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# Evidencing 98 secondary metabolites of *Penicillium verrucosum* using substrate isotopic labeling and high-resolution mass spectrometry

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## Highlights

- Double stable isotope labeling of substrate for labeling the fungal metabolites
- *Evidencing of 98 secondary metabolites produced by Penicillium verrucosum on wheat*
- 82 unknown secondary metabolites detected on *Penicillium verrucosum* by HPLC-HRMS
- Detailed MS and MS/MS data of all detected unknown metabolites

**ABSTRACT:** Industrial applications of fungal compounds, coupled with the emergence of fungal threats to natural ecosystems and public health, have increased interest in filamentous fungi. Among all pathogenic fungi, *Penicillium verrucosum* is one of the most common mold-infecting stored cereals in temperate regions. However, it is estimated that 80% of fungal secondary metabolites remain unknown. To detect new *P. verrucosum* compounds, an untargeted metabolomic approach was applied to fungus

grown on wheat grains labeled with stable isotopes: (i) natural grains (99%  $^{12}\text{C}$ ); (ii) grains enriched with 97% of  $^{13}\text{C}$ ; and (iii) grains enriched with 53% of  $^{13}\text{C}$  and 97% of  $^{15}\text{N}$ . Analyses performed by high-performance liquid chromatography coupled with high-resolution mass spectrometry (HPLC–HRMS) enabled the specific detection of fungal metabolites, and the unambiguous characterization of their chemical formulas. In this way, 98 secondary metabolites were detected and their chemical formulas were determined. Of these, only 18 identifications could be made based on databases, the literature and mass spectrometry fragmentation experiments, with the result that 80 were totally unknown. Molecular networks were generated to analyze these results, leading to the characterization by  $\text{MS}^n$  experiments of a new fungisporin produced by *P. verrucosum*. More generally, this article provides precise mass spectrometric data about all these compounds for further studies of the *Penicillium* metabolome.

**Key words:** fungus; *Penicillium verrucosum*; mass spectrometry; stable isotope labeling; secondary metabolite; molecular networking

## 1. Introduction

Filamentous fungi are well-known for their ability to produce a wide range of secondary metabolites. These molecules are mostly involved in fungal defense and environmental adaptation systems [1]. Since the discovery of penicillin as the first antibiotic of fungal origin used for human health [2], natural compounds have attracted great interest in a variety of industrial areas. Today, 42% of the metabolites adapted for human health applications are derived from fungal compounds [3]. In the agri-food field, they are commonly used as plant-growth hormones [4] or food additives [5]. On the other hand, fungal secondary metabolites known as ‘mycotoxins’ produce deleterious effects directly on their vegetal hosts, but also indirectly through human food or livestock feed contamination. Mycotoxins are considered the most important chronic dietary risk factor, higher than pesticide residues [6]. Various types of toxicity are associated with fungal metabolites. Some of them display carcinogenic potential, such as aflatoxin, [7] or nephrotoxicity, such as ochratoxin A [8]. Others are estrogenic mycotoxins such as zearalenone,

which is involved in reproductive disorders of farm animals and in hyperestrogenic syndromes in humans [9]. Moreover, contaminations by mycotoxins produce major economic consequences, due to the destruction of crops and stocks [10]. Today, although several hundreds of secondary metabolites are characterized as mycotoxins and about 30 are considered of human and animal health concern, only six of them are regulated. Furthermore, fungal genome observations suggest that 80% of their secondary metabolome remains unknown, highlighting the great proportion of compounds still to be discovered [11].

*Penicillium verrucosum* is one of the most common molds infecting stored cereals in temperate regions [12]. Its ability to grow at mild temperatures can lead to the infection of grains during their storage, particularly in case the drying or re-moistening process fails [8,13]. In Canada, annual economic losses due to the elimination and destruction of infected cereals stocks are estimated to be over 260 million Canadian dollars [14]. Moreover, ochratoxin A, the major mycotoxin of *P. verrucosum*, is considered to be the most potent renal carcinogen of natural origin [15], and is classified as a possible human carcinogen (Group 2B) [16]. According to some studies, ochratoxin A could be the cause of Balkan Endemic Nephropathy (BEN), a fatal disease first recorded in 1956 [17–19]. Some other secondary metabolites of *P. verrucosum* have been characterized, such as citrinin (nephrotoxic) and ochratoxin B (an unchlorinated intermediate of ochratoxin A biosynthetic pathway), which are considered as mycotoxins, and some metabolites belonging to the families of verrucolones and verrucines [20].

Among the relevant analytical techniques for the characterization of secondary metabolites, untargeted metabolomics using High-Resolution Mass Spectrometry (HRMS) are particularly interesting [21]. This technique enables the detection of both known and unknown metabolites, and the determination of their exact mass, and contributes to their structural identification. However, data interpretation remains complex due to the non-specific detection of compounds including both metabolites and contaminants, and the difficulty of achieving unambiguous chemical formulas, even with highly accurate mass measurement [22]. To overcome these difficulties, a number of original analytical approaches using stable isotope labeling (SIL) have been developed. Bueschl *et al.* pointed out the benefits provided by this

strategy for the specific detection of the metabolites, as well as for the characterization of the global biochemical composition of biological samples [23]. One SIL strategy consists of growing a fungus on the non-labeled glucose medium ( $^{12}\text{C}$ ) as well as on the fully  $^{13}\text{C}$  labeled glucose. The two extracts are then pooled and analyzed by High-Performance Liquid Chromatography (HPLC) coupled with HRMS [24,25]. The detection of two mirror isotopic distributions enables the rapid determination of the number of carbon atoms in all the detected fungal metabolites, facilitating the determination of their chemical formula. However, for high-molecular weight compounds, a single isotope labeling might not be enough to obtain a unique chemical formula and another stable isotope labeling would be required.

Moreover, fungi modulate the synthesis of their secondary metabolites depending on their needs for the medium colonization. This principle, commonly called OSMAC (One Strain Many Compounds), means that every small change in growth conditions can completely alter the fungal secondary metabolism. This implies that the fungal metabolome should be studied in culture conditions as close as possible to its natural growth environment [26]. As stressed by Hallas-Moller *et al.* [27], the study of cereal-associated species such as *P. verrucosum* has indeed been chemically investigated, but only by outdated analytical systems and on laboratory culture media. Thus, a SIL method based on the growth of plant pathogens on labeled cereals was previously developed in our laboratory [28]. To avoid the formula ambiguity for high-molecular weight metabolites, two isotopic labelings were used by the HPLC-HRMS analysis of carbon 13 ( $^{13}\text{C}$ ) and nitrogen 15 ( $^{15}\text{N}$ ) labeled wheats. Below, following the successful validation of the method on the well-documented metabolism of *Aspergillus fumigatus* [28], we present the application to the saprophytic fungus *P. verrucosum*, with the aim of detecting unknown metabolites produced during wheat grain contamination.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents.

The formic acid, and solvents used for sample preparation (ethyl acetate and methanol) and for the analyses (acetonitrile) were purchased from Fisher Scientific (Thermo Fisher Scientific, Illkirch, France). The ethyl acetate and methanol were HPLC grade and the acetonitrile was MS (mass spectrometry) grade. Ultrapure water was generated from a Milli-Q system (Millipore, Saint Quentin en Yvelines, France). Standards of ochratoxin A, citrinin and cyclopiazonic acid were purchased from Sigma (Sigma-Aldrich, Saint-Quentin Fallavier, France), and ochratoxin B was purchased from Fermentek (Fermentek Ltd, Jerusalem, Israel).

## 2.2. Identification of *Penicillium verrucosum*.

The study was performed on the strain NRRL 5571. Morphological identification was performed as recommended in the monograph of the *Penicillium* genus [29]. Genomic identification was conducted after DNA extraction. After 5 days of culture on YES medium (2.5 g of yeast extract, 10 g of glucose and 500 ml of distilled water), fungal DNA was extracted. After grinding, the mycelium was incubated in an extraction buffer (CTAB 1%, Tris-HCl pH 8 100 mM, EDTA pH8 20 mM and NaCl 1.4 M), Chloroform, phenol, and isopropanol were successively added. The pellet was washed with ethanol 70% and suspended in water. Amplification was performed with ITS (ITS 4 and 5) and  $\beta$ -tub (a and b) primers according to Adjovi *et al.* [30] and the PCR result was subjected to gel electrophoresis. Each isolated fragment was then purified with a GenElute PCR Clean-Up Kit (Sigma-Aldrich) and the DNA concentrations were measured with Nanodrop (ND-1000 program, Thermo Fisher Scientific, Illkirch, France). Then, they were sequenced by the dye terminator technology on an AB3130XL sequencer (Applied Biosystems). The DNA sequences of ITS and  $\beta$ -tubulin genes were finally compared by sequence alignment and deposited in GenBank under KY347870 and KY347871 accession numbers respectively.

## 2.3. Production of $^{13}\text{C}$ - and $^{13}\text{C}/^{15}\text{N}$ Labeled Wheats

Uniform isotopic enrichments were performed on the variety Caphorn of *Triticum aestivum* as previously described [31]. Briefly, two different enrichments were achieved in hermetically sealed

chambers (750 L) during a complete growth cycle, i.e. 6 months, with  $^{13}\text{C}$ -labeled  $\text{CO}_2$  and  $^{15}\text{N}$ -labeled nitrate and ammonium salts: one nearly 100%  $^{13}\text{C}$  enriched wheat (referred to as  $^{13}\text{C}$  wheat) and one nearly 50%  $^{13}\text{C}$  and nearly 100%  $^{15}\text{N}$  wheat (referred to as  $^{13}\text{C}/^{15}\text{N}$  wheat). The isotopic enrichments were measured with a Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, Illkirch, France):  $96.8\% \pm 0.15\%$  for the  $^{13}\text{C}$  wheat,  $53.4\% \pm 0.3\%$   $^{13}\text{C}$  and  $96.8\% \pm 0.07\%$   $^{15}\text{N}$  for the  $^{13}\text{C}/^{15}\text{N}$  wheat.

#### 2.4. Fungal Strain and Growth Conditions

The natural and labeled wheat grains were soaked in water and autoclaved twice. The water activity ( $a_w$ ) of the grains was then measured with an HC2-AW measurement device (Rotronik AG, Basserdorf, Switzerland), and sterile water was added where necessary to reach an  $a_w$  of 0.98 before inoculation. Thirty grams of each type of sterilized labeled wheat grains were placed in thin layers into sterile 140 mm diameter Petri dishes (Sarstedt, Marnay, France) to create a total of 4 culture media: one dish with  $^{13}\text{C}$  wheat, one dish with  $^{13}\text{C}/^{15}\text{N}$  wheat and two dishes with  $^{12}\text{C}$  wheat. The first three plates were inoculated with 250  $\mu\text{L}$  of a spore solution (at  $10^5$  spore/mL) prepared from the PDA culture of the fungus. The second  $^{12}\text{C}$  wheat Petri plate was used as a control and incubated in the same conditions as the other samples but without fungus. The fungal cultures were then placed in the dark without shaking for 1 month at  $25^\circ\text{C}$ .

#### 2.5. Metabolite Extraction

After incubation, the content of each Petri dish (wheat grains and mycelium) was extracted with 200 mL of ethyl acetate. After 36 h of shaking on a shaking table IKA Labor technik HS501 (IKA, Staufen, Germany) at 180 rpm, the extract was filtered and evaporated to dryness. The resulting residue was dissolved in 400  $\mu\text{L}$  of methanol and filtered with 0.45  $\mu\text{m}$  Whatman filters (Whatman, GE Healthcare, Kent, U.K.) before HPLC–HRMS analysis.

