New Natural Products from Endophytic Fungi from Mangrove Plants – Structure Elucidation and Biological Screening

Neue Naturstoffe aus endophytischen Pilzen aus Mangroven – Strukturaufklärung und Evaluierung der biologischen Aktivität

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Für meine Lieben

Die Entdeckung, dass es so einfach nicht ist, wie man gedacht hat,

ist als Gewinn anzusehen.

Carl Friedrich von Weizsäcker (1912-2007)

Erklärung

Ich versichere, dass ich die vorliegende Dissertation mit dem Titel " Neue Naturstoffe aus endophytischen Pilzen aus Mangroven – Strukturaufklärung und Evaluierung der biologischen Aktivität" selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die eingereichte Arbeit habe ich in dieser oder ähnlicher Form noch keinem anderen Prüfungsausschuss vorgelegt.

Düsseldorf, den 25.11.2009

Julia Kjer

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1. Introduction

1.1 Natural products in drug discovery

Medicinal application of natural products can be traced back several milllenia in human history. Natural products have been an integral part, in one form or another, of several indigenous therapeutic systems including traditional Chinese medicine (TCM) and Ayurveda (Liu and Wang 2008). In traditional medicine, natural products, mainly botanical drugs, were and are used on the basis of empirical experiences rather than pharmacological knowledge. With the increase of this knowledge and the better understanding of the mode of action of different remedies, more and more distinct drugs were used and chemically modified. Additionally, scores of novel active compounds were introduced to medicine that are produced by means of traditional or combinatoral synthetic chemistry. Still many of the most renowned drugs used today are of natural origin, like the famous analgesic morphine (**1**) (from *Papaver somniferum*), the antipyretic and antimalarial active alkaloid quinine (**2**) (from *Cinchona pubescens*) or the cardiac glycoside digitoxin (**3**) (from *Digitalis purpurea*) (Teuscher 1997). Even more drugs are derived from natural products, like the world-famous aspirin (**4**). Its lead structure salicin (**5**), first isolated from willow trees (*Salix* spp.) has been modified to improve the activity and reduce side-effects. Aspirin is only one, albeit the best known example for the prosperous interaction of synthetic and natural products chemistry.

In the development of novel lead compounds for drugs, cosmetics or agrochemicals, researchers can still find a plethora of promising natural products with unique structures and remarkable biological activities. Despite new methods such as molecular modelling and combinatorial chemistry techniques, more than 60% of the newly approved drugs in the years 1981-2006 were of natural origin or derived from natural lead compounds (Newman and Cragg 2007). Especially in the fields of immunosuppressant and antibacterial drug discovery, natural products are playing a pivotal role because of the occurrence of great numbers of compounds with these kinds of activities. This circumstance can be understood easier if the ecological advantage these components confer to their producers is taken into consideration. The biosynthesis of secondary metabolites requires a high input of energy by the producing organism and therefore is only performed if the metabolites exhibit a certain evolutionary benefit for the producer. The metabolites in one way or another improve the evolutionary fitness or competitiveness of the producing organism in its natural environment, where the defense against microbial invaders plays a decisive role (Butler 2008).
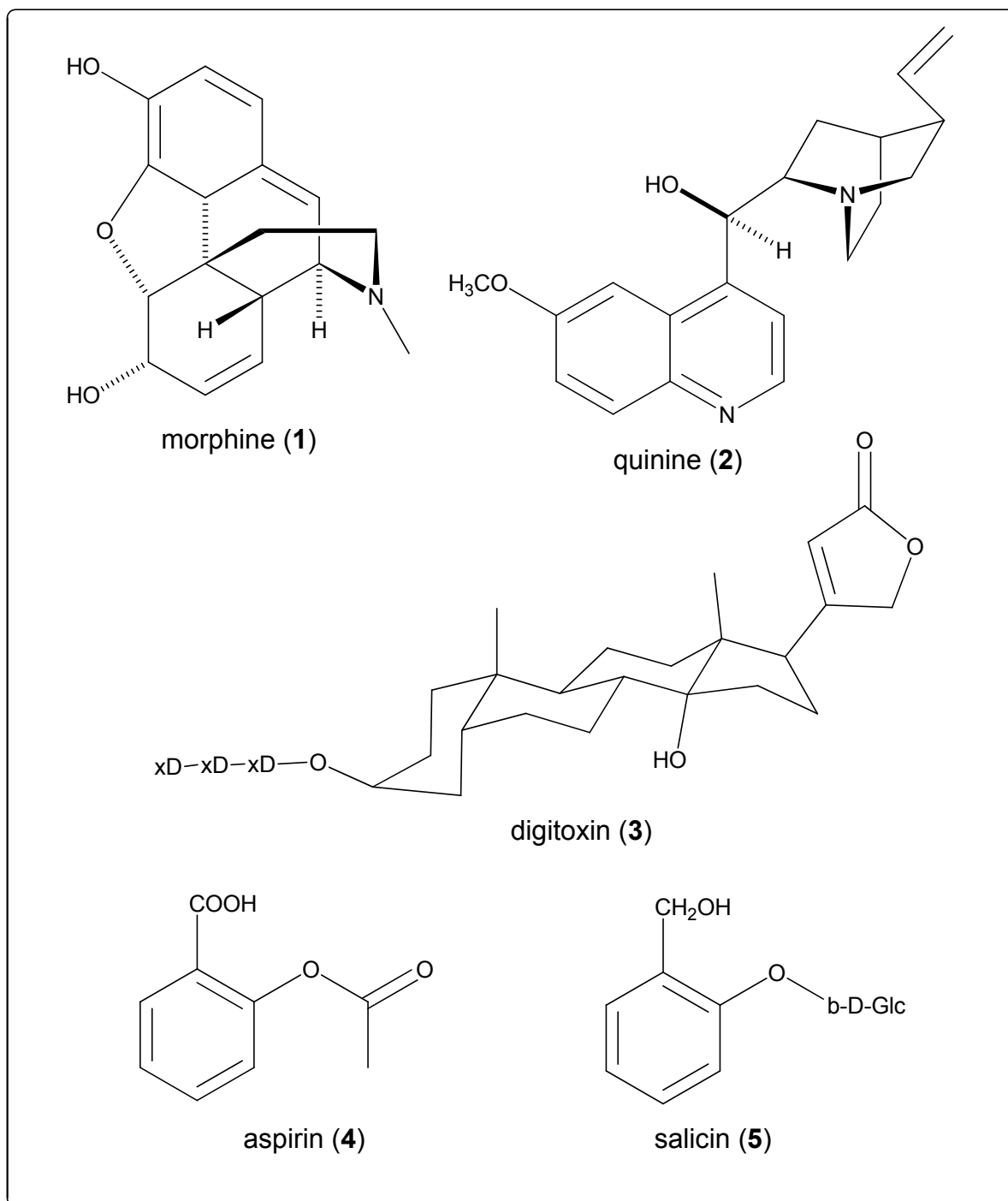
Figure 1.1. Natural products of medicinal importance.

1.2   Fungi as sources of bioactive products

Fungi are ubiquitous occurring, eukaryotic, heterotrophic organisms. Beside the well-known mushrooms, fungal life is found worldwide, in soil samples as well as deep sea vents and arctic ice, and often reveals symbiotic traits. Similar to plants, there is a long history of the utilisation of fungi by mankind as remedies and in every-day life. Nearly 3000 years ago the Mayans used fungi to treat intestinal ailments (Strobel et al. 2004). Without deeper knowledge about the mode of action the transformation by fungi has been used for food production since Neolithic times. The earliest types of fermented food were beer, wine, and leavened bread, followed by the early Chinese who produced fermented soy foods. Since Pasteur's discovery that fermentation is caused by living cells, investigation of microbes as natural products resources sprung up. But it was not until the discovery of penicillin (**6**) isolated from *Penicillium notatum* by Sir Alexander Fleming in 1928 which resulted in a breakthrough in the treatment of bacterial infections, that fungi became an important source of drugs for the treatment of a variety of diseases.

Since then, especially fungi isolated from soil samples have been identified as a rich source of biologically active secondary metabolites. Beside other well known antimicrobial agents like fusidic acid (**7**) (Godtfredsen et al. 1962) and griseofulvin (**8**) (Grove et al. 1952), novel semisynthetic antifungal drugs like anidulafungin (**9**) (Eraxis®) and caspafungin (Cancidas®) are likewise derived from fungal metabolites (Butler 2004). With the discovery of cyclosporine (**10**, also known as ciclosporin or cylosporin) isolated from *Tolypocladium inflatum* in 1971, an important step in immunopharmacology was made because this substance prevents rejection after organ or tissue transplantations. Improvements in the field of organ transplantations and treatment of autoimmune diseases are still in progress with the discovery that also known substances such as the fungal metabolite mycophenolic acid (**11**) (Myfortic®) possess immunosuppressive activities. Cyclosporine exhibits, in addition to its potent immunosuppressant activity pronounced antiviral activity. Therefore it furthermore served as a model for the design of substances like Debio-025, a potential antiviral drug that has successfully passed clinical trials (Butler 2004, Flisiak et al. 2008).

Probably the most economical important fungal metabolites represent antilipidemic drugs collectively known as "statins", with their parent compounds mevastatin and lovastatin (**12**) isolated from *Penicillium citrinum* and *Aspergillus terreus*, respectively. Statins reduce blood cholesterol levels by inhibiting the rate-limiting enzyme HMG-CoA reductase in the mevalonate pathway of cholesterol synthesis, and are used for the treatment of cardiovascular diseases (Butler 2004, Dewick 2006).

Fungal metabolites are, however, not only indispensable for medicine but are also important for plant protection as demonstrated by the discovery of the strobilurines (**13**), that were first isolated from *Strobilurus* sp. and served as lead compounds for synthetic fungicidals such as trifloxystrobin (**14**) (Flint®) (Balba 2007). The demand for new highly effective agricultural agents to control farm pests and pathogens is enormous, and partly arises from the removal of synthetic compounds from the market because of their toxicity towards the environment, but also from the rising need for food due to a stringent population growth.

penicillin G (**6**)

fusidic acid (**7**)

griseofulvin (**8**)

anidulafungin (**9**)

cyclosporine (**10**)

mycophenolic acid (**11**)

lovastatin (**12**)
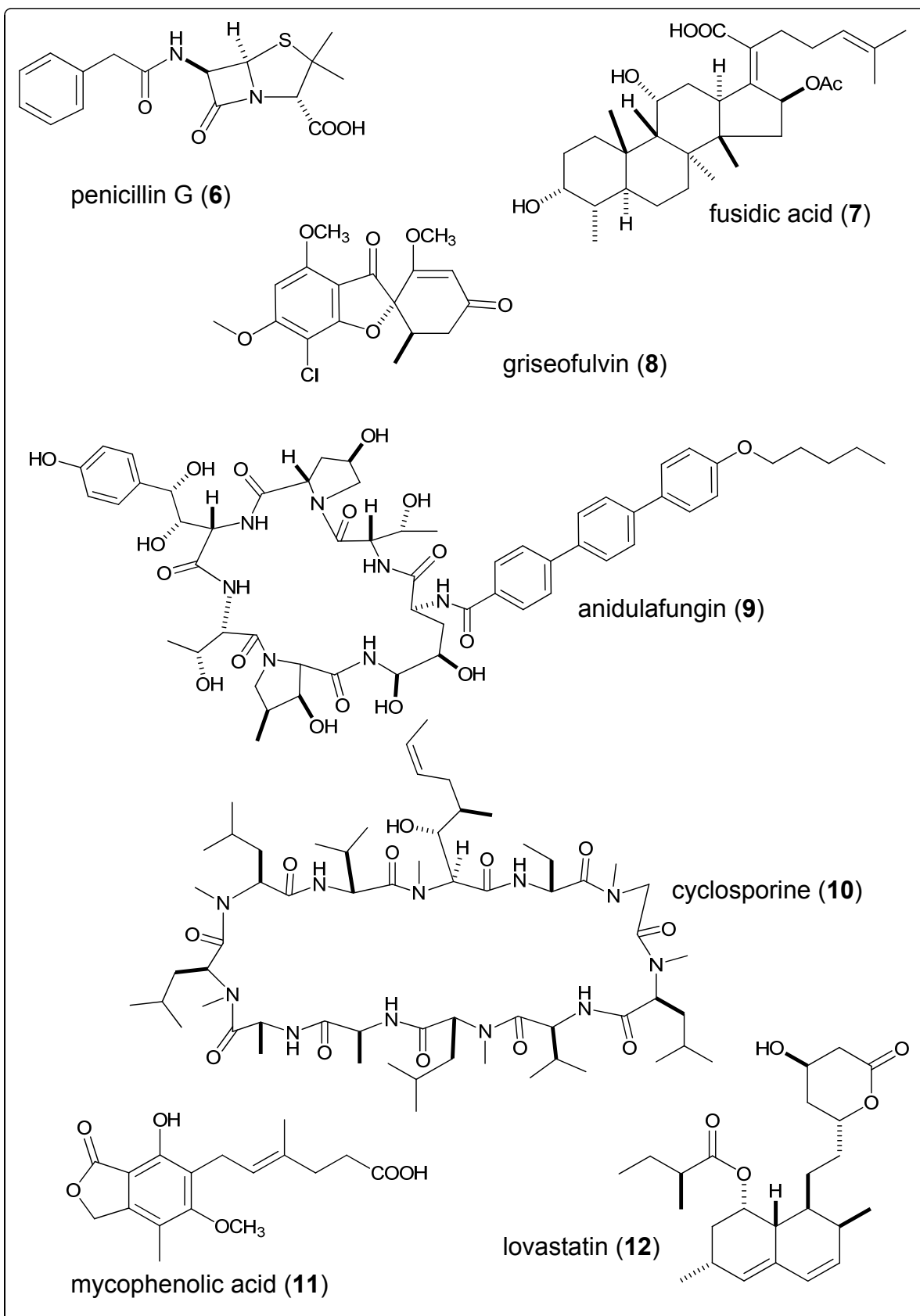
Figure 1.2a. Bioactive fungal metabolites used in medicine.
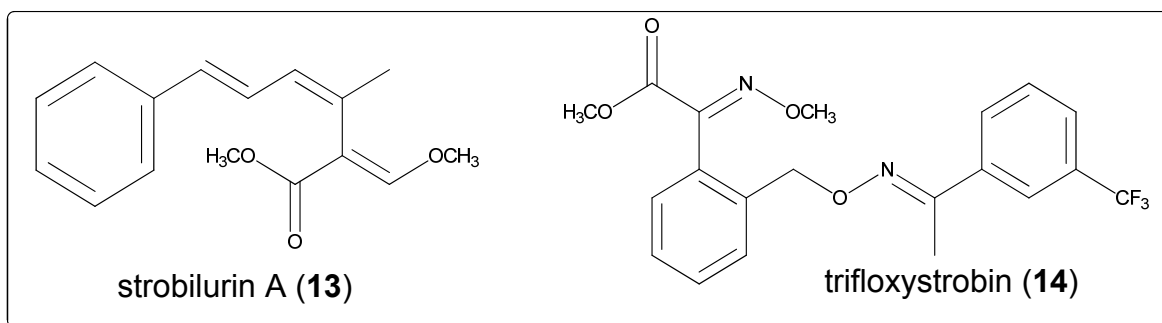
Figure 1.2b. Fungicides based on fungal natural products.

However, it has been stated that "the rediscovery of high numbers of previously described metabolites has to some extend precluded the study of traditional terrestrial sources of fungi" (Bugni and Ireland 2004). For this reason, interest of natural products chemists and pharmacologists alike has recently turned to so far less investigated habitats and ecological niches. Examples for such scarcely explored ecological niches that offer a plenitude of novel bioactive compounds are the oceans and rainforests, while as far as the biological sources are concerned, in particular microorganisms inhabiting these distinct biotopes such as marine and endophytic fungi have become the focus of attention.

1.3 Endophytic fungi

Endophytes are microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects (Bacon 2000). As almost all vascular plant species appear to be inhabited by endophytic bacteria or fungi, these represent important components of microbial diversity. The relationship between the host plant and its endophyte shows symbiotic characteristics as the endophytic occupant usually obtains nutritients and protection from the host plant and in return profoundly enhances the fitness of the host by producing certain functional metabolites (Tan and Zou 2001). Still, if the host plant is weakened, the endophyte can also become an aggressive saprophyte and thereby reveal the smooth transition between symbiont and opportunistic pathogen (Schulz and Boyle 2005). Fungal endophytes are a polyphyletic group of primarily ascomycetous fungi, whereas basidiomycetes, deuteromycetes and oomycetes are rarely found (Saikonnen et al. 1998, Arnold 2007). Although they do not show host specifity, certain fungal lineages appear with greater frequency in plants representing particular families and thus denote host preference (Cannon and Simmons 2002, Arnold 2007).

Consistent with the tremendous diversity of endophytic fungi and their ecological roles is the astounding chemical variety of their secondary metabolites, which often display promising pharmaceutically or agrochemically exploitable activities when tested in various bioassays (Strobel et al. 2004). Due to the world's urgent need for new antibiotics, chemotherapeutic agents and agrochemicals to cope with the growing medicinal and environmental problems facing mankind, growing interest is taken into the research on the chemistry of endophytic fungi. Whereas between 1987 and 2000 approximately 140 new natural products were isolated from endophytic fungi (Tan and Zou 2001), a similar number was subsequently characterised in half of this time span, i.e. between 2000 and 2006 (Zhang et al. 2006). Many of these exhibit interesting activity profiles. Cryptocin (**15**), for example, is an tetramic acid isolated from the endophytic fungus *Cryptosporiopsis quercina*, an endophyte of *Tripterigeum wilfordii*, that possesses potent activity against the world's worst plant pests *Pyricularia oryzae* and other plant pathogenic fungi, advocating it for possible agrochemical usage (Li et al. 2000). From the medicinal plant *Erythrina crista-galli* the endophyte *Phomopsis* sp. was isolated, which produced the anti-inflammatoriy as well as antifungally and antibacterially active polyketide lactone, phomol (**16**) (Weber et al. 2004).
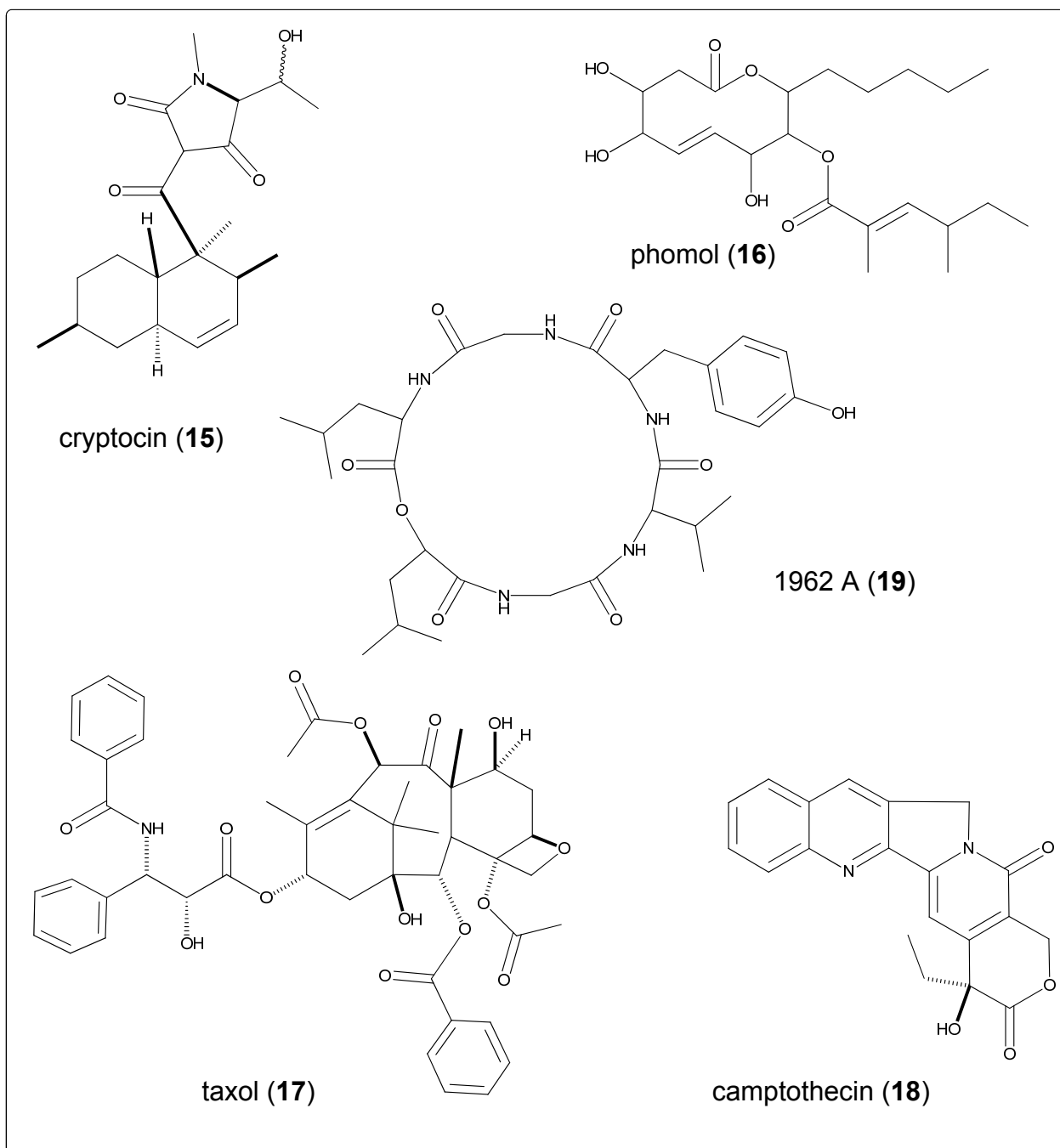
Figure 1.3. Bioactive metabolites from endophytic fungi.

But it is not only new compounds being isolated from endophytes that are promising. The well known plant metabolite taxol (**17**), the "world's first billion-dollar anticancer compound" (Strobel 2004), was originally isolated from the bark of the endemic Pacific yew tree, *Taxus brevifolia*. It interferes with the normal function of microtubule breakdown. Specifically, taxol binds to the β-subunit of tubulin and thereby interrupts the dynamic rearrangement of this important component of the cytoskeleton. This adversely affects cell function because the shortening and lengthening of microtubules is necessary for their function as a mechanism to transport other cellular components, e. g. during mitosis. Thus taxol affects dividing cells, especially fast dividing ones like cancer cells. For the treatment of one patient suffering from cancer, 2 g taxol are required, which represents an amount equivalent to twelve trees and thereby posing a challenge to the limited natural resources, since the isolation from the inner bark implies the destruction of yew trees. Thus, the

demand for taxol greatly exceeds the supply that can be sustained by isolation from its natural source and alternative sources of the drug have been sought for a long time. Although the highly functionalized, polycyclic diterpene has been prepared by total synthesis, the process is too complex and not economically feasible. Currently, the supply of the compound is achieved by a successfully implied partial synthetic route based on baccatin III or its 10-deacetyl congener, which are isolated from the needles of other *Taxus* species and thus from a renewable resource. However, the extraction process of these precursors is tedious and costly. In the ongoing search for alternative sources of taxol, the group of Gary Strobel discovered taxol production in a hitherto undescribed endophytic fungus associated with *Taxus brevifolia*, identified as *Taxomyces andreanae* (Stierle et al. 1993). Although initially controversial, these findings prompted further studies, and it is nowadays an emerging picture that the ability to produce taxol upon fermentation seems to be a rather widespread feature among endophytic fungi. So far, more than 10 different fungal strains from at least 6 different host plants, most of them only distantly (if at all) related to *Taxus*, have been identified. However, it is worth mentioning that in all cases the resulting yields are minuscule, so far preventing any commercial exploitation (Strobel et al. 2004).

Similar to the taxol case, the endophytic fungus *Entrophospora* sp. associated with *Nothapodytes foetida* was found to produce the cytotoxic plant alkaloid camptothecin (**18**) (Puri et al. 2005). The substance which was first described from the Chinese medicinal plant *Camptotheca acuminata* in 1966, exhibits remarkable anticancer activity by inhibition of the DNA enzyme topoisomerase I. Due to its low solubility and adverse drug reaction it is not used as an anticancer drug itself, but served as a drug lead and precursor for the semi-synthetic antiproliferatic drugs topotecan and irinotecan. Optimization of the fermentation conditions of the endophytic fungus may lead to the development of an economically and eco-friendly process for the production of camptothecin that could overcome the ever demanding supply problem (Puri et al. 2005)

Thus, the ability to produce pharmacologically important natural products previously only known from plant sources is occasionally also inherent to endophytic fungi, whith further examples including podophyllotoxin (Puri et al. 2006, Kour et al. 2008) which will undoubtedly prompt future research into endophytic fungi.

1.4   Selection of promising sources for the isolation of endophytic fungi

When working with endophytic fungi in order to discover bioactive metabolites, the choice of the host is of particular importance. The exploration of endophytic fungi is still an emerging field and all plants seem to deliver fungi with auspicious contents and activities. However, certain microbial metabolites seem to be characteristic of certain biotopes (Schulz 2001) and a rationale for selecting promising plant sources has recently been proposed (Strobel et al. 2004). Of particular interest are plants which themselves are used as medicinal plants or are known to produce bioactive metabolites. Other favourable sources of endophytic fungi include plants that populate distinct biotopes and have to cope with extreme living conditions like cold, heat or multitudinous competing organisms in their natural environment, for example inhabitants of rainforests or mangrove forests. In these ecosystems, where the evolutionary race to survive is most pronounced and requires constant innovations by the plants, i. e. morphological and physiological adaptations as well as chemical variation, the chance to find novel compounds with high bioactivities is most probable (Strobel et al. 2004).

1.5 Mangroves

The term "mangrove" in itself is used in different contexts. Strictly speaking, it describes trees or shrubs growing in saline coastal habitats, called "mangrove forest" or "mangrove swamp". However, occasionally it is also used to describe all woody plants in such an environment, or alternatively, to refer to the habitat as a whole. True mangroves are a diverse group of approximately 70 plant species belonging to several botanical families, among which Rhizophoraceae, Combretaceae, Lythraceae and Avicenniaceae are the most prominent examples. In almost all cases, they represent facultative halophytes that thrive in the intertidal zone in tropical or subtropical climates, acting as a bridge between the marine and the terrestrial habitat. Mangroves are highly adapted to their environmental conditions and are able to cope with numerous physical stress factors including sharp variation in moisture or salt concentration, changing tides, or biological stress factors such as abundant microorganisms or herbivorous insects. The most eye-catching adaptation is the development of an intricate root system of pneumatophors or prop roots to ensure oxygen exchange despite the anaerobic sediments in which they sprout. A less visible feature is the aptitude to tolerate various levels of salinity by developing special tissues to prevent the uptake of salt or concentrate salt in leaves, particular glands to excrete salt crystals or discardable salt hairs.

Mangrove forests represent an ecosystem of high biodiversity (Bandaranayake 2002, Macintosh and Ashton 2002). It has been stated previously that biological diversity would imply chemical diversity, because the constant evolutionary race to survive would be most active (Strobel et al. 2004). Thus, in addition to their refined morphological and physiological adaptations, the production of bioactive secondary metabolites might play an important role in the constant competition of mangroves with other plants, animals and microorganisms for the limited resources in their habitat. In fact, the capability of mangroves to produce a wide array of bioactive compounds is reflected in numerous publications which describe the high chemical diversity of their metabolites, despite the fact that intensive research on mangrove metabolites only sprung up in the last two decades (Wu et al. 2008, Li et al. 2008).

Moreover, their unique living conditions are thought to predestine mangroves as promising sources for the isolation of endophytic fungi. This type of symbiosis has been demonstrated to offer multiple advantages to both endophytes and their respective host plants. It has been suggested that the association of fungi as endophytes with mangrove roots would confer protection from adverse environmental conditions and would allow the latter to successfully compete with saprophytic fungi that decompose senescent roots (Anada and Sridhar 2002). Moreover, mangrove forests are considered an open interface ecosystem connecting upland terrestrial and coastal estuarine ecosystems, and likewise, the endophytic fungi in mangroves constitute a consortium of soil, marine and freshwater fungi (Anada and Sridhar 2002, Sengputa and Chaudhuri 2002). Additionally, endophyte assemblage varies with different parts (leaves, twigs, roots) and age of the host plant and with different season (Pang et al. 2008, Liu et al. 2007).

More than 200 species of endophytic fungi have been isolated and identified from mangrove trees and have, despite the short period of research on the chemistry of mangrove endophytes, already been proven to be a well-established source for structurally diverse and biologically active secondary metabolites (Li et al. 2009, Pang et al. 2007). An interesting example is the cyclic depsipeptide 1962A (**19**) that was isolated from an unidentified fungus associated with the mangrove plant

Kandelia candel. It exhibited cytotoxic activity against human breast cancer MCF-7 cells when tested in the MTT assay (Huang et al. 2007). Other examples are new polyketides that were isolated from a *Penicillium* sp. associated with the mangrove plant *Aegiceras corniculatum*. Upon biological screening, two of them exhibited cytotoxic activities (Lin et al. 2007).

These examples illustrate the enormous chemical diversity and biological potential of endophytic, particularly mangrove-derived, endophytic fungi. Thus they represent an interesting source of new lead structures for medical and agrochemical applications.

1.6 Regulation of secondary metabolite production by endophytic fungi

Fungi are known to synthesize a wide variety of secondary metabolites, low molecular weight molecules that are thought to aid the fungus in competing successfully with other organisms in its natural habitat. Many of these substances exhibit bioactive properties and have therefore been adopted by humans for use as pharmaceuticals such as antibiotics, cholesterol-lowering agents, tumor inhibitors and immunosuppressants for organ transplantations. Hence there is a particular desire to achieve high productivity of fungal fermentation. However, typical fungal fermentation methods such as axenic shake or static cultures on artificially defined media are poor surrogates for mimicking an organism's native habitat. The majority of these compounds, especially those having toxic or inhibitory effects to other organisms, is not required for growth or development of the producing organism under laboratory conditions. Fungi are able to turn off the energetically costly process of secondary metabolite production under certain environmental conditions (Shwab 2007).

In order to exploit the true biochemical potential of fungi, the effect of physiological growth conditions have long been studied. However, they are still far from completely explained. Optimal conditions for biosynthesis of secondary metabolites have been found to be not necessarily identical to those for growth, and to vary among different microorganisms and different metabolic pathways. The regulation of fungal secondary metabolism appears to be responsive to general environmental factors, such as carbon and nitrogen sources, temperature, light, and pH (Knight 2003).

An attempt to describe and take advantage of this phenomenon is the so-called "OSMAC"-approach (**O**ne **S**train, **Ma**ny **C**ompounds). The idea of this approach is that the systematic alteration of easily accessible cultivation parameters like media composition, aeration and temperature increases the number of secondary metabolites available from one microbial source (Bode et al. 2002). The growth of mangrove-derived endophytic fungi in particular has been demonstrated to be dependent on the salinity of the fermentation medium (Teuscher 2005). Another example is the advantageous effect of treatment with metal ions, especially zinc, on *Aspergillus flavus*: It was shown in an experiment to not only stimulate the fungal growth, but also its aflatoxin production (Cuero et al. 2003).

The goal of investigation of microorganisms is not only a high production of known metabolites, but even more the detection of new bioactive compounds. Accordingly, strategies that discover and exploit the full metabolic potential of newly investigated microorganisms are being developed in order to maximize their chemical diversity. Investigations of bacteria have revealed that antibiotics act not only as growth inhibitors of bacteria, but that subinhibitory doses can also exhibit more diverse effects on other microorganisms. The addition of specific antibiotics, namely tobramycin, tetracycline and norfloxacin, in concentrations below their minimal inhibitory concentrations (MICs), to cultures of *Pseudomonas aeruginosa* was proven to induce the formation of biofilm*,* a behavior that could be beneficial for the microbe in its natural environment (Linares 2006). Comparable mechanisms have been shown for other bacteria and antibiotics in recent years. More and more evidence is found to prove the suggestion that antibiotics, especially when applied at subinhibitory concentrations, could promote metabolic variability of bacteria (Couce 2009), possibly mimicking the presence of other competing microorganisms. A recent study investigated the effect of supplement with subinhibitory concentrations of antibiotics on the metabolism of a marine *Streptomyces* sp. The pattern of secondary

metabolites produced depended on the applied antibiotic. New phenazines were produced by the actinomycete, and one of them, streptophenazine E, only in the presence of tetracycline. Upon addition of tetracycline streptoephenazines F and G were induced, while mainly streptophenazine H was formed when applying bacitracin (Mitova et al. 2008).

Similar effects were examined in cultures of *Candida albicans*. Exposure to subinhibitory concentrations of the antifungal agent fluconazole resulted in enhanced extracellular production of secreted aspartyl proteinase (SAP) and thus in an increased virulence of the fungus (Wu et al. 2000, Barelle et al. 2008). Green fluorescent protein (GFP)-tagged promoters showed that the presence of subinhibitory concentrations of fluconazole also lead to an increase of the fluorescence in the filaments.

Recent advances in molecular biology, bioinformatics and comparative genomics have revealed that the number of putative biosynthetic pathways in fungi exceeds the sum total of secondary metabolites observed under laboratory culture conditions, and that the genes encoding specific fungal secondary metabolites are clustered. Gene expression is known to be controlled by various mechanisms such as nuclear transcriptional regulators ("epigenetic modifiers"). Manipulation of these regulators could result in amplified gene expression as has been described for LaeA, a methyltransferase which controls secondary metabolite production in *Aspergillus* (Shwab and Keller 2008, Keller et al. 2005, Pazin 1997). Furthermore, researchers succeeded in stimulating the production of new secondary metabolites by a marine-derived fungal strain, systematically applying various small molecule inhibitors of DNA methyltransferase and histone deacetylase and thereby proving the concept of or the correlation between transcriptional repression and regulation by epigenetic mechanisms (see also figure 1.6). They showed that it is indeed possible to abolish the repression of hidden gene clusters, and to use their inherent genetic information for the production of novel natural products by fermentation (Williams et al. 2008).
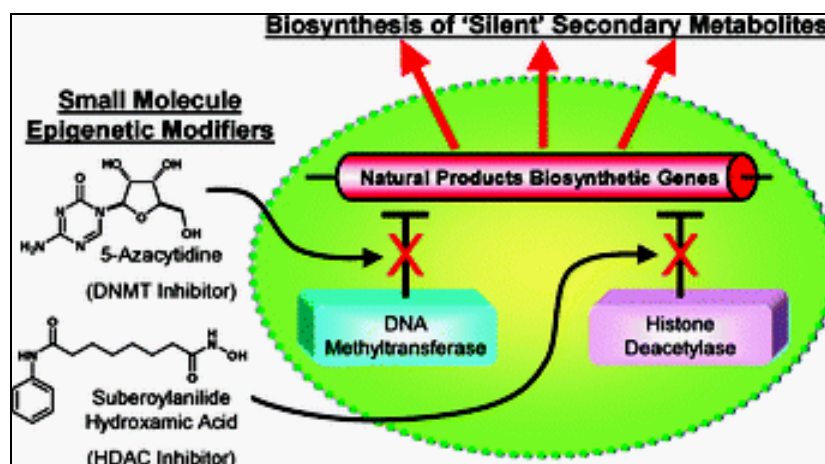
Figure 1.6. Influence of HDAC- and DNM-inhibitors on gene expression and metabolite production (from Williams 2008).

1.7   Aim and scope of this study

Mangrove-derived fungi so far are still poorly investigated and thus represent a promising source of chemically new compounds with huge pharmaceutical and agrochemical potential.

The aim of this study was the investigation of bioactive metabolites of endophytic fungi derived from mangrove plants of the South Chinese Sea. In order to select the most promising fungal strains, the isolated fungi were grown on a small scale basis in liquid Wickerham medium and extracted with EtOAc. Based on the results of chemical and biological screening, five fungal strains, *Cladosporium sphaerospermum*, *Fusarium incarnatum*, *Nigrospora* sp., *Alternaria* sp. and *Bionectria ochroleuca* were chosen for further workup.

The selected strains were grown in liquid Wickerham medium and on solid rice medium and extracted with organic solvents, followed by fractionation and purification using various chromatographic techniques. Fractions and pure compounds were analyzed by HPLC-DAD and LSMS for their purity, UV spectra, molecular weights and fragmentation patterns. The structures of the metabolites were elucidated using state-of-the-art one- and two-dimensional NMR techniques. Additionally, selected compounds were derivatized to identify their absolute stereochemistry.

Pure compounds were subjected to various bioassays such as antimicrobial, cytotoxic and protein kinase inhibitory activities to determine their pharmaceutical potential. Bioassays were conducted in cooperation with SeaLife Pharma, Austria, Prof. U. Hentschel, Würzburg, Prof. W. E. G. Müller, Mainz and ProQinase, Freiburg.

In order to evaluate the effects of altering fermentation conditions on the productivity of endophytic fungi, substances that are known to be epigenetic modifiers were added to the cultures of two selected strains.

2. Materials and Methods

2.1 Biological material

2.1.1 Field trip and sample collection

All mangrove species were collected in "Dong Zhai Gang Mangrove Garden" near Haikou on Hainan Island, China during research stays at the "National Research Laboratory of Natural and Biomimetic Drugs". All mangroves with the code "MGC" were collected by Dr. Franka Teuscher between 02.06. - 04.06.2004; all plants named "JCM" were collected by the author between 07.10. - 09.10.2005. Small parts of twigs, leaves and fruit were directly put in paper bags and stored dark and at 4°C until the isolation procedure of the fungi was performed two days later.

Figure 2.1.1 Collection area of mangrove plants.

2.1.2 Taxonomy of collected mangrove plants

Table 2.1.2a. Collected mangrove plant samples.

Code No.	Organ	Plant species	Family
MGC 4.2	leaf	*Bruguiera sexangula*	Rhizophoraceae
MGC 12.4	leaf	*Aegiceras corniculatum*	Myrsinaceae

		(Blanco)	
JCM 1	leaf	*Heritiera littoralis*	Malvaceae
JCM 2A	twig	*Rhizophora mucronata*	Rhizophoraceae
JCM 3	leaf	*Rhizophora apiculata*	Rhizophoraceae
JCM 3A	twig	*Rhizophora apiculata*	Rhizophoraceae
JCM 4	leaf	*Rhizophora candelaria*	Rhizophoraceae
JCM 5	leaf	*Rhizophora longissima* (Blanco)	Rhizophoraceae
JCM 5A	twig	*Rhizophora longissima* (Blanco)	Rhizophoraceae
JCM 6	leaf	*Excoecaria agallocha*	Euphorbiaceae
JCM 6A	twig	*Excoecaria agallocha*	Euphorbiaceae
JCM 7	leaf	*Xylocarpus granatum*	Meliaceae
JCM 7A	twig	*Xylocarpus granatum*	Meliaceae
JCM 8	leaf	*Avicennia marina*	Avicenniaceae
JCM 8A	twig	*Avicennia marina*	Avicenniaceae
JCM 9	leaf	*Sonneratia alba*	Sonneratiaceae
JCM 9A	twig	*Sonneratia alba*	Sonneratiaceae
JCM 10	leaf	*Sonneratia caseolaris*	Sonneratiaceae
JCM 10A	twig	*Sonneratia caseolaris*	Sonneratiaceae
JCM 11A	twig	*Sonneratia ovata*	Sonneratiaceae
JCM 12	leaf	*Aegiceras corniculatum*	Myrsinaceae
JCM 12A	twig	*Aegiceras corniculatum*	Myrsinaceae
JCM 13	leaf	*Ceriops tagal*	Rhizophoraceae
JCM 13A	twig	*Ceriops tagal*	Rhizophoraceae
JCM 13B	fruit	*Ceriops tagal*	Rhizophoraceae
JCM 14A	twig	*Bruguiera gymnorrhiza*	Rhizophoraceae
JCM 15A	twig	*Bruguiera sexangula*	Rhizophoraceae
JCM 16	leaf	*Bruguiera sexangula var. Rhynchopetala*	Rhizophoraceae
JCM 16A	twig	*Bruguiera sexangula var. Rhynchopetala*	Rhizophoraceae

JCM 17	leaf	*Kandelia candel*	Rhizophoraceae
JCM 18	leaf	*Lumnitzera littorea*	Combretaceae
JCM 19	leaf	*Lumnitzera racemosa*	Combretaceae
JCM 19A	twig	*Lumnitzera racemosa*	Combretaceae
JCM 20	leaf	*Barringtonia racemosa Roxb.*	Lecythidaceae
JCM 20A	twig	*Barringtonia racemosa Roxb.*	Lecythidaceae
JCM 21	leaf	*Nypa futicans*	Arecaceae

Table 2.1.2b. Collected mangrove associated plant samples.

Code No.	Organ	Plant species	Family
JCM 22	leaf	*Pluchea indica*	Asteraceae
JCM 23	leaf	*La gong mu*	

2.1.3 Taxonomy of isolated fungi

Table 2.1.3. Taxonomy of isolated fungi.

Code No.	Fungal species	Synonyms	Family
MGC 4.2	*Nigrospora* sp.	*Khuskia* sp., *Basisporium* sp., *Dichotomella* sp.	Trichosphaeriaceae
MGC 12.4	*Cladosporium sphaerospermum*		Davidiellaceae
JCM 2A4	*Pestalotiopsis* sp.	*Pestalotia sp., Broomella sp., Monochaetia sp., Truncatella sp.*	Amphisphaeriaceae
JCM 3A3	*Pestalotiopsis* sp.		Amphisphaeriaceae
JCM 9.2	*Alternaria* sp.	*Lewia* sp.	Pleosporaceae
JCM 9.4	*Phoma* sp.	*Leptosphaerulina* sp., *Didymella* sp., *Pseudoplea* sp*.	Pleosporaceae
JCM 10.2	*Pestalotiopsis virgatula*	*Pestalotia virgatula*	Amphisphaeriaceae
JCM 10.3	*Bionectria* sp.		Hypocreaceae
JCM 10.4	*Glomerella cingulata*	*Colletotrichum gloeosporioides*	Glomerellaceae
JCM 19.1	*Alternaria* sp.	*Lewia* sp.	Pleosporaceae
JCM 19.2 JCM 19.7	*Phomopsis* sp.	*Diaporthe* sp.	Pleosporaceae
JCM 19.4	*Glomerella* sp.	*Colletotrichum sp.*	Glomerellaceae
JCM 19.6	*Corynespora* sp.		Corynesporascaceae
JCM 19.8	*Neofusicoccum parvum*	*Fusicoccum parvum*	Botryosphaeriaceae
JCM 22.1	*Fusarium*	*F. semitectum, F.*	Hypocreaceae

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	incarnatum	*pallidoroseum*	
JCM 23.3	*Corynespora cassiicola*	*Helminthosporium vignicola*	Corynesporascaceae

2.1.4 Endophytic fungi worked on during the study
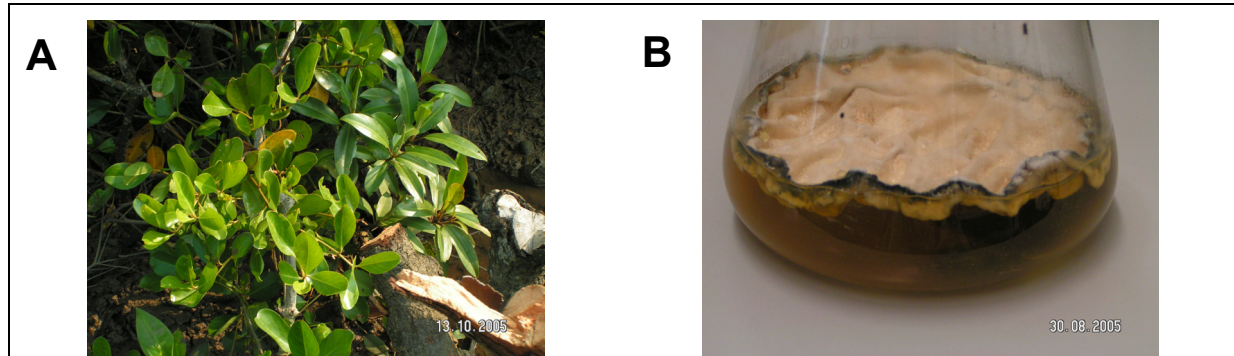
Figure 2.1.4a. *Cladosporium sphaerospermum* (**A**: *Aegiceras corniculatum* **B**: pure strain in liquid Wickerham medium).
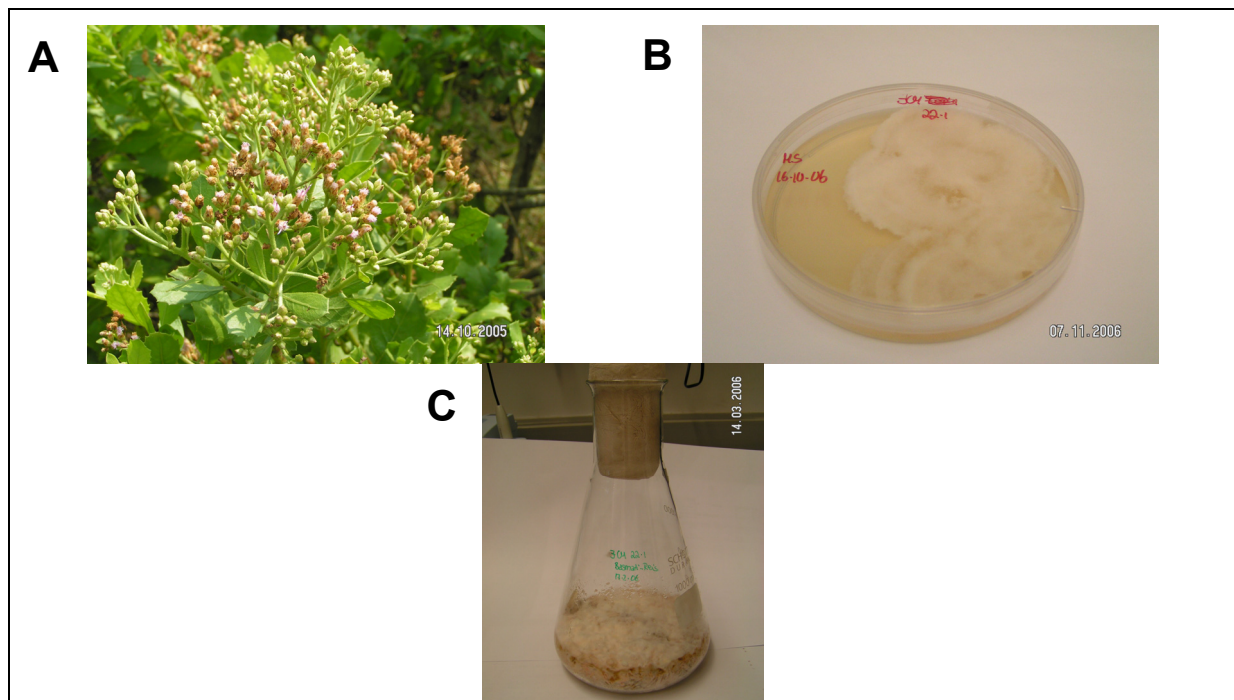
Figure 2.1.4b. *Fusarium incarnatum* (**A**: *Pluchea indica* **B**: pure strain on agar plate **C**: culture on solid rice medium).
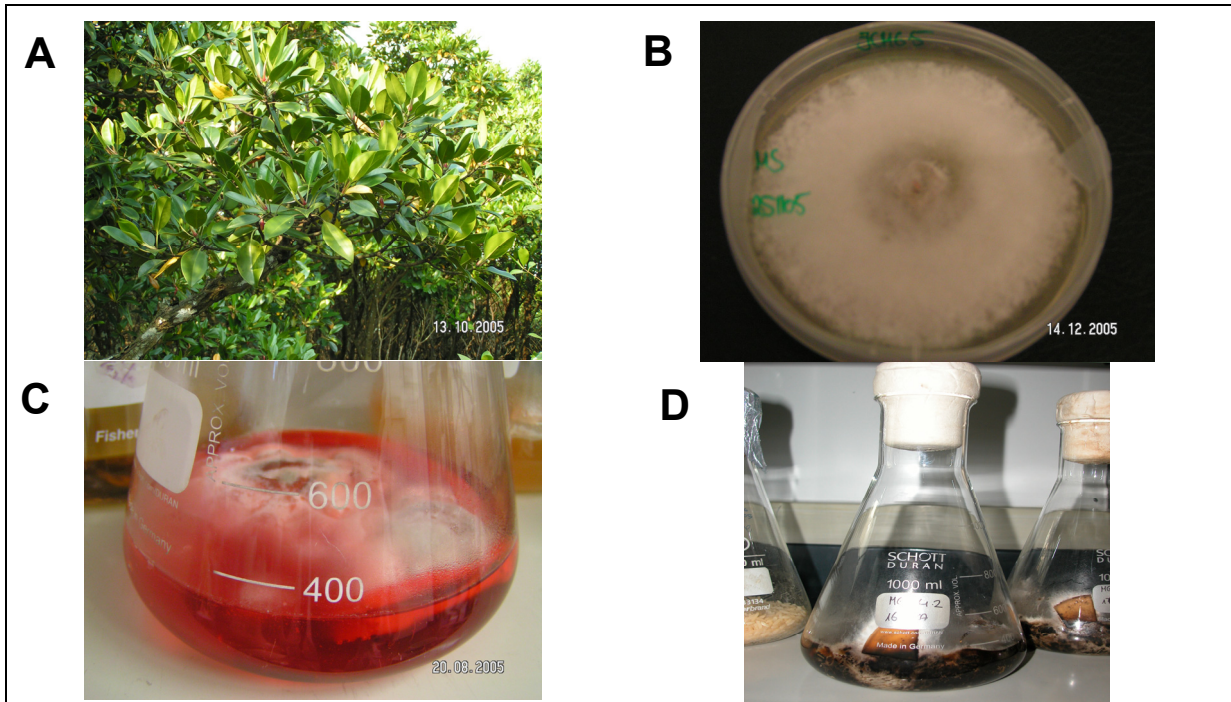
Figure 2.1.4c. *Nigrospora* sp. (**A**: *Bruguiera sexangula* **B**: pure strain on agar plate **C**: culture in liquid Wickerham medium **D**: culture on solid rice medium).
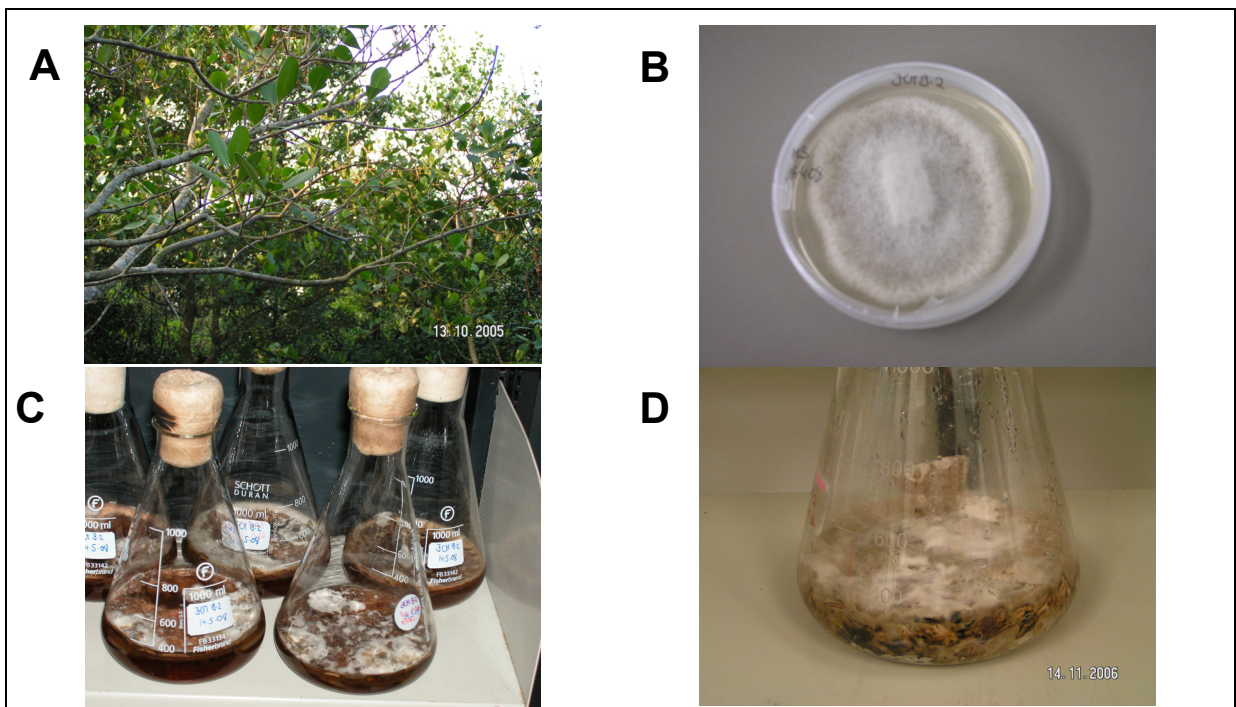
Figure 2.1.4d. *Alternaria* sp. (**A**:*Sonneratia alba* **B**:pure strain on agar plate **C**:culture in liquid Wickerham medium **D**: culture on solid rice medium).
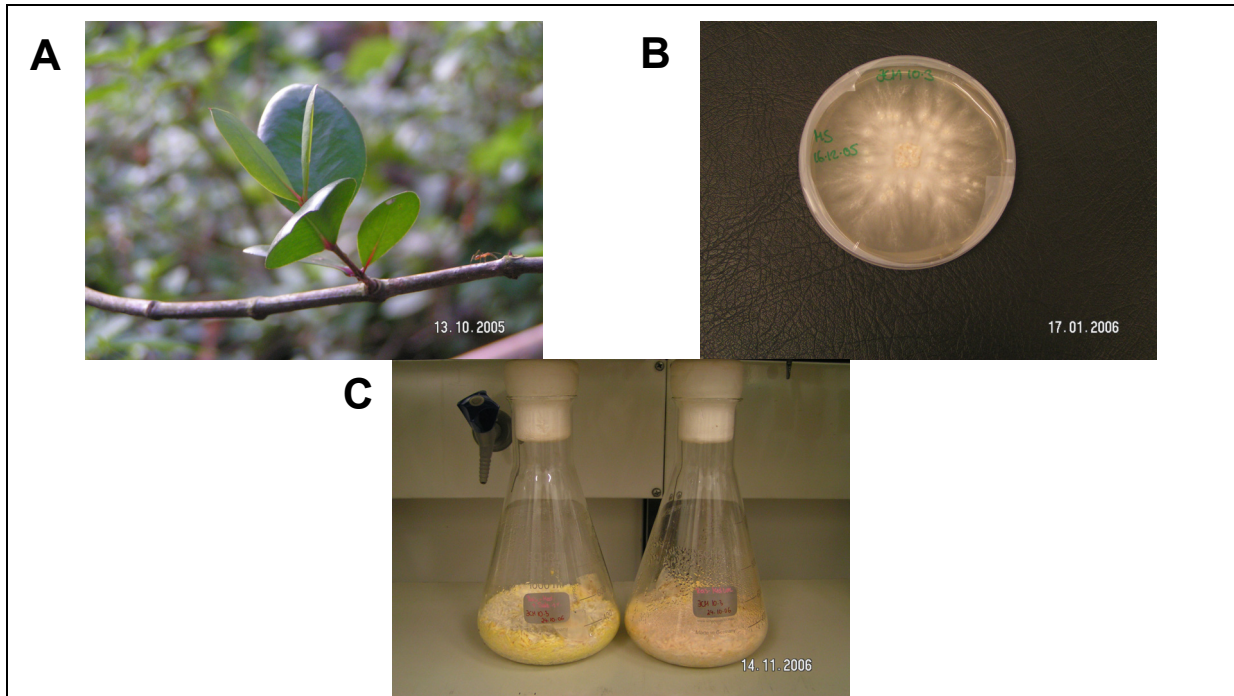
Figure 2.1.4e. *Bionectria ochroleuca* (**A**: *Sonneratia caseolaris* **B**: pure strain on agar plate **C**: culture on solid rice medium).

2.2 Isolation and cultivation of endophytic fungi

2.2.1 Composition of media

Medium for isolation of fungal strains from mangrove plants

Bacto agar (Galke)	15.0 g
Malt extract (Merck)	15.0 g
Artificial sea salt (Sera)	10.0 g
Chloramphenicol (Sigma)	0.2 g
pH	7.4-7.8 (adjusted with NaOH/HCl)
Dem. Water	ad 1000 mL

Medium for purification and short term storage of fungal strains

Bacto agar (Galke)	15.0 g
Malt extract (Merck)	15.0 g
Artificial sea salt (Sera)	10.0 g
pH	7.4-7.8 (adjusted with NaOH/HCl)
Dem. water	ad 1000 mL

MexA medium for long term storage

Malt extract (Merck)	20.0 g
Yeast extract (Sigma)	0.1 g
Glycerin (Roth)	50.0 g
Artificial sea salt (Sera)	10.0 g
Bacto Agar (BD)	13.0 g
Dem. water	ad 1000 mL

Liquid Wickerham medium

Yeast extract (Sigma)	3.0 g
Malt extract (Merck)	3.0 g
Peptone (Merck)	5.0 g
Glucose (Caelo)	10.0 g
Artificial sea salt (Sera)	10.0 g
pH	7.2-7.4 (adjusted with NaOH/HCl)
Dem. water	ad 1000 mL

For the experiments in enhancing the fungal productivity of strains MGC 4.2 and JCM 9.2, different HDAC-inhibiting substances in varying concentrations were added to the liquid medium, one inhibitor per flask. After fermentation for one week and two weeks, the same concentration of each substance was added to the medium again:

- Psammaplin A: 10 µg/300 mL (0.05 µM), 100 µg/300 mL (0.5 µM), 200 µg/300mL (1 µM)
- 5-Azacytidine (Hybri-Max™, Sigma-Aldrich): 74 µg/300 mL (1 µM), 366 µg/300 mL (5 µM)
- Sodiumvalproate (Ergenyl™, Sanofi-Aventis): 43 mg/300 mL (1mM), 173 mg/300 mL (4 mM)

For the search of bacteria in the fungal strain JCM 9.2, the antibiotics chloramphenicol or penicillin G (Sigma) were added at concentrations of 0.7 g/300 mL or 1.0 g/300 mL, respectively.

Solid rice medium

Rice	100 g
Dem. Water	110 mL

For first experiments in search of the most suitable medium, artificial sea salt was added in a concentration of 2 g per flask.

2.2.2 Isolation of pure fungal strains

The plant samples were washed with demineralised water, dried and immersed in EtOH 70% for 60-120 s for surface sterilisation. They were dried with sterile cotton cloth to stop the sterilisation process and carefully struck over the surface of a first petri dish containing isolation medium with sterilized tweezers to ensure that no surface contaminating microbes were isolated. A piece of tissue was then cut it into smaller segments with a sterile razor blade and put on a second petri dish containing isolation medium so that the freshly cut edges were in direct contact with the agar surface. After several days first fungal growth was observable. If different fungal strains developed from one sample, the individual strains were isolated by transferring hyphal tips growing out of the cut tissue pieces with a sterile loop onto a fresh malt agar dish. For purification of the fungal strains this step was repeated several times until the colony was deemed uniform.

2.2.3 Long term storage of pure fungal strains

For long term storage, pieces of pure fungal strains were transferred to 10 mL BD Falcon® tubes containing approx. 5 mL MexA medium. When growth could be observed (after approx. 3 days, depending on the fungal strain), the strain was placed in a deep freezer at -80°C.

2.2.4 Cultivation for screening and isolation

For small scale and large scale fermentation, a pure fungal strain was inoculated in a 1000 mL Erlenmeyer flask containing either 300 mL of liquid Wickerham medium or 210 g of solid rice medium. For this purpose, a strain that nearly covered the surface of a petri dish (after 1-2 weeks growth on malt agar medium) was cut into small pieces and these were transferred to an Erlenmeyer flask containing the sterilised medium. Cultivation was performed at room temperature under static conditions and daylight. Depending on the fungal growth, cultures on liquid medium were incubated for 3-4 weeks, on rice for 4-6 weeks. The fermentation was brought to an end by adding 250 mL EtOAc to the culture flask and standing closed for at least 24 hours.

Small scale fermentation was carried out in one Erlenmeyer flask to gain enough extract for first bioactivity screening. For mass growth to gain enough fungal extract for isolation of secondary metabolites, 20 flasks of rice or Wickerham medium were inoculated.

For the experiment to enhance transcriptional activation of gene clusters and thus the fungal productivity by the addition of various DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors, to liquid media were added the following substances in two different concentrations:

- Psammaplin A   0.05 μM, 0.5 μM to MGC4.2
                                 0.5 μM, 1 μM to JCM9.2
- 5-Azacytidine   1 μM, 5 μM to MGC4.2
                                 1 μM, 5 μM to JCM9.2
- Valproic acid   1 mM, 4 mM to MGC 4.2.

Two culture flasks were prepared of each concentration. The fermentation time was three weeks. After each week a sample was aseptically taken for screening by the Dionex HPLC system and each substance added to the culture flask again in the respective concentration. Three control flasks without addition of any substance were fermented of each fungal strain.

2.3   Identification of microbes

Fungi that showed promising results in biological and chemical screening were identified by molecular biology methods. These methods were also used to examine the fungal culture JCM 9.2 for bacterial growth.

2.3.1   Fungal identification

Taxonomic identification of the fungal strains was achieved by DNA amplification and sequencing of the fungal ITS region. For this purpose, a piece of fungal mycelium (0.5 cm^2) was sampled from an agar plate, lyophilized in a freeze dryer and powderised in a mixer mill after adding a tungsten carbide bead (Qiagen).

DNA isolation was performed using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. The procedure included cell lysis, digestion of RNA by RNase A, removing of precipitates and cell debris, DNA shearing, DNA precipitation and purification. This was followed by DNA amplification using Hot StarTaq Master Mix Taq polymerase (Qiagen) and the primer pair ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (Invitrogen) in an iCycler thermocycler. Each sample contained 25 µL Taq polymerase master mix, 3 µL primer mix (each 10 pmol/µL), 3 µL template and 19 µL RNA-free water. PCR was carried out according to the following protocol:

Table 2.3.1. PCR program for amplification of fungal DNA using primer ITS1 and ITS4.

1.	15:00 min	95°C	initial denaturation	
2.	1:00 min	95°C	denaturation	These steps
3.	0:30 min	56°C	annealing	were repeated
4.	1:00 min	72°C	extension	35 times
5.	10:00 min	72°C	final extension	
6.	∞	4°C	storage until workup	

10 µL of the PCR product together with 2µL loading buffer were loaded onto agarose gel (2% agarose (Sigma) in 100 mL TBE buffer (Merck) with 10 µl SybrSafe (Invitrogen) for DNA staining). For comparison of the size of the isolated DNA fragments, a DNA ladder was loaded onto the electrophoresis gel as well. After electrophoresis at 70 V for 60 min, the band due to the PCR product (approx. size 550 bp) was isolated from the gel slice using the PerfectPrep® Gel Cleanup Kit (Eppendorf) following the manufacturer's protocol.

Pure PCR products together with the primer ITS1 (10 pmol/µL) were submitted for sequencing to the BMBF, Heinrich-Heine-Universität Düsseldorf). The base sequences were compared with publicly available databases with the help of Blast-Algorithmus on the BLAST homepage (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the option "blastn".

2.3.2   Investigation for bacteria

The strain JCM 9.2 was examined for bacterial growth inside the hyphal cells by isolation and amplification of bacterial DNA. For this purpose, the same protocol as for the fungal identification was used. The only difference was the use of bacterial

primers 149r (GGTTACCTTGTTACGACTT) and 27f (AGAGTTTGATCCTGGCTCAG) and a PCR protocol suitable for these primers:

Table 2.3.2. PCR program for amplification of bacterial DNA using primer 27f and 149r.

1.	5:00 min	95°C	initial denaturation	
2.	1:00 min	95°C	denaturation	These steps
3.	1:00 min	56°C	annealing	were repeated
4.	1:30 min	72°C	extension	30 times
5.	5:00 min	72°C	final extension	
6.	∞	4°C	storage until workup	

The Gram-positive bacterium *Bacillus subtilis* and the Gram-negative *Escheirichia coli* served as positive controls.

2.4 Extraction of fungal cultures

2.4.1 Extraction of small scale cultures grown in liquid Wickerham medium

The content of the culture flask including the added 250 mL of EtOAc was thoroughly mixed in an Ultraturrax at 4000 u/min for cell destruction and extraction for 10 min and filtered under vacuum using a Buchner funnel. The mycelium was discarded and the culture filtrate transferred to a separation funnel. The EtOAc and H_2O phases were separated and the aqueous phase extracted two more times with 300 mL EtOAc each. The combined EtOAc phases were washed with 100 mL dem. water to eliminate any remaining sea salt.

2.4.2 Extraction of large scale cultures grown in liquid Wickerham medium

For the workup of the large scale fermentation of fungal strain JCM 9.2 grown in liquid medium, mycelia were separated from culture media, covered with approx. 5 L MeOH and left standing overnight followed by extraction for 10 min at 4000 u/min using an Ultraturrax. The mixture was filtered under Vacuum using a Buchner funnel and the extraction repeated two times until exhaustion. The obtained extract was taken to dryness and re-extracted successively with *n*-hexane, EtOAc, BuOH and dem. water. The culture media were extracted in the same manner as described for the extraction of small scale cultures to obtain the EtOAc extract.

2.4.3 Extraction of fungi grown on solid rice medium

The culture medium containing the mycelium was cut into small pieces to allow exhaustive extraction with EtOAc. The contents were filtered under vacuum using a Buchner funnel and the extraction repeated two times with EtOAc until exhaustion. The combined EtOAc phases were washed with 300 mL demineralised water to eliminate remaining sugar and starch.

All obtained extracts were taken to dryness under reduced pressure at 40°C and partitioned between 90% MeOH and *n*-hexane to remove the fatty acids. After evaporating the solvent, these crude extracts were submitted to TLC, analytical HPLC, LC-MS and also to bioactivity screening including cytotoxicity and antimicrobial assays.

Based on these results, promising extracts were processed in order to isolate and identify pure secondary products.

2.4.4 Extraction and fractionation of mangrove plant material

Leaves from *Sonneratia alba* (JCM 9) were extracted overnight with Ethanol 96% (V/V) and the resulting extract was dried. The dried residue was partitioned between EtOAc and water. The EtOAc soluble fraction was dried again and subjected to fractionation over Diaion HP-20 using H_2O:MeOH step gradient elution.

2.5 Isolation of secondary natural products

For the isolation of natural products different chromatographic methods depending on the nature of the product can be used. A chromatographic system comprises two phases, a stationary phase absorbing the compounds according to their physical properties, e. g. polarity (silica gel, normal or reversed phase), size (Sephadex LH-20) or charge (Diaion), whereas the mobile phase moves through the stationary phase and gradually elutes the compounds according to their affinity from it.

2.5.1 Thin layer chromatography (TLC)

Analytical TLC is an inexpensive, fast and qualitatively relevant technique for screening of the chemical profiles of crude extracts or fractions as well as for choosing appropriate conditions for column chromatography.

During this work, TLC was performed on pre-coated TLC plates using the following systems:

TLC on silica gel 60 F_{254}, layer thickness 0.2 mm (Merck):

- *n*-hexane:EtOAc [90:10, 80:20 and 70:30 (V/V)] and *n*-hexane:MeOH [95:5 and 90:10 (V/V)] for non-polar compounds
- DCM:MeOH [95:5, 90:10, 85:15, 80:20 and 70:30 (V/V)] for semi-polar compounds
- EtOAc:MeOH:H2O[30:5:4, 30:65 and 30:7:6 (V/V)] for polar compounds

TLC on Diol F_{254}S, layer thickness 0.25 mm (Merck):

- *n*-hexane:EtOAc [90:10, 80:20 and 70:30 (v/v)] EtOAc:acetone [95:5 and 90:10 (V/V)]

TLC on reversed phase RP-18, F_{254} S, layer thickness 0.25 mm (Merck):

- MeOH:H_2O (90:10, 80:20, 70:30 and 60:40 (V/V).

The band separation on TLC was observed under a UV lamp at 254 or 366 nm, followed by spraying TLC plates with anisaldehyde/sulphuric acid or ninhydrin spraying reagent and heating plates at 110°C.

The different compounds could be compared and identified due to their specific retention factors in defined chromatographic systems. These so-called R_f-values can be calculated as

$$R_f = \frac{\text{migration distance of compound}}{\text{migration distance of solvent front}}$$

With ninhydrin spray reagent (0.2 % in MeOH, m/V) amino acids, amines and amino sugars can be detected. The composition of anisaldehyde/sulphuric acid spray reagent, useful for the detection of phenols, steroids, sugars and terpenes, was

Methanol	85 mL
Glacial acetic acid	10 mL
Conc. sulphuric acid	5 mL
Anisaldehyde	0.5 mL

2.5.2 Vacuum liquid chromatography (VLC)

VLC is a useful method as an initial isolation step employing extracts or fractions exceeding amounts of 1 g. Silica gel is packed to a hard cake under applied vacuum. The sample to be separated was adsorbed onto a small amount of silica gel using appropriate organic solvents (mostly MeOH). After evaporation of the solvent, the resulting sample mixture was packed onto the top of the column. By step gradient elution starting with a non-polar solvent (e. g. *n*-hexane or DCM) and increasing amounts of polar solvents (e. g. EtOAc or MeOH) successive fractions were collected. The flow was produced by applying vacuum with the help of a pump. The column was allowed to run dry after each collected fraction.
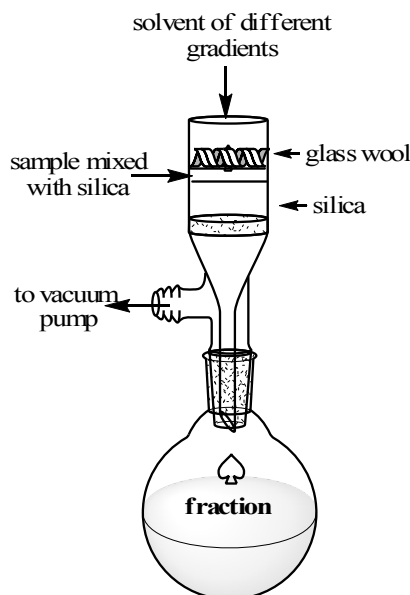
Figure 2.5.2 VLC setup.

Three different gradient systems were used:

Table 2.5.2a. Proportion of solvent mixtures used for VLC of ethyl acetate-extract of rice culture of *Nigrospora* sp.

Fraction	*n*-hexane [mL]	EtOAc [mL]	DCM [mL]	MeOH [mL]
I	500	-	-	-
II	375	125	-	-
III	250	250	-	-
IV	125	375	-	-
V	-	-	500	-
VI	-	-	375	125
VII	-	-	250	250
VIII	-	-	125	375
IX	-	-	-	500

Table 2.5.2b. Proportion of solvent mixtures used for VLC of ethyl acetate-extract of liquid and rice culture of *Alternaria* sp.

Fraction	DCM [mL]	MeOH [mL]
I	500	-
II	360	40
III	320	80
IV	280	120
V	240	160
VI	200	200
VII	160	240
VIII	120	280
IX	80	320
X	40	360
XI	-	500

Table 2.5.2c. Proportion of solvent mixtures used for VLC of ethyl acetate-extract of liquid and rice culture of *Bionectria* sp.

Fraction	*n*-hexane [mL]	EtOAc [mL]	DCM [mL]	MeOH [mL]
I	500	-	-	-
II	320	80	-	-
III	240	160	-	-
IV	160	240	-	-
V	80	320	-	-
VI			500	-
VII	-	-	340	60
VIII	-	-	280	120
IX			220	180
X			160	240
XI	-	-	100	300
XII	-	-	-	500

2.5.3 Low pressure liquid chromatography (LC)

Open column chromatography was used to separate fractions of different amounts and polarities and to ultimately yield purified natural products. Appropriate stationary phases and mobile phase solvent systems were previously determined and optimized by TLC and were adjusted for a column of suitable size for the mixture. The following separation systems were used:

- **Normal phase** chromatography using a polar stationary phase, typically silica gel (Silica Gel 60, 0.04 – 0.063 mm mesh size , Merck) or Diol (LiChroprep® Diol (40 – 63 µm) for liquid chromatography, Merck), in conjunction with a non-polar mobile phase (e.g. *n*-hexane, DCM) or with a gradually increasing amount of a polar solvent (e.g. EtOAc or MeOH). Hydrophobic compounds elute quicker than hydrophilic compounds.
- **Reversed phase (RP)** chromatography using a non-polar stationary phase and a polar mobile phase (e.g. H_2O or MeOH). The stationary phase consists of reversed phase silica material. For instance, RP-18 stands for an octadecyl ligand in the matrix of the RP-18, 0.04 – 0.0.63 mm mesh size (Merck) material that was used. The more hydrophobic the matrix, the greater the tendency of the column to retain hydrophobic compounds. Thus, hydrophilic

compounds elute more quickly than do hydrophobic compounds. Elution was performed using H_2O with gradually increasing amounts of MeOH or acetonitrile.

- **Size exclusion** chromatography involves separations based on molecular sizes of analyzed compounds. The stationary phase consists of porous beads (Sephadex LH-20, 0.25 – 0.1 mm mesh size, GE Healthcare). Compounds having larger molecular diameter are excluded from the interior of the bead and thus elute firstly, while compounds with smaller molecular diameters enter the beads and elute according to their ability to exit from the small sized pores where they are trapped. Elution was performed using MeOH or MeOH:DCM [1:1 (V/V)] as mobile phases.
- **Ion exclusion** chromatography uses ion exchange resin beds (Diaion HP-20, Supelco) that act as a charged solid separation medium. The components of the processed sample have different electrical affinities to this medium and consequently are differently retained by the resin according to their different affinities.

The eluted fractions were collected by an automate fraction collector and combined according to TLC results.

2.5.4 High pressure liquid chromatography (HPLC)

High pressure liquid chromatography (HPLC) is a high resolution chromatographic technique, where the mobile phase is forced through the column containing the stationary pharse by a pump, resulting in a fast separation. The high resolution is achieved by the use of small particle size of the absorbent material, which mostly is RP silica material. Adjacent to the HPLC a UV detector is connected for monitoring the separation of the eluted compounds shown in a chromatogram. The method can be used for semi preparative or analytical purposes.

2.5.4.1 Semi preparative HPLC

This technique was used for isolation and purification of compounds from fractions previously pre-purified using column chromatographic separation. The most appropriate solvent systems were determined by analytical HPLC before running the preparative HPLC separation. The mobile phase consisted of MeOH and nanopure H_2O. Each injection consisted of 1–3 mg of the fraction dissolved in 100 µL 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.

2.5.4.2 Analytical HPLC

All extracts, fractions and pure compounds were monitored by anylytical HPLC to determine the composition of the fractions, the purity of the isolated substances and the optimum conditions for semi preparative HPLC. Additionally, known substances could be identified by comparison of the obtained UV spectra with the internal UV-spectra library using the online software. For the routine analytical HPLC detection a solvent system of nanopure water adjusted to pH 2.0 by addition of

phosphoric acid (eluent A) and MeOH (eluent B) with a flow rate of 1 mL/min employing the standard gradient system (see table 2.5.4.2) was used.

Table 2.5.4.2. Standard gradient for analytical HPLC.

Time (min)	Eluent A (%)	Eluent B (%)
0	90	10
5	90	10
35	0	100
45	0	100
46	90	10
60	90	10

In case of insufficient separation the gradient was adjusted adequate to the substances to be separated.

