Proceedings

Naturally-Produced Beauvericins and Divergence of *BEAS* Gene among *Fusarium* and *Trichoderma* Species †

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Abstract: Beauvericin (BEA) and its analogs are non-ribosomal cyclodepsipeptide mycotoxins produced by a wide range of fungal species, including saprotrophs, plant, and insect pathogens, particularly belonging to *Fusarium*, *Trichoderma*, *Beauveria*, and *Isaria* genera. Most beauvericin analogs were described among *Beauveria* and *Isaria* genera as "unnatural" beauvericins by adding amino acid precursors to the growing media. The aim of the study was to find BEAs naturally-synthesized by *Fusarium* species and tentatively determine their structures using mass spectrometry. Moreover, because of the unknown ability to produce beauvericin by *Trichoderma* fungi, we carried out the quantitative analysis using UPLC-MS. We also analyzed the polymorphism of the *BEAS* gene by sequencing partial *BEAS* regions from *Trichoderma* and *Fusarium* species. We screened five fungal cultures from the *Fusarium* genus cultivated on rice grain for the presence of the new natural beauvericins. The peptide sequence data of beauvericin analogs were established using MS/MS experiments as well as amino acid and hydroxy acid analysis following acid hydrolysis. Ten cyclodepsipeptide analogs described earlier were tentatively identified in the extract. In addition, two so far undescribed tyrosine-containing beauvericin analogs were tentatively identified in the cultures. Moreover, a quantitative analysis of beauvericin was performed using a UPLC-MS in eleven *Trichoderm* and six *Fusarium* rice cultures. The phylogenic analyses of beauvericin synthase (*BEAS*) divergence were performed on the basis of sequenced PCR-amplified fragments from *Trichoderma* and *Fusarium* fungi and partial reference genes from the GenBank database (representing *Beauveria*, *Fusarium*, and *Trichoderma* genera). This study demonstrates the high variability of naturally-produced new types of beauvericins, such as tyrosine-containing analogs in *Fusarium* fungi. It also shows that fungi belonging to the *Trichoderma* genus possess the ability to produce beauvericin.

Keywords: beauvericin; *Fusarium*; *Trichoderma*; cyclodepsipeptide

1. Introduction

Beauvericin (BEA) (Figure 1) and its analogs are non-ribosomal cyclodepsipeptide mycotoxins produced by a wide range of fungal species, including saprotrophs, plant, and insect pathogens, particularly belonging to *Fusarium*, *Trichoderma*, *Beauveria*, and *Isaria*

genera [1–4]. Most beauvericin analogs were described among *Beauveria* and *Isaria* genera as "unnatural" beauvericins (BEAs), synthesized by adding amino acid precursors to the growing media [5–7]. The aim of the study was to find BEAs naturally-synthesized by *Fusarium* species and tentatively determine their structures using mass spectrometry. Moreover, because of the unknown ability to produce beauvericin by *Trichoderma* fungi, we carried out the quantitative analysis using UPLC-MS. We also analyzed the polymorphism of *BEAS* gene by sequencing partial *BEAS* regions from *Trichoderma* and *Fusarium* species.
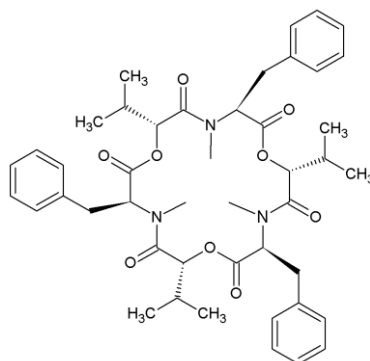

Figure 1. Chemical structure of beauvericin [3].

2. Methods

2.1. Fungal Strains, Media and Growth Conditions

For our study we investigated twenty two *Fusarium* and *Trichoderma* strains (Table 1), which were identified earlier [3,8–12] and deposited in the fungal strain collection of the Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland. For purification and genomic DNA extraction, the individual fungi strain were cultivated on potato dextrose agar medium (PDA, Oxoid, Basingstoke, UK) for seven days. The qualitative and quantitative BEAs analyses were conducted using fourteen-day-old pure rice cultures of each fungal strain. The samples for chemical analyses were prepared in three replications [1,3,4].

