“Chemical characterization of antioxidative compounds of the root-associated endophytic fungi *Phialophora lignicola* found in *Pinus sylvestris* seedlings”

Eduardo Luis León-Denegri Zevallos

Master’s thesis

Laboratory of Organic Chemistry, Department of Chemistry, University of Helsinki, P.O. Box 55, FI-0014 Helsinki, Finland

May 2015
ACKNOWLEDGEMENTS

It is a pleasure to find the chance to show my gratitude and all my regards to Prof. Kristiina Wähälä for her instructive supervision, her kind help and her continuous support and encouragement throughout the completion of this work.

I would like to express my cordial thanks and gratitude to PhD. Tytti Sarjala and PhD. Harri Latva-Mäenpää for giving me the opportunity to pursue my master research in collaboration with the Natural Resources Institute Finland (Luke), as well as for their valuable suggestions, their fruitful discussions, their unforgettable support and for the excellent work facilities at Luke, Parkano.

My special thanks to PhD. Harri Koskela for his constructive advises, NMR measurements, sharing his expertise in NMR data interpretation as well as for his help and support.

I also appreciate the sincere cooperation of PhD. Petri Heinonen and PhD. Sami Heikkinen for their instructions and training in mass spectrometric and NMR techniques.

I would like to thank my present friends and colleagues Daniel Rico del Cerro, Mika Berg, Tiina Laaksonen, Oleksndr Zagorodko, Keith Biggart and Antoine Corbin, and all others for the nice multicultural time I spent with them, for their help and assistance whenever I needed it.

I would like to thank my family who supported me and made it possible for me to set my own goals and to reach them.

Finally, special thanks to İpek Yükselen who was always there for me to help me and assist me in the most challenging times.
“Chemical characterization of antioxidative compounds of the root-associated endophytic fungi *Phialophora lignicola* found in *Pinus sylvestris* seedlings”

Many diseases are associated with oxidative stress caused by free radicals. Fungal endophytes are microbes that inhabit host plants without causing disease and have been recognized as potential sources of pharmaceutically valuable compounds. The extract of the endophytic fungi, *Phialophora lignicola* found in roots of *Pinus sylvestris* (Scots pine) seedlings has revealed protective bioactivity on human retinal pigment epithelial cells (hRPE) against oxidative stress which can lead to obtain compounds for the treatment or prevention of Age-related macular degeneration (AMD). The current research was directed towards finding the naturally-occurring antioxidant(s) in *P. lignicola* and to characterize and identify them by NMR spectroscopy and MS analysis.

The bioactive aqueous extract of *P. lignicola* was fractionated by preparative HPLC and the antioxidant activity of each sample was evaluated by H₂O₂ scavenging test and bioassay with model cells of hRPE cells. The bioactive fractions were characterized by 500 Mhz NMR spectroscopy, performing both one- and two-dimensional NMR experiments, including ¹H NMR, COSY, TOCSY, HSQC, ROESY, ³¹P-NMR and ³¹P-¹H correlation. ESI-TOF MS in the positive mode was used for the determination of molecular weights found in the fractions. Additionally, LC-MSⁿ was used for the separation of compounds and mass analysis to complement the information given by ESI-TOF MS.

The present study provides evidence that two bioactive fractions of *P. lignicola* possessed the presence of antioxidative activity. The NMR experiments suggested arginine and polysaccharides. ESI-TOF MS results established the presence of arginine, a hexose-arginine conjugate and other compounds that based on NMR and mass spectrometry literature they could be chitin oligomers. Furthermore, LC-MSⁿ identified fragments typical of L-arginine. For future research, some drawbacks of this study such as concentration of samples and chromatographic purification method should be improved to optimize the NMR and mass spectrometry analysis.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
</tr>
<tr>
<td>COSY</td>
<td>correlation spectroscopy</td>
</tr>
<tr>
<td>°C</td>
<td>centigrade</td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift</td>
</tr>
<tr>
<td>D₂O</td>
<td>deuterium oxide</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>DAD</td>
<td>diode array detector</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>half maximal effective concentration</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>ESI-TOF MS</td>
<td>electrospray ionization-time of flight mass spectrometry</td>
</tr>
<tr>
<td>et al.</td>
<td>et alera (and others)</td>
</tr>
<tr>
<td>eV</td>
<td>electronvolt</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>ha</td>
<td>hectares</td>
</tr>
<tr>
<td>¹H-NMR</td>
<td>proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HILIC</td>
<td>hydrophilic interaction chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>Hr</td>
<td>hour</td>
</tr>
<tr>
<td>hRPE</td>
<td>human retinal pigment epithelium</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>HCl</td>
<td>chlorhydric acid</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single-quantum-coherence</td>
</tr>
<tr>
<td>ITS</td>
<td>internal transcribed spacer</td>
</tr>
<tr>
<td>J</td>
<td>coupling constant</td>
</tr>
<tr>
<td>K</td>
<td>kelvin</td>
</tr>
<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>[M+H]⁺</td>
<td>molecular ion</td>
</tr>
<tr>
<td>MALDI-TOF MS</td>
<td>matrix assisted laser desorption ionization-time of flight mass spectrometry</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MHz</td>
<td>mega Hertz</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>m/z</td>
<td>mass per charge</td>
</tr>
<tr>
<td>mΩ</td>
<td>milliohms</td>
</tr>
<tr>
<td>N₂</td>
<td>nitrogen</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NH₃</td>
<td>ammonia</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>³¹P-NMR</td>
<td>phosphorus nuclear magnetic resonance</td>
</tr>
<tr>
<td>³¹P-¹H</td>
<td>phosphorus-proton correlation</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pH</td>
<td>potential of hydrogen</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>q</td>
<td>quartet</td>
</tr>
<tr>
<td>ROESY</td>
<td>rotating frame Overhauser effect spectroscopy</td>
</tr>
<tr>
<td>RP 18</td>
<td>reversed phase C 18</td>
</tr>
<tr>
<td>s</td>
<td>singlet</td>
</tr>
<tr>
<td>T</td>
<td>tesla</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>TOCSY</td>
<td>total correlation spectroscopy</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μL</td>
<td>microliter</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>V</td>
<td>voltage</td>
</tr>
</tbody>
</table>
LIST OF CONTENTS

1. INTRODUCTION ........................................................................................................... 1
   1.1. Endophytic fungi ........................................................................................................ 1
   1.2. Interaction endophyte-host plant ............................................................................. 2
   1.3. Choice of plants for the isolation of endophytes ..................................................... 3
   1.4. Pharmaceutical potential of fungal natural products ................................................. 5
   1.5. Bioactive natural products of endophytic fungi ....................................................... 6
       1.5.1. Anticancer natural products of endophytic fungi ............................................... 7
       1.5.2. Antibiotic natural products of endophytic fungi ............................................... 9
   1.6. Antioxidant natural products of endophytic fungi .................................................. 10
   1.7. The forests as resource of endophytes .................................................................... 11
   1.8. Age-related macular degeneration ......................................................................... 12
   1.9. Aim and scopes of the study .................................................................................... 13

2. EXPERIMENTAL SECTION .......................................................................................... 14
   2.1. Materials .................................................................................................................. 15
       2.1.1. Biological material .............................................................................................. 15
       2.1.2. Growth medium ................................................................................................. 15
       2.1.3. Solvents for HPLC ............................................................................................. 15
       2.1.4. Solvent for NMR ................................................................................................ 15
       2.1.5. Solvents for ESI-TOF MS ................................................................................. 16
       2.1.6. Solvents for LC-MS^n ....................................................................................... 16
   2.2. Methods ................................................................................................................... 16
       2.2.1. Isolation of fungal strains ...................................................................................... 16
       2.2.2. Identification of root-associated fungi .................................................................... 17
           2.2.2.1. Fungal identification ......................................................................................... 17
           2.2.2.2. Taxonomy ...................................................................................................... 17
       2.2.3. Cultivation and storage of pure fungal strain of Phialophora lignicola ............... 18
       2.2.4. Extraction of fungal culture .................................................................................. 18
       2.2.5. Bioactivity test on human retinoplastic endothelium cells against oxidative stress
              by extracts of root-associated fungi .................................................................. 19
       2.2.6. Fractionation by preparative HPLC ................................................................... 20
       2.2.7. Mass spectrometry (MS) ..................................................................................... 21
           2.2.7.1. Electrospray ionization-time of flight mass spectrometry (ESI-TOF MS) .... 21
2.2.7.2. Liquid chromatography-electrospray ionization mass spectrometry (LC-ESI MSⁿ)

2.2.8. Nuclear magnetic resonance spectroscopy (NMR)

3. RESULTS AND DISCUSSION

3.1. Bioactivity test results for fractions from the endophytic fungi Phialophora lignicola

3.2. Chromatographic fractionation

3.3. Nuclear magnetic resonance spectroscopic analysis

3.3.1. 1D NMR

3.3.2. 2D NMR

3.4. Electrospray ionization time-of-flight mass spectrometry analysis (ESI-TOF MS)

3.5. Liquid chromatography electrospray ionization mass spectrometry analysis (LC-MSⁿ)

3.6. HPLC retention time of L-arginine

3.7. The occurrence hexose-arginine conjugate

4. CONCLUSIONS

5. LIST OF REFERENCES
1. INTRODUCTION
1.1. Endophytic fungi

There is an estimation that there may be approximately 1.5 million different fungal species on Earth and around 80,000 species having been described (Hawksworth 1991, 2001; Hawksworth et al., 1995). Fungi plays a vital ecological role in almost all ecosystems. Saprotrophic fungi have key role important in the cycling of nutrients, especially the carbon that is sequestered in wood and other plant tissues. Pathogenic and parasitic fungi attack practically all types of organism, including bacteria, plants, fungi and animals, including also humans. Other fungal species act as mutualistic symbionts, for example, mycangial associates of insects, mycorrhizae, lichens and endophytes. From these symbioses, fungal organism have implemented a diversity of other organisms to exploit novel habitats and resources (Lutzoni et al., 2004). More than 100 years of research suggests that almost every plant in natural ecosystems is symbiotic with mycorrhizal fungi and/or fungal endophytes (Petrini 1986). Fossil records indicate that plants have been associated with endophytic and mycorrhizal fungi for more than 400 million years and were likely associated when plants colonized land, thus playing an important role in driving the evolution of life on land (Krings et al., 2007; Redecker et al., 2001).

