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THE CYTOTOXICITY OF SOME TRANSFORMATION PRODUCTS OF DIACETOXYSCIRPENOL

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Abstract—The cytotoxicity of eighteen relatives and transformation products of diacetoxyscirpenol, an important mycotoxin from *Fusarium sp.* implicated in mouldy corn toxicosis and fescue foot disease of cattle, has been investigated.

The results are consistent with the hypothesis that toxicity is associated with the presence of the 12, 13-epoxytrichothecane nucleus, but other molecular features contribute to the manifestation of high toxicity.

A method for the detoxication of diacetoxyscirpenol follows from the observation that non-toxic products result from the acid-catalysed re-arrangement of the 12, 13-epoxytrichothec-9-ene nucleus to the 10 → 13-cyclotrichothecane or apotrichothec-9-ene systems.

THE NATURALLY-OCCURRING esters of the group of sesquiterpene alcohols containing the tetracyclic 12, 13-epoxytrichothec-9-ene nucleus[1] (I; $R^1{=}R^2{=}R^3{=}R^4{=}H$) are produced by a wide range of soil fungi. Trichodermin (I; $R^1{=}R^2{=}R^4{=}H$, $R^3{=}OAc$) has been isolated[2] from *Trichoderma viride*; diacetylverrucarol[3] (I; $R^1{=}R^4{=}H$, $R^2{=}R^3{=}OAc$) and the verrucarins and roridins, of which the macrolide verrucarin A[4,5] (I; $R^1{=}R^4{=}H$, $R^2R^3{=}O.CO.CHOH.CHMe.CH_2.CH_2.O.CO.CH{\leq}.CH.CH{\leq}CH.CO.O$—) the major constituent of the antibiotic glutinosin,[6] is the principal member, are produced by a number of strains of *Myrothecium verrucaria* and *M. roridum*; diacetoxyscirpenol[7,8] (I; $R^1{=}H$, $R^2{=}R^3{=}OAc$, $R^4{=}OH$) has been obtained from a number of closely related *Fusarium sp.*, notably *F. equiseti*, *F. sambucinum*, *F. scirpi* and *F. tricinctum*;[9–11] trichothecin[2] (II; $R^1{=}R^2{=}R^4{=}H$, $R^3{=}O.CO.CH{\leq}CH.Me$) is obtained from *Trichothecium roseum*[12] and crotocin (III; $R{=}O.CO.CH{\leq}CH.Me$) from *Cephalosporium crotocinigenum*.[13]

These esters show marked selectivity and specificity of biological activity. Selective toxicity is an outstanding property of the group: thus, all the esters inhibit, in low concentration, the growth of various cell types in tissue culture, but show only weak or negligible antibacterial activity *in vitro*. With the notable exception of diacetoxyscirpenol all the esters show antifungal properties; but diacetoxyscirpenol, as well as trichothecin, is strongly phytotoxic. Exceptionally, verrucarin A is insecticidal.[14] The high relative activity of diacetylverrucarol against *Trichophyton asteroides*[3] and of trichothecin against *Penicillium digitatum*[15] are examples of the specificity of antifungal activity in this series.

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High mammalian toxicity, coupled with a powerful local irritant action on topical application of the compound, has been reported for trichothecin, LD_{50} 10 mg/kg, i.v. in mouse; for diacetoxyscirpenol, LD_{50} 0·75 mg/kg, and a co-metabolite[16] (II; $R^1=R^4=OH$, $R^2=R^3=OAc$) LD_{50} 1·2 mg/kg, i.p. in rat; and particularly for verrucarin A, LD_{50} 1·5 mg/kg, i.v. in mouse.

Diacetoxyscirpenol and its 8α-(3-methylbutyryloxy)-derivative (I; R^1 = .O.CO.CH_2.$CHMe_2$, $R^2=R^3=OAc$, $R^4=OH$) have been implicated[11,17] in mouldy corn toxicosis and fescue foot disease of cattle. Interest has therefore been directed towards the study of simple chemical reactions of the 12, 13-epoxytrichothec-9-ene system, particularly hydrolytic fission, which lead to detoxication of the molecule. Although the epoxide group is protected from rearside nucleophilic attack by rings B/C and is relatively stable in basic media, protonation of the epoxide in acidic media is followed by intramolecular re-arrangement to products having a different carbon skeleton.[18] Thus, the 10→13 cyclo-structures (VI) and (VII) and the apotrichothec-9-ene compound (VIII) were obtained (after partial reacetylation) from diacetoxyscirpenol via the mechanistic processes a, aa′ and b respectively[10,19,20] the toxicities of these compounds, together with the toxicities of a number of other derivatives and transformation products of diacetoxyscirpenol, have now been investigated.

