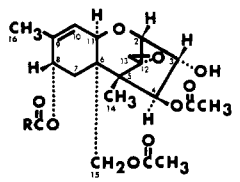
A New Trichothecene Mycotoxin Isolated from *Fusarium tricinctum*

John A. Lansden,* Richard J. Cole, Joe W. Dorner, Richard H. Cox, Horace G. Cutler, and J. Derrell Clark

Fusarium tricinctum was isolated from peanuts remaining in the soil after harvest. The mold produced a toxic metabolite belonging to the Trichothecene family of mycotoxins. The toxin was shown to be 3α-hydroxy-4β,8α,15-triacetoxy-12,13-epoxytrichothec-9-ene and assigned the trivial name neosolaniol monoacetate. The mean lethal dose of the toxin in 1-day-old cockerels was 0.789 mg/kg. The toxin was also shown to be a potent plant growth inhibitor active to 10^{-6} M in wheat coleoptile tests and possesses similar dermal toxicity to T-2 toxin in rabbit skin tests.

A significant amount of peanuts are left in the soil after peanuts are dug and harvested. Efforts to utilize these peanuts by allowing hogs or other livestock to scavenge fields after harvest was a common practice in the South. However, the practice has been essentially discontinued. Recently, it has been suggested that these peanuts could be salvaged by mechanical equipment and returned to the normal utilization channels (edible, seed and oil stock). In analyzing the quality of these salvaged peanuts, Duke (1971) tested for aflatoxin contamination.

We became interested in the possibility that other toxigenic microflora might be present. By using a screening program for toxigenic fungi from peanuts developed by Kirksey and Cole (1974), a toxigenic fungus was isolated from Florunner peanuts remaining in the soil after the 1976 harvest. Guided by bioassay, we isolated a new toxic metabolite belonging to the Trichothecene group of mycotoxins. We report here the isolation, identification, and characterization of neosolaniol monoacetate (Ib).



Ia, R = C_5H_9

Ib, R = CH_3

MATERIALS AND METHODS

Isolation of Toxigenic Fungus. The toxigenic *Fusarium tricinctum* (accessed as NRRL A-23377) was isolated from field-loss peanuts after sterilization with 1.0% sodium hypochlorite for 2 min and incubation on Difco potato dextrose agar (PDA) plates for 7 days at 27 °C. The isolate was maintained on PDA plates at 5 °C. The toxigenicity of the fungus was ascertained using the methods of Kirksey and Cole (1974).

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Production and Isolation of Toxin. The mold was grown in liquid culture (15% sucrose; 5% mycological broth, pH 4.8; 2% yeast extract) for 32 days at 27 °C. The toxin was extracted from 13 L of mycelia and culture medium by homogenization with an equal volume of chloroform in a Waring blender. The emulsified mass was strained through two layers of cheesecloth into 4-L separatory funnels. After phase separation, the chloroform extract was vacuum filtered through anhydrous sodium sulfate and concentrated at 60 °C on a rotary evaporator.

The particulate material trapped on the cheesecloth was reextracted with an equal volume of chloroform. The second chloroform extract was vacuum filtered through anhydrous sodium sulfate, concentrated, and combined with the first extract.

The crude extract was chromatographed on a 4.5 cm i.d. × 65 cm column packed with silica gel 60 (Brinkmann Instruments, Westbury, N.Y.). The column was eluted sequentially with 2 L of benzene, 2 L of chloroform, 2 L of ethyl acetate, 3 L of acetone, and 1 L of methanol. The toxic fractions (ethyl acetate, acetone, methanol) were combined, concentrated, and chromatographed on a 4.5 cm i.d. × 65 cm silica gel 60 column. The column was eluted with 1.5 L of benzene, 1.5 L of benzene–ethyl acetate (1:1), 1 L of ethyl acetate, 1 L of acetone, and 1 L of methanol. Most of the toxin was eluted with ethyl acetate as determined by bioassay and by thin-layer chromatography (TLC) (93:7 $CHCl_3$–acetone).

The ethyl acetate fraction was concentrated, then chromatographed on 2 cm i.d. × 50 cm silica gel 60 column with a linear gradient from benzene to ethyl acetate. The toxin was located in tubes 65 to 71 (17-mL fractions); these were combined and evaporated to dryness. The residue was dissolved in a minimum amount of benzene and an equal volume of heptane was added to form a precipitate. The precipitate was aged overnight at 5 °C and collected by vacuum filtration to yield ca. 250 mg of pure compound as determined by TLC analysis. Crystallization was effected by dissolving the pure toxin in benzene and adding an equal volume of heptane. The crystals were collected after standing for 24 h at 5 °C.

