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Combination of a new sample preparation strategy with an accelerated high-performance liquid chromatography assay with photodiode array and mass spectrometric detection for the determination of destruxins from *Metarhizium anisopliae* culture broth

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Abstract

A method is presented allowing the qualitative and quantitative analysis of destruxins (dtxs) in fungal culture broth. Sample preparation was carried out by ultrafiltration over a commercially available acetylated cellulose (CTA) membrane with a M_r 10000 cut-off. The developed high-performance liquid chromatography assay with diode array detection (HPLC–DAD) cuts down the analysis time by 50% compared to most of the currently applied methods (retention times: dtx A = 8.3 min, dtx B = 8.9 min, dtx E = 7.5 min) and enables dtx detection down to sub-ppm range (limits of detection: dtx A = 0.19 mg/l, dtx B = 0.41 mg/l, dtx E = 0.10 mg/l). Stability of dtx E in filtrated culture broth was found to be much lower than anticipated (half-life time = 64.5 ± 1.7 h). Thus, the detoxification of this metabolite is an abiotic process. Coupling of the HPLC–DAD system to an ion trap mass spectrometer with an electrospray ionization source operating in the positive mode allowed identification of most dtxs encountered by utilizing multiple stage MS–MS experiments and retention time rules.
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Keywords: *Metarhizium anisopliae*; Destruxins; Ultrafiltration; CTA membranes; HPLC–DAD–MS–MS; ESI-MS; Ion-trap MS; Fragmentation

1. Introduction

Destruxins (dtxs) are a class of hexadepsipeptides produced by different soil fungi [1]. Most of the more than 35 characterized congeners have been described from *Metarhizium anisopliae*, an entomopathogenic anamorphic fungus utilized as biological pest control agent (BCA). Dtxs can be classified into main series, depending on the nature of the pentanoic acid side chain and into further sub-series according to differences in the amino acid substitution pattern (Fig. 1). Amongst these, dtxs A, B, and E are predominating in quantitative terms [2,3]. They are considered to be important pathogenicity determinants of some *Metarhizium* strains [4–6]. Furthermore, dtxs have also been reported to prevent the formation of osteoblasts [7], inhibit the growth of certain cancer cell lines [8,9], induce erythropoietin production [10], and effect the contraction (positive inotropic, negative chronotropic) of isolated rat heart tissue [11]. Their mode of action is still under investigation but dtxs are known to inhibit vacuolar ATPase activity [12,13], form trans-membrane ion-channels [14] and influence calcium flux dependent processes [15,16]. Factors influencing dtx production include the fungal species and strain, cultural conditions, certain mutagens and biomass [6,17,18]. The production, synthesis and bioactivity profile of dtxs has been reviewed by Pedras et al. [1] and Vey et al. [19]. For the identification and quantification of dtxs from fungal cultures or infested host organisms, analyti-

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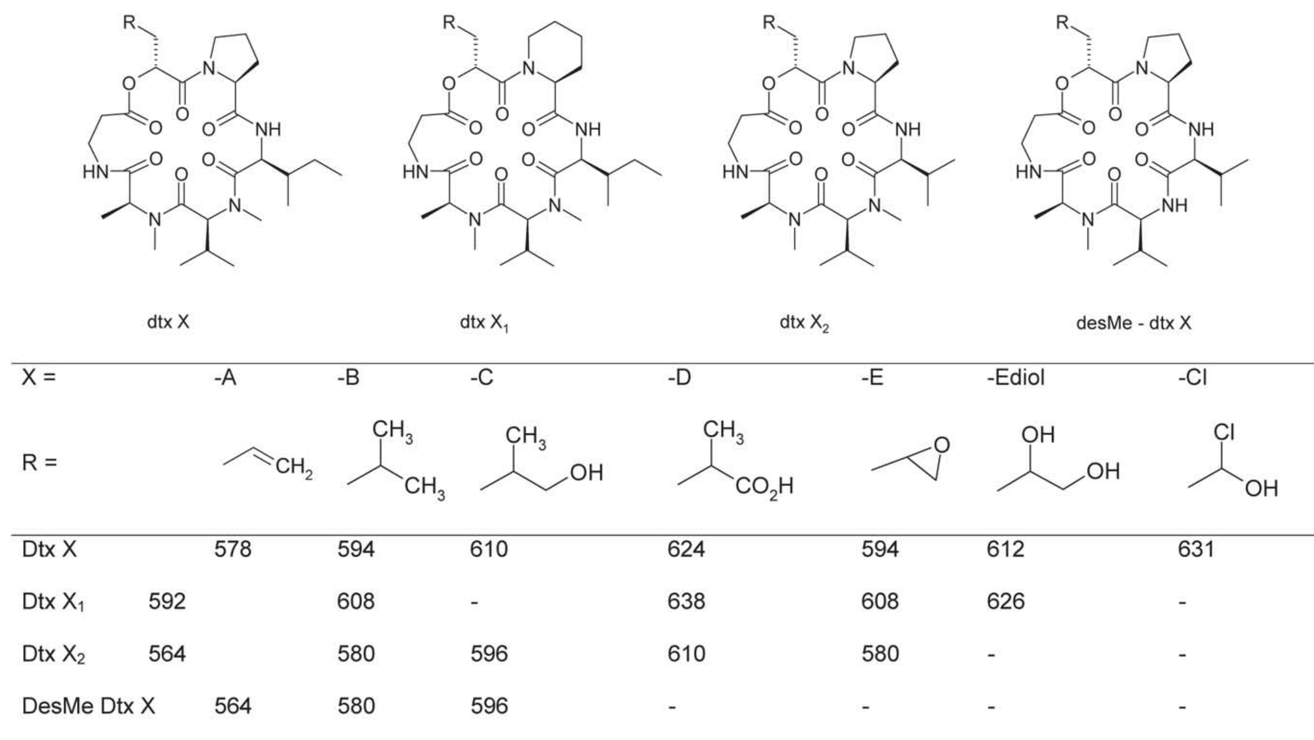


