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Peptaibols from Two Unidentified Fungi of the Order Hypocreales with Cytotoxic, Antibiotic, and Anthelmintic Activities

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Abstract

As part of an ongoing investigation of filamentous fungi for anticancer leads, an active culture was identified from the Mycosynthetix library (MSX 70741, of the order Hypocreales, Ascomycota). The fungal extract exhibited cytotoxic activity against the H460 (human non-small cell lung carcinoma) cell line, and bioactivity-directed fractionation yielded peptaibols **1–12** and harzianums A (**13**) and B (**14**). Structure elucidation of **1–12** was facilitated by high-resolution MS/MS obtained on a Thermo LTQ Orbitrap XL using Higher-Energy Collisional Dissociation (HCD) and by high field NMR (950 MHz). The absolute configuration was determined by Marfey's analysis of the individual amino acids; the time required for such analysis was decreased via the development of a 10 min UPLC method. The isolated peptaibols (**1–12**), along with three other peptaibols isolated and elucidated from a different fungus (MSX 57715) of the same Order (**15–17**), were examined for activity in a suite of biological assays, including those for cytotoxic, antibacterial, and anthelmintic activities.

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Keywords

peptaibols; cytotoxicity; anthelmintic; Hypocreales; Higher-Energy Collisional Dissociation (HCD)

Introduction

By most measures of scientific progress, peptaibols have been investigated rather extensively. An entire book [1] and an issue of a two separate journals [2, 3] have been devoted to the subject, and various aspects have been reviewed extensively, especially when dealing with a common source (*Trichoderma* sp.) [4] or the most well studied class of peptaibols (the alamethicins) [5]. However, in the course of a collaborative project to identify anticancer leads from diverse natural product sources [6, 7], extracts of filamentous fungi from the Mycosynthetix library, representing over 55,000 accessions, have not yielded peptaibols to date. In fact, most of the compounds discovered in this program, which is driven by bioactivity-directed fractionation guided by a suite of cytotoxicity and mechanism of action based assays, have been of a molecular weight well under 1000 a.m.u. [8–11]. Hence, uncovering a series of both new and known compounds of significantly greater molecular weight was of interest, both from the standpoint of evaluating their biological activity in assays that pertain to anticancer activity and from examining their chemical diversity relative to the library of fungal isolates. In the course of this research, state-of-the-art technologies were applied to the structure elucidation processes, thereby developing tools that could be applied to research on peptaibols or related compounds. For example, determining the sequence of residues was facilitated via the use of UPLC coupled to high-resolution MS/MS using Higher-Energy Collisional Dissociation (HCD) on a Thermo LTQ Orbitrap XL. This was complemented by the resolution enhancements observed when analyzing the TOCSY and NOESY spectra on a 950 MHz NMR spectrometer. Moreover, the time required to determine the absolute configuration of the residues using Marfey's analysis was decreased by the development of a 10 min UPLC procedure, compared to 30 to 40 min run times for similar analyses of peptaibols by HPLC [12, 13]. In short, the bioactivity-directed fractionation study of fungus MSX 70741 resulted in the isolation and characterization of a series of peptaibols (1–12; compounds 1–7 and 12 being new) and two known trichothecene analogues [harzianum A (13) and harzianum B (14)]. The isolated peptaibols (1–12), along with three other known peptaibols (15–17) isolated from a different fungus of the same order (MSX 57715) were examined for cytotoxicity, antibacterial and anthelmintic activities, and activity in a mitochondrial transmembrane potential assay. Figure 1 illustrates the structures/sequences for the isolated peptaibols (1–12 and 15–17).

Materials and Methods

General Experimental Procedures

NMR experiments were conducted in CD₃OH with presaturation of the OH peak at δ_{H} 4.9 ppm. NMR instrumentation was a Bruker Ultrashield Plus with Avance III console, Topspin software version 2.1, and a QNP style Cryoprobe (operating at 950.30 MHz for ¹H). For comparison of these NMR data to that of a 500 MHz spectrometer a JEOL ECA-500 was utilized. HRESIMS was performed on a Thermo LTQ Orbitrap XL system equipped with HCD cell. UPLC was carried out on a Waters Acquity system with data collected and analyzed using Empower software (build 2154). Preparative HPLC was performed on Varian Prostar HPLC systems equipped with Prostar 210 pumps and a Prostar 335 photodiode array detector (PDA), with data collected and analyzed using Galaxie Chromatography Workstation software (version 1.9.3.2). For preparative HPLC, a Phenomenex Synergi Max-RP 80 (4 μm ; 250 $\times$ 21.2 mm) column was used at a 15 mL/min

flow rate, while for UPLC, a BEH C18 (1.7 μm ; 50 $\times$ 2.1 mm) column was used with a 0.61 mL/min flow rate (0.5 mL/min for Marfey's analysis), both monitored at 205 nm (340 nm for Marfey's analysis). Flash chromatography was performed on a Teledyne ISCO CombiFlash Rf using a 40 g Silica Gold column and monitored by UV and evaporative light-scattering detectors. Reference standards of amino acids and Marfey's reagent were obtained from Sigma-Aldrich. All other reagents and solvents were obtained from Fisher Scientific and were used without further purification.