Fungal endophytes are microorganism that live intercellularly or intracellularly within plants tissues, without causing any visible manifestation of damage (Saikkonen 1998; Bacon and White 2000). The existence of fungi inside the organs of asymptomatic plants has been known since the end of the 19th century (Guerin 1898), and the term “endophyte” was proposed in 1866 (de Bary 1866). Unlike mycorrhizal fungi that colonize plant roots and grow into rhizosphere, endophytes typically are found in above-ground plant tissues like stems and leaves, but also occasionally in roots, and are distinguished from mycorrhizae by lacking external hyphae or mantles. Endophytic fungi living asymptotically have been found in almost all plant species (Kusari et. al., 2012; Saikkonen et al., 1998). It is noteworthy that, of the nearly 300,000 plant species that exist on the Earth, each plant is the host to one or more endophytes (Strobel & Daisy, 2003). It is estimated that there may be as many as 1 million different endophyte species (Petrini 1991), which enables the great opportunity to find new and targeting natural products from interesting endophytic microorganisms among hosts of plants in different ecosystems (Guo et al., 2008). During this association, none of the interacting partners is apparently harmed, and the individual
benefits depend on both the cooperating partners (Souvik et al., 2012). The harmony of such complex interaction can be represented between extremely dedicated mutualism and ardent parasitism or saprophytism or exploitation, which might bear the potential to shift variably or progressively toward a more specialized interaction (Millet et al., 2010; Zuccaro et al., 2011).

**1.2. Interaction between endophyte and host plant**

Fungal life-styles can be saprophytic, symbiotic or both (Hudson 1986). Although symbiotic associations can be either parasitic, mutualistic, or commensalistic, the genetic bases of these different life-styles remain mysterious (Freeman & Rodriguez, 1993). The interaction between a fungal endophyte and the plant host is defined by a finely tuned equilibrium between fungal virulence and plant defense. If this balance is disturbed by either a reduction in plant defense or an increase in fungal virulence, disease develops (Schulz et al., 2002). The endophytes receive nutrition and protection from the plant while the host plant might benefit from enhanced competitive abilities and increased resistance to herbivores, pathogens and various abiotic stress (Saikkonen et al., 2002). Endophytes’ reproduction has showed that they are more likely to be mutualistic when reproducing vertically by growing into seeds and antagonistic to the host when transmitted horizontally via spores (Scharld et al., 1991). The existence of a genetic system is also a possibility that allow endophytes to transfer nucleic acids from endophytes to plant, or vice versa. Some rare organic molecules made by higher plants can be produced by certain endophytic microbes as well (Strobel 2002).

Fungal endophytes protect host plants from natural enemies by mutualistic interaction with their host plant by increasing resistance to herbivores, and have been termed “acquired plant defenses”. Endophytes produce mycotoxins that poison herbivores and pathogenic microbes (Carrol 1988; Faeth & Fagan, 2002). In grasses, endophytic fungal species (family Clavicipitaceae, Ascomycetes) produce physiologically active alkaloids in the tissues of the host plant, which turns grass toxic to domestic mammals and improves resistance to insect herbivores (Clay 1988). Similarly, an endophytic fungus found in conifer needles produce toxic metabolites to the spruce budwirm (Calhoun et al., 1992). In woody plants, endophytic fungi provide a defensive role for the host plant since they produce a wide array of
mycotoxins and enzymes that can inhibit growth of microtubes and invertebrate herbivores (Saikkonen et al., 1998; Petrini et al., 1992).

In the mutualistic relationship the endophyte can enhance the plant’s growth and contribute to its adaptation to stress. The fungi improve tolerance to abiotic stress such as drought, heavy metals toxicity, high soil salinity, heat, cold and oxidative stress (Lamabam et al., 2011). The plants with fungal symbionts often grow faster than non-infected ones. This is due to the endophyte’s production of phytohormones such as indole-3-acetic acid (IAA), cytokines, or other plant promoting compounds, or to the fact that endophytes may enhance the hosts’ uptake of nutrients such as nitrogen and phosphorus (Tan & Zou, 2001).

In turn, the host plant offers to the endophytic fungus a spatial structure, protection from desiccation and supply of nutrients for the hyphal growth and, dissemination to the next generation of hosts (for vertical-transmitted endophytes) (Saikkonen et al., 1998; Faeth & Fagan 2002; Hinton and Bacon 1985; Rudgers et al., 2004). There may be a possibility that the plant supplies compounds that stimulates the growth of the endophyte or for the completion of its life cycle (Metz et al., 200; Strobel, 2002a).

The variation of endophytic-host plant interaction may also be originated by genotypic combinations between the plant and the endophyte (Faeth & Fagan, 2002). Moreover, little information exists in relation to the biochemistry and physiology of the interactions of the endophyte with its host plant. Most probably many aspects varying in the host as related to the season and age, environment, and location could influence in the biology of the endophyte. Further research at the molecular level must be developed to understand better the interactions between endophytes and ecology. These interactions are possibly chemically mediated for some motive in nature. An ecological approach of the role of these organisms play in nature will provide the best evidence for targeting particular types of endophytes with the most promising bioprospecting (Strobel & Daisy, 2003).

1.3. Choice of plants for the isolation of endophytes

Endophytes are seen as an outstanding source of bioactive natural products because there are so many of them inhabiting literally millions of unique biological niches (higher plants) growing in so many unusual environments. It seems that these biotypical factors can be
important in plant selection, since they may lead the uniqueness and biological activity of the products associated with endophytic microbes (Strobel & Daisy, 2003). Since the number of plant species in the world is great, creative and imaginative strategies must be used to quickly narrow the search for endophytes that reveal bioactivity (Mittermeier 1999).

As the endophytes as live in close association with living plant tissues, it is important to understand the methods and choices used to isolate endophytic microorganisms. To acquire endophytes, in the first place it is important to select plant species that may be of interest because of its unusual biology, age, endemism, ethnobotanical history (used by indigenous people), possessing novel strategies for survival and unique environmental setting. This is a vital step in the whole process because it represents a biological foundation in plant selection and will help to eliminate the work involved in doing a random search of all flora in any given area. The challenge is to properly choose those among thousands of plant species on the planet that are most profitable to study. It appears that endemic plants growing in moist, warm, and geologically isolated climates are among the first choices for study. It seems that microorganism competition in such area would be hostile given the abundance of both water and plants. The number and diversity of natural products produced by microbes surviving in such area would be massive. Therefore, plants in extremely moist conditions (like aquatic plants), or those growing in rainforests (at a more or less constant 90 to 100% relative humidity) are susceptible to attack by a certain group of extremely pathogenic fungi and mechanisms of defense are essential for survival. These defenses may be offered normally by the endophyte associated with the host plant. Plants growing in areas of great biodiversity also have the prospect of housing endophytes with great biodiversity (Strobel 2002b; Strobel & Daisy, 2003).

Additionally, it is notable that some plants producing bioactive natural products have associated endophytes that produce the same natural products. Such is the case with paclitaxel, a highly functionalized diterpenoid and well-known anticancer agent that is found in each of the world’s yew tree species (Taxus spp.) (Suffnes 1995). In 1993, a novel paclitaxel-producing fungus, Taxomyces andreanae, from the yew Taxus brevifolia was isolated and characterized (Strobel et al., 1993).
1.4. Pharmaceutical potential of fungal natural products

Since the discovery of penicillin G from *Penicillium* species in 1928, fungal microorganism suddenly became a hunting ground for novel drug leads (Demain 1999). Following the success of penicillin, drug pharmaceutical companies and research groups soon assembled large microorganisms’ culture collections in order to discover new antibiotics (Butler 2004). In 1955 an antibacterial agent, cephalosporin C, a hydrophilic antibiotic, was isolated from the fungus species from the genus *Cephalosporium* showing promising antibacterial properties (Newton & Abraham, 1955). Griseofulvin, isolated from the mycelium *Penicillium griseofulvum* was one of the first antifungal natural products characterized by its unique biological activity (Grove et al., 1952). Cyclosporine revolutionized the management of rejection in solid organ transplantation. The immunosuppressive properties of cyclosporine were discovered in 1972. It is found naturally as a cyclic polypeptide which is isolated from the mycelia of two strains of fungi, *Cylindocarpom lucidum* and *Tolypocladium inflatum* (Chaudhuri et al., 1997; Borel & Kis, 1991). Another immunosuppressive fungal natural product used for organ transplantations or autoimmune diseases is mycophenolic acid. It is produced by five *Penicillia*, one *Aspergillus*, one *Byssoschlamys* and one *Septoria* species (Larsen et al., 2005). It is also an antibacterial, antifungal and antiviral compound (Bentley 2000). Moreover, other compounds discovered were echinocandin B and pneumocandin B, from *Aspergillus rugulosus* and *Glarea lozoyensis*, respectively, which were lead compounds and templates for the semi synthesis of the antifungal drug anidulanfungin and caspofungin (Butler 2014) (Fig. 1).

