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„Investigation of antibiotically active compounds produced by newly isolated endophytic fungi *Aspergillus westerdijkiae, Akanthomyces muscarius* and *Alternaria* sp.“

verfasst von / submitted by

Havva Işıkoğlu

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1. Zusammenfassung/ Abstract

1.1. Zusammenfassung

Durch die Zunahme der Antibiotikaresistenzen wird es immer schwieriger, wirksame Antibiotika gegen Infektionskrankheiten bereitzustellen. Die Suche nach neuen Wirksubstanzen ist zeitaufwändig und teuer und stellt eine große Herausforderung für die Pharmaindustrie dar.

Daher werden dringend neue Quellen für Antibiotika gesucht, um den Bedarf an neuen Wirksubstanzen decken zu können. In dieser Hinsicht sind Endophyten eine vielversprechende neue Quelle. Endophyten sind kommunikative Mikroorganismen, die im Inneren von Pflanzen leben und daher ein hohes Potential für interaktive Substanzen besitzen. Sie produzieren zahlreiche sekundäre Metabolite, unter anderem auch antimikrobielle Substanzen.

Aus diesen Gründen wurden in dieser Diplomarbeit Pilzendophyten aus der Medizinalpflanze Edelweiß (*Leontopodium nivale* subsp. *alpinum*) auf ihre antimikrobielle Aktivität gegen verschiedene Testorganismen, wie z.B. *Escherichia coli*, *Bacillus subtilis*, *Sacharomyces cerevisiae*, *Fusarium graminearum* und *Aspergillus niger*, untersucht.

Kulturmedien wurden mit Endophytenreinkulturen beimpft und inkubiert. Die Ansätze wurden mit geeigneten Lösungsmitteln extrahiert und mit Hilfe von Agardiffusionstests auf ihre antibiotische Wirkung getestet. Aktive Extrakte wurden mittels Hochleistungsflüssigkeitschromatografie (HPLC) analysiert und anschließend mithilfe von semi-präparativer HPLC aufgetrennt und fraktioniert, um Reinsubstanzen zu erhalten. Schließlich wurden aktive Substanzen mittels hochauflösende Massenspektrometrie (HRMS) und Kernspinresonanzspektroskopie (NMR) identifiziert.

Fünf aus Edelweiß isolierte Pilzendophyten wurden untersucht, wobei zwei Isolate antibiotische Aktivitäten zeigten. Das Isolat LN733 wurde als *Aspergillus westerdijkiae* und LN303 als *Akanthomyces muscarius* identifiziert.

Das Gesamtextrakt von *A. westerdijkiae* zeigte antibiotische Aktivitäten gegen die Testorganismen *B. subtilis*, *E. coli* und *S. cerevisiae*. Bioassays der Fraktionen wiesen die antibiotische Aktivität gegen *S. cerevisiae* den Fraktionen 9 und 10 zu, während die Fraktionen 12 und 13 Aktivitäten gegen *E. coli* und *B. subtilis* zeigten. Des Weiteren wies Fraktion 16 eine antibiotische Aktivität gegen *B. subtilis* auf. Massenspektrometriesche Untersuchen zeigten, dass in den aktiven Fraktionen v.a.

ungesättigte und gesättigte langkettige Fettsäuren enthalten waren. Untersuchungen der Fraktion 12 mittels HRMS und NMR ergaben, dass Linolsäure für die antibiotische Aktivität gegen *B. subtilis* verantwortlich war.

Das Gesamtextrakt von *A. muscarius* war aktiv gegen *E. coli, S. cerevisiae*, *F. graminearum* und *A. niger*. Mithilfe semi-präparativer HPLC konnte durch Fraktionierung und anschließender Subfraktionierung der aktiven Fraktion eine Reinsubstanz gewonnen werden. NMR Analysen zufolge handelt es sich hierbei um die Substanz Emestrin A. Agardiffusionstest der Reinsubstanz zeigten eine komplette Inhibition bei den Testorganismen *E. coli* und *S. cerevisiae*, während bei den filamentösen Pilzen *F. graminearum* und *A. niger* nur eine partielle Inhibition beobachtet wurde. Aufklärungen mittels HRMS und Analyse der absoluten Konfiguration waren nicht Teil dieser Arbeit.

1.2. Abstract

The increase of antibacterial resistance makes it difficult to provide effective antibiotics against infectious diseases. Furthermore, the search for new active substances is time-consuming and expensive and for these reasons represents a major challenge for the pharmaceutical industry.

Therefore, new sources for antibiotics are being searched to meet the need for new drugs. In this regard, endophytes are a promising source for the discovery of new antibiotics. Endophytes are communicative microorganisms, which are living inside plants and have therefore a high potential for interactive substances. They produce numerous secondary metabolites, including antimicrobial compounds.

In this Diploma Thesis, fungal endophytes from the medicinal plant *Leontopodium nivale* subsp. *alpinum* (Edelweiss) were tested for their antimicrobial activity against various test organisms such *e.g*. *Escherichia coli*, *Bacillus subtilis*, *Sachharomyces cerevisiae*, *Fusarium graminearum* and *Aspergillus niger*.

Media were inoculated and incubated with pure cultures of the endophytes. The cultures were extracted with appropriate solvents and tested for their antibiotic activity by diffusion disc assays. Active extracts were analyzed by high performance liquid chromatography (HPLC) and then fractionated with semi-preparative HPLC to obtain pure substances. Finally, active substances were identified by high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) spectroscopy.

Five isolated endophytes were investigated, whereby two showed an antibiotic activity. The fungus LN733 was identified as *Aspergillus westerdijkiae* and LN303 as *Akanthomyces muscarius*.

The total extract of *A. westerdijkiae* showed antibiotic activity against the test organisms *B. subtilis, E. coli* and *S. cerevisiae*. Bioassays of the fractions showed antibiotic activity against *S. cerevisiae* for fractions 9 and 10, while fractions 12 and 13 showed activity against *E. coli* and *B. subtilis*. Furthermore, fraction 16 had antibiotic activity against *B. subtilis*. Mass spectrometry revealed that in the active fractions unsaturated and saturated long-chain fatty acids were prominent. Analyses of fraction 12 with HRMS and NMR spectroscopy revealed that linoleic acid was responsible for the antibiotic activity against *B. subtilis*.

The total extract of *A. muscarius* was active against *E. coli, S. cerevisiae, F. graminearum* and *A. niger*. By means of semi-preparative HPLC a pure substance could be obtained by fractionation followed by subfractionation of the active fraction.

According to NMR analyzes, the active substance was emestrin A. Diffusion disc assays of the pure substance showed complete inhibition against the test organisms *E. coli* and *S. cerevisiae*, whereas partial inhibition was observed testing against the filamentous fungi *F. graminearum* and *A. niger*. Elucidations using HRMS and analyses of the absolute configuration were not part of this work.

2. Introduction

2.1. *Leontopodium nivale* subsp. *alpinum*

Leontopodium nivale subsp. *alpinum* (Cass.) Greuter belongs to Asteraceae family and is commonly known as “Alpen-Edelweiß”. The plant prefers calcareous soils and grows mostly on grassy and rocky slopes at altitudes up to 3140 m. It is native in the mountains of Balkan, the European Alps, the Tatra and the Pyrenees (Meusel & Jäger, 1992).

L. nivale subsp. *alpinum* is a perennial plant with a plant height of 2-45 cm (Hook, 1993). The leaves are lanceolate and hairy and built a basal rosette. The capitulae is 5 mm long and forms a cyme which is surrounded by the characteristic white upper leaves, which have a tomentuose hairy surface. The blossoms are yellowish- white and are located in the middle of the capitulae central of the radial bracts (Hegi, 1965) (**Figure 1**).

Figure 1: Flowering plant of *Leontopodium nivale* subsp. *alpinum* is shown on its natural habitat. Photograph was taken by Claudia Hager.

In folk medicine this plant was commonly used. Recordings from 1582 reported that for oral use the plant was extracted in wine and then mixed with milk to treat angina and dysentery. For treating breast cancer, it was used topically as a compress (Tabernaemontanus, 1582). Furthermore, extracts were used to treat abdominal aches, bronchitis, angina pectoris, tonsillitis, cancer, dysentery, diarrhea and fever (Hegi, 1935; Bitschnau, 1991; Kiene, 1992; Knechtl, 1992; Pickl-Herck, 1995; Wieser, 1995). In polish folk medicine, it was used against cancer diseases, especially breast cancer (Hartwell, 1968).

Drobner *et al*. (2003) reported about the antibacterial activity of the dichloromethane extracts against *B. subtilis*, *E. coli*, *Staphylococcus aureus* and *S. pyogenes* and *Pseudomonas aeruginosa*. Recent studies of Drobner *et al.* (2004) have shown that *L. nivale* subsp. *alpinum*, in addition, inhibits the leukotriene synthesis which causes the anti-inflammatory effect of the plant.

In Austria, *L. nivale* subsp. *alpinum* is under nature-protection since 1886 and native plant collection is forbidden (Drobner *et al*., 2004). For this reason, plants used within this thesis were harvested with a governmental permission.

2.2. Fungal endophytes

Wilson (1995) gave a basic definition for the term endophyte: “endo” stands for within and “phyte” for plant. In dependence of this definition, endophytes are microorganisms, usually bacteria and fungi, which are living within the plant (Wilson, 1995). However, the most common definition stems from Petrini (1991): Endophytes are microorganisms, which invaded plant tissues at some period of their life cycle without causing any harm or disease for the host.

Endophytes appear in all plant tissues and benefit from the multifarious nutrients of the plant (Petrini and Müller, 1979), in which three different association types between endophyte and host exist. The cohabitation can be either parasitic (the endophyte benefits from the host), mutualistic (the plant benefits from the endophyte) or commensalistic (the endophyte does not affect the host in any way). Increasing tolerance to salt, heat or drought, and increasing resistance against pathogens and diseases are some of the profits the host plants get from endophytes (Rodriguez and Redman, 2008).

Endophytic fungi are widespread. While the host plant feeds and protects the fungus, the endophyte produces bioactive compounds, *inter alia*, to enhance the plants growth and assures its host`s survival in the nature (Gunatilaka, 2006).

Metabolites are products of metabolism and are defined as small molecules. Primary metabolites are present in every organism and are indispensable for growth and molecular function of organisms. In contrary, secondary metabolites are not essential for the survival of the organism, but they have different roles *e.g.* as signaling molecules, differentiation and defensive compounds (Durairaj *et al*., 2018).

Fungi produce many secondary metabolites which are not mandatory for their survival (Nisa *et al.*, 2015), but are essential for the signaling between plant and fungal endophyte. Metabolites that are isolated from endophytes are very divers, including phenols, terpenoids, isocoumarins, steroids, xanthones *etc.* (Schulz and Boyle, 2005).

In all higher plants, fungal endophytes are present universally and they provide an untapped resource for novel bioactive natural products (Strobel, 2003). Starting with the discovery of penicillin from *Penicillium* sp. in 1928 by Sir Alexander Fleming, the

focus of the pharmaceutical industry shifted more and more from plants to microorganism to find new medicinal drugs (Nisa *et al*, 2015).

Regarding this, an important example is the anti-cancer drug Paclitaxel (Strobel, 2002). It is produced by the yew tree species (*Taxus sp.*) and used to treat tissue proliferating diseases. A few years ago, a fungal endophyte, *Taxomyces andreanae*, which is producing Paclitaxel, was discovered and isolated from *Taxus brevifolia* (Strobel, 2003). As a result, it is no longer necessary to harvest the yew tree which is growing slow and is relatively uncommon (Strobel, 2002).

As endophytes provide a resource for novel natural products (Strobel, 2003), fungal endophytes were isolated from *L. nivale* subsp. *alpinum* at the Department of Pharmacognosy for further investigations, of which this Thesis is an aspect.

2.3. Isolated fungal endophytes from *Leontopodium nivale* subsp. *alpinum*

2.3.1. Aspergillus westerdijkiae

Molecular barcoding assigned one of the isolates from *L nivale* subsp. *alpinum* (strain number LN733) to *Aspergillus westerdijkiae* (data provided by Mag. Dr. Martina Oberhofer).

This filamentous fungus is a member of *Aspergillus* section *Circumdati* (Frisvad *et al.*, 2004) and is related to *Aspergillus ochraceus*, but a differentiation is possible with DNA-based (Morello *et al*., 2007) and sequence- based analyses (Frisvad *et al*., 2004). *A. westerdijkiae* infects grains and several food products like coffee beans, beer, milk, wine and oranges (Han *et al*., 2016).

Like other members of genus *Aspergillus*, *A. westerdijkiae* produces several secondary metabolites, particularly mycotoxins (Frisvad *et al*., 2004). *A. westerdijkiae* has the highest rate of ochratoxin A production (Han *et al*., 2016), which has a high carcinogenic potency. Ochratoxin A (**Figure 2**) is a polyketide which can induce an oxidative damage of the DNA (Palma *et al*., 2007). This mycotoxin causes hepatocellular carcinomas and renal adenocarcinomas in rodents (Pfohl-Leszkowicz, 1994).

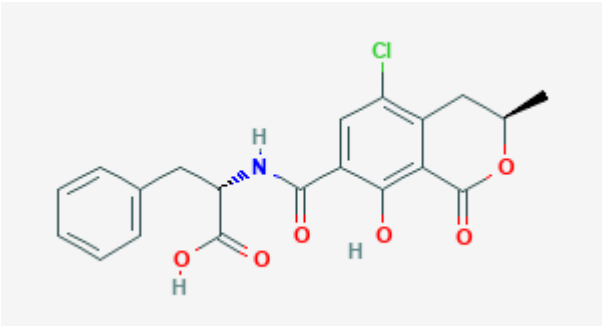


Figure 2: This figure depicts the molecular structure of ochratoxin A (PubChem).

Other secondary metabolites which are produced by *A. westerdijkiae* are penicillic acid, xanthomegnins, aspergamides, neohydroxyaspergillic acid, etc. (Visagie *et al*., 2014).

2.3.2. Akanthomyces muscarius

The isolate with the strain number LN303 was identified as *Akanthomyces muscarius* (Cordycipitaceae) by barcoding and BLAST search in Genbank (data provided by Mag. Dr. Martina Oberhofer). This fungus is entomopathogenic (Vinit *et al*., 2018), meaning it is a specialized pathogen of insects, and as such has an important potential as a biocontrol organism (Gibson *et al*., 2014).

Typically, the pathogenic pathway of an entomopathogenic fungus is as follows: with their conidia they adhere to the cuticle of the insect, where the germination occurs. The next step is to degrade the cuticula and produce blastospores. Then they attack the tissues by branching out mycelia, which means death for the host. Finally, stomata are formed on the insects surface, which contain asci with ascospores (Goettel *et al*., 2008).

2.4. Antibiotics and bacterial resistance

According to the WHO (Worlds Health Organization), antibiotics are drugs affecting bacteria and are used to combat bacterial infections (WHO, 2019). Since the discovery of penicillin by Alexander Fleming (Fleming, 1929), antibiotics have become one of the most important remedies against infectious diseases in the world and are the most used drugs in pharmacy (Forth *et al*., 2009). Nowadays, some earlier used antibiotics cannot be used in antibacterial therapy anymore because of their discovered strong toxicity (Mariottini and Grice, 2016).

Yim *et al*. (2007) defines antibiotics as “small molecules identified by a pharmaceutical company, which have therapeutic activity in inhibiting or killing microbes.” Furthermore, he underlines that naturally produced antibiotics are signaling molecules under natural conditions. They belong to small molecules, which affect at sub-inhibitory concentrations the transcription of many cellular functions (Yim *et al*., 2007).

According to their antibacterial spectra, antibiotics are either bactericidal or they only prevent the increase of bacteria populations (bacteriostatic). They either act against certain strains of bacteria or are broad-spectrum antibiotics (Aktories *et al.*, 2008).

Different types of antibiotics have specific molecular targets in their target organisms, accordingly antibiotics are classified in different modes of actions (Aktories *et al.*, 2008).

Antibiotic classes defined by their molecular targets are: inhibitors of the cell wall biosynthesis (*e.g*. ß- lactame- and glycopeptide antibiotics), inhibitors of bacterial protein synthesis (*e.g*. Chloramphenicol, tetracycline, makrolides, lincomycine, aminoglycoside antibiotics), inhibitors of folic acid metabolism (*e.g*. sulfonamide, trimethoprime), inhibitors of RNA replication (*e.g*. Rifampicin) or DNA replication (*e.g*. Chinolone) (Aktories *et al*., 2008).

In this Thesis however, the term antibiotics will be used furtheron in a broader term to describe compounds that act antimicrobial against organisms belonging to bacteria, fungi and yeast.

Bacterial resistance

In nature, resistance genes against antibiotics are common, because every bacteria producing antibiotics has to be able to survive this process (Yim *et al*., 2007).

Bacteria can have a natural resistance to antibiotic molecules that they produce themselves or the resistance can be acquired by horizontal gene transfer or mutation. Horizontal gene transfer is the process, in which a plasmid is exchanged, thus the resistance can be transferred to other bacterial species (Aktories *et al*., 2008). Resistance genes encode molecular mechanisms to disarm the effects of antibiotics. For example, bacteria can produce enzymes to inactivate antibiotic compounds, pump them out with efflux pumps or mutate the targets (Brauner *et al*., 2016).

Therefore, infections caused by antibiotic resistant bacteria are an increasing problem (Mehl *et al*., 2017). As a result of the rapidly increasing resistances, the need of new effective antibiotics with new action mechanisms is increasing (Laxminarayan *et al.*, 2013).

2.5. Bioactivity- guided fractionation

In order to investigate new bioactive natural products, often bioactivity-guided fractionation is used. This method generally consists of: A total extraction of an organism fermented on a growth medium using appropriate solvents, performing a bioassay to test for activity. If activity is found, the total extract will be treated by chromatographic fractionation, then bioassays of each fraction will be done and after

isolation of bioactive substance(s) (Nothias *et al*., 2018) they will be identified using different spectrometric methods (Atanasov *et al.*, 2015).

Extracts of natural products consist of a complex mixture of compounds and there is often an interaction between these substances. For that reason, a strong activity of an extract could result from synergistic effects of many lesser active compounds. In that case, fractionation would lead to a loss of activity. Another possibility could be that the extract does not show any activity because the concentration of the bioactive substance(s) is too low (Atanasov *et al.*, 2015).

The disc diffusion assay is one of the most commonly used bioassay method for testing the antimicrobial activity. In this assay filter paper disks will be soaked with the extract of interest and then placed on a nutrient agar plate, which has been inoculated with the test organism. In addition, discs with negative and positive controls will be captured on the same plate. Subsequently, the plates will be incubated overnight at an appropriate temperature for the test organism. If the extract contains of an antimicrobial active compound, an inhibition zone around the filter paper disc occurs, because the active compound diffuses through the agar and acts on the test organism. Disc diffusion assays can differ by used test organism, incubation time, conditions and medium (Driscoll *et al*., 2012).

Bioactivity-guided fractionation was used within this Diploma Thesis to investigate new antibiotically active compounds from fungal endophytes.

2.6. High- performance liquid chromatography (HPLC)

2.6.1. Analytical HPLC

Chromatographic methods are the most commonly used methods of analysis in drug discovery and development (Kazakevich *et al.,* 2007). They are used for separation, detection, and quantification of substances.

HPLC is a kind of column liquid chromatography (Lundanes *et al.*, 2014). The technique is based on the separation of samples between a stationary phase and a liquid mobile phase. A column is used as stationary phase, while the mobile phase is liquid (Czaplicki, 2014). After injecting the sample, it is transported with the mobile phase *via* pumps through the column. Depending on the strength of interactions between substances and stationary phase, the components need different amounts of time for eluting (Czaplicki, 2014), which results in different retention times. The stronger

the interaction between molecule and stationary phase, the higher is the retention time of the molecule (Kazakevich *et al.*, 2007).

The measurements can be done using isocratic elution, where the mobile phase has a constant composition, or using a time-gradient, where the composition is changing (Lundanes *et al.,* 2014). Depending on the sample to be analyzed, the solvents of the mobile phase are chosen, whereby the most common combinations are a mixture of water and acetonitrile (ACN) or methanol (MeOH) (Malviya *et al*., 2010).

Principles

One of the separation principles is Normal-Phase HPLC, at which the stationary phase is polar and the mobile phase non-polar. The stationary phase is commonly packed with silica, which is suitable as HPLC packing material because of its compactness. In Normal- Phase HPLC, substances with polar functional groups like -NH, -NO, -CO, -OH, *etc*. have a higher retention time as compounds lacking these groups (Lundanes *et al*., 2014).

Another separation principle represents the opposite of Normal-Phase HLPC, the so-called Reversed-Phase HPLC. Here, the columns are packed with silica supplemented with an organic surface consisting of phenyl-, C2-, C8- and C18- groups bound to the silica. For this reason, non-polar analytes retain stronger because of hydrophobic interactions. Commonly used mobile phases are again mixtures of water with ACN or MeOH (Lundanes *et al*., 2014).

Essential instruments of HPLC

The HPLC system consists of one or more solvent reservoirs for the mobile phase, one or more pumps, an inlet for the sample, which can be an autosampler or manual injector, one or more columns, a detector (commonly an UV-Visible detector) and an equipment for data acquisition (Lundanes *et al*., 2014). **Figure 3** shows a schematic representation of a HPLC system.

The columns are commonly made of stainless steel, because this is one of the most inert materials. Mostly, packing materials have a diameter of 3-5 µm and the columns are 3-25 cm long with an inner diameter of 2-5 mm (Czaplicki, 2014).

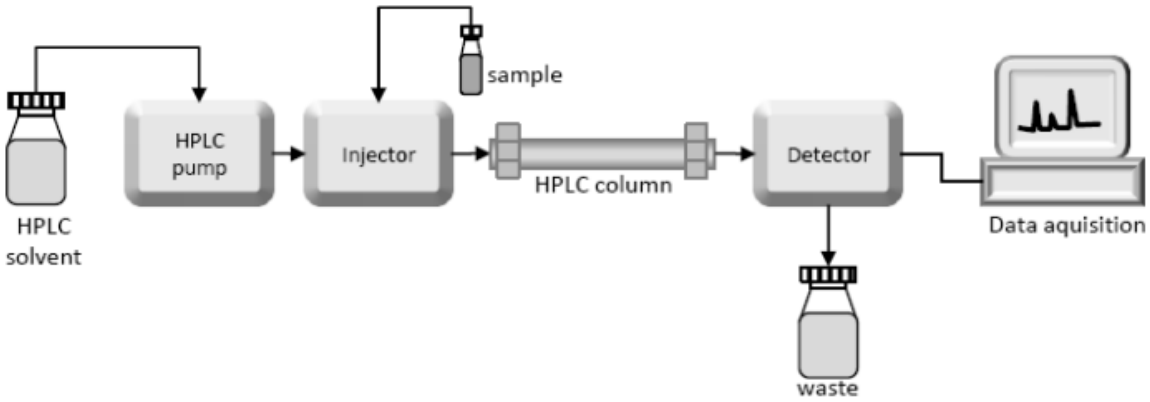


Figure 3: A schematic representation of a HPLC system is demonstrated (Czaplicki, 2014). The HPLC system consists of solvent reservoirs, an HPLC pump, a sample inlet which can be an autosampler or manual injector, an HPLC column, detector and equipment for data acquisition.

2.6.2. Semi-preparative HPLC

While analytical HPLC is used to investigate and separate small amounts of samples, preparative HPLC is employed for separating larger amounts of samples. The leading difference of these two chromatography methods is the diameter and thus the volume of the packing material of the used columns. While analytical HPLC systems are commonly run with a column size of 4.6 x 250 mm, the semi-preparative system is run with a 25 x 250 mm column (McMaster, 2007).

3. Material and methods

3.1. Isolation of fungal endophytes

Five *L. nivale* subsp. *alpinum* plants were harvested on August 8th, 2016. Plants were transported under cooled conditions and stored at 4°C until the following day. Plants were rinsed under tap water and surface sterilized with an optimized protocol (Claudia Haager, Mag. Dr. Martina Oberhofer). Afterwards, plants were separated in different plant tissues and each tissue per plant individual was macerated and serially diluted before plating them on different isolation media. Two of these media were specifically targeting fungi. These were potato- dextrose- agar (PDA) and synthetic nutrient-poor agar (SNA). Endophyte isolation was performed before this Thesis and fungal material was provided by Mag. Dr. Martina Oberhofer.

55 strains were isolated from five different *L. nivale* subsp. *alpinum* plants. Preselection of bioactive endophytes for this work were based on a co-culture interaction assay. As organism we used *B. subtilis*, which was inoculated in the center, surrounded with two inocula from the fungus under investigation (**Figure 4**).

Five strains were pre-selected based on their bioactivity (personal communication, Mag. Dr. Martina Oberhofer). Those strains were identified with the Basic Local Alignment Search Tool Genbank (BLAST Genbank) by Mag. Dr. Martina Oberhofer at the Department of Pharmacognosy. The results of this analysis are shown in **Table 1**. Especially, the fungal endophytes *A. westerdijkiae* and *A. muscarius* built the main part of this work.

Table 1: With BLAST Genbank identified fungal endophytes, their query covery and identity are depicted.

Strain number	Blast genbank	Query covery [%]	Query identity [%]
LN733	*Aspergillus westerdikijae*	100	99
LN303	*Akanthomyces muscarius*	100	99
LN487	*Pleosporales*	94	99
LN255	*Mycochaetophora gentianae*	100	99
LN525	*Trichoderma* sp.	Identified morphologically (**Figure 5**)	

Figure 4: Co-culture plate of LN303 with *B. subtilis* as test organism in the middle of the plate is shown in this figure. Photograph was taken by Mag. Dr. Martina Oberhofer.

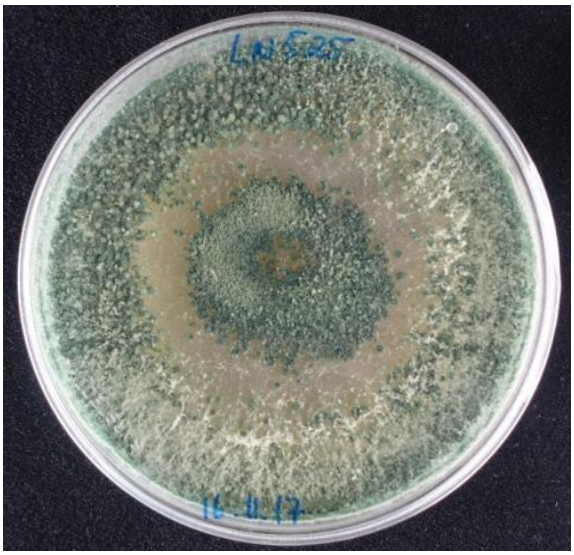


Figure 5: This figure shows the inoculated PDA plate of the endophyte LN525 after 23 days of incubation. Photograph taken by Mag. Dr. Martina Oberhofer.

3.2. Media preparation

Two different media were used to cultivate the active endophytes. For PDA medium 19.5 g PDA (Sigma- Aldrich, Spain) was mixed with 500 mL of distilled water (dH_2O) and put into 1 L Schott flask, autoclaved and stored at +8°C in the cold room.

For the rice medium, 10 g rice (Billa Himalaya Basmatireis biologisch) was added with 25 mL of dH_2O into 250 mL Erlenmeyer flaks and closed with a cotton plug. After placing aluminum foil to cover the plug, the medium was autoclaved and stored in the cold room at +8°C.

For the test microbe stocks and the bioassays two different liquid and agar media were prepared (**Table 2**). For each medium, the components were mixed for a volume of 500 mL and prepared in 1 L Schott flasks, autoclaved and finally stored at 8°C in the cold room.

Table 2: Following media and their components were used for producing microbial test organism stocks for the bioassays.

	Compounds	**Amount**
LB liquid medium	Bacto tryptone (Becton, Dickinson and Company, France)	5 g
	Bacto yeast extract (Becton, Dickinson and Company, France)	1.25 g
	NaCl (Sigma- Aldrich, USA)	2.5 g
	dH_2O	250 mL
L- Agar (LA)	Bacto typtone	5 g
	Bacto yeast extract	2.5 g
	NaCl	2.5 g
	Agar (Sigma-Aldrich, Ash 2.0-4.5%)	7.5 g
	dH_2O	500 mL
YPD liquid medium	Bacto peptone	10 g
	Bacto yeast extract	2.5 g
	D-(+)– Glucose (Sigma- Aldrich; USA)	5 g
	dH_2O	250 mL

YPD agar medium	Bacto peptone	10 g
	Bacto yeast extract	5 g
	D-(+)– Glucose	10 g
	Agar (Sigma-Aldrich, Ash 2.0-4.5%)	7.5 g
	dH_2O	500 mL

3.3. Preparing stocks of test organisms

Five microorganisms were used to test the antibiotic activities of fungal extracts. These included Gram- positive and Gram- negative bacteria, a yeast and two filamentous fungi (**Table 3**).

Table 3: Used test organisms and their strain numbers are listed in this table.

Species	Strain number	Type of organism
Bacillus subtilis	168	Gram-positive bacterium
Escherichia coli	DH5-alfa	Gram-negative bacterium
Saccharomyces cerevisiae	BY4742	Yeast
Aspergillus niger	CBS 110271	Filamentous fungus
Fusarium graminearum	CBS 112.30	Filamentous fungus

Stocks for *E. coli*, *B. subtilis* and *S. cerevisiae*

In order to produce a stock of an organism, a pre-existing stock was thawed on ice and 100 µL were added to 20 mL liquid media. This culture was incubated in the rotary shaking incubator at 200 rpm. *E. coli* and *B. subtilis* were incubated at 37°C and *S. cerevisiae* at 28°C. LA liquid medium was used as medium for *E. coli* and *B. subtilis*, YPD liquid medium for *S. cerevisiae*.

After inoculation, each sample was split and transferred aseptically into two sterile 15 mL falcon tubes in the sterile bench. After centrifugation for 12 min at 4000 rpm, the supernatant was removed using a pipette, and the cells re-suspended in 5 mL of 20% glycerol. The glycerol stocks were pipetted into cryo-tubes with aliquots of 500 µL each, and then stored at -80°C.

Stocks for *A. niger* and *F. graminearum*

From an already existing fungal plate, a small square (~1x1 cm) of fungal material was harvested and transferred into an Eppendorf tube. Subsequently, it was macerated

with a sterile pistil that fits precisely into a 1.5 mL tube. Sterile petri dishes were poured with PDA, solidified and finally supplemented with autoclaved cellophane (Celloclair, Switzerland) on the surface using sterile utensils. After the media were overgrown, a small portion of fungal material was harvested and put into an Eppendorf tube and macerated with a sterile pistil. Finally, 150 µL of 20% glycerol were added into each tube and frozen at -80°C.

3.4. Cultivation of bioactive fungal endophytes

Sterile petri dishes were poured with PDA and then solidified in the sterile bench. After that, the plates were supplemented with autoclaved cellophane on the surface, which was superimposed with sterile utensils. Each experimental fungus was transferred from the co-culture plates to a new PDA plate to get pure cultures of the fungal endophytes (**Figure 6 + 7**). Each plate was incubated for 6 days. However, strains LN255 and LN525 did not grow on cellophane, so they were transferred again to PDA plates without cellophane and incubated for 23 days.

Figure 6: This figure shows the PDA plate of the endophyte LN303 after 6 days of incubation. The plate was supplemented with autoclaved cellophane on the surface before inoculation.

Figure 7: This figure shows the PDA plate of the endophyte LN733 after 6 days of incubation. The plate was supplemented with autoclaved cellophane on the surface before inoculation.

In order to transfer the endophytes to rice medium, a small rectangle (~1 x 3-4 cm) of each fungus was harvested from the PDA plate and put into 1,5 mL Eppendorf tubes.

The rice medium was prepared as described in **chapter 3.2.** Then the fungal material was macerated and filled up to 1000 µL with autoclaved water. Each macerate was poured into an Erlenmeyer flask with sterilized rice medium and incubated in an incubator at room temperature in darkness.

3.5. Preparing fungal material for extraction

Each endophyte needed a different amount of time to grow on rice (**Table 4**). After the fungi were grown through the rice completely, they were transferred together with the fully overgrown rice into Schott flasks. The bottles were covered with a tea filter (Profissimo Teefilter DM, Gr.3) to protect the samples and the lyophilizer from cross contamination. Then all strains except LN255 were frozen at -20°C for 3 h and then lyophilized (-86,6°C, 0,000 mbar) for 24 h. LN255 was frozen and lyophilized for 72 h over the weekend.

Table 4: The times of fungal growth on rice media and lyophilizing time for each endophyte strain is depicted. Each fungus needed a different amount of time to grow.

Strain number	Growth time [days]	Freezing time [hours]	Lyophilizing time [hours]
LN733	23	3	24
LN303	35	3	24
LN487	23	3	24
LN255	20	72	72
LN525	35	3	24

3.6. Extraction of low scale extract

At first, low scale extracts with 100 mL solvent were prepared to test the bioactivity of the endophytes. In order to prepare low scale extracts of the endophytes, each fungal lyophilizate was extracted with 100 mL solvent (dichloromethane/ MeOH 2:1) in the rotary shaking incubator for 1 h at 200 rpm at 28°C. After transferring the extracts into 50 mL falcon tubes, they were centrifuged for 10 min at 4000 rpm at 20°C. Then the supernatants were decanted into round bottom flasks and evaporated until all solvent was evaporated. Dry material was subsequently re-dissolved in specific volumes of MeOH for each fungus using an ultrasonic bath. Finally, the extracts were transferred into Eppendorf tubes, closed with parafilm and stored in the freezer at -20°C.

3.7. Diffusion disc assay

Diffusion disc assays were performed in order to find out, which endophyte has an antibiotic activity. Generally, diffusion disc assays are performed as described in **chapter 2.5.** However, assays can differ in used test organism, incubation time, conditions and medium (Driscoll *et al*., 2012). **Table 5** shows the details for the diffusion disc assays, which were used within this Thesis. Before starting with the assays, the extracts were centrifuged at 200 rpm for 5 min, so the precipitate was condensed to a pellet at the bottom of the Eppendorf tube.

Table 5: For diffusion disc assay used test organisms, their incubation temperature and time and the used media are depicted.

Microorganism	Medium for inoculation	Incubation temperature and time
Bacillus subtilis	LA	37°C, 24h
Escherichia coli	LA	37°C, 24 h
Saccharomyces cerevisiae	YPD	28°C, 24 h
Aspergillus niger	PDA	Room temperature, 48 h
Fusarium graminearum	PDA	Room temperature, 48 h

Media were poured into sterile petri dishes and while they were drying, a sterile filter disc with 6 mm diameter (Oxoid antimicrobial susceptibility test discs, United Kingdom) was placed on an empty sterile petri dish in the hood. 50 µL of fungal extract was slowly pipetted onto the disc, at which first 30 µL and after drying 20 µL were applied. The extract was not pipetted at once because MeOH has a low surface tension and the extract would leak from the disc otherwise. The negative control was prepared the same way using pure MeOH. Nystatin (NY) served as positive control for the filamentous fungi at a concentration of 10 µg/mL applying 50 µL to the disc. After pipetting, the discs were left for drying in the hood. Afterwards, 100 µL of the microbial stocks were pipetted on each plate, streaked out with a sterile drigalski spatula and left for drying. Subsequently, the discs were placed with the pipetted side towards the test organism in the marked area of the culture plates with a flame sterilized forceps and pressed on the agar with a little pressure (**Figure 8**).

