University of South Bohemia in České Budějovice, Faculty of Science

Profiles of beauverolide peptides isolated from entomopathogeneous fungi

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Bachelor Thesis

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Annotation

The aim of this thesis was to review biochemical properties of cyclodepsipeptide secondary metabolites called beauverolides (BVDs) produced by some entomopathogenic fungi, to extract and characterize beauverolides in four strains of the genus Isaria by liquid chromatography - mass spectrometry (LC-MS) and estimate relative abundance of beauverolides in mycelium.

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1 Abstract

Some strains of entomopathogenous fungi produce cyclodepsipeptides called Beauverolides (BVDs). An LC-MS method was developed for characterization of BVDs in fungal mycelium. Separation of beauverolides was performed on a C18 Gemini column, the cyclodepsipeptides were detected by positive ion electrospray ionization mass spectrometry. A calibration curve for beauverolide I(BVD-I) was constructed and diluted BVD extracts were analyzed against an available BVD-I standard by external calibration. The described LC-MS method was found suitable to study BVD profiles in mycelium. At least 15 BVDs were detected in two Isaria and some other in a Bassiana strain. BVD-I cyclopeptidedominates in Isaria strains in accordance with literature. However, BVDs were not detected in a new Isaria isolate If CCM 8367. Quantitative analysis of the BVD-I in the studied two Isaria strains, Apopka and PFR-97, shows that the peptide is present in mycelium at low $\mu g/g$ concentration. The developed method enables quantification of BVD-I in the concentration range 0.1 -5 ppm.

2 Introduction

Fungi are common pathogens of various organisms including insects. It was reported about 1 000 species of fungi which colonize insects, spiders or mites and some of them have been used as biopesticides, particularly against insects. Fungi penetrate the cuticle and insect body orifices directly and their action is different than that of bacteria and viruses [33]. Some fungi are able to produce infectious conidia capable to penetrate cuticle and germinate [13].

Fungi produce a variety of secondary metabolites, which have, depending on the species of the fungus, different functions [15]. The entomopathogenous fungus *Isaria fumosorosea*(formerly Paecilomyces fumosorosea) and Beauveria bassiana, which have been extensively used in America and Europe against various species, particularly against whiteflies [15], produce interesting secondary peptidic metabolites called beauverolides (BVDs) [9]

The genera *Beauveria* and *Isaria* produce BVDs in relatively high amounts [in $\mu g/\mathrm{g}$] in a culture broth. However, their exact biological role remains unknown [6, 19]. BVDs exhibit immunomodulatory activities [13], inhibit the macrophage acyl-CoA; cholesterol acyltransferase (ACAT) activity in order to block the synthesis of cholesteryl ester (CE) [20, 26, 30].

These features may be attractive to study for regulation of the β-amyloid peptides, $(A\beta)$, which are present in brain and responsible for cognition. If too many of them accumulate in the brain, they form amyloid-plaque. It is assumed that this plaque significantly influences the progress of the Alzheimer disease (AD) and needs to me minimized [32]. The BVDs action has been tested against the treatment against atherosclerosis and may be useful for the senile plaque reduction in Alzheimer‘s disease [32]

As the role of BVDs in the fungi, the fungi-host insect interactions and their pharmacologic properties have not been well understood, there is increased demand for their study in biological material and environment. Development of an appropriate method for the BVDs analysis represents first step to accomplish this goal. Liquid chromatography-mass spectrometry (LC-MS) has been most perspective tool for BVD analysis [1, 13, 21, 30]. In this work, an LC-MS method has been developed and applied to characterization of BVDs in three *Isaria* strains.

3 Current status of knowledge

3.1 Importance of entomopathogenous fungi

The word entomogenous is from Greek: *"emtomon"* stands for insects and *"genes"* means arising in. As a consequence, entomogenous microorganisms are "microorganisms, that arise in insects". As one of the first organisms entomopathogenic fungi were used in biological control of pests. As these fungi are virulent, persistent in environment for long time and infect their hosts by contact, they represent perspective natural pesticides useful in pest control [28].

Entomopathogenic fungi do not have a perfect stage, therefore they are called *Fungi imperfecti* (order *Moniliales* and family *Stilbaceae*).

The fungi examined in this work are *Isaria fumosorosea* (Hypocreales: Cordycipitacea) and *Beauveria bassiana* and are entomopathogenic fungi which have been registered and employed as mycoinsecticide in North America, Europe and Brazil. They are used in greenhouses to control different pest populations reducing thus economical damages [16, 17].

Application of fungi in order to decrease pests density, aiming decreased crop damage in biological processes is called "myco-biocontrol". Due to the complex way of action of biopesticides, pests cannot develop resistance against it as they do against chemical pesticides [28]. The fungi have been tested for biological control of many species. Interestingly, an Isaria isolate is also a pathogen of tick larvae [7].

Entomopathogenic organisms can be used against adult mosquitoes too. Kanzok et al. [11] reported about possible application of the fungus *Beauveria bassiana* in order to fight against the malaria parasite. It takes the fungus around two weeks to multiply and kill

the host, which is approximately the time for development of the malaria parasite [11]. Isaria genus has been isolated form several insects already in diverse orders worldwide [2, 14].