Conventionally, microorganisms have been isolated from soil samples and studied for any pharmaceutical or biological activity which might be useful for medicinal or agrochemical application. Currently, the discovery of novel compounds from soil fungi has shown a decrease compared to endophytic fungus’ finds. By 2012, 51% of biologically active substances isolated from endophytic fungi were previously unknown, this compares with 38% of novel substances from the soil (Schulz et al., 2002; Strobel 2003). Consequently, in the search for new sources of pharmaceutical agents, endophytic fungi associated with plants have emerged as a big unexploited reservoir of metabolic diversity producing an extensive group of new and valuable bioactive secondary metabolites, compared to soil fungi (Schulz et al., 2002).
1.5. Bioactive natural products of endophytic fungi

Despite the fact that endophytes were first described from the darnel (*Lolium temulentum*) in the 1904 (Freeman 1904), they started to draw considerable attention after the discovery of paclitaxel (Taxol®) produced by the endophytic fungi, *Taxomyces andreanae*, the latter which was isolated from *Taxus brevifolia*, the original source of the multibillion dollar anticancer drug (Stierle *et al.* 1993, 1995) (Fig. 2). This discovery aroused the interest in endophytes as potential new sources for therapeutic agents (Aly *et al.*, 2012).

Novel and unusual organic substances may be discovered that benefit to the host plant-endophyte relationship while at the same time provides new and interesting bioactive compounds that may find applicability in medicine, industry and agriculture (Strobel 2002a). The number of secondary metabolites produced by fungal endophytes is bigger than of any other endophytic microorganism class. The reason could be due to the high frequency of isolation of fungal endophytes from plants. Secondary metabolites from fungal endophytes have a broad spectrum of biological activity, and they can be classified into various types, alkaloids, steroids, tephenoinds, isocoumarins, quinones, phenylpropanoids and lignans, phenol and phenolic acids, aliphatic metabolites, lactones, etc. and can be used for curing many diseases (Zhang *et al.*, 2006; Tejesvi *et al.*, 2007). Certainly, endophytic fungi are a very promising source of novel compounds when testing larger number of bioactivities or...
chemical profiles (Schulz et al., 2002). In general, most of the natural products from endophytes are antibiotics, anticancer agents, immunomodulatory, antioxidant, antiparasitic, antiviral, antituberculosis, insecticidal, etc. for use in the pharmaceutical and agrochemical industries (Guo et al., 2006; Kaul et al., 2012; Tan & Zou, 2001).

1.5.1 Anticancer natural products of endophytic fungi

Paclitaxel (Taxol®), produced the endophytic fungi Taxomyces andreanae, impulsed the interest in endophytes as potential new sources of potential pharmaceutical agents. (Strobel et al., 1993; Stierle & Strobel, 1995). Taxol is a very potent anticancer agent, isolated for the first time from the bark of the pacific yew tree (Taxus brevifolia). The FDA determined and proved the potential of taxol for the treatment of ovarian and breast cancer (Kaul et al., 2012; Cremasco et al., 2009). Its high cost makes it inaccessible to many people in the world. Consequently, alternative sources are needed since although total organic chemical synthesis has been accomplished, it is not yet economically feasible (Nicolau et al., 1994). After efforts were launched to discover if other species of Taxus produce taxol, the isolation of taxol have been successful from endophytic fungi from all yew species, and a myriad of trees in each of the major rain forests of the world (Li et al., 1996; Strobel et al., 1996).

Torreyanic acid, a quinone dimer, is produced by the endophyte Pestalotiopsis microsporum isolated from Torreya taxifolia. It is a potent cytotoxic agent and found to be more efficient in cell lines that are sensitive to protein kinase C agonists; it causes cell death by apoptosis (Lee et al., 1996). The alkaloid camptothecin is a potent antineoplastic agent from the endophytic fungus Entrophospora infrequens inhabiting Nothapodytes foetida. By checking the biological activity of this compound by in vitro cytotoxic assays against human cancer cell lines (A-459 for lung cancer, HEP-2 for liver cancer, OVCAR-5 for ovarian cancer) resulted in promising results (Puri et al., 2005). Two potent anticancer compounds, topotecan and irinotecan were extracted from the endophytic fungus Fusarium solani inhabiting Camptotecha acuminata (Kusari et al., 2009). Another relevant anticancer agent is podophyllotoxin, a non-alkaloid lignin, its analogues are clinically relevant mainly due to their antiviral and anticancer activities, further they are the precursors of many other useful anticancer drugs including etoposide, teniposide and etopophos phosphate (Kour et al., 2008). Podophyllotoxin and other related aryl tetralin lignans have also been reported to be produced by another endophytic fungi, Tramates hirsute with anticancer potential (Puri et al., 2005).
Several microbial sources of podophyllotoxin include *Aspergillus fumigatus* from *Juniperus communis* (Kusari et al., 2009), *Phialocephala fortinii* from *Podophyllum peltatum* (Eyberger et al., 2006) and *Fusarium oxysporum* from *Juniperus recurve* (Kour et al., 2008). Additionally, ergoflavin, a novel anticancer agent was isolated from the leaf endophytes of an Indian medicinal plant *Mimusops elengi*. It is a dimeric xanthene linked at position-2, belonging to the ergochrome class of compounds (Deshmukh et al., 2009) (Fig. 2). Currently, there are larger numbers of anticancer agents produced by fungal endophytes inhabiting different medicinal plants (Kaul et al., 2012).

![Figure 2. Anticancer compounds from endophytic fungi from medicinal plants.](image-url)
1.5.2 Antibiotic natural products of endophytic fungi

Antibiotics are defined as low molecular weight organic natural products made by microorganisms. They are bioactive at low concentrations against any other microorganisms (Demain 1981). The most bioactive natural products with antibiotic action are from endophytes. Coronamycin is a complex peptide antibiotic with activity against pythiaceous fungi and the human fungal pathogen Cryptococcus neoformans, produced by a verticillate Streptomyces sp. isolated as an endophyte from an epiphytic vine Monstera sp. It was also active against Plasmodium falciparum, with an IC 50 (inhibitory concentration 50%) of 9.0 ng mL⁻¹ (Ezra et al., 2004). Phomol, a novel antibiotic was isolated from fermentations of an endophytic fungal Phomopsis species from the medicinal plant Erythrina crista. It was characterized as a polyketide lactone (Weber et al., 2002). Two fusicoccane diterpenes, named periconicins A and B, with antibacterial activities were isolated by bioassay-guided fractionation from the endophytic fungi Periconia sp. from the branches of Taxus cuspidata (Kim et al., 2004). Moreover, phomodione, found in the endophytic fungi, Phoma sp. from the Saurauia scaberrinae, showed to be effective at a minimum inhibitory concentration of 1.6 μg/mL against Staphylococcus aureus (Hoffmann et al., 2008) (Fig. 3).

Figure 3. Antibiotic compounds from endophytic fungi from medicinal plants.
1.5.3 Antioxidant natural products of endophytic fungi

Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals. Free radical reactions are linked to degenerative diseases such as cancer, Alzheimer, age-related macular degeneration, etc. Due to the fact that only few antioxidants are approved for clinical applications, there is a necessity to search for novel and effective antioxidants (Kaul et al., 2012). The discovery of pestacin and isopestacin as antioxidant compounds from the endophytic fungi Pestalotiopsis microspore residing in Terminalia morobensis led to the exploration of antioxidant potential in this group of fungi. Pestacin occurs naturally as a racemic mixture and acts by cleaving a reactive C-H bond and through O-H abstraction to a minor extent (Harper et al., 2003). Isopestacin acts as an antioxidant by scavenging both superoxide and hydroxyl free radicals (Strobel et al., 2002). Furthermore, graphislactone A isolated from Cephalosporium sp., and endophytic fungi found in Trachelospermum jasminoides. The natural product had a strong antioxidant activity in vitro as compared to butylated hydroxytoluene and ascorbic acid which were used as positive control (Song et al., 2005). Cajaninstilbene acid has been reported from a Fusarium sp. endophyte of Cajanus cajan (Pigeon pea) (Zhao et al., 2012). Another example was the strong antioxidant activity exhibited by the extract of Xylaria sp. isolated from Gingkgo biloba, due to the presence of phenolics and flavonoids (Lui et al., 2007) (Fig. 4)

![Figure 4. Antioxidative compounds from endophytic fungi from medicinal plants.](image)
1.6. The forests as resource of endophytes

One of the major problems for the future of endophyte biology is the rapidly diminishing forests, which hold the greatest possible resource for acquiring novel and unusual microorganisms and their products (Strobel 2003). Nowadays, the total land-mass of the world that currently includes rainforests is about equal to the area of the United States (Mittermeier et al., 1999). It is estimated that only 40-50%, of what the original rainforests were existing 1000-2000 years ago, are currently present on Earth. The beginning of major negative pressures on them from human activities appears to be eradicating entire life forms at a frightening rate, not considering what would happen regarding the potential loss of microbial diversity as entire plant species disappear. It can only be predicted that this loss is also happening, possibly with the same frequency as the loss of mega-life forms, particularly since microorganism might have developed unique specific symbiotic relationships with their host-plant. As a consequence, when a plant species disappears, also the associated endophytes will. Efforts are needed to secure information about life forms before they continue to be lost. Regions of the planet that represent unique places storing biodiversity need immediate preservation. There is a need for establishing information of the biodiversity and collection of microorganism that live in these areas. Endophytes are only an example of life form source.

