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.

Keywords: *Nectriopsis* Maire (Bionectriaceae) | Hypocreales | Fungal metabolites | Peptaibols | Threonine | *In situ* mass spectrometry

Article:

1. Introduction

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

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, Toniolo and Brückner, 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, Sica et al., 2015, Sica et al., 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, Kertesz and Van Berkel, 2013, Kertesz and Van Berkel, 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, Sica et al., 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 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, Bhushan and Brückner, 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, Sica et al., 2015, Sica et al., 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).

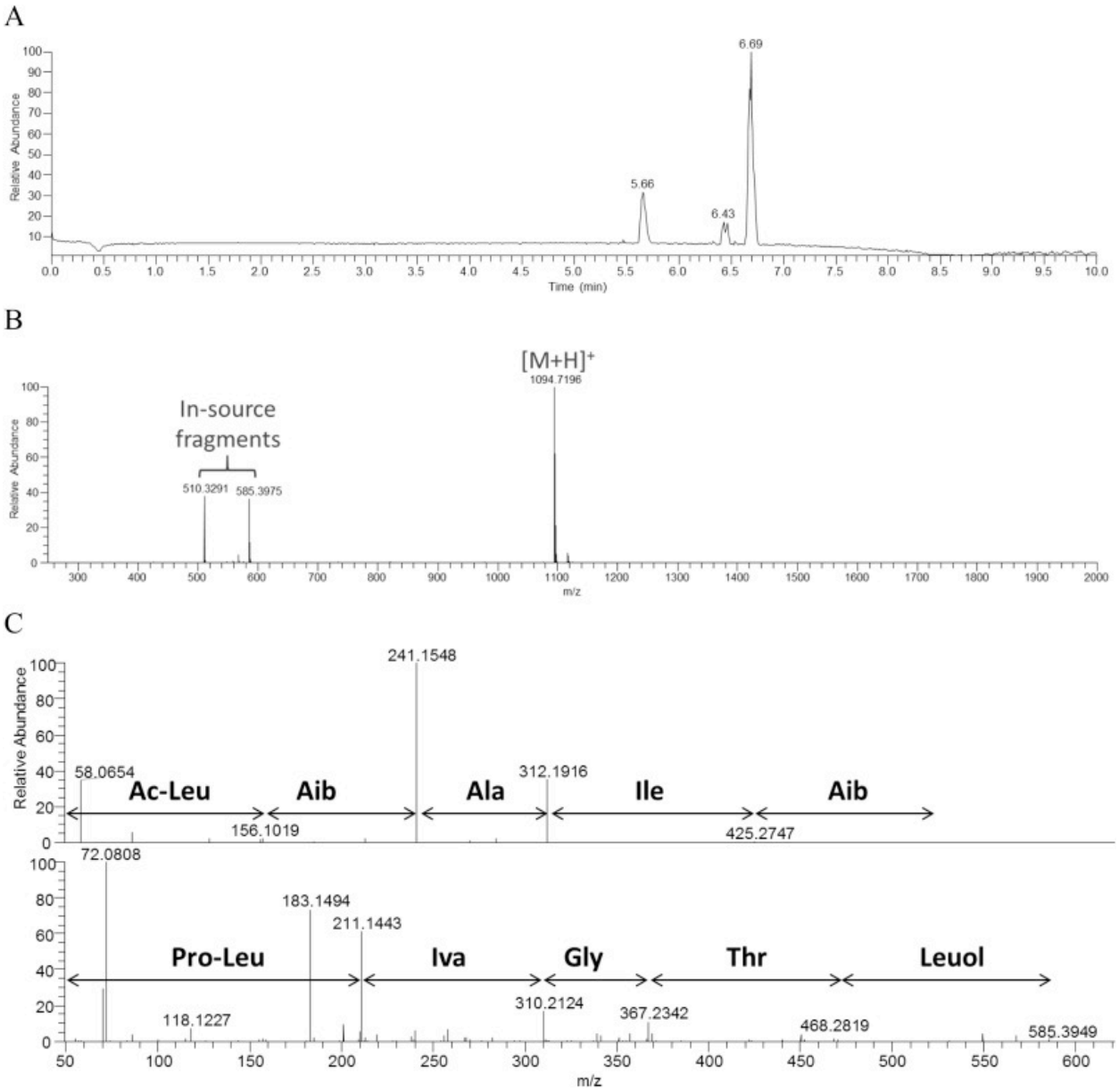

Fig. 1. (A) The chromatographic data obtained from the surface sampling screening protocol. (B) The mass spectrum for the peak at 5.66 min, indicating the presence of a peptaibol by in-source fragments for the b-type (510.3291) and y-type (585.3975) ions. (C) The tandem mass spectrum from each fragment (*m/z* 510.3291, top; *m/z* 585.3975, bottom), providing enough information to begin characterizing the structure from *in situ* data; all the fragments connected by arrows in this panel are b-type ions.

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.

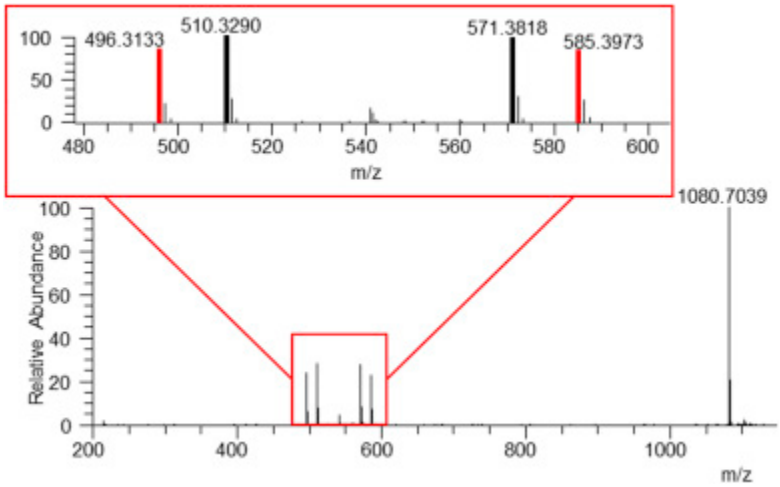

Fig. 2. Two pairs, colored red and black, of in-source fragments for *m/z* 1080.7039 that indicated co-eluting isomers.