X =		-A	-B	-C	-D	-E	-Ediol	-Cl
R =		CH_2	CH_3 CH_3	CH_3 OH	CH_3 CO_2H	O	OH OH	Cl OH
Dtx X		578	594	610	624	594	612	631
Dtx X_1	592		608	-	638	608	626	-
Dtx X_2	564		580	596	610	580	-	-
DesMe Dtx X		564	580	596	-	-	-	-

Fig. 1. Formula schemes and molecular masses (MH^+) of major dtxs.

cal methods combine a sample preparation step with high-performance liquid chromatography (HPLC) using RP-18 stationary phases [2,3,5,20–27]. Sample preparation usually entails dichloromethane (or solvents of related polarity) extraction of culture broth or fungal biomass [2,3,17,22,24–27]. In some cases solid-phase extraction (SPE) protocols have been used [4,5,21]. Most of the chromatographic assays used binary water–acetonitrile mobile phase gradient systems with or without acidic modifiers [3,17,23,24,26,27]. Isocratic systems have also been used successfully [2,4,5,22,25]. Only a limited number of studies have used a hyphenation of HPLC with mass spectrometry to aid the identification of dtxs [22,26]. Most studies use UV or diode array detection (DAD) in combination with reference materials to identify and quantify specific dtxs (mostly dtxs A and B) [2,3,5,17,23,25,27]. The retention time for the most lipophilic major metabolite, dtx B, exceeded 20 min in the majority of presented assays. Exception is the isocratic approaches of Liu et al. [25] and Kershaw et al. [5]. Most studies fail to provide details of the validation parameters such as the limit of detection (LOD) and quantification (LOQ), the analyte recovery rate or precision and accuracy to allow for comparative analysis. Of the few exceptions, the most notable studies include those of Chen et al. [23], Hsiao and Ko [17], and Loutelier et al. [2]. Recently, a capillary electrophoresis assay was presented with a retention time of about 14 min for dtx B and without giving validation parameters [28]. Based on this knowledge and being confronted with the need to quantify dtxs in a great number of fungal culture broth samples, the aim of this study was (i) to establish a simpler sample workup procedure and (ii) an accelerated HPLC–DAD assay. The need to replace the usually used time consuming, cost intensive, and environmentally burdening (chlorinated solvents involved) technique of liquid–liquid extraction of the culture broth was addressed by using an ultrafiltration approach. Over the last two decades, this technique has been successfully applied in the fields of fermentation based bioproduct manufacture [29–31], environmental analysis [32–35], and biofluid analysis [36–39]. In all cases, the swift removal of cellular debris and macromolecular matrix constituents as proteins and polysaccharides was the crucial step in the sample workup scheme directed toward low-molecular-mass analytes. An additional intention of this study was to detect and identify minor dtx congeners by using a HPLC–DAD system coupled to an ion trap mass spectrometer equipped with an electrospray ionization (ESI) source (HPLC–DAD–MS–MS).

2. Experimental

2.1. Reagents

Reference standards were obtained either from Sigma (dtx A; Sigma Aldrich, Vienna, Austria) or were kindly provided by Dr. A. Vey (dtxs B and E; INRA, France). Acetonitrile, agar, methanol (both gradient grade), agar, Sabouraud 2% glucose (S2G) medium and Tween-80 were purchased from Merck (Darmstadt, Germany). Nitrogen (99.995%) for mass spectrometry was produced by a nitrogen generator (Peak

Scientific Instruments, Fountain Crescent, UK). Water for the HPLC was produced by reverse osmosis followed by distillation.

2.2. Cultivation of *M. anisopliae* and sample preparation

A single-spore isolate of *M. anisopliae* var. *anisopliae*, BIPESCO 5 (equals KVL 275 or V275, obtained from the Royal Veterinary and Agricultural University, Department of Ecology, Frederiksberg C, Denmark) was maintained in a culture chamber on S2G agar at 25 °C and 80% relative humidity. Conidia, harvested from 14–20-day-old cultures were suspended in sterile 0.1% (w/v) aqueous Tween 80, were used to inoculate 100 ml Erlenmeyer flasks containing 20 ml S2G liquid medium. The final concentration per flask was of 7.6×10^5 conidia/ml. Cultures were incubated at 25 °C on a gyratory shaker (250 rpm; 80% relative humidity) and four flasks were sampled each day over a period of 8 days. Before harvesting the culture broth, the liquid lost due to evaporation was estimated. The mycelium was pelleted by centrifugation (Sorvall R5, 7000 rpm, 15 min) and dried at 105 °C until the mass was constant. The supernatant was filtered using a cellulose acetate filter (0.22 μm, Sartorius, Göttingen, Germany) before determining the pH. The resulting culture filtrates were stored at −20 °C until needed. Final purification was achieved by centrifugation (Hermle Z383 centrifuge (Hermle Labortechnik, Wehingen, Germany), 2500 rpm, 45 min, room temperature) of 2 ml culture filtrate over an acetylated cellulose (CTA) M_r 10000 cutoff membrane (Vivaspin2, Sartorius, Göttingen, Germany). The obtained ultrafiltrate was transferred to HPLC vials and either analysed by HPLC–DAD or stored at −20 °C until required.

