Mycopathologia 98: 21–26 (1987)

A new mycotoxin from *Aspergillus candidus* Link isolated from rough rice

S. K. Chattopadhyay,[1] B. Nandi,[1] P. Ghosh[2] & S. Thakur[2]
[1]*Mycology and Plant Pathology Laboratory, Department of Botany, Burdwan University, Burdwan, IND-713 104;* [2]*Natural Product Laboratory, Department of Chemistry, Burdwan University, Burdwan, IND-713 104, India*

Keywords: *Aspergillus candidus* Link, new mycotoxin: toxicological studies on rats

Abstract

A new mycotoxin (AcT_1) was obtained from the mycelium of *Aspergillus candidus* Link isolated from rough rice stored under tropical conditions. AcT_1 with the mol-formula $C_{28}H_{40}O$ showed bright greenish blue fluorescence under uv (254 nm). The spectral and other characteristics indicated that the compound was a new one. The LD_{50} of the toxin on white rats was found to be 4.5 mg/Kg when injected intraperitoneally.

Introduction

Aspergillus candidus Link is a common mould of food grains stored under hot and humid conditions prevailing in tropical countries. A number of toxic compounds have been reported from *A. candidus* such as candidusins (8), Kojic acid (2, 4, 11), citrinin (1, 7, 9), toxin A (8) and ochratoxin complex (6). Detailed studies of a toxin isolated from *A. candidus,* obtained from rice in an earlier work (3), was undertaken. The purified compound does not appear to be the same as any known mycotoxins and has been assigned the name AcT_1.

Materials and methods

Organism

Aspergillus candidus Link was isolated as a dominant colonizer of rough rice in natural storage (3) and was maintained on salt (NaCl, 10%) malt (2%) agar (2%) slants.

Production of the toxin

Production of the toxin (AcT_1) was initially carried out by growing the organism at 30±2 °C on rough rice in which the moisture level was elevated to 16% following Nandi & Haggblom (5). Further studies of the toxin production were, however, carried out by growing the organism in six Erlenmayer's flasks, each containing 500 ml of glucose peptone broth (glucose, 20.0 g; peptone, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; distilled water, 1.0 l) and incubated in still condition at 30±2 °C for 21 days. The mycelium was then harvested, and air dried at 80 °C for 48 h to get constant dry weight.

Extraction and purification of the toxin

Dry mycelium (10 g) was extracted with 500 ml of chloroform-methanol (5:3, v/v) in a Waring Blendor for 10 min. The extract was filtered through Whatman No. 4, the filtrate evaporated to dryness and a brownish mass thus obtained was then chromatographed over a silica gel (60–120 mesh, BDH, 100 gm) column (2.5×48 cm). The column was eluted with 300 ml each of petroleum ether (60–80 °C), petroleum ether-benzene (1:1, v/v), benzene, benzene-chloroform (1:1, v/v) and 800 ml of chloroform in succession. The fractions were monitored for the AcT_1 by thin layer chromatography (TLC) on silica gel G coated (0.5 mm wet thickness) plates using

benzene-ethyl acetate (1:1, v/v) as the developing solvent. The toxin (AcT_1) was eluted only from the chloroform fraction and showed bright greenish blue fluorescence under UV lamp (254 nm). This fraction was evaporated to dryness and the yellowish residue (4 gm) thus obtained was rechromatographed over silica gel (60–120 mesh, DBH, 40 gm) column (2×40 cm) using benzene-chloroform (1:4, v/v) as eluant. The flow rate was adjusted to 2 ml/min and 5 ml fractions were collected in each tube. AcT_1 was found to be present in the tubes between 75 and 105. These fractions (75–105) were then mixed together, evaporated to a small volume (near dryness) and subjected to preparative layer chromatography (1.5 mm wet thickness) on silica gel G using benzene-ethyl acetate (5:1, v/v) as the mobile phase. The plates were then air-dried, and the process of development repeated twice in order to achieve successful separation of the toxin. The plates were observed under UV lamp (254 nm) and the band having bright greenish blue fluorescence was scraped off from the plates and eluted with chloroform to furnish the pure toxin (AcT_1) for further studies.

