universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„Characterization of antimicrobial compounds from endophytic fungi“

verfasst von / submitted by

Martin Aichner, Bakk. rer. nat. MSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magister der Pharmazie (Mag.pharm.)

Wien, 2018 / Vienna, 2018

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	ao. Univ.-Prof. Mag. Dr. Ernst Urban
Mitbetreut von / Co-Supervisor:	Mag. Dr. Martina Oberhofer

2018

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

……………………………   ………………………………………………..

date   (signature)

Acknowledgement

I would like to thank Prof. Mag. Dr Ernst Urban for giving me the opportunity to do my Diploma Thesis at the Department of Pharmaceutical Chemistry and for his support during the thesis. I also want to thank Mag. Dr. Judith Wackerlig for her guidance through the work. I thank Univ.-Prof.Dr. Sergey B. Zotchev for enabling this Thesis and I want to thank Mag. Dr. Martina Oberhofer for providing all biological materials, providing the disc diffusion assays and her advice. I am grateful for the support and the trust I received during this work. Thanks also to the team of Department of Pharmaceutical Chemistry for advices and helpful hands.
Finally, I want to express my appreciation for my parents Rosa and Alois Aichner for their encouragement and support. This work is dedicated to them.

Abstract

Resistances to antibiotics and antimycotics are a worldwide rising issue and the misuse of antimicrobials created diverse multi-resistant germs. Natural sources can provide unknown substances and fungal endophytes are a relative unused origin for molecules with antimicrobial activities. Therefore, this Diploma Thesis deals with the discovery of new antimicrobials derived from fungal endophytes.

Bioactivity-guided fractionation, a method that is largely used for bioactivity screenings of new molecules, was applied within this work to fractionize fungal extracts and to isolate the compounds of interest. Dichloromethane/methanol (2:1) was used for the extraction of the fungi Ab1 (*Preussia* sp.), Ab11 (*Nemania* sp.) and Bl73 (Helotiales). High performance liquid chromatography (HPLC) was utilized as chromatography system. Analytical HPLC was used to investigate the extracts, while semi - preparative HPLC was applied for fractionation of larger amounts of the extracts. For antimicrobial activity testings of the extracts and their fractions disc diffusion assays were performed at the Department of Pharmacognosy (not part of this work). By liquid chromatography - mass spectrometry (LCMS) analysis the molecules of interest were identified and characterized through their mass within the fractions. The compounds of interest, which were determined by LCMS, were subsequently gained by subfractionation of the according fractions.

For this Diploma Thesis fungal extracts of Ab1, Ab11 and BL73 were provided for intial antibiotic activity testing. The provided extracts of Ab1 and Ab11 both inhibited *B. subtilis*, while Bl73 extract inhibited *B. subtilis*, *S. cerevisiae*, *A. niger* and *F. graminearum* in disc diffusion assays. The extracts, which were produced in context of this work, all showed the same antimicrobial activities, except Ab11 extract, which lost activity against *B. subtilis.* Consequently Ab11 extract was excluded for the rest of this work.

After analytical HPLC, Ab1 extract was fractionized by semi - preparative HPLC and some fractions showed inhibition of *B. subtilis*. In the subsequent work on Ab1 extract, however, problems in the dissolution of the extract occurred, which lead to the loss of the antimicrobial effect on *B. subtilis* and therefore the work on this extract was stopped. Following analytical HPLC of Bl73 the extract was also fractionized by semi - preparative HPLC and various fractions inhibited *B. subtilis*, *S. cerevisiae*, *A. niger* and *F. graminearum*.

In the follow up of the work on BI73 extract the LCMS analysis of these active fractions revealed two compounds with mass $[M+Na]^+$ 495.28 [m/z] and mass $[M+Na]^+$ 323.06 [m/z]. These substances were supposed to be responsible for the antimicrobial effects on the test organisms. The isolation by semi - preparative HPLC of the compound with the mass $[M+Na]^+$ 495.28 [m/z] resulted in an unknown pure substance, (which was determined by NMR spectroscopy and was not part of this work). The antibacterial effect of the substance with mass $[M+Na]^+$ 495.28 [m/z] on *B. subtilis* was confirmed by a disc diffusion assay. However, the inhibition of *B. subtilis* was very weak both in the according fraction and pure substance. By the isolation of the substance with mass $[M+Na]^+$ 323.06 [m/z] antifungal properties against *S. cerevisiae*, *A. niger* and *F. graminearum* were confirmed by disc diffusion assays. Nevertheless at the end of this work no information about the purity or the structure of the compound was available. Moreover the inhibitory effect on the fungi was not as good as the effect of the corresponding fraction.
As a conclusion by the isolation of the substance with mass $[M+Na]^+$ 495.28 [m/z] one particular compound of BI73 extract could be gained that inhibited *B. subtilis.* Subsequently to this work the structural analysis of the substance with mass $[M+Na]^+$ 323.06 [m/z] could be done. Furthermore the analysis of other molecules in the fraction containing substances 495.28 [m/z] and 323.06 [m/z] should be done to confirm whether a synergistical effect of inhibition is present.

Keywords: antimicrobials, fungal endophytes, analytical and semi - preparative HPLC, bioactivity-guided fractionation;

Zusammenfassung

Resistenzen gegen Antibiotika und Antimykotika sind ein weltweites Problem und durch den falschen Einsatz von antimikrobiellen Substanzen sind verschiedene multiresistente Keime entstanden. Unbekannte Substanzen können aus natürlichen Ressourcen gewonnen werden und endophytische Pilze stellen eine relativ unbenutzte Quelle für Moleküle mit antimikrobellen Eigenschaften dar. Aus diesen Gründen beschäftigt sich diese Diplomarbeit mit der Entdeckung von neuen antimikrobiellen Substanzen aus endophytische Pilzen.

Bioaktivitätsgeleitete Fraktionierung, eine Methode die bei Bioaktivitäts-Screenings von neuen Stoffen breit angewendet wird, wurde in dieser Arbeit genutzt um Pilz-Extrakte zu fraktionieren und um Reinsubstanzen *zu gewinnen.* Dichlormethan/ Methanol (2:1) wurde für die Extraktion von den Pilzen Ab1 (*Preussia* sp.), Ab11 (*Nemania* sp.*)* und Bl73 (Helotiales) benutzt.

Hochleistungsflüssigkeitschromatographie (HPLC) wurde als Chromatographie - Methode gewählt. Mit analytischer HPLC wurden die Extrakte untersucht, während semi - präparative HPLC für die Fraktionierung von größeren Mengen der Extrakte verwendet wurde. Um die antimikrobielle Aktivität der Extrakte und deren Fraktionen zu überprüfen wurden Plattendiffusionstests am Department für Pharmakognosie durchgeführt (nicht Teil dieser Arbeit). Unter der Verwendung von Flüssigchromatographie mit Massenspektrometrie - Kopplung (LCMS) wurden die wichtigen Moleküle in den Fraktionen identifiziert und hinsichtlich der Masse charakterisiert. Diese speziellen Substanzen, die durch LCMS bestimmt wurden, wurden darauf durch Subfraktionierung der entsprechenden Fraktionen gewonnen. Für diese Diplom Arbeit wurden bereits existierende Pilz Extrakte von Ab1, Ab11 und Bl73 zur Verfügung gestellt. Die zur Verfügung gestellten Extrakte von Ab1 und Ab11 inhibierten beide *B. subtilis*, während der Bl73 Extrakt das Wachstum von *B. subtilis*, *S. cerevisiae*, *A. niger* und *F. graminearum* in den Plattendiffusionstests inhibieren konnte. Die Folgeextrakte, welche im Rahmen dieser Diplomarbeit produziert wurden, zeigten dieselben antimikrobiellen Aktivitäten. Ausgenommen war der Ab11 Extrakt, welcher *B. subtilis* nicht inhibieren konnte und dadurch wurde dieser Extrakt nicht weiter bearbeitet. Nach der HPLC Analyse wurde der Ab1 Extrakt mit semi - präparativer HPLC fraktioniert und einige Fraktionen zeigten Wirkung gegen *B. subtilis*. In der darauffolgenden Arbeit beim Ab1 Extrakt traten Probleme mit der

Löslichkeit auf, welche zum Verlust der antimikrobiellen Aktivität gegen *B. subtilis* führten. Daraufhin wurde die Arbeit an diesem Extrakt gestoppt.

Nach der analytischen HPLC Untersuchung des BI73 Extraktes wurde dieser ebenfalls fraktioniert und verschiedene Fraktionen von BI73 inhibierten *B. subtilis*, *S. cerevisiae*, *A. niger* und *F. graminearum.*

In der weiteren Bearbeitung des BI73 Extraktes wurden über die LCMS Analyse der aktiven Fraktionen die Moleküle mit der Masse $[M+Na]^+$ 495.28 [m/z] und der Masse $[M+Na]^+$323.06 [m/z] gefunden, von denen man sich die antimikrobielle Wirkung gegen die Testorganismen erwartete. Die Isolierung des Moleküls mit der Masse $[M+Na]^+$ 495.28[m/z] resultierte in einer unbekannten Reinsubstanz, (welche durch NMR Spektroskopie bestimmt wurde und nicht Teil dieser Arbeit war). Der antimikrobielle Effekt der Substanz mit der Masse $[M+Na]^+$ 495.28 [m/z] auf *B. subtilis* wurde durch einen Plattendiffusionstest bestätigt. Die Inhibierung von *B. subtilis* war jedoch sehr schwach und entsprach der Inhibierung durch die entsprechende Fraktion. Durch die Isolierung der Substanz mit der Masse $[M+Na]^+$ 323.06 [m/z] wurden antifungale Eigenschaften gegen *S. cerevisiae*, *A. niger* und *F. graminearum* durch Plattendiffusionstests bestätigt. Am Ende dieser Arbeit war jedoch noch keine Information über die Reinheit oder die Struktur dieses Moleküls bekannt. Darüber hinaus war der inhibierende Effekt auf die Pilze schwächer als bei der entsprechenden Fraktion.

Als Schlussfolgerung konnte mit der Isolierung der Substanz mit der Masse von $[M+Na]^+$ 495.28 [m/z] eine bestimmte Komponente des BI73 Extraktes gewonnen werden, welche *B. subtilis* inhibierte. Nachfolgend zu dieser Arbeit könnte die Strukturanalyse der Substanz mit der Masse $[M+Na]^+$ 323.06 [m/z] vorgenommen werden. Weiters sollten andere Moleküle in der Fraktion der Substanzen 495.28 [m/z] und 323.06 [m/z] analysiert werden, um zu bestätigen, ob ein synergistischer Effekt der Inhibition vorhanden ist.

Schlagwörter: antimikrobielle Substanzen, endophytische Pilze, analytische und semi - präparative HPLC, bioaktivitätsgeleitete Fraktionierung;

Content

1 Introduction……………………………………………………………………………......1

1.1. Antimicrobial Resistance……………………………………………………………1

1.2. Plant associated microorganisms...1

1.2.1 Definition of endophytes ………………………………...…........……….......1

1.2.2 *Preussia sp*………………………………………………………………….......2

1.2.3 *Nemania sp*…………………… ……………………..…………………….....3

1.2.4 Helotiales…………………………………………………………………….3

1.3 Bioactivity-guided fractionation and disc diffusion assays………………………4

1.4 Test organisms for this diploma thesis……………………..…………….……….5

1.4.1 *Bacillus subtilis*……………………………………………………………......6

1.4.2 *Escherichia coli*………………………………………………………..……..7

1.4.3 *Saccharomyces cerevisiae*……………………………………………………8

1.4.4 *Aspergillus niger*…………………………………..………………………..9

1.4.5 *Fusarium graminearum*…………………………………………..…………10

1.5 Analytical and semi - preparative HPLC…………………………………………10

1.6 Aim of this work…………………………………………………………………12

2 Materials and Methods……………………...……………………………………12

2.1 Chemicals……………………...12

2.2. Origin of fungi and provided fungal extracts…...13

2.3 Test organisms of this work.……………………………………........................14

2.4 Diffusion disc assay……………………………………………….....................14

2.5 Extraction of fungi ………………………………………………......................14

2.6 HPLC………………………...15

2.6.1 Analytical HPLC……...15

2.6.2 Semi - preparative HPLC and fractioning of Ab1…………….…………16

2.6.3 Semi - preparative HPLC and fractioning of Bl73…………..……........18

2.6.4 Semi - preparative HPLC and subfractioning of Bl73
Fraction 10 and Fraction14/15.3…………………………..……………….20

2.7 Liquid chromatography mass spectrometry (LCMS………..…………….……..23

2.8 Additional equipment ……………………….………..25

3 Results………………………….………………...……………..……………...............25

3.1 Disc diffusion assay of the provided extracts ……………………………………25

3.2 Extraction of fungi and diffusion disc assays …...25

3.3 Analytical HPLC and fractionation of Ab1 extract ……………….……..........27

3.3.1 Analytical HPLC of Ab1 extract….……………………..............................27

3.3.1.1 Provided Ab1 extract ………………….….…………….........27

3.3.1.2 Second Ab1 extract ………………………………….….............28

3.3.1.3 Semi - preparative HPLC of Ab1 extract..……….………….......30

3.3.1.4 Results of disc diffusion assays of
fractionation of Ab1 Extract ….……………………………….…..32

3.3.1.5 Subsequent fractionation of Ab1 Extract ………..…………..... 33

3.4 Analytical HPLC and fractionation of Bl73 extract………………..……….........34

3.4.1 Analytical HPLC of second Bl73 extract………....................….........34

3.4.2 Semi - preparative HPLC of second Bl73 extract ………………….......35

3.4.3 Results of disc diffusion assays of Fractionation ……………….........39

3.4.4 .Analytical HPLC of Bl73 Fractions..……………………………..….......40

3.5 LC-MS of Bl73 Fractions …………………………………..…………………....48

3.5.1 BL73 Fraction 3 - LCMS run analysis ……………………………..….......49

3.5.2 BL73 Fraction 8 - LCMS run analysis ……………...…..……..................52

3.5.3 BL73 Fraction 9 - LCMS run analysis………………………………..........54

3.5.4 BL73 Fraction 10 - LCMS run analysis…………….....………….........57

3.5.5 BL73 Fraction 11 - LCMS run analysis……………………….......……63

3.5.6 BL73 Fraction 14 - LCMS run analysis…………….….…….................68

3.5.7 BL73 Fraction 15 - LCMS run analysis……………...............................73

3.6. Subfractionation of Bl73 fractions………………………………………..........78

3.6.1 Subfractionation of Bl73 Fraction 10………………….........................78

3.6.1.1 Analytical HPLC of Bl73 Fraction 10……………………...............78

3.6.1.2 Semi - preparative HPLC and subfractionation of Bl73 Fraction 10………………………………….………………...79

3.6.1.3 Weights of subfractionation of Bl73 Fraction 10…………….....80

3.6.1.4 Analysis of Direct injection MS of Bl73 Subfraction F10.11.......81

3.6.1.5 Analysis of Direct injection MS of Bl73 Subfraction F10.3.........82

3.6.2 Subfractionation of Bl73 Fraction 14 and 15…………………..……....84

3.6.2.1 Semi - preparative HPLC and subfractionation of Bl73 Fraction14/15……………………………………………………84

3.6.2.2. Disc diffusion assays of subfractionation of Bl73 Fraction 14 and 15…………………………….…………....85

4 Discussion……………………………….……………………………........………..86

4.1 Endophytes and test organisms of this work ………………………….........86

4.2 Bioactivity-guided fractionation ………………..87

4.3 Ab1 extract………..……………………………………………..………...........88

4.3.1 Disc diffusion assays of Ab1 extract……………….……………………88

4.3.2 Fractionation of Ab1 extract ……………………………….....................89

4.4 Ab11 extract……………..…………..90

4.5 Bl73 extract……………………..……………………………………………..……..90

4.5.1 Disc diffusion assays of Bl73 extract…………….…………………..………90

4.5.2 Fractionation of Bl73 extract……………………………………………….91

4.5.3. LCMS analysis of Bl73 extract…………………………………………….93

4.5.4 Semi - preparative HPLC of Bl73 Subfraction 10………………………...94

4.5.5 Semi - preparative HPLC of Bl73 Subfractions 14 and 15………………97

5 Conclusion……………………………………………………………………….....98

6 Outlook……………………………………………........……………………..98

7 Literature……………………………........…………………..……..................................99

8 Appendage…..........………………………………...110

1. Introduction

1.1. Antimicrobial Resistance

Antimicrobials are essential medicinal agents and since the last past-half century they have saved millions of patients from life threatening infections. Penicillin by Fleming was the first antibiotic that was largely used in clinical treatment. However, soon microbial resistances were observed. Since then the misuse of antimicrobials has created multi resistant germs like Methicillin- resistant *Staphylococcus aureus* (MRSA), which are a worldwide problem. In the last decade only few new antimicrobials were developed and thus the need for new antimicrobials, that overcome this issue, arise [Cantas *et al.*, 2013].

Generally there are two strategies for development and discovery of new antibiotics. The first is related to in silicio design of new compounds and the second involves the isolation of bioactive molecules from natural sources [Mapperson *et al.*, 2014]. While the computational development deals with in silicio methods regarding functional structure analysis [Tavares et. al, 2013], the development of antimicrobials from natural sources includes the isolation of compounds produced by plants [Cowan, 1999] or microorganisms [Gunatilaka, 2006].

1.2 Plant associated microorganisms

1.2.1 Definition of endophytes

Microorganisms provide a great source of low molecular weight products including immune-suppressants, agents against cancer, antibiotics, etc. They colonize various biomes on earth *e.g*. the Arctic, the Antarctic, deserts and alpine regions. It is supposed that only 1 % of the bacterial and 5% of the fungal species are known [Gunatilaka, 2006] with very recent estimations of 2.2 to 3.8 million different fungal species [Hawksworth and Lücking, 2017].
The plant microbiome comprises different microbial communities found in various parts of plants [Egamberdieva, 2017], forming mutualistic relationships [Gunatilaka, 2006].

These endophytes penetrate and reside within the plants without harming them. The host plant feeds and protects the endophytes, while the microorganism produces secondary metabolites that enhance the growth of the host plant and protects it from pathogens, and deliver a plethora of other benefits [Gunatilaka, 2006; Fridlender *et al.*, 2015; Nisa *et al.*, 2015].

Endophytic fungi are predominately consisting of Ascomycetes [Gunatilaka, 2006; Arnold, 2007] and Fungi Imperfecti [Gunatilaka, 2006; Nisa *et al.*, 2015]. Endophytes were discovered in all plant species, that were investigated and it was also reported that a plant that does not comprise any endophyte is rarely found [Santoyo *et al.*, 2016]. Secondary metabolites build by the endophytic fungi can have medicinal effects [Jia *et al.*, 2016] and moreover fungal endophytes are a hardly tapped resource for biomolecules [Gunatilaka, 2006; Nisa *et al.*, 2015].
Secondary metabolites are defined as molecules that are not essential for normal growth [Nisa *et al.*, 2015], albeit they serve for defense or the communication between endophytes and plants. They may consist of diverse classes of chemical substances like steroids, xanthones, phenols, etc. [Nisa *et al.*, 2015]. Hence, fungal endophytes can serve as source for new chemotherapeutic agents and antibiotics [Nisa *et al.*, 2015]. Often the discovery of unknown chemicals and antimicrobials is associated with fungi that were isolated within medical plants [Jia *et al.*, 2016].
As an example, the roots of *Atropa belladonna* were used for the isolation of antifungal and antibacterial compounds preusomerrins J, K, L from fungi of the form group mycelia sterilia [Razzaghi-Abyaneh and Rai, 2013]. Therefore, previously to this work, fungal endophytes were isolated from *Atropa belladonna* and *Bergenia pacumbis* at the Department of Pharmacognosy. By molecular sequencing of the ITS barcoding region, which was done by Mag. Dr. Martina Oberhofer using the ITS1, 5.8S, ITS2 and the LSU region, these isolates resulted in three taxa: *Preussia* sp., *Nemania* sp. and Helotiales order.

1.2.2 *Preussia* sp.

Preussia sp. appears in various ecosystems and the fungi within this genus have different lifestyles as they for example can exist as saprophytes or as endophytes [Gonzalez-Menendez *et al.*, 2017]. Most of the fungi occur on dung, albeit they were

also found in wood, soil, etc. It is a genus that is hardly examined [Gonzalez-Menendez *et al.*, 2017, Mapperson *et al.*, 2014] and morphological characterizations in order to distinguish the fungi from *Sporomiella* sp. and *Spororominula* sp. are difficult. By DNA sequencing of the ITS barcoding region sequence led to the taxonomic re-assignment of the new genus *Preussia* [Mapperson *et al.*, 2014]. *Preussia* species produces various kinds of bioactive secondary metabolites with antimicrobial compounds among them, especially preussomerins [Mapperson et al, 2014; Gherbawy and Elhariry, 2016]. Preusomerins were isolated from *Preussia isomera* and antifungal properties were reported [Herz *et al*., 2002]. It was also shown that extracts of *Preussia* species can affect various microorganisms *e.g. Bacillus cereus*, *Escherichia coli*, Candida *albicans*, etc. [Mapperson *et al.*, 2014].

1.2.3 *Nemania* sp.

Nemania sp. comprises approximately 37 species and it is considered as a plurivorous genus of fungi [Tang *et al.*, 2007]. Previously it was considered that species of this genus belong to *Hypoxylon*, as there were problems in the classification of the fungi. By now, however, these fungi are seen as own genus [Tang *et al.*, 2007]. *Nemania* sp. belongs to the family of Xylariaceae [Ju and Rogers, 2002] and Xylariaceae belongs to Ascomycota [Cruz and Cortez, 2015].
It is found on rotting wood of angiosperms and these fungi were also found as endophytes [Tang *et al.*, 2007]. Moreover *Nemania* sp. is known as producer of antimicrobials [Liu *et al.*, (2016]. For instance Liu *et al.*, (2016) isolated fungi of traditional Chinese medicinal plant *Cephalotaxus hainanensis* and the fungi from genera *Nemania* sp. inhibited various microorganism *e.g. B. subtilis* or *Fusarium oxysporum.*

1.2.4 Helotiales

Helotiales belongs to the class of Leotiomycetes, which is a lichen-forming division of Ascomycota. They occur in diverse habitats of nature and also have different lifestyles. For instance some of the species occur as plant pathogens, whereas some of them have mutualistic relationships to plants and appear as endophytes [Wang Z *et al.*, 2006]. As an example, *Pseudogymnoascus* sp. strains found in antarctic and

sub-antarctic regions, were shown to possess certain antimicrobial activity against Gram-positive and Gram-negative bacteria [Yogabaanu *et al.*, 2017].

The fungal endophytic strains from *Preussia* sp., *Nemania* sp. and Helotiales order served as source for possible antimicrobials. The production of extracts after cultivation of fungi and the subsequent analysis and purification was the main concept in this Diploma Thesis. This is a method widely applied in research, which deals with the isolation of molecules from natural sources and it is linked with the term "bioactivity-guided fractionation" [Atanasov *et al.*, 2015].

1.3 Bioactivity-guided fractionation and disc diffusion assays

Bioactivity-guided fractionation is often synonymously used for bioassay-guided fractionation, biochemical detection and bioactivity screening. Predominantly it is associated to the discovery of drugs [Weller, 2012].
The concept of bioactivity-guided fractionation is to separate complex mixtures of substances derived from extracting entire organisms or from fermented substrates by microbes through fractionation [Atanasov *et al.*, 2015].This is done to reduce the complexity of the samples and sometimes multiple separation steps coupled with bioactivity screenings are required to finally isolate the molecule of interest [Atanasov *et al.*, 2015; Weller, 2012]. This was the main concept applied within this diploma thesis.
Bioactivity guided fractionation involves appropriate methods for extraction [Atanasov *et al.*, 2015] and extraction is one of the most important procedures within the isolation of the desired chemical compound. For the extraction, the chemical properties of the molecules are important. Thus for hydrophilic components polar solvents as methanol, ethanol or ethylacetat are used, whereas for the isolation of more hydrophobic molecules, dichloromethan or a dichloromethane/methanol mixture is applied [Sasidharan *et al.*, 2011]. In context of this Diploma Thesis a mixture of dichloromethane/methanol (2:1) was used for the extraction of the fungi. This was done because in former extraction procedures within the Department of Pharmacognosy this combination gave the best results and yields.

For the segregation of a molecule from an extract the utilization of a chromatography system is necessary. Various chromatography strategies are available which include among other things Gas Chromatography and Liquid Chromatography. For Liquid Chromatography several techniques are accessible *e.g.* Low-Pressure LC, HPLC and Ultra-Performance Liquid Chromatography (UPLC) [Weller, 2012]. As HPLC is very common in the pharmaceutical research [Ahuja and Dong, 2005] for this Diploma Thesis HPLC was used in order to analyze and fractionize the fungal extracts.

Another important part in bioactivity-guided fractionation is an appropriate biological testing system, by which the activity of the collected fractions is analyzed [Weller, 2012]. Antimicrobial activity testings are widely applied in biological assays [Driscoll *et al*., 2012] and disc diffusion assays are commonly used for detection of new antimicrobial molecules [Driscoll *et al.*, 2012; Mbah *et al.*, 2012].
Generally in the disc diffusion assays, agar plates are inoculated with a test organism at a standardized concentration. Subsequently filter paper discs, which comprise the molecules of interest at a certain concentration, are placed on the agar surface and cultivated under proper conditions for the test organism. The test compound diffuses into the agar and inhibits the growth of the microorganism. In the end the size of possible inhibition zones are measured. For optimal growth of the microorganism, it is cultivated under optimum conditions, which predominantly includes the optimal growth medium and growth temperature [Balouiri *et al.*, 2016].
Thus disc diffusion assays were selected to determine the antimicrobial activity of the extracts and their fractions. They were performed by the co-supervisor of this Diploma Thesis, Mag. Dr. Martina Oberhofer from the Department of Pharmacognosy. Therefore, in methods and materials and results disc diffusion assays are not going to be discussed in detail.

1.4 Test organisms for this diploma thesis

In the context of this Diploma Work antimicrobial activity testings were performed in laboratory facilities of the Department of Pharmacognosy. As there are four classes of risk group assessments there are certain safety requirements for the work with certain microorganisms. The laboratories of the Department of Pharmacognosy cover

safety level 1, which allows the work on microbes that belong to risk group 1. Bacteria that are allocated to this group there is no or a negligible safety-risk by the current state of scientific knowledge and techniques (§ 1 Z 1 GTG). [GENTECHNIKBUCH: 3. KAPITEL LISTE RISIKOBEWERTETER MIKROORGANISMEN FÜR GENTECHNISCHE ARBEITEN - TEIL 1: BAKTERIEN, Bundesministerium für Gesundheit und Frauen]. On account of this all strains of test organisms, which were tested in context of this Diploma Thesis, belonged to risk group one. In the following the test organisms are presented.

1.4.1 *Bacillus subtilis*

Bacillus subtilis is an ubiquitous bacterium which can be isolated from terrestrial and aquatic environments. It can also be found around plants and even in gastrointestinal tracts of animals. It forms spores and it is also forming biofilms. Moreover it is a Gram - positive bacterium. [Brenner and Miller, 2013; Earl *et al*, 2008].

As a Gram-positive bacterium, *B. subtilis* has a certain structure of cell wall. The cell wall of Gram -positive bacteria predominately includes a broad layer of peptidoglykan with teichoic and lipoteichoic acids. Moreover they do not have an outer membrane and periplasmatic space [Black, 2012; Rosenthal, 2016]. Fig.1 gives a schematic overview of the cell wall structure of Gram - positive bacteria. These are important aspects, because the cell wall has an essential influence on the activity of antimicrobial agents. As far as Gram - positive bacteria is concerned, the cell wall structure makes it very permeable for most antibiotics [Rosenthal, 2016].

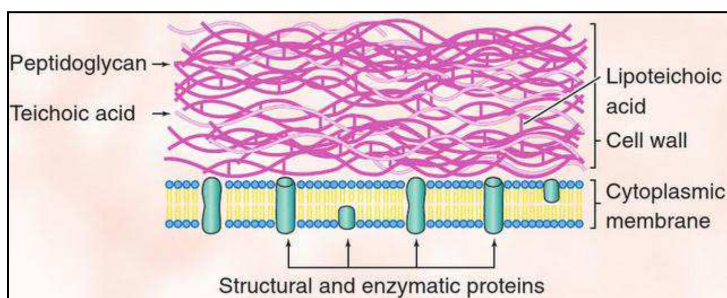

Fig. 1 Schematic overview of the Gram - positive cell wall [source: (Rosenthal, 2016)].

Another important aspect about Gram-positive bacteria is that they are commonly associated with growth on skin. They can appear as commensals, but can also be responsible for infections. Therefore Gram-positive bacteria are often responsible for

cutaneous and systemic infections [Chiller *et al.*, 2001]. In contrast to this *B. subtilis* is not considered as pathogen and is seen as a save species [Brenner and Miller, 2013]. Therefore *B. subtilis* was chosen for antimicrobial activity testings, because it is a Gram - positive bacterium and because Gram - positive bacteria often cause infections [Chiller *et al.*, 2001].

Tab.1: Antibiotic susceptibilities of *B subtilis* strain 168, MIC = Minimal inhibitory concentration; [modified from (Tanaka et al., 2013)].

Antibiotic	Major target	MIC (μg/ml) 168
Actinomycin D	DNA	0.08
α-Amanitin	RNAP	>50
Ampicillin	Cell wall	0.08
Antimycin A	Respiratory chain	>100
Apramycin	Ribosome	0.8
Bacitracin	Cell wall	>100
Cephalexin	Cell wall	0.2
Decoyinine	Nucleotide synthesis	>1,000
Erythromycin	Ribosome	0.1
Fidaxomicin	RNAP	5 (10)
Formycin	Nucleotide synthesis	>200
Fusidic acid	Ribosome	0.3
Geneticin	Ribosome	0.8
Gentamicin	Ribosome	0.1
Gramicidin	Membrane	>100
Griseofulvin	Tubulin microtubules	>100
Hygromycin	Ribosome	20
Kanamycin	Ribosome	0.5
Kasugamycin	Ribosome	2,000
Lincomycin	Ribosome	30
Neomycin	Ribosome	0.2
Novobiocin	DNA gyrase	0.8
Oligomycin	Respiratory chain	>100
Paromomycin	Ribosome	0.3
Penicillin G	Cell wall	0.1
Polymyxin B	Membrane	20
Puromycin	Protein synthesis	20
Rifampin	RNAP	0.3 (0.4)
Rifamycin SV	RNAP	4 (7)
Spectinomycin	Ribosome	30
Spiramycin	Ribosome	5
Streptomycin	Ribosome	10
Streptothricin	Ribosome	3
Tetracycline	Ribosome	8
Thiostrepton	Ribosome	0.03
Tubercidin	Nucleotide synthesis	>100
Vancomycin	Cell wall	0.3

Furthermore the bacterium is widely used in biotechnology for genetic research and it serves as model organism for the development of chemical agents as sporicides (substances that kill spores [Brenner et al., 2013]) and antibiotics [Tanaka et al., 2013]. Table 1 gives an overview of the antibiotic susceptibilities of *Bacillus subtilis* strain 168, a widely used strain in research [Zeigler et al., 2008]. This was also the strain, which was used within the disc diffusion assays of this Diploma Thesis.

Moreover it was also reported that strains with antibiotic resistance against erythromycin, chloramphenicol, tetracycline, rifampicine and streptomycin [Adimpong et al., 2012] exist.

1.4.2. *Escherichia coli*

E. coli is a Gram - negative bacillus that is the most well researched organism to date. It is used as model organism in research [Blount, 2015]. *E. coli* is a Gram - negative bacterium and the cell wall structure differs from Gram - positive bacteria in certain aspects. The Gram - negative call wall includes an outer membrane attached with Lipopolysaccharide. It features a thin layer of peptidoglycan and the cell wall also contains a periplasmatic space [Black, 2012; Rosenthal, 2016]. Fig.2 gives a

schematic overview of the cell wall structure of Gram - negative bacteria. As far as the permeability of antibiotics is concerned, the structure of cell wall protects Gram - negative from many antibiotics [Rosenthal, 2016].

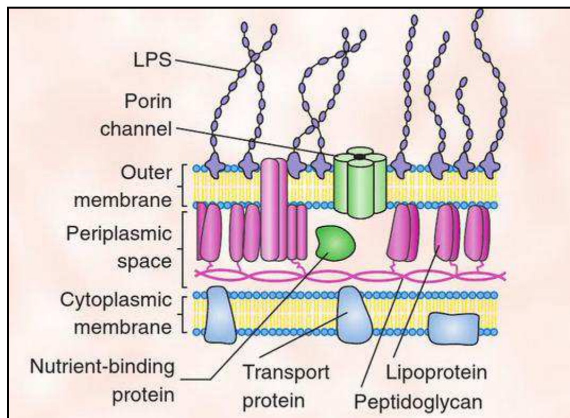

Fig. 2: Schematic overview of the Gram - negative cell wall;
[Source: (Rosenthal, 2016)]

E. coli is a commensal and therefore rarely causes diseases. [Allocati *et al.*, 2013] However, pathogenic variants are responsible for diseases including urinary tract infection, gastroenteritis, meningitis, etc. [Tadesse *et al.*, 2012]. Thus the reasons for testing *E. coli* in disc diffusion assays were that on the hand it is a Gram - negative bacterium and on the other *E. coli* can be connected to infections.
Resistances to antibiotics were reported and those resistances are depending on the isolate of *E. coli*. The most common resistance phenotype includes older drugs as tetracyclines, sulfonamide, streptomycin and ampicillin [Tadesse *et al.*, 2012].

1.4.3 *Saccharomyces cerevisiae*

S. cerevisiae is a yeast that is used for food industry and biotechnology research [Barchiesi *et al.*, 1998]. Yeasts belong to the kingdom of fungi [Fell and Kurtzman, 2005]. It usually grows by budding and it is associated with formation of biofilms [Bojsen *et al.*, 2012]. It is non pathogenic [Barchiesi *et al.*, 1998; Pilehvar-Soltanahmadi *et al.*, 2014] whereas it was reported to affect hospitalized immunosuppressive patients [Barchiesi *et al.*, 1998]. *S. cerevisiae* is an eukaryote, thus it is used to evaluate cytotoxicity of compounds in research [Degrandi *et al.*, 2010]. This was an important aspect for this work and the main reason why *S. cerevisiae* was tested in antimicrobial assays.

Furthermore the fungus is an important model organism for research in human disease [Hiren *et al.*, 2010; Botstein *et al.*, 1997], and it is also used for testing and research of new antifungal agents [Pilehvar-Soltanahmadi *et al.*, 2014].

Generally *S. cerevisiae* is affected by triazole agents [Pfaller *et al.*, 1997] like itraconazole, fluconazole and by amphotericin B and flucytosine [Barchiesi, *et al.*, 1998; Pfaller *et al.*, 1997]. A more recent triazole agent is posaconazol, also known as Noxafil or SCH 56592 [Nomeir et al. 2008]. Table 2 gives an overview of the antifungal susceptibilities of S*. cerevisiae* compared to *Candida albicans,* a pathogenic yeast.

Tab.2: Antifungal susceptibilities of *C. albicans* and *S. cerevisiae,* MIC = Minimal inhibitory concentration; [modified from (Pfaller et al., 1997)].

Organism (no. of isolates)	Antifungal agent	MIC (µg/ml)		
		Range	50%	90%
Candida albicans (166)	Sch 56592	0.03–>4.0	0.06	0.25
	Itraconazole	0.015–>8.0	0.03	0.25
	Fluconazole	0.12–>128	0.25	2.0
	Amphotericin B	0.25–1.0	0.5	1.0
	5-FC	0.06–>128	0.25	4.0
Saccharomyces cerevisiae (22)	Sch 56592	0.12–1.0	0.5	0.5
	Itraconazole	0.03–0.5	0.5	0.5
	Fluconazole	0.5–16	2.0	16
	Amphotericin B	0.5–1.0	1.0	1.0
	5-FC	0.06–0.12	0.06	0.12

Recent reported chemicals that harm *S. cerevisiae* are 3-Cyclohexan propionic acid and 4-phenyl butyric acid [Pilehvar-Soltanahmadi *et al.*, 2014]. It is also affected by other chemicals like alkenals (acyclic, unsaturated aldehydes) [Kubo *et al.*, 2003] and essential oils like linalool, carvacrol or thymol [Kuorwel *et al.* 2011].

1.4.4 *Aspergillus niger*

A. niger is ubiquitous in worldwide nature and is predominantly found in soil, in compost and on rotting plant material. [Hendrickx *et al.*, 2012; Tokarzewski *et al.*, 2012] The filamentous fungus [Martos *et al.*, 2010] is insensitive to temperature and pH and has a high production of air-distributed conidia. Furthermore it is responsible for spoilage of food [Hendrickx *et al.*, 2012].

Aspergillus sp. is associated with the development of allergies against mold [Twaroch *et al.*, 2015]. When there is enough humidity, bad ventilation and organic nutrient sources, then the fungi grow inside of buildings [Rogawansamy *et al.*, 2015;

Twaroch *et al.*, 2015]. Due to these and the spread through the air-distributed spores [Hendrickx *et al*., 2012] *A. niger* was selected for antimicrobial diffusion disc tests. Generally the species is seen as save [Hendrickx *et al.*, 2012; Tokarzewski S, *et al.,* 2012].

Apergillus spp. was reported to be sensitive for amphotericin B, itraconazole, voriconazole, posaconazole , andravuconazole [Baddley *et al.*, 2009]. Examples for echinocandins are micafungin [Martos *et al.*, 2010; Baddley *et al.*, 2009], caspofungin [Baddley *et al.*, 2009] and anidulafungin [Martos *et al.*, 2010].