2.3. HPLC–DAD conditions

HPLC analyses were performed using a HP 1090 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a DAD system, an automatic injector, an auto sampler and a column oven. Separations were performed on a Zorbax SB-C_{18} column (150 mm × 4.6 mm), particle size 3.5 μm (Agilent Technologies) with a solvent gradient of water (A) and acetonitrile (B). A LiChroCART 4–4 (Merck, Darmstadt, Germany) column (4 mm × 4 mm) filled with LiChrospher 100 RP-18 (particle size 5 μm) material was used as guard column. Gradient: $t = 0$ min 95% A; $t = 6$ min 50% A; $t = 8$ min 2% A; $t = 12$ min 2% A. Between runs the column was equilibrated with 95% A for 8 min. The system was operated at a flow rate of 1 ml/min at room temperature (thermo-stated, 23 °C). The injection volume was 10 μl. Chromatograms were recorded at 210 nm (sample bandwidth 4 nm, reference wavelength 300 nm, reference bandwidth 60 nm). DAD spectra between 190 and 600 nm were stored for all peaks exceeding a threshold of 0.1 mAU. Optimization of these detection parameters was achieved out using an experimental array with detection wavelengths between 194 nm and 218 nm and different reference wavelengths conditions. Experiments were performed with a reference sample of dtx A in methanol (3.10 mg/l, peak height 10–20 mAU). The signal to noise ratio (S/N) was determined graphically by dividing the measured peak height with peak to peak noise measured over a 1-min interval.

2.4. HPLC–DAD–MS–MS conditions

HPLC–DAD–MS–MS experiments for the identification of dtxs were performed with the HPLC method described above. A HP1100 liquid chromatograph (Agilent Technologies) coupled to an Esquire 3000^{plus} ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) was used. Experiments were performed in positive ESI-mode with the following ionizer and transfer optics parameters: spray voltage 4500 V; nebulizer gas (N_2) flow set to 3.5 bar; capillary exit voltage 116.9 V; dry gas (N_2) flow 10 l/min with a temperature of 300 °C; skimmer voltage 40 V; octopole 1 dc 12 V; partition 6.8 V; octopole 2 dc 1.7 V; octopole RF amplitude 150 V peak-to-peak (p–p), lenses 1 and 2 (at the trap entrance) voltage −5 and −60 V, respectively. A spectral scan range 100–1000 *m/z* with a maximum accumulation time of 50 ms and an ion charge control (ICC) target setting of 20,000 were applied. Background helium gas was used as collision gas for the MS–MS experiments. A fragmentation voltage ramp from 0.3 to 2.0 V was chosen and the activation time was 40 ms.

2.5. Calibration functions and assay validation

Calibration functions were obtained for methanolic dilution series of reference standards of dtxs A, B, and E and for a dilution series of dtx A in S2G medium serving as a matrix model. All calibration levels were measured five times. Calibration levels exceeding the upper linear detector response limit were excluded from the calibration function as well as levels close to or below the limit of quantification determined by visual signal to noise estimation. Calibration functions were obtained by linear regression of the respective dtx concentration (c_A) against the peak area (A) resulting in the calibration function $A = kc_A + d$. LOD and LOQ values for the HPLC–DAD method were calculated for all dilution series from the the lowest four fortification levels. In all cases sample volumes were 1 ml. The LOD equals the mean value of the intercepts of the respective calibration curve (blank mean d_b) plus three times the standard deviation of the intercept (blank s_b) whereas the LOQ equals the mean value of the intercepts of the respective calibration curve (blank mean d_b) plus ten times the standard deviation of the intercept (blank s_b) [40]. The precision of the method was determined as the relative standard deviation (R.S.D.) of the calibration levels, the accuracy was determined as deviation of the measured mean from the fortification level expressed as percentage of the fortification level. The recovery rate from the *M. anisopliae* culture

broth was measured for dtx A at two different spiking levels. Fungal culture samples from day 4 were used to assess the recovery from developed fungal cultures. Younger cultures containing only traces of dtx A (day 1) were used to assess the recovery rate at trace levels. In both cases, culture broth samples were spiked with an amount of dtx A approximately equivalent to the amount already present in the sample by dilution of appropriate dtx A standard solutions in methanol. The reference samples used were diluted with an equal volume of methanol. The intra-day and inter-day repeatability of the method was measured on a developed *M. anisopliae* culture broth sample. Measurements were performed on three different days in two day intervals with five replications per day.

2.6. Dtx identification

Dtxs A, B, and E were identified using reference material. Further congeners were identified by their relative retention times and their MS–MS fragmentation pattern.

3. Results and discussion

3.1. Sample preparation

Improved separation of media or fungal-derived debris through combined use of centrifugation and filtration steps helped accelerate preparation of samples for HPLC–DAD analysis. The culture broth was worked up in three steps. After removal of the mycelium by centrifugation a crude filtration step over a 0.22 μm membrane allowed to remove most of fungal biomass. The final purification was carried out by ultrafiltration using a CTA membrane with a M_r 10000 cut-off. Two ml of culture filtrate were applied to the Vivaspin centrifugation device equipped with a vertical membrane. Under the centrifugation conditions used 0.4–1.2 ml ultrafiltrate were collectable even if fungal spores were still visibly present in the pre-cleaned filtrate. It was possible to process 32 samples in parallel, thus allowing the workup of 96 samples within a working day. Up to now several hundreds of culture filtrate samples have been processed and no failure of the ultrafiltration device had to be observed. The high recovery rate of dtx A from culture broth (see below) did prove that no analyte loss had to be encountered at the CTA membrane.

