# MARINE-DERIVED FUNGI: A SOURCE FOR STRUCTURALLY NEW AND BIOACTIVE SECONDARY METABOLITES

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# Abbreviations

| | |
|---|---|
| $[\alpha]_D^T$ | specific rotatory power; Sodium D-line (589 nm); T: temperature |
| °C | degrees Celsius |
| 1D | one dimensional |
| 2D | two dimensional |
| Å | Ångström |
| δ | NMR chemical shift [ppm] |
| λ | wavelength [nm] |
| μ | micro ($10^{-6}$) |
| ν | wave number ($cm^{-1}$) |
| ACN | acetonitrile |
| ASW | artificial seawater |
| ATR | attenuated total reflection |
| *B. m.* | *Bacillus megaterium* |
| br | broad (in connection with NMR data) |
| c | concentration [g/100 mL] |
| C-18 | C-18 modified silica gel |
| calcd | calculated |
| CC | column chromatography |
| CD | circular dichroism |
| $CDCl_3$ | chloroform-$d_1$ |
| CdC25A | cell division cycle 25A, oncogen protein phosphatase of CdC25 family |
| *C. f.* | *Chlorella fusca* |
| $CHCl_3$ | chloroform |
| $CH_2Cl_3$ | dichloromethane |
| CoA | coenzyme A |
| Conc. | concentration |
| COSY | correlated spectroscopy |
| CYS | cysteine |
| d | doublet (in connection with NMR data) |
| Da | Dalton |
| DAD | diode array detector |
| DCM | dichloromethane |
| DEPT | distortionless enhancement by polarization transfer |

| | |
|---|---|
| DNA | deoxyribonucleic acid |
| DPPH | α,α-diphenyl-β-picrylhydrazyl |
| DSM | Deutsche Sammlung von Mikroorganismen |
| *E. c.* | *Escherichia coli* |
| e. g. | for example |
| EI | electron impact |
| ESI | electronspray ionization |
| *E. r.* | *Eurotium repens* |
| *et al*. | et alii [Lat.]: and others |
| EtOAc | ethyl acetate |
| EtOH | Ethanol |
| eV | electron Volt |
| FAB | fast atom bombardment (in connection with mass spectrometry) |
| g | gram |
| GC | gas chromatography |
| GI | growth inhibition |
| $GI_{50}$ | growth inhibition (drug concentration causing 50 % growth inhibition) |
| GPCR | G-protein coupled receptor |
| HIS | histidine |
| HLE | human leucocyte elastase |
| HMBC | heteronuclear multiple-bond correlation |
| HPLC | high performance liquid chromatography |
| HR | high resolution |
| hrs | hours |
| HSQC | heteronuclear single quantum correlation |
| Hz | Hertz |
| $IC_{50}$ | Inhibition concentration (drug concentration causing 50 % inhibition) |
| $IC_{70}$ | Inhibition concentration (drug concentration causing 70 % inhibition) |
| $IC_{90}$ | Inhibition concentration (drug concentration causing 90 % inhibition) |
| IR | infrared |
| *J* | spin-spin coupling constant [Hz] |
| K | Kelvin |
| kcal | kilocalories |
| L | liter |

| | |
|---|---|
| *laeA* | gene encoding LaeA |
| LaeA | global regulator protein in *Aspergillus* spp. |
| *lck* | gene encoding lymphocyte tyrosine kinase $p56^{lck}$ |
| m | multiplet (in connection with NMR data) |
| *M. tuberculosis* | *Mycobacterium tuberculosis* |
| *M. v.* | *Microbotyrum violaceum* |
| m/z | mass-to-charge ratio (in connection with mass spectrometry) |
| MCPBA | *meta*-chloroperbenzoic acid |
| Me | methyl |
| MeOH | methanol |
| mg | $10^{-3}$ gram |
| MHz | megahertz |
| min | minute |
| mL | $10^{-3}$ liters |
| mm | $10^{-3}$ meters |
| mM | $10^{-3}$ Molar |
| MPtpA | protein tyrosine phosphatase A from *Mycobacterium tuberculosis* |
| MPtpB | protein tyrosine phosphatase B from *Mycobacterium tuberculosis* |
| MS | mass spectrometry |
| $NH_4Ac$ | ammonium acetate |
| ng | $10^{-9}$ gram |
| nm | $10^{-9}$ meter |
| NMR | nuclear magnetic resonance |
| no. | number |
| NOE | nuclear Overhauser effect |
| NOESY | nuclear Overhauser effect spectroscopy |
| NRPS | non-ribosomal peptide synthetase |
| OSMAC | one strain many compounds |
| $p56^{lck}$ | protein 56 kilo Dalton, lymphocytic kinase |
| Pa | probability of a molecule to be active (in connection with PASS) |
| PacC | $CYS_2HIS_2$ zinc finger transcription factor in *Aspergillus* spp. |
| PASS | prediction of activity spectra for substances |
| PE | petroleum ether |
| pH | potentia hydrogenii |

| | |
|---|---|
| Pi | probability of a molecule to be inactive (in connection with PASS) |
| PKS | polyketide synthase |
| PP | protein phosphatase |
| ppm | parts per million |
| PTP | protein tyrosine phosphatase |
| PTP1B | non-receptor protein tyrosine phosphatase 1b |
| q | quartet (in connection with NMR data) |
| qC | quaternary carbon |
| $R_f$ | retention factor |
| RNA | ribonucleic acid |
| ROESY | rotating frame Overhauser effect spectroscopy |
| RP | reversed phase |
| RPM | rounds per minute |
| RT | room temperature |
| s | singlet (in connection with NMR data) |
| SAM | S-adenosyl-methionine |
| SAR | structure activity relationship |
| sec | second |
| Si | silica gel |
| sp. | species |
| spp. | species (Plural) |
| t | triplet (in connection with NMR data) |
| TLC | thin layer chromatography |
| TMSH | trimethylsulfonium hydroxide |
| UV | ultraviolet |
| VE-PTP | vascular endothelial-protein tyrosine phosphatase |
| ver. | version |
| VHR | *Vaccinia* H1 related protein phosphatase |
| VIS | visible |
| VLC | vacuum-liquid chromatography |

# 1 Introduction

## 1.1 Natural products in therapeutic use

Throughout many years, natural products have been playing a major role in the search for novel drugs or drug candidates. They are an ongoing and inspiring source for researchers due to their enormous structural diversity and complexity. Today, more than 50 % of the drugs prescribed in the USA are natural products or semisynthetic derivatives thereof (Schneider *et al.*, 2008). In addition, a number of chemicals used in crop protection are also of natural origin.

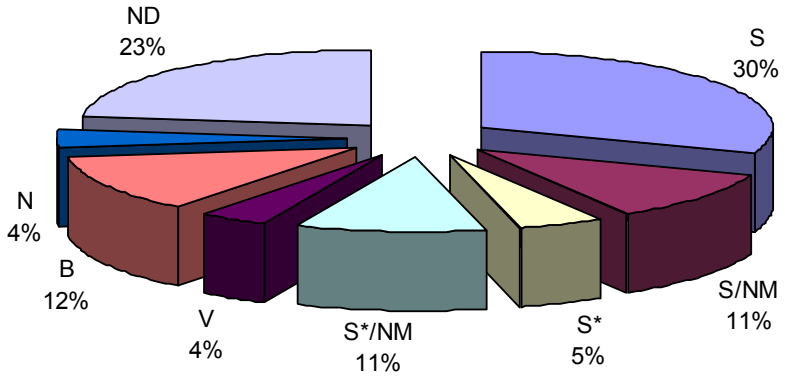
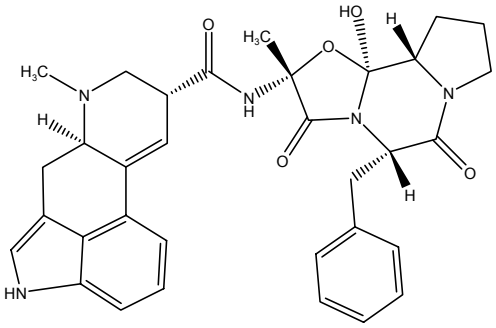



**Figure 1-1-1:** New chemical entities in the time frame 1981 – 2006
**"B" Biological; usually a large (> 45 residues) peptide or protein, generally isolated from an organism/cell line or produced by biotechnological means in a surrogate host; "N" Natural product; "ND" Derived from a natural product, usually a semisynthetic modification; "S" Totally synthetic drug, mostly found by random screening or modification of an existing agent; "S*" Made by total synthesis, but the pharmacophore is/was from a natural product; "V" Vaccine; "NM" Natural Product mimic. (Taken from Newman & Cragg, 2007)**

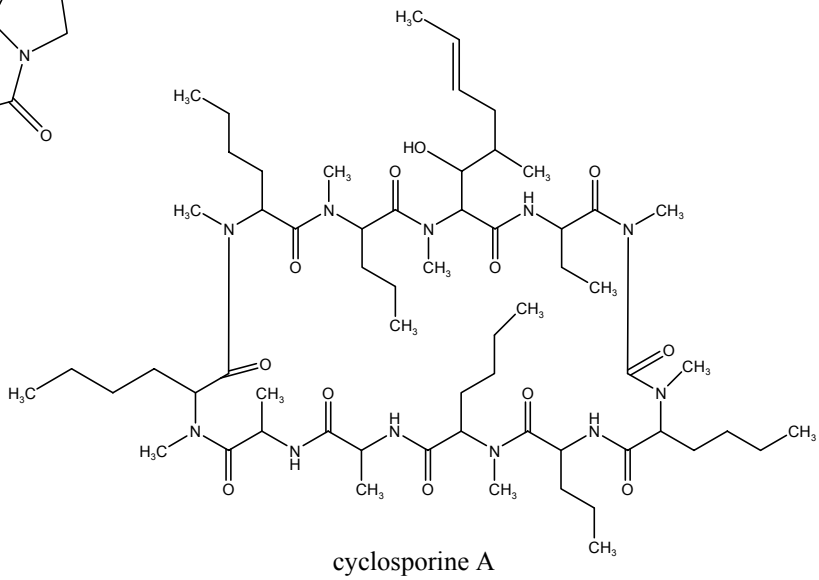
The most prominent producers of natural products can be found within three groups of organisms: plants, bacteria, mainly actinomycetales, myxobacteria and cyanobacteria, and fungi. From all these groups, a plethora of exciting compounds belonging to diverse biogenetic families has been isolated. While plants and plant extracts have been playing an important role in traditional medicine for thousands of years, the discovery and investigation of bacterial and fungal natural products had only been made possible with the development of

natural sciences during the 20th century. However, especially since the discovery of penicillin in 1929 and the following "Golden Age of Antibiotics", as the period from 1940 to the 1970s has often been described, metabolites of microbial origin are well known as valuable supply of new compounds and lead structures in the quest for drug candidates against infectious diseases, cancer and many other illnesses (Newman & Cragg, 2007; Koehn & Carter, 2005). Among them are therapeutically used alkaloids like ergotamine, the immunosuppressive peptide cyclosporine A, other peptidic compounds like the penicillins and cephalosporins, the polyketide lovastatin and the antibacterial terpenoid fusidic acid (Fig. 1-1-2).

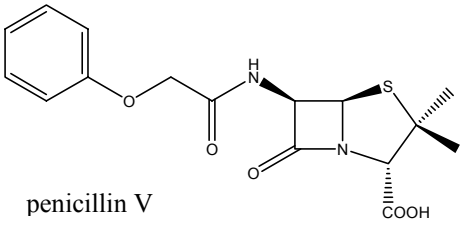
During the last decades, more and more efforts have been undertaken by researchers in the search for drugs against cancer, seeing that the disease is becoming a major cause of death among the population of developed countries (Szekeres & Novotny, 2002). The various forms of cancer require multiple approaches for their treatment, which opens a wide field of research that has to be explored. Natural products have therefore been recognized as one promising source for antitumor compounds. A substantial amount of research into cytotoxic natural products has been carried out in the last 50 years, and significant advances in cancer treatment have been achieved (da Rocha *et al.*, 2001).



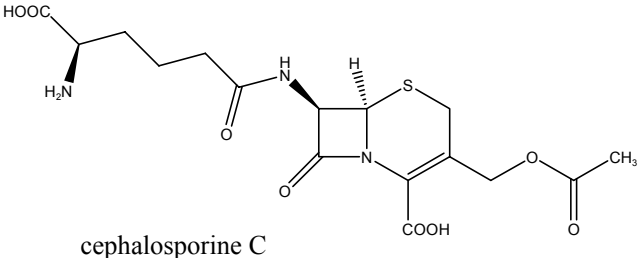
ergotamine



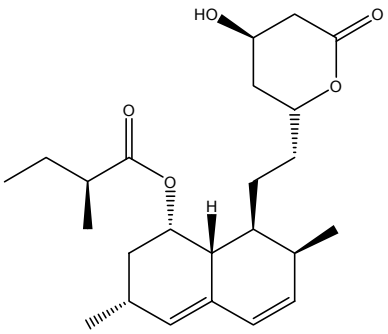
cyclosporine A



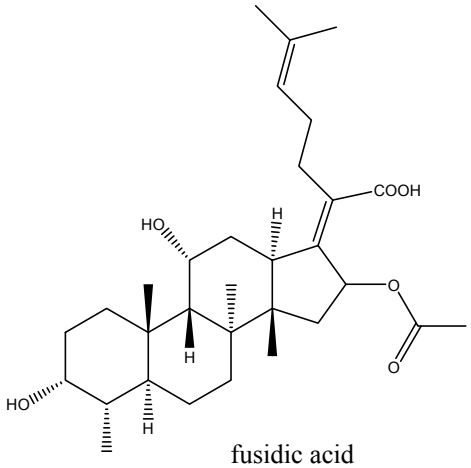
penicillin V



cephalosporine C



lovastatine



fusidic acid

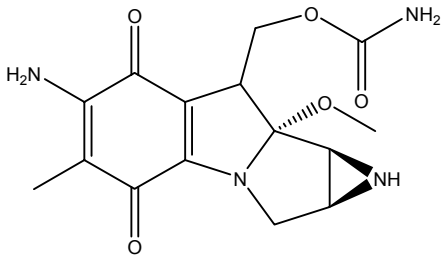
**Figure 1-1-2:** fungal secondary metabolites used as therapeutic agents

## 1.2 Cytotoxic natural products

### 1.2.1 Cytotoxic natural products in therapeutic use

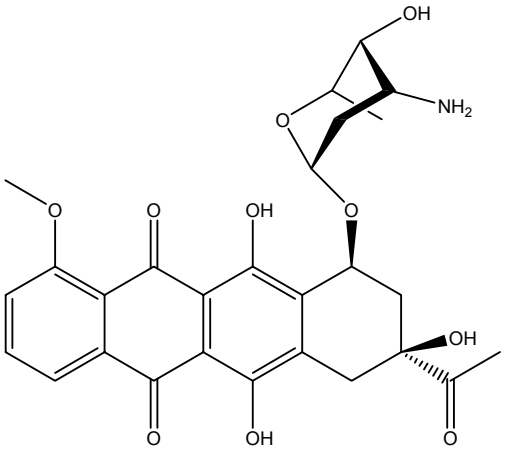
Cytotoxic compounds that are in clinical use as antitumor drugs represent an immense variety of structural types, according to the many different targets focused on in the drug therapy of cancer. Within this group of compounds, natural products still play an important role and have been proven to be valuable tools for the development of new anti-cancer drugs, but also for the antitumor treatment itself.

The alkylating agents represent the biggest group of cytotoxic compounds used in cancer treatment. They possess the ability to form reactive carbenium-ions that attack nucleophilic centers of the DNA. The natural product mitomycin C has been isolated from the actinobacterium *Streptomyces caespitosus* in 1956 by Hata and co-workers and is a great example for a perfect "drug design" by nature. Mitomycin C is completely inactive *in vitro*, but shows alkylating activity after enzymatic reduction of the chinone to the hydrochinone. After elimination of methanol and carbaminate, the nucleophilic attack takes place, while undergoing ring opening of the aziridin system. In addition to the cytotoxic activity, mitomycin C also possesses antibiotic and antiviral properties (Crooke & Bradner, 1976).



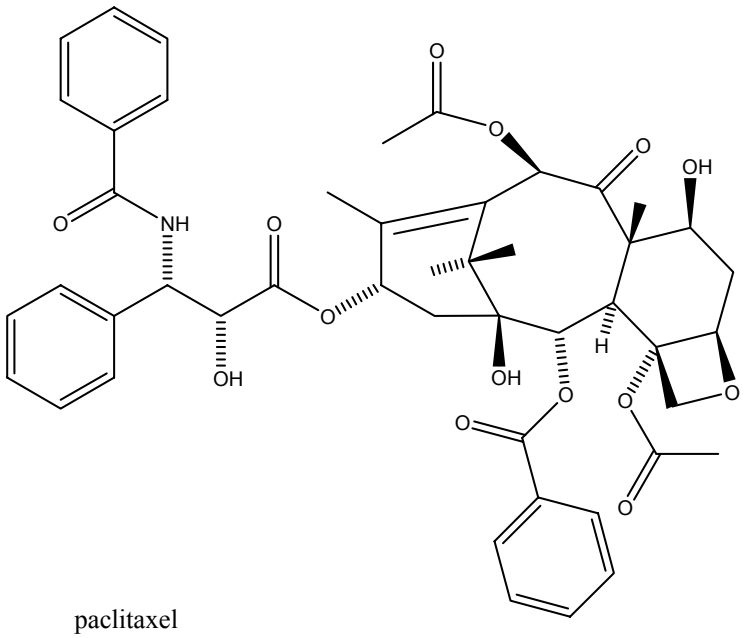

mitomycin C

The anthracyclines, e. g. daunorubicin or doxorubicin, form a whole series of natural and natural-derived compounds that have also been isolated from *Streptomyces* spp.. They are very commonly used in cancer treatment and act through several mechanisms, e. g. by intercalation of DNA, inhibition of DNA synthesis, interaction with the RNA polymerase and inhibition of DNA-topoisomerase II (Minotti *et al.*, 2004). Inhibition of type I or type II topoisomerases interferes with both transcription and replication of DNA by upsetting proper DNA supercoiling.




daunorubicin

The very complex structure of paclitaxel (Taxol®) has been isolated in the 1960s from *Taxus brevifolia* (Wani *et al.*, 1971). It is an inhibitor of the mitosis process in cells. Paclitaxel interferes with the normal function of microtubule breakdown and arrests the microtubule function by hyper-stabilization of their structure. This destroys the cell's ability to use its cytoskeleton in a flexible manner. The resulting microtubule / paclitaxel complex does not have the ability to disassemble (McGrogan *et al.*, 2008).

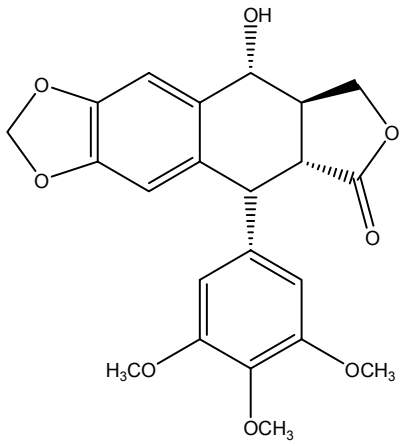



paclitaxel

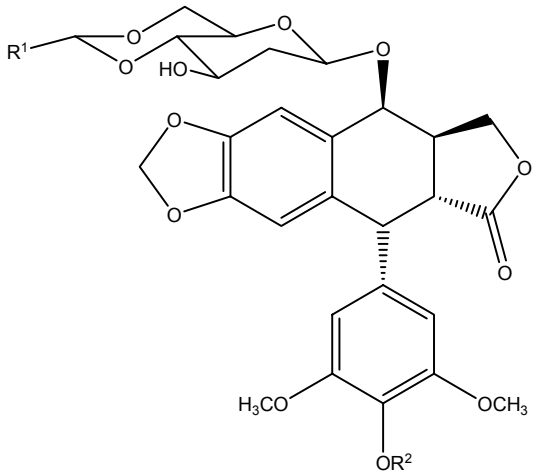
Due to its unique structure containing no less than eleven chiral centers, a fast and cost-effective total synthesis of paclitaxel has not been succeeded yet. In 1993, Stierle and co-workers discovered that taxol was coincidentally produced in a newly described fungus living in the yew tree (Stierle *et al.*, 1993). Since, it has been found in a number of other endophytic fungi, opening the possibility of taxol production by culturing one of these fungal species and

thus reducing time and cost for production of the compound (Ge *et al.*, 2004; Yuan *et al.*, 2006). Paclitaxel can thus be considered as the first cytotoxic fungal secondary metabolite in clinical use.

Comparably to paclitaxel, the aryltetralignan podophyllotoxin has been reported to be produced by endophytes, namely *Phialocephela fortinii*, isolated from the rhizomes of the host plant *Podophyllum peltatum* (Eyberger *et al.*, 2006).

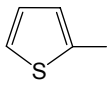



podophyllotoxin




etoposide: $R^1 = CH_3$, $R^2 = H$

etoposide phosphate: $R^1 = CH_3$, $R^2 = PO(OH)_2$

teniposide: $R^1 =$ 

, $R^2 = H$

Podophyllotoxin, which was originally isolated from the rhizoma of *Podophyllum peltatum*, is a valuable natural product as precursor for several therapeutic agents, including the anticancer drugs etoposide, teniposide and etoposide phosphate (Canel *et al.*, 2000). There is an urgent demand for other sources than the primary source plant, whose wild populations are being destroyed more and more and the pharmaceutical demand will not be covered anymore if no other sources are made available (Eyberger *et al.*, 2006).

The cytotoxic podophyllotoxin analogues in clinical use have been shown to delay transit of cells through the S phase and arrest cells in the S phase or early G2 phase. Etoposide is a topoisomerase II inhibitor and appears to cause DNA stand breaks (Hainsworth and Greco, 1995).

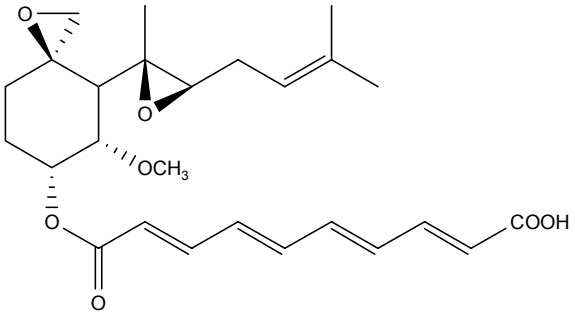
### 1.2.2 Cytotoxic fungal metabolites as future antitumor agents

Fungal secondary metabolites play a major role within the large group of natural products originating from microorganisms and show a wide range of different bioactivities, such as antiinfective, antiproliferative, immunosuppressive, and many more (Misiek & Hoffmeister, 2007; Schneider *et al.*, 2008). Some substances of fungal origin are not applicable for therapeutical use but are known as mycotoxins, e. g. the aflatoxins or the trichothecenes (Sweeney & Dobson, 1998). Furthermore, the functions of many molecules have not been discovered yet, even though they possess remarkable scaffolds, often including amazing stereochemical variations. A large number of fungal secondary metabolites is published possessing cytotoxic properties. Like their plant- or bacterium-derived relatives, some of them have entered clinical trials while others serve as lead structures in the search for clinically applicable antitumor drugs (Siddiq & Dembitsky, 2008).

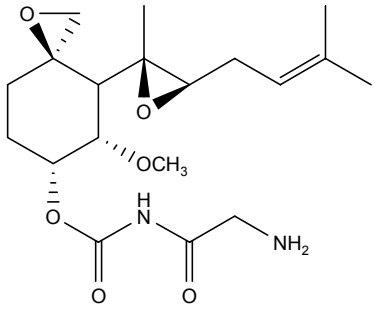
A considerable number of natural products have been isolated from marine fungi, albeit that the number of bioactive secondary metabolites from fungi in the terrestrial habitat is still dominating the diverse spectrum of fungal secondary metabolites. This is because they are more thoroughly investigated than their marine relatives and not due to a higher biodiversity within the terrestrial habitat (Saleem *et al.*, 2007). However, those fungal secondary metabolites already obtained are exemplary for the structures still waiting to be discovered.

#### 1.2.2.1 Cytotoxic fungal metabolites from the terrestrial habitat

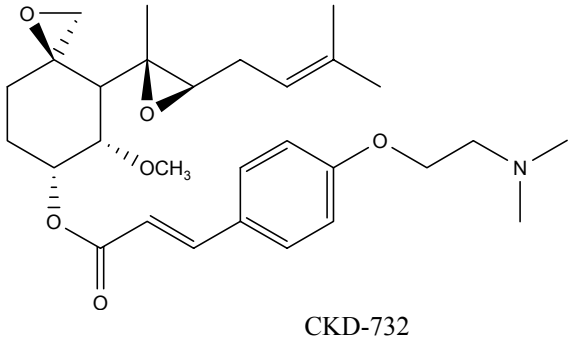
One of the best evaluated fungal metabolites in oncological research is fumagillin. It has been isolated from the fungus *Aspergillus fumigatus* and has created researchers' interest because of its anti-angiogenetic properties (Lu *et al.*, 2006; Kim *et al*., 2004; Lee *et al.*, 2007). Many semisynthetic fumagillin analogues derived from the lead structure have been synthesized in order to increase potency and at the same time decrease toxicity. Of these, TNP-470 has entered phase I and phase II clinical studies for the treatment of breast, prostate, and brain cancer, as well as Kaposi sarcoma (Kruger & Figg, 2000). A second analogue, CKD-732, has also entered clinical trials, being even more potent and less toxic than TNP-470 (Lee *et al.*, 2004; Kim *et al*., 2007).




fumagillin



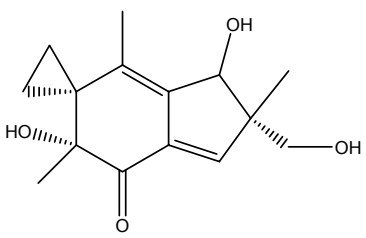

TNP-470



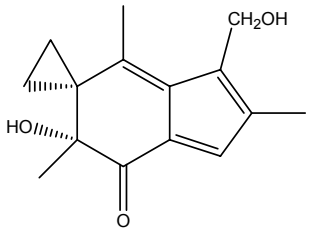

CKD-732

TNP-470 acts through an irreversible binding to methionine aminopeptidase 2, which then leads to intracellular signalling interference (Zhang *et al.*, 2006; Lu *et al.*, 2006).
As the same antiproliferative mechanism of action might be used for the treatment of rheumatoid arthritis, the fumagillin derivative PPI-2485 has been investigated for its effect on this. Furthermore, fumagillin and another analogue, ovalicin, have been proven to be highly active as antimicrosporidial agents in mice (Didier *et al.*, 2006) and have been found to inhibit HIV-1 viral protein R (VPR) activity (Asami *et al.*, 2006).

Illudin S is a highly toxic substance belonging to the sesquiterpenes, which are extremely rare in fungi. It was isolated from the homobasidiomycete *Omphalotus illudens*. Semi-synthetic derivatives like irofulvene showed less severe side effects and toxicity whilst still being cytotoxic in the MV522 lung carcinoma cell assay with an $IC_{50}$ value of 73 nM for irofulvene (McMorris *et al.*, 2001). Irofulvene possesses a greater efficacy than illudin S due to its slower and more selective action. It has entered phase I and II clinical trials against several types of cancer, e. g. prostate and ovarian cancer (Seiden *et al.*, 2006). Several other acylfulvenes have been isolated or synthetized and are likely to enter clinical studies as well (McMorris *et al.*, 1999a; McMorris *et al.*, 1999b).



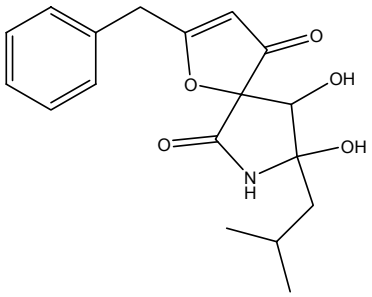

illudin S




irofulvene

Irofulvene is an alkylating agent, which is attacked by cellular nucleophiles after being activated by reduction of the α,β-unsaturated ketone (Gregerson *et al.*, 2003). The resulting adducts lead to inhibition of DNA synthesis, DNA stand breaks, cell cycle arrest and, finally, apoptosis. Surprisingly, irofulvene is not affected by DNA repair mechanism enzymes and also acts very selective against several human carcinoma cells. This selectivity is due to a fast, energy-dependent uptake mechanism existing in sensitive cells (McMorris *et al.*, 2001).

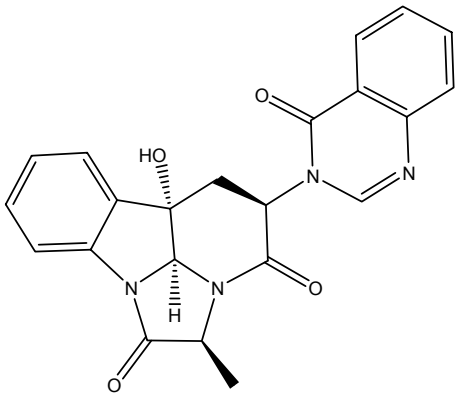
Besides compounds submitted to clinical studies, several fungal metabolites are known to date whose promising *in vitro* antitumor activity makes them interesting candidates for preclinical and clinical studies. Stierle and co-workers have isolated the berkeleyamides A-D from a deep water *Penicillium rubrum* (Stierle *et al*., 2008). The producing strain was derived from a flooded open-pit copper mine containing acidic water (pH 2.5) contaminated with high concentrations of metal sulphates (iron, copper, aluminium, zinc).




berkeleyamide D (configuration not published)

Berkeleyamide D shows a very unusual azaspirocyclic ring system and exhibits promising cytotoxic activities with an $IC_{50}$ value of 0.61 µM. It has been submitted to the NCI/NIH Development Therapeutics Program for further preclinical testing (Stierle *et al.*, 2008).

Jiao and co-workers have isolated the tripeptide-derived alkaloid chaetominine from an endophytic *Chaetomium* sp. in 2006. Chaetominine possesses an unprecedented structural framework, which is not comparable to any isolated compounds from the host plant *Adenophora axilliflora*, thus underlining the fungal origin of the substance. The compound is active against the human leukemia K562 and colon cancer SW1116 cell lines with $IC_{50}$ values of 21 and 28 nM, respectively, which is more potent than the frequently prescribed antitumor drug 5-fluorouracil (Jiao *et al.*, 2006).

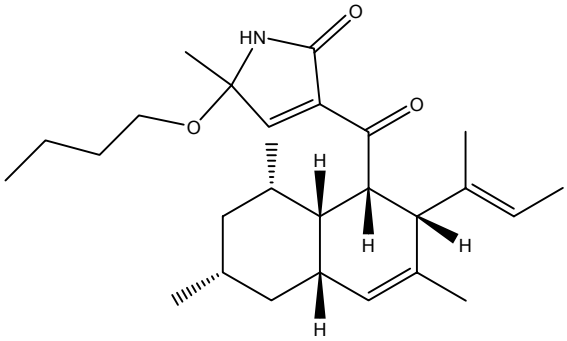



chaetominine

### 1.2.2.2 Cytotoxic fungal metabolites from the marine habitat

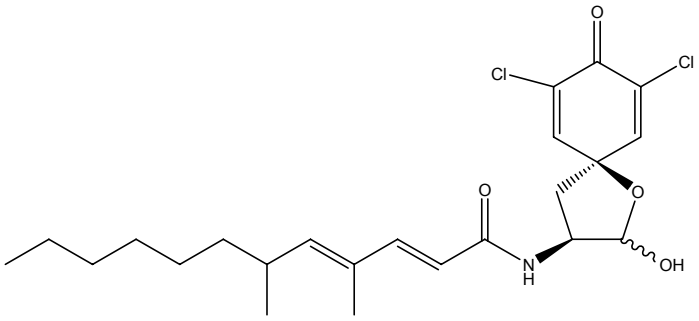
As the need for new anticancer substances continues, and many well-investigated approaches such as combinatorial chemistry only led to minor innovations in drug therapy, the investigation of new, rather unexplored sources is one logical consequence in the process of searching for new bioactive natural products. The marine ecosystem, covering more than 70 % of the planet's surface, is home to an immense diversity of life. Though, due to the extreme depth of the oceans, they represent more than 99 % of our planet's natural habitat when setting the terrestrial habitat up to 100 m of height (Schuh, 2008). Compared to the research done into terrestrial microorganisms, there is extremely little knowledge about their marine relatives. Even though the important antibiotic cephalosporin C has been isolated from a marine-derived fungus in the 1940s, research focused on the discovery of marine natural products was rare until the 1990s. The almost exponential growth of studies of marine fungi led to more than 300 new natural products (Bugni & Ireland, 2004). Most metabolites known to date are produced by *Aspergilli* and *Penicillia*. Reasons therefore might be that both species are somewhat ubiquitous, salt tolerant and fast growing. Several interesting structures have been isolated from obligate marine fungi restricted to the marine environment, e. g. the antimicrobial alkaloid ascosalipyrrolidinone A from *Ascochyta salicorniae* (Osterhage *et al.*,

2000). However, many facultative marine species that might also occur in freshwater or terrestrial habitats often produce compounds similar to those isolated from other environments.



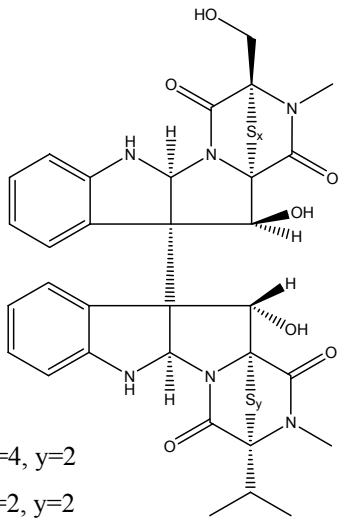

Ascosalipyrrolidinone A

The gymnastatins, isolated by Numata and co-workers from a sponge-derived *Gymnascella dankaliensis* are among the first cytotoxic metabolites isolated from a marine-derived fungus. Five substructures of these unusual chlorinated polyketides are known to date, that exhibit cytotoxic activity in a P388 lympholeukemia test system with an $ED_{50}$ value of 18 ng $mL^{-1}$ for the most potent gymnastatin A (Numata *et al*., 1997). It is presumed that the gymnastatins act as cytotoxins due to a Michael-acceptor function.



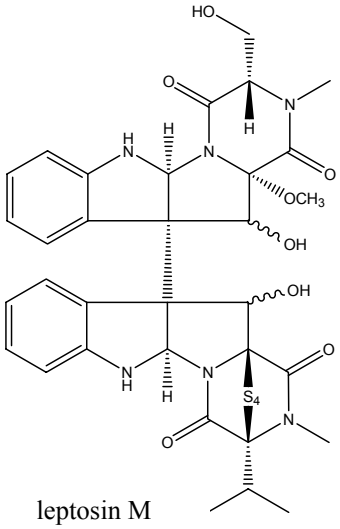

gymnastatin A

The leptosin family with 19 members known to date is one of the largest classes of cytotoxic fungal metabolites (Bugni & Ireland, 2004). The leptosins are dimeric diketopiperazines possessing one or even two polythiobridges, and are related to the terrestrial metabolite chaetocin. Their cytotoxic potential against a P388 leukemia cell line ranges from 1.75 ng $mL^{-1}$ to low µg $mL^{-1}$ values, depending on the number of the polythiobridges and stereochemical effects.



leptosin A: x=4, y=2

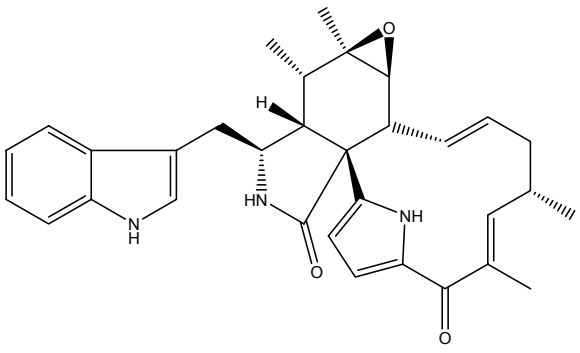
leptosin B: x=2, y=2



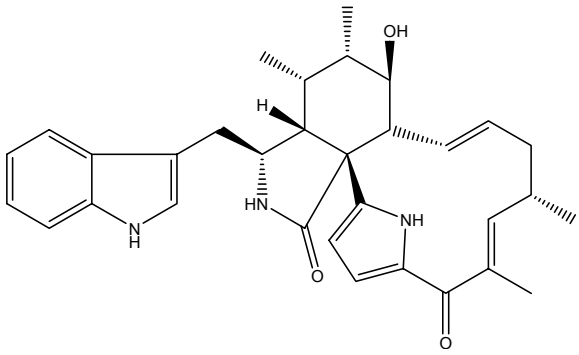
leptosin M

Leptosins A and C showed significant antitumor activity in mice with Sarcoma-180 ascites while leptosin M revealed to be a potent inhibitor of topoisomerase II, and two kinases, PTK and CaMKIII (Yanagihara *et al.*, 2005; Yamada *et al.*, 2002).

The penochalasins belong to the class of cytochalasins, more precisely to the subgroup of chaetoglobosins. Cytochalasins are remarkably cytotoxic metabolites because of their effective mode of action, which includes the movement inhibition and cytoplasmic cleavage of mammalian cells by inactivation of F-actin. Chaetoglobosins differ from the cytochalasins in the presence of a tryptophan moiety rather than phenylalanine.



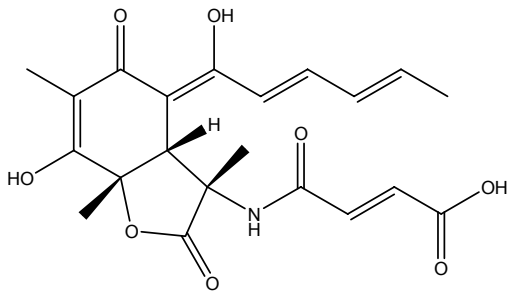
penochalasin A



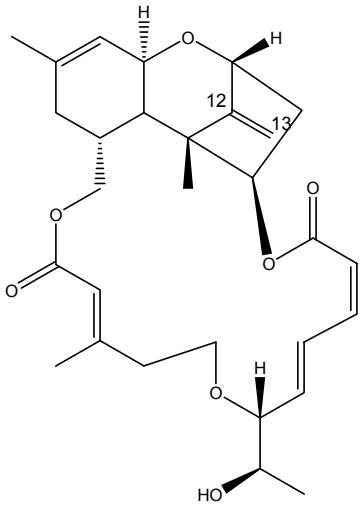
penochalasin B

The penochalasins A-D are particularly unique as they include a pyrrole moiety in their skeleton. They have been isolated from a *Penicillium* sp. and exhibit cytotoxic activity in the low micromolecular range, while those compounds bearing a pyrrole moiety are significantly more potent than those without (Iwamoto *et al.*, 2001).

The potent antileukemic alkaloid sorbicillactone A was isolated from a sponge-derived *Penicillium chrysogenum* by Bringmann and co-workers in 2003. It belongs to the class of sorbicillin derivatives and is the first nitrogen-containing natural representative of this structural class. It displayed strong cytostatic activity (2.2 µg $mL^{-1}$) against murine leukemic lymphoblast cells (L5178y) whilst showing relatively low toxicity to cervical carcinoma HeLa S3 cells and pheochromocytoma PC12 cells (Baker *et al*., 2007). Other bioactivities such as an anti-HIV-activity have been observed as well. The production of sorbicillactone A has been increased from 4 mg $L^{-1}$ up to more than 500 mg $L^{-1}$ to make it available for clinical studies (Bringmann *et al.*, 2007).




sorbicillacton A




12, 13-deoxyroridin E

The roridins belong to the group of the mycotoxins trichothecenes. They are well-known plant pathogens; however their ability to serve as antitumor substances in humans is subject of ongoing discussions (Murakami *et al.*, 2001, and others). 12,13-deoxyroridin E, which has been isolated from a marine-derived *Myrothecium roridum* indeed showed promising cytotoxic activity. The derivative roridin E, was even about 80-fold more potent than 12,13-deoxyroridin E (Namikoshi *et al.*, 2001). These results clearly show that substances that are originally known as human pathogens might also be used for clinical use in the future.

### 1.2.3 New sources for cytotoxic secondary metabolites?

Whether compounds that have been isolated from algae, plants, sponges or corals are possibly not only produced by the macroorganisms, but by associated endosymbionts as well, has been – and still is – subject of debates (König *et al.*, 2006; Piel *et al.*, 2004). By definition, these endosymbionts, mostly fungi and bacteria (including actinomycetes), colonize the living internal tissue of their hosts, typically without causing any apparent symptoms of disease (Bacon & White, 2000). Endosymbionts usually remain harmless and only become parasitic when their host is stressed. The mutual relationship between endosymbiontic microorganisms and their hosts often leads to the production of many bioactive metabolites, including known as well as new substances, possessing a wide variety of biological activities such as antibiotic, antitumor, anti-inflammatory, antioxidant, etc. (Firáková *et al.*, 2007). Currently, more and more examples are being published where indeed natural products are produced in one species, excreted, and then assimilated by a second species (Simmons *et al.*, 2008). Prominent examples for this phenomenon are paclitaxel and podophyllotoxin (see 1.2), or polyketides belonging to the pederin family, that have been isolated from endosymbiontic bacteria (Piel *et al.*, 2005). Particularly marine invertebrates such as sponges and soft corals are well recognized sources for the isolation of endosymbiontic fungi. Strains isolated of these often produce unique structures that are likely to be useful for both host and endosymbiont.

## 1.3 Discovery of cytotoxic secondary metabolites

How was it possible to discover the biological activities for the above-mentioned compounds? For a long time the discovery of novel secondary metabolites exhibiting cytotoxic activities was based more on coincidence and luck rather than on logical approaches. There are countless examples when biological activities were discovered only years after the isolation of the substances themselves. On one hand, this is due to the advances that have been made in science: many bioassays that are well established today for the investigation of the isolated compounds were much less sensitive or not even available at the time the structures have been isolated. On the other hand, often a focus on specific activities was not set and compounds whose structural features were not familiar to the researcher have been overlooked.
However, there are urgent needs for the development of novel antitumor drugs. For this purpose, the establishment of rational strategies is inevitable to undertake a specific and effective screening for cytotoxic active metabolites.

### 1.3.1 Bioassay-guided isolation

Bioassay-guided isolation is a widely used rational approach for the discovery of cytotoxic compounds. It combines the separation of compounds from a mixture, by using various analytical methods, with results obtained from biological testing (Rimando *et al.*, 2001). The process begins with testing of the extract to confirm its activity, followed by crude separation of the compounds in the matrix and testing of the crude fractions. Further fractionation is carried out on the fractions that are found to be active, at a given concentration threshold. The inactive fractions are set aside or discarded. The process of fractionation and biological testing is repeated until pure compound(s) are obtained. Structural identification of the pure compound and, if required, further classification of the bioactivity (e. g. mechanism of action, exact target, etc.) is performed afterwards. This methodology precludes overlooking novel compounds that remain often undetected in studies where the investigator only identifies compounds he is familiar with. Moreover, the possibility of discovering an unknown molecular site of action is maximized (Duke *et al.*, 2000).

As mentioned above, bioassays play a very important role in today's natural products chemistry research. They must fulfil the following criteria (Sjögren, 2005):

1. Relevant targets
2. Reliable and reproducible results
3. Sensitive
4. Fast
5. Simple
6. Standardized detection method, preferably an instrumental method.

