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Further Biological Properties of Trichothecin, an Antifungal Substance from *Trichothecium roseum* Link, and its Derivatives

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SUMMARY: Production of trichothecin in submerged aerated cultures of a strain of *Trichothecium roseum* is described. The antibiotic is active *in vitro* against certain fungi pathogenic in man, including the causative organisms of some generalized infections and several of the common skin pathogens. In general, the sensitive pathogens were inhibited at trichothecin concentrations of 16 p.p.m. or less and the antifungal activity was not influenced by the presence of blood serum (5 %) in the medium. Trichothecin is toxic to laboratory animals; single doses at the rate of 250 mg./kg. intravenously in mice and subcutaneously in rats resulted in death. The antibiotic also produced a reaction when brought into contact with the skin.

The antifungal activity of a series of esters related to trichothecin was of the same order as that of the parent substance. With the exception of esters in which the ethylenic double bond of trichothecolone had been saturated, none of the known derivatives or fission products exhibits more than a very slight activity. The presence of a large excess of β -indolylacetic acid did not affect the antifungal activity of trichothecin. This suggests that the effect of the latter in inhibiting plant growth regulating activity of β -indolylacetic acid and related substances is not due to molecular combination of the two substances.

The presence of an antifungal compound in culture filtrates of *Trichothecium roseum* was reported by Brian & Hemming (1947), and the substance, trichothecin, responsible for the antifungal activity was isolated and some of its chemical and biological properties described by Freeman & Morrison (1949*a, b*). In addition to its antifungal action, trichothecin was reported by Hessayon (1951) to give well-marked stimulation of mycelial growth, at exceedingly small concentrations. Other metabolic products of the mould have been described by Robertson, Smithies & Tittensor (1949) and by Freeman, Morrison & Michael (1949). The activity of trichothecin as an inhibitor of plant virus infectivity and isolation from culture filtrates of *T. roseum* of a polysaccharide which behaved as an inhibitor, was described by Bawden & Freeman (1952). Freeman & Gill (1950) showed that trichothecin is the *isocrotonyl* ester ($C_{16}H_{24}O_5$) of the ketonic alcohol trichothecolone ($C_{15}H_{20}O_4$). Darpoux & Faivre-Amiot (1952) reported the presence in culture filtrates of *T. roseum* of a gummy substance (irritant substance) which gives rise to burns when placed on the skin. The presence of this irritant substance has been confirmed by the present author (unpublished observations), but it has not yet been obtained in a crystalline state. Yasue (1948, 1949) found that

a culture filtrate of *Cephalothecium roseum* (*T. roseum*) inhibited growth of *Staphylococcus aureus*. The active substance was not characterized, but Freeman, Morrison & Michael (1949) showed that rosein II had weak anti-bacterial activity.

The present paper is concerned with further biological properties of trichothecin including inhibition of growth of certain fungi pathogenic to man and toxicity in animals. The antifungal activity of various esters related to trichothecin and of some derivatives is reported.

EXPERIMENTAL

Preparation of trichothecin

Trichothecin was prepared from *Trichothecium roseum* strain no. F 292 in our laboratory collection (originally isolated at the London School of Hygiene and Tropical Medicine from tomato by Mrs Marcus; LSHTM catalogue no. 95). This strain gave maximum trichothecin yields of about 200 mg./l. in the culture filtrate after 21 days of incubation. The method of isolation closely followed that described by Freeman & Morrison (1949*a*); the most important modifications were: (a) addition of zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g./l.) to the culture medium which increased the yield of antibiotic and promoted improved sporulation of the fungus; (b) use of carbon tetrachloride, instead of chloroform, as a more specific solvent for extraction of antifungal substance from the culture filtrate. The crude residue from the carbon tetrachloride extract contained about 50 % trichothecin; approximately half of this was recovered as the pure crystalline substance by the chromatographic method previously described.

Fermentations carried out with the above strain in submerged culture showed that trichothecin yields of 200 mg./l. were reached after 90 hr. incubation at 25° in a medium based on that of Freeman & Morrison (1949*a*) in which the initial pH value was adjusted to 7.0 by addition of sodium hydroxide. The fermentations were carried out in a stirred aerated medium (1.2 l.) in 2 l. glass bolthead flasks. The cultures were stirred with paddle stirrers (blade, 4.5 × 2 cm.) rotated at 500 r.p.m. and aerated through a porcelain filter (Aerox) with air at 0.5 vol./vol./min. The inoculum for each culture was a suspension containing *c.* 10×10^6 spores from a 7-day culture on beer wort agar. Good mycelial growth was established after 48 hr. incubation. The mycelium was pink in colour; microscopical examination showed that the hyphae were rough and pitted and there was abundant sporulation. Foaming was controlled by manual addition of octadecanol (2 %) in arachis oil. The trichothecin yield remained practically constant for a further 80 hr. after the maximum concentration had been reached and the pH did not change appreciably during this period.

