New Metabolic Products of *Fusarium culmorum*: Toxic Trichothec-9-en-8-ones and 2-Acetylquinazolin-4(3*H*)-one

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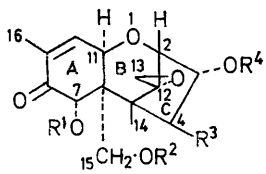
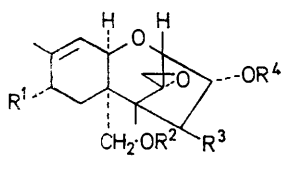
4β,15-Diacetoxy-3α,7α-dihydroxy-12,13-epoxytrichothec-9-en-8-one, previously obtained from other *Fusarium* spp., has been isolated from a toxic strain of *F. culmorum*. The new fungal metabolic products 3α-acetoxy-7α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one and 2-acetylquinazolin-4(3*H*)-one have been obtained from a second such strain. The known sesquiterpene culmorin was produced by both strains.

A NUMBER of *Fusarium* spp. associated with pathogenic conditions in vertebrates have been shown to produce toxic 12,13-epoxytrichothec-9-enes when cultured *in vitro* on a chemically defined medium.[1] Because many of the trichothecane-producing Fusaria have been reported as being pathogenic to insects [2] we have been extending our studies [3] of structure–activity relationships in this group of compounds with a view to revealing more examples of selectivity of biological action.[4]

Several toxic strains of *F. culmorum* associated with cereal crops causing sickness in dairy cattle have been described,[5,6] and chromatographic evidence for the formation of diacetoxyscirpenol (II; $R^1 = R^4 = H$, $R^2 = Ac$, $R^3 = OAc$), neosolaniol (II; $R^1 = OH$, $R^2 = Ac$, $R^3 = OAc$, $R^4 = H$), T_2-toxin (II; $R^1 = Bu^iCO_2$, $R^2 = Ac$, $R^3 = OAc$, $R^4 = H$), and HT_2-toxin (II; $R^1 = Bu^iCO_2$, $R^2 = Ac$, $R^3 = OH$, $R^4 = H$) by this fungus has been adduced.[7] We have examined two strains of *F. culmorum* producing toxic culture filtrates and insecticidal secondary metabolites. The sesquiterpene culmorin (III) [8,9] was isolated from both strains, but was not responsible for the toxicity.



(I)



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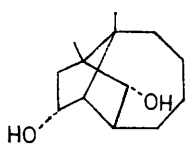
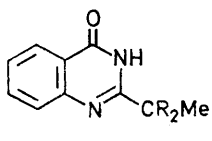
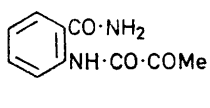


(III)



(IV)



(V)

The first strain, number 53 in our collection, was obtained from a dairy pasture in the Republic of Ireland. Preparative t.l.c. of a toxic extract of the culture filtrate led to the isolation of the enone (I; $R^1 = R^4 = H$,

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R^2 = Ac, R^3 = OAc), previously known as a metabolic product of *F. equiseti* [10,11] and *F. nivale*.[12]

Two new fungal products, $C_{10}H_8N_2O_2$ and $C_{17}H_{22}O_7$, were isolated by column chromatography of a toxic extract from the second strain, number 34. The former was identified as 2-acetylquinazolin-4(3*H*)-one (IV; R_2 = O), from spectroscopic evidence. It has been described as a transformation product of chrysogine (IV; R_2 = H,OH),[13] from *Penicillium chrysogenum*, and of 2-pyruvoylaminobenzamide (V), from *P. chrysogenum*,[14] *P. notatum*,[14] and *Colletotrichum lagenarium*,[15] but has not previously been recorded as a natural product. Although it was isolated from the *P. chrysogenum* fermentation, it was considered in that instance [14] to be an artefact.