All plates were incubated overnight. *E. coli* and *B. subtilis* were incubated at 37°C, *S. cerevisiae* at 28°C and both, *A. niger* and *F. graminearum* at room temperature. The plates of *E. coli*, *B. subtilis* and *S. cerevisiae* were evaluated after 24 hours and the plates of the filamentous fungi after 48 hours of incubation. **Table 6** shows the results of the assays at a glance. Based on these results only LN303 and LN733 were further investigated.

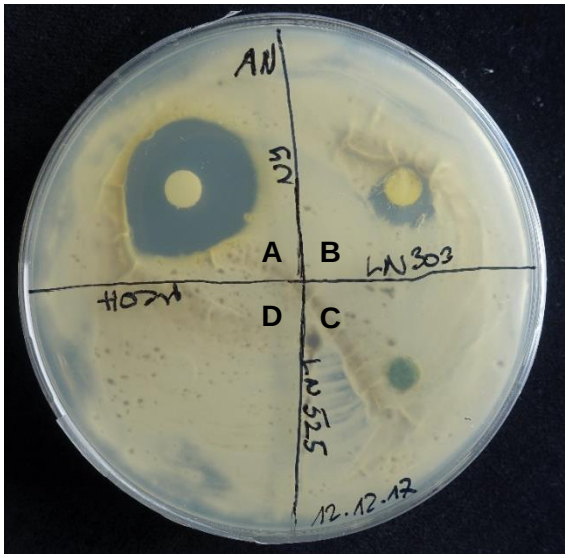


Figure 8: Diffusion disc bioassay testing the low scale extracts of LN303 and LN525 for activity against *A. niger* with nystatin (A) as positive and MeOH (D) as negative control. LN303 (B) showed an inhibition and is an example for a positive reaction, and LN525 (C) lacking an inhibition of the test organism is an example for a negative result.

Table 6: This table shows the first results of the bioassays of the low scale extracts.

Extract	*E. coli*	*B. subtilis*	*S. cerevisiae*	*A. niger*	*F. graminearum*
LN733	**+***	**+**	**+**	**-***	**+**
LN303	**+**	**-**	**+**	**+**	**+**
LN487	**-**	**-**	**-**	**-**	**-**
LN255	**-**	**-**	**-**	**-**	**-**
LN525	**-**	**+**	**-**	**-**	**-**

*+ represents positive and - represents negative bioassay.

3.8. Preparing upscale extracts of LN303 and LN733

Upscales of the endophyte were prepared after the biotic activity of the low scale extracts was confirmed and methods for fractionation established. Upscales were prepared for the active endophytes to gain higher extract yields for further investigations. Each endophyte was inoculated into five Erlenmeyer flasks with rice media as described before. Rice media for LN733 were prepared as described in **chapter 3.4.** LN303 was supplemented with peptone into the rice medium, because it was assumed that the endophyte growths faster and produces more substances with the added peptone. Therefore 0.15 g bacto peptone (Becton, Dickinson and Company, France, LOT: 7073543) was added to 25 g rice (Billa Himalaya Basmatireis, biologisch) in one 250 mL Erlenmeyer flask and mixed with 25 mL distilled water. Furthermore, 0.125 mL trace element solution (**Table 7**) was added to provide the endophyte with the needed trace elements. Finally, the Erlenmayer flask was left at room temperature overnight and autoclaved the next day.

The inoculation and lyophilizing were done as described in **chapter 3.5**., whereby LN733 was inoculated for 22 days and LN303 for 36 days. The extraction method is as described in **chapter 3.6**. This time, more solvent was needed to re-dissolve the dry material, because for the upscale 5 Erlenmeyer flasks per fungus were inoculated and more dry material of the fungus was achieved (**Table 30** in results).

Table 7: Protocol for the applied trace element solution (total volume of 1 L) is depicted.

Compounds	Amount
$CaCl_2$ x H_2O	3 g/L
Fe- III- citrate	1 g/L
$MnSO_4$	0.2 g/L
$ZnCl_2$	0.1 g/L
$CuSO_4 \cdot 5\ H_2O$	0.025 g/L
Sodium tetra borate	0.2 g/L
$CoCl_2 \cdot 6\ H_2O$	0.004 g/L
Sodium molybdate	0.01 g/L

Dry matter yield of LN733 was 14928.25 mg, which was re-dissolved in 28 mL MeOH. Dry matter of LN303 was estimated as 847.02 mg and was re-dissolved in 14 mL MeOH.
All further investigations of LN303 and LN733 were done with the upscale extracts.

3.9. Upscale extract of Ab5

Earlier investigations from Mag. Dr. Martina Oberhofer have shown that the endophyte Ab5 (*Alternaria* sp.), isolated from ***A****tropa* ***b****elladonna*, has a distinguished bioactivity against *B. subtilis*, but detailed further analyses were not yet done. Hence, it was part of this work to analyze this fungal endophyte extract further. For the upscale, three Erlenmeyer flasks with rice media were inoculated with the fungus as described in **chapter 3.4.** and then incubated for 8 days at room temperature by Mag. Dr. Martina Oberhofer.
After freezing and lyophilizing, the endophyte was extracted with 100 mL solvent (acetone/ MeOH 1:1) per flask in the rotary shaking incubator for 1 h at 200 rpm at 28°C. After transferring the extract into 50 mL falcon tubes, it was centrifuged for 10 min at 4000 rpm at 20°C. Then the supernatant was decanted into round bottom flasks and evaporated until an oily residue was left

3.10. Analytical and semi-preparative HPLC

Two different HPLC systems were used to investigate the fungal extracts. At first analytical HPLC (**Table 8**) separation was used to gain first insights and to explore methods for separation of the extracts. Subsequently, semi-preparative HPLC (**Table 10**) was used to fractionate the extracts and achieve pure active substance(s).

3.10.1. Chromatographic analyses of LN733

3.10.1.1. Analytical HPLC analyses of LN733

Before the experiments were performed, the low scale extract was diluted with MeOH (1:10) to a final concentration of 20.93 mg/mL and then centrifuged for 5 min at 4000 rpm. The supernatant was transferred into an HPLC vial, so possible precipitates were removed. This was necessary so that the column did not get clogged. The analytical experiment was performed with following analytical HPLC system and settings (**Table 8**).

Table 8: Analytical HPLC system and settings used for LN733 are depicted.

HPLC	**Shimadzu LC-20AD**
Column	Shimadzu Shimpack GIS 5 µm C18 4.6 x 250 mm
Mobile phase	Gradient of acetonitrile ACN/ H_2O Pump A: H_2O, Pump B: ACN
Flow speed	1 mL/ min
Injection volume	5 µL
Detection wavelength	254 nm
Temperature of column oven	20°C

For the first experiment, a linearly increasing gradient of ACN/ H_2O was chosen. The concentration of ACN increased from 5% to 95% in 45 min and was then maintained constantly at 95% for 20 min, in order to purge the column and to be sure that no more substances were eluting (**Figure 9**).

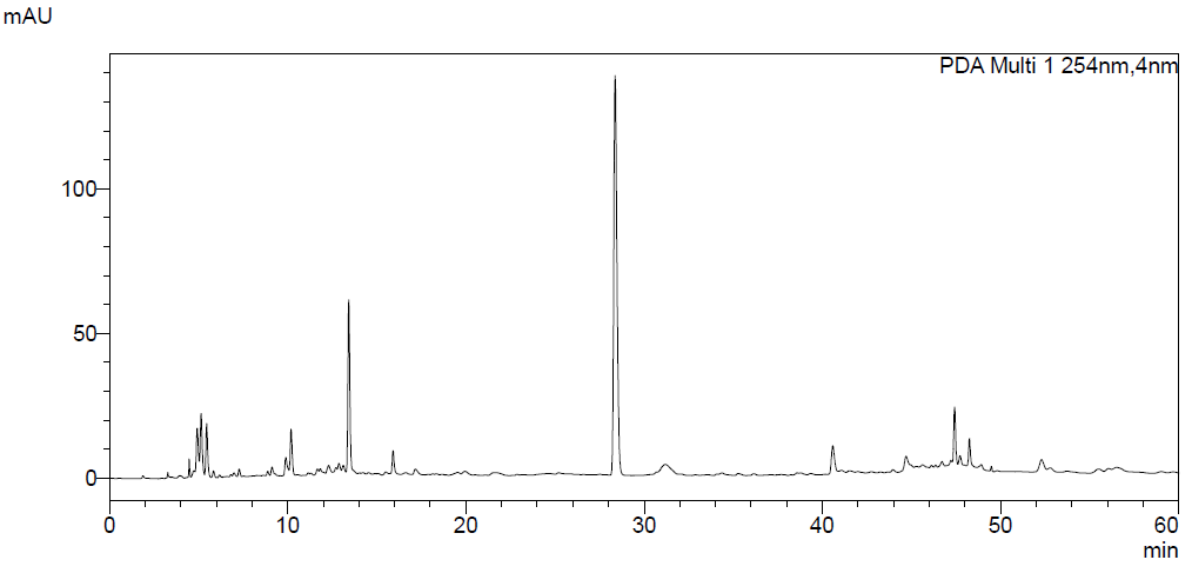


Figure 9: Analytical HPLC separation of LN733 low scale extract (20.93 mg/mL) using initial linear increasing gradient. Analysis was detected at wavelength 254 nm. Injection volume was 5 µL.

Applying this gradient, overlapping peaks especially in the first 20 min were observed (**Figure 9**). The gradient was optimized (**Table 9**) in order to obtain a better resolution of the peaks. At the beginning, the concentration of ACN was set to 5% and was increased to 20% in 15 min. Afterwards it was increased linearly to 95% until minute 50 and was then maintained constantly at 95% for 30 min. Furthermore, the run was extended to 80 min to be sure, that no more substances were eluting after minute 60.

Table 9: Optimized ACN/water gradient used for LN733 is depicted. Pump B represents the concentration of ACN. Analysis started with a concentration of 5% ACN. The concentration was increased to 20% in the first 15 min, then linearly to 95% until 50 min and was finally maintained constantly at 95% for 30 min.

Time [min]	Command	Value [%]
0.01	Pump B Conc.	5
15.00	Pump B Conc.	20
50.00	Pump B Conc.	95
80.00	Pump B Conc.	95

3.10.1.2. Semi-preparative HPLC analyses of LN733

In order to fractionate the fungal extract, a semi-preparative HPLC system was used (**Table 10**) at which the column allows injection of higher volumes of the extract. The optimized gradient (**Table 9**) tested with the analytical HPLC system could be directly transferred to the semi-preparative HPLC system, although the semi-preparative system is lacking a column oven. The extract was diluted with MeOH (1:10) to a final concentration of 20.93 mg/mL and centrifuged for 5 min at 4000 rpm.

The injection volume was 2 mL, corresponding to 41.86 mg extract and before injecting, the extract was centrifuged to precipitate larger particles, which could clog the column while fractionation. In order to compare the analytical and the semi-preparative HPLC run, the ACN/ H_2O gradient of the analytical experiment, as described in **Table 9**, was used.

Table 10: Semi-preparative HPLC system and settings used for LN733 are depicted.

	HPLC	Shimadzu LC- 8A
	Column	Shimadzu Shimpack GIS 10 µm C18 20 x 250 mm
	Mobile phase	Gradient of ACN/ H_2O
	Flow speed	20 mL/min
	Injection volume	2 mL
	Detection wavelength	254 nm
	Detector A	Model: SPD- 10Avp Lamp: D2 Polarity: + Response: 1.0 sec Wavelength CH1: 254 nm
	Fraction collector	Delay volume: 200 µL Rack type: 3 Vial volume: 50 mL

Up to min 40, 16 fractions (fraction 0-15) were collected into 50 mL vials (1 fraction every 2.5 min). Fraction 16 was collected as one single fraction from minute 40 to 55 and fraction 17 from minute 55 to 80 (**Figure 10**).

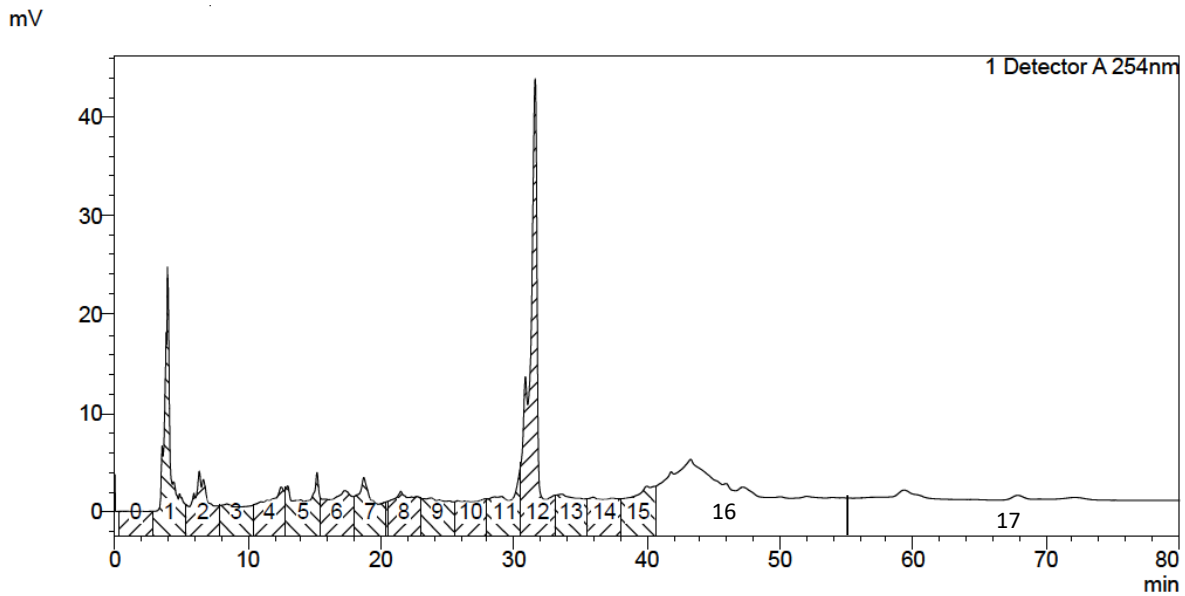


Figure 10: Semi-preparative HPLC separation of LN733 low scale extract (20.93 mg/mL) using the gradient depicted in Table 9. Analysis was detected at wavelength 254 nm. Injection volume was 2 mL. 18 fractions were collected.

After fractionation, fractions 0-7 were evaporated on the rotavapor at 40 °C of the water bath to remove the ACN proportion. Afterwards, the residual yield of water was frozen at -80°C for 3h and subsequently, the fractions were dried by lyophilizing. Fractions 8-17 were completely evaporated to dryness on the rotavapor. Finally, the residues of all 17 fractions were re-dissolved in MeOH (for used amounts of MeOH see **Table 33** in results).

In order to test, which fraction contains the active compound, diffusion disc assay of every single fraction was done as described in **chapter 3.7**. After semi-preparative HPLC separation, all the activities were lost, although the extract previously had an activity against the test organisms *E. coli, B. subtilis, S. cerevisiae. and F. graminearum*.

Considering the low intensity of the peaks of the semi-preparative HPLC analysis and the results **(Figure 10)**, it was assumed that diluting the extract 1:10 resulted in a very low concentration of active compounds. Consequently, the fractionation was repeated with a 1:2 dilution of the extract with a final concentration of 104.65 mg/ mL, using the same semi-preparative HPLC system and conditions as before (**Table 10**).

Fractionation of LN733 low scale extract (1:2 dilution (104.65 mg/mL))

Again, 17 fractions were collected and treated the same way as dilution 1:10 (20.93 mg/mL). Bioassays were performed after re-dissolving the fractions in MeOH (used amounts of MeOH are depicted in **Table 33** in results).

This time, the concentrations of the fractions were high enough to achieve activities at the bioassays. Fractions 9, 10, 12, 13 and 16 showed activities against the test organisms and were further investigated.

Fractionation of the upscale extract of LN733

In order to achieve a higher yield of the active fractions, the fractionation was repeated with the upscale extract of LN733, which had a concentration of 533.15 mg/mL. 2 mL of the 1:2 dilution corresponding to 533.15 mg upscale extract was injected, and the conditions were the same as in the experiments before (**Table 10**).

Ultimately, 3 runs were done in which only the interesting fractions (9, 10, 12, 13) were collected (**Table 11**). Subsequently, fraction 12 was subfractionated using a semi-preparative HPLC system. The other active fractions were further analyzed using NMR and HRMS.

Table 11: Dry matter yields of the active fractions of LN733 upscale extract are depicted in mg.

Fraction	Dry matter yields [mg]
9	2.9
10	4.3
12	4.76
13	3.1

3.10.1.3. Analytical HPLC analyses and subfractionation of LN733_F12

Fraction 12 was further analyzed because the focus was primarily on investigation antibiotic activities. First, analytical HPLC experiments were conducted to infer the optimal gradient for the mobile phase (**Figure 11**). Considering that the interesting fraction 12 eluted at min 30.5 at the semi-preparative separation, which had an ACN concentration of approximately 40%, the analytical analysis was started with this solvent concentration (**Table 12**).

Table 12: Optimized gradient for analytical HPLC of LN733_F12 is depicted. Pump B represents the concentration of ACN. Analysis started at a concentration of 40% ACN, which was increased to 50% in 10 min. In the next 10 min, the concentration was increased to 65% and then to 75% in the last 5 min.

Time [min]	Command	Value [%]
0.01	Pump B Conc.	40
10.00	Pump B Conc.	50
20.00	Pump B Conc.	65
25.00	Pump B Conc.	75

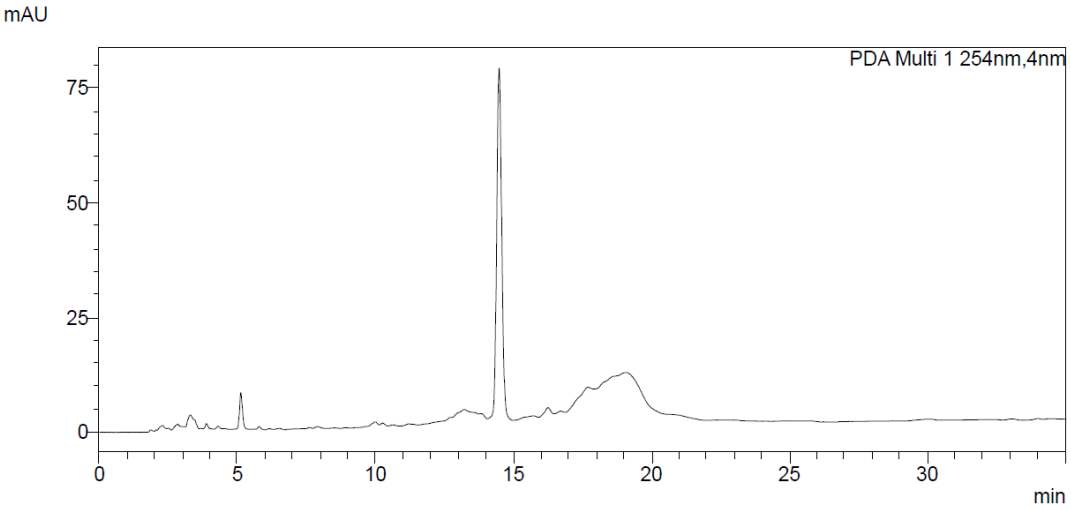


Figure 11: Analytical HPLC separation of LN733_F12 (0.79 mg/mL) using optimized gradient (Table 12) is shown. Analysis was detected at wavelength 254 nm. Injection volume was 10 µL.

For subfractionation, the same semi-preparative HPLC system from former experiments was used (**Table 10**). The optimized gradient from the analytical run (**Table 12**) was used as the mobile phase.

The dry matter yield of LN733_F12 was about 4.76 mg in 6 mL. Consequently, 3 runs of each 2 mL were performed and the main peak with the retention time 16.91 min from fraction 6 was collected manually (**Figure 12**).

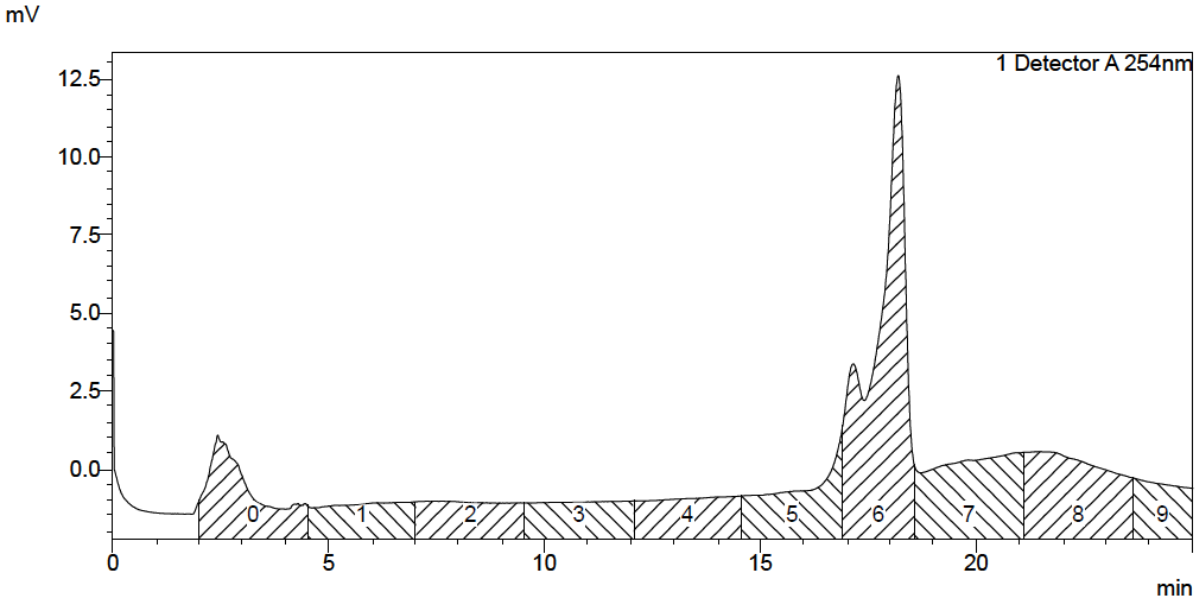


Figure 12: Chromatogram of the semi-preparative HPLC separation of LN733_F12 (0.79 mg/mL) using the optimized gradient (Table 12) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 2 mL. 10 fractions were collected.

Fraction 6 (LN733_F12.6) yield about 1.9 mg dry matter and was further analyzed with HRMS and NMR and finally tested against *E. coli* and *B. subtilis* in diffusion disc assays.

Based on the results of NMR and HRMS analyses (see **chapter 4.3.**), LN733_F12.6 and LN733_F13 were chemically treated using the Stas-Otto separation process (**Figure 13**). 1 mL of the subfraction was evaporated to remove MeOH. Subsequently, it was re-dissolved in 1 mL ether and was separated in the separating funnel by shaking out with 1 mL $NaHCO_3$. Afterwards, the lower aqueous layer was removed, and the ether phase was again treated with 1mL $NaHCO_3$ two more times as described above. Then, the initial ether phase (E1) was removed and all three aqueous phases were decanted together.

The aqueous phase was acidulated with 2N HCl and separated in the separating funnel by shaking out with 1 mL ether. This process was repeated twice with each 1 mL ether and the final ether phases were decanted together (E2). At least both ether phases were analyzed by diffusion disc assays, NMR- and mass spectroscopy.

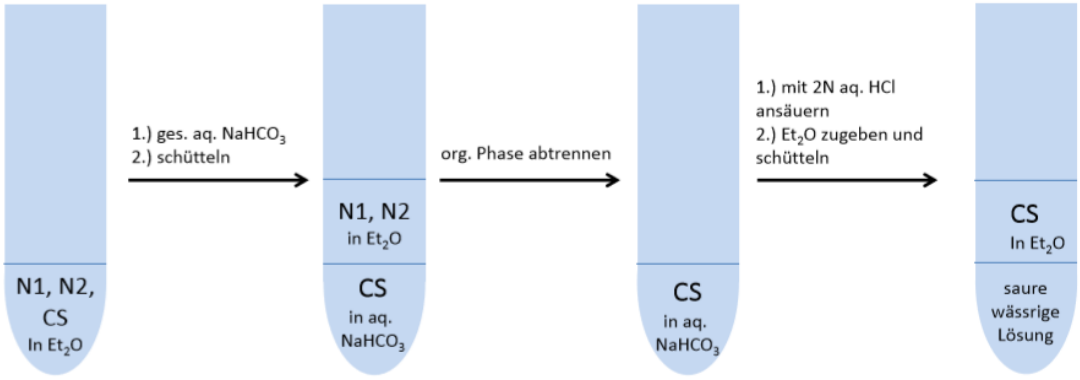


Figure 13: Stas- Otto separation process is represented. The sample was dissolved in 1 mL ether and then separated by shaking out with 1 mL $NaHCO_3$. The lower aqueous phase was removed, and the ether phase was treated two more times with 1 mL $NaHCO_3$. The aqueous phases were decanted together and acidulated with 2N HCl and was shaken out with 1 mL ether. This was repeated two more times and at least the final ether phases were decanted together.
N1 and N2… natural substance 1 and 2; CS… carbonic acid
http://www.ichemlab.at/info/Presentation_organische_Analyse_2017.pdf (accessed on April 5th, 2018)

3.10.2. Chromatographic analyses of LN303

3.10.2.1. Analytical HPLC analyses of LN303

The upscale extract of LN303 was diluted with MeOH (1:2) to a final concentration of 30.25 mg/mL and then centrifuged for 5 min at 4000 rpm, so that larger particles of the extract could sediment at the bottom of the Eppendorf tube. This was necessary to avoid that the column gets clogged during the analyses. Then, the supernatant was transferred into a HPLC vial and the analytical analysis was conducted with the following analytical HPLC system and settings (**Table 13**).

Table 13: Analytical HPLC system and settings used for the upscale extract of LN303 are depicted.

HPLC	**Shimadzu LC-20AD**
Column	Shimadzu Shimpack GIS 5 µm C18 4.6 x 250 mm
Mobile phase	Gradient of acetonitrile ACN/ H_2O (**Table 14**) Pump A: H_2O Pump B: ACN
Flow speed	1 mL/ min
Injection volume	10 µL
Detection wavelength	254 nm
Temperature of column oven	20°C

Table 14: Initial ACN/water gradient used for analytical HPLC separation of the upscale extract of LN303 is depicted. Pump B represents the concentration of ACN. Analysis started with a concentration of 5% ACN, which was increased to 30% in the first 10 min. In the next 30 min, the concentration was increased to 70% and then to 95% in 5 min. Finally, the concentration was maintained constantly at 95% for 40 min.

Time [min]	Command	Value [%]
0.01	Pump B Conc.	5
10.00	Pump B Conc.	30
40.00	Pump B Conc.	70
45.00	Pump B Conc.	95
85.00	Pump B Conc.	95

As overlapping peaks at the analytical separation with the initial gradient were observed (data not shown), the mobile phase was acidulated with 0.1% formic acid (**Figure 14**). However, there was no recognizable difference between the two runs. Therefore, all further investigations were performed without acid addition.

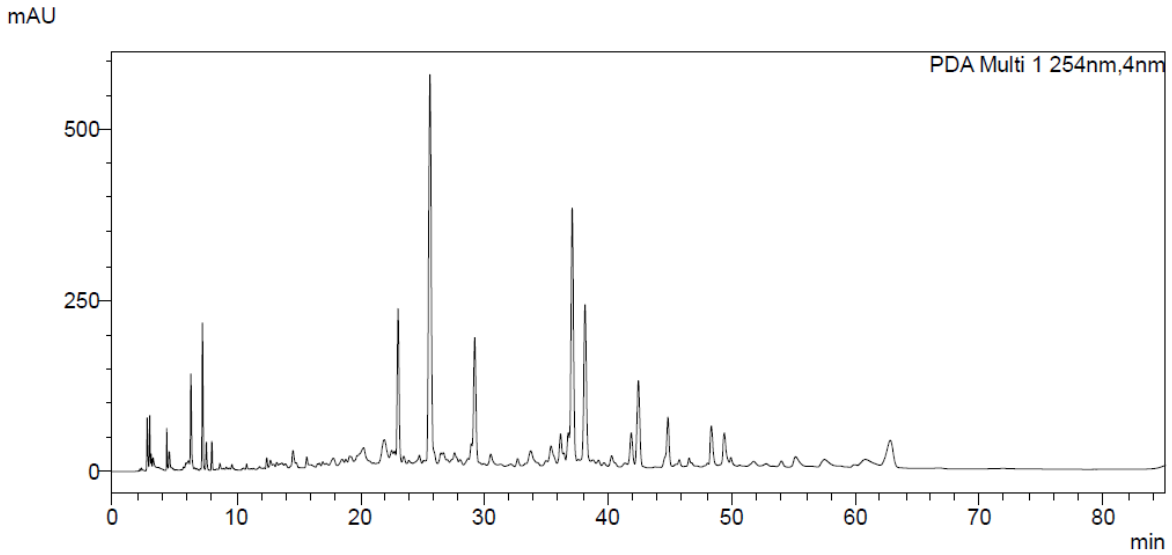


Figure 14: Analytical HPLC separation of the upscale extract of LN303 (30.25 mg/mL) using initial gradient is depicted. Analysis was detected at wavelength 254 nm. The injection volume was 10 µL.

3.10.2.2. Semi-preparative HPLC analyses of LN303 upscale extract

For fractionation of the fungal upscale extract, a semi-preparative HPLC system was used (**Table 15**). For the initial fractionation, a 1:2 dilution with a final concentration of 30.25 mg/mL was applied, to prevent overcharging of the column. The injection volume was 2 mL, corresponding to 60.50 mg upscale extract, and before injecting, the extract was centrifuged so possible precipitates were removed and could not clog the column while fractionation. The ACN/ H2O gradient of the analytical experiment (**Table 14**) was used to be able to compare the analytical and the semi-preparative HPLC run.

Table 15: Semi-preparative HPLC system and settings used for the upscale extract of LN303 are depicted.

HPLC	**Shimadzu LC- 8A**
Column	Shimadzu Shimpack GIS 10 µm C18 20 x 250 mm
Mobile phase	Gradient of ACN/ H_2O
Flow speed	20 mL/ min
Injection volume	2 mL
Detection wavelength	254 nm

Every 2.5 min, one fraction was collected into a 50 mL vial for 40 min summing up to 16 fractions (fraction 0-15). Fraction 16 was collected as one single fraction from minute 40 to 60 and fraction 17 from minute 60 to 85 (**Figure 15**). After fractionation all fractions were dried by evaporation on the rotavapor at 40°C of the water bath and the residues were subsequently re-dissolved in MeOH.

In order to test which fraction obtains the active compound, diffusion disc assay of every single fraction was done as described in **chapter 3.7**. Fraction 11 had an activity against *S. cerevisiae*, *F. graminearum* and *A. niger*. None of the fractions had an activity against *E. coli*.

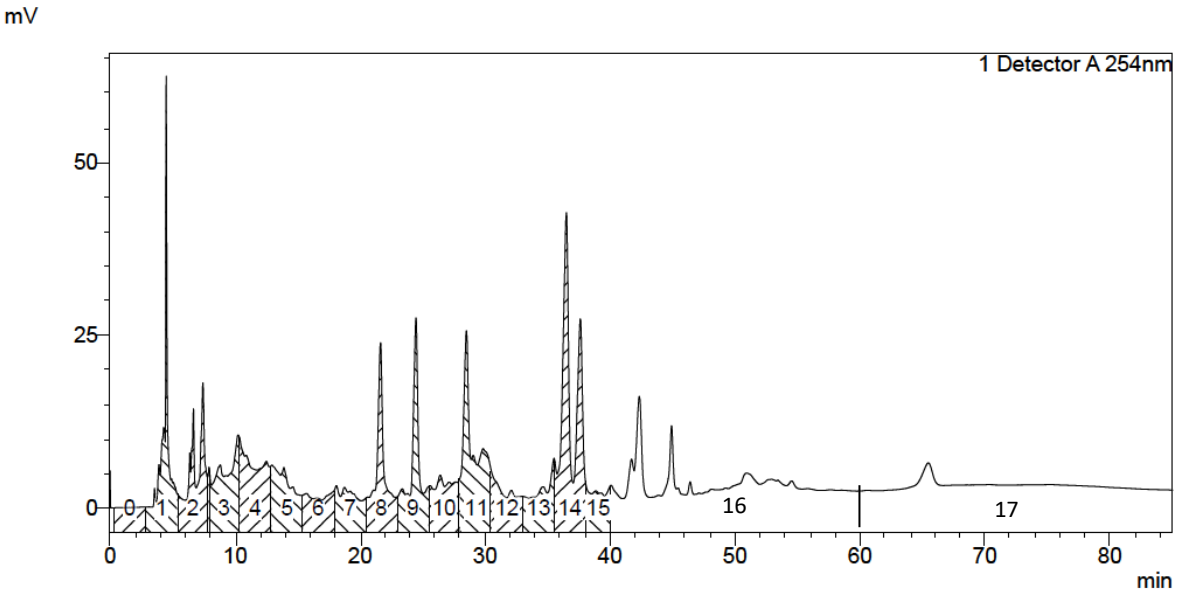


Figure 15: Semi-preparative HPLC fractionation of the upscale extract of LN303 (LN303_A_) (30.25 mg/mL) using initial gradient (Table 14) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 2 mL. 18 fractions were collected.

Considering the intensity of the peaks of the semi-preparative HPLC run and the results of the bioassays it was concluded, that the concentration of the extract was too low to observe an activity against *E. coli*.

Preparing LN303 upscale extract for second fractionation

The fractionation was repeated with the non-diluted extract of the upscale. Chemical pre- treatments were executed to prevent a clogging of the column and to prevent precipitating compounds when the extract encounters the mobile phase. Therefore, H_2O was added to 1 mL extract in excess and left sitting for 1 h at room temperature. Then the precipitate was centrifuged for 20 min at 4000 rpm. The same was done with another 1 mL of the extract with ACN in excess. The supernatants of each approach were transferred into a round bottom flask and evaporated. However, foaming of the supernatants prevented their evaporation. For this reason, 600 µL of 5% formic acid were added to each. This prevented the foaming of the supernatants and they were evaporated until an oily residue was left. Subsequently, the residue was re-dissolved in MeOH. Diffusion disc assays were performed in order to see if the pre-treatments had an influence of the bioactivity.

Bioassays were performed using *S. cerevisiae*, *E. coli* and *A. niger*. Again, the extract showed activity against *S. cerevisiae* and *A. niger* but not against *E. coli*.

Consequently, the pre- treatment was repeated with some changes. 1 mL H_2O was added to 1 mL extract, left for 1 h at room temperature and subsequently, the precipitate was centrifuged for 20 min at 4000 rpm. Then the supernatant was removed, and the precipitate was resuspended in MeOH. Afterwards the aqueous supernatant as well as the resolved precipitate were tested against *E. coli* and *S. cerevisiae*. Bioassays showed that the aqueous supernatant is active against both microorganisms while the precipitate is not. Finally, the supernatant was evaporated again, and the residue was re-dissolved in MeOH.