#### 2.6. Analytical Parameters



The extracts were analyzed by High-Performance Liquid Chromatography (HPLC) using an Ultimate 3000 system (Thermo Fisher Scientific, Les Ulis, France). A gradient program of 0.1% formic acid in water (phase A) and 100% acetonitrile (phase B) was used as follows (flow rate, 0.2 mL/min): 0 min 20% of B, 30 min 50% of B, from 35 to 45 min 90% of B, from 50 to 60 20% of B. A volume of 10  $\mu$ L was injected on a reversed-phase Luna C18 column (150 mm  $\times$  2.0 mm, 5  $\mu$ m) (Phenomenex, Torrance, CA, U.S.A.). HPLC was coupled to a LTQ Orbitrap XL hybrid high-resolution mass spectrometer (Thermo Fisher Scientific, Les Ulis, France) using electrospray ionization (ESI) in the positive and negative modes. The ionization parameters were as follows: spray voltage +4.5 kV, capillary temperature 350  $^{\circ}$ C, sheath gas ( $N_2$ ) flow rate 40 au (arbitrary units), auxiliary gas ( $N_2$ ) flow rate 6 au for the positive mode, and spray voltage  $-3.7$  kV, capillary temperature 350  $^{\circ}$ C, sheath gas flow rate 30 au ( $N_2$ ), auxiliary gas flow rate 10 au ( $N_2$ ) for the negative mode. The full HRMS spectra were acquired at a resolution of 60,000 from  $m/z$  50 to  $m/z$  800. The high-resolution mass analyzer was calibrated in each ionization mode based on the supplier's protocol and calibration mixtures (Thermo Fisher Scientific, Les Ulis, France). The targeted MS/MS spectra were performed according to the following parameters: [Parameters for MS/MS by collision-induced dissociation (CID): resolution= 7500,  $MS^n$  maximum ion time= 200 ms, isolation width= 1.5 Da, Act. Q= 0.250 and activation time= 30 ms]. [Parameters for MS/MS by higher energy collision dissociation (HCD): resolution= 7500,  $MS^n$  maximum ion time= 200 ms, isolation width= 1.5 Da and activation time= 30 ms]. HCD and CID energies were adjusted to obtain appropriate fragmentation and maximum structural information. Since the analyses were performed to identify metabolites from a strictly qualitative point of view, neither quality control samples, nor replicates were necessary.

Analysis by HPLC coupled with diode-array-detection (Ultimate 3000 system, Thermo Fisher Scientific, Les Ulis, France) were performed to identify anacine. The chromatographic system and parameters were the same as for HPLC-HRMS analyses. The presence of anacine was monitored at a wavelength of 220 nm.

### 2.7. Identification of metabolites

Comparisons of proposed  $^{12}\text{C}$ ,  $^{13}\text{C}$  and  $^{13}\text{C}^{15}\text{N}$  formulas were performed thanks to the MassCompare program developed in-house (<http://www.toulouse.inra.fr/axiommm>) using a measurement accuracy of 5 ppm. C, H, O, N, S, P, Cl, K and Na were the only accepted elements.

Identifications of metabolites were classified according to the Metabolomics Standard Initiative [32]. Metabolites identified at level 1 in our results displayed the same retention time, chemical formula and MS/MS fragmentation pattern as the standard compound (section 2.1) measured in a parallel analysis in the same analytical conditions. The metabolites identified at level 2 in our results displayed an MS/MS or UV spectrum in accordance with the bibliography. The metabolites annotated as compounds known to be produced by *P. verrucosum* and presenting coherent MS/MS spectra were identified at the level 3A. The metabolites annotated as compounds never characterized on *P. verrucosum* and presenting coherent MS/MS spectra were identified at the level 3B. Complementary *in silico* fragmentations were performed using CFM-ID [33] and compared with experimental MS/MS spectra of annotated metabolites of level 3.

A molecular network generated using GNPS [34] was created from MS/MS spectra in the CID mode of all annotated secondary metabolites. The software parameters were set as follows: ion mass tolerance= 0.0036 Da, cosine score= 0.7, minimum match fragment ion= 1, maximum connected components= 100 and Top-K= 100, filter peaks removed. The resulting molecular network was visualized in Cytoscape 2.8.2 [35].

### 3. Results and Discussion

#### 3.1. Certification of *Penicillium verrucosum*

The identification of fungal strains could be problematic for secondary metabolome studies due to the lack of robust classification criteria [20], despite the availability of genetic classification. For example, species such as *Penicillium nordicum* and *P. verrucosum* were considered as *Penicillium viridicatum* for some time and some of their secondary metabolites are still referenced as *P. viridicatum* compounds, therefore misleading studies on these fungi. Given the confusion between these similar fungi, it was

crucial for our study to ensure the identification of *P. verrucosum*. Three different approaches were investigated. Firstly, a morphological characterization was achieved and displayed both macroscopic and microscopic *Penicillium* properties, namely terverticillate conidiophores, rough-walled and globose conidia. At this stage, no differentiation could be made between *P. verrucosum* and *P. nordicum* as these two species display the same characteristics. Then, cultures on YES agar were performed and showed a red-brown reverse color characteristic of *P. verrucosum* under some sporulation areas. This observation is the only morphological point enabling differentiation between *P. verrucosum* and *P. nordicum* [29]. Secondly, the fungal secondary metabolite analysis achieved by HPLC-HRMS led to the identification of ochratoxin A and citrinin, which were validated by comparison with the standards. Ochratoxin A is known to be produced by both *P. nordicum* and *P. verrucosum* but, according to the *Penicillium* classification of Larsen *et al.* [20], only *P. verrucosum* is known to produce citrinin in addition to ochratoxin A. Finally, a genetic identification was conducted. Since Samson *et al.* [36] estimated that  $\beta$ -tubulin sequences are relevant species markers, we compared the  $\beta$ -tubulin DNA sequences of the studied fungus with the standard sequences of *P. verrucosum* and *P. nordicum*. As a result,  $\beta$ -tubulin sequences showed strong similarities with those of *P. verrucosum*. ITS DNA sequences are well-conserved sequences also used as species markers in fungal studies. Comparison of the ITS sequences confirmed the strong similarity between the studied strain (NRRL 5571) and the standard sequences of *P. verrucosum*. To conclude, the three identification steps (morphological observations, metabolite characterizations and genetic validation) were in accordance with the identification of *P. verrucosum*. NRRL 5571 was also classified as *P. verrucosum* by Larsen *et al.* in a study characterizing 48 *P. verrucosum* strains [20].

### 3.2.HPLC-HRMS Analysis of the Three Wheat Cultures

The characterization of the fungal secondary metabolome was carried out according to the protocol previously developed in our group [28]. Natural wheat,  $^{13}\text{C}$  wheat and  $^{13}\text{C}^{15}\text{N}$  wheat were infected with *P. verrucosum* under adapted culture conditions (*i.e.* culture in the dark at 25°C for 30 days). A control sample, corresponding to non-infected natural wheat, was placed in the same environment. The secondary

metabolites were then extracted and the samples analyzed separately by HPLC-HRMS. The extracts were not pooled in order to limit the complexity of MS spectra due to the complex isotopic compositions used in our protocol and to avoid possible overlapping signals despite the high-resolution power provided by the Orbitrap analyzer. The total ion current chromatograms obtained in the positive and negative ionization modes are presented in Figure 1. First, the chromatogram and mass spectrum obtained from the  $^{13}\text{C}^{15}\text{N}$  labeling experiment were analyzed to specifically detect all the secondary metabolites. The isotopic labeling with 50%  $^{13}\text{C}$  and 100%  $^{15}\text{N}$  provides an easily recognizable signal, since it generates a specific ion pattern corresponding to a normal distribution of  $^{12}\text{C}/^{13}\text{C}$  on the mass spectrum (Figure 2C). These specific signals could be exclusively attributed to either the labeled substrate or the fungal metabolites, enabling the removal of any contaminant or chemical used during incubation or extraction. Here, 91 and 130 specific isotopic patterns were detected by positive (ESI+) and negative (ESI-) electrospray ionization modes respectively. Among these signals, 33 adducts and in-source fragment ions were rejected in the ESI+ mode (68 were rejected for the ESI - mode) in order to consider only molecular ions for each secondary metabolite [37]. This led to a final listing of 58 signals in the positive ionization mode and 62 in the negative mode. The same approach was applied to the  $^{13}\text{C}$  labeling experiment, in which secondary metabolites displayed another specific pattern (Figure 2B) due to the 3% of remaining  $^{12}\text{C}$ . For each previously detected  $^{13}\text{C}^{15}\text{N}$  labeled ions, the  $^{13}\text{C}$  equivalent signal was found in HRMS spectra. Each secondary metabolite was finally searched in the HRMS spectra obtained from the natural  $^{12}\text{C}$  experiment (Figure 2A). To validate the detection of similar metabolites in the three parallel HPLC-HRMS analyses ( $^{12}\text{C}$ ,  $^{13}\text{C}$  and  $^{13}\text{C}^{15}\text{N}$ ), chromatographic retention times and MS/MS spectra were compared. As displayed in Figure 2, MS/MS spectra were conducted on precursor ions detected at the same retention time (26.7 min in the example given in Figure 2), evidencing that the same metabolite was detected in the three labeled substrates. Finally, wheat molecules were rejected by comparing extract and control samples. In this way, 3 and 13 compounds were eliminated from the ESI+ and ESI - results, respectively. A final list of the 98 secondary metabolites detected from *P. verrucosum* is reported in Table 1. The metabolites detected both in ESI+ and ESI- are displayed as a single entry in Table 1.

For further identification of the detected metabolites, the first step was the determination of their chemical formulas. Each secondary metabolite, measured  $m/z$  in  $^{12}\text{C}$ ,  $^{13}\text{C}$  and  $^{13}\text{C}^{15}\text{N}$  extracts was compared using MassCompare, a software developed in-house. This tool calculates all the possible chemical formulas for each  $^{12}\text{C}$ ,  $^{13}\text{C}$  and  $^{13}\text{C}^{15}\text{N}$  metabolites, selects the common ones, and calculates the number of carbon and nitrogen atoms by subtractions between  $^{12}\text{C}$ ,  $^{13}\text{C}$  and  $^{13}\text{C}^{15}\text{N}$   $m/z$  values. In this way, MassCompare provides the unique chemical formula of each metabolite. Concerning the example illustrated in Figure 2, the mass difference between the  $m/z$  ratio of the  $^{12}\text{C}$  ion ( $m/z$  370.1285) and the  $^{13}\text{C}$ -labeled ion ( $m/z$  390.1951) was used to determine the number of carbon atoms ( $n_{\text{C}}= 20$ ). The mass differences between the  $m/z$  of the  $^{12}\text{C}$  ion, the  $^{13}\text{C}$ -labeled ion and the  $^{13}\text{C}/^{15}\text{N}$ -labeled ion ( $m/z$  382.1667) enabled the calculation of the number of nitrogen atoms ( $n_{\text{N}}= 1$ ) by MassCompare. With this information, the software determined a single chemical formula per metabolite ( $\text{C}_{20}\text{H}_{19}\text{O}_6\text{N}$  for the example of Figure 2). As displayed in Table 1, the metabolite formulas are almost homogenous in terms of heteroatom composition with a majority of oxygenated structures, and only seven non-oxygenated metabolites. Over half of the 98 metabolites contain at least one nitrogen atom. These observations were in agreement with the general composition of fungal secondary metabolites. These extrolites are synthesized by two major types of enzymes. The first, polyketide synthases (PKS), synthesize secondary metabolites from acetyl or malonyl groups to generate oxygenated compounds [38]. The second, nonribosomal peptide-synthetases (NRPS) ensure the incorporation of amino acids, leading to the synthesis of nitrogenous secondary metabolites. Among all detected metabolites, only three display other heteroatoms: one chlorinated and two sulfured formulas. The chlorinated compound was identified as the well-known ochratoxin A. To the best of our knowledge, it is the first time that sulfur containing metabolites has been detected in *P. verrucosum*.

### 3.3. Dereplication of Known Secondary Metabolites

Experimental MS/MS spectra were compared with the open-access GNPS library of natural products [34]. In this study, only one match was found but the MS/MS spectra were not sufficiently similar

to allow its identification. Alternatively, the database of natural compounds Antibase (2012) [39], coupled with the literature on fungal secondary metabolites, was used to successfully annotate several compounds according to their chemical formulas.

Ochratoxin A (compound 1 in Figure 3) is considered as the major toxin of *P. verrucosum* involved in the sanitary and economic troubles caused by this fungus [40,41]. This metabolite was suspected to be detected at 32.4 min at  $m/z$  404.0897 with a chlorinated isotopic pattern after positive ionization of the  $^{12}\text{C}$  sample. Separate observation of the  $^{12}\text{C}$  spectrum, with consideration of the chlorine atom, led to eight possibilities of chemical formulas using the nitrogen rules. The comparison of the three lists of possible formulas from the three different wheat cultures and calculation of the number of carbon and nitrogen atoms finally led to a unique chemical formula:  $\text{C}_{20}\text{H}_{19}\text{O}_6\text{NCl}$ . This result was confirmed by corresponding data obtained in the negative mode. The comparison of chromatographic and MS/MS patterns of the metabolite with those of the standard of ochratoxin A validated the identification at level 1 [32] (see section 2.7). Similarly, analysis of the standard of citrinin, a well-known *P. verrucosum* metabolite (compound 3 in Figure 3) [20,42], confirmed its production by the studied strain. Ochratoxin B (compound 2 in Figure 3) was also identified at a level 1 ( $\text{C}_{20}\text{H}_{20}\text{O}_6\text{N}$ ) at  $R_T=$  26.7 min, in accord with other works which noticed the excretion of this dechlorinated intermediate of the biosynthesis pathway of ochratoxin A by *P. verrucosum* [20,29]. Cyclopiazonic acid, never observed in *P. verrucosum*, was proposed for annotation of the metabolite  $\text{C}_{20}\text{H}_{20}\text{O}_3\text{N}_2$  but the chromatographic and MS/MS results obtained from the reference compound ruled out this hypothesis.