2.2. DNA Extraction, PCR Primers, Cycling Profiles and DNA Sequencing

The genomic DNA extraction was carried out according to Gorczyca et al. [13]. The beauvericin synthase gene (*BEAS*) was partially amplified using a degenerate primer pair BEA_F2/BEA_R2 (5′-TGGACDTCHATGTAYGAYGG-3′/5′-GGCTCRACRAGMARYT-CYTC-3′) designed in previous work [3].

Polymerase chain reactions (PCRs) were conducted using Phire II HotStart Taq DNA polymerase (Thermo Scientific, Espoo, Finland) and the conditions of the reactions were described by Tomczyk et al. [14].

For sequence analysis, PCR amplicons were purified with exonuclease I (Thermo Scientific, Espoo, Finland) and FastAP shrimp alkaline phosphatase (Thermo Scientific, Espoo, Finland). The purified products of PCR were labeled using forward primer and the BigDyeTerminator 3.1 kit (Applied Biosystems, Foster City, CA, USA) according to Kozłowska et al. [15].

The phylogeny reconstruction were done using MEGA7 software [16] (maximum parsimony approach, enabled closest neighbor interchange heuristics with default settings, 1000 bootstrap iterations).

2.3. Mycotoxin Analyses

2.3.1. Extraction, Purification and Liquid Chromatography Mass Spectrometry Analyses

The extraction and purification of beauvericin from pure fungal rice cultures (15 g) was carried out according to by Stępień and Waśkiewicz [11]. The final methanolic solution (2 mL) was filtered using a 0.20 μm Waters HV membrane filter before injection into the UPLC-triple quadrupole mass spectrometer (TQD) system for quantitative analysis. The analytical methods with conditions of quantitative beauvericin analysis using of the Aquity UPLC chromatograph (Waters, Manchester, MA, USA), electrospray ionization triple quadrupole mass spectrometer (TQD) (Waters, Manchester, MA, USA) were described earlier by Urbaniak et al. [3]. For data processing Empower™ 2 software was used (Waters, Manchester, UK).

2.3.2. Liquid Chromatography High-Resolution Mass Spectrometry (HRMS)

To determine the elemental composition of individual beauvericins analogs, extracts were analyzed using a Q-Exactive Fourier-transform high-resolution mass spectrometer (Thermo Fisher Scientific), which was interfaced to a Dionex UltiMate 3000 UHPLC (Thermo Fisher Scientific). The chromatographic method and condition of qualitative analysis was described earlier by Urbaniak et al. [1]. Elemental compositions were calculated using Xcalibur, version 2.3. (Thermo Fisher Scientific).

2.3.3. Chromatographic Fractionation, Acid Hydrolysis, and Amino and Hydroxy Acid Analysis

The dried extracts were conditioned with 2 mL of chloroform and applied to 100-mg silica gel columns (Phenomenex, Torrance, CA, USA). All of the fractions were prepared according to Urbaniak et al. [1]. For acid hydrolysis, the appropriate fractions were evaporated and dissolved in 200 μL of acetonitrile, followed by the addition of 200 μL of 6 M of hydrochloric acid. The preparation of the samples for acid hydrolysis of the individual fractions was described earlier by Urbaniak et al. [1].

3. Results and discussion

We screened five fungal cultures from the *Fusarium* genus cultivated on rice grain for the presence of the new natural beauvericins. The peptide sequence data of beauvericin analogs were established using MS/MS experiments as well as amino acid and hydroxy acid analysis following acid hydrolysis. Twelve cyclodepsipeptide analogs described earlier were tentatively identified in the extract. In addition, a so far undescribed two tyrosine-containing beauvericin analogs were tentatively identified in the cultures. Moreover, quantitative analysis of beauvericin was performed using a UPLC-MS in eleven *Trichoderma* and six *Fusarium* rice cultures. The characterization of twenty two fungal strains and their metabolic profile has been show in Table 1.

Table 1. Characterization of twenty two fungal strains and their metabolic profile.