Fractionation of the pre-treated upscale extract of LN303

The pre-treated extract was fractionated with the same semi-preparative HPLC system (**Table 15**). The extract had a concentration of 30.25 mg/mL and the injection volume was 2 mL, corresponding to 60.50 mg of the upscale extract. Conditions, mobile phase and gradient were the same as the fractionation before (**Table 14**) except for a shorter running time of 60 min (**Figure 16**).

16 fractions (fraction 0-15) were collected until min 40, fraction 16 was collected from minute 40 to 50 and fraction 17 from minute 50 to 60.

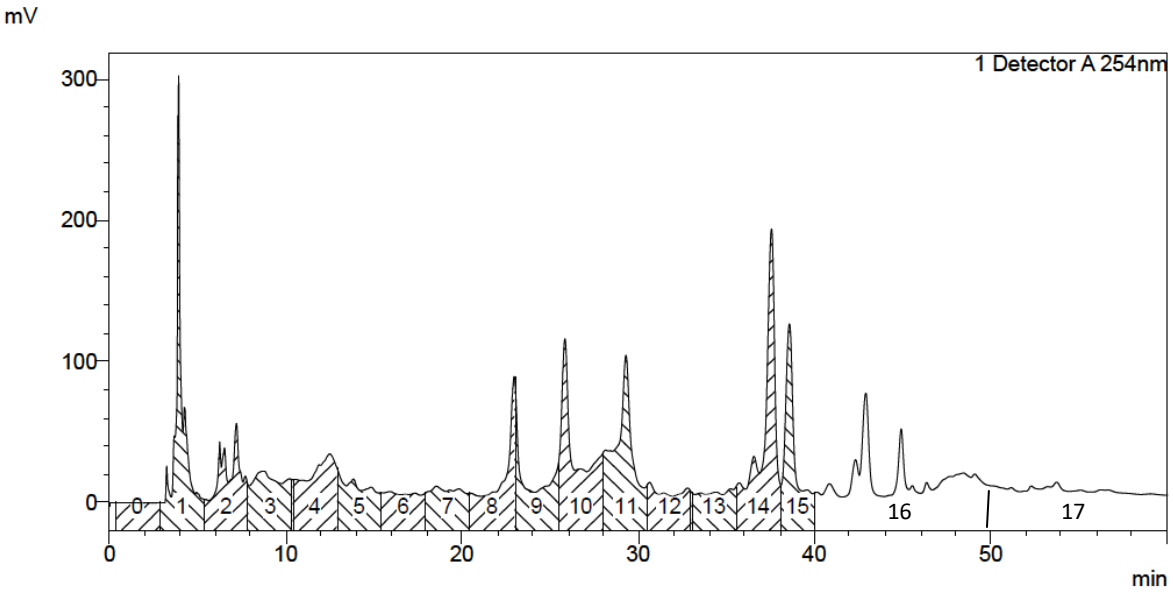


Figure 16: Semi-preparative HPLC separation of the pre-treated upscale extract of LN303 (LN303_B_) (30.25 mg/mL) using the initial gradient (Table 14) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 2 mL. 18 fractions were collected.

Fractions were pooled and renumbered before evaporating (**Table 16**). Then the pooled fractions were decanted into round bottom flasks and evaporated on the rotavapor until a dry residue was left. Finally, the residues were re-dissolved in MeOH and thus ready for performing bioassays.

Table 16: Re-numbering of the pooled fractions of LN303_B_ is represented.

Fraction number old	Pooled fraction number
0, 1, 2	1
3, 4, 5, 6	2
7, 8, 9	3
10, 11, 12	4
13, 14, 15	5
16	6
17	7

Bioassays of the fractions showed that fraction 4 is the only active fraction showing an activity against *E. coli*, *S. cerevisiae*, *F. graminearum* and *A. niger*. Consequently, LN303_B_F4 was further investigated.

3.10.2.3. Analytical HPLC analyses and subfractionation of LN303_B_F4

Analytical HPLC runs were conducted to gain first insights and to explore methods for separation of the extracts. The same HPLC system and column from former analyses was used (**Table 13**). Considering that the interesting fraction 4 eluted at minute 25 at the semi-preparative experiments, which had an ACN concentration of approximately 50%, the analytical run was started with this solvent concentration (**Table 17**).

Table 17: Optimized ACN/ water gradient used for the analytical HPLC analysis of LN303_B_F4 is depicted. Pump B represents the concentration of ACN. Analysis started with a concentration of 50% ACN, which was increased to 52% in the first 5 min. In the next 10 min, the concentration was increased to 53% and then linearly to 95% in 5 min. Finally, the concentration was maintained constantly at 95% for 5 min.

Time [min]	Command	Value [%]
0.01	Pump B Conc.	50
05.00	Pump B Conc.	52
15.00	Pump B Conc.	53
20.00	Pump B Conc.	95
25.00	Pump B Conc.	95

For subfractionation, the same semi-preparative HPLC system from former experiments was used (**Table 15**). The gradient of the mobile phase was the optimized one from the analytical run (**Table 17**). The yield of fraction 4 was 4.66 mg, which was dissolved in 1 mL MeOH for the subfractionation.

Since the semi-preparative run only took 20 min, 11 fractions (fraction 0-10) were collected in which the visible two peaks were collected manually with the objective of getting pure compounds (**Figure 17**).

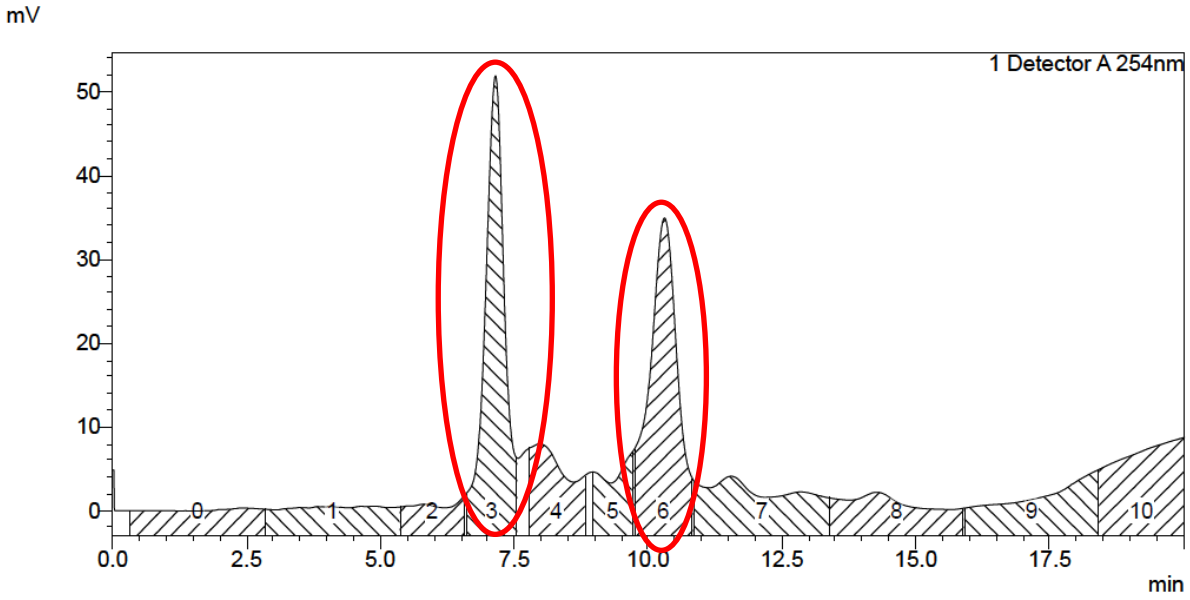


Figure 17: Semi-preparative HPLC separation of LN303_B_F4 (4.66 mg/mL) using optimized gradient (Table 17) is depicted. Fraction number 3 and 6 were collected manually. Analysis was detected at wavelength 254 nm. Injection volume was 1 mL. 11 fractions were collected.

Before evaporating some fractions were pooled into round bottom flasks and renumbered (**Table 18**). The pooled fractions were evaporated on the rotavapor until a dry residue was left. Finally, the residues were re-dissolved in MeOH and diffusion disc assays were prepared.

Table 18: Re-numbering of the pooled fractions of LN303_B_F4 is depicted.

Fraction number old	Pooled fraction number
0, 1, 2	1
3	2
4, 5	3
6	4
7, 8	5
9, 10	6

3.10.2.4. Preparation and analyses of second upscale extract of LN303

As the concentrations of the fractions were not sufficient for further analyses, the endophyte LN303 was cultivated in a second upscale approach as described in **chapter 3.8**.

After fermentation, harvest, freezing, lyophilizing and extraction, the concentration of the second upscale extract (LN303_Ups.2) averaged 58.57 mg/mL. Before fractionation, analytical HPLC was done to get an overview of upscale extract 2 and to see if it is comparable to the first upscale extract. After it was verified, that both upscale extracts were nearly identical, LN303_Ups.2 was pre-treated and then fractionated using the same methods and the same semi-preparative HPLC system as in the upscale extract 1 fractionation. Also, the gradient of the mobile phase was identical to the former one, except that the run was stopped after 60 min because no more active substances were eluting (**Table 19**).

Table 19: Optimized ACN/ water gradient used for semi-preparative HPLC of LN303_Ups.2 is depicted. Pump B represents the concentration of ACN. Analysis started with a concentration of 5% ACN, which was increased to 30% in the first 10 min. In the next 40 min, the concentration was increased to 70% and then to 95% in 5 min. Finally, the concentration was maintained constantly at 95% for 15 min.

Time [min]	Command	Value [%]
0.01	Pump B Conc.	5
10.00	Pump B Conc.	30
40.00	Pump B Conc.	70
45.00	Pump B Conc.	95
60.00	Pump B Conc.	95

The injection volume was 2 mL, corresponding to 117 mg of LN303_Ups.2. Again, 16 fractions (fraction 0-15) were collected until minute 40, fraction 16 was collected from minute 40 to 50 and fraction 17 from minute 50 to 60 (**Figure 18**). Fractions were pooled, renumbered and decanted into round bottom flasks for evaporating (**Table 20**). Finally, the fractions were evaporated on the rotavapor to dryness and re-dissolved in MeOH.

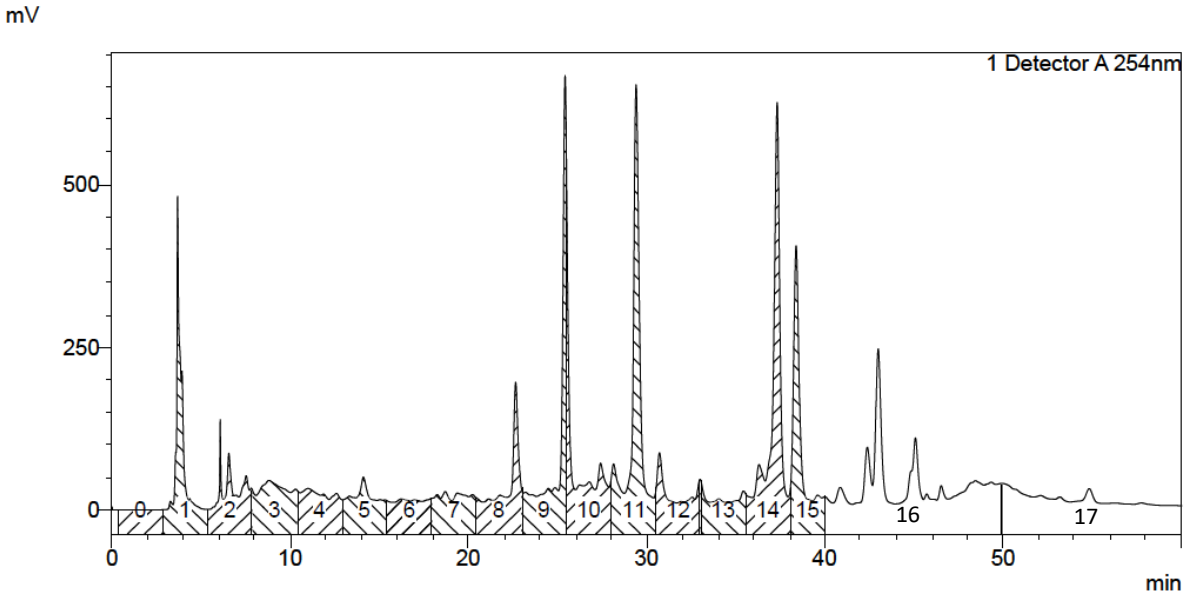


Figure 18: Semi-preparative HPLC separation of LN303_Ups.2 (58.57 mg/mL) using optimized gradient (Table 19) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 2mL. 18 fractions were collected.

Table 20: Re-numbering of the pooled fractions of LN303_Ups.2_A is depicted.

Fraction number old	Pooled fraction number
0, 1, 2	1
3, 4, 5, 6	2
7, 8	3
9, 10, 11, 12	4
13, 14, 15	5
16	6
17	7

Semi-preparative separation of LN303_Ups.2_F4

The active fraction 4 was subfractionated the same way as in former experiments using the same ACN/ H_2O as described in **Table 17** as mobile phase.

This time, the injection volume was 2 mL, corresponding to 35.5 mg of LN303_Ups.2_F4, and 13 fractions (fraction 0-12) were collected into 50 mL vials in which the two peaks with the retention time min 6.90–7.44 and min 9.93–10.66 were collected manually. Before evaporating, some fractions were pooled together, and the fractions were renumbered (**Table 21**).

Table 21: Re-numbering of the pooled fractions of LN303_Ups.2_A_F4 is depicted.

Fraction number old	Pooled fraction number
0, 1, 2	1
3	2
4, 5	3
6	4
7	5
8, 9, 10, 11, 12	6

After evaporation, all subfractions were re-suspended in MeOH except of subfraction 4 (LN303_Ups.2_A_F4_4). Its dry yield averaged 2 mg which was enough for NMR analysis. After NMR analysis, subfraction 4 was dissolved in MeOH and diffusion disc assay was done to confirm the previously observed bioactivity against the microorganisms.

3.11. Flash chromatography and semi-preparative HPLC of Ab5

In former analyses of Ab5 performed by Mag. Dr. Martina Oberhofer, flash column chromatography (**Table 22**) was used to fractionate the extract. Furthermore, it was already determined in which fraction the active compound(s) against *B. subtilis* was (were). For this reason, same methods and settings were used to analysis the newly extracted upscale to be able to compare it with the former one. Flash column chromatography of Ab5 was kindly performed by Dr. Ammar Tahir, MSc. at the Department of Pharmacognosy.

Flash column chromatography was performed (**Figure 19**) with a linear increasing gradient of MeOH and H_2O (**Table 23**) using a C18 column. For dry load of the cartridge, 350 mg of the extract was mixed with 700 mg silica gel (Kieselgel 60, Merck®). Detailed setting and parameters are depicted in **Table 22**.

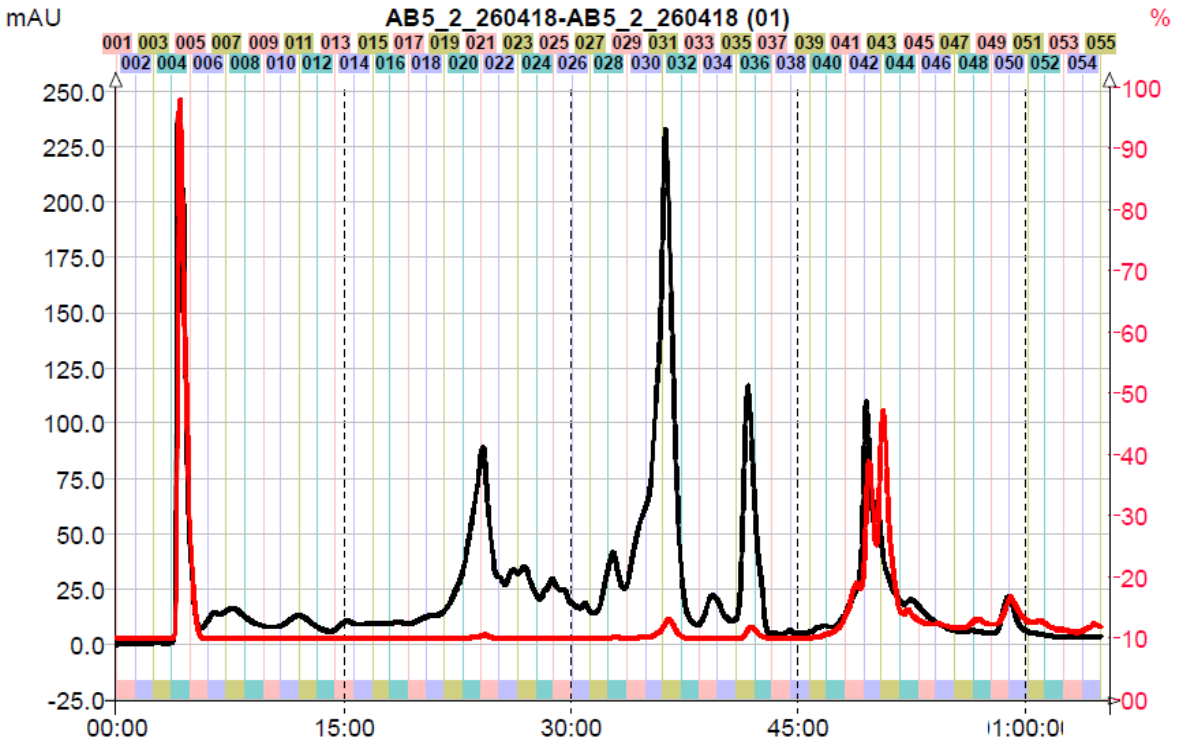


Figure 19: Flash chromatography separation of Ab5 with time gradient as depicted in Table 22.

Table 22: Re-numbering of the pooled fractions of LN303_Ups.2_A_F4 is depicted.

Flash chromatography	**Interchim puriFlash 4250**
Column	PURIFLASH Column 15 C18 HQ – 35.0g (22 bar)
Mobile phase	Gradient of MeOH/ H_2O (**Table 23**)
Detection by UV	Scan 205- 600 nm
Fractions collected	55 à 18 mL

Table 23: Used time gradient of the mobile phase of flash chromatography (MeOH/ H2O) with a flow rate of 15 mL/min is depicted. The analysis started with a concentration of 5% MeOH, which was maintained constantly for 10.40 min. The concentration was increased then linearly to 98% until minute 45 and was finally maintained constantly for 20 min.

Time [min]	**Flow rate [mL/min]**	**MeOH [%]**	**H_2O [%]**
0.01	15.0	5	95
10.40	15.0	5	95
45.00	15.0	98	2
65.00	15.0	98	2

Before evaporating, some fractions were pooled together and got a re-numbering. After evaporation the residues were re-dissolved in MeOH. **Table 24** shows the dry matter yields of the fractions and the amounts of dissolved MeOH.

Table 24: Re-numbering of the pooled fractions of Ab5, their dry matter yields and the amounts of used MeOH for re-dissolving are depicted.

Fraction number old	Pooled fraction number	Dry matter yields [mg]	MeOH [mL]
001- 004	1	155.36	1.5
005- 007	2	21.56	1.5
008- 011	3	6.1	1.5
012- 018	4	14.2	1.5
019- 021	5	7.64	1
022- 023	6	3.58	1
024- 027	7	6.4	1
028- 032	8	13.95	1.5
033- 034	9	2.55	1
035- 036	10	4.59	1.5
037- 040	11	4.87	1.5
041- 042	12	28.22	1.3
043- 044	13	25.6	1.5
045, 046	14	6.59	1.2
047- 050	15	16.4	1
051- 055	16	14.46	1.5

Subsequently, diffusion disc assays against *B. subtilis* were performed as described in **chapter 3.7.** Based on the results **(Table 52)** the fraction with the highest activity (Ab5_F12) was further investigated. Other active fractions were analyzed with analytical HPLC to see if there is any similarity with the fraction of the highest activity. The fraction with the highest activity (Ab5_F12) eluted at the same retention time like in former analyses done by Mag. Dr. Martina Oberhofer. Consequently, the gradient from former analyses (**Table 25**) was used for analytical HPLC separation, at which H_2O was acidulated with 0.1% formic acid. As the absorbance maximum of the peaks was observed at 264nm, the analyses were detected at this wavelength. Detailed parameters about the analytical HPLC system are depicted in **Table 26**.

Table 25: Optimized ACN/ water gradient used for Ab5_F12 is depicted. Pump B represents the concentration of ACN. The analysis started with a concentration of 80% ACN, which was increased to 90% in the first 5 min. In the next 10 minutes the concentration was increased 1% and then to 95% until minute 20. Finally, the concentration was maintained constantly at 95% for 5 min.

Time [min]	Command	Value [%]
0.01	Pump B Conc.	80
5.00	Pump B Conc.	90
15.00	Pump B Conc.	91
20.00	Pump B Conc.	95
25.00	Pump B Conc.	95

Table 26: Analytical HPLC system and settings used for Ab5_F12 separation are depicted.

HPLC	**Shimadzu LC-20AD**
Column	Shimadzu Shimpack GIS 5 µm C18 4.6 x 250 mm
Mobile phase	Gradient of ACN/ acidulated H_2O Pump A: H_2O + 0.1% formic acid Pump B: ACN
Flow speed	1 mL/ min
Injection volume	10 µL
Detection wavelength	264 nm
Temperature of column oven	20°C
Concentration of extract	21.7 mg/ mL

Semi-preparative separation of Ab5_F12

After analytical HPLC analysis the active fraction was separated with following semi-preparative HPLC system and settings (**Table 27**) using the same gradient as in analytical separation (**Table 25**). The injection volume was 1 mL, corresponding to 21.7 mg of Ab5_F12, and before injecting, the extract was centrifuged for 5 min at 4000 rpm to eliminate unsolved particle.

Table 27: Semi-preparative HPLC system and settings used for Ab5_F12 separation are depicted.

HPLC	Shimadzu LC- 8A
Column	Shimadzu Shimpack GIS 10 µm C18 20 x 250 mm
Mobile phase	Gradient of ACN/ H_2O (+0.1% formic acid)
Flow speed	20 mL/ min
Injection volume	1 mL
Detection wavelength	200 nm

Fractions were collected for 25 min into 50 mL vials while the detected peaks were collected manually (**Figure 20**). Especially the main peak with a retention time of 15.32–16.30 min was collected manually. Before evaporating, some fractions were pooled together and subsequently dried by evaporation. **Table 28** shows the re-numbering and dry matter yields of the fractions.

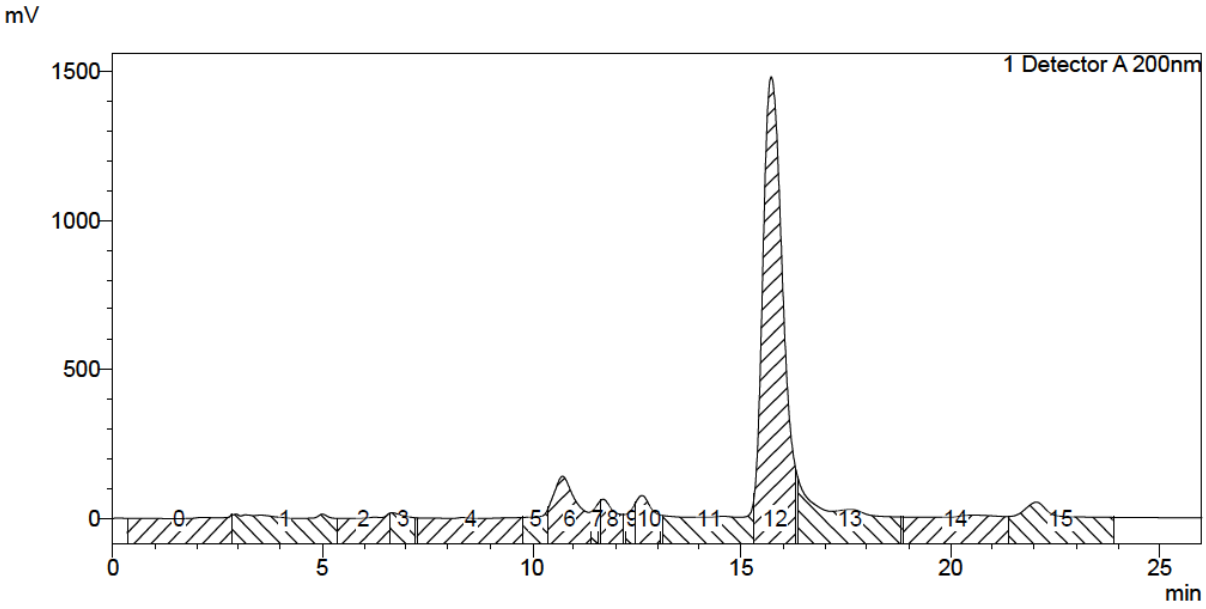


Figure 20: Semi-preparative HPLC separation of Ab5_F12 (21.7 mg/mL) using the optimized gradient (Table 25) is depicted. Analysis was detected at wavelength 200 nm. Injection volume was 1 mL. 16 fractions were collected.

Table 28: Re-numbering of the pooled subfractions of Ab5_F12, their dry matter yields and the amounts of used MeOH for re-dissolving are depicted.

Fraction number old	Pooled fraction number	Dry matter yields [mg]	MeOH [µL]
0 – 5	1	2.75	1200
6	2	1.37	500
7 – 8	3	0.23	500
9 – 10	4	0.58	500
11	5	0.82	500
12	6	7.13	-
13	7	1.34	500
14	8	0.86	500
15	9	2.98	500

All residues except of subfraction 6 (Ab5_F12_6) were subsequently re-dissolved in MeOH for diffusion disc assays. As subfraction 6 consisted of one single peak it was re-dissolved in d_6DMSO and analyzed using NMR spectroscopy.
After NMR analysis fraction F12_6 was dried by lyophilizing and then re-dissolved in MeOH for HRMS analyses.

3.12. Mass spectrometry (MS)

In order to achieve information about the molar mass (and to some extend the sum formula) of possible active compounds, HRMS (direct injection) of the most interesting bioactive fractions were done. For the measurements, maXis HD ESI- Qq-TOF mass spectrometer (Bruker Corporation, Bremen, Germany) and Bruker Apollo II Electrospray Source (Bruker Corporation, Bremen, Germany) were applied. Following settings were used for ESI ion source: Capillary voltage 3.5 kV; nebulizer 0.8 bar N2; dry gas flow rate 7.0 L/min N2 and dry temperature 200 ºC.
All HRMS analyses were kindly prepared and applied by Daniel Dobusch MSc. at the Department of Pharmaceutical Chemistry.

3.13. NMR- spectroscopy

For analyzing the structure of the active substances, NMR analyses were performed. NMR measurements were executed on Bruker Avance 500 NMR spectrometer (UltraShield) using a 5 mm switchable probe (TCI Prodigy Kryo-probe head, 5 mm, tripelresonance-invers-detection probe head) with z axis gradients and automatic tuning and matching accessory (Bruker BioSpin). For ^{1}H-NMR the resonance frequency was 500.13 MHz and for ^{13}C-NMR 125.75 MHz.

For the analyses the dry substances were dissolved in fully deuterated dimethlysulfoxide (d_6DMSO) or MeOH (d_4MeOH), and all measurements were performed at 298 K. Standard 1D and gradient-enhanced (ge) 2D experiments, like double quantum filtered (DQF) correlated spectroscopy (COSY), nuclear overhauser enhancement spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC), were used as supplied by the manufacturer. Chemical shifts are referenced internally to the residual, non-deuterated solvent signal for d_6DMSO ^{1}H (δ 2.50 ppm) or d_6MeOH (δ 3.31 ppm) and to the carbon signal of the solvent for d_6DMSO ^{13}C (δ 39.50 ppm) or d_6MeOH (δ 49.00 ppm).

All NMR- spectra were recorded, evaluated and interpreted kindly by ao. Univ.-Prof. Mag. Dr. Ernst Urban at the Department of Pharmaceutical Chemistry.

3.14. Chemicals

- LiChrosolv® Reag. Ph Eur **Acetonitrile** gradient grade for liquid chromatography (Merck KGaA)
- LiChrosolv® Reag. Ph Eur **Methanol** gradient grade for liquid chromatography (Merck KGaA)
- HiPerSolv CHROMANORM® **Methanol** for HPLC LC-MS grade, VWR PROLABO® chemicals
- AnalaR NORMAPUR® **Dichloromethane**, VWR PROLABO® chemicals
- **Formic acid** (Carl Roth GmbH + Co. KG)

3.15. Technical equipment

- Eppendorf Centrifuge 5804 R
- Ultrasonic bath: Branson 3210/ 5200
- Rotavapor: Heidolph VV2011
- Lyophilizer: CHRIST Alpha 2-4 LDplus
- Lyophilizer: VaCo 5-II, Zirbus technology GmbH, Germany
- Rotary shaking incubator: Infors HT Shaker, Multitron Pro, Switzerland
- Analytical balance: Sartorius Lab Instruments GmbH & Co. KG, Germany

4. Results and discussion

4.1. Low scale and upscale extracts

For first investigations, one 250 mL Erlenmeyer filled with 10 g of rice medium per fungus was inoculated (low scale extract) (**Table 29**). After identification of active fungal strains with diffusion disc assays, upscales were prepared and extracted as described in **chapter 3.8**. to achieve more dry matter of produced compounds (**Table 30**).

Table 29: Colors of the low scale extracts, their dry matter yields and the amounts of used MeOH for re-dissolving are depicted.

Low scale extract	Color	Dry matter yield [mg]	MeOH [mL]
LN733	reddish	418.6	2
LN303	fulvous	113.44	3
LN487	yellow	118.64	3
LN255	green grey	178.22	3
LN525	dark green	381.37	3.5

Table 30: Dry matter yields of the upscale extracts from active fungi and the amounts of used MeOH for re-dissolving are depicted.

Upscale	Dry matter yield [mg]	MeOH [mL]
LN733	14928.25 mg	28
LN303	1. upscale: 847.02 mg	14
	2. upscale: 937.00 mg	16
Ab5	609.09 mg	3

4.2. Diffusion disc assays

Bioactivity investigations were done with diffusion disc assays as depicted in **chapter 3.7**. First assays were prepared with the low scale extracts. Based on these results (**Table 31**) it was determined which endophytes should be further investigated for this Thesis. All bioassays showed activities against *E. coli, B. subtilis* and *S. cerevisiae* after 24 hours and activities against *F. graminearum* and *A. niger* after 48 hours.

Table 31: Detailed results of the bioassays of the low scale extracts are represented. Diameters of the inhibition zones were measured in mm.

Extract	*E. coli*	*B. subtilis*	*S. cerevisiae*	*A. niger*	*F. graminearum*
LN733	9 mm (i), (pi)	8.5 mm (i)	9.5 mm (pi)*	-	9.5 mm (pi)
LN303	9 mm (i)*	-	7 mm (i)	10 mm (i)	25 mm (pi)
LN487	-	-	-	-	-
LN255	-	-	-	-	-
LN525	-	9 mm (pi)	-	(+)**	-

*(i) represents complete inhibition and (pi) partial inhibition of the test organism (**Figure 21**).

**LN525 showed a weak pi (+) against *A. niger*, which could not be measured in mm.

LN733 was analyzed microscopically because two different types of inhibitions appeared (**Figure 22**). Microscopic analysis showed that in the first inhibition zone (1.5 mm width), total inhibition with lysed cells occurred and the second zone (1.5 mm width) showed a partial inhibition (observed by Mag. Dr. Martina Oberhofer, personal communication).

Based on these first results, the endophytes LN487 and LN255 were excluded from further investigations. Considering the aspect that LN525 showed only a partial inhibition singly against *B. subtilis*, its analysis was no longer part of this thesis. Consequently, LN733 and LN303 were further investigated.

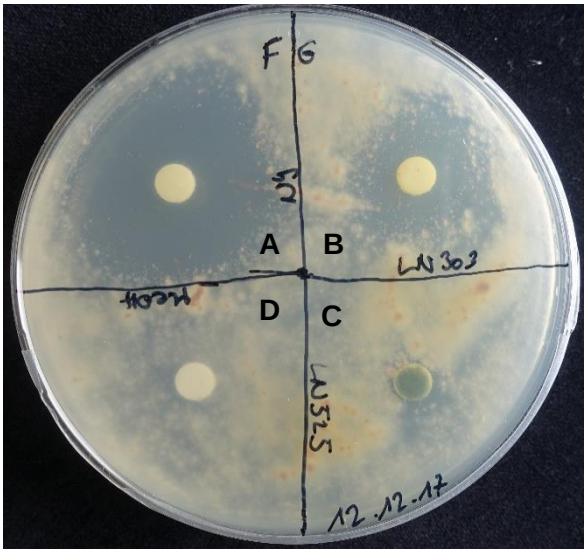


Figure 21: Diffusion disc bioassay testing the low scale extracts of LN303 and LN525 for activity against *F. graminearum* with nystatin (A) as positive and MeOH (D) as negative control. LN303 (B) showed an inhibition and is an example for a positive reaction, and LN525 (C) lacking an inhibition of the test organism is an example for a negative result.

Figure 22: Diffusion disc bioassay testing the low scale extracts of LN733 and LN487 for activity against *E. coli* with MeOH (C) as negative control. LN733 (B) showed an inhibition and is an example for a positive reaction, and LN487 (A) lacking an inhibition of the test organism is an example for a negative result.

For the upscale extracts LN733 was inoculated the same way as the low scale extract, but to accelerate the growth and to get a higher yield of LN303, peptone enriched rice media as described in **chapter 3.8.** were used for the upscale extract. After extraction, the yellow color of the upscale was more intensive than the low scale extract, probably because of the difference in the concentrations of the extracts.

As LN303 was inoculated with peptone supplementation for the upscale (LN303_Ups.2), its bioactivity was tested again by using diffusion disc assays (**Table 32**) to see, if peptone addition had an impact on bioactivity. To see an immediate comparison between the low scale extract and the upscale extract, both were tested at one petri dish. Based on the results of the bioassays it was determined that the addition of peptone did not have an effect on the fungus in terms of higher bioactivity. On the contrary bioassays showed that the upscale has a lower activity against the test microorganisms (**Figure 23**).

Table 32: Results of the bioassays of LN303 low scale extract and LN303_Ups. are depicted by comparison. Diameters of the inhibition zones were measured in mm.

Extract	*E. coli*	*S. cerevisiae*	*A. niger*	*F. graminearum*
LN303 (low scale extract)	9 (i)*	9 (i)	13 (pi)*	14 (pi)
LN303_Ups.	7 (i)	7 (i)	10 (pi)	12 (pi)

*(i) represents complete inhibition and (pi) partial inhibition of the test organism.

