The Isolation and Chemical Properties of Trichothecin, an Antifungal Substance from Trichothecium roseum Link

BY G. G. FREEMAN AND R. I. MORRISON
Imperial Chemical Industries, Ltd., Nobel Division, Research Department, Stevenston, Ayrshire

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Antagonism between Trichothecium roseum Link and various fungi pathogenic to plants has been reported by Whetzel (1909), Boning (1933), Koch (1934) and Greaney & Machacek (1935). Culture filtrates of T. roseum have been shown to inhibit germination of Botrytis allii conidia (Brian & Hemming, 1947). A preliminary account of the isolation and properties of trichothecin, an antifungal compound from Trichothecium roseum, has been given by Freeman & Morrison (1948). The present paper gives a detailed description of the isolation and properties of the antifungal compound.

EXPERIMENTAL

Strains of Trichothecium roseum

The strains used in this work were typical cultures of T. roseum and were isolated in the laboratory mainly from dead wood collected locally. The strains are distinguished by their laboratory catalogue numbers. The cultures are described in detail by Freeman & Morrison (1949).

Isolation of trichothecin

Medium. The following medium was used for production of trichothecin. It is based on the formula of Czapek (as modified by Dox, 1910); ammonium tartrate has been substituted for NaN03 as source of N, and corn steep liquor has been added as a supplementary nutrient (Freeman & Morrison, 1949).

Ammonium tartrate 2-0 g.
MgSO4·7H2O 0-5 g.
K2HPO4 1-0 g.
KCl 0-5 g.
FeSO4·7H2O 0-01 g.
Glucose 50 g.
Corn steep liquor 10 ml.
Water to 1000 ml.

Crude commercial glucose ('glucose chips') was found to be satisfactory for this purpose. The medium (800 ml.) was sterilized by autoclaving in 'Glaxo' bottles (Clayton, Hems, Robinson, Andrews & Hunwicke, 1944). The reaction was adjusted to pH 5-0. Each bottle was inoculated with 1 ml. of a spore suspension from a test-tube slope culture (about 7 days old) of T. roseum F 227 on beer-wort agar medium. The cultures were incubated at 25° for 28 days in darkness.

Extraction and isolation. At the time of harvesting, trichothecin concentration in the culture filtrates was determined by the Penicillium digitatum spore-germination method (Freeman & Morrison, 1949). The cultures were filtered, the mycelium washed with a little water and the filtrates and washings from a batch of bottles (usually about 40) combined for chloroform extraction. The filtrates were clear and brown in colour; they were readily filtered through paper. The filtrate in lots of 1 l. was twice extracted with chloroform (200 ml.). The extracted liquor was free from antifungal activity as determined by the P. digitatum spore-germination method. The chloroform extracts were combined and evaporated to dryness under reduced pressure. The dried extract from 1 l. of culture filtrate was obtained as a brown gummy solid (2-165 g.) which was fractionated as shown in Fig. 1; further details of the chromatographic technique are given below.

In one experiment, the combined extracts from 84 l. (7-885 g.) were dissolved in ether (25 ml.) and poured on to a column (30 cm. long × 2-2 cm. diam.) of activated alumina. The column was developed with ether and the eluate collected in 100 ml. fractions. Practically the whole of the antifungal substance was contained in fractions 1, 2 and 3, which gave dry weights of 1-392, 3-196 and 0-433 g. respectively. The fractions were dissolved in a 9:1 (v/v) mixture of light petroleum (b.p. 60–80°) and chloroform (100 ml.) on a steam bath. After 18 hr. at 20°, crystalline precipitates of an inactive compound (II) were separated off. On evaporation to dryness, the combined filtrates gave an almost colourless syrup (4-292 g.). The latter was dissolved in carbon tetrachloride (25 ml.), and poured on to an alumina column similar to that described above. The chromatogram was developed with a mixture of chloroform and carbon tetrachloride (1:1) and the eluate collected in 50 ml. fractions. On evaporation to dryness, the trichothecin fractions crystallized as fine, colourless needles (3-472 g.). Recrystallization from hot light petroleum (b.p. 60–80°), gave pure trichothecin (3-015 g.), m.p. 118°. (All melting points are corrected.) Yield about 36 mg./l. of culture filtrate. Details of the recovery from three batches are given in Table 1.

