Secondary metabolites from the saprotrophic fungus *Granulobasidium vellereum*

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2014

Acta Universitatis agriculturae Sueciae
2014:88

ISSN 1652-6880
ISBN (print version) 978-91-576-8124-9
ISBN (electronic version) 978-91-576-8125-6

Print: SLU Service/Repro, Uppsala 2014

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Abstract

The production of secondary metabolites by the saprotrophic fungus *Granulobasidium vellereum* was investigated. *G. vellereum* is a wood decomposing basidiomycete fungus that arrives at dead trunks of fallen hard wood trees at a later stage of the decomposition process and hence need to outcompete the organisms already colonizing the site. It was hypothesised that part of the fungus competitive ability was based on the production of secondary metabolites.

In total, 33 secondary metabolites, all of sesquiterpenoid origin and comprising structures with the protoilludane, illudane, illudalane or cerapicane carbon skeletal types, were isolated by chromatographic methods and characterized by spectroscopic techniques. Twenty-two of these compounds had previously not been described and of the 11 known compounds two were not previously described as natural products. Four of the new compounds showed potent cytotoxic effects against the Huh7 and MT4 tumour cell lines. The mechanism of action was most likely similar to that of the known alkylating agent illudin M and based on chemical reactivity rather than enzymatic affinity. None of these cytotoxic compounds had any potent anti-fungal effects, perhaps due to them not being able to penetrate the cell walls of the fungi. The compound that had the most anti-fungal effects was the known compound radulone A and thus possibly a part of the molecular defence system of *G. vellereum*. One of the compounds was found to increase the elongation of lettuce seedlings.

G. vellereum was co-cultivated with seven other wood-decay fungi with the aim of investigating the secondary metabolite response of *G. vellereum* towards other fungi, with regard to up-regulation and *de novo* production of secondary metabolites. Samples from the co-cultures were taken after 7 to 12 days of incubation and subsequently analysed by LC-HRMS followed by PCA of the data. Nineteen metabolites from *G. vellereum* were up-regulated in all seven interactions, of these were six identified as previously known metabolites from *G. vellereum*, one of which was the highly cytotoxic 3S,7S-illudin M. The response of *G. vellereum* towards the different fungi varied, indicating that *G. vellereum* has the ability to adapt its response slightly depending on the interacting species.

Keywords: *Granulobasidium vellereum*, wood decomposing, secondary metabolites, sesquiterpenes, cytotoxic, co-cultivation, up-regulation

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Dedication

To my parents

O Fortuna
velut luna
statu variabilis
semper cresis
aut decrecis;
vita detestebilis
nunc obdurat
et tunc curat
ludo mentis aciem,
egestatem
potestatem
dissolvit ut glaciem

Medieval poem

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Christina L. Nord, Audrius Menkis, Rimvydas Vasaitis and Anders Broberg (2013). Protoilludane sesquiterpenes from the wood decomposing fungus *Granulobasidium vellereum* (Ellis & Cragin) Jülich. *Phytochemistry* 90, 128-134.

II Christina L. Nord, Audrius Menkis, Christofer Lendel, Rimvydas Vasaitis and Anders Broberg (2014). Sesquiterpenes from the saprotrophic fungus *Granulobasidium vellereum* (Ellis & Cragin) Jülich. *Phytochemistry* 102, 197-204.

III Christina L. Nord, Audrius Menkis and Anders Broberg (2014). Cytotoxic illudalane sesquiterpenes from the wood-decay fungus *Granulobasidium vellereum* (Ellis & Cragin) Jülich. *Molecules* 19, 14195-14203.

IV Christina L. Nord, Audrius Menkis and Anders Broberg. Cytotoxic illudane sesquiterpenes from the fungus *Granulobasidium vellereum* (Ellis & Cragin) Jülich. (manuscript).

V Christina L. Nord, Audrius Menkis, Mattias Fredriksson and Anders Broberg. *In vitro* metabolic response of the wood-decomposing fungus *Granulobasidium vellereum* to seven competing fungal species. (manuscript).

The contribution of Christina Nord to the papers included in this thesis was as follows:

I Planning the work together with the co-authors and reviewing the literature. All chemical work, including the interpretation of the data and writing the majority of the chemically related parts of the paper. Overall conclusions were made together with the co-authors.

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V Planning the work together with the co-authors and reviewing the literature. All chemical work and writing the chemically related parts of the paper. Overall evaluation of the data analysis and overall conclusions were made together with all co-authors.

Abbreviations

CD	circular dichroism
$CDCl_3$	deuterated chloroform
COSY	correlation spectroscopy
CPL	circularly polarized light
DMAPP	dimethylallyl diphosphate
ESI	electrospray ionization
FDA	food and drug administration
FID	free induction delay
FPP	farnesyl diphosphate
GPP	geranyl diphosphate
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HR	high resolution
HSQC	heteronuclear single quantum coherence
IPP	isopentyl diphosphate
m/z	mass-to-charge ratio
MeCN	acetonitrile
MeOD	deuterated methanol
MEP	methylerythritol phosphate
MVA	mevalonic acid
MS	mass spectrometry
NCE	new chemical entity
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
NRPS	non-ribosomal peptide synthetases
PCA	principal component analysis
ppm	parts per million
Q	quadrupole

ROESY	rotating-frame nuclear Overhauser effect spectroscopy
RP	reversed phase
SPE	solid phase extraction
TOF	time of flight
t_R	retention time
δ	chemical shift

1 Introduction

Secondary metabolites are a large and structurally diverse group of compounds produced by organisms for a variety of different reasons such as toxic molecules for defence and pheromones for communication etc. The production of secondary metabolites by the organism is under constant evolutionary pressure, and since the production costs resources it will continue only if the total benefits exceed the costs.

Presently most studies of secondary metabolite production are bioassay guided studies aimed on finding compounds with specific biological effects e.g. cytotoxicity, antimicrobial activity and antidiabetic. This is of course a valid method for the purpose of finding compounds with potential use for humans such as new drugs, but it will perhaps not result in any deeper understanding of the ecological roles of the secondary metabolites for the species that produce them (O'Brien and Wright, 2011). Lately the interest to find the ecological functions of these compounds and hence gain further understanding of our environment has grown. The discovery of silent biosynthesis genes within the organisms has also showed that many secondary metabolites are not produced under normal laboratory conditions.

The competition between the different wood decay organisms for the wooden substrate is harsh and the different species are often found to live in well-defined areas of the wooden tissue often separated from each other by a dark interaction zone, which indicates the production of chemical warfare (Rayner & Boddy 1988; Owens *et al.* 1994).

But how does the chemical interactions between the wood-decay fungi work? There is still much to learn about the molecular mechanisms underlying the antagonism between the different species.

1.1 Aim of the thesis

The aim of this thesis is to isolate and characterize the secondary metabolites produced by the wood decomposing fungus *Granulobasidium vellereum* (Ellis & Cragin) Jülich, with the objectives of investigating the potential ecological roles of the compounds as well as the chemical response of *G. vellereum* towards competing species of wood-decay fungi.

2 Wood-decay fungi

A tree stump in the forest might at first glance seem to be a tranquil place, but it is actually the site of fierce competition between a wide array of different wood decomposing microorganism. The most important wood decay organisms in terrestrial ecosystems are fungi, and then in particular white-rot and brown-rot fungi from the phylum Basidiomycotina (Cooke & Rayner 1984; Owens *et al.* 1994).

In a living tree the physiologically active wood tissue is protected against invasion of most species of different wood decomposing microorganisms. There are though some pathogenic species that are able to colonize living tree tissue as well, for example the root rotting fungus *Heterobasidion annosum* s.l., which is the economically most important forest pathogen in the northern hemisphere (Asiegbu 2005; Cooke & Rayner 1984). When a tree (or even just parts of it) dies, the woody substrate will however lose its defence mechanisms and will consequently be colonized by a wide variety of different saprotrophic fungi and other microorganisms. All saprothrops will however not arrive at the newly available woody substrate simultaneously. The first fungi to arrive are ruderal species that grow fast but have weak combative ability, sometimes called primary saprotrophs. They are later replaced by more combative fungal species, also known as secondary saprotrophs (Cooke & Rayner 1984; Owens *et al.* 1994) (Fig. 1). The succession of the different fungal species on the wood is however difficult to predict and is highly dependent on both the host and the environment (Frankland 1998).

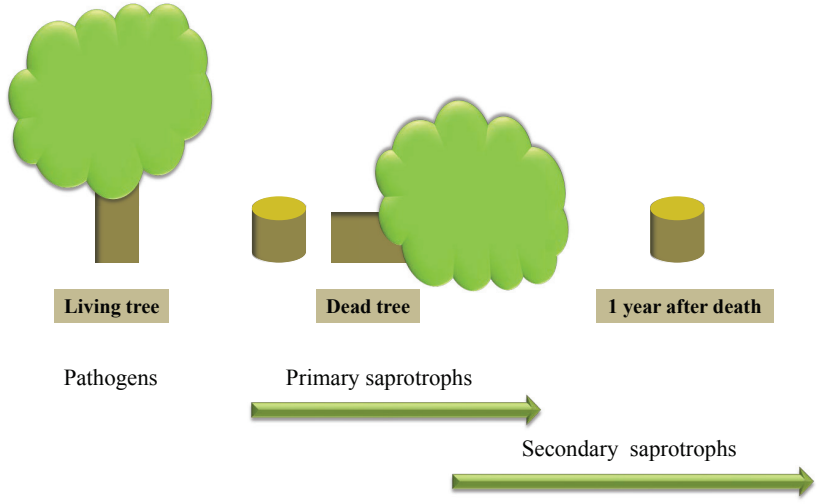


Figure 1. In the living tree the wooden substrate is well protected against the invasion of wood decomposing fungi, but some pathogenic species are however able to colonize living tree tissue. When a tree dies the first species to arrive will be fast growing ruderal fungi, known as primary saprotrophs. The primary saprotrophs will later be replaced by more combative secondary saprotrophic fungi.

The competitive pressure between the different fungal species on the wood tissue is immense and mutualistic interaction occurs only rarely or not at all. The mycelium of each fungus occupies its own distinct area or region, also known as decay columns. The regions between the different species are often delineated by dark zone lines, which might be an indication of the release of defence substances (Rayner & Boddy 1988; Owens *et al.* 1994). Much remains to be discovered about the mechanisms behind the interactions between different wood-decay fungi in their natural environment, though the use of various antibiotic compounds has been implied as a likely mechanism (Bertrand 2013; O'Brien & Wright 2011; Peiris *et al. 2008*).

2.1 *Granulobasidium vellereum*

Granulobasidium vellereum (prev. *Hypochnicium vellereum*) is a saprotrophic wood decomposing fungus from the family Cyphellaceae and the phylum Basidiomycotina, which is one of the five subdivisions of Eumycota (true fungi) (Larsson 1997). The fungus is rather rare though geographically widely spread and might be found in deciduous forests throughout Europe, East Asia and North America, where it lives on decaying trunks of fallen trees and dead branches of mainly elms. On the tree trunks *G. vellereum* forms mildly pink widespread fruiting bodies, with a smooth surface (Larsson 1997).

G. vellereum belong to white-rot fungi which unlike the brown-rot fungi are able to decompose all three wood polymers: lignin, cellulose and hemicellulose (Ginns 1986). *G. vellereum* is also a secondary saprotrophic species and will hence arrive on dead wood tissue at a later stage of the decomposition process. Secondary saprotrophic organisms are often characterized by their strong combative abilities, since they need to be able to outcompete the species that already inhabit the wooden substrate before they can colonize it themselves (Owens *et al.* 1994). The combative abilities of secondary saprotrophs are hypothesised to be linked to their ability to produce antimicrobial secondary metabolites. It might consequently be assumed that *G. vellereum* is able to produce bioactive compounds though no secondary metabolites so far have been described for this species.

3 Natural products

3.1 Secondary metabolites

Primary metabolites are often defined as compounds which are of crucial importance for life and its upholding. Their structures have remained highly conserved within large groups of organisms throughout evolution. Primary metabolites include compounds such as fats, proteins, carbohydrates and nucleic acids. Secondary metabolites are in contrast molecules of adaptation used by the organisms for various different reasons such as defence, hunting, communication and many more. They are also unlike the primary metabolites generally produced exclusively by a genus or a single species (Dewick 2009; O'Brien & Wright, 2011). The distinction between primary and secondary metabolism as two separate processes has lately began to be challenged, due to the lack of evolutionary basis and the fact that many important metabolites do not fit in either category (Firn & Jones 2009). In this thesis the traditional distinction between primary and secondary metabolism will be used, even though some problems with this distinction will be addressed.

