Research Article

Production and Identification of Destruxins in Crude Extract of the Local Fungus *Metarhizium anisopliae* by Liquid Chromatography -Electrospray time-of-flight Mass Spectrometry

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ABSTRACT

The entomopathogenic fungus *Metarhizium anisopliae* (Metchnicoff) Sorokin has been used as bio-control for long time. It produces a family of cyclic peptide toxins, destruxins (DTXs), which exhibit various insecticidal activities. Characterization of insecticidal cyclodepsipeptides, destruxins, from *Metarhizium anisopliae* was performed. Electro spray ionization time-of-flight mass spectrometry (ESI-Q-TOF-MS) has been used for the detection and identification of destruxins (cyclodepsipeptides) in a crude extract of the fungus *Metarrhizium anisopliae*. Twelve DTXs have been separated and identified by high resolution mass measurement and tandem mass spectrometry.

Keywords: Destruxins, ESI-TOF-MS, HPLC, Identification, *Metarhizium anisopliae*, Toxins.

INTRODUCTION

Metarhizium anisopliae is a genus of entomopathogenic fungi used worldwide as an alternative to chemical insecticides in agricultural pest and disease vector control programs.

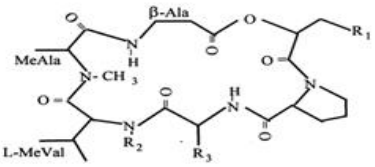
The extensive utilization of *Metarhizium anisopliae* as a bio-insecticides had increased interest in its basic biology including its production of fungal metabolites. Secondary metabolite production is a complex process that is often coupled with the morphological development of filamentous fungi.[1] *Metarhizium anisopliae* as been widely used for the biosynthesis of cyclic hexadepsipeptides, also known as Destruxins (DTXS).

Structurally destruxins are cyclic hexadepsipeptides composed of one α-hydroxy acid and five amino acid residues. The general formula of destruxins is cyclo (-D-HA-L-PRO-L-Ile-L-MeVal-L-MeAla-β-Ala-), where HA is a D-α hydroxyl acid residue (Figure 1).

To date 39 different destruxins have been reported,[3] they were found from different fungi,[4,5] but the most extensively reported fungus was *Metarhizium anisopliae*.[6-11]

Some destruxins, especially destruxin A, E and B (DA, DE, DB) showed insecticidal activities..[12-15] DB and Desmethyl-DB were phytotoxic to the plants of Brassica.[16,17]

In this paper, we report a high performance liquid chromatographic/mass spectrometry (HPLC/MS) method that permits the separation and identification of the majority of destruxins present in extracts of fungal fermentation broth. A conventional gradient HPLC method was used to Setup the elution parameters and to obtain qualitative analytical data based on the exact mass measurement and tandem mass spectrometry. Then the HPLC system was connected to the mass spectrometer via electro spray ionization (ESI) interface and Atmospheric Pressure Chemical Ionization interface (APCI). Twelve destruxins from the fungi *Metarhizium anisopliae* are described.



R_1	R_2	R_3	Destruxin
$-CH=CH_2$	$-CH_3$	$-CH(CH_3)CH_2CH_3$	A
$-CH(CH_3)_2$	$-CH_3$	$-CH(CH_3)CH_2CH_3$	B
$-CH(CH_2OH)CH_3$	$-CH_3$	$-CH(CH_3)CH_2CH_3$	C
$-CH(CHOOH)CH_3$	$-CH_3$	$-CH(CH_3)CH_2CH_3$	D
$-CH-CH_2$ (O)	$-CH_3$	$-CH(CH_3)CH_2CH_3$	E
$-CH(CH_3)_2$	$-H$	$-CH(CH_3)CH_2CH_3$	DMDB
$-CH=CH_2$	$-CH_3$	$-CH(CH_3)_2$	A_2
$-CH-CH_2$ (O)	$-CH_3$	$-CH(CH_3)_2$	E_2

Figure 1: Representative structure of destruxins[2]

MATERIALS AND METHODS

Microorganism and cultivation

Metarhizium anisopliae was isolated from an agricultural soil of Constantine (east Algeria). The culture was maintained on PDA medium (potato 200 g, glucose 20 g, agar 16 g and water 1000 mL). The pre-culture medium

which we have used was Czapek-Dox broth plus 0.5% peptone: NaNO3 3g, K2HPO4 1g, MgSO4 7H2O 0.5 g, KCl 0.5 g, FeSO4 7H2O 0.01 g, glucose 30 g, peptone 5 g, and water 1000mL.

