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TRICHOTHECENE MYCOTOXINS FROM *FUSARIUM SULPHUREUM*

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Key Word Index—*Fusarium sulphureum*; fungi; sesquiterpene: 12,13-epoxytrichothec-9-ene; mycotoxin.

Abstract—Four mycotoxins isolated from moulded maize cultures of *Fusarium sulphureum* have been characterized as 3α,4β,15-triacetoxy-12,13-epoxytrichothec-9-ene, 4β,15-diacetoxy-3α-hydroxy-12,13-epoxytrichothec-9-ene, 15-acetoxy-3α,4β-dihydroxy-12,13-epoxytrichothec-9-ene and 4β-acetoxy-3α,15-dihydroxy-12,13-epoxytrichothec-9-ene.

INTRODUCTION

As part of a programme on the occurrence of mycotoxin-producing fungi in food in high incidence areas of oesophageal cancer, potatoes were collected in the Gonbad area of Iran [1, 2] and examined for fungi. Five species were isolated of which *Fusarium sulphureum* Schlechtendahl, the conidial state of *Gibberella cyanogena* (Desm.) Sacc., proved to be dominant.

Maize cultures of *F. sulphureum* (MRC 514) cause acute and chronic toxicoses in day-old ducklings and rats when fed at different dietary levels. The gross- and histo-pathological lesions observed in rats were similar to those caused by the trichothecene mycotoxins [3–5]. The trichothecenes, a class of related biologically active metabolites are produced by members of the fungus genera *Myrothecium*, *Stachybotrys*, *Fusarium*, *Cephalosporium*, *Trichoderma* and *Trichothecium* [4]. These biologically active metabolites have been implicated in a variety of mycotoxicoses involving both animals and humans, the most notably that of alimentary toxic aleukia [5, 6]. The production of the trichothecene, 4β,15-diacetoxy-3α-hydroxy-12,13-epoxytrichothec-9-ene(1)by *Fusarium sambucinum* Fuck [7] and *F. solani* var. coeruleum (Sacc.) Booth [8], two pathogens which occur on potatoes, has been reported.

RESULTS AND DISCUSSION

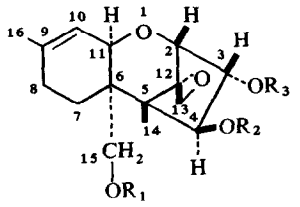
Extraction of *F. sulphureum* moulded maize meal (5 kg) with $CHCl_3$–MeOH and systematic fractionation of the extract, guided by bio-assay using skin tests on rats [9], led to the isolation of 4 related trichothecenes. The main toxin (**1**), (3.02 g) had mp 163–165° and analysed for $C_{19}H_{26}O_7$. The MS lacked a M^+ peak but showed a peak at *m/e* 306 ($C_{17}H_{22}O_5$) corresponding to the facile loss of HOAc from the M^+. Chemical ionization MS of the compound confirmed the MW as 366. The toxin was identified as 4β,15-diacetoxy-3α-hydroxy-12,13-epoxytrichothec-9-ene (**1**) on the basis of its IR ($\nu_{max}^{CHCl_3}$ 3540 and 1720 cm^{-1}) and UV (end absorption only) as well as its PMR [7] and ^{13}C-NMR [10, 11].

A minor product (**2**) (150 mg), mp 124–126° analysed for $C_{21}H_{28}O_8$. The IR spectrum lacked absorption in the OH region but showed strong absorption at 1730 (acetate CO) cm^{-1}. The PMR spectrum is in agreement with the 3α,4β,15-triacetoxy-12,13-epoxytrichothec-9-ene structure (**2**). Acetylation of 4β,15-diacetoxy-3α-hydroxy-12,13-epoxytrichothec-9-ene gave (**2**) identical with the natural product.

The third mycotoxin was identified by IR ($\nu_{max}^{CHCl_3}$ 3430 and 1730 cm^{-1}), chemical ionization MS (M^+ 324) and PMR as 15-acetoxy-3α,4β-dihydroxy-12,13-epoxytrichothec-9-ene (**3**). This toxin was recently isolated from maize infected with *F. roseum* Gibbosum [12]. Mild alkaline hydrolysis of (**1**) in N NH_4OH–MeOH yielded (**3**) identical with the natural toxin.