R_f values

Purified AcT_1 was subjected to analytical silica gel TLC (0.5 mm wet thickness) using various developing solvents viz., toluene-ethyl acetate-formic acid (6:3:1, v/v), ethyl acetate-toluene (3:1, v/v), chloroform-methanol (19:1, v/v), benzene-chloroform-methanol (10:10:1, v/v), benzene-ethyl acetate (1:1 and 5:1, v/v) for determining its homogeneity and the R_f values. The homogeneity was confirmed by charring the chromatoplates after spraying with 50% methanolic sulphuric acid.

Spraying reagent

The developed plates were observed under UV (254 nm) in three different conditions: (i) without using any additional reagent; (ii) using 15% H_2SO_4 in MeOH; (iii) using NH_4OH solution.

Thermostability

The plates developed as before were exposed to different temperatures ranging between 80 and 206 °C for 1 h and the thermostability of the compound was tested by TLC technique, fluorescence under UV lamp (254 nm), spraying reagents and toxicity response.

Solubility

Solubility of the compound was tested in various organic solvents (petroleum ether, benzene, chloroform, acetone, ethyl acetate, toluene, methanol and ethanol) and water.

Spectroscopic studies

Ultraviolet (UV), infrared (IR), and high resolution mass spectra of AcT_1 were carried out.

2,4-Dinitrophenyl hydrazone (DNP) derivative

2,4-dinitrophenyl hydrazine (10 mg) was dissolved by heating a mixture of ethanol (5 ml) and hydrochloric acid (2 drops). AcT_1 (10 mg) in ethanol was added to it and warmed for 5 minutes on a water bath followed by cooling to room temperature. The orange-red precipitate was then filtered and the derivative was recrystallised from ethanol. The homogeneity of the DNP derivative was checked through TLC on silica gel G coated plates using solvent systems, chloroform (R_f 0.69), benzene:ethyl acetate (3:1, R_f 0.93), benzene:chloroform:methanol (50:50:5, R_f 0.55) and keeping them in a iodine chamber over night.

Animal toxicity

Animal toxicity of AcT_1 was studied on white rats (*Rathus norvegicus*) ranging in body weight from 105 to 108 g. The single median lethal dose (LD_{50}) for white rats was determined by intraperitoneal (ip) injection of the toxin in propylene glycol (0.5 ml).

In another set, the toxin was applied through feeding the rats with the toxin (10 mg/100 g body weight) mixed with a commercial rat ration. The toxin-contaminated material was ingested in a single feeding. Subsequently, the treated animals were maintained on standard ration. Necessary controls for the two treatments, intraperitoneal injection and feeding tests, were maintained.

Results and discussion

About 120 mg of AcT_1 was isolated from 10 g of dry mycelium. The pure toxin was a colourless, thick liquid showing R_f values that varied from 0.21 to 0.85 (Table 1) depending on the developing solvents. On TLC plates developed with benzene-ethyl acetate (1:1, v/v), the compound which initially showed bright greenish blue fluorescence under UV (254 nm), changed to light yellow when sprayed with 15% H_2SO_4 in methanol and to intense blue with NH_4OH solution.

Table 1. R_f values of the toxin (AcT_1 in different mobile phases on TLC plates coated with silica gel G).

Mobile phases	R_f
Benzene-ethyl acetate (5:1, v/v)	0.21
Benzene-ethyl acetate (1:1, v/v)	0.66
Benzene-chloroform-methanol (50:50:5, v/v)	0.68
Ethyl acetate-toluene (3:1, v/v)	0.74
Toluene-ethyl acetate-formic acid (6:3:1, v/v)	0.77
Chloroform-methanol (95:5, v/v)	0.85

Long exposure to temperatures between 80° and 206 °C did not reduce the toxicity. The compound was soluble in all the organic solvents tested but the highest solubility was recorded in chloroform and the lowest in petroleum ether. The compound was insoluble in water.