Physical and Chemical Analyses. Ultraviolet spectra (UV) of the toxin in methanol (Mallinckrodt U.V. grade) were recorded with a Beckman Model DB-G recording spectrophotometer. Infrared spectra (IR) were recorded with a Perkin-Elmer Model 257 recording spectrophotometer equipped with a 4X beam condenser. Samples for analysis were coated on KBr windows as a thin film from methanol solution. Low-resolution (LRP) and high-resolution (HRP) mass spectra were made with an A.E.I. MS-9 mass spectrometer. Samples were introduced by direct probe and ionization was effected by electron

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impact at 70 eV. Melting points were determined with a Kofler micro-melting point apparatus and are uncorrected.

Proton NMR spectra were obtained for a $CDCl_3$ solution using a Varian Associates XL-100 spectrometer. Homonuclear spin-decoupling experiments were carried out to aid in the assignment of the protons. Fourier transform, natural abundance, proton-decoupled carbon-13 NMR spectra were obtained for a $CDCl_3$ solution on a JEOL PFT-100 spectrometer equipped with the EC-100 data system. FID's were collected into 8K data points using a pulse angle of ~30° with a repetition rate of 2 s and a filter corresponding to a sweep width of 6250 Hz. A single-frequency, off-resonance, proton decoupled carbon-13 spectrum was obtained by setting the proton decoupling frequency 1000 Hz downfield from the center of proton absorptions.

TLC plates were coated with 0.5 mm of silica gel GH-R (Macherey Nagel, Brinkmann Instruments) and developed with either chloroform–acetone (93:7 v/v) or toluene–ethyl acetate–formic acid (5:4:1 v/v/v). The toxin was visualized by spraying with 50% ethanolic sulfuric acid and heating for 10 min at 120 °C.

Base hydrolysis of the toxin and T-2 toxin was effected by dissolving several milligrams of each in 2 mL of 1% ethanolic potassium hydroxide in test tubes. Complete solution was achieved by the addition of 2–3 drops of methanol. The solutions were heated in a boiling water bath for 1 h under nitrogen atmosphere and then partitioned between equal volumes of water and ethyl acetate. Most of the ethyl acetate layer was withdrawn by pipet, and the water layer was extracted with an additional 5 mL of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness with a stream of N_2 gas. The residues were dissolved in acetone prior to TLC analysis.

Vertebrate Bioassay. One-day-old Dekalb cockerels (38 g average) were used for bioassay of the crude, semipurified, and pure fractions. Bioassay was accomplished by crop intubation of 1 mL of corn oil preparations. The fractions were dissolved in acetone and mixed with corn oil, and then the acetone was evaporated under vacuum at 70 °C. Median lethal dose determinations (LD_{50}) were made simultaneously on the toxin and on T-2 toxin at concentrations of 200, 100, 50, and 25 μg/cockerel. Ten cockerels were dosed for each concentration. Mortality counts were taken up to 6 days after dosing. Weil's tables were used for calculating the LD_{50} (Weil, 1952).

The dermal toxicity of the toxin and of T-2 toxin was compared on the denuded back skin of rabbits, guinea pig, and rats. The method used was that proposed by Chung et al. (1974). The toxins were dissolved in nanograde ethyl acetate (Mallinckrodt) to give the desired concentrations. The animals used in these tests were: rabbits, 2–3-kg New Zealand White, female (Redbrook Farms, Loganville, Ga.); rats, 135–150-g Sprague-Dawley descended male (Charles River Breeding Labs, Wilmington, Mass.); guinea pigs, 600–800-g Hartley, male (Goods and Sons, Talmo, Ga.).

Five animals of each species were used. The hair on the backs of the animals was clipped with an electric clipper, and a depilatory, Neet (Whitehall Laboratories, Inc., N.Y.), was applied to the clipped skin 24 h prior to testing. Rabbits which showed uneven hair growth with red rings or spots after clipping were not used. Rabbits were pretested and those which showed no response to 0.05 μg of T-2 toxin were not used. The clipped backs of the test animals were marked with a median line following the spine and with seven approximately even-spaced cross lines. This provided 12 blocks on each animal for the application of toxin or control blanks.

