

Antifungal Antibiotics from Potential Biocontrol Microorganisms

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Doctoral thesis

Swedish University of Agricultural Sciences

Uppsala 2006

Acta Universitatis Agriculturae Sueciae

2006: 47

ISSN 1652-6880
ISBN 91-576-7096-X
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Tryck: SLU Service/Repro, Uppsala 2006

Abstract

Pohanka, A., 2006. Antifungal Antibiotics from Potential Biocontrol Microorganisms.
Doctor's dissertation
ISSN 1652-6880, ISBN 91-576-7096-X

This thesis presents isolation and structure elucidation of twenty-five antifungal antibiotics. The compounds were isolated from four different microorganisms, which had all been found to suppress fungal pathogens in bioassays.

Eight cyclic depsipeptides, enniatins, were isolated from the fungus *Fusarium* sp. F31. Four of them have not previously been isolated from natural sources. They inhibited *Botrytis cinerea* spore germination at levels down to 100 µg/ml. From the bacterium *Pseudomonas* sp. MF381-IODS, two linear polyene polyketides were isolated, pseudotrienic acids A and B, together with the known antifungals didehydro-deepoxirhizoxin, and pyrrolnitrin. The pseudotrienic acids inhibited Gram-positive bacteria at levels down to 70 µg/ml. The *Pseudomonas* sp. Ki19 strain produced four different dialkylresorcinols, of which two were new analogues. All four were active against fungi at levels down to 100 µg/ml, and also against Gram-positive bacteria down to 5 µg/ml. The actinomycete *Kutzneria* sp. 744 was found to produce a spectrum of nine di- and trichlorinated cyclic depsipeptides, containing several unusual amino acids. The trichlorinated compounds were significantly more active and inhibited fungi down to 60 µg/ml and Gram-positive bacteria down to 5 µg/ml.

A robust sample work-up method was developed for isolation of antifungal metabolites from both fungi and bacteria. It consisted of bioassay-guided isolation by solid phase extraction (SPE) and HPLC. The bioassay was based on inhibition of fungal spores or bacterial cells and was able to detect compounds with a range of antifungal activities. Such a multi-target functional bioassay was used in order to isolate compounds with several types of antifungal mechanisms. Bacteria were included in the bioassays in order to gain a broader knowledge of the antibiotic spectrum.

The structural elucidation of the compounds relied on NMR and MS techniques together with chemical degradation and derivatization methods, and GCMS analysis for stereochemical investigation. The absolute configuration was determined for all previously unpublished compounds.

Keywords: antifungal activity, depsipeptides, polyketides, dialkylresorcinols, Marfey's method, enantiomeric resolution, *Pseudomonas*, *Fusarium*, *Kutzneria*, bioassay-guided isolation

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Appendices

A Taxonomic names of organisms

B Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

- I Pohanka, A., Capieau, K., Broberg, A., Stenlid, J., Stenström, E., and Kenne, L. 2004. Enniatins of *Fusarium* sp. strain F31 and their inhibition of *Botrytis cinerea* spore germination. *Journal of Natural Products*, 67 (5), 851-857.
- II Pohanka, A., Broberg, A., Johansson, M., Kenne, L., and Levenfors, J. 2005. Pseudotrienic acids A and B, two bioactive metabolites from *Pseudomonas* sp. MF381-IODS, *Journal of Natural Products*, 68 (9): 1380-1385.
- III Pohanka, A., Levenfors, J., and Broberg, A. 2006. Antimicrobial dialkylresorcinols from *Pseudomonas* sp. Ki19. *Journal of Natural Products*, 69 (4). 654-657.
- IV Pohanka, A., Menkis, A., Levenfors, J., and Broberg, A. Low abundance kutznerides from *Kutzneria* sp. 744 and antimicrobial activities of kutznerides 1-9. Manuscript.

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Contributions for Papers I-IV:

The projects were all co-operations between chemists and biologists. All biological work, such as growth of organisms, bioassays, and sequencing was done by the biologist co-authors. The HRMS measurements in Papers I-IV were performed by Suresh Gohil.

- I I initiated and designed the project in collaboration with Kristof Capieau. All chemical work was performed by me and also the major part of the writing.
- II I initiated and designed the project in collaboration with Maria Johansson. I performed all chemical work except absolute configuration of the amino acid moiety of pseudotrienic acid, which was done by Anders Broberg. The major part of the writing was performed by me.
- III I initiated and designed the project in collaboration with Jolanta Levenfors. All chemical work was performed by me and also the major part of the writing.
- IV I took part in expansion of the project leading to the paper. All chemical work was performed by me and also the major part of the writing.

Abbreviations and symbols

Abs	absorbance
AA	amino acid
AWB	asexual white basidiomycete
AU	arbitrary units
BCA	biocontrol agent
BuOH	butanol
CD	circular dichroism
COSY	correlation spectroscopy
δ	chemical shift
DNA	deoxyribonucleic acid
EF2	elongation factor 2
EIMS	electron impact mass spectrometry
ESIMS	electrospray ionisation mass spectrometry
FAB	fast atom bombardment
FT-ICR	Fourier transform ion cyclotron resonance
GC	gas chromatography
GRAS	generally regarded as safe
HA	hydroxy acid
HPLC	high performance liquid chromatography
HMBC	heteronuclear multiple bond correlation
HSQC	heteronuclear single quantum coherence
IODS	isolates having characteristic optically denser spots
ITS	internal transcribed spacer
IPC	inositol phosphoceramide
<i>J</i>	coupling constant
MALDITOF	matrix assisted laser desorption/ionisation time-of-flight
MeCN	acetonitrile
MeOH	methanol
MHz	megahertz
MIC	minimal inhibitory concentration
mRNA	messenger RNA
MS	mass spectrometry
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
NRPS	nonribosomal peptide synthetase
ODS	octadecyl-bonded silica
OSMAC	one strain – many compounds

PPFM	pink pigmented facultative methylotroph
PSD	post source decay
pv.	pathovar
RI	refractive index
RNA	ribonucleic acid
ROE	rotating frame nuclear Overhauser effect
ROESY	rotating frame nuclear Overhauser effect spectroscopy
RP	reversed phase
rRNA	ribosomal RNA
sp.	species (singular)
SPE	solid phase extraction
spp.	species (plural)
TLC	thin layer chromatography
UV	ultraviolet

1. Introduction

Fungi have fundamental functions in terrestrial ecosystems, in degradation of organic matter and in nutrient uptake of plants through mycorrhizal interactions. From a human point-of-view, there are both good and bad fungi. There are quite some delicious edible fungi and other are used in production of food like soy sauce, tempe, and bread. They are also a source of important drugs like the penicillins, the cholesterol-lowering lovastatin, and the cyclosporins, which counteracts the rejection of transplanted organs.

On the downside, fungi cause enormous economic loss in agriculture and food industry by destroying crops and plants in the field and during storage. In addition, many also produce mycotoxins which are harmful to humans and livestock. To a certain extent, pathogens can be controlled by sound agriculture and silviculture, *e.g.* maximum use of suppressive soils, crop rotation and removal of potential inocula, but all these have a limited effect. When combating fungal pathogens a wide array of antifungal agents have been and are being used. They include inorganic and synthetic organic compounds, but also antifungal antibiotics and compounds based on natural products. Many of these can keep fungal infections at an acceptable level but there are several concerns. Some, mainly synthetic agents, can accumulate in nature if biodegradation is slow or even missing, other are toxic not only to fungi but to other organisms as well, including humans, and all have the potential of losing their effect as the targeted pathogens develop resistance. This means that there will probably never be a perfect antifungal agent, a *magic bullet*, which kills the pathogen every time and leaves the treated organism and the rest of the environment unharmed. There will always be a need for new agents and new ways of controlling fungal pathogens.

One alternative way to maintain populations of pathogenic fungi at low levels is by biocontrol agents, *i.e.* organisms that act as natural enemies to the fungi. Various microorganisms have been investigated as potential antifungal biocontrol agents (BCAs) and several have been launched on the market.¹

The high expectations put on BCAs are the result of several factors. The agents can have multiple modes of action, which can make the pathogen less prone to develop resistance. Modes of action of BCA that have been put forward are competition for nutrients and space, induction of the hosts own defense against pathogens (induced systemic resistance), interference with the chemical signaling of the pathogen, parasitism, and production of antifungal metabolites.^{2,3} The mode(s) of action that are the most important ones probably depends on the specific host plant, biocontrol agent, and pathogen combination.

Another benefit of BCAs is that they can be able to propagate in field. Fewer treatments might then be needed during a growth season compared to chemicals. If antifungal antibiotics are produced by the agent, there will be less risk of bioaccumulation, as paths of biodegradation will be present in nature.

When developing potential biocontrol agents it is crucial to investigate the production of any possible antifungal antibiotics. New antifungal metabolites may have new modes of action and, even if the investigated organisms are not suitable for development to a BCA product, the metabolites can still be potential lead

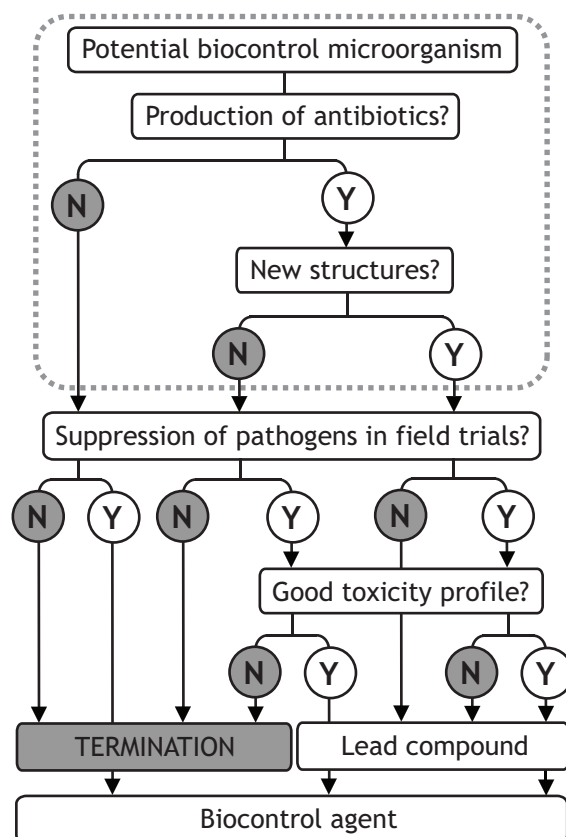


Fig. 1. Work flow for chemical investigation of potential biocontrol microorganisms, with the possible outcomes at the bottom. The work in this thesis deals with the area marked by the dotted rectangle.

compounds for new compound-based antifungal agents. If the potential biocontrol organism is suitable for development to a product, registration of a biocontrol product within the European Union requires investigation of mode of action, production of any antifungal metabolites and their toxicity profile, and a corresponding investigation of *other significant metabolites*.^{4,5} For BCA registration in the USA, discussions and experimental findings along the same lines need to be presented.⁶⁻⁸

This thesis deals with chemical investigation of potential biocontrol microorganisms and is a part of the research program Microbial Antagonism against Fungi (MAaF), which started in 1996. The goal of the program was to find potential antifungal biocontrol agents for the food and feed industry and in agriculture and silviculture. Chemical investigation of potential biocontrol strains is a venture which has several goals, that all have to be weighted against each other. In the end, a new BCA may be developed or a new lead compound may be fed into the agrochemical or pharmaceutical industry, or possibly both (Fig. 1).

2. Scope of the thesis

The overall scope of this thesis was to isolate antifungal compounds from microorganisms exhibiting antifungal activity.

Selection of microbial strains for chemical investigation was mainly based on activity in antifungal bioassays. As several different strain collections were utilized, it was expected that both fungi and bacteria were to be investigated. In order to make this possible, it was needed to develop a robust bioassay-guided isolation method that could function with both fungal and bacterial strains (applied in Papers I-IV).

In this thesis, four projects are included. They are based on isolation of antifungal antibiotics from phyllosphere fungi (Paper I), rhizosphere bacteria (Papers II, III), and from mycorrhizal root bacteria (Paper IV)

3. Definitions

The title of this thesis contains the terms antibiotic, biocontrol and microorganism which all have a broad use in different scientific fields. The mycologist Selman Waksman defined the noun *antibiotic* in 1947 as

*“a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms. The action of an antibiotic against micro-organisms is selective in nature”*⁹

This definition is still valid and will be used in the thesis. The addition of antifungal in *antifungal antibiotic* highlights the antibiotics ability to be detrimental to one or several fungal taxa. The more-inclusive term *antifungal compound* can be used when referring to both natural products and synthesized antifungal compounds. The even broader term *antifungal agent* also includes antifungal biocontrol agents.

The term *biocontrol* will be used in the definition put forward by Paul DeBach in 1964:

*“The action of parasites, predators, or pathogens in maintaining another organism’s population density at a lower average than would occur in their absence.”*¹⁰

There is no scientific definition of *microorganism*, although the term can be clarified to a certain degree. A usable circumscription of a microorganism is that it is a microscopic organism with no or low cell differentiation capable of living as a single cell in its natural habitat. With this definition, bacteria, unicellular algae, protozoae and microscopic fungi, including yeasts, all count as microorganisms. However, some of these organisms can aggregate to form multicellular differentiated macroscopic structures, especially fungi and algae. All microorganisms dealt with in this thesis are either bacteria or fungi.

An individual microorganism can most often be determined to belong to a specific genus. Species definition, however, is never straightforward in any organism group and for microorganisms such as fungi, and especially bacteria, it can be even harder due to their genome flexibility. If unambiguous identification can not be done, an organism can instead be given an individual *strain* designation for identification. In this thesis, *strain* is used in the meaning of a microorganism isolate originating from a single isolated cell or spore. The strain can in turn be identified to the level of family or genus.

The taxonomy of bacteria and fungi is under constant revision. Species and genera can be split, clumped together, moved to other families or be established on new taxonomic levels. Old well-known species names are sometimes used although they have no standing in nomenclature, as new names can be hard to implement. In this thesis, the scientific names in the original references are conserved for ease of recognition, and any taxonomical name changes as of 2005 are included in Appendix A.

4. Antifungal antibiotics

Natural products are an important source of new bioactive compounds and for the drugs launched over the period 1981-2002, Newman *et al.*¹¹ found that 40% were either natural products as is, or modified natural products. In the case of antifungals the share was much lower, 14%, mainly because of the azole, allylamine and morpholine synthetic antifungal compounds. In agriculture, Pachlatko estimated that natural products had only about 10% of the market for crop-protection¹², and thus, the share for antifungal antibiotics was even smaller.

4.1 Control of fungal pathogens on plant and man

Research on new agents for controlling fungi has been a concern both for the agricultural and pharmaceutical industry. This is not least seen in the mergers of pharmaceutical and agrochemical companies like the formation of Novartis in 1996 from Ciba-Geigy and Sandoz and the merger of Astra and Zeneca to AstraZeneca in 1999. Although the knowledge on antifungal antibiotic compounds is shared between the two areas there are important differences.

4.1.1 Pathogens

Important pathogens on humans are *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*¹³ while important pests in agriculture are *Fusarium* spp., *Botrytis* spp., and *Rhizoctonia solani* together with the oomycetes *Phytophthora infestans* and *Pythium* spp. The activity of many antifungal antibiotics is specific and inhibition of one fungal pathogen does not immediately imply that it can inhibit another.

4.1.2 Compound stability and physical properties

Agricultural products need to be compatible with automated administration and sufficiently stable to sun irradiation. In developing drugs for humans, other criteria have to be met, such as formulation and stability to the gut environment.

4.1.3 Costs

In agriculture, the investment spent on controlling fungal pathogens needs to be balanced by the effect on the harvest. A rough estimate of the maximum possible investment in 1998 was 25-50 US dollars/hectare.¹² In contrast, treatment of human mycosis in industrialized countries is more limited by the choice of available drugs than their costs. In comparison, development of a new agrochemical is estimated to take at least four and as much as ten years and cost 150-250 million US dollars^{14,15} while a new drug takes ten to fifteen years with an average cost of 800 million US dollars.¹⁶ Development of a biocontrol agent has been estimated to take as little as three years at an average cost of 6 million US dollars.¹⁷ Note that costs for agrochemicals and biocontrol agents are estimates from industrial sources, while the drug cost is based on academic evaluation.

4.1.4 Human toxicity

In developing new pharmaceuticals, toxicity to humans is closely followed, not least if the goal would be an oral drug (systemic drug). For treatment of critical illness, toxicity can be accepted as for the standard antifungal agent amphotericin B, nicknamed “ampho-terrible”¹⁸, which cause severe kidney injuries.

In agriculture, low toxicity is also a priority, not only to humans but also towards plants and other non-harmful organisms in the field. Human toxicity of agricultural agents can partly be dealt with by careful handling and letting the last treatment have time to wash away or degrade before harvest. In silviculture, however, the main risk is the actual handling of the agent. Biocontrol agents have a potential for controlling fungal infections in the field, and for humans, similar broad prophylactic treatment is feasible. For example, the use of *Lactobacilli* as probiotics is based on their assumed biocontrol ability. Treatment of developed systemic human fungal infections with BCAs, does not seem likely in the near future as the treatment of an infection with essentially another infection is still a too unknown system to control.

