Secondary Metabolites associated with Plant Disease, Plant Defense and Biocontrol

Studies of *Hymenoscyphus pseudoalbidus*, *Fraxinus excelsior* and *Pseudomonas brassicacearum* MA250

Pierre F. Andersson
Faculty of Natural Resources and Agricultural Sciences
Department of Chemistry
Uppsala

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Abstract

Secondary metabolites of the fungal ash dieback pathogen *Hymenoscyphus pseudoalbidus* were investigated. Nine steroidal compounds were isolated from, or detected in, liquid cultures of the fungus. Three were the previously known compounds viridiol, viridin and demethoxyviridiol. Six were the previously unreported compounds 1-deoxyviridiol, 1-deoxy-2-demethylviridiol, 3-dihydrovirone, B-norviridin enol, B-norviridiol lactone and 1β-hydroxy-2α-hydroasterogynin A. All of the compounds are structurally related to each other, although the three latter are B-norsteroids, which are rare in nature. Viridiol displayed phytotoxic activity to seedlings of the natural host *Fraxinus excelsior*. The amount of damage caused by viridiol was higher on ash seedlings of clones, classed as susceptible to *H. pseudoalbidus*.

The metabolomes of resistant clones of *F. excelsior* were compared to those of susceptible clones. No differences were detected that were common to all clones within the groups. Metabolomes of ash seedlings treated with viridiol were compared to those of untreated ash seedlings. A number of significant changes in the concentrations of compounds were observed at the clone level. No change common to all resistant or susceptible clones was observed.

Secondary metabolites from the bacterial biocontrol agent *Pseudomonas brassicacearum* MA250 were investigated. The known compounds piliferolide A and SB-253514, as well as, the previously unreported compound piliferoic acid A were isolated from the bacterium. The compounds displayed growth inhibition of the fungal pathogen *Microdochium nivale* at concentrations down to 180 μM, which indicates that they may contribute to the biocontrol effect of *P. brassicacearum* MA250 on *M. nivale*.

Keywords: Chalara fraxinea, *Hymenoscyphus pseudoalbidus*, secondary metabolites, steroids, biocontrol, *Michrodochium nivale*, *Pseudomonas*.

Author's address: Pierre Andersson, SLU, Department of Chemistry,
P.O. Box 7015, 750 07 Uppsala, Sweden
E-mail: Pierre.Andersson@ slu.se

Dedication

To Adalaura

Why do you look so sad and forsaken?
When one door is closed, don't you know another is open?
Bob Marley

Contents

List of Publications

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

I Andersson, P.F., Johansson, S.B.K., Stenlid, J. & Broberg, A. (2010). Isolation, identification and necrotic activity of viridiol from *Chalara fraxinea*, the fungus responsible for dieback of ash. *Forest pathology* 40(1), 43-46.

II Andersson, P.F., Bengtsson, S., Cleary, M.R., Stenlid, J. & Broberg, A. Viridin-like steroids from *Hymenoscyphus pseudoalbidus* (submitted to Phytochemistry, under revision).

III Andersson, P.F., Bengtsson, S., Stenlid, J. & Broberg, A. (2012). B-norsteroids from *Hymenoscyphus pseudoalbidus*. *Molecules* 17(7), 7769-7781.

IV Cleary, M.R., Andersson, P.F., Broberg, A., Daniel, G. & Stenlid, J. Clonal differences in sensitivity of *Fraxinus excelsior* to the phytotoxin viridiol associated with *Hymenoscyphus pseudoalbidus*, the causal agent of ash dieback (manuscript).

V Andersson, P.F., Levenfors, J. & Broberg, A. (2012). Metabolites from *Pseudomonas brassicacearum* with activity against the pink snow mould causing pathogen *Microdochium nivale*. *Biocontrol* 57(3), 463-469.

The contribution of Pierre F. Andersson to the papers included in this thesis was as follows:

I Initiation of the project in collaboration with the co-authors. All chemical work and the majority of the writing.

II Initiation of the project in collaboration with the co-authors. All chemical work and the majority of the writing.

III Initiation of the project in collaboration with the co-authors. All chemical work and the majority of the writing.

IV Initiation of the project in collaboration with the co-authors. Sample preparation and LC-HRMS analyses. The interpretation of the results of the chemical analyses was done in collaboration with Anders Broberg. Writing of the parts of the manuscript related to performed chemical analyses in collaboration with Anders Broberg.

V All chemical work and the majority of the writing.

Abbreviations

ABA	abscisic acid
ACP	acyl carrier protein
CoA	coenzyme A
COSY	^{1}H-^{1}H correlation spectroscopy
DMAPP	dimethylallyl diphosphate
Enz	enzyme
FID	free induction decay
FPP	farnesyl diphosphate
GC	gas chromatography
GGPP	geranylgeranyl diphosphate
HMBC	heteronuclear multiple bond correlation
HPLC	high-performance liquid chromatography
HSQC	heteronuclear single quantum coherence
IPP	isopentenyl diphosphate
KS	β-ketoacylsynthase
m/z	mass to charge ratio
MeCN	acetonitrile
MeOH	methanol
MIC	mininum inhibitory concentration
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NMR	nuclear magnetic resonance
NMV	net magnetization vector
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
NRPS	non-ribosomal peptide synthases
P	phosphate
PCP	peptidyl carrier protein

PEP	phosphoenolpyruvate
PKS	polyketide synthase
PLP	pyridoxal 5'-phosphate
PLS-DA	partial least squares – discriminant analysis
PP	diphosphate
ppm	parts per million.
Q	quadrupole
ROESY	rotating-frame nuclear Overhauser effect spectroscopy
TOCSY	total correlation spectroscopy
TOF	time-of-flight
t_R	retention time
δ	the chemical shift

1 General introduction and objectives

1.1 Secondary metabolites

Secondary metabolites are small organic compounds (molecular masses generally less than 3000 Da), which, as opposed to primary metabolites have no function in the life cycle of cells. The production of specific secondary metabolites varies between species or genera and is thus, apart from appearance and size *etc*., an aspect of characterization of a species. The boundary between primary and secondary metabolites is not well defined and the areas overlap. The difference between the meanings of the terms *natural product* and *secondary metabolite* is also difficult to define, since most compounds dealt with in natural products research are secondary metabolites. The exact definition of a secondary metabolite, however, is not crucial for this thesis and can be studied elsewhere (Solecka *et al.*, 2012; Dewick, 2009; Bennett & Wallsgrove, 1994).

From a chemical point of view secondary metabolites are interesting for various reasons *e.g*. their structural diversity, their potential as drug candidates or as natural pesticides. The structural diversity of secondary metabolites is astonishing and there are examples of compounds produced in nature with structures so complex that no chemist could invent them, let alone synthesize them.

1.2 Function and use of secondary metabolites

Various hypotheses of the function of secondary metabolites and why they are produced have been presented in the past. One hypothesis states that they are waste or detoxification products (Williams *et al.*, 1989). This hypothesis, however, might just reflect that most research effort up until then had been put into applications of secondary metabolites for human benefit rather than what

their intended functions in nature are. Today, it is widely accepted that many of them are involved in interactions between organisms, for example in plant defense against pathogens, in toxicity of pathogens or attraction of organisms beneficial for the producer (Hartmann, 2007; Kimura *et al.*, 2001; Bennett & Wallsgrove, 1994). The functions of the vast majority of secondary metabolites, however, are still unknown.

The uses of secondary metabolites for humans are many and include uses as pharmaceuticals, agrochemicals, food additives and as ingredients in cosmetics. The impact of natural products research on medicinal chemistry is indisputable with widely known examples such as penicillins, paclitaxel, morphine and acetylsalicylic acid. The fraction of approved drugs between 1981 and 2010 that were of natural origin or in some manner derived from a natural product was as much as 50% (Newman & Cragg, 2012). The corresponding fraction of new active ingredient registrations for pesticides between 1997 and 2010 was 42% (Cantrell *et al.*, 2012).

1.3 Plant pathology

Plants comprise the only group of higher organisms that can transform the energy of sunlight into stored energy as carbohydrates. Animals thus depend on plants to take advantage of the sun's constant input of energy in the shape of food (Agrios, 2005). Plants further convert carbon dioxide to oxygen, making them important to prevent global warming and provide oxygen to the atmosphere.

Many microorganisms, as they also need to live and reproduce, also want to profit from the nutrients that plants can provide, thus choose to live in, or on, plants. Some live in symbiosis with the host and some off the dead plant. In a competition between a number of plant decomposing microorganisms, ways to gain advantages in the hunt for nutrients include arriving early to a dying plant, growing fast on the surface to prevent other contestants from reaching the nutrients or to produce antibiotic secondary metabolites. In an evolutionary perspective, the plant decomposing microorganisms that arrive earlier would thus grow and reproduce more, and in consequence out-compete the later arriving ones. In nature, however, there is more than one way to compete. If a microorganism cheats and arrives before the plant is dying and attacks it, it obviously arrives before other plant decomposers. The plant, in turn, gets diseased (Gordon & Leveau, 2010). The groups of microorganisms that cause disease in plants, pathogens, are similar to those that cause disease in humans, and include bacteria, fungi, viruses *etc*. Fungi is the group of microorganisms

that cause the most economical damage on crops (Horbach *et al.*, 2011), which makes it reasonable to assume that fungal pathogens have severe implications for both wild and cultivated plants.

The studies of organisms that induce disease in plants, plant responses to disease and plant protection against disease all compose parts of plant pathology, similar to the aspects studied in medicine for humans (Agrios, 2005).

1.4 The pathological system *Hymenoscyphus pseudoalbidus – Fraxinus excelsior*

Hymenoscyphus pseudoalbidus is a fungal pathogen, which causes dieback of European or common ash, *Fraxinus excelsior* L. The disease has been observed since 1995 in Poland (Przybyl, 2002), but is now widely spread in Europe (Gross *et al.*, 2012). It was first described in its asexual form, as *Chalara fraxinea* by Kowalski (2006), and shortly after, its sexual stage was identified as *Hymenoscyphus albidus* (Kowalski & Holdenrieder, 2009), normally a non-pathogenic fungus known since 1850. It was later established that *H. albidus* s.l. is comprised of the non-pathogenic *H. albidus* as well as a novel pathogenic species, which was ascribed the name *H. pseudoalbidus* (Queloz *et al.*, 2011). The symptoms of the disease include necrotic spots, wilting and premature shedding of leaves as well as top and shoot dieback (Bakys *et al.*, 2009). The susceptibility and resistance of different ash trees has been shown to be strongly related to the genotype (*e.g.* McKinney *et al.*, 2011).

1.5 Biocontrol of *Microdochium nivale* by bacterium *Pseudomonas brassicacearum* MA 250

The fungus *Microdochium nivale* is a plant pathogen that causes severe damage on turf grass and winter cereals. It is often referred to as pink snow mould and is common in humid and cold climates, as in northern Europe. The fungus grows well on the host under a cover of snow and often leads to death of the host after the snow melts (Levenfors *et al.*, 2008). The controlling of the fungus by synthetic pesticides may lead to the accumulation of chemicals that are toxic to the environment, which has led to the development of environmentally friendly alternatives (Boulter *et al.*, 2002; Gerhardson, 2002). One such alternative is the use of other organisms to suppress the disease, so called biocontrol agents (Compant *et al.*, 2005; Dowling & Ogara, 1994). The bacterial isolate MA250 of *Pseudomonas brassicacearum* has been shown to

have biocontrol effect on *M. nivale (Levenfors et al., 2008)*. The mechanism of the observed biological control effect is, however, unknown.

1.6 Objectives

The aim of this thesis was to investigate the involvement of secondary metabolites in the observed plant-pathogen and biocontrol agent-pathogen interactions in the above described systems. The objectives of the individual projects are listed below.

- To characterize the fungal pathogen *H. pseudoalbidus* with regard to its secondary metabolite production.
- To investigate if isolated compounds from *H. pseudoalbidus* show phytotoxic activity towards ash.
- To investigate whether the different susceptibility of ash genotypes towards *H. pseudoalbidus* is reflected on the metabolome level.
- To investigate if susceptible and resistant ash genotypes show different responses to stress associated with *H. pseudoalbidus*.
- To investigate if *P. brassicacearum* MA250 produces secondary metabolites which contribute to its biocontrol effect of fungal pathogen *M. nivale*.

2 Structural classes of secondary metabolites

Secondary metabolites are commonly divided into structural classes related to their biosynthesis. This classification has its limitations because some compounds have building blocks from more than one biosynthetic pathway and some compounds that appear closely related can have completely different biosynthetic origins. Secondary metabolites are the results of secondary metabolism, as well as primary metabolism, since primary metabolites such as amino acids, carbohydrates and acetyl-coenzyme A (CoA) are used as building blocks in secondary metabolites. Some metabolic transformations in primary metabolism also have counterparts in secondary metabolism. For further reading, consult for example Dewick (2009).