Samples were applied topically as described by Wei et al. (1972). A 2-μL sample was delivered to a wire loop applicator from the tip of a Hamilton microsyringe. The filled loop was placed on the clipped skin of a test animal. After each use, the loop was washed several times in methanol. Each animal received the same treatment, using the same aliquots of diluted toxins. Ethyl acetate control blanks were applied to the two most cranial blocks. Progressing caudally, the following amounts of toxins, T-2 on the left, unknown toxin on the right, were applied: 0.01, 0.02, 0.04, 0.08, and 1.6 μg.

The degree of redness or erythema of the skin reactions was graded as: no reaction, 0; trace of redness (barely visible), tr; slight red reaction, ±; fairly distinct red reaction with clear demarcations, +; appreciably red reaction but seldom with edema or induration, ++; strong red reaction, often with a slight edema, induration, or necrosis (or a slightly blanched center), +++; and very strong red reaction with inflaming or beet redness, accompanied most of the time by a distinctly raised edema, induration, or necrosis (or strongly blanched center), ++++.

Plant Bioassay. Wheat coleoptiles (*Triticum aestivum* L. cv. "Wakeland") were cut 4 mm long from 4-day-old etiolated seedlings and assayed in duplicate tests. Ten coleoptiles were added to each test tube with 2 mL of a buffered solution (pH 5.6) that was 2% sucrose and 10^{-3}, 10^{-4}, 10^{-5}, or 10^{-6} M toxin. Stock 10^{-3} M solutions were made by dissolving the purified toxin in acetone (Cutler, 1968). Coleoptiles were incubated at 21 °C for 24 h in the dark and then measured (Hancock et al., 1964).

RESULTS AND DISCUSSION

Physical and Chemical. The toxin crystallized as long white needles (mp 190–190.5 °C) from benzene–heptane (1:1 v/v) solutions. Elemental analysis gave C, 59.78; H, 6.88; O, 33.34 (calcd for $C_{21}H_{28}O_9$: C, 59.4; H, 6.6; O, 33.9).

UV analysis of the toxin in methanol showed only end absorption. The major IR bands and probable assignments are 3370 (OH), 3010 (C=CH), 1730 (ester), 1230 (ester), 1370 (CH_3), 1460 (CH_2), 1070 (ring ether), 3040 (epoxy), 1265 (8 μ band), 920 (11 μ band), and 799 (12 μ band) cm^{-1}.

The HRP mass spectral analysis showed a molecular ion (M^+) at m/e 424.1735 (calcd for $C_{21}H_{28}O_9$, 424.1733). Fragment ions appeared at m/e 422 (H_2), 406 (H_2O), 393 (hydroxymethyl), 365 (-acetate), 306 (2 acetate), 247 (3 acetate).

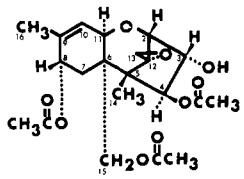
The above data suggested that the toxin might belong to the trichothecene class of mycotoxins; therefore, proton and carbon-13 NMR spectra were run and compared with reported spectra of Trichothecenes.

The proton and carbon-13 NMR spectra of the toxin (Table I) are similar in many respects to those of the trichothecene, T-2 toxin (Ia) (Bamburg et al., 1969; Ellison and Kotsonis, 1976). However, it is clear from the proton and carbon-13 spectra that the unknown toxin does not possess the isovaleryl group on position 8. The proton spectrum indicated the presence of three acetyl methyl groups. This is confirmed by the appearance of three ester carbonyl peaks and three methyl carbon peaks in the carbon-13 NMR spectrum.

The remaining question concerning the structure of the toxin was the location of the three acetate groups on the trichothecene carbon skeleton. Comparison of the carbon-13 chemical shifts (Table I) with those of other trichothecenes (Ellison and Kotsonis, 1976) unequivocally established the location of the acetate groups at C-4, C-8, and C-15. It also established that the toxin contained a methylene group at C-7 similar to T-2 toxin.

Alkaline hydrolysis of the toxin and T-2 toxin produced

Table I. Proton and Carbon-13 Chemical Shifts for Neosolaniol Monoacetate[a,b]



Carbon	δ ^{13}C[c]	δ ^{1}H[d,e]
2	78.7 (d)	3.69 (5.0)
3	78.3 (d)	4.18 (5.0, 3.0)
4	84.5 (d)	5.30 (3.0)
5	47.1 (s)	
6	43.0 (s)	
7	27.4 (t)	1.99 (15.0), 2.36 (15.0, 5.5)
8	68.5 (d)	5.27 (5.5)
9	136.1 (s)	
10	123.8 (d)	5.81 (6.0)
11	67.4 (d)	4.31 (6.0)
12	64.3 (s)	
13	48.6 (t)	2.81 (4.0), 3.06 (4.0)
14	7.0 (q)	0.91
15	64.4 (t)	4.08 (13.4), 4.30 (13.4)
16	20.3 (q)	1.76
C=O C_4	170.0 (s)	
C=O C_8	172.5 (s)	
C=O C_{15}	170.6 (s)	
CH_3 C_4	21.0 (q)	2.05
CH_3 C_8	21.0 (q)	2.14
CH_3 C_{15}	21.0 (q)	2.05