All of the parameters listed above should be considered when developing or selecting a bioassay. Generally the limiting factor for discovering natural products is the small amount available for bioassay and structural analysis from the isolation procedure.
Applying a high number of bioassays requires considerably more material during the isolation procedure than testing towards just one target. As a result, usually a compromise between time, target and quantity of isolated material used for the bioassay has to be made.

### 1.3.2 Established bioassays for discovery of cytotoxic activity

The bioassays for cytotoxic activity established by Oncotest GmbH are examples for efficient test systems fulfilling the requirements as mentioned above (Fig. 1-3-1). The monolayer test permits finding the most potent and tumor specific fractions and compounds during screening and bioassay-guided isolation. Selected promising compounds can then be subjected to the clonogenic assay and, in the case of a positive result, to *in vivo* studies.

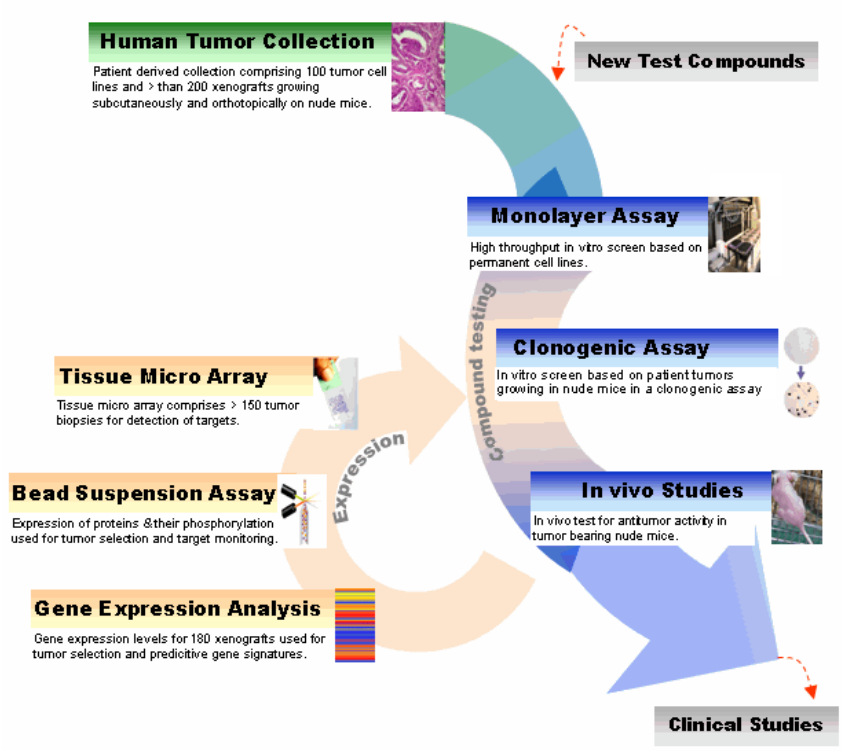
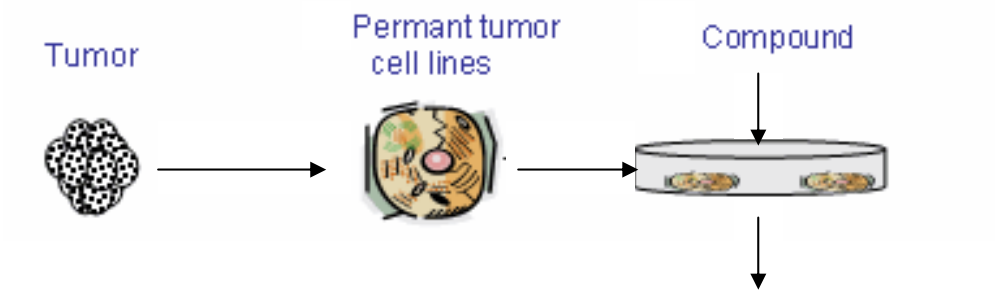



**Figure 1-3-1:** Testing principle of Oncotest GmbH (taken from www.oncotest.com).

The monolayer-in-vitro-assay determines the capacity of compounds to inhibit growth of permanent tumor cell lines. The testing principle is based on an analysis of the viable cell number after four days of incubation (Fig. 1-3-2). Tumor derived cell lines are incubated together with the test compounds. Proliferation inhibition is then determined by measuring the DNA content with propidium iodide or other appropriate markers. Compounds are typically tested at different concentrations and evaluated for their potency and tumor selectivity (Fiebig *et al.*, 1992; Boven *et al.*, 1992). Most of the cell lines for this assay are established from tumor xenografts derived from patients. *In vivo* counterparts are available for further testing.
For the screening for cytotoxic compounds, the substances are tested in a six cell-line panel in an automated set-up and read-out, which allows a rapid testing of up to 4000 compounds or fractions per week. The monolayer-in-vitro-test thus is an eligible tool for bioassay-guided isolation of cytotoxic secondary metabolites.




IN-VITRO ANTITUMOR ACTIVITY OF MNEB433F IN HUMAN TUMOR CELL LINES (Monolayer Assay, PI)

| TUMOR/ PASSAGE NO. | EXP. NO. | CTRL FLUOR. UNITS | Distribution of IC70 related to Mean log.scaled axis *0.01 *0.1 Mean 6.220 *10 *100 | IC50 ug/ml | IC70 ug/ml | IC90 ug/ml |
|---|---|---|---|---|---|---|
| GXF | | | | | | |
| 251L | IF025RT | 1439 | | 12.728 | 20.775 | 33.908 |
| LXF | | | | | | |
| 529L | IG025RT | 3872 | | 2.335 | 6.965 | 21.418 |
| MAXF | | | | | | |
| 401NL | IF026RT | 2338 | | 1.306 | 2.476 | 56.214 |
| MEXF | | | | | | |
| 462NL | IG026RT | 2045 | | 1.828 | 3.000 | 29.999 |
| RXF | | | | | | |
| 486L | IF027RT | 2557 | | 1.601 | 3.665 | 27.142 |
| UXF | | | | | | |
| 1138L | IG027RT | 4421 | | 7.683 | 14.698 | 28.115 |
| Mean | | n=6 | 6.220 | 3.091 | 6.220 | 31.268 |

**Figure 1-3-2:** Principle of the monolayer assay (taken from www.oncotest.com).

As for a screening for new and bioactive compounds, the automated and fast monolayer assay is the appropriate choice. The clonogenic assay would then be the next step for pure compounds showing positive results during the screening (Fiebig *et al.*, 2004). It evaluates the inhibition capacity of compounds to form three-dimensional colonies which consist of tumor cells that possess the potential for anchoring independent growth in semisolid media (Fig. 1-3-3). Compounds are tested at different concentrations against up to 40 different tumors. Evaluation of the activity data from the monolayer assay allows a specific selection of suitable tumors.

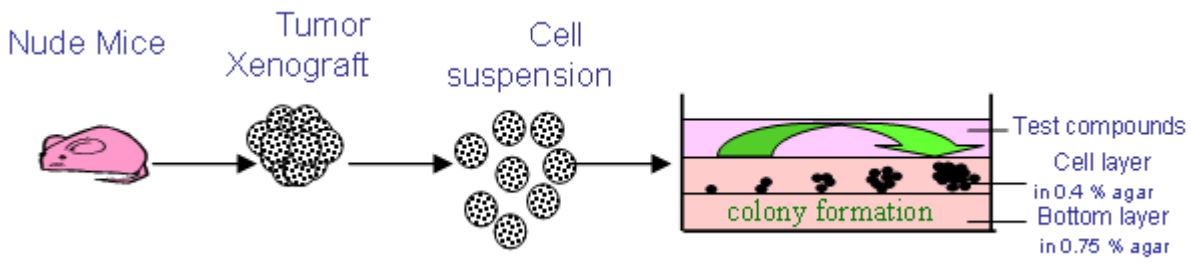



**Figure 1-3-3:** Principle of the clonogenic assay (taken from www.oncotest.com).

The clonogenic assay is based on human xenografts and combines the advantages of a high drug response correlation to the same tumor growing in vivo or in the patient with moderate time and running expenditures (Fiebig *et al.*, 2004). Compounds that have been successfully tested in the clonogenic assay would then be subjected to *in vivo* studies as promising candidates for future antitumor therapy.

## 1.4 Heading forward – future prospects for the identification of cytotoxic natural products

The need for new anticancer drugs will not cease in the future. Bearing in mind the relatively high toxicity of antitumor drugs in clinical use on one hand and the continuing problem of resistance against these compounds on the other, there is a high demand for substances that can fulfil the requirements for an activity that acts as broadly as possible while minimizing undesired side effects. With the recent developments in analytical chemistry, together with the identification and availability of more and more valid molecular targets such as specific proteins and receptors or DNA, the possibilities to specifically search for cytotoxic compounds increase (Teicher, 2008).

In the first half of the 20$^{th}$ century, when deeper knowledge about cellular features and DNA structure was only beginning to be developed, only very few and unspecific bioassays towards cytotoxic activity were established. Nowadays, more sensitive and more diverse bioassays exist, corresponding to the many newly characterized targets and key pathways like the epidermal growth factor (EGFR) pathway, the insulin-like growth factor (IGF) pathway, or microRNAs (Boshoff, 2007), to name just a few. They can be included in bioassay-guided isolation of new cytotoxic natural products and might also open the door for the immense library of already known natural products to be investigated for a new "career" as antitumor agent.

# 2 Scope of the present study

There is high demand for innovative lead structures in order to develop novel drugs for the treatment of cancer and other menacing diseases. Resistance of tumor cells, pathogenic bacteria and viruses is steadily increasing and many established drug therapies are not effective anymore. Natural products have been shown to possess the potential to be excellent lead structures and to serve as a basis for promising therapeutic agents in the area of anticancer treatment. The marine environment is a rather unexplored source in the quest for new natural products. The present study was thus devoted to the investigation of cytotoxic properties of marine fungal extracts and to the isolation and characterization of the secondary metabolites responsible for this activity. It was intended to place special emphasis on structurally novel compounds. Furthermore, the characterization of the secondary metabolite spectrum of fungi without prominent cytotoxic activity, but with other important biological activities, e. g. antimicrobial or human leucocyte elastase inhibitory activity, or with interesting chemical properties, was performed. This project thus comprises the following tasks:

- Biological and chemical screening of fungal extracts
- Chemical investigation of interesting fungal strains and bioassay-guided isolation
- Biological testing of isolated secondary metabolites.

## 2.1 Biological and chemical screening of fungal extracts

For screening purposes, selected fungal strains isolated from marine macroorganisms such as sponges and algae were cultured in small scale and extracted with EtOH. The crude extracts were chemically and biologically evaluated. Biological tests consisted of cytotoxic assays in a panel of six cancer cell lines in a monolayer culture, and agar diffusion assays for antibacterial, antifungal and antialgal activity. Investigations by $^{1}$H-NMR spectroscopy and LC-MS measurements were used for the chemical characterization of the fungal extracts. The results obtained with these methods were used for the selection of strains which were subsequently subjected to detailed chemical and biological analyses.

## 2.2 Chemical investigation of interesting fungal strains and bioassay-guided isolation

The selected strains from the screening were cultivated on a large scale and the EtOH extracts were subjected to chromatographic methods, mainly HPLC, for the isolation of secondary metabolites. It was intended to perform bioassay-guided isolation, using the same cytotoxicity test systems as for the screening. Chromatographic steps and cytotoxicity assays were repeated until pure compounds were obtained. Structure elucidation of the pure compounds was performed by spectroscopic measurements including 1D and 2D NMR techniques and mass spectral data. Physico-chemical and physical methods completed the chemical characterization of the pure compounds.

## 2.3 Biological testing of isolated secondary metabolites

Pure compounds isolated in this study were tested for their cytotoxic activities and, in second priority, for antibacterial, antifungal and antialgal activity. Additionally, other bioassays were performed when the isolated material was available in sufficient quantities. In these cases, the compounds were subjected to further assays to evaluate their ability to inhibit several enzymes or antiviral and antiprotozoal activity.

# 3 Materials and methods (general procedures)

## 3.1 Origin and taxonomy of fungi

The strains described in this study were identified by the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, or by the Leibnitz Insitut für Meeres-wissenschaften, Kiel, Germany.

*Trichoderma saturnisporium* (strain 54) was isolated from the sponge *Agelas dispar* collected around the Caribbean island of Dominica.

*Beauveria bassiana* (strain 321) was isolated from the sponge *Myxilla incrustans* collected around the island of Helgoland (Germany, North Sea).

*Alternaria japonica* (strain 194) was isolated from an unidentified algae sample collected around the Island of Tenerife.

*Trichoderma harzianum* (strain 714) was isolated from the green alga *Chaetomorpha linum* collected from the waters around Büsum (Germany, North Sea).

*Microdiplodia* sp. (strain 405) was isolated from the green seaweed *Enteromorpha* sp. collected from the Baltic See waters around the island of Fehmarn (Germany, Baltic Sea).

## 3.2 Isolation of fungal strains

All chemically investigated fungal strains described in this study were obtained from fungal culture collection of Professor G. M. König (Institute for Pharmaceutical Biology, University of Bonn), and were isolated by Ekaterina Eguereva or by the former Ph.D. students Dr. Ulrich Höller, Dr. Claudia Osterhage and Dr. Christine Klemke.

### 3.2.1 Isolation from algal material

After sterilization of the algal material with 70 % ethanol, algal samples were rinsed with sterile water and pressed onto agar plates to detect any residual fungal spores on their surface. The water used for media was artificial sea water (ASW) containing the following salts: [(g $L^{-1}$): KBr (0.1), NaCl (23.48), $MgCl_2$ x $H_2O$ (10.61), $CaCl_2$ x $2H_2O$ (1.47), KCl (0.66), $SrCl_2$ x $6H_2O$ (0.04), $Na_2SO_4$ (3.92), $NaHCO_3$ (0.19), $H_3BO_3$ (0.03)]. Sterilized algae were then cut into pieces and placed on agar plates containing isolation medium: biomalt 20 g $L^{-1}$, 15 g $L^{-1}$ agar, 1 L ASW, benzyl penicillin and streptomycin sulphate (250 mg $L^{-1}$). Fungal colonies growing out of the algal tissue were transferred onto a sporulation medium consisting of 15 g $L^{-1}$ agar and 20 g $L^{-1}$ biomalt extract in artificial sea water (Klemke, 2004).

### 3.2.2 Isolation from sponge material

Fungal strains were isolated by inoculating small pieces of the inner tissue of the sponge on glucose peptone yeast extract agar at room temperature: glucose 1 g $L^{-1}$, peptone from Soya 0.5 g $L^{-1}$, yeast extract 0.1 g $L^{-1}$, streptomycin sulphate 250 mg $L^{-1}$, agar 15 g $L^{-1}$, 1 L ASW (Höller *et al.*, 2000). Fungal colonies growing out of the tissue of the sponge were transferred to medium for sporulation (15 g $L^{-1}$ agar, 20 g $L^{-1}$ biomalt extract, 1 L ASW).

## 3.3 Cultivation of fungal strains

### 3.3.1 Preparation and maintenance of stock cultures

The fungal strains were kept in cryogenic polypropylene vials at -80° C on a Mex A medium (see 3.9.2).

### 3.3.2 Pre-cultivation

For the first pre-cultivation, the fungal strains were inoculated on Petri dishes with BM20 A and incubated at 25° C for 4 weeks.

The second pre-cultivation consisted of inoculation of material taken from the first pre-cultivation and cultivation of the strain in cultivation tubes containing 10 mL of BM20 A each at 25° C for 3 – 4 weeks.

### 3.3.3 Large scale cultivation of the fungal strains

Fungal strains were cultivated in Fernbach flasks at room temperature for one to three months (specific cultivation conditions see chapter 4). The solid media used for the cultivation were (a) biomalt agar medium (15 g $L^{-1}$ biomalt, 15 g $L^{-1}$ agar and ASW), or (b) malt-yeast agar medium (4 g $L^{-1}$ yeast extract, 10 g $L^{-1}$ malt extract, 4 g $L^{-1}$ glucose, 15 g $L^{-1}$ agar and ASW, pH 7.3). For the screening examinations fungal strains were cultivated in Petri dishes for one month on three different media: (a) biomalt agar medium, (b) malt-yeast agar medium, or (c) czapek agar medium (35 g $L^{-1}$ czapek solution agar, 15 g $L^{-1}$ agar and ASW).

## 3.4 Extraction of fungal biomass

Cultivation medium and mycelia were homogenized using an Ultra Turrax T45. The homogenate was extracted in 1 L or 2 L round-bottomed flasks with ethyl acetate (3 × 300 mL per 300 mL homogenate). The organic fractions were combined and the solvent removed under reduced pressure at 30° C to yield crude extracts.

## 3.5 Chromatography

### 3.5.1 Thin layer chromatography (TLC)

Stationary phase for thin layer chromatography was either TLC aluminium sheets silica gel 60 $F_{254}$ (Merck) or TLC aluminium sheets RP-18 $F_{254}$ (Merck). Standard chromatograms of fungal extracts and fractions thereof were prepared at room temperature using PE/acetone/EtOAc 50:25:25 or MeOH/$H_2O$ 50:50 as mobile phase under saturated conditions. The chromatograms were detected using daylight and UV light (254 nm and 366 nm) as well as vanillin-$H_2SO_4$-reagent (0.5 g vanillin dissolved in a mixture of 85 mL MeOH, 10 mL acetic acid and 5 mL $H_2SO_4$, TLC plate heated at 95° C after spraying).

### 3.5.2 Open column (CC) and vacuum liquid chromatography (VLC)

Sorbent for CC was Sephadex LH-20 (0.018-0.111 mm, Pharmacia Biotech AB; size exclusion material), using MeOH as eluent. The column was wet packed with MeOH before applying the sample solution.

VLC was carried out using silica gel 60 (0.040-0.063 mm, Merck), silica gel 60 (0.063-0.200 mm, Merck) or Polygoprep 60 $C_{18}$ (0.05 mm, Macherey-Nagel; reversed phase). After being filled with the appropriate sorbent, columns were compressed under vacuum and soaked with PE or MeOH. The columns were equilibrated with the first designated eluent before the sample solution was applied.

### 3.5.3 High performance liquid chromatography (HPLC)

HPLC was performed on either a Merck-Hitachi system equipped with an L-6200A pump, an L-4500A photodiode array detector, a D-6000A interface with D-7000 HSM software and a Rheodyne 7725i injection system or a Waters system, controlled by Waters millennium software, consisting of a 717 plus auto sampler, 600 controller pump with in-line degasser and a 996 photodiode array detector. A third system used for HPLC separations was equipped with a Rheodyne 7725i injection system, a Waters 6000A pump, a Knauer differential refractometer and a Linseis L 200 E recorder. Columns used were either:

A: Macherey-Nagel Nucleodur Sphinx C-18 EC (5 µm, 250 × 4.6 mm)
B: Phenomenex Luna C-18 (2) 100 Å (5 µm, 250 × 8 mm)
C: Knauer Si Eurospher-100 Si (5 µm, 250 × 8 mm)
D: Knauer $C_{18}$ Eurospher-100 (5 µm, 250 × 8 mm)
E: Knauer $C_8$ Eurospher-100 (5 µm, 250 × 8 mm)
F: Phenomenex Hydro RP-18 (5 µm, 50 × 4.6 mm)
G: Waters X-Terra C-18 (5 µm, 250 × 4.6 mm)

Typical flow rates were 1.7 or 2.0 mL $min^{-1}$ (250 × 8 mm column), or 1.0 mL $min^{-1}$ (250 × 4.6 mm column). All solvents, except $H_2O$, were distilled prior to use. The eluents were degassed under reduced pressure.

## 3.6 Structure elucidation

For structure elucidation, one- and two-dimensional NMR techniques were used in combination with various mass spectrometric methods. In addition, optical rotations as well as the UV and IR spectroscopic properties were determined. The elucidation of the relative and absolute stereochemistry of the new compounds was accomplished using the following methods: CD spectroscopy, molecular modelling experiments (see 3.6.7) and various NOESY experiments. Literature searches were carried out using the MarinLit® database, Antibase® and the SciFinder® database. Structures were designated as novel if they could not be found in any of the databases.

### 3.6.1 NMR spectroscopy

All NMR spectra were recorded either on a Bruker Avance 300 DPX operating at 300 MHz ($^{1}$H) and 75 MHz ($^{13}$C) or on a Bruker Avance 500 DRX spectrometer operating at 500 MHz ($^{1}$H) and 125 MHz ($^{13}$C).

NMR spectra were processed using Bruker 1D WIN-NMR, 2D WIN-NMR or XWIN-NMR Version 2.6, 3.1 and 3.5 software. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 3.35/49.0 for $CD_3OD$, $\delta_{H/C}$ 7.20/128.0 for $C_6D_6$ and $\delta_{H/C}$ 2.04/29.8 for $(CD_3)_2CO$. Multiplicity of carbons was deduced by DEPT experiments. Structural assignments were based on spectra resulting from one or more of the following NMR experiments: $^{1}$H, $^{13}$C, DEPT135, $^{1}$H homonuclear decoupling, $^{1}$H-$^{1}$H COSY, $^{1}$H-$^{13}$C direct correlation (HSQC), $^{1}$H-$^{13}$C long-range correlation (HMBC), $^{1}$H-$^{1}$H NOESY, $^{1}$H-$^{1}$H ROESY and selective gradient NOE measurements.

### 3.6.2 Mass spectrometry

EI, HR-EI and HR-ESI mass spectral measurements were performed by Ms. C. Sondag, Department of Chemistry, University of Bonn, Germany, using a Kratos MS 50 and a Finnigan MAT 95 spectrometer.

HPLC-ESI-MS measurements were conducted by Dr. A. Krick and E. Eguereva, Institute for Pharmaceutical Biology, Bonn, Germany, employing an Agilent 1100 Series HPLC with a

Macherey-Nagel Nucleodur C-18 100 (5 µm, 125 mm × 2 mm) and a 2 mmol $NH_4Ac$ buffered MeOH/$H_2O$ gradient system, increasing MeOH from 10 to 100 % over 20 min, holding steady for 10 min at 0.25 mL $min^{-1}$. Separation was monitored by absorption at 250 nm with a photodiode array detector. The HPLC system was coupled with an API 2000, Triple Quadrupole, LC/MS/MS, Applied Biosystems/MDS Sciex with ESI source. For injection into the HPLC-ESI-MS system, the samples were solved in MeOH (1 mg $mL^{-1}$).

### 3.6.3 UV measurements

UV spectra were recorded on a Perkin-Elmer Lambda 40 with UV WinLab Version 2.80.03 software, using 1.0 cm quartz cells. Compounds were measured in methanol. The molar absorption coefficient was determined in accordance with the Lambert-Beer-Law:

$$A = \varepsilon \times c \times b \Leftrightarrow \varepsilon \left[ \frac{L}{mol \times cm} \right] = \frac{A}{c \left[ \frac{mol}{L} \right] \times b[cm]}$$

A = absorption at peak maximum
c = concentration
b = layer thickness of solution

### 3.6.4 IR spectroscopy

IR spectra were recorded using a Perkin-Elmer FT-IR Spectrum BX spectrometer interfaced with a Specac Golden Gate Diamond ATR system. Analysis and reporting were performed with Spectrum v3.01 software.

### 3.6.5 Optical rotation

Optical rotation measurements were conducted on a Jasco model DIP-140 polarimeter (1 dm, 1 $cm^3$ cell) operating at λ=589 nm corresponding to the sodium D line at room temperature. Specific optical rotation $[\alpha]_D^T$ was calculated according to:

$$[\alpha]_D^T = \frac{100x\alpha}{c \times l}$$

T: temperature [°C]

D: sodium D line at λ=589 nm

c: concentration [g/100 mL]

d: cell length [dm]

The compounds were dissolved either in MeOH or $CHCl_3$. The rotation angles α were determined as an average value based on at least 10 measurements.

### 3.6.6 CD spectroscopy

CD spectra were recorded in MeOH or ACN at room temperature using an AVIV 62DS spectropolarimeter with the kind help of Prof. Dr. G. Raabe, Institute for Organic Chemistry, University of Aachen, Germany. The concentration of the samples was $2.5 \times 10^{-3}$ mol $L^{-1}$ and the path length was 0.05 cm. A background correction was performed by subtracting the spectrum of the neat solvent recorded under identical conditions

### 3.6.7 Molecular modelling

The configurations of trichodermanones A-C (see 4.2) were determined by conformation search (Boltzmann jump) using the CVFF1.01 force field refined with 500 iterations of smart minimization as implemented in the Cerius$^2$ 4.0 (MSI) molecular modelling software package. Calculations were performed using a Silicon Graphics $O_2$ workstation (Irix 6.5.6).

## 3.7 *In silico* screening (PASS)

*In silico* screening was carried out via the www interface (http://www.ibmc.msk.ru/PASS/) of the PASS (Prediction of Activity Spectra for Substances) software (Poroikov *et al.*, 2001; Poroikov *et al.*, 2000; Lagunin *et al.*, 2000).

## 3.8 Biological screening

### 3.8.1 Cytotoxicity test against human cancer cell lines

All cytotoxicity data were provided by Dr. G. Kelter and Dr. A. Maier, Oncotest GmbH, Institute for Experimental Oncology, Freiburg, Germany. For screening purposes cytotoxicity of fungal extracts against the following six cell lines was evaluated according to Roth and co-workers (Roth *et al.*, 1999): GXF 251L (gastric), LXFL 529L (large cell lung carcinoma), MEXF 462NL (melanoma), RXF 486L (renal), UXF 1138L (uterus carcinoma), MAXF 401NL (breast carcinoma). Extracts were tested in concentrations of 10 $\mu g\ mL^{-1}$.
The cytotoxicity of pure compounds was tested at Oncotest GmbH using 36 human tumor cell lines. The origin of the donor xenografts was described by Fiebig and co-workers (Fiebig *et al.*, 1992):

| | |
|---|---|
| Bladder | BXF 1218L, BXF T24 |
| CNS | CNXF 498NL, CNXF SF268 |
| Colorectal | CXF HCT116, CXF HT29, CXF SW620 |
| Gastric | GXF 251L |
| Lung | LXF 1121L, LXF 289L, LXF 526L, LXF 529L, LXF 629L, LXF H460 |
| Mammary | MAXF 401NL, MAXF MCF7 |
| Melanoma | MEXF 276L, MEXF 394NL, MEXF 462NL, MEXF 541L, MEXF 520L |
| Ovary | OVXF 1619L, OCXF 899L, OVXF OVCAR3 |
| Pancreas | PAXF 1657L, PAXF PANC1 |
| Prostate | PRXF 22RV1, PRXF DU145, PRXF LNCAP, PRXF PC3M |
| Pleuramesothelioma | PXF 1752L |
| Renal | RXF 1781L, RXF 393NL, RXF 486L, RXF 944L, RXF UO31 |
| Uterus body | UXF 1138L |

Cell death of ≥ 70 % was regarded as active while moderate activity was defined as 50-70 % cell death.

### 3.8.2 Agar diffusion assays

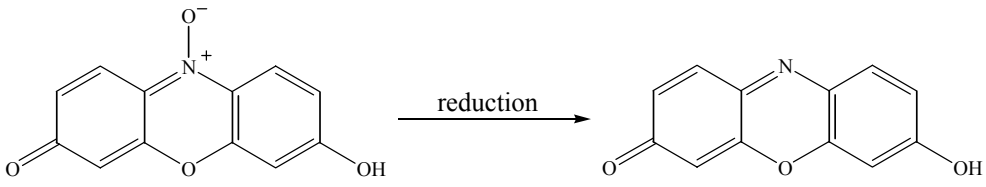
The tests of VLC fractions and pure compounds for antimicrobial activity *in vitro* were performed by E. Neu, Institute for Pharmaceutical Biology, University of Bonn, Germany, following the method described by Krohn and co-workers (Schulz *et al.*, 2002). The bacteria *Bacillus megaterium* de Bary (Gram-positive) and *Escherichia coli* (Migula) Castellani & Chambers (Gram-negative), the fungi *Eurotium rubrum* (formerly *E. repens*) König, Spieckermann & Bremer (Ascomycetes) and *Mycotypha microspora* Fenner (Zygomycetes) were used as test organisms.

Sample solutions contained 1 mg $mL^{-1}$ per test sample. 50 µL (equivalent to 50 µg) of each solution were pipetted onto a sterile antibiotic filter disk (diameter: 9 mm, Schleicher & Schuell 2668), which was then placed onto the appropriate agar medium and sprayed with a suspension of the test organism. Growth media, preparation of spraying suspensions, and conditions of incubation were carried out according to Schulz and co-workers (Schulz *et al.*, 1995). For tested samples, a growth inhibition zone ≥ 3 mm or a complete inhibition ≥ 1 mm, measured from the edge of the filter disk, were regarded as a positive result; growth inhibition: growth of the appropriate test organism was significantly inhibited compared to a negative control; complete inhibition: no growth at all in the appropriate zone. Benzyl penicillin (1 mg $mL^{-1}$ MeOH), streptomycin (1 mg $mL^{-1}$ MeOH) and miconazole (0.5 mg $mL^{-1}$ DCM) were used as positive controls.

### 3.8.3 Antiprotozoal assay

The antiprotozoal tests were performed by Prof. Dr. R. Brun (Swiss Tropical Institute, Basel, Switzerland). Antiplasmodial activity was determined against the K1 strain of *Plasmodium falciparum*, using a modified [$^3$H] hypoxanthine incorporation assay. Briefly, infected human erythrocytes were exposed to serial drug dilutions in microtiter plates for 48 h at 37° C in a gas mixture with reduced oxygen and elevated $CO_2$. [$^3$H] hypoxanthine was added to each well and after further incubation for 24 h the wells were harvested on glass fiber filters and counted in a liquid scintillation counter. From the sigmoidal inhibition curve the $IC_{50}$ value was calculated. Chloroquine was used as positive control in each test series.

Activity against *Trypanosoma brucei rhodesiense* (strain STIB 900), the causative agent of African sleeping sickness, was evaluated according to Räz and co-workers, 1997. Parasites were grown axenically in culture medium supplemented with horse serum. Following a 3-day exposure to test compounds, the viability of tryptomastigote parasites was quantified using the dye Almar Blue® by monitoring the reductive environment of living cells (Fig. 3-8-1). Fluorescence development was expressed as percentage of the control, and $IC_{50}$ values were calculated. Melarsoprol was included as positive control.

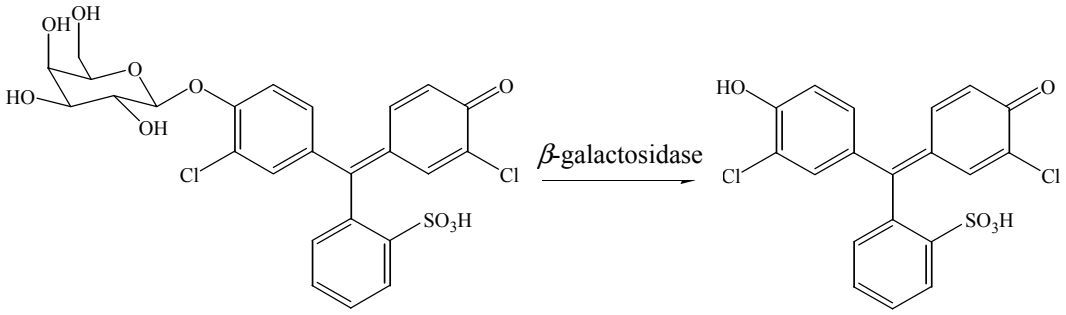



Resazurin (Alamar Blue®) — blue, non-fluorescent

Resorufin — pink, fluorescent

**Figure 3-8-1:** Alamar Blue® reaction.

Activity against *Trypanosoma cruzi*, the causative agent of Chagas disease, was determined according to Buckner and co-workers, 1996. Briefly, the strain Tulahuen C4 of *T. cruzi*, which had been transfected with the galactosidase *lac*-Z gene, was cultivated for 4 days on rat skeletal myoblasts (5 % $CO_2$, 37° C) in the presence of drug. For measurement of the $IC_{50}$ the substrate chlorophenol red-$\beta$-D-galactopyranoside was added. The colour reaction (Fig. 3-8-2) that developed during the following 2-4 h was quantified photometrically employing an ELISA reader. Benznidazole was included in each test series as positive control.




chlorophenol red-β-D-galactopyranoside (yellow)

chlorophenol-red

**Figure 3-8-2:** Chlorophenol-red reaction.

Evaluation of antileishmanial activity was carried out in mouse peritoneal macrophages. The ratio of infection with *Leishmania donovani* (strain MHOM-ET-67/L82), the causative agent of Kala-Azar disease, was determined microscopically after exposure to test compounds, incubation and staining with Giemsa. $IC_{50}$ values were calculated by linear regression. Miltefosine was used as positive control.

Cytotoxicity was evaluated in rat skeletal myoblasts (L6-cells), using podophyllotoxin as positive control.

### 3.8.4 Acetylcholine esterase assay

Inhibitor experiments with acetylcholine esterase were carried out by the research group of Prof. Dr. M. Gütschow (Institute for Pharmaceutical Chemistry, University of Bonn, Germany). The general protocol involved a conversion of 5,5´-dithio-bis-2-nitrobenzoic acid (DTNB) with thiocholine, originating from the enzymatic degradation of acetylthiocholine, to give a product with strong UV absorption at $\lambda = 412$ nm (Fig. 3-8-3). Thus, concentration shifts of thiocholine were monitored by spectroscopic methods after addition of test compounds (25 µM) or extracts (100 µg $mL^{-1}$). The assay was run in duplicate and repeated at least once. Samples that inhibited the enzyme activity greater than 85 % or more relative to a negative control were regarded as active.

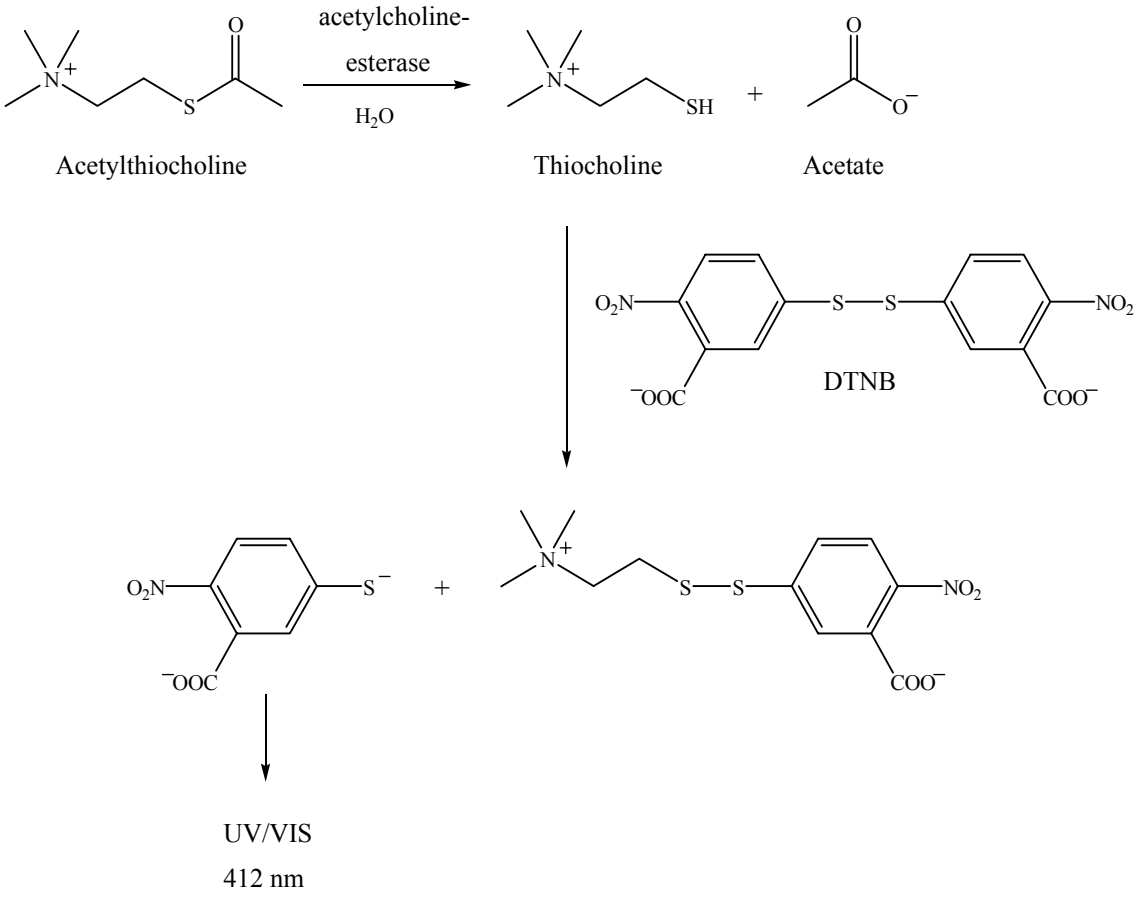



**Figure 3-8-3:** Test principle of acetylcholinesterase inhibition assay.

### 3.8.5 Cysteine protease papain inhibition assay

Investigations on the inhibitory potential towards papain were performed by the research group of Prof. Dr. M. Gütschow (Institute for Pharmaceutical Chemistry, University of Bonn, Germany). Using Z-phenylalanyl-arginine-p-nitroanilide (Z-Phe-Arg-pNA) as substrate for papain, the enzyme activity was monitored by the formation of free p-nitroaniline. Concentration shifts were measured spectrophotometrically at $\lambda = 405$ nm after addition of bacterial extracts (100 µg $mL^{-1}$) and expressed as percentage of a negative control. The assay was run in duplicate and repeated at least once.

### 3.8.6 Trypsine inhibition assay

The inhibitory potential towards trypsine was investigated in cooperation with the research group of Prof. Dr. M. Gütschow (Institute for Pharmaceutical Chemistry, University of Bonn, Germany). Using Succinyl-alanyl-alanyl-propyl-arginine-p-nitroanilide (Suc-Ala-Ala-Pro-Arg-NHNp) as substrate for trypsine, the enzyme activity was monitored by the formation of free p-nitroaniline. Concentration shifts were measured spectrophotometrically at $\lambda = 405$ nm after addition of bacterial extracts (100 µg $mL^{-1}$) and expressed as percentage of a negative control. The assay was run in duplicate and repeated at least once.

### 3.8.7 HLE inhibition assay

Investigations on the inhibitory potential towards human leucocyte elastase were performed by the research group of Prof. Dr. M. Gütschow (Institute for Pharmaceutical Chemistry, University of Bonn, Germany). Human leukocyte elastase was assayed spectrophotometrically at 405 nm at 25° C. Assay buffer was 50 mM sodium phosphate buffer (500 mM NaCl, pH 7.8). A stock solution of the chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-NHNp was prepared in DMSO and diluted with assay buffer. Final concentration of DMSO was 1.5 %, the final concentration of the chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-NHNp was 100 µM, unless stated otherwise. Assays were performed with a final HLE concentration of 50 ng $mL^{-1}$, which corresponded to an initial rate of 0.7 µM $min^{-1}$, when 100 µM of the substrate was used. An inhibitor solution (10 µL) and the substrate solution (50 µL) were added to a cuvette that contained the assay buffer (890 µL), and the solution was thoroughly mixed. The reaction was initiated by adding the HLE solution (50 µL) and was followed over 10 min. $IC_{50}$ values were calculated from the linear steady-state turnover of the substrate. The HLE inhibition by the crude extracts was determined in the presence of DMSO (0.5 %) and acetonitrile (1 %).

### 3.8.8 Antiviral assay

Replication of viruses leads to a complete lysis of host cells, known as cytopathic effect (CPE). Antiviral activity of pure compounds was evaluated by Dr. M. Schmidtke (Institute for Virology, University of Jena, Germany) in CPE inhibitory assays using crystal violet staining on HeLa Ohio cells (human cervix carcinoma; ATCC No. CCL-2) infected with coxsackie

virus B3 (CVB3), MDCK cells (Madin-Darby canine kidney; Institute of Influenza viruses, St. Petersburg, Russia) infected with influenza virus A and GMK cells (green monkey kidney; Schaper and Brümmer, Salzgitter, Germany) infected with herpes simplex virus type 1 (HSV-1) according to the method described by Schmidtke and co-workers, 2001.

### 3.8.9 Protein phosphatase assays

The enzyme assays were conducted in cooperation with Dr. H. Prinz, research group of Prof. Dr. H. Waldmann of the Max-Planck-Institute of Molecular Physiology, Dortmund, Germany. Pure compounds were tested against the protein phosphatases Cdc25A, PTP1B, MPtpA, MPtpB, PTP-SHP2, PTPN2 TC-PTP, VE-PTP, PP1 and VHR using a concentration of 100 µM $L^{-1}$ (Brohm *et al.*, 2001). If significant inhibition could be observed, $IC_{50}$ values were determined.

## 3.9 Chemicals

### 3.9.1 Chemicals and solvents

| | |
|---|---|
| Acetic acid | Merck (Germany) |
| Acetone-$d_6$ 99.8 % | Deutero GmbH (Germany) |
| Acetonitrile | Merck (Germany) |
| Agar-Agar | Fluka (Switzerland) |
| Ammonium acetate | Merck (Germany) |
| Benzene-$d_6$ 99.8 % | Dr. Glaser (Switzerland) |
| Benzyl penicillin | Fluka (Switzerland) |
| Biomalt extract | Villa Natura GmbH (Germany) |
| $CaCl_2 \times 2\ H_2O$ | Merck (Germany) |
| Chloroform | Merck (Germany) |
| Chloroform-$d_1$ 99.8 % | Deutero GmbH (Germany) |
| *meta*-Chloroperbenzoic acid (MCPBA) | Fluka (Switzerland) |
| $CuSO_4$ | Fluka (Switzerland) |
| Czapek Solution Agar | Becton Dickinson (MD, USA) |
| Dimethylsulfoxide | AppliChem (Germany) |
| D-(+)-Glucose | Serva (Germany) |

| | |
|---|---|
| Glycerine | Roth (Germany) |
| $H_3BO_3$ | Serva (Germany) |
| HCl, 37 % | Merck (Germany) |
| KBr | Merck (Germany) |
| KCl | Merck (Germany) |
| $KH_2PO_4$ | Merck (Germany) |
| Malt-extract | Roth (Germany) |
| 3-Hydroxy-2-methylbutanoic acid | Prof. Brunet, University of Madrid (Spain) |
| Methanol Lichrosolv, LC-MS grade | Merck (Germany) |
| Methanol-$d_4$ 99.8 % D | Deutero GmbH (Germany) |
| *S*-(+)-2-Methylbutyric acid | Fluka (Switzerland) |
| (+/-)-2-Methylbutyric acid | Fluka (Switzerland) |
| N-methyl-N-(trimethyl-silyl) trifluoroacetamide | WGA GmbH (Germany) |
| $MgCl_2 \times 6\ H_2O$ | Merck (Germany) |
| $MgSO_4 \times 7\ H_2O$ | Merck (Germany) |
| Miconazole | ICN Biomedicals (CA, USA) |
| $MnCl_2 \times 4\ H_2O$ | Merck (Germany) |
| NaCl | Merck (Germany) |
| $NaHCO_3$ | Merck (Germany) |
| $NaNO_3$ | Merck (Germany) |
| NaOH | Merck (Germany) |
| $Na_2SO_4$ | KMF (Germany) |
| Penicillin/Streptomycin | Biomol GmbH (Germany) |
| D-(+)-Saccharose | Roth (Germany) |
| Sephadex® LH-20 | Pharmacia Biotech (Sweden) |
| Soyapeptone | Merck (Germany) |
| $SrCl_2 \times 6\ H_2O$ | Merck (Germany) |
| Streptomycin sulphate | Fluka (Switzerland) |
| Trimethylsulfonium hydroxide (TMSH) | WGA GmbH (Germany) |
| Tween 80 | Roth (Germany) |
| Yeast extract | Fluka (Switzerland) |
| Vanillin | Merck (Germany) |

All other chemicals were supplied by Merck (Germany), Fluka (Switzerland), Roth (Germany) and Sigma-Aldrich (Germany). All other solvents were research grade and supplied by Infracor or BASF (Germany). Acetone, $CHCl_3$, $CH_2Cl_2$, EtOAc, MeOH and PE were distilled prior to use. Water for HPLC was de-ionized using a Millipore system (milli-Q® academic).