Since many natural products are unavailable as reference compounds [37], some secondary metabolites already known to be produced by *P. verrucosum* were detected without it being possible to validate their identifications. The quinazolines verrucine A (compound 6 in Figure 3) [20,43], verrucine B (compound 7 in Figure 3) [20,43] and verrucine F (compound 9 in Figure 3) [44] were annotated. Their MS/MS spectra displayed a loss of ammonia and a consecutive loss of carbon monoxide in accordance with the structures of verrucines A, B and F. Furthermore, the fragmentation patterns of verrucines A and

B have already been reported [43], allowing the identification of verrucines A and B at a level 2. Since standards were not available, *in silico* fragmentations were also performed using CFM-ID [33]. Although it should not be considered as proof of identification, this innovative tool provides relevant supporting information. The comparison between experimental and simulated MS/MS spectra is shown in Figure 4A for the formally identified ochratoxin B as an example of successful simulation. Application of CFM-ID to the putative quinazoline structures showed that *in silico* spectra were in accord with the annotation of the three verrucines. Indeed, the same two major fragment ions at  $m/z$  360.2 and  $m/z$  332.2 were detected in both experimental and *in silico* MS/MS spectra of verrucines A and B (Figure 4B). The same result was obtained with the annotated verrucine F ( $m/z$  375.1450), which shared two fragment ions at  $m/z$  358.2 and  $m/z$  330.2 with the simulated MS/MS spectrum (Figure 4C). Verrucolone, also known as arabenoic acid (compound 5 in Figure 3) and referenced as a typical *P. verrucosum* metabolite [45], was attributed to the detected  $C_6H_{10}O_4$  formula. The experimental MS/MS spectrum was compared to CFM-ID simulations and some common fragment ions were observed at  $m/z$  129.0, 117.0, 115.0 and 103.0. Verrucine F and verrucolone were therefore identified with a level 3A (see section 2.7).

Some secondary metabolites detected in our extracts have already been characterized in *Penicillium* fungi but not in the *P. verrucosum* strain. Unfortunately, no standards of these supposed secondary metabolites were available. In this case, the only supporting information came from the MS/MS or UV spectra available from the literature and from comparison with *in silico* fragmentations. Anacine (compound 8 in Figure 3) is known to be produced by *Penicillium nordicum*, one of the closest fungi of *P. verrucosum* [20]. This compound is a quinazoline belonging to the verrucines family [46]. In this study, anacine was identified at a level 2 since its UV spectrum was in accordance with the spectrum from literature [43]. Moreover, the unsaturated analog of anacine, namely aurantiomide C (compound 10 in Figure 3) was proposed for the annotation of  $C_{18}H_{21}O_3N_4$ . To our knowledge, this secondary metabolite was characterized in *Penicillium aurantiogriseum* [47] but never in *P. verrucosum*. Although its MS/MS spectrum was not available in literature, an *in silico* simulation was performed and compared with the experimental MS/MS fragmentation. Both spectra displayed a major fragment ion at  $m/z$  324.1. In the

same way, brevianamide F (compound 11 in Figure 3), a metabolite of *Penicillium brevicompactum* [39] and a precursor of fumitremorgins in *Aspergillus fumigatus* [48], was proposed for the annotation of  $C_{16}H_{17}O_2N_3$ . In this case again, no MS/MS information was available in the literature, and the *in silico* MS/MS simulation displayed almost all the fragment ions observed on the experimental spectrum. LL-P880 $\beta$  (compound 4 in Figure 3), another *Penicillium* metabolite whose fragmentation pattern was not published, was suspected based on database researches [39]. This pestalotin analog, also named hydroxy-pestalotin, was reported to be synthesized by *Penicillium sp.* without any precision about the species [39]. Moreover, in a study of the pestalotins family, Rahbaek *et al.* reported that PC-2, LL-P880 $\gamma$  and two verrucosapyrones displaying very similar structures, were secreted by several strains of *P. verrucosum* [45]. Consequently, *P. verrucosum* likely forms part of the *Penicillium* species able to produce LL-P880 $\beta$ . Finally, several cyclic tetrapeptides of the fungisporin family were suspected to be produced by *P. verrucosum*. Indeed, the metabolite  $C_{28}H_{36}O_6N_4$  showed a fragmentation pattern in accordance with a tetrapeptide structure and was identified as fungisporin C (compound 12 in Figure 3). This natural compound was previously described by Klitgaard *et al.* in *Aspergillus nidulans* but the study did not report the fragmentation spectrum [49]. Furthermore, the degradation products of fungisporin A (compound 13 in Figure 3), fungisporin B (compound 14 in Figure 3), fungisporin D (compound 15 in Figure 3) and cyclo-(Tyr-Trp-Val-Val) (compound 16 in Figure 3) were observed. The MS/MS spectrum of the degradation products of fungisporin A was presented by Ali *et al.* in their study characterizing fungisporins in *Penicillium chrysogenum* [50]. It was therefore possible to identify at a level 2 the compound 13 (Table 1) as a hydrolysis product of fungisporin A.

### 3.4. Unknown Secondary Metabolites

Finally, 85 formulas could not be associated with known *Penicillium* secondary metabolites. This observation is in accord with the estimation based on genomic data claiming that more than 80% of fungal secondary metabolomes remain uncharacterized [11]. All detected secondary metabolites are listed in Table 1 with their structural hypotheses. Moreover, detailed lists of the major MS/MS fragment ions



generated from each secondary metabolite by higher energy collision dissociation (HCD) (Table 2) as well as by collision-induced dissociation (CID) (Table S1, supplementary data) are provided together with their relative intensity. This complementary information is presented as an argument for further studies into these secondary metabolites. CID and HCD modes provide complementary information useful for structural analyses of fungal secondary metabolites. The resonant excitation mode used in CID within the linear ion trap rarely leads to consecutive fragmentations, whereas the non-resonant excitation of HCD could generate this kind of informative fragmentations. On the other hand, the CID mode is useful to study the filiation between the fragment ions formed in the consecutive MS<sup>n</sup> experiments. Unlike CID, the HCD mode is carried out without the low mass cut-off restriction, allowing the detection of low mass fragment ions.

In Table 1, several metabolites, including those identified, present the same chemical formula but are detected at different retention times. Since two isomers may display very different structures, MS/MS experiments were carried out. Two metabolites with the chemical formula C<sub>18</sub>H<sub>20</sub>N<sub>4</sub>O<sub>3</sub> were detected in the positive ionization mode (Table 1: PVer\_341.16\_20.1 and PVer\_341.16\_15.1). This formula was annotated as aurantiomide C (compound 10 in Figure 3), and the two isomers displayed very similar MS/MS spectra (three same fragment ions in similar relative abundances, Table 2). Similarly, two isomers of C<sub>18</sub>H<sub>22</sub>N<sub>4</sub>O<sub>3</sub> with very similar MS/MS spectra and UV spectra (*data not shown*) (Table 2) were detected in positive ionization mode and annotated as anacine (compound 8 in Figure 3) (Table 1: PVer\_343.17\_12.7 and PVer\_343.17\_17.9).

Conversely, several metabolites with different molecular weights may share a common structure. For example, C<sub>6</sub>H<sub>10</sub>O<sub>4</sub>, annotated by Antibase as verrucolone (PVer\_147.06\_3.1), and C<sub>6</sub>H<sub>8</sub>O<sub>3</sub> (PVer\_129.05\_7.6) shared three common ions observed in their MS/MS spectra (Table 2), including two specific fragmentations:  $m/z=97.0280$  and  $m/z=87.0437$ . The metabolite C<sub>6</sub>H<sub>8</sub>O<sub>3</sub> (PVer\_129.05\_7.6) was therefore suspected to be a dehydroxylated form of verrucolone.

To overcome the complex manual comparison of hundreds of fragmentation patterns, some bioinformatics tools are available, such as MS2LDA [51] or the GNPS Molecular Networking system [34] which was developed for the study of natural products. This latter system highlights ions according to the similarity of their MS/MS spectra. In this way, a molecular network was generated from the MS/MS spectra of all annotated secondary metabolites (Figure 5). This network displays already detected similar compounds such as ochratoxins A and B, or isomers, and ions linked through non-specific neutral losses: H<sub>2</sub>O between ochratoxin B and the node 299.09 or NH<sub>3</sub> and CO between verrucines and the node 391.14. The other groups consist of unknown molecules with the same formula.

More interestingly, the two metabolites at  $m/z= 511.29$  shared seven common ions, suggesting a high similarity. The corresponding fragmentation patterns were scrutinized to explain the structural differences between the compound at  $R_T= 18.0$ , annotated as the hydrolyzed fungisporin A, and the metabolite at  $R_T= 18.7$ . The MS/MS spectra of the two metabolites are shown in Figure 6. MS<sup>3</sup> and MS<sup>4</sup> experiments were performed to validate that the common ions displayed the same structure, and to obtain more structural information (Figure S2). Both the MS/MS fragmentation patterns of the two metabolites show a dipole ion at  $m/z 247.1435$  and  $m/z 265.1539$  and consecutive losses of 99 and 147. Thus, it was suggested that  $m/z= 247.1435$  consisted of a valine and a phenylalanine residue at the N-terminal extremity (Figure 6, ions b<sub>2</sub>) and that the  $m/z 265.1539$  consisted of a valine and a phenylalanine residue in the C-terminal extremity of the two peptides (Figure 6, ions y<sub>2</sub>). Moreover, the MS<sup>3</sup> spectrum of the  $m/z 265$  fragment ion of hydrolyzed fungisporin A showed a  $m/z 118.0860$  (C<sub>5</sub>H<sub>12</sub>NO<sub>2</sub>) corresponding to a C-terminal valine (Figure S2 A), whereas the same MS<sup>3</sup> of PVer\_511.29\_18.7 displayed a  $m/z 166.0860$  suggesting a C-terminal phenylalanine (Figure S2 B). This was confirmed by the presence of a fragment ion at  $m/z 346.2113$  on its MS<sup>2</sup> spectrum (Figure 6, ion b<sub>3</sub>). Finally, the MS<sup>3</sup> spectra of the  $m/z 247.1435$  fragment ion of both hydrolyzed fungisporin A and PVer\_511.29\_18.7 were perfectly similar, suggesting that the N-terminal extremities of the two metabolites are identical. In this way, the linear tetrapeptide Val-Phe-Phe-Val was confirmed at  $R_T= 18.0$  min. According to Ali *et al.*, this molecule is suspected to result from the hydrolysis of a cyclic tetrapeptide, namely fungisporin A. Furthermore, structural analyses

allowed the identification of its isomer detected at  $R_T = 18.7$  min as the tetrapeptide Val-Phe-Val-Phe. Based on the assumption that this linear compound also resulted from the hydrolysis of a cyclic peptide [50], a cyclic Val-Phe-Val-Phe structure was suspected.

### 3.5. Conclusion

The use of two isotope labelings of fungal substrate and HPLC-HRMS analyses enabled the detection of 98 secondary metabolites produced by *P. verrucosum* grown on wheat grains, and led to the unambiguous determination of all their chemical formulas. Among these metabolites, 18 structural hypotheses were proposed from databases, at least nine of which were observed for the first time in *P. verrucosum*: anacine, aurantiomide C, brevianamide F, LL-P880 $\beta$  and at least six fungisporins. The lack of available reference compounds remains problematic for natural product studies. This can be partially balanced by using informatics tools for the simulation of fragmentation spectra. In this study, CFM-ID was successfully used for several *P. verrucosum* metabolites, providing relevant supporting information about the metabolite annotations. However, 79 secondary metabolites remained unidentified. A molecular network was generated with the GNPS system, enabling the detection of a metabolite of interest. This new metabolite was characterized by MS<sup>n</sup> experiments as a linear tetrapeptide, most likely resulting from the hydrolysis of the cyclic Val-Phe-Val-Phe. In any case, the 98 metabolites detected in this study constitute a valuable source of information for further works focused on identification of new metabolites. These data will be useful for purification and structural analyses with the help of complementary analyses by NMR for example, for application to the particular *P. verrucosum* metabolome, or more generally to other *Penicillium* metabolomes.