Producing Organisms and Fermentations

Mycosynthetix fungal strain MSX 70741 was isolated in April 1993 from wood collected in a humid mountain forest and strain MSX 57715 was isolated in October 1991 from leaf litter from a predominately oak, humid forest, both by Dr. Barry Katz of MYCOsearch and later acquired by Mycosynthetix. DNA analyses were performed by MIDI Labs, Inc. (Newark, DE), and the D2 variable region of the large subunit (LSU) rRNA was sequenced and compared to their database; in both cases, the closest match could only determine that these fungi were of the order Hypocreales, Ascomycota; these data were deposited in Genbank (accession Nos. JN377382 and JN377381, respectively). The cultures were stored on malt extract slants and were transferred periodically. Fresh cultures were grown on a similar slant, and a piece was transferred to a medium containing 2% soy peptone, 2% dextrose, and 1% yeast extract (YESD media). Following incubation (7 d) 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 H₂O, in a 250 mL Erlenmeyer flask. This was incubated at 22 °C until the cultures showed good growth (approximately 14 d) to generate the screening cultures. The scale-up cultures, used for isolation of the peptaibols, were grown in a 2.8 L Fernbach flask containing 150 g rice and 300 mL H₂O and were inoculated using a seed culture grown in YESD medium. These were incubated at 22 °C for 14 d.

Extraction and Isolation

To the large scale solid fermentation of MSX 70741 was added 500 mL of 1:1 MeOH:CHCl₃. The mixture was shaken for 16 h then filtered, and the solvent was evaporated. The material (2.07 g) was then dissolved in 1:1 CHCl₃:MeOH and adsorbed onto Celite 545 and fractionated by flash silica-gel chromatography. The solvent conditions were 100% hexane to 100% CHCl₃ over 10 column volumes (CV), then 100% CHCl₃ for 7 CV, followed by increasing amounts of MeOH in CHCl₃ from 0–10% over 20 CV, 10–20% over 5 CV, 20–100% over 2 CV, and 100% MeOH for the remaining 8 CV, all at 40 mL/min. The peptaibol-enriched material (100% MeOH; 600 mg) was purified via six separate injections on preparative HPLC using a gradient that initiated with 40:60 CH₃CN:H₂O and increased linearly to 100:0 CH₃CN:H₂O over 30 min.

UPLC-HRMS of 1–12 and 15–17

High resolution UPLC-MS was carried out on a Waters Acquity UPLC system coupled to Thermo LTQ Orbitrap XL. Sample injections of 3 μL of 0.5 mg/mL were separated via UPLC at 0.61 mL/min; an in-line column flow tee was used to divert the mobile phase 1:1 between the electrospray source and waste. UPLC conditions were 20% CH₃CN in 0.1% aqueous formic acid for 0.5 min., then 20–100% CH₃CN from 0.5–3.0 min. Peptaibols have a labile bond between the central Aib and Pro residues that is cleaved under conventional electrospray ionization conditions [14]. As a result, two fragments (referred to as in-source fragments hence forth) are commonly observed at higher abundance than that of the monoisotopic parent ion. Mass spectra were collected in positive mode ESI with the following source parameters: capillary temp. = 275°C, sheath gas = 15, auxiliary gas = 5, sweep gas = 2, source voltage = 4.50 kV, capillary voltage = 46 V, tube lens = 115 V. The

MS/MS fragmentation was performed using an instrument method comprised of four scan events. During scan event 1, a full scan mass spectrum of mass range 75–2000 a.m.u. was collected; scan event 2 was used to conduct HCD fragmentation of the low molecular weight (MW) fragment (in-source) at an optimized collision energy of 22 (Table 1); scan event 3 was used to perform HCD fragmentation of the high MW fragment (in-source) at a collision energy of 15; scan event 4 was for HCD fragmentation of the high MW fragment (in-source) at a collision energy of 35.

Collision energies selected in this study were optimized using the LTQ Tune software's automatic tuning feature. To do so, a pure solution of a selected peptaibol was infused directly under UPLC flow conditions. In-source fragments were then subjected to a range of collision energies (10–35 NCE; normalized collision energy is a resonance excitation process used for inducing fragmentation while compensating for mass dependence). Fragmentation energies used in this study were selected to generate a broad molecular weight range of fragments of the highest signal possible. MSⁿ of the large (in-source) fragment was collected at two different fragmentation energies (15 and 35 NCE) during two different scan events because we were not able to produce all of the observed fragments at a single NCE value.