2.1.1 Polyketides

Polyketides are a structurally diverse group of compounds with, for example, antibiotic, cholesterol-lowering and antitumor activities. They are synthesized via the acetate pathway, which refers to the fact that they are mainly constructed by units of acetic acid, or more accurately, by units of acetyl-CoA and malonyl-CoA. Regarding chemical mechanisms, biosynthesis of polyketides is similar to fatty acid biosynthesis. Polyketides are synthesized by enzymes called polyketide synthases (PKS), which vary in size, architecture and function depending on the type of organism. The general biosynthesis (*Figure 1*) of polyketides starts with a starter unit, commonly acetyl-CoA, from which the acetyl group is transferred to a β-ketoacyl synthase (KS) unit of the PKS. In a similar transacylation, an extender unit, commonly malonyl-CoA, is transferred to an acyl carrier protein (ACP). This is followed by a Claisen-type reaction between the malonyl-ACP and the acetyl-KS during which a decarboxylation of the malonyl-ACP takes place (the exact mechanism of the generation of the electron pair that makes the nucleophilic attack on the acetyl-

KS is unproven). This generates a β-ketoacyl-ACP, which then takes one of two different pathways depending on the kind of PKS. Pathway A transfers the β-ketoacyl moiety to another KS, and the generated β-ketoacyl-KS enters the cycle again, which after a number of cycles generates a poly β-keto thioester. Pathway B, on the other hand, takes the β-ketoacyl moiety through one, two or three optional reductive steps, before entering the cycle again. Generally, pathway A ultimately leads to aromatic polyketides such as anthraquinones, while pathway B leads to macrolides and polyethers for example (*Figure 2*).

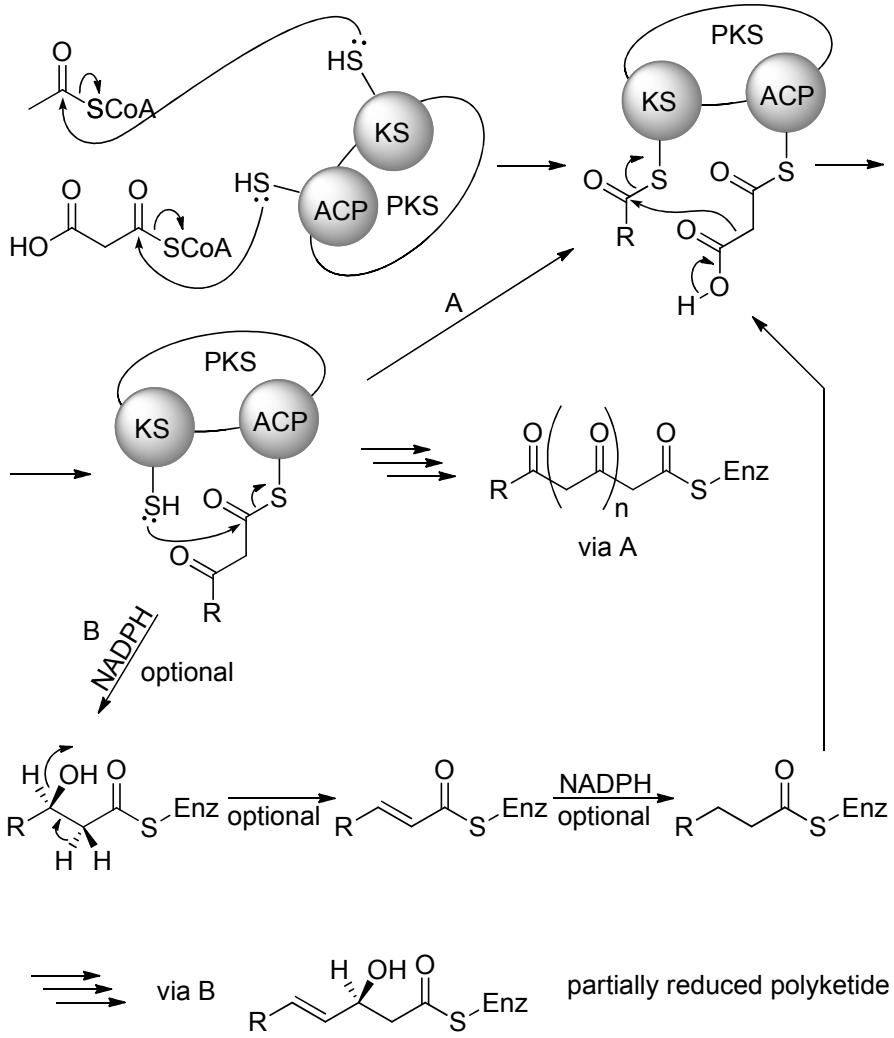
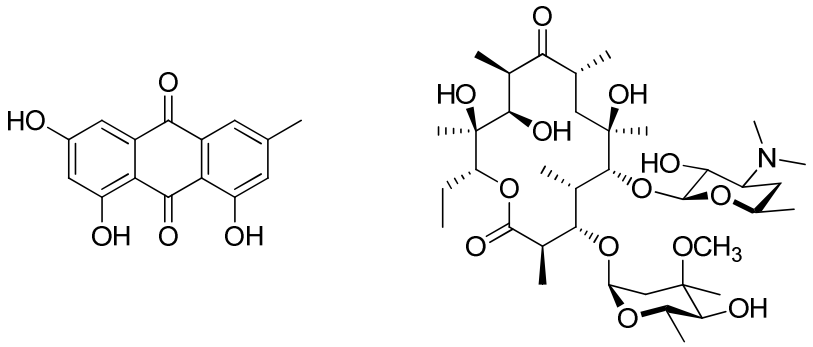


Figure 1. Basic mechanism for the biosynthesis of polyketides. Pathway A generates a poly β-keto thioester, while pathway B generates a partially reduced enzyme bound polyketide.

Once the backbone of the polyketide is finished it is released from the PKS by, for example, hydrolysis or lactonization. After the release from the PKS the polyketide can undergo cyclizations, rearrangements and further tailoring reactions such as methylations, alkylations or glycosylations. This, in combination with the fact that some PKSs utilize other starter and extender units than acetyl-CoA and malonyl-CoA, makes the structural diversity of the polyketides impressive (Dewick, 2009; Hertweck, 2009).



emodin (anthraquinone)

erythromycin A (macrolide)

Figure 2. Examples of polyketides.

2.1.2 Terpenoids

Terpenoids comprise the group of natural products with most known examples, more than 35000. The group of terpenoids includes compounds with, for example, antitubercular (Garcia *et al.*, 2012), anxiolytic, analgesic, mutagenic (Sterner *et al.*, 1987) and anticancer activities (Dewick, 2009). Many of the more volatile terpenoids give flavour and fragrance to herbal teas and essential oils. Terpenoids are sometimes referred to as isoprenoids, as they are derived from five carbon isoprene units, although isoprene (*Figure 3*) itself is not involved in the biosynthesis.

isoprene dimethylallyl diphosphate isopentenyl diphosphate

Figure 3. Molecular structures of isoprene, dimethylallyl diphosphate and isopentenyl diphosphate.

Terpenoids are divided into classes depending on the number of isoprene units that are used to generate the compound. These are; hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}) and tetraterpenes (C_{40}). Isoprene units can also be attached to compounds from other structural classes (prenylation) and these are sometimes referred to as meroterpenoids. There are, however, examples of modified terpenoids possessing a carbon skeleton that does not contain a multiple of five carbons *e.g.* the steroids, which are modified triterpenes (*Figure 4*).

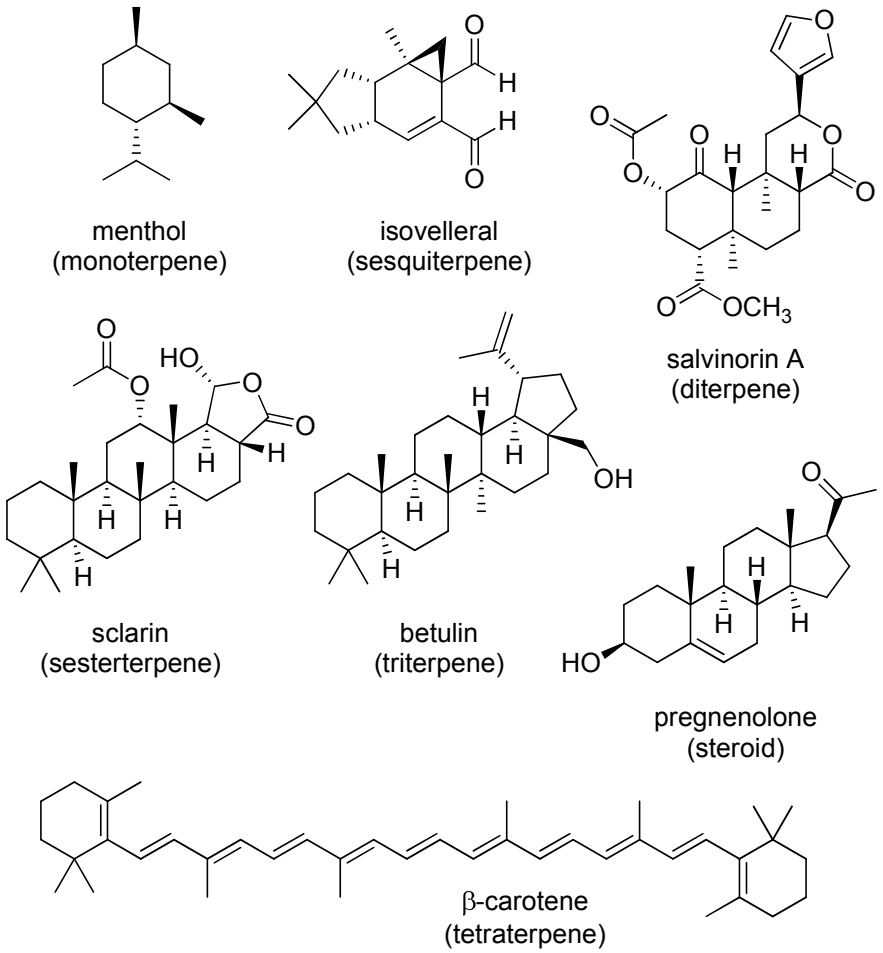


Figure 4. Examples of chemical structures of terpenoids.

The two fundamental building blocks to terpenoids are (*Figure 3*) dimethylallyl diphosphate (DMAPP [1]) and isopentenyl diphosphate (IPP). These can be produced by either the mevalonate pathway or the methylerythritol phosphate pathways depending on the producing organism and the compound. In the mevalonate pathway, DMAPP and IPP are derived from acetyl-CoA, while in the methylerythritol phosphate pathway they are derived from pyruvic acid and glyceraldehyde 3-phosphate. Hemiterpenes, of which there are only a small amount of examples, are formed from one DMAPP.

The precursor of monoterpenes, geranyl diphosphate, is formed from one DMAPP and one IPP, which are joined in a head-to-tail fashion. The DMAPP forms a resonance stabilized dimethylallylic cation, which is electrophilic, by the leaving of OPP. An electrophilic addition of the dimethylallyl cation to the double bond of IPP generates a tertiary cation, from which a proton is stereospecifically lost, forming geranyl PP (*Figure 5*).

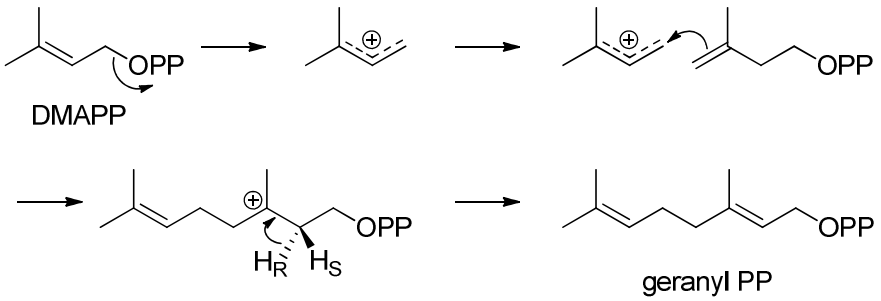


Figure 5. The chemical mechanism of the formation of geranyl PP from DMAPP.

The geranyl PP can then either go on to form monoterpenes or, by the leaving of OPP and an analogous addition to another IPP, form farnesyl PP (FPP), the precursor of sesquiterpenes. The analogous sequential additions of one or two IPP to farnesyl PP lead to the respective formations of geranylgeranyl PP (GGPP) and geranylfarnesyl PP, which are the precursors of diterpenes and sesterterpenes, repectively (*Figure 6*).