Strain	Species	Source/Host	Metabolic profile	References
1337	*F. avenaceum*	wheat	BEA	[1,4]
41/9/3	*F. acuminatum*	pea	BEA, BEA C, BEA D, BEA G$_1$	[1,4]
P35	*F. concentricum*	pineapple	BEA, BEA B, BEA C, BEA D, BEA E, BEA F/A, BEA J, BEA G$_1$, BEA G$_2$, BEA K, BEA L	[1,4]
RT6.7	*F. proliferatum*	rice	BEA, BEA B, BEA C, BEA D, BEA E, BEA F/A, BEA J, BEA G$_1$, BEA G$_2$, BEA K	[1,4]
MU12	*F. verticillioides*	banana	BEA, BEA D, BEA G$_1$, BEA K	[1,4]
KF3566	*F. proliferatum*	rice	BEA	[3,11]
KF3386	*F. oxysporum*	pineapple	ND	[3, 12]
KF3406	*F. concentricum*	pineapple	BEA	[3,12]
KF3564	*F. polyphialidicum*	pineapple	ND	[3,12]

KF337	*F. nygamai*	pigeon pea	BEA	[3,11]
KF3327	*F. guttiforme*	pineapple	BEA	[3,12]
AN240	*T. atroviride*	decaying wood	BEA	[3,8]
AN255	*T. viride*	decaying wood	BEA	[3,9]
AN251	*T. koningiopsis*	decaying wood	BEA	[3,8]
AN143	*T. koningiopsis*	decaying wood	BEA	[3,10]
AN242	*T. viride*	decaying wood	BEA	[3,8]
AN327	*T. gamsii*	decaying wood	ND	[3,8]
AN359	*T. longipile*	decaying wood	ND	[3,8]
AN421	*T. viride*	decaying wood	ND	[3,8]
AN528	*T. atroviride*	decaying wood	BEA	[3]
AN494	*T. paraviridescens*	decaying wood	ND	[3,8]
AN550	*T. gamsii*	decaying wood	ND	[3,10]

BEA—beauvericin; ND—not detected.

3.1. Non-Ribosomal Peptide Synthase Genes Divergence

The received PCR-amplified sequences represented one region of the beauvericin synthase gene, which were then analyzed using ClustalW algorithm. Then, a dendrogram was calculated for the beauvericin synthase (*BEAS*) gene fragments obtained with the BEA_F2/BEA_R2 primers in various genotypes of beauvericin-producers and non-producers, belonging to the *Hypocreales* order (Figure 2). Additionally, the reference gene fragments were downloaded from GenBank database for beauvericin synthase. Moreover the sequence of enniatin synthase from *F. scirpi* (GenBank Acc. CAA79245.2) and *F. oxysporum* (GenBank Acc. KP000028.1) were added to the analysis as outgroup.

Three divided groups can be observed, the first consisting of *Trichoderma* strains, the second of *Fusarium* strains and the third of *Beauveria bassiana* strains. The phylogenic analysis show the high similarities (between 91% and 100%) of sequences among *Trichoderma* spp. While, the sequences of *Fusarium* spp. are more divergent, only the PCR amplicons acquired from *F. proliferatum* (KF3566) and *F. concentricum* (KF3406) strains showed high nucleotide similarity (about 99% of identical bases). The reference sequence of *BEAS* from *F. oxysporum* (GU294760.1), such as obtained *BEAS* sequence from *F. polyphialidicum* (KF3564) showed some variance, grouping with the reference enniatin synthetases from *F. equiseti* and *F. oxysporum* (Figure 2). This would suggest that not only are there distinguishable alleles of *BEAS* and *ESYN* but also that both variants can be found in the strains of the same species.

