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MYCOTOXINS OF THE TRICHOTHECANE FAMILY PRODUCED BY *FUSARIUM TRICINCTUM* AND *TRICHODERMA LIGNORUM* [1]

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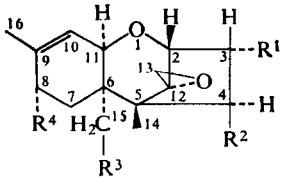
Abstract—In addition to diacetoxyscirpenol (I) and T-2 toxin (II) the fungus *Fusarium tricinctum*, strain T-2, when grown at 24°, produces a new trichothecane derivative, HT-2 toxin, which has been isolated and characterized as 3,4-dihydroxy-15-acetoxy-8-(3-methylbutyryloxy)-12,13-epoxy-Δ^9-trichothecene (IV). Another toxin-producing fungus, *Trichoderma lignorum*, isolated from moldy corn, also produces T-2 toxin (II). This is the first time that fungi of different genera have been shown to produce T-2 toxin.

INTRODUCTION

TOXIN-producing strains of *Fusarium tricinctum* and *Trichoderma lignorum* are frequently isolated from moldy cereals in Wisconsin.[2] The isolation and characterization of two toxic metabolites of *F. tricinctum* grown at 8° have been reported.[3] They are trichothecane derivatives commonly called diacetoxyscirpenol (4,15-diacetoxy-12,13-epoxy-Δ^9-trichothecen-3-ol) (I) and T-2 toxin (4,15-diacetoxy-8-(3-methylbutyryloxy)-12,13-epoxy-Δ^9-trichothecen-3-ol) (II). Trichodermin (4-acetoxy-12,13-epoxy-Δ^9-trichothecene) (III) has been the only trichothecane-type metabolite reported for *T. lignorum*.[4]



	R^1	R^2	R^3	R^4
(I)	OH	OAc	OAc	H
(II)	OH	OAc	OAc	$OOCCH_2CH(CH_3)_2$
(III)	H	OAc	H	H
(IV)	OH	OH	OAc	$OOCCH_2CH(CH_3)_2$

Growth studies with *F. tricinctum* at various temperatures between 8 and 24° led to the discovery that at 24° *F. tricinctum*, strain T-2, produced a toxic compound that chromatographed differently from either I or II.[5] The present report describes the identification of the

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[2] J. R. BAMBURG, W. F. O. MARASAS, N. V. RIGGS, E. B. SMALLEY and F. M. STRONG, *Biotech. Bioeng.* **10**, 445 (1968).

[3] J. R. BAMBURG, N. V. RIGGS and F. M. STRONG, *Tetrahedron* **24**, 3329 (1968).

[4] W. O. GODTFREDSEN and S. VANGEDAL, *Acta Chem. Scand.* **19**, 1088 (1965).

[5] J. R. BAMBURG, Mycotoxins of the Trichothecane Family Produced by Cereal Molds, Ph.D. Thesis University of Wisconsin, Madison, Wisconsin (1969).

new compound, designated HT-2 toxin, as 3,4-dihydroxy-15-acetoxy-8-(3-methylbutyryloxy)-12,13-epoxy-Δ^9-trichothecene (IV), i.e. 4-desacetoxy II. The possibility that IV is an artefact arising from partial, non-enzymic hydrolysis of II is unlikely as II is stable for extended periods at the pH values and temperatures encountered during growth of the mold cultures. IV is more probably an excreted metabolite or is produced by the action of a specific esterase on II.

The major mycotoxin from a strain of *T. lignorum* was also isolated and proved to be identical with II. This is the first report of II being produced by fungi of different genera.

RESULTS AND DISCUSSION

A summary of the extraction and chromatographic procedures used to isolate IV is shown in Fig. 1. The yield of purified IV, obtained as a pale yellow oil, was 109 mg from sixty cultures (115 g dry weight) of *Fusarium tricinctum*. Even after many months of experimenting with various solvents and conditions the oil could not be induced to crystallize.