2.5.5 Isolation and purification of fungal extracts
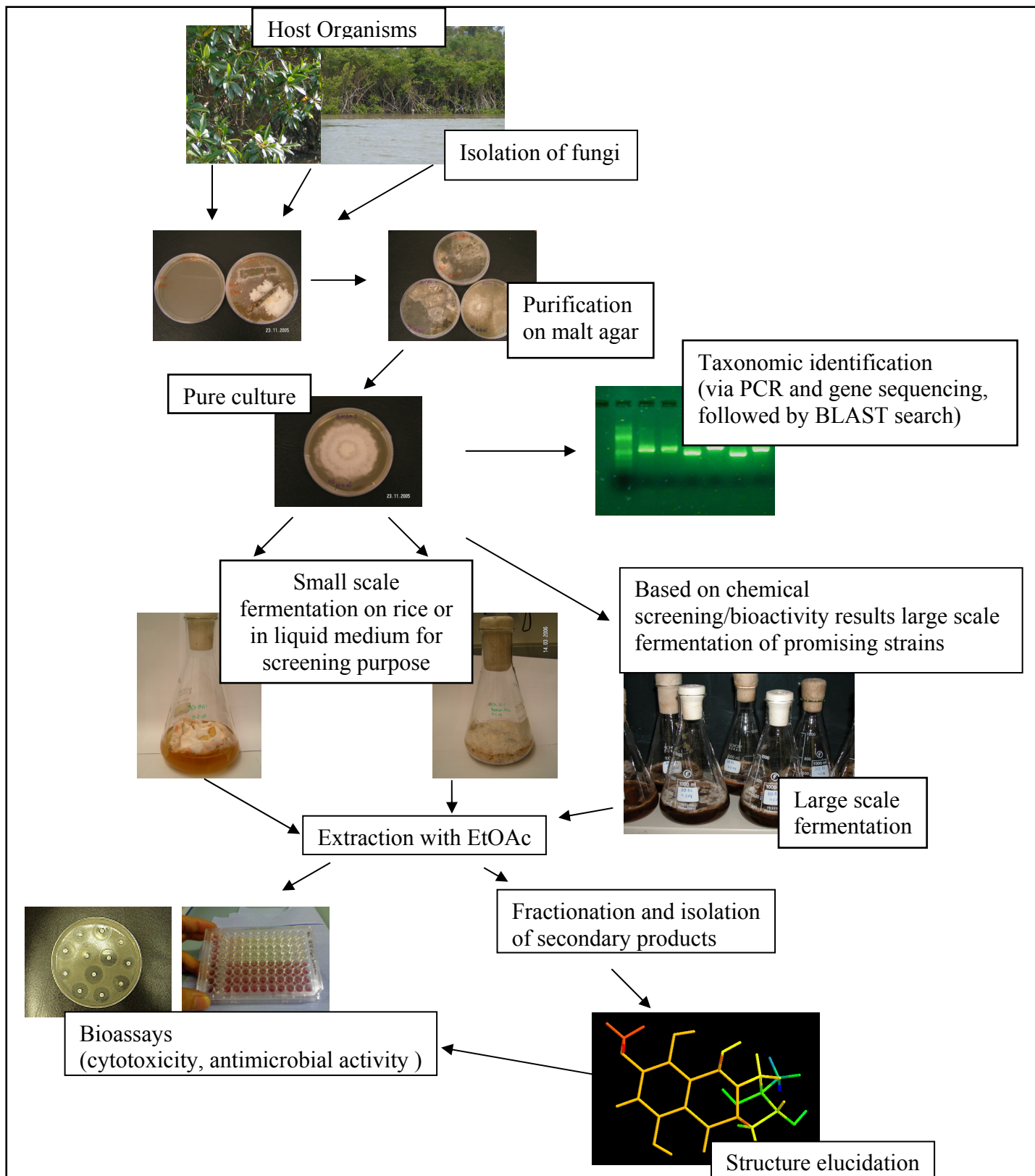
Figure 2.5.5. Schematic overview of important steps involved in the isolation of fungi from mangroves and in the isolation and identification of their secondary metabolites.

2.5.5.1 Secondary metabolites isolated from fermentation of *Cladosporium sphaerospermum* in liquid Wickerham medium

```
                    Crude EtOAc extract
                         670 mg
                            |
                    solvent fractionation
              _____|_____
             |                                 |
     90% MeOH extract                   n-hexane extract
         393 mg                             165 mg
            |
  Sephadex column (60% MeOH:40% CHCl₃)
    _____|_____
   |                                 |
Fraction 5                       Citrinin
   |                              82 mg
 semi preparative HPLC
   |
Quinolactacin A1 and A2
       1.1 mg
```

Sephadex column (60% MeOH:40% CHCl$_3$)
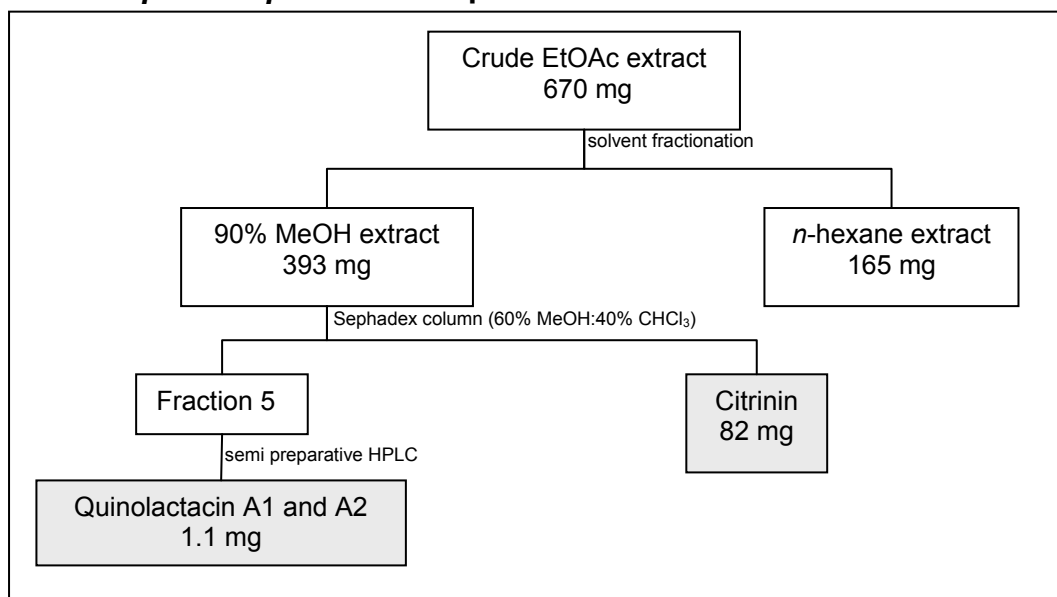
2.5.5.2 Secondary metabolites isolated from fermentation of *Fusarium incarnatum* on solid rice medium

```
                    Crude EtOAc extract
                        2680 mg
                            |
                    solvent fractionation
              _____|_____
             |                                 |
     90% MeOH extract                   n-hexane extract
        2150 mg                             458 mg
            |
           VLC
            |
        fraction 6
         252 mg
            |
  Diol column (60% n-hexane/40% acetone)
            |
        Equisetin
         47 mg
```

Diol column (60% *n*-hexane/40% acetone)
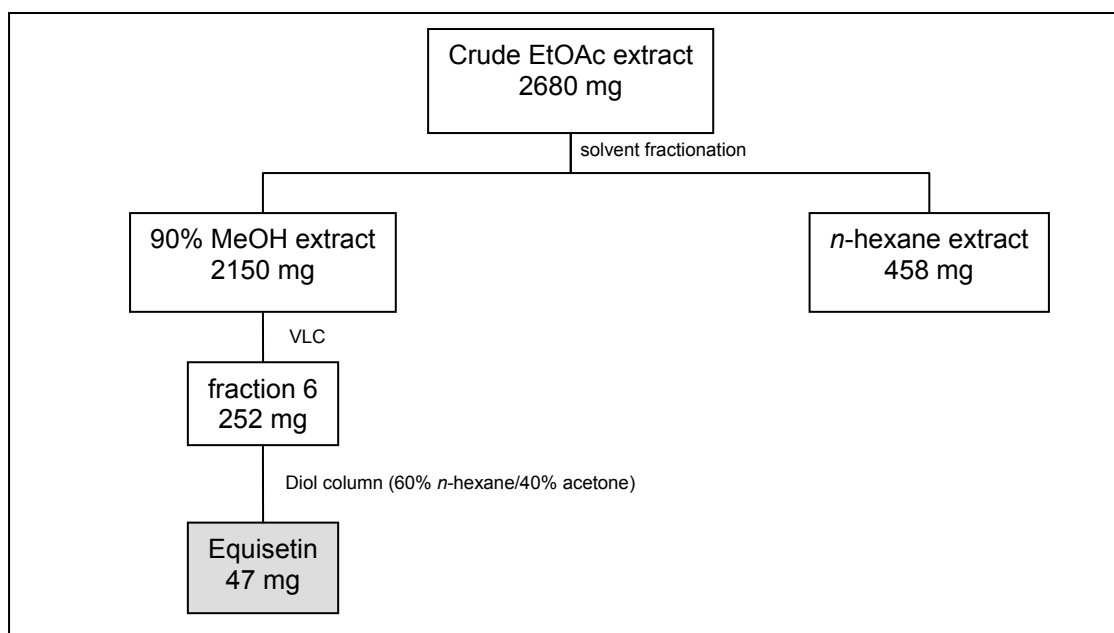
2.5.5.3 Isolation of secondary metabolites from *Nigrospora* sp.

2.5.5.3.1 Secondary metabolites isolated from fermentation of *Nigrospora* sp. in liquid Wickerham medium

```
                        ┌─────────────────────┐
                        │  Crude EtOAc extract │
                        │       300 mg         │
                        └─────────────────────┘
                                   │ solvent fractionation
                   ┌───────────────┴───────────────────┐
        ┌──────────────────┐              ┌──────────────────┐
        │  90% MeOH extract│              │  n-hexane extract│
        │      126 mg      │              │       90 mg      │
        └──────────────────┘              └──────────────────┘
                 │ Sephadex column 100% MeOH
      ┌──────────┼────────────────────────┐
┌───────────┐ ┌──────────────────────────┐ ┌──────────────────┐
│Fraction D │ │1-Deoxytetrahydrobostrycin│ │  Deoxybostrycin  │
│  11.8 mg  │ │         8.0 mg           │ │      2.4 mg      │
└───────────┘ └──────────────────────────┘ └──────────────────┘
      │ Semi preparative HPLC
┌───────────┐
│  Aloesol  │
│  1.8 mg   │
└───────────┘
```

2.5.5.3.2 Secondary metabolites isolated from fermentation of *Nigrospora* sp. on solid rice medium

```
                        Crude EtOAc extract
                            7350 mg
                               |
                               | solvent fractionation
              ┌────────────────┴────────────────┐
        90% MeOH extract                  n-hexane extract
           393 mg                            165 mg
              |
              | VLC
      ┌───────┼───────────────┐
  Fraction VII       Fraction VIII  +  Fraction IX
    1741 mg            2449 mg          1755 mg
      |                     |
      | Silica column       | Sephadex 100% MeOH
      | (gradient DCM:MeOH)
  ┌───┼──────────┐      ┌────────┴────────┐
Fraction 8  Fraction 11  Fraction 16   Fraction 8   Fraction 9
 6.6 mg      76 mg        742 mg         23 mg        76 mg
   |           |            |              |            |
   | Semi      | RP column  | HP-20 column | Semi       | Semi
   | preprative|            |              | preprative | preprative
   | HPLC      |            |              | HPLC       | HPLC
Indole-3-carbaldehyde              Bostrycin    Bostrycin  Tenuazonic acid
   0.4 mg                          14.8 mg       0.8 mg      14.1 mg
             Fraction 3
               4.5 mg
                  |
                  | Semi preprative HPLC
         Cyclo(threonylisoleucinyl)
                1.6 mg
```

2.5.5.4 Isolation of secondary metabolites from *Alternaria* sp.

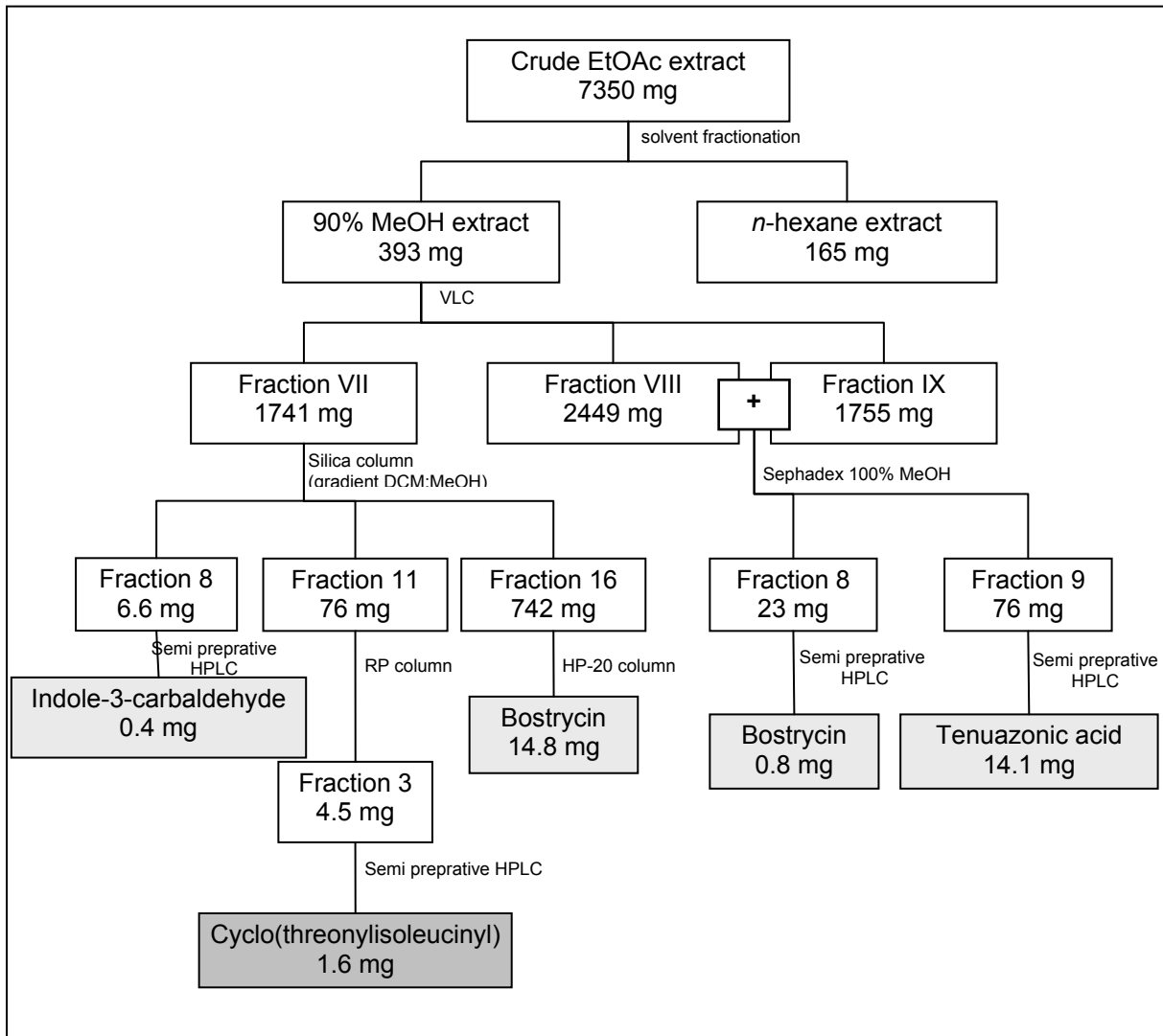## 2.5.5.4.1 Secondary metabolites isolated from fermentation of *Alternaria* sp. in liquid Wickerham medium
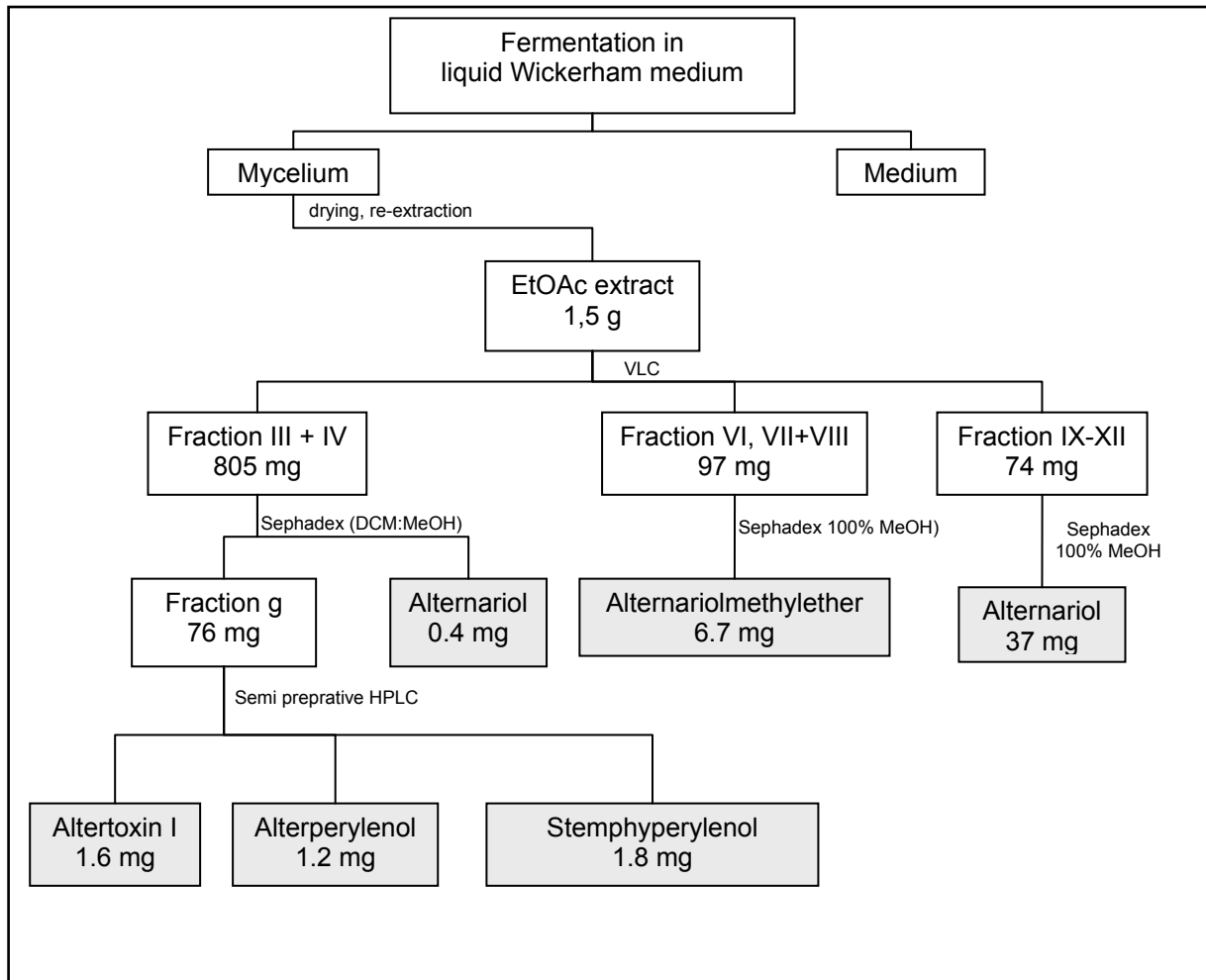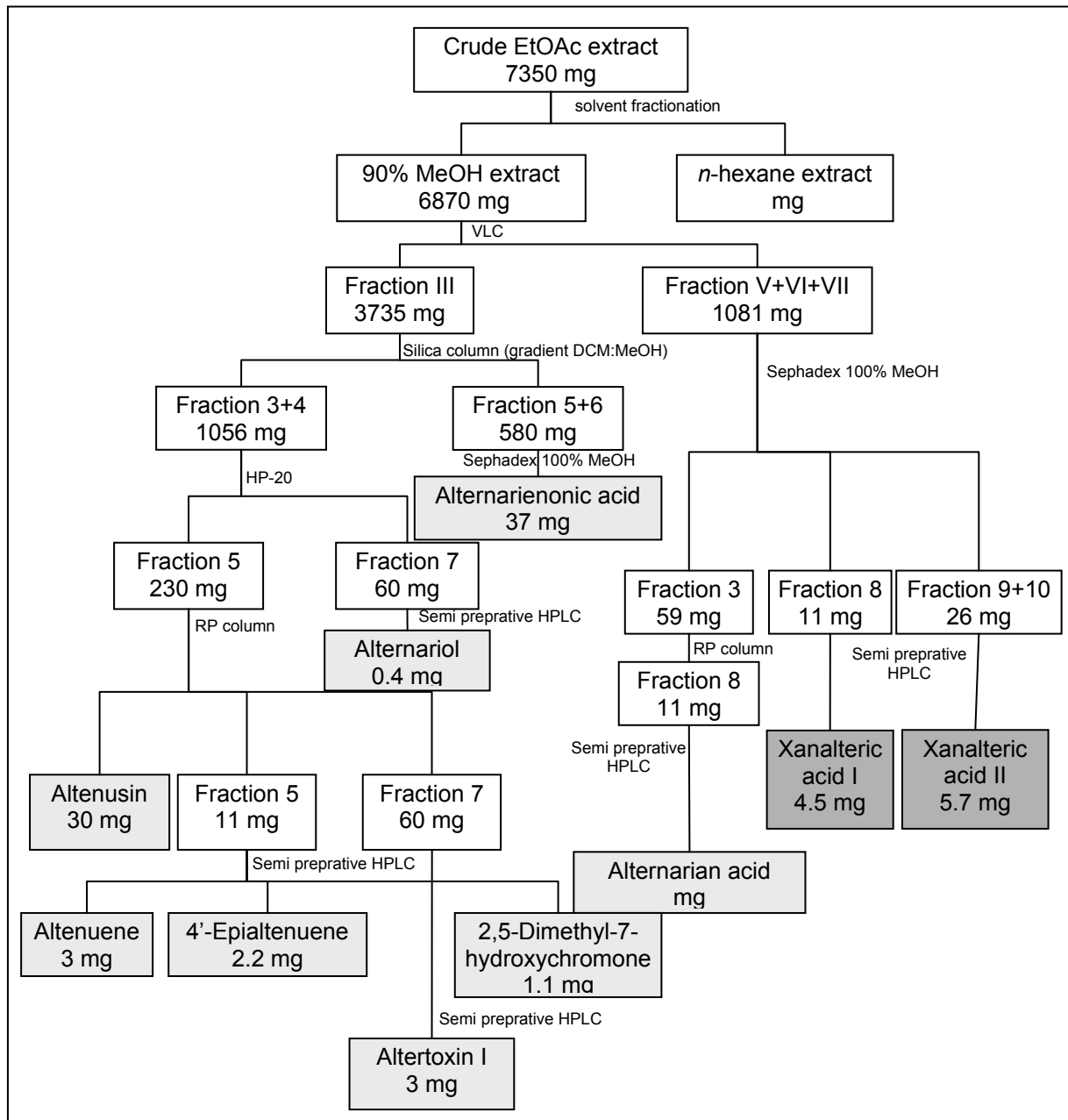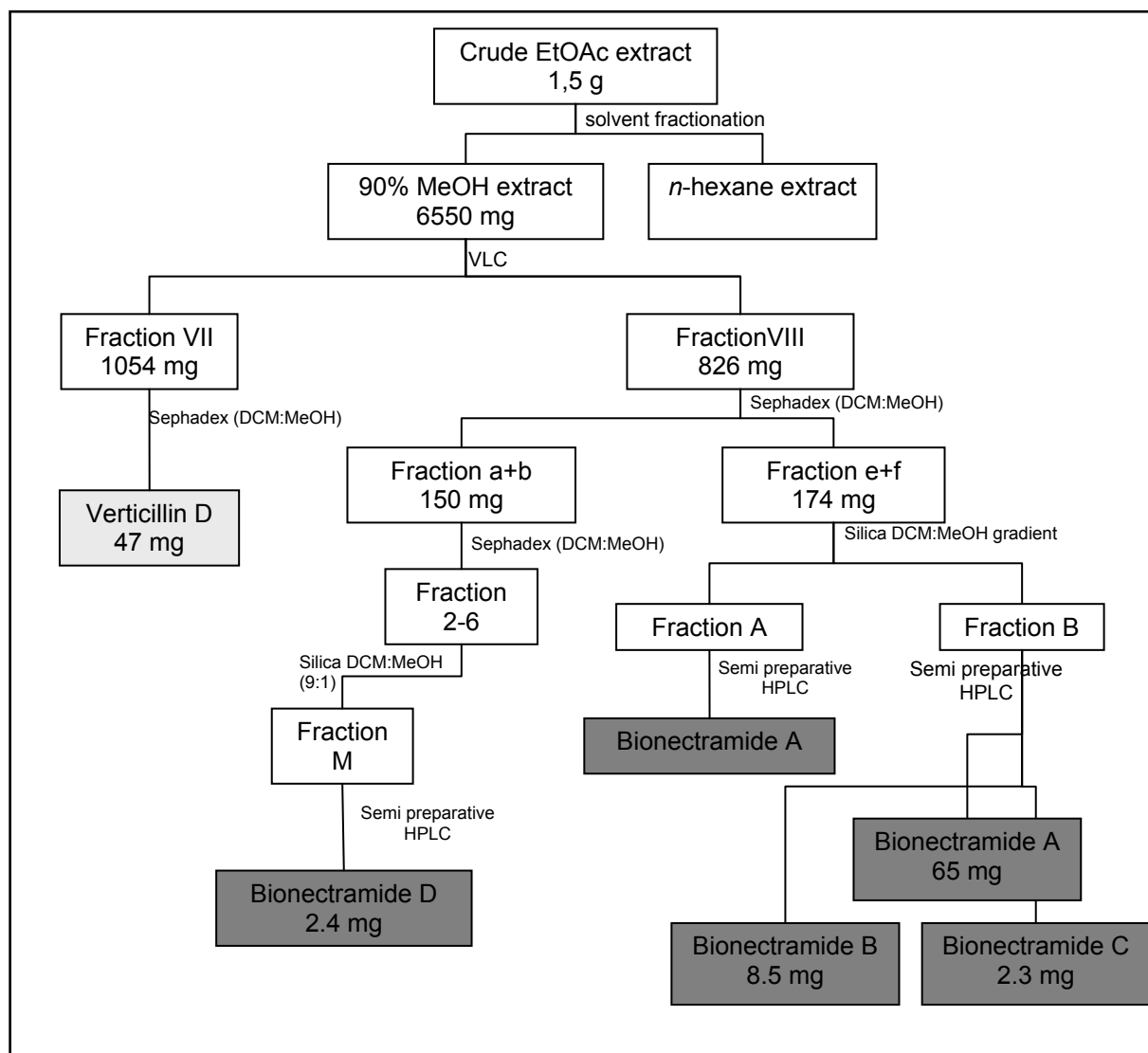
2.5.5.4.2 Secondary metabolites isolated from fermentation of *Alternaria* sp. on solid rice medium

```
                        Crude EtOAc extract
                             7350 mg
                                │ solvent fractionation
              ┌─────────────────┴─────────────────┐
      90% MeOH extract                      n-hexane extract
          6870 mg                                  mg
              │ VLC
      ┌───────┴────────────┐
  Fraction III          Fraction V+VI+VII
    3735 mg                 1081 mg
      │ Silica column (gradient DCM:MeOH)       │ Sephadex 100% MeOH
  ┌───┴──────────┐
Fraction 3+4    Fraction 5+6
  1056 mg          580 mg
      │ HP-20         │ Sephadex 100% MeOH
              Alternarienonic acid
                    37 mg
  ┌───────┴──────┐
Fraction 5    Fraction 7
  230 mg        60 mg
      │ RP column    │ Semi preprative HPLC
              Alternariol
                0.4 mg
```

Fraction 3 — 59 mg
Fraction 8 — 11 mg
Fraction 9+10 — 26 mg
(RP column)
Fraction 8 — 11 mg
(Semi preprative HPLC)
Xanalteric acid I — 4.5 mg
Xanalteric acid II — 5.7 mg

Altenusin — 30 mg
Fraction 5 — 11 mg
Fraction 7 — 60 mg
(Semi preprative HPLC)

Alternarian acid — mg

Altenuene — 3 mg
4'-Epialtenuene — 2.2 mg
2,5-Dimethyl-7-hydroxychromone — 1.1 mg
(Semi preprative HPLC)

Altertoxin I — 3 mg

2.5.5.5 Secondary metabolites isolated from fermentation of *Bionectria ochroleuca* on solid rice medium

```
                    Crude EtOAc extract
                          1,5 g
                             |
                    solvent fractionation
                    |                    |
          90% MeOH extract        n-hexane extract
             6550 mg
                |
               VLC
        |                              |
   Fraction VII                   FractionVIII
     1054 mg                         826 mg
        |                              |
  Sephadex (DCM:MeOH)           Sephadex (DCM:MeOH)
        |                    |                    |
   Verticillin D        Fraction a+b         Fraction e+f
      47 mg               150 mg                174 mg
                             |                    |
                    Sephadex (DCM:MeOH)    Silica DCM:MeOH gradient
                             |              |               |
                         Fraction       Fraction A      Fraction B
                           2-6             |               |
                             |       Semi preparative  Semi preparative
                    Silica DCM:MeOH       HPLC              HPLC
                        (9:1)             |
                             |       Bionectramide A
                         Fraction
                            M                      Bionectramide A
                             |                        65 mg
                    Semi preparative
                         HPLC
                             |       Bionectramide B   Bionectramide C
                    Bionectramide D      8.5 mg           2.3 mg
                         2.4 mg
```

2.6 Structure elucidation of the isolated fungal metabolites

The structure elucidation of the isolated compounds followed a standard scheme. Firstly, the data obtained from analytical HPLC were compared with the in-house substance library regarding their retention times at the standard gradient program and their UV spectra. Comparable hits indicated at the class belonging of the compound. From LCMS measurement the mass of the compound and from ^{1}H-NMR measurement substructures could be compiled. With these information, together with the identity of the fungus, a literature search using the latest versions of Dictionary of Natural Products (DNP,Chapman and Hall, 2005-2009), Antibase (2002-2007) and SciFinder was performed. In some cases these data were insufficient and additional measurements, especially one and two dimensional NMR experiments were necessary to finally identify the secondary metabolite.

2.6.1 Mass spectrometry (MS)

Mass spectrometry (MS) determines the molecular weights of pure compounds or compounds in a mixture. It is a very sensitive technique and even from micro gram amounts good spectra can be obtained. The sample is ionized in the ionization source and the rising ions ions are sorted and separated according to their mass (m) to charge (z) ratio (m/z) in the mass analyser. Both negative and positive charged ions can be observed. The molecular ion (parent ion) has to be identified giving the molecular weight of the compound. From the fragmentation patterns of each compound information about substructures can be attained.

2.6.1.1 Electron spray ionization mass spectrometry (ESIMS)

ESIMS is a gentle ionization method where the sample is passed through a high voltage metal capillary. At the end of this capillary it is sprayed by a flow of nitrogen gas at atmospheric pressure to form an aerosol. Together with heating, the nitrogen evaporates the emerging droplets forcing the ions in each droplet together until repulsion causes them to eject from the surface. The ions are extracted into the vacuum of the mass analyzer where they are detected. Additionally to the molecular ion peaks $[M+H]^+$ or $[M-H]^-$ fragments of these can detected.

2.6.1.2 Liquid chromatography mass spectrometry (LC-MS)

The combination of HPLC to an MS enables the recording of mass spectra of components of mixtures that elute successively from the LC column. Thus, quick characterisation of the components is greatly facilitated. Typically this LC-MS system also contains a UV detector so that from each peak found in the chromatogram a mass spectrum and a UV spectrum can be obtained. For the routine measurements a solvent system of 0.1% formic acid (eluent A) and acetonitrile (eluent B) with a flow rate of 0.4 mL/min employing the standard gradient system (see table 2.6.1) was used. Detection was achieved by an ion trap mass filter together with a UV photodiode array detector.

Table 2.6.1. Standard gradient for LCMS system.

Time (min)	Eluent A (%)	Eluent B (%)
0	90	10
5	90	10
35	0	100
45	0	100
46	90	10
60	90	10

2.6.1.3 High resolution mass spectrometry (HR-MS)

Combination of mass filters (double focusing) gives rise to high resolution measurement with an accuracy of about 1ppm. Thus not only a specific molecular mass value is provided, but also the molecular formula of an unknown compound can be established.

2.6.2 Nuclear magnetic resonance spectroscopy (NMR)

This technique utilizes the atomic nuclei spinning behavior of atoms with an odd number of nucleons, e. g. [1]H and [13]C. An NMR spectrum is acquired by varying the magnetic field that is applied to the sample dissolved in a deuterated solvent over a small range while observing the resonance signal from the sample. Depending on the electron density around each proton they obtain different shielding and deshielding effects appearing in different parts of the resulting NMR spectrum and thus provide information about the environment of each proton. The resulting frequency where the nuclei resonate, the so-called chemical shift, is given in ppm and the coupling constants between adjacent nuclei in Hertz (Hz). NMR experiments can be conducted in a one (1D) or two (2D) dimensional manner. 2D NMR spectra can be either measured between two equal (H, H-COSY, correlated spectroscopy) or two different (H;C-COSY) frequency axes. For the H-H 2D experiments the connection between two adjacent protons (COSY), between two protons through space (NOESY, nuclear Overhauser enhancement spectroscopy; ROESY, rotating-frame enhancement spectroscopy) or between all protons in one spin system (TOCSY, total correlation spectroscopy) are given. H-C 2D experiments measure the direct correlation between a proton and a carbon (HMQC, heteronuclear multiple quantum correlation) or the connection of protons over two, three and even four bonds to carbon atoms, so-called long range coupling (HMBC, heteronuclear multiple bond correlation). Correlations are shown as cross peaks in the plane between two axes containing the 1D NMR shifts.

Most NMR spectra were measured at Institut für Anorganische Chemie und Strukturaufklärung, Heinrich-Heine Universität, Düsseldorf with a Bruker ARX-500, by Dr. Peters and co-workers. Further measurements were at Helmholtz Zenter für Infektionsforschung, Braunschweig with Bruker AM-300, ARX-400 and DMX-600 by Dr. Wray. Citrinin was measured at Beijing Normal University, Beijing, China with a Bruker 500 Ultra Shield by Prof. Deng.

2.6.3 Optical activity

Molecules that possess at least one chiral center interact with linearly polarized light. Enantiomeres can be differentiated as the D- or (+)-isomer of a molecule rotates the orientation of linearly polarized light clockwise whereas the L- or (-) isomer counterclockwise. Thus it allows the determination of the absolute stereochemistry.

The specific optical rotation at the wavelength of the sodium D-line, 589 nm at 20°C can be calculated as

$$[\alpha]_D{}^{20} = \frac{100 * \alpha}{l * c}$$

where α = the measured angle of rotation in degrees °

l = the length in dm of the polarimeter tube (typically = 1)

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

Pure substances were dissolved in appropriate spectroscopic grade solvent. The optical rotation of known compounds was compared to literature data.

2.6.4 Determination of the absolute configuration of amino acids by Marfey's analysis

Marfey's method was used to determine the absolute configurations of the dipeptide cyclothreonylisoleucin and the bionectramides A – D.

In general, Marfey's reagent (FDAA = 1-fluor-dinitrophenyl-5-L-alanine amide, TCI) is used as reagent for derivatization of D- and L-amino acids that are obtained after hydrolysis of cyclic or linear peptides in order to determine their absolute configuration. The obtained diastereoisomers can be easily differentiated and identified by their retention times following HPLC analysis on RP columns and comparison with commercially available D- and L-amino acids (ICN) that have been treated in the same way (Marfey 1984, Ashour 2006).
50 µl of 50 mM of each commercially available standard amino acid (D- or L-form) that is of interest in H_2O was mixed with 100 µl of 1% Marfey's reagent in acetone and heated at 40°C for one hour. The reaction was stopped by addition of 10 µl of 2M HCl and the derivatized product dried in a freeze dryer, re-dissolved in MeOH and analysed by HPLC and by LC-MS.
The isolated peptide was hydrolysed (0.5 – 1 mg) with 1 – 2 ml 6N-HCl at 110°C for 24 h under N_2 atmosphere. The hydrolysate containing a mixture of free amino acids was cooled, dried and re-dissolved in water. Derivatization was achieved in the same manner as applied to standard amino acids.

The retention times of the derivatized standard amino acids and of the derivatized amino acids obtained following hydrolysis of the peptide were compared to distinguish D- and L-amino acids.

2.6.5 Determination of absolute stereochemistry by Mosher reaction

The reaction was planned according to a modified Mosher ester procedure described before (Su et al. 2002) to assign the absolute stereochemistry of compound **7**.
In preparation for this method, other hydroxyl groups than the wanted had to be protected as acetonides or esters according to the following procedures:

Formation of acetonides
10 mg of the compound were treated with 0.75 mL acetone containing 0.4% (V/V) H_2SO_4 at ice temperature for 90 min and another 90 min at room temperature. The reaction product was extracted into EtOAc from aqueous solution (Stoessel and Stothers 1983).

Formation of aromatic ester by Schotten-Baumann reaction
10 mg of the compound, well-dried under nitrogen atmosphere and dissolved in 1M NaOH, were stirred with 25 mg benzoyl chloride at ice temperature for 5 hrs and the reaction products extracted with EtOAc.

As both methods failed to be successful, the actual Mosher reaction to assign the absolute conformation of compound **7** was not performed.

2.7 Biological assays

2.7.1 Antimicrobial and antifungal activity

Extracts and pure substances were tested in a 96 well plates primary screening assay against the following resistant pathogens:
Escherichia coli (VR), Enterococcus faecium (BR) , Staphylococcus aureus (MRSA), Streptococcus pneumonia (BR), Pseudomonas aeruginosa (VR), Klebsiella pneumonia (VR), Candida albicans (KR), Candida krusei (KR), Aspergillus fumigatus (VR), Aspergillus faecius (VR).
Pure compounds were diluted from 250 to 62.5 µg/mL and extracts from 1250 to 312 µg/mL in Müller Hinton Bouillon (Merck, Germany) for bacterial screening and in RPMI (PAA, Austria), enriched with 2% glucose (PAA, Austria), for fungal screening. Afterwards the substance/extract solution was overlaid with the microbes (10^5 CFU/mL) and cultivated for bacteria 24 h for fungi 48h at 35°C. As negative control an antibiotic/antimycotic mix (PAA, Austria) was used in addition to a non treated infected control (positive). The test was analysed by checking the microbial growth with the visible eye and by measurement of the turbidity at 650 nm. All procedures were done under aseptic conditions in a sterile laminar air flow according to good laboratory practice.

Substances with an activity around 125 µg/ml and extracts with 625µg/ml were considered as possible candidates for further antimicrobial screening. With these positive candidates a MIC (minimal inhibition concentration) assay was performed to identify the exactly minimal inhibition concentration. Therefore the substances/extracts were diluted from 250µg/mL to 0.24µg/mL and screened in the same manner as in the primary screening.

2.7.2 Antiviral activity

Antiviral properties of several pure compounds at concentrations from 250 to 3.9µg/mL were tested against two different groups of respiratory virus (HRV = human rhino virus; RSV = respiratory syncytial virus). In these two groups the followed serotypes were tested: HRV 2, HRV 8, HRV 16, HRV39, RSV B and RSV A2.
As host cell line HeLa cells were cultivated in DMEM (PAA, Austria), with 2% fetal calve serum (PAA, Austria), 1% antibiotic/antimycotic solution (PAA, Austria) and 1mMol $MgCl_2$ in a 37°C incubator (5% CO_2, 95% humidity). The cells were sowed in a 96 well plate so that they had a confluence of 70-80% at the day of the experiment. The cells were infected with 100 µL of a viral/substance solution. This viral/substance solution was prepared before by mixing virus (MOI = 10; [Multiplicity of Infection = ratio of infectious virus particles to cells]) with different substances at concentrations between 250 and 3.9µg/mL. Before infecting the cells the mixture was incubated for 20 minutes to allow virus/substance interaction. The infected cells were incubated for 72h at 37°C (5% CO_2, 95% humidity). Additionally non-infected cells (negative control) and infected cells (positive control) were included.
Data were analyzed by checking the grade of infection in the wells with optical evaluation and staining of the living cells with crystal violet at the end of the test. The stained cells were analyzed by followed measurement of the optical density at 490 nm.

2.7.3 Toxicity

Compound with a promising bioactivity were examined for their toxicity profile. HeLa cells were sowed the day before the start of the experiment in a 96 well plate to give a confluence of approximately 60-70%. The cells were overlaid with the substances diluted in medium (250, 125 and 62 µg/mL) and incubated for 48 hours. Viability of cells was defined by staining with crystal violet and measuring at 450 nm. Data points were taken five times to calculate an average. The toxicity profile was deemed positive if the concentration of the living cells reached 80% of the negative control.

Antimicrobial, antifungal, antiviral and toxicity tests were performed by Dr. Alexander Pretsch at SeaLife Pharma GmbH in Tulln, Austria.

2.7.4 Biofilm inhibition and anti-infective assay

Both tests were performed in the laboratory of Prof. U. Hentschel, Zentrum für Infektionsforschung, Würzburg.

Biofilm formation was determined by an adhesion assay in polystyrene microtiter plates. For this purpose, cultures of *Staphylococcus epidermidis* were diluted appropriately in TBS medium (17 g peptone from casein, 3 g peptone from soy meal, 2.5 g glucose 5 g NaCl and 2.5 g K_2HPO_4 in 1 L demineralised water with an adjusted pH of 7.3), pipetted into the wells and incubated at 37°C for 18 hours. Samples were added to growing or already formed biofilm in concentration of 50µg/mL (n=8). After incubation, the wells were carefully emptied and rinsed prior to heat –fixing of the remaining biofilm on a hotplate at about 60°C and subsequent staining with crystal violet. After washing off the excess dye and drying, the optical density of the adhering biofilm was determined at 490 nm. *S. epidermidis* RP62A (wild type) served as positive, *S. carnosus* TM300 as negative control.

2.7.5 Cytotoxicity test: Microculture tetrazolium (MTT) assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is a yellow coloured compound that is converted by mitochondrial reductase into a blue formazan derivative.

MTT assay was performed following the method described 1987 by Carmichael (Carmichael et al. 1987), and % cell viability was determined by spectrophotometric determination of accumulated formazan derivative in treated cells at 560 nm in comparison to control cells (Ashour et al. 2006).

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 units/mL streptomycin. The cells were maintained in a humidified atmosphere at 37°C with 5% CO_2.

Of the test samples to be analyzed in the bioassay, stock solutions in EtOH 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 in 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 for initial experiments should be 3 and 10 µg/ml of pure compounds, fractions or crude extracts.

The test plates were incubated at 37°C with 5% CO_2 for 72 h. A solution of MTT was prepared at 5 µg/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 were pipetted into each well.

The yellow MTT penetrated the cells and in the presence of mitochondrial dehydrogenases MTT was 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 at 210 x g) with 200 µl DMSO and the cells were lysed to liberate the formazan product.

After thorough mixing, the absorbance was measured at 520 nm. The color intensity could be correlated with the number of healthy living cells and cell survival was calculated using the formula:

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

All experiments were carried out in triplicate and repeated three times. As negative controls, media with 0.1% (v/v) EtOH were included in all experiment. As positive controls, compounds with known cytotoxicity such as kahalalide derivatives were used[33].

MTT assays were carried out by co-workers of Prof. W. E. G. Müller at the Institut für Physiologische Chemie, Johannes-Gutenberg Universität, Mainz.

2.7.6 Protein kinase assay

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 extra cellular environment. Consequently, they are major targets for the development of novel drugs to treat diseases such as cancer or inflammatory disorders.

Protein kinase assays were performed by Dr. Michael Kubbutat and co-workers at ProQinase GmbH, Freiburg.

Protein kinases (see table 2.7.6) were expressed in Sf9 insect cells as human recombinant GST-fusion proteins or His-tagged proteins by the baculovirus expression system. Purification of the kinases was achieved by affinity chromatography on agarose and checked by SDS-PAGE/silver staining. The identity of each kinase was verified by western blot or mass spectrometry.

A proprietary protein kinase assay ([33]Panqinase® Activity Assay) was carried out by a robotic system (BeckmanCoulter/Sagain) in 96-well FlashPlates® from Perkin Elmer/NEN at an end concentration of the tested compounds of 1 µL/mL in 1% DMSO at a volume of 50 µL (n=1). The reaction cocktail was made up of 20µL assay buffer, 5µL aqueous ATP solution, 5 µL test compound and 10 µL substrate/ 10µL enzyme solution. The premixed enzyme solution contained 60 mM HEPES-NaOH (pH 7.5), 3mM $MgCl_2$, 3mM $MnCl_2$, 3µM Na-orthovandanate, 1.2mM DTT, 50 µg/mL PEG_{20000}, 1 µM [γ-^{33}P]ATP (approx. $5*10^5$ cpm per well).

Reaction mixtures were incubated at 30°C for 80 minutes and the reaction was stopped by addition of 50 µL of H2PO4 2% (V/V). Thereafter the plates were aspirated and washed twice with 200µL of NaCl 0.9% (m/V). The 33Pi incorporation was determined with a microplate scintillation counter (Microbeta Trilux, Wallac). Protein kinase inhibition could be calculated as

$$\text{residual activity \%} = 100 * \frac{\text{cpm of compound – cpm of low control}}{\text{cpm of high control – cpm of low control}}$$

with low control reflecting the unspecific binding of radioactivity to the plate and high control full activity in the absence of any inhibitor.

Table 2.7.6. List of the applied amounts of protein kinases and their substrates used in the assay.

Family	Protein kinase	conc. [ng/50µL]	Substrate	conc. [ng/50µL]	Onclologically relevant mechamism
Serine / threonine kinase	ATK1	100	GSK3(14-27)	1000	Apoptosis
	ARK5	100	Autophosphorylation	-	Apoptosis
	Aurora-A	50	tetra(LRRWSLG)	Proliferation	500
	Aurora-B	50	tetra(LRRWSLG)	Proliferation	250
	B-RAF VE	20	MEK1 KM(Lot013)	Proliferation	250
	COT	400	Autophosphorylation	Proliferation	-
	SAK	200	Autophosphorylation	Proliferation	-
	CDK2/cycA	100	Histone H1	Proliferation	125
	CDK4/CycD1	50	Rb-CTF(Lot010)	Proliferation	500
	CK2-alpha1	200	p53-CTM	Proliferation	200
	PLK-1	200	Casein	Proliferation	1000
Receptor tyrosine kinase	EGF-R	25	Poly (Glu, Tyr)$_{4:1}$	Proliferation	125
	EPHB4	10	Poly (Glu, Tyr)$_{4:1}$	Angiogenesis	125
	EPHB2	200	Poly (Glu, Tyr)$_{4:1}$	Proliferation	125
	FLT3	200	Poly (Ala,Glu,Lys,Tyr)$_{6:2:4:1}$	Proliferation	125
	IGF1-R	20	Poly(Glu, Tyr)$_{4:1}$	Apoptosis	125
	INS-R	25	Poly (Ala,Glu,Lys,Tyr)$_{6:2:4:1}$	"counter kinase"	125
	MET	20	Poly (Ala,Glu,Lys,Tyr)$_{6:2:4:1}$	Metastasis	125
	PDGFR-beta	100	Poly (Ala,Glu,Lys,Tyr)$_{6:2:4:1}$	Proliferation	125
	TIE-2	200	Poly(Glu, Tyr)$_{4:1}$	Angiogenesis	250
	VEGF-R2	50	Poly(Glu, Tyr)$_{4:1}$	Angiogenesis	125
	VEGF-R3	100	Poly(Glu, Tyr)$_{4:1}$	Angiogenesis	125
Soluble tyrosine kinase	FAK	200	Poly(Glu, Tyr)$_{4:1}$	Metastasis	125
	SRC	10	Poly(Glu, Tyr)$_{4:1}$	Metastasis	125

2.8   Laboratory instruments and chemicals

2.8.1   Laboratory instruments

Analytical HPLC

Pump	P580A LPG, Dionex
Autosampler	ASI-100 (standard injection volume 0 20µL), Dionex
Detector	UVD 340S (photodiode array detector), Dionex
Column oven	STH 585, Dionex
Column	Eurosphere 100-C18 (5 µM; 125 x 4 mm), with integrated pre-column
Software	Chromeleon 6.30

Semi preparative HPLC
      Pump   L-7100, Merck/Hitachi
      Detector   UV-L7400 (photodiode array detector), Merck/Hitachi
      Column   Eurosphere 100-C18 (10 µm; 300 x 8 mm), Knauer
      Precolumn   Eurosphere 100-C18 (10 µm; 30 x 8 mm), Knauer
      Printer   Chromato-Integrator D-2000, Merck/Hitachi

LC-MS (ESI-MS)
      HPLC System   Agilent 1100 series (pump, photodiode array UV-detector, autosampler and injector), Agilent
      MS spectrometer Finnigan LCQ^{Deca}, Thermoquest
      Column   Eurosphere 100-C18 (5 µM; 227 x 2 mm), Knauer
      Vacuum pump   Edwards 30, BOC
      Software   Xcalibur, version 1.3

High resolution ESI-MS   Qtof 2, Micromass
Polarimeter   241 MC, Perkin-Elmer

Fungal identification
      Laminar air flow   Herasafe HS15, Heraeus
      Autoclave   Varioklav, H&P
      pH meter   inoLab, pH electrode Sen Tix 21, WTW
      Microcentrifuge   Biofuge pico, Heraeus
      PCR machine   iCycler, Bio-Rad
      UV transluminator   Syngene GVM 20
      Mixer mill   MixerMill MM30
      Power Supply for electrophoresisPowerPac 300, Bio-Rad

General instruments
      Freeze dryer   Lyovac GT2, Steris; pump Trivac D10E, Savant
      Speedvac   SPD 111V with cooling trap RVT 400, Savant
      Ultra Turrax®   T18 basic, IKA
      Vacuum pump   4EKF56CX-4, Greiffenberger Antriebstechnik
      Balance   BL1500, Sartorius
      Analytical balance   MC-1, Sartorius
      Desiccator   Glaswerk Wertheim
      Hot plate and magnetic stirrer   IKA-Combimag RCH, Janke & Kunkel KG
      Drying oven   ET6130, Heraeus
      Ultra sonnicator   RK 510H, Bandelin
      Rotary evaporator   RV 05-ST, Vaccubrand
      Membrane vacuum pump   Vaccubrand
      UV lamp   Camag (wave length 254 and 366 nm)
      Nitrogen generator   UHPN 3001, Nitrox

2.8.2 Laboratory chemicals

General solvents
      Acetone p.a.   Merck
      n-Butanol p. a.   Merck

Methanol, ethyl acetate, dichloromethane and *n*-hexane were purchased as technical solvents and distilled before using.

HPLC solvents
Methanol   LiChroSolv HPLC grade, Merck
Acetonitrile   LiChroSolv HPLC grade, Merck
Distilled and heavy metal free water was obtained by passing through nano- and ion-exchange filter cells (Barnstead) to yield nanopure water.
Solvents for optical rotation
Chloroform   Spectroscopic grade, Sigma
Methanol   Spectroscopic grade, Sigma

Solvents for NMR
Acetone-d_6 Uvasol, Merck
Chloroform-d Uvasol, Merck
DMSO-d_6 Uvasol, Merck
Methanol-d_4 Uvasol, Merck

General laboratory chemicals
Acetic acid   Merck
Anisaldehyde   Merck
Formic acid   Sigma
Hydrochloric acid   Merck
Ninhydrin   Sigma
Ortho-phosphoric acid Merck
Potassium hydroxide Merck
Sodium hydroxide   Sigma
Sulphuric acid   Merck

3. Results

The focus of this work, which includes the quest for new and potentially bioactive secondary metabolites, their structure elucidation and examination of their bioactivities, is presented in this section. In the first instance pure fungal strains that were isolated from mangrove plants were grown on small scale basis and screened for their antibiotic and cytotoxic properties as well as for protein kinase inhibition. Based on these results and in conjunction with chemical screening by HPLC and LC-MS, certain fungal strains were chosen for further examination. MGC 12.4 (*Cladosporium sphaerospermum*) and JCM 9.2 (*Alternaria* sp.) showed interesting chemical profiles, and extracts of MGC 4.2 (*Nigrospora* sp.), JCM 22.1 (*Fusarium incarnatum*), and JCM 10.3 (*Bionectria ochroleuca*) exhibited promising activities in first bioactivity screenings.

3.1 Bioactivity screening of fungal extracts

Table 3.1a. Results of the bioactivity screening of selected extracts.

Extract	L5178Y survival rate in % (@ 10 µg/mL)	Survival rate in % (@ 312 µg/mL)					
		E. c.	K. p.	E. f.	S. a.	S. p.	P. a.
MGC 4.2	-2.2	90	50	85	10	10	90
MGC12.4	80.2	85	80	75	50	80	80
JCM 9.2	14.2	90	100	80	85	85	85
JCM 9.4	64.5	100	90	70	40	25	75
JCM 9A2	13.1	100	80	100	70	65	95
JCM 9A3	-8.9	100	80	100	80	75	90
JCM 10.2	-6.4	95	100	100	60	100	90
JCM 10.3	-0.4	100	100	70	95	95	100
JCM 10.6	-5.3	95	100	20	5	10	100
JCM 10.7	-5.7	100	85	100	60	5	90
JCM 10.8	31.2	100	100	100	95	100	95
JCM 10A1	41.3	100	100	100	90	20	95
JCM 10A2	44.8	95	100	100	80	20	90
JCM 12.3	88.8						
JCM 22.1	7.9	75	100	0	5	10	55
JCM 23.3	65.4	100	100	100	100	100	90

E. c. *Escherichia coli*, K. p. *Klebsiella pneumoniae*, E. f. *Enterococcus faecium*, S. a. *Staphylococcus aureus*, S. p. *Streptococcus pyogenes*, P. a. *Pseudomonas aerigunosa*

Table 3.1b. Results of screening of fungal extracts against 24 different protein kinases. Inhibitory potentials of extracts were determined in biochemical protein kinase activity assays. Listed are the residual activities at a concentration of 1µg/mL in %.

Extract	AKT1	ARK5	Aurora-A	Aurora-B	B-RAF-VE	CDK2/CycA	CDK4/CycD1	COT	EGF-R	EPHB4	ERBB2	FAK	IGF1-R
MGC4.2	88	93	93	90	67	110	101	82	82	89	90	84	82
JCM9A2	99	99	100	90	73	130	90	97	85	102	85	95	89
JCM10.3	99	99	105	100	72	156	102	90	90	96	92	104	91
JCM10.6	103	94	106	98	98	104	109	101	109	87	108	99	102
JCM22.1	102	103	79	83	117	244	117	96	72	100	81	114	70
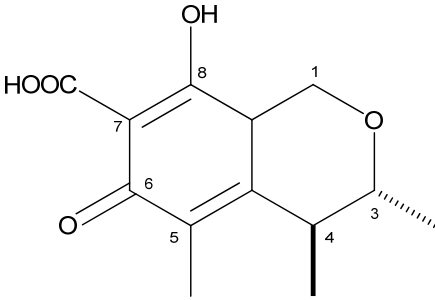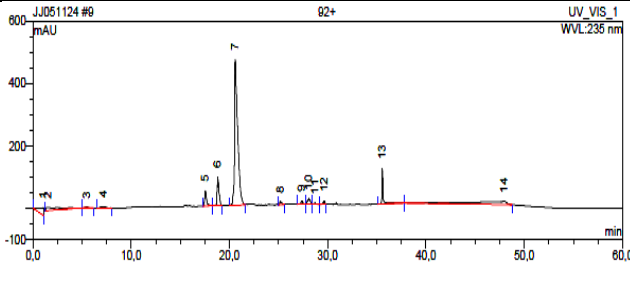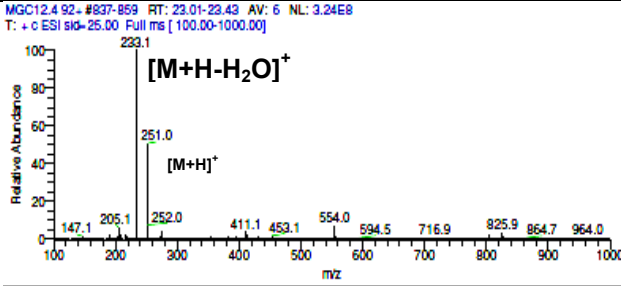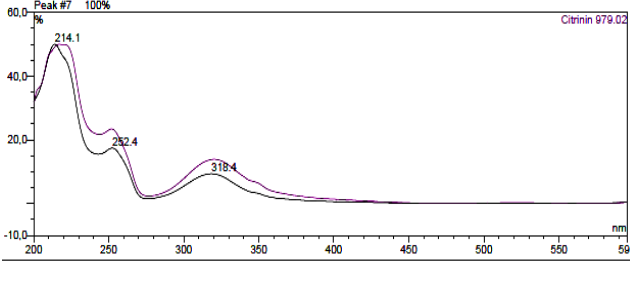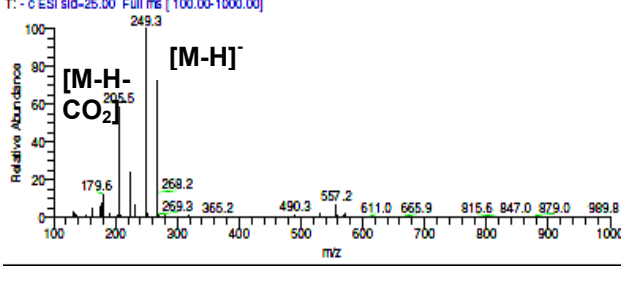
Extract	SRC	VEGF-R2	VEGF-R3	FLT3	INS-R	MET	PDGF-Rβ	PLK1	CK2-α1	SAK	TIE2
MGC4.2	59	86	84	110	114	112	93	85	97	84	94
JCM9A2	84	90	93	134	112	115	101	95	97	100	95
JCM10.3	86	94	104	146	102	106	101	100	103	97	98
JCM10.6	94	99	105	105	108	106	101	102	100	98	97
JCM22.1	101	93	97	115	120	95	111	109	111	105	95

3.2 Compounds isolated from the endophytic fungus *Cladosporium sphaerospermum*

The ethyl acetate extract obtained after fermentation of *C. sphaerospermum* was isolated from the mangrove plant *Aegiceras corniculatum*. Fermentation of the fungus in liquid medium yielded three known fungal metabolites. Beside the major compound citrinin (**1**) an inseparable mixture of the diastereomers quinolactacin A1 (**2**) and quinolactacin A2 (**3**) was obtained.

3.2.1 Citrinin (1, known compound)

Citrinin	
Synonym(s)	(3*R*,4*S*)-8-hydroxy-3,4,5-trimethyl-6-oxo-4,6-dihydro-3*H*-isochromene-7-carboxylic acid
Sample code	MGC 12.4 92+
Biological source	*Cladosporium sphaerospermum* (from *Aegiceras corniculatum*)
Sample amount	65 mg
Physical description	yellow, amorphic powder
Molecular formula	$C_{13}H_{14}O_5$
Molecular weight	250 g/mol
Optical rotation $[\alpha]_D^{20}$	-36° (*c* 0.05, CHCl$_3$)
Retention time HPLC	20.6 min (standard gradient)

The yellow citrinin was the main metabolite produced by *C. sphaerospermum*. It showed UV maxima at 214, 252 and 318 nm. Its molecular weight was determined as 250 amu based on the pseudomolecular ions observed at *m/z* 251.0 [M+H]$^+$ and 249.3 [M-H]$^-$ upon positive and negative ionization by ESI-MS, respectively. Furthermore, analysis of the fragmentation patterns revealed the presence of both a hydroxyl group, evident from the peak at *m/z* 233.1 [M+H-H$_2$O]$^+$, and a carboxylic acid function, demonstrated by the loss of CO_2 in the negative ionization mode (*m/z* 205.5). These findings were confirmed by the ^{1}H NMR spectrum (see table 3.2.1), displaying two sharp signals for hydroxyl groups at δ 15.11 (OH-8) and δ 15.88 (COOH-7). Their considerable downfield shifts resulted from the strong hydrogen bonds to the neighboring carbonyl functions. For 7-COOH, this hydrogen bond was

also evident from the HMBC correlations to C-5 (δ 123.1) and to C-7 (δ 100.3). The ^{1}H NMR spectrum also revealed one aromatic proton at δ 8.23 (H-1), two aliphatic protons at δ 4.78 (H-3) and δ 2.98 (H-4) and three methyl groups. Both ^{1}H and ^{13}C NMR data as well as the optical rotation were in excellent agreement with reported data (Barber et al. 1987). The compound was first isolated from *Penicillium citrinum* (Wyllie 1946) and later also reported from other sources like other *Penicillium* spp. or *Aspergillus* spp. (Bennett and Klich 2003).
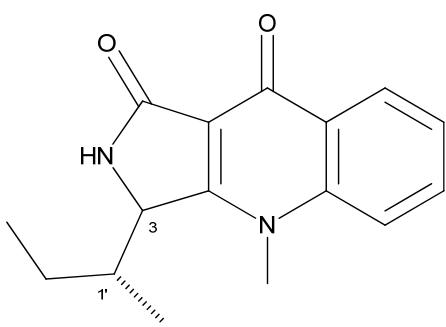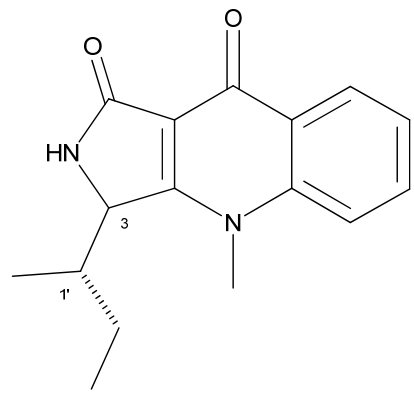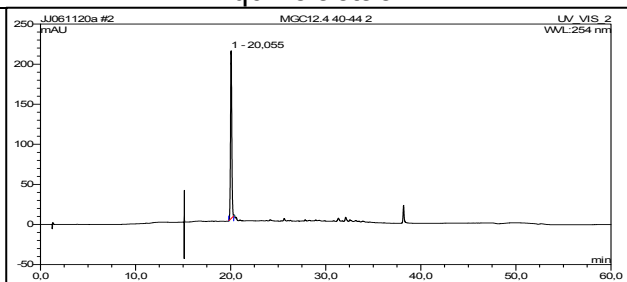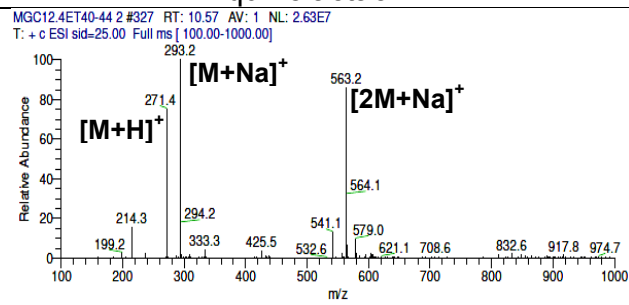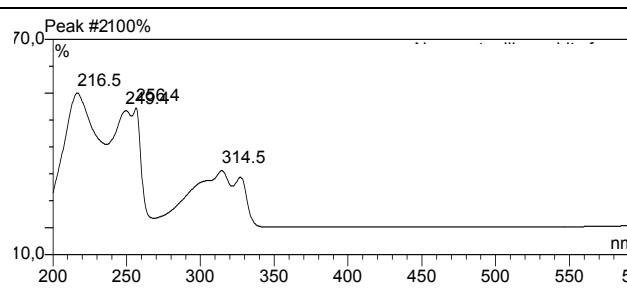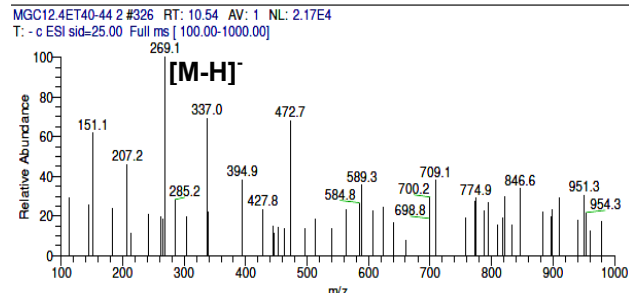
Table 3.2.1. NMR data of citrinin (**1**) at 500 (^{1}H) and 125 MHz (^{13}C), measured in CDCl$_3$.

	1			**Literature**[a]	
	δ_H (*J* Hz)	δ_C	**HMBC**	δ_H (*J* Hz)	δ_C
1	8.23 s	162.7	3, 4a, 8, 8a, COOH-7	8.24	162.0
3	4.78 q (6.9)	81.6	1, 4, 4a, CH$_3$-3	4.78 q (6.8)	80.9
4	2.98 q (7.3)	34.6	4a, 5, 8a, CH$_3$-4	2.99 q (7.3)	33.7
4a		139.0			139.2
5		123.1			122.0
6		183.8			182.7
7		100.3			99.4
8		177.2			176.2
8a		107.4			106.4
CH$_3$-3	1.34 d (6.4)	18.2	3, 4	1.35 d (6.8)	17.8
CH$_3$-4	1.22 d (7.3)	18.5	3, 4, 4a	1.23 d (7.3)	17.6
CH$_3$-5	2.01 s	9.4	4a, 5, 6, 8a	2.02	8.7
COOH-7	15.88 s	174.5	5, 7, COOH-7		173.5
OH-8	15.11 s		7, 8a		

[a] Barber et al. 1987

3.2.2 Quinolactacin A1 (2, known compound) and quinolactacin A2 (3, known compound)

Quinolactacin A1 and quinolactacin A2	
Synonym(s)	(*S*)-3-sec-butyl-4-methyl-2,3-dihydro-1H-pyrrolo[3,4-b]quinoline-1,9(4*H*)-dione
Sample code	MGC 12.4 40-44.2
Biological source	*Cladosporium sphaerospermum* (from *Aegiceras corniculatum*)
Sample amount	1.1 mg
Physical description	light yellow, amorphic powder
Molecular formula	$C_{16}H_{18}N_2O_2$
Molecular weight	270 g/mol
Retention time HPLC	20.1 min (standard gradient)

quinolactacin A1   quinolactacin A2

Quinolactacin A1 and A2 were obtained as an inseparable mixture (ratio approx. 1:1) upon purification by column chromatography with Sephadex LH-20. Attempts to separate the mixture via semi-preparative HPLC proved unsuccessful. Only one peak was visible in analytical HPLC, displaying UV maxima at 217, 240, 256 and 315 nm and high similarity with the one of quinolactacin of the institute's own database. ESI-MS gave pseudomolecular ions at *m/z* 271 [M+H]$^+$ and 269 [M-H]$^-$ upon positive and negative ionization, respectively, indicating a molecular weight of 270 amu.

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However, the ^{1}H NMR spectrum (see table 3.2.2) revealed the presence of two substances in an approximate ratio of 1:1, as evident from the integration of the peaks. While most signals, especially in the aromatic region more or less completely overlapped, signals for the isobutyl moiety in both compounds could be differentiated. Signals for H_2-2' and H_3-3' of quinolactacin A2 were shifted upfield in comparison to quinolactacin A1, while 1'-CH_3 was shifted downfield. The obtained NMR data for both compounds in this study were in accordance with literature data (Takashi et al. 2000, Kim et al. 2001). Both compounds were obtained from *Penicillium* species, and in 2001, successfully separated, for the first time, by chiral HPLC (Kim et al. 2001).

Table 3.2.2. NMR data of compounds **2** and **3** at 500 MHz.

	2		**Literature**	**3**	**Literature**
	$\delta_H{}^a$ (*J* Hz)	$\delta_H{}^b$ (*J* Hz)	$\delta_H{}^c$ (*J* Hz)	$\delta_H{}^a$ (*J* Hz)	$\delta_H{}^d$ (*J* Hz)
3	4.81 d (1.8)	4.82 bs	4.80 d (2.5)	4.89 d (1.5)	4.84 s
5	7.81 bd (6.1)[e]	7.82 bd	7.47 d (8.2)	7.81 bd (6.1)[e]	7.83 d (8.4)
6	7.81 bd (6.1)[e]	7.82 m	7.66 ddd (8.2; 7.5; 1.4)	7.81 bd (6.1)[e]	7.81 dd (8.4; 6.8)
7	7.48 m	7.48 m	7.29 dd (7.6; 1.4)	7.48 m	7.48 dd (7.2; 6.8)
8	8.25 d (7.3)	8.25 bd (7.6)	8.23 dd (7.6; 1.4)	8.25 d (7.3)	8.26 d (7.2)
1'	1.92 m	2.16 m	2.13 m	2.16 m	2.19 m
2'	1.59 m	1.58 m	1.62 m	0.85 m[e]	0.88 m
	1.39 m	1.39 m	1.48 m	0.82 m[e]	0.83 m
3'	1.00 t (7.4)	0.98 t (7.6)	1.09 t (7.4)	0.65 t (7.4)	0.65 t (7.4)
CH_3-1'	0.43 d (6.6)	0.43 d (6.6)	0.51 d (6.7)	1.13 d (6.7)	1.14 d (6.8)
CH_3-4	3.81 s	3.81 s	3.80 s	3.83 s	3.86 s
NH-2	8.06 s	8.07 s	7.37 bs	8.13 s	8.17 s

[a] in DMSO-d_6 [b] Indriani, 2007 (in DMSO-d_6)   [c] Kim *et al*, 2001 (in CDCl$_3$)
[d] Takahashi et al. 2000 (in DMSO-d_6)   [e] overlapping signals

3.2.3 Bioactivity test results for compounds isolated from *Cladosporium sphaerospermum*

The cytotoxic and antibiotic potentials of the crude EtOAc extracts of *Cladosporium spaerospermum* as well as the isolated citrinin were determined. Neither showed any cytotoxicity. Whereas the antibiotic activity of the crude extract was only marginal, citrinin exhibited promising growth inhibition against *S. pyrogenes*. The obtained results of the biochemical protein kinase assay showed hardly any inhibition potential.

Table 3.2.3 a. Biological screening test results for EtOAc extracts of *Cladosporium spaerospermum* and citrinin (**1**).

Extract or compound	L5178Y survival rate in % (@ 10 µg/mL)	Survival rate in % (@ 312 µg/mL / <u>MIC</u> [µg/mL])					
		E. c.	K. p.	E. f.	S. a.	S. p.	P. a.
MGC 12.4 EtOAc	80.2	90	80	75	50	85	80
Citrinin (**1**)	77.1	95	75	100	40	5 /<u>31.2</u>	65
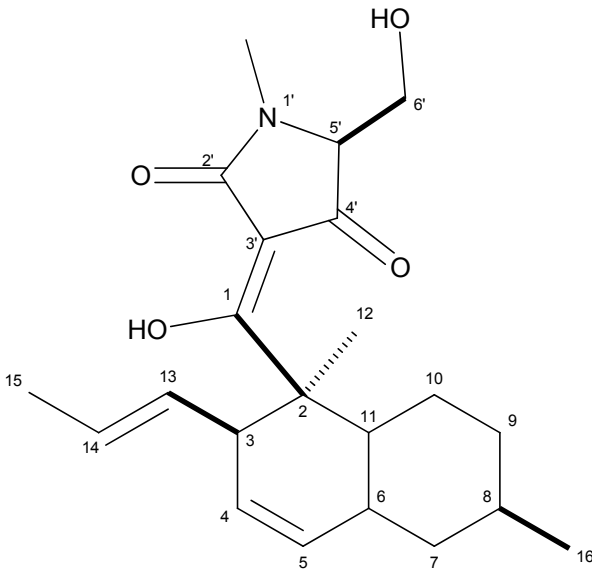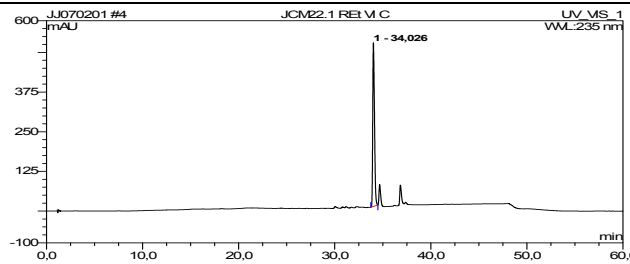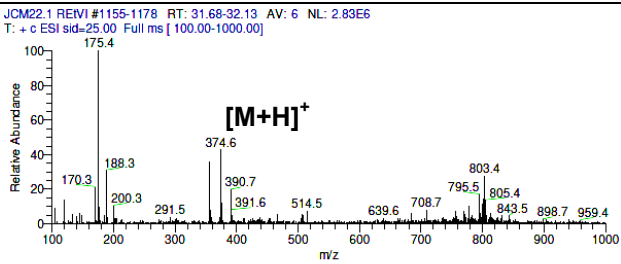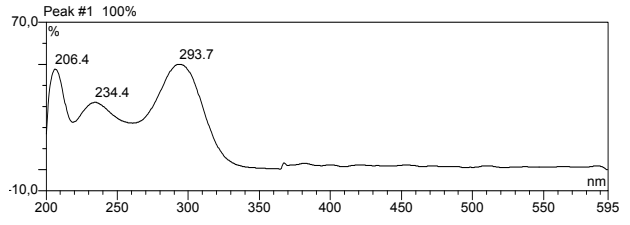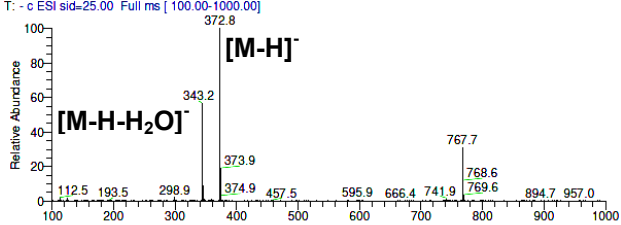
E. c. *Escherichia coli*, K. p. *Klebsiella pneumoniae*, E. f. *Enterococcus faecium*, S. a. *Staphylococcus aureus*, S. p. *Streptococcus pyrogenes*, P. a. *Pseudomonas aerigunosa*

Table 3.2.3b. Results of screening of citrinin (**1**) against 24 different protein kinases. Inhibitory potentials of extracts were determined in biochemical protein kinase activity assays. Listed are the residual activities at a concentration of 1µg/mL in % and IC_{50} values in g/mL.

	AKT1	ARK5	Aurora-A	Aurora-B	B-RAF-VE	CDK2/CycA	CDK4/CycD1	COT	EGF-R	EPHB4	ERBB2	FAK
%	100	100	89	77	90	95	106	100	95	93	76	78
IC_{50}	>1E-05	>1E-05	>1E-05	5,7E-06	>1E-05	>1E-05	>1E-05	>1E-05	>1E-05	>1E-05	>1E-05	2,6E-06

	IGF1-R	SRC	VEGF-R2	VEGF-R3	FLT3	INS-R	MET	PDGF-Rβ	PLK1	CK2-α1	SAK	TIE2
%	94	80	72	117	105	110	109	98	79	51	73	67
IC_{50}	>1E-05	7,3E-06	>1E-05	2,3E-06	>1E-05	>1E-05	>1E-05	>1E-05	>1E-05	8,0E-06	2,5E-06	3,6E-06

3.3 Compound isolated from the endophytic fungus *Fusarium incarnatum*

3.3.1 Equisetin (4, known compound)

Equisetin	
Synonym(s)	(S,Z)-3-(((1S,2R,4aS,6R,8aR)-1,6-dimethyl-2-((E)-prop-1-enyl)-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl)(hydroxy)-methylene)-5-(hydroxymethyl)-1-methylpyrrolidine-2,4-dione
Sample code	JCM 22.1 VI C
Biological source	*Fusarium incarnatum* (from *Pluchea indica*)
Sample amount	25 mg
Physical description	brownish oily substance
Molecular formula	$C_{22}H_{31}NO_4$
Molecular weight	373 g/mol
Optical rotation $[\alpha]_D^{20}$	-259° *(c* 0.1, $CHCl_3$)
Retention time HPLC	34.0 min (standard gradient)

Fusarium incarnatum isolated from *Pluchea indica* was chosen for further workup due to its remarkable activity found in a first bioassay screening.

Fermentation in liquid Wickerham medium and on solid rice medium both gave extracts that displayed one prominent peak around 34 min in the HPLC chromatogram. It displayed UV maxima at 206, 234 and 294 nm. Isolation of the compound from the extract obtained from fermentation on rice was accomplished by initial separation by VLC and subsequent column chromatography over Diol with acetone and *n*-hexane as mobile phases. LC-MS showed pseudomolecular ions at *m/z* 374.6 [M+H]$^{+}$ upon positive and 372.8 [M-H]$^{-}$ upon negative ionization, revealing a molecular weight of 373 amu. The presence of an uneven number of nitrogen atoms implied by the molecular weight and interpretation of the NMR data suggested the molecular formula $C_{22}H_{31}NO_4$.