3.2 Mode of action

Entomopathogenic fungi penetrate an insect host through the cuticle and its body orifices. The insect host is damaged by several factors combined: mechanical damage because of invasion of the tissue, toxicosis through producing toxins inside the insect and because the fungi share the insects nutrients. As soon as the nutrients have been used up, hyphal bodies will grow towards the surface of the host as mycelial threads. Unfortunately, the exact interaction mechanism between the fungal spores and the hosts cuticle have not been well understood yet [4, 28].

Fungi have been studied extensively since 1836 as they were thought to be mycoinsecticides [8]. The insects cuticle is the target at which the entomopathogenous fungus attacks in the following way: The conidium (the infective unit of the fungus) is supposed to penetrate the cuticle of the target organism directly by applying mechanical pressure and leading to enzymatic degradation of the cuticle. After penetration, germinating spores are introduced into the insect body by overcoming the defense mechanisms. As a consequence, changes in the growth morphology are observed and the fungus multiplies by budding, during which its hyphal bodies replicate. After infesting the haemocoel, which is important for the oxygen transport within the insect, the onset of death will occur within 3-14 days [12]. Mechanical damage, which leads to water loss, as well as nutrient exhaustion and toxicosis cause the insect´s death. Depending on the relative humidity, which should be high for effective sporulation, the fungus turns into its mycelial growth form after the host died. In this stadium, the whole process of penetration is repeated. If humidity is low, the fungus can survive several months inside the host. The whole process of penetration just shows local discoloration at the point at which the fungus entered the insect, but no other obvious signs [8].

Several fungal strains have been exploited for the insect pest control. Among them

Isaria, Beauveria, which are known to produce BVD peptides [8]. They are thought to perturb the cholesterol homeostasis by reducing A-production and the secretion in vitro through inhibiting ACAT [20, 21]. Oshiro et al. [25] reported about the inhibition of the cholesteryl ester synthesis (CE) of BVD derivatives.

3.3 The production and structure of beauverolides (BVDs)

The first isolated beauverolide I has been described by Grove et al. (1977). The group of Jegorov et al. designated the BVDs as beauverolides which is shown in Table 3.2.

Cyclic compounds, which bear alternating amino and hydroxy acid residues, are called cyclodepsipeptides (cyclic peptido-lactones) [4, 6]. The positions 2-4 shown in Fig. 3.1 are occupied by different amino acids, which are specific for the type of BVD. Position 1 is occupied by a fatty acid. In beauverolides B, C, E and F, isoleucine is the amino acid present in those peptides. Additionally, some beauverolides have a chiral Ile isomer allo-isoleucine instead of isoleucine and are marked with the subscript a, for example L_a [5]. These peptides are reported to belong to a group of lipophilic and neutral cyclotetradepsipeptides that contain C_9 or $C_{11} - \beta$-hydroxy acid residues, linear and branched [10, 13].

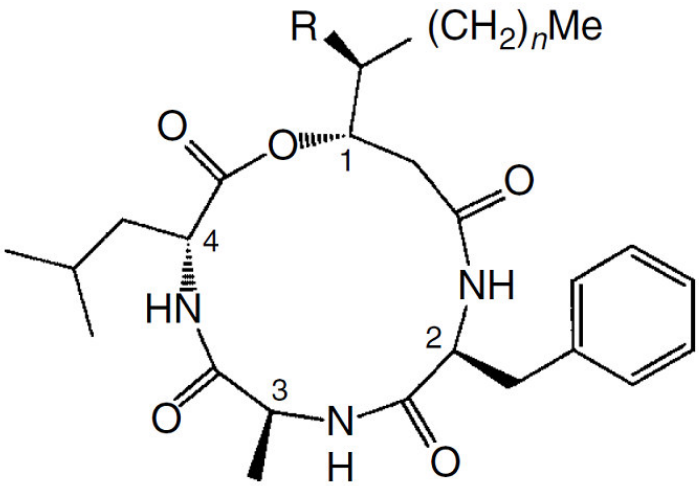


Figure 3.1: The general structure of beauverolides. In the case of Beauverolide I, n =6 and residue is a hydrogen atom. However, methyl moiety is dominant here in the BVD structures [13].