3.2. HPLC–DAD method development

Chromatographic method development was carried out with a representative *M. anisopliae* culture broth sample. Of all the RP-C_{18} columns available, the Zorbax SB-C_{18} was chosen due to its low particle size and proven ability to separate lipophilic analytes in complex biogenic matrices [41,42]. The gradient mobile phase developed facilitated an efficient separation of dtxs from the more polar constituents of the culture broth (Fig. 2). Dtxs were eluted in a retention time window of approximately 3 min and showed baseline separation in most cases – even for minor derivatives. Addition of acidic additives such as 0.1% acetic acid resulted in an increase of the dtx peak widths and was not further pursued as a mobile phase modifier option. Operating the separation system at elevated temperatures (33–45 °C) did not show any significant improvement of the separation and was also abandoned. Optimization of the detection parameters did result in a detection wavelength (WL) of 210 nm. At this WL, obtained S/N ratios are significantly better than for WLs < 210 nm (one-sided *t*-test $p < 0.05$) independently from reference wavelength and reference bandwidth chosen. These results are in contrast to results presented by Krska et al. [43] for the structurally similar depsipeptide beauvericin, where – in an isocratic assay – a wavelength optimum of 192 nm was obtained. Transferring the method from the HP1090 chromatograph to the HP1100 chromatograph for the LC–MS experiments did not alter the peak shape chromatographic resolution, but increased the retention times (e.g. dtx A from 8.4 to 10.0 min, dtx B from 8.9 to 10.6 min), presumably due to different post column dead volumes on the HP1100 system. Several Zorbax SB-C_{18} columns tested showed similar retention behaviour patterns except the heavily used columns (>5000 injections of complex plant matrices) which exhibited peak distortion effects (e.g. fronting) not visible with other applications. This effect may be due to column metal ion contamination leading to complexes forming with dtxs.

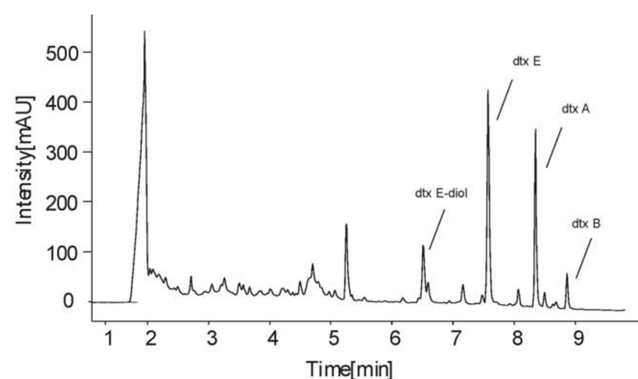


Fig. 2. Representative HPLC–DAD chromatogram of a *Metarhizium anisopliae* culture broth sample recorded at 210 nm. Dtx derivatives elute within a chromatographic window of less than 3 min. Dtx concentrations determined from this sample are 116 mg dtx A/l culture broth, 32 mg dtx B/l culture broth, and 209 mg dtx E/l culture broth.

3.3. HPLC–DAD method validation and dtx E stability

Assay validation was performed either with fully developed *M. anisopliae* culture broth samples (analyte recovery, intra-day and inter-day repeatability) or with dilution series of reference material in methanol or S2G medium. For reference standard dilution series of dtxs A, B, and E in methanol, linear calibration functions were obtainable over about 2.5 orders of magnitude. Due to the limited amount of dtxs B and

Table 1
Calibration function parameters slope (k), intercept (d), coefficient of determination (R^2), calculated LOD and LOQ for dtxs A, B, and E in methanol and dtx A in S2G medium

	Calibration range (mg dtx/l)	Y (peak area) = kc_A (mg dtx/l) + d		R^2	LOD (mg/l)	LOQ (mg/l)
		Slope (k)	Intercept (d)			
Dtx A[a]	1.2–445.5	9.46 ± 0.02	3.1 ± 3.6	0.9998	0.19	0.65
Dtx B[a]	0.5–113.3	7.62 ± 0.05	3.4 ± 1.1	0.9997	0.41	1.38
Dtx E[a]	0.4–310.0	10.64 ± 0.02	1.9 ± 2.7	0.9999	0.10	0.34
Dtx A[b]	0.6–14.9	11.37 ± 0.06	1.9 ± 0.4	0.9991	0.14	0.46

[a] Dtx dilution in methanol.
[b] Dtx dilution in S2G medium.