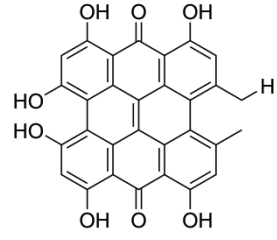
Secondary metabolites are often small organic compounds and even though their structural diversity is enormous they are generally produced from only a few different biosynthetic pathways within all organisms. Normally secondary metabolites are considered to be produced by so-called secondary metabolism (natural products chemistry) but the building blocks might also come from primary metabolism (biochemistry), resulting in that the border between secondary and primary metabolites in some cases might be somewhat blurry (Dewick 2009).

Secondary metabolites are divided into different groups or classes depending on certain characteristic structural features that arise from the respective biosynthetic origin of the different compounds. Some secondary metabolites may consist of moieties and features from more than one class

making the classification more difficult. There is unfortunately no general consensus or agreement on how the different types of secondary metabolites should be divided, but the following six different classes are sometimes used (Dewick 2009; Hanson 2003):

- Polyketides and fatty acids
- Phenylpropanoids and aromatic amino acids
- Terpenoids
- Alkaloids
- Peptides, proteins and other amino acid derivates
- Specialized carbohydrates

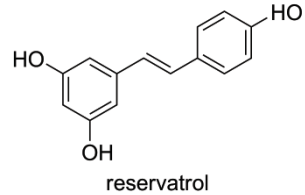
Polyketides and fatty acids represent a large and diverse class of secondary metabolites, which are formed from the acetate pathway in the cell through the stepwise coupling of acetate units to each other. Members of this class include among others the macrolide antibiotics, statins and the antidepressant components of St John's wort (Fig. 2) (Dewick 2009; Kirakosyan *et al.* 2004).



hypericin

Figure 2. Structure of hypericin, a secondary metabolite produced by St John's wort that displays antidepressant effects on humans.

Phenylpropanoids and aromatic amino acids are formed from a pathway exclusive to plants and microorganisms namely the shikimate pathway, in which the shikimic acid is an intermediate hence the name. Members of this class includes compounds such as folic acid, the anti-inflammatory and analgesic salicylic acid, the mildly hallucinogenic myristicin and the famous antioxidant found in red wine resveratrol (Fig. 3) (Dewick 2009; Corder *et al*. 2003).



reservatrol

Figure 3. Structure of resveratrol, an antioxidant found in red wine.

The terpenoids constitute another large group of secondary metabolites and are formed through the head-to-tail linkage of isoprene units (C_5). The terpenoids are further classified by the number of isoprene units they consist of (i.e. number of carbons): hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), tetraterpenes (C_{40}). Since *G. vellereum* was found to produce sesquiterpenes this terpenoid type will be discussed further in section 3.1.1. Noteworthy members of the terpenoid class include compounds such as menthol, steroids, the anticancer drug taxol and β-carotene which contribute to the orange colour of carrots (Fig. 4) (Dewick 2009; Hanson 2003).

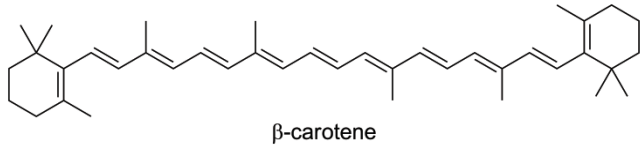


Figure 4. Structure of β-carotene.

The nitrogen containing alkaloids constitute perhaps the most famous class of secondary metabolites, including notable compounds such as cocaine, atropine, nicotine, caffeine, morphine, strychnine and many others (Fig. 5). The nitrogen in the alkaloids originates from different amino acids, and unlike the previously mentioned classes of secondary metabolites alkaloids are not produced by one specific biosynthetic pathway, but can be produced in several different ways (Dewick 2009; Hanson 2003).

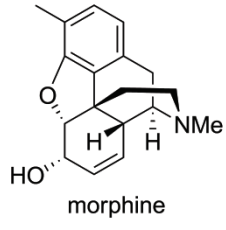


Figure 5. Structure of morphine.

Peptides and proteins exist in the grey zone between primary and secondary metabolites, in that some are produced with only minor variations throughout a large number of organisms whereas other such as snake and spider venoms are produced in just one or a few species. Many secondary metabolites derived from amino acids are not produced via the typical ribosomal peptide biosynthesis sequences but instead are produced by large multifunctional enzymes known as non-ribosomal peptide synthetases (NRPSs). NRPSs are

arranged in modules, where the linear sequence of modules corresponds to the amino acid sequence in the product (Fig. 6). Examples of a compounds produced by NRPS enzymes include the immunosuppressant ciclosporin and β-lactam antibiotics among others (Kalb *et al.* 2013; Dewick 2009).

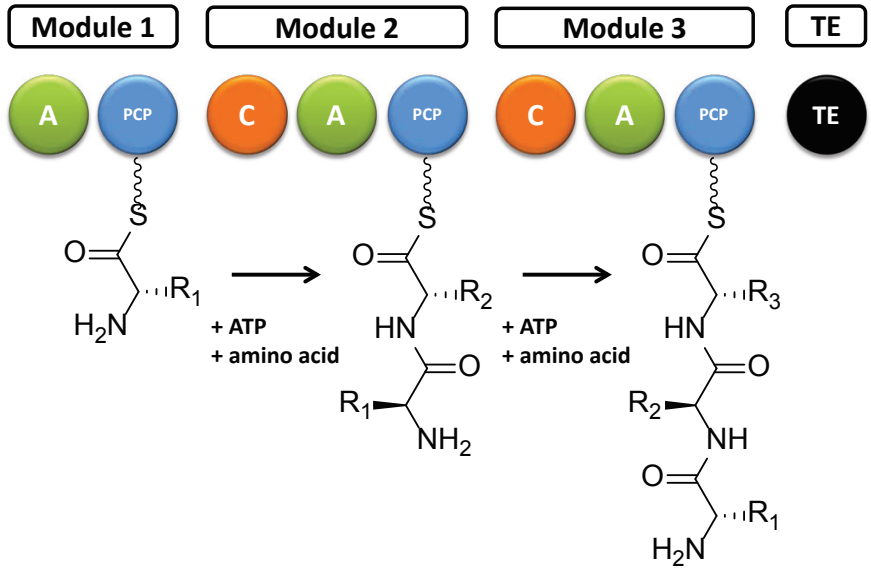


Figure 6. NRPSs are arranged in modules, where the linear sequence of modules corresponds to the amino acid sequence in the product. A: Adenylation domain. PCP: Peptidyl carrier protein domain. C: Condensation domain. TE: thioesterase.

Just as peptides and proteins, carbohydrates might be described as either primary or secondary metabolites depending on to what extent their structures have been retained within large groups of organisms. In some cases the differentiation is difficult to make since the compounds do not perfectly fit any of the two categories, examples including such compounds as ascorbic acid (vitamin C) and the blood anticoagulant heparin (Dewick 2009).

3.1.1 Biosynthesis of sesquiterpenes

Sesquiterpenes consist as mentioned above of three isoprene (C_5) units that are linked together and consists consequently of a 15 carbon structural backbone. The isoprene units can be derived from two different biosynthetic pathways, namely the mevalonic acid (MVA) pathway and the methylerythritol phosphate (MEP) pathway. The MVA pathway was discovered more than half a century ago, and was until the MEP pathway was discovered in the 1990s believed to be the only source for isoprene units. The MEP pathway is mainly utilized by

bacteria, green algae and higher plants, whereas the MVA pathway has been identified in plants, archaea, animals and fungi, though there are examples of organisms that are able to produce isoprene units from both these pathways (Eisenreich *et al.* 2004; Chang *et al.* 2013).

Both the MVA and the MEP pathways will result in the formation of the same products, namely the precursors in the terpene biosynthesis, the nucleophilic isopentyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). The biosynthesis of terpenes is initiated by the leaving of DMAPP's diphosphate moiety, resulting in the formation of an electrophilic allylic carbocation. This carbocation will then be subjected to a nucleophilic attack by the double bond of IPP in the enzyme catalysed formation of geranyl diphosphate (GPP). In the biosynthesis of sesquiterpenes another IPP unit will then be added to GPP resulting in the formation of the 15 carbon sesquiterpene precursor farnesyl diphosphate (FPP) (Fig. 7). The FPP might then give rise to a vast number of different natural sesquiterpenes, either linear or more commonly cyclized. In fact, the diversity of different sesquiterpenes is immense and more than 200 skeletal types have been identified so far (Dewick 2009; Mann *et al.* 1994).

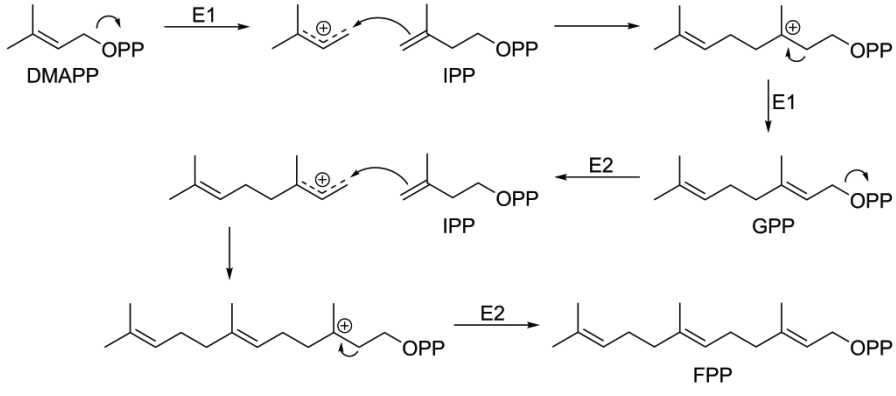


Figure 7. Biosynthesis of farnesyl diphosphate (FPP) from the addition of isopentenyl diphosphate (IPP) to dimethylallyl diphosphate (DMAPP) and gernayl diphosphate (GPP) respectively. The biosynthesis of GPP from IPP and DMAPP is catalyzed by geranyl diphosphate synthase (E1) and the elongation of GPP with an additional IPP unit is catalyzed FPP farnesyl diphosphate synthase (E2).

Fungi from the phylum Basidiomycotina, such as *G. vellereum*, are known to produce a wide range of different terpenoid metabolites of which the majority are sesquiterpenes. The largest group among these sesquiterpenes are those that are derived from humulene, which are formed through a specific cyclization of E,E-FPP (Fig. 8) (Dewick 2009; Ayer & Brown 1981).

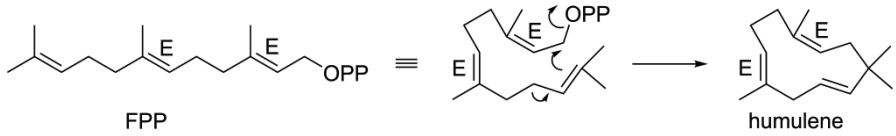


Figure 8. Biosynthesis of humulene from E,E-FPP.

Humulene can then be further cyclized to the type of compounds that have been isolated from *G. vellereum* in paper I-IV of this thesis namely the protoilludane, illudane, illudalene and cerapicane types (Fig. 9).

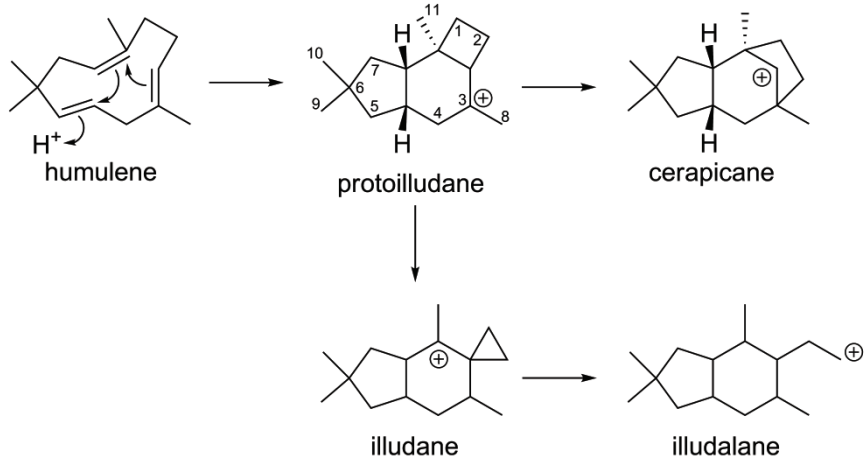


Figure 9. Formation of the protoilludane, cerapicane, illudane and illudalane carbon skeletons from humulene.