### 3.9.2 Media

Mex A: biomalt extract 20 g $L^{-1}$, yeast extract 0.1 g $L^{-1}$, glycerin 50 g $L^{-1}$, 80 % artificial seawater, agar-agar 13 g $L^{-1}$.
BM20 A: biomalt extract 20 g $L^{-1}$, 80 % artificial seawater, agar-agar 20 g $L^{-1}$.
BM20: biomalt extract 20 g $L^{-1}$, 80 % artificial seawater.
BM50: biomalt extract 50 g $L^{-1}$, 80 % artificial seawater.
SPG: soyapeptone 10 g $L^{-1}$, glucose 4 g $L^{-1}$, $KH_2PO_4$ 1 g $L^{-1}$, 80 % artificial seawater.
YMG: yeast extract 4 g $L^{-1}$, biomalt extract 1 g $L^{-1}$, glucose 4 g $L^{-1}$, 80 % artificial seawater.
CA: $NaNO_3$ 3 g $L^{-1}$, $KH_2PO_4$ 1 g $L^{-1}$, KCl 0.5 g $L^{-1}$, $MgSO_4 \times 7\ H_2O$ 0.01 g $L^{-1}$, saccharose 30 g $L^{-1}$, 80 % artificial seawater.

Artificial seawater (ASW, g $L^{-1}$) KBr 0.1, NaCl 23.48, $MgCl_2 \times 6\ H_2O$ 10.61, $CaCl_2 \times 2\ H_2O$ 1.47, KCl 0.66, $SrCl_2 \times 6\ H_2O$ 0.04, $Na_2SO_4$ 3.92, $NaHCO_3$ 0.19 and $H_3BO_3$ 0.03.

# 4 Results and discussion

## 4.1 Screening of fungal extracts for structurally interesting and biologically active metabolites

The focus on the present study was the investigation of marine fungi towards their secondary metabolism with an emphasis on structurally novel and biologically active compounds, mainly from cytotoxicity bioassays. The fungal strains taken for the cytotoxicity screening program of the Institute for Pharmaceutical Biology have been isolated from marine macroorganisms such as algae and sponges. For screening purposes the strains were cultivated on 500 mL of solid media in Petri dishes. Each strain was cultivated on three different media: biomalt agar medium (BMS), malt-yeast agar medium (MYA) and Czapek agar medium (CZ). After one month of cultivation, fungal biomass, together with the medium, was homogenized and extracted with ethyl acetate. The crude extracts were then evaluated by $^{1}$H-NMR and LC-MS data and investigated for biological activities. The major criterion for selection of the fungal strains was significant cytotoxic activity, tested in a six cell line panel at a concentration of 10 µg mL$^{-1}$. Additionally, agar diffusion tests for antimicrobial and antialgal activity were performed. The extracts were tested against the bacteria *Escherichia coli* (*E.c.*), *Bacillus megaterium* (*B.m.*), the fungi *Microbotryum violaceum* (*M.v.*), *Eurotium rubrum* (*E.r.*) and *Mycotypha microspora* (*M.m.*) as well as towards the alga *Chlorella fusca* (*C.f.*).

Of all evaluated fungal strains, five were chosen for further investigation according to the results of the antitumor assay and $^{1}$H-NMR and LC-MS data as well as, in second priority, promising antimicrobial activity (Table 4-1-1).

| No.[1] | Taxonomy | Medium[2] | Crude Extract (g $L^{-1}$) | NMR | MS | Agar diffusion test (growth inhibition in mm, conc. 1mg $mL^{-1}$) | | | | | | Cytotox[3] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *E.c.* | *B.m.* | *M.v.* | *E.r.* | *M.m.* | *C.f.* | x/ 6 |
| **54** | ***Trichoderma saturnisporium*** | **BMS** | **0.113** | **+++** | **+** | **0** | **0** | **0** | **0** | **0** | **0** | **1** |
| 54 | *T. saturnisporium* | CZ | 0.013 | - | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 54 | *T. saturnisporium* | MYA | 0.417 | - | + | 0 | 0 | 0 | 0 | 0 | 0 | 1&1 |
| 71 | *Phoma* sp. | BMS | 0.245 | ++ | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 71 | *Phoma* sp. | CZ | | - | | 0 | 0 | 0 | 0 | 0 | 0 | - |
| 71 | *Phoma* sp. | MYA | 0.138 | - | | 0 | 0 | 0 | 0 | 0 | 0 | 6&5 |
| 74 | *Spicellum roseum* | BMS | 0.461 | + | + | 0 | 0 | 0 | T5 | T11 | 0 | 6&6 |
| 74 | *S. roseum* | CZ | 0.733 | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 5&4 |
| 74 | *S. roseum* | MYA | 0.137 | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 6&6 |
| 96 | *Microsphaeropsis* sp. | BMS | 0.164 | + | | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| 96 | *Microsphaeropsis* sp. | CZ | 0.457 | - | | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| 96 | *Microsphaeropsis* sp. | MYA | 0.447 | - | | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| 193 | *Paecilomyces lilacinus* | BMS | 0.143 | +++ | ++ | 0 | 0 | 0 | 0 | 0 | 0 | 6&4 |
| 193 | *P. lilacinus* | CZ | 0.187 | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 193 | *P. lilacinus* | MYA | 0.082 | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 194 | *Alternaria japonica* | BMS | 0.065 | - | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 194 | *A. japonica* | CZ | 0.082 | - | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **194** | ***A. japonica*** | **MYA** | **0.008** | **++** | **+** | **0** | **0** | **0** | **0** | **0** | **0** | **5** |
| 714 | *Trichoderma harzianum* | BMS | 0.060 | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 714 | *T. harzianum* | CZ | 0.028 | - | - | / | / | / | / | / | / | 2 |
| **714** | ***T. harzianum*** | **MYA** | **0.183** | **-** | **-** | **0** | **0** | **0** | **0** | **0** | **0** | **2&3** |
| 738 | *Acremonium sclerotigenum* | BMS | 0.544 | | + | 0 | 0 | 0 | 0 | 0 | 0 | 6&6 |
| 738 | *A. sclerotigenum* | CZ | 0.092 | | | 0 | 0 | 0 | 0 | 0 | 0 | 4&5 |
| 738 | *A. sclerotigenum* | MYA | 0.113 | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 741 | *Phoma macrostoma* | BMS | 0.246 | ++ | ++ | 0 | 0 | 0 | 0 | 0 | 0 | 4&5 |
| 741 | *P. macrostoma* | CZ | 0.101 | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 741 | *P. macrostoma* | MYA | 0.038 | + | ++ | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 240 | *Chaetomium* sp. | BMS | 0.614 | +++ | +++ | 0 | T7 | 0 | 0 | 0 | 0 | 6 |
| 240 | *Chaetomium* sp. | CZ | 0.065 | + | ++ | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| 240 | *Chaetomium* sp. | MYA | 0.766 | | | 0 | W2 | 0 | 0 | 0 | 0 | 6 |
| **321** | ***Beauveria bassiana*** | **BMS** | **0.226** | **++** | **++** | **0** | **0** | **0** | **0** | **0** | **0** | **6 & 5** |
| 321 | *B. bassiana* | CZ | 0.231 | + | ++ | 0 | T3 | T6 | 0 | 0 | 0 | 0 |
| 321 | *B. bassiana* | MYA | 0.228 | + | ++ | 0 | 0 | T4 | 0 | 0 | 0 | 0 |
| 405 | *Microdiploida* sp. | BMS | 0.544 | | | 0 | 0 | 0 | 0 | 0 | 0 | 2 & 1 |
| 405 | *Microdiploida* sp. | CZ | 0.042 | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **405** | ***Microdiploida* sp.** | **MYA** | **0.641** | | | **0** | **T3** | **0** | **0** | **0** | **0** | **3** |
| 418 | *Wardomyces anomalus* | BMS | 0.117 | | | 0 | 0 | 0 | 0 | 0 | 0 | 4&3 |
| 418 | *W. anomalus* | CZ | 0.077 | | | 0 | 0 | 0 | 0 | 0 | 0 | 2&3 |
| 418 | *W. anomalus* | MYA | 0.241 | | | 0 | 0 | 0 | 0 | 0 | 0 | 4 |

[1]Number refers to the fungal collection (Institute for Pharmaceutical Biology).

[2] BMS, biomalt-agar medium; MYA, malt-yeast medium; CZ, czapek medium.

[3] Number of cell lines showing cytotoxicity in six cell line panel. One or two runs performed, conc. 10 µg $mL^{-1}$.

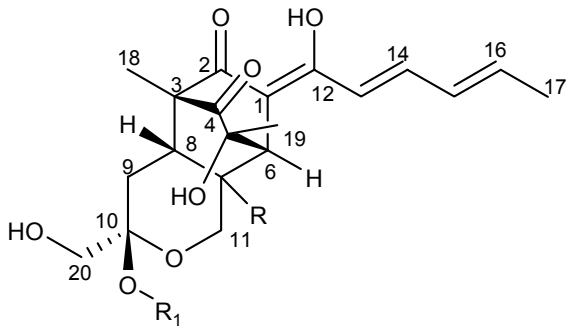
**Table 4-1-1:** Overview of the screening results for selected fungal strains. Those chosen for further investigation are marked in red.

Of the marine fungi selected for further investigations during this study, two have been isolated from sponges while three are alga-derived. High antitumor activity came from the BMS extract of strain 321, a sponge-derived *Beauveria bassiana*. Together with interesting signals in the $^{1}$H-NMR and LC-MS spectra, the strain represented a highly interesting organism for detailed investigation. Data of the cytotoxicity bioassay was also determining for the choice of strain 194, *Alternaria japonica* isolated from an alga, which was active against five cell lines in the cytotoxicity screening. For strain 405 (*Microdiploida* sp.), positive results in both bioassays led to the choice for detailed investigation of this fungus, showing cytotoxic activity against three cancer cell lines and antibiotic properties against *Bacillus megaterium*. The MYA-extract of the alga-derived fungus *Trichoderma harzianum* (screening No. 714) also proved to be active in the cytotoxicity bioassay, showing relatively high antitumor activity, particularly against the cancer cell line UXF 1138L. Similar data came from the sponge-derived fungus *Trichoderma saturnisporium* (strain 54), which was active towards the same uterus cancer cell line and additionally indicated the presence of structurally interesting compounds from the $^{1}$H-NMR and LC-MS data. This fungus had been investigated before by the former Ph.D. student A. Abdel-Lateff (Abdel-Lateff, 2004) and had revealed the presence of several known as well as new structures belonging to the class of the sorbicillin derivatives. As the stereochemistry of these compounds had remained unclear, confirmation of the planar structure and determination of the absolute stereochemistry was subjected to further investigation which is part of the present study.

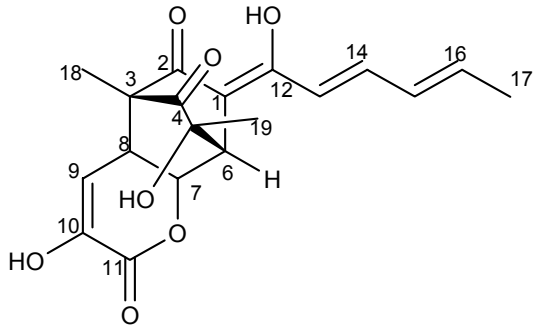
## 4.2 Novel sorbicillin derivatives with an unprecedented carbon skeleton from the sponge-derived fungus *Trichoderma saturnisporium*

Fungi of the genus *Trichoderma* are widespread in terrestrial and marine environments, e. g. soil and barks. They also live in association with higher plants and sponges. *Trichoderma* spp. are noted for their diverse secondary metabolite chemistry that is not characterized by any clear pattern concerning the structural types encountered. To date, more than 500 compounds have been reported from fungi belonging to this genus, including the bicyclic dodecanone koninginin G (Cutler *et al.*, 1999), the carotane derivatives trichocaranes A–D (Macias *et al.*, 2000), and the cyclopentenones pentenocins A and B (Matsumoto *et al.*, 1999).

The current secondary metabolite investigation focused on a marine *Trichoderma saturnisporium*, isolated from the Caribbean sponge *Agelas dispar*. The fungus was cultivated on a solid glucose biomalt medium containing artificial sea water (ASW). Successive fractionation of the ethyl acetate extract by vacuum liquid chromatography (VLC) and HPLC yielded four novel sorbicillinoid polyketide derivatives, compounds **1**–**4** (Fig. 4-2-1), and the monomer rezishanone C (**5**), a possible product of a Diels-Alder reaction between sorbicillinol and a dienophile not related to the sorbicillinoids. Additionally, the known sorbicillinoid monomers vertinolide (**6**) (Trifonov *et al.*, 1982) and epoxisorbicillinol (**7**) (Sperry *et al.*, 1998) and the dimers trichodimerol (**8**) (Andrade *et al.*, 1992), bislongiquinolide (**9**) (Andrade *et al.*, 1997) and bisvertinol (**10**) (Trifonov *et al.*, 1986), had been isolated; their presence could be confirmed by observation of the corresponding masses and several $^{1}$H- and $^{13}$C-NMR signals in the spectra of several fractions and comparison with data previously obtained within our group (Abdel-Lateff, 2004).




**trichodermanone A (1):** R = α-OH, $R_1$ = $CH_3$
**trichodermanone B (2):** R = ß-OH, $R_1$ = $CH_3$
**trichodermanone C (3):** R = ß-OH, $R_1$ = H




**trichodermanone D (4)**

**Figure 4-2-1:** Structures of trichodermanones A-D.

Sorbicillines are structurally unusual natural products and were discovered in several species of marine and terrestrial fungi, namely *Trichoderma* spp., *Verticillium* spp., and *Penicillium* spp (Abe *et al.*, 2000). This class of hexaketides and dimers thereof have been found to exhibit a broad range of biological activities including inhibition of lipopolysaccharide-induced production of tumor necrosis factor alpha (TNF α) in human monocytes, radical scavenging and cytotoxicity (Barnes-Seeman & Corey, 1999; Abe *et al.*, 1999; Bringmann *et al.*, 2005).

### 4.2.1 Cultivation, extraction and isolation of secondary metabolites from strain 54

The sponge *Agelas dispar* J. was collected in December-January of 1993 by divers using SCUBA from the waters around the Caribbean Island of Dominica, Windward Islands. The sponge was identified by Dr. R. Desqueroux-Faundez, Musée d'Histoire Naturelle, Geneva, Switzerland. The fungus was isolated as described in chapter 3.2.2. The fungal strain was identified as *Trichoderma saturnisporium* by Dr. J. Wiese, Leibnitz Institut für Meereswissenschaften, Kiel, Germany.

In order to increase the amount of the interesting structural types, variations of the culture conditions were performed and the fungus was grown in a small scale (0.3 L medium) on six different solid media and one liquid medium as shown in table 4-2-1 (Medium 1-7). All solid media contained 15 g $L^{-1}$ agar. After extraction with EtOAc, the $^1$H-NMR spectra, LC-MS data and the amounts of the crude extracts were compared. The addition of glucose to the

medium resulted in increased secondary metabolite production, which became apparent by the amount of biomass and by the intensified yellow colour of the extract. From the mass spectra, the focus was set on the presence of unusual molecular mass values in the region of 300-500 Da, while the $^1$H-NMR spectra were evaluated especially with respect to low field resonances in the δ 6–8 region. Medium 2 gave the best results regarding a high amount of crude extract together with interesting LC-MS data and NMR signals, and therefore was chosen for mass cultivation.

| | | **biomalt** | **glucose** | **ASW** | **crude extract (g L$^{-1}$)** | **$^1$H-NMR** |
|---|---|---|---|---|---|---|
| Medium 1 | solid | 50 g L$^{-1}$ | - | yes | 0.21 | ++ |
| Medium 2 | solid | 50 g L$^{-1}$ | 15 g L$^{-1}$ | yes | 0.33 | +++ |
| Medium 3 | solid | 15 g L$^{-1}$ | - | yes | 0.09 | + |
| Medium 4 | solid | 15 g L$^{-1}$ | 15 g L$^{-1}$ | yes | 0.21 | +++ |
| Medium 5 | solid | 15 g L$^{-1}$ | - | no | 0.17 | ++ |
| Medium 6 | solid | 15 g L$^{-1}$ | 15 g L$^{-1}$ | no | 0.56 | ++ |
| Medium 7 | liquid | 15 g L$^{-1}$ | 15 g L$^{-1}$ | no | 0.08 | ++ |

**Table 4-2-1:** Comparison of medium composition, amounts and results of $^1$H-NMR data during medium optimization attempts for strain 54.

The fungus was thus cultivated at room temperature for two months in 5.75 L (23 Fernbach flasks) of solid medium containing 50 g L$^{-1}$ biomalt, 15 g L$^{-1}$ glucose, 15 g L$^{-1}$ agar in artificial sea water (ASW). Mycelia and medium were homogenized and the resulting mixture was exhaustively extracted with EtOAc and filtered. The filtrate was evaporated to yield 2.5 g of a yellowish crude extract. This extract was fractionated by reversed phase vacuum liquid chromatography (RP-VLC), employing gradient elution from 10:90 $H_2O$/MeOH to MeOH, to yield 9 fractions. According to the differences in composition of the fractions as detected by $^1$H-NMR spectroscopy, especially with respect to the resonances in the δ 6–8 region as stated above, fractions 4, 5 and 6 seemed promising for further investigation. The three fractions were combined and fractionated by NP-VLC, using gradient elution from petroleum ether to EtOAc, followed by MeOH, to yield 15 fractions. Fraction 4.6.4 gave a very prominent single spot on TLC and was undertaken to further spectroscopic investigation without additional purification. The spectral data revealed the compound to be pure **5** (24 mg; Fig. 4-2-2).

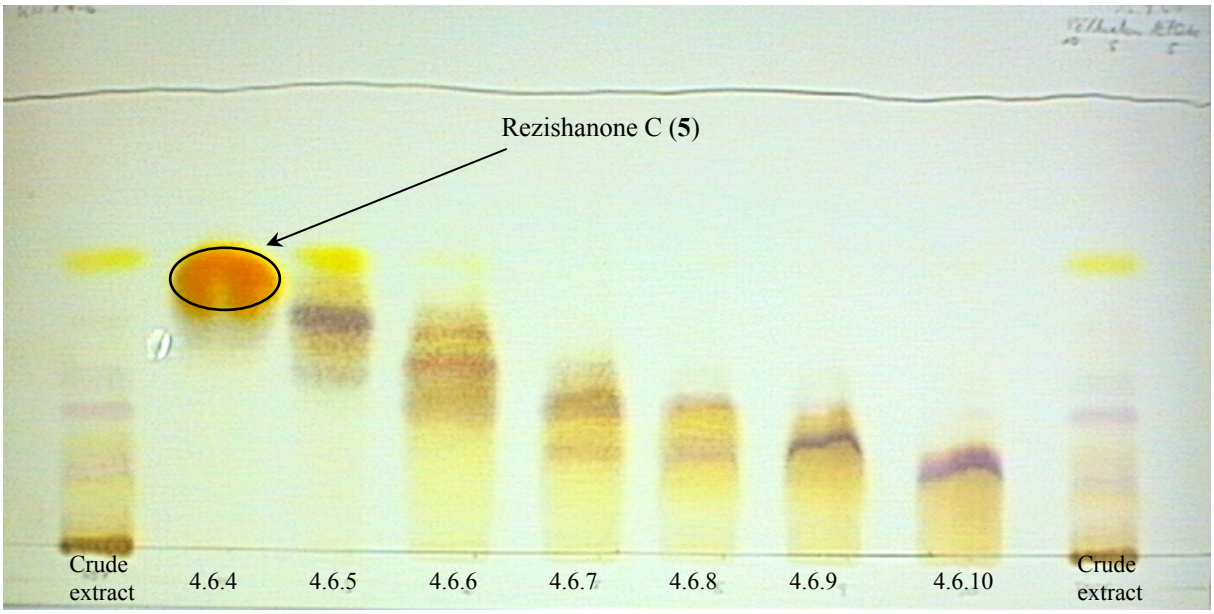



**Figure 4-2-2:** TLC of fractions 4.6.4 to 4.6.10 after detection with vanillin-$H_2SO_4$-reagent. Fraction 4.6.4 is pure **5**.

Fraction 4.6.5 yielded 32.0 mg of semi-pure **8**. Fractions 4.6.7 and 4.6.8 were combined and subjected to RP-18 HPLC (eluent 65:35 MeOH/$H_2O$), to yield seven fractions that were further purified by HPLC (eluent 50:25:25 PE/acetone/EtOAc), to yield 14 mg of **2** ($t_R$ = 9 min).

Compounds **1** (5.2 mg, $t_R$ = 37 min) and **3** (11.9 mg, $t_R$ = 27 min) were purified from fraction 4.6.9, after two RP-18 HPLC separations (eluent 60:40 MeOH/$H_2O$ for the first and 45:55 MeOH/$H_2O$ for the second HPLC). From fraction 4.6.9 we also isolated semi-pure **6** ($t_R$ = 30.4 min) and semi-pure **9** ($t_R$ = 41 min).

The extract from a second cultivation of the fungus was fractionated by VLC, employing a gradient elution from PE to acetone, to yield five fractions (PE/acetone 1:0, 7.5:2.5, 5:5, 2.5:7.5, 0:1, 200 mL each). According to the low field resonances in the δ 6-8 region of the $^1$H-NMR spectral data, fractions 2 and 3 were further investigated. VLC fraction 3 was fractionated over NP-Si by VLC employing gradient elution from PE to EtOAc, to yield 11 fractions (PE/EtOAc 1:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:1; 10 mL each). Fraction 3.4 was then purified by RP-18 HPLC, employing a gradient elution from 9:1 $H_2O$/MeOH to MeOH in 45 min, 2 mL min$^{-1}$, to yield **6** ($t_R$ = 32 min, 4 mg), semi-pure **7** ($t_R$ = 29 min) and semi-pure **9** ($t_R$ = 41 min). Fraction 3.6 was purified by RP-18 HPLC, using a gradient of 6:4 $H_2O$/MeOH to MeOH in 60 min, 2 mL min$^{-1}$, and yielded four fractions (3.6.4 – 3.6.7; $t_R$ = 25 min, 26 min, 29 min, 35 min, respectively). The first of these fractions was purified using

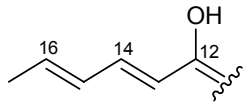
RP-HPLC, employing a gradient elution from 65:35 $H_2O$/MeOH to MeOH in 35 min, 1 mL $min^{-1}$, to give 10 mg of **3** ($t_R$ = 21 min) and 2 mg of **4** ($t_R$ = 25 min). HPLC peak 3.6.5 was purified using RP-18 HPLC (gradient elution 65:35 $H_2O$/MeOH to 20:80 $H_2O$/MeOH in 35 min, 1 mL $min^{-1}$) to yield 4 mg of **2** ($t_R$ = 22 min), and 1 mg of **3** ($t_R$ = 21 min). HPLC peak 3.6.6 was further purified employing RP-18 HPLC (gradient elution from 7:3 $H_2O$/MeOH to 3:7 $H_2O$/MeOH in 30 min, 1 mL $min^{-1}$) to yield 4 mg of **1** ($t_R$ = 23.5 min). Fraction 3.6.7 was subjected to RP-18 HPLC, using gradient elution from 55:45 $H_2O$/MeOH to MeOH in 60 min, 2 mL $min^{-1}$ to yield 10 mg of **3** ($t_R$ = 23 min).

VLC fraction 2 was subjected to normal phase VLC, using a gradient elution from PE to EtOAc, to yield five fractions (PE/EtOAc 1:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:1, 200 mL each. Fraction 2.4 was then purified by RP-HPLC, employing gradient elution from 7:3 $H_2O$/MeOH to MeOH in 50 min, 2 mL $min^{-1}$, and yielded **1** ($t_R$ = 29 min, 2 mg), **2** ($t_R$ = 32 min, 2 mg) and **10** ($t_R$ = 44 min, 2 mg).

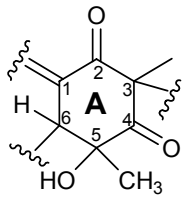
### 4.2.2 Structure elucidation

Accurate mass measurement and $^1$H- and $^{13}$C-NMR analysis of compound **1** showed it to have the molecular formula $C_{21}H_{28}O_8$. Its $^{13}$C-NMR spectral data allowed five of the eight elements of double-bond unsaturation to be attributed to three carbon-carbon double bonds and two carbonyl groups (Table 4-2-2); the molecule thus had to be tricyclic. $^1$H- and $^{13}$C-NMR spectra showed the presence of two $sp^3$ hybridized methine groups, four $sp^2$ hybridized methine groups, three methylene groups, two of them attached to oxygen, one allylic methyl group, two methyl groups attached to quaternary carbons, one methoxyl group and eight quaternary carbons. The NMR data further enabled all but four hydrogen atoms of **1** to be attached to carbons; hence it was evident that the remaining four are present in the molecule as hydroxyl functions, a deduction supported by IR data ($\nu_{max}$ 3315 $cm^{-1}$). One of them was assigned in the $^1$H-NMR spectrum to the resonance at $\delta$ 14.2, and showing it to probably hydrogen-bond to a keto-function.

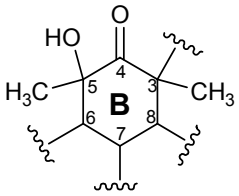
The planar structure of **1** was deduced by interpretation of its $^1$H-$^1$H COSY and $^1$H-$^{13}$C HMBC 2D shift correlated NMR spectral data. Initially, four partial substructures were deduced (Fig. 4-2-3).



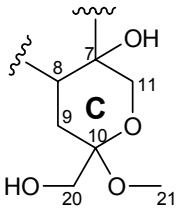

**Fragment 1**




**Fragment 2**




**Fragment 3**




**Fragment 4**

**Figure 4-2-3:** Fragments of compound **1** deduced from $^{1}$H-$^{1}$H COSY and $^{1}$H-$^{13}$C HMBC data.

Fragment 1, a sorbyl unit, was elucidated based on a $^{1}$H-$^{1}$H spin system from $H_3$-17 to H-13, as well as $^{1}$H-$^{13}$C long range correlations between H-13 and C-12. The chemical shift of C-12 ($\delta_C$ 167.8) evidenced this carbon to bear a hydroxyl group. Fragment 2 (ring A) was characterized by the presence of many quaternary carbons. Heteronuclear long range couplings were observed from the resonance of H-6 to those of C-1, C-2, C-4 and C-5, between $H_3$-19 and C-4, C-5 and C-6, and between $H_3$-18 and C-2, C-3 and C-4, and evidenced these methyl groups to be bonded to C-5 and C-3, respectively. These correlations also made clear that C-5 was bound to C-6 and C-4, and C-3 to C-2 and C-4. The chemical shifts for C-2 and C-4 in the $^{13}$C-NMR spectrum of **1** placed the carbonyl functionalities at these positions, while C-1 was part of a carbon-carbon double bond and C-5 hydroxylated. From the spectral data it was evident that fragment 3 (ring B) partly overlapped with fragment 2, i. e. concerning C-3, C-4, C-5 and C-6. Long range C-H correlations between the resonances of H-8 and C-3 implied that C-8 is directly bonded to C-3, and C-7 is located next to C-6 because of HMBC correlations between H-6 and C-7. According to its characteristic chemical shift, C-7 ($\delta_C$ 92.7) must also bear a hydroxyl group. For fragment 4 (ring C), proton-proton couplings between H-8 and H-$9_{ax}$ proved that C-8 has to be bonded directly to C-9. The latter carbon is linked to C-10 based on HMBC couplings observed between $H_2$-9 and C-10. Long range correlations between $H_2$-20 and C-9 and C-10 then led to the linkage between C-10 and the hydroxy methylene functionality being established. The $^{13}$C-NMR chemical shifts of C-10 ($\delta$ 110.6, s) and C-11 ($\delta$ 69.7, t) indicated C-10 to be attached to two

and C-11 to one oxygen atom. One of these oxygens connected to C-10 proved to be part of the methoxyl group C-21, based on the diagnostic long range correlation observed between the resonances of $H_3$-21 and C-10. Subsequently, C-10 and C-11 were concluded to be connected via an ether bridge. Finally, long range correlations observed between the resonances of H-11 and C-7 showed C-11 to be attached directly to C-7, completing ring C. Applying the ACD / Chem Sketch software, the calculated $^{1}$H- and $^{13}$C-NMR shifts for fragments 2, 3 and especially 4 were found to be in good accordance to the experimental data and strengthened the suggestion of an ether functionality between C-10 and C-11. Fragments 1 to 4 were then connected making use of further HMBC correlations. Heteronuclear couplings between H-6 and C-12 and between H-13 and C-1 proved that fragment 1 is attached to fragment 2 via C-1. Extension of fragment 2 was achieved by connecting C-6 to C-7 and C-3 to C-8 (shown in fragment 3), and results in a bridged ring system composed of rings A and B. Fragments 3 and 4 must then be linked via C-7 and C-8, since these carbons are common to both substructures. Further proof for this structural assignment came from long range $^{1}$H-$^{1}$H spin couplings between $H_2$-11 and H-6 and between $H_3$-18 and $H_2$-9.

| Position | $\delta_C$ in ppm | $\delta_H$ in ppm, mult., *J* (Hz) | $^1H$-$^1H$ COSY-Correlations | HMBC-Correlations | NOE-Correlations |
|---|---|---|---|---|---|
| 1 | 112.3 C | | | | |
| 2 | 199.0 C | | | | |
| 3 | 64.1 C | | | | |
| 4 | 210.9 C | | | | |
| 5 | 74.7 C | | | | |
| 6 | 49.1 CH | 3.38, s | | 1, 2, 4, 5, 7, 8, 12 | $11_{eq}$, 13, 19 |
| 7 | 92.7 C | | | | |
| 8 | 50.2 CH | 2.56, d, 10.7 | $9_{ax}$ | 2, 3, 4, 9, 10, 11 | $9_{eq}$, $11_{ax}$, 18 |
| 9 | 38.1 $CH_2$ | ax: 2.64, dd, 10.7, 13.6 | 8, $9_{eq}$ | 3, 7, 8, 10 | 8, $9_{eq}$, $20_a$ |
| | | eq: 1.75, d, 13.6 | 8, $9_{ax}$ | 3, 7, 8, 10 | 8, $9_{ax}$, 18 |
| 10 | 110.6 C | | | | |
| 11 | 69.7 $CH_2$ | ax: 4.35, d, 11.8 | 6, $11_{eq}$ | 7, 8 | 8, $11_{eq}$, 21 |
| | | eq: 3.71, d, 11.8 | $11_{ax}$ | | 6, $11_{ax}$ |
| 12 | 167.8 C | | | | |
| 13 | 120.4 CH | 6.57, d, 15.0 | 14, 15 | 1, 12, 14, 15 | 6, 14, 15, 19 |
| 14 | 141.4 CH | 7.22, dd, 10.9, 15.0 | 13, 15, 16, 17 | 12, 16 | 13, 15, 16 |
| 15 | 132.1 CH | 6.38, dd, 10.9, 15.0 | 13, 14, 16 | 16, 17 | 13, 14, 16, 17 |
| 16 | 138.7 CH | 6.18, dq, 15.0, 6.8 | 13, 15, 17 | 14, 15, 17 | 14, 17 |
| 17 | 18.8 $CH_3$ | 1.80, d, 6.8 | 13, 15, 16 | 15, 16 | 15, 16 |
| 18 | 10.9 $CH_3$ | 1.10, s | 8, $9_{ax}$ | 2, 3, 4, 8 | 8, $9_{ax}$ |
| 19 | 27.0 $CH_3$ | 1.14, s | | 4, 5, 6 | 6, 13 |
| 20 | 62.7 $CH_2$ | a: 3.82, d, 10.9 | $20_b$ | 9, 10 | $9_{eq}$, $11_{eq}$, $20_b$, 21 |
| | | b: 3.40, d, 10.9 | $20_a$ | | $9_{eq}$, $11_{eq}$, $20_a$ |
| 21 | 48.8 $CH_3$ | 2.98, s | | 10 | $20_a$, $20_b$, $11_{ax}$ |
| 12-OH | | 14.20, s | | | |

**Table 4-2-2:** NMR-spectral data of **1** measured in [$D_6$]acetone at 300 and 500 MHz.

2D NOESY and selective gradient NOE data, energy-minimized 3D models and proton coupling constant analysis and calculations, as well as CD measurements were used to assign the absolute configuration. First the sorbicillin part of the molecule was investigated. The bridged ring system consisting of rings A and B is only possible if H-6 and $CH_3$-18 are disposed equatorially. NOE enhancements of H-13 and H-6 were observed by irradiation of $H_3$-19, showing the C-19 methyl group to be oriented towards the sorbyl side chain. With this information, the relative configuration of this part of the molecule was designated as 3*R**, 5*S**, 6*R**. A CD spectrum of **1** gave proof for the 3*R*, 5*S*, 6*R* absolute configuration of the molecule (Fig. 4-2-4). Characteristic Cotton effects with a minimum at 295 nm and a maximum at 335 nm were observed that corresponded well with those reported for rezishanone C (294 and 335 nm, Maskey *et al.*, 2005), which contains a similar sorbicillin-substructure to the one reported here.

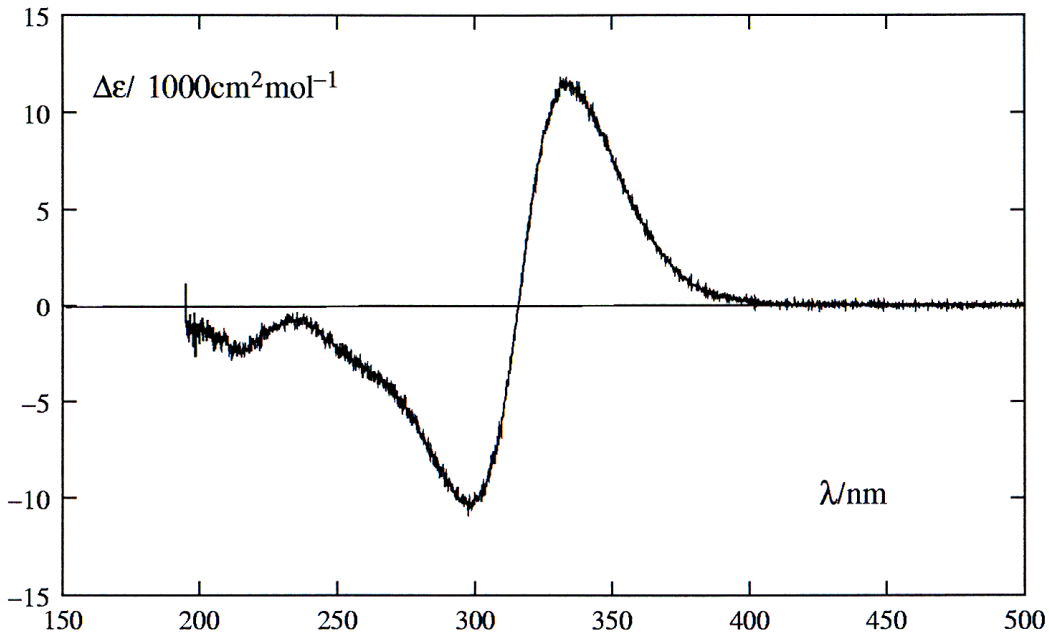
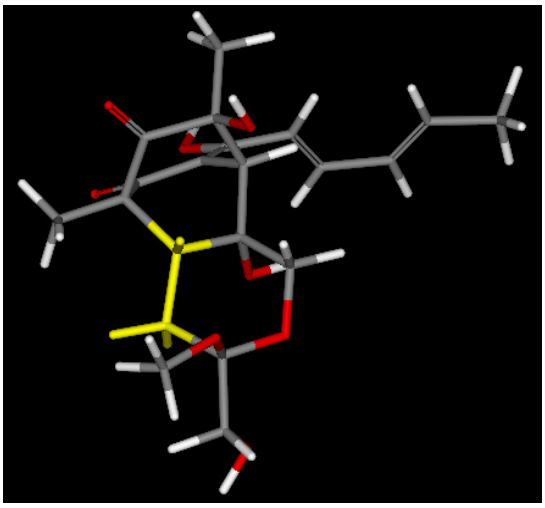



**Figure 4-2-4:** CD spectrum of compound **1** (MeOH, conc. $1.0 \times 10^{-3}$ mol $L^{-1}$).

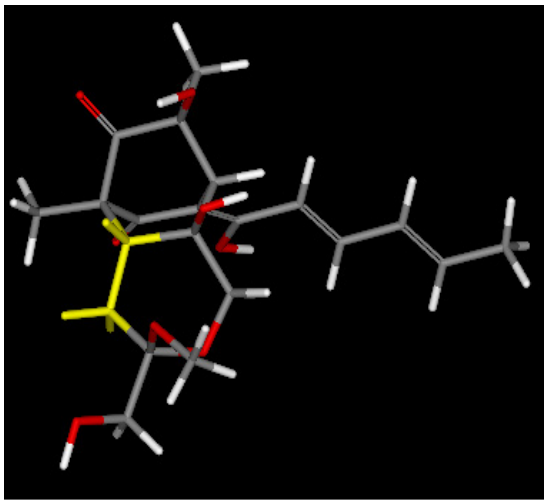
Selective NOE experiments also proved to be helpful for deducing the configuration of the non-sorbicillin part of **1**. NOE enhancements observed for H-8 and of $CH_3$-21 upon irradiation of the H-$11_{ax}$ resonance (δ 4.35) indicated the methoxyl group and H-8 to have axial orientation, and to be located on the same side of the molecule. Consequently, H-11 (δ 3.71) must have equatorial orientation, a finding that also explains the enhancement of H-6 by irradiation of H-$11_{eq}$ (δ 3.71). Ring C thus adopts a chair conformation.

Still missing at this point was information concerning the relative configuration at C-7. Furthermore, the relative configuration of ring A (C-3, C-5 and C-6) could not yet be related to that of ring C (C-7, C-8, C-10). NOE experiments were not useful in this case due to the quaternary nature of C-7. It was suspected however that the conformation of ring C and thus $^{1}$H-$^{1}$H coupling constants were dependent on the configuration at C-7. The magnitude of proton-proton coupling constants deduced from $^{1}$H-NMR experiments were thus considered and compared with those obtained from molecular modelling calculations. It was clear from the large coupling constant measured for $J_{8/9ax}$ (10.7 Hz) that, according to the Karplus equation (Karplus, 1959; Karplus, 1963; Haasnot *et al.*, 1980), these two protons must have a torsion angle of close to 180°. The absence of $^{1}$H-$^{1}$H couplings between H-8 and H-9$_{eq}$ indicated the dihedral angle between these two protons to be close to 90°. Four models were calculated for compound **1** taking into account all possible configurations at C-7, C-8 and C-10; i. e. 7*R*, 8*R*, 10*S*; 7*S*, 8*R*, 10*S*; 7*S*, 8*S*, 10*R* and 7*R*, 8*S*, 10*R*, with configurations at C-3, C-5 and C-6 taken as *R*, *S* and *R*, respectively (Fig. 4-2-5).

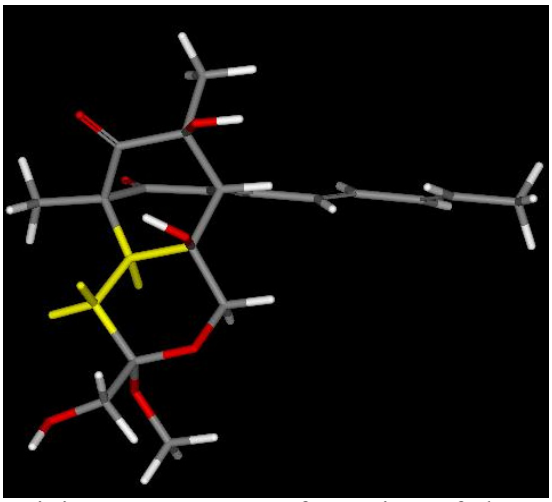
Model #4, having 7*R*, 8*S*, 10*R* configuration, was considered not appropriate, as ring C does not have the required chair conformation. When comparing the remaining models that all displayed ring C in a chair conformation, a significant difference was noted concerning the size of the dihedral angles between H-8 and H-9$_{ax}$/H-9$_{eq}$. These torsion angles were calculated for the energy minimized structures #1 to #3 and used in the Karplus equation to calculate the expected $^{1}$H coupling constants.



Minimum energy conformation of the 7*R*, 8*R* epimer of **1** (model #1)



Minimum energy conformation of the 7*S*, 8*R* epimer of **1** (model #2)



Minimum energy conformation of the 7*S*, 8*S* epimer of **1** (model #3)

Minimum energy conformation of the 7*R*, 8*S* epimer of **1** (model #4)

**Figure 4-2-5:** Minimum energy conformation of epimers of **1** (minimization program see 3.6.7).

As shown in table 4-2-3, the calculated coupling constants for the minimized 7*R*, 8*R*, 10*S* epimer corresponded best with the actual NMR values and thus led to the conclusion that **1** possesses a trans-junction between the sorbicillin part and the non-sorbicillin part of the molecule, with the hydroxyl group at C-7 having α orientation. Based on this evidence, the absolute configuration of **1** is proposed as 3*R*, 5*S*, 6*R*, 7*R*, 8*R*, 10*S*. For **1**, the trivial name trichodermanone A is proposed.

| | Torsion angle | | Coupling constant calculated (Hz) | Compound | | Coupling constant measured (Hz) |
|---|---|---|---|---|---|---|
| Model #1[a] | H-8/H-$9_{eq}$ | 78.6° | 1.2 | **1** | H-8/H-$9_{eq}$ | 0.0 |
| | H-8/H-$9_{ax}$ | 171.4° | 12.1 | | H-8/H-$9_{ax}$ | 10.7 |
| Model #2[a] | H-8/H-$9_{eq}$ | 29.3° | 8.0 | **2** | H-8/H-$9_{eq}$ | 7.3 |
| | H-8/H-$9_{ax}$ | 145.8° | 9.1 | | H-8/H-$9_{ax}$ | 9.9 |
| Model #3[a] | H-8/H-$9_{eq}$ | 62.0° | 2.7 | **3** | H-8/H-$9_{eq}$ | 7.0 |
| | H-8/H-$9_{ax}$ | 179.8° | 12.4 | | H-8/H-$9_{ax}$ | 9.9 |
| Model #4[a] | H-8/H-$9_{eq}$ | 45.3° | 5.3 | | | |
| | H-8/H-$9_{ax}$ | 160.8° | 11.3 | | | |

[a] Implied configuration for all models is 3*R*, 5*S*, 6*R*

**Table 4-2-3:** Calculated $^{1}$H-$^{1}$H coupling constants for models #1–4 and measured coupling constants for compounds **1** – **3**.