E reference material available, only a dtx A dilution series was performed in S2G medium. This series covers a reduced concentration range, but both accuracy and precision values of the highest fortification level indicated that the upper limit of linearity will be similar to the methanolic dilution series of dtx A. For all but the lowest fortification levels the assay precision was found to be <5.0% R.S.D. and the accuracy ranged between −10 and 7%. LODs of dtxs A, B, and E ranged between 0.1 and 0.4 mg dtx/l, depending on the chromophore of the respective metabolite. Deterioration of accuracy and precision of the lowest fortification levels confirmed the calculated LOQ values. In all cases, calibration levels close to the calculated LOQ show strongly reduced accuracy, the respective dtx content is underestimated by approximately 20%. The precision of the measurement is lowered, too; relative standard deviations exceeding 5% are common. The LOD of dtx A in methanolic solution and in S2G medium were comparable, no additional matrix effects were observed. Therefore, and backed by the similarity of calibration functions published by Loutelier et al. [2] the methanolic calibration functions were used for the quantification of dtxs A, B, and E in culture broth samples. Calibration function parameters, LOD and LOQ values are summarized in Table 1. The recovery of dtxs from fungal culture broth was addressed by spiking experiments using dtx A. Two fortification levels were chosen to (i) address the recovery of dtxs from fully developed culture broth samples and (ii) to prove the capability of the sample workup scheme to measure sub-ppm quantities of dtx A. Analyte recovery was determined as 92 ± 7 % (n = 3) at higher dtx A concentrations (46 mg dtx A/l culture broth) and 91 ± 20% (n = 5) in the range of the LOQ (0.5 mg dtx A/l culture broth). These value obtained for fully developed cultures is comparable with recovery levels reported by Loutelier et al. [2] who found 93 ± 2% (n = 4) recovery for dtx A by peak height comparison. The practical suitability of the assay was finally evaluated with samples from a cultivation experiments (four parallels) performed with the BIPESCO 5 strain of *M. anisopliae*. In this context, intra-day and inter-day repeatability of the HPLC–DAD method was assessed on a culture broth sample containing 76 mg dtx A/l, 34 mg dtx B/l, and 26 mg dtx E/l. Assay stability was addressed by monitoring the retention times of dtxs A, B, and E. Deviations were found to be lower than 0.1 min (<0.1% R.S.D.) in all cases. Hence, the assay was considered stable. Analyte stability was found to be sufficiently high for dtxs A and B (inter-day R.S.D. 0.6 and 2.4%, respectively) whereas the epoxy derivative dtx E proved to be instable. Dtx E degradation was paralleled by an increase of a more polar component, assignable as dtx E-diol by HPLC–DAD–MS–MS experiments (see below for details). Dtx E-diol is the hydrolysis product of dtx E. Assuming first order kinetics for the reaction of dtx E to dtx E-diol the rate constant for the degradation and the half-life time of dtx E can be determined as $k = 0.0107 \pm 0.0003\,\mathrm{h}^{-1}$ and $64.5 \pm 1.7\,\mathrm{h}^{-1}$, respectively. Even in ultrafiltrated culture broth samples stored 6 months at −20 °C dtx E, unlike dtx A or B, was degraded. A decrease in dtx E in older cultures paralleled by a late onset of dtx E-diol production has been already reported by several authors [2,3,6] but in all cases liquid–liquid extraction protocols have been used to retrieve the analytes from the culture broth. In these extracts (e.g. dichloromethane) as well as in methanolic solutions dtx E is stable. Until now, dtx E degradation in fungal culture broth has not been addressed directly without using extraction protocols. In contrast to the liquid–liquid extraction approaches the sample preparation by ultrafiltration does not change matrix properties as pH or ionic strength. The sample is still in the buffered environment created by fungal growth. This matrix lacks proteins and other macromolecules as well as fungal biomass. Therefore, it can be concluded, that dtx E degradation by diol formation is most likely an abiotic process and is neither promoted by the fungus itself, nor by the targeted host insect as result of enzymatic hydrolysis [2,44]. These results are backed by the dtx E degradation experiments performed by Dudley et al. [45] who investigated the long time stability of isolated dtx E and dtx E containing dichloromethane culture broth extract under different storage conditions. Up to 92% dtx E breakdown was observed with dtx E-diol as major reaction product. Consequently, it was concluded, that dtx E degradation is a non-enzymatic process. However, hydrolysis in the culture filtrate mirrors the reactivity of the epoxide group towards nucleophilic attack and helps to explain the reported biological activities of this particular dtx [15,46,47]. Dtx E-diol formation also may be the first and crucial step in the detoxification cascade of dtx E, since the side-chain hydroxylation seems to be – besides linearization of the depsipeptide core – a possible

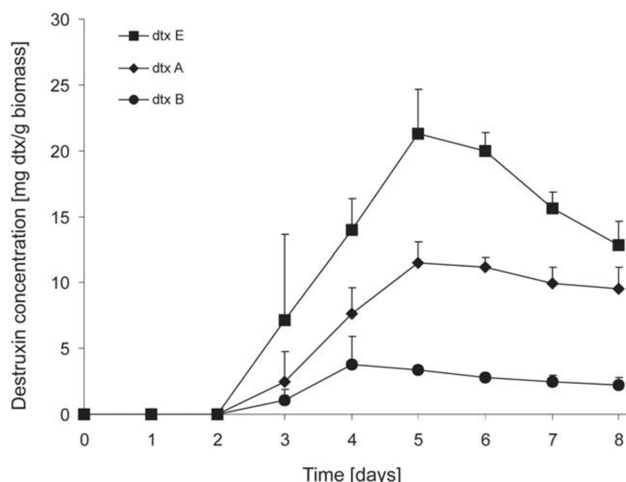


Fig. 3. Dtx secretion in a submerged *M. anisopliae* culture. Time course of averaged dtxs A, B, and E concentrations referred to the fungal biomass. Error bars represent the standard deviation ($n = 4$).

key step both in plant and insect dtx detoxification processes [48–51].