A delay of 5–6 hr was reported[9] between injection of diacetoxyscirpenol and the development of toxic symptoms in rat and it was suggested that a metabolite, possibly

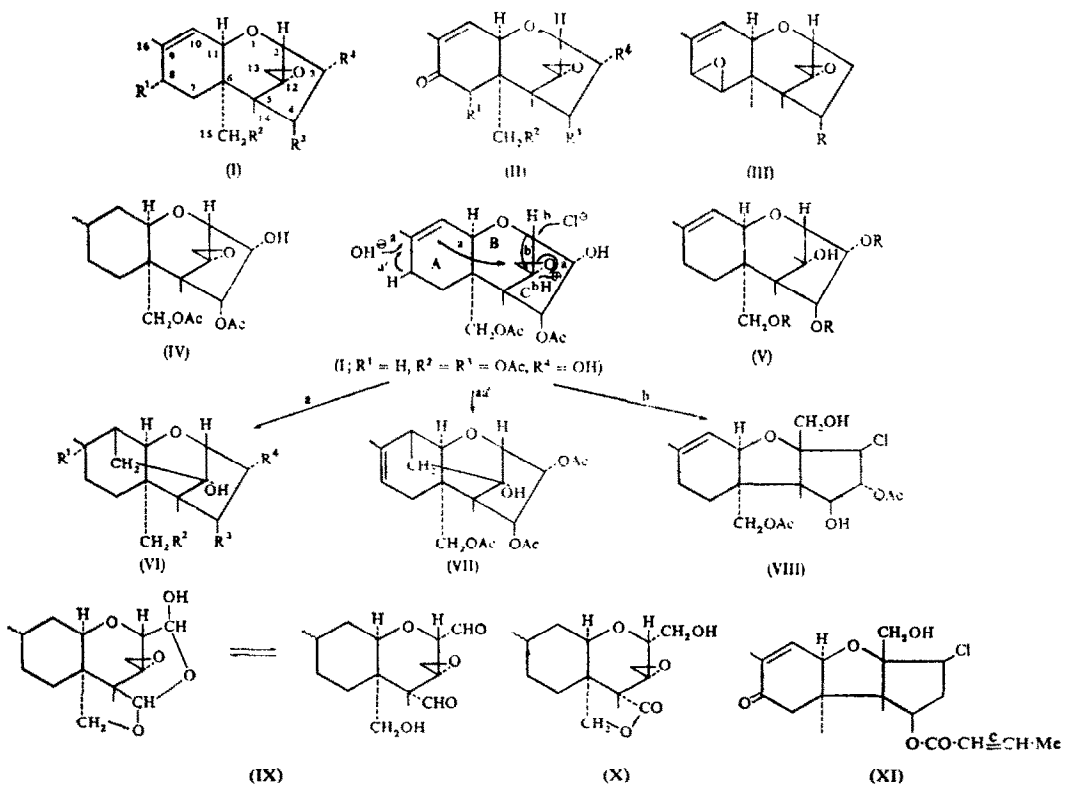

TABLE 1. MELTING POINTS AND STRUCTURES OF RELATIVES AND TRANSFORMATION PRODUCTS OF DIACETOXYSCIRPENOL

Compound	Structure	m.p.(°C)
4β, 15-Diacetoxy-12, 13-epoxytrichothec-9-en-3α-ol (diacetoxyscirpenol)	I; R^1=H, R^2=R^3=OAc, R^4=OH	162
3α, 4β, 15-Triacetoxy-12, 13-epoxytrichothec-9-ene	I; R^1=H, R^2=R^3=R^4=OAc	124
12, 13-Epoxytrichothec-9-en-3α, 4β, 15-triol (scirpentriol)	I; R^1=H, R^2=R^3=R^4=OH	192
4β, 15-Diacetoxy-12, 13-epoxytrichothecan-3α-ol	IV	147
4β, 15-Diacetoxy-12, 13-epoxy-3α, 7α-dihydroxytrichothec-9-en-8-one	II; R^1=R^4=OH, R^2=R^3=OAc	136
12, 13-Epoxy-3α, 4β, 7α, 15-tetrahydroxytrichothec-9-en-8-one	II; R^1=R^2=R^3=R^4=OH	189 dec.
Trichothec-9-en-3α, 4β, 12, 15-tetraol	V; R=H	228
3α, 4β, 15-Triacetoxytrichothec-9-en-12-ol	V; R=Ac	78
3α, 4β, 15-Triacetoxy-10→13-cyclotrichothecan-9α, 12-diol	VI; R^1=OH, R^2=R^3=R^4=OAc	163 and 178
10→13 Cyclotrichothecan-3α, 4β, 9α, 12, 15-pentaol	VI; R^1=R^2=R^3=R^4=OH	180–205
3α, 4β, 15-Triacetoxy-10→13-cyclotrichothec-8-en-12-ol	VII	178
3α, 15-Diacetoxy-2β-chloroapotrichothec-9-en-4β, 13-diol	VIII	166
12, 13-Epoxy-15-hydroxy-3, 4-secotrichothecan-3, 4-dione	IX	135–145
12, 13-Epoxy-3, 15-dihydroxy-3, 4-secotrichothecan-4-oic acid 4→15 lactone	X	glass
12, 13-Epoxy-4β-hydroxytrichothec-9-en-8-one (trichothecolone)	II; R^1=R^2=R^4=H, R^3=OH	170–5
Trichothecin	II; R^1=R^2=R^4=H, R^3=OCO.CH[c]=CH.Me	110
2β-Chloro-4β-(cis-crotonoyloxy)-13-hydroxyapotrichothec-9-en-8-one (trichothecin chlorhydrin)	XI	129
12, 13-Epoxytrichothec-9-en-4β, 15-diol (verrucarol)	I; R^1=R^4=H, R^2=R^3=OH	156
Verrucarin A		>300

the deacetylated compound (I, R^1=H, R^2=R^3=R^4=OH) which showed mammalian toxicity of the same high order (LD_{50} 0·81 mg/kg, i.p. in rat), was responsible for the toxicity. To exclude these, and other complications inherent in animal testing, and also to conserve valuable material, an *in vitro* cytotoxicity test[21] was used. Such tests provide more reliable information on the relation between chemical structure and intrinsic biological activity in a closely related group of compounds.

MATERIALS AND METHODS