1.4.5 *Fusarium graminearum*

F. graminearum is a major cause for Fusarium head blight, which is a destructive disease on cereals [Becher *et al*., 2010; Gamba *et al*., 2016; Chen and Zhou, 2009]. Becher *et al.,* (2010) reported that *F. graminearum* produces several potent mycotoxins such as deoxy- nivalenol, nivalenol and the estrogenic poly-ketide zearalenone that also harm human and animal health.

Hyphae of the fungus grow from hyphae fragments [personal observation of Mag. Dr. Martina Oberhofer] and account of this *F. graminearum* was selected for antimicrobial tests in disc diffusion assays.

F. graminearum was reported to be resistant against many fungicides. Generally resistances to azoles and other demethylation inhibitors like piperazine, pyridine, and pyrimidine fungicides were reported [Becher *et al.*, 2010].

1.5 Analytical and semi - preparative HPLC

HPLC belongs to column chromatography and it is a method that is used for the isolation and quantification of molecules within samples. In broad terms it consists of a column, called stationary phase, a pump and a detector. The pump drives the mobile phase through the column and the detector views the retention time of the analytes. [Malviya et al, 2010]. Fig. 3 gives a schematic overview of the modules of a HPLC system.

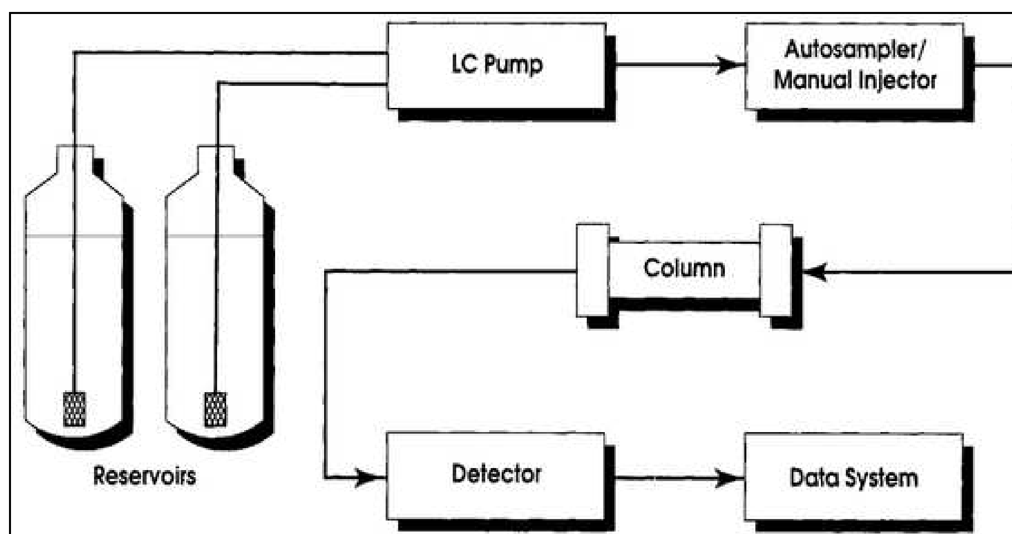

Fig 3: Schematic overview of a HPLC system; [Source: (Ahuja and Dong, 2005)].

For pharmaceutical purposes HPLC often includes a multi-solvent pump, a column oven, an autosampler and an UV/VIS detector and/or a photodiode array detector. All modules are regulated by a computational system [Ahuja and Dong, 2005]. The components of the sample, which is injected to the mobile phase, chemically or physically interact with the stationary phase. This results into retention of the molecules to the stationary phase and the retention time depends on the chemistry of the analyte and the type of chromatography which is used. Normal phase chromatography utilizes a polar stationary phase and a non polar-mobile phase, whereas in reverse phase chromatography an apolar stationary phase and a polar mobile phased is used [Malviya et al, 2010]. Within this work reversed phase chromatography was applied.

Common solvents for the mobile phase are combinations of water and organics like acetonitrile and methanol. For separation and elution of the analytes often the composition of the mobile phase is changed during the analysis. The choice of the mobile phase and gradient depends on the sample to be analyzed [Malviya et al, 2010]. Within this Diploma Work gradients of acetonitrile and water were applied in order to achieve the best separation of the substances.
Analytical HPLC is used when a small amount of material is sufficient for analytic purposes, whereas preparative HPLC is used when larger amounts of materials need to be separated.

The difference between analytical and preparative HPLC also includes the use of columns of different sizes. Typically analytical columns have a size of about 4.6x250mm, a semi - preparative column has a size of about 10x250mm and preparative columns have a size of about 25x250mm [McMaster, 2007].
As far as this Diploma Work is concerned analytical HPLC was performed to get an overview of the peak density of the extracts, whereas semi - preparative HPLC was performed to gain the fractions and subfractions of the corresponding extracts.

1.6 Aim of this work

This Diploma Thesis deals with analysis of fungal extracts showing antimicrobial properties in disc diffusion assays. Such extracts shall be investigated and analyzed by HPLC. Subsequently to this by LCMS analysis the most important molecules shall be analyzed in order and isolate the molecules responsible for antimicrobial effect *via* semi - preparative HPLC.

2 Materials and Methods

2.1 Chemicals

The chemicals, which were used within this Thesis, were as follows:
Acetonitrile J.T Baker, Ultra gradient HPLC grade; Deventer, The Netherlands
Acetonitrile J.T Baker LC- MS grade; Deventer, The Netherlands
Methanol LiChrosolv Reag. Ph Eur, gradient grade for liquid chromatography; Darmstadt, Germany
Methanol HiPerSolv CHROMANORM for HPLC LC-MS grade, VWR PROLABO CHEMICALS; Leuven, Belgium
Dichloromethane AnalaR NORMAPUR, VWR PROLABO CHEMICALS; Fontenay-sous-Bois, France

2.2. Origin of fungi and provided fungal extracts

Isolation of fungi from plants, cultivating, barcoding, fermentation of media, from which fungal extracts were yielded, along with the preliminary bioactivity monitoring were kindly provided by Dr. Mag. Martina Oberhofer. Tab. 3 gives an overview about the origins of fungal endophytes, which were relevant for this Diploma Thesis (personal communication, Oberhofer M.). Tab. 4 displays the dry weight and the volume of methanol for dissolution of the provided extracts, which were produced by cultivation of isolates from *Preussia* sp., *Nemania* sp. and Helotiales (personal communication, Oberhofer M.). The isolates of the fungi were named according to their initials. As example Ab1 was named after *Atropa belladonna*, isolate 1. The isolate Bl73 was named after *Bergenia ligulata*, which is a synonym for *Bergenia pacumbis* [Source: Catalogue of Life: 2017 Annual Checklist].

Tab. 3: Fungal isolates, their origins and blast results.[1]

Fungal isolate	**Plant origin**	**Host tissue**	**Media**	**Blast result**
Ab1	**A**tropa **b**elladonna	petiole	TSA	*Preussia sp.*
Ab11	**A**tropa **b**elladonna	petiole	HV	*Nemania sp.*
Bl73	Bergenia pacumbis (**B**ergenia **l**igulata)	root	PDA	Helotiales

Tab. 4: Fungal extracts as provided by Mag. Dr. Martina Oberhofer with dry weight and dissolution volume.

Fungal extract	**Dry weight [mg]**	**Vol. methanol [mL]**
Ab1	106	2
Ab11	62	1
Bl73	466	2

[1] TSA- Tryptic Soy Agar HV -Humic Acid-Vitamin Agar (for selective isolation of soil actinomycetes), PDA- Potato Dextrose Agar

2.3 Test organisms of this work

The extracts, their successive fractionations and isolates were tested by disc diffusion assays on five organisms. Tab. 5 displays all testing microorganisms and the name of the strains.

Tab.5: Test organisms of this work.

Species	Strain
Bacillus subtilis	168
Saccharomyces cerevisiae	BY4742
Aspergillus niger	CBS 110271
Fusarium graminearum	CBS 112.30
E. coli	DH5-alfa

2.4 Diffusion disc assay

All diffusion disc assays were performed at the Department of Pharmacognosy by Mag. Dr. Martina Oberhofer.

2.5 Extraction of fungi

The inoculation of fungi on the rice media and the cultivation was done by Mag. Dr. Martina Oberhofer. Five approaches per fungus were inoculated; the subsequent extraction was done by me. The detailed procedure of the extraction was as follows:

Fungi were cultivated until they spread through the whole rice media and kept for one more week before extraction. For extraction, the fungi infested media were cut into small pieces and then transferred to 0.5 L Schott bottles. In order to prepare the fungi for lyophilisation the bottles were sealed with tea filter and then frozen at -20°C for 1 hour. The lyophilisation was performed for 48 h, and then the bottles were extracted with 100 mL of 2 parts dichloromethane/ 1 part methanol under agitation for 1h. Afterwards the extracts were transferred into 50 mL falcon tubes for centrifugation under 2500 rpm and the supernatant was poured into round bottom flasks for evaporation. After evaporation the oily residues were dissolved by ultrasonic bath in methanol.

To distinguish these extracts from the provided ones, they were named "second" - Ab1, Ab11 and Bl73 extracts. The concentrations of the gained extracts were as follows:

Second Ab1 extract - 13.5 µg/µl

Second Ab11 extract - 25.8 µg/µL

Second Bl73 extract - 18.9 µg/µL

2.6 HPLC

In this Diploma Work two variants of HPLC were performed. The first variant was analytical HPLC, which was done for prior analysis of the extracts. The second one was semi - preparative HPLC, which was done subsequently to the analytical runs.

2.6.1 Analytical HPLC

For analytical HPLC the provided Ab1 extract, the second Ab1 extract and the second Bl73 extract were investigated. The analysis of Ab1 and Bl73 extracts was performed with two different analytical columns. All other equipment and settings stayed the same. The injection volume for all analytical runs was 5 µL. The concentrations of the extracts were 53 µg/µl for the provided extract, 13.5 µg/µl and 38 µg/µL for the second Ab1 extract and 18.9 µg/µL for the second Bl73 extract. Tab. 6 gives an overview about the general equipment and parameters of analytical HPLC. In Tab. 7 relevant detailed settings are displayed.

Tab.6: General equipment and settings for analytical HPLC.

HPLC	SHIMADZU UFLC XR
Column for Ab1	NUCLEODUR C18 HTec 4x 125mm
Column for BI73	Shimadzu Shimpack GIS 4.6 x250 mm
Mobile phase	Gradient of acetonitrile/H_2O
Flow speed	1mL/min
Temperature in column oven	25°C
Detection wavelength	254 nm

Detailed HPLC settings

Tab.7: Detailed settings of the analytical HPLC.

PDA		
	Model	SPD-M20A
	Lamp	D2&W
	Cell temp.	40°C
	Start wavelength	180 nm
	End wavelength	800 nm
	Sit Width	1.2 nm
Column oven		
	Model	CTO-20C
	Oven temp.	25°C

2.6.2 Semi - preparative HPLC and fractionation of Ab1

For the fractionation of the Ab1 extract the same HPLC system and the same column were used as in the analytical experiments. This was possible because the SHIMADZU UFLC XR allowed semi - preparative runs with a maximum injection volume of 100 µL. For the semi - preparative work the second Ab1 extract with a concentration of 38µg/µL was used. The injection volume was 50 or 100 µL. Tab. 8

gives an overview of the equipment and parameters, which were used for the experiments. Tab.9 views detailed settings for semi - preparative HPLC.

Tab.8: General equipment and settings for semi - preparative HPLC applied for second extract of Ab1.

HPLC	SHIMADZU UFLC XR
Column	NUCLEODUR C18 HTec;
Mobile phase	Gradient of acetonitrile/H_2O
Flow speed	1mL/min
Temperature setting oven	25°C
Detection wavelength	254 nm

Detailed HPLC settings

Tab.9: Detailed settings for semi - preparative HPLC applied for second extract of Ab1.

PDA			
	Model		SPD-M20A
	Lamp		D2&W
	Cell temp.		40°C
	Start wavelength		180 nm
	End wavelength		800 nm
	Sit Width		1.2 nm
Column oven			
	Model		CTO-20C
	Oven temp.		25°C
Fraction collector			
	Model		FRC - 10A
	Vial volume		1.5 mL
	Delay volume		220 µL
	Rack type		1

Procedure of fractionation

The gradient that was used for the semi - preparative work was obtained in analytical experiments with Ab1 extract, see below. 1.5 mL fractions were collected from minute 0 to 40.5 (fractions 0-26= 27 fractions). Minute 40.5 to 70 was collected in one single fraction = Fraction 27. The 1.5 mL fractions were collected in 2 mL Eppendorf Tubes and subsequently the fractions underwent centrifugal freeze drying. After freeze drying the yield was confirmed by weight and the same Eppendorf Tubes were then used for separating another amount of the extract. The solid and solvent-free fractions were stored at - 20°C. Fraction 27 was collected in one single round bottom flask and the solvent was evaporated after each fractionation. A total volume of 350 µL Ab1 extract was fractionized following this procedure.

Gradient for fractionation:

0.0 min Solvent Acetonitrile Conc. 5%
10.0 min Solvent Acetonitrile Conc. 15%
30.0 min Solvent Acetonitrile Conc. 95%
70.0 min Solvent Acetonitrile Conc. 95%

2.6.3 Semi - preparative HPLC and fractionation of BI73

For the fractionation of BI73 extract a new semi - preparative HPLC column was available that allowed injection of higher volumes of fungal extracts. Therefore the utilization of another HPLC system was necessary, which was compatible with the bigger column. This allowed processing a much larger volume of extract. Thus the injection volume for the semi - preparative runs was 2 mL with a concentration of 18.9 µg/µL. However, the applied semi - preparative HPLC system was not equipped with a column oven. Tab. 10 displays the general equipment and settings for these operations. Tab. 11 shows relevant detailed settings of the HPLC system.

Tab.10: General equipment and settings for semi-preparative HPLC of second BI73 extract.

HPLC	Shimadzu LC-8A
Column	Shimadzu Shimpack GIS20x250mm
Mobile phase	Gradient of acetonitrile/H_2O
Flow speed	20 mL/min
Detection wavelength	254 nm

.

Detailed HPLC settings

Tab.11: Detailed settings for semi-preparative HPLC of second BI73 extract.

Detector A			
	Model		SPD-10Avp
	Lamp		D2
	Polarity		+
	Response		1.0 sec
	wavelength CH1		254 nm
	Out put		
		Intensity Unit	Volt
		Auxiliary Range	1.0 AU/V
		Recorder Range	1.0
Fraction collector			
	Delay volume		200 µL
	Sig. Delay Volume		0 µL
	Rack Type		3

Procedure of fractionation

The optimised gradient that was obtained in former analytical experiments was applied for fractionation, (see next page). However, the applied semi-preparative HPLC system is not equipped with a column oven.

16 fractions (Fraction 0-15) were collected from minute 0 to 40 in 50 mL vials corresponding to 2.5 minutes per fraction. Fraction 16 was collected from minute 40 to 70 as one single fraction.
After semi - preparative HPLC the collected Fractions 0 - 7 were dried by a rotary evaporator in order to remove the amount of acetonitrile. The remaining water was then removed by lyophilisation.
Fractions 8- 16, which predominantly contained acetonitrile, were directly dried by rotary evaporation. After drying, the yield was determined by weighing and the residues were dissolved in 500 - 1400 µL methanol. In order to obtain higher amounts of the solid compounds of the fractions, the separation was repeated three times.

Gradient for fractionation:

0.0 min Solvent Acetonitrile Conc. 15%
30.0 min Solvent Acetonitrile Conc. 95%
70.0 min Solvent Acetonitrile Conc. 95%

2.6.4 Semi - preparative HPLC and subfractioning of Bl73 Fraction 10 and Fraction 14/15

For semi - preparative HPLC of the Bl73 fractions the same semi - preparative column from former experiments was used. However, for the subfractionation of the Bl73 fractions a new HPLC system was available, which was used in order to gain the optimal conditions for the procedure. Due to different yields in the fractionation of Bl73, for these operations different injection volumes were applied. The injection volume ranged from 600 - 1400 µL. Similarly to Bl73 fractionation the applied semi - preparative HPLC system was not equipped with a column oven. Tab. 12 gives an overview about the general equipment and parameters, which were used for the subfractioning. Tab. 13 displays important detailed settings for the operations.

Tab. 12 General equipment and settings for semi-preparative HPLC of BI73 Fraction 10 and Fraction14/15.

HPLC	Shimadzu prominence Liquid Chromatograph DGU-20A/LC-20AR/ CBM- 20A
Column	Shimadzu Shimpack GIS 20x250 mm
Mobile phase	Gradient of acetonitrile/H_2O
Flow speed	10 mL/min or 20 mL/min
Detection wavelength	254 nm

Detailed HPLC settings

Tab.13: Detailed settings for semi-preparative HPLC of BI73 Fraction 10 and Fraction 14/15.

Detector A			
	Model		SPD-10Ai
	Lamp		D2
	Polarity		+
	Response		0.1 sec
	Wavelength CH1		254 nm
	Out put		
		Intensity Unit	Volt
		Auxiliary Range	1.0 AU/V
		Recorder Range	1.0
Fraction collector			
	Model		FRC - 10A
	Delay volume		200 µL
	Sig. Delay Volume		0 µL
	Rack Type		3

Procedure of fractionation

BI73 Fraction 10 Subfractions

The optimized gradient that was obtained in former analytical experiments with Fraction 10 was used (see below). The difference between this semi - preparative procedure and the previous one was the mode of fractionation. The initial BI73 fractionation was done automatically. In the subfractionation, however, BI73 Subfractions 10 were collected manually. Consequently the volume for each collected fraction was different. The subfractions were dried by rotary evaporation.

Gradient for subfractionation:

0.0 min Solvent Acetonitrile Conc. 60%
5.0 min Solvent Acetonitrile Conc. 60%
10.0 min Solvent Acetonitrile Conc. 70%
30.0min Solvent Acetonitrile Conc. 83%

BI73 Fraction 14 and 15 Subfractions

For the semi - preparative HPLC of Fraction 14 and 15 of the BI73 extract an automated fractionation procedure was applied. The optimized gradient that was obtained in former analytical experiments with Fraction 14 and 15 was used (see below). By this operation 16 vials were collected from minute 0 to 40, vial 0 - 15 were collected in 50 mL vials corresponding to 2.5 minutes per fraction. In order to obtain 8 subfractions in total, two vials were mixed together, respectively (Tab.14).

Gradient for subfractionation of Fraction 14

0.0 min Solvent Acetonitrile Conc. 60%
5.0 min Solvent Acetonitrile Conc. 60%
10.0 min Solvent Acetonitrile Conc. 70%
20.0 min Solvent Acetonitrile Conc. 80%
25.0 min Solvent Acetonitrile Conc. 87%
40.0 min Solvent Acetonitrile Conc. 87%
60.0 min Solvent Acetonitrile Conc. 95%

Gradient for subfractionation of Fraction 15

0.0 min Solvent Acetonitrile Conc. 50%
5.0 min Solvent Acetonitrile Conc. 50%
10.0 min Solvent Acetonitrile Conc. 70%
20.0 min Solvent Acetonitrile Conc. 80%
25.0 min Solvent Acetonitrile Conc. 85%
35.0 min Solvent Acetonitrile Conc. 85%
40.0 min Solvent Acetonitrile Conc. 87%
60.0 min Solvent Acetonitrile Conc. 87%

Tab. 14: Scheme for BI73 Subfractions of F14 and F15.

	Vial	Added vial
Subfraction		
0	0	1
1	2	3
2	4	5
3	6	7
4	8	9
5	10	11
6	12	13
7	14	15

Furthermore, Subfraction 8 was collected from minute 40 to 70 in one single fraction. After semi - preparative HPLC the collected Subfractions 0 - 7 were evaporated in order to remove the acetonitrile. The remaining water then underwent lyophilisation. Subfraction 8 was dried by rotary evaporation. The residues were dissolved in about 500 µL of methanol and subsequently reduced in volume to 50- 100 µL at 40 °C in an Eppendorf thermomixer under agitation. These subfractions were then tested in disc diffusion assays by Mag. Dr. Martina Oberhofer.

2.7 Liquid chromatography mass spectrometry (LCMS)

LCMS analysis was done in order characterize the masses of substances within the fractions and therefore same column as for analytical BI73 HPLC runs was used. The injection volume was 3 or 5 µL. Tab. 15 gives an overview of the setup of the LC part. Tab. 16 displays important equipment and settings for the MS.

Tab. 15: General equipment and settings for LC.

LC	UltiMate 3000 RSLC (Dionex; Thermo Fisher Scientific, Inc., Germering, Germany)
Temperature	25°C
Mobile phase	gradient of acetonitrile/H_2O
Flow speed	mL/min
Detection wavelength	190 nm (254 nm not available due to technical issues)
Column	Shimadzu Shimpack GIS 4.6 x250 mm

Tab.16: General equipment and settings for MS.

LC	UltiMate 3000 RSLC (Dionex; Thermo Fisher Scientific, Inc., Germering, Germany)
Temperature	25°C
Mobile phase	gradient of acetonitrile/H_2O
Flow speed	1 mL/min
M/z-range	190 nm (240 nm not available due to technical issues)
Column	Shimadzu Shimpack GIS
MS	4.6 x250 mmmaXis HD ESI-Qq-TOF (Bruker Corporation, Bremen, Germany
Capillary voltage	3500 V
Nebulizer	0.8 bar
Dry gas flow rate	7.0 l/min
Detection wavelength	50 to 2800 m/z

The running gradients for all BI73 fractions are stated in the results. LCMS was performed twice with separately collected fractionations of BI73. Tab. 17 outlines the way LCMS samples were named. As an example, for F10 the first and second run (fractionation) were mixed together, F10_2 represents the third run (see results pages 35, 36 and 37).

MS was performed in positive mode (except LCMS run in Fig. 59, page 77 which was done in negative mode).

Tab.17: Nomenclature of the LCMS samples and injection volume.

Fraction	**Run**	**Injection vol. (µL)**
F3	1 +2	5
F3_2	3	5
F8	1+2	5
F8_2	3	5
F9	1 +2	3
F9_2	3	3
F10	1+2	3
F10_2	3	3
F11	1 +2	5
F11_2	3	5
F14	1+2	5
F14_2	3	5
F15	1 +2	5
F15_2	3	5

2.8 Additional equipment

Centrifuge for centrifugal freeze drying: CHRIST

Eppendorf Centrifuge 5804 R

Eppendorf Thermomixer comfort

Laboratory scale: Sartorius Extend

Laboratory shaker: GFL 3005

Lyophilisator: CHRIST Alpha 2-4 LDplus

Rotavapor: Heidolph VV2011

Ultrasonic bath: Branson 3210/5200

3 Results

3.1 Diffusion disc assay of the provided extracts

Tab.18: Results of diffusion disc assays of the provided extracts, ++.... radius = 2.5 mm, +.... radius = 0.75 mm, (+)….radius = 0.6 mm (data provided by Mag. Dr. Martina Oberhofer)

	B. subtilis	*S. cerevisiae*	*A. niger*	*F. graminearum*	*E. coli*
Ab1	+	-	-	-	-
Ab11	+	-	-	-	-
BI73	++	+	(+)	(+)	-
Methanol	-	-	-	-	-

In the disc diffusion assays Ab1 and Ab11 showed an inhibition zone of 0.75 mm radius on *B. subtilis.* BI73 extract displayed the best results of the tests as an inhibition zone with radius size of 2.5 mm was observed for B*. subtilis*. An inhibition zone of 0.75mm radius was found for *S. cerevisiae*. Furthermore, BI73 showed a very slight effect on *A. niger* and *F. graminearum. E. coli* was not affected by any fungal extract.

3.2 Extraction of fungi and disc diffusion assays

In order to gain a higher amount for the subsequent semi - preparative work on the fungal extracts of Ab1, Ab11 and BI73, 5 Erlenmeyer flasks per fungi were cultivated. Thereon the extraction with dichloromethane/methanol (2:1) was performed. After the extraction the according fungal extracts were pooled and dried by rotary evaporation.

Ab1 Extract

19 mL of methanol was added to the total extract yield of 558 mg and subsequently centrifuged for disc diffusion assays and HPLC usage. Centrifugation was used to remove the insoluble parts of the extract with a total remaining volume of 16.5 mL (concentration: 13.5 µg/µl). Ab1 was concentrated for further working steps as the native extract did not show any antimicrobial activity. 4 mL of the processed extract were dried by rotary evaporation and then dissolved in methanol by 100 - 200 µL steps until the whole residue was in solution. A final concentration of 38 µg/µL was obtained.

Concentration of the concentrated second Ab1 extract: 38 µg/µL

Tab.19: Results of diffusion disc assays of the concentrated second Ab1 extract, +.... radius = 0.75 mm (data provided by Mag. Dr. Martina Oberhofer).

	B. subtilis	***S. cerevisiae***	***A. niger***	***F. graminearum***
Ab1	+	-	-	-
Methanol	-	-	-	-

The second fungal extract of Ab1 showed weaker antimicrobial activity in comparison to the provided extract, as prior no antimicrobial activity was observed. When the extract was concentrated, it showed similar antibacterial properties as the provided extract. All other disc diffusion assays gave the same results as the provided Ab1 extract.

Ab11 Extract

5.5 mL methanol was added to the total extract yield of 142.7 mg from the round bottom flasks and after centrifugation final volume was 5.5 mL (25.8 µg/µL). The solid residues were minimal, therefore volume stayed the same. Ab11 was concentrated for further working steps as the native extract showed no antimicrobial activity. However, even a concentration of 81, 1 µg/µL showed no antimicrobial activity as it had no inhibitional activity against *B. subtilis*. All other microorganisms were not tested. Thus, the work on this extract was stopped.

BI73 Extract

BI73 was cultivated for 13 days after the fungus had spread through the rice media, then the extraction was performed. 35 mL methanol was added to the total extract of 2030.6 mg. Subsequently, the extract was centrifuged and the dry weight of the supernatant was determined. The dry weight of supernatant was 473.5 mg. This residue of the second BI73 extract was diluted with methanol to a final concentration of 18.9 µg/µL.

Tab.20: Results of diffusion disc assays of the second BI73 extract. ++.... radius = 2.5 mm, +.... radius = 0.75mm (data provided by Mag. Dr. Martina Oberhofer).

	B. subtilis	*S. cerevisiae*	*A. niger*	*F. graminearum*	*E. coli*
BI73	++	+	+	+	-
Methanol	-	-	-	-	-

The second extract of BI73 showed significant antimicrobial activity against all test organisms, except *E. coli*. The inhibition zones of *B. subtilis and S. cerevisiae* were of the same size as for the provided BI73 extract. However, the efficacy of the second extract of BI73 against the fungi was better than the provided one as the second showed stronger activity against *A. niger* and *F. graminearum* in disc diffusion assays.

3.3 Analytical HPLC and fractionation of Ab1 extract

3.3.1 Analytical HPLC of Ab1 extract

3.3.1.1 Provided Ab1 extract

At the beginning of HPLC analysis the provided Ab1 extract was investigated. For the mobile phase a gradient of acetonitrile/water was applied. The best separation of peaks was achieved with a gradient that started at 5% acetonitrile, with a slow increase to 15% acetonitrile until minute 10 and a gradient from 15 - 95% acetonitrile until 30 minutes. The duration of the run was extended to 70 min with a concentration of 95% acetonitrile from minute 30 to 70. Fig. 4 shows an analytical HPLC chromatogram with the optimised gradient.

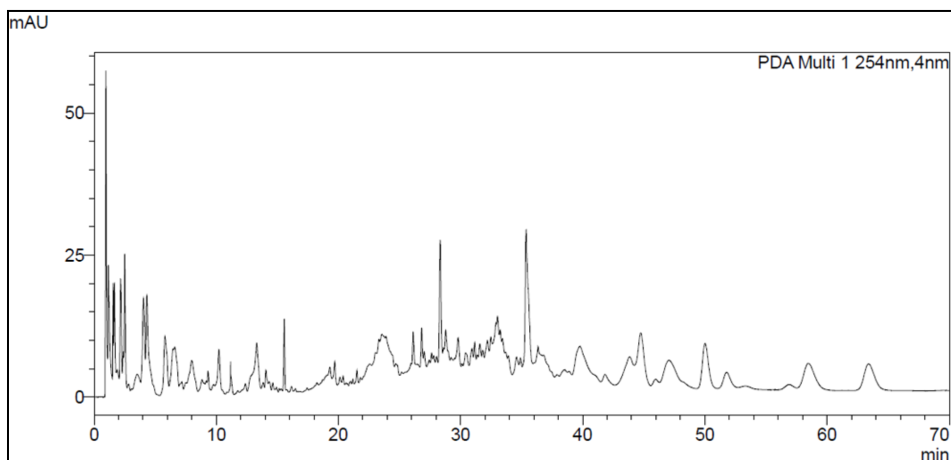

Fig.4: Analytical HPLC run, provided Ab1 extract, optimised gradient.

Final optimized gradient

0.0 min Solvent Acetonitrile Conc. 5%
0.0 min Solvent H2O Conc. 95%

10.0 min Solvent Acetonitrile Conc. 15%
10.0 min Solvent H2O Conc. 85%

30.0 min Solvent Acetonitrile Conc. 95%
30.0 min Solvent H2O Conc. 5%

70.0 min Solvent Acetonitrile Conc. 95%
70.0 min Solvent H2O Conc. 5%

3.3.1.2 Second Ab1 extract

An analytical HPLC run was done with the provided extract applying the optimised gradient that was obtained previously. Fig. 5 views a comparison between the extracts.

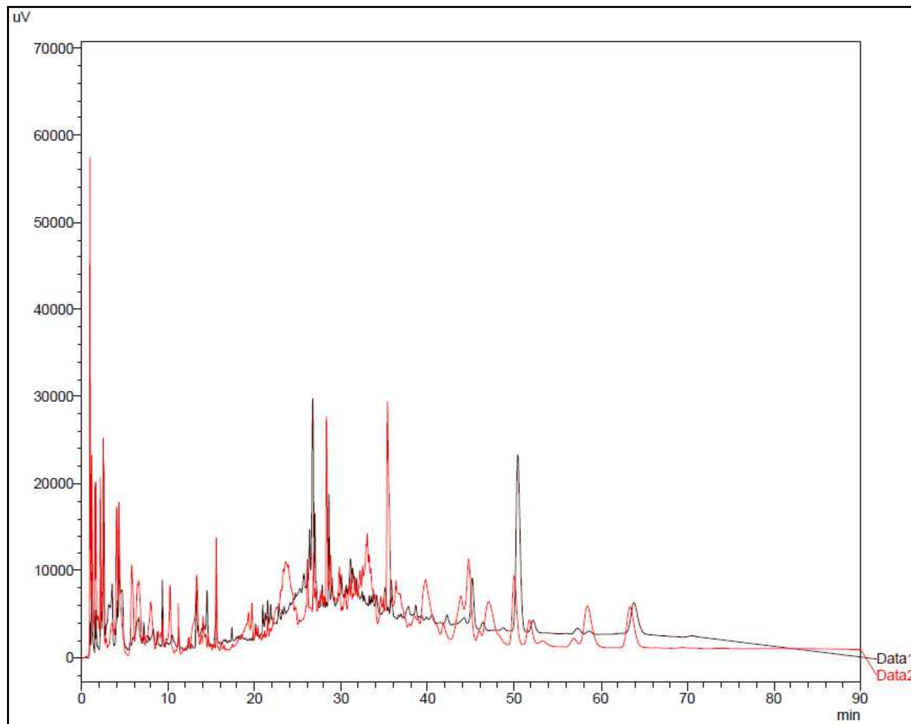

Fig.5: Comparison of the provided and the second Ab1 extract, black line = second Ab1 extract, red line = provided Ab1 extract, uV = µV.

Comparing the two extracts the peaks and peak sizes vary. Only few similarities can be found, which predominantly is at end of the runs at minute 50. Nevertheless, one has to consider that the concentration of the extracts is not standardized (provided Ab1 extract 53 µg/µl; second Ab1 extract 13.5 µg/µl). Thus, the peak size can vary. Fig. 5, however, outlines that the two extracts, although cultivated and extracted under same conditions, have a different qualitative composition too, as the peaks in the chromatograms did not fit up.

As the second Ab1 extract with a concentration of 13.5 µg/µl was not antimicrobial active, it was further concentrated. Therefore, in the next step an analysis of the higher concentrated second Ab1 extract (38 µg/µL) was done. Fig 6 displays the chromatogram of the concentrated Ab1 extract.

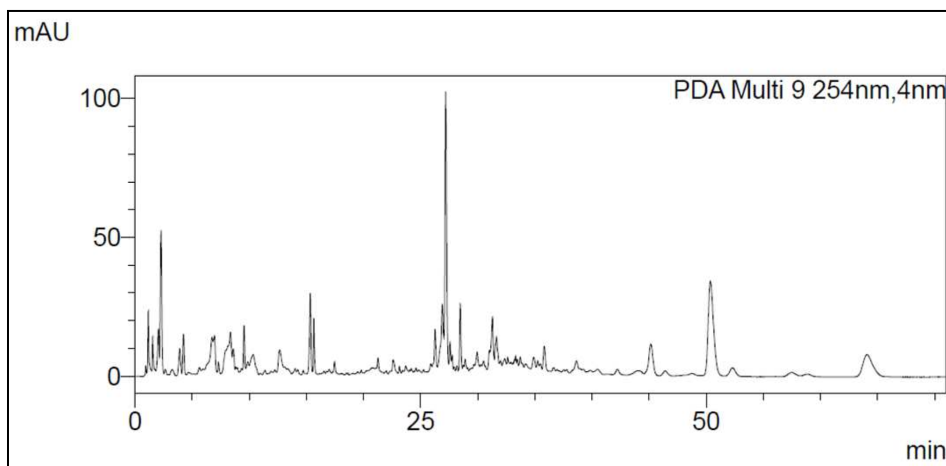

Fig.6: Analytical HPLC run, concentrated second Ab1 extract.

3.3.1.3 Semi - preparative HPLC of Ab1 extract

Subsequently to the analytical work on the Ab1 extract, semi - preparative HPLC was done. Fig. 7 illustrates the HPLC chromatogram with the collected fractions. Fraction 0 and 17 showed the highest absorbance at 254 nm. Altogether 350 µL of the second concentrated extract were separated.

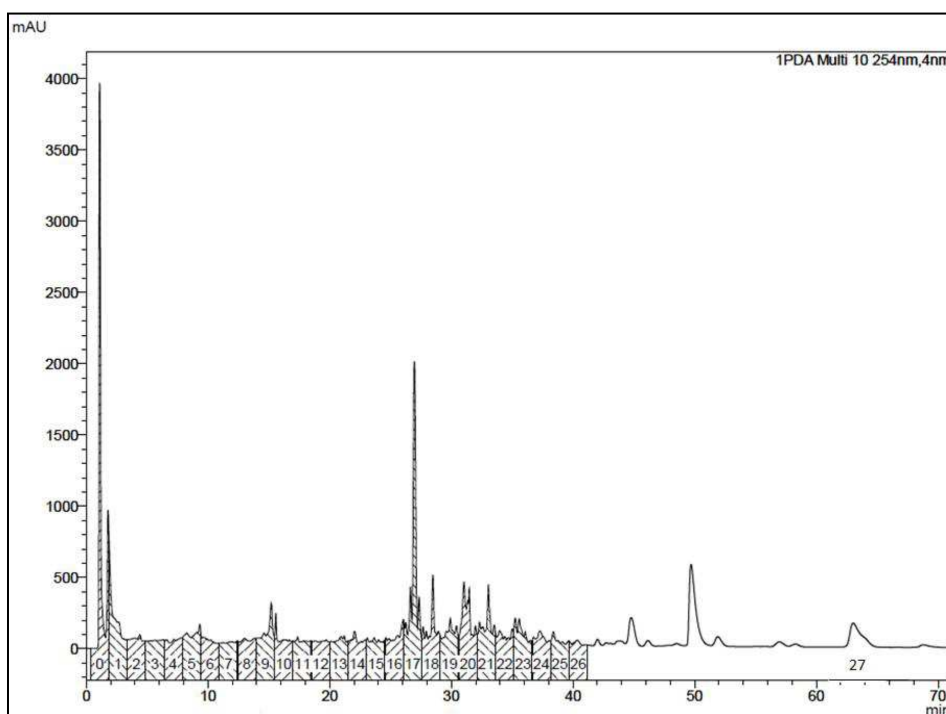

Fig.7: Preparative HPLC run, concentrated second Ab1 extract, 100 µL injection volume.

Weights:

Tab.21: Total yield after fractionation of 350 µL Ab1 extract, respectively.

Weight [mg]			
F0	3.3	**F14**	0,0
F1	0.5	**F15**	0.0
F2	0.3	**F16**	0.4
F3	0.2	**F17**	0.5
F4	0.3	**F18**	0.1
F5	0.4	**F19**	0.3
F6	0.3	**F20**	1.3
F7	0.6	**F21**	2.2
F8	0.2	**F22**	0.3
F9	0.1	**F23**	0.3
F10	0.0	**F24**	0.3
F11	0.0	**F25**	0.4
F12	0.0	**F26**	0.0
F13	0.3	**F27**	3.1

Tab. 21 displays the yields of the stepwise fractionation. Although residues were visible within the Eppendorf tubes in some fractions no weight could be obtained. This can largely be due to the small prepared volumes of the fractionation and also weighing errors.

Dissolution of samples:

In the next step the obtained Ab1 fractions were prepared for antimicrobial activity tests. Fraction 0 (F0) was dissolved in 80 µL methanol and 20 µL H_20 dest. Water was added for a better dissolution. Fractions 1- 26 (F1-26) and fraction 27 (F27) were dissolved in 80 µL and 160 µL methanol, respectively. Tab. 22 shows the concentration of Ab1 extracts. Subsequently, the fractions of Ab1 extract were tested for antimicrobial activity against *Bacillus subtilis*.