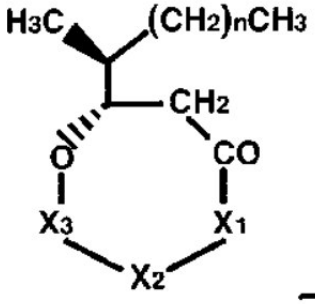
Beauverolide	*n*	R	AA2	AA3	AA4	MW	Composition	*m/z* $[M+H]^+$	*m/z* $[M+Na]^+$	Ref.
Beauverolide A	5	Me	Phe	Val	Val	529	$C_{30}H_{47}N_3O_5$	530	552	20
Beauverolide B	5	Me	Val	Phe	Ile	543	$C_{31}H_{49}N_3O_5$	544	566	20
Beauverolide B_a[a]	5	Me	Val	Phe	allo-Ile	543	$C_{31}H_{49}N_3O_5$	544	566	18,21
Beauverolide C	5	Me	Phe	Phe	Ile	591	$C_{35}H_{49}N_3O_5$	592	614	20
Beauverolide C_a	5	Me	Phe	Phe	allo-Ile	591	$C_{35}H_{49}N_3O_5$	592	614	18,20
Beauverolide D	5	Me	Phe	Val	Val	501	$C_{28}H_{43}N_3O_5$	502	524	20
Beauverolide E	3	Me	Val	Phe	Ile	515	$C_{29}H_{45}N_3O_5$	516	538	20
Beauverolide E_a	3	Me	Val	Phe	allo-Ile	515	$C_{29}H_{45}N_3O_5$	516	538	20
Beauverolide F	3	Me	Phe	Phe	Ile	563	$C_{33}H_{45}N_3O_5$	564	586	20, 21
Beauverolide F_a	3	Me	Phe	Phe	allo-Ile	563	$C_{33}H_{45}N_3O_5$	564	586	20
Beauverolide I	6	H	Phe	Ala	Leu	515	$C_{29}H_{45}N_3O_5$	516	538	19
Beauverolide H	4	H	Phe	Ala	Leu	487	$C_{27}H_{41}N_3O_5$	488	510	19
Beauverolide J_a	3	Me	Trp	Phe	allo-Ile	602	$C_{35}H_{46}N_4O_5$	603	625	18
Beauverolide K_a	5	Me	Trp	Phe	allo-Ile	630	$C_{37}H_{50}N_4O_5$	631	653	18
Beauverolide L	5	Me	Phe	Ala	Ile	515	$C_{29}H_{45}N_3O_5$	516	538	1
Beauverolide L_a	5	Me	Phe	Ala	allo-Ile	515	$C_{29}H_{45}N_3O_5$	516	538	1
Beauverolide M	3	Me	Val	Ala	Leu	439	$C_{23}H_{41}N_3O_5$	440	462	this work
Beauverolide N	3	Me	Tyr	Ala	Leu	503	$C_{27}H_{41}N_3O_6$	504	526	this work
Beauverolide P	5	Me	Val	Ala	Lxx	467	$C_{25}H_{45}N_3O_5$	468	490	this work
Beauverolide I	3	Me	Phe	Ala	Leu	487	$C_{27}H_{41}N_3O_5$	488	510	14
Beauverolide II	5	Me	Phe	Ala	Leu	515	$C_{29}H_{45}N_3O_5$	516	538	14
Beauverolide III	3	Me	Phe	Ala	allo-Ile	487	$C_{27}H_{41}N_3O_5$	488	510	16,17

[a] Also named beauverilide A.

Figure 3.2: The structures of beauverolides characterized by Jegorov et al. AA stands for amino acid [13]. R=H or Me; n=6 or 3.

Jegorov et al. [10] isolated the new BVDs L and L_a for the first time from the mycelium of the entomopathogenic fungi *Beauveria brogniartii* and *Isaria fumosorosea*. By using

preparative reversed-phase liquid chromatography he designated them as beauverolides L and L_a. Through using mass spectrometry (MS) and tandem mass spectrometry (MS^n), three new beauverolides were found and described in the fermentation broth of *Beauveria bassiana*. They were tested that they do not exhibit insecticidal, bactericidal or fungicidal effects [13]. Further two BVDs were described in extracts of a fungal strain of *I. fumosorosea* and characterized by tandem mass spectrometry, namely beauverolides Q and R [9]. Beauveriolides were discovered in 1999 [18, 24]. Mochizuki et al. [19] reported about beauveriolide II. Tomoda et al [30] characterized same cyclodepsipeptides and desiguated them as beauveriolide I and III, which were originally isolated from *Bauveria sp.* FO-6979 [32]. Table 3.3 summarizes these beauveriolides.



		Amino acid			
Beauveriolide	n	X_1	X_2	X_3	BVD
I	3	*L*-Phe	*L*-Ala	*D*-Leu	BVD-I
III	3	*L*-Phe	*L*-Ala	*D-allo*-Ile	BVD-Ia
IV	3	*L*-Val	*L*-Ala	*D*-Val	
V	3	*L*-Val	*L*-Ala	*D-allo*-Ile	BVD-Ma
VI	3	*L*-Val	*L*-Ala	*D*-Leu	BVD-M
VII	3	*L*-Phe	*L*-Ala	*D*-Val	
VIII	5	*L*-Val	*L*-Ala	*D-allo*-Ile	
IX	3	*L*-Phe	*L*-Phe	*D-allo*-Ile	BVD-Ca

Figure 3.3: General skeleton, as described by Tomoda et al., of beauveriolides and different substituents for beauveriolides I - IX [30].

The structure contains a unique (R,S-system as seen in figure 3.3) 3-hydroxy-4-methyl C9 or C11 carboxylic acid, two L-amino acids and one D-amino acid in a 13-membered ring. Tomoda et al estimated the complete structure of beauverolides including the hydroxy acid. Thus, Beaverolide La stucture was deduced as cyclo-[(3S,4S)-3-hydroxy-4-methyloctanoyl-L-phenylalanyl-L-alanyl-D-allo-isoleucyl]. They reported about the new beauveriolides IV - XII, but beauveriolides VI and IX already identified as beauverolides M [13] and Fa [5]. Nagai et al. [20, 21] described a synthetic route to beauverolides and Tian et al. [29] described the first total synthesis of beauveriolide I.

Review of the references indicates that beauverolides and beauveriolides represent the

identical cyclodepsipeptides described independently by two different research groups. The different nomenclature arises from the fact that Jegorov et al.[9, 10] were not able to describe the chirality of the 3-OH-acid while the Japanese researchers managed this task. As a result, beauverolides as well as beauveriolides represent the same cyclopeptides, which is reported in this work by the term beauverolide (abbreviation, BVD). The novel cyclodepsipeptide beauverolide III was isolated, as well as the already known beauveriolide I, from the broth of a strain of *Beauveria sp.*FO-6979 [22, 23, 24].