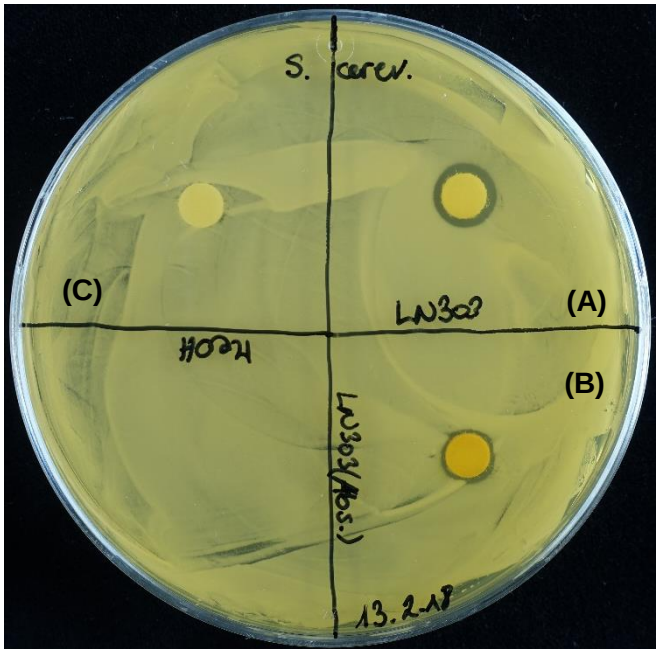


Figure 23: Diffusion disc bioassay testing the low scale extract of LN303 (A) and LN303_Ups.2 (B) for activity against *S. cerevisiae* by comparison. MeOH (C) represents negative control. Both showed an inhibition and are examples for a positive reaction.

4.3. Results and discussion of LN733

A 1:10 dilution of the low scale extract with a final concentration of 20.93 mg/mL was used at the beginning of the investigations. With a linear increasing gradient of ACN from 5% to 95% in 45 min, several overlapping peaks were observed (**Figure 9**). Thus, an optimized gradient (**Table 9**) was determined and used for further analyses for a better base line separation of the peaks (**Figure 24**).

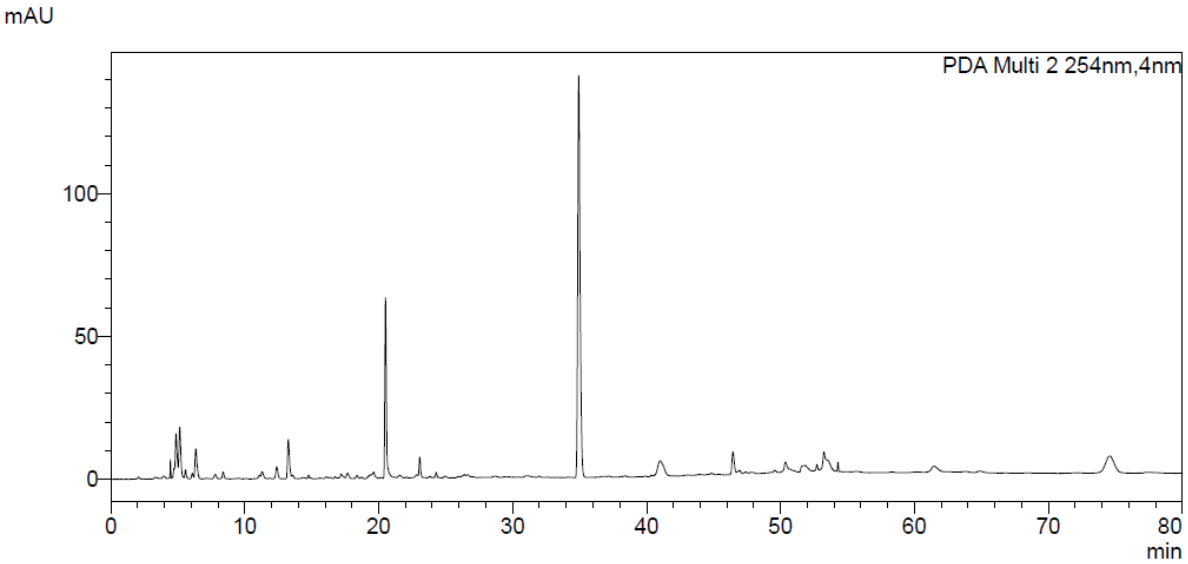


Figure 24: Analytical HPLC separation of LN733 low scale extract (20.93 mg/mL) using the optimized gradient (Table 9) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 5 µL.

<u>Semi-preparative HPLC of LN733 low scale extract and bioassays</u>

Subsequently, semi-preparative separation with a 1:10 dilution of LN733 low scale extract with a final concentration of 20.93 mg/mL was performed using the optimized gradient from analytical separation (**Figure 25**).

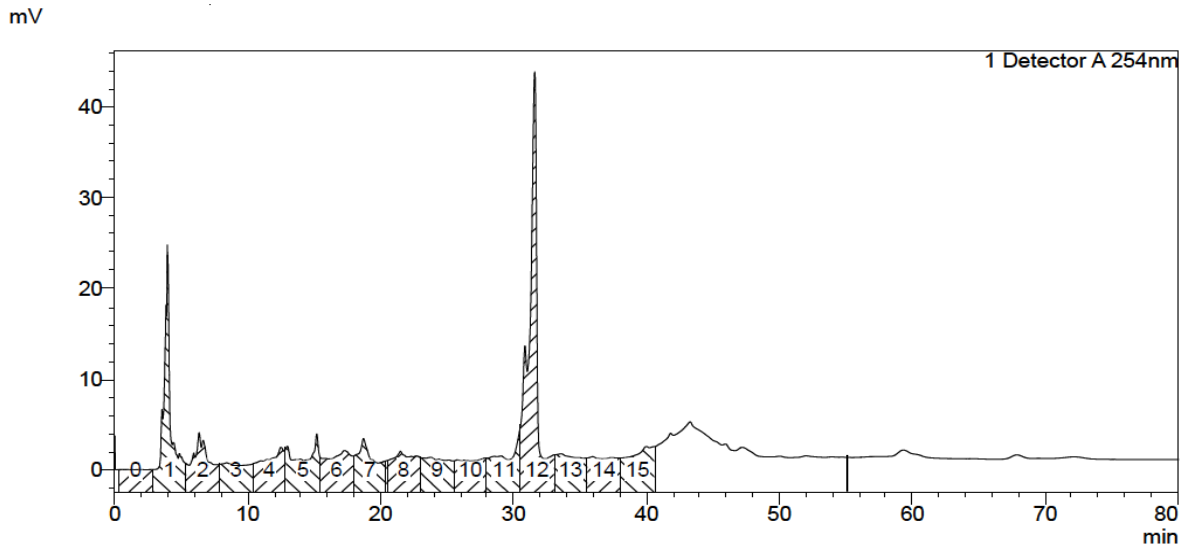


Figure 25: Semi-preparative HPLC separation of LN733 low scale extract (20.93 mg/mL) using optimized gradient (Table 9) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 2 mL. 18 fractions were collected.

As the absorbance maximum of the substances was detected at 254 nm in the analytical run, the semi-preparative run was analyzed at this wavelength. For fractionation, 2 mL corresponding to 41.86 mg of the low scale extract, was injected. Bioassays were conducted, but none of the fractions showed an antibiotic activity. Based on these results, the separation was repeated with a 1:2 dilution of the low scale extract, which had a concentration of 104.65 mg/mL (**Figure 26**).

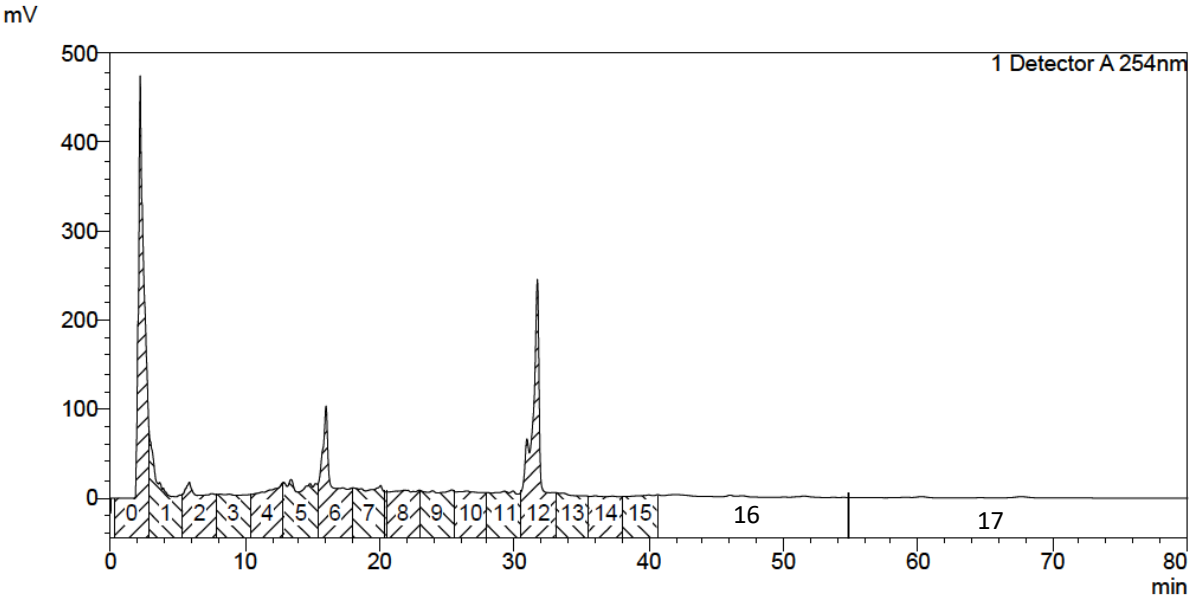


Figure 26: Semi-preparative HPLC separation of LN733 low scale extract (104.65 mg/mL) using the optimized gradient (Table 9) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 2 mL. 18 fractions were collected.

The fractions were dried by a combination of evaporation on the rotavapor and lyophilizing for remnant water. Then the residues were re-dissolved in MeOH. **Table 33** shows the dry matter yields of the fractions of both dilutions by comparison. Due to a measuring error of the analytical balance negative yields arose at some fractions, although a residue was visible after evaporation, and the fractions of the separation using the 1:10 dilution (20.93 mg/mL) seemed to have a higher yield. Although, due to the results of the bioassays (**Table 34**) it can be concluded that the fractions of the 1:2 dilution (104.65 mg/mL) must have had a higher yield because this time the bioactivity of the fractions occur.

Table 33: Dried crude extracts had different weights in respect to dilution and fraction. Negative yields are because of a measuring error of the analytical balance.

Fractions	Dried crude extract 1:10 dilution (20.93 mg/mL) [mg]	Dried crude extract 1:2 dilution (104.65 mg/mL) [mg]	Fractions	Dried crude extract 1:10 dilution (20.93 mg/mL) [mg]	Dried crude extract 1:2 dilution (104.65 mg/mL) [mg]
0	3.2	33.1	**9**	1.8	0.5
1	11.4	11.6	**10**	2.1	0.3
2	6.2	0.9	**11**	3.5	1.8
3	7	0.2	**12**	3.3	-0.5
4	5.1	-0.6	**13**	5.5	8
5	3	0.7	**14**	4	0.6
6	7.3	0.5	**15**	3.8	0.8
7	5.1	-0.8	**16**	6.4	12.1
8	1.5	2.3	**17**	12.8	1.3

Table 34: Inhibition zones caused by different fractions and dilutions in diffusion disc assays of LN733 low scale extract (1:2 dilution, 104.65 mg/mL). Diameters of the inhibition zones were measured in mm.

Fraction	*E. coli*	*B. subtilis*	*S. cerevisiae*	*F. graminearum*
1:2 dilution	9 (pi)*	10 (i)*	7 (pi)	-
0	-	-	-	-
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	10 (pi)	-
10	-	-	11 (pi)	-
11	-	-	-	-
12	10 + 2 (pi)	8 (i)	-	-
13	9 (pi)	6 (i)	-	-
14	-	-	-	-
15	-	-	-	-
16	-	9 (i)	-	-
17	-	-	-	-

*(i) represents complete inhibition and (pi) partial inhibition of the test organism.

Based on the results of the bioassays, it is conspicuous that diluting the extract had the consequence that the activity against *F. graminearum* got lost. As the focus of this Thesis was primarily investigating antibiotic activities, this aspect was not further elaborated.

Only fraction 12 and 13 had an activity against both bacteria **(Table 34)** and based on these results, these two fractions were selected for further analyses. The other active fractions 9, 10 and 16 were analyzed with HRMS.

However, before HRMS analyses, the semi-preparative separation was repeated with the upscale of LN733 at which only the active fractions were collected. **Table 35** shows the dry matter yield of the antibacterial active fractions.

Table 35: Dry matter yields of the active fractions of LN733 upscale extract are depicted in mg.

Fraction	Dry matter yield [mg]
9	2.9
10	4.3
12	4.76
13	3.2

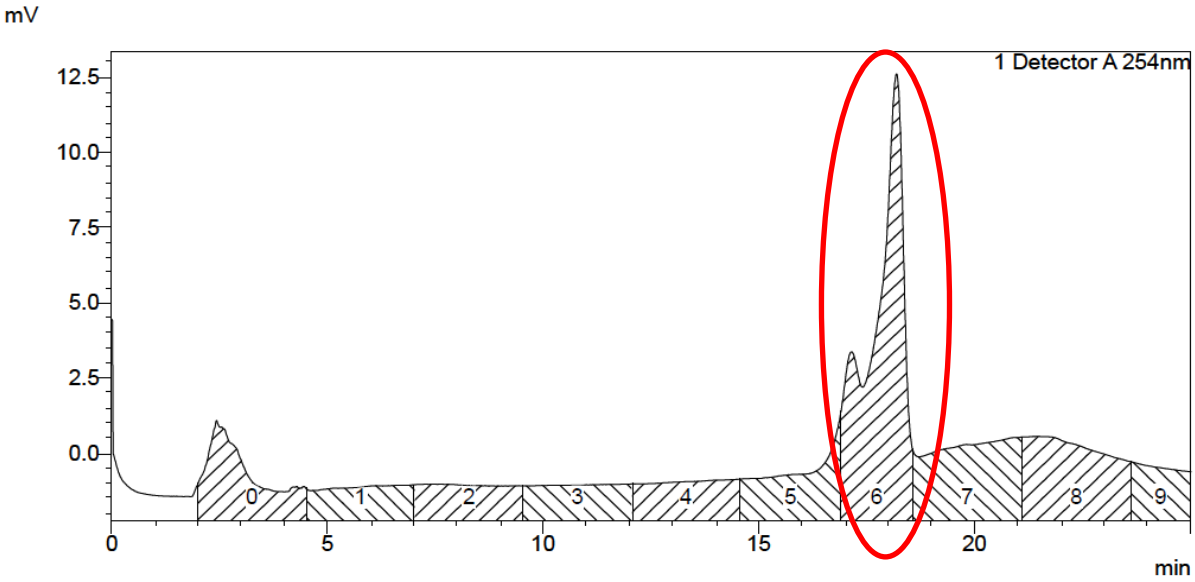


Figure 27: Chromatogram of the semi-preparative HPLC separation of LN733_F12 (0.79 mg/mL) using the optimized gradient (Table 12) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 2 mL. 10 fractions were collected, while fraction 6 was collected manually.

Considering the aspect, that two overlapping peaks occurred in fraction 12 at semi-preparative separation, fraction LN733_F12 was used for subfractionation (**Figure 27**) using the optimized gradient showed in **Table 12**. The yield of LN733_F12 was about 4.76 mg in 6 mL. Consequently, 3 runs of each 2 mL were performed, while the main peak with the retention time 16.91 min was collected manually.

Subsequently, diffusion disc assays against *E. coli* and *B. subtilis* were performed (**Table 36**).

Table 36: Inhibition zone diameters in mm of the diffusion disc assays of LN733_F12 and LN733_F12.6 tested against *E. coli* and *B. subtilis* are depicted.

	E. coli	***B. subtilis***
LN733_F12	10 + 2 (pi)*	8 (i)*
LN733_F12.6	-	11 (i)

*(i) represents complete inhibition and (pi) partial inhibition of the test organism.

Comparing the activity of LN733_F12.6 with the whole fraction 12 **(Table 36)**, it was obvious that the isolated fraction F12.6 had a higher activity against the test organism *B. subtilis.* For *E. coli*, it was quite the contrary. While the whole fraction 12 had a notable activity, it got lost after subfractionation so that the isolated fraction 12.6 did not show any activity against this bacterium. Consequently, diffusion disc assays were performed for all subfractions of LN733_F12 to test whether the active substance against *E. coli* was within the previous fractions (0-5), or the following fractions (7-9). No activities could be detected.

It can be presumed, based on these results that the activity against *E. coli* was a result of synergistic effects caused by multiple unknown substances within the extract, since it was lost as a consequence of the fractionation.

NMR results of LN733_F12.6

The yield of LN733_F12.6 was 1.9 mg. The substance was dissolved in deuterated MeOH (d_4MeOH) and ^{1}H- and ^{13}C- NMR spectra were recorded and analyzed kindly by ao. Univ.-Prof. Mag. Dr. Ernst Urban at the Department of Pharmaceutical Chemistry.

Finally, the substance LN733_F12.6 was identified as linoleic acid (**Figure 28**). The molecular formula is $C_{18}H_{32}O_2$ and it has a molecular weight of 280,4472 g/mol (PubChem).

NMR analyses detailed that the substance had a sufficient purity for structure analysis but there were some signals appearing, which did not belong to linoleic acid.

^{1}H- and ^{13}C- NMR spectra can be found in the appendix.

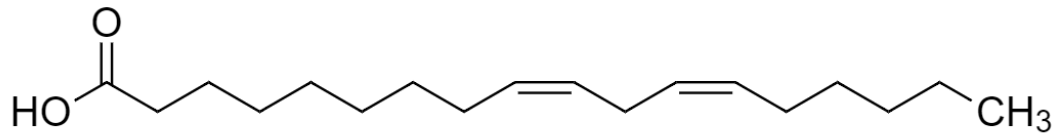


Figure 28: This figure shows the structural formula of linoleic acid, with the molecular formula $C_{18}H_{32}O_2$ and the molecular weight of 280.4472 g/mol (PubChem). Structure drawn with ChemDraw.

HRMS results of LN733_F12.6

In negative and positive mode linoleic acid was the main compound. In negative mode, linoleic acid occurred with the *m/z* = 279.2327, missing one hydrogen atom, with an intensity of approximately 7 x 10^6 (**Figure 29**). In positive mode the fatty acid is present as hydrogen adduct at *m/z* = 303.2296 with an intensity of approximately 1.8 x 10^6 (**Figure 30**). Linoleic acid had a higher intensity in negative mode because its carbonic acid function can be easily deprotonated in negative mode. In positive mode more signals are shown, because possible contaminations of the sample are empirically substances, which can be ionized rather in positive mode than in negative mode (personal communication, Mag. Dr. Judith Wackerlig).

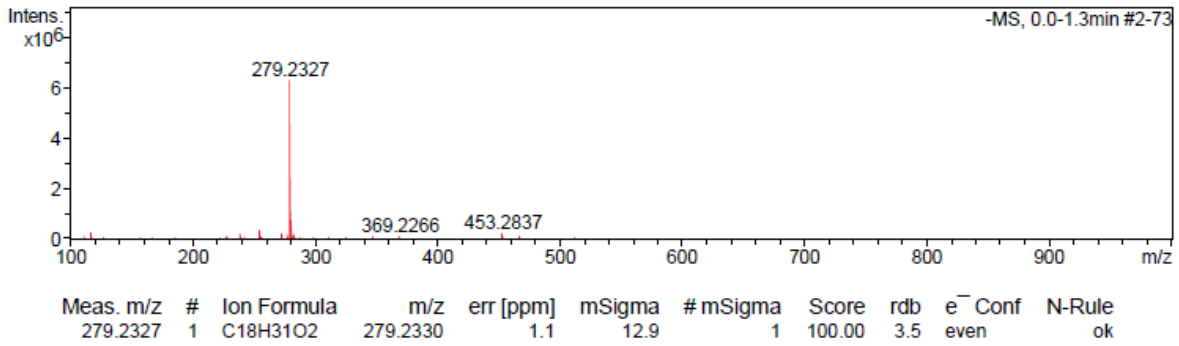


Figure 29: This figure shows the HRMS data of LN733_F12.6 in negative mode $[M-H]^-$. *m/z* 279.2327 depicts the mass of linoleic acid missing one hydrogen atom. It has an intensity of approximately 7 x 10^6.

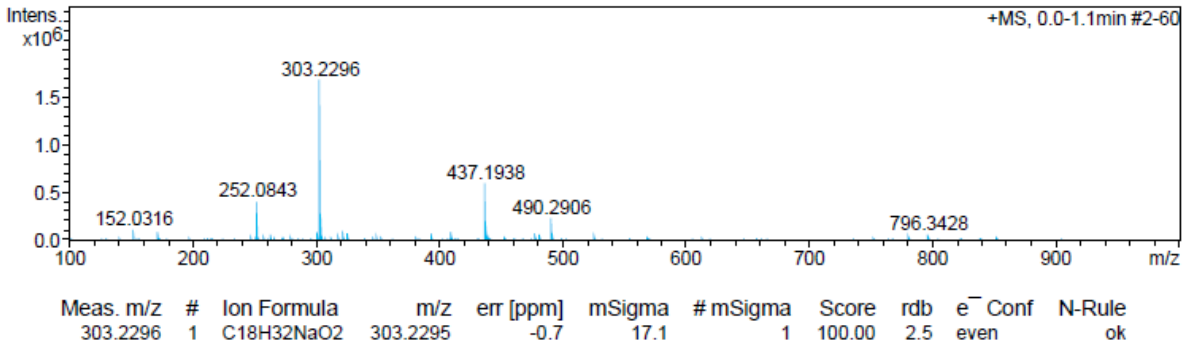


Figure 30: This figure shows the HRMS data of LN733_F12.6 in positive mode $[M+H]^+$. *m/z* 303.2296 depicts the mass of linoleic acid as sodium adduct. It has an intensity of approximately 1.8 x 10^6.

Results of Stas-Otto separation

Stas- Otto separation process was used to separate linoleic acid from the other unknown substances, because NMR analysis showed that linoleic acid was not a pure and the only substance within F12.6.

F13 was treated the same way, because it had the same activities as F12.6 and linoleic acid as one compound.

According to the description of Stas-Otto separation, linoleic acid should be in the final ether phase (E2) at the end of the separation process, while the other unknown compounds should remain in the initial ether phase (E1).

HRMS analyses in negative mode of the ether phases produced following results (**Table 37**), at which the ether phases of both fractions (F12.6 and F13) were nearly identical and in the initial ether phases (F12.6_E1 and F13_E1) linoleic acid was the main component. The ether phases contained many fatty acids as, *e.g*. linoleic acid, palmitic acid, stearic acid, *etc*. **(Table 37)**. As these substances have carbonic acid as functional group, they were ionized rather in negative mode than in positive mode.

Table 37: Results of the HRMS analyses of the ether phases of LN733_F12.6 and LN733_F13 are depicted. The calculated *m/z* values, their intensities and proposed formulas in negative mode $[M-H]^-$ are represented.

Fraction	*m/z* $[M-H]^-$	Intensity	Proposed formula	Substances
F12.6_E1	279.2330	1.7×10^5	$C_{18}H_{31}O_2$	Linoleic acid
	599.4727	1.8×10^4	$C_{36}H_{63}O_4$	Dilinoleic acid
F13_E1	255.2327	5.2×10^4	$C_{16}H_{31}O_2$	Palmitic acid
	279.2328	3.7×10^5	$C_{18}H_{31}O_2$	Linoleicacid
	559.4719	1.40×10^4	$C_{36}H_{63}O_4$	Dilinoleic acid
F12.6_E2	223.1452	3×10^2	$C_{12}H_{19}N_2O_2$	Aspergillic acid
	239.1401	4.6×10^2	$C_{12}H_{19}N_2O_3$	Hydroxyaspergillic acid
	255.2330	2.8×10^4	$C_{16}H_{31}O_2$	Palmitic acid
	279.2330	1.3×10^4	$C_{18}H_{31}O_2$	Linoleic acid
	283.2643	3.2×10^4	$C_{18}H_{35}O_2$	Stearic acid
F13_E2	223.1452	0.8×10^4	$C_{12}H_{19}N_2O_2$	Aspergillic acid
	239.1401	2.3×10^3	$C_{12}H_{19}N_2O_3$	Hydroxyaspergillic acid
	255.2330	1.7×10^4	$C_{16}H_{31}O_2$	Palmitic acid
	279.2330	1.7×10^4	$C_{18}H_{31}O_2$	Linoleic acid
	283.2643	1.6×10^4	$C_{18}H_{35}O_2$	Stearic acid
	559.4715	2.5×10^2	$C_{36}H_{63}O_4$	Dilinoleic acid

In view of the results of the HRMS analysis, the Stas-Otto separation process was not delivering results as expected, because there were other carbonic acids within the unknown compounds. Thus, it was not possible to separate linoleic acid from them. Furthermore, linoleic acid was not only in the final ether phase (E2) as expected, but in both ether phases.

Furthermore, NMR analyses were performed of all ether phases with following results: at both initial ether phases (F12.6_E1 and F13_E1) linoleic acid was the main component, at which again other signals of unknown compounds were visible. Final ether phases (F12.6_E2 and F13_E2) contained an organic compound as main component, which was considered a contamination.

Bioassays of the ether phases

As shown in **Table 38**, only the initial ether phases of the subfractions had an activity against *B. subtilis*, while F13 had a stronger activity than F12.6.

Table 38: Results of the diffusion disc assays of the ether phases of LN733_F12.6 and LN733_F13 against *B. subtilis* and *E. coli* are depicted. Diameters of the inhibition zones were measured in mm.

	E. coli	*B. subtilis*
LN733_F12.6_E1	-	7 (i)
LN733_F12.6_E2	-	-
LN733_F13_E1	-	10 (i)
LN733_F13_E2	-	-

(i) represents complete inhibition of the growth of the test organisms.

The stronger activity of F13 could be explained with the HRMS results (**Table 37**). In F13_E1 linoleic acid had a higher intensity than in F12.6_E1, and besides linoleic acid, palmitic acid occurs in F13. It is possible that both carbonic acids had a synergistic effect regarding the activity against the bacterium.

HRMS results of LN733

For HRMS, the fractions were diluted with MeOH so that they had a final concentration about 0.1 mg/mL. HRMS measurements of following fractions were performed at positive and negative mode: F9, F10, F12, F13 and F16.

Fraction 9 and 10

HRMS measurements were performed, because Fraction 9 and 10 had an activity against *S. cerevisiae*.

In positive mode, the mass peak 318.279 [*m/z*] had the highest intensity (F9= 1.7×10^6, F10= 6×10^4) and was as well present in fractions 12 and 13. This signal was not detected in fractions F7, F11, F15 and F16.

Furthermore, two masses were interesting because they occurred also in negative mode. **Table 39** shows the results of the HRMS analysis. Further investigations are being performed at the Department of Pharmacognosy.

Table 39: Results of the HRMS analysis of LN733_F9 and LN733_F10 are depicted. The calculated *m/z* values, their intensities and proposed formulas in negative mode $[M-H]^-$ are represented.

Fraction 9 + 10	*m/z*	Proposed formula	Substance
Negative mode $[M-H]^-$	223.1452	$C_{12}H_{19}N_2O_2$	Aspergillic acid
	239.1401	$C_{12}H_{19}N_2O_3$	Hyrdoxyaspergillic acid
	279.2317	$C_{18}H_{31}O_2$	Linoleic acid

Fraction 12 and 13

Measurements of Fractions 12 and 13 were performed in positive and negative mode. The results are depicted in **Table 40**. In positive mode again, *m/z* = 318.2791 was conspicuous with an intensity of 6.0×10^4 in F12 and 1.0×10^4 in F13.

Table 40: Results of the HRMS analysis of LN733_F12 and LN733_F13 are depicted. The calculated *m/z* values, their intensities and proposed formulas in negative mode $[M-H]^-$ are represented.

	m/z	Proposed formula	Substances
Faction 12	223.1447	$C_{12}H_{19}N_2O_2$	Aspergillic acid
	239.1397	$C_{12}H_{19}N_2O_3$	Hyrdoxyaspergillic acid
Fraction 13	223.1451	$C_{12}H_{19}N_2O_2$	Aspergillic acid
	239.1401	$C_{12}H_{19}N_2O_3$	Hyrdoxyaspergillic acid
	255.2320	$C_{16}H_{31}O_2$	Palmitic acid
	279.2319	$C_{18}H_{31}O_2$	Linoleic acid

Fraction 16

Mass spectrometry of F16 was performed in positive and negative mode, at which in positive mode the mass peak at *m/z* = 303.2294 with the highest intensity was proposed as sodium adduct of linoleic acid.

In negative mode, again the masses of aspergillic acid and hydroxyaspergillic acid occurred as in F9, 10, 12 and 13. In addition, saturated and unsaturated fatty acids as, *e.g.,* palmitic acid, linoleic acid, oleic acid, *etc*. were detected (**Table 41**).

Table 41: Results of the HRMS analysis of LN733_F16 are depicted. The calculated *m/z* values, their intensities and proposed formulas in positive $[M+H]^+$ and negative mode $[M-H]^-$ are represented.

Fraction 16	*m/z*	Proposed formula	
Negative mode $[M-H]^-$	223.1441	$C_{12}H_{19}N_2O_2$	Aspergillic acid
	239.1388	$C_{12}H_{19}N_2O_3$	Hyrdoxyaspergillic acid
	255.2321	$C_{16}H_{31}O_2$	Palmitic acid
	279.2321	$C_{18}H_{31}O_2$	Linoleic acid
	281.2476	$C_{18}H_{33}O_2$	Oleic acid
Positive mode $[M+H]^+$	247.1409	$C_{12}H_{20}N_2NaO_2$	Aspergillic acid
	263.1368	$C_{12}H_{20}N_2N_aO_3$	Hyrdoxyaspergillic acid
	303.2296	C18H32NaO$_2$	Linoleic acid

Discussion of LN733

The endophytic fungus with strain number LN733 was identified as *Aspergillus westerdijkiae*. Within this Thesis, it was possible to identify the antimicrobial activity of the fungal extract *via* bioactivity guided fractionation. Regarding to diffusion disc assays the endophyte inhibits the growth of the test organisms *E. coli*, *B. subtilis* and *S. cerevisiae*.

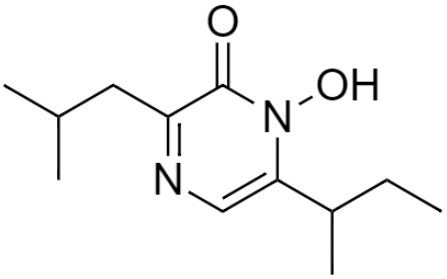


Figure 31: This figure shows the molecular structure of aspergillic acid. Structure drawn with ChemDraw.

HRMS analyses showed that the endophyte produces many unsaturated and saturated fatty acids like linoleic acid, palmitic acid, oleic acid and stearic acid. In addition, the masses of aspergillic acid (**Figure 32**) and hydroxyaspergillic acid occurred in HRMS analyses.

It is known that some *Aspergillus* sp. are producing aspergillic and hydroxyaspergillic acids, which are antibiotic active substances (Dutcher, 1947; MacDonald, 1961). Additionally, Visagie *et al*. (2014) could prove neohydroxyaspergillic acid ($C_{12}H_{20}N_2O_3$) (**Figure 31**) as secondary metabolite produced by *A. westerdijkiae*. However, it is not detailed against which bacteria the antibiotic activity is directed.

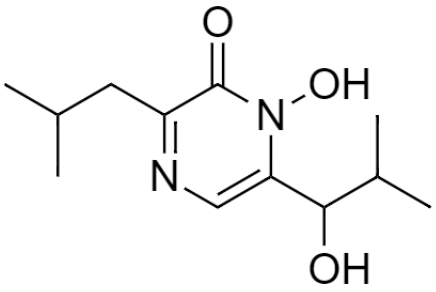
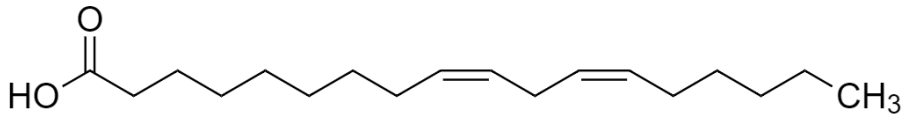
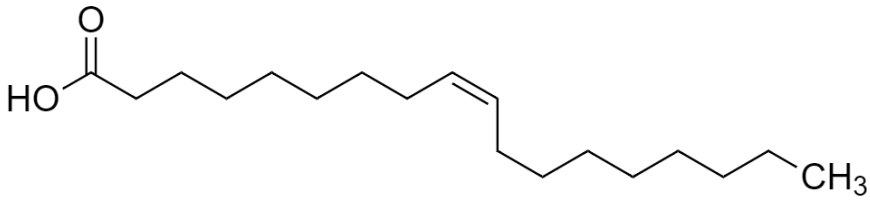
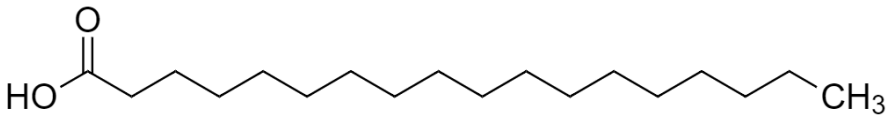
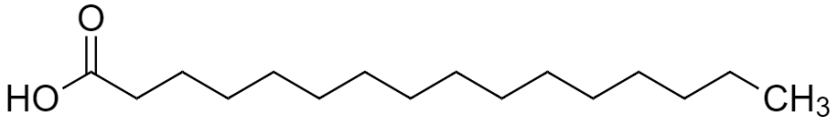


Figure 32: This figure shows the molecular structure of neohydroxyaspergillic acid. Structure drawn with ChemDraw.

Zheng *et al.* 2005 reported about the bactericidal actions of fatty acids. Especially unsaturated long- chain fatty acids like linoleic acid, linolenic acid and oleic acid have a strong antibacterial bioactivity against Gram- positive bacteria. Furthermore, saturated long- chain fatty acids like palmitic acid and stearic acid have a lower activity against Gram- positive bacteria. None of these fatty acids have an activity against Gram- negative bacteria (**Table 42**).

Table 42: Structural formulas of fatty acids produced by *A. westerdijkiae* are represented (Structures drawn with ChemDraw).

	**Linoleic acid–** unsaturated long- chain fatty acid
	**Oleic acid**– unsaturated long- chain fatty acid
	**Stearic acid** – saturated long- chain fatty acid
	**Palmitic acid** – saturated long- chain fatty acid

Within this Thesis it could be determined that long- chained fatty acids produced by *A. westerdijkiae* especially inhibited the growth of *B. subtilis*. Furthermore, there were fractions like 12 and 13, which had an antimicrobial activity against *B. subtilis* as well as *E. coli.* Fractions 9 and 10 had an antimicrobial activity against *S. cerevisiae* **(Table 34)**. It is known, that long- chained fatty acids do not have an antimicrobial activity

against Gram- negative bacteria. Based on the investigation results, it can be expected that these two activities (against *E. coli* and *S. cerevisiae*) were caused by synergistic effects of the contained compounds such as aspergillic acid, hydroxyaspergillic acid and other substances which were not identified. It is already known, that both aspergillic acid and hydroxyaspergillic acid are antimicrobial active against *E. coli*.However, aspergillic acid showed a stronger activity against *E. coli* than Hydroxyaspergillic acid (Dutscher *et al*., 1957).

Additional investigation on the fungal endophyte *A. westerdijkiae* were performed in summer 2018 by Mag. Dr. Judith Wackerlig at the Department of Pharmaceutical Chemistry. Further investigations are going on by Mag. Dr. Martina Oberhofer at the Department of Pharmacognosy.