The compound $C_{17}H_{22}O_7$ (I; $R^1 = R^2 = R^3 = H$, R^4 = Ac) showed dimorphism and was a neutral hydroxy-ester ($\nu_{max.}$ 3500, 3420, and 1740 cm^{-1}). It contained one acetoxy-group (τ 7·90) and was hydrolysed by sodium hydroxide to an alcohol $C_{15}H_{20}O_6$ (I; $R^1 = R^2 = R^3 = R^4 = H$) which had no ester carbonyl absorption in the i.r. The diacetyl derivative (I; $R^1 = R^2 = R^4$ = Ac, $R^3 = H$) showed no OH absorption. The u.v. spectrum [$\lambda_{max.}$ 226 nm (ε 8100)] of this triacetate was consistent with the presence of an $\alpha\beta$-unsaturated ketone grouping ($\nu_{max.}$ 1697 and 1680 cm^{-1}) and its n.m.r. spectrum (Table 1) contained signals characteristic of the naturally occurring trichothecenes, particularly at τ 7·0 (12-spiro-oxiran) and τ 9·0 [tertiary C(14)H_3]. Double-resonance experiments (τ 3·3 and 5·2) confirmed the presence of the partial structure –O·CHR·CH=CMe·CO– present [11] in ring A of the enone (I; $R^1 = R^4 = H$, R^2 = Ac, R^3 = OAc).

Double-resonance experiments (τ 4·7, 6·1, and 7·7) with the ester (I; $R^1 = R^2 = R^3 = H$, R^4 = Ac) revealed the presence of the partial structure –O·CHR·CH(OAc)·CH_2R, also present in ring c of calonectrin (II; $R^1 = R^3 = H$, $R^2 = R^4$ = Ac),[16] and an α-configuration for the 3-acetoxy-substituent was deduced from the coupling constant $J_{2,3}$ (4·5 Hz). Deshielding of the C-14 protons (τ 8·8) is consistent [11] with a 7-hydroxy-substituent in the α-configuration, confirmed by observation of long-range W-coupling between the C-15 protons (τ 6·14) and a 7β-proton (τ 5·12).

TABLE 1

Chemical shifts (τ values) and coupling constants (J/Hz in parentheses) for protons in the enone (I; $R^1 = R^2 = R^3 = H$, R^4 = Ac) and related compounds

Compound	Position 2	3	4	7	10	11	13	14	15	16	OAc	OH
(I; $R^1 = R^2 = R^3 = H$, R^4 = Ac)	6·10d (4·5)	4·72m	*ca.* 7·75m	5·12d (1·5)	3·36dd (5·5, 1·5)	5·29d (5·5)	6·84s	8·81s	6·14d (1·5)	8·12s	7·90	6·15 8·0
(I; $R^1 = R^2 = R^4$ = Ac, $R^3 = H$)	6·01d (5)	4·70m	*ca.* 7·75m	3·86s	3·36d (5·5)	5·18d (5·5)	7·00AB (4)	9·00s	5·59s	8·08s	8·04 7·80 7·74	
(I; $R^1 = R^4 = H$, R^2 = Ac, R^3 = OAc) [11]	6·13d (4·6)	5·69dd (4·6, 3)	4·70d (3)	5·07s	3·27dd (6, 1·6)	5·20d (6)	6·86s	8·88s	5·67AB (12)	8·09d (1·6)	8·07 7·83	
(I; $R^1 = R^2 = R^4$ = Ac, R^3 = OAc) [11]	6·08d (4·6)	4·84dd (4·6, 3)	4·24d (3)	4·02s	3·52dd (6, 1·6)	5·42dd (6, 0·8)	7·12AB (4)	9·18s	5·63AB (12)	8·20dd (1·6, 0·8)	7·83 7·87 7·93 8·07	
(II; $R^1 = R^2 = R^3 = H$, R^4 = Ac) [16]	6·27d (5)	4·82m			4·53d (5)	6·02d (5)	7·02AB (4)	9·08s	6·44AB (12)	8·27s	7·91	8·45

The compound $C_{17}H_{22}O_7$ therefore bears the same relationship to 15-de-*O*-acetylcalonectrin [16] (II; $R^1 = R^2 = R^3 = H$, R^4 = Ac) as the enone (I; $R^1 = R^4 = H$, R^2 = Ac, R^3 = OAc) bears to diacetoxyscirpenol (II; $R^1 = R^4 = H$, R^2 = Ac, R^3 = OAc). The organism described [16] as *Calonectria nivalis* (≡ *F. nivale*), which produces calonectrin and 15-de-*O*-acetylcalonectrin, is now considered to be *F. culmorum*.[17]

EXPERIMENTAL

M.p.s were taken on a Kofler hot-stage apparatus and are corrected. Unless otherwise stated, i.r. spectra were determined for mulls in Nujol and u.v. spectra and optical rotations for solutions in methanol. N.m.r. spectra were obtained at 100 MHz for solutions in deuteriochloroform with tetramethylsilane as internal standard. Molecular weights were determined by mass spectroscopy. Light petroleum had b.p. 60—80°. Merck silica gel 7734 was used in column chromatography. Unless stated to the contrary, t.l.c. R_F values refer to Merck H_{254} silica gel developed in chloroform–methanol (95 : 5).

