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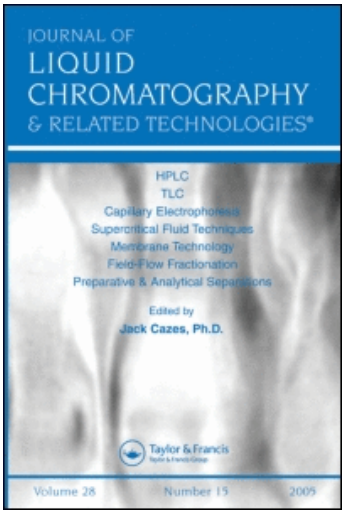

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Preparative Isolation of Destruxins from *Metarhizium Anisopliae* by High Performance Liquid Chromatography

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PREPARATIVE ISOLATION OF DESTRUXINS FROM METARHIZIUM ANISOPLIAE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method has been developed for preparative isolation of destruxins, a group of major insecticidal cyclodepsipeptides from culture broth of *Metarhizium anisopliae*. Prepurification of the crude extract by flash chromatography on silica gel followed by HPLC on reverse phase C18 column using linear gradients of acetonitrile-water permitted the isolation of ten destruxins.

INTRODUCTION

Destruxins are insecticidal cyclodepsipeptides which were first isolated from the entomogenous fungus *Metarhizium anisopliae* (1,2), which is pathogenic on several different insects. The lipid soluble destruxins are of interest because of their structural uniqueness as well as their insecticidal activity (3-5). Structurally destruxins consist of four L-∝-amino acids (proline, isoleucine, valine and alanine or their analogs), one ß-amino acid (ß-alanine) and one D-∝-hydroxy acid as the constituent moieties. Esterification between the carboxyl group of ß-alanine and the hydroxyl group of the ∝-hydroxy acid

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results in the formation of a macrocylic lactone--the depsipeptide. There may be a direct link between the pathogenicity of the fungus and the production of destruxins as these insect toxins have been isolated from infected silkworms shortly after death (6). Seventeen different naturally occuring destruxins have so far been isolated from three different sources: *Metarhizium anisopliae* (7,8), *Alternaria brassicae* (9,10) and *Trichothecium inflatum* (11,12). Recently destruxin B was isolated as the chlorosis-causing active principle from *Alternaria brassicae* (9,10), which is a fungus pathogenic on certain *Brassica* plants. Roseotoxin B, the *trans*-3-methylproline analog of destruxin A, was isolated as the toxic component from *Trichothecium roseum* (11,12). Figure 1 lists the structures of the destruxins isolated in the present investigation. During a program to screen extracts of entomopathogenic fungi for insecticidal activity, the extract from one of the isolates of *Metarhizium anisopliae* showed strong activity over a wide range of insects (13). Follow-up, bioassay-guided fractionation resulted in the isolation of ten insecticidal destruxins from this extract. In the present communication we report an efficient method for the preparative isolation of major neutral destruxins from the culture broth of *Metarhizium anisopliae*. After initial fractionation on a flash silica gel column, excellent resolution of destruxins could be achieved by reverse phase (C18) HPLC (high performance liquid chromatography). Previous investigations have reported considerable difficulty in resolving this complex mixture of closely related compounds (8). More destruxins were resolved by the method described below than a combination of ion exchange columns followed by HPLC (14).

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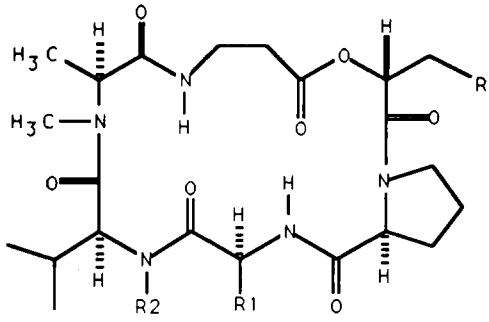
MATERIALS AND METHODS

Extraction and Fractionation

The isolate of *Metarhizium anisopliae* (ARSEF number 1095) used in the present studies was obtained from the collection of entomopathogenic fungi, USDA-ARS, Plant Protection Research Unit, Boyce Thompson Institute at Cornell university, Ithaca. This isolate was originally obtained from an infested *Carpocapsa pomonella* (Lepidoptera: Olethrentidae) from Austria. The fungus was grown in sterilized Czapek-Dox medium enriched with 0.5% bactopeptone. The inoculum was made in 6x1L Fernbach flasks and the culture was allowed to grow at ambient temperature for ten days on a rotary shaker