4.4. Results and discussion of LN303

For the investigation of LN303, a 1:2 dilution of the upscale extract with a final concentration of 30.25 mg/mL was used with the initial gradient as described in **Table 14**. As there were many overlapping peaks, the measurement was repeated at which the mobile phase was acidulated with 0.1% formic acid. **Figure 33** show that there was no apparent difference between the analytical separations.

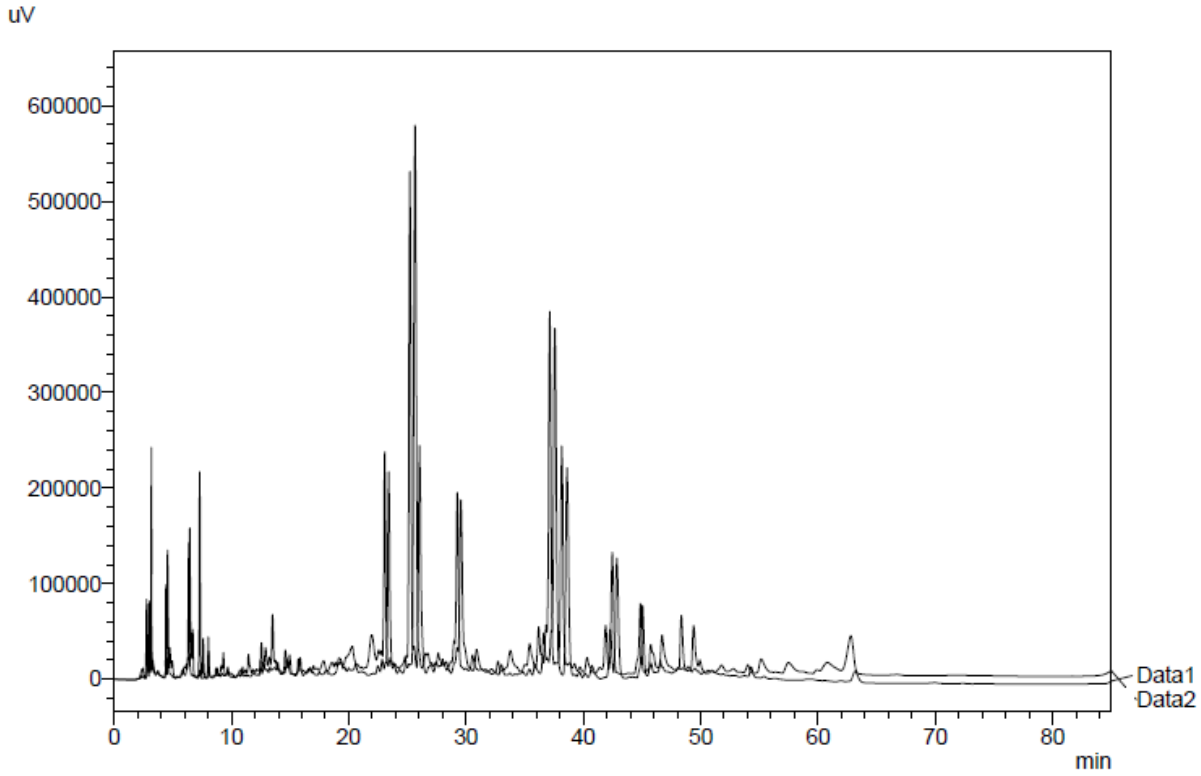


Figure 33: Comparison of two analytical HPLC separations of LN303 upscale extract (30.25 mg/mL) using initial gradient (Table 14) is depicted. At Data1 the mobile phase was acidulated with 0.1% formic acid. Data2 shows the analytical run without acidulated mobile phase. Analyses were detected at wavelength 254 nm. Injection volumes were 10 µL.

As a result, semi-preparative fractionation was performed with a 1:2 dilution (30.25 mg/mL) of the upscale extract, corresponding to 60.50 mg of the upscale extract, without acid addition, using the same gradient as in the analytical separation (**Figure 34**).

Table 43 shows the yields of the fractions and the amounts of MeOH, which was used for re-dissolving. Because of the measuring error of the analytical balance, negative

yields arose at some fractions although a residue was visible in the round bottom flask after evaporation.

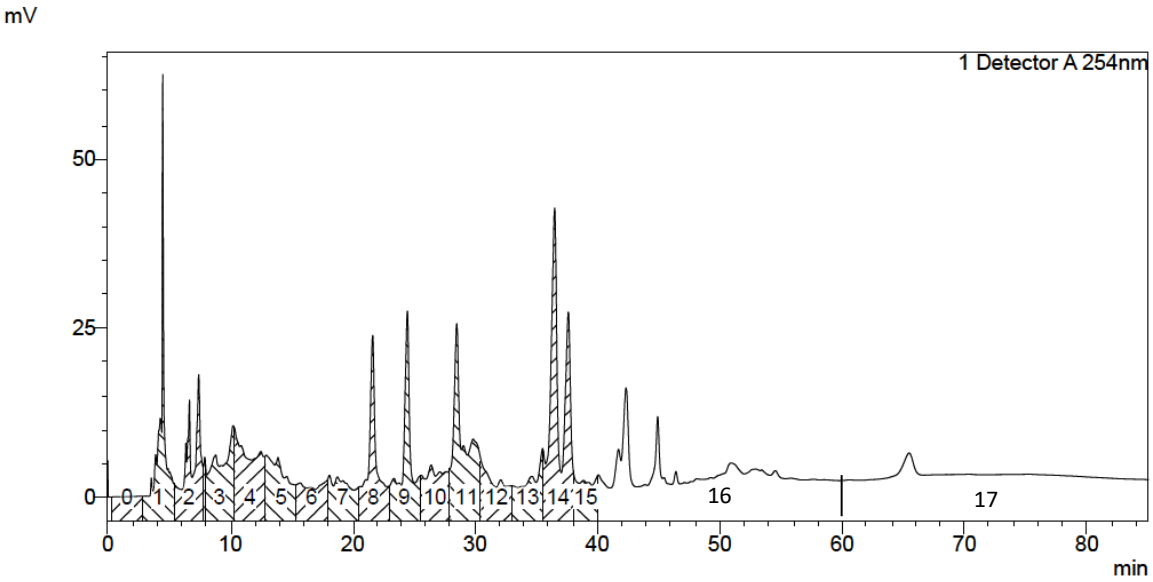


Figure 34: Semi-preparative HPLC fractionation of the upscale extract of LN303 (LN303_A_) (30.25 mg/mL) using initial gradient (Table 14) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 2 mL. 18 fractions were collected.

Table 43: Dry matter yields of the fractions of LN303 upscale extract (60.50 mg/mL) and the amounts of used MeOH for re-dissolving are depicted.

Fractions	Dry matter yield [mg]	MeOH [µL]	Fractions	Dry matter yield [mg]	MeOH [µL]
0	- 0.7*	350	**9**	- 0.22*	400
1	5.56	300	**10**	0.24	400
2	0.86	300	**11**	0.43	400
3	0.36	400	**12**	- 0.02*	300
4	0.7	400	**13**	- 0.9*	300
5	0.8	400	**14**	0.44	300
6	- 0.72*	400	**15**	0.22	300
7	0.24	400	**16**	5.02	1500
8	0.62	350	**17**	11.40	1300

* Negative yields because of a measuring error of the analytical balance.

The low intensity of the peaks was suggestive that the concentration of the diluted extract, which was 30.25 mg/mL, was not enough to achieve positive results at the bioassays. As a result, only one fraction had an activity against *S. cerevisiae*,

F. graminearum and *A. niger* at diffusion disc assays (**Table 44**), although the undiluted extract was active against *E. coli*, *S. cerevisiae*, *F. graminearum* and *A. niger*.

Table 44: Results of the diffusion disc assays of LN303 upscale extract (LN303_A_) (30.25 mg/ mL) are depicted. Diameters of the inhibition zones were measured in mm.

Fractions	*E. coli*	*S. cerevisiae*	*A. niger*	*F. graminearum*
Undiluted upscale extract	7 (i)*	7 (i)	10 (pi)*	12 (pi)
0	-	-	-	-
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
11	-	9 (i)	8 (pi)	10.5 (pi)
12	-	-	-	-
13	-	-	-	-
14	-	-	-	-
15	-	-	-	-
16	-	-	-	-
17	-	-	-	-

*(i) represents complete inhibition and (pi) partial inhibition of the test organism.

Because the 1:2 dilution was too low in concentration (30.25 mg/ mL) to achieve inhibitions in the diffusion disc assay against *E. coli*, it was decided to use the undiluted extract of the upscale for fractionation, which had a concentration of 60.50 mg/mL. Diffusion disc assays were done against *S. cerevisiae* and *E. coli* (**Figure 35 and 36**) in order to compare if the undiluted upscale and the 1:2 dilution had a difference relating to the activity against these microorganisms. The bioassays showed that the

undiluted extract (60.50 mg/mL) and its 1:2 dilution (30.25 mg/mL) had the same dimension of bioactivity against *E. coli* and no significant difference against *S. cerevisiae* (**Table 45**). As result, all further analyses were performed using the undiluted extract of the upscale.

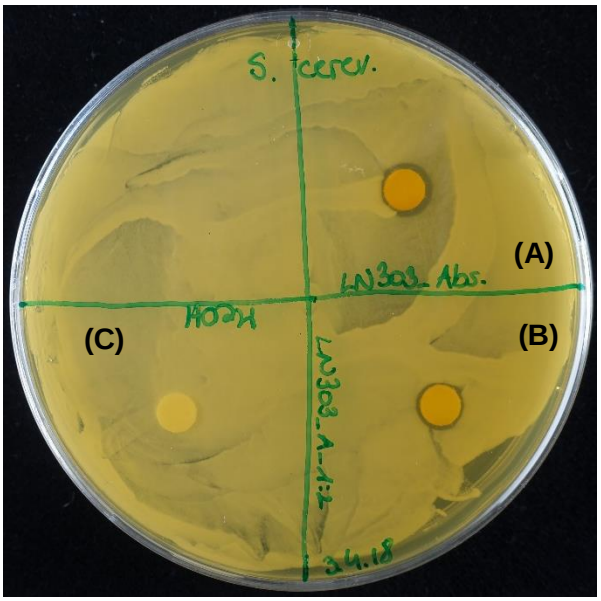


Figure 35: Diffusion disc bioassay testing the upscale extracts of LN303 (60.50 mg/mL) and its 1:2 dilution (30.25 mg/mL) for activity against *S. cerevisiae* by comparison. MeOH represents negative control. Both showed an inhibition and are examples for a positive reaction.

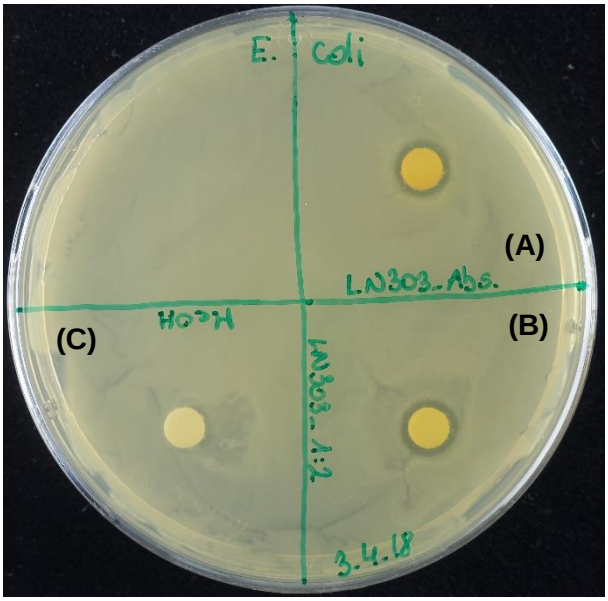


Figure 36: Diffusion disc bioassay testing the upscale extracts of LN303 (60.50 mg/mL) and its 1:2 dilution (30.25 mg/mL) for activity against *E. coli* by comparison. MeOH represents negative control. Both showed an inhibition and are examples for a positive reaction.

Table 45: Results of the diffusion disc assays of LN303 Upscale extract (60.50 mg/mL) and its 1:2 dilution (30.25 mg/mL) against *E. coli* and *S. cerevisiae* are depicted by comparison. Diameters of the inhibition zones were measured in mm.

	E. coli	***S. cerevisiae***
LN303_Ups.	8 (i)*	8 (i)
LN303_1:2	8 (i)	7 (i)

*(i) represents complete inhibition of the test organism.

Considering the aspect, that some substances could precipitate when the undiluted extract encounters the mobile phase, chemical pre-treatments were executed. This should prevent a clogging of the column and loss of sample. This pre-treatment was

not necessary for the diluted extract, because some substances precipitated by diluting it.

The extract was pre-treated in two different ways: 1. The pre-treated extract was acidulated with 5% formic acid before evaporation (**Figure 37**) and 2. the pre-treated extract was not acidulated (**Figure 38**) as described in **chapter 3.10.1.2**. The analytical HPLC analyses did not differ. However, the acid addition had a consequence on the activity of the extract (**Table 46**).

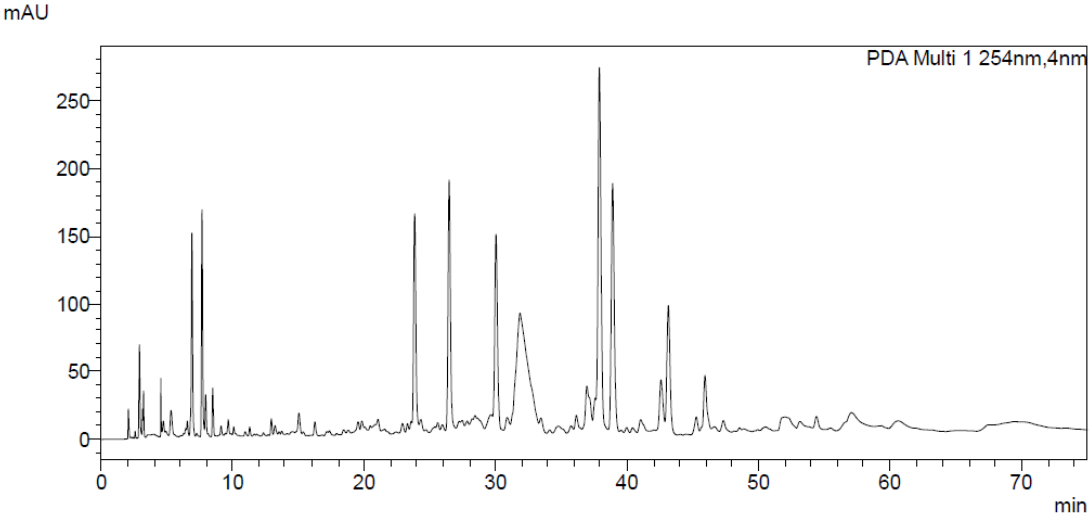


Figure 37: Analytical HPLC analysis of pre- treated upscale extract of LN303 (30.25 mg/mL) with acid addition before evaporating is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 10 µL. Initial gradient (Table 14) was used.

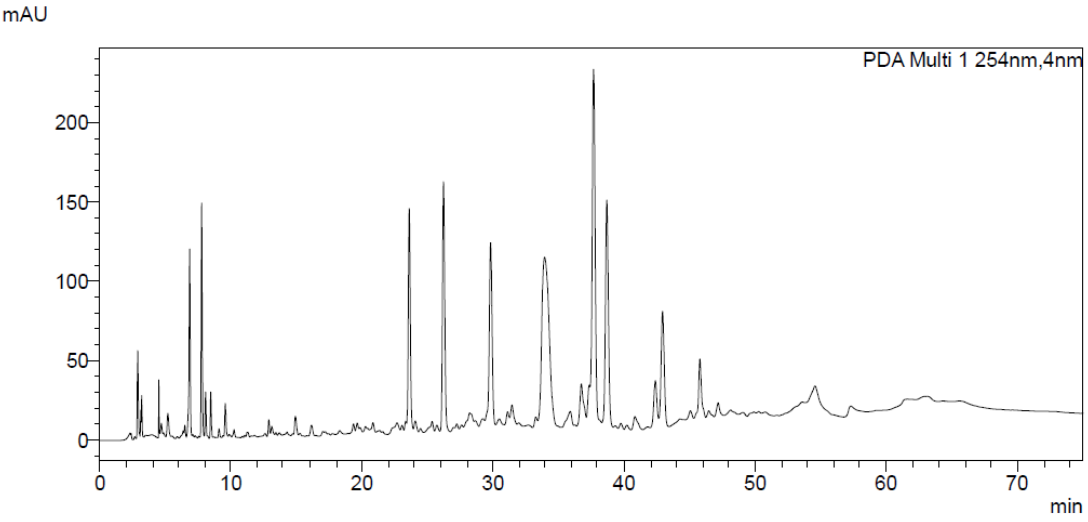


Figure 38: Analytical HPLC analysis of pre-treated upscale extract of LN303 (30.25 mg/mL) without acid addition is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 10 µL. Initial gradient (Table 14) was used.

The pre- treated extract with acid addition had no activity against *E. coli* while the pre-treated extract without acid addition had one. Consequently, it can be said that due the addition of formic acid the activity against *E. coli* was lost while the activity against *S. cerevisiae* persisted at which the activity was weakened slightly.

The precipitate of the pre- treated extract without acidulating were also tested to confirm that the active substances had not precipitated from the aqueous phase. The precipitate was dissolved in MeOH before testing (**Table 46**).

Table 46: Results of the diffusion disc assays of the pre- treated upscale extracts of LN303 against *E. coli* and *S. cerevisiae* are depicted. Diameters of the inhibition zones were measured in mm.

	E. coli	***S. cerevisiae***
Pre- treated extract with acid addition	-	7 (i)*
Pre- treated extract without acid addition – aqueous phase	8 (i)	8 (i)
Pre- treated extract without acid addition – precipitate	-	-

*(i) represents complete inhibition of the test organism.

Semi-preparative HLPC fractionation of the pre-treated upscale extract

After pre-treatment, semi-preparative separation was repeated with the undiluted extract using the same gradient while the analysis lasted for 60 min because no more substances were eluting after this time (**Figure 39**). As it was determined that diluting the extract did not influence the activity against *E. coli* (**Table 45**), other measures were taken to find out, in which fraction the activity was present. For this reason, some fractions were pooled before evaporation and the fractions got a re-numbering (**Table 47**). This measure was supposed to increase the concentration of the substances and in this way to appear the activity against *E. coli,* which was tested with diffusion disc assays (**Table 48**).

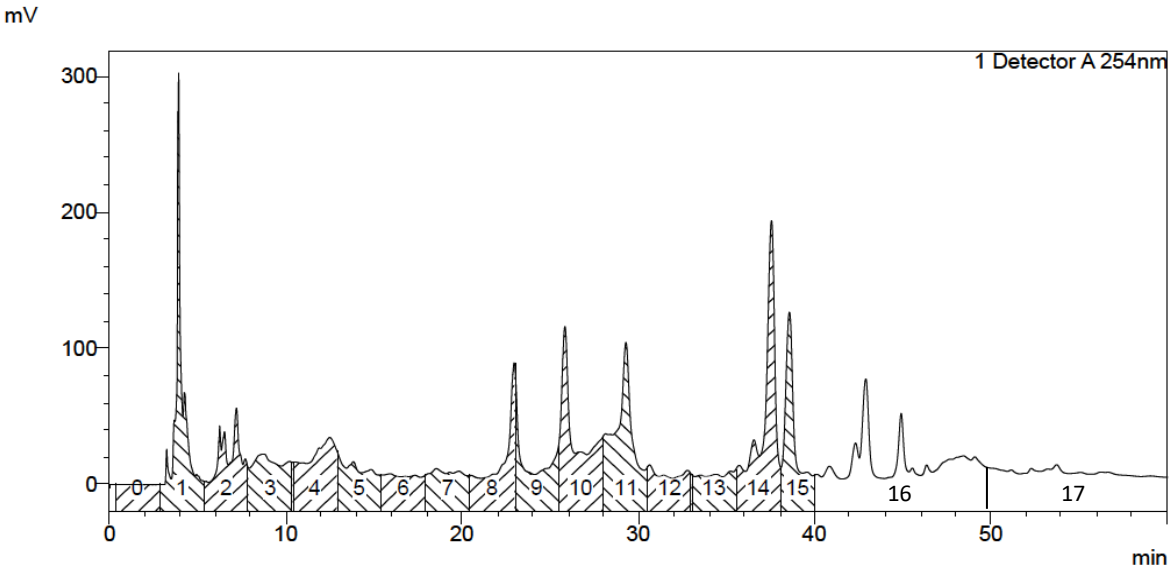


Figure 39: Semi-preparative HPLC separation of the pre-treated extract of LN303 (LN303_B_) using initial gradient (Table 14) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 2 mL. 18 fractions were collected.

Although the dilution did not impact the activity of the substances it can be concluded that the dilution had an effect on the concentration of the substances of the extract. This is obvious by comparing the semi-preparative chromatograms **Figure 34** and **Figure 39**. In the undiluted upscale extract (60.50 mg/mL) the total area of the peaks between retention time 22.95 – 38.57 min is 4.3 times greater than in the diluted upscale extract (30.25 mg/mL).

Table 47: Re-numbering of the pooled fractions of the pre-treated upscale extract of LN303, their dry matter yields and the amounts of used MeOH for re-dissolving are depicted.

Fraction number old	Pooled fraction number	Dry matter yields [mg]	MeOH [mL]
0, 1, 2	1	28.3	1
3, 4, 5, 6	2	1.34	1
7, 8, 9	3	0.72	1
10, 11, 12	4	0.32	1.3
13, 14, 15	5	-0,1*	1.5
16	6	0.66	3
17	7	12.44	2.5

* Negative yields due to a measuring error of the analytical balance.

Table 48: Results of the diffusion disc assays of LN303_B_ against *E. coli*, *S. cerevisiae, A. niger and F. gramineraum* are depicted. Diameters of the inhibition zones were measured in mm.

Fractions	*E. coli*	*S. cerevisiae*	*A. niger*	*F. graminearum*
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	9 (i)*	7 (i)	11 (pi)*	15 (pi)
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-

*(i) represents complete inhibition and (pi) partial inhibition of the test organism.

By combining the fractions, the activity against *E. coli* occurred again. Fraction 4 (LN303_B_F4) was the only active fraction. It had an activity against *E. coli, S. cerevisiae*, *A. niger* and *F. graminearum* like the low scale extract. The dry matter yield of fraction 4 was only 0.32 mg, although more residues were visible after evaporation.

Results of LN303_B_F4

A 1:2 dilution of the active fraction 4, with a final concentration of 0.16 mg/mL, was analyzed with analytical HPLC using the gradient as described in **Table 17** (**Figure 40**).

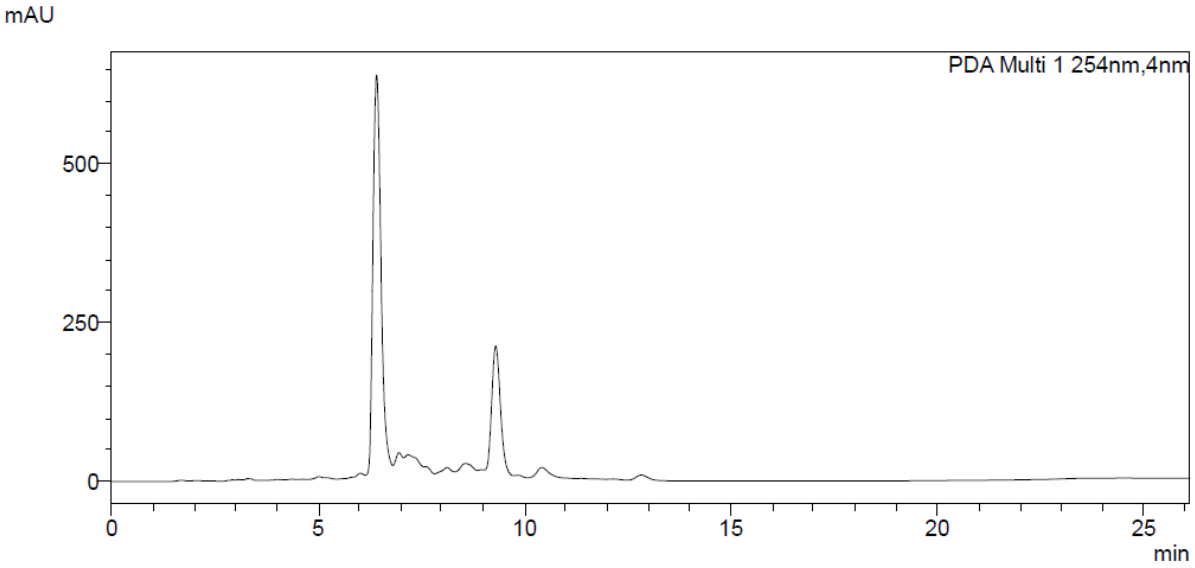


Figure 40: Figure: Analytical HPLC separation of LN303_B_F4 (0.16 mg/mL) using the initial gradient (Table 17) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 10 µL.

Afterwards, fraction 4 was subfractionated with the same gradient as for the analytical separation **(Table 17)**, at which the two peaks were collected manually with the objective of getting pure compounds for further analyses (**Figure 41**).

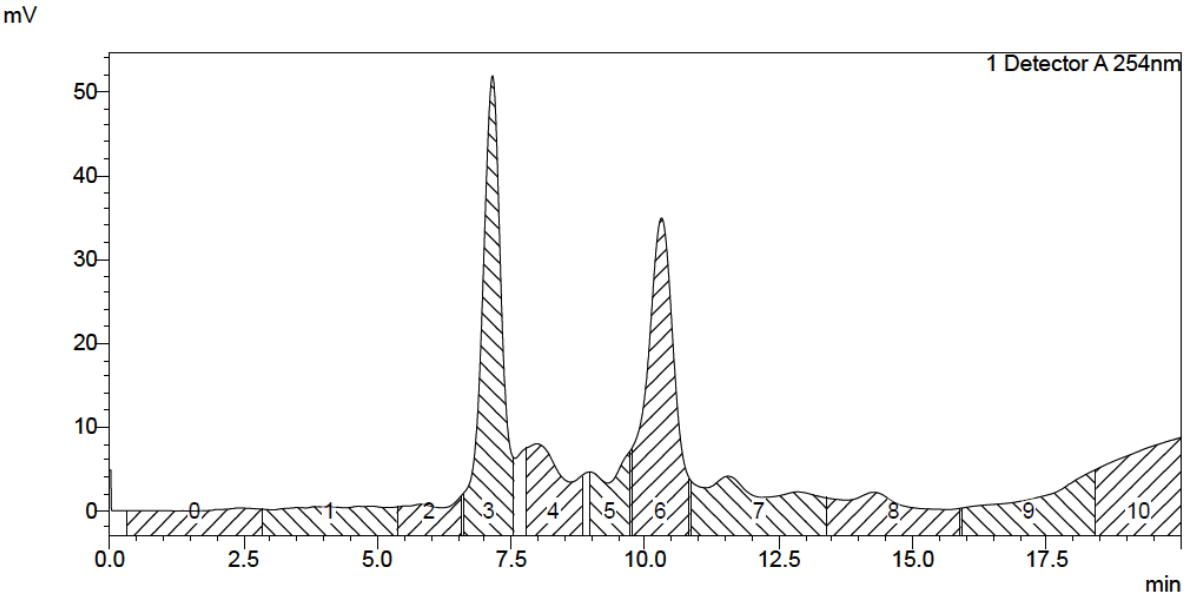


Figure 41: Figure: Semi-preparative HPLC separation of LN303_B_F4 (0.16 mg/mL) using initial gradient (Table 17) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 2 mL. 11 fractions were collected at which fraction 3 and 6 were collected manually.

Subsequently, diffusion disc assays were done (**Table 50**). The peak with the retention time 6.60–7.55 and the second peak with the retention time 9.73–10.83 were tested against *E. coli, S. cerevisiae, A. niger* and *F. graminearum* as single fractions and the other fractions were pooled (**Table 49**).

Table 49: Re-numbering of the pooled fractions of LN303_B_F4, their dry matter yields and the amounts of used MeOH for re-dissolving are depicted.

Fraction number old	Pooled fraction number	Dry matter yields [mg]	MeOH [µL]
0, 1, 2	1	0.00	500
3	2	1.00	500
4, 5	3	0.00	500
6	4	0.55	500
7, 8	5	0.00	500
9, 10	6	0.00	500

Table 50: Results of the diffusion disc assays of LN303_B_F4 against *E. coli*, *S. cerevisiae, A. niger and F. gramineraum* are depicted. Diameters of the inhibition zones were measured in mm.

Fractions	*E. coli*	*S. cerevisiae*	*A. niger*	*F. graminearum*
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	7 (i)*	10 (i)	10 (pi)*	13 (pi)
5	-	-	-	-
6	-	-	-	-

*(i) represents complete inhibition and (pi) partial inhibition of the test organism.

The subfraction 4 (LN303_B_F4_4) was the only one with an activity against all tested microorganisms. As a result, subfraction 4 was collected manually and sent to NMR analysis. LN303_B_F4_4 was a pure substance, but the concentration was too low for structure determination (personal communication, ao. Univ.-Prof. Mag. Dr. Ernst Urban).

Results of Upscale extract 2 of LN303

In order to achieve a larger amount of the active compound in subfraction 4, a second upscale fermentation (LN303_Ups.2) of the fungus on a rice culture was prepared and extracted as described in **chapter 3.10.1.4.** Before fractionation the second upscale extract was analyzed with analytical HPLC to see if it is comparable to the first upscale (**Figure 42**). Both analyses **Figure 33** and **Figure 42** were nearly identical except that some peaks of the second upscale extract showed higher absorbance at 254 nm.

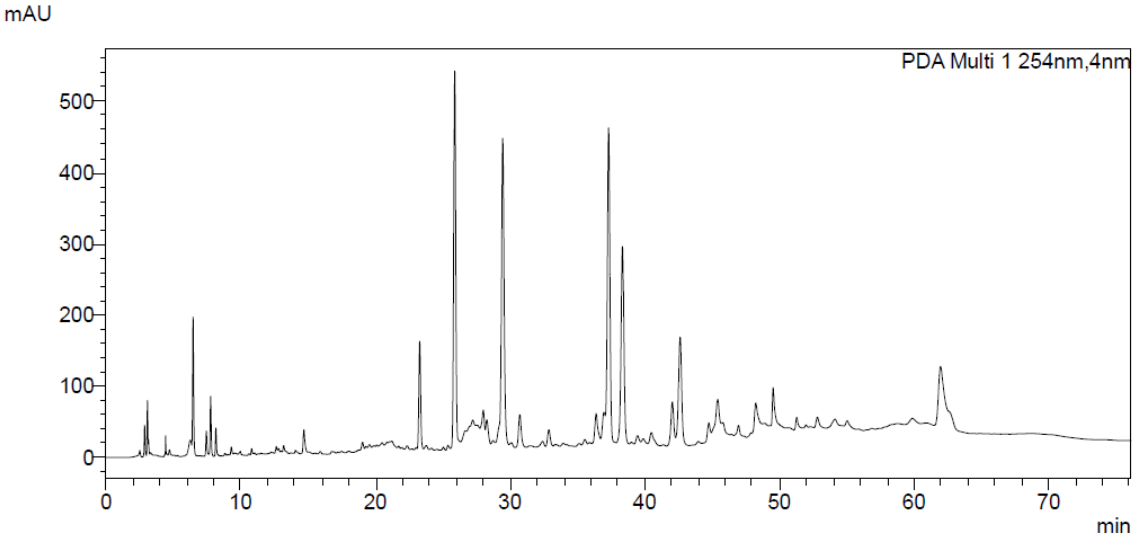


Figure 42: Analytical HPLC separation of LN303_Ups.2 (29.3 mg/mL) using initial gradient (Table 14) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 10 µL.

For semi-preparative separation 2 mL, corresponding to 117.14 mg of the second upscale extract was injected (**Figure 43**). The gradient from the analytical analysis **(Table 14)** was used.

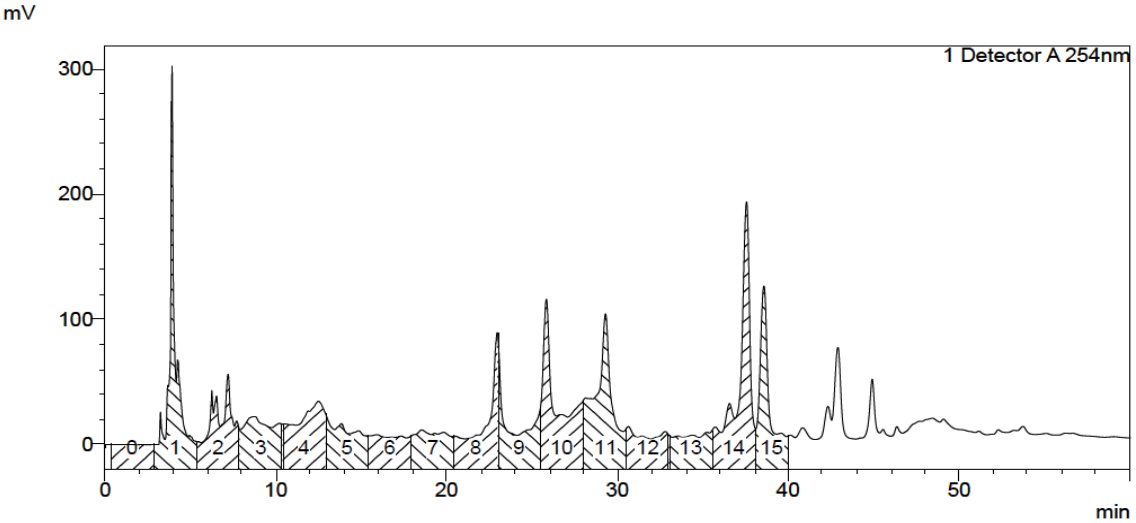


Figure 43: Semi-preparative HPLC separation of LN303_Ups.2 (58.57 mg/mL) using initial gradient (Table 14) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 2 mL. 18 fractions were collected.

By comparison with **Figure 34**, the substances in Ups.2 possessed higher absorbance at 254 nm based on the higher concentration of the extract. The relevant range starting at the retention time 22.97 until 33.02 (vial number 9-12) were collected as one fraction and numbered as fraction 4. As this was the fraction with antimicrobial activity, its dry matter yield was determined, which averaged 17.74 mg. Subsequently LN303_Ups.2_F4 was subfractionated at which 2 mL was injected (**Figure 44**). Compared with the semi-preparative separation of the first upscale extract (LN303_B_F4) (**Figure 41**), it is obvious that the total area of the two peaks (at minute 7.15 and 10.30) is 9.2 times greater than in the second upscale extract.