The fourth metabolite (**4**), an oil which could not be induced to crystallize analysed for $C_{17}H_{24}O_6$. The IR spectrum showed absorption at 3480 (OH) and 1720 (acetate CO) cm^{-1}. Chemical ionization MS indicated a MW of 324. The location of the single OAc group at C-4 was evident from the chemical shift of the C-4 proton (δ 5.53, $J \approx 3.5$ Hz) in the PMR spectrum. To our knowledge this is the first time that 4β-acetoxy-3α,15-dihydroxy-12,13-epoxytrichothec-9-ene (**4**) has been isolated as a natural product.

Acetylation of **1**, **3** and **4** with Ac_2O and pyridine in each case yielded the triacetate (**2**). Mild alkaline hydrolysis with 0.3 N NaOH of each of the four metabolites



(**1**) $R_1 = R_2 = Ac, R_3 = H$
(**2**) $R_1 = R_2 = R_3 = Ac$
(**3**) $R_1 = Ac, R_2 = R_3 = H$
(**4**) $R_1 = R_3 = H, R_2 = Ac$
(**5**) $R_1 = R_2 = R_3 = H$

(1–4) yielded the common product, 3α,4β,15-trihydroxy-12,13-epoxytrichothec-9-ene (5). In the PMR of 5 the protons of the C-3, C-4 and C-15 OH groups appeared as a doublet (δ 4.64, J = 5 Hz), a doublet (δ 5.03, J = 4 Hz) and a quartet (δ 3.83, J = 4 Hz), respectively due to spin–spin coupling with the respective C-3, C-4 and C-15 protons.

Four isolates of *F. sulphureum* were obtained from Dr W. Gerlach, Berlin. The origin of these isolates is shown in Table 1. 4β,15-Diacetoxy-3α-hydroxy-12,13-epoxytrichothec-9-ene (1) was isolated in all cases from maize meal infected with each of these isolates of *F. sulphureum*.

Table 1. Toxicity of different isolates of *F. sulphureum* grown on maize, to ducklings*

Isolate no.	No. died/no. tested	Average days to death
MRC 514†	4/4	5
MRC 845 (B.B.A. 10899)‡	4/4	4
MRC 846 (B.B.A. 11124)§	4/4	3
MRC 847 (B.B.A. 11125)§	4/4	4
MRC 848 (B.B.A. 11126)§	4/4	3

* Moulded maize was incorporated into a commercial chicken mash on a 50% wt basis and fed *ad libitum*.
† Isolated from potatoes, Gonbad area, Iran, 1976.
‡ Isolated from sugar beet, Germany, 1968.
§ Isolated from potatoes, Iran, 1968.

EXPERIMENTAL

Mps are uncorr. IR spectra were measured for solns in $CHCl_3$. PMR spectra were recorded on an HA-100 spectrometer for solns in $CDCl_3$ with TMS as int. stand.

Isolation and culture of fungus. Potatoes were surface sterilized (80% EtOH for 3–5 min) after which small pieces were excised, macerated and plated out on potato dextrose agar containing albamycin. Plates were incubated at 25° for 5 days and the fungi that developed most numerously, isolated in pure culture. 5 Species were isolated of which *F. sulphureum* Schlechtendahl, designated MRC 514, proved to be dominant. Spore suspensions of *F. sulphureum* were used to inoculate whole yellow maize in 2 l. fruit jars. The maize (400 g maize: 400 ml H_2O), previously autoclaved for 1 hr on 2 consecutive days at 121°, was incubated at 25° for 21 days after inoculation. The material was subsequently dried in a forced-draught oven at 50° for 24 hr, milled to a fine meal and stored at 5°.

Isolation of trichothecenes. Maize meal (5 kg) was continuously extracted with $CHCl_3$–MeOH (1:1) for 24 hr in a Soxhlet. The extract was concd to a small vol. and partitioned between aq. 90% MeOH and *n*-hexane. The aq. MeOH soln was concd and the residue partitioned between $CHCl_3$ and H_2O. Bioassay of the different fractions using skin tests on rats. [9] indicated biological activity almost exclusively in the $CHCl_3$ fraction. The $CHCl_3$ soln was dried (Na_2SO_4), filtered and evapd to dryness. The residue (25 g) was further fractionated and purified by column chromatography on Merck Si gel, Type H (1 kg) using $CHCl_3$–MeOH (19:1) as eluant. The column was developed under 1 kg/cm² pres.; 10 ml fractions were collected. Appropriate fractions (TLC:Si gel, $CHCl_3$–MeOH, 19:1) were combined to yield the trichothecenes.