In vitro activity of trichothecin against fungi pathogenic to man

The growth of twenty-seven representative non-pathogenic Fungi Imperfecti, Zygomycetes and Ascomycetes was inhibited to some degree by trichothecin (Freeman & Morrison, 1949*b*). *In vitro* tests were carried out with

eighteen cultures of pathogenic fungi, including some dermatophytes, in the presence of trichothecin concentrations of 0.64–80 mg./l. Growth of the following fungi was inhibited at 16 mg. trichothecin/l. or less: *Blastomyces dermatitidis*, *Torulopsis neoformans*, *Candida albicans*, *Hormodendrum langeroni*, *Histoplasma capsulatum*, *Epidermophyton floccosum*, *Trichophyton rubrum*, *T. interdigitale*, *T. sulphurum*, *T. mentagrophytes* and *Microsporium canis* (Table 1). Where tests were made, the antifungal activity was not significantly influenced by the presence of blood serum (5%) in the culture medium.

Table 1. In vitro activity of trichothecin on the growth of certain pathogenic fungi

The fungi were seeded on to the surface of agar media containing a series of trichothecin concentrations. After 5 days of incubation at 25° growth was compared with that obtained on control plates containing no antifungal substance. No growth, 0; trace of growth, 1; slight growth, 2; good growth, 3; abundant growth (as control), 4.

Organism	Laboratory catalogue no.	Medium	Trichothecin concentration (mg./l.)			
			80	16	3.2	0.64
			Relative growth			
<i>Actinomyces asteroides</i> (Epp.) Gasp.	278	Nutrient agar + 5 % serum	4	4	4	4
<i>Coccidioides immitis</i> Stiles	279	Beer wort agar	4	4	4	4
<i>Blastomyces dermatitidis</i> (Gilchrist and Stokes)	280	Nutrient agar + 5 % serum	1	1	1	3
<i>B. dermatitidis</i> (yeast-like form)	291	Nutrient agar + 1 % glucose	0	1	2	3
<i>Torulopsis neoformans</i> Lodder	281	Nutrient agar	0	0	2	3
<i>T. neoformans</i> Lodder	281	Nutrient agar + 5 % serum	0	0	2	3
<i>Candida albicans</i> (Robin) Berkh.	282	Nutrient agar	0	0	3	4
<i>C. albicans</i> (Robin) Berkh.	282	Nutrient agar + 5 % serum	0	0	3	4
<i>Hormodendrum langeroni</i>	283	Beer wort agar	0	1	3	4
<i>Sporotrichum tropicale</i>	286	Nutrient agar + 1 % glucose	2	3	4	4
<i>S. schencki</i> Matr.	287	Nutrient agar + 1 % glucose	3	4	4	4
<i>S. beurmanni</i> Matr. & Ramond	288	Nutrient agar + 1 % glucose	2	3	4	4
<i>Geotrichum cutaneum</i>	289	Nutrient agar + 1 % glucose	0	3	4	4
<i>Histoplasma capsulatum</i> Darling	290	Nutrient agar + 1 % glucose	0	0	0	3
<i>Epidermophyton floccosum</i> (Harz) Lang. and Miloch.	294	Beer wort agar	0	1	2	3
<i>Trichophyton rubrum</i> Semon	295	Beer wort agar	0	0	4	4
<i>T. interdigitale</i> Priestley	296	Beer wort agar	0	1	4	4
<i>T. sulphureum</i> Sab.	297	Beer wort agar	0	1	2	3
<i>T. mentagrophytes</i> Geddoelst	298	Beer wort agar	0	0	2	4
<i>Microsporium canis</i> Bodin	299	Beer wort agar	0	0	3	3