50% of the European forests are mainly coniferous, quarter a predominantly broadleaved and a quarter are mixed. About 87 percent of the European forests are categorized as semi-natural. Undisturbed forests and plantations cover 4% and 9%, respectively, of the forest area in Europe. The forest area in Finland is wider than in any other European country, representing about 11% of the forest area in Europe (210 million ha.). Three fourths of the land area, around 23 million hectares (76%), is covered by forests (Figure 4). Furthermore, there are cleared land areas where there are only few trees, for example open peatland and areas of exposed bedrock, approximately 3 million hectares altogether in Finland. Near 90% of the forests are mainly coniferous forests of pine or spruce. Almost all (96%) Finnish forests are classified as semi-natural forests displaying signs of human impact. The sum of undisturbed forests is 4%, of which nearly 60% are located in protected areas (Natural Resources Institute Finland, 2012).
Age-related macular degeneration (AMD) is the leading cause of legal blindness in elderly individuals in the developed world, affecting 30–50 million people worldwide (Osaki et al., 2014). The annual direct cost of medical care for AMD, was estimated at US$255 billion in 2010 with an additional economic impact of US$88 billion due to lost productivity and the burden of family and community care for visual disability (Blenkinsop et al., 2012). It causes deterioration to the macula located in the center of the retina, needed for sharp and central vision (Randolph 2014). The presence of drusen, extracellular protein aggregates that accumulate under the retinal pigment epithelium (RPE), is a major pathological hallmark in the early stages of the disease (Osaki et al., 2014). Most visual loss occurs in the late stages of the disease due to one of two processes: neovascular (wet) age-related macular degeneration and geographic atrophy (dry). In neovascular AMD, choroidal neovascularization breaks through to the neural retina, leaking fluid, lipids and blood and leading to fibrous scarring. In geography atrophy, progressive atrophy of the retinal pigment epithelium, choriocapillaris, and photoreceptors occurs. Most grave visual loss from AMD is caused by these advanced forms of the disease (Lim et al., 2012).

The cause of AMD is unknown. Nevertheless, there are risk factors that may contribute to the development of AMD. The primary risk factor is high age, particularly older than 60.
years. Other risks factors are smoking, genetic component, race (AMD is more common in whites), gender (females are more affected), obesity, hypertension, and hypercholesterolemia (National Eye Institute 2013).

1.8. Aim and scopes of the study

There is limited research about fungal endophytes, which are indisputably an abundant source of bioactive natural products with enormous pharmaceutical potential. The aim of this study was the isolation of an endophytic fungal *Phialophora lignicola* strain to pure culture from the roots of Scots pine (*Pinus sylvestris*) found in the Finnish peatland and the isolation, identification and chemical characterization of the biologically active compounds from the extracts of this endophytic fungi. The aqueous extract of *Phialophora lignicola*, presented significant antioxidative bioactivity on the protection of human retinoplastic endothelium cells (hRPE) against oxidative stress.

To obtain the biological active compounds, the fungus was grown on modified Hagem medium with agar. The culture was allowed to grow for 4 weeks and refrigerated at -20°C, followed by grinding and successive extraction of the fungal mass with water at 95-100°C. The obtained extracts were then fractionated using preparative HPLC-DAD and the fractions were subjected to bioassay to determine their antioxidative potential. ESI-TOF MS and LC-MS^n were used for the analysis of the molecular weight. Also, the fractions were submitted to analysis by one- and two- dimensional NMR techniques for structure elucidation.

The collection of the biological material, identification and chromatographic fractionation were conducted in Natural Resources Institute Finland (Luke) by the research group of Ph.D. Tytti Sarjala and group, Parkano, Finland. Biological assays with RPE cells were carried out by the research group of Professor Hannu Uusitalo, University of Tampere.
2. EXPERIMENTAL SECTION

Figure 5. General protocol developed for the identification of antioxidative compounds of Phialophora lignicola found in the roots of Pinus sylvestris.
2.1. Materials

2.1.1. Biological material

Roots of Scots pine trees (*Pinus sylvestris*) were collected from the peatland forest Haukilampi (latitude North: 62° 0’ 48.373”; longitude East: 23° 15’ 34.555’’), field experimental area and cleaned from soil for isolation of the fungi (Fig. 6).

![Collection area of Pinus sylvestris seedlings (Haukilampi).](image)

2.1.2. Growth medium

The modified Hagem medium contained per Liter, CaNO3 (0.5 g.), d-glucose (3.0 g.), KH2PO4 (0.5 g.), MgSO4.7H2O (0.15 g.), CaCl2 (0.05 g.), NaCl (0.025 g.), 0.1 % w/w solution of thiamine HCl (1 mL), 1% w/w solution of FeCl3 (1.2 mL) and 997.8 mL of deionized H2O, pH 4.5.

2.1.3. Solvents and reagents for HPLC

- Methanol
- HPLC-grade water (18 mΩ)
- L-arginine hydrochloride ≥98% (Sigma-Aldrich, USA)

2.1.4. Solvent for NMR

- Deuterium oxide (D2O)

LiChroSolv HPLC grade (VMR).
Prepared with a Milli-Q purification system from Millipore (Milford, MA, USA).
Eurosi-top (Saint-Aubin, France).
2.1.5. Solvents for ESI-TOF MS

- Acetonitrile
- LiChroSolv HPLC grade (VMR).
- HPLC-grade water (18 mΩ)
- Prepared with a Milli-Q purification system from Millipore (Milford, MA, USA).

2.1.6. Solvents for LC-MS

- Methanol
- LiChroSolv HPLC grade (VMR).
- Acetic acid
- LiChroSolv HPLC grade (Merck).
- HPLC-grade water (18 mΩ)
- Prepared with a Milli-Q purification system from Millipore (Milford, MA, USA).

2.2. Methods

2.2.1. Isolation of fungal strains

Root surface sterilization was performed by dipping the root segments briefly (about 15 seconds) in 70% ethanol, followed by washing with sterile water, soaking in 30% hydrogen peroxide for 1 min 30 seconds, and washing with sterile water. Sterilized root tips were transferred to isolation medium (2% water agar, glucose + Hagemagar + Streptomycin) (Fig.7,8,9) (Viitala et al., 2011).

Figure 7. Scots pine roots (Photo by T.Sarjala, Luke).

Figure 8. Washing of the roots (Photo by T.Sarjala, Luke).

Figure 9. Root tip in isolation medium (Photo by T.Sarjala, Luke).
2.2.2. Identification of root-associated fungi

2.2.2.1. Fungal identification

Identification of the fungal isolated was done in Luke by amplifying fungal specific ITS-region with PCR using ITS1F-ITS4 primer pair. This primer pair have been designed by Gardes and Bruns (1993) and by White et al. (1990). High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) was used to purify amplified DNA and direct sequencing of the purified PCR samples was performed with CEQ 8000 DNA analysis system and Quick Start Kit (Beckman Coulter Inc., Fullerton, California, USA). (Viitala et al., 2011).

Sequences were compared to the information in Genbank to identify Phialophora lignicola. This fungus was maintained on modified Hagem medium with agar.

2.2.2.2. Taxonomy

The fungus Phialophora lignicola was isolated from roots of an 8 years old Pinus sylvestris L. (Scots Pine) seedling (Fig. 10). The roots of Pinus sylvestris were growing on the drained peatland Haukilampi field experimental area.

Kingdom: Fungi  
Division: Ascomycota  
Class: Eurotiomycetes  
Subclass: Chaetothyriomycetidae  
Order: Chaetothyriales  
Family: Herpotrichiellaceae  
Genus: Phialophora  
Species: Phialophora lignicola (Nannf.) Goid. 1937
2.2.3. Cultivation and storage of pure fungal strain of *Phialophora lignicola*

Agar (15 g/L) was used for the solidification of the culture media. A moist cellophane membrane (P 400, Visella Oy, Valkeakoski, Finland) was used on the agar medium to prevent the fungus penetrating into the agar medium. One to three plugs, each 5 mm in diameter and cut from the margin of a 1-month-old fungal colony were then placed on the cellophane membrane and cultivated in the dark at room temperature.