3.4. Dtx quantification from culture broth

The time course of dtx production observed for the BIPESCO 5 strain (Fig. 3) is similar to that reported for other strains of *M. anisopliae* [2,3,5,47], which show peaking in 7 day-old cultures. In this study, a maximum of 289 ± 63 mg/l dtx E, 161 ± 35 mg/l dtx A, and 40 ± 7 mg/l dtx B ($n = 4$) was achieved. This is similar in range to that reported by Loutelier et al. for a different strain of *M. anisopliae* (Ma23) grown in Czapek Dox medium [2], but approximately 5- to 10-folds higher than the levels reported by Wang et al. [3], using the same strain under different culture conditions (Czapek Dox medium, 100 ml cultures in 250 ml flasks).

3.5. Identification of dtxs with HPLC–DAD–MS–MS

Besides dtxs A, B, and E which were identified using reference material, thirteen more derivatives were tentatively identified by HPLC–DAD–MS–MS experiments in combination with retention time information (Fig. 4). The overall retention times of the basic dtx series are governed by the variable in the pentanoic acid side chain resulting in the elution order D < C < E < A < B. This pattern is superimposed by the relative retention within a series depending on the nature of the amino acids 2 and 3. Dtxs of the dtxX (Pro^2–Ile^3) sub-series are proceeded by the dtxX_2 sub-series (Pro^2–Val^3) lacking one methylene group equivalent and are followed by the dtxX_1 sub-series (Pip^2–Ile^3) bearing an additional –CH_2– element [22]. Linearization of dtxs occurs mostly between amino acid 4 (Val) and 5 (MeAla), the primary cation is preferentially formed at the methylated amide of amino acid 5. It is followed by a series of fragmentation reactions including oxazolon ring formations and rearrangement processes as described in detail by Jegorov et al. [26] (Fig. 5B). The degradation process leads to the consecutive loss of amino acids 4 and 3. In HPLC–DAD–MS–MS experiments starting at the respective parent ion [MH^+], the loss of these fragments can be observed in most cases (Table 2). Combining the above described retention time rules with the observed breakdown pattern even allowed to de-replicate isobaric groups of analytes. In the case of dtx E_2, desMe-dtx B, dihydro-dtx A, and dtx B_2 (all $MH^+ = 580$ *m/z*), with the latter three eluting within one peak group between dtxs A and B (Fig. 5) the differentiation between dtxs E_2 and B_2, showing identical breakdown patterns due to identical amino acid sequences in positions 3 (Val) and 4 (Val), is aided by the retention time rules explained above, which also allows to discriminate dtxs E_1 and B_1 ($MH^+ = 608$ *m/z*) in a similar manner. Differentiation of the remaining three isobaric metabolites desMe-dtx B, dihydro-dtx A, and dtx B_2 is facilitated by the sequential

Table 2
HPLC–MS–MS fragmentation of dtxs recorded on an ESI-ion trap

	t_R	Parent ion MH^+	Ion type (*m/z* (rel. int. %))				
			$[MH–H_2O]^+$	$[MH–CO]^+$ $^{4–5}b_5$	$[MH–AA4]^+$ $^{4–5}a_5$	$[MH–AA4–CO]^+$ $^{4–5}b_4$	$[MH–AA4–AS3]^+$ $^{4–5}a_4$
Dtx A	10.0	578	–	550 (<10)	465 (100)	437 (58)	352 (<10)
Dtx B	10.6	594	–	566 (<10)	481 (100)	453 (71)	368 (<10)
Dtx C	8.9	610	–	582 (19)	497 (100)	469 (73)	384 (34)
Dtx D	8.2	624	–	596 (21)	511 (100)	483 (82)	–
Dtx E	8.9	594	–	566 (23)	481 (54)	453 (100)	368 (14)
Dtx E-diol	7.5	612	594 (100)	–	499 (64)	471 (34)	–
Dtx Cl	9.1	631	612 (56)	602 (<10)	517 (100)	489 (48)	–
Dtx B_1	10.9	608	–	–	495 (100)	467 (72)	–
Dtx A_2	9.8	564	–	536 (100)	451 (33)	423 (23)	–
Dtx B_2	10.4	580	–	552 (100)	467 (27)	439 (35)	368 (39)
Dtx C_2	8.5	596	578 (97)	–	483 (44)	465 (100)	–
Dtx D_2	8.7	610	592 (88)	–	497 (100)	469 (83)	–
Dtx E_2	8.4	580		552 (24)	467 (72)	439 (100)	368 (< 10)
Dihydro-dtx A	10.3	580		552 (<10)	467 (100)	439 (70)	354 (<10)
desMe-dtx A	9.4	564		536 (100)	465 (94)	437 (41)	352 (<10)
desMe-dtx B	10.2	580		552 (76)	481 (83)	453 (100)	368 (11)

Retention times (t_R), parent ions (MH^+) and most prominent observed fragment ions (MH^+) are given.