Toxicity tests in animals

Unfortunately the antifungal activity of trichothecin is of no practical importance in treating infections in animals owing to the high toxicity of the antibiotic. The following tests of toxicity of trichothecin on intravenous injection into mice were carried out by Dr A. R. Martin. Trichothecin (0.25 g.) was dispersed in 2.5 ml. of a solution containing 'Dispersol' OG (5%) and methyl ethyl cellulose (0.1%) by milling overnight with steel balls. Further dilutions of the resulting suspension (100 mg./ml.) were made with the above

solution of dispersing agents. Groups of three mice (18–22 g.) were injected intravenously with 0.1 ml. trichothecin suspension; each injection was made at a steady rate over a period of 15 sec. The results noted in Table 2 were observed. To confirm that the immediate collapse observed after injection of trichothecin was not due to mechanical blockage of capillaries by particles of the antibiotic, suspensions of sulphathiazole, milled under conditions similar to those used for trichothecin, were injected into mice. Milling of the sulphathiazole suspension was arrested when the particles of the drug were of the same order of size as those of the trichothecin suspension. Injection of 10 mg. of sulphathiazole in this form did not cause any immediate collapse comparable with that produced by the injection of 1 mg. trichothecin. Injection of a second trichothecin suspension, which was ball-milled for 40 hr. and contained a smaller proportion of relatively large particles than that described above gave results not significantly different from those of the previous preparation. It was concluded that the observed toxicity was an intrinsic property of the antibiotic, independent of its physical form.

Table 2. *Results of toxicity tests of trichothecin in mice*

Dose of trichothecin		Effect
(mg./20 g. mouse)	(mg./kg.)	
0.1	5	None
0.25	12.5	Transient flaccid paralysis of hind legs lasting c. 20 sec.
0.5	25	Paralysis of hind legs followed by unsteady gait, recovery after 2–5 min.
1.0	50	Collapse immediately after injection. Hind legs paralysed for about 5 min. Animals recovered
2.5	125	Collapse followed by recovery
5.0	250	Initial collapse. Two mice began to recover after 4 min., third mouse died. Normal gait in 2 survivors after 10 min.
10.0	500	Collapse followed by death of all mice within 30 sec.

Three rats (100 g.) were injected subcutaneously with a solution of trichothecin (25 mg.) in arachis oil (0.5 ml.) (dose, 250 mg./kg.). The animals became ill after 1.5 hr. with severe diarrhoea and excessive micturition and were dead after 16 hr. Post-mortem examination confirmed the observation of diarrhoea by the complete absence of formed faeces in the large intestine. The livers were paler than normal.

When trichothecin (5 mg.) as a 1 % solution in arachis oil was applied daily to shaved guinea-pig skin, reddening and soreness followed by cracking and pin-point haemorrhages were observed after five applications. Similar treatment of the inner surface of a rabbit's ear resulted in reddening of the skin followed by scaly patches after three applications. One drop of the 1 % solution applied to the skin of the human head caused definite irritation. Four drops of the same solution (two drops at 2 hr. intervals) introduced into a rabbit's eye caused slight inflammation of the conjunctiva, nictitating mem-

brane and lower eyelid. Gorlenko (1948) reported the presence of endotoxins in the mycelium of *Trichothecium roseum* and other moulds. This toxicity does not appear to be associated with that of trichothecin since the medium upon which the mould was grown was free from the toxin.

Antifungal activity of various esters related to trichothecin

Trichothecin ($C_{19}H_{24}O_5$) is the *isocrotonyl* ester of the ketonic alcohol trichothecolone ($C_{15}H_{20}O_4$; Freeman & Gill, 1950). The following synthetic esters related to trichothecin were prepared: acetyltrichothecolone, acetyldihydrotrichothecolone, crotonyltrichothecolone and butyryltrichothecolone (Table 3). Hydrolysis of trichothecin with boiling aqueous sodium hydroxide gave *isocrotonic* acid and a ketonic alcohol, *isotrichothecolone*, isomeric with trichothecolone (Freeman & Gill, 1950). The corresponding acetyl ester, acetyl *isotrichothecolone*, was prepared and its biological properties examined.

Functional groups in the trichothecolone portion of the molecule include an ethylenic double bond and an endomethylene oxy bridge. The ethylenic double bond is saturated in dihydrotrichothecolone and the corresponding tetrahydrotrichothecin. The endomethylene oxy bridge may be opened with the addition of the elements of water and of hydrogen chloride with formation of trichothecin glycol and trichothecin chlorohydrin. Details of the preparation and properties of these esters and derivatives will be published elsewhere. Their antifungal activities against six test fungi were determined by plate tests in which the substances were dissolved in the beer wort agar (Table 3).

The results showed that acetyltrichothecolone, butyryltrichothecolone and crotonyltrichothecolone were of the same order of activity as trichothecin. Trichothecolone, acetyldihydrotrichothecolone, and tetrahydrotrichothecin exhibited very slight activity against some of the test fungi and none against others which were less sensitive. *Isotrichothecolone*, acetyl*isotrichothecolone*, trichothecin hydrate and trichothecin chlorohydrin were inactive.