The fungal mass was collected after growth of four weeks and stored refrigerated at -20°C until careful grinding in mortar. The ground fungus was divided into aliquots in polypropylene test tubes, weighed, and refrigerated at -80°C before further processing. One aliquot for each fungal sample was weighed on a plastic Petri dish and dried at 45°C to determine the percentage of moisture of the samples (Viitala *et al.*, 2011).

2.2.4. Extraction of fungal culture

The extraction of the fungal mass was performed by deionized water extraction. The fungal samples were first vortexed rigorously for 1-2 minutes with 5 mL of deionized water per 1-3 g of fungal mass in sealed test tubes. Thereafter, the extraction tubes were incubated in a water bath of 95°C to 100°C for 15 minutes, cooled in an ice-water bath, and vortexed.
rigorously for 1-2 minutes. In the end, the tubes were centrifuged 7000 per g for 10 minutes at 4°C.

The supernatants of the extractions were poured off the fungal pellets before next extraction cycle. There were 2-3 extraction cycles for each sample. The supernatants of the extractions were poured off the fungal pellets before next extraction cycle. There were 2-3 extraction cycles per each sample. The supernatants (2-3 x 5 mL) were combined, mixed thoroughly and centrifuged again as before. The final supernatants were filtered with a nylon syringe filter (diameter 25 mm, pore size of 0.2 μm). A known volume (e.g. 10 mL) from each filtrate was dried using a vacuum centrifuge at 25-45°C and dissolved thereafter in Dulbecco’s Modified Eagle Medium DMEM/F-12 (Ham) 1 X (1:1, Gibco). The rest of the filtered supernatants were divided into aliquots and refrigerated at -80°C.

The samples dissolved in DMEM/F-12 were sterilized in a laminar flow chamber with PES syringe filters (diameter 25 mm, pore size 0.1 μm). Thereafter, these filtrates were used for bioactivity tests including, but not limited to, the cell model of age related macular degeneration (Uusitalo et al., 2013; Viitala et al., 2011).

2.2.5. Bioactivity test on human retinoplastic endothelium cells against oxidative stress by extracts of root-associated fungi

Bioactivity tests with RPE cells were conducted in the University of Tampere by the research group of prof. Hannu Uusitalo. Human ARPE-19 retinal pigment epithelial cells (RPE) were obtained from American Type Culture collection (USA). The cells were grown to confluency in a standard incubator in appropriate Dulbecco’s MEM/Nut MIX F-12 medium (Gibco, UK). Oxidative stress was applied to the cells (1 x 10⁴ cells/well) by using various concentrations of H₂O₂. Cytotoxicity was evaluated by two cytotoxicity tests, WST-1 test. WST-1 test reagent was purchased from Roche, Basel, Switzerland. WST-1 test is based on the cleavage of the tetrazolium salt WST-1 (4-*3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate, pale red) to formazan (dark red) by various mitochondrial dehydrogenase enzymes (Fig. 10). The mean optical density values corresponding to the non-treated controls were taken 100%. The results were expressed as percentages of the optical density of treated vs. untreated controls. Dose-response curves were drawn from the results expressed as mean ± standard error of mean (SEM) by GraphPad
Prism software (GraphPadTM, San Diego, US) using non-linear regression analysis. The EC₅₀ values, the concentrations of H₂O₂ and extracts that decreased the WST-1 reduction values to 50% of the controls, were determined when possible from the dose-response curves. The statistical significance of the differences between the cultures exposed to H₂O₂ and tested extracts without or with serum was determined with the Student’s two-tailed t-test (GraphPad Prism). Differences were considered significant when P< 0.05 (Uusitalo et al., 2013; Viitala et al., 2011).

![Figure 10. Cleavage of the tetrazolium salt WST-1 to Formazan by mitochondrial dehydrogenase.](image)

### 2.2.6. Fractionation by preparative HPLC

Fractionation of the extract from the fungal mass of *P. lignicola* was carried out in Like by using preparative HPLC-DAD. A Shimadzu Prominence Liquid Chromatograph system UFLC system with a LC-20AP pumps, degasser, autosampler, column oven and a Prominence Photo diode array SPD-M20A detector (Shimadzu, USA). The HPLC separation was performed in a Waters XBridge C18 reverse-phase column (4.6 x 150 mm, 5 μm) (Waters Corporation, Milford, MA, USA), using with H₂O: Methanol gradient elution (0.01 min, 100:0; 1.50 min 100:0; 9.50 min, 50:50; 10.00 min, 50:50; 12.00 min, 100:00) at a flow rate of 15 mL/min during 15 min. The UV profiles of eluates were monitored with the DAD over the range of 220–400 nm. Detection was carried out at 220, 260, 280 and 300 nm. The injection volume of the extract solution was 100 μL. The same procedure was used for the determination of the retention time of L-arginine hydrochloride by using a standard solution of 0.01 M of this reagent.
2.2.7. Mass spectrometry (MS)

2.2.7.1. Electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS)

For the ESI-TOF MS analysis, a Bruker Daltonics microTOF-Q I (Bruker Daltonics, Bremen, Germany) was used. Fraction samples from the P. lignicola were diluted in 100 μL of HPLC-grade H₂O. An aliquot of each (1 μL) was diluted in 1000 μL of H₂O: Acetonitrile (1:1). Then 200 μL of each solution was injected to the equipment. The data was processed by means of DataAnalysis 3.2 (Bruker Daltonics, Bremen, Germany). The positive ion ESI-TOF MS analysis was conducted using source conditions as follows: scan range of 0 - 3000 m/z; dry temperature, 180 °C; Nitrogen was used as the drying gas, 4.0 L/min; nebulizer, 0.4 bar. Voltages used were: nebulizer end plate 500 V, capillary exit 100 V and capillary potential 4.5 kV.

2.2.7.2. Liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS")

No sample preparation was required other than pouring samples into vials and placing them in the correct position of the HPLC autosampler. Hewlett-Packard Series 1100 HPLC coupled to a Bruker Esquire 3000 plus ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) was used with an Atlantis C18 (Waters, Ireland) column with 3 μm particle size and dimensions of 2.1 x 150 mm. Sample injection volume was 10 μL with flow rate 0.25 mL/min creating a back pressure of 280 bar. The overall run time was 31 minutes, 27 minutes for the run and 4 minutes post run to allow the solvent ratios to realign and to flush the column. The two solvent mixes used were MeOH with 0.1 % acetic acid and H₂O with 0.1 % acetic acid and their mixing ratios were optimized to achieve desirable separation.

The MS instrument was connected to the HPLC system outlet via peek tubing and a T splitter to direct 1/4 of the eluate to the MS and the remaining 3/4 parts to waste. The HPLC coupled to the MS instrument was controlled with HyStar 1.2 software (Bruker Analytik, Rheinstetten, Germany). Data were processed by means of DataAnalysis 3.2 (Bruker Daltonics, Bremen, Germany). Nitrogen was used as the drying gas at 7.5 L/min at 300 °C and as the nebulizer gas with a pressure of 20 psi. Voltages used were: nebulizer end plate 500 V, capillary exit 160 V and capillary 4000 V. The mass spectrometer was used in positive ion mode with a scan range of 50 - 2000 m/z HPLC-ESI-MS-MS analysis was
completed for the two most intense m/z values of the original MS spectrum for each chromatographic peak.

2.2.8. Nuclear magnetic resonance spectroscopy (NMR)

The NMR analysis were carried out at 11.75 T using Bruker Avance III 500 a NMR spectrometer. The NMR spectrometer was controlled with TopSpin 1.3 (Bruker, Karlsruhe, Germany). Each sample was dissolved in 50 μL of D₂O and 40 μL of the aliquots were transferred into 1.7 mm NMR microtubes for one- and two dimensional NMR measurements.

All NMR spectra were acquired at 300°C. The ¹H-NMR spectra were recorded with water-solvent peak suppression. ³¹P-NMR and two-dimensional NMR spectra were recorded using standard pulse programmes (Correlation Spectroscopy (COSY), Total Correlation Spectroscopy (TOCSY), Heteronuclear Single-Quantum-Coherence (HSQC) and Nuclear Overhauser Effect Spectroscopy (ROESY) and ³¹P-¹H (Phosphorus-proton correlation) . ¹H chemical shifts was referenced to residual solvent signals of D₂O, δ (¹H) 4.79 ppm. The observed chemical shift (δ) values are given in ppm and the coupling constants (J) in Hz.
3. RESULTS AND DISCUSSION

3.1. Bioactivity test results for fractions from the endophytic fungi *Phialophora lignicola*

The extract of *Phialophora lignicola* protected hRPE cells against oxidative stress statistically significantly (Fig. 11), showing that the level of protection against oxidative stress was dose dependent (detailed information is provided in Uusitalo et al., 2013). Quercetin (QUE) was used as control.

![Figure 11. Protection level given by P. lignicola (Uusitalo et al., 2013).](image)

3.2. Chromatographic fractionation

The endophytic fungal strain, *Phialophora lignicola* was isolated from the roots of *Pinus sylvestris* growing in Finland. The pure fungal strain was cultivated on a modified Hagem medium. 184.7 g. of fungal mass was collected and extracted with deionized water at 95-100°C. The yield of the extraction was 2.34% (4.372 g.) and after Strata-x-SPE purification yield was 0.67% (1.2494 g.). Strata-x-SPE purifies the extract by removing unwanted contaminants such as phospholipids (Fig. 12). Furthermore, after subjecting the preliminary fractions to biological screening assay (H₂O₂ scavenging), it was determined that the bioactivity appears in the region of the chromatogram appearing at 0.65 – 1.20 min. (Fig. 13). This peak was fractionated by preparative HPLC yielding 12 fractions, where fractions 6 and 7 were the ones that held the bioactivity (Fig. 14).
Figure 12. Fraction yields after chromatographic fractionation.