The ^{1}H NMR spectrum (see table 3.3.1) revealed the presence of an *N*-methyl group (δ 3.05), four protons between δ 5.2 - δ 5.4, indicative of double bonds, three protons connected to heteroatom-bearing carbon atoms between δ 3.3 - δ 4.1, and several overlapping signals in the aliphatic region around δ 0.9 - δ 2.0. The COSY spectrum showed the presence of three distinct spin systems (see figure 3.3.1), one of them due to a CH-CH$_2$OH substructure (H-5' and H$_2$-6'). The second spin system consisted of two double bonds with a bridging aliphatic proton and a terminal methyl group (H-5, H-4, H-3, H-13, H-14, and H$_3$-15). The third spin system represented a cyclohexane ring system to which a methyl group was connected (H$_2$-7, H-6, H-11, H$_2$-10, H$_2$-9, H-8, and H$_3$-16). Based on these substructures and the molecular weight, compound **4** was readily identified as the known equisetin, previously described from *Fusarium equiseti* (Burmeister *et al.* 1979, Phillips *et al*., 1989). This was confirmed by comparison of the UV, MS and NMR data. Measurement of the optical rotation, which also concurred with the one that has been described for equisetin before ([α]$_D^{22}$ -278° *c*, 0.77 in CHCl$_3$) allowed the assignment of the absolute stereochemistry. At room temperature the tetramic acid part of the structure is subject to dynamic enolization. Thus the ^{1}H NMR spectrum not only gave abnormally broad lines, but also did not allow the assignment of the enol proton. A low temperature ^{1}H NMR experiment at −20° C revealed a signal at δ 16.05 (see table 3.3.1).
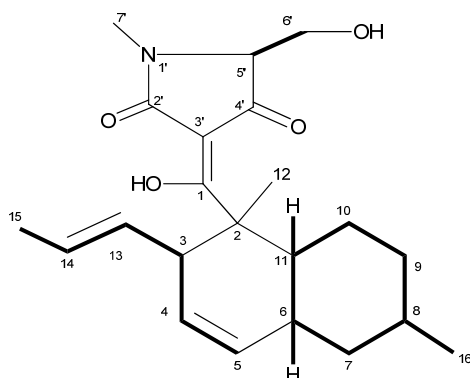
Figure 3.3.1. COSY correlations observed for equisetin (**4**).

Table 3.3.1. NMR data of equisetin (**4**) at 500 MHz measured in $CDCl_3$.

	4			**Literature**[a]
	δ_H (*J* Hz) (20° C)	δ_H (*J* Hz) (−20° C)	**COSY**	δ_H (*J* Hz) (−20°C)
3	3.34 m	3.31 m	4; 13	3.32 bd (9.0)
4	5.40 bs	5.40 s	3; 5	5.40 bs
5	5.40 bs	5.40 s	4	5.40 bs
6	1.78 m	1.78 m	7α, 7β; 11	1.84 bt (12)
7 α	0.91 m	0.87 m	6; 7β; 8	0.90 q (12) ax.
β	1.78 m	1.78 m	6; 7α; 8	1.81 bd (12) eq.
8	1.55 m	1.43 m	7α; 7β; 9α; 9β; 16	1.48 m
9 α	1.08 m	1.06 m	8; 9β; 10β	1.11 q (12) ax
β	1.75 m	1.78 m	8; 9α; 10α	1.76 bd (12) eq
10α	1.97 m	1.92 bd (9)	(9β); 9α; 10β; (11)	1.96 bd (12) eq
β	1.08 m	1.06 m	9α; 9β; 10α; 11	1.04 q (12)
11	1.67 m	1.64 bt (10.5)	6; 10α; 10β	1.66 bd (11)
12	1.55 m	1.41 m		1.46 s
13	5.25 m	5.13 m	3;14; (15)	5.17 dd (15, 9.0)
14	5.25 m	5.2 m	13; 15	5.25 dq (15, 6.5)
15	1.55 m	1.5 m	14	1.56 d (6.5)
16	0.91 d (6.5)	0.88 d (5.5)	8	0.92 d (6.5)
5'	3.63 t (4.4)	3.65 m	6α; 6β	3.67 dd (5.0,3.5)
6' A	4.04 dd (11.0;3.5)	4.03 dd (10.8, 3.6)	6B	4.07 dd (12, 3.5)
B	3.97 m	3.89 dd (10.8, 4.2)	6A	3.90 dd (12, 5.0)
7'	3.05 s	3.04 s		3.07 s
OH-1		16.05 s		17.3 s

[a] Phillips et al. 1989

3.3.2 Bioactivity test results for the isolated compound from *Fusarium incarnatum*

The EtOAc extract of *Fusarium incarnatum* cultured on solid rice medium displayed remarkable cytotoxic activity. This activity was obviously not due to equisetin as the isolated compound did not exhibit cytotoxicity against the tested cell line L5178Y.

The inactivity in the protein kinase assay of equisetin mirrored the lack of inhibitory effects of the crude extract against protein kinases.

On the other hand antibiotic potency against *E. faecium*, *S. aureus* and *S. pyrogenes* was observed which was related to the isolated substance, because the activity profile of equisetin corroborated the one of the crude extract.

Table 3.3.2a. Biological screening test results for EtOAc extracts from rice cultures of *Fusarium incarnatum* and equisetin (**4**).

Extract/ compound	L5178Y survival rate in % (@ 10 μg/mL)	Survival rate in % [@ 312 μg/mL] (MIC [μg/mL])					
		E. c.	K. p.	E. f.	S. a.	S. p.	P. a.
JCM22.1 rice EtOAc	7.9	75	100	0	5	10	55
Equisetin (**4**)	107.1	70	75	0 (7.8)	30 (3.9)	5 (7.8)	75

E. c. *Escherichia coli*, K. p. *Klebsiella pneumoniae*, E. f. *Enterococcus faecium*, S. a. *Staphylococcus aureus*, S. p. *Streptococcus pyrogenes*, P. a. *Pseudomonas aerigunosa*

Table 3.3.2b. Results of screening of equisetin (**4**) against 24 different protein kinases. Inhibitory potentials of extracts were determined in biochemical protein kinase activity assays. Listed are the residual activities at a concentration of 1μg/mL in %.

AKT 1	ARK 5	Aurora-A	Aurora-B	B-RAF-VE	CDK2/ CycA	CDK4/ CycD1	COT	EGF-R	EPHB4	ERBB2	FAK
105	92	95	92	109	115	109	97	90	97	81	113

IGF1-R	SRC	VEGF-R2	VEGF-R3	FLT3	INS-R	MET	PDGF-R-β	PLK1	CK2-α1	SAK	TIE2
93	109	97	101	96	102	100	99	89	98	97	102

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

The endophyte *Nigrospora* sp. was isolated from the mangrove plant *Bruguiera sexangula*. Fermentation in liquid Wickerham medium and on solid rice medium afforded ethyl acetate extracts of different composition. Grown in liquid medium, the fungus yielded two known compounds, aloesol (**5**) and deoxybostrycin (**7**), and the new natural product 8-hydroxytetrahydroaltersolanol B (**6**). Fermentation on rice medium rendered three known secondary metabolites, bostrycin (**8**), tenuazonic acid (**9**) and indole-3-carbaldehyde (**10**) and the new cyclic dipeptide cyclo(threonylisoleucinyl) (**11**).

Comparison of the HPLC chromatograms of the ethyl acetate extracts (see figure 3.4) revealed a difference in the chemical patterns, whereas deoxybostrycin (**7**) and 8-hydroxytetrahydroaltersolanol B (**6**) were the main constituents of the liquid EtOAc extract, bostrycin (**8**), tenuazonic acid (**9**) were found to be the main components of the rice EtOAc extract.

Both crude extracts were submitted to cytotoxicity and antibacterial screening. They both exhibited a strong inhibition against L5187Y cell line. The antibacterial profile on the other hand, revealed slight differences. No inhibition to marginal inhibition was found against *E. coli*, *K. pneumoniae*, *E. faecium* and *P. aeruginosa*. The liquid extract also exhibited only moderate antibiotic activity against *S. aureus* and *S. pyrogenes*, whereas the rice extract showed strong inhibition against these germs.
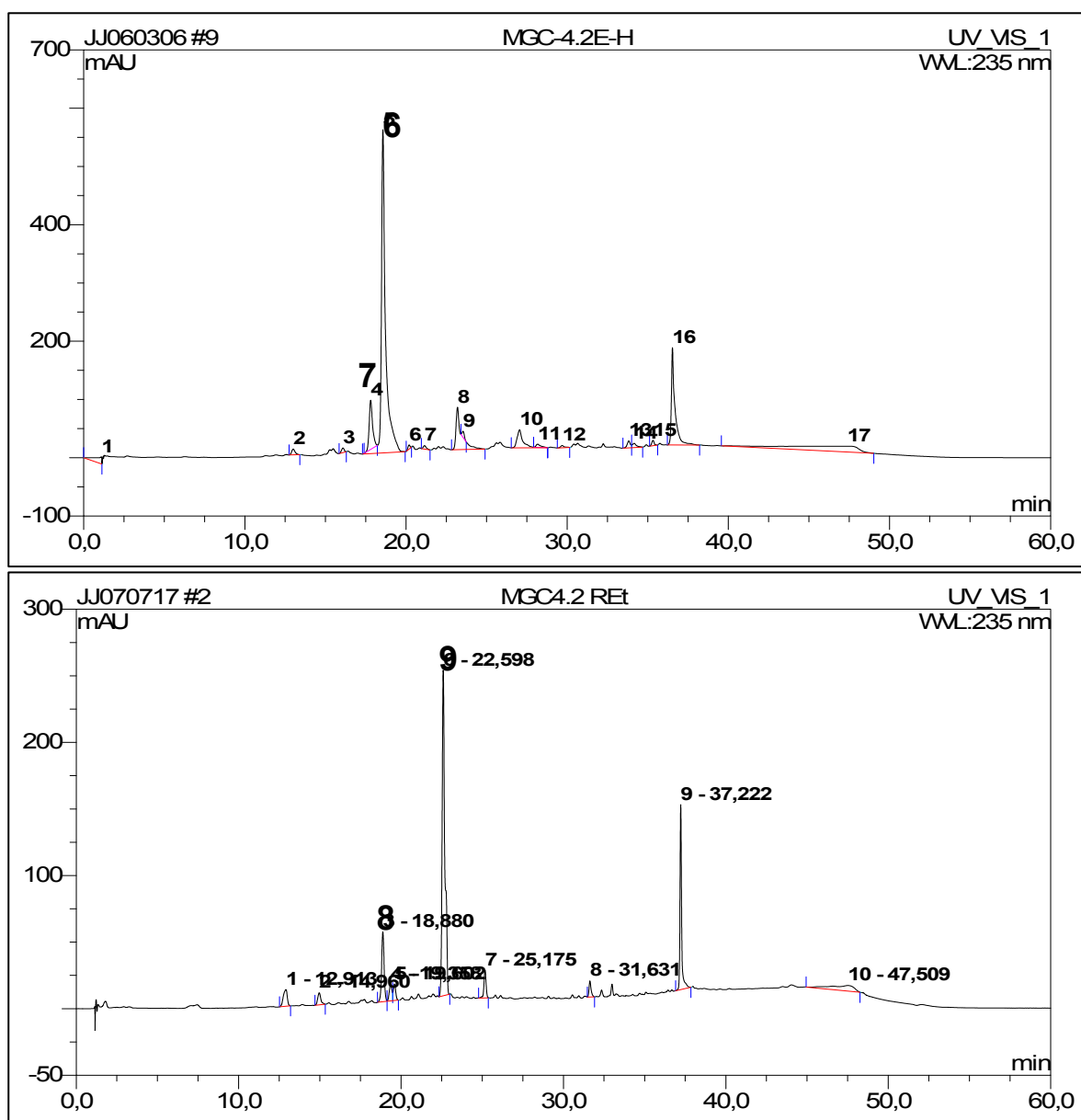
Figure 3.4. EtOAc chromatograms of liquid (above) and rice (below) cultures of *Nigrospora* sp.

Table 3.4. Biological screening test results for EtOAc extracts from rice or liquid cultures of *Nigrospora* sp.

Tested extract	L5178Y survival rate in % (@ 10 μg/mL)	Survival rate in % (@ 312 μg/mL)					
		E. c.	K. p.	E. f.	S. a.	S. p.	P. a.
MGC4.2 liq. EtOAc	-2.2	80	100	100	45	35	100
MGC4.2 rice EtOAc	-1.4	90	50	85	10	10	90

E. c. *Escherichia coli*, K. p. *Klebsiella pneumoniae*, E. f. *Enterococcus faecium*, S. a. *Staphylococcus aureus*, S. p. *Streptococcus pyogenes*, P. a. *Pseudomonas aerigunosa*

3.4.1 Aloesol (5, known compound)

Aloesol	
Synonym	7-hydroxy-2-(2'-hydroxypropyl)-5-methyl-4H-chromen-4-one
Sample code	MGC 4.2 D6
Biological source	*Nigrospora* sp. (from *Bruguiera sexangula*)
Sample amount	1.8 mg
Physical description	whitish powder
Molecular formula	$C_{13}H_{14}O_4$
Molecular weight	234 g/mol
Optical rotation $[\alpha]_D^{20}$	0° (*c* 0.3, MeOH)
Retention time HPLC	21.1 min
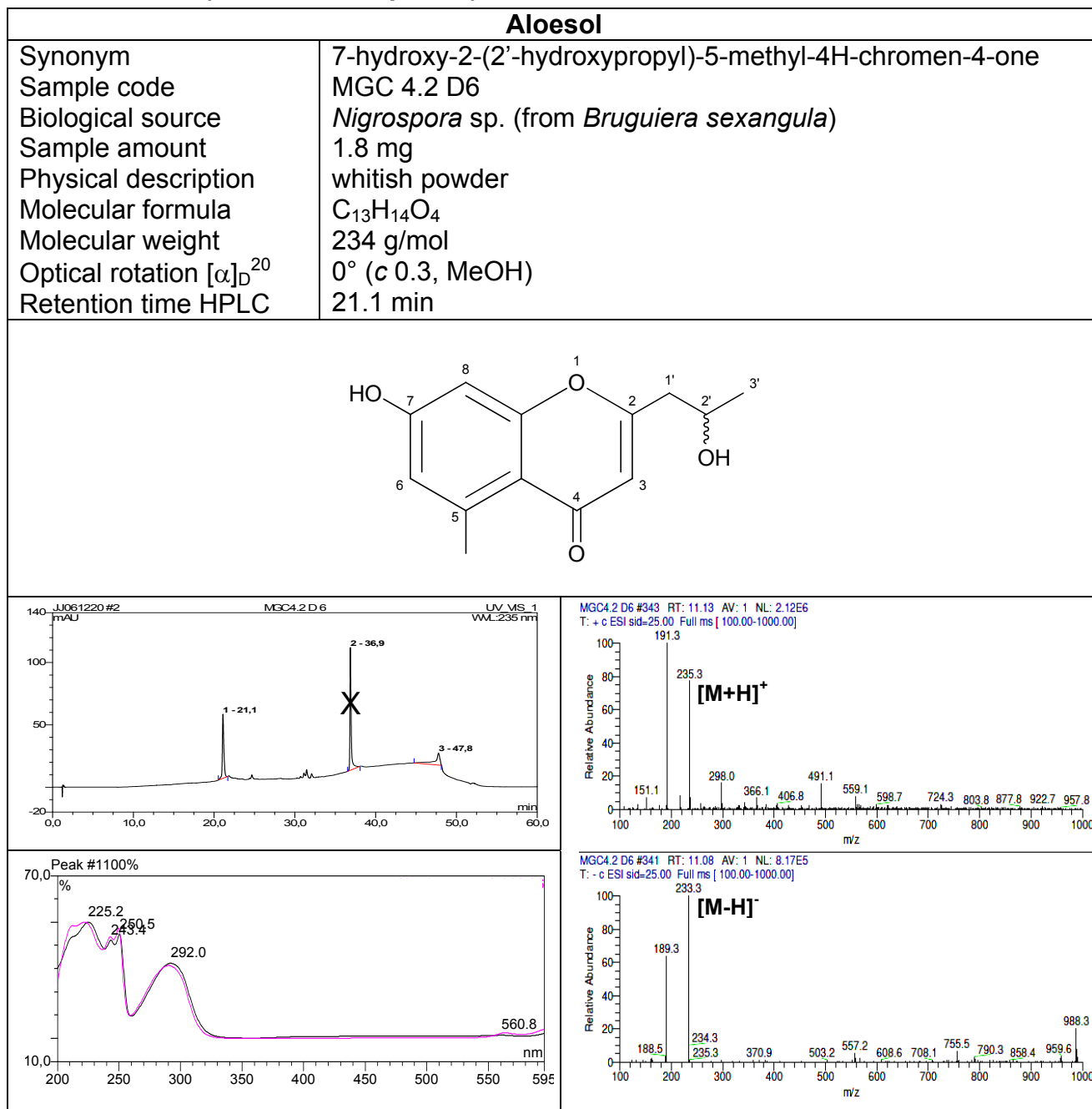
Aloesol was obtained as a racemic mixture from the EtOAc extract of liquid culture of *Nigrospora* sp. after fractionation over Sephadex followed by semi preparative HPLC. It displayed a UV spectrum characteristic of a 7-hydroxy-4-chromone with maxima at 225, 243, 251 and 292 nm. Positive and negative ESI-MS showed pseudomolecular ions at *m/z* 235.3 [M+H]+ and 233.3 [M-H]-, respectively, indicating a molecular weight of 234 amu. The ^{1}H NMR spectrum exhibited signals due to a 2-hydroxyisopropyl moiety consisting of protons H_2-1', H-2' and H_3-3' (δ 2.57, δ 4.02 and δ 1.12), a methyl group (δ 2.65, CH_3-5), a hydroxyl group (δ 8.46, OH-7), an olefinic proton (δ 5.96, H-3) plus two aromatic protons (δ 6.60, 6.62, H-6 and H-8). The ROESY experiment allowed the assignment of the structure due to the correlations of H-6 to the methyl group residing at C-5, and H_2-1' to H-3. All spectroscopic data were in good agreement with published data by for the S-

enantiomer of the compound, originally isolated from *Rhei rhizoma* (Kashiwada et al. 1984). However, in the current study aloesol (5) was probably obtained as a racemic mixture, since its optical rotation amounted to 0°C. This is the first report about isolation of the compound from a fungal source.

Table 3.4.1. NMR data of aloesol (**5**) at 500 MHz.
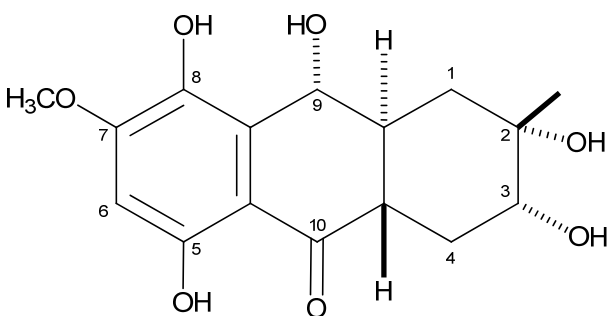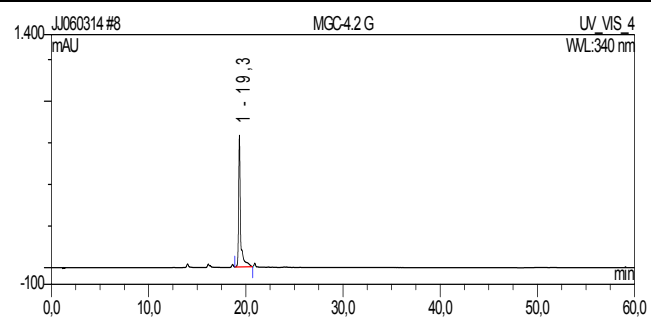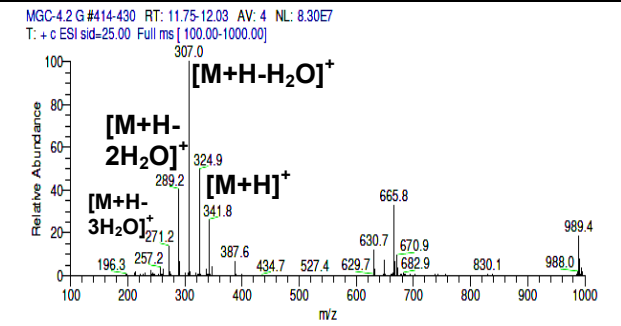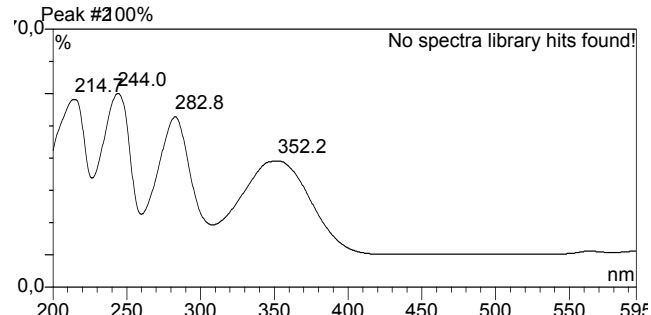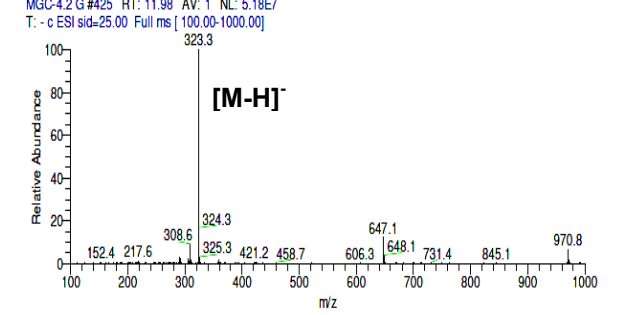
	5			**Literature[c]**
	$\delta_H{}^a$ (*J* Hz)	$\delta_H{}^b$ (*J* Hz)	**ROESY**	$\delta_H{}^b$ (*J* Hz)
3	5.97 s	5.96 s	1'A; 1'B	5.97 s
6	6.55 d (1.6)	6.60 bs	CH$_3$-5	6.61 s
8	6.57 bs	6.62 d (2.3)		6.61 s
1'	2.57 m	A 2.60 dd (14.3; 5.3)	3; 3'	2.58 d (7)
		B 2.55 dd (14.3; 7.7)	3; 3'	
2'	4.09 m	4.02 m		4.02 m
3'	1.18 d (6.0)	1.12 d (6.2)	1'A; 1'B	1.15 d (7)
CH$_3$-5	2.62 s	2.65 s	6	2.66 s
OH-7		8.46 bs		10.51

[a] in MeOD
[b] in DMSO-d_6
[c] Kashiwada 1984

3.4.2 8-Hydroxytetrahydroaltersolanol B (6, new compound)

8-Hydroxytetrahydroaltersolanol B	
Synonym(s)	(2R,3S,4aS,9aS,10R)-2,3,5,8,10-pentahydroxy-6-methoxy-3-methyl-1,3,4,4a,9a,10-hexahydroanthracen-9(2H)-one, 1-deoxytetrahydrobostrycin
Sample code	MGC 4.2 G
Biological source	*Nigrospora* sp. from *Bruguiera sexangula*
Sample amount	8.4 mg
Physical description	red powder
Molecular formula	$C_{16}H_{20}O_7$
Molecular weight	324 g/mol
Optical rotation $[\alpha]_D^{20}$	-67° (*c* 0.005, MeOH)
Retention time HPLC	19.3 min (standard gradient)

8-Hydroxytetrahydroaltersolanol B was isolated as a red powder from the EtOAc extract of liquid culture of *Nigrospora* sp. after fractionation over Sephadex material. It showed UV maxima at 214, 244, 283 and 352 nm. HRMS measurement indicated a molecular formula of $C_{16}H_{20}O_7$ according to a mass of *m/z* 325.1282 amu $[M+H]^+$ (calcd. for $C_{16}H_{21}O_7$ 325.1287). Upon positive ionization, the spectrum of the substance displayed losses of 18, 36 and 54 mass units, indicating the subsequent loss of 3 water moieties due to the presence of hydroxyl groups. This was confirmed

by the ^{1}H NMR spectrum of **6** recorded in DMSO-d_6 that exhibited two aliphatic alcohol groups at δ 4.08 and 4.46 (OH-2, OH-3), two phenol groups at δ 6.51 and 9.47 (OH-9, OH-8) and one hydrogen bonded OH-group at δ 12.66 (OH-5). A singlet corresponding to an aliphatic methyl group at δ 1.15 was assigned to CH_3-2. Moreover, a methoxy group at δ 3.88 and an aromatic proton at δ 6.44 appeared as singlets. In the COSY spectrum one spin system was discernible consisting of H-1α (δ 1.20), H-1β (δ 2.15), H-9a (δ 2.08), H-9 (δ 4.69), OH-9, H-4a (δ 2.43), H-4α (1.48), H-4β (δ 2.07), H-3 (δ 3.43) and OH-3 (figure 3.4.2a.). Interpretation of the HMBC data allowed assignment of the skeletal structure (figure 3.4.2b.). Of particular importance were correlations of H-6 and H-4α to the carbonyl group C-10 in the middle ring at δ 205 and the correlation of the methoxy group to C-7 at δ 155.4 where it is attached to the ring system.
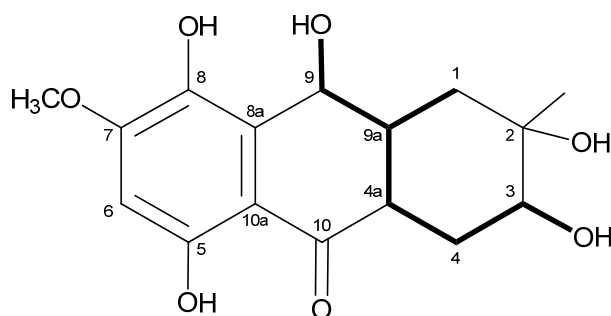
Figure 3.4.2a. COSY correlations observed for 8-hydroxytetrahydroaltersolanol B (**6**).
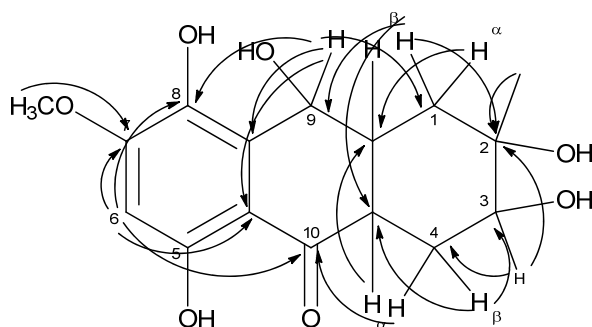
Figure 3.4.2b. Important HMBC correlations of 8-hydroxytetrahydroaltersolanol B (**6**).

The relative stereochemistry of 8-hydroxytetrahydroaltersolanol B could partly be derived from the coupling constants observed in the ^{1}H NMR spectrum. The large value of $J_{4\alpha\text{-}3}$ (12.4 Hz) indicated a trans-diaxial coupling. This was confirmed by a ROESY experiment, which did not show a correlation between 3 and 4α but between 3 and 4β. Moreover, correlations between H-3 and CH_3-2, H-1α, H-4β, H-4a and H-9 that were observed in the ROESY spectrum showed that these protons and the methyl group must be residing on the same face of the ring system.

Since from the ROESY experiment the absolute stereochemistry could not be assigned, the so-called Mosher method was attempted to assign the absolute stereochemistry. In this well-established procedure, a chiral alcohol of unknown absolute configuration is brought into reaction separately with both R-methyl-phenyl-trifluormethylacetochloride (MPTA) and its (S)-enantiomer (Dale and Mosher 1973). The resulting diastereomers will give distinguishable NMR spectra and in particular will display differentiated upfield chemical shifts at positions in vicinity to the chiral center, due to the shielding by the aromatic ring introduced with the coupling reagent,

on the basis of which the absolute stereochemistry of the chiral carbon atom bearing the OH-group can be assigned.

Table 3.4.2. NMR data of 8-hydroxytetrahydroaltersolanol B (**6**) at 500 MHz.

			6					**Literature**	
	$\delta_H{}^a$ (*J* Hz)	$\delta_H{}^b$ (*J* Hz)	$\delta_C{}^a$	COSY[b]	HMBC[a] (H→C)	HMBC[b] (H→C)	ROESY[b]	$\delta_H{}^b$ (*J* Hz)	$\delta_C{}^a$
1 α ax	1.34 dd (12.0; 1.9)	1.20 dd (12.6;12.3)	41.8	1β; 9a; CH₃-2	2; 4a 9;; 9a	2; 3; 9a	3; 4a; 9	1.20 dd (13.2; 12.0)	40.7
β eq	2.35 dd (6.6;2.8)	2.15 dd (13.6;3.8)		1α; 3; 9a	2; CH₃-2; 4a; 9a	2; 3; 9a	2-CH₃	2.15 dd (13.2; 3.8)	
2			71.1						69.4
3	3.43 dd (11.7;4.6)	3.43 dd (11.7;4.6)	75.1	4α/β; 1A; OH-3	2; 4; CH₃-2		1α, 4β, 4a; CH₃-2	3.25 ddd (12.0; 6.3; 5.6)	73.2
4 α ax.	1.65 dd (12.6;12.0)	1.48 dd (12.0)	29.7	3; 4β; 4a	2; 3; 4a; 10	3; 4a	9a	1.48 ddd (12.4; 12.2;12.0)	29.2
β eq	2.27 ddd (12.9; 4.1; 4.1)	2.07 m*			2; 3; 4a; 9a			2.05 ddd (12.2; 5.6; 4.0)	
4a	2.37 dd (8.0;3.9)	2.43 dd (12.1;3.8)	47.3	1α; 4α/β, 9a	4; 9a; 10	10	1B; 3; 9	2.42 dd (13.2; 9.3)	52.1
5			157.2						157.2
6	6.44 s	6.47 s	99.8		8; 10a; 5; 7; 10(LR)	5, 7, 8, 10a		6.49 s	99.2
7			155.4						155.6
8			137.3						137.5
8a			129						127.7
9	4.74 d (10.1)	4.69 d (10.1)	73.3	9a, OH-9	1; 8a; 8; 10a		1α; 4a	4.76 dd (9.8; 6.8)	71.8
9a	2.15 m (21.9;12.8;3.1)	2.08 m*	41.8		9		?3; 4α?	2.05 m	40.7
10			205						202.7
10a			109						107.6
OCH₃-7	3.88 s	3.88 s	56.4			7		3.81 s	55.9
CH₃-2	1.3 s	1.15s	26.8		1; 2; 3	1; 2, 3	3	1.15 s	26.9
OH-2		4.08 s						3.96 s	
OH-3		4.46 s		3				4.47 d (6.3)	
OH-5		12.66 s				5, 6 10a		12.67 s	
OH-8		9.47 s						9.49 brs	
OH-9		6.51 s		9, (9a)	9			6.51 brs	

[a] in MeOD
[b] in DMSO-d_6
* overlapping signals

As 8-hydroxytetrahydroaltersolanol B possesses 5 OH-groups and thus was expected to yield a complex mixture of MTPA ester, the aliphatic hydroxyl groups attached at C-2 and C-3 were planned to be protected in order to prevent them from

reacting with (R)- or (S)-MPTA chloride. Initially, it was attempted to achieve this aim by acetonization of these two aliphatic hydroxy groups as outlined in figure 3.4.2c (Stoessl and Stothers 1983).
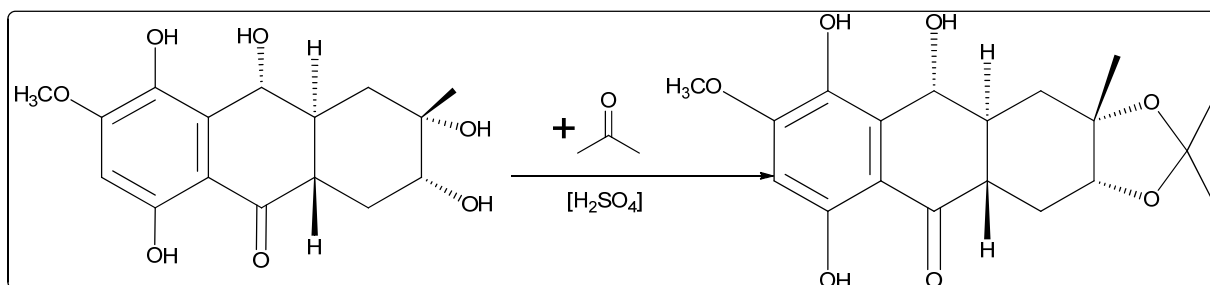
Figure 3.4.2c. Proposed acetonization of **6**.
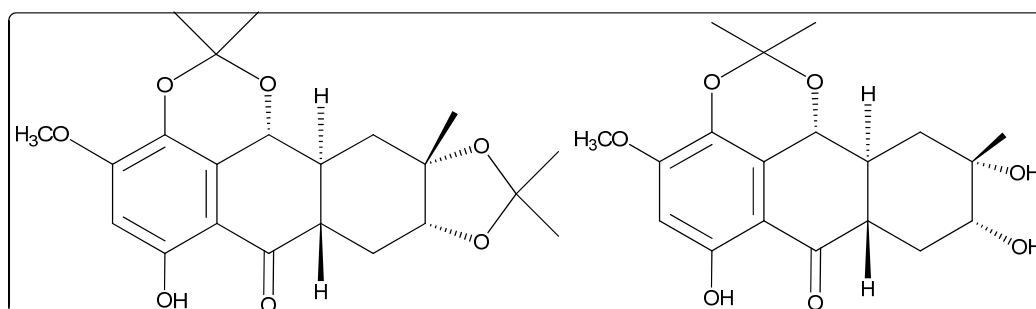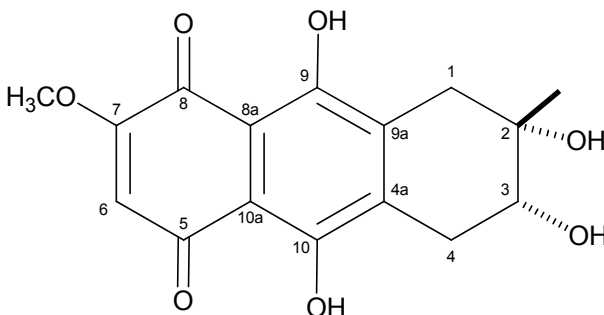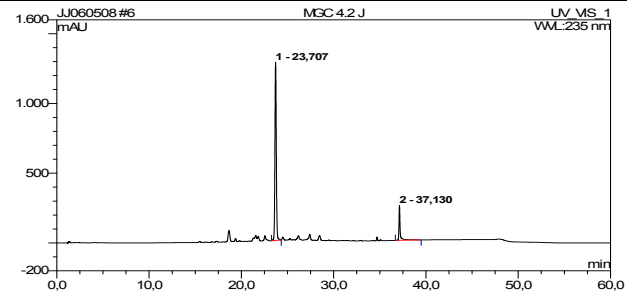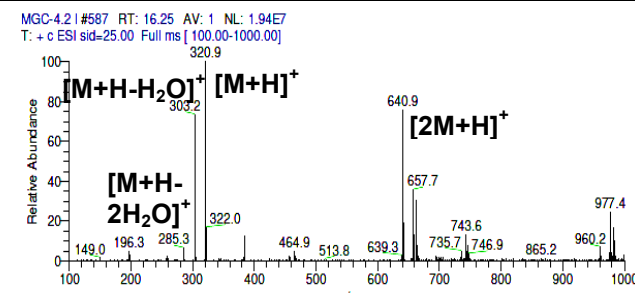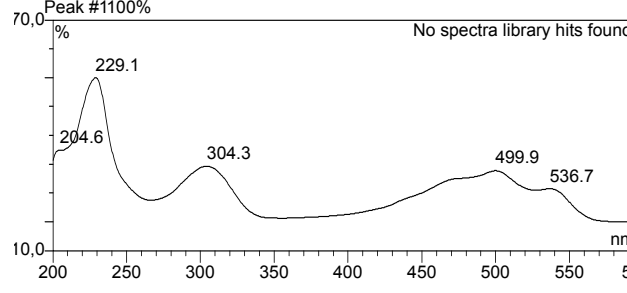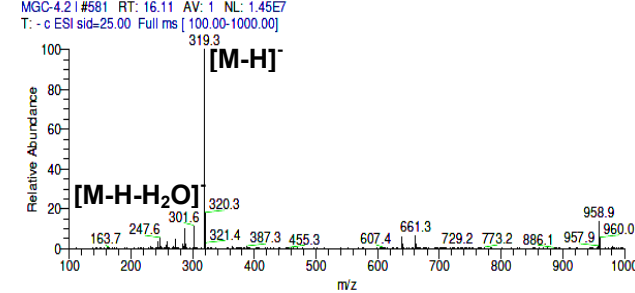
Figure 3.4.2d. Reaction products of the acetonization of **6**.

However, the phenolic groups unexpectedly also reacted with acetone and instead yielding solely 2,3-acetonide, other acetonides were also isolated by preparative HPLC after purification of the reaction mixture (figure 3.4.2d). Subsequently, an attempt was made to protect the phenolic groups as benzoylic esters by the so-called "Schotten-Baumann"-reaction. Despite trying various reaction conditions, this experiment ultimately also proved unsuccessful. In all cases, experiments were hampered by the small amounts of the natural product availabe, and it was not possible to assign the absolute stereochemistry of **6**.

After the isolation and structure elucidation of 8-hydroxytetrahydroaltersolanol B (**6**) was completed during this study, the isolation of the same compound, described as "1-deoxytetrahydrobostrycin", was reported from a marine-derived *Aspergillus* sp. (Xu et al. 2008). UV, NMR, MS data and optical rotation proved virtually identical to those found in this study and thus confirmed the identicalness of both compounds.

3.4.3 Deoxybostrycin (7, known compound)

Deoxybostrycin	
Synonym(s)	(6R,7S)-6,7,9,10-tetrahydroxy-2-methoxy-7-methyl-5,6,7,8-tetrahydroanthracene-1,4-dione
Sample code	MGC 4.2 J
Biological source	*Nigrospora* sp. from *Bruguiera sexangula*
Sample amount	2.4 mg
Physical description	maroon powder
Molecular formula	$C_{16}H_{16}O_7$
Molecular weight	320 g/mol
Optical rotation $[\alpha]_D^{20}$	-47° (*c* 0.01, MeOH)
Retention time HPLC	21.1 min (standard gradient)

Deoxybostrycin (**7**) was isolated from fermentation broth of *Nigrospora* sp. by EtOAc extraction as a dark red powder. It exhibited UV maxima at 229, 304, 500 and 537 nm. Its molecular weight was determined as 320 amu based on the pseudomolecular ions observed at *m/z* 320.9 [M+H]$^+$ and 319.3 [M-H]$^-$ upon positive and negative ionisation by ESI-MS, respectively. Moreover, the presence of hydroxyl groups was indicated by the facile loss of water in the positive ionization mode.

^{1}H NMR spectrum showed the presence of two hydrogen bonded OH-groups at δ 12.67 and 13.23 (OH-8, OH-5) and two aliphatic hydroxyl groups at δ 4.08 and 4.80 (OH-2 and OH-3). Beside an aromatic proton at δ 6.49, a methyl group at δ 1.17 and

a methoxy group at δ 3.90 were also observable. Compared to the spectrum of **6**, with otherwise very similar chemical shifts and coupling constants, one proton signal and one hydroxyl signal were missing. Together with the difference of 14 amu found in the MS, this indicated a higher oxygenized substance. Based on these results and the high similarity with data found in literature (Noda et al. 1970), **7** was identified as the deoxybostrycin. This phytotoxin was previously reported from *Bostryconema alpestre* and endophytic *Alternaria eichorniae* (Noda et al. 1970, Charudattan 1982)

3.4.4 Bostrycin (8, known compound)

Bostrycin	
Synonym(s)	(5S,6R,7S)-5,6,7,9,10-pentahydroxy-2-methoxy-7-methyl-5,6,7,8-tetrahydroanthracene-1,4-dione
Sample code	MGC 4.2 VIII 8.4, VII 16.60%
Biological source	*Nigrospora* sp. (from *Bruguiera sexangula*)
Sample amount	8.4 mg
Physical description	red powder
Molecular formula	$C_{16}H_{16}O_8$
Molecular weight	336 g/mol
Optical rotation $[\alpha]_D^{20}$	-112° (*c* 0.0085, MeOH)
Retention time HPLC	18.7 min (standard gradient)

Bostrycin (**8**) was obtained from *Nigrospora* sp. after fermentation on solid rice medium, extraction with EtOAc, separation over Sephadex material and final purification via semi-preparative HPLC as a dark red pigment. In the UV spectrum, three maxima at 228, 302 and 501 nm were observable. Pseudomolecular ions observed at *m/z* 337.0 [M+H]+ and 335.0 [M-H]- upon positive and negative ionization by ESI-MS, respectively indicated a molecular weight of 336 amu with an increase of 16 mass units compared to deoxybostrycin (**7**) and thus supporting a molecular formula of $C_{16}H_{16}O_8$. Moreover, the presence of an additional hydroxyl group was indicated by the facile loss of three molecules of water upon positive ionization. The ^{1}H NMR spectrum measured in CDCl3 showed two hydrogen bonded OH-groups at δ

12.7 and 13.0 (OH-5, OH-8) and three aliphatic hydroxyl groups at δ 4.30, 4.46 and 4.92 (OH-2, OH-4, OH-3). One aromatic proton at δ 6.41 (H-6), two carbinolic protons at δ 3.50 and 4.73 (H-3, H-4) and two overlapping proton signals at δ 2.65 (H$_2$-1) were observed as well as two singlets of a methoxy group at δ 3.89 (OCH$_3$-7) and of a methyl group at δ 1.22 (CH$_3$-2). The replacement of a proton adjacent to C-4 by a hydroxyl group in comparison to compound **7** was evident from the COSY spectrum, revealing clear correlations from OH-4 to H-4, which was also shifted downfield, and OH-3 to H-3. The identification of **8** as bostrycin was further corroborated by the very similar experimental UV, NMR and mass spectral data and [α]$_D$ value of published data for bostrycin (Noda et al. 1970, Stevens *et al*. 1979, Kelly *et al*. 1985). Bostrycin was first identified from *Bostryconema alpestre* (Noda et al. 1970), but later also from other fungi like *Nigrospora oryzae* and *Alternaria eichorniae* (Charudattan and Rao 1982).

Table 3.4.4. NMR data of deoxybostrycin (**7**) and bostrycin (**8**) at 500 MHz.

	7			**Lit.**	**8**				**Lit.**
	δ$_H$^{a} (*J* Hz)	**COSY**^{b}	**HMBC**^{a} **(H→C)** (δ$_H$ ppm)	δ$_H$^{ad} (*J* Hz)	δ$_H$^{a} (*J* Hz)	δ$_c$^{c}	**COSY**	**HMBC**^{c} **(H→C)**	δ$_H$^{ad} (*J* Hz)
1 α	2.62 d (11.4)	1β		2.62 – 2.8 m	2.65 m	35.3		2, 3, 4a, 9a	2.68 "s" (2H)
β	2.784 d (18.3)	1α							
2						70.4			
3	3.62 ddd(11.7; (6.6)	4α/β		3.55 m	3.50 brd	76.7	4; 3-OH	2	3.51 d (4.5)
4 α	2.67 dd (18.3;6.8)	3; 4β		2.62 – 2.8 m	4.73 d (4.1)		3; 4-OH	3, 4a, 9a	4.73 d (4.5)
β	2.8 dd (18.8; 4.9)	3; 4α							
4a						138.1			
5						177.4			
6	6.49 s			6.38 s	6.41 s	108.1		5, 7, 10a	6.41 s
7						160.5			
9a						135.6			
10a						109.5			
CH$_3$-2	1.17 s		2(35);1;3(69/70)	1.18 s	1.22 s			1, 2, 3	1.23 s
OCH$_3$-7	3.90 s		7 (159)	3.88 s	3.89 s	56.6		7	3.90 s
OH-2	4.08			4.22 s	4.30				4.45
OH-3	4.80 d (3.8)			4.72 s	4.92 s		3		4.70
OH-4					4.46 s		4		5.16
OH-5	13.23 s			12.90 s	13.0				13.12
OH-8	12.67 s			12.37 s	12.7				12.42

^{a} in DMSO-d_6
^{b} in MeOD
^{c} in CDCl$_3$
^{d} Noda et al., 1970

3.4.5 Tenuazonic acid (9, known compound)

Tenuazonic acid	
Synonym(s)	(S)-3-acetyl-5-sec-butyl-4-hydroxy-1H-pyrrol-2(5H)-one
Sample code	MGC 4.2 VIII 9.10a; -11a
Biological source	*Nigrospora* sp. (from *Bruguiera sexangula*)
Sample amount	14.1 mg
Physical description	viscous brown oil
Molecular formula	$C_{10}H_{15}NO_3$
Molecular weight	197 g/mol
Optical rotation $[\alpha]_D^{20}$	-60° (*c* 0.42, $CHCl_3$)
Retention time HPLC	22.6 min (standard gradient), 13.8 min (half gradient)

Tenuazonic acid (**9**) was isolated from the EtOAc extract of *Nigrospora* sp. obtained after fermentation on solid rice medium through semi-preparative HPLC. It exhibited UV maxima at 219 and 277 nm. Its molecular weight was determined as 197 amu based on the pseudomolecular ion peaks at *m/z* 198.2 [M+H]$^+$ and 196.2 [M-H]$^-$ upon positive or negative ionization, respectively. This suggested the presence of an uneven number of nitrogen atoms and the molecular formula of $C_{10}H_{15}NO_3$. In the ^{1}H NMR spectrum, three methyl groups were observable. One

singlet was shifted downfield to δ 2.29 (H-7) due to an adjacent carbonyl group, a doublet was located at δ 0.89 (H-11) adjacent to a CH (H-8, δ 1.76) and a triplet adjacent to a CH$_2$ (H-9A, δ 1.24 and H-9B, δ 1.08) appeared at δ 0.75. Proton 5 was shifted downfield to δ 3.65 due to the neighboring NH-group, the proton of which appeared as a doublet at δ 8.45. The hydroxyl group was barely notable as a very broad signal at δ 10.51. Comparison of UV, optical rotation, mass spectral and ^{1}H NMR data with literature data (Hassan 2008, Nolte et al. 1980) as well as co-elution on the analytical Dionex-HPLC system together with an authentic reference present at our laboratory confirmed compound **10** to be tenuazonic acid. This tetramic acid was first isolated from *Alternaria tenuis* (Rosett et al. 1957), but was since then found to be a widespread fungal metabolite, for example in other *Alternaria* species, but also in *Aspergillus* and numerous other genera (Hassan 2008)

Table 3.4.5. NMR data of tenuazonic acid (**9**) at 500 MHz
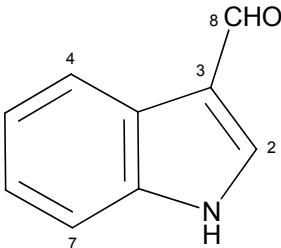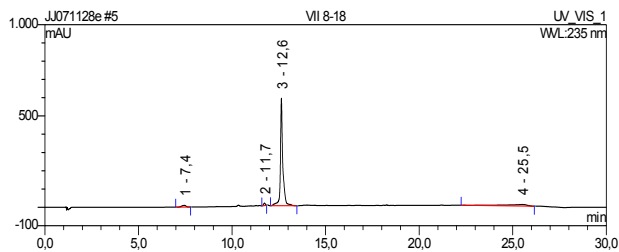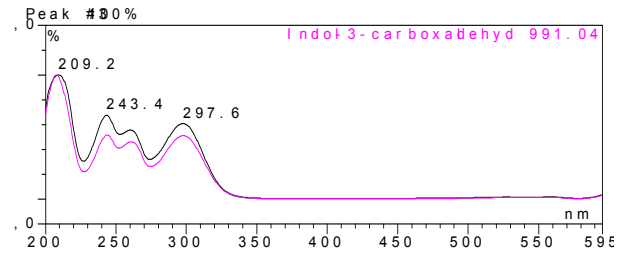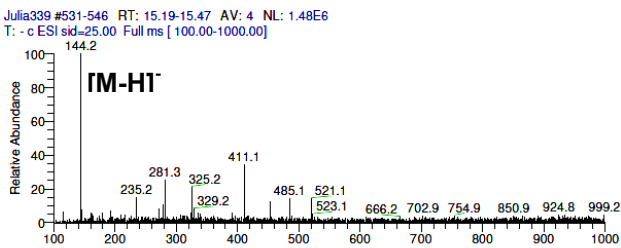
	9		**Lit.**
	$\delta_H{}^a$ (*J* Hz)	$\delta_H{}^b$ (*J* Hz)	$\delta_H{}^{b,c}$ (*J* Hz)
5	3.84 d(1.9)	3.65 brs	3.77 d(2.8)
7	2.43 s	2.29 s	2.33 s
8	1.93 brs	1.76 brs	1.78 m
9	A: 1.39 m	A: 1.24 m	1.24 m
	B: 1.25 m	B: 1.08 m	1.09 m
10	0.92 t (7.4)	0.75 t (7.2)	0.8 t (7.2)
11	1.01 d (7.0)	0.89 d (6.9)	0.9 d(6.9)
NH-1		8.45 d (8.7)	8.74 brs
OH-4		10.51 s	11.1 brs

[a] in MeOD
[b] in DMSO-d_6
[c] Hassan, 2008

3.4.6 Indole-3-carbaldehyde (10, known compound)

Indole-3-carbaldehyde	
Synonym(s)	1*H*-indole-3-carbaldehyde; 3-formylindole
Sample code	MGC 4.2 REt VII 8-18
Biological source	*Nigrospora* sp. (from *Bruguiera gymnorrhizza*)
Sample amount	0.4 mg
Physical description	brown powder
Molecular formula	C_9H_7NO
Molecular weight	145 g/mol
Retention time HPLC	19.8 min (standard gradient)

No ionisation in positive mode.

Indole-3-carbaldehyde (**10**) was obtained as a brown powder from the EtOAc extract after fermentation of *Nigrospora* sp. on solid rice medium, fractionation via VLC and purification over silica and semi-preparative HPLC. Its UV spectrum showed maxima at 209, 243, 297 nm. Upon positive ionization, no clear pseudomolecular ion peak was visible, but upon negative ionization, a pseudomolecular peak at *m/z* 144.2 [M-H]⁻ indicated a molecular weight of 145 amu and a molecular formula of C_9H_7NO. The ¹H NMR spectrum showed seven proton resonances including an aromatic ABCD spin system at δ 8.08 (dd; *J*=8.0 Hz; *J*=1 Hz. H-4), δ 7.50 (brd, *J*=8.1 Hz, H-8), δ 7.24 (td, *J*=8.0 Hz; *J*=1.0 Hz, H-7) and δ 7.20 (td, *J*=8.0 Hz; *J*=0.9 Hz, H-6) typical for indole rings. A further aromatic proton appeared as a singlet at δ 8.27 (H-2). The downfield shift of H-8 to δ 9.92 could be explained by the aldehyde function. Comparison of the obtained UV, MS and ¹H NMR data with reported data (Hiort 2002, Chowdhury et al. 1981) established compound **10** as the well-known metabolite indole-3-carbaldehyde.

Table 3.4.6. ^{1}H NMR data of indole-3-carbaldehyde (**10**) at 500 MHz.

	10 $\delta_H{}^a$ (*J* Hz)	**Lit.** $\delta_H{}^{a,b}$ (*J* Hz)
2	8.27 s	8.27 s
4	8.08 dd (8.0; 0.9)	8.07 dd (7.6; 1)
5	7.20 td (7.5; 0.9)	7.20 td (6.9; 1.3)
6	7.24 td (8.0; 1.0)	7.25 td (6.9; 1.3)
7	7.50 brd (8.1)	7.50 dd (8.2; 1)
8	9.92 s	9.92 s
NH	12.13 s	12.11 s

[a] in DMSO-d_6
[b] Hiort, 2002

3.4.7 Cyclo(threonylisoleucinyl) (11, new compound)

Cyclo(threonylisoleucinyl)	
Synonym(s)	(3S,6S)-3-sec-butyl-6-((R)-1-hydroxyethyl)piperazine-2,5-dione, cyclo-L-threonyl-L-isoleucine
Sample code	MGC 4.2 VII-11-3-16
Biological source	*Nigrospora* sp. (from *Bruguiera sexangula*)
Sample amount	14 mg
Physical description	whitish powder
Molecular formula	$C_{10}H_{18}N_2O_3$
Molecular weight	214 g/mol
Optical rotation $[\alpha]_D^{20}$	13.5° (*c* 0.0085, MeOH)
Retention time HPLC	18.0 min (standard gradient)

Cyclo(threonylisoleucinyl) (**11**) was isolated as a whitish amorphous substance from the extract of *Nigrospora* sp. grown on solid rice medium after fractionation over silica gel and purification by semi preparative HPLC. Its UV spectrum showed maxima at 201, 235 and 293 nm. The MS exhibited two prominent peaks, in the positive ionization mode at *m/z* 197.2 and in the negative ionization mode at *m/z* 195.2. The first suggestion was that these were the pseudomolecular ion peaks, but analysis of the NMR data revealed that they represented $[M+H-H_2O]^+$ and $[M-H-H_2O]^-$, respectively. The pseudomolecular ion peaks were not detectable because of the facile loss of water. Also HRMS measurement only revealed a mass of *m/z* 197.1284 amu $[M+H-H_2O]^+$ (calcd. for $C_{10}H_{16}N_2O_2$, 197.1290). However, measurement of the compound in DMSO-*d₆* revealed two nitrogen-bonded protons at

δ 7.68 (NH-4) and δ 5.37 (NH-1) as well as a hydroxyl signal at δ 3.74 (OH-1'). NH-4 was part of a 1-amino-2-methylbutyl-moiety showing a coupling constant of 9.0 Hz to the neighboring H-3 at δ 4.28. H-3 coupled also with H-1'' at δ 1.81, as evident from the COSY spectrum. Moreover, correlations of H-1'' to H-2''B at δ 1.15 and CH₃-1'' at δ 0.82 were likewise observed in the COSY spectrum. Correlations between H-2''B and H-2''A (δ 1.40) and H₃-3'' (δ 0.84) completed this spin system. A second spin system comprised a heteroatom bearing CH₃-CH-CH-fragment with the terminal methyl group at δ 1.03 (H₃-1'), a carbinolic proton at δ 3.81 (H-1') and a further proton at a heteroatom-bearing carbon at δ 4.28 (H-6). Although the second amine proton did not show correlations in the COSY spectrum, the presence of two NH-groups together with the downfield chemical shifts of H-3 and H-6 between δ 4 and 4.5 suggested the presence of a dipeptide. This assumption was affirmed by a TOSCY experiment exhibiting two spin systems (see figure 3.4.7a) and the ^{13}C NMR spectrum revealing two carbonyl carbons at δ 171.5 and 172.5. An HMBC experiment showed correlations between NH-4 and C-5 as well as H-3 and C-2. Although no correlation could be observed between C-5 and the threonine part of the structure, the presence of a diketopiperazine ring was confirmed by HRMS.

Table 3.4.7a. NMR data of cyclo(threonylisoleucinyl) (**11**) at 500 MHz.

11

	δ$_H$^{a} (*J* Hz)	δ$_H$^{b} (*J* Hz)	δ$_C$^{a}	COSY^a	HMBC^a (H→C)	NOESY^a	TOSCY^a
2			171.5				
3	4.28 dd (7.2; 3.5)	4.46 d (5.7)	55.5	1'', NH-4	3, 1'', 2'', CH₃-1''	1'', 2''A, 2''B, 3''	1'', 2''A, 2''B, 3'', CH₃-1'', NH-4
5			172.5				
6	4.5 d (7.2)	3.89 d (3.5)	75	1'	1', 2'	1'	1', 2', NH-1, OH-1'
1'	3.81 m	4.01 m	67.5	6, 2'		6, 2'	6, 2', NH-1, OH-1'
2'	1.03 d (4)	1.22 d (6.3)	19	1'	6, 1'	1', OH-1'	6, 1', NH-1, OH-1'
1''	1.81 m	1.92 m	36.5	3, 2''B, CH₃-1''	CH₃-1''	3, 2''A, 2''B, 3'', CH₃-1'', NH-4	3, 2''A, 2''B, 3'', CH₃-1'', NH-4
2''	A: 1.40 m	1.48 m	24.5	2''B, 3''	3, 1'', 3'', CH₃-1''	3, 1'', 2''B, 3'', CH₃-1'', NH-4	3, 1'', 2''B, 3'', CH₃-1'', NH-4
	B: 1.15 m	1.23 m		1'', 2''A, 3''	3, 1'', 3'', CH₃-1''	3, 1'', 2''B, 3'', CH₃-1'', NH-4	3, 1'', 2''A, 3'', CH₃-1'', NH-4
3''	0.84 m	0.93 t (7.3)	11	2''A, 2''B	1'', 2''	3, 1'', 2''A, 2''B	3, 1'', 2''A, 2''B, CH₃-1'', NH-4
CH₃-1''	0.82 m	0.93 d (6.9)	15	1''	3, 1'', 2''	1'', 2''A, 2''B	3, 1'', 2''A, 2''B, 3'', NH-4
NH-1	5.37 s						6, 1', 2', OH-1'
NH-4	7.68 d (9.0)			3	5	3, 6, 1',2', 1'', 2''A, 2''B, CH₃-1'', OH-1'	3, 1'', 2''A, 2''B, 3'', CH₃-1''
OH-1'	3.74 s					6, 2',NH-4	6, 1', 2', NH-1

^{a} in DMSO-d_6
^{b} in MeOD
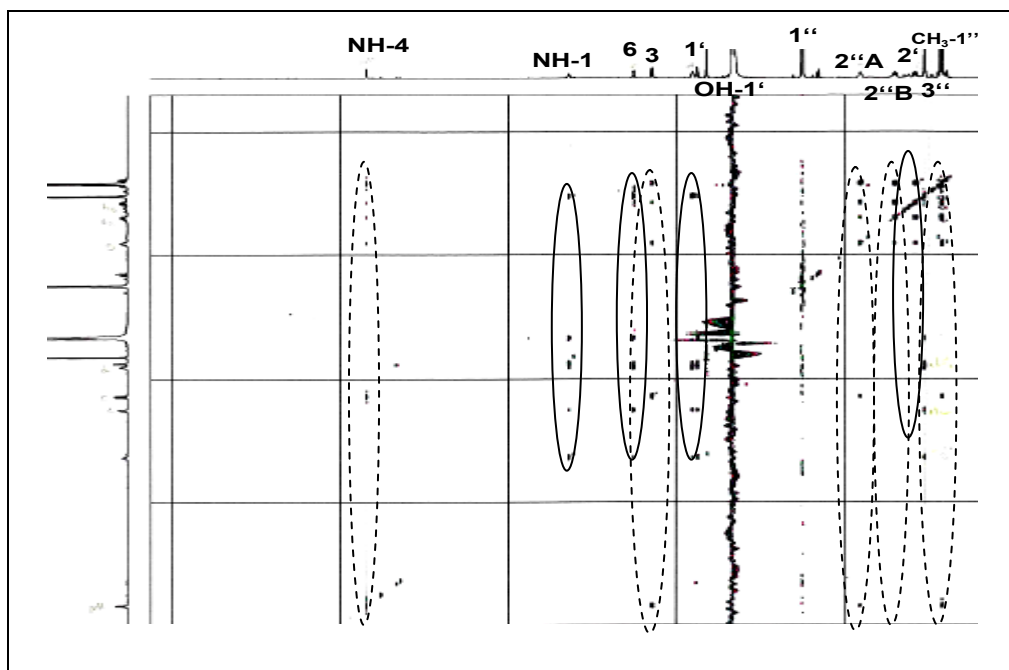
Figure 3.4.7a. TOCSY spectrum of (**11**) in DMSO-*d₆*, indicating spin systems for ile (dashed line) and thr (continuous line).
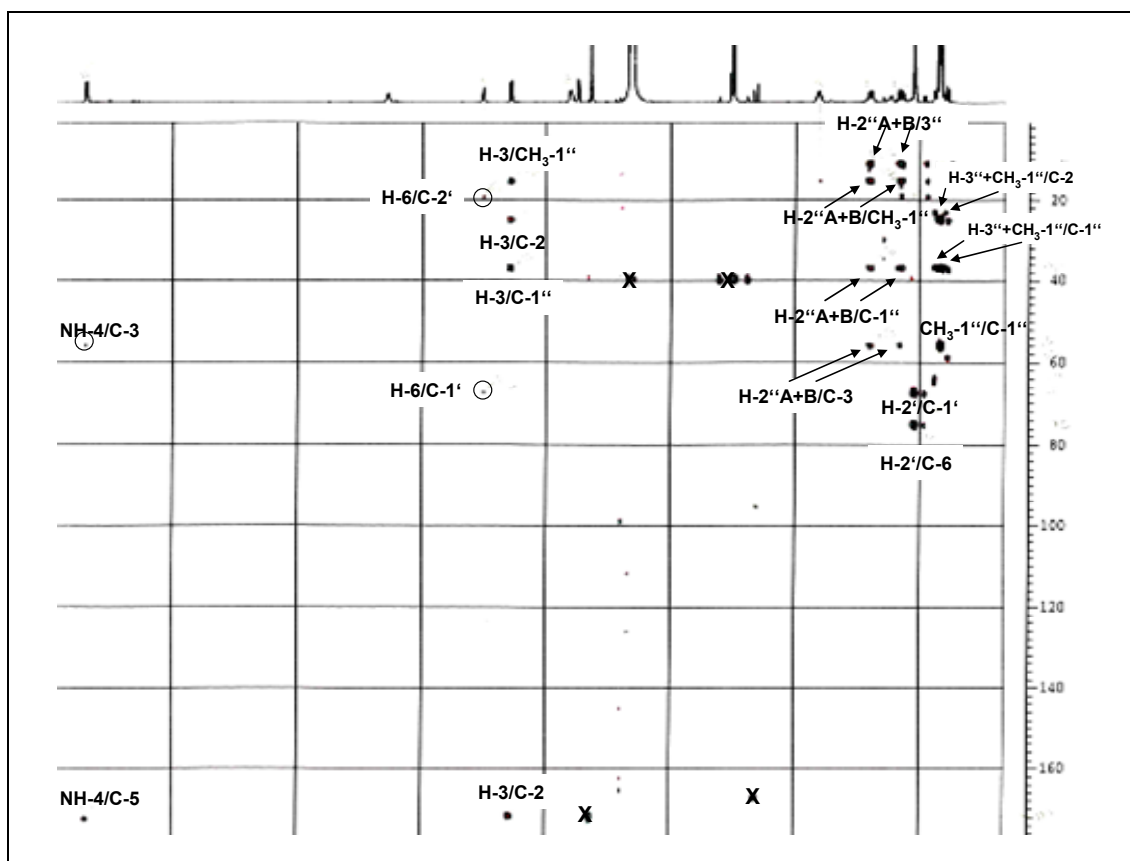
Figure 3.4.7b. HMBC spectrum of (**11**) in DMSO-*d₆*.

For determination of the absolute stereochemistry, a Marfey experiment was performed. After hydrolysis of the dipeptide the hydrolyzate was transformed to diastereomeric reaction products by adding the diastereomeric reagent FDAA (1-fluoro-dinitrophenyl-5-L-alanineamide). These reaction products were submitted to analytical HPLC and LC-MS. Moreover, the commercially available amino acids

isoleucine, *allo*-isoleucine and threonine were used as authentic standards, both as the respective L-enantiomers and racemates, and subjected to similar conditions for the same reaction and analysis. By comparison of the retention times of the obtained reaction products of the hydrolyzate and the amino acid standards the configuration of the dipeptide was obtained. From the Dionex (HPLC-DAD) as well as the LCMS spectra it was obvious, that threonine must have "L"- or 2R,3S-conformation. For the second amino acid, this analysis proved more complicated due to the similarity of the retention times for L-*allo*-isoleucine and L-isoleucine. Thus, a co-elution analysis by LCMS with a modified gradient program was performed, which ultimately gave two peaks when co-eluting with L-*allo*-isoleucine, but only one for L-isoleucine, thereby proving the conformation as L-isoleucine (2S,3S) (see figure 3.4.7c)

Table 3.4.7b. Retention times of standard amino acid derivatives and derivatives of the hydrolyzate of cyclo(threonylisoleucinyl) (**11**) in HPLC-DAD and LCMS. The respective retention time of FDAA is given as a relation.

amino acid	retention time HPLC-DAD	FDAA ret. time HPLC-DAD	retention time LCMS	FDAA ret. time LCMS
D-ile	40.3	32.8	34.96	22.46
L-ile	37.7	32.8	31.22	22.46
D-*allo*-ile	40.3	32.9	34.86	22.45
L-*allo*-ile	37.9	32.9	31.09	22.45
D-thr			24.42	22.59
L-thr	30.6	33.0	20.94	22.59
11-3-16a	30.8	32.9	21.70	22.56
11-3-16b	38.0	32.9	31.29	22.56
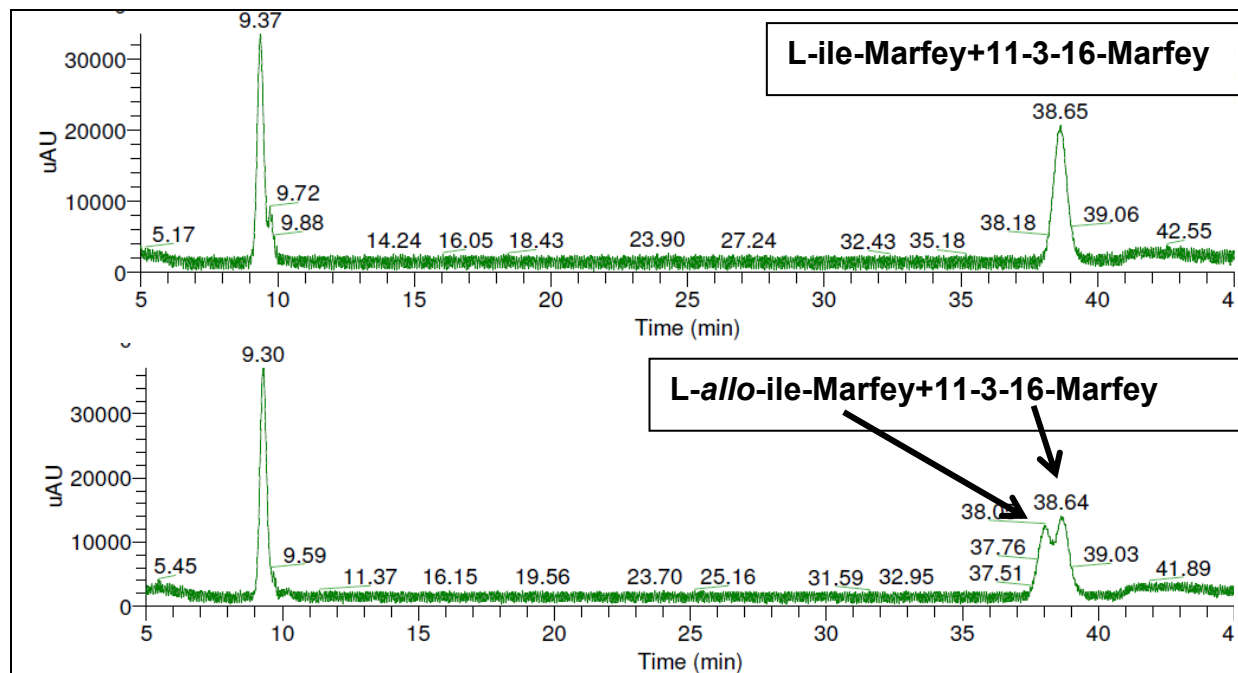
Figure 3.4.7c. LCMS chromatograms of co-elution of Marfey reaction products of L-ile with hydrolysate of MGC 4.2 VII-11-3-16 (above) and of L-*allo*-ile hydrolysate of MGC 4.2 VII-11-3-16 (below).

3.4.8 Bioactivity test results for compounds isolated from *Nigrospora* sp.

Looking at the cytotoxicity against L5178Y, the strong cytotoxicity observed for the EtOAc extracts could be explained by the high growth inhibition potential of the new compound 8-hydroxytetrahydroaltersolanol B (**6**) and bostrycin (**8**) (see also table 3.4). The moderate antibiotic potential against *S. aureus* and *S. pyrogenes* by the extract obtained after cultivation in liquid medium also seemed to stem from 8-hydroxytetrahydroaltersolanol B (**6**). The antimicrobial activity of the extract obtained after cultivation on rice medium, however, could not be explained by the activity profile of one of the isolated compounds. Although bostrycin showed good inhibition against *S. aureus*, the inhibition by the crude extract was even stronger, and the formidable inhibition against *S. pyrogenes* could not be observed in any of the isolated substances. Thus, beside possible synergistic effects, it is also possible that the main antibiotically active principle was not obtained. Deoxybostrycin (**7**) did not exhibit good antibiotic activity. However, tested for biofilm inhibition against *Staphylococcus epidermidis*, it exhibited – as the only isolated compound - a 60% inhibiting activity at a concentration of 50 µg/mL.

Analyzing antiviral activities, compounds **6** and **8** exhibited good prophylactic effects against human Rhinoviruses. But due to their toxicity they were not further investigated.

Neither the extract nor any isolated compound exhibited mentionable inhibitory potential against any tested protein kinase in the activity assay.

Table 3.4.8a. Biological screening test results for isolated compounds of *Nigrospora* sp.

Compound	L5178Y survival rate in %(@ 10 µg/mL)	Survival rate in % (@ 312 µg/mL / <u>MIC</u> [µg/mL])						Conc. exhibiting prophylactic effect against viral infection [µg/mL]		
		E.c.	K.p.	E.f	S.a.	S.p.	P.a.	HRE2	HRE8	HRE16
8-Hydroxy-tetrahydroalter-solanol B (**6**)	1.7	100	100	95	15 <u>>15.6</u>	65 <u>>15.6</u>	85	>3.9	>3.9	>7.8
Deoxybostrycin (**7**)	27.9	85	85	90	70	100	80			
Bostrycin (**8**)	-1.5	80	85	80 <u>>125</u>	20 <u>>62.5</u>	100	100	>3.9	>3.9	>7.8
Tenuazonic acid (**9**)	40.0	85	65	90	70	100	80			

E. c. *Escherichia coli*, K. p. *Klebsiella pneumoniae*, E. f. *Enterococcus faecium*, S. a. *Staphylococcus aureus*, S. p. *Streptococcus pyogenes*, P. a. *Pseudomonas aerigunosa; HRE2, 8 and 16 Human Rhinovirusses 2, 8 and 16.*

Table 3.4.8b. Results of screening of isolated substances from *Nigrospora* sp. against 24 different protein kinases. Inhibitory potentials of extracts were determined in biochemical protein kinase activity assays. Listed are the residual activities at a concentration of 1μg/mL in %.

Tested cmd.	AKT1	ARK5	Aurora-A	Aurora-B	B-RAF-VE	CDK2/CycA	CDK4/CycD1	COT	EGF-R	EPHB4	ERBB2
(**7**)	108	100	90	94	99	114	102	119	100	110	90
MGC4.2 HAc 6[a]	105	97	98	102	113	104	103	97	103	99	93
MGC4.2 HAc 11[a]	97	97	100	94	113	102	88	101	104	93	89
(**9**)	98	103	93	87	97	103	103	122	96	78	94
(**10**)	86	99	88	85	95	104	101	126	97	105	85

[a]Two reaction products obtained after acetonization of compound **6** were also tested for comparison their potential activities to compound **7**. As no activities were observed, no attempts were made towards further examination of these derivatives.

Tested cmd.	FAK	SRC	VEGF-R2	VEGF-R3	FLT3	INS-R	MET	PDGF-R-β	PLK1	CK2-α1	SAK	TIE2
(**7**)	93	106	108	106	97	97	94	103	99	94	97	89
MGC4.2 HAc 6[a]	99	102	109	108	98	104	98	105	78	90	97	90
MGC4.2 HAc 11[a]	102	102	106	109	103	100	104	100	80	101	103	91
(**9**)	90	90	104	105	93	97	97	96	89	92	98	92
(**10**)	99	87	89	109	97	94	120	84	103	77	92	73

[a]Two reaction products obtained after acetonization of compound **6** were also tested for comparison their potential activities to compound **7**. As no activities were observed, no attempts were made towards further examination of these derivatives.

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

Alternaria sp. was isolated as an endophytic fungus from the mangrove plant *Sonneratia alba*. Extracts of *Alternaria* sp. when grown on solid rice medium yielded the known fungal metabolites 2,5-dimethyl-7-hydroxychromone (**12**), alternarian acid (**13**), altenusin (**14**), altenuene (**15**), 4'-epialtenuene (**16**), alternarienonic acid (**17**) alternariol (**18**) and altertoxin I (**20**) together with two new carboxylic acids, for which the author proposes the names xanalteric acid I (**23**) and II (**24**). When the fungus was grown in liquid medium, compounds **14**, **15**, **18**, **20**, **23** and **24** were likewise detected via HPLC/DAD and LC-MS. Additionally, the known compounds alternariol-5-*O*-methylether (**19**) and the perylene derivatives alterperylenol (**21**) and stemphyperylenol (**22**) were obtained. The latter compounds were not detected when the fungus was cultivated on rice medium.

The difference in the chemical patterns that was also observed by comparison of the HPLC chromatograms of the crude ethyl acetate extracts (see figure 3.5) was mirrored by the results of the bioactivity screening. The crude extracts were submitted to cytotoxicity screening, with both displaying activity against the cell line L5178Y. The growth inhibition of the extract obtained after fermentation in liquid Wickerham medium was larger than that of the crude extract obtained from rice cultures. Moreover, the liquid extract when subjected to testing for antibiotic activity, exhibited only mild activity.
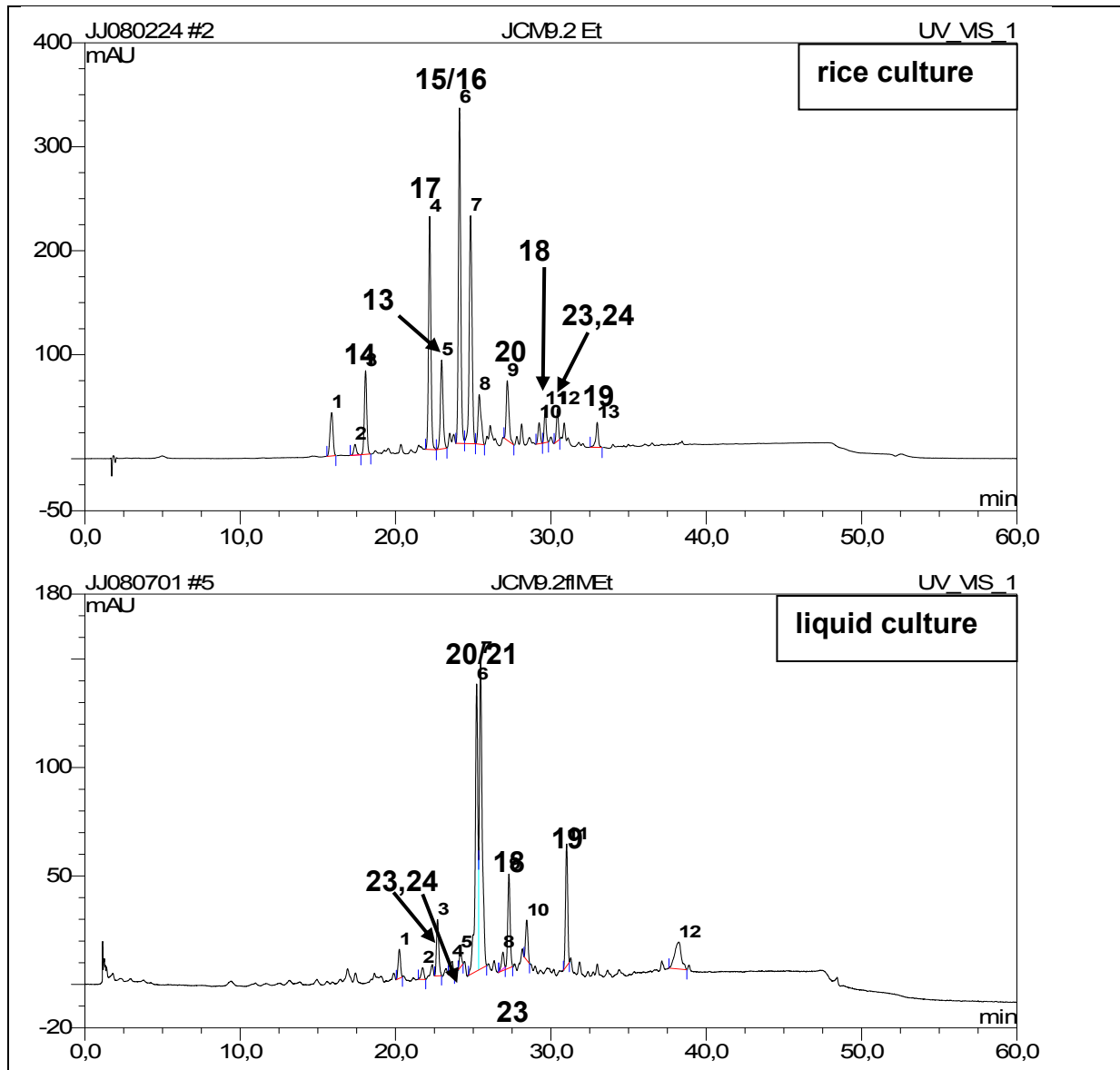
Figure 3.5: EtOAc chromatograms of rice (above) and liquid (below) cultures of *Alternaria* sp.