This hypothesis may be in line with the findings described in the previous work of Stępien and Waśkiewicz [11]. This study showed that Esyn1/Esyn2 and beas_1/beas_2 primers were used to obtain sequences of two different regions of the enniatin synthase gene (*Esyn1*) in various genotypes of *Fusarium* fungi. The phylogenetic analysis clearly showed the divided groups on enniatin and beauvericin producers, and revealed that the majority of the strains produced a mixture of BEA and ENNs. Nowadays, numerous partial regions of the *Esyn1* from various fungal species have been published [11,17], however, only a few reports are available on the structure of the divergent *BEAS*. Recently, *B. bassiana* [18,19], *F. venenatum* [20] and *F. proliferatum* [21] are the fungal species, which produce BEA and possess described *BEAS* gene. For *Trichoderma*, only one research paper has been published, where authors described the reference sequence as "similar to the *BEAS* gene" [22].
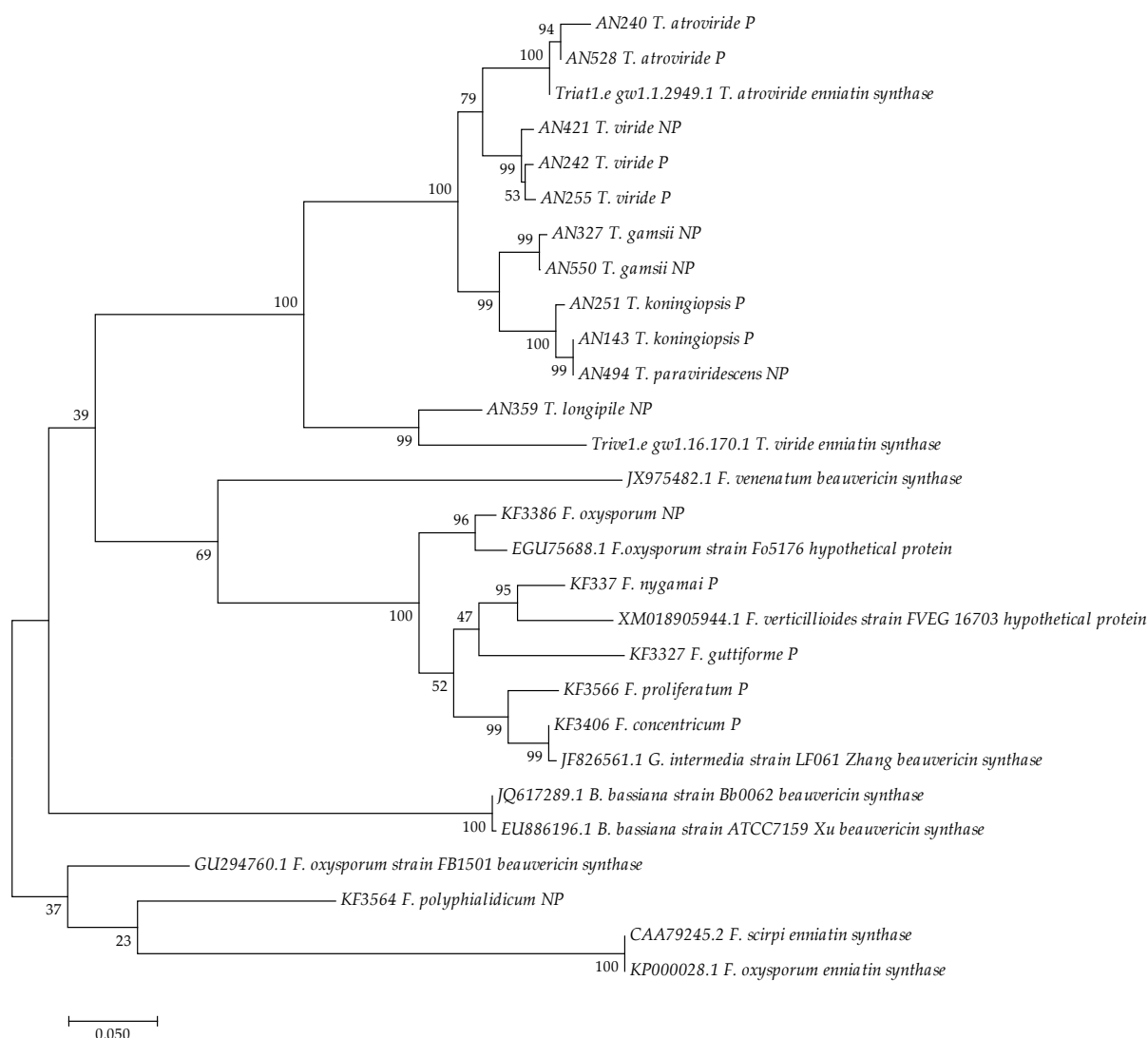
Figure 2. The most parsimonious tree created for a partial beauvericin synthase (*BEAS*) gene sequence obtained with BEA_F2 andBEA_R2 primers from 17 strains of *Fusarium* and *Trichoderma* species. *B. bassiana* (GenBank Acc. EU886196.1; JQ617289.1), *F. proliferatum* (GenBank Acc. JF826561.1 - *G. intermedia*), *F. scirpi* (GenBank Acc. CAA79245.2), *F. venenatum* (GenBank Acc. JX975482.1), *F. verticillioides* (GenBank Acc. XM018905944.1), *F. oxysporum* (GenBank Acc. KP000028.1; GU294760.1; EGU75688.1), *T. atroviride* (JGI ID: Triat1.e_gw1.1.2949.1) and *T. viride* (JGI ID: Trive1.e_gw1.16.170.1) sequences were included as the reference, as well as for outgrouping. The maximum parsimony approach and bootstrap test were applied (1000 replicates). "P" producer or "NP" non-producer of beauvericin [3].