The lack of absorption bands in the u.v. spectrum of IV indicated that the compound contained no aldehyde or ketone carbonyl groups and no conjugated systems of unsaturation. The presence of hydroxyl, aliphatic CH, ester carbonyl and olefinic groups was indicated by absorption maxima in the i.r. spectrum at 2·9, 3·4, 5·8, and 6·15 μ (3400, 2950, 1720 and 1635 cm^{-1}) respectively. An acetate ester was indicated by the large peak at 8·05 μ (1240 cm^{-1}). The i.r. spectra of I, II, and IV were all very similar.[3, 6]

Analysis of the NMR spectrum of IV (Fig. 2) provided evidence needed for structure assignment. A comparison of the NMR spectra of IV before and after D_2O exchange showed a reduction in the size of two peaks at δ 3·6 and 4·0, indicating the presence of two exchangeable protons. The general appearance of the spectrum strongly suggested that IV was a 12,13-epoxy-Δ^9-trichothecene compound. The *AB* quartet at δ 2·75 and 3·04 due to signals from the protons of the spiroepoxy group and the methyl singlets at δ 0·8 and 1·75 provide a key for recognizing the naturally occurring trichothecanes.

The very close similarity between II and IV became apparent by closer examination of their NMR spectra. The new compound contained one less acetyl group than II, a single acetate ester group being indicated by the strong three-proton singlet at δ 2·05. The only other major difference between the NMR spectra of II and IV is the position of the signal from the proton H4. This signal has moved upfield from δ 5·50 to δ 4·35 in going from the T-2 toxin spectrum to that of the new compound. This position must therefore be the site of deacetylation.

As final proof of structure, IV was acetylated and the product was compared directly to acetylated II. As expected, a comparison of the NMR spectra and i.r. spectra of the two compounds showed them to be identical.

Cultures of *Trichoderma lignorum* were extracted with ethyl acetate and the crude toxic extract was purified by column and thin-layer chromatography. The i.r. spectrum of the purest preparation of the toxin was very similar to the i.r. spectra of the other members of the trichothecane family, especially II. The NMR spectrum of the *T. lignorum* toxin also showed the characteristic *AB* quartet at δ 2·75 and 3·04 arising from the spiroepoxy protons, and the methyl singlets, which identify the compound as a member of the trichothecane family. Except for peaks at δ 1·3 and δ 0·85, the NMR spectrum of the *T. lignorum* toxin was identical to

[6] P. W. BRIAN, A. W. DAWKINS, J. F. GROVE, H. G. HEMMING, D. LOWE and G. L. F. NORRIS, *J. Exp. Botany* **12**, 1 (1961).