Marfey's Analysis of 1–12

Our method was based on Kjer *et al.* [15] with the following modifications. Approximately 0.2 mg of each amino acid standard was weighed into separate glass 2 mL reaction vials. To each standard was added 50 μ L of H₂O, 20 μ L of 1 M NaHCO₃, and 100 μ L 1% Marfey's reagent in acetone. The reaction mixtures were agitated at 40°C for 1 h. The reactions were halted by the addition of 10 μ L of 2N HCl. The product of the reactions was dried under a stream of air and dissolved in ~1.7 mL of MeOH. Each derivatized standard was injected individually (0.7 μ L) onto the UPLC. Also, aliquots of all of the derivatized standards were combined to give a mixed standard, which was injected just prior to the digested and derivatized peptaibols (see below). UPLC conditions were 10–70% MeOH in H₂O over 10 min on the aforementioned BEH column and eluent monitored at 340 nm.

To generate the digested and derivatized peptaibols, approximately 0.2–0.3 mg of compounds 1–12 were weighed separately into 2 mL reaction vials, to which was added 0.5 mL of 6N HCl. The compounds were hydrolyzed at 110°C for 24 h, at which time they were evaporated under a stream of air. To each hydrolysis product was then added 25 μ L H₂O, 10 μ L 1 M NaHCO₃, and 50 μ L of 1% Marfey's reagent in acetone. The reaction mixtures were agitated at 40°C for 1 h. The reactions were halted by the addition of 5 μ L of 2N HCl. The mixtures were dried under a stream of air and brought up in ~200 μ L of MeOH and injected onto the UPLC using the same conditions as for the standards.

Cytotoxicity Assays

The cytotoxicity measurements against the MCF-7 [16] human breast carcinoma (Barbara A. Karmanos Cancer Center), NCI-H460 [17] human large cell lung carcinoma (HTB-177, American Type Culture Collection (ATCC)), and SF-268 [18] human astrocytoma (NCI Developmental Therapeutics Program) cell lines were performed as described previously [19, 20]. Moreover, a second cytotoxicity assay was performed on only the isolated compounds using the MDA-MB-435 [21] human melanoma (HTB-129, ATCC) cell line as described previously [8] and with the following modifications. After treating the MDA-MB-435 cells with test substances and 96 h incubation at 37°C, the cells were evaluated for viability with a commercial absorbance assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega Corp, Madison, WI). The compounds were also tested in the IMR90 fibroblast cell line (ATCC CCL-186) [22], a normal diploid cell line that proliferates

in culture for approximately 58 generations prior to senescence. Positive control data for all cell lines are provided in the legend of Table 2.

Antimicrobial Assay

The compounds were screened initially for antimicrobial activity using an agar plate diffusion assay. Overnight cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Mycobacterium smegmatis*, *Candida albicans*, and *Bacillus subtilis* were used to inoculate molten LB media or Middlebrook 7H9 media (Difco) with 1% glycerol, containing 1.5% agar and kept at 50°C; these were then used to prepare assay plates. Samples (dissolved in 10 µL MeOH) were applied to the surface of the assay dish, and positive controls were treated in a similar manner (penicillin G, gentamicin, novobiocin, and streptomycin; all from Sigma). The bioassay plates were incubated overnight at 37°C. Biological activity of the standards could be detected to 1 µg/mL (except that penicillin G was active against *E. coli* at 100 µg/mL only).

Measurement of Antibiotic Activity against Methicillin-resistant *S. aureus* (MRSA)

The samples were tested against a suite of methicillin-resistant *S. aureus* (MRSA) isolates (data not shown); only representative data are shown in Table 2 against *S. aureus* (ATCC 6538) and an unrelated MRSA strain (ATCC 43300), which were both acquired from Danville Community Hospital (Danville, VA, USA). Minimal inhibitory concentration (MIC) measurements were performed as described previously [23, 24]. All measurements were made in duplicate and susceptibilities of the strains to vancomycin as a positive control were measured in parallel.