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 (71.0368), and Aib (85.0527). The final fragment at *m/z* 156.1016 was attributed

to Lxx and the presence of an acetylated N-terminus, a feature that is commonly observed for peptaibols (Degenkolb et al., 2003, Neumann et al., 2015). HCD fragmentation of *m/z* 585.3966 resulted in the sequential losses of Lxxol (*m/z* 117.1153), Thr (101.0477), Gly (57.0216), and Vxx (99.0682). The diagnostic fragment *m/z* 211.1438 was attributed to Pro-Lxx with the Pro presumed to be adjacent to the cleavage point. At this point, the sequence for compound **1**was identified as Ac-Lxx^{1}-Aib^{2}-Ala^{3}-Lxx^{4}-Aib^{5}-Pro^{6}-Lxx^{7}-Vxx^{8}-Gly^{9}-Thr^{10}-Lxxol.
Compounds **2** (Fig. S6), **3** (Fig. S7), and **4** (Fig. S8) were analyzed in similar fashion (Table 1).

Table 1. The high resolution tandem mass spectrometry (HRMS/MS) data for peptaibols **1**–**4**.

Compound	**$[M+H]^+$**	**In-source Fragment[a]**	**HRMS/MS of in-source fragment[b]**			
Necthreonin A (**1**)	1094.7196	510.3264	425.2752	312.1911	241.1543	156.1016
		585.3966	468.2813	367.2336	310.2120	211.1438
Necthreonin B (**2**)	1080.7018	510.3282	425.2751	312.1911	241.1543	156.1015
		571.3807	454.2653	353.2177	296.1963	211.1438
Necthreonin C (**3**)	1080.7015	496.3122	411.2595	312.1910	241.1540	156.1017
		585.3962	450.2705	367.2333	310.2118	211.1437
Necthreonin D (**4**)	1080.7010	496.3119	411.2594	298.1752	277.1389	156.1015
		585.3959	468.2806	367.2329	310.2118	211.1435

[a]In this column, the top number indicates the b-type ion fragment and the bottom number indicates the y-type ion fragment.
[b]In this column, all fragments are the b-type ions after applying MS/MS to the in-source fragment in the adjacent column.

2.3.2. Amino acid analysis using Marfey's reagent

The absolute configuration of the individual amino acids in **1** was assigned by acid hydrolysis and subsequent derivatization with *Nα*-(2,4-dinitro-5-fluorophenyl)-L-alaninamide [Marfey's reagent (Bhushan and Brückner, 2004, Bhushan and Brückner, 2011)]. Traditionally, this method compares the retention time of the amino acid-Marfey's derivatives with those of derivatized D and L standards for each amino acid, using LC-UV to monitor the eluents at a wavelength of 340 nm (Ayers et al., 2012, Figueroa et al., 2013, Rivera-Chávez et al., 2017). Compound **1** was examined in a similar fashion, but also incorporated MS to add mutually supportive data to this confirmation (Table S1). For instance, the chromatographic method resulted in both D-Val and L-Leu eluting at the same time (Table S1). Thus, UV data alone would not allow for differentiating the two without optimizing the chromatography and re-running the samples. Furthermore, the use of MS was beneficial over UV alone, since the α-substituted amino acids (Aib, Iva, etc.) were over a magnitude lower in UV and MS signal. Thus, the ability to perform extracted ion chromatograms (±5 ppm) provided a more confident comparison of retention times with the standards without the need to increase the sample's concentration and perform re-analysis.

Additionally, the data showed that there was both Leu and Ile residues present, which was something that could not be differentiated for the intact peptaibol by MS. Based on the integration under each peak, it was determined that the Leu peak was about three times larger than that of the Ile peak. Thus, one of the four MS fragments that indicated a loss of Lxx (*m/z* 113 for Lxx and 117 for Lxxol) must be an Ile residue.

2.3.3. Nuclear magnetic resonance

The structure of peptaibol **1**, including the position of the Ile residue, was confirmed by 1D (Fig. S9) and 2D NMR (Table 2). A TOCSY experiment readily identified the protonated amide associated with each amino acid residue (Fig. S10). As expected, the Pro, Aib, and Iva residues do not present any correlations due to the lack of protons on the α-carbon. The Ile residue was distinguished from the Leu residues by the integration of the α-H (H), β-H (H), γ_1-H (2H), γ_2-H (3H), and δ-H (3H), the TOCSY correlations (Fig. S11A) of the NH (δ_H 7.66) with the α-H (δ_H 4.17), β-H (δ_H 1.95), γ_1-H (δ_H 1.35 and 1.57), γ_2-H (δ_H 0.97), and δ-H (δ_H 0.86), and the HMBC correlations (Fig. S11B) of the NH (δ_H 7.66) with the α-C (δ_C59.0) and β-C (δ_C 36.8). The position of the Ile was determined by the HMBC correlations of the NH (δ_H 7.66, Ile^4) with the carbonyl of Ala^3 (δ_C 174.6) and the NH (δ_H 8.06, Aib^5) with the carbonyl of Ile^4 (δ_C 173.6).

Table 2. NMR spectroscopic data for compound **1** (^{1}H, 700 MHz and ^{13}C 175 MHz in $CDCl_3$).[a]