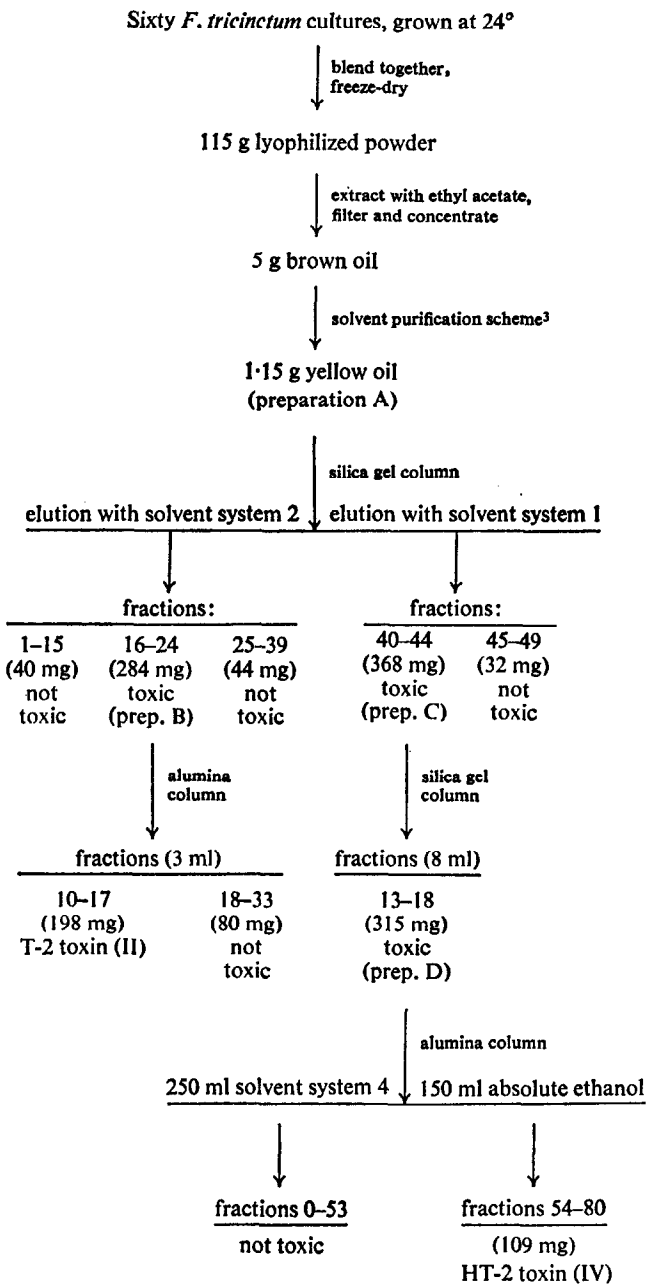

FIG. 1. FLOW CHART SUMMARIZING THE ISOLATION OF TOXINS FROM SIXTY CULTURES OF *F. tricinctum*, STRAIN T-2, GROWN AT 24° FOR 14 DAYS. TOXICITY ESTIMATIONS WERE DONE BY THE RAT SKIN TEST.

that of II run under the same conditions. This similarity suggested that the *T. lignorum* toxin is identical to II; the extraneous peaks probably arise from contaminants in the sample.

The identity of the *T. lignorum* metabolite with II was further substantiated by TLC on

silica gel G and alumina plates in three solvents in each of which both compounds had the same R_fs. A small amount of a second component in the *T. lignorum* sample was also observed on TLC which would explain the extraneous peaks in the NMR spectrum.

Attempts to isolate more of the T-2 toxin from this same strain of *T. lignorum* several months later failed because the culture had lost all of its toxin-producing abilities upon storage in the laboratory.