Anthelmintic Assay

The compounds were screened against *Haemonchus contortus* infective larvae (*HcL3*) in a L3 motility assay; this assay evaluates the effect of the compounds on the body wall musculature since this is not a feeding stage. Third stage larvae of an isolate of *H. contortus* maintained in goats were obtained from the laboratory of Dr. Raymond Kaplan (University of Georgia, Athens, GA). The nematode L3 bioassay was as described [25] with slight modifications. Briefly, in a 15 mL centrifugation tube, *HcL3*s were incubated at rt in a 0.15% sodium hypochlorite solution for 25 min, centrifuged at 100 rcf for 5 min, and then the supernatant was removed and rinsed with distilled H₂O. *HcL3*s were concentrated in phosphate buffered saline to contain approximately 800 *HcL3*s/mL. The L3 motility assay was conducted in triplicate in 96-well plates; each well had a total volume of 75 µL and contained roughly 50 exsheathed *HcL3*s. Plates were incubated in a humidified chamber at 25–30°C for 72 h and percent immobility was calculated. Ivermectin (positive control) and DMSO only (negative control) wells were included on every plate, and percent immobility was calculated using the mean counts from three replicates. The peptaibols were dissolved in DMSO and evaluated at 20 µg/mL in triplicate; compounds were considered active if they had any immobilizing effects on *HcL3*s. Active compounds were evaluated further in dose response, and IC₅₀ values were calculated using a log probit regression analysis.

Mitochondria Transmembrane Potential Assay

The mitochondrial transmembrane potential assay was performed as described previously [8].

Results

The crude 1:1 CHCl₃:MeOH extract of fungus MSX 70741 was partitioned with 4:1:5 CHCl₃:MeOH:H₂O. The organic soluble material was active (>95% growth inhibition of

H460 cells at 20 $\mu\text{g/mL}$), and was fractionated initially by flash silica-gel chromatography. Harzianum A (**13**, 6.8 mg) [26] and harzianum B (**14**, 0.9 mg) [27] eluted in the 4–5% MeOH in CHCl_3 fraction (>96% growth inhibition of H460 at 2 $\mu\text{g/mL}$); these were purified by HPLC and their NMR data were in excellent agreement with the literature (see Figure S1 for structures of **13** and **14**) [27]. The peptaibols eluted in the flash chromatography system in the 100% MeOH fraction (>98% growth inhibition of H460 at 20 $\mu\text{g/mL}$). Final purification of the peptaibols was accomplished by preparative scale RP-HPLC (see Figure S2). Compound **1** eluted at 12.0 min (1.1 mg), **2** at 13.1 min (3.3 mg), **3** at 14.0 min (0.7 mg), **4** at 14.3 min (2.0 mg), **5** at 15.7 min (2.6 mg), **6** at 17.7 min (6.7 mg), **7** at 18.7 min (12.4 mg), **8** between 19–21 min (242.5 mg), **9** between 21.3–22.3 min (77.6 mg), **10** between 22.3–23.5 min (52.6 mg), **11** between 23.5–24.5 min (28.8 mg), and **12** at 25.3 min (4.1 mg). Using the exact same conditions as above, fungus MSX 57715 was extracted and fractionated to isolate peptaibols **15** (205.6 mg), **16** (93.6 mg), and **17** (7.5 mg) with HPLC elution times of 17–19 min, 19–20.5 min, and 21.1 min, respectively. Compounds **8–11** were the major isolates and were known peptaibols, while **1–7** and **12** were new. The numbering of these compounds corresponds to their elution order on RP-HPLC: **1** eluted earliest and **12** eluted latest.

The high-resolution protonated monoisotopic precursor ion data, $[\text{M} + \text{H}]^+$, and the resultant molecular formulae for compounds **1–12**, are listed in Table 1; the structure for **8** and the sequence for the other peptaibols are displayed in Figure 1. An important characteristic of the MS spectra for typical 20-residue peptaibols was in-source fragmentation between the Aib13 and Pro14 residues, leaving two major fragments (Figure 2) [14]. Other diagnostic peaks were the doubly- and triply-charged species, as well as the triply-charged dimer (Figure 2). The MS data for the two major fragments (termed here, “low MW fragment” and “high MW fragment”) of **1–12** are listed in Table 1; note that **7** had three major in-source fragments. These major fragments were then subjected to MS/MS to elucidate the sequence.

The MS/MS data, in conjunction with NMR data, were used to sequence the major metabolite (**8**), identified as the known peptaibol alamethicin F50 (or atroviridin A) [28]. The absolute configuration of **8** was confirmed by Marfey’s analysis. As with all of the peptaibols isolated in this study, the individual amino acids had the L- configuration except for isovaline, which was D-configuration. Compounds **9–11** were likewise found to be the known compounds atroviridin B (**9**) [29], polysporin B (**10**) [30], and alamethicin II (**11**) [31].

The sequences and structures of compounds **1–7** and **12** were elucidated in a similar fashion by MS/MS, NMR, and Marfey’s analysis. Examples of MS/MS analysis (Figure 3), of ^1H NMR and NOESY spectra (Figures 4 and 5, respectively), and of Marfey’s analysis (Figure 6) are provided. Due to paucity of material, quality NMR data for compounds **1** and **3** could not be obtained; however, MS/MS and Marfey’s analysis was sufficient for the complete structure elucidation for **1**. Alternatively, structure **3** should be considered tentative because the locations of valine vs isovaline could not be determined unequivocally, since both were present in the Marfey’s analysis. It is highly likely that residues 9 and 15 were Val and residue 17 was Iva, due to similarity with known peptaibols; however, this could not be proven.