The precursor of triterpenes, squalene, is not formed by adding five IPPs to a DMAPP in a head-to-tail fashion analogously to the C_{10}-C_{25} terpenoids. Instead, squalene is formed by joining two FPP, by the generation of a farnesyl cation from one FPP, which adds to the double bond closest to the PP in the other, generating a tertiary cation. A formation of a cyclopropyl moiety is then accomplished by the loss of a proton. The remaining diphosphate leaves,

1. PP is short for diphosphate.

forming a primary cation, which by a 1,3-alkyl shift generates a different cyclopropyl ring and a more stable tertiary cation. A bond cleavage in the three-membered ring subsequently generates an allylic cation, which is discharged by a hydride ion from NADPH.

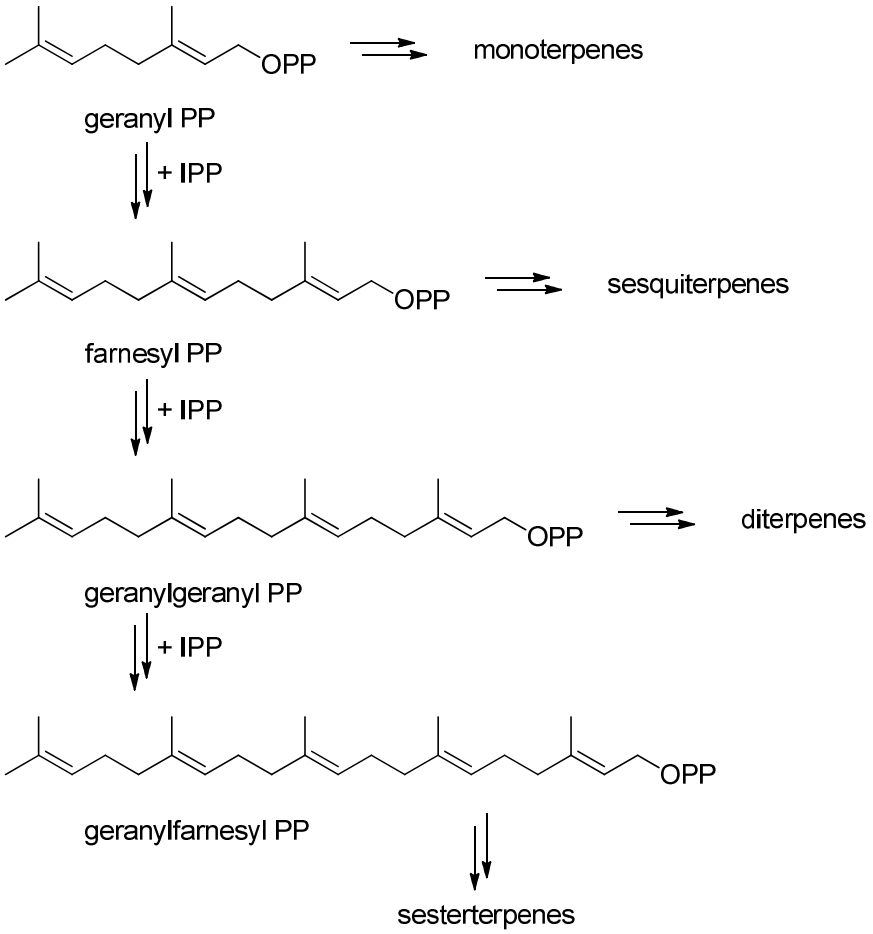


Figure 6. Schematic view of the formation of the precursors of mono-, sesqui- , di- and sester-terpenes.

Phytoene, the precursor of tetraterpenes, is formed in a similar way as squalene, but with two entities of GGPP instead of FPP. The allylic cation, however, which is quenched by a hydride ion in the forming of squalene, is, when forming phytoene, discharged by the loss of a proton, and this leaves a double bond. The configuration of the double bond is generally Z in plants and fungi, while in bacteria, it is E (*Figure 7*).

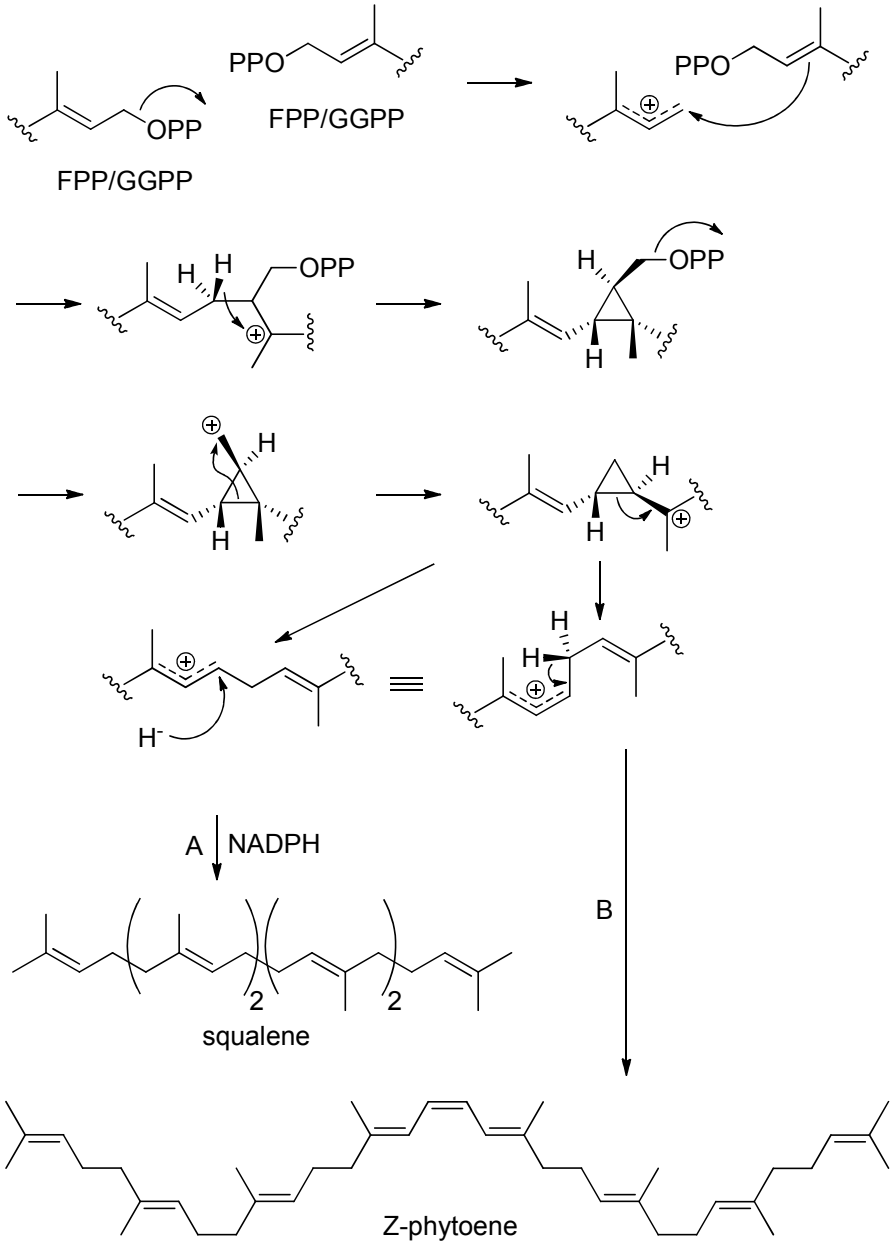


Figure 7. Mechanism of formation of squalene (A) from farnesyl PP and Z-phytoene from geranylgeranyl PP.

The precursors of the different terpenoids often go through an array of reactions to form the actual products *e.g.* cyclizations, rearrangements, oxidations and glycosylations. There are often different modes of cyclization and different rearrangements for the same precursor, which contribute to the structural diversity of this group. The beautiful formation of lanosterol, a precursor of many steroids in animals and fungi, is used in *Figure 8* as an example. After the formation of squalene oxide from squalene, three sequential electrophilic cyclizations occur. The formed five-membered ring is rearranged to form a six-membered ring and another electrophilic cyclization follows to result in the formation of a protosteryl cation. Lanosterol is then formed after a sequence of hydride shifts and methyl shifts and a final double bond formation (Dewick, 2009; Dewick, 2002; Harrison, 1990).

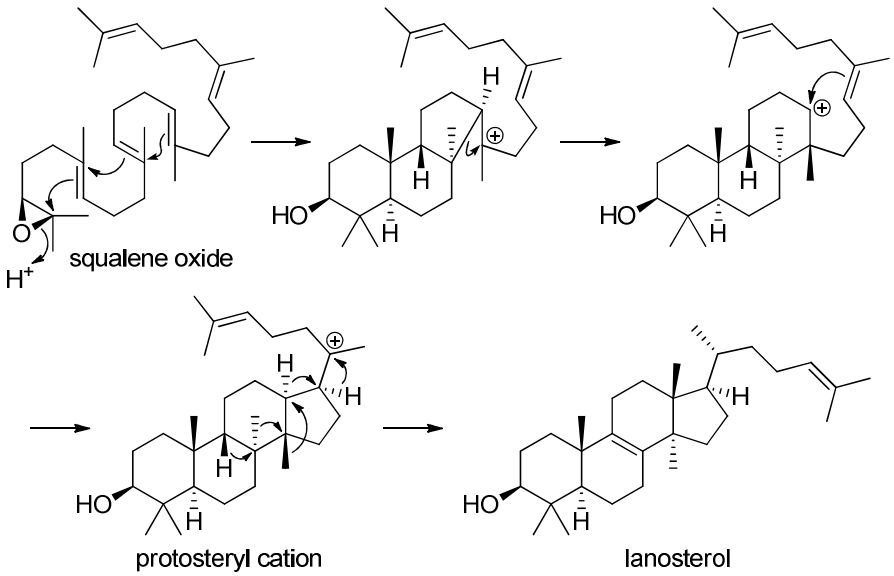


Figure 8. A somewhat shortened mechanism of the formation of lanosterol from squalene oxide.

2.1.3 Phenyl propanoids

Phenylpropanoids are biosynthesized via the shikimate pathway, which is only used by plants and microorganisms. The shikimate pathway produces the aromatic amino acids phenylalanine and tyrosine, which is why animals need to ingest plants, or perhaps microorganisms for that matter, in order to obtain these essential amino acids. Via cinnamic acid and/or 4-coumaric acid an array of biosynthetic routes can be taken to form lignans, lignin, coumarins, volatile phenylpropenes, flavonoids and stilbenes (*Figure 9*).

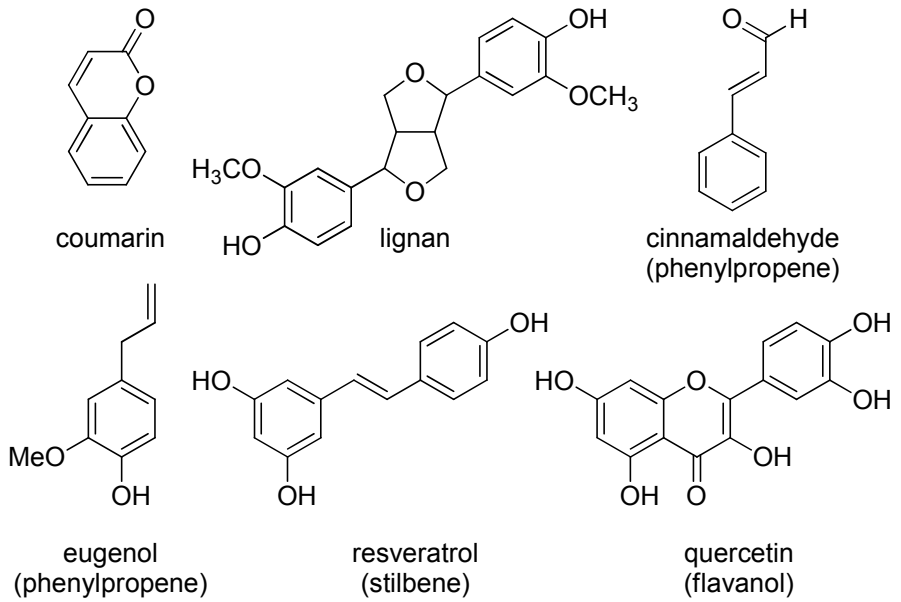


Figure 9. Examples of phenylpropanoids.

In plants, phenylpropanoids contribute to flavour, smell and colour to interact with pathogens and animals. These properties are used by man as some spices, *e.g.* cloves and cinnamon, contain volatile phenylpropenes, while many fruits contain flavonoids for flavour and antioxidant activity. Pharmaceutical activities of phenylpropanoids include anti-tumor, anti-viral, hypertensive (Kim, 2010) and anxiolytic (Karim *et al.*, 2012; Kahnberg *et al.*, 2002) activities.