Mass spectral analysis of **2** showed it to have the same molecular formula as **1**. Analysis of its NMR data, in particular the $^{1}$H-$^{1}$H COSY and $^{1}$H-$^{13}$C HMBC spectral data, revealed **2** and **1** to have identical planar structures meaning the two molecules must differ in configuration at one or more of their chiral centers (Table 4-2-4). NOE correlations for **2** were observed between H-6, H-13 and $H_3$-19 as in **1**. A CD spectrum of **2** showed it to be almost congruent to that measured for **1**, proving the configuration in the sorbicillin part of the molecule to be the same as for **1**. Ring C of **2** must also have a chair conformation due to mutual NOE enhancements of $H_3$-21 and H-8 with $H_2$-11, respectively. Irradiation of H-8 caused enhancements of both H-$9_{ax}$ (δ 1.69) and H-$9_{eq}$ (δ 2.17), indicating that the torsion angles between H-8 and the protons at C-9 are different from those in **1**. The proton coupling constants measured for $J_{8/9ax}$ (9.9 Hz) and $J_{8/9eq}$ (7.3 Hz) confirmed this deduction. With this information in hand the measured $^{1}$H-$^{1}$H coupling constants of **2** were compared to those calculated for models #1 to #3 (Table 4-2-3). The actual coupling constants obtained by experimental measurement were almost identical to those calculated for model #2, that has a cis-junction of rings B and C, and C-7 and C-8 having *S* and *R* absolute configurations, respectively. For **2** the absolute configuration of 3*R*, 5*S*, 6*R*, 7*S*, 8*R*, 10*S*, and the trivial name trichodermanone B are proposed.

| Position | $\delta_C$ in ppm | $\delta_H$ in ppm, mult., *J* (Hz) | $^1H$-$^1H$ COSY-Correlations | HMBC-Correlations | NOE-Correlations |
|---|---|---|---|---|---|
| 1 | 111.5 C | | | | |
| 2 | 200.2 C | | | | |
| 3 | 64.5 C | | | | |
| 4 | 210.9 C | | | | |
| 5 | 74.7 C | | | | |
| 6 | 49.1 CH | 3.51, s | | 1, 2, 4, 5, 7, 8, 12 | 11, 13, 19 |
| 7 | 90.3 C | | | | |
| 8 | 49.7 CH | 2.56, dd, 7.3, 9.9 | $9_{eq}$, $9_{ax}$ | 2, 3, 4, 9, 11, 18 | $9_{eq}$, $9_{ax}$, 11, 18 |
| 9 | 40.9 $CH_2$ | ax: 2.17, dd, 9.9, 13.9 | 8, $9_{eq}$ | 3, 8, 10, 20 | 8, $9_{eq}$, 18 |
| | | eq: 1.69, dd, 7.3, 13.9 | 8, $9_{ax}$ | 3, 7, 8, 10 | 8, $9_{ax}$, 18 |
| 10 | 111.0 C | | | | |
| 11 | 68.4 $CH_2$ | 3.91, br m | $20_a$ | 6, 7 | 6, 8, 21 |
| 12 | 168.9 C | | | | |
| 13 | 120.0 CH | 6.44, d, 15.0 | 14, 15, 16 | 12, 15 | 6, 14, 15, 16, 19 |
| 14 | 142.1 CH | 7.25, dd, 11.0, 15.0 | 13, 15, 16, 17 | 12, 15, 16 | 16 |
| 15 | 132.0 CH | 6.39, dd, 11.0, 15.0 | 13, 14, 16, 17 | 16, 17 | 13, 17 |
| 16 | 139.5 CH | 6.23, dq, 15.0, 7.0 | 13, 15, 17 | 14, 15, 17 | 14 |
| 17 | 18.8 $CH_3$ | 1.86, d, 7.0 | 13, 15, 16 | 13, 15, 16 | 15, 16 |
| 18 | 11.8 $CH_3$ | 1.08, s | 8, $9_{eq}$ | 2, 3, 4, 8 | 8, $9_{eq}$ |
| 19 | 26.5 $CH_3$ | 1.14, s | | 4, 5, 6 | 6, 11, 13 |
| 20 | 62.2 $CH_2$ | a: 3.85, d, 11.0 | $20_b$ | 10 | $9_{eq}$, $20_b$, 21 |
| | | b: 3.01, d, 11.0 | $20_a$ | | $20_a$, 21 |
| 21 | 48.9 $CH_3$ | 3.20, s | | | 11, $20_b$ |

**Table 4-2-4:** NMR-spectral data of **2** measured in [$D_6$]acetone at 300 and 500 MHz.

The molecular formula and NMR data of **3** revealed it to have the same planar structure as **2** except for the functionality at C-10, being -$OCH_3$ in **2** and -OH in **3**. The $^{13}$C-NMR data for the two compounds were almost identical with the exception of the resonance frequencies associated with C-10 and C-20 (Table 4-2-5).

| Position | $\delta_C$ in ppm | $\delta_H$ in ppm, mult., *J* (Hz) | $^1$H-$^1$H COSY-Correlations | HMBC-Correlations | NOE-Correlations |
|---|---|---|---|---|---|
| 1 | 111.6 C | | | | |
| 2 | 200.0 C | | | | |
| 3 | 64.4 C | | | | |
| 4 | 211.0 C | | | | |
| 5 | 74.7 C | | | | |
| 6 | 49.6 CH | 3.37, s | 19 | 1, 2, 4, 5, 7, 8, 12 | $11_{ax}$, $11_{eq}$, 13, 19 |
| 7 | 90.3 C | | | | |
| 8 | 49.9 CH | 2.71, dd, 7.0, 9.9 | $9_{eq}$, $9_{ax}$ | 2, 3, 4, 9, 11, 18, 20 | $9_{eq}$, $11_{ax}$, $11_{eq}$, 18 |
| 9 | 40.3 $CH_2$ | ax: 2.17, dd, 9.9, 14.1 | 8, $9_{eq}$ | 3, 7, 8, 10 | 8, $9_{eq}$ |
| | | eq: 1.74, dd, 7.0, 14.1 | 8, $9_{ax}$ | 3, 6, 8, 10 | $9_{ax}$, 20 |
| 10 | 107.5 C | | | | |
| 11 | 68.2 $CH_2$ | ax: 4.28, d, 11.7 | $11_{eq}$ | 6 | 8, $11_{eq}$ |
| | | eq: 3.76, d, 11.7 | $11_{ax}$ | 6, 7, 8 | 6, $11_{ax}$ |
| 12 | 168.8 C | | | | |
| 13 | 120.1 CH | 6.45, d, 15.0 | 14, 15, 16, 17 | 12, 15 | 6, 15, 16 |
| 14 | 142.1 CH | 7.24, dd, 10.6, 15.0 | 13, 15 | 12, 16 | 16 |
| 15 | 132.0 CH | 6.36, dd, 10.6, 15.0 | 14, 16 | | 13, 14, 16, 17 |
| 16 | 139.3 CH | 6.25, dq, 15.0, 6.5 | 13, 15, 17 | 14, 17 | 14, 17 |
| 17 | 18.8 $CH_3$ | 1.85, d, 6.5 | 15, 16 | 15, 16 | 15, 16 |
| 18 | 11.0 $CH_3$ | 1.09, s | | 2, 4, 8 | 8, $9_{ax}$ |
| 19 | 26.9 $CH_3$ | 1.14, s | 6 | 2, 4, 5, 6 | 6 |
| 20 | 67.4 $CH_2$ | 3.29, s | | 10 | |

**Table 4-2-5:** NMR-spectral data of **3** measured in [$D_6$]acetone at 300 and 500 MHz.

The configuration of the sorbicillin part of the molecule was solved by taking the mutual NOE correlations between H-6, H-13 and H-19 into account, and by comparison of the CD spectra of **3** with those of **1** and **2**, and found to be as shown in **3**. The mutual NOE correlations observed between H-8 and H-$11_{ax}$ indicated ring C to have a chair conformation, similar to that found in **1** and **2**. Calculation of the $^1$H-$^1$H coupling constants for $J_{8/9ax}$ and $J_{8/9eq}$ yielded values comparable to those measured for model #2, showing that **3** has a *cis* junction between rings B and C, leading to the absolute configuration of **3** being best described as 3*R*, 5*S*, 6*R*, 7*S*, 8*R*, 10*S*. The trivial name trichodermanone C is proposed for **3**.

Mass spectral analysis of **4** showed it to have the molecular formula $C_{19}H_{20}O_7$. Extensive comparison of $^1H$, $^{13}C$, $^1H$-$^1H$ COSY, and $^1H$-$^{13}C$ HMBC NMR spectral data of **1** and **2** with those of **4** showed that the data were in good agreement concerning the sorbyl unit, and rings A and B (Table 4-2-6). Differences between the compounds were, however, evident from the spectral data associated with ring C. From $^1H$-$^1H$ COSY correlations observed between H-8 and H-9, and the HMBC couplings observed between H-9 and C-8, it was clear that C-9 had to be attached to C-8. HMBC couplings between H-9 and C-10 and C-11 enabled the C-C bond from C-9 to C-10 to be established. Linkage between C-7 and C-11 via an oxygen and the completion of ring C was probable taking into account the $^{13}C$-NMR shift of C-7 (δ 80.9, d), which clearly differs from the equivalent atom in **2** (δ 90.3, s). Comparison of the NOESY correlations and the CD spectra of **2** and **4** showed the configuration for this pair of molecules to be the same for the stereogenic centers C-3, C-5 and C-6; 3*R*, 5*S*, 6*R*. The large $^1H$-$^1H$ NMR coupling constants associated with H-8 and H-7 ($J_{H7,\ H8}$ = 10.6 Hz) showed these protons to have either a dihedral angle of about 180° or close to 0°; mutual NOESY correlations between H-8 and H-7 revealed the angle to be close to 0° meaning the junction between rings B and C had to be *cis*. In order to obtain information on the absolute configurations at C-7 and C-8, molecular modelling experiments with the two possible isomers (7*S*, 8*R* and 7*R*, 8*S*) were performed. The two models unfortunately did not show significant differences in the torsion angles for H-7 and H-8 and so the absolute configuration of **4** remains unresolved.

| Position | $\delta_C$ in ppm | $\delta_H$ in ppm, mult., *J* (Hz) | $^1H$-$^1H$ COSY-Correlations | HMBC-Correlations | NOE-Correlations |
|---|---|---|---|---|---|
| 1 | 107.8 C | | | | |
| 2 | 198.9 C | | | | |
| 3 | 64.7 C | | | | |
| 4 | 202.3 C | | | | |
| 5 | 74.5 C | | | | |
| 6 | 47.6 CH | 3.74, d, 3.7 | 7 | 1, 2, 4, 5, 7, 8, 12 | 13, 19 |
| 7 | 80.9 CH | 5.57, dd, 3.7, 10.6 | 6, 8 | 1, 3 | |
| 8 | 53.0 CH | 3.58, dd, 2.9, 10.6 | 7, 9 | 2, 3, 4, 6, 9 | 9, 18 |
| 9 | 109.6 CH | 5.75, d, 2.9 | 8 | 7, 8, 10, 11 | 8, 18 |
| 10 | 151.7 C | | | | |
| 11 | 161.0 C | | | | |
| 12 | 170.9 C | | | | |
| 13 | 120.2 CH | 6.55, d, 15.0 | 14, 15, 16 | 1, 12, 15 | 6, 15, 17 |
| 14 | 143.4 CH | 7.29, dd, 11.0, 15.0 | 13, 15 | 12, 16 | 16 |
| 15 | 132.6 CH | 6.44 dd, 11.0, 15.0 | 14, 16 | 16, 17 | 13, 17 |
| 16 | 140.1 CH | 6.26, dq, 15.0, 7.0 | 13, 15, 17 | 15, 17 | 14, 17 |
| 17 | 19.2 $CH_3$ | 1.88, d, 7.0 | 15, 16 | 15, 16 | 15 |
| 18 | 10.6 $CH_3$ | 1.19, s | | 2, 3, 4, 8 | 8, 9 |
| 19 | 23.9 $CH_3$ | 1.25, s | | 4, 5, 6 | 6 |

**Table 4-2-6:** NMR-spectral data of **4** measured in [$D_6$]acetone at 300 and 500 MHz.

The structure of compound **5** was elucidated by 1D and 2D NMR spectroscopy, LC-MS data and comparison with literature data. Rezishanone C has only been known for a few years. As the presumed precursor for the formation of the structure, ethyl vinyl ether has never been isolated as a natural product so far, **5** is discussed to be an artefact which is formed during the isolation process (Maskey *et al.*, 2005). Rezishanone is described to exhibit weak antibiotic activity against *Staphylococcus aureus* and *Bacillus subtilis*.

Investigation of NMR data and mass spectroscopy of the VLC fractions and comparison with previous work from our group also revealed the presence of the known compounds **6-10**, which have been isolated by the former Ph.D. student A. Abdel-Lateff (Fig. 4-2-6; Abdel-Lateff, 2004).

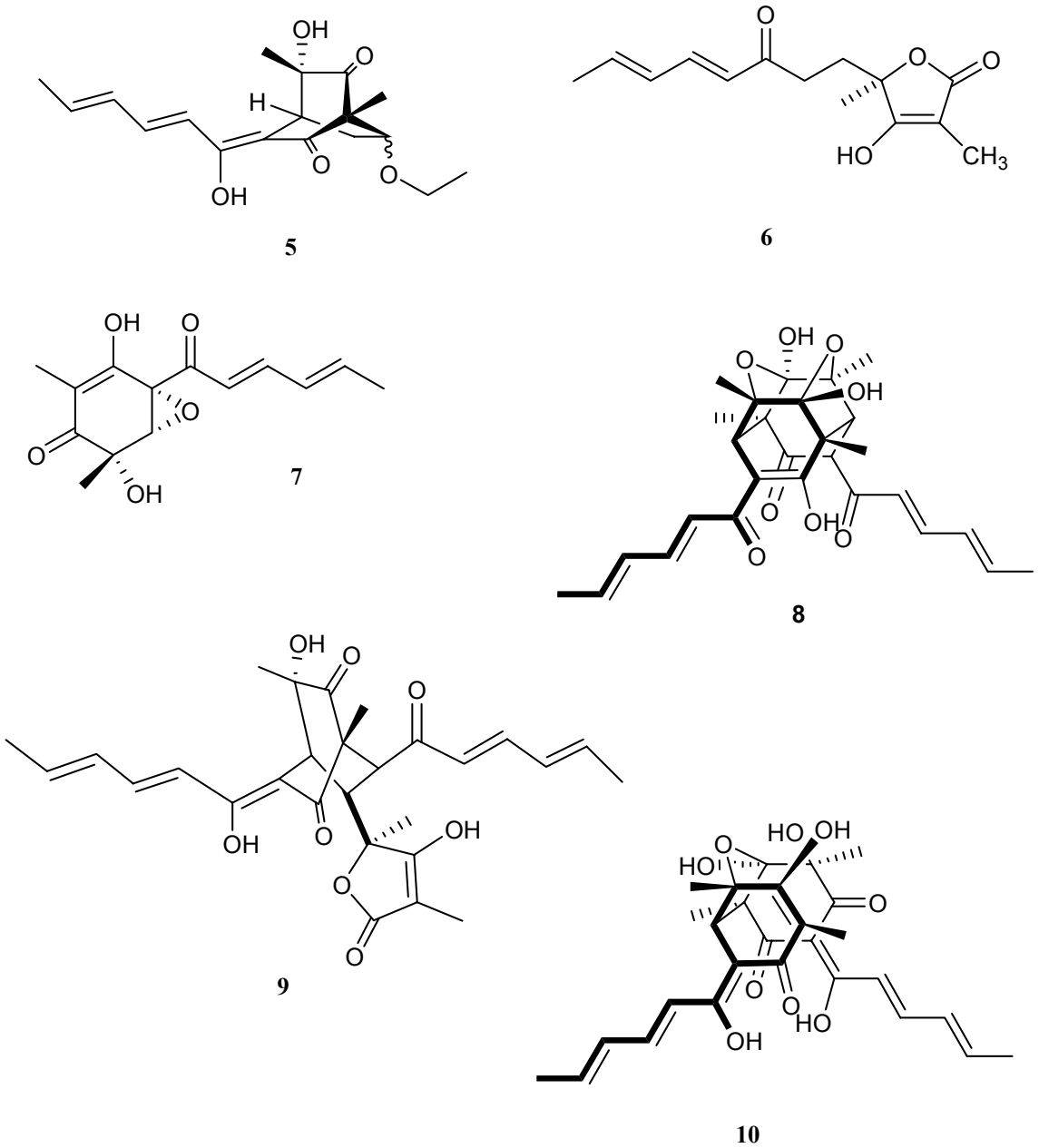



**Figure 4-2-6:** Secondary metabolites produced by *Trichoderma saturnisporium*.

Vertinolide (**6**) is the only compound isolated from *T. saturnisporium* that does not show the typical sorbicillin substructure, but possesses a cyclopentanone instead of the cyclohexanone moiety. The structure has been deduced by X-ray analysis by Trifonov and co-workers (Trifonov *et al.*, 1982).

Epoxisorbicillinol (**7**) was first isolated in 1998 from a sponge-derived *Trichoderma* species (Sperry *et al.*, 1998) and until today represents the only sorbicillin derivative possessing an epoxide function. No biological activities have been observed to date.

Trichodimerol (**8**) is probably the best investigated compound belonging to the sorbicillinoids. It was isolated for the first time by Andrade and co-workers from a terrestrial *Trichoderma longibrachiatum* (Andrade *et al*., 1992). Structure confirmation via the crystallographic analysis was published in 1995 (Gao *et al.*, 1995). Trichodermanone has demonstrated inhibition of lipopolysaccharide (LPS)-stimulated tumor necrosis factor-alpha secretion in various in vitro macrophage models (human and murine) including primary and tumor cell lines (Mazzucco & Warr, 1996).

Compound **9** (bislongiquinolide), which has been isolated from *Trichoderma longibrachiatum* as well, (Andrade *et al.*, 1997) is a bisorbicillinoid exhibiting antioxidant properties, which is very common among sorbicillin derivatives.

Bisvertinol (**10**) also represents the group of the bisorbicillinoids, characterized by the presence of two sorbicillin substructures within the molecule. It was first obtained from *Verticillium intertextum* as a yellow amorphous powder (Trifonov *et al*., 1986). As for other sorbicillinoids and bisorbicillinoids, a weak radical scavenging activity has been observed in a DPPH assay.

**Trichodermanone A (1):**

$C_{21}H_{28}O_8$ (408.18)

Yellowish viscous oil (5.2 mg); $[\alpha]^{22}_D$ = + 203.0 (MeOH, *c* 0.24).

**$^1$H- and $^{13}$C-NMR**: see table 4-2-2;

**UV** (MeOH) $\lambda_{max}$ (log ε): 360 (4.6), 248 (4.2), 203 (4.2) nm;

**CD** (MeOH) Δε= 335 (+ 1.990), 293 (- 1.945);

**IR** (film): ν = 3315, 2920, 2851, 1732, 1630, 1602, 1565, 1455, 1381 cm$^{-1}$;

**EIMS** m/z (% rel int), 408 (10) $[M]^{\bullet+}$, 376 (100), 101 (80), 95 (40), 59 (90);

**Cytotoxicity assay:** not active;

**Agar diffusion assay:** not active;

**Acetylcholine esterase assay:** not active;

**Trypsine inhibition assay:** not active;

**Phosphatase assays:** not active;

**Trichodermanone B (2):**

$C_{21}H_{28}O_8$ (408.18)

Yellowish viscous oil (14 mg); $[\alpha]^{22}_D$ = + 251.0 (MeOH, *c* 0.2).

**$^1$H- and $^{13}$C-NMR**: see table 4-2-4;

**UV** (MeOH) $\lambda_{max}$ (log ε): 358 (4.7), 248 (4.2), 202 (4.4) nm;

**CD** (MeOH) Δε = 348 (+5.436), 310 (-6.950);

**IR** (film) ν = 3393, 2938, 1733, 1630, 1600, 1561, 1383 cm$^{-1}$;

**EIMS** m/z (% rel int), 408 (22) $[M]^{\bullet+}$, 376 (80), 359 (50), 305 (20), 287 (28), 101 (82), 95 (70), 59 (76);

**Cytotoxicity assay:** not active;

**Agar diffusion assay:** not active;

**Acetylcholine esterase assay:** not active;

**Trypsine inhibition assay:** not active;

**Phosphatase assays:** not active;

**Trichodermanone C (3):**

$C_{20}H_{26}O_8$ (394.16)

Yellowish viscous oil (11.9 mg); $[\alpha]^{22}_D$ = + 265.7 (MeOH, *c* 0.5).

**$^1$H- and $^{13}$C-NMR:** see table 4-2-5;

**UV** (MeOH) $\lambda_{max}$ (log ε): 360 (4.3), 248 (3.8) nm;

**CD** (MeOH) Δε = 333 (+ 11.678), 297 (- 10.631);

**IR** (film) ν = 3330, 2937, 1731, 1627, 1597, 1556, 1380 cm$^{-1}$;

**EIMS** m/z (% rel int), 394 (20) $[M]^{\bullet+}$, 376 (100), 305 (22), 287 (20), 101 (37), 95 (65), 59 (37);

**Cytotoxicity assay:** not active;

**Agar diffusion assay:** not active;

**Acetylcholine esterase assay:** not active;

**Trypsine inhibition assay:** not active;

**Phosphatase assays:** not active;

**Trichodermanone D (4):**

$C_{19}H_{20}O_7$ (360.13)

Yellowish viscous oil (2 mg); $[\alpha]^{22}_D$ = + 51.5 (MeOH, *c* 0.08).

**$^1$H- and $^{13}$C-NMR:** see table 4-2-6;

**UV** (MeOH) $\lambda_{max}$ (log ε) 360 (4.3), 250 (4.2), 204 (4.1) nm;

**IR** (film) ν = 3383, 2923, 2359, 1729, 1599, 1404 $cm^{-1}$;

**ESIMS**: positive ion m/z 361 $[M + H]^+$, negative ion m/z 359 $[M – H]^+$;

**Rezishanone C** (**5**):

$C_{18}H_{24}O_5$ (320.38)

Yellow amorphous powder (24 mg);

**$^1$H-NMR [300 MHz, $(CD_3)_2CO$]:** δ = 1.06 (3H, dd, *J* = 6.9, 8.5 Hz, $H_3$-10), 1.15 (3H, s, $H_3$-17), 1.19 (3H, s, $H_3$-18), 1.64 (1H, m, H-2a), 1.85 (3H, d, *J* = 6.9 Hz, $H_3$-16), 2.87 (1H, d, *J* = 8.1 Hz, H-2b), 3.24 (1H, s, H-3), 3.34 (1H, m, H-9a), 3.56 (1H, m, H-9b), 3.62 (1H, t, *J* = 8.1 Hz, H-1), 6.23 (1H, m, *J* = 6.9, 14.5 Hz, H-15), 6.39 (1H, dd, *J* = 10.9, 14.5 Hz, H-14), 6.52 (1H, d, *J* = 14.9 Hz, H-12), 7.24 (1H, dd, *J* = 10.9, 14.9 Hz, H-13);

**$^{13}$C-NMR [75.5 MHz, $(CD_3)_2CO$]:** δ = 9.5 (q, C-18), 15.4 (q, C-10), 18.8 (q, C-16), 24.0 (q, C-17), 31.1 (t, C-2), 40.9 (d, C-3), 65.6 (t, C-9), 68.0 (s, C-6), 74.2 (s, C-8), 79.1 (d, C-1), 112.1 (s, C-4), 119.7 (d, C-12), 132.0 (d, C-14), 139.1 (d, C-15), 142.0 (d, C-13), 166.9 (s, C-11), 198.0 (s, C-5), 209.4 (s, C-7);

**ESIMS:** positive ion m/z 321 $[M + H]^+$, negative ion m/z 319 $[M - H]^+$;

**Agar diffusion assay:** not active;

**Literature:** Maskey *et al*., 2005

Compounds **6-10** have been described by Abdel-Lateff (Abdel-Lateff, 2004).

### 4.2.3 *In silico* screening of the trichodermanones (1-4)

In order to identify the possible biological activity of the trichodermanones (**1-4**), the compounds were investigated in an *in silico* screening approach using the PASS software (Poroikov *et al.*, 2000; Poroikov *et al*., 2001). The results indicated that the probability was highest for respiratory analeptic and TNF α inhibitory activity (respiratory analeptic: Pa 0.761, Pi 0.005; TNF α: Pa 0.752, Pi 0.004; values for compound **2**).

### 4.2.4 Biological testing of the trichodermanones (1-4)

Compounds **1**–**3** were tested in variety of bioassays including agar diffusion tests for antimicrobial properties, tests for antiparasitic and cytotoxic activity, phosphatase inhibiting activity, acetylcholine esterase inhibition and trypsin inhibition. In each case no activity was observed for the tested compound. Due to the small amount of **4** obtained during isolation it could not be tested in any of the assays.

### 4.2.5 Discussion

Natural products belonging to the sorbicillin class are hexaketide-derived metabolites. All of these compounds are composed of a cyclohexanone ring to which a sorbyl, i. e. hexa-2,4 dien-one, chain is attached. Structural variations within this structural class concern the substitution of the cyclohexanone ring, which is often further methylated and hydroxylated. The sorbyl side chain, however, in most cases remains unchanged with exception of a few 2,3-dihydrosorbyl-derivatives (Maskey *et al.*, 2005). More than 30 different sorbicillin monomers have been found to date. For the recently published rezishanones it is postulated that they originate from sorbicillinol and butylvinyl or ethylvinyl ethers attached to sorbicillinol via a Diels-Alder reaction. The only known nitrogen-containing sorbicillinoids are the anti-cancer agents sorbicillactone A and B, whose biosynthesis also involves sorbicillinol, the amino acid alanine and presumably a fumaryl-related $C_4$ unit (Bringmann *et al.*, 2005). Sorbicillinol itself was also shown to be the common precursor for many dimeric sorbicillin derivatives giving rise to a high structural diversity within this group of natural products (Trifonov *et al.*, 1982). The dimers are biosynthetically generated from sorbicillinol through two ways of dimerization, i. e. Diels-Alder cycloaddition, as in the case of bisorbicillinol (Nicolaou *et al*., 1999), and Michael addition, as suggested for trichodimerol (Nicolaou *et al*., 2000).

Compounds **1**–**4**, even though sharing the basic sorbicillin part of their structures with that of other sorbicillinoids, possess an unprecedented tricyclic ring system. They are unique with regard to ring C, which may arise biosynthetically by combining a non-sorbicillinoid polyketide with the hexaketide-derived sorbicillinol. A biosynthetic pathway proposed for compounds **1**–**4** is shown in figure 4-2-7. Our proposal is similar to that of Trifonov and co-workers (Trifoniv *et al.*, 1986) for the bisvertinols concerning the first steps of biosynthesis up to the formation of sorbicillinol: In their concept all known sorbicillinoid structures derive

from 2,4-dimethyldodecyl units yielding sorbicillin, an assumption confirmed by Abe and co-workers via biosynthetic studies with labelled acetate units (Abe *et al.*, 2001). According to Trifonov and co-workers sorbicillin undergoes an epoxidation, a process also proposed as a key step in the biosynthesis of penicillic acid from orsellinic acid (Al-Rawi *et al.*, 1974). Opening of the epoxide ring would then lead to the quinol sorbicillinol. It is probable that compounds **1**–**4** were produced from sorbicillinol via a Diels-Alder cycloaddition of another polyketide, followed by cyclization and hydroxylation to give compounds **1**–**3**, or oxidation and decarboxylation to yield compound **4** (Fig. 4-2-7). This combination of two polyketide precursors for the formation of secondary metabolites is especially exciting, as this is a very rare case in nature.

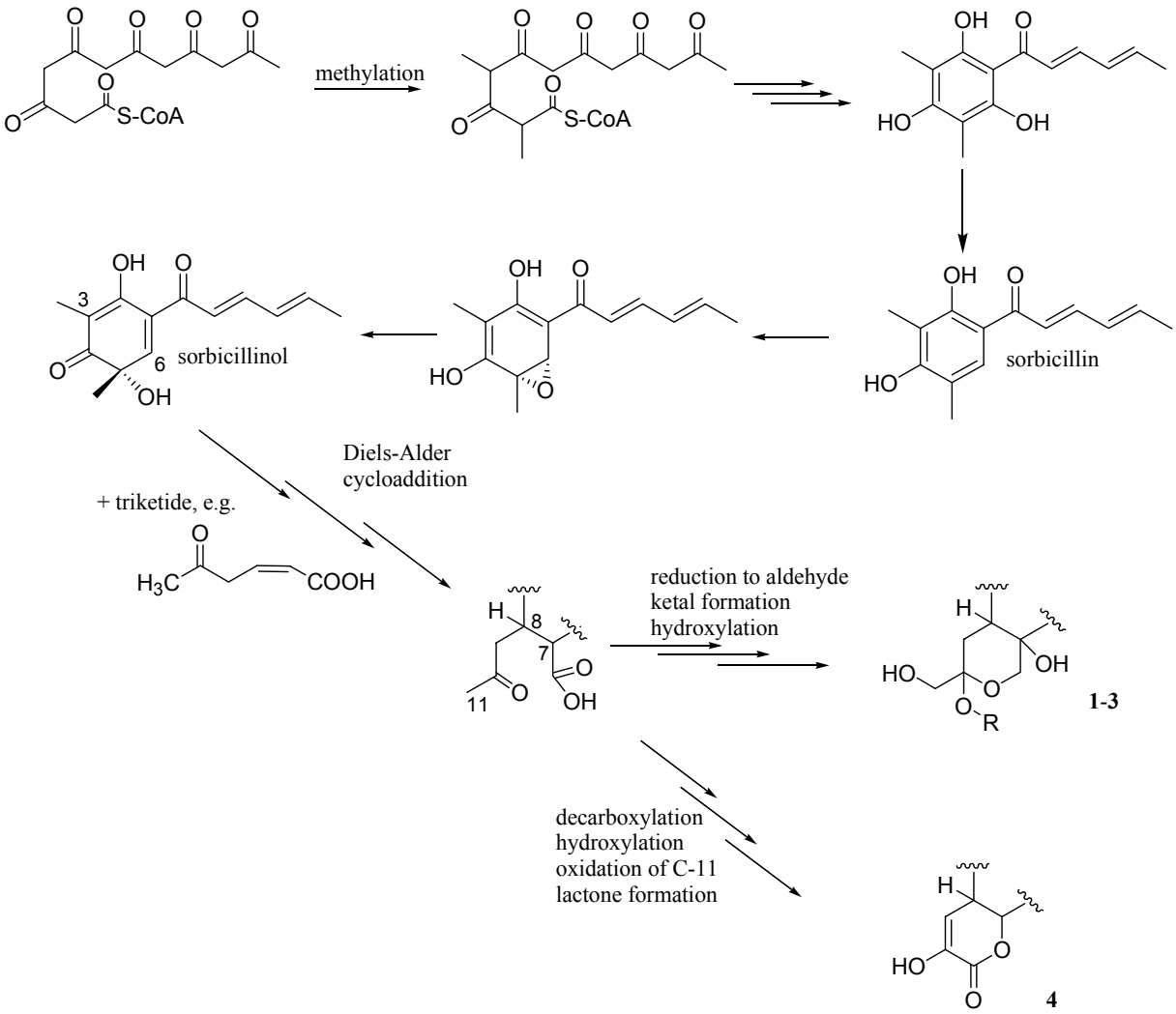


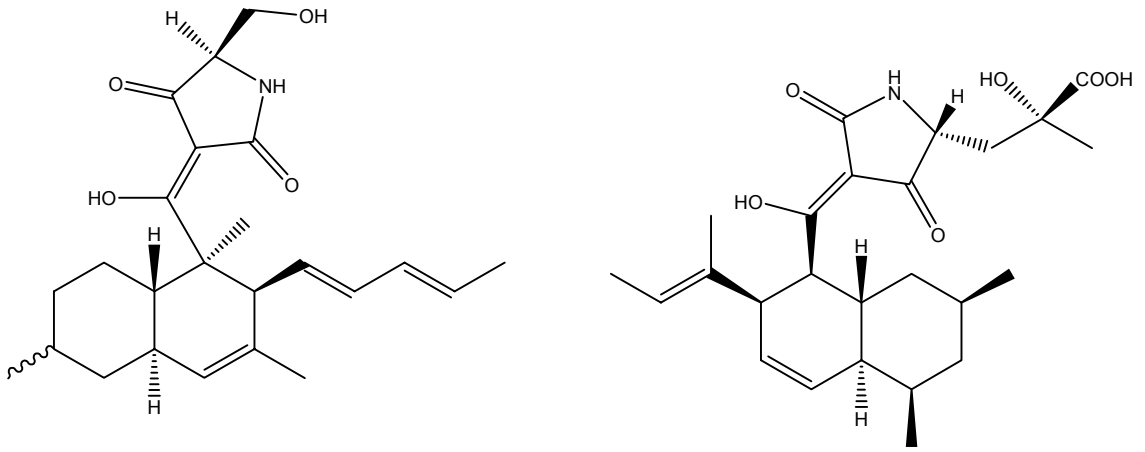

**Figure 4-2-7:** Proposed biosynthesis of the trichodermanones.

Involvement of biological Diels-Alder reactions is presumed in the biosynthesis of a variety of secondary metabolites since many years (Katayama *et al.*, 2008; and other). Among the candidates for such a biogenetic path are the alkaloid presecamines (Bazan *et al.*, 1978), the terpenoid heliocides (Stipanovic *et al*., 1977), or the solanapyrones (Oikawa, 1989).

For a few years, there have been discussions arising about the presence of enzymes or other cellular features that catalyze Diels-Alder cycloadditions in microorganisms, namely the Diels-Alderases (Katayama *et al.*, 2008; Oikawa, 2005). Indeed, the function and catalytic mechanism of natural Diels-Alderases would be of great interest due to the diversity of molecular skeletons in natural Diels-Alder adducts. The discovery of several ribozyme Diels-Alderases catalyzing such reactions has been published in the last years (Keiper *et al.*, 2004). It is thus possible that such an enzyme is involved in the biosynthesis of the trichodermanones as well. However, the possibility cannot be excluded that an enantioselective enzymatic activation of the precursor molecule sorbicillinol leads to spontaneous cyclization and forming of the compounds. Attempts isolating catalytic enzymes involved in the [4+2] addition of the second polyketide to sorbicillinol should thus be performed in order to obtain more evidence of the biosynthetic pathway of the trichodermanones.

## 4.3 *Beauveria bassiana* (strain 321) and *Microdiplodia* sp. (strain 405): Cytotoxic and HLE-inhibitory tetramic acid derivatives from marine-derived fungi

Tetramic acid derivatives are an important class of nitrogen heterocycles with a pyrrolidine-2,4-dione core structure as a key structural motif. They show a wide range of biological activities including antibiotic (Royles, 1995; Hopman *et al.*, 2002), antiviral (Karwowski *et al.*, 1992), antifungal (Wolf *et al*., 1999), cytotoxic (Lang *et al.*, 2006) and enzyme inhibitory activities against bacterial DNA-directed RNA polymerases (Reusser, 1976). Tetramic acid derivatives with an attached bicyclic hydrocarbon moiety are designated as equisetin-like. Equisetin itself has been found in a *Fusarium* species (Vesonder *et al.*, 1979) while other equisetin derivatives came from *Trichoderma* (Marfori *et al.*, 2002a), *Paecilomyces* (Lang *et al.*, 2005) or *Coniochaeta* spp. (Segeth *et al.*, 2003). In this chapter, two tetramic acid derivatives (**11**, **15**; Fig. 4-3-1) from the marine-derived fungi *Beauveria bassiana* and *Microdiplodia* sp., with cytotoxic and enzyme inhibitory (human leucocyte elastase) activity are described.




**11** **15**

**Figure 4-3-1:** Chemical structures of compounds **11** and **15**.

The fungus *Beauveria bassiana* is a well-known insect pathogenic mitosporic fungus and the most widely used mycopesticide for biological control of insect pests (Devi *et al.*, 2006). It has been shown to produce several secondary metabolites, including the antibiotic and cytotoxic cyclic peptides beauvericin (Hamill *et al.*, 1969), bassianolide (Nakajyo *et al.*, 1982) and beauveriolides (Namatame *et al.*, 1999), the antiviral 2-pyridone tenellin (Wat *et al.*, 1977) and the dibenzoquinone oosporein (Wainwright *et al.*, 1986).

Fungi of the genus *Microdiplodia* are little investigated. Some of these species are known to be plant pathogens that cause stem rot (Humphreys-Jones, 1981). Only a few secondary metabolites were isolated from *Microdiplodia* spp.. Among these are the xanthone derivatives 2-hydroxyvertixanthone, vertixanthone and barleriaquinone II, the anthraquinone chrysophanol and the steroid ergosterol (Hussain *et al.*, 2007).

During our screening for new and cytotoxic fungal metabolites, we embarked onto a study of the marine-derived fungus *B. bassiana* that was isolated from the sponge *Myxilla incrustans*, collected from the North Sea. The crude extract showed significant cytotoxicity and was thus chosen for further examination (Fig. 4-3-2).

| | | | | 1. run | | | | | | | | | 2. run | | | | | | both runs | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | cell line: Tumor Typ / Name / FU control | | | | | | | | | cell line: Tumor Typ / Name / FU control | | | | | | 1. + 2. Run | |
| | | mean | s.d. | GXF | LXFL | MAXF | MEXF | RXF | UXF | | mean | s.d. | GXF | LXFL | MAXF | MEXF | RXF | UXF | mean | tot. |
| compound | % | T/C [%] | [%] | 251L | 529L | 401NL | 462NL | 486L | 1138L | % | T/C [%] | [%] | 251L | 529L | 401NL | 462NL | 486L | 1138L | T/C | act.** |
| P321BMS | 100 | 12 | 6 | 22 | 4 | 11 | 14 | 14 | 7 | 83 | 16 | 10 | 34 | 7 | 15 | 15 | 12 | 9 | 14 | 11 |

Test/control (T/C) value smaller than 30 %, green; 30 – 50 %, yellow, higher than 50 %, red.

**Figure 4-3-2:** Screening results of cytotoxicity bioassay of the crude extract of *B. bassiana* (P321BMS). Conc. of all samples: 10 µg $mL^{-1}$.

Bioassay-guided isolation led to the discovery of a new equisetin-like tetramic acid derivative, beauversetin (**11**). To our knowledge, **11** is the first equisetin analogue isolated from a *Beauveria* sp. The structure elucidation was based on $^{1}H$- and $^{13}C$-NMR spectroscopy, mass spectrometry, UV and CD measurements. Additionally, the two known furan derivatives 5-hydroxymethyl-furan-2-carboxylic acid (**12**; Klemke, 2004) and 2-furoic acid (**13**; Hong *et al.*, 1982), and the depsipeptide beauvericin (**14**; Hamill *et al.*, 1969) were identified.

From *Microdiplodia* sp., we have isolated the tetramic acid derivative sch210972 (**15**; Yang *et al.*, 2006a). The compound showed good activity in the HLE inhibition assay with a mean $IC_{50}$ value of 1.04 µg $mL^{-1}$. Furthermore the phenolic structures 4-oxo-phenyl-butyric acid (**16**; Tomassini *et al.*, 1994) and WF-3681 (**17**; Namiki *et al.*, 1987) were identified by various spectroscopic methods. Compound **15** represents a new structural type for the development of HLE inhibitors.

Human leukocyte elastase (HLE) belongs to the chymotrypsin family of serine proteinases. HLE is located in the azurophilic granules of polymorphonuclear leukocytes and aids in the migration of neutrophils from blood to various tissues such as the airways in response to chemotactic factors (Varga *et al.*, 2003). It is suggested that HLE may play a role in several diseases, such as pulmonary emphysema (Janoff, 1985), chronic bronchitis (Llewellyn-Jones *et al.*, 1996), acute respiratory distress syndrome (Lee *et al.*, 1981), and other inflammatory diseases (Adeyemi *et al.*, 1985).

### 4.3.1 Cultivation and isolation

The sponge *Myxilla incrustans* was collected from the waters around the island of Helgoland. The fungus *Beauveria bassiana* was isolated from the sponge by the former Ph.D. student U. Höller as described in chapter 3.2.2.

The fungus *Microdiplodia* sp. was isolated from the sea weed *Enteromorpha* sp. collected from the Baltic See waters around the island of Fehmarn. *Microdiplodia* sp. was isolated from the sea weed by the former Ph.D. student C. Klemke (see 3.2.1). Briefly, sea weed sample pieces were placed on isolation agar after sterilization. Fungal colonies were transferred to a special alga agar, and pure cultures were then transferred to biomalt agar and biomalt salt agar. Agar for sporulation was used to identify the fungus.

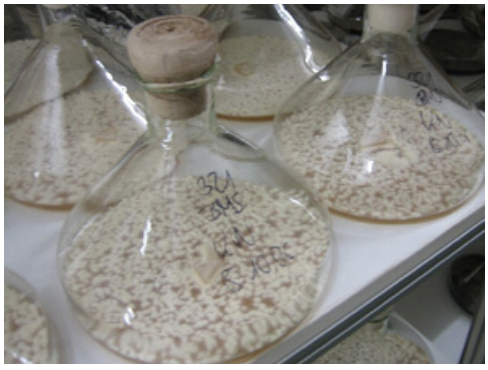


**Figure 4-3-3:** Culture of *B. bassiana* on solid biomalt-glucose medium.

*Beauveria bassiana* was cultivated at room temperature for five weeks in 11 L (44 Fernbach flasks) of solid biomalt-glucose medium (Fig. 4-3-3). Mycelia and medium were homogenized using an Ultra-Turrax. The resulting mixture was exhaustively extracted with EtOAc (3 × 7 L) and filtered. After evaporation of the organic phase 7.25 g of yellowish

oil was obtained. This was fractionated by VLC, using gradient elution from PE to EtOAc, followed by acetone and MeOH, to yield 15 fractions. Due to its promising cytotoxic activity, fraction 10 was chosen for further investigation. It was fractionated on RP-VLC, employing gradient elution from 10:90 $H_2O$/MeOH to MeOH, to yield six fractions (10A-10F). The hydrophilic fraction 10A was separated via HPLC (eluent 40:10:10 PE/EtOAc/acetone) to yield three fractions. Fraction 10A2 (30 mg) revealed to be a non-separable mixture of **12** and **13**. Observing cytotoxic activity in fractions 10 D-F, these were further examined. After purification via RP-HPLC (eluent 75:25 MeOH/$H_2O$ to 100 % MeOH in 20 min), fraction 10F yielded 2.6 mg of **11**. On the basis of TLC results, VLC fractions 5 and 6 were combined and then purified via RP-HPLC (eluent 35:65 MeOH/$H_2O$ to 75:25 MeOH/$H_2O$ in 20 min, holding steady for 10 min) to yield 20 mg of **14**. Fraction 7 was purified via RP-HPLC (eluent 65:35 MeOH/$H_2O$ to 75:25 MeOH/$H_2O$ in 10 min and to 100 % MeOH in further 20 min) and yielded further 1.6 mg of **14**.

*Microdiplodia* sp. was cultivated at room temperature for eight weeks in 10.5 L (42 Fernbach flasks) of solid malt-yeast agar medium. Mycelia and medium were homogenized with an Ultra-Turrax. The resulting mixture was exhaustively extracted with EtOAc (3 × 7 L) and filtered. After evaporation of the organic phase 5.9 g of a brown crude extract was obtained. This extract was fractionated by vacuum VLC, using gradient elution from PE to EtOAc, followed by acetone and MeOH, to yield 11 fractions. Due to moderate antibiotic activity, fraction 2 was chosen for further investigation. It was separated by NP-VLC (gradient elution from PE to EtOAc, followed by MeOH) and yielded 10 fractions (C2.1-C2.10). Fraction C2.2 was further separated via a Sephadex LH-20 column (eluent MeOH) to give 6 fractions. Of these, Fraction C2.22 and C2.23 were subjected to HPLC (eluent 40:60 $H_2O$/MeOH) for purification and yielded **15** (~100 mg) and **16** (8 mg). Fraction C2.3 also looked promising for further investigation from $^1$H-NMR spectroscopy and was subjected to a Sephadex LH-20 column, with MeOH as eluent to give further 14 fractions. Purification of fraction 2.32 via RP-HPLC (eluent 80:20 MeOH/$H_2O$) led to pure **15** (83.5 mg) while fraction 2.35 gave **17** (18 mg) after purification via RP-HPLC (eluent 60:40 MeOH/$H_2O$).