Some peptides originally obtained other names, for example beauvellide produced by *Beauveria tenella* (now the fungus is called *Beauveria brongiarti*), is a mixture of beauverolide peptides (H and I in this case) [10]. Vilcinskas et al. [31] reported already 15 different BVDs in species of *Beauveria* and *Isaria*. Besides several other beauverolides, beauverolide I is the principal component in all *Isaria* isolates [9].

The entomopathogenic fungi *Beauveria brogniartii* and *Isaria fumosorosea* are known to produce beauverolides L and L_a. It was found out that beauverolide L and beauveriolide II (reported by Mochizuku et al. [19]) have identical structure [10]. It was reported about new natural beauverolides [13].

Through the addition of specific amino acids to the beauveriolide producing strain in a selective medium, it is possible to selectively produce a beauveriolide peptide. For example, if the amino acid added is L-leucine, then the resulting Beauveriolide peptide will be Beauveriolide I. If the amino acid is L-isoleucine, the produced Beauveriolide would be Beauveriolide III, which is very similar to I in its physicochemical properties [27]. Several microorganisms, like *Beauveria bassiana, Bauveria brogniartii, Beauveria vermicoria* and *Isaria fumosorosea*, produce beauverolides.

3.4 Biomedical aspect

BVDs represent highly interesting peptide structures produced as a homologous peptide mixture by means of the non-ribosomal peptide synthesis. Being produced in considerable amount in the mycelia, they can be isolated relatively easy in milligram-gram amounts and thus disposable to testing of their biochemical and biomedical properties. Although they

directly do not possess any evident pathogenous activity, interesting biochemical action has been described [27]. The biological activity of beauverolides requires the presence of an aromatic amino acid in the second position. A bulky, aromatic amino acid in the second position is typical for the genus *Isaria*, whereas incorporation of valine in the second and isoleucine in the fourth position is typical for strains of *Beauveria bassiana* [9]. For the treatment of atherosclerogenesis, the inhibition of lipid droplet accumulation in macrophages is essential. Beauverolides I and III were reported to inhibit the ACAT (acyl-CoA:cholesterol acyltransferase) activity and exerted the antiatherogenic activity without negative effects in mouse models [21, 30].

Two pathological hallmarks, intracellular accumulation of hyperphosphorylated tau and extracellular accumulation of plaques in specific regions of the brain, characterize Alzheimer‘s disease (AD). Plaques are derived from proteolytic cleavages of amyloid precursor protein (APP) and are aggregates of amyloid beta (Aβ) peptides [3]. Free cholesterol is converted to cholesterol ester by Acyl-CoA:Cholesterol Acyltransferase (ACAT), which is one of the key enzymes in cellular cholesterol metabolism. The inhibition of ACAT could be a possible strategy against Alzheimer‘s disease. Treatment of AD means the slowing down of the progression of the disease or even preventing it. [3].

3.5 The beauverolide analysis

BVDs have been characterized by various methods including FAB, MS, NMR [4, 5]. Because BVDs are produced by fungi as a mixture of homologous cyclopeptides, prior separation of the BVDs by LC with atmospheric mass spectrometry has been the most suitable tool for their analysis. Jegorov et al. [9, 10, 13] were one of the first who analyzed the BVDs in various fungal strains. Atmospheric pressure electrospray ionization (ESI) as an ion source and MS and MS/MS analysis enable studies of the BVD profiles in biological material [4, 5].

4 The goals of this study

The purpose of this study was:

(1) To develop a convenient HPLC-MS method for the determination of BVD-I.

(2) To characterize the BVD profiles in four fungal strain isolates and estimate relative BVD abundance in their mycelium.

(3) To estimate the BVD-I level in the mycelium against the available BVD-I standard.

5 Experimental

5.1 Biological material

The strain of *Isaria fumosorosea* Apopka was a commercial specimen from Certis, USA . The *Isaria fumosorosea* PFR-97 straom was provided by prof. Dr. Zdenek Landa, PhD, Uni South Bohemia. Further two strains,*Isaria fumosorosea* CCM 8367 (isolated and cultivated by Dr. Rostislav Zemek, PhD, Institute of Entomology, CAS) and *Beauveria bassiana* botanyguard, gha (cultivated by Dr. Katerina Simackova,PhD, Institute of Entomology, CAS, respectively).

5.2 Chemicals and reagents

0.1 % formic acid in acetonitrile, 0.1% formic acid in deionized water, MeOH (95%) and all of the other used chemicals and reagents were purchased from Sigma-Aldrich (Praha, Czech Republic). The Beauverolide I standard, containing about 15 % of BVD-Ia, was isolated at the Department of Analytical Biochemistry and Metabolomics from the *Isaria fumosorosea* PFR-97 strain.

5.3 Extraction of the BVD peptides from mycelium of *Isaria fumosorosea*

From the mycelium of the fungal strain PFR-97 of *Isaria fumosorosea*, a yellowish peptide mixture containing different BVDs, was obtained through extracting the mycelium with

methanol and further vacuum filtrating the extract as the peptides precipitated from the solution.