DESTRUXIN	R	R1	R2
A	$-CH=CH_2$	$-CH(CH_3)CH_2CH_3$	CH_3
A2	$-CH=CH_2$	$-CH(CH_3)_2$	CH_3
B	$-CH(CH_3)_2$	$-CH(CH_3)CH_2CH_3$	CH_3
B2	$-CH(CH_3)_2$	$-CH(CH_3)_2$	CH_3
E	$-CH-CH_2$ (O)	$-CH(CH_3)CH_2CH_3$	CH_3
E2	$-CH-CH_2$ (O)	$-CH(CH_3)_2$	CH_3
C	$-CH(CH_3)CH_2OH$	$-CH(CH_3)CH_2CH_3$	CH_3
C2	$-CH(CH_3)CH_2OH$	$-CH(CH_3)_2$	CH_3
DMDB	$-CH(CH_3)_2$	$-CH(CH_3)CH_2CH_3$	H
Chl	$-CH(OH)CH_2Cl$	$-CH(CH_3)CH_2CH_3$	CH_3

FIGURE 1. Structures of Destruxins isolated from *Metarhizium anisopliae*

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(150 rpm). The culture was then filtered under suction through filter paper (Whatman #1) and the clear filtrate was treated with dil. HCl to adjust the pH to 5.5. The aqueous solution was then fractionated according to the flow chart as shown in Figure 2. Residues I-V, so obtained, were tested for insecticidal activity to first instar tobacco budworm larvae (*Heliothis virescens).* Only residue I showed significant activity and this fraction was therefore selected for further chemical analysis.

Flash Silica Gel Chromatography

The methylene chloride-soluble residue I (530 mg) was flash chromatographed (15) on a column of silica gel (flash chromatography silica gel, Woelm, particle size

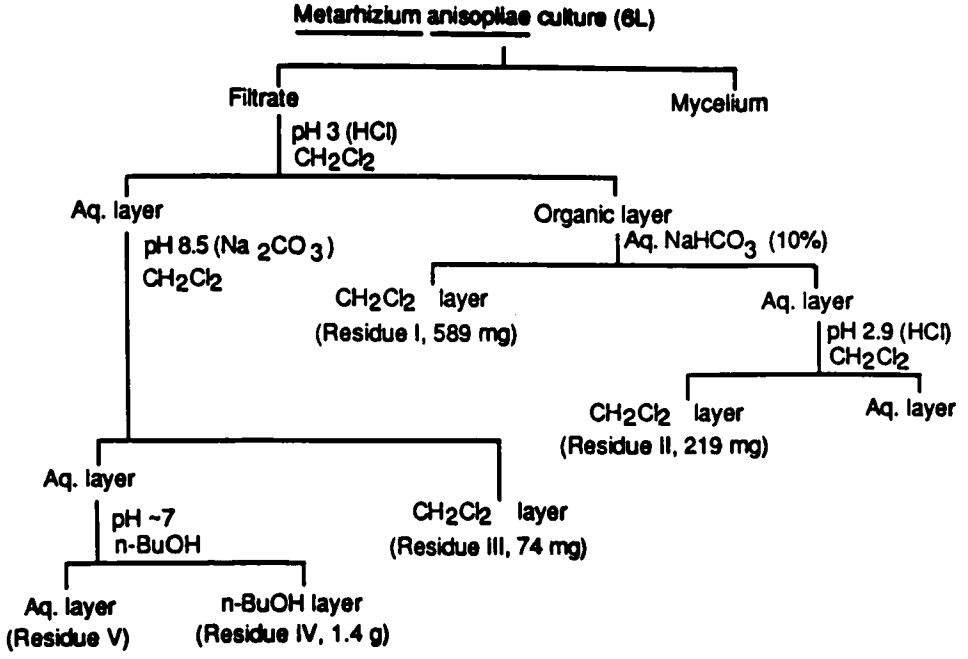

FIGURE 2. Fractionation of *Metarhizium anisopliae* broth

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29-63 µm, 30g). The column was first eluted with methylene chloride and then with increasing methanol in methylene chloride. The solvent flow through the column was maintained under pressure with dry nitrogen. Elution was monitored by thin layer chromatography and fractions containing the same components were combined to afford nine major fractions. These fractions were subjected to bioassay and three fractions (4, 5 and 6 according to the order of elution) were found to be active. Weak activity was also detected in fraction 8. Fraction 4 (195.8 mg) was eluted with methylene chloride-methanol (99:1) and fractions 5 and 6 (75.9 and 65.3 mg respectively) were eluted with methylene chloride-methanol (98:2). Elution of fraction 8 (28 mg) was achieved with methylene chloride-methanol (95:5).