Figure 13. Chromatogram of aqueous extract of *P. lignicola*.
3.3. Nuclear magnetic resonance spectroscopic analysis

$^1$H NMR spectra of the 12 fractions yielded in the fractionation of the bioactive peak were obtained using a 500 MHz NMR spectrometer. 2D NMR spectra were examined for the bioactive fraction F6 and F7. Experiments and time for each sample is given in Table 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Experiment</th>
<th>Experiment time</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>$^1$H NMR</td>
<td>12 min.</td>
</tr>
<tr>
<td>F2</td>
<td>$^1$H NMR</td>
<td>12 min.</td>
</tr>
<tr>
<td>F3</td>
<td>$^1$H NMR</td>
<td>12 min.</td>
</tr>
<tr>
<td>F4</td>
<td>$^1$H NMR</td>
<td>12 min.</td>
</tr>
<tr>
<td>F5</td>
<td>$^1$H NMR</td>
<td>12 min.</td>
</tr>
<tr>
<td>F6</td>
<td>$^1$H NMR</td>
<td>12 min.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 h.</td>
</tr>
<tr>
<td></td>
<td>COSY</td>
<td>6 h.</td>
</tr>
<tr>
<td>------------------</td>
<td>------------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>TOCSY</td>
<td>48 h.</td>
</tr>
<tr>
<td></td>
<td>HSQC</td>
<td>6 h.</td>
</tr>
<tr>
<td></td>
<td>ROESY</td>
<td>5 h.</td>
</tr>
<tr>
<td></td>
<td>31P-NMR</td>
<td>4 h.</td>
</tr>
<tr>
<td></td>
<td>31P-1H</td>
<td></td>
</tr>
<tr>
<td>F7</td>
<td>1H NMR</td>
<td>12 min.</td>
</tr>
<tr>
<td></td>
<td>COSY</td>
<td>3 h.</td>
</tr>
<tr>
<td></td>
<td>TOCSY</td>
<td>6 h.</td>
</tr>
<tr>
<td></td>
<td>HSQC</td>
<td>48 h.</td>
</tr>
<tr>
<td></td>
<td>ROESY</td>
<td>6 h.</td>
</tr>
<tr>
<td></td>
<td>31P-NMR</td>
<td>5 h.</td>
</tr>
<tr>
<td></td>
<td>31P-1H</td>
<td>4 h.</td>
</tr>
<tr>
<td>F8</td>
<td>1H NMR</td>
<td>12 min.</td>
</tr>
<tr>
<td>F9</td>
<td>1H NMR</td>
<td>12 min.</td>
</tr>
<tr>
<td>F10</td>
<td>1H NMR</td>
<td>12 min.</td>
</tr>
<tr>
<td>F11</td>
<td>1H NMR</td>
<td>12 min.</td>
</tr>
<tr>
<td>F12</td>
<td>1H NMR</td>
<td>12 min.</td>
</tr>
</tbody>
</table>

3.3.1. 1D NMR
3.3.1.1. 1H NMR

The 1H NMR spectra of fractions 1 to 12 are given in Fig. 15. The area between δH 5.5 ppm and δH 3.2 ppm appears to indicate the presence of polysaccharide(s). These signals are stronger in F6 to F12. Signals around δH 3.60, 1.75, 1.50 and 3.10 ppm are characteristic of L-arginine (Fig. 16). A singlet appearing between δH 1.85-1.90 ppm indicates the presence of an acetyl group, which is predominant from F6 to F12. Fractions 1 to 3 shows the high intensity signals belonging to L-arginine, in comparison with the low intensity of the polysaccharide region signals (δH 5.50-3.20 ppm).
Figure 15. Stacked NMR spectra of all fractions.

![Figure 16. L-arginine.](image)

L-arginine $^1$H NMR was compared with the $^1$H NMR spectra of F1, F6, F7 and F12, revealing the signals with same multiplicities as those of L-arginine, present in these fractions (Fig. 17). Clear signals and multiplicities of L-arginine for F6 and F7 are shown in Fig. 18 and 19. A proton triplet at $\delta_H$ 3.60 or 3.62 was assigned to H-2, a proton triplet at $\delta_H$ 3.09-3.10 was assigned to H-5, proton multiplicity at $\delta_H$ 1.75-1.76 was assigned to H-3 and proton multiplicity at $\delta_H$ 1.53 was assigned to H-4.
Figure 17. Fractions 1, 6, 7 and 12 compared to $^1$H NMR of L-arginine.

Assignments of the signals’ position of L-arginine in F6 and F7, integration, type of multiplicity and coupling constant ($J$) are given in Tab. 2.

Table 2. $^1$H NMR spectral data for F6 and F7 (L-arginine signals) at 500 Mhz.

<table>
<thead>
<tr>
<th>Position</th>
<th>F6</th>
<th>F7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\delta_H$</td>
<td>$\delta_H$</td>
</tr>
<tr>
<td></td>
<td>(D$_2$O)</td>
<td>(D$_2$O)</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>3.60 ($1H, t, J=6.33$ Hz)</td>
<td>3.62 ($1H, t, J=6.29$ Hz)</td>
</tr>
<tr>
<td>3</td>
<td>1.75 ($2H, m$)</td>
<td>1.76 ($2H, m$)</td>
</tr>
<tr>
<td>4</td>
<td>1.53 ($2H, m$)</td>
<td>1.53 ($2H, m$)</td>
</tr>
<tr>
<td>5</td>
<td>3.09 ($2H, t, J=6.94$ Hz)</td>
<td>3.10 ($2H, t, J=6.89$ Hz)</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*All spectra were recorded in D$_2$O. Chemical shifts are given by residual peak of D$_2$O at 4.79 ppm.
Figure 18. Arginine signals from F6.

Figure 19. Arginine signals from F7.
3.3.1.2. $^{31}$P NMR

$^{31}$P is the only naturally occurring isotope of phosphorus. Phosphorus is targeted in this study as it is found in nature as inorganic forms of phosphorus and in organic phosphines, phosphites, phosphonium salts, phosphorus ylides, etc. Low intensity signals of $^{31}$P were obtained for F6 and F7. This showed that any phosphorus of inorganic or organic nature is a minor or trace component of the samples. Signals obtained for $^{31}$P NMR are given in Tab. 3. The chemical shift range for $^{31}$P is rather larger and generalization is unreliable. The only signals that have been characterized are -2.03 (F6) and -1.99 (F7) which may correspond to O=P (OR)$_3$ (which appears from 0 to -20 ppm). Further studies are not necessary because phosphorus compounds in the bioactive samples appear to be less noteworthy.

<table>
<thead>
<tr>
<th>Table 3. $^{31}$P NMR signals data for F6 and F7.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>δP</td>
</tr>
<tr>
<td>139.83, 103.61, -2.03, -21.69</td>
</tr>
</tbody>
</table>

Figure 20. $^{31}$P-NMR signals from F6.
3.3.2. 2D NMR

The complete assignment of protons corresponding to L-arginine H-2, H-3, H-4 and H-5 was clearly evident in the COSY spectrum of both F6 and F7 samples. In the COSY spectrum the less shielded proton H-2 exhibited correlation with H-3, H-3 correlates with H-4 and finally H-4 correlates with H-5 (Tab. 4) (Fig. 22, 23). This correlation was confirmed by TOCSY which demonstrated the continuous correlation for the 4 type of protons from H-2 to H-5 (Fig. 24, 25).

Table 4. COSY spectral data for F6 and F7 (L-arginine signals) at 500 Mhz.

<table>
<thead>
<tr>
<th>Position</th>
<th>F6</th>
<th>F7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>COSY</td>
<td>COSY</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>2,4</td>
<td>2,4</td>
</tr>
<tr>
<td>4</td>
<td>3,5</td>
<td>3,5</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*All spectra were recorded in D2O. Chemical shifts are given by residual peak of D2O at 4.79 ppm.
Figure 22. COSY of fraction 6.

Figure 23. COSY of fraction 7.
Figure 24. TOCSY fraction 6.

Figure 25. TOCSY fraction 7.
The signals appearing from $\delta_H$ 3.20 to 5.50 ppm that correspond to the saccharide region are not clearly visible in COSY nor TOCSY. This may be due to the presence of a mixture of polysaccharides and low concentration of the samples. HSQC and ROESY experiments for F6 and F7 were not suitable for further analysis due to the lack of signals. A low intensity signal was obtained in $^{31}$P-$^1$H NMR in both F6 and F7 samples. This may be a correlation between a proton appearing in low intensity in $^1$H NMR of both F6 and F7 ($\delta_H \sim$5.25) with the phosphorus ($\delta_P$ -2.03, F6; $\delta_P$ -1.99, F7) (Fig. 26, 27). Once again, the presence of this phosphoric compound appears not to be relevant in the samples studied and it does not require further studies.