The shikimate pathway is named after the compound shikimic acid, which is produced in a number of steps from phosphoenolpuryvate (PEP) and D-erythrose-4-phosphate. The shikimic acid pathway is in this way linked to primary metabolism, since these two compounds are intermediates in the glycolysis and the pentose phosphate pathway, respectively. Shikimic acid, after its formation, is 3-phosphorylated, and the 5-hydroxy group of the resulting shikimic acid 3-phosphate makes a nucleophilic attack on the phosphorylated carbon of a protonated PEP. The phosphate (P) group originating from the recently attached PEP is β-eliminated and the 3-P group is removed by a 1,4-elimination forming chorismic acid (*Figure 10*).

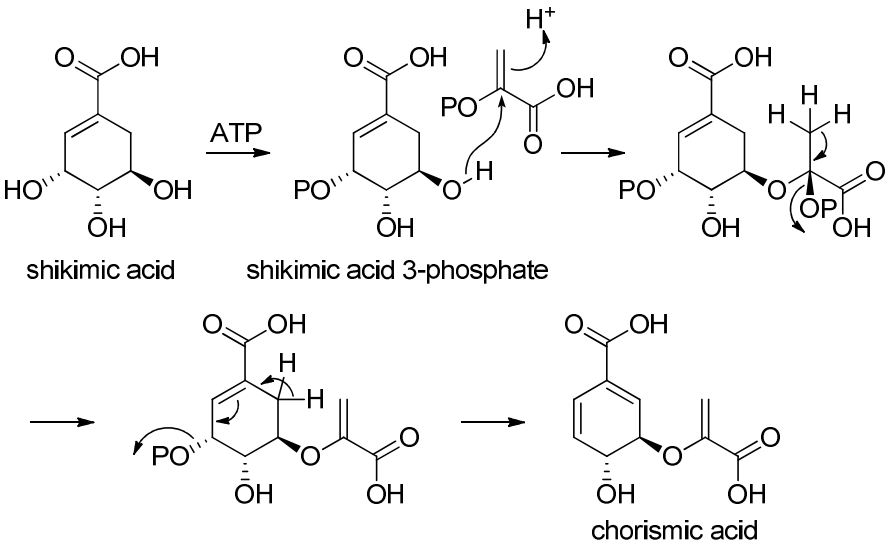


Figure 10. The conversion of shikimic acid to chorismic acid.

A Claisen rearrangement then turns chorismic acid to prephenic acid. The transformation of prephenic acid into phenylalanine can be done in more than one way, but only one was chosen to be depicted here. Via a transamination reaction, involving pyridoxal 5'-phosphate (PLP) and an amino acid that provides the amino group (possibly glutamic acid), prephenic acid is turned into L-arogenic acid. L-phenylalanine can then be formed by a decarboxylation, whilst tyrosine is formed is by a decarboxylative reaction, which retain the hydroxy group, for which there is no proposed mechanism (Tzin & Galili, 2010; Dewick, 2009). By E2 mechanisms L-phenylalanine and L-tyrosine are turned into cinnamic acid and *p*-coumaric acid, respectively (*Figure 11*). As mentioned above, these are precursors to many phenylpropanoids, which can often be noted on the characteristic C_6C_3 scaffold present in most phenylpropanoids.

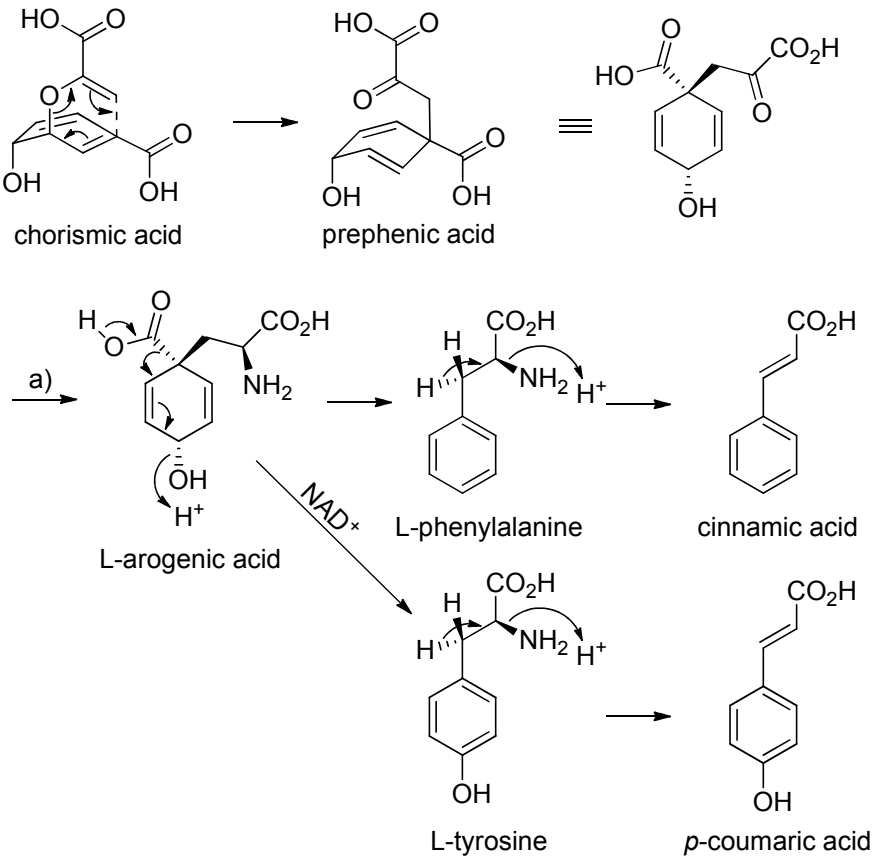


Figure 11. Mechanism of the transformation of chorismic acid to cinnamic acid and *p*-coumaric acid. a) transamination involving pyridoxal 5'-phosphate and an amino acid that donates the amino group.

2.1.4 Amino acids, peptides

Proteins, peptides and enzymes are commonly produced in the ribosome and are not seen as secondary metabolites. There are, however, ribosomal peptides, which can be seen as secondary metabolites although their molecular mass sometimes is more than 3000 Da. These include toxins from mushrooms, venoms from snakes and endorphins. Here, the focus will be on non-ribosomal peptides and small molecules derived from amino acids, such as penicillins and cephalosporins, out of which many have antibacterial properties.

Non-ribosomal peptides are synthesized by non-ribosomal peptide synthases (NRPS), which assemble peptides from amino acids in a manner similar to the assembly of polyketides by PKSs. NRPSs are enzymes with modules that each connect another amino acid to the peptide. The first of the modules, naturally, loads the first amino acid to a peptidyl carrier protein

(PCP) domain, whereas the last module releases the peptide either by cyclization or by hydrolysis (*Figure 12*).

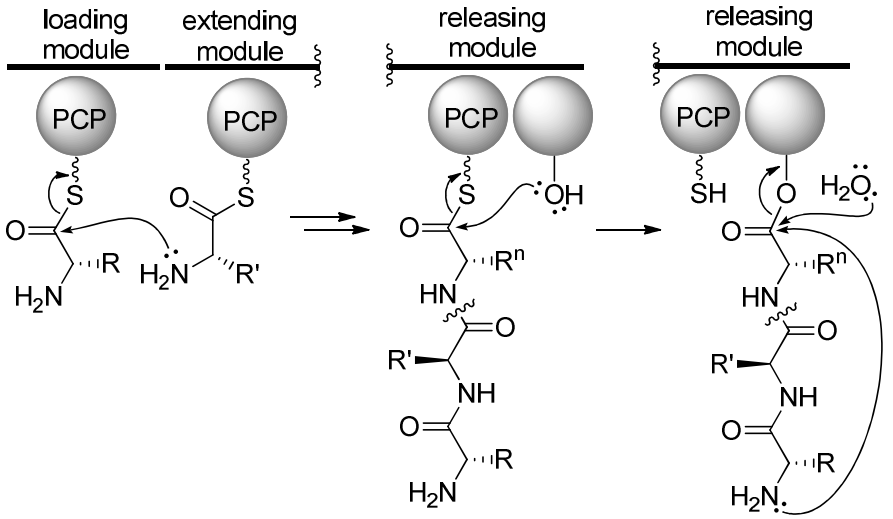
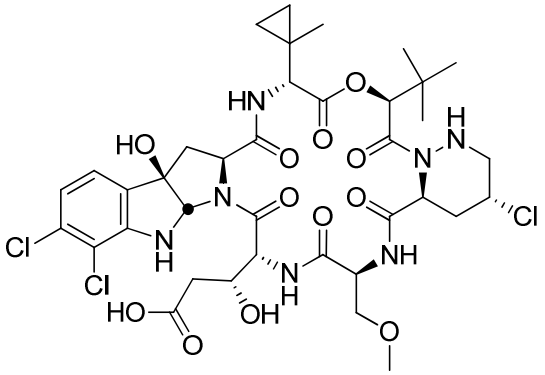


Figure 12. Brief illustration of the formation of a non-ribosomal peptide.

The repertoire of the NRPSs is much larger than this, however, as the description above only leads to formation of linear and cyclic peptides. There are examples of NRPSs that have PKS modules to incorporate polyketides in the peptides. Some NRPSs also incorporate unusual amino acids or hydroxylated acids, resulting in ester linkages instead of some of the amide bonds, so called depsipeptides (Evans *et al.*, 2011; Dewick, 2009; Xu *et al.*, 2008). One example in *Figure 13*.

Penicillins and cyclosporins are β-lactam antibiotics. An overview of the biosynthetic routes will be exemplified by benzylpenicillin and cephalosporin C. They are produced by the assembly of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine by different NRPSs, since they are produced by different species. This tripeptide goes through a number of transformations to form isopenicillin N. After the formation of isopenicillin N, the biosyntheses diverge, as the penicillin scaffold is ready, while to produce the cephalosporin scaffold, the thiazolidine ring in penicillin N has to be transformed to a dihydrothiazine ring. Benzylpenicillin is then produced by replacing the α-aminoadipic acid with a phenylacetic acid (*Figure 14*). A number of therapeutic penicillins are produced by replacing this acyl side-chain by adding the suitable carboxylic acid to the medium. This can alter the spectrum of activity and the stability towards acid of the drug.



Kutzneride 2
(cyclic depsipeptide)

Figure 13. Chemical structure of a depsipeptide containing unusual amino acids (Broberg *et al.*, 2006).

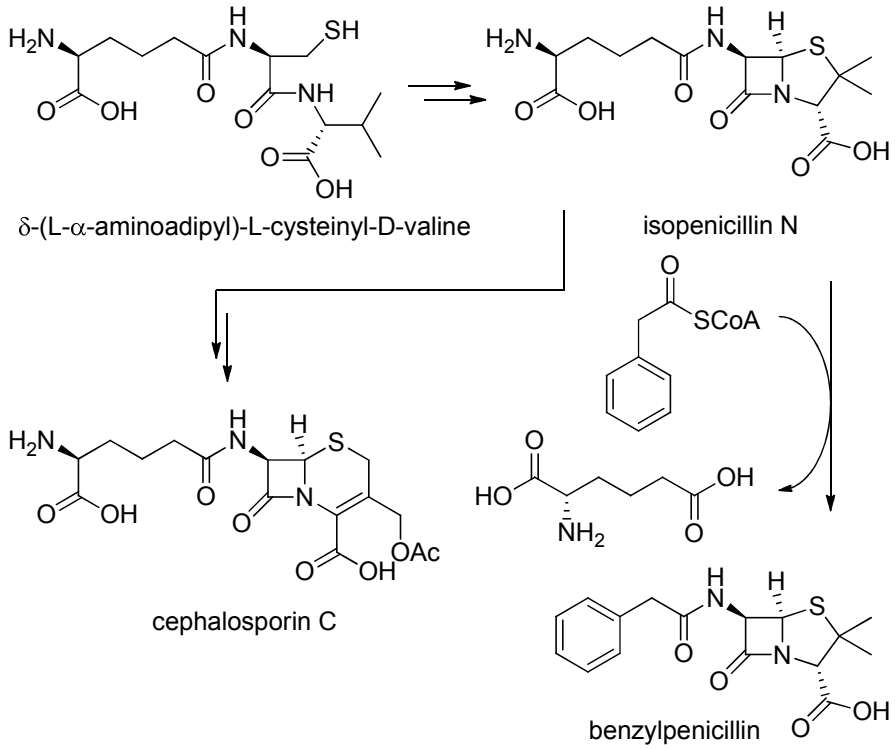


Figure 14. Overview of benzylpenicillin and cephalosporin C biosynthesis.