In work of a similar vein from another fungus from the Mycosynthetix library (specifically, MSX 57715), other known peptaibols were isolated and elucidated, along with trichodermin, which is a simple trichothecene. These peptaibols were identified as trichokonin VI (**15**) [32], trichokonin VII (**16**) [32], and longibranchin BIII (**17**) [33, 34]. These peptaibols were evaluated in the same bioassays as **1–12** to determine the significance of Pro2 vs. Ala2.

The isolated peptaibols were evaluated in a series of biological assays. With respect to anticancer activity, they were tested for cytotoxicity against a panel of human tumor cell lines and a human fibroblast cell line (Table 2). They were also examined for activity in a mitochondria transmembrane potential assay but found inactive (data not shown). Based on literature precedent for antimicrobial activity for some peptaibols [35], they were tested against a battery of assays, including those for Gram positive and Gram negative bacteria, and *C. albicans*. The most promising antimicrobial activity was with respect to *Staphylococcus aureus*, which spawned further examination against methicillin-resistant *S. aureus* (Table 2). Finally, a few peptaibols have been reported to have anthelmintic activity [36, 37], and thus the compounds were examined in an assay for larval motility against *Haemonchus contortus* infective stages. Larval motility was inhibited completely by four of the peptaibols (**8**, **9**, **16** and **17**) when tested at 20 $\mu\text{g/ml}$; these were evaluated further for dose response with a top concentration of 8.3 $\mu\text{g/ml}$ (Table 2). In summary, the biological potential of these compounds were examined extensively.

Discussion

In this work, a single injection on UPLC-MS/MS using HCD on a Thermo LTQ Orbitrap XL gave high-resolution fragmentation and nearly complete sequence data in a three minute run. HCD fragmentation is becoming a routine tool in proteomics research, especially for quantitation studies that utilize various isotopic-labeling methods and for peptide sequencing studies [38]; to the best of our knowledge, this technique has not been applied previously to research peptaibols. The advantages of the HCD collision cell in the LTQ Orbitrap XL system include its ability to generate “rich” fragmentation spectra that include low m/z values and Orbitrap (high resolution/high mass accuracy) detection of mass fragments, allowing for molecular formula assignment of a given m/z value [39].

Compound **1** was identical to the major metabolite **8** except for the substitution of Ala1 for Aib1. To our knowledge, the only other peptaibol that has been reported to have Ala1 is trichokonin IIb [40], which was identical to **1** except for the replacement of Pro2 with Ala2. The result of substituting Aib1 with Ala1 on the cytotoxic activity was interesting, as **1** was inactive compared to **8** (Table 2).

Compounds **2–6** were also structurally related to alamethicin F50 (**8**; Fig. 1). Compounds **2**, **4**, **5**, and **6** differed from **8** by a single amino acid residue substitution. Residue 6 appeared to be the most variable site, with substitution of Ser, Gly, or Aib for Ala. Compound **2** was identical to **8** except for substitution of Ser6 for Ala6. Compound **3** likely included Iva17 instead of Aib17 in addition to Ser6 versus Ala6, though the structure elucidation of **3** was incomplete due to paucity of material. Compound **5** was identical to **8** except for substitution of Ser4 for Ala4. Compound **6** was identical to **8** except for substitution of Val12 for Leu12. Compound **12** was more closely related to alamethicin II (**11**), and differed from **11** only by substitution of Iva17 for Aib17. Due to their similarity to the known atroviridins, compounds **1–6** and **12** were ascribed the trivial names of atroviridin D through atroviridin J (Fig. 1), respectively.

Compound **7** was unique with respect to the other peptaibols isolated from MSX 70741 (Fig. 1), in that **7** contained 11 amino acid residues whereas the others were 20-mers. Also, the HRMS of **7** showed three major in-source fragments instead of two, as with the other peptaibols (see Fig. 7). The ^1H NMR also showed a methyl triplet at δ_{H} 0.85 (see Fig. 8), and from 2D TOCSY and NOESY spectra this triplet was due to isoleucine (Ile) at residue 4. Isoleucine was not present in any of the other peptaibols in this study. Compound **7** was most closely related to the trichobrachin [41, 42], hypomurocin [43], and trichobrevin [44, 45] subclasses of 11-mer peptaibols. The effect of a shorter chain on cytotoxic activity was