![Figure 26. $^{31}$P-$^1$H Heteronuclear correlation of fraction 6.](image1)

![Figure 27. $^{31}$P-$^1$H Heteronuclear correlation of fraction 7.](image2)
3.4. Electrospray ionization time-of-flight mass spectrometry analysis (ESI-TOF MS)

As shown in Figures 28 to 39, ESI-TOF MS of all spectra from fractions 1 to 12 showed characteristics \( m/z \) ions 175, 337, 425, 561, 675, 769 and 811 (Tab. 5). Knowing that the exact mass of L-arginine is 174.11168 Da and the evidence of its occurrence in the NMR analysis, 175 \( m/z \) was identified as the molecular ion \([M+H]^+\) of L-arginine. 337 \( m/z \) appears to be an arginine conjugate with a hexose. Hexoses’ molecular weight is 180.063385 Da and when a hexose is linked to arginine it loses a \( H_2O \) molecule (18 Da). The difference between 175 \( m/z \) and 337 \( m/z \) is 162 \( m/z \), supporting the possible presence of a unit of saccharide (Fig. 33 and 34). The molecular ions \( m/z \) 425, 769 and 811 were identified as possible chitin oligomers based on literature survey. 425 \( m/z \) could be di-\(N\)-acetylchitobiose which is a dimer of \( \beta\)-1,4-linked glucosamine units (Fig. 40). The chitin oligomer di-\(N\)-acetylchitobiose was detected from the commonly known fungus Aspergillus niger as a sodium adduct \([M+Na]^+\) 447 \( m/z \) by MALDI-TOF MS (Kittur et al., 2005). By MALDI-TOF-MS analysis of a mixture of hetero-chitosan oligomers obtained after enzymatic degradation of chitosan, 769 and 811 \( m/z \) were detected as sodium adducts (Fig. 41a,b) (Se-Kwon Kiro et al., 2010). The singlet appearing between \( \delta_H \) 1.85- 1.90 ppm indicates the presence of an acetyl group, which is predominant from F6 to F12 and is present in the structure of these chitin oligomers (Fig. 17). The other minor detected \( m/z \) ions in ESI-TOF MS remain unidentified.

Chitin is more widely distributed in the microbial world than chitosan and can be found in fungi (Gooday 1990). Fungal cell walls and septa of Ascomycetes, Zygomycetes, Basidiomycetes, and Deuteromycetes contain mainly chitin (Peter 2002). \( P. \) lignicola belongs to the division Ascomycetes.

Furthermore, the obvious similarities in the obtained ions \( m/z \) in each fraction from F1 to F12 is evidence that the isolation and fractionation procedure should be improved to avoid overlapping between compounds, as the retention time for the compound(s) of interest is very short (less than 1 min.). By changing the type of column to one that retains the compound(s) of interest for a longer time could improve this crucial step in the present study.
Table 5. Molecular weights found in the fractions of *P. lignicola* by ESI-TOF MS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Significant ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>175, 337, 425, 561, 675, 769, 811</td>
</tr>
<tr>
<td>F2</td>
<td>175, 337, 425, 561, 675, 811</td>
</tr>
<tr>
<td>F3</td>
<td>175, 337, 425, 561, 675, 811</td>
</tr>
<tr>
<td>F4</td>
<td>175, 337, 425, 561, 675, 769, 811</td>
</tr>
<tr>
<td>F5</td>
<td>175, 337, 425, 457, 561, 675, 769, 811</td>
</tr>
<tr>
<td>F6</td>
<td>175, 337, 425, 561, 675, 769, 811</td>
</tr>
<tr>
<td>F7</td>
<td>175, 337, 425, 561, 675, 779, 811</td>
</tr>
<tr>
<td>F8</td>
<td>175, 337, 397, 561, 739, 811</td>
</tr>
<tr>
<td>F9</td>
<td>175, 337, 425, 561, 675, 811</td>
</tr>
<tr>
<td>F10</td>
<td>175, 337, 425, 561, 675, 779, 811</td>
</tr>
<tr>
<td>F11</td>
<td>175, 337, 425, 561, 675, 769, 811</td>
</tr>
<tr>
<td>F12</td>
<td>175, 337, 425, 561, 675, 769, 811</td>
</tr>
</tbody>
</table>

Figure 28. ESI-TOF MS of fraction 1.
Figure 29. ESI-TOF MS of fraction 2.

Figure 30. ESI-TOF MS of fraction 3.
Figure 31. ESI-TOF MS of fraction 4.

Figure 32. ESI-TOF MS of fraction 5.
Figure 33. ESI-TOF MS of fraction 6 (Bioactive).

Figure 34. ESI-TOF MS of fraction 7 (Bioactive).
Figure 35. ESI-TOF MS of fraction 8.

Figure 36. ESI-TOF MS of fraction 9.
Figure 37. ESI-TOF MS of fraction 10.

Figure 38. ESI-TOF MS of fraction 11.
Figure 39. ESI-TOF MS of fraction 12.

Figure 40. di-\textit{N}-acetylchitobiose.

Figure 41. a) 769 m/z [M+Na]⁺ and b) 811 m/z [M+Na]⁺.
3.4.1. Isotopic pattern calculation of $175 \, m/z \text{ and } 337 \, m/z$

The isotopic pattern of ions obtained in MS helped to establish the elemental component formula on each ion. DataAnalysis 3.2 software (Bruker Daltonics, Bremen, Germany) provides a method for predicting the elemental component formulas of molecular ions using isotope patterns. The method is based on the estimation of the intensities of the isotope peaks to calculate the maximal and minimal numbers of each element. The method usually includes the following processes: candidates generation, which exhaustively enumerates all possible elemental component formulas corresponding to a given mass and tolerant mass error and filtering, which excludes formulas violating chemical constraints (Zhang, et al., 2005). The obtained results for the isotopical pattern $175 \, m/z$ yielded 5 possible candidates (Tab. 6). $C_6H_{15}N_4O_2 \left[M+H\right]^+$, matched and agreed with the previous results of NMR analysis as the molecular formula of L-arginine is $C_6H_{14}N_4O_2$.

**Table 6.** Molecular formula generation based of isotopic pattern calculation of $175 \, m/z$.

<table>
<thead>
<tr>
<th>Sum Formula</th>
<th>Sigma</th>
<th>$m/z$</th>
<th>Err (ppm)</th>
<th>Mean Err (ppm)</th>
<th>N rule</th>
<th>e-</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_9H_{10}O_3$</td>
<td>0.15</td>
<td>175.1329</td>
<td>37.32</td>
<td>41.59</td>
<td>ok</td>
<td>even</td>
</tr>
<tr>
<td>$C_8H_{17}N_1O_3$</td>
<td>0.16</td>
<td>175.1203</td>
<td>-34.49</td>
<td>-30.52</td>
<td>odd</td>
<td></td>
</tr>
<tr>
<td>$C_7H_{17}N_3O_2$</td>
<td>0.19</td>
<td>175.1315</td>
<td>29.65</td>
<td>32.65</td>
<td>ok</td>
<td>odd</td>
</tr>
<tr>
<td>$C_6H_{15}N_4O_2$</td>
<td>0.20</td>
<td>175.1190</td>
<td>-42.16</td>
<td>-39.62</td>
<td>ok</td>
<td>even</td>
</tr>
<tr>
<td>$C_5H_{15}N_6O_1$</td>
<td>0.20</td>
<td>175.1302</td>
<td>21.99</td>
<td>23.30</td>
<td>ok</td>
<td>even</td>
</tr>
</tbody>
</table>
In the case of 337 m/z the obtained results for the isotopical pattern yielded 5 possible candidates (Tab. 7). After reviewing the literature and contrasting the chemical structures found in the survey with the NMR and MS analysis, C_{12}H_{25}N_{4}O_{7}[M+H]^+ appears to be the most probable candidate. C_{12}H_{26}N_{5}O_{7} seems to be a hexose-arginine conjugate. The following compounds found in the literature share the same molecular weight and structural characteristics: D-arginine D-galactos-6'-yl ester (Melisi et al., 2006), L-arginine D-galactos-6'-yl ester (Melisi et al., 2006), D-fructose-L-arginine (Wang et al., 2008), N^2-β-D-fructopyranos-1-yl-arginine (Jang, 2012), N^{ω}-glucopyranosyl-L-arginine (Hamamura 1956), α-D-Fructose-L-arginin (Heyns & Noack, 1962) and α-D-Glucose-L-arginin (Heyns,K. & Noack,H., 1962).
Table 7. Molecular formula generation based of isotopic pattern calculation of 337 m/z.