Fermentation medium

The cultivation was performed with a 250ml Erlenmeyer flask contain fermentation medium (Maltose 2.55%, peptone 0.75%, b-alanine 0.02%, and glucose 0.50%).[8]

The conidia spores from PDA culture were suspended into a concentration of 10^7 spores/mL. One mL of conidia suspension was poured into a 150-mL-flask with 49mL pre-culture broth, and incubated for 4 days at 150 r/min and 28°C. For fermentation culture, 10mL of pre-cultured broth was inoculated into 90mL of fermentation medium, and incubated for 14 days at 28°C and 150 r/min. Each strain was replicated 3 times.

Isolation of Destruxins

The destruxins were isolated from submerged fermentation culture, after 14 days on a rotary incubator at 150 rpm in 28°C by realizing organic extraction and using methylene dichloride as solvent.

The liquid culture was blended for 30s. After centrifugation at 6000g during 30min in order to eliminate the mycelium and impurities from fermentation broth, the supernatant was confronted three times successively to methylene dichloride with equal volume (CH_2Cl_2). The organic layer was then collected and concentrated with a vacuum rotary evaporator, then recuperated by dissolving it in 1ml absolute Methanol (MeOH). The extract solution was filtered through a 0.22µm membrane disc. The extract components were then separated by reverse-phase HPLC. The experiments were realized twice in triplicates.

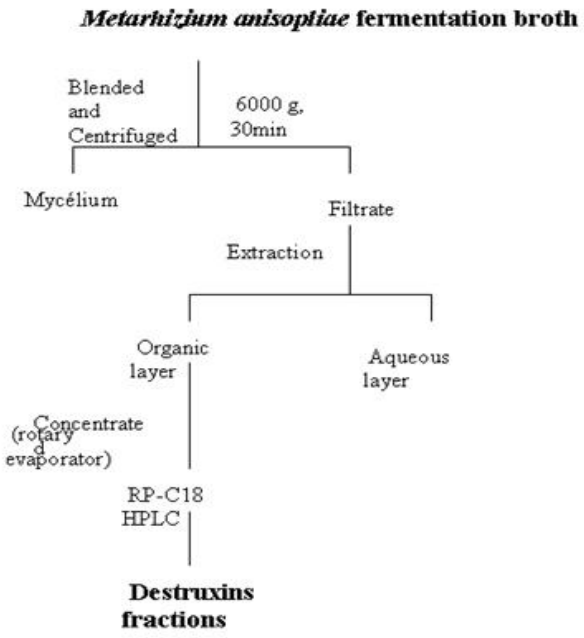


Figure 2: The purification scheme of *M.anisopliae* fermentation broth

HPLC analysis

Extracts of fungal cultures were separated by preparative HPLC (Pump. Agilent G 1312A binary, C_{18} column). Gradient combination of Acetonitrile-Water was employed as following: 0min (4% Acetronile), 12min (30% Acetronile), 30min (95% Acetronile), 40min (4% Acetronile). The eluting solvent with a flow-rate of 0.3ml/ml was employed.

HPLC MS QTOF analysis

The high resolution mass spectra were recorded in a positive ion mode on a hybrid quadrupole time of flight mass spectrometer (Micro TOF-QII Bruker Daltonics, Bremen). With an Electro spray Ionization (ESI) source and atmospheric pressure chemical ionization (APCI) interface. The gas flow of spray gas is 3 bar and the capillary voltage is +/- 4.5 KV. The mass range of the analysis is 50-1000m/z and the calibration was done with PEG (300+400). The solutions are infused at 400µl/min via an HPLC system (Agilent 1200 series). The HPLC column is a C18PolarTec (Magerey Nagel) and a Gradient of Acetonitrile-Water were used as following: 0min (4% Acetronile), 12min (30% acetronile), 30min (95% Acetronile), 40min (4% Acetronile).