Table 3.5 a. Biological screening test results for EtOAc extracts from rice or liquid cultures of *Alternaria* sp.

Extract	L5178Y survival rate in % (@ 10 μg/mL)	Survival rate in % (@ 312 μg/mL)					
		E. c.	K. p.	E. f.	S. a.	S. p.	P. a.
JCM9.2 rice EtOAc	14.2	n. t.	n. t.	n. t.	n. t.	n. t.	n. t.
JCM9.2 liq. EtOAc	-1.4	90	100	85	85	85	85

E. c. *Escherichia coli*, K. p. *Klebsiella pneumoniae*, E. f. *Enterococcus faecium*, S. a. *Staphylococcus aureus*, S. p. *Streptococcus pyogenes*, P. a. *Pseudomonas aerigunosa*

3.5.1 2,5-Dimethyl-7-hydroxychromone (12, known compound)

2,5-Dimethyl-7-hydroxychromone	
Synonym(s)	7-Hydroxy-2,5-dimethyl-4*H*-chromen-4-one
Sample code	JCM9.2 III 3+4.5.5.6
Biological source	*Alternaria* sp. (from *Sonneratia alba*)
Sample amount	1.1 mg
Physical description	light brown solid
Molecular formula	$C_{11}H_{10}O_3$
Molecular weight	190 g/mol
Retention time HPLC	22.8 min (standard gradient)

2,5-Dimethyl-7-hydroxychromone (**12**) was obtained as a beige powder from the EtOAc extract of solid rice cultures of *Alternaria* sp. after several purification steps. It displayed a UV spectrum characteristic of a 7-hydroxy-4-chromone with maxima at 225, 242, 250 and 292 nm. Positive and negative ESI-MS showed pseudomolecular ions at *m/z* 191.3 [M+H]$^+$ and 189.3 [M-H]$^-$, indicating a molecular weight of 190 amu. The ^{1}H NMR spectrum (table 3.5.1) also displayed characteristics of a 7-hydroxy-4-chromone, notably the olefinic proton H-3 (δ 6.00) together with two *meta*-coupled aromatic protons H-6 (δ 6.63) and H-8 (δ 6.64). These signals, together with the one due to a methyl group (δ 2.71, CH$_3$-5) resembled the signals found in the ^{1}H NMR spectrum of aloesol (**5**) that was isolated from *Nigrospora* sp. The 2-hydroxyisopropyl moiety attached to C-2 in **5** was replaced by a methyl group (δ 2.31). The proposed structure of **12** was confirmed by comparison of UV, ^{1}H NMR and mass spectra with published data for 2,5-dimethyl-7-hydroxychromone

(Kashiwada et al. 1984). The substance was first identified as a plant metabolite of *Rhei rhizoma*, but was also described from endophytic *Alternaria* sp. (Hassan 2007).

Table 3.5.1. NMR data of 2,5-dimethyl-7-hydroxychromone (**12**) at 500 MHz.

	12	**Literature**[c]
	$\delta_H{}^a$ (*J* Hz)	$\delta_H{}^b$ (*J* Hz)
3	6.00 s	5.96 s
6	6.63 d (0.6)	6.60 s
8	6.64 d (2.4) s	6.60 s
CH$_3$-2	2.31 s	2.28 s
CH$_3$-5	2.71 s	2.66 s

[a] in MeOD
[b] Kashiwada 1984; in DMSO-d_6

3.5.2 Alternarian acid (13, known compound)

Alternarian acid	
Synonym(s)	3-(2-carboxy-3-hydroxy-5-methoxyphenyl)-4-methyl-2-oxo-2H-pyran-6-carboxylic acid
Sample code	JCM9.2 V.2.2.2
Biological source	*Alternaria* sp. (from *Sonneratia alba*)
Sample amount	15.1 mg
Physical description	brown disks
Molecular formula	$C_{11}H_{10}O_3$
Molecular weight	320 g/mol
Retention time HPLC	20.6 min (standard gradient)

Alternarian acid (**13**) was obtained as brown disks from the EtOAc extract obtained after fermentation on solid rice cultures of *Alternaria* sp., fractionation over VLC, silica gel and repeated purification by semi preparative HPLC. It displayed an UV spectrum with maxima at 219, 259 and 306 nm. Positive and negative ESI-MS showed pseudomolecular ions at *m/z* 321.0 $[M+H]^+$ and 319.0 $[M-H]^-$ indicating a molecular weight of 320 amu. The ^{1}H NMR spectrum displayed an aromatic singlet at δ 6.93 (H-5) and a pair of *meta*-coupled protons at δ 5.90 (H-12) and δ 6.24 (H-10). The location of the methyl group (δ 1.83) at C-4 was confirmed by HMBC correlation of its protons to C-2, C-3, C-4 and C-5. The location of the methoxy group (δ 3.71) at C-11 was confirmed by the HMBC correlation of its protons with C-11 and the ROESY correlations between its protons with H-10 and H-12. HMBC correlations of

85

H-10 to C-8, C-9, C-11 and C-12 and from H-12 to C-8, C-10 and C-11 allowed assignment of the aromatic ring. The correlation from H-12 to C-3 revealed the connection to the pyrone ring. The structure was confirmed by comparison of UV, mass spectra, ^{1}H NMR and ^{13}C NMR with published data for alternarian acid (Chadwick et al. 1984). The substance was originally isolated from *Alternaria mali* and its structure determined by X-ray diffractometry (Chadwick et al. 1984).

Table 3.5.2. NMR data of alternarian acid (**13**) at 500 MHz.

	13				**Literature**	
	$\delta_H{}^a$ (*J* Hz)	$\delta_C{}^b$	**HMBC**[a] (H→C)	**ROESY**[a]	$\delta_H{}^c$ (*J* Hz)	$\delta_C{}^c$
2		171.3				
3		132.4				
4		144.1				
5	6.93 s	112.3			7.15 s	
6		160.6				
7		137.9				
8		111.2				
9		166.1				
10	6.24 brs	100.2	8, 9, 11, 12	11-OCH$_3$	6.35 d (0.3)	101.8
11		161.3				
12	5.90 brs	105.4	3, 8, 10, 11	11-OCH$_3$	6.52 d (0.3)	105.3
CH$_3$-4	1.83 s	19.3	2, 3, 4, 5		2.06 s	19.8
OCH$_3$-11	3.71 s	54.9	11		3.86 s	56.2
COOH-6/ -8		161.1/161.3				

[a] in DMSO-d_6
[b] confirmed from HMBC
[c] Chadwick 1984., no solvent reported

3.5.3 Altenusin (14, known compound)

Altenusin	
Synonym(s)	3,4',5'-Trihydroxy-5-methoxy-2'-methyl[1,1'-biphenyl]-2-carboxylic acid
Sample code	JCM9.2III3+4.5.3
Biological source	*Alternaria* sp. from *Sonneratia alba*
Sample amount	19 mg
Physical description	brown prisms
Molecular formula	$C_{15}H_{14}O_6$
Molecular weight	290 g/mol
Retention time HPLC	22.4 min (standard gradient)

Altenusin (**14**) was isolated as brown prisms from EtOAc extract from solid rice cultures of *Alternaria* sp. and repeated purification. It displayed an UV spectrum with maxima at 203, 216, 256 and 292 nm. Positive and negative ESI-MS showed pseudomolecular ions at *m/z* 291.1 [M+H]$^+$ and 289.1 [M-H]$^-$ indicating a molecular weight of 290 amu. Furthermore, upon positive ionization loss of up to 3 molecules of water and upon negative ionization decarboxylation was observed, suggesting the presence of three hydroxyl and one carboxyl group. ^{1}H NMR revealed two aromatic singlets at δ 6.47 (H-2') and δ 6.55 (H-5'), a pair of *meta*-coupled aromatic protons at δ 6.40 (H-4) and δ 6.03 (H-6) with a coupling constant of 2 Hz, a methyl group at δ 1.88 (CH₃-6') and a methoxy group at δ 3.74 (OCH₃-5). These observations were in good accordance with published data for altenusin (Hassan, 2007). The substance

was first reported from *Alternaria* (Coombe et al. 1970), but later also from *Penicillium* species (Nakanishi et al. 1995).
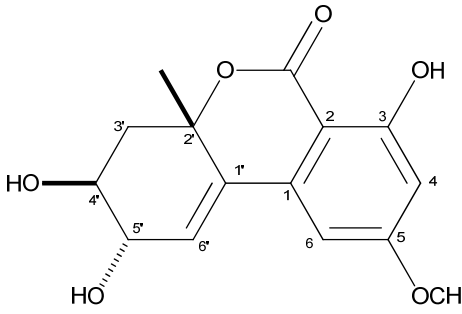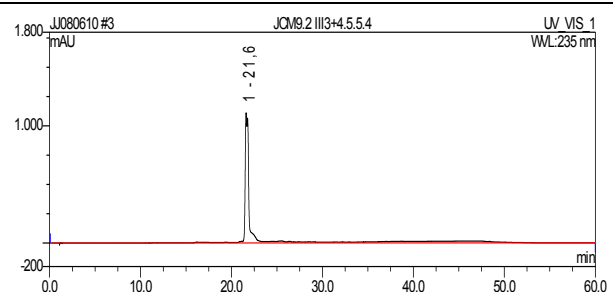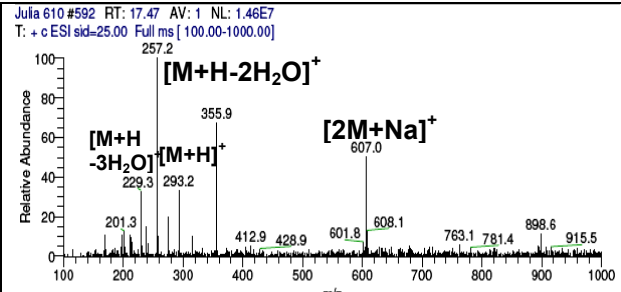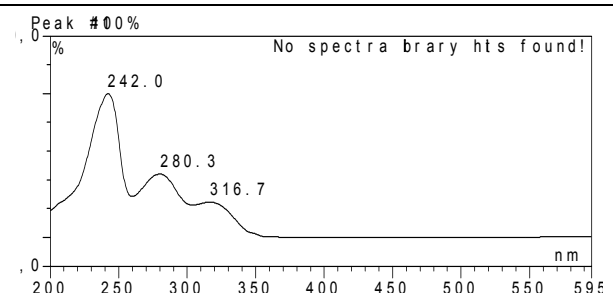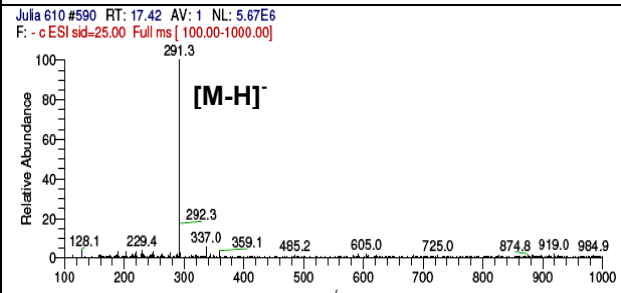
Table 3.5.3. NMR data of altenusin (**14**) at 500 MHz.

	14	**Literature**
	$\delta_H{}^a$ (*J* Hz)	$\delta_H{}^{a,b}$ (*J* Hz)
4	6.40 d (1.9)	6.42 d (2.5)
6	6.03 d (2.2)	6.15 d (2.5)
2'	6.47 s	6.47 s
5'	6.55 s	6.57 s
CH_3-6'	1.88 s	1.89 s
OCH_3-5	3.74 s	3.79 s

[a] in MeOD
[b] Hassan 2007

3.5.4 Altenuene (15, known compound)

Altenuene	
Synonym(s)	(2S,3S,4aS)-2,3,7-trihydroxy-9-methoxy-4a-methyl-4,4a-dihydro-2H-benzo[c]chromen-6(3H)-one; 2,3,4,4a-Tetrahydro-2,3,7-trihydroxy-9-methoxy-4a-methyl-6H-dibenzo[b,d]pyran-6-one
Sample code	JCM9.2III3+4.5.5.4
Biological source	*Alternaria* sp. from *Sonneratia alba*
Sample amount	3 mg
Physical description	beige powder
Molecular formula	$C_{16}H_{16}O_6$
Molecular weight	292 g/mol
Optical Rotation$[\alpha]_D^{20}$	-62° *(c* 0.02, CHCl$_3$)
Retention time HPLC	21.6 min (standard gradient)

Altenuene (**15**) was isolated as a light brown amorphous substance from the EtOAc extract of rice cultures of *Alternaria* sp. after fractionation over VLC, HP-20, RP-18 and final purification by semi preparative HPLC. It displayed an UV spectrum with maxima at 242, 280 and 317 nm. Positive and negative ESI-MS showed pseudomolecular ions at *m/z* 293.2 [M+H]$^+$ and 291.3 [M-H]$^-$, indicating a molecular weight of 292 amu. Furthermore, upon positive ionization loss of up to 3 molecules of water was observed, suggesting the presence of three hydroxyl groups in the molecule. In the ^{1}H NMR spectrum a methyl group at δ 1.50, a methoxy group at δ

3.87 and two *meta*-coupled aromatic protons at δ 6.47 (H-4) and δ 6.66 (H-6) are visible. Moreover, the spectrum revealed a spin system consisting of five protons. A vinyl proton at δ 6.22 (H-6') shared a coupling constant of 2.8 Hz with a carbinolic proton at δ 4.07 (H-5'). H-5' also showed a coupling of 6.0 Hz to the second carbinolic proton in the molecule, resonating at δ 3.78 (H-4'). From the coupling constants of the diastereotopic methylene protons CH_2-3' to H-4' their orientation was deduced. H-3'α (δ 2.41, 3.8 Hz) was found to be situated equatorial, while H-3'β (δ 1.97, 9.2 Hz) was oriented axial. These data were in accordance with previously published data for altenuene (see table 3.5.5) (Bradburn 1994, Hassan 2007), revealing the half-chair conformation of the molecule. The structure was furthermore confirmed by matching UV, MS and α_D data. Altenuene was first isolated from *Alternaria tenuis* and its configuration assigned by X-ray crystallographic data (McPhail 1973). Previously the compound was also described from a freshwater fungus belonging to the family Tubeufiaceae (Jiao 2006).

3.5.5 4'-Epialtenuene (16, known compound)

4'-Epialtenuene	
Synonym(s)	(2S,3R,4aS)-2,3,7-trihydroxy-9-methoxy-4a-methyl-4,4a-dihydro-2H-benzo[c]chromen-6(3H)-one
Sample code	JCM9.2III3+4.5.5.5
Biological source	*Alternaria* sp. from *Sonneratia alba*
Sample amount	2.5 mg
Physical description	beige powder
Molecular formula	$C_{16}H_{16}O_6$
Molecular weight	292 g/mol
Optical Rotation$[\alpha]_D^{20}$	-144° (*c* 0.02; CHCl$_3$)
Retention time HPLC	22.3 min (standard gradient)

4'-Epialtenuene (**16**) was isolated as a light brown amorphous substance from the EtOAc extract of rice cultures of *Alternaria* sp. after fractionation over VLC, HP-20, RP-18 and final purification by semi preparative HPLC. It displayed an UV spectrum with maxima at 245, 282 and 321 nm, similar to the spectrum of altenuene (**16**). Positive and negative ESI-MS showed pseudomolecular ions at *m/z* 293.2 [M+H]$^+$ and 291.3 [M-H]$^-$, in agreement with a molecular weight of 292 amu. Furthermore, upon positive ionization loss of up to 3 molecules of water was observed, suggesting the presence of three hydroxyl groups in the molecule and the molecular formula $C_{16}H_{16}O_6$, reminiscent of compound **16**. Compounds **15** and **16**

displayed very similar retention times and were only separable after several purification steps. In the ^{1}H NMR spectrum of purified **16**, a clear difference in comparison to **15** was observable. Whereas the ^{1}H NMR signals of the aromatic part and the methyl group were comparable, the ABX-type spin system comprising H-3'α, H-3'β and H-4' exhibited significantly different resonances and coupling constants, hinting at a difference in conformation. Whereas H-3'α was shifted upfield to δ 2.15 and showed a larger coupling of 12.4 Hz to H-4' (δ 3.73), H-3'β was shifted downfield to δ 2.25 and shared a smaller coupling constant with H-4' of 3.8 Hz, indicating axial orientation of H-4'. This was corroborated by comparison with previously reported NMR data for 4'-epialtenuene (Aly et al. 2008) and the observed difference in the optical rotation. 4'-Epialtenuene was reported before from an endophytic *Alternaria* strain, but only as an inseparable mixture with altenuene (Aly et al. 2008), so that the current work represents the first report of the isolated compound and its optical rotation.
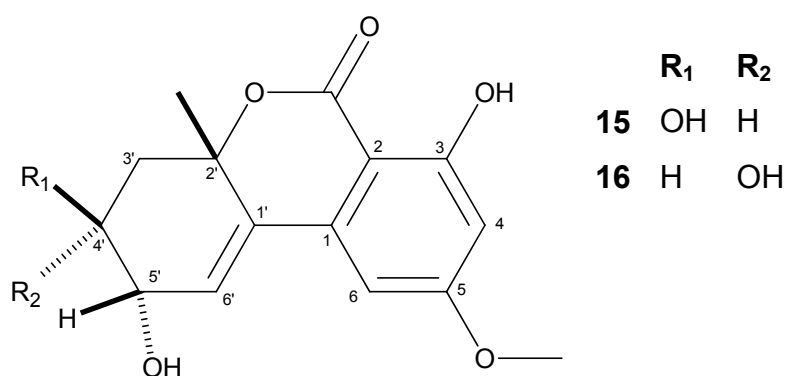
Table 3.5.5. NMR data of altenuene (**15**) and 4'-epialtenuene (**16**) at 500 MHz.

	15	**Literature**		**16**
		15	**16**	
	$\delta_H{}^a$ (*J* Hz)	$\delta_H{}^{a,b}$ (*J* Hz)	$\delta_H{}^{a,b}$ (*J* Hz)	$\delta_H{}^a$ (*J* Hz)
4	6.47 d (2.2)	6.45 d (2.2)	6.46 d (2.2)	6.48 d (2.2)
6	6.66 d (2.2)	6.64 d (2.2)	6.62 d (2.2)	6.65 d (2.2)
3'α	2.41 dd (3.8, 14.5)	2.40 dd (3.7, 14.5)	2.15 brt (12.3)	2.16 t (12.4)
3'β	1.97 dd (9.2, 14.5)	1.96 dd (9.1, 14.5)	2.25 dd (3.7, 11.9)	2.26 dd (3.8, 12.0)
4'	3.78 ddd (3.8, 6.0, 9.0)	3.77 ddd (3.7, 5.6, 9.1)	3.73 ddd (3.7, 8.2 12.3)	3.73 ddd (4.1, 8.2, 12.3)
5'	4.07 dd (2.8, 6.0)	4.06 dd (2.8, 5.6)	4.20 dd (2.5,8.2)	4.11 dd (2.3, 8.0)
6'	6.22 d (2.8)	6.21 d (2.8)	6.17 d (2.5)	6.18 d (2.2)
CH$_3$-2	1.50 s	1.49 s	1.54 s	1.55 s
OCH$_3$-5'	3.87 s	3.86 s	3.85 s	3.86 s

[a] in MeOD
[b] Hassan, 2008.

3.5.6 Alternarienonic acid (17, known compound)

Alternarienonic acid	
Synonym	2-hydroxy-6-(4-hydroxy-2-methyl-5-oxocyclopent-1-enyl)-4-methoxybenzoic acid
Sample code	JCM9.2III5+6.5
Biological source	*Alternaria* sp. (from *Sonneratia alba*)
Sample amount	31.3 mg
Physical description	beige substance
Molecular formula	$C_{14}H_{14}O_6$
Molecular weight	278 g/mol
Optical Rotation$[\alpha]_D^{20}$	+63°(*c* 1.0, MeOH)
Retention time HPLC	19.7 min (standard gradient)

Alternarienonic acid (**17**) was isolated from the EtOAc extract of rice cultures of *Alternaria* sp. as a brown solid substance. Its UV spectrum exhibited maxima at 231, 249 and 302 nm. Besides pseudomolecular ions at *m/z* 279.1 [M+H]+ and 277.1 [M-H]-, indicative of a molecular weight of 278 amu, ESI-MS showed loss of water in the positive ionization and decarboxylation in the negative ionization mode, hinting at the presence of hydroxyl and carboxyl groups in the compound. This was affirmed by ^{1}H NMR measurement in DMSO-d_6, where one alcohol group was observed at δ 5.38 (OH-4') that showed a small coupling with a carbinolic proton resonating at δ 4.23 (H-4'). Moreover, two methylene protons at δ 2.82 and δ 2.37 attributed to H-3'A and H-

3'B and a signal for a methyl group at δ 1.82 were visible. 2D NMR experiments allowed assignment of the methyl group at C-2 because of the correlation of its protons to C-2 as found in the HMBC spectrum and to the methylene protons H_2-3 as observed in the ROESY spectrum. The two *meta*-coupled aromatic protons at δ 5.79 (H-4) and δ 6.21 (H-6) and a methoxy group at δ 3.68 resembled the aromatic part of compound **14**, although the signal for OH-7 was missing. All spectroscopic data as well as retention time at standard HPLC conditions fitted very well with data reported for alternarienonic acid. This metabolite was first described by our group from an endophytic *Alternaria* sp. isolated from *Polygonum senegalense* (Aly et al. 2008).

Table. 3.5.6. NMR data of alternarienonic acid (**17**) at 500 MHz.
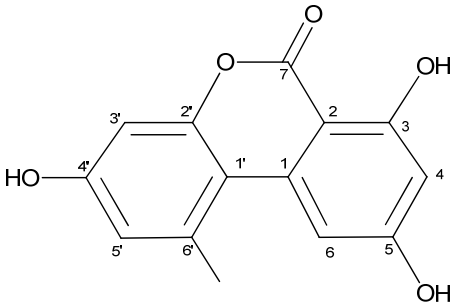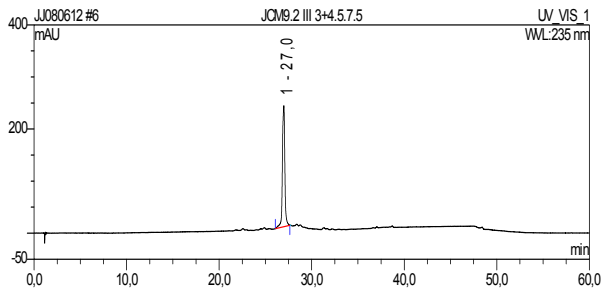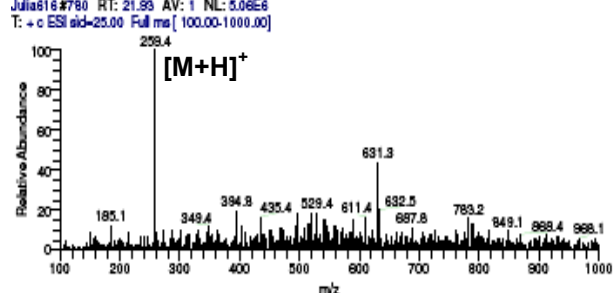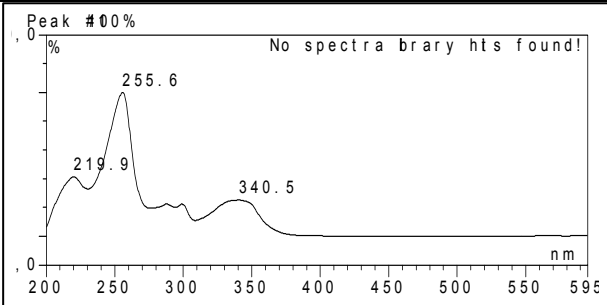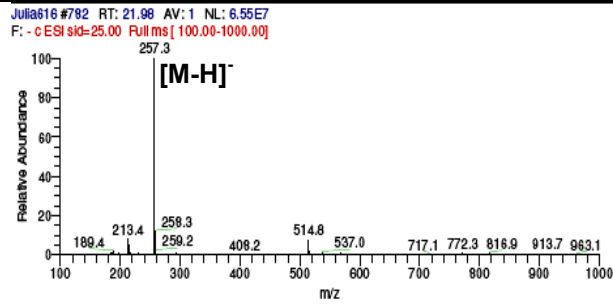
	17				Literature	
	$\delta_H{}^a$ (*J* Hz)	$\delta_C{}^b$	HMBC[a] (H→C)	ROSY[a]	$\delta_H{}^c$ (*J* Hz)	$\delta_C{}^c$
1						164.3
2		111				109.9
3						137.3
4	5.79 brs	106	2		6.07 d (2.5)	110.2
5		161				164.3
6	6.21 brs	100	2		6.40 d (2.5)	101.4
7						166.0
1'		142				142.5
2'		159				165.2
3'	A: 2.82 dd (7.3, 17.3)	40	4'	3'B, 4', CH₃-2'	A: 3.00 dd (6.9, 17.3)	41.8
	B: 2.37 brd (15.5)		2', 4'	3'A, 4', CH₃-2'	B: 2.49 brd (17.0)	
4'	4.23 brs	72		3'A, 3'B, OH-4'	4.38 dd (2.8, 6.3)	73.2
5'						209.0
CH₃-2'	1.82 s	17	1', 2'	3'A, 3'B	1.99 s	17.8
OCH₃-5	3.68 s	55	5		3.78 s	55.8
OH-7						
OH-4'	5.38 d (4.1)			4'		

[a] in DMSO-d_6
[b] confirmed from HMBC
[c] MeOD, Hassan, 2008.

3.5.7 Alternariol (18, known compound)

Alternariol	
Synonym(s)	3,7,9-Trihydroxy-1-methyl-[6H]-dibenzo(b,d)pyran-7-one
Sample code	JCM9.2III3+4.7.5, 92IX-XIIf
Biological source	*Alternaria* sp. (from *Sonneratia alba*)
Sample amount	38.5 mg
Physical description	light orange powder
Molecular formula	$C_{14}H_{10}O_5$
Molecular weight	258 g/mol
Retention time HPLC	27.0 min (standard gradient)

Alternariol was isolated from the EtOAc extracts from cultures of *Alternaria* sp. after fermentation both on solid rice and in liquid Wickerham medium as a light orange substance. Its UV spectrum revealed three maxima at 220, 256 and 341 nm. Upon positive and negative ionization, pseudomolecular ions at *m/z* 259.4 $[M+H]^+$ and 257.3 $[M-H]^-$, consistent with a molecular weight of 258 amu, were detected. The ^{1}H NMR spectrum revealed five peaks, one indicative of a methyl group adjacent to an aromatic carbon at δ 2.64 (CH_3-6') and two pairs of *meta*-coupled aromatic protons. The first pair showed resonances at δ 7.26 and δ 6.36 and a coupling constant of 2.0 Hz, representing H-6 and H-4, and the second pair was detected at δ 6.70 and δ 6.71 with a slightly larger coupling constant of 2.7 Hz, consisting of H-5'

and H-3'. The spectral data were found to be identical to published data of alternariol (Raistrick et al. 1953, Coombe et al. 1970, Hassan 2007) and thus proved the structure of compound **18**. Alternariol was first reported from *Alternaria tenuis* but since then described from several *Alternaria* species (Raistrick et al. 1953, Coombe et al. 1970, Bradburn et al 1994).

3.5.8 Alternariol-5-methylether (19, known compound)

Alternariol-5-methylether	
Synonym(s)	3,7,9-Trihydroxy-1-methyl-[6H]-dibenzo(b,d)pyran-7-one, djalonensone
Sample code	92III+VIi
Biological source	*Alternaria* sp. (from *Sonneratia alba*)
Sample amount	6.7 mg
Physical description	yellow-orange substance
Molecular formula	$C_{15}H_{12}O_5$
Molecular weight	272 g/mol
Retention time HPLC	30.4 min (standard gradient)

Alternariol-5-methylether (**19**) could be isolated as a light orange substance from the EtOAc extract from liquid cultures of *Alternaria* sp. Its UV spectrum revealed five maxima at 218, 257, 287, 298 and 340 nm. Upon positive and negative ionization, pseudomolecular ions at *m/z* 273.4 [M+H]$^+$ and 271.3 [M-H]$^-$, suggesting a molecular weight of 272 amu, were detected. Both MS spectra showed high similarities with the ones of alternariol, with a mass difference of 14 amu, indicative of an additional methyl group. The ^{1}H NMR spectrum corroborated this suggestion, because as described above for alternariol, again two pairs of *meta*-coupled aromatic protons, a methyl group and an additional signal of a methoxy group (δ 3.92, OCH$_3$-5') were detected. The observed spectral data fitted also to results previously described for alternariol-5-methylether (Onocha et al. 1995, Hassan 2007). This

metabolite, which is also known as djalonensone, has been described from several *Alternaria* species as well as from the African medicinal plant *Anthocleista djalonensis* (Onocha et al. 1995).
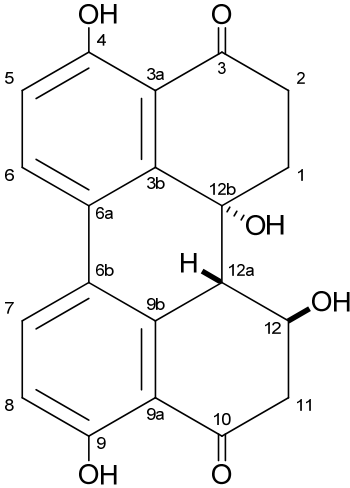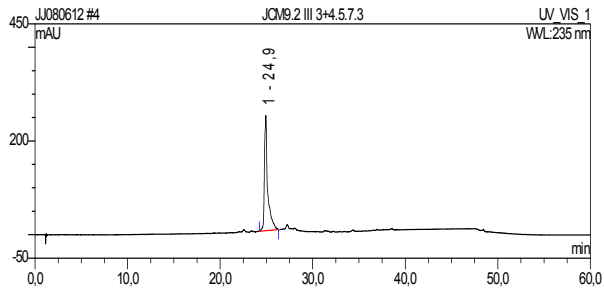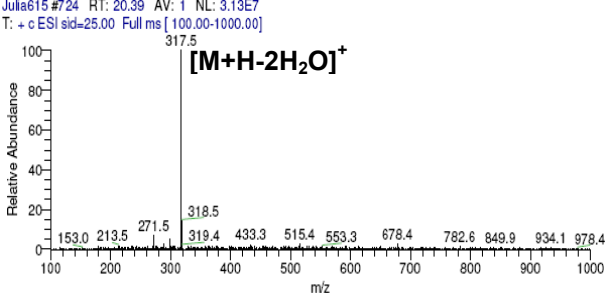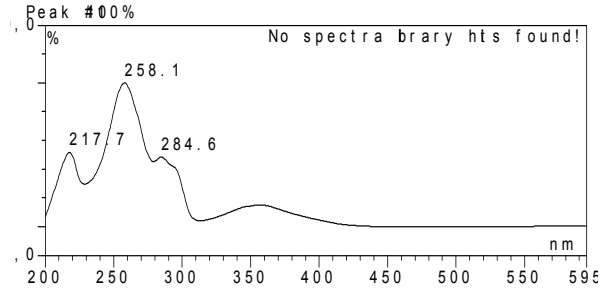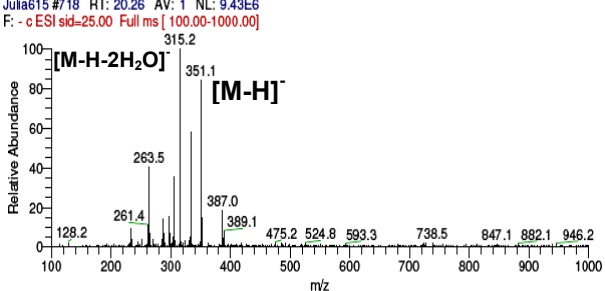
Table 3.5.8. NMR data of alternariol (**18**) and alternariol-5-methylether (**19**) at 500 MHz.

| | **18** | **Literature** | | **19** |
| | | **18** | **19** | |
	$\delta_H{}^a$ (*J* Hz)	$\delta_H{}^{a,\,b}$ (*J* Hz)	$\delta_H{}^{a,\,b}$ (*J* Hz)	$\delta_H{}^a$ (*J* Hz)
4	6.36 d (2.2)	6.32 d (2.0)	6.54 d (1.8)	6.56 d (2.2)
6	7.26 d (1.9)	7.20 d (2.0)	7.28 d (1.8)	7.30 d (2.1)
3'	6.61 d (2.8)	6.55 d (2.5)	6.61 d (2.5)	6.62 d (2.5)
5'	6.70 d (2.6)	6.65 d (2.5)	6.70 d (2.5)	6.71 d (2.3)
CH_3-6'	2.64 s	2.71 s	2.76 s	2.77 s
OCH_3-5'			3.91 s	3.92 s

[a] in MeOD
[b] Hassan, 2008.

3.5.9 Altertoxin I (20, known compound)

Altertoxin I	
Synonym(s)	(1S,12aR,12bS)-1,4,9,12a-tetrahydroxy-1,2,12,12a-tetrahydroperylene-3,10(11H,12bH)-dione, Dihydroalterperylenol
Sample code	JCM9.2 III 3+4.5.7.3, 92IIIgV
Biological source	*Alternaria* sp. from *Sonneratia alba*
Sample amount	4.6 mg
Physical description	maroon powder
Molecular formula	$C_{20}H_{16}O_6$
Molecular weight	352 g/mol
Optical Rotation$[\alpha]_D^{20}$	+365 (*c* 0.01, MeOH)
Retention time HPLC	24.9 min (standard gradient)

Similar to alternariol, Altertoxin I was isolated from the EtOAc extracts from cultures of *Alternaria* sp. after fermentation both on solid rice and in liquid Wickerham medium. The UV spectrum of the reddish colored substance exhibited three maxima at 218, 258 and 285 nm. Upon negative ionization, a pseudomolecular ion at *m/z*

351.1 [M-H]⁻ indicative of a molecular weight of 258 amu, was detected. Facile loss of water upon positive and negative ionization implied the presence of at least two hydroxyl groups in the molecule. The ¹H NMR spectrum revealed two sets of *ortho*-coupled aromatic protons (δ 7.05/8.07 and 8.00/6.95; H-5/H-6 and H-7/H-8, respectively), two hydrogen-bonded phenolic protons (δ 12.32, OH-9 and δ 12.72, OH-4), two hydroxyl signals (δ 5.27, OH-12 and δ 5.35, OH-12b) and six protons resonating at δ 2.30 – 3.10. The positioning of the protons was deduced partly from their coupling constants and mainly by interpretation of two dimensional NMR experiments. COSY experiment allowed the assignment of the four spin systems H-1α/H-1β/H-2α/H-2β, H-5/H-6, H-7/H-8 and H-11α/H-11β/H-12. In the ROESY spectrum correlations of OH-4 to H-5 and OH-9 to H-8 afforded the position of the hydrogen bonded protons, and correlations of H-6 to H-7 and H-1β to H-12 the connection of the ring system, which was also confirmed by HMBC correlations. Moreover, the relative stereochemistry could be established from the ROESY spectrum. H-1α, H-2α, H-11α, H-12α and OH-12 are situated on one face of the molecule, H-1β, H-2β, H-11β and H-12 at the opposite face. The optical rotation as well as the other spectral data were in good accordance with literature data for altertoxin I (Stack et al. 1986, Hassan 2007). Hence, the structure of compound **20** was confirmed. This fungal metabolite was previously described from several *Alternaria* species, primarily from *Alternaria mali* (Pero et al. 1973).
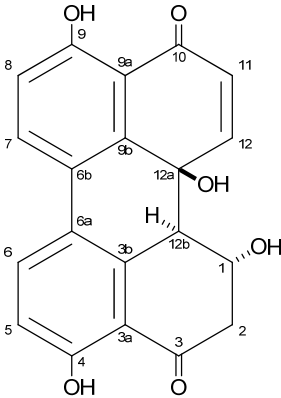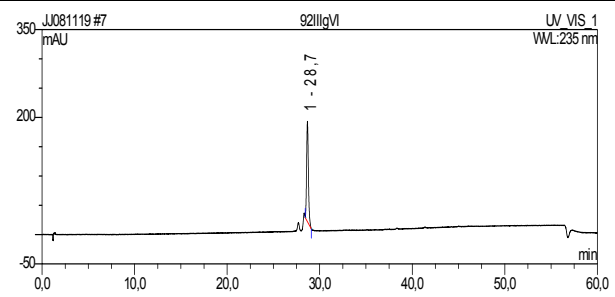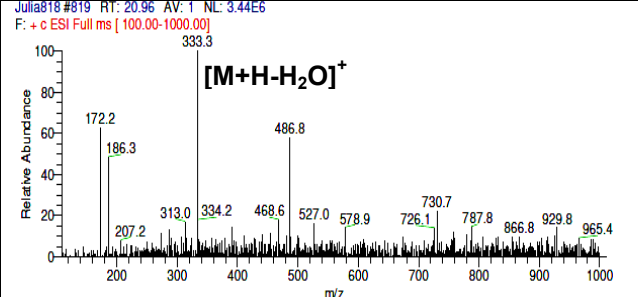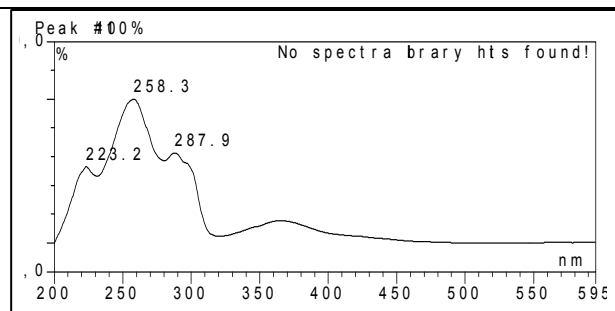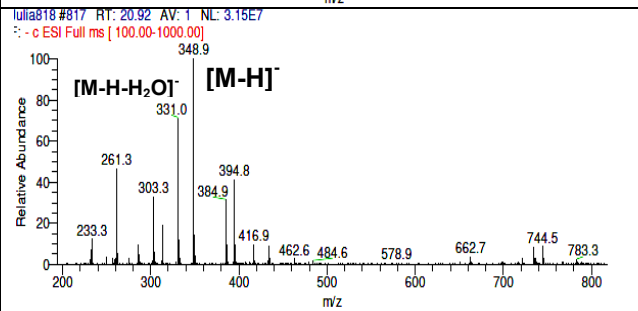
Table 3.5.9. NMR data of altertoxin I (**20**) at 400 MHz.

		20				**Literature**	
	δ$_H$^{a} (*J* Hz)	δ$_C$^{a}	COSY^a	ROESY^a	HMBC^a	δ$_H$^{a,b} (*J* Hz)	δ$_C$^{a,b}
1α	2.56 t (3.2)		1β, 2α, 2β	2α	3,, 3a	2.30 dt	
1β	2.98 ddd (4.0; 8.0; 15.0)	34.7	1α, 2α, 2β	2β, 12	3, 3b, 12	ca. 3 m	34.8
2α	3.1 dq (4.6; 17.5)	33.4	1α, 1β, 2β	1α	3, 1	ca. 3 m	33.5
2β	2.30 td (14.3; 3.5)		1α, 1β, 2α	1β		2.59 dt	
3		206.0					206
3a		113.7					113.8
3b		140.7					138.4
4		160.9					161.0
5	7.05 d (8.7)	117.8	6	OH-4	3a, 4, 6a		117.8
6	8.07 d (8.8)	132.6	5		3a, 3b, 4, 6b,	7.1 d	132.9
6a		123.4				8.1 d	123.5
6b		124.7					124.8
7	8.00 d (8.7)	132.6	8		6a, 9, 9a, 9b	8.0 d	132.5
8	6.95 d (8.6)	115.5	7	OH-9	6b, 9, 9a	6.9 d	115.5
9		160.4					160.4
9a		116.5					116.5
9b		138.3					140.7
10		204.1					204.2
11α	2,96 m	47.4	11β, 12			ca. 3 m	47.5
11β	2.86 td (4.6;15.8)		11α, 12	1β, 12	12	2.86 m	
12	4.54 m	64.6	11α, 11β, 12a	1β, 11β		4.5 m	64.7
12a	2.97 d (8.9)	51.3	12	6b, 9a, 9b, 12, 12b			51.4
12b		67.6				2.86 m	68.0
OH-4	12.72 s			5	3a, 4, 5	12.7	
OH-9	12.32 s			8	8, 9	12.3	
OH-12	5.27 brs						
OH-12b	5.35 brs						

^{a}measured in DMSO-*d$_6$*
^{b}Stack et al. 1986

3.5.10 Alterperylenol (21, known compound)

Alterperylenol	
Synonym(s)	(12R,12aR,12bS)-4,9,12,12b-tetrahydroxy-12,12a-dihydroperylene-3,10(11H,12bH)-dione, Alteichin
Sample code	92IIIgVI
Biological source	*Alternaria* sp. (from *Sonneratia alba*)
Sample amount	1.3 mg
Physical description	red substance
Molecular formula	$C_{14}H_{10}O_5$
Molecular weight	350 g/mol
Optical Rotation$[\alpha]_D^{20}$	-145° (*c* 0.01, MeOH)
Retention time HPLC	28.1 min (standard gradient)

Alterperylenol was obtained after several purification steps from the EtOAc extract of liquid cultures of *Alternaria* sp. The red pigment showed an UV spectrum with maxima at 223, 258 and 288 nm. ESI-MS revealed a pseudomolecular ion peak at *m/z* 348.9 [M-H]⁻ upon negative ionization, indicative of a molecular weight of 350 amu. As facile loss of water was observed both upon negative and positive ionization, the molecule was expected to contain several hydroxyl groups. Both UV and mass spectra indicated a structural analogy to altertoxin I (**20**) with a difference of 2 mass

units. This was confirmed by ^{1}H NMR and two dimensional NMR experiments. Two sets of *ortho*-coupled aromatic protons and two hydrogen bonded protons were observed, similar to the aromatic part of altertoxin I. The carbinolic proton H-1 and the protons H-2α and H-2β were equal to H-12, H-11α and H-11β. Instead of four aliphatic protons, only two protons, shifted downfield in comparison to **20**, were attached at C-11 (δ 6.34, H-11) and C-12 (δ 7.89, H-12) and exhibited a coupling constant of 10.4 Hz, consistent with a *cis*-double bond. The relative stereochemistry could be deduced after close examination of the ROESY spectrum. H-1 (δ 4.51) showed correlations to both hydroxyl groups OH-1 (δ 5.71) and OH-12b (δ 5.73), but not to H-12b (δ 3.11). All data were in accordance with literature data of the plant pathogenic and antifungal metabolite alterperylenol, also known as alteichin, which was previously reported from several *Alternaria* species (Okuno et al. 1983, Robeson et al. 1984, Stack et al. 1986, Hradil et al. 1989). Comparison with literature data is also the reason for the inconsistent numbering of the similar compounds **20** and **21**.

Table 3.5.10. NMR data of alterperylenol (**21**) at 400 MHz.

	21			**Literature**	
	$\delta_H{}^a$ (*J* Hz)	**COSY**[a]	**NOESY**[a]	$\delta_H{}^{a,b}$ (*J* Hz)	$\delta_H{}^{a,c}$ (*J* Hz)
1	4,51 m	2α, 2β, 12b, OH-1	12, OH-1, OH-12a	4,74 td (10, 5)	4.50 m
2α	2.95 d (11.9)	1, 2β		3.01 m	2.97 dd (16, 6.3)
2β	2.90 dd (5.0; 15.8)	1, 2α	1	3.26 t (10)	2.89 dd (16, 11.7)
5	7.06 d (8.7)	6		7.04 d (8.7)	7.06 d (8.8)
6	8.02 d (8.7)	5		8.01 d (8.7)	8.02 d (8.8)
7	8.08 d (8.6)	8		8.05 d (8.7)	8.08 d (8.8)
8	6.96 d (8.5)	7		6.95 d (8.7)	6.96 d (8.8)
11	6.34 d (10.4)	12		6.31 d (10)	6.35 d (10.5)
12	7.89 d (10.4)	11	1, 11, OH-1, OH-12a	8.04 d (10)	7.89 d (10.5)
12b	3.11 d (9.5)			3.01	3.10 dd (9.7, 1.7)
OH-1	5.71 brs		1, 12		5.73 d (6.3)
OH-4	12.39 s			12.4	12.36 s
OH-9	12.47 s			12.5	12.72 s
OH-12a	5.73 s		1, 12		5.76 d (1.7)

[a]measured in DMSO-d_6
[b]Robeson et al. 1984
[c]Okuno et al. 1983

3.5.11 Stemphyperylenol (22, known compound)

Stemphyperylenol	
Synonym(s)	(1R,6bR,7R,12bR)-1,4,7,10-tetrahydroxy-1,2,7,8-tetrahydroperylene-3,9(6bH,12bH)-dione
Sample code	92IIIgVII
Biological source	*Alternaria* sp. (from *Sonneratia alba*)
Sample amount	5 mg
Physical description	dark red powder
Molecular formula	$C_{20}H_{16}O_6$
Molecular weight	252 g/mol
Optical Rotation$[\alpha]_D^{20}$	+39° (*c* 0.1, MeOH)
Retention time HPLC	28.9 min (standard gradient)

Stemphyperylenol was obtained as a red pigment from the EtOAc extract of liquid cultures of *Alternaria* sp. Its UV spectrum exhibited maxima at 229, 262 and 342 nm. Pseudomolecular ions were observed at *m/z* 365.2 [M+Na]$^+$ and 351.0 [M-H]$^-$ upon positive and negative ionization, implying a molecular weight of 352 amu. The substance was found to be a symmetrical dimer because the ^{1}H NMR and ^{13}C

NMR spectra only revealed signals for eight protons and ten carbons. A pair of aromatic protons (δ 8.04, H-6/H-12 and δ 6.86, H-5/H-11) exhibited an *ortho*-coupling (J = 8.7 Hz), while a chelated phenolic hydroxyl proton (OH-4/OH-10) resonanted at δ 12.02, and another hydroxyl proton at δ 3.3 (OH-1/OH-7) was part of an ABMXY spin system comprising signals at δ 4.60 (H-1/H-7), δ 3.73 (H-6b/H-12b), δ 3.15 (H-2b/H-11b) and δ 2.92 (H-2a/H-11a). The spin systems that were deduced from the ^{1}H NMR spectrum could be connected after examination of COSY, HMBC and ROESY spectra. ^{13}C NMR also revealed typical signals of a reduced perylenequinone derivative with similarities to altertoxin I (**20**). Comparison of the obtained results with literature data identified compound **22** as stemphyperylenol, a metabolite that has been described from *Stemphylium botryosum* and several *Alternaria* species (Arnone et al. 1986, Hradil et al 1989).

Table 3.5.11. NMR data of stemphyperylenol (**22**) at 400 MHz.

				22				**Literature**	
	$\delta_H{}^a$ (*J* Hz)	$\delta_H{}^b$ (*J* Hz)	COSY[b]	HMBC[b]	$\delta_C{}^a$	$\delta_C{}^b$	NOESY[b]	$\delta_H{}^{c,d}$ (*J* Hz)	$\delta_C{}^{a,d}$
1	4.63 d (8.8)	4.60 m	2α, 2β, 12b		68.2	67.0	2β,11, 12	4.76	68.32
2α 2β	3.01 d (3.2)	2.92 dd (4.5; 15.9) 3.15 d (12.7)	1, 2β 1, 2α	1, 3, 3a, 12b 1, 3, 12b	47.4	47.5	1 12b	3.07 3.17	47.84
3						201.5			204.01
3a					115.8	115.5			115.92
3b					144.2	143.5			
4					161.4	159.5			161.03
5	6.61 d (8.8)	6.86 d (8.7)	6	3a, 3b, 4, 6a	115.8	115.0	6b, 7	6.81	115.55
6	8.03 d (8.8)	8.04 d (8.7)	5	3a, 3b, 4, 6b	135.4	135.5	6b, 7, OH-7	8.14	135.58
6a					131.3	130.0			130.90
6b	3.70 d(8.9)	3.73 d (8.9)	7	7, 6a, 9b	45.9	45.0	5, 6, 8β	3.75	46.05
7	4.63 d (8.8)	4.60 m	6b, 8α, 8β		68.2	67.0	8α	4.76	68.32
8α 8β	3.01 d (3.2)	2.92 dd (4.5; 15.9) 3.15 d (12.7)	7, 8β 7, 8α	6b, 7, 9,9a 6b, 7, 9	47.4	47.5	7 6b	3.07 3.17	47.84
9						201.5			204.01
9a					115.8	115.5			115.92
9b					144.2	143.5			143.73
10					161.4	159.5			161.03
11	6.61 d (8.8)	6.86 d (8.7)	12	9b, 10, 12a, 12b	115.8	115.0	1, 12b	6.81	115.55
12	8.03 d (8.8)	8.04 d (8.7)	11	9a, 9b, 10, 12b	135.4	135.5	1, OH-1, 12b	8.14	135.58
12a					131.3	130.0			130.9
12b	3.70 d(8.9)	3.73 d (8.9)	1	1, 3b, 12a	45.9	45.0	1α, 11, 12	3.75	46.05
OH-1		3.3					12	4.97	
OH-4		12.02						12.09	
OH-7		3.3					6	4.97	
OH-10		12.02						12.09	
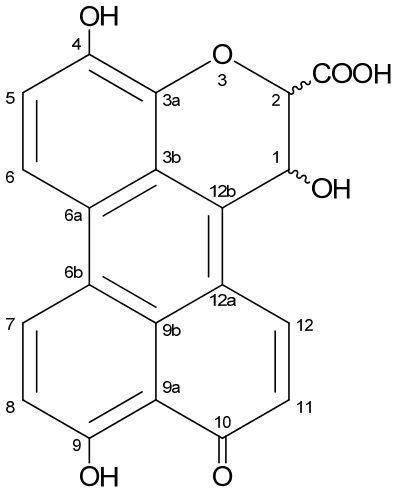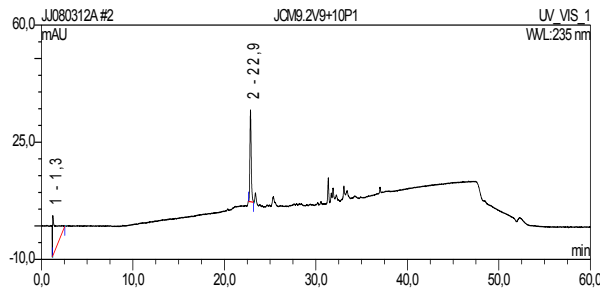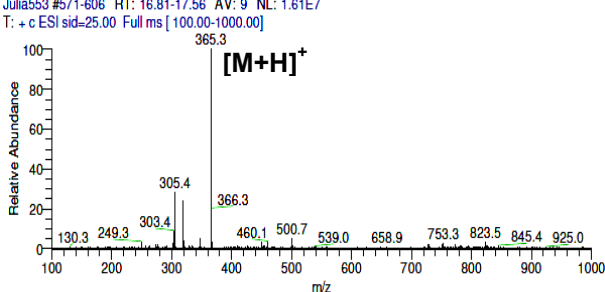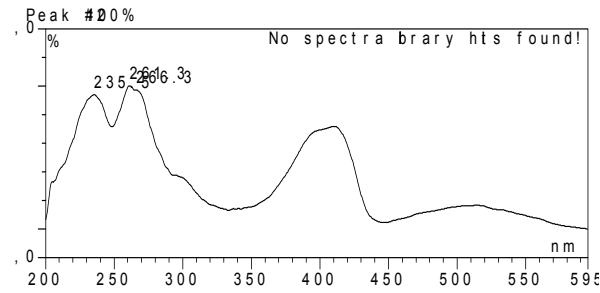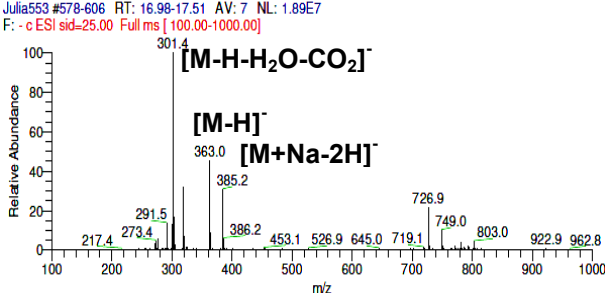
[a]measured in MeOD
[b]measured in DMSO-d_6 at 300 MHz
[c]in acetone-d_6
[d]Arnone et al.1986
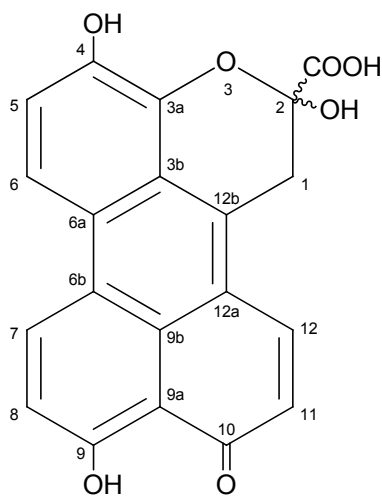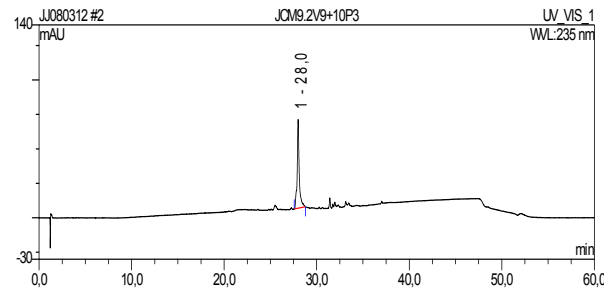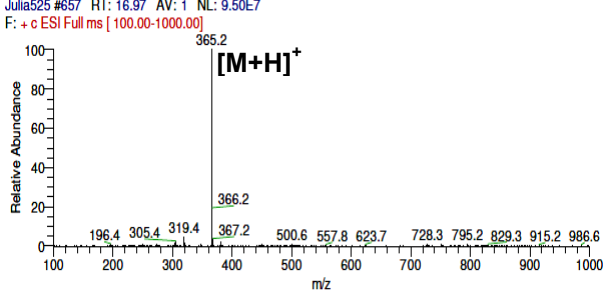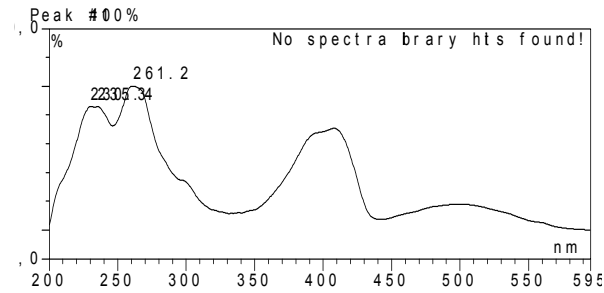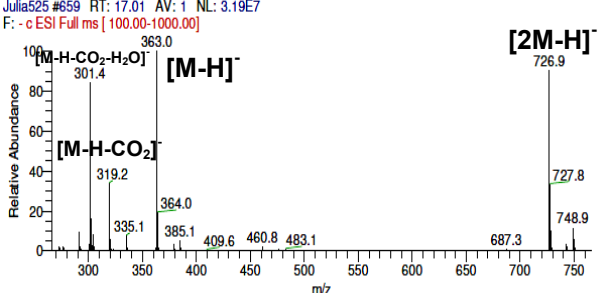
3.5.12 Xanalteric acid I (23, new compound)

Xanalteric acid I	
Synonym(s)	1,4,9-trihydroxy-10-oxo-2,10-dihydro-1H-phenaleno[1,2,3-de]chromene-2-carboxylic acid
Sample code	JCM9.2V8.3.7; JCM9.2V9+10.1
Biological source	*Alternaria* sp. (from *Sonneratia alba*)
Sample amount	4.6 mg
Physical description	dark red pigment
Molecular formula	$C_{14}H_{10}O_5$
Molecular weight	364 g/mol
Optical Rotation$[\alpha]_D^{20}$	-117° (*c* 0.03, MeOH)
Retention time HPLC	22.9 min (standard gradient)

Xanalteric acid I was obtained from the EtOAc extracts of *Alternaria* sp. after fermentation on solid rice medium, and it was also identified in the EtOAc extract of the liquid medium. The UV/Vis spectrum of the red pigment exhibited maxima at 236, 261, 266, 413 nm and 514 nm. Taken together with the molecular formula $C_{20}H_{12}O_7$ as determined by HRESIMS (*m/z* 365.0656 [M+H]⁺, calcd. for $C_{20}H_{13}O_7$ 365.0661),

corresponding to fifteen double bond equivalents, these data suggested that it featured a complex aromatic ring system. This was in accordance with three sets of *ortho*-coupled aromatic protons (δ 7.46/8.24, 9.00/7.38 and 6.94/8.74; H-5/H-6, H-7/H-8 and H-11/H-12, respectively) that were observed in the ^{1}H NMR spectrum. Two vicinally coupled protons at δ 6.17 and 5.06 (H-1/H-2) formed a fourth spin system. When the ^{1}H NMR spectrum was measured in DMSO-d_6, four hydroxyl protons (δ 3.3, 5.75, 8.73 and 15.12; OH-1, OH-4, COOH and OH-9) were detected. The striking downfield shift of OH-9 was typical of a strong hydrogen bond, which is in accordance with the downfield shift of C-10 (δ 191.0) as observed in the ^{13}C NMR spectrum that was indicative of an aromatic carbonyl group. The COSY spectrum revealed the correlation between OH-1 (overlapping with the water peak in the ^{1}H NMR spectrum) and H-1 (δ 5.93). HMBC data allowed the connection of the different spin systems to be deduced. The quaternary carbons C-9a (δ 112.9) and C-9b (δ 126.4) showed correlations to the aromatic doublets H-8 (δ 7.38) and H-11 (δ 6.94), and H-7 (δ 9.00) and H-12 (δ 8.74), respectively. Moreover, H-7 and H-5 (δ 7.46) showed correlations with C-6a (δ 126.9), whereas H-8 and H-6 (δ 8.24) correlated with C-6b (δ 122.9). Both H-6 and H-1 exhibited correlations to C-3b**.** The correlations of H-11 and H-1 to C-12a, and of H-12 and H-2 to C-12b, respectively, provided the remaining connections of the five ring core structure of **23**. NOESY correlations that were observed between the aromatic protons H-6 and H-7, and between H-12 and H-1 confirmed this result. The presence of oxygen in the heterocyclic ring system was corroborated by the downfield shifts of the aromatic carbons C-3a (δ 140.6) and C-2 (δ 81.7). The presence of a carboxyl group at C-2 was indicated by the downfield shift of its carbonyl carbon (δ 174.7) which correlated with H-1 as well as with H-2 in the HMBC spectrum. ESI-MS/MS experiments supported this structural feature by the observed facile decarboxylation of **23** in the negative as well as in the positive ionization mode.

3.5.13 Xanalteric acid II (24, new compound)

Xanalteric acid II	
Synonym(s)	2,4,9-trihydroxy-10-oxo-2,10-dihydro-1H-phenaleno[1,2,3-de]chromene-2-carboxylic acid
Sample code	JCM9.2V9+10.3, JCM9.2V9+11.2
Biological source	*Alternaria* sp. (from *Sonneratia alba*)
Sample amount	5 mg
Physical description	dark red substance
Molecular formula	$C_{14}H_{10}O_5$
Molecular weight	364 g/mol
Optical Rotation$[\alpha]_D^{20}$	+43° (*c* 0.019, MeOH)
Retention time HPLC	27.0 min (standard gradient)

Xanalteric acid II, also obtained as a red powder, displayed physical characteristics comparable to those of compound **23**. The HRESIMS again exhibited a peak at *m/z* 365.0656 [M+H]$^+$, and in the MS/MS experiment a facile decarboxylation of the compound was observed. ^{1}H-NMR and HMBC spectra revealed very similar signals for the three spin systems of the aromatic protons H-

5/H-6, H-7/H-8 and H-11/H-12 as found for xanalteric acid I (**23**), whereas the fourth spin system due to its upfield shift (compared to **23**, see table 3.5.13) and the large coupling constant of 17.3 Hz indicated the presence of geminal protons. This observation was confirmed by a HMQC experiment, which displayed correlations of protons H-1A and H-1B to C-1 at δ_C 34.8. Accordingly, **24** (2,4,9-trihydroxy-10-oxo-2,10-dihydro-1*H*-phenaleno[1,2,3-*de*]chromene-2-carboxylic acid) is an isomer of **23**, for which the name xanalteric acid II was proposed.

Table 3.5.13. NMR data for xanalteric acid I (**23**) and II (**24**).

	21						22			
	δ_H (*J* Hz)[a]	δ_H (*J* Hz)[b]	COSY[b]	HMBC[a]	δ_C mult.[a]	NOESY[a]	δ_H (*J* Hz)[a]	δ_H (*J* Hz)[b]	HMBC	δ_C mult.[a]
1	6.17 d (3.0)	5.93 d (2.3)	2, 1-OH	3b, 12a, 12b, COOH-2	65.1 CH	12	A: 4.22 d (17.3) B: 3.66 d (17.3)	A: 3.88 d (18.3) B: 3.71 d (18.0)	3b, 2, 12a, 12b 2, 3b, 9b, 12a, 12b, COOH-2	34.8 CH$_2$
2	5.06 d (3.0)	4.74 brs	1	1, 3a, 12b, COOH-2	81.7 CH					97.0 qC
3a					140.6 qC					138.8 qC
3b					119.3 qC					120.0 qC
4					143.6 qC					144.4 qC
5	7.46 d (9.1)	7.35 d (7.4)	6	3a, 4, 6a	123.3 CH		7.43 d (8.8)	7.42 d (8.9)	3a, 4, 6a	122.8 CH
6	8.24 d (8.8)	8.16 d (9.3)	5	3a, 3b, 4, 6a, 6b	115.9 CH	7	8.24 d (9.1)	8.24 d (8.8)	3b, 4, 6b, 12b	116.3 CH
6a					126.9 qC					126.7 qC
6b					122.9 qC					122.1 qC
7	9.00 d (9.1)	9.04 d (7.5)	8	6a, 6b, 9, 9a, 9b, 12a	134.0 CH	6	8.98 d (9.5)	9.07 d (8.6)	6a, 9, 9b	134.1 CH
8	7.38 d (9.1)	7.35 d (7.4)	7	6b, 9, 9a, 9b	119.6 CH		7.38 d (9.1)	7.37 d (9.2)	6b, 9, 9a	118.7 CH
9					167.1 qC					167.4 qC
9a					112.9 qC					113.2 qC
9b					126.4 qC					126.7 qC
10					191.0 qC					190.5 qC
11	6.94 d (10.0)	6.94 d (9.4)	12	9, 9a, 9b, 12a	127.3 CH		6.92 d (9.8)	6.89 d (9.8)	9a, 12a	126.9 CH
12	8.74 d (10.1)	8.62 d (7.3)	11	6b, 9a, 9b, 10, 12a, 12b	140.4 CH	1	8.53 d (10.0)	8.62 d (9.8)	9b, 10, 12a, 12b	139.7 CH
12a					121.6 qC					122.0 qC
12b				3b, 12a, 12b, COOH-2	137.1 qC					138.7 qC
OH-1		3.3	1							
OH-2										
COOH-2		8.73 s			174.7			9.35 s		174.7
OH-4		5.75 s						5.75 s		

[a] in MeOD [b] in DMSO-*d$_6$*

3.5.14 Bioactivity test results for compounds isolated from *Alternaria* sp.

The isolated compounds were subjected to cytotoxicity and antibacterial assays. Table 3.5.14 gives an overview on the results. No compound exhibited strong cytotoxicity against the tested cell line, only the new metabolite xanalteric acid I (**23**) inhibited the growth of the cells moderately.

Antibiotic properties were more prevalent among the isolated substances. Altenusin (**14**) exhibited good inhibition against *E. faecium*, *S. aureus and S. pyogenes* with MIC of 31.25 to 62.5 μg/mL. *S. aureus* was also found to be sensitive towards alternarienonic acid (**17**) and xanalteric acid I (**23**) and II (**24**).

Table 3.5.14. Biological screening test results for isolated compounds of *Alternaria* sp.

Compound	L5178Y survival rate in % (@ 10 μg/mL)	Survival rate in % (@ 312 μg/mL / <u>MIC</u> [μg/mL])						Conc. exhibiting prophylactic effect against viral infection [μg/mL]		
		E. c.	K.p.	E. f.	S. a.	S. p.	P.a.	HRV2	HRV8	HRV16
2,5-Dimethyl-7-hydroxychromone (**11**)		75	70	90	45	60	75			
Alternarian acid (**13**)	99.2	80	65	90	85	65	65			
Altenusin (**14**)		90	75	95/ <u>62.5</u>	25/ <u>31.25</u>	10/ <u>62.5</u>	75	>3.9	>3.9	>7.8
Altenuene (**15**)		95	70	100	90	70	85			
5'-Epialtenuene (**16**)		85	100	95	90	80	80			
Alternarienonic acd (**17**)		75	80	100	35	55	95			
Alternariol (**18**)		95	100	100	100	90	80			
Alternariol-5-methylether (**19**)		90	50	95	100	65	80			
Xanalteric acid I (**23**)	45.0	90	85	70	30/ <u>125</u>	70	65			
Xanalteric acid II (**24**)	87.5	100	70	60	30/ <u>250</u>	70	65			

E. c. *Escherichia coli*, K. p. *Klebsiella pneumoniae*, E. f. *Enterococcus faecium*, S. a. *Staphylococcus aureus*, S. p. *Streptococcus pyogenes*, P. a. *Pseudomonas aerigunosa; HRE2,8, 16 Human Rhinoviruses 2, 8 and 16*

3.6 Proof of the fungal origin of the new compounds xanalteric acid I and II

To prove the fungal origin of the new compounds xanalteric acid I (**23**) and II (**24**), two different approaches were undertaken. Firstly, *Alternaria* sp. was fermented in liquid Wickerham medium upon addition of two different antibiotics, penicillin G or chloramphenicol. The obtained extracts still contained the two new compounds that could be unambiguously identified by their characteristic UV and mass spectra. In a second experiment PCR with DNA fragments of freeze-dried pieces of the fungal strain JCM9.2 (*Alternaria* sp.) was performed using the bacterial primers 27f and 149r. No bacterial DNA was identified in the sample of *Alternaria* sp.

3.6.1 Metabolite profiling of *Alternaria* sp. upon addition of different antibiotics

To suppress potential bacterial growth within the fungal culture, either chloramphenicol at a concentration of 200 mg/mL or penicillin G at a concentration of 500 mg/mL was added to liquid fermentation flasks of *Alternaria* sp. Negative controls without addition of antibiotics were included. After fermentation for three weeks, the cultures were extracted with EtOAc and subjected to chemical screening by HPLC-DAD and LC-MS. As the typical UV spectra were not observable in any of the extracts, they were all fractionated over HP-20 material to obtain 8 fractions of each extract.

In fractions IV (88% H_2O:12% MeOH) and V (100% MeOH) the new compounds were identified in fractions obtained from negative control as well as from both fermentations with antibiotic supplement by their characteristic UV and mass spectra.
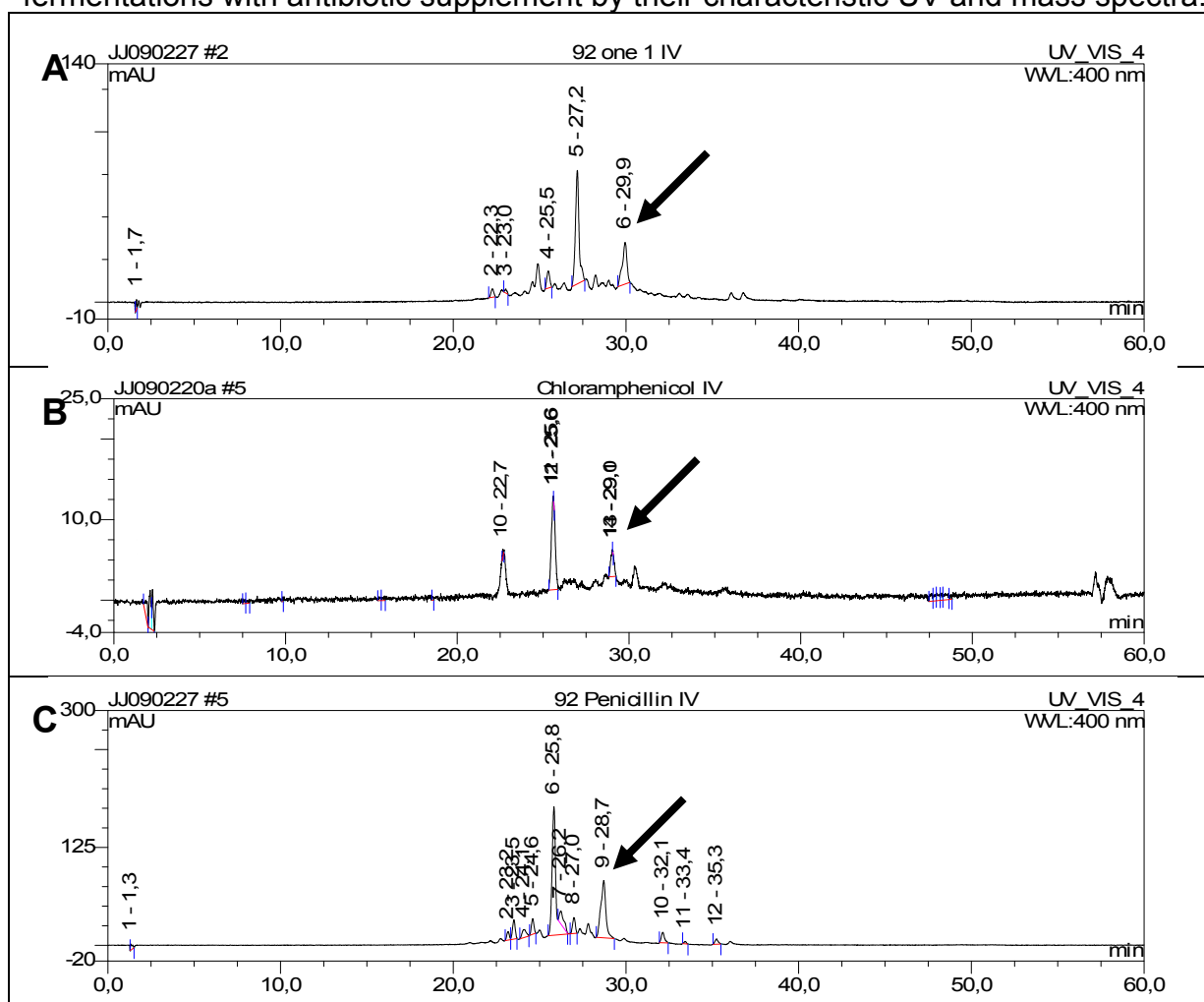
Figure 3.6.1. HPLC chromatograms with marked peaks of xanalteric acid of extracts of *Alternaria* sp. obtained after fermentation **A**: without antibiotics, **B**: upon addition of chloramphenicol, **C**: upon addition of penicillin G.

3.6.2 Results of the investigation for bacterial DNA in *Alternaria* sp. via PCR

Freeze-dried pieces obtained from cultures of JCM9.2 on agar plates were subjected to the DNA isolation process and amplified using the bacterial primers 27f and 149r. As positive controls, the gram-negative bacterium *E. coli* and the gram-positive *B. subtilis* were included in the experiment. The applied conditions proved suitable for the isolation and identification of bacterial DNA as both controls afforded good results and showed sharp bands after staining and gel electrophoresis. No bacterial DNA could be found in the sample from the fungal culture.

1	2	3	4	5	6	7
B. subtilis	*B. subtilis*	JCM9.2	DNA ladder	JCM9.2	*E. coli*	*E. coli*
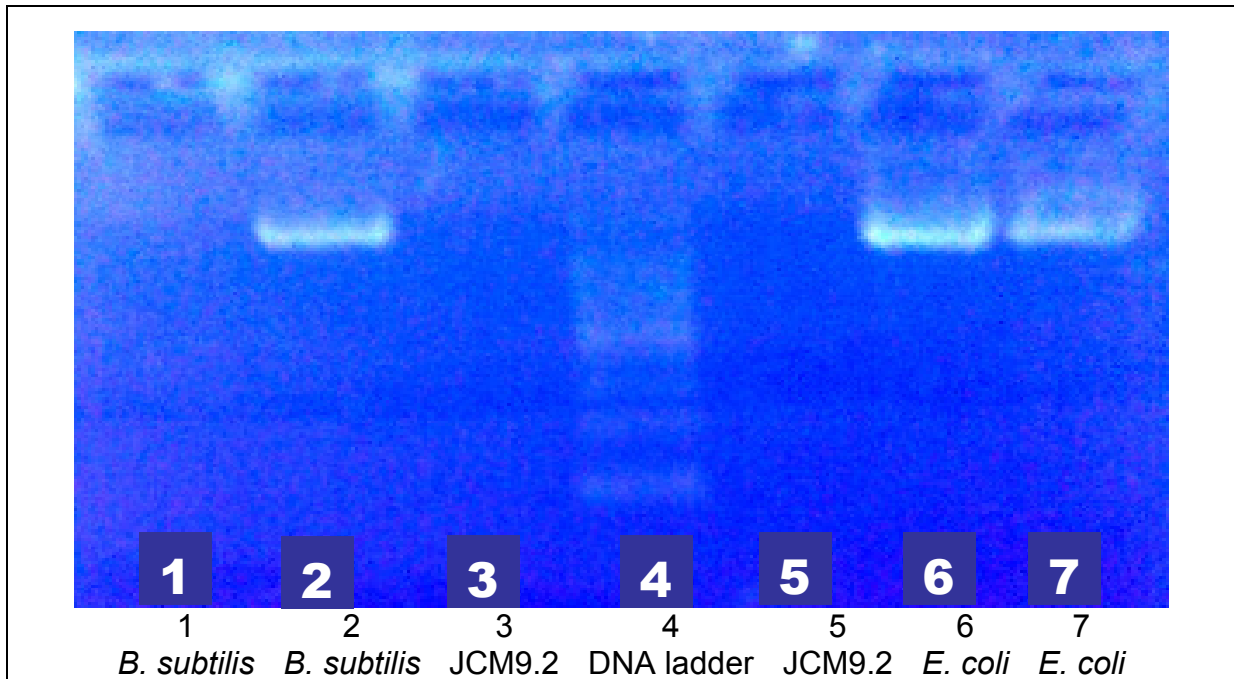
Figure 3.6.2. PCR products of *Alternaria* sp. (JCM9.2).

3.7 Tracing of fungal metabolites in the corresponding plant extracts

All of the compounds isolated from *Alternaria* sp., besides **12** and **22**, were detected in the crude extracts of the fungus, either from liquid fermentation or from fermentation on rice medium. As our group was recently able to detect fungal compounds in their respective host plants, the crude extract of *Sonneratia alba* was also analyzed for fungal substances. As no positive results were obtained after analysis of the crude extract of the plant (Hassan 2007), a fractionation over Diaion material was performed in order to concentrate the potential compounds for further investigation. However, it was not possible to detect any of the secondary metabolites in any of the host-plant derived fractions.

3.8 Results of the fermentation experiments upon addition of epigenetic modifiers

Two fungal strains, *Nigrospora* sp. (MGC4.2) and *Alternaria* sp. (JCM9.2) were treated with substances known to inhibit histone deacetylase (HDAC) or DNA methyltransferase (DNMT) with the intention to enhance transcriptional activation of normally "silent" biogenetic gene clusters and thus the chemical productivity of the fungus.

Liquid cultures of *Nigrospora* sp. were treated with the nucleoside analog 5-azacytidine, (a DNMT inhibitor), the marine sponge metabolite psammaplin A or the anticonvulsant drug valproic acid (both HDAC inhibitors) at different concentrations. Control cultures were grown under the same fermentation conditions, but without addition of the inhibitor.

In comparison with the extracts of the control flasks, the dry weights of all EtOAc extracts, except for addition of the lower concentration of 5-azacytidine, were larger.

The outcome of the examination of extracts of *Alternaria* sp. was slightly different. The amount of the EtOAc extract was larger *a priori* and could only be boosted by a concentration of 5 μM of 5-azacytidine and 0.5 μM of psammaplin A.