Four different fungal strains (Apopka (If), 8367(If), PFR-97 (If) and Bota (Bb) were examined regarding their BVD content. First, the culture broth of each fungus sample, cultivated on a petri dish, was filtrated by vacuum filtration using paper filter for collecting the peptides. The filter cake was washed with distilled water and dried on the filter paper at 30 °C . 100 mg of the dried filtrate were dissolved in 1 mL of 95 % Methanol. The sample was placed in an ultrasonic bath for 10 min, extracted at 50 °C in a water bath and placed in an ultrasonic bath for another 10 minutes. A 700 μL aliquot of the extract was then centrifuged for 30 min at 20 °C . The supernatant was diluted 1:50 with 95 % Methanol prior to the LC-MS analysis.
In the case that no BVDs were found in the examined strain extract with the dilution of 1:50, a second sample with 1:1 dilution was prepared from the supernatant and analyzed. This procedure was used for the BVD extraction from all four examined strains and the results were compared.

5.4 BVD instrumental analysis

For LC-MS analysis an Accela autosampler and Accela 600 LC pump were used (both Thermo Scientific, San Jose, USA). Separation of BVDs was performed on a 3 μm Gemini 150 x 2m ID column (Phenomenex, Torrance, USA). 5 μL were chosen as the injection volume. The temperature of the column, which is stable up to 60 °C , was set to 50 °C , although usually it is between 30 – 40 °C . For the separation a mobile phase, consisting of acetonitrile (A) and water (B) enriched in 0.1% formic acid with a flow rate of 0.25 mL/min was chosen. The linear gradient elution A:B used was starting with 55:45. Within four minutes it reached 95:05, it was hold for three minutes and then switched back to the starting elution. Because of the solubility of the samples, the temperature of the autosampler was set to 40 °C .

The analysis of the different BVD peptides in the samples was accomplished on a linear ion trap LTQ-XL mass spectrometer (Thermo Scientific, USA) coupled to an Accela

autosampler (Thermo Scientific, USA) and Accela 600 (Thermo Scientific, USA) liquid chromatograph. The mass spectrometer was equipped with an electrospray HESI II ion source. The positive ions were detected in two scan modes, which were alternating each other: the full scan mass range (400 – 850 Da) and the CID MS/MS scan type with the m/z ratio of BVD-F.

An ion source temperature of 300 °C and a capillary temperature of 350 °C were used in this measurement. A sheat gas flow of 25 and a Aux gas flow of 7 were set, while the Sweep gas flow (going in the opposite direction to sheat and aux gas flow) was set to 1. The source voltage (spray voltage) was 2kV and the capillary voltage was set to 40 V. The tube lens had 110 V. The collision energy was 20%. For the precursior M^+H^+ ion an isolation mass width was set to 3 Da. The speed of the scanning of the mass spectrometer was different in full MS and tandem MS/MS. In full MS it was 1 scan/50 ms and in MS/MS it was 1 scan/100 ms.

5.5 Calibration and the BVD analysis

0.7 mg of the standard of the BVD I peptide were dissolved in one mL of methanol to get a concentration of 0.7mg/mL. Six serial dilutions were perfomed with MeOH (95%) in the following way: At first, the standard was diluted with 95% MeOH to get a concentration of 10 000 ng/mL. Then further dilutions were done to get the following concentrations: 5 000, 2 000, 1 000, 400 and 80 ng/mL (n=3). Five μL of the calibration solution of each level were injected and measured. A calibration curve was constructed with the concentration range depending on the concentration level of every solution of the BVD I peptide standard.

The data were processed by the XCalibur Software. The BVD-I amount in the mycelium was estimated by using external calibration after sample dilution. The sample extract volumes were 5 μL, each at a concentration of 20 μg/mL. The dissolved BVD sample dissolved in MeOH to the mentioned concentration and then directly injected into a mobile phase flow into the ESI ion source of the LTQ mass spectrometer and scanned in the 150 - 1000 Da mass range every 1.2 seconds.

6 Results and Discussion

6.1 The BVD extraction

BVDs are relatively non-polar peptides and are very badly soluble in aqueous solutions and also not easy in polar solvents. However, BVDs are thermally stable and thus their solubility can be increased by an elevated temperature. Therefore, warm methanol and ethanol solvents (40-50 °C) were used for the extraction. Fig. 6.1 and 6.1 show the fresh mycelium the remaining peptide mixture after extraction and further evaporation of the solvent.

Figure 6.1: The mycelium fungal material of the *Isaria* strain PFR-97 **before** extraction of the BVDs.

Figure 6.2: The mycelium fungal material of the *Isaria* strain PFR-97 **after** extraction and before filtration of the BVDs.

Mycelium analysis: as BVDs are present in mycelium at high levels, the samples can be severally diluted prior to the LC-MS analysis. The dilution of the sample extracts enables to detect BVDs within the dynamic response range of the MS and minimizes the matrix effects on the BVD peak area response.

The experimental A/x weighed calibration curve shows different dilutions of the BVD I standard: 5000, 2000, 1000, 400 and 80 ng/mL.