<table>
<thead>
<tr>
<th>Sum Formula</th>
<th>Sigma</th>
<th>m/z</th>
<th>Err (ppm)</th>
<th>Mean Err (ppm)</th>
<th>N rule</th>
<th>e-</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₉H₁₇N₁₄O₁</td>
<td>0.04</td>
<td>337.1704</td>
<td>-1.54</td>
<td>8.50</td>
<td>ok</td>
<td>even</td>
</tr>
<tr>
<td>C₁₀H₂₃N₇O₆</td>
<td>0.04</td>
<td>337.1704</td>
<td>-1.53</td>
<td>3.00</td>
<td>-</td>
<td>odd</td>
</tr>
<tr>
<td>C₁₁H₁₉N₁₁O₂</td>
<td>0.03</td>
<td>337.1718</td>
<td>2.44</td>
<td>8.00</td>
<td>-</td>
<td>odd</td>
</tr>
<tr>
<td>C₁₁H₂₉O₁₁</td>
<td>0.04</td>
<td>337.1704</td>
<td>-1.51</td>
<td>-2.50</td>
<td>ok</td>
<td>even</td>
</tr>
<tr>
<td>C₁₂H₂₅N₄O₇</td>
<td>0.03</td>
<td>337.1718</td>
<td>2.46</td>
<td>2.50</td>
<td>ok</td>
<td>even</td>
</tr>
</tbody>
</table>

Figure 43. Isotopic peaks of 337 m/z taken from fraction 6.
3.5. Liquid chromatography electrospray ionization mass spectrometry analysis (LC-MS)°

The LC-MS analysis of samples F6 and F7 confirmed the ions 175 m/z and 337 m/z (Fig. 44 and 45). 99 m/z is a contaminant from the column used and appears at 1.3 min which is more visible in the LC-MS of F6. The compound of interest appears at 1.5 min in F6 and 1.4 min in F7. The type of column used was Atlantis C18 (Waters, Ireland), the same used for the fractionation of the samples. The separation of components was not accomplished in this LC-MS and would require a modification in the method, beginning with the change of column type for one that retains for a longer time the compound of interest to obtain better MS analysis.

Figure 44. LC-MS of fraction 6 at 1.5 min.
LC-MS/MS analysis was done for F6 and F7. Tab.8 shows the signals obtained for the fragmentation of L-arginine. 337 m/z low intensity and fragmentation could not be observable in any of the samples. The fragmentation of L-arginine agrees with the preceding observations (Shek et al., 2006). NH3 (-17 Da) is a loss from the side chain to create an ion at 158 m/z. Another loss is the complete cleavage of the guanidinyl group HN=C(NH₂)₂ (-59 Da) from 175 m/z; and forming 116 m/z in this reaction a cyclic ion, protonated proline is formed. Additionally, the ion at 130 m/z originates from the neutral losses of NH3 (-17 Da) and CO (-28 Da). Furthermore signal at 70 m/z is formed by the losses of H₂O (-18 Da) and CO (-28 Da) and 60 m/z corresponds to the protonated species of the guanidinyl group [H₂N=C(NH₂)₂]⁺. These results in combination with the previous analysis confirmed the identity of L-arginine in the samples F6 and F7.
Table 8. Fragmentations peak characteristic of L-arginine.

<table>
<thead>
<tr>
<th>Sample</th>
<th>[M+H]</th>
<th>[M+H-17]</th>
<th>[M+H-45]</th>
<th>[M+H-59]</th>
<th>[M+H-105]</th>
<th>60 m/z (Base peak)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F6</td>
<td>175</td>
<td>158</td>
<td>130</td>
<td>116</td>
<td>70</td>
<td>+</td>
</tr>
<tr>
<td>F7</td>
<td>175</td>
<td>158</td>
<td>130</td>
<td>116</td>
<td>70</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 46. LC-MS-MS spectra of fraction 6 at 1.4 min.

Figure 47. LC-MS-MS spectra of fraction 7 at 1.5 min.
3.6. HPLC retention time of L-arginine

L-arginine is a common metabolite in fungi and a nitrogen molecule. The synthesis of arginine in fungi has three main components: synthesis of ornithine, synthesis of carbamoyl phosphate, and the transformation of these two into arginine. Arginine is a relatively good nitrogen source and it can be hydrolyzed to ornithine and urea. Urea is converted to carbon dioxide and ammonia and ornithine to glutamate (Davis 1986).

The retention time of the standard compound, L-arginine hydrochloride 0.01M, was 1.8 minutes. Compared to the bioactive fraction in the aqueous extract of *P. lignicola* showed in the chromatogram of the whole extract (Fig. 14), the peak of interest appears between 0.65 min and 1.20 min. Although the retention times are different between the bioactive peak and L-arginine, there could be a poor separation of L-arginine produced by the fungus and the compound(s) of interest. This overlapping could be overcome by, instead of using a C18 reverse-phase column in the purification procedure, using columns that retain the compounds of interest (e.g. HILIC silica) for a longer time and isolate properly the content of L-arginine in the samples.

![Figure 48. LC-MS-MS spectra of fraction 7 at 1.5 min.](image-url)
3.7. The occurrence hexose-arginine conjugate (337 m/z)

N-substituted 1-amino-1-deoxyketoses are known as Amadori compounds, which are originated in the first phase of the Maillard reaction by and Amadori rearrangement representing an important class of Maillard intermediates (Hodge1955; Wrodnigg & Eder 2001). The Maillard reaction is a non-enzymatic interaction between reducing sugars and amino acids (Ulrich & Cerami, 2001).

Fructosyl-amino acids, such as fructosyl-arginine were found in the fungal cells, yeast and Aspergillus terreus. MALDI-TOF-MS experiments detected m/z 337 in the mycelial extracts of these fungi, which indicated the presence of the Amadori compound derived from arginine and glucose followed by Amadori rearrangement (Yoshida et al., 2004). Characterizing Amadori compounds is a difficult task, because they are numerous and their structures are similar. Electrospray ionization tandem mass spectrometry could be the best option for characterizing these organic molecules. So far little has been developed on research of mass spectrometry of Amadori compounds under electrospray ionization. ESI-MS/MS experiments were done in 18 Amadori compounds, including fructosyl-arginine (337 m/z, [M+H]⁺), determining characteristic fragment ions at m/z [M+H-18]⁺, [M+H-36]⁺, [M+H-46]⁺, [M+H-54]⁺, [M+H-64]⁺, [M+H-82]⁺, [M+H-84]⁺, [M+H-150]⁺ and [M+H-162]⁺. It was established that these were typical fragmentations pathways rules for Amadori compounds and an excellent method for the study of Amadori compounds (Wang 2008).

Furthermore, Na-(1-Deoxy-D-fructos1-yl)-L-arginine (Fru-Arg) found in aged garlic extract was identified as a major antioxidant which was comparable to that of ascorbic acid, scavenging hydrogen peroxide totally at 50 μmol/L and 37% at 10 μmol/L. The aged garlic extract may contain Maillard reaction products because garlic contains considerable amounts of amino acids and reducing sugars (Ryu et al., 2001). The hexose-arginine detected in the following studied by NMR analysis and ESI-MS had the same characteristics as these Amadori compounds. The fungal origin and the antioxidant activity are also matches. For further studies, it would be necessary to improve the isolation and purification of this Hexose-arginine to determine the complete structure by NMR and MS experiment, like ESI-MS/MS method to identify and detect the characteristic fragment ions of this compound.
4. CONCLUSIONS

Endophytic fungi, microorganism that live internally in the tissues of living plants are relatively unstudied and have proved to be an alternative source of novel natural products. These secondary metabolites show specific biological activities and have shown to be useful for novel drug discovery. The aim of this study was the characterization of the antioxidant compounds of the pure strain of the endophytic fungus, *Phialophora lignicola* found in root association in 8 years old seedlings of *Pinus sylvestris*, obtained from the Finnish peatland (Haukilampi). The aqueous extract of *Phialophora lignicola* showed antioxidative bioactivity on the protection of human retinoplastic endothelium cells (hRPE) against oxidative stress.

Samples studied were obtained by preparative HPLC fractionation. Structure elucidation of the natural products in the fractions obtained was performed using ESI-TOF MS, LC-MS$^8$ and one and two-dimensional NMR analysis.

The $^1$H NMR experiments indicate the presence of L-arginine and polysaccharides. The 2D NMR, COSY and TOCSY revealed clearly the correlation signals for L-arginine. However, due to the isolation and purification method they also expose that the active fractions were composed of a mixture. $^{31}$P NMR demonstrated the existence of phosphorus compounds but in extremely low quantities. Due to the low concentration of samples no further 2D NMR experiments were achievable.

ESI-TOF MS revealed the presence of L-arginine at 175 m/z, a hexose-arginine conjugate at 337 m/z, and probably chitin oligomers at 425, 769 and 811 m/z. Additionally, by using the isotopic pattern calculation, the molecular formulas: C$_6$H$_{15}$N$_4$O$_2$ for 175 m/z and C$_{12}$H$_{25}$N$_4$O$_7$ and for 337 m/z were obtained. LC-MS was performed to detect the peaks corresponding to L-arginine and the hexose-arginine. Furthermore, LC-MS-MS confirmed definitely the existence of L-arginine based on its characteristic fragmentation ions. Due to the absence of signal for 337 m/z in MS/MS, no fragmentation ions were obtained for these ion.

According to the literature survey, the hexose-arginine conjugate might be responsible for the antioxidant activity. For further studies, it is necessary to improve the purification and isolation method and to obtain more concentrated samples for NMR and MS analysis.
5. LIST OF REFERENCES

2. Bacon, C. W. and White, J. F. in Microbial endophytes, Marcel Dekker, Inc., New York, USA, 2000, chapter 1, p. 4-5.
19. Freeman, E.M. *Phil. Trans. R. Soc. B* (1904) 196.
24. Guerin, P. *J. Botanique.* 12 (1898)
27. Harper JK, Arif AM, Ford EJ, Strobel GA, Porco JA, Tomer DP,