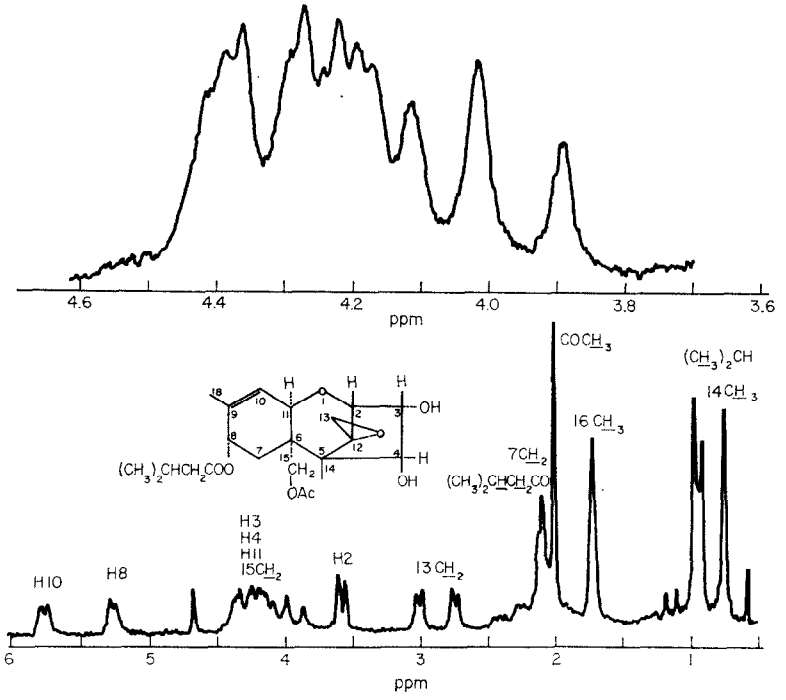

FIG. 2. ONE HUNDRED MC NMR SPECTRUM OF HT-2 TOXIN (IV) IN $CDCl_3$ AFTER D_2O EXCHANGE WITH TETRAMETHYLSILANE AS INTERNAL STANDARD. EXPANDED SCALE OF SIGNALS AROUND 4·2 ppm IS SHOWN IN TOP SCAN.

EXPERIMENTAL

NMR spectra were measured on a Varian Associates HA-100 spectrometer equipped for time averaging and double resonance measurements. Silica gel G and H, and neutral alumina used for TLC and column chromatography were supplied by Brinkmann Instruments. All solvents used were analytical grade reagents. The following solvents (ratios given by volume) were used: (1) EtOH–EtOAc–Me_2CO (1:4:4); (2) toluene–EtOAc (1:3); (3) EtOH–$CHCl_3$–Me_2CO (1:4:4); (4) EtOH–benzene–Me_2CO (1:3:3); (5) Skellysolve B–benzene–Me_2CO (2:1:1). The toxicity of all metabolite fractions was followed with the rat skin test.[7]

Isolates of *Fusarium tricinctum* and *Trichoderma lignorum* were grown as previously described[3] in 500-ml Erlenmeyer flasks containing 100 ml of Gregory's medium.[8] Cultures were incubated at either 8 or 24° for 2–3 weeks and then freeze-dried in a 7 l. capacity Virtis Freeze-Dryer.

Fusarium tricinctum *Toxins*

Purification of II and IV from *F. tricinctum* grown at 24° for 14 days is shown in Fig. 1. The cultures were extracted and worked up to the point of column chromatography exactly as described for T-2 toxin purification.[3] From 115 g of lyophilized mold powder, 5 g of crude oil was obtained which yielded 1·15 g of solvent-purified oil (preparation A).

[7] M. W. GILGAN, E. B. SMALLEY and F. M. STRONG, *Archs Biochem. Biophys.* **114**, 1 (1966).
[8] K. F. GREGORY, O. N. ALLEN, A. J. RIKER and W. H. PETERSON, *Am. J. Botany* **39**, 405 (1952).

Preliminary examination of preparation A by TLC followed by charring with H_2SO_4 revealed the presence of several components of which at least two gave a positive response in the rat skin test. Accordingly, preparation A (1·15 g) was dissolved in a small volume of solvent 2 and applied to a column (2 × 70 cm) packed with 0·05–0·2 mm silica gel. Elution of the column with three retention volumes of solvent 2 yielded a mixture of compounds active in the rat skin assay (preparation B). Solvent 1 was then used to elute a second band of organic material as detected by spotting fractions on a silica gel plate, spraying with conc. H_2SO_4 and charring. This second mixture (preparation C) contained at least one toxic compound (rat skin test).

Preparation B (284 mg) contained two major components which were separated on a column (1·5 × 30 cm) packed with neutral alumina. Following elution with solvent 1, the fractions (3 ml) containing similar material (as determined by TLC) were combined and solvent removed by slow evaporation. Fractions 10–17 yielded a crystalline compound (198 mg) that had R_f 0·60 on alumina TLC plates in solvent 1 and was shown to be identical with II by a comparison of NMR spectra and by mixed m.p. (151–152°). Fractions 18–33 yielded a second crystalline compound (80 mg) which melted at 248–250° and was non-toxic in the rat skin assay. No further work on the characterization of this compound was performed.