[a] In $CDCl_3$ solution. [b] In ppm downfield from internal Me_4Si. [c] Single-frequency, off-resonance decoupling results in parentheses. [d] Proton-proton coupling constants in Hertz given in parentheses. [e] Chemical shift of the 3-OH proton is 3.36 ppm.

the same two compounds which were located on TLC (toluene–ethyl acetate–formic acid) at R_f 0.09 and 0.26. IR spectra of the hydrolysis products indicated the expected loss of the ester functions. Major IR absorption bands were 3380 (OH), 1455 (CH_2), 1363 (CH_3), 1240, 955, 715 (epoxy), and 1020 (ether) cm^{-1}.

From the above data, the toxin has been shown to be 3α-hydroxy-4β,8α,15-triacetoxy-12,13-epoxytrichothec-9-ene (Ib), and we have assigned it the trivial name "neosolaniol monoacetate".

Vertebrate Toxicity. LD_{50} determinations for neosolaniol monoacetate and T-2 toxin determined simultaneously on cockerels were 0.789 and 1.84 mg/kg, respectively. Mortalities at low dosage levels were preceded by increasing lethargy; however, in some cases the onset of clinical signs did not occur for several days. Mortalities at higher dosage levels usually occurred within 24 h and the onset of signs could be observed within the first 6 h.

In side-by-side comparison on the same animals, T-2 toxin and neosolaniol monoacetate have similar dermal toxicity. For both toxins the lower practical limit of detection was 0.02 μg. The average scores for five animals of each species is presented in Table II. The results indicate that, on the average, rats are slightly more sensitive to both T-2 toxin and neosolaniol monoacetate. However, the hair of the rats grew so rapidly that it was necessary to clip the animals every 24–48 h. Consequently, trace (tr) and slight reactions (±) were difficult to read.

The scores presented in Table II were those read 24 h after application. Scores read 48 h after application were not significantly different. By 72 h, the reactions were visually subsiding and healing was evident after 96 h.

Plant Toxicity. Wheat coleoptile growth was inhibited 87, 69, 45, and 16% ($P < 0.01$) at 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M, respectively, relative to controls (Figure 1). It should

Table II. Comparative Toxicity of T-2 and Neosolaniol Monoacetate to the Back Skin of Rabbit, Guinea Pig, and Rat

Toxin	Dose, μg	Animals[a] Rabbit	Guinea pig	Rat
Neosolaniol monoacetate	0.01	tr	0	±
	0.02	±	0	+
	0.04	+	±	+++
	0.08	++	++	++++
	0.16	+++	++	++++
T-2 toxin	0.01	0	0	tr
	0.02	+	0	±
	0.04	++	±	+++
	0.08	+++	+	++++
	0.16	++++	+	++++

[a] The scores represent the averages for five animals. Scoring was as follows: 0, normal; tr, trace; ±, slight reaction; +, distinct reaction; ++, appreciable reaction but no edema; +++, strong reaction with slight edema, ++++, very strong reaction with distinct edema (see text for complete description).

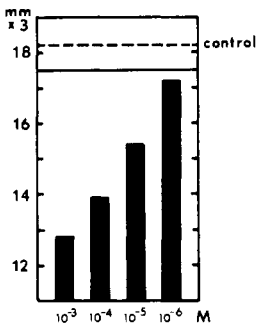


Figure 1. Growth regulating activity of neosolaniol monoacetate in wheat coleoptile bioassays (*Triticum aestivum* L. cv. "Wakeland"): control, dotted line; significant inhibition, below solid line ($P < 0.01$).

be noted that although neosolaniol monoacetate is biologically active at 10^{-6} M, it did not have the expected 100% inhibition of growth at 10^{-3} M. Brian et al. (1961) reported that a structurally similar trichothecene, diacetoxyscirpenol, caused a slight inhibition of pea seedlings at 2.73×10^{-6} M and a severe inhibition at 2.73×10^{-5} M but did not cause inhibition of wheat seedlings.