Tab.22: Concentration of Ab1 extract fractions.

Concentration [µg/µL]			
F0	3.3	**F14**	-
F1	6.2	**F15**	-
F2	3.8	**F16**	3.8
F3	2.5	**F17**	6.2
F4	3.8	**F18**	1.3
F5	5	**F19**	3.8
F6	3.8	**F20**	16.3
F7	7.5	**F21**	27.5
F8	2.5	**F22**	3.8
F9	1.3	**F23**	3.8
F10	-	**F24**	3.8
F11	-	**F25**	5
F12	-	**F26**	-
F13	3.8	**F27**	19.4

3.3.1.4 Results of disc diffusion assays of fractionation of Ab1 Extract

Tab.23: Results of bioassay tests against *B. subtilis*, ++....radius = 2.5 mm, +....radius = 0.75 mm (provided through personal communication by Oberhofer M.)

Results disc diffusion assays			
F0	-	**F14**	-
F1	-	**F15**	-
F2	-	**F16**	-
F3	-	**F17**	++
F4	-	**F18**	+/-
F5	-	**F19**	+
F6	-	**F20**	+
F7	-	**F21**	-
F8	-	**F22**	-
F9	-	**F23**	-
F10	-	**F24**	-
F11	-	**F25**	-
F12	-	**F26**	-
F13	-	**F 27**	-

F 17, F19 and F20 were tested positively with small inhibition zones, F18 remained unclear. Methanol control was negative. As nearly all fractions from F17 to F20 inhibited *B. subtilis*, the compound eventually was carried through several fractions.

This means that it is possible that same substance was responsible for the positively tested fractions, although this cannot be assured properly.

3.3.1.5 Subsequent fractionation of Ab1 Extract

For another yield of Ab1 fractions, 4 mL of the Ab1 extract were evaporated and dissolved in 1700 µL methanol (concentration 38 µg/µL).

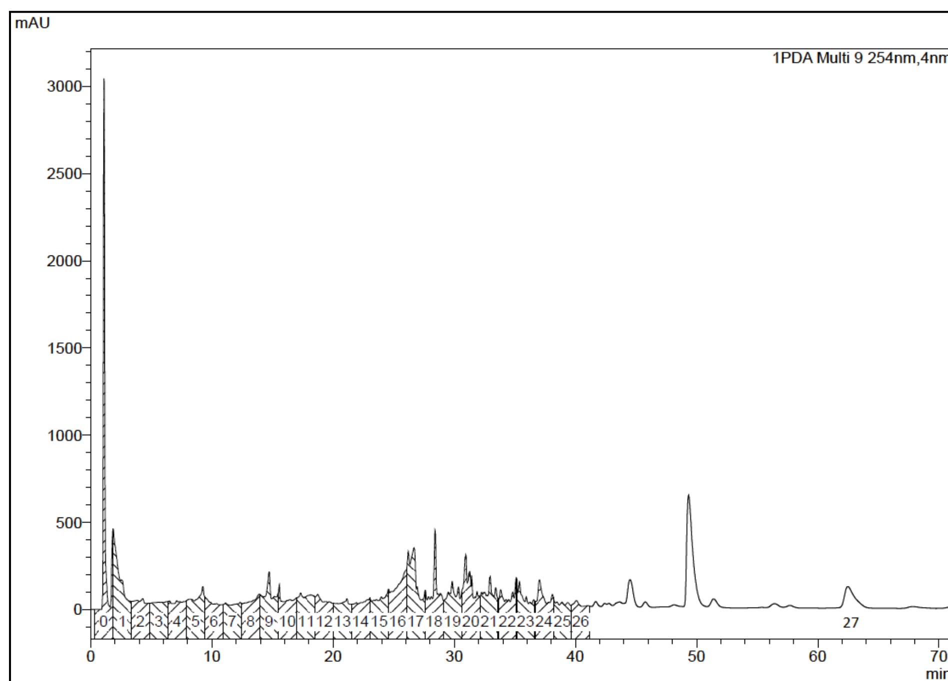

Fig.8: Semi - preparative HPLC run, concentrated second Ab1 extract, 100 µL injection volume.

Fraction 17 of Ab1 showed a very minor UV absorbance in comparison to former fractionations (Fig.7). Therefore, the activity was tested on *B. subtilis*, which did not show the antimicrobial effect from former disc diffusion assays. Comparing Fig. 7 and 8 at pages 30 and 33 it can be seen that Fraction 17, containing one of the highest peaks in the HPLC chromatogram, decreased to a very minor one in the other chromatogram. That means that a high amount of the active compound in Fraction 17 precipitated. Eventually the storage and subsequent evaporation was one reason for that, as by storage at -25 °C it is not unlikely certain amounts can precipitate. Subsequently the work on Ab1 extract was stopped.

3.4 Analytical HPLC and fractionation of Bl73 extract

3.4.1 Analytical HPLC of second Bl73 extract

A good separation of the peaks was achieved by starting with 15% acetonitrile, and an increase to 95% until minute 30. The duration of the run was extended to 70 minutes with gradient constant concentration of 95% acetonitrile. Fig. 9 shows a chromatogram of Bl73 with the optimised gradient.

Final optimized gradient

0.0 min Solvent Acetonitrile Conc. 15%
0.0 min Solvent H_2O Conc. 85%

30.0 min Solvent Acetonitrile Conc. 95%
30.0 min Solvent H_2O Conc. 5%

70.0 min Solvent Acetonitrile Conc. 95%
70.0 min Solvent H_2O Conc. 5%

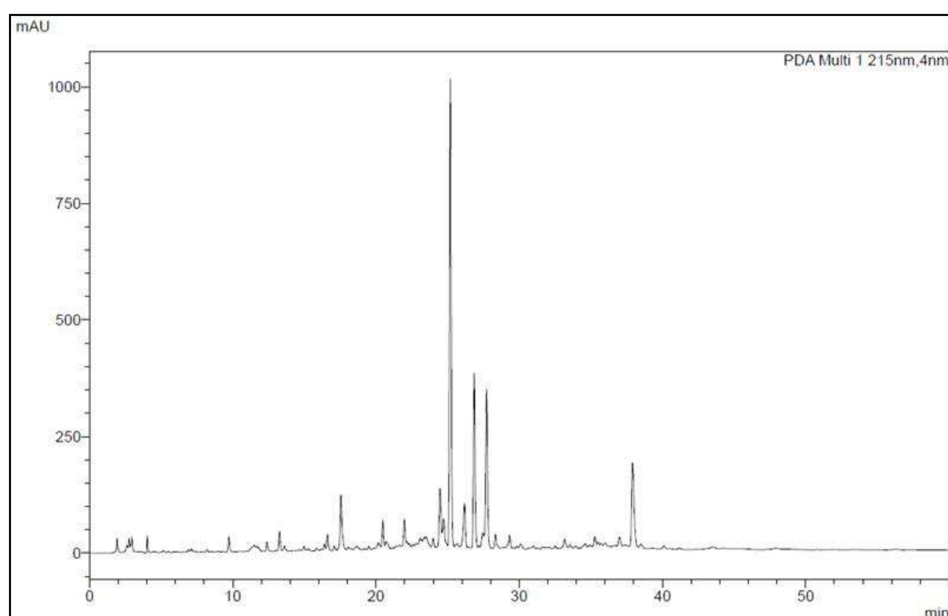

Fig.9: Analytical HPLC run, second Bl73 extract, optimised gradient.

The first peaks appeared at about 5 minutes of the chromatogram. The peaks with the highest UV absorbance at 215 nm appeared between minute 24 and 28 of the run. After minute 40 the UV absorbance at this wavelength was minimal.

3.4.2 Semi - preparative HPLC of second BI73 extract

Subsequently to the analytical experiments, semi - preparative HPLC was performed in order to fractionize the extract. The optimised gradient that was obtained in analytical experiments was applied, see page 34. For each fractionation of the extract 2000 µL were injected.

1. Yield

The starting pressure was about 80 bar. Fraction 9 and 10 showed the highest absorbance at 254 nm.
These fractions corresponded to the highest peaks, observed between minute 24 and 28 of the analytical run. Fractions 11 until 15 hardly showed any UV Absorbance, which also correlated to the analytical HPLC experiments of the extract.

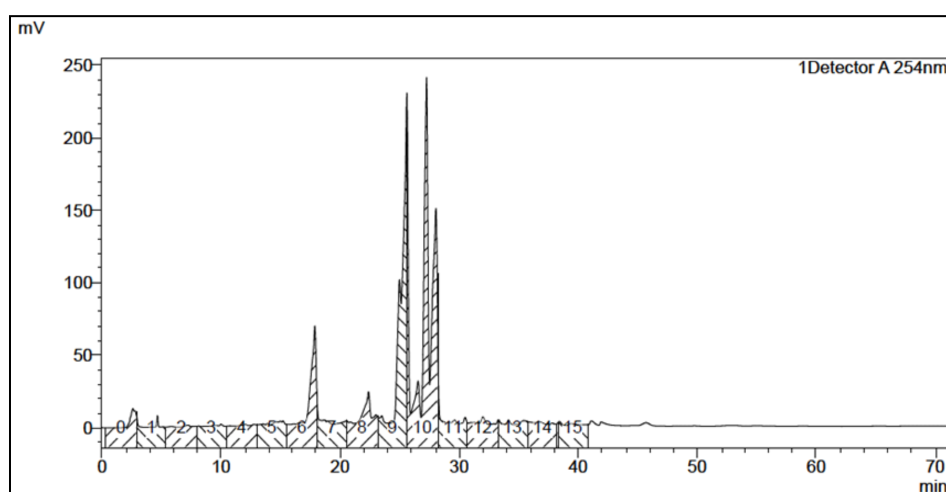

Fig.10: Semi - preparative HPLC run, second BI73 extract.

.

2. Yield

The starting pressure in the second fractionation was 79bar and the running time was decreased from 70 minutes to 60 minutes (Fig. 11). This was done because after 50 minutes no peaks appeared. The chromatograms of the first and second preparation were nearly identical. Fig. 12 displays the overlaid chromatograms of the first and second run. As they were nearly completely identical, the dissolved residues were mixed together.

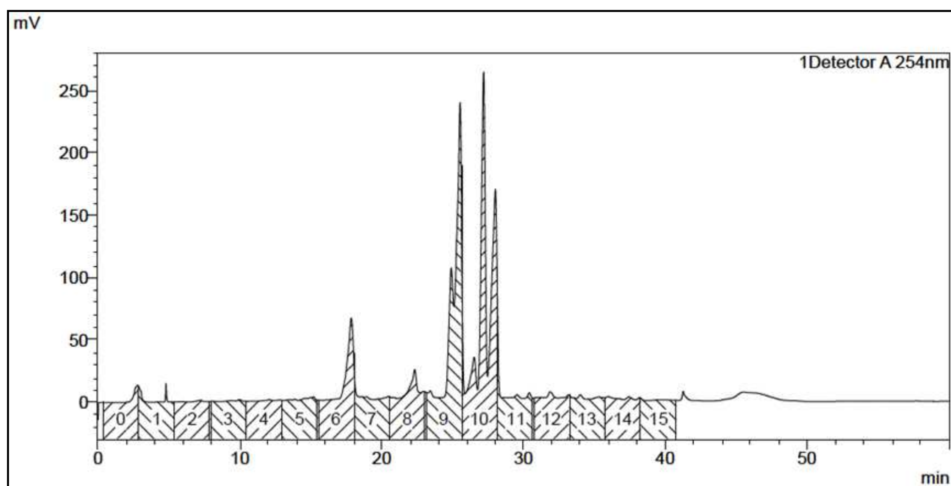

Fig.11: Semi - preparative HPLC run, second Bl73 extract.

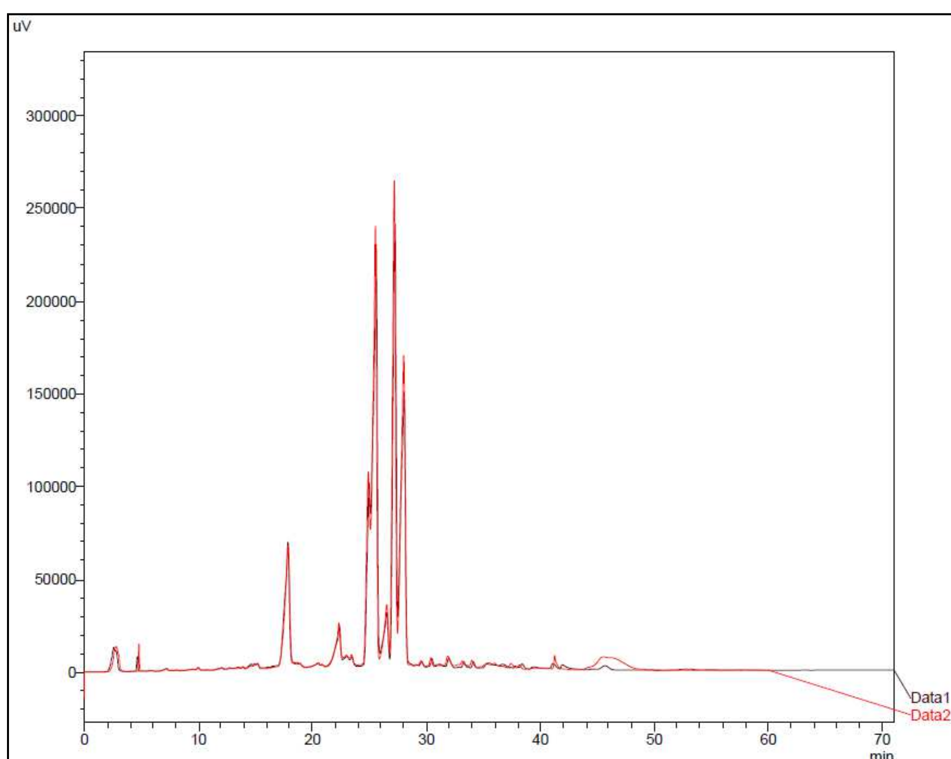

Fig.12: Comparison of the first and second semi - preparative HPLC run, second Bl73 extract, black line = first yield, red line = second yield, uV = µV.

3. Yield

At the third yield the starting pressure was 72 bar due to increased room temperature. Fig.14 outlines that in the third yield the retention time of the peaks is shifted due to the decrease in starting pressure. Therefore, the collected fractions were not mixed together with the yields from the first and second fractionation.

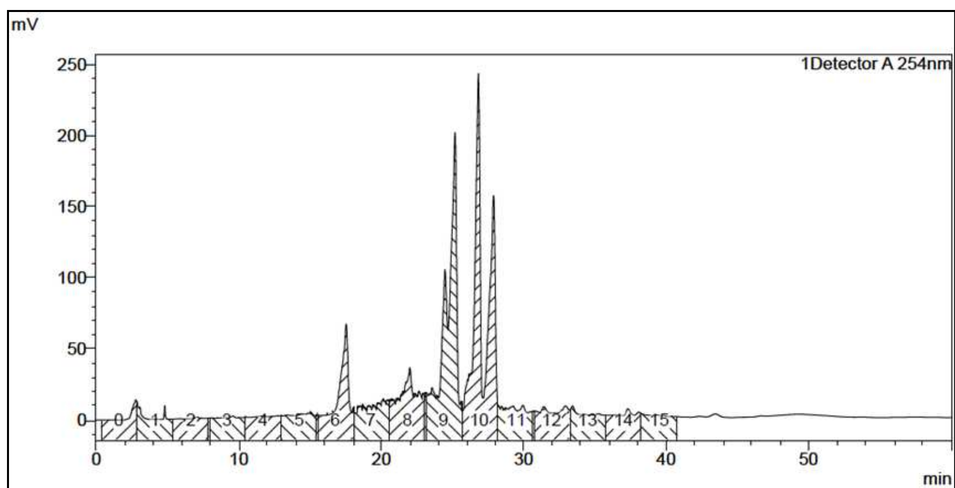

Fig.13: Semi - preparative HPLC run, second Bl73 extract.

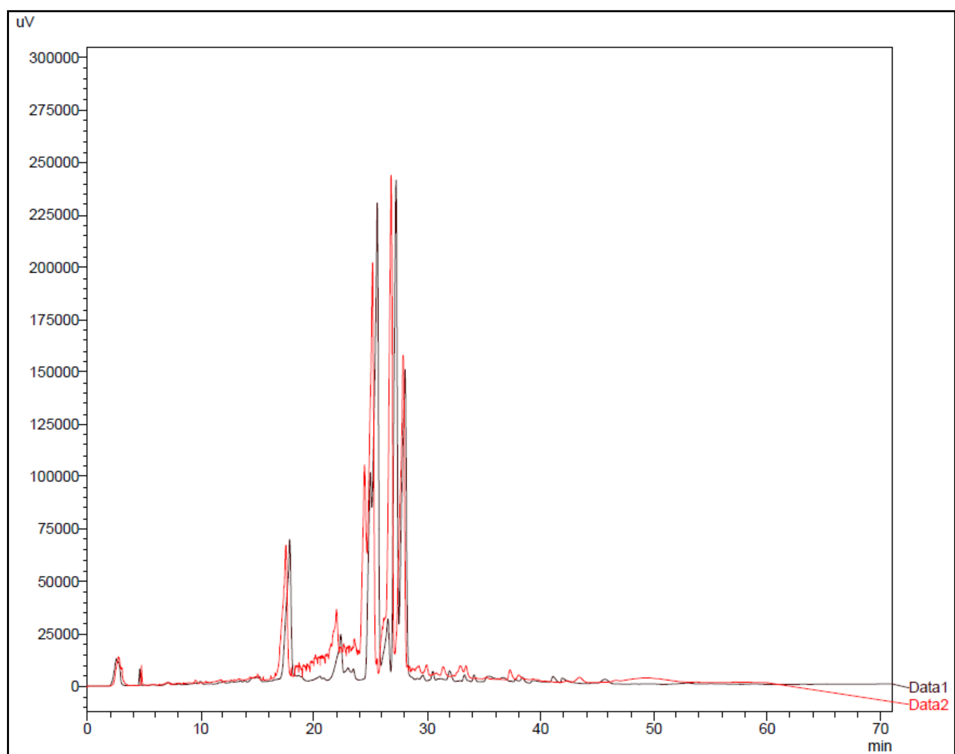

Fig.14: Comparison of the first and third semi - preparative HPLC run, second Bl73 extract, black line = first yield, red line = third yield, uV = µV.

Weights and Concentration

Tab.24: Yields of semi - preparative HPLC, at the third yield only antimicrobial active fractions were collected.

BI73	1. yield Weight [mg]	2. yield Weight [mg]	3. yield Weight [mg]
F0	11.5	10.2	
F1	3	2	
F2	2.9	2.3	
F3	3.6	1.3	2.4
F4	3.7	1.2	
F5	3.8	2.3	
F6	4.5	4.6	
F7	3.2	2.1	
F8	4.4	1	3.2
F9	3,2	1.4	1
F10	2.1	3.1	2.6
F11	0.6	1.7	0.5
F12	1.2	0.7	
F13	1.2	1	
F14	1.2	2.9	2.6
F15	1.6	0.8	1.2
F16	5,9	6,4	2,8

Tab.25: Concentration of the obtained BI73 fractions.

BI73	1. yield [µg/µL]	2. yield [µg/µL]	3. yield [µg/µL]
F0	11.5	10.2	
F1	5	3.3	
F2	5.8	4.6	
F3	5.1	1.9	3
F4	7.4	2.4	
F5	7.6	3.8	
F6	7.5	7.6	
F7	6.4	2.1	
F8	7.3	1.7	3.2
F9	6.4	2	1
F10	2.1	2.8	1.9
F11	1.2	2.8	0.6
F12	2.4	1.2	
F13	2	1.4	
F14	2.4	4.8	3.3
F15	3.2	1.3	1.5
F16	11.8	10.7	2,5

Tab. 24 views the weight of the yielded fractionations. These residues were dissolved in different volumes (500 - 1400 µL) of methanol by use of supersonic bath. The concentration of the obtained fractions is displayed in Tab. 25.

3.4.3 Results of disc diffusion assays of Fractionation

After the first preparative HPLC run, the dissolved fractions (see Tab. 26) with concentrations between 1.2 and 11.5 [µg/µL] were tested in bioassays for antimicrobial activity.

Tab.: Results of disc diffusion assays. +++.... radius = 3 mm ++.... radius = 2.5 mm, +.... radius = 0.75mm, (+)….radius = 0.6 mm.

	B. subtilis	***S. cerevisiae***	***F. gramineum***	***A. niger***
F0	-	-	-	-
F1	-	-	-	-
F2	-	-	-	-
F3	+	-	-	-
F4	-	-	-	-
F5	-	-	-	-
F6	-	-	-	-
F7	-	-	-	-
F8	-	-	-	(+)
F9	+	+++	+	+
F10	+	++	+	++
F11	+	-	-	-
F12	-	-	-	-
F13	-	-	-	-
F14	+++	-	-	-
F15	++	-	-	-
F16	-	-	-	-
Methanol	-	-	-	-

In the disc diffusion assays six fractions of BI73 indicated antibacterial and antifungal active compounds. Fraction 3 and 11 were active against *B. subtilis*, with an inhibition zone of about 0.75 mm. Fraction 8 showed a very slight effect on *A. niger*, whereas *S. cerevisiae* and *F. graminearum were* not inhibited. Fractions 9 and 10 showed the greatest potential of all because they were active against all four test organisms. Fraction 9 and 10 nearly had the same efficacy, as Fraction 9 was only slightly more

active against *S. cerevisiae*. Fraction 14 and 15 showed a strong effect on *B. subtilis*, whereby the inhibition zone of F14 was a little bigger than the one of F15.

In contrast to Ab1 extract these active fractions were separated by inactive ones. As an example Fraction 11 and 14 were separated by two inactive parts, which suggested that although both are active against *B. subtilis* they should not be the same compound. Fraction 9, 10 showed a similar efficacy, which meant that is was likely, that the active substance was split within these two ones. Fraction 11, however only inhibited *B. subtilis* which lead the assumption that another substance stopped the growth of this bacterium. Fraction 14 and 15 were also supposed to have the same antimicrobial, whereas the substance in Fraction 3 was assumed to be unique. Fraction 8 only showed a slight effect on *A. niger*, it was supposed that traces of the substance in Fraction 9 made the effect.

3.4.4. Analytical HPLC of BI73 Fractions

Subsequently to the disc diffusion assays, analytical HPLC was performed on the antimicrobial active fractions in order to optimise the acetonitrile/water gradients for LCMS.

Fraction 3

Time program analytical HPLC

0.0 min Solvent Acetonitrile Conc. 30%
10.0 min Solvent Acetonitrile Conc. 30%
15.0 min Solvent Acetonitrile Conc. 50%
25.0 min Solvent Acetonitrile Conc. 80%
35.0 min Solvent Acetonitrile Conc. 95%
50.0 min Solvent Acetonitrile Conc. 95%

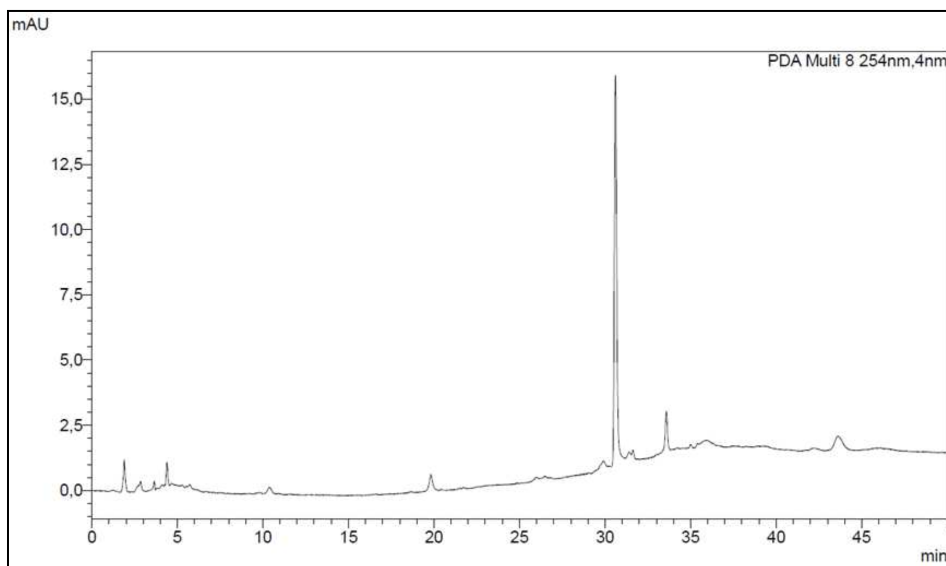

Fig.15: Analytical HPLC run, Fraction 3, optimized gradient.

Fig. 15 shows the optimized gradient for Fraction 3. Starting at 30% acetonitrile the best separation was achieved, with the biggest peak occurring at about 31 minutes. The final gradient for LCMS remained the same.

Final time program LCMS

0.0 min Solvent Acetonitrile Conc. 30%
10.0 min Solvent Acetonitrile Conc. 30%
15.0 min Solvent Acetonitrile Conc. 50%
25.0 min Solvent Acetonitrile Conc. 80%
35.0 min Solvent Acetonitrile Conc. 95%
50.0 min Solvent Acetonitrile Conc. 95%

Fraction 8

Time program analytical HPLC

0.0 min Solvent Acetonitrile Conc. 58%
5.0 min Solvent Acetonitrile Conc. 58%
10.0 min Solvent Acetonitrile Conc. 62%
20.0 min Solvent Acetonitrile Conc. 62%

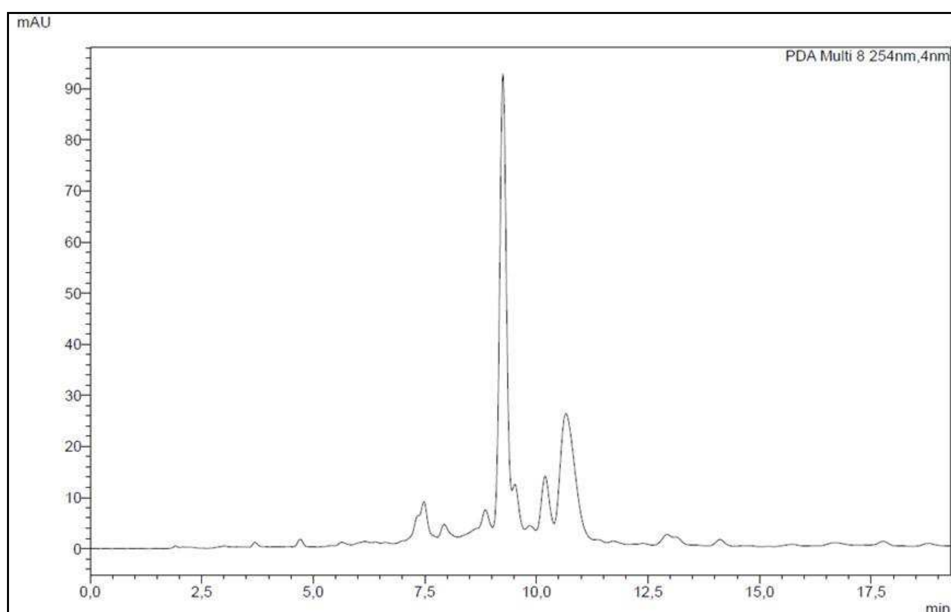

Fig.16: Analytical HPLC run, Fraction 8, optimized gradient.

Fraction 8 in the semi - preparative run occurred at 68 to 75 percent acetonitrile. For a better separation of the peaks a higher amount of water was chosen for starting the run. This was done for several analytical gradients. In Fraction 8 the analytical run started at 58 percent and was raised to 62 percent acetonitrile.

Final time program LCMS

0.0 min Solvent Acetonitrile Conc. 58%
5.0 min Solvent Acetonitrile Conc. 58%
10.0 min Solvent Acetonitrile Conc. 62%
20.0 min Solvent Acetonitrile Conc. 62%

Fraction 9

Time program analytical HPLC

0.0 min Solvent Acetonitrile Conc. 62%
30.0 min Solvent Acetonitrile Conc. 62%

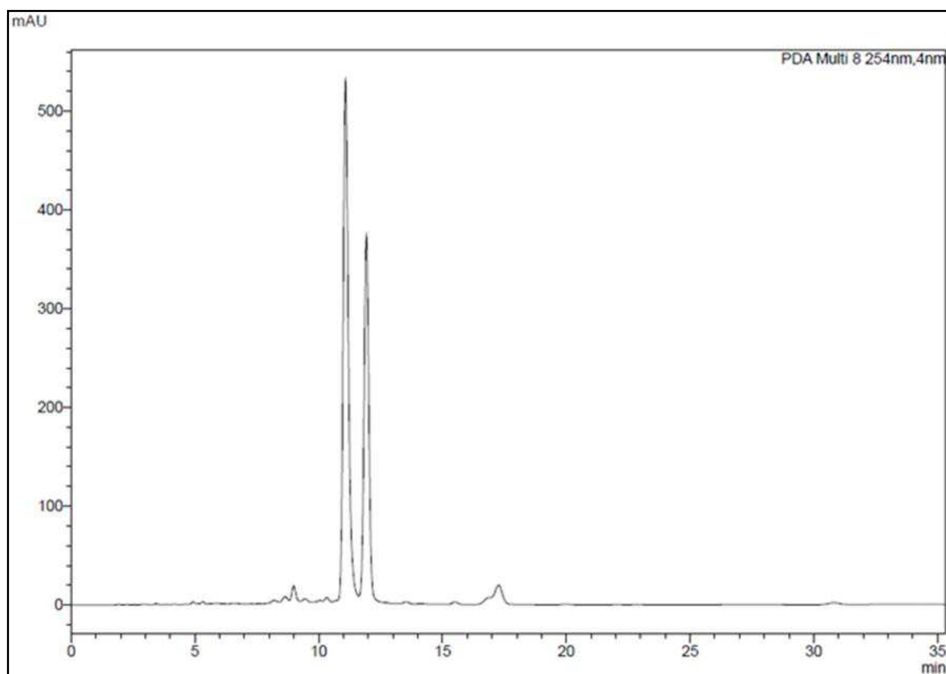

Fig.17: Analytical HPLC run, Fraction 9, optimized gradient.

For Fraction 9 an isocratic mobile phase of 62 % acetonitrile was chosen for ideal separation conditions. It resulted in two big peaks, which occurred at about 11 minutes in the analytical run. The actual run time in Fig. 17 was 35 minutes. However, for the LCMS analysis, the running time was decreased to 30 minutes.

Final time program LCMS

0.0 min Solvent Acetonitrile Conc. 62%
30.0 min Solvent Acetonitrile Conc. 62%

Fraction 10

Time program analytical HPLC

0.0 min Solvent Acetonitrile Conc. 60%
5.0 min Solvent Acetonitrile Conc. 60%
10.0 min Solvent Acetonitrile Conc. 70%
30.0min SolventAcetonitrile Conc. 83%

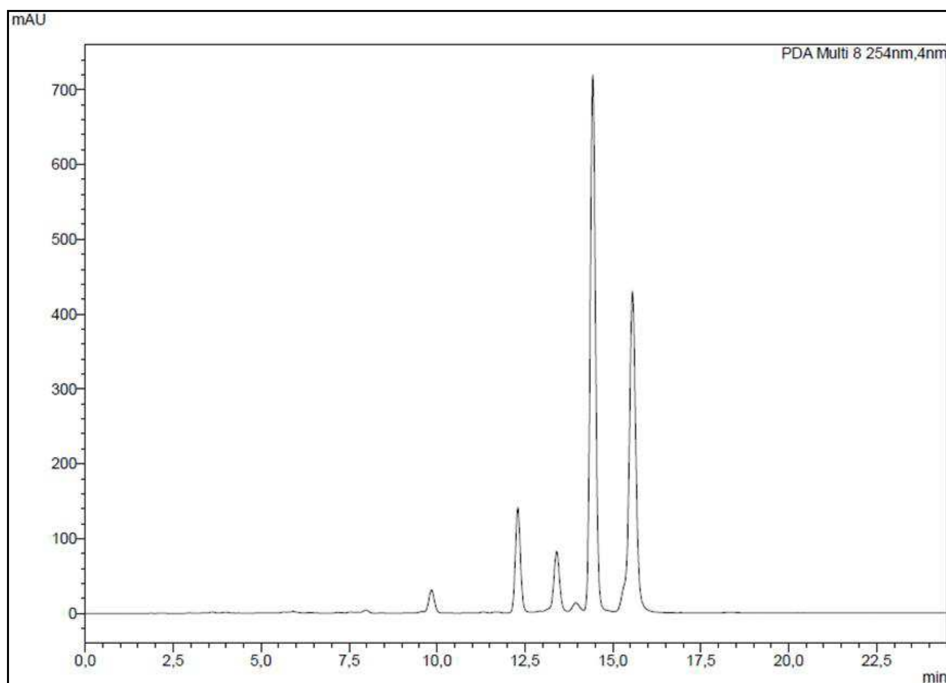

Fig.18: Analytical HPLC run, Fraction 10, optimized gradient.

The gradient for Fraction 10 started at 60 percent acetonitrile and was raised to 83 percent until minute 30. The main peaks occurred after 12.5 minutes. The actual run time in Fig. 18 was about 25 minutes; it was aborted after the main peaks occurred in the chromatogram.

Final time program LCMS

0.0 min Solvent Acetonitrile Conc. 60%
5.0 min Solvent Acetonitrile Conc. 60%
10.0 min Solvent Acetonitrile Conc. 70%
30.0 min Solvent Acetonitrile Conc. 83%

Fraction 11

Time program analytical HPLC

0.0 min Solvent Acetonitrile Conc. 70%
20.0 min Solvent Acetonitrile Conc. 70%
25.0 min Solvent Acetonitrile Conc. 95%
40.0 min Solvent Acetonitrile Conc. 95%

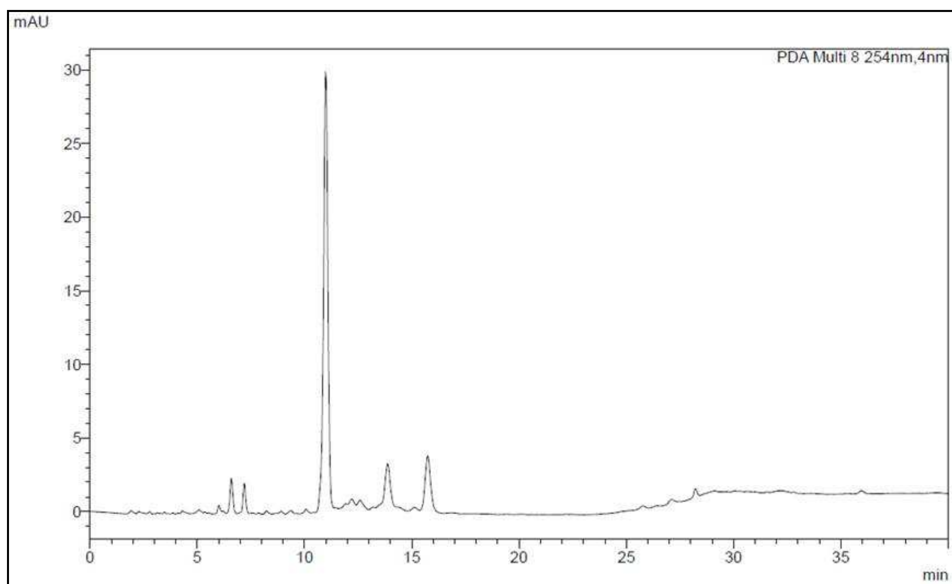

Fig.19: Analytical HPLC run, Fraction 11, optimized gradient.

In Fraction 11 the run started with 70 percent acetonitrile. The main peak at 254 nm appeared at about 11 minutes (Fig.19). Between 25 and 36 minutes still small peaks occurred, that was the reason for choosing a gradient that extended to 40 minutes.

Final time program LCMS

0.0 min Solvent Acetonitrile Conc. 70%
20.0 min Solvent Acetonitrile Conc. 70%
25.0 min Solvent Acetonitrile Conc. 95%
40.0 min Solvent Acetonitrile Conc. 95%

Fraction 14

Time program analytical HPLC

0.0 min Solvent Acetonitrile Conc. 60%
5.0 min Solvent Acetonitrile Conc. 60%
10.0 min Solvent Acetonitrile Conc. 70%
20.0 min Solvent Acetonitrile Conc. 80%
25.0 min Solvent Acetonitrile Conc. 87%
40.0 min Solvent Acetonitrile Conc. 87%
60.0 min Solvent Acetonitrile Conc. 95%

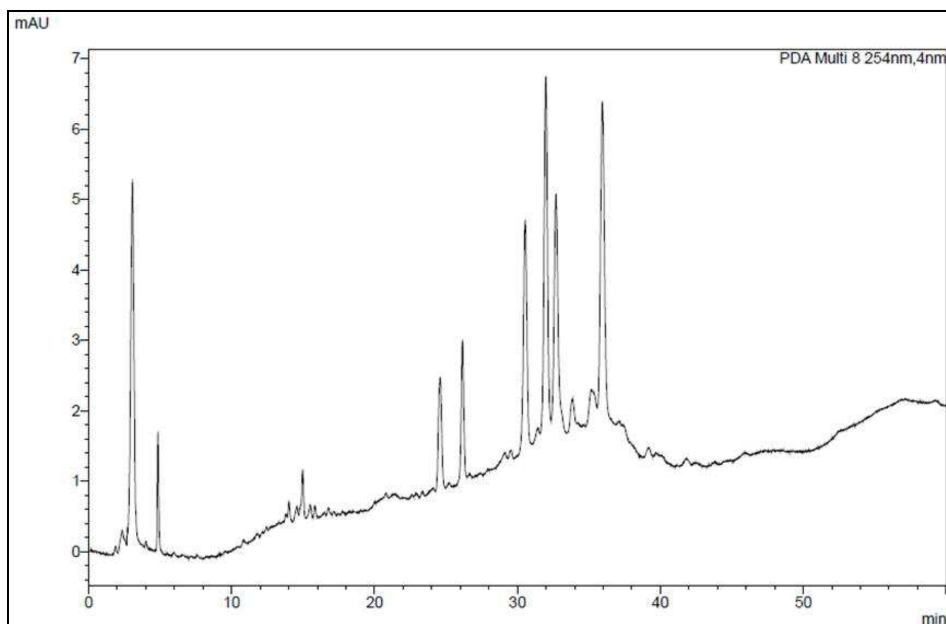

Fig.20: Analytical HPLC run, Fraction 14, optimized gradient.