3.2. In vitro BEA Biosynthesis

The concentrations of beauvericin in each sample of fungal rice culture have been shown in Table 2. The most efficient producers among *Hypocreales* fungi were the strains from *Fusarium* genus.

Table 2. Mean concentrations of beauvericin (µg/g) produced in vitro by studied *Fusarium* and *Trichoderma* strains along with standard deviations [3].

Species	Strain	Concentration of beauvericin [µg/g]	References
T. atroviride	AN240	8.78 ± 0.92	[3]
T. viride	AN255	3.02 ± 0.41	[3]
T. koningiopsis	AN251	3.85 ± 2.77	[3]

T. koningiopsis	AN143	4.22 ± 0.39	[3]
T. viride	AN242	2.74 ± 0.35	[3]
T. gamsii	AN327	ND	[3]
T. longipile	AN359	ND	[3]
T. viride	AN421	ND	[3]
T. atroviride	AN528	5.54 ± 0.46	[3]
T. paraviridescens	AN494	ND	[3]
T. gamsii	AN550	ND	[3]
F. proliferatum	KF 3566	90.85 ± 10.21	[11]
F. oxysporum	KF 3386	ND	[12]
F. concentricum	KF 3406	0.51 ± 0.06	[12]
F. polyphialidicum	KF 3564	ND	[12]
F. nygamai	KF 337	22.86 ± 2.66	[11]
F. guttiforme	KF 3327	7.70 ± 1.15	[12]

The most efficient beauvericin producers were *F. proliferatum* (KF3566) and *F. nygamai* (KF337), which synthesized this mycotoxin in amounts of 90 µg/g and 22.86 µg/g, respectively. In *F. oxysporum* (KF 3386) and *F. polyphialidicum* (KF 3564) cultures BEA was not detected. The lack of the mycotoxin presence in fungal samples can be explained by the fact that *F. oxysporum* and *F. polyphialidicum* may become non-pathogenic fungi by changing the niches between plant and soil [23,24]. Among *Trichoderma* strains beauvericin was detected at low concentrations in *T. atroviride* (AN240), *T. viride* (AN255), *T. koningiopsis* (AN251), *T. koningiopsis* (AN143), *T. viride* (AN242) and *T. atroviride* (AN528) rice cultures. *Trichoderma* fungi are mostly opportunistic, non-pathogenic and possess the ability to form mutualistic endophytic relationships with plants [25], this might suggest that the production of mycotoxin and that's why BEA was found in minor amounts.

3.3. Mass Spectrometry and Tentative Structure Determination of Beauvericins

The family of regular cyclodepsipeptides is represented by BEA, composed of three *N*-methyl amino acids and three hydroxy acid groups (D-Hiv). Most of the BEA analogs consist of three groups of *N*-methyl-phenylalanine and BEAs J, K, and L are the exceptions, containing one, two, or three groups of *N*-methyl-tyrosine, respectively [1]. Moreover, BEA D and E are composed of demethylated amino acids—phenylalanine and leucine [1,4]. BEAs can also differ in hydroxy acids possession, like BEA A/F, B, and C, having in their structures D-2-hydroxy-3-methylpentanoic acid (D-Hmp), whereas BEA G_1 and G_2 have D-2-hydroxybutyric acid (D-Hbu). On the other hand, BEA and BEA D, E, J, K, and L possess D-2-hydroxyisovaleric acid (D-Hiv) [1,4]. Some of BEA analogs, such as BEA B, C, J, K, L, G_1, G_2 were described earlier as amino acid precursor-directed compounds, detected in fungal cultures of *Beauveria* sp., *Acremonium* sp., and *Paecilomyces* sp. [5,7,26]. We have proven that all mentioned BEAs were naturally produced by phytopathogenic fungi from the *Fusarium* genus [1,4].

