spectrum (see Table 3.31) and the ESI-MS as well as by comparison with published data for dehydrocurvularin (Munro *et al.*, 1967; Lai *et al.*, 1989) and curvularin (Ghisalberti *et al.*, 1993). Again, the $[\alpha]_D$ value obtained in this study suggested the *S*-configuration at C-15 (Munro *et al.*, 1967). Dehydrocurvularin was previously reported from *Curvularia* sp. (Munro *et al.*, 1967), *Penicillium* sp. (Lai *et al.*, 1989), *Alternaria* sp. (Arai *et al.*, 1989; Robeson and Strobel, 1981, 1985) and *Stemphylium radicinum* (Grove, 1971).

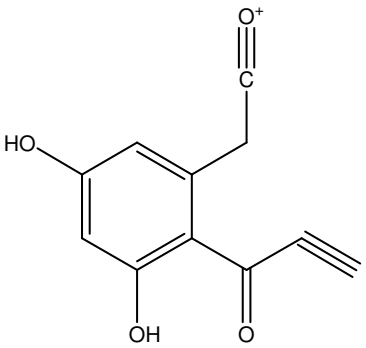


32 Curvularin

33 Dehydrocurvularin



32a
$C_{11}H_9O_4$
m/z 205



33a
$C_{11}H_7O_4$
m/z 203

Table 3.31: ^{1}H-NMR, COSY and HMBC data of compounds **32** and **33** at 500 MHz

Nr.	32			32[a]	33		
	δ_H (MeOD)	COSY	HMBC	δ_H (Acetone-d_6)	δ_H (MeOD)	COSY	HMBC
1							
2A	3.85, d (15.7)	2B,4	1,3,4,8	3.77, d (15.7)	3.72, d (16.7)	2B,4	1,3,4,8
2B	3.61, d (15.7)	2A,4	1,3,4,8	3.69, d (15.7)	3.44, d (16.7)	2A,4	1,3,4,8
3							
4	6.21, d (2.2)	2A/B,6	2,6,8	6.33, d (2.3)	6.28, d (2.0)	2A/B,6	2,5,6,8
5							
6	6.24, d (2.2)	4	4,5,8	6.37, d (2.3)	6.24, d (2.0)	4	4,5,8
7							
8							
9							
10	A 3.20, ddd (15.2, 8.8, 2.8)	10B,11A/B		A 3.10, ddd (15.4, 8.7, 3.0)	6.49, d (15.4)	11	8,9,11,12
	B 2.73, ddd (15.2, 9.7, 2.8)	10A,11A/B		B 2.75, ddd (15.4, 9.7, 2.9)			
11	A 1.74, m	10A/B,11B,12A/B		A 1.73, m	6.57, ddd (15.4, 8.2, 5.6)	10,12A/B	9,10,12,13
	B 1.57, m	10A/B,11A,12A/B		B 1.52, m			
12A	1.39, m	11A/B,12B,13A/B		1.41, m	2.38, m	11,12B,13A/B	10,11,13,14
12B	1.26, m	11A/B,12A,13A/B		1.25, m	2.29, m	11,12A,13A/B	10,11,13,14
13A	1.47, m	12A/B,13B,14A/B		1.45, m	1.96, m	12A/B,13B,14A/B	11,12,14,15
13B	1.30, m	12A/B,13A,14A/B		1.28, m	1.56, m	12A/B,13A,14A/B	12,15
14A	1.59, m	13A/B,14B,15		1.58, m	1.85, m	13A/B,14B,15	CH_3,12,13
14B	1.43, m	13A/B,14A,15		1.43, m	1.58, m	13A/B,14A,15	12,13
15	4.92, m	CH_3,14A/B		4.90, m	4.79, m	CH_3,14A/B	1,13,14
CH_3	1.12, d (6.3)	15	14,15	1.10, d (6.4)	1.18, d (6.6)	15	14,15

a) Ghisalberti *et al.*, 1993.

Table 3.32: ^{13}C-NMR data of compounds **32** and **33** at 125 MHz

Nr.	32	32[a]	33
	δ_C (MeOD)[b]	δ_C (MeOD)	δ_C (MeOD)
1	173.0	172.8	173.0
2	40.6	40.5	42.4
3	137.5	137.2	137.0
4	112.6	112.2	112.2
5	161.5	161.2	162.0
6	102.9	102.7	102.8
7		159.5	162.2
8	121.0	120.9	117.8
9		209.7	200.0
10		44.6	133.3
11		23.8	154.3
12		27.7	34.2
13		24.9	25.4
14	33.0	32.9	35.1
15	73.9	73.7	74.2
CH_3	20.5	20.4	20.3

a) Ghisalberti *et al.*, 1993.
b) Derived from HMBC spectrum.

3.3.5. Bioactivity test results for compounds isolated from the endophytic fungus *Stemphylium botryosum*

The isolated compounds were subjected to bioassays aimed to determine their cytotoxicity and their protein kinase inhibitory profiles. The results are shown in Tables 3.33 and 3.34.

Table 3.33: Cytotoxicity test results for the compounds isolated from *Stemphylium botryosum* rice extracts

Nr.	Compound tested	L5178Y growth in %* (Conc. 10 µg/mL)	EC_{50}* (µg/mL)	EC_{50} (µmol/L)
30	Tetrahydro-altersolanol B	38.9		
31	Stemphyperylenol	31.6		
32	Curvularin	6.6	4.7	16.0
33	Dehydrocurvularin	- 3.8	0.43	1.4

* Data provided by Prof. W. E. G. Müller, Mainz.

Curvularin and dehydrocurvularin showed high activity against the L5178Y cell line, whereas tetrahydroaltersolanol B and stemphyperylenol were moderately active.

Table 3.34: Protein kinase assay results for the compounds isolated from *Stemphylium botryosum* rice extract

Compound tested (Conc. 1µg/mL)	Activity on various protein kinases based on IC_{50} [g/mL]*																												
	EGF-R	EPHB4	ERBB2	FAK	IGF1-R	SRC	VEGF-R2	VEGF-R3	AKT1	ARK5	Aurora-A	Aurora-B	PAK4	PDK1	CDK2/CvcA	CDK4/CvcD1	CK2-alpha1	FLT3	INS-R	MET	PDGFR-beta	PLK1	SAK	TIE2	COT	B-RAF-VE
Tetrahydro-altersolanol B	0	0	0	M	0	0	0	0	0	0	M	M	0	0	0	0	0	M	0	0	0	A	0	0	0	0
Stemphyperylenol	0	0	0	0	0	0	0	0	0	0	M	0	0	0	0	0	0	M	0	0	0	0	0	0	0	0
Curvularin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dehydrocurvularin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

S: strongly active, A: active, M: moderately active, 0: not active
* Data provided by ProQinase, Freiburg.

The results of the protein kinase assay showed a different activity profile than the cytotoxicity assay results. Tetrahydroaltersolanol B was active against a few protein kinases whereas stemphyperylenol inhibited only two of the tested enzymes. On the other hand, curvularin and dehydrocurvularin were inactive for all tested protein kinases in spite of their pronounced activity in the cytotoxicity assay.

3.4. Compounds isolated from the endophytic fungus *Chaetomium* sp.

This undescribed endophytic fungal strain of the genus *Chaetomium* was isolated from fresh stems of *Otanthus maritimus* growing in Egypt. The pure fungal strain was cultivated on liquid Wickerham medium and rice solid medium. Preliminary biological and chemical screening studies indicated slight differences between *Chaetomium* extracts obtained from liquid and rice cultures. Comparison of the HPLC chromatograms of the EtOAc extracts of both cultures showed that both extracts had cochliodinol (**35**) as their main component. While this was the only peak observed in extracts obtained from rice cultures, liquid culture extracts showed additional peaks for orsellinic acid (**39**) and aureonitolic acid (**34**) (see Figure 3.26A-B). Similar to observations made with other fungal strains throughout this thesis, the yield of rice cultures was higher than that of liquid cultures with a ratio of 2:1 of dried extract, respectively. Furthermore, extracts obtained from rice cultures were slightly more active in preliminary biological screening tests compared to the liquid culture extracts (see Table 3.35).

In this part results of investigating the natural products produced by *Chaetomium* sp. when grown in liquid medium or on solid rice medium are presented.

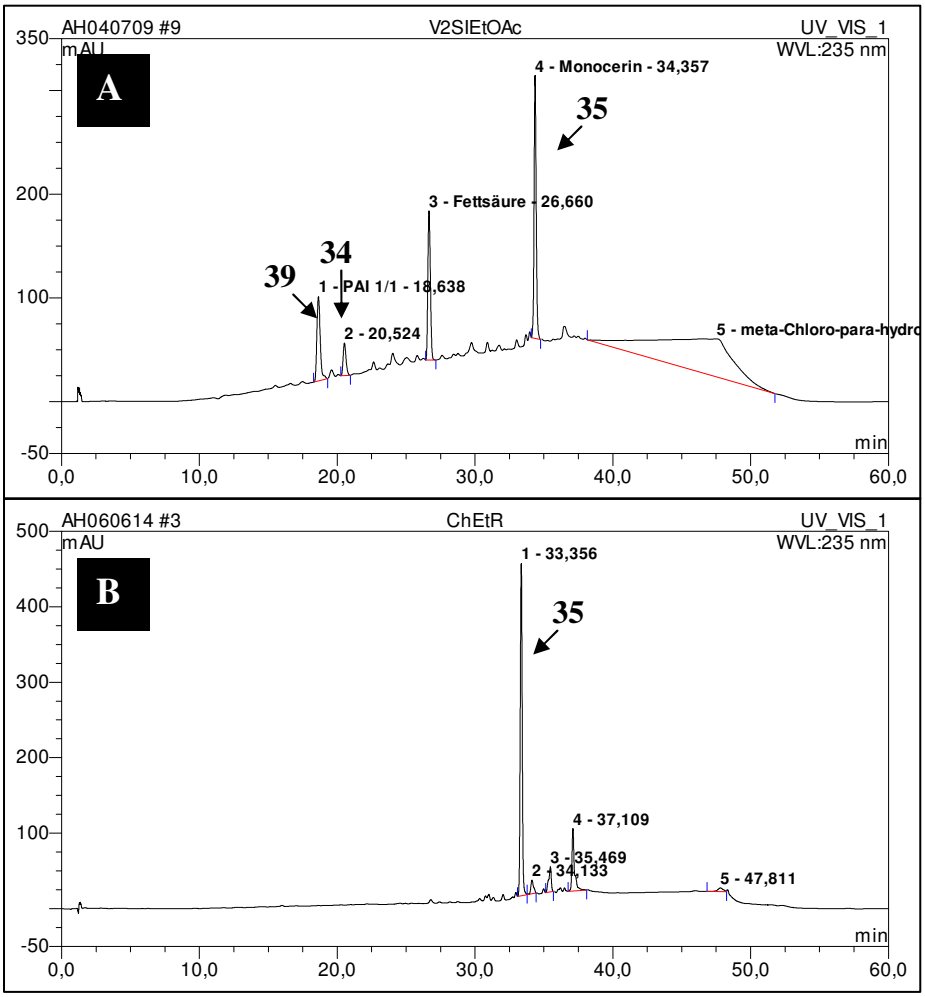


Figure 3.26A-B: EtOAc extracts of *Chaetomium* sp. cultures. **A:** HPLC chromatogram of EtOAc extract of liquid cultures (Wickerham medium). **B:** HPLC chromatogram of EtOAc extract of rice cultures. **34:** Aureonitolic acid. **35:** Cochliodinol. **39:** Orsellinic acid.

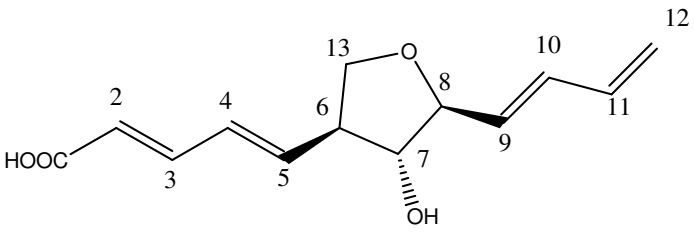
Table 3.35: Biological screening test results for *Chaetomium* liquid and rice extracts

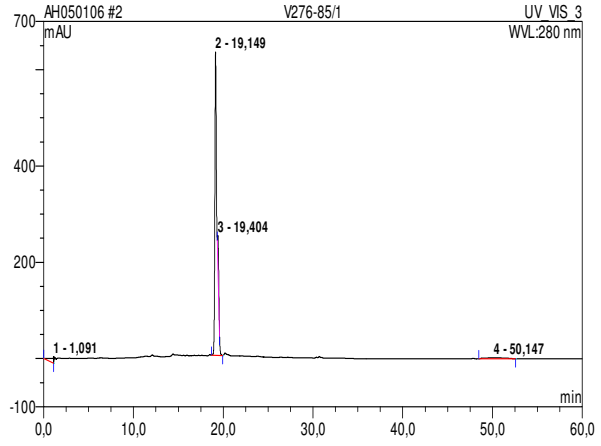
Extracts tested	**L5178Y growth in %** (Conc. 10 µg/mL)	**Protein kinase activity** (Conc. 1 µg/mL)	**Antimicrobial activity** IZ [mm], 0.5 mg		
			BS	SC	CH
Chaetomium liquid *n*-BuOH	99.5		0	0	0
Chaetomium liquid EtOAc	72.4	Active	0	0	0
Chaetomium rice EtOAc	52.6	Active	10	0	0

BS: *B. subtilis*, SC: *S. cerevisiae*, CH: *C. herbarum*.

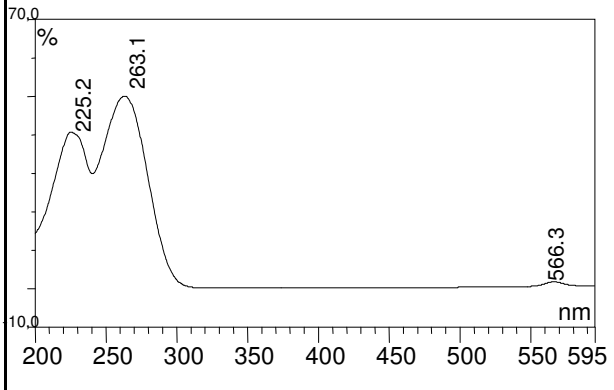
3.4.1. Aureonitolic acid (34, new compound)

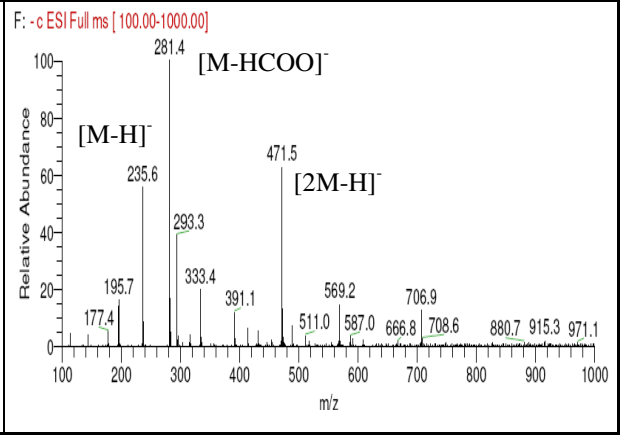
Aureonitolic acid	
Synonym(s)	(2E,4E)-5-((3S,4R,5S)-5-((E)-buta-1,3-dienyl)-4-hydroxy-tetrahydrofuran-3-yl)penta-2,4-dienoic acid
Sample code	ChE2.2
Biological source	*Chaetomium* sp. (from *Otanthus maritimus*)
Sample amount	1.7 mg
Physical Description	viscous colourless oil
Molecular Formula	$C_{13}H_{16}O_4$
Molecular Weight	236 g/mol
Optical Rotation $[\alpha]_D^{20}$	- 5.0 (*c* 0.5, MeOH)
Retention time HPLC	19.1 min (standard gradient)





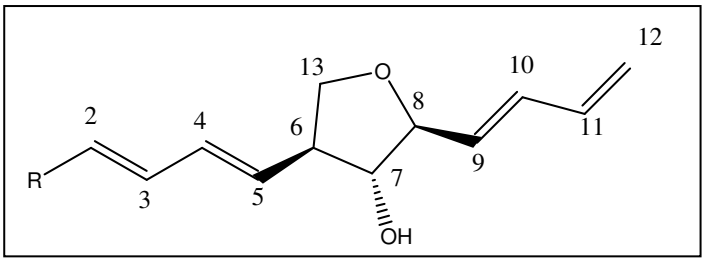
(+)-ESI-MS: no ionization





Aureonitolic acid (**34**) was isolated from the EtOAc extracts of liquid cultures of *Chaetomium* sp. as viscous colourless oil (1.7 mg). It showed UV absorbances at λ_{max} (MeOH) 225.2 and 263.1 nm. The HRESI-MS exhibited a strong peak at *m/z* 259.0940 $[M+Na]^+$ indicating a molecular formula of $C_{13}H_{16}O_4$ (calculated 259.0946, Δ 0.0006). The ^{1}H NMR and COSY spectra (see Table 3.36 and Figure 3.27) showed two major spin systems, which on the basis of the observed coupling constants were shown to consist of two conjugated double bonds each (C-2 through C-5 as well as C-9 through C-12, respectively). Both were connected to a central 3-hydroxytetrahydrofuran moiety at its positions 4 and 2, respectively. The coupling constants observed for the terminal methylene protons H-12A and H-12B indicated mutual geminal as well as vicinal couplings to H-11 as in aureonitol (**34a**, see below). The ^{13}C NMR spectrum (see Table 3.37) showed two oxymethine carbons at δ_C 82.3 and 86.2, as well as one oxymethylene carbon at δ_C 71.7 assigned to C-7, C-8 and C-13, respectively, which would correspond to positions 3, 2 and 5 of the central 3-hydroxytetrahydrofuran ring. Signals for H-7 and H-13B overlapped at δ_H 3.80, H-8 and H-13A at δ_H 4.05 in the ^{1}H NMR spectrum (see Table 3.36). The carbon framework of **34** which was basically already evident from the COSY spectrum was confirmed by inspection of the HMBC spectrum (see Figure 3.28). Key correlations include H-2 to C-1, C-3 and C-4, H-5 to C-3, C-4, C-6, C-7 and C-13, H-6 to C-4, C-5, C-7 and C-13, H-7 to C-5, C-6, C-8 and C-9, H-8 to C-7 and C-10, H-9 to C-8 and C-11 as well as those of C*H*$_2$-13 to C-5, C-7 and C-8.

In order to determine the relative configuration of the compound, 1D NOE difference spectra were acquired. Irradiation of H-3, H-6 and H-9 gave enhancements as listed in Table 3.36. Most importantly, H-6 exhibited a pronounced NOE with H-8, and correspondingly, H-9 with H-7. These results together with the coupling constants observed in the ^{1}H NMR spectrum are in agreement with a *syn* configuration of the two carbon chains and a *trans* configuration of the hydroxy group at the ether ring, as well as an all *trans* configuration of the double bonds in the side chains. All spectroscopical data obtained for **34** were in agreement with the corresponding signals reported for aureonitol (Abraham and Arfmann, 1992; Bohlmann and Ziesche, 1979; Seto *et al.*, 1979), except for the fact that the terminal methyl group in one of the side chains of the latter was replaced by a carboxylic acid group, as indicated by the signal at δ_C 174.5 in the ^{13}C NMR spectrum and the presence of a fragment at *m/z* 190.8 $[M-CO_2-H]^-$ in the negative mode ESI-MS. Thus, **34** was identified as a new natural product for which we propose the name aureonitolic acid. Aureonitol was previously reported from *Chaetomium* species (Abraham and Arfmann, 1992; Seto *et al.*, 1979) as well as from *Helichrysum aureo-nitens* (Bohlmann and Ziesche, 1979).



Nr.	Compound	R
34	Aureonitolic acid	COOH
34a	Aureonitol	CH_3

Table 3.36: ^{1}H NMR, COSY, NOE and HMBC data of compound **34** at 500 MHz

Nr.	34				34a[a]
	δ_H (MeOD)	COSY	NOE	HMBC	δ_H ($CDCl_3$)
1					1.74, dd (6.5, 1.5)
2	5.89, d (15.1)	3		1,3,4	5.65, dq (14.5, 6.5)
3	7.00, dd (14.8, 10.7)	2,4	5	1,4,5	6.00, ddq (14.5, 10.0, 1.5)
4	6.30, dd (15.3, 10.7)	3,5		2,3,6	6.12, dd (15.0, 10.0)
5	5.89, dd (15.3, 8.5)	4,6		3,4,6,7,13	5.40, dd (15.0, 9.0)
6	2.82, q (7.8)	5,7,13A,13B	4,8,13A>>5,7	4,5,7,13	2.84, dddd (9.0, 8.5, 7.5)
7	3.80, dd (6.8)	6,8		5,6,8,9	3.72, dd (7.5)
8	4.05, dd (6.8)	7,9		7,10	4.12, dd (7.5)
9	5.70, dd (14.8, 6.9)	8,10	7,11	8,11	5.68, dd (14.5, 7.5)
10	6.28, m	9		8,12	6.32, m (14.5, 10.0)
11	6.29, m	12A,12B		12	6.32, m (17.0, 10.0)
12A	5.21, d (16.1, 1.7)	11,12B		10	5.22, d (17.0)
12B	5.08, d (9.4, 1.7)	11,12A		10,11	5.10, d (10.0)
13A	4.05, dd (8.4)	6,13B		5,7,8	4.10, dd (8.5)
13B	3.80, dd (8.4)	6,13A		5,7,8	3.70, dd (8.5)

a) Abraham and Arfmann, 1992.

Table 3.37: ^{13}C NMR data of compound **34** at 125 MHz

Nr.	34	34a[a]
	δ_C (MeOD)	δ_C ($CDCl_3$)
1	174.5	18.0
2	128.0	128.3
3	139.0	130.9
4	132.5	133.1
5	138.1	129.3
6	52.4	51.6
7	82.3	81.7
8	86.2	84.8
9	133.1	133.2
10	134.1	131.0
11	137.7	136.2
12	118.0	118.1
13	71.7	71.1

a) Abraham and Arfmann, 1992.

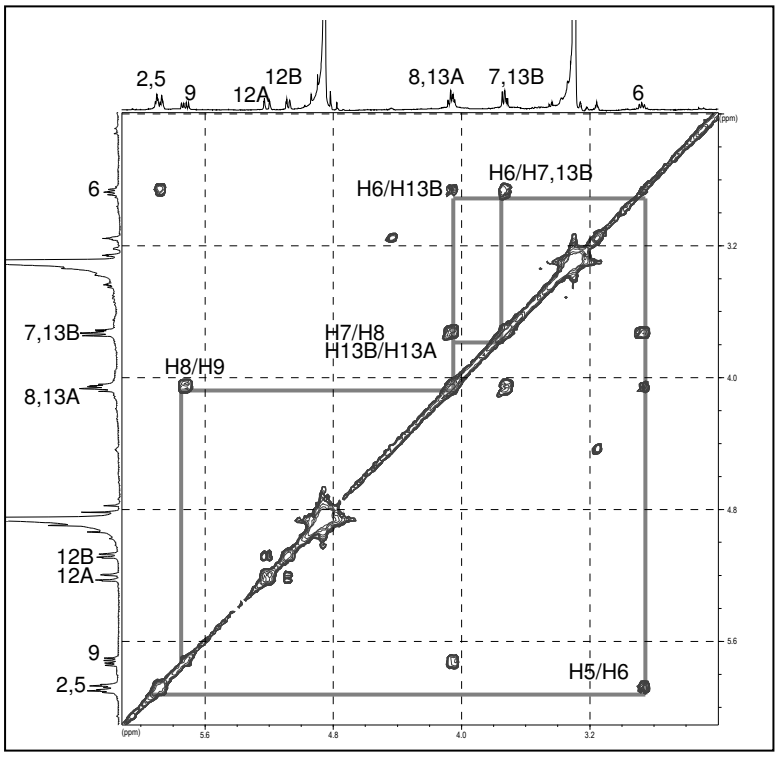


Figure 3.27A: Expansion of the COSY spectrum of compound **34**.

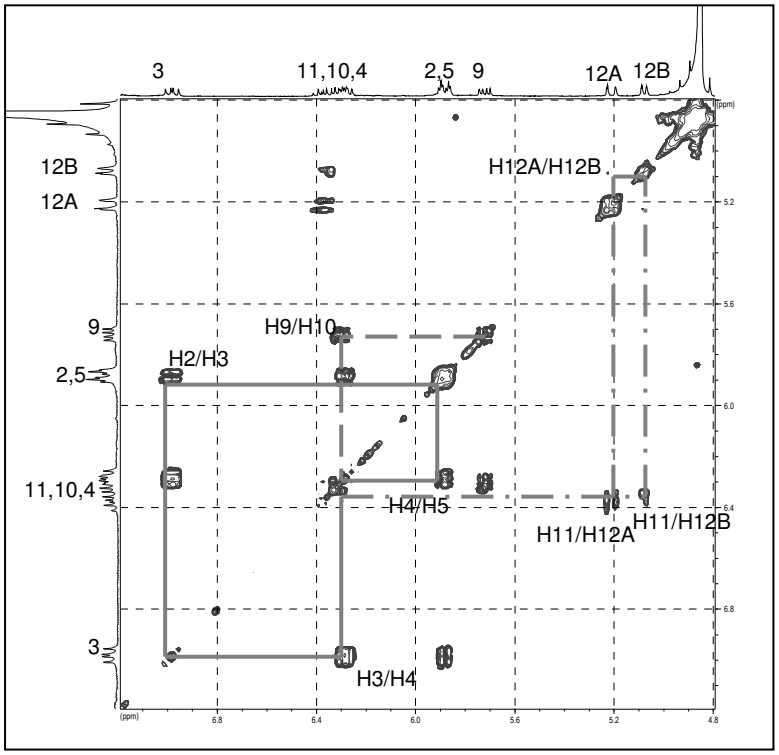


Figure 3.27B: Expansion of the COSY spectrum of compound **34**.

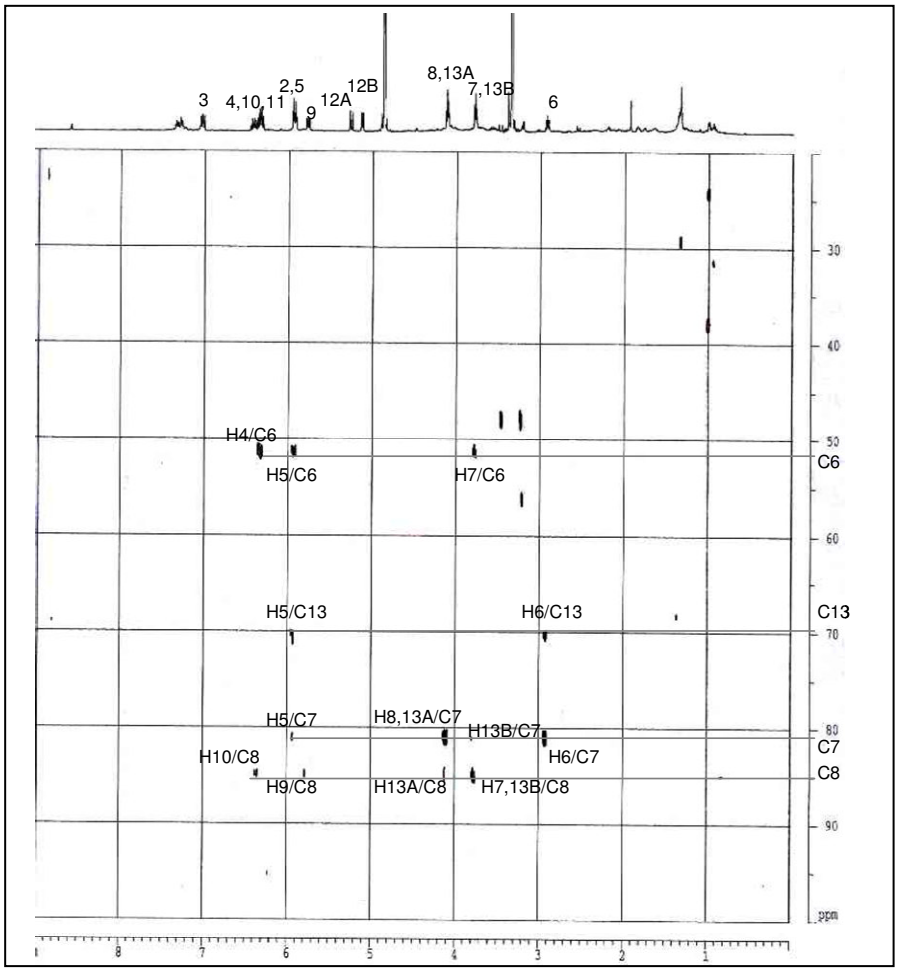


Figure 3.28A: Expansion of the HMBC spectrum of compound **34**.

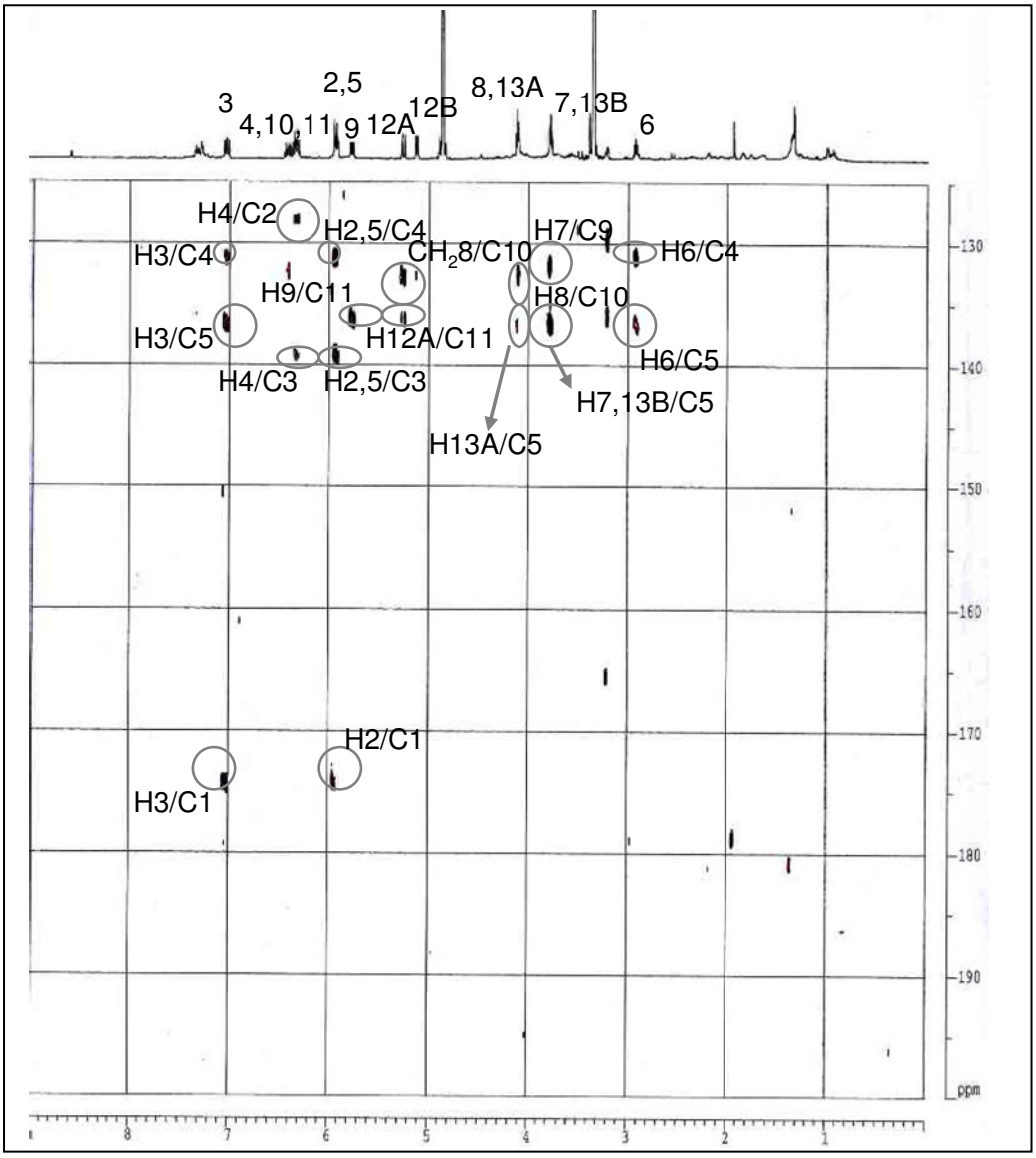
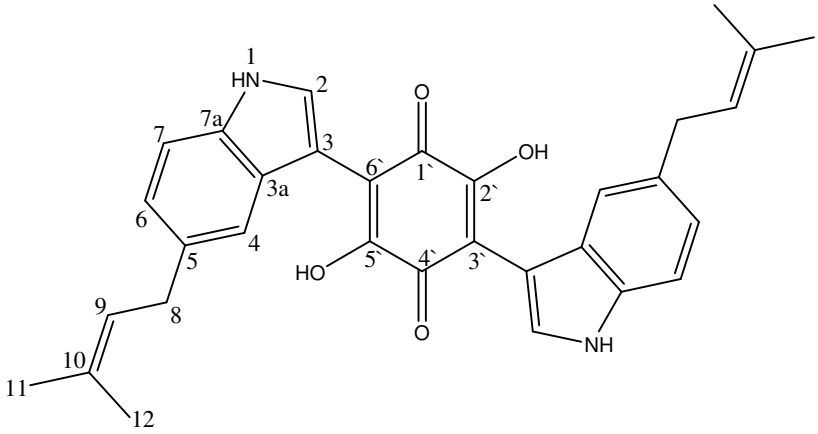
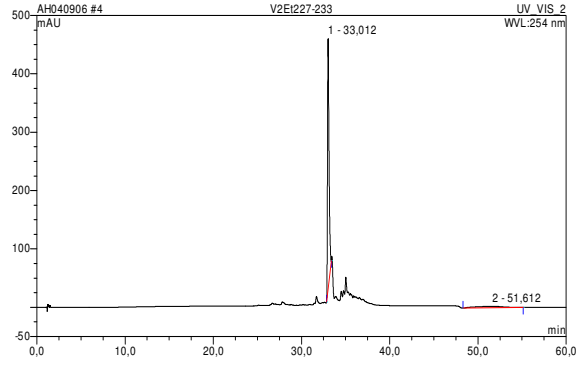


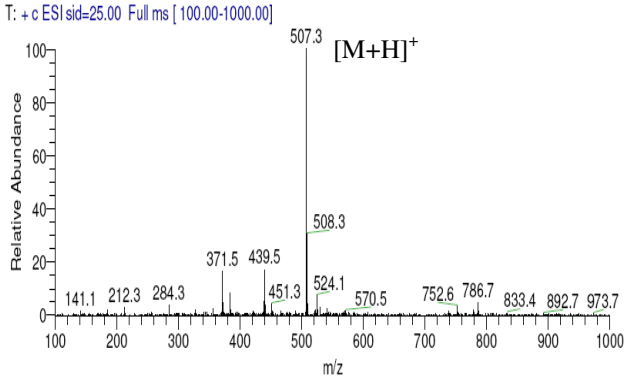
Figure 3.28B: Expansion of the HMBC spectrum of compound **34**.

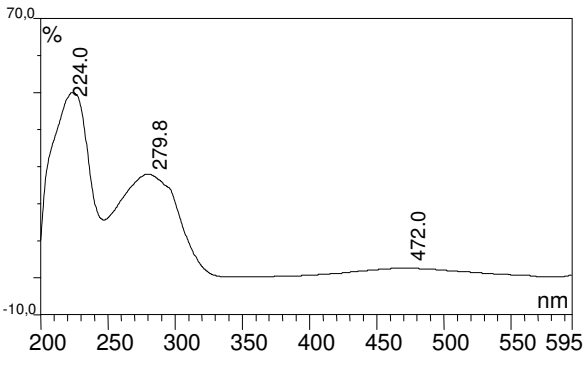
3.4.2. Cochliodinol (35, known compound)

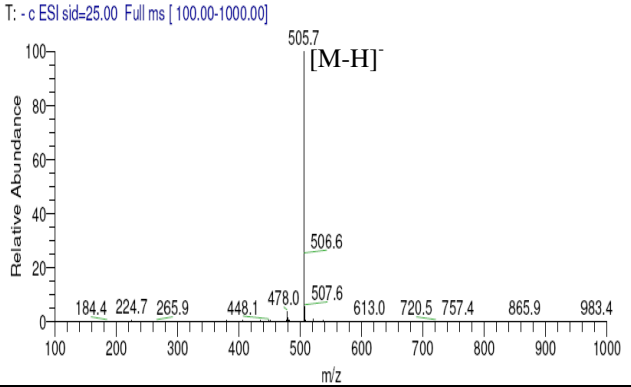
Cochliodinol	
Synonym(s)	2,5-Dihydroxy-3,6-bis[5-(3-methyl-2-butenyl)-1H-indol-3-yl]-2,5-cyclohexadiene-1,4-dione
Sample code	ChE8.1, ChR3
Biological source	*Chaetomium* sp. (from *Otanthus maritimus*)
Sample amount	455.7 mg
Physical Description	purple crystals
Molecular Formula	$C_{32}H_{30}N_2O_4$
Molecular Weight	506 g/mol
Retention time HPLC	33.0 min (standard gradient)









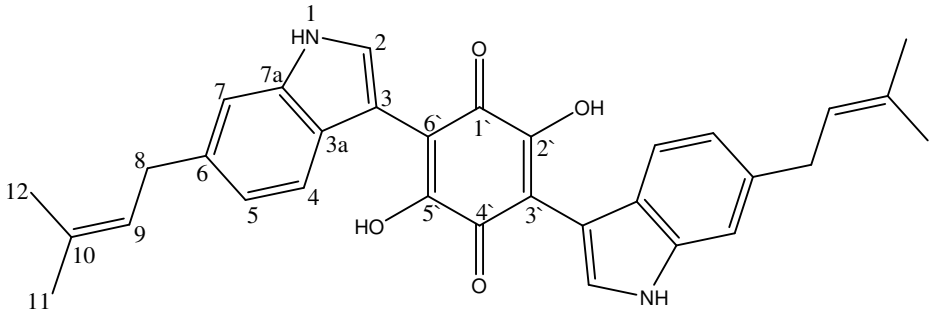


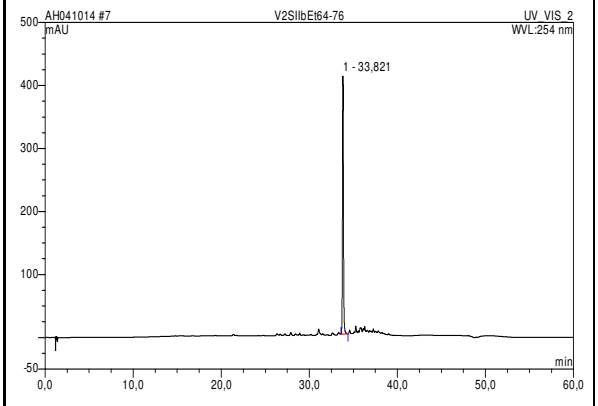
Cochliodinol (**35**) was isolated from the EtOAc extracts of liquid and rice cultures of *Chaetomium* sp. as purple crystals (455.7 mg). The UV spectrum showed λ_{max} (MeOH) at 224.0, 279.8 and 472.0 nm, indicative of an indole chromophore. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 507.3 $[M+H]^+$ (base peak) and *m/z* 505.7 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 506 g/mol. Together with inspection of the NMR spectra, a molecular formula of $C_{32}H_{30}N_2O_4$ was derived. However, the ^{1}H NMR spectrum only gave signals in a ratio of 4:1:2:6 for aromatic, olefinic, methylene and methyl protons, respectively. All resonances therefore had to originate from pairs of chemically equivalent groups due to symmetry in the molecule. Furthermore, aromatic proton and carbon resonances had chemical shifts and multiplicities consistent with the presence of either a 3,5- or a 3,6-disubstituted indole residue (see Table 3.38 and 3.39), since the aromatic multiplet signals clearly indicated a 1,2,4-trisubstituted phenyl substructure. The presence of a 2-methyl-but-2-enyl (= isoprenyl) substituent was established by a metastable ion observed in the mass spectrum indicating the loss of a neutral fragment of 69 mass units. In addition, the ^{1}H NMR spectrum indicated that the olefinic proton (H-9) at δ_H 5.37 was allylically coupled to the methyl protons at δ_H 1.74 and 1.72, and vicinally coupled to the methylene group (C*H*$_2$-8) at δ_H 3.40. From the COSY spectrum, the attachment of the isoprenyl side chain to the aromatic side of the indole substructure (either at C-5 or C-6) was evident by long range correlations of C*H*$_2$-8 to both H-4 and H-6. Accordingly, correlations of H-2 to both C-3a and C-7a indicated that a further substituent had to reside at C-3 in the heteroaromatic ring of the indole moiety. Most diagnostic for unambiguously deducing the position of the isoprenyl side chain proved a correlation of H-4 to C-3 in the HMBC spectrum, the corresponding proton signal was the one exhibiting the *meta*-coupling, thus representing the proton immediately adjacent to the substituent. Based on consideration of symmetry as outlined above, two of these 3,5-disubstituted indole systems were present in **35**. However, in the ^{13}C NMR spectrum only one signal (δ_C 111.9, C-3`/C-6`) was observed belonging to the central unit connecting these two substructures. The molecular weight indicated this to be a symmetrical 2,5-dihydroxy-1,4-quinone ring, thus suggesting that **35** was identical to the known cochliodinol. According to the literature, the non-appearance of the signal for C-1`, C-2`, C-4` and C-5` (due to the rapid interconversion of two equivalent tautomeric forms only one instead of two signals is to be expected) seems to be a typical feature of this substructure. The identification of **35** as 2,5-dihydroxy-3,6-bis[5-(3-methyl-2-butenyl)-3-indolyl]-benzoquinone was confirmed by comparison of UV, ^{1}H, ^{13}C NMR and mass spectral data with published data for cochliodinol (Jerram *et al.*, 1975; Sekita, 1983). Cochliodinol was previously reported from

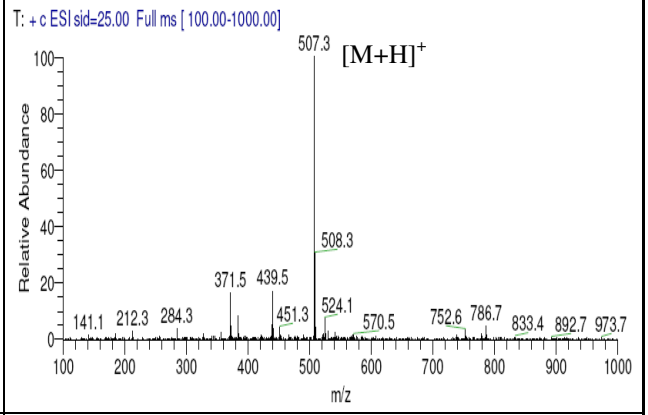
several *Chaetomium* species (Jerram *et al.*, 1975; Sekita *et al.*, 1981; Sekita, 1983; Brewer *et al.*, 1984).