Properties of trichothecin

Biological. The antifungal activity of trichothecin is exhibited against Fungi Imperfecti, Zygomyceotes and Ascmyoeotes. The growth of each of some 26 species belonging to the above classes was in some degree inhibited (Freeman & Morrison, 1949). At a concentration of 400 mg./l. and pH 7-0, trichothecin was inactive against Staphylococcus aureus, Bacillus subtilis and Escherichia coli.

Chemical and physical. Trichothecin crystallizes from light petroleum in long fibrous needles, m.p. 118°. It is readily soluble in chloroform, ethanol, acetone and benzene,
Culture filtrate (16 l.)

Chloroform extraction

Extract (2-165 g.)
Dissolved in chloroform (5 ml.)
and treated with excess ether
(100 ml.)

Extract
Evaporated to dryness gave
2-149 g. residue, dissolved in
chloroform (5 ml.) and diluted
with light petroleum, b.p. 60–80°
(100 ml.)

Residual liquor rejected

Precipitate rejected

Evaporated to dryness gave clear
yellow syrup (1-337 g.)

Evaporated to dryness gave 0-340 g. residue

Extract Evaporated to dryness gave clear
yellow syrup (1-337 g.)

Precipitate (I)
Dissolved in chloroform (5 ml.)
and diluted with light
petroleum (100 ml.)

Combined extracts (1-677 g.)
Chromatographed in ether
solution on Al₂O₃

Combined antifungal fractions (1-068 g.)
Dissolved in boiling light
petroleum-chloroform and
allowed to cool

Evaporated to dryness gave
0-913 g. residue. Dissolved in
CCl₄ and chromatographed on
Al₂O₃

Crystalline precipitate (II)
(0-155 g.)

Trichothecin (0-738 g.)

Fig. 1. Isolation of trichothecin.

Table 1. Summary of data on recovery of trichothecin from batches of culture filtrate

<table>
<thead>
<tr>
<th>Batch no.</th>
<th>Time of incubation (days)</th>
<th>Vol. of culture filtrate (l.)</th>
<th>Dry wt. of mycelium (g.)</th>
<th>Trichothecin assay (mg./l.)</th>
<th>Total trichothecin calc. (g.)</th>
<th>Wt. of chloroform extract (g.)</th>
<th>Wt. of trichothecin isolated (g.)</th>
<th>Trichothecin yield from culture filtrate (mg./l.)</th>
<th>Assay (%)</th>
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<td>68-5</td>
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</table>
slightly soluble in light petroleum and only slightly soluble in water (400 mg./l. at 25°). It is optically active,\(\beta\) 1\(^{57}\) + 44\(^{\circ}\) (c, 1 in chloroform). The compound contains no halogen, S or N. Micro-analyses (Weiler and Strauss): Found: C, 68-8, 68-7, 68-8; H, 7-2, 7-3, 7-4; mol. wt. (Rast), 278. C\(_{13}\)H\(_{18}\)O\(_{4}\) requires C, 68-2; H, 7-5; mol. wt. 264. C\(_{13}\)H\(_{18}\)O\(_{4}\) requires C, 68-7; H, 6-9%; mol. wt. 262.

There is no evidence of the presence of free carboxyl, hydroxyl, alkali or aldehydic groups in the molecule. The presence of a ketonic group is indicated by reactions with hydroxylamine, semicarbazine and 2,4-dinitrophenylhydrazine. The oxide and semicarbazone were not well characterized crystalline derivatives.