### 4.3.2 Results and Discussion

#### 4.3.2.1 Structure elucidation of tetramic acid derivatives from *B. bassiana* and *Microdiplodia* sp.

The structure of beauversetin (**11**) was determined by extensive ESI-mass spectrometry as well as one and two dimensional NMR studies, including $^{1}$H- and $^{13}$C-NMR DEPT, $^{1}$H-$^{1}$H COSY, $^{1}$H-$^{13}$C HMBC, $^{1}$H-$^{13}$C HSQC, ROESY and selective NOE spectra.

ESI mass spectral analysis indicated a molecular weight of 399, which was confirmed by high resolution mass measurement to give the molecular formula $C_{24}H_{33}NO_4$, implying 9 double bond equivalents. The $^{13}$C-NMR and DEPT spectra however, only showed 21 carbon atoms (Table 4-3-1). Four of these could be determined as methyl groups, two of which ($CH_3$-17 and $CH_3$-18) had to be bound to a double bond. The resonance signal for $CH_3$-19, showing a doublet and possessing a chemical shift of δ 0.91 in the $^{1}$H-NMR spectrum, was located on a $sp^3$ hybridized carbon and $CH_3$-12 was bounded to a quaternary carbon. Four of the $^{13}$C-NMR resonances were due to $CH_2$ moieties, ten were assigned as methines and three as quaternary carbons as indicated by the DEPT spectrum. At this point, it was anticipated that the three missing carbon resonances, according to the molecular formula were non detectable due to their quaternary nature and tautomerizations.

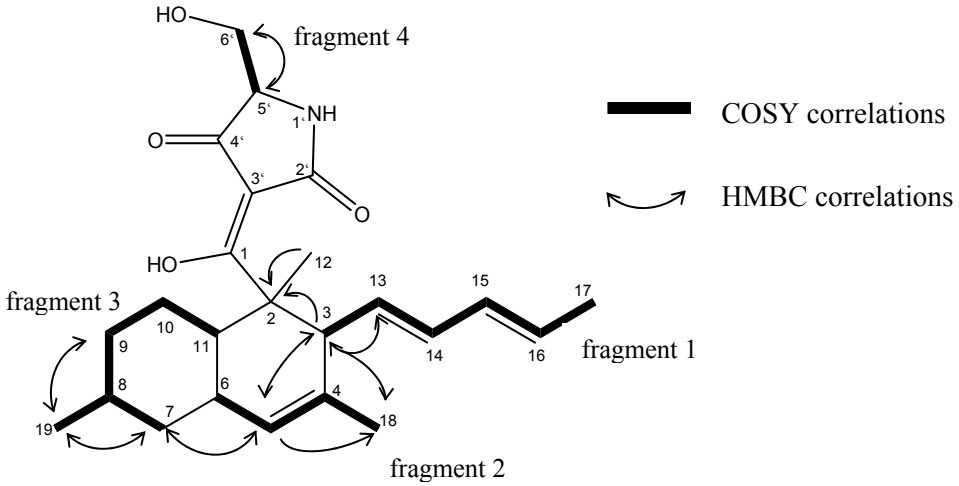



**Figure 4-3-4:** Important $^{1}$H-$^{1}$H COSY and $^{1}$H-$^{13}$C HMBC correlations for **11**.

The clear deduction of homo- and heteronuclear couplings proved to be extremely difficult due to many overlapping signals, however, the elucidation of the planar structure was possible based on a few distinctive $^{1}$H-$^{1}$H COSY and $^{1}$H-$^{13}$C HMBC correlations. Interpretation of the $^{1}$H-$^{1}$H COSY spectrum enabled us to elucidate four fragments of the structure of **11** (Fig. 4-3-4). Thus, the partial structure CH-3 to $CH_3$-17 was established due to $^{1}$H-$^{1}$H couplings from H-3 through to $H_3$-17. Connection of CH-5 and CH-6 was also evident from mutual correlations in the $^{1}$H-$^{1}$H COSY data, and H-5 additionally coupled to $CH_3$-18. Furthermore, the $^{13}$C-NMR chemical shift of C-4 ($\delta_C$ 133.5) indicated this quaternary carbon to be $sp^2$-hybridized and thus to be connected to C-5 via a double bond. C-4 is consequently placed between C-5 and $CH_3$-18, resulting in fragment 2. The third fragment, $CH_2$-7 to CH-11, was established because of $^{1}$H-$^{1}$H COSY correlations between $H_2$-7 and H-8, $CH_3$-19 to H-8, and couplings from H-8 through to H-11. The downfield shifts in the $^{1}$H-NMR spectrum for the protons at C-6' and C-5' ($\delta$ 3.58 and 3.90 for H-6' and $\delta$ 3.69 for H-5') indicated both carbons to bear a heteroatom each. Furthermore, $^{1}$H-$^{1}$H COSY and $^{1}$H-$^{13}$C HMBC correlations between CH-5' and $CH_2$-6' evidenced that CH-5' must be linked to $CH_2$-6' to form fragment 4.

Proof for the connection of the described fragments came from interpretation of $^{1}$H-$^{13}$C HMBC data. The linkage of fragments 1 and 2 was clear from HMBC correlations observed from H-3 to C-5 and C-18. HMBC long range correlations between H-$7_a$ and C-5 led to the linkage of fragments 2 and 3 via $CH_2$-7. C-2 and C-3 are connected due to an observed HMBC coupling from H-3 to C-2, and $CH_3$-12 must be linked to C-2, as interpreted from the $^{1}$H-$^{13}$C HMBC correlations for $H_3$-12 to C-2. Ring closure was done by connection of CH-11 and C-2 because of an observed ROESY correlation between H-11 and H-13, indicating a spatial closeness of these protons, suggesting a decaline system within **11**, which was secured from further HMBC, and also ROESY correlations, as well as comparison of NMR data with those of similar structures (Singh *et al.*, 1998). Thus connection of C-6 and C-11 was confirmed by comparison of the chemical shifts for these carbons with those published for phomasetin (Singh *et al.*, 1998), showing close to identical data, which makes a bicyclic decaline moiety as shown in figure 4-3-4 most likely.

| Atom number | $\delta_C$ in ppm | $\delta_H$ in ppm, mult., *J* [Hz]* | $^1$H-$^1$H COSY-Correlations | HMBC-Correlations | ROESY-Correlations |
|---|---|---|---|---|---|
| 1 | n.d. | | | | |
| 2 | 52.4 (C) | | | | |
| 3 | 48.1 (CH) | 3.93, d, 10.0 | 13, 14 | 2, 5, 12, 14, 18 | 12, 14, 18 |
| 4 | 133.5 (C) | | | | |
| 5 | 127.1 (CH) | 5.15, s | 6, 18 | 3, 18 | |
| 6 | 40.9 (CH) | 1.75* | 5 | 5, 18 | |
| 7 | 44.3 ($CH_2$) | a: 0.74, d, 12.3 | 7b, 8 | 5, 19 | |
| | | b: 1.76, d, 12.3 | 7a | | |
| 8 | 34.9 (CH) | 1.43, m | 7a, 7b, 9b,19 | 19 | |
| 9 | 37.7 ($CH_2$) | b: 0.96* | 9a | 19 | |
| | | a: 1.57* | | | 11 |
| 10 | 30.8 ($CH_2$) | a: 0.75* | 9a, 10b | | |
| | | b: 1.79* | 10a, 11 | | |
| 11 | 41.8 (CH) | 1.58* | 10b | | |
| 12 | 13.7 ($CH_3$) | 1.28, s | | 2 | 3, 6 |
| 13 | 133.7 (CH) | 5.26, dd, 10.0, 14.1 | 3, 14 | 3 | 11 |
| 14 | 132.3 (CH) | 5.82, dd, 10.0, 14.1 | 13, 15 | 16 | 3, 16 |
| 15 | 133.5 (CH) | 5.89, dd, 10.0, 14.8 | 13, 16, 17 | 13, 14 | |
| 16 | 127.6 (CH) | 5.48, dd, 6.3, 14.8 | 15, 17 | 14, 17 | 14 |
| 17 | 18.1 ($CH_3$) | 1.69, d, 6.3 | 15, 16 | | 13, 15 |
| 18 | 22.7 ($CH_3$) | 1.58, s | 3, 5, 6 | | 3, 5, 14 |
| 19 | 23.1 ($CH_3$) | 0.91, d, 6.6 | 8 | 8 | |
| 1’ | - | | | | |
| 2’ | n.d. | | | | |
| 3’ | 102.9 (C) | | | | |
| 4’ | n.d. | 3.69, dd, 5.3, 9.1 | 6’a, 6’b | 6’b | |
| 5’ | 63.2 (CH) | a: 3.90, d, 5.3 | 5’, 6’b | 5’ | |
| 6’ | 65.2 ($CH_2$) | b: 3.58, dd, 5.3, 9.1 | 5’, 6’a | 5’ | |

*multiplicities and coupling constants partly not identifiable due to overlapping signals.

n.d. = not detected

**Table 4-3-1:** NMR-spectral data of **11** measured in MeOD at 300 and 500 MHz.

Remaining to be assigned at this point were the nitrogen atom as well as the quaternary carbon resonating at δ 102.9, and three further probably quaternary carbons not detectable in the $^{13}$C-NMR spectrum. Furthermore, fragment 4 had to be incorporated into the structure of **11**. The weak $^{13}$C-NMR signal and the unusual chemical shift of C-3' ($\delta_C$ 102.9) indicated that C-3' was part of a double bond in conjugation with carbonyl or carbon-hydroxyl functionalities allowing tautomerization reactions. These features, including the presence of the nitrogen atom suggested a tetramic acid moiety with ring closure via C-5' ($\delta_C$ 63.2). C-1, the third quaternary carbon in neighbourhood to C-3', together with an attached hydroxyl group is then part of the tautomeric system neighbouring C-3', linking the tetramic acid moiety to the decaline system. This suggestion also matched with the number of double bond equivalents remaining to be accounted for and the accurate mass determined for **11**. The possibility of keto-enol tautomerization within this substructure explains the absence of signals for C-2', C-4' and C-1 and the only weak signal of C-3' in the $^{13}$C-NMR spectrum of **11**. For other "Setins" it had also been reported that $^{13}$C-NMR signals for the carbons C-2', C-4' and C-1 were non-detectable (Marfori *et al.*, 2002a; Lang *et al.*, 2005), which in a few cases could be solved by measuring the sample at -20° C, thus minimizing tautomerism reactions.

Comparison of the NMR data for **11** with those of other equisetin-like derivatives, e. g. phomasetin and equisetin, revealed almost identical spectroscopic data and thus confirmed the deduced structure for **11** (Singh *et al.*, 1998; Phillips *et al.*, 1989). Significant differences in the $^{1}$H- and $^{13}$C-NMR chemical shifts for phomasetin (Singh *et al.*, 1998) and **11** were only present for the tetramic acid fragment. Thus, taking into consideration the lack of the N-methyl signal in the $^{1}$H- and $^{13}$C-NMR spectra of **11**, the methyl group connected to the nitrogen N-1', as it is the case for phomasetin, was replaced by a hydrogen atom in **11**. This is also consistent with the mass spectral data, showing a mass reduction of 14 Da compared to the molecular weight of phomasetin.

The relative configuration of **11** was mainly determined by interpretation of the observed ROESY correlations and CD measurements. Mutual ROESY correlations between $H_3$-12 and H-3 as well as correlations from $H_3$-12 to H-6 established $CH_3$-12, H-3 and H-6 to be on the same side of the molecule (Fig. 4-3-5). H-11 is placed on the other side of the molecule, i. e. β, due to the ROESY correlations between CH-13 and CH-11, resulting in a trans-connection of the rings of the decaline moiety. The 6, 11-trans ring fusion of the decaline ring is further

supported by the absence of a cross peak between H-6 and H-11 in the ROESY experiment. Mutual ROESY correlations between H-14 and H-16, as well as between H-13, H-15 and $H_3$-17 proved the double bonds in the side chain to be E-configurated. Unfortunately, due to overlapping signals in the $^1$H-NMR spectrum, selective NOE measurements did not give any further information regarding the relative configuration at C-8. However, to date, all "Setins" reported in the literature possess the same relative configuration at C-8, with $CH_3$-19 being β and at the same side of the molecule as the proton at C-11.

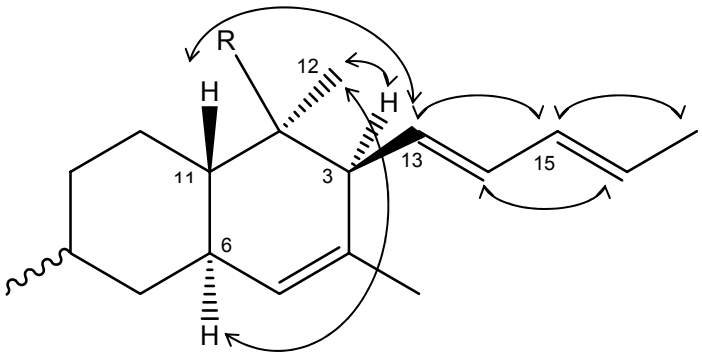



**Figure 4-3-5:** Important ROESY correlations for **11**.

Due to the lack of signals in the ROESY spectrum, the relative stereochemistry of the tetramic acid part could not be solved by NMR measurements. Thus a CD spectrum was recorded that showed Cotton effects with maxima at 201 nm (Δε = - 2.4), 236 (+ 6.9), 258 (+ 1.2) and 278 (+ 4.8). Also, the specific optical rotation (+ 122) was measured, and all chiroptical data were compared with the values reported for phomasetin and equisetin (Singh *et al.*, 1998; Phillips *et al.*, 1989). The measured data proved to be comparable to those for phomasetin, and were opposite to those of equisetin. This led to the conclusion that C-5' is most probably S-configurated, as in phomasetin (Table 4-3-2).

| $[\alpha]_D$ ($CHCl_3$) of **11** | $[\alpha]_D$ ($CHCl_3$) of phomasetin | $[\alpha]_D$ ($CHCl_3$) of equisetin | Cotton effects in the CD spectrum of **11** ($CH_3CN$) | Cotton effects in the CD spectrum of phomasetin ($CH_3OH$) | Cotton effects in the CD spectrum of equisetin ($CH_3OH$) |
|---|---|---|---|---|---|
| $[\alpha]^{25}_D$ = +122 | $[\alpha]^{22}_D$= +93.9 | $[\alpha]^{22}_D$ = -273 | Δε = 201 (-2.4) | Δε = 225 (+3.2) | Δε = 227 (-5.5) |
| (c = 0.77) | (c = 1.05) | (c = 0.77) | Δε = 236 (+6.9) | Δε = 232 (+4.4) | Δε = 235 (-7.5) |
| | | | Δε = 258 (+1.2) | Δε = 260 (+1.0) | Δε = 260 (- 3.0) |
| | | | Δε = 278 (+4.8) | Δε = 290 (+5.2) | Δε = 290 (-8.9) |

**Table 4-3-2**: Chiroptical data of compound **11** in comparison with published values of equisetin and phomasetin given in the literature.

Extensive evaluation of one and two dimensional NMR and mass spectrometric data for the *Microdiplodia*-derived compound **15** indicated the presence of a tetramic acid structural part and a hydrocarbon-type subunit and thus to possess a similar chemical structure as compound **11**. The obtained NMR data as well as the measured optical rotation ($[\alpha]^{22}_D$ = + 55.5) were thus compared with values published in the literature for other, structurally similar tetramic acid derivatives. The spectroscopic data of compound **15** revealed to be identical to those of the tetramic acid derivative sch210972 (Yang *et al.*, 2006). The structure of compound **15** is thus determined to be identical to that of sch210972.

**Beauversetin (11):**

$C_{24}H_{33}NO_4$ (399.24)

Yellow amorphous powder (2.6 mg), $[\alpha]^{22}_D$ = + 122 ($CHCl_3$, *c* 0.24).

**$^1$H- and $^{13}$C-NMR:** see table 4-3-1;

**ESI-MS:** m/z (% rel int), 400.1 (100) $[M + H]^+$, HRESIMS m/z 422.2301 $[M + Na]^+$ (calcd. for $C_{24}H_{33}NO_4Na^+$ m/z 422.2302).

**UV:** ($CH_3CN$) $\lambda_{max}$ (log ε): 285 (4.6), 235 (4.2) nm;

**CD:** ($CH_3CN$) $\lambda_{max}$ (Δε): 330 (0), 278 (+ 4.8), 258 (+ 1.2), 236 (+ 6.9), 201 (- 2.4);

***In vitro* antitumor assay:** mean $IC_{50}$ = 3.09 µg $mL^{-1}$

**Agar diffusion test:** not active

**Sch210972 (15):**

Amorphous white powder (83.5 mg); $[\alpha]^{22}_D$ = + 55.5 (MeOH, *c* 0.1)

**$^1$H-NMR [300 MHz, $(CD_3)_2CO$]:** δ = 0.84 (3H, d, $H_3$-16, *J* = 6.5), 0.91 (3H, d, $H_3$-15, *J* = 6.5), 0.98 (1H, d, H-$6_a$, *J* = 13.1), 1.39 (1H, dd, H-9, *J* = 4.4; 8.8), 1.49 (2H, s, $H_3$-14 / $H_3$-24), 1.56 (3H, s, $H_3$-12), 1.64 (1H, d, H-$8_a$, *J* = 10.8), 1.75 (1H, dd, H-$22_a$, *J* = 4.4; 10.3), 1.83 (1H, d, H-5, *J* = 10.8), 1.91 (1H, d, H-$6_b$, *J* = 13.1), 2.50 (1H, dd, H-$22_b$, *J* = 2.9; 13.1), 3.00 (1H, t, H-2, *J* = 7.7), 3.80 (1H, dd, H-20, *J* = 2.9; 10.3), 3.94 (1H, dd, H-1, 7.7; 8.8), 5.19 (1H, m, H-13), 5.66 (2H, s, H-3 / H-4);

**$^{13}$C-NMR [75.5 MHz, $(CD_3)_2CO$]:** δ = 14.3 (q, C-14), 16.6 (q, C-12), 21.9 (q, C-16), 23.2 (q, C-15), 28.2 (q, C-24), 33.8 (d, C-7), 40.4 (d, C-9), 41.7 (d, C-5), 43.1 (t, C-6), 43.2 (t, C-22), 47.1 (t, C-8), 47.4 (d, C-1), 48.9 (d, C-2), 50.3 (s, C-10), 61.4 (d, C-20), 75.7 (s, C-23), 101.9 (s, C-18), 122.7 (d, C-13), 129.9 (d, C-3), 134.9 (d, C-4), 136.7 (s, C-11), 177.1 (s, C-19), 177.8 (s, C-25), 194.0 (s, C-21), 195.4 (s, C-17);

**ESI-MS:** m/z 446 $[M + H]^+$;

**HLE inhibition assay:** active (mean $IC_{50}$ = 1.04 µg $mL^{-1}$)

***In vitro* antitumor assay:** not active

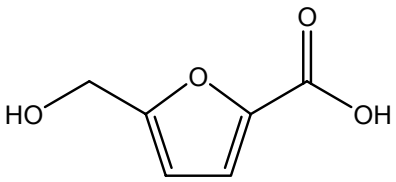
**Agar diffusion test:** moderate activity against *B. megaterium* (GI 1.5 mm)

**Literature:** Yang *et al.*, 2006.

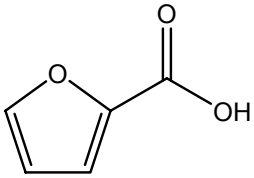
### 4.3.2.2 Other secondary metabolites from *Beauveria bassiana* and *Microdiplodia* sp.

a) *Beauveria bassiana*

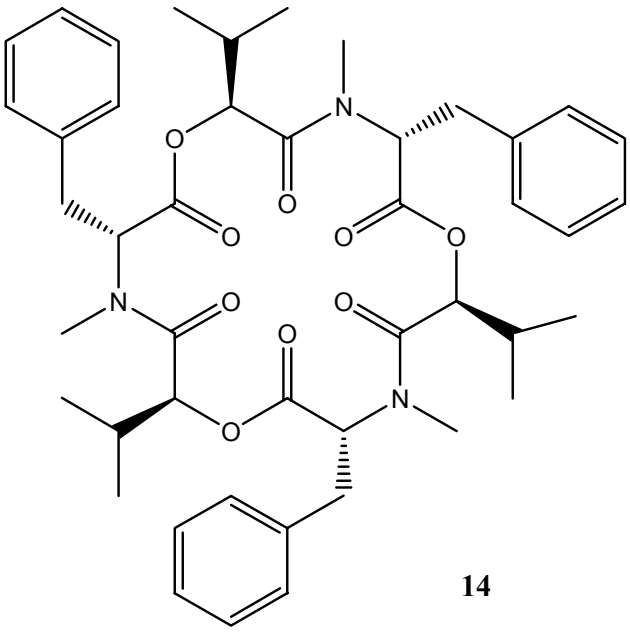
From *B. bassiana*, the two furane derivatives, 5-hydroxymethyl-furan-2-carboxylic acid (**12**) and 2-furoic acid (**13**) were elucidated by one and two dimensional NMR spectroscopy and comparison with previous results from our group (Klemke, 2004). They are very common compounds that are used in synthesis work, but have also been isolated from many microorganisms, such as *Aspergillus* spp., *Gibberella fujikuroi* or *Pseudomonas* spp. (Sumiki *et al.*, 1929; Kawarada *et al.*, 1955; and other). 2-Furoic acid has often been used in feeding experiments, serving as carbon source and precursor for secondary metabolites (Jones *et al.*, 1968; Trugdill, 1969; Kitcher *et al.*, 1970). Compound **14** (beauvericin) was first isolated by Hamill and co-workers and has been found to be a major secondary metabolite of *Beauveria bassiana* as well as several *Paecilomyces* and *Fusarium* spp. (Klaric *et al.*, 2005). The structure was determined by spectroscopic measurements and comparison with published data (Hamill *et al.*, 1969).




**12**




**13**




**14**

**Figure 4-3-6:** Structures of compounds **12-14**, isolated from *Beauveria bassiana*.

**5-Hydroxymethyl-furan-2-carboxylic acid (12):**

Colourless powder

**$^{1}$H-NMR [300 MHz, $CD_3OD$]:** δ = 4.56 (2-H, s, H-6), 6.41 (1-H, d, H-5, *J* = 3.29), 7.03 (1-H, d, H-4, 3.29);

**$^{13}$C-NMR [300 MHz, $CD_3OD$]:** δ = 57.6 (t, C-6), 110.0 (d, C-5), 117.36 (d, C-4), 158.72 (s, C-2);

**ESI-MS:** m/z 142.5

**Literature:** Klemke, 2004.

**2-Furoic acid (13):**

White crystalline powder

**$^{1}$H-NMR [300 MHz, $CD_3OD$]:** δ = 6.55 (1-H, dd, H-5, *J* = 1.46, 3.29), 7.08 (1-H, d, H-4, 3.29), 7.65 (1-H, d, H-2, 1.46);

**$^{13}$C-NMR [300 MHz, $CD_3OD$]:** δ = 112.5 (d, C-5), 116.4 (d, C-4), 145.9 (s, C-3), 146.2 (d, C-2), 157.4 (s, C-1);

**ESI-MS:** m/z 112.5

**Literature:** Hong *et al.*, 1981.

**Beauvericin (14):**

Yellow amorphous powder (21.4 mg); $[\alpha]^{22}_{D}$ = + 33.2 ($CHCl_3$, *c* 0.27; Lit: + 65.8, MeOH, *c* 0.1).

**$^{1}$H-NMR [500 MHz, $CD_3OD$]:** δ = 0.27 (9-H, d, $H_3$-15, *J* = 6.59), 0.90 (9-H, d, $H_3$-14, 6.59), 1.83 (3-H, m, H-13), 3.06 (3-H, dd, H-$11_b$ , 12.08, 14.27), 3.17 (9-H, s, $H_3$-12), 3.43 (3-H, dd, H-$11_a$, 4.39, 14.27), 4.87 (3-H, d, H-9, 8.78), 5.83 (3-H, dd, H-10, 4.39, 12.08), 7.23 (3-H, m, H-8), 7.29 (6-H, s, H-6/H-7), 7.31 (6-H, s, H-4/H-5);

**$^{13}$C-NMR [500 MHz, $CD_3OD$]:** δ = 17.1 (q, C-15), 19.1 (q, C-14), 31.2 (d, C-13), 32.2 (q, C-12), 35.4 (t, C-11), 57.8 (d, C-10), 77.2 (d, C-9), 128.0 (s, C-8), 129.7 (6-C, d, C-6/C-7), 129.8 (6-C, d, C-4/C-5), 138.1 (s, C-3), 170.9 (s, C-2), 173.1 (s, C-1);

**ESI-MS:** m/z 783.5

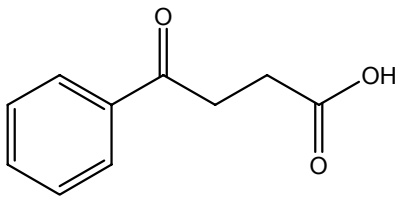
***In vitro* antitumor assay:** mean $IC_{50}$ = 3.09 µg $mL^{-1}$

**Agar diffusion test:** not active

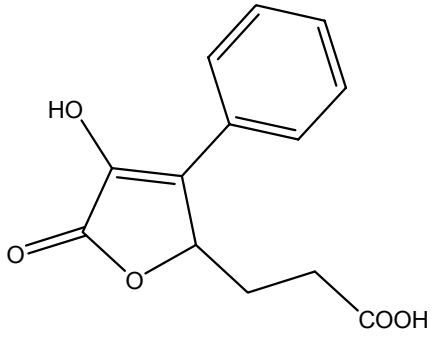
**Literature:** Hamill *et al.*, 1969.

b) *Microdiplodia* sp.

Compounds **16** and **17** have been isolated from *Microdiplodia* sp. in addition to compound **15**. From $^{1}$H- and $^{13}$C-NMR and LCMS spectral data and comparison of the obtained data with the values published in the literature, it was evident that **16** is identical with 4-oxo-phenyl-butyric acid. The structure is well known from synthesis studies (Tomassini *et al.*, 1994) and is most probably an intermediate product in fungal secondary metabolism. Compound **17** proved to be the propionic acid derivative WF-3681 (Nishikawa *et al.*, 1987; Namiki *et al.*, 1987) after interpretation of the $^{1}$H- and $^{13}$C-NMR spectral data and comparison with the published NMR values. It has first been isolated from the sphaeropsidaceous fungus *Chatomella raphigera* Swift No. 3681. **17** is optically inactive, which supports the assumption to be present as a racemic mixture made by Nishikawa and co-workers.




**16**




**17**

**Figure 4-3-7:** Secondary metabolites isolated from *Microdiplodia* sp.

**4-Oxo-phenyl-butyric acid (16):**

Yellow oil (8 mg);

**$^{1}$H-NMR [300 MHz, $(CD_3)_2CO$]:** δ = 2.76 (2-H, dd, $H_2$-2, *J* = 5.9 / 6.6), 3.18 (2-H, dd, $H_2$-3, 5.9 / 6.6), 7.56 (2-H, dd, H-7 / H-9, 7.3 / 8.4), 7.71 (1-H, d, H-8, 7.3), 8.00 (2-H, d, H-6 / H-10, 8.4);

**$^{13}$C-NMR [75.5 MHz, $(CD_3)_2CO$]:** δ = 28.2 (t, C-2), 34.2 (t, C-3), 130.2 (d, C-7 / C-9), 131.5 (d, C-6 / C-10), 133.6 (s, C-5), 135.9 (d, C-8), 174.4 (s, C-1), 193.5 (s, C-4);

**ESI-MS:** m/z 179 $[M + H]^+$;

**HLE inhibition assay:** active (mean $IC_{50}$ = 1.78 µg $mL^{-1}$)

**Agar diffusion test:** moderate activity against *B. megaterium* (GI 3 mm)

***In vitro* antitumor assay:** not active

**Literature:** Tomassini *et al.*, 1994

**WF-3681 (17):**

Amorphous yellowish powder (18 mg);

**$^{1}$H-NMR [300 MHz, $(CD_3)_2CO$]:** δ = 1.72 (2-H, m, $H_2$-5), 2.50 (2-H, m, $H_2$-6), 5.60 (1-H, dd, H-4, *J* = 1.8 / 8.8), 7.40 (1-H, m, H-11), 7.50 (2-H, m, H-10 / H-12), 7.85 (2-H, m, H-9 / H-13);

**$^{13}$C-NMR [75.5 MHz, $(CD_3)_2CO$]:** δ = 29.6 (t, C-5), 30.6 (t, C-6), 79.0 (d, C-4), 128.9 (d, C-C-9 / C-13), 130.0 (d, C-11), 130.1 (d, C-10 / C-12), 130.4 (s, C-8), 132.0 (s, C-3), 139.3 (s, C-2), 170.0 (s, C-1), 174.6 (s, C-7);

**ESI-MS:** m/z 249 $[M + H]^+$;

**Agar diffusion test:** not active

***In vitro* antitumor assay:** not active

**Literature:** Namiki *et al.*, 1987.

### 4.3.3 Biological activities

A sample of compound **11** (80 % pure) revealed moderate activity in a cytotoxicity test using a six cell line panel for a monolayer assay (mean $IC_{50}$ 3.09 µg $mL^{-1}$; Fig. 4-3-8). Cytotoxic activity for **11** is in good accordance with published data for similar structures like paecilosetin, which shows an $IC_{50}$ of 3.1 µg $mL^{-1}$ (Lang *et al.*, 2005).

IN-VITRO ANTITUMOR ACTIVITY OF MNEB433F IN HUMAN TUMOR CELL LINES (Monolayer Assay, PI)

| TUMOR/ PASSAGE NO. | EXP. NO. | CTRL FLUOR. UNITS | Distribution of IC70 related to Mean log.scaled axis *0.01 *0.1 Mean 6.220 *10 *100 | IC50 ug/ml | IC70 ug/ml | IC90 ug/ml |
|---|---|---|---|---|---|---|
| GXF 251L | IF025RT | 1439 | | 12.728 | 20.775 | 33.908 |
| LXF 529L | IG025RT | 3872 | | 2.335 | 6.965 | 21.418 |
| MAXF 401NL | IF026RT | 2338 | | 1.306 | 2.476 | 56.214 |
| MEXF 462NL | IG026RT | 2045 | | 1.828 | 3.000 | 29.999 |
| RXF 486L | IF027RT | 2557 | | 1.601 | 3.665 | 27.142 |
| UXF 1138L | IG027RT | 4421 | | 7.683 | 14.698 | 28.115 |
| Mean | | n=6 | 6.220 | 3.091 | 6.220 | 31.268 |

CHEMICAL NAME: KN321E10F

Cell line origin: GXF gastric cancer; LXF lung cancer; MAXF, mammary carcinoma; MEXF, melanoma; RXF renal cell cancer; UXF uterine cancer.

**Figure 4-3-8:** Cytotoxic activity of beauversetin in human tumor cell lines in monolayer culture.

Compound **15** was first isolated by Yang and co-workers from the fermentation broth of *Chaetomium globosum* (Yang *et al.*, 2006). It is reported to inhibit the chemokine receptor CCR-5. This receptor, belonging to the super-family of seven-transmembrane G-protein coupled receptors (GPCRs), has been identified to play an important role in viral attachment to and entry into the human host cell in the initial stage of HIV-I infection (Samson *et al.*, 1996; Berger *et al.*, 1999). Compound **15** showed good activity in the HLE inhibition assay with a mean $IC_{50}$ value of 1.04 µg $mL^{-1}$. To our knowledge, this activity is reported for the first time for a tetramic acid. Furthermore, **15** showed moderate antibiotic activity against *B. megaterium* (growth inhibition zone of 1.5 mm at a concentration of 50 µg/disk), an activity often reported for tetramic acid derivatives. The mechanism for the antibiotic activity of tetramic acids has been explained by the ability to strongly chelate biochemically

indispensable metal ions and to mimic phosphate groups in the binding site of kinases (Peikert *et al.*, 2008).

Compound **16** showed moderate antibiotic properties against *B. megaterium*. Additionally, the compound was active in the HLE inhibition assay, with an $IC_{50}$ value of 1.78 µg $mL^{-1}$.

For compound **17**, an inhibition of aldose reductase is described (Nishikawa *et al.*, 1990). Aldose reductase is an enzyme that catalyzes the conversion of glucose to sorbitol and thus plays an important role in the pathogenesis of diabetic complications. In our test systems, **17** did not show significant bioactivities.

### 4.3.4 Biosynthetic considerations for the isolated tetramic acids

The tetramic acid derivatives isolated during this study are characterized by a decaline ring system connected to a pyrrol-2,4-dione moiety and thus can be described as equisetin-like tetramic acids. Structural variations for this group of secondary metabolites mostly occur in the methylation pattern and in the length of the side chain that is connected to the decaline system. We propose that **11** as well as **15** originate from two separate biogenetic units, a polyketide part and an amino acid. Additionally, methylation reactions would form the final structure. Marfori and co-workers proposed a biogenetic pathway for trichosetin via an octaketide intermediate directly derived from eight acetate units attached by a head-to-tail fashion, which is typical for the polyketide pathway. The assumption was confirmed by incorporation studies with labelled acetate (Marfori *et al.*, 2002b). Consequently, **11** would be formed by a nonaketide, increasing the side chain by two carbon atoms to be a five-membered chain, while for **15** an octaketide as for trichosetin would be the starting point. For the formation of the tetramic acid moiety, the incorporation of an amino acid is proposed, which is also proven for the equisetin biosynthesis (Schobert, 2007). Serine would then be the precursor for the tetramic acid moiety of **11** and 4-hydroxy-4-methylglutamic acid for **15**. The methyl groups are likely to originate from the important biological methylation agent S-adenosylmethionine (SAM).

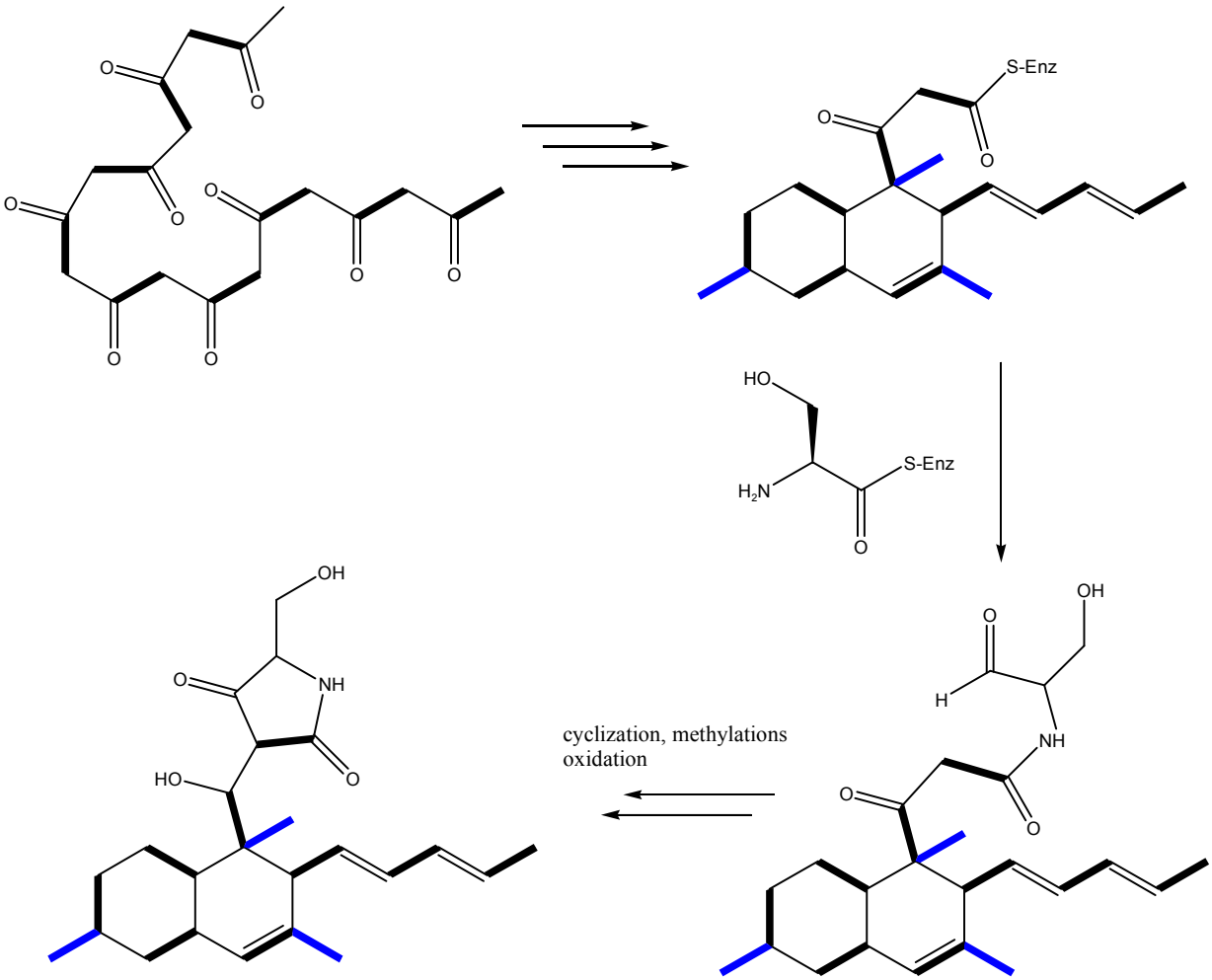



**Figure 4-3-9:** Proposed biosynthetic pathway for compound **11**. Blue: methylation via SAM.

Recently, an iterative polyketide-synthase-peptide synthetase (PKS/NRPS) hybrid gene has been identified in the genome of *Fusarium heterosporum*, the main producer of equisetin. The gene contains all necessary domains for the formation of a polyketide chain and the covalent linkage to an amino acid via an amide (Sims *et al.*, 2004). In accordance to the proposed biosynthesis for equisetin, the intermediates of compounds **11** and **15** undergo spontaneous cyclization and oxidation reactions to yield the tetramic acid, respectively (Fig. 4-3-9).

## 4.4 *Alternaria japonica* (strain 194)

Fungi belonging to the genus *Alternaria* are well-known due to their ubiquitous occurrence. They are a natural part of the fungal flora almost everywhere and known as plant pathogens. Some species however are also used as biocontrol agents against invasive plant species (Shabana *et al.*, 1996). Terrestrial as well as facultative marine species have been investigated, and the major pathogenic compounds produced by *Alternaria* spp., such as alternariol or altertoxin, are well characterized (Fehr *et al.*, 2008; Andersen *et al.*, 2008).

*Alternaria japonica* has been chosen for cultivation and bioassay-guided investigation due to its good results during the screening for cytotoxic activity and interesting $^{1}$H-NMR spectra and LC-MS data. The dominating compound produced by the investigated fungus was pachybasin (**18**, Shibata *et al.*, 1955). In minor quantities, the presence of chrysophanol (**19**, Howard & Raistrick, 1950) was observed. Both compounds are well-known anthraquinone derivatives.

| | | | | 1. run | | | | | | | | | 2. run | | | | | | both runs | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | cell line: Tumor Typ / Name / FU control | | | | | | | | | cell line: Tumor Typ / Name / FU control | | | | | | 1. + 2. Run | | |
| | | mean | s.d. | GXF | LXFL | MAXF | MEXF | RXF | UXF | | mean | s.d. | GXF | LXFL | MAXF | MEXF | RXF | UXF | mean | tot. | % |
| compound | % | T/C [%] | [%] | 251L | 529L | 401NL | 462NL | 486L | 1138L | % | T/C [%] | [%] | 251L | 529L | 401NL | 462NL | 486L | 1138L | T/C | act.** | act.* |
| P194MYA | 83 | 18 | 9 | 32 | 13 | 16 | 18 | 20 | 6 | 0 | 49 | 11 | 44 | 44 | 52 | 50 | 69 | 37 | 33 | 5 | 42 |

Test/control (T/C) value smaller than 30 %, green; 30 – 50 %, yellow, higher than 50 %, red.

**Figure 4-4-1:** Screening results of cytotoxicity bioassay of the crude extract of *A. japonica* (P194MYA). Conc. of all samples: 10 µg mL$^{-1}$.

### 4.4.1 Cultivation and secondary metabolite isolation

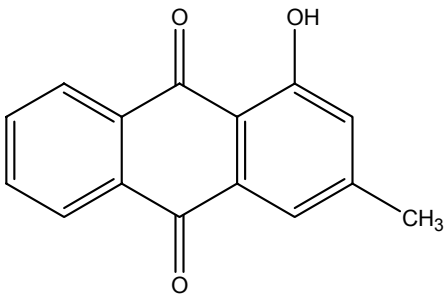
The fungus *Alternaria japonica* was isolated by the former Ph.D. student U. Höller from an unidentified alga collected from the waters around the Island of Tenerife. The isolation procedure was carried out as described in chapter 3.2.1.

The fungus was cultivated at room temperature for two months in 6.75 L (27 Fernbach flasks) of solid malt-yeast agar medium. The fungal biomass, including the medium, was homogenized and the resulting mixture was exhaustively extracted with EtOAc (3 × 5 L) and filtered. The filtrate was evaporated to yield 779.6 mg of a brown crude extract. This extract

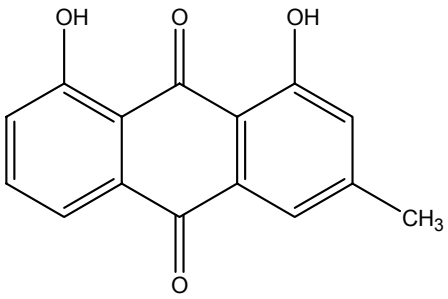
was fractionated by VLC, using gradient elution from PE to EtOAc, followed by acetone and MeOH, to yield 18 fractions. Fraction 2 revealed to be pure **18** (63.8 mg). Fraction 4 was subjected to RP-18 HPLC (eluent 50:10 MeOH/$H_2O$) and 42.3 mg of semi-pure **19**. Fractions 6 and 7 were combined and subjected to RP-HPLC (eluent 90:10 MeOH/$H_2O$ to 100 % MeOH in 15 min) to give four fractions. Structure elucidation of these fractions could not be performed due to the small amount and instability of the isolated substances.

### 4.4.2 Results and discussion

Structure elucidation of **18** and **19** was accomplished by $^{1}H$- and $^{13}C$-NMR spectroscopy, LC-MS analysis and comparison with literature values. From interpretation of the spectroscopic data, it was clear that both structures must belong to the groups of anthraquinones. Comparison of the measured values with those obtained from a literature search for anthraquinone derivatives confirmed compound **18** to be identical to pachybasin (Shibata *et al.*, 1955) and conmpound **19** to be chrysophanol (Howard & Raistrick, 1950).




**18** (pachybasin)




**19** (chrysophanol)

**Figure 4-4-2:** Chemical structures of **18** and **19**.

Pachybasin (**18**) was formerly isolated as the colouring agent of the fungus *Pachybasium candidum* by Shibata and co-workers (Shibata *et al.*, 1955) while chrysophanol (**19**) has been found in many plants as well as fungi (Howard & Raistrick, 1950). Chrysophanol is a highly active mycotoxin against powdery mildew, one of the most serious plant diseases causing

large yield losses in a number of crops (Choi *et al.*, 2004). Chrysophanol is known to act synergistically together with other anthraquinones such as physcion (Yang *et al.*, 2006b).