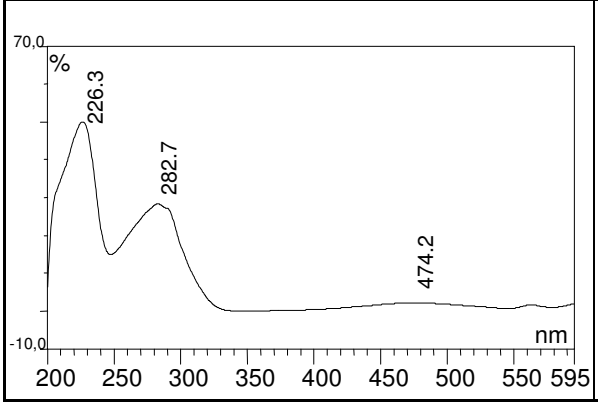
3.4.3. Isocochliodinol (36, known compound)

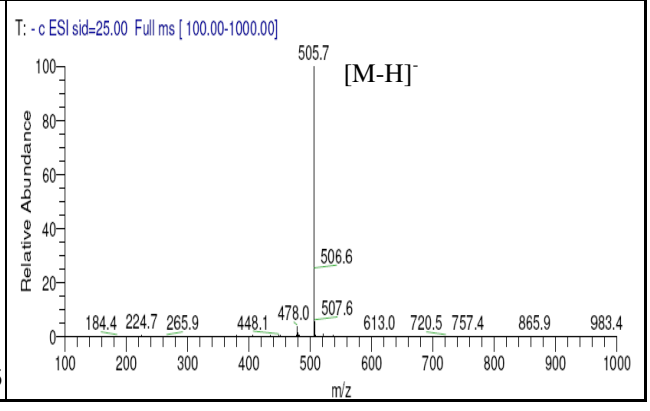
Isocochliodinol	
Synonym(s)	2,5-Dihydroxy-3,6-bis[6-(3-methyl-2-butenyl)-1H-indol-3-yl]-2,5-cyclohexadiene-1,4-dione
Sample code	ChE8.2, ChR5
Biological source	*Chaetomium* sp. (from *Otanthus maritimus*)
Sample amount	8.8 mg
Physical Description	purple crystals
Molecular Formula	$C_{32}H_{30}N_2O_4$
Molecular Weight	506 g/mol
Retention time HPLC	33.8 min (standard gradient)



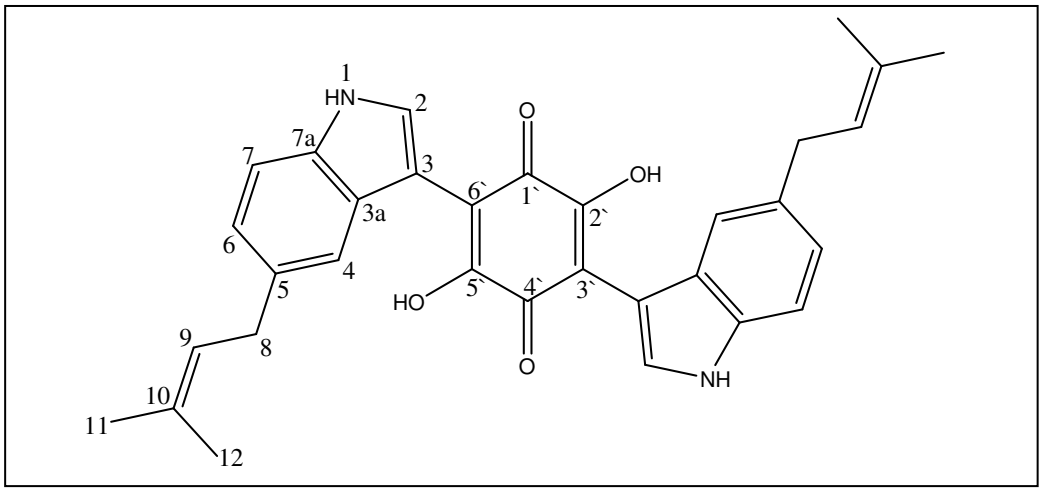




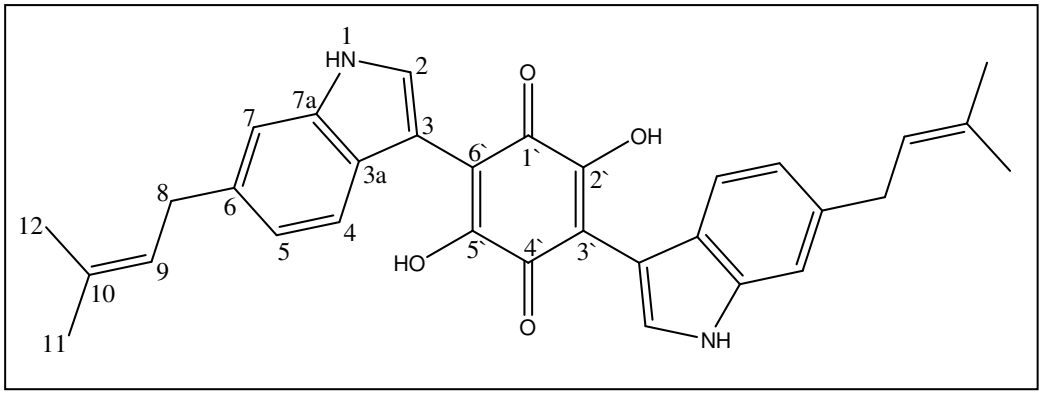




Isocochliodinol (**36**) was isolated from the EtOAc extracts of liquid and rice cultures of *Chaetomium* sp. as purple crystals (8.8 mg). Its UV spectrum showed λ_{max} (MeOH) at 226.3, 282.7 and 474.2 nm, with very high similarity to that of **35**. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 507.3 $[M+H]^+$ (base peak) and *m/z* 505.7 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 506 g/mol and thus identical to that of cochliodinol (**35**). The ^{1}H NMR spectrum disclosed identical spin systems as described above for **35**, with the mass spectrum also supporting the presence of a 2-methyl-but-2-enyl group. Thus, **36** had to represent a symmetrical isomer of cochliodinol (**35**), leaving a positional isomer with the prenyl groups attached to C-6 as the most probable alternative. As in the case of cochliodinol, the COSY spectrum (see Table 3.38) confirmed that the isoprenyl group was attached to the aromatic side of the indole substructure since C*H*$_2$-8 exhibited long range correlations to both H-7 and H-5. In addition, in the HMBC spectrum (see Table 3.38) a correlation of H-4 to C-3 was detected. However, in the case of **36**, this proton signal was the one exhibiting the *ortho*-coupling, proving that the side chain was situated *meta* with regard to H-4 and thus resided at C-6. Based on these observations, **36** was identified as the known isocochliodinol which was confirmed by comparison of its UV, ^{1}H, ^{13}C NMR and mass spectral data with published data (Sekita, 1983). The compound was previously obtained from several *Chaetomium spp*. (Sekita *et al.*, 1981; Sekita, 1983; Brewer *et al.*, 1984).



35 Cochliodinol



36 Isocochliodinol

Table 3.38: ^{1}H NMR, COSY and HMBC data of compounds **35** and **36** at 500 MHz

Nr.	35			35[a]	36			36[a]
	δ_H (MeOD)	COSY	HMBC	δ_H (THF-d_8)	δ_H (DMSO-d_6)	COSY	HMBC	δ_H (THF-d_8)
NH				10.30, br s	11.21,s	2	2,3,3a,7a	10.14, br s
2	7.50, s		3,3a,7a	7.51, d (2.4)	7.43, d (2.5)	NH	3,3a,7a	7.39, d (2.4)
3								
4	7.32, d (0.9)	6,8	3,6,7a,8	7.39, br s	7.32, d (8.2)	5	3,6,7a	7.38, d (8.3)
5					6.81, br d (8.2)	4,7,8	3a,7,8	6.73, dd (8.3, 1.4)
6	6.94, dd (8.5, 1.5)	4,7,8	4,7a,8	6.90, dd (8.3, 1.4)				
7	7.29, d (8.2)	6	3a,5	7.19, d (8.3)	7.16, br s	5,8	3a,5,8	7.01, d (1.4)
8	3.40, d (7.2)	4,6,9,11,12	4,5,6,9,10	3.40, d (6.8)	3.42, d (7.2)	5,7,9,11,12	5,6,7,9,10,	3.32, d (7.3)
9	5.37, tq (7.2, 1.5)	8,11,12	8,11,12	5.36, tqq (6.8, 1.4, 1.4)	5.35, br t (7.2)	8,11,12	8,11,12	5.29, tqq (7.3, 1.5, 1.5)
10								
11	1.74, br s	8,9	9,10,12	1.72, br s	1.71, br s	8,9	9,10,12	1.639, d (1.5)
12	1.72, d (0.95)	8,9	9,10,11	1.72, br s	1.71, br s	8,9	9,10,11	1.636, d (1.5)
OH				9.72, s				9.56, s

a) Sekita, 1983.

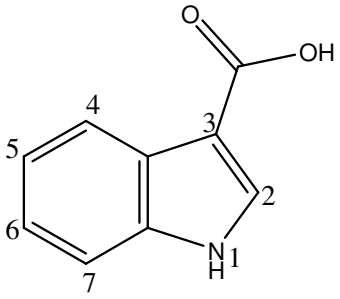
Table 3.39: ^{13}C NMR data of compounds **35** and **36** at 125 MHz

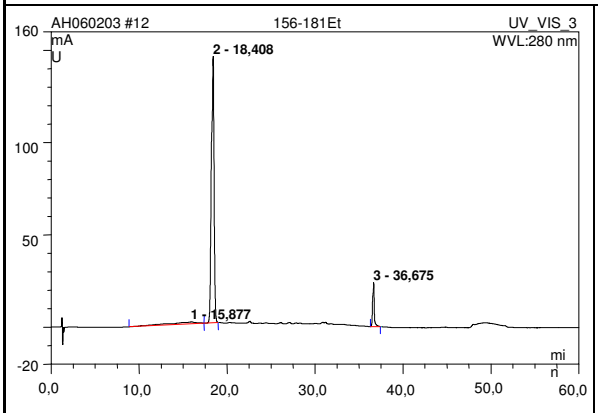
Nr.	35	35[a]	36
	δ_C (MeOD)[b]	δ_C (THF-d_8)	δ_C (DMSO-d_6)[b]
1`,4`		168.5	
2`,5`		168.5	
3`,6`	111.9	112.1	
2		128.4	128.0
3	106.6	105.5	105.8
3a	128.3	132.7	126.0
4	121.8	122.8	
5	133.5	127.9	121.1
6	123.0	122.0	135.8
7	111.7	111.5	111.6
7a	135.5	135.8	135.2
8	35.6	35.6	35.0
9	126.1	126.2	126.0
10	131.0	131.0	131.2
11	17.9	17.9	18.0
12	25.9	25.9	26.0

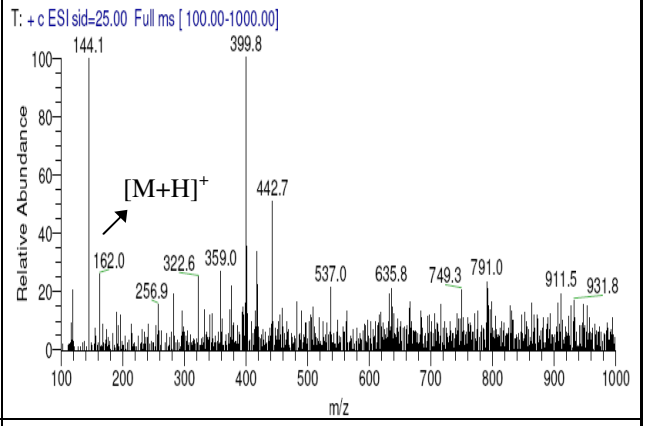
a) Sekita, 1983.
b) Derived from HMBC spectra.

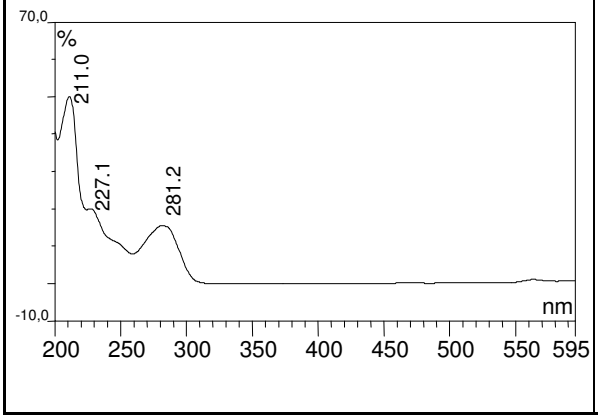
3.4.4. Indole-3-carboxylic acid (37, known compound)

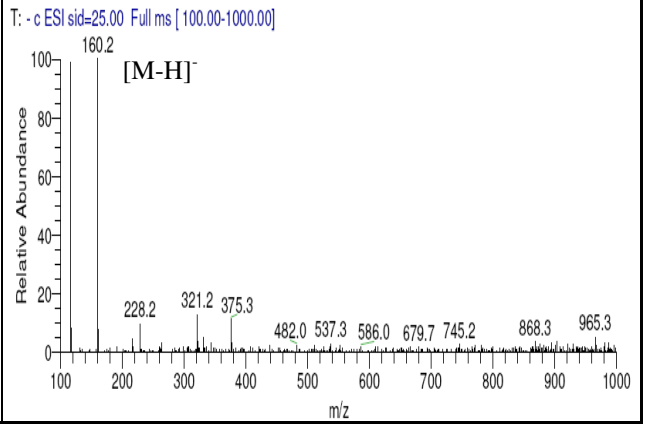
Indole-3-carboxylic acid	
Synonym(s)	1*H*-indole-3-carboxylic acid
Sample code	ChE6
Biological source	*Chaetomium* sp. (from *Otanthus maritimus*)
Sample amount	20.1 mg
Physical Description	brown needles
Molecular Formula	$C_9H_7NO_2$
Molecular Weight	161 g/mol
Retention time HPLC	18.4 min (standard gradient)



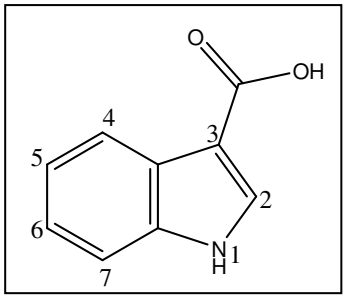








Indole-3-carboxylic acid (**37**) was isolated from the EtOAc extracts of liquid cultures of *Chaetomium* sp. as brown needles (20.1 mg). It had UV absorbances at λ_{max} (MeOH) 211.0, 227.1 and 281.2 nm, indicative of an indole chromophore. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 162.0 $[M+H]^+$ and *m/z* 160.2 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 161 g/mol and suggesting the molecular formula $C_9H_7NO_2$. The presence of a fragment at *m/z* 116.0 $[M-CO_2-H]^-$ in the negative ESI-MS indicated that the compound contained a carboxylic acid function. The ^{1}H NMR spectrum (see Table 3.40) showed 7 proton resonances that included an aromatic ABCD spin system at δ_H 8.06 (ddd, *J*=6.9, 2.2, 0.6 Hz, H-4), 7.42 (ddd, *J*=6.9, 1.2, 0.6 Hz, H-7), 7.18 (dt, *J*=6.9, 2.2 Hz, H-6), 7.15 (dt, *J*=6.9, 1.2 Hz, H-5), as well as a proton resonance at δ_H 7.93 (s, H-2). The obtained UV, ^{1}H NMR and mass spectral data were identical to those reported for indole-3-carboxylic acid (Aldrich, 1992; Hiort, 2002).



37 Indole-3-carboxylic acid

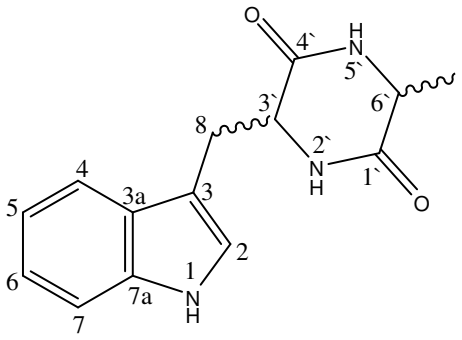
Table 3.40: ^{1}H NMR data of compound **37** at 500 MHz

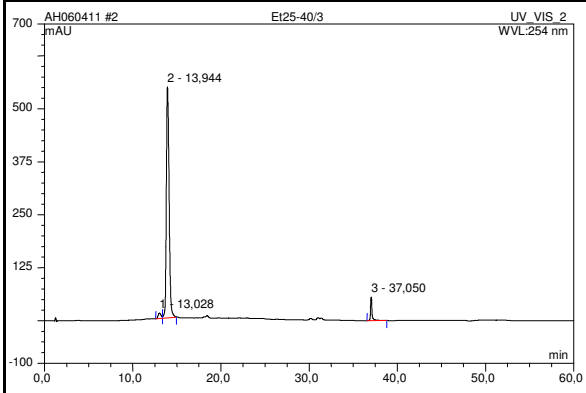
Nr.	**37**	**37**[a]
	δ_H (MeOD)	δ_H (DMSO-d_6)
1		11.43, s
2	7.93, s	7.70, s
4	8.06, ddd (6.9, 2.2, 0.6)	8.14, d (7.6)
5	7.15, dt (6.9, 1.2)	7.02, dt (6.9, 1.3)
6	7.18, dt (6.9, 2.2)	7.05, dt (6.9, 1.3)
7	7.42, ddd (6.9, 1.2, 0.6)	7.35, d (7.6)

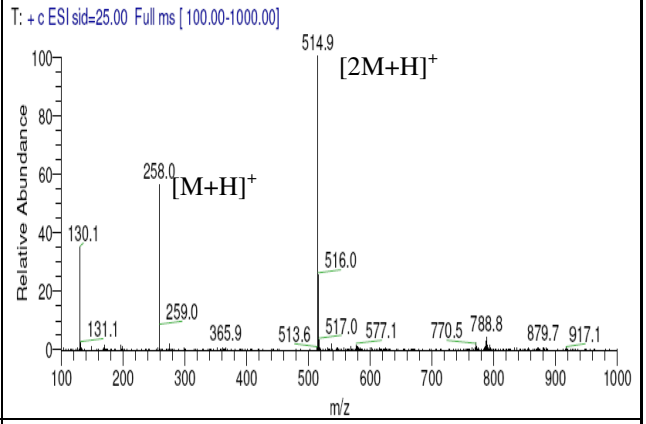
a) Hiort, 2002.

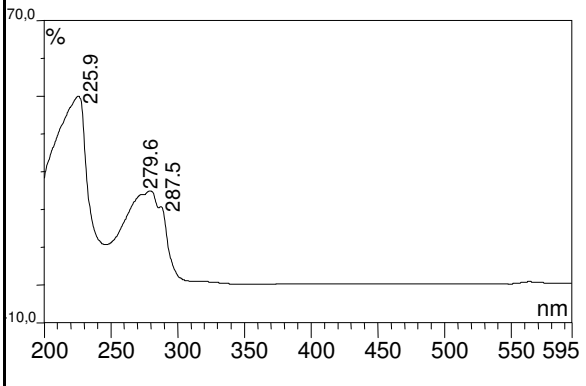
3.4.6. Cyclo(alanyltryptophane) (38, known compound)

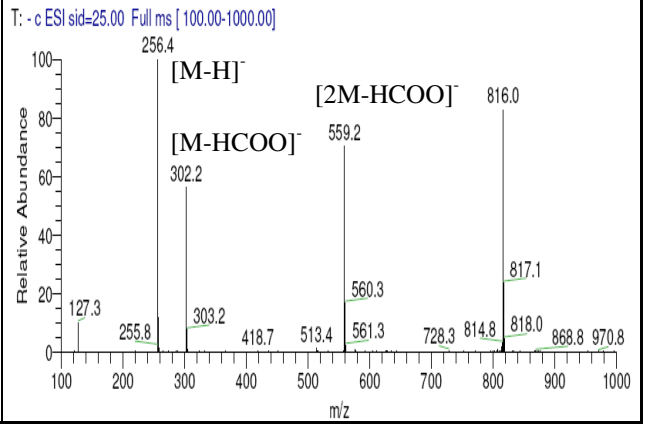
Cyclo(alanyltryptophane)	
Synonym(s)	3-(1*H*-Indol-3-ylmethyl)-6-methyl-2,5-diketopiperazine
Sample code	Ch2.1
Biological source	*Chaetomium* sp. (from *Otanthus maritimus*)
Sample amount	5.4 mg
Physical Description	white crystals
Molecular Formula	$C_{14}H_{15}N_3O_2$
Molecular Weight	257 g/mol
Retention time HPLC	13.9 min (standard gradient)



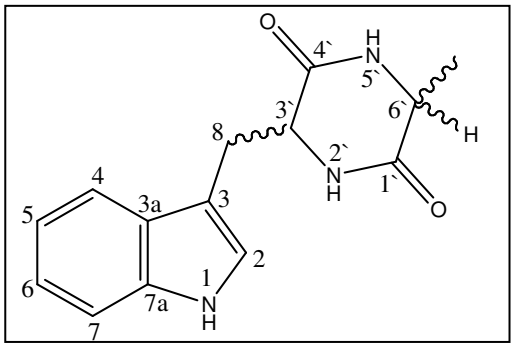








Cyclo(alanyltryptophane) (**38**) was isolated from the EtOAc extracts of liquid cultures of *Chaetomium* sp. as white crystals (5.4 mg). It showed UV absorbances at λ_{max} (MeOH) 225.9, 279.6 and 287.5 nm, indicative of an indole chromophore. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 258.0 $[M+H]^+$ and *m/z* 256.4 $[M-H]^-$ (base peak), respectively, revealing a molecular weight of 257 g/mol and suggesting the molecular formula $C_{14}H_{15}N_3O_2$. The ^{1}H NMR spectrum showed the characteristic 3-substituted indole signals, as also observed in the spectrum of **37** (see Tables 3.40 and 3.41), namely the aromatic ABCD spin system appearing at δ_H 7.55 (br d, *J*=7.7 Hz, H-4), 7.29 (br d, *J*=7.7 Hz, H-7), 7.01 (br t, *J*=7.7 Hz, H-6), 6.92 (br t, *J*=7.7 Hz, H-5), as well as two proton singlets at δ_H 10.90 and 7.02, assigned to NH-1 and H-2, respectively. Additionally, the protons of an aliphatic methylene group (C*H*$_2$-8) were observed at δ_H 3.22 (*J*=14.3, 3.9 Hz) and 2.99 (*J*=14.3, 4.5 Hz). In the COSY spectrum (see Table 3.41), this methylene group was found to be part of a saturated spin system coupling to a signal at δ_H 4.09 (br s, H-3`) and a signal at $\delta_H$ 8.02 (br s, NH-2`). Interpretation of the HMBC spectrum (see Table 3.41) showed that C*H*$_2$-8 correlated to C-3, C-3a as well as C-3`, confirming the 3-substituted indole structure. Moreover, both C*H*$_2$-8 and H-3` showed HMBC correlations to an oxygenated carbon signal at δ_C 166.5 (C-4`), thereby establishing the C3`-C4` bond and the tryptophane substructure. Furthermore, the COSY spectrum showed another saturated spin system composed of an aliphatic methyl group detected at $\delta_H$ 0.38 (d, *J*=6.8 Hz), a broad quartet at $\delta_H$ 3.57 (*J*=6.8 Hz, H-6`) and a signal at δ_H 7.90 (brs, NH-5`). All the latter proton signals showed HMBC correlations to an oxygenated carbon atom appearing at $\delta_C$ 167.8 (C-1`), thus confirming an alanine substructure. The HMBC correlation between NH-5` and C-3` as well as the pronounced upfield shift of the methyl group indicated that both amino acids were arranged into a cyclic dipeptide or diketopiperazine. The obtained UV, ^{1}H, ^{13}C NMR and mass spectral data were identical to published data for cyclo(alanyltryptophane) (Hiort, 2002) previously isolated from *Aspergillus* sp. (Marchelli *et al.*, 1975). Since all possible stereoisomers of this simple metabolite have already been found in nature, and moreover its origin as a true natural product, rather than an artifact resulting for example from the amino acid-containing culture medium and formed upon autoclaving, is debatable, no efforts were undertaken to elucidate the absolute stereochemistry of **38**.



38 Cyclo(alanyltryptophane)

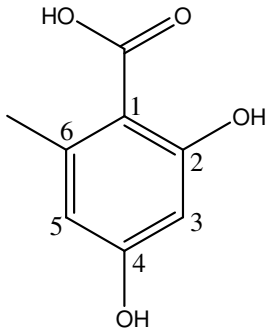
Table 3.41: ^{1}H, ^{13}C NMR, COSY and HMBC data of compound **38** at 500 (^{1}H) and 125 MHz (^{13}C)

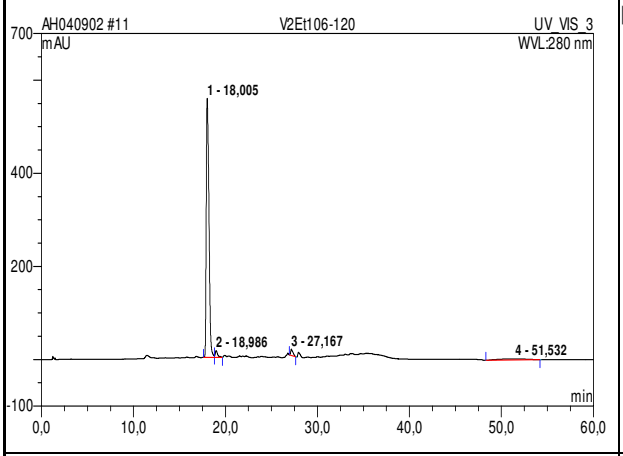
Nr.	**38**				**38**[a]	
	δ_H (DMSO-d_6)	COSY	HMBC	δ_C (DMSO-d_6)[b]	δ_H (DMSO-d_6)	δ_C (DMSO-d_6)
1	10.90, s	2,4	2,3,3a,7a		10.88, s	
2	7.02, d (0.6)	1,8A,8B	3,3a,7a	124.5	7.05, m	124.7
3				108.1		108.3
3a				127.5		127.7
4	7.55, br d (7.7)	1,5	3,6,7a	118.6	7.55, d (7.6)	118.9
5	6.92, br t (7.7)	4,6	3a,7	118.0	6.90, m	118.2
6	7.01, br t (7.7)	5,7	4,7a	120.4	7.05, m	121.1
7	7.29, br d (7.7)	6	3a,5	110.9	7.29, d (8.1)	110.7
7a				135.5		135.9
8	A 3.22, dd (14.3, 3.9)	2,8B,3`	3,3a,3`		3.23, dd (14.5, 4.1)	28.7
	B 2.99, dd (14.3, 4.5)	2,8A,3`	2,3,3a,3`,4`		3.00, dd (14.5, 4.4)	
1`				167.8		168.0
2`	8.02, brs	3`	4`,6`		7.99, d (1.3)	
3`	4.09, brs	8A,8B,2`,6`	4`	55.4	4.09, brs	55.3
4`				166.5		166.7
5`	7.90, brs	6`	1`,3`		7.89, d (1.3)	
6`	3.57, brq (6.8)	CH_3,3`,5`	CH_3,1`	49.5	3.58, brq (6.9)	49.3
CH_3	0.38, d (6.8)	6`	1`,6`	19.0	0.41, d (6.9)	18.8

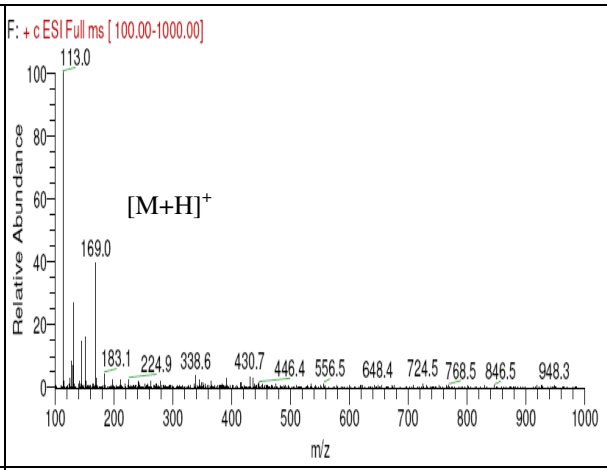
a) Hiort, 2002.
b) Derived from HMBC spectrum.

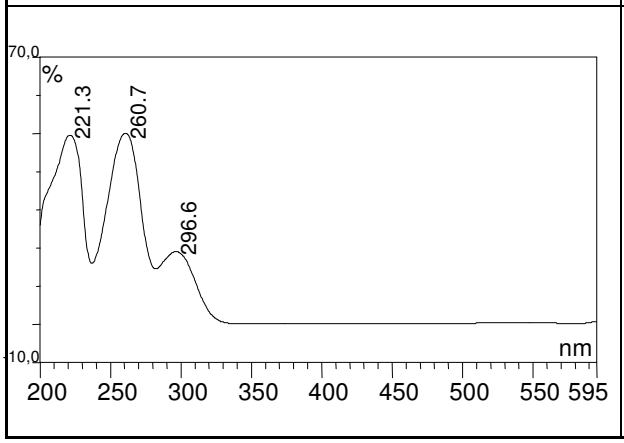
3.4.7. Orsellinic acid (39, known compound)

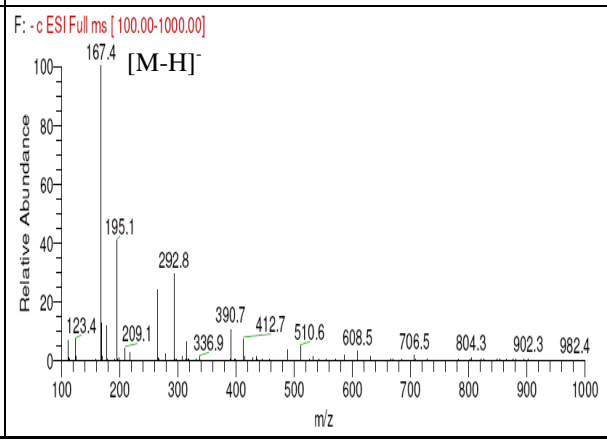
Orsellinic acid	
Synonym(s)	2,4-Dihydroxy-6-methylbenzoic acid
Sample code	ChE4
Biological source	*Chaetomium* sp. (from *Otanthus maritimus*)
Sample amount	49.6 mg
Physical Description	brown needles
Molecular Formula	$C_8H_8O_4$
Molecular Weight	168 g/mol
Retention time HPLC	18.0 min (standard gradient)



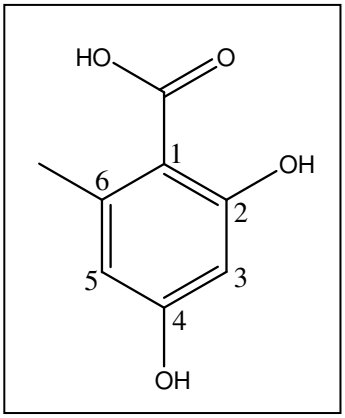








Orsellinic acid (**39**) was isolated from the EtOAc extracts of liquid cultures of *Chaetomium* sp. as brown needles (49.6 mg). It displayed UV absorbances at λ_{max} (MeOH) 221.3, 260.7 and 296.6 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 169.0 $[M+H]^+$ (base peak) and *m/z* 167.4 $[M-H]^-$ (base peak), respectively, revealing a molecular weight of 168 g/mol. ^1H and ^{13}C NMR spectra (see Table 3.42) indicated the presence of an aromatic methyl group at δ_H 2.38 and δ_C 23.4 as well as a pair of *meta*-coupled protons at δ_H 6.16 and 6.11 (each d, *J*=2.2 Hz) assigned to H-3 and H-5, respectively. Additionally, the ^{13}C NMR spectrum (see Table 3.42) contained 6 aromatic carbon signals, two of which were hydroxylated, and a signal at δ_C 173.2 indicative of an aromatic carboxylic acid function. This was further confirmed by a fragment at *m/z* 123.0 $[M-CO_2-H]^-$ in the negative ESI-MS. The ^{13}C, ^1H NMR and mass spectra supported a molecular formula of $C_8H_8O_4$. The obtained UV, ^1H NMR and mass spectral data were found to be almost identical to published data for orsellinic acid (Evans and Staunton, 1988). Orsellinic acid was previously isolated from lichens (Maass, 1975) as well as cultures of *Penicillium* sp. and other microorganisms (Birkinshaw and Gowlland, 1962).



39 Orsellinic acid

Table 3.42: ^1H and ^{13}C NMR data of compound **39** at 500 (^1H) and 125 MHz (^{13}C)

Nr.	39		39[a]
	δ_H (DMSO-d_6)	δ_C (DMSO-d_6)	δ_H (MeOD)
1		104.8	
2		161.9	
3	6.11, d (2.2 Hz)	100.4	6.20, br s
4		164.4	
5	6.16, d (2.2 Hz)	110.9	6.20, br s
6		142.8	
CH_3	2.38, s	23.4	2.54, s
COOH		173.2	
2-OH	12.63, br s		
4-OH	10.13, br s		

a) Evans and Staunton, 1988.

3.4.8. Bioactivity test results for compounds isolated from the endophytic fungus *Chaetomium* sp.

The isolated compounds were subjected to bioassays aimed to determine their cytotoxicity and their protein kinase inhibitory profiles. The results are shown in tables 3.43 and 3.44.

Table 3.43: Cytotoxicity test results for the compounds isolated from *Chaetomium* sp. rice extracts

Nr.	Compound tested	L5178Y growth in %* (Conc. 10 µg/mL)	EC_{50}* (µg/mL)	EC_{50} (µmol/L)
34	Aureonitolic acid	79.6		
35	Cochliodinol	- 2.3	7.0	14.0
36	Isocochliodinol	75.8		
37	Indol-3-carboxylic acid	93.7		
38	Cyclo(alanyl-tryptophane)	104.7		
39	Orsellinic acid	1.0	2.7	16.0

* Data provided by Prof. W. E. G. Müller, Mainz.

Orsellinic acid and cochliodinol proved to be highly active against the L5178Y cancer cell line, whereas a weak activity was observed for isocochliodinol and aureonitolic acid.

Table 3.44: Protein kinase assay results for the compounds isolated from *Chaetomium* sp. liquid extract

Compound tested (Conc. 1µg/mL)	Activity toward selected protein kinases*																											
	EGF-R	EPHB4	ERBB2	FAK	IGF1-R	SRC	VEGF-R2	VEGF-R3	AKT1	ARK5	Aurora-A	Aurora-B	PAK4	PDK1	CDK2/CycA	CDK4/CycD1	CK2-alpha1	FLT3	INS-R	MET	PDGFR-beta	PLK1	SAK	TIE2	COT	B-RAF-VE
Aureonitolic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cochliodinol	A	M	M	0	A	A	A	M	0	A	M	A	0	0	M	M	0	A	A	A	M	M	A	A	A	A
Isocochliodinol	A	M	M	0	A	A	A	M	0	0	M	M	0	0	M	M	0	M	M	M	M	M	M	M	M	M
Orsellinic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

S: strongly active, A: active, M: moderately active, 0: not active

* Data provided by ProQinase, Freiburg.

The results of the protein kinase assay showed a different activity profile in comparison to the cytotoxicity assay. Cochliodinol and isocochliodinol were active on various protein kinases, while aureonitolic acid and orsellinic acid were inactive for all tested protein kinases, in spite of the high activity of orsellinic acid in the cytotoxicity assay.

3.5. Tracing of fungal metabilites in the corresponding plant extracts

With the different compounds isolated from endophytic fungal strains in the course of this thesis at hand, extracts of their respective host plants were screened to detect the presence of these metabolites. To this aim, crude plant extracts were prepared and divided into subfractions by liquid-liquid partitioning and subsequent fractionation over Diaion HP-20 using H_2O:MeOH and MeOH:acetone gradient elution. Each of these fractions were then analyzed by LC-MS, with specifically preparing mass chromatograms obtained at the respective base or other characteristic intense peaks for the individual isolated fungal metabolites. If matching mass spectra were suspected, co-elution studies with the corresponding metabolite and the respective plant fraction were carried out to compare the corresponding retention times.

3.5.1. Tracing of *Alternaria* metabolites in *Polygonum senegalense* fractions

All of the compounds isolated from *Alternaria* sp. were detected in the crude extract of this fungus. Most remarkably, alternariol, alternariol-5-*O*-methyl ether and altenusin were also traced in subfractions of the *Polygonum senegalense* extract (see Table 3.45 and Figures 3.29-31), the host plant from which the fungal strain was originally obtained.

Table 3.45: Compounds detected in *P. senegalense* fractions

Compound	Fraction*	Polarity of eluting solvent
Altenusin	2	50% MeOH:H_2O
Alternariol	3	75% MeOH:H_2O
Alternariol monomethyl ether	4	100% MeOH

* The 90% MeOH fraction was fractionated over Diaion HP-20 using H_2O:MeOH and MeOH:acetone gradient elution.