High resolution mass spectrum (Fig. 4) registered a molecular ion peak at m/z 392. 3088) ionizing voltage 70 eV and ion source 170 °C) having as molecular formula $C_{28}H_{40}O$. The ultraviolet spectrum in chloroform (Fig. 1) exhibited $\lambda_{max}^{CHCl_3}$ nm 324, 275 and 285 (sh). The IR spectrum in KBr (Fig. 2) showed peaks at 2955, 2920 and 2840 (C−H stretching), 1720 (> C=O stretching), 1280 (> C−H bending) and 740 (> C=C < cis-olefinic C−H bending) cm^{-1}. The most prominent peak at 1720 cm^{-1} clearly indicates the presence of a saturated 6-membered ring ketone in AcT_1. The formation of a 2,4-dinitrophenyl hydrazone derivative also confirmed that the only hetero atom present in the molecule is a carbonyl function (Fig. 3).

Literature survey revealed that Ergosta-4,6,8[14], 22-tetraen-3-one (10) is the only known fungal metabolite having the same molecular formula, $C_{28}H_{40}O$. Some of the characteristic differences

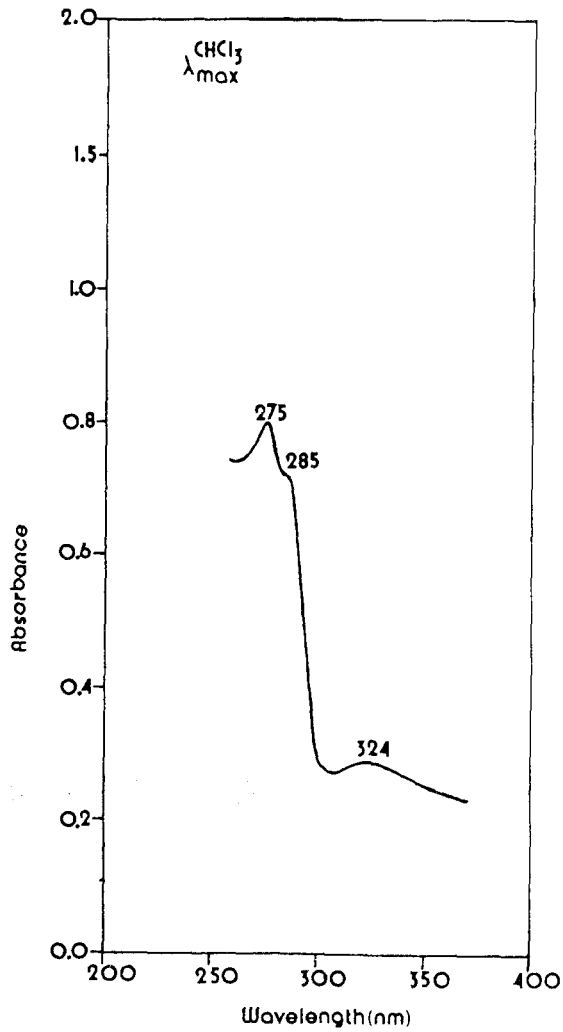

Fig. 1. Ultraviolet spectrum of AcT_1. (Instrument: UV spectrophotometer, Beckman, Model 26; Solvent: Chloroform; Scan speed 50 nm/min.)

between the Ergosta-4,6,8[14],22-tetraen-3-one and the AcT are placed in Table 2, which shows that these two compounds are not identical.