2.1.5 Alkaloids

The alkaloids are cyclic nitrogen containing organic compounds with limited distribution in nature (Pelletier, 1983). The alkaloids got their collective name in 1814 by Meissner from their alkaline-like (basic) properties. Thus, they were conveniently extracted by an acidic aqueous extraction due to their increased solubility in water upon protonation of the nitrogen. The basicity criterion for the classification as an alkaloid is today, however, of less importance and compounds containing only amide nitrogens, can still be called alkaloids. The alkaloids often have physiological effects on the central nervous system with examples of anxiolytic (Lager *et al.*, 2006), analgesic and hallucinogenic effects (Dewick, 2009). It is probably the group of compounds with most famous and infamous examples known to the wider population *e.g.* caffeine, nicotine, cocaine and morphine (*Figure 15*). In spite of the negative connotations of these examples, research on the alkaloids has provided valuable knowledge on the physiology of the central nervous system. Furthermore, 50% of the plant-derived natural product pharmaceuticals are alkaloids (Samuelsson, 2004). In animals, endogenous alkaloids serve as neurotransmitters *e.g.* melatonin, which is involved in sleep regulation. In plants, on the other hand, alkaloids are often used as protection against animals, as many of them are toxic by affecting neurotransmission (Konno, 2011).

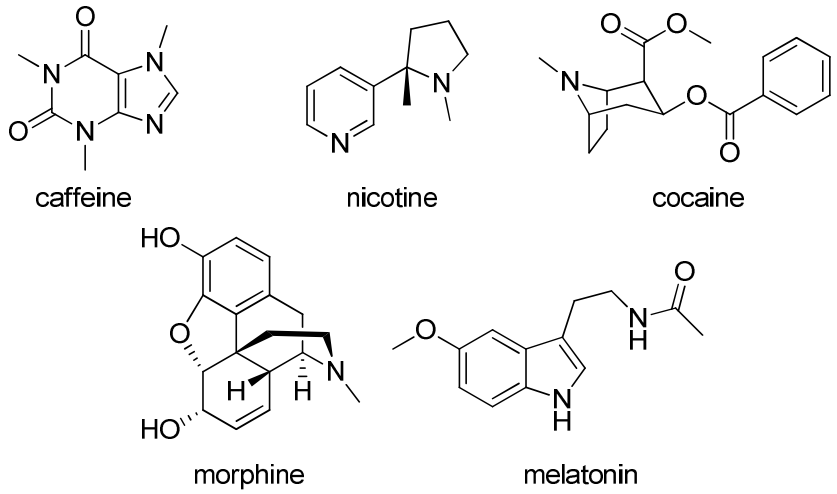


Figure 15. Chemical structure of some widely known alkaloids.

As the definition above states, the alkaloids were not grouped due to any specific biosynthetic origin, as opposed to the other described structural classes in this thesis. The biosynthesis of alkaloids often uses building blocks from other structural classes *e.g.* terpenes and phenylpropanoids and combines these with a nitrogen containing cyclic moiety, which is often derived from an amino acid *e.g.* ornithine, lysine, tryptophan or tyrosine. An example of an alkaloid derived from ornithine and phenylalanine is the hallucinogen scopolamine. Ornithine is decarboxylated to form putrescine, of which one of the amino groups is methylated by S-adenosyl methionine. A diamine oxidase subsequently transforms the remaining amino group to an aldehyde. An attack of the methylated amino group on the aldehyde and a subsequent loss of water leads to an iminium cation formation. To the formed electrophilic carbon, two consecutive additions of acetyl-CoA are achieved by a Mannich reaction and a Claisen reaction, respectively. The pyrrolidin in the formed compound is oxidized again to form an electrophilic iminium cation and the thioester function is hydrolized to an acid function. Via a possible decarboxylative generation of an enolate a tricyclic compound is formed, which after a stereospecific reduction of the remaining ketone forms tropine. The tropine is then esterified by phenyllactic CoA, which is derived from phenylalanine, resulting in littorine. After an unusual rearrangement of the phenyllactate moiety and a two-step epoxidation of the tropinyl moiety, scopolamine is formed (*Figure 16*). Note the tropinyl like moiety in cocaine as well.

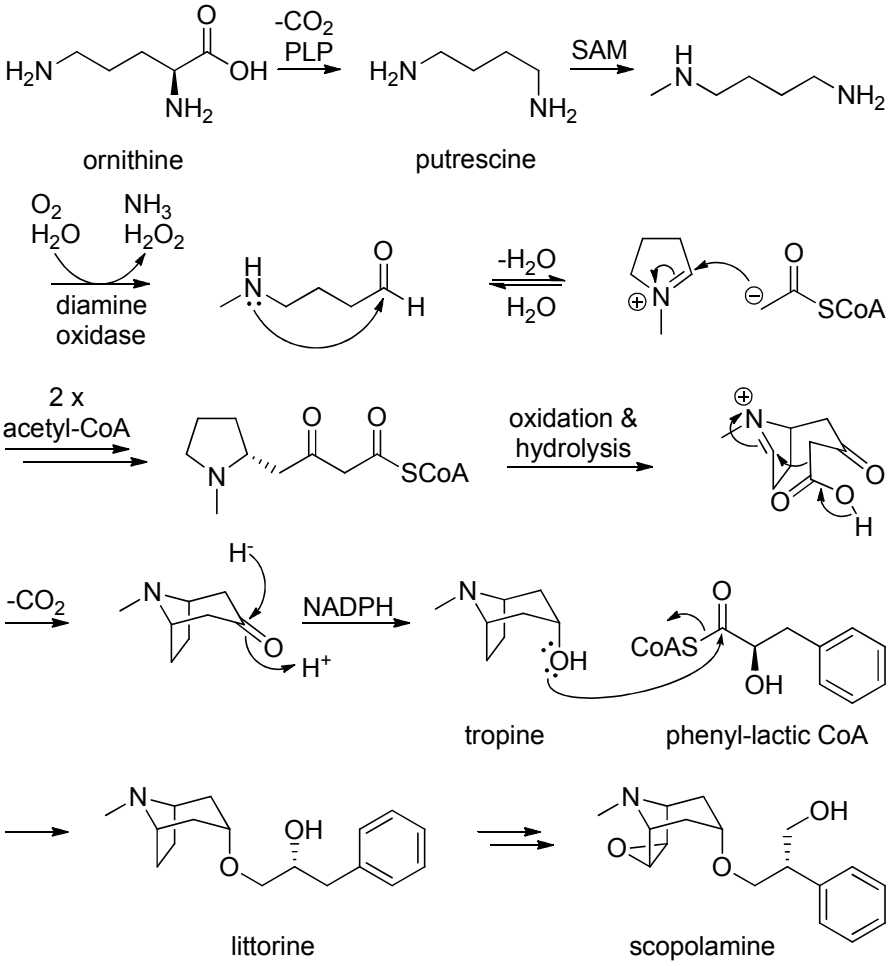


Figure 16. Undetailed mechanism of the biosynthesis of scopolamine. SAM is short for S-adenosyl methionine

3 Applied methods for isolation and structure elucidation

This section contains brief descriptions of the techniques used in this research for compound isolation and structure elucidation. The emphasis is thus on the applications relevant for this thesis.

3.1 Solid phase extraction

Traditionally lipophilic compounds have been extracted from aqueous solution *e.g.* liquid fungal cultures, by liquid-liquid extraction, where the aqueous mixture is shaken in a separating funnel, with an organic solvent, which is immiscible with water. When the two solvent phases are subsequently separated, the lipophilic components end up in the organic phase, while hydrophilic components end up in the aqueous phase. Solid phase extraction (SPE) has the same objective, but relies on principles from liquid chromatography. The corresponding separation with SPE can be achieved by passing the aqueous mixture through a short column packed with silica particles, to which alkyl chains are bound *e.g.* octadecyl (C_{18}). Lipophilic components are adsorbed and thus retained, while hydrophilic components are rinsed out. The lipophilic components are subsequently eluted using a polar organic solvent such as acetonitrile (MeCN). Extensive development of automation methods, adsorbent materials and hyphenation of SPE between chromatography and spectroscopic methods has led to SPE being seen today as a very common sample preparation technique in areas such as environmental trace analysis, biological sample analysis and natural products chemistry (Wubshet *et al.*, 2012; Hennion, 1999).

3.2 Reversed-phase high-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a separation technique that uses the ability of different compounds to interact to different extents with a certain packing material, a stationary phase. The stationary phase is packed in a column, through which a mobile phase is continuously pumped. After the injection of a sample, containing a number of compounds, the different compounds bind with different strengths to the stationary phase and thus elute after different amounts of time, the so called retention time (t_R). HPLC techniques can be divided into groups by the predominant force that accomplishes their separation. These include normal phase-, reversed phase (RP)-, ion exchange- and size exclusion-HPLC and their areas of use depend on the types of compounds that are supposed to be separated. In this work only RP-HPLC has been used and will thus solely be discussed. In RP-HPLC the stationary phase is hydrophobic, while the mobile phase is hydrophilic, which makes the compounds elute roughly with decreasing polarity. The most common stationary phase in RP-HPLC is based on silica particles, to which C_{18} chains are covalently bound, while the mobile phase is a mixture between water, buffered or not, and an organic solvent miscible with water *e.g.* MeCN or methanol (MeOH). There are also stationary phases with alkyl chains of different lengths, phenyl groups as well as substituted alkyl chains with for example an amino group or a diol, which slightly changes the elution order. The retention mechanism of RP-HPLC has been debated for some time. The debate is roughly about whether the stationary phase should be seen as a solid surface or a liquid. If the stationary is a solid surface, the retention would be accomplished by adsorption of the analytes on that surface. The compounds adsorbing stronger thus elute later. The competing hypothesis is that the retention is accomplished by partition between the hydrophobic stationary phase and the hydrophilic mobile phase. In both cases the result, however, is roughly that less polar compounds elute later, although the exact order of elution is difficult to predict (Kazakevich & Lobrutto, 2007; Kazakevich, 2006; Skoog *et al.*, 1998). In this work both analytic and preparative HPLC has been utilized. The difference of the two, apart from different amounts of loaded samples and different equipments, is the objective. The objective of analytical HPLC is to separate compounds and detect them, while preparative HPLC aims to collect the separated compounds in fractions.

3.2.1 Gradient elution

With increasing the fraction of organic solvent in the mobile phase the overall retention of compounds is reduced. Hydrophopic analytes thus require a large fraction of the so called organic modifier in order to have reasonable t_Rs. On

the other hand, a too large fraction of the organic modifier can result in short t_R so that analytes with comparable affinity to the column are not separated, they co-elute. This is especially an issue when working with complex samples, such as fungal cultures, with a large number of components with varying polarities. To overcome this, the amount of organic modifier is kept low in the beginning of the analysis and is then gradually increased *i.e.* a concentration gradient of the organic modifier is used. The proportions of the two mobile phases are programmed in advance, and can be optimized to accomplish separations of rather complex mixtures.

3.2.2 Isocratic elution

Gradient elution is a common first step to purify compounds. However, sometimes two components co-elute and are difficult to separate with a gradient of one specific organic modifier. One possibility to separate the two components would then be to change the organic modifier, which is somewhat time-consuming. An alternative is to employ isocratic elution, meaning the mixture of the mobile phase is constant. The amount of organic modifier can be optimized, in order to get sufficient separation in a reasonable amount of time. Excessive time spent by analytes in a column leads to peak broadening, which in the case of preparative HPLC means large volumes of solvent and possible contamination from surrounding compounds present in small amounts.

3.3 Mass spectrometry

A mass spectrometer measures the mass to charge ratio (*m/z*) of ions, which in turn gives information about the mass of the analyte. Mass spectrometry (MS) is an enormous field of research, which includes many disciplines. It includes construction and development of mass spectrometers, method development and the finding of new applications for mass spectrometry as well as the actual usage of mass spectrometry to answer physical, chemical and biological questions. MS is often used as a detector after a chromatographic separation. On some mass spectrometers there is a possibility to record a mass spectrum following the induced fragmentation of an ion, so called MS/MS. The ions are produced in a part of the mass spectrometer called the ion source and analyzed in the mass analyzer (de Hoffman & Stroobant, 2007). The type of ionization process and the resulting ion depends on the type of ion source. In this work, ion sources using electro-spray ionization (ESI) and electron ionization (EI) have been used. The mass analyzers used have been ion-trap, quadrupole (Q) or time-of flight (TOF) analyzers. When running gas chromatography-mass spectrometry (GC-MS), the combination EI-quadrupole was used. LC-MS was

run on an ESI-ion-trap or on a Q-TOF, which is a combination of an analytical quadrupole, a collision cell and a TOF analyzer.

