RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2009; **23**: 1426–1434
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.4018

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Mass spectrometry as a tool for the selective profiling of destruxins; their first identification in *Lecanicillium longisporum*

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Received 4 February 2009; Revised 4 March 2009; Accepted 6 March 2009

Mass spectrometry was applied to the identification of the destruxins (dtxs), cyclic peptides that are commonly produced by the fungal insect-pathogen, *Metarhizium anisopliae*. The aim of the study was to optimise a methodology in order to firstly determine whether these compounds were present in other species and to determine the effect of differing growth conditions upon the dtx content detected. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF-MS) was initially used to analyse the dtxs, but limitations were indicated. Nano-scale high-performance liquid chromatography/electrospray ionisation mass spectrometry (HPLC/ESI-MS) and automated 'data-dependent' tandem mass spectrometric (MS/MS) analysis were also applied, utilising characteristic neutral losses during fragmentation to confirm the presence of the dtxs. This latter approach distinguished the dtx E and B isoforms by retention time and diagnostic neutral losses during fragmentation allowing extraction of the destruxin data from a complex dataset. This process revealed the presence of a number of dtxs in the fungal species *Lecanicillium longisporum*, a species previously not known to produce dtxs, and dtx production in this species was shown to be significantly higher in aerated cultures compared with still cultures. Copyright © 2009 John Wiley & Sons, Ltd.

Destruxins (dtxs) are cyclic peptides composed of an α-hydroxy acid and five amino acid residues joined by amide and ester linkages.[1] Many dtxs have been identified to date and these are placed in five major groups (A–E) and several sub-groups with dtxs A, B and E usually being the predominant ones (Fig. 1). Most dtxs and their analogues have been isolated from cultures of the insect-pathogenic fungus *Metarhizium anisopliae*; their production is less well documented in other fungi. Dtx B, desmethyldtx B and homodtx B have been reported as being produced by the plant pathogen *Alternaria brassicae* [2] whilst *Ophiosphaerella herpotricha*, a plant pathogen on Bermuda grass, produces dtx B.[3] Dtx A4, A5 and homodtx B are found in cultures of the insect-pathogen *Aschersonia* sp.,[4] while the coprophilous fungus *Nigrosabulum globosum* is known to produce pseudodtxs A and B.[5] The exact role of dtxs produced by insect and plant pathogenic fungus has not been fully elucidated; however, they are considered to be important determinants of pathogenicity.[6,7] The toxicity of dtxs to insects is well documented,[8] with low concentrations being sufficient to temporarily paralyse an insect host[6] and to suppress its cellular defences.[9] Dtxs exhibit other biological activities which make them interesting tools that may be used to study cellular processes and possibly as lead compounds for the development of novel pharmaceuticals to treat cancer, osteoporosis, and hepatitis B.[8,10–13]

The level and type of dtxs secreted by *M. anisopliae* are dependent on the species, strain and culture conditions such as pH, substrate and aeration.[14–16] Despite many previous studies that have identified these compounds in different species and utilised mass spectrometry to study their occurrence[17,18] and breakdown products,[19] a robust and reliable method for the selective analysis of these compounds from the complete metabolite profile of such organisms has yet to be developed. Therefore, in the study reported this paper we investigated two mass spectrometric protocols for the determination of dtxs from organisms and compared their

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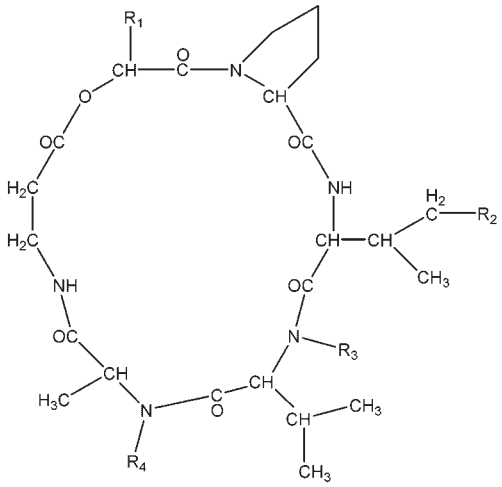
Contract/grant sponsor: European Commission, Quality of Life and Management of Living Resources Programme (QoL), Key Action 1 on Food, Nutrition and Health; contract/grant number: QLK1-2001-01391.



Destruxin	R_1	R_2	R_3	R_4
E	C_3H_5O	CH_3	CH_3	CH_3
B	C_4H_9	CH_3	CH_3	CH_3
A	C_3H_5	CH_3	CH_3	CH_3
D	$C_3H_5O_2$	CH_3	CH_3	CH_3
E_2	C_3H_5O	H	CH_3	CH_3
B_2	C_4H_9	H	CH_3	CH_3
DesMeE	C_3H_5O	CH_3	H	CH_3
DesMeB	C_4H_9	CH_3	H	CH_3

Figure 1. Structures of common destruxins.

effectiveness. This optimised technique was then used to study whether the entomopathogenic fungus *Lecanicillium longisporum*, formerly known as *Verticillium lecanii*,[20] produces dtxs and whether these compounds are produced in higher levels in aerated cultures compared to still cultures. This report details the first identification of these compounds produced by *L. longisporum* and describes the analytical process used to selectively study the dtxs. This optimised and selective analytical protocol can now be utilised for the further selective study of these compounds in this and other species.

