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Liquid chromatography–electrospray time-of-flight mass spectrometry for on-line accurate mass determination and identification of cyclodepsipeptides in a crude extract of the fungus *Metarrhizium anisopliae*

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Abstract

Electrospray ionisation time-of-flight mass spectrometry (ESI-TOF-MS) has been used for the detection and identification of destruxins (cyclodepsipeptides) in a crude extract of the fungus *Metarrhizium anisopliae*. HPLC–MS analyses were performed with a post-column addition of erythromycin as a reference compound (lock mass procedure). Seven destruxin derivatives could be identified on-line from their accurate masses (deviation from calculated values <5.5 ppm) through elemental composition calculations. As a highly sensitive and accurate method, ESI-TOF-MS proved to be very powerful for the analysis and dereplication of natural products in complex mixtures.

Keywords: *Metarrhizium anisopliae*; Cyclodepsipeptides; Peptides; Destruxins; Time-of-flight mass spectrometry

1. Introduction

Natural products represent an untapped reservoir of novel pharmacophors or scaffolds for combinatorial chemistry. However, to cope with the constraints of modern drug research and to keep natural products competitive in a high throughput screening environment, rapid identification and effective dereplication procedures are required. During the last decade, HPLC–MS has become the method of choice for the identification of natural products in complex mixtures [1–3]. Most of the work has been performed on quadrupole instruments giving information on nominal mass and fragmentation patterns. The coupling of time-of-flight mass spectrometry (TOF-MS) with electrospray ionization (ESI) [4,5] has been achieved only recently. This method combines high accuracy and sensitivity due to the high frequency sampling of all ions simultaneously across the full mass range. Up until now it has been mostly applied to protein and peptide investigations [6,7] and has only scarcely been used for the detection of low-molecular compounds. To our knowledge no report has been published about its application in the field of natural product research.

We report here on the on-line accurate mass determination and identification of destruxin deriva-

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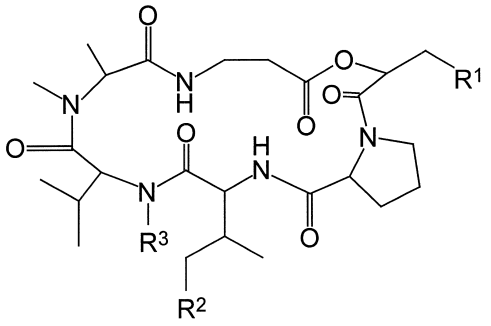

	R^1	R^2	R^3
Destruxin A	$CH=CH_2$	CH_3	CH_3
Dihydrodestruxin A	CH_2CH_3	CH_3	CH_3
Destruxin B	$CH(CH_3)_2$	CH_3	CH_3
N-Demethyldestruxin B	$CH(CH_3)_2$	CH_3	H
Destruxin B2	$CH(CH_3)_2$	H	CH_3
Destruxin D	$CH(CH_3)COOH$	CH_3	CH_3
Destruxin E	$CHOCH_2$	CH_3	CH_3
Destruxin E diol	$CHOHCH_2OH$	CH_3	CH_3
Destruxin E chlorohydrin	$CHOCH_2Cl$	CH_3	CH_3

Fig. 1. Structures of destruxins.

tives in a crude extract of the entomopathogenic fungus *Metarrhizium anisopliae* using ESI-TOF-MS.

Destruxins are cyclodepsipeptides known to possess various biological properties, in particular insecticidal and fungicidal activities [8–11]. The structure of the destruxins is shown in Fig. 1. Up until now, fast atom bombardment has been the most commonly used ionisation method for the detection and investigation of destruxins [12–14]. Recently, destruxins have also been analysed by HPLC–MS using an atmospheric pressure chemical ionization interface and collisionally induced dissociation [15].

2. Experimental

2.1. ESI-MS

Experiments were performed on a Micromass LCT instrument equipped with a Z-spray atmospheric pressure ionization source for ESI (Micromass, Manchester, UK). Source temperature: 100°C; desolvation temperature: 150°C; capillary: 3500 V; sample cone: 35 V; extraction cone 8 V. Spectra were recorded in the positive ion mode over the range m/z 250–900 with a 1.0 s integration time per spectrum. The MS was controlled and data were acquired with MassLynx 3.2 software. Accurate calibration of the instrument was performed with a solution of polyethylene glycol (PEG) 600 (1 nl/ml) and PEG 300 (1 nl/ml) in 2 m*M* aq. NH_4OAc–MeCN (1:1).