3α,4β,15-Triacetoxy-12,13-epoxytrichothec-9-ene (2). Fraction A (463 mg) was filtered through a short column of Al_2O_3, act II–III (50 g) using $CHCl_3$. The yellow oil obtained from the filtrate was recrystallized from C_6H_6–*n*-hexane to give colourless crystals of 2 (150 mg), mp 124–126° (lit. [7], 123–125°); ν_{max} 1730 (acetate CO) cm^{-1}; PMR, δ 0.76 (*s*, 3H, C-14 Me), 1.71 (*s*, 3H, C-16 Me), 2.04 (*s*, 3H, C-15 OAc), 2.09 (*s*, 3H, C-3 OAc), 2.12 (*s*, 3H, C-4 OAc), 2.77 and 3.05 (each *d*, 1H, $J_{13,13}$ = 4 Hz, C-13 H), 3.84 (*d*, 1H, $J_{2,3}$ = 5 Hz, C-2 H), 3.99 (*d*, 1H, $J_{10,11}$ = 5 Hz, C-11 H), 4.03 and 4.25 (each *d*, 1H, $J_{15,15}$ = 12 Hz, C-15 H), 5.17 (*dd*, 1H, $J_{3,4}$ = 3.5 Hz, $J_{2,3}$ = 5 Hz, C-3 H), 5.46 (*br d*, 1H, $J_{10,11}$ = 5 Hz, C-10 H) and 5.74 (*d*, 1H, $J_{3,4}$ = 3.5 Hz, C-4 H). (Found: C, 61.89; H, 6.68. Calc. for $C_{21}H_{28}O_8$: C, 61.75; H, 6.91%).

4β,15-Diacetoxy-3α-hydroxy-12,13-epoxytrichothec-9-ene (1). Fraction C (4.9 g) was filtered through a short column of Al_2O_3 act II–III (250 g) with $CHCl_3$–MeOH (19:1). The solid material obtained from the filtrate was recrystallized from C_6H_6–*n*-hexane to give (1) (3.02 g), mp 163–165° (lit. [7], 162–164°), ν_{max} 3540 (OH), 1720 (acetate CO) cm^{-1}, PMR δ 0.81 (*s*, 3H, C-14 Me), 1.72 (*s*, 3H, C-16 Me), 2.04 (*s*, 3H, C-15 OAc), 2.13 (*s*, 3H, C-4 OAc), 2.78 and 3.06 (each *d*, 1H, $J_{13,13}$ = 4 Hz, C-13 H), 3.67 (*d*, 1H, $J_{2,3}$ = 5 Hz, C-2), 4 and 4.19 (each *d*, 1H, $J_{15,15}$ = 12 Hz, C-15 H), *ca* 4.1 (2H, C-3 H and C-11 H), 5.23 (*d*, 1H, $J_{3,4}$ = 3 Hz, C-4 H), 5.53 (*br d*, 1H, $J_{10,11}$ = 5 Hz, C-10 H) and 3.41 (*d*, 1H, J = 3 Hz, disappears on addition of D_2O, C-3 OH). (Found: C, 62.58; H, 7.20. Calc. for $C_{19}H_{26}O_7$: C, 62.28; H, 7.15%).

4β-Acetoxy-3α,15-dihydroxy-12,13-epoxytrichothec-9-ene (4). Fraction H (895 mg) was filtered through a short column of Al_2O_3, act II–III (100 g) using $CHCl_3$–MeOH (19:1). 4 was obtained as a colourless oil (600 mg) which could not be induced to crystallize (cf. ref. [7]). ν_{max} 3480 (OH), 1720 (acetate CO) cm^{-1}; PMR δ 0.84 (*s*, 3H, C-14 H), 1.74 (*s*, 3H, C-16 Me), 2.15 (*s*, 3H, C-4 OAc), 2.78 and 3.06 (each *d*, 1H, $J_{13,13}$ = 4 Hz, C-13 H), 3.64 and 3.8 (each *d*, 1H, $J_{15,15}$ = 12 Hz, C-15 H), 3.67 (*d*, 1H, $J_{2,3}$ = 5 Hz, C-2 H), *ca* 4.25 (2H, C-3 H and C-11 H), 5.48 (1H, partly obscured, C-10 H) and 5.53 (*d*, 1H, $J_{3,4}$ = 3.5 Hz, C-4 H). (Found: C, 63.02; H, 7.91. Calc. for $C_{17}H_{24}O_6$: C, 62.95; H, 7.46%).