The relative activity of these substances was determined more accurately by measurement of their inhibition of germination of *Penicillium digitatum* conidia. The basis of the method was described by the Committee on Standardization of Fungicidal Tests of the American Phytopathological Society (1947). Its use for trichothecin determination was described by Freeman & Morrison (1949*b*); the results are summarized in Table 4. It is concluded that the antifungal activity of trichothecin is a function of the molecule as a whole. Saturation of the ethylenic double bond in the trichothecolone portion of the molecule (as in acetyldihydrotrichothecolone and tetrahydrotrichothecin) destroyed the activity almost completely. Complete destruction of the activity followed isomerization with hot alkali (*isotrichothecolone* and the corresponding ester, acetyl*isotrichothecolone*) or opening of the endomethylene-oxy bridge by addition of the elements of water or of hydrogen chloride (as in trichothecin glycol and trichothecin chlorohydrin, respectively).

Table 3. *Antifungal activity of various esters related to trichothecin and trichothecin derivatives*

The test fungi were seeded on to the surface of beer wort agar containing different concentrations of the compounds. Period of incubation 5 days at 25°. Scoring of growth as in Table 1.

Details of fungi tested: *Aspergillus niger* van Tiegh. F160; *Mucor erectus* Bain. F266;
Penicillium digitatum Sacc. F196; *P. nigricans janczewskii* series F211;
Saccharomyces carlsbergensis Hansen B55;
Trichothecium roseum Link F227.

Substance	Relative growth of test fungi at concentrations of trichothecin derivatives															
	<i>Aspergillus niger</i>				<i>Mucor erectus</i>				<i>Penicillium digitatum</i>				<i>P. nigricans janczewskii</i>			
	80	16	3.2	0.6	80	16	3.2	0.6	80	16	3.2	0.6	80	16	3.2	0.6
Trichothecin	0	4	4	4	0	1	3	4	0	0	1	2	3	4	4	4
Acetyltrichothecolone	4	4	4	4	2	4	4	4	0	1	2	4	4	4	4	4
Butyryltrichothecolone	1	2	4	4	3	3	4	4	0	1	2	3	4	4	4	4
Crotonyltrichothecolone	2	3	4	4	3	4	4	4	0	0	2	4	4	4	4	4
Trichothecolone	4	4	4	4	4	4	4	4	1	2	4	4	3	4	4	4
Acetyldehydrotrichothecolone	4	4	4	4	4	4	4	4	0	1	4	4	3	4	4	4
Tetrahydrotrichothecin	4	4	4	4	4	4	4	4	1	4	4	4	4	4	4	4
	Relative growth															
	0	4	4	4	0	1	3	4	0	0	1	2	3	4	4	4
	4	4	4	4	2	4	4	4	0	1	2	4	4	4	4	4
	1	2	4	4	3	3	4	4	0	1	2	3	4	4	4	4
	2	3	4	4	3	4	4	4	0	0	2	4	4	4	4	4
	4	4	4	4	4	4	4	4	1	2	4	4	3	4	4	4
	4	4	4	4	4	4	4	4	0	1	4	4	3	4	4	4
	4	4	4	4	4	4	4	4	1	4	4	4	4	4	4	4
	0	4	4	4	0	1	3	4	0	0	1	2	3	4	4	4
	4	4	4	4	2	4	4	4	0	1	2	4	4	4	4	4
	1	2	4	4	3	3	4	4	0	1	2	3	4	4	4	4
	2	3	4	4	3	4	4	4	0	0	2	4	4	4	4	4
	4	4	4	4	4	4	4	4	1	2	4	4	3	4	4	4
	4	4	4	4	4	4	4	4	0	1	4	4	3	4	4	4
	4	4	4	4	4	4	4	4	1	4	4	4	4	4	4	4
	0	4	4	4	0	1	3	4	0	0	1	2	3	4	4	4
	4	4	4	4	2	4	4	4	0	1	2	4	4	4	4	4
	1	2	4	4	3	3	4	4	0	1	2	3	4	4	4	4
	2	3	4	4	3	4	4	4	0	0	2	4	4	4	4	4
	4	4	4	4	4	4	4	4	1	2	4	4	3	4	4	4
	4	4	4	4	4	4	4	4	0	1	4	4	3	4	4	4
	4	4	4	4	4	4	4	4	1	4	4	4	4	4	4	4

*Iso*trichothecolone, acetyl*iso*trichothecolone, trichothecin glycol and trichothecin chlorohydrin were inactive against the above test fungi.

