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In situ mass spectrometry monitoring of fungal cultures led to the identification of four peptaibols with a rare threonine residue

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ABSTRACT

Peptaibols are an intriguing class of fungal metabolites due both to their wide range of reported bioactivities and to the structural variability that can be generated by the exchange of variable amino acid building blocks. In an effort to streamline the discovery of structurally diverse peptaibols, a mass spectrometry surface sampling technique was applied to screen the chemistry of fungal cultures *in situ*. Four previously undescribed peptaibols, all containing a rare threonine residue, were identified from a fungal culture (MSX53554), which was identified as *Nectriopsis* Maire (Bionectriaceae, Hypocreales, Ascomycota). These compounds not only increased the known threonine-containing peptaibols by nearly 20%, but also, the threonine residue was situated in a unique place compared to the other reported threonine-containing peptaibols. After the initial *in situ* detection and characterization, a large-scale solid fermentation culture was grown. The four peptaibols were isolated and characterized by mass spectrometry. In addition, one of the peptaibols was fully characterized by NMR and amino acid analysis using Marfey's reagent and exhibited moderate *in vitro* anticancer activity.

1. Introduction

Peptaibols are a class of fungal secondary metabolites that are typically comprised of 5–20 amino acid units, including one to several α-aminoisobutric acid (Aib) residues, and have an acylated N-terminus with an amino-alcohol C-terminus (Degenkolb et al., 2003; Degenkolb and Bruckner, 2008; Neumann et al., 2015; Otto et al., 2016; Toniolo and Brückner, 2007, 2009; Whitmore and Wallace, 2004). These small peptides have been reported to possess a wide variety of bioactivities, such as antimicrobial (Figueroa et al., 2013), anthelmintic (Schiell et al., 2001; Thirumalachar, 1968), and cytotoxic (He et al., 2006). As part of a multidisciplinary program to identify new anticancer leads from nature (Ayers et al., 2012; Figueroa et al., 2013; Kinghorn et al., 2016), a protocol was implemented to monitor the chemistry of fungal cultures *in situ* using a recently described droplet–liquid microjunction–surface sampling probe (droplet–LMJ–SSP) coupled to an ultraperformance liquid chromatography–high resolution mass spectrometry (UPLC–HRMS) system (Kertesz and Van Berkel, 2010; Paguigan et al., 2016; Sica et al., 2016b, 2015, 2016c).

The droplet–LMJ–SSP system is a modified autosampler with the ability to manually control the syringe to act as a surface sampling probe. This system is designed to perform microextractions on the surface of a sample and directly inject the extract into LC-MS (Kertesz and Van Berkel, 2010, 2013, 2014). Recently, this technique has been applied towards *in situ* analyses of fungal and plant metabolites with various goals, such as dereplication of fungal cultures (Sica et al., 2015), spatial and temporal mapping of the biosynthesis of targeted leads in fungal cultures (Paguigan et al., 2016; Sica et al., 2016b, 2016c), and the characterization of complex mixtures in a suite of plant tissues (Sica et al., 2016a). A major benefit of this technique is the ability to monitor the biosynthesis of fungal secondary metabolites, both with respect to space and time, without having to extract the entire culture. Recently, Ifa and colleagues demonstrated the detection of peptaibols from fungal

Abbreviations: droplet–liquid microjunction–surface sampling probe, droplet–LMJ–SSP; ultraperformance liquid chromatography, UPLC; high resolution mass spectrometry, HRMS; higher-energy collisional dissociation, HCD; internal transcribed spacer, ITS.

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cultures using ambient ionization mass spectrometry (Tata et al., 2015); however, there has yet to be a protocol reported for both the detection and characterization of peptaibols from fungal cultures *in situ*.

Since peptaibols are readily identified by mass spectrometry due to a few characteristic signals, an *in situ* mass spectrometry technique possesses great potential for early stage prioritization. When using electrospray ionization (ESI), peptaibols often undergo in-source fragmentation, effectively splitting the compound into two or more product ions (b-type and y-type ions) adjacent to any proline residues that are present (Ayers et al., 2012; Degenkolb et al., 2003). Subsequently, high resolution tandem mass spectrometry (HRMS/MS) on each fragment provides the accurate masses of each amino acid as they cleave sequentially. Due to these characteristics, the droplet–LMJ–SSP can not only detect the presence of peptaibols, but also allows for *in situ* characterization (Sica et al., 2016a) of them prior to performing any fermentation and isolation procedures (Ayers et al., 2012; Figueroa et al., 2013; Rivera-Chávez et al., 2017).

During the *in situ* evaluation of the biosynthesis of fungal metabolites in cultures (Sica et al., 2015), a series of novel, threonine-containing peptaibols were identified, which was notable because only a small percentage of peptaibols are known to contain a threonine (Thr) residue. According to the online peptaibiotics database, as of 2017 (Neumann et al., 2015), only about 1.5% (21 of 1390) of peptaibols and peptaibol-like compounds contained threonine, including those with antibiotic properties (emerimicins and zervamicins (Rinehart et al., 1981)) and with anthelmintic activity (cephaibols (Schiell et al., 2001)). Of these peptaibols from the database (Neumann et al., 2015), all are either 16- or 17-residue long sequences with the Thr in the sixth position of the amino acid sequence (Andersson et al., 2009; Rinehart et al., 1981; Schiell et al., 2001; Sharman et al., 1996). The peptaibols reported herein contained the Thr residue in position 10 of a sequence that was only eleven residues long. Upon *in situ* detection and identification of Thr-containing peptaibols, a larger scale fermentation of the fungal culture was prepared and extracted to isolate and fully characterize their structures using HRMS/MS, NMR, and Marfey's analysis (Bhushan and Brückner, 2004, 2011; Kjer et al., 2010; Marfey, 1984). Additionally, one of the isolated peptaibols showed moderate cytotoxicity.