The high cytotoxicity found in the screening could not be recovered from the extract of the fungus. Only VLC fraction 13 showed weak cytotoxic activity with an average $IC_{50}$ value of 30.6 µg $mL^{-1}$, but was not further purified because of its small quantity (4 mg). Based on its interesting $^{1}$H-NMR data, fraction 11 was further investigated. After separation, the obtained fractions exhibited weak cytotoxicity suggesting an accumulation of potentially cytotoxic metabolites. Unfortunately, the purified compounds were not stable enough to determine their structure.

**Pachybasin (18):**

$C_{15}H_{10}O_3$ (238.28)

Yellow amorphous solid (63.8 mg);

**$^{1}$H-NMR [300 MHz, $CD_3Cl$]:** δ = 2.44 (3H, s, $H_3$-15), 7.07 (1H, s, H-6), 7.60 (1H, s, H-4), 7.78 (2H, m, H-11 / H-12), 8.26 (2H, m, H-10 / H-13), 12.54 (1H, s, OH-16);

**$^{13}$C-NMR [75.5 MHz, $CD_3Cl$]:** δ = 22.3 (q, C-15), 114.0 (s, C-2), 120.8 (d, C-4), 124.08 (d, C-6), 126.7 (d, C-13), 127.3 (d, C-10), 133.0 (s, C-9), 133.2 (s, C-14), 133.5 (s, C-7), 134.1 (d, C-11), 134.4 (d, C-12), 148.6 (s, C-5), 162.7 (s, C-3), 182.6 (s, C-8), 188.0 (s, C-1);

**ESI-MS:** m/z 255.4 $[M - H + H_2O]^-$;

**Literature:** Shibata *et al*., 1955;

**Chrysophanol (19):**

$C_{15}H_{10}O_4$ (254.24)

Yellow solid (42.3 mg), semi-pure;

**$^{1}$H-NMR [300 MHz, $(CD_3)_2CO$]:** δ = 2.49 (s, $H_3$-19), 7.13 (s, H-12), 7.21 (d, H-4, *J* = 6.6), 7.24 (t, H-5, *J* = 6.6), 7.61-7.63 (br m, H-14), 8.15 (d, H-6, *J* = 6.6);

**ESI-MS:** m/z 252.9 $[M - H]^-$;

**Literature:** Howard and Raistrick, 1950.

## 4.5 *Trichoderma harzianum* (strain 714)

Fungi belonging to the genus *Trichoderma* are very common findings in the terrestrial as well as in the marine habitat. *T. harzianum* is one of the fungal species most studied for biocontrol purposes (Hanson, 2005). The biocontrol potential is assumed to rely on the production of peptaibol molecules which, combined with the production of hydrolytic enzymes, strongly inhibit growth of fungal plant pathogens (Peltola *et al.*, 2004).

In continuation of our projects aimed at finding new natural products with biological activity and novel chemical structures from marine-derived fungi, a *Trichoderma harzianum* strain isolated from the green alga *Chaetomorpha linum* collected around the Island of Helgoland, Germany, was investigated. The crude extract showed promising cytotoxic activity, inhibiting tumor cell growth, especially the UXF (uterus carcinoma) and the MAXF (melanoma xenografts) cell lines.

| | | | | 1. run | | | | | | | | | 2. run | | | | | | both runs | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | cell line: Tumor Typ / Name / FU control | | | | | | | | | cell line: Tumor Typ / Name / FU control | | | | | | 1. + 2. Run | | |
| | | mean | s.d. | GXF | LXFL | MAXF | MEXF | RXF | UXF | | mean | s.d. | GXF | LXFL | MAXF | MEXF | RXF | UXF | mean | tot. | % |
| compound | % | T/C [%] | [%] | 251L | 529L | 401NL | 462NL | 486L | 1138L | % | T/C [%] | [%] | 251L | 529L | 401NL | 462NL | 486L | 1138L | T/C | act.** | act.* |
| P714MYA | 33 | **47** | **33** | 49 | 39 | 19 | 57 | 104 | 12 | 50 | **41** | **29** | 36 | 22 | 26 | 41 | 99 | 24 | **44** | **5** | **42** |

Test/control (T/C) value smaller than 30 %, green; 30 – 50 %, yellow, higher than 50 %, red.

**Figure 4-5-1:** Screening results of cytotoxicity bioassay of the crude extract of *T. harzianum* (P714MYA). Conc. for all samples: 10 µg $mL^{-1}$.

LC-MS spectra of the crude extract displayed series of ions at m/z $[M]^+$ 960 to 1250, indicating the presence of a peptidic mixture. Characteristic signals in the $^1$H-NMR spectrum, showing signals in the region typical for amino acid α-protons at δ 4-5 ppm underlined this assumption. Bioassay-guided isolation led to a mixture of peptaibols that are structurally similar or identical to the trichorovins (Iida *et al.*, 1995).

### 4.5.1 Cultivation and bioassay-guided isolation of secondary metabolites

The fungus *Trichoderma harzianum* was isolated from the alga *Chaetomorpha linum* by E. Eguereva. The isolation procedure was done as described in chapter 3.2.1: the alga was sterilized and then cut into pieces and placed on agar plates containing isolation medium. Fungal colonies growing out of the algal tissue were then transferred to medium for sporulation in order to enable taxonomy of the isolates. The fungal strain was identified as *Trichoderma harzianum* by the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

**Figure 4-5-2:** Culture of *T. harzianum* in Fernbach flasks.

The fungus was cultivated at room temperature for eight weeks in 10.5 L (42 Fernbach flasks; Fig. 4-5-2) of solid malt-yeast agar medium. Mycelia and medium were homogenized using an Ultra-Turrax. The resulting mixture was exhaustively extracted with EtOAc (3 × 7 L) and filtered. The filtrate was evaporated to yield 1.08 g of a brown crude extract. This extract was fractionated by VLC with a PE – EtOAc – MeOH gradient, to yield ten fractions, with fractions 8, 9 and 10 showing moderate cytotoxic activity. Fraction 8 was then separated on a Sephadex LH-20 column, with MeOH as eluent to give 14 fractions. Of these, fraction 8.3 was subjected to RP-18 HPLC employing gradient elution from 25:75 $H_2O$/MeOH to 5:95 $H_2O$/MeOH in 10 min, followed by 100 % MeOH for further 10 min, to yield six fractions. Fraction 5 ($t_R$ = 14 min) gave a mixture of peptaibols (**21**, Iida *et al.*, 1995). Fraction 4 ($t_R$ = 13.5 min) was further attempted to purify by RP-HPLC (eluent 29:71 $H_2O$/MeOH) and gave five fractions. Of these, fraction 4 showed to be a mixture of peptaibols as well (**20**).

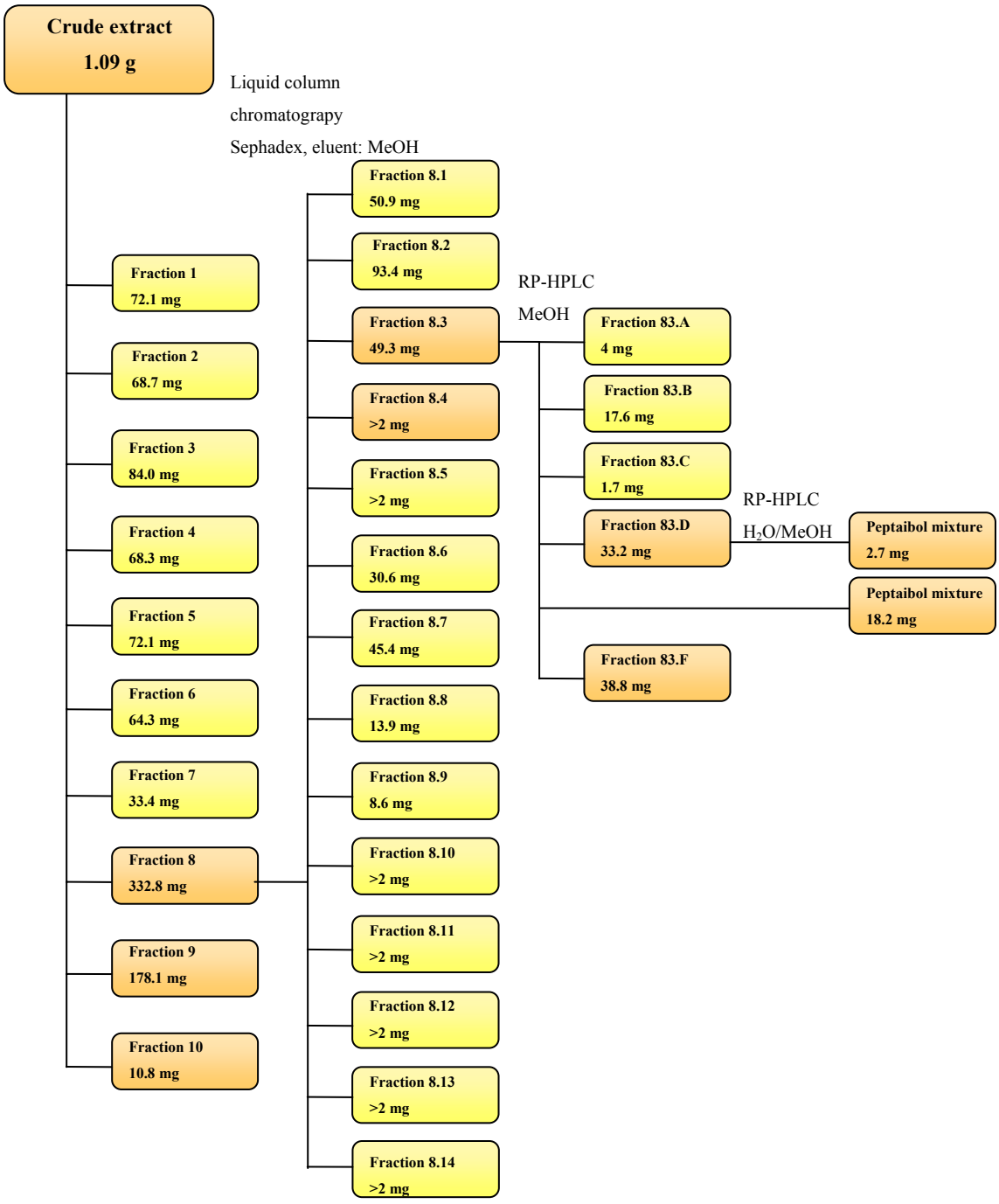



**Figure 4-5-3:** Isolation scheme for bioassay-guided isolation of **20** and **21**. Fractions showing cytotoxic activity are marked in orange.

### 4.5.2 Results and discussion

The $^{1}$H-NMR spectrum of the peptidic mixtures **20** and **21** showed several interesting resonances in the region of 2.5 ppm to 3 ppm and between 4 and 5 ppm, indicating the presence of α - and β-protons of a peptidic structure. This assumption was underlined by the $^{13}$C-NMR spectrum, showing characteristic carbonyl signals in the $\delta_C$ 170-180 ppm region, and α-carbon signals between 50 and 70 ppm. Furthermore, a signal corresponding to an acetyl group was observed in the $^{1}$H-NMR spectrum at $\delta_H$ 2.0 ppm. From LC-MS measurements, molecular mass values in the range of 960 to 1280 m/z were observed. These data suggested **20** and **21** to be mixtures of 11-residue peptides similar to the trichorovins and the trichorozins (Iida *et al.*, 1995; Wada *et al.*, 1995).

Trichorovins and trichorozins have been isolated from two *Trichoderma* species as series of peptaibols. Typical for this group of substances is the absence of free carboxyl groups and the acetylation of the N-terminal amino acids, as well as the incorporation of α-aminoisobutyric acid (Aib) residues into the molecule. Both, trichorovins and trichorozins are groups of 11-residue linear peptaibols, including 3 Aib residues at the positions 1, 5 and 9. The primary structures of the trichorovins are shown in figure 4-5-4.

1 2 3 4 5 6 7 8 9 10 11

Ac–Aib–W–X–Y–Aib–Pro–Lxx–Lxx–Aib–Pro–Z

| TV | W | X | Y | Z | m/z |
|---|---|---|---|---|---|
| I | Asn | Val | Lxx | Vol | 1147 |
| II | Asn | Val | Val | Lxxol | 1147 |
| III | Gln | Val | Val | Lxxol | 1161 |
| IV | Gln | Val | Val | Lxxol | 1161 |
| V | Asn | Val | Lxx | Lxxol | 1161 |
| VI | Asn | Val | Lxx | Lxxol | 1161 |
| VII | Asn | Lxx | Val | Lxxol | 1161 |
| VIII | Gln | Val | Lxx | Lxxol | 1175 |
| IX | Gln | Val | Lxx | Lxxol | 1175 |
| X | Gln | Lxx | Val | Lxxol | 1175 |
| XI | Asn | Lxx | Lxx | Lxxol | 1175 |
| XII | Asn | Ile | Ile | Iol | 1175 |
| XIII | Gln | Lxx | Lxx | Lxxol | 1189 |
| XIV | Gln | Lxx | Lxx | Lxxol | 1189 |

Lxx: Leu or Ile

Lxxol: leucinol or isoleucinol

Lol: leucinol; Iol: isoleucinol; Vol: valinol

**Figure 4-5-4:** Structures of trichorovins I – XIV.

The trichorovins are described in literature as a mixture of peptaibols with molecular masses between 1147 and 1187. The mass differences of 14 Da in the structural analogues stem from the replacement of leucine or isoleucine by valine or from the exchange of asparagine by glutamine. They display characteristic fragmentation patterns that result from cleavage at the unstable Aib-Pro bonds in the molecule. As shown in figure 4-5-5 for trichorovin XII, cleavage between Aib-9 and Pro-10 leads to a fragment of m/z 961. This is further fractionated into two fragments of 553 and 409 Da, respectively.

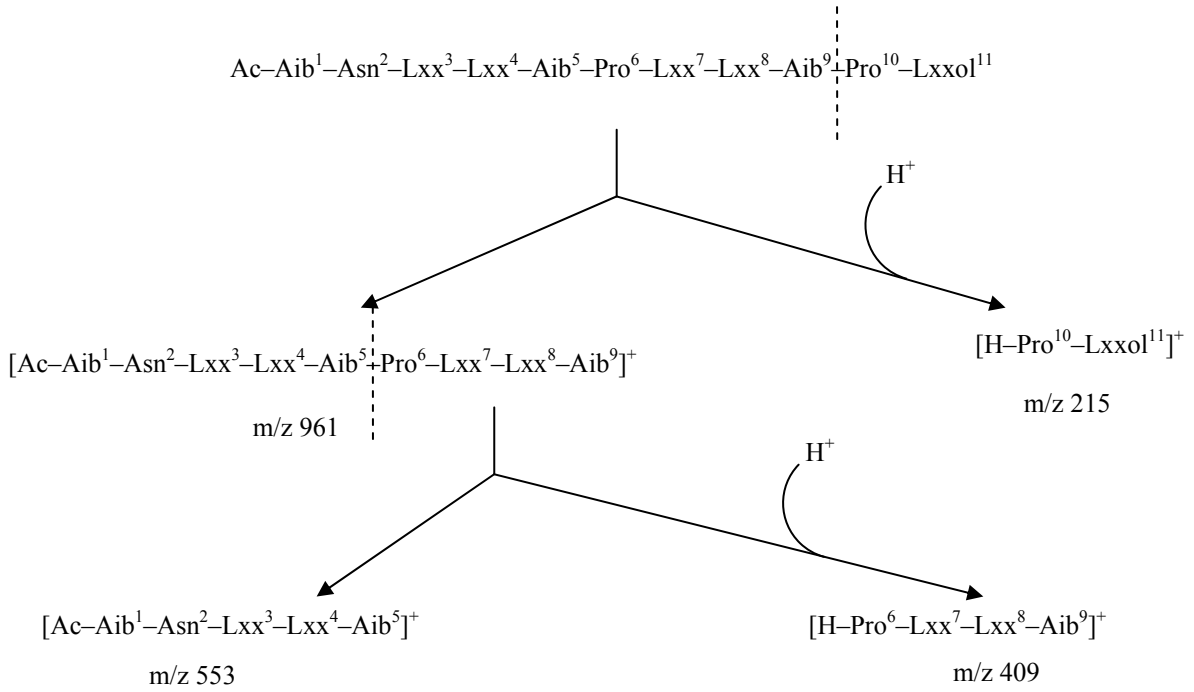
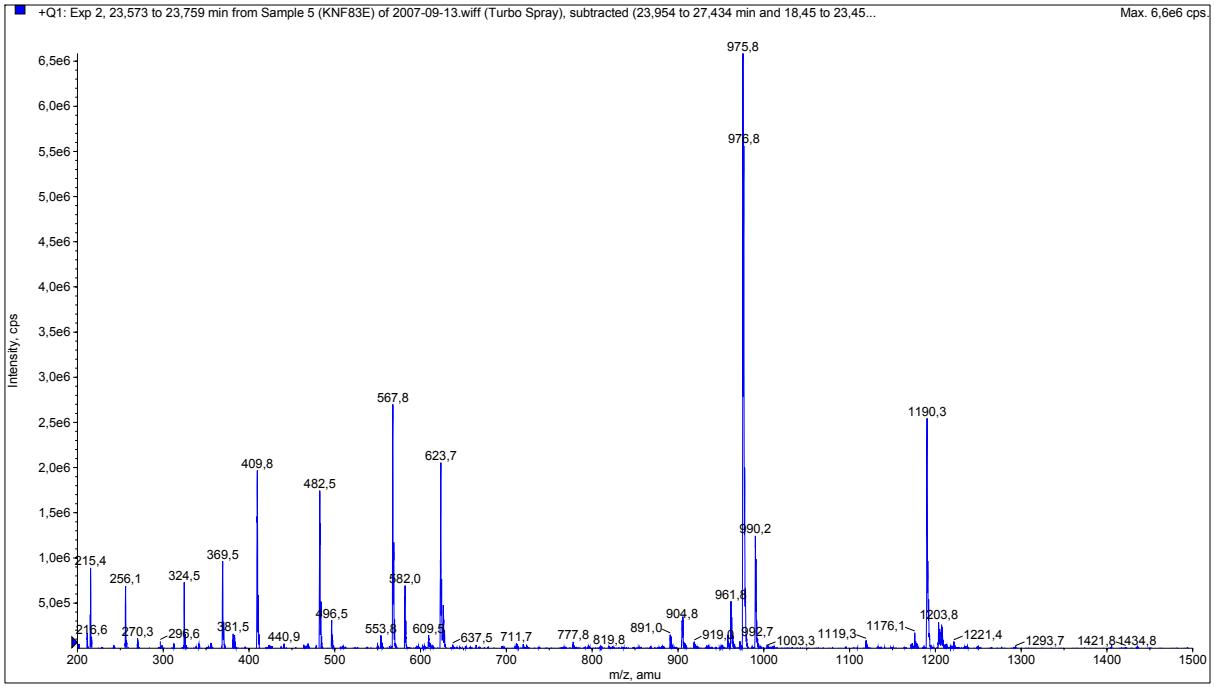



**Figure 4-5-5:** Fragmentation pattern of trichorovin XII during FAB-MS measurement (Wada *et al.*, 1995).

The ESI-MS spectrum of fraction **20** showed a series of ions at m/z 1175, 961, 553 and 409, which can be deduced from cleavage reactions between Aib and Pro as described above (Fig. 4-5-5), suggesting the presence of structures similar to those of trichorovins VIII to XII. The molecular ion of m/z 1161 could also be observed in minor quantities, indicating the presence of further trichorovins or related structures. The fragmentation pattern of **21** m/z 1189, 975, 567 and 409 shows analogy to that of trichorovins XIII and XIV. The difference of 1 Da in the molecular weight visible in the spectra is explainable by the presence of the carboxyl form of glutamine or asparagine in the structure (Fig. 4-5-6). The exact structures of the compounds in **20** and **21** should be confirmed by further investigations.

A




B

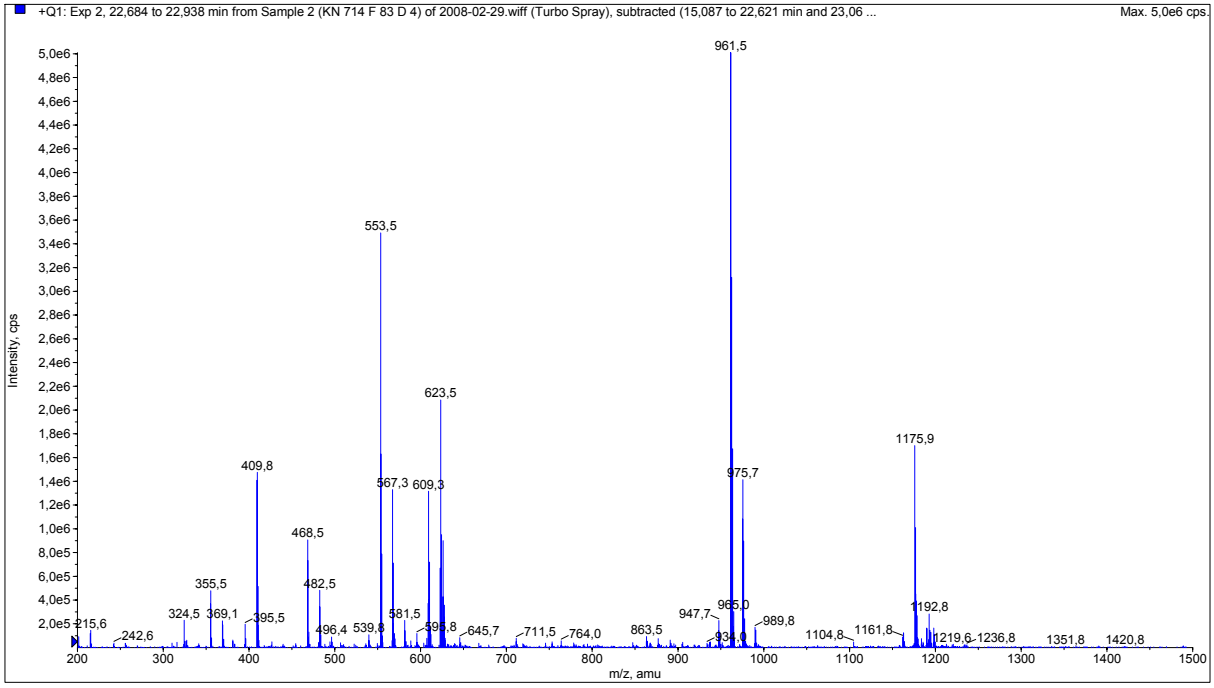



**Figure 4-5-6:** ESIMS spectra of peptaibol mixtures **20** (A) and **21** (B) in positive mode.

### 4.5.3 Biological activities

Based on the screening results, the VLC fractions obtained after separation of the crude extract were investigated for cytotoxic activity. Fractions 8, 9 and 10 showed moderate activities with mean $IC_{70}$ values in the range of 14.8 to 21.4 µg $mL^{-1}$. Bioassay-guided isolation led to the above-described mixtures of peptaibols. Fractions 83.D1 through 83.D4 were not tested due to the small amount obtained. Fraction 83.D, however exhibited an $IC_{70}$ value of 17.6 µg $mL^{-1}$ (Fig. 4-5-7). The mixture of peptaibols **21**, being identical with fraction 83.E, showed a mean $IC_{70}$ value of 6.7 µg $mL^{-1}$. Mixture **21** additionally showed moderate antifungal activity against *Microbotryum violaceum* (GI 2.5 mm, conc. 50 µg/disk).

A

IN-VITRO ANTITUMOR ACTIVITY OF MNEB551 IN HUMAN TUMOR CELL LINES (Monolayer Assay, PI)

| TUMOR/ PASSAGE NO. | EXP. NO. | CTRL FLUOR. UNITS | Distribution of IC70 related to Mean log.scaled axis *0.01 *0.1 Mean 17.576 *10 *100 | IC50 ug/ml | IC70 ug/ml | IC90 ug/ml |
|---|---|---|---|---|---|---|
| GXF 251L | IF374OT | 2906 | | 9.342 | 17.263 | 31.899 |
| LXF 629L | IG374OT | 3204 | | 7.086 | 13.095 | 24.198 |
| MAXF 401NL | IF375OT | 2769 | | 9.224 | 16.174 | 28.361 |
| MEXF 462NL | IG375OT | 2608 | | 11.595 | 17.691 | 26.992 |
| RXF 486L | IF376OT | 2394 | > | >30.000 | >30.000 | >30.000 |
| UXF 1138L | IG376OT | 2115 | | 9.003 | 15.193 | 25.641 |
| Mean | | n=6 | 17.576 | 11.141 | 17.576 | 27.729 |

B

IN-VITRO ANTITUMOR ACTIVITY OF MNEB552 IN HUMAN TUMOR CELL LINES (Monolayer Assay, PI)

| TUMOR/ PASSAGE NO. | EXP. NO. | CTRL FLUOR. UNITS | Distribution of IC70 related to Mean log.scaled axis *0.01 *0.1 Mean 6.750 *10 *100 | IC50 ug/ml | IC70 ug/ml | IC90 ug/ml |
|---|---|---|---|---|---|---|
| GXF 251L | IF374OT | 2906 | | 2.774 | 8.682 | 28.280 |
| LXF 629L | IG374OT | 3204 | | 1.745 | 3.965 | 16.010 |
| MAXF 401NL | IF375OT | 2769 | | 1.567 | 2.828 | 18.185 |
| MEXF 462NL | IG375OT | 2608 | | 2.721 | 7.159 | 19.922 |
| RXF 486L | IF376OT | 2394 | | 13.155 | 23.227 | 41.013 |
| UXF 1138L | IG376OT | 2115 | | 2.147 | 5.842 | 19.629 |
| Mean | | n=6 | 6.750 | 2.890 | 6.750 | 22.566 |

Cell line origin: GXF gastric cancer; LXF lung cancer; MAXF, mammary carcinoma; MEXF, melanoma; RXF renal cell cancer; UXF uterine cancer.

**Figure 4-5-7:** Cytotoxicity of the fractions containing mixture **20** (A), and **21** (B) in human tumor cell lines in monolayer culture.

Like other peptaibols, trichorovins were reported to exhibit antibiotic properties, caused by an ion-channel formation in membranes of mitochondria and adrenalin-containing vesicles of the sympathetic nervous system, the adrenal chromaffin cells (Mathew *et al.*, 1981, Tachikawa *et al.*, 1991). However, this activity could not be confirmed in the agar diffusion test. Iida and co-workers suggest that the channel-forming abilities of these rather short peptides may be related to a head-to-tail dimerization forming long helical rods that are long enough to span membranes (Iida *et al.*, 1995).

# 5 General discussion

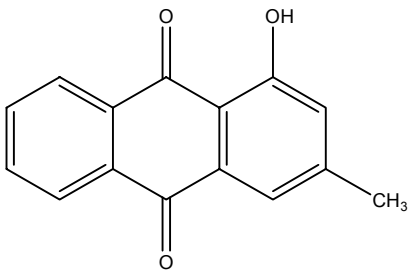
Marine fungi are recognized as a valuable source for new and bioactive secondary metabolites that have the potential to lead to innovations in drug therapy (Haefner, 2003). The purpose of this study was thus to investigate marine fungal strains towards their secondary metabolism in order to find new and preferably bioactive compounds with an emphasis on cytotoxic and antimicrobial activity. From the screening program, five fungal strains with promising cytotoxic or antimicrobial activity were cultivated on large scale, the secondary metabolites produced were chemically characterized and their potential concerning cytotoxicity and antibacterial activity was investigated using bioassay-guided isolation.

## 5.1 Exploring the chemical diversity of marine-derived fungi

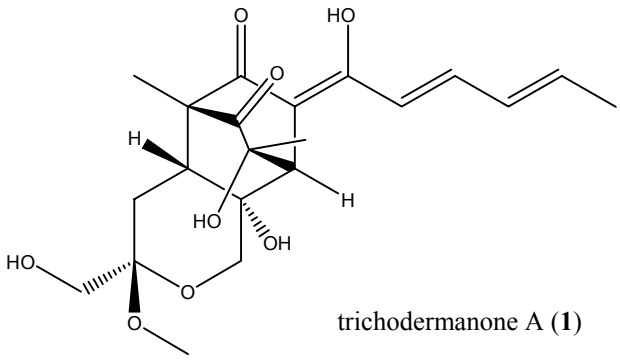
The spectrum of fungal metabolites ranges from polyketides and peptides to terpenes and heteroaromatic compounds which illustrates the extraordinary structural diversity of secondary metabolites produced by these microorganisms (Schneider *et al.*, 2008). This is impressively demonstrated by the fact that metabolites belonging to different compound classes have been found in this project, including several new structures with very unusual carbon skeletons and complex stereochemistry. Even though a remarkable number of fungal natural products is already known, the potential of these microbes is far from being fully exploited.

Investigations of fungal secondary metabolism show that the majority of the compounds produced by marine fungi belong to the class of polyketides and peptides (Keller *et al.*, 2005), which precisely reflects the results of this study. While the anthraquinone derivatives pachybasin (**18**; Shibata *et al.*, 1955) and chrysophanol (**19**; Howard & Raistrick, 1950) isolated from *Alternaria japonica* / strain 194 are rather small and simple polyketides formed by eight acetate units (see 4.4), the trichodermanones (**1-4**) found in *Trichoderma saturnisporium* / strain 54 possess a complicated, bridged ring system, most likely originating from two different polyketide units that have undergone a Diels-Alder cycloaddition (see 4.2). Peptidic structures have been found as well, e. g. beauvericin (**14**; Hamill *et al.*, 1969) in *Beauveria bassiana* (see 4.3) and peptides similar or identical to the trichorovins (**20**, **21**) in

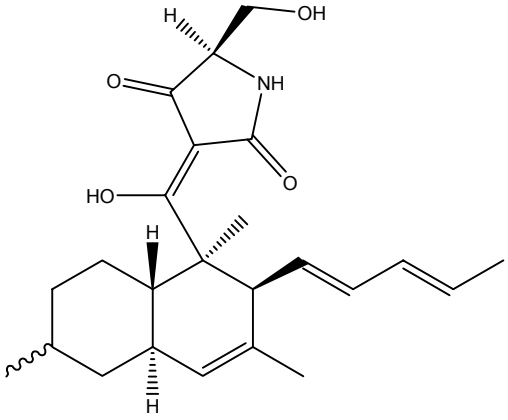
*Trichoderma harzianum* / strain 714 (see 4.5). Furthermore, tetramic acid derivatives like the newly found beauversetin (**11**) from *Beauveria bassiana* / strain 321 or sch210972 (**15**; Yang *et al.*, 2006) from *Microdiplodia* sp. / strain 405 (see 4.3) are examples for heteroaromatic, alkaloid compounds originating from two separate biogenetic units, a polyketide part and an amino acid part (Fig. 5-1-1) .



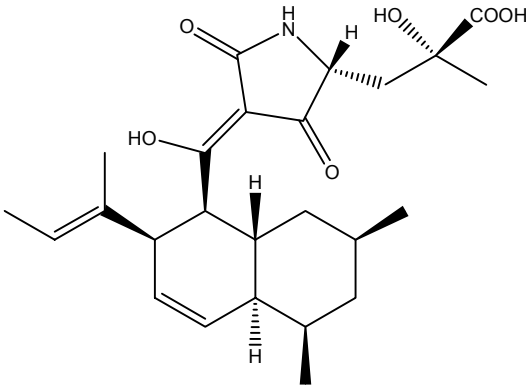

pachybasin (**18**)




trichodermanone A (**1**)




beauversetin (**11**)




sch210972 (**15**)

**Figure 5-1-1:** Examples for polyketides and PKS/NRPS derived compounds isolated during this study.

All structural classes described in this study were found in several fungal organisms. However, a single fungus is also capable of producing secondary metabolites via different biosynthetic pathways, each of which seems to be regulated depending on the nutritional offers from its surrounding and the ecological needs of the microbe, e. g. defence. Attempts to optimize culture conditions for strain 54 (see 4.2.1) clearly showed that this also applies to laboratory conditions, i. e. variation of culture media can result in a significant change of secondary metabolite production and thus in changes in the $^{1}$H-NMR spectra. In fact, it is

known that fungi possess the ability to adapt to the surrounding ecosystem in an extremely fast manner (Turner, 1971). Hence, the chemical diversity observed for fungal strains is related to a high degree to the situation the microbes grow in.

Regarding the incredible variability of the secondary metabolites produced by fungi that are known to date and the evidence for genetic regulation mechanisms (Shwab *et al.*, 2008; Stadler & Keller, 2008), it is undoubtedly the case that fungi – marine as well as terrestrial – possess the ability to produce a plethora of many more different compounds than those that are known to date.

## 5.2 Evaluation of the screening and isolation procedure – variations in fungal secondary metabolism

Bioassay-guided isolation of secondary metabolites represents a rational and time-effective tool for the discovery of new and bioactive compounds. Further advantages of this method are obvious: it prevents novel compounds from being overlooked that often remain undetected in studies where the investigator only identifies compounds he is familiar with (Duke *et al.,* 2000; Rimando *et al.*, 2001). Moreover, it can allow detecting unknown bioactivities or side effects of existing compounds or compound mixtures. Such substances or extracts may have been noted for different bioactivities before, but have not been tested for the target(s) used for bioassay-guided isolation. On the other hand, the bioassays used in this study possess a rather general validity and do not target specific functions. The monolayer tumor assay for example only considers the percentage of surviving cells and not the exact mechanism of action of the test compounds. When a specific cellular function is recognized as a target for potential therapeutics and used for screening, there are more chances for the isolation of compounds that serve as tools for biological research or lead structures for clinical medicines.

During this study, fungal strains were first cultured on a small scale for screening purposes. After extraction with EtOAc, their crude extracts were investigated by spectroscopic methods and biological assays, e. g. cytotoxic and antimicrobial tests. Those strains showing bioactivities and promising analytical data from the screening were then cultivated again on a large scale, and investigated based on the results of the screening extract. Isolation of the secondary metabolites was done bioassay-guided in the cases where the results from the screening could be reproduced during large scale cultivation.

Bioassay-guided isolation has proven to be successful for the isolation of the secondary metabolites from *Trichoderma harzianum* (see 4.5), where the causing agents of the cytotoxicity could be determined as the peptaibol mixtures **20** and **21**. However, for several fungi subjected to this study, e. g. *Microdiplodia* sp. and *Alternaria japonica*, the promising cytotoxic results from the screening could not be re-gained after large scale cultivation and fractionation of the crude extract, which made the approach of bioassay-guided isolation of cytotoxic compounds not possible. This phenomenon is frequently observed under laboratory conditions and is due to the above-mentioned adaptation of the investigated microorganisms to their surrounding conditions and the resulting change in secondary metabolism (Williams *et al.*, 2008), since culture conditions cannot be strictly controlled in the laboratory. There are several explanations for this change of metabolism, even though the exact mechanisms of how fungi regulate their secondary metabolite production are not understood in every detail yet. Fungi are extremely variable organisms – strains obtained from different sources, although appearing morphologically identical, will not necessarily behave in the same way biologically (Turner, 1971). Variation can already occur within one given strain: frequent mutations in the genome can determine a complete change in secondary metabolite production. Additionally, and presumably even more frequently, the expression of bioactive secondary metabolite genes can be downregulated when those compounds are no longer needed for survival of the organism, which is often the case under standard laboratory conditions (Casselton & Zolan, 2002). Thus, the secondary metabolites will not be produced anymore even though their genetic information is still intact. The number of putative natural product biosynthetic pathways found in fungal genomes typically exceeds the total of natural products observed under laboratory culture conditions (Williams *et al.*, 2008). Pertinent examples highlighting this phenomenon are found among *Aspergillus* spp. (Nierman *et al*., 2005), *Neurospora crassa* (Galagan *et al*., 2003), and *Magnaporthe grisea* (Dean *et al*., 2005). These biosynthetic gene clusters can be described as “silent” genes.

Evidence for the regulation of biosynthetic gene clusters on the molecular level came from studies indicating that responses to environmental signals are transmitted through pathway-specific regulatory genes; so-called narrow- and broad-domain transcription factors (Keller *et al*, 2005). Narrow pathway-specific regulators are usually found in the gene cluster and positively regulate gene expression. Those transcription factors encoded in the biosynthetic gene cluster are often $Zn(II)_2Cys_6$ zinc binuclear cluster proteins and so far only found in fungi (Proctor *et al.*, 1995; Woloshuk *et al.*, 1994; Fernandes *et al.*, 1998). Responses to environmental signals are transmitted through broad-domain factors that are often $Cys_2His_2$ zinc-finger global transcription factors mediating carbon, nitrogen or pH signalling (Tudzynski *et al.*, 1999; Ehrlich *et al.*, 2003; Tilburn *et al.*, 1995). Broad transcription factors can positively or negatively regulate secondary metabolite production and are conserved in all fungi. Regulation by both narrow- and broad-domain transcription factors ensures that secondary metabolite pathways can respond to the demands of general cellular metabolism and the presence of specific pathway inducers.

In order to keep variations of the fungal strains as small as possible and thus to obtain reproducible results, the cultivation conditions for screening and large scale cultivation have been optimized and adjusted. Nevertheless, in some cases a significant change in secondary metabolism was observed after large scale cultivation, resulting in different results from cytotoxicity tests of the crude extracts compared to the screening results. Obviously, minor and unintentional changes of the culture conditions beyond the simple fact of medium composition can be responsible for such changes, e. g. minimal light or temperature differences. This fact impressively underlines how capriciously fungal strains can behave and how fast they adapt to their surroundings.

In conclusion, bioassay-guided isolation is a valuable approach for target-oriented work, but depends heavily on the metabolic stability of the investigated organisms when they have to be re-cultured. This includes mutations, but also regulation of secondary metabolism within the microorganism. If this requirement cannot be met, other approaches for the isolation of bioactive secondary metabolites must be considered, possibly including methods of bioinformatics or molecular biology and genetics.

## 5.3 Biological activity

One of the goals of natural products research including the present study is to find substances which are suitable as lead structures in the development of drugs or crop protection products. Moreover, a characterization of the selected fungal strains in terms of chemical diversity and biological activity was intended.

One of the products isolated during this study exhibited promising bioactivities that have not been published before. The tetramic acid derivative sch210972 (**15**) from *Microdiplodia* sp., which has been published to be a chemokine receptor antagonist (Yang *et al.*, 2006) revealed a significant inhibition of human leucocyte elastase as well as antimicrobial properties against *B. megaterium* in the bioassays performed during this study. The antibiotic activity corresponds well with published data for similar structures containing the tetramic acid moiety, like tenuazonic acid (active against *B. megaterium*; Gitterman, 1965), pachydermin (mild activity against *B. subtilis*; Lang *et al.*, 2006) or ravenic acid (active against *S. aureus*; Michael *et al.*, 2004). HLE inhibition however, is reported here for the first time. Tetramic acids thus represent a new structural type for the development of HLE inhibitors. Additionally, the HLE inhibitory bioactivity further enlarges the broad spectrum of bioactivities known for tetramic acids so far.

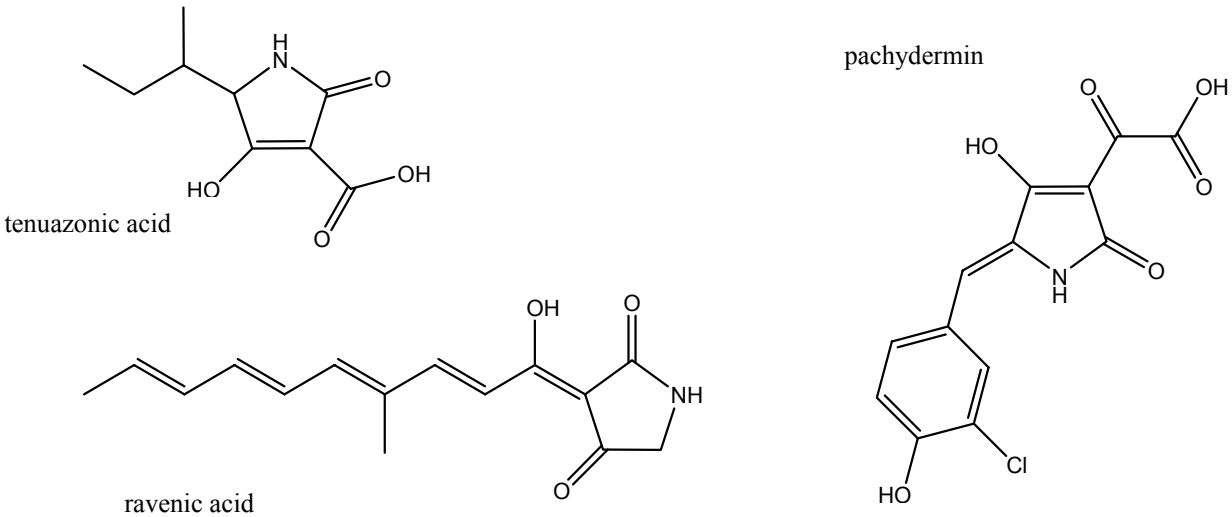



**Figure 5-1-2:** Examples for tetramic acid derivatives possessing antibiotic activities.

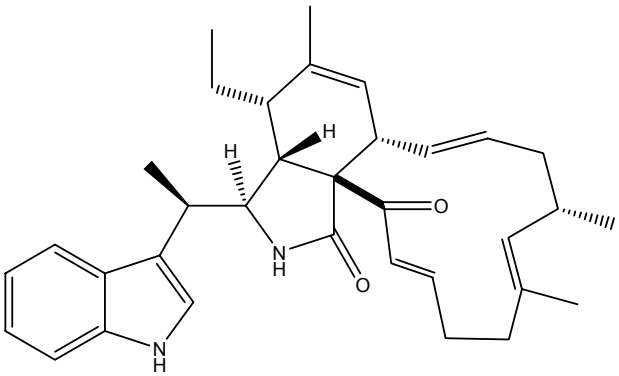
Bioassay-guided isolation of the cytotoxic crude extact of *Trichoderma harzianum* revealed the presence of cytotoxic peptaibol mixtures (see 4.5). Moreover, the new compound beauversetin (**11**), which has been isolated from a cytotoxic fraction of *B. bassiana*, is likely to possess the desired activity as well (see 4.3). Unfortunately, due to the very small amount isolated (2.6 mg) the activity could not be entirely verified. The structurally novel trichodermanones (**1-4**) elucidated during this study were tested in several bioassays, but did not show significant bioactivities (see 4.2). However, an in-silico-screening revealed the probability of the trichodermanones to possess TNF α-inhibitory potential. This should be verified in further studies.

The results obtained help to complete the chemical characterization of the investigated fungal strains. Furthermore, the bioactivity spectrum of several compounds that have been isolated from other strains before could be enlarged by the discovery of new bioactivities. Thus, even the re-isolation of already known compounds (dereplication) can be useful in order to gain more insight into natural products characteristics, e. g. their bioactivities. These results, the discovery of novel secondary metabolites and new bioactivities will lead to a better understanding of fungal secondary metabolite production.