Table 3.8. Yields of *Nigrospora* sp. and *Alternaria* sp. after fermentation of epigenetic modifiers.

Substance [conc.]	control	aza [1 μM]	aza [5 μM]	pA [0.05 μM]	pA [0.5 μM]	pA [1 μM]	va [1 mM]	va [4 mM]
MGC4.2EtOAc [mg]	78.1	71.3	229.5	93.7	205.5	n. t.	147.1	236.3
JCM9.2 EtOAc [mg]	163.2	128.8	170.1	n. t.	197.9	99.0	n. t.	n. t.

aza: 5-azacytidine, pA: psammaplin A, va: valproic acid
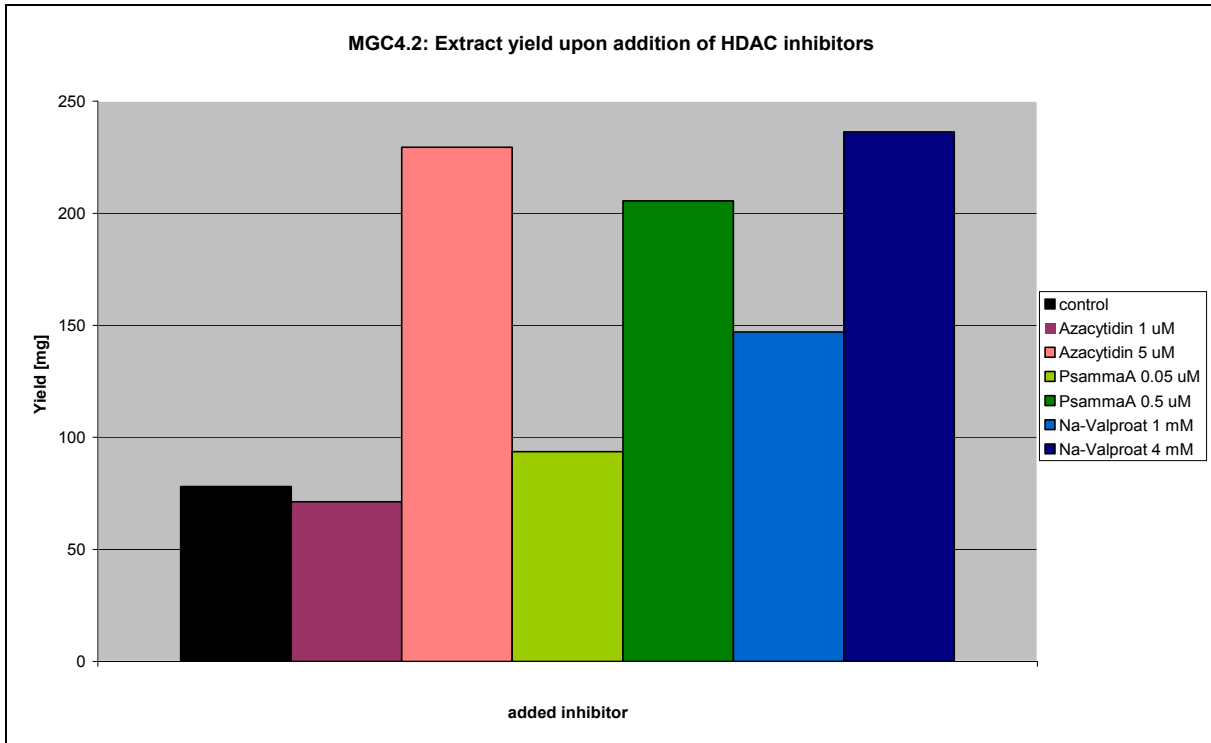
Figure 3.8a. Diagram of extract yields of *Nigrospora* sp. after fermentation with epigenetic modifiers.
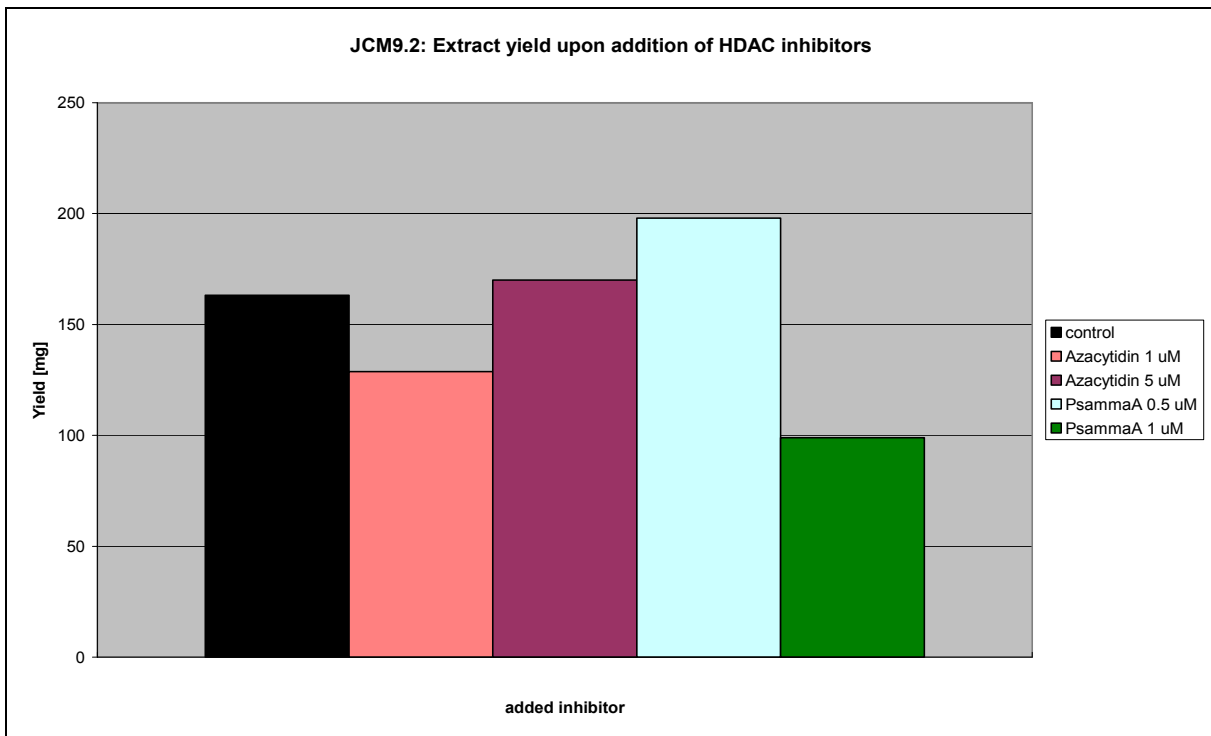
Figure 3.8b. Diagram of extract yields of *Alternaria* sp. after fermentation with epigenetic modifiers.

The obtained chromatograms of the extracts of *Nigrospora* sp. showed that the concentration of the individual metabolites, in comparison to the control flasks, varied upon addition of the respective inhibitors, adumbrating a varying gene expression. Upon addition of 5-azacytidin, for example, one main metabolite of the control disappeared and two minor constituents of the control became the major

metabolites of the extract. Upon treatment with psammaplin A the result was reversed. Treatment with a high concentration of valproic acid afforded an additional peak that could not be found in any of the other treated or untreated extracts (see also figure 3.8c). The assumption that this additional compound could originate from an activation of a silent natural product biosynthetic pathway could not be resolved in the course of this thesis, since it was not possible to isolate the natural product.

Such changes in the composition of the crude EtOAc extracts were not observable in the chromatograms of *Alternaria* sp. (see figure 3.8d). All chromatograms displayed similar compositions of the extracts.
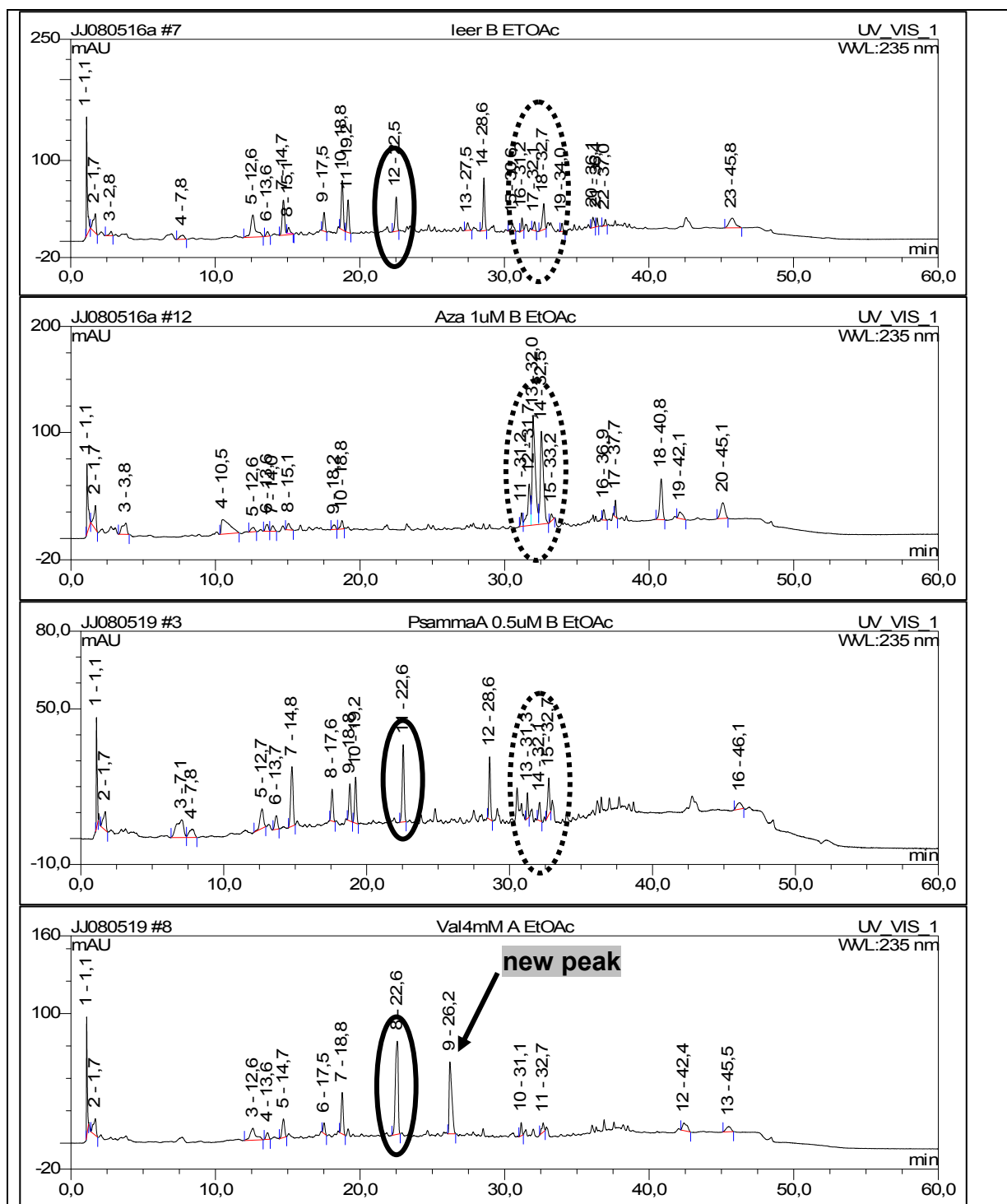
Figure 3.8c. HPLC chromatograms of EtOAc extracts of *Nigrospora* sp. after fermentation with epigenetic modifiers.
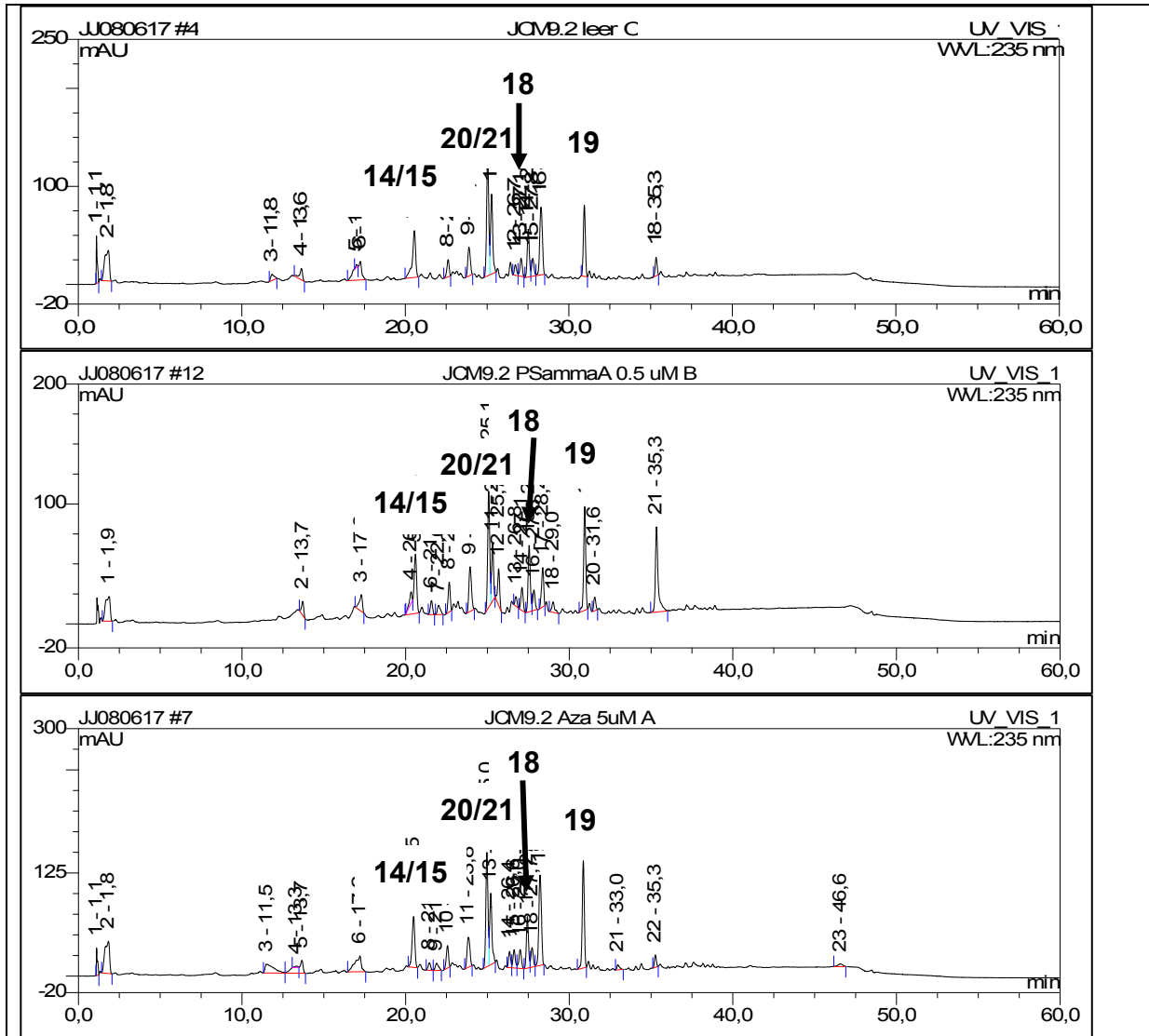
Figure 3.8d. HPLC chromatograms of EtOAc extracts of *Alternaria* sp. after fermentation with epigenetic modifiers (numbers of detected compounds).

3.9 Compounds isolated from the endophytic fungus *Bionectria ochroleuca*

Bionectria ochroleuca was isolated as an endophytic fungus from the mangrove plant *Sonneratia caseolaris*. The fungus was fermented on a small scale basis on solid rice medium in two Erlenmeyer flasks. In one flask, the rice medium was supplemented with 1% artificial sea salt in order to survey the strain's response to altered conditions. As no difference could be observed for the obtained extracts, the fermentation without salt addition was scaled up.

From the EtOAc extract of *Bionectria ochroleuca* the known fungal metabolite verticillin D (**25**) together with four new depsipeptides, bionectriamides A – D (**26**-**29**), was isolated.
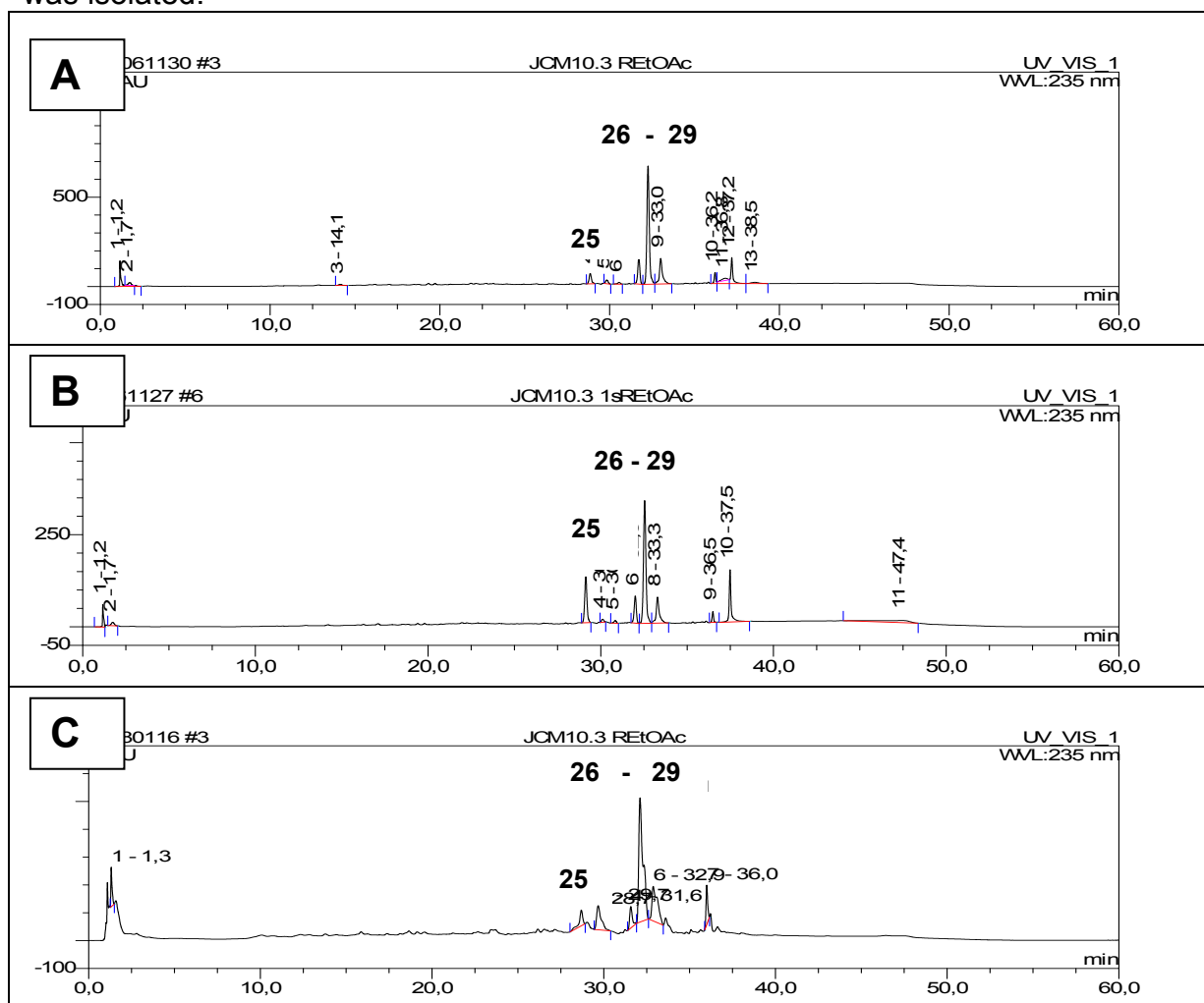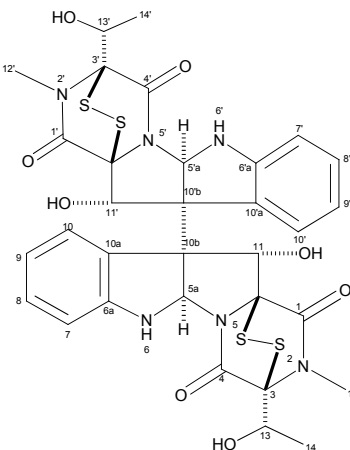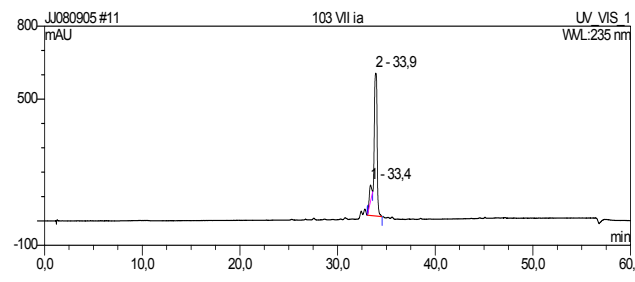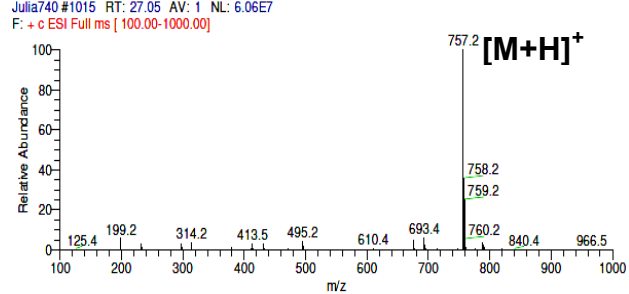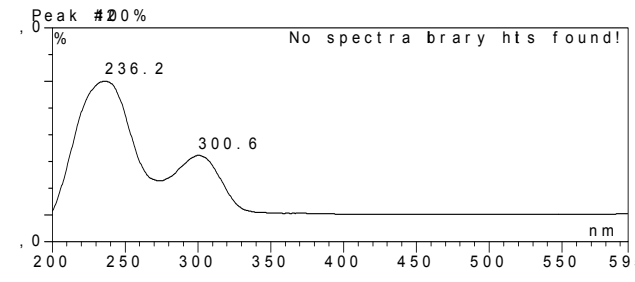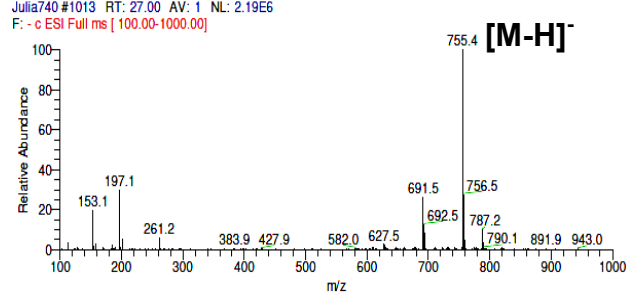
Figure 3.9. HPLC Chromatograms of EtOAc extracts of *Bionectria ochroleuca* after fermentation on **A**: rice without salt, **B**: rice with 1% artificial sea salt, **C**: rice without salt (large scale).

3.9.1 Verticillin D (25, known compound)

Verticillin D	
Synonym(s)	(3S,3'S,6R,6'R,14S,14'S,15S,15'S,16S,16'S)-15,15'-dihydroxy-3'-((R)-1-hydroxyethyl)-3-((S)-1-hydroxyethyl)-2,2'-dimethyl-2,2',3,3',6,6',7,7'-octahydro-1H,1'H-[14,14'-bi(3,11a-epidithiopyrazino[1',2':1,5]pyrrolo[2,3-b]indole)]-1,1',4,4'(15H,15'H)-tetraone
Sample code	JCM10.3 III i, ia
Biological source	*Bionectria ochroleuca* (from *Sonneratia caseolaris*)
Sample amount	47 mg
Physical description	white powder
Molecular formula	$C_{32}H_{32}N_6O_8S_4$
Molecular weight	756 g/mol
Optical rotation $[\alpha]_D^{20}$	305° (*c* 0.1, MeOH)
Retention time HPLC	33.9 min (standard gradient)

Verticillin D was obtained as a white powder from the EtOAc extracts of *Bionectria ochroleuca* after fermentation on solid rice medium. The UV/Vis spectrum of the compound exhibited maxima at 236 and 301 nm. Its molecular weight was determined as 756 amu based on the pseudomolecular ions observed at *m/z* 757.2

[M+H]⁺ and 755.4 [M-H]⁻ upon positive and negative ionization by ESI-MS, respectively. However, the ^{1}H NMR spectrum revealed only a relatively limited number of signals, which indicated the presence of a symmetric molecule. It was possible to attribute the signals resonating between δ 6.7 – 7.8 (H-10, H-8, H-9 and H-7) to an ABCD system of a 1,2-disubstituted aromatic ring, three methine protons attached to heteroatoms at δ 5.74 (H-11), δ 5.10 (H-5a) and δ 4.47 (H-13), an *N*-methyl group at δ 3.17 (CH$_3$-12), an aliphatic methyl signal at δ 1.64 (H$_3$-14) and a hydroxyl group at δ 3.31 (OH-13). The HMBC experiment allowed assembling of two distinct parts of the molecule. Correlations of the aromatic protons and protons 5a and 11 suggested a hexahydropyrrolo[2,3-b]indole ring, while the *N*-methyl group showed a correlation to an amide carbonyl at δ 166.5 (C-1) and shared another cross peak to a quaternary sp^3 carbon (C-3) with the aliphatic methyl group. The latter in turn shared a correlation with the hydroxyl to C-13 at δ 67.5. The chemical shifts of C-3 and C-11a were consistent with C-α carbons of amino acids. On this basis, it was assumed that the two discrete systems obtained from NMR were part of a substituted diketopiperazinopyrroloindole structure, while the considerable difference in molecular weight between the substructures identified so far and the actual compound could only be explained by the presence of "NMR silent" subsituents, which most likely could be attributed to a total of four sulphur atoms.

This hypothesis was verified by detailed literature search, which revealed compound **25** to be a member of the epidithiodioxopiperazine class of fungal metabolites. This class of fungal-derived antibiotics has been prevalently isolated from members of the family Bionectriaceae. Due to the agreement of its spectral data with previously reported data, compound **25** was readily identified as verticillin D. This natural product was first reported from *Gliocladium catenulatum*, a mycoparasite of *Aspergillus flavus* (Joshi et al. 1999).

Table 3.9.1. NMR data for verticillin D (**25**).

	25				**Literature**[c]	
	δ_H (*J* Hz)[b]	COSY[b]	HMBC[a]	δ_C mult.[a] (from HMBC)	δ_H (*J* Hz)[c]	δ_C mult.**
1, 1'				166.5		167.9
3, 3'				77.0		82.4
4, 4'						162.6
5a, 5a'	5.10 s		6a, 10a, 10b, 11a	82.0	5.88 s	84.0
6a, 6a'				148.0		151.3
7, 7'	6.69 d (7.7)	8	8, 9, 10a	110.5	6.66 brd (7.8)	110.6
8, 8'	7.18dt (1.0; 7.6)	7, 9	10, 6a	130.0	7.07 dd (7.8; 7.8)	130.5
9, 9'	6.87 dt (0.8; 7.8)	8, 10	7, 8, 10, 10a	120.5	6.72 dd (7.8; 7.8)	119.9
10, 10'	7.83 brd (7.4)	9	8, 6a	128.5	8.32 brd (7.8)	129.1
10a, 10a'				129.5		131.3
10b, 10b'				66.0		67.7
11, 11'	5.74 d (7.5)		5a, 10b, 11a	82.0	6.38 brs	83.3
11a, 11a'				75.5		79.0
12, 12'	3.17 s		1, 3	27.0	3.45 s	30.0
13, 13'	4.47 td (6.6; 17.6)	14, 13-OH		67.5	5.00 m	68.0
14, 14'	1.64 d (6.6)	13	3, 13	19.5	1.51 d (5.8)	20.5
11-, 11'-OH					8.29 brs	
13-,13'-OH	3.31 brd (11.5)		13			
6-, 6'-NH					7.45 s	

[a] in MeOD
[b] in CDCl$_3$
[c] in pyridine-*d$_5$*, Joshi et al. (1999)

3.9.2 Bionectriamide A (26, new compound)

Bionectiramide A	
Synonym(s)	(3S,6S,9S,12S,20aR)-15-benzyl-6-sec-butyl-3-(hydroxymethyl)-5,11,12-trimethyl-9-(4-(3-methylbut-2-enyloxy)benzyl)octahydro-1H-pyrrolo[1,2-d][1,4,7,10,13,16]oxapentaazacyclooctadecine-1,4,7,10,13,16(15H,18H,19H,20H,20aH)-hexaone
Sample code	JCM10.3 eB3
Biological source	*Bionectria ochroleuca* (from *Sonneratia caseolaris*)
Sample amount	65 mg
Physical description	white crystals
Molecular formula	$C_{42}H_{57}N_5O_9$
Molecular weight	775 g/mol
Optical rotation $[\alpha]_D^{20}$	-36° (*c* 1.0, MeOH)
Retention time HPLC	38.5 min (standard gradient)
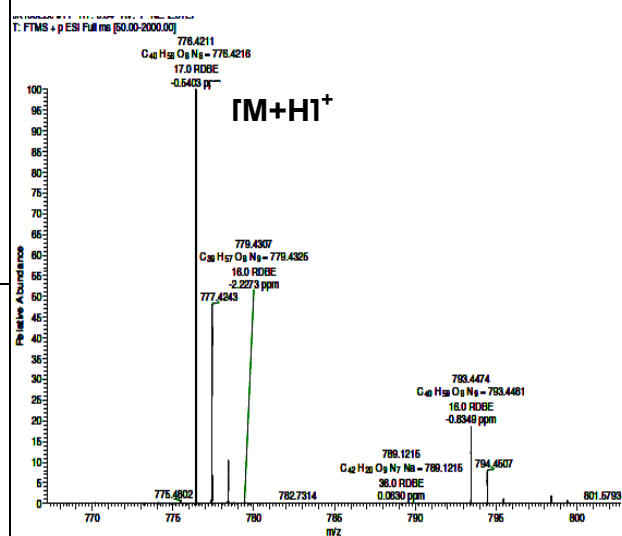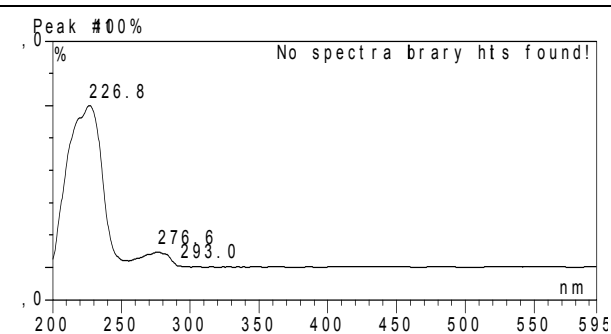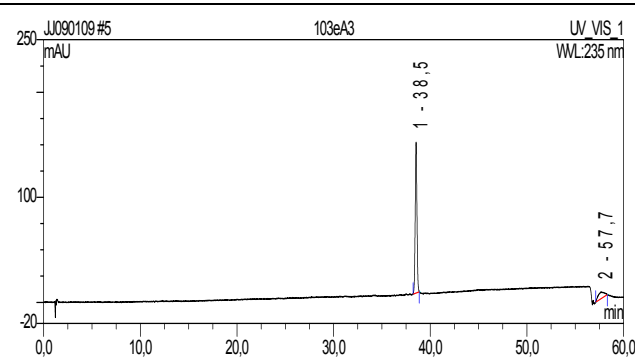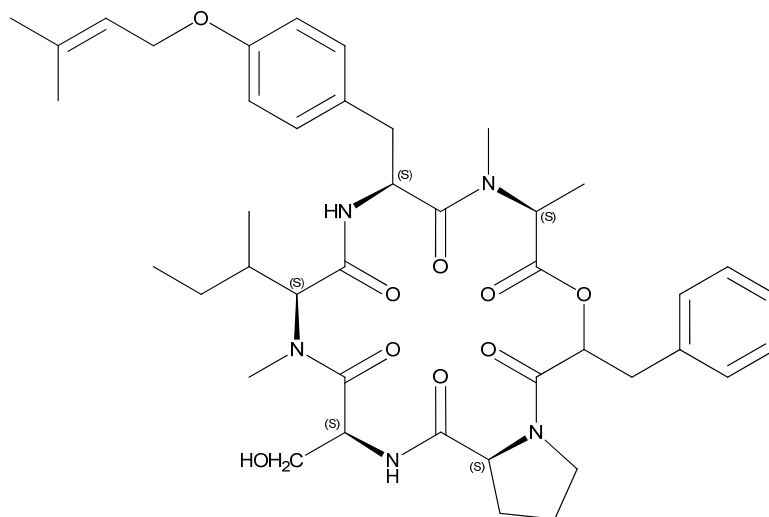
Bionectriamide A was obtained as crystals from the EtOAc extract of the large scale fermentation of *Bionectria ochroleuca.* Its UV maxima at 227 and 277 nm concurred with its colorless appearance. From the HRESIMS exhibiting a peak at *m/z* 776.4211 [M+H]+ the molecular formula $C_{42}H_{57}N_5O_9$ was determined (calcd. for $C_{42}H_{57}N_5O_9$ 776.4235). The number of hydrogen and carbon atoms observed in the ^1H and ^{13}C NMR spectra was in agreement with this molecular formula, indicating that **26** was a hexadepsipeptide composed of one 2-hydroxycarboxylic acid and five amino acid residues. Correspondingly, one ester carbonyl carbon (3-Phe-Lac-CO, δ 167.9) and five amide carbonyl carbons (δ 168.1, 168.9, 169.5, 171.5, 171.8) were discernible. Since only two NH proton signals (δ 8.21 and 9.47) and two *N*-methyl groups ($δ_H$ 2.94 and 2.49) were observed, the fifth amino acid was assumed to represent proline. This assumption was corroborated by analysis of the TOCSY spectrum, which in addition allowed for assigning the spin systems characteristic for tyrosine, serine, isoleucine, alanine and phenylalanine, and furthermore hinted at the presence of an *O*-prenyl rest. The positions of the *N*-methyl groups, the prenyl rest, the sequence of the amino acid residues and the 2-hydroxycarboxylic acid were established by extensive analysis of the HMBC and ROESY data (see figure 3.9.2a). The 2-hydroxycarboxylic acid was assigned to be 3-phenyllactic acid (3-Ph-Lac), on the basis of an oxymethine (δ 72.2) attached to a methylene at δ 36.5, which in turn was adjacent to a monosubstituted phenyl group. The prenyl moiety was linked to the tyrosine *via* an ether, as perceptible from the HMBC correlation from the methylene protons (δ 4.44) of the prenyl group to the quaternary carbon of the tyrosine (δ 157.4) as well as a ROESY cross-peak between these protons and $H_ε$ of the tyrosine (δ 6.79). The overall sequence of **26** was established as *O*-prenyl-Tyrl, *N*-Me-Ile, Ser, Pro, 3-Ph-Lac and *N*-Me-Ala (see figure 3.9.2a).
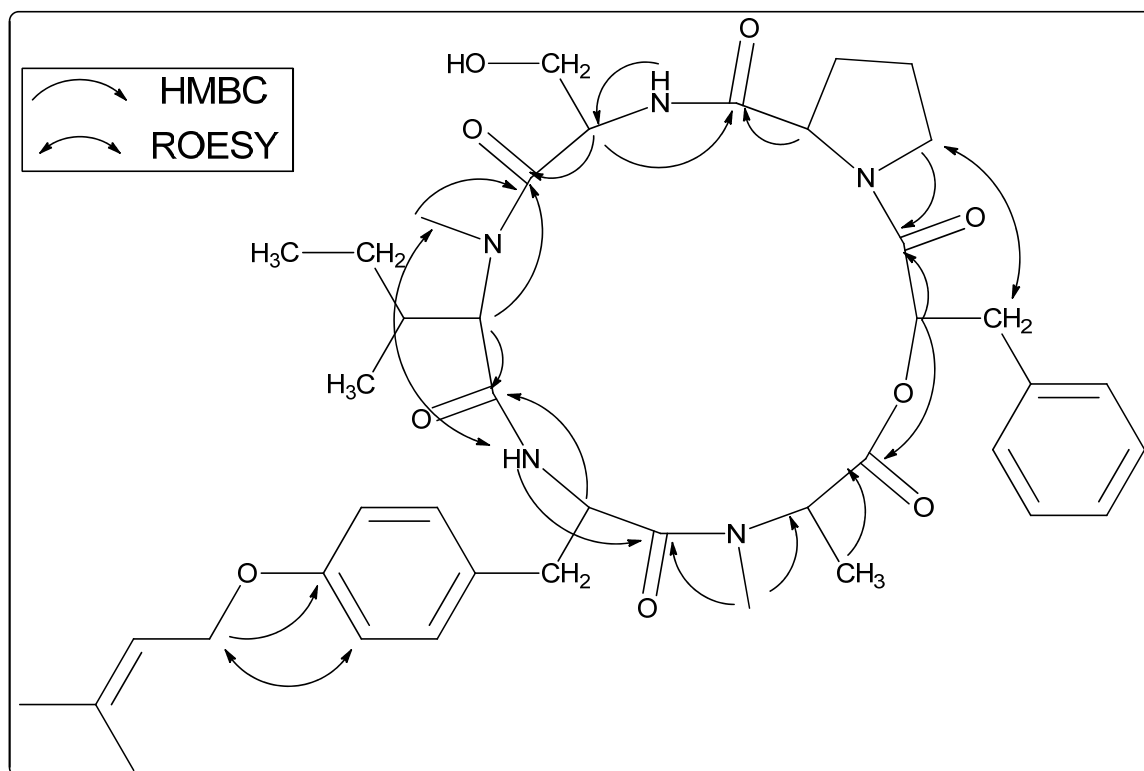
Figure 3.9.2a. Selected HMBC and ROESY correlations of bionectriamide A.

Table 3.9.2a. NMR data for bionectriamide A (**26**) in CDCl$_3$.

Unit	C/H no.	δ$_C$ mult.	δ$_H$ (*J* Hz)	COSY	HMBC	ROESY
O-prenyl-Tyr	H$_N$		9.47, d (9.0)	H$_α$	CO, C$_α$	H$_α$, H$_α$(*N*-Me-Ile)
	CO	168.9			C$_α$	
	C$_α$	51.7, CH	4.88 m	H$_N$, H$_β$	*O*-prenyl-Tyr -CO, N-Me-Ile-CO, C$_β$, C$_γ$	
	C$_β$	37.3, CH$_2$	A 3.12, dd (13.5; 7.3)	H$_β$, H$_α$	*O*-prenyl-Tyr -CO, C$_α$, C$_γ$, C$_δ$	H$_δ$
			B 2.58, dd (13.8; 6.5)	H$_β$, H$_α$	*O*-prenyl-Tyr -CO, C$_γ$, C$_δ$	H$_δ$
	C$_γ$	128.5, qC				
	C$_δ$	130.6, CH	7.16, d (8.6)	H$_β$, H$_ε$	C$_δ$, C$_ε$, C$_ζ$	H$_β$
	C$_ε$	114.3, CH	6.79, d (8.7)	H$_δ$	C$_γ$, C$_ε$, C$_ζ$	H$_1$
	C$_ζ$	157.4, qC				
	1	64.6, CH$_2$	4.44, d (6.8)	H$_2$, H$_4$, H$_5$	C$_ζ$, C$_2$, C$_3$	H$_ε$, H$_5$
	2	119.8, CH	5.46, t (5.5)	H$_1$, H$_4$, H$_5$	C$_1$, C$_2$, C$_3$	H$_1$, H$_4$
	3	137.9, qC				
	4	25.2, CH$_3$	1.79, s	H$_2$	C$_2$, C$_3$, C$_5$	H$_2$
	5	18.1, CH$_3$	1.72, s	H$_2$	C$_2$, C$_3$, C$_4$	H$_1$
N-Me-Ala	CH$_{3-N}$	36.5, CH$_3$	2.94, s		*O*-prenyl-Tyr -CO, C$_α$	
	CO	169.5				
	C$_α$	59.4, CH	3.42, q (6.7)	H$_β$	*O*-prenyl-Tyr -CO, N-Me-Ala-CO, CH$_{3-N}$, C$_β$	H$_β$
	C$_β$	13.4, CH$_3$	1.32, d (6.7)	H$_α$	*N*-Me-Ala-CO, C$_α$	H$_α$
3-Ph-Lac	CO	167.9				
	C$_α$	72.2, CH	5.73, dd (10.0; 4.6)	H$_β$	*N*-Me-Ala-CO, 3-Phe-Lac-CO, C$_β$, C$_γ$	H$_δ$, H$_δ$(Pro)
	C$_β$	36.5, CH$_2$	A 3.24, dd (12.8; 10.3)	H$_β$, H$_α$	3-Phe-Lac-CO, C$_δ$, C$_γ$	
			B 3.05, dd (12.8; 4.7)			
	C$_γ$	135.4, qC				
	C$_δ$	129.7, CH	7.24, m*	H$_α$, H$_β$, H$_ε$	C$_γ$, C$_δ$	H$_α$
	C$_ε$	128.5, CH	7.24, m*	H$_δ$, H$_ε$		H$_α$ (Pro- H$_β$)
	C$_ζ$	126.9, CH	7.22, m	H$_δ$		
Pro	CO	171.8				
	C$_α$	59.8, CH	4.27, dd (6.2; 3.2)	H$_β$	Pro-CO, C$_β$, C$_γ$	
	C$_β$	29.2, CH$_2$	2.06, m*	H$_α$, H$_γ$	Pro-CO, C$_γ$	
	C$_γ$	24.5, CH$_2$	2.06, m*	H$_γ$, H$_δ$	C$_α$	
			1.93, m*			
	C$_δ$	47.3	A 3.95, m	H$_δ$, H$_γ$	3-Phe-Lac-CO, C$_β$, C$_γ$	H$_γ$(3-Phe-Lac)

Ser	H_N	CH_2	B 3.25, m 8.21, d (8.2)	H_α		H_α(Pro)
	CO	171.5				
	C_α	52.5, CH	4.83, t (3.1)	H_δ	Ser-CO	H_α(*N*-Me-Ile)
	C_β	63.1, CH_2	A 4.00, dd (13.2; 6.5) B 3.69, dd (12.3; 1.7)	H_β, H_α	Ser-CO, C_α	H_γ(*N*-Me-Ile), H_β(*N*-Me-Ile)
N-Me-Ile	$CH_{3\text{-N}}$	28.6 CH_3	2.49, s		Ser-CO, C_α	H_β
	CO	168.1 C=O				
	C_α	65.5 CH	4.63, d (10.9)	H_β	Ser-CO, *N*-Me-Ile-CO, C_γ, C_γ, $CH_{3\text{-N}}$,	
	C_β	32.3 CH	2.12, m	H_α, H_γ	C_γ, C_γ, C_δ	$CH_{3\text{-N}}$, H_δ
	C_γ	25.2 CH_2	A 1.33, m* B 0.90, m	H_β, H_γ, H_δ	C_β, C_γ, C_δ	H_α(Ser)
	C_γ	16.3 CH_3	0.95, d (6.3)	H_δ	C_α, C_β, C_γ	H_α
	C_δ	12.1 CH_3	0.98, t (8.0)	H_γ	C_α, C_β, C_γ	H_β

* overlapping signals

The ESI fragmentation pattern supported this analysis by showing the loss of the following moieties from the [M+Na]$^+$ pseudomolecular ion, *N*-Me-Ile+*O*-prenyl-Tyr, *O*-prenyl-Tyr+*N*-Me-Ala, Ser+*N*-Me-Ile and Ser+Pro.
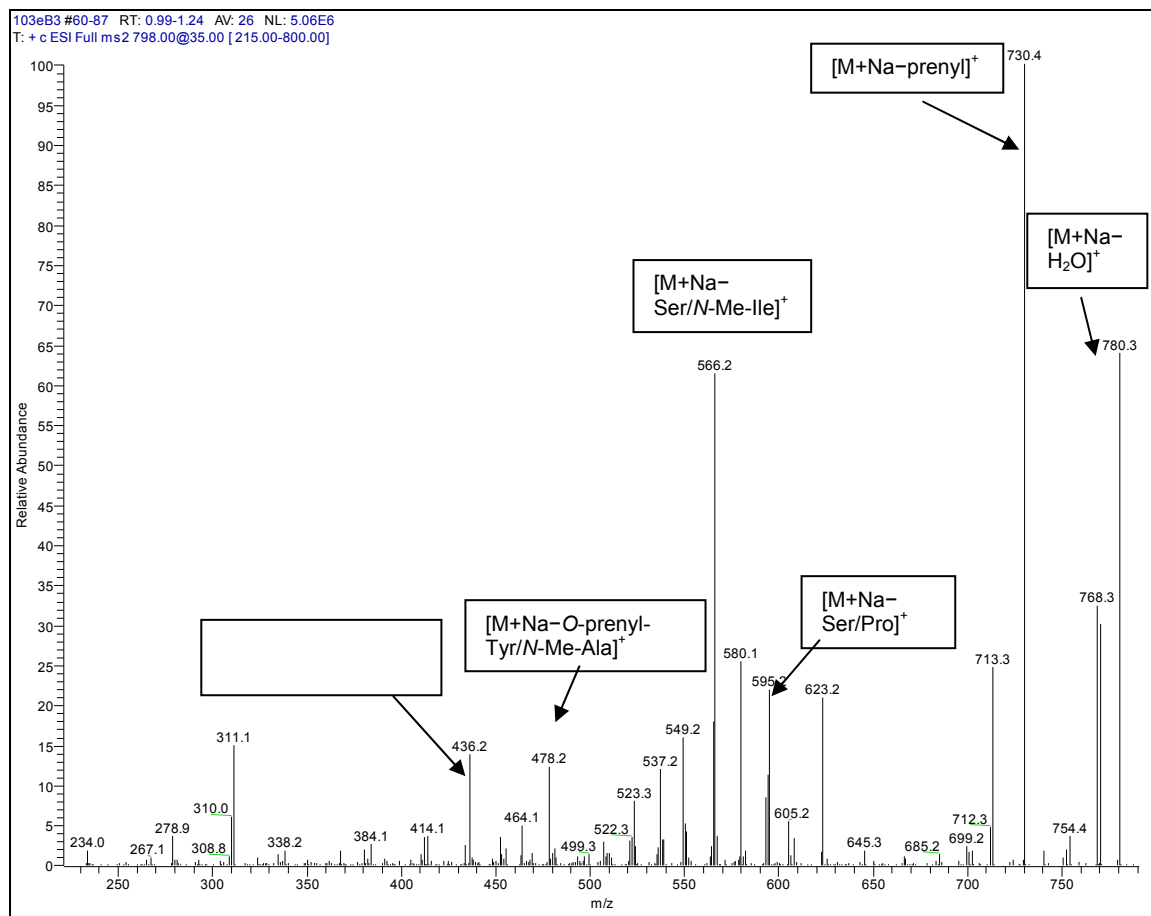
Figure 3.9.2b. ESI-MS fractionation pattern of bionectriamide A (**25**).
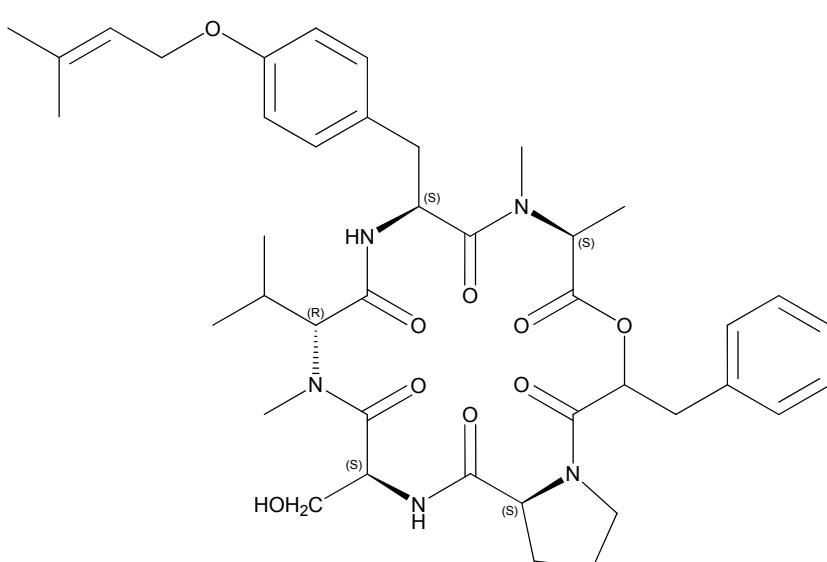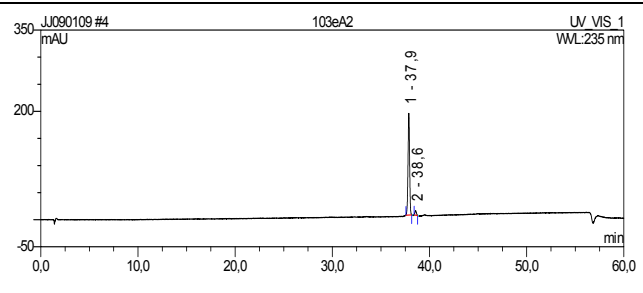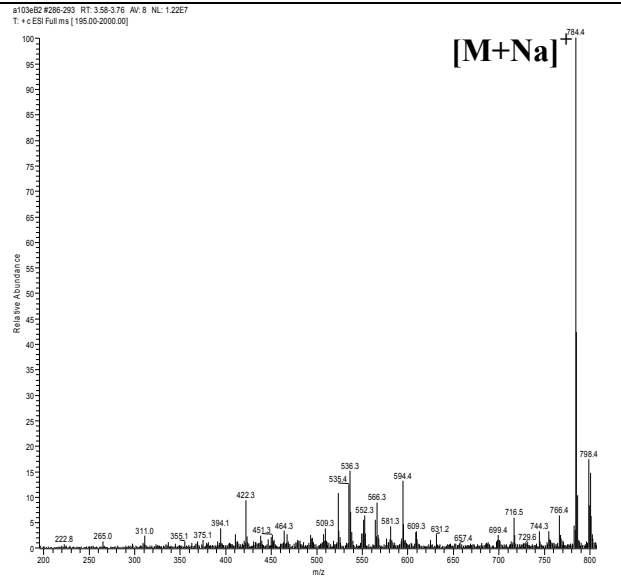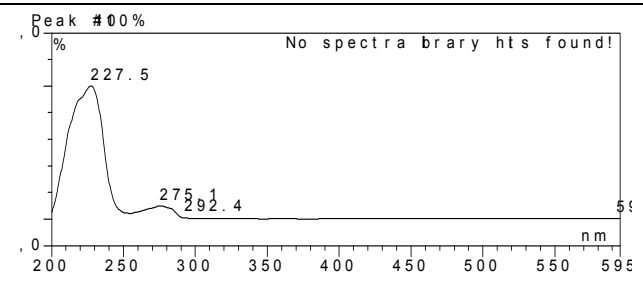
To determine the stereochemistry of the amino acid residues, the so-called advanced Marfey's method was employed (Harada et al. 1995). After hydrolysis of **26** in 6 M HCl for 24 h, the hydrolyzate was transformed to diastereomeric reaction products by adding the reagent FDAA. The reaction products thus obtained were submitted to analytical HPLC and LCMS. Moreover, the commercially available amino acids tyrosine, *N*-methyl-isoleucine, serine, proline and *N*-methyl-alanine were used as authentic standards, both as the respective L-enantiomers and racemates, and subjected to similar conditions for the derivatisation and analysis. By comparison of the retention times of the reaction products of the hydrolyzate and the amino acid standards, the configuration of the respective amino acid was determined. As tyrosine lost its prenyl rest during hydrolysis, it was not necessary to provide *O*-prenyl tyrosine derivatives. On this basis, the configurations of the amino acids in **26** were determined as L-*O*-prenyl-Tyr, *N*-Me-L-Ile, L-Ser, L-Pro and *N*-Me-L-Ala (see table 3.9.2b).

Table 3.9.2b. Retention times of standard amino acid derivatives and derivatives of the hydrolyzate of bionectriamide A (**26**) in HPLC-DAD and LCMS. The respective retention time of FDAA is given as a reference.

Amino acid	Retention time HPLC-DAD	FDAA ret. time HPLC-DAD	Retention time LCMS	FDAA ret. time LCMS
D-Tyr	24.3	20.3	26.9	21.0
L-Tyr	22.8	20.3	24.5	21.0
26	22.8	20.4	24.5	21.0
N-Me-D-Ile				
N-Me-L-Ile	26.7	20.4	32.5	21.9
26	26.7	20.4	32.5	21.8
D-Ser	19.1	20.4	20.7	22.8
L-Ser	19.6	20.4	20.8	22.8
26	19.6	20.4	19.3	21.0
D-Pro	21.4	20.4	23.1	20.9
L-Pro	21.2	20.4	22.5	20.9
26	21.2	20.4	22.5	21.0
N-Me-D-Ala	21.9	20.4	25.0	21.8
N-Me-L-Ala	22.1	20.4	25.2	21.8
26	22.1	20.4	24.1	21.0

3.9.3 Bionectriamide B (27, new compound)

Bionectriamide B	
Synonym(s)	(3S,6R,9S,12S,20aS)-15-benzyl-3-(hydroxymethyl)-6-isopropyl-5,11,12-trimethyl-9-(4-(3-methylbut-2-enyloxy)benzyl)octahydro-1H-pyrrolo[1,2-d][1,4,7,10,13,16]oxapentaazacyclooctadecine-1,4,7,10,13,16(15H,18H,19H,20H,20aH)-hexaone
Sample code	JCM10.3 eB2
Biological source	*Bionectria ochroleuca* (from *Sonneratia caseolaris*)
Sample amount	8.8 mg
Physical description	white powder
Molecular formula	$C_{41}H_{55}N_5O_9$
Molecular weight	761 g/mol
Optical rotation $[\alpha]_D^{20}$	-61° (*c* 0.5, MeOH)
Retention time HPLC	37.9 min (standard gradient)

Bionectriamide B was isolated as a white powder after several chromatographic purification steps of the EtOAc extract of *Bionectria ochroleuca*. It

displayed UV maxima at 228 and 275 nm, with the shape of the spectrum resembling that of bionectriamide A. The compound showed a pseudomolecular peak at 784 $[M+Na]^+$ upon positive ionization by ESI-MS, 14 amu less than bionectriamide A and thus indicating a molecular formula of $C_{41}H_{55}N_5O_9$. The one- and two-dimensional NMR spectra also exhibited great analogy to spectra obtained for bionectriamide A (**26**).

Table 3.9.3a. NMR data for bionectriamide B (**27**) in $CDCl_3$.

Unit	C/H no.	δ_C mult.	δ_H (*J* Hz)	COSY	HMBC	ROESY
O-prenyl-Tyr		168.8 C=O				
	α	51.8 CH	4.86 td (8.6; 5.4)	H_N, $H_β$	*O*-prenyl-Tyr -CO, N-Me-Val-CO, $C_γ$, $C_β$	$H_δ$, N-CH$_3$ (*N*-Me-Ala)
	β	37.3 CH$_2$	3.12 m	$H_α$, $H_β$	*O*-prenyl-Tyr -CO $C_α$, $C_γ$, $C_δ$	
			2.59 brd (4.3)	$H_α$, $H_β$	*O*-prenyl-Tyr -CO $C_α$, $C_γ$, $C_δ$	$H_β$ (*N*-Me-Val)
	γ	128.6 qC				
	δ	130.6 CH	7.15 d (8.5)	$H_ε$	$C_β$, $C_γ$, $C_ε$, $C_ζ$	$H_α$, $H_β$ (*N*-Me-Ala)
	ε	114.4 CH	6.78 d (8.6)	$H_δ$	$C_γ$, $C_ε$, $C_ζ$	H_1, H_2
	ζ	157.4 qC				
	1	64.7 CH$_2$	4.4 d (7.8)	H_2, H_4, H_5	$C_ζ$, C_2, C_3	$H_ε$, H_4, H_5
	2	119.7 CH	5.45 dt (1.3; 5.4)	H_1, H_4, H_5	C_4, C_5	$H_ε$, H_4, H_5
	3	138.0 qC				
	4	25.8 CH$_3$	1.77 s	H_1, H_2	C_2, C_3, C_5	H_1, H_2
	5	18.2 CH$_3$	1.73 s	H_1, H_2	C_2, C_3, C_4	H_1, H_2
	NH		9.36 d (8.9)	$H_α$	$C_α$	$H_α$ (*N*-Me-Val), N-CH$_3$ (*N*-Me-Val)
N-Me-Ala		169.9 C=O				
	α	59.6 CH	3.43 q (6.7)	$H_β$	*N*-Me-Ala-CO *O*-prenyl-Tyr -CO, N-Me-Val-CO, $C_β$, N-CH$_3$	N-CH$_3$
	β	13.4 CH$_3$	1.29 d (6.8)	$H_α$	N-Me-Ala-CO, *O*-prenyl-Tyr -CO, $C_α$	$H_δ$ (*O*-prenyl-Tyr), N-CH$_3$
	N-CH$_3$	36.5 CH$_3$	2.96 s		N-Me-Ala-CO, *O*-prenyl-Tyr -CO, $C_α$	$H_α$, $H_β$
3-Ph-Lac		167.6 C=O				
	α	72.2 CH	5.73 dd(10.0; 4.4)	$H_β$	*N*-Me-Ala-CO, 3-Ph-Lac-CO, $C_β$, $C_γ$	$H_δ$, $H_δ$ (Pro)
	β	36.4 CH$_2$	3.24 m	$H_α$, $H_β$	3-Ph-Lac-CO, $C_α$ $C_γ$, $C_δ$	$H_δ$
			3.03 dd (12.7;4.5)	$H_α$, $H_β$	3-Ph-Lac-CO, $C_α$ $C_γ$, $C_δ$	$H_δ$
	γ	135.5 qC				
	δ	129.6 CH	7.26 m*	$H_δ$, $H_ζ$	$C_α$, $C_ε$, $C_ζ$	$H_α$, $H_β$
	ε	128.6	7.26 m*	$H_ε$	$C_δ$, $C_ζ$	$H_γ$ (Pro)

Pro	ζ	CH 126.9 CH	7.22 m*	H_ε	C_δ, C_ε	
		171.9 C=O				
	α	59.5 CH	4.32 d (7.3)	H_β	Pro-CO, C_δ, C_β, C_γ	NH (Ser), H_β, H_γ
	β	29.4 CH$_2$	2.03 m*	H_α, zf	Pro-CO, C_δ, C_γ	H_α
			2.03 m*	H_α, zd	Pro-CO, C_δ, C_γ	H_α, $H_{\delta(A)}$
	γ	24.4 CH$_2$	A: 2.03 m*	H_β, H_γ, H_δ	C_α, C_β, C_γ	H_α
			B: 1.92 m	H_β, H_γ, H_δ	C_α, C_β, C_γ	$H_{\delta(B)}$
	δ	47.1 CH$_2$	A: 3.95 m*	H_δ, H_γ	C_β, C_γ	H_δ, H_γ
			B: 3.26 m	H_δ, H_γ	C_β, C_γ	H_δ, H_γ
Ser		171.9 C=O				
	α	51.3 CH	4.80 brs	NH, H_β	Pro-CO, C_β	$H_{\beta(B)}$, H_α (*N*-Me-Val), H_β (*N*-Me-Val)
	β	62.8 CH$_2$	A: 3.97 m*	H_α, H_β	Pro-CO	$H_{\beta(B)}$, H_δ (*N*-Me-Val)
			B: 3.73 dd (12.3; 2.2)	H_α, H_β	Pro-CO	H_α, H_β , H_γ (*N*-Me-Val), H_δ (*N*-Me-Val), N-CH$_3$
	NH		7.72 brs	H_α		H_α
N-Me-Val		167.9 C=O				
	α	65.9 CH	4.57 d (10.9)	H_β	*N*-Me-Val-CO, Pro-CO, C_β, C_γ, C_δ, NCH$_3$	NH (*O*-prenyl-Tyr), H_γ, H_δ, H_α (Ser)
	β	25.9 CH	2.33 m	H_α, H_γ, H_δ	N-Me-Val-CO, C_α, C_γ, C_δ, NCH$_3$	H_γ, H_δ
	γ	19.8 CH$_3$	0.98 d (15.7)	H_β	C_α, C_β, C_δ	H_α, H_β
	δ	19.1 CH$_3$	0.85 d (6.9)	H_β	C_α, C_β, C_γ	H_α, H_β, NCH3
	N-CH$_3$	28.7 CH$_3$	2.54 s		C_α, Pro-CO	H_δ, NH (*O*-prenyl-Tyr)

* overlapping signals

Whereas *O*-prenyl-tyrosine, *N*-methyl-alanine, 3-phenyllactic acid, proline and serine showed similar peaks, only one major difference was observed in the NMR spectra, pertaining to the remaining amino acid. Instead of an *N*-methylated isoleucine, *N*-methyl-valine was observed with two methine protons at δ 4.57 and 2.33 and two methyl groups at δ 0.98 and 0.85. As in the case of bionectriamide A (**26**), the sequence of **27** was deduced from ROESY and HMBC data and was confirmed by the ESI fragmentation pattern as shown in figure 3.9.3.
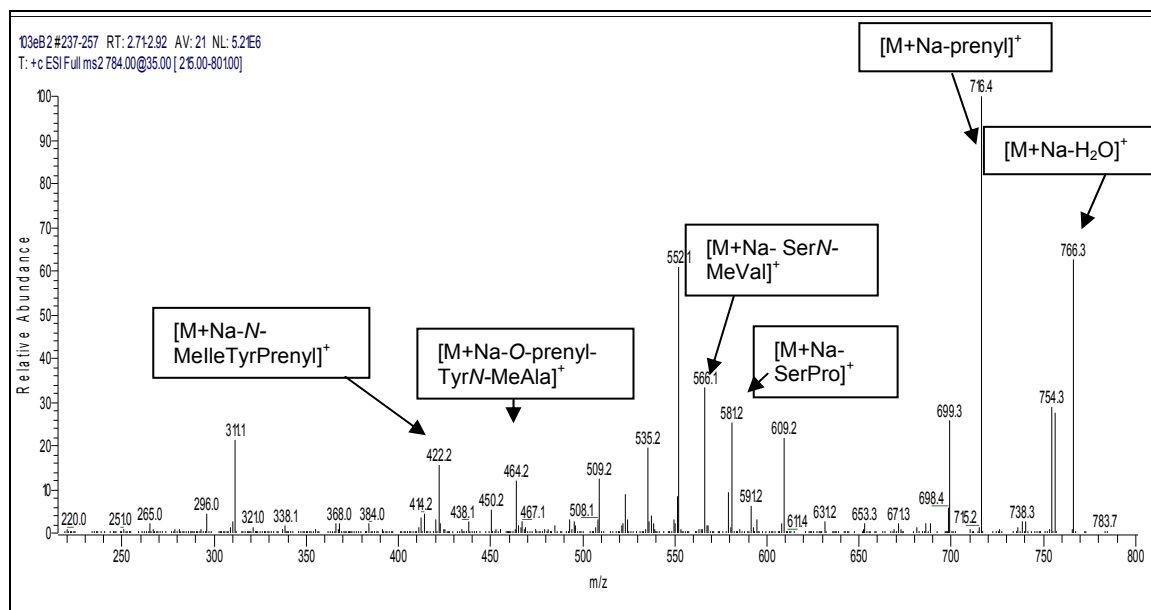
Figure 3.9.3. ESI-MS fractionation pattern of bionectriamide B (**27**).
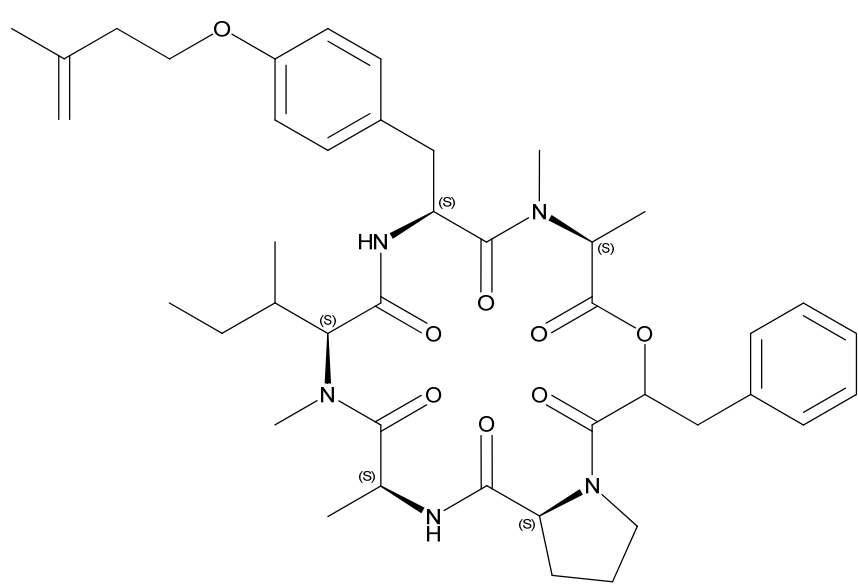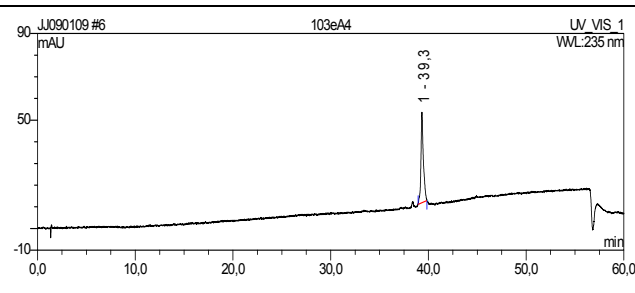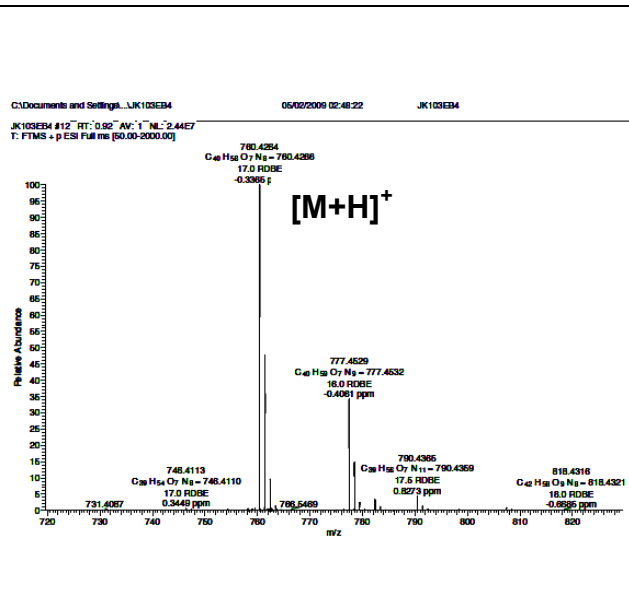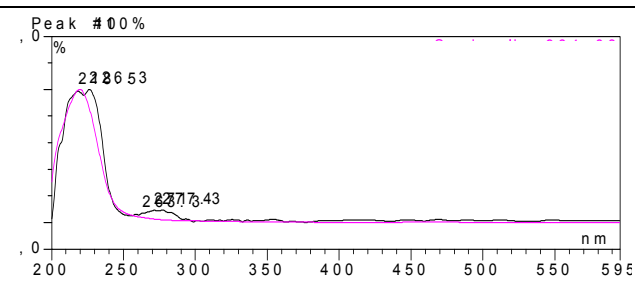
For determination of the stereochemistry of the amino acids, a Marfey analysis was performed after hydrolysis of the depsipeptide to generate diastereometric reaction products. From comparison of their LCMS data with those of the standards, the configuration of the amino acids in **27** were determined as L-*O*-prenyl-Tyr, L-Ser, L-Pro and N-Me-L-Ala (see table 3.9.3b). *N*-methyl-valine could not be elucidated, but as all other amino acids show L configuration, it is assumed that this amino acid also has L-configuration. Moreover, comparison of the retention times of derivatized valine and isoleucine in the LCMS showed that the D-derivative eluted approx. 4 min later than the L-derivative. Comparison of the *N*-methylated isoleucine with its unmethylated congener revealed a difference in the retention times of approx. 1 minute. Thus, a retention time of 30.3 min of the *N*-methylated valine of bionectriamide B (see table 3.9.3b) would be in agreement with the hypothesis of all amino acids belonging to the L-series.

Table 3.9.3b. Retention times of standard amino acid derivatives and derivatives of the hydrolyzate of bionectriamide B (**27**) in LCMS. The respective retention time of FDAA is given as a reference.

Amino acid	Retention time LCMS	FDAA ret. time LCMS
D-Tyr	28.1	21.8
L-Tyr	25.6	21.8
27	25.1	21.0
D-Ser		22.8
L-Ser	20.1	22.8
27	20.2	21.8
D-Pro	24.3	
L-Pro	23.6	
27	23.5	21.8
N-Me-D-Ala	24.9	
N-Me-L-Ala	25.1	
27	25.2	21.8
D-Val	33.1	22.6
L-Val	29.4	22.6
D-Ile	35.1	22.5
L-Ile	31.3	22.5
L-*N*-Me-Ile	32.5	21.8
27 (*N*-Me-Val)	30.3	21.8

3.9.4 Bionectriamide C (28, new compound)

Bionectramide C	
Synonym(s)	(3S,6S,9S,12S,20aS)-15-benzyl-6-sec-butyl-3,5,11,12-tetramethyl-9-(4-(3-methylbut-3-enyloxy)benzyl)octahydro-1H-pyrrolo[1,2-d][1,4,7,10,13,16]oxapentaazacyclooctadecine-1,4,7,10,13,16(15H,18H,19H,20H,20aH)-hexaone
Sample code	JCM10.3 eB4
Biological source	*Bionectria ochroleuca* (from *Sonneratia caseolaris*)
Sample amount	4.5 mg
Physical description	white powder
Molecular formula	$C_{42}H_{57}N_5O_8$
Molecular weight	759 g/mol
Optical rotation $[\alpha]_D^{20}$	-77° (*c* 0.7, CHCl$_3$)
Retention time HPLC	39.3 min (standard gradient)

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Bionectriamide C was obtained as a white powder from the EtOAc extract of the large scale fermentation on rice of *Bionectria ochroleuca*. The UV/Vis spectrum of the compound exhibited maxima at 219, 226 and 277 nm. The molecular formula $C_{42}H_{57}N_5O_8$ was derived from the HRESIMS exhibiting a peak at *m/z* 760.4264 (calcd. for $C_{42}H_{58}N_5O_8$ 760.4285) and indicated a close similarity to compounds **26** and **27**, with one oxygen less than bionectriamide A. Extensive analysis of the obtained NMR data showed that the serine residue in **26** was replaced by an alanine residue in **28**, as evident from the upfield shifts of the two proton signals to δ 4.65 of the α proton and to δ 1.21 of the aliphatic methyl group resulting in a alanine residue. Its NH group at δ 8.89 showed correlations to the methyl group and the carbonyl groups of proline, indicating that both amino acids were adjacent.

But beside the location of the interchanged amino acid, the NMR spectra also revealed an alteration in the prenyl rest of the tyrosine. Instead of two methyl groups as in compounds **26** and **27** only one was found at δ 1.80, sharing a COSY cross peak with a CH_2 at δ 5.20. Both proton signals also correlated with a quaternary carbon at δ 141.3. Thus, instead of a double bond between carbon 2 and 3, bionectriaminde C (**28**) featured an exomethylene group and thus a rearranged prenyl rest, herein referred to as an "isoprenyl" moiety.

Table 3.9.4a. NMR data for bionectriamide C (**28**) in CDCl$_3$.