Fractions 14 and 15 were collected at 95 % acetonitrile. However, for a better separation of the peaks, the mobile phase started at 60 percent acetonitrile. In analytical tests the running time was 60 min, in the final LCMS gradient it was extended to 70 min. This was done to be sure, that all substances that occurred after 50 minutes were analysed within the LCMS procedure.

Final time program LCMS

0.0 min Solvent Acetonitrile Conc. 60%
5.0 min Solvent Acetonitrile Conc. 60%
10.0 min Solvent Acetonitrile Conc. 70%
20.0 min Solvent Acetonitrile Conc. 80%
25.0 min Solvent Acetonitrile Conc. 87%
40.0 min Solvent Acetonitrile Conc. 87%
60.0 min Solvent Acetonitrile Conc. 95%
70.0 min Solvent Acetonitrile Conc. 95%

Fraction 15

Time program analytical HPLC

0.0 min Solvent Acetonitrile Conc. 50%
5.0 min Solvent Acetonitrile Conc. 50%
10.0 min Solvent Acetonitrile Conc. 70%
20.0 min Solvent Acetonitrile Conc. 80%
25.0 min Solvent Acetonitrile Conc. 85%
35.0 min Solvent Acetonitrile Conc. 85%
40.0 min Solvent Acetonitrile Conc. 87%
60.0 min Solvent Acetonitrile Conc. 87%

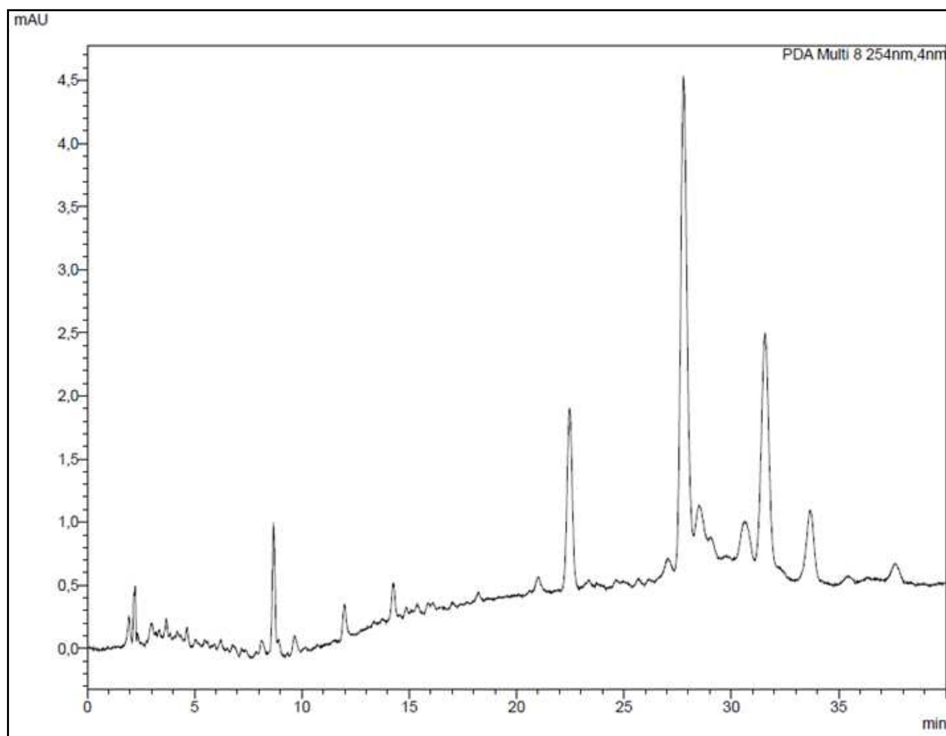

Fig.21: Analytical HPLC run, Fraction 15, optimized gradient

The actual running time in Fig. 21 was 40 min; the run was aborted after the main peaks occurred until 35 min. Equal to Fraction 14, in the final LCMS gradient it was extended to 70 min. As Fig. 21 outlines the UV absorbance at 254 nm was very small in comparison to all other fractions (except Fraction 14). As an example the highest peak in Fraction 10 had an absorbance of 700 mAU (see page 44), whereas the highest peak in Fraction 15 only had an absorbance of 4.5 mAU. The best absorbance was determined under UV 190 nm; however, as Fig. 22 shows this wavelength did not provide appropriate results.

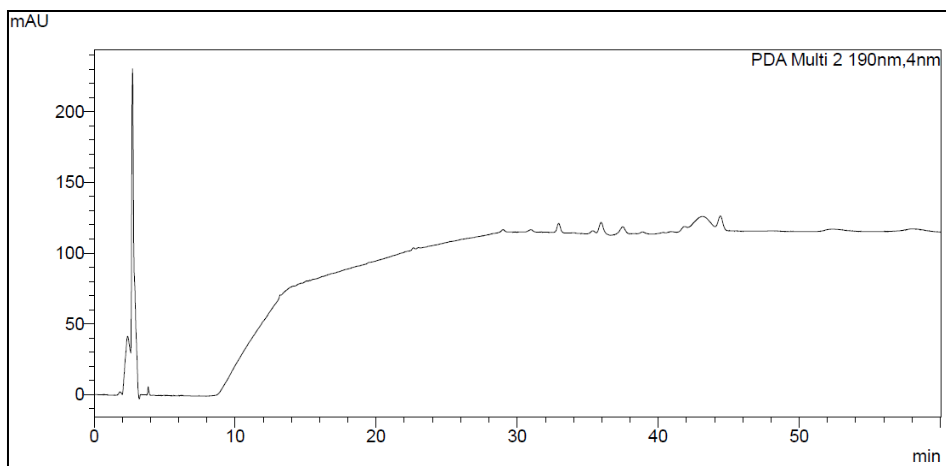

Fig.22: Analytical HPLC run of Fraction 15 at 190nm.

Final time program LCMS

0.0 min Solvent Acetonitrile Conc. 50%
5.0 min Solvent Acetonitrile Conc. 50%
10.0 min Solvent Acetonitrile Conc. 70%
20.0 min Solvent Acetonitrile Conc. 80%
25.0 min Solvent Acetonitrile Conc. 85%
35.0 min Solvent Acetonitrile Conc. 85%
40.0 min Solvent Acetonitrile Conc. 87%
60.0 min Solvent Acetonitrile Conc. 87%
70.0 min Solvent Acetonitrile Conc. 95%

3.5 LCMS of BI73 Fractions

After the analytical HPLC of the BI73 fractions LCMS was done in order to analyse the masses of the substances within the fractions of interest. All fractions of BI73 extract comprised a high number of substances. In the following the most relevant mass peaks are presented.

3.5.1 BI73 Fraction 3 - LCMS run analysis

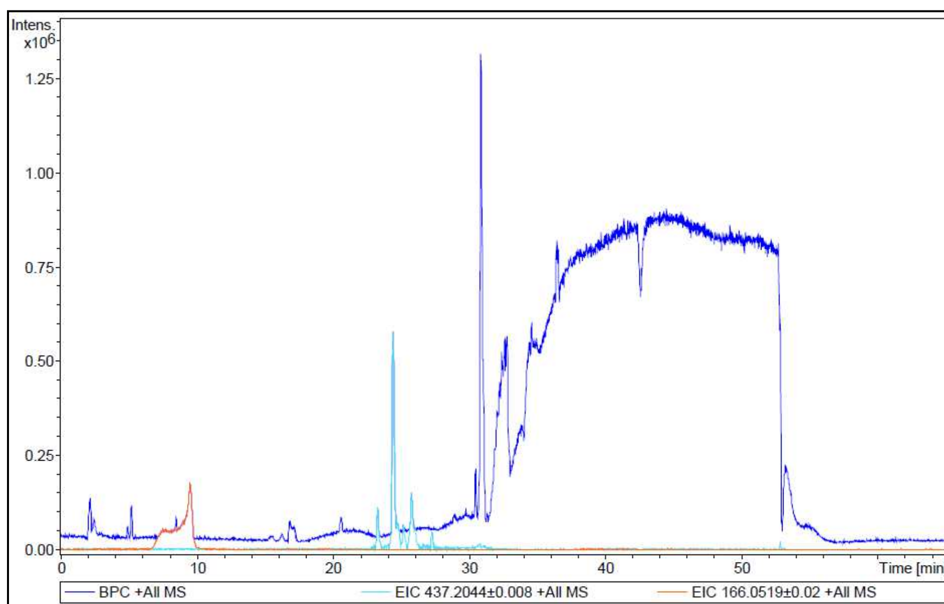

Fig.23: Complete LCMS run of F3 with most relevant extracted ion chromatograms (EIC).

In Fraction 3 mass $[M+H]^+$166.0519/166.0538 [m/z] and mass $[M+Na]^+$ 437.2030/437.2012 [m/z] were notable substances. The compound with mass 166.0519/166.0538 [m/z] was unique for Fraction 3, whereas the compound 437.2030/437.2012 [m/z] was ubiquitous in all other fractions of BI73. The EICs in Fig.23 and 24 highlight the masses in the complete LCMS chromatograms of F3 and F3_2.

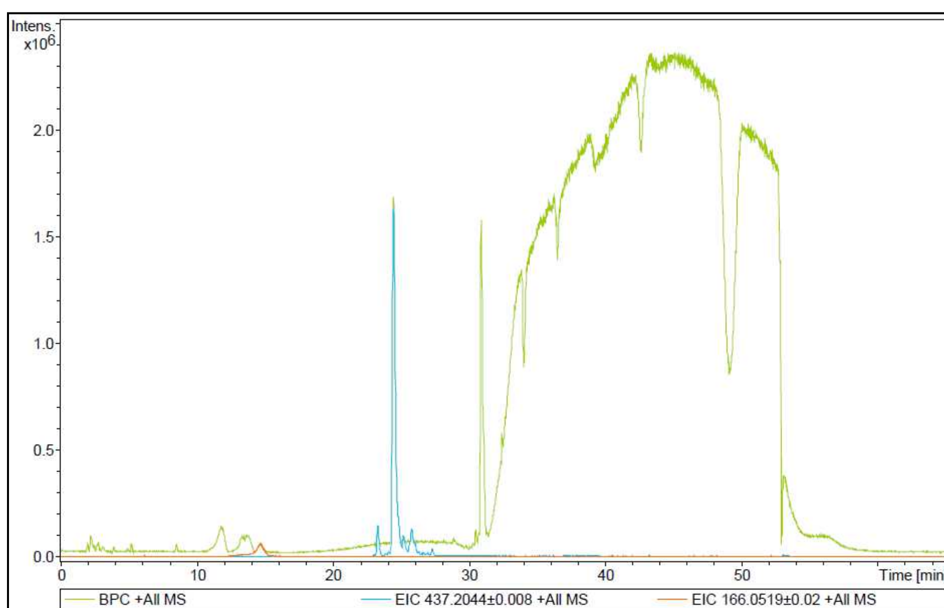

Fig.24: Complete LCMS run of F3_2 with most relevant EICs.

Mass peak at 9.5 min

Extracted mass $[M+H]^+$166.0519/166.0538 [m/z]

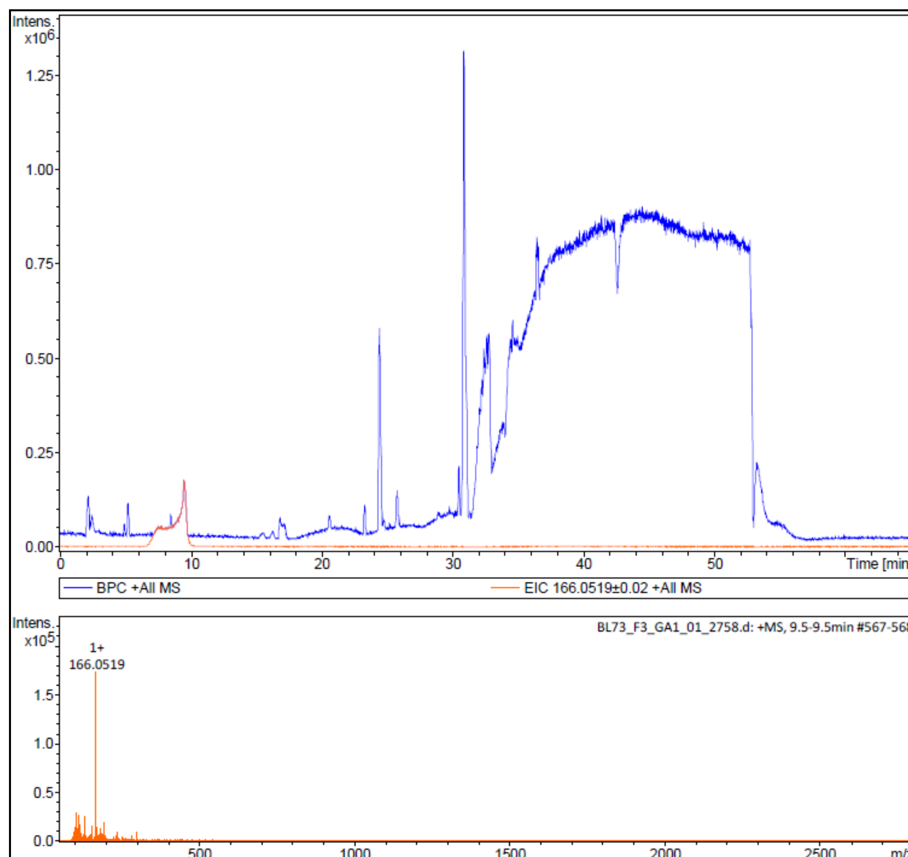

Fig.25: Complete LCMS run of F3 with EIC of mass 166.0519 +/- 0.02; the lower part of the figure displays mass 166.0519 [m/z].

166.0519 [m/z] and 166.0538 [m/z] were the masses detected in F3 and F3_2, respectively. The substance was not found in other fractions and not in rice nor methanol blank. The predicted mass formula for both 166.0519 [m/z] and 166.0538 [m/z] was $C_8H_8NO_3$. The predicted formulas depend on scores; the highest possible score is 100. The LCMS analysis was done twice for each fraction; therefore, the most appropriate formula for the substances, depending on the scores, was determined. For masses 166.0519 [m/z] and 166.0538 [m/z] for both the score was 100. As the molecule only occurred in Fraction 3, it eventually was responsible for the activity against *B. subtilis*; however Fraction 3 was not considered in subsequent subfractionations.

Mass peak at 24.4 min

Extracted mass $[M+Na]^+$ 437.1980/437.2044 [m/z]

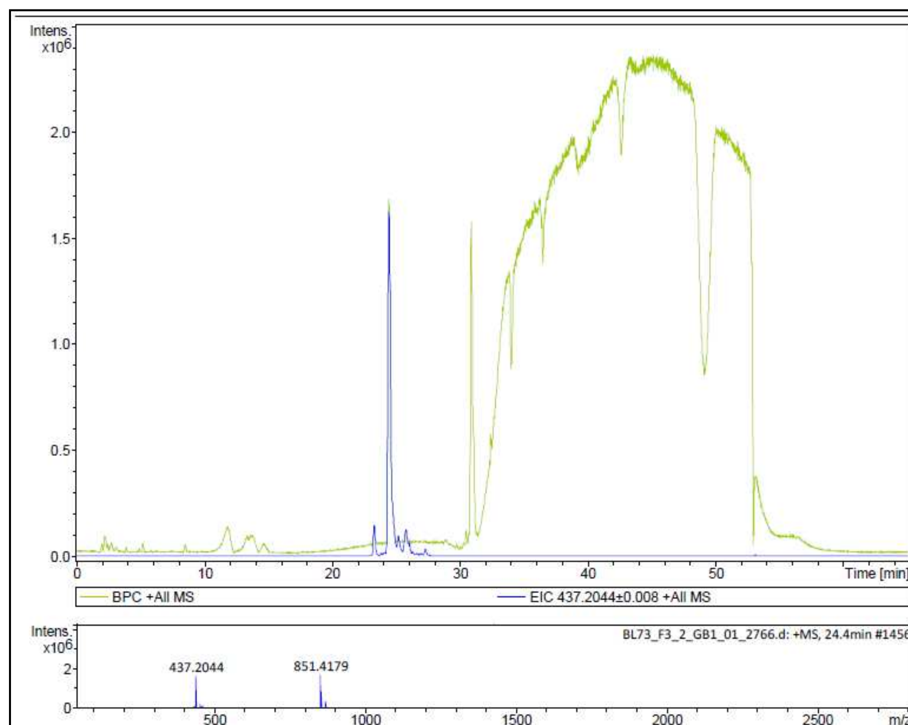

Fig.26: Complete LCMS run of F3_2 with EIC of mass 437.2044 +/- 0.008,
the lower part of the figure displays mass 437.2044[m/z].

437.1980 [m/z] and 437.2044 [m/z] were the masses found in F3 and F3_2, respectively. The chromatogram in Fig.26 also shows mass 851.4179 [m/z], which is a dimere of mass 437.2044 [m/z].

This mass was existent in nearly all other fractions with a signal intensity of 10^5- 10^6 and showed the same pattern in all fractions. It was also found in methanol and rice blank, however with a lower intensity of $2.5*10^4$ for both of them. The lower intensity in the blanks can be due to the lower injection volume, which was 1 µL. The best fitting overall formula for 437.1980 [m/z] and 437.2044 [m/z] was $C_{18}H_{30}N_4NaO_7$ (scores 49.02 and 7.46, respectively).

Common contaminations with mass 437.23572[m/z] and mass 851.57025[m/z] are polyethylen glycol and Triton reduced, respectively [Keller *et al.*, 2008]. In conclusion, it was not considered as substance of interest.

3.5.2 BI73 Fraction 8 - LCMS run analysis

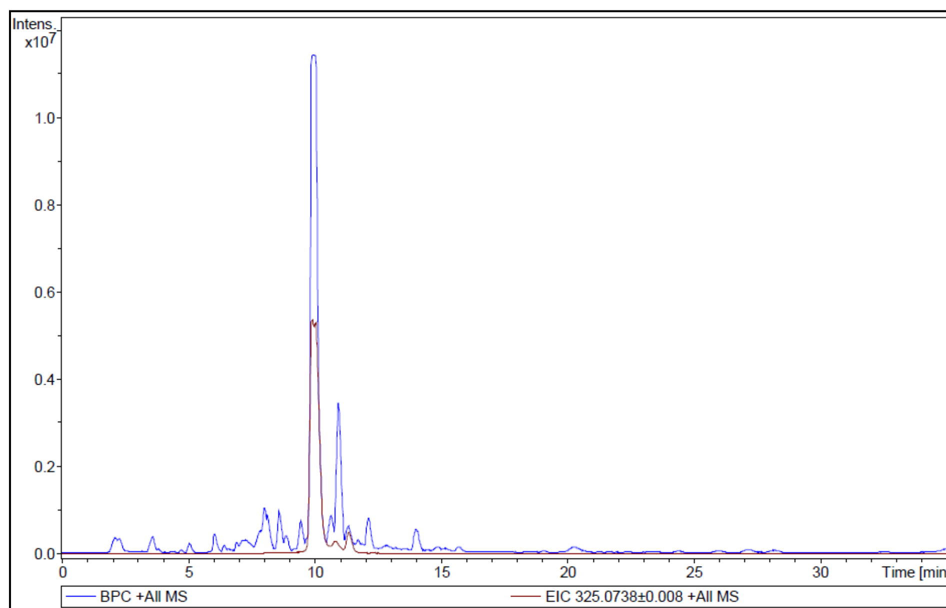

Fig.27: Complete LCMS run of F8 with most relevant EIC.

The most significant compound in Fraction 8 was mass $[M+Na]^+$ 325.0738 /325.0754 [m/z], which is indicated by Fig. 27 and 28.

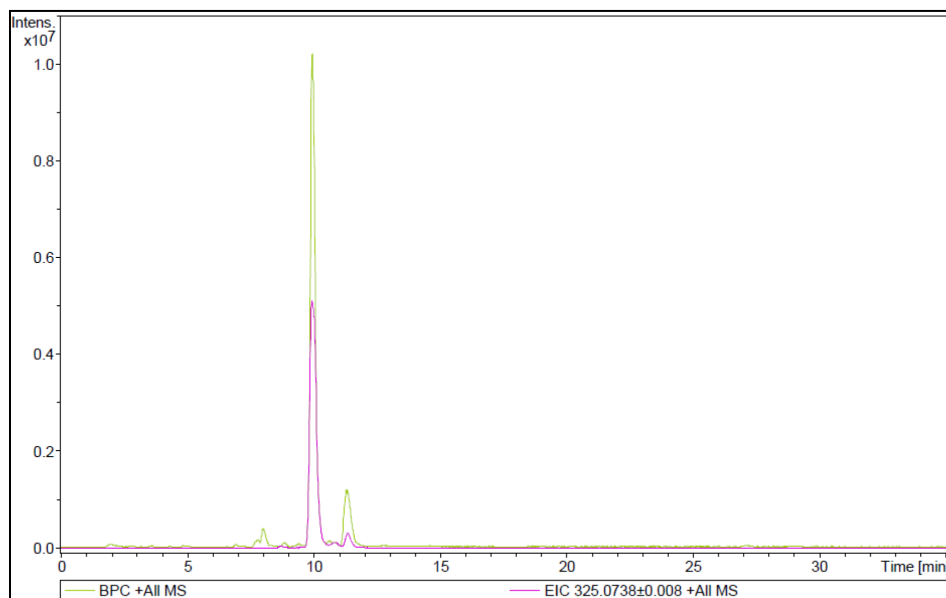

Fig.28: Complete LCMS run of F8_2 with most relevant EICs.

Mass peak at 10.0 min

Extracted mass $[M+Na]^+$ 325.0738/325.0754 [m/z]

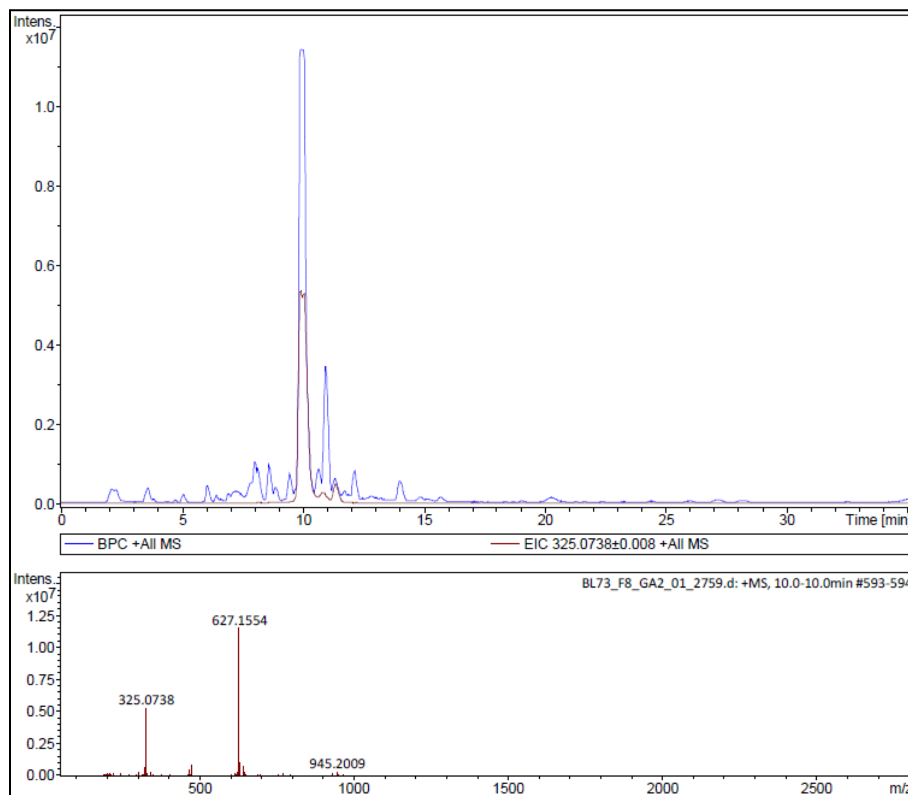

Fig.29: Complete LCMS run of F8 with EIC of mass 325.0738 +/- 0.008, the lower part of the figure displays mass 325.0738[m/z] and 627.1554[m/z].

Masses 325.0738 [m/z] and 325.0754 [m/z] were determined for F8 and F8_2, respectively. This molecule also occurred as dimer. This compound is present in F9 (intensity= $2.4*10^5$), F9_2 (intensity = $8*10^4$), F10 (intensity = $6*10^4$) and F10_2 (intensity = $5.5*10^5$) and was not detected in other fractions. Although in Fraction 8 the molecule predominately occurred as dimer, in other fractions the signal for the dimer was lower: F9 (intensity = $5*10^4$), F9_2 (intensity = $2.7*10^4$), F10 (intensity = $1.8*10^4$) and F10_2 (intensity = 1500). The proposed formula for both masses 325.0738 [m/z] and 325.0754 [m/z] (score 100 for both molecules) was $C_9H_{18}NaO_{11}$.

In Fraction 8 the substance with mass 325.07 [m/z] was dominant, but as the fraction showed the lowest activity against all test organisms compared to the other active fractions, it was not considered as an important substance. Moreover, traces of Fraction 9 were found within it and were seen as responsible for activity against *A. niger*. Noticeable, however, was that the molecular weight of this compound was

similar to substance with mass 323.06 [m/z], which will be discussed later (see page 57).

3.5.3 BI73 Fraction 9 - LCMS run analysis

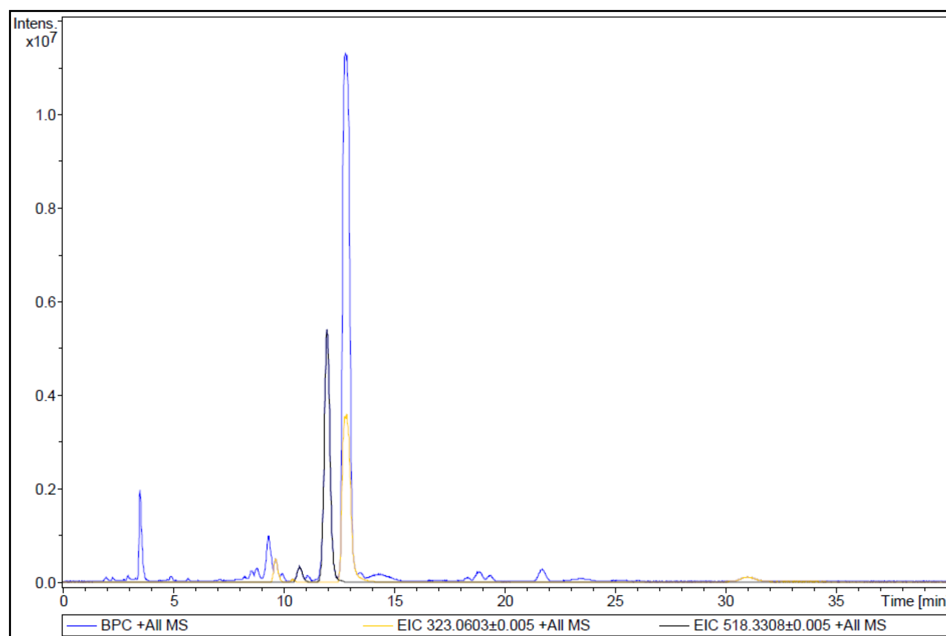

Fig.30: Complete LCMS run of F9 with the most relevant EICs.

The most important molecules that were found within Fraction 9 were mass $[M+H]^+$ 518.3307 [m/z] and mass $[M+Na]^+$ 323.0590/323.1279 [m/z]. Fig. 30 and 31 display the full LCMS runs with the EICs of the mentioned compounds.

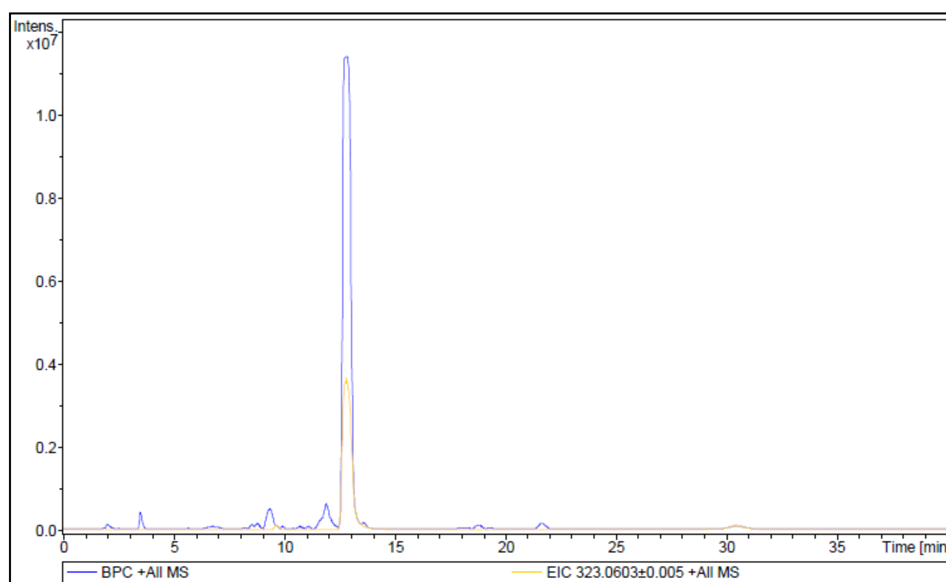

Fig.31: Complete LCMS run of F9_2 with most relevant EIC.

Mass peak at 12.0 min

Extracted mass $[M+H]^+$ 518.3307 [m/z]

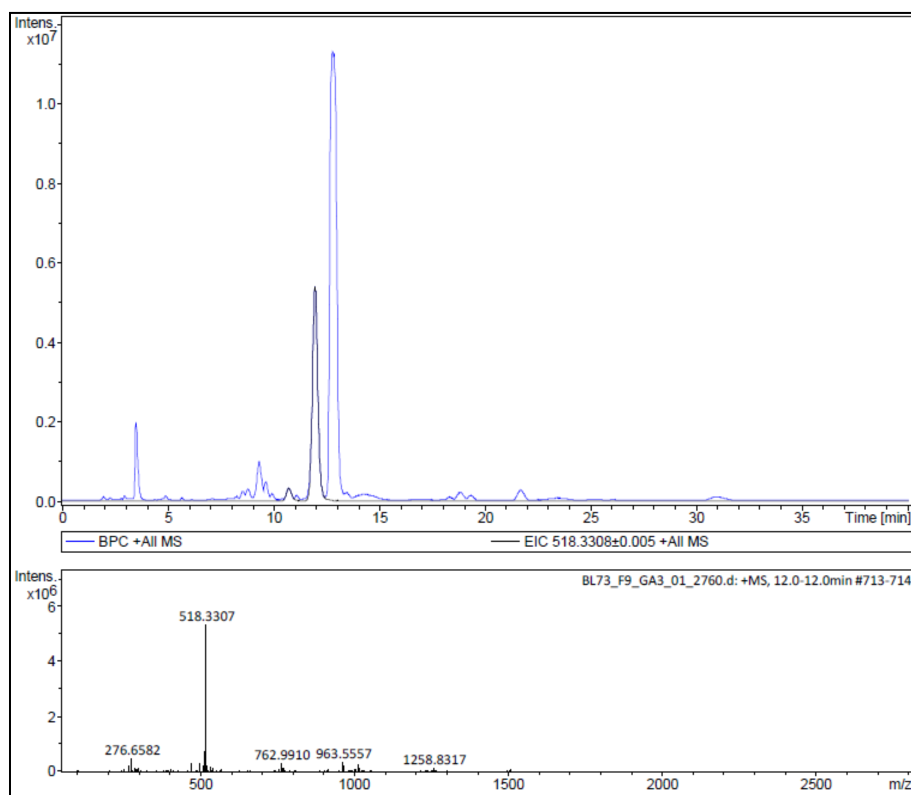

Fig.32: Complete LCMS run of F9 with the EIC of mass 518.3307+/- 0.008, the lower part of the figure displays mass 518.3307 [m/z].

The substance was also detected in F10_2 (intensity = $3.2*10^6$) and a minor amount of it was found in F9_2 (intensity = $3*10^5$). It was not present in other fractions or in the blanks. This substance was one of the main peaks in F10_2, it will be discussed more detailed later (see Page 59).

Mass peak at 12.8 min

Extracted mass $[M+Na]^+$ 323.0591/323.1279 [m/z]

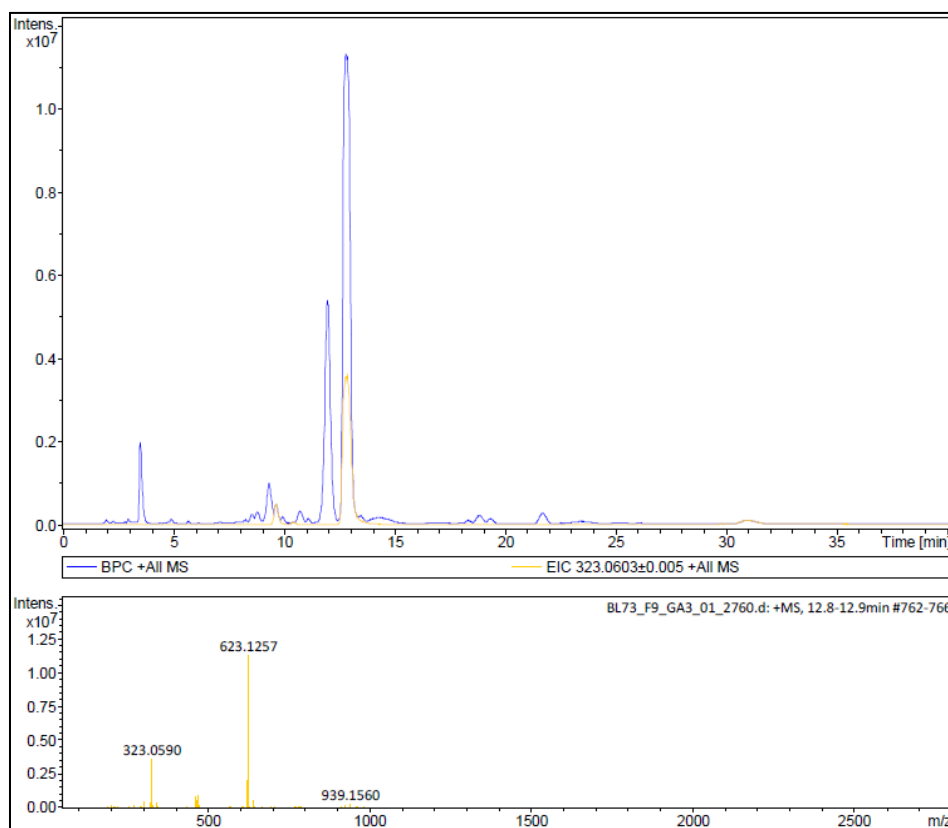

Fig.33: Complete LCMS run of F9 with the EIC of mass 323.0603+/- 0.005, the lower part of the figure displays mass 323.0591 [m/z] and 623.1257 [m/z].

Masses 323.0591 [m/z] and 323.1279 [m/z] were apparent in F9 and F9_2, respectively.

623.1257 [m/z] is the dimer of mass 323.0603 m/z]. Fig.31 outlines that the molecule mainly appeared as dimer. The proposed formula for mass 323.0591 [m/z] and mass 323.1279 [m/z] (scores 86.0 and 99.90, respectively) was $C_{10}H_{12}N_4NaO_7$. Anticipating the results of F10, the proposed formula in F10 for 323.0602 [m/z] also was $C_{10}H_{12}N_4NaO_7$ (score 100).

The substance with mass 323.0603+/-0.005 [m/z] was also found in F8 (intensity = $8*10^5$), F8_2 (intensity = $4*10^5$) and F10 (intensity = $1*10^7$), where it is one of the main peaks. In F11 and F11_2 a minor intensity of $5.5*10^4$ and $4.5*10^4$, respectively, was observed. In other fractions it was not existent. Furthermore, it was not detected

in rice or methanol blank. The compound was highly apparent in F9 and F10, therefore, it was considered as molecule of interest.
As mentioned before the substance with the mass 325.07 [m/z] found in F8 (predicted formula $C_9H_{18}NaO_{11}$), might be a derivate of the compound with mass 323.0591/ 323.1279 [m/z]. Eventually it comprises two additional hydrogen atoms. Thus, the corresponding formula for 325.07 [m/z] would be $C_{10}H_{10}N_4NaO_7$.