The biosynthesis of the protoilludane and the cerapicane carbon skeletons are stereospecific, resulting in a 4aR, 7aS and 7bS configuration in these structures (Dewick 2009; Abraham 2001).

3.2 Ecology of secondary metabolites

Whilst studying natural products with the aim of finding new potential drugs has been a popular subject for decades traditionally not much emphasis has been put into finding the actual role of the compounds for the organism that produces them (O'Brien & Wright 2011). Secondary metabolite production is the results of hundreds of millions of years of evolution and since the production of secondary metabolites take energy and resources from the species that produce them, the production must have given producers competitive advantages compared to non-producers in order for genes connected to secondary metabolite production to be passed on through the

generations. The evolutionary advantages might include ways for the species to defend themselves from being eaten, ways to defend their habitat for example by inhibiting the growth of competitors or as inter or intracellular signalling systems (Brakhage & Schroeckh 2011). The secondary metabolites play hence an important role within the biological environment and in recent years more and more emphasis has been put into finding their true role in the ecosystem (reviewed by O'Brien & Wright 2011).

3.2.1 Effects of secondary metabolites in nature

In some cases the functions of the secondary metabolites for the species that produces them are quite easily comprehensible as for example the production of polypeptide venoms by some snake species, which are usually used for immobilization and digestion of pray (Dewick 2009). But the roles of other secondary metabolites especially those produced by microbes are harder to understand and in some cases their roles have been showed to be more complex than first thought, as exemplified with antibiotic compounds as described below.

Secondary metabolites with antibiotic effects have long been believed to be a way for the organisms that produces them to kill or inhibit the growth of their competitors and hence win advantages, especially in nutrient poor environments (O'Brien & Wright 2011). Soil-dwelling bacteria are known to produce a multitude of different antibiotic substances, and it has been shown that both producers and non-producers within these environments have developed a resistome towards various antibiotics. The development of resistance mechanisms in antibiotic rich natural environments supports the hypothesis that these compounds really are used as agents of chemical warfare (D'Costa *et al.* 2006). This discovery can also be of clinical importance since there is evidence that resistance genes from the environment can transfer into pathogenic species (D'Costa *et al.* 2006; Wright 2010). The problem with this hypothesis is that antibiotic compounds rarely are produced or distributed in concentration high enough to kill or inhibit the growth of competing organisms in the environment. Instead it has been proposed that antibiotics in subinhibitory concentrations might act as signalling molecules (Aminov 2009). Studies have showed that antibiotics in low concentrations can alter the expression of different genes resulting for example in either increased or decreased virulence depending on which antibiotics that have been used. They also indicate that the antibiotics might have different targets at subinhibitory concentrations even though they have the same target at higher lethal concentrations (Goh *et al.* 2002; Bagge *et al.* 2004; Skindersoe *et al.* 2008). In conclusion the ecological role of antibiotic secondary metabolites is not as

clear cut as it first may seem and their roles are likely quite complex and still not fully understood.

3.3 Regulation of secondary metabolite production

During the last twenty years the understanding of the biosynthesis of secondary metabolites has increased immensely through better knowledge about the biosynthetic pathways within the organisms and the genes that control them (Brakhage & Schroeckh 2011). In fungi and bacteria the genes involved in biosynthesis of secondary metabolites are most commonly located in large clusters, which may contain one or more biosynthesis genes that encode for the enormous multidomain, multimodular enzymes that are responsible for the production of the secondary metabolites within the organisms (Brakhage 2013). The number of these secondary metabolite gene clusters within the total genome varies substantially between different fungal species. In some fungi, generally those with large genomes such as the *Aspergillus* spp., the genome might contain more than 50 secondary metabolite clusters, whereas other species have significantly fewer (von Döhren 2009; Burmester *et al.* 2011).

Interestingly, the number of secondary metabolites identified from most organisms does not even come close to match the possible number of compounds these gene clusters are able to code for. This indicates that a large proportion of the secondary metabolite biosynthesis genes is silent under standard conditions, hence are these gene clusters sometimes referred to as orphan or cryptic (Gross 2007; Brakhage 2013).

It is also well-known among natural products chemist that wild-type microorganisms cultivated in laboratory environment normally only produces very low amount of secondary metabolites (Chiang *et al.* 2011). This strongly supports the notion that many genes involved in secondary metabolite production are indeed silent under standard laboratory cultivation condition. Developing methods for activating these cryptic biosynthetic pathways are of great interest, since it is hypothesized that this might lead to the production of novel bioactive compounds. These new bioactive compounds might then become potential new leads in drug discovery but also give new insights to the chemical ecology of these organisms (Brakhage 2013).

The complex regulatory systems involved in the biosynthesis of secondary metabolites are known to respond to various stimuli from the environment (stress factors) such as e.g. the composition of the growth media (carbon and nitrogen sources, amino acids, iron availability etc.), pH and temperature. Optimization of these conditions can result in increased production of

secondary metabolites by the organisms (Sørensen *et al.* 2012). Unfortunately the response to such stimuli is highly species dependent and consequently there is no universal method that will increase the production of secondary metabolites within a large spectrum of organisms in a predictable way. The stimuli are also only relevant for some gene clusters and only the production of some compounds will be up-regulated or *de novo* produced (Brakhage 2013).

Another environmental stimulus based approach that has proven to be successful regarding the up-regulation and *de novo* production of secondary metabolites involves the co-cultivation of two or several microorganisms in a single environment, also known as interspecies crosstalk (Fig. 10) (Brakhage & Schroeckh 2011; Bertrand *et al.* 2013).

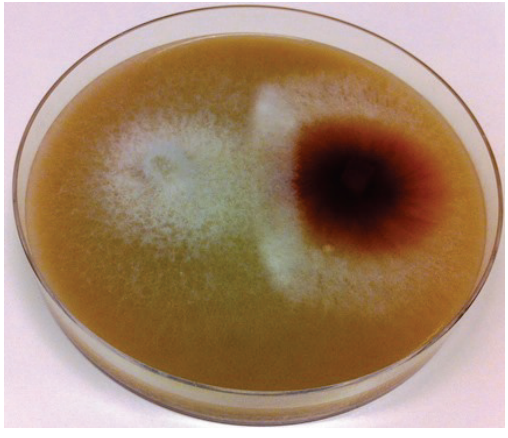


Figure 10. Co-cultivation of two or more different species of microorganisms in a single environment can result in up-regulation and de novo production of secondary metabolites by the organisms. Here a fungal-fungal co-culture between the wood-decay fungi G. vellereum and F. oxysporum. Photo: Christina Nord.

In nature organisms evolve in constant interaction with each other and consequently it is logical that they have produced different compounds involved in interspecies communication. The biosynthetic pathways that control the production of these molecules might only be activated by specific triggers and would hence have remained silent under traditional mono-culture cultivation conditions. It can also be hypothesized that the co-cultivation of organisms might to a greater extent contribute to the understanding of the ecological aspects of secondary metabolism, since the compounds are produced in direct response to other organisms and not to artificial triggers (Brakhage & Schroeckh 2011; O'Brien & Wright 2011). Results from co-cultivation studies do also indicate that many of these *de novo* produced compounds are previously undescribed in the literature and that some possess interesting biological activities, such as antimicrobial and anti-cancer (Bertrand *et al.* 2013; Cueto *et al.* 2001; Zuck *et al.* 2011).

There are also other methods than those described here that might be used to up-regulate the secondary metabolism in microorganism e.g. genetic

engineering. These will however not be considered within the scope of this thesis but are previously described in several reviews (e.g. Bergman *et al.* 2007; Brakhage 2013; Winter *et al.* 2011).

3.3.1 Explaining the chemical diversity within organisms

The discovery of silent biosynthesis gene clusters indicates that most microorganisms are able to produce many more secondary metabolites than those that have been isolated from a single species (Brakhage 2013). But only a small proportion of all compound isolated from different organism possess any known biological activity. The production of secondary metabolites evidently takes resources, so why would evolution favor organisms that produce compounds with no evident beneficial effects (Firn & Jones 2003)?

There are several hypotheses that try to explain this paradox. In some cases the compounds may actually have important effects that just have not been discovered so far. The metabolites or the genes that encode for them might also be evolutionary artefacts, which previously have played important roles for the organisms but have for one reason or another lost their effect or importance (Firn & Jones 2003). Others possibilities includes that the compounds might have synergistic effects and their true effects cannot be correctly understood in the usual biological assays were only a single compound is tested for its individual biological activity (Nelson & Kursar 1999; Richards *et al.* 2012). Yet another possibility is that they are intermediates in the biosynthetic route to the active end products. While all of these hypotheses might be true in some cases, it must be considered if these theories are fully able to explain the large multitude of in some cases very similar compounds produced by some organisms.

In 1991 Jones & Firn proposed that the production of these apparently inactive compounds might be the result of unspecific secondary metabolite biosynthesic enzymes that allows the organism to at a relatively low cost produce a multitude of different compounds. This hypothesis is based on the notion that biological activity is a rare property and consequently the sheer number of different compounds will increase the likelihood for the organism to produce a bioactive compound, which might increase its possibility of survival. Consequently will evolution favor organisms that are able to at a relatively low cost produce large amount of secondary metabolites, since they will be able to faster adapt to new threats and situations as long as the total benefit of the production exceeds the total cost of the production. This screening for activity of a large variety of compounds might be seen as an analogy of the screening programs for new drugs carried out in pharmaceutical

research, hence the name the Screening Hypothesis (Jones & Firn 1991; Firn & Jones 2003; Firn & Jones 2009). The Screening Hypothesis is not unchallenged and Pichersky *et al.* (2006) argue that while not all secondary metabolites confer with a specific selective advantage for the producer, the possibility of other unknown functions has not fully been taken into consideration by Firn and Jones. Pichersky *et al.* (2006) also argue that according to their experience natural selection does not operate based on potential future benefits but on providing the species with competitive advantage in their current situation.

In conclusion whilst many of these seemingly inactive compounds can be explained with the Screening Hypothesis and unspecific enzymatic pathways, others may very well have various not yet discovered roles and activities. It would hence be unwise to totally ignore the multitude of apparently inactive compounds produced by various organisms on the basis that they merely are evolutionary artefacts. If we want to fully understand and appreciate the fascinating nature of secondary metabolism, we need to expand our views and realize that the ecological roles of secondary metabolism are complex and not just accept the easy answers.

3.4 Natural products in drug discovery

The science that deals with the discovery and potential of natural products as pharmaceuticals or poisons are often called pharmacognosy. The term is derived from the Greek words *pharamakon* and *gnosis* which translates to drug and knowledge, respectively. In most of human history has nature been the sole source of drugs, mainly in the form of different herbal extracts, and it was not until the development of industrial synthetic chemistry in the 20th centuries that synthetic drugs came onto the market (Dhami 2013; Samuelsson 2004).

Through the years many different naturally derived drugs have been identified. In some cases the organisms were used as in medical remedies long before the actual active components were discovered. The flower purple foxglove (*Digitalis purpurea*) has for example been used in folk medicine as a remedy for heart failure for at least 200 years, but the compound responsible for the effect digitoxin was not obtained pure until later (Belz *et al*. 2001). Other species such as the fungus *Penicillium crysogenum*, which produces the antibiotic penicillin, have on the other hand never been used in folk medicine (Dewick 2009).

In the mid-20th century pharmacognosy was beginning to grow out of fashion as a research field, mainly due to predictions stating that, in the future new drugs would be produced synthetically rather than come from nature. The process of isolation and structural elucidation of natural products at the time were also a slow and tedious process, and it could in some cases take years to fully elucidate the structure of a single compound. During the past decades the development of better analytical techniques such as high performance liquid chromatography (HPLC), mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy has speed up and improved the process of isolation and structural elucidation of secondary metabolites substantially. Improved work methods such as bio-assay guided isolation have also been developed. Some of the new synthesis based work methods (e.g. high throughput screening) for drug discovery have also not been quite as successful as first expected. Together, this has resulted in that more interest is once again directed towards natural products as a potential source of new drugs (Phillipson 2007; Newman & Cragg 2012).

Presently drugs of natural origin or directly derived therefrom compose approximately 40 % of the drugs approved by FDA between the years 1981 to 2010, and if only the timespan between 2000 to 2010 is considered, roughly 50 % of all approved small molecular new chemical entities (NCE) were of natural origin (Newman & Cragg 2012). The diversity of secondary metabolites in nature is indeed enormous and much remains to be discovered.

4 Experimental

This chapter aims to give a short overview of the most important techniques used for isolation and structural elucidation of the secondary metabolites described in this thesis.