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## FIGURE CAPTIONS

**Fig. 1.** Total ion current chromatograms obtained in the positive (A) and negative (B) ionization modes from the extract of wheat grains infected by *Penicillium verrucosum*.

**Fig. 2.** Detection of ochratoxin B: mass spectra detected at  $R_T = 26.7$  min after positive ESI-HPLC-MS from extract of *Penicillium verrucosum* grown (A) on natural 99%  $^{12}\text{C}$  wheat grains, (B) on grains labeled with 97% of  $^{13}\text{C}$  and (C) on grains labeled with 53% of  $^{13}\text{C}$  and 97% of  $^{15}\text{N}$ . Right-hand panels display the corresponding CID MS/MS spectra from culture (D) on natural 99%  $^{12}\text{C}$  wheat grains, (E) on grains labeled with 97% of  $^{13}\text{C}$  and (F) on grains labeled with 53% of  $^{13}\text{C}$  and 97% of  $^{15}\text{N}$ .

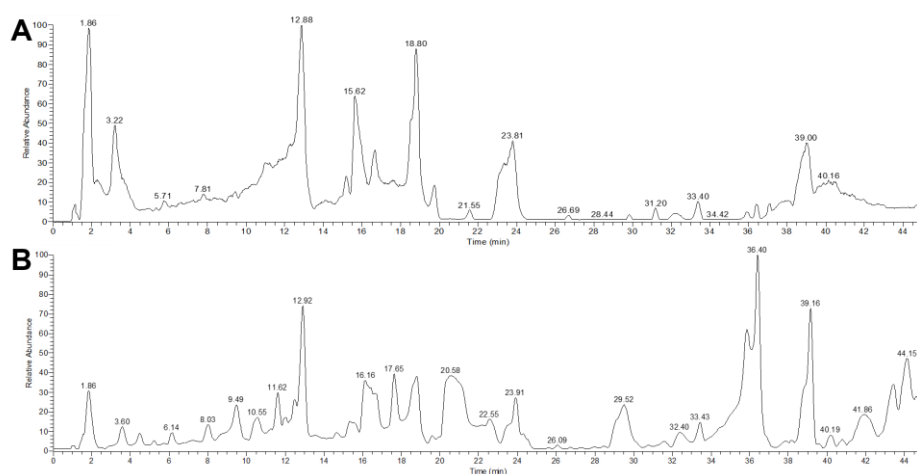
**Fig. 3.** Secondary metabolites identified from *Penicillium verrucosum*. The chemical formulas were calculated after comparison of the  $m/z$  ratios obtained from cultures of *P. verrucosum* in  $^{12}\text{C}$  wheat, 97%  $^{13}\text{C}$  wheat and 53%  $^{13}\text{C}$  / 97%  $^{15}\text{N}$  wheat. <sup>a</sup> Identification at level 1, <sup>b</sup> at level 2, <sup>c</sup> at level 3 of compounds known to be produced by *P. verrucosum*, <sup>d</sup> at level 3 of compounds never characterized in *P. verrucosum* but in other *Penicillium* species.

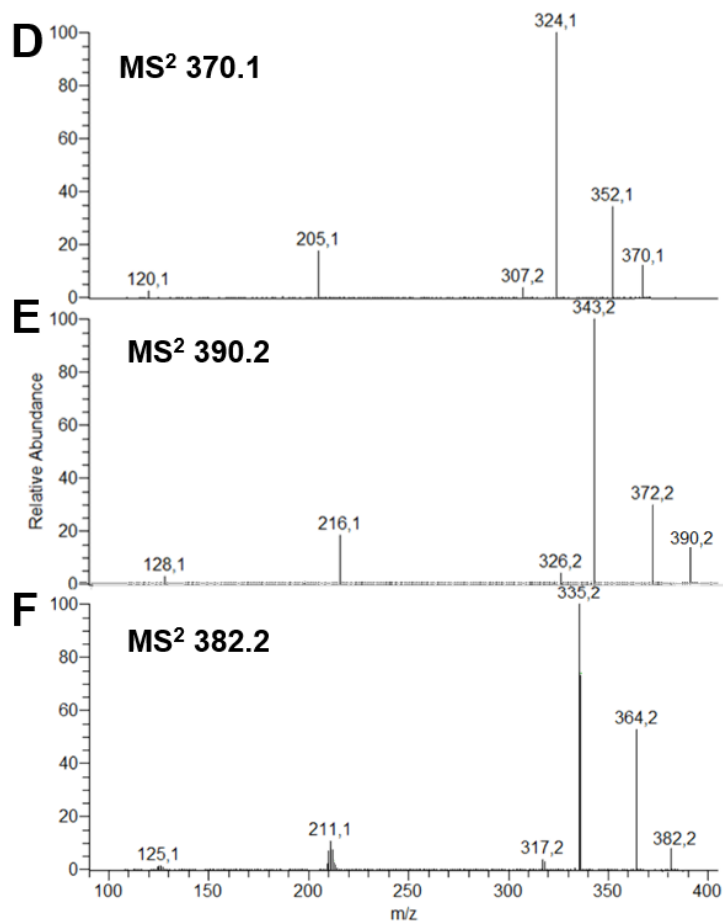
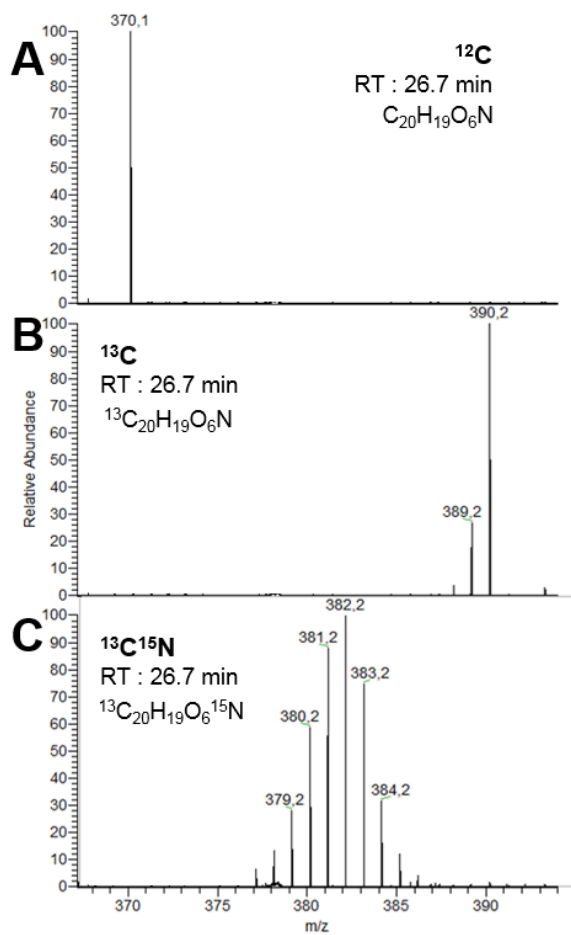
**Fig. 4.** Comparison between experimental MS/MS spectra (ESI<sup>+</sup> high-resolution CID) of three secondary metabolites of *Penicillium verrucosum* and the simulated MS/MS spectra of their respective structural hypotheses. (A) Successful *in silico* simulation: experimental MS/MS spectrum of ochratoxin B (upper part) (identified by homology to an authentic standard) and theoretical MS/MS generated by CFM-ID (lower part). (B) MS/MS spectrum obtained from the secondary metabolite  $\text{C}_{21}\text{H}_{21}\text{N}_4\text{O}_3$  detected at  $R_T = 18,4$  min (upper part) and *in silico* fragmentation of verrucines A and B using CFM-ID (lower part). (C) MS/MS spectrum obtained from the secondary metabolite  $\text{C}_{21}\text{H}_{19}\text{N}_4\text{O}_3$  detected at  $R_T = 18,7$  min (upper part) and *in silico* fragmentation of verrucine F using CFM-ID (lower part).

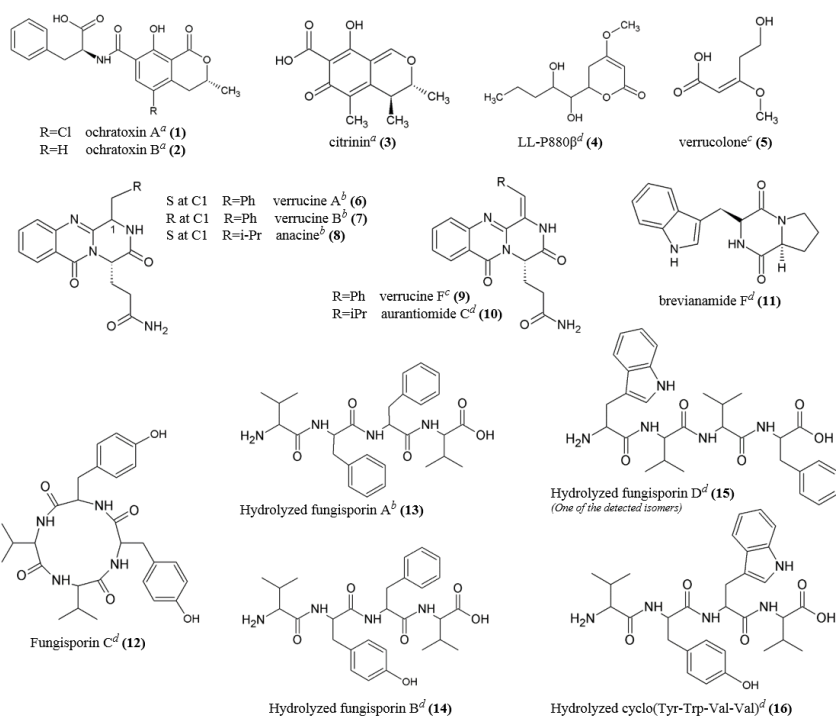
**Fig. 5.** Molecular network of the secondary metabolome of *Penicillium verrucosum*, created with the GNPS software from the targeted MS/MS spectra (ESI<sup>+</sup> high-resolution CID): nodes name:  $m/z$  ratio and retention time of the precursor ion, node size: absolute intensity in a logarithmic scale of the precursor ion

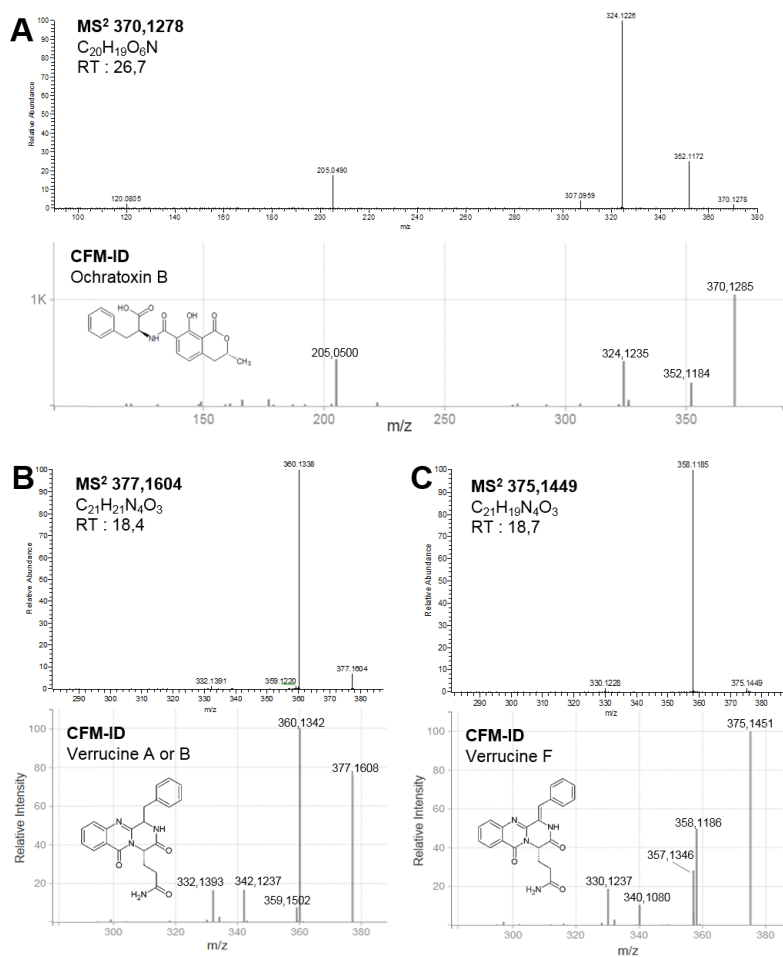
in the HRMS spectrum, black edges: connected MS/MS spectra according to GNPS software, red dotted edges: manually connected isomers which were not detected by GNPS.