## 5.4 The future of natural products research on marine-derived fungi

In many cases the biosynthetic capability of microbes is by far not reflected in the observed metabolite spectrum when they are cultivated under standard conditions (Bergmann *et al.*, 2007). Thus, strategies must be elaborated to explore such secondary metabolites, whose biosynthesis is encoded by "silent" genes (Williams *et al.*, 2008). While under certain laboratory conditions, fungi may produce highly cytotoxic secondary metabolites, in different surroundings (e. g. provoked by a slightly different medium composition, or simply changes of light or temperature) the secondary metabolite production can change rapidly and the resulting compounds are completely different, concerning both the structures as well as the bioactivities. This high potential of variations in secondary metabolite production among fungi, while being disadvantageous for bioassay-guided isolation, on the other hand opens new possibilities of finding new and bioactive natural products and can be used to push secondary metabolite production into a desired direction.

Based on this evidence, Zeeck and co-workers have developed the OSMAC (One Strain Many Compounds) approach, a rather undirected, but successful tool to gain access to "hidden" natural products. It is based on the alteration of cultivation conditions with the aim of achieving different secondary metabolite profiles (Bode *et al.*, 2002; Bode *et al.*, 2000). This can be the change of media components, differentiation of pH value, temperature, or duration of cultivation, but also co-cultivation with another microorganism or the addition of biosynthetic precursors or enzyme inhibitors to the growth medium. The latter was successfully realized by Crews and co-workers. Applying the OSMAC approach by incubating the fungus together with the potent F-actin inhibitor jasplakinolide as specific pathway inducer, new cytotoxic chaetoglobosins from the sponge-derived fungus *Phomopsis asparagi* have been isolated. The most potent compound, chaetoglobosin-510, disrupted actin microfilaments at 1 µg $L^{-1}$ (Christian *et al.*, 2005).



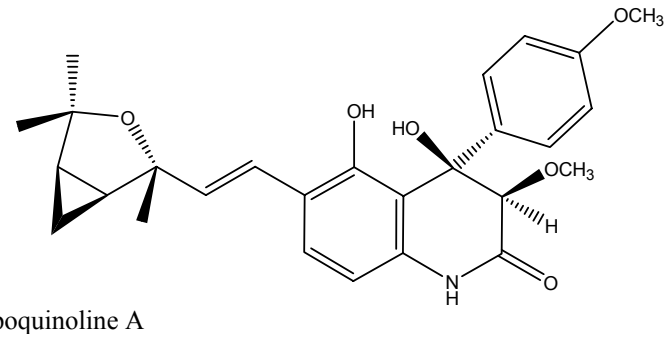

chaetoglobosin-510

**Figure 5-1-3:** The cytotoxic chaetoglobosin-510, isolated via the OSMAC approach (Christian *et al.*, 2005).

Until recently, research into fungal secondary metabolism was dominated by establishing screening programmes for chemically new and biologically active products, as was done in this study as well. Biochemical and genetic studies were generally rare due to the formidable technical challenges: the enzymes that synthesize secondary metabolites are often only present in minute quantities, and many of the producing fungal species lacked the sexual systems that were required for classical genetic analysis and mapping. Only after the finding of recombination via horizontal gene transfer, the first molecular genetic analysis and reverse genetics became possible (Casselton & Zolan, 2002).

Enormous advances in molecular biology, bioinformatics and genomics have enabled the development of new techniques that can overcome these challenges, and revealed that the genes encoding specific fungal enzymes responsible for the building of secondary metabolites are clustered and often located near telomeres (Keller *et al*., 2005). Hence, new strategies for the isolation of fungal secondary metabolites are nowadays shifting away from the purely chemical analysis towards making use of the genomic information in order to find metabolites which are encoded by "silent" (also referred to as "orphan" or "cryptic") gene clusters (Gross, 2007). To date, a significant number of fungal PKS and NRPS gene clusters have been identified although it is suspected that an intriguingly high number of gene clusters is still waiting to be discovered.

The detection of silent pathways can be achieved by different methods. A very successful way is a genomics-guided approach which consists of an *in silico*-screening of a complete genome sequence in order to find consensus motifs, e. g. of certain PKS or NRPS modules, which are known to be the major biosynthetic gene clusters found in fungi (Gross, 2007). This way, Scherlach and co-workers have isolated four novel prenylated quinoline alkaloids, the aspoquinolines, from the filamentous fungus *Aspergillus nidulans* that are only produced under defined growth conditions (Scherlach *et al.*, 2006). By scanning the publicly available genome sequence of the fungus, the presence of several gene copies of a putative anthranylate synthase led to re-investigation of the microbe by analytical methods and resulted in the discovery of the novel secondary metabolites.




Aspoquinoline A

**Figure 5-1-4:** Chemical structure of aspoquinoline A, which has been discovered by a genomics-guided approach (Scherlach *et al.*, 2006).

Without the availability of complete sequence data of a microorganism, a PCR based search for distinct modules can be undertaken. As soon as a silent genomic region has been identified, it can be further analyzed to unveil the corresponding secondary metabolite. This

can be done for example by inactivation studies or the genomisotopic approach (Kunze *et al.*, 2005; Gross *et al.*, 2007). These methods have been successfully applied for bacteria and myxobacteria, and will hopefully also help to discover new fungal secondary metabolites in the near future.
Metagenomics is another key approach for the unravelling of silent pathways, especially to those microorganisms that cannot be cultivated under laboratory conditions (Schloss & Handelsman, 2005). Heterologous expression would be an elegant way to uncover the products correlated to the genomic information.

The field of chemical and analytical methods that can be described as the "classical" approach in natural products research has rendered outstanding services to the finding of new and bioactive natural products in the last years, and continue to be invaluable for this issue. Recently, new methods in the field of molecular biology and genomics have made enormous advances and significantly contributed to the field of natural products research. However, to continue the successful work, both methods should not be regarded as separated approaches. For example, it is not yet possible to predict the structure of any polyketide of a fungal PKS through analysis of domain and motif structures alone (Keller *et al*., 2005). Heterologous expression and chemical analysis of the produced compound are needed to completely describe the natural product in question. Thus, the strategy to combine both methods of molecular biology and natural products chemistry will open new horizons in natural products research and provide new resources for the discovery of novel and bioactive natural products.

# 6 Summary

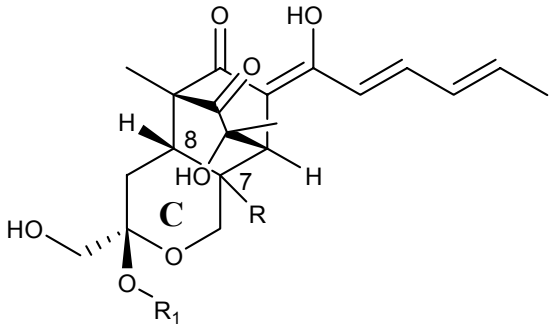
Many drugs in clinical use are natural products or natural product-derived. Among the most important natural products in drug therapy are many antibiotics, lipid-lowering agents based on the structure of lovastatin and anticancer drugs like paclitaxel and derivatives thereof. The need for new lead structures for the treatment of severe diseases like cancer or inflammatory ailmens continues, since established therapies are inadequate or lose their efficacy due to drug resistance.

The marine habitat has been proven to be a rich source for both macro- and microorganisms that produce novel and often highly bioactive natural products. Hence, the investigation of the secondary metabolite spectrum of marine fungi represents a valuable approach in the search for new and bioactive natural products. The present study thus focused on the investigation of marine-derived fungi with the aim to isolate bioactive secondary metabolites via a bioassay-guided isolation strategy. Special emphasis was placed on new structural types and their ability to act as antitumor or, in second priority, antibiotic agents.

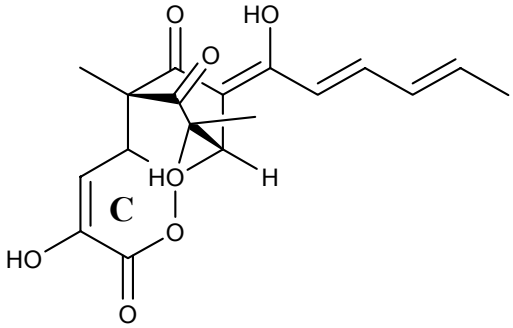
From the cytotoxicity screening program undertaken within the Institute for Pharmaceutical Biology, five fungal strains with cytotoxic activity towards cancer cells and interesting chemical features, i. e. $^{1}$H-NMR and LC-MS data, were chosen for detailed investigation. Two of these strains were sponge-derived while three have been isolated from the inner tissue of marine algae. From these fungal cultures altogether 19 compounds and two peptidic mixtures have been isolated and their structures characterized. They belonged to different structural classes, e. g. polyketides (pachybasin, chrysophanol, and more), peptides (beauvericin, peptaibols) and PKS/NRPS derived compounds (beauversetin and sch210972).

The sorbicillin derivatives trichodermanones A-D (**1**-**4**) were obtained from the sponge-derived fungus *Trichoderma saturnisporium*. The trichodermanones are remarkable because of their unprecedented carbon skeletons, most likely originating from two different polyketide units that have been connected via a Diels-Alder reaction during biosynthesis. One of these units can be described as sorbicillin-like, characterized by a cyclohexanone ring with an attached six-membered side chain (sorbyl moiety). The second structural part which is a pyrane ring in **1**-**3** and a lactone ring in **4** is possibly triketide-derived (ring C). The structures

of the compounds, including the absolute stereochemistry, were determined by interpretation of their spectroscopic data (1D and 2D NMR, CD, MS, UV and IR) and molecular modelling calculations. The relative configuration of the sorbicillin part of the molecule as well as of ring C could be solved by selective NOE measurements, respectively. A CD spectrum gave evidence for the absolute configuration of the sorbicillin part. However, the spatial relationship between the sorbicillin substructure and ring C and thus the configuration at C-7 and C-8 remained unclear at first. This problem could be solved by molecular modelling calculations. Four preferred conformations were calculated for the trichodermanones A-C taking into account all possible configurations at C-7 and C-8. As it was clear that the conformation of the non-sorbicillin part of the structures and thus $^{1}H$-$^{1}H$ coupling constants were dependent on the configuration at C-7, the magnitude of proton-proton coupling constants were considered and compared with those calculated for the modelled molecules by the Karplus equation. The results obtained finally led to the deduction of the absolute configuration of the trichodermanones 3*R*, 5*S*, 6*R*, 7*R*, 8*R*, 10*S* for trichodermanone A, 3*R,* 5*S*, 6*R*, 7*S*, 8*R*, 10*S* for trichodermanone B and 3*R*, 5*S*, 6*R*, 7*S*, 8*R*, 10*S* for trichodermanone C.



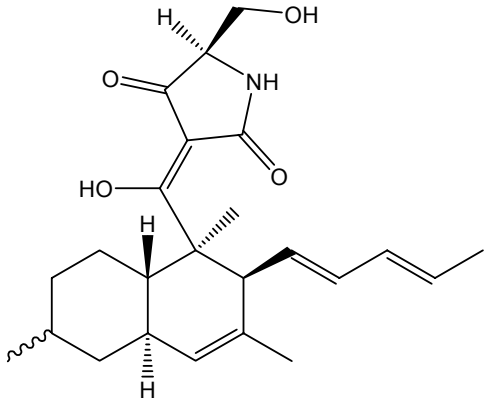

**trichodermanone A (1):** R = α-OH, $R_1 = CH_3$
**trichodermanone B (2):** R = ß-OH, $R_1 = CH_3$
**trichodermanone C (3):** R = ß-OH, $R_1$ = H



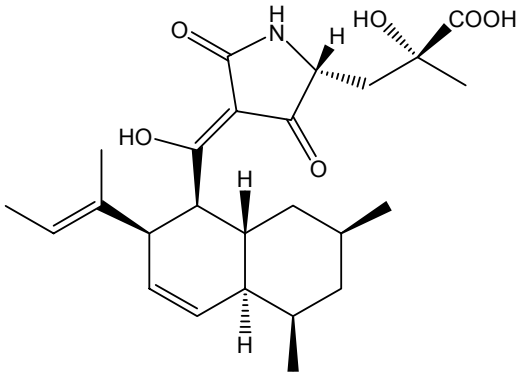

**trichodermanone D (4)**

Tetramic acid derivatives are an important class of nitrogen heterocycles with a pyrrolidine-2,4-dione core structure as a key structural motif and a wide range of bioactivities. Bioassay-guided isolation of the cytotoxic crude extract of *Beauveria bassiana* led to the isolation of the new cytotoxic tetramic acid derivative beauversetin. A compound of the same structural type, the tetramic acid sch210972 was found to be produced by the sea weed-derived fungus *Microdiplodia* sp. and showed strong human leucocyte elastase (HLE) inhibitory activity with an $IC_{50}$ value of 1.04 µg $mL^{-1}$. HLE plays a role in several inflammatory diseases such as pulmonary emphysema or chronic bronchitis and thus represents an important target for drug

research. Our results show that tetramic acids represent a new lead structure for the development of potent HLE inhibitors.




beauversetin (**11**)




sch210972 (**15**)

The compounds isolated during this study impressively demonstrate the structural diversity of fungal secondary metabolites. The trichodermanones are a formidable example for unique structures from marine-derived fungi. Furthermore, the discovery of tetramic acids as inhibitors of HLE showed that the marine habitat is a rich source for structurally novel compounds which can serve as lead structures for the development of novel and innovative drugs.

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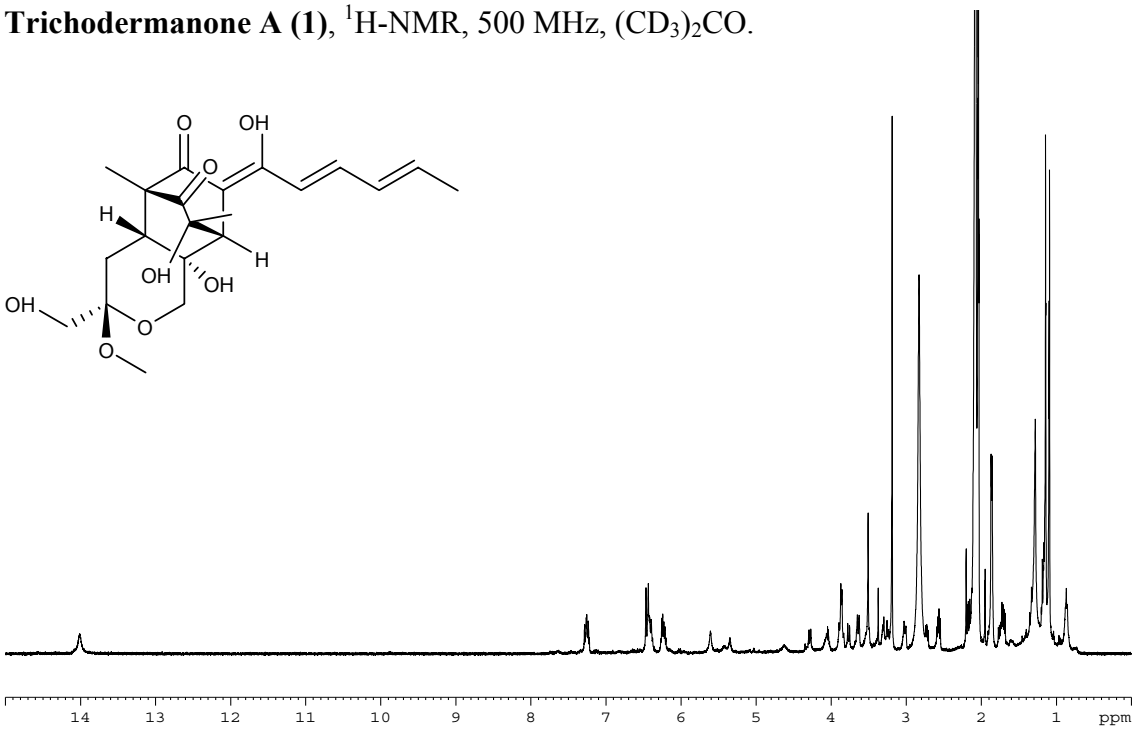
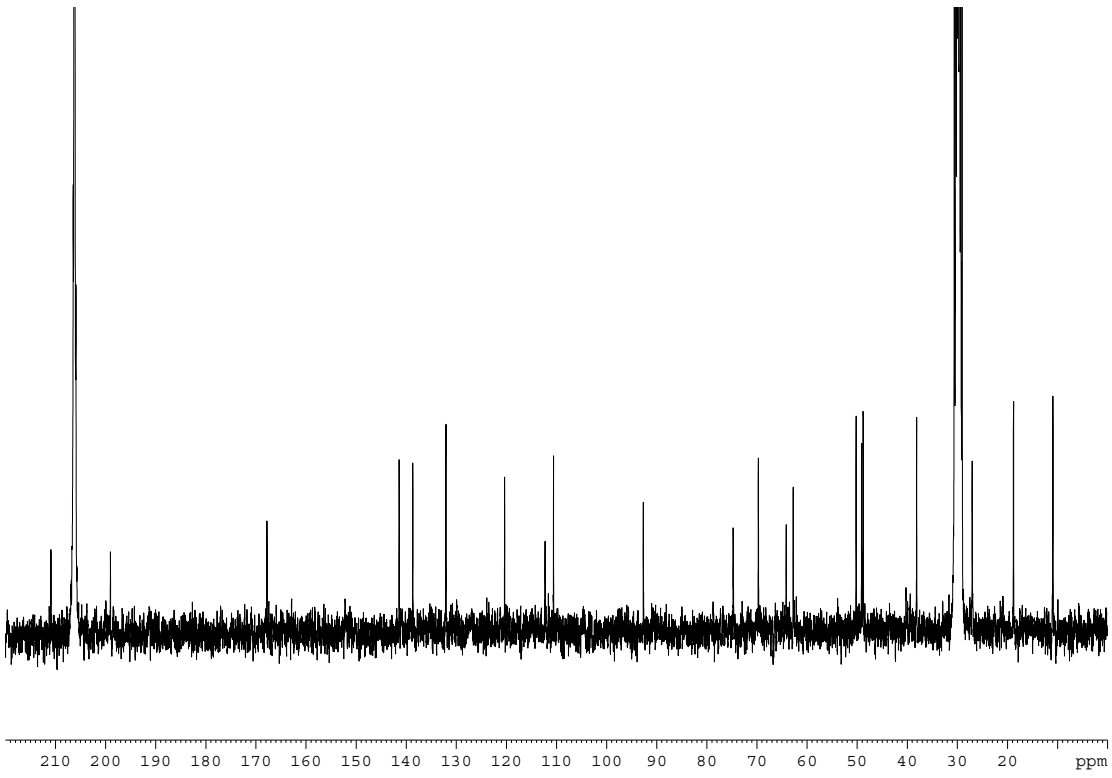
# 8 Appendix

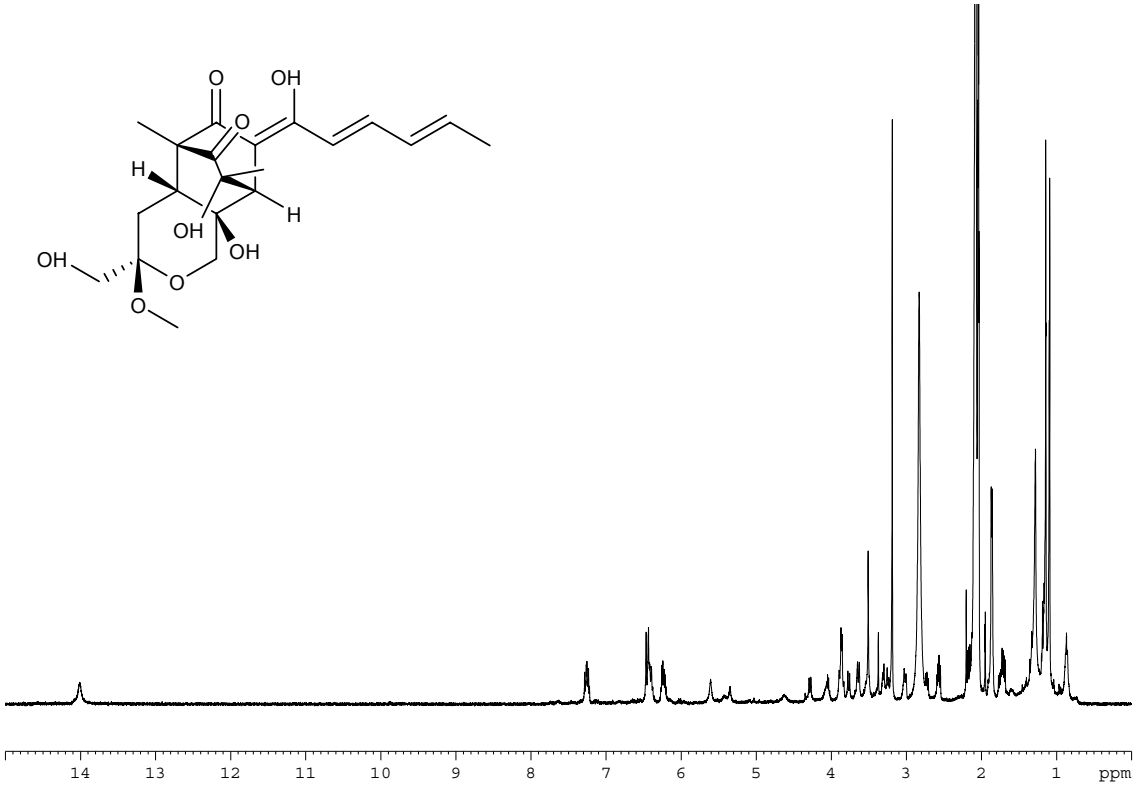
**Trichodermanone A (1)**, $^{1}$H-NMR, 500 MHz, $(CD_3)_2CO$.




$^{13}$C-NMR, 75 MHz, $(CD_3)_2CO$.



**Trichodermanone B (2)**, $^{1}$H-NMR, 500 MHz, $(CD_3)_2CO$.

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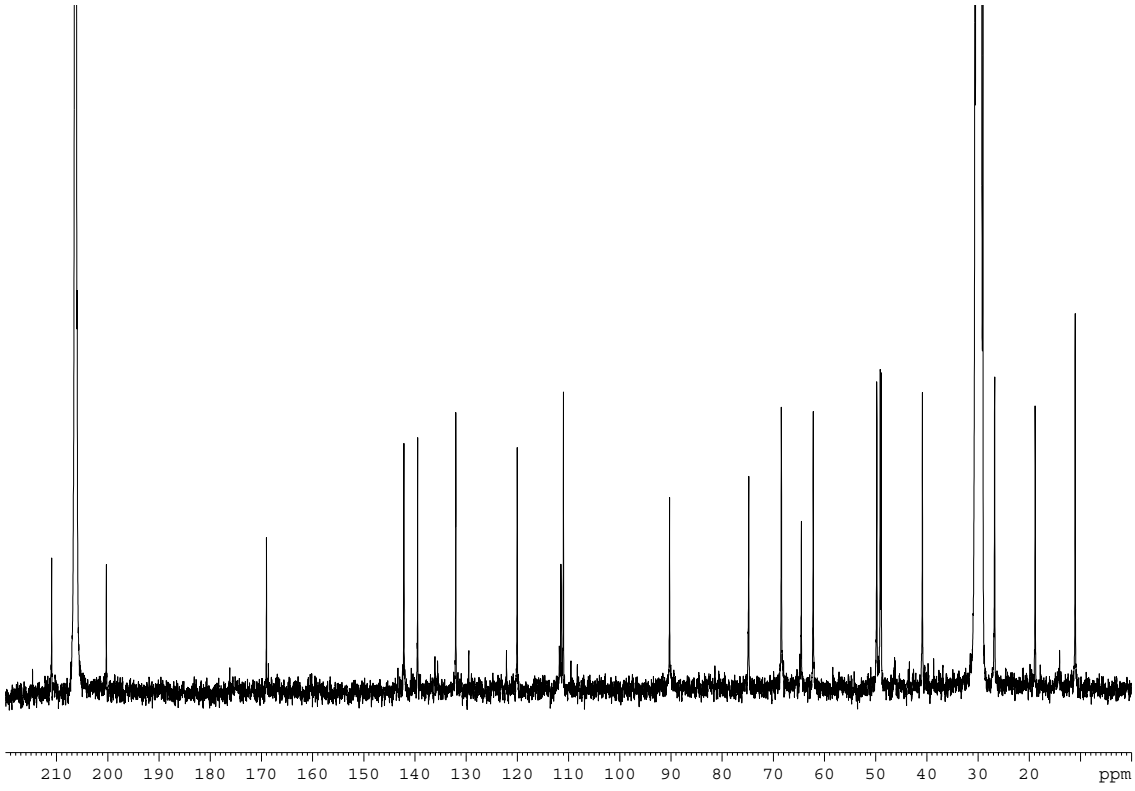
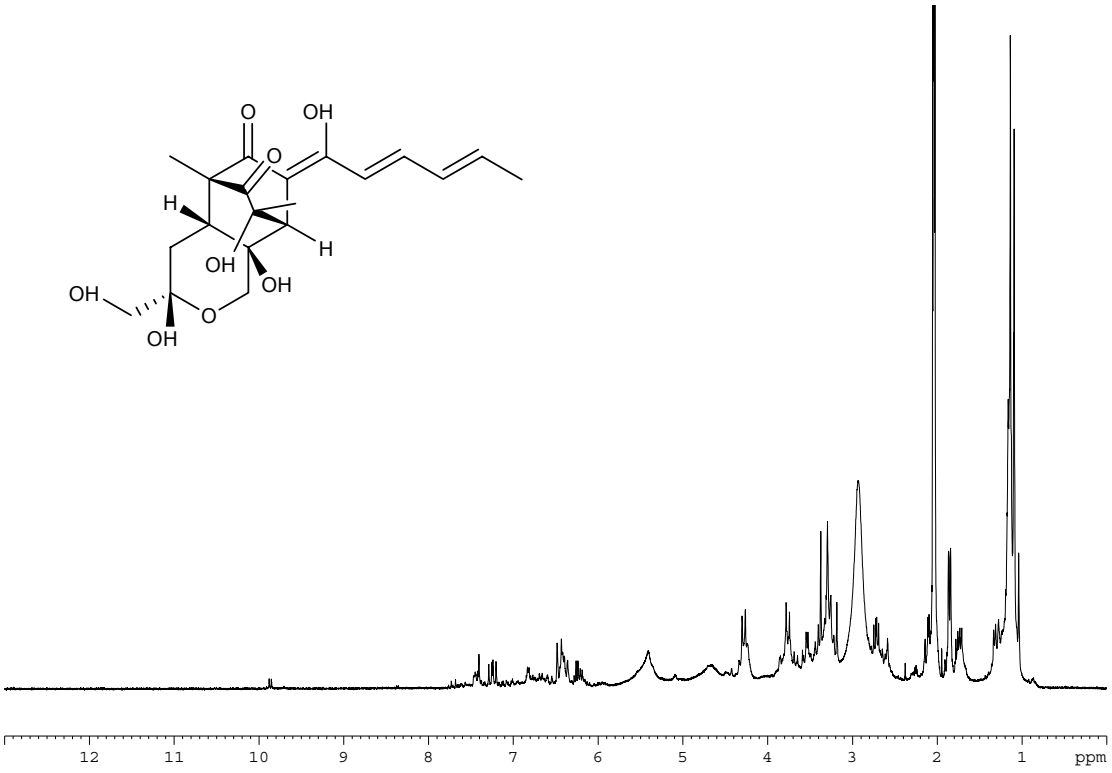


**Trichodermanone C (3)**, $^{1}$H-NMR, 500 MHz, $(CD_3)_2CO$.

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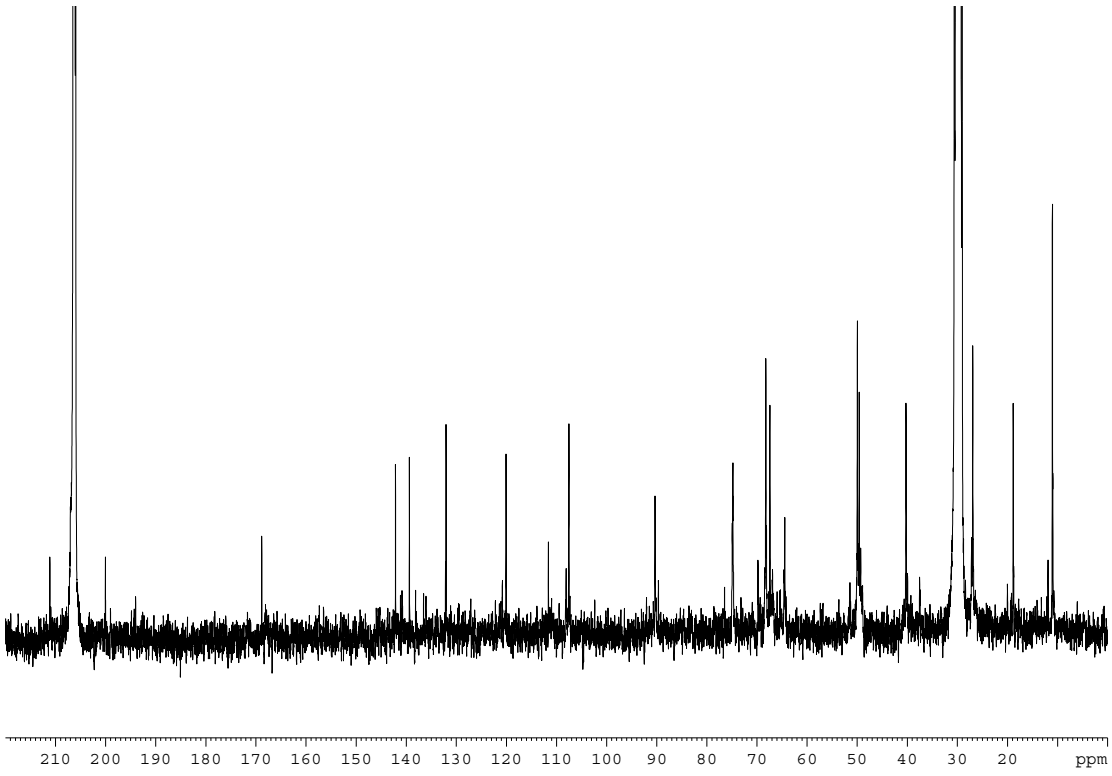
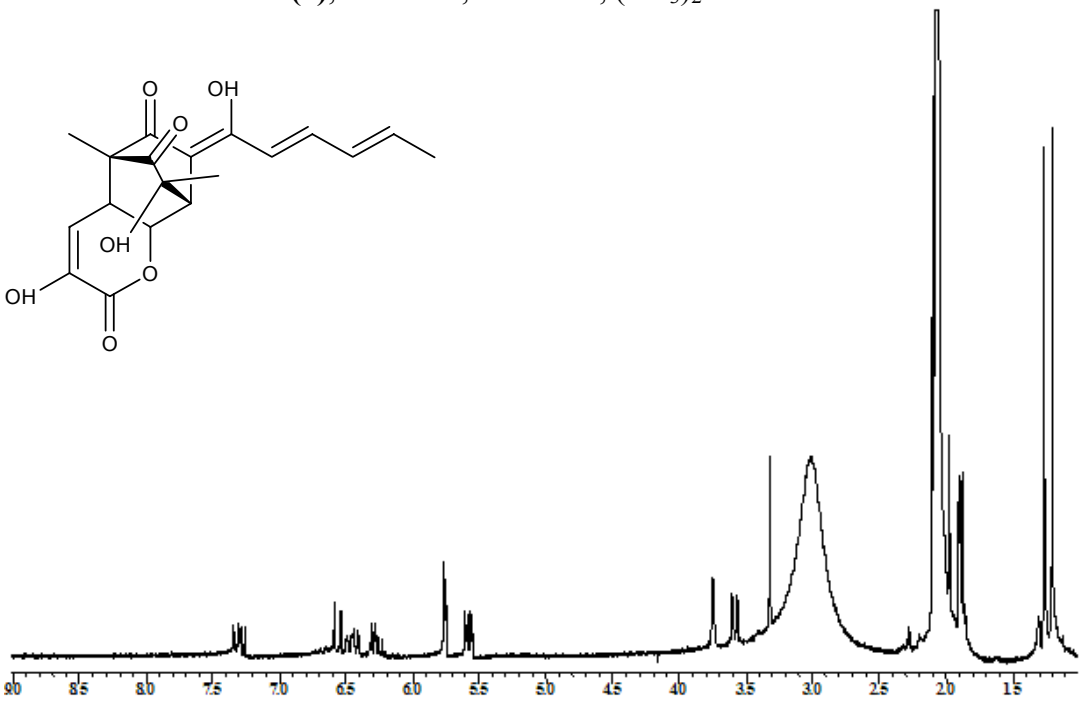
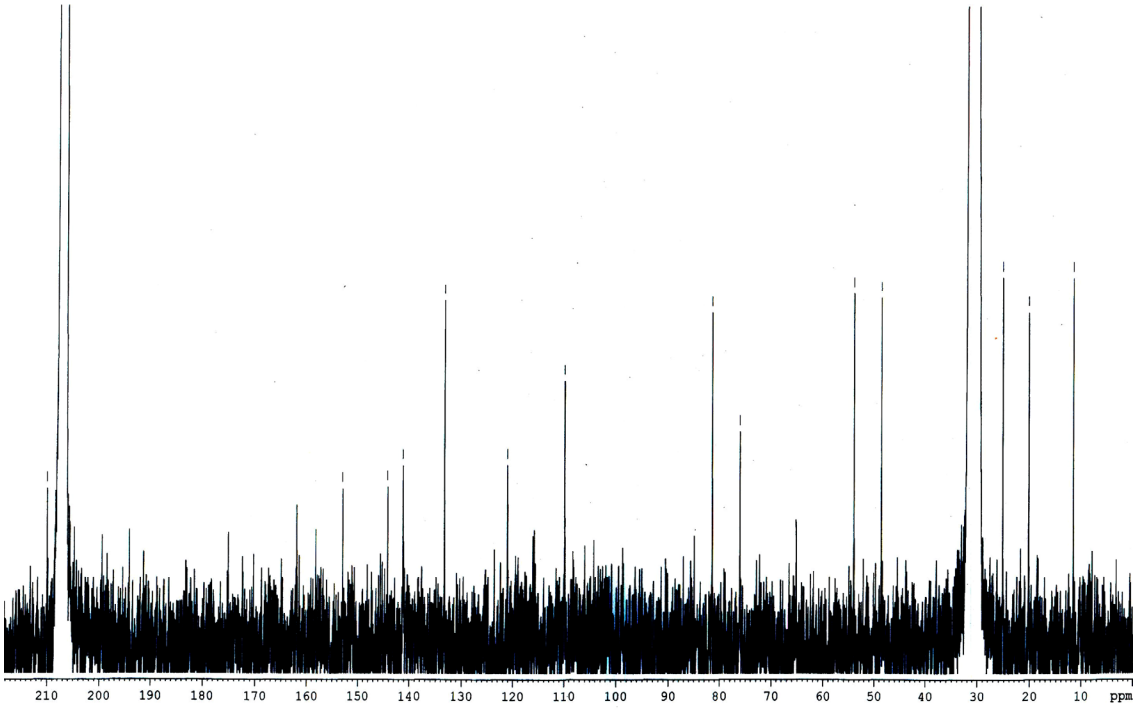


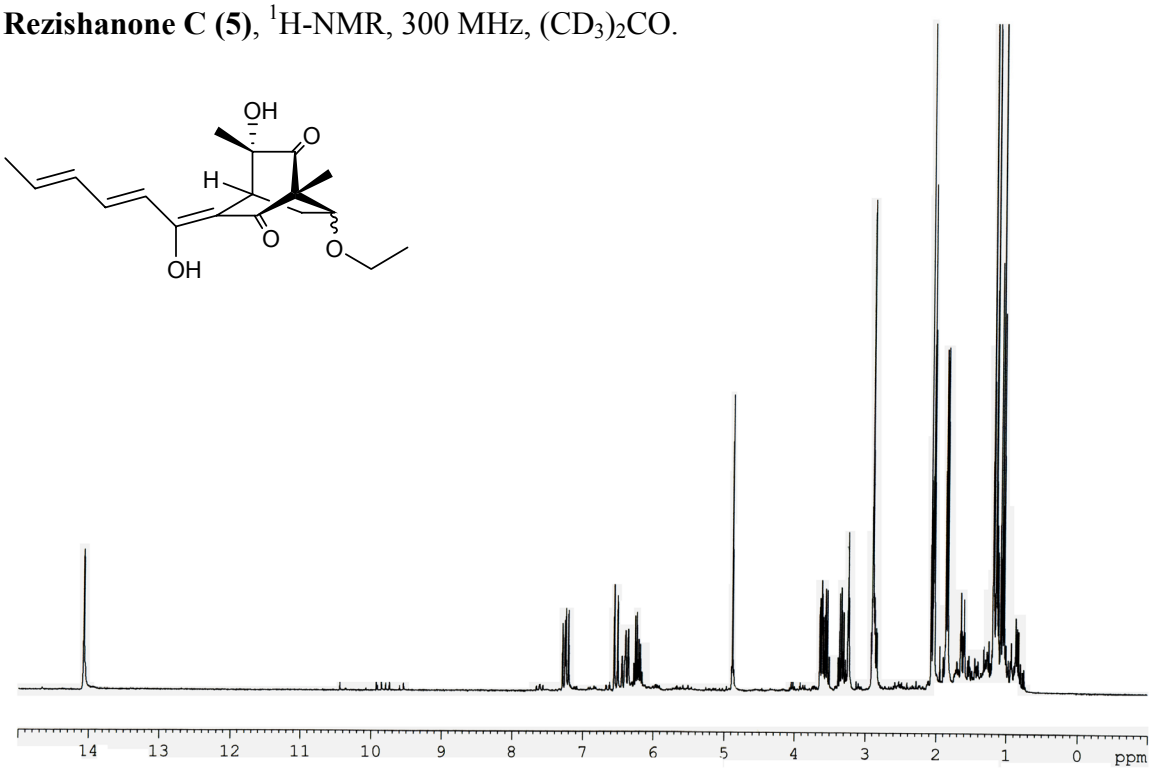
**Trichodermanone D (4)**, $^{1}$H-NMR, 500 MHz, $(CD_3)_2CO$.




$^{13}$C-NMR of **4**, 75 MHz, $(CD_3)_2CO$.