Compounds. These were prepared by literature methods[10,19,20] and were purified by chromatography, followed by recrystallization, until no impurities could be detected as dark spots in u.v. light after thin-layer chromatography (TLC) on silica gel HF_{254} (Merck) in chloroform–methanol (9:1) and benzene–methanol (3:1). The systematic names[1] of the compounds and the m.p.'s of the purified specimens are recorded in Table 1.

Tissue culture cells. Cultures of the continuous cell lines HEp2, of human origin, and BHK, from baby hamster kidney, were used. The methods of culture and of interpreting the tests have been described previously.[22] Non-toxic concentrations (0·5% v/v) of ethanol or acetone were used to dissolve compounds which were insoluble in water.

RESULTS

The results are contained in Table 2 where the Lowest Toxic Dose listed represents the lowest concentration of each compound at which cytopathogenic effects could be clearly recognised. The effects were closely similar with all compounds found to be cytotoxic. Briefly, at the edges of cell sheets, individual cells became detached from the glass at their extremities and progressively shortened until they were isolated from each other. Eventually each degenerating cell presented a condensed, granular appearance. Swelling of cells and cytostatic effects could often be noted at lower concentrations than those listed but, for purposes of comparison, the clearcut cytopathogenic effect was taken as the endpoint.

The compounds inhibited the development of the two cell lines at comparable concentrations.

DISCUSSION

At 1 ng/ml the *in vitro* toxicity of verrucarin A, (I; R^1=R^4=H, R^2R^3=O.CO.CHOH.CHMe.CH_2.CH_2.O.CO CH≦CH.CH.≦CH.CO.O.) to HEp2 and BHK cells was comparable with that of the mycotoxin sporidesmin,[22] and is of the same order as the LD_{50} values given for verrucarin A against a number of normal and malignant human and rodent cell lines.[23] Trichothecin (II; R^1=R^2=R^4=H, R^3=O.CO.CH≦CH.Me) was somewhat less toxic. By contrast, the parent alcohols verrucarol, (I;R^1=R^4=H, R^2=R^3=OH) and trichothecolone (II; R^1=R^2=R^4=H, R^3=OH), were relatively non-toxic. These results may indicate that the greater lipoid solubility and lipoid-water partition ratio of the esters is of importance in effecting penetration of the cells in these test systems. As one example of this effect it has been shown[24] that the *in vitro* toxicities of a homologous series of 5-alkyl-2,4,6-trichloropyrimidines to a KB cell culture passed through a maximum[25] as the series was ascended.