Unit	C/H no.	δ$_C$ mult.	δ$_H$ (*J* Hz)	COSY	HMBC	ROESY
O-iso prenyl-Tyr		168.1 C=O				
	α	51.2 CH	4.72 td (8.8; 4.4)	H$_β$, NH	*N*-Me-Ile-CO	H$_α$, *N*-Me-Ala-CH$_3$
	β	35.8 CH$_2$	2.96 dd (12.8: 4.0) 2.59 brd (4.3)	H$_β$, H$_α$ H$_α$, H$_β$	C$_α$, C$_δ$, *N*-Me-Ile-CO C$_α$, C$_δ$, *N*-Me-Ile-CO	H$_β$, H$_δ$(*N*-Me-Ile) H$_α$, H$_β$
	γ	128.5 qC				
	δ	130.2 CH	7.04 d (8.6)	H$_ε$	C$_β$, C$_γ$, C$_ε$, C$_ζ$	H$_α$, H$_β$
	ε	114.4 CH	6.82 d (8.6)	H$_δ$	C$_δ$, C$_ε$, Cζ	H$_1$
	ζ	156.2 qC				
	1	69.7 CH$_2$	4.17 ddd (11-12; 7; 1-2)	H$_2$	C$_2$, C$_3$, C$_ζ$	H$_2$, H$_5$
	2	63.0 CH	4.86 dt (2.0; 6.5)	H$_1$	C$_1$, C$_3$, C$_4$, C$_5$	H$_1$, H$_4$
	3	141.3 qC				
	4	126.6 CH$_2$	5.20 s 5.02 s	H$_4$, H$_5$ H$_4$, H$_5$	C$_2$, C$_3$, C$_5$ C$_2$, C$_5$	H$_2$, H$_1$, H$_4$,H$_5$ H$_4$
	5	17.6 CH$_3$	1.80 s	H$_4$	C$_2$, C$_3$, C$_4$	H$_1$, H$_2$, H$_4$
	NH		9.53 d (8.7)	H$_α$ (*O*-iso prenyl-Tyr)	C$_α$ (*O*-isoprenyl-Tyr)	H$_β$, H$_α$ (*N*-Me-Ile)
N-Me-Ala		169.5 C=O				
	α	58.4 CH	3.71 q (5.0)	H$_β$	C$_β$, N-CH3, *O*-isoprenyl-Tyr-CO	H$_β$, N-CH$_3$
	β	15.9 CH$_3$	1.05 d (6.6)	H$_α$	C$_α$, CO	H$_α$, N-CH$_3$
	N-	32 CH$_3$	2.93 s		C$_α$	H$_α$, H$_β$,H$_α$ (*O*-

	CH₃					isoprenyl-Tyrl)
3-Ph-Lac		165.4 C=O				
	α	71.5 CH	5.51 dd(8.7; 5.1)	H$_\beta$	H$_\gamma$, CO, *N*-Me-Ala-CO	H$_\beta$, H$_\delta$ (Pro), H$_\alpha$ (*N*-Me-Ala)
	β	33 CH₂	3.12 dd (12.2; 3.2)	H$_\beta$, H$_\alpha$	C$_\alpha$,C$_\delta$, C$_\gamma$, CO	H$_\beta$
			2.81 dd (13.1; 4.8)	H$_\beta$, H$_\alpha$	C$_\alpha$,C$_\delta$,C$_\gamma$, CO	H$_\alpha$, H$_\beta$
	γ	136.4 qC				
	δ	129.5 CH	7.19 dd (8.8; 2.0)*	H$_\varepsilon$	C$_\beta$, C$_\varepsilon$, C$_\zeta$	H$_\alpha$, H$_\beta$
	ε	126.6 CH	7.25 d (7.2)*	H$_\delta$, H$_\zeta$	C$_\gamma$, C$_\delta$, C$_\zeta$,	
	ζ	128.4 CH	7.26 m*	H$_\varepsilon$	C$_\delta$, C$_\varepsilon$	
Pro		171.8 C=O				
	α	58.4 CH	4.31 dd (8.6; 2.1)	H$_\beta$	C$_\beta$, C$_\gamma$, C$_\delta$	H$_\beta$
	β	29.1 CH₂	1.69 m	H$_\beta$	C$_\gamma$	H$_\alpha$, H$_\beta$
			2.00 m*	H$_\alpha$, H$_\beta$	CO	H$_\alpha$, H$_\beta$
	γ	23.7 CH₂	1.82 m*	H$_\beta$, H$_\delta$		
			1.77 m			
	δ	45.8 CH₂	3.75 dt (8.5; 5.2)*	H$_\delta$, H$_\gamma$	C$_\beta$	H$_\gamma$, H$_\delta$, H$_\alpha$, H$_\delta$, H$_\delta$
			3.21 q (8.5)	H$_\delta$, H$_\gamma$	C$_\gamma$	
Ala		173.8 C=O				
	α	44.4 CH	4.65 q (6.3)	H$_\beta$, NH	C$_\beta$, CO, Pro-CO	H$_\beta$
	β	17.2 CH₃	1.21 d (6.6)	H$_\alpha$	C$_\alpha$, CO	H$_\alpha$, N-CH₃ (*N*-Me-Ile), H$_\beta$ (Pro)
	NH		8.89 d (4.3)	H$_\alpha$	C$_\beta$, CO (Pro)	H$_\alpha$, H$_\beta$, H$_\alpha$ (Pro), H$_\beta$ (Pro)
N-Me-Ile		168.0 C=O				
	α	64.8 CH	4.53 d (8.8)	H$_\beta$	C$_\beta$, C$_{\gamma CH3}$, N-CH₃, CO, Ala-CO	H$_\gamma$, H$_\delta$, H$_\alpha$(Ala)
	β	32.0 CH₂	2.00 m*	H$_\gamma$		H$_\gamma$, H$_\delta$, N-CH₃
	γ	24.3 CH₃	1.23 m*	H$_\gamma$, H$_\delta$		
			0.90 m*			
	γ	17.2 CH₃	0.90 d (6.6)	H$_\beta$	C$_\beta$, C$_\gamma$	H$_\alpha$, H$_\beta$
	δ	13.0 CH₃	0.91 d (6.3)	H$_\gamma$	C$_\alpha$, C$_\beta$, C$_\gamma$	H$_\alpha$, H$_\beta$, H$_\gamma$, H$_\beta$ (*O*-isoprenyl-Tyr),
	N-CH₃	28.4 CH₃	2.40 s		CO, CO(Ala)	H$_\alpha$, H$_\beta$, H$_\gamma$

* overlapping signals
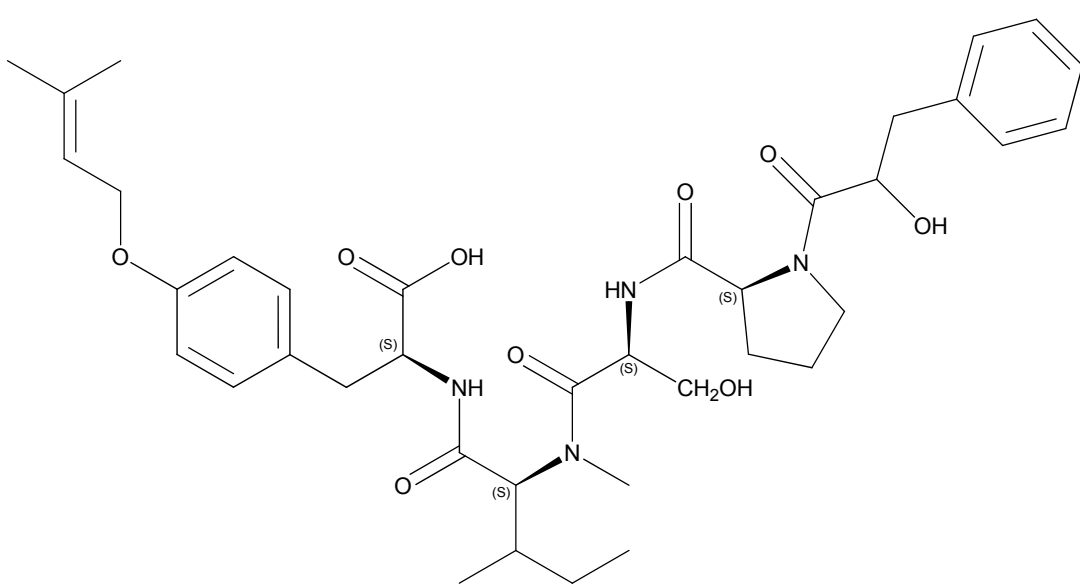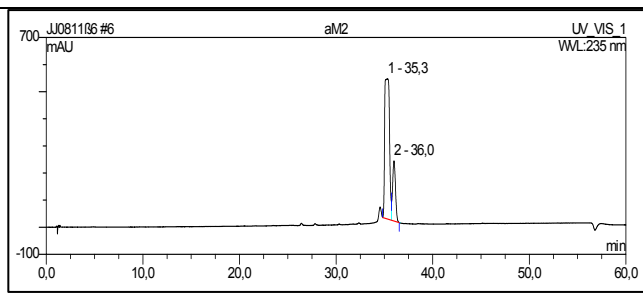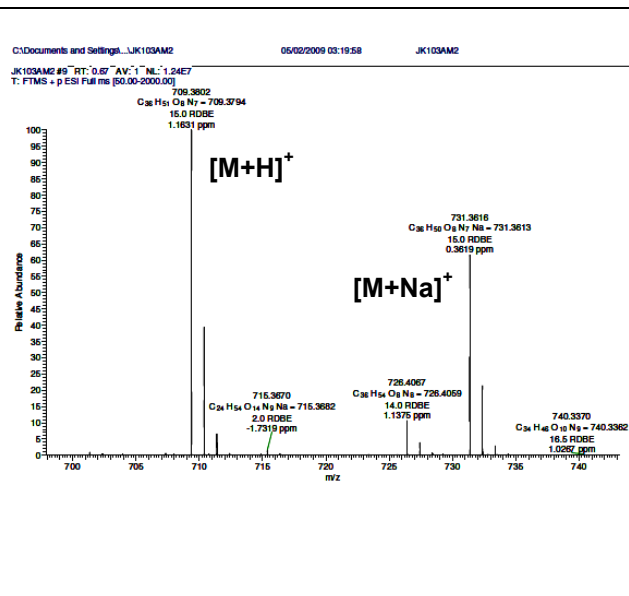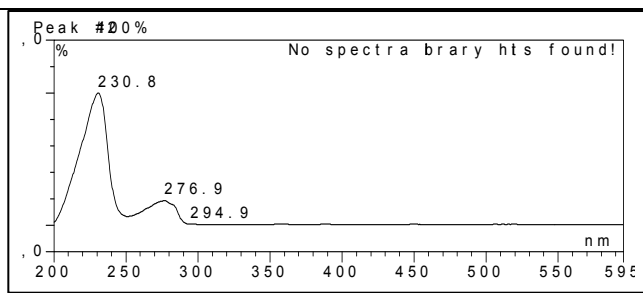
As for the previous bionectriamides, the stereochemistry of the amino acids in **28** was established after a Marfey experiment with the hydrolysate of the depsipeptide followed by the comparison of the retention times of the reaction products with those of commercially available standards. The result was L-Tyr, *N*-Me-L-Ile, L-Ala, L-Pro and *N*-Me-L-Ala as shown in table 3.9.4b.

Table 3.9.4b. Retention times of standard amino acid derivatives and derivatives of the hydrolyzate of bionectriamide C (**28**) in LCMS. The respective retention time of FDAA is given as a reference.

Amino acid	Retention time LCMS	FDAA ret. time LCMS
D-Tyr	28.1	22.1
L-Tyr	25.6	22.1
28	25.6	21.9
N-Me-L-Ile	32.4	22.7
28	31.2	22.3
D-Ala	27.4	22.8
L-Ala	24.2	22.8
28	23.3	21.9
D-Pro	24.3	22.0
L-Pro	23.6	22.0
28	23.6	21.9
N-Me-D-Ala	25.0	21.8
N-Me-L-Ala	25.2	21.8
28	25.2	21.9

3.9.5 Bionectriamide D (29, new compound)

Bionectriamide D	
Synonym(s)	(2S)-2-((2S)-2-((2R)-3-hydroxy-2-((2S)-1-(2-hydroxy-3-phenylpropanoyl)pyrrolidine-2-carboxamido)-N-methylpropanamido)-3-methylpentanamido)-3-(4-(3-methylbut-2-enyloxy)phenyl)propanoic acid
Sample code	JCM10.3 aM2
Biological source	*Bionectria ochroleuca* (from *Sonneratia caseolaris*)
Sample amount	2.4 mg
Physical description	white powder
Molecular formula	$C_{38}H_{52}N_4O_9$
Molecular weight	708 g/mol
Optical rotation $[\alpha]_D^{20}$	-140° *c* (0.5, MeOH)
Retention time HPLC	35.7 min (standard gradient)

The linear depsipeptide bionectriamide D (**29**) was obtained from *Bionectria ochroleuca* after purification of the EtOAc extract of the large scale fermentation. The compound exhibited an UV/Vis spectrum with maxima at 226 and 275 nm, resembling those of the previous bionectriamides. From the HRESIMS exhibiting a peak at *m/z* 709.3602 [M+H]$^+$, the molecular formula $C_{38}H_{52}N_4O_9$ was determined (calcd. for $C_{38}H_{53}N_4O_9$ 709,3813), indicating the presence of only four amino acids. When preparing for NMR measurement, the compound revealed more polar characteristics than the other bionectriamides, as it did not be dissolved in CDCl$_3$, but only in DMSO-*d$_6$*. Signals of the amino acid residues for proline, serine, *N*-methylated isoleucine and *O*-prenyl-tyrosine were observed, as well as signals for 3-phenyllactic acid, proving the absence of *N*-methylated alanine. A conspicuous feature of the NMR spectra was the upfield shift of H$_\alpha$ of 3-Ph-Lac to δ 4.1 as compared to δ 5.7 in the other depsipeptides, indicating the presence of an alcohol function instead of an ester moiety. This finding and the lack of correlations between the α-hydroxycarboxylic acid and the *O*-prenyl-tyrosine indicated that bionectriamide D was a linear and not a cyclic peptide. This finding was also consistent with the molecular formula obtained from HRESIMS. Moreover, it also explained the increase in polarity of **29** in comparison to bionectriamides A-C (**26**-**28**).

Table 3.9.5a. NMR data for bionectriamide D (**29**) in DMSO-*d$_6$*.

Unit	C/H no.	δ_C mult.	δ_H (*J* Hz)	COSY*	HMBC°
3-Ph-Lac		165.4 C=O			
	α	71.5 CH	4.1 d	H$_\beta$	
	β	41 CH$_2$	2.85 m*	H$_\alpha$	C$_\gamma$, C$_\delta$, CO
			2.7 m*		C$_\gamma$, C$_\delta$, CO
	γ	138 qC			
	δ	129 CH	7.19 dd (8.8; 2.0)*	H$_\varepsilon$	C$_\gamma$, C$_\zeta$
	ε	126 CH	7.25 d (7.2)*	H$_\delta$	C$_\gamma$, C$_\delta$, C$_\varepsilon$
	ζ	128 CH	7.26 m*		
Pro		171.5 C=O			
	α	52 CH	4.7 m		C$_\beta$, C$_\gamma$
	β	32 CH$_2$	3.48 m*	H$_\beta$, H$_\gamma$	C$_\alpha$
			3.2 m*	H$_\beta$, H$_\gamma$	
	γ	22 CH$_2$	1.8 m*	H$_\beta$, H$_\gamma$,	
			1.75 m	H$_\gamma$, H$_\delta$	
	δ	32 CH$_2$	1.75-1.8	H$_\gamma$	
Ser		171.8 C=O			
	α	44.4 CH	4.8 m		C$_\beta$, CO(Pro)
	β	62 CH$_2$	3.65 m		CO
			3.48		CO
	NH		8.18 d (4.3)		CO
N-Me-Ile		168.5 C=O			
	α	61 CH	4.51 m		CO, CO(Ser)
	β	31 CH	1.9 m*	H$_{\gamma(CH3)}$	
	γ	24 CH$_2$		H$_\gamma$, H$_\delta$	
				H$_\gamma$, H$_\delta$	C$_\beta$, C$_\delta$
	γ	15.5 CH$_3$	0.80 m	H$_\beta$	C$_\alpha$, C$_\beta$, C$_\gamma$
	δ	10 CH$_3$	0.75 m*	H$_\gamma$	C$_\beta$, C$_\gamma$
	N-CH$_3$	28.4 CH$_3$	2.85 s		C$_\alpha$, CO(Ser)
O-prenyl-Tyr		172 C=O			
	α	51.2 CH	4.72 td (8.8; 4.4)		C$_\beta$, C$_\gamma$, CO, CO(*N*-Me-Ile)
	β	35.8 CH$_2$	2.96 dd (12.8: 4.0)	H$_\beta$, H$_\delta$	C$_\gamma$, C$_\delta$, CO
			2.59 brd (4.3)	H$_\beta$, H$_\delta$	C$_\gamma$, C$_\delta$, CO
	γ	131 qC			

δ	130.5 CH	7.04 d (8.6)	H_β, H_ε	C_β, C_δ, C_ε, C_ζ
ε	116 CH	6.82 d (8.6)	H_δ	C_γ
ζ	156.5 qC			
1	51 CH_2	4.49 d (6.4)	H_2	C_ζ, C_2, C_3
2	120.5 CH	5.53 brs	H_1, H_4, H_5	C_4, C_5
3	137 qC			
4	25 CH_3	1.72 s	H_2	C_2, C_3, C_5
5	18 CH_3	1.76 s	H_2	C_2, C_3, C_4
NH_2		8.45 d (8.7)		CO

* overlapping signals

The depsipeptide was hydrolyzed and the stereochemistry of the amino acids analyzed *via* Marfey's method. On this basis, the configurations of the amino acids in **29** were determined as L-Tyr, *N*-Me-L-Ile, L-Ser and L-Pro (see table 3.9.5b).

Table 3.9.5b. Retention times of standard amino acid derivatives and derivatives of the hydrolyzate of bionectriamide D (**29**) in LCMS (modified program). The respective retention time of FDAA is given as a reference.

Amino acid	Retention time LCMS	FDAA ret. time LCMS
D-Tyr	45.5	8.2
L-Tyr	43.5	8.2
29	43.0	8.2
N-Me-L-Ile	41.5	8.2
29	41.8	8.2
D-Ser	4.9	8.2
L-Ser	5.1	8.2
29	5.2	8.2
D-Pro	10.9	8.2
L-Pro	9.4	8.2
29	9.8	8.2

To summarize, the sequences of all four bionectriamides are shown in table 3.9.5c.

Table 3.9.5c. Amino acid sequences of the isolated bionectriamides A-D (**26**-**29**)

	Amino acid residue											
A (**26**) (cyclic)	3-Ph-Lac	→	L-Pro	→	L-Ser	→	*N*-Me-L-Ile	→	L- *O*-prenyl Tyr	→	*N*-Me-L-Ala	
B (**27**) (cyclic)	3-Ph-Lac	→	L-Pro	→	L-Ser	→	*N*-Me-Val	→	L- *O*-prenyl Tyr	→	*N*-Me-L-Ala	
C (**28**) (cyclic)	3-Ph-Lac	→	L-Pro	→	L-Ala	→	*N*-Me-L-Ile	→	L-*O*- isoprenyl Tyr	→	*N*-Me-L-Ala	
D (**29**) (linear)	3-Ph-Lac	→	L-Pro	→	L-Ser	→	*N*-Me-L-Ile	→	L- *O*-prenyl Tyr			

3.9.6 Bioactivity test results for compounds isolated from *Bionectria ochroleuca*

Among the isolated depsipeptides, only bionectriamide A (**26**) was subjected to antibiotic screening, and as the test results were negative and the amount of the other bionectriamides was low, further tests were ommitted. All isolated compounds were subjected to a cytotoxicity assay, which is summarised in table 3.9.6. Verticillin D (**25**) showed pronounced cytotoxic activities against the tested cell line. Antiproliferative properties were prevalent among the cyclic depsipeptides bionectriamides A-C (**26**-**28**) with EC_{50} values ranging between 0.1 and 6.7 µg/mL, whereas the linear bionectriamide D (**29**) did not exhibit any cytotoxic activity.

Shortly before the submission of this thesis, we obtained the results of antitrypanosomal testing, carried out by Carol Clements at University of Strathclyde, Glasgow. Preliminary results indicate a pronounced activity against *T. brucei* with an MIC of 3.12 µg/mL. However, due to the limited amounts of pure compounds available, these results were obtained with an enriched fraction containing a mixture of bionectriamides A-D (**26**-**29**), but lacking verticillin D (**25**). Currently, further tests with the isolated pure compounds are under way.

Table 3.9.6. Biological screening test results for isolated compounds of *Bionectria ochroleuca*.

Compound	L5178Y survival rate in % (@ 10 µg/mL)	EC_{50} [µg/mL]
Verticillin D (**25**)	0.5	<0.1
Bionectriamide A (**26**)	1.7	2.6
Bionectriamide B (**27**)	21.7	6.7
Bionectriamide C (**28**)	15.6	5.6
Bionectriamide D (**29**)	114.3	>10

4. Discussion

4.1 Endophytic fungi isolated from Chinese mangrove plants

Mangrove plants from the South Chinese Sea proved to be a very fruitful source of endophytic fungi. From 20 different plants, more than 90 fungal strains could be isolated. Due to that enormous amount, only the most promising fungi could be identified. Despite the possibility that some of these isolates might actually represent the same fungal strains, halotolerant plants were thus confirmed as a reliable source for the isolation of endophytes.

4.2 Isolated compounds from endophytic fungi

From the selected species, 29 compounds, including alkaloids, polyketides and depsipeptides, were isolated. A closer look will be taken at their structural features, their biogenetic origin, and biological activities.

4.2.1 Alkaloids

4.2.1.1 Quinolactacin A1 and A2

The diastereomeric pyrroloquinolone alkaloids were first isolated from an insect-derived *Penicillium* sp. and were shown to possess inhibitory activity against tumour necrosis factor (TNF) (Takahashi et al. 2000). Their biosynthesis was investigated by feeding experiments using ^{13}C-labelled precursors and proven to comprise several steps, including incorporation of acetate-, isoleucine-, methionine- and glucose units (Sasaki et al. 2006).

4.2.1.2 Tetramic acids: Tenuazonic acid and equisetin

Tetramic acids, i.e. substituted pyrrolidine-2,4-diones acylated at the 3-position, constitute an important group of secondary metabolites exhibiting diverse bioactivities. In the course of this study, I was able to isolate two tetramic acids, tenuazonic acid (**9**) from *Nigrospora* sp. and equisetin (**4**) from *Fusarium incarnatum*.

Tenuazonic acid has been found in several *Alternaria*, *Aspergillus* and *Sphaeopsidales* strains. The pyrrolidine-2,4-dione is biosynthesized from L-isoleucine and 2 molecules of acetate, by elimination of 2 molecules of water (Nolte et al. 1980, Royles 1995). Equisetin is frequently found in *Fusarium* species. It is composed of a partially reduced polyketide and a serine residue. Both substances display keto-enol tautomery as depicted in figure 4.2.1.2, and these different tautomeric forms of the tetramic acids in solution must be considered in the course of the structure determination of the substances. Low temperature measurement made possible the assignment of the enol structure of the tetramic acid heterocycle in equisetin, which was not amendable to ^{1}H NMR analysis at room temperature.

Both compounds are notorious for causing several diseases in crops like rice and tobacco such as sheet rot disease, but have also been described as possessing insecticidal, antibacterial, antineoplastic and antiviral activity (Vesonder et al 1979, Cole et al. 1981, Phillips et al. 1989, Royles 1995).
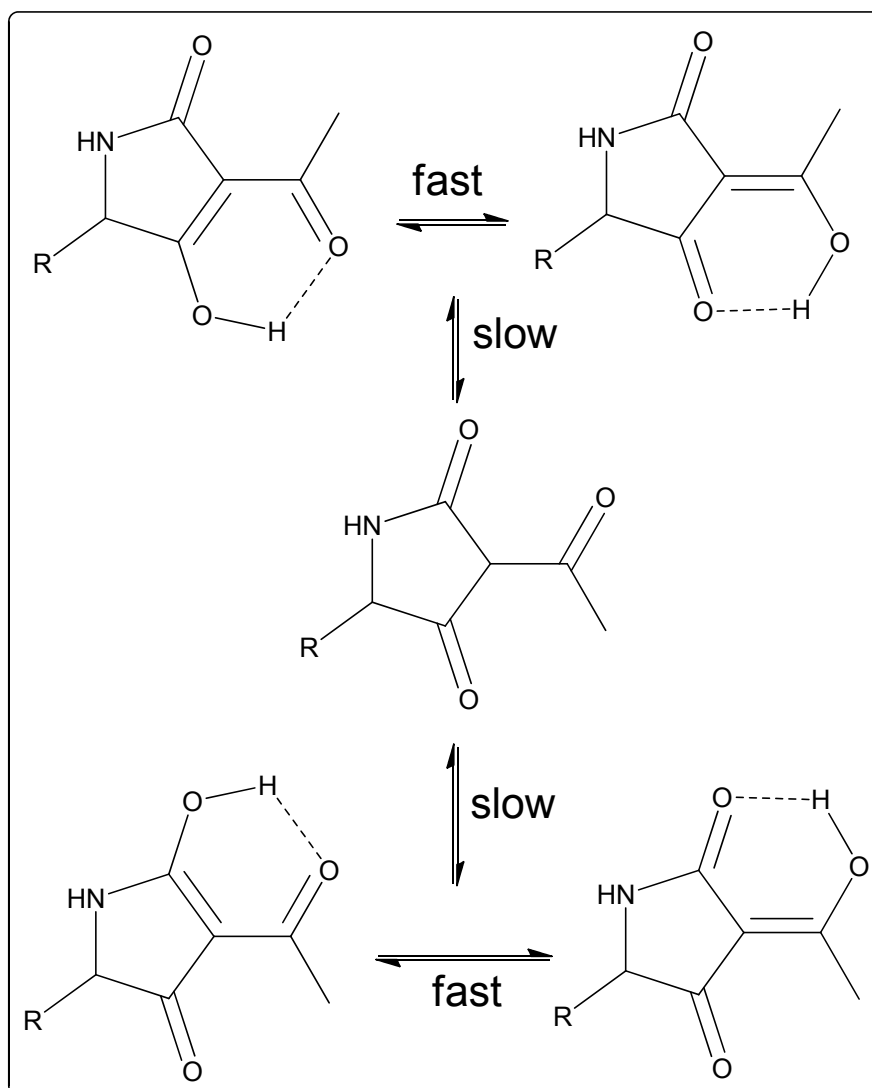
Figure 4.2.1.2. Tautomerism of 3-acylated pyrrolidine-2,4-diones (Nolte et al. 1980).

Due to their pronounced toxicities they are considered unsafe for potential pharmaceutical use. However, the described cytotoxic effect of tenuazonic acid on mouse lymphoma cells (Royles 1995) could not be observed in this study, as the isolated compound only mildly affected the growth of the L5178Y cell line. On the other hand, the antibiotic activity observed for equisetin in this study, mirrored the antibiotic spectrum described in literature.

4.2.2 Polyketides

4.2.2.1 Citrinin

Citrinin is a pentaketide that is biosynthetically derived from five acetate units, which are thrice methylated, aromatized *via* an aldol reaction, reduced and dehydrated to give the quinonemethide system of citrinin. Three *C*-methyl substituents are inserted into the acetate-derived skeleton by *S*-adenosylmethionine (SAM), one of these introduced methyls is oxidized into a carboxyl. The methyls were found to be introduced into the polyketide prior to the release of the first aromatic intermediate, which undergoes reduction, cyclization via a hemiacetal and dehydration to the quinonemethide system (Barber et al 1981, Dewick 2002).

This mycotoxin's previously reported antibiotic activity (Barber et al 1987) has been confirmed in the course of this study, exhibiting a MIC of 31.2 µg/mL against *Streptococcus pyogenes*. Furthermore, the compound has been demonstrated to possess a number of chronic toxic effects, which is the reason why it cannot be used as antibiotic drug. Citrinin is a well-known contaminant of a number of agricultural products and hence several studies have been carried out on its detoxification via degradation. Detoxification could be achieved by heating of the dissolved citrinin up to 140 °C and thereby degenerating the compound via decarboxylation and hydrolytic ring opening to the non-toxic "citrinin H2" (Hirota et al. 2002, Clark et al. 2006).
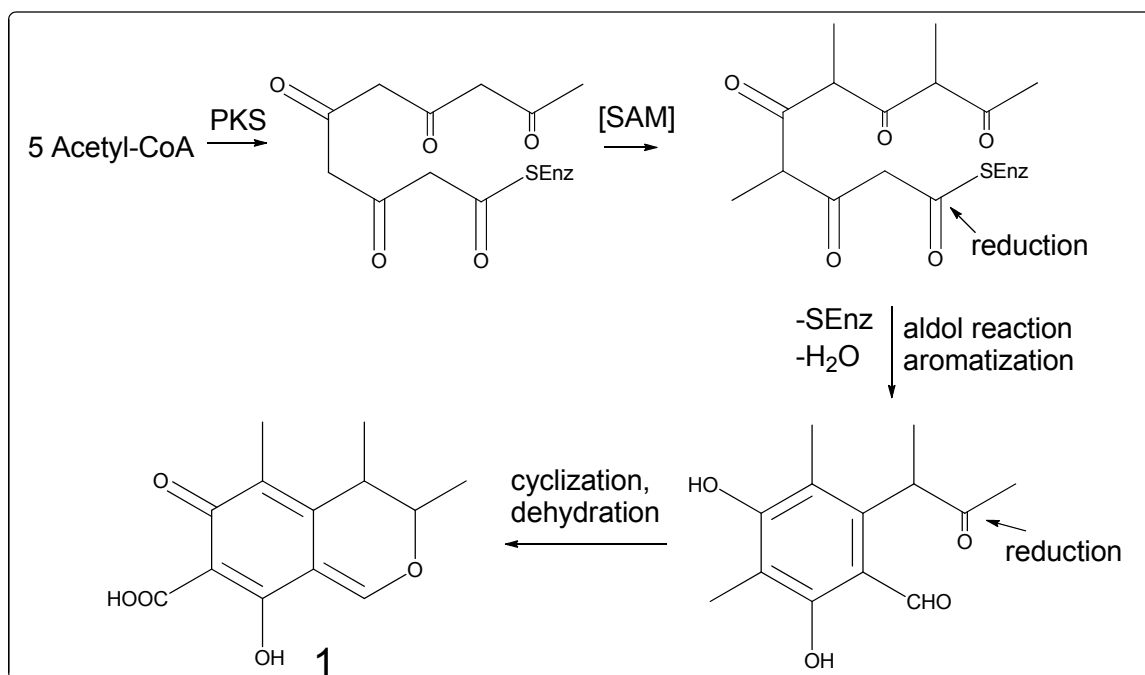
Figure 4.2.2.1. Biosynthesis of citrinin (Barber et al. 1981, Dewick 2002).

4.2.2.2 Chromones: Aloesol and 4,5-dimethyl-7-hydroxychromone

The chromones 4,5-dimethy-7-hydroxy-chromone (**12**) and aloesol (**5**), are, unlike citrinin, synthesized *via* a hexaketide or a heptaketide precursor, respectively as shown in figure 4.2.2.2. The aromatic ring is formed from a poly-β-keto-chain by aldol reaction followed by loss of water and enolization, while decarboxylation causes the shortening of the side chain. Lactonization gives the chromones **12** or **5** (Kashiwada et al. 1984, Ayer and Racock 1990). Furthermore, aloesol was found to be a decomposition product of aloesin (5-methyl-7-hydroxy-2-propionyl-8-glucosyl-chromone), to the effect that cleavage of the C-glycolsyl bond and reduction of the acetonyl side chain by intestinal bacteria yielded **5** (Che et al. 1991).

Both compounds have previously been described as plant metabolites. Whereas 4,5-dimethyl-7-hydroxychromone was described as a fungal product before, this is the first report of aloesol as a secondary metabolite of fungal origin. Although aloesol was not reported from the host plant *Bruguiera sexangula*, this discovery affirms the hypothesis of plant-endophyte interactions, which might also include horizontal transfer of biogenetic gene clusters.
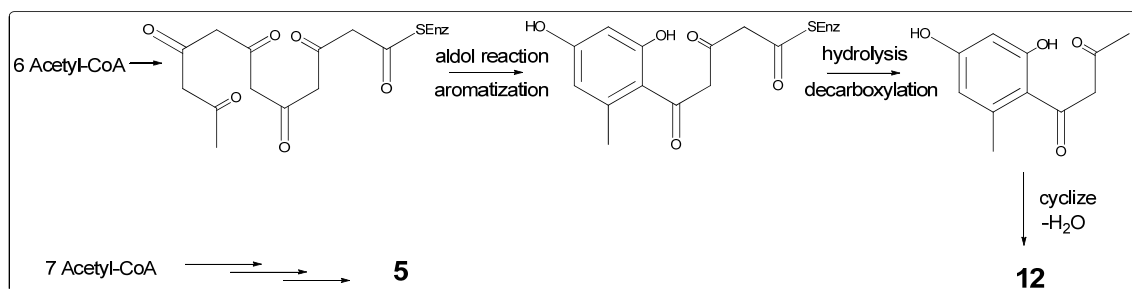
Figure 4.2.2.2. Biosynthesis of chromones **12** and **5** (Kashiwada et al. 1984, Ayer and Racock 1990).

4.2.2.3 Anthraquinone derivatives

Bostrycin (**8**), its 4-deoxy-derivative (**7**) and the further reduced 8-hydroxytetrahydroalterlolanol B (**6**) belong to a group of modified anthraquinones, which are well known to be produced by fungi from an octaketide precursor. A linear head-to-tail arrangement of the monomeric subunits, catalyzed by a fungal polyketide synthase (PKS), is followed by decarboxylation at the terminal unit at C-3 and oxidation at C-10. However, the detailed sequence of the individual steps is not well known (Stoessel et al. 1987). Subsequent oxidation at C-8 and reduction at C-1 would yield bostrycin. Extensive spectroscopic studies together with X-ray chrystallographic analysis have proven structure **8** instead of its tautomer **8a** to be the predominant one (Noda et al. 1970, Charudattan et al 1979, Kelly and Saha 1985). Similar observations apply for deoxybostrycin (**7**).

The red pigments **7** and **8** both were shown to possess phytotoxic, antibiotic and cytotoxic activities (Chadurattan et al. 1982, Ge et al. 2005, Xu et al. 2008). The present study confirmed these findings in particular for bostrycin. Studies on reduced anthraquinones have shown that a related compound, tetrahydroaltersolanol B, lacked antibiotic activity against e. g. *S. aureus* (Okamura et al. 1996), hinting at the necessity of a complete anthraquinone system for proper antibiotic activity. In this study, however, it was shown that the reduced metabolite 8-hydroxytetrahydroaltersolanol B (**6**) also exhibited considerable antibiotic activity, even though not against all bacteria tested, but especially against *S. aureus*. Moreover, all three metabolites exhibited pronounced cytotoxic properties, with anthraquinone **8** displaying the strongest and the reduced compound **6** the second strongest activity. Hence, structure-activity relationships of modified anthraquinones seem to be more complex than merely just depending on the oxidation status of the core anthraquinone system.
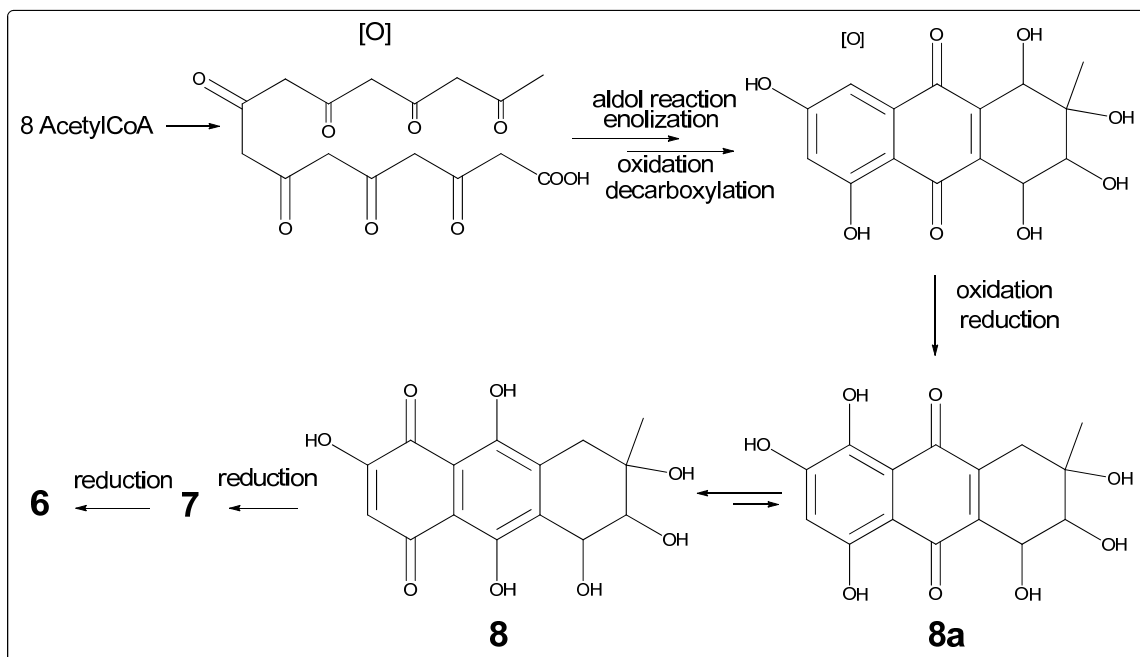
Figure 4.2.2.3. Proposed biosynthesis of modified anthraquinones **6**, **7** and **8** (after Stoessel et al. 1987).

4.2.2.4 Dibenzopyrones and biphenic acids

The α-dibenzopyrones **15**, **16**, **18** and **19** are typical *Alternaria* mycotoxins. They are all derived from a heptaketide chain composed of six malonyl-CoA and one acetyl-CoA (Abell et al. 1982, Dasenbrock and Simpson 1987, Ayar and Racock 1990, Dewick 2002). The biphenic acids **13**, **14** and **17** stem from the same precursor. Whereas in the case of alternariol and its derivatives a fungal polyketide synthase catalyzes aromatizing and cyclizing, no lactonization takes place in altenusin (Ayer and Racock 1990). Altenusin itself was previously assumed to be the precursor of alternarienonic acid (Aly et al. 2007), and a similar pathway can be assumed for alternarian acid.
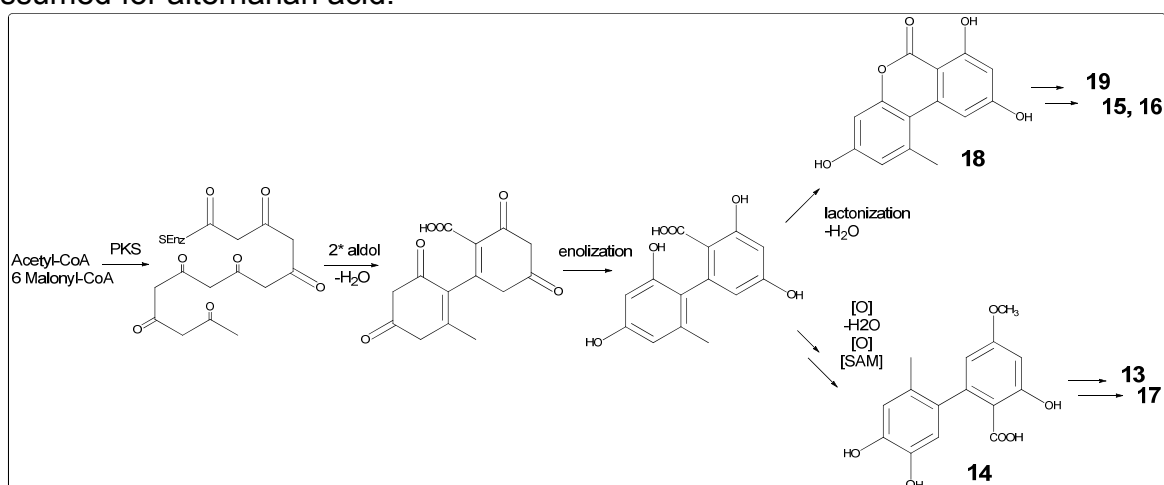
Figure 4.2.2.4. Biosynthesis of α-dibenzopyrones and biphenic acids

The growth inhibiory profiles of the α-dibenzopyrones against the cell line L5178Y have recently been assessed in our group, and these studies have affirmed the known cytotoxic nature of alternariol and its methyl ether, and the lack of

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cytotoxicity of altenuene, its 4'-epimer and alternarienonic acid (Aly et al. 2007). Hence, inhibitory tests were not repeated during this study. The cytotoxicity of alternariol is attributed to its mutagenic and potentially clastogenic profile. Moreover, it was proven to exhibit a similar estrogenicity as the known phytoestrogen daidzein (Lehmann 2006, Brugger 2006). Although altenuene has been described to exhibit mild antibiotic activity against *S. aureus* before (Jiao 2006), the lack of profound antibiotic activities of altenuene and 4'-epialtenuene as observed in the present study mirrors the literature data for these two compounds. The same applies to alternarian acid and alternarienonic acid. Both compounds did neither show cytotoxicity nor antimicrobial properties.

Altenusin, on the other hand, exhibited a broad spectrum of activity. The substance, as well as its desmethylated derivative, has been demonstrated as a cytotoxic and protein kinase inhibiting compound (Aly et al. 2007). Here it has been shown to also possess antibiotic activity and, for the first time, considerable antiviral properties, which makes it a promising natural product for further investigation.

4.2.2.5 Reduced perylenequinones

Like alternariol derivatives, perylenequinones **20**-**22** belong to the prevalently isolated *Alternaria* mycotoxins. Their biosynthesis most probably involves a phenolic oxidative coupling of two identical 1,8-dihydroxynaphthalenes, derived from a linear pentaketide precursor, further phenolic oxidative coupling to yield dihydroxyperylene-3,10-quinone, followed then either by hydroxylation or epoxidation in different positions as shown in figure 4.2.2.5. This assumption was confirmed by incorporation experiments with ^{13}C-labeled sodium acetate (Okubo 1975, Okuno 1983, Arnone 1986; Schlörke 2005).
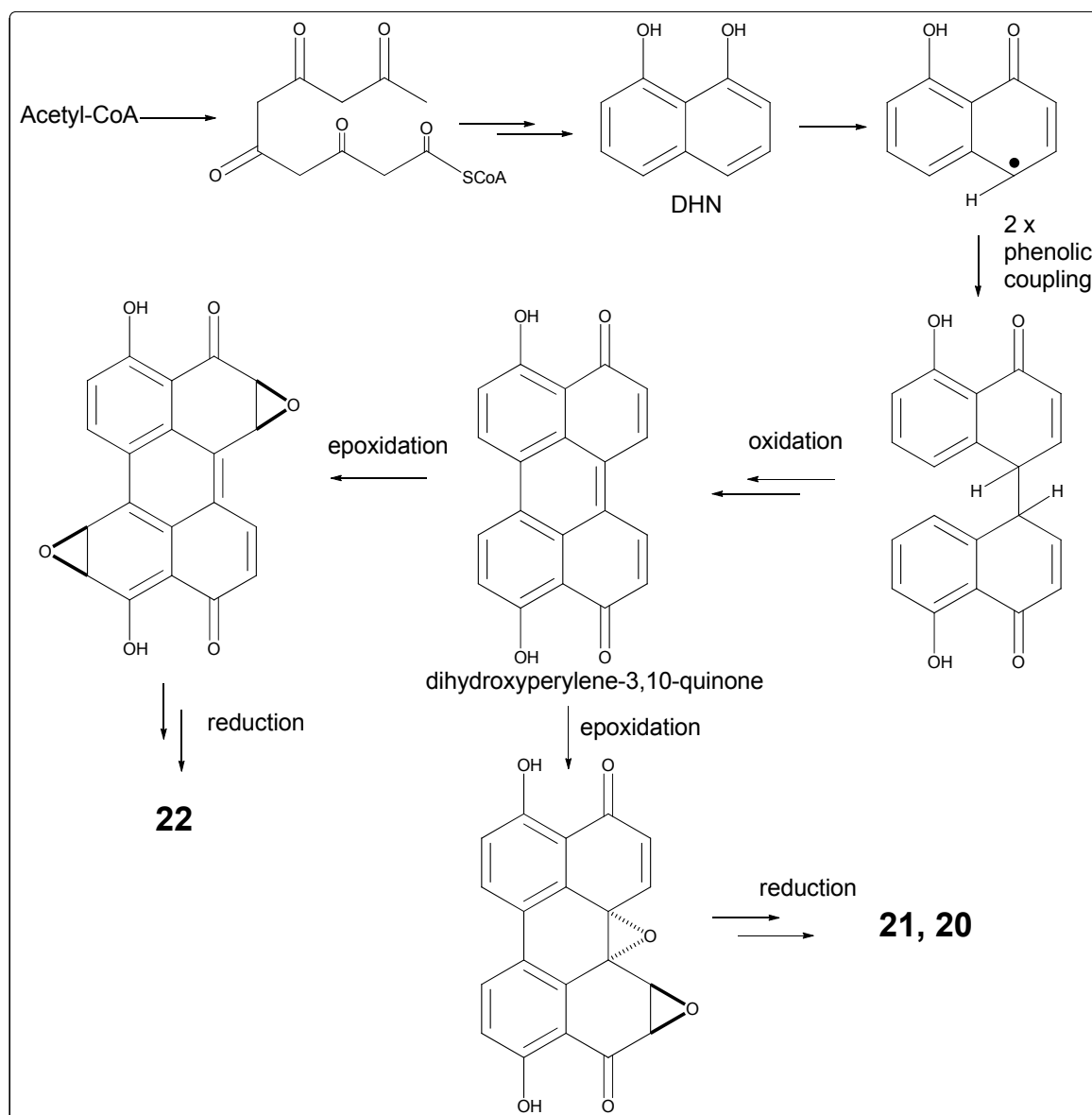
Figure 4.2.2.5. Proposed biosynthetic pathway of reduced perylenequinones (Obuko 1975, Schlörke 2005).

All three derivatives have been described as phytotoxic and antifungal agents. Furthermore, altertoxin I exhibited mutagenic effects in the Ames test (Stierle 1989). Because of the low amounts of compounds available in this study, their bioactivities could not be investigated further.

4.2.2.6 New xanalteric acids

The two new compounds xanalteric acid I and II show some degree of structural similarity to previously isolated perylenequinones. However, instead of a carbonyl function at position 3, an oxygen atom is included in the molecule. This is the first report of fungal substances featuring such a 10*H*-phenaleno[1,2,3-*de*]chromene skeleton. Interestingly, intensive literature search afforded a compound with a similar carbon skeleton, xanosporic acid. This substance was found as a bacterial degradation product of the fungal metabolite cercosporin. Cercosporin is a perylenequinone produced by members of the plant pathogenic fungal genus

Cercospora. It acts as a photosensitizer by using light energy to produce activated oxygen species and thus causing damaging leaf spot and blight diseases. As a possible method to control *Cercospora* diseases in crops, biodegradation of the pathogenic substance was investigated. Out of a total of more than 200 bacteria tested, seven species, all belonging to the genus *Xanthomonas*, were found to render cercoporin innocuous by catabolizing it to the harmless xanosporic acid (Mitchell 2002, 2003, Taylor 2006). Xanosporic acid itself proved instable and could only be isolated as its lactone xanosporolactone. It was suggested that the catabolism was initiated by Bayer-Villiger-type, cytochrome P-450 monooxygenase-mediated insertion of an oxygen atom between C-3 and C-3a, thereby expanding the A ring. Hydrolysis of this seven-membered lactone ring was followed by spontaneous recyclization with the elimination of methanol as shown in figure 4.2.2.6a.
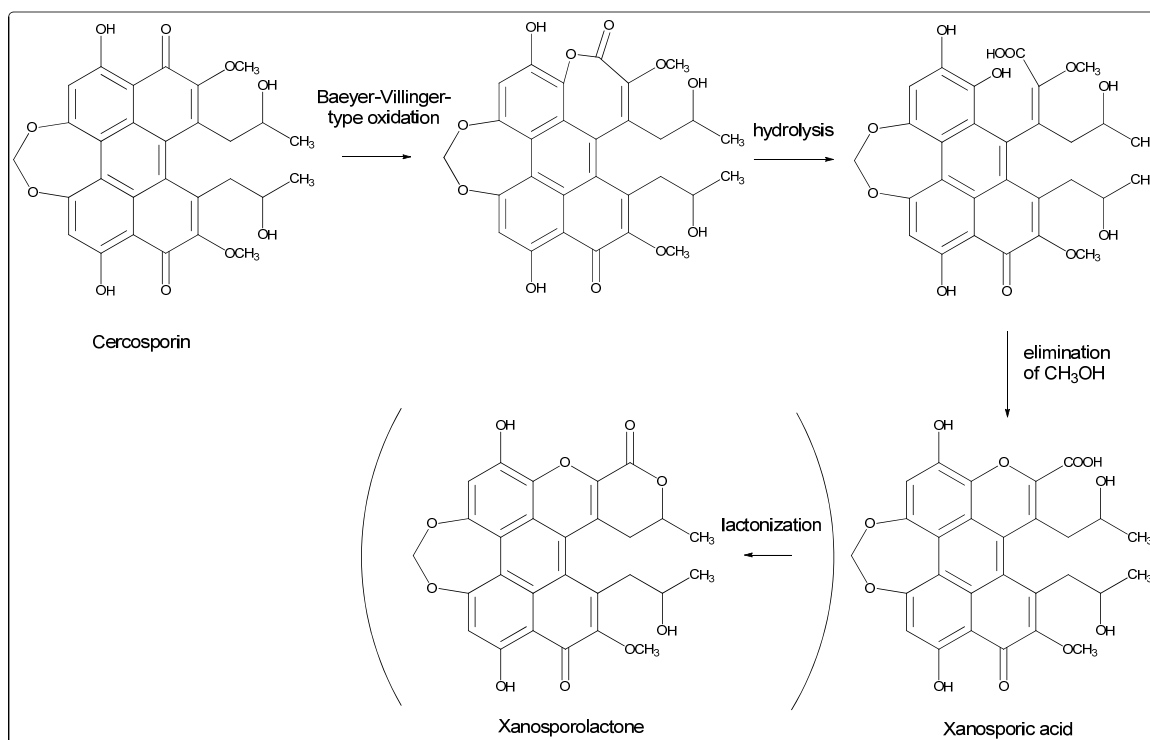
Figure 4.2.2.6a. Proposed pathway of oxidative transformation of cercosporin into xanosporic acid (Mitchell et al. 2003).

Because of the close structural relationship of cercosporin to the perylenequinones isolated from *Alternaria* sp. on the one hand, and between xanosporic acid and xanalteric acids I and II on the other hand, a similar biosynthetic pathway is proposed for xanosporic acid I and II. Biogenetically they could stem from the same pentaketide as **20**, **21** and **22**. Two possible biosynthetic pathways are shown in figure 4.2.2.6b. The one shown above would require epoxidation at positions 1 and 2 after phenol coupling of two pentaketide-derived moieties, followed by addition of one molecule of water. The accomplished reduced perylenequinone then would undergo ring expansion to a seven membered lactone. Upon loss of one molecule of water recyclization would yield xanosporic acid I. In the second possible pathway, phenolic coupling of two pentaketide-derived moieties, would accordingly accomplish dihydroxyperylene-3,10-quinone. This then would undergo a Bayer-Villiger-type ring expansion to a seven membered lactone as described above. Elimination of water would result in recyclization. Regiospecific addition of water at

the heterocyclic ring would finally yield xanosporic acid I or II as shown in figure 4.2.2.6b.

Although it was not possible during this study to isolate the proposed precursors of **23** and **24**, this pathway seems very plausible because the oxidoreductase required for the insertion of the oxygen in ring A is not exclusively found in bacteria but also present in fungi (Taylor et al. 2006).
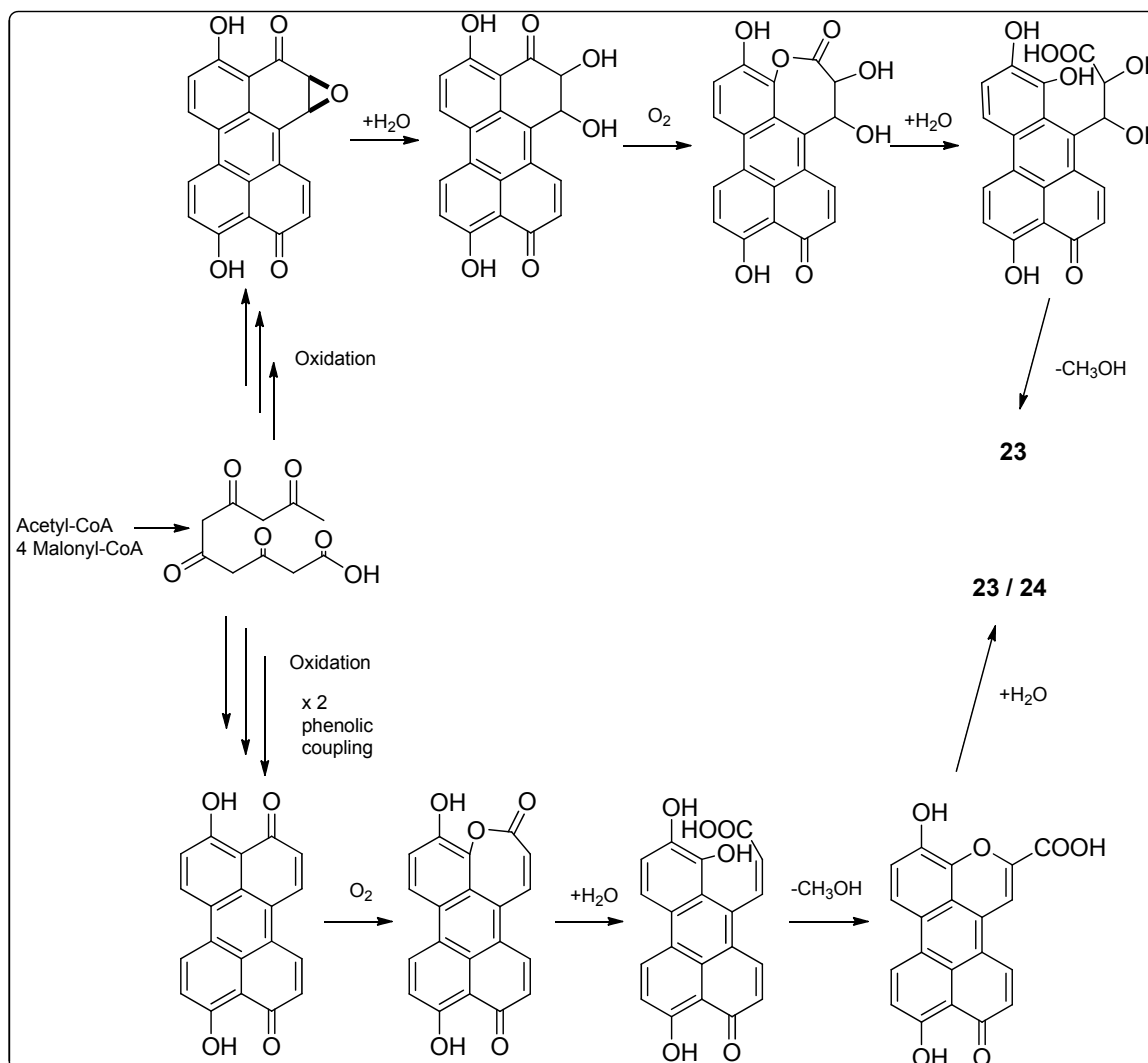
Figure 4.2.2.6b. Proposed biosynthetic pathway of xanalteric acid I (**23**) and II (**24**).

Both new metabolites exhibited moderate activity in the biological screening. Noteworthy is the activity found against *S. aureus* with MIC of 125 and 259 µg/mL. It is interesting to note that for the structurally analogous pair of compounds, cercosporin and xanosporic acid, significantly lower phytotoxicity has been described for the latter (Mitchell 2002). However, since no phytotoxicity tests were carried out, it cannot be judged at this point whether this is also the case for xanalteric acids in comparison to compounds **20**-**22** obtained during this study.

4.2.3 Depsipeptides

Protein biosynthesis is known to take place on the ribosomes, where a biological production line interprets the genetic code. This biosynthetic pathway, however, is not the sole source of all peptides. The biosynthetic origin of many peptides, especially those containing non-protein amino acids, D-amino acids, hydroxyl acids and other unusual constituents is connected to a more individualistic enzyme system referred to as nonribosomal peptide synthase (NRPS), representing huge multi-functional enzyme complexes that range among the largest proteins known so far. These enzymes first directly activate the amino acid or hydroxyl acid by cleavage of the α,β-phosphate of ATP, leading to a amino or hydroxyl acyladenylate, followed by a sequential series of peptide bond formation until the peptide is finally released from the enzyme. The enzymes normally possess other domains that are responsible for further chemical modifications such as epimerizing L-amino acids to D-amino acids, *N*-methylation or cyclization (Kleinkauf und Döhren 1990, Krause et al. 2001).

Although no studies analyzing the biosynthesis of the isolated bionectriamides were performed during this work, it can be assumed that the biosynthesis of the newly isolated depsipeptides likewise occurs *via* NRPS, in analogy to the fungal-derived enniatins or beauvericins.

Biologically active peptides are known to feature a variety of alterations in the sequence and residues. They are of enormous pharmaceutical interest because of their diverse activities, including antifungal (enniatins), antibacterial (polymyxin, baitracin), immunosuppressive (cyclosporine) and anticancer (bleomycin) properties. Their activity is thought to be dependent on the substitution pattern of the amino acid residues and the consequent secondary and tertiary structures.

During the present study, three cyclic and one linear depsipeptide were studied for their biological potential. Whereas all cyclic bionectramides exhibited profound cytotoxic properties towards the tested cell line, the linear derivative bionectriamide D has proved inactive. This result is in good conformance with the recently reported investigation of related depsipeptides such as paecilodepsipeptide A (Isaka et al. 2007). This study fungal-derived cyclic depsipeptide which also featured an *O*-prenylated tyrosine (see figure 4.2.3) exhibited cytotoxicity towards cancer cell lines, whereas its linear analogues proved inactive. Because of the similarity of these fungal metabolites, it can thus be hypothesized that cyclisation is an important requirement for cytotoxic properties of this group of depsipeptides.

Enniatin A

Bleomycin A2

Paecilodepsipeptide A

Bionectramide A
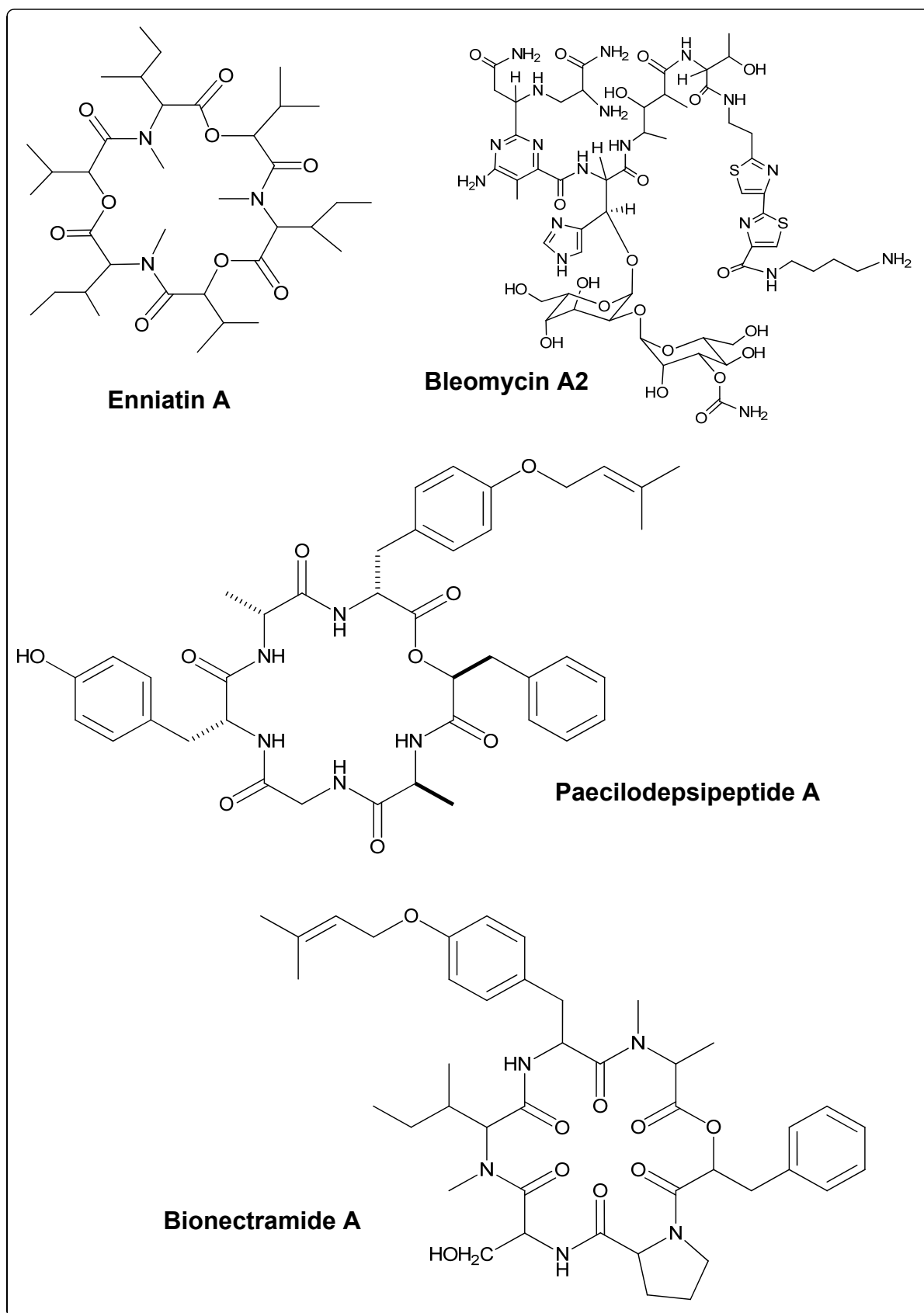
Figure 4.2.3. Examples of bioactive cyclic peptides.

4.3 Endosymbiont-host interactions

During the workup of the extract obtained from *Alternaria* sp. the question emerged whether or not bacteria could be the actual producers of some of the compounds. This hypothesis was based on ubiquitous endosymbiotic bacterial-fungal interactions that have been described for more than three decades in general (Kobayashi and Crouch 2009), and the discovery of an endosymbiotic bacterium producing a "fungal" toxin in particular (Partida-Martinez and Hartweck 2005). However, the presence of bacteria residing inter- or intracellularly in *Alternaria* sp. could be excluded, a hypothesis that came to mind when considering the structures of xanalteric acids, which exhibit a close similarity to the bacterial compound cercosporin, itself derived from metabolization of a fungal precursor. Fermentation upon addition of antibiotics still afforded the metabolites, and in addition, PCR amplification of total DNA extracted from the culture with bacteria-specific primers gave no sign of bacterial growth in the fungal culture

Interactions between host plant and endophyte are far from being understood, and the same applies for interactions between bacteria and fungi. Culture experiments together with the benefits of studying these systems at the molecular level would help to understand these interactions.

4.4   Regulation of secondary metabolite production by endophytic fungi

In the course of this study it was found that the productivity and metabolite profiles of the isolated endophytic fungi *Alternaria* sp. and *Nigrospora* sp. changed upon fermentation in different media. This observation confirms the concept of the OSMAC (**O**ne **S**train, **Ma**ny **C**ompounds) approach (Bode et al. 2002) and suggested these two fungal strains to be ideal candidates for exemplary investigation of the effect of the supplementation of the fermentation medium. Among the various biotic and abiotic culture parameters that have been described in the literature, the application of epigenetic modifiers, namely histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors (Williams et al. 2008), was chosen.

The nucleoside analogue 5-azacytidine is a DNMT-inhibitor that is mainly used in the treatment of several kinds of leukemia. It was shown to elicit *de novo* synthesis of several oxylipins in *Cladosporium cladosporioides* (Williams et al. 2008). In the present study, *Alternaria* sp. was not responsive to addition of 5-azacytidine, whereas *Nigrospora* sp. revealed a more than doubled total extract yield (see table 3.8.1 and figures 3.8.1-3.8.2) upon exposure to a concentration of 5 μM of the substance, and a distinct shift in the metabolite profile towards an induced synthesis of more lipophilic metabolites (see figure 3.8.3, around 30 min). As the UV spectra corresponding to these compounds (peaks 12-15) show high similarities it is reasonable to assume that they are chemically closely related compounds and might possibly be derived from the same biogenetic gene cluster and are thus co-regulated.

Due to the low extract yields obtained after fermentation of only one Erlenmeyer flask, however, it was not possible to isolate and elucidate the structures of the newly produced metabolites.

In the context of the development of new anticancer agents, the sponge metabolite psammaplin A was proven to be a potent inhibitor of histone deacetylase at nanomolar concentrations (Kim 2007). In the present study, the exposure to a concentration of 0.5 μM of this marine metabolite resulted in an increase of total extract in both fungal strains, whereas at a lower concentration only a slight increase of the extract amount was observed. At a higher concentration, even a decrease in the extract amounts was detected (see table 3.8.1). As the HPLC chromatograms of the extracts of both investigated endophytes did not exhibit an altered metabolite profile, it can be assumed that in this particular case, psammaplin A might boost a more general effect on gene transcription rather than deregulating individual biogenetic gene clusters.

Valproic acid is a short-chained fatty acid widely used as an anticonvulsant and mood stabilizer. In recent years it has attracted scientists' interest because it was shown to activate gene expression by inhibition of histone decetylase *in vitro* and thus commending itself as potential anticancer drug (Phiel 2001, Segura-Pacheo 2007). Application of 1 and 4 mM of this anticonvulsant drug to the liquid medium resulted in an up to threefold increase of obtained yields of the fungal strain *Nigrospora* sp. Moreover, a new peak appeared in the HPLC chromatogram. This promising result was only diminished by the need of the application of a rather high concentration of the compound. Nevertheless the activating effect of this compound of comparably low toxicity should warrant further investigation in this specific field.
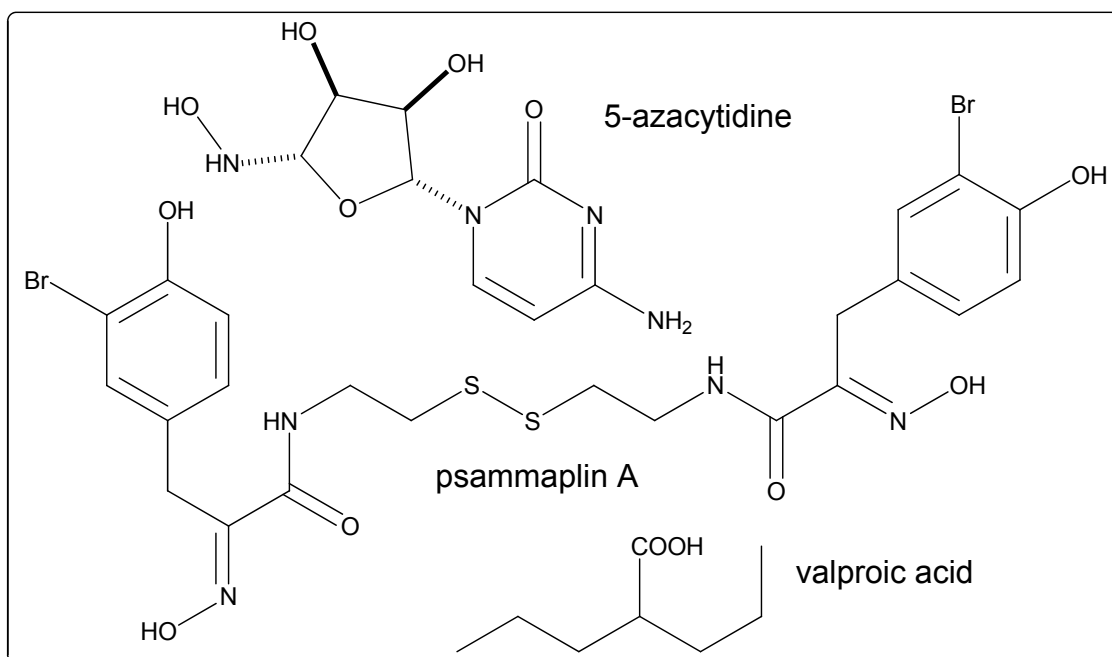
Figure 4.4. Epigenetic modifiers applied in the present study.

To summarize, it can be stated that epigenetic modifiers are not only a promising group for development of new anti-cancer drugs but also in enhancing microbial productivity by increased gene expression although not all biosynthetic pathways seemed to be activated by these small molecules in general and different fungal strains responded differently to the application of the modifiers under investigation. Further studies using other epigenetic modifiers are needed to affirm their potential as an alternative methodology for rationally inducing the expression of silent natural product biosynthetic pathways.

5. Summary

Natural products play a major role in the objective to meet the general demand for new pharmaceutically or biologically active substances. This is particularly true for metabolites from microorganisms, especially endophytic fungi, microorganisms that reside in the tissues of living plants, and which are known to produce a plethora of secondary metabolites. Many of these compounds exhibit biological activities in pharmaceutically relevant bioassay systems and thus represent potential lead structures which could be optimized for medicinal or agrochemical applications.

This thesis focused on the isolation of secondary metabolites from mangrove-derived endophytic fungi, followed by structure elucidation and examination of their pharmacological potential. After biological and chemical screening, five of the more than ninety obtained fungal strains were chosen for detailed examination. Therefore they were grown in liquid Wickerham medium and on solid rice medium for three to five weeks and extracted with organic solvents, followed by fractionation and purification of the secondary metabolites using various chromatographic techniques.

Structure elucidation was performed using state-of-the-art analytical techniques, including mass spectrometry (MS) and one- and two-dimensional nuclear magnetic resonance (NMR) experiments. Furthermore, selected optically active compounds were derivatized in order to determine their absolute configuration.

Pure compounds were subjected to various bioassays to examine their antimicrobial, antiviral, antifungal, cytotoxic and protein kinase inhibitory properties. Finally, two fungal strains were fermented upon addition of substances that are known to be epigenetic modifiers in order to evaluate the effects of altering growth conditions on the chemical productivity of endophytic fungi.

A total of twenty-nine secondary metabolites were isolated in this study, seven of which were identified as new natural products. Known and new compounds were tested for their biological activities using different bioassay systems.

1. *Cladosporium sphaerospermum*
Cladosporium sphaerospermum, isolated from the mangrove plant *Aegiceras corniculatum*, was grown in liquid medium and yielded three known fungal metabolites. Beside the major compound citrinin, which showed antibiotic activity against *Streptococcus pyrogenes*, an inseparable mixture of the diastereomers quinolactacin A1 and quinolactacin A2 was obtained.

2. *Fusarium incarnatum*
Equisetin was isolated as the main metabolite of *Fusarium incarnatum* that was isolated from *Pluchea indica*. The compound exhibited pronounced antibiotic activity against several selected bacterial strains.

3. *Nigrospora sp.*
Nigrospora sp. was isolated from *Bruguiera sexangula.* Beside five known metabolites, one new diketopiperazine and one new anthraquinones derivative were isolated. Two of the anthraquinones exhibited moderate antibacterial and good prophylactic effects against human rhinoviruses, but also profound cytotoxic properties.

4. *Alternaria sp.*

Thirteen metabolites were obtained from *Alternaria* sp., which was isolated from *Sonneratia alba.* Most of them represent derivatives of alternariol or perylenequinones, while in addition two new natural products were obtained. They feature a 10*H*-phenaleno[1,2,3-*de*]chromene skeleton hitherto unknown for fungal metabolites, displaying structural similarity to the perylenequinones, but with an oxygen atom incorporated into the ring system. The new compounds showed antibacterial properties against *S. aureus*. Altenusin was likewise active, but exhibited a broader antibacterial spectrum and additionally, prophylactic effects against infection by selected human rhinoviruses.

5. *Bionectria ochloleuca*

Bionectria ochroleuca obtained from the mangrove *Sonneratia caseolaris* was investigated and yielded several new depsipeptides, featuring an *O*-prenylated tyrosine which is most unusual for fungal secondary metabolites. Whereas the linear derivative bionectriamide D proved inactive when tested against L5178Y mouse lymphoma cell line, the remaining three cyclic depsipeptides bionectriamides A-C displayed pronounced cytotoxic activities with EC_{50} values between 2.5 and 8 µg/mL. In addition, an enriched fraction containing all four depsipeptides exhibited antitryponosomal activity against *T. brucei* with an MIC of 3.12 µg/mL.

Furthermore, the fungal strains *Nigrospora* sp. and *Alternaria* sp. were selected for fermentation upon addition of substances that are known to be epigenetic modifiers in order to evaluate the effects of altering fermentation conditions on the chemical productivity of endophytic fungi. For both strains examined, alterations of the amounts of the obtained extracts and their composition were observed. The most effective additive was valproic acid, inducing up to threefold increase of obtained yields of the fungal strain *Nigrospora* sp. and the appearance of an additional peak in the HPLC chromatogram.

6. Zusammenfassung

Im Bestreben, den ausgeprägten Bedarf an neuen pharmazeutisch und biologisch aktiven Substanzen zu decken, spielen Naturstoffe eine große Rolle, was im Besonderen für mikrobielle Sekundärstoffe gilt. Vor allem endophytische Pilze, die im Gewebe lebender Pflanzen wachsen, produzieren eine Fülle von Sekundärstoffen, von denen zahlreiche viel versprechende biologische Aktivitäten in pharmazeutisch relevanten Testsystemen aufweisen und sich so als zu optimierende Leitstrukturen für medizinische oder landwirtschaftliche Nutzung anbieten.

Der Schwerpunkt dieser Doktorarbeit lag in der Isolierung von Sekundärstoffen aus endophytischen Pilzen aus Mangroven, ihrer Strukturaufklärung und der Untersuchung ihres pharmakologischen Potentials. Nach ersten Aktivitätsuntersuchungen und chemischem Screening wurden fünf der über neunzig isolierten Pilzstämme zur eingehenden Untersuchung ausgewählt. Dafür wurden diese für drei bis fünf Wochen in Flüssigmedium oder auf Reis kultiviert, die Kulturen mit organischen Lösungsmitteln extrahiert und die Reinsubstanzen mittels verschiedener chromatographischer Methoden isoliert.

Die Strukturaufklärung erfolgte mit Hilfe moderner Analysemethoden, darunter Massenspektrometrie und Kernspinresonanzspektroskopie. Zudem wurden ausgewählte optisch aktive Substanzen derivatisiert, um ihre absolute Konformation zu ermitteln.
Die Reinsubstanzen wurden verschiedenen biologischen Testsystemen, darunter auf antibiotische, antivirale, cytotoxische Aktivität sowie Inhibierung verschiedener Proteinkinasen, unterworfen.

Außerdem wurden zwei der Pilzstämme ausgewählt und unter Zugabe von Substanzen fermentiert, die als epigenetische Modifikatoren der Genregulation beschrieben wurden, um den Effekt veränderter Wachstumsbedingungen auf die chemische Produktivität der Endophyten zu untersuchen.

Insgesamt wurden in dieser Arbeit neunundzwanzig Sekundärstoffe isoliert, von denen sieben neue Naturstoffe darstellen. Sowohl die neuen als auch die bekannten Stoffe wurden mittels unterschiedlicher Testsysteme auf ihre biologische Aktivität hin untersucht.

1. *Cladosporium sphaerospermum*
C. sphaerospermum aus der Mangrovenpflanze *Aegiceras corniculatum* wurde auf Flüssigmedium fermentiert und lieferte drei Naturstoffe. Neben der Hauptsubstanz Citrinin, welche antibiotische Aktivität gegen *Streptococcus pyrogenes* aufwies, wurde eine nicht weiter aufzutrennende Mischung der diastereomeren Quinolactacin A1 und Quinolactacin A2 erhalten.

2. *Fusarium incarnatum*
Das Alkaloid Equisetin war der Hauptinhaltsstoff von *Fusarium incarnatum*, welcher aus *Pluchea indica* isoliert wurde. Die Substanz zeigte eine ausgeprägte antibiotische Aktivität gegen verschiedene Bakterienstämme.

3. *Nigrospora* sp.
Nigrospora sp. wurde aus der Mangrove *Bruguiera sexangula* erhalten. Neben fünf bekannten Substanzen wurden ein neues Anthrachinonderivat und ein neues Diketopiperazinderivat isoliert. Zwei der isolierten Anthrachinone zeigten moderate

antibakterielle Aktivität und wirkten prophylaktisch gegenüber Infektionen mit Rhinoviren. Sie wiesen auch eine starke Zytotoxizität auf.

4. *Alternaria* sp.
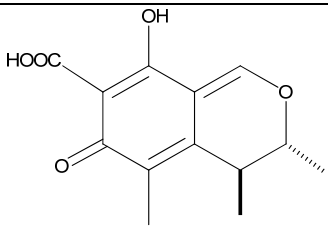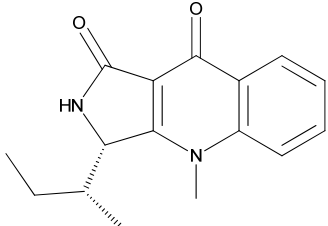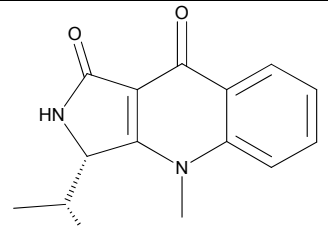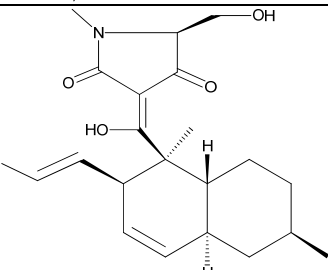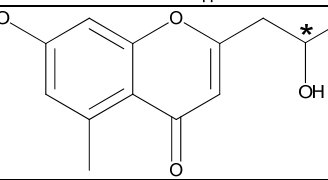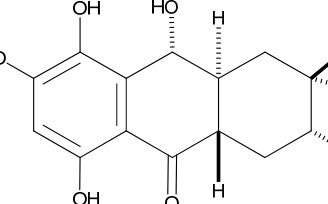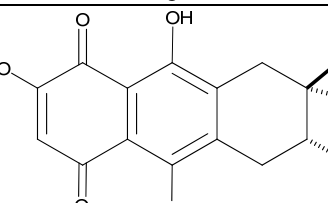
Aus dem Endophyten *Alternaria* sp. aus der Mangrovenpflanze *Sonneratia alba* wurden dreizehn Sekundärstoffe erhalten, darunter Alternariolderivate und Perylenchinone sowie zwei neue Naturstoffe. Diese weisen ein 10*H*-phenaleno[1,2,3]chromen-Grundgerüst auf, das bisher für Naturstoffe aus Pilzen noch nie beobachtet wurde, und das strukturelle Verwandtschaft zu den Perylenchinonen aufweist, wobei sich allerdings ein Sauerstoffatom im Ringsystem befindet. Die neuen Naturstoffe zeigten antibakterielle Eigenschaften gegenüber *S. aureus*. Dies wurde auch für Altenusin beobachtet, das allerdings noch ein breiteres antibiotisches Spektrum und zudem prophylaktische Wirkung gegenüber Infektion mit Rhinoviren besaß.

5. *Bionectria ochroleuca*
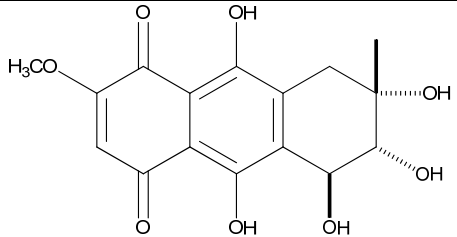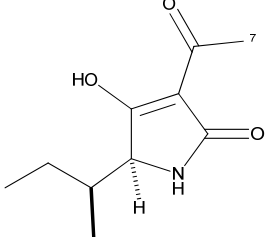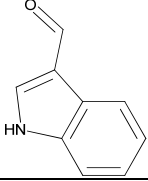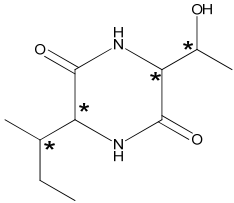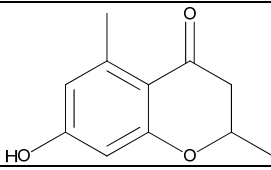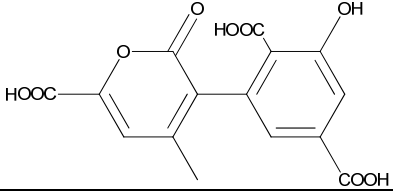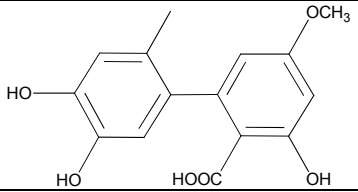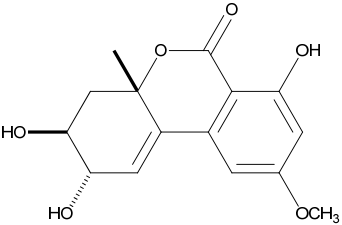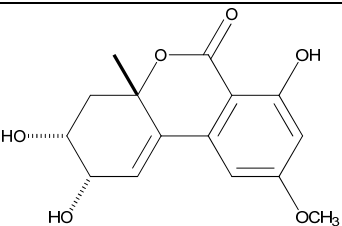
Außerdem wurde *Bionectria ochroleuca* aus *Sonneratia caseolaris* untersucht. Aus diesem Endophyten wurden vier neue Depsipeptide isoliert, die ein für Naturstoffe aus Pilzen äußerst ungewöhnliches *O*-prenyliertes Tyrosin enthalten. Während das lineare Depsipeptid Bionectriamid D keinen inhibierenden Effekt auf das Wachstum der untersuchten Mauslymphomzelllinie L5178Y entwickelte, zeigten die drei zyklischen Derivate Bionectriamid A-C eine ausgeprägte Zytotoxizität mit EC_{50}-Werten zwischen 2.5 und 8 μg/ml. Zudem wurde für ein angereichertes Gemisch der vier Depsipeptide eine antitrypanosomale Aktivität gegenüber *T. brucei* mit einer MIC von 3.12 μg/ml festgestellt.