	δ_C	**type**	**δ_C mult. (*J* in Hz)**	**HMBC ($^n J_{HC}$)**	**TOCSY ($^n J_{HH}$)**
Ac					
CO	173.4	C	–	–	–
Me	23.0	CH_3	2.11, s	CO	–
Leu1					
CO	174.5	C	–	–	–
NH	–	–	7.99	–	–
α	55.9	CH	3.91, td (8.0, 3.1)	CO, β	β, γ, δ′
β	40.0	CH_2	1.68, m	CO, α, γ, δ, δ′	α, γ, δ′
γ	24.9	CH	1.74, m	CO, α, β, δ, δ′	α, β, δ, δ′
δ	22.4	CH_3	0.94, d (6.5)	β, γ, δ′	α, β, γ, δ′
δ′	22.4	CH_3	0.97, d	β, γ, δ	α, β, γ, δ
Aib2					
CO	176.0	C	–	–	–
NH	–	–	8.23, s	CO (L1), CO, α, β	–
α	56.6	C	–	–	–
β_1	27.7	CH_3	1.43, s	CO, α, β_2	–
β_2	23.4	CH_3	1.51, s	CO, α, β_1	–
Ala3					
CO	174.6	C	–	–	–
NH	–	–	7.38, d (6.5)	CO (U2), α, β	α, β
α	51.6	CH	4.21, m	CO, β	NH, β
β	17.5	CH_3	1.51, d (7.0)	CO, α	NH, α
Ile4					
CO	173.6	C	–	–	–
NH	–	–	7.66, d (7.8)	CO (A3), α, β	α, β, γ_1, γ_2, δ
α	59.0	CH	4.17, t (7.4)	CO, β, γ_1, γ_2	NH, β, γ_1, γ_2, δ
β	36.8	CH	1.95, m	α, β, γ_1, γ_2, δ	NH, α, γ_1, γ_2, δ
γ_1	26.0	CH_2	1.35, m 1.57, m	β, γ_2, δ	NH, α, β, γ_1, γ_2, δ
γ_2	15.7	CH_3	0.97, d, overlapped	α, β, γ_1	NH, α, β, γ_1
δ	11.5	CH_3	0.86, t (7.4)	β, γ_1	β, γ_1, γ_2, δ
Aib5					
CO	173.8	C	–	–	–
NH	–	–	8.06, s	CO (I4), α, β_2	–
α	56.9	C	–	–	–
β_1	26.4	CH_3	1.48, s	CO, α, β_2	–

	δ_C	type	δ_C mult. (*J* in Hz)	HMBC ($^nJ_{HC}$)	TOCSY ($^nJ_{HH}$)
β_2	23.5	CH_3	1.55, s	CO, α, β_1	–
Pro6					
CO	175.7	C	–	–	
α	63.6	CH	4.36, t (7.5)	CO, β, γ, δ	β, γ, δ
β	28.9	CH_2	1.79, m 2.23, m	CO, α, γ, δ	α, β, γ, δ
γ	26.4	CH_2	1.95, m 2.12, m	α, β, δ	α, β, γ, δ
δ	49.3	CH_2	3.71, m	β, γ	α, β, γ
Leu7					
CO	175.7	C	–	–	–
NH	–	–	7.73, d (6.4)	CO (P6), α, β	α, β, γ, δ, δ′
α	55.5	CH	4.06, dd (7.3)	CO, β, γ	NH, β, γ, δ, δ′
β	39.4	CH_2	1.88, m	CO, α, γ, δ	NH, α, γ, δ, δ′
γ	25.0	CH	1.74, m	CO, α, β	NH, α, β, δ, δ′
δ	22.1	CH_3	0.92, m	Nothing	α, β, γ, δ′
δ′	22.6	CH_3	0.98, m	Nothing	α, β, γ, δ
Iva8					
CO	178.4	C	–	–	–
NH	–	–	7.95, s	CO (L7), CO α, β_2	–
α	59.7	C	–	–	–
β_1	25.3	CH_2	2.37, dd (7.5) 1.78, m	CO α, β_2, γ	β_1, γ
β_2	23.1	CH_3	1.47, s	CO α, β_1	–
γ	7.1	CH_3	0.79, t (7.4)	α, β_1	β_1
Gly9					
CO	171.3	C	–	–	–
NH	–	–	7.82, d (7.7)	CO (J8), α, α′	α, α′
α	44.9	CH_2	3.70, m	CO	NH, α′
α′	44.9	CH_2	4.00, dd (16.7, 4.6)	CO	NH, α
Thr10					
CO	171.4	C	–	–	–
NH	–	–	7.81, d (7.7)	CO (G9), α, β	α, β, γ, OH
α	63.5	CH	4.11, t (6.4)	CO, β, γ	NH, β, γ, OH
β	67.0	CH	4.30, m (6.5)	γ	NH, α, γ, OH
γ	20.0	CH_3	1.35, d (6.4)	α, β	NH, α, β, OH
OH	–	–	4.75, d (7.7)	β, γ	NH, α, β, γ
Leuol					
NH	–	–	6.95, d (9.1)	CO (T10)	α, β_1, β_2, γ, δ, OH
α	50.1	CH	4.12, m		NH, β_1, β_2, γ, δ, OH
β_1	39.7	CH_2	1.22, m 1.54, m	β_1, γ, δ	NH, α, β_2, γ, δ
β_2	65.8	CH_2	3.55, m 3.64, t (8.8)	α, β_1	NH, α, β_1, β_2, γ, δ, OH
γ	24.9	CH	1.68, m	α, β_1	NH, α, β_1, δ
δ	22.1	CH_3	0.90, m	β_1, γ	NH, α, β_1, β_2, γ
OH	–	–	3.85, t	β_2	NH, α, β_1, β_2, γ

[a]L = Leu, U = Aib, I = Ile, A = Ala, P = Pro, J = Iva, G = Gly, T = Thr.

Threonine was also confirmed via NMR, by integration of the α-H (H), β-H (H), and γ-H (3H) along with the OH. The TOCSY signals correlated (Fig. S11C) from the NH (δ_H 7.81) to the α-H

(δ_H 4.11), β-H (δ_H 4.30), γ-H (δ_H 1.35), and OH (δ_H 4.75). Furthermore, the coupling constants of the α-H (J = 6.4 Hz), β-H (J = 6.5 Hz), and γ-H (J = 6.4 Hz) were all in agreement with each other. The HMBC data correlated (Fig. S11C) the NH (δ_H 7.81) with the α-C (δ_C 63.5.0) and β-C (δ_C 67.0), as well as with the carbonyl of the adjacent Gly (171.3) residue. Further confirming the presence of the threonine, the OH displayed correlations to the α-H (δ_H 4.11), β-H (δ_H 4.30), γ-H (δ_H 1.35), and NH (δ_H 7.81) and to the β-C (δ_C 67.0) and γ ___C (δ_C 20.0) in the TOCSY and HMBC spectra, respectively. The position of the Thr was determined by the HMBC correlations of the NH (δ_H 7.81, Thr[10]) with the carbonyl of Gly[9] (δ_C 171.3) and the NH (δ_H 6.95, Leuol) with the carbonyl of Thr[10](δ_C 171.4). Interestingly, this is the first reported case where the Thr residue was in the tenth position of an 11-residue long sequence. In all other Thr-containing peptaibols, this residue was located in the sixth position of a 16- or 17-residue peptaibol (Andersson et al., 2009, Neumann et al., 2015, Rinehart et al., 1981, Schiell et al., 2001, Sharman et al., 1996). All other amino acids and their respective positions were determined (Fig. 3) via TOCSY, HMBC, and NOESY in a similar fashion (Table 2), and compound **1** was ascribed the trivial name necthreonin A.