negligible (Table 2), although it seemed to diminish antibacterial activity (Table 2) and was inactive in the *HcL3* motility assay at 20 $\mu\text{g/mL}$. The NMR data for **7** illustrated the benefit of higher field on resolution (see Fig. 8). The Ile4 methyl triplet for **7** was fully resolved at 950 MHz, whereas at 500 MHz, the triplet could not be identified due to overlap with the adjacent methyl doublets from the Leu8 residue. Four methyl doublets from δ_{H} 0.90–0.95 were also completely resolved at 950 MHz, whereas at 500 MHz there was too much overlap to distinguish the signals. The 950 MHz NMR data was not only well-resolved for the methyl peaks of compound **7**, but it also resulted in greatly improved resolution of NH–NH correlations, which were essential for sequencing. While it is well appreciated in the natural products community that higher field provides better resolution, the example of 500 vs 950 MHz NMR spectra of compound **7** provides a cogent example of how this can be applied to structure elucidation of peptaibols. Compound **7** most closely resembled trichobrevin B–IIb [45], having the same “rough” amino acid sequence; meaning that the leucine/isoleucine and valine/isovaline residues were not unambiguously determined for trichobrevin B–IIb. As compound **7** also closely resembled the trichobranchin peptaibol series, it was ascribed the trivial name trichobranchin D–I, to differentiate it from the trichobranchin A series (which all have Asp2, Aib9, and Pro10), the trichobranchin B series (which contain Asp2, Val9, and Pro10), and the trichobranchin C series (which have Gln2, Aib9, and Pro10). All trichobranchins, including **7**, are also characterized by Aib5 and Pro6 [41, 42].

Compounds **2**, **3**, and **5** were novel in that they contain Ser at positions 4 or 6, which, to the best of our knowledge, has not been reported previously in 20-mer peptaibols. While Ser2 has been reported in a number of classes of 18-mer peptaibols, such as the hypomurocin B series [43], the trichokindins [46], the trichorzin MA series [47], and the trichorzin PA series [33, 34], the presence of Ser2 is rare for 11-mer peptaibols (as in compound **7**), having only been reported in the trichobrevin B class [44, 45]. The presence of Ser in **2**, **3**, **5**, and **7**, according to the MS/MS fragmentation work, was confirmed by the TOCSY NMR spectra (except for **3**) as well as the Marfey’s analyses, which also confirmed the stereochemistry as L-Ser (see Figure 6).

Three known peptaibols were isolated from a second *Mycosynthetix* fungus (MSX 57715; Fig. 1). Trichokonin VI (**15**) was the major peptaibol isolate, and was identical to alamethicin F50 (**8**), except for replacement of Pro2 with Ala2; compounds **8** and **15** had nearly the same cytotoxic activity profile (Table 2). Trichokonin VII (**16**) was analogous to atroviridin B (**9**) with the same substitution of Ala2 for Pro2; these two compounds were essentially equipotent in the cytotoxicity and antibacterial assays (Table 2). Longibranchin BIII (**17**) substituted Glu18 for Gln18 of trichokonin VII (**16**); the former was slightly more potent in both cytotoxicity and antibacterial assays (Table 2), although possibly within experimental error of the assays.

A range of bioactivities were observed for the isolated peptaibols (Table 2). Several of the peptaibols exhibited not only cytotoxicity, but cancer cell selectivity (Table 2). Selectivity was assessed via the IMR90 cell lines, and they are non-transformed but proliferating cells. For example, the positive control camptothecin exhibited 3.6-fold selectivity in MCF-7 cells to 23-fold selectivity in H460 cells relative to IMR90 cells. Alamethicin II (**11**), atroviridin J (**12**), trichokonin VII (**16**), and longibranchin BIII (**17**) were most noteworthy in exhibiting 3.6-fold, 5.2-fold, 3.9-fold, and 5.8-fold selectivity, respectively, in MCF-7 vs. IMR90 cells. These data were consistent with a previous report of the growth inhibitory activity of trichokonin VI (**15**) against hepatocellular carcinoma cells, in which **15** did not obviously affect normal liver cells at lower concentrations [48]. Three of these four compounds contained Iva17 and were the more hydrophobic peptaibols. A general trend of higher activity with higher hydrophobicity has been reported previously [42], although the effect

was much less pronounced in our work. With respect to antibacterial activity, the compounds were essentially equipotent to both *S. aureus* and MRSA (or within experimental error), except compound **8**, which was approximately four times more potent against *S. aureus* than MRSA. Compound **8** was also the most potent in the assay for larval motility against *Haemonchus contortus* infective stages, although still two orders of magnitude less potent than the positive control.