Animal toxicity

By intraperitoneal application of the toxin, the animals became lethargic within 3 to 5 mins followed by a symptom of convulsion of limb muscles and often of the entire body, the cardiac rate increased, asphyxia developed. Death occurred within 15 mins to 48 h depending upon the dosage show-

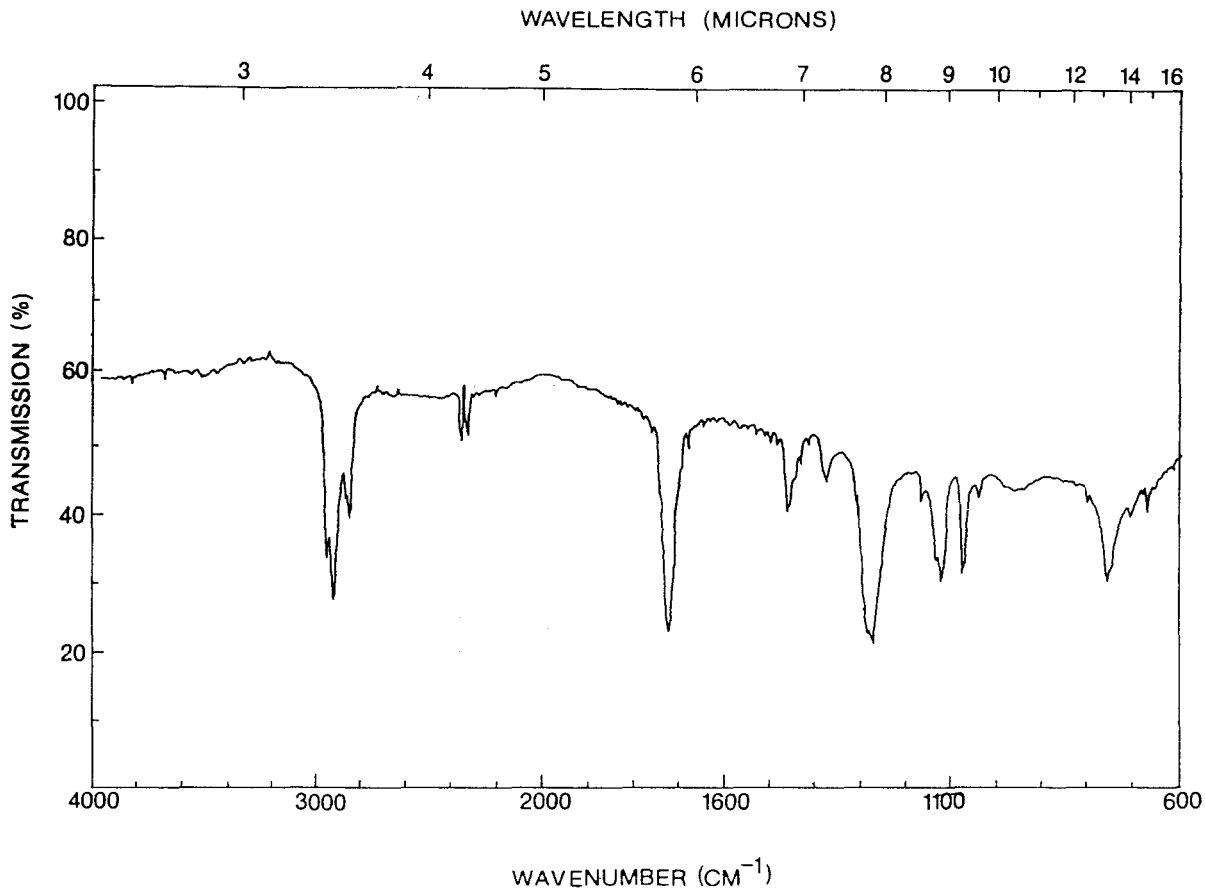

Fig. 2. Infrared spectrum of AcT_1. (Instrument: IR spectrometer, Beckman, Acculab 10.)

Table 2. Comparison between Ergosta-4,6,8[14] 22-tetraen-3-one (I) (10) and AcT_1.