3.5.4 BI73 Fraction 10 - LCMS run analysis

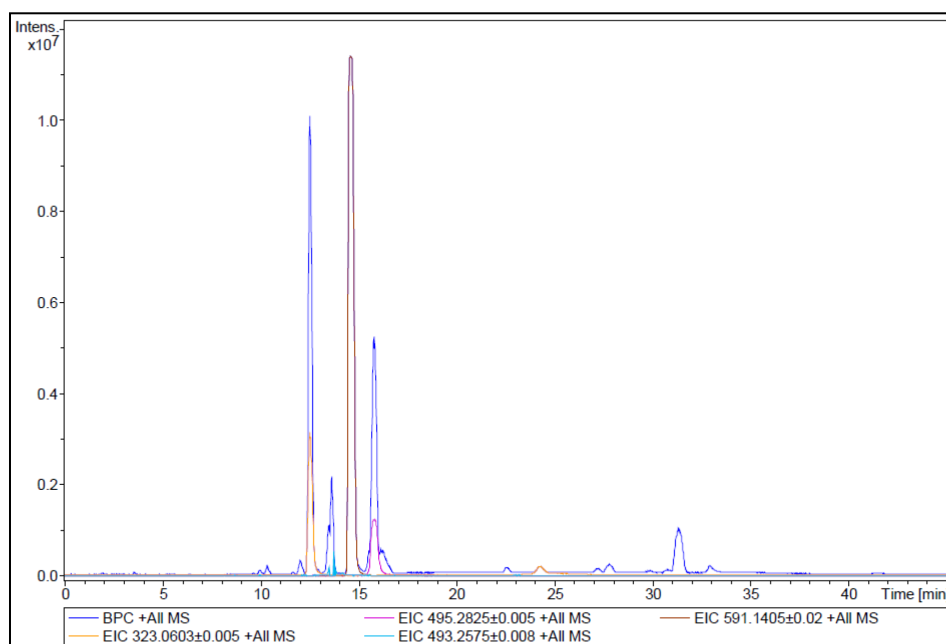

Fig.34: Complete LCMS run of F10 with most relevant extracted ion chromatograms (EIC).

The most important compounds within Fraction 10 were mass $[M+Na]^+$ 493.2659/493.2645 [m/z], mass $[M+Na]^+$ 495.2820/495.2799 [m/z] $^+$] and mass $[M+Na]^+$ 591.1378/591.1367 [m/z]. As it can be seen in Fig. 34 and 35, there are differences between F10 and F10_2. In F10 mass $[M+Na]^+$ 323.0602 [m/z] was found, albeit in F10_2 mass $[M+Na]^+$ 518.3312 [m/z] was detected.

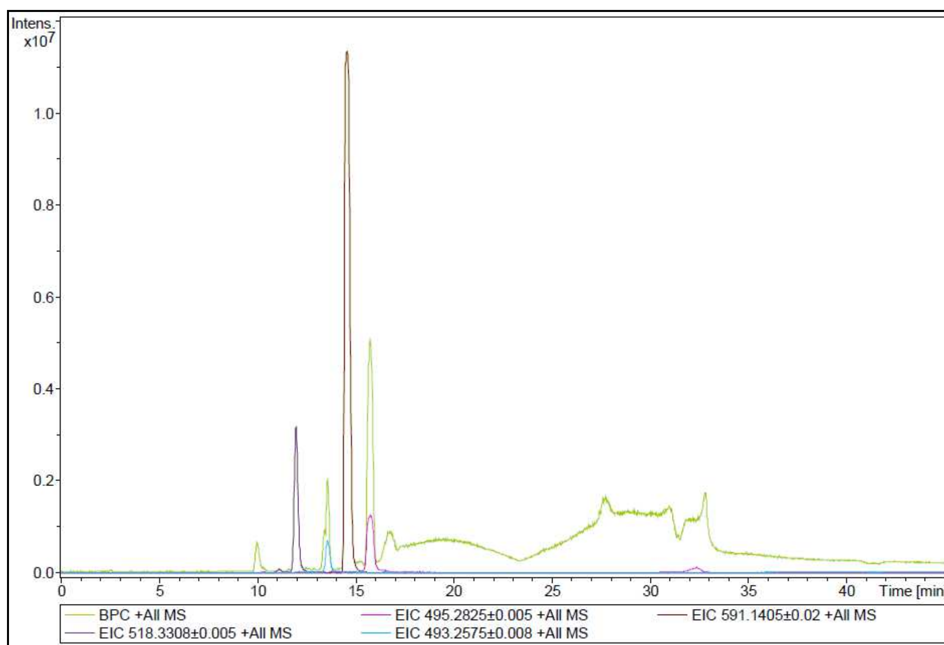

Fig.35: Complete LCMS run of F10_2 with most relevant EICs.

Mass peak at 12.0 min (F10_2)

Extracted mass $[M+H]^+$ 518.3312 [m/z]

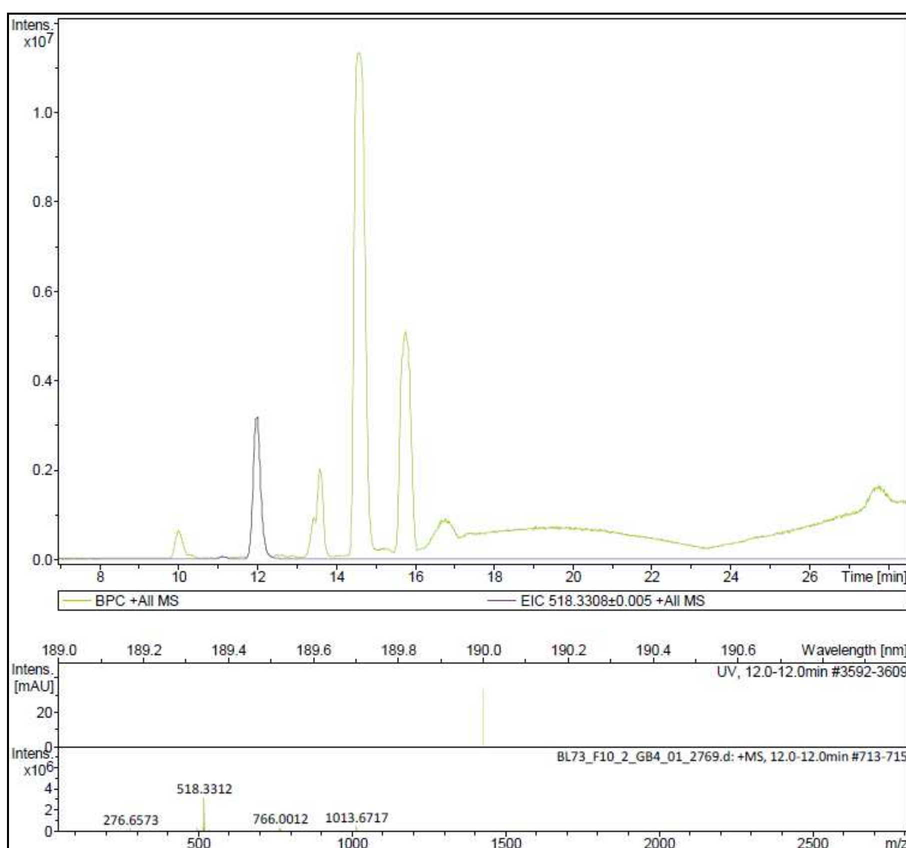

Fig.36: Section from LCMS run of F10_2 with the EIC of mass 518.3308+/- 0.005, the lower part of the figure displays mass 518.3312[m/z].

This substance with a mass of 518.3308+/-0.005 m/z was predominately existent in F10_2, albeit it was nearly absent in F10. The substance also occurred in F9 and F9_2 with intensities of $5*10^6$ and $3*10^5$, respectively. It was not determined in other fractions of Bl73 or methanol - or rice blank.

As the substance was one of the main peaks in F9 with a mass of 518.3307 [m/z] the proposed formula for 518.3307 [m/z] and 518.3312 [m/z] was $C_{22}H_{44}N_7O_7$ (scores 72.72 and 54.24, respectively)

This substance was one of the main peaks in F9, in F10 however, it was hardly detected. In the disc diffusion assays Fraction 9 and Fraction 10 (not F10_2) were tested and both of them had a similar effect on the test organisms. Therefore, this compound was not considered as important antimicrobial molecule.

Mass peak at 12.5 min (F10)

Extracted mass $[M+Na]^+$ 323.0602 [m/z]

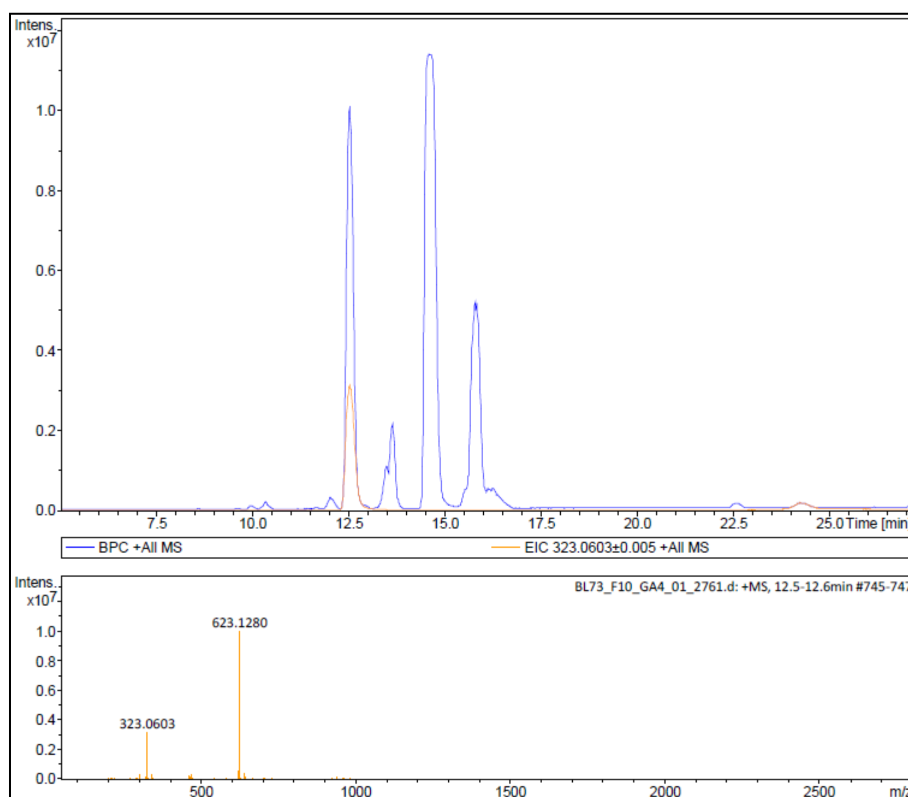

Fig.37: Section from LCMS run of F10 with the EIC of mass 323.0603+/- 0.005, the lower part of the figure displays mass 323.0602[m/z] and 623.1280 [m/z].

The substance with mass 323.0603 [m/z] was only found in F10, it was not apparent in F10_2. 623.1280 [m/z] is the dimer of mass 323.0603 [m/z]. As can be seen in Fig. 37, the molecule predominantly appeared as dimer. The substance is one of the two main peaks in Fraction 9; hence, it was already discussed (see page 56).

Mass peak at 13.6 min

Extracted mass $[M+Na]^+$ 493.2659/493.2645 [m/z]

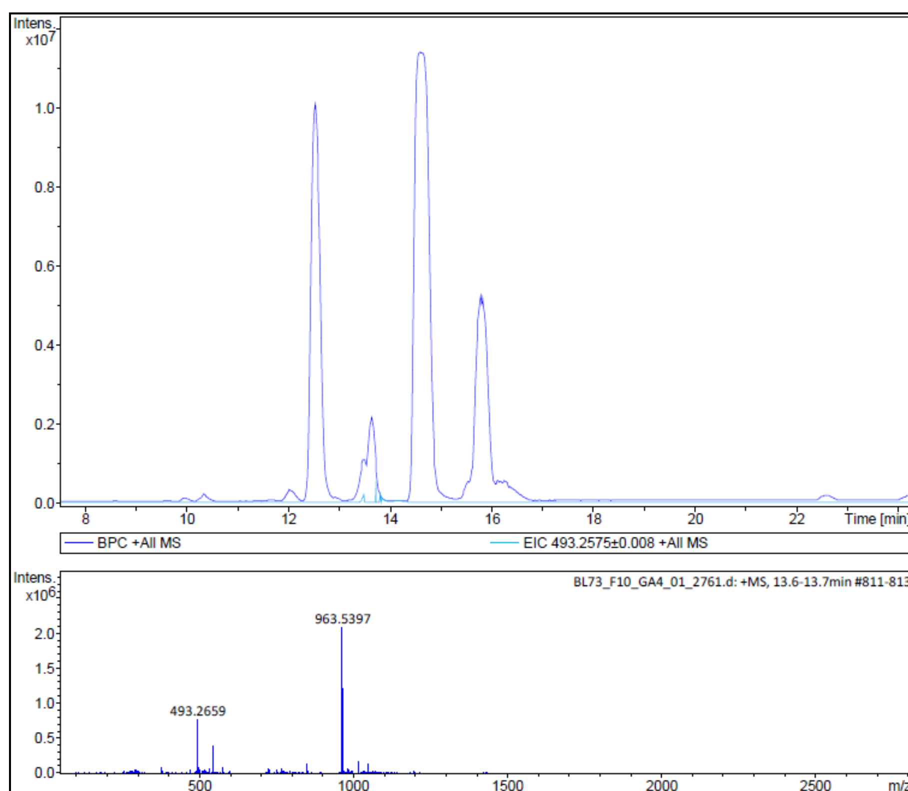

Fig.38: Section from LCMS run of F10 with the EIC of mass 493.2575 +/- 0.008, the lower part of the figure displays mass 493.2659[m/z] and 963.5397[m/z].

The masses 493.2699 [m/z] and 493.2645 [m/z] were found in F10 and F10_2, respectively.

963.5397/963.5396 [m/z] is the dimer of 493.2699/493.2645 [m/z] and shows an about two times higher intensity compared to 493.2699 [m/z] and 493.2645 [m/z]. The mass 493.2575+/- 0.008 was also found in F8 (intensity = $5.5*10^4$), F8_2 (intensity = $2.1*10^4$), F9 (intensity = $3.8*10^4$), F9_2 (intensity = $2.4*10^4$), F14

(intensity = $4*10^4$) and F14_2 (intensity = $4.5*10^4$) with a minor intensity. It was not detected in rice nor methanol blank.

The proposed formula for 493.2699 [m/z] and 493.2645 [m/z] was $C_{23}H_{34}N_8NaO_3$ (scores 77.84 and 100, respectively).

Mass peak at 14.6 min

Extracted mass $[M+Na]^+$ 307.0651/307.0635 [m/z]

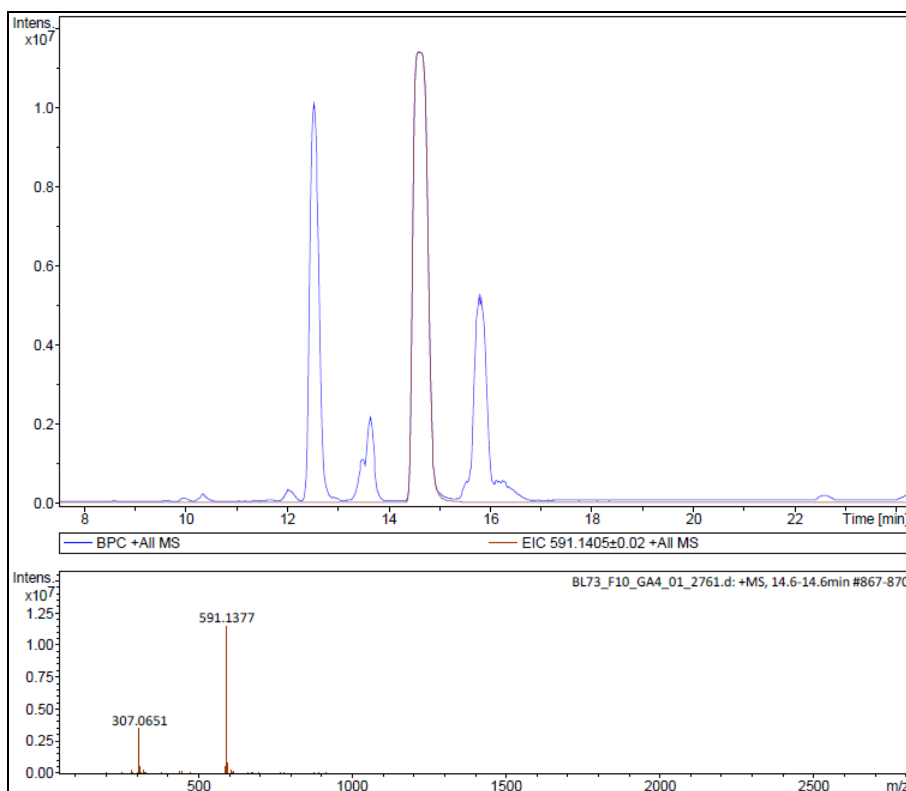

Fig.39: Section from LCMS run of F10 with the EIC of mass 591.1405+/- 0.02, the lower part of the figure displays mass 591.1377 [m/z] .

The substance with a mass of 307.0651 [m/z] (F10) and 307.0635 [m/z] (F10_2), predominantly occurred as dimere (Fig. 39). The mass 591.1377/591.1367 [m/z], present in F10 and F10_2, respectively, was rarely available in other fractions. Traces were found in F11 (intensity = $3.3*10^4$) and F11_2 (intensity = $1.8*10^4$). It was not found in the methanol - or rice blank.

Mass peak at 15.7 min

Extracted mass $[M+Na]^+$ 495.2820/ 495.2799 [m/z]

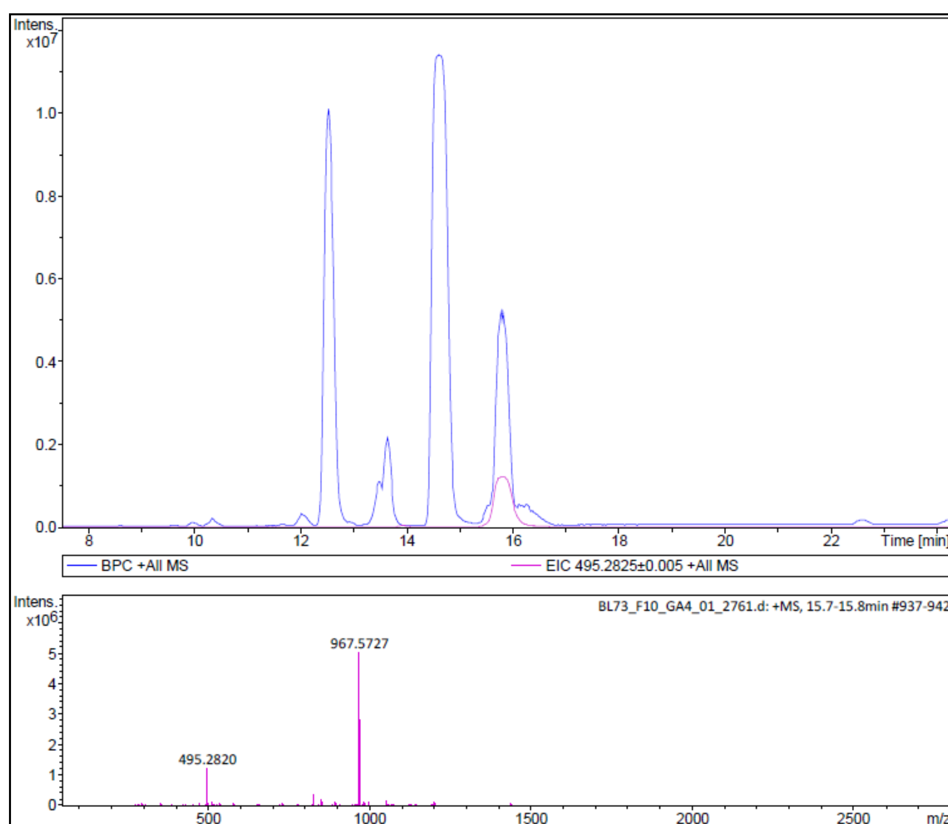

Fig.40: Section from LCMS run of F10 with the EIC of mass 495.2825±0.005, the lower part of the figure displays mass 495.2820 and 967.5727[m/z].

495.2820 [m/z] and 495.2799 [m/z] were dominant masses for F10 and F10_2. Furthermore, it is one of the main masses in F11, whereas it is absent in F11_2. A pretty minor amount was found in F9, F9_2, (both with intensities in the low range of 10^5) and F15 (intensity = $2.5*10^4$). It was also apparent in F14 (intensity $1.2*10^6$), whereas it did not appear in F14_2, F15_2. As the compound appeared in F14, it eventually was responsible for the better effect of F14 compared to F15 on *B. subtilis*. The molecule was not found in methanol - or rice blank.

The proposed formula for 495.2820 [m/z] was $C_{27}H_{40}N_2NaO_5$ (score 100). Anticipating the results of F11 the proposed formula for mass 495.2825 [m/z] also was $C_{27}H_{40}N_2NaO_5$ (score 100). For F10_2 and for F11_2 the proposed formula was $C_{23}H_{43}O_{11}$ (scores 70.22 and 97.44, respectively).

On account of this, it was supposed that the compound could be important for antimicrobial activity.

Summing up the LCMS analysis of BI73 Fractions 9 and 10 it was determined that a particular compound was existent in both fractions. The substance with the mass 323.06 [m/z] occurred with similar high signal intensity in both fractions. Therefore, it was suspected that this compound is responsible for the inhibition of the test organisms. As already discussed, another substance which occurred in these two fractions was the substance with mass 518.33 [m/z]. In F10, however, the amount of it was very small in comparisons to F9 and thus, it was not considered as important antimicrobial component.

3.5.5 BI73 Fraction 11 - LCMS run analysis

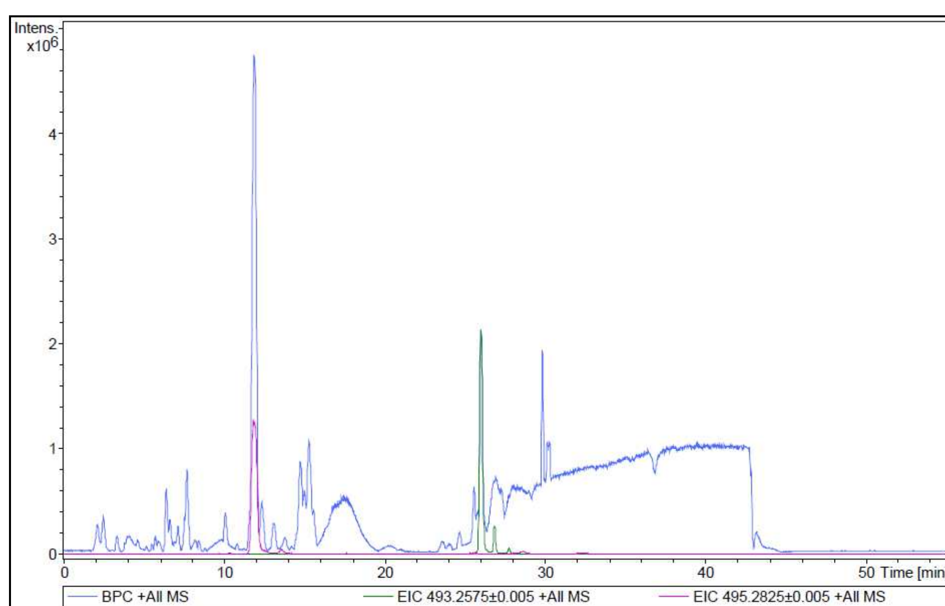

Fig.41: Complete LCMS run of F11 with most relevant EICs.

Fig. 41 and 42 give an overview of the most important EICs with correlating masses of F11 and F11_2. Substances with mass $[M+Na]^+$ 495.2825 [m/z] and mass $[M+Na]^+$ 493.2575 [m/z] were present in F11. In F11_2 the compounds with mass $[M+Na]^+$ 495.2788 [m/z] and $[M+Na]^+$ 493.2533 [m/z] were detected too, albeit with much lower intensities (1.5 $*10^5$ and 2.2$*10^5$ instead of 1.5$*10^6$ and 2$*10^6$, respectively.

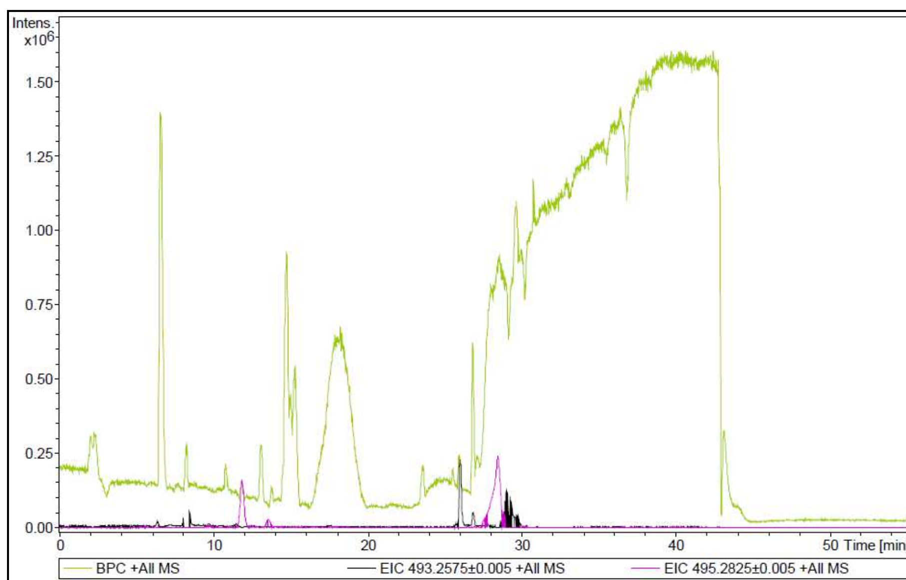

Fig.42: Complete LCMS run of F11_2 with most relevant EICs.

Mass peak at 11.9 min

Extracted mass $[M+Na]^+$ 495.2825/495.2785 [m/z]

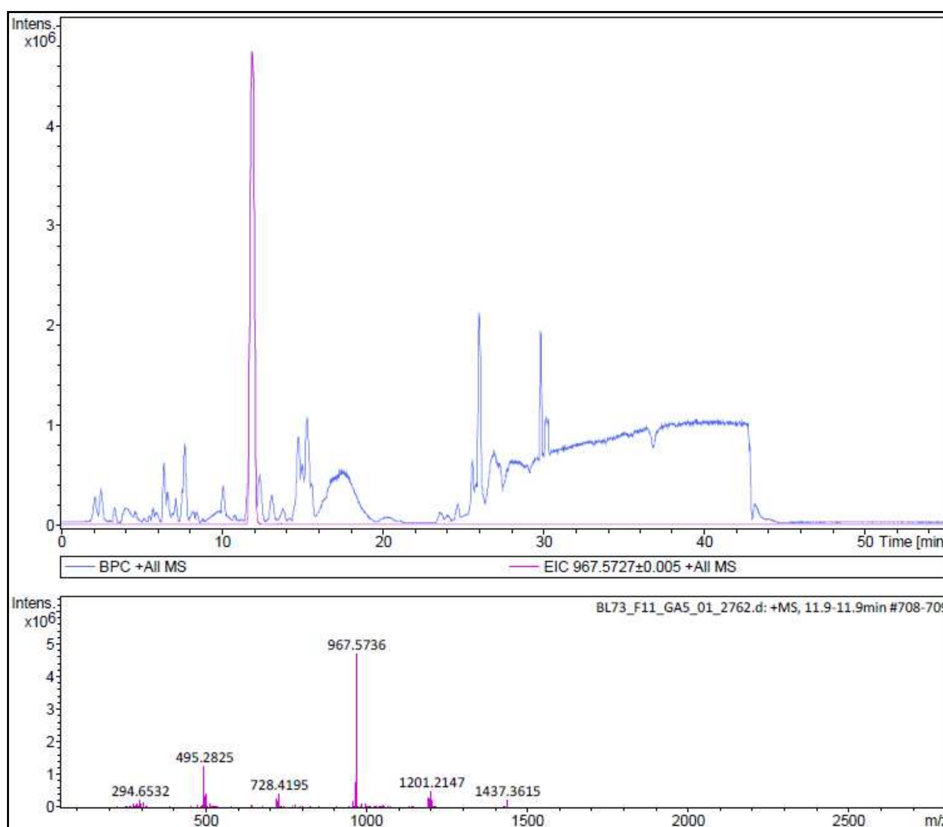

Fig.43: Complete LCMS run of F11with EIC of mass 967.5736+/- 0.005, the lower part of the figure displays mass 495.2825 [m/z] and 967.5736 [m/z].

495.2825 [m/z] and 495.2786 [m/z] were the masses determined for F11 and F11_2, respectively.
Mass 967.5736 [m/z] in F11 and the according mass 967.5646 [m/z] in F11_2 are dimers of mass 495.2825/495.2788 [m/z]. The molecule was primarily detected as dimer at 967.5727+/-0.005 m/z as can be seen in Fig. 43.

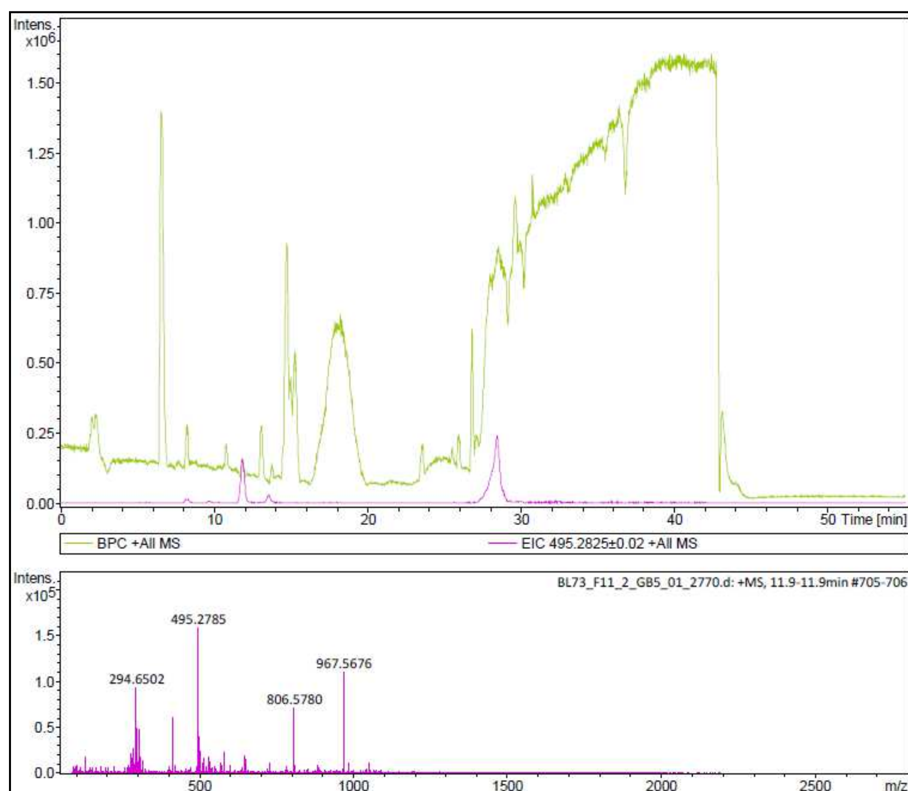

Fig.44: Section from LCMS run of F11_2 with EIC of mass 495.2825 +/- 0.005, the lower part of the figure displays mass 495.2788 [m/z] and 967.5736 [m/z].

Figure 44 outlines that in F11_2 hardly any of the substance 495.2785 [m/z] was found and moreover it also occurred at 28.4 min of the chromatogram. In the third yield of Bl73 fractions (see Fig. 14, page 37) the peaks occurred premature in comparison to the first and second yield (see Fig. 12, page 36). Eventually this explains the lack of substance 495.2786 in F11_2.

As discussed previously, the substance was one of the most relevant compounds in Fraction 10 (F10 and F10_2; see Fig 38). On account of these findings, it was suggested that the substance with mass 495.2825/495.2786 [m/z] is one substance of interest in Fraction 11.

As already mentioned, the determined formula for the substance in F11 was $C_{27}H_{40}N_2NaO_5$, whereas for F11_2 the proposed formula was $C_{23}H_{43}O_{11}$.

Mass Peak at 26.0 min

Extracted mass $[M+Na]^+$ 493.2576/493.2530 [m/z]

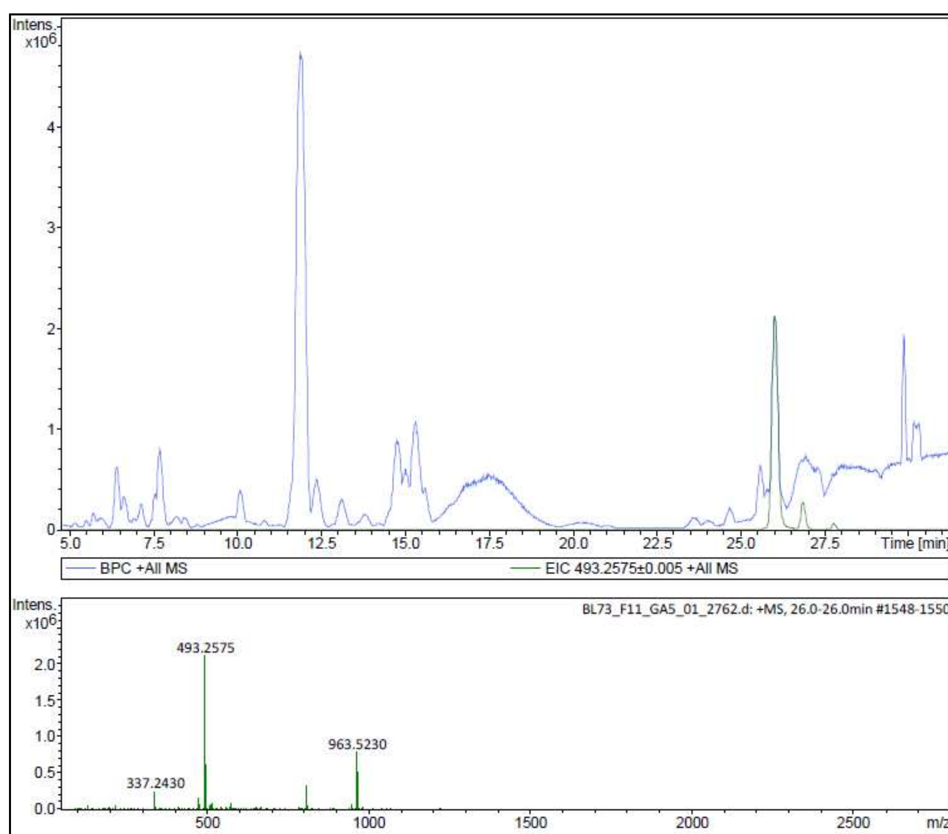

Fig.45: Section from LCMS run of F11 with the EIC of mass 493.2576+/- 0.005, the lower part of the figure displays mass 493.2576 [m/z].

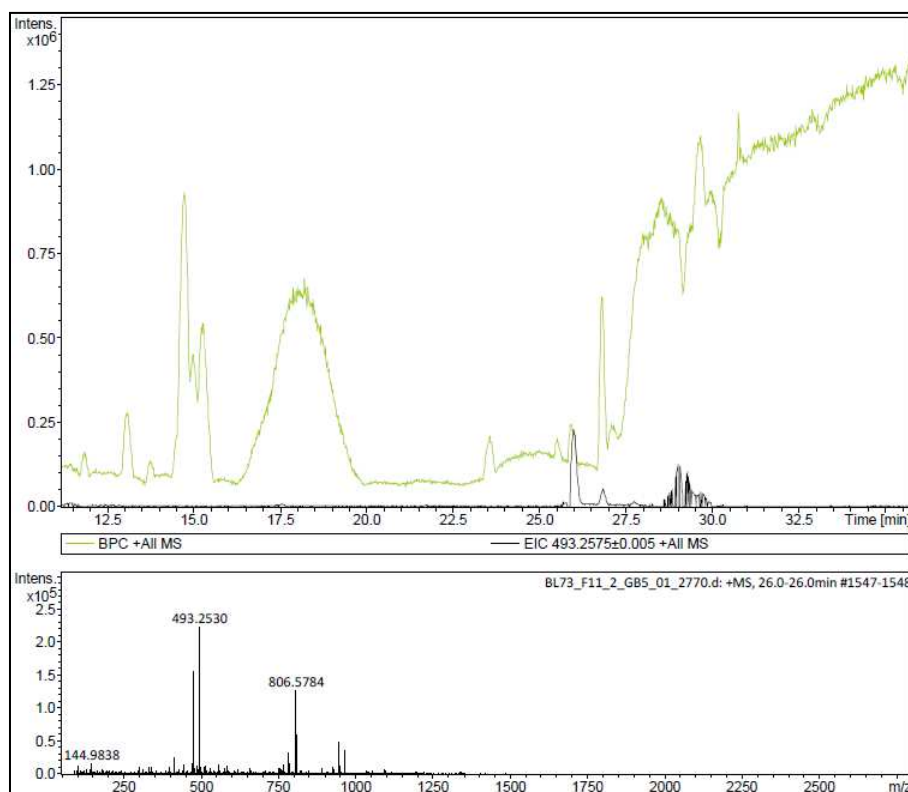

Fig.46: Section from LCMS run of F11_2 with the EIC of mass 493.2576+/- 0.005, the lower part of the figure displays mass 493.2533 [m/z].

The detected mass of this substance was 493.2575 [m/z] and 493.2533 [m/z] in F11 and F11_2, respectively. It was apparent in F10 and F10_2 with an intensity of $2*10^6$. The molecule was one of the most relevant molecules of Fraction 10. The compound was also present in F8 (intensity = $5.5*10^4$), F8_2 (intensity = $2.1*10^4$), F9 (intensity = $3.8*10^4$), F9_2 (intensity = $2.4*10^4$), F14 (intensity = $4*10^4$) and F14_2 (intensity = $4.5*10^4$). However, these signals were negligible low. The molecule was not found in methanol - or rice blank.