3.3.1 Electron ionization

EI, or electron impact as it was originally called, is usually hyphenated with gas chromatography (GC) as it only works for ionization in the gas-phase. The gaseous analytes that enter the source pass perpendicularly through a beam of electrons. Energy of an electron in the beam can be transferred to the analyte molecule, which with enough energy expels an electron resulting in a molecular cation radical. The energy of the mentioned radical is quite high, which leads to instability and fragmentation. The fragments, as they are products of unimolecular chemical reactions and not random processes, give structural information. EI is a so called hard ionization technique, since it gives rise to extensive fragmentation and often no peak corresponding to the molecular ion (de Hoffman & Stroobant, 2007).

3.3.2 Electrospray ionization

ESI is a soft ionization technique that can be used to produce gas-phase ions from analytes in solution. It can be used to produce either positive or negative ions. It produces ions from molecules by adduct formation *e.g.* $[M+H]^+$, $[M+Na]^+$ or by deprotonation $[M-H]^-$. Large molecules such as proteins get multiply charged. Some fragmentation occurs in the source *e.g.* loss of water $[M+H-H_2O]^+$.

In positive mode, the solution reaches the tip of a positively charged capillary. At a distance (1-3 cm) in front of the capillary is a negatively charged plate with an orifice leading eventually to the mass analyzer. This creates a concentration of positively charged ions on the surface of the meniscus at the capillary tip. The meniscus is destabilized due to the attraction of the positive ions towards the negatively charged plate, leading to the formation of a cone and eventually, the emission of droplets with excesses of positive charges. The droplets, in turn, produce smaller droplets with even higher charge density and so forth, until gas-phase ions are produced, which can be analyzed by the mass analyzer (de Hoffman & Stroobant, 2007; Cole, 1997).

3.3.3 Quadrupole

The Q, originally described by Paul and Steinwedel (1953), is the most prevalent and economic mass analyzer in GC-MS, LC-MS and LC-MS/MS mass spectrometers. The Q has insufficient resolution and mass accuracy to use

to determine the elemental composition of ions. It can be seen as a mass filter, which, at a given time, only leads ions of a specific *m/z* to the detector. A Q is generally made up of four circular, or ideally hyperbolical, parallel rods to which varying electrical fields are applied. Ions will travel along the rods due to a constant electric field, but will only reach the detector if they have a stable trajectory and don't discharge on the rods. As the Q can work as a mass filter, which only allows ions of a certain *m/z* to pass, it is often used in MS/MS spectrometry, before a fragmentation step followed by a second MS step. One such example used in this work is the Q-TOF (de Hoffman & Stroobant, 2007).

3.3.4 Ion-trap

One characteristic feature of the ion-trap, referring to the Paul ion trap (Paul, 1990), is the possibility to run more than two MS steps in a row, so called MS^n. It can be seen as a circular Q, in which ions of different *m/z* are orbiting at different radii by a varying 3D electrical field. The ion-trap is quite versatile as it can store ions of a specific *m/z* or a range of *m/z*, expelling the undesired ions. For detection, ions are expelled by applying a specific radio frequency voltage, which makes ions of a specific *m/z* resonate. Once expelled, the ions are detected instead of discarded. When using the ion-trap in MS/MS mode, ions are selected and fragmented in the trap, by applying its specific radiofrequency voltage, making it resonate and collide with helium gas, a so called collision-induced dissociation. The resulting fragments can subsequently be detected by scanning a range of *m/z* (de Hoffman & Stroobant, 2007).

3.3.5 Time-of-flight

TOF analyzers use instantaneous acceleration of ions, giving them the same kinetic energy, and thus different velocities to ions of different *m/z*. The ions then travel through a field-free region at different velocities and arrive at the detector at different times. Newer TOF analyzers have rather high resolution and mass accuracy, allowing for the determination to the elemental composition of the analyte. The Bruker maXis impact (Bruker, 2011) used in this work uses pulsed orthogonal acceleration, which means the ions have no or little velocity in the trajectory of the path of the TOF. This focuses the ions travelling at the same velocity, resulting in increased resolution. Another feature of the used TOF is a dual reflector field, which further focuses the ions with the same *m/z*. The exact function of the dual reflector is rather complex, but the general principle for a reflector is the following: Since ions with the same *m/z* may have slightly different velocities prior to the orthogonal acceleration, there may be a slight difference in their kinetic energy, resulting in lower resolution. The reflector compensates for this since ions with slightly

dissimilar velocities penetrate to different extents in the reflector field and thus reach the detector more focused (de Hoffman & Stroobant, 2007; Mamyrin *et al.*, 1973).

3.4 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) is a technique used for many purposes *e.g.* structure determination, the detection and characterization of molecular interactions and quantification. It gives more detailed information about the structure than MS *e.g.* it can often differentiate, for example, different types of carbons. On the other hand, it is much less sensitive than MS, in spite of extensive probe development by NMR manufacturers. NMR is quite an expensive technique, both to purchase and to maintain, which often makes it unavailable to smaller laboratories.

3.4.1 NMR spectral features for structure elucidation

The introduction of NMR below is a brief description of the concepts of NMR relevant to this work and is intended to give an intuitive understanding without going into quantum mechanics. For a more comprehensive description, please consult Keeler (2004) or Levitt (2001).

NMR has, in this work, been used for structure elucidation *i.e.* the determination of the connectivity as well as relative configuration of stereogenic carbons. NMR has been run in deuterated solvents with detection on two nuclei ^{1}H and ^{13}C. Both of these nuclei have a property called spin and they both have two spin states, out of which one has a slightly higher energy when the nuclei are put in the strong applied magnetic field used in NMR. Thus, when adding up all of the magnetic moments of the nuclei in an NMR sample it will result in a small net magnetization vector (NMV) aligned with the applied magnetic field (*Figure 17*).

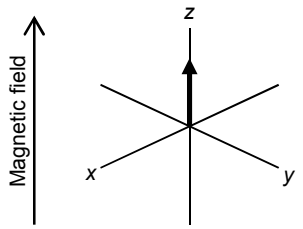


Figure 17. Net magnetization vector of nuclei in an applied magnetic field.

If the NMV were to be tilted away from the *z* axis it would precess or rotate around the direction of the magnetic field at the resonance frequency of the

nuclei and gradually return to be along the *z* axis again (equilibrium). Thus, if the magnetic field was to be replaced momentarily by one along the *x* axis the NMV would rotate around the *x* axis towards the *y* axis. This can be accomplished in NMR by applying a magnetic field, which is oscillating at a frequency close to the resonance frequency of the nuclei, along the *x* axis for a short amount of time (a pulse). Once that temporary magnetic field is turned off, the NMV rotates around the *z* axis gradually returning to equilibrium. When the x component of that rotation is detected, it will look like a decaying sinus wave. This is called a free induction decay (FID). A mathematical operation, called a Fourier transformation, turns the FID of the described situation into a peak at the resonance frequency of the nuclei (*Figure 18*).

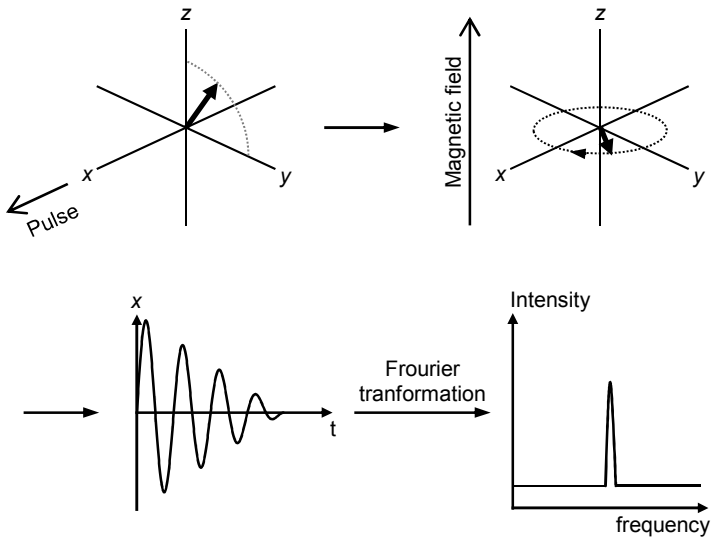


Figure 18. Illustration of the application of a pulse and the subsequent rotating net magnetization vector, the detected free induction decay turned into a NMR peak by Fourier transformation.

^{1}H NMR spectra in most cases consist of various signals at different frequencies and with different shapes. The fact that different ^{1}H nuclei, commonly referred to as protons, in a molecule have slightly different resonance frequencies is one of the fundamental properties in NMR from which it is possible to draw conclusions about the chemical environments of the protons. The resonance frequencies of different protons in a spectrum are commonly given in relation to the resonance frequency of tetramethylsilane (TMS), in parts per million (ppm). This is referred to as the chemical shift (δ), which has the feature of being independent of the strength of the used NMR magnet.

There are basically four fundamental physical features in NMR that are exploited in order to gain information about the analyte: The chemical shift, integrals, scalar couplings and the nuclear Overhauser effect (NOE). The chemical shift, as previously stated, provides information about the chemical environments of the nuclei *e.g.* an aromatic carbon has a higher chemical shift than an aliphatic one. Detailed studies of the dependence of the chemical shifts of protons and carbons have led to rather elaborate tables, books and software which can be helpful when attempting structure determination (for example Lodewyk *et al.*, 2011; Breitmaier & Voelter, 1990).

The integral *i.e.* the area of a signal, is related to the number of nuclei that give rise to the signal. This means that a signal, corresponding to three magnetically equivalent protons *e.g.* methyl protons will have an integral three times the integral of a signal corresponding to one proton within the same molecule. The integral can also be helpful for overlapping signals.

The scalar couplings, or spin-spin couplings, are detected between spins that are within a few chemical bonds from each other. The size of the spin-spin coupling depends on a number of factors, *e.g*. number of bonds and dihedral angle between the spins (Karplus, 1963), and can thus be used in both configurational and conformational analysis of compounds. Coupled spins affect the energies of the spin states of each other. Consider first one spin with two spin states, with different energies. The flipping between these states thus corresponds to an energy gap, related to a frequency *i.e*. the resonance frequency of the spin. Now consider the two coupled spins, 1 and 2, with each one lower and a higher spin state, between which there is a coupling J_{12}. The energy gap corresponding to the flipping of spin 1 will then be slightly different depending on whether spin 2 is in its higher or lower state. This gives rise to a split signal, a doublet, centered at the resonance frequency of spin 1, with the distance between the two components of J_{12}. There is a corresponding doublet originating from the flipping of spin 2.

The NOE detects proximity in space under the right conditions. In the discussion above regarding how a basic NMR experiment is acquired, the returning of the NMV to equilibrium is mentioned. It does so by processes called relaxation. One such relaxation process is cross-relaxation where a spin can exchange magnetization with surrounding spins. This results for small molecules in non-viscous solutions in an increase of intensity for surrounding spins.

3.4.2 NMR experiments

A one dimensional ^{1}H NMR experiment is the fastest and the most basic of all in organic chemistry. Within seconds an idea is obtained about the number of

protons in your molecule as well as their chemical shifts. The extraction of the coupling constants requires a bit more work if highly coupled or overlapping protons are present. As two protons with a mutual coupling share coupling constant it is often possible to deduce which protons are coupled to each other. An often faster mean to acquire this information is to perform a ^{1}H-^{1}H correlation spectroscopy (COSY) experiment, a 2D experiment which shows cross-peaks between coupled protons. In case of severe overlapping of proton signals, it can be useful to run a total correlation spectroscopy (TOCSY) experiment. A phase-sensitive heteronuclear single quantum coherence (HSQC) can then be used indicate the chemical shift of the carbon to which the respective proton is attached or that it is not attached to a carbon *i.e.* it detects one bond HC couplings. Further, a phase-sensitive HSQC shows the opposite signs of CH_2 carbons compared to CH or CH_3. To show two to four bond HC couplings, an experiment called heteronuclear multiple bond correlation (HMBC) can be performed. This is a very powerful technique to fit fragments of a molecule together and to obtain the chemical shifts of carbons, which have no protons directly bound to them and will thus not appear in HSQC.

There are, however, non-proton carrying carbons that have no correlations neither in HMBC nor HSQC. These can be detected by a one dimensional ^{13}C NMR experiment, but it requires more material than HSQC and HMBC. In this work the ^{13}C spectra were acquired with decoupling of the protons during acquisitions. This has the advantages of seeing carbon signals as singlets and improving the sensitivity of the experiment due to NOE from neighboring protons. At this point, in most cases, the connectivity of the analyzed compound can be established. In order to determine the relative configurations of stereogenic carbons the experiments rotating frame nuclear Overhauser effect spectroscopy (ROESY) or nuclear Overhauser effect spectroscopy (NOESY) have been employed, which in a 2D spectrum show cross-peaks between protons close in space. In one case selective NOE experiments were used to detect key NOEs.