2.2. Liquid chromatography

The HPLC system consisted of a Waters 2690 Alliance chromatograph coupled with a Waters 996 photodiode array detector (Waters, Milford, MA, USA). Analyses were performed on a Symmetry C_{18} column (5 μm, 150×3.9 mm I.D.) (Waters) with a linear MeCN–0.01% aq. AcOH gradient of 5:95–90:10 (v/v) in 28.6 min; flow-rate was 1 ml/min. No precolumn was used. The oven temperature was

35°C, and UV data were recorded in the range 240–500 nm. The eluent was split at a 1:4 ratio after the diode array detector and before the mass spectrometer. A solution of erythromycin (Sigma, M_r 734.4690; 10 μg/ml in MeOH–water, 1:1) was added post column, beyond the splitting, at a flow-rate of 0.4 ml/h, with a syringe pump 11 (Harvard Apparatus, Holliston, MA, USA). Samples to be analyzed were dissolved in MeOH–water at a concentration of ca. 10 μg/ml, centrifuged before injection and then 15 μl were injected.

2.3. Destruxins

Metarrhizium anisopliae, isolate BI-0747 was isolated from a rain forest soil sample collected in Kalimatan (Indonesia). The strain was cultured in 18 baffled 500-ml erlenmeyer flasks each containing 100 ml of a medium consisting of 2% maltose, 0.4% potato starch and 0.1% soybean peptone (pH 5.5). XAD 16 (final concentration was 1%, m/v) was added after 2 days. The fermentation was carried out at 23°C for 7 days. The mycelium was collected together with the XAD 16 resin by filtration and soaked with Me_2CO (115 ml). After 1 h, 300 ml of MeOH was added and the mixture was stirred at 4°C overnight. The mycelium and the resin were filtered off and the extract was evaporated to dryness to provide 3.03 g. The extract was solubilised in MeOH–water (10 ml) and insoluble material removed by centrifugation. The solution was then chromatographied on a Sephadex LH-20 column (70×4 cm) with MeOH (0.4 ml/min) to yield an enriched mixture of destruxins. Destruxins B and E were isolated from the mixture by preparative HPLC (Waters PrepLC, Nova-Pak Radial-Pak C_{18} column (6 μm, 100×25 mm I.D.) with MeCN–water (5:95–90:10) in 28.6 min (28 ml/min); detection 210 nm; compound elution: destruxin B: 19.0–19.5 min, destruxin E: 13.5–14.0 min. The final purification of both compounds was achieved by HPLC on a SymmetryPrep C_{18} column (7 μm, 150 mm×7.8 mm I.D.) with MeCN–water (20:80–35:65) (4 ml/min) in 30 min (destruxin E, elution 17.0–18.5 min) and MeCN–water (5:95–90:10) (4 ml/min) in 28.6 min (destruxin B, elution 17.5–18.0 min), respectively.

The identity of destruxin A was confirmed by comparison with a commercial sample (Sigma). The identities of destruxins B and E were definitively established from their ^1H and ^{13}C NMR data [8]. Signal assignments were supported by ^1H–^1H COSY (correlated spectroscopy), ROESY (rotating frame Overhauser spectroscopy) HSQC (heteronuclear single-quantum correlation) and HMBC (heteronuclear multiple-bond correlation) two-dimensional NMR spectra.

3. Results and discussion

3.1. HPLC–MS analysis with accurate mass determination

Determination of accurate masses requires an internal reference to compensate drifts occurring over the course of instrument operation. Post-column addition of PEG gives very accurate results over a broad mass range. However, spectra are complicated by the numerous peaks, which can obscure the compounds to be analyzed or lead to isobaric interferences. Recent software implementations (calibration curve of polynomial order 5; dead time correction) enable accurate mass determination using a single point mass correction (lock mass procedure). Provided there is no isobaric interference, the deviations from calculated values are below 10 ppm and in the majority of cases even below 5 ppm.

HPLC–MS analysis of the crude MeOH–Me_2CO extract of the mycelium of *Metarrhizium anisopliae* was performed on a Symmetry C_{18} column with a linear gradient of MeCN–aq. 0.01% AcOH at a flow-rate of 1 ml/min. The mobile phase was split at a ratio of 1/4 before the mass spectrometer. Erythromycin, giving a strong $[M+H]^+$ pseudomolecular ion at m/z 734.4690, was used for mass correction and added as a 10 μg/ml methanolic solution post column after the splitting. Accurate mass calibration of the instrument was previously performed with PEG. The HPLC–UV–MS chromatogram is presented in Fig. 2. In the 250–900 u total ion current chromatogram, peaks are obscured by the continuous background flow of erythromycin. Destruxins can be detected in the 250–700 u chromatogram, as well as in the chromatogram obtained by selecting masses corresponding to pseudomolecular $[M+H]^+$ ions of