Schließlich wurden die Pilzstämme *Nigrospora* sp. und *Alternaria* sp. unter Zugabe von sogenannten epigenetischen Modifikatoren fermentiert, um die Auswirkung dieser Substanzen auf die chemische Produktivität der Pilzkulturen zu ermitteln. Bei beiden untersuchten Pilzkulturen zeigten sich Veränderungen in der Menge der erhaltenen Extrakte sowie in deren Zusammensetzung. Die stärksten Veränderungen bewirkte die Zugabe von Valproinsäure, die für die dreifache Extraktmenge aus *Nigrospora* sp. und das Auftauchen eines zusätzlichen Peaks im HPLC-Chromatogramm verantwortlich zeichnete.
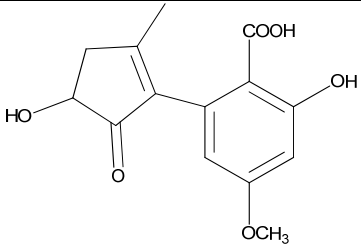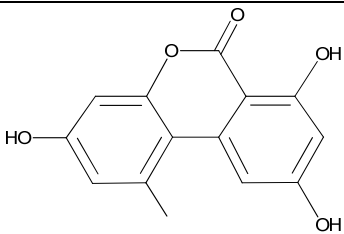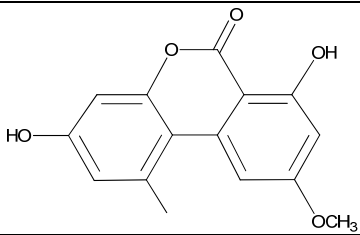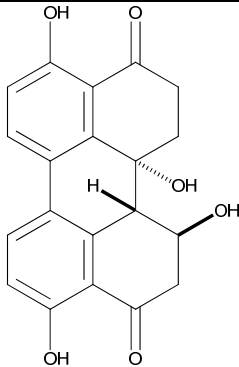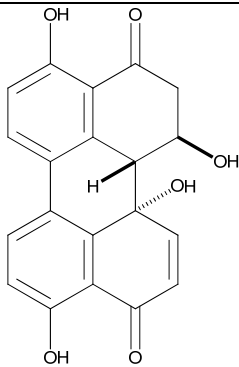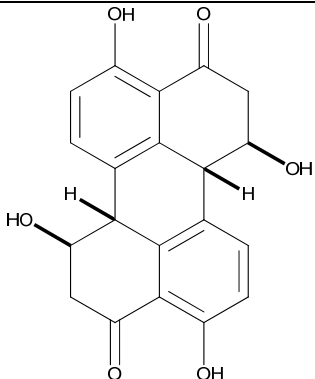
7. List of the isolated compounds

Compound	Structure	Herkunft	Comment
Citrinin		*Cladosporium sphaerospermum*	known
Quinolactacin A1		*Cladosporium sphaerospermum*	known
Quinolactacin A2		*Cladosporium sphaerospermum*	known
Equisetin		*Fusarium incarmatum*	known
Aloesol		*Nigrospora* sp.	known
8-Hydroxy-tetrahydroaltersolanol B		*Nigrospora* sp.	new
Deoxybostrycin		*Nigrospora* sp.	known
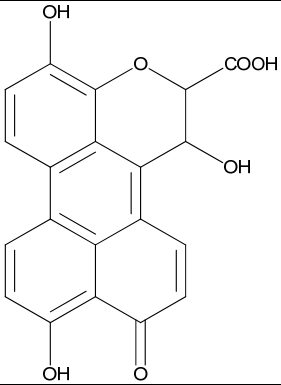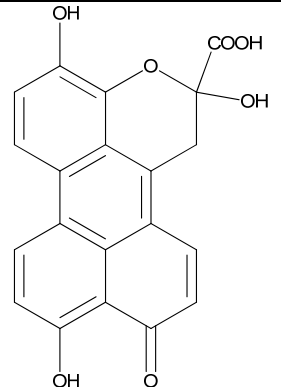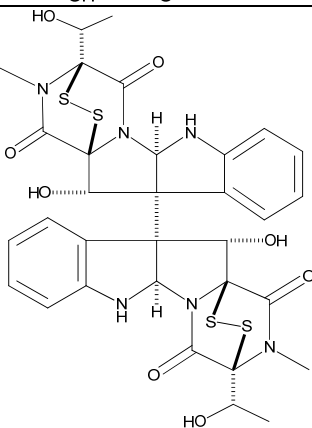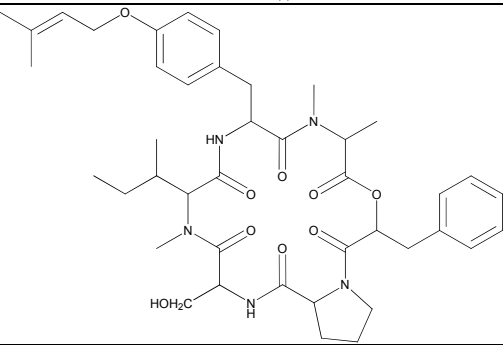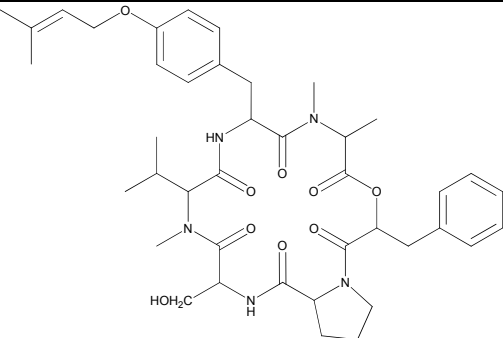
Bostrycin		*Nigrospora* sp.	known
Tenuazonic Acid		*Nigrospora* sp.	known
Indol-3-carbaldehyde		*Nigrospora* sp.	known
Cyclo(threonylisoleucyl)		*Nigrospora* sp.	new
2,5-Dimethyl-7-hydroxychromone		*Alternaria* sp.	known
Alternarian Acid		*Alternaria* sp.	known
Altenusin		*Alternaria* sp.	known
Altenuene		*Alternaria* sp.	known
4'-Epialtenuene		*Alternaria* sp.	known
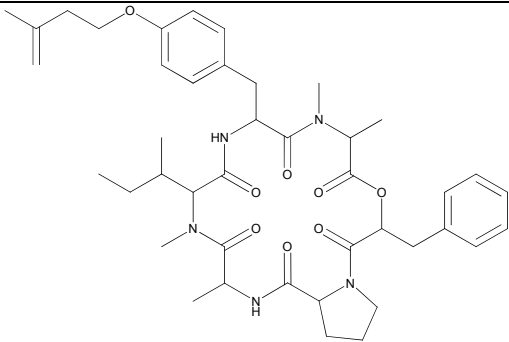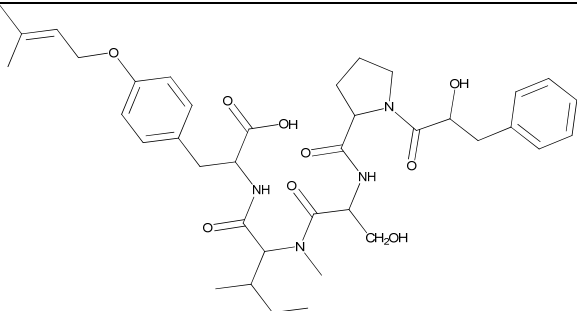
Alternarienonic Acid		*Alternaria* sp.	known
Alternariol		*Alternaria* sp.	known
Alternariol-5-methylether		*Alternaria* sp.	known
Altertoxin I		*Alternaria* sp.	known
Alterperylenol		*Alternaria* sp.	known
Stemphyperylenol		*Alternaria* sp.	known

Xanalteric acid I		*Alternaria* sp.	new
Xanalteric acid II		*Alternaria* sp.	new
Verticillin D		*Bionectria ochroleuca*	known
Bionectramide A		*Bionectria ochroleuca*	new
Bionectramide B		*Bionectria ochroleuca*	new

| Bionectramide C | | *Bionectria ochroleuca* | new |
| Bionectramide D | | *Bionectria ochroleuca* | new |

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9. Abbreviations

$[\alpha]_D^{20}$	Specific rotation at the sodium D-line at 20 °C
ACN	Acetonitrile
BLAST	Basic local alignment search tool
bp	Base pairs
br	Broad
$CDCl_3$	Deuterated chroloform
COSY	Correlation spectroscopy
d	Doublet
δ	Chemical shift
DCM	Dichloromethane
dd	Doublet of doublet
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyle sulfoxide
DMSO-d_6	Deuterated dimethyle sulfoxide
DNMT	DNA methyltransferase
DNA	Deoxyribonuceic acid
EC	Effective concentration
ED	Effective dose
ESI	Electron spray ionization
et al.	*et alii* (and others)
EtOAc	Ethyl acetate
EtOH	Ethanol
g	Gram
HDAC	Histone deacetylase
HHU	Heinrich-Heine-Universität
HMBC	Heteronuclear multiple bond connectivity
HMQC	Heteronuclear multiple quantum coherence
HPLC	High performance liquid chromatography
HPLC-DAD	HPLC with diode array detector
hr	Hour
HRESI	High resolution electron spray ionisation
Hz	Hertz
Da	Dalton
IC	Inhibition concentration
ITS	Internal transcribed sequence
L	Liter
LC	Liquid chromatography
M	Molar
m	Multiplet
MeOD	Deuterated methanol
MeOH	Methanol
mg	Miligram
MHz	Megahertz
MIC	Minimal inhibition concentration
min	Minute
mL	Mililiter
MS	Mass spectrometry
MPTA	Methyl-phenyl-trifluormethylacetochloride ("Mosher reagent")

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
m/z	mass per charge
μg	Microgram
NaCl	Sodium chloride
ng	Nanogram
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser and exchange spectroscopy
PCR	Polymerase chain reaction
ppm	Parts per million
q	Quartet
rDNA	Ribosomal DNA
ROESY	Rotating frame Overhauser enhancement spectroscopy
RP	Reversed Phase
s	Singulet
sp.	Species
t	Triplet
TLC	Thin layer chromatography
TOCSY	Totally related spectroscopy
UV	Ultraviolet
VLC	Vacuum liquid chromatography

10.   Attachment

Attachment 1: ^{1}H NMR spectrum of citrinin (**1**) (CDCl$_3$, 500 MHz)
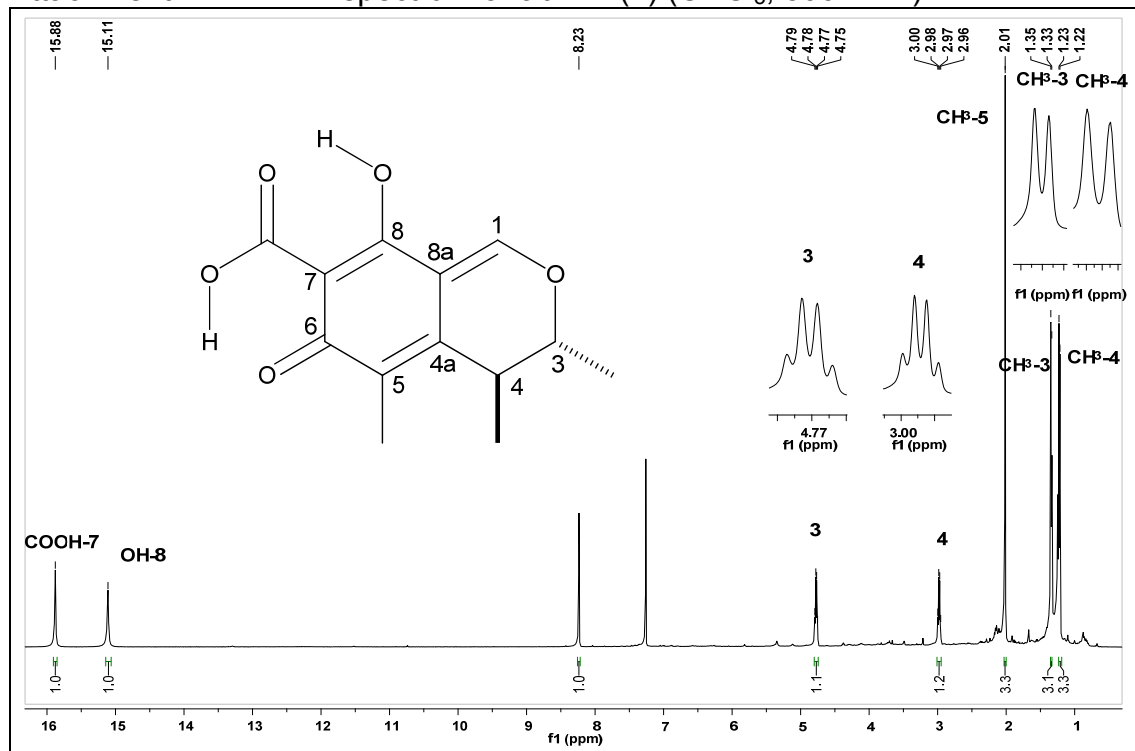
Attachment 2: ^{1}H NMR spectrum of compounds **2** and **3** (DMSO-d_6, 500 MHz)
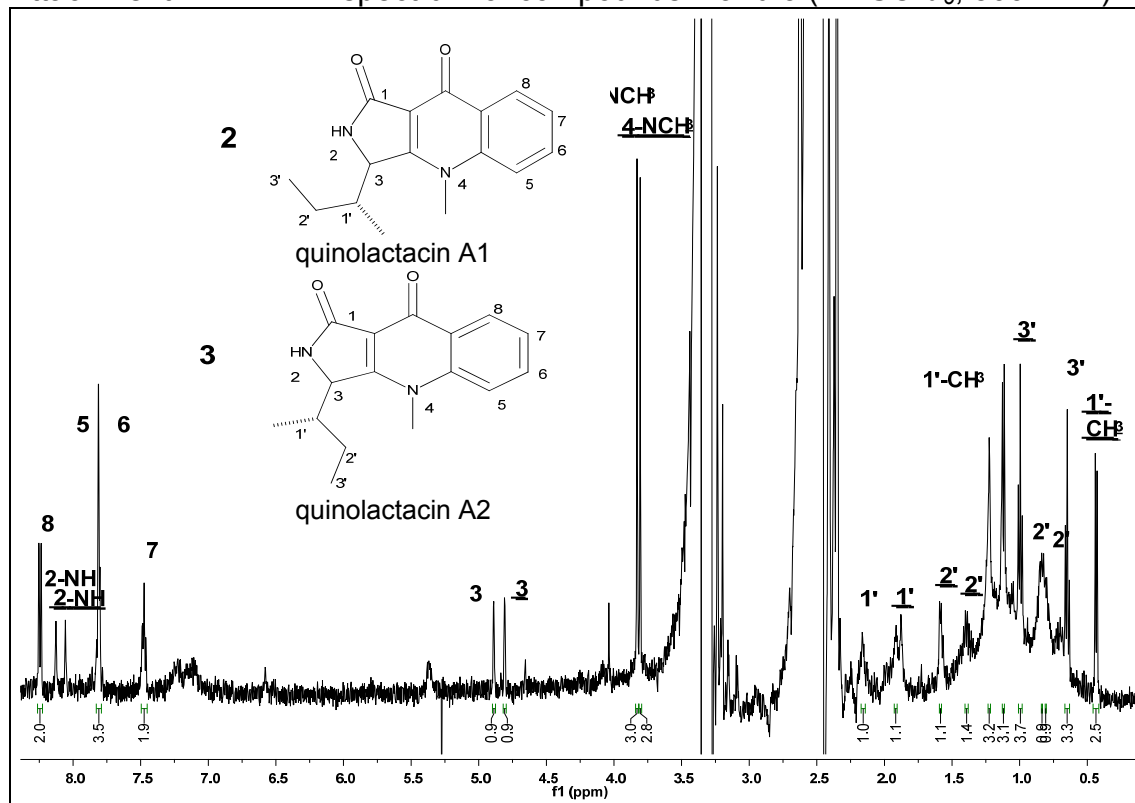
Attachment 4a: ^{1}H NMR spectrum of equisetin (**4**) (CDCl$_3$, 500 MHz), measured at 20 ° C
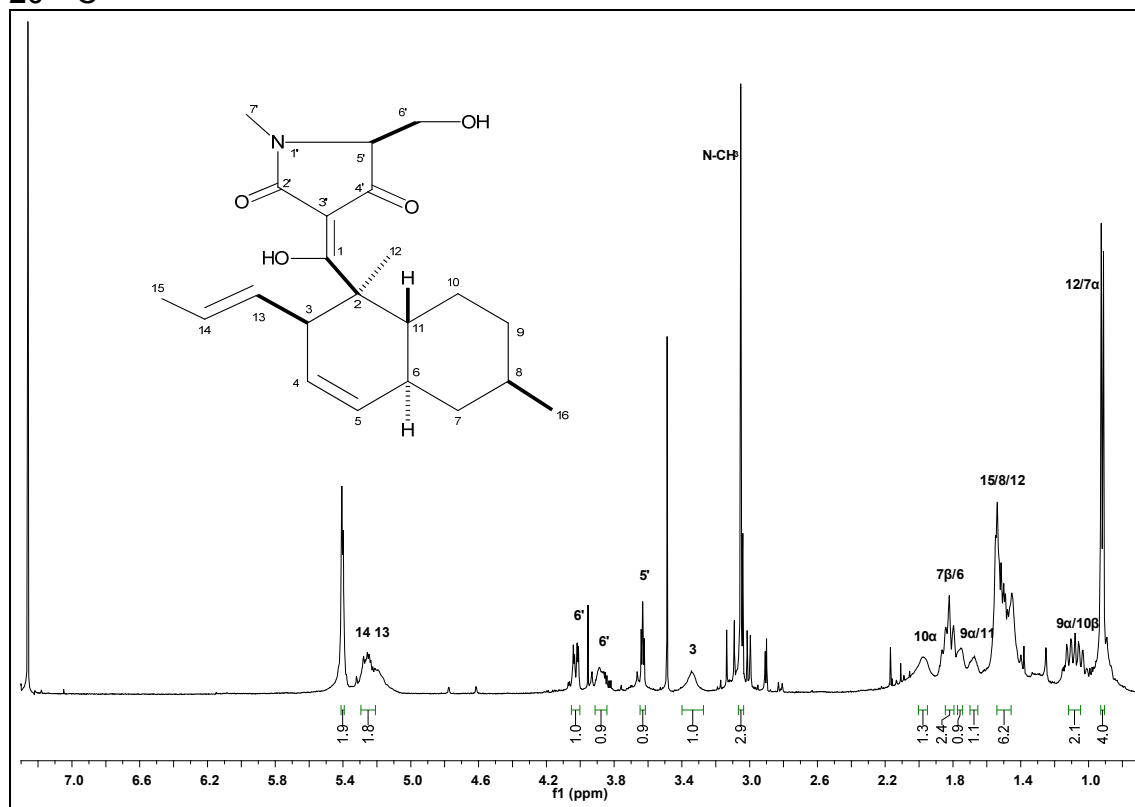
Attachment 4b: ^{1}H NMR spectrum of equisetin (**4**) (CDCl$_3$, 500 MHz), measured at -20 ° C.
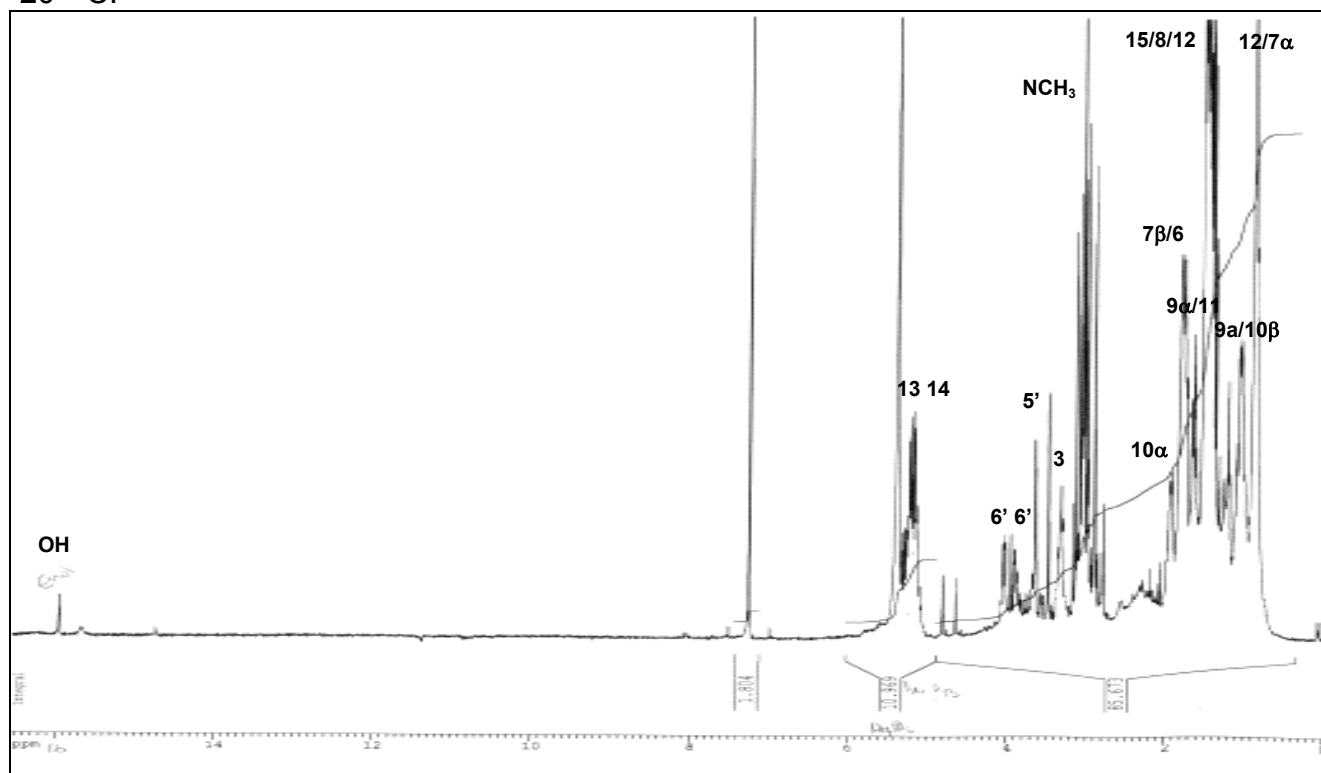
Attachment 5a: ^{1}H NMR spectrum of aloesol (**5**) (DMSO-d_6, 500 MHz)
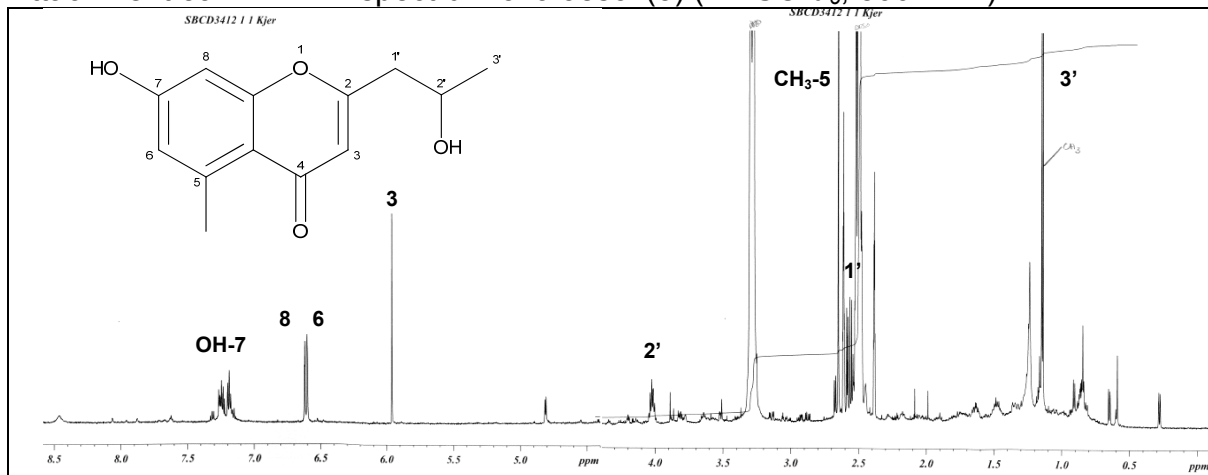
Attachment 5b: ROESY spectrum of aloesol (**5**) (DMSO-d_6, 500 MHz)
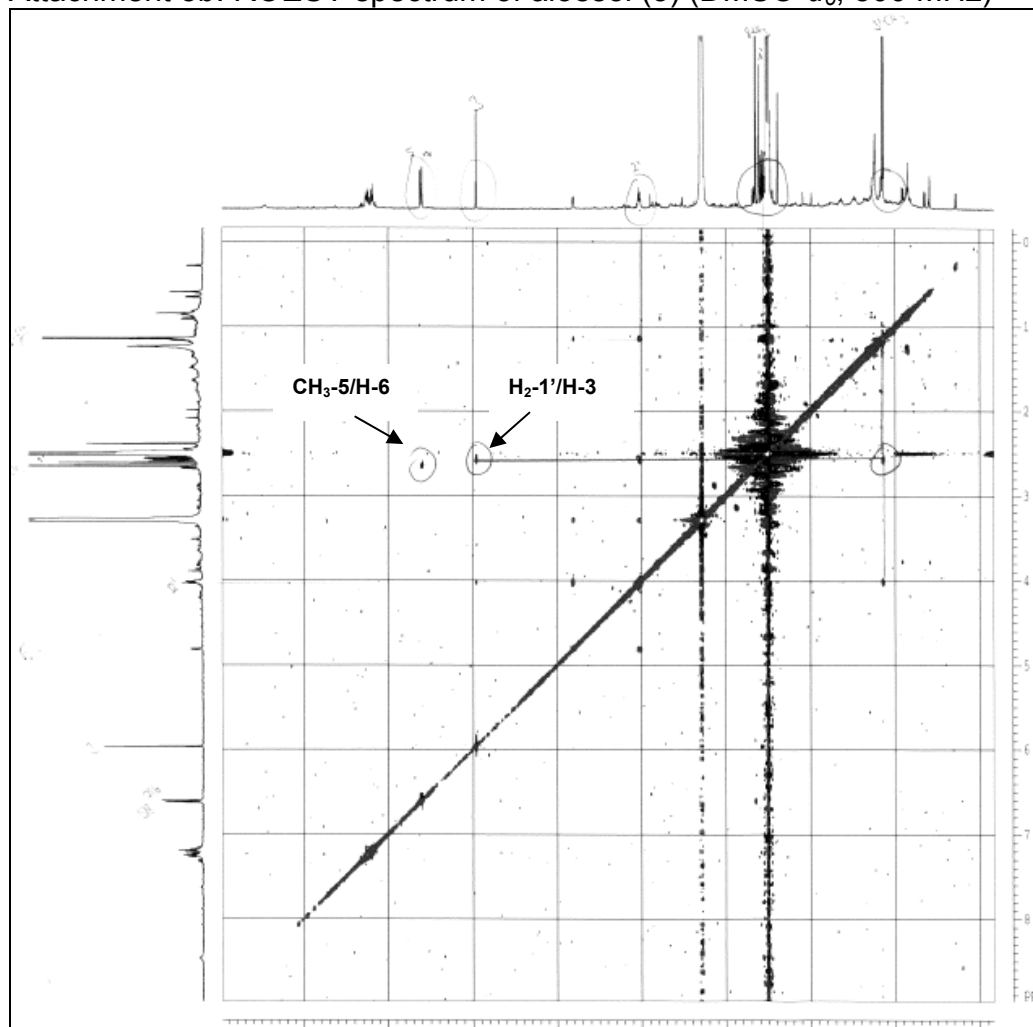
Attachment 6a: ^{1}H NMR spectrum of 8-hydroxytetrahydroaltersolanol B (**6**) (DMSO-d_6, 500 MHz)
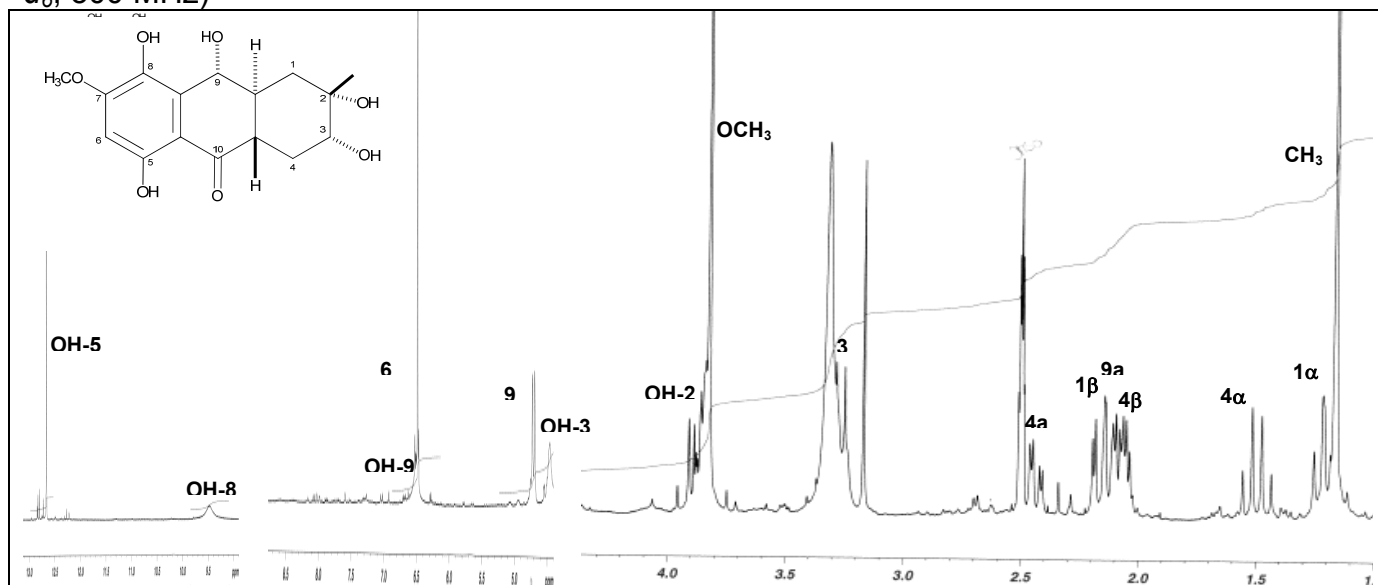
Attachment 6b: COSY spectrum of 8-hydroxytetrahydroaltersolanol B (**6**) (DMSO-*d₆*, 500 MHz)
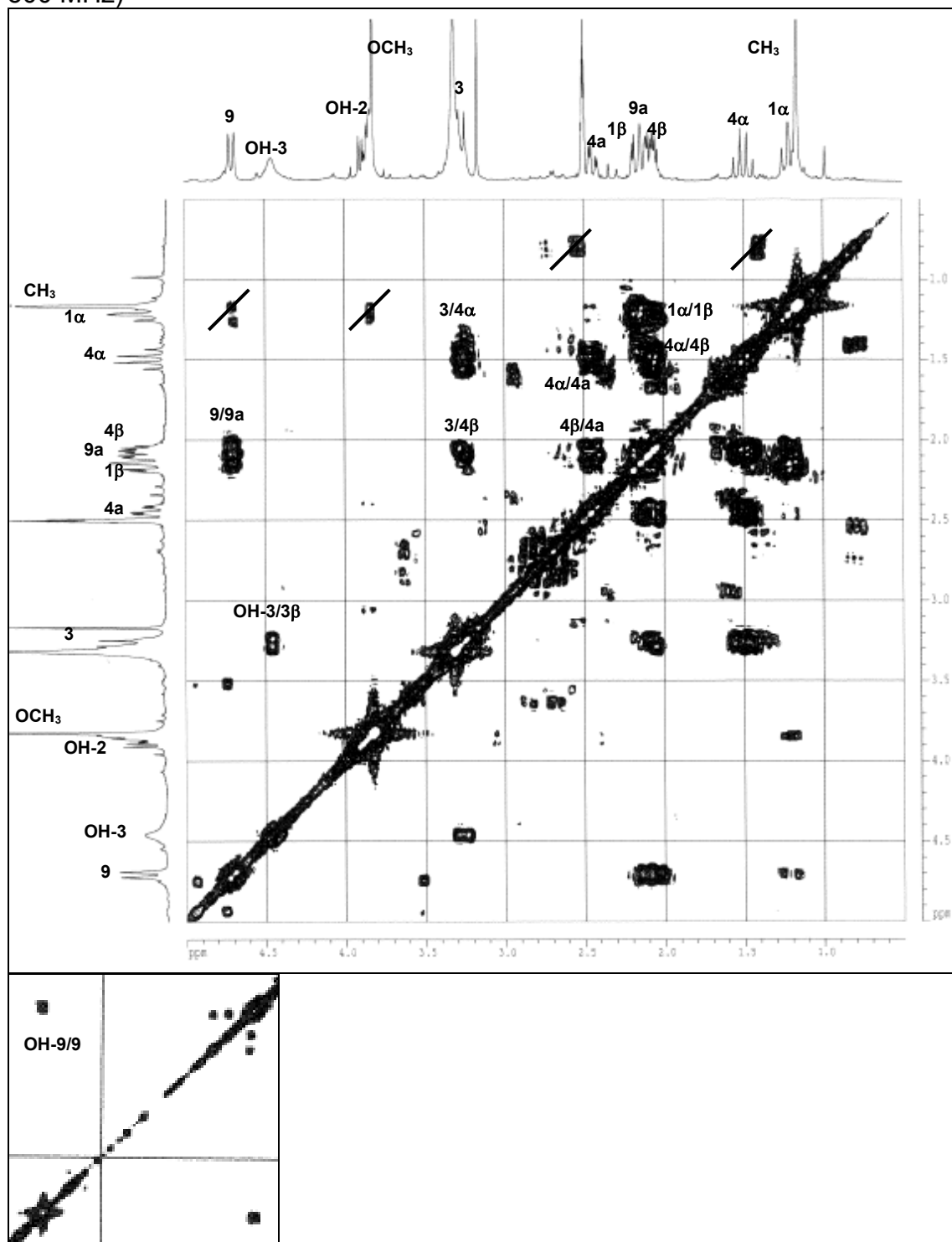
Attachment 6c: NOESY spectrum of 8-hydroxytetrahydroaltersolanol B (**6**) (DMSO-*d₆*, 500 MHz)
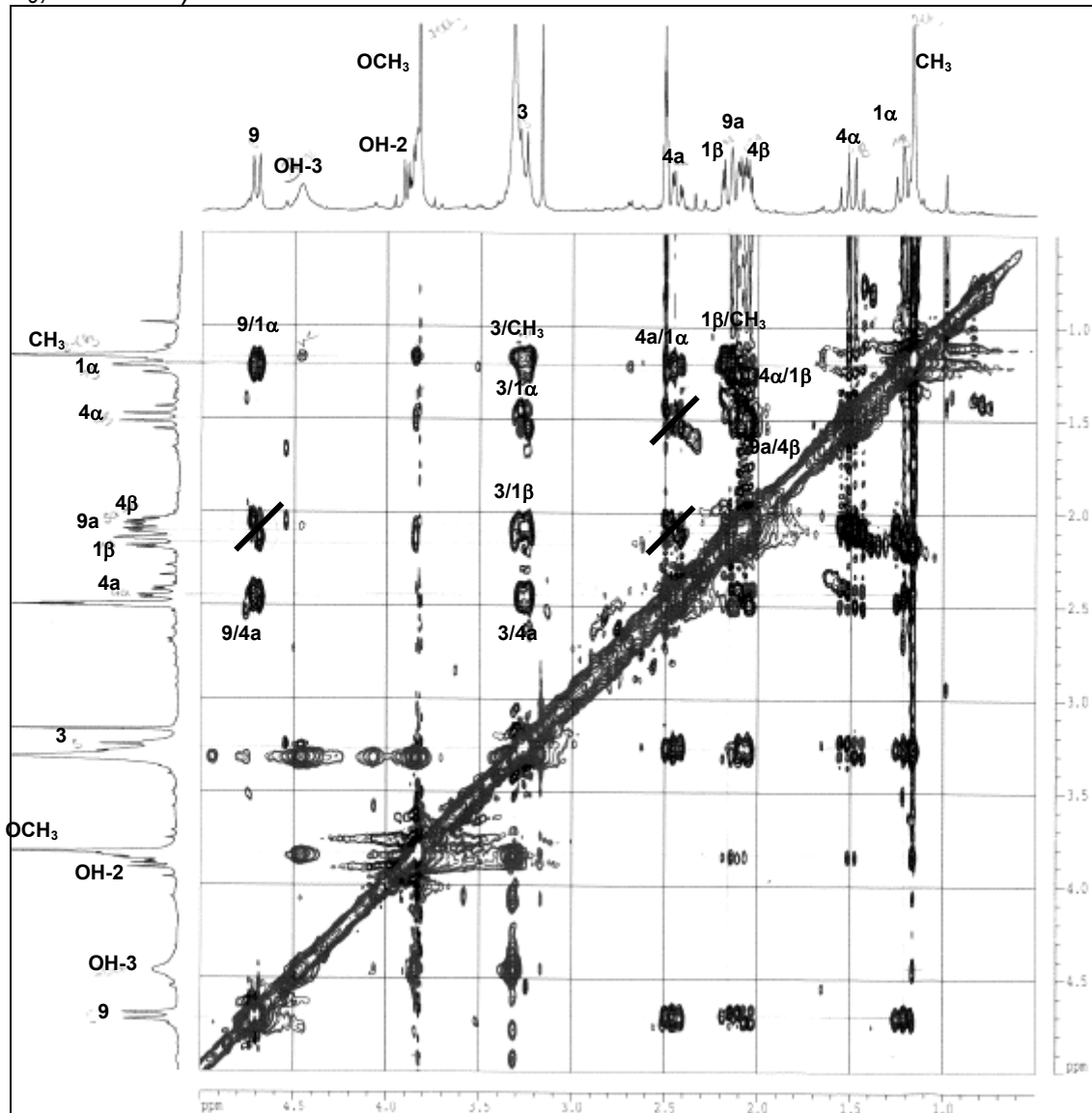
Attachment 6d: HMBC spectrum of 8-hydroxytetrahydroaltersolanol B (**6**) (DMSO-*d₆*, 500 MHz)
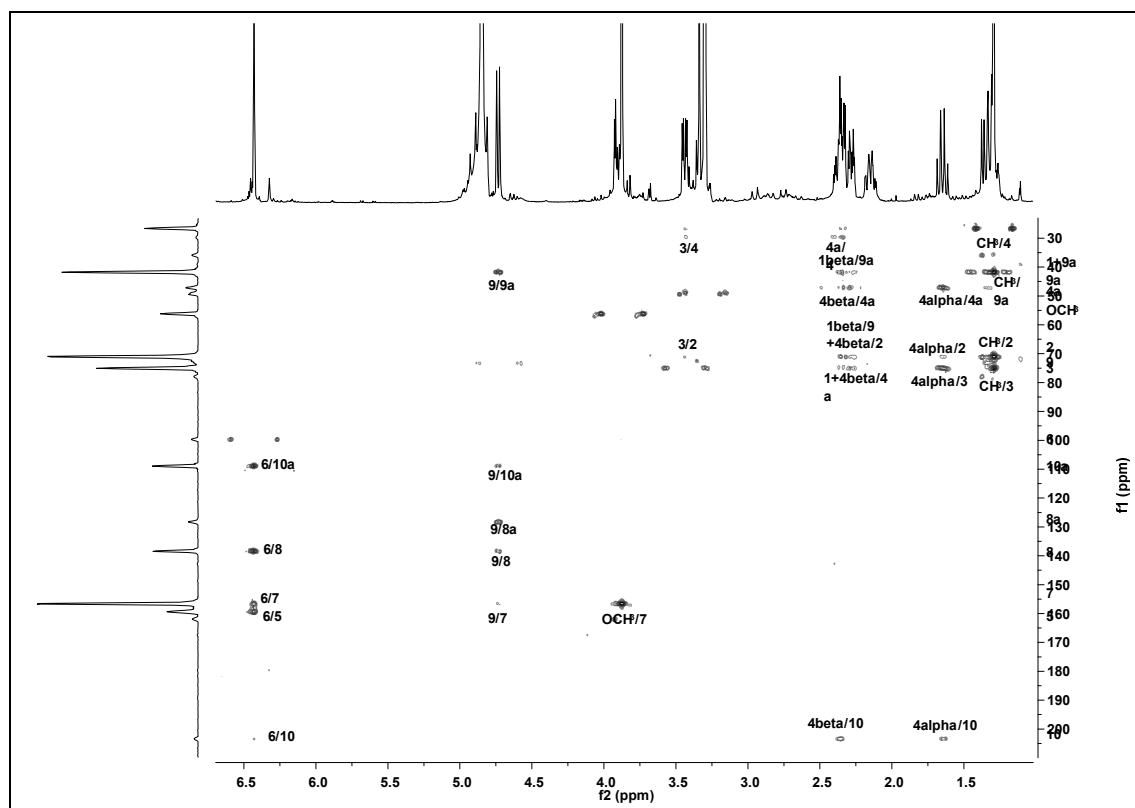
Attachment 7a: ¹H NMR spectrum of deoxybostrycin (**7**) (DMSO-*d₆*, 500 MHz)
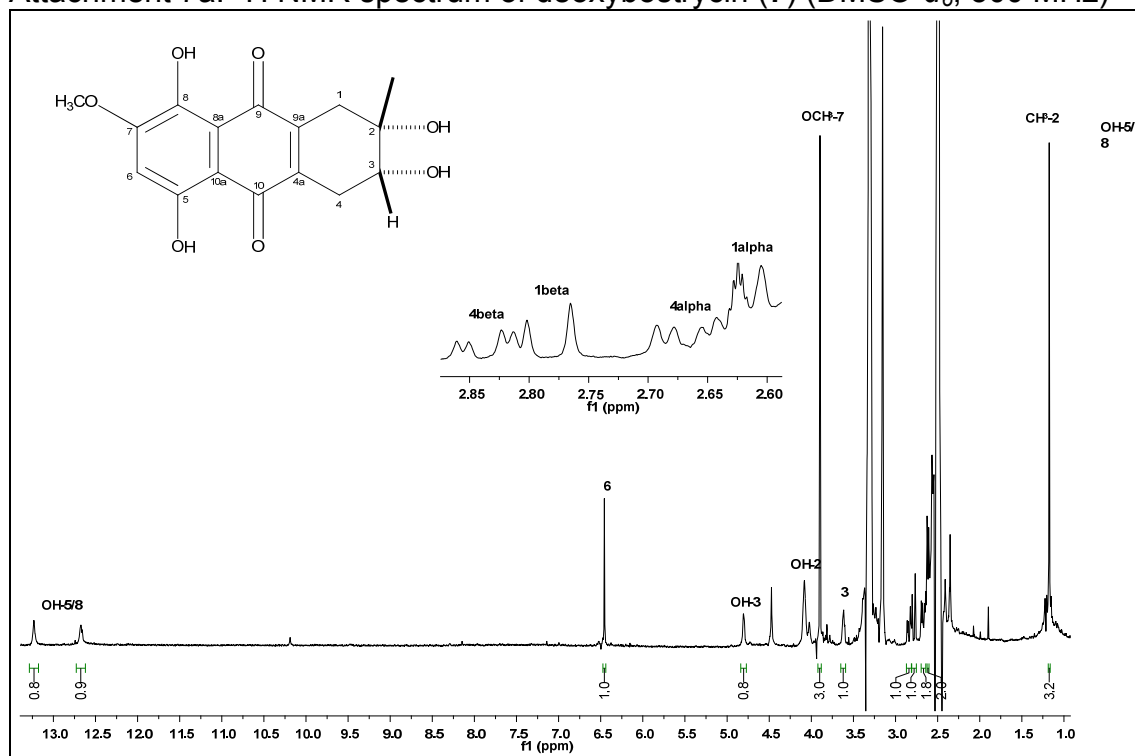
Attachment 7b: COSY spectrum of deoxybostrycin (**7**) (MeOD, 500 MHz)
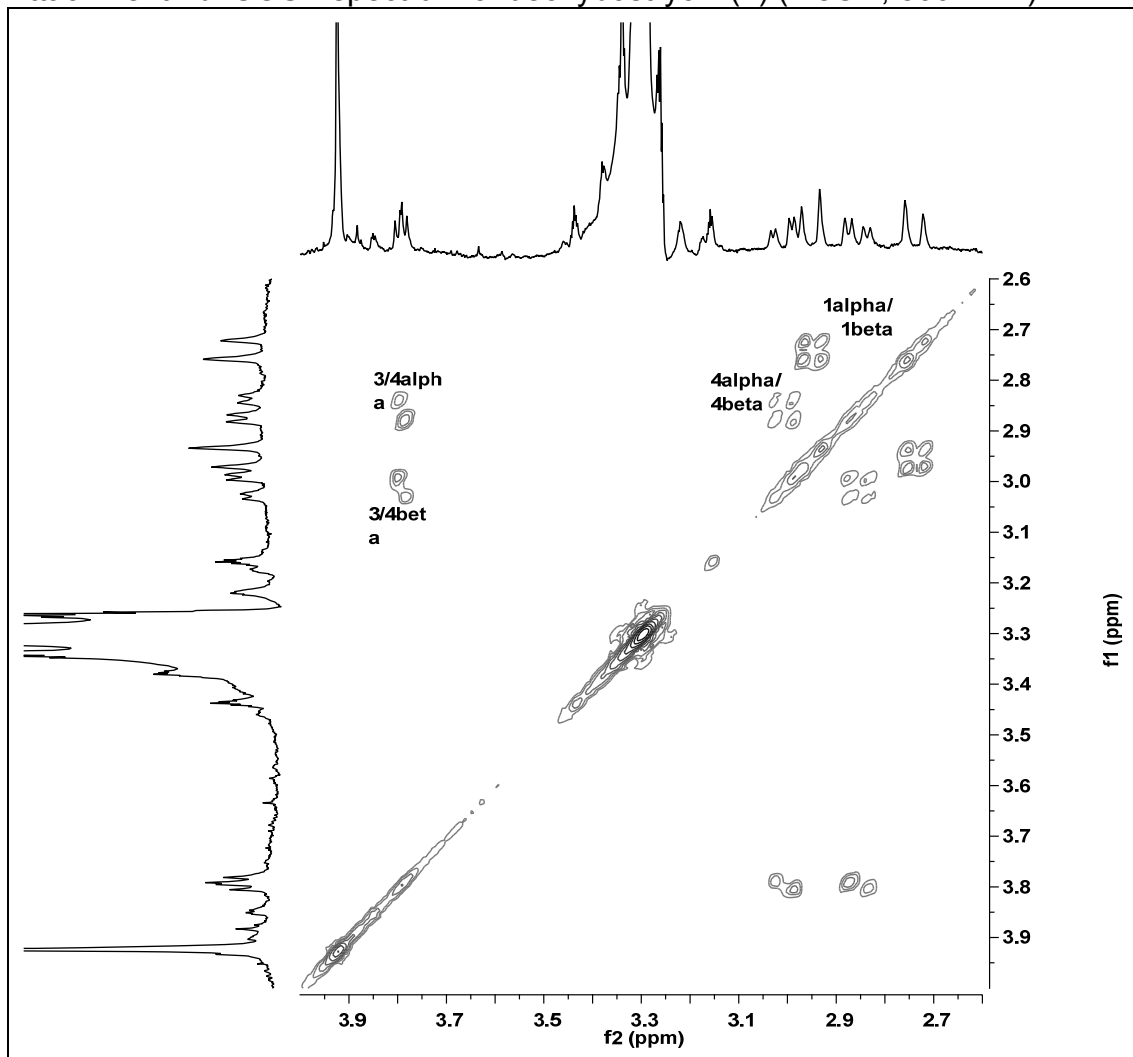
Attachment 7c: HMBC spectrum of deoxybostrycin (**7**) (DMSO-*d₆*, 500 MHz)
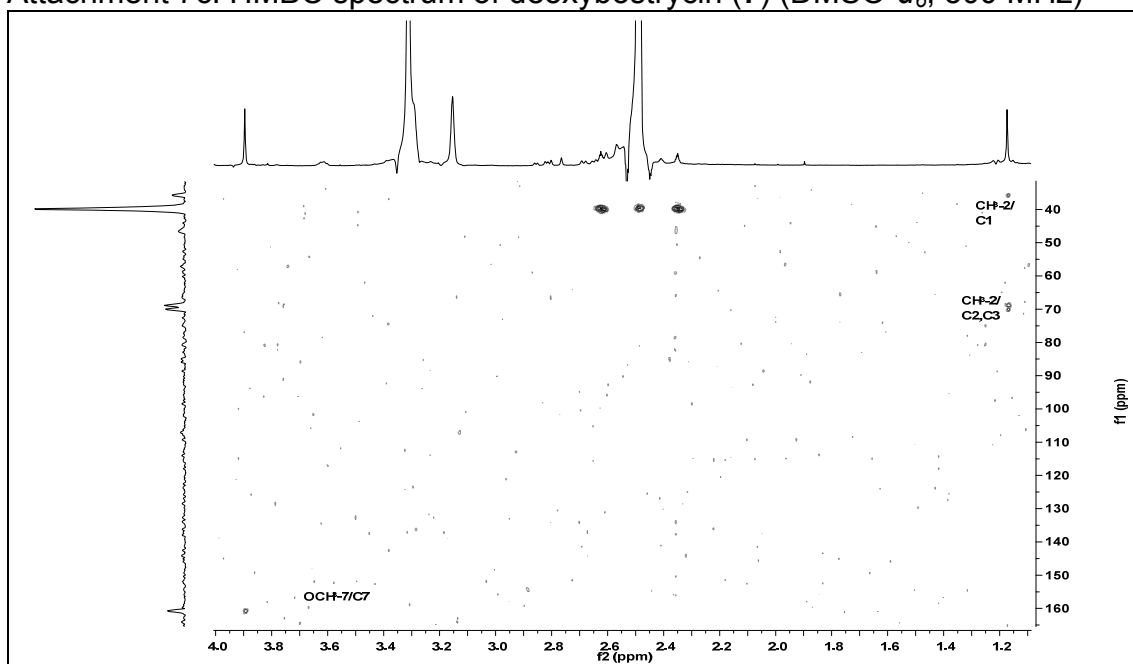
Attachment 8a: ^{1}H NMR spectrum of bostrycin (**8**) (DMSO-d_6, 500 MHz)
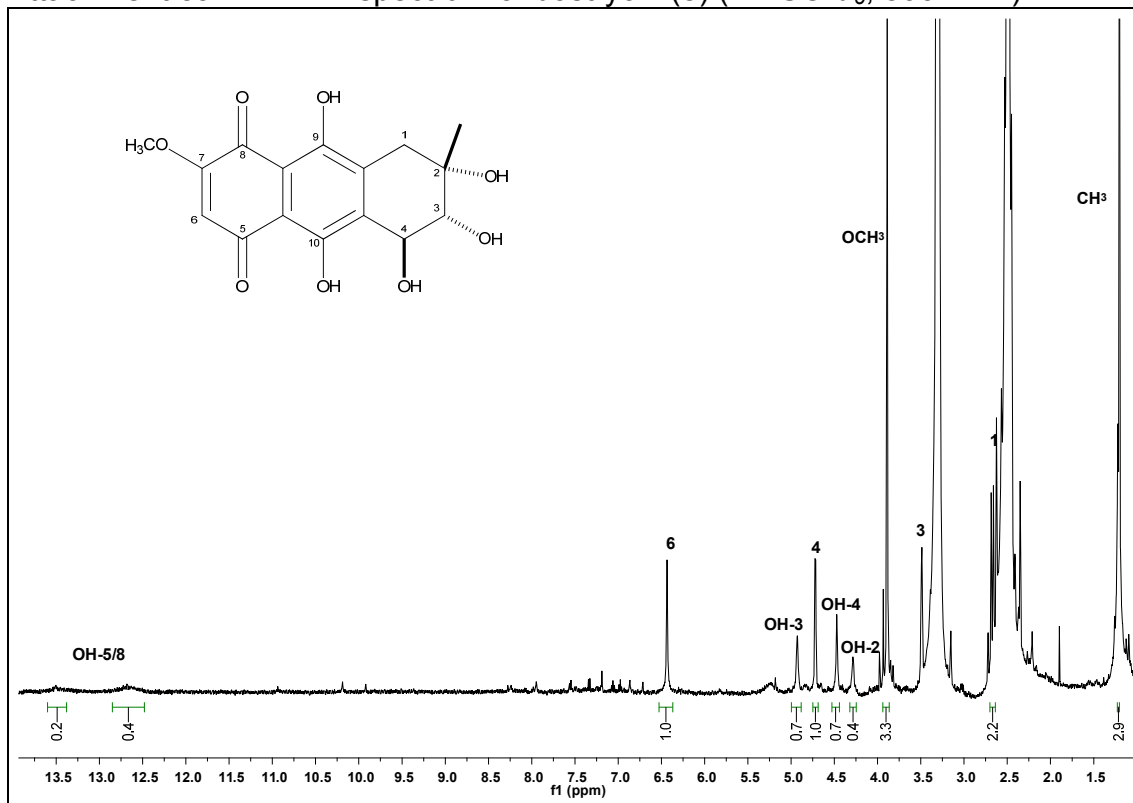
Attachment 8b: COSY spectrum of bostrycin (**8**) (DMSO-d_6, 500 MHz)
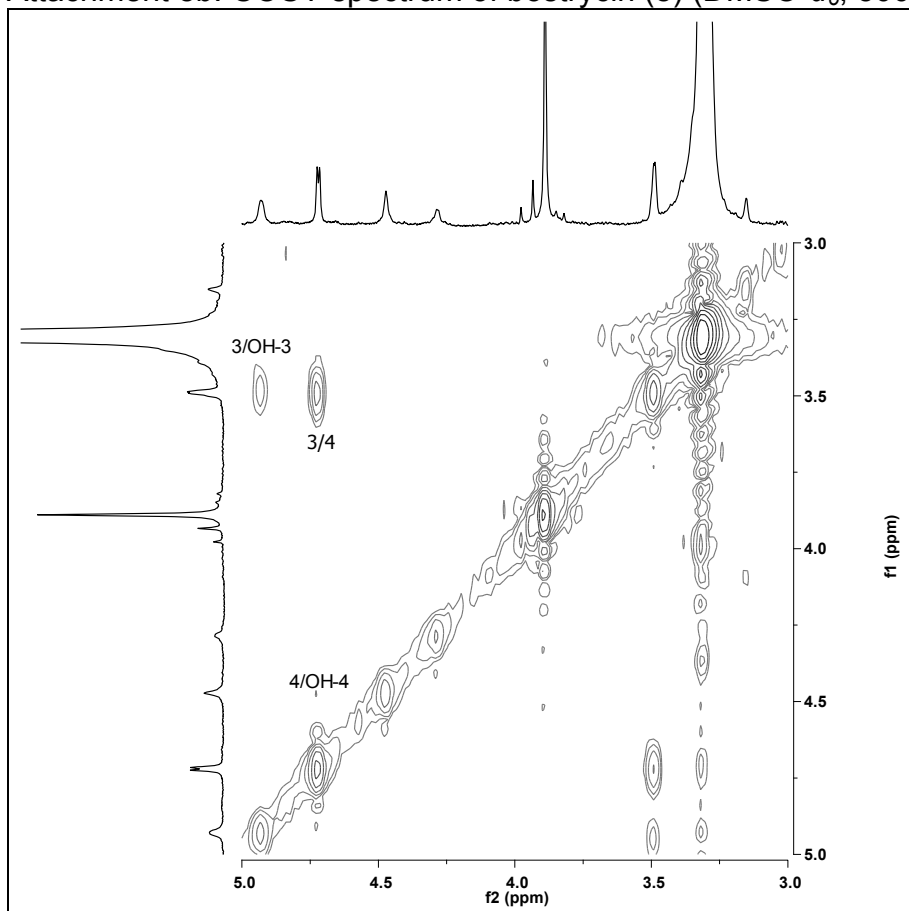
Attachment 8c: HMBC spectrum of bostrycin (**8**) (CDCl$_3$, 500 MHz)
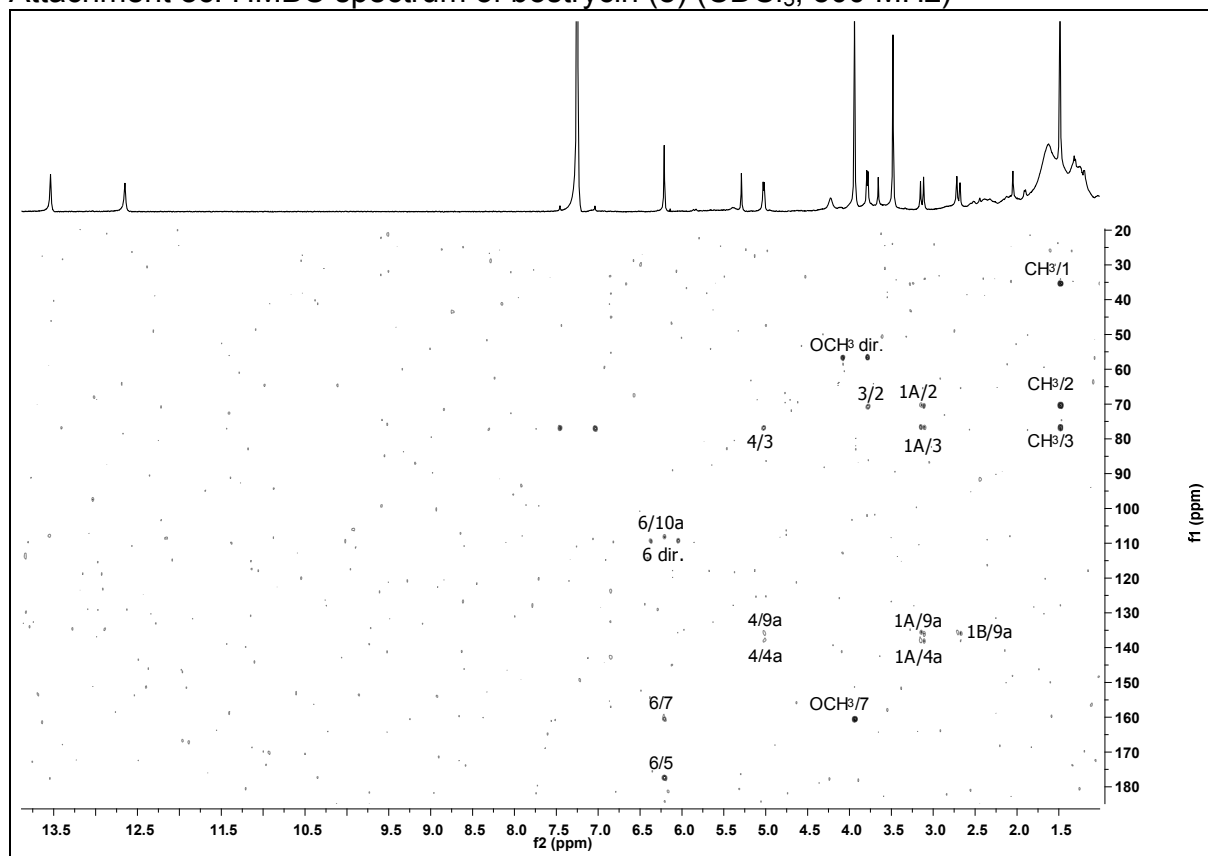
Attachment 9: ^{1}H NMR spectrum of tenuazonic acid (**9**) (MeOD, 500 MHz)
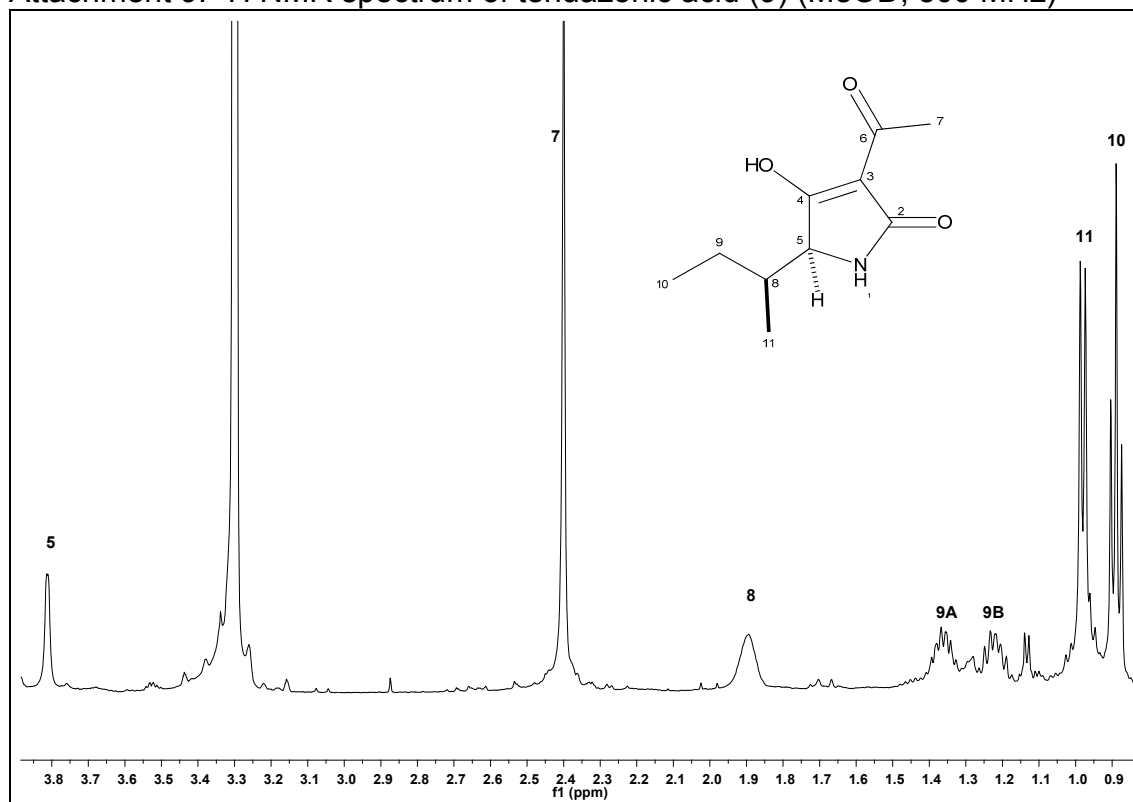
Attachment 10: ^{1}H NMR spectrum of indole-3-carboxaldehyde (**10**) (DMSO-d_6, 500 MHz)
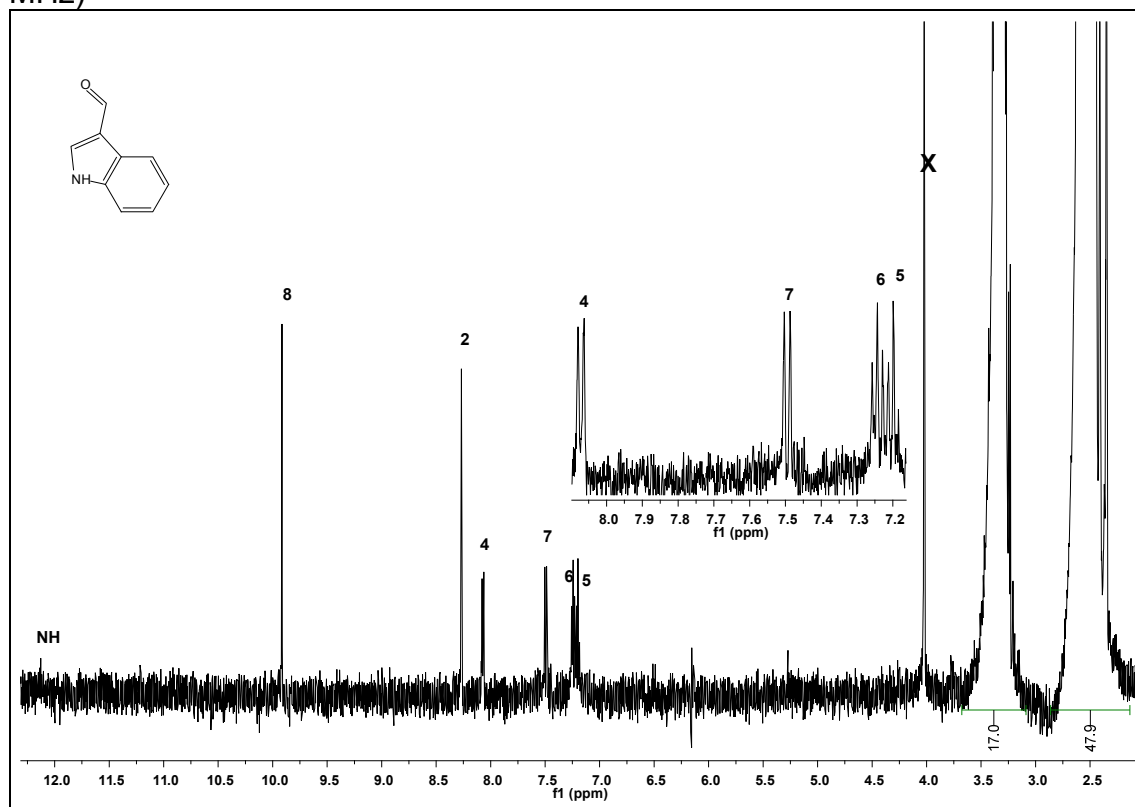
Attachment 11a: ^{1}H NMR spectrum of Cyclo(threonylisoleucine) (**11**) (DMSO-d_6, 500 MHz)
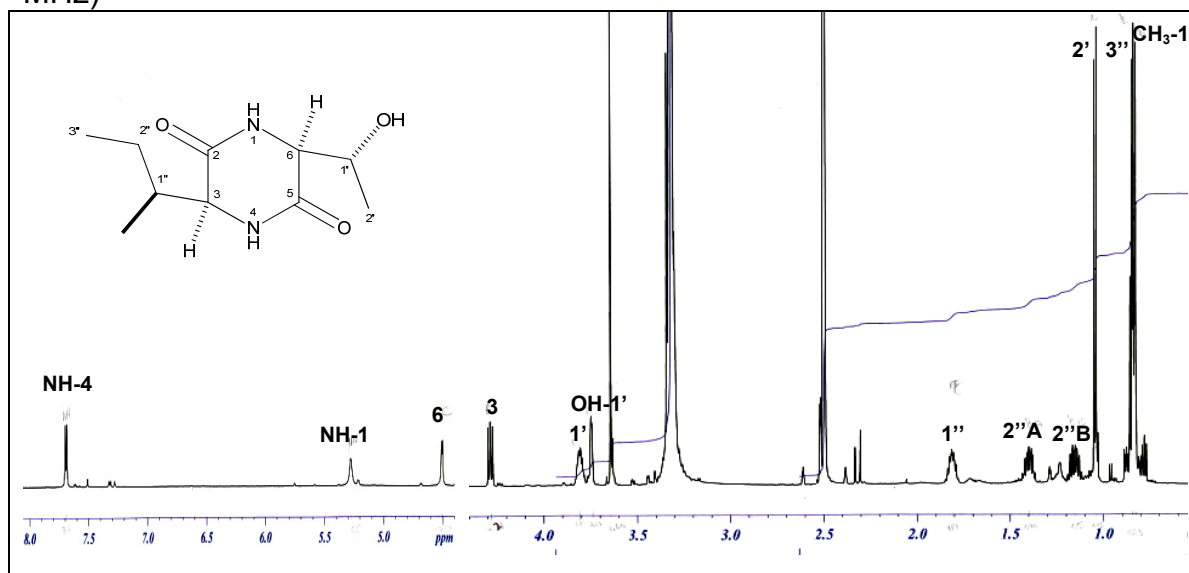
Attachment 11b: ^{13}C NMR spectrum of Cyclo(threonylisoleucine) (**11**) (DMSO-d_6, 500 MHz)
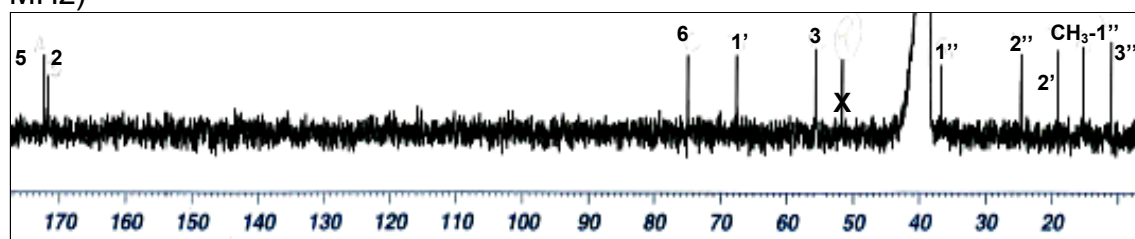
Attachment 12: ^{1}H NMR spectrum 2,5-dimethyl-7-hydroxychromone (**12**) (MeOD, 500 MHz)
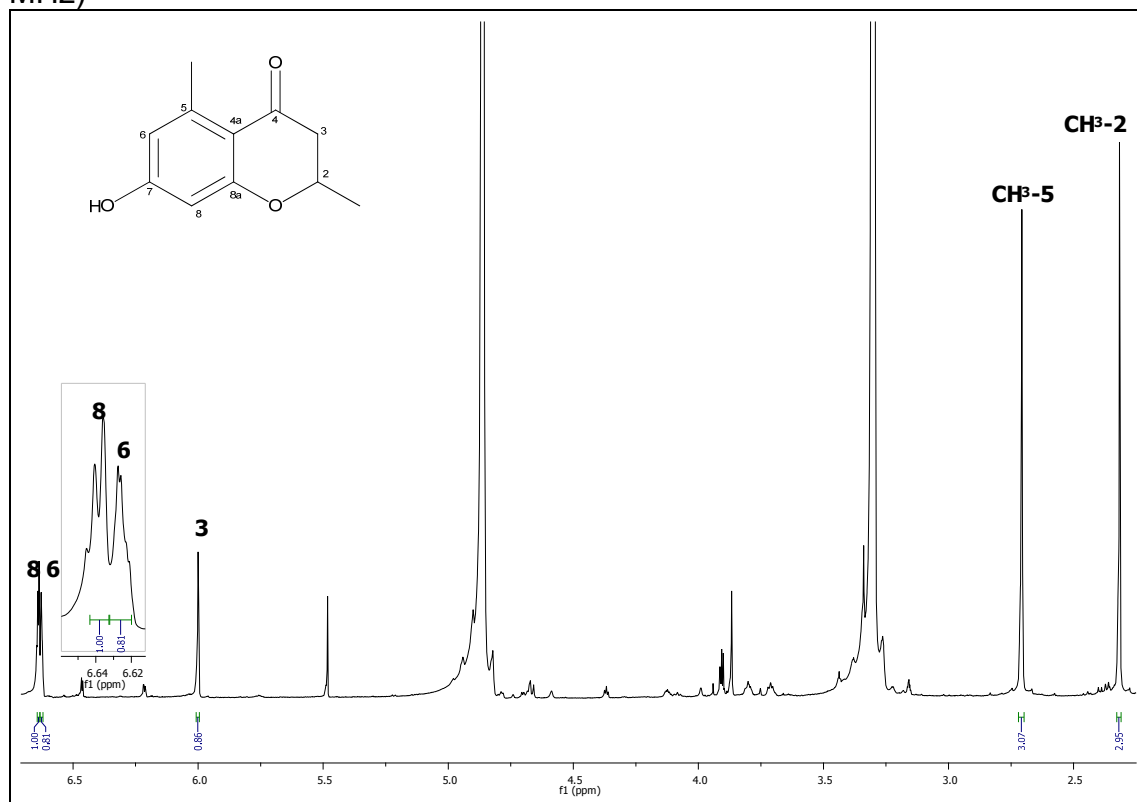
Attachment 13a: ^{1}H NMR spectrum alternarian acid (**13**) (MeOD, 500 MHz)
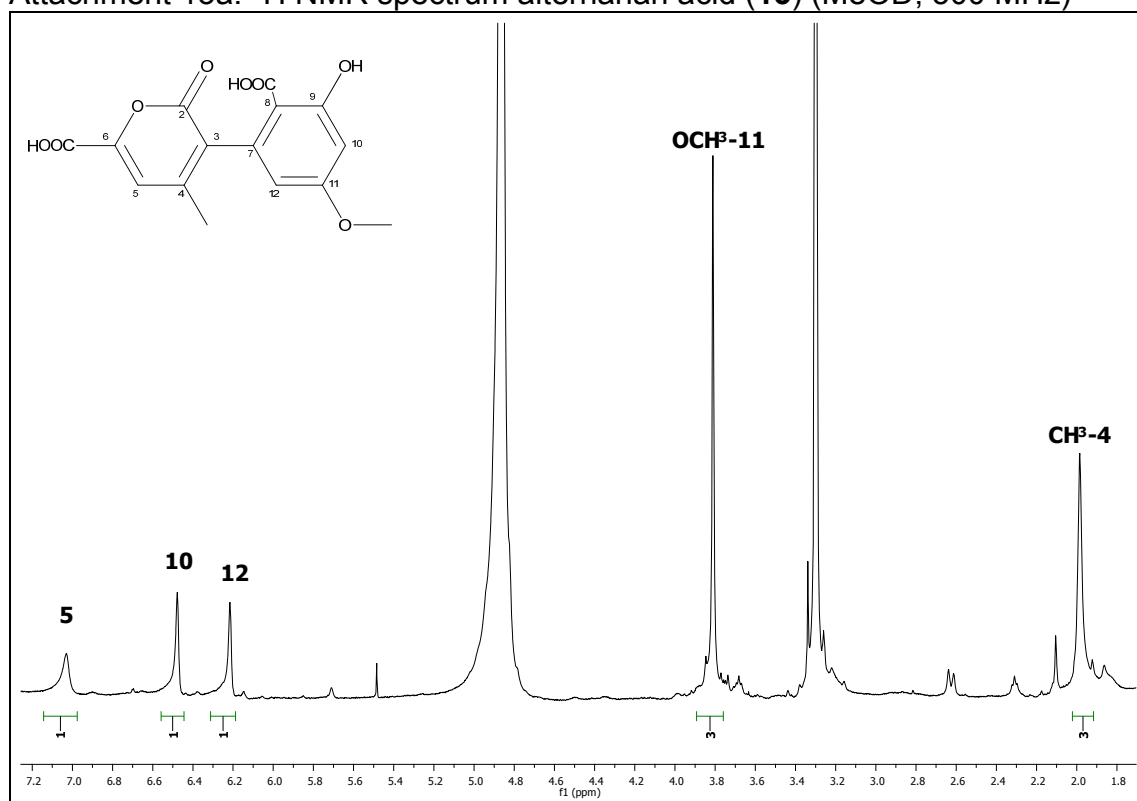
Attachment 13b: ROESY spectrum alternarian acid (**13**) (DMSO-*d₆*, 500 MHz)