**Fig. 6.** Comparison of the MS/MS spectra (ESI<sup>+</sup> high-resolution CID) of two secondary metabolites produced by *Penicillium verrucosum* grown on wheat grains. (A) MS/MS spectra of C<sub>28</sub>H<sub>38</sub>N<sub>4</sub>O<sub>5</sub> detected at R<sub>T</sub>= 18.0 min (PVer\_511.29\_18.0). (B) MS/MS spectra of C<sub>28</sub>H<sub>38</sub>N<sub>4</sub>O<sub>5</sub> detected at R<sub>T</sub>= 18.7 min (PVer\_511.29\_18.7).

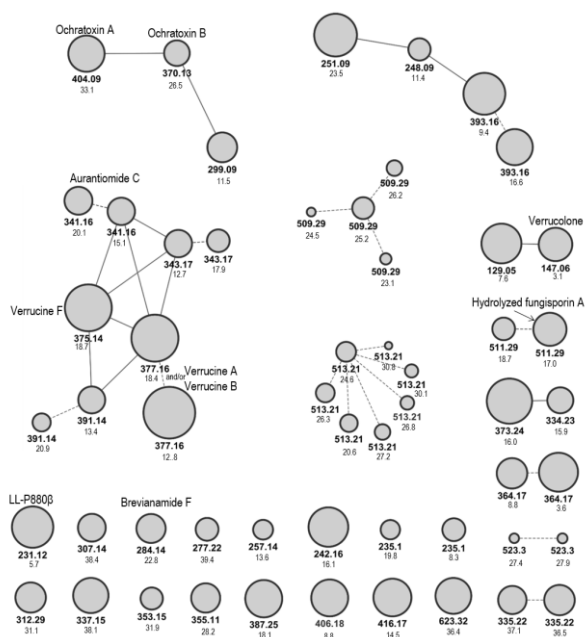
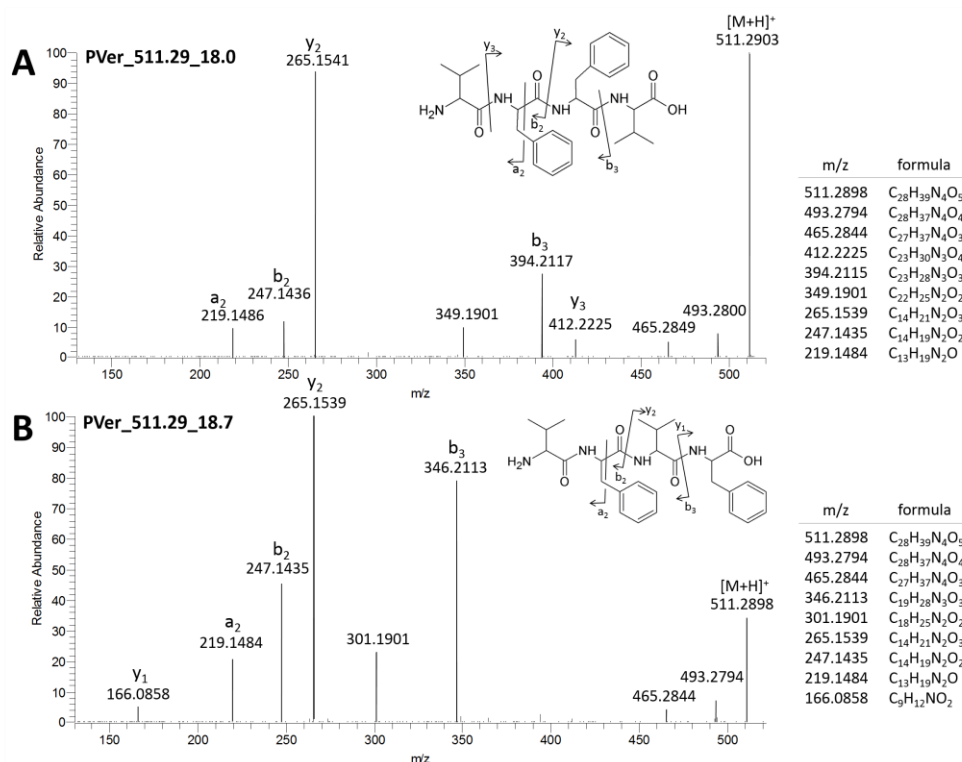












## TABLE CAPTIONS

**Table 1.** List of the secondary metabolites detected in *Penicillium verrucosum* grown on wheat grains. <sup>a</sup> The chemical formulas were calculated after comparison of the *m/z* ratios obtained from the culture of *P. verrucosum* in <sup>12</sup>C wheat, 97% <sup>13</sup>C wheat and 53% <sup>13</sup>C/96% <sup>15</sup>N wheat. <sup>b</sup> Identification at level 1, <sup>c</sup> at level 2, <sup>d</sup> at level 3 of compounds known to be produced by *P. verrucosum*, <sup>e</sup> at level 3 of compounds never characterized in *P. verrucosum* but in other *Penicillium* species, <sup>f</sup> secondary metabolites identifier (PVer\_m/z\_RT).

molecular formula <sup>a</sup>	Identifier <sup>f</sup>	proposed identification	molecular formula <sup>a</sup>	Identifier <sup>f</sup>	proposed identification	molecular formula <sup>a</sup>	Identifier <sup>f</sup>	proposed identification
C <sub>6</sub> H <sub>8</sub> O <sub>3</sub>	PVer_129.05_7.6		C <sub>18</sub> H <sub>24</sub> O <sub>7</sub>	PVer_351.14_29.1		C <sub>24</sub> H <sub>28</sub> N <sub>4</sub>	PVer_373.24_16.0	
C <sub>6</sub> H <sub>10</sub> O <sub>4</sub>	PVer_147.06_3.1	Verrucolone <sup>d</sup>	C <sub>18</sub> H <sub>24</sub> O <sub>7</sub>	PVer_351.14_24.1		C <sub>25</sub> H <sub>30</sub> N <sub>4</sub>	PVer_387.25_18.0	
C <sub>11</sub> H <sub>18</sub> O <sub>5</sub>	PVer_231.12_5.6	LL-P880β <sup>e</sup>	C <sub>18</sub> H <sub>28</sub> O <sub>2</sub>	PVer_277.21_39.4		C <sub>25</sub> H <sub>30</sub> N <sub>4</sub>	PVer_385.24_35.7	
C <sub>12</sub> H <sub>12</sub> O <sub>5</sub>	PVer_235.06_24.2		C <sub>18</sub> H <sub>32</sub> O <sub>4</sub>	PVer_335.22_36.2		C <sub>25</sub> H <sub>30</sub> N <sub>4</sub>	PVer_385.24_37.2	
C <sub>13</sub> H <sub>13</sub> NO <sub>4</sub>	PVer_248.09_11.4		C <sub>18</sub> H <sub>32</sub> O <sub>4</sub>	PVer_335.22_36.8		C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	PVer_513.21_20.6	
C <sub>13</sub> H <sub>14</sub> O <sub>4</sub>	PVer_235.10_8.3		C <sub>19</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>	PVer_307.14_38.5		C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	PVer_513.21_24.6	
C <sub>13</sub> H <sub>14</sub> O <sub>4</sub>	PVer_235.10_19.8		C <sub>19</sub> H <sub>18</sub> N <sub>2</sub> O <sub>3</sub> S	PVer_355.11_28.1		C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	PVer_513.21_26.3	
C <sub>13</sub> H <sub>14</sub> O <sub>5</sub>	PVer_251.09_23.5	Citrinin <sup>b</sup>	C <sub>19</sub> H <sub>26</sub> O <sub>6</sub>	PVer_371.15_22.5		C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	PVer_513.21_26.7	
C <sub>13</sub> H <sub>16</sub> O <sub>5</sub>	PVer_295.09_4.6		C <sub>19</sub> H <sub>37</sub> NO <sub>2</sub>	PVer_312.29_31.9		C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	PVer_513.21_27.1	
C <sub>13</sub> H <sub>16</sub> N <sub>2</sub> O <sub>6</sub>	PVer_251.09_40.0		C <sub>20</sub> H <sub>18</sub> NO <sub>6</sub> Cl	PVer_404.09_33.1	Ochratoxin A <sup>b</sup>	C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	PVer_513.21_30.1	
C <sub>13</sub> H <sub>16</sub> O <sub>6</sub>	PVer_267.09_9.2		C <sub>20</sub> H <sub>18</sub> NO <sub>6</sub> Cl	PVer_402.07_33.1		C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	PVer_513.21_31.4	
C <sub>13</sub> H <sub>16</sub> O <sub>6</sub>	PVer_267.09_6.5		C <sub>20</sub> H <sub>19</sub> NO <sub>6</sub>	PVer_370.13_26.5	Ochratoxin B <sup>b</sup>	C <sub>28</sub> H <sub>36</sub> N <sub>4</sub> O <sub>6</sub>	PVer_523.25_25.8	Fungisporin C <sup>e</sup>
C <sub>13</sub> H <sub>16</sub> O <sub>6</sub>	PVer_267.09_21.9		C <sub>20</sub> H <sub>20</sub> N <sub>2</sub> O <sub>3</sub>	PVer_337.15_38.1		C <sub>28</sub> H <sub>38</sub> N <sub>4</sub> O <sub>5</sub>	PVer_511.29_18.0	Hydrolyzed fungisporin A <sup>c</sup>
C <sub>13</sub> H <sub>20</sub> O <sub>5</sub>	PVer_257.14_13.6		C <sub>20</sub> H <sub>20</sub> N <sub>2</sub> O <sub>3</sub>	PVer_353.15_32.0		C <sub>28</sub> H <sub>38</sub> N <sub>4</sub> O <sub>5</sub>	PVer_509.28_18.0	
C <sub>14</sub> H <sub>12</sub> N <sub>4</sub> O <sub>4</sub>	PVer_299.08_3.5		C <sub>20</sub> H <sub>20</sub> N <sub>2</sub> O <sub>4</sub>	PVer_395.17_15.0		C <sub>28</sub> H <sub>38</sub> N <sub>4</sub> O <sub>5</sub>	PVer_511.29_18.7	New tetrapeptide
C <sub>15</sub> H <sub>11</sub> O <sub>3</sub>	PVer_238.06_11.5		C <sub>20</sub> H <sub>28</sub> O <sub>8</sub>	PVer_349.20_22.5		C <sub>28</sub> H <sub>38</sub> N <sub>4</sub> O <sub>5</sub>	PVer_509.28_18.7	
C <sub>15</sub> H <sub>19</sub> N <sub>3</sub>	PVer_242.16_16.1		C <sub>20</sub> H <sub>30</sub> O <sub>5</sub>	PVer_375.14_18.7	Verrucine F <sup>d</sup>	C <sub>28</sub> H <sub>38</sub> N <sub>4</sub> O <sub>6</sub>	PVer_525.27_13.0	Hydrolyzed fungisporin B <sup>e</sup>
C <sub>16</sub> H <sub>12</sub> O <sub>3</sub>	PVer_251.07_7.9		C <sub>21</sub> H <sub>18</sub> N <sub>4</sub> O <sub>3</sub>	PVer_373.13_17.0		C <sub>30</sub> H <sub>38</sub> N <sub>4</sub> O <sub>2</sub>	PVer_509.29_26.2	
C <sub>16</sub> H <sub>12</sub> O <sub>3</sub>	PVer_251.07_11.6		C <sub>21</sub> H <sub>18</sub> N <sub>4</sub> O <sub>3</sub>	PVer_391.14_13.5		C <sub>30</sub> H <sub>38</sub> N <sub>4</sub> O <sub>2</sub>	PVer_509.29_25.2	
C <sub>16</sub> H <sub>17</sub> N <sub>3</sub> O <sub>2</sub>	PVer_284.14_22.8	Brevianamide F <sup>e</sup>	C <sub>21</sub> H <sub>18</sub> N <sub>4</sub> O <sub>4</sub>	PVer_389.12_13.5		C <sub>30</sub> H <sub>38</sub> N <sub>4</sub> O <sub>2</sub>	PVer_509.29_23.0	
C <sub>17</sub> H <sub>14</sub> O <sub>5</sub>	PVer_299.09_11.5		C <sub>21</sub> H <sub>18</sub> N <sub>4</sub> O <sub>4</sub>	PVer_391.12_13.5		C <sub>30</sub> H <sub>38</sub> N <sub>4</sub> O <sub>2</sub>	PVer_509.29_24.4	
C <sub>17</sub> H <sub>14</sub> O <sub>5</sub>	PVer_297.08_11.5		C <sub>21</sub> H <sub>18</sub> N <sub>4</sub> O <sub>4</sub>	PVer_391.14_21.0		C <sub>30</sub> H <sub>39</sub> N <sub>5</sub> O <sub>5</sub>	PVer_548.29_17.4	Isomers of hydrolyzed fungisporin D <sup>e</sup>
C <sub>17</sub> H <sub>34</sub> O <sub>5</sub>	PVer_317.23_41.0		C <sub>21</sub> H <sub>20</sub> N <sub>4</sub> O <sub>3</sub>	PVer_377.16_18.4	Verrucine A and/or B <sup>c</sup>	C <sub>30</sub> H <sub>39</sub> N <sub>5</sub> O <sub>5</sub>	PVer_548.29_18.0	
C <sub>18</sub> H <sub>14</sub> N <sub>2</sub> O <sub>2</sub>	PVer_289.10_24.4		C <sub>21</sub> H <sub>20</sub> N <sub>4</sub> O <sub>3</sub>	PVer_377.16_12.8		C <sub>30</sub> H <sub>39</sub> N <sub>5</sub> O <sub>5</sub>	PVer_548.29_19.1	
C <sub>18</sub> H <sub>15</sub> N <sub>3</sub> O <sub>2</sub>	PVer_304.11_36.5		C <sub>21</sub> H <sub>20</sub> N <sub>4</sub> O <sub>4</sub>	PVer_393.15_9.4		C <sub>30</sub> H <sub>39</sub> N <sub>5</sub> O <sub>6</sub>	PVer_564.28_12.6	Hydrolyzed cyclo-(YWVV) <sup>e</sup>
C <sub>18</sub> H <sub>15</sub> N <sub>3</sub> O <sub>2</sub>	PVer_304.11_38.1		C <sub>21</sub> H <sub>20</sub> N <sub>4</sub> O <sub>4</sub>	PVer_391.14_9.4		C <sub>31</sub> H <sub>20</sub> O <sub>11</sub>	PVer_567.09_26.3	
C <sub>18</sub> H <sub>15</sub> N <sub>3</sub> O <sub>2</sub>	PVer_304.11_36.4		C <sub>21</sub> H <sub>20</sub> N <sub>4</sub> O <sub>4</sub>	PVer_393.15_15.5		C <sub>31</sub> H <sub>20</sub> O <sub>11</sub>	PVer_567.09_25.1	
C <sub>18</sub> H <sub>16</sub> N <sub>2</sub> O <sub>6</sub> S	PVer_387.06_3.8		C <sub>21</sub> H <sub>21</sub> N <sub>3</sub> O <sub>3</sub>	PVer_364.16_3.6		C <sub>31</sub> H <sub>40</sub> N <sub>4</sub> O <sub>2</sub>	PVer_523.30_27.7	
C <sub>18</sub> H <sub>20</sub> N <sub>4</sub> O <sub>3</sub>	PVer_341.16_20.1	Aurantioamide C <sup>e</sup>	C <sub>21</sub> H <sub>21</sub> N <sub>3</sub> O <sub>3</sub>	PVer_364.16_8.8		C <sub>31</sub> H <sub>40</sub> N <sub>4</sub> O <sub>2</sub>	PVer_523.30_27.4	
C <sub>18</sub> H <sub>20</sub> N <sub>4</sub> O <sub>3</sub>	PVer_341.16_15.1	and/or isomer(s)	C <sub>21</sub> H <sub>30</sub> N <sub>4</sub> O <sub>4</sub>	PVer_401.22_25.8		C <sub>32</sub> H <sub>36</sub> N <sub>4</sub> O <sub>2</sub>	PVer_507.28_38.5	