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High Performance Liquid Chromatography

HPLC samples were prepared by passing the material through RP C18 cartridges [JT Baker, Octadecylsilane (C18) solid phase extraction columns, 3ml, 40μm apd 60 angstrom] (16). The samples were dissolved in methanol:water (40:60) and adsorbed on the preconditioned column. After washing the column with the same solvent system (3x bed volume), the toxins were eluted with methanol followed by filtration through a 0.45 μm filter (Acrodisc-CR, Gelman). HPLC was performed using a Waters HPLC system equipped with a 600E system controller and multisolvent delivery system, a Waters U6K injector and Waters 490 programmable multiwavelength detector. A reverse phase C18 column (Varian Micropak MCH-10, semipreparative column, 0.8 x 50 cm, particle size 10 μm, monomeric ODS, bonded to Lichrosorb) equipped with a C18 guard cartridge (Varian) was used for the analysis. A gradient combination of acetonitrile-water was used as the eluting solvent with a flow rate of 3.3 ml/min. HPLC grade acetonitrile (UV cutoff 190 nm) and methanol (UV cutoff 205 nm) were purchased from Fisher Scientific and the water for HPLC was obtained from a Millipore Milli-Q water system. All solvents were filtered through a 0.22 μm filter before use. A typical linear gradient solvent system was acetonitrile:water (30:70) to acetonitrile:water (70:30) in 45 minutes (Waters curve # 6). The system operated with a pressure of 1200 (±100) psi under these conditions. The detector monitored absorption at 215 and 254 nm with a sensitivity of 0.2 aufs for analytical runs and 2.5 aufs for preparative runs. For analytical runs, sample solutions of concentration 5 μg / μl in acetonitrile were prepared and 10 μl of the solution was injected. Figures 3-7 represent the analytical runs of different destruxin containing samples. The destruxins absorbed at 215 nm while the major contaminants, co-eluting yellow pigments, showed absorption at 254 nm also.

Thin Layer Chromatography

Thin layer chromatography of the destruxins was performed on glass precoated high performance silica gel TLC plates (Whatman silica gel, HPTLC, LHP-KF) using chloroform: methanol (97:3) as the developing solvent system. Spots were visualized by iodine vapors (2), by 0.1% acidic potassium permanganate solution or by chlorine--ortho-tolidine spray reagent (17).

Identification of Destruxins

Isolated destruxins were identified by their physical and spectral data (mp, UV, IR NMR, MS), chemical conversions and comparison of the data with the literature values (8). Complete proton and carbon NMR assignments for destruxins A and B were accomplished using different 1D and 2D NMR techniques and this information was used for the identification of other destruxins. Details of these studies will be published elsewhere.

Bioassay

Bioassays for the insecticidal activity were performed with tobacco budworm (*Heliothis virescens*). Samples were formulated in acetone-5% Triton X155 (Rhom & Haas, 1:1) and sprayed to run-off on whole garbanzo leaves which were kept on moist filter paper in petri dishes. The leaves were then infested with 1st instar larvae and the effects were evaluated after three days.

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RESULTS AND DISCUSSION

Mycelium from the ten-day-old culture of *Metarhizium anisopliae* was separated by filtration and the broth was fractionated by liquid-liquid partitioning as shown in Figure 2. Residues I-V were subjected to bioassay and most of the activity was found to be concentrated in the neutral residue I. Subsequent studies as described below revealed that this fraction contained a mixture of neutral destruxins. The different destruxins could be well resolved on reverse phase HPLC. Weak activity was also detected in residue III but it was ascribed to the presence of residual destruxins A and B in this fraction as detected by HPLC.