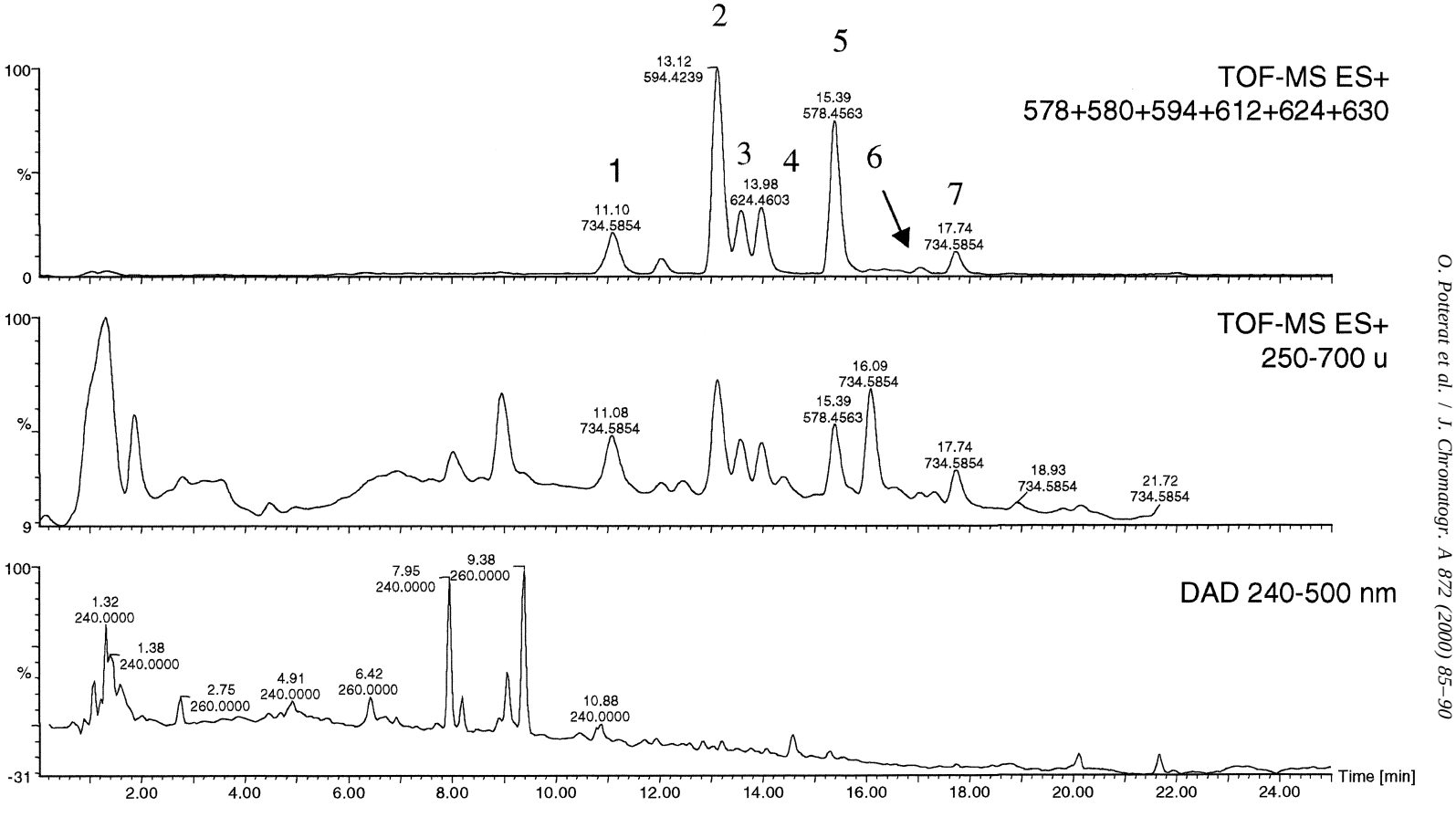

Fig. 2. HPLC–UV–MS analysis of the crude extract of *Metarrhizium anisopliae*. (A) Reconstructed ESI-TOF-MS trace obtained by combining masses corresponding to destruxin pseudomolecular $[M+H]^+$ ions; (B) ESI-TOF-MS chromatogram (250–700 u); (C) diode array chromatogram (240–500 nm). Analysis was performed on a Symmetry C_{18} column (5 μm, 150×3.9 mm I.D.) with a linear MeCN–0.01% aq. AcOH gradient of 5:95–90:0 (v/v) in 28.6 min; flow-rate 1 ml/min. The sample was dissolved in MeOH–water at a concentration of ca. 10 mg/ml and then 15 μl were injected (see Section 2).