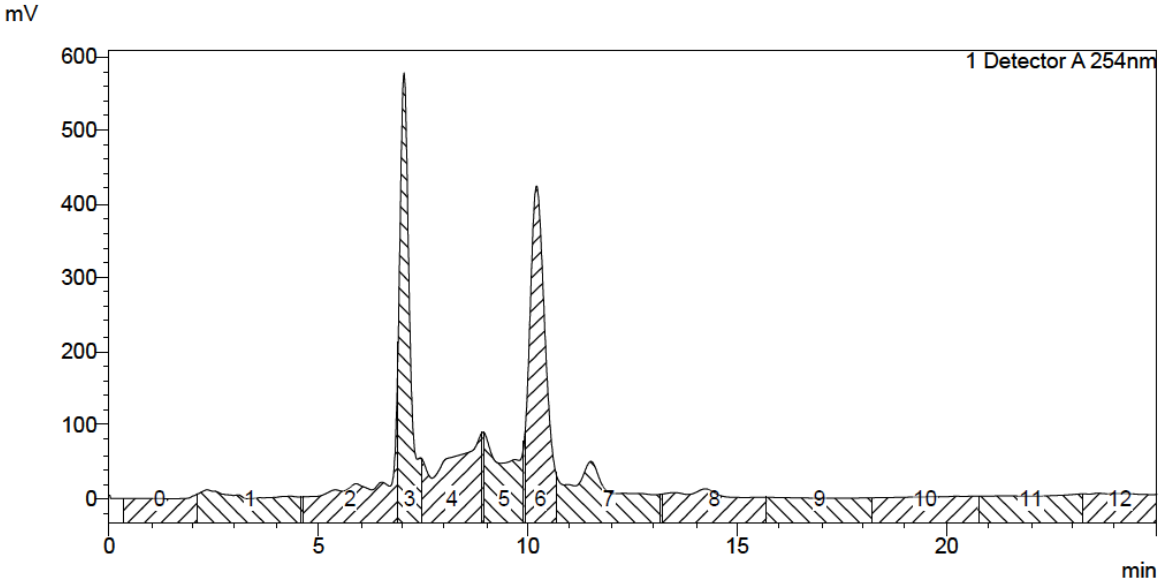


Figure 44: Semi-preparative HPLC separation of LN303_Ups.2_F4 (17.75 mg/mL) using initial gradient (Table 17) is depicted. Analysis was detected at wavelength 254 nm. Injection volume was 2 mL. 13 fractions were collected at which fraction 6 was collected manually. Fraction 6 was renumbered as LN303_Ups.2_F4_4.

Again, some fractions were pooled and the relevant fraction (vial number 6) with the retention time 9.93–10.66 was numbered as fraction 4 (LN303_Ups.2_F4_4) and was collected manually.

Diffusion disc assays of LN303_Ups.2_F4_4

In order to reveal the intensity of the antimicrobial effect of the pure substance, bioassays were done (**Table 51**). The substance was additionally tested against *Pseudomonas fluorescence* (*P. fluorescence*). The diameter of the inhibition zone

against *F. graminearum* was remarkable (inhibition zone: 22 mm), because it was bigger than the inhibition zone of the positive control nystatin (inhibition zone: 17.5 mm).

Table 51: Results of the diffusion disc assays of LN303_Ups.2_F4_4 (17.75 mg/ mL) against *E. coli*, *S. cerevisiae, A. niger and F. gramineraum* and *P. fluorescence* by comparison to LN303_B_F4_4 are depicted. Diameters of the inhibition zones were measured in mm.

	LN303_Ups.2_F4_4	**LN303_B_F4_4**
E. coli	7 (i)*	7 (i)
S. cerevisiae	7 (i)	10 (i)
A. niger	10 (pi)*	10 (pi)
F. graminearum	22 (pi)	13 (pi)
P. fluorescence	-	x**

*(i) represents complete inhibition and (pi) partial inhibition of the test organism.
**LN303_B_F4_4 was not tested against P. fluorescence.

The activity of both approaches LN303_Ups.2_F4_4 and LN303_B_F4_4 were comparable when tested against *E. coli* and *A. niger*, although their concentrations differed significantly: The concentration of LN303_B_F4_4 was 0.55 mg/mL and the concentration of LN303_Ups.2_F4_4 2.0 mg/mL. **(Table 51)**. Those subfractions were considered to be pure substances, however, since they result from different upscale approaches, the two substances may not be the same. The substance with the higher concentration (LN303_Ups.2_F4_4) had a lower activity against *S. cerevisiae* (**Figure 45 + 46**) and a considerable activity against *F. graminearum.* Alternatively, a reason for this result could be, that the substances did not have the same purity. NMR analyses of LN303_Ups.2_F4_4 revealed that the substance has a purity of at least 95%, while the degree of purity of the other substance could not be determined because of its low concentration (0.55 mg). This assumption was confirmed by the color of the dissolved substances. While LN303_Ups.2_F4_4 was completely colorless after re-dissolving in MeOH, LN303_B_F4_4 had a slight touch of yellow. The possibility that the first substance consists of other unknown substances which could have a synergistic or antagonistic effect together, may explain the results of the diffusion disc assays.

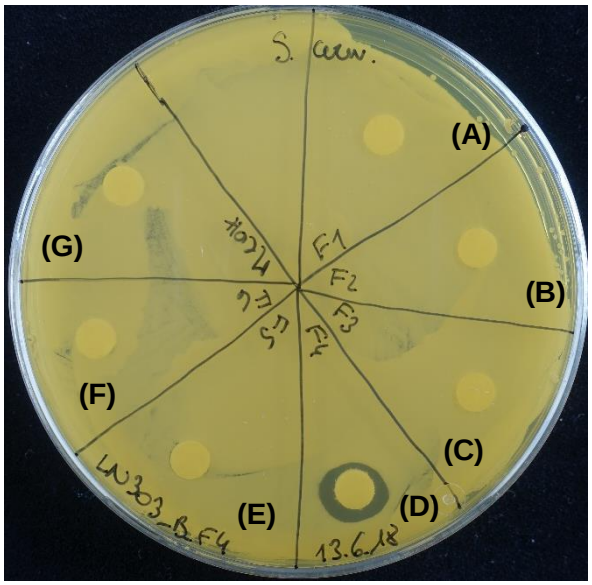


Figure 46: Diffusion disc bioassay testing the fractions of LN303_B_F4 for activity against *S. cerevisiae*. MeOH (G) represents negative control. Subfraction 4 (D) showed an inhibition with 10 mm in diameter and is an example for a positive reaction. All other fractions (A, B, C, E, F) are examples for a negative reaction.

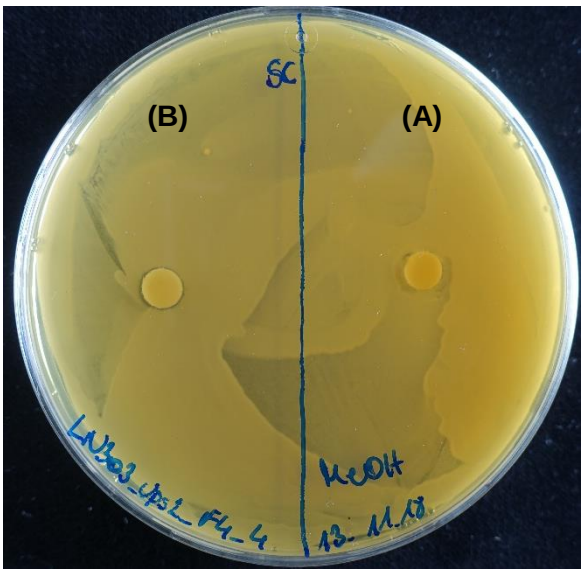


Figure 45: Diffusion disc bioassay testing the fractions of LN303_B_F4 for activity against *S. cerevisiae*. MeOH (B) represents negative control. LN303_Ups.2_F4_4 (A) showed an inhibition with 7 mm in diameter and is an example for a positive reaction.

NMR analyses

The dry matter yield of LN303_Ups.2_F4_4 was 2 mg. The compound was re-dissolved in deuterated MeOH and ^{1}H- and ^{13}C- NMR spectra were recorded and analyzed kindly by ao. Univ.-Prof. Mag. Dr. Ernst Urban at the Department of Pharmaceutical Chemistry.

NMR analyses detailed that the substance has a degree of purity of at least 95%. Finally, the substance LN303_Ups.2_F4_4 was identified as emestrin A (**Figure 47**). ^{1}H- and ^{13}C- NMR spectra can be found in the appendix.

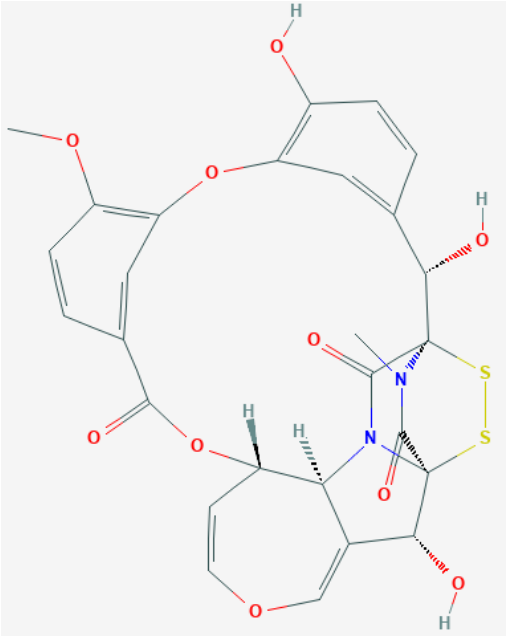


Figure 47: This figure shows the structure of emestrin A with the molecular formula $C_{27}H_{22}N_2O_{10}S_2$. Emestrin A has a molecular weight of 598.597 g/mol (PubChem).

Summary of the results and discussion of LN303

The active compound of the fungal endophyte *A. muscarius* (LN303) was identified as the macrocyclic epidithiodioxopiperazine (ETP) emestrin A (Seya *et al*., 1985). ETP are mycotoxins which are only produced by fungi.Producers are found within five ascomycetes classes: *Dothideomycetes, Eurotiomycetes, Lecanoromycetes, Saccharomycetes* and *Sordariomycetes* (Gardiner *et al.*, 2005).

In former studies, in which emestrin was isolated from *Emericella striata*, was demonstrated that this mycotoxin has a strong antifungal activity (Seya *et al.,* 1985). Furthermore, it has a potent toxicity to lymphocytes, liver and heart by deformation of mitochondria (Kawai *et al*., 1988) and anticancer activity (Nursid *et al*, 2011).

Seya *et al*. (1985) mentioned that emestrin had a potent antifungal activity, but no details are given, and no other publication describing the antimicrobial activity of emestrin was found.

In this Thesis, two filamentous fungi (*A. niger* and *F. graminearum*) were used to test the antifungal activity of the substances. Diffusion disc assays of emestrin A showed that it has an antifungal activity against both fungi, at which the activity against *F. graminearum* is stronger. Furthermore, it was found that emestrin A had an antimicrobial activity against the Gram- negative bacterium *E. coli* and the yeast *S. cerevisiae*, while it did not have an activity against the Gram- negative bacterium *P. fluorescens* and the Gram- positive bacterium *B. subtilis*.

4.5. Results and discussion of Ab5

Diffusion disc assay of Ab5 fractions

After separating the extract with flash column chromatography diffusion disc assays were performed against *B. subtilis* (**Figure 48**). As depicted in **Table 52**, 6 fractions out of 16 fractions had an activity against *B. subtilis*, at which fraction 12 had the highest activity with an inhibition zone of 26 mm in diameter.

Table 52: Results of the diffusion disc assays of the subfractions of Ab5 against *B. subtilis* are depicted. Diameters of the inhibition zones were measured in mm. All active fractions showed complete inhibition of the test organism.

Fraction	*B. subtilis*	Fraction	*B. subtilis*
1	-	**9**	7
2	-	**10**	-
3	-	**11**	12
4	-	**12**	26
5	-	**13**	14
6	-	**14**	16
7	-	**15**	7.5
8	-	**16**	-

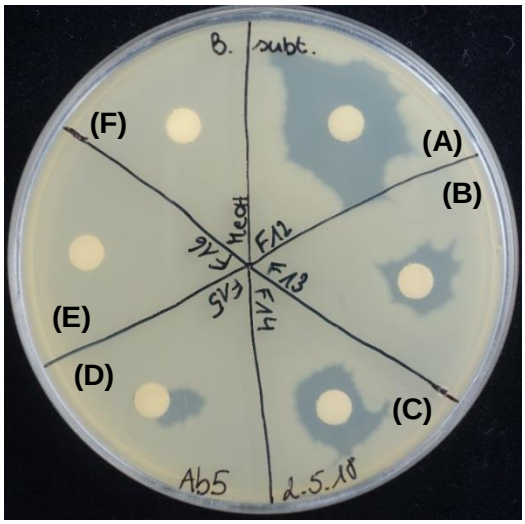


Figure 48: Diffusion disc bioassay testing five fractions of Ab5 for activity against *B. subtilis*. MeOH (F) represents negative control. Subfractions 12 (A), 13 (B), 14 (C) and 15 (D) showed complete inhibition of the test organism and are examples for positive reaction. Fraction 16 (E) showed no activity and is an example for a negative bioassay.

Analytical and semi-preparative HPLC

Analytical measurements were performed for all active fractions to reveal possible similarity between the fractions. **Figure 49** shows the analytical separation of fraction 12 with the highest activity against the test organism.

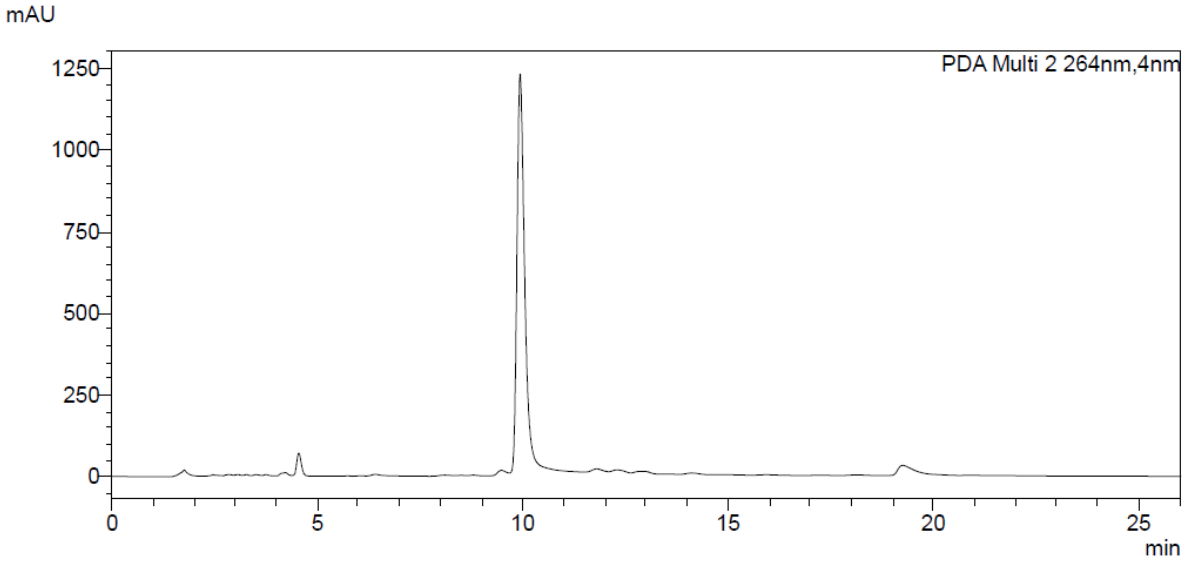


Figure 49: Analytical HPLC separation of Ab5_F12 using optimized gradient (Table 25). Analysis was detected at wavelength 264 nm. Injection volume was 10 µL.

Comparing all chromatograms, it was conspicuous that two peaks with retention times 4.5 min and 9.9 min appeared in all active fractions, but with different peak areas. As the absorbance maximum of the peaks was given at 264 nm, the analyses were detected at this wavelength.

As fraction 12 had the strongest activity, semi-preparative separation was performed using the optimized gradient as described in **Table 25** (**Figure 50**). Before evaporating some fractions were pooled and subsequently dried by evaporation. The re-numbering and dry matter yields of the fractions are depicted in **Table 53**.

The peak with the highest absorbance rate with retention time 15.32–16.30 min was collected manually and numbered as subfraction 6 (Ab5_F12_6).

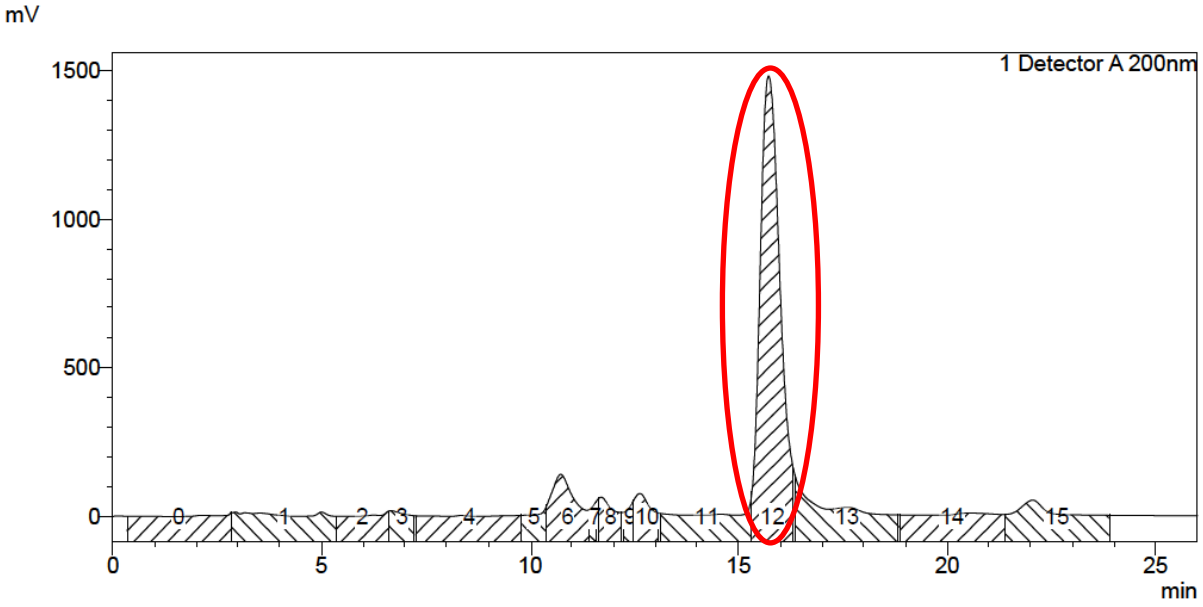


Figure 50: Semi-preparative HPLC separation of Ab5_F12 using the optimized gradient (Table 25). Analysis was detected at wavelength 264 nm. Injection volume was 1 mL. 16 fractions were collected at which fraction 12 was collected manually.

Table 53: Re-numbering of the pooled subfractions of Ab5_F12, their dry matter yields and the amounts of used MeOH for re-dissolving are depicted.

Fraction number old	Pooled fraction number	Dry matter yields [mg]	MeOH [µL]
0 – 5	1	2.75	1200
6	2	1.37	500
7 – 8	3	0.23	500
9 – 10	4	0.58	500
11	5	0.82	500
12	6	7.13	-
13	7	1.34	500
14	8	0.86	500
15	9	2.98	500

Diffusion disc assays of Ab5_F12 subfractions

After separation with semi-preparative HPLC, all subfractions except of F12_6 were tested in diffusion disc assays against *B. subtilis.* As subfraction 6 was prepared for

NMR analysis it was not previously tested in bioassays. The results were unexpected, because all subfractions had an activity against the test organism (**Figure 51 + 52**) and especially the activities of the fractions 2, 3, 4 and 5 were so strong that they overlap each other. For this reason, the individual inhibition zone could not be defined for every fraction (**Table 54**).

Table 54: Results of the bioassays of Ab5_F12 subfractions. Diameters in mm. i… complete inhibition; -… inhibition zone not defined.

Fraction	*B. subtilis*	Fraction	*B. subtilis*
1	10 (i)	**6**	not tested
2	-	**7**	28 (i)
3	-	**8**	19 (i)
4	-	**9**	13 (i)
5	-	**10**	18 (i)

Figure 51: Diffusion disc bioassay testing subfractions 1-5 of Ab5_F12 for activity against *B. subtilis*. MeOH represents negative control. All tested fractions have shown complete inhibition of the test organism and are examples for positive reaction. At some fractions (F2, F3, F4, F5) the inhibition zones have overlapped each other.

Figure 52: Diffusion disc bioassay testing subfractions 7-10 of Ab5_F12 for activity against *B. subtilis*. MeOH represents negative control. All tested fractions have shown complete inhibition of the test organism and are examples for positive reaction.

NMR analyses

As subfraction 6 was collected manually as one single peak it was subsequently dissolved in DMSO and analyzed using NMR spectroscopy by ao. Univ.-Prof. Mag. Dr. Ernst Urban at the Department of Pharmaceutical Chemistry.

Based on the NMR results, Ab5_F12_6 was identified as linoleic acid (**Figure 53**) with a high degree of purity. The molecular formula is $C_{18}H_{32}O_2$ and it has a molecular weight of 280,4472 g/ mol (PubChem).

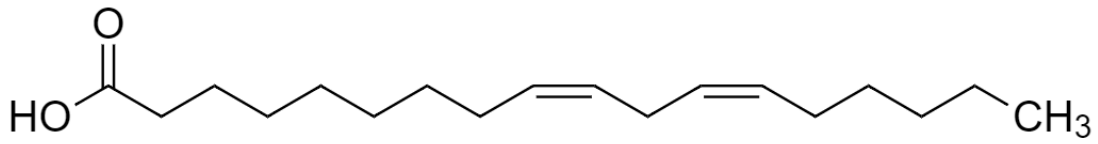


Figure 53: This figure shows the structural formula of linoleic acid, with the molecular formula $C_{18}H_{32}O_2$ and the molecular weight of 280.4472 g/mol (PubChem). Structure drawn with ChemDraw.

Furthermore, subfraction 3 and 4, which had a strong activity against *B. subtilis,* were as well investigated with NMR spectrometry. However, no clear structure analysis could be made, because the fractions did not consist of pure substances. Again, linoleic acid was the main compound.

HRMS results

HRMS analyses were performed for Ab5_F12 and Ab5_F13, because the analytical HPLC analyses of both were nearly identical (**Figure 54**), except that in Ab5_F12 the area of the main peak with the retention time 9.9 min was 2.19 times greater than in Ab5_F13. Both had a strong activity against *B. subtilis* in the bioassay.

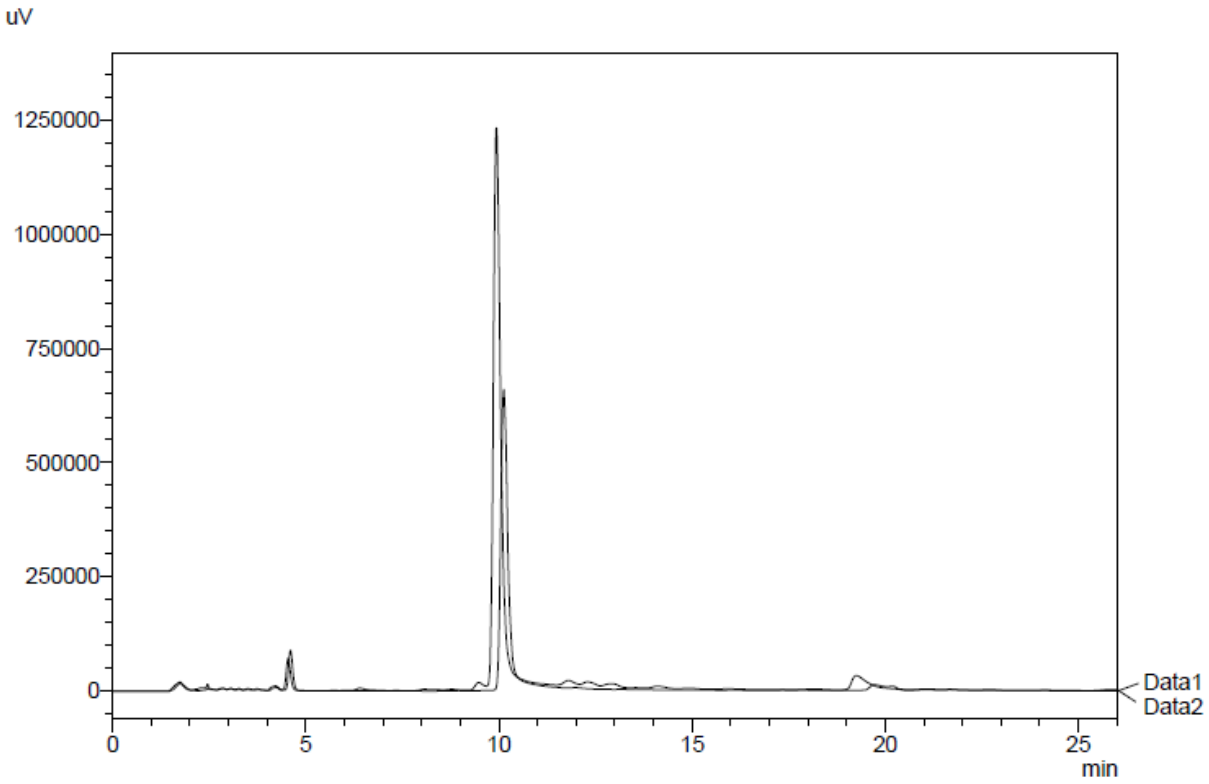


Figure 54: Comparison of the analytical HPLC separations of Ab5_F12 (Data 1) and Ab5_F13 (Data 2) using optimized gradient (Table 25) is depicted. Analyses were detected at wavelength 264 nm. Injection volumes were 10 µL. In Ab5_F12 the area of the main peak with the retention time 9.9 min is 2.19 times greater than in Ab5_F13.

Both measurements were performed in positive mode because linoleic acid was a compound and could be ionized easily in positive mode. There was one mass striking which occurred in both fractions: *m/z* = 422.2302. The proposed molecular formula for this mass was $C_{24}H_{33}NNaO_4$, which belongs to the molecular formula of the substance altersetin (**Figure 55**) as sodium adduct.

Furthermore, the highest signal in fraction 12 was found to be the substance with a m/z = 303.2303, for which the molecular formula $C_{18}H_{32}NaO_2$ is proposed. This formula represents linoleic acid as sodium adduct.

The highest signal in F13 with *m/z* = 305.2451 belongs to the molecular formula $C_{18}H_{34}NaO_2$, which constitutes oleic acid as sodium adduct.

In both fractions, other masses were present which were not defined yet.

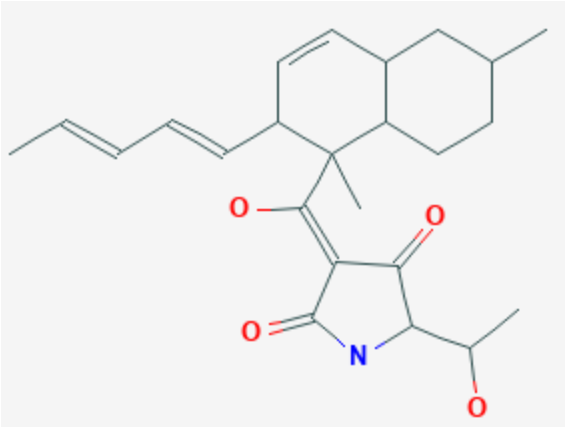


Figure 55: This figure shows the structural formula of altersetin with the molecular formula $C_{24}H_{33}NO_4$. Altersetin has a molecular weight of 369.505 g/mol (PubChem).

Discussion of Ab5

Within this thesis, linoleic acid is the main compound of the endophyte and had a strong activity against the Gram- positive bacterium *B. subtilis*.

Ab5_F12 was the fraction with the highest activity, in which only linoleic acid could be detected with HRMS as well as with NMR spectroscopy.

According to HRMS analyses, altersetin could be a compound of the endophyte Ab5. Furthermore, the strong activity of the endophyte against the test bacterium *B. subtilis* may result from the antimicrobial potential of the substance altersetin (Helwig *et al.*, 2002). Linoleic acid as well as altersetin were contained in the fraction with the strongest activity (Ab5_F12) and former analyses showed that both substances possess an antibiotic activity (Helwig *et al*., 2002; Zheng *et al*., 2005). Synergistic effects of both substances could explain the strong activity of the fraction.

Furthermore, Ab5_F13 had an intensive activity against the test organism. According to HRMS analyses, the main compounds of this fraction are supposed to be altersetin and oleic acid. Oleic acid is a fatty acid and possess an antibacterial activity such as linoleic acid (Zheng *et al*., 2005).

Additional investigation on the fungal endophyte *A. westerdijkiae* were performed by Mag. Dr. Judith Wackerlig at the Department of Pharmaceutical Chemistry. Further investigations are going on by Mag. Dr. Martina Oberhofer at the Department of Pharmacognosy.

5. References

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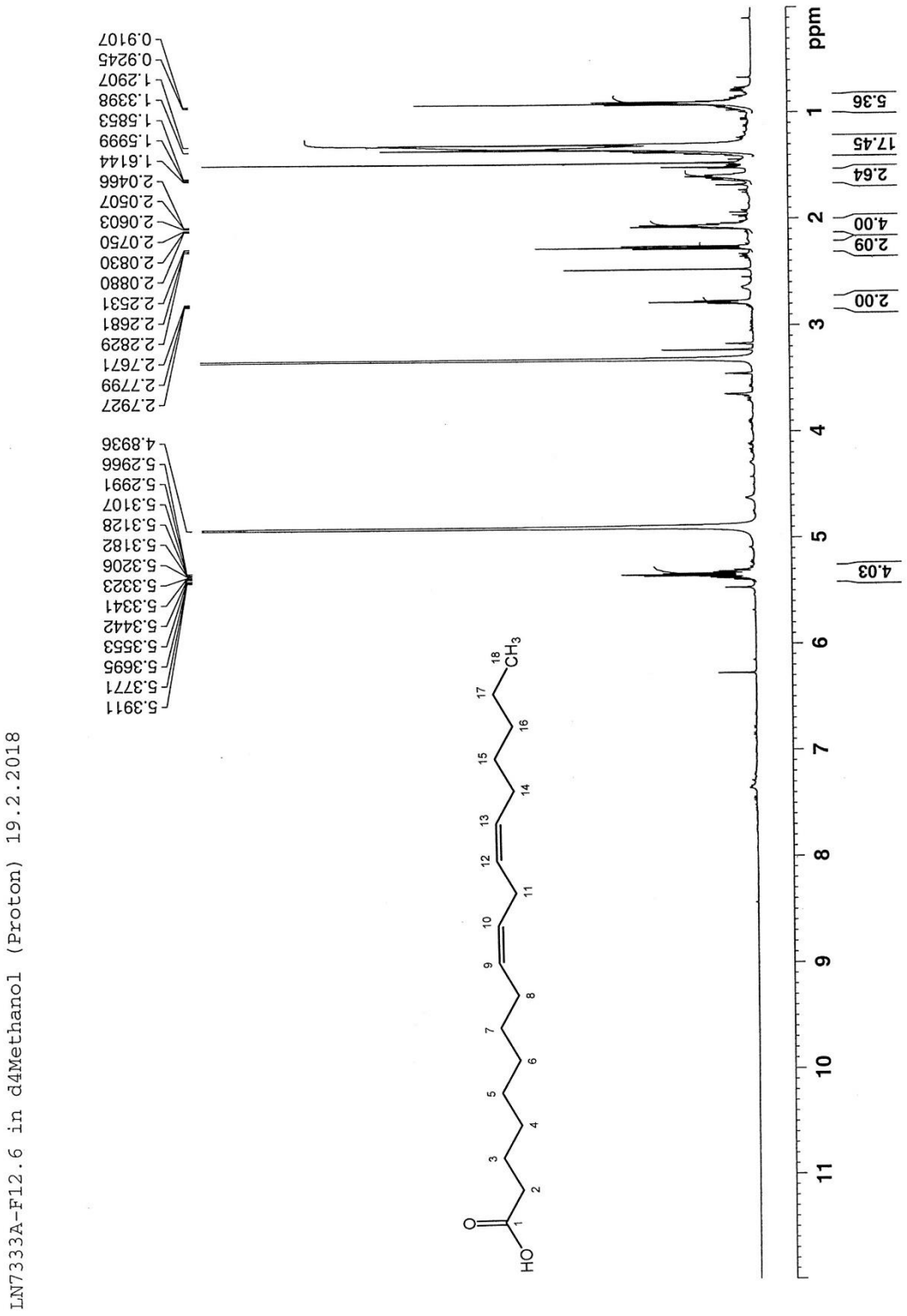
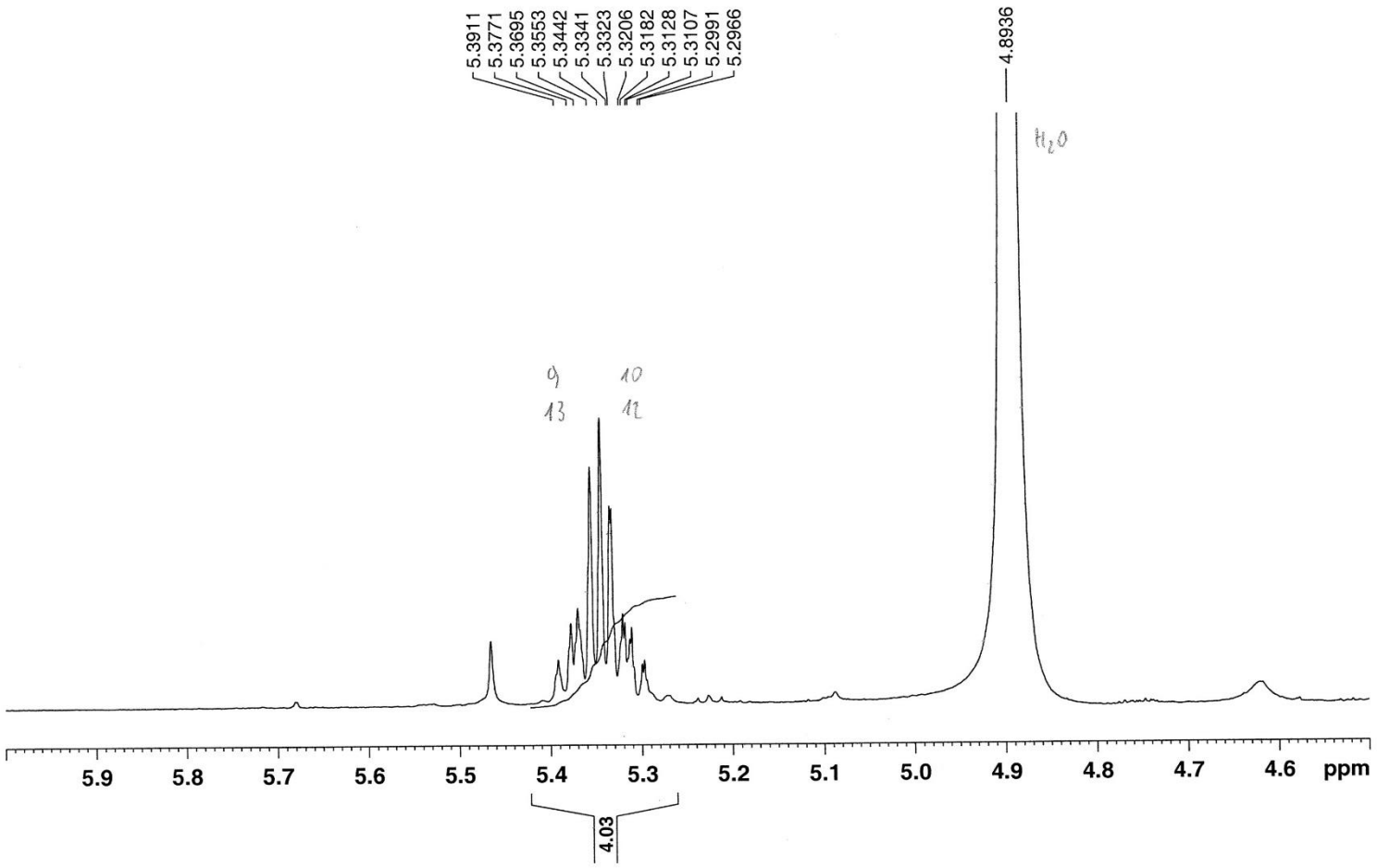
6. Appendix