RESULTS AND DISCUSSION

In this study the fermentation broth from 14 days-old culture of *M. anisopliae* was blended, and separated by centrifugation. The supernatant was extracted three times by equal volume of methylene dichloride. A method for the separation and purification of destruxins were followed as shown in Figure 2. The extract obtained after centrifugation and confrontation to the CH_2Cl_2 was analyzed by an analytical RP-C HPLC column achieved with a water–acetonitrile gradient over a period of 40 min in order to separate the various destruxins present in the extract. Figure 3 shows the HPLC chromatogram of the crude extract. From the chromatogram (figure 3) demonstrating the separation of destruxins by reverse phase HPLC, it can be seen that 12 compounds were detected. The retention times of molecules separated are: 17.1, 18.2, 18.9, 20.9, 21.9, 22, 22.2, 23.4, 24.8, 25.5, 26.4 and 27.8. As shown in figure 3: Destruxins peaks 6 and 8 have highest intensity and peak area.

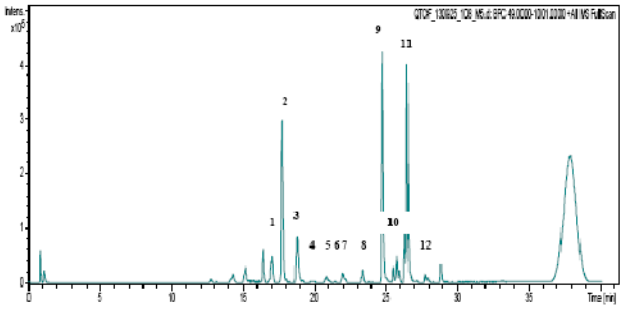


Figure 3: Different HPLC fractions of crude extracts from culture filtrates of *Metarhizium anisopliae*

The distinction between about 12 expected destruxins is not possible using a simple gradient HPLC method. The development of liquid chromatography coupled with mass spectrometry (LC-MS) permits the relatively rapid characterization of complex natural DTX. Positive ion electrospray ionization (ESI) was chosen for LC/MS experiments. Destruxins peaks (1-12) were identified as dtx : dtx E-diol ([MH]+ 612 *m/z*), dtx F ([MH]+ 596 *m/z*), E-diol 1 ([MH]+ 626 *m/z*), dtx C ([MH]+ 610 *m/z*), dtx E Chlorohydrin ([MH]+ 630 *m/z*), dtx E ([MH]+ 594 *m/z*), dtx D ([MH]+ 624 *m/z*), A2 ([MH]+ 578 *m/z*), A ([MH]+ 578 *m/z*) , a dtx derivative of the [MH]+ 580*m/z* series (either desMe-dtx B, dihydro-dtx A or dtx B2), dtx B ([MH]+594 *m/z*) and dtx B1 ([MH]+608 *m/z*). The MS data and respective identification of peaks are presented in table 1. Mass deviations from calculated values for destruxin derivatives' are between 1.1 and 4.6ppm. The LC/MS showed that extraction procedures provided a high Dtx A and B concentration (peaks 6 and 8).

Table 1: HPLC-ESI-T0F-MS data and identification of peaks 1-8

Peak	t_R	$[M+H]^+$			Molecular formula	Compound
		Measured	Calculated	Difference/ppm		
1	17.1	612.3585	612.3603	1.8	$C_{29}H_{49}N_5O_9$	Destruxin Ed
2	18.2	596.3644	596.3654	1.7	$C_{29}H_{50}N_5O_8$	Destruxin F
3	18.8	626.3739	626.3760	3.2	$C_{30}H_{50}N_5O_8$	Destruxin Ed1
4	20.9	610.3804	610.3810	1.1	$C_{29}H_{47}N_5O_8$	Destruxin C
5	21.9	630.3232	630.3253	3.2	$C_{29}H_{48}N_5O_8Cl$	Destruxin E Chlorohydrin
6	22	594.3489	594.3497	1.4	$C_{29}H_{47}N_5O_9$	Destruxin E
7	22.2	624.3590	634.3603	1.3	$C_{30}H_{50}N_5O_9$	Destruxin D
8	23.4	564.3375	564.3392	1.7	$C_{28}H_{45}0_7N_5$	Destruxin A2
9	24.8	578.3533	578.3548	2.6	$C_{29}H_{47}N_5O_7$	Destruxin A
10	25.5	580.3678	580.3705	4.6	$C_{29}H_{49}N_5O_7$	Destruxin B2, Dihydrodestruxin A, N-Demethyldestruxin B
11	26.4	594.3845	594.3861	2.8	$C_{30}H_{51}N_5O_7$	Destruxin B
12	27.8	608.3996	608.4018	3.6	$C_{31}H_{54}N_5O_8$	Destruxin B1