15-Acetoxy-3α,4β-dihydroxy-12,13-epoxytrichothec-9-ene (3). Fraction L (1.1 g) was filtered through a short column of Al_2O_3, act II–III (100 g) using $CHCl_3$–MeOH (19:1). The colourless oil was recrystallized from EtOAc to give 3 (380 mg), mp 170–172° (lit. [7, 12], 170–172°; 172–173°); ν_{max} 3430 (OH), 1730 (acetate CO) cm^{-1}; PMR δ 0.8 (*s*, 3H, C-14 Me), 1.7 (*s*, 3H, C-16 Me), 2.03 (*s*, 3H, C-15 OAc), 2.72 and 3.01 (each *d*, 1H, $J_{13,13}$ = 4 Hz, C-13 H), 3.61 (*d*, 1H, $J_{2,3}$ = 4 Hz, C-2 H), 3.84 and 4.2 (each *d*, 1H, $J_{15,15}$ = 12 Hz, C-15 H) and 5.49 (*br d*, 1H, $J_{10,11}$ = 5 Hz, C-10 H). (Found: C, 63.21; H, 7.22. Calc. for $C_{17}H_{24}O_6$: C, 62.95; H, 7.46%).

Acetylation of 1. A soln of 1 (1 g) in Ac_2O (30 ml) and Py (30 ml) was stirred at room temp for 12 hr. The crude product was recrystallized from C_6H_6–*n*-hexane to give 2 (885 mg), mp 124–126° (lit. [7], 123–125°).

Hydrolysis of 1. (a) A soln of 1 (366 mg) in MeOH (36 ml) and N NH_4OH (36 ml) was stirred at room temp. for 8 hr. The solvents were evaporated *in vacuo* and the residue extracted with EtOAc to give a colourless glass. Recrystallization from EtOAc gave 3 (254 mg), mp 171–173° (lit. [7, 12], 170–172, 172–173°). (b) A soln of 1 (1 g) in MeOH (20 ml) and 0.3 N NaOH (60 ml) was stirred at room temp. for 15 min and acidified with HOAc (pH 5). The solvents were removed *in vacuo* and the residue partitioned between $CHCl_3$ and H_2O. The product obtained from the $CHCl_3$ soln was recrystallized from EtOAc to give colourless crystals of 3α,4β,15-trihydroxy-12,13-epoxytrichothec-9-ene (5) (630 mg), mp 198–200° (lit. [7, 13], 189–191°; 192–197°); PMR δ (DMSO-d_6) 0.68 (*s*, 3H, C-14 Me), 1.72 (*s*, 3H, C-16 Me), 2.56 and 2.79 (each *d*, 1H, $J_{13,13}$ = 4 Hz, C-13 H), 3.17 (*d*, 1H, $J_{2,3}$ = 5 Hz, C-2 H), 3.18 and 3.52 (each 1H, *dd*, $J_{15,15}$ = 12 Hz, J = 4 Hz, C-15 H), 3.7 (*d*, 1H, $J_{10,11}$ = 5 Hz, C-11 H), 4.08 (*m*, 1H, C-3 H), 4.27 (*q*, 1H, $J_{3,4}$ = 3.5 Hz, J = 4 Hz, C-4 H), 5.27 (*d*, 1H, $J_{10,11}$ = 5 Hz, C-10 H), 3.83 (*q*, 1H, J = 4 Hz, C-15 OH), 4.64 (*d*, 1H, J = 5 Hz, C-3 OH) and 5.03 (*d*, 1H, J = 4 Hz, C-4 OH) (the last 3 signals disappear on addition of D_2O). (Found: C, 63.67; H, 7.75. Calc. for $C_{15}H_{22}O_5$: C, 63.81; H, 7.85%).

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