4.1 Isolation and purification

4.1.1 Solid phase extraction

In paper I-IV of this thesis the fungi were cultivated in liquid growth media. After a certain cultivation period the supernatants were filtered to obtain cell-free samples. The obtained filtrates still contained besides the desired secondary metabolites unconsumed components from the growth media as well as other by-products. Many of these residues are however more hydrophilic than most secondary metabolites. There are several possible methods to separate the desired products from these impurities such as liquid-liquid extraction but in this thesis solid phase extraction (SPE) was used.

The principles for SPE are quite simple. Basically a SPE-column consists of a chromatographic stationary adsorption medium contained within a small cartridge. When a dissolved sample is applied to the column some of the components within the sample will adsorb to the stationary phase whereas others will not, depending on the chemical properties of both the stationary adsorption medium and the sample components. The column might then be washed to remove even more undesired impurities, before the products of interest are eluted from the column (for further reading see e.g. Harris 2002)

4.1.2 Liquid chromatography

Liquid chromatography (LC) was the main chromatographic technique used for the separation and isolation of the compounds described in this thesis. The principles for LC are similar to those of SPE, but allow separation of more similar compounds than SPE due to better efficiency of the separation.

The separation in a LC system depends on the interaction of a sample mixture with a stationary phase packed in a column, through which a mobile phase is pumped. Compounds that to a large extent interacts (i.e. are able to form hydrogen bonds or van der Waals interactions) with the stationary phase will be retained longer in the column than those that do not interact with the stationary phase, resulting in them being separated from each other. The time it takes for a compound to pass through the column is known as its retention time (t_R). The choice of stationary phase and mobile phase will affect the retention time of the compound.

There are different types of techniques that can be chosen depending on the kind of compounds to be separated. The main types are normal phase-, reversed phase- (RP), size exclusion and ion exchange chromatography. Since mainly reversed phase was used in this work, only this type will be discussed more thoroughly. In RP chromatography a nonpolar stationary phase is used in combination with a polar mobile phase, usually consisting of a mixture of water and an organic solvent that is miscible with water. The most popular stationary phase is ODS (octadecylsilane) that consists of C_{18} alkyl chains covalently bond to silica particles (Fig. 11). When a RP column is used the t_R will increase with the lipophilicity of the compound.

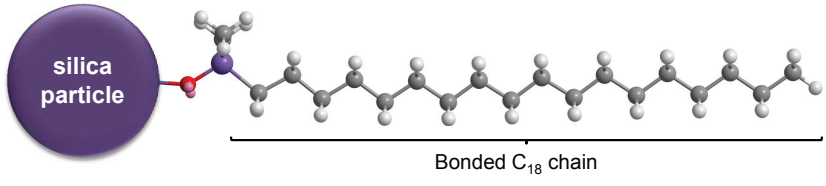


Figure 11. The octadecylsilane (ODS) stationary phase consists of C_{18} alkyl chains covalently bond to silica particles.

To be able to separate a mixture of compounds with very different chemical properties which often is the case for natural products, the concentration of the organic solvent in the mobile phase is increased with time (gradient elution) compared to using the same mobile phase during the separation (isocratic elution). Gradient elution will increase the efficiency of the separation for

complex mixtures as well as it allows the separation to be performed in a shorter time span. A disadvantage is that the column needs to be reconditioned to the initial concentration before a second separation can be started. To be able to monitor the separation, the LC-system is often connected to a detector. Common detectors include the cheap and reliable UV-detector and the more expensive but more information rich mass spectrometry (MS) detector (a combination referred to as LCMS).

Originally LC was performed in a gravity flow system, using loosely packed columns with relatively large particles. Decreasing the size of the stationary phase particles and packing it more tightly will result in a better efficiency and shorter run times for the separation, but the pressure will be higher in the system. To be able to handle this increased pressure, pumps that can handle high pressure have been constructed. This high pressure method is distinguished from normal LC and is called high-performance LC (HPLC) and was the method of choice for most of the work within this thesis (for further reading see e.g. Harris 2002 or Miller 2009).

4.2 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is perhaps alongside mass spectrometry (MS) the most important technique for structure elucidation of secondary metabolites, and much of the work in this thesis has been performed using NMR.

NMR spectroscopy is based on a property of certain atomic nuclei called nuclear spin. Best suited for NMR analyses are nuclei with a spin quantum number (I) of ½, since they will only have two so called spin states positive (+) and negative (-). Nuclei with $I > 1$ have several different spin states which makes them more disadvantageous for NMR analyses while atoms with nuclei that lack spin e.g. ^{12}C cannot be analysed by NMR. Fortunately for the natural products chemist, many of the most abundantly occurring elements in secondary metabolites have at least one isotope with $I = ½$ e.g. ^{1}H, ^{13}C and ^{15}N.

Without influence of outside forces both spin states of the nuclei ($I = ½$) will have the same energy level. In NMR spectroscopy a strong external magnetic field (B_0) is applied to the nuclei, which will make the spin of the nuclei orient themselves in either the direction of B_0 (+½) or in the opposite direction (-½). The spin state in the direction of B_0 has a slightly lower energy than that in the opposite direction and consequently more nuclei will be in the +½ state. It is this difference that is the bases for NMR spectroscopy. The energy difference between the two spin states is dependent on the strength of

the B_0 field as well as on the isotope species. The ^1H isotope has a larger energy difference between the two spin states than for example ^{13}C and thus ^1H NMR will be more sensitive.

In an NMR experiment a second magnetic field (B_1) at radio frequencies is then applied perpendicular to the first (B_0) field. When the B_1 field reaches the resonance frequency of the nuclei the spin states will start to flip and since more nuclei are initially in the lower energy +½ state, this will result in a net absorption of energy at certain frequencies. The resonance frequency is dependent on the chemical environment of the nuclei through a phenomenon known as shielding, making nuclei in a molecule possible to distinguish from each other (for further reading see e.g. Lambert & Mazzola 2003 or Hesse *et al.* 1997).

4.2.1 NMR experiments

The most common and simplest NMR experiment is the one-dimensional (1D) proton (^1H) NMR experiment. ^1H NMR is very useful due to its high sensitivity and consequently spectra with a high signal-to-noise ratio can be obtained in short time on relatively small amounts of sample. The ^1H NMR spectrum gives information about the number of hydrogen in the molecule as well as the chemical environment of the individual protons from their chemical shifts and coupling constants. Another important isotope for NMR experiments in organic chemistry is ^{13}C. ^{13}C NMR is less sensitive than ^1H NMR and since the ^{13}C-isotope also only constitutes 1 % of all naturally occurring carbon, ^{13}C NMR require larger amounts of sample and longer experimental times than ^1H NMR. In this thesis all one-dimensional ^{13}C NMR experiments have been acquired with proton decoupling, which increases the sensitivity and makes the spectra easier to interpret since all carbon signals will be obtained as singlets.

To be able to fully elucidate the structure of a molecule is it often not enough with only 1D NMR experiments and additional information can be obtained from two-dimensional (2D) NMR experiments. The simplest 2D NMR experiment is ^1H-^1H correlation spectroscopy (COSY), which shows correlation between protons on adjacent carbons. Heteronuclear single quantum coherence (HSQC) is another commonly used experiment which shows correlations between hydrogens and carbons connected by one chemical bond. A third very useful technique for structural elucidations is heteronuclear multiple bond correlation (HMBC), which show correlations between hydrogen and carbon connected by two to four chemical bonds . Correlations between nuclei that are close in space can be obtained through the so-called

nuclear Overhauser effect (NOE) which may then give information about the relative configuration of the compound. Two common experiments used to determine NOE effects are nuclear Overhauser effect spectroscopy (NOESY) and rotating frame nuclear Overhauser effect spectroscopy (ROESY) (for further reading see e.g. Lambert & Mazzola 2003).

4.2.2 Chiral reagents in NMR spectroscopy

The relative configuration of a compound may be determined from ROESY and NOESY NMR experiments but they cannot be utilized to determine the absolute configuration of the structure. To resolve the absolute configuration of a compound, other techniques must be applied.

One such NMR based approach involves the derivatization of the compound with enantiomerically pure chiral reagents. In most cases the compound are derivatized with both the (S)- and (R)-isomer of the derivatization reagent, respectively. When the (S)- and (R)-derivate of the compound are analysed with NMR spectroscopy this might result in chemical shifts differences between the two derivatives. These chemical shift differences are in some cases predictable, for example if previous studies on structurally similar compounds have displayed consistent empirical trends. If the shift differences are predictable, the results may be used to determine the absolute configuration of the compound (Wenzel & Chisholm 2011).

There are several different NMR based methods available that might be used to determine the absolute configuration of different compounds depending on the structural features. In this thesis the so-called Mosher's test was used to determine the absolute configuration of secondary alcohols (Wenzel & Chisholm 2011; Ohtani et al. 1991).

4.3 Mass spectrometry

Mass spectrometry (MS) is an enormously versatile analytical method used for numerous different applications, due to its high sensitivity and the low amount of sample needed for the analysis.

In MS the mass to charge ratio (m/z) of gas phase ions are measured, which consequently means that the compounds must be ionized and transferred to gas phase before they can be analysed in the mass analyser. This has led to the construction of various different kinds of ion sources, some of these can only be used for molecules that are already in gas phase and are hence limited to sufficiently volatile compounds, whereas other can be used for liquid or even solid samples. The ion sources can also be divided into soft and hard

techniques, where hard ion sources cause the molecules to fragment to a greater extent than soft methods. In this thesis, the electrospray ionization (ESI) ion source has been used. ESI is a fast, sensitive soft technique that is used on samples in solution and is hence often the ion source of choice for LCMS-systems. In ESI, the sample in solution is passed through a capillary tube while being subjected to a strong electric field and a heated drying gas. This will result in the formation of increasingly smaller charged droplets that eventually becomes free ions that can be injected to the MS analyser (Fig. 12).

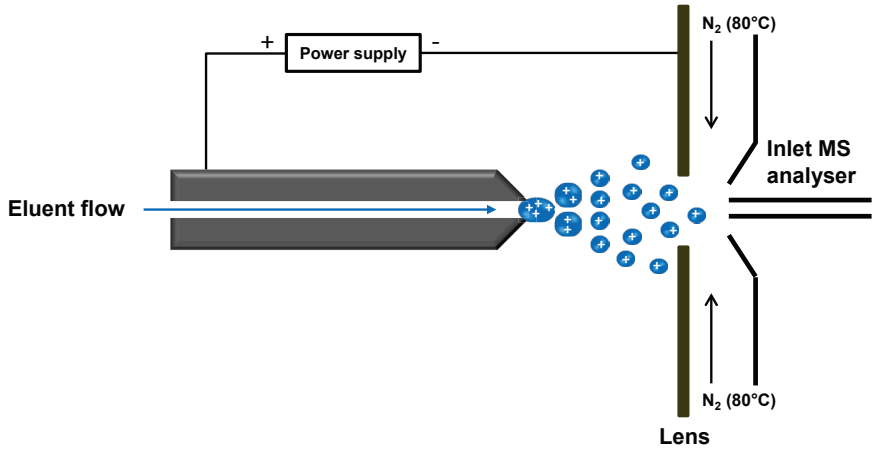


Figure 12. Illustration of the electrospray ionization (ESI) process.

Just as there are different ion sources there are several different mass analysers available on the market such as the quadrupole (Q) and time-of-flight (TOF) mass analysers. The Q consist of four rods positioned parallel to each other on which radio frequency (RF) and direct current (DC) voltage are applied creating an oscillating electric field. Depending on the applied potential the trajectory of an ion with a certain *m*/*z* can be either stabilized allowing it to pass through the rods and be detected or destabilized causing the ion to collide with the rods. In a TOF analyser ions with different *m*/*z*-ratio are separated by their time of flight, hence the name. Ions with a lower *m/z* will travel faster than those with larger *m/z* resulting in a separation. Recently, tandem mass spectrometers where different mass analysers are combined within one instrument have become increasingly more popular e.g. triple quadropole (QqQ) and hybrid instruments such as the Q-TOF. The combination of analysers makes it possible to run MS/MS experiments, which might give additional structural information compared to just a MS analysis.

Mass spectrometers which can be used to determine the mass of ions and their isotopes with enough accuracy to allow the determination of elemental composition of the ion are known as high-resolution (HR) instruments (for further reading see e.g. de Hoffman & Stroobant 2007).

4.4 Circular dichroism spectroscopy

Circular dichrosim (CD) spectroscopy is perhaps best known for its uses within the field of biochemistry, but it can also be used to determine the configuration of small organic compounds as described in paper I and II of this thesis.