4 Results and discussion

4.1 Secondary metabolites from *Hymenoscyphus pseudoalbidus* (Papers I-III)

The purpose of this project was to characterize the recently described fungus *H. pseudoalbidus* with regard to its secondary metabolite production. The project began with small scale experiments in which some parameters were varied for the growth of the fungus, in liquid medium, in order to maximize the amounts of as many compounds as possible. Parameters that were varied were time, growth medium and fungal isolate. It was also tested whether it was beneficial to stir the cultures during incubation or not as well as whether the presence of a piece of ash would have a positive effect on secondary metabolite production. The results were then evaluated by LC-MS and the evaluation suggested that a stirred culture in Hagem medium (Stenlid, 1985) was the best choice. Over time the results showed that the state of the *inoculum* had a large impact on the secondary metabolite production. Older *inocula* produced fewer metabolites in lower amounts and in consequence we used isolates recently collected from infected ash trees. This explains why different isolates were used in the different papers. It is important to mention that all compounds were detected in at least three separate batches to avoid irreproducible results.

Once a well-producing isolate was found it was grown in a larger scale (1-6 liters). The production of secondary metabolites was monitored by taking a sample in a sterile fashion once a week and then evaluating it with LC-MS. When the amounts of metabolites were deemed large enough the culture was harvested *i.e.* filtered and extracted by SPE. After some method development on LC-MS, the organic phase was fractionated by preparative HPLC and the largest peaks (λ = 254 nm) were collected and analyzed by ^{1}H NMR to check if the compounds were pure enough and in sufficient amounts. Once the compounds were pure, their structures (*Figure 19*) were elucidated by data

from techniques such as ^{1}H NMR, ^{1}H-^{1}H COSY, ^{13}C NMR, HSQC-DEPT, HMBC, ROESY and HRMS.

The first isolate of *H. pseudoalbidus* investigated was Ö3 and the major compound was identified as the known steroidal phytotoxin viridiol (Moffatt *et al.*, 1969). It was identified prior to its full structure elucidation with aid by Antibase, a searchable database of structures of natural products isolated by microorganisms and higher fungi, using MS and NMR data. A literature search on viridiol gave information about various related compounds (Hanson, 1995) and, guided by LC-MS, the closely related known compound viridin (Brian & McGowan, 1945) was isolated and identified (Paper I).

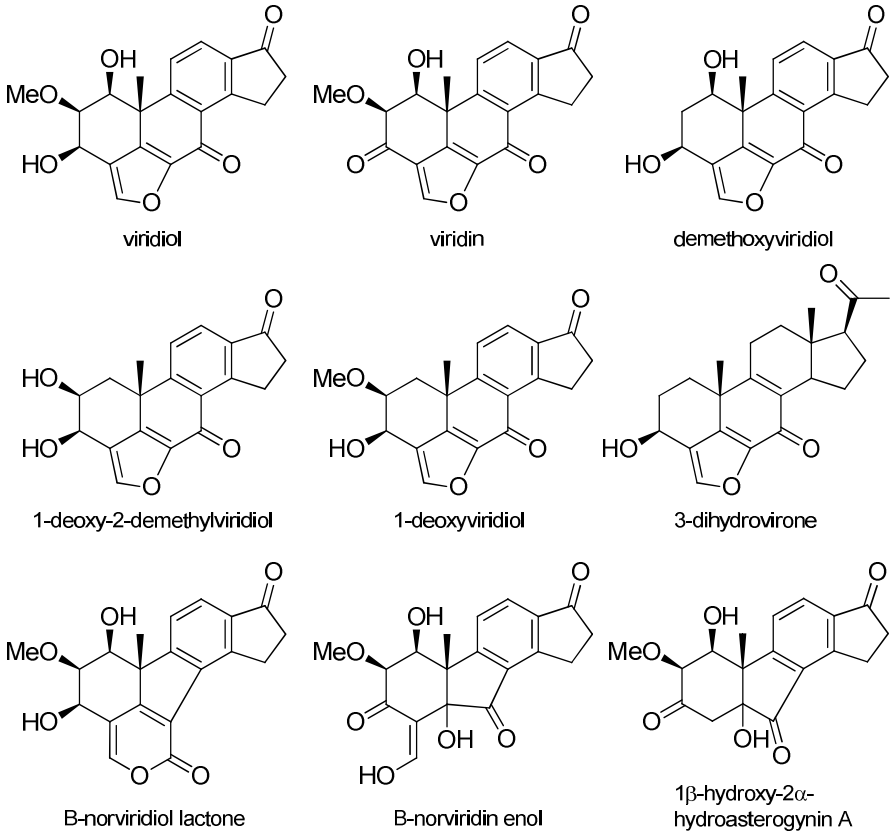


Figure 19. Chemical structures of the compounds isolated from, or detected in, cultures of *H. pseudoalbidus*.

Due to insufficient amounts of other minor compounds, Ö3 was abandoned and the work was continued with studying the isolate nf3, which produced few compounds in large amounts. These were the previously described

demethoxyviridiol (Aldridge *et al.*, 1975) and the previously undescribed 1-deoxy-2-demethylviridiol, 1-deoxyviridiol and 3-dihydrovirone (Paper II).

An LC-MS analysis of the isolate 24c showed the presence of two compounds, which in cultures of other isolates were only produced in small amounts. The compounds were isolated and their structures, which are viridin related, were determined. Both compounds were shown to be steroids with a five-membered B-ring, so called B-norsteroids (Paper III), of which there are only a small amount of reported examples (for example Anke *et al.*, 2004; Lin *et al.*, 1998). One of the compounds, B-norviridiol lactone, further possessed a 2-pyrone ring fused with rings A and B *i.e.* instead of the furan ring in viridiol. Coincidentally, the compound had the equivalent connectivity as the inaccurate structure proposed for the compound TAEMC161 (Sakuno *et al.*, 2000), which was later reassigned as viridiol (Wipf & Kerekes, 2003). B-norviridin enol, the other isolated compound, also lacked the furan ring, which otherwise is characteristic for the viridin family of steroids. Further, the compound had a β-keto enolyl moiety in which the hydroxy proton was strongly hydrogen bound to the keto oxygen. This presented itself as an HMBC coupling from the mentioned proton through the hydrogen bond to the carbon α to the ketone, which through covalent bonds is five bonds away (*Figure 20*).

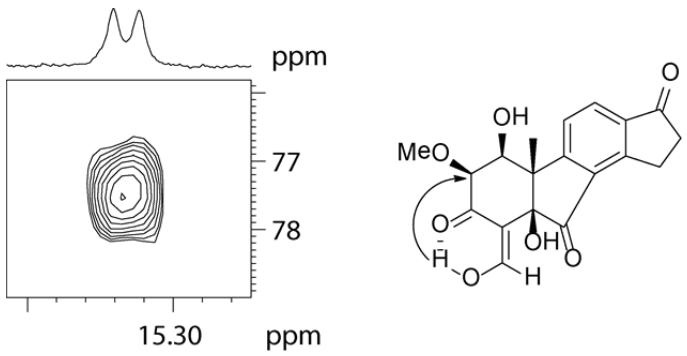


Figure 20. The HMBC spectrum correlation and the corresponding correlation in the structure of B-norviridin enol.

Due to difficulties determining the relative configuration of B-norviridin enol, in spite of extensive efforts, using NOESY and ROESY at different temperatures and with a variety of solvents, slow crystallization of the compound was attempted. This resulted in a degradation of the compound to the previously undescribed 1β-hydroxy-2α-hydroasterogynin A, which in turn, had degraded in part to the previously described compound asterogynin A (Cao *et al.*, 2010). The reported configuration of asterogynin A and the determination of the configuration of 1β-hydroxy-2α-hydroasterogynin A

indirectly gave the configuration of B-norviridin enol. Revision of the original LC-MS analysis of the fungal culture indicated the presence of 1β-hydroxy-2α-hydroasterogynin A in small amounts, which suggests that it is also a secondary metabolite of *H. pseudoalbidus*.

4.2 Phytotoxicity of compounds isolated from *Hymenoscyphus pseudoalbidus* towards ash seedlings (Paper I & IV)

As the compound viridiol is a known phytotoxin (Howell & Stipanovic, 1984), its activity on ash seedlings was tested. Separate 11-day old ash seedlings were treated with solutions of viridiol in concentrations ranging from 1.64 mM to 1.64 µM. Control samples were prepared in the same way, but with solutions lacking viridiol. Viridiol was found to cause similar brown necrotic lesions on the treated cytoledons as the lesions associated with ash dieback caused by the fungus (Paper I, *Figure 21*).

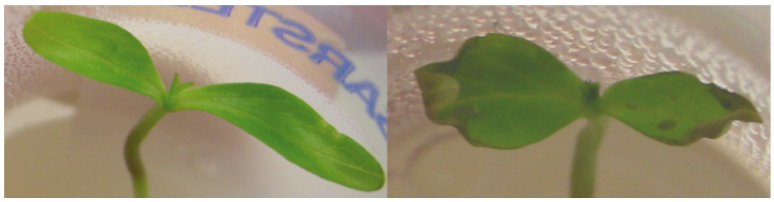


Figure 21. Ash seedlings treated with aqueous 50% MeOH (blank) and 1.64 mM viridiol in aqueous 50% MeOH, respectively.

A modified test for the toxicity towards ash for the compounds viridiol, 1-deoxyviridiol, 1-deoxy-2-demethyl, 3-dihydrovirone, B-norviridin enol and B-norviridiol lactone was done. Five 1-year old ash seedlings from the susceptible clone 101 were used as replicates. Four solutions with different concentrations were prepared for every compound as well as a solution without viridiol. Different concentrations of the compounds were tested on four separate leaflets. On each leaflet, solutions of all compounds were applied on separate leaves. On a sixth leaf on all leaflets, the control solution was applied. The results were evaluated after 24 and 48 hours, which showed no visible effects for any of the compounds. This suggests that the cuticle of the leaves of the 1-year old seedling is thicker, since viridiol has shown phytotoxic effects on younger seedlings and can thus be seen as a positive control.

As differences between genotypes in susceptibility towards *H. pseudoalbidus* has been shown, the same was then investigated towards viridiol. Three susceptible clones and five resistant clones were selected for the experiment. Four treatments were tested for each clone in five replicates. Three

of the treatments were with solutions containing viridiol in different concentrations and one was a control treatment with a solution not containing viridiol. The damage was then evaluated after 24 and 48 hours on a scale from zero to four. The results showed that the damage scores of susceptible clones were significantly higher than those on resistant clones (*Figure 22*). The damage detected included brown necrotic spots and wilting, which are both symptoms associated with ash dieback caused by *H. pseudoalbidus* (Paper IV).

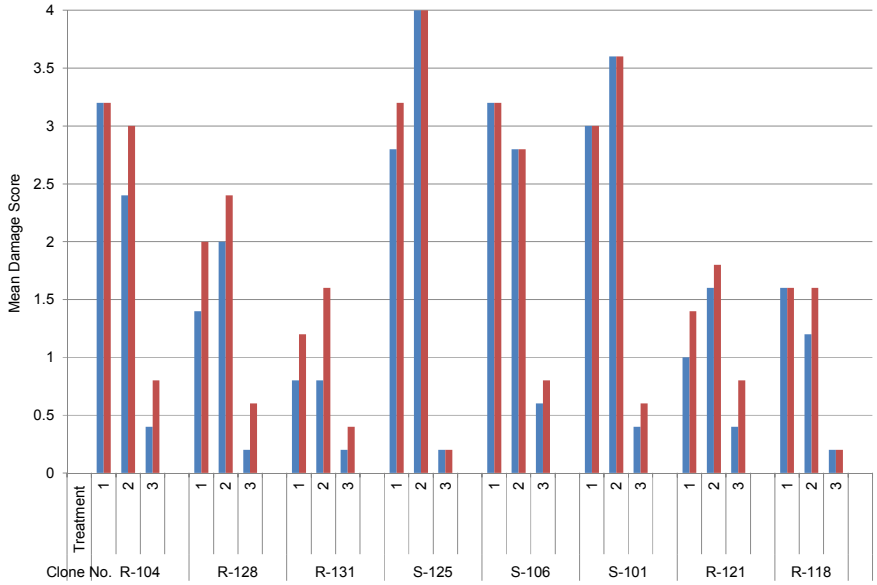


Figure 22. Mean damage score on resistant (R) and susceptible (S) clones after 24 h (blue) and 48 h (red) treated with solutions of viridiol in concentration 1.47 mM (1), 147 µM (2) or 14.7 µM (3). Columns without label are control samples.

4.3 Metabolomic comparison of clones of *Fraxinus excelsior* with different susceptibility to *Hymenoscyphus pseudoalbidus* (Paper IV)

To protect themselves from pathogens plants have different defense mechanisms. On the metabolome level, a plant´s first line of defense against a pathogen may be metabolites that are present at all times, constitutive metabolites. These metabolites can, for example, repel or kill pathogens. If this proves to be ineffective the plant may produce metabolites as a response to the pathogen attack, induced metabolites (Eyles *et al.*, 2010).