EXPERIMENTAL

Materials

All reagents were obtained from Sigma unless stated otherwise. Dichloromethane (CH_2Cl_2) and ethyl acetate (EtOAc) were of analytical grade while acetonitrile (MeCN) and methanol (MeOH) were of HPLC grade. Ultra-pure analytical grade water ($r > 18\,M\Omega/cm$) was produced by a Milli-Q Plus® water system (Millipore, UK). Standards for dtxs E and B were purified from *M. anisopliae* culture broth as described by Pais *et al*.[21]

Organisms and cultivation

The fungi *M. anisopliae* and *L. longisporum* KV71 (IMI 179172; HRI 1-72, Ve2), active ingredient of the commercial agent Vertalec® (Koppert, NL), were grown in Czapek Dox liquid medium (30 g sucrose, 2 g sodium nitrate, 1 g dipotassium phosphate, 0.5 g magnesium sulphate, 0.5 g potassium chloride, 0.01 g ferrous sulphate and 20 g yeast extract/L deionised water). A starter culture was initiated by inoculating 50 mL medium in a 250 mL conical flask with 100 μL spore suspension (10^7 spores/mL deionised water) and incubating for 3 days on an orbital shaker (Sanyo Gallenkamp, UK) at $25 \pm 3^\circ C$ and 100 rpm. The main cultures (100 mL medium in 500 mL conical flasks) were inoculated with 1.5 mL of starter culture and incubated for 16 days at $25 \pm 3^\circ C$ as still (no shaking) and shake cultures (100 rpm).

Metabolite extraction

Culture filtrate from shaken and still cultures was separated by filtration through a Whatman No. 1 filter paper under vacuum and extraction of the culture filtrate was based on the methodology of Starratt and Loschiavo.[22] Briefly, a separating funnel was charged with culture filtrate and CH_2Cl_2 (1:4, v/v) and left for 5 h. The solvent phase was washed with deionised water before evaporation of the solvent in a fume cupboard and the residues re-dissolved in CH_2Cl_2/MeOH (1:1, v/v). Extracts were stored at $-20^\circ C$ until required for analysis.

Mass spectrometry

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF-MS)

MALDI-ToF-MS analysis was performed using a Voyager DE-STR MALDI mass spectrometer (Applied Biosystems, Warrington, UK) equipped with a nitrogen laser (emission wavelength 337 nm, pulse duration 4 ns). Spectra were recorded in positive ion mode using 20 kV acceleration voltage, delayed extraction (100 ns) and reflectron mode. External calibration was carried out using the protonated molecules of leucine-enkephalin, substance P, neurotensin and ACTH (fragment 18-39). MALDI targets were prepared using cyano-4-hydroxycinnamic acid (CHCA) as matrix and the dried-droplet method was used for sample/matrix co-crystallisation. Briefly, 1 μL of a 100-fold diluted sample was mixed with 1 μL of the matrix solution (10 mg/mL in MeOH/MeCN); then 1 μL of the mixture was deposited on the MALDI target and allowed to dry under vacuum.

Nano-scale high-performance liquid chromatography/electrospray ionisation mass spectrometry (nano-HPLC/ESI-MS)

A reversed-phase C18 HPLC column (75 μm × 10 cm, Pepmap C18 stationary phase; Dionex, Camberley, UK) was prepared in-house by loading a piece of fused silica with a pulled tip with the packing slurry until the desired length of silica had been packed. An Ultimate pump and a FAMOS autosampler (Dionex, Camberlay, UK) were used to pump mobile phase over the column at a flow rate of 200 nL/min and inject 5 μL of the culture extract onto the column. The mobile phases used were 2% MeCN/98% water/0.1% formic acid (A) and MeCN with 0.1% formic acid (B). After sample loading, the mobile phase was maintained at 100% A for 10 min, a gradient between 100% A and 40:60 A/B was then applied over 1 h; following this the gradient was increased to

DOI: 10.1002/rcm

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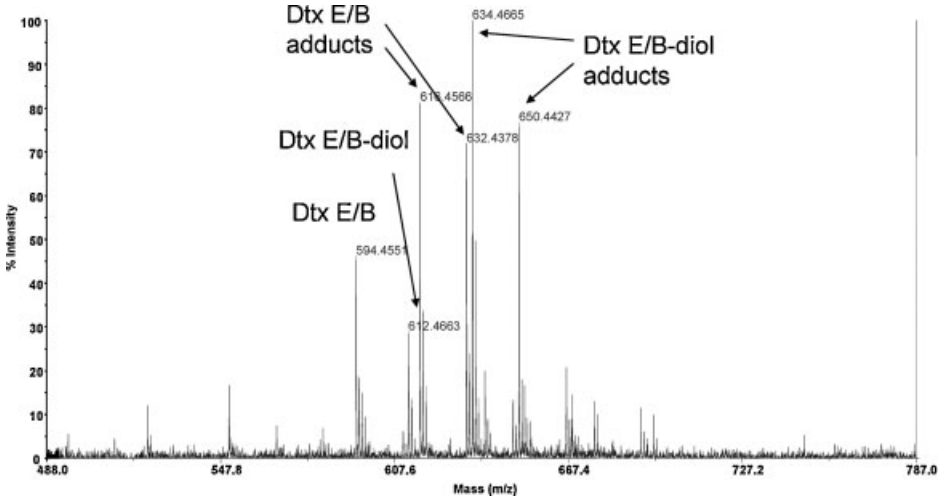

Figure 2. MALDI-ToF mass spectrum of dtxs E (or B – *m/z* 594) and E-diol (*m/z* 612) from *M. anisopliae*.