A chiral molecule will render in a difference in absorption between right and left circularly polarized light (CPL), this effect is called CD. When CPL are transmitted through a sample containing a chiral compound it will cause either the right or the left CPL to be absorbed at a greater extent than the other, resulting in a positive or negative signal. The CD effects are normally measured over several wavelengths within the visible or ultra violet (UV) region, creating the CD spectrum (Fig. 13) (For further reading see e.g. Applied photophysics ltd. 2011 or Berova *et al*. 2000).

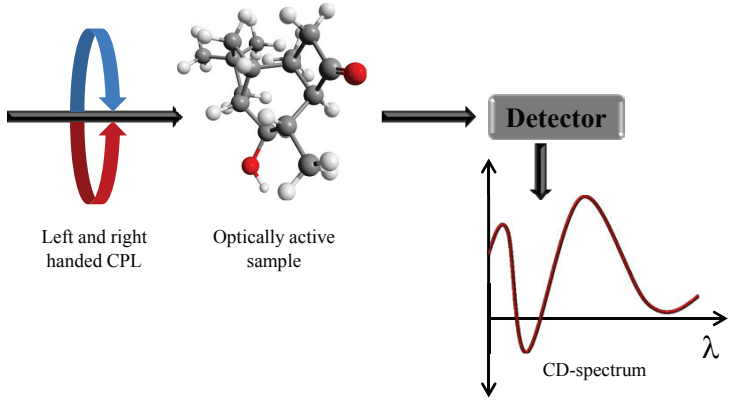


Figure 13. In CD spectroscopy left and right circularly polarized light (CPL) are emitted through a optically active sample, which result in that either the right or the left CPL will be absorbed at a greater extent than the other.

There are several chirality rules that can be used to assign the configuration of a molecule from CD spectroscopy, the one being used depending on the molecular features of the compound. Here only the two rules that were used in

the work in this thesis will be described. From the octant rule, the configuration of a saturated alkyl ketone can be deduced, if the conformation of the compound is previously known. This rule involves the placement of the compound into a coordinate system with the carbonyl group placed along the Z axis. The space around the carbonyl is then divided into eight octants. Depending on which octant that will be populated by the heaviest groups the sign (positive or negative) of the ketone carbonyl n→π* transition may be predicted (Fig. 14) (Berova *et al*. 2000).

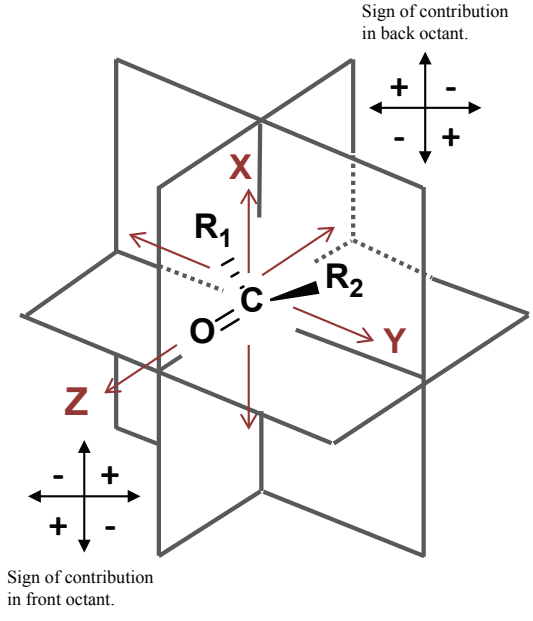


Figure 14. The octant rule can be applied to help assign the configuration of saturated alkyl ketones, if the conformation of the compound is previously known. The compound is placed into a coordinate system with the carbonyl moiety placed along the Z axis. The space around the carbonyl is then divided into eight octants. Depending on which octant that will be populated by the heaviest groups the sign (positive or negative) of the ketone carbonyl n→π transition may be predicted.*

The configuration of non-planar conjugated transoid dienes can also be deduced from CD spectroscopy. The compound are placed into the coordinate system with C-1, C-2 and C-3 of the diene in one plane, with C-2 and C-3 aligned along the Y axis and C-1 in a negative X direction, the sign of the long-wavelength π→π* transition of trans dienes can then be predicted from the position of C-4 on the Z axis (Berova *et al*. 2000; Charney *et al*. 1965).

5 Results and discussion

5.1 Secondary metabolites from *G. vellereum* (paper I-IV)

The purpose with this project was to identify and characterize the secondary metabolites produced by the wood decomposing saprotrophic fungus *G. vellereum*.

G. vellereum was one of seven wood decomposing fungal species that were screened for antifungal activity in a bioassay; the other species were *Sarea resinae*, *Cylindrobasidium evolvens*, *Gloeoporus taxicola*, *Sarea difformis, Pesotum fragrans* and *Postia stiptica*. The assay showed that *G. vellereum* was the fungi with the most potent antifungal activity (unpublished data). When LCMS data of four week liquid cultures from the different fungi were compared, *G. vellereum* was also showed to be the fungus that produced the highest amount of secondary metabolites (Fig. 15), hence was *G. vellereum* chosen for further studies.

G. vellereum was cultivated in liquid Hagem medium and different growth conditions were tested to optimize the secondary metabolite production. Thus, shaken cultures were preferred to still cultures and most compounds had reached their maximum production after four weeks.

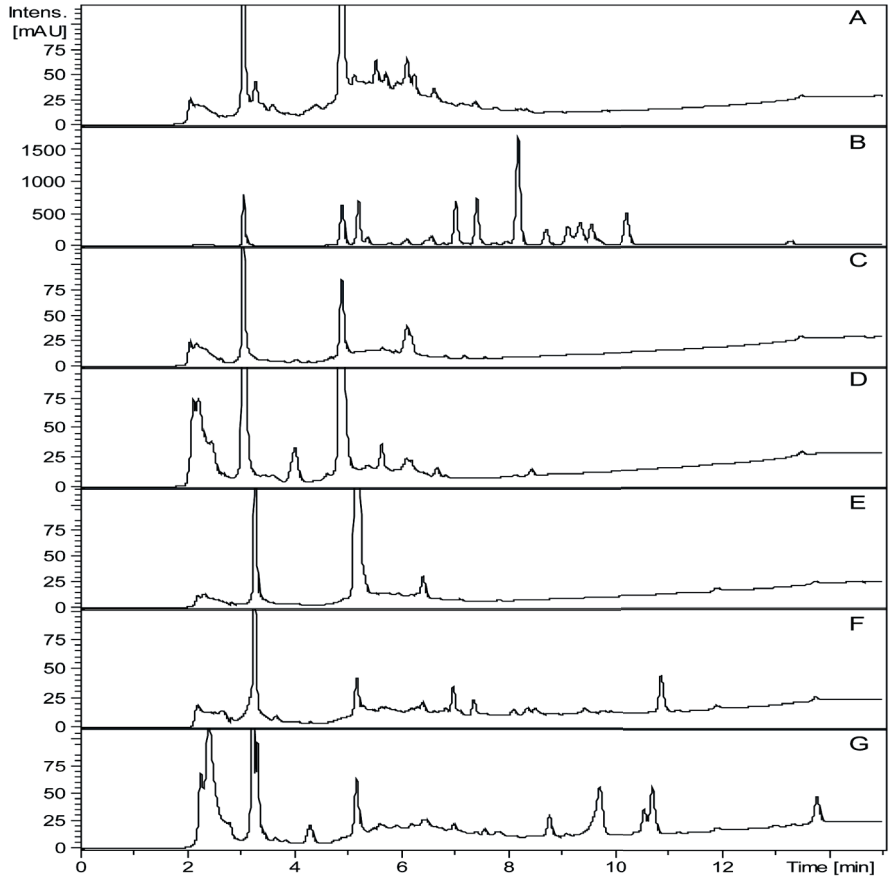


Figure 15. UV-chromatograms (254 nm) from the wood decay fungi; A Sarea resinae, B: Granulobasidium vellereum, C: Cylindrobasidium evolvens, D: Gloeoporus taxicola, E: Sarea difformis, F: Pesotum fragrans and G: Postia stiptica. Note the scale difference on the y-axis.

Even though the same isolate was used for inoculation with identical growth conditions, the production of secondary metabolites did vary between different batches. The main metabolites of the fungus (i.e. those produced with the highest yields) were produced in most cultures though in varying amounts, whereas other minor metabolites were not always produced. Only compounds that were obtained from at least two different cultures were included in the papers, due to the need for reproducibility. The reason for the difference in secondary metabolite production between the different batches is likely the up or down regulation of the genes that control the enzymatic pathways that produces the different types of secondary metabolites. In some cultures a lot of illudane

type sesquiterpenes were produced whereas in others there were hardly any, indicating up and down regulation of the genes involved in the biosynthesis of these compounds. The structures of compounds that were found in only one culture were in some instances partially or fully elucidated. The elucidated structures represented all sesquiterpene skeletal types previously found to be produced by the fungi except for the cerapicane and no specific structural trends among these compounds were detected (Fig. 16). The reason why different genes are turned off and on when the same inoculum is used and the liquid cultures treated identically is not clear, but might be the result of the different growth stages of the fungi and hence the age of the inoculum.

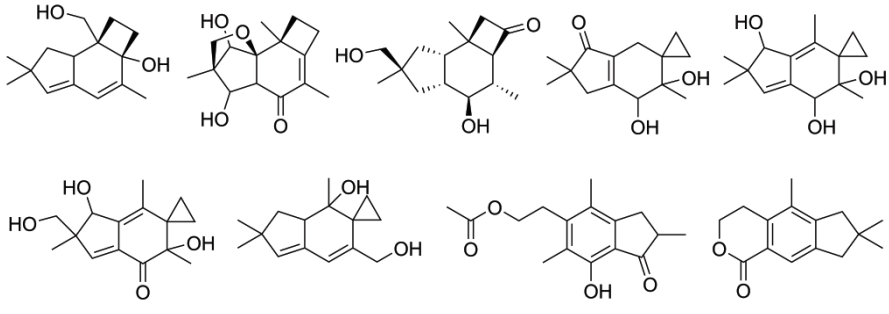


Figure 16. Structures of some compounds only found in isolated cultures of G. vellereum. In some instances the absolute configuration were not elucidated.

5.1.1 Structure elucidation

The secondary metabolites produced by *G. vellereum* were isolated and extracted using SPE and chromatographic techniques as described in paper I-IV. The structures and the relative configuration of the compounds were then determined using ^{1}H, ^{13}C, COSY, HSQC, HMBC and ROESY NMR experiment combined with the elemental composition obtained from HRMS. The absolute configuration of the compounds was determined from biosynthetic considerations as well as from CD spectroscopy and Mosher´s test, when the structures comprised suitable structural elements.

G. vellereum was showed to produce sesquiterpenes comprising four different skeletal types, namely protoilludane, illudane, illudalane as well as one compound from the rare cerapicane type. In total 33 secondary metabolites were isolated and characterized of which 22 were not previously described (Fig. 17). Of the 11 previously known compounds (Fig. 18), two (coprinolone diol and pterosin M) were not previously described as natural products (paper I-IV). Interestingly, five of these known compounds had

previously been isolated from the same fungus, namely the basidiomycete *Radulomyces confluens*, from which they also constitute all known secondary metabolites (Fabian *et al.* 1998). *R. confluens* might according to Larsson (1997) sometimes be mistaken for *G. vellereum*, but the identity of the *G. vellereum* isolate used in our studies has been established genetically and not only on morphological basis (paper I; Lygis *et al.* 2005).