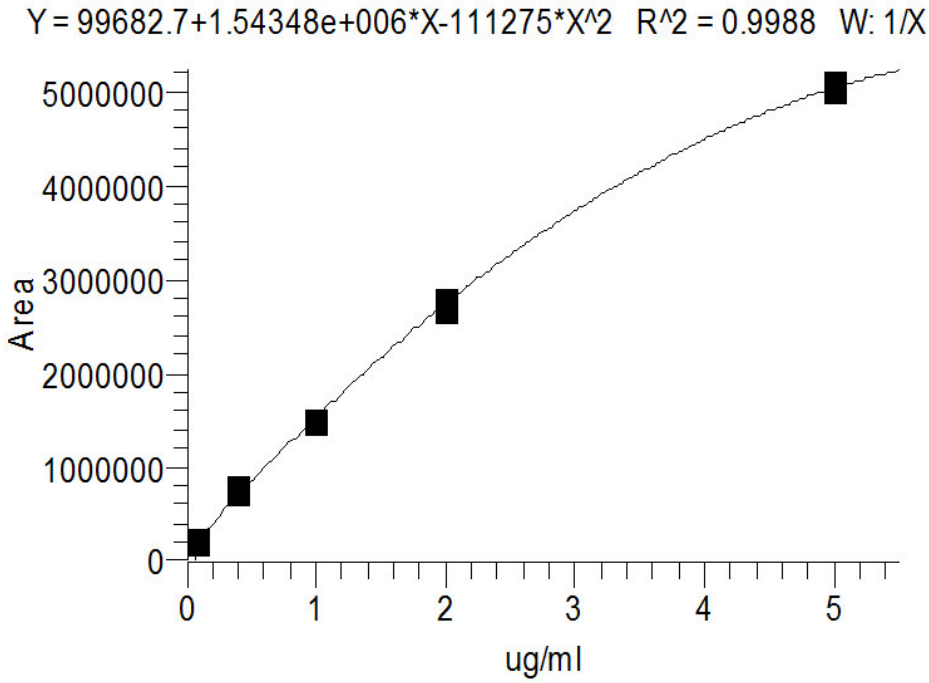


Figure 6.3: The calibration line for BVD I was linear from 0.1 - 5 ppm. The lowest point was a point of the LLOQ (lower limit of quantitation). The quadratic 1/x weighed function approached best the calibration regression.

6.2 Mass spectral analysis

HPLC-MS with positive electrospray ionization has been found sensitive and selective well for the BVD-analysis. It provides good chromatographic separation of components and sensitive, highly specific detection of BVD-I.

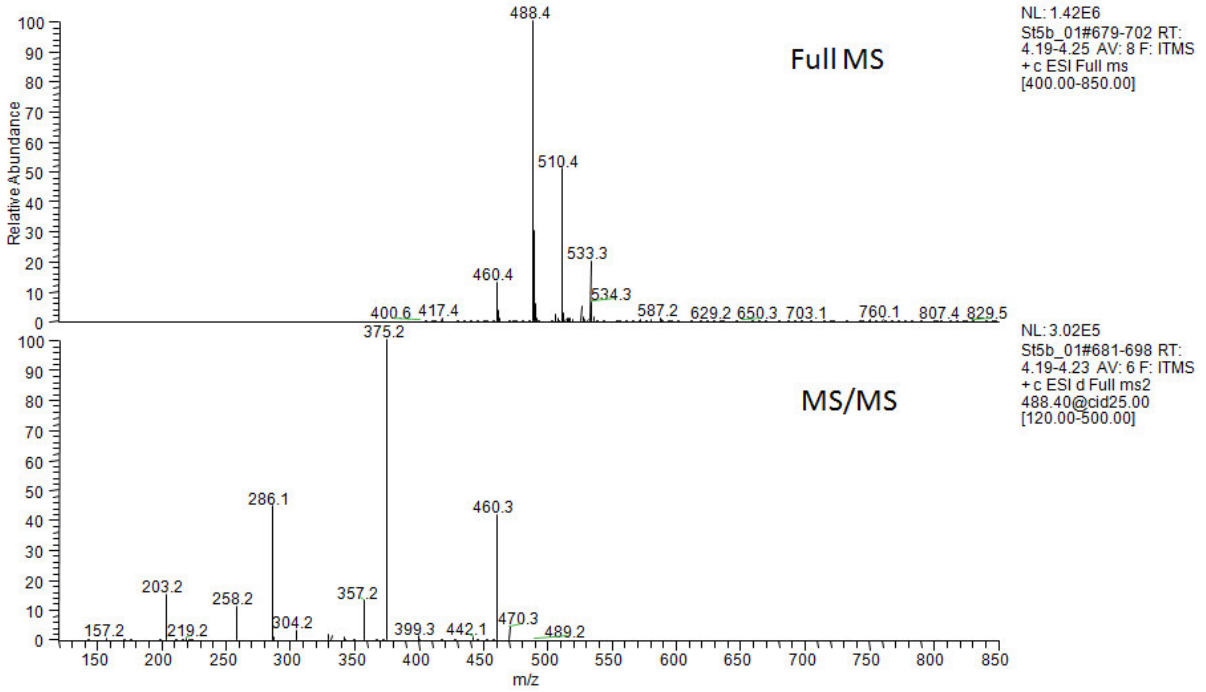


Figure 6.4: +ESI mass spectrum of BVD I contained in first extract with MeOH. The top panel was obtained by the full scan regime (120 - 850 amu), the bottom panel by the tandem MS/MS.