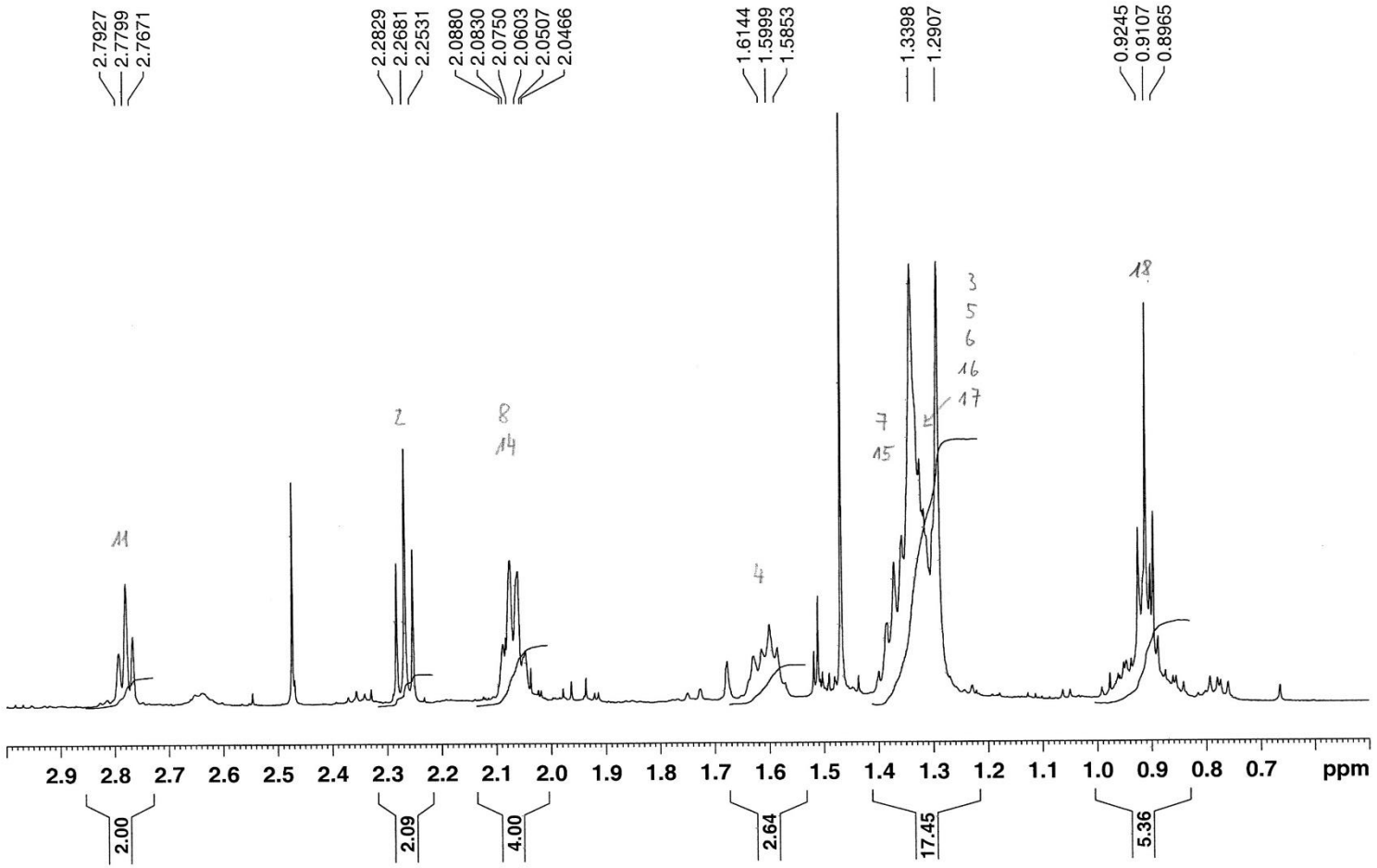
6.1. NMR chromatograms of LN733_F12.6

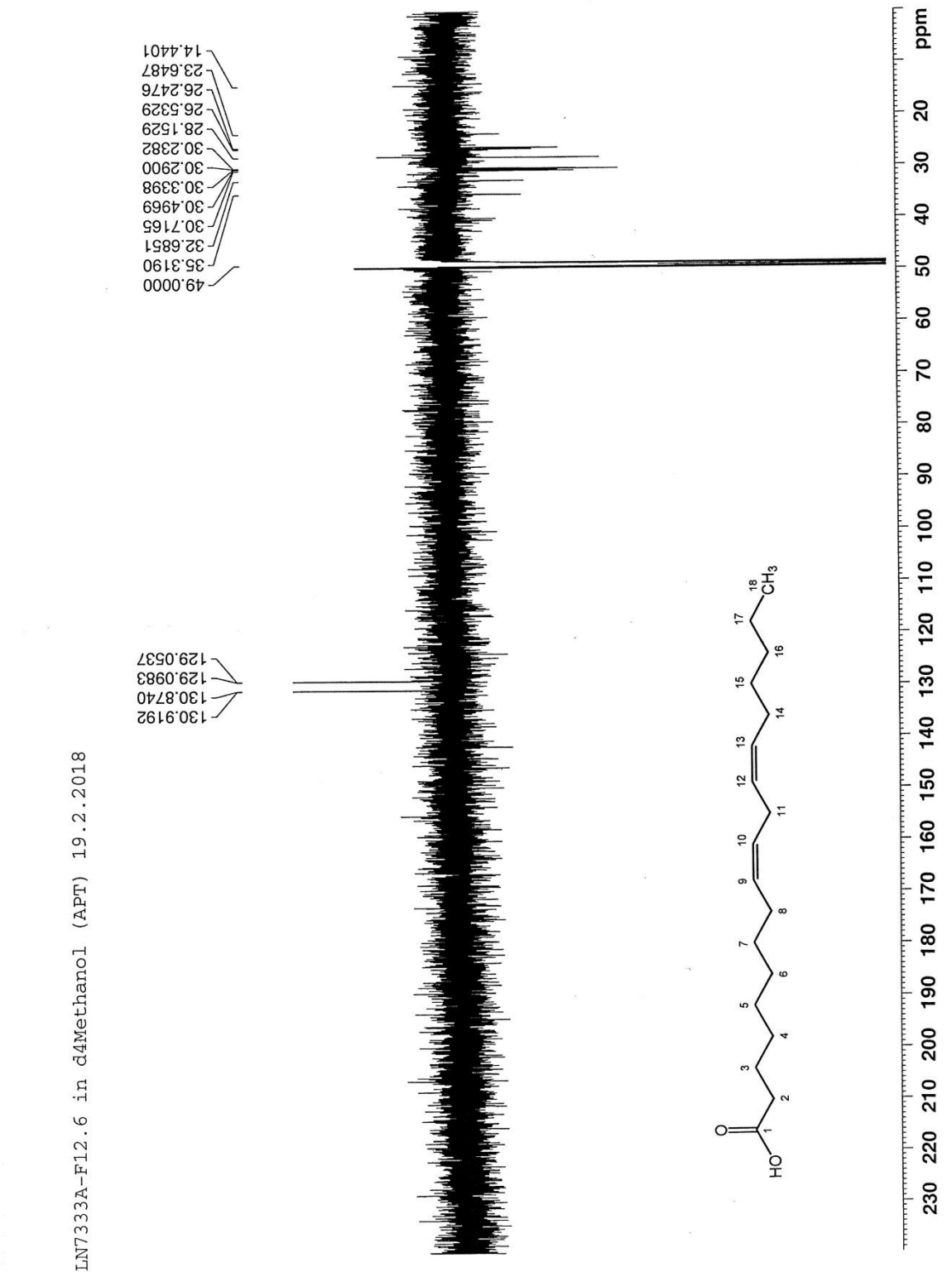


LN7333A-F12.6 in d4Methanol (Proton) 19.2.2018

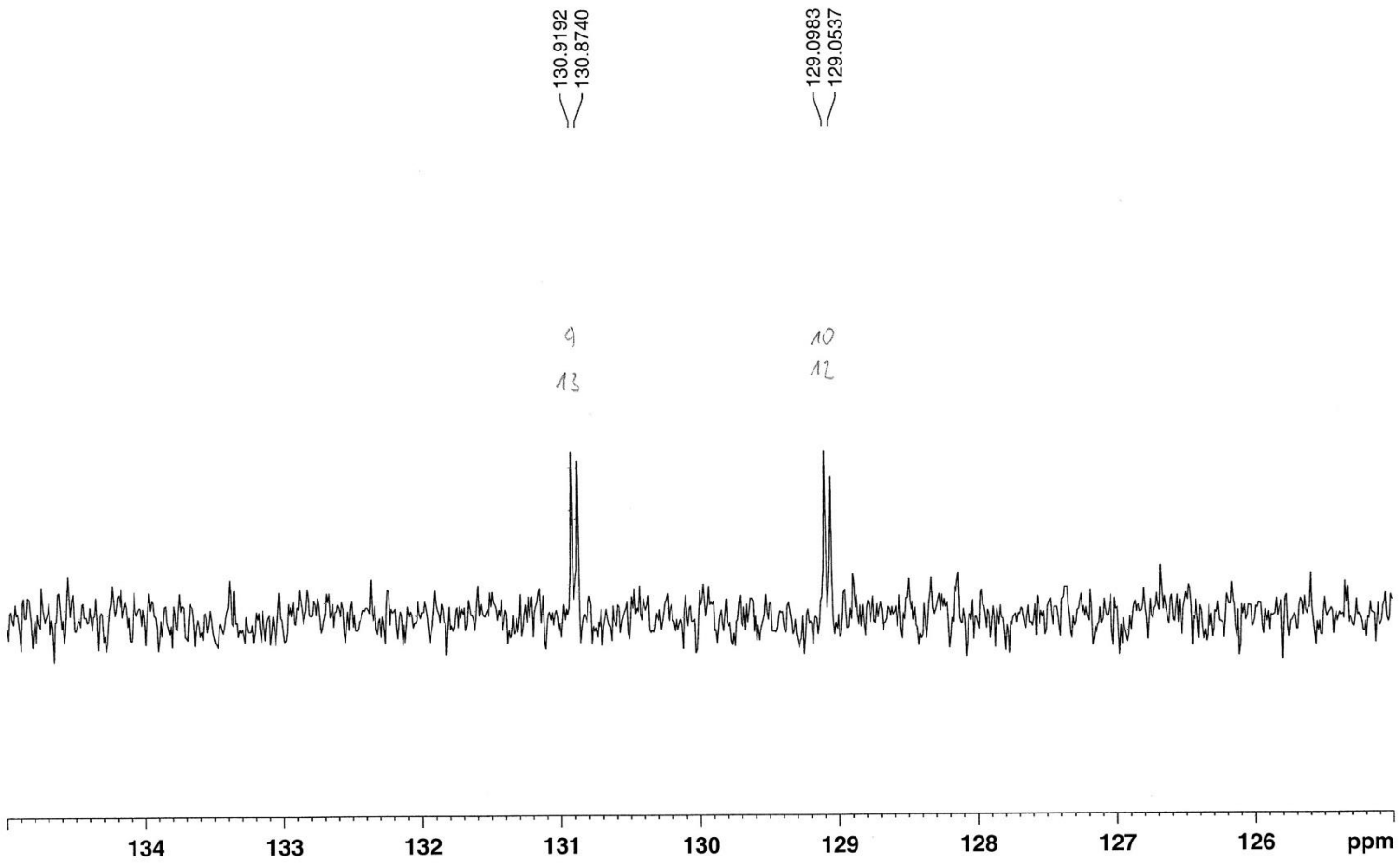


LN7333A-F12.6 in d4Methanol (Proton) 19.2.2018




LN7333A-F12.6 in d4Methanol (APT) 19.2.2018
14.4401
23.6487
26.2476
26.5329
28.1529
30.2382
30.2900
30.3398
30.4969
30.7165
32.6851
35.3190
49.0000
129.0537
129.0983
130.8740
130.9192
HO
O
CH3
ppm

LN7333A-F12.6 in d4Methanol (APT) 19.2.2018



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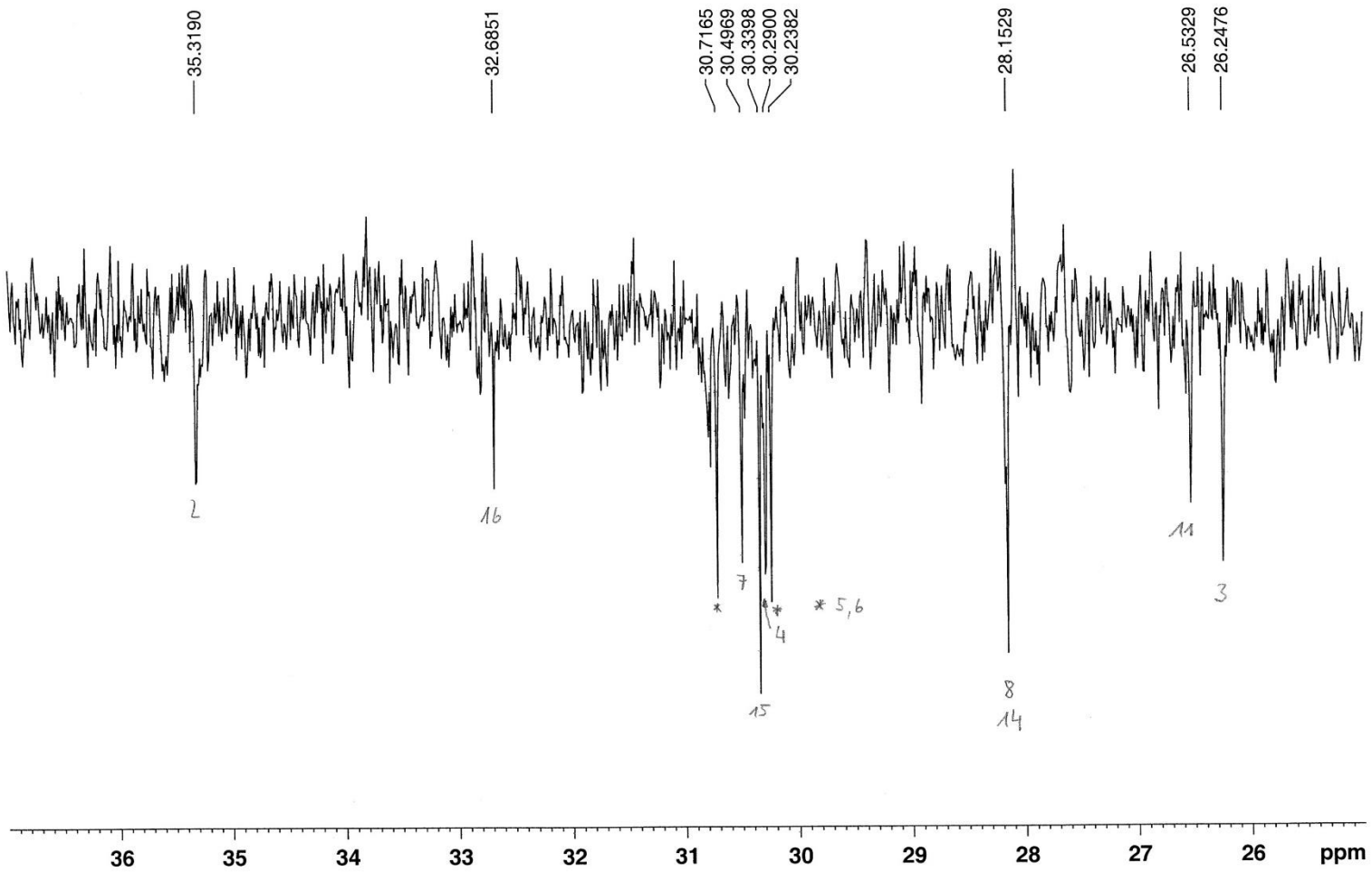

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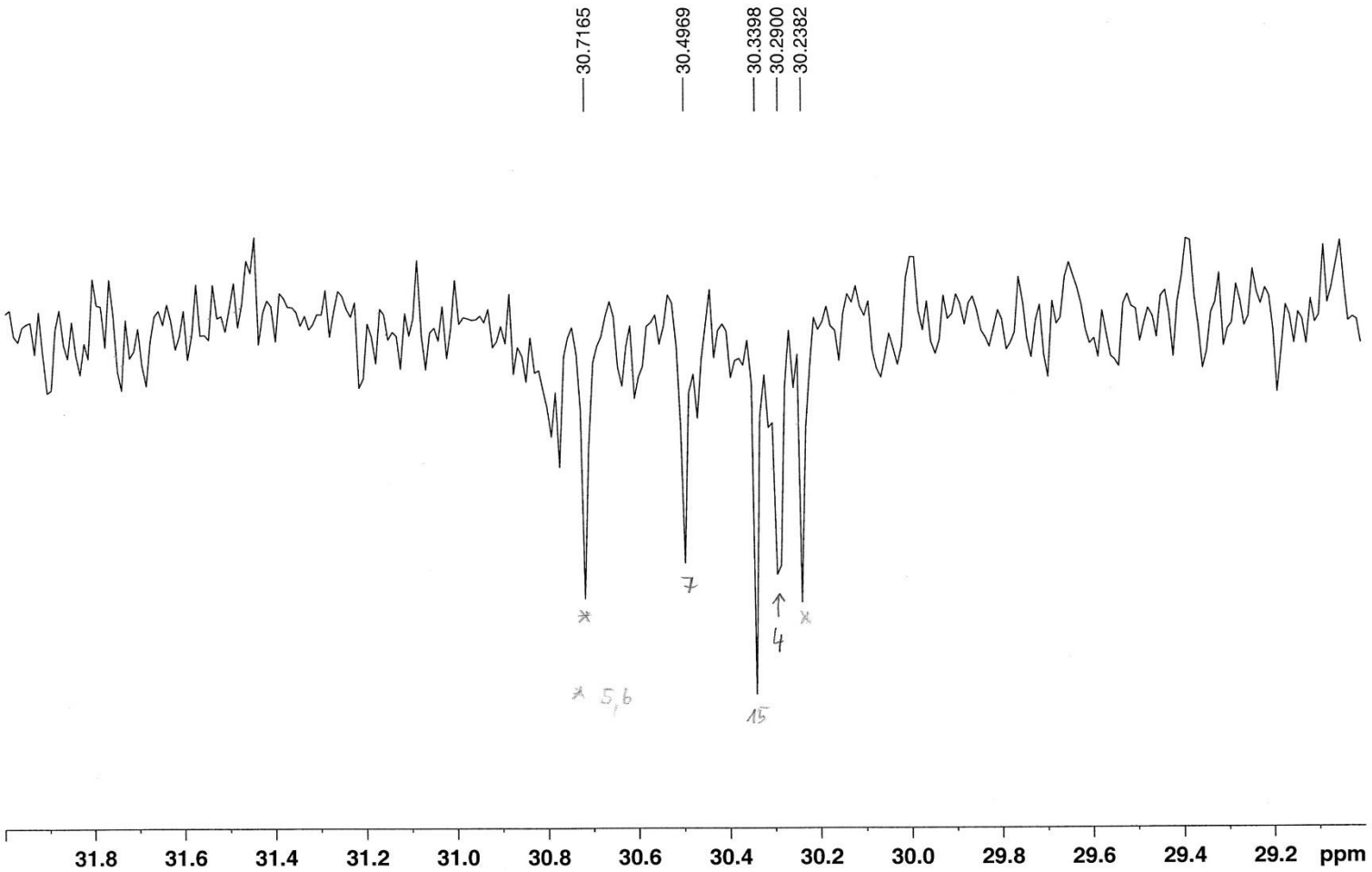

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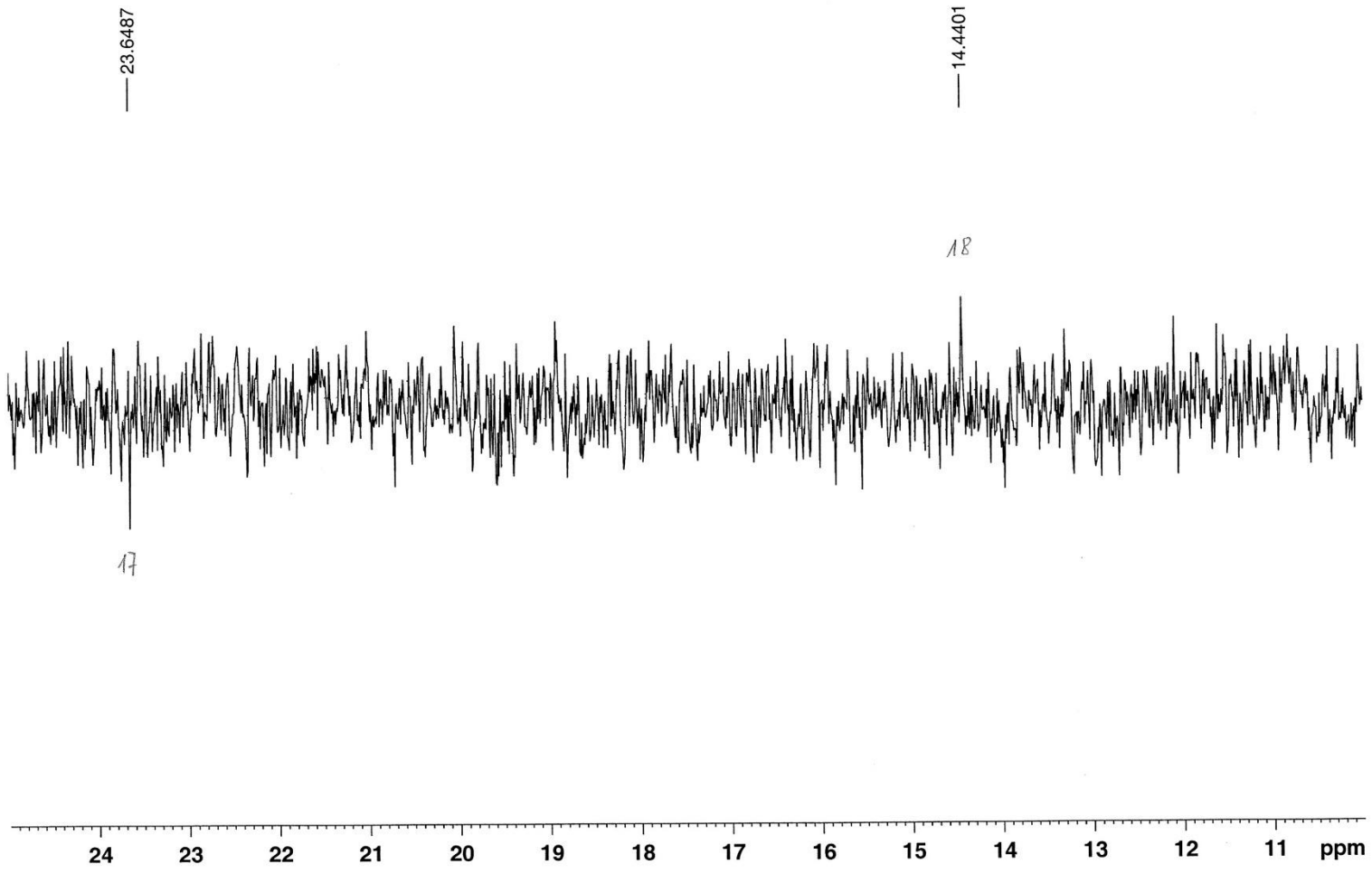
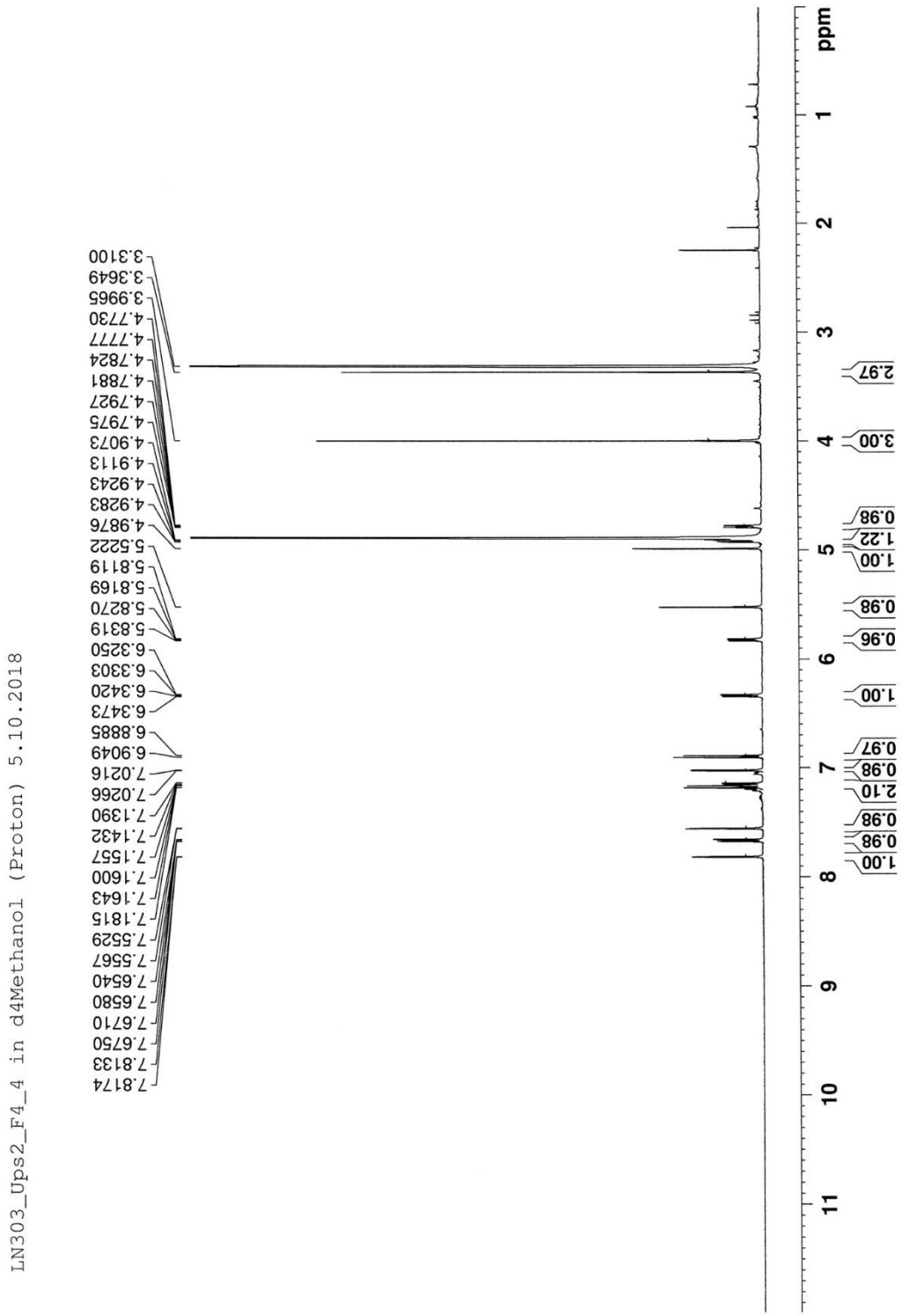


6.2. NMR chromatograms of LN303_Ups.2_F4_4



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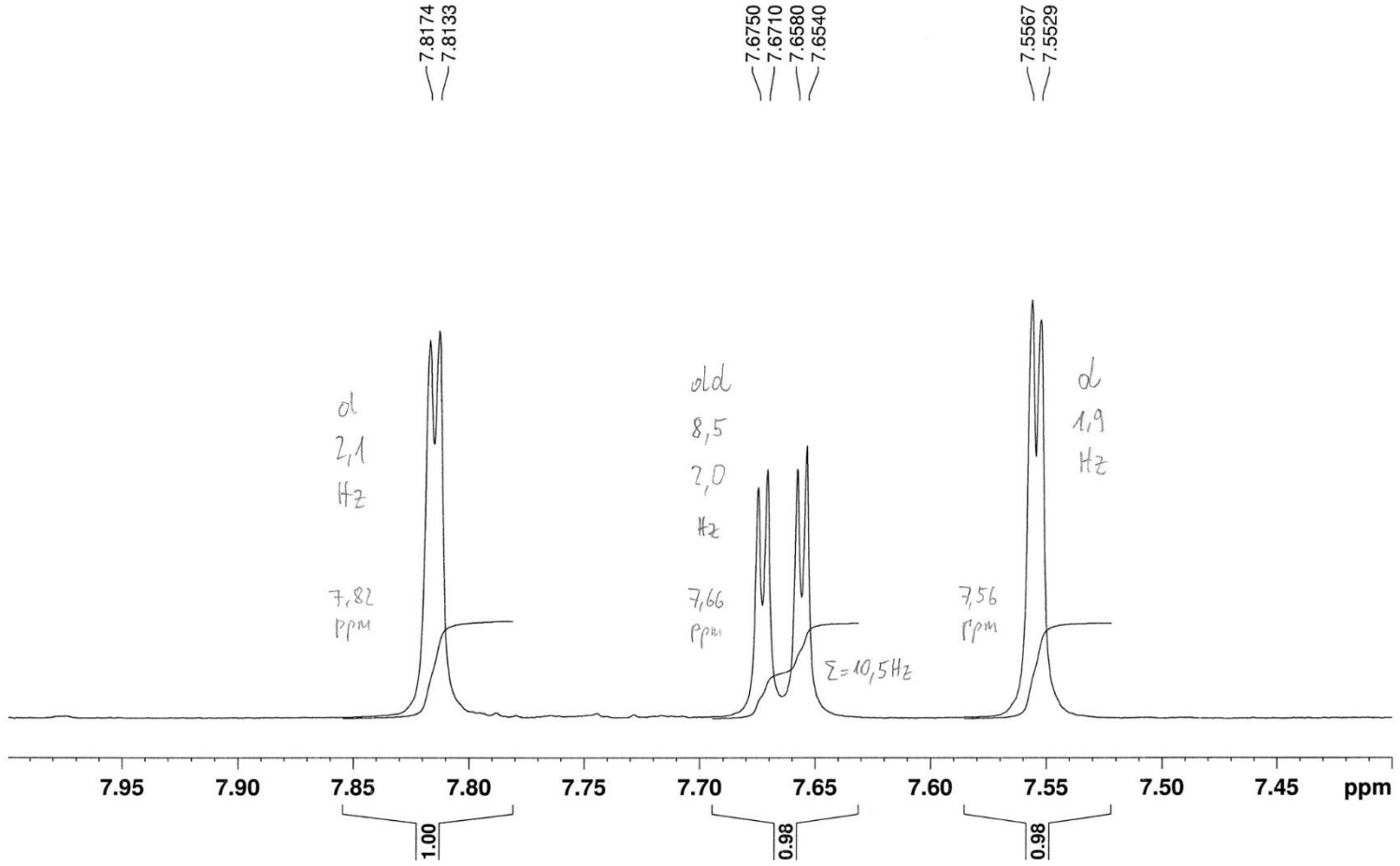
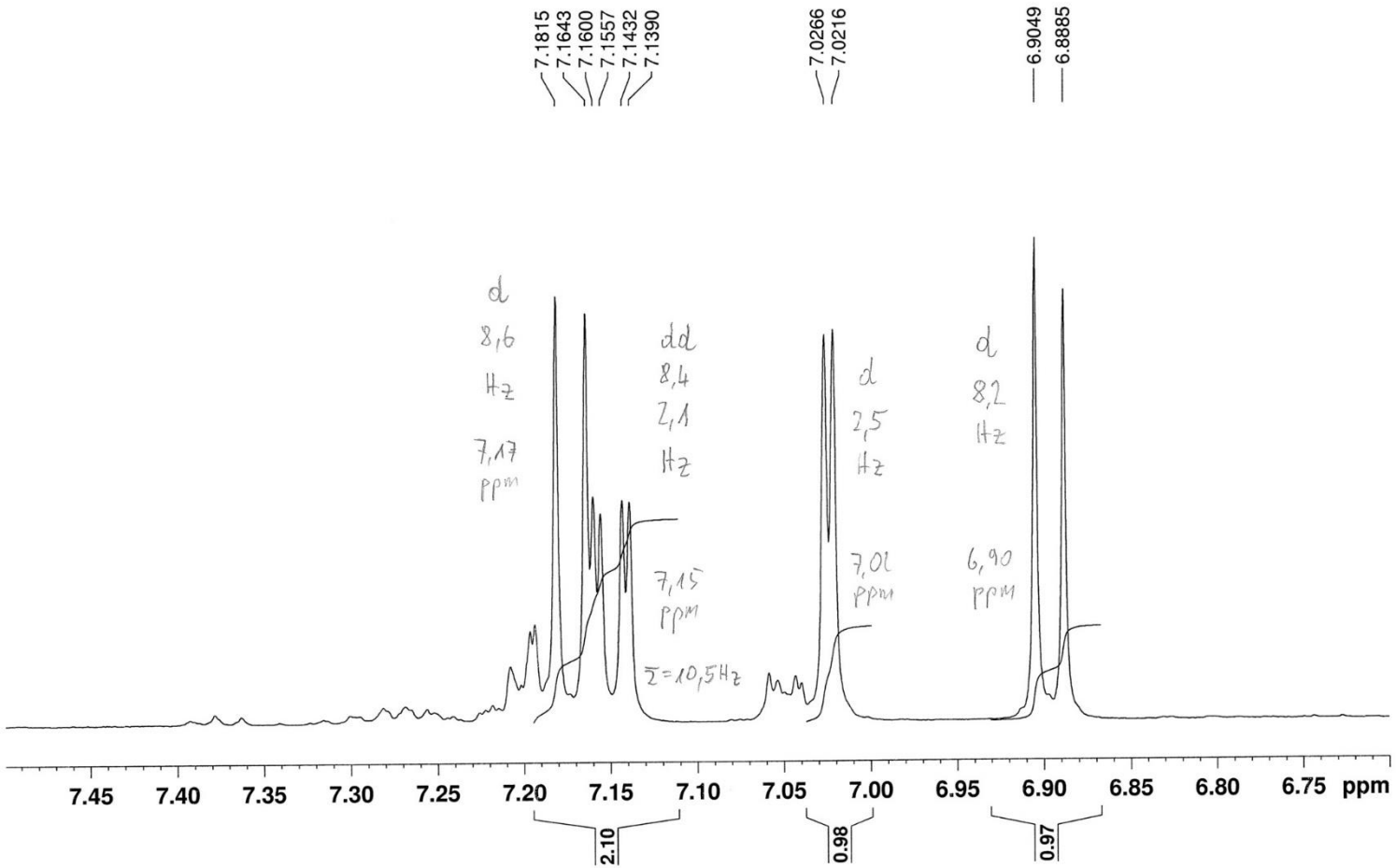
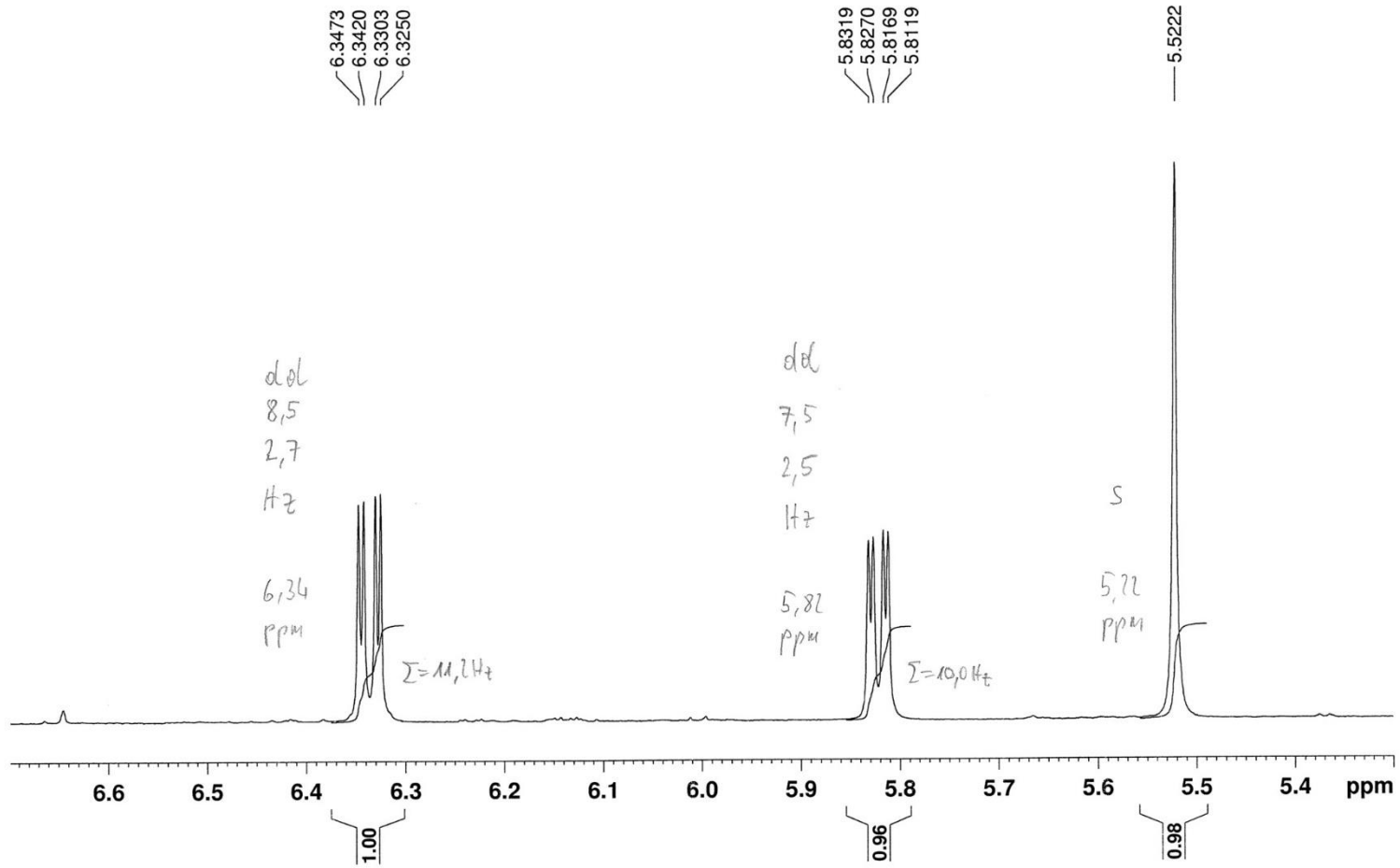