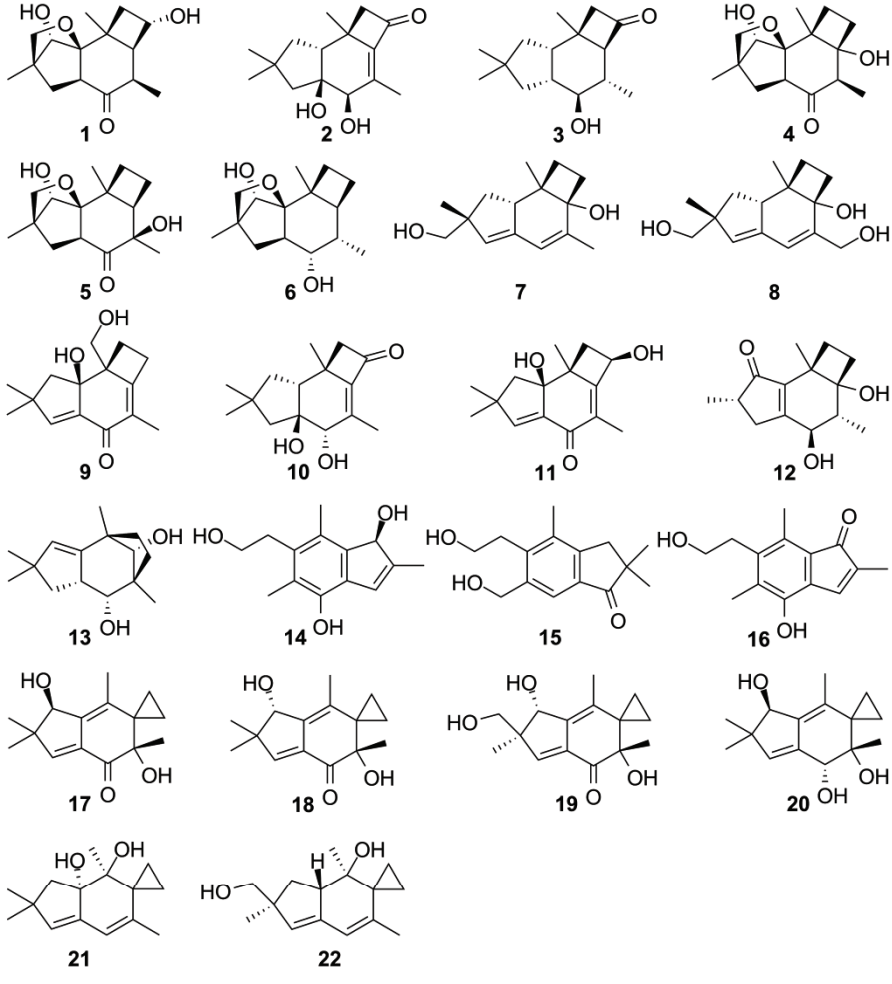


Figure 17. Structures of compound ***1-22****.*

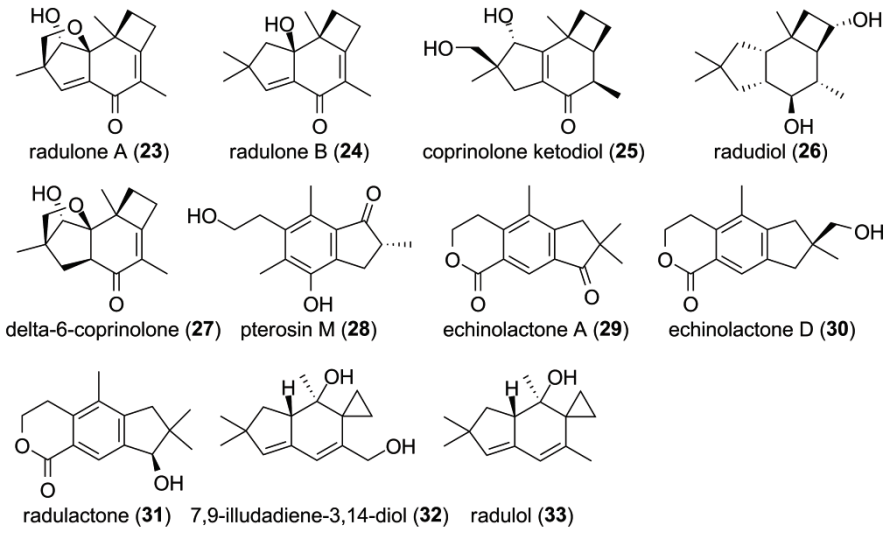


*Figure 18. Structures of compounds **23-33**.*

5.1.2 Biologic activity

The potential biological activities of the secondary metabolites obtained from *G. vellereum* were tested in a series of bioassays, with the aim of finding bioactive compounds and if possible also link the activity to potential ecological roles. The bioassays comprised antifungal, cytotoxicity and growth promoting studies. All compounds were not tested in all assays but were selected with regard to their availability and expected effects based on the activity of similar compounds.

At first a literature review of the eleven previously known compounds, regarding their potential biological activities was done. This review showed that radulone A (**23**) has potent antibacterial, antifungal, cytotoxic as well as platelet aggregation inhibiting effects (Fabian *et al.* 1998). Antifungal activity were also displayed by the illudane 7,9-illudadiene-3,14-diol (**32**), which had previously been obtained from both the fungi *Agrocybe aegerita* and *Russula delica* (Stránský *et al.* 1992; Clericuzio *et al.* 1998). The illudalane sesquiterpenes echinolactone A and D (**29** and **30**), previously obtained from the wood decaying pathogenic fungus *Echinodontium japonicum*, did induce the elongation of lettuce seedlings (Suzuki *et al.* 2005; Suzuki *et al.* 2006). For the remaining seven previously described compounds (**24-28**, **31** and **33**) no or only moderate biological effects had been attributed (Fabian *et al.* 1998; Hasegawa *et al.* 1974; Starratt *et al.* 1989). It must be noticed that illudin M, which is the enantiomer of **17** and diasteromer of **18**, and illudin S (Fig. 19),

which is the diasteromer of **19**, are known as very potent cytotoxic agents, and since their mechanism of action is proposed to be based on chemical reactivity rather than enzymatic activity is it likely that compounds **17-19** also will display cytotoxic activity (McMorris & Anchel, 1965; McMorris *et al.* 1989; McMorris *et al.* 1990; McMorris *et al.* 1992).

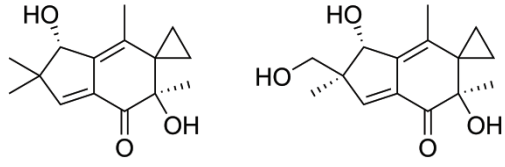


Figure 19. Structures of illudin M and S.

The ability of the compounds[1] to inhibit the condia germination of seven different species of wood-decay fungi were tested against: *Heterobasidion occidentale* (tested compounds **1-15**, **17**, **18**, **20**, **23-25** & **32**), *Fusarium oxysporum* (tested compounds **1-13**, **23** & **24**), *Penicillium canescens* (tested compounds **1-13**, **23** & **24**), *Bjerkandera adusta* (tested compounds **1-3**, **14**, **15**, **17**, **18**, **20**, **23-25** & **32**), *Coniothyrium sporulosum* (tested compounds **1-3**, **14**, **15**, **17**, **18**, **20**, **23-25** & **32**), *Coniophora puteana* (**1-3**, **14**, **15**, **17**, **18**, **20**, **23-25** & **32**) and *Phlebiopsis gigantea* (tested compounds **3**, **14**, **17**, **18**, **20**, **23** & **24**) at concentrations up to 1.0 mM (papers I-II and unpublished data). Compound **23** inhibited the growth of *H. occidentale*, *C. puteana* and *P. gigantea* at concentrations down to 100 µM, 500 µM and 10 µM respectively, which was expected since this compound has previously been described with having antifungal properties (Fabian *et al.* 1998). Surprisingly compound **32** did not inhibit the growth of any of the four fungal species it was tested against, even though it previously has been found to inhibit the growth of the yeast fungus *Candida kefyr* (Stránský *et al.* 1992). Of the new secondary metabolites, compound **18** was able to inhibit the growth of *H. occidentale* and *P. gigantea* at concentrations down to 500 µM in both cases, which has to be considered as rather moderate inhibitions. None of the other compounds had any growth inhibiting effects on the tested fungal species.

[1] Not all compounds are tested against all species. Compounds **16**, **19**, **21** and **22** were not tested due to lack of available material and of the previously known compounds only **23-25** and **32** were tested.

Compounds **29** and **30** have previously been shown to promote the radial elongation of lettuce seedlings and it was thus of interest to investigate if the other illudalane sesquiterpenes obtained from *G. vellereum* displayed similar effects. Since growth promoting effects also have been observed for protoilludane and cerapicane type sesquiterpenes a selection of these compounds was also tested (Suzuki *et al.* 2005; Suzuki *et al.* 2006; Hirota *et al.* 2003). The effects of compounds **4-15**, **26**, **27** and **31** on the growth of lettuce seedlings were evaluated at concentrations of 100 μM[2] during a time span of 72 h. The results showed that compound **31**, that display large structural resemblance to both known growth promoters **29** and **30**, did unexpectedly decrease the total length of the lettuce seedling with 40 % though their total mass were not significantly changed. Compound **9** did increase the total length of the seedlings with 34 %, though the biomass of the seedlings was not significantly changed. The other compounds did not increase or decrease the length or the biomass of the lettuce seedlings significantly (paper II and unpublished data).

*Table 1. CC_{50} (μM) values for compounds **16-20**, **22** and **32** against the two tumor cell lines Huh7 and MT4*

Cell Line	CC_{50} (μM)								
	14	**15**	**16**	**17**	**18**	**19**	**20**	**22**	**32**
Huh7	>400	>400	6.7	1.3	0.38	0.098	160	>400	240
MT4	55	180	0.15	0.12	0.014	0.023	45	>400	110

The cytotoxic activity of compounds **14-20**, **22** and **32** were evaluated against the Huh7 and MT4 cancer cell lines. Compounds **16-19** showed potent cytotoxic effects against both cell lines, whereas the others had no or only moderate activity at concentrations up to 400 μM (Table 1). Interestingly, compound **18** was roughly ten times more potent than its diasteromer **17**, with CC_{50} values of 0.38 μM (Huh7) and 0.014 μM (MT4) for compound **18** compared to 1.3 μM (Huh7) and 0.12 μM (MT4) for compound **17**. The cytotoxic effects of illudin M and illudin S have been proposed to be dependent on chemical reactivity rather than on enzymatic affinity. The proposed mechanism of action in the cell involve a two-step reaction, initiated by a Michael type addition of thiols in e.g. amino acids to the α,β-unsaturated

[2] The test concentration was chosen based on the concentrations used by Suzuki *et al.* (2005 & 2006) on **29** and **30**.

carbonyl moiety resulting in unstable intermediate followed the creation of a more stable aromatic product through the loss of the tertiary hydroxyl group and the opening of the cyclopropane moiety through a nucleophilc attack by e.g. H_2O, DNA, protein (Fig. 20) (McMorris *et al.* 1989; McMorris *et al.* 1990; McMorris *et al.* 1992; McMorris *et al.* 1996). This chemical reaction has also proven to be highly pH dependent with a maximum reactivity at pH values between 5 and 6.5 (McMorris *et al.* 1990).

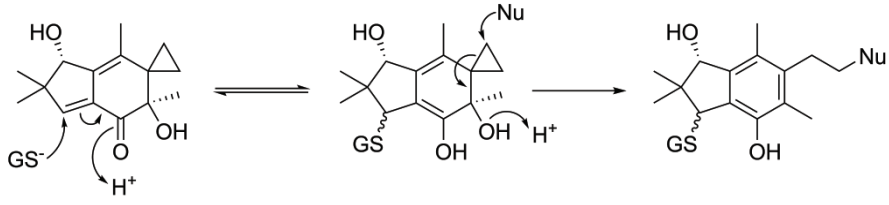


Figure 20. Proposed mechanism of action of the alkylating agent illudin M, possible nucleophiles (Nu) might include H_2O, DNA and proteins.

To test if compounds **17** and **18** reacted in a similar fashion as described for their isomer illudin M, they were reacted with cysteine at pH 5.4. Compound **17** did indeed react in a similar though not identical fashion and rate as described for illudin M forming a 2:1 mixture of two isomeric products with a $t_{1/2}$ of 27 min. Compound **18** reacted at a higher rate ($t_{1/2}$ 5.6 min) and did also almost exclusively form only one of the expected reaction products (McMorris *et al.* 1990). These results indicate that the difference in cytotoxicity observed between **17** and **18** can be explained on the basis of chemical reactivity and that the cis configuration of OH-3 and OH-7 increases the reactivity compared to the trans configuration. The chemical mechanism behind the observed change in reactivity is quite puzzling and has not yet been given a satisfactory explanation.

The low or absent cytotoxic effects of compounds **14**, **15**, **20**, **22** and **32**, might be explained by them not being able to facilitate a Michael type reaction. This hypothesis is strengthened by the fact that the cytotoxic illudalane **16** like compounds **17-19** has an α,β-unsaturated carbonyl moiety and hence the ability to facilitate a Michael type reaction. Compound **16** reacted also similarly with cysteine as **17** and **18**, indicating that they may share a similar mechanism of action.

Both compounds **23** and **24** comprises also α,β-unsaturated carbonyl moieties, but only compound **24** has potent cytotoxic activity (Fabian *et al.* 1998). This might suggest that compounds with this type of protoilludane

carbon skeletons may have a somewhat different mechanism of action. An interesting observation is that compound **24** unlike compound **23** forms at low pH several degradation products through a Michael type addition of water at C-2a followed by a S_N1 type opening of the ether moiety and the addition of water to C-7a. This indicates a general increase in reactivity of **24** compared to **23**.