$C_{18}H_{22}N_4O_3$	PVer_343.17_12.7	Anacine <sup>c</sup> and/or isomer(s)	$C_{21}H_{30}N_4O_4$	PVer_401.22_21. 9	$C_{32}H_{42}O_7$	PVer_537.29_39.5
$C_{18}H_{22}N_4O_3$	PVer_343.17_17.9		$C_{22}H_{27}N_3$	PVer_334.23_15. 8	$C_{32}H_{42}O_8$	PVer_553.28_38.7
$C_{18}H_{24}O_7$	PVer_351.14_18.1		$C_{22}H_{27}N_3$	PVer_332.21_35. 6	$C_{32}H_{42}O_8$	PVer_553.28_38.3
$C_{18}H_{24}O_7$	PVer_351.14_29.0		$C_{23}H_{21}N_5O_3$	PVer_416.17_14. 6	$C_{37}H_{42}N_4O_5$	PVer_623.32_36.3
$C_{18}H_{24}O_7$	PVer_351.14_24.6		$C_{23}H_{23}N_3O_4$	PVer_406.17_8.8	$C_{38}H_{42}N_4O_1$	PVer_713.28_33.4

**Table 2.** Secondary metabolites detected from *Penicillium verrucosum* after culture on wheat grains and their MS/MS fragmentation in higher energy collision dissociation (HCD) mode. The fragmentation spectra are presented as a list of fragment ions with their relative intensity. <sup>a</sup> The chemical formulas were calculated after comparison of the  $m/z$  ratios obtained from the culture of *P. verrucosum* in <sup>12</sup>C wheat, 97% <sup>13</sup>C wheat and 53% <sup>13</sup>C / 96% <sup>15</sup>N wheat. <sup>b</sup> Secondary metabolites identifier (PVer\_m/z\_RT).

Identifier <sup>f</sup>	molecular formula <sup>a</sup>	parent ion	<sup>12</sup> C m/z (Da)	R <sub>T</sub> (min)	MS/MS mode (Normalized Collision Energy)	m/z of major fragment ions (% relative intensity)
PVer_129.05_7.6	C <sub>6</sub> H <sub>8</sub> O <sub>3</sub>	[M+H] <sup>+</sup>	129.0544	7.6	HCD (20)	129.0544(100) 87.0437(12) 97.0281(8) 55.0176(6) 111.0439(3)
PVer_147.06_3.1	C <sub>6</sub> H <sub>10</sub> O <sub>4</sub>	[M+H] <sup>+</sup>	147.0655	3.1	HCD (20)	147.0655(5) 129.0543(100) 117.0543(32) 97.0280(17) 85.0280(17) 99.0437(7) 87.0437(7) 75.0437(6) 55.0176(5) 71.0488(5) 115.0387(3)
PVer_231.12_2.5	C <sub>11</sub> H <sub>18</sub> O <sub>5</sub>	[M+H] <sup>+</sup>	231.1229	5.6	HCD (20)	231.1227(100) 181.0857(70) 127.0387(27) 213.1120(26) 195.1014(25) 81.0332(22) 139.0750(21) 155.1064(21) 141.0543(13) 151.1116(10)
PVer_235.06_24.2	C <sub>12</sub> H <sub>12</sub> O <sub>5</sub>	[M-H] <sup>-</sup>	235.0616	24.2	HCD (60)	235.0616(100) 217.0510(85) 191.0718(70) 163.0770(5) 189.0562(2)
PVer_248.09_11.4	C <sub>13</sub> H <sub>13</sub> N <sub>4</sub> O <sub>4</sub>	[M+H] <sup>+</sup>	248.0916	11.4	HCD (20)	248.0914(100) 230.0810(87)
PVer_235.10_8.3	C <sub>13</sub> H <sub>14</sub> O <sub>4</sub>	[M+H] <sup>+</sup>	235.0965	8.3	HCD (20)	235.0964(100) 217.0687(2) 191.0699(2)
PVer_235.10_19.8	C <sub>13</sub> H <sub>14</sub> O <sub>4</sub>	[M+H] <sup>+</sup>	235.0965	19.8	HCD (20)	235.0963(47) 193.0855(100) 165.0907(7) 217.0854(6) 207.1014(4) 139.1114(4) 211.0958(2) 137.0955(3)
PVer_251.09_23.5	C <sub>13</sub> H <sub>14</sub> O <sub>5</sub>	[M+H] <sup>+</sup>	251.0912	23.5	HCD (20)	251.0915(100) 233.0807(45)
PVer_295.09_4.6	C <sub>13</sub> H <sub>16</sub> N <sub>2</sub> O <sub>6</sub>	[M-H] <sup>-</sup>	295.0936	4.6	HCD (60)	295.0936(15) 221.0573(100) 251.1039(20) 236.0807(7) 193.0622(25) 249.0885(6) 122.0252(9) 137.0361(9) 208.0859(8)
PVer_251.09_40.0	C <sub>13</sub> H <sub>16</sub> O <sub>5</sub>	[M-H] <sup>-</sup>	251.0924	40.0	HCD (60)	251.0924(100) 233.0823(9) 163.0769(9) 189.0562(5) 207.1030(6)
PVer_267.09_9.2	C <sub>13</sub> H <sub>16</sub> O <sub>6</sub>	[M-H] <sup>-</sup>	267.0871	9.2	HCD (60)	267.0871(100) 249.0772(6) 207.0666(16) 163.0769(5) 193.0875(4) 237.0773(3) 205.0875(2)
PVer_267.09_6.5	C <sub>13</sub> H <sub>16</sub> O <sub>6</sub>	[M-H] <sup>-</sup>	267.0871	6.5	HCD (60)	267.0871(100) 239.0721(5) 249.0563(1)
PVer_267.09_21.9	C <sub>13</sub> H <sub>16</sub> O <sub>6</sub>	[M-H] <sup>-</sup>	267.0871	21.9	HCD (60)	267.0871(95) 221.0825(100) 249.0773(10) 177.0926(10) 203.0719(4)
PVer_257.14_13.6	C <sub>13</sub> H <sub>20</sub> O <sub>5</sub>	[M+H] <sup>+</sup>	257.1381	13.6	HCD (35)	257.1382(65) 157.0493(100)
PVer_299.08_3.5	C <sub>14</sub> H <sub>12</sub> N <sub>4</sub> O <sub>4</sub>	[M-H] <sup>-</sup>	299.0783	3.5	HCD (60)	299.0783(35) 256.0731(100) 228.0421(15) 145.0413(30) 213.0673(5) 238.0627(3) 185.0361(3)
PVer_238.06_11.5	C <sub>15</sub> H <sub>11</sub> O <sub>3</sub>	[M-H] <sup>-</sup>	238.0640	11.5	HCD (60)	238.0637(100) 210.0694(2)
PVer_242.16_16.1	C <sub>15</sub> H <sub>19</sub> N <sub>3</sub>	[M+H] <sup>+</sup>	242.1647	16.1	HCD (20)	242.650(100) 111.0915(33)
PVer_251.07_7.9	C <sub>16</sub> H <sub>12</sub> O <sub>3</sub>	[M-H] <sup>-</sup>	251.0712	7.9	HCD (60)	251.0712(100) 250.0646(13) 223.0771(6) 211.0405(4)
PVer_251.07_11.6	C <sub>16</sub> H <sub>12</sub> O <sub>3</sub>	[M-H] <sup>-</sup>	251.0712	11.6	HCD (60)	251.0712(100) 233.0612(3) 223.0770(2)
PVer_284.14_22.8	C <sub>16</sub> H <sub>17</sub> N <sub>3</sub> O <sub>2</sub>	[M+H] <sup>+</sup>	284.1388	22.8	HCD (20)	284.1390(100) 130.0648(30) 170.0597(3) 132.0804(3)
PVer_299.09_11.5	C <sub>17</sub> H <sub>14</sub> O <sub>5</sub>	[M+H] <sup>+</sup>	299.0907	11.5	HCD (20)	299.0912(60) 205.0493(100) 281.0807(6) 189.0543(6) 253.0857(3) 107.0490(3) 187.0388(3) 159.0438(1) 263.0702(1)
PVer_297.08_11.5		[M-H] <sup>-</sup>	297.0773		HCD (60)	297.0773(10) 253.08874(100) 238.0641(6) 159.0456(4) 109.0300(4) 93.0350(4) 226.0626(2)
PVer_317.23_3_41.0	C <sub>17</sub> H <sub>34</sub> O <sub>5</sub>	[M-H] <sup>-</sup>	317.2327	41.0	HCD (60)	317.2327(100) 257.2127(52) 299.2229(6) 115.0770(3) 59.0143(18) 183.1395(4) 195.1759(4) 255.2334(4) 85.0663(1)
PVer_289.10_24.4	C <sub>18</sub> H <sub>14</sub> N <sub>2</sub> O <sub>2</sub>	[M-H] <sup>-</sup>	289.0977	24.4	HCD (60)	289.0977(100) 116.0510(33) 261.1039(24)
PVer_304.11_36.5	C <sub>18</sub> H <sub>15</sub> N <sub>3</sub> O <sub>2</sub>	[M-H] <sup>-</sup>	304.1087	36.5	HCD (60)	304.1087(26) 213.0549(100) 261.1037(29) 183.0567(8) 286.0988(6) 159.0568(3) 145.0410(3) 212.0470(3) 262.0989(3)
PVer_304.11_38.1	C <sub>18</sub> H <sub>15</sub> N <sub>3</sub> O <sub>2</sub>	[M-H] <sup>-</sup>	304.1087	38.1	HCD (60)	304.1087(100) 213.0548(100) 261.1038(25) 286.0998(5) 183.0569(7)
PVer_304.11_36.4	C <sub>18</sub> H <sub>15</sub> N <sub>3</sub> O <sub>2</sub>	[M-H] <sup>-</sup>	304.1087	36.4	HCD (60)	304.1102(3) 213.0548(100) 261.1040(25) 286.0992(5) 183.0569(7) 159.0569(4)
PVer_387.06_3.8	C <sub>18</sub> H <sub>16</sub> N <sub>2</sub> O <sub>6</sub> S	[M-H] <sup>-</sup>	387.0655	3.8	HCD (60)	387.0662(100) 176.0356(75) 353.0784(55) 202.0149(50) 269.0570(52) 121.0296(20) 231.0414(16) 277.0297(15) 121.0296(21) 270.0646(16)
PVer_341.16_20.1	C <sub>18</sub> H <sub>20</sub> N <sub>4</sub> O <sub>3</sub>	[M+H] <sup>+</sup>	341.1604	20.1	HCD (20)	341.1605(55) 324.1338(100) 296.1389(10)
PVer_341.16_15.1	C <sub>18</sub> H <sub>20</sub> N <sub>4</sub> O <sub>3</sub>	[M+H] <sup>+</sup>	341.1604	15.1	HCD (20)	341.1601(60) 324.1337(100) 296.1388(8) 151.1114(2) 195.1013(1)
PVer_343.17_12.7	C <sub>18</sub> H <sub>22</sub> N <sub>4</sub> O <sub>3</sub>	[M+H] <sup>+</sup>	343.1747	12.7	HCD (20)	343.1758(45) 326.1496(100) 298.1546(5)
PVer_343.17_17.9	C <sub>18</sub> H <sub>22</sub> N <sub>4</sub> O <sub>3</sub>	[M+H] <sup>+</sup>	343.1747	17.9	HCD (20)	343.1758(45) 326.1495(100) 298.1545(5) 270.0869(2)
PVer_351.14_18.1	C <sub>18</sub> H <sub>24</sub> O <sub>7</sub>	[M-H] <sup>-</sup>	351.1448	18.1	HCD (60)	351.1448(100) 307.1190(60) 249.0773(60) 333.1351(9) 205.0874(11) 101.0248(4) 219.1028(3)
PVer_351.14_29.0	C <sub>18</sub> H <sub>24</sub> O <sub>7</sub>	[M-H] <sup>-</sup>	351.1448	29.0	HCD (60)	351.1450(65) 201.0924(100) 245.1188(80) 277.1449(42) 263.1293(21) 231.1033(21) 233.1188(19) 213.0923(6)
PVer_351.14_24.6	C <sub>18</sub> H <sub>24</sub> O <sub>7</sub>	[M-H] <sup>-</sup>	351.1448	24.6	HCD (60)	351.1448(55) 321.1347(100) 307.1190(55) 245.0825(40) 201.0924(35) 233.1186(5) 259.0980(5)
PVer_351.14_29.1	C <sub>18</sub> H <sub>24</sub> O <sub>7</sub>	[M-H] <sup>-</sup>	351.1448	29.1	HCD (60)	351.1448(60) 201.0925(100) 245.1189(80) 277.1448(45)
PVer_351.14_24.1	C <sub>18</sub> H <sub>24</sub> O <sub>7</sub>	[M-H] <sup>-</sup>	351.1448	24.1	HCD (60)	351.1450(100) 321.1349(45) 249.0773(30) 307.1191(25) 245.0826(18) 201.0924(17) 101.0614(19)
PVer_277.11_39.4	C <sub>18</sub> H <sub>28</sub> O <sub>2</sub>	[M+H] <sup>+</sup>	277.1158	39.4	HCD (20)	277.2164(100) 179.1431(12) 135.1168(5) 259.2060(3) 121.1011(3) 235.1696(1) 161.1325(1)
PVer_335.22_36.2	C <sub>18</sub> H <sub>32</sub> O <sub>4</sub>	[M+Na] <sup>+</sup>	335.2187	36.2	HCD (35)	335.2187(100) 317.2079(3) 190.1235(3)
PVer_335.22_36.8	C <sub>18</sub> H <sub>32</sub> O <sub>4</sub>	[M+Na] <sup>+</sup>	335.2187	36.8	HCD (35)	335.2187(100) 190.1232(20)
PVer_307.14_38.5	C <sub>19</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>	[M+H] <sup>+</sup>	307.1427	38.5	HCD(35)	307.1439(75) 229.0971(100) 201.1022(12) 111.0553(10) 292.1207(2)