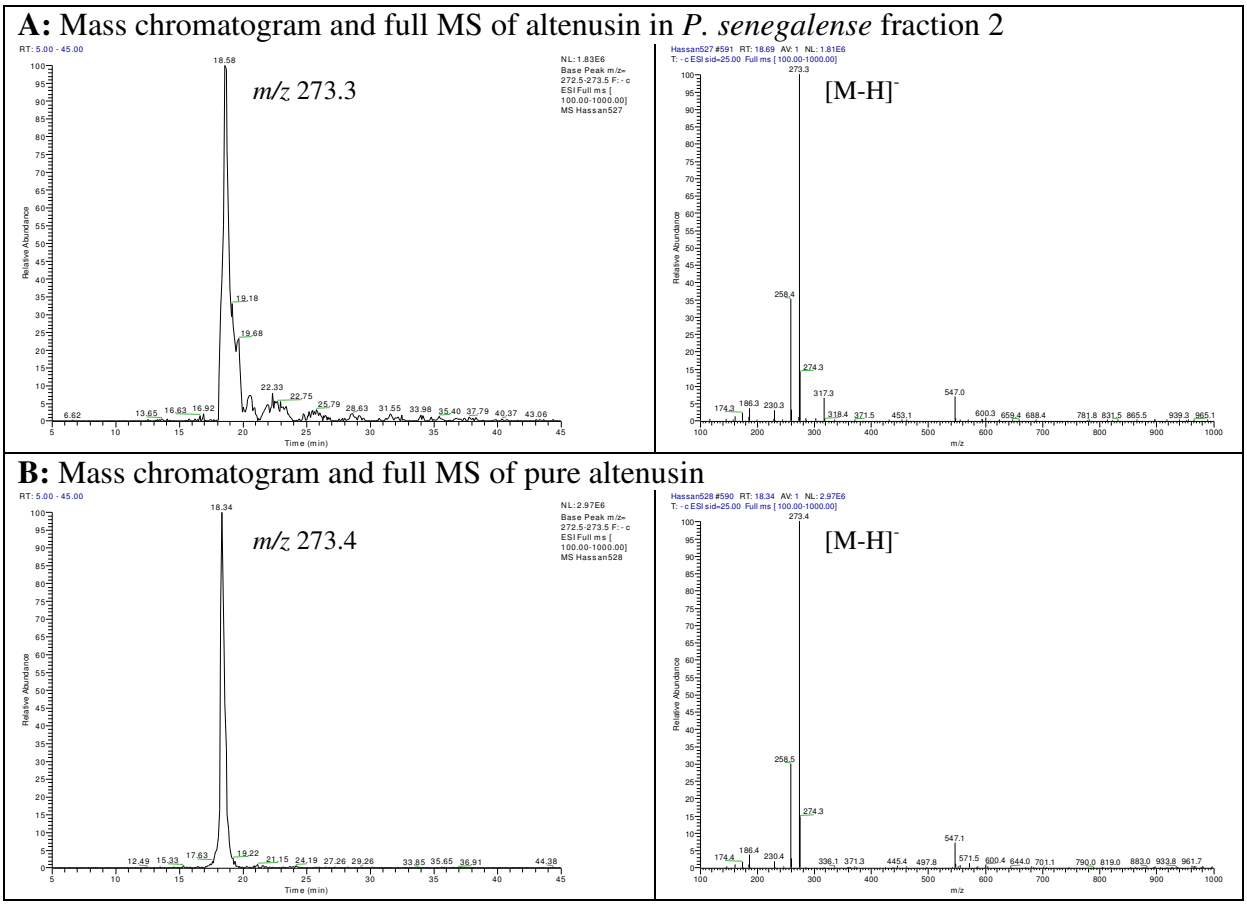
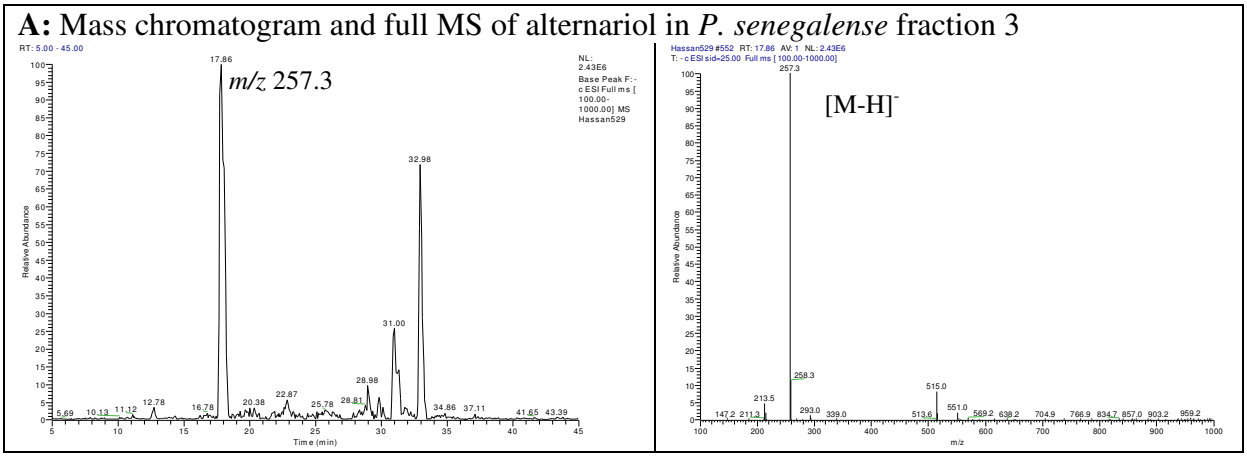


Figure 3.29: MS detection of altenusin (**A:** in *P. senegalense* fraction 2, **B:** pure compound).



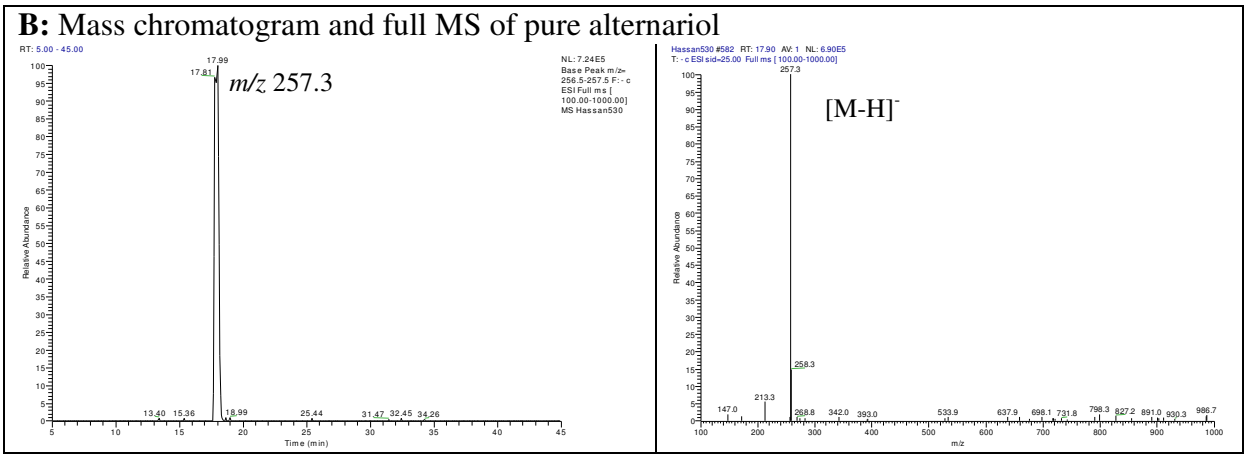


Figure 3.30: MS detection of alternariol (**A:** in *P. senegalense* fraction 3, **B:** pure compound).

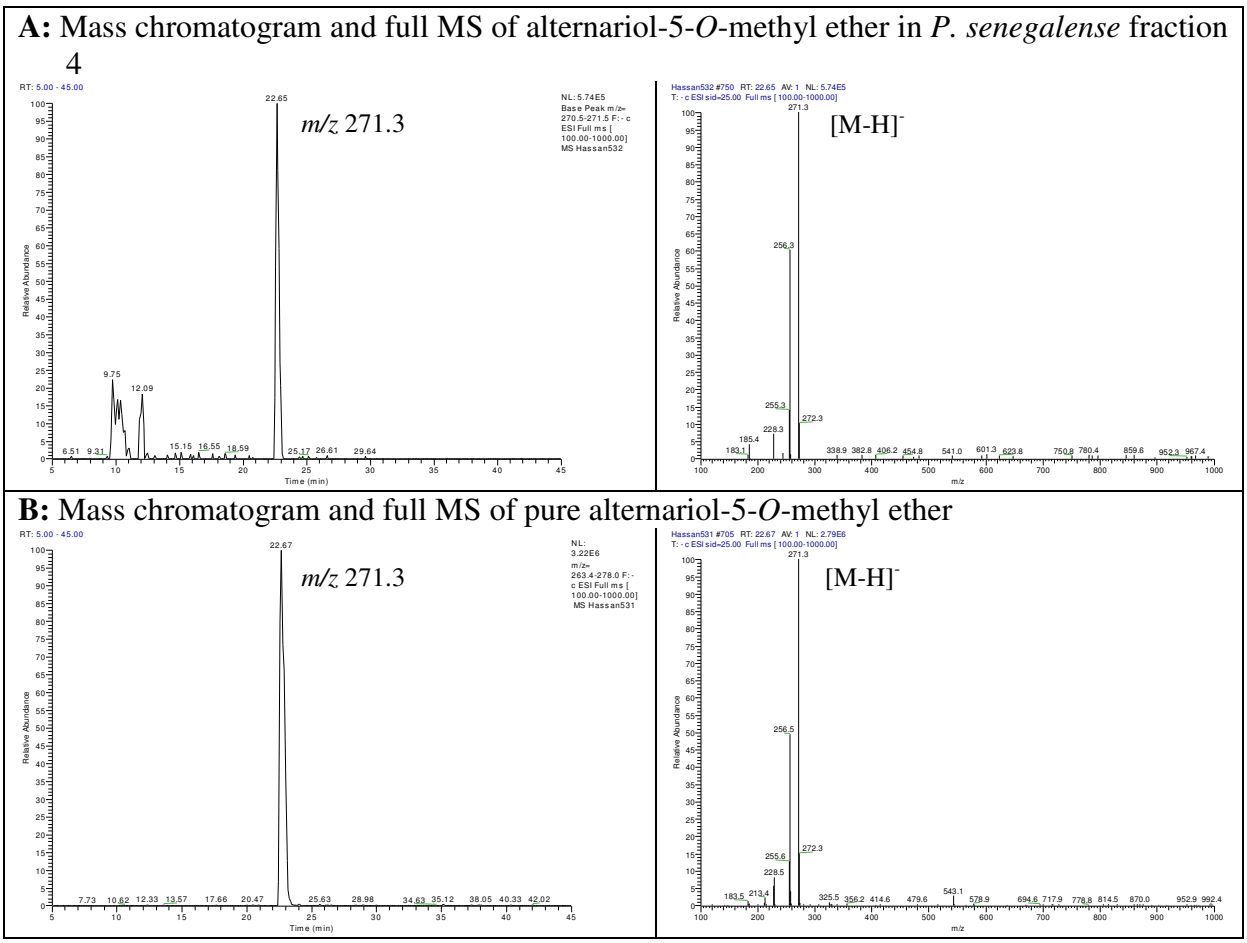


Figure 3.31: MS detection of alternariol-5-*O*-methyl ether (**A:** in *P. senegalense* fraction 4, **B:** pure compound).

3.5.2. Tracing of *Ampelomyces* metabolites in *Urospermum picroides* fractions

All of the compounds isolated from *Ampelomyces* sp. were detected in the crude extract of this fungus. Most remarkably, macrosporin and 3-*O*-methylalaternin were also traced in subfraction 5 (eluted by 100% acetone) of the *Urospermum picroides* extract (see Figures 3.32-33), the host plant from which the fungal strain was originally obtained.

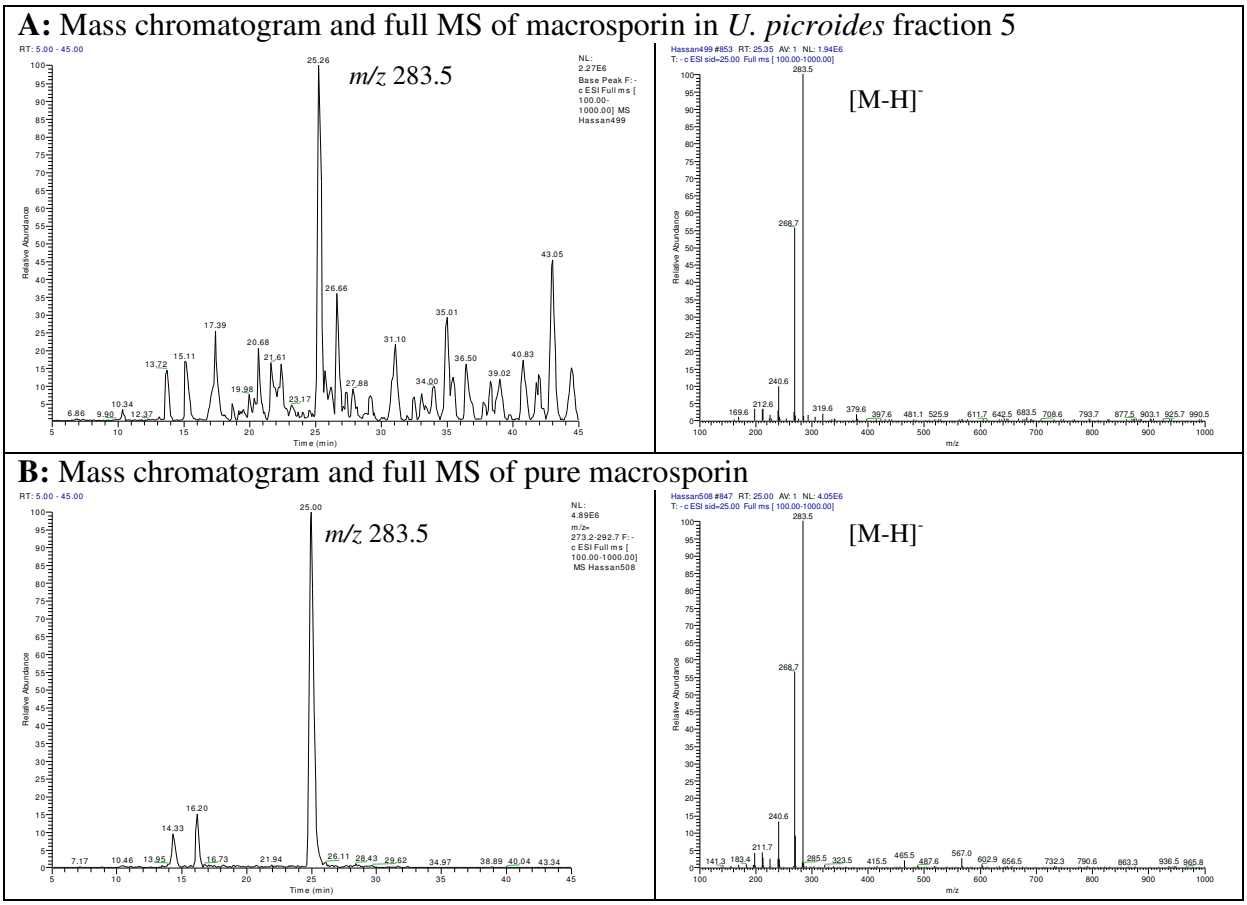


Figure 3.32: MS detection of macrosporin (**A:** in *U. picroides* fraction 5, **B:** pure compound).

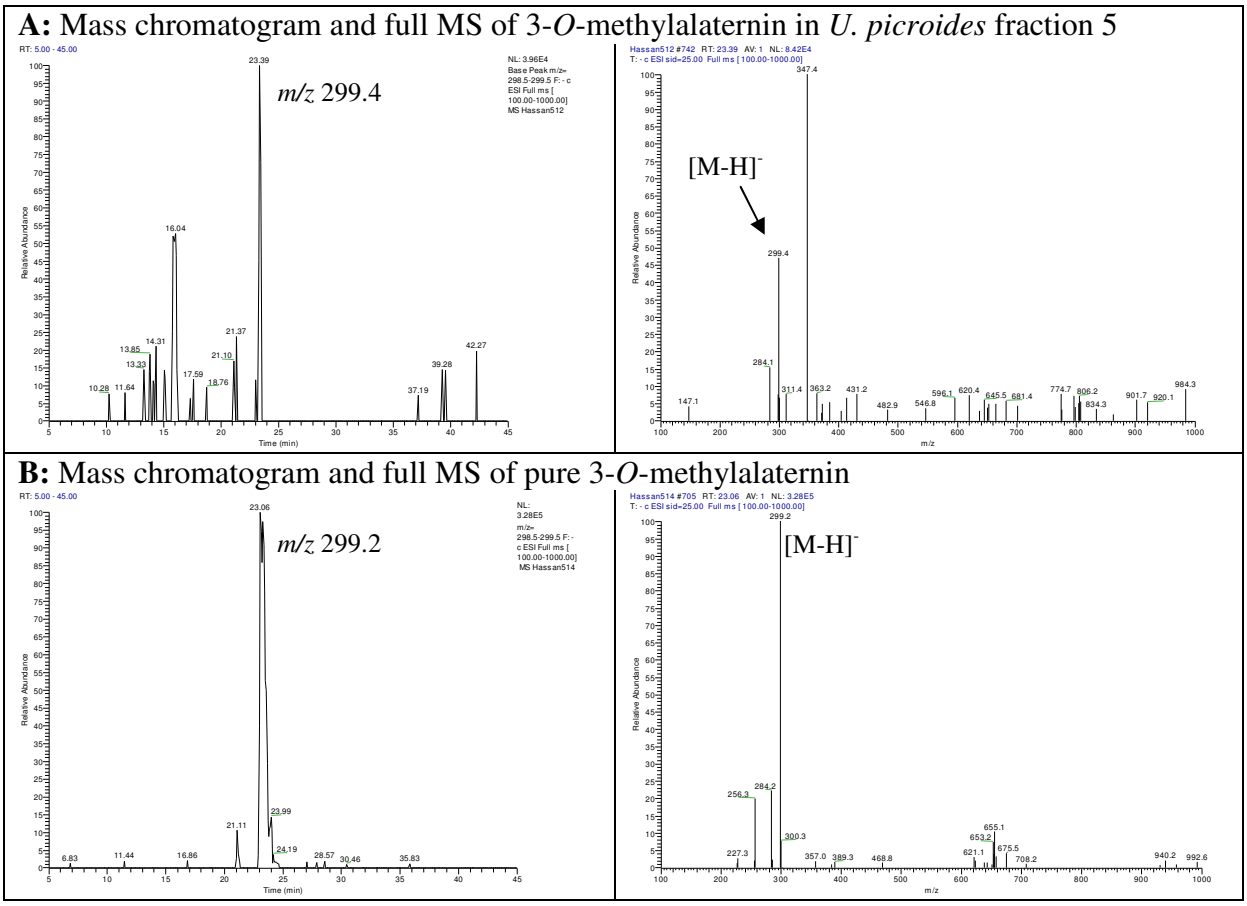


Figure 3.33: MS detection of 3-*O*-methylalaternin (**A:** in *U. picroides* fraction 5, **B:** pure compound).

3.5.3. Tracing of *Stemphylium botryosum* metabolites in *Chenopodium album* fractions

All of the compounds isolated from *Stemphylium botryosum* were detected in the crude extract of this fungus. Most remarkably, tetrahydroaltersolanol B, curvularin, stemphyperylenol and macrosporin were also traced in subfractions of the *Chenopodium album* extract (see Table 3.46 and Figures 3.34-37), the host plant from which the fungal strain was originally obtained.

Table 3.46: Compounds detected in *C. album* fractions

Compound	Fraction*	Polarity of eluting solvent
Tetrahydroaltersolanol B	2	50% MeOH:H_2O
Curvularin	4	100% MeOH
Stemphyperylenol	4	100% MeOH
Macrosporin	5	100% Acetone

* The 90% MeOH fraction was fractionated over Diaion HP-20 using H_2O:MeOH and MeOH:acetone gradient elution.

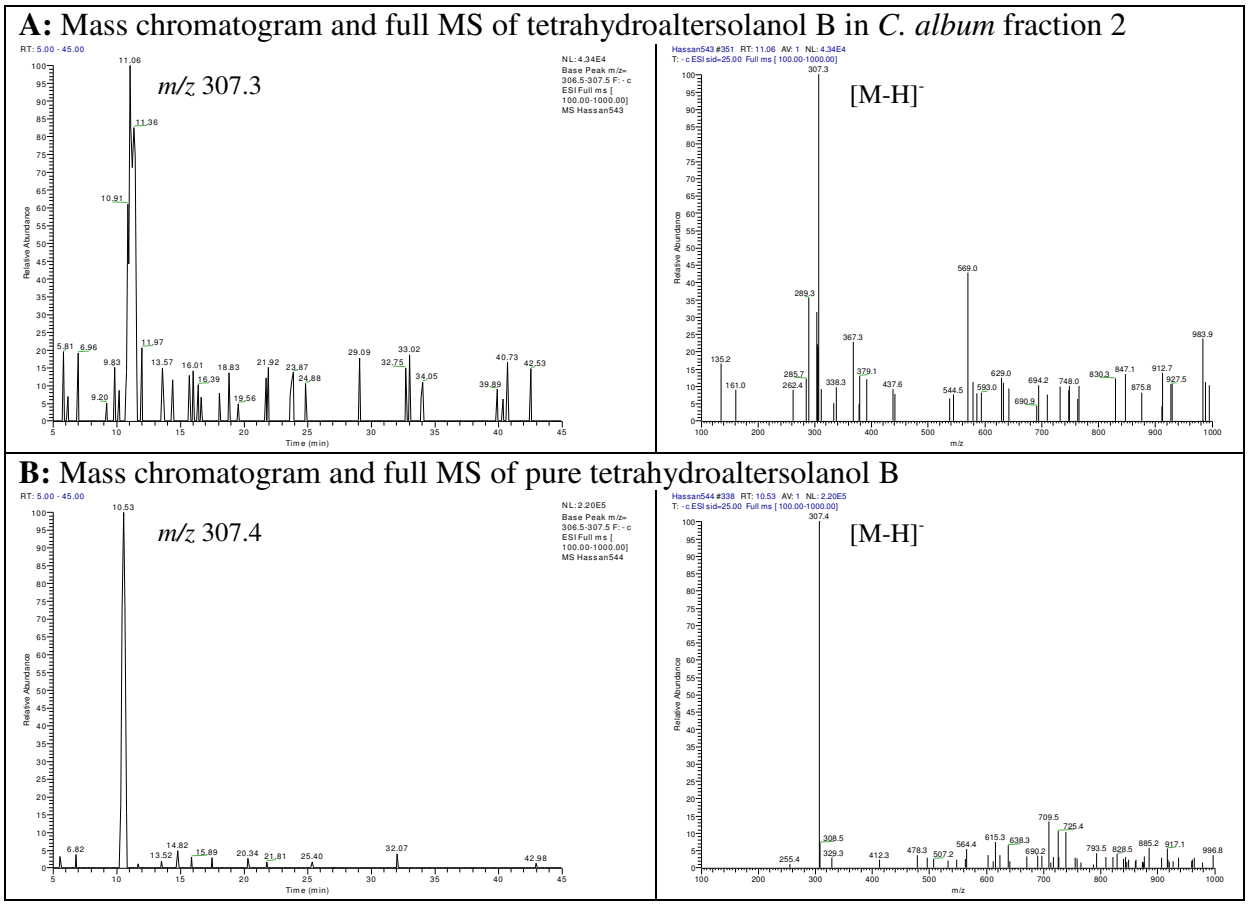
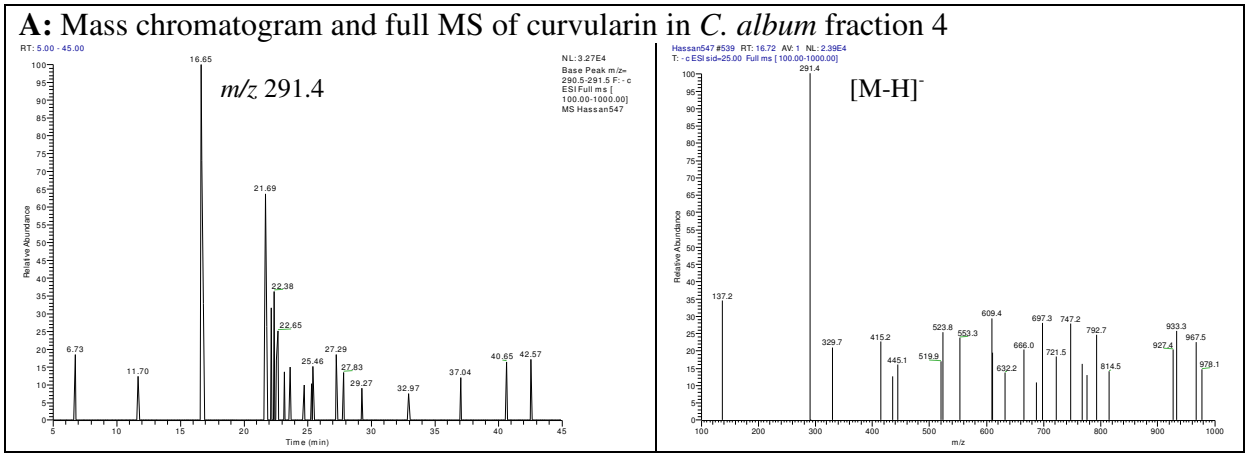


Figure 3.34: MS detection of tetrahydroaltersolanol B (**A:** in *C. album* fraction 2, **B:** pure compound).



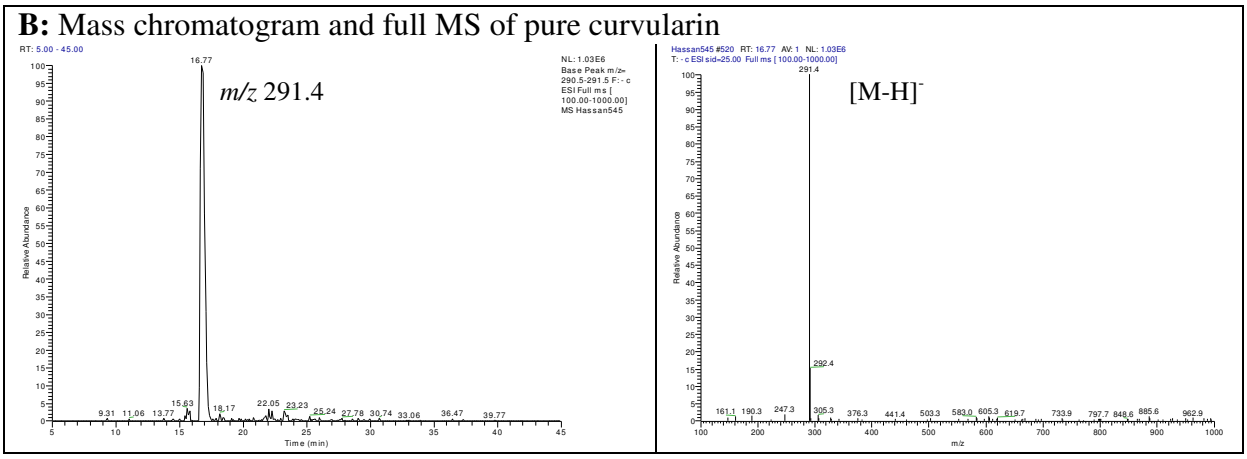


Figure 3.35: MS detection of curvularin (**A:** in *C. album* fraction 4, **B:** pure compound).

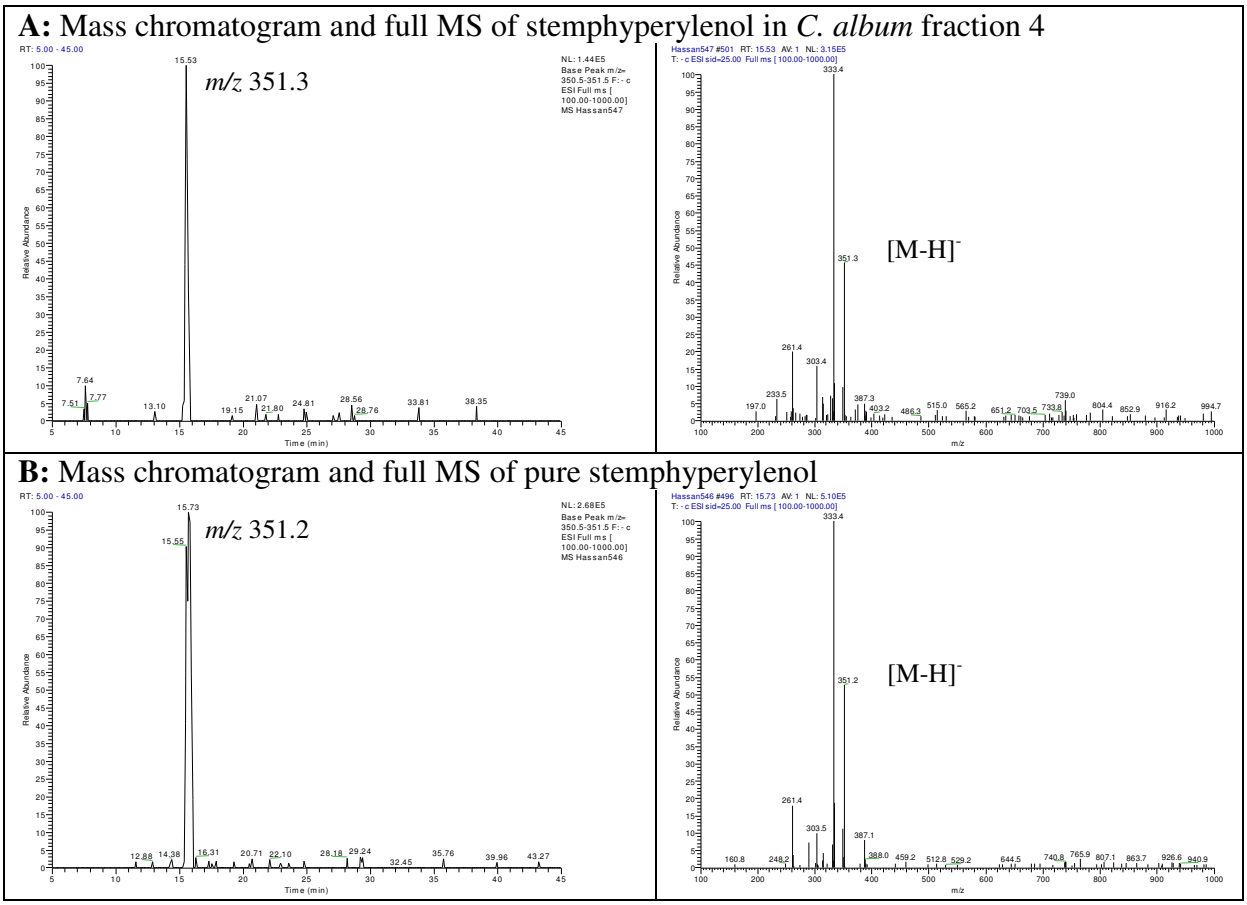


Figure 3.36: MS detection of stemphyperylenol (**A:** in *C. album* fraction 4, **B:** pure compound).

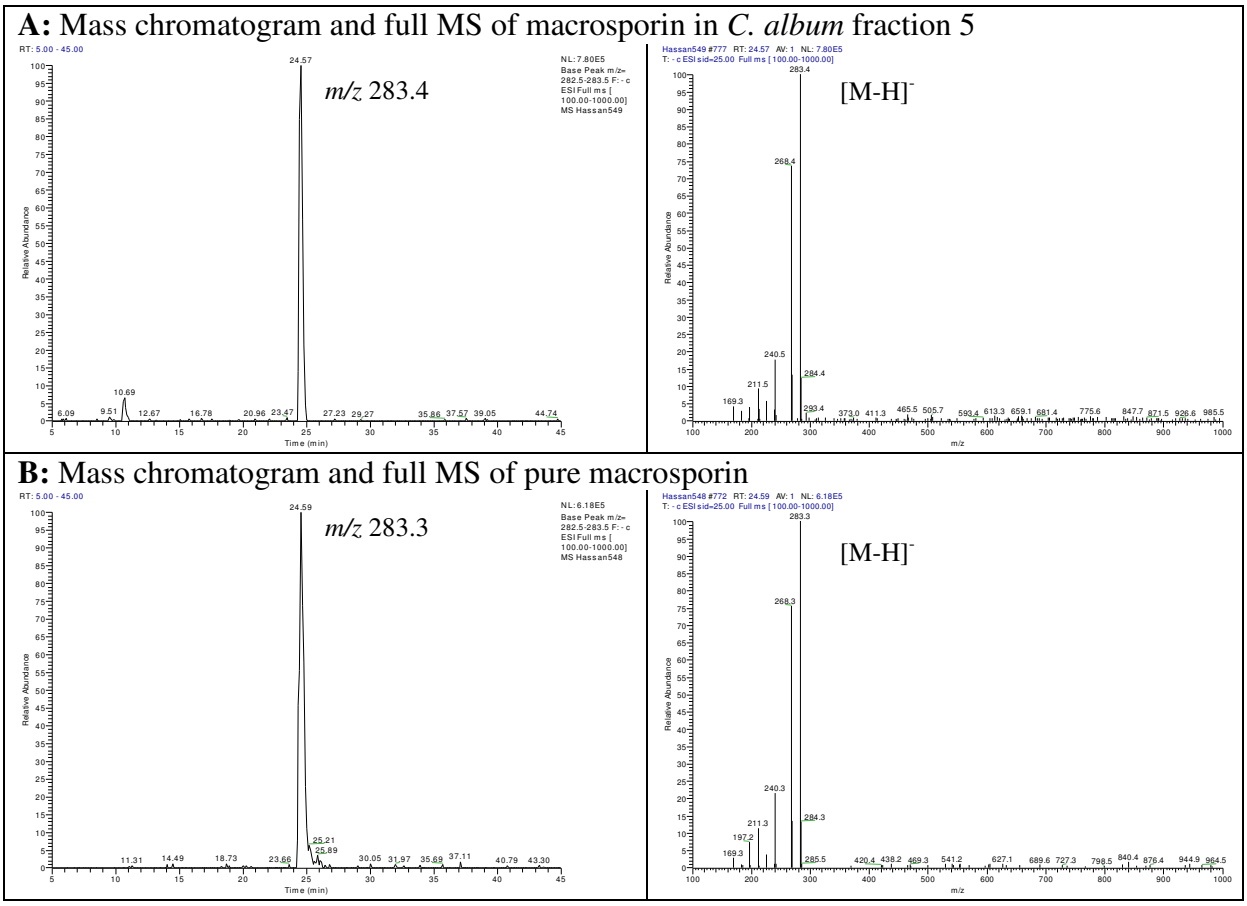


Figure 3.37: MS detection of macrosporin (**A:** in *C. album* fraction 5, **B:** pure compound).

3.5.4. Tracing of *Chaetomium* sp. metabolites in *Otanthus maritimus* fractions

In a manner completely analogous to the procedures described above for *Polygonum senegalense*, *Urospermum picroides* and *Chenopodium album*, similar investigations were also carried out with extracts of *Otanthus maritimus*, from which the endophytic fungus *Chaetomium* sp. was isolated. For these studies, intense MS peaks for cochliodinol, isocochliodinol, orsellinic acid and aureonitolic acid were used. However, it was not possible to detect any of these secondary metabolites in any of the host-pant-derived fractions, even though all of them were detectable in the crude extract of the fungus.

4. Discussion

4.1. Choice of culture media

The physiology of secondary metabolism has often been neglected and still few of the regulatory features involved in the biosynthesis of natural products have been elucidated. One of the factors having great impact on growth and production of secondary metabolites from microorganisms is medium composition and culture conditions (Bills, 1995). Thus it may be necessary to use several media and growth conditions when strains are to be investigated for their full metabolic potential in order to generate conditions that will allow the expression of as broad a range of secondary metabolites as possible for a given strain to increase the chance to generate novel drug candidates (Larsen *et al.*, 2005). Furthermore, some natural products are only produced under certain environmental conditions and if all trace metals, phosphate and other medium factors are present in certain ranges of concentrations (Knight *et al.*, 2003). Thus, optimal media for good metabolite production can change for different genera being investigated (Larsen *et al.*, 2005).

Different and relatively easy to control conditions to investigate in a discovery programme include growing cultures at both solid and liquid conditions, incubation at two or more temperatures, incubation at two or more shaker speeds, incubation for at least two different time periods, media with at least two different pH levels, choosing carbon and nitrogen sources at different concentrations, high- or low phosphate content, adding trace minerals *etc.* (Knight *et al.*, 2003).

Some authors strongly argue in favour of using solid substrate fermentations in studies of fungal metabolites since fungi, unlike other microorganisms, typically grow in nature on solid substrates such as wood, roots and leaves of plants (Nielsen *et al.*, 2004). On the other hand, some believe and argue that all metabolites can be expressed in liquid culture by varying carbohydrate composition, nitrogen source, oxygen tension, pH, redox potential, water activity, as the right conditions will produce intracellular conditions that will trigger production of a certain metabolite. It was found that metabolites associated to spore or sclerotia formation are often produced under solid conditions, whereas the production of other metabolites is enhanced under liquid conditions (Larsen *et al.*, 2005, Nielsen *et al.*, 2004).

Of course, in a screening effort aimed at the discovery of novel natural products which includes a variety of different strains, one has to focus on a limited set of parameters, since otherwise the sheer number of extracts generated will easily be overwhelming. Thus, in this study chosen fungal strains were cultured in liquid (Wickerham) medium as well as on solid

rice medium. Bioactivity and chemical profiles of the obtained extracts from both cultures were compared and subjected to further investigation. HPLC chromatograms of the EtOAc extracts of liquid and rice cultures showed different chemical patterns for all the fungal strains investigated in this study. Moreover, EtOAc extracts of liquid and rice cultures showed different antimicrobial and cytotoxic activities in preliminary biological screening tests which was in accordance with the different chemical picture. It was also observed as a general trait that the yield of dry extract obtained from rice cultures was higher than that of liquid cultures with varying rations in all investigated fungal strains. However, it cannot be excluded that this finding, at least to a certain degree, was due to the fact that more polar material, e.g. sugars or amino acids, were extracted from the culture medium in the case of the solid rice cultures compared to the liquid medium

4.2. Strategies and methodologies for metabolite profiling

In order for natural product chemistry to continue to be competitive with purely synthetic based discovery methods, natural product research needs to continually improve the efficiency of the selection, screening, dereplication, isolation and structure elucidation processes (Butler, 2004). Hence, talented microbial strains can be selected to be included in screening programs, which together with the use of spectroscopic methods in combination with chemoinformatics can be used as part of an effective dereplication strategy (Larsen *et al.*, 2005). In fact the chemical diversity and the resources of natural products are immense and nowhere near fully exploited. Fungi are known to produce species specific profiles of natural products which can be used as efficient tools to select some representative strains for biological testing. Thus, extracts for chemical fractionation were selected based on the biological activity and chemical profiles of the crude extract. Metabolite profiling is not an easy task to perform since natural products display a very high structural diversity. Consequently a single analytical technique does not exist, which is capable of profiling all secondary metabolites in the biological source (Wolfender *et al.*, 2005). However, advanced analytical and spectroscopic methods, like the hyphenated techniques coupled to HPLC, can give a good idea about the different substructures and/or functional groups of the structure.

4.2.1. HPLC/UV

With the advancement of HPLC as well as much more stable and better columns for high resolution separation, combined with fast UV diode array detectors it has become easy to acquire the UV spectrum of practically every single component from an extract, provided a

suitable chromophore. Consequently the UV spectrum has turned into one of the most readily accessible pieces of information related to structure of natural products which increased the interest in exploiting its usefulness (Cannall, 1998).

In the present study, a lot of chemical compounds that share similar chromophoric functions were examined by the hyphenated technique HPLC/UV-photodiode array detection (LC/UVDAD) which showed very often this also translated into similar UV spectra, even though there were significant differences in terms of additional non-chromophoric functions or molecular weights (*e.g.* alternariol derivatives (**1**-**5**), altenusin and desmethyl altenusin (**6**-**7**), altenuene and 4`-epialtenuene (**11**-**12**), isocoumarin derivatives (**18**-**20**), ampelanol (**26**) and tetrahydroaltersolanol B (**30**) and the cochliodinol derivatives (**35**-**36**)).

4.2.2. HPLC/ESI-MS

With the arrival of electrospray ionization mass spectrometry (ESI-MS) and the associated techniques about 25 years ago the scientific community obtained a highly versatile tool for studies of natural products. ESI-MS has the advantage of being a soft and sensitive ionization technique which can be optimized to produce mainly protonated or sodiated ions (assuming positive ESI) from a very broad range of natural products (Smedsgaard and Frisvad, 1996). Moreover, modern LC-MS system allow to switch between positive and negative ionization very quickly, i.e. in the order of 1 second per spectrum, thus allowing to obtain complimentary information to securely identify the molecular weight of an analyte, or to obtain molecular weight information for compounds not ionizing upon positive, but only upon negative ionization. Hence, ESI-MS represents a rapid method to differentiate and estimate the presence of secondary metabolites in microbial extracts.

Furthermore, this method is also helpful in establishing the relation between closely related natural products. In the context of the present study, this proved extremely valuable in detecting the characteristic loss of 80 mass units from the new sulphated alternariol derivatives (**2** and **4**) and anthraquinone derivatives (**22** and **24**) indicating the presence of a sulphate group. It is difficult to come to a definite conclusion, but there is some degree of probability that at least some members of this series of sulphated compounds had simply overlooked in previous studies.

4.2.3. Dereplication and partial identification of natural products by UV-based techniques

Apart from exact structural formulae structural databases usually also contain physical and chemical data including UV maxima and minima characteristic of the included compounds. Moreover, modern HPLC-DAD systems allow to generate a library of UV spectra in a rather straightforward manner which in turn is extremely valuable for dereplication of compounds previously isolated. Many natural products such as polyketides and alkaloids derived from aromatic amino acids have characteristic UV spectra due to their polyunsaturated nature. In addition many such natural products often have one or more carbonyl groups as part of ketone, carboxylic acid, ester or amide functional groups. Thus, the UV based library established at the Institute of Pharmaceutical Biology at Düsseldorf in the last 10 years was extensively used for dereplication purposes to investigate isolated fungal strains for production of natural products but also as an approach to discover possible novel bioactive metabolites with structural features similar to that of already known bioactive compounds.

4.3. Isolation of natural products

Chromatographic techniques were used to isolate and purify the chemically most interesting substances. Ideally the structurally unusual or novel compounds are also responsible for the activity of the extract. The approach proved indeed efficient with respect to isolating numerous new compounds, many of which probably being responsible for most of the biological activity observed for the crude extract (*e.g.* alternariol and altenusin derivatives in *Alternaria* extracts, altersolanol A in *Ampelomyces* rice culture extract, curvularin derivatives in *Stemphylium* rice culture extract as well as cochliodinol and orsellinic acid in *Chaetomium* liquid culture extract).