**Trichothecin**

Trichothecin (0-13 g., 0-5 mmol.) was dissolved in ethanol (2 ml.) and mixed with 2,4-dinitrophenylhydrazine (0-1 g.) which had been dissolved in conc. H\(_2\)SO\(_4\) (0-3 ml.) and diluted with ethanol (2 ml.). After 18 hr. at 20° the mixture was diluted with 2\(_{\text{N}}\)-H\(_2\)SO\(_4\) (10 ml.) and extracted with ether (20 ml.). The extract was washed with 2\(_{\text{N}}\)-H\(_2\)SO\(_4\) then several times with water and dried over anhydrous Na\(_2\)SO\(_4\). After filtration and concentration to 10 ml. the solution was chromatographed on a column of activated alumina. The main fraction (orange band) was eluted with ether and recrystallized from industrial spirit as orange prisms, m.p. 193°. A second recrystallization from ethyl alcohol gave the hydrazide, m.p. 200°. Found: C, 57-8, 58-6; H, 5-3, 5-5; N, 10-6, 12-6. C\(_{13}\)H\(_{18}\)O\(_{4}\)N\(_2\) requires C, 56-9; H, 5-4; N, 12-6%.

The presence of ethylenic unsaturation was indicated by reduction of KMnO\(_4\) in cold acetone solution of trichothecin. There was, however, no reaction with Br\(_2\) in carbon tetrachloride. Microhydrogenations of trichothecin in acetic acid solution were carried out in the presence of Pd black catalyst and Adam's platinum oxide catalyst. With the Pd catalyst, trichothecin (3-255 mg.) absorbed 0-570 ml. H\(_2\) at 744 mm./18°; absorption was complete in 35 min. Number of double bonds = 1-89. With the Adam's platinum oxide catalyst, trichothecin (3-240 mg.) absorbed 0-600 ml. H\(_2\) at 742 mm./20°, absorption being complete in 20 min. Number of double bonds = 1-98. (The authors are grateful to Prof. J. W. Cook, in whose laboratory these determinations were made.) Two molecules of H\(_2\) were absorbed in the presence of each of the catalysts. This is consistent with the presence of conjugated carbonyl and ethylenic groups.

Trichothecin (1-00 g.) in absolute ethanol (50 ml.) was catalytically reduced at 760 mm./20° in the presence of Adam's platinum oxide catalyst (0-02 g.). Hydrogenation was complete after 35 min. when 220 ml. of H\(_2\) (119% of the theoretical) had been absorbed. The product (1-021 g.) was a colourless syrup, which did not crystallize.

Determination of methyl groups attached to carbon (Weiler and Strauss) gave \(\text{C}(\text{CH})\), 16-4; C\(_{13}\)H\(_{18}\)O\(_{4}\)(CH\(_2\)) requires 17-0%. Methoxyl determination gave OCH\(_3\), 0-24%. Theoretical for 1 OCH\(_3\)/mol. (C\(_{13}\)H\(_{18}\)O\(_{4}\)), 11-8%.

**Alkaline hydrolysis of trichothecin.** Trichothecin (0-100 g.) was heated under reflux with ethanolic KOH (20 ml., approx. 0-1N). After 3 hr. the solution was cooled, diluted with water (10 ml.) and titrated with 0-1N-HCl using phenolphthalein as indicator. The titration difference was 3-75 ml. 0-1N-acid, which corresponds to an equivalent of 266 (theoretical for 1 potential carbonyl group per molecule (C\(_{13}\)H\(_{18}\)O\(_{4}\)), 264).

Excess acid was added and the bulk of the ethanol removed by distillation under reduced pressure. The residue was diluted with water (10 ml.) and twice extracted with chloroform. The extract was washed, dried over anhydrous Na\(_2\)SO\(_4\) and evaporated to dryness. The residue (0-091 g.) was a pale yellow syrup which crystallized on cooling. On recrystallization from a mixture of benzene and light petroleum, b.p. 60–80°, it was obtained as colourless prisms, m.p. 181°. The product was readily soluble in chloroform, and only slightly soluble in ether. The reactions of the compound showed that a ketonic group was present. Found: C, 67-8, 67-9; H, 7-5, 7-3; mol. wt. (Rast) 212, 299. C\(_{13}\)H\(_{18}\)O\(_{4}\) requires C, 68-2; H, 7-5; mol. wt. 264.