100% B over 20 min, held at 100% B for 15 min before being returned to 100% A over 15 min. The HPLC column also acted as the nano-ESI tip in the source of the mass spectrometer for an LCQ-XP plus ion trap mass spectrometer (Thermo Finnigan, Hemel Hempstead, UK). The ion trap was operated in positive ion mode with a spray voltage of 1.6 kV, a capillary temperature of 165°C and a capillary voltage of 10 V. The mass spectrometer was utilised in a 'data-dependent scanning mode' in which the most abundant ion in the initial full scan mass spectra was automatically isolated and fragmented in order to generate tandem mass (MS/MS) spectra. From these MS/MS spectra, the two most abundant product ions were automatically isolated and further fragmented generating data-dependent MS^3 spectra. A dynamic exclusion parameter was applied which excluded any product ion that had been analysed twice in 1 min from further analysis for a period of 2 min in order to allow low-abundance compounds to be examined. This detailed analysis afforded structurally relevant information on the precursor ions that were present, without the prerequisite of knowing from which compounds they were isolated. The data was analysed using the Xcalibur software package

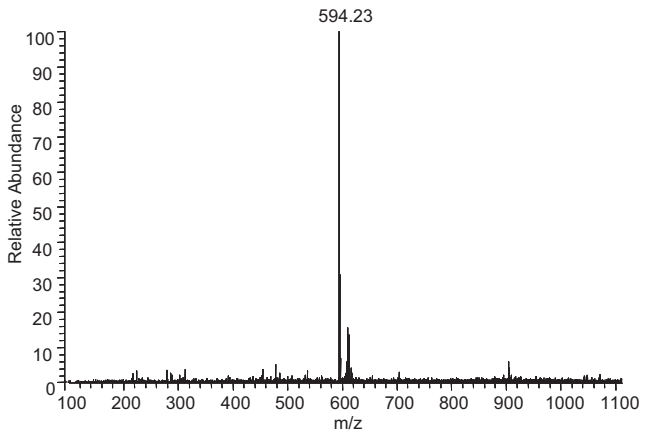

Figure 3. Full scan HPLC mass spectrum at 68.5 min indicating the protonated molecule of dtx E from *M. anisopliae*.

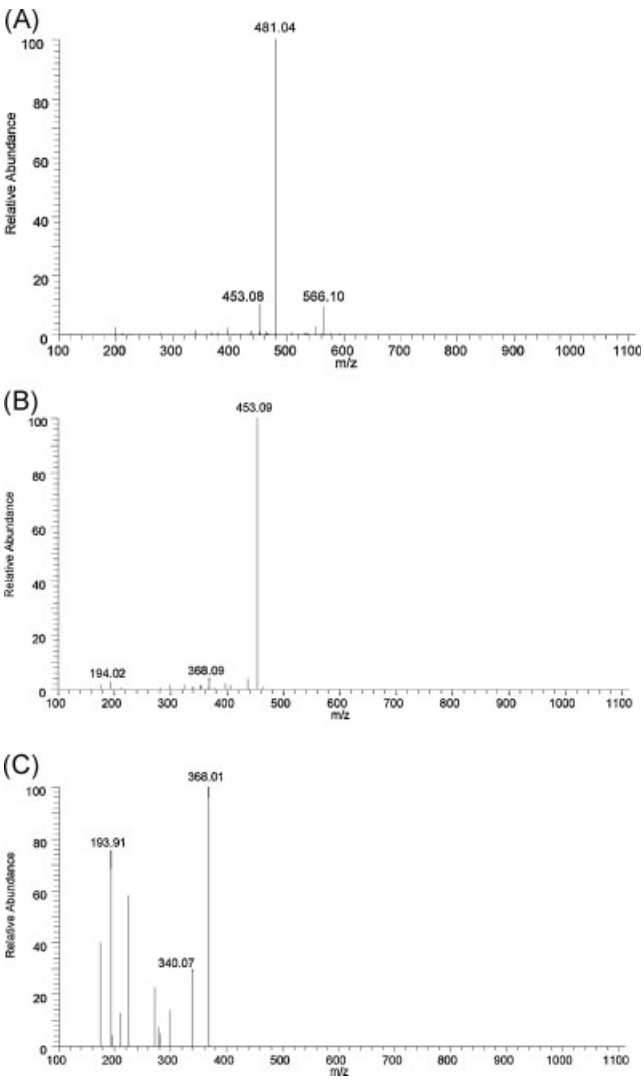

Figure 4. Data acquired by data-dependent fragmentation analysis of dtx E from *M. anisopliae*: (A) MS/MS of *m/z* 594; (B) MS^3 analysis of the *m/z* 481 product ion of MS/MS of *m/z* 594; and (C) MS^3 analysis of the *m/z* 453 product ion of MS/MS of *m/z* 594.