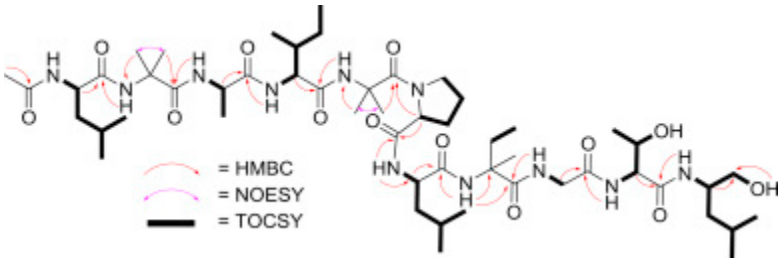

Fig. 3. The overall structure for compound **1** was confirmed by TOCSY, HMBC, and NOESY NMR. This figure illustrates key correlations.

2.3.4. Sequence determination

Compounds **2**–**4**, which were ascribed the trivial names necthreonin B, necthreonin C, and necthreonin D, respectively, were characterized only by HRMS/MS due to the paucity of sample. Fortunately, their fragmentation patterns were very similar to that of compound **1**, with only one amino acid difference for each. Compared to compound **1**, compound **2** had Aib[8] instead of Iva[8], compound **3** presumably had Val[4] instead of Ile[4], and compound **4** had Ala[2] instead of Aib[2] (Table 3).

Table 3. The proposed sequences of the four peptaibols from fungal culture MSX53554, *Nectriopsis* sp. The amino acid residues that are in **bold** were the positions that differed from compound **1**. For compounds **2**–**4**, the structures of isobaric amino acids (underlined) could not be confirmed due to paucity of sample and are proposed based on biosynthetic considerations relative to the fully characterized **1**. Those amino acids could also be labeled as Lxx, indicating Leu or Ile, and Vxx, indicating Val or Iva.

Compound	**Sequence**
Necthreonin A (**1**)	Ac–Leu–Aib–Ala–Ile–Aib–Pro–Leu–Iva–Gly–Thr–Leuol
Necthreonin B (**2**)	Ac–Leu–Aib–Ala–Ile–Aib–Pro–Leu–**Aib**–Gly–Thr–Leuol
Necthreonin C (**3**)	Ac–Leu–Aib–Ala–**Val**–Aib–Pro–Leu–Iva–Gly–Thr–Leuol
Necthreonin D (**4**)	Ac–Leu–**Ala**–Ala–Ile–Aib–Pro–Leu–Iva–Gly–Thr–Leuol

While only compound **1** was confirmed by NMR and derivatization with Marfey's reagent, the amino acids for compounds **2**–**4** were presumed to contain the same isomeric ratios. For instance, Ile in position 7 for compound **1** was presumed to be Ile in position 7 for compounds **2** and **4** rather than Leu. The Val in position 4 for compound **3** was presumed to be Val, as opposed to Iva, due to its structural similarity to Ile (Fig. 4A). This assumption is based on similar instances observed for the other compounds. For example, the Iva in position 8 for compound **1** was found to be Aib in compound **2**, which is only a methylenedifferent (Fig. 4B). Similarly, the Ala in position 2 for compound **4** is only a methylene different from the Aib in position 2 for compound **1** (Fig. 4C). However, we stress that the identification of the isobaric amino acids could not be confirmed for compounds **2**–**4** (i.e., Val^4 instead of Iva^4 for compound **3**), even though the structural trends between the compounds supported these hypotheses.

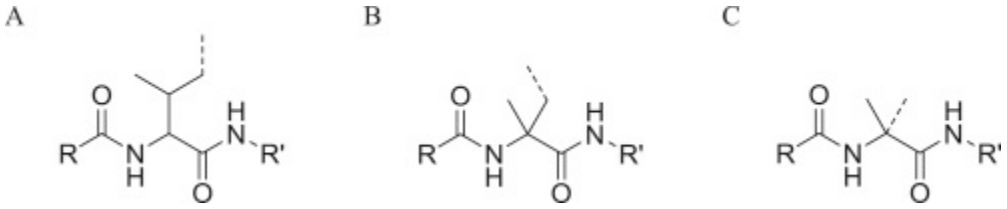

Fig. 4. The dashed lines indicate the difference of CH_2 between (A) position 4 for compounds **1** and **3**, (B) position 8 for compounds **1** and **2**, and (C) position 2 for compounds **1** and **4**. The inclusion of the dashed line represents compound **1** in all cases above.

2.4. Anticancer bioactivity

The purified peptaibols (**1**–**4** (Fig. S12)) were tested against human melanoma cancer cells(MDA-MB-435), human breast cancer cells (MDA-MB-231), and human ovarian cancer cells (OVCAR3). Compound **1** was moderately active against the human cancer cell lines, with IC_{50} values ranging between 15 and 23 μM (Table 4); the other three peptaibols were inactive, with IC_{50} values > 25 μM.

Table 4. The bioactivity for the four peptaibols in the breast (MDA-MB-435 and MDA-MB-231) and ovarian (OVCAR3) cancer cell lines.

Compound	**IC_{50} (μM)**		
	MDA-MB-435	**MDA-MB-231**	**OVCAR3**
Necthreonin A (**1**)	23.3	15.1	22.6
Necthreonin B (**2**)	>25	>25	>25
Necthreonin C (**3**)	>25	>25	>25
Necthreonin D (**4**)	>25	>25	>25
taxol	0.004	0.011	0.006

3. Conclusion

Using the droplet–LMJ–SSP, it is feasible to scan fungal cultures *in situ* for compounds of interest, thereby prioritizing samples for scale-up fermentations. The droplet–LMJ–SSP coupled to UPLC–HRMS provided the chromatographic resolution necessary to separate mixtures and characterize fungal secondary metabolites *in situ*. By performing this screening protocol, a fungal culture was rapidly identified as a peptaibol-producing fungus, and subsequently, four

new threonine-containing peptaibols were characterized (Table 2), thereby increasing the number of these known in the literature by nearly 20%.

