A rapid method for the isolation and analysis of destruxins from *Metarhizium anisopliae* culture broth

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INTRODUCTION

Destruxins (dtxs) are a substance class mainly produced by the enthomopathogenic fungus *Metarhizium anisopliae* (Pedras et al. 2002). Currently about 35 derivatives of these cyclic hexadepsipeptides are known. They belong to different sub-series, differing in the nature of the D-α-hydroxyacid HA (dtx A-F), and the amino acids 2 (subscript 1), 3 (subscript 2), and 4 (desmethyl series).

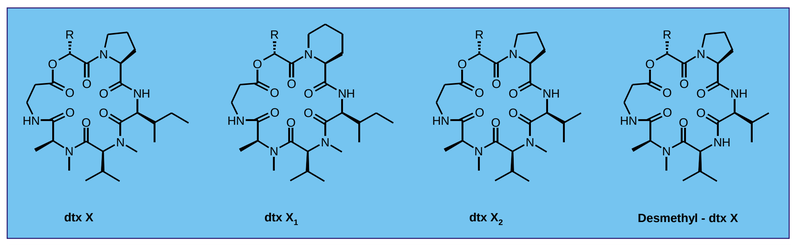
Destruxins are highly potent insecticides and it has been proven unequivocally that their presence is correlated with fungal virulence (Kershaw et al. 1999). They are, like most fungal depsipeptides, forming ion channels across membranes (Hinaje et al. 2002). Their ability to inhibit the bone-resorbing activities of osteoclasts has been shown recently (Nakagawa et al. 2003).

Photographs by H. Strasser, M. Kirchmair (ELMI), and M. Traugott (top left)

RESULTS

- A new sensitive (LODs <0.5 ppm), robust (reprod. <2.5% RSD), and fast (t_R dtxB = 9 min) method for the detection and quantification of destruxins has been developed.
- The sample preparation was simplified, an ultrafiltration step replaces more time consuming procedures.
- Identification of a broad variety of destruxin derivatives was achieved by MS/MS experiments using a HPLC-ESI-iontrap coupling.
- The developed method was successfully applied to the quantification of destruxins from fungal culture broth.
- The *in situ* instability of one of the most bioactive derivatives, destruxin E, was monitored. A half-life time of 64 hours was determined.

STRUCTURES and MASSES



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

Table of the most common destruxin derivatives and their MH^+ molecular ions.

HPLC-DAD METHOD DEVELOPMENT AND VALIDATION

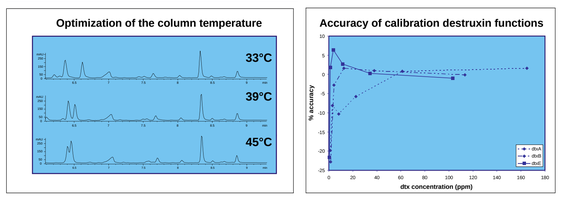
VALIDATION DATA

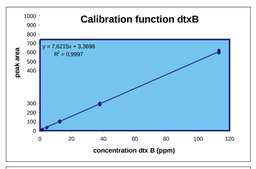
	dtxA	dtxB	dtxE
LOD (ppm):	0.19	0.41	0.10
LOQ (ppm):	0.65	1.38	0.34
Repeatability			
intraday (% RSD):	<0.4	<2.3	<3.2
interday (% RSD):	<0.6	<2.4	n.d.

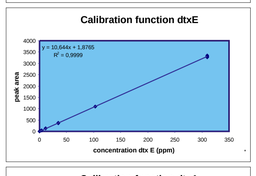
HPLC-DAD METHOD

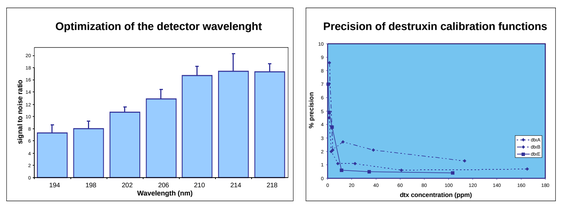
Instrumentation: Agilent HP1090 liquid chromatograph.
Stationary phase: Zorbax SB-C18 (4.6 mm x 150 mm), particle size 3.5 µm.
Mobile phase: H_3O(A)-ACN(B) at 1 ml/min at 23°C. Elution profile: t= 0 min 5% B, t = 6 min 50 % B, t= 8 min 98 % B, t = 12 min 98 % B; 8 min post time.
Detection: UV-DAD 210 nm. Injection volume: 10 µl.

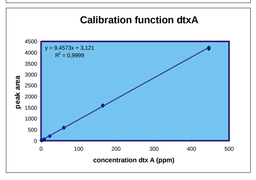
AcOH (pH = 2) lowers the resolution.
Temperature has no influence.
90.7±20.4 % dtxA recovery at LOD.
92.1±6.6 % dtxA recovery at 46 ppm.