As genetic variance has been shown to be related to susceptibility to *H. pseudoalbidus* (McKinney *et al.*, 2011), differences could also be displayed on the metabolome level. The seedlings used in the study of damage caused by viridiol on resistant and susceptible clones were thus used to study such differences. The seedlings (treated with viridiol and the controls, all in five replicates *Figure 22*) were extracted and analyzed by LC-HRMS. The identification of compounds corresponding to major peaks in the base peak chromatogram was done tentatively by LC-HRMS/MS. The identification was at times supported by comparison with authentic standards or by a comparison of the ^{1}H NMR spectrum of the pooled samples to data taken from previous literature. The seedlings were found to contain a number of secoirodoids, some secondary metabolites related to abscisic acid (ABA) and two flavonol glycosides. Surprisingly, most detected compounds seemed to be compounds previously undetected in European ash.

To detect differences in the metabolome between two groups of samples, LC-HRMS data of these two groups were compared by partial least square – discriminant analysis (PLD-DA) and Welch's t-test. The PLS-DA was used to point out metabolites that are important for separating the two groups and Welch's t-test was used to determine whether the concentrations of such metabolites are significantly different between the groups.

A comparison of the resistant and the susceptible clones, to find metabolites part of a constitutive defense, showed no consistent differences for all clones within the groups. However, the concentration of one compound with the molecular formula $C_{19}H_{23}NO_7$ was significantly lower in the resistant clones, while compounds with the *formulae* $C_{30}H_{46}O_4$ and $C_{20}H_{28}O$ were higher in concentration. The two compounds higher in concentrations in the resistant clones may contribute to a constitutive defense, where they are present.

A similar comparison done on the viridiol treated seedlings showed that one unidentified compound and two compounds, with MS/MS data consistent with the ABA related compounds xanthoxin ($C_{15}H_{22}O_3$) and ABA-cysteine ($C_{18}H_{25}NO_5S$), were significantly higher in the susceptible clones, although this was not consistent for all clones.

At the clone level, the viridiol treated seedlings were compared to the control to find significant changes resulting from viridiol treatment (*Table 1*). As stated above, there were no shared changes in concentration of any metabolites detected for all clones within the groups of resistant and susceptible clones. There were, however, more examples of up-regulated ABA-related compounds in the susceptible clones. The role of ABA in plants is rather complex and somewhat contradictory, although it is known that it is involved in plant response to biotic and abiotic stress.

Table 1. *List of significant differences in compound concentration, between viridiol treated seedlings and controls of each clone, deemed important for group separation in PLS-DA.*

Formula	*m/z*[1]	Compound[2]	Clone	Type	Fold change	p-value[3]
$C_{14}H_{20}O_7$	121	tyrosol-*Hex*[4]	118	R	0.3	0.021
$C_{11}H_{12}N_2O_2$	205	tryptophane	128	R	0.4	0.041
$C_{20}H_{24}O_5$	293	unknown	118	R	0.3	0.037
$C_{12}H_{17}NO_5$	164	unknown	104	R	8.9	0.034
			106	S	0.2	0.0022
$C_{24}H_{30}O_{13}$	137	demethyloleuropein	118	R	15.0	0.031
$C_{15}H_{16}O_8$	325	umbelliferone-*Hex*	125	S	0.3	0.023
$C_{27}H_{30}O_{16}$	303	quercetin-*Hex*-*dHex*	128	R	0.4	0.0043
n.d.[6]	475	unknown	131	R	2.2	0.027
n.d.	519	unknown	101	S	0.1	0.040
$C_{25}H_{32}O_{14}$	137	10-hydroxyoleuropein	104	R	0.2	0.0053
$C_{24}H_{30}O_{12}$	121	demethylligstroside	118	R	6.5	0.0022
$C_{27}H_{30}O_{15}$	287	kaempferol-*Hex*-*dHex*[5]	128	R	0.5	0.048
n.d.	473	unknown	101	S	0.1	0.035
n.d.	279	unknown	101	S	0.3	0.044
n.d.	293	unknown	101	S	0.3	0.038
			118	R	0.2	0.029
$C_{19}H_{23}NO_7$	164	unknown	104	R	4.0	0.014
			106	S	0.2	0.018
$C_{25}H_{32}O_{13}$	165	oleuropein	104	R	0.5	0.018
$C_{19}H_{23}NO_6$	164	unknown	104	R	7.7	0.011
			106	S	0.2	0.029
$C_{18}H_{25}NO_5S$	368	ABA-cysteine	101	S	4.2	0.00026
$C_{12}H_{15}NO_4$	164	unknown	104	R	11.0	0.018
			106	S	0.3	0.013
$C_{14}H_{16}O_3$	233	unknown	125	S	0.1	0.0039
$C_{19}H_{21}NO_6$	164	unknown	104	R	4.5	0.012
			106	S	0.2	0.027
$C_{19}H_{21}NO_5$	164	unknown	104	R	9.6	0.019
			106	S	0.2	0.031
$C_{15}H_{20}O_3$	231	abscisic aldehyde	101	S	17.6	0.048
$C_{15}H_{22}O_3$	251	xanthoxin	101	S	21.4	0.012
			106	S	13.0	0.012
			131	R	4.0	0.041

1, *m/z* used for relative quantification. 2, Tentative identification. 3, from Welch´s t-test. 4 Hexose 5, deoxyhexose. 6, not determined.

The elevated levels of ABA-related compound most likely mean that the plant is under stress due to the presence of viridiol. However, there are studies that have shown increased susceptibility due to ABA response (Cao *et al.*, 2011; Ton *et al.*, 2009). This could mean that, not only are the susceptible clones sensitive to viridiol, but they also to a greater extent respond to the phytotoxin by producing a compound, ABA, which increases their susceptibility to pathogen attacks.

4.4 Secondary metabolites involved in biocontrol effect of *Pseudomonas brassicacearum* MA 250 on *Microdochium nivale* (Paper V)

In this study bioassay guided fractionation was used to look for bioactive compounds produced by the strain MA250 of the bacterium *Pseudomonas brassicacearum*. Screening was done for growth inhibitory activity towards the plant pathogen *Microdochium nivale* and the human pathogens *Staphylococcus aureus* and *Candida albicans*. Although the main target was *M. nivale*, screening for antibacterial and anti-yeast activity against human pathogens is always of interest.

The first batch of culture supernatant was extracted by SPE and both the aqueous phase and the aqueous 95% MeCN phase were tested for activity. Activity was indicated in the organic phase, which was fractioned by preparative HPLC using a fast gradient of MeCN in water. Aliquots of each fraction were tested for activity. Three active fractions were chosen for structure analysis by NMR and LC-MS, due to their growth inhibition of all three pathogenic organisms. The main compounds in the selected fractions were found to be three not previously described monoacylglycerols of 3-hydroxy fatty acids (*Figure 23*). However, when attempting to repeat the isolation of these compounds the following batch had a very different metabolic profile and the compounds were not produced in any of the following batches.

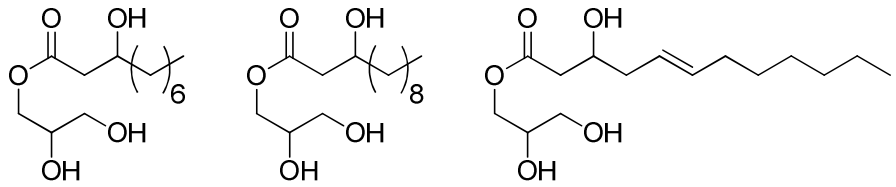


Figure 23. Molecular structures of the isolated monoacylglycerols.

Following SPE and HPLC fractionation of the second batch of culture supernatant, coinciding bioactivities against *M. nivale* and *S. aureus* were found in three fractions. Two of the active fractions were found to contain the two known compounds SB-253514 (*Figure 24*) and piliferolide A, as determined by LC-MS, NMR and some aid from Antibase. SB-253514 is an imide of a bicyclic carbamate and a 3-*O*-rhamnosyl-tetradecanoic acid, which had been previously isolated from *Pseudomonas fluorescens* DSM 11579 (Busby *et al.*, 2000). Piliferolide A is a γ-lactone of an unsaturated fatty acid, which was originally isolated from the fungus *Ophiostoma piliferum* (Ayer & Khan, 1994). NMR and LC-MS performed on the third active fraction showed that it contained a mixture of piliferolide A and its previously not described open acid form, which is thus given the name piliferoic acid A. The fact that piliferolide A eluted well after the currently analyzed fraction excluded the possibility of co-elution, which led to the conclusion that piliferoic acid A had, in part, lactonized during the drying of the fraction. Using milder conditions during the drying, piliferoic acid A was later successfully re-isolated.

The minimum inhibitory concentrations (MIC) of the three compounds against a number of fungi and bacteria and the results are summarized in *Table 2*. No growth inhibition of *C. albicans* was detected with the used concentrations.

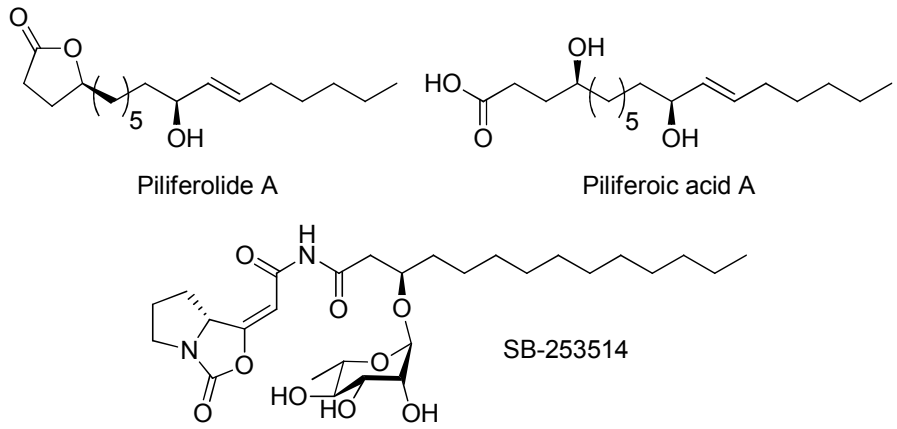


Figure 24. Chemical structures of compounds isolated from *P. brassicacearum* MA250.

Table 2. *Determined MIC values (μM) for compounds isolated from P. brassicacearum MA250.*

Organism	Organism type	SB-253514	Piliferolide A	Piliferoic acid A
Erwinia carotovora	Bacterium	91	-	-
Pseudomonas syringae	Bacterium	(180[a])	-	-
Staphylococcus aureus	Bacterium	91	-	-
Heterobasidion annosum	Fungus	180 (91)	-	-
Aspergillus fumigatus	Fungus	180 (91)	400 (200)	-
Fusarium culmorum	Fungus	91(18)	-	-
Microdochium nivale	Fungus	180 (91)	200 (40)	330 (160)

[a]Values within parenthesis denote concentrations (lower than MIC) at which partial inhibition was detected, if it was.

"-" Denotes that the MIC was >180 for SP-253514, >400 for piliferolide A and >330 for piliferoic acid A.

5 Conclusions

Eight secondary metabolites were isolated from liquid cultures of *H. pseudoalbidus*. One additional was detected in the liquid culture. Three of the nine compounds were the known compounds viridiol, viridin and demethoxyviridiol. The other six compounds were the previously undescribed 1-deoxyviridiol, 1-deoxy-2-demethylviridiol, 3-dihydrovirone, B-norviridin enol, B-norviridiol lactone and 1β-hydroxy-2α-hydroasterogynin A. Viridiol may contribute to the phytotoxicity of *H. pseudoalbidus* on *F. excelsior* as it displayed such properties on seedlings of the plant. Further, viridiol caused higher damage scores for clones, classed as susceptible to the pathogen, than for resistant clones.

The comparison between the metabolomes of clones that are resistant and susceptible to *H. pseudoalbidus* showed no consistent differences. Thus, there seems to be no constitutive metabolites responsible for the difference in susceptibility.

On the clone level there was no common response to viridiol treatment for the resistant or the susceptible clones.

The three compounds piliferolide A, piliferoic acid A and SB-253514 were isolated from *P. brassicacearum* MA250. All three showed moderate activity to *M. nivale* and they may thus contribute to the biological control effect of *P. brassicacearum* MA250 on *M. nivale*.

5.1 Proposals for future studies

- Characterize more secondary metabolites of *H. pseudoalbidus* and test for toxicity towards *F. excelsior*.
- Identify the target and investigate the mechanism of viridiol.
- Investigate the responses of older ash plants of different genotypes towards an infection of *H. pseudoalbidus*.

- Investigate a possible use of viridiol in the screening process for resistant trees for breeding.

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