Fermentations with F. culmorum.—(a) *Strain* 53. Conical culture flasks (20) containing glucose–ammonium nitrate medium [18] (200 ml) were inoculated and incubated at 25° for 14 days. The fermentation was harvested and the culture filtrate was extracted with chloroform. A portion of the recovered extract (150 mg) produced a necrotic skin reaction when applied to the shaved backs of 21-day old

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albino rats. This skin reaction was not observed when the medium of Gregory *et al.*[19] was used instead of glucose–ammonium nitrate.

The remainder (120 mg) of the extract was subjected to preparative t.l.c. on silica gel (Merck $HF_{254+366}$; 40 × 20 × 0·05 cm) in chloroform–methanol (97 : 3) and bands detected at R_F 0·2 and 0·43 in u.v. light were eluted with rigorously dried acetone.

The material recovered from the band at R_F 0·2 was a solid (56 mg; 20 mg l^{-1}) which crystallised from ethyl acetate in rhombs, m.p. 178—179° (183—185° in sealed tube), $[\alpha]_{546}^{21}$ −14° (*c* 0·63 in $CHCl_3$) (Found: *M*, 238·1946. Calc. for $C_{15}H_{26}O_2$: *M*, 238·1933), identified as culmorin (III) by mixed m.p. and i.r. spectral comparison with an authentic specimen (lit.,[8,9] m.p. 178—179°, $[\alpha]_{546}^{20}$ −14°).

The band at R_F 0·43 yielded semi-solid material (8 mg) which crystallised from ethyl acetate–light petroleum in prisms (6 mg; 2 mg l^{-1}), m.p. 134—135° of the (+)-enone (I; $R^1 = R^4 = H$, $R^2 = Ac$, $R^3 = OAc$), identified by mixed m.p. and i.r. spectral comparison with an authentic specimen.[10]

(b) *Strain* 34. In a typical example, Roux bottles (50) containing Czapek-Dox medium (200 ml) were inoculated with a spore suspension (prepared from potato dextrose agar slopes) of the fungus and incubated at 25° in artificial light. At intervals, aliquot portions (10 ml) of the culture fluid were removed under sterile conditions for determination of the pH and optical rotation (*l* 10 cm) (Table 2) and for extraction with ethyl acetate (2 × 3 ml) and t.l.c. of the product. Spots were seen at R_F 0·70, 0·55, 0·36, 0·29, 0·20 (I_2), and 0·10 (I_2).

TABLE 2

Course of a typical fermentation with *F. culmorum* strain 34 on Czapek-Dox medium

Days	0	5	10	15	21
pH	6·4	6·8	7·0	7·4	7·3
Optical rotation (°)	2·40	0·60	−0·27	−0·57	−0·64

After 10 days the fermentation was harvested and the culture filtrate (6·6 l) was extracted with ethyl acetate (2 × 2 l) at the natural pH (7·3). Evaporation of the dried extract (Na_2SO_4) *in vacuo* furnished a brown oily residue (1·13 g) which gave a positive necrotic skin reaction and was chromatographed in benzene–chloroform (1 : 1; 20 ml) on a column of silica gel (30 g; 20 × 1·8 cm) made up in benzene.

After intractable gummy fractions (46 mg) had been eluted with benzene–chloroform (3 : 1; 300 ml), a brown band was eluted with the same solvent giving a gum (22 mg) which crystallised from ethyl acetate in plates (4 mg; 0·6 mg l^{-1}), m.p. 200° (subl.). Recrystallisation furnished prisms (3 mg), m.p. 205°, R_F 0·78, of 2-acetylquinazolin-4(3*H*)-one (Found: C, 63·8; H, 4·35; N, 14·9%; *M*, 188. Calc. for $C_{10}H_8N_2O_2$: C, 63·8; H, 4·3; N, 14·9%; *M*, 188), $\nu_{max.}$ 3165w, 3065w, 1710, 1670s, and 1600 cm^{-1}, $\lambda_{max.}$ 302 nm (ε 11,600), τ 0·3br (NH), 1·7—2·6 (4H, m, ArH), and 7·25 (3H, s, COMe) [lit.,[14] m.p. 202—205°, $\nu_{max.}$ 3160w, 1706, 1666, and 1594 cm^{-1}, $\lambda_{max.}$ 303 nm (ε 8800)].