**Rezishanone C (5)**, $^{1}$H-NMR, 300 MHz, $(CD_3)_2CO$.

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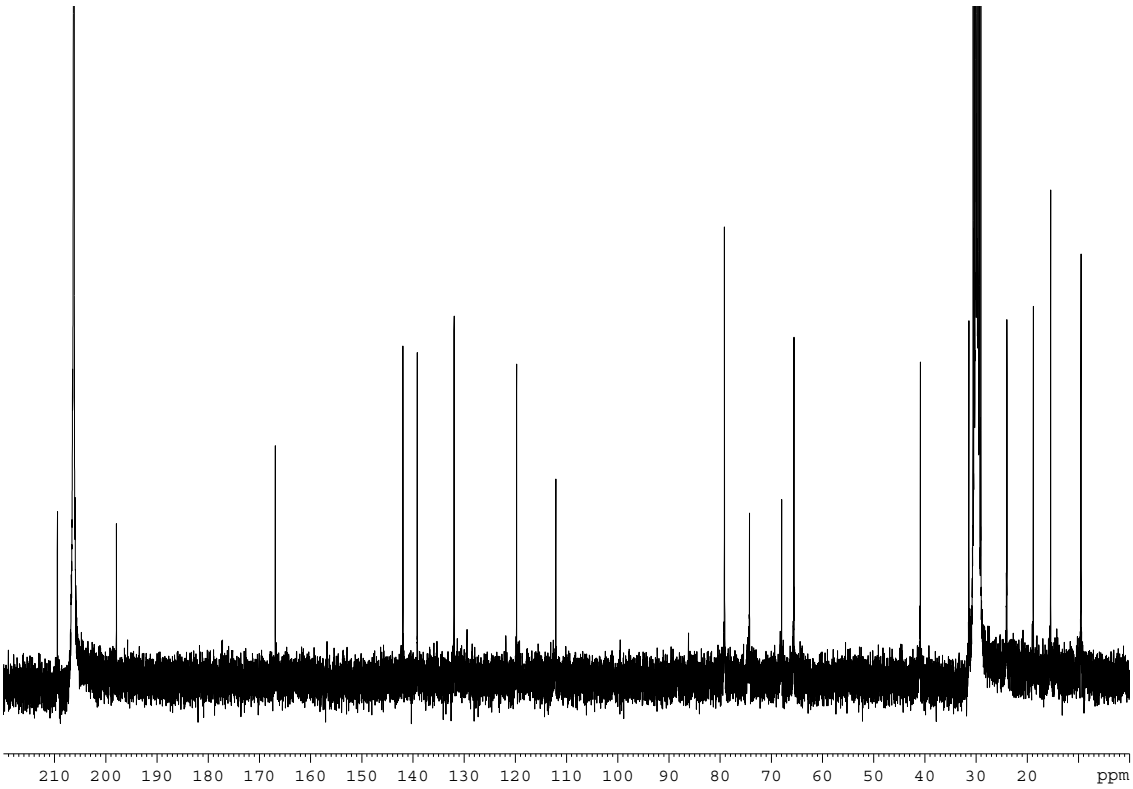
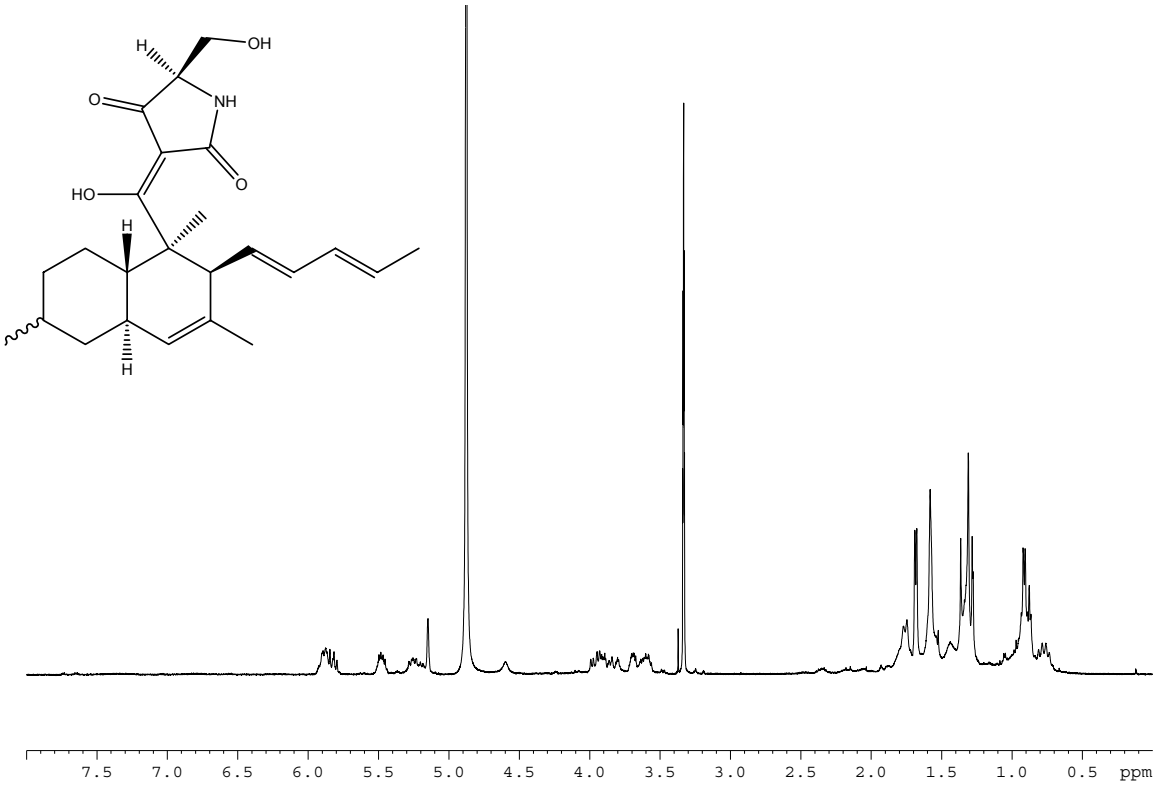
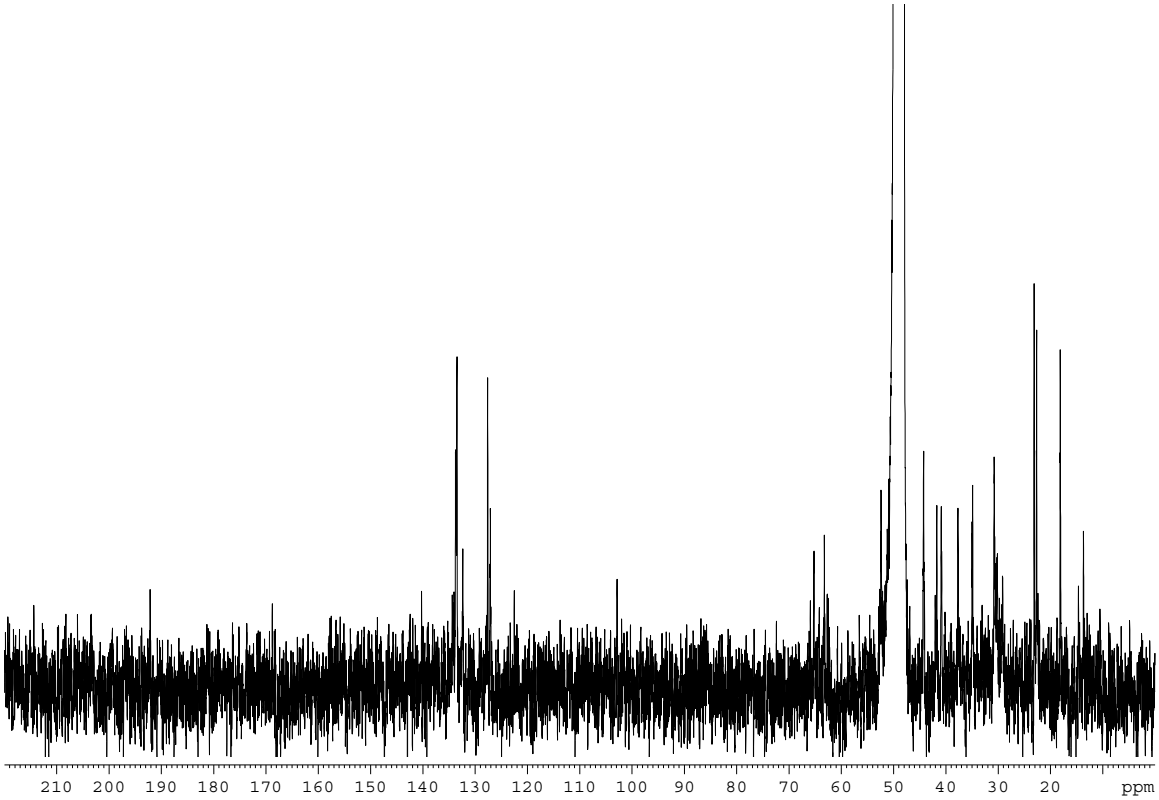


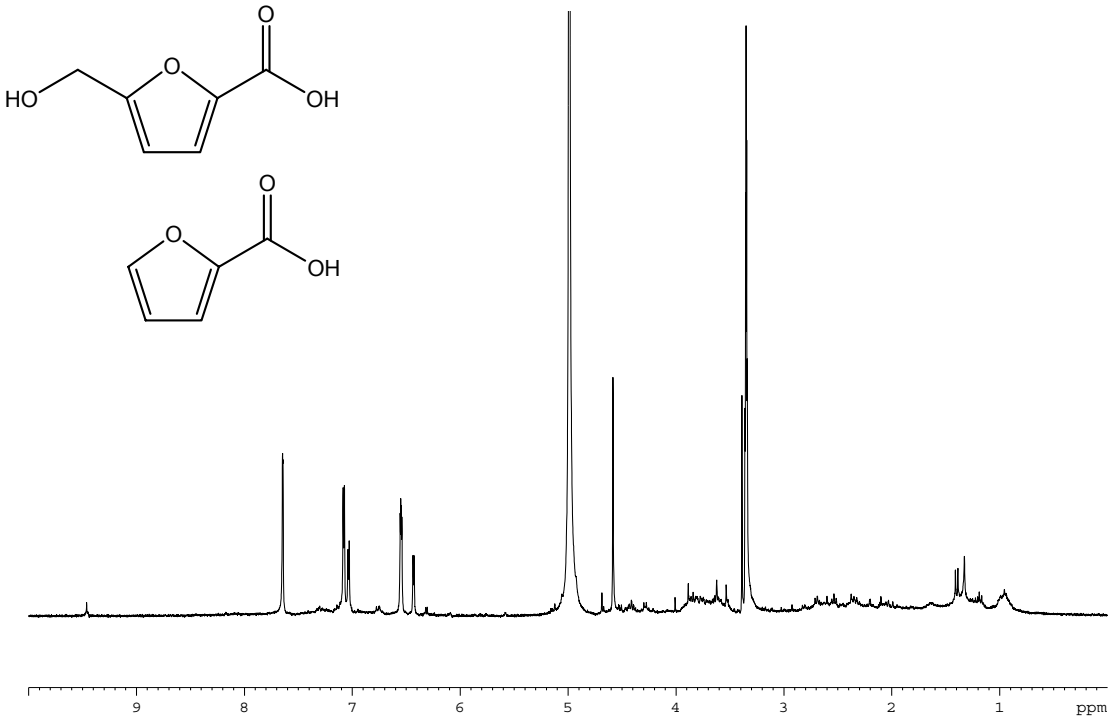
**Beauversetin (11)**, $^{1}$H-NMR, 500 MHz, $CD_3OD$.



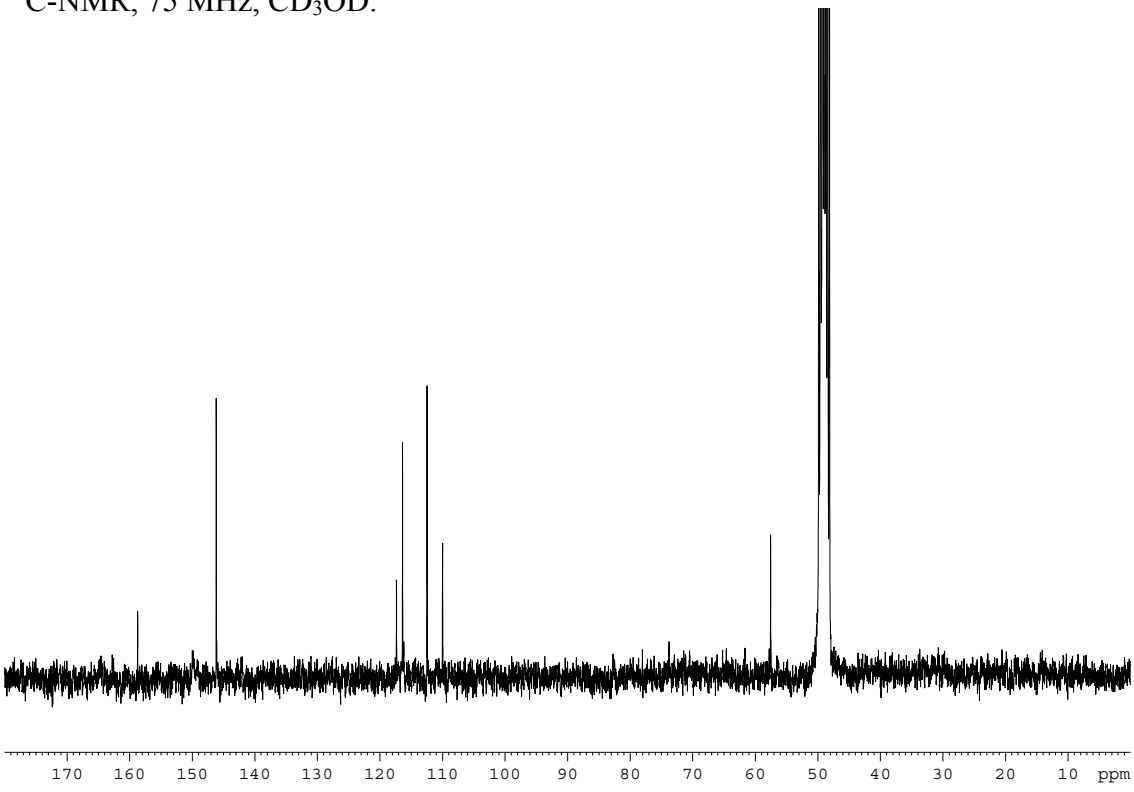

$^{13}$C-NMR, 75 MHz, $CD_3OD$.



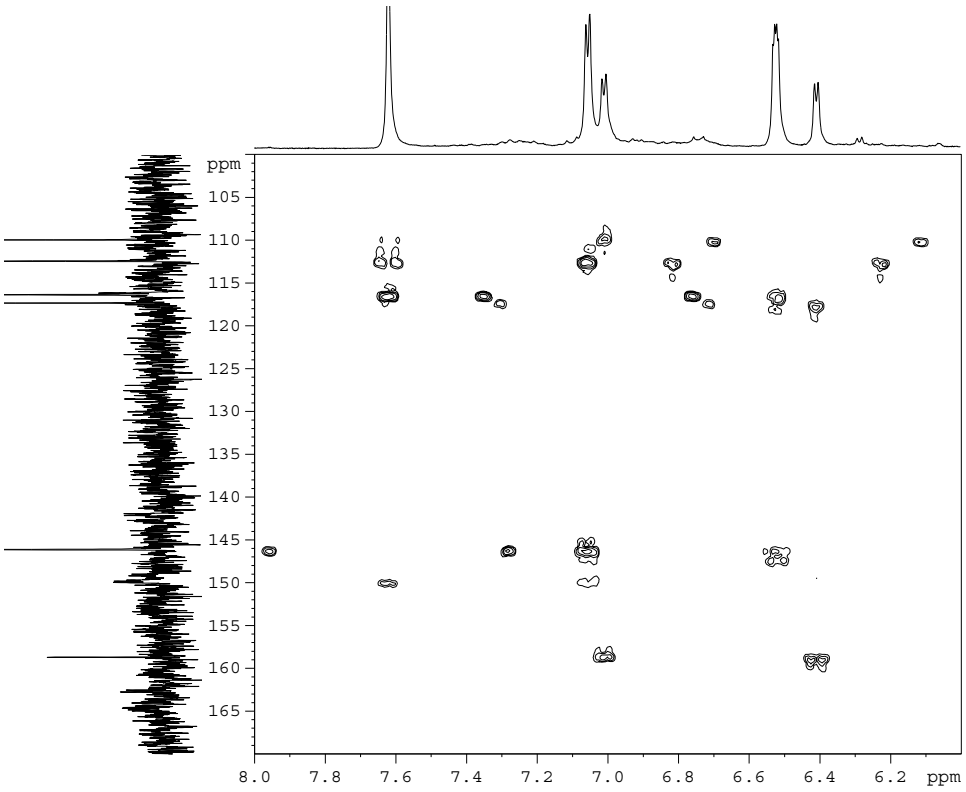
**Mixture of furane derivatives (12, 13)**, $^{1}$H-NMR, 500 MHz, $CD_3OD$.



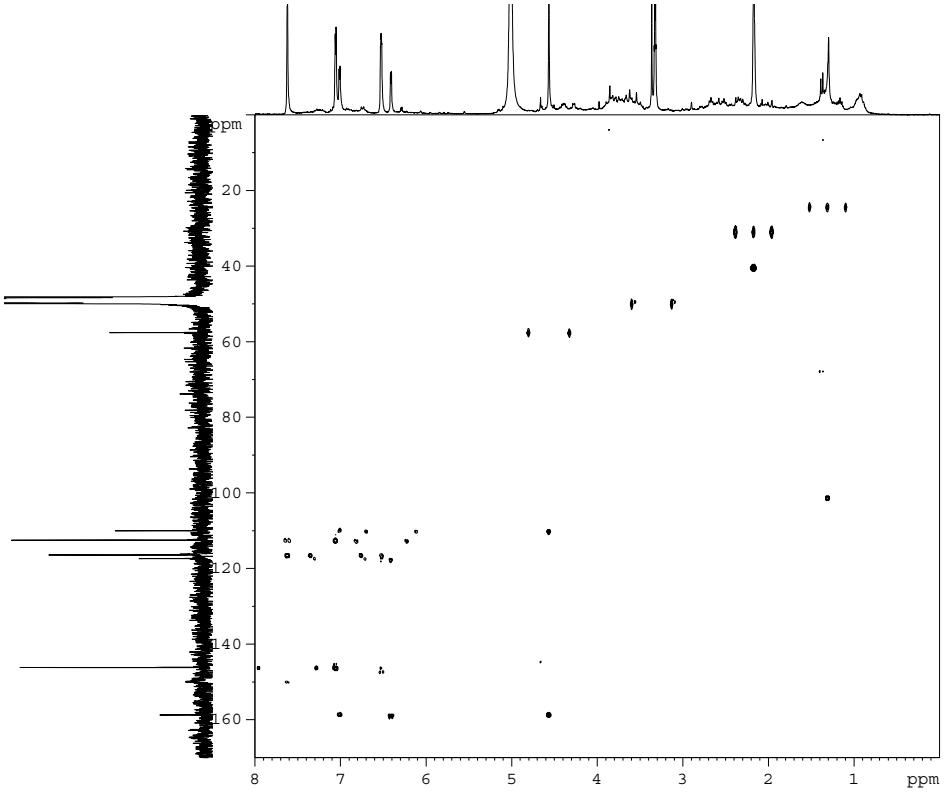

$^{13}$C-NMR, 75 MHz, $CD_3OD$.



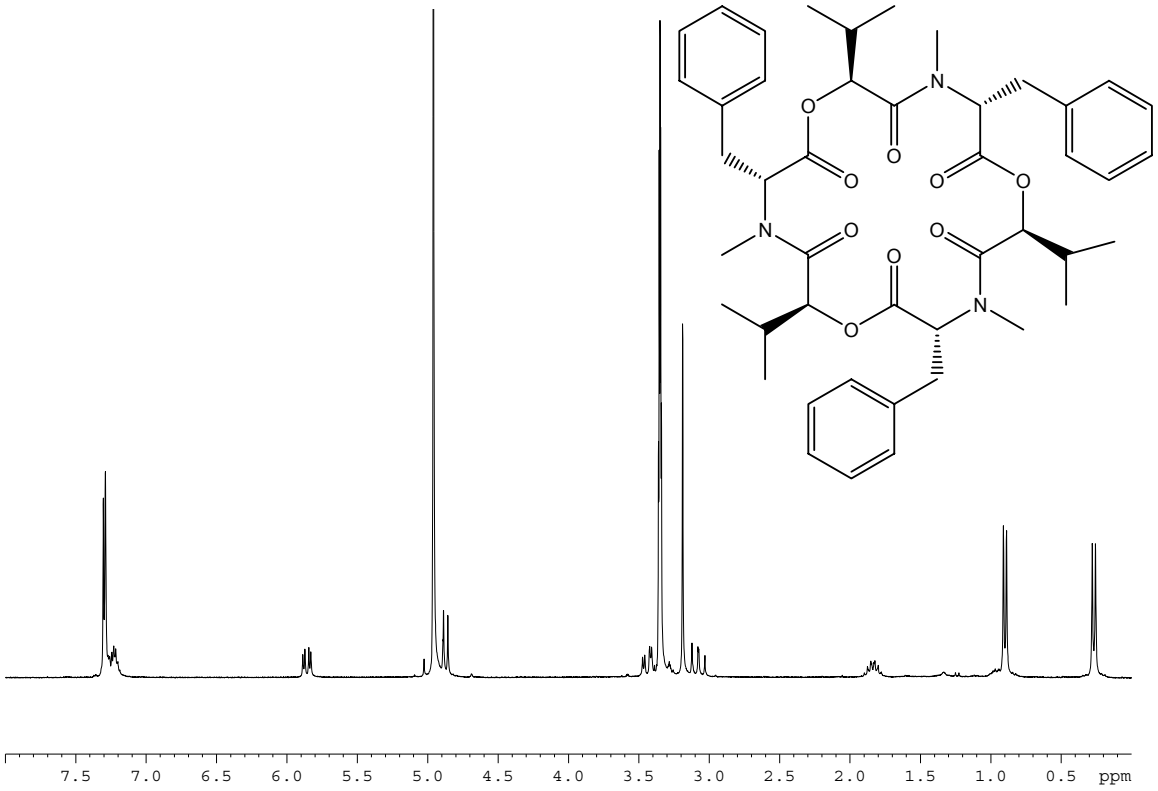
HMBC of **12, 13**, 300 MHz, $CD_3OD$.



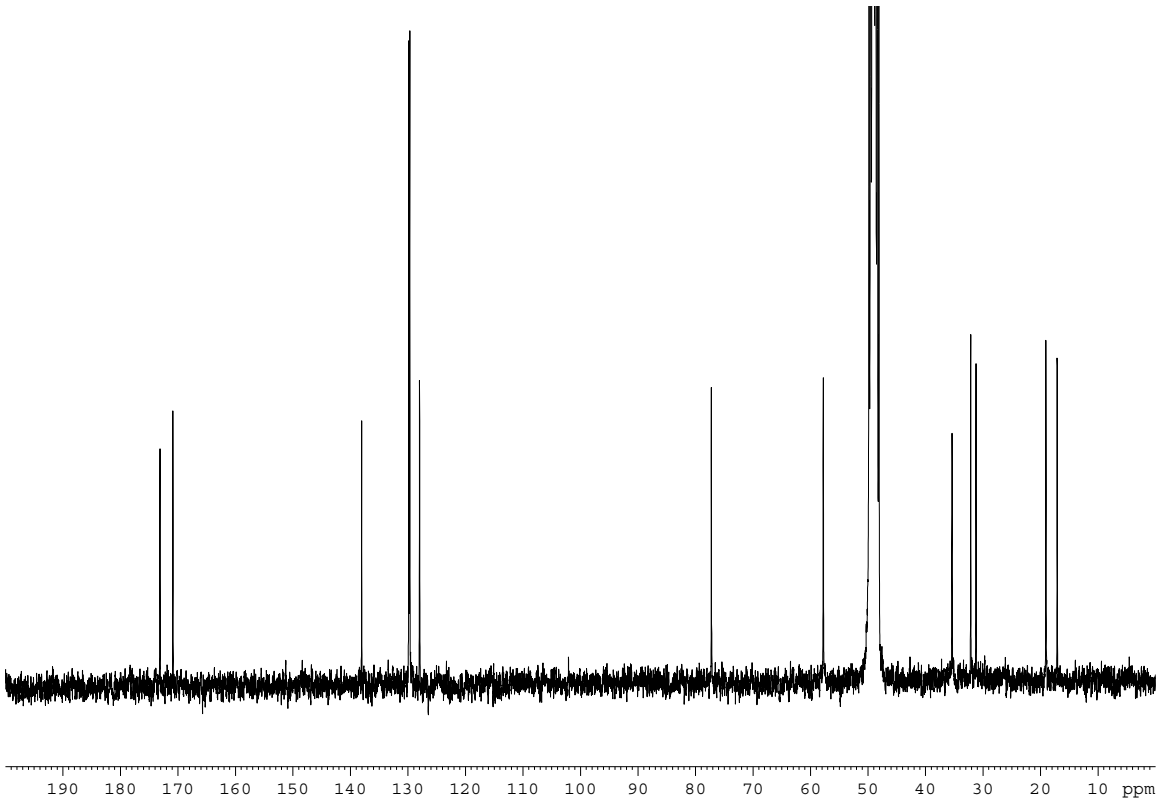

$^1H$-$^1H$ COSY of **12, 13**, 300 MHz, $CD_3OD$.



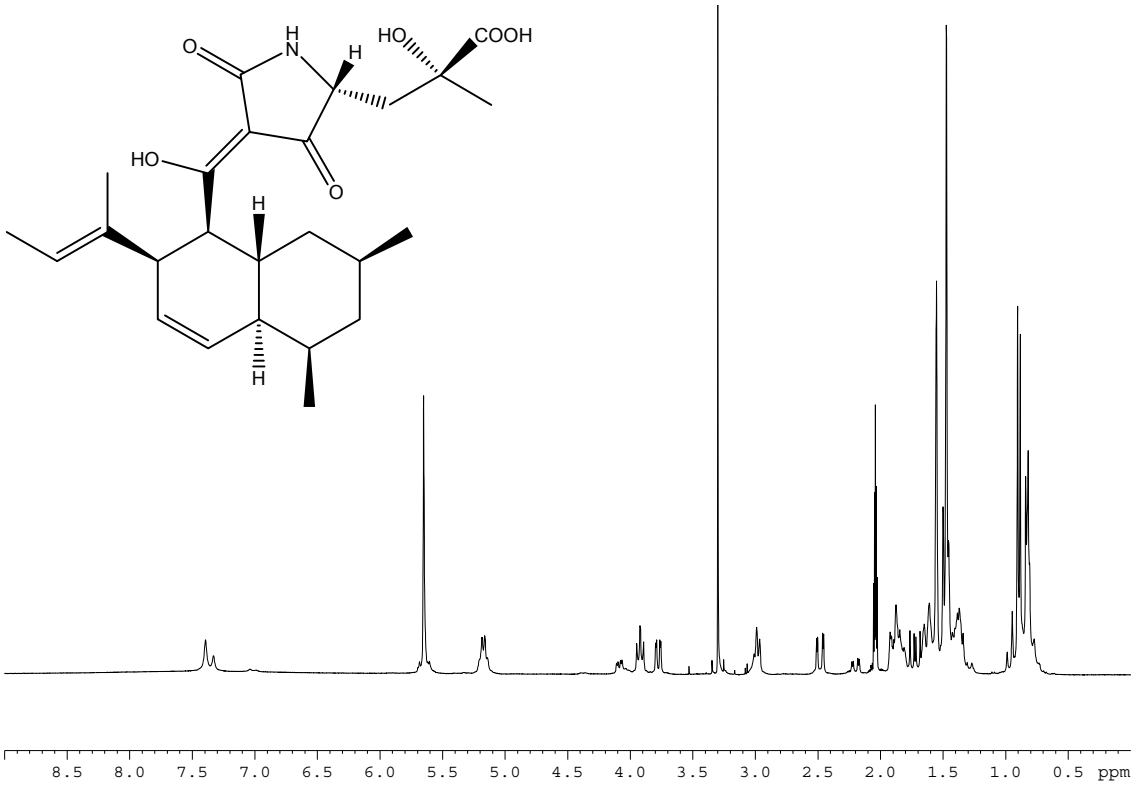
**Beauvericin (14)**, $^{1}$H-NMR, 300 MHz, $CD_3OD$.



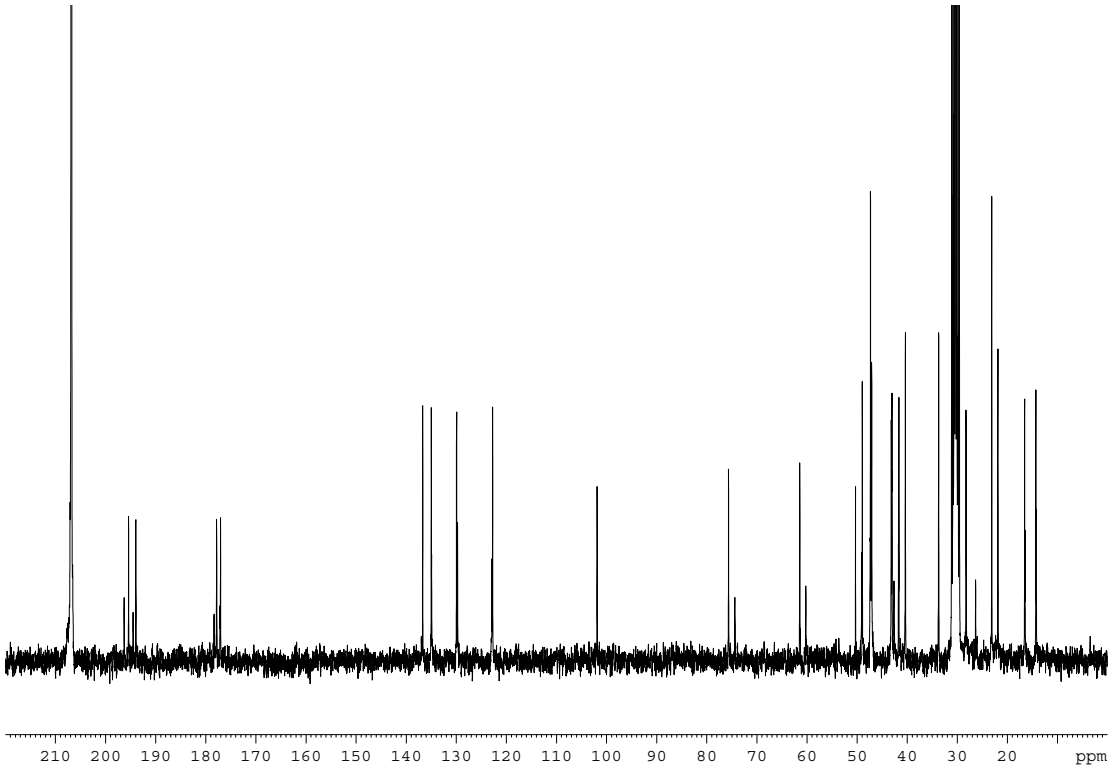

$^{13}$C-NMR, 75 MHz, $CD_3OD$.



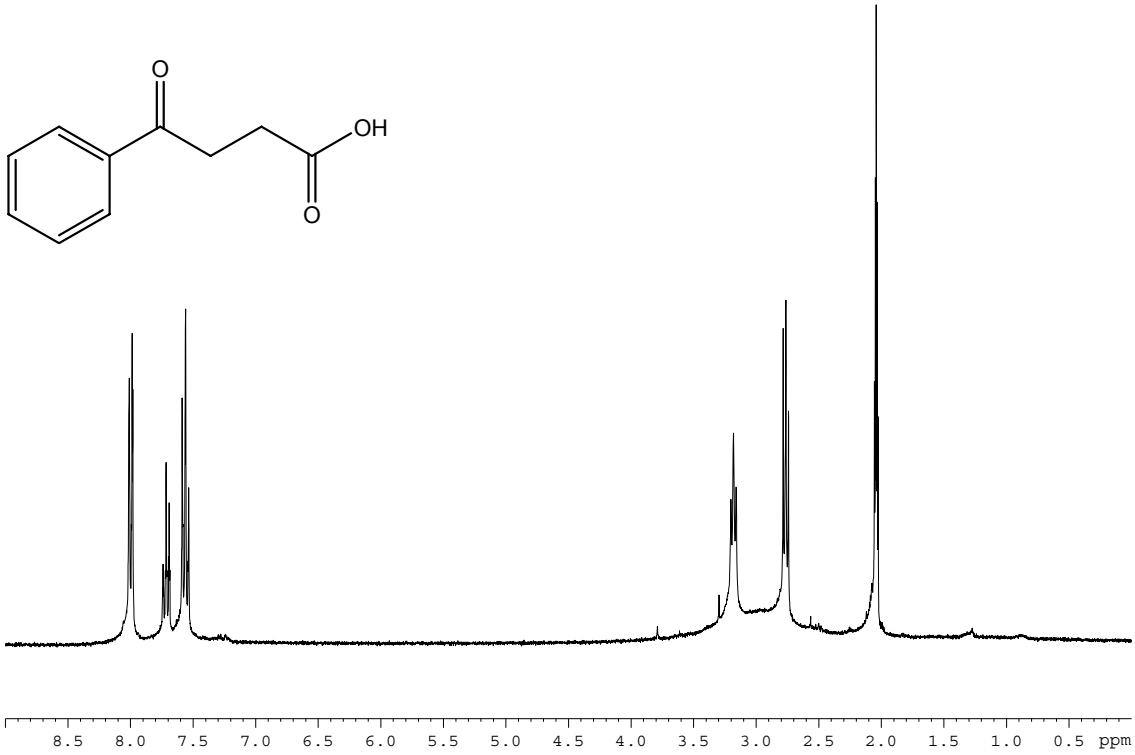
**Sch210972 (15)**, $^{1}$H-NMR, 300 MHz, $(CD_3)_2CO$).



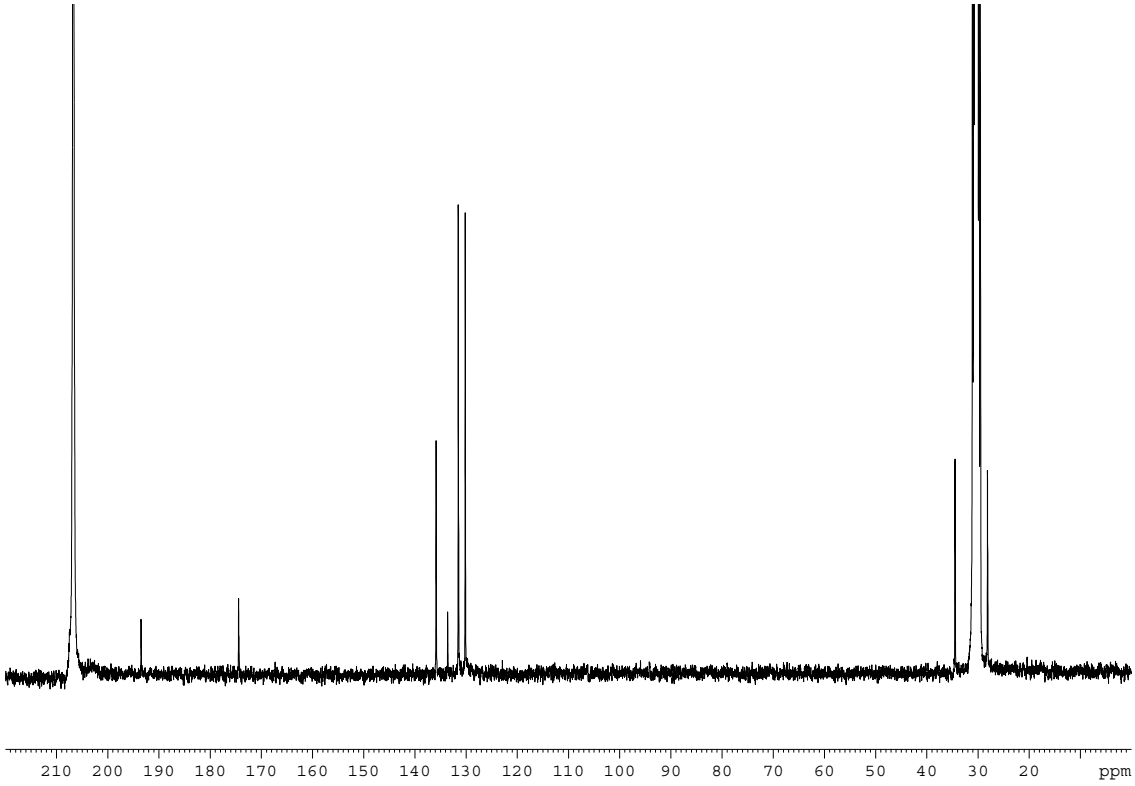

$^{13}$C-NMR, 75 MHz, $(CD_3)_2CO$.



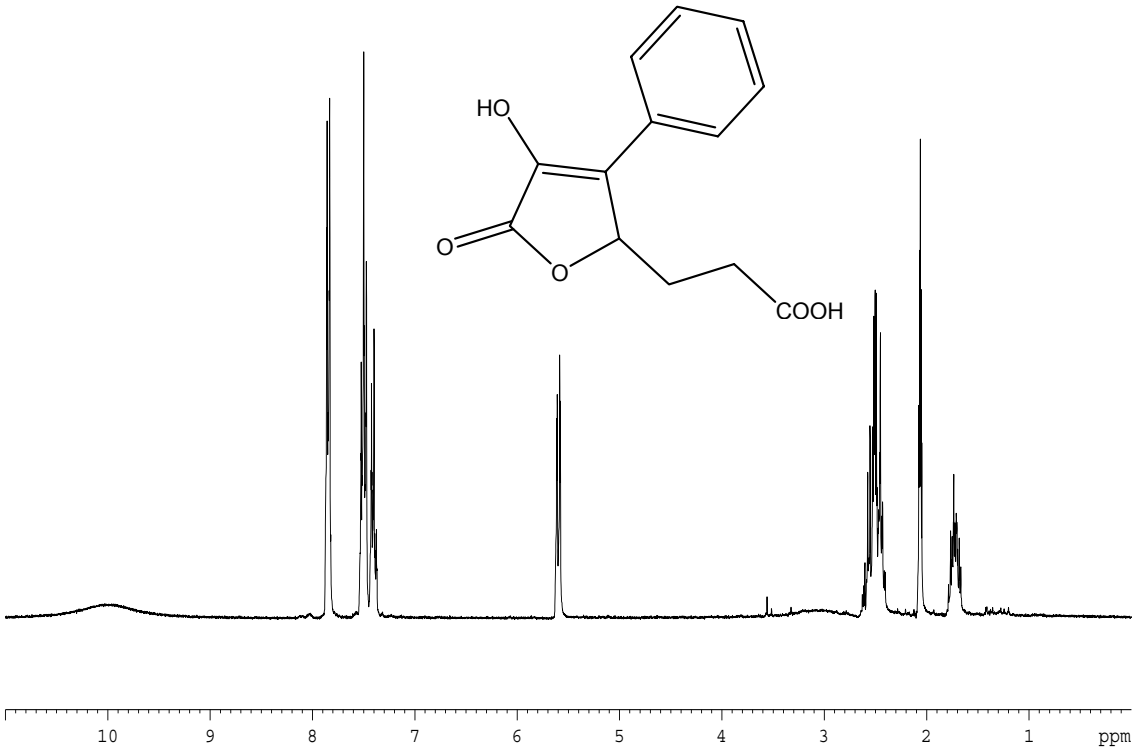
**4-oxo-benzenebutanoic acid (16)**, $^{1}$H-NMR, 300 MHz, $(CD_3)_2CO$).



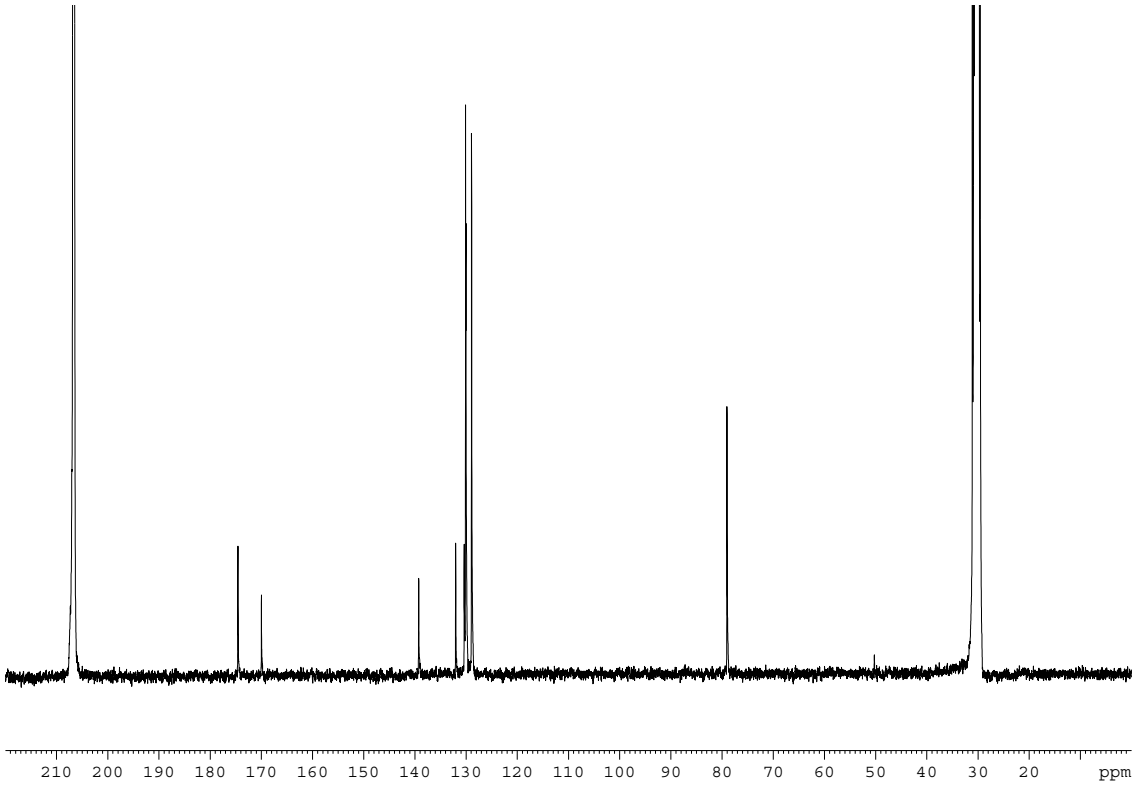

$^{13}$C-NMR, 75 MHz, $(CD_3)_2CO$.



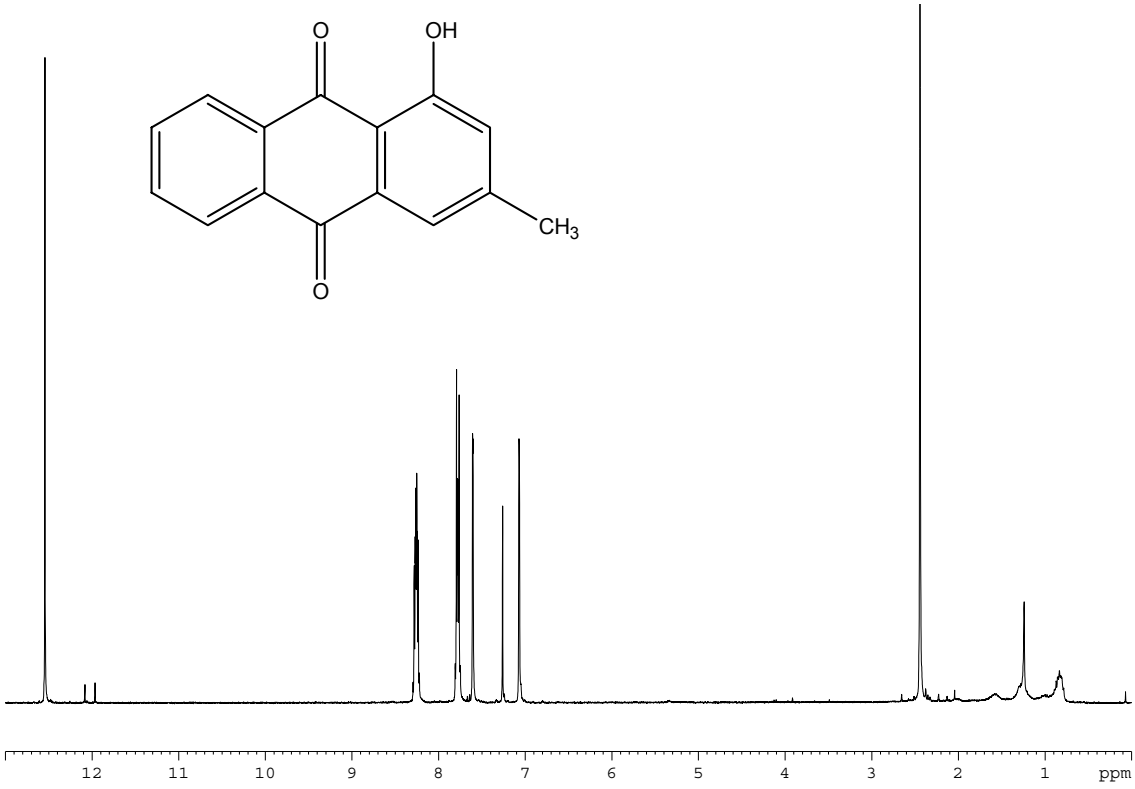
**WF-3681 (17)**, $^{1}$H-NMR, 300 MHz, $(CD_3)_2CO$).



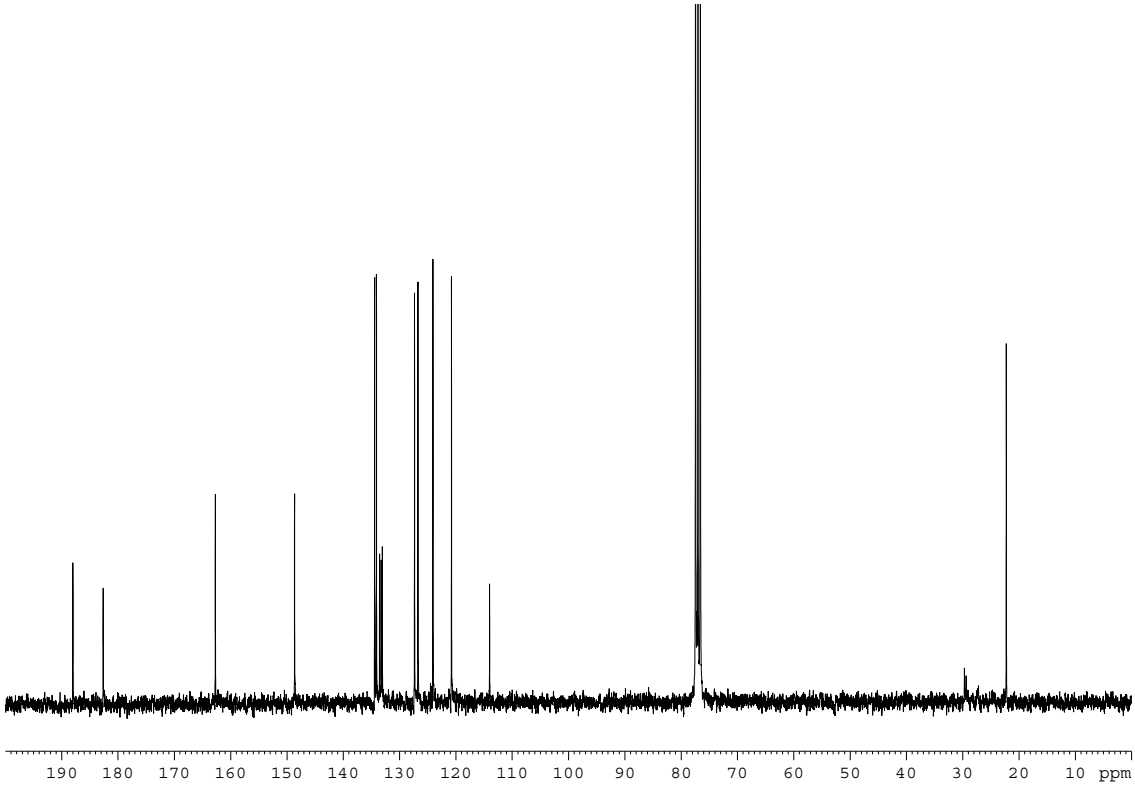

$^{13}$C-NMR, 75 MHz, $(CD_3)_2CO$.



**Pachybasin (18)**, $^{1}$H-NMR, 300 MHz, $CDCl_3$.




$^{13}$C-NMR, 75 MHz, $CDCl_3$.



## Erklärung

Hiermit versichere ich, die vorliegende Arbeit selbständig und unter ausschließlicher Verwendung der angegebenen Hilfsmittel verfasst zu haben, wobei ich die von mir verwendeten Quellen als solche gekennzeichnet habe.