DOI: 10.1002/rcm

(Thermo Finnigan, Hemel Hempstead, UK) with the neutral loss facility being utilised in order to monitor characteristic losses post-acquisition. The output generated by the analysis presented total ion current (TIC)-like chromatograms for all precursor ions that, under fragmentation conditions, exhibited the loss of the masses under study in any given experiment. Such chromatograms mimic those generated by multiple reaction monitoring (MRM) analyses.

RESULTS

MALDI-ToF-MS was first applied for the dtx determination. Figure 2 indicates the MALDI spectrum obtained from the analysis of dtxs purified from a culture extract of *M. anisopliae*. Dtx E or B (*m/z* 594) and E (or B)-diol (*m/z* 612) are the only dxts detected by the analysis and the spectrum obtained is further complicated by the presence of sodium and potassium adducts of both ions. For example, the $[M+H]^+$ ion at *m/z* 594 also exhibits the sodium adduct $[M+Na]^+$ at *m/z* 616 and a potassium adduct $[M+K]^+$ at *m/z* 632. Further study using metastable decomposition, in a post-source decay (PSD) mode, generated data that was not informative enough to determine the structural identity of the dtxs present and in many cases indicated low mass accuracy. A further disadvantage of MALDI-ToF-MS analysis of the dtxs became apparent in that isobaric dtxs, such as dtx E and B (*m/z* 594) and their derivatives (primarily dxt E_2, B_2, DesMeE and DesMeB all with *m/z* 580), could not be distinguished. Hence, despite MALDI-ToF-MS being a very rapid analytical technique, it was not capable of determining the relative quantities of these isobaric dtxs. Therefore, the application of nano-HPLC/ESI-MS was undertaken as an alternative approach to the study of dtxs.

As expected, from previously unpublished data undertaken by the group, for the *M. anisopliae* extract, the dxt E ion represented the most abundant dxt ion present in the sample during HPLC/MS analysis. The full scan ESI mass spectrum at 68.5 min clearly shows an abundant protonated molecule (dxt E), $[M+H]^+$ at *m/z* 594 (Fig. 3), and without any other adducts. The collision-induced dissociation (CID) fragmentation data, generated by the data-dependent analysis procedure, is shown for the *m/z* 594 ion (dtx E, Figs. 4(A)–4(C)) and an overview of this fragmentation data in relation to the structural fragmentation of the protonated molecule of dtx E is shown in Fig. 5. The CID-MS^n fragmentation data was used to determine its potential to selectively quantify the isobaric dtxs from complex mixtures. The main consideration was whether the combined HPLC separation and data-dependent fragmentation data collection could overcome some of the problems represented by the initial MALDI-ToF-MS data. The first challenge to this study was that some of the dtxs (such as dtx E and B) are isobaric and hence would generate identical *m/z* ions in this HPLC/ESI-MS system. Furthermore, the fragmentation data generated for these two precursor ions is also identical, sine they only differ in a side chain (referred to here as R_1) present on the cyclic peptide structure. This side chain is not lost during fragmentation of the cyclic peptide and henceforth does not interfere with the CID fragmentation. However, due to the differences in polarity of the various R_1 side chains present in dtxs E and B,

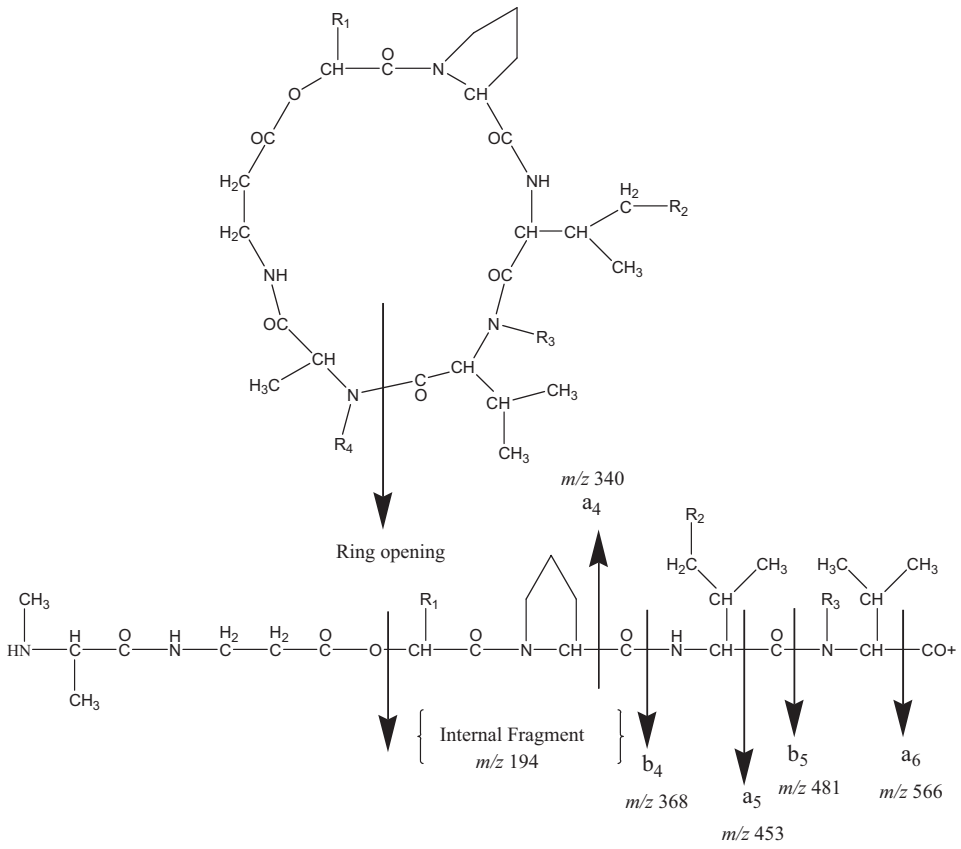

Figure 5. Fragmentation map of dtx E.