BVD I showed the highest abundance, followed by BVD F and BVD M. These peptides were contained to a higher degree in the extract of the fungi than all the others, but still they were present. BVD I provides a typical +ESI spectrum with [M+H]+ of 489.3. This is the peak of BVD-I, from which we had the standard. A water loss results in a peak at 460.4 in the spectrum. The m/z 510.4 is a sodiated BVD-I adduct [MH + Na+]. The [MH+] precursor ion at 488.3 was collisionally decomposed into several characteristic product fragments through tandem MS/MS. The mass to charge ratio of these fragments (375.2, 286.1, 203.2 and 460.3) was then used to find out the quantity of the peptide in the extract. The tandem CID (collisionally induced decomposition) MS/MS spectrum of the MH+ = 488.3 shows several distinct fragment ions which assign mostly the cleavage of the peptide bond. The lactone ring is preferably cleaved with a consequent preferable loss of an amino acid residue from position 4. Here, a Leu-loss is giving a fragment m/z = 375 (486 - 113) [10]. Homologous BVDs provide similar ESI spectra as shown in figure 6.5. Their molecular mass can easily be deduced from their LC-MS analysis.

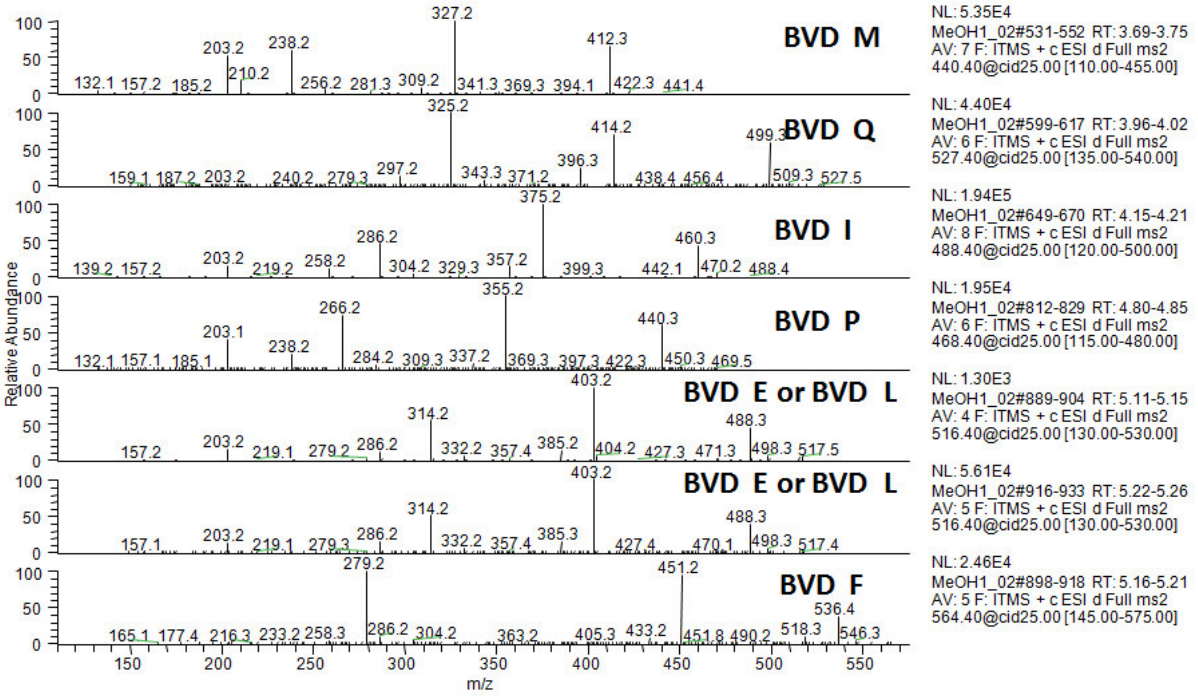


Figure 6.5: Mass spectra of different Beauverolide peptides present in the first peptide extract with MeOH. The relative abundance in dependence of the mass to charge ratio is shown. Again, just the most abundant BVDs were chosen.

6.3 Chromatographic separation of beauverolide peptides

Figure 6.6 clearly indicates very good separation of the BVDs on the reversed phase LC column. Even geometrical isomers of BVDs can be very well separated as shown in the case of the BVD-E and BVD-L having the same MH+ = 515.3.