Figure 3 shows the HPLC chromatogram of the crude methylene chloride-soluble residue I. The peaks representing the individual destruxins have been marked on the chromatogram. The region around destruxin E (retention time 23.5 minutes) is unresolved because the yellow pigments (absorbing at 254 nm) co-elute at about the same time. The column was eluted with a linear gradient of acetonitrile-water (30:70) to acetonitrile over a 60 minute period.

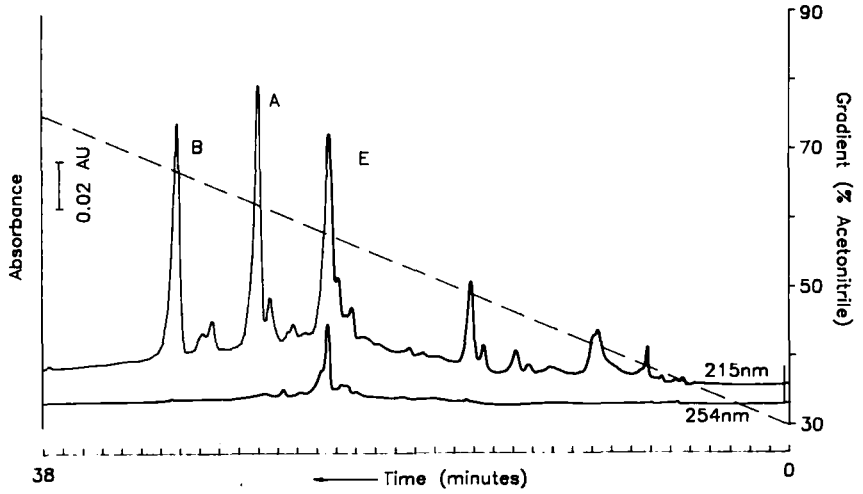

FIGURE 3. HPLC Chromatogram of methylene chloride soluble fraction I Acetonitrile:Water (30:70) --> Acetonitrile, 60 min

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The crude extract was first subjected to flash silica gel chromatography (15) as a prepurification step. The column was eluted with methylene chloride and then with increasing proportions of methanol in methylene chloride. Nine fractions were collected essentially on the basis of thin layer chromatography. Fractions 4 (eluted with methylene chloride-methanol 99:1) and 5-6 (eluted with methylene chloride:methanol 98:2) were found to be active when bioassayed for insecticidal activity on tobacco budworm. Fraction 8 (eluted with methylene chloride:methanol 95:5) also showed weak acitvity under similar conditions and a total of ten insecticidal destruxins (18) were isolated from these fractions as described below.

Figure 4 represents the analytical HPLC chromatogram of fraction 4. The sample was run on a RP C18 column (Varian MCH10, 0.8 x 50 cm) and separated with a linear gradient of acetonitrile:water (30:70) to acetonitrile:water (70:30) during 45 minutes. Major peaks were collected and compounds were identified. Four destruxins (B, B2, A and A2) were isolated from this fraction. As evident from the chromatogram these destruxins showed excellent resolution on RPHPLC under the conditions described.

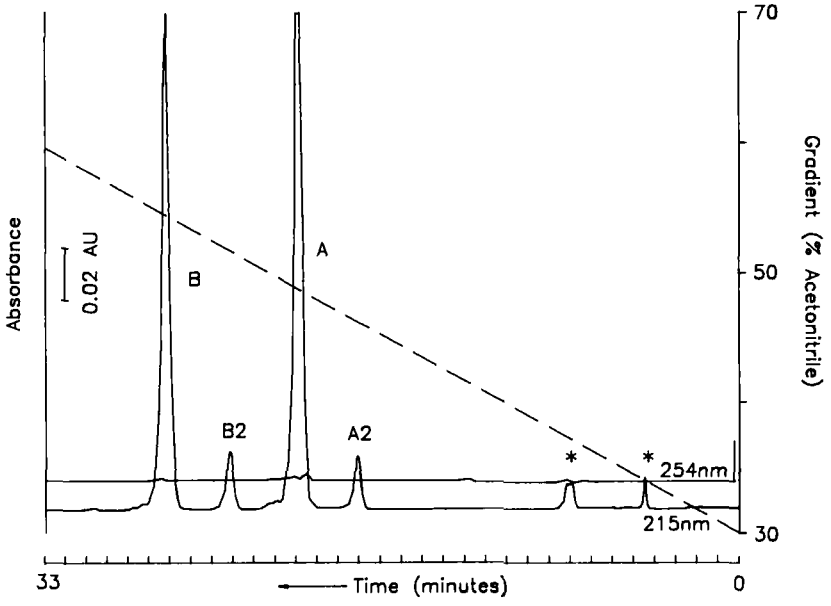

FIGURE 4. HPLC Chromatogram of fraction 4. * Nondestruxins Acetonitrile:Water (30:70) --> Acetonitrile:Water (70:30), 45 min