4.2 Microbial sources

In the early history of antibiotics, a variety of bacterial and fungal strains were investigated with a majority coming from rhizosphere samples from temperate regions. When streptomycin was discovered in 1944, the main focus turned to streptomycetes. They were found to produce a wide spectrum of structurally diverse bioactive secondary metabolites. During the fifties and sixties, as much as 70% of the reported antibiotics were isolated from this genus. The main change since then has been an increase of compounds isolated from fungi and rare actinomycetes (*i.e.* non-*Streptomyces* actinomycetes).¹⁹ In addition, investigation

of organisms from new habitats or organism groups, and new selection strategies has been presented for improving isolation of new chemical entities. Organisms that inhabit unique niches or areas of high biodiversity should have a high probability of producing unique metabolites. One such area is the marine environment. Although there is much focus today on marine natural products chemistry, large scale investigation of marine microorganisms started as late as in the seventies. Since then, many antifungal compounds of marine origin have been reported and there are probably many more to come.^{20,21}

Microorganisms which live under harsh conditions, so called extremophiles, also have a potential for producing unique metabolites.²² They can be found in, for example, deep sea vents (black smokers), in permafrost, in alkaline or saline lakes, or hot springs. Their adaptations to extreme physical environments have inevitably affected their biosynthesical abilities.

Another specialized habitat is inside other organisms. It has been shown that many plants harbor endophytic microorganisms that can contribute to the metabolic content of the plant tissue.²³ These endophytes have a high degree of specialization, and it is possible that many plant species may have their own endemic species. There is thus a vast biochemical diversity to investigate in these microorganisms.

Although isolation of microorganisms from previously uninvestigated biotopes will yield many new bioactive compounds, estimates propose that only a small fraction (0.1-1%) of all microorganisms can be isolated and propagated with standard culturing conditions.²⁴ There is thus a large genetic diversity that might not be realized. This can be approached by specializing isolation and growth conditions²⁵ or by metagenomics, *i.e.* by inserting biosynthesis genes of uncultivable microorganisms in modified producer organisms.^{26,27}

Genetic methods can also be useful for evaluating the biosynthesis potential of microorganisms. They can be screened for known biosynthesis genes or gene regions and in the best-case scenario, qualified suggestions can be made of which compounds the strain can produce. This has been done successfully for polyketide synthase gene sequences. The approach will improve as more sequencing data of known producer strains will become present.^{28,29}

Nevertheless, one should not abandon the more easily accessible microorganisms. Some of these, like streptomycetes and pseudomonads, are capable of producing a spectrum of chemically diverse metabolites, and can be said to be metabolically talented. By changing the culturing conditions for such strains, different metabolic outputs can be obtained, and even new compounds can be isolated. This approach is known as OSMAC (one strain – many compounds).^{30,31}

4.3 Structural diversity of antifungal antibiotics

Antifungal antibiotics display a wide structural diversity and their modes of action target a number of fungal enzymes and physical structures. In the overview below, they are presented according to compound class. Original articles for isolation and structure elucidation are cited as far as possible.

4.3.1 Carbohydrates

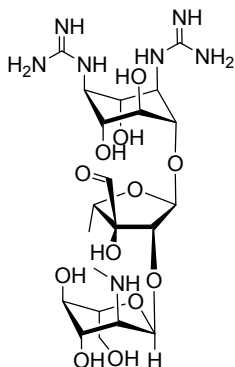


Fig. 2. Streptomycin.

The streptomycins (Fig. 2) were isolated for their antibacterial properties and proved effective against the much feared human pathogen *Mycobacterium tuberculosis*, the cause of tuberculosis. The bactericidal effect is based on inhibition of protein synthesis. Streptomycin binds to the ribosome, and causes misreading of the genetic code.³² This, in turn, permits even more influx of the compound to the bacterium cytosol. They were originally isolated from strains of *Streptomyces griseus* in 1944³³, and were also found to be effective for controlling fungal pests on pears, apples and tobacco.³⁴

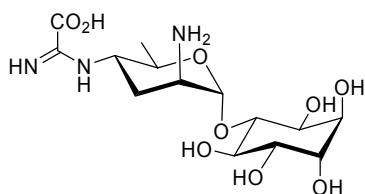


Fig. 3. Kasugamycin.

The disaccharide kasugamycin (Fig. 3) was isolated from *Streptomyces kasugaensis* in 1965³⁵ and the structure was elucidated the year after.³⁶ It blocks protein synthesis and shows low toxicity to humans.¹² Kasugamycin is used for treatment of *Pyricularia oryzae* but as the pathogen rapidly develops resistance to the compound, it is used combination with other fungicides.¹

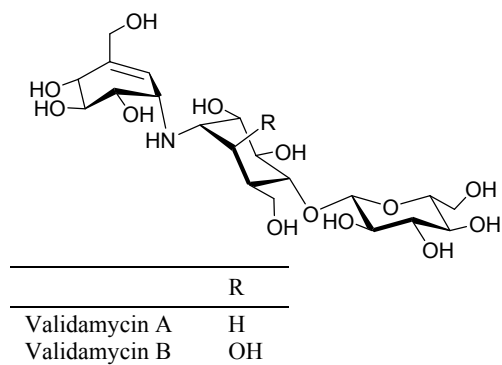


Fig. 4. Validamycins.

The validamycin group of antifungal antibiotics (Fig. 4) were discovered from a culture of *Streptomyces hygroscopicus* in 1970.^{37,38} The target pathogen was *Rhizoctonia solani* and validamycin A and B were later found to control *R. solani* on cucumbers in the field. Structure elucidation revealed that they were aminotrisaccharides.³⁹⁻⁴¹ The compounds are metabolized in fungal cells to validoxylamines, trehalose analogues (Fig. 5), which inhibit the enzyme trehalase.⁴² The trehalase enzyme is needed for regulation of trehalose levels in the fungal cell. Trehalose is thought to be critical in stress metabolism, and for cell wall stability.^{43,44}

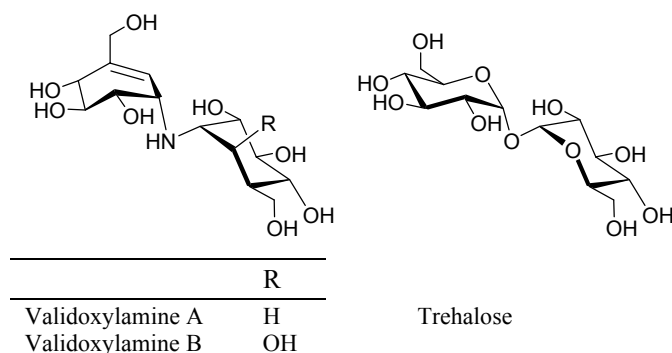


Fig. 5. Validoxylamines, trehalose analogues which inhibit the enzyme trehalase.

4.3.2 Amino acids and peptides

Single amino acids as well as peptides and proteins have been found to have antifungal activities. The cyclic β -amino acid cispentacin (Fig. 6) was isolated from *Bacillus cereus* in 1989⁴⁵ and the year after under the name FR109615 from *Streptomyces setonii*.^{46,47} Cispentacin acts by interfering with amino acid metabolism as well as by inhibiting isoleucin-tRNA synthase.⁴⁸ The synthetic cispentacin analogue BAY 10-8888/PLD-118 (Fig. 6) has entered clinical trials for treatment of candidiasis.⁴⁹

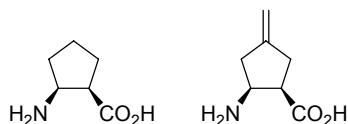


Fig. 6. The amino acid cispentacin (left) and its synthetic analogue BAY 10-8888/PLD-118 (right).

A large group of cyclic peptides, lipopeptides and lipodepsipeptides (containing one or several ester bonds) have been isolated on their antifungal activity, and they act by several different modes of action. Of the antifungal cyclic peptides, aureobasidin has gained much attention since its discovery in 1991.⁵⁰

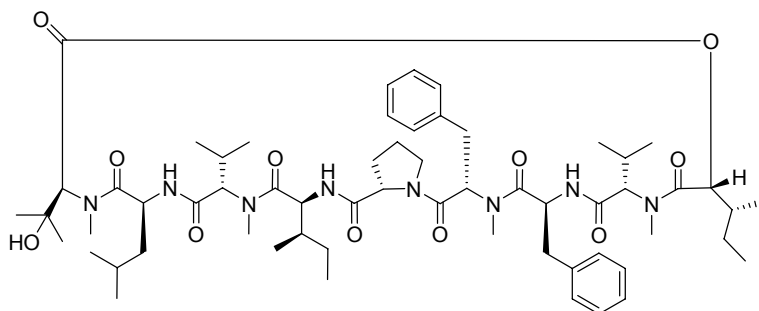
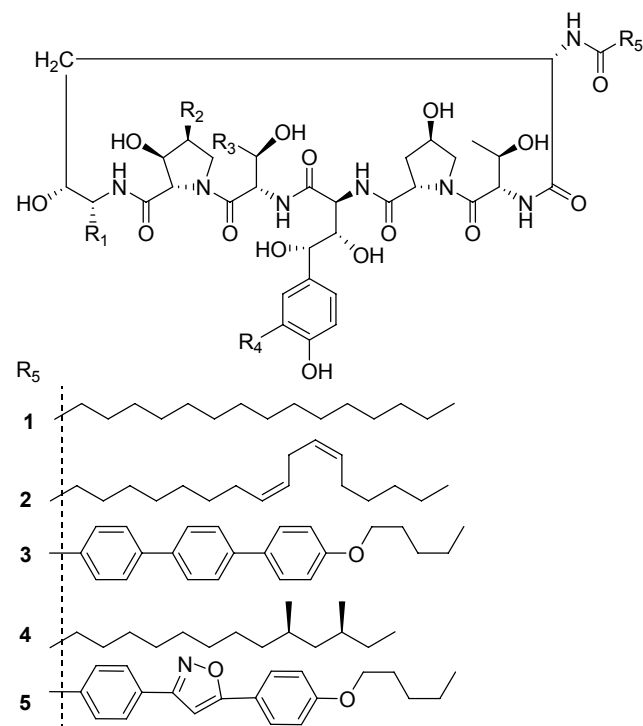


Fig. 7. Aureobasidin.

Aureobasidin (Fig. 7) was isolated from the fungus *Aureobasidium pullulans* and was found to inhibit the enzyme inositol phosphoceramide (IPC) synthase which is critical in fungal sphingolipid biosynthesis. Sphingolipids, in turn, are vital for the function of the cell membrane for fungi and humans alike. The IPC enzyme, on the other hand, is lacking in mammals, which means that all compounds selectively inhibiting IPC are highly interesting for development of antifungal drugs.

The echinocandins are cyclic lipohexapeptides (Fig. 8) which interfere with the fungal glucan synthesis through inhibiting the β -1,3-D-glucan synthase enzyme complex.⁵¹ β -1,3-D-glucan is a major constituent of the outer parts of the fungal cell wall. The earliest member of the class to be isolated was echinocandin B. The compound was discovered from a strain of the fungus *Aspergillus nidulans* var. *echinulatus* in 1972 in a random screening at Ciba-Geigy.⁵² Researchers at Sandoz

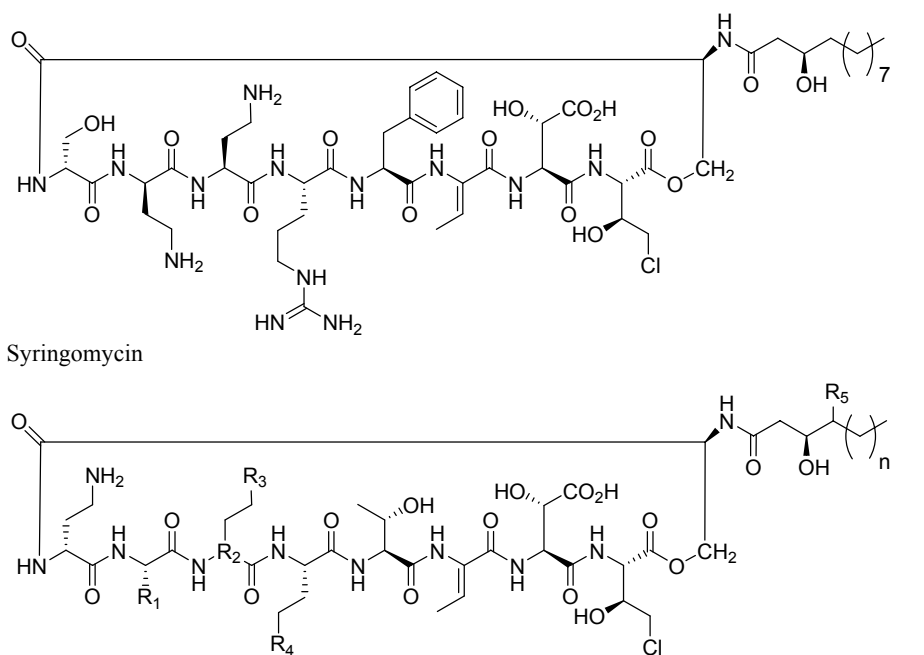
had independently isolated echinocandin B at about the same time and they published the full structure from a sample produced by *Aspergillus rugulosus*.⁵³



	R ₁	R ₂	R ₃	R ₄	R ₅
Aculeacin A _γ	OH	CH ₃	CH ₃	H	1
Echinocandin B	OH	CH ₃	CH ₃	H	2
Anidulafungin	OH	CH ₃	CH ₃	H	3
Pneumocandin B ₀	OH	H	CH ₂ CONH ₂	H	4
Caspofungin	NHCH ₂ CH ₂ NH ₂	H	CH ₂ CH ₂ NH ₂	H	4
WF11899A ⁵⁴	OH	CH ₃	CH ₂ CONH ₂	OSO ₃ H	1
Micafungin	OH	CH ₃	CH ₂ CONH ₂	OSO ₃ H	5

Fig. 8. Echinocandin-type antifungal antibiotics and their semisynthetic derivatives. Anidulafungin is a semisynthetic derivative of echinocandin B while caspofungin is based on a pneumocandin core. Micafungin is a semisynthetic derivative of WF11899A.

Echinocandins and related compounds (Fig. 8) like pneumocandins^{55,56} and aculeacins⁵⁷ have been used as lead structures in drug development. Three semisynthetic derivatives have been marketed as systemic antifungal drugs for human use, caspofungin in 2001, micafungin in 2002, anidulafungin in 2006. The two first are targeted against invasive aspergillosis, while anidulafungin is used for treatment of candidiasis.



Syringomycin

	R ₁	R ₂	R ₃	R ₄	R ₅	n
Syringostatin A	CH ₂ CH ₂ NH ₂	R	OH	CH ₂ NH ₂	H	9
Syringostatin B	CH ₂ CH ₂ NH ₂	R	OH	CH ₂ NH ₂	OH	9
Syringotoxin B	H	R	OH	CH ₂ NH ₂	H	9
Pseudomycin A	CH ₂ COOH	S	CH ₂ CH ₂ NH ₂	NH ₂	OH	9
Pseudomycin B	CH ₂ COOH	S	CH ₂ CH ₂ NH ₂	NH ₂	H	9
Pseudomycin C'	CH ₂ COOH	S	CH ₂ CH ₂ NH ₂	NH ₂	H	11

Fig. 9. Examples of cyclic lipodepsinonapeptides produced by *Pseudomonas syringae*.

The bacterium *Pseudomonas syringae* pv. *syringae* has been the source of a number of lipodepsinonapeptides: syringomycins,^{58,59} syringotoxins,^{60,61} syringostatins,⁶² and pseudomycins (Fig. 9).^{63,64} They are thought to interact with the fungal cell membrane by pore formation, which results in fatal electrolytic leakage.⁶⁵ The structures were elucidated in the late eighties and early nineties⁵⁹ and they show activity against *Candida* spp. and *Aspergillus* spp.

For a more detailed account of antifungal and antimicrobial peptides and lipopeptides, see the reviews of Balkovec,⁶⁶ de Lucca and Walsh,⁶⁷ and Chen.¹³ For an introduction to antifungal proteins, see the review of Theis and Stahl.⁶⁸

4.3.3 Terpenes

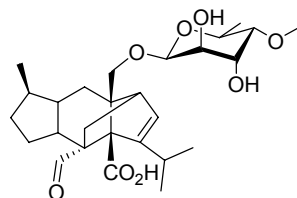


Fig. 10. Sordarin.