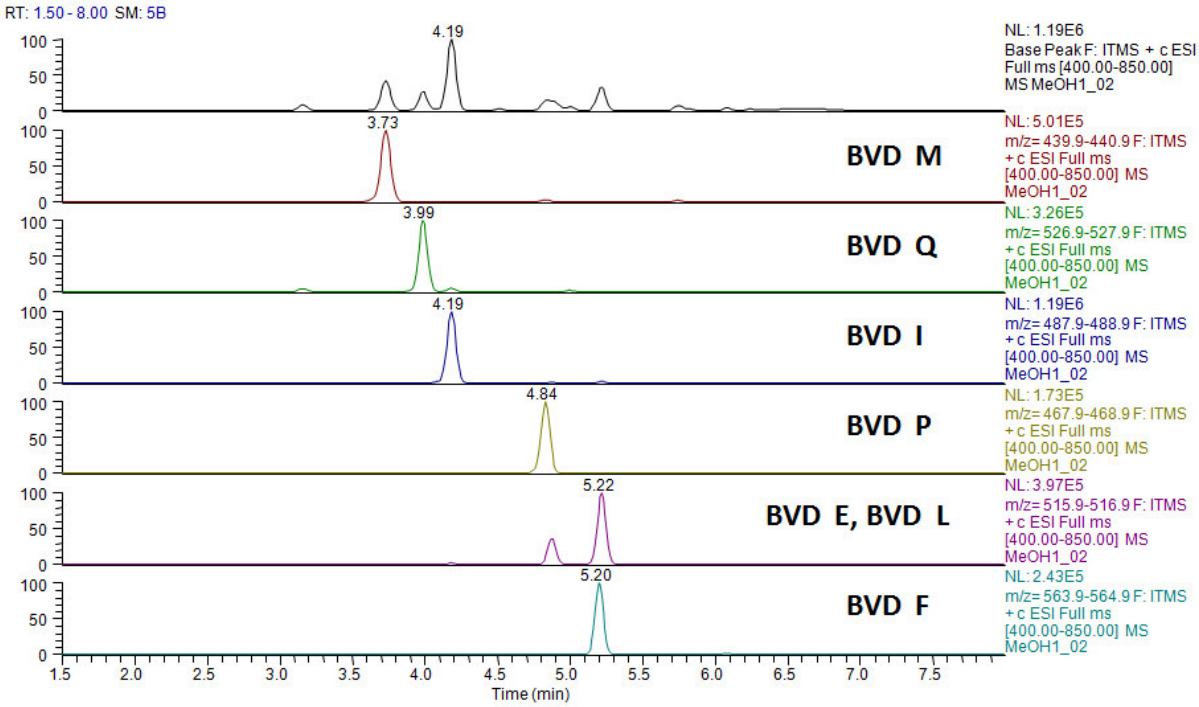


Figure 6.6: +ESI extracted chromatogram trace of the particular detected BVDs. Top trace: Base peak chromatogram of the HPLC-MS analysis. Other panels extracted chromatograms of the BVDs showing their excellent separation on a RP-HPLC column. BVDs E and L possess the same m/z.

6.4 BVD analysis in the Isaria strains

From the four different examined fungal strains (three from genera *Isaria fumosorosea* and one from *Beauveria bassiana*), two were found to contain BVD-I. The strain 8367, although it shows Isaria taxonomic characteristics, does not contain BVDs. Two cultures of PFR-97 were measured and the approximate content of BVD-I was 1.30 mg/g of mycelium. As the BVD-I standard is the only available BVD standard, no information about the amount of the other BVDs was obtained.

The following two Tables 6.1 and 6.2 give an overview of all the detected BVDs in the different fungal strains. Their relative abundance, calculated as peak area obtained from +ESI [M+Na] adducts is listed in the same tables.

Different BVDs	B.b. strain (Bota), 50x dil (Area)	%	I.f. strain (PFR-97), 50xdil (Area)	%
BVD_N	NF		299612.59	1.81
BVD_M	NF		2589064.73	15.67
BVD_Q	NF		2784467.77	16.85
BVD_I_FS	NF		7045635.65	42.64
BVD_D	NF		NF	
BVD_P	NF		581800.76	3.52
BVD_E	2156134.63	40.22	199411.73	1.21
BVD_R	NF		505584.89	3.06
BVD_J	504562.25	9.41	239478.29	1.45
BVD_L	NF		1370935.54	8.30
BVD_F	546021.77	10.19	691819.05	4.19
BVD_A	NF		NF	
BVD_B	1648893.50	30.76	NF	
BVD_K	12993.44	0.24	58488.41	0.35
BVC_C	492371.46	9.18	156918.40	0.95
	5360977.05	100.00	16523217.81	100.00

Table 6.1: Part 1: Results of the analysis of four different fungal strains (NF = not found).

Different BVDs	I.f. strain (Apopka), 50x dil (Area)	%	I.f. strain (8367), 50xdil (Area)
BVD_N	110241.69	0.75	NF
BVD_M	1561378.87	10.60	NF
BVD_Q	1224227.55	8.31	NF
BVD_I_FS	4346753.34	29.50	NF
BVD_D	116995.02	0.79	NF
BVD_P	500709.39	3.40	NF
BVD_E	685567.59	4.65	NF
BVD_R	312938.45	2.12	NF
BVD_J	208376.00	1.41	NF
BVD_L	1413535.39	9.59	NF
BVD_F	2939701.21	19.95	NF
BVD_A	33199.94	0.23	NF
BVD_B	207563.22	1.41	NF
BVD_K	75491.99	0.51	NF
BVC_C	997106.07	6.77	NF
	14733785.72	100.00	

Table 6.2: Part 2: Results of the analysis of four different fungal strains (NF = not found).

7 Conclusion

An LC-MS method was developed for characterization of BVD producing mycelia. Separation of BVDs is performed on a C18 Gemini column, the cyclodepsipeptides are detected by +ESI MS and tandem MS/MS. A calibration curve for BVD-I was constructed and diluted BVD extracts were analysed against an available BVD-I standard by external calibration. The described LC-MS method was suitable to study BVD profiles in culture broths. At least 15 BVDs were successfully detected in two Isaria strains, where BVD-I dominates in accordance with literature. In the strain of B.b., BVD-I was not detected. Quantitative analysis of the BVD-I in the studied two Isaria strains, Apopka and PFR-97, shows that the peptide is present at about 1-2 $\mu g/g$ of mycelium.

Using the method, quantification of BVD-I is amenable in the concentration range 0.1 - 5 ppm.

The work thus indicates that the HPLC-MS method has been a good outcome for the characterization of BVDs in a diverse biological material and the method can be useful for future BVD metabolite investigations.

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