5.1.3 Possible ecological roles

In this section some potential ecological roles for the growth promoting, growth inhibiting, anti-fungal and cytotoxic effects of the secondary metabolites produced by *G. vellereum* will be discussed. The production of the multitude of apparently inactive sesquiterpenes by the fungus will also be addressed.

The growth promoting effects observed on lettuce seedlings by compounds **9**, **29** and **30** are hard to explain. The natural environment of wood-decay fungi is very competitive and synergistic relationships are rarely if ever detected (Owens *et al.* 1994). Considering that *G. vellereum* is also a saprotrophic species and consequently live on dead tree tissue, it seems unlikely that the fungus would produce compounds that are involved in synergistic interactions with either its host or the microorganisms that share the same environment. The conclusion to the detected growth promoting effect might instead be that *G. vellereum* uses the compounds to control its own growth, that the compounds have other not yet detected effects or that the production just is a result of a unspecific enzymatic system and that the effects are mere coincidences.

Surprisingly compounds **17** and **18**, even though they displayed potent cytotoxic effects, have no or only moderate growth inhibiting effects on the other fungi. A possible reason might be that the fungi tested have evolved resistance against these types of compounds. It is also possible that these compounds aren't able to get through the cell membranes and hence cannot enter the cells were they would have effect. Kramer and Abraham (2012) suggest that volatile sesquiterpenes might facilitate the passage of toxins through membranes. If a similar mechanism is involved here it would explain why **17** and **18** alone would not inhibit the growth of fungi in the bioassays though highly cytotoxic. *G. vellereum* produces indeed some rather volatile sesquiterpenes, such as compound **33**. To confirm this hypothesis further studies are needed.

Only few of the 33 secondary metabolites obtained from *G. vellereum* displayed any potent activity in the bioassays or have been described as having

potent biological effects in the literature. Normally fungi in monocultures do not produce a lot of secondary metabolites but the production of sesquiterpenes by this isolate of *G. vellereum* is an exception. It produces large amounts of different sesquiterpenes even in monocultures and among the most abundant compounds **1**, **14**, **17**, **18** and **20**, **23** and **24**, only **17**, **18** and **23** have potent biological activity. So why would this species produce such large quantities of mainly inactive structurally closely related sesquiterpenes? Have all of the compounds without detected biological activities unknown roles for the fungi? Some compounds might very well have unknown effects such as assisting the passage of toxins through cell membranes as previously discussed, but in most cases they probably do not. The large production of sesquiterpenes by *G. vellereum* can instead most likely be explained by the Screening hypothesis (Jones and Firn 1991) indicating that *G. vellereum* has unspecific enzymatic biosynthesis machinery and as long as the benefit of the production exceeds the cost, the production will continue. Firn and Jones (2003) suggested that compounds that are themselves inactive but that might be transformed into active compounds even at low rates might still be beneficial for the organism to produce. Compound **14** might in fact be an example of this since it is in itself rather inactive but forms the cytotoxic compound **16** by an autooxidation process, though only at a rather low reaction rate.

5.2 Metabolic response to competing fungal species (paper V)

The aim of this project was to investigate the secondary metabolite response of *G. vellereum*, with regard to the up-regulation and the *de novo* production of different compounds, when interacting with competing fungal species in dual culture fungal fungal systems. The hypothesis was that the regulation of the genes that encode for the enzymatic machineries involved in the biosynthesis of secondary metabolites will be affected by the interaction with other fungal species, resulting in the up-regulation and *de novo* production of secondary metabolites. The hypothesis was also that the up-regulated or *de novo* produced compounds are involved in fugal-fungal interspecies communication to a larger extent than those not up-regulated and as such might possess interesting biological activities.

Seven different species of wood-decay fungi representing three different life strategies in nature were chosen for this study, including the plant pathogens *Eutypa lata*, *Fusarium oxysporum* and *Heterobasidion occidentale*, the wood decay fungi *Coniophora puteana* and *Phlebiopsis gigantea* and the generalist

saprotrophs *Apiospora montagnei* and *Xylaria cubensis*. The isolates of four of the selected fungal species (*E. lata*, *X. cubensis*, *A. montagnei* and *C. puteana*) had been obtained from the same substrate together with *G. vellereum* during a study, indicating that these species indeed interacts with *G. vellereum* in nature (Vasiliauskas & Stenlid 1998).

The fungal-fungal co-cultures were established by placing inocula of the fungi roughly 3 cm apart on ash sapwood agar medium. After 7 to 12 days of incubation, depending on the growth rates of the fungi, sample plugs (Ø 1 cm) were taken from the interaction zone between the fungi as well as reference samples from the opposite side of the inoculation points. The agar plugs were then extracted in methanol and subsequently analysed by LC-HRMS followed by principal component analysis (PCA) of the data.

5.2.1 Outcome of the co-cultures

The interaction patterns between the fungi were established visually at the point of harvest. In the co-cultures between *G. vellereum* and *E. lata*, *X. cubensis*, *A. montagnei*, *C. puteana* and *P. gigantea* the fungi had formed contact inhibition zones between each other, whereas the two pathogenic species *H. occidentale* and *F.oxysporum* had been overgrown by *G. vellereum* (Fig. 21).

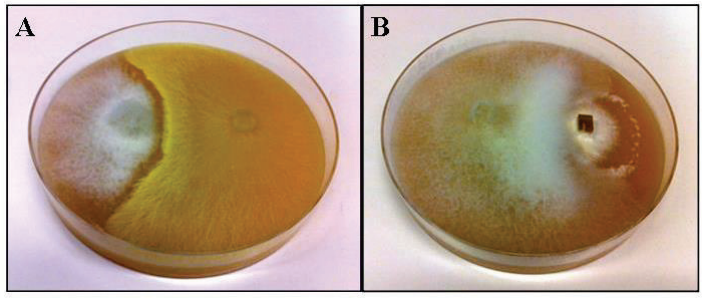


Figure 21. A: In the co-culture between G. vellereum (left) and C. puteana (right) had a contact inhibition zone been formed. B: In the co-culture between G. vellereum (left) and H. occidentale (right) had G. vellereum overgrown H. occidentale.

The data from LC-HRMS analysis were at first analysed with the aim of identifying secondary metabolites that had been up-regulated or *de novo* by *G. vellereum* in all seven interactions. A compound was considered to be up-regulated or *de novo* produced if the peak area of the compound (identified by automatic peak picking) in the interaction zone samples were at least 1.5 times higher than the median area of the same compound in the *G. vellereum*

reference samples. In total 19 generally up-regulated or *de novo* produced compounds were identified (Table 2).

Table 2. Secondary metabolites from G. vellereum up-regulated at least 1.5 times in all seven fungal fungal dual cultures

No.	$t_{R\ (min)}$	*m/z*	adduct	formula	compound	fold (min. –max.)
1	7.29	233.1533[3]	$[M+H]^+$	$C_{15}H_{20}O_2$	unknown [4]	4.6 - 26.2
2	8.62	249.1483	$[M+H]^+$	$C_{15}H_{20}O_3$	**27**	2.7 - 94.9
3	8.71	271.1301	$[M+Na]^+$	$C_{15}H_{20}O_3$	unknown[4]	2.5 - 81.3
4	8.77	217.1583[3]	$[M+H]^+$	$C_{15}H_{20}O$	unknown[4]	4.5 - 22.6
5	8.92	231.1376	$[M+H-H_2O]^+$	$C_{15}H_{20}O_3$	**18**	3.5 - 54.5
6	9.15	233.1532	$[M+H-H_2O]^+$	$C_{15}H_{22}O_3$	**2**	2.7 - 21.2
7	9.42	217.1584[3]	$[M+H]^+$	$C_{15}H_{20}O$	unknown[4]	6.4 - 16.2
8	9.60	231.1377	$[M+H]^+$	$C_{15}H_{18}O_2$	unknown[4]	3.0 - 49.2
9	10.04	233.1538	$[M+H]^+$	$C_{15}H_{20}O_2$	**24**	2.3 - 18.8
10	10.37	177.1635[3]	$[M+H]^+$	$C_{13}H_{20}$	unknown	4.0 - 135.1
11	10.48	255.1352	$[M+Na]^+$	$C_{15}H_{20}O_2$	unknown[4]	3.4 - 148.7
12	10.74	219.1740	$[M+H-H_2O]^+$	$C_{15}H_{24}O_2$	**3**	1.7 - 21.5
13	10.94	231.1379	$[M+H]^+$	$C_{15}H_{18}O_2$	unknown[4]	20.6 - 172.8
14	11.45	219.1740[3]	$[M+H-H_2O]^+$	$C_{15}H_{24}O_2$	unknown[4]	4.7 - 26.7
15	11.84	231.1376[3]	$[M+H]^+$	$C_{15}H_{18}O_2$	unknown[4]	1.9 - 10.0
16	12.77	215.1427	$[M+H]^+$	$C_{15}H_{18}O$	unknown[4]	3.7 - 57.6
17	13.14	201.1635[3]	$[M+H-H_2O]^+$	$C_{15}H_{22}O$	**33**	1.8 - 8.8
18	13.30	217.1582	$[M+H]^+$	$C_{15}H_{20}O$	unknown[4]	2.5 - 49.3
19	13.81	203.1791[3]	$[M+H]^+$	$C_{15}H_{22}$	unknown[4]	2.8 - 21.1

Six of the compounds were identified as previously known secondary metabolites from *G. vellereum* and of the remaining unidentified compounds were at least twelve likely also of sesquiterpenoid origin, since the HRMS data indicated that they all contained 15 carbons. Among the identified compounds only one, namely the highly cytotoxic 3S,7S-illudin M (**18**), had known potent biological activity.

To investigate if the secondary metabolite response by *G. vellereum* was dependent on the interacting fungal species, the LC-HRMS data from the seven different interactions were analysed individually by PCA. In most score-

[3] Adduct cannot be conclusively determined.
[4] The compound is most likely of sesquiterpenoid origin.

plots the interaction zone samples were well separated from the reference samples within the first two components (Fig. 22 & 23). The grouping of the interaction zone samples was rather poor in some of the interactions, and especially in those where *G. vellereum* had overgrown the other fungi (*H. occidentale* and *F. oxysporum*). From the loading plots, the compounds considered most important for the grouping of the interaction zone samples were selected. Many of the compounds considered most important for the separation were also among those generally up-regulated by *G. vellereum*, but not all. In total 23 compounds that were not among those generally up-regulated by *G. vellereum* were identified, of which radulone A (**23**) and an unpublished isomer to 3S,4R,7R-dihydroilludin M (**19**) were identified as metabolites previously obtained from *G. vellereum*. Compound **23** though important for the separation in the score plot for several of the interactions (*C. puteana*, *F. oxysporum*, *H. occidentale*, *P. gigantea* and *X. cubensis*) were never significantly up-regulated. The most likely explanation to this is that the slightly concentration sensitive Pareto scaling method was used and compound **23** was always produced in very high concentrations by *G. vellereum*. In some cases it was not possible to determine which fungi that had produced the compounds, since some of them were only obtained in the interaction zones and not in any of the reference samples. Thus, some of the unidentified compounds may be produced by the other fungi rather than *G. vellereum*.