NMR, HRMS, and Marfey's analysis supported the screening results and confirmed the structures of the new compounds. The inclusion of HRMS data into the Marfey's analysis provided mutually supportive data and removed any uncertainty that remained from using UV data alone. Ultimately, this study incorporated advanced mass spectrometry techniques to discover and elucidate new bioactive compounds from fungi.

4. Experimental

4.1. General experimental procedures

In situ sampling of fungal cultures was performed by the droplet–liquid microjunction–surface sampling probe (droplet–LMJ–SSP). This system was constructed as detailed previously (Kertesz and Van Berkel, 2010, Kertesz and Van Berkel, 2013, Sica et al., 2015). The droplet–LMJ–SSP was coupled to the Waters Acquity ultraperformance liquid chromatography (UPLC) and Thermo QExactive Plus mass spectrometry system. The UPLC separations were performed on an Acquity BEH C_{18} column (50 mm × 2.1 mm i.d., 1.7 μm) equilibrated at 40 °C and operated at a flow rate of 0.3 ml/min. The mobile phase consisted of Fisher Optima LC−MS grade CH_3CN—H_2O (acidified with 0.1% formic acid), starting at 15% CH_3CN and increasing linearly to 100% CH_3CN over 8 min. The system was held at 100% CH_3CN for 1.5 min before returning to starting conditions for re-equilibration. The photodiode array detector (PDA) was set to acquire from 200 to 500 nm with 4 nm resolution.

HPLC separations were performed using Varian ProStar HPLC system connected to a ProStar 335 PDA with UV detection set at 210 and 254 nm. Preparative HPLC purifications were performed on an Atlantic T3 C_{18} column (250 × 19 mm i.d., 5 μm) at a flow rate of 15.0 ml/min. The semi-preparative HPLC purification of isomers was performed on a YMC C_{18} column (250 × 10 mm i.d., 5 μm) at a flow rate of 4.7 ml/min. Flash column chromatography was carried out with a Teledyne ISCO Combiflash Rf connected to ELSD and PDA detectors with UV detection set at 200–400 nm.

The NMR data were collected on an Agilent 700 MHz NMR spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a cryoprobe operating at 700 MHz and 175 MHz for ^{1}H and ^{13}C, respectively. The chemical shift values were referenced to the residual solvent signals for $CDCl_3$ (δ_H/δ_C 7.26/77.16). HRMS data were collected using a Thermo QExactive Plus mass spectrometer (ThermoFisher, San Jose, CA, USA) in positive electrospray ionization mode coupled to a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA). The spray voltage was set to 4.0 kV, resulting in the in-source fragmentation of the precursor ions for the peptaibols. The higher-energy collisional dissociation (HCD) fragmentation used a normalized collision energy of 25 for all compounds to obtain the tandem mass spectrometry (MS/MS) data of each in-source generated fragment. In general, the b-ions were the predominant fragment ions.

Reference standards of amino acids and Marfey's reagent were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and solvents were obtained from Fisher Scientific and were used without further purification.

4.2. *In situ* chemical analysis of fungal cultures

Mass spectrometry monitoring of fungal secondary metabolites *in situ* can address important ecological information that could be lost via traditional extraction process (Sica et al., 2016b, Sica et al., 2015, Sica et al., 2016c). To better understand the fungal metabolites biosynthesized in a living axenic culture, we analyzed a culture of strain MSX53554 by the droplet–LMJ–SSP system. Briefly, the culture was grown in a Petri dish containing nutrient agar, such as potato dextrose agar (Difco), and allowed to grow for 3 weeks prior to analysis.

4.3. Identification of fungal strain (MSX53554)

Mycosynthetix fungal strain MSX53554 was isolated from leaf litter collected in a baboon sanctuary in 1991 by Dr. Barry Katz. Identification of strain MSX53554 was based on morphological, cultural, and molecular data. Colonies of strain MSX53554 on potato dextrose agar were fast growing, reaching a diameter of 30 mm in 3 weeks; milky white, reverse colony of same color (Fig. S1A); conidiophores branched, phialidic; conidia oblong to short cylindrical, in slimy heads, cyanophilic (blue in aqueous cotton blue stain) (Fig. S1B & C). Thus, based on morphological examination of the strains grown on potato dextrose agar, we were able to identify the strain as belonging to the genus *Nectriopsis* Maire (Zare and Gams, 2016).

Molecular identification followed protocols that have been described in detail recently (Raja et al., 2017b). Briefly, DNA was extracted from the fungal culture using a Zymo Research Fungal/Bacterial DNA Extraction Kit. For PCR, we sequenced portions of three gene regions: ITS1, 5.8S, ITS2 rDNA (Internal transcribed spacer region), partial 28S rDNA large subunit (LSU), and partial fragment of translation elongation factor 1-alpha (*tef1*). The ITS region was PCR amplified using primers ITS5 and ITS4 (White et al., 1990), the partial LSU region was amplified using LROR and LR6 (Vilgalys and Hester, 1990), while the *tef1*region, specifically intron 4 in combination with intron 5, which is useful for species-level identification, was obtained using primer combinations 983F/728F and 2218R (Carbone and Kohn, 1999, Jaklitsch et al., 2005). Protocols for Sanger sequencing were as outlined previously (Raja et al., 2015, Raja et al., 2017a). All three sequences were subject to a BLAST search in NCBI GenBank to verify their identity. BLAST search was evaluated only with published, authentic sequences. Based on a BLAST search of NCBIs GenBank nucleotide database, the closest hit using the ITS sequence was *Nectriopsis rexiana* (≡ *Verticillium* rexianum) culture-collection CABI:IMI320287 (GenBank JQ647443; Identities = 492/500 (98%), Gaps = 1/1122 (0%)), followed by *Nectriopsis exigua* strain G.J.S. 98-32 (GenBank HM484865.1; Identities = 462/463 (99%), Gaps = 0/463 (0%). The LSU data showed high sequence similarity to *Nectriopsis violacea* strain CBS 424.64 (GenBank AY489719; Identities = 994/1004 (99%), Gaps = 0/1004 (0%)), followed by *Nectriopsis violacea* strain MUCL40056 (GenBank AF193242; Identities = 956/969 (99%), Gaps = 2/969 (0%). A BLAST search with partial *tef1* region also showed a high sequence similarity with *Nectriopsis exigua* strain G.J.S. 98-32 (GenBank HM484852; Identities = 834/837 (99%), Gaps = 0/837(0%). To elucidate the phylogenetic placement of strain