In the mass spectra of destruxins, intense [M+H]+ and weaker [M+Na]+ pseudo molecular ions were detected. The ES mass spectrum of destruxins A and B is shown in (figure 4). In figure 4 we observed DTX adducts with sodium cations, i.e. m/z 600 for sodium adducts DTX-A and 616 for sodium adduct of DTX-B. In the other hand, we observed the production of protonated molecules $[M+H]^+$, i.e. m/z 578 for DTX-A and 594 for DTX-B.

Positive –ion APCI was chosen for LC/MS experiments in view of the production of abundant protonated molecules [M+ H]+ (figure 5). In contrast to the APCI spectra the $[M+ H]^+$ and $[M+ Na]^+$ ions were the dominant species in the positive ion electro spray ionization mass spectra.

The collision-induced dissociation (CID) fragmentation data, generated by the data-dependent analysis procedure, is shown for the m/z 578 ion and m/z 594 (dtx A and B, Figure 6).

The relative abundance ratios of dominant ions are similar for all destruxins, which permits a fast orientation in the spectra (Fig. 6). The dominating sequence ions are $^{4-5}b_5$, $^{4-5}a_5$, $^{4-5}b_4$, $^{4-5}a_5$ $^{4-5}b_3$ and D^+.

Metarhizium anisopliae metabolites are toxic to a broad range of animals and microbes, including insects, fungi, bacteria and viruses.[1] Most of these metabolites were isolated from mycelia or fermentation extracts. There is much less information regarding secondary metabolites exclusively or uniquely present in *Metarhizium* conidia.[18,19] In fact, the dtxs are the most prevalent secondary metabolites produced by *M. anisopliae* during fermentation. Furthermore they are the most exhaustively researched toxins in this entomopathogenic fungus. The medium used in the fermentation (submerged culture) studies of *Metarhizium anisopliae* has been focused on the combination of czapek dox broth and peptone with no exception in the literature.

