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Toxic 12,13-Epoxytrichothecenes from Anise Fruits Infected with *Trichothecium roseum*

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Four 12,13-epoxytrichothecenes, viz., 4-*O*-acetyltrichothecolone (1), 4-*O*-cinnamoyltrichothecolone (2), trichothecolone (3), and trichothecin (4), were isolated and characterized from anise fruits infected in fields with *Trichothecium roseum* van Beyma (CMI-IMI 225229). Among these compounds, 1 and 2 are naturally occurring new 12,13-epoxytrichothecenes. From the in vitro culture extracts of the fungus, only 1, 3, and 4 were isolated and identified. Thus, the host–parasite interaction was suggested for the formation of 2, its cinnamoyl moiety being a metabolite of the host species. The total chloroform extractives of the moldy anise fruits produced dermatitic skin reactions in albino rats on external application and caused listlessness, anorexia, diarrhea, and stunted growth when ingested. The circumstantial evidence suggests toxin risk in man from prolonged ingestion of moldy anise fruits.

In connection with our work on mycotoxins in food and feed materials (Ghosal et al., 1976, 1979a,b), the fungal infection of anise (*Pimpinella anisum* Linn.; family, Umbelliferae) in fields was detected for the first time. Anise is widely cultivated in India in the northern parts, in Uttar Pradesh, and in Orissa, and is liberally consumed by Indians as a chewing spice, in food preparations, and also in medications. Extract of anise fruits is used in Ayurvedic system of medicine (Chopra et al., 1956) as a diuretic, a carminative in the treatment of colic, and, in combination with licorice roots, an anti-ulcerogenic agent (Revers, 1946). During a field survey, by the present authors in 1977, in the Mirzapur District of Uttar Pradesh, fruit-bearing anise herbs were found to be heavily infested with a fungus. The fungus was isolated from the moldy anise fruits by the single-spore isolation method (Funder, 1961) and was identified as *Trichothecium roseum* van Beyma. The

identity of the strain (CMI-IMI 225229) was confirmed by the Commonwealth Mycological Institute, Kew, England.

Although report of any toxicosis in humans or animals ingesting moldy anise is not known so far, this investigation was thought warranted for a number of reasons: (1) highly toxic isolates were reported from *T. roseum* (Joffe, 1971); (2) *T. roseum* species is a well-known producer of 12,13-epoxytrichothecenes (Achilladelis and Hanson, 1969); (3) 12,13-epoxytrichothecenes, on prolonged ingestion, are known to produce degeneration of nerve cells in the brain and central nervous system of laboratory animals (Tatsuno, 1968); (4) initial experiments with a chloroform extract of the moldy anise fruits, in the authors' laboratory, suggested the presence of 12,13-epoxytrichothecenes when examined according to a published procedure (Ghosal et al., 1978). Albino rats when fed in their diet with the chloroform extractives of the moldy anise fruits developed anorexia and diarrhea. Common people in all parts of India consume sizable amounts of anise fruits (ranging from ca. 0.1–1 g person^{-1} day^{-1}) in different preparations. Prolonged ingestion of moldy anise may produce neurological disorders or predispose man to other ailments. Chemical and

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Toxic 12,13-Epoxytrichothecenes from Anise Fruits

J. Agric. Food Chem., Vol. 30, No. 1, 1982   107

Table I. Effect[a] of Toxins of Moldy Anise on Albino Rats

| compd no.	test samples[b]	dose, mg/rat	skin reactions[c]
1	chloroform extractives in vitro culture of *T. roseum*	0.5	no observable reaction
		1.0	skin reddening followed by formation of dry area on eight rats
		2.0	appreciable inflammation on all rats
2	chloroform extractives of moldy anise fruits (collected from field)	0.5	no observable reaction
		1.0	skin reddening followed by formation of dry area on all rats
		2.0	appreciable inflammation and heavy scab formation on all rats
3	chloroform extractives of stored moldy anise fruits	0.5	swollen reddish weals on all rats
		1.0	appreciable inflammation developing into a heavy scab on all rats
		2.0	severe edema accompanied by a marked subdermal hemorrhaging in the affected zone on all rats
4	mixture of 1-4	0.5	no observable reaction
		1.0	skin reddening on six rats
		2.0	dry area on all rats

[a] Ten animals in each experiment. [b] The test samples were dissolved in chloroform (0.2 mL) and applied in a single dose to the shaven back of albino rats (about 80 g of body weight) with a wire loop. [c] The animals were observed for a period of 1 week.

biological investigations of the moldy anise fruits were therefore carried out. The details of the findings constitute the subject of this paper.