Further inspection of the dose response curve in Figure 1 showed that the response was linear rather than curvilinear. However, Bamburg (1972) reported a curvilinear response for T-2 toxin on pea seedlings with 40% length reduction at ca. 10^{-5} M.

At present, we are unable to propose a mode of action which would account for the high potency and linear dose response of neosolaniol monoacetate.

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Relationship between Fungal Growth and Aflatoxin Production in Varieties of Maize and Groundnut

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Available methods to quantitate fungal growth in infected grains are far from satisfactory. A chemical method to estimate fungal growth in infected plant tissue has been successfully used to estimate accurately the somatic amount of *Aspergillus parasiticus* on infected grains. Varieties of maize and of groundnuts were studied with respect to their capacity to promote fungal growth and aflatoxin production. No direct correlation was observed between fungal growth and aflatoxin production by the fungus among varieties of maize and groundnut, suggesting that genotypes support different amounts of fungal growth and also different amounts of aflatoxin production per unit growth of the fungus.

Contamination of food grains by some storage fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* is known to occur very widely. During their growth, these fungi produce a group of toxic metabolites collectively known as aflatoxins which are potent hepatotoxins and carcinogens. Consumption of such contaminated foodstuffs have been shown to be hazardous to a variety of animals including the monkey (Butler, 1974; Gopalan et al., 1972; Tilak, 1975) and more recently to man (Krishnamachari et al., 1975a,b).

Considerable efforts have therefore been directed toward preventing aflatoxin contamination of food grains. Among new approaches to the problem has been an attempt to identify and develop varieties which are resistant to aflatoxin production. Earlier work reported from this Institute (Rao and Tulpule, 1967; Nagarajan and Bhat, 1972, 1973) and from elsewhere (Mixon and Rogers, 1973; LaPrade, 1973) have shown that varietal differences do occur among genotypes of maize and groundnut with respect to their capacity to support production of aflatoxins by *A. flavus* and *A. parasiticus*. It has also been observed that under experimental conditions, aflatoxin production appreciably increased in some common food grains following their exposure to ^{60}CO irradiation (Priyadarshini and Tulpule, 1976). The basis for these differences in aflatoxin production, however, is not clear. It may be due to differences in fungal growth, to differences in the amount of toxin produced per unit growth of the fungus, or to both. Methods available so far to quantitate fungal growth in natural substrates are far from satisfactory. Recently, Ride and Drysdale (1972) have successfully developed a chemical method for quantitating the growth of *Fusarium* strains in infected leaflets of the tomato plant, using chitin as a biochemical marker. Chitin, a β, 1,4 linked linear polymer of 2-*N*-acetyl-D-glucosamine, cannot be detected in higher plants but forms a major constituent of most fungi and green algae. Chitin cannot be estimated directly, but Ride and Drysdale (1972) have suggested an alkali treatment which partially depolymerizes and deacetylates chitin to produce chitosan units, which can then be assayed for glucosamine by the modified method of Tsuji et al. (Ride and Drysdale, 1972).

Using this method, wherein glucosamine values can be used to determine fungal growth, studies were undertaken to quantitate the growth of *A. parasiticus* and to determine the correlation between fungal growth on the one hand and the amount of aflatoxin produced on the other.

EXPERIMENTAL SECTION

Aspergillus parasiticus NRRL 2999 strain was maintained on Potato Dextrose Agar slants. To determine the conversion factor (i.e., translational unit of glucosamine content into fungal growth) an in vitro glucosamine estimation of the fungus grown on a standard synthetic medium was done. Spores from a 4-day-old culture were inoculated into sterilized flasks containing 50 mL of standard synthetic medium (Adye and Mateles, 1964) and incubated at 27 °C for 7 days. At the end of the incubation period, the well-formed fungal mycelial mat was thoroughly washed with distilled water and homogenized to a known volume in a mechanical homogenizer. The dry weight of the fungal mycelial mat was determined by transferring 3-mL aliquots of the homogenate into preweighed aluminium foil boats and drying in an oven at 115 °C to constant weight. Suitable aliquots from the same homogenate were processed and assayed for glucosamine as described by Ride and Drysdale (1972), with a few minor variations. The initial alkali hydrolysis was run for 2 h at 130 °C. All centrifugations were run for 20 min at 2000*g*. These variations were introduced to ensure complete hydrolysis and better separation of the particles. To determine varietal differences, 10-g lots of 13 varieties of maize and six varieties of groundnut in duplicates were infected in the laboratory with a uniform spore suspension

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