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The identities of these compounds were established on the basis of physicochemical and spectral data. Retention times and percent yields of the isolated destruxins are listed in Table 1. For preparative isolation, as much as 10 mg of the material could be chromatographed on this column in one injection with fairly good resolution.

Figure 5 shows the HPLC chromatogram of fraction 5 from the silica column. The C18 column was eluted with a linear gradient of acetonitrile: water (30:70) to acetonitrile: water (70:30) in 45 minutes. The major peaks were found to be of destruxins B, A, A2, E and E2. The two early eluting peaks (marked by an asterisk in the chromatogram) were found to be contaminants unrelated to the destruxibns. Figure 6 shows the HPLC chromatogram of fraction 6 from the silica column using the same solvent gradient as for fraction 5. The major components of this fraction were desmethyldestruxin B (DMDB), destruxin E and the chlorohydrin (Chl), which were well resolved under these HPLC

Table 1

Retention Times and % Yields of Destruxins Isolated from *M. anisopliae*

Destruxin	% yield	Retention time (minutes)
B	15.9	27.3
B2	1.7	24.2
A	23.8	20.9
A2	1.6	18.1
E	7.7	14.4
E2	0.4	11.8
Chlorohydrin	1.4	13.2
Desmethyldestruxin B	1.1	22.3
C	2.5	11.7*
C2	0.4	10.3*

* Retention times for destruxin C and C2 were obtained with a different elution gradient (see discussion for Figure 7)

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conditions. Destruxins A and B2 and desmethyldestruxin B, which have close retention times on reverse phase (C18) HPLC were well separated by flash chromatography on silica gel. The non-destruxin peaks are marked by an asterisk in the chromatogram.

Destruxins C and C2 were isolated from fraction 8 from the silica column. It is interesting to note that destruxins C and C2 (which have alcoholic functionality in the side chain of the α-hydroxy acid moiety) could be well separated from other destruxins by

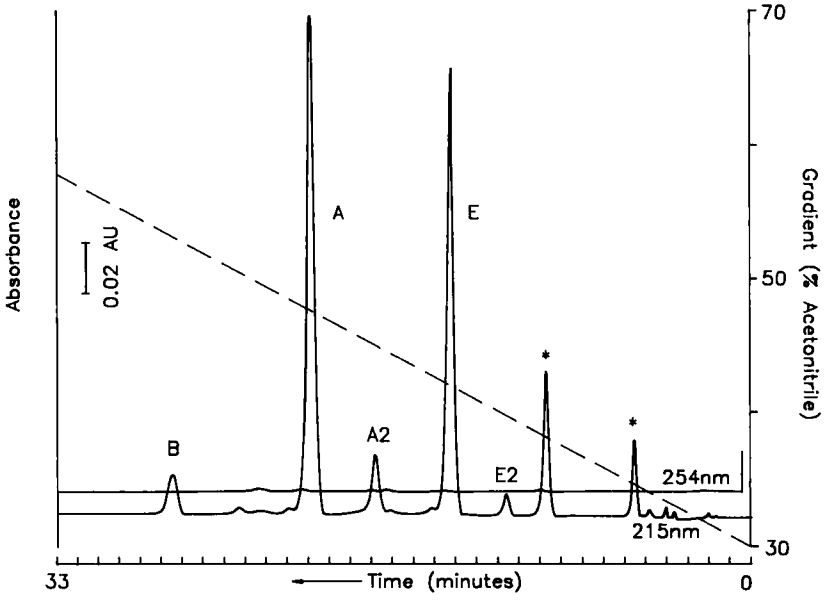

FIGURE 5. HPLC Chromatogram of fraction 5. * Nondestruxins Acetonitrile:Water (30:70) --> Acetonitrile:Water (70:30), 45 min