DOI: 10.1002/rcm

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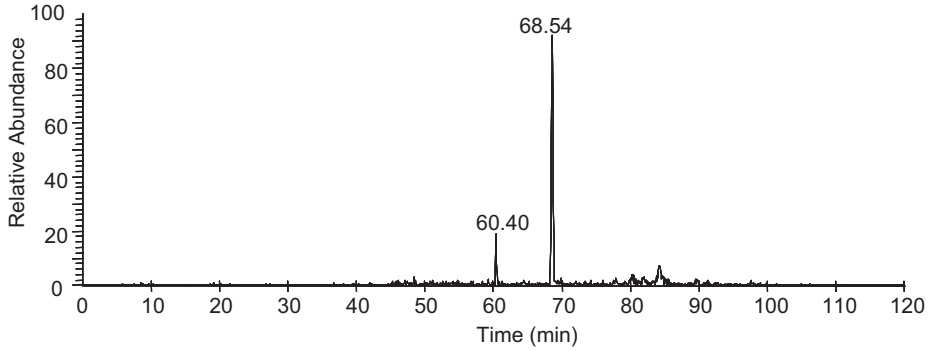

Figure 6. Extracted ion chromatogram from HPLC/MS analysis of *m/z* 594 (dtx E or B) from *M. anisopliae*.

the two dtxs exhibit different retention times on a reversed-phase C18 HPLC column with dtx B isomers always eluting before the dtx E isomer (observed in unpublished data). Figure 6 shows an extracted ion chromatogram (generated post-acquisition from full scan ESI data) of *m/z* 594 for the *M. anisopliae* sample in which both dtx E and B can be seen (E at 68.5 min and B at 60 min) and dtx E is found at a more elevated level than dtx B. Therefore, the application of the HPLC separation prior to on-line MS analysis allows the distinction of dtx E and B isoforms.

From the data obtained from the analysis of dtx E, it could be seen that ring opening of these cyclic peptides is followed by a series of characteristic losses during CID-MS/MS similar in nature to non-cyclic peptide fragmentation. Hence the product ions of the 'B-like' series are predominantly present in the MS/MS data (retaining the C-terminus of the opened peptide); the alternative 'Y-like' product ion series is present in too low an abundance to provide any structural information. The 'b_5' ion varies in its relation to the precursor ion depending on the state of the side chain at R_3, which is usually a methyl group or a hydrogen, therefore resulting in a loss of 113 Da or 99 Da, respectively (Fig. 6), and this difference should therefore allow the differentiation of dtxs that have different R groups at this point. Furthermore, a loss

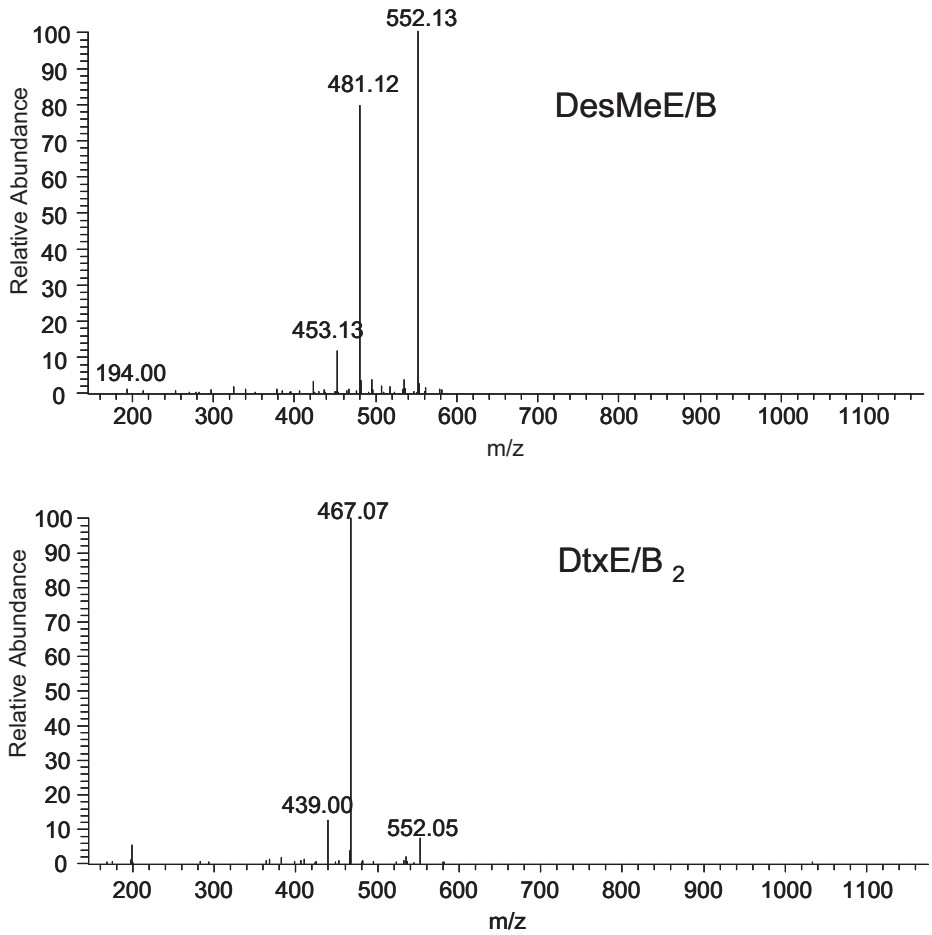

Figure 7. Different MS/MS spectra from dtx DesMe E or B (upper trace) and dtx E_2 or B_2 (lower trace).