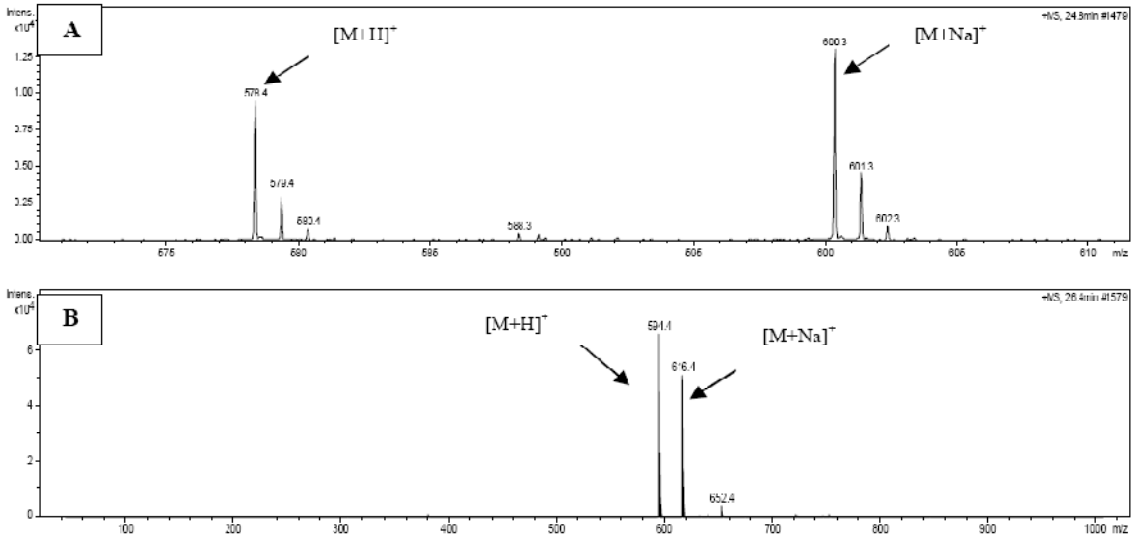


Figure 4: (A) HPLC-ESI-TOF-MS of Destruxin A, (B) HPLC-ESI-TOF-MS of Destruxin B

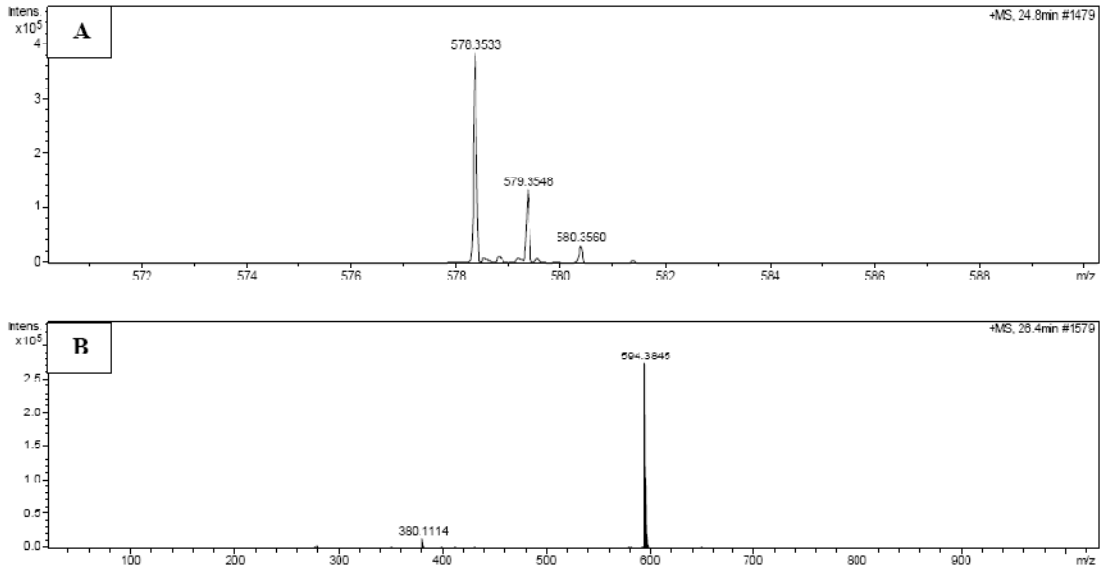


Figure 5: (A) HPLC-APCI-TOF-MS of Destruxin A,(B) HPLC-APCI-TOF-MS of Destruxin B