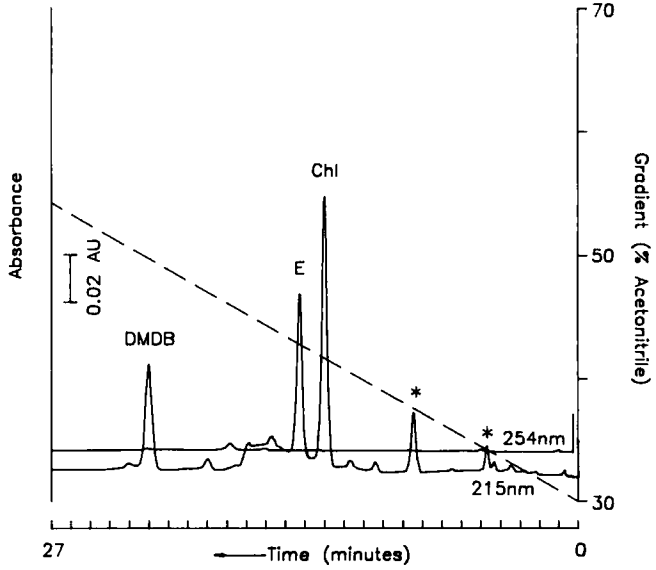

FIGURE 6. HPLC Chromatogram of fraction 6. * Nondestruxins Acetonitrile:Water (30:70) --> Acetonitrile:Water (70:30), 45 min

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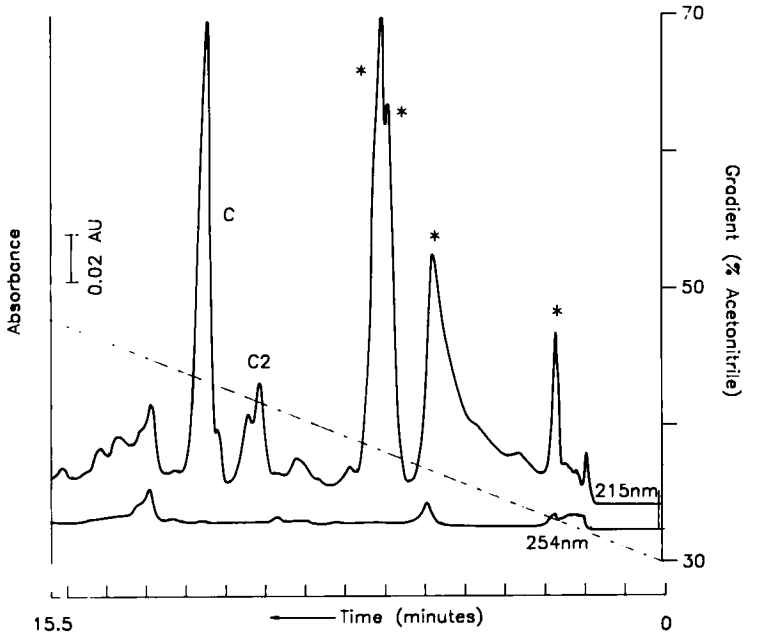

FIGURE 7. HPLC Chromatogram of fraction 8. * Nondestruxins Acetonitrile:Water (30:70) --> Acetonitrile:Water (70:30), 35 min

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normal phase flash silica gel column chromatography, but were poorly resolved on reverse phase (C18) HPLC. The HPLC chromatogram of fraction 8 is shown in Figure 7. The column was eluted with a gradient of acetonitrile:water (30:70) to acetonitrile:water (70:30) in 35 minutes. The peaks corresponding to destruxins have been marked on the chromatogram. The peaks marked with an asterisk were found to be compounds unrelated to destruxins.

Percent yields (calculated from the weight of the crude methylene chloride-soluble residue I) and retention times of all the different destruxins isolated in the present investigation are listed in Table 1. Destruxins constituted about 57% of the total crude extract. Destruxins B, A and E are the major compounds present in an approximate ratio of 2:3:1 accounting for about 83% of the total destruxins. Destruxin E2 and the chlorohydrin were new compounds isolated for the first time from the fungal extract.

Details of the identification of these compounds will be published elsewhere. Chlorohydrin is obviously an artefact of the isolation procedure resulting from the use of methylene chloride for the extraction. Chlorohydrin can be formed by the addition of one molecule of hypochlorous acid to the exocyclic double bond of destruxin A.

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