4.4. Compounds isolated from purified fungal strains

4.4.1. Compounds isolated from the endophytic fungus *Alternaria* sp.

Alternaria sp. was isolated from leaves of *Polygonum senegalense* growing wild in Egypt. External application of an extract of the fresh leaves of this plant is reported in folk medicine to be highly effective in treating skin troubles. Species of *Polygonum* are known in traditional medicine for their diuretic, cholagic, antihemorragic and antiseptic actions

(Smolarz, 2002). In addition, it was found that crude aqueous methanol extract of *P. senegalense* exhibited molluscicidal activity (Dossaji and Kubo, 1980).

Alternaria species have a widespread distribution. Many species are plant pathogens, which cause both pre- and post-harvest decay. A number of metabolites of polyketide origin, including many *α*-dibenzopyrones such as alternariol and alternariol monomethyl ether have been isolated from species of *Alternaria* (Stinson *et al.*, 1986, Onocha *et al.*, 1995). Many of these metabolites were reported to be toxic to mammalian systems, nevertheless, our interest in the metabolites produced by *Alternaria* was stimulated by the recent report of the estrogenic potential of alternariol in cultured mammalian cells (Lehmann *et al.*, 2006).

Chemical investigation of the ethyl acetate extract of the fungus grown in liquid culture lead to the isolation of new sulphated derivatives of alternariol and its monomethyl ether (**2** and **4**) as well as the known compounds alternariol (**1**), alternariol-5-*O*-methyl ether (**3**), altenusin (**6**), 2,5-dimethyl-7-hydroxychromone (**13**), altertoxin I (**14**), tenuazonic acid (**15**) and adenosine. When grown on solid rice medium the fungus yielded four new compounds, identified as 3`-hydroxyalternariol-5-*O*-methyl ether (**5**), desmethylaltenusin (**7**), alterlactone (**8**) and alternaric acid (**10**) in addition to the known compounds **1**, **3** and **6**, talaroflavone (**9**) and altenuene (**11**). Furthermore, we isolated a new altenuene isomer that was given the trivial name 4`-epialtenuene (**12**).

4.4.1.1. Alternariol derivatives

4.4.1.1.1. Biosynthesis of alternariol derivatives

The biosynthesis of alternariol had been reported to involve assembling a heptaketide chain, folded as shown in Figure 4.1, aromatizing, cyclizing, and release of alternariol in a single step. The enzyme responsible for the production of alternariol is a fungal polyketide synthetase. The central theme of polyketide biosynthesis is that iterative decarboxylative Claisen condensations of malonyl thiolesters result in a growing carbon chain (Liu *et al.*, 1998). The folding of the polyketide chain was established by labeling studies, feeding $^{13}C_2$-labelled acetate to the appropriate organism and establishing the location of labeled intact C_2 units in the final product by ^{13}C NMR spectrometry. Whilst the precise sequence of reactions involved is not known, the essential features would include two aldol condensations followed by enolization in both rings to give a biphenyl, and lactonization would then lead to alternariol. The oxygenation pattern in alternariol shows alternate oxygens on both aromatic rings, and an acetate origin is readily presumed, even though some oxygens have been

consumed in ring formation processes. The lone methyl 'start of chain' usually obvious in acetate-derived compounds is also detected, though the carboxyl 'end of chain' reacted with a convenient hydroxyl function, which may have arisen through enolization, to form the lactone function. The formation of alternariol monomethyl ether from alternariol is known to occur catalyzed by a O-methyltransferase in a transmethylation reaction involving S-adenosyl methionine (Stinson *et al.*, 1986, Dewick, 2006).

An alternative mechanism for alternariol biosynthesis has been also suggested, which would proceed through the rearrangement of a norlichexanthone intermediate, in analogy to a well-documented step in aflatoxin biosynthesis. In this case the polyketide chain is assembled on the surface of the enzyme in a configuration that facilitated the formation of norlichexanthone (see Figure 4.1). Then oxidative cleavage of the aromatic phloroglucinol ring occurs, followed by limited rotation of the aryl fragment and ring closure to produce the coupling pattern observed in alternariol (Stinson *et al.*, 1986).

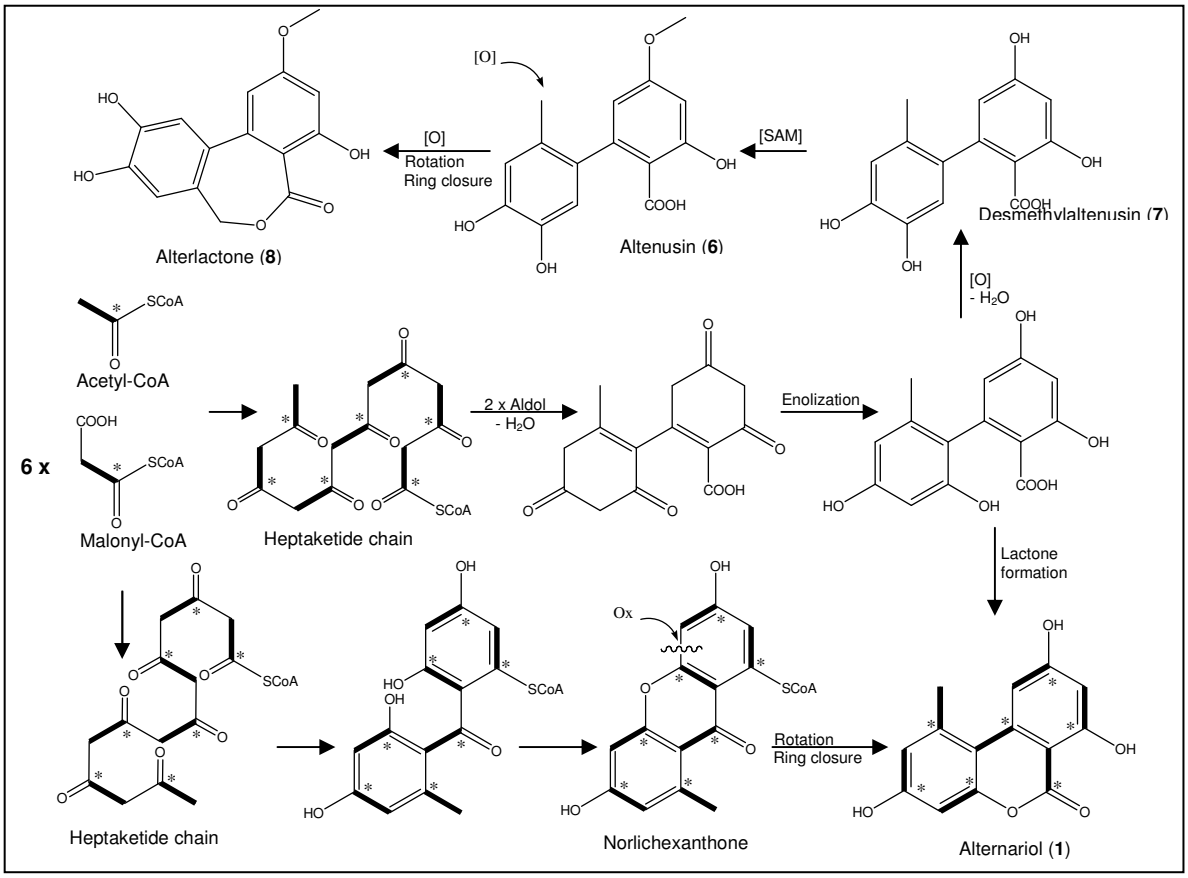


Figure 4.1: Postulated biosynthesis of alternariol derivatives (Stinson *et al.*, 1986, Dewick, 2006).

4.4.1.1.2. Biosynthesis of biphenic acids and related compounds

The formation of altenusin (**6**) and the previously unreported desmethylaltenusin (**7**) may be envisaged as proceeding by cyclization of a heptaketide precursor to give the biphenyl derivative shown in Figure 4.1 (similar to alternariol biosynthesis). An oxidation and reduction sequence, and methylation of the phenolic hydroxyl, in case of **6**, affords the biphenyl metabolites **6** and **7**. The previously unreported alterlactone (**8**) is most probably biosynthetized through the same pathway from altenusin (**6**) by oxidation of the aromatic methyl group, rotation and closure of the lactone ring at a different site compared to alternariol derivatives (**1**-**5**), namely at the aromatic hydroxymethyl group.

The heptaketide biphenyl intermediate in the biosynthesis of biphenic acids **6** and **7** is also believed to be the precursor to the structurally and biosynthetically interesting spirocyclic metabolite, talaroflavone (**9**), previously isolated from *Talaromyces flavus* (Ayer and Racok, 1990a), as well as the new natural product, alteric acid (**10**). Feeding ^{13}C-labelled sodium acetate to *T. flavus* resulted in the incorporation of label at six carbons of **9**. A biosynthetic pathway that accounts for this is illustrated in Figure 4.2 (Ayer and Racok, 1990b).

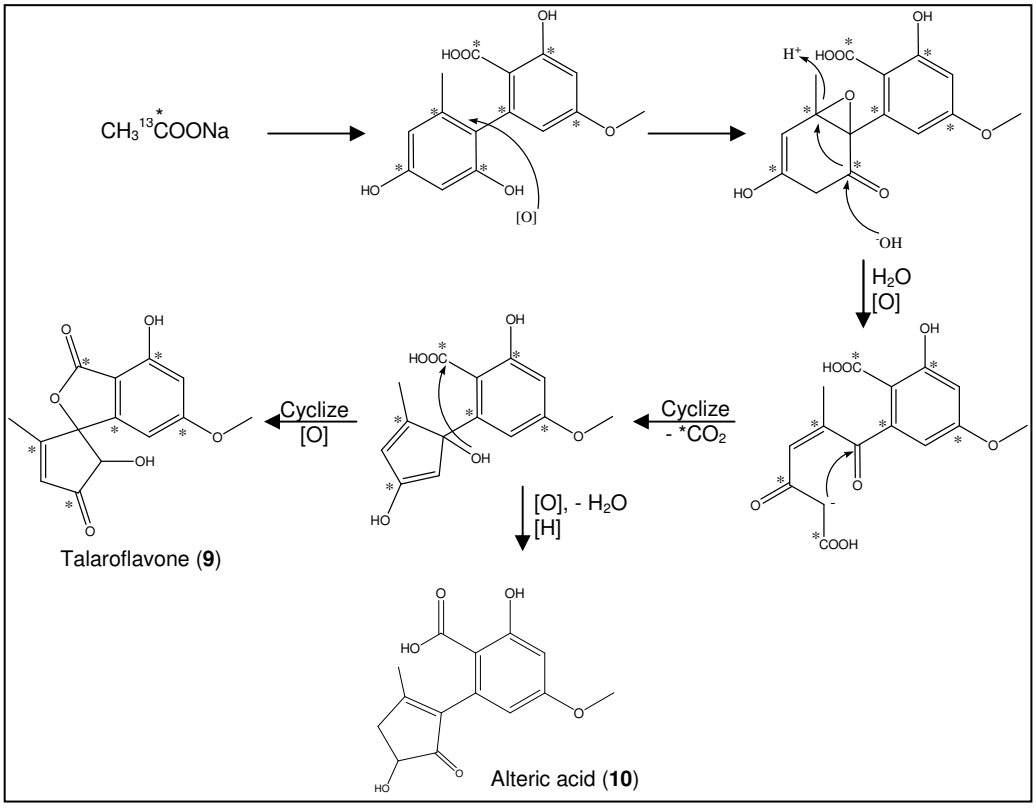


Figure 4.2: Postulated biosynthesis of talaroflavone (Ayer and Racok, 1990b).

4.4.1.1.3. Bioactivity and structure activity relationship of alternariol and biphenic acid derivatives

Alternariol (**1**) was found to have fairly powerful activity against some Gram-positive and Gram-negative bacteria. Furthermore, the compound had no phytotoxic effect when sprayed on to young carrot seedlings and applied to their roots (Raistrick *et al.*, 1953, Freeman, 1966).

Recently, alternariol (**1**) was reported to show estrogenic potential in cultured mammalian cells. Furthermore, it inhibited cell proliferation by interference with the cell cycle (Lehmann *et al.*, 2006). The results of our cytotoxicity test of the isolated alternariol derivatives from the endophytic fungus *Alternaria* sp. toward L5178Y (mouse lymphoma) cell line (see Table 3.13), strongly suggest that the free hydroxyl group at C-4`, which is present in all strongly active alternariol derivatives (**1**-**3**), plays an important role in the cytotoxic activity. Upon substitution of the 4`-OH by a sulphate group in alternariol monomethyl ether sulphate (**4**) activity was greatly decreased. Moreover, presence of a hydroxyl substituent at C-3` reduced the activity of hydroxyalternariol monomethyl ether (**5**) to only moderate. Substitution of the 5-OH by a sulphate group in alternariol sulphate (**2**) or a methyl group in alternariol monomethyl ether (**3**) had no effect on the activity of the compounds (see Figure 4.3). On the other hand, altenusin (**6**) and desmethylaltenusin (**7**) showed strong activities as well. Substitution of the 5-OH by a methyl group in desmethylaltenusin (**7**) had no effect on the activity of the compound. Moreover, alterlactone (**8**) was also active in the cytotoxicity assays. Interestingly, these results were in accordance with the results of protein kinase inhibition assay except for hydroxyalternariol monomethyl ether (**5**) which showed a protein kinase inhibitory activity comparable to that of alternariol (**1**) (see Table 3.14). Both altenusin (**6**) and desmethylaltenusin (**7**) were active against several protein kinases tested in the assay, which is in accordance with the recent report of altenusin as potent protein kinase inhibitor (Oyama *et al.*, 2004).

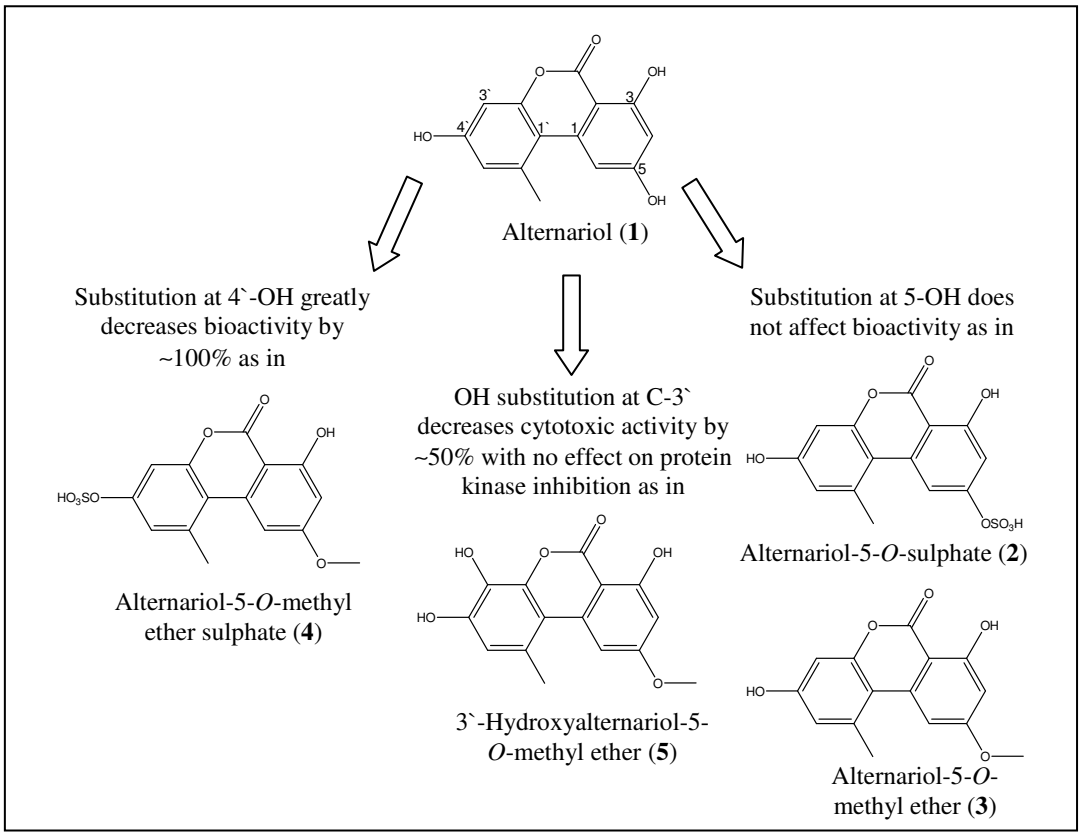


Figure 4.3: Structure-activity relationship for alternariol derivatives.

4.4.1.1.4. Toxicity studies of alternariol derivatives

The chicken embryo assay is often used as a convenient assay for toxicity of mycotoxins as well as other compounds. Investigation of the effects in the chicken embryo assay for alternariol (**1**), alternariol monomethyl ether (**3**) and altenuene (**11**) showed that these compounds exhibited no toxic effect at doses up to 1.000, 500, and 100 µg per egg, respectively, when administered to 7-day-old chicken embryos by yolk sac injection. In addition to the lack of mortality, these metabolites exerted no teratogenic effect in the developing embryo (Griffin and Chu, 1983). In another study, one day-old chicks were fed a standard diet supplemented with purified **3** at levels of up to 100 mg/kg of feed for 4 weeks exhibiting no mortality or significant loss in performance, indicating a general lack of toxicity of **3** in poultry systems (Griffin and Chu, 1983). Similarly, alternariol (**1**) and alternariol monomethyl ether (**3**) were shown to be nonmutagenic to *Salmonella* strains in the Ames *Salmonella typhimurium* assay (Davis and Stack, 1994). Thus, the finding of other investigators that alternariol monomethyl ether (**3**) was weakly mutagenic could have resulted from the presence of a small amount of one of the highly mutagenic altertoxins in the sample of alternariol monomethyl ether originally tested (Davis and Stack, 1994).

4.4.1.1.5. Occurrence of sulphated metabolites

In the plant kingdom, sulphated products, mainly flavonoids, have been isolated from more than 250 species, including dicotyledons and monocotelydons (Barron *et al.*, 1988). Sulphated phlorotannins were also detected in marine algae (Glombitza and Knöss, 1992). In fungi, however, few studies of sulphate conjugates appear in the literature. Choline sulphate is produced by *Aspergillus nidulans* presumably as sulphur reserve (Hussey *et al.*, 1965). The sulphate conjugation of aromatic hydrocarbons in liquid fermentation by *Cunninghamella elegans* was also reported (Cerniglia *et al.*, 1982). Furthermore, zearalenone sulphate was detected in four species of *Fusarium* as well as a sterol sulphate in corn cultures of *F. graminearum* (Vesonder *et al.*, 1990, Plasencia and Mirocha, 1991). However, the mechanism of sulphate conjugation in *Fusarium* sp. is unknown (Plasencia and Mirocha, 1991). In this study sulphated derivatives of alternariol and its monomethyl ether (**2** and **4**, respectively) were isolated from the *n*-BuOH extract of *Alternaria* liquid culture. In addition, the sulphated anthraquinones, macrosporin sulphate (**22**) and methylalaternin sulphate (**24**), were isolated from MeOH and EtOAc extracts of *Ampelomyces* liquid and rice cultures, respectively (see 4.4.2.).

4.4.1.2. Biosynthesis of 2,5-dimethyl-7-hydroxychromone

2,5-Dimethyl-7-hydroxychromone (**13**) has been isolated from the roots of *Polygonum cuspidatum*, a plant used in Chinese and Japanese traditional medicine (Kimura *et al.*, 1983), from the aerial parts of *Hypericum perforatum* (Yin *et al.*, 2004), and from a Japanese commercial rhubarb sample (Kashiwada *et al.*, 1984). The first report for its isolation from a fungal source, *Talaromyces flavus*, was in 1990 (Ayer and Racok, 1990a). The biosynthesis of **13** did not seem to follow the pattern of most chromones that arise from a pentaketide precursor but probably originates from a hexaketide precursor as illustrated in Figure 4.4 (Ayer and Racok, 1990a).

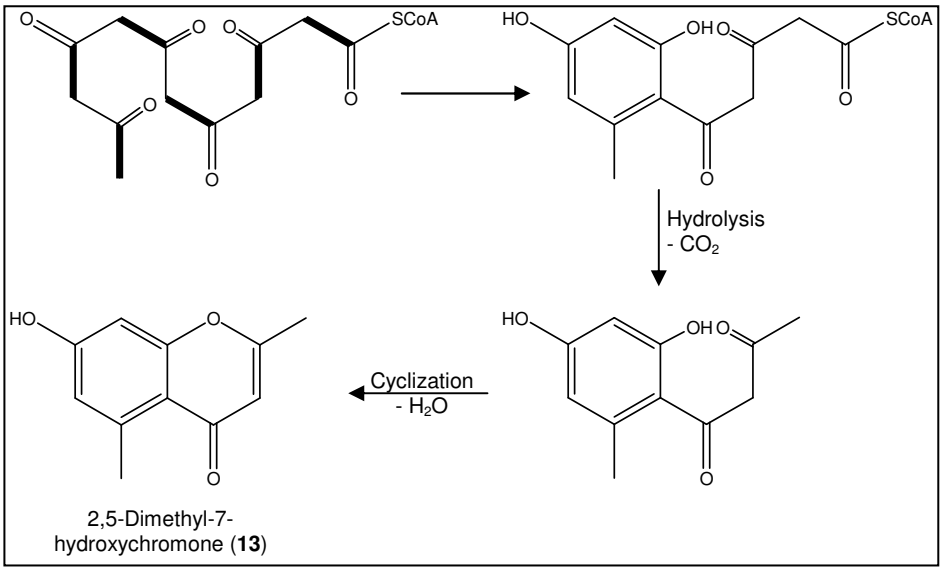


Figure 4.4: Postulated biosynthesis of 2,5-Dimethyl-7-hydroxychromone (Ayer and Racok, 1990a).

4.4.1.3. Biosynthesis of reduced perylenequinones

Altertoxin I (**14**) is an example of reduced perylenequinones so far identified in fungi of the morphologically closely related genera *Alternaria* and *Stemphylium*. The biosynthesis of these compounds occurs most probably *via* oxidative coupling of two molecules of a tetralone derivative, which in turn is synthesized from a pentaketide derivative (Okuno *et al.*, 1983) by so-called head-to-head coupling, followed by reduction and hydroxylation in different positions (Arnone *et al.*, 1986). The proposed biosynthetic pathway was confirmed by an incorporation experiment of ^{13}C-labelled sodium acetate and may be depicted as shown in Figure 4.5 (Okuno *et al.*, 1983).

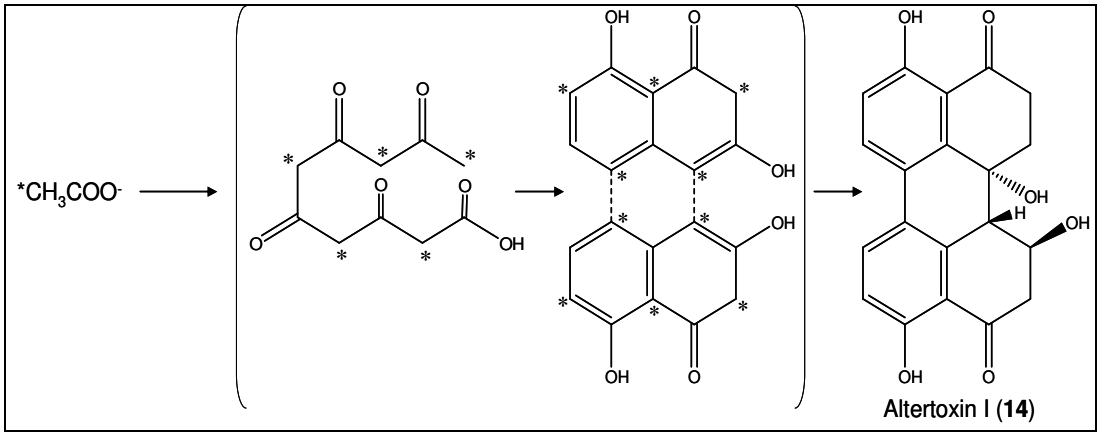


Figure 4.5: Proposed biosynthetic pathway of reduced perylenequinones (Okuno *et al.*, 1983).

4.4.1.4. Tautomerism and biosynthesis of tenuazonic acid

The tautomeric behavior of 3-acylpyrrolidine-2,4-diones (*e.g.* tenuazonic acid (**15**)) involves two sets of rapidly interchanging internal tautomers (a↔b) and (c↔d), where each set arises through proton transfer along the intramolecular hydrogen bond, together with two pairs of slowly interconverting external tautomers (ab↔cd), arising from the rotation of the acyl side chain (see Figure 4.6). It was found that the internal tautomerization occurs too rapidly to be detected on the time scale of an NMR experiment, the external tautomerism, however, occurs at a rate which can be measured on the NMR time scale. In non polar solvents (*e.g.* $CDCl_3$) the interconversion between the external enolic tautomers (ab↔cd) was found to be a comparatively slow process, while interconversion between the pairs of internal tautomers (a↔b, c↔d) was found to be fast. Thus, the two sets of resonances observed in the NMR spectra are attributed to the external tautomers (ab) and (cd). In polar solvents (*e.g.* CD_3OD) the two external pairs were found to interconvert at a much faster rate and, therefore, the NMR signals of the external tautomers coalesce (Nolte, 1980; Royles, 1995).

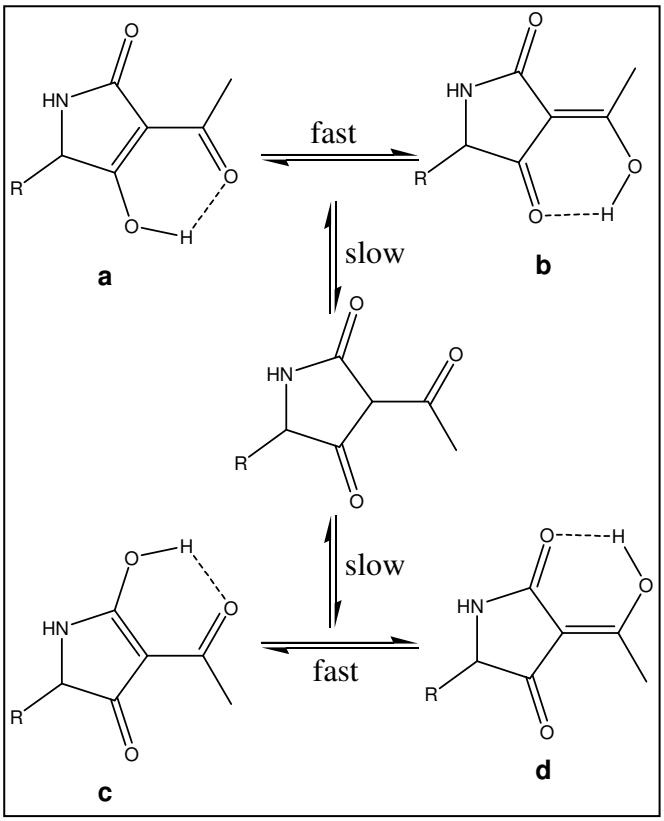


Figure 4.6: Tautomerism of 3-acylpyrrolidine-2,4-diones (Nolte, 1980).

From biosynthetic studies on tenuazonic acid (**15**) established by feeding experiments using ^{14}C-labelled acetate it was concluded that the biosynthesis occurs *via* cyclization of *N*-acetoacetyl-L-isoleucine to produce tenuazonic acid as shown in Figure 4.7 (Royles, 1995).

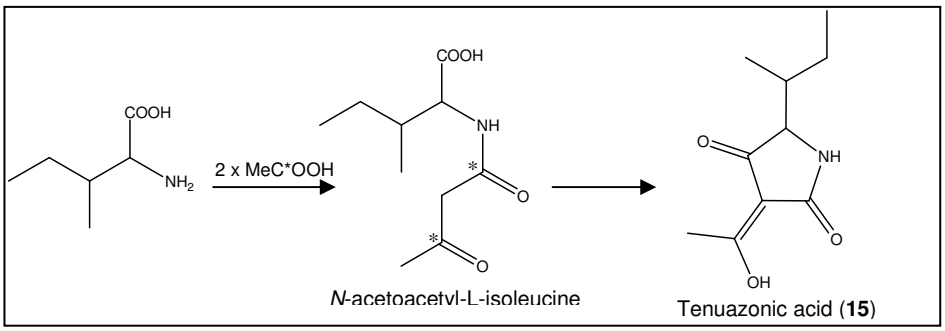


Figure 4.7: Postulated biosynthesis of tenuazonic acid (Royles, 1995).

4.4.1.5. Bioactivity of selected *Alternaria* metabolites

Altertoxin I (**14**) had been previously isolated from a number of *Alternaria* spp. (Okuno *et al.*, 1983, Stack *et al.*, 1986, Hradil *et al.*, 1989). The compound showed a high level of phytotoxicity toward corn (B73), as measured by the size of necrotic lesions 72 h after application of 1 µg of compound, with strong selectivity as well (Hradil *et al.*, 1989).

Tenuazonic acid (**15**) was originally isolated from the culture filtrate of *Alternaria tenuis* and subsequently other species have also been found to produce it. The compound exhibited a low level of antibacterial activity and showed an inhibitory effect on several viruses including poliovirus MEF-1, ECHO-9, parainfluenza-3, vaccinia, and herpes simplex (HF) (Miller *et al.*, 1963). It also showed the ability to inhibit human adenocarcenoma growing in the embryonated egg with a proposed mode of action involving the inhibition of amino acid incorporation into microsomal proteins. However, the compound has been of limited value due to its extreme toxicity (Royles, 1995).

In this study both compounds **14** and **15** were tested for cytotoxicity toward L5178Y cell line where they showed weak and moderate activities, respectively. In the protein kinase inhibition assay, however, **14** showed to be active on various protein kinases, while **15** showed no activity.

4.4.2. Compounds isolated from the endophytic fungus *Ampelomyces* sp.

The *Ampelomyces* sp. strain investigated was isolated from flowers of *Urospermum picroides* growing wild in Egypt. *U. picroides* is typical for the traditional Mediterranean diet and its extract shows anti-inflammatory activities (Strzelecka, *et al.*, 2005).

Historically, pycnidial fungi belonging to the genus *Ampelomyces* were among the first mycoparasites to be studied in detail and were also the first fungi used as biocontrol agents of plant parasitic fungi (Yarwood, 1932; Sundheim and Krekling, 1982). The interactions between host plants, powdery mildew fungi and *Ampelomyces* mycoparasites are one of the most evident cases of tritrophic relationships in nature, in which organisms on three different trophic levels integrate functionally through host-parasite interactions (Kiss *et al.*, 2004). While it seems likely that fungal metabolites are involved in many reported interspecies interactions, *Ampelomyces* mycoparasites attracted our attention because they have rarely been studied chemically.

Extracts of the fungus grown in liquid culture afforded a new pyrone, ampelopyrone (**17**), two new sulphated derivatives of macrosporin (**22**) and methylalaternin (**24**) together with the known compounds methyltriacetic lactone (**16**), citreoisocoumarin (**20**), macrosporin (**21**), methylalaternin (**23**), ergosterol and cerebroside C. From extracts of the fungus grown on solid rice medium we obtained two new isocoumarins, desmethyldiaportinol (**18**) and desmethyldichlorodiaportin (**19**) and a new hexahydroanthronol, ampelanol (**26**), as well as compounds **21**-**24**, altersolanol A (**25**), the atropisomers, alterporriols D and E (**27** and **28**, respectively), and altersolanol J (**29**).

4.4.2.1. Anthraquinones and modified anthraquinones

4.4.2.1.1. Biosynthesis of anthraquinones and modified anthraquinones

Fungi are known to form anthraquinones by linear head-to-tail combination of acetate and malonate, namely, octaketide chains, catalyzed by a fungal polyketide synthase, followed by the loss of carboxylic acid carbon from the terminal unit at C-3, but the detailed sequence of condensation, dehydration and hydroxylation steps is not well known (see Figure 4.8). The periphery of the carbon skeleton is constructed by folding the octaketide chain, and then the ring at the centre of the fold is formed first, followed in turn by the next two rings (Ohnishi *et al.*, 1991, Dewick, 2006). The validity of the octaketide pathway was confirmed by spectroscopic studies on the biosynthesis of macrosporin (**21**) utilizing single and double labeled acetates (Stoessl *et al.*, 1983, Suemitsu *et al.*, 1989, Ohnishi *et al.*, 1992).

Altersolanol A (**25**) is clearly closely related to its anthraquinone co-metabolites **21**-**24** since they have the same *meta*-substituted aromatic ring and a *C*-methyl group in their biogenetic 2-position. It is indeed possible that the anthraquinones are formed from altersolanol derivatives by dehydration brought about enzymatically or by acid catalysis within the mold tissue. However, the reverse may also be true, *i.e.* that **25** is derived from anthraquinone precursors. At any rate, it is very probable that altersolanol A (**25**) and macrosporin (**21**) share a common biogenetic origin (Stoessl, 1969b).

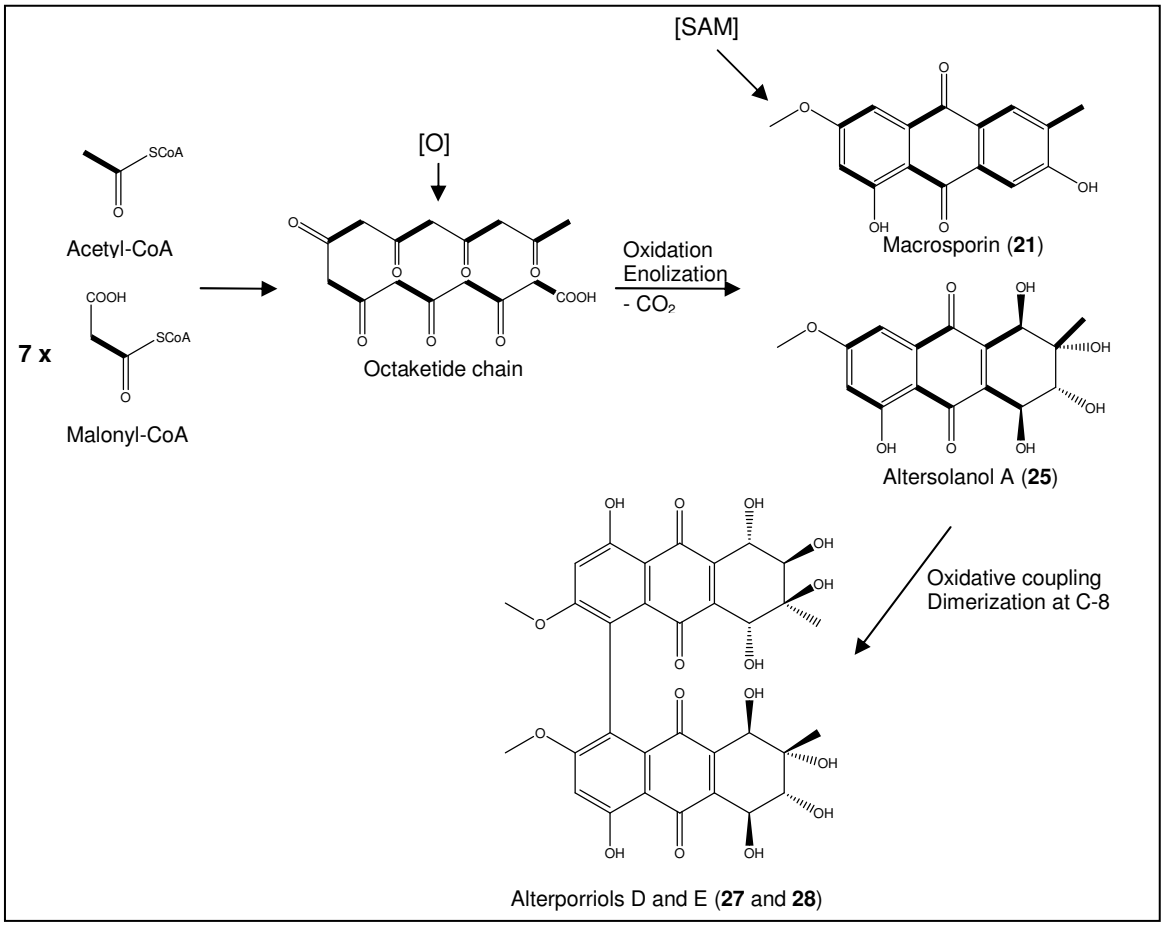


Figure 4.8: Postulated biosynthesis of anthraquinones and modified anthraquinones (Stoessl *et al.*, 1983, Suemitsu *et al.*, 1989, Ohnishi *et al.*, 1991, 1992).

4.4.2.1.2. Bioactivity of anthraquinones and modified anthraquinones

Altersolanol A (**25**) inhibited the growth of Gram-positive bacteria and *Pseudomonas aeruginosa* IFO 3080 when tested by the broth dilution method (Yagi *et al.*, 1993). It was found that the compound acts as an electron acceptor in the bacterial membrane to inhibit bacterial growth (Haraguchi *et al.*, 1992). Moreover, **25** was found to be highly phytotoxic

when injected into tomato leaves. However, using this assay, the two altersolanol A dimers, **27** and **28**, were only very weakly phytotoxic (Lazarovits *et al.*, 1988).

The anthraquinones and modified anthraquinones isolated from the endophytic fungus *Ampelomyces* sp. in this study were tested for cytotoxicity toward L5178Y (mouse lymphoma) cell line (see Table 3.26). The obtained results showed that the tetrahydroanthraquinone, altersolanol A (**25**), was the most active compound. The anthranol derivatives, ampelanol (**26**), altersolanol J (**29**) and tetrahydroaltersolanol B (**30**) showed moderate to weak activities, suggesting that the *para*-quinone moiety is of great importance for the cytotoxic activity. Furthermore, the monomer **25** showed much higher activity than its dimers **27** and **28**. Methylalaternin (**23**) was the most active under the anthraquinone derivatives, while macrosporin (**21**) only showed moderate activity. Sulphate substitution at 7-OH in **22** and **24** reduced the activity indicating the possible contribution of this hydroxy group to the activity (see Figure 4.9). Furthermore, results of protein kinase inhibition assay showed a similar pattern of activity, with altersolanol A (**25**) being the most active compound inhibiting various protein kinases in the protein kinase inhibition assay, while methylalaternin (**23**) and macrosporin (**21**) were less active (see Table 3.27).

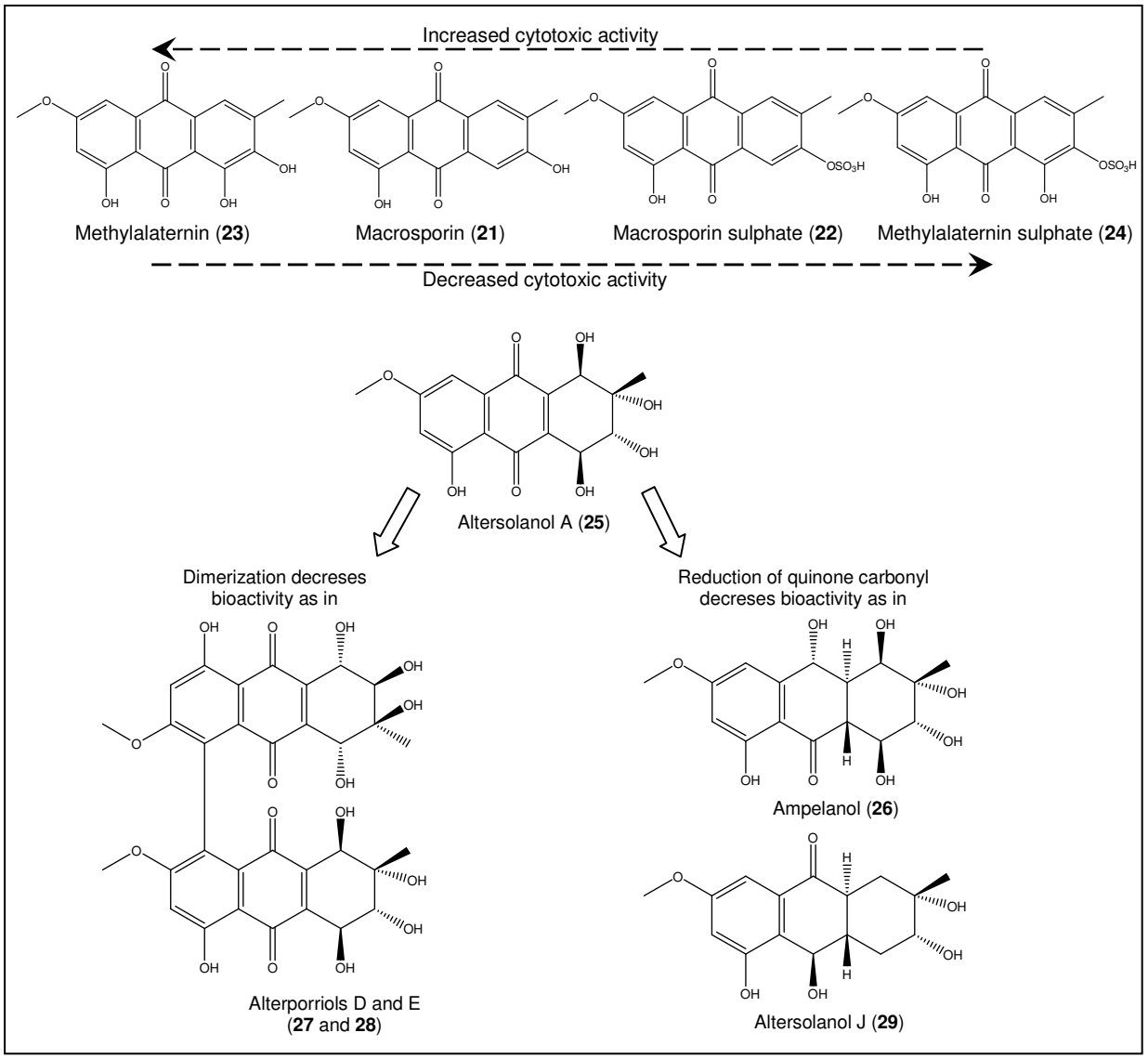


Figure 4.9: Structure-activity relationship of anthraquinones and modified anthraquinones.