After further intractable gummy fractions (51 mg) had been eluted with benzene–chloroform (1 : 1; 300 ml), elution with chloroform furnished (i) (300 ml) a solid (619 mg) (see later) followed by (ii) (100 ml) a gum (42 mg) which crystallised from ethyl acetate–light petroleum in prisms (6 mg; 0·9 mg l^{-1}), m.p. 179—180°, R_F 0·25 (I_2), $\nu_{max.}$ 3320 cm^{-1}, of culmorin (III) (Found: C, 75·6; H, 11·1. Calc. for $C_{15}H_{26}O_2$: C, 75·6; H, 11·0%), identified by i.r. spectral comparison with an authentic specimen.

The pattern of t.l.c. spots observed in u.v. light and in iodine vapour was not significantly altered and the yield of the major metabolite was not significantly changed when the fermentation time was extended from 10 to 21 days, or when the fermentation was conducted in darkness or in light, or when Raulin-Thom medium was used in place of Czapek-Dox.

3α-Acetoxy-12,13-epoxy-7α,15-dihydroxytrichothec-9-en-8-one (I; $R^1 = R^2 = R^3 = H$, $R^4 = Ac$) (340 mg; 52 mg l^{-1}), R_F 0·55, was obtained from the solid fraction (i) of the chloroform eluate by three recrystallisations from ethyl acetate–light petroleum. It formed (*a*) needles, m.p. 135—136°, $\nu_{max.}$ 3500, 3420, 1740, 1683, and 1668 cm^{-1}, or (*b*) needles or plates, m.p. 187—188°, $\nu_{max.}$ 3480br, 1750, 1690, 1672, and 1660 cm^{-1} (Found: C, 60·4; H, 7·0%; *M*, 338·1360. $C_{17}H_{22}O_7$ requires C, 60·35; H, 6·55%; *M*, 338·1365), $\lambda_{max.}$ 220 nm (ε 7100), $[\alpha]_D^{20}$ +43° (*c* 0·28). A melt of the low m.p. form reset and remelted at 187—188° on seeding with the high m.p. form.

The *diacetate* (I; $R^1 = R^2 = R^4 = Ac$, $R^3 = H$), prepared with acetic anhydride in pyridine at room temperature for 2 days, formed prisms, m.p. 155—156°, R_F 0·80 (from ethyl acetate–light petroleum) (Found: C, 60·0; H, 6·5%; *M*, 422·1547. $C_{21}H_{26}O_9$ requires C, 59·7; H, 6·2%; *M*, 422·1557), $\nu_{max.}$ (OH absent) 1750, 1740 (ester C=O), 1697, and 1680 cm^{-1} (C=C–C=O), $\lambda_{max.}$ 226 nm (ε 8100).

Hydrolysis of the Ester (I; $R^1 = R^2 = R^3 = H$, $R^4 = Ac$). —The ester (40 mg) in ethanol (0·5 ml) and 0·1N-sodium hydroxide (3·00 ml) was set aside at room temperature for 24 h. After potentiometric back-titration to pH 7 with 0·1N-hydrochloric acid (1·05 mol. equiv. acetic acid liberated), the solution was extracted with ethyl acetate. Recovery afforded a gum (26 mg) which, after preparative t.l.c. (R_F 0·14), crystallised from ethyl acetate in (*a*) prisms, m.p. >250° (decomp.) (Found: *M*, 296), $\nu_{max.}$ (C=O absent) 1620 cm^{-1}, of the hemiacetal form or (*b*) rhombs, m.p. 160° (Found: *M*, 296·1261. Calc. for $C_{15}H_{20}O_6$: *M*, 296·1260), $\nu_{max.}$ 1680 and 1630 cm^{-1}, of the solvated keto-form of the triol (I; $R^1 = R^2 = R^3 = R^4 = H$).

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