Preparation C (368 mg) was further purified by chromatography on a column (1·6 × 70 cm) packed with 0·05–0·2 mm silica gel and developed with solvent 3. Fractions (8 ml) of the eluate were collected and assayed for desired material by analytical TLC on silica gel G plates developed in solvent 3. Plates were sprayed with conc. H_2SO_4 and charred and those fractions (13–18) containing material with an R_f between 0·5 and 0·65 were combined and evaporated (315 mg, preparation D). Preparation D was further purified by chromatography on an alumina column (1·5 × 30 cm). The column was developed with 250 ml of solvent 4, which removed inert material, and then with 150 ml of absolute ethanol. Evaporation of the ethanol effluent *in vacuo* gave 109 mg of a pale yellow oil, IV, which appeared homogeneous on silica gel G and alumina TLC plates developed in four solvents. The R_fs for II and IV on silica gel G TLC plates were 0·67 and 0·55, 0·42 and 0·17, 0·69 and 0·56, and 0·74 and 0·60 in solvents 1–4, respectively.

The u.v. spectrum of IV in 95% EtOH (1 mg/ml) showed only end-absorption. The i.r. spectrum was measured on a 5% solution in $CHCl_3$ in 0·1 mm cells. The spectrum differed only in minor detail from that of II.[3] A $CDCl_3$ solution (0·4 ml) of about 40 mg of IV with 5% tetramethylsilane as internal standard was used for measuring the NMR spectrum (Fig. 2). The toxicity of IV as measured by the rat skin test[3,5] was approximately the same as that of II. Doses of IV of 1·78, 0·71, 0·35 and 0·17 mg tested on two rats at each dose level gave responses of 5, 4, 4, and 3, respectively.[9]

Acetylation of HT-2 Toxin

A mixture of IV (10 mg), pyridine (1 ml) and Ac_2O (1 ml) was held at room temp. for 24 hr, then ethanol (1 ml) was added and the solution dried to an oil *in vacuo*. The oil was applied to a preparative silica gel H plate (20 × 20 cm) and developed in solvent 2. The edge of the plate was sprayed with conc. H_2SO_4 and charred over a hot plate to locate the organic material (R_f 0·61–0·76) and the uncharred portion of the band was eluted with ethanol. Evaporation of the eluate gave a clear oil which was identical in its i.r. and NMR spectra to acetylated II.

Trichoderma lignorum *Toxin*

The freeze-dried mold powder was extracted in 10-g portions with EtOAc (2 × 250 ml), filtered, and the filtrate concentrated *in vacuo*. The crude extract from about twenty cultures (1·4 g) was chromatographed on a silica gel column (1·5 × 70 cm) packed and eluted with solvent 2. Fractions (10 ml) were collected and those containing the toxic material (24–30) were combined and concentrated. A small portion of the residue (7·9 mg) was examined by TLC on silica gel G in five solvents against a trichodermin standard.[10] Localization of compounds on the developed plates by spraying with H_2SO_4 and charring showed that there was no trichodermin in this partially purified preparation. The remainder of the partially purified extract (7 mg) was applied to a preparative silica gel H plate (2 × 8 in.) and developed in solvent system 5. Organic material was localized as before with H_2SO_4 spray and charring the edge of the plate. Bands were removed, eluted with ethanol and the concentrates tested for toxicity in the rat skin test. Only one toxic band (R_f 0·18–0·27) was observed.

The i.r. spectrum of the material in this toxic band was identical to that of II. A time-averaged NMR spectrum (24 scans) was run in $CDCl_3$. A portion of the residue was shown to be toxic in the rat skin test and the remainder of the material was chromatographed along with II on silica gel G and alumina in solvents 1, 2,

[9] These ratings[3,5] correspond to the following responses: 3, severe edema and heavy scab formation; 4, like 3 plus subcutaneous hemorrhaging; 5, death.

[10] The authors gratefully acknowledge the gift of trichodermin from Dr. W. O. Godtfredsen, Leo Pharmaceutical Products, Ballerup, Denmark.

and 5. The R_fs of the *T. lignorum* metabolite and II were identical in all systems. A minor component of the *T. lignorum* toxin was also visible on TLC after spraying with H_2SO_4 and charring.

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