These considerations do not apply with the same force, apparently, to the toxic metabolic products of *F. equiseti*: the triol (I; R^1=H, R^2=R^3=R^4=OH) (scirpentriol) and tetraol (II; R^1=R^2=R^3=R^4=OH) are more water soluble than the diol (I; (R^1=R^4=H, R^2=R^3=OH) but they were considerably more toxic, though appreciably less so than their diacetyl derivatives (I; R^1=H, R^2=R^3=OAc, R^4=OH) (diacetoxyscirpenol) and (II; R^1=R^4=OH, R^2=R^3=OAc) respectively. The cytotoxicity of diacetoxyscirpenol approached that of verrucarin A but was diminished on complete acetylation to (I; R^1=H, R^2=R^3=R^4=OAc). The triacetate (I; R^1=H,

TABLE 2.

Compound	Cell line: HEp2 Lowest toxic dose‡ (ng/ml)	Cell line: Baby hamster kidney (BHK) Lowest toxic dose (ng/ml)
(I; R^1 = H, R^2 = R^3 = OAc, R^4 = OH)	5§	1·5‖
(I; R^1 = H, R^2 = R^3 = R^4 = OAc)	25	25
(I; R^1 = H, R^2 = R^3 = R^4 = OH)	75	25
(IV)	100	100
(II; R^1 = R^4 = OH, R^2 = R^3 = OAc)	30	18
(II; R^1 = R^2 = R^3 = R^4 = OH)	225	200
(V; R = H)	>20,000¶	>5000
(V; R = Ac)*	5000	5000
(VI; R^1 = OH, R^2 = R^3 = R^4 = OAc)	3000¶	2500
(VI; R^1 = R^2 = R^3 = R^4 = OH)	10,000¶	10,000
(VII)*	>10,000	>20,000
(VIII)	>20,000	>20,000
(IX)*	>10,000	10,000
(X)*	>20,000	>10,000
(II; R^1 = R^2 = R^4 = H, R^3 = OH)	5000	2500
(II; R^1 = R^2 = R^4 = H, R^3 = OCO.CH = CH.Me)*	75¶	75
(XI)*	>10,000	>10,000
(I; R^1 = R^4 = H, R^2 = R^3 = OH)	7500	5000
Verrucarin A†	1¶	1

* In presence of ethanol.
† In presence of ethanol and acetone.
‡ Mean of 2 determinations except where stated to the contrary.
§ Mean of 8 determinations, range 2–10 ng/ml.
‖ Mean of 2 determinations, range 1–2 ng/ml.
¶ Mean of 3 determinations.

R^2=R^3=R^4=OAc) is hydrolyzed *in vitro* to the triol (I; R^1=H, R^2=R^3=R^4=OH) at least as readily as diacetoxyscirpenol.[19] It is unlikely therefore that the triol, if formed by hydrolysis after penetration of the lipoid soluble ester into the cell, is the common toxic molecular species in this series, unless some highly specific esterase is involved. Both diacetoxyscirpenol and the parent triol appear to be cytotoxic molecular species *per se*.

The *in vitro* toxicity of diacetoxyscirpenol was diminished by reduction of the 9-ene to give the ester (IV), and eliminated, compared with the triol (I; R^1=H, R^2=R^3=R^4=OH), by reductive opening of the 12, 13-epoxy group to give the tetraol (V; R=H). The triacetate (V; R=Ac) of this alcohol was also non-toxic. The 10→13-cyclo-rearrangement products (VI; R^1=OH, R^2=R^3=R^4=OAc),

(VI; $R^1=R^2=R^3=R^4=OH$) and (VII) and the apotrichothecene (VIII) were likewise non-toxic; as also, compared with the parent ester (IV), were the ring C *seco*-compounds (IX) and (X) in which the 12, 13-epoxy group is retained but is now accessible to rearside nucleophilic attack.

These results are consistent with the hypothesis that toxicity is associated with the presence of the protected 12, 13-epoxy group on the trichothecane system; but additional molecular features, notably the presence of a 9-ene and esterification of some, though not all, of the hydroxyl substituents are important factors in the manifestation of high toxicity.

Acid catalysed hydrolytic opening of the epoxide group with accompanying rearrangement of the carbon skeleton to the 10→13-cyclo-system (VI) is the method of choice for the detoxication of diacetoxyscirpenol and its near relatives of structure (I). This reaction is inhibited by the presence of an 8-keto group,[20] and stronger acid treatment, giving the apotrichothecene system, is required for detoxication of mycotoxins of structure (II). The apotrichothec-9-en-8-one (XI) (trichothecin chlorohydrin), like (VIII), was non-toxic.

In connection with these *in vitro* toxicity results, it is of interest that the antifungal activity of trichothecin is diminished by hydrolysis of the ester group and is eliminated on rearrangement to the apotrichothecene system.[26]

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