In summary, a series of twelve structurally-related peptaibols (**1–12**) were isolated from MSX 70741, including eight new peptaibols, four of which featured the incorporation of serine. These peptaibols were sequenced by MS/MS using HCD. The method was designed to enable full scan intact mass analysis followed by sequential fragmentation of the low and high MW fragments from a single UPLC injection. This streamlined approach enabled nearly complete amino acid sequencing capabilities of peptaibols from a small volume of sample, over a 10-fold decrease in separation time, and accurate mass measurements to confirm amino acid identities. Fragmentation of peptaibols by high-resolution was important due to the fact that some peptaibols contain both leucine (and/or isoleucine) as well as hydroxyproline residues, an example of which is clonostachin [49]. Leucine, isoleucine, and hydroxyproline fragments have the same nominal mass (113); however, hydroxyproline has a different formula, and therefore can only be distinguished in MS by high-resolution measurements. Leucine and isoleucine have the same formula and therefore have to be distinguished by NMR. The NMR experiments confirmed the sequencing of the peptaibols and were critical for distinguishing the locations of constitutional isomers that were present in the same compound, such as leucine and isoleucine in compound **7** or valine and isovaline in compound **12**. The Marfey's analysis was also a good demonstration of the power of UPLC compared to HPLC, where analyses were completed in 10 min with excellent resolution of the amino acid-Marfey derivative standards. Finally, from a mycological perspective, both organisms were prolific producer of peptaibols, generating >400 mg (MSX 70741) and >300 mg (MSX 57715) of peptaibols per solid phase culture in a 2.8 L Fernbach flask, using standard techniques with no growth optimization studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Compound	Sequence	Name
1	Ac-Ala-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol	Atroviridin D
2	Ac-Aib-Pro-Aib-Ala-Aib-Ser-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol	Atroviridin E
3*	Ac-Aib-Pro-Aib-Ala-Aib-Ser-Gln-Aib-Vxx-Aib-Gly-Leu-Aib-Pro-Vxx-Aib-Vxx-Gln-Gln-Pheol	Atroviridin F
4	Ac-Aib-Pro-Aib-Ala-Aib-Gly-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol	Atroviridin G
5	Ac-Aib-Pro-Aib-Ser-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol	Atroviridin H
6	Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Val-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol	Atroviridin I
7	Ac-Aib-Ser-Val-Ile-Aib-Pro-Leu-Leu-Aib-Pro-Valol	Trichobrachin D-I
8	Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol	Alamethicin F50
9	Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Iva-Gln-Gln-Pheol	Atroviridin B
10	Ac-Aib-Pro-Aib-Ala-Aib-Aib-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol	Polysporin B
11	Ac-Aib-Pro-Aib-Ala-Aib-Aib-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Pheol	Alamethicin II
12	Ac-Aib-Pro-Aib-Ala-Aib-Aib-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Iva-Glu-Gln-Pheol	Atroviridin J
15	Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol	Trichokonin VI
16	Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Iva-Gln-Gln-Pheol	Trichokonin VII
17	Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Iva-Glu-Gln-Pheol	Longibrachin BIII

*Structure tentative. Vxx = valine or isovaline.

Figure 1.
Structure of Alamethicin F50 (**8**) and sequences of the other isolated peptaibols.

Figure 2.

Full-scan HRMS (single scan) of atroviridin D (**1**). The two diagnostic in-source fragments are apparent at 774.4487 (low MW fragment) and 1175.6748 (high MW fragment). The multiply charged species are apparent at 650.7113 $[M + 3H]^{3+}$, 975.5646 $[M + 2H]^{2+}$, and 1300.4180 $[2M + 3H]^{3+}$.

Figure 3.

HR-MS/MS by HCD of Atroviridin D (**1**). a) HCD of 774 peak, CE = 22; b) HCD of 1175 peak, CE = 15; c) HCD of 1175 peak, CE = 35.

Figure 4.

^1H NMR Spectrum of Atroviridin J (**12**), (CD_3OH , 950 MHz). Spectrum was obtained with presaturation of the -OH peak at δ_{H} 4.9.

Figure 5. NOESY spectrum of Atroviridin J (**12**), highlighting the correlations proving the position of the Iva (J) residue (CD₃OH, 950 MHz).

Figure 6.

Marfey's Analysis of Atroviridin E (2). Note that the peak in the sample hydrolysate at 3.4 min that appears to indicate the presence of D-serine is an artifact of the derivatization reaction that was present in all hydrolysate chromatograms. The retention times, while close, do not match. For procedure and chromatographic conditions, see Section 2.5.

Figure 7.
Full-scan HRMS (sum of 8 mass spectra) of Trichobrachin D-I (7).

Figure 8.

Comparison of the Upfield Methyl Region of the 500 MHz vs 950 MHz ¹H NMR Data of Trichobrachin D-I (7).