Sordarin (Fig. 10) was isolated from the fungus *Sordaria araneosa* and patented in 1969 through Sandoz. Two years later, the isolation procedure and structure was published.⁶⁹ The compound consists of the tetracyclic diterpene aglycone sordaricin and a glycosyl moiety. Several natural analogues with different glycosylation units were isolated in the nineties and showed potent activity against certain *Candida* spp.^{70,71} The compounds interrupt protein synthesis in the fungus by inhibiting one of the protein elongation factors, EF2. Mammalian cells are not effected.⁷²

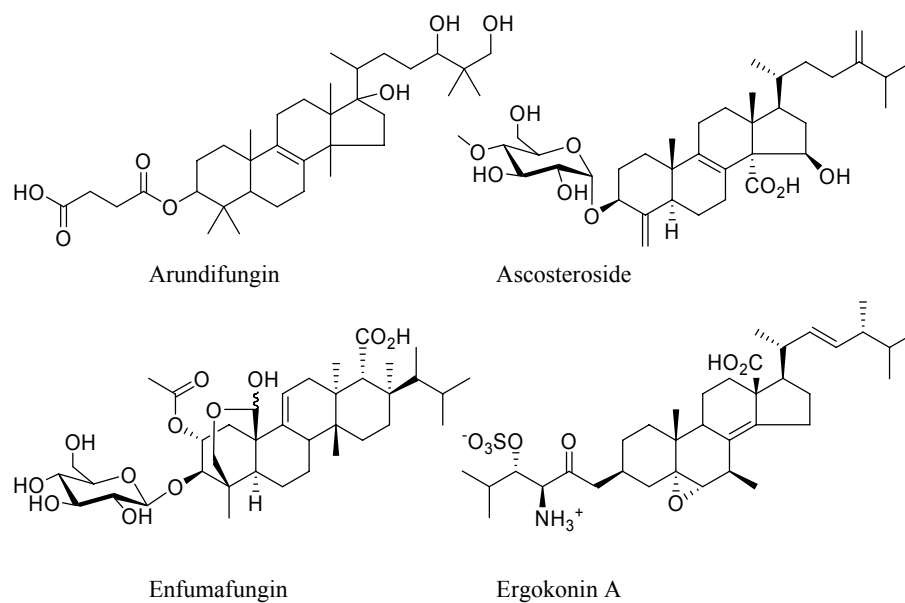


Fig. 11. Polar triterpenes inhibiting the enzyme β -1,3-D-glucan synthase.

A group of polar triterpenes were reported in year 2000, from a screening program aimed at finding inhibitors of β -1,3-D-glucan synthase (Fig. 11). They include the fungal metabolites enfumafungin from a *Hormonema* sp.,^{73,74} ascosteroside from *Ascotricha amphitricha*,⁷⁵ arundifungin from *Arthrinium arundinis*^{75,76} and ergokonins from *Trichoderma koningii*.⁷⁷

4.3.4 Polyketides

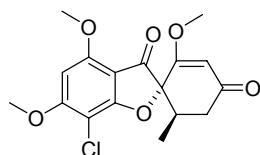


Fig. 12. Griseofulvin.

The polyketide griseofulvin (Fig. 12) is considered as the first compound isolated on its antifungal properties. It was isolated from *Penicillium griseofulvum* in 1939,⁷⁸ with structure elucidation performed some years later.⁷⁹ The compound was shown to be active against *Botrytis alli*, which causes neck rot in onions, and it has been used for treating *Botrytis* infections in agriculture.³⁴ The compound was launched in 1958 as the first systemic fungicide in treatment of human mycosis.⁸⁰ It is fungistatic and inhibits cell division at higher concentrations through destabilization of the microtubuli, possibly in the same way as other antimetabolic compounds such as vinblastine and paclitaxel.⁸¹

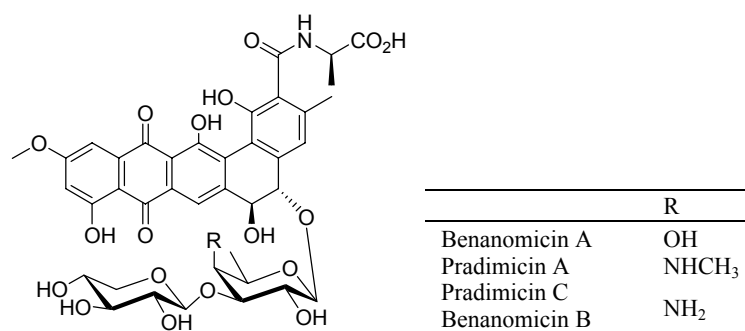


Fig. 13. Pradimicins and benanomicins.

Pradimicins,⁸² and benanomicins^{83,84} are glycosylated benzonaphthacene-quinones (Fig. 13) and were independently isolated by two research groups in 1988 from actinomycetes. They bind to the mannan part of mannoproteins which is present at the cell surface on many fungi. Binding is followed by rupture of the fungal cell membrane. The glycone is responsible for mannan association while the carboxylic acid is needed for fungal cell rupture.⁸⁵

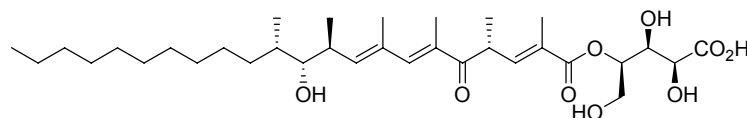


Fig. 14. Khafrefungin.

The linear polyketide khafrefungin (Fig. 14) was isolated from an unidentified deuteromycete^{86,87} in 1997 and was found to interfere with sphingolipid biosynthesis by inhibiting inositol phosphoceramide (IPC).

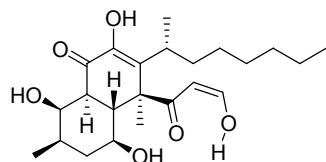


Fig. 15. Australifungin.

Australifungin (Fig. 15), from *Sporormiella australis*,^{88,89} was isolated in the same screening project as khafrefungin, but as an inhibitor of ceramide synthase. This enzyme is placed one step earlier than IPC in sphingolipid biosynthesis.

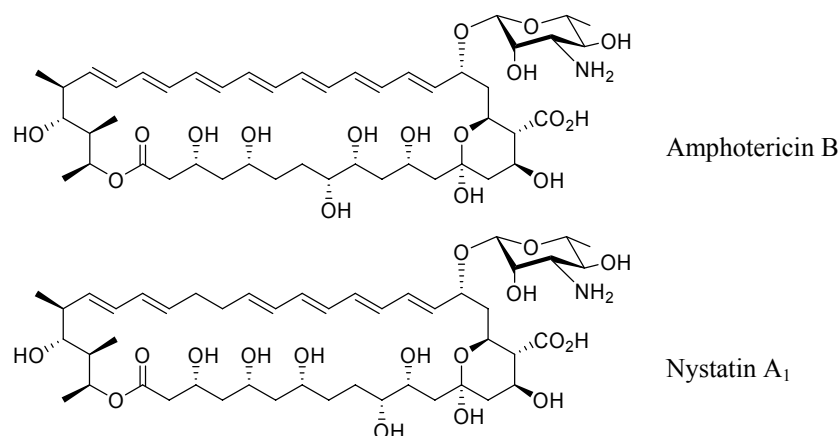


Fig. 16. The antifungal macrolides amphotericin B and nystatin A₁. Commonly, nystatin A₁ is called only nystatin.

Polyketide macrolides and polyene macrolides have been much investigated for antifungal activity and the latter contain some of the most important antifungal drugs for human use, amphotericin B and nystatin (Fig. 16).

Nystatin was isolated from a soil actinomycete in 1950 by Hazen and Brown.⁹⁰ They named it fungicidin after its prominent property, but later renamed it to nystatin. It could be used to treat systemic fungal infections in humans as well as be used as a topical antifungal, but finding a good formulation for oral administration has been a problem till this day. In 1956, amphotericin B was isolated by Gold *et al.*⁹¹ and this compound was launched as an oral antifungal drug in 1960. This was to be the standard treatment of systemic mycosis until the eighties, when synthetic antifungal antibiotics such as imidazoles and triazoles were launched. Both nystatin and amphotericin B are 37-membered macrolactones containing a cyclic hemiketal.⁹²⁻⁹⁴ The polyenes bind irreversibly to ergosterol, which is a major constituent of the fungal cell wall. The cell wall is destabilized, possibly by pore formation by polyene octomers, which causes cell leakage.⁹⁵ Many more antifungal polyenes have been isolated from natural sources,⁹⁶ but few

have been applied as agricultural antifungal agents because of their photosensitivity and easy oxidation.³⁴

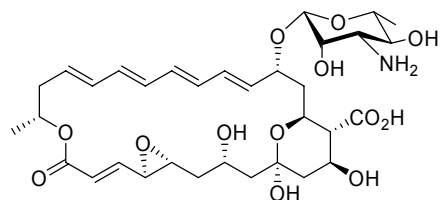


Fig. 17. Natamycin.

The epoxide polyene natamycin (Fig. 17) is an exception.⁹⁷ It was isolated in 1957 from *Streptomyces natalensis* under the name pimaricin⁹⁸ and from *Streptomyces chattanoogensis* under the name tennecetin.⁹⁹ It is used for treating *Fusarium oxysporum* basal rots in ornamental plants.¹

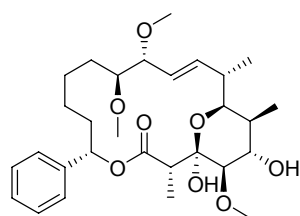


Fig. 18. Soraphen A.

Soraphen A (Fig. 18) is an 18-membered macrolide isolated from the myxobacterium *Sorangium cellulosum* in 1994.¹⁰⁰ Several agricultural pathogens could be controlled by the compound, including *Erysiphe graminis*, *Gerlachia nivalis* and *Botrytis cinerea*. It was found to inhibit the fungal acetyl coenzyme A carboxylase¹⁰¹ but had no effect on the corresponding plant enzyme. However, it *did* inhibit the corresponding mammalian enzyme. Together with an discouraging toxicity profile, this disqualified it as an agricultural antifungal antibiotic.¹²

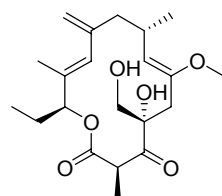


Fig. 19. Rustmicin or galbonoid.

Rustmicin¹⁰²/galbonolid^{103,104} (Fig. 19) is a 14-membered macrolide found to inhibit the sphingolipid IPC synthase. It has been isolated from the bacteria *Micromonospora chalcea* and *Streptomyces galbus*

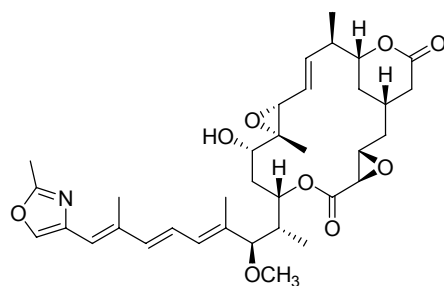
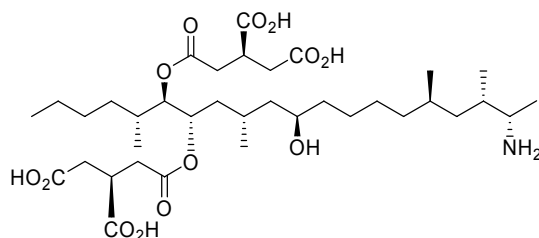


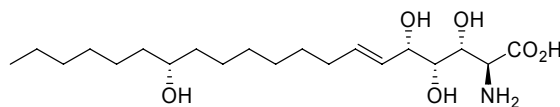
Fig. 20. Rhizoxin.

The antifungal activity of the 15-membered macrolide rhizoxin (Fig. 20) is based on interference with microtubuli dynamics.¹⁰⁵ Rhizoxin and analogues were isolated from a fungal *Rhizopus chinensis* strain^{106,107} but have also been found in cultures of *Pseudomonas* (Paper II).

4.3.5 Fatty acids and fatty acid mimics



Fumonisin B₁



SpHINGOFUNGIN B

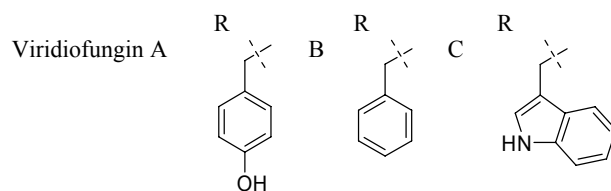
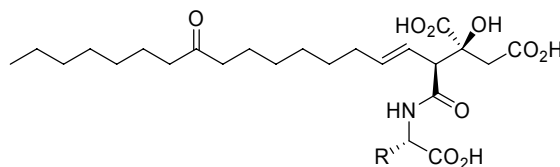


Fig. 21. SpHINGOLIPID biosynthesis inhibitors.

Several structures with fatty acid or fatty acid-like moieties have been isolated as antifungals, specifically in conjunction with screening for inhibition of enzymes connected with spHINGOLIPID biosynthesis.¹⁰⁸ These include the fumonisins from

Fusarium moniliforme,^{109,110} the sphingofungins from *Aspergillus fumigatus*,¹¹¹⁻¹¹³ viridifungins from *Trichoderma viride*¹¹⁴⁻¹¹⁶ and myriocin¹¹⁷/ISP-I^{118,119} from *Myriococcum albomyces*/*Isaria sinclairii* (Fig. 21).

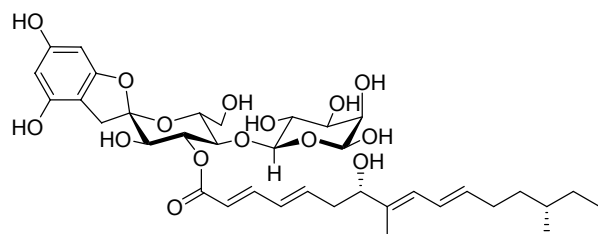


Fig. 22. Papulacandin D, a representative of the papulacandin glycolipids.

The papulacandin glycolipids (Fig. 22), on the other hand, inhibit glucan biosynthesis. They were isolated from a *Aspergillus aculeatus* strain in 1977.¹²⁰⁻¹²²

4.3.6 Nucleoside analogues

Although the following compounds all can be described as nucleoside analogues, the suggested mechanism of antifungal activity is not in all cases interference with DNA/RNA dynamics.

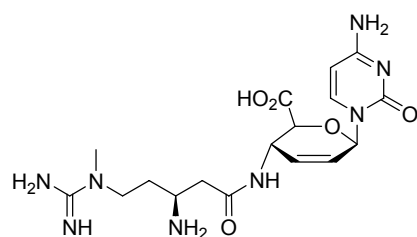


Fig. 23. Blasticidin S.

Blasticidin S (Fig. 23), a metabolite of *Streptomyces griseochromogenes*, was isolated in 1958¹²³ and has been used commercially for control of *Pyricularia oryzae* from the early sixties up till today.^{1,124} The compound blocks protein synthesis in both prokaryotic and eukaryotic cells.¹²⁵ Blasticidin S has toxic and phytotoxic effects and the use dropped when alternatives like kasugamycin became available.¹²

Polyoxin A (Fig. 24) was isolated in 1965 on a sample from a *Streptomyces cacaoi* strain.¹²⁶ The structure of polyoxin A and several analogues were published shortly afterwards.^{127,128} They selectively inhibit the fungal enzyme chitin synthetase, which polymerizes *N*-acetylglucosamine to chitin.¹²⁵ Chitin is a major constituent of the cell wall core in most fungi, This specific mechanism of the polyoxins is highly interesting for drug development as chitin is not present in human cells. In agriculture the polyoxins are effective for treatment of a variety of fungal pathogens, especially *Alternaria* spp. and *Botrytis cinerea* [polyoxin B (Fig. 24)], and *P. oryzae* [polyoxin D (Fig. 24)].¹

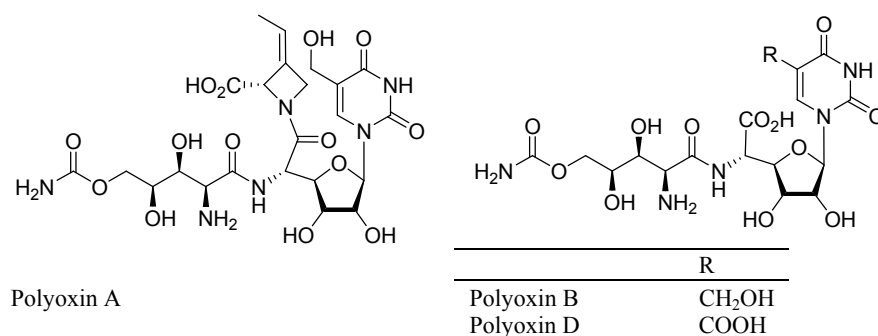


Fig. 24. Polyoxins from *Streptomyces cacaoi*.

A polyoxin-like group of antifungal (and bonus acaricidal) compounds with similar mechanism of action are the nikkomycins. The first compound, nikkomycin Z (Fig. 25), was isolated from *Streptomyces tendae* in 1970 with structure elucidation some years later.¹²⁹ Nikkomycins were developed as promising agricultural antifungals but research was terminated for cost reasons.¹²

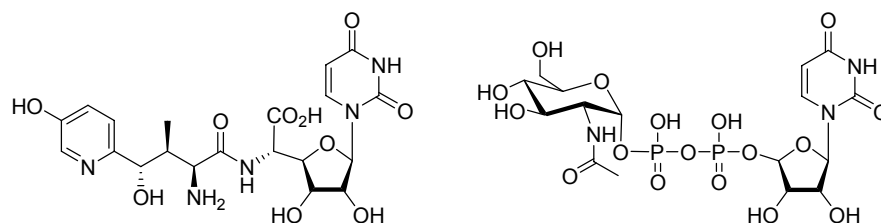


Fig. 25. Nikkomycin Z (left), is a mimic of UDP-*N*-acetyl-D-glucosamine (right) which is a building block in chitin synthesis.

Despite large efforts in drug development, no candidate has yet entered clinical trials although nikkomycin Z shows promising preclinical results in combination with fluconazole.¹³⁰ For an in-depth picture of the development of the polyoxins and nikkomycins, see the review of Zhang and Miller.¹³¹

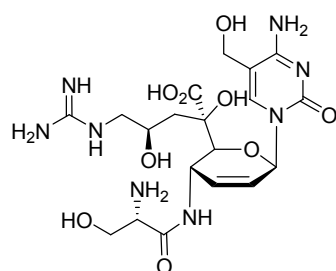


Fig. 26. Mildiomycin.