The proposed formula for mass 493.2576 [m/z] and 493.2533 [m/z] was $C_{28}H_{38}NaO_6$ (scores 87.94 and 21.36, respectively).

Consequently, this mass was also seen as possible substance of interest because it eventually was a derivate of the component with mass 495.28 [m/z]. Eventually the molecule with mass 493.25 [m/z] might be a derivate of] 495.28 [m/z] without two H^+. Consequently, the substances should have similar formulas and the most appropriate overall formulas (due to scores) for the compounds with mass 495.28 [m/z] and 493.25 [m/z] were $C_{23}H_{36}N_8NaO_3$ and $C_{23}H_{34}N_8NaO_3$, respectively.

Summing up LCMS of Fraction 11, the most important substances of interest were the compounds with masses 495.2825/495.2788 [m/z] and 493.2576/493.2533 [m/z]. LCMS of Fraction 11 outlined that the substance with mass 495.28 [m/z], that was existent in Fraction 10, was also located here. During the analysis it became obvious that there were differences between LCMS results of F11 and F11_2. While F11 comprised a large amount of the particular substance F11_2 nearly lacked it.
Similar to the substance with mass 495.28 [m/z], mass 493.25 [m/z] was also found in F11 and Fraction 10 (F10 and F10_2), albeit only a minor amount was found in F11_2.
On account of this Fraction F11_2 was tested on *B. subtilis*, which lacked the inhibition of organism. This was proof that the activity of Fraction 11 was bound to one of these substances.

3.5.6 BI73 Fraction 14 - LCMS run analysis

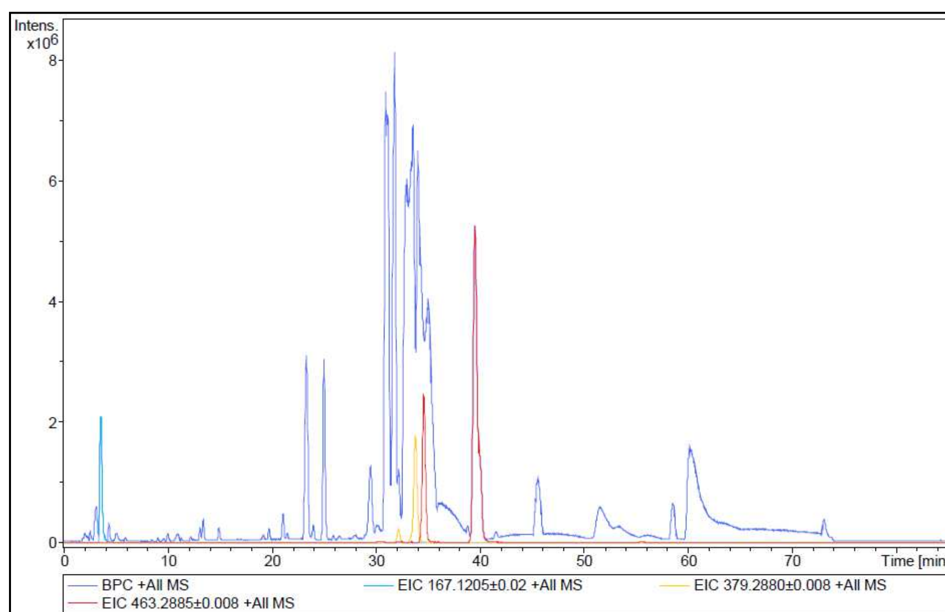

Fig.47: Complete LCMS run of F14 with most relevant EICs.

As it can be seen in Fig. 47 and 48 the LCMS analysis of Fraction 14 revealed a lot of masses. In Fraction 14 the substances with mass [M+Na$^+$] 167.1205/167.1177 [m/z], mass [M+Na$^+$] 379.2880/379.2930 [m/z] and mass [M+Na$^+$] 463.2934/ 463.2885 [m/z] were considered as relevant molecules. They were considered as relevant molecules because they were not found in other fractions than in F14 or F15.

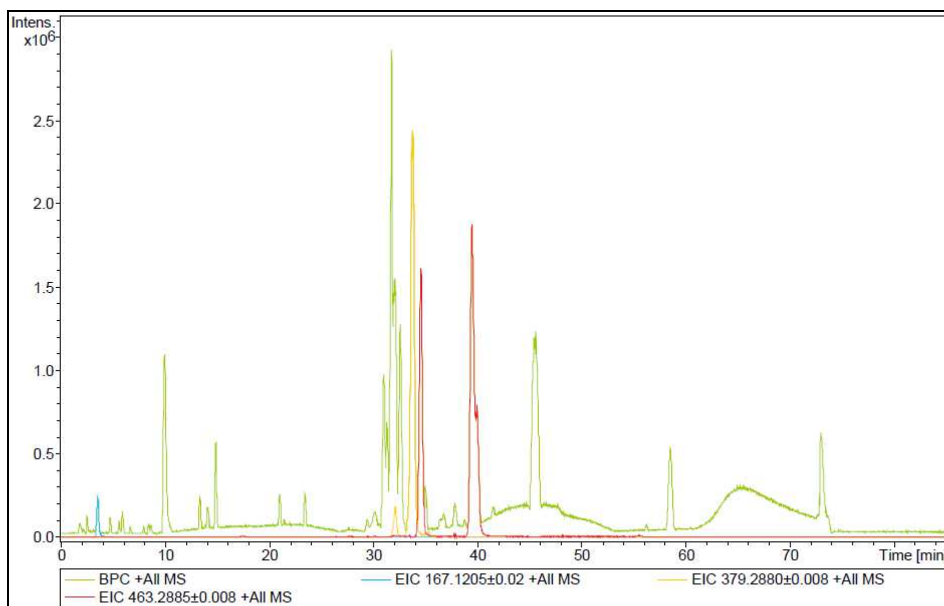

Fig.48: Complete LCMS run of F14_2 with most relevant EICs.

Mass peak at 3.6 min

Extracted mass $[M+Na]^+$ 167.1205/167.1177 [m/z]

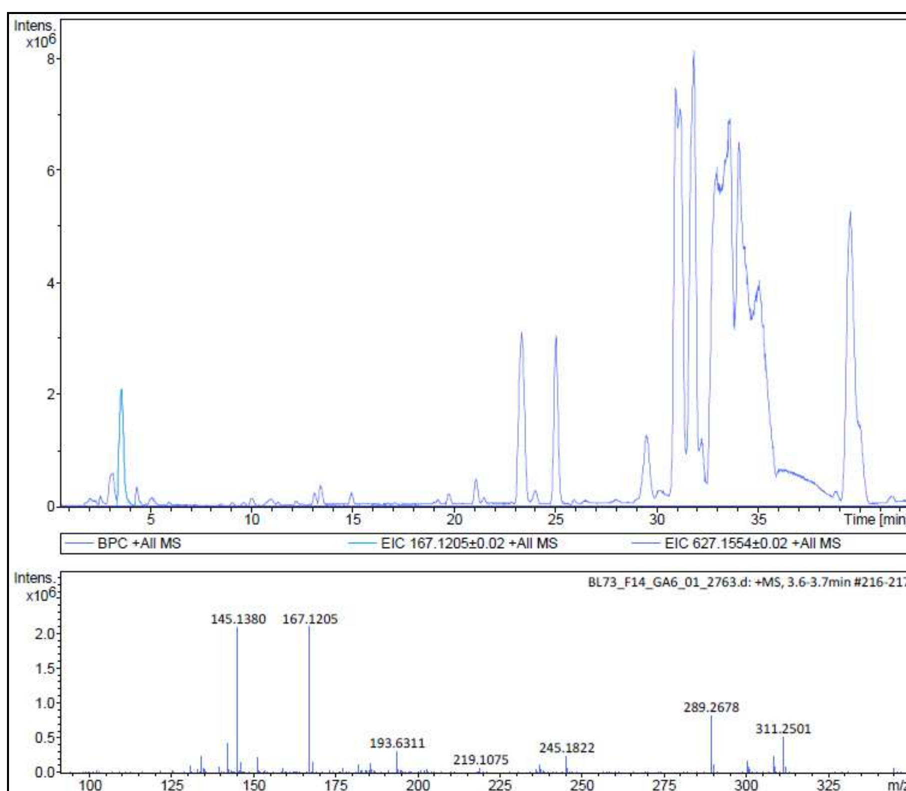

Fig.49: Section from LCMS run of F14 with the EIC of mass 167.1205 +/- 0.02, the lower part of the figure displays mass 167.1205 [m/z] and 145.1380 [m/z].

The substance with mass 145.1380 [m/z] was the compound with mass 167.1205 [m/z] without sodium but with H^+. The signal intensity for the molecule 167.12 [m/z] and 145.13 [m/z] in both F14 and F15 was equal. In F14_2 and F15_2 the substance with mass 167.12 [m/z] was about two times higher than the corresponding mass 145.13 [m/z]. This substance also occurred in Fraction 15 (in F15 the intensity was $1.9*10^6$; in F15_2 the intensity was $5.9 *10^5$). The molecule did not absorb at 190 nor 254 nm and it was not found in methanol - or rice blank or in other fractions. As the molecule appeared in Fraction 14 and 15 the overall fitting formula for the compound was $C_7H_{16}N_2NaO$ (scores 85.66, 100, 79.55 and 100 for 167.1205 [m/z] (F14), 167.1177 [m/z] (F14_2), 167.1206 [m/z] (F15) and 167.1175 [m/z] (F15_2), respectively).

Mass peak at 33.7 min

Extracted mass $[M+Na]^+$ 379.2920/379.2880 [m/z]

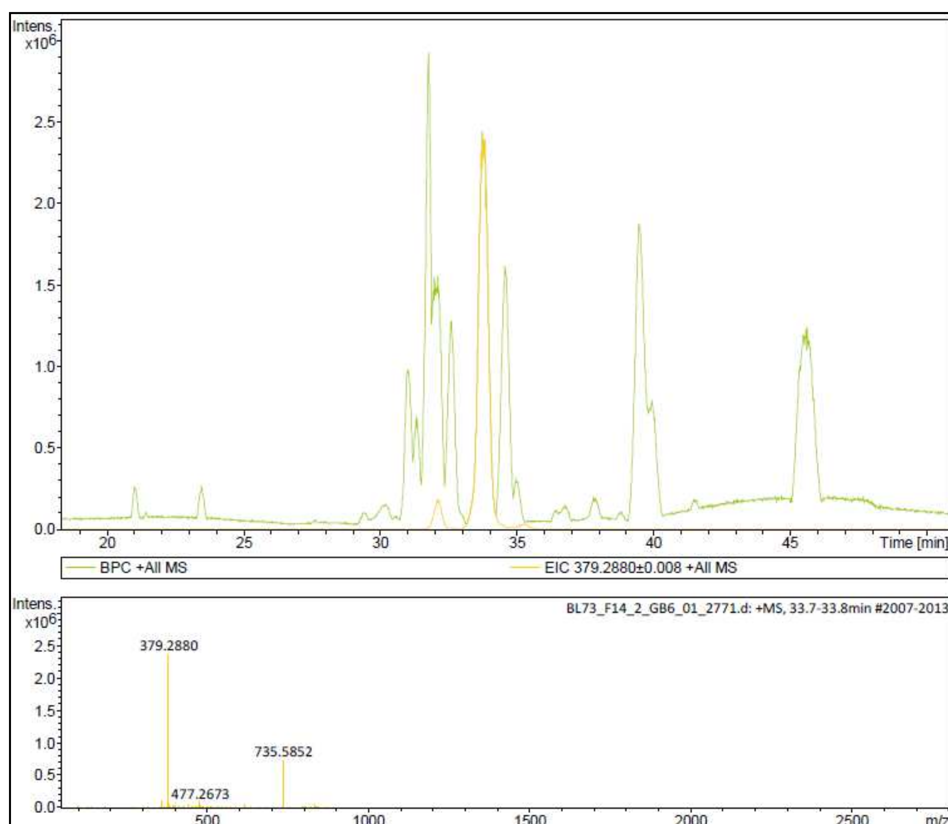

Fig.50: Section from LCMS run of F14_2with the EIC of mass 379.2880+/ - 0.008, the lower part of the figure displays mass 379.2880 [m/z].

Mass 379.2920 [m/z] and mass 379.2880 [m/z] were present in F14 and F14_2, respectively.
This mass was a dominant mass in F14_2 (intensity = 2.4* 10^6), but it also existed in F14 (intensity = 1.9*10^6).
The substance with the mass 379.2880+/-0.008 [m/z] was found in F14 and F15 with intensity of 1.9*10^6 and 1.4*10^6, respectively. It was not apparent in other fractions. The best fitting overall formula for the molecule in F14, F14_2 and F15 with the masses 379.2920 [m/z], 379.2880 [m/z] and 379.2926 [m/z] was $C_{16}H_{36}N_8NaO$ (scores 90.03, 100 and 44.32, respectively). The substance was unique for Fraction 14 and F15, thus it was considered as a possible antimicrobial component.

Mass peaks at min 34.3/39.2 min

Extracted mass $[M+Na]^+$ 463.2934/463.2885 [m/z]

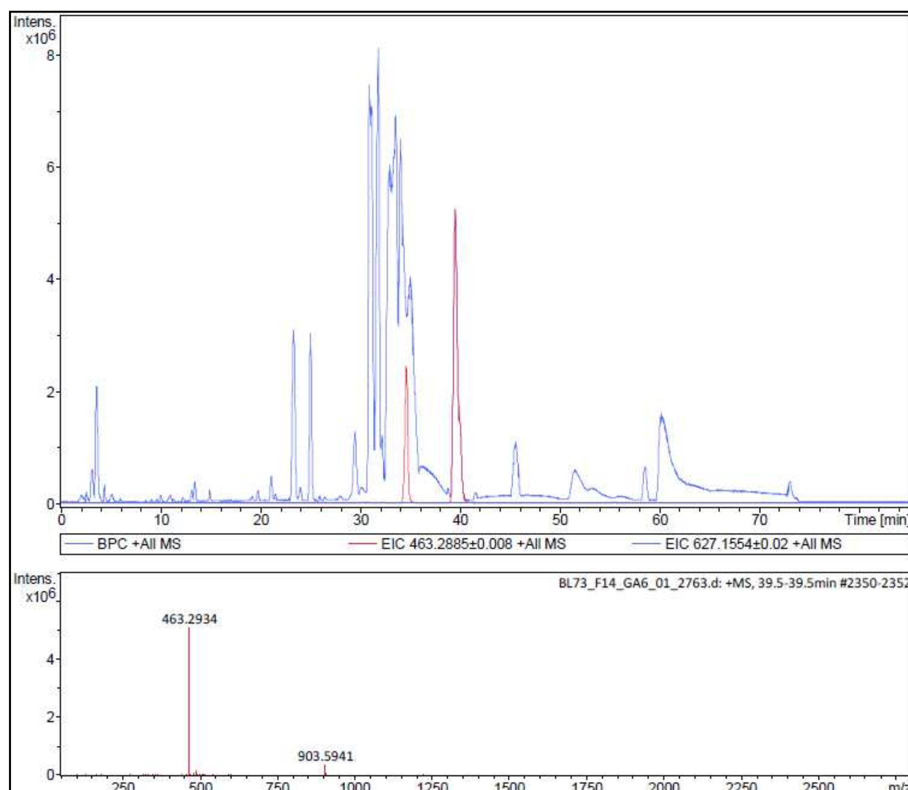

Fig.51 Complete LCMS run of F14, with the EIC of mass 463.2885+/ - 0.008, the lower part of the figure displays mass 463.2885 [m/z].

This mass was detected in F14 (463.2934 [m/z]) and F14_2 (463.2885 [m/z]). It was one of the mass peaks with the highest intensity in F15 (intensity = 2.5*10^6), albeit a

small in F15_2 (intensity = $4*10^5$). A minor signal was seen in F3_2 (intensity = $1.25*10^4$), in all other fractions it was not apparent. As it can be seen in Fig. 51 it occured as two mass peaks in the chromatogram. It was not found in rice - or methanol blank. The proposed formula for the mass 463.2934 [m/z] (F14) and 463.2946 [m/z] (F15) was $C_{27}H_{40}N_2 NaO_3$ (scores 100 and 96.03), whereas for 463.2885 [m/z] (F14_2) and 463.2866 [m/z] (F15_2) the formula $C_{21}H_{44}NaO_9$ was determined (scores 93.71 and 100, respectively).
The substance occurred in Fraction 14 and 15 and thus, like the other discussed substances of Fraction 14, it was considered as possible antimicrobial compound. Generally, the entire fraction showed low UV absorbance in comparison to other fractions.

Fig. 52 demonstrates that the UV 190 nm absorbance of many substances within F14 in comparison to components in F10 (Fig.53) is low.

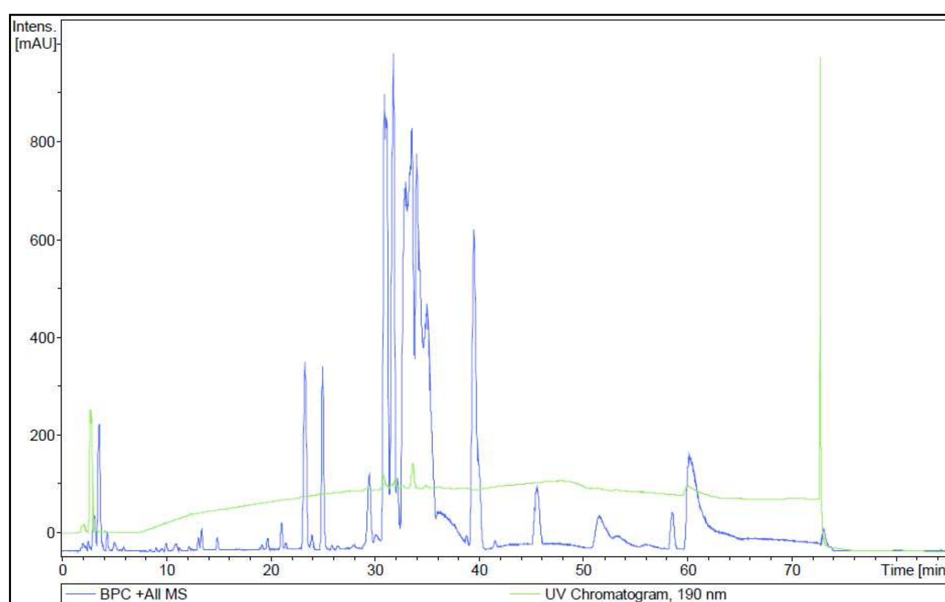

Fig.52: Complete LCMS run of F14 with relevant EICs and UV Chromatogram, 190nm

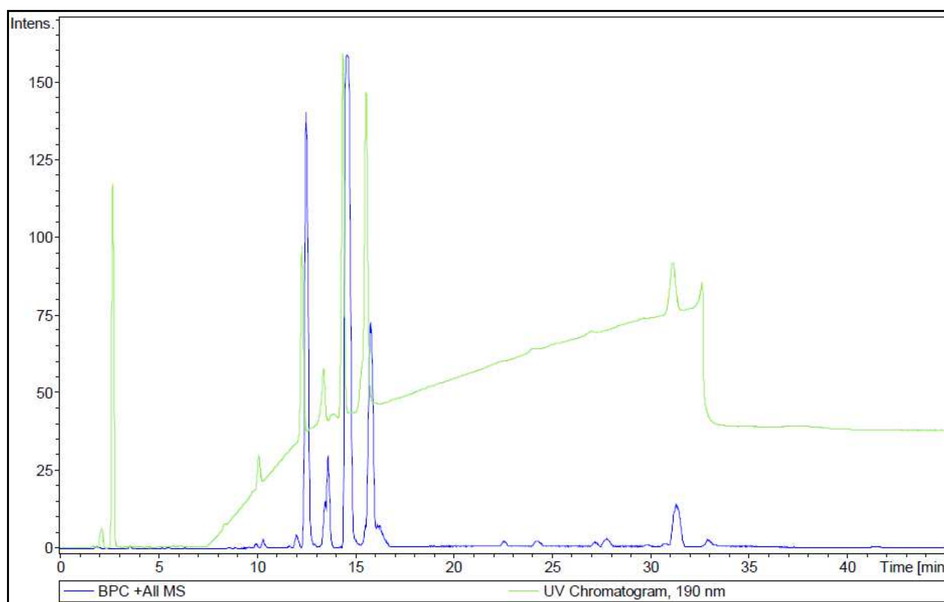

Fig.53: Complete LCMS run of F10 with relevant EICs and UV Chromatogram, 190nm

Because the UV absorbance in Fraction 14 was low, a further time based fractionation of it was chosen in order to separate the compounds.

3.5.7 BI73 Fraction 15 - LCMS run analysis

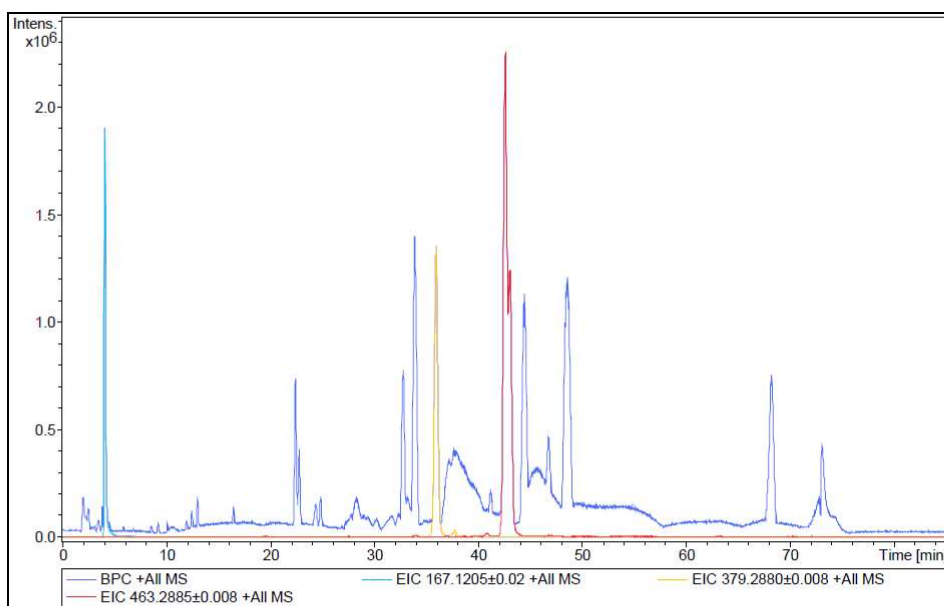

Fig.54: Complete LCMS run of F15 with most relevant EICs.

Equal to Fraction 14 LCMS analysis of Fraction 15 comprised a high number of various substances. The experiments revealed the molecules with mass

[M+Na^{+}]167.1207/167.1157 [m/z], mass [M+Na]$^{+}$ 379.2926 [m/z] and mass [M+H]$^{+}$463.2946/463.2866 [m/z]. The substances were considered to be relevant, because they were present in Fraction 14 and 15.

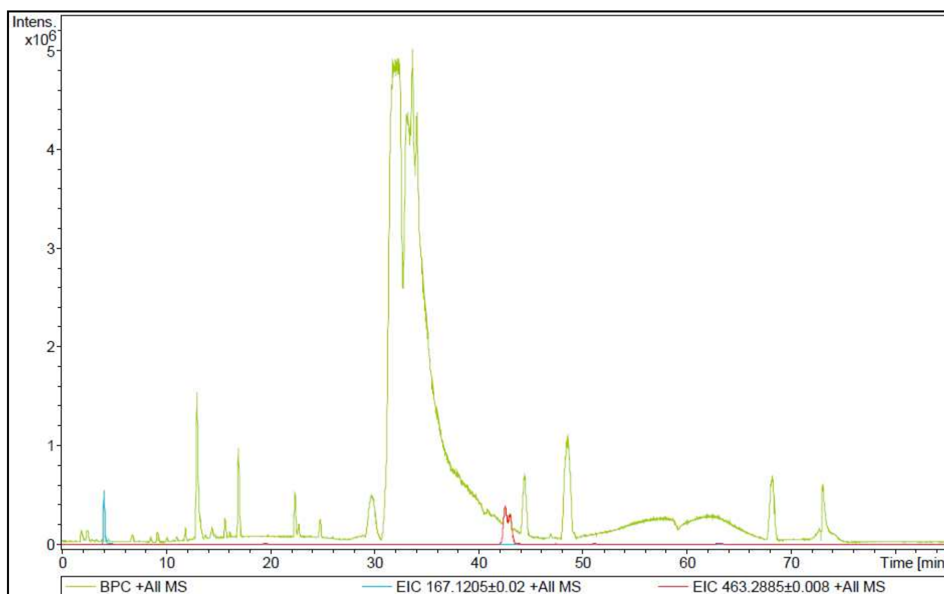

Fig.55: Complete LCMS run of F15_2 with most relevant EICs.

Mass peak at 4.1 min

Extracted mass [M+Na]$^{+}$ 167.1207/167.1157 [m/z]

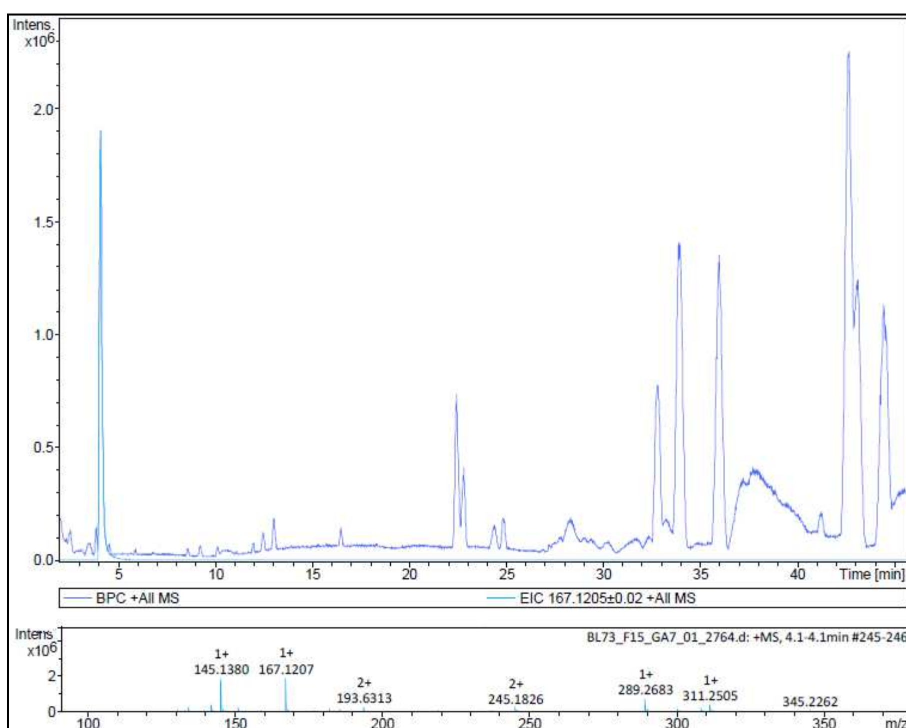

Fig.56: Section from LCMS run of F15 with the EIC of mass 167.1205 +/- 0.02, the lower part of the figure displays mass 167.1205 [m/z] and 145.1380 [m/z].

The masses present in F15 and F15_2 were 167.1207 [m/z] and 167.1157 [m/z], respectively.
The substance with mass 145.1380 [m/z] was the compound with mass 167.1207 [m/z] without sodium but with H^+. The substance was already discussed in Fraction 14.

Mass peak at 35.9 min

Extracted mass $[M+Na]^+$ 379.2926 [m/z]

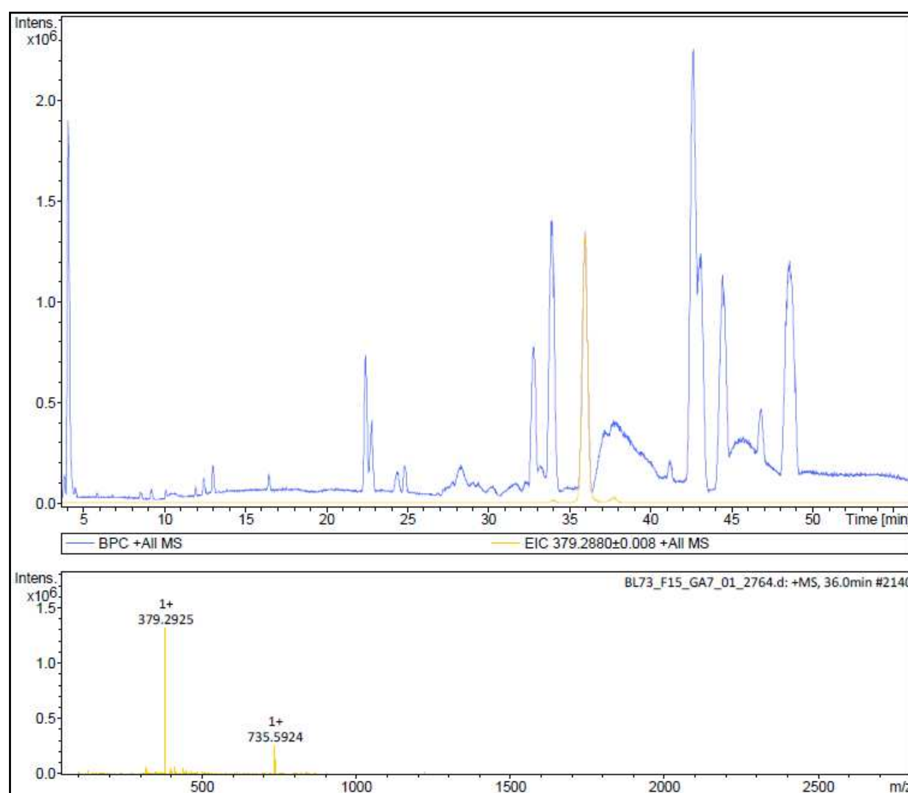

Fig.57: Complete LCMS run of F15 with the EIC of mass 379.2880+/- 0.008, the lower part of the figure displays mass 379.2925 [m/z].

This substance with mass 379.2926 [m/z] was not found in F15_2 but in F14 and F14_2 and was already discussed at page 71.

Mass peak at min 42.6

Extracted mass $[M+H]^+$ 463.2946/ 463.2866 [m/z]

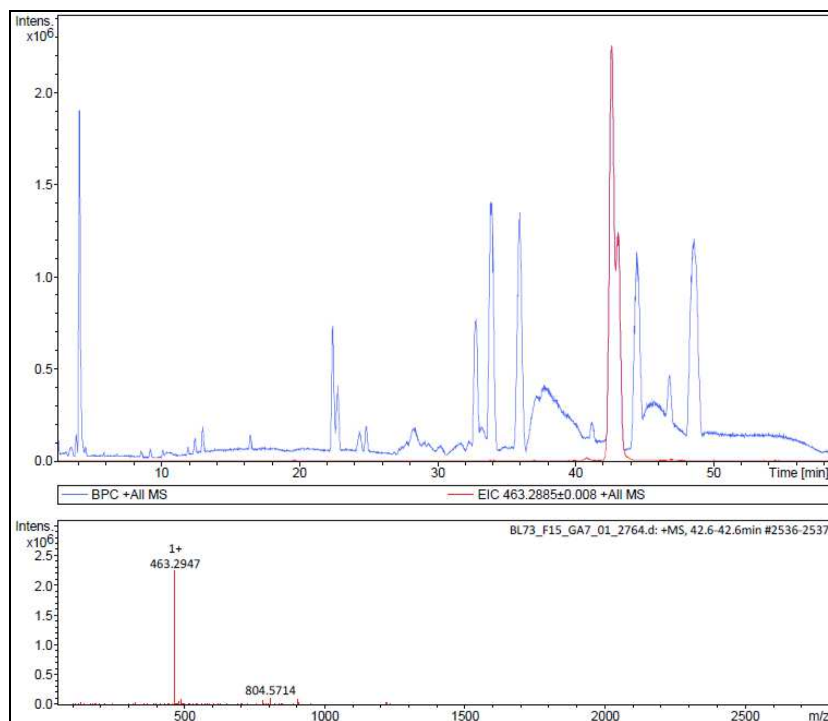

Fig.58: Complete LCMS run of F15 with the EIC of mass 463.2885±0.008, the lower part of the figure displays mass 463.2946 [m/z].

Mass 463.2946 [m/z] and 463.2866 [m/z] was determined in F15 and F15_2, respectively. It was also present in Fraction 14, where the molecule appeared as two mass peaks in the chromatogram (see Fig. 51 page 71). In Figure 58 from F15 the molecule was found as one single mass peak. The compound was already discussed at page 72 and as it occurred in Fraction 14 and 15 an antimicrobial effect was considered.

Summing up LCMS of Fraction 14 and 15 showed that these fractions comprised a large number of mass peaks and the analysis revealed the substances with mass 167.12 [m/z], 379.29 [m/z] and 463.29 [m/z] that were supposed to be active compounds, because they occurred in both fractions. All other masses which occurred in Fraction 14 and 15 were not relevant, because they were not present in both Fractions 14 and 15.

Furthermore it was also considered that in these two fractions fatty acids could be responsible for the antibacterial effect. Fatty acids are commonly known for antimicrobial activities [McGaw *et al.*, 2002; Desbois and Lawlor, 2013]. Furthermore they are often isolated within bioassay-guided fractionation of plant extracts [McGaw *et al.*, 2002]. Therefore, an LCMS analysis of F15 in negative mode in order to detect fatty acids was performed.

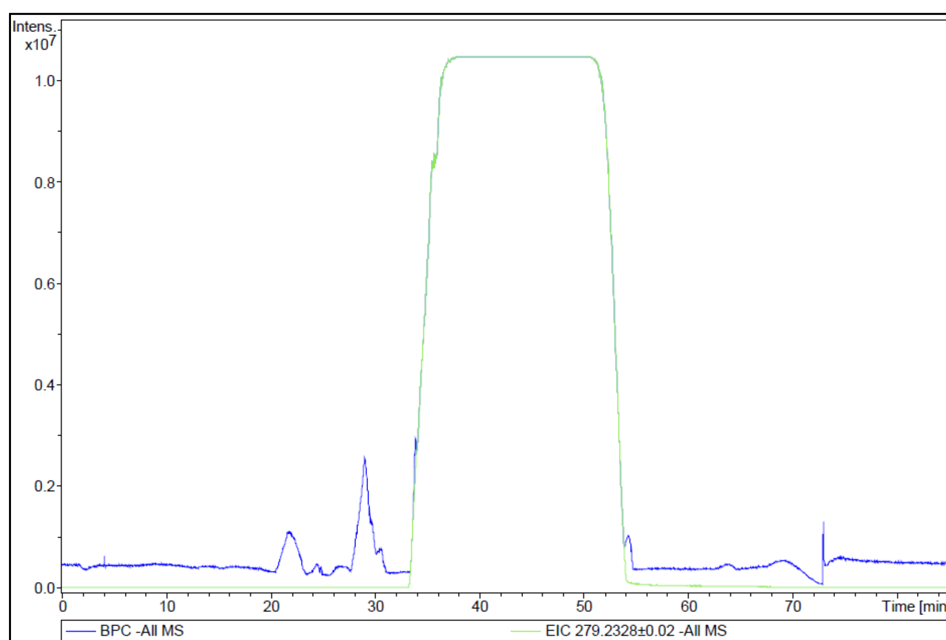

Fig.59: Complete LCMS run of F15 with the EIC line 279.2328+/- 0.008.

Fig.59 indicates a high prevalence of the substance with mass 279.2328 [m/z] from 33 to 54 minutes. The proposed formula was $C_{18}H_{31}O_2$, which was determined as fatty acid. The molecule eventually is linolenic acid [source: Pubchem; PubChem CID 5280450], which will be discussed later, see page 97.

Other relevant molecules with high prevalence that were found, were masses 255.2307 [m/z] and 283.2626 [m/z].

The compound with mass 255.2307 [m/z] was detected at minute 24 (intensity $=2.8*10^4$), minute 28 (intensity $= 3*10^4$) and minute 29 (intensity $= 3*10^4$), which were negligible amounts. At minute 33-35 an intensity of $2.3*10^6$ and at minute 52-54 (intensity $= 6*10^5$) was apparent. The substance was also detected at minute 62-64 (intensity $= 7.1 *10^4$) and 67-69 (intensity $= 5*10^4$). The predicted formula for 255.2307 [m/z] was $C_{16}H_{31}O_2$**.** The molecule eventually is palmitic acid [source: Pubchem; PubChem CID 985]

The substance with mass 283.2626 [m/z] was apparent at minute 24 (intensity = $1.7*10^4$), minute 62-64 (intensity = $4.3*10^5$) and minute 67-69 (intensity = $3.5*10^4$]. The proposed formula for the substance was $C_{18}H_{35}O_2$. The compound eventually is stearate [source: Pubchem; PubChem CID 3033836].
Similar to Faction 14, as the UV absorbance was low, a time base fractionation method was selected for the subsequent work on Fraction 15.

3.6. Subfractionation of BI73 fractions

3.6.1 Subfractionation of BI73 Fraction 10

3.6.1.1 Analytical HPLC of BI73 Fraction 10

In order to isolate the substance of interest Fraction 10 was chosen for the semi - preparative HPLC runs. However, for the semi - preparative work a better baseline separation between the UV peaks was necessary. Hence, analytical HPLC was done prior to the semi - preparative work. The analytical HPLC runs were done with F10_2.

To achieve a better baseline separation the flow rate was decreased from 1 mL/min to 0.5 mL/min. The time program was the gradient for Fraction 10 obtained in former experiments (see page 43). The experimental setup from former analytical runs of BI73 extract stayed the same.