MSX53554, we downloaded ITS and LSU sequences of *Nectriopsis* from a recent morphological and molecular study of *Nectriopsis* spp. (Zare and Gams, 2016). Since the ITS and LSU sequences were unequally available for different strains, we did not combine the two datasets. Multiple sequence alignment were performed using MUSCLE (Edgar, 2004) for separate ITS and LSU datasets, and phylogenetic analysis was performed using Maximum Likelihood (PHYML) with General Time Reversible model employing 1000 bootstrap searches in Seaview v.4.5.4 (Gouy et al., 2010). In the ITS phylogeny, MSX53554 was grouped with *Nectriopsis exigua* strain G.J.S. 98-32 with 87% PHYML bootstrap support (Fig. S2). The LSU phylogeny also showed that MSX53554 grouped with *Nectriopsis exigua* strain G.J.S. 98-32 with 72% PHYML bootstrap support (Fig. S3). *Nectriopsis exigua* has recently been synonymized as *Nectriopsis rexiana* (Sacc.) Rossman, L. Lombard & Crous, comb. nov. (MycoBank MB810977) (Lombard et al., 2015). Although, *Nectriopsis exigua* is same as *Nectriopsis rexiana* as accepted by Lombard et al. (2015). and more recently by Zare and Gams (2016), our Maximum Likelihood analyses with separate ITS and LSU data does show grouping of the newly obtained sequences of *Nectriopsis rexiana* by Zare and Gams (2016) with MSX53554 and strain G.J.S. 98-32. Due to the contradiction in our analysis with those of recently published studies (Lombard et al., 2015, Zare and Gams, 2016), we take a more conservative approach and identify MSX53554 as *Nectriopsis* Maire (Bionectriaceae, Hypocreales, Ascomycota). The sequences obtained in this study were deposited in GenBank (ITS: KY471442; LSU: KY471443; *tef1*: KY471444).

4.4. Fermentation, extraction, and isolation

The fungal fermentations were performed using procedures reported previously (Ayers et al., 2011, Ayers et al., 2012, El-Elimat et al., 2014, Figueroa et al., 2012). Briefly, fresh cultures were inoculated in a medium containing 2% soy peptone, 2% dextrose, and 1% yeast extract (YESD media). Following incubation (7 days) at 22 °C with agitation, the cultures were used to inoculate 50 ml of a rice medium, prepared using rice to which was added a vitamin solution and twice the volume of rice with H_2O in a 250-ml Erlenmeyer flask. This was incubated at 22 °C until the cultures showed good growth (approximately 14 days) to generate the screening cultures. The one scale-up culture used for isolation of the peptaibols was grown in a 2.8 l Fernbach flask containing 150 g of rice and 300 ml of H_2O and were inoculated using a seed culture grown in YESD medium. It was incubated at 22 °C for 14 days.

The scale-up fungal culture was extracted using a previously reported procedure (Ayers et al., 2011, Ayers et al., 2012, El-Elimat et al., 2014, Figueroa et al., 2012). Briefly, the culture was extracted by adding 500 ml of MeOH–$CHCl_3$ (1:1) to a 2.8 l fernbach flask containing 150 g of rice with fungal growth. The culture was chopped with a spatula before shaking overnight (∼16 h) at ∼100 rpm at room temperature. Using vacuum filtration, the sample was filtered, and the remaining residue was washed with MeOH. To the filtrate, 900 ml of $CHCl_3$ and 1500 ml of H_2O were added. The mixture was stirred for 30 min and then transferred into a separatory funnel. The organic layer was drawn off and evaporated to dryness. The dried organic extract was re-constituted in 300 ml of MeOH–CH_3CN (1:1) and 300 ml of hexanes and transferred to a separatory funnel. The biphasic solution was shaken vigorously. After settling, the MeOH–CH_3CN layer was evaporated to dryness under vacuum (480.81 mg).

The dried extract was dissolved in $CHCl_3$, adsorbed onto Celite 545, and fractionated via normal phase flash chromatography on a CombiFlash Rf system using a 12 g RediSep Rd Si-gel Gold column (Teledyne-ISCO, Lincoln, NE, USA). The gradient solvent system was hexane–$CHCl_3$–MeOH at a flow rate of 30 ml/min with 70.0 column volumes over 39.2 min. This afforded three fractions: fraction 1 (21.52 mg), fraction 2 (277.07 mg) and fraction 3 (118.55 mg).

Fraction 3, the peptaibol-enriched material as noted by mass spectrometry, was dissolved in 500 µl MeOH and purified via preparative HPLC using a gradient that initiated with 60:40 CH_3CN/H_2O and increased linearly to 80:20 CH_3CN/H_2O over 30 min. This afforded two sub-fractions. Sub-fraction 1 afforded compound **1** (15 mg), but sub-fraction 2 was a mixture of three positional isomers. The isomers were separated from each other by semi-preparative HPLC using an isocratic method of 40:60 CH_3CN/H_2O, yielding compounds **2**(1.81 mg), **3** (0.44 mg), and **4** (0.56 mg).

4.5. Amino acid analysis using Marfey's reagent

Compound **1** was analyzed using an optimization of a procedure we reported previously (Ayers et al., 2012). Briefly, to each reaction vial, approximately 0.2 mg of each amino acid standard was mixed with 50 µl of H_2O, 20 µl of 1M $NaHCO_3$, and 100 µl 1% Marfey's reagent ((*Nα*-(2,4-dinitro-5-fluorophenyl)-L-alaninamide; Acros Organics) in acetone. The mixtures were stirred at 40 °C for 1 h, then halted by the addition of 10 µl of 2N HCl. The product of the reactions was dried under a stream of air and dissolved in 2 ml of MeOH. Each derivatized standard was injected individually (0.7 µl) onto the UPLC-MS system. The UPLC conditions were 10–70% MeOH in H_2O over 10 min on the aforementioned BEH column and eluent monitored at 340 nm (Ayers et al., 2012).