The fragmentation of the sodiated molecular ions [M + Na]$^+$ of beauvericin and its analogs have been shown previously as a method of high diagnostic value for structure determination, allowing the sequencing of depsipeptides [5,26]. BEA was used as a model molecule for the fragmentation patterns with the putative beauvericin analogs comparison. BEA contains *N*-methyl-phenylalanine and D-Hiv moieties, and this fact we confirmed by the loss of −161 Da and −100 Da due to *N*-methyl-phenylalanine and D-Hiv, respectively (Figure 3), which we observed on the HRMS/MS spectrum from higher-energy collision dissociation (HCD). For BEA J and BEA K we observed the loss of −177 Da and −161 Da products ions, which are *N*-methyl-tyrosine and *N*-methyl-phenylalanine, respectively. The amino acid tyrosine is equivalent to the 4-hydroxy-phenylalanine; thus we hypothesized that the additional oxygen in BEA J and BEA K was due to the partial exchange of *N*-methyl-phenylalanine by *N*-methyl-tyrosine.
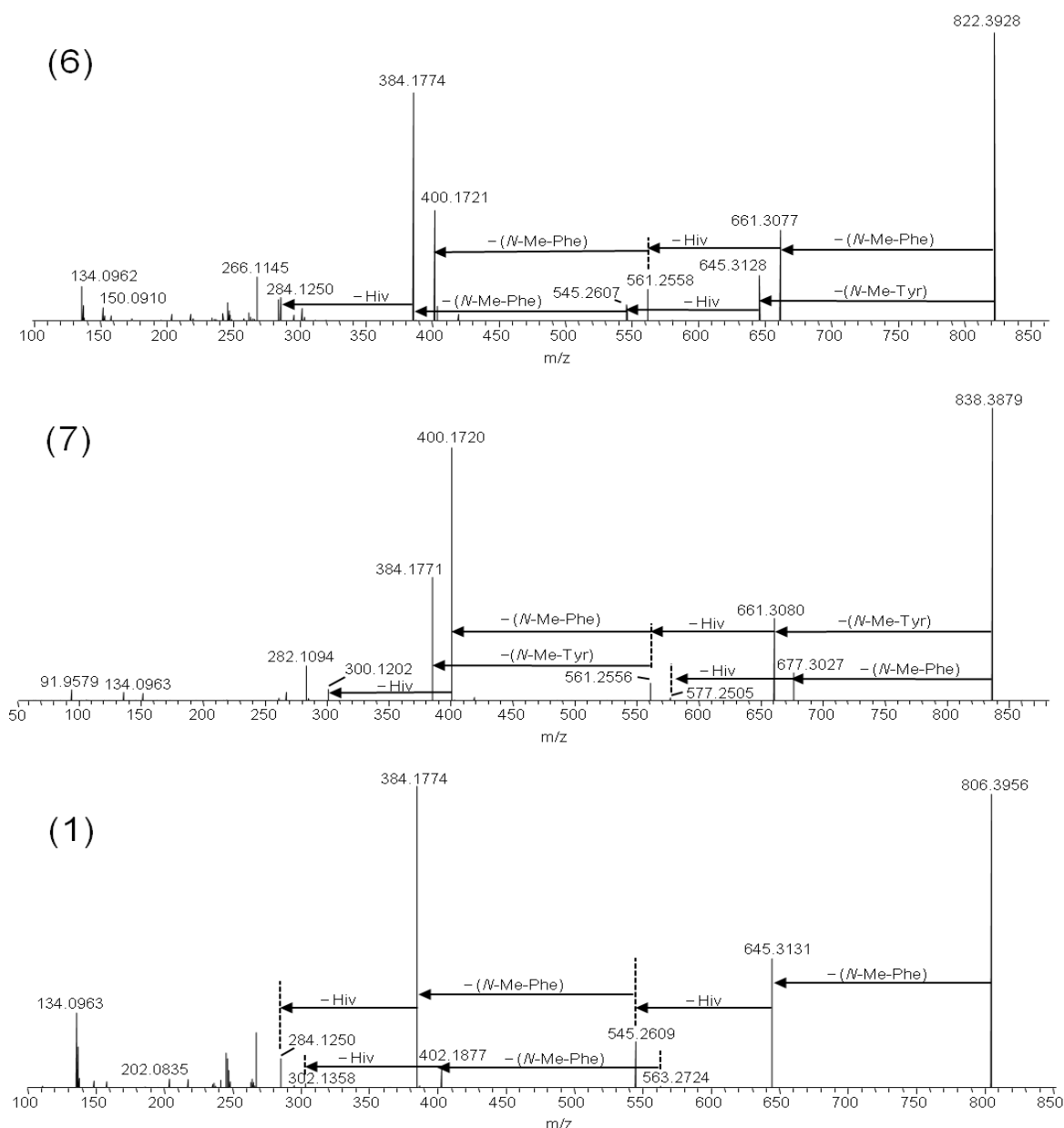
Figure 3. LC–HRMS/MS spectra from higher-collision dissociation of the [M + Na]+ ions of beauvericin analogues containing *N*-methyl-tyrosine: beauvericin J (m/z 822.3928) and beauvericin K (m/z 838.3879), compared to beauvericin (m/z 806.3956) [1].