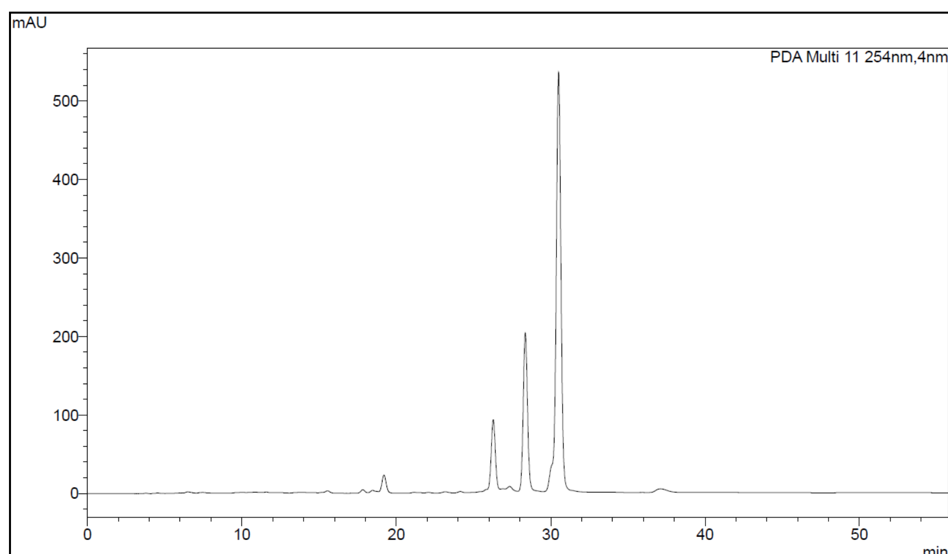

Fig.60: Analytical HPLC run of F10_2, flow rate 0.5 mL/min.

Comparison to a chromatogram of F10_2 with flow rate with 1 mL/min:

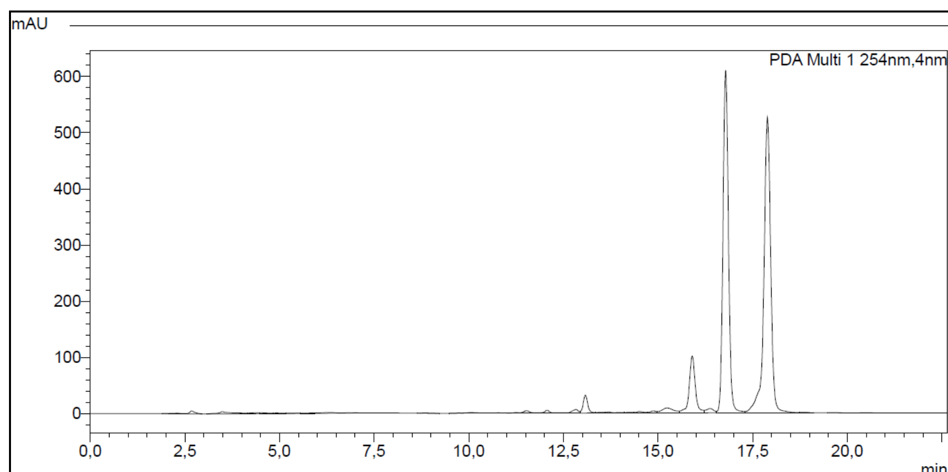

Fig.61: Analytical HPLC run of F10_2, flow rate 1 mL/min.

In Fig. 60 with a flow rate of 0.5mL/min the baseline separation of the last two peaks increased for about two times in comparison with the same sample in Fig. 61 with a flow rate of 1 mL/min. This was predominantly important for the last two peaks in the chromatogram, because the last peak resembled the substance with mass 495.28 [m/z]. In order to isolate the compound as pure substance a good baseline separation was needed.

3.6.1.2 Semi - preparative HPLC and subfractionation of BI73 Fraction 10

Semi - preparative HPLC runs of Fraction 10 were done with F10 and F10_2. The time program was the gradient for Fraction 10 obtained in former experiments (see page 43). The flow rate was decreased to 10 mL/min.

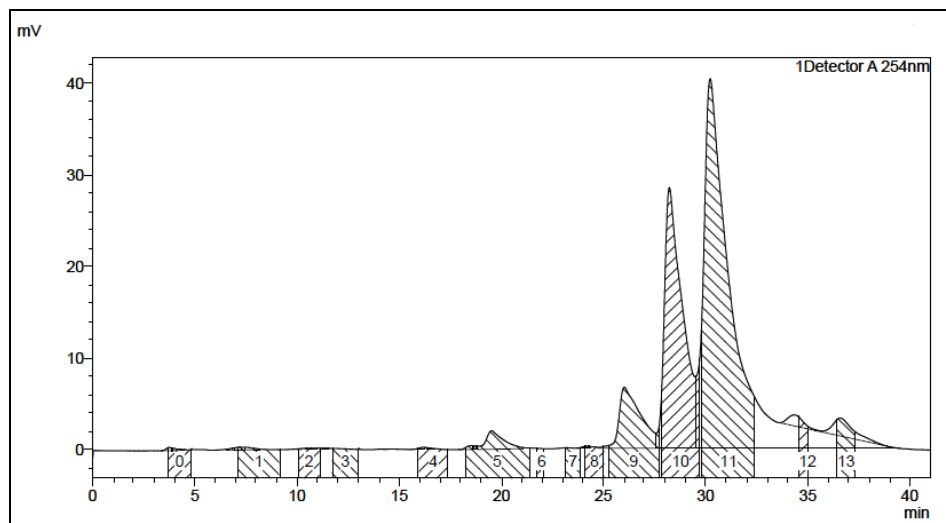

Fig.62: Semi - preparative HPLC run of F10_2, second BI73 extract, 600 µL injection volume, flow rate 10 mL/min.

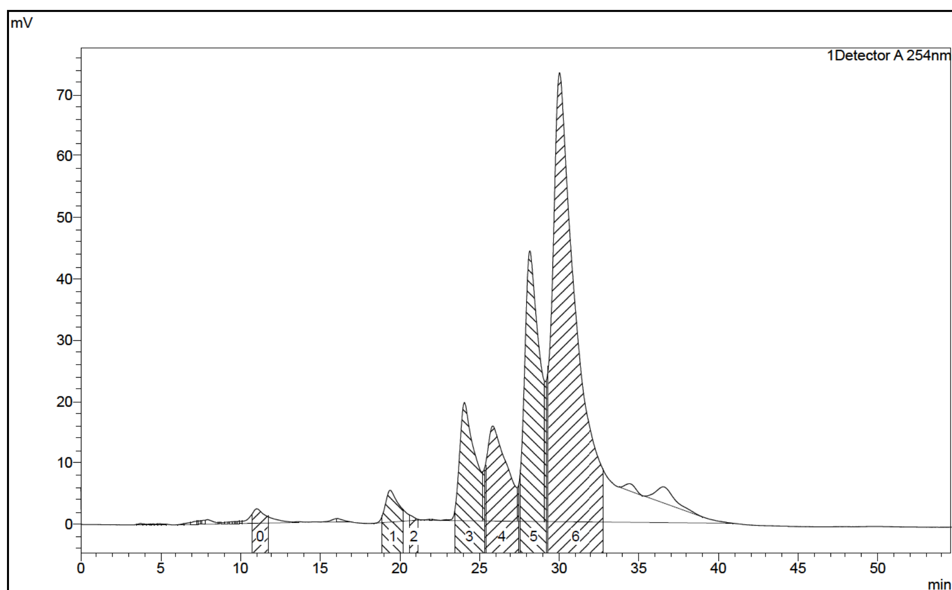

Fig.63: Semi -preparative HPLC run of F10, second BI73 extract, 1600 µL injection volume, flow rate 10 mL/min.

Fig. 62 and Fig. 63 show the results of the semi - preparative HPLC runs. The relevant peaks that were collected within F10_2 were Subfraction 9, 10 and 11 which occurred at about 25 min of the chromatogram. For F10 relevant collected peaks were Subfractions 3, 4, 5 and 6, which occurred at about 23.5 min of the chromatogram. Although the flow rate was decreased to 10 ml /min, the baseline separation did not comply with the one in analytical HPLC runs (see Fig. 60).

The main peaks collected for F10_2:
Subfractions 9, 10 and 11 were named F10.9, F10.10 and F10.11, respectively.

The main peaks collected for F10:
Subfractions 3, 4, 5, 6 were named F10.3, F10.4, F10.5 and F10.6, respectively.

3.6.1.3 Weights of subfractionation of BI73 Fraction 10

Tab.27: Weights of F10_2 Subfractions

	Weight [mg]
F10.9	1.7
F10.10	1.7
F10.11	2.7

Tab.28: Weights of F10 Subfractions

	Weight [mg]
F10.3	1.0
F10.4	3.0
F10.5	1.8
F10.6	1.0 mg

Tab. 27 and 28 depict the yields of the subfractionation of Bl73 F10 and F10_2. After the yield the samples were stored dry at -20°C.

3.6.1.4 Analysis of Direct injection MS of Bl73 Subfraction F10.11

The direct injection to MS of the gained subfractions was done to verify that the molecule of interest was collected. For direct injection F10.11 was dissolved into 1ml methanol to a concentration of 2.7 µg/µL and diluted to 1:500 with methanol. Subsequently the sample was dried by rotary evaporation and again stored dry at -20°C.

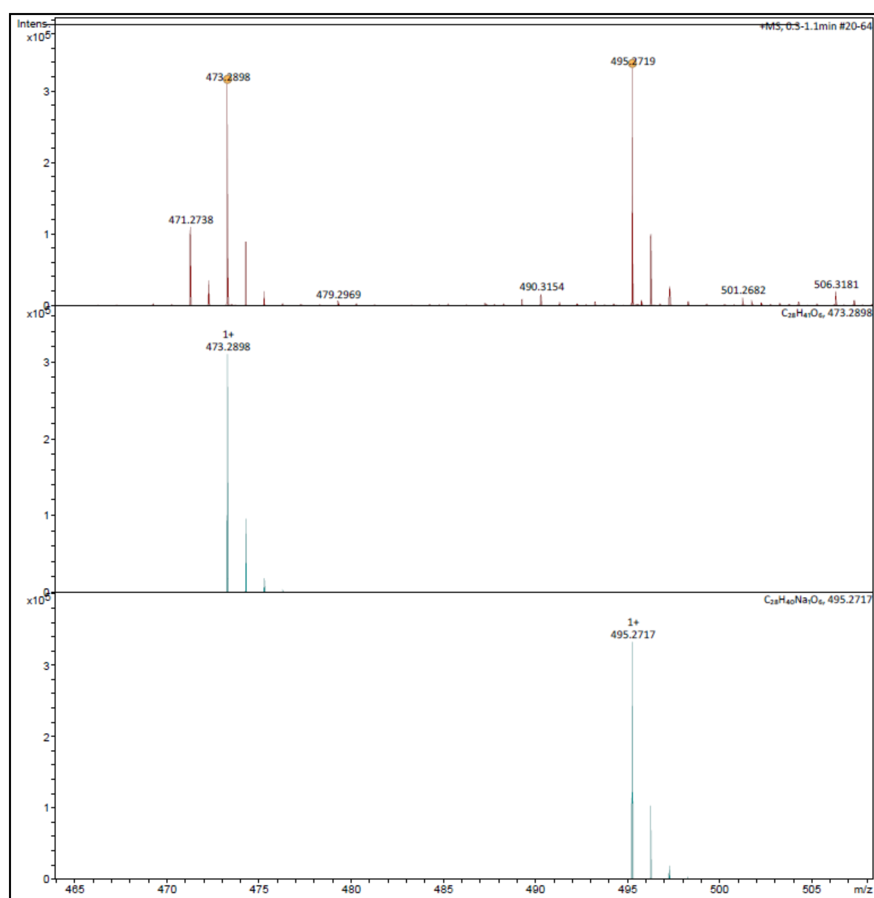

Fig.64: Direct Injection Mass spectrometry of Bl73 Subfraction F10.11.

Fig. 64 indicates that the substance of interest with mass $[M+Na]^+$ 495.28 [m/z] was yielded by the fractionation. It displays the compound with mass $[M+H]^+$ 473.2898 [m/z] which is the molecule of interest with H^+. The figure also outlines the substance with mass $[M+Na]^+$ 495.1719 [m/z], which is the molecule of interest with sodium.

The compound with mass 495.28 [m/z] was a powdery substance with yellow stain. Subsequently, the substance was analyzed by NMR (not part of this work). This analysis outlined that the substance with mass 495.28 [m/z] was a pure substance with unknown structure. Thereon the molecule was tested in disc diffusion assays for antimicrobial activity against *B. subtilis, S. cerevisiae*, *A. niger*, *F. graminearum and E. coli.* F10.11 was dissolved into 300 µL to a concentration of 9 µg/µL.
The substance was positively tested against *B. subtilis* with an inhibition zone of 0.75 mm radius. Thus a light inhibition was observed. The disc diffusion tests against the fungi and *E. coli* were negative, which however was expected, because Fraction 11 did not inhibit them.

3.6.1.5 Analysis of Direct injection MS of BI73 Subfraction F10.3

Equal to the substance with 495.28 [m/z] a control of the substance with mass 323.06 [m/z] was needed. For direct injection to MS F10.3 was dissolved into 1ml methanol to a concentration of 1 µg/µL and diluted to 1:500 with methanol. Subsequently the sample was dried by rotary evaporation and again stored dry at -20°C.

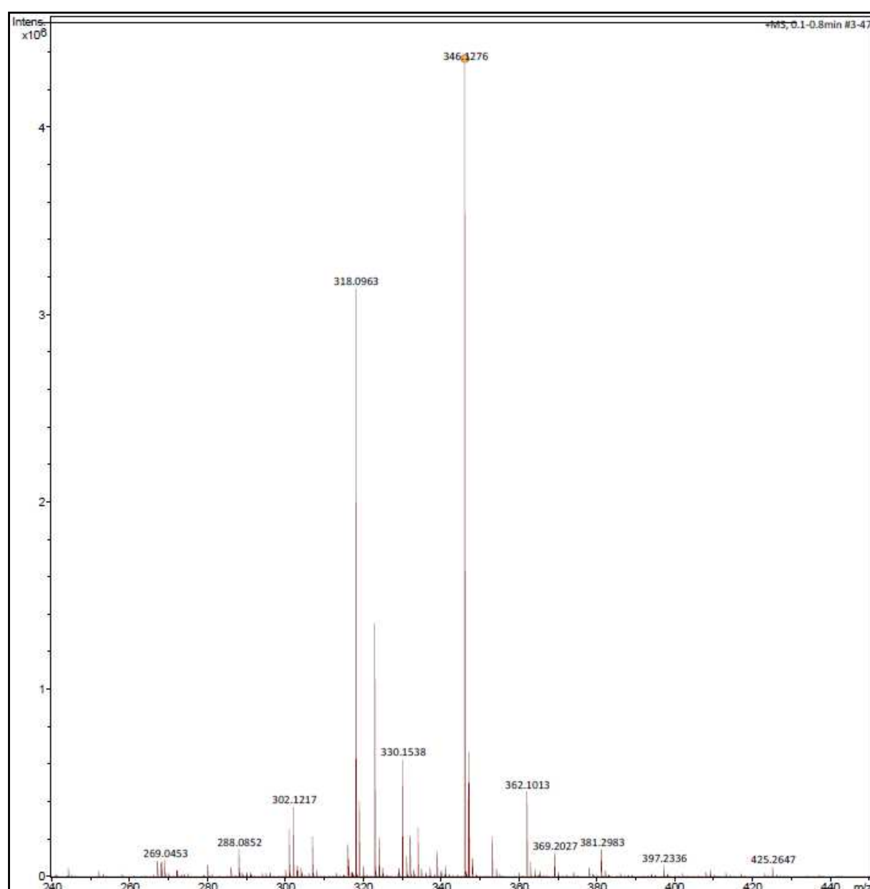

Fig.65: Direct Injection Mass spectrometry of BI73 Subfraction F10.3.

Fig. 65 indicates that the molecule of interest with mass $[M+Na]^+$ 323.06 [m/z] was yielded by the fractionation. The figure, however, displays the compound with mass 346.1276 [m/z]. This is the mass $[M+Na]^+$ 323.06 [m/z] with an additional sodium.

The isolation of the component resulted in an oily, slightly brownish substance. Subsequently, the substance was tested in disc diffusion assays for antimicrobial activity against *B. subtilis*, *S. cerevisiae*, *A. niger* and *F. graminearum.* For disc diffusion assays F10.3 was dissolved into 300 µL to a concentration of 3 µg/µL. The substance was tested positively with an inhibition zone of 1 mm radius for *S. cerevisiae*, *A. niger* and *F. graminearum*. Thus, a light inhibition was observed and the antimicrobial effect was not as good as for Fraction 10. Furthermore the inhibition of *A. niger* and *F. graminearum* was only visible for some days. That means the concentration of substance with mass 323.06 [m/z] was too less, or it was a volatile compound. At the end of this Diploma Thesis no information was already available about the pureness of the substance and NMR structure analysis.

In context with this work the other collected subfractions of Fraction 10_2 (including F10.9 and F10.10) could not be isolated pure enough for NMR analysis. Furthermore in disc diffusion assays no inhibition was observed. Consequently the concentration of the subfractions was too low or the compounds within in these subfractions did not have antimicrobial activities. At the end of this Diploma work no information about NMR results of subfractions of F10 (including F10.4, F10.5) was already available and the compounds were not tested in disc diffusion assays.

3.6.2 Subfractionation of BI73 Fraction 14 and 15

3.6.2.1 Semi - preparative HPLC and subfractionation of BI73 Fraction 14/15

For BI73 Fraction 14 and 15 no prior analytical runs were needed.

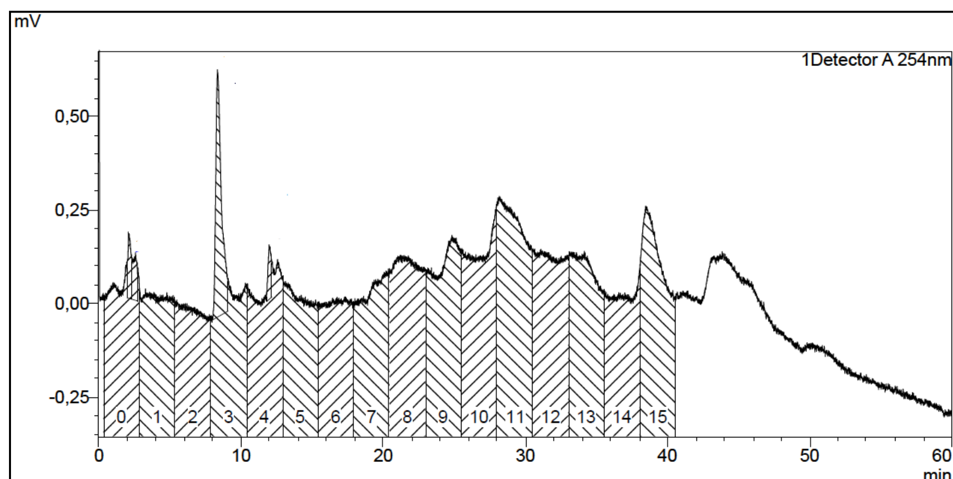

Fig.66: Semi - preparative HPLC run of F15, second BI73 extract, 800 µL injection volume, flow rate 20 mL/min.

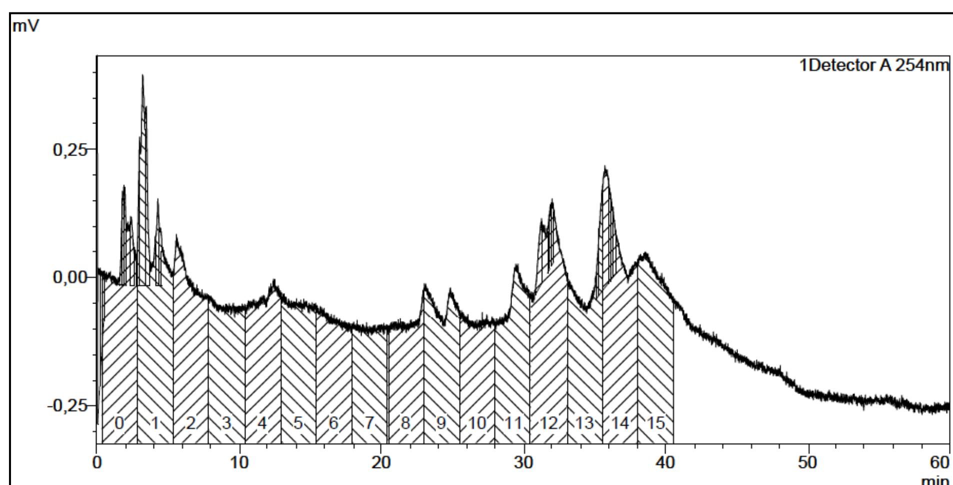

Fig.67: Semi - preparative HPLC run of F14, second BI73 extract, 500 µL injection volume, flow rate 20 mL/min.

Fig. 66 and 67 illustrate the subfractions collected by semi - preparative HPLC. 15 subfractions were collected. The UV absorbance of the compounds within Fraction 14 and 15 is low. As example in Fig. 66 the Subfraction 3 had the highest absorbance of the whole chromatogram (about 0.6 mV). For comparison the Subfraction 10.6 in Fig 63 at page 80 had an absorbance of 70 mV.

3.6.2.2. Disc diffusion assays of subfractionation of BI73 Fraction 14 and 15

Tab.29: Results of disc diffusion assays of Fraction14 subfractions, (data provided by Mag. Dr. Martina Oberhofer).

F0	-	**F5**	-
F1	-	**F6**	-
F2	-	**F7**	-
F3	-	**F8**	+/-
F4	-	**Methanol**	-

Tab.30: Results of disc diffusion assays of Fraction15 subfractions,+.... radius = 0.75mm

F0	-	**F5**	-
F1	-	**F6**	-
F2	-	**F7**	-
F3	-	**F8**	+
F4	-	**Methanol**	-

For Fraction 14 and 15 another time based fractioning procedure was chosen due to the minimal UV absorbance of the compounds located in these fractions. As a result Subfraction F15.8 was tested positively in disc diffusion tests against *B. subtilis,* while F14.8 remained unclear. The effect, however, was not as good as in Fractions 14 and 15 as the inhibition zone was smaller. The according subfractions were collected by minute 40 to 70 and the LCMS analysis of F15 in negative mode demonstrated that fatty acids are present in this section. Eventually the concentration of the fatty acids in Subfraction F15.7, which extends from minute 35 until minute 40 of the run, was not high enough as a particular fatty acid was predominantly found after minute 33.

A special problem that occurred was the reduced solubility after the subfractionation. While Fractions 14 and 15 were easily dissolved in methanol, subfractions, especially dried by lyophilisation, were nearly insoluble. As a conclusion the compounds within Fraction 14 and 15 probably enhanced their own solubility.

As a consequence the analysis and the procedure of cleaning up the subfractions would not pay off as on the one hand the effect was worse compared to according

fractions and on the other hand it might be fatty acids responsible for the antibacterial effect. Fatty acids are considered to be used as therapeutic antimicrobial agents [Desbois and Lawlor, 2013], however the aim of this work was to identify new antimicrobials and thus the work on F14 and F15 was stopped.

4. Discussion

4.1 Endophytes and test organisms of this work

Natural sources as plants or microorganisms often provide new bioactive compounds and thus many reports about isolation of various biomolecules exist [Balouir *et al.*, 2016]. Fungal endophytes can provide new antimicrobial agents [Gunatilaka, 2006] and due to the issue of rising resistances to antibiotics [Cantas *et al.*, 2013], this Diploma Thesis dealt with the discovery of new antimicrobials from fungal endophytes.

The fungal endophytes from *Preussia* sp., *Nemania* sp. and Helotiales order were used to gain extracts that were essential for this work. *Preussia* sp. was reported to be a relative unexplored fungal genus [Mapperson *et al.*, 2014]. Mapperson *et al.*, 2014 published their results about antimicrobial active *Preussia* sp., which originated from Australian Dry Rainforest. In comparison to this work the strains of *Preussia sp.* derived from *A. belladonna*. Gherbawy and Elhariry, (2016) also isolated antimicrobial active *Preussia* sp. from *Juniperus procera*.

The second fungal endophyte of this Diploma Thesis was a genus from *Nemania* sp. It was also isolated from *A. belladonna*. Recently Liu *et al.*, (2016) reported strains of this genus from traditional Chinese medicinal plant *Cephalotaxus hainanensis*, which showed antimicrobial properties.

The last fungal isolate belonged to Helotiales order. Unfortunately the genus could not be determined, which meant that it likely was an unknown species. The fungal strain was isolated from *Bergenia pacumbis* (Saxifragaceae). Helotiales involves several taxa and families [Wang Z *et al.*, 2006], which includes Lachnum *sp*. [Ye *et al*, 2006] - a genus with large antimicrobial potential [Stadler and Anke, 1993, Matsumoto *et al.*, 2011]. Stadler and Anke, (1993) isolated *Lachnum papyraceum* and Matsumoto *et al.*, 2011 isolated *Lachnum palmae* from *Livistona sp.,* which is a plant genus from Arecaceae [Carlile *et al.*, 2012].

As this work dealt with the discovery of new antimicrobial compounds, test organisms for antimicrobial activity tests were needed. The choice of microorganisms derived from certain requirements for the tests. The most important aspects for the choice of the test organisms were as follows: A Gram-positive and a Gram -negative bacterium was needed for the evaluation of the extracts, as the different structure of cell wall influences the activity of antimicrobial agents [Rosenthal, 2016]. Therefore *B. subtilis* was chosen as it is a Gram - positive bacterium and *E. coli* was selected as Gram - negative bacterium. Another test organism selected was *S. cerevisiae* since it is a eukaryote [Degrandi *et al.*, 2010] in order to get hints about possible cytotoxicity of the compounds.

A. niger was selected because it is a filamentous fungus, that spreads through high production of air-distributed conidia, [Hendrickx *et al*., 2012], whereas *F. graminearum* was taken due its growth through hyphae fragments [personal observation of Mag. Dr. Martina Oberhofer].

4.2 Bioactivity-guided fractionation

The experimental approach in context of this work included the extraction of cultivated fungal endophytes and the subsequent fractionation of the extracts. The extraction was performed with dichloromethane/methanol (2:1), as this mixture gained the best results in former extraction experiments within the Department of Pharmacognosy. Subsequently HPLC was used to separate compounds within this extract.

This main concept was based on bioactivity-guided fractionation, a method commonly used for the detection of novel plant derived products and drugs. By bioactivity-guided fractionation many secondary plant metabolites were analyzed. [Atanasov *et al.*, 2015] However, there are certain issues that have to be considered within this methodology.

Generally the procedure of creating an extract is cheap and does not consume much time. Nevertheless the choice of the extraction method is crucial as it can have a great impact on the composition of compounds within the extract and its biological effect. By the procedure of fractionation, inactive fractions are discarded and active fractions are subsequently separated into other parts. These subfractions in turn

must undergo activity tests too. Finally when the compound of interest is isolated pure enough, spectrometric methods are needed for structure analysis. Therefore the entire procedure consumes much time and money. Moreover it can also lead into the detection of already known substances with known biological effects [Atanasov *et al.*, 2015]. In turn it is also possible that a known structure leads into unknown bioactivities [Katiyar *et al.*, 2012].

Furthermore, as there are many compounds existent in extracts, there are often interactions between the molecules. Thus a highly active initial extract can result into the isolation of several low active components. This means a synergistical effect is observed [Atanasov *et al.*, 2015]. This was a possible effect within this Diploma work, which will be discussed more detailed later. If the single compounds fail the observed effect, a solution might be to develop the whole extract as a drug [Katiyar *et al.*, 2012].

Additionally when the concentration of the molecules in the extract is not high enough this can result into missing a bioactive effect. It was also reported, that molecules as chlorophylle or polyphenols can sophisticate those biological tests [Atanasov *et al.*, 2015]. Subsequently all results from bioactivity- and fractionations tests will be discussed in detail.

4.3 Ab1 extract

4.3.1 Disc diffusion assays of Ab1 extract

Preussia sp. served for the production of the Ab1 extracts and in the disc diffusion tests the provided and second extract showed activity against *B. subtilis.* However, no effect on the other test organisms could be observed. The second extract initially showed no activity, but this problem could be solved by concentrating the extract.

Gherbawy and Elhariry, (2016) reported different extracts from isolates of *Preussia* sp. to inhibit *S. aureus, Klebsiella pneumoniae, C. albicans* and *Fusarium solani*. Comparing the results to this Diploma Thesis the extracts fom Gherbawy and Elhariry, (2016) were additionally active against a Gram - negative bacterium (*Klebsiella pneumoniae)*, a yeast (*C. albicans)* and also against a species from *Fusarium* genus. Unfortunately the group did not test *B. subtilis.* However, the

extracts of Gherbawy and Elhariry, (2016) demonstrated a much stronger antimicrobial potential than the Ab1 extracts from this Diploma Thesis.
Another study that was done on *Preussia* sp. was the work of Mapperson *et al.,* (2014). The research team produced several extracts from *Preussia* sp., which originated from different host plants from the Australian Dry Rainforests. They reported the most effective extracts from this genus to inhibit *B. cereus, E. faecalis*, MRSA and *C. albicans*.

In comparison similarities can be found as the extracts from Mapperson *et al.,* (2014) and Ab1 showed antimicrobial activity against *Bacillus* sp. Moreover no inhibition of *E. coli* was observed.
In contrast to the Ab1 extracts the extracts of Mapperson *et al.,* (2014) also affected *C. albicans*. Furthermore activity against MRSA was observed. Thus the *Preussia* sp. extracts of Mapperson *et al*., (2014) demonstrated a high antimicrobial potential.

4.3.2 Fractionation of Ab1 extract

In the subsequent work on Ab1, the extract was fractionized and in the disc diffusion assays the activity was still existent. In the results of these tests Fraction 17, 19 and 20 were positive against *B. subtilis*, while Fraction 18 remained unclear. The fractions occurred at 26 min until 32 min of the HPLC chromatogram, which means the substances eluted at 83% until 95% acetonitrile. This means that the compounds were lipophilic.
As mentioned previously Mapperson *et al*., (2014), did studies on *Preussia* sp. Similar to this Diploma Thesis the work team fractionized their extracts by HPLC. A gradient of (70:30) methanol/ water with 1% trifluoroacetic acid was used as mobile phase to collect fractions [Camp *et al*., 2012]. Certain fractions of various *Preussia* sp. extracts showed activity against MRSA and *C. albicans.*
The team suggested the secondary metabolites within the extracts to be lipophilic. Furthermore Mapperson *et al.*, (2014) reported that up to 6 metabolites from their isolate BSH2.9 (*Preussia aff. africana*) eventually would be unknown polyketide-derived compounds.
5 fractions within 5 minutes were collected by Mapperson *et al.*, (2014); whereas in this Diploma work 27 fractions were collected within a total run time of 70 mins. This

means much fewer compounds were present in extracts of Mapperson *et al.*, (2014). While in this work rice media was used to culture the fungi, Mapperson *et al.,* (2014) cultivated their fungi with malt extract broth.
One problem that occurred in subsequent fractionations with Ab1 extract was that certain amounts of it precipitated and remained insoluble in methanol. Mapperson *et al.*, (2014) used ethyl acetate to dilute their extracts; eventually the use of this solvent would have solved the issues with Ab1 extract.
Unfortunately no further information about outcomes of studies from Mapperson *et al.*, (2014) was found.

.

4.4 Ab11 extract

The provided extract of Ab11 affected *B. subtilis,* whereas the second extract did not inhibit the organism. The extract originated from *Nemania* sp*.* As mentioned previously Liu *et al.*, (2016) isolated fungi from genera *Nemania* sp. which inhibited amongst other things *E. coli, B. subtilis and Fusarium oxysporum.* Thus the *Nemania* sp. isolate of Liu *et al.* showed a much better potential than the *Nemania* isolates of this work.
Generally the production of secondary metabolites is highly influenced by the growth conditions [VanderMolen *et al*., 2013]. In the case of the second Ab11 extract, although cultivated under same conditions as the provided one, probably the production of antimicrobial active secondary metabolites was not sufficient enough. Thus the work on this extract was stopped.

4.5 Bl73 extract

4.5.1 Disc diffusion assays of Bl73 extract

Bl73 extract was gained through the cultivation of an unknown species from Helotiales. In the disc diffusion assays activity against *B. subtilis, S. cerevisiae*, *A. niger* and *F. graminearum* was observed. The efficacy of the second extract of Bl73 was better than the provided one as the second showed stronger activity against *A. niger* and *F. graminearum* in disc diffusion assays.
Elias *et al*., (2006) reported in their work on a *Penicillium* species, that the qualitative and quantitative generation of secondary metabolites was influenced by the

cultivation time. As the second Bl73 extract was cultivated for additional 6 days eventually the amount of secondary metabolites, which harm *A. niger* and *F. graminearum*, was greater than in the provided extract.
Another aspect to mention is, that the amount of fungal inoculum (from *A. niger* and *F. graminearum*) is not standardized, therefore the fungal growth on the plates can differ.

Stadler and Anke, (1995) determined antimicrobial metabolites from *Lachnum papyraceum* with large antimicrobial activities against bacteria including *B. subtilis, Bacillus brevis,* etc. and yeasts including *S. cerevisiae*, *C. albicans*, etc. These tests were done by serial dilution assays. Furthermore they reported antifungal activities in disc diffusion assays against fungi amongst other things *Penicillium notatum*.
Most of the detected substances were already known; however the research team also published two new compounds, called lachnumon and lachnumol A [Stadler and Anke, 1995], which will be discussed more detailed later (see page 95). In comparison to this work Bl73 extract and the substances of Stadler and Anke, (1995) both affected *B. subtilis* and *S. cerevisiae*. Moreover antifungal properties were found for Bl73 extract and the molecules of the research team.

In another study by Matsumoto *et al.*, (2011) novel substances from *Lachnum palmae* were identified inhibiting various microorganisms including *B. subtilis, E. coli, A. niger, C. albicans, S. cerevisiae,* etc. in disc diffusion assays. The research team isolated the pure substances palmaenones A and B, which will be discussed more detailed later (see page 95). Comparing the disc diffusion assays from this Diploma Thesis and from the work of Matsumoto *et al.,* (2011) inhibition of *B. subtilis*, *S. cerevisiae* and *A. niger* can be determined. In contrast to Bl73 extract, Palmaenone A and B also inhibited *E. coli*.

4.5.2 Fractionation of Bl73 extract

In the subsequent semi - preparative treatment of Bl73 extract a time based HPLC fractionation technique was applied. Seven fractions of Bl73 indicated antibacterial and antifungal compounds. Fractions 9 and 10 showed the greatest effects of all because they were active against all test organisms but *E. coli*.

Fraction 9 and 10 showed similar inhibitory effects, which meant that is was likely that the same substance was responsible for the effect. Fraction 11, however only inhibited *B. subtilis,* which lead to the assumption that another substance stopped the growth of this bacterium. Fraction 9, 10 and 11 occurred at about 75% until 95% of acetonitrile. Thus the molecules were seen as lipophilic ones.
Fraction 14 and 15 showed a good inhibition of *B. subtilis* and they were also supposed to have the same antimicrobials. They occurred at 95% of acetonitrile and this meant the compounds of Fraction 14 and 15 were lipophilic too.
The compounds in Fraction 3 affected *B. subtilis* and Fraction 8 only showed a very slight effect on *A. niger*. It was supposed that traces of the substances in Fraction 9 were responsible for the inhibition. Consequently they were not further investigated and therefore they are not considered in the discussion.

Stadler and Anke, (1993), isolated their molecules from extracts of *Lachnum papyraceum* by flash chromatography on silicia gel 60 by a cyclohexane - ethyl acetate gradient and subsequent HPLC. The HPLC involved mobile phases with isopropanol and mixtures of cyclohexane and tert- butylmethylether. This meant the compounds compared to this Bl73 extract were lipophilic too.
The research team gained their molecules through fermentation of certain media with the fungus. They determined the antimicrobial activity via disc diffusion assays by taking aliquots at certain times during the cultivation [Stadler and Anke, 1993]. A direct comparison to the fractionation methodology of this Diploma Thesis therefore is not possible.

Matsumoto *et al.*, (2011) gained their extracts by fermentation of certain media with *Lachnum palmae* and subsequent extraction with ethyl acetate. The ethyl acetate extract was then applied to silicia gel column chromatography and antimicrobial tests were done against *Penicillium* sp. and *Botrytis* sp. Subsequently reversed-phase HPLC by 1:1 methanol/ water was applied to yield their lipophilic molecules Palmaenones A and B [Matsumoto *et al.*, 2011]. Thus, the methodology to collect and test fractions distinguished from the one done within this Diploma Thesis.

4.5.3. LCMS analysis of BI73 extract

LCMS analysis of BI73 Fractions 9 and 10 revealed that a particular compound was existent in both fractions. Substance with the mass $[M+Na]^+$ 323.06 [m/z] occurred with similar high signal intensity in both fractions. Therefore, it was suspected that this compound is responsible for the inhibition of the test organisms. The proposed formula for the molecule in Fraction 9 and 10 was $C_{10}H_{12}N_4NaO_7$.

LCMS of Fraction 11 outlined that the substance with mass $[M+Na]^+$ 495.28 [m/z], that was existent in Fraction 10, was also located here. During the analysis it became obvious that there were differences between LCMS results of F11 and F11_2. While F11 comprised a large amount of the particular substance, F11_2 nearly lacked it. The proposed formula for the molecule in F10 and F11 was $C_{27}H_{40}N_2NaO_5$, for F10_2 and for F11_2 the proposed formula was $C_{23}H_{43}O_{11}$.
Additionally the substance with mass $[M+Na]^+$ 493.25 [m/z] was found in F11 and Fraction 10 (F10 and F10_2), albeit only a minor amount was found in F11_2. The proposed formulas for the molecule $[M+Na]^+$ 493.25 in F10 and F10_2 was $C_{23}H_{34}N_8NaO_3$, whereas for F11 and F11_2 $C_{28}H_{38}NaO_6$ was determined.