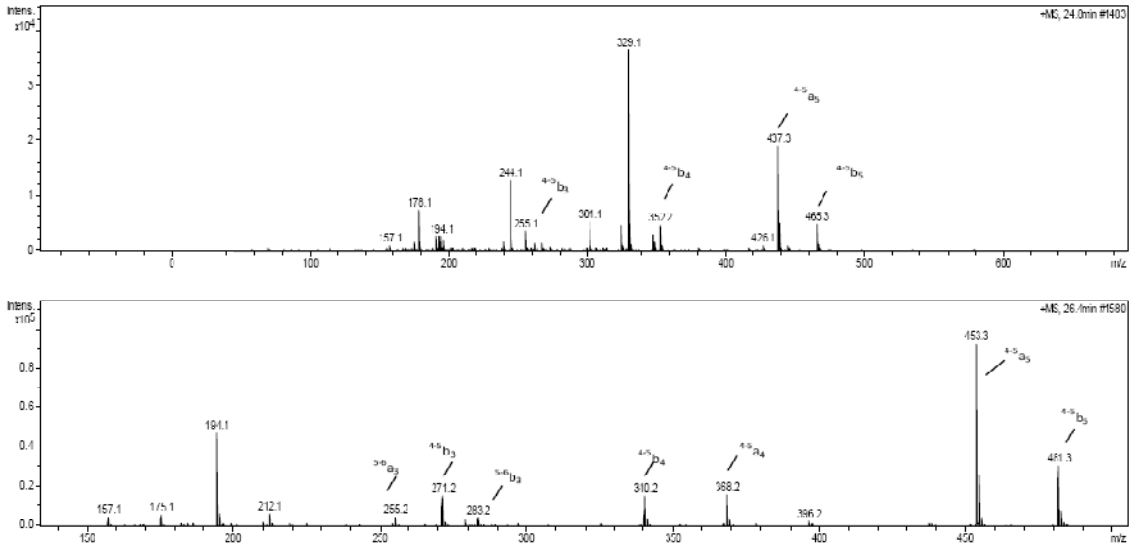


Figure 6: Data acquired by data-dependent fragmentation analysis of dtx A and B from *M. anisopliae*: (A) MS/MS of m/z 578; (B) MS/MS of m/z 594

A modified medium was used to grow this fungus, *Metarhizium anisopliae* was cultured on a maltose medium containing peptone in a flask shake at 150rpm, Liu[8] reported that maltose and peptone were the best carbon and nitrogen sources with the addition of amino acid-β alanine for the production of destruxins by *Metarhizium anisopliae*. Campbell[20] also reported that glucose and maltose induced good spore production for *M. anisopliae* Destruxins are cyclic peptide toxins with high hydrophobicity. Many organic solvents have been used for extraction of destruxins (EtOAc, CCl_4, CH_2Cl_2) following bioassay-guided fractionation; both silica gel chromatography and reversed-phase HPLC have been utilized.[21-28] Fast atom bombardment– mass spectrometry (FAB–MS) has been a very useful method for analysis of destruxins in crude fungal extracts.[29-32]

The development of liquid chromatography procedures coupled with mass spectrometry (LC-MS) permits the relatively rapid characterization of complex natural destruxins. More recent methods include LC-MS with APCI and LC-ESI.[33,34]

Destruxins are quite disparate compounds which may occur as isomers or structurally similar compounds. This compound may exist in many forms. So far, different structurally related destruxins have been identified from different fungal sources; most forms are produced by *Metarhizium anisopliae*.[35]

Investigations on dtxs production by *Metarhizium anisopliae* in this study showed that eight destruxins could be detected in liquid medium in a lab-scale flask. In this experiment, the LC/MS showed that extraction procedures provided a high dtx A and B concentration, since they are the most common and important destruxins.[21,3] The first systematic study of toxin production by fungal entomopathogens in vitro was conducted on *M. anisopliae* and led to the discovery of two novel insecticidal substances destruxin A ($C_{29}H_{47}N_5O_7$) and destruxin B ($C_{30}H_{51}N_5O_7$).[6] The structure was confirmed by total synthesis[36] and NMR spectra by Suzuki.[37] Later, three compounds, desmethyl destruxin B, D and C, were isolated from *M. anisopliae* culture filtrates by Suzuki.[38] Several destruxins were separated by Pais[21] including: destruxins E, Al, A2, B1, B2, C2, D1, D2 and E1. The structure of these compounds was established by NMR and mass spectrometry.

CONCLUSION

Fungal toxins are thought to be involved during pathogenesis for certain host / pathogen relationships. These toxins are normally classified as non enzymic, low molecular weight non antigenic compounds, many of which have been isolated from the culture filtrates of entomopathogenic fungi.[39,11] However, the destruxins produced by *Metarhizium anisopliae* are the only toxins to have been detected in moribund insects in sufficient quantities to cause death.[40-43] A number of qualitative and quantitative approaches based on mass spectrometry have been reviewed and proposed for the analysis of cyclic peptides and depsipeptides. Practically, there have been several approaches that have been developed to detect and identify destruxins from different strains of *M. anisopliae*. The techniques described here enable further detailed studies of these interesting peptides.

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