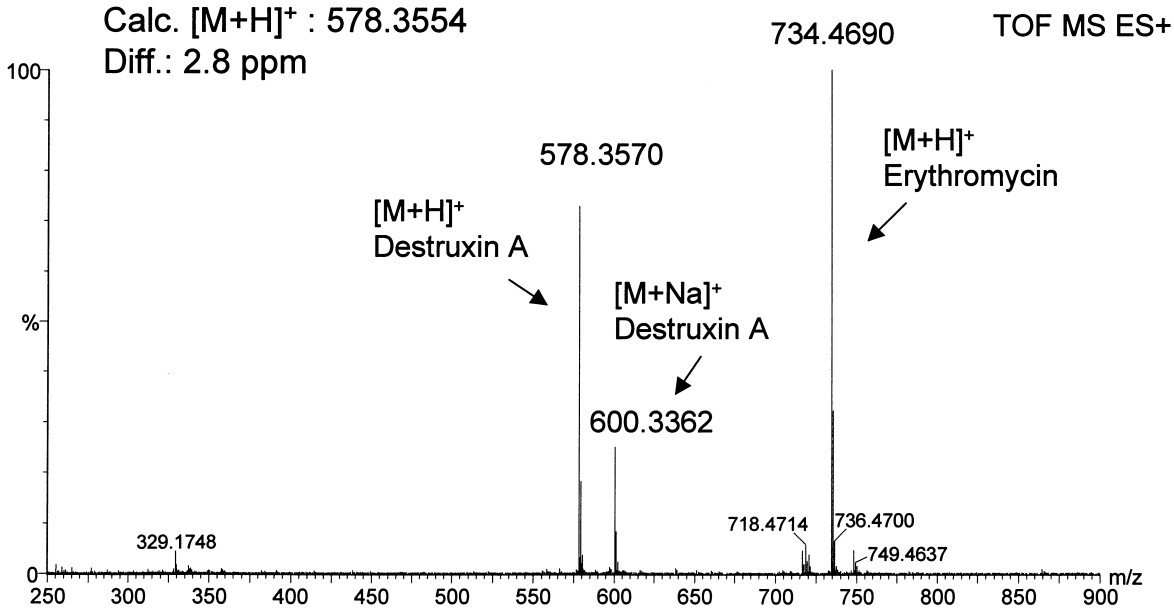

Fig. 3. HPLC–ESI-TOF-MS of destruxin A (lock mass corrected with erythromycin).

destruxins. In the mass spectra of destruxins, intense $[M+H]^+$ and weaker $[M+Na]^+$ pseudomolecular ions were detected. The ES mass spectrum of destruxin A (**5**) (lock mass corrected) is shown in Fig. 3. The MS data and respective identification of peaks **1**–**7** are presented in Table 1. Mass deviations from calculated values for destruxin derivatives are between 2.2 and 5.5 ppm.

3.2. Identification through elemental composition calculation

Identification of the destruxins were made by means of elemental composition calculations from the $[M+H]^+$ pseudomolecular ions and a subsequent database search. Calculation limits were introduced using a maximum of double bond equivalents of 15, the presence of C (1–50), H (10–80), N (0–10) and O (0–18) atoms, and a mass tolerance of 10 ppm. In the case of peak **3**, one Cl atom was detected in the mass spectrum. Database search in Registry (Chemical Abstracts) using the complete set of calculated molecular formula hypotheses allowed identification of peaks **1**–**7** as destruxin derivatives. For example, in the case of peak **5** (m/z 578.3570), six molecular formula hypotheses were calculated, corresponding

Table 1
HPLC–ESI-TOF-MS data and identification of peaks **1**–**7**

Peak	t_R (min)	$[M+H]^+$			Molecular formula	Compounds
		Measured	Calculated	Difference (ppm)		
1	11.10	612.3622	612.3609	2.2	$C_{29}H_{49}N_5O_9$	Destruxin E diol
2	13.12	594.3519	594.3503	2.8	$C_{29}H_{47}N_5O_8$	Destruxin E
3	13.54	630.3292	630.3270	3.5	$C_{29}H_{48}N_5O_8Cl$	Destruxin E chlorohydrin
4	13.98	624.3643	624.3609	5.5	$C_{30}H_{49}N_5O_9$	Destruxin D
5	15.39	578.3570	578.3554	2.8	$C_{29}H_{47}N_5O_7$	Destruxin A
6	17.06	580.3732	580.3710	3.8	$C_{29}H_{49}N_5O_7$	Dihydrodestruxin A *N*-Demethyldestruxin B Destruxin B2
7	17.74	594.3895	594.3867	4.7	$C_{30}H_{51}N_5O_7$	Destruxin B