12/OCH₃-11

10/OCH₃-11
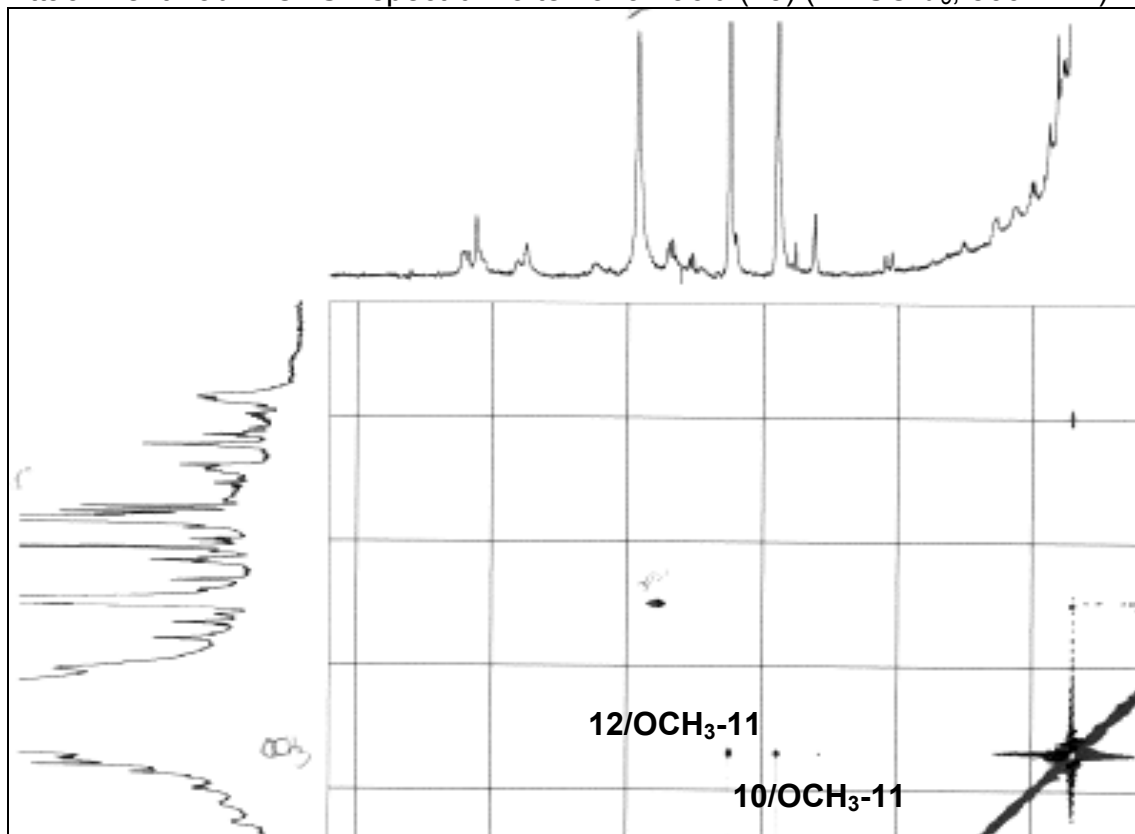
Attachment 14: ¹H NMR spectrum altenusin (**14**) (MeOD, 500 MHz)
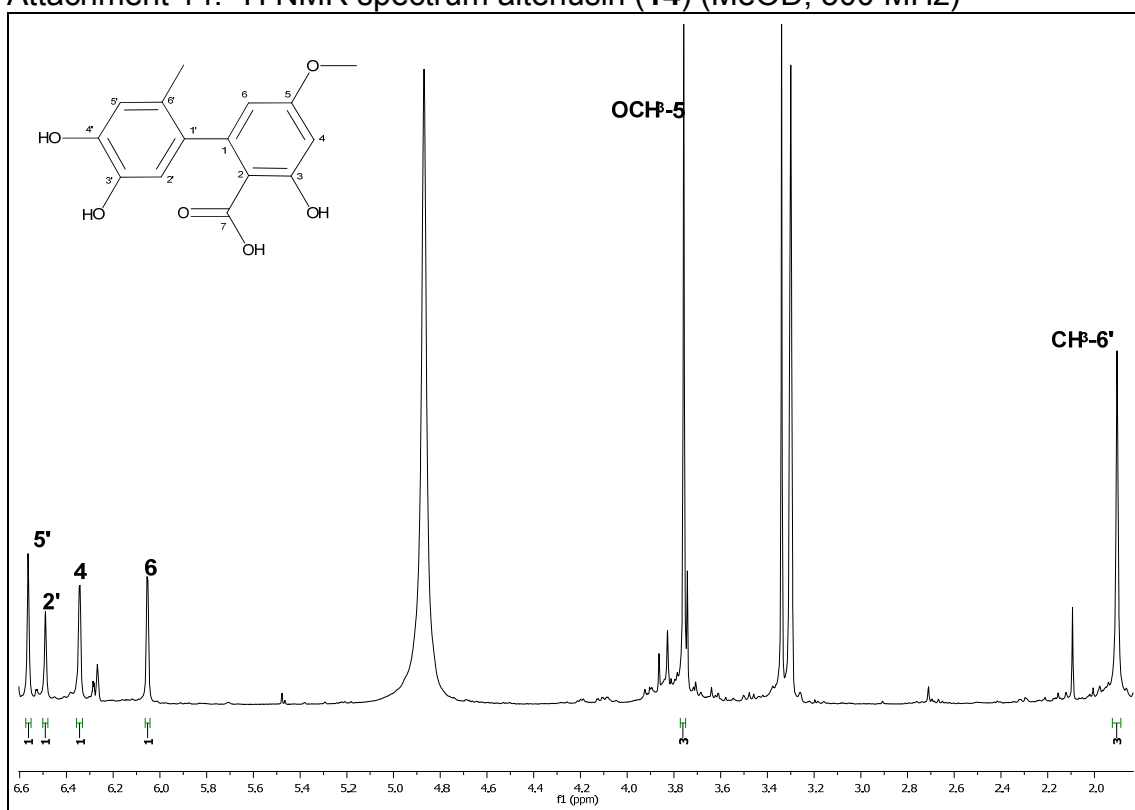
Attachment 15: ¹H NMR spectrum of altenuene (**15**) (DMSO-*d₆*, 500 MHz)
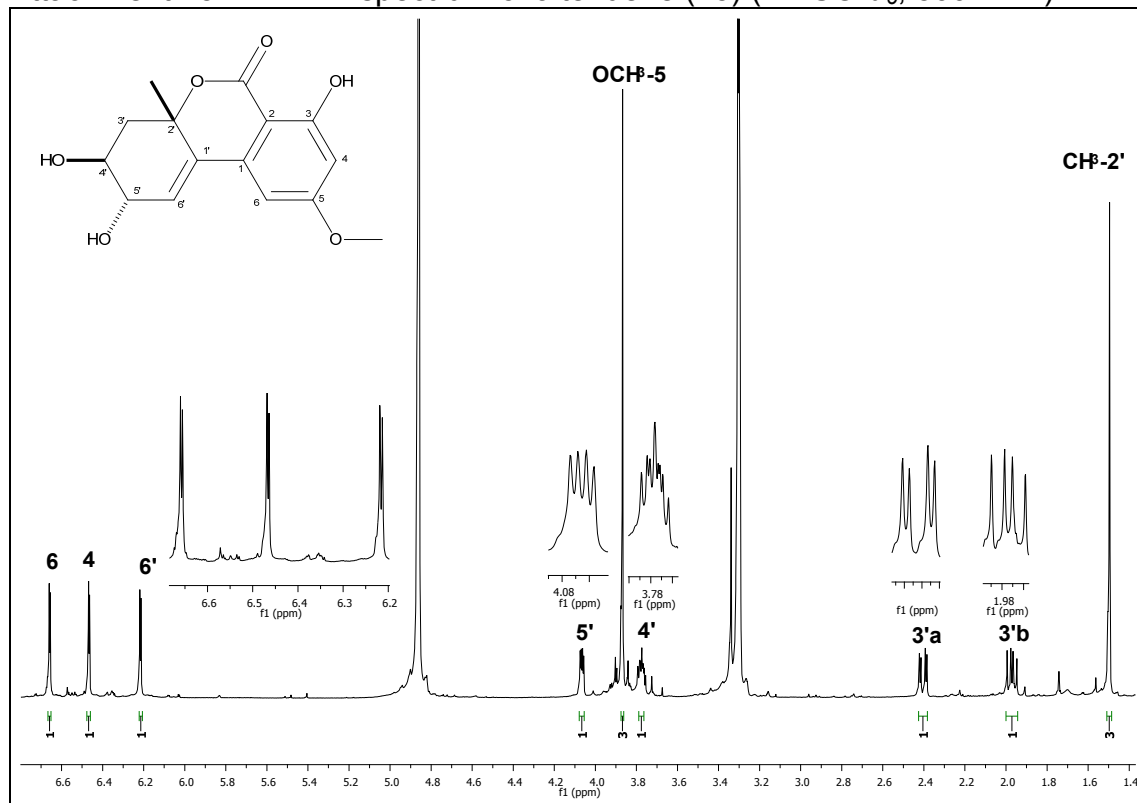
Attachment 16: ¹H NMR spectrum of 4'-epialtenuene (**16**) (DMSO-*d₆*, 500 MHz)
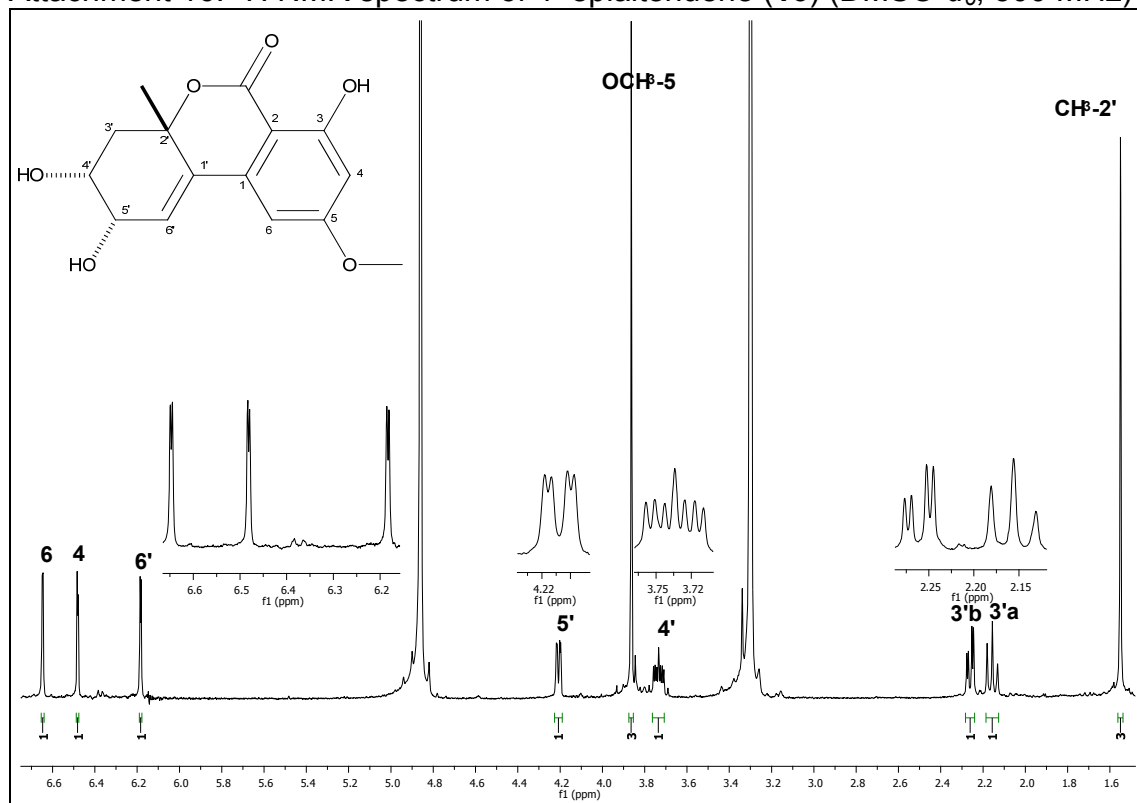
Attachment 17: ^{1}H NMR spectrum of alternarienonic acid (**17**) (DMSO-d_6, 500 MHz)
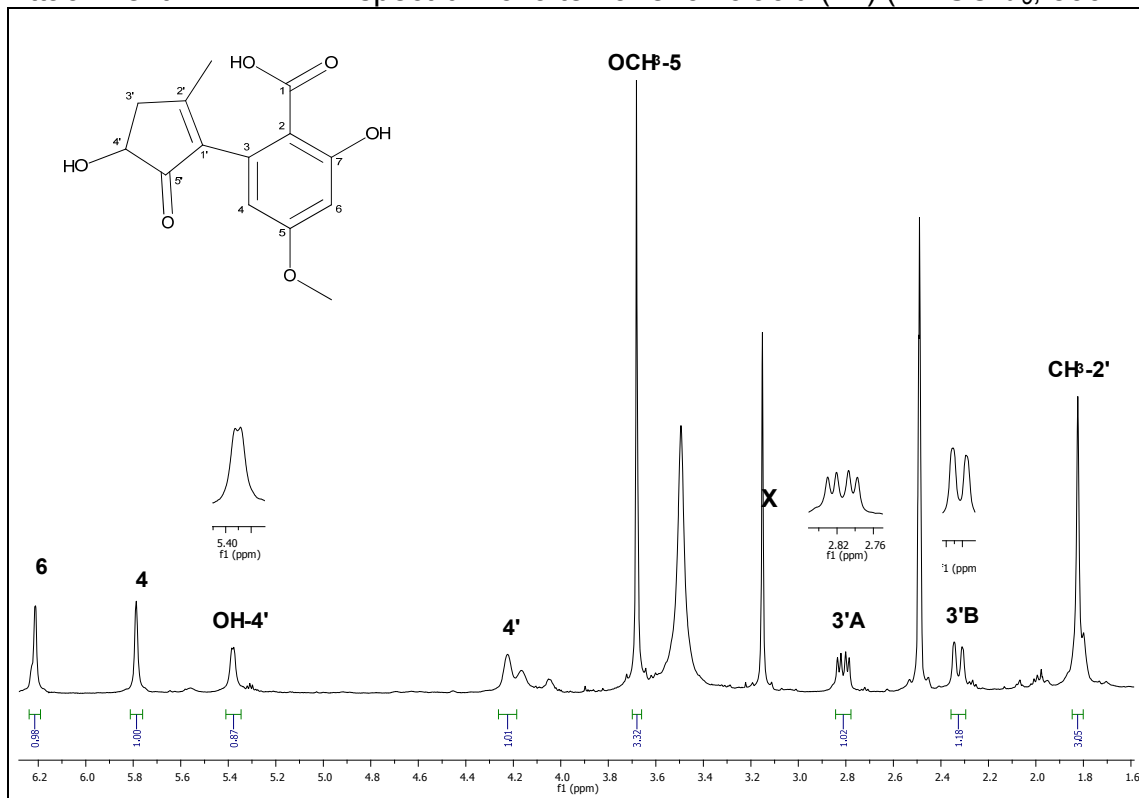
Attachment 18: ^{1}H NMR spectrum of alternariol (**18**) (MeOD, 500 MHz)
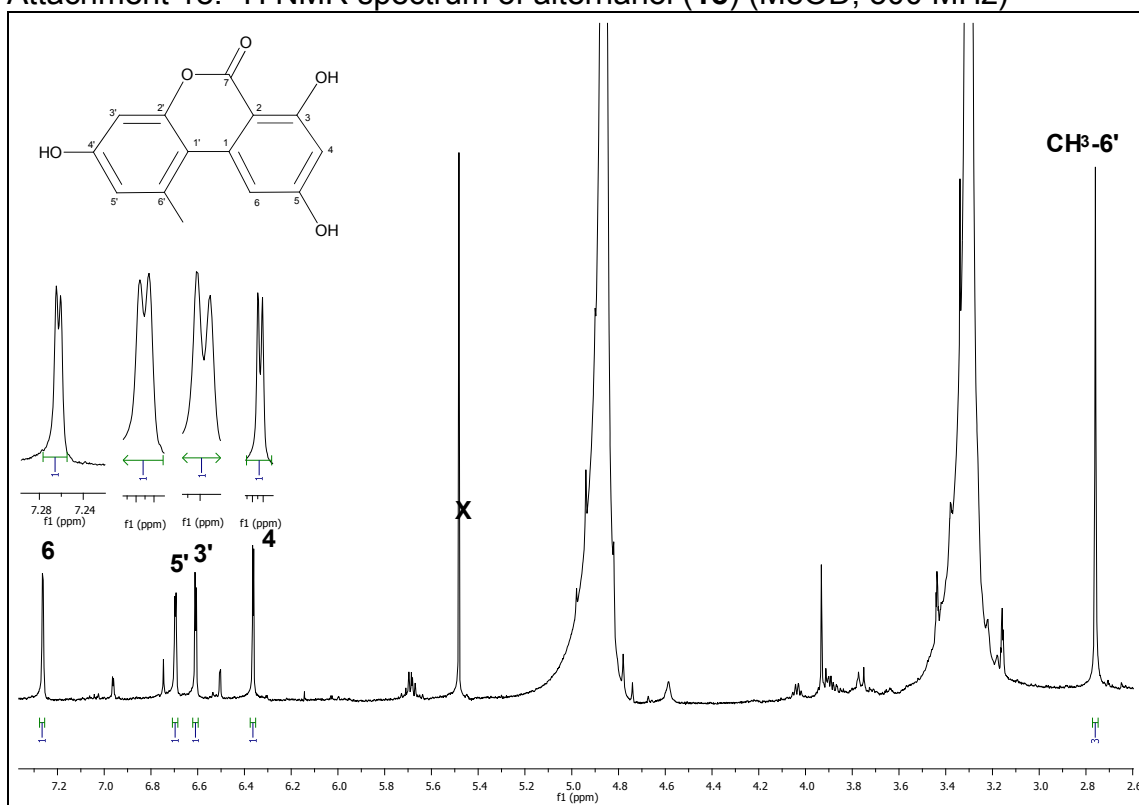
Attachment 19: ^{1}H NMR spectrum of alternariol-5-methylether (**19**) (MeOD, 500 MHz)
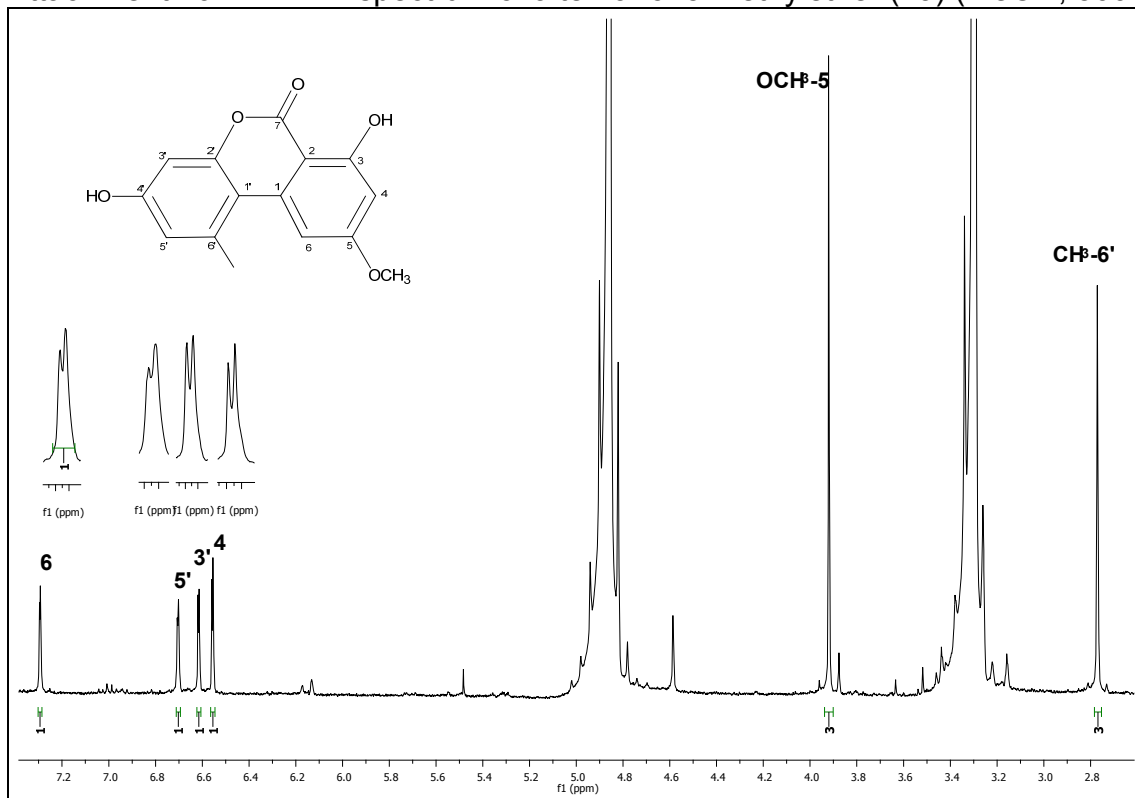
Attachment 20: ^{1}H NMR spectrum of altertoxin I (**20**) (DMSO-d_6, 600 MHz)
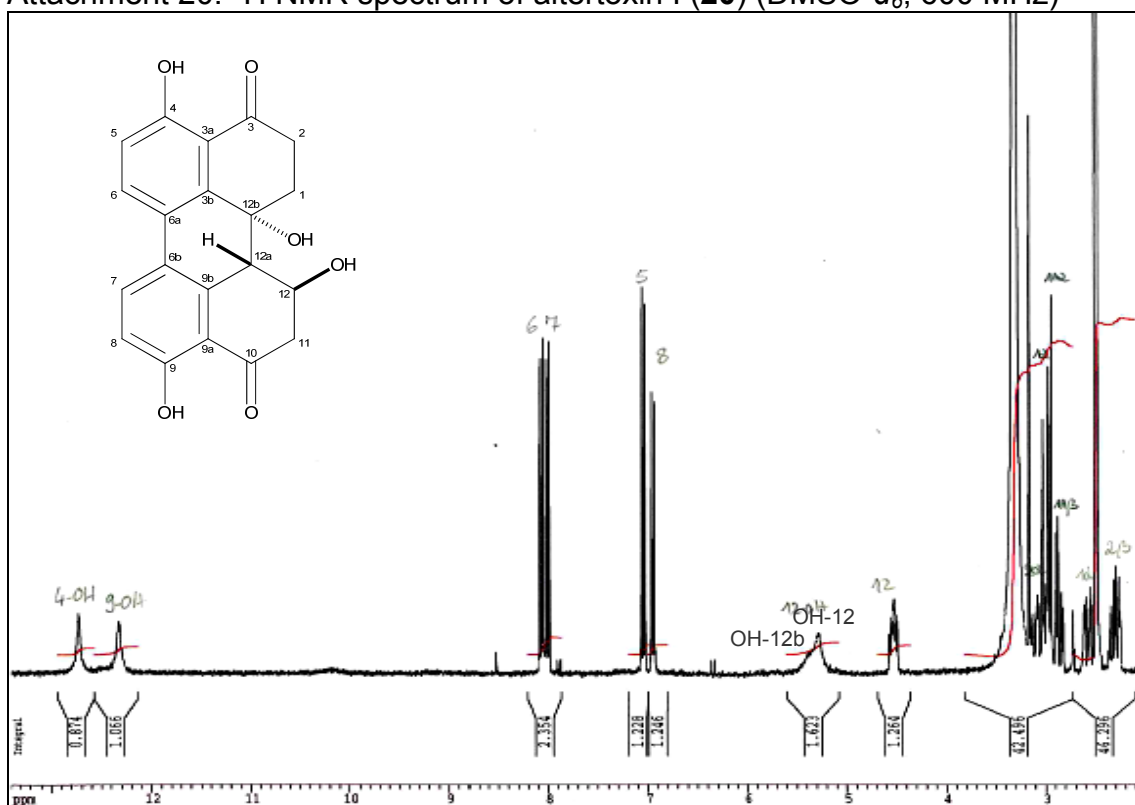
Attachment 21a: ^{1}H NMR spectrum of alterperylenol (**21**) (DMSO-d_6, 400 MHz)
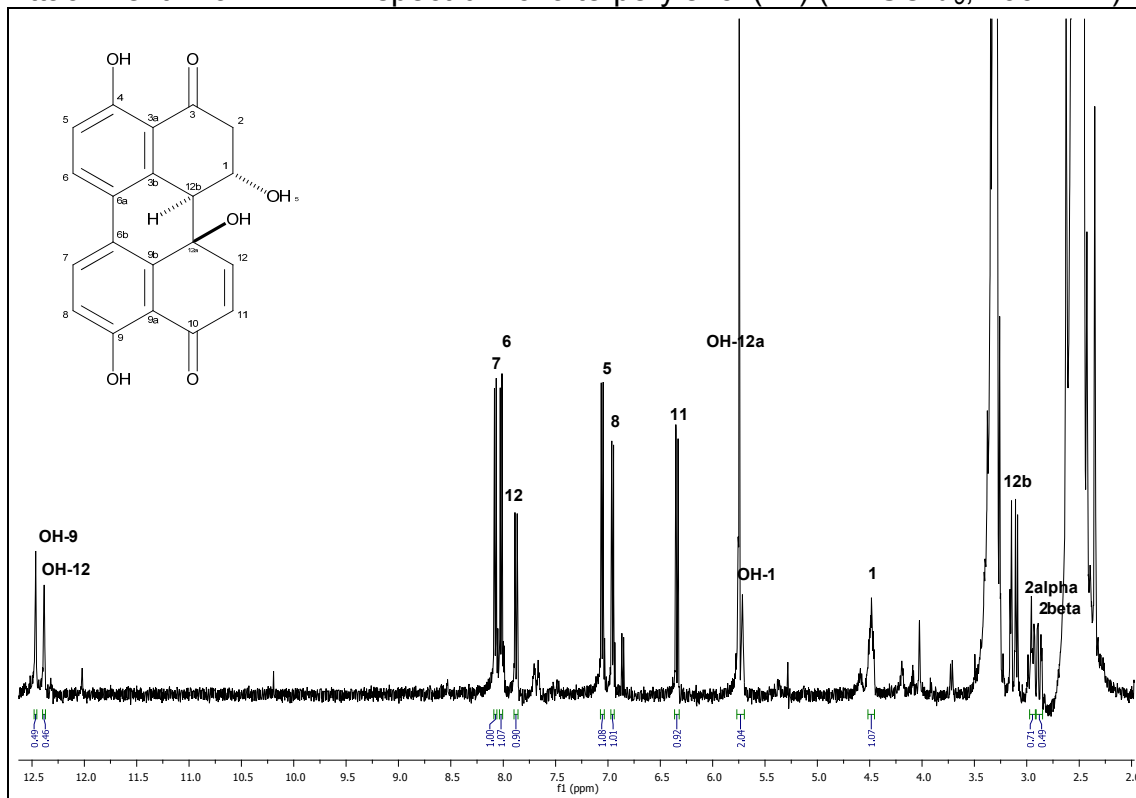
Attachment 21b: ROESY spectrum of alterperylenol (**21**) (DMSO-d_6, 400 MHz)
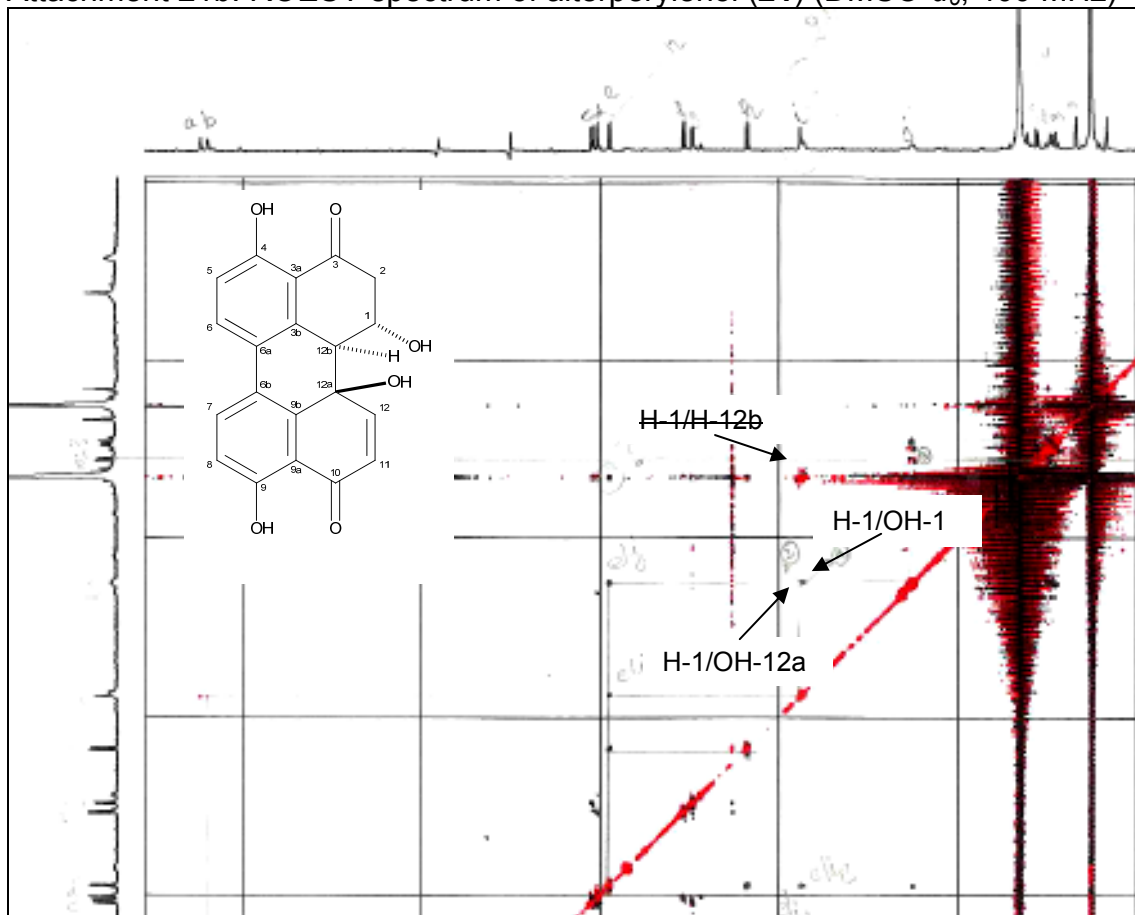
Attachment 22: ROESY spectrum of stemphyperylenol (**22**) (DMSO-*d₆*, 400 MHz)
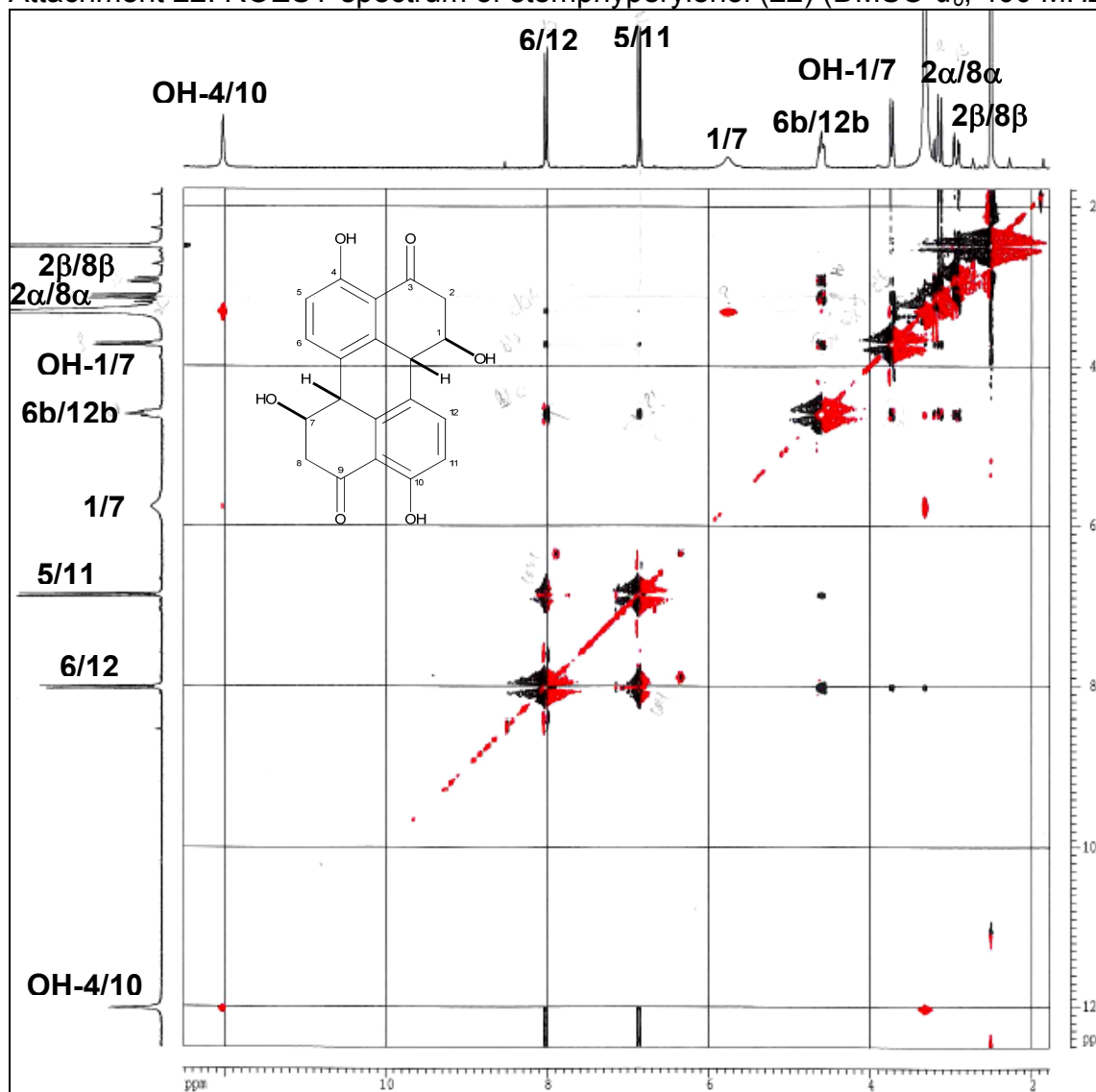
Attachment 23: ^{1}H NMR spectrum of xanalteric acid I (**23**) (DMSO-d_6, 600 MHz)
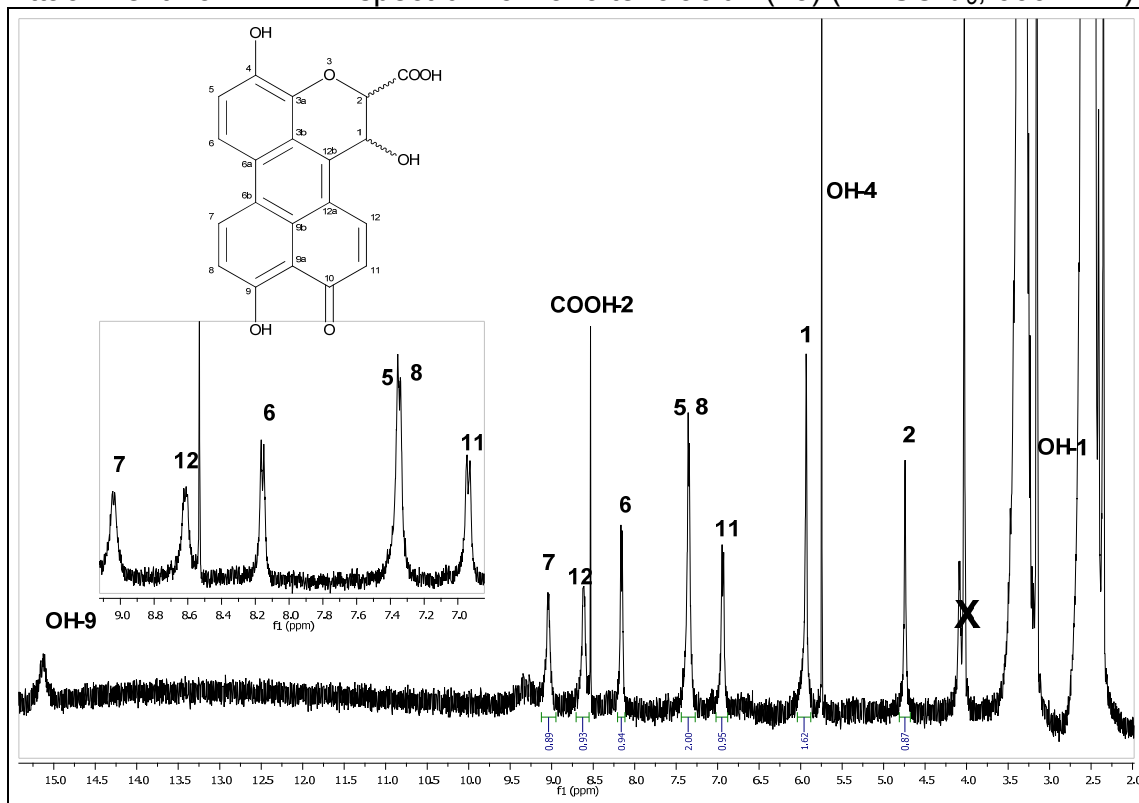
Attachment 24: ^{1}H NMR spectrum of xanalteric acid II (**24**) (DMSO-d_6, 600 MHz)
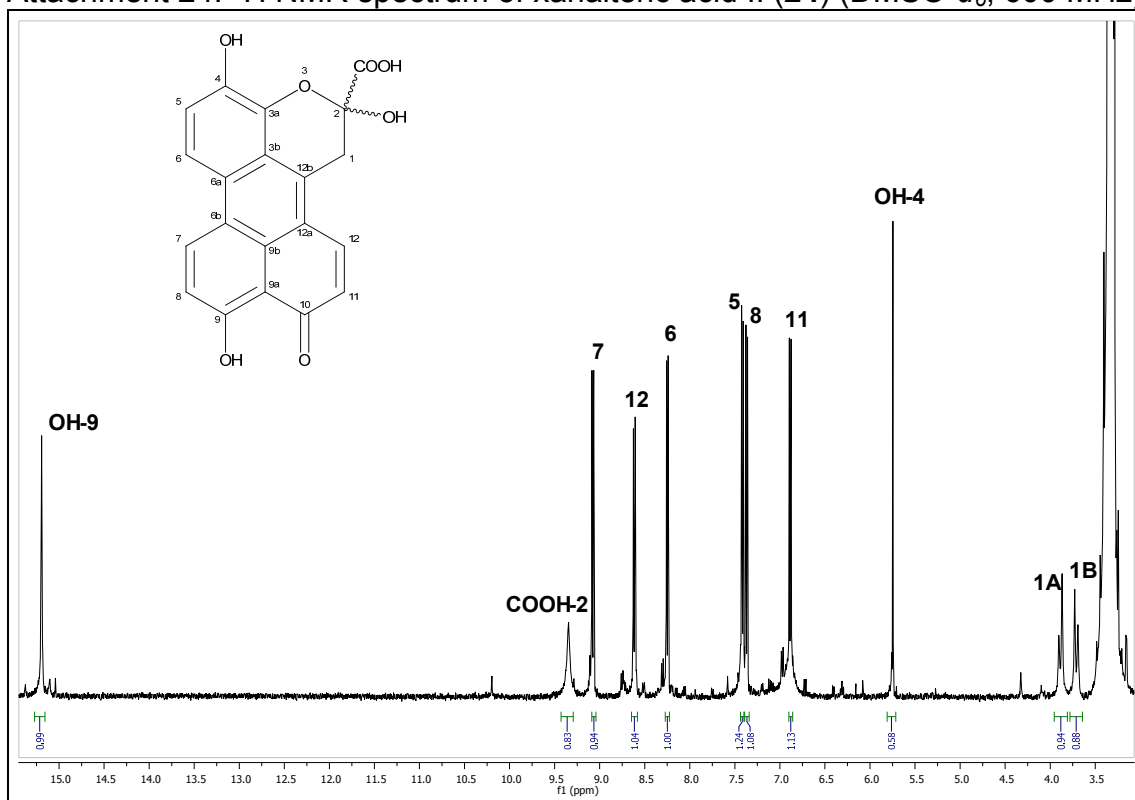
Attachment 25: ^{1}HNMR spectrum of verticillin D (**25**) (CDCl$_3$, 500 MHz)
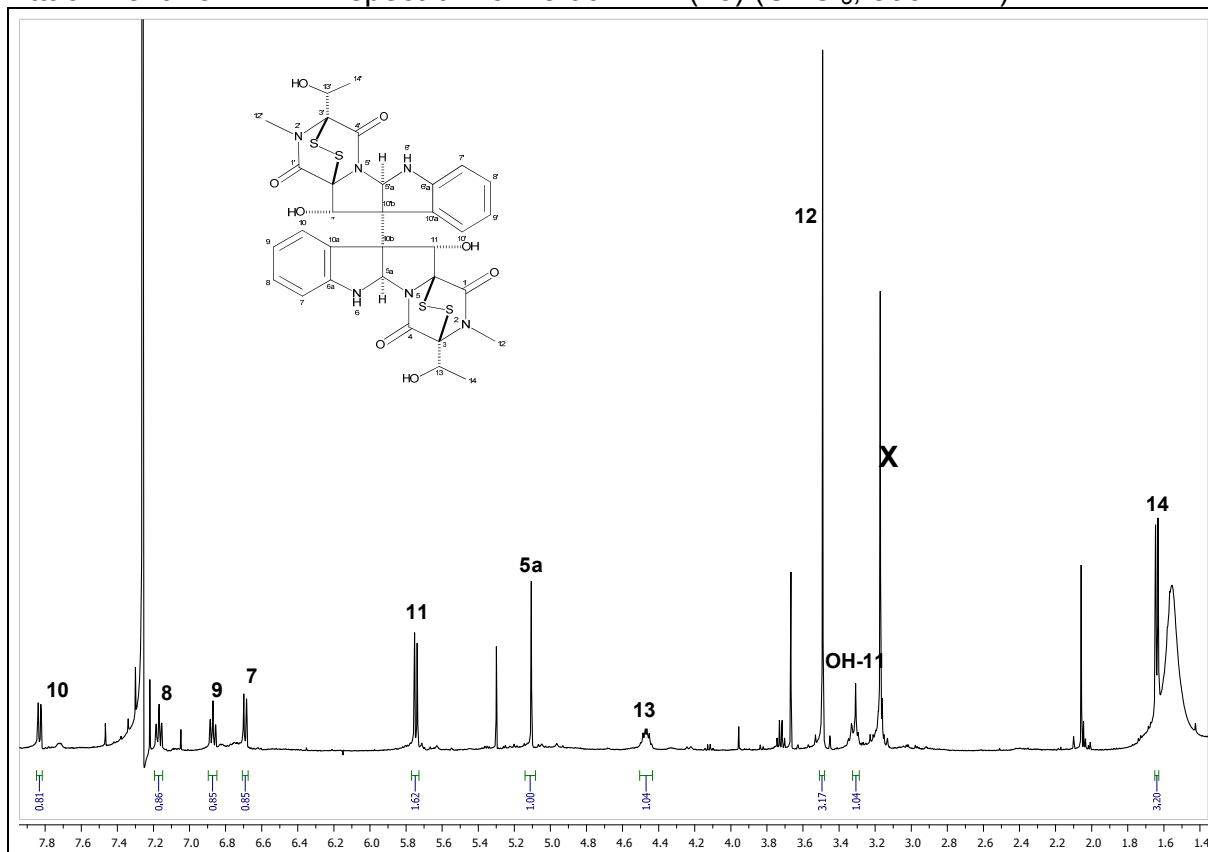
Attachment 26a: ^{1}HNMR spectrum of bionectriamide A (**26**) (CDCl$_3$, 500 MHz)
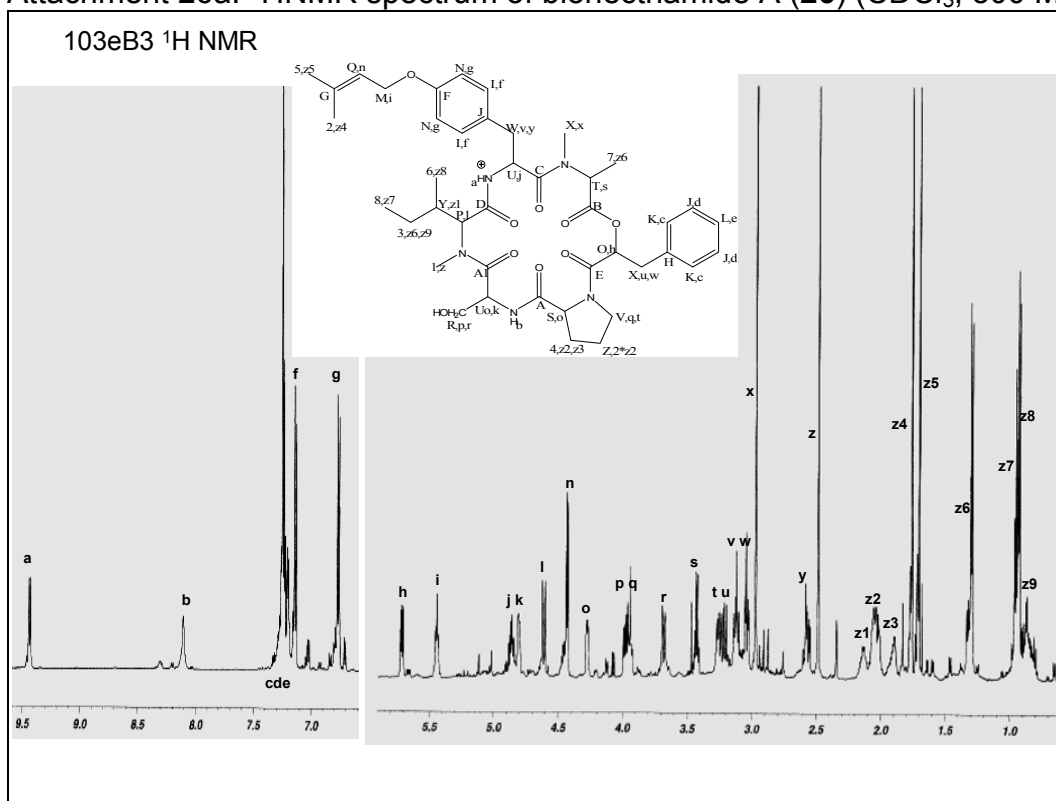
Attachment 26b: HMQC spectrum of bionectriamide A (**26**) (CDCl$_3$, 500 MHz)
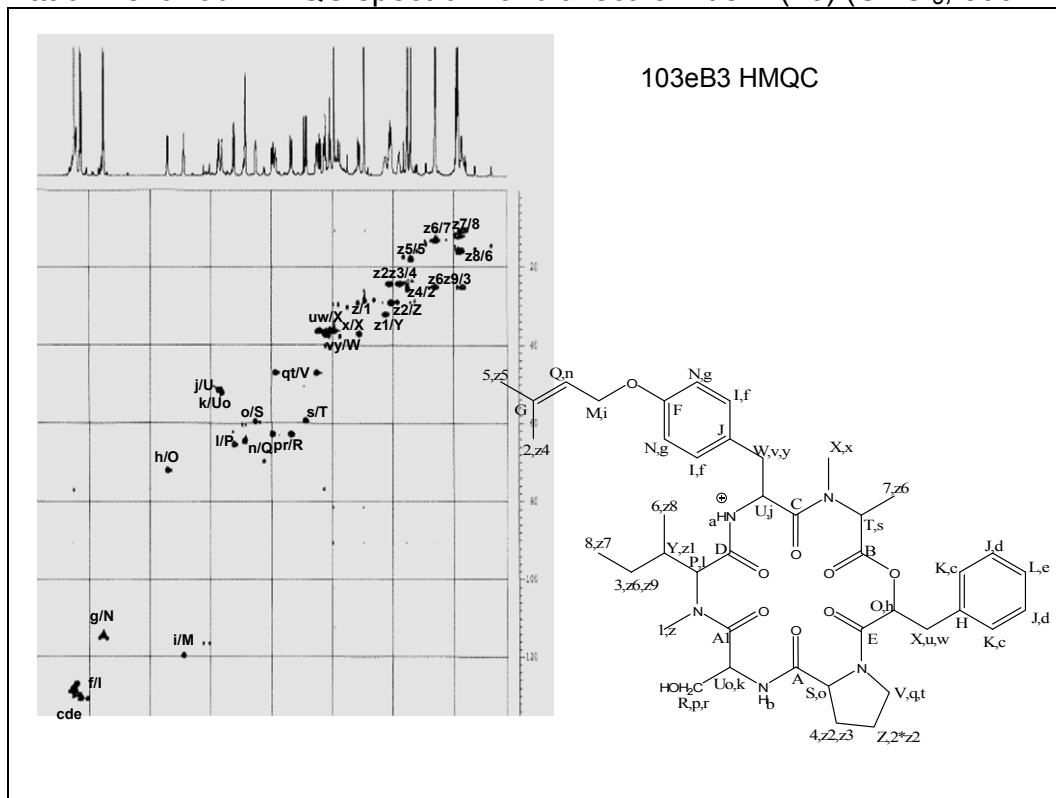
Attachment 26c: HMBC spectrum of bionectriamide A (**26**) (CDCl$_3$, 500 MHz)
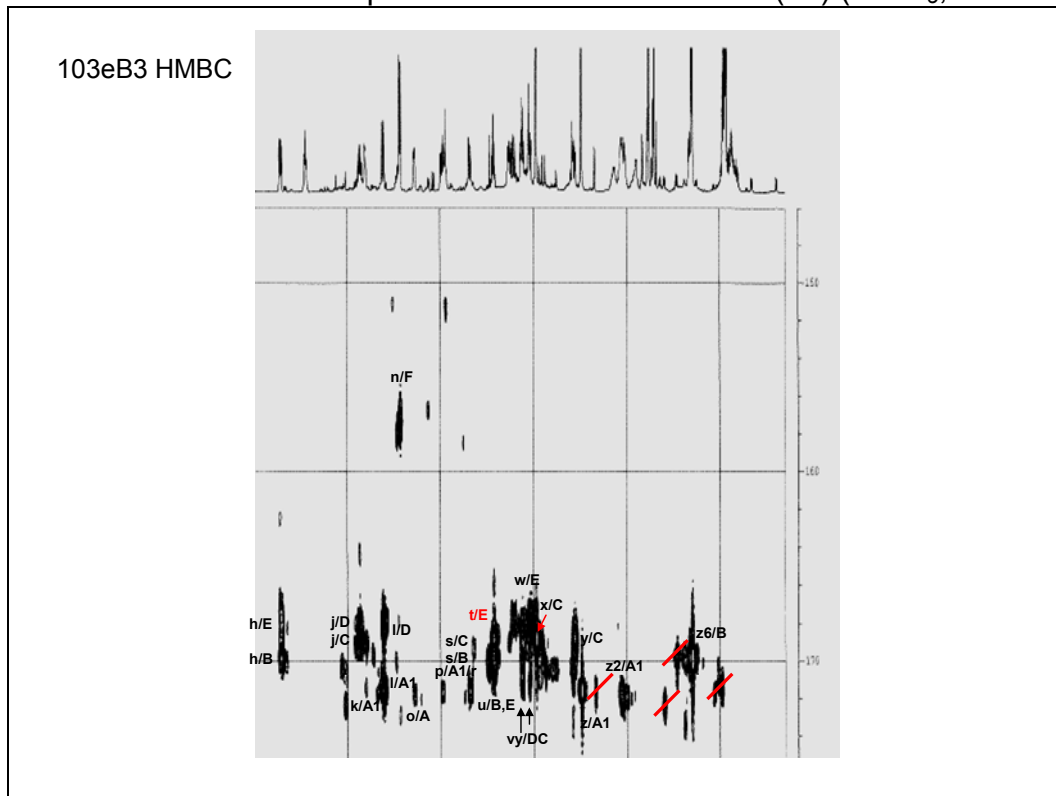
Attachment 26d: TOCSY spectrum of bionectriamide A (**26**) (CDCl$_3$, 500 MHz)
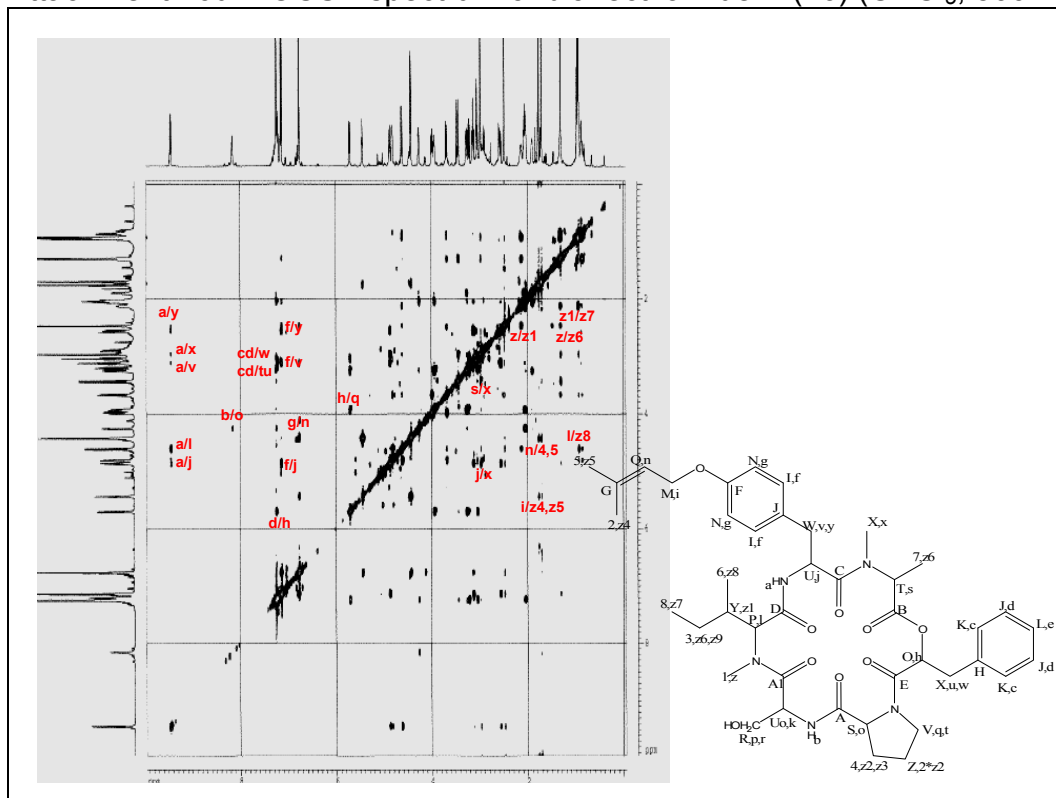
Attachment 27a: ^{1}HNMR spectrum of bionectriamide B (**27**) (CDCl$_3$, 500 MHz)

103eB2 ^{1}H NMR
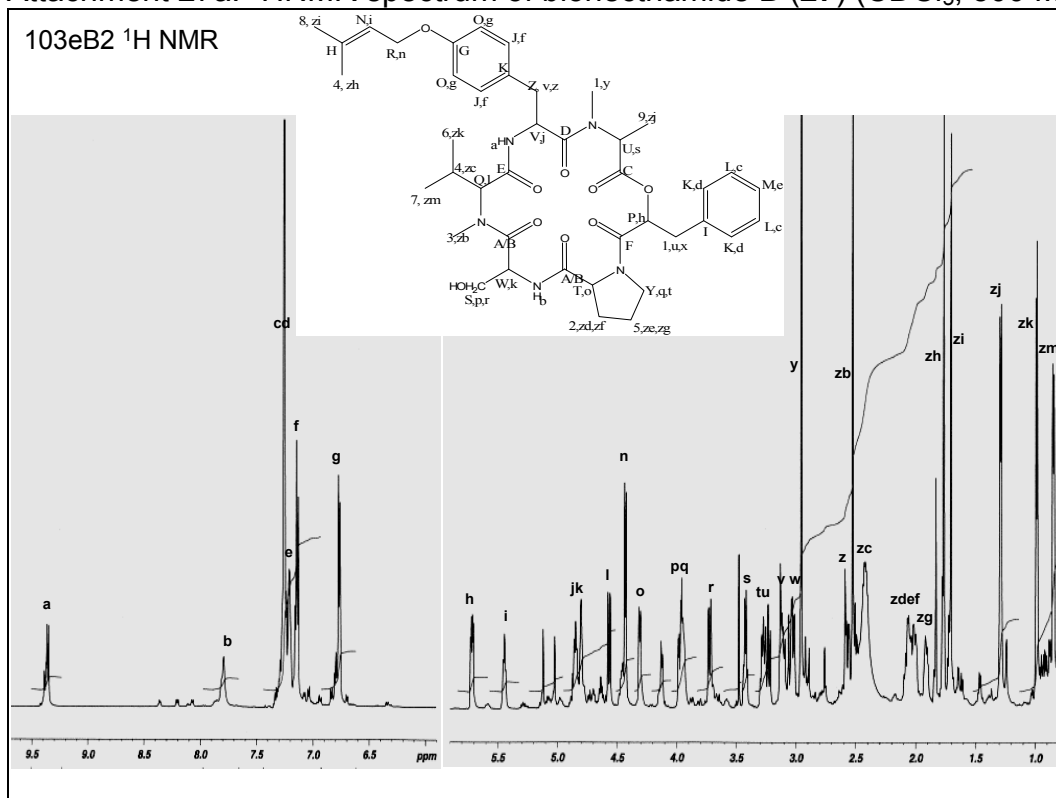
Attachment 27b: HMBC spectrum of bionectriamide B (**27**) (CDCl$_3$, 500 MHz)
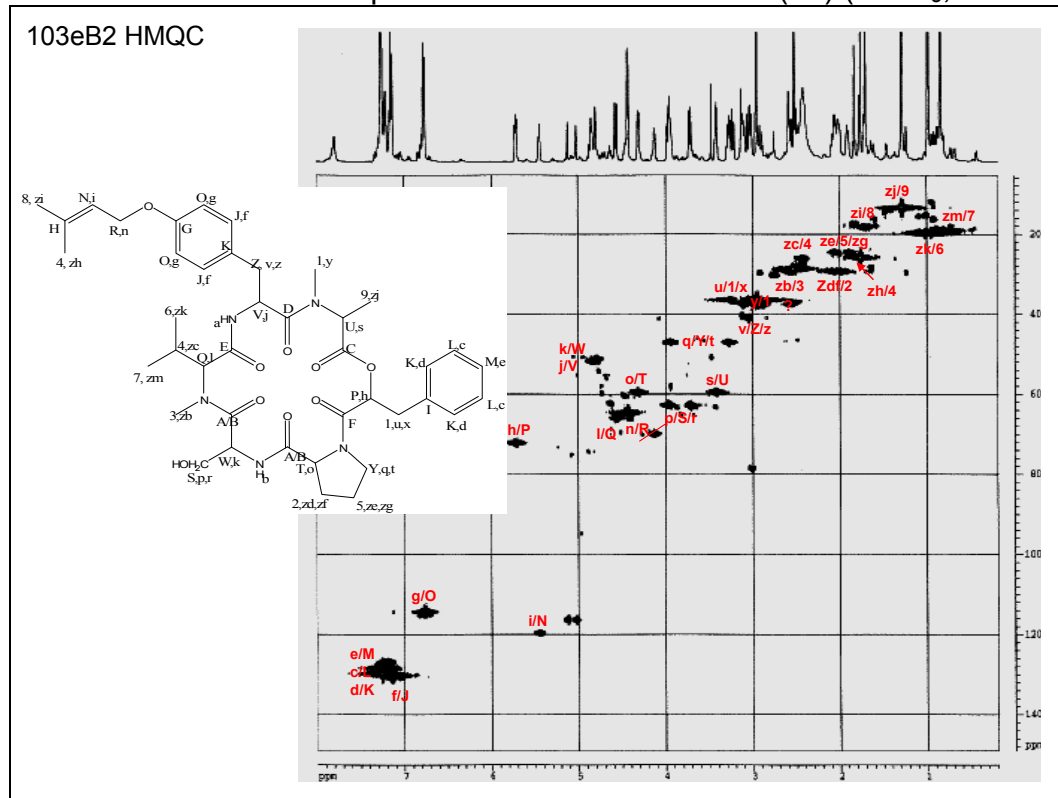
Attachment 27c: HMBC spectrum of bionectriamide B (**27**) (CDCl$_3$, 500 MHz)
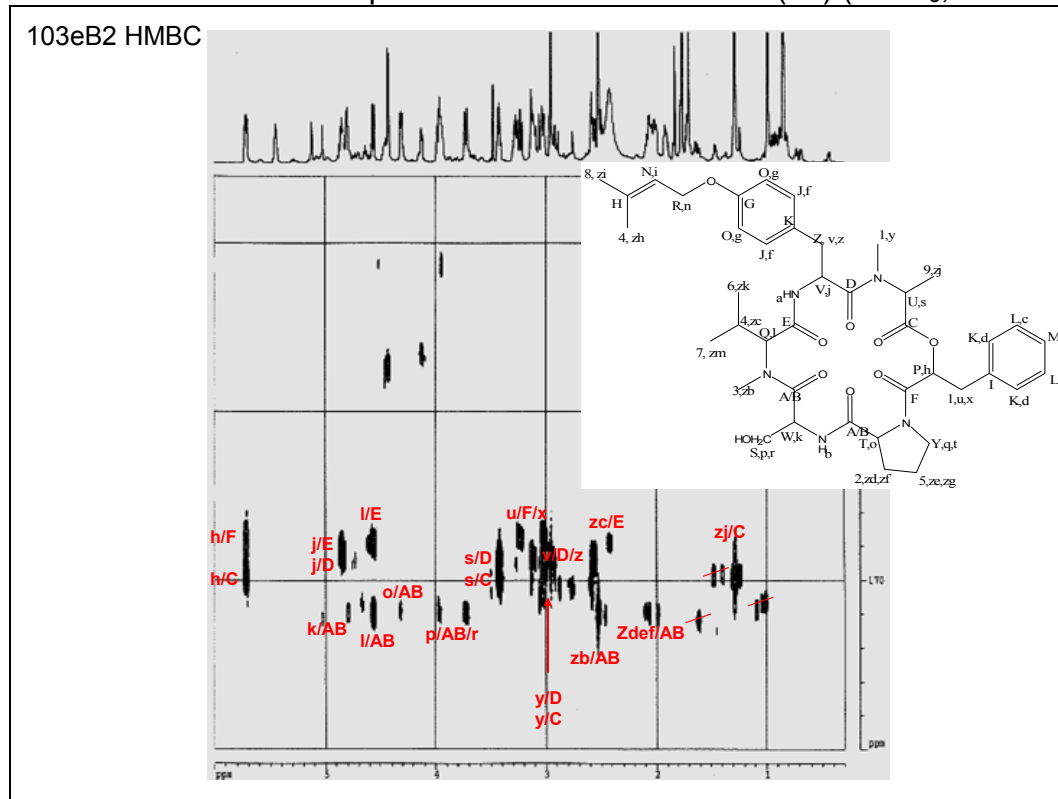
Attachment 27d: ROESY spectrum of bionectriamide B (**27**) (CDCl$_3$, 500 MHz)
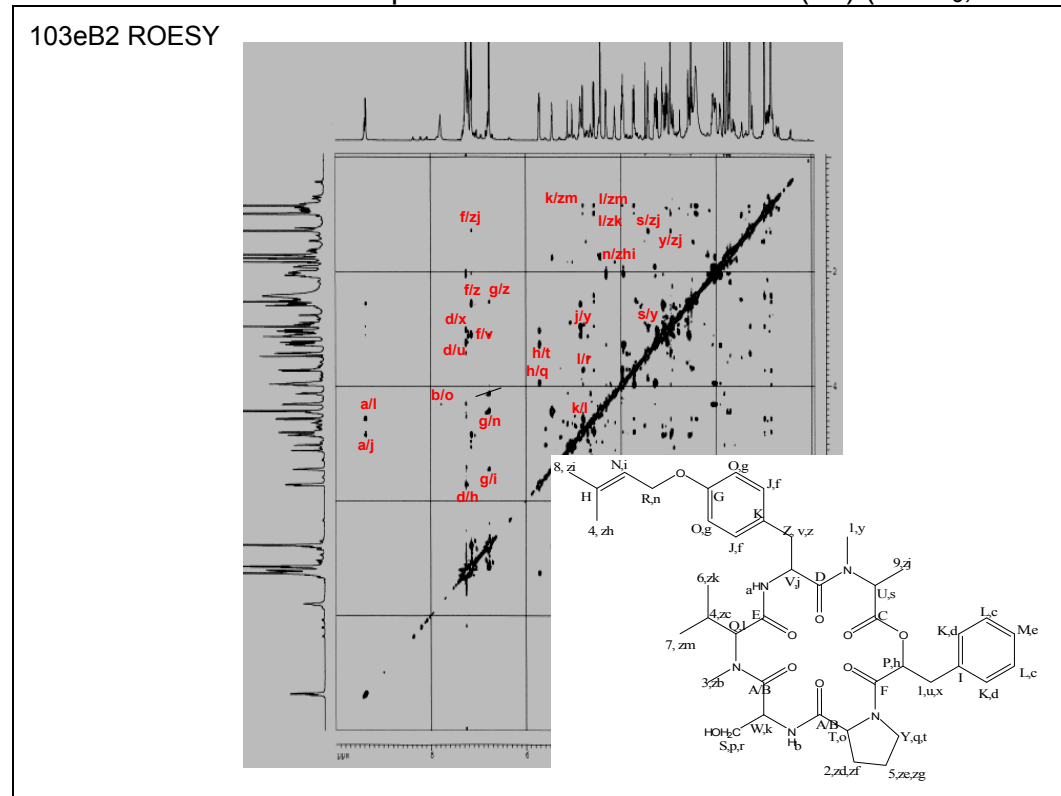
Attachment 27e: TOCSY spectrum of bionectriamide B (**27**) (CDCl$_3$, 500 MHz)
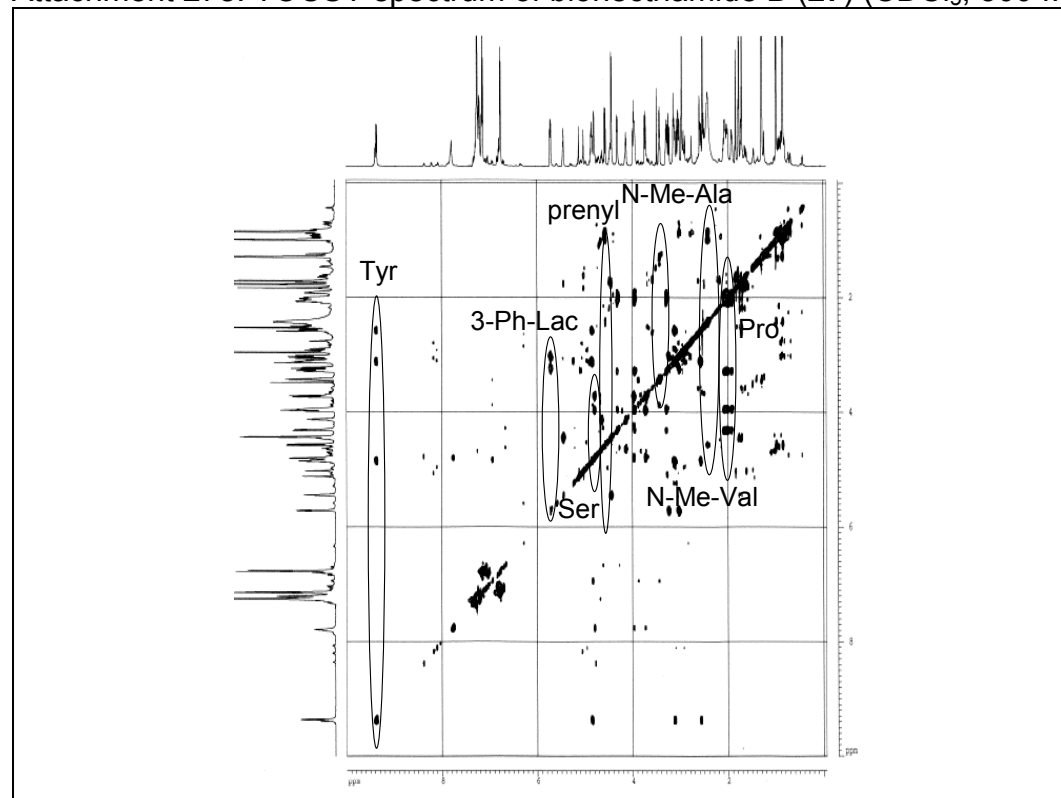
Attachment 28: ^{1}HNMR spectrum of bionectriamide C (**28**) (CDCl$_3$, 500 MHz)
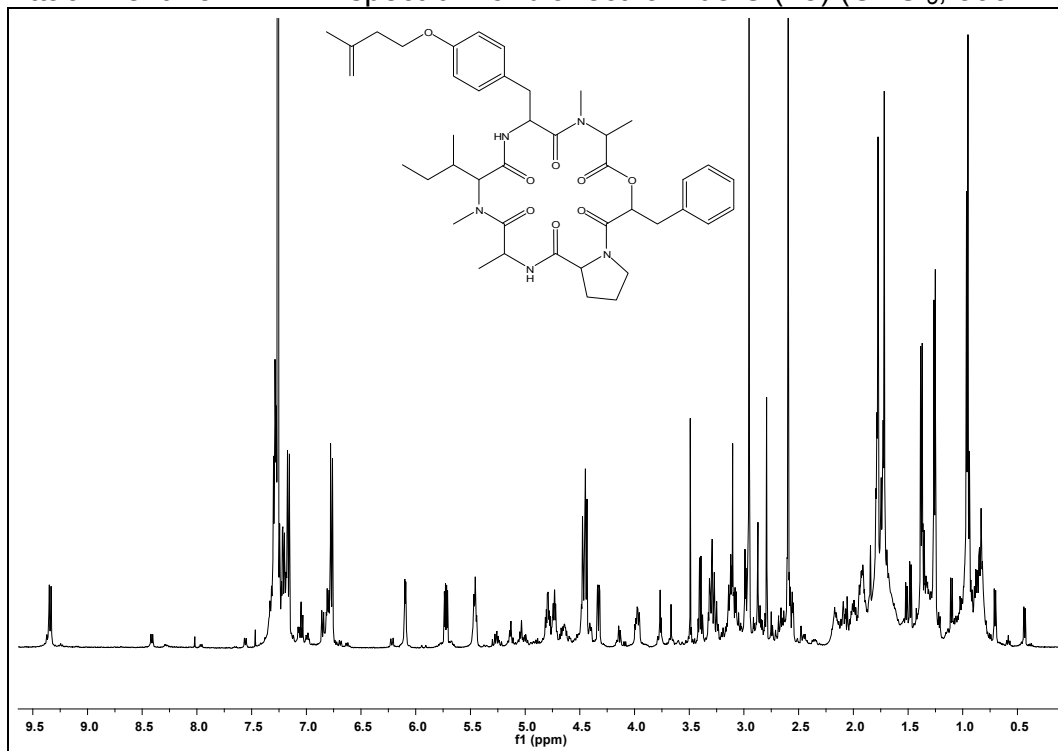
Attachment 29a: ^{1}HNMR spectrum of bionectriamide D (**29**) (DMSO-d_6, 500 MHz)
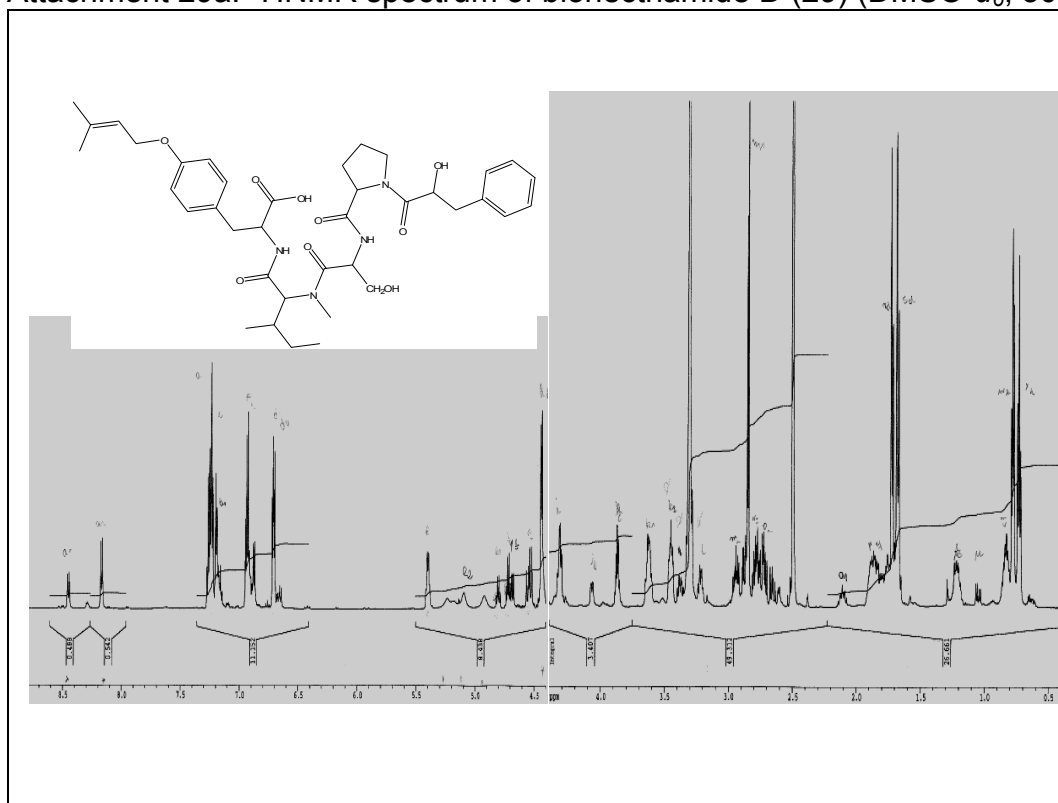
Attachment 29b: TOCSY spectrum of bionectriamide D (**29**) (DMSO-*d₆*, 500 MHz)

11.   Acknowledgments

12.   CURRICULUM VITAE

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Nationality   deutsch
Civil status   married

Education

1983 – 1987   Schlossschule, Neuhof (Primary school)
1987 – 1996   Winfriedschule, Fulda (Secondary school)
1997 – 2001   Julius-Maximilians-Universität, Würzburg
                    Studies of Pharmacy
24.07.2002   License to practise pharmacy

Practical Work

Jan. - March 2000   Julius-von-Sachs-Institut, Würzburg
                    Scientific assistant in pharmaceutical development and
                    validation
September 2000   Julius-Maximilians-Universität, Würzburg – Institut für
                    Pharmakologie und Toxikologie
                        Traineeship in the research of gene-toxicity of
                        phytoestrogenes
Nov. 01 - April 02 Pharmacy Department, RD & E Hospital, Exeter, England
2001-2005   Work as pharmacist in retail pharmacies
Since June 05   Heinrich-Heine-Universität, Düsseldorf – Institut für
                    Pharmazeutische Biologie und Biotechnologie
                        PhD studies, isolation und examination of endophytic fungi
Since February 09   Staatlich anerkannte Lehranstalt für pharmazeutisch-technische
                    Assistenten, Essen (assistant headteacher)
Since October 09   Rats Apotheke, Recklinghausen

Work abroad

October 2005   National Research Laboratories of Natural and Biometric
                Drugs, Beijing, China
March 2007   Vietnamese Academy of Science and Technology, Institute of
                Chemistry, Hanoi, Vietnam
November 2007   TERI - The Energy and Resources Institute Bioresources and
                Biotechnology Division, New-Delhi, India

Congresses

September 2007 European Conference on Marine Natural Products, Ischia, Italy
                    (Oral presentation)
September 2007 1st Sino-German Workshop on Bioactive Marine Products,
                Düsseldorf (Oral presentation)
February 2008   20. Irseer Naturstofftage (Poster presentation)

Publications

Kjer, J.; Wray, V.; Edrada-Ebel, R. A.; Ebel, R.; Pretsch, A.; Lin, W.; Proksch, P. Xanalteric Acids I and II and Related Phenolic Compounds from an Endophytic Alternaria sp. Isolated from the Mangrove Plant Sonneratia alba. *J. Nat. Prod*. (Oct. 2009, epub ahead of print).

Kjer, J.; Debbab, A.; Aly, A. H.; Proksch, P. Methods for Isolation of Marine-derived Endophytic Fungi and Their Bioactive Secondary Products *Nat. Protoc*., accepted.

Xu, J.; Kjer, J.; Sendker, J.; Wray, V.; Guan, H.; Edrada, R. A.; Lin, W.; Wu, J.; Proksch, P. Chromones from the Endophytic Fungus Pestalotiopsis sp. Isolated from the Chinese Mangrove Plant Rhizophora mucronata. *J. Nat. Prod*. **72**, 662-665 (2009).

Xu, J.; Kjer, J.; Sendker, J.; Wray, V.; Guan, H.; Edrada, R. A.; Müller, W. E. G.; Bayer, M.; Lin, W.; Wu, J.; Proksch, P. Cytosporones, coumarins, and an alkaloid from the endophytic fungus Pestalotiopsis sp. isolated from the Chinese mangrove plant Rhizophora mucronata. *Bioorg. Med. Chem*. **17**, 7362-7367 (2009).