2. Results and discussion

2.1. In situ analysis of fungal metabolites

Traditionally, the structures of peptaibols are characterized by mass spectrometry. This technique allows for the assembly of the amino acids in a molecule by analysis of the sequential fragmentation of b-type and y-type ions generated in-source. However, this method does not allow the differentiation between isobaric amino acids (e.g., leucine and isoleucine, etc.) and enantiomers (i.e., D or L). Given the ability to study peptaibols by mass spectrometry, coupled with their interesting biological activities (Figueroa et al., 2013; He et al., 2006; Schiell et al., 2001; Thirumalachar, 1968), we sought to study fungal cultures *in situ* toward the discovery of peptaibols by using the droplet–LMJ–SSP, (Kertesz and Van Berkel, 2010; Paguigan et al., 2016; Sica et al., 2016b, 2015, 2016c). The fungal culture, coded MSX53554, which was identified as *Nectriopsis* Maire (Bionectriaceae), was readily noted as a peptaibol producer based on the mass spectral data (Fig. 1). The key indicators of peptaibols in the HRMS and MS/MS spectra were the presence of in-source fragments around the proline residue, the presence of Aib residues, a C-terminus that was reduced to an alcohol, and an acetylated N-terminus. Upon further analysis of the MS/MS data (Fig. 1C), it was determined that strain MSX53554 biosynthesized peptaibols with a Thr residue, which was quite rare (Degenkolb et al., 2003; Degenkolb and Bruckner, 2008; Neumann et al., 2015; Whitmore and Wallace, 2004).

2.2. Isolation

A solid phase culture of fungal culture MSX53554 was prepared for the purpose of isolation and identification of the peptaibols detected via the surface sampling system. The culture was extracted and subjected to fractionation by normal phase flash chromatography. The peptaibol-containing fraction was further processed by reverse phase HPLC to isolate the peptaibols. While compound **1** was readily isolated from the fraction, compounds **2**–**4** co-eluted, appearing as a single peak. Initially thought to be a single compound, the mixture of **2**–**4** was run on LC-MS to begin the characterization process. The full scan MS quickly showed the presence of isomeric peptaibols by the detection of two in-source fragment pairs of *m/z* 510/571 and *m/z* 496/585 (Fig. 2).

To further purify this mixture, analytical HPLC of the mixture of **2**–**4** was performed, and the extracted ion chromatograms (±5 ppm) of the in-source fragments were monitored to optimize the separation of the isomers (Fig. S4). A 40% acetonitrile isocratic method revealed three isomeric peptaibols and provided the best separation of these compounds for the isolation of pure compounds. Enough material for characterization by HRMS and MS/MS was purified via semi-preparatory HPLC.

2.3. Structure elucidation

The complete structure of compound **1** was elucidated using three orthogonal approaches: (1) MS/MS fragmentation patterns to determine the amino acid sequence; (2) derivatization with Marfey's reagent to identify the chirality of each amino acid; and (3) 2D NMR experiments to confirm the structure. However, for the positionally isomeric compounds (**2**–**4**), their structure elucidation was limited to MS/MS analysis due to paucity of the samples.

2.3.1. Mass spectrometry

In-source fragmentation of compound **1** resulted in two ions that were further fragmented by higher-energy collisional dissociation (HCD), providing high resolution fragments that indicated which amino acids were present. Furthermore, the b-type ions were most abundant, which accelerated the identification of the sequence. The spectra obtained from this analysis of a pure compound isolated from a large-scale culture matched the spectra previously obtained via the screening process of a droplet from the culture in a Petri dish. This confirmed the viability of the screening process for *in situ* detection and characterization of peptaibols. It also demonstrated that the fungal culture continued to biosynthesize this unique class of threonine-containing peptaibols during the scale-up process.

The pseudomolecular ion $[M+H]^+$ of the intact peptaibol **1** was observed at *m/z* 1094.7186, which confirmed the molecular formula of $C_{53}H_{95}N_{11}O_{13}$ (Δ *m/z* 0.2 ppm). The two diagnostic in-source fragments were *m/z* 510.3264 and *m/z* 585.3966, suggesting molecular formulae of $C_{25}H_{43}N_5O_6$ and $C_{28}H_{52}N_6O_7$, respectively (Fig. S5). As is typical, the fragmentation was presumed to be adjacent to the proline residue, which was later confirmed by NMR experiments. Since the isobaric amino acid isomers cannot be differentiated by MS, some amino acid identifications remained ambiguous (e.g., Lxx indicates either Leu or Ile; Vxx indicates Val or Iva). HCD fragmentation of *m/z* 510.3264 resulted in the sequential losses of Aib (85.0512), Lxx (113.0841), Ala