Additionally, approximately 0.5 mg of compound **1** was hydrolyzed with 0.5 ml of 6N HCl in a reaction vial at 110 °C for 24 h. The solvent was evaporated under a stream of air. To the hydrolysis product, 25 µl H_2O, 10 µl 1M $NaHCO_3$, and 50 µl of 1% Marfey's reagent in acetone was added. The reaction mixture was agitated at 40 °C for 1 h. The reaction was quenched by the addition of 5 µl of 2N HCl followed by drying under a stream of air. The dried product was reconstituted in 200 µl of MeOH and injected onto the UPLC with the use of the same conditions as for the standards.

4.6. Cytotoxicity bioassay

Human melanoma cancer cells MDA-MB-435, human breast cancer cells MDA-MB-231, and human ovarian cancer cells OVCAR3 were purchased from the American Type Culture Collection (Manassas, VA). The cell lines were propagated at 37 °C in 5% CO_2 in RPMI 1640 medium, supplemented with fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100 µg/ml) (Life Technologies, Grand Island, NY). Cells in log phase growth were harvested by trypsinization followed by two washes in PBS to remove all traces of the enzyme. A total of 5000 cells were seeded per well in a 96-well clear, flat-bottom plate (Microtest 96®, Falcon) and incubated overnight (37 °C in 5% CO_2). Samples dissolved in DMSO were then diluted and added to the appropriate wells. The cells were incubated in the presence of test substance for 72 h at 37 °C and evaluated for viability with a commercial

absorbance assay (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega Corp, Madison, WI). IC_{50} values are expressed in μM relative to the solvent (DMSO) control. All IC_{50} determinations were conducted with three biological replicates that were each performed in triplicated wells.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2017.07.004.

References

Andersson, M.A., Mikkola, R., Raulio, M., Kredics, L., Maijala, P., Salkinoja-Salonen, M.S., 2009. Acrebol, a novel toxic peptaibol produced by an Acremonium exuviarum indoor isolate. J. Appl. Microbiol. 106, 909e923.

Ayers, S., Ehrmann, B.M., Adcock, A.F., Kroll, D.J., Carcache de Blanco, E.J., Shen, Q., Swanson, S.M., Falkinham, J.O., Wani, M.C., Mitchell, S.M., Pearce, C.J., Oberlies, N.H., 2012. Peptaibols from two unidentified fungi of the order Hypocreales with cytotoxic, antibiotic, and anthelmintic activities. J. Pept. Sci. 18, 500e510.

Ayers, S., Graf, T.N., Adcock, A.F., Kroll, D.J., Matthew, S., Carcache de Blanco, E.J., Shen, Q., Swanson, S.M., Wani, M.C., Pearce, C.J., Oberlies, N.H., 2011. Resorcylic acid lactones with cytotoxic and NF-kB inhibitory activities and their structure-activity relationships. J. Nat. Prod. 74, 1126e1131.

Bhushan, R., Brückner, H., 2004. Marfey's reagent for chiral amino acid analysis: a review. Amino Acids 27, 231e247.

Bhushan, R., Brückner, H., 2011. Use of Marfey's reagent and analogs for chiral amino acid analysis: assessment and applications to natural products and biological systems. J. Chromatogr. B 879, 3148e3161.

Carbone, I., Kohn, L.M., 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91, 553e556.

Degenkolb, T., Berg, A., Gams, W., Schlegel, B., Gr€afe, U., 2003. The occurrence of peptaibols and structurally related peptaibiotics in fungi and their mass spectrometric identification via diagnostic fragment ions. J. Pept. Sci. 9, 666e678.

Degenkolb, T., Bruckner, H., 2008. Peptaibiomics: towards a myriad of bioactive peptides containing C(alpha)-dialkylamino acids? Chem. Biodivers. 5, 1817e1843.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792e1797.

El-Elimat, T., Raja, H.A., Day, C.S., Chen, W.L., Swanson, S.M., Oberlies, N.H., 2014. Greensporones: resorcylic acid lactones from an aquatic Halenospora sp. J. Nat. Prod. 77, 2088e2098.

Figueroa, M., Graf, T.N., Ayers, S., Adcock, A.F., Kroll, D.J., Yang, J., Swanson, S.M., Munoz-Acuna, U., Carcache de Blanco, E.J., Agrawal, R., Wani, M.C., Darveaux, B.A., Pearce, C.J., Oberlies, N.H., 2012. Cytotoxic epipolythiodioxopiperazine alkaloids from filamentous fungi of the Bionectriaceae. J. Antibiot. 65, 559e564.

Figueroa, M., Raja, H., Falkinham, J.O., Adcock, A.F., Kroll, D.J.,Wani, M.C., Pearce, C.J., Oberlies, N.H., 2013. Peptaibols, tetramic acid derivatives, isocoumarins, and sesquiterpenes from a Bionectria sp. (MSX 47401). J. Nat. Prod. 76, 1007e1015.

Gouy, M., Guindon, S., Gascuel, O., 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27, 221e224.

He, H., Janso, J.E., Yang, H.Y., Bernan, V.S., Lin, S.L., Yu, K., 2006. Culicinin D, an antitumor peptaibol produced by the fungus Culicinomyces clavisporus, strain LL-12I252. J. Nat. Prod. 69, 736e741.

Jaklitsch,W.M., Komon, M., Kubicek, C.P., Druzhinina, I.S., 2005. Hypocrea voglmayrii sp. nov. from the Austrian Alps represents a new phylogenetic clade in Hypocrea/Trichoderma. Mycologia 97, 1365e1378.

Kertesz, V., Van Berkel, G.J., 2010. Liquid microjunction surface sampling coupled with high-pressure liquid chromatography-electrospray ionization-mass spectrometry for analysis of drugs and metabolites in whole-body thin tissue sections. Anal. Chem. 82, 5917e5921.

Kertesz, V., Van Berkel, G.J., 2013. Automated liquid microjunction surface sampling-HPLC-MS/MS analysis of drugs and metabolites in whole-body thin tissue sections. Bioanalysis 5, 819e826.

Kertesz, V., Van Berkel, G.J., 2014. Sampling reliability, spatial resolution, spatial precision, and extraction efficiency in droplet-based liquid microjunction surface sampling. Rapid Commun. Mass Spectrom. 28, 1553e1560.

Kinghorn, A.D., Carcache de Blanco, E.J., Lucas, D.M., Rakotondraibe, H.L., Orjala, J., Soejarto, D.D., Oberlies, N.H., Pearce, C.J., Wani, M.C., Stockwell, B.R.,

Burdette, J.E., Swanson, S.M., Fuchs, J.R., Phelps, M.A., Xu, L., Zhang, X., Shen, Y.Y., 2016. Discovery of anticancer agents of diverse natural origin. Anticancer Res. 36, 5623e5637.