Mildiomycin (Fig. 26) is yet another peptidyl nucleoside. It was isolated from a strain of *Streptovercillium rimofaciens* in a project targeted against *Erysiphe graminis*.¹³²⁻¹³⁵ and it acts by inhibiting fungal protein synthesis.¹²⁵

4.3.7 Other biosynthetic origin

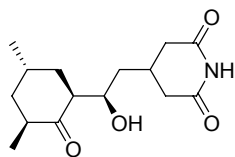


Fig. 27. Cycloheximide.

The compound cycloheximide (Fig. 27), also called actidione, was isolated as a minor component in a streptomycin-producing strain of *Streptomyces griseus* in 1946¹³⁶ and its structure was published two years later.^{137,138} The compound selectively inhibits protein synthesis in eukaryotic cells through blocking translation of mRNA. It has been used in agriculture and silviculture but due to its high phytotoxicity it is a less-than-ideal antifungal agent.^{34,124}

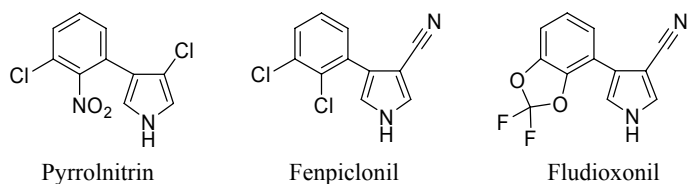


Fig. 28. Pyrrolnitrin and two synthetic analogues, fenpiclonil and fludioxonil.

The small tryptophan derived antifungal antibiotic pyrrolnitrin¹³⁹ (Fig. 28) was isolated in 1964 from a strain of *Pseudomonas pyrrocina*.¹⁴⁰ The compound was found to be sensitive to light and analogues were synthesized at Ciba-Geigy to enhance its photostability. The efforts resulted in the two agricultural commercial compounds fenpiclonil and fludioxonil (Fig. 28) used in seed coating.¹² Pyrrolnitrin acts by inhibiting mitochondrial respiration.^{141,142}

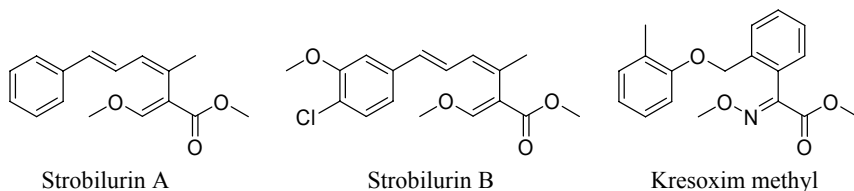


Fig. 29. Strobilurin A and B and the synthetic analogue kresoxim methyl.

The β -methoxyacrylic acid strobilurin A (Fig. 29) was first isolated in 1967 from a strain of *Oudemansiella mucida* under the name mucidin¹⁴³ and later independently isolated from *Strobilurus tenacellus* together with strobilurin B (Fig. 29).¹⁴⁴ The strobilurins inhibit mitochondrial respiration in fungi and were found to control several important plant pathogens but the natural compounds suffered from photoinstability. Synthetic modifications of the strobilurins have resulted in more stable and active compounds and the first product for agricultural use, kresoxim methyl (Fig. 29), was launched by BASF in 1996. The strobilurins are at present an important group of agricultural fungicides.¹⁴⁵

4.3.8 Diversity of structures and targets

This background on antifungal antibiotics is in no way comprehensive but does present the major groups of natural products to which hopes of controlling fungal pathogens have been put. The survey also shows that many fungal subcellular processes can be targets for antifungal compounds (Fig. 30). Basically, any protein that is necessary for the growth of a fungal pathogen is a potential antifungal target. In the model fungus *Saccharomyces cerevisiae*, over 1100 genes have been found to be essential for growth on rich media.¹⁴⁷ This gives an indication that there are many potential targets which could be used for antibiotic screening. There are also efforts for finding genetic targets that are not part of basic fungal metabolism.

One approach could be to target virulence factors. These are many times pathogen specific, and by only focusing on the mechanism of infection for pathogen control, the pressure for development of resistance is lower.¹⁴⁸ However, bioassays based on sub-cellular targets, such as interference with protein production or inhibition of enzymes, investigates only one range of antifungal modes of action. Monitoring physical interactions with existing fungal cell structures can also be of value, exemplified by the cell membrane association of the benanomicins, all syringo-peptides and the macrolides. None of these antibiotics would have been discovered in an enzyme-based approach.

Major components of the fungal cell wall:

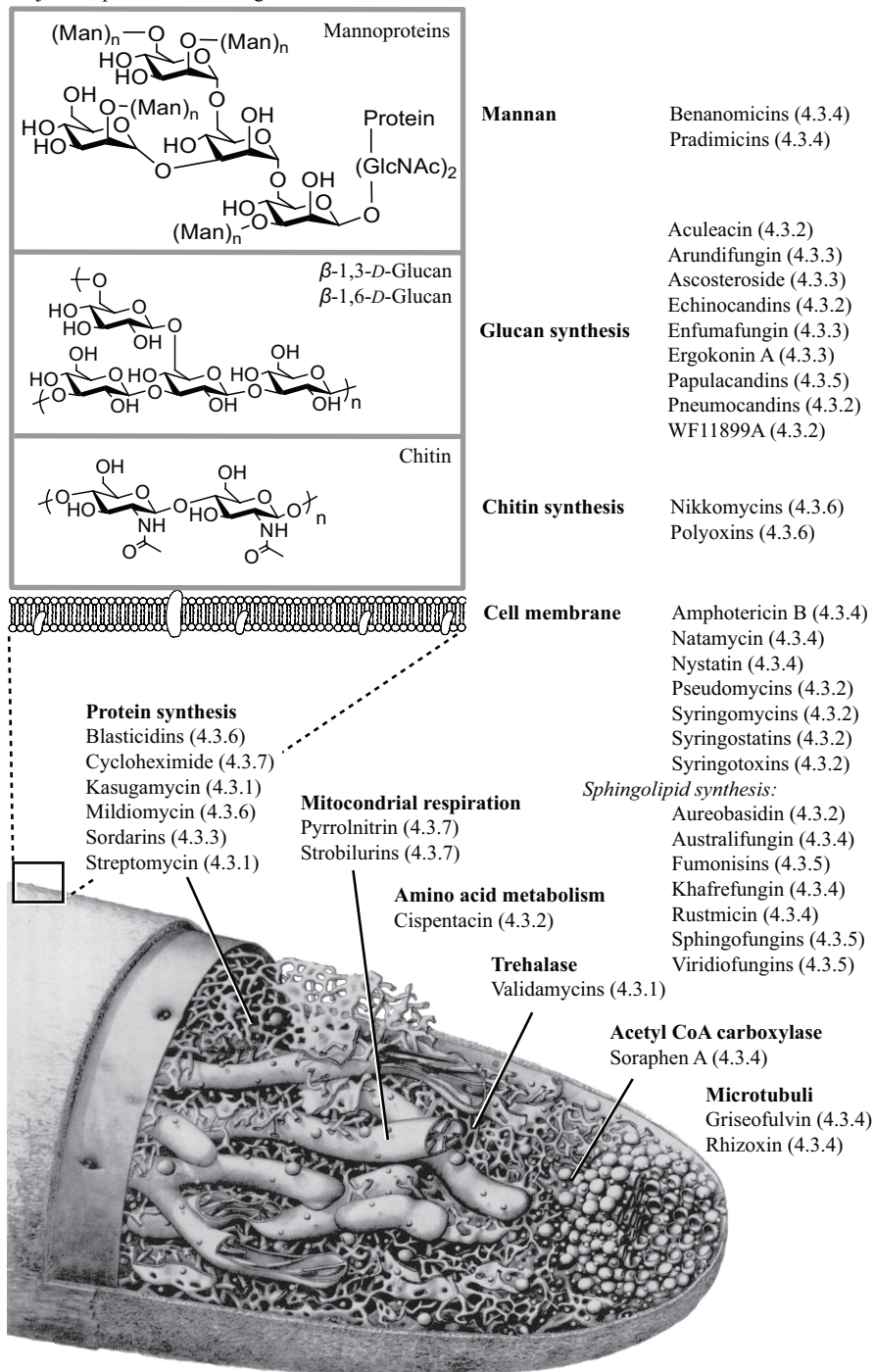


Fig. 30. Antifungal antibiotics grouped according to fungal target. Numbers in parenthesis refer to chapters in the thesis (hyphal tip after Girbardt, ref 146).

5. Isolation of antifungal antibiotics

This chapter presents an introduction to a selection of useful methods and techniques associated with natural product chemistry. Any thorough descriptions of the methods was outside the scope of the thesis, and the interested reader will find further reading in the review articles and books cited in this section.

5.1 Bioassays

In 1972, the first antifungal bioassay based on a sub-cellular target was published by Ragsdale and Sisler.¹⁴⁹ They presented an assay monitoring ergosterol inhibition in the smut *Ustilago maydis*. Although they did not target a specific enzyme in ergosterol biosynthesis, the approach was definitely new, in comparison with earlier assays which monitored inhibition of the whole pathogen cell, or spore. They presented a *single-target specific bioassay* in contrast to the earlier *multi-target functional bioassays*.¹⁵⁰

Another way to categorize bioassays is into *in vivo* and *in vitro* bioassays, where the *in vivo* assays more closely resembles the system where the tested agent should be used.¹⁵¹ In agriculture, an *in vivo* assay could mean spraying a potential antifungal agent on living plants infected with a fungal pathogen, or compare incidence of natural infection between non-treated fields with treated fields. Clearly, *in vivo* assays of this kind are not well suited for rapid bioassay-guided isolation of antifungal antibiotics. Too much material is needed, they are very space consuming, and assay cycle time might be very long. Instead, different types of *in vitro* assays are used. Taking an antifungal agent all the way to the market does, however, include *in vivo* assays but keeping them to a minimum will save both time and money.

The *in vitro* assays can be divided into diffusion and dilution assays.¹⁵² In diffusion assays, the sample needs to diffuse through a solid medium which also contains the test organism. The classical diffusion assay consists of placing a paper disk soaked with the test compound on an agar plate inoculated or plated with fungal spores, or pipetting a solution of the sample to a small well in the agar plate. The size of any mycelium-free zone around the sample after inoculation is used as the bioassay reading. Agar is compatible with hydrophilic and moderately lipophilic compounds but highly lipophilic compounds and charged compounds do not diffuse well. For lipophilic compounds normal phase TLC plates have been used as solid support,¹⁵³ although it has been proposed that minimal inhibitory concentration (MIC) values from this method might be overestimated in comparison with other assays.¹⁵¹ Diffusion assays are very versatile and do not require expensive equipment. However, the observed biological activity is hard to correlate to exact concentrations of the test compounds as the inhibition zones depend on concentration gradients. The relative large size of the individual assays also makes it laborious to test many samples.

In dilution assays the test organism or sub-cellular target is suspended in a solution where the sample concentration can be controlled. Solubility of the samples may pose a problem and can be improved by additions of detergents or

organic solvents at levels that do not affect the assay organism too much. Dilution assays are easily adapted to automation and can be used with microtiter plates in the 96 (Papers I-IV), 384, and even 1536 format. Most enzyme-based assays belong to this category, as well as the dilution microtiter bioassay used in the work of this thesis (see Chapter 7.3), where testing is done on cell free supernatant of microbial liquid cultures.

5.1.1 Bioassays in chemical investigation of BCAs

Chemical investigation of potential BCAs is aimed at identifying known, as well as new antibiotics. An ideal bioassay should therefore be non-specific in terms of mode of action. It should be able to detect antifungal and fungistatic effects regardless of what fungal enzyme might be inhibited or whatever physical barrier in the fungus that is affected, *i.e.* it should be a multi-target functional bioassay. This can be achieved by basing an assay on inhibition of fungal cell growth or fungal spore germination.

5.2 Dereplication

Dereplication in natural product screening means early elimination of uninteresting entities. By removing them from investigation, time is saved. The process can be done at different stages in the screening process, both in selection of strains and during isolation. Entities that should be removed are non-active strains, excess numbers of metabolically similar strains *etc.* In isolation, known compounds are the main target for dereplication. Strain and compound dereplication is many times overlapping. To speed up dereplication, a reference database is very useful, may it be commercial or set up by the individual research group.

5.2.1 Strain dereplication

Genetic similarity of microorganisms is expected to mirror similarity in metabolite production, virulence and overall performance. For bacteria, the preferred part of the genome to base comparison on is the gene for the 16s subunit of the ribosome (16s rDNA) or the corresponding RNA (16s rRNA). This most often provides resolution at the genus level. For fungi, the internal transcribed spacer (ITS) region is widely used. Any obtained sequence can be compared in-house and with online database collections such as GenBank.¹⁵⁴

Strain dereplication can also be achieved by looking at the expression of metabolites directly, *i.e.* metabolic profiling. Methods used includes screening with ESIMS of extracted agar plugs,¹⁵⁵ GC-MS of volatile metabolites,¹⁵⁵ direct injection in ESIMS of SPE concentrated liquid supernatant,¹⁵⁶ liquid-liquid extracted supernatant¹⁵⁷ and MALDITOF of whole bacterial cells¹⁵⁸. Several NMR screening protocols have also been applied.^{159,160} The data can be interpreted in two different ways. Either the data is analyzed for presence of specific known metabolites and a decision is taken whether the strains are interesting for further investigation, or the data is used for grouping strains according to metabolic similarity, and strains are selected depending on their position in the cladogram, PCA plot *etc.* However, the most important dereplication process when working

with potential BCAs, is selection for strains that perform well in bioassays, thus deselecting strains without biological activity.

5.2.2 Compound dereplication

In compound dereplication there is always a trade-off between speed and accuracy. The less data used to identify a compound, the faster will dereplication proceed but the risk of discarding interesting hits will also increase. Identification could be based on mass spectra, MS fragmentation patterns, HPLC retention times, UV absorption spectra, ¹H NMR spectra or any combination of these. Large databases exist for on-line identification of compounds by EIMS but as EI ion sources are not suitable for HPLC coupling they are not of much help for rapid dereplication of strains grown in liquid culture. Useful databases include Antibase, MarinLitt, CASonline and the Dictionary of Natural Products. However, dereplication depends on manual comparison of own data with the databases and any automation is normally achieved by building an own in-house database.¹⁶¹ Efforts towards standardization or cross-platform compatibility would be much appreciated.

5.2.3 Dereplication in search of BCAs

For a microbial biocontrol agent, the performance in the field is the most important trait. The organism needs to survive long enough to exert its effect on any pathogen. One way of improving the chances for this is to collect strains for screening from sites that are similar to the sites where a possible end-product will be used. When targeting agricultural pathogens in a temperate climate this approach rules out the use of extremophiles, and to a certain extent also endophytes. Instead, the best places to look are the classical rhizosphere and phyllosphere. In order to obtain representative or unique strain collections from these sources, one must apply unique isolation protocols. This could include use of specific isolation media, physical thresholds or selection on morphological traits, as well as early field trials or greenhouse trials.

If specific isolation protocols are used when collecting strains, there is a high probability of isolating many similar strains, which means that strain dereplication is essential before chemical investigation. However, a collection of similar isolates can also be an asset as minor genetic differences can influence traits as viability, tolerance of storage or virulence.

5.3 Sample preparation and isolation

Bioactive natural products are often present in low concentrations. In extreme cases, metabolites can accumulate up to a few percent of organism dry weight or growth medium, but often the concentrations are much lower, usually at the ppm or sub-ppm level. Different types of sample preparation are needed for removing the bulk matrix and concentrating the metabolites prior to chromatographic separation.¹⁶² Traditionally, liquid-liquid extraction has been used for extracting metabolites and the method is well suited for working with extracts from solid or liquid matrix. The main drawbacks are high consumption of organic solvents and

difficulties in automatization. When working with liquid microbial cultures, solid phase extraction (SPE) is a good alternative.

5.3.1 Solid phase extraction

Solid phase extraction (SPE)¹⁶³ relies on the same principles as all other chromatographic techniques; the distribution of the analyte between a stationary phase and a mobile phase. There exists a plethora of stationary phases for SPE, spanning most chromatographic separation mechanisms. In natural products isolation, octadecyl-bonded silica (ODS, or more commonly C₁₈) is a useful material for isolating analytes of a wide lipophilic range. This can be complemented with graphitized carbon or short-chain alkyl-bonded silica for less lipophilic compounds. The solid phase comes in pre-packed disposable columns and in bulk for packing columns of desired size.

The aqueous sample solution is passed through the column and the analytes are adsorbed on the stationary phase. The material is then desorbed with a small amount of organic solvent. This results in a concentrated lipophilic fraction and a hydrophilic fraction with the same size as the loading volume. SPE has very low resolution when compared to HPLC and is used as a kind of digital or discrete chromatography. Either a specific compound is adsorbed on the column during sample loading (ON), or passes right through. The column is then eluted with different solvent, and the adsorbed compound is either displaced (OFF) or is still bound to the column. Any intermediate results are unwanted. The technique is easily automated¹⁶⁴ and makes use of small amounts of organic solvents.