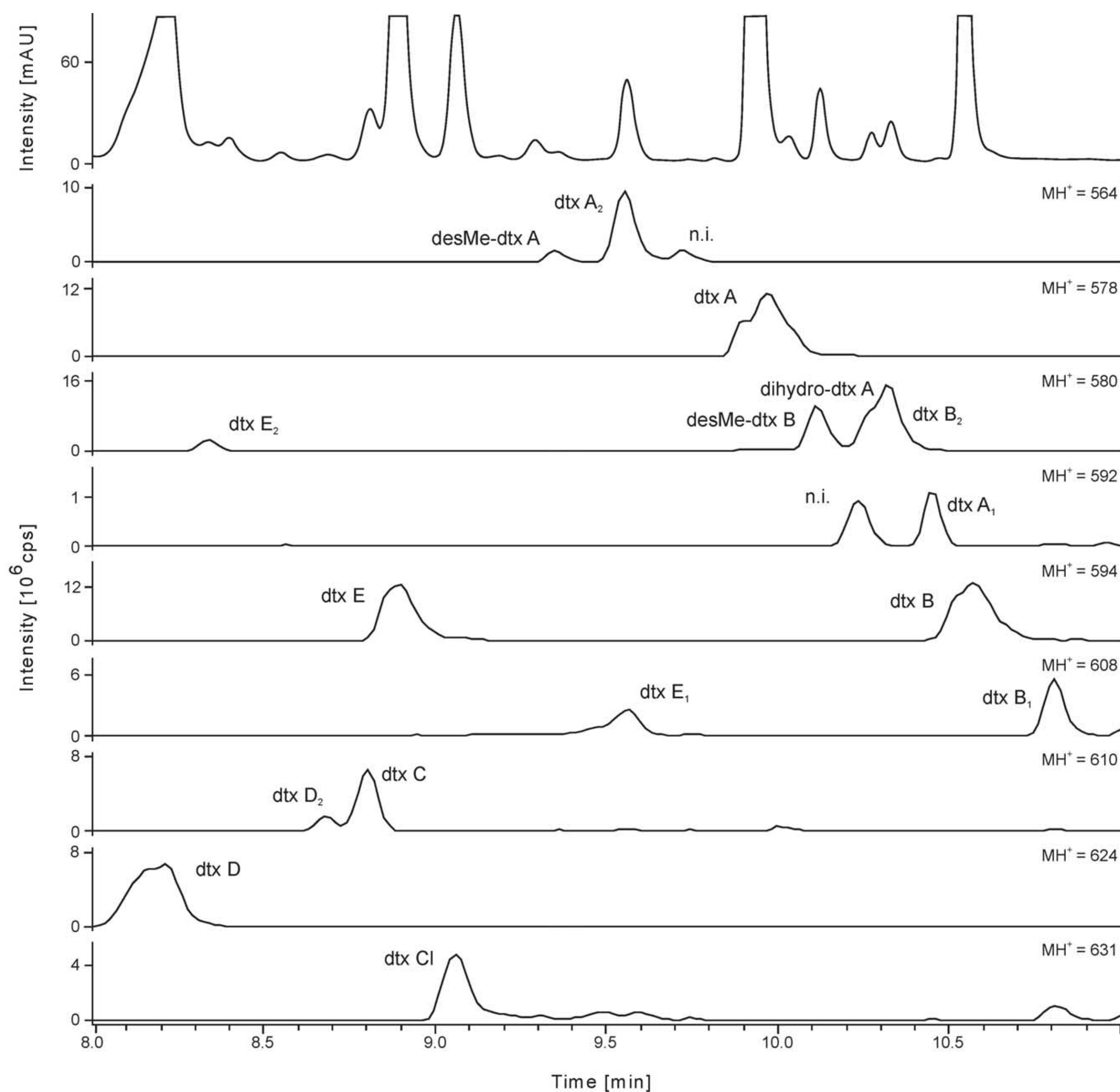
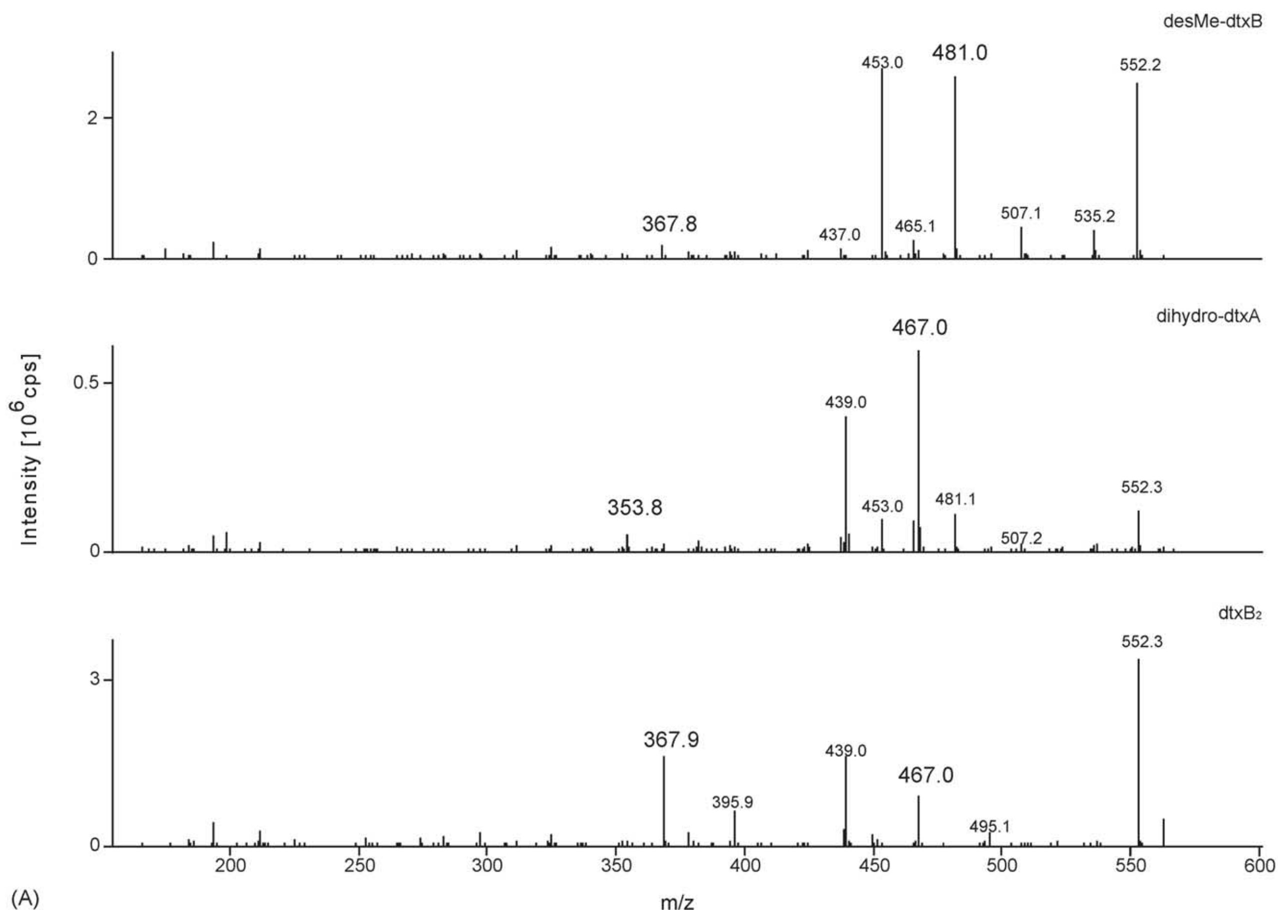