**Oxidative degradation.** Attempts to obtain characteristic degradation products by oxidation with various reagents such as KMnO\(_4\), K\(_2\)Cr\(_2\)O\(_7\), KBrO, lead tetra-acetate and SeO\(_2\) have so far been unsuccessful. Although oxidation usually took place, the products resisted attempts at crystalization or purification. Further work is being done on these lines.

**Stability of trichothecin.** The reaction of aqueous solutions of trichothecin (20 mg./l.) was adjusted to pH 1 and 10 by addition of HCl and NaOH, respectively. After suitable intervals, the solutions were neutralized and trichothecin concentration determined by the Penicillium digitatum spore-germination method. There was no significant loss of activity at 20° after 48 hr. at pH 1 and 10. At pH 12, the antifungal activity was completely destroyed in 6 hr. at 20°. The rate of inactivation of trichothecin at pH 12 and 20° corresponded to that of a first-order reaction in which \(k\) was approximately 0-01. Aqueous solutions of the antifungal substance at pH 7 showed no detectable loss of activity after 1 hr. at 100°.

Cavallito & Bailey (1944) reported that a number of antibiotics such as gliotoxin, patulin and penicillic acid were inactivated by cysteine. Addition of cysteine (30–1000 mg./l.) to a solution of trichothecin (0-625 mg./l.), which permitted germination of 5% of P. digitatum spores in 18 hr., had no effect on the number of spores which germinated.

**Ultraviolet absorption spectrum.** The following main absorption bands were observed with chloroform or hexane solutions of trichothecin, (i) broad shallow band at 334 m\(\mu\), with molar extinction coefficient \(\epsilon, 44\); (ii) intense band at 220 m\(\mu\); \(\epsilon, 10^4\). Ethanol solutions exhibited the following absorption bands, (i) broad shallow band at 325 m\(\mu\); \(\epsilon, 37\); (ii) intense band at 230 m\(\mu\); \(\epsilon, 10^4\).

The alkaline hydrolysis product from trichothecin (in chloroform or ethanol) exhibited the following absorption bands, (i) intense band at 230 m\(\mu\); \(\epsilon, 10^4\) (based on a mol. wt. of 262); (ii) shallow, broad band at 339 m\(\mu\); \(\epsilon, 49\). The position of the bands was not altered by a change of solvent from ethanol to the less polar solvent chloroform. Band (i) is attributed to the presence of an ethylenic linkage and band (ii) to a ketonic group.

**Infrared absorption spectrum.** The infrared absorption spectrum showed a group of characteristic bands at 815, 847, 970, 1080, 1180 and 1290 cm\(^{-1}\). These should be useful in characterizing the compound.

C—H bond bendings were shown at 1367 and 1460 cm\(^{-1}\). Strong absorption in the region 1645–1700 cm\(^{-1}\) indicated the presence of carboxyl groups and an ethylenic group. Separate bands occurred at 1645 cm\(^{-1}\) (unsaturation), and 1677 and 1698 cm\(^{-1}\) (carboxyl groups). C—H bond stretching, near 2900 cm\(^{-1}\), was shown, but there was no indication of the presence of hydroxyl groups. There was no trace of benzenoid absorption.
DISCUSSION

It is concluded that trichothecin is responsible for the antifungal properties exhibited by culture filtrates of *Trichothecium roseum*. Trichothecin isolated by the process described accounts for about 70% of the fungistatic activity of the culture filtrates as determined by the *Penicillium digitatum* spore-germination method (cf. Table 1). None of the fractions obtained in the fractionation described in Fig. 1 exhibited significant antifungal activity apart from the trichothecin-containing fractions. Crystalline products have been isolated from fractions I and II and will be described in a later paper; they were devoid of antitumour or antibacterial activity. In the chromatographic separations, only the trichothecin-containing fractions inhibited germination and growth of *P. digitatum*.