One very promising new approach for developing novel antibiotics is based on the fact that bacterial colonization and pathogenesis seemingly depends on the ability of the bacteria to communicate and thereby coordinate the behavior of the entire population (Hall-Stoodley *et al.*, 2004). Population activity such as biofilm formation is coordinated by simple communication systems which in many Gram-negative bacteria is based on homoserine lactone (HSL) signals, which have been described in numerous pathogens (Costerton *et al.*, 1999). HSL systems are referred to as quorum sensing (QS) systems, *i.e.* they express target genes in relation to the quorum size (or density) of the population and in most known cases control expression of virulence factors. A screening strategy aiming at inhibition of QS is therefore not targeting bacterial growth but instead blocking the coordination of bacterial population activity. This means that a quorum sensing inhibiting (QSI) drug is not generating

a selective pressure on the bacteria, and it is therefore unlikely that bacteria will develop resistance towards a given QSI compound (Larsen *et al.*, 2005). Upon testing the anthraquinones and modified anthraquinones for inhibition of biofilm formation of *Staphylococcus epidermidis*, methylalaternin (**23**) showed very high activity with a MIC of 12.5 μg/mL and complete inhibition of biofilm formation, whereas altersolanol A (**25**) having MIC of >50 μg/mL inhibited biofilm formation by 50%.

4.4.2.2. Pyrone and isocoumarin derivatives

4.4.2.2.1. Biosynthesis of pyrone and isocoumarin derivatives

From a biogenetic point of view, *β*-polyketo carboxylic acids are expected to convert into both the corresponding pyrones and phenolic compounds according to the mode of enzymatic cyclization as shown in Figure 4.10 (Lai *et al.*, 1991).

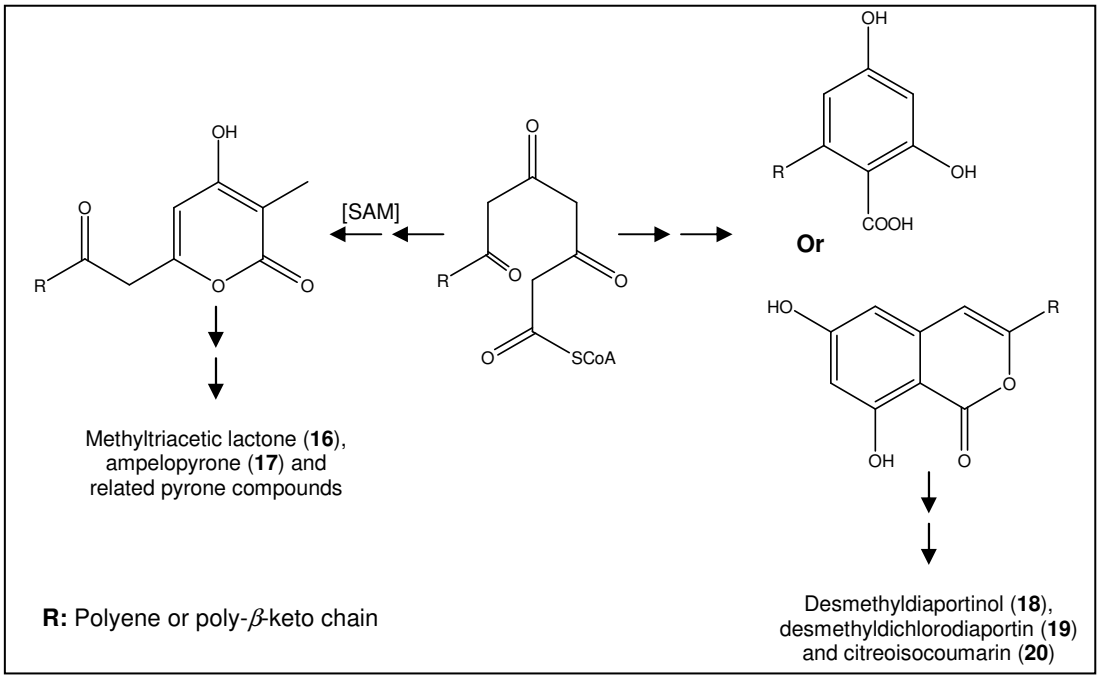


Figure 4.10: Biosynthetic pathways of pyrones and isocoumarins (Lai *et al.*, 1991).

The isocoumarin derivatives **18**-**20** are typical heptaketide compounds with oxygen atoms located at alternate carbons. The carbonyl carbons in the side chain (R) might be reduced by fungal reductases to hydroxyl groups (Watanabe *et al.*, 1998).

On the other hand, recently naturally occurring organohalogen compounds have assumed an important role in the field of natural products. The number of natural

organohalogen compounds has multiplied about 250 times in the past 40 years. Fungi and lichens are known to be a bountiful source of such metabolites, and the earliest examples of natural chlorine-containing metabolites include the fungal metabolites griseofulvin and chloramphenicol. The mechanism for the formation of organohalogen compounds was reported to initially involve the oxidation of halide by a peroxidase enzyme and hydrogen peroxide (Gribble, 1998). A more recent study presented evidence that NADH-dependent halogenases rather than haloperoxidases are the enzymes that actually do the chlorination (Hohaus *et al.*, 1997).

4.4.2.2.2. Bioactivity of pyrone and isocoumarin derivatives

Pyrone and isocoumarin derivatives were subjected to cytotoxicity testing toward L5178Y (mouse lymphoma) cell line (see Table 3.26). Desmethyldiaportinol (**18**) was the most active of the compounds, whereas desmethyldichlorodiaportin (**19**) and citreoisocoumarin (**20**) were found to be moderately active and inactive, respectively. This indicated that presence of bulky substituents on the side chain attached to the isocoumarin structure may result in reduction and loss of activity (see Figure 4.11). On the other hand, the pyrone compounds **16** and **17** were found to be inactive in the test (see Figure 12). Furthermore, all compounds were inactive when tested for protein kinase inhibition, which suggested that desmethydiaportinol cytotoxic acitivity did not involve interaction with protein kinases.

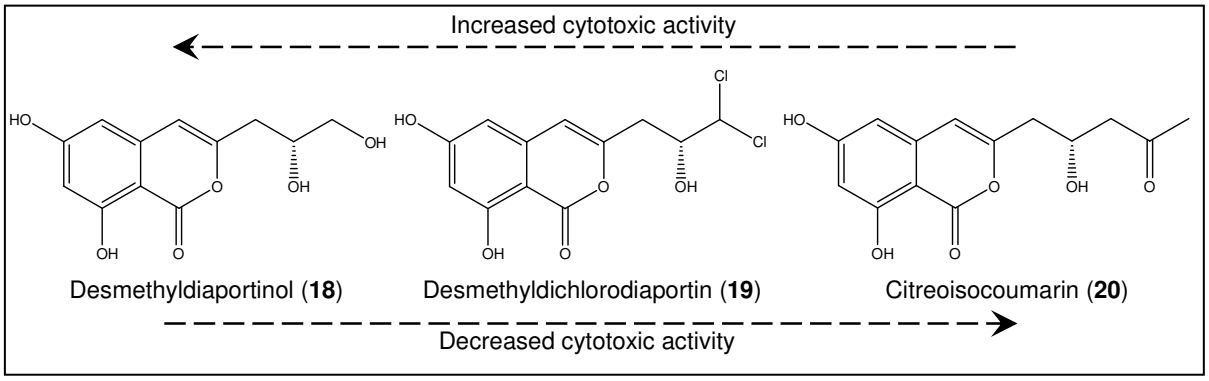


Figure 4.11: Structure-activity relationship of isocoumarin derivatives.

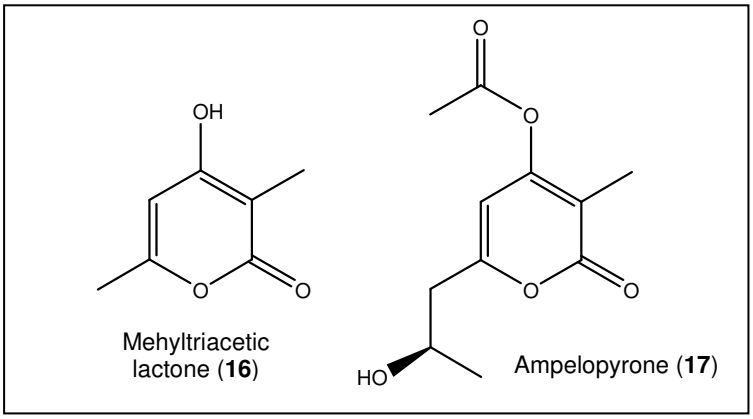


Figure 4.12: Structures of inactive pyrones.

4.4.3. Compounds isolated from the endophytic fungus *Stemphylium botryosum*

Stemphylium botryosum was isolated from *Chenopodium album*, a plant growing wild in Egypt. *C. album* is reported in folk medicine to possess anthelmintic properties and the seed oil is effective against many forms of intestinal parasites. The plant was also used in the past as oral contraceptive (Laszlo and Henshaw, 1954). It is also used in the Indian Himalayan Region for treating liver diseases (Samant and Pant, 2006).

Stemphylium botryosum is a mould which causes leaf spot of lettuce, a disease of economic importance in many countries. Both saprotrophic and pathogenic forms of *Stemphylium* occur on a wide range of plants. Many species of *Stemphylium* are economically important pathogens of agricultural crops. Usually, the toxicity of moulds is related to the production of one or more phytotoxins, which is the case in *Stemphylium* species that are reported to produce a wide array of toxins (Arnone and Nasini, 1986, Camara *et al.*, 2002).

Chemical investigation of the EtOAc extract of *Stemphylium botryosum*, grown on solid rice medium, yielded altersolanol A (**25**), tetrahydroaltersolanol B (**30**), stemphyperylenol (**31**), as well as the macrocyclic lactones, curvularin (**32**) and dehydrocurvularin (**33**), in addition to macrosporin (**21**).

4.4.3.1. Biosynthesis of stemphyperylenol

The biosynthesis of stemphyperylenol (**31**) occurs as described above in the context of other reduced perylenequinones (see 4.4.1.3.). However, it is remarkable that, whereas all the compounds so far found appear to derive from so-called head-to-head coupling, stemphyperylenol (see Figure 4.13) seems to be an unusual example of a head-to-tail coupling of pentaketide-derived moieties (Arnone and Nasini, 1986).

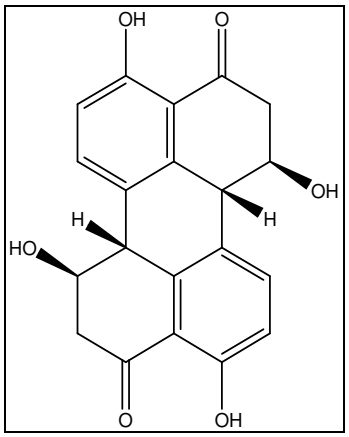


Figure 4.13: Structure of stemphyperylenol (**31**).

4.4.3.2. Biosynthesis of macrocyclic lactones

The fungal metabolite curvularin (**32**) is a macrolide octaketide produced by some *Curvularia* (Coombe *et al.*, 1968), *Alternaria* (Robeson and Strobel, 1981) and *Penicillium* (Lai *et al.*, 1989) species. Interestingly, curvularin-type metabolites had never been previously described for the chemically well-investigated *Stemphylium botryosum*. Curvularin (**32**) and dehydrocurvularin (**33**) are lactones containing a twelve membered ring. The acyl-resorcinol fragment is probably formed by cyclization of an intermediate containing a carbon chain formed by serial head-to-tail linkage of acetate units typical of polyketide biogenesis (Liu *et al.*, 1998). Mould incorporation of labeled ^{13}C- or ^{14}C-acetate units into **32** or **33** indicated that the C_{16} metabolites are derived from eight acetate units, very probably through linear precursors (Birch *et al.*, 1959, Arai *et al.*, 1989). More recent studies (see Figure 4.14) also showed that diketides I and its reduced congener II are the precursors in the biosynthetic pathway, and additionally, that the double bond of the tetraketide intermediate III is essential for incorporation. This strongly supports the hypothesis that the unsaturated tetraketide III represents the final oxidation state achieved on the polyketide synthase prior to addition of the next C_2 unit. Since the subsequent penta-, hexa-, hepta-, and octaketide intermediates require no reduction, this suggested that dehydrocurvularin (**33**) is the initial polyketide synthetase product (Liu *et al.*, 1998). Curvularin (**32**) could be obtained by reduction of dehydrocurvularin (**33**) (Arai *et al.*, 1989). The proposed biosynthetic pathway is illustrated in Figure 4.14.

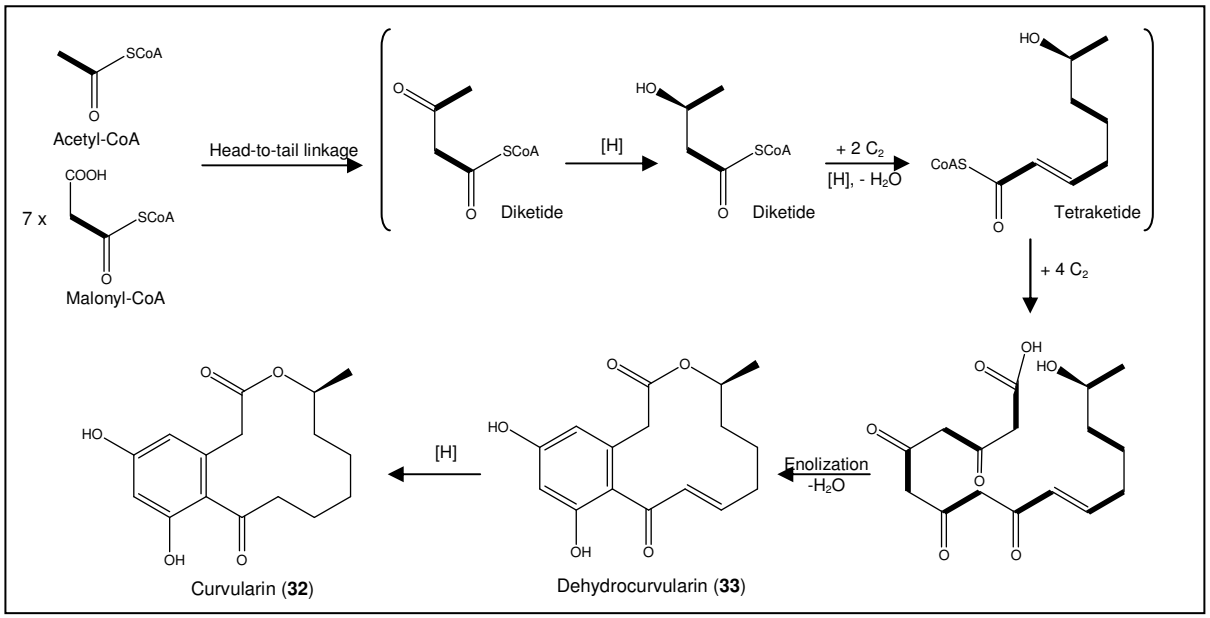


Figure 4.14: Biosynthesis of macrocyclic lactones (Birch *et al.*, 1959, Liu *et al.*, 1998).

4.4.3.3. Bioactivity of selected *Stemphylium* metabolites

Stemphyperylenol (**31**) was reported to show antibacterial activity *in vitro* against *Bacillus subtilis*, *B. cereus* and *E. coli* (Arnone and Nasini, 1986). In the present study, this compound showed moderate activity when tested for cytotoxicity toward L5178Y cell line.

Curvularin (**32**) and dehydrocurvularin (**33**) exhibited antifungal and antibacterial activity as well as non-specific phytotoxicity (Robeson and Strobel, 1981). More interestingly, curvularin (**32**) showed remarkable cytotoxic activity towards sea urchin embryogenesis, blocking cell division at concentrations of 2.5 µg/mL by specifically disordering microtubule centers and inducing barrel-shaped spindles (Kobayashi *et al.*, 1988). The results obtained by testing **32** and **33** for cytotoxic activity toward L5178Y (mouse lymphoma) cell line (see Table 3.33) were in accordance with the data reported in literature. Dehydrocurvularin (**33**) was found to be highly active with an EC_{50} value of 0.43 µg/mL, and curvularin (**32**) was also very active having an EC_{50} value of 4.7 µg/mL. However, both compounds were inactive in the protein kinase inhibition assay, which also strongly points to a mechanism of cytotoxic activity not involving interactions with protein kinases.

4.4.4. Compounds isolated from the endophytic fungus *Chaetomium* sp.

Chaetomium sp. was isolated from fresh stems of *Otanthus maritimus* growing wild on the sandy Mediterranean coast in Egypt. The genus *Otanthus*, found mainly in the Mediterranean region, belongs to family Asteraceae and is represented by a single species.

Otanthus maritimus has been reported in the past to exhibit a significant array of biological and pharmacological activities including the treatment of dysentery and inflammation of the urinary bladder (Muselli *et al.*, 2007). Dry specimens of *O. maritimu*s have been traditionally used as decoration and at the same time as a means of repelling flying insects from household areas (Christodoulopoulou *et al.*, 2005). Moreover, it was reported to be used by the Bedouins for treating asthmatic bronchitis (Jakupovic *et al.*,1988).

The genus *Chaetomium* is a member of the subphylum Ascomycotina, family Chaetomiaceae. Members of this family are cellulolytic and occur naturally on paper and cotton fabrics (Alexopoulous *et al.*, 1996). *Chaetomium* species are reported to be widespread in soil and plant debris, where they are important agents of cellulose degradation (Abbott *et al.*, 1995, Carlile *et al.*, 2001). As pathogens of crop plants, timber and ornamental trees, they received comprehensive attention with regard to the production of mycotoxins (Alexopoulous *et al.*, 1996).

The EtOAc extract of *Chaetomium* sp. liquid cultures afforded the previously unreported aureonitolic acid (**34**), as well as the known compounds cochliodinol (**35**), isocochliodinol (**36**), indole-3-carboxylic acid (**37**), cyclo(alanyltryptophane) (**38**) and orsellinic acid (**39**).

4.4.4.1. Biosynthesis of tetrahydrofurans

The previously unreported tetrahydrofuran aureonitolic acid (**34**) is structurally very similar to the fungal metabolite aureonitol (**34a**), isolated for the first time in 1967 from the culture broth of *Chaetomium coarctatum* (Abraham and Arfmann, 1992). Thus, both compounds are presumably biosynthesized as illustrated in Figure 4.15. The epoxide intermediate **a**, comprising seven acetate units, is rearranged to the aldehyde **b** which is then reduced to the alcohol **c**. **C** is then epoxidized again to the intermediate **d** which is subsequently opened intramolecularly by the hydroxyl group to **34a** (Seto *et al.*, 1979, Abraham and Arfmann, 1992). This biosynthetic pathway was established by feeding experiments using ^{13}C-labelled precursors (Seto *et al.*, 1979).

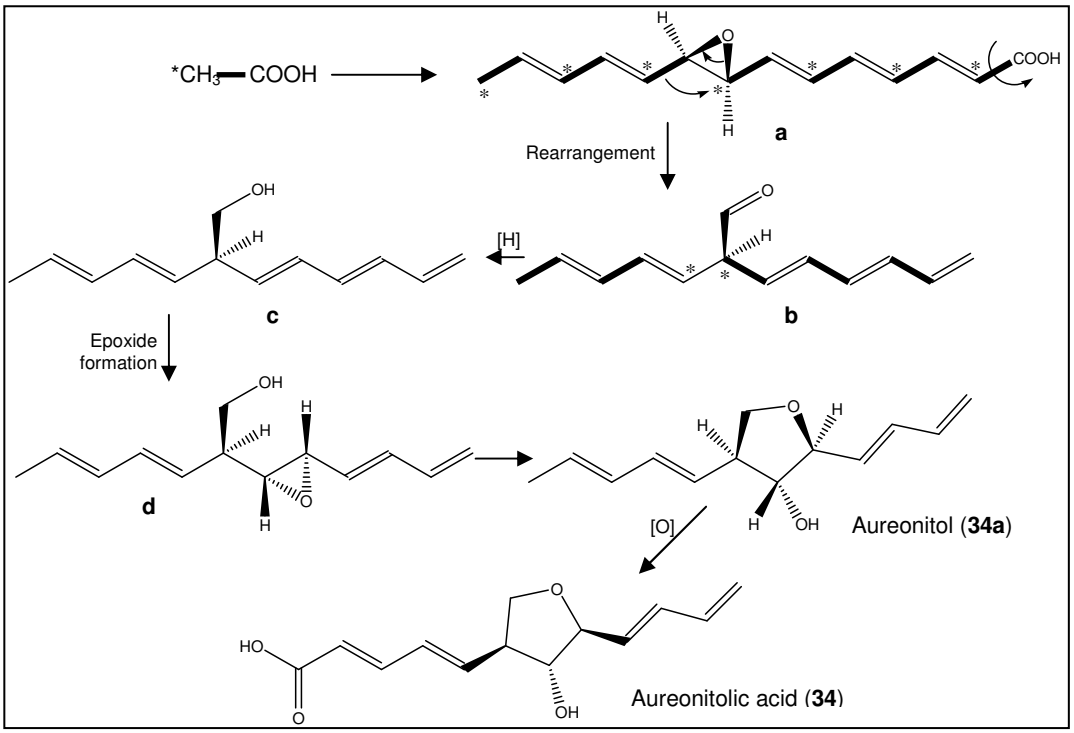


Figure 4.15: Proposed biosynthesis of aureonitol and aureonitolic acid (Seto *et al.*, 1979, Abraham and Arfmann, 1992).

4.4.4.2. Biosynthesis of bis-(3-indolyl)-benzoquinones

The purple pigment cochliodinol (**35**) and related compounds were found to be common metabolic products of the genus *Chaetomium* (Sekita *et al.*, 1981). As suggested by the chemical structure (see Figure 4.16), cochliodinol (**35**) and isocochliodinol (**36**) are presumed to be biosynthesized from tryptophane and and isopentenyl unit derived from mevalonic acid. To confirm the participation of tryptophane and mevalonate, administration experiments were attempted using ^{13}C- and ^{14}C-precursors. Very surprisingly, the results indicated that tryptophane was also incorporated into the oxygenated carbon atoms of the benzoquinone ring of cochliodinol (Yamamoto *et al.*, 1976; Taylor and Walter, 1978).

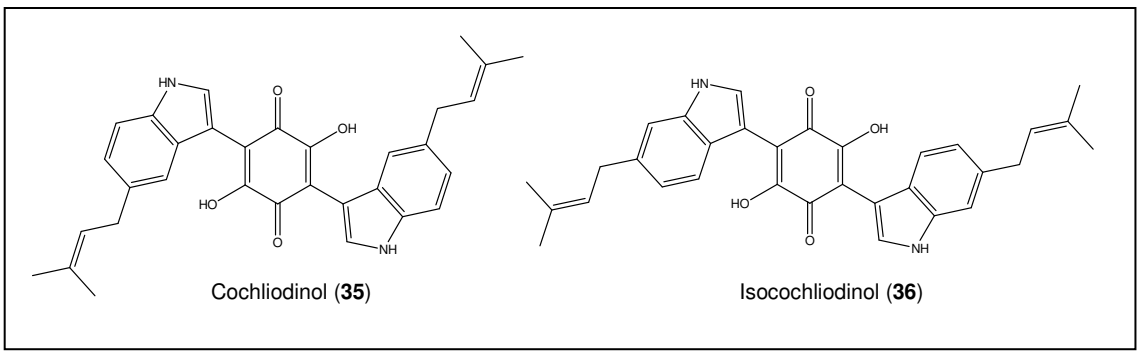


Figure 4.16: Structures of bis-(3-indolyl)-benzoquinones.

4.4.4.3. Bioactivity of selected *Chaetomium* metabolites

Cochliodinol (**35**) and related compounds are produced by several *Chaetomium* species. It was found that these quinonoid metabolites inhibit the growth and metabolism of a range of bacterial genera (Brewer *et al.*, 1984). In this study cochliodinol (**35**) and isocochliodinol (**36**) were tested for cytotoxic activity against L5178Y (mouse lymphoma) cell line (see Table 3.43). Interestingly, **35** was found to be very active with an EC_{50} value of 7.0 µg/mL, while **36** was only weakly active inhibiting L5178Y growth to 75.8 % at a concentration of 10.0 µg/mL. Thus, it may be concluded that cytotoxic activity was affected by the position of prenyl substituents at the indole rings.

Furthermore, results of brine shrimp lethality test showed that chain elongation (increase in lipophilicity) caused a rise in the cytotoxic activity of orsellinates. In addition, the reduction of activity upon substitution at 4-OH suggested that the hydroxy group at the C4 position causes effect in the cytotoxic activity of these compounds (Gomes *et al.*, 2006). In our study orsellinic acid (**36**) was found to be very active in the cytotoxic test against L5178Y (mouse lymphoma) cell line (see Table 3.43). It inhibited L5178Y growth to only 1.0 % at a concentration of 10.0 µg/mL, with an EC_{50} value of 2.7 µg/mL. On the other hand, orsellinic acid was inactive in the protein kinase inhibition assay, indicating that the mechanism of cytotoxic activity was most probably not due to interaction with protein kinases.

4.5. Detection of fungal metabolites in the host plant fractions

The fungal metabolite alternariol monomethyl ether (**3**) was isolated for the first time from a plant source, *Anthocleista djalonensis*, in 1995 (Onocha *et al.*, 1995). This plant is of West African origin and is used in traditional medicine for the treatment of various diseases (Onocha *et al.*, 1995). Furthermore, 2,5-dimethyl-7-hydroxychromone (**13**) has been isolated from *Polygonum cuspidatum* (Kimura *et al.*, 1983), *Hypericum perforatum* (Yin *et al.*, 2004) and *Rhei rhizoma* (Kashiwada, Nonaka and Nishioka, 1984). Aureonitol (**34a**), a fungal metabolite isolated from *Chaetomium* species, was isolated from an extract of *Helichrysum aureo-nitens* (Bohlmann and Ziesche, 1979). These reports of isolation of fungal metabolites from higher plants by circumstantial evidence evoked our interest to see if the metabolites we isolated from the fungal species, investigated in this study, were detectable in fractions of the corresponding host plants.

The fungal metabolites were traced in the host plant subfractions using LC/MS, an analytical technique that provides high sensitivity and specificity even for very complex extracts or fractions. Moreover, to increase the specificity of the method co-elution studies

with the corresponding pure metabolites and the respective plant fractions were carried out and spectra were evaluated for matching of retention times, presence of the molecular ion of the target compounds, patterns of MS and MS/MS spectra of the pure substances and the substances detected in the host plant fractions. Compared with LC/UV the LC/MS method was found to be approximately twenty-five times more sensitive, with the lower limit of quantification twenty-five times lower than that of LC/UV (10 ng/mL) for equivalent sample volumes (Baldrey *et al.*, 2002). Another difference between the two methods was that the LC/UV method needs a cleaner extract as it is less specific and any endogenous compounds with a similar retention time and similar maximum of absorption would interfere (Baldrey *et al.*, 2002). This illustrated the fact that LC/MS is in many aspects superior to LC/UV, and thus, for the current study where sensitivity is an issue and limited amounts of substances or complex fractions were to be investigated, would be the method of choice.

As a result, LC/MS analysis showed the presence of the *Alternaria* metabolites alternariol (**1**), alternariol monomethyl ether (**3**) and altenusin (**6**) in the fractions of the host plant, *Polygonum senegalense*. The *Ampelomyces* metabolites macrosporin (**21**) and methylalaternin (**23**) were also detected in the fractions of *Urospermum picroides*. Similarly, the *Stemphylium* metabolites macrosporin (**21**), tetrahydroaltersolanol B (**30**), stemphyperylenol (**31**) and curvularin (**32**) were detected in *Chenopodium album*. Interestingly, the substances detected were found in both liquid and rice cultures of the endophytic fungus, while the substances obtained from only one of both cultures were not detected in the host plant fractions. These results suggest the possible production of such metabolites by the endophytic fungus under its normal physiological conditions of growth within the tissues of the healthy plants, implying their possible contribution to the mutualistic interaction between the endophyte and its host plant and proving the contribution of the fungal endophyte to the chemical composition of the host plant. In the case of secondary metabolites of the endophytic fungus *Chaetomium* sp., it was not possible to detect any of the isolated secondary metabolites in any of the fractions of *Otanthus maritimus*, even though all of them were detectable in the crude extract of the fungus. Thus, it could be concluded that the fungal metabolites were either not produced *in planta* or present in very minute quantities beyond the limit of detection of the very sensitive technique applied.

This evidence for presence of the fungal metabolites in the corresponding host plant was quite surprising and encourages a quantification study which would be of great significance. It is worth mentioning that, apart from a few studies more or less by chance reporting the isolation of typical fungal metabolites from plant sources, the presence of

secondary metabolites of endophytic fungi in the same host plants from which they had originally been isolated has rarely been investigated. The reason could also be the use of less sensitive methods of detection (*e.g.* LC/UV) resulting in lack of positive evidence. Recently, it was demonstrated that *Neotyphodium uncinatum*, the common endophyte of *Festuca pratensis*, had the full biosynthetic capacity for some of the most common loline alkaloids, which were formerly exclusively found in endophyte-infected grasses. The identity of the alkaloids was confirmed by GC/MS and ^{13}C-NMR spectroscopic analysis (Blankenship *et al.*, 2001). Intensive studies of grass-endophyte associations, however, showed that endophytes in the grasses produce physiologically active alkaloids in the tissues of their host, which cause toxicosis to grazing livestock, increase resistance to invertebrate herbivores and pathogenic microorganisms, and may inhibit germination and growth of other grasses. Experiments demonstrated that plant growth and seed production can be increased by infection as well. Ecologically, certain loline analog alkaloids have been demonstrated to contribute to the allelopathic properties of host grasses (Clay, 1988; Joost, 1995; Siegel and Bush, 1997; Tan and Zou, 2001). Moreover, endophyte-infected grasses usually possess an increased tolerance to drought and aluminium toxicity (Malinowski and Belesky, 2000).

Thus, the present study has proved, for the first time, that the postulated, and hitherto only for grass-endophyte associations proven hypothesis, that endophyte infection enhances host plant fitness and competitiveness in stressful environments by producing functional metabolites, could be also the case in other plant-endophyte associations, supported by the unequivocal detection of fungal metabolites in three out of four investigated host plants indicating that this could actually be a general case. It may also be hypothesized that fungal metabolites reported previously from other plants presumably also originate from endophytic fungi colonizing these plants. This finding supplies an important contribution to the question of the ecological function of secondary metabolites produced by endophytic fungi, which could lead to a better understanding of this interesting group of organisms as well as help in the specific search for new bioactive substances with pharmaceutical potential to assist in solving not only human, but also animal and plant health problems.

5. Conclusion

Plant endophytic fungi produce natural products with a large diversity of chemical structures which might prove to be suitable for specific medicinal or agrochemical applications. Most of these secondary metabolites show biological activities in pharmaceutically relevant bioassay systems and thus represent potential lead structures which could be optimized to yield effective therapeutic and bioactive agents.

The aim of this work was the isolation of secondary metabolites from endophytic fungi, followed by structure elucidation and examination of their pharmocological potential. Four endophytic fungal strains (*Alternaria* sp., *Ampelomyces* sp., *Stemphylium botryosum* and *Chaetomium* sp.), obtained from Egyptian medicinal plants, were selected as biological sources. The fungi were grown in liquid Wickerham medium as well as in solid rice medium for a period of three to four weeks. The extracts obtained were then subjected to different chromatographic separation techniques in order to isolate the secondary metabolites.

Structure elucidation of secondary metabolites was performed using state-of-the-art analytical techniques, including mass spectrometry (MS) and nuclear magnetic resonance (NMR) experiments. In addition, in the case of selected optically active natural products, chiral derivatisation methods were applied in order to determine their absolute configuration. Finally, the isolated compounds were subjected to various bioassays to examine their anti-microbial, antifungal and cytotoxic activities as well as inhibitory profiles towards selected protein kinases and their potential suppression of *Staphylococcus epidermidis* biofilm formation.

1. *Alternaria* sp.

Three new alternariol derivatives were obtained from *Alternaria* sp., isolated from *Polygonum senegalense*. Moreover, four new compounds including desmethylaltenusin, 4`-epialtenuene, alterlactone and alternaric acid were isolated. The alternariol derivatives and some of the structurally related compounds showed high cytotoxic activity when tested against L5178Y mouse lymphoma cell line as well as pronounced protein kinase inhibitory activity

2. *Ampelomyces* sp.

Ampelomyces sp. was isolated from *Urospermum picroides*. Six new compounds were obtained from this fungal strain, including a new pyrone, two new isocoumarines, two new

sulphated anthraquinones and a new hexahydroanthronol. Desmethyldiaportinol, altersolanol A and methylalaternin showed cytotoxic activity against L5178Y cells. Moreover, altersolanol A and methylalaternin inhibited *S. epidermidis* biofilm formation.

3. *Stemphylium botryosum*

The fungal strain *Stemphylium botryosum* was isolated from *Chenopodium album*. From this fungus curvularin derivatives, showing high cytotoxic activity against L5178Y, were isolated.

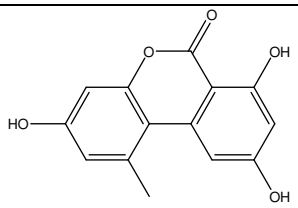
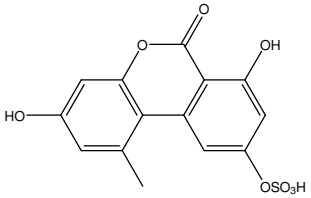
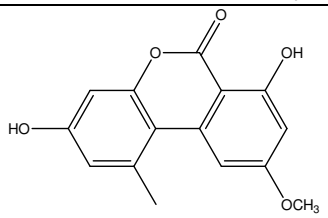
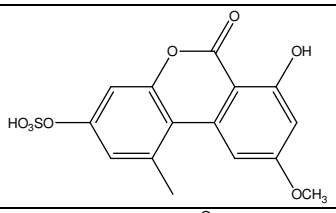
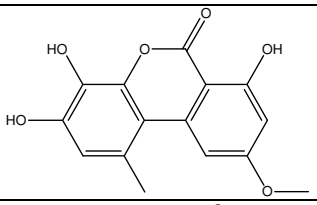
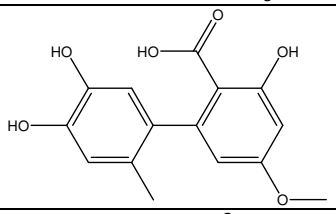
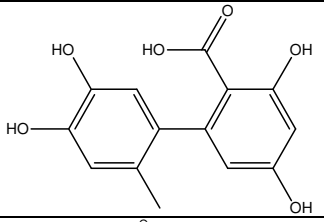
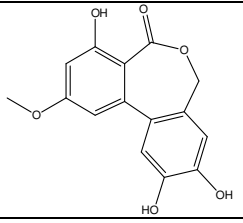
4. *Chaetomium* sp.

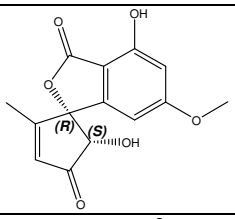
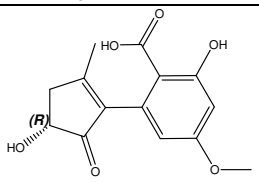
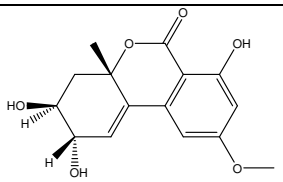
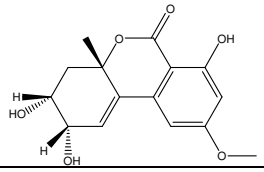
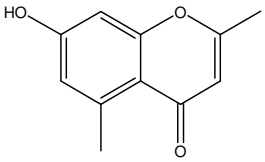
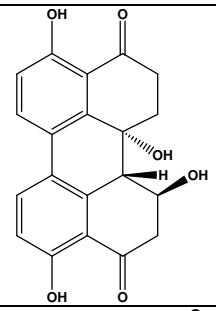
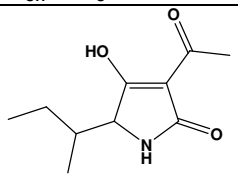
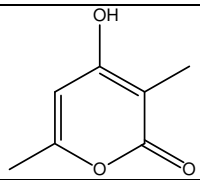
Finally, the fungal strain *Chaetomium* sp., isolated from *Otanthus maritimus*, was investigated. A new tetrahydrofuran derivative as well as known cochliodinol derivatives and orsellinic acid were obtained from extracts of this fungus. The cochliodinol derivatives inhibited various protein kinases tested in the bioassay. Furthermore, cochliodinol and orsellinic acid showed high cytotoxic activity when tested against L5178Y lymphoma cell line.

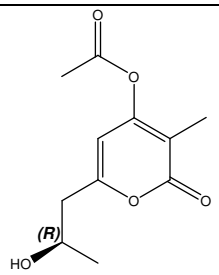
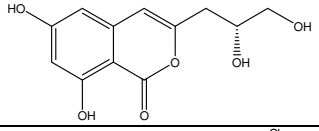
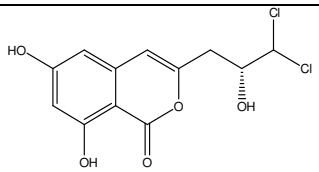
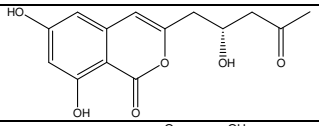
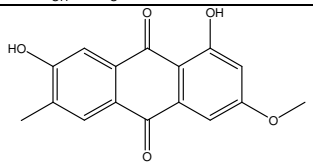
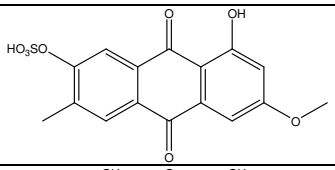
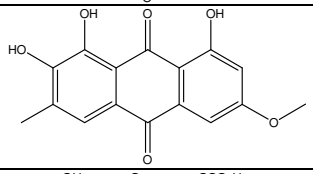
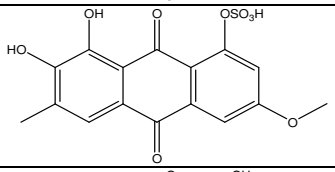
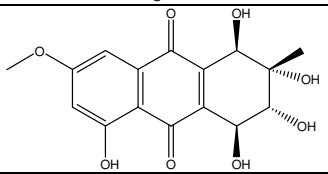
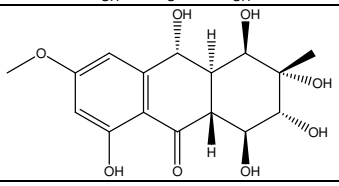
A total of forty-two compounds were isolated in this study, fourteen of which were identified as new natural products. Both known and new compounds were tested for their biological activities using different bioassay systems.