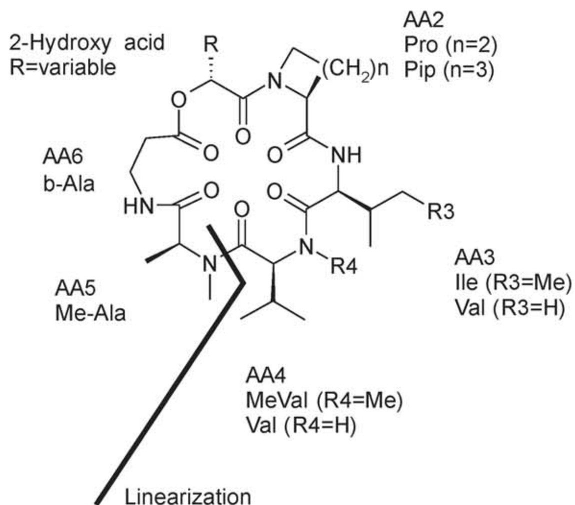
Fig. 4. HPLC–DAD–MS analysis of a *Metarhizium anisopliae* culture broth sample. HPLC–DAD chromatogram recorded at 210 nm (top) and HPLC–DAD–MS selective ion traces (ESI, positive mode) of the isobaric derivative groups. Identified dtxs are named and unidentified ones are denoted n.i. (not identified).

arrangement and nature of the amino acids 3 and 4 of these derivatives. Observed MS–MS spectra for these constituents, breakdown rules, and theoretical breakdown pattern is summarised in Fig. 5. The dtx E chlorohydrine derivate dtx Cl, discussed by Dudley et al. [44] as degradation product arising from dtx E was present in the ultrafiltrate solution which has neither been in contact with any chlorinated extraction solvent. This supports previous contributions describing dtx Cl as biogenic entity [52]. However, it can not be excluded, that this derivative is – as dtx E diol – an inorganic dtx E degradation product already formed in the fungal culture broth.

3.6. Conclusive remarks

The methods described in this study facilitate an accelerated qualitative and quantitative analysis of dtxs in fungal culture broth. High throughput of samples is achieved by avoiding time consuming and cost intensive extraction schemes for sample preparation. The simplified sample workup procedure utilizing ultrafiltration technologies removes macromolecular components but retains polarity, pH and ion strength of the matrix. The HPLC–DAD assay reduces analysis time by 50% compared to current methods and allows dtx detection in the sub-ppm range. The stability of one of the most promi-





	desm-dtx B	dihydro-dtx A	dtx B_2
linear [dtx-H]$^+$	580	580	580
↓ [dtx-H] $^+$-CO	552	552	552
↓ [dtx-H] $^+$-AA4	481 (-Val)	467 (-MeVal)	467 (-MeVal)
↓ [dtx-H] $^+$-AA4-CO	453	439	439
↓ [dtx-H] $^+$-AA4-AA3	368 (-Ile)	354 (-Ile)	368 (-Val)
↓ Residual Fragment			

(C)

Fig. 5. Identification of isobaric dtxs from a *Metarhizium anisopliae* culture broth sample. (A) Online HPLC–DAD–MS–MS spectra for the isobaric ($MH^+ = 580$) constituents desMe-dtx B, dihydro-dtx A, and dtx B_2 eluting between $t_R = 9.9$ min and $t_R = 10.4$ min. Discriminating ions are given in bold letters. (B) General dtx breakdown pathway scheme according to Jegorov et al. [26]. (C) Corresponding fragment (MH^+) ions of desMe-dtx B, dihydro-dtx A, and dtx B_2.

nent dtx congeners, dtx E, was lower than expected due to its degradation which can be considered an abiotic process. HPLC–DAD–MS–MS derived breakdown pattern combined with empirical retention time rules allowed identification of a wide range of dtx derivatives.

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