Kjer, J., Debbab, A., Aly, A.H., Proksch, P., 2010. Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. Nat. Protoc. 5, 479e490.

Lombard, L., van der Merwe, N.A., Groenewald, J.Z., Crous, P.W., 2015. Generic concepts in nectriaceae. Stud. Mycol. 80, 189e245.

Marfey, P., 1984. Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. Carlsb. Res. Commun. 49, 591e596.

Neumann, N.K.N., Stoppacher, N., Zeilinger, S., Degenkolb, T., Brückner, H., Schuhmacher, R., 2015. The peptaibiotics database - a comprehensive online resource. Chem. Biodivers. 12, 743e751.

Otto, A., Laub, A., Wendt, L., Porzel, A., Schmidt, J., Palfner, G., Becerra, J., Krüger, D., Stadler, M., Wessjohann, L., Westermann, B., Arnold, N., 2016. Chilenopeptins A and B, peptaibols from the chilean Sepedonium aff. chalcipori KSH 883. J. Nat. Prod. 79, 929e938.

Paguigan, N.D., Raja, H.A., Day, C.S., Oberlies, N.H., 2016. Acetophenone derivatives from a freshwater fungal isolate of recently described Lindgomyces madisonensis (G416). Phytochemistry 126, 59e65.

Raja, H.A., Baker, T.R., Little, J.G., Oberlies, N.H., 2017a. DNA barcoding for identification of consumer-relevant mushrooms: a partial solution for product certification? Food Chem. 214, 383e392.

Raja, H.A., El-Elimat, T., Oberlies, N.H., Shearer, C.A., Miller, A.N., Tanaka, K., Hashimoto, A., Fournier, J., 2015. Minutisphaerales (Dothideomycetes, Ascomycota): a new order of freshwater ascomycetes including a new family, Minutisphaeraceae, and two new species from North Carolina, USA. Mycologia 107, 845e862.

Raja, H.A., Miller, A.N., Pearce, C.J., Oberlies, N.H., 2017b. Fungal identification using molecular tools: a primer for the natural products research community. J. Nat. Prod. 80, 756e770.

Rinehart Jr., K.L., Gaudioso, L.A., Moore, M.L., Pandey, R.C., Cook Jr., J.C., Barber, M., Sedgwick, R.D., Bordoli, R.S., Tyler, A.N., Green, B.N., 1981. Structures of eleven zervamicin and two emerimicin peptide antibiotics studied by fast atom bombardment mass spectrometry. J. Am. Chem. Soc. 103, 6517e6520.

Rivera-Chávez, J., Raja, H.A., Graf, T.N., Burdette, J.E., Pearce, C.J., Oberlies, N.H., 2017. Biosynthesis of fluorinated peptaibols using a site-directed building block incorporation approach. J. Nat. Prod. 80, 1883e1892.

Schiell, M., Hofmann, J., Kurz, M., Schmidt, F.R., Vertesy, L., Vogel, M., Wink, J., Seibert, G., 2001. Cephaibols, new peptaibol antibiotics with anthelmintic properties from Acremonium tubakii DSM 12774. J. Antibiot. 54, 220e233.

Sharman, G.J., Try, A.C., Williams, D.H., Ainsworth, A.M., Beneyto, R., Gibson, T.M., McNicholas, C., Renno, D.V., Robinson, N., Wood, K.A., Wrigley, S.K., 1996. Structural elucidation of XR586, a peptaibol-like antibiotic from Acremonium persicinum. Biochem. J. 320, 723e728.

Sica, V.P., El-Elimat, T., Oberlies, N.H., 2016a. In situ analysis of Asimina triloba (paw paw) plant tissues for acetogenins via the droplet-liquid microjunction-surface sampling probe coupled to UHPLC-PDA-HRMS/MS. Anal. Meth. 8, 6143e6149.

Sica, V.P., Figueroa, M., Raja, H.A., El-Elimat, T., Darveaux, B.A., Pearce, C.J., Oberlies, N.H., 2016b. Optimizing production and evaluating biosynthesis in situ of a herbicidal compound, mevalocidin, from Coniolariella sp. J. Ind. Microbiol. Biotechnol. 43, 1149e1157.

Sica, V.P., Raja, H.A., El-Elimat, T., Kertesz, V., Van Berkel, G.J., Pearce, C.J., Oberlies, N.H., 2015. Dereplicating and spatial mapping of secondary metabolites from fungal cultures in situ. J. Nat. Prod. 78, 1926e1936.

Sica, V.P., Rees, E.R., Tchegnon, E., Bardsley, R.H., Raja, H.A., Oberlies, N.H., 2016c. Spatial and temporal profiling of griseofulvin production in Xylaria cubensis using mass spectrometry mapping. Front. Microbiol. 7, 544.

Tata, A., Perez, C., Campos, M.L., Bayfield, M.A., Eberlin, M.N., Ifa, D.R., 2015. Imprint desorption electrospray ionization mass spectrometry imaging for monitoring secondary metabolites production during antagonistic interaction of fungi. Anal. Chem. 87, 12298e12305.

Thirumalachar, M.J., 1968. Antiamoebin, a new antiprotozoal-anthelmintic antibiotic. I. Production and biological studies. Hindustan Antibiot. Bull. 10, 287e289.

Toniolo, C., Brückner, H., 2007. Peptaibiotics. Chem. Biodivers. 4, 1021e1022.

Toniolo, C., Brückner, H., 2009. In: Peptaibiotics: Fungal Peptides Containing adialkyl a-amino Acids. Wiley VCH, Zurich.

Vilgalys, R., Hester, M., 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. J. Bacteriol. 172, 4238e4246.

White, T.J., Bruns, T., Lee, S.H., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J.,

White, T.J. (Eds.), PCR Protocols: a Guide to Methods and Application. Academic Press, New York, pp. 315e322.

Whitmore, L., Wallace, B.A., 2004. The peptaibol database: a database for sequences and structures of naturally occurring peptaibols. Nucleic Acids Res. 32, D593eD594.

Zare, R., Gams, W., 2016. More white verticillium-like anamorphs with erect conidiophores. Mycol. Prog. 15, 993e1030.