The molecular structure of trichothecin is not yet known. The analytical data are in good agreement with the molecular formulae, $C_{18}H_{16}O_4$ and $C_{18}H_{20}O_4$, of which the latter is preferred. There is at present insufficient evidence to decide between these alternatives. The molecule has been shown to contain one ketonic group, one ethylenic group and three methyl groups attached to carbon. Other functional groups, such as free carboxyl, hydroxyl, alkoxyl and aldehydeic groups, have been shown to be absent. On hydrolysis with ethanolic potassium hydroxide, trichothecin combined with one equivalent of the alkali, indicating the liberation of one carboxyl group. It has not been possible to isolate the corresponding acid, since on acidification a neutral compound, probably isomeric with trichothecin, was formed. This compound, which had no antifungal properties, differed from trichothecin in containing unconjugated ketonic and ethylenic groups.

The ultraviolet absorption spectrum of trichothecin contained two main bands which corresponded closely with the bands in the spectra of unsaturated aldehydes and ketones referred to by Morrison (1947) as the $R$ and $K$ bands. In conjugated systems of this type the position of the bands is influenced by the polarity of the solvent. Change from a relatively non-polar solvent such as hexane or chloroform to a polar solvent such as ethanol results in a shift of the $R$ band to shorter wave lengths whilst the $K$ band moves towards the longer (Morrison, 1947). This was the case with the trichothecin spectra, which indicates that the ethylenic and carbonyl groups in the molecule are conjugated with respect to each other.

The alkaline hydrolysis product, on the other hand, had a spectrum in which the corresponding bands were not altered in position by a change in solvent from chloroform to ethanol. These bands are attributed to the presence of unconjugated ethylenic and ketonic groups. The chemical evidence denoted the presence of a ketonic group. The effect of alkali is interpreted as due to the opening of a lactone ring with formation of the potassium salt. The acid equivalent of the alkali used in the reaction corresponded to the formation of one carboxyl group per molecule. On reacidification, a new neutral compound was formed, isomeric with trichothecin, in which unconjugated ketonic and ethylenic groups were present.

Infrared absorption-spectrum observations confirmed the conclusions drawn from the chemical and ultraviolet data that carbonyl groups and an ethylenic group were present. The infrared absorption data also indicated the absence of hydroxyl groups and benzenoid structures. The presence of carbon-methyl groups and ether linkages was suggested.

Trichothecin has been shown to be fairly stable at pH 1–10 at ordinary temperatures. At pH 12, hydrolysis, following a unimolecular reaction course, took place with virtually complete loss of antifungal activity in 6 hr. at 20°. The inactivation was irreversible. At pH 7–8, trichothecin was not destroyed in 1 hr. at 100°. These data suggest that the antifungal compound may persist sufficiently long under natural conditions to influence the growth of certain fungi and lead to the antagonism between *Trichothecium roseum* and other fungi mentioned in the introduction.

The antifungal activity of trichothecin does not appear to depend upon its intervention in a metabolic reaction involving SH groups, since its activity is unaffected by the presence of a large excess of cysteine. Further work on the structure of trichothecin is proceeding.

SUMMARY

1. The isolation of trichothecin from the culture filtrate of *Trichothecium roseum* is described. The antifungal compound was extracted with chloroform and purified by fractional precipitation and chromatographic separation on alumina.

2. Trichothecin crystallized from light petroleum in long fibrous needles, m.p. 118°, $[\alpha]_D^{12} + 44°$ (c, 1 in chloroform). The analytical data were consistent with the molecular formula $C_{18}H_{18}O_4$ or $C_{18}H_{20}O_4$.