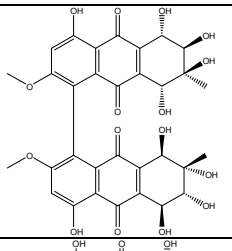
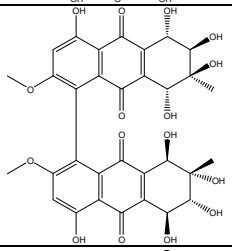
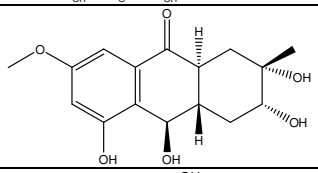
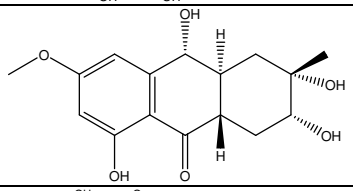
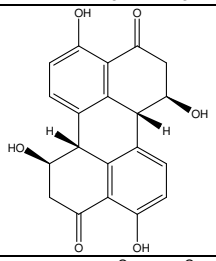
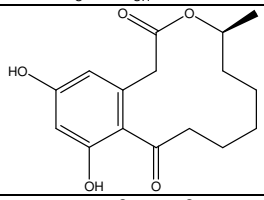
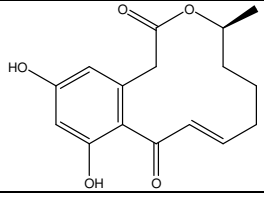
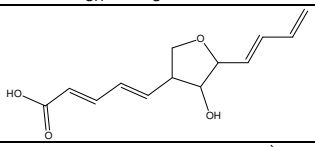
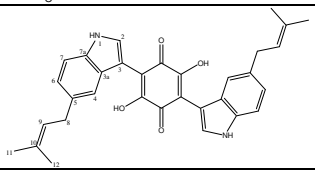
Furthermore, the fungal metabolites were traced in the corresponding host plant subfractions using LC/MS. None of secondary metabolites of the endophytic fungus *Chaetomium* sp. was found in any of the fractions of *O. maritimus*. On the other hand, major compounds of the remaining fungal extracts could unequivocally be detected in fractions of the respective host plants *P. senegalense*, *U. picroides* and *C. album*.

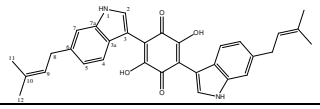
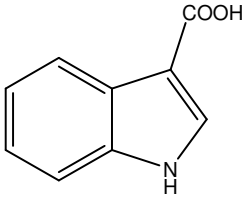
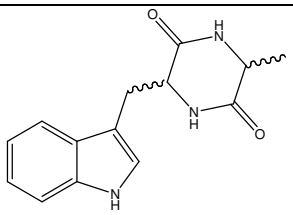
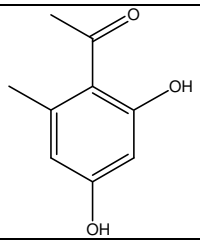
Table 5.1: Summary of the isolated compounds

Compound name	**Structure**	**Source**	**Comment**
Alternariol		*Alternaria* sp.	Known
Alternariol-5-*O*-sulphate		*Alternaria* sp.	New
Alternariol-5-*O*-methyl ether		*Alternaria* sp.	Known
Alternariol-5-*O*-methylether-4`-*O*-sulphate		*Alternaria* sp.	New
3`-Hydroxyalternariol-5-*O*-methylether		*Alternaria* sp.	New
Altenusin		*Alternaria* sp.	Known
Desmethylaltenusin		*Alternaria* sp.	New
Alterlactone		*Alternaria* sp.	New

Compound name	**Structure**	**Source**	**Comment**
Talaroflavone		*Alternaria* sp.	Known
Alternaric acid		*Alternaria* sp.	New
Altenuene 4`-Epialtenuene	 	*Alternaria* sp.	Known New
2,5-Dimethyl-7-hydroxychromone		*Alternaria* sp.	Known
Altertoxin I		*Alternaria* sp.	Known
Tenuazonic acid		*Alternaria* sp.	Known
Methyltriacetic lactone		*Ampelomyces* sp.	Known

Compound name	**Structure**	**Source**	**Comment**
Ampelopyrone		*Ampelomyces* sp.	New
Desmethyldiaportinol		*Ampelomyces* sp.	New
Desmethyldichlorodiaportin		*Ampelomyces* sp.	New
Citreoisocoumarin		*Ampelomyces* sp.	Known
Macrosporin		*Ampelomyces* sp. and *Stemphylium botryosum*	Known
Macrosporin sulphate		*Ampelomyces* sp.	New
3-*O*-Methylalaternin		*Ampelomyces* sp.	Known
3-*O*-Methylalaternin sulphate		*Ampelomyces* sp.	New
Altersolanol A		*Ampelomyces* sp. and *Stemphylium botryosum*	Known
Ampelanol		*Ampelomyces* sp.	New

Compound name	**Structure**	**Source**	**Comment**
Alterporriol D		*Ampelomyces* sp.	Known
Alterporriol E (atropsiomer of alterporriol D)		*Ampelomyces* sp.	Known
Altersolanol J		*Ampelomyces* sp.	Known
Tetrahydroaltersolanol B		*Stemphylium botryosum*	Known
Stemphyperylenol		*Stemphylium botryosum*	Known
Curvularin		*Stemphylium botryosum*	Known
Dehydrocurvularin		*Stemphylium botryosum*	Known
Aureonitolic acid		*Chaetomium* sp.	New
Cochliodinol		*Chaetomium* sp.	Known

Compound name	**Structure**	**Source**	**Comment**
Isocochliodinol		*Chaetomium* sp.	Known
Indole-3-carboxylic acid		*Chaetomium* sp.	Known
Cyclo(alanyltryptophane)		*Chaetomium* sp.	Known
Orsellinic acid		*Chaetomium* sp.	Known

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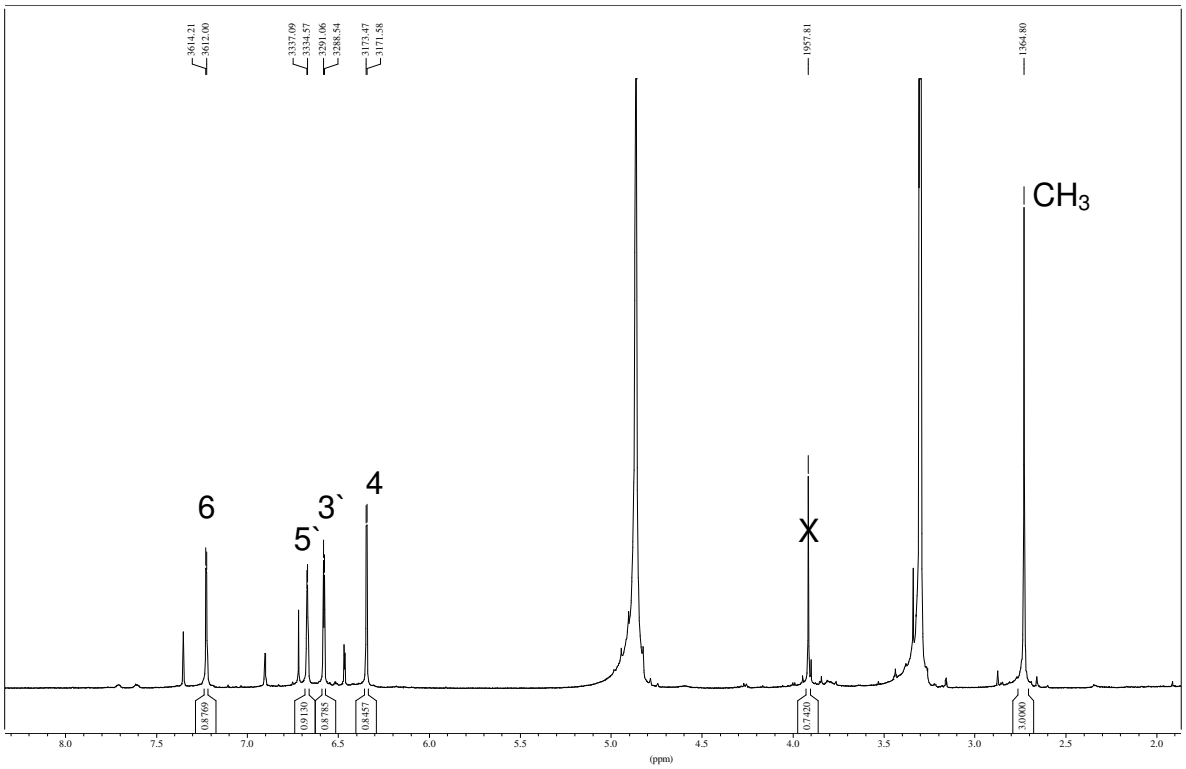
7. List of Abbreviations

$[\alpha]_D$	specific rotation at the sodium D-line
br	broad signal
$CDCl_3$	deuterated chloroform
$CHCl_3$	chloroform
CI	chemical ionization
COSY	correlation spectroscopy
d	doublet
DCM	dichloromethane
dd	doublet of doublet
DEPT	distortionless enhancement by polarization transfer
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ED	effective dose
EI	electron impact ionization
ESI	electrospray ionization
et al.	*et altera* (and others)
EtOAc	ethyl acetate
eV	electronvolt
FAB	fast atom bombardment
g	gram
HMBC	heteronuclear multiple bond connectivity
HMQC	heteronuclear multiple quantum coherence
H_2O	water
HPLC	high performance liquid chromatography
H_3PO_4	phosphoric acid
hr	hour
HR-MS	high resolution mass spectrometry
Hz	Herz
IZ	inhibition zone
L	liter
LC	liquid chromatography

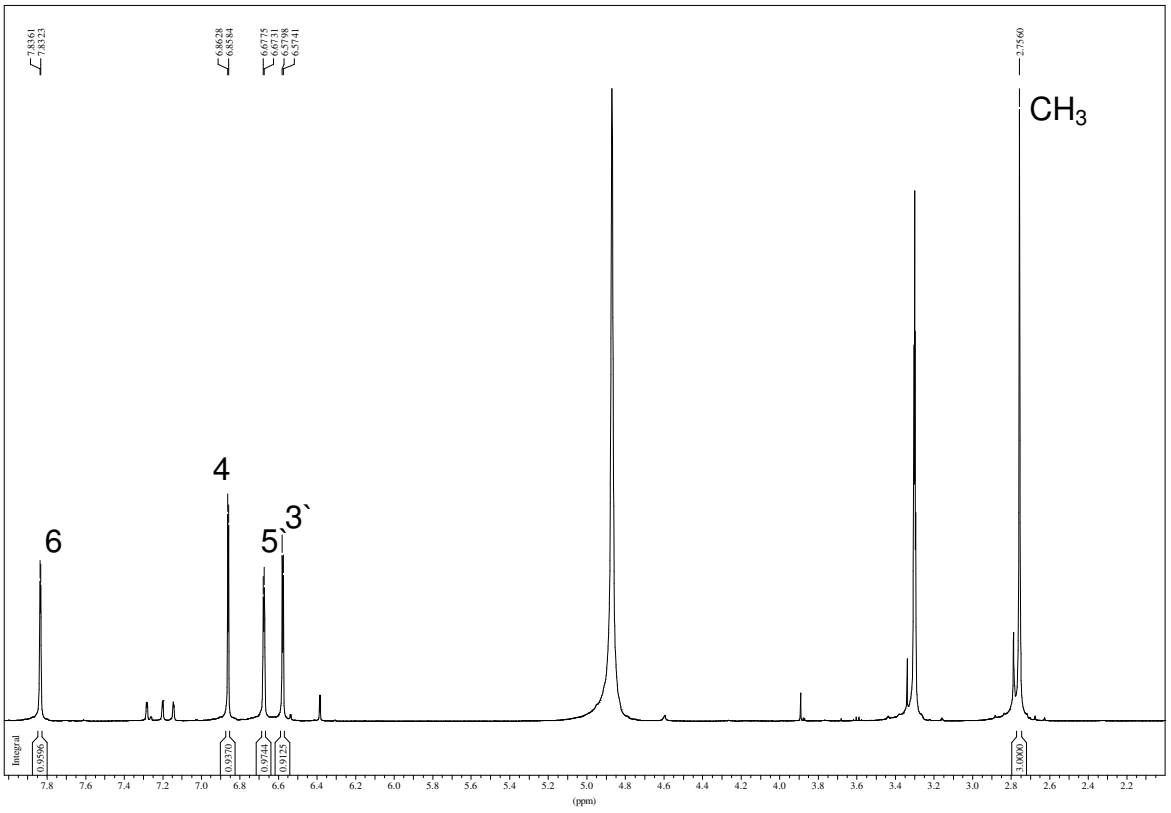
LC/MS	liquid chromatography-mass spectrometery
m	multiplet
M	molar
MeOD	deuterated methanol
MeOH	methanol
mg	milligram
MHz	mega Herz
min	minute
mL	milliliter
mm	millimeter
MS	mass spectrometry
MTT	microculture tetrazolium assay
m/z	mass per charge
µg	microgram
µL	microliter
µM	micromol
NaCl	sodium chloride
ng	nanogram
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser and exchange spectroscopy
PCR	polymerase chain reaction
ppm	parts per million
q	quartet
ROESY	rotating frame overhauser enhancement spectroscopy
RP 18	reversed phase C 18
s	singlet
t	triplet
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
UV	ultra-violet
VLC	vacuum liquid chromatography

8. Attachments

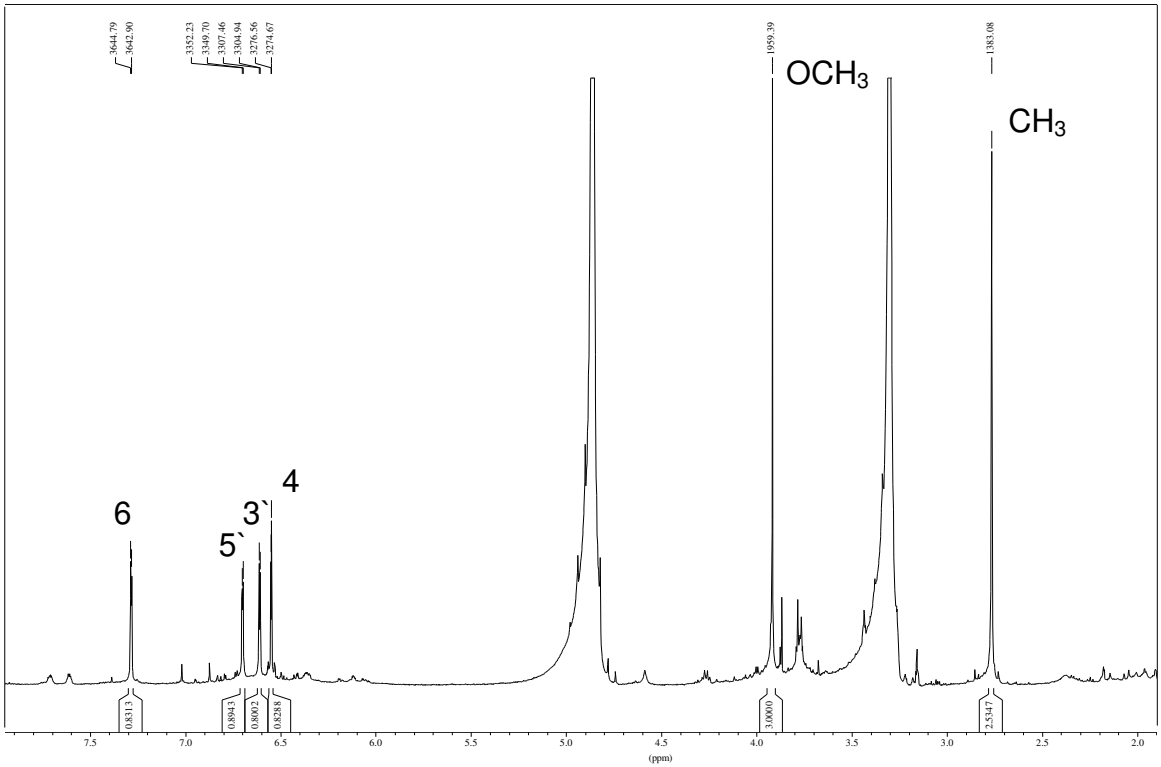
Attachment 1: The ^{1}H NMR spectrum of alternariol (**1**).



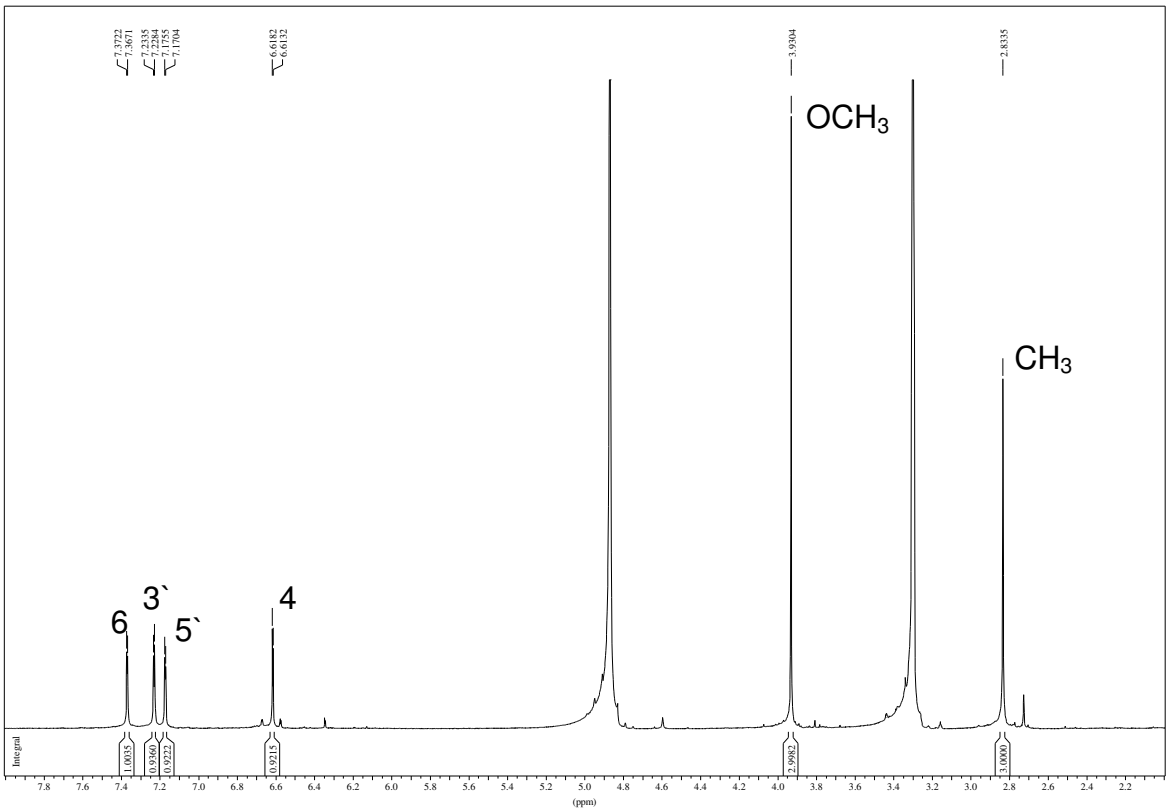
Attachment 2: The ^{1}H NMR spectrum of alternariol-5-*O*-sulphate (**2**).



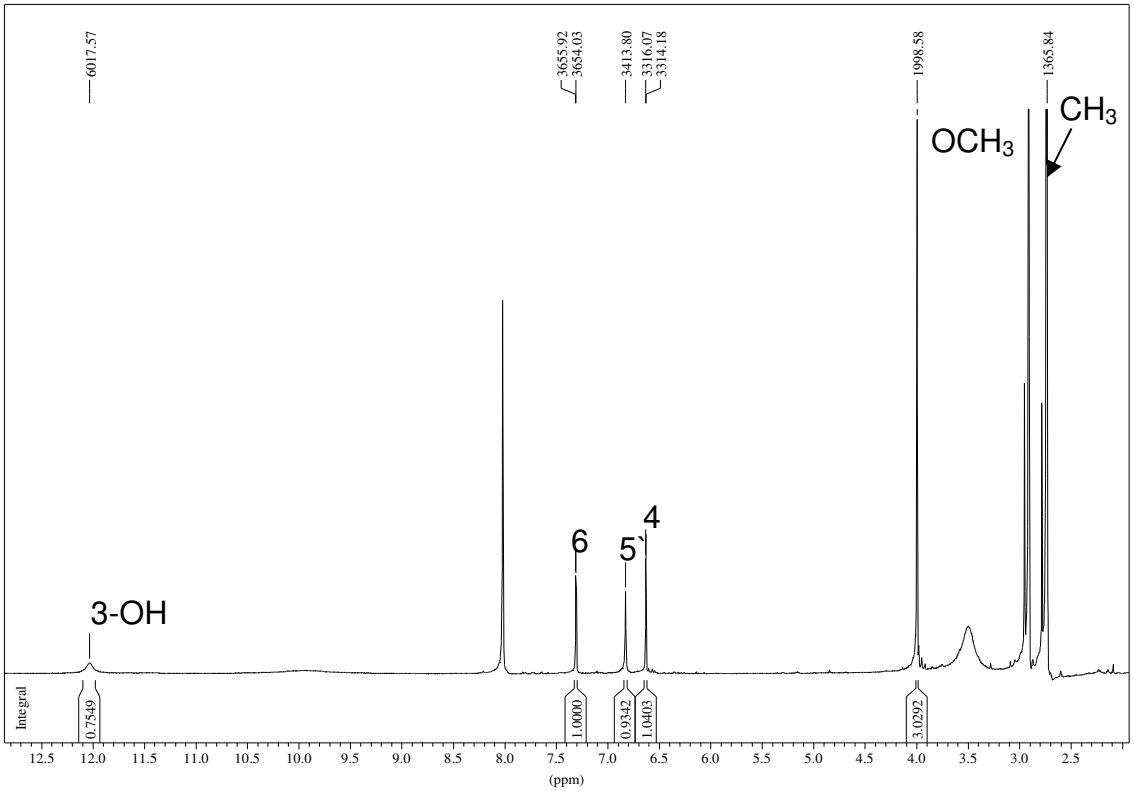
Attachment 3: The ^{1}H NMR spectrum of alternariol-5-*O*-methylether (**3**).



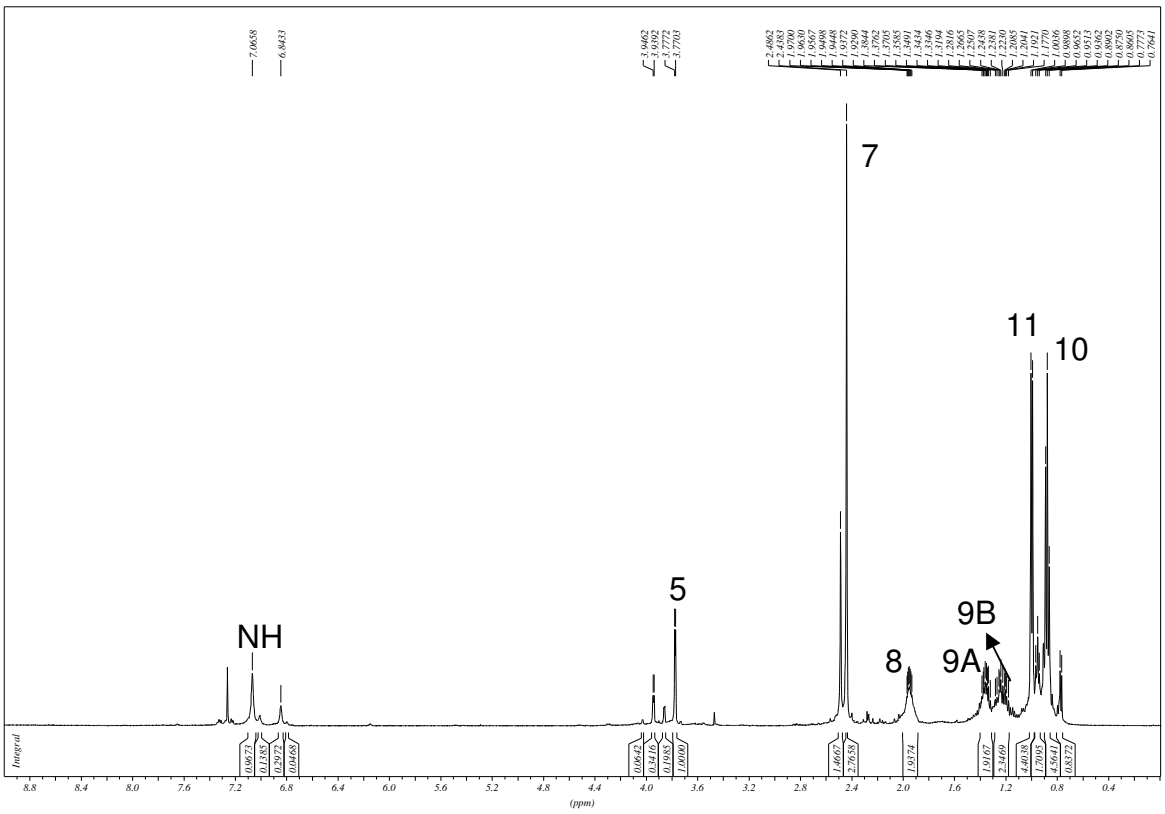
Attachment 4: The ^{1}H NMR spectrum of alternariol-5-*O*-methylether-4`-*O*-sulphate (**4**).



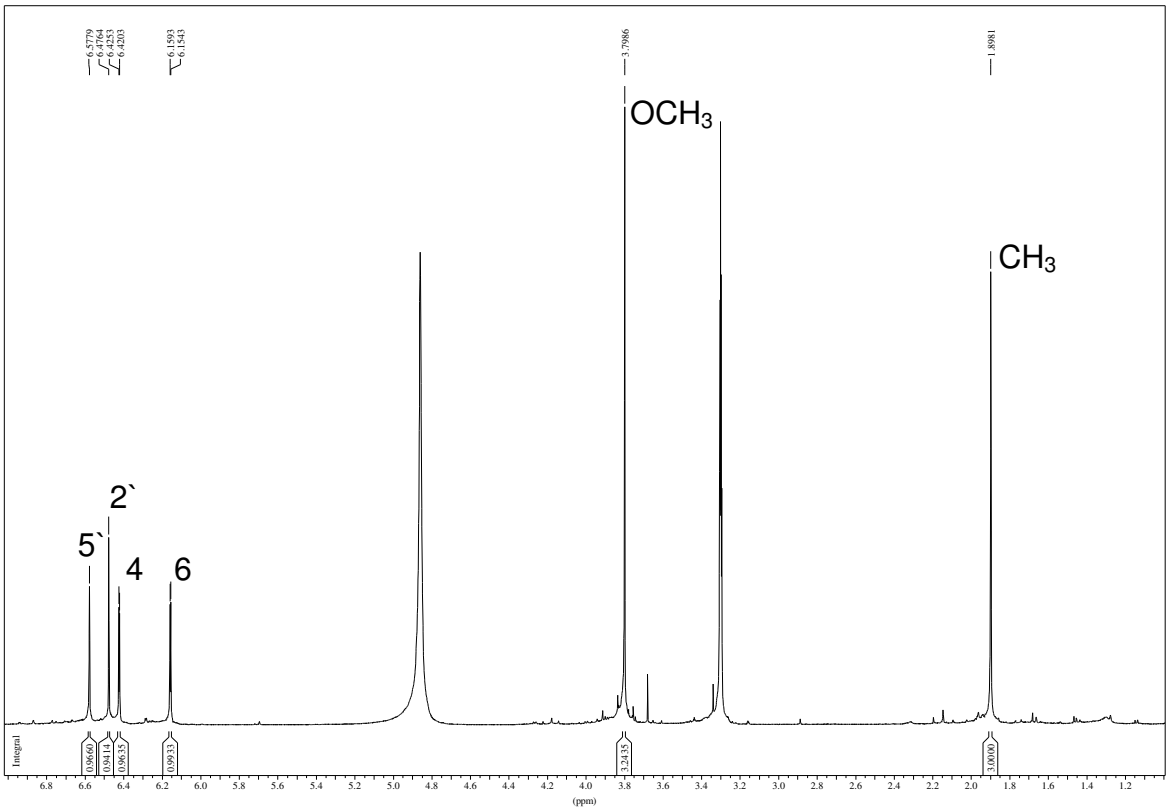
Attachment 5: The ^{1}H NMR spectrum of 3`-hydroxyalternariol-5-*O*-methylether (**5**).



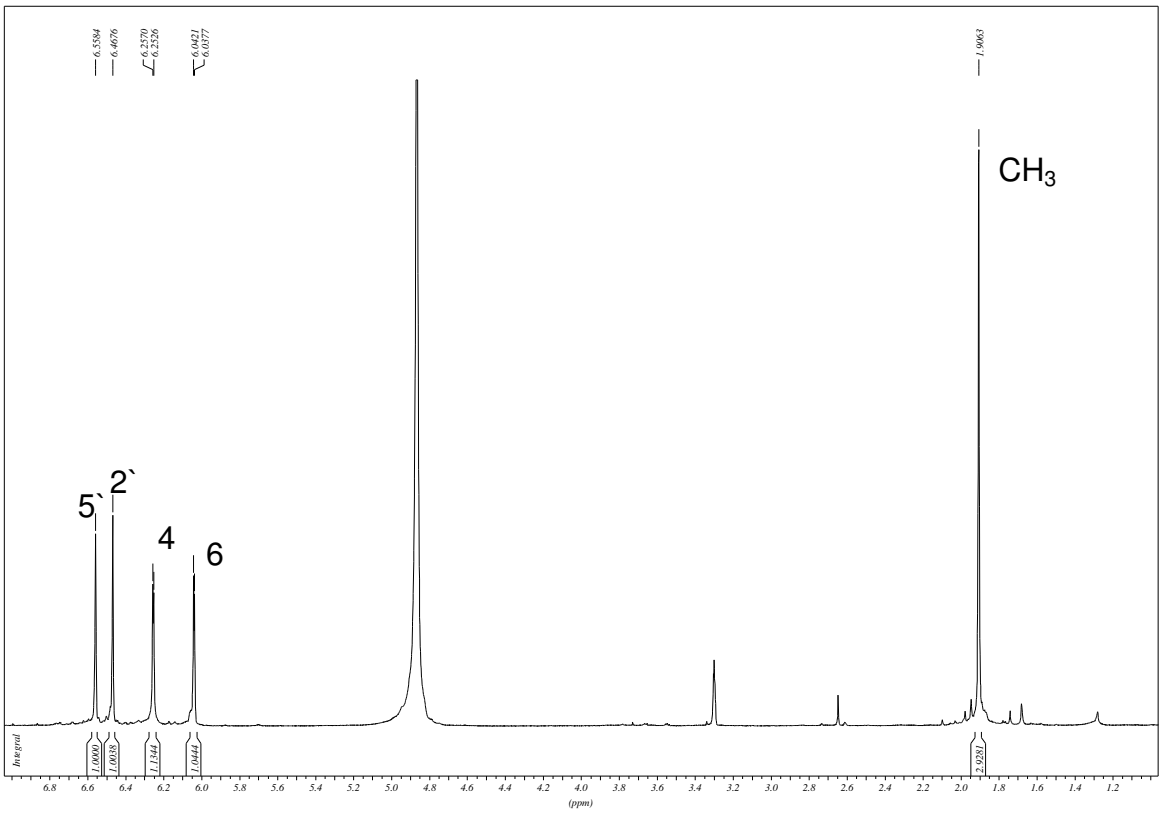
Attachment 6: The ^{1}H NMR spectrum of tenuazonic acid (**15**).



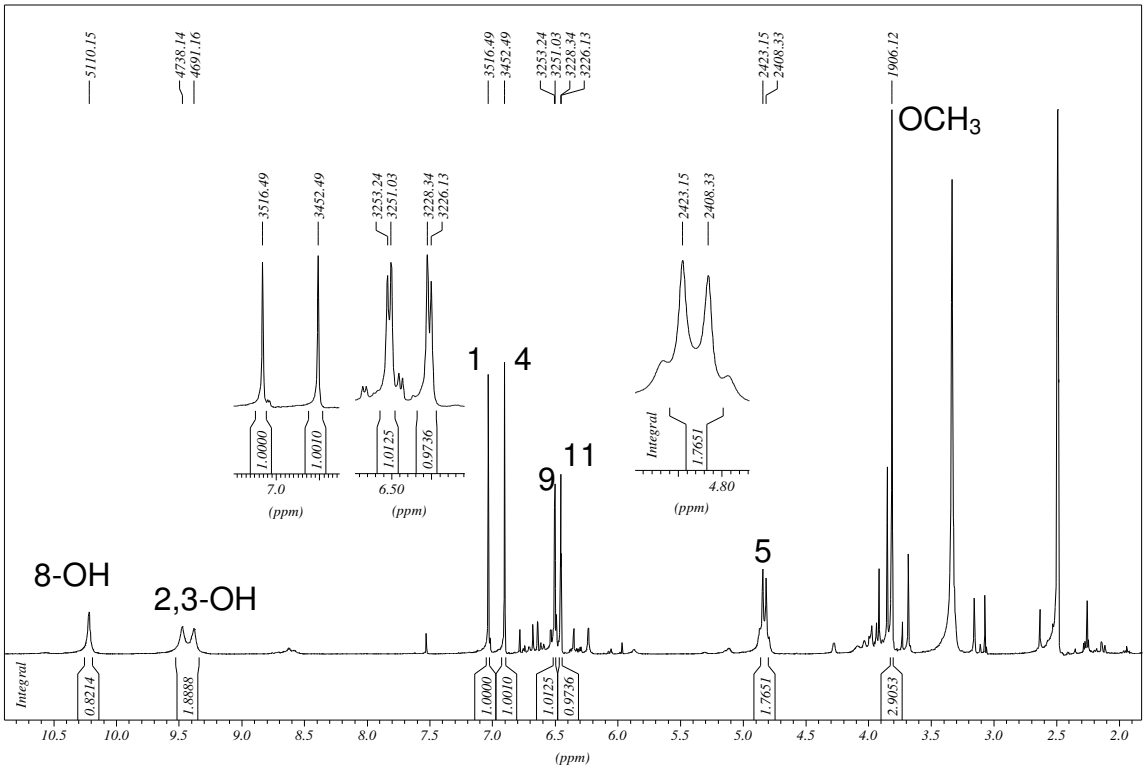
Attachment 7: The ^{1}H NMR spectrum of altenusin (**6**).



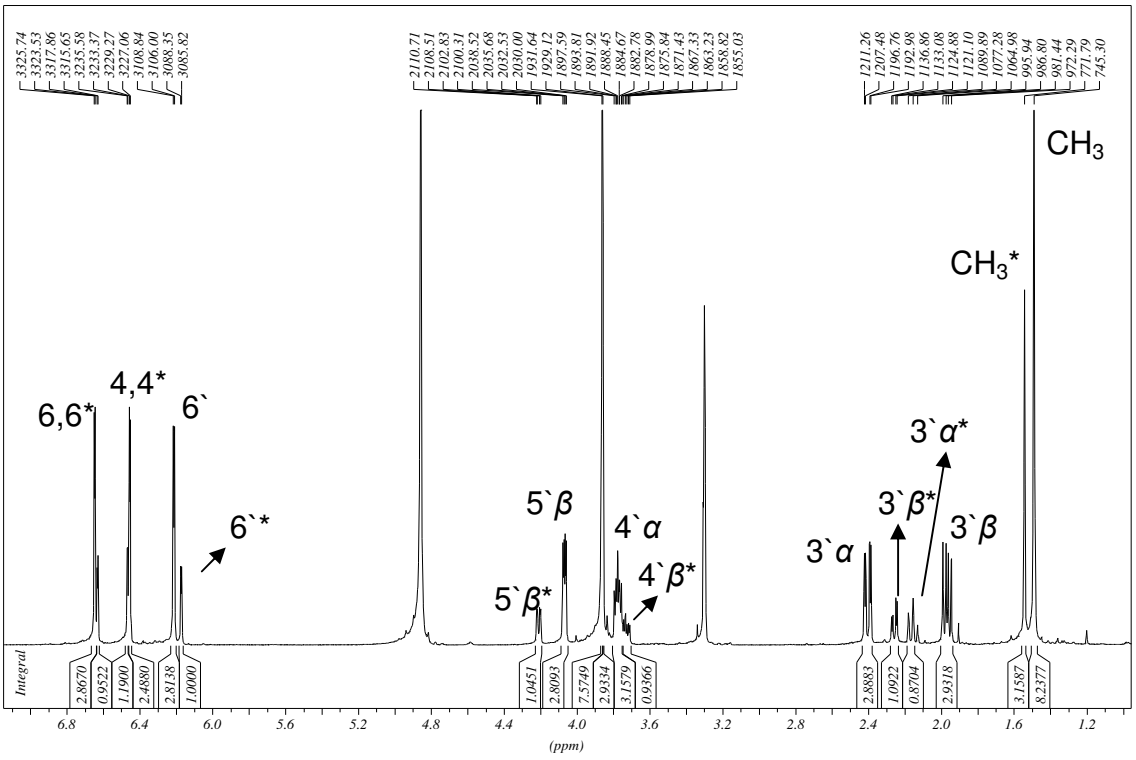
Attachment 8: The ^{1}H NMR spectrum of desmethylaltenusin (**7**).



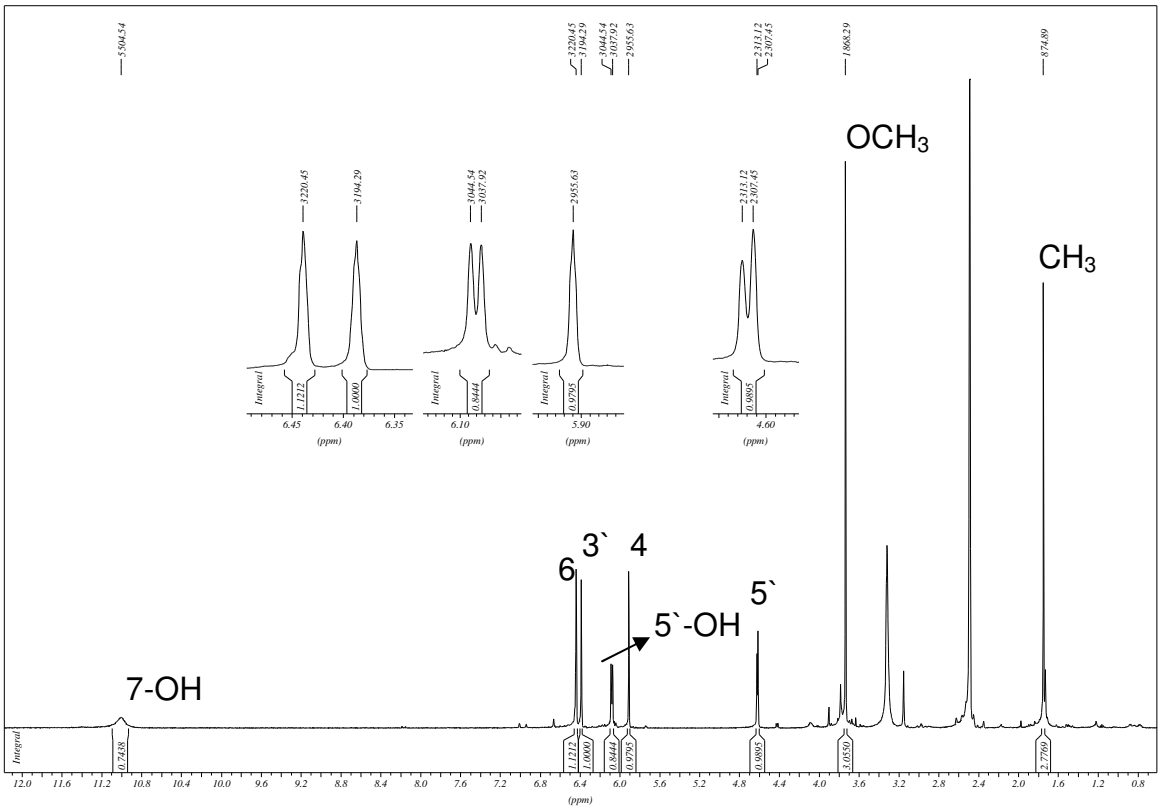
Attachment 9: The ^{1}H NMR spectrum of alterlactone (**8**).



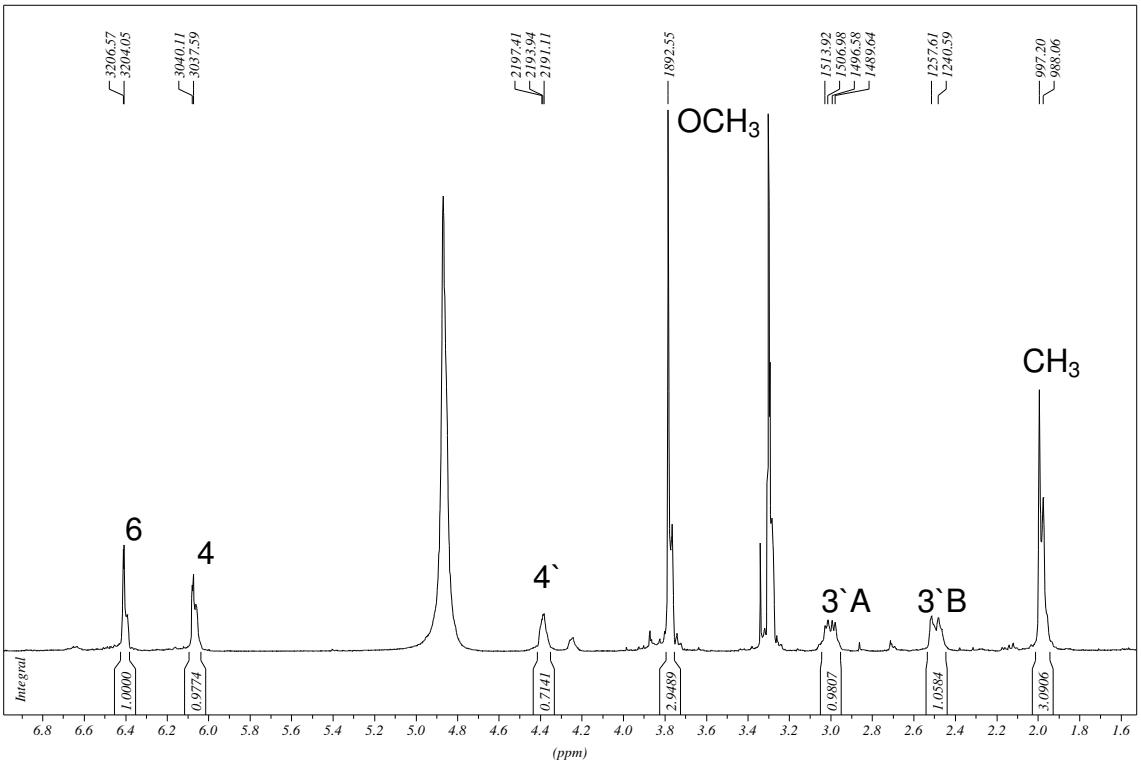
Attachment 10: The ^{1}H NMR spectrum of altenuene (**11**) and 4`-epialtenuene (**12***).