to a total of 43 compounds registered in the Chemical Abstracts. Among them however, destruxin A was the only natural product. UV spectral data and taxonomy of the producer confirmed the identification. The same procedure was applied for the identification of the other destruxins. In fact, destruxins were found to be, together with a further cyclodepsipeptide, bursaphelocide A ($C_{30}H_{51}N_5O_7$) [16], the only natural products with a molecular formula agreeing with the mass measured, respectively. The isobaric destruxins E ($C_{30}H_{51}N_5O_7$) and B ($C_{29}H_{47}N_5O_8$) could also be unambiguously distinguished and only peak **6** could not be assigned to one of the isomeric structures dihydrodestruxin A, *N*-demethyldestruxin B and destruxin B2. Identity of destruxin A was confirmed by comparison with an authentic sample, while identities of destruxins B and E were definitively established by thorough NMR analysis after isolation.

3.3. Discussion

Rapid and reliable identification of compounds in complex extracts is a prerequisite for successful natural product research. When dealing with unknown substances, as is typically the case during the screening and dereplication processes, nominal mass and fragmentation information provided by quadrupole instruments often proves to be difficult to use for database searches and is insufficient for compound identification. On the other hand, the high accuracy of masses determined by HPLC–ESI-TOF-MS enables calculations of elemental composition hypotheses, thus allowing direct and efficient searches of large chemical databases such as the Chemical Abstracts. By combining molecular formula information with additional data (UV spectral data, taxonomy of the producer), identification of a great number of substances is possible at a very early stage of an investigation. Moreover, using the lock mass facility, the accurate masses of an unlimited number of sample constituents can be obtained in a single analysis, thus speeding up the dereplication process. Isolation work can then focus on promising compounds, which results in more efficient follow-up strategies. HPLC–ESI-TOF-MS has been successfully applied in our laboratory for the identification of various microbial metabolites in crude extracts.

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References

[1] M.A. Strege, J. Chromatogr. B 725 (1999) 67.
[2] H.L. Constant, C.W.W. Baecher, Nat. Prod. Lett. 6 (1995) 193.
[3] J.L. Wolfender, K. Hostettmann, F. Abe, T. Nagao, H. Okabe, T. Yamauchi, J. Chromatogr. A 712 (1995) 155.
[4] D. Waidelich, LaborPraxis (March 1997) 14.
[5] I.V. Chernushevich, W. Ens, K.G. Standing, Electrospray ionization time-of-flight mass spectrometry, in: R.B. Cole (Ed.), Electrospray Ionization Mass Spectrometry, Wiley, New York, Chichester, 1997, pp. 203–234.
[6] K. Seta, H. Fukuda, M. Fukuda, N. Kobayashi, M. Taoka, T. Isolbe, Chromatography 18 (1997) 258, C.A. 128 (1997) 112623.
[7] K. Seta, H. Fukuda, M. Funahashi, B. Carlisle, F. Shinkai, M. Taoka, T. Isolbe, Chromatography 19 (1998) 342, C.A. 130 (1997) 278881.
[8] S. Gupta, D.W. Roberts, J.A.A. Renwick, J. Chem. Soc., Perkin Trans. I (1989) 2347.
[9] A. Jegorov, V. Matha, P. Sedmera, D.W. Roberts, Phytochemistry 31 (1992) 2669.
[10] M. Wahlman, B.S. Davidson, J. Nat. Prod. 56 (1993) 643.
[11] H.C. Chen, S.F. Yeh, G.-T. Ong, S.-H. Wu, C.-M. Sun, C.-K. Chou, J. Nat. Prod. 58 (1995) 527.
[12] C. Lange, C. Mulheim, A. Vey, M. Pais, Biol. Mass Spectrom. 21 (1992) 33.
[13] C. Loutelier, J.-C. Cherton, C. Lange, M. Traris, A. Vey, J.Chromatogr. A 738 (1996) 181.
[14] M. Hubert, F. Cavelier, J. Verducci, J.-C. Cherton, A. Vey, C. Lange, Rapid. Commun. Mass Spectrom. 13 (1999) 860.
[15] A. Jegorov, V. Havlicek, P. Sedmera, J. Mass Spectrom. 33 (1998) 274.
[16] K. Kawazu, T. Murakami, Y. Ono, H. Kanzaki, A. Kobayashi, T. Mikawa, N. Yoshikawa, Biosci. Biotechnol. Biochem. 57 (1993) 98.