LN303_Ups2_F4_4 in d4Methanol (Proton) 5.10.2018



LN303_Ups2_F4_4 in d4Methanol (Proton) 5.10.2018



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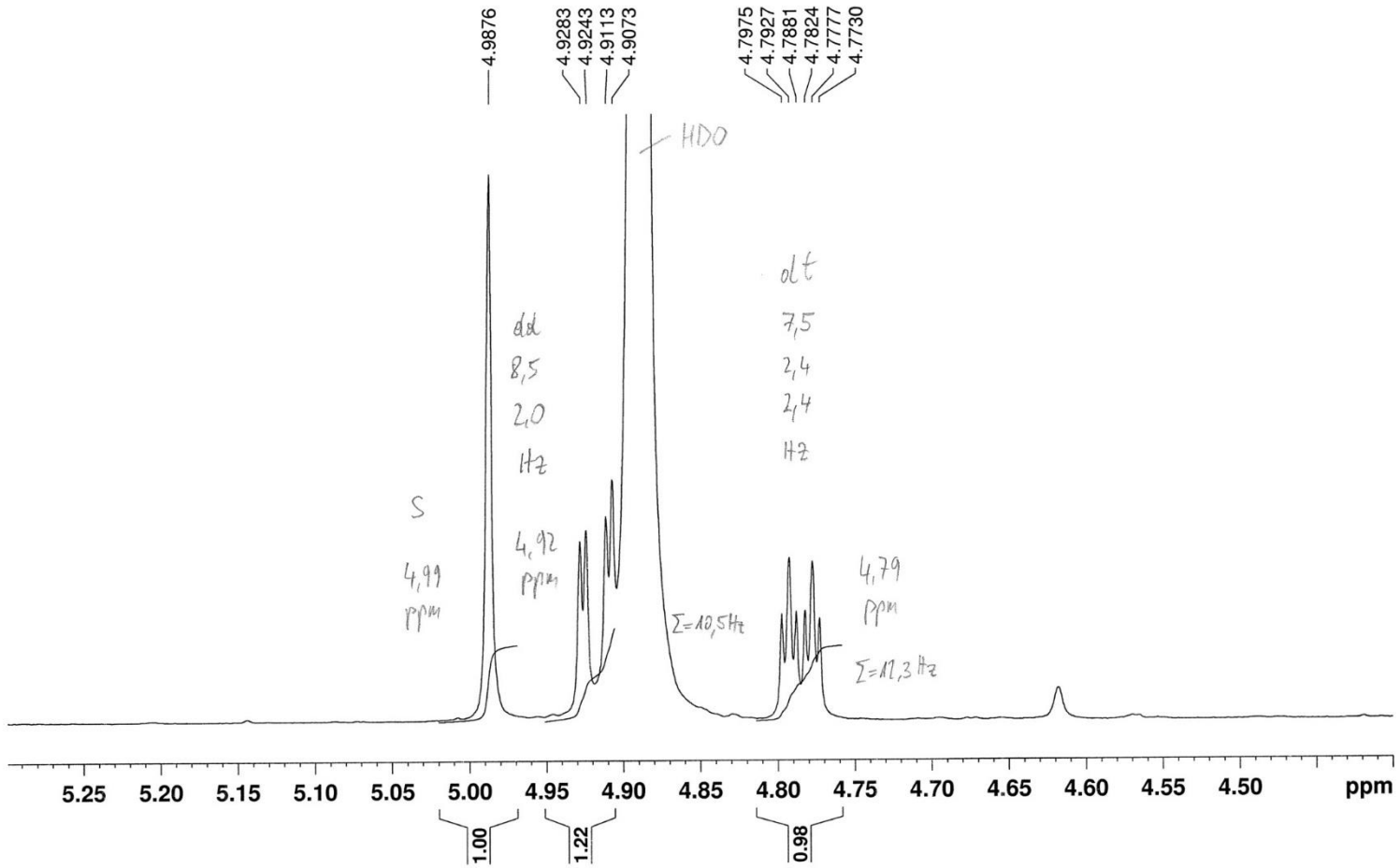
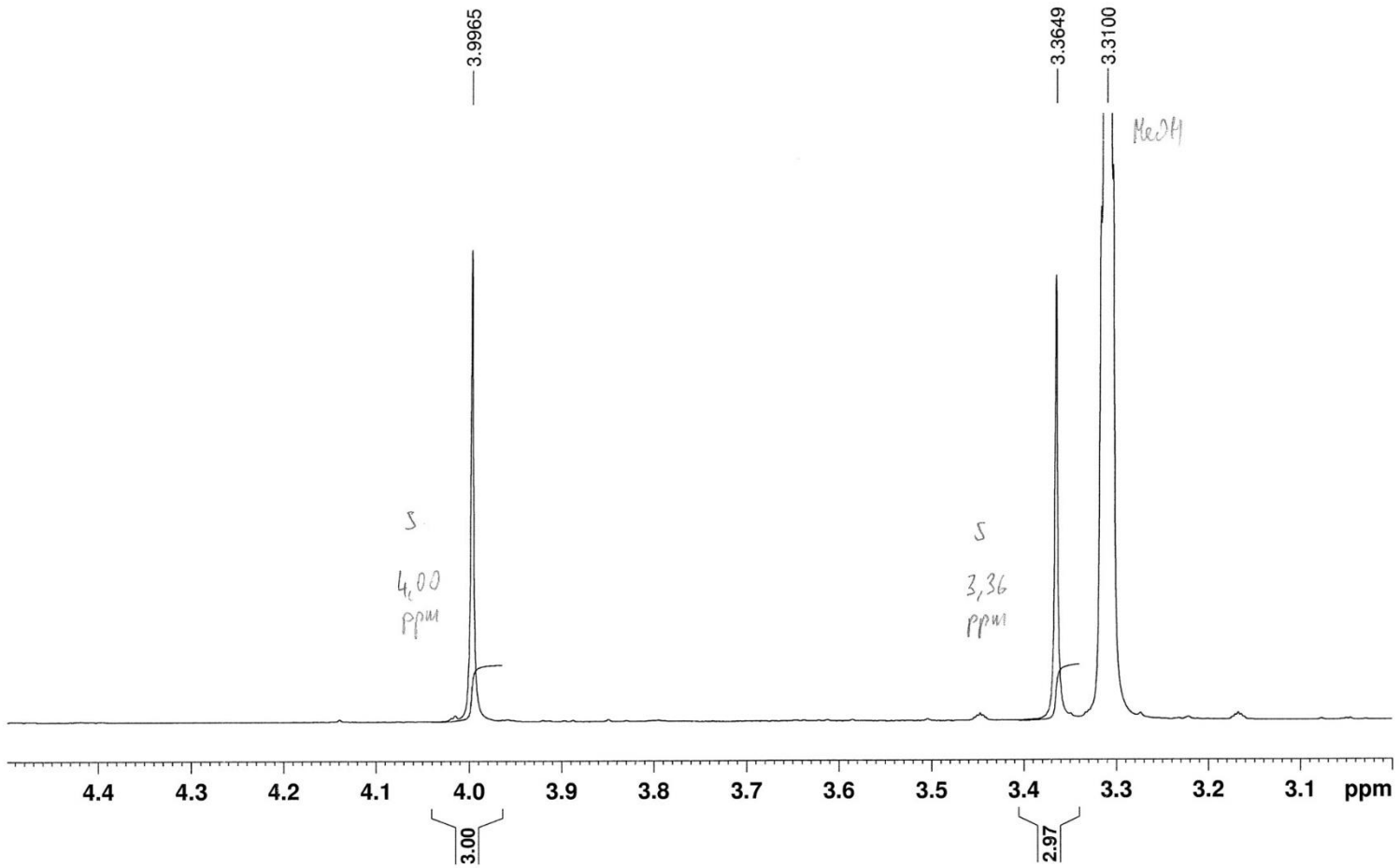
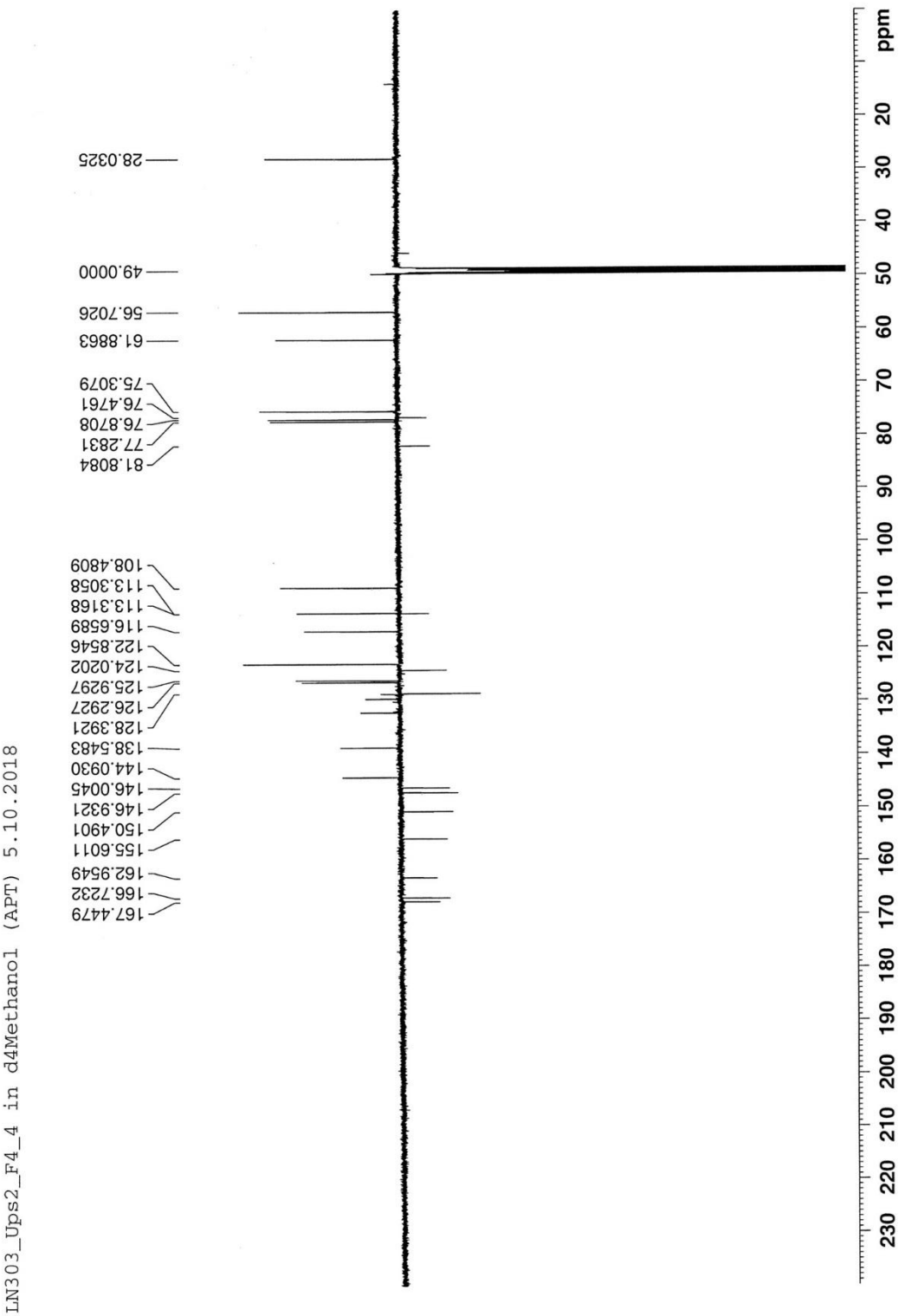
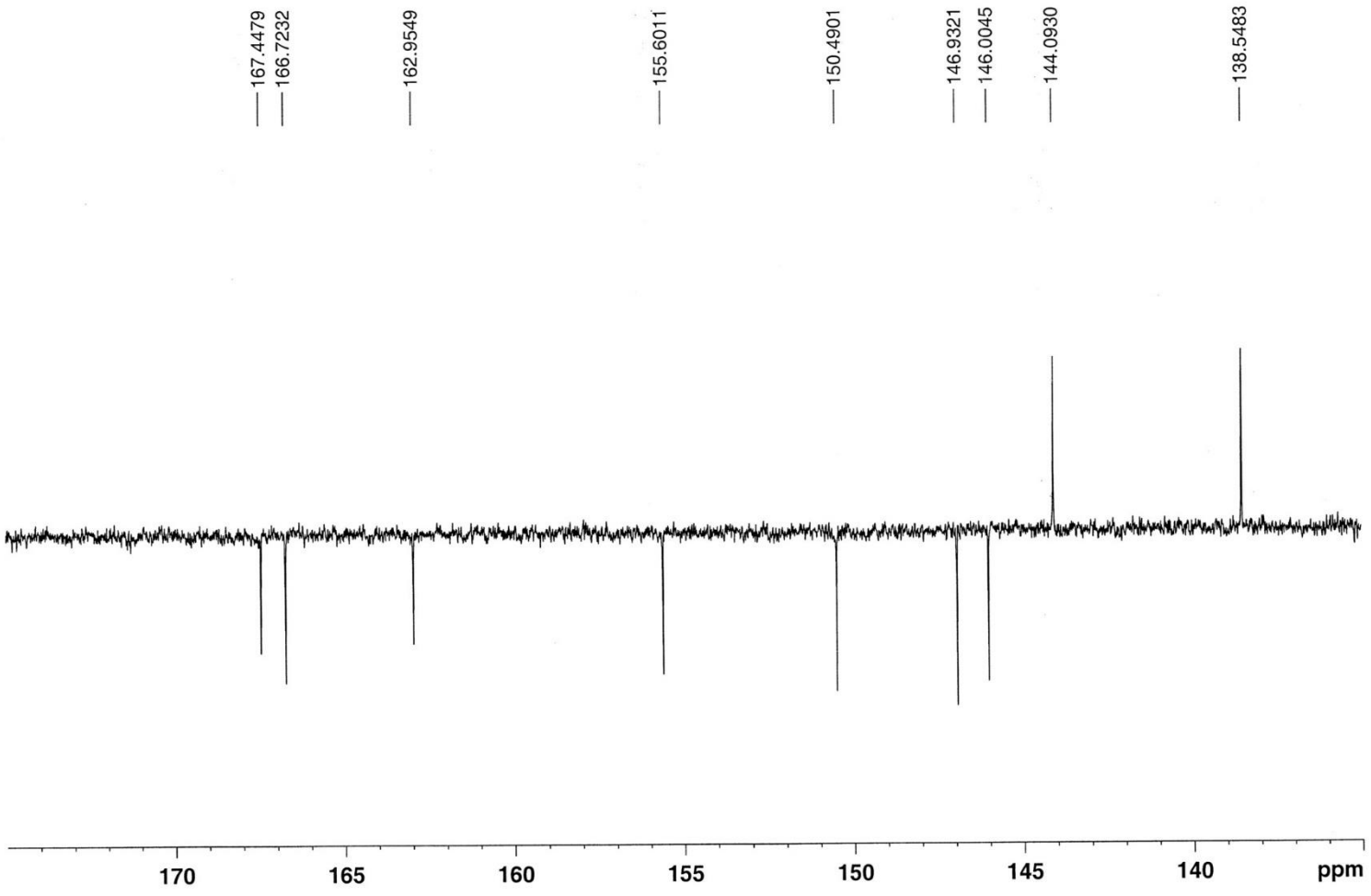


LN303_Ups2_F4_4 in d4Methanol (Proton) 5.10.2018




LN303_Ups2_F4_4 in d4Methanol (APT) 5.10.2018
28.0325
49.0000
56.7026
61.8863
75.3079
76.4761
76.8708
77.2831
81.8084
108.4809
113.3058
113.3168
116.6589
122.8546
124.0202
125.9297
126.2927
128.3921
138.5483
144.0930
146.0045
146.9321
150.4901
155.6011
162.9549
166.7232
167.4479
ppm

LN303_Ups2_F4_4 in d4Methanol (APT) 5.10.2018



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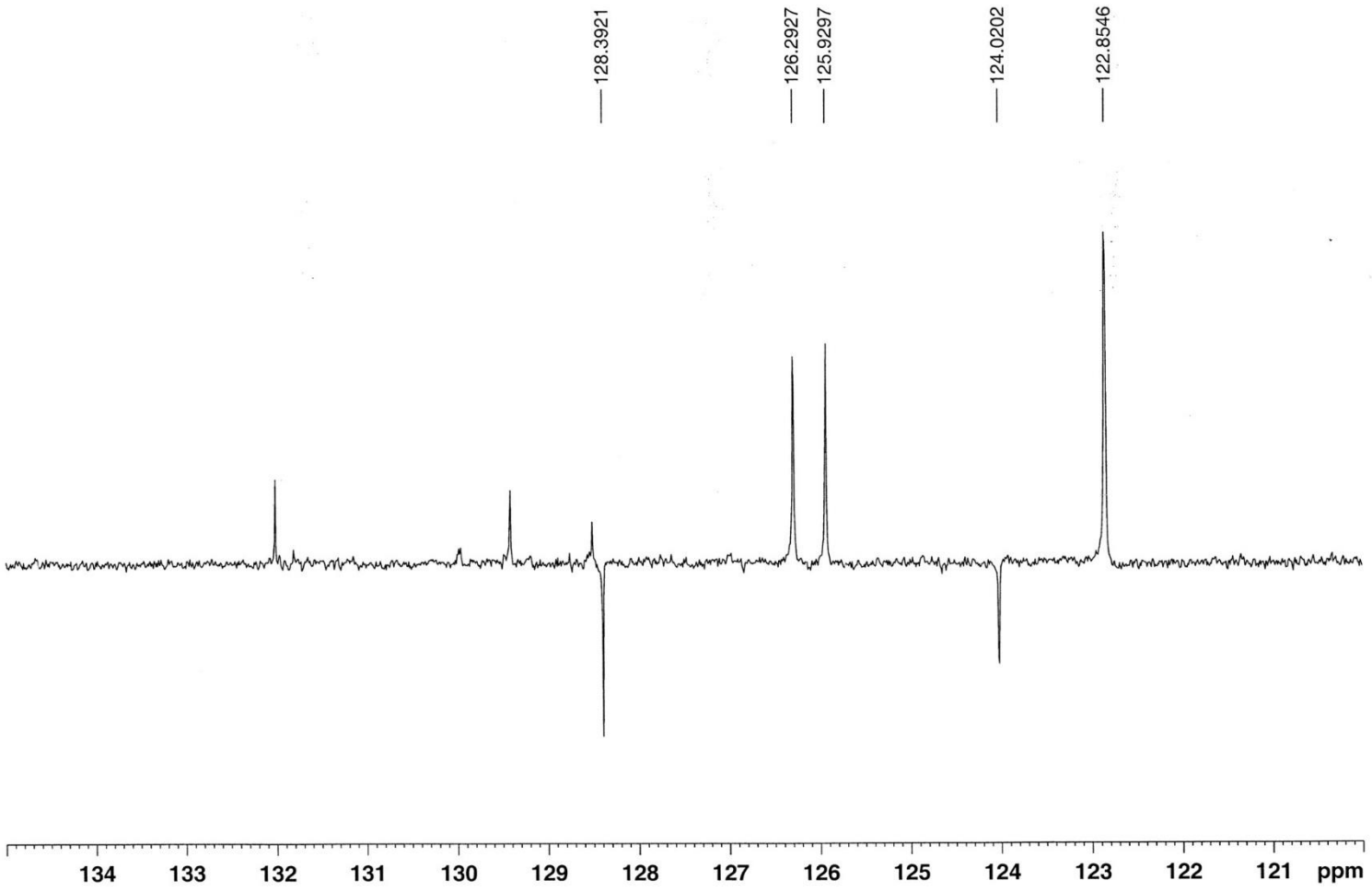
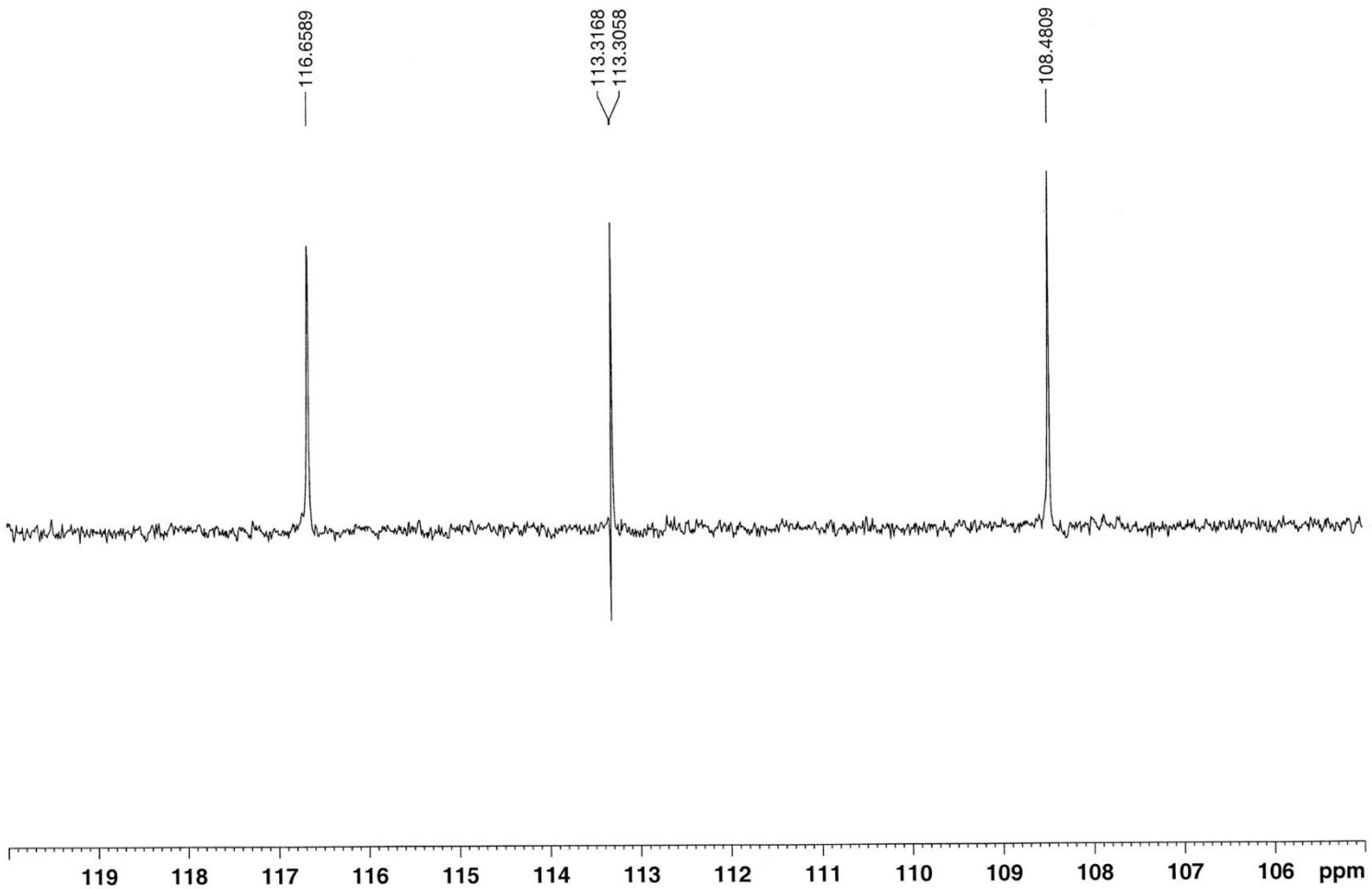
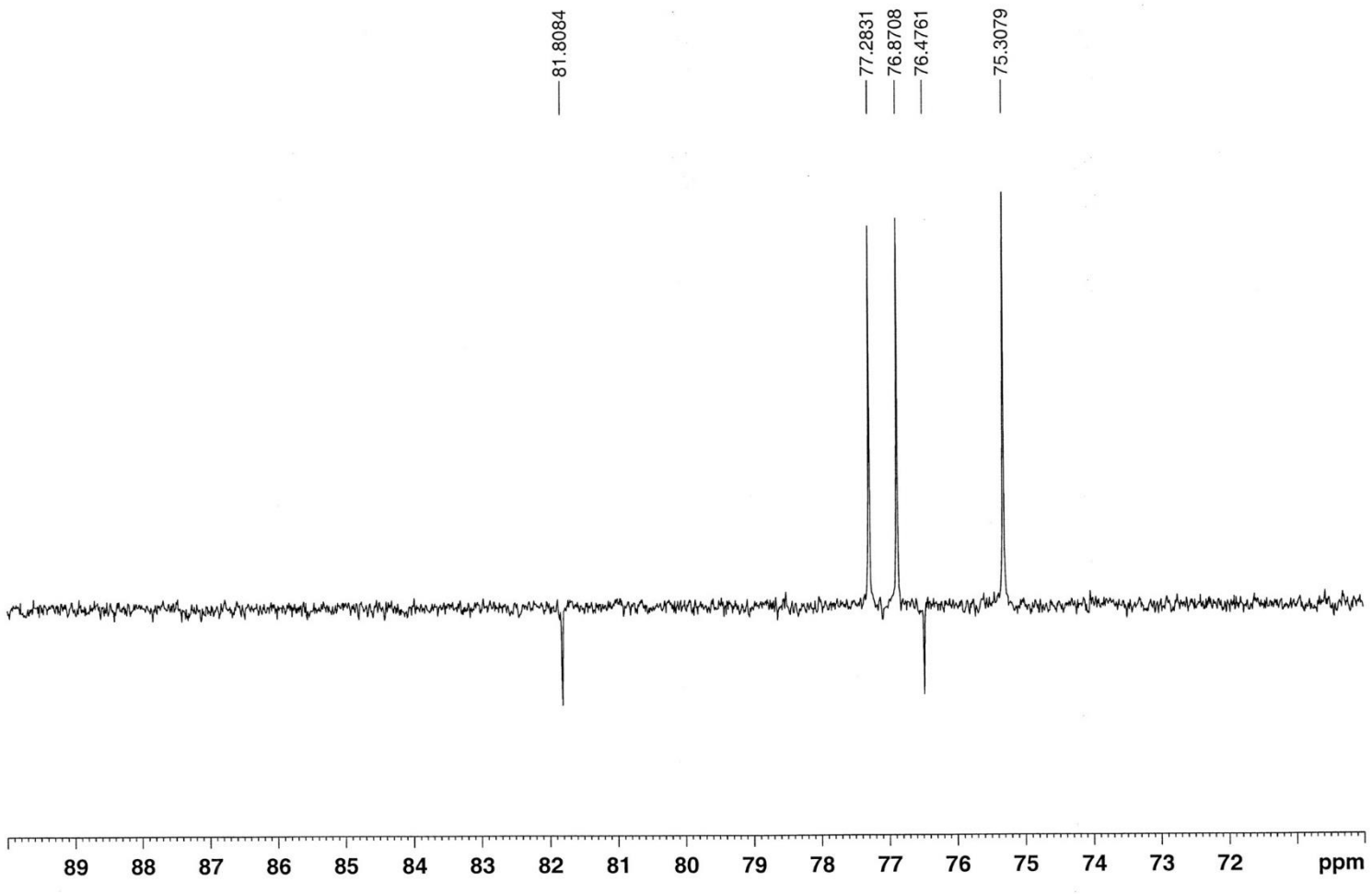
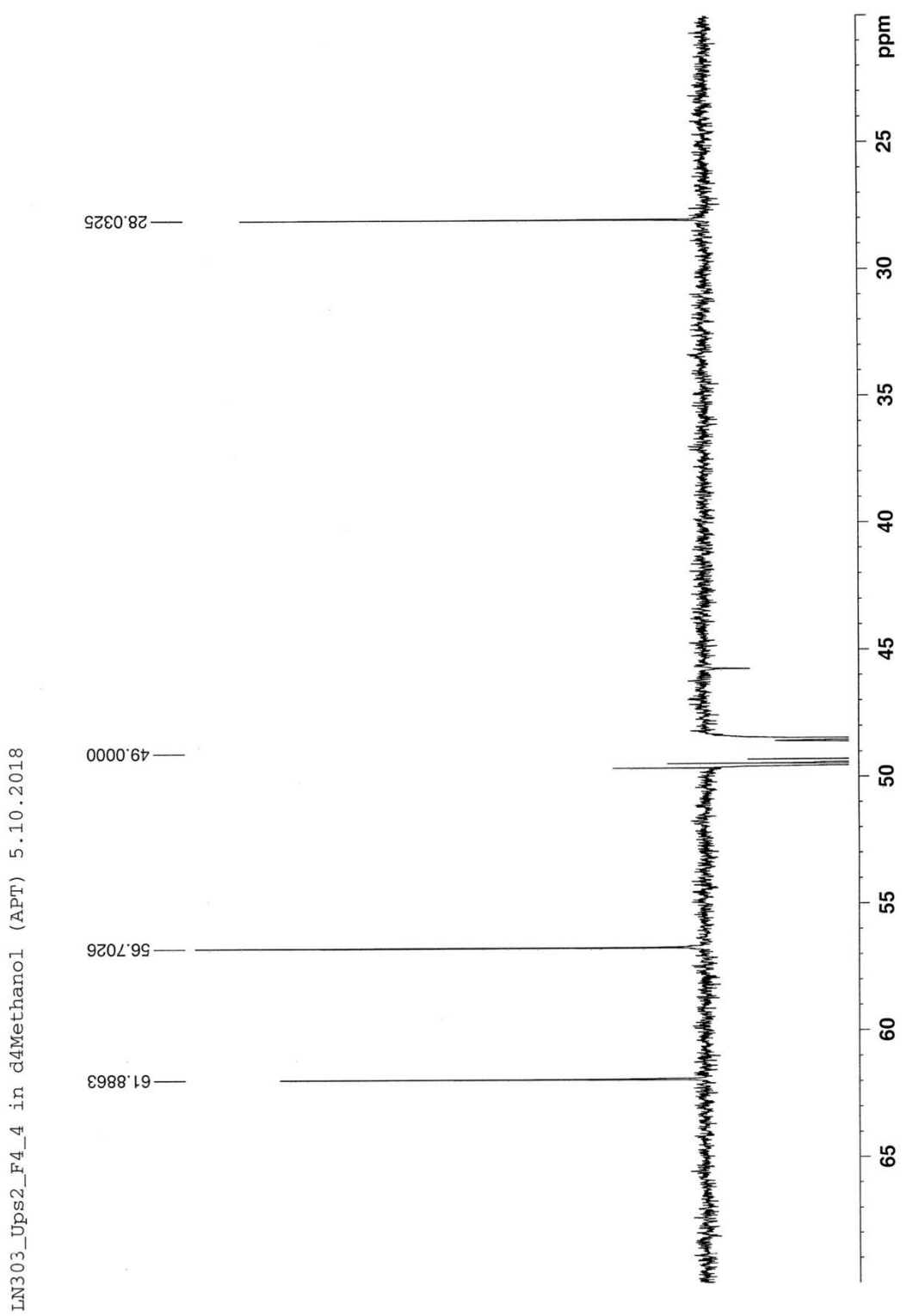


LN303_Ups2_F4_4 in d4Methanol (APT) 5.10.2018



LN303_Ups2_F4_4 in d4Methanol (APT) 5.10.2018




LN303_Ups2_F4_4 in d4Methanol (APT) 5.10.2018
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49.0000
56.7026
61.8863
ppm
25
30
35
40
45
50
55
60
65