DOI: 10.1002/rcm

of 28 Da (loss of CO) is commonly detected in the CID-MS/MS fragmentation of these cyclic peptides. The next challenge in studying the dtxs was therefore to determine if the fragmentation profiles were able to distinguish the E/B_2 isoforms from the DesMeE/B isoforms (isobaric yet differing in R_2 and R_3 side chains). During MS/MS analysis, the differing losses of 99 Da from the DesMeE isoform (*m/z* 580 to 481) and 113 Da from the E/B_2 isoforms (*m/z* 580 to 467), due to the nature of the R_3 side chain, can easily distinguish the two dtx types (Fig. 7). Again the different retention times, due to side-chain polarity, offer the only distinction between the specific dtx E and B isomers due to the lack of fragmentation within the side-chain groups. However, this difference in retention time is more than sufficient to separately identify and quantitate the two isomers. It was concluded that the combination of the nano-HPLC separation with ESI-MS/MS and the study of the characteristic neutral losses produced during fragmentation (loss of 28, 99 and 113 Da) should allow the selective and detailed study of the dtxs from the secreted metabolome of the fungi under study. Therefore, this technique was applied to the study of the species *L. longisporum* in order to study whether it also produced dtxs. Figure 8(a) indicates the base peak (representing the most abundant ion at each retention