5.3.2 High performance liquid chromatography

High performance liquid chromatography (HPLC)¹⁶⁵ is a standard analytical and preparative technique for fractionating complex samples to obtain pure analytes. Separation of samples in HPLC depends on the choice of stationary phase, mobile phase, temperature and flow rate. The C₁₈ stationary phase is, as in SPE, a default starting point in sample isolation. The mobile phase is usually a combination of water and a miscible organic solvent such as MeOH or MeCN. A common modification of the mobile phase is control of pH by acids or bases and organic or inorganic buffers. The HPLC system can be coupled to a number of detectors depending on the type of samples analyzed. Common detectors are refraction index (RI), UV/VIS, and MS detectors, of which the two latter are often combined. The more information present of the analyte, the more specific detection methods can be used in order to increase sensitivity.

Purification of complex samples on HPLC, such as SPE-fractions from culture broths, is preferentially started with a standardized gradient run that will give a coarse fractionation of the sample based on lipophilicity. The next HPLC step (or steps) is concentrated on developing an isocratic or gradient method that will separate the compounds into pure fractions.

6. Structure elucidation

A compound is described by its atoms and how they are connected to each other. The bonding pattern (compound skeleton) and the atoms' spatial arrangement (absolute configuration) need to be elucidated. Compound conformation is normally not necessary for unambiguous description. When the antibiotic era started, structure elucidation depended heavily on compound degradation and derivatization, but since the sixties spectroscopic and spectrometric methods are dominating. In natural products chemistry, NMR and MS are often sufficient to determine the compound skeleton. IR, which can identify functional groups and carbon hybridization level, has been phased out, and is mainly used as a compound characterization method, together with optical rotation, UV absorbance and melting point. In determination of absolute configuration, the method of choice is dependent on the properties of the investigated compound. A short presentation of MS, NMR, and two approaches to determine absolute configuration, is listed below.

6.1 Mass spectrometry

In mass spectrometry (MS) the investigated species is ionized and detected by its specific mass to charge ratio (m/z). The output data gives information on the monoisotopic mass of the investigated species. If the ionization technique is soft, like in electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI), the molecular ion or pseudomolecular ion is present at high intensity. Soft ionization is often combined with one or several fragmentation step on selected molecular ions to yield more information on the species. In high energy ionization like electron impact (EI), most molecular ions are fragmented and fragment ions are presented in the mass spectrum. The high fragmentation pattern reproducibility in EIMS makes it possible to use the compound mass spectrum as a fingerprint.

If the mass analyzers have sufficiently high resolution, the data can be used to calculate the elemental composition of the species, because of the minute differences in mass of neutrons and protons, together with the small mass addition of electrons. Mass analyzers with high resolution include sector instruments, some time-of-flight (TOF)-instruments, and the gold standard Fourier transform ion cyclotron resonance (FT-ICR) instruments.

Very often the mass spectrometer is hyphenated to a chromatographic system, for separation of complex samples before MS analysis. For HPLC, the standard is coupling to an ESIMS while GC is compatible with EIMS. Mass spectrometry is a destructive technique but most variants are very sensitive, requiring only micrograms or nanograms for analysis.

In compound isolation and identification, mass spectrometry fills many functions. It can be used for scanning complex samples for specific compounds, either directly in mixture, or by using the mass spectrometer as a detector in a chromatographic system (HPLC-ESIMS, GC-MS). It can also indicate (ESIMS, EIMS, MALDITOF) or verify (high resolution techniques) compound elemental

composition. Multiple stage mass spectrometry can aid structure elucidation [ion-trap (IT) ESIMSⁿ, post source decay (PSD) MALDITOF and Q-TOF], *e.g.* in sequencing of peptides.

6.2 Nuclear magnetic resonance spectroscopy

Certain nuclei have a magnetic spin, which can be manipulated by an external magnetic field. This is made use of in nuclear magnetic resonance (NMR) spectroscopy. The spin possessing nuclei of main interest in natural product structure elucidation are ¹H, ¹³C, ¹⁵N and to some extent ³¹P. By using one and two-dimensional NMR techniques the carbon-hydrogen bonding pattern can be elucidated. NMR is a non-destructive technique but sensitivity is lower than for MS. One way to achieve higher sensitivity, is to use a stronger magnetic field. At present, field strengths corresponding to a proton frequency of 400-900 MHz are widely used. If the sample tube is narrow, the sensitivity will be high, as less solvent can be used and the receiving coils can be placed closer to the sample. This is the reason for development of probes with smaller and smaller sample volumes. Use of a cryo-probe, which reduces noise by cooling the electronics, will also boost sensitivity significantly. With a 600 MHz NMR spectrometer, equipped with a 2.5 mm non-cryo microprobe (45 μl sample volume), less than a micromole is often sufficient for elucidation.

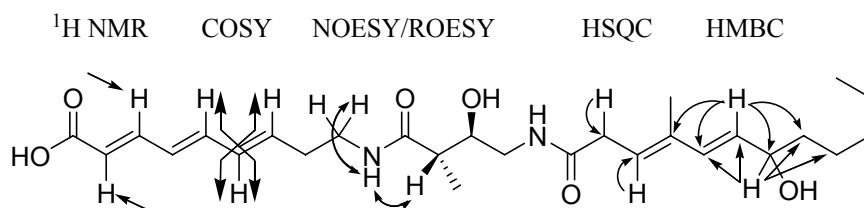


Fig. 31. Different ¹H-¹H and ¹H-¹³C correlations achieved by standard NMR pulse programs, exemplified on pseudotrienic acid B (Paper II).

A standard set of NMR experiments is normally used to elucidate an unknown structure (Fig. 31). One-dimensional ¹H NMR gives a picture of the hydrogens of the sample and proton-proton coupling constants. Two-dimensional homonuclear (¹H, ¹H) experiments show proton spin systems (COSY and TOCSY experiments) or can identify through-space correlations between protons (ROESY and NOESY experiments). Two-dimensional heteronuclear (¹H, ¹³C) NMR will display one-bond correlations between carbons and protons (HSQC experiments) or two-bond to four-bond correlations between carbons and protons (HMBC experiments).

There are many other experiment to produce specific data (two-bond to four-bond proton-carbon coupling constants, chemical exchange *etc.*). A good introduction article on how to apply NMR in natural products chemistry is the review article by Reynolds and Enriquez.¹⁶⁶

6.3 Absolute configuration

Many biochemical processes depend on chirality, *i.e.* that the reaction has a defined stereochemistry. One enantiomer will be able to participate in the reaction while the other might not react. This is exemplified by the consistent inclusion of only L-amino acids in human proteins. Certain microorganisms, on the other hand, make use of only the L form of some amino acids and only the D form of other. The handedness of biochemistry makes it logical to expect that a metabolite containing chiral centers should be biosynthesized in only one configuration. Determination of absolute configuration is thus an important part of structure elucidation. However, stereocenters in isolated natural products *can* lack enantiomeric excess due to rearrangements, non-stereospecific hydrolysis or oxygenation *etc.*, which could occur spontaneously or be induced during the isolation process.

Two approaches in investigation of absolute configuration are enantiomeric resolution methods and different NMR techniques. In addition to these, which are presented below, there are also other ways to determine absolute configuration. X-ray crystallography is the gold standard, if one only can produce crystals of sufficient quality of the compound. Circular dichroism and optical rotation can be calculated from hypothesized structures¹⁶⁷ and compared with experimental data. Another approach is through stereoselective synthesis and then physico-chemical comparison with the natural material. If the natural product holds for comparison, it is often seen as the definitive proof of correct structure assignment.¹⁶⁸

6.3.1 Enantiomeric resolution methods

Enantiomeric resolution is achieved by derivatizing a chiral compound of interest with an enantiomerically pure reagent. The derivatized compound is then compared with standards to verify absolute configuration. The *raison* for enantiomeric resolution is that diastereomers do not have identical physico-chemical properties. They can thus often be separated by chromatography on a non-chiral system. The need for standards means that natural products are often too complex to analyze directly, and have to be degraded to yield small chiral parts for analysis. However, caution is recommended when degrading a compound, as to not induce racemization of the chiral centers. One examples of an enantiomeric resolution methods is the advanced Marfey's method^{169,170} for defining absolute configuration of amino acids (Paper I). It is based on derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) and subsequent analysis with HPLC-UV or HPLC-ESIMS. A second example is the method for 2-hydroxy and 3-hydroxycarboxylic acids used in Paper II.¹⁷¹

6.3.2 NMR

When several stereogenic carbons are immediately bound to each other, or if they are part of a rigid ring, there is often a possibility to determine the *relative* configuration by NMR. This can be done by using the relationship between three-bond coupling constants between protons, two-bond couplings between protons and carbons, and the dihedral angle described by the Karplus equation. This

method has been named *J*-based configuration analysis¹⁷² and specific pulse programs can be applied measuring the size of small two-bond proton-carbon couplings¹⁷³. Determination of relative configuration is also helped by NOESY and ROESY experiments which give information on relative proximity in space between protons. When relative configuration is solved, only one chiral center in the cluster has to be determined to solve the stereochemistry of all connected centers, thus saving a lot of time.

The Mosher method is a type of enantiomeric resolution method for defining absolute configuration of stereogenic carbons with an attached hydroxyl group. Mosher ester derivatization¹⁷⁴ and the modified Mosher method¹⁷⁵ are based on derivatizing the alcohol with both *R* and *S* enantiomers of arylmethoxy acids. Comparing the chemical shifts of the *R* and *S* diastereomeric derivatives with a model of preferred conformations will indicate whether the hydroxylated carbon is *R* or *S*. For a review on the Mosher method, see the review article of Kusumi and Ohtani¹⁷⁶. In analogy with the Mosher method, NMR can also be used for determination of absolute configuration in amines and carboxylic acids.¹⁷⁷

7. Results and discussion

Chapters 7.1-7.4 describe the microbial material and the bioassay-guided isolation method, and chapters 7.5-7.8 present the isolated antifungal antibiotics. For a more detailed account of the antifungal antibiotics, the reader is referred to the individual Papers I-IV.

7.1 Microbial sources

The starting point for all projects were different collections of organism strains exhibiting antifungal effect in one or several types of bioassays. For each project, five to ten strains were selected for chemical investigation. As the collections and biological data present for the individual strains differed, it made it possible to see if the construction of the collections would influence the outcome of chemical investigation. Four different collections were used.

1. A diverse collection of phyllosphere microorganisms undergoing identification at the same time as chemical investigation (Paper I).
2. A collection of rhizosphere bacteria isolated by selection for growth under cold conditions, identified to the genus level (Paper II).
3. A collection of rhizosphere microorganisms identified to the genus or species level (Paper III).
4. A collection of mycorrhizal root inhabiting microorganisms identified to the genus or species level (Paper IV).

For collection one, the five strains showing highest inhibition in the bioassays were selected. In collection two, ten strains with high results in the bioassays were investigated. In order to avoid sister isolates they were selected through colony

morphological traits. For collection three, eleven strains were selected on bioassay performance, and avoiding sister isolates while eight strains from collection four were selected in the same way.

7.2 Sample preparation and isolation

A robust non-specific sample preparation method was developed, which could be applied to both fungi and bacteria (Fig. 32). It was based on the use of liquid biological material, SPE, HPLC and a dilution microtiter bioassay (Papers I-IV).

All organisms were grown in liquid nutrient media after which the organism was mechanically removed. Bacteria were removed by centrifugation at 4 °C (Papers II and III) or by filtration under reduced pressure (Paper IV), and filamentous fungi by filtration under reduced pressure (Paper I). The organisms were stored as reference at -20 or -70 °C for possible later extraction while the supernatant was immediately processed or stored at +6 °C (Paper IV), -20 °C (Paper I) or -70 °C (Papers II and III).

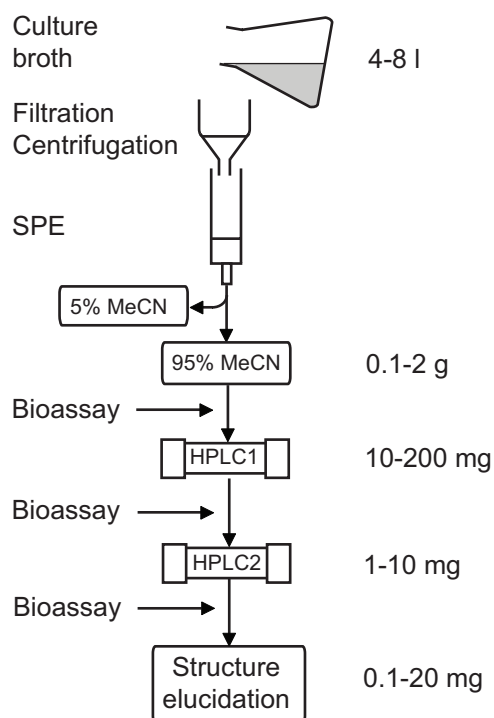


Fig. 32. Schematic view of the bioassay-guided sample preparation and isolation method. Numbers on the right indicate typical amounts of sample in each step.

To prepare the biological material for HPLC, it was subjected to C_{18} SPE to produce one hydrophilic fraction and one lipophilic fraction. About 20-40 g SPE material was used per liter supernatant, depending on whether defined or complex liquid medium had been used. The SPE fractionation was done either with prepacked 10 g-columns or columns of sufficient size prepared with bulk SPE

material. After loading the supernatant on the column, the hydrophilic compounds were washed out with aqueous 5% MeCN and pooled with the loading eluate. Then the lipophilic compounds were eluted with aqueous 95% MeCN. The use of aqueous 95% MeCN instead of 100% MeCN in eluting the lipophilic fraction was to avoid elution of compounds that later would adsorb irreversibly to the HPLC columns.

Both SPE fractions were tested for biological activity. An active lipophilic fraction was subjected to gradient HPLC on a C₁₈-column which was followed by gradient or isocratic HPLC on active fractions with C₁₈ or graphitized carbon column. The endpoint was an isolated pure compound. In this thesis, all isolated compounds came from the lipophilic fraction. All HPLC eluates were collected in 96-well plates with a 2 ml well capacity at five fractions per minute.

In re-isolation of compounds, the scheme was sped up by relying on the first isolation parameters; bioassays were routinely skipped, the SPE elutions were modified to obtain less material for HPLC preparation, and when possible, isocratic HPLC systems were applied directly after SPE to save one fractionation cycle.

7.3 Bioassays

The biological activity of sample fractions was followed by a previously described dilution microtiter bioassay.¹⁷⁸⁻¹⁸⁰ It was based on the 96-well microtiter format where cells or fungal spores were co-inoculated with samples in liquid nutrient media. After inoculation, inhibition of cell growth and spore germination was estimated by the naked eye or by an automated plate reader.

In the bioassays of chromatographic fractions, replicates of the 2 ml 96-well plates from preparative HPLC were done by transferring aliquots to microtiter plates, from which the solvents were evaporated in a fume hood overnight. To each well was added 100 µl of spore or cell suspension in appropriate culture medium. Due to the concentration achieved in the SPE step, the transferred sample amount corresponded to 100-400 times higher sample concentration in the bioassay than in the culture supernatant.

Although the projects were aimed at isolation of antifungal antibiotics, several pathogenic bacteria were also used as bioassay microorganisms, in both isolation and determination of MIC values. This was done in order to obtain information on the specificity of any antimicrobial activity. In total, eleven different pathogens were used in the projects (Table 1.).

Table 1. Microorganisms used in bioassay-guided isolation, and determination of minimal inhibitory concentrations (MIC)

Pathogen	Type:		Use:	
	Organism	Indication ^a	Isolation	MIC
<i>Aspergillus fumigatus</i>	Fungus	A, H		II, III, IV
<i>Botrytis cinerea</i>	Fungus	A	I	I
<i>Candida albicans</i>	Fungus	H	III	II, III, IV
<i>Drechslera sorokiniana</i>	Fungus	A	II	II, III, IV
<i>Erwinia carotovora</i>	Bacterium	A		IV
<i>Fusarium culmorum</i>	Fungus	A	II, III	II, III, IV
<i>Heterobasidion annosum</i>	Fungus	A		II
<i>Microsporum canis</i>	Fungus	H		II
<i>Pseudomonas sevastanoi</i>	Bacterium	A		II, III, IV
<i>Pseudomonas syringae</i>	Bacterium	A		II
<i>Staphylococcus aureus</i>	Bacterium	H	II, III,	II, III, IV

^aH = human pathogen, A = agricultural pathogen

7.4 Project priorities

There were three main parameters that influenced how to prioritize microbial strains during chemical investigation; lack or loss of antifungal activity, isolation of known antifungal metabolites and the limitations of lab-scale cultivation.

7.4.1 Stability of the biological activity

Some strains did not show detectable inhibition after SPE fractionation and for others, the activity found after SPE was lost after the first or second HPLC step. Thus, there was need for a fast routine to check if any found activity was reproducible. If antifungal activity was verified up to HPLC fractionation for three consecutive batches, investigation of the strain was continued.