	(I)	AcT_1
(a) Source	*Fomes officinalis*	*Aspergillus candidus*
(b) Melting point	113–114 °C	Thick liquid
(c) TLC on silica gel G [benzene-chloroform-methanol (50:50:5)]	R_f: 0.65	R_f: 0.68
(d) Visualisation: using 15% H_2SO_4 in MeOH followed by observation under UV	Intense green fluorescence	Greenish blue fluorescence
(e) UV maxima (nm)	Ethanol 348, 282, 238	Chloroform 324, 285(sh), 275
(f) UV maximum of 2,4-dinitrophenyl hydrazone derivative	Ethanol 409	Ethanol 414
(g) IR peaks (cm^{-1})	1670, 1640, 1595, 968, 873, 760, 695	2955, 2920, 2840, 1720, 1280, 1120, 1070, 742
(h) Toxicity data	Not reported	LD_{50}, 4.5 mg/Kg white rats

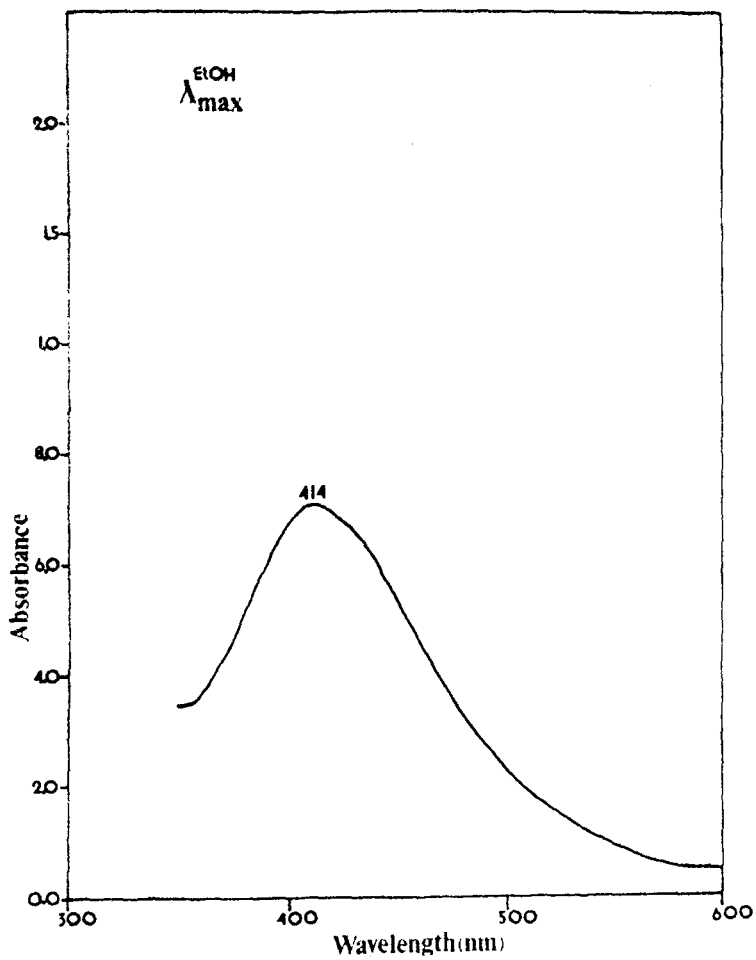

Fig. 3. Ultraviolet spectrum of 2,4-dinitrophenyl hydrazone derivative of AcT_1. (Instrument: UV spectrophotometer, Beckman, Model 26; Solvent: Ethanol; Scan speed 20 nm/min.)