Table 4. Activity of various esters and trichothecin derivatives as inhibitors of germination of *Penicillium digitatum* conidia

Germination of *Penicillium digitatum* conidia in Czapek-Dox medium containing 5 % (w/v) glucose at pH 4.2 was observed after 18 hr. incubation at 25° in the presence of a series of concentrations of the substances. The concentration which permitted 50 % germination (ED 50) was determined graphically.

Substance	ED 50 (mg./l.)	Activity as % that of trichothecin
Trichothecin*	0.38	100
Acetyltrichothecolone†	0.47	81
Crotonyltrichothecolone‡	1.6	23.7
Butyryltrichothecolone‡	1.9	20
Acetyldihydrotrichothecolone	6.6	5.8
Tetrahydrotrichothecin	23	1.7
Trichothecolone	34	1.1
Isotrichothecolone	> 50	< 0.8
Acetylisotrichothecolone	> 50	< 0.8
Trichothecin glycol	> 50	< 0.8
Trichothecin chlorohydrin	> 50	< 0.8

* Germination virtually completely inhibited by 0.78 mg./l.; 100 % germination at 0.19 mg./l.

† Germination virtually completely inhibited by 1 mg./l.; 100 % germination at 0.26 mg./l.

‡ Germination virtually completely inhibited by 3 mg./l.; 100 % germination at 0.77 mg./l.

Antifungal activity of trichothecin in the presence of β -indolylacetic acid

Dr W. G. Templeman has observed that trichothecin inhibits the activity of β -indolylacetic acid and other plant growth regulators (private communication). About two molecular proportions of trichothecin are required for complete inhibition of the activity of one molecule of β -indolylacetic acid. None of the known reactions of trichothecin suggests that combination with β -indolylacetic acid in these proportions does, in fact, occur. As shown above, all the known modifications to the trichothecin molecule, with the exception of substitution of other acidic groups for the *isocrotonyl* radical in the ester, result in complete or almost complete destruction of the antifungal activity. Incubation of as much as 60 equivalents of β -indolylacetic acid with trichothecin had no effect on the germination of *P. digitatum* conidia in presence of the mixture, as compared with conidia incubated with the corresponding quantities of trichothecin (Table 5). This evidence suggests that no interaction takes place between β -indolylacetic acid and trichothecin in aqueous solution, under these conditions, since it is regarded as unlikely that combination would take place without affecting the antifungal activity.

DISCUSSION

The work reported above on the biological activity of trichothecin and its derivatives has not revealed any close relationship between the functional groups in the molecule and its antifungal activity. The evidence so far as it goes indicates that the activity is dependent upon the molecular structure as a whole, since all the modifications which it has been possible to introduce,

with the exception of substitution of the *isocrotonyl* radical by other acidic groups in the ester, have resulted in complete or almost complete destruction of the activity.

Table 5. *Germination of conidia of Penicillium digitatum in the presence of mixtures of trichothecin and β -indolylacetic acid*

The experimental conditions were similar to those described in Table 4. Solutions containing twice the concentrations of trichothecin and β -indolylacetic acid, shown below, were incubated at 25° for 1.5 hr. before addition of equal volumes of the spore suspension.

There was 100 % germination in presence of β -indolylacetic acid (up to 50 mg./l.) alone.

β -indolylacetic acid (mg./l.)	Germination of conidia in presence of trichothecin (mg./l.)					ED 50 (mg./l.)
	1.25	0.625	0.313	0.156	0.078	
	% germination					
Nil	0	50	80	100	100	0.50
0.025	0	60	80	100	100	0.52
0.25	0	40	80	100	100	0.48
2.5	0	60	80	100	100	0.52
25	1	50	80	100	100	0.52

Data are now beginning to accumulate on the action of trichothecin, and in some cases also of its derivatives, towards a number of widely different biological systems including fungi, viruses, flowering plants and mammals. Different molecular functions are probably responsible for the activity in certain of these fields. Thus, whereas the antifungal activity of trichothecin is practically lost in its derivatives, with the exception of certain esters, trichothecolone and acetyltrichothecolone more effectively prevented infection of *Nicotiana glutinosa* by two test viruses than did trichothecin (Bawden & Freeman, 1952). Similar comparisons of the activities of trichothecin and its derivatives towards plant growth regulators and comparisons of their toxicities in animals may lead to interesting conclusions.

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