There are several possible explanations for loss of activity. The antifungal activity after SPE or the first crude HPLC step could be due to synergistic effects of co-eluting metabolites. As they would be separated by further (isocratic) chromatography, synergy would be lost. Consecutive isocratic HPLC steps in itself lowers concentration of an active metabolite in the bioassay as it produces broader peaks. The material is then eluted over a larger number of fractions. Degradation or modification of the active component during the isolation process is also a possible cause for vanishing activity. This means that strains in which the activity was lost could still be worthwhile investigating by applying more specialized sample work-up methods.

Loss of activity was found for all types of organisms. Bacterial examples include pseudomonads from the Paper II collection and several strains of pink pigmented facultative methylotrophs (PPFMs) from the collection used in Paper III. Examples of fungi include several asexual white basidiomycete (AWB) strains (3035 and 3041),¹⁸¹ and a *Chaetomium hispanicum* strain 704 from the collection used in Paper IV.

7.4.2 Previously isolated compounds

In several of the projects, previously described antifungal compounds were isolated. Some of them were published together with new compounds found from the same strain (Papers I-IV). Other were duly noted after which the investigation of the strain was closed. In one case, citrinin, a production optimization study of the compound was done.¹⁸²

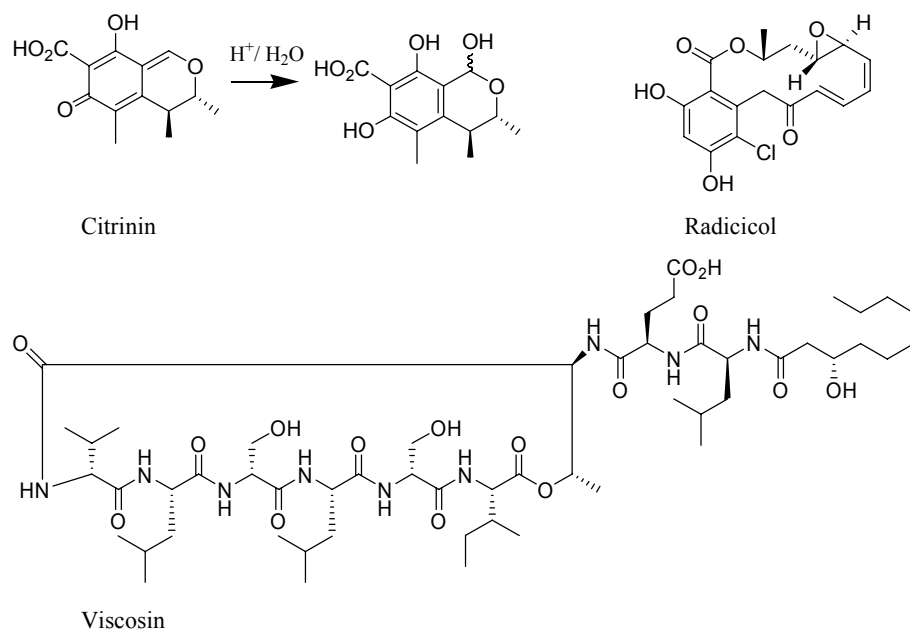


Fig. 33. Previously reported antifungal antibiotics, which were isolated in the projects.

Radicol

Radicol or monorden (Fig. 33), was isolated from *Cylindrocarpon* sp. F23¹⁸³ from the Paper I collection, on its activity against *Botrytis cinerea*. Radicol was first isolated in 1953¹⁸⁴ and its structure was elucidated on material isolated from *Cylindrocarpon radicol* and *Neocosmospora tenuicristata*.^{185,186} Radicol has been reported to be active against several fungal pathogens, including *Fusarium*.

Citrinin

Citrinin (Fig. 33) was isolated from a *Penicillium janczewskii* strain found to inhibit the root-rot pathogen *Heterobasidion annosum*.¹⁸² Citrinin was isolated as early as 1931 and is produced by *Penicillium citrinum* and also by several *Aspergillus* spp.¹⁸⁷⁻¹⁹⁰ This polyketide is known for its antifungal activity.¹⁹¹ If citrinin is isolated in an aqueous environment, it is hydrated and exhibits a characteristic doubled 1H NMR spectrum due to the $\sim 2:1$ ratio of the formed diastereomers.

Viscosin

Viscosin (Fig. 33) was isolated on its activity against *Fusarium culmorum* from a *Pseudomonas fluorescens* MF588¹⁹² from the Paper II collection. Viscosin¹⁹³⁻¹⁹⁵ is a lipodepsinonapeptide, *i.e.* it has the same basic structure as the pseudomycins and syringo-peptides. It has mainly been investigated for its surfactant properties, but has also been reported as an antifungal and antibacterial.¹⁹⁶

7.4.3 Material priorities

In the projects, the maximum volume of liquid supernatant that could be produced and handled under standard conditions was four to eight liters, and if additional material was needed, new batches were started. During a project, the total amount of supernatant to be processed was at the most a few tens of liters. In characterization of a compound, the bottleneck was to produce enough material for heteronuclear NMR-experiments. The amounts needed for this would also be enough for MIC determinations on several pathogens at the 50 $\mu\text{g/ml}$ level. No strain investigation was stopped due to too small amounts of material.

7.5 Compounds from phyllosphere fungi (Paper I)

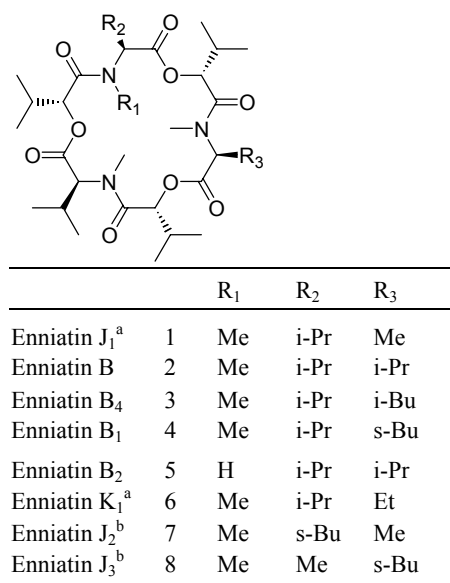


Fig. 34. Enniatins isolated from *Fusarium* sp. F31. ^aNot previously isolated from a natural source. ^bNot previously reported.

7.5.1 Source

The fungal strain *Fusarium* sp. F31 (Nectriaceae) was isolated from needles of a *Pinus sylvestris* L. (Pinaceae) plant collected in a Swedish forest nursery. The fungus was grown in liquid Hagem medium on a rotary shaker. The cell-free culture was subjected to SPE followed by gradient preparative HPLC (Fig. 35). Further purification yielded eight enniatins (Fig. 34).

7.5.2 Structure elucidation

The compounds were found to consist of three alternating *N*-methylated amino acids (AA) and three hydroxy acids (HA) each. The primary sequence of the compounds was not deducible from NMR due to lack of N-hydrogens and overlapping signals in the HMBC spectra. Instead, multiple stage MS was performed to elucidate the primary sequence. Multiple stage mass spectrometry of the pseudomolecular ion $[M+Na]^+$ revealed the sequential loss of any included AA, one HA, and any remaining AA. As ring-opening of depsipeptides in the presence of sodium has been found to take place at an ester bond,¹⁹⁷ this proved the alternating sequence of HA and AA in the compounds.

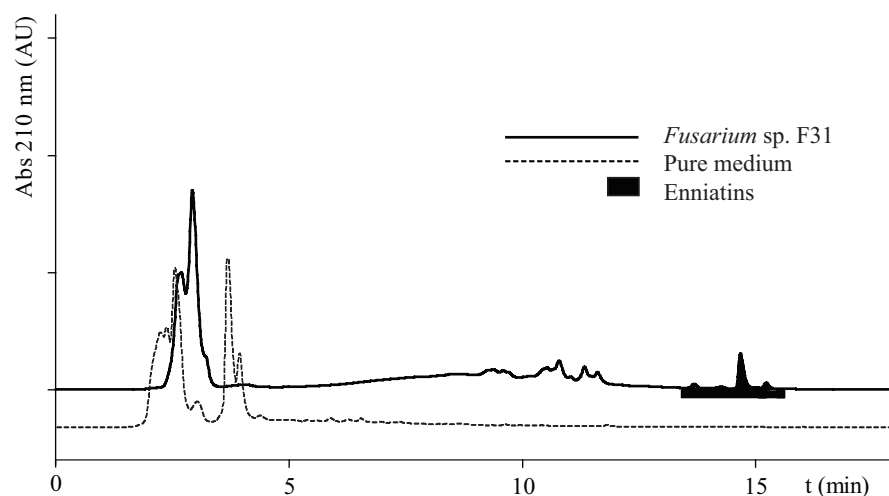


Fig. 35. Preparative gradient HPLC chromatogram of the 95% MeCN SPE-fraction from *Fusarium* sp. F31. Pure medium subjected to the same sample work-up is included for reference (both runs 10 to 100% MeCN in ten minutes with a hold at 100% for eight minutes).

One isolated fraction was found to contain two different enniatins, J₂ and J₃, containing the same moieties and only differing in primary sequence. Attempts to isolate the compounds were unsuccessful and MSMS of the mixture was not applicable for determining the primary sequence as resulting fragments from both enniatins would have the same mass. In order to verify the alternating sequence of J₂ and J₃, the mixture was partially hydrolyzed, and the resulting linear depsipeptides were isolated. Multiple stage MS then proved the opposite primary sequences of J₂ and J₃.

Determination of the absolute configuration of enniatins K₁, J₁, J₂, and J₃ was performed in two steps. The amino acids were investigated with the advanced Marfey's method,^{169,198} and the hydroxy acids were determined with an enantiomeric resolution method based on derivatization with (*S*)-2-butanol and analysis with GCMS.¹⁹⁹

7.5.3 Biological activity

The enniatins were tested for inhibition of *Botrytis cinerea* spore germination and showed moderate activity, with detectable inhibition down to 25 µg/ml, and total inhibition down to 100 µg/ml. Enniatins are reported to have phytotoxic,²⁰⁰ antimicrobial²⁰¹ and insecticidal²⁰² activities and they also inhibit the enzyme acyl-Co-A:cholesterol acyltransferase.²⁰³ In addition, they have been found to be toxic to mammalian cell lines and mitochondria.²⁰⁴

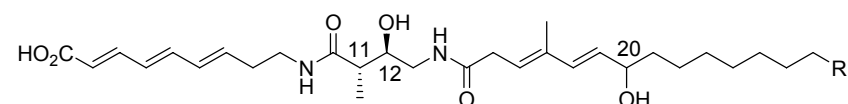
In agriculture, enniatins are seen as a potential toxic hazard as pathogenic *Fusarium* species infect small-grain cereals, which results in mycotoxin accumulation, including enniatins.²⁰⁵ On the other hand, a mixture of enniatins, fusafungine, is sold as a nasal spray under the name Locabital as an antibiotic and anti-inflammatory agent for treatment of nose and throat infections.²⁰⁶ The broad

activity of enniatins have been coupled to the compounds well established ionophoric properties which can destroy membrane potentials.²⁰⁷

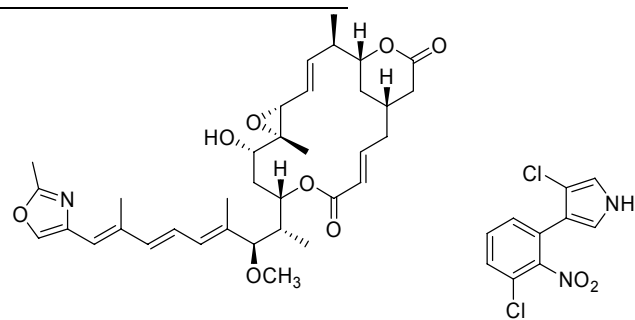
7.5.4 Remarks

Enniatins are produced by the multifunctional nonribosomal peptide synthetase (NRPS) enniatin synthetase which is present in many *Fusarium* spp.²⁰⁸ Enniatin synthetases show different specificity for certain amino acids,²⁰⁹ which can explain the range of enniatins isolated from *Fusarium* and other sources. The substrate specificity for the *Fusarium* sp. F31 enzyme was found to be similar to *Fusarium scirpi* enzymes.²¹⁰

7.6 Compounds from rhizosphere bacteria (Paper II)



	R
Pseudotrienic acid A	CH ₂ CH ₃
Pseudotrienic acid B	H



2,3-Deepoxy-2,3-didehydrorhizoxin (DDR)

Pyrrolnitrin

Fig. 36. Antimicrobial compounds isolated from *Pseudomonas* sp. MF381-IODS.

7.6.1 Source

The bacterial strain *Pseudomonas* sp. MF381-IODS (Pseudomonadaceae) was isolated from the roots of a *Brassica rapa* subsp. *rapa* L. (Brassicaceae) plant collected in Switzerland.²¹¹ It was grown on a Mineral Medium for *Pseudomonas* on a rotary shaker. The cell-free culture was subjected to SPE followed by gradient preparative HPLC (Fig. 37). Further purification yielded pseudotrienic acid A and B, 2,3-deepoxy-2,3-didehydrorhizoxin (DDR), and pyrrolnitrin (Fig. 36).

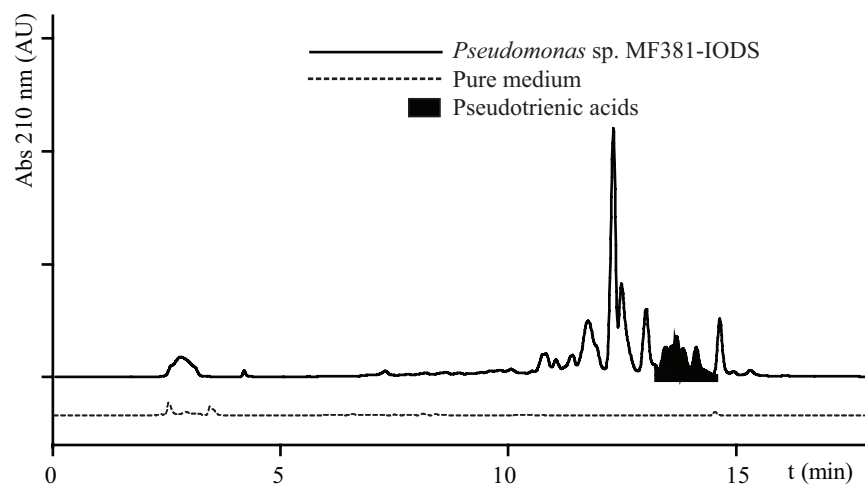


Fig. 37. Preparative gradient HPLC chromatogram of the 95% MeCN SPE-fraction from *Pseudomonas* sp. MF381-IODS. Pure medium subjected to the same sample work-up is included for reference (both runs 10 to 100% MeCN in ten minutes with a hold at 100% for eight minutes).

7.6.2 Structure elucidation

The carbon skeleton of the pseudotrienic acids was elucidated by NMR and MS. The *trans* configuration of all double bonds was indicated by NOE experiments and by the size of the three bond coupling constants. NOE correlations also help to define the relative configuration of C-11 and C-12 to (*R,S*)*.

The (11*S*,12*R*) configuration was determined with an enantiomeric resolution method on the central γ -amino acid, which was released through acidic hydrolysis. The amino acid was derivatized with (*S*)-2-butanol, pentafluoropropionic anhydride and analyzed by GCMS (Fig. 38). A hydrolysed authentic sample of Bistramide A, which contains the γ -amino acid moiety with a (2*S*,3*R*) configuration, was used as a standard.

The stereogenic C-20 was found to be present as *R* and *S* in equal amounts. This was determined by an enantiomeric resolution method for determination of 2-OH and 3-OH carboxylic acids. The method was based on *O*-methylation and derivatization with (*S*)-phenylethylamine.¹⁷¹ To access the C-20 as part of a hydroxy acid pseudotrienic acid B was ozonolysed followed by oxidative work-up. This released a 2-hydroxynonanoic acid with the C-2 corresponding to the pseudotrienic acid C-20. The ozonolysis mixture was derivatized with (*S*)-phenylethylamine and compared with a racemic 2-hydroxynonanoic acid standard subjected to the same procedure (Fig. 38.).

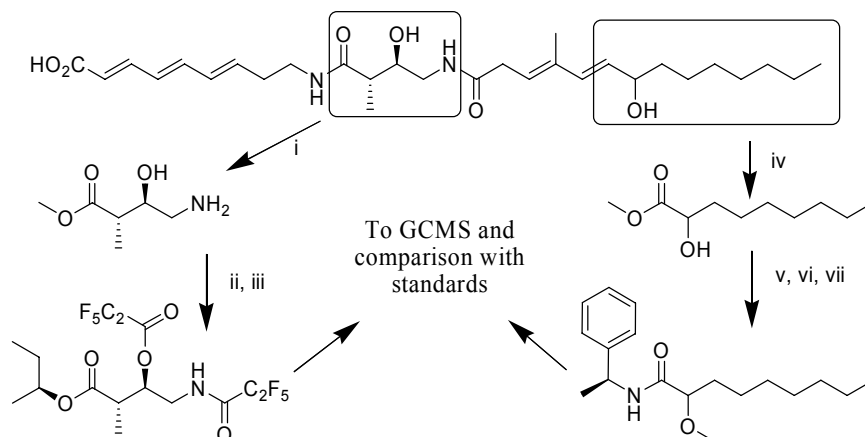


Fig. 38. Determination of absolute configuration of pseudotrienic acid B. Reagents and conditions: i) 6M HCl, 110 °C, 20 h; ii) (*S*)-2-BuOH/AcCl, 100 °C, 40 min; iii) $O(COCF_2CF_3)_2$, 100 °C, 40 min; iv) O_3 , MeOH, -20 °C, 10 min, then H_2O_2 , AcOH, 50 °C, 18 h, and finally MeOH/AcCl, 80 °C, 30 min; v) trimethylsilyldiazomethane, BF_3 , diethyl ether, 0 °C, 15 min; vi) KOH, MeOH/ H_2O 60 °C, 2 h; vii) $SOCl_2$, benzene, 30 °C, 30 min, then (*S*)-phenylethylamine, benzene, 30 °C, 25 min.