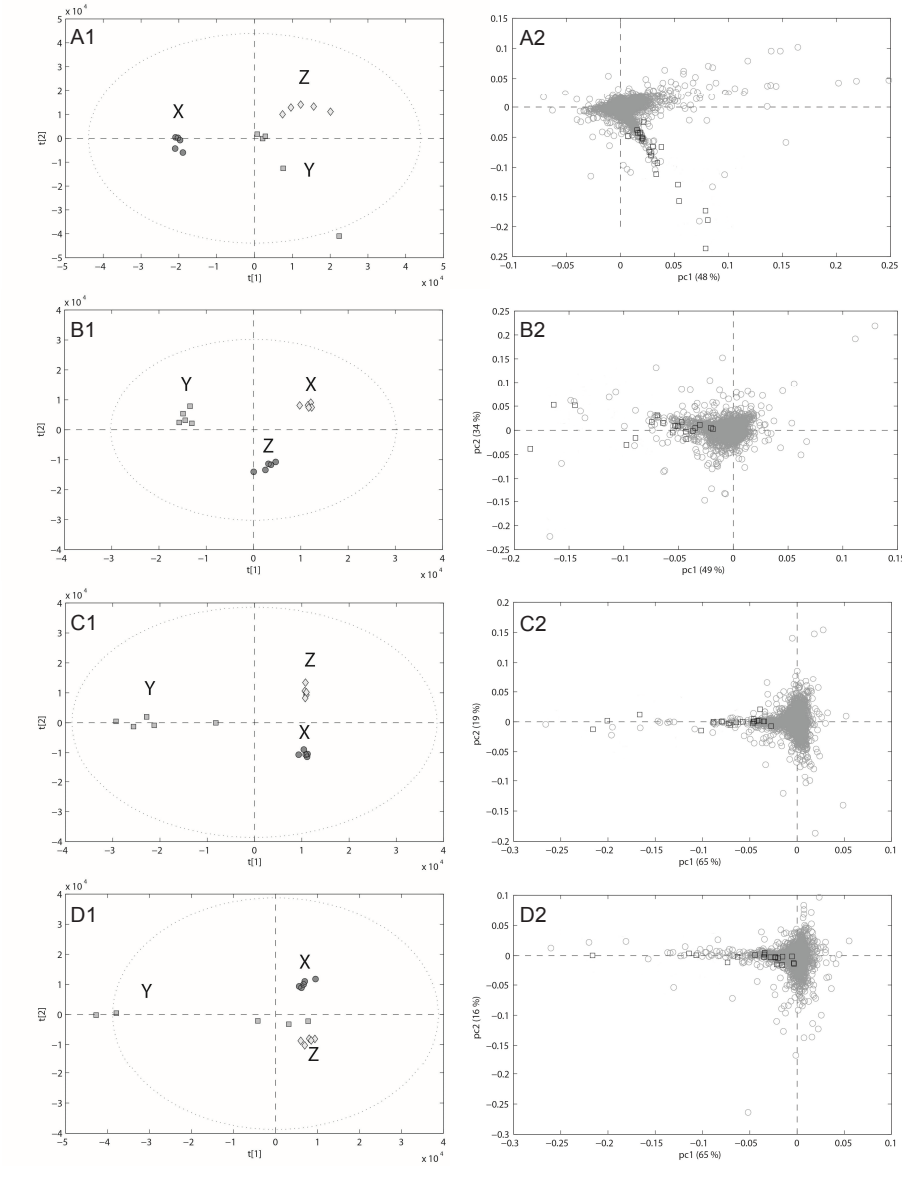


Figure 22. Score plots (1) and loading plots (2) obtained from the PCA analysis of LC-HRMS data from the fungal fungal interactions between G. vellereum and A. montagnei (A), C. puteana (B), E. lata (C) and F.oxysporum (D). Y: Samples from the interaction zones. X: Reference samples for G. vellereum. Z: Reference samples for the competing fungi.

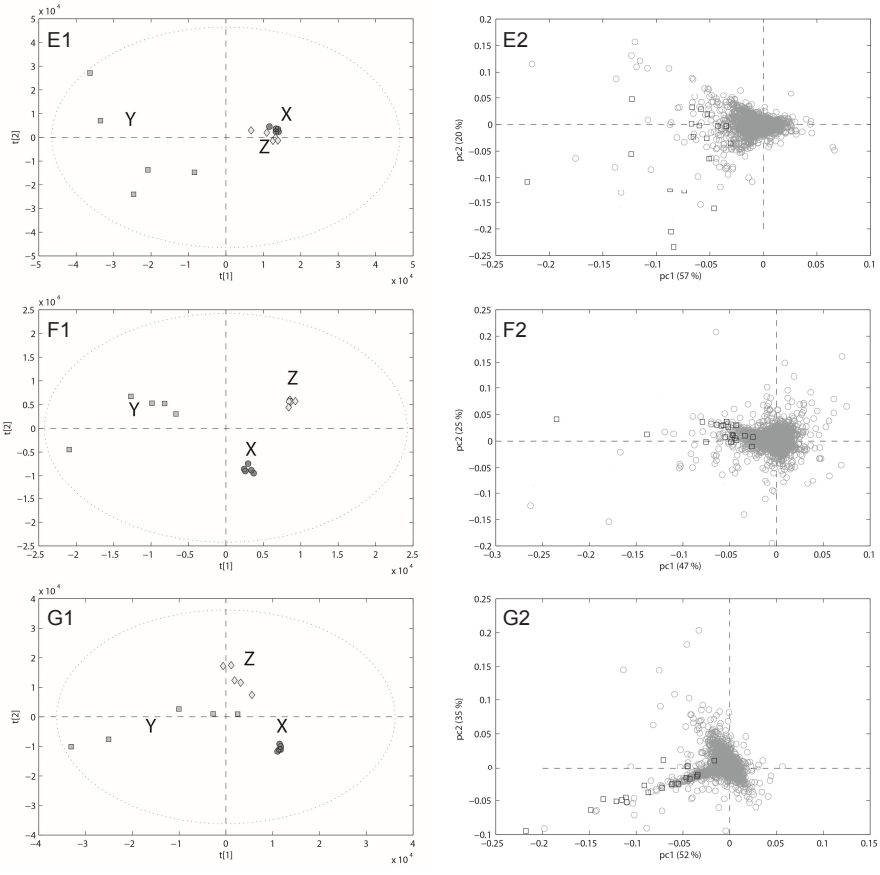


Figure 23. Score plots (1) and loading plots (2) obtained from the PCA analysis of LC-HRMS data from the fungal fungal interactions between G. vellereum and H. occidentale (E), P. gigantea (F) and X. cubensis (G). Y: Samples from the interaction zones. X: Reference samples for G. vellereum. Z: Reference samples for the competing fungi.

In conclusion, the results show that *G. vellereum* indeed up-regulates and *de novo* produces compounds when interacting with other fungi and that the majority of these compounds likely were of sesquiterpenoid origin. Among the 19 generally up-regulated metabolites six compounds were identified as previously known metabolites from *G. vellereum*, of which one was a known bioactive agent. We had hypothesized that the up-regulated compounds would be involved in the interspecies communication e.g. possess antifungal effects and it was hence surprising that only one of the identified compounds had any potent biological activity. Thirteen of the generally up-regulated compounds have not been identified yet and it is still possible that there are compounds

with strong antifungal effects among these. Some of the up-regulated compounds were also quite lipophilic, and might as suggested in section 5.1.3. help the more potent toxins to enter the cell by effecting the permeability of the cell membrane rather than having toxic effects themselves. The cytotoxic and antifungal compound **23** is also though the production did not increase in the interaction zones always produced at very high amounts. The six identified sesquiterpenes (**2**, **3**, **18**, **24**, **27** & **33**) were of either illudane or protoilludane type with varying degrees of oxidation and position of double bonds. Thus, could no conclusions to which type of biosynthesis enzymes that are activated by *G. vellereum* be made based on their structures.

The secondary metabolite response of *G. vellereum* towards other fungi varied somewhat between the different interactions. No clear trends in which type of fungus that elicits which type of response from *G. vellereum* were however observed. There were though some very interesting metabolite responses in the interactions, which would be interesting to investigate further.

6 Concluding remarks

In total 33 sesquiterpenoid secondary metabolites were isolated from liquid cultures of the, with regard to secondary metabolites, previously uncharacterized fungus *G. vellereum*. Among them, 22 were previously not described. Of the 11 previously known metabolites, two had not previously been obtained as natural products. Four of the previously not described compounds granuloinden B (**16**), 3S,7R-illudin M (**17**), 3S,7S-illudin M (**18**) and 3S,6S,7R-illudin S (**19)** were showed to have potent cytotoxic activity against the Huh7 and MT4 cancer cell lines. The cytotoxic effect of the compounds were determined to most likely be dependent on chemical reactivity rather than enzymatic affinity, and a mechanism of action similar to that of the alkylating agents illudin M and S were proposed for all four compounds. 3S,7S-Illudin M and radulone A (**23**), one of the previously obtained compounds, displayed also growth inhibiting effects on some wood-decaying fungal species. The new metabolites granulone A (**9**) increased the total length of lettuce seedlings in a bioassay, whereas the previously known radulactone (**31**) decreased the total length.

The multitude of different sesquiterpenes produced by *G. vellereum* was proposed to be to a large extent the effect of unspecific biosynthesis enzymes. The production of secondary metabolites by *G. vellereum* was up-regulated when the fungus was co-cultivated with other wood decomposing fungal species. Nineteen secondary metabolites from *G. vellereum* were up-regulated or *de novo* produced in all fungal co-cultures, of these were six identified as previously known metabolites from *G. vellereum*. Only one of the identified up-regulated compounds 3S,7S-illudin M had any known potent biological activity. The chemical response of *G. vellereum* varied somewhat depending on with which species it interacted. No clear trends regarding the metabolic response towards the different fungal types were however observed.

6.1 Proposal for further studies

As discussed in the thesis further studies are necessary if the potential ecological roles of the secondary metabolites produced by *G. vellereum* are to be better understood. An interesting experiment would be to investigate if the addition of any of the volatile sesquiterpenes obtained from *G. vellereum* might enhance the anti-fungal effects of any one of the compounds with cytotoxic effects also obtained from *G. vellereum*.

Only six of the 19 compounds that were generally up-regulated or *de novo* produced have been identified, hence it would be of interest to elucidate the structures of the unknown compounds as well as to test their potential biological activities. It would also be of interest to investigate the chemical response of *G. vellereum* towards other organisms it may encounter in the environment such as bacteria or moss.

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7 Acknowledgments

Som de flesta andra har jag lyckats spara skrivandet av den här delen av avhandlingen till allra sista minuten. Med tanke på att det är den enda delen av avhandling som de flesta av er kommer att läsa, kan det ju förefalla korkat men det är som det är. Tyvärr vet jag inte heller om min hjärna fungerar riktigt optimalt för tillfället, så därför vill jag redan nu passa på att be om ursäkt om jag har glömt någon.

I would like to express my sincere gratitude towards all of you that have been of importance during these years.

Anders Broberg, my supervisor, for all your support during these years. Whenever I got stuck you always came up with some good idea that helped solve the problem. You did also always take the time to answer my questions and help me with malfunction equipment. Thank you for taking me in as a PhD student, it has been a pleasure working here and I have learned a lot.

Audrius Menkis, my assistant supervisor, for your patience in trying to explain the strange world of fungi to me (something I know nothing about before I started here) and for always taking the time to do yet another culture.

Corine Sandström, my assistant supervisor, for showing interest in my work and your help with my thesis.

The late Lennart Kenne, you were always a joy to be around and an inspiration to us all.

I would like to thank Christofer Lendel, Rimvys Vasaitis and Mattias Fredriksson for your help with the papers.

Jag skulle också vilja tacka alla fina kollegor på institutionen, så väl nya som gamla för trevligt sällskap under dessa år. Vi har haft många underliga

diskussioner i fikarummet genom åren, om homeopatiskt knark och annat konstigt. Jag skulle dock vilja passa på att tacka några av er lite extra.

Sonja för all din hjälp med alla administrativa ärenden och att du tagit dig tid att utreda och svara på alla frågor som uppstått p.g.a. Proceedo.

Bernt tack för ditt tålamod med alla oss virriga doktorander som lallat runt på kurslab. genom åren, ställt dumma frågor och stökat till i största allmänhet.

Anders S. och Daniel för att ni alltid är villiga att diskutera undervisningen och då i synnerhet hur laborationerna kan förbättras.

Janne och Suresh för att ni är en outsinlig källa till kunskap om MS som ni gärna delar med er av.

Alla doktorander som började i samma veva som jag: Pierre, David, Eric, Gustav, Kai, Shahin, Johan M. och Lars för alla glada stunder under dessa år.

Frida för att du alltid är så glad och positiv, men inte minst för att du bakar fantastiska tårtor.

Ali och Mattias, mina rumskamrater, hoppas ni har överlevt stanken av lakrits i rummet under tiden jag skrev den här avhandlingen.

Jag skulle också vilja ge ett extra tack och en stor kram till Lena för att du är en god vän och tillika en äkta hästnörd precis som jag. Jag hoppas jag kan vara ett lika stort stöd för dig när du skriver din avhandling som du har varit för mig.

Ett tack till alla trevliga människor i stallet som jag träffat under åren och ett speciellt till Anna för att du är en så fin människa och för att du hjälper mig med att ta hand om min häst när jag inte själv hinner.

Sedan måste jag också få tacka min fina men synnerligen bortskämda hästtant Financiera, för att du alltid får mig att total kunna släppa allt annat och bara leva i nuet. Min älskade lilla hund Lotus, tänk att du föddes samma år som jag började doktorera här, vad åren går, men du är fortfarande och kommer alltid vara min lilla valp.

Jag skulle också vilja tacka min mamma Monica och pappa Ulf. Ni finns alltid där för mig och en bättre föräldrar kan man knappast ha! Tack också Magnus, för att du stått ut med en ettrig lillasyster och för alla dina råd i datorfrågor.