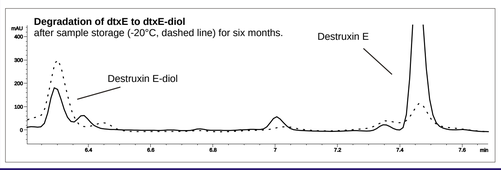
METHOD APPLICATION TO CULTURE BROTH

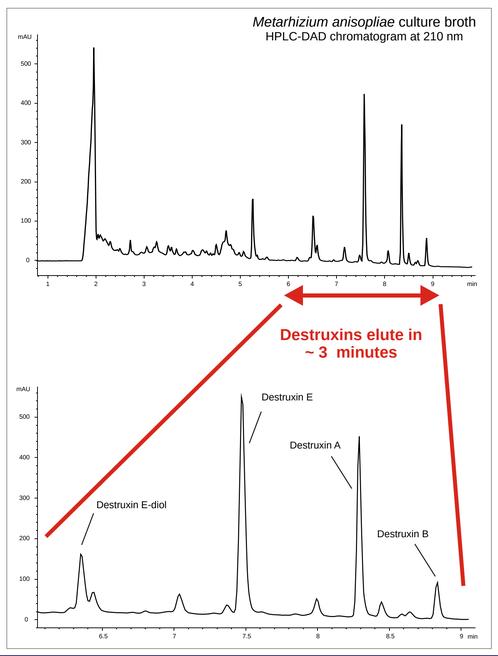
CULTIVATION CONDITIONS AND SAMPLE PREPARATION

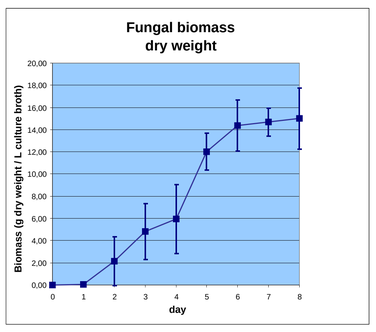
Metarhizium anisopliae strain BIPESCO5 (KVL275) cultures were grown on S2G medium petri dishes until sporulation. Spores were sampled with a 0.1% Tween-80 solution with a germination activity of 98.6 ± 2.5 %. 100 mL Erlenmeyer flasks (four parallels, 20 ml S2G medium) were incubated with 7.6 ± 0.4 x 10^5 spores per culture and incubated on a rotary shaker (200 rpm) at 25 °C and 80 % relative humidity. Samples were drawn in daily intervals. Culture medium (culture supernatant) and mycelium were separated by filtration over a 0.22 µm cellulose acetate filter. The biomass content was determined and the pH of the supernatant was measured. The culture filtrates were stored at -20°C until further workup. Two mL of stored culture filtrates were finally purified by centrifugation over a 10kDa membrane (Vivaspin2, CTA membrane, Sartorius, Göttingen, Germany) and used for HPLC analysis without further dilution. The recovery rate of the filtration step was found to be 93.4±2.0 % for dtxA at 3ppm. All HPLC solutions were stored at -20°C.

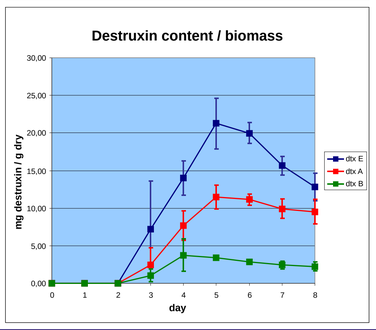
DESTRUXIN E DEGRADATION

The epoxide derivative destruxin E degrades readily to destruxin E-diol in the culture filtrate. At room temperature a half-life time of 64±2 hours was determined. Even under storage conditions (-20°C) the degradation of this derivative is remarkably.



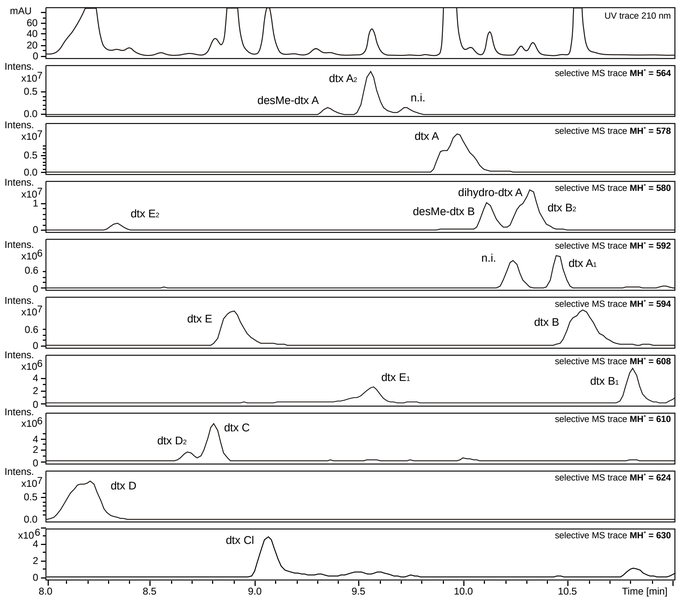






DERIVATIVE IDENTIFICATION WITH HPLC-MS/MS

IDENTIFIED DESTRUXINS



HPLC-DAD/MS METHOD

Instrumentation:
Agilent HP1100 liquid chromatograph.
Bruker Esquire3000^{plus} ion trap MS.
Chromatography identical HPLC-DAD.
Retention time shift ~1.6 min.
Mass spectrometry parameters:
Ion source: ESI, positive mode
Spray voltage: 4500 V
Nebulizer gas: N_2, 40 psi
Dry gas: N_2, 10 l/min 350°C
Scan range: 100-1000 *m/z*