The pseudotrienic acids may derive from conjugated ring opening of a macrolactone. This would explain the lack of enantiomeric excess at C-20 as ring opening would proceed through an achiral intermediate, and such lactones have been isolated from a *Pseudomonas fluorescens* strain.²¹² In acidic environment, the pseudotrienic acids were found to lactonize, which further indicates the relationship between the linear and lactonized forms.

7.6.3 Biological activity

The pseudotrienic acids inhibited the growth of Gram-positive and Gram-negative bacteria at a concentration of 70 $\mu\text{g/ml}$, but did not inhibit yeasts or fungi. The corresponding pseudotrienic acid lactones have been reported to have immunosuppressant activity.^{212,213}

7.6.4 Remarks

Although the pseudotrienic acids did not inhibit any fungal pathogens at 100 $\mu\text{g/ml}$ they were still isolated in the bioassay-guided fractionation. This happened because of contamination by pyrrolnitrin or DDR in the crude pseudotrienic acid fraction, producing a false positive.

7.7 Compounds from rhizosphere bacteria (Paper III)

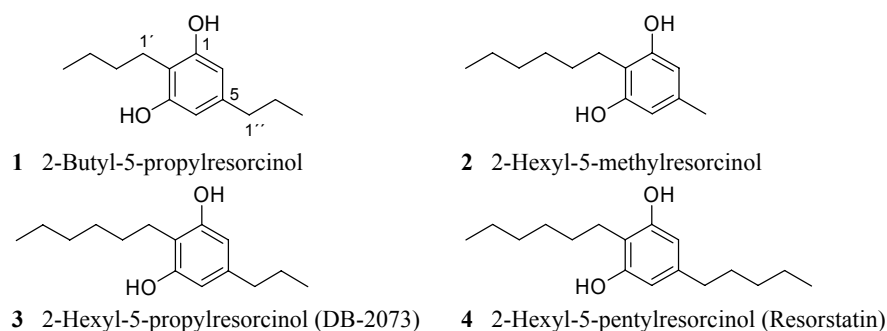


Fig. 39. Dialkylresorcinols isolated from *Pseudomonas* sp. Ki19. Compounds **1** and **2** have not been previously reported.

7.7.1 Source

The bacterial strain *Pseudomonas* sp. Ki19 (Pseudomonadaceae) was isolated from the roots of a crowberry plant [*Empetrum nigrum* L., (Empetraceae)] collected nearby Kiruna in northern Sweden. It was grown on a Mineral Medium for *Pseudomonas* on a rotary shaker. The cell-free culture was subjected to SPE followed by gradient preparative HPLC (Fig. 41). Further purification yielded four dialkylresorcinols (Fig. 39).

7.7.2 Structural elucidation

Elucidation of the compounds was performed by NMR and ESIMS. A key indication was the chemical shift of C-2 (δ 141) of compounds 1-4 indicating the position between the two hydroxy groups. Assignment of the alkyl group positions was done by HMBC correlations between the alkyl alpha protons and the ring carbons. In addition, a benzoquinone oxidation product of compound **4** was isolated and described (Fig. 40).

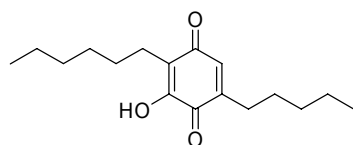


Fig. 40. 2-Hexyl-3-hydroxy-5-pentylbenzo-1,4-quinone, a benzoquinone oxidation product of resorstatin (**4**).

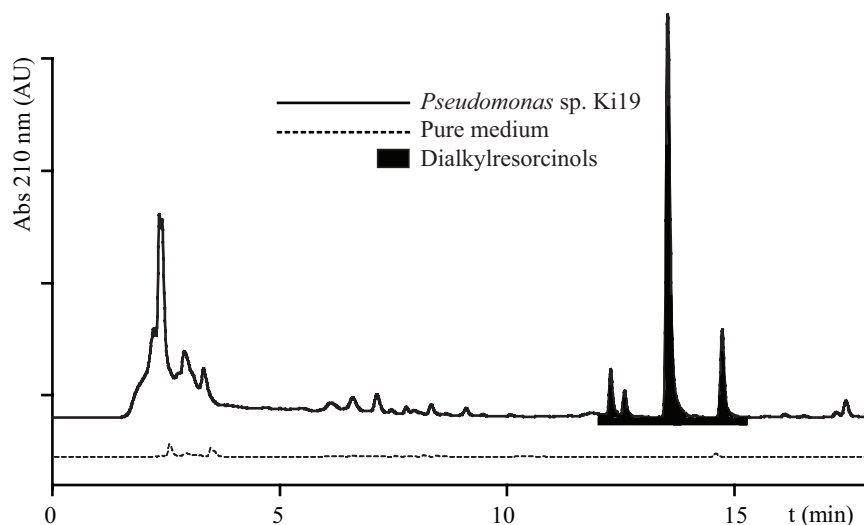


Fig. 41. Preparative gradient HPLC chromatogram of the 95% MeCN SPE-fraction from *Pseudomonas* sp. Ki19. Pure medium subjected to the same sample work-up is included for reference (both runs 10 to 100% MeCN in ten minutes with a hold at 100% for eight minutes).

7.7.3 Biological activity

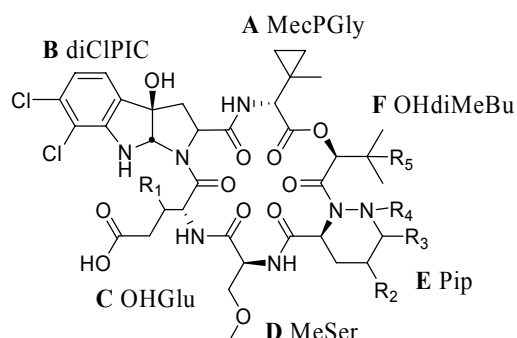
The compounds were found to be moderately active against fungi with full inhibitions down to 50 $\mu\text{g/ml}$. For Gram-positive bacteria, *Staphylococcus aureus* was completely inhibited at 2 $\mu\text{g/ml}$, with detectable inhibition at 1 $\mu\text{g/ml}$ while *Pseudomonas savastanoi* pv. *savastanoi* was more or less unaffected at the 50 $\mu\text{g/ml}$ level. The benzoquinone oxidation product inhibited *Candida* and *Staphylococcus* at 100 $\mu\text{g/ml}$, *i.e.* it was less potent than its mother compound. Investigation of formation of the benzoquinone indicated that it was not formed in the bioassay. This result suggests that the antimicrobial activity of the dialkylresorcinols does not depend on formation of benzoquinones during the bioassay. However, it does not rule out the possibility that other antimicrobial compounds are metabolized from the dialkylresorcinols.

A possible mechanism for formation of benzoquinones involves oxidation over trihydroxylated intermediates. The oxygen radicals formed during oxidation has been proposed to be responsible for the DNA degradation activity found for some alkylresorcinols.^{214,215} The DNA strand scission of alkylresorcinols has been shown *in vitro* in the presence of Cu(II) at alkaline pH, but has not been shown *in vivo*. Dialkylresorcinols have been reported for diverse biological activities, such as antibacterial and antifungal,^{216,217} cytotoxic²¹⁸ and radical scavenging activity.²¹⁹

7.7.4 Remarks

The dialkylresorcinols lack antifungal specificity, and the possible degradation to benzo-1,4-quinones with alkylating properties rise some questions regarding human toxicity. On the other hand, cereals contain resorcinolic lipids in amounts of 0.01-0.1%, which imply that they can be generally regarded as safe (GRAS).

7.8 Compounds from mycorrhizal root bacteria (Paper IV)



	R ₁	R ₂	R ₃	R ₄	R ₅
Kutzneride 1 ^a	HO _⋮	H	H	H	CH ₃
Kutzneride 2 ^a	HO _⋮	Cl	H	H	CH ₃
Kutzneride 3 ^a	HO _⋮	H	H	H	CH ₃
Kutzneride 4 ^a	HO _⋮	H	π -bond		CH ₃
Kutzneride 5	HO _⋮	H	H	H	H
Kutzneride 6	HO _⋮	OH	π -bond		CH ₃
Kutzneride 7	HO _⋮	H	H	H	H
Kutzneride 8	HO _⋮	Cl	H	H	CH ₃
Kutzneride 9	HO _⋮	H	π -bond		CH ₃

Fig. 42. Kutznerides isolated from *Kutzneria* sp. 744. ^aPreviously reported from this strain.

7.8.1 Source

The actinomycete *Kutzneria* sp. 744 (Pseudonocardiaceae) was isolated from the mycorrhizal roots of a *Picea abies* (L.) H. Karst (Pinaceae) plant, collected in a Lithuanian forest nursery. The bacterium was grown on a rotary shaker in liquid modified Melin Norkrans medium. The cell-free culture was subjected to SPE followed by gradient preparative HPLC (Fig. 43). Further purification yielded nine kutznerides (Fig. 42).

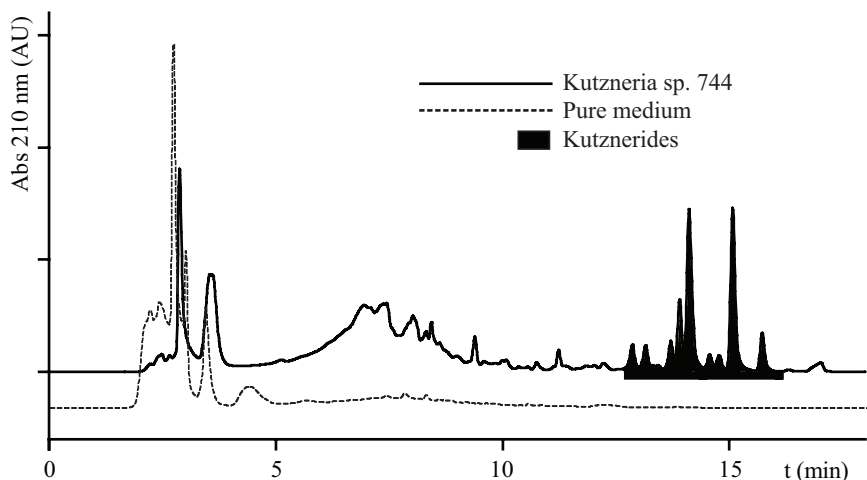


Fig. 43. Preparative gradient HPLC chromatogram of the 95% MeCN SPE-fraction from *Kutzneria* sp. 744. Pure medium subjected to the same sample work-up is included for reference (both runs 10 to 100% MeCN in ten minutes with a hold at 100% for eight minutes).

7.8.2 Structure elucidation

The compounds **5-9** were determined to be cyclohexadepsipeptides containing several unusual amino acid moieties.

Subunit A was determined as 2-(1-methylcyclopropyl)-D-glycine, subunit B as a 6,7-dichloro-3 α -hydroxy-1,2,3,3 α ,8,8 α -hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid with a (2*S*,3 α *R*,8 α *S*) configuration, subunit C as either *threo* (**7**, **9**) or *erythro*-3-hydroxy-D-glutamic acid (**5**, **6**, **8**), subunit D as a straightforward *O*-methyl-L-serine, subunit E as a L-piperazic acid (**5**, **7**), (4*R*)-5,6-didehydro-4-hydroxy-L-piperazic acid (**6**), *R*-4-chloro-L-piperazic acid (**8**), or 5,6-didehydro-L-piperazic acid (**9**), and finally subunit F as (*S*)-2-hydroxy-3-methylbutyric acid (**5**, **7**) or (*S*)-2-hydroxy-3,3-dimethylbutyric acid (**6**, **8**, **9**).

Carbon-nitrogen skeletons of the individual subunits were determined from COSY, HSQC and HMBC experiments, while the primary sequence of the subunits could be elucidated by ROESY correlations. To determine the absolute configuration of subunits A, C, D, E and F, **5-9** were subjected to hydrolysis, hydrogenation, derivatization with (*S*)-2-BuOH followed by derivatization by pentafluoropropionic acid anhydride and subsequent analysis by GCMS. Hydrogenation reduced the cyclopropyl ring in subunit A, presenting it as a *tert*-leucine, while the piperazic acid (subunit E) was reduced to ornithine. Subunit D was demethylated during hydrolysis, transforming it to serine.

For subunit B, ROESY experiments together with previously performed molecular modeling made it possible to determine the absolute configuration. ROESY also helped also helped determining the configuration of the different Pip residues of subunit E.

7.8.3 Biological activity

Kutznerides **1-9** were found to inhibit Gram-positive bacteria down to 5 µg/ml and the fungi down to 60 µg/ml. The compounds displayed a vast span in biological activity with the trichlorinated kutznerides **2** and **8** being most potent and kutzneride **6**, containing a hydroxylated piperazic acid, being least.

Other cyclic peptides, which share one or several of the unusual amino acids with the kutznerides, have been reported to have diverse biological activities. In subunit B, the H-8a and OH at C-3a have a *cis* relation and they are both *trans* to H-2. This basic configuration is also present in the cytotoxic compounds himastatin,²²⁰ kapakahine C²²¹, phakellistatin 3²²², and the nematocidal omphalotins B, C, and D.²²³ Interestingly, kapakahine D and isophakellistatin 3, which instead have an all-*cis* relation of H-8a, OH and H-2, are reported as non-cytotoxic.

Hydroxylated Pip residues are found in the mentioned himastatin, as well as in the cytotoxic polyoxypeptin.²²⁴ Unsaturated piperazic acids are found in the antimicrobial and cytotoxic aurantimycins,²²⁵ and, albeit some of them also 3-hydroxylated or 3-*O*-acetylated, in the antimicrobial luzopeptins²²⁶

7.8.4 Remarks

The kutznerides are most likely produced by a NRPS. The reported compounds show variation in the three subunits C, E and F. Four types of piperazic acid were found, while the OHGlu was present as its *threo*- and *erythro*-form. Finally, both di and monomethylated 2-hydroxybutyric acid was found. In theory, all combinations of the found variations would yield 24 different kutznerides. Even though there might be biosynthetic barriers for formation of some of the hypothetical compounds, trace amounts of several other kutznerides were detected in ESIMS. Growth optimization might succeed in isolating some of the missing variants.

8. Concluding remarks

The investigated organisms in this thesis were selected from four different strain collections (see Chapter 7.1). In all cases, the first criterion of selection was strong pathogen suppression in bioassays, while the second was to avoid genetic similarities. Antifungal antibiotics were isolated from strains from all four collections, indicating that their biocontrol ability could be due to production of antibiotics.

The structural diversity of the twenty-five isolated compounds spans several groups: depsipeptides, lipopeptides, polyketides, and resorcinols. Some of them could be suspected to be antibiotic precursors, in analogy with pro-drugs, meaning that the activity is related to a chemical modification of the structure. This could be argued for the resorcinols (Paper III) where oxidation products have been shown to degrade DNA *in vitro*. In the case of the pseudotrienic acids, the likely presence of both the linear and lactonized compounds in a biocontrol situation calls for investigations to pinpoint what activities are exhibited by which form.

Many of the isolated compounds have not previously been reported in the literature. This was the case for the kutznerides **5-9** from *Kutzneria* sp. 744 in paper IV. There are few reports on chemical investigations of *Kutzneria* so the probability of isolating new metabolites from this genus might be thought to be higher than for thoroughly investigated organisms like the pseudomonads. However, Papers I-III present new compounds isolated from such well-investigated taxa: *Fusarium* and *Pseudomonas*. Such metabolically diverse organisms are still a good source for new bioactive metabolites. This fact also stresses that strains should not be disregarded in dereplication just because they belong to a well-known genus or species, as there is metabolic diversity between microorganisms assigned to the same taxon. Instead, dereplication should be based on homology of biosynthesis gene regions or actual metabolic output, *i.e.* the metabolome. If these options are not available, a selection solely based on bioassay pathogen suppression is to prefer.

Many antifungal compounds reported in the last twenty years have been discovered in enzyme-based assays. The assays detect a narrow spectrum of structures with a defined mode of action. This type of screening has been successfully applied in, for example, the isolation of inhibitors of fungal sphingolipid biosynthesis (see Chapter 4.3.5) Enzyme-based assays and also the promising use of genome-based screening (see Chapter 4.1) need to be based on known modes of action. In a way, these approaches only find what is already there. The methodology is useful, but will become even more so, as novel modes of action are described, and new biosynthesis genes are sequenced. In this thesis, a different approach was applied by using a bioassay based on inhibition of the whole pathogen. The use of such a multi-functional assay helps to isolate a wide array of antibiotics, in comparison with a specific enzyme inhibition assay, and can also detect compounds with novel antifungal modes of action.