Table 1

High-Resolution Mass Data for Compounds 1–12

Compound	[M + H] ⁺	Theoretical [M + H] ⁺	Mass Error (ppm)	Formula	Low MW Fragment	High MW Fragment
1	1949.1166	1949.1219	-2.7	C ₉ H ₁₄ N ₂ O ₂₄	774.4487	1175.6748
2	1979.1276	1979.1324	-2.4	C ₉ H ₁₅ N ₂ O ₂₅	774.4483	1205.6868
3	1993.1443	1993.1480	-1.9	C ₉ H ₁₅ N ₂ O ₂₅	788.4641	1205.6873
4	1949.1136	1949.1219	-4.2	C ₉ H ₁₄ N ₂ O ₂₄	774.4479	1175.6745
5	1979.1300	1979.1324	-1.2	C ₉ H ₁₅ N ₂ O ₂₅	774.4482	1205.6870
6	1949.1145	1949.1218	-3.8	C ₉ H ₁₄ N ₂ O ₂₄	774.4482	1175.6758
7	1120.7307	1120.7340	-3.0	C ₅ H ₉ N ₁ O ₁₃	512.3060/609.4315/920.5790 *	
8	1963.1321	1963.1375	-2.8	C ₉ H ₁₅ N ₂ O ₂₄	774.4478	1189.6898
9	1977.1481	1977.1532	-2.6	C ₉ H ₁₅ N ₂ O ₂₄	788.4638	1189.6911
10	1977.1468	1977.1532	-3.2	C ₉ H ₁₅ N ₂ O ₂₄	774.4478	1203.7051
11	1978.1309	1978.1371	-3.2	C ₉ H ₁₅ N ₂ O ₂₅	775.4318	1203.7056
12	1992.1476	1992.1528	-2.6	C ₉ H ₁₅ N ₂ O ₂₅	789.4475	1203.7075

* Three major in-source fragments were present with compound 7

Table 2

Biological Activities of Peptaibols 1–12 and 15–17.

Compound	Cytotoxicity IC ₅₀ values (in μ M) ^a						MIC ^b (μ g/mL)		IC ₅₀ ^c (μ g/mL)
	MCF-7	H460	SF268	IMR90	MDA-MB-435	NT ^d	S. aureus	MRSA	
Atroviridin D (1)	>10	>10	>10	>10	NT ^d	NT	NT	NT	NT
Atroviridin E (2)	3.3	3.0	6.8	7.7	NT	NT	NT	NT	NT
Atroviridin F (3)	3.8	3.2	5.3	>10	NT	NT	NT	NT	NT
Atroviridin G (4)	2.7	1.3	1.9	5.9	NT	NT	NT	NT	NT
Atroviridin H (5)	1.8	2.5	4.4	6.1	NT	NT	NT	NT	NT
Atroviridin I (6)	2.1	2.3	5.1	9.8	NT	NT	44	44	NT
Trichobrachin D-I (7)	3.4	4.1	7.0	>10	7.3	113	113	113	NT
Alamethicin F50 (8)	2.2	3.4	2.3	4.8	8.9	35	140	140	0.2
Atroviridin B (9)	1.3	2.5	1.9	3.6	4.2	6	13	13	0.4
Polysporin B (10)	1.3	2.0	1.5	2.9	3.2	102	102	102	NT
Alamethicin II (11)	1.1	1.6	1.7	4.0	6.2	12	23	23	NT
Atroviridin J (12)	1.0	1.0	2.6	5.2	NT	9	18	18	NT
Trichokonin VI (15)	2.3	2.7	2.0	4.5	3.8	21	43	43	NT
Trichokonin VII (16)	1.3	2.2	1.5	5.1	3.0	8	17	17	>8.3
Longibrachin BIII (17)	0.8	0.8	1.4	4.6	NT	4	8	8	3.0
Camptothecin ^e	0.05	0.008	0.03	0.18	NT	NT	NT	NT	NT
Vinblastine ^e	NT	NT	NT	NT	r	NT	NT	NT	NT
Vancomycin ^e	NT	NT	NT	NT	NT	1.5	0.8	0.8	NT
Ivermectin ^e	NT	NT	NT	NT	NT	NT	NT	NT	0.006

^aIC₅₀ values were determined as the concentration required to reduce cellular proliferation by 50% relative to untreated controls following 72 h of continuous exposure (96 h for MDA-MB-435).

^bMinimal inhibitory concentration (MIC) is the lowest concentration of compound completely inhibiting growth as expressed in μ g/mL [19].

^cIC₅₀ values were determined as the concentration required to inhibit larval motility by 50% relative to untreated controls following 72 h of continuous exposure as expressed in μ g/mL.

^dIndicates 'not tested'.

^ePositive controls.

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f_i The positive control for MDA-MB-435 was vinblastine tested at 2.0 nM and 1.0 nM, which resulted in 41% and 68% viable cells after treatment, respectively.