3. The compound was a neutral, unsaturated ketone in which the carbonyl and ethylenic groups were conjugated.

4. Hydroxyl and alkoxyl groups were shown to be absent. The presence of three carbon-methyl groups per molecule has been established.

5. Trichothecin was found to be relatively stable in acid solution and at pH 10, but at pH 12 hydrolysis took place with liberation of a carboxyl group; subsequent acidification led to formation of an inactive neutral ketone.

We wish to express our thanks to Dr N. F. H. Bright for the ultraviolet absorption data, to Mr K. S. Tetlow for the infrared absorption data, and to Mr A. J. Baillie and Miss A. McCann for their assistance in the preparation of the trichothecin used in this work.
The Nucleoprotein Content of Fibroblasts Growing in vitro

4. CHANGES IN THE RIBONUCLEIC ACID PHOSPHORUS (RNAP) AND DEOXYRIBONUCLEIC ACID PHOSPHORUS (DNAP) CONTENT

By J. N. DAVIDSON, I. LESLIE and CHARITY WAYMOUTH (Beit Memorial Research Fellow), Biochemistry Department, St Thomas's Hospital Medical School, London, S.E. 1

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In the earlier papers of this series (Davidson & Waymouth, 1943, 1945, 1946) an account has been given of the conditions under which it is possible to record an increase in the nucleic acid content of chick-heart fibroblasts growing in vitro. Using the roller-tube technique of Willmer (1942), appreciable increases in the total nucleic acid phosphorus (NPP) of the fibroblasts have been obtained when the cultures were planted in fowl plasma and allowed to grow in chick-embryo extract. Defatted chick-embryo extract and fowl plasma, either together or separately, produced smaller increases in nucleic acids measured over 48 hr. periods, and led to a more rapid deterioration of the cultures (Davidson & Waymouth, 1946). Loss of nucleic acid was shown to occur over the 2 days following planting, and was greater for cultures in Tyrode solution than for those in embryo extract. However, the increase following addition of embryo extract to the cultures in Tyrode was as great 2 days later as in the case of cultures provided with embryo extract throughout. As a rule increases in NPP were greater for higher than for lower concentrations of embryo extract.

Schmidt & Thannhauser in 1945 published their method for determining the amounts of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in tissues (Schmidt & Thannhauser, 1945). This method has been adapted for estimating the phosphorus content of each acid after separation, and it has been possible to follow changes in these two components in the fibroblast cultures, and to investigate the relationship between growth and the total nucleic acid phosphorus (NPP), the ribonucleic acid phosphorus (RNAP) and the deoxyribonucleic acid phosphorus (DNAP). Although Willmer (1942) and Cunningham & Kirk (1942) proposed that growth in tissue cultures could be measured by determining the NPP content of cultures at different stages of development, they did so before it had become clear that there were two types of nucleic acids in each cell, the more abundant one usually being the ribonucleic acid of the cytoplasm and nucleolus, the other, the deoxyribonucleic acid, being apparently always confined to the nucleus.

In the tissue-culture technique which we have employed, it is possible to select a basal starting level of NPP according to the number and size of the cultures employed. From the time that growth-promoting medium is added to the cultures, changes in the amounts of phosphorus from both types of nucleic acids can be measured at varying intervals of time. Since the tissues are in a resting state before addition of growth-promoting medium, and the production of new tissue is in abeyance, it is reasonable to suppose that if one nucleic acid is the precursor of the other, then it will be the first to appear in the growing cultures. In this work we have been able to show that an increase in the RNAP always precedes by relatively long intervals any increase in DNAP. If the assumption that the P content of the nucleic acids is invariable is justified (as has been questioned; Wiam, 1946), the synthesis of RNA appears to be established before that of DNA.

Our observations on the changes occurring in both the RNAP and the DNAP during growth of fresh embryo-heart cultures have confirmed the general findings of Bruess, Rathbun & Cohn (1944) and have enabled us to find satisfactory conditions for growth