Although the main goal in this thesis was isolation of *antifungal* antibiotics, many compounds were also found to have antibacterial activity, and in the case of the pseudotrienic acids, even found to have *only* antibacterial activity. The reason for inclusion of pathogenic bacteria in the isolation bioassay (Papers II and III)

was that it quickly established a picture of the specificity of the biologically active compounds. When the pure compounds were obtained, pathogenic bacteria were included when feasible (Papers II-IV) in order to gain a broader knowledge of the antibiotic spectrum. There is certainly a risk that compounds isolated on a specific activity are regarded as “done”, and any additional bioactivity is left unexplored. But if there is plenty of an isolated compound, why not test it in all possible obtainable bioassays? From this aspect, the compound myriocin (see Chapter 4.3.5) is an interesting example. It was isolated both as an inhibitor of fungal sphingolipid biosynthesis and, independently, on its strong immunosuppressant activity. Along the same lines, the pseudotrienic acids in Paper II were isolated as antibiotics while the lactonized forms were isolated as immunosuppressants by another research group.

In conclusion, this thesis shows that microorganisms able to inhibit fungal pathogens are good sources for antifungal antibiotics. The use of a bioassay based on pathogen growth makes it possible to isolate a wide range of compounds, with the potential of finding compounds with new antifungal modes of action. As a bonus, the strains in which no antifungal metabolites were found can still be investigated as potential biocontrol agents.

8.1 Proposal for further studies

The potential of the strain collections used in this work is far from exhausted. The Papers I-IV can be seen as a preliminary sampling and when further isolation of compounds will be done, it would benefit much to be performed on a larger scale. A first step would be to make the collections compatible. There exists a large amount of sequence data which needs to be merged for all libraries, while other types of data need to be produced. A rapid metabolomic screening on all strains based on ESIMS could be one way of producing the latter, and another could be to screen all strains against an extended set of pathogens. If a screening program is sufficiently large, it can also be argued that additional bioassays should be included, in order to find stray hits. This could include assays aimed at cancer, immunosuppression and inflammation.

On a more specialized level, there are several areas adjacent to the scope of this thesis that would benefit from further research. In Paper II, the relation between the pseudotrienic acids and the lactonized forms needs to be clarified, both in terms of biosynthesis and bioactivity. The enniatins, and probably the kutznerides, are biosynthesized by nonribosomal peptide synthetases. Investigation of amino acid specificity and directed biosynthesis could in the kutzneride case possibly yield even further structures with antimicrobial activity.

The compounds in Papers I-IV inhibit pathogens *in vitro*. The next logical questions are: do the compounds inhibit pathogens in the field, and are the compounds biosynthesized when the producing organism is applied in the field?

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10. Acknowledgments

My supervisor **Anders Broberg** has helped me in all ways possible, not least through his sharp reasoning. Your way of treating me as a research colleague from the very start, and not as the inexperienced M. Sc. (only in biology even!) student I was back in the beginning, was truly valuable for me!

Lennart Kenne, my assistant supervisor, was very good help in writing and in strategic research decisions. You also had the impeccable ability to ask exactly the questions that made my research findings tremble. This is a gift you have Lennart! Use it wisely (and not on my thesis at this stage!)

My colleague and friend **Jörgen Sjögren**. For me, you were a born and raised analytical chemist, and it felt good to be at least two chemists amidst the massive biological attack!

In writing the thesis, I had help with suggestions from **Anders** and **Lennart**. **Daniel** took many turns scrutinizing the language and suggested typographical possibilities. Thanks for lending me your critical eye! Last but not least, brother **Anders** did a final readability test.

If it wasn't for my biologist co-operators I wouldn't have had much to work with at all!

Maria Johansson, with whom I started the first, and longest! project, and who all along continued to believe in MF381 until it started to behave! It was a tough project and our cooperation put me on track.

Kristof Capicau. The thorough investigation of *Fusarium* came out fine and I really enjoyed our discussions and side projects.

Jolanta Levenfors, with whom I've worked with on several projects, whether you were on Agrivir or MASE. Your inability to say no to work has helped me a lot when I needed bioassays or new strains with short notice!

Audrius Menkis. I always needed just a final batch.....No problem!

Juxian, **Jamshid**, **Olga** and **Xinmei** also gave me material for several projects that didn't wind up in the thesis.

The **MAaF people** made the first years nice with skiing at Enaforsholm, full board conferences, kick-offs and other "real-work" stuff. All of them students are Ph.Ds now.....what happened, what went right?

My colleagues at the Department of Chemistry have made my PhD time pleasant. **Lena** for helping me with all administration, **Rolf** and **Suresh** for keeping the instruments running and always willing to discuss MS and NMR-related problems. **Bernt** for his friendly ways and his *laissez faire* attitude when I snuck in and borrowed things from the students' lab (or perhaps you never noticed?). Although chemistry today is a high-tech venture, most of the *really* important knowledge is passed on by oral tradition, just like in biblical times. Daniel, Gunnar, Birgit, Johan, Ingmar, Janne, Anders S, Corine, Frank, Thomas, Lars N. Lasse A. Semiha, Somer, Ianric, Susanna, Tomek and many more, you all contributed to what I know today!

Hesham El-Seedi and **Premila Perera**, who supervised back at pharmacognosy in Uppsala, influenced me a lot, and their enthusiasm had a great part in that I shifted from working with secondary metabolites as a biologist, to working as a chemist.

At SLU, **Peter** was one of the few people I knew with a decent taste of music. I enjoyed the breaks we took once in a while! I spent many nice evenings with **Ove L.**, who also taught me a lot about how the academy *really* works!

My parents **Ragnhild** and **Antonin**! I was always free in my choices and you've supported me throughout my life. My brother **Anders** showed me the way to Uppsala and always cared for my well-being. The other members of my family, both Öckermans and Arnqvists, also have their fare share in caring for me. The last year was short on time, but **Margareta** relieved pressure for me and Eva. Without your help, things wouldn't have worked out so well!

I was invited, or invited myself to bed-and-breakfast on innumerable occasions when Stockholm seemed too far away. Thanks for letting me in: **Anders**, **Eva** and **Embla**, **Ove L.**, **Peter**, **Daniel** and **Julia**, **Fredrik** and **Emilia** (and on the last occasions also **Maja**), and **Maria A.** I realize I must have been a boring guest, coming too late in the evening and rising too early for work.

Eva, your support and belief in me has helped me do this, and if I hadn't been working as an assistant in *your* work once in a while, I would have become way too square!

Och så **Mina** så klart! Allt gott till dig!

This work was supported by the Foundation for Strategic Environmental Research (MISTRA). The SLU fund for internationalization of post-graduate studies, and the Royal Swedish Academy of Agriculture and Forestry (KSLA) gave financial support for visiting conferences in New Dehli and Kyoto.

A Taxonomic names of organisms

A.1 Bacteria

Organism ^a	Type of human impact	Family ^a
<i>Actinomadura hibisca</i> Tomita et al. 1991	Pradimicin producer	Termomonosporaceae
<i>Bacillus cereus</i> Frankland and Frankland 1887	Cispentacin producer	Bacillaceae
<i>Erwinia carotovora</i> (Jones 1901) Bergey <i>et al.</i> 1923	Agricultural pathogen	Enterobacteriaceae
<i>Kutzneria</i> sp.	Paper IV. Kutznerid producer	Pseudonocardiaceae
<i>Lactobacillus</i> sp.	Used as probiotics	Lactobacillaceae
<i>Micromonospora chalcea</i> (Foulerton 1905) Ørskov 1923	Rustmicin/galbonolid producer	Micromonosporaceae
<i>Mycobacterium tuberculosis</i> (Zopf 1883) Lehmann and Neumann 1896	TBC	Mycobacteriaceae
<i>Pseudomonas fluorescens</i> Migula 1895	Paper II collection. Viscosin producer	Pseudomonadaceae
<i>Pseudomonas</i> sp. Ki19	Paper III. Dialkylresorcinol producer	Pseudomonadaceae
<i>Pseudomonas</i> sp. MF381-IODS	Paper II. Pseudotrienic acid producer	Pseudomonadaceae
<i>Pseudomonas pyrrocina</i> Imanaka et al. 1965	Pyrrrolnitrin producer	Pseudomonadaceae
<i>Pseudomonas savastanoi</i> (Janse 1982) Gardan <i>et al.</i> 1992	Agricultural pathogen	Pseudomonadaceae
<i>Pseudomonas syringae</i> pv. <i>syringae</i> van Hall 1902	Agricultural pathogen source of syringomycins syringotoxins syringostatins pseudomycins	Pseudomonadaceae
<i>Sorangium cellulosum</i> Reichenbach 2002 Current name: <i>Polyangium cellulosum</i> (ex Imshenetski and Solntseva 1936) Brockman, 1989	Soraphen producer	Polyangiaceae
<i>Staphylococcus aureus</i> Rosenbach 1884	Human pathogen	Staphylococcaceae
<i>Streptomyces cacaoi</i> (Waksman 1932) Waksman and Henrici 1948	Polyoxin producer	Streptomycetaceae
<i>Streptomyces chattanoogenis</i> Burns and Holtman 1959	Natamycin producer	Streptomycetaceae

Organism ^a	Type of human impact	Family ^a
<i>Streptomyces galbus</i> Frommer 1959	Rustmicin/galbonolid producer	Streptomycetaceae
<i>Streptomyces griseochromogenes</i> Fukunaga 1955	Blasticidin producer	Streptomycetaceae
<i>Streptomyces griseus</i> (Krainsky 1914) Waksman and Henrici 1948	Streptomycin producer	Streptomycetaceae
<i>Streptomyces hygroscopicus</i> (Jensen 1931) Waksman and Henrici 1948	Validamycin producer	Streptomycetaceae
<i>Streptomyces kasugaensis</i> Hamada et al. 1995	Kasugamycin producer	Streptomycetaceae
<i>Streptomyces natalensis</i> ^b	Natamycin producer	Streptomycetaceae
<i>Streptomyces setonii</i> (Millard and Burr 1926) Waksman 1953	Cispentacin producer	Streptomycetaceae
<i>Streptomyces tendae</i> Ettliger et al. 1958	Nikkomycin producer	Streptomycetaceae
<i>Streptoverticillium rimofaciens</i> ^c	Mildiomycin producer	

^aAuctors, family names and species names were retrieved from the National Centre for Biotechnology Information (Tax Browser: <http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/>) 20060420, and J.P. Euzéby: List of Prokaryotic Names with Standing in Nomenclature (<http://www.bacterio.cict.fr/index.html>) 20060420.

^bThis species name has no standing in nomenclature

^cThis species name has no standing in nomenclature. *Streptoverticillium* was transferred to *Streptomyces* 1991.

A.2 Fungi

Organism ^a	Type of human impact	Family ^a	Division ^b
<i>Alternaria</i> spp.	Agricultural pathogen	Pleosporaceae	Ascomycete
<i>Arthrinium arundinis</i> (Corda) Dyko & B. Sutton Current name: <i>Apiospora montagnei</i> Sacc.	Arundifungin producer	Apiosporaceae	Ascomycete
<i>Ascotricha amphitricha</i> (Corda) S. Hughes	Ascosteroside producer	Xylariaceae	Ascomycete
<i>Aspergillus aculeatus</i> Iizuka	Papulacandin producer	Trichocomaceae	Ascomycete
<i>Aspergillus fumigatus</i> Fresen.	Human/agricultural pathogen	Trichocomaceae	Ascomycete
<i>Aspergillus nidulans</i> var. <i>echinulatus</i> Fennell & Raper	Echinocandin producer	Trichocomaceae	Ascomycete

Organism ^a	Type of human impact	Family ^a	Division ^b
<i>Aspergillus rugulosus</i> Thom & Raper	Echinocandin producer	Trichocomaceae	Ascomycete
<i>Aureobasidium pullulans</i> (de Bary) G. Arnaud	Aureobasidin producer	Dothioraceae	Ascomycete
<i>Botrytis</i> sp.	Agricultural/silvicultural pathogen	Sclerotiniaceae	Ascomycete
<i>Botrytis alli</i> Munn	Agricultural pathogen	Sclerotiniaceae	Ascomycete
<i>Botrytis cinerea</i> Pers.	Agricultural/silvicultural pathogen	Sclerotiniaceae	Ascomycete
<i>Candida albicans</i> (C. P. Robin) Berkhout	Human pathogen	Saccaromycetaceae	Ascomycete
<i>Chaetomium hispanicum</i> Guarro & Arx	Paper IV collection	Chaetomiaceae	Ascomycete
<i>Cryptococcus neoformans</i> (San Felice) Vuill.	Human patogen	Tremellaceae	Basidiomycete
<i>Cylindrocarpon</i> sp.	Sphingofungin producer Paper I collection. Monorden producer	Nectriaceae	Ascomycete
<i>Cylindrocarpon radicum</i> Wollenw. Current name: <i>Nectria radicum</i> Gerlach & L. Nilsson	Monorden producer	Nectriaceae	Ascomycete
<i>Drechslera sorokiniana</i> (Sacc.) Subram. & B. L. Jain Current name: <i>Cochliobolus sativus</i> (S. Ito & Kurib.) Drechsler ex Dastur	Agricultural pathogen	Pleosporaceae	
<i>Erysiphe graminis</i> DC	Agricultural pathogen	Erysiphaceae	Ascomycete
<i>Fusarium</i> sp.	Agricultural pathogen Paper I. Enniatin producers Enniatin producer	Nectriaceae	Ascomycete
<i>Fusarium scirpi</i> F. Lamb. & Fautrey Current name: <i>Gibberella acuminata</i> C. Booth		Nectriaceae	Ascomycete
<i>Fusarium moniliforme</i> J. Sheld.	Fumonisin producer	Sclerotiniaceae	Ascomycete
<i>Fusarium oxysporum</i> Schltdl.	Agricultural pathogen	Nectriaceae	Ascomycete
<i>Gerlachia nivalis</i> (Ces. ex Berl. & Voglino) W. Gams & E. Müll. Current name: <i>Monographella nivalis</i> var. <i>nivalis</i> (Schaffnit) E. Müll.	Agricultural pathogen	Of uncertain position	Ascomycete
<i>Heterobasidion annosum</i> (Fr.) Bref.	Silvicultural pathogen	Bondarzewiaceae	Basidiomycete
<i>Hormonema</i> sp.	Enfumafungin producer	Of uncertain position	Deuteromycete
<i>Isaria sinclairii</i> (Berk.) Lloyd	Myriocin/ISP-1 producer	Clavicipitaceae	Ascomycete
<i>Microsporium canis</i> E. Bodin ex Guég	Human pathogen	Arthrodermataceae	Ascomycete

Organism ^a	Type of human impact	Family ^a	Division ^b
<i>Myriococcum albomyces</i> Cooney & R. Emers. Current name: <i>Melanocarpus albomyces</i> (Cooney & R. Emers.) Arx 1975	Myriocin/ISP-1 producer	Of uncertain position	Ascomycete
<i>Neocosmospora tenuicristata</i> S. Ueda & Udagawa	Monorden producer	Nectriaceae	Ascomycete
<i>Oudemansiella mucida</i> (Schrad.:Fr.) Höhn.	Strobilurin producer	Marasmiaceae	Basidiomycete
<i>Penicillium citrinum</i> Thom	Citrinin producer	Trichocomaceae	Ascomycete
<i>Penicillium griseofulvum</i> Dierckx	Griseofulvin producer	Trichocomaceae	Ascomycete
<i>Penicillium janczewskii</i> K.M. Zalesky	Citrinin producer	Trichocomaceae	Ascomycete
<i>Pyricularia oryzae</i> Cavara Current name: <i>Magnaporthe grisea</i> (T.T. Hebert) M.E. Barr	Agricultural pathogen	Magnaporthaceae	Ascomycete
<i>Pythium</i> sp.	Agricultural pathogen	Pythiaceae	Oomycete
<i>Phytophthora infestans</i> (Mont.) de Bary	Agricultural pathogen	Pythiaceae	Oomycete
<i>Rhizoctonia solani</i> J.G. Kühn Current name: <i>Thanatephorus cucumeris</i> (A.B. Frank) Donk	Agricultural pathogen	Ceratobasidiaceae	Basidiomycete
<i>Rhizopus chinensis</i> Saito Current name: <i>Rhizopus microsporus</i> var. <i>chinensis</i> (Saito) Schipper & Stalpers	Rhizoxin producer	Mucoraceae	Zygomycete
<i>Saccharomyces cerevisiae</i> Meyen ex E.C. Hansen	Bakers yeast, type yeast	Saccharomycetaceae	Ascomycete
<i>Sordaria araneosa</i> Cain	Sordarin producer	Sordariaceae	Ascomycete
<i>Sporormiella australis</i> (Speg.) S.I. Ahmed & Cain	Australifungin producer	Sporormiaceae	Ascomycete
<i>Strobilurus tenacellus</i> (Pers.) Singer	Strobilurin producer	Marasmiaceae	Basidiomycete
<i>Trichoderma koningii</i> Ouderm.	Ergokonin producer	Hypocreaceae	Ascomycete
<i>Trichoderma viride</i> Pers.	Viridofungin producer	Hypocreaceae	Ascomycete
<i>Ustilago maydis</i> (DC.) Corda	Agricultural pathogen	Ustilaginacea	Basidiomycete

^aAuctors, family names and species names were retrieved from Index Fungorum (<http://www.indexfungorum.org>) 20060420

^bDivision affiliations based on common usage.