PVer_355.1 1_28.1	C <sub>19</sub> H <sub>18</sub> N <sub>2</sub> O <sub>3</sub> S	[M+H] +	355.1 105	28.1	HCD (20)	355.1135(10) 289.0967(100) 337.1000(45) 307.1074(43) 308.1150(32) 279.1125(15) 290.1047(12) 236.1068(11) 261.1019(10) 327.1156(10)
PVer_371.1 5_22.5	C <sub>19</sub> H <sub>26</sub> O <sub>6</sub>	[M- 2H+Na ] -	371.1 466	22.5	HCD (60)	371.1466(100) 353.1375(26)
PVer_312.2 9_31.9	C <sub>19</sub> H <sub>37</sub> N O <sub>2</sub>	[M+H] +	312.2 895	31.9	HCD(20)	312.2895(5) 294.2792(100) 276.2684(5) 86.0598(5) 259.2417(5) 277.2524(1) 264.2683(1)
PVer_404.0 9_33.1	C <sub>20</sub> H <sub>18</sub> N O <sub>6</sub> Cl	[M+H] +	404.0 897	33.1	HCD (20)	404.0892(30) 358.0836(100) 239.0103(66) 341.0572(13) 257.0209(11) 120.0804(10) 386.0786(5)
PVer_402.0 7_33.1		[M-H] -	402.0 750		HCD (60)	402.0756(10) 358.0855(100) 211.0171(42) 254.0229(12) 239.0122(6) 322.1090(6) 314.0587(6) 270.0696(4)
PVer_370.1 3_26.5	C <sub>20</sub> H <sub>19</sub> N O <sub>6</sub>	[M+H] +	370.1 285	26.5	HCD (20)	370.1286(30) 205.0492(100) 324.1226(37) 352.1172(7) 223.0598(7) 307.0959(6) 120.0805(6)
PVer_368.1 1_26.5		[M-H] -	368.1 130		HCD (60)	368.1146(100) 324.1245(100) 220.0622(15) 177.0561(7) 280.0981(6) 164.0720(4)
PVer_337.1 5_38.1	C <sub>20</sub> H <sub>20</sub> N <sub>2</sub> O <sub>3</sub>	[M+H] +	337.1 552	38.1	HCD (20)	337.1552(100) 306.1360(54) 336.1952(10) 305.1281(7) 277.1331(6) 167.0851(3) 91.0539(4)
PVer_353.1 5_32.0	C <sub>20</sub> H <sub>20</sub> N <sub>2</sub> O <sub>4</sub>	[M+H] +	353.1 480	32.0	HCD (20)	353.1489(100) 305.1278(56) 336.1460(7) 118.0409(4) 275.1020(2) 91.0538(1) 322.1309(1)
PVer_395.1 7_15.0	C <sub>20</sub> H <sub>28</sub> O <sub>8</sub>	[M-H] -	395.1 708	15.0	HCD (60)	395.1708(46) 261.1866(100) 349.1661(62) 305.1762(60) 243.1760(36) 287.1656(29) 307.1919(25) 245.1553(17) 289.1813(9) 137.0612(7)
PVer_349.2 0_22.5	C <sub>20</sub> H <sub>30</sub> O <sub>5</sub>	[M-H] -	349.2 012	22.5	HCD (60)	349.2021(9) 305.1761(100) 261.1865(15) 137.0612(7) 287.1656(5)
PVer_375.1 4_18.7	C <sub>21</sub> H <sub>18</sub> N <sub>4</sub> O <sub>3</sub>	[M+H] +	375.1 450	18.7	HCD (20)	375.1449(42) 358.1183(100) 330.1231(11)
PVer_373.1 3_17.0	C <sub>21</sub> H <sub>18</sub> N <sub>4</sub> O <sub>3</sub>	[M-H] -	373.1 295	17.0	HCD (60)	373.1304(62) 302.0940(100) 262.0991(8) 145.0413(6) 355.1206(3)
PVer_391.1 4_13.5	C <sub>21</sub> H <sub>18</sub> N <sub>4</sub> O <sub>4</sub>	[M+H] +	391.1 392	13.5	HCD (20)	391.1398(55) 374.1130(100) 346.1182(15)
PVer_389.1 2_13.5		[M-H] -	389.1 258		HCD (60)	389.1264(100) 318.0897(99) 83.0145(15) 278.0948(7) 145.0417(5)
PVer_391.1 4_21.0	C <sub>21</sub> H <sub>18</sub> N <sub>4</sub> O <sub>4</sub>	[M+H] +	391.1 392	21.0	HCD (20)	391.1396(60) 374.1129(100) 346.1182(10) 149.0230(9) 328.1078(1) 263.0810(1)
PVer_377.1 6_18.4	C <sub>21</sub> H <sub>20</sub> N <sub>4</sub> O <sub>3</sub>	[M+H] +	377.1 604	18.4	HCD (20)	377.1603(45) 360.1336(100) 332.1392(5) 91.0539(1)
PVer_377.1 6_12.8	C <sub>21</sub> H <sub>20</sub> N <sub>4</sub> O <sub>3</sub>	[M+H] +	377.1 604	12.8	HCD (20)	377.1603(43) 360.1339(100) 332.1389(5)
PVer_393.1 5_9.4	C <sub>21</sub> H <sub>20</sub> N <sub>4</sub> O <sub>4</sub>	[M+H] +	393.1 554	9.4	HCD (20)	393.1551(18) 358.1181(100) 375.1448(75) 376.1284(55) 330.1227(7) 120.0441(6) 274.1180(3)
PVer_391.1 4_9.4		[M-H] -	391.1 400		HCD(60)	391.1154(100) 250.1088(47) 294.0985(37) 373.1052(22) 300.0863(17) 276.0863(12) 302.0943(12)
PVer_393.1 5_15.5	C <sub>21</sub> H <sub>20</sub> N <sub>4</sub> O <sub>4</sub>	[M+H] +	393.1 554	15.5	HCD (20)	393.1541(3) 358.1181(100) 375.1447(65) 330.1230(13) 376.1286(7) 348.1341(1)
PVer_364.1 6_3.6	C <sub>21</sub> H <sub>21</sub> N <sub>3</sub> O <sub>3</sub>	[M+H] +	364.1 656	3.6	HCD (20)	364.1649(100) 308.1026(10) 200.1066(6) 346.1544(3) 196.1115(2)
PVer_364.1 6_8.8	C <sub>21</sub> H <sub>21</sub> N <sub>3</sub> O <sub>3</sub>	[M+H] +	364.1 656	8.8	HCD (20)	364.1650(100) 308.1027(20) 177.0654(3) 200.1067(2) 294.1232(1) 346.1543(1) 145.1280(1)
PVer_401.2 2_25.8	C <sub>21</sub> H <sub>30</sub> N <sub>4</sub> O <sub>4</sub>	[M-H] -	401.2 185	25.8	HCD (60)	401.2200(100) 262.1562(35) 172.1094(62) 214.1565(15) 245.1300(13) 228.1036(10) 198.0888(10) 203.0825(4) 137.0724(9) 290.1066(7)
PVer_401.2 2_21.9	C <sub>21</sub> H <sub>30</sub> N <sub>4</sub> O <sub>4</sub>	[M-H] -	401.2 185	21.9	HCD (60)	401.2200(100) 262.1562(35) 172.1094(60) 214.1565(25) 245.1301(20) 203.0833(20) 155.0830(20) 137.0724(18) 228.1033(15) 198.0890(16) 146.0617(16)
PVer_334.2 3_15.8	C <sub>22</sub> H <sub>27</sub> N <sub>3</sub>	[M+H] +	334.2 271	15.8	HCD (20)	334.2274(100) 203.1540(55) 217.1696(1)
PVer_332.2 1_35.6	C <sub>22</sub> H <sub>27</sub> N <sub>3</sub>	[M-H] -	332.2 126	35.6	HCD (60)	332.2126(100) 264.1148(10) 240.1504(3)
PVer_416.1 7_14.6	C <sub>23</sub> H <sub>21</sub> N <sub>5</sub> O <sub>3</sub>	[M+H] +	416.1 712	14.6	HCD (20)	416.1709(40) 399.1445(100) 130.0648(56) 270.0870(45) 287.1136(40) 242.0919(1)
PVer_406.1 7_8.8	C <sub>23</sub> H <sub>23</sub> N <sub>3</sub> O <sub>4</sub>	[M+H] +	406.1 758	8.8	HCD (20)	406.1753(21) 346.1544(100) 364.1649(62) 306.1232(19) 308.1027(10) 290.0921(4)
PVer_373.2 4_16.0	C <sub>24</sub> H <sub>28</sub> N <sub>4</sub>	[M+H] +	373.2 385	16.0	HCD (20)	373.2381(100) 242.1649(55) 111.0914(7) 256.1802(1)
PVer_387.2 5_18.0	C <sub>25</sub> H <sub>30</sub> N <sub>4</sub>	[M+H] +	387.2 536	18.0	HCD (50)	387.2542(3) 130.0652(100) 187.1232(10) 156.0810(10) 72.0807(7) 144.0809(6) 113.1074(4) 257.1889(2) 58.0650(2)
PVer_385.2 4_35.7	C <sub>25</sub> H <sub>30</sub> N <sub>4</sub>	[M-H] -	385.2 391	35.7	HCD (60)	385.2404(99) 230.1668(100) 256.2821(65) 116.0508(24) 156.0823(16)
PVer_385.2 4_37.2	C <sub>25</sub> H <sub>30</sub> N <sub>4</sub>	[M-H] -	385.2 391	37.2	HCD (60)	385.2391(85) 230.1667(100) 256.1822(72) 116.0511(27) 156.0824(25) 142.0667(10)
PVer_513.2 1_20.6	C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	[M+Na] +	513.2 119	20.6	HCD (20)	513.2129(60) 496.1858(100) 227.1063(25) 351.1674(4)
PVer_513.2 1_24.6	C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	[M+Na] +	513.2 119	24.6	HCD (20)	513.2129(45) 496.1856(100) 468.1894(6)
PVer_513.2 1_26.3	C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	[M+Na] +	513.2 119	26.3	HCD (20)	513.2129(100) 496.1858(65) 468.1930(4)
PVer_513.2 1_26.7	C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	[M+Na] +	513.2 119	26.7	HCD (20)	513.2134(92) 496.1855(100)
PVer_513.2 1_27.1	C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	[M+Na] +	513.2 119	27.1	HCD (20)	513.2128(63) 496.1852(100)
PVer_513.2 1_30.1	C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	[M+Na] +	513.2 119	30.1	HCD (20)	513.2129(40) 496.1857(100) 351.1769(10)
PVer_513.2 1_31.4	C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>5</sub>	[M+Na] +	513.2 119	31.4	HCD (20)	513.2134(55) 496.1863(100)
PVer_523.2 5_25.8	C <sub>28</sub> H <sub>36</sub> N <sub>4</sub> O <sub>6</sub>	[M-H] -	523.2 555	25.8	HCD (60)	523.2562(5) 401.2204(100) 262.1565(16) 135.0455(16) 172.1097(10) 214.1570(4)
PVer_511.2 9_18.0	C <sub>28</sub> H <sub>38</sub> N <sub>4</sub> O <sub>5</sub>	[M+H] +	511.2 909	18.0	HCD (20)	511.2906(10) 265.1542(100) 219.1488(30) 247.1438(21) 120.0804(16) 394.2120(10) 412.2228(9) 349.1902(3) 295.1438(3) 267.1484(2)
PVer_509.2 8_18.0		[M-H] -	509.2 769		HCD (60)	509.2769(17) 116.0722(100) 276.1033(66) 115.0882(55) 202.1442(32) 146.0616(22) 263.1405(16) 164.0722(12) 262.1566(11) 246.1141(8) 174.0565(4)