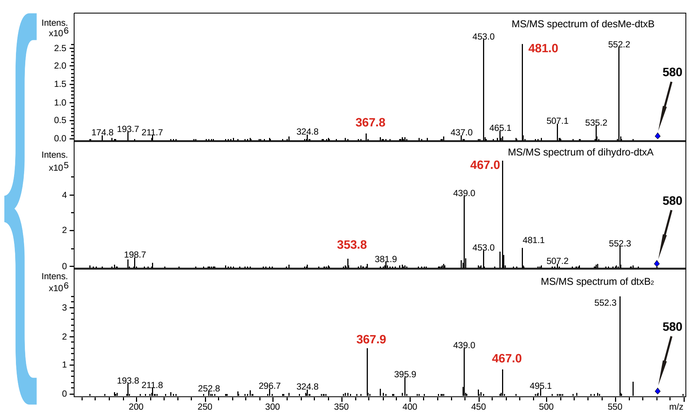
RETENTION RULES

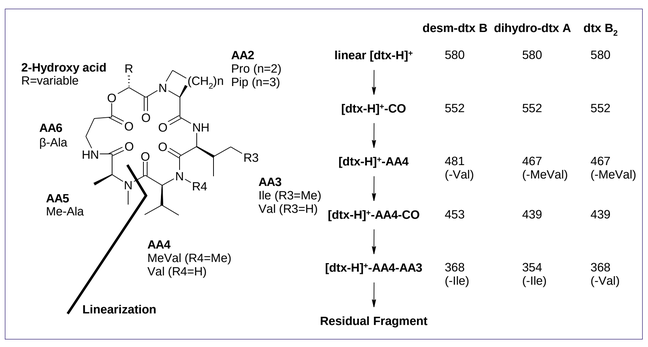
Series	dtx_2	dtx	dtx_1
E	8.3	8.6	9.6
A	9.5	9.9	10.4
B	10.3	10.5	10.8

AS2	Pro	Pro	Pip
AS3	Val	Ile	Ile
+ CH_2	0	+1	+2

The relative retention within a destruxin series (e.g. A, B, E) depends on the nature of AA 2 and 3.

DECONVOLUTION OF ISOBARIC GROUPS





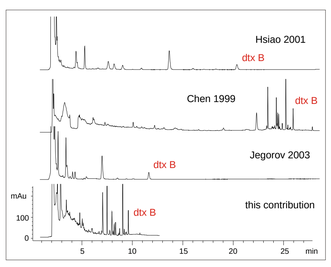
	desm-dtx B	dihydro-dtx A	dtx B_2
linear $[dtx-H]^+$	580	580	580
$[dtx-H]^+$-CO	552	552	552
$[dtx-H]^+$-AA4	481 (-Val)	467 (-MeVal)	467 (-MeVal)
$[dtx-H]^+$-AA4-CO	453	439	439
$[dtx-H]^+$-AA4-AA3	368 (-Ile)	354 (-Ile)	368 (-Val)
Residual Fragment			

Primary ionization occurs typically between AA5 and AA4. The linerarized amino acid chain is degraded by a series of fragmentation reactions (Jegorov 2003 and 1998). The loss of AA4 and AA3 is detectable in a single MS/MS experiment starting with the protonated linerarized destruxin derivative. Differences in the amino acid sequence allow the assignment of isobaric derivatives with similar retention behaviour.

COMPARISON WITH OTHER METHODS

	extraction	HPLC method	t_R dtx B	LOD
this contribution	none	gradient / C-18	9 min	<0.5 ppm
Jegorov 2003	liquid / liquid	gradient / C-18	24 min	-
Hsiao 2001	liquid / liquid	gradient / C-18	24 min	>50 ppm
Kershaw 1999	SPE	isocratic / C-18	10 min	-
Chen 1999	not given	gradient / C-18	18 min	>50 ppm
Jegorov 1998	liquid / liquid	isocratic / C-18	-	-
Loutelier 1996	liquid / liquid	isocratic / C-18	37 min	>3 ppm

All literature methods use time consuming sample preperation procedures, mostly dichloromethan extractions. In most cases, retention times for dtxB are at least twice the retention time achieved by the presented method. All methods utilized RP-C18 stationary phases and ACN/H_2O mixtures as moblie phase. For method comparison literature methods were adapted to the stationary phase used in this contribution.



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