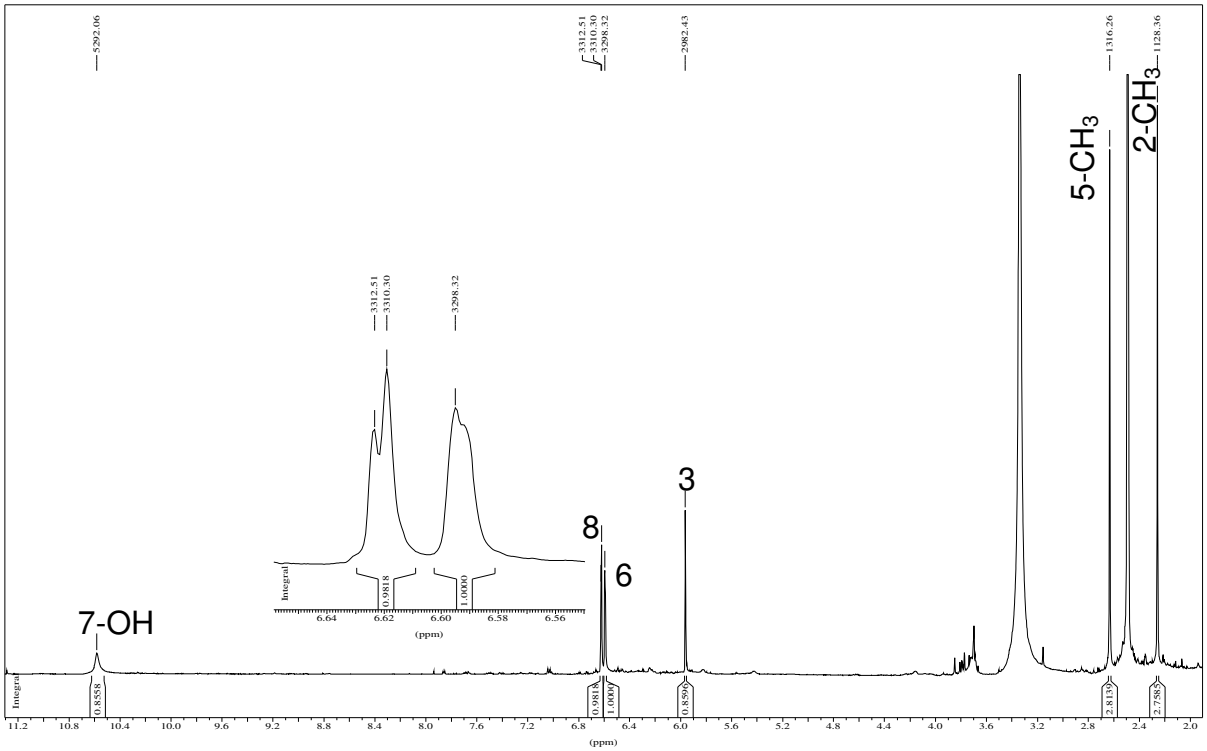
Attachment 11: The ^{1}H NMR spectrum of talaroflavone (**9**).



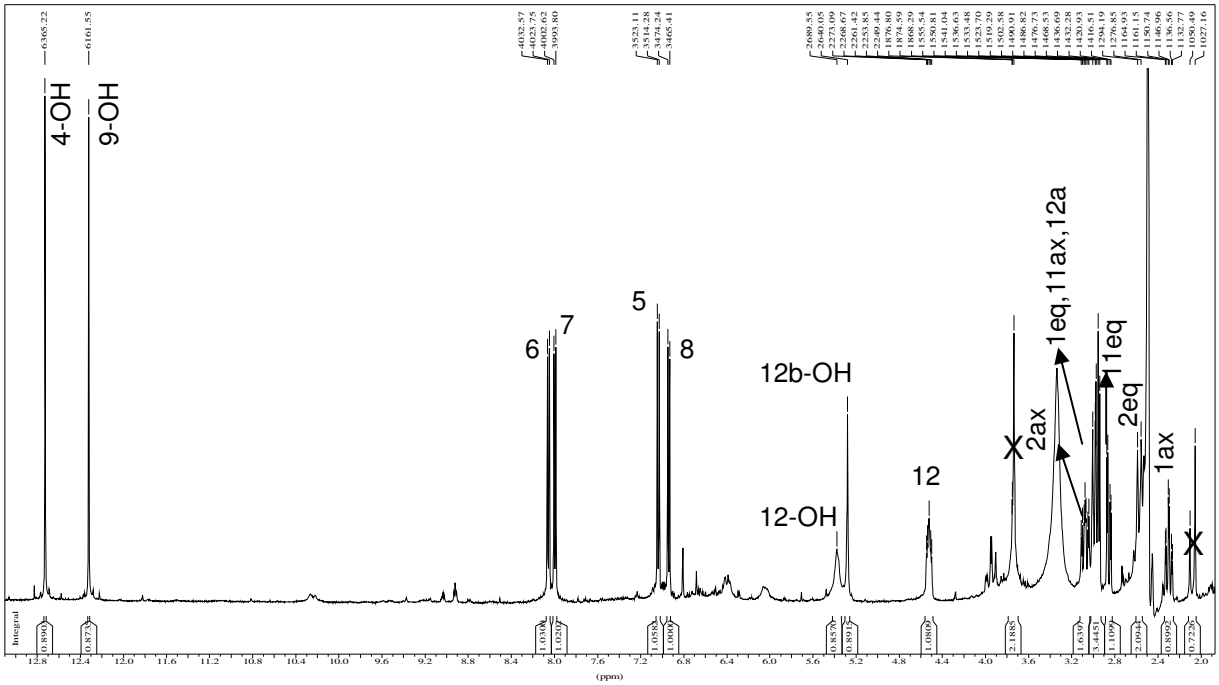
Attachment 12: The ^{1}H NMR spectrum of alternaric acid (**10**).



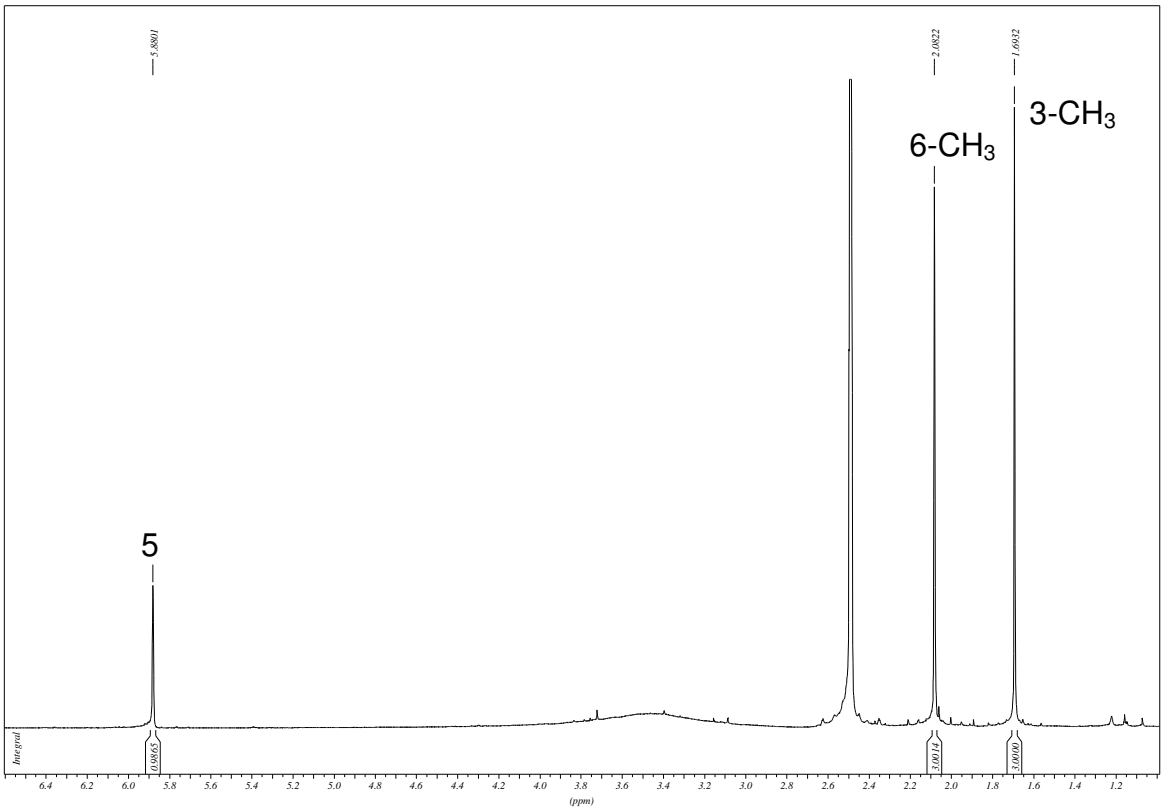
Attachment 13: The ^{1}H NMR spectrum of 2,5-dimethyl-7-hydroxychromone (**13**).



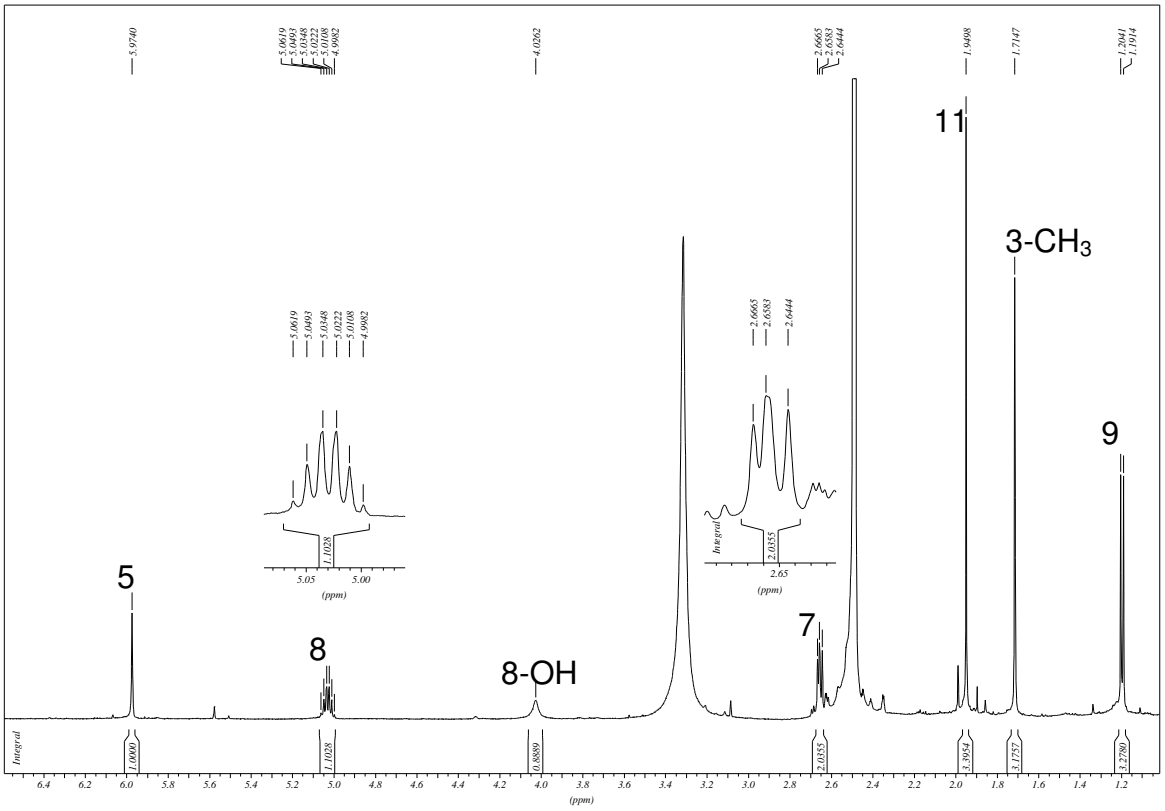
Attachment 14: The ^{1}H NMR spectrum of altertoxin I (**14**).



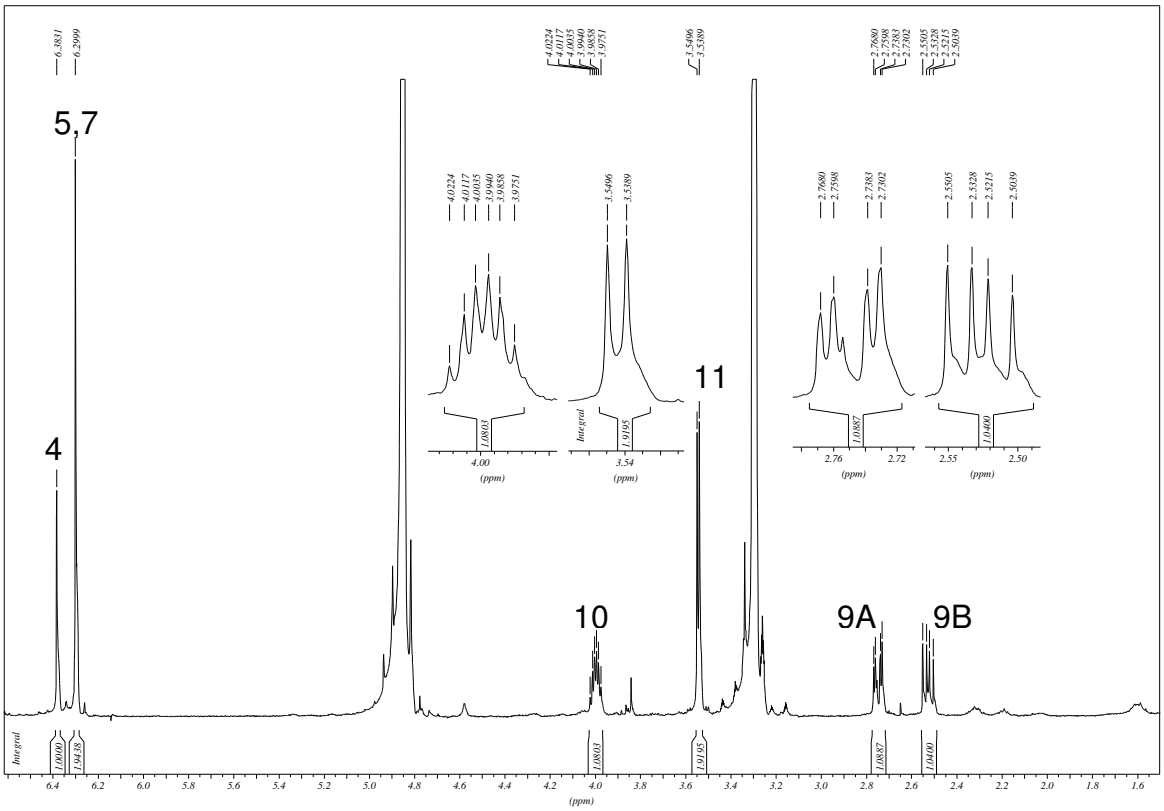
Attachment 15: The ^{1}H NMR spectrum of methyltriacetic lactone (**16**).



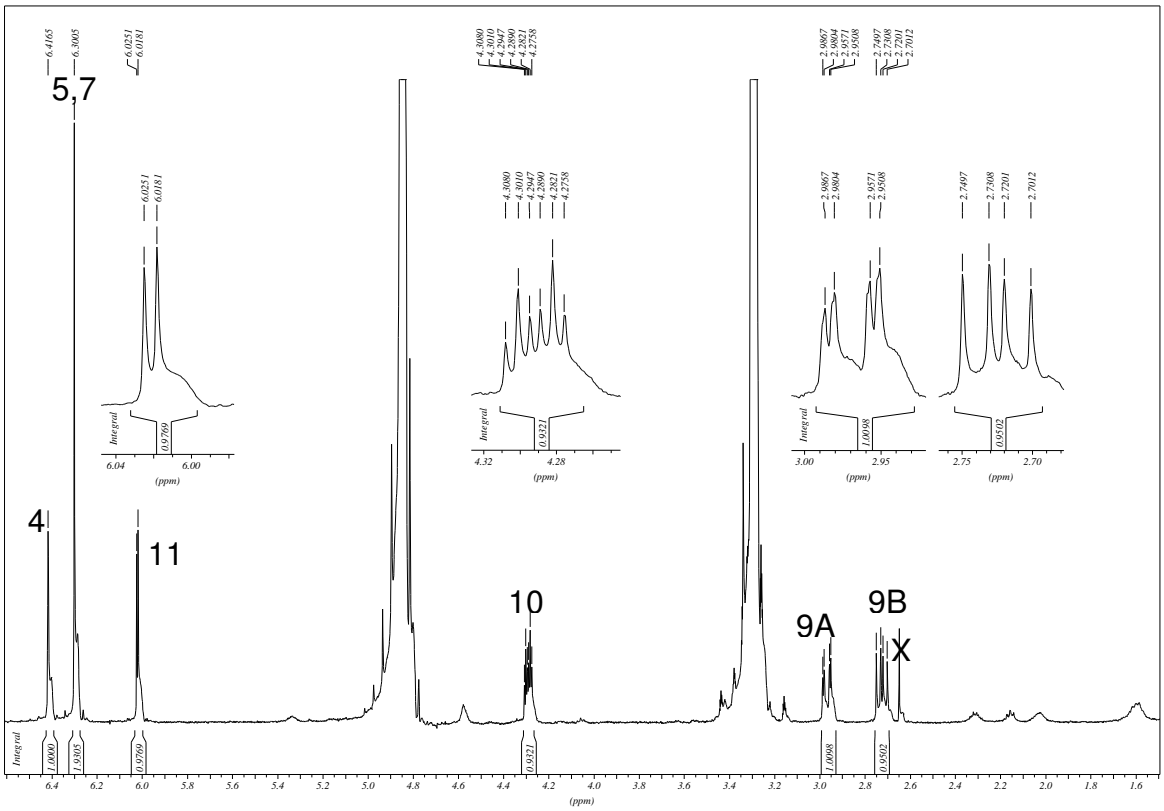
Attachment 16: The ^{1}H NMR spectrum of ampelopyrone (**17**).



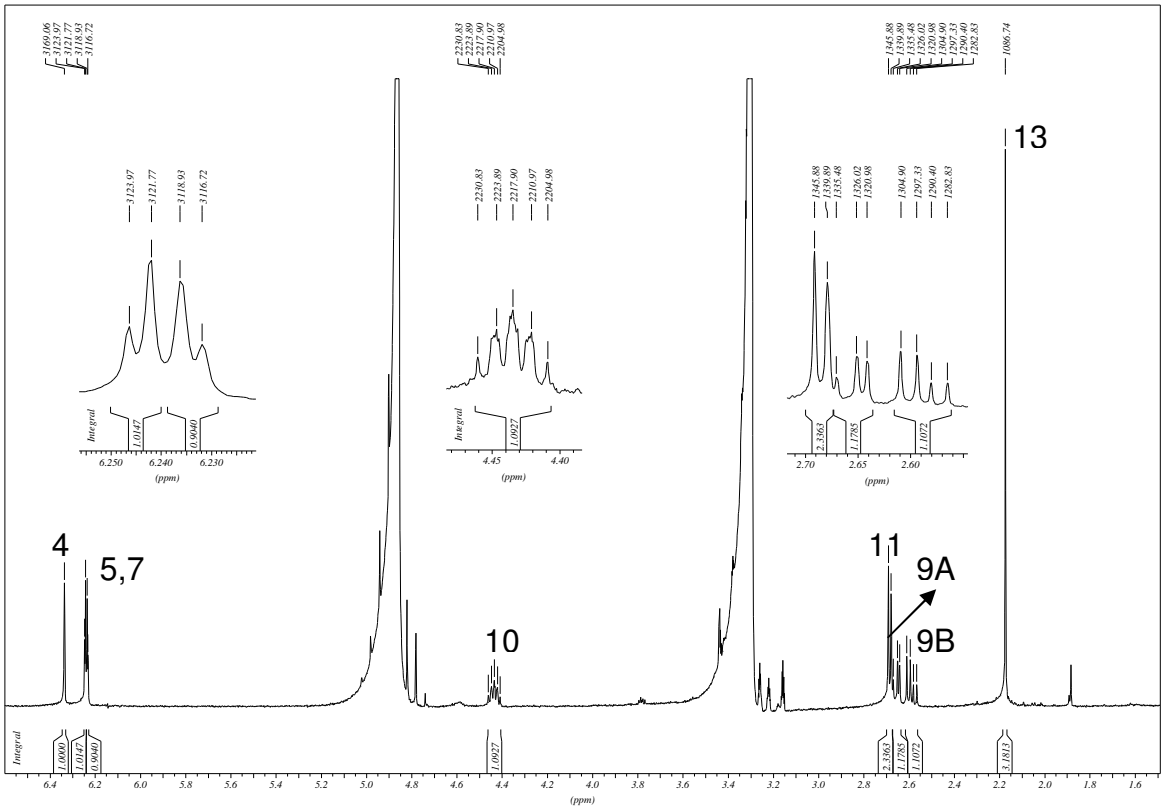
Attachment 17: The ^{1}H NMR spectrum of desmethyldiaportinol (**18**).



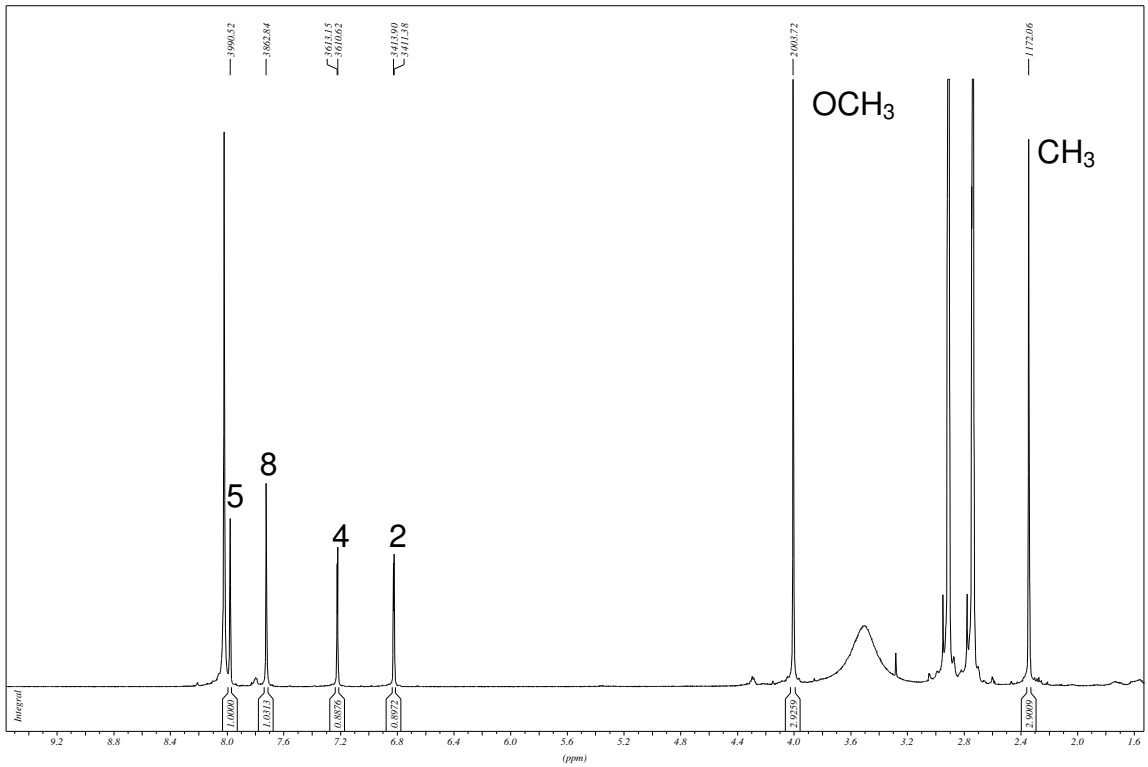
Attachment 18: The ^{1}H NMR spectrum of desmethyldichlorodiaportin (**19**).



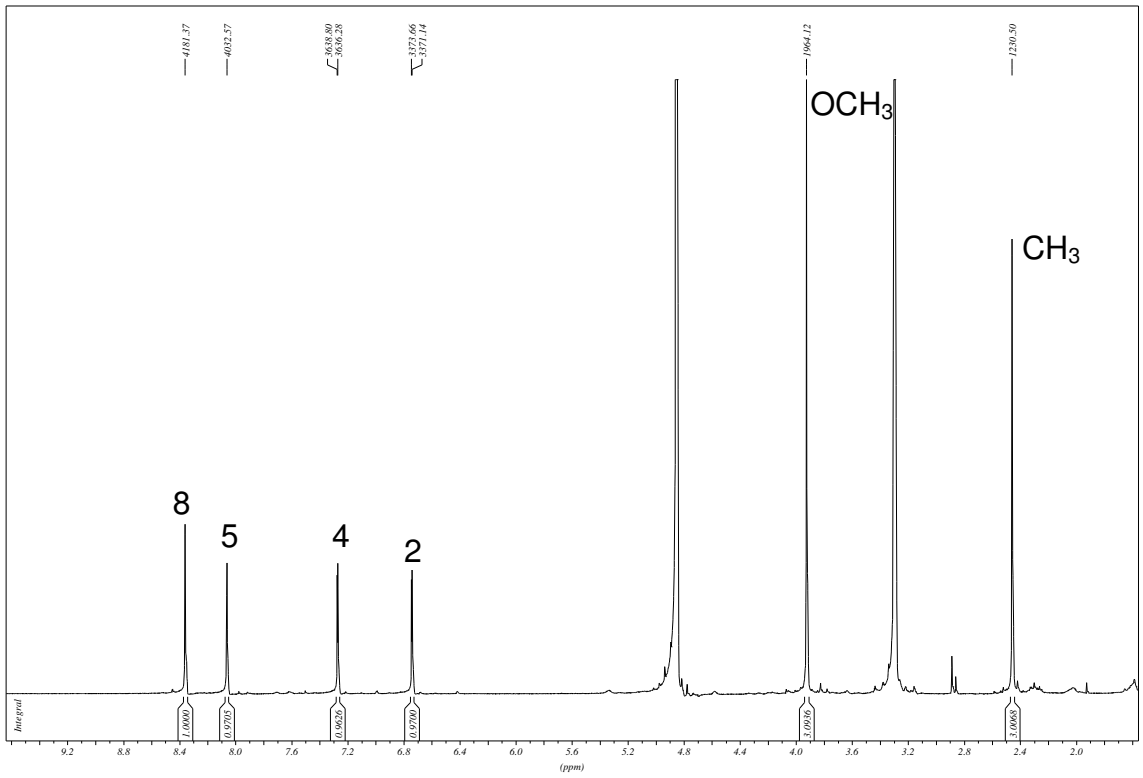
Attachment 19: The ^{1}H NMR spectrum of (+)citreoisocoumarin (**20**).



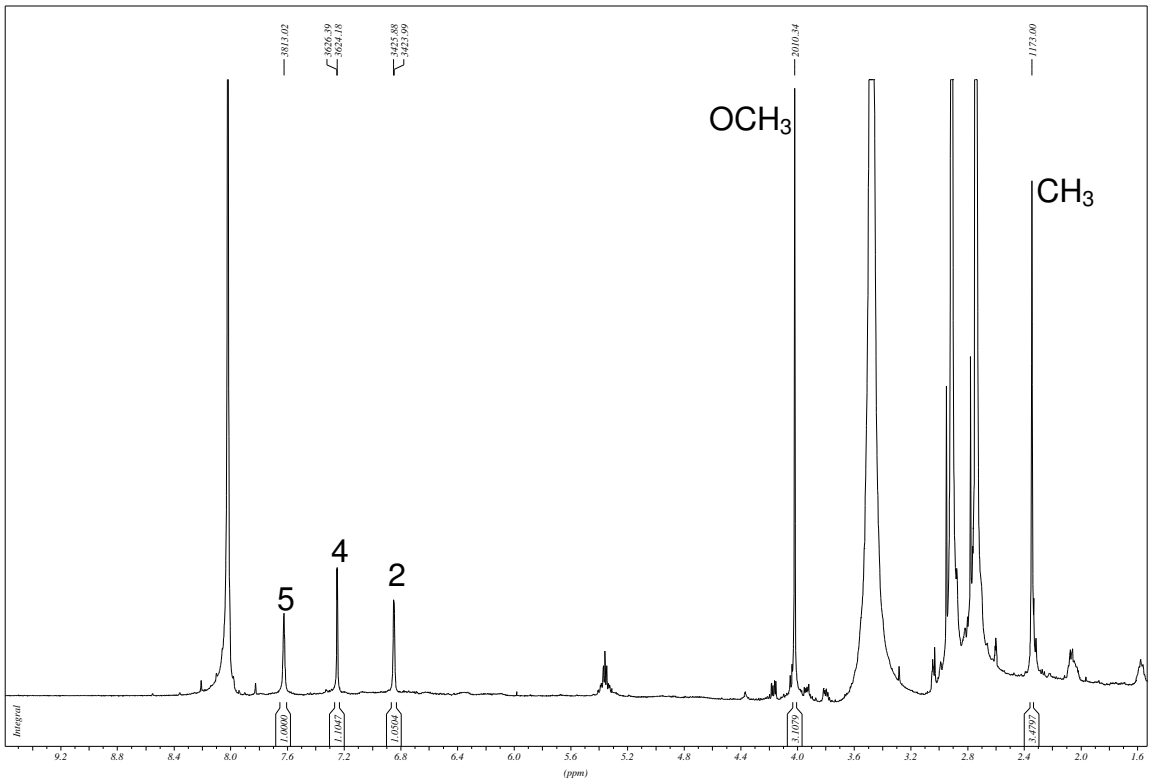
Attachment 20: The ^{1}H NMR spectrum of macrosporin (**21**).



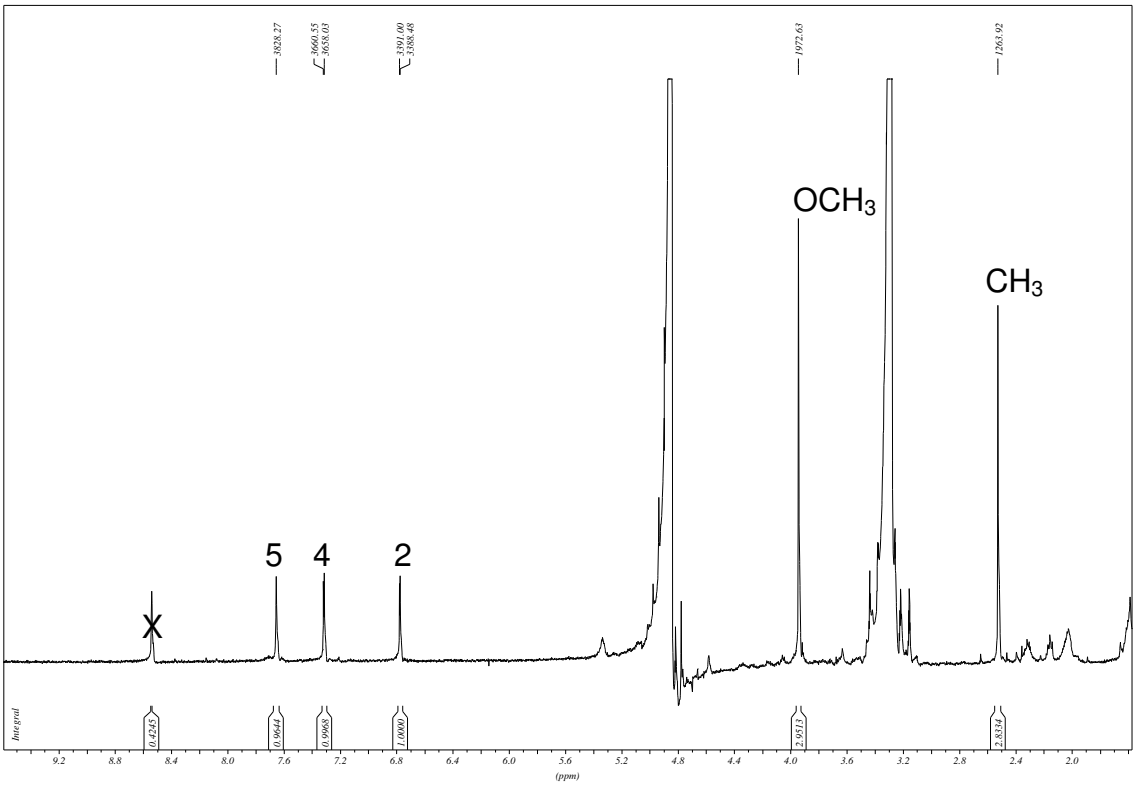
Attachment 21: The ^{1}H NMR spectrum of macrosporin-7-*O*-sulphate (**22**).



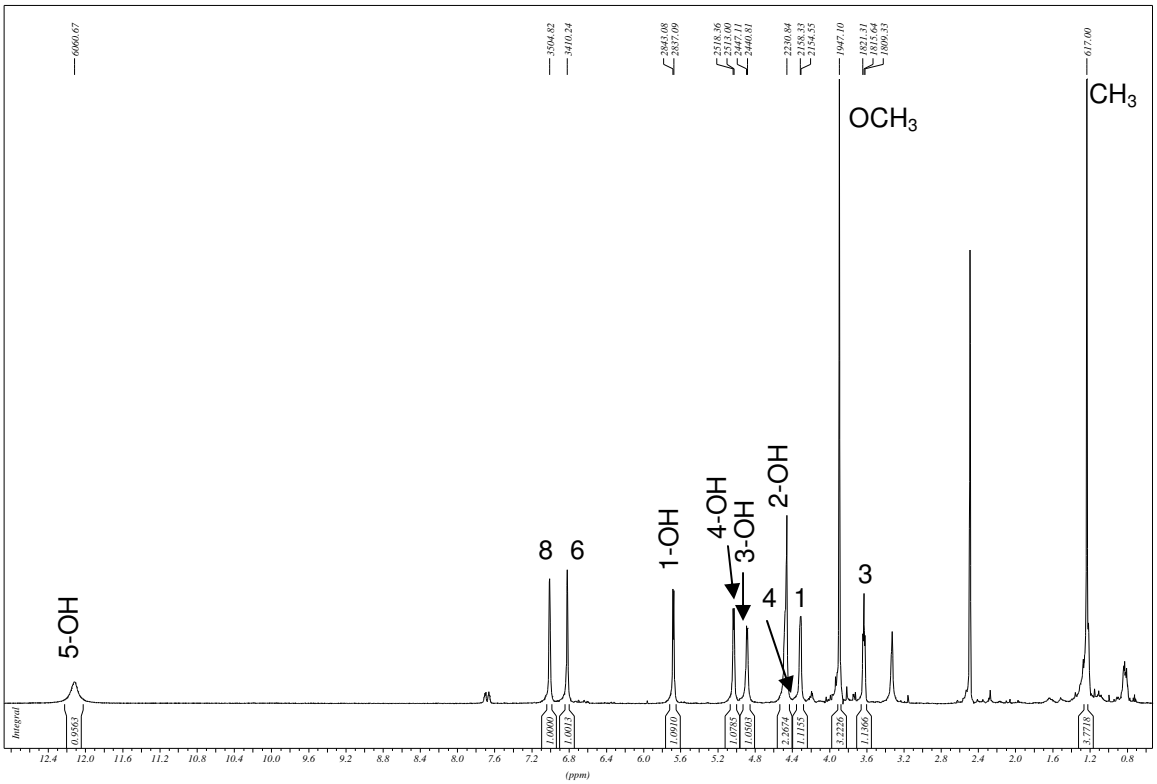
Attachment 22: The ^{1}H NMR spectrum of 3-*O*-methylalaternin (**23**).



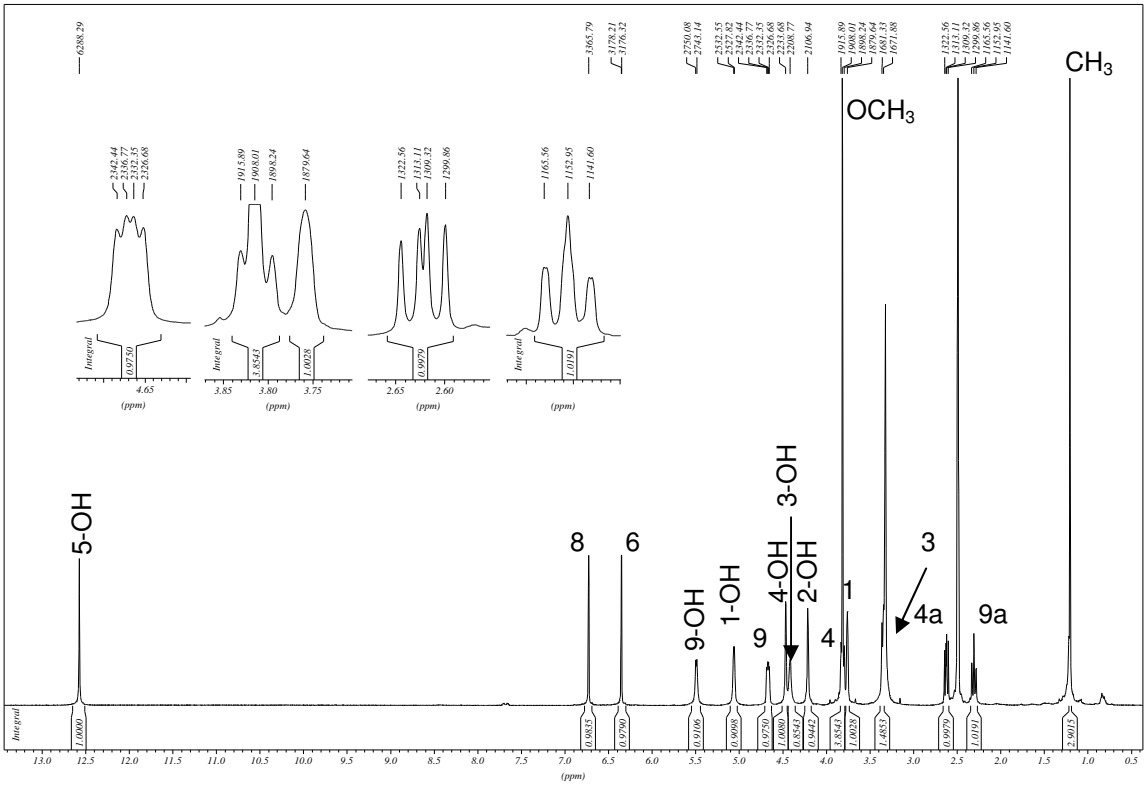
Attachment 23: The ^{1}H NMR spectrum of 3-*O*-methylalaternin-7-*O*-sulphate (**24**).