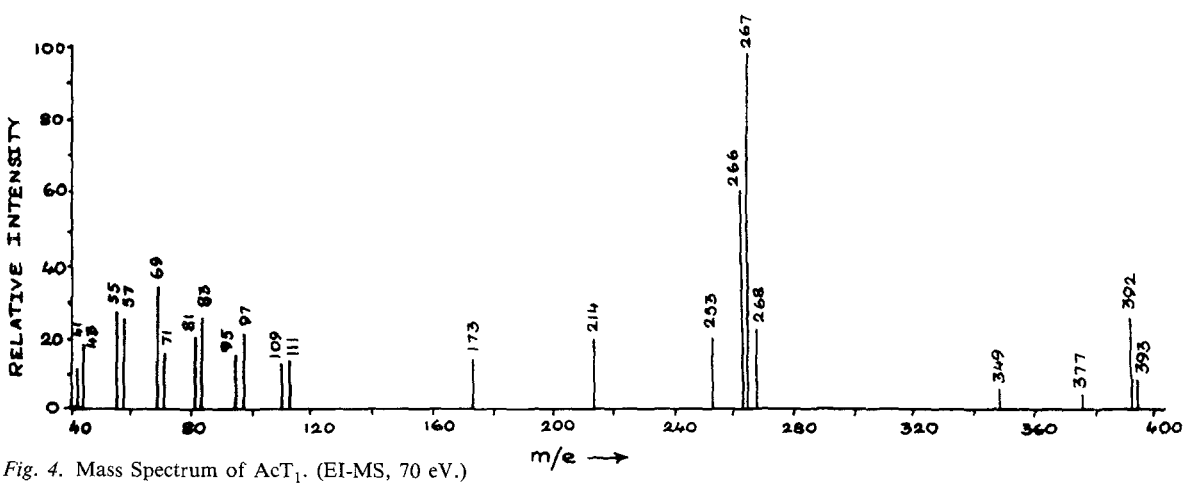

Fig. 4. Mass Spectrum of AcT_1. (EI-MS, 70 eV.)

ing a sympton of violent tossing about in pain. On opening the body cavity immediately after death it was found that the finer blood vessels were ruptured throughout the body cavity. The single median lethal dose (LD_{50}) was 0.45 mg/100 g of white rats, which corresponded to 4.5 mg/Kg of white rats.

The toxin was less toxic in oral application than when administered intraperitoneally. The lower toxicity in oral administration was most probably due to either partial detoxification in the digestive track or partial failure of the toxin to be transported across the intestinal wall or both. The rats fed on the toxin gradually became lethargic, showed a progressive loss of appetite, decrease in body weight followed by death (showing the same symptom of violent tossing about in pain) within 3 to 4 months.

Acknowledgements

We thank Prof. T. Matsumoto and M. Aimi, College of Science and Technology, Nihon University, Japan for high resolution MS.

References

1. Birch AJ, Fitton P, Pride E, Ryan AJ, Smith H, Whalley WB: Studies in relation to biosynthesis. Part XVII. Sclerotiorin, Citrinin and Citromycetin. J Chem Soc 4576–4581, 1958.
2. Hesseltine CW: Natural occurrence of mycotoxins in cereals. Mycopathol Mycol Appl 53:141–153, 1974.
3. Mallick AK, Nandi B: Role of moisture content in deterioration of rough rice in storage. Seed Sci Technol 7:423–429, 1979.
4. Morton HE, Kocholaty W, Junowicz-Kocholaty R, Kelner A: In: Cole RJ & Cox RH (eds) Handbook of toxic fungal metabolites. Academic Press, New York, 1981, pp 759–760.
5. Nandi B, Haggblom P: Production of aflatoxin in rough rice under different storage conditions. Acta Agric Scand 34:128–132, 1984.
6. Neergaard P: Seed pathology, Vol. I. The Macmillan Press Ltd, London, 1977, p 291.
7. Timonin MI, Rouatt JW: Production of citrinin by *Aspergillus* sp. of the *Candidus* group. Can J Pub Health 35:80–88, 1944.
8. Turner WB, Aldridge DC: Fungal metabolites II. Academic Press, London, 1983, pp 14 & 500.
9. Saito M, Enomoto M, Tatsuno T: In: Ciegler A, Kadis S, & Ajl SJ (eds) Microbial Toxins, Vol. 6. Academic Press, New York, 1971, p 358.
10. Schulte KE, Rucker G, Fachmann H: Ergosta-4,6,8[14],22-tetraenon-(3) AIS Inhalts-Stoff Des Larchenschwammes. Tetrahedron Lett 46:4763–4764, 1968.
11. Wilson BJ: In: Ciegler A, Kadis S, Ajl SJ (eds) Microbial Toxins, Vol. 6. Academic Press, New York, 1971, p 235.