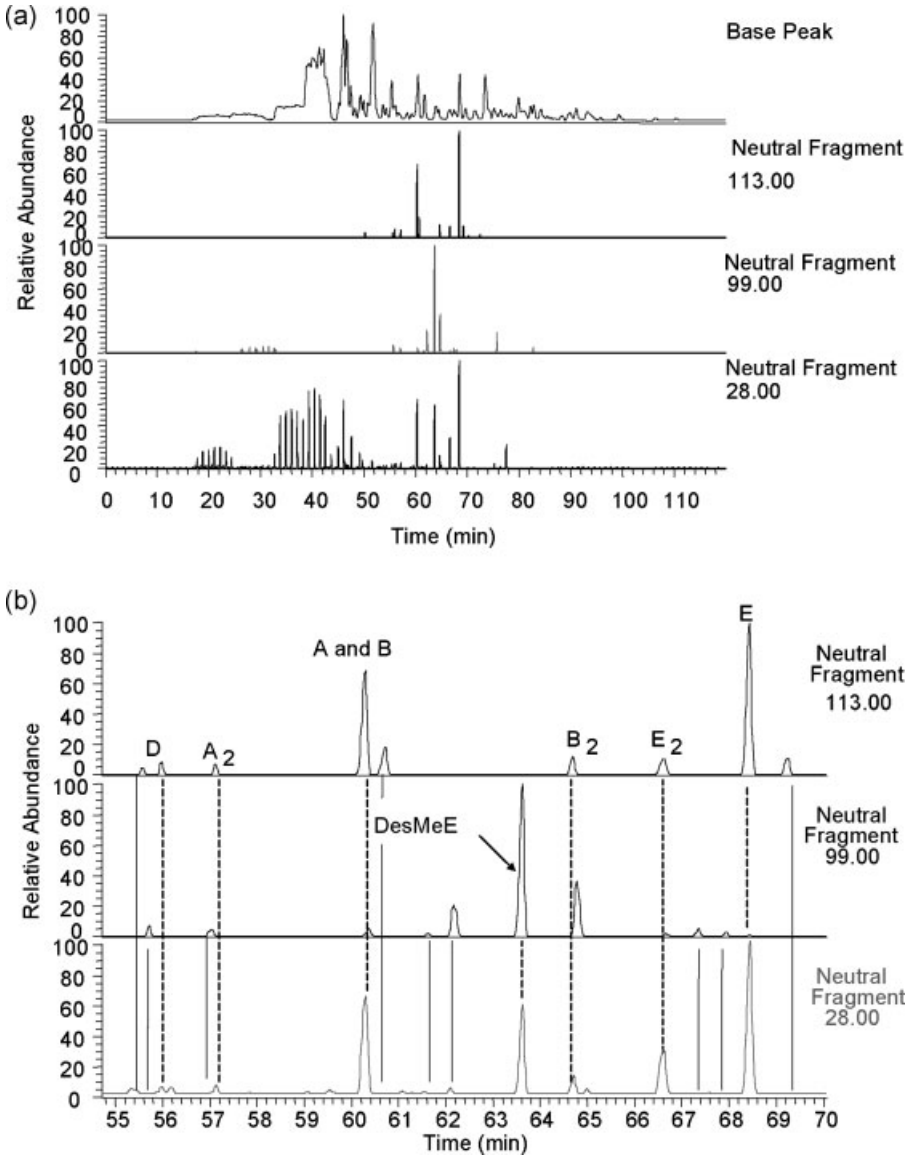

Figure 8. (a) Base peak (most abundant ion – upper trace) from HPLC/DDA-MS mode analysis, ions that undergo loss of 113 Da during analysis in HPLC/DDA-MS mode (second trace), ions that undergo loss of 99 Da during analysis in HPLC/DDA-MS mode (third trace), ions that undergo loss of 28 Da during analysis in HPLC/DDA-MS mode (bottom trace). (b) Zoom-in of ions that undergo loss of 113 Da during analysis in HPLC/DDA-MS mode (upper trace), ions that undergo loss of 99 Da during analysis in HPLC/DDA-MS mode (second trace), ions that undergo loss of 28 Da during analysis in HPLC/DDA-MS mode (bottom trace). Full lines represent 'false' identifications whilst broken lines represent accurate identifications with −28 Da loss and also either loss of 99 or 113 Da.

DOI: 10.1002/rcm

time point) and those compounds that exhibited the loss of 113, 99 and 28 Da during data-dependent fragmentation analysis of the secreted metabolome from *L. longisporum*. Firstly, from the upper trace of Fig. 8(a) (the base peak), it can be seen that the purification process is non-selective and that a large number of compounds are extracted by the methodology used for purification, hence demonstrating the requirement for selective MS/MS analysis. The analysis of compounds which exhibited the selected characteristic losses indicated a number of peaks and these peaks and their confirmed identifications are shown in Fig. 8(b). Although the highlighting of these losses improves the detection of the dtxs above the background signal or base peak, it can be seen that the loss of CO (28 Da) is not diagnostic for dtxs (Fig. 8(a)) because a large number of ions (other than dtxs) exhibiting this loss were determined. Therefore, a more detailed study was made of the peaks indicating the loss of 99 and 113 Da during fragmentation. Studying these losses, a number of dtxs were identified for the first time as being produced by the Vertalec sample (a commercial fungus, *L. longisporum*). For these compounds the *m/z* of the protonated molecular ion, the retention time and the fragmentation data due to fragmentation from both the MS/MS and MS^n data confirmed the identification of the dtxs that are summarised in Table 1. Closer examination of the data (Fig. 8(b)) however identified a number of chromatographic peaks in the TIC profiles of ions which exhibited the required loss of either 99 or 113 Da but which were shown, by examining the full MS/MS and MS^n spectra generated by data-dependent analysis, to not be dtxs. Therefore, it was considered whether the loss of 28 Da taken in conjunction with the other two losses might be diagnostic for identification of dtxs. The 28 Da loss, although not unique to dtxs, is always determined as a neutral loss in dtx fragmentation and hence any dtx-derived ion, identified by the loss of 113 or 99 Da, should also give a response in the −28 Da post-acquisition analysis. The application of this approach to the data confirms the presence of the previously identified dxts (by the loss of 113 or 99 Da), and also eliminates other product ions that also lose 113 or 99 Da (Fig. 8(b)). Alternative diagnostic losses were considered; however, none indicated the specificity or required intensity to allow low levels of dtxs to be identified. Such analysis requires good HPLC resolution of the individual components of the extract (Fig. 8(b)) where the peaks obtained are approximately 12 s wide peaks. Therefore, if the loss of 28 Da and the retention time do not precisely match that of the other specified loss (either 99 or 113 Da), the peak can be discounted. For example, the loss of 113 Da at 64.7 min co-elutes with a loss of 28 Da, indicated by a dotted line, and this peak matches exactly the retention time of dtx B_2. However, the peak that indicates a loss of 99 Da at 64.85 min does not precisely co-elute with a signal

Table 1. Dtxs identified by HPLC/MS and the ions that confirmed their presence

							Fragment ions detected in MS/MS and MS^n analysis					
Destruxin	Rt	R_1	R_2	R_3	R_4	*m/z* MH^+	a_6	b_5	a_5	b_4	a_4	Internal fragment
E	68.5	C_3H_5O	CH_3	CH_3	CH_3	594	√	√	√	√	√	√
D	56	$C_4H_7O_2$	CH_3	CH_3	CH_3	624	√	√	√	√	√	√
B	60.2	C_4H_9	CH_3	CH_3	CH_3	594	√	√	√	√	√	X
A	60.2	C_3H_5	CH_3	CH_3	CH_3	578	√	√	√	√	√	√
E_2	66.6	C_3H_5O	H	CH_3	CH_3	580	√	√	√	√	√	√
B_2	64.7	C_3H_5O	H	CH_3	CH_3	580	√	√	√	√	X	√
desMeE	63.7	C_3H_5O	CH_3	H	CH_3	580	√	√	√	√	X	√
A_2	57.2	C_3H_5	H	CH_3	CH_3	564	√	√	√	√	√	√

Table 2. Relative peak areas for the dtxs under shaken culture conditions and still culture conditions of Vertalec (*L. longisporum*) versus levels detected in *M. anisopliae*

			Comparative peak areas		
				Vertalec	
Destruxin	MW	Rt	*M. anisopliae*	Shaken	Still
E	593	68.5	58391	28232	1554
B	593	60.2	8128	2762	488
A	577	60.2	51866	11176	798
D	623	56.0	6068	3486	183
Ediol	611	51.8	13585	n.d.	n.d.
E2	579	66.6	7646	3814	300
B2	579	64.7	11120	7005	369
A2	563	57.2	5972	1306	110
DesMeE	579	63.7	11061	6425	225
DesMeB	579	57.0	963	n.d.	n.d.
ProtoB	565	60.0	1200	n.d.	n.d.

n.d.: not detected.