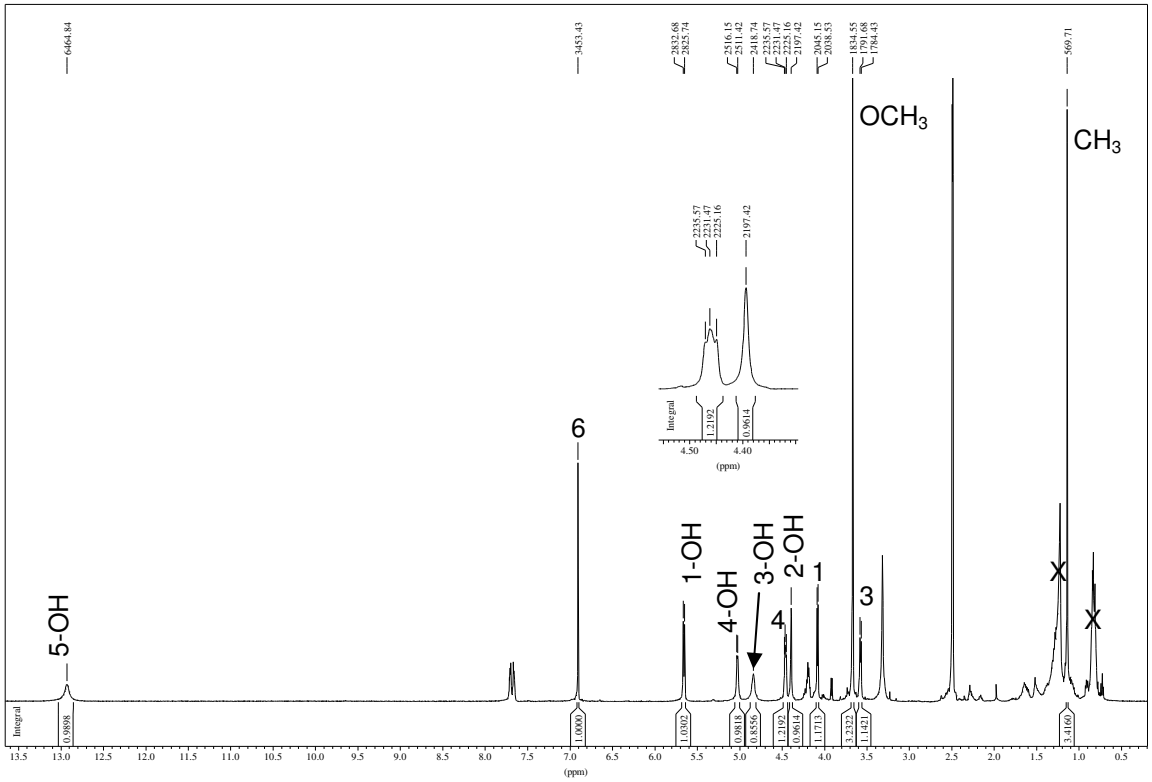
Attachment 24: The ^{1}H NMR spectrum of altersolanol A (**25**).



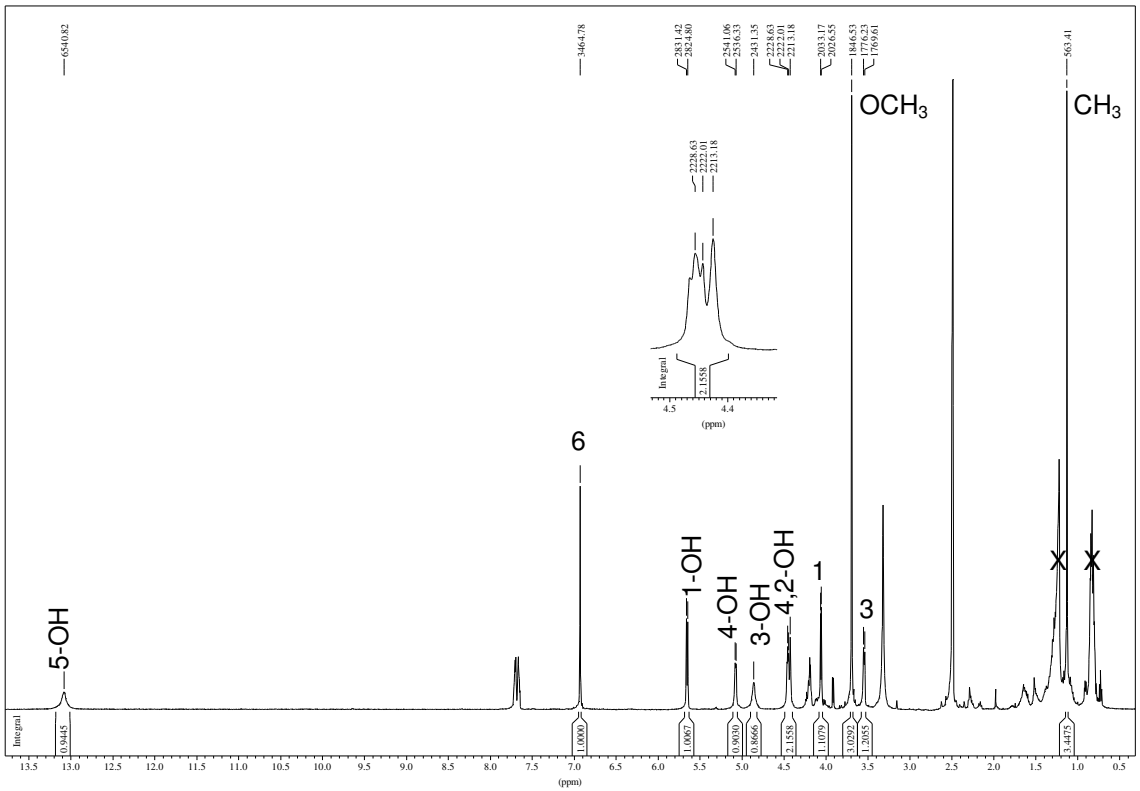
Attachment 25: The ^{1}H NMR spectrum of ampelanol (**26**).



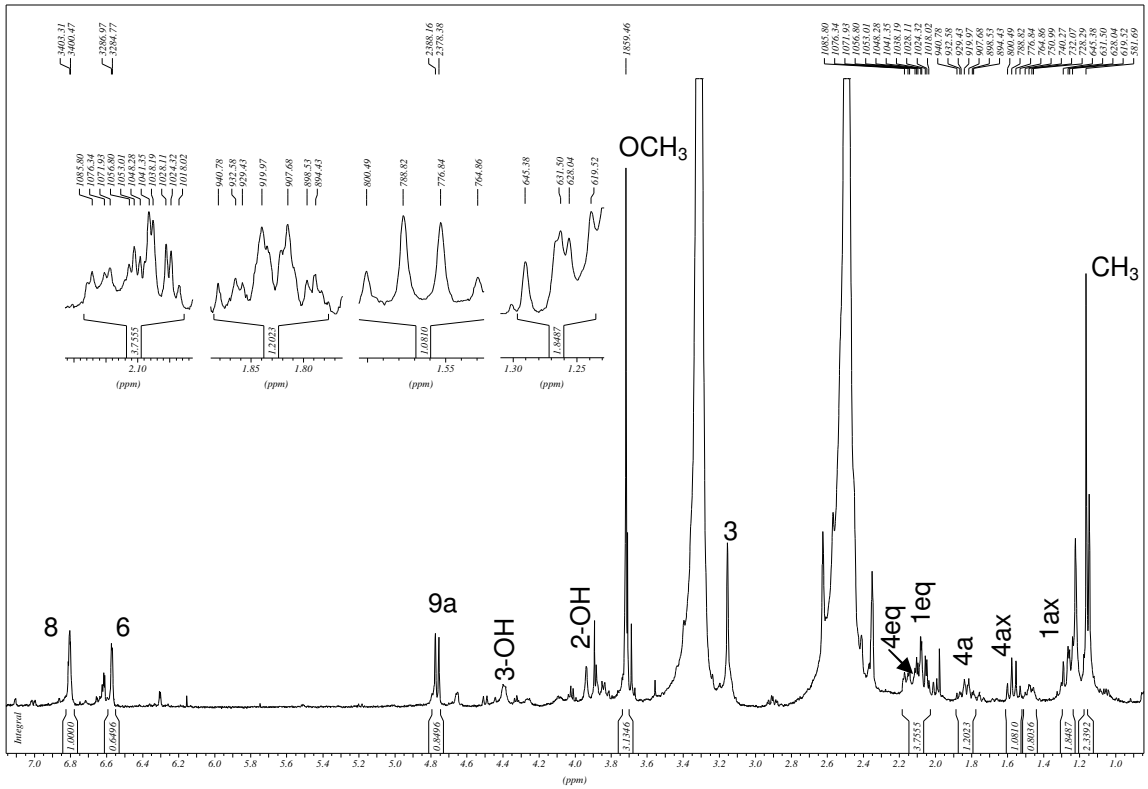
Attachment 26: The ^{1}H NMR spectrum of alterporriol D (**27**).



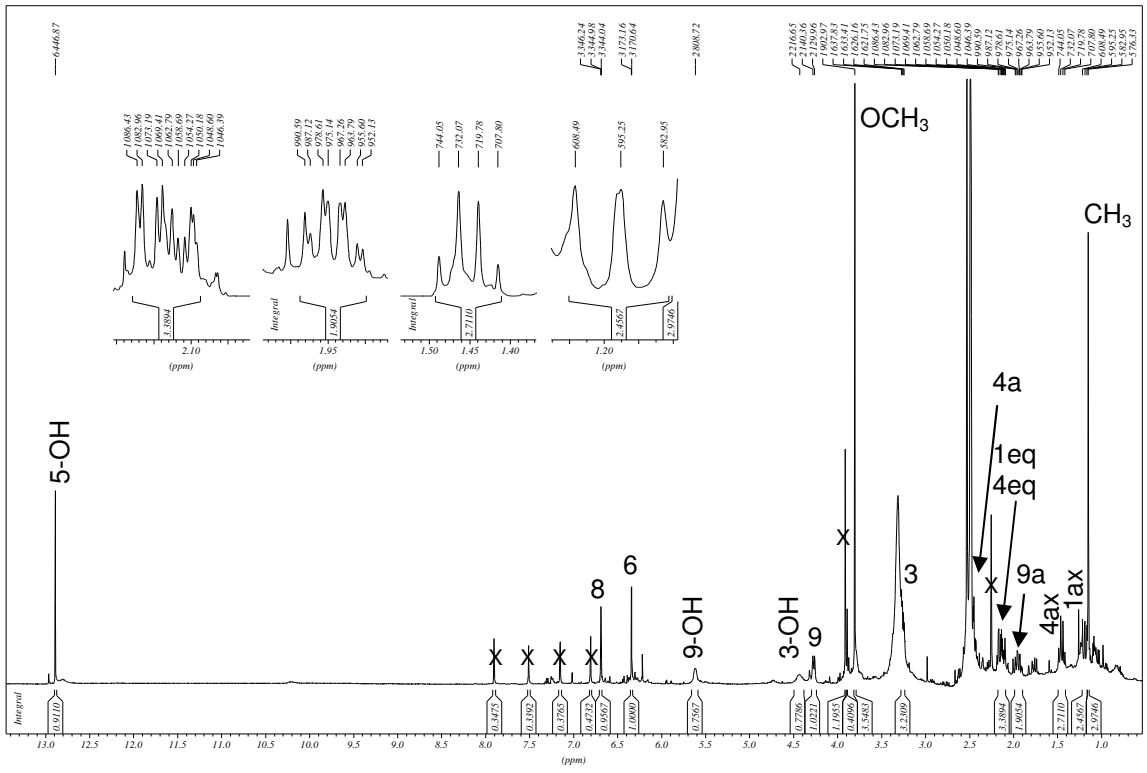
Attachment 27: The ^{1}H NMR spectrum of alterporriol E (**28**).



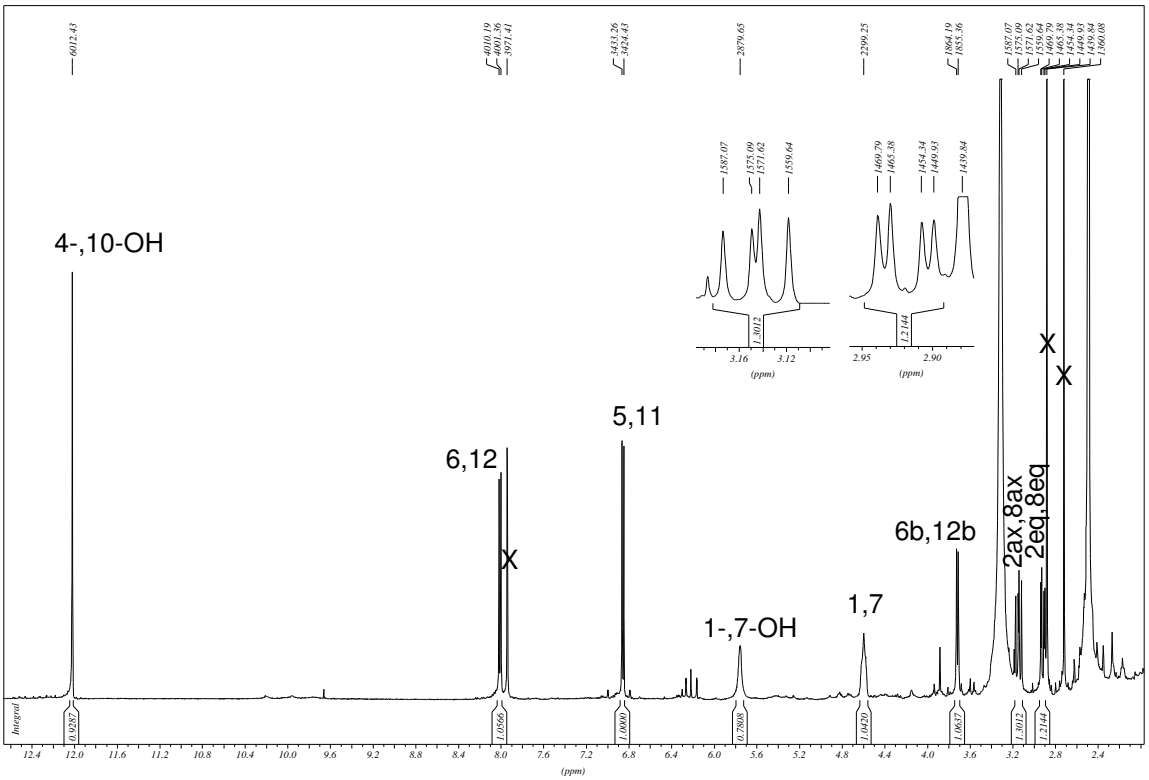
Attachment 28: The ^{1}H NMR spectrum of altersolanol J (**29**).



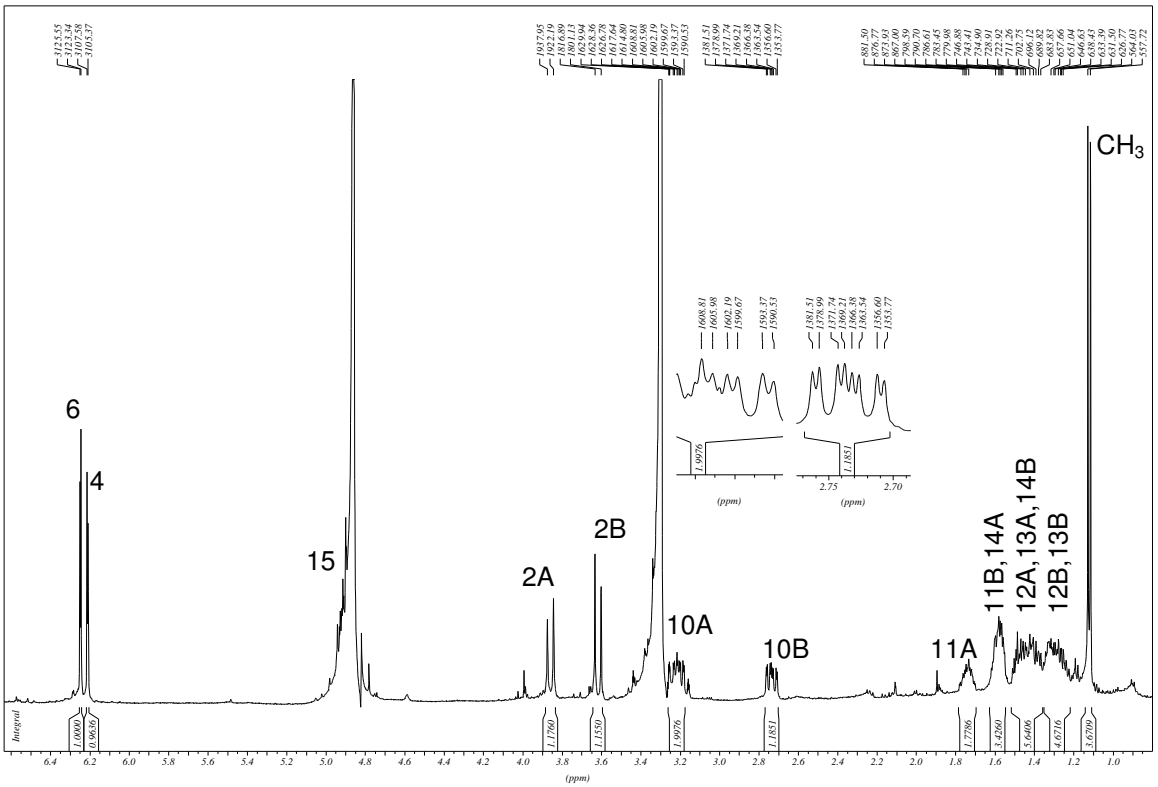
Attachment 29: The ^{1}H NMR spectrum of tetrahydroaltersolanol B (**30**).



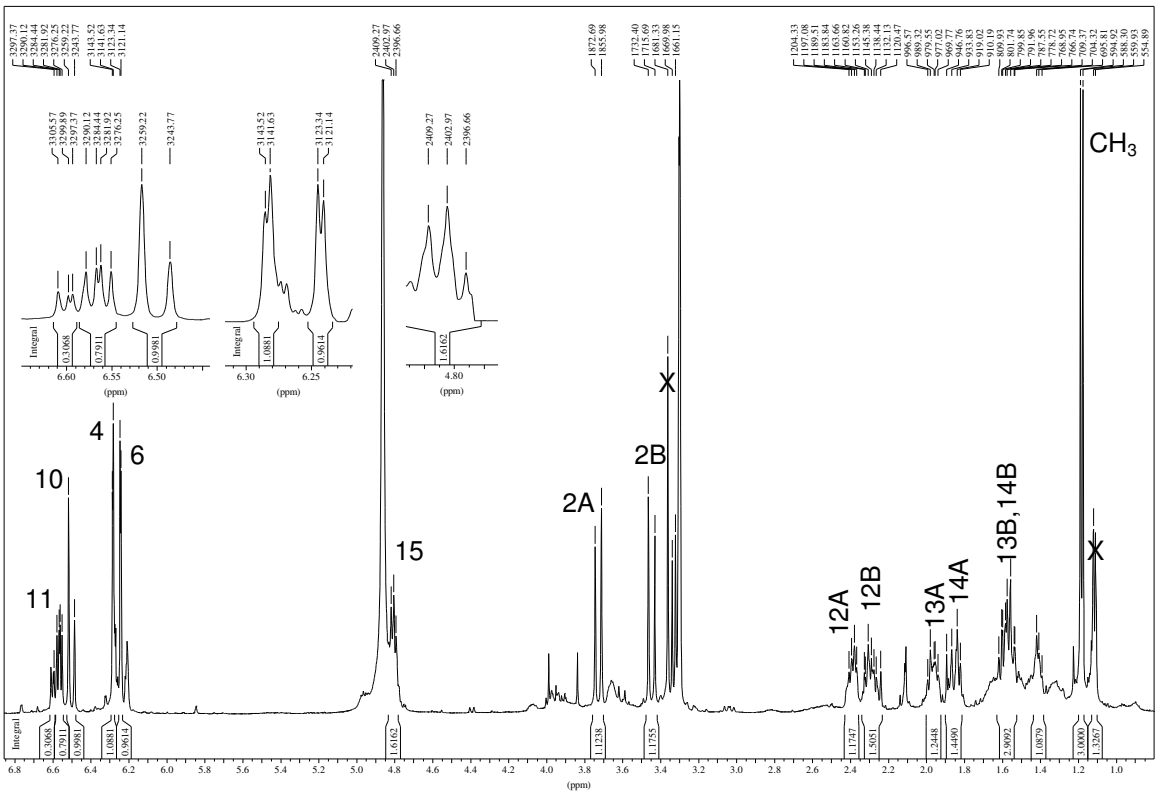
Attachment 30: The ^{1}H NMR spectrum of stemphyperylenol (**31**).



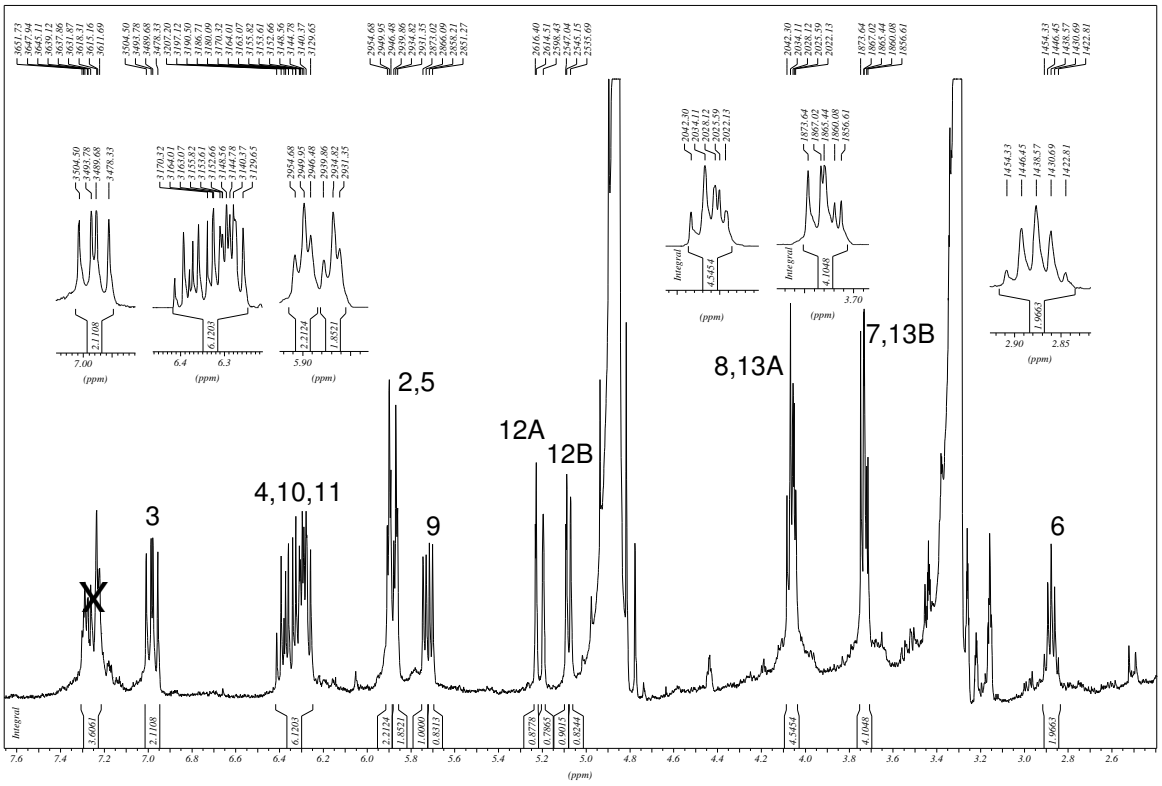
Attachment 31: The ^{1}H NMR spectrum of curvularin (**32**).



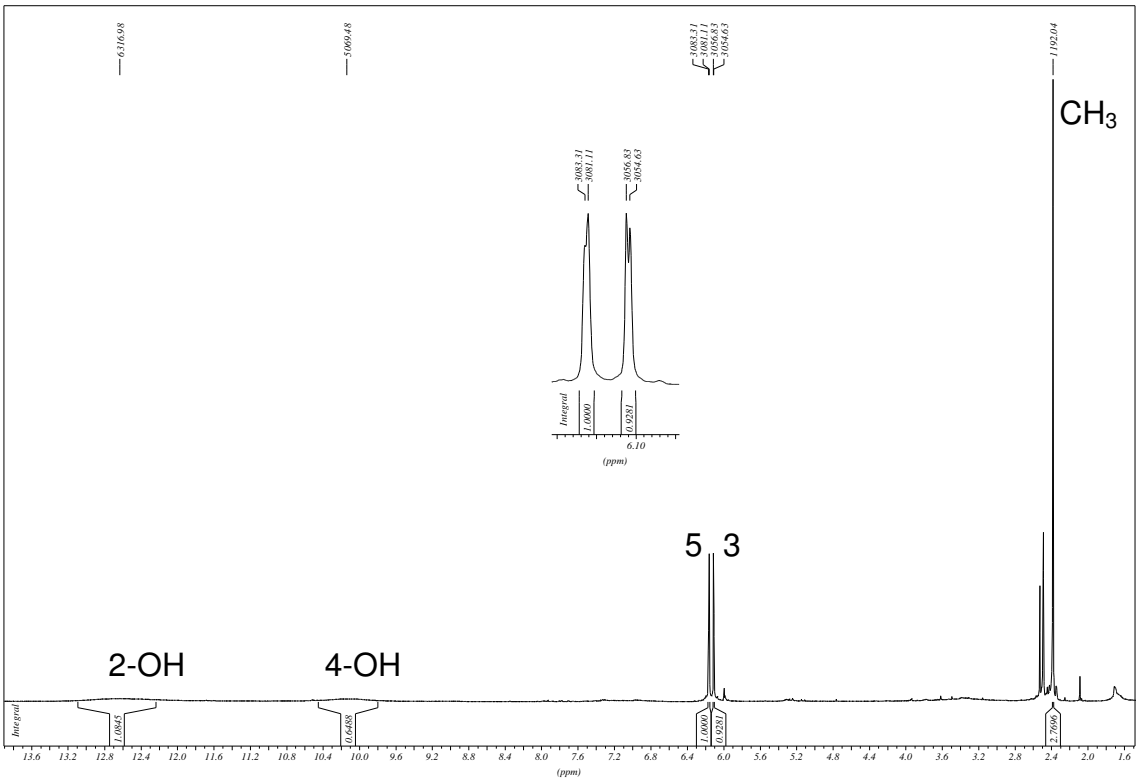
Attachment 32: The ^{1}H NMR spectrum of dehydrocurvularin (**33**).



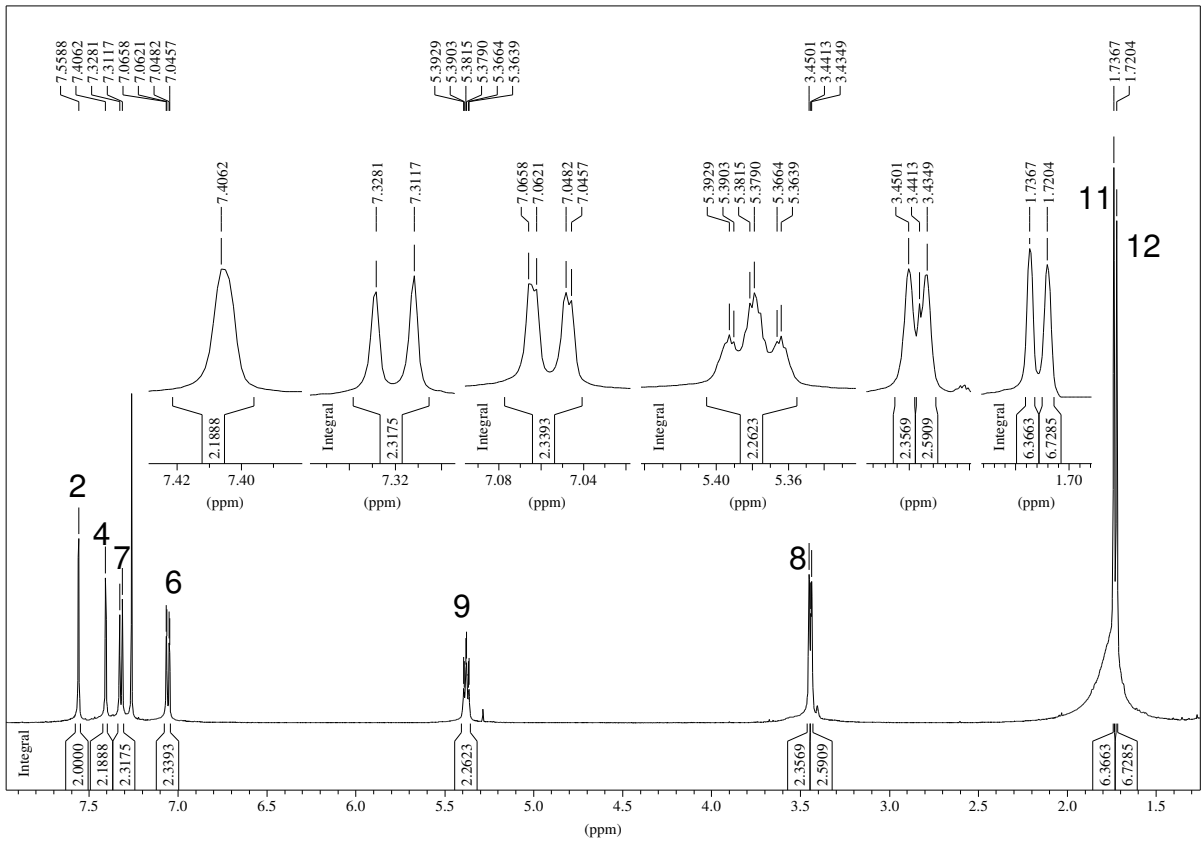
Attachment 33: The ^{1}H NMR spectrum of aureonitolic acid (**34**).



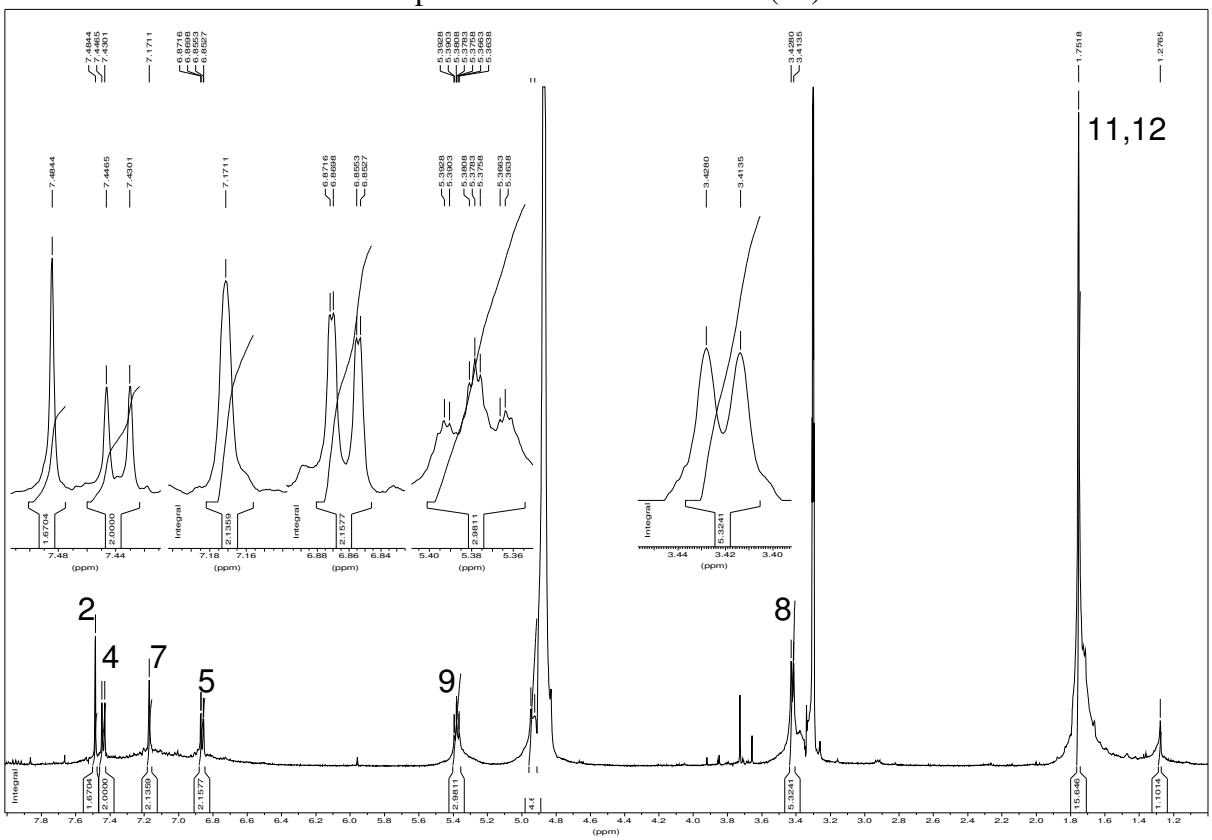
Attachment 34: The ^{1}H NMR spectrum of orsellinic acid (**39**).



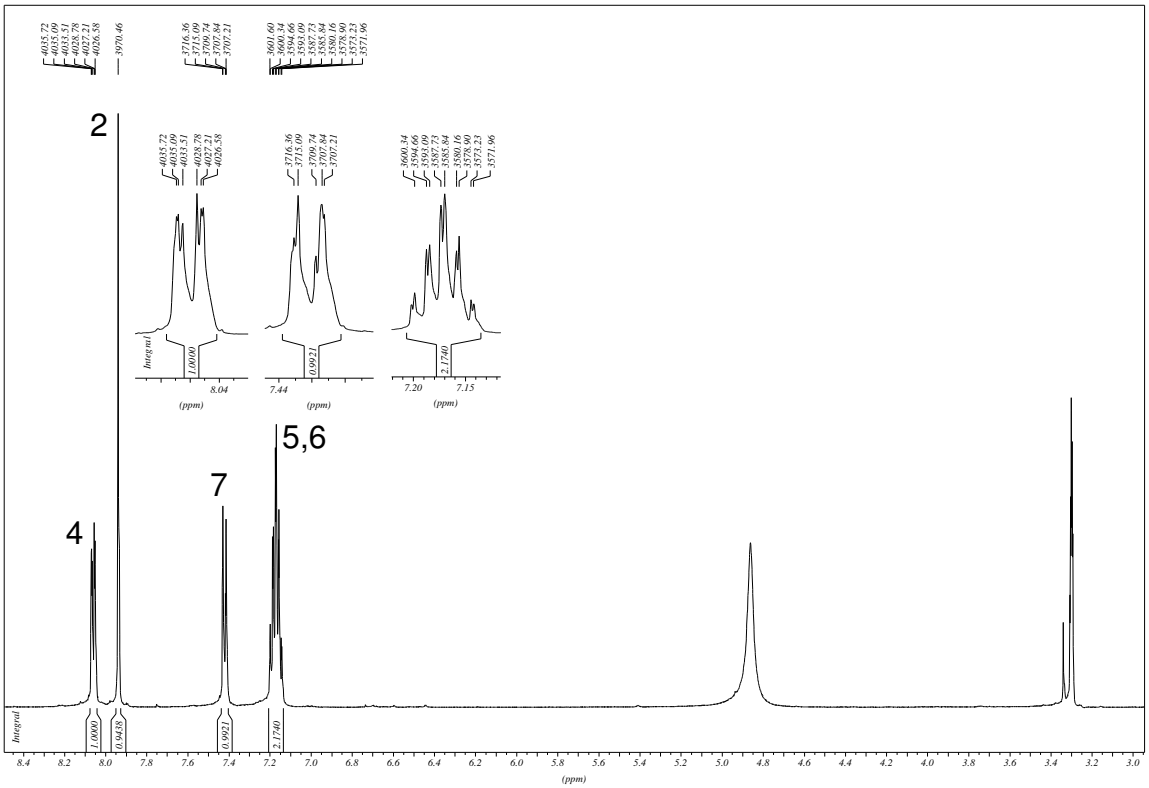
Attachment 35: The ^{1}H NMR spectrum of cochliodinol (**35**).



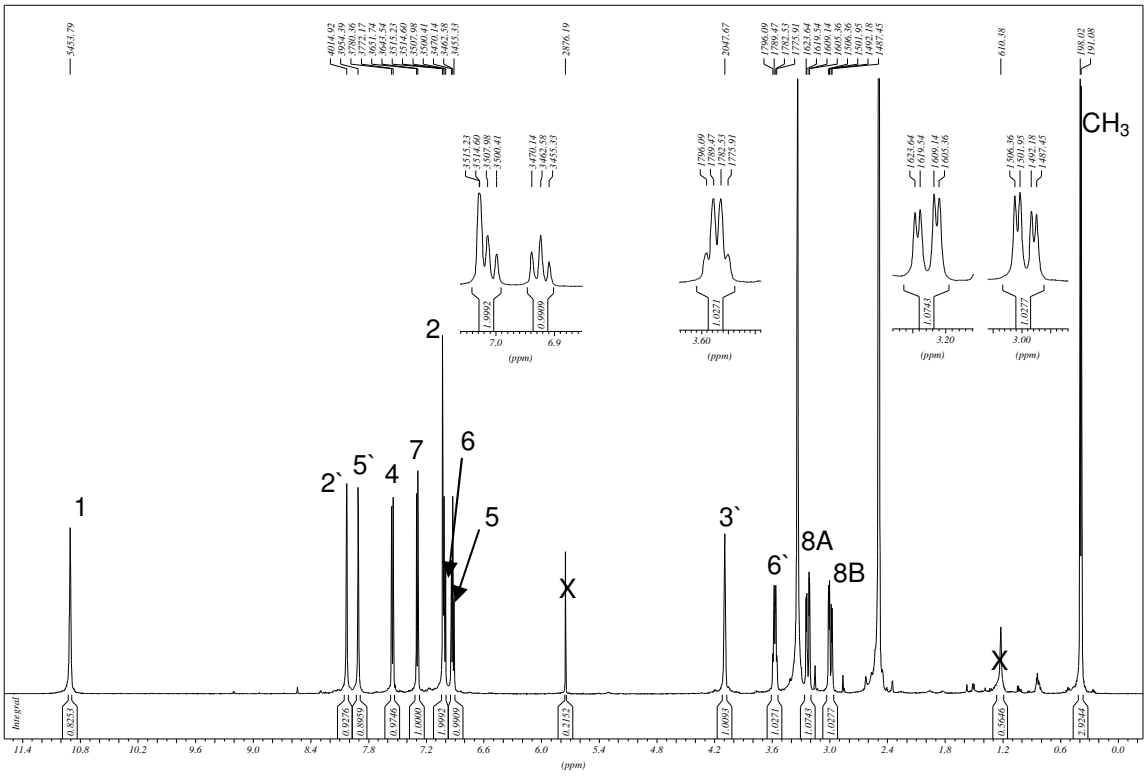
Attachment 36: The ^{1}H NMR spectrum of isocochliodinol (**36**).



Attachment 37: The ^{1}H NMR spectrum of indol-3-carboxylic acid (**37**).



Attachment 38: The ^{1}H NMR spectrum of cyclo(alanyltryptophane) (**38**).



Curriculum Vitae

Name : Amal El-Sayed Hassan Abbas Hassan Aly

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Nationality : Egyptian

Course of Education

- Postgraduate studies at the Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf, Germany, since 2003
- Courses in Mass Spectrometry at the Institut für Pharmazeutische Chemie, Heinrich-Heine-Universität Düsseldorf, Germany, 2005
- Courses in Nuclear Magnetic Resonance Spectroscopy at the Institut für Pharmazeutische Chemie, Heinrich-Heine-Universität Düsseldorf, Germany, 2005-2006
- Receiving the M. Sc. degree in Pharmaceutical Sciences (Pharmacognosy) from the Faculty of Pharmacy, Alexandria University, Egypt, in 2002
- Postgraduate studies at the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University, Egypt, 1999-2002
- Second part of M. Sc. degree in Pharmaceutical Sciences (Pharmacognosy), grade distinction, in 2001
- First part of M. Sc. degree in Pharmaceutical Sciences (Pharmacognosy), grade distinction, in 2000
- Graduation with B. Sc. in Pharmacy, grade distinction, from the Faculty of Pharmacy, Alexandria University, Egypt, in 1998
- Faculty of Pharmacy, Alexandria University, Egypt, from 1994-1998
- Graduation with secondary school certificate, in 1993
- Deutsche Schule der Borromäerinnen in Alexandria, Egypt, from 1980-1993

Scientific Experience

- Ph.D. student at the at the Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf, Germany, since 2003
- Teaching practical courses in pharmaceutical biology for fifth semester students at the Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf, Germany, from 2004-2006
- Assistant lecturer at the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University, Egypt, from 2002-2003

- Supervising and teaching practical courses in phytochemistry for seventh and eighth semester students at the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University, Egypt, from 2002-2003
- Demonstrator at the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University, Egypt, from 1999-2002
- Supervising and teaching practical courses in pharmacognosy and phytochemistry for fifth and sixth semester students at the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University, Egypt, from 2000-2002
- Assistant at the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University, Egypt, from 1998-1999
- Teaching practical courses in pharmacognosy and phytochemistry for third to sixth semester students at the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University, Egypt, from 1998-1999

Publications

- El-Ghazooly, Maged G., El-Lakany, Abdalla M., Abou-Shoer, Mohamed I., Aly, Amal H.. Chemical constituents of *Helichrysum conglobatum* growing in Egypt. *Natural Product Sciences* (2003), **9**(4), 213-219.

Conferences

- Oral and Poster presentation at the International Congress on the Medicinal and Aromatic Plants, March 2007, Fes, Morocco.
- Poster presentation at the Pharmaceutical Sciences World Congress, April 2007, Amsterdam, Netherlands.
- Poster presentation at the International Congress and 55th Annual Meeting of the Society for Medicinal Plant Research, September 2007, Graz, Austria.