However, as already discussed in the results, the substance with mass 493.25 [m/z] might be a derivate of 495.28 [m/z] without two H^+. Thus the most appropriate overall formulas for 495.28 [m/z] and 493.25 [m/z] was $C_{23}H_{36}N_8NaO_3$ and $C_{23}H_{34}N_8NaO_3$, respectively.
Similarly in F8 a substance with mass 325.07 [m/z] was detected (proposed formula $C_9H_{18}NaO_{11}$), which eventually was a derivate of 323.06 [m/z] without two H^+.

Stadler and Anke, (1993), as discussed previously, did studies on *Lachnum papyraceum* extracts, and characterized the isolation of their substances, which included mass spectroscopy analysis of the extracts. The team published high resolution electron impact - mass spectrometry (HREI- MS) results of their newly found molecules lachnumon and lachnumol A. The observed mass for lachnumon and lachnumol A was $[M]^+$ 263.9946 [m/z] and $[M]^+$ 266.0133 [m/z] respectively. The calculated formula for 263.9956 [m/z] was $C_{10}H_{10}O_4{}^{35}Cl_2$, the calculated formula for 266.0112 [m/z] was $C_{10}H_{12}O_4{}^{35}Cl_2$. Through their research data the team supposed that a carbonyl group in lachnumon was replaced by an alcohol in lachnumol A. The

team proved it via reducing lachnumon by sodium borohydride to lachnumol A [Stadler and Anke, 1993].
Compared to this Diploma Thesis substances 325.07 [m/z] / 323.06 [m/z] and 495.28 [m/z] / 493.25 [m/z] might be derivates too. Eventually the molecules 325.07 [m/z] and 495.28 [m/z] have an hydroxyl group, albeit 323.06 [m/z] and 493.25 [m/z] have a carbonyl group at this site.
Matsumoto *et al.*, (2011), as discussed prior, isolated two new molecules from *Lachnum palmae* and published the mass spectroscopy data of palmaenones A and B. In the high resolution electrospray ionization mass spectrometry (HR - ESI-MS) in negative mode the mass of palmaenone A was $[M -H]^-$ 296.9483 [m/z] with the calculated formula $C_{10}H_8{}^{35}Cl_3O_4$ of 296.9488 [m/z]. The data for palmaenone B was $[M -H]^-$ 296.9483 [m/z] with the calculated formula $C_{10}H_8{}^{35}Cl_3O_4$ of 296.9488 [m/z] [Matsumoto *et al.*, 2011].

LCMS of Fraction 14 and 15 showed that these fractions comprised a large number of mass peaks and the analysis revealed certain substances including mass 167.12 [m/z] and 463.29[m/z]. However, fatty acids are commonly isolated within fractionations of plant extracts [McGaw *et al.*, 2002] and due to the limited UV absorbance of fatty acids, the detection of fatty acids by HPLC is difficult [Tarola *et al.*, 2012]. Moreover fatty acids in reverse phase chromatography elute with very lipophilic mobile phases [Guarrasi et al., 2010] and therefore it was assumed that in these two fractions fatty acids were responsible for the antibacterial effect.
In the LCMS analysis of F15 in negative mode several fatty acids were found. One particular with the mass 279.2335 [m/z] and the proposed formula $C_{18}H_{31}O_2$ was highly apparent at the end of LCMS run. The molecule eventually was linoleic acid [source: Pubchem; PubChem CID 5280450]. Linoleic acid was reported to have antibacterial effects on Gram - positive bacteria [Dilika *et al.*, 2000], which will be discussed more detailed later, see page 97.

4.5.4 Semi - preparative HPLC of BI73 Subfraction 10

In order to gain the desired compounds determined by LCMS it was decided to collect the molecules manually by semi - preparative HPLC. This was possible because the wanted substances with masses 323.06 [m/z] and 495.28 [m/z] had a

good UV absorbance at 254nm. Therefore substances with a poor UV absorbance could not be collected by this method.
Compounds with masses 323.06 [m/z] and 495.28 [m/z] were found in Fraction 10 and 11. Hence it was decided to collect the compounds of interest from Fraction 10 manually by semi - preparative HPLC. Other substances *e.g.* the one with mass 493.25 [m/z], which might be derivates of the wanted ones, were collected too. As a result of the semi - preparative work Subfraction F10.11 revealed the wanted molecule with mass 495.28 [m/z], a powdery substance with yellow stain. It was chemically pure enough for subsequent NMR structure analysis (which was not part of this work), which revealed an unknown molecule. Subsequently the pure substance was tested against *B. subtilis* in disc diffusion assays, a light inhibition was observed.
The isolation of mass 323.06 [m/z] resulted in an oily, slighty brownish substance. However, at the end of this Diploma Thesis no information was yet available about the pureness of the substance and NMR structure analysis. The substance was tested against *S. cerevisiae* and a light inhibition was observed. Other tests against *A. niger* and *F. graminearum* were also positive, however as already discussed, the inhibition was not steady.

Stadler and Anke, (1993) isolated lachnumon and lachnumol A from *Lachnum papyraceum*. Lachnumon was described as colorless crystals, lachnumol A as colorless oil. Subsequently Stadler and Anke, (1995) reported that their substance lachnumon was positively tested against *Penicillium notatum* in disc diffusion assays, whereas tests against the fungi *Mucor miehei* and *Paecilomyces variotii* were negative.
Compared to this Diploma Thesis the pure substance 495.28 [m/z] affected *B. subtilis*, but it did not inhibit the fungi *A. niger*, *F. graminearum, S. cerevisiae* and *E. coli*. This, however was expected as F11 had no effect on the fungi. In contrast to this the substance with mass 323.06 [m/z], affected all fungi, whereas no effect on *B. subtilis* was observed.

Matsumoto *et al.*, (2011) isolated two new molecules palmaenones A and B of *Lachnum palmae*. The substances were described as colorless crystals (palmaenone A) and colorless powder (palmaenone B). The two molecules were reported to be *Cis*

-*trans* isomeres. Palmaenone A showed better antimicrobial activities than palmaenone B and affected *B. subtilis, S. aureus, E. coli, Mycobacterium smegmatis, Mucor racemosus, A. niger, C. albicans and S. cerevisiae* in disc diffusion assays. Comparing Palmaenone A and the pure substance with the component with mass 495.28 [m/z] both compounds inhibited *B. subtilis*. The molecule with mass 323.06 [m/z] and palmaenone A both inhibited *S. cerevisiae* and *A. niger*.

Other collected fractions were not pure enough or the collected volume was not sufficient enough for structure analysis or disc diffusion assays. The problem in semi - preparative runs of Fraction 10 was that the peaks were not completely separated at the baseline (see Fig. 61 and 62). As mentioned before Stadler and Anke, (1993) used isopropanol and mixtures of cyclohexane and tert- butylmethylether to separate their peaks in HPLC chromatograms. Eventually the use of these mobile phases would have solved the problem.
Matsumoto *et al.*, (2011) used a mixture of 1:1 methanol/ water to collect their molecules. In this Diploma work gradients of methanol water were applied in analytical runs to improve the separation. Nevertheless, it even diminished the baseline separation of the peaks in comparison to the usual gradient with acetonitrile/water (details see supplementary data).

As a conclusion of the isolated substances 495.28 [m/z] and 323.06 [m/z] both molecules showed antimicrobial activity. While the effect of the molecule 495.28 [m/z] on *B. subtilis* corresponded to the one of Fraction 10, the overall effect of the component 323.06 [m/z] was not as good as the inhibitory activity of Fraction 10. As Atanasov *et al.*, (2015) reported, components in an extract can interact with each other and can have a synergistic effect. Eventually this was the case for the molecules of Fraction 10. However, as the other subfractions of Fraction 10 could not be analyzed, this remains unclear.

The substance 495.28 [m/z] inhibited *B. subtilis* but it did not suppress the growth of *E. coli*. Eventually this can be due to the different structure of the cell wall of Gram - positive and Gram - negative bacteria. Maybe 495.28 [m/z] did not inhibit *E. coli* because the substance could not penetrate through the cell wall [Rosenthal, 2016].

The compound 323.06 [m/z] affected all fungi from this Diploma Thesis, whereas it did not affect *B. subtilis.* As it inhibited *S. cerevisiae* eventually a cytotoxic effect was existent. Moreover as the substance also affected *A. niger* and *F. graminearum* the substance possibly affected the spore formation [Hendrickx *et al*., 2012] and hyphae formation [Wang Q *et al*., 2015], respectively, of the fungi. Possibly the compound also influences the cell membrane like other antifungal compounds like Amphotericin B [Mesa-Arango *et al*., 2016].

4.5.5 Semi - preparative HPLC of BI73 Subfractions 14 and 15

In disc diffusion tests against *B. subtilis* Subfraction F15.8 was tested positively, while F14.8 remained unclear. As already discussed, the effect was not as good as in Fractions 14 and 15. Due the LCMS analysis of F15 in negative mode it was suspected that fatty acids were responsible for the antibacterial effect.
By bioactivity screenings of a dichloromethane extract from leaves of *Helichrysum pedunculatum* Dilika *et al.*, (2000) isolated linoleic and oleic acids. The team reported antibacterial effects against Gram -positive bactera, whereas Gram- negative bacteria were not inhibited. In this study *B. subtilis* was inhibited by linoleic acid. Furthermore a synergistic effect between the two fatty acids was observed *against S. aureus* and *Micrococcus kristinae* [Dilika *et al.*, 2000].
In comparision to the findings of Dilika *et al.*, (2000) in this Diploma Thesis the compound with mass 279.2335 [m/z] and the proposed formula $C_{18}H_{31}O_2$ was suspected to be linoleic acid [source: Pubchem; PubChem CID 5280450]. It was strongly present in Subfraction 8 of F15 which inhibited *B. subtilis* in the disc diffusion assay. As mentioned before the effect of the Subfraction F15.8 was not as good as the overall effect of Fraction 15. Compared to the findings of Dilika *et al.,* (2000) this probably is a synergistic effect of fatty acids in F15. As the aim of this Diploma Thesis was to isolate new antimicrobial compounds, the work on F14 and F15 was stopped.

5 Conclusion

BI73 extract comprised several antibacterial and antifungal components and some of them could be analyzed within this work. Particular substances $[M+Na]^+$ 323.06 [m/z] and $[M+Na]^+$ 495.28 [m/z] were suspected to have antimicrobial qualities. The predicted formula for $[M+Na]^+$ 323.06 [m/z] was $C_{10}H_{12}N_4NaO_7$, the proposed formula for $[M+Na]^+$ 495.28[m/z was $C_{23}H_{36}N_8NaO_3$.

The compound with mass $[M+Na]^+$ 495.28 [m/z] was isolated as pure substance and after NMR experiments, which were not part of this work here and where it was outlined that it was a new unknown chemical compound, the antimicrobial activity was confirmed by disc diffusion assays against *B. subtilis*. For the molecule $[M+Na]^+$ 323.06 [m/z] antifungal properties were shown, however at end of this work the purity and structure of the substance remained unclear. In conclusion within the isolation of mass $[M+Na^+]$495.28 [m/z] one particular compound of the BI73 extract was isolated.

6 Outlook

Subsequently to this work, the component of BI73 extract with mass $[M+Na]^+$ 323.06 [m/z] collected as pure substance in order to do NMR structure analysis might pay off, because it also likely is an unknown compound. Furthermore all other molecules within BI73 Fraction 10 should be analyzed and tested whether in F10 a synergistical effect of different compounds or one single potent component is responsible for the antimicrobial effect.

The compound with mass $[M+Na^+]$ 495.28 [m/z] could be further tested in cytological tests, whether the substance also affects human cells. Moreover it could be tested against Gram - positive pathogens like *Staphylococcus aureus.*

7 Literature

Adimpong DB, Sørensen KL, Thorsen L, Stuer-Lauridsen B, Abdelgadir WS, Nielsen DS, Derkx PM, Jespersen L, 2012. Antimicrobial susceptibility of *Bacillus* strains isolated from primary starters for African traditional bread production and characterization of the bacitracin operon and bacitracin biosynthesis. *American society for microbiology*. Volume 78 (22), Pages 7903-7914. DOI: 10.1128/AEM.00730-12

Ahuja S and Dong MW, 2005. Handbook of pharmaceutical analysis by HPLC. Pages 48-49. ISBN: 0080455182, 9780080455181

Allocati N, Masulli M Alexeyev FM Di Ilio C, 2013. *Escherichia coli* in Europe: An overview. *International Journal of Environmental Research and Public Health*. Volume 10 (12), Pages 6235-6254. DOI: 10.3390/ijerph10126235

Arnold E, 2007. Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal biology reviews*. Volume 21 (2-3), Pages 51-66. DOI: 10.1016/j.fbr.2007.05.003

Atanasov AG, Waltenberger B, Pferschy-Wenzig EM, Linder T, Wawrosch C, Uhrin P, Temml V, Wang L, Schwaiger S, Heiss EH, Rollinger JM, Schuster D, Breuss JM, Bochkov V, Mihovilovic MD, Kopp B, Bauer R, Dirsch VM, Stuppner H, 2015. Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnology advances*. Volume 33 (8), Pages 1582-1614. DOI: 10.1016/j.biotechadv.2015.08.001

Baddley WJ, Marr AK, Andes RD, Walsh JT, Kauffman AC,Kontoyiannis PD, Ito IJ, Balajee SA, Pappas GP, Moser AS, 2009. Patterns of susceptibility of *Aspergillus* isolates recovered from patients enrolled in the transplant-associated infection surveillance network. *Journal of clinical microbiology*. Volume 47 (10), Pages 3271-3275. DOI: 10.1128/JCM.00854-09

Balouiri M, Sadiki M, Ibnsouda SK, 2016. Methods for in vitro evaluating antimicrobialactivity: A review. *Journal of pharmaceutical analysis*. Volume 6, Pages 71-79. DOI: https://doi.org/10.1016/j.jpha.2015.11.005

Barchiesi F, Arzeni D, Compagnucci P, Di Francesco LF, Giacometti A , Scalise G, 1998. In vitro activity of five antifungal agents against clinical isolates of *Saccharomyces cerevisiae. Medical mycology*. Volume 36, Pages 437-440

Becher R, Hettwer U, Karlovsky P, Deising HB, Wirsel SG, 2010. Adaptation of *Fusarium graminearum* to tebuconazole yielded descendants diverging for levels of fitness, fungicide resistance, virulence, and mycotoxin production. *Phytopathology*. Volume 100 (5), Pages 444-453. DOI:10.1094/PHYTO-100-5-0444

Black JG, 2012. Microbiology: Principles and explorations. Pages 84-86. ISBN: 978-1118285954, 1118285956

Bojsen RK, Andersen KS, Regenberg B., 2012. *Saccharomyces cerevisiae*-a model to uncover molecular mechanisms for yeast biofilm biology. *FEMS immunology and medical microbiology*.
Volume 65 (2), Pages 169-182. DOI: 10.1111/j.1574-695X.2012.00943.x

Botstein D, Chervitz AS, Cherry JM, 1997. Yeast as a model organism. Science. Volume 277(5330), Pages 1259-1260

Blount DZ, 2015. The natural history of model organisms: The unexhausted potential of *E. coli*. *eLife* 2015;4:e05826. DOI: 10.7554/eLife.05826.

Brenner S and Miller J, 2013. *Bacillus subtilis*. Brenner's encyclopedia of genetics (Second Edition). Pages 246-248. ISBN: 9780080961569, 9780123749840.

Camp D, Rohan AD, Campitelli M, Ebdon J, Quinn J. Ronald, 2012. Drug-like properties: Guiding principles for the design of natural product libraries. *Journal of natural products*. Volume 75 (1), Pages 72-81. DOI: 10.1021/np200687v

Cantas L, Shah SQ, Cavaco LM, Manaia CM, Walsh F, Popowska M, Garelick H, Bürgmann H, Sørum H,2013. A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota. *Frontiers in microbiology*. Volume 4 (96). Pages 1- 14.
DOI: 10.3389/fmicb.2013.00096

Carlile N, Priddel D, Auld TD, and Morrison DA, 2012. Determining the factors affecting seed germination in Livistona australis (Arecaceae) for the recovery of fragmented populations. *Australian journal of botany*. Volume 60 (7), Pages 575-581. DOI: https://doi.org/10.1071/BT12056

Catalogue of Life: 2017 Annual Checklist: http://www.catalogueoflife.org/annual-checklist/2017/details/species/id/11d5a27f453983b425218d520370228a

Chen Y and Zhou MG, 2009. Characterization of *Fusarium graminearum* isolates resistant to both carbendazim and a new fungicide JS399-19. *Phytopathology*. Volume 99(4), Pages 441-446. DOI: 10.1094/PHYTO-99-4-0441

Chiller K, Selkin BA, Murakawa GJ, 2001. Skin microflora and bacterial infections of the skin. *Journal of investigative dermatology symposium proceedings*. Volume 6(3), Pages 170-174. DOI: 10.1046/j.0022-202x.2001.00043.x

Cowan MM, 1999. Plant products as antimicrobial agents. *Clinical microbiology reviews*. Volume 12(4), Pages 564–582.

Cruz KS and Cortez VG, 2015. *Hypoxylon* (Xylariaceae, Ascomycota) from Western Paraná, Brazil.
Brazilian journal of botany. Volume 38(4). Pages 889-901. DOI: https://doi.org/10.1007/s40415-015-0189-z

Degrandi TH, de Oliveira IM, d'Almeida GS, Garcia CR, Villela IV, Guecheva TN, Rosa RM, Henriques JA. 2010. Evaluation of the cytotoxicity, genotoxicity and mutagenicity of diphenyl ditelluride in several biological models. *Mutagenesis*. Volume 25 (3), Pages 257-269. DOI:10.1093/mutage/geq002

Desbois PA and Lawlor CK, 2013. Antibacterial activity of long-chain polyunsaturated fatty acids against *Propionibacterium acnes* and *Staphylococcus aureus. Marine drugs*. Volume 11 (11), Pages 4544-4557. DOI: 10.3390/md11114544

Dilika F, Bremner PD, Meyer JJ. 2000. Antibacterial activity of linoleic and oleic acids isolated from *Helichrysum pedunculatum*: a plant used during circumcision rites. *Fitoterapia*. Volume 71 (4), Pages 450-452. DOI: 10.1016/S0367-326X(00)00150-7

Driscoll JA, Bhat N, Karron AR, O'Brien LK, Murdoch RD, 2012. Disk diffusion bioassays for the detection of antibiotic activity in body fluids: Applications for the pneumonia etiology research for child health project. *Clinical infectious disease*. Volume 54 (2), Pages 159-164. DOI: 10.1093/cid/cir1061

Earl MA, Losick R, Kolter R, 2008. Ecology and genomics of *Bacillus subtilis*. *Trends in microbiology*. Volume 16 (6), Pages 269-275. DOI: 10.1016/j.tim.2008.03.004

Egamberdieva D, Wirth S, Behrendt U, Ahmad P, Berg G, 2017. Antimicrobial activity of medicinal plants correlates with the proportion of antagonistic endophytes. *Frontiers in microbiology*. Volume 8 (199), Pages 1-11. DOI: 10.3389/fmicb.2017.00199

Elias BC, Said S, de Albuquerque S, Pupo MT, 2006. The influence of culture conditions on the biosynthesis of secondary metabolites by *Penicillium verrucosum* Dierck. *Microbiological research*. Volume 161 (3). Pages 273-280

Fell WJ and Kurtzman PC, 2005. Yeast systematics and phylogeny — implications of molecular identification methods for studies in ecology. *Biodiversity and ecophysiology of yeasts*. Pages 11-30. DOI: 10.1007/3-540-30985-3_2

Fridlender M, Kapulnik Y, Koltai H, 2015. Plant derived substances with anti-cancer activity: from folklore to practice. *Frontiers in plant science*. Volume 6 (799), Pages 1-9. DOI: 10.3389/fpls.2015.00799

Gamba RR, De AG, Peláez AL, 2016. Whey permeate fermented with kefir grains shows antifungal effect against *Fusarium graminearum*. *The Journal of dairy research*. Volume 83 (2), Pages 249-255. DOI: 10.1017/S0022029916000121

GENTECHNIKBUCH: 3. KAPITEL LISTE RISIKOBEWERTETER MIKROORGANISMEN FÜR GENTECHNISCHE ARBEITEN - TEIL 1: BAKTERIEN, Bundesministerium für Gesundheit und Frauen

Gherbawy YA and Elhariry HM, 2016. Endophytic fungi associated with high-altitude Juniperus trees and their antimicrobial activities. *Plant Biosystems*. Volume 150 (1), Pages 131-140. DOI: 10.1080/11263504.2014.984011

Gonzalez-Menendez V Martin JSiles JA Gonzalez-Tejero MR Reyes F, Platas G, Tormo JR, Genilloud O. 2017. Biodiversity and chemotaxonomy of *Preussia* isolates from the Iberian Peninsula. *Mycological Progress*. Volume 16 (7), Pages 713–728. DOI: https://doi.org/10.1007/s11557-017-1305-1

Guarrasi V, Mangione MR, Sanfratello V, Martorana V, Bulone D, 2010.Quantification of underivatized fatty acids from vegetable oils by HPLC with UV detection. *Journal of chromatographic science*. Volume 48 (8), Pages 663-668.

Gunatilaka AA , 2006. Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. *Journal of natural products.* Volume 69 (3), Pages 509-526. DOI: 10.1021/np058128n

Hawksworth LD and Lücking R, 2017. Fungal diversity revisited: 2.2 to 3.8 million species. *Microbiology spectrum*. Volume 5 (4). DOI: 10.1128/microbiolspec.FUNK-0052-2016

Hendrickx M, Beguin H, Detandt M, 2012. Genetic re-identification and antifungal susceptibility testing of *Aspergillus* section *Nigri* strains of the BCCM/IHEM collection. *Mycoses*. Volume 55 (2), Pages 148-55. DOI: 10.1111/j.1439-0507.2011.02049.x

Herz W, Falk H, Kirby G.W, 2002. Fortschritte der Chemie organischer Naturstoffe / Progress in the chemistry of organic natural products. Pages 23-24. ISBN: 3211837833, 9783211837832

Hiren K, Vilaprinyo E , Sorribas A, 2010. *Saccharomyces cerevisiae* as a model organism: A comparative study*. Public library of science one*. Volume 6 (2), e16015. DOI: 10.1371/journal.pone.0016015

Jia M, Chen L, Xin HL, Zheng CJ, Rahman K, Han T, Qin LP, 2016. A friendly relationship between endophytic fungi and medicinal plants: A systematic review. *Frontiers in microbiology*. Volume 7 (906), Pages 1 -14 . DOI: 10.3389/fmicb.2016.00906

Ju YM and Rogers JD, 2002. The genus *Nemania*(Xylaraiceae). *Nova Hedwigia*. Volume 74(1-2) Pages 75-120. DOI: https://doi.org/10.1127/0029-5035/2002/0074-0075

Katiyar C, Gupta A, Kanjilal S, Katiyar S, 2012. Drug discovery from plant sources: An integrated approach. *Ayu*. Volume 33 (1), Pages 10-19. DOI: 10.4103/0974-8520.100295

Keller OB, Jie S, Young AB, Whittal MR, 2008. Common mass spectrometry contaminants and their sources. *Analytica chimica acta*. Volume 627 (1), Pages 71-81

Kubo I, Fujita KI, Kubo A, Nihei KI, and Lunde SCr, 2003. Modes of antifungal action of (2E)-alkenals against *Saccharomyces cerevisiae*. Journal of agricultural and food chemistry. Volume 51 (14), Pages 3951-3957. DOI: 10.1021/jf0211884

Kuorwel KK, Cran JM, Sonneveld K, Miltz J, Bigger WS, 2011. Antimicrobial activity of natural agents against *Saccharomyces.* Packaging technology and science. Volume 24 (5), Pages 299-307. DOI: 10.1002/pts.939

Liu YH, Hu XP, Li W, Cao XY, Yang HR, Lin ST, Xu CB, Liu SX, Li C F, 2016. Antimicrobial and antitumor activity and diversity of endophytic fungi from traditional Chinese medicinal plant *Cephalotaxus hainanensis* Li. *Genetics and molecular research*. Volume 15 (2), Pages 1-11. DOI: 10.4238/gmr.15028633

Malviya R, Bansal V, Pal OP, Sharma PK, 2010. High performance liquid chromatography: A Short review. *Journal of global pharma technology*. 2010 June, Pages 21-26

Mapperson RR, Kotiw M, Davis AR, Dearnaley DWJ, 2014. The Diversity and antimicrobial activity of *Preussia* sp. endophytes Isolated from Australian Dry Rainforests. *Current microbiology*. Volume 68 (1), Pages 30-37. DOI: 10.1007/s00284-013-0415-5

Matsumoto T , Hosoya A, Tomoda H, Shiro M, Shigemori H, 2011. Palmaenones A and B, two new antimicrobial chlorinated cyclopentenones from discomycete *Lachnum palmae. Chemical & pharmaceutical bulletin*. Volume 59 (12), Pages 1559-1561. DOI: 10.1248/cpb.59.1559

Martos AI, Romero A, Gonzalez MT, Gonzalez A, Serrano C, Castro C, Peman J, Canton E, Martin ME, 2010. Evaluation of the Etest method for susceptibility testing of *Aspergillus* spp. and *Fusarium* spp. to three echinocandins. *Medical mycology*. Volume 48 (6), Pages 858-861. DOI: 10.3109/13693781003586943

Mbah AJ, Ngemenya NM, Abawah AL, Babiaka BS, Nubed NL, Nyongbela DK, Lemuh ND, Efange SM, 2012. Bioassay-guided discovery of antibacterial agents:in vitro screening of *Peperomia vulcanica*, *Peperomia fernandopoioana* and *Scleria striatinux*. *Annals of clinical microbiology and antimicrobials.* Volume 10 (11), Pages 1-10. DOI: 10.1186/1476-0711-11-10

McGaw LJ, van Staden J, Jäger AK, Houghton PJ, 2002. Antibacterial effects of fatty acids and related compounds from plants. *South african journal of botany*. Volume 68 (4), Pages 417-423. DOI: 10.1080/01635581.2018.1412484

McMaster C. Marvin, 2007. HPLC: a practical user's guide. Page 138. ISBN 0470079088, 9780470079089

Mesa-Arango AC, Rueda C, Román E, Quintin J, Terrón MC, Luque D, Netea MG, Pla J, Zaragoza O, 2016. Cell wall changes in Amphotericin B-resistant strains from *Candida tropicalis* and relationship with the Immune responses elicited by the host. *Antimicrobial agents and chemotherapy*. Volume 60 (4) Pages: 2326-2335. DOI: 10.1128/AAC.02681-15

Nisa H, Kamili AN, Nawchoo IA, Shafi S, Shameem N, Bandh SA, 2015. Fungal endophytes as prolific source of phytochemicals and other bioactive natural products: A review. *Microbial pathogenesis*. Volume 82, Pages 50-59. DOI: 10.1016/j.micpath.2015.04.001

Nomeir AA, Pramanik BN, Heimark L, Bennett F, Veals J, Bartner P, Hilbert M, Saksena A, McNamara P, Girijavallabhan V, Ganguly AK, Lovey R, Pike R, Wang H, Liu YT, Kumari P, Korfmacher W, Lin CC, Cacciapuoti A, Loebenberg D, Hare R, Miller G, Pickett C, 2008. Posaconazole (Noxafil, SCH 56592), a new azole antifungal drug, was a discovery based on the isolation and mass spectral characterization of a circulating metabolite of an earlier lead (SCH 51048). J*ournal of mass spectrometry*. Volume 43 (4), Pages 509-517. DOI: 10.1002/jms.1341

Pfaller MA , Messer S, and Jones RN, 1997. Activity of a new triazole, Sch 56592, compared with those of four other antifungal agents tested against clinical Isolates of *Candida* spp. and *Saccharomyces cerevisiae*. *Antimicrobial agents and chemotherapy*. Volume 41 (2), Pages 233-235

Pilehvar SY, Panahi Y, Andalib S, Balaghi-IM, Alipour S, Moazzez-Lalaklo N, Sandoghchian-Shotorbani S, Sadat-Hatamnejad L, 2014. Two small molecule lead compounds as new antifungal agents effective against *Candida albicans* and *Saccharomyces cerevisiae. Novelty in biomedicine*. Volume 2, Pages 47-52

PubChem CID 3033836:
https://pubchem.ncbi.nlm.nih.gov/compound/Octadecanoate

PubChem CID 5280450: https://pubchem.ncbi.nlm.nih.gov/compound/linoleic_acid

PubChem CID 985: https://pubchem.ncbi.nlm.nih.gov/compound/palmitic_acid

Razzaghi-Abyaneh M and Rai M, 2013. Antifungal metabolites from plants. Page 1892. ISBN 978-3-642-38076-1

Rogawansamy S, Gaskin S, Taylor M, Pisaniello D, 2015. An evaluation of antifungal agents for the treatment of fungal contamination in indoor air environments. *International journal of environmental research and public health.* Volume 12 (6). Pages 6319-6332. DOI: 10.3390/ijerph120606319.

Rosenthal KS, 2016. Medical microbiology and immunology flash cards e-book. Page 47. ISBN 0323478506, 9780323478502

Santoyo G, Moreno-Hagelsieb G, Orozco-Mosqueda Mdel C, Glick BR, 2016. Plant growth-promoting bacterial endophytes. *Microbiological research*. Volume 183, Pages 92-99. DOI: 10.1016/j.micres.2015.11.008

Sasidharan S, Chen Y, Saravanan D, Sundram KM, Yoga Latha L, 2011. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African journal of traditional, complementary, and alternative medicines*. Volume 8(1). Pages 1-10.

Stadler M and Anke H, 1993,. Lachnumon and Lachnumol A, new metabolites with nematicidal and antimicrobial activities from ascomycete *Lachnum papyraceum*. (KARST.) KARST.; I. Producing organism, fermentation, isolation and biological activities. *The journal of antibiotics*. Volume 46 (6), Pages 961-967. DOI:10.7164/antibiotics.46.961

Stadler M and Anke H, 1993. Lachnumon and Lachnumol A, new metabolites with nematicidal and antimicrobial activities from ascomycete *Lachnum papyraceum*. (KARST.) KARST.; II. Structural elucidation. *The journal of antibiotics*. Volume 46 (6), Pages 968-971. DOI: 10.7164/antibiotics.46.968

Stadler M and Anke H, 1995. Metabolites with nematicidal and antimicrobial activities from the ascomycete *Lachnum papyraceum* (Karst.) Karst V. Production, isolation and biological activities of bromine-containing mycorrhizin and lachnumon derivatives and four additional new bioactive metabolites. *The journal of antibiotics*. Volume 48 (2), Pages 149-153

Tadesse AD, Zhao S, Tong E, Ayers S, Singh A, Bartholomew JM and McDermott FP, 2012. Antimicrobial drug resistance in *Escherichia coli* from humans and food animals. Emerging infectious diseases. Volume 18 (5), Pages 1950-2002. DOI: 10.3201/eid1805.111153

Tanaka Y, Nanamiya H, Yano K, Kakugawa K, Kawamura F, Ochi K, 2013. rRNA (rrn) Operon-engineered *Bacillus subtilis* as a feasible test organism for antibiotic discovery. *American society for microbiology*. Volume 57 (4), Pages 1948- 1951. DOI:10.1128/AAC.02604-12

Tang AMC, Jeewon R, Hyde DK., 2007. Phylogenetic relationships of *Nemania plumbea* sp. nov. and related taxa based on ribosomal ITS and RPB2 sequences. *Mycological research*. Volume 111 (4), Pages 392-402. DOI:10.1016/j.mycres.2007.01.009

Tavares S, Silva CSF, de Souza VC, da Silva VL, Diniz CG, Santos MO, 2013. Strategies and molecular tools to fight antimicrobial resistance: resistome, transcriptome, and antimicrobial peptides. *Frontiers in microbiology*. Volume 4 (412), Pages 1 -11. DOI: 10.3389/fmicb.2013.00412

Tarola AM, Girelli AM, Lorusso S, 2012. High Performance Liquid Chromatography Determination of Fatty Acids in Drying Oils Following Lipase Action. *Journal of chromatographic science*. Volume 50 (4), Pages 294-300. DOI: 10.1093/chromsci/bms005

Tokarzewski S, Ziółkowska G, Nowakiewicz A, 2012. Susceptibility testing of *Aspergillus niger* strains isolated from poultry to antifungal drugs-a comparative study

of the disk diffusion, broth microdilution (M 38-A) and EtestR methods. Polish journal of veterinary sciences. Volume 15 (1), Pages 125- 133.

Twaroch ET, Curin M, Valenta R, Swoboda I, 2015. Mold allergens in respiratory allergy: From structure to therapy. *Allergy, asthma & immunology research*. Volume 7(3). Pages 205-220. DOI:10.4168/aair.2015.7.3.205

Vandermolen KM, Raja HA, El-Elimat T, Oberlies NH, 2013. Evaluation of culture media for the production of secondary metabolites in a natural products screening program. *AMB Express*. Pages 1-7. Doi: 10.1186/2191-0855-3-71

Wang Q, Vera Buxa S, Furch A, Friedt W, Gottwald S., 2015. Insights into triticum aestivum seedling root rot caused by *Fusarium graminearum*. *Molecular plant-microbe interactions*. Volume 28 (12). Pages 1288-1303. DOI: 10.1094/MPMI-07-15-0144-R

Wang Z, Johnston PR, Takamatsu S, Spatafora JW, Hibbett DS, 2006. Toward a phylogenetic classification of the Leotiomycetes based on rDNA data. Mycologia. Volume 98 (6), Pages 1065-1075

Weller GM, 2012. A unifying review of bioassay-guided fractionation, effect-directed analysis and related techniques. *Sensors*. Volume 12, Pages 9181-9209. DOI: 10.3390/s120709181

Ye M, Cao SQ, Jiang ST, Pan LJ, Luo SZ, Li XJ, 2006. Species diversity of *Lachnum* (Helotiales, Hyaloscyphaceae) from temperate China. *Journal of zhejiang university. Science. B.* Volume 7(1), Pages 20-27. DOI: 10.1631/jzus.2006.B0020

Yogabaanu U, Faizal FJW, Convey P, Rizman-Idid M, Alias AS , 2017. Antimicrobial properties and the influence of temperature on secondary metabolite production in cold environment soil fungi. *Polar science*. Volume 14, Pages 60-67. DOI: 10.1016/j.polar.2017.09.005

Zeigler DR, Prágai Z, Rodriguez S, Chevreux B, Muffler A, Albert T, Bai R, Wyss M, Perkins JB, 2008. The origins of 168, W23, and other *Bacillus subtilis* legacy strains. *Journal of bacteriology*. Volume 190 (21), Pages 6983-6995. DOI: 10.1128/JB.00722-08

8 Appendage

List of abbreviations

e.g.- exempli gratia
EIC - Extracted ion chromatogram
et al.- et alteri
Fig.- figure
g/l - grams per liter
HPLC - High performance liquid chromatography
LCMS - Liquid chromatography-mass spectrometry
mAU - milli absorbance unit
ml - milliliter
mm - milimetre
MRSA - Methicillin- resistant *Staphylococcusaureus*
mV-minivolt
[m/z] - Mass-to-charge ratio
PDA - Photo diode array
uV - microvolt
µg/µl - micrograms per microliter
µl - microliter

Supplementary data

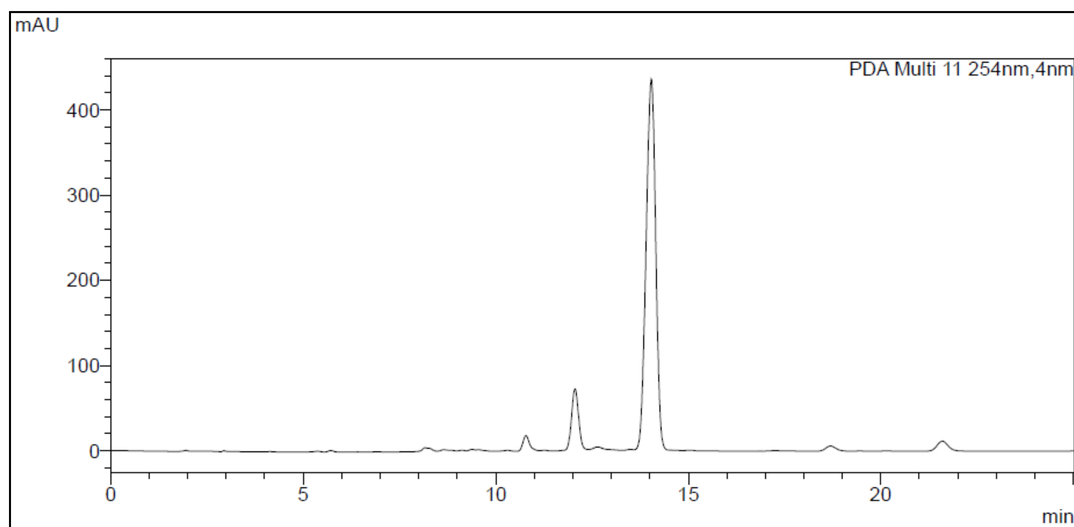

Fig.: Analytical HPLC run of F10_2, Methanol/H_2O gradient, flow rate 1 mL/min.

Gradient:

0.0 min Solvent Methanol Conc. 70%
5.0 min Solvent Methanol Conc. 80%
20.0 min Solvent Methanol Conc. 85%
25.0min Solvent Methanol Conc. 95%