PVer_511.2 9_18.7	C <sub>28</sub> H <sub>38</sub> N <sub>4</sub>	[M+H] <sup>+</sup>	511.2 909	18.7	HCD (20)	511.2904(15) 265.1554(100) 219.1487(65) 247.1437(52) 346.2117(35) 120.0804(10) 72.0804(7) 166.0858(6) 301.1904(6)
PVer_509.2 8_18.7	O <sub>5</sub>	[M-H] <sup>-</sup>	509.2 769		HCD (60)	509.2769(16) 164.0722(100) 219.1506(20) 147.0457(19) 318.2191(10) 465.2877(15) 263.1404(6) 214.1564(7) 343.2139(6) 361.2249(5)
PVer_525.2 7_13.0	C <sub>28</sub> H <sub>38</sub> N <sub>4</sub> O <sub>6</sub>	[M-H] <sup>-</sup>	525.2 720	13.0	HCD (60)	525.2730(18) 116.0722(100) 292.0982(87) 172.1096(59) 278.1515(22) 162.0566(24) 263.1405(20) 115.0882(18) 248.1087(13) 142.0667(13) 202.1242(12)
PVer_509.2 9_26.2	C <sub>30</sub> H <sub>38</sub> N <sub>4</sub> O <sub>2</sub>	[M+Na] <sup>+</sup>	509.2 894	26.2	HCD (20)	509.2899(40) 242.1651(100) 111.0915(20) 378.2171(12) 351.1741(6)
PVer_509.2 9_25.2	C <sub>30</sub> H <sub>38</sub> N <sub>4</sub> O <sub>2</sub>	[M+Na] <sup>+</sup>	509.2 894	25.2	HCD (20)	509.2895(100) 242.1651(83) 111.0915(16) 378.2171(40)
PVer_509.2 9_23.0	C <sub>30</sub> H <sub>38</sub> N <sub>4</sub> O <sub>2</sub>	[M+Na] <sup>+</sup>	509.2 894	23.0	HCD (20)	509.2895(73) 242.1347(100) 378.2168(13) 111.0913(13)
PVer_509.2 9_24.4	C <sub>30</sub> H <sub>38</sub> N <sub>4</sub> O <sub>2</sub>	[M+Na] <sup>+</sup>	509.2 894	24.4	HCD (20)	509.2898(50) 242.1648(100) 378.2159(19) 111.0913(19) 478.0047(5)
PVer_548.2 9_17.4	C <sub>30</sub> H <sub>39</sub> N <sub>5</sub> O <sub>5</sub>	[M-H] <sup>-</sup>	548.2 892	17.4	HCD (60)	548.2892(10) 116.0722(100) 259.1457(65) 186.0565(45) 315.1144(30) 419.2307(20) 271.1252(15) 302.1515(15)
PVer_548.2 9_18.0	C <sub>30</sub> H <sub>39</sub> N <sub>5</sub> O <sub>5</sub>	[M-H] <sup>-</sup>	548.2 892	18.0	HCD (60)	548.2892(15) 116.0722(100) 172.1094(100) 315.1141(50) 258.1600(22) 271.1242(18) 186.0565(15) 419.2306(5)
PVer_548.2 9_19.1	C <sub>30</sub> H <sub>39</sub> N <sub>5</sub> O <sub>5</sub>	[M-H] <sup>-</sup>	548.2 892	19.1	HCD (60)	548.2892(12) 164.0722(100) 202.0990(40) 375.2409(32) 147.0457(18) 219.1505(10) 271.1780(8) 318.2193(8) 301.1675(7) 253.1677(5)
PVer_564.2 8_12.6	C <sub>30</sub> H <sub>39</sub> N <sub>5</sub> O <sub>6</sub>	[M-H] <sup>-</sup>	564.2 828	12.6	HCD (60)	553.2814(15) 116.0721(100) 202.0513(72) 285.1937(55) 119.0507(45) 331.1091(30) 435.2257(20) 302.1512(12) 278.1515(12) 155.0831(11) 241.1352(8)
PVer_567.0 9_26.3	C <sub>31</sub> H <sub>20</sub> O <sub>1</sub> 1	[M-H] <sup>-</sup>	567.0 941	26.3	HCD (60)	567.0941(100) 523.1042(97) 257.0462(86) 265.0510(55) 309.0402(30) 256.0376(20)
PVer_567.0 9_25.1	C <sub>31</sub> H <sub>20</sub> O <sub>1</sub> 1	[M-H] <sup>-</sup>	567.0 941	25.1	HCD (60)	567.0941(60) 523.1042(100) 439.0817(17)
PVer_523.3 0_27.7	C <sub>31</sub> H <sub>40</sub> N <sub>4</sub> O <sub>2</sub>	[M+Na] <sup>+</sup>	523.3 047	27.7	HCD (20)	523.3038(12) 266.1168(100)
PVer_523.3 0_27.4	C <sub>31</sub> H <sub>40</sub> N <sub>4</sub> O <sub>2</sub>	[M+Na] <sup>+</sup>	523.3 047	27.4	HCD (20)	523.3058(62) 266.1170(100)
PVer_507.2 8_38.5	C <sub>32</sub> H <sub>36</sub> N <sub>4</sub> O <sub>2</sub>	[M-H] <sup>-</sup>	507.2 760	38.5	HCD (60)	507.2762(100) 236.0728(70)
PVer_537.2 9_39.5	C <sub>32</sub> H <sub>42</sub> O <sub>7</sub>	[M-H] <sup>-</sup>	537.2 867	39.5	HCD (60)	537.2867(3) 71.0507(100) 95.0507(80) 147.0821(55) 159.1185(30) 417.2810(25) 321.2230(15) 399.2701(12)
PVer_553.2 8_38.7	C <sub>32</sub> H <sub>42</sub> O <sub>8</sub>	[M-H] <sup>-</sup>	553.2 814	38.7	HCD (60)	553.2814(25) 175.1133(100) 121.0663(35) 97.0300(23) 253.1602(6)
PVer_553.2 8_38.3	C <sub>32</sub> H <sub>42</sub> O <sub>8</sub>	[M-H] <sup>-</sup>	553.2 814	38.3	HCD (60)	553.2814(5) 175.1133(100) 121.0663(72) 97.0300(66) 201.0926(15) 393.1443(10) 135.0820(19) 119.0507(19)
PVer_623.3 2_36.3	C <sub>37</sub> H <sub>42</sub> N <sub>4</sub> O <sub>5</sub>	[M+H] <sup>+</sup>	623.3 215	36.3	HCD (20)	623.3226(2) 373.2386(100) 242.1653(25) 251.0916(13) 111.0916(3)
PVer_621.3 0_36.3	[M-H] <sup>-</sup>	621.3 073	HCD (40)		621.3091(5) 371.2249(100) 249.0773(62)	
PVer_713.2 8_33.4	C <sub>38</sub> H <sub>42</sub> N <sub>4</sub> O <sub>10</sub>	[M-H] <sup>-</sup>	713.2 847	33.4	HCD (60)	713.2847(2) 190.0276(100) 191.0355(65) 309.0411(22) 161.0249(17) 311.0567(12) 325.0723(9) 400.2126(2)