DOI: 10.1002/rcm

representing the loss of 28 Da and hence this 99 Da loss peak, despite being very close in retention time to the 28 Da loss peak, is not defined as a dtx in this analysis. This small difference in retention time is sufficient to be diagnostic for the identification of dtx signals from 'false' signals and these findings were substantiated by studying in detail the fragmentation data obtained.

This optimised HPLC/ESI-MS analysis was next used in order to study the levels of dtxs produced with differing growth conditions and if this affected the production of the dtxs in fungi where they were previously undetected. The MS-derived chromatographic peak areas due to the identified cyclic peptides from *M. anisopliae* compared to the levels produced by *L. longisporum* in the aerated and still cultures are represented in Table 2. The first conclusion is that the production of the dtxs by *L. longisporum* is much reduced compared to that of *M. anisopliae*, with some dtxs being absent from the analysis of the *L. longisporum* samples (e.g. dtx E-diol). Furthermore, without exception, the dtxs are found to be elevated in the aerated culture compared to the still culture for *L. longisporum*. The increase in levels between the two culture types varies between 5.7- and 28.6-fold. The difference in levels is not consistent across all the dtxs determined suggesting that certain dtxs may, as a percentage of the total dtxs present, be produced preferentially in still cultures. Repeated extraction and analysis of dxts from *L. longisporum* indicated that the production of dxts by this species is more variable compared to production by *M. anisopliae*, suggesting that other factors also play a role in the levels of the dtxs produced (data not shown). The methodology for the MS analysis we developed may allow the study of the various growth parameters (media conditions, number of sub-cultures, etc.) leading to more fully understanding all the factors that affect production of dtxs in the different fungal species.

CONCLUSIONS

The utilisation of two mass spectrometric techniques, MALDI-ToF-MS/MS (with PSD analysis) and nano-scale HPLC/ESI-MS/MS, were evaluated in order to determine the levels of the biologically important group of insecticidal cyclic peptides, the dtxs. The sensitivity and potential specificity of this optimised MS process was then used to determine if these compounds were present in a strain of fungus from which they had not been previously identified. MALDI-ToF-MS analysis can identify ions that are characteristic of some common dtxs; however, the analyses could not distinguish between isobaric dtxs and PSD analysis of these structures gave little information. Nano-scale HPLC/ESI-MS and data-dependent analysis (as described in the Experimental section) was also used for the characterisation and quantification of dtxs. Whilst in the case of the dtxs E and B (and their derivatives) the isomers could still not be distinguished by MS/MS and MS^n analysis (due to the lack of fragmentation of the isobaric side chain), the differing retention characteristics of the two isomers allowed their separation and thus provided their determination and comparative quantitation. The nano-scale HPLC/ESI-MS with data-dependent fragmentation analysis also allowed the metabolomic data produced to be 'mined' for dtx-specific data. This took advantage of the fact that the dtxs preferentially undergo ring opening at a specific point in the cyclic structure and this forms a linear peptide intermediate which then fragments in a specific fashion generating fragment ions that are characteristic for the dtxs. Whilst the loss of CO (a characteristic neutral loss) was rather non-specific for the dtxs, the losses of 99 and 113 Da were more useful in extracting dtx-specific information. The combination of the loss of 99 and 113 Da as a first screen and the second screen with loss of 28 Da allowed the removal of any false-positive peaks detected by this process. A final consideration is the time taken for each analysis; whilst the nano-HPLC/ESI-MS experiment produced more informative information, it required a lengthy HPLC separation in order to distinguish certain isobaric dtxs. In contrast, the MALDI-ToF-MS analysis gave less information regarding the nature and quantity of the isobaric dtxs and is thought to be realistically only semi-quantitative in nature. However, the analysis time for the MALDI analysis is markedly reduced (minutes for multiple samples). Therefore, MALDI-ToF-MS, for the purpose of an initial screening of a large number of samples for dtxs, does have an advantage and may be used as a 'first-pass' analysis in order to determine which samples of a large set requires further detailed analysis. HPLC/ESI-MS could then be used for the quantitation of dtxs. These techniques were utilised here in order to show, for the first time, that dtxs are produced by the genus *L. longisporum* and that the dtxs identified were produced at elevated levels under aerated conditions compared to the same species grown under still conditions. Previously, aeration has generally been shown to be important for optimal production of biomass and secondary metabolites,[23] although the role of aeration in the production of such metabolites remains ambiguous.[16] The optimised MS investigation of dtxs, outlined in this report, therefore affords the identification of dtxs from different fungal species, and permits the relationship between this class of organisms to be studied at the metabolomic level. It also permits the investigation of the produced toxins and their specific target organisms.

Acknowledgements

This work was supported by the European Commission, Quality of Life and Management of Living Resources Programme (QoL), Key Action 1 on Food, Nutrition and Health QLK1-2001-01391 (RAFBCA).

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