To verify the identity of the amino acid *N*-methyl-tyrosine in BEA J, K and L we conducted the acid hydrolysis of the molecules (Figure 4c2) and compared the amino acid composition to the standards (Figure 4c1). Hydrophilic interaction chromatography (HILIC) coupled with ion trap mass spectrometry of the hydrolysates, in comparison with reference standards, showed the presence of *N*-methyl-tyrosine in the enriched fractions (Figure 4).
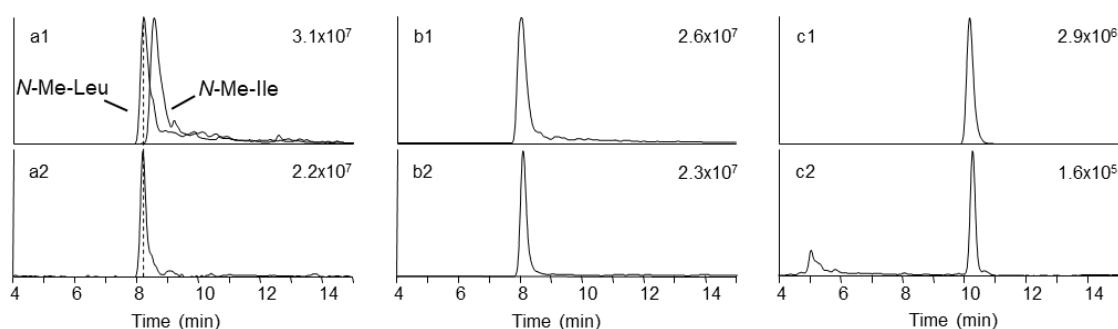
Figure 4. Extracted ion chromatograms from amino acid analysis of hydrolyzed peptide mixtures containing either *N*-methyl-tyrosine containing beauvericins or beauvenniatin L using hydrophilic interaction chromatography ion trap mass spectrometry. Upper traces represent chromatograms from pure reference standards, while lower traces are from hydrolyzed depsipeptide mixtures: (**a1**, **a2**) *N*-methyl-leucine and *N*-methyl-isoleucine; (**b1**, **b2**) *N*-methyl–phenylalanine; and (**c1**, **c2**) *N*-methyl–tyrosine. Individual chromatograms are scaled to the highest peak (number in the top right-hand corner of each chromatogram) [1].

4. Conclusions

This study demonstrates the high variability of naturally-produced new types of beauvericins, such as tyrosine-containing analogs in *Fusarium* fungi. It also shows that fungi belonging to the *Trichoderma* genus possess the ability to produce beauvericin.

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