RESULTS AND DISCUSSION

Anise fruits infected with *T. roseum* were collected from fields near the Windaum region of Mirzapur Distinct during Feb 1977 (temperature 10–22 °C; relative humidity 55–75%). The field survey in subsequent years revealed that *T. roseum* infection of anise has been a recurring phenomenon in the region and in adjoining areas. The infection was observed on all parts of the herb *P. anisum*, e.g., tender roots, stems, young leaves, and mature fruits. Interestingly, *T. roseum* was the only fungus detectable in the moldy anise fruits. This could be due to the fact that the host species produces liberal amounts of phenolic constituents, e.g., phenolic acids and coumarin derivatives (Ghosal et al., 1979c), which prevent the ingress of common fungal parasites. In a recent survey from the Caribbean Industrial Research Institute, it was reported (Imbert, 1980) that common spices, e.g., cinnamon and nutmeg, which contained abundant quantities of cinnamaldehyde and eugenol, had pronounced fungistatic properties. The mentioned spices completely prevented the growth of several species of *Aspergillus* and *Penicillium* commonly found in foodstuffs. The present observation, however, suggested that despite appreciable abundance of phenolic constituents in anise the attack and proliferation of *T. roseum* on this species remained unabetted.

Extraction of moldy anise fruits, infected with *T. roseum*, with chloroform followed by partitioning of the chloroform extractives in solvents of graded polarity, column chromatography, and preparative layer chromatography (PLC) afforded four 12,13-epoxytrichothecenes, viz., 4-*O*-acetyltrichothecolone (1), 4-*O*-cinnamoyltrichothecolone (2), trichothecolone (3), and trichothecin (4), in quantities sufficient for their complete characterization. Among these, 1 and 2 are new naturally occurring compounds.
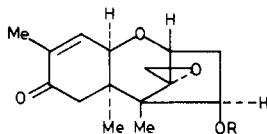
1, R = OCCH₃
2, R = OCCH=CHPh
3, R = H
4, R = OCCH=CHCH₃

The amounts of 1–4 in the naturally infected anise fruits were estimated as 14, 35, 19, and 62 μg/g, respectively. The intracellular (mycelium) and extracellular (culture filtrate) chloroform extractives of the fungus, grown in Richard's medium (Young and Bennett, 1921), provided 1, 3, and 4 in smaller amounts.

The identity of the known compounds (3 and 4) was established by direct comparison with reference samples. The characterization of only the new compounds (1 and 2) is described here.

4-*O*-Acetyltrichothecolone (1). This compound, $C_{17}H_{22}O_5$ (M⁺, 306), responded to 2,4-dinitrophenylhydrazine (DNP) reagent on analytical TLC. It exhibited ¹H NMR spectrum characteristic of an 8-ketoacetoxy-12,13-epoxytrichothecene. In its IR spectrum, in addition to the α,β-unsaturated keto carbonyl, a saturated ester carbonyl band appeared (in lieu of the 4-OH function). Saponification of the compound gave trichothecolone and sodium acetate. Thus, structure 1 was assigned to this compound.

4-*O*-Cinnamoyltrichothecolone (2). This compound, $C_{24}H_{26}O_5$ (M⁺, 394), also responded to DNP reagent. It showed UV absorption maxima, in ethyl alcohol, characteristic of a cinnamic ester. The IR spectrum exhibited bands due to α,β-unsaturated keto and α,β-unsaturated ester carbonyl functions. The ¹H NMR spectrum of the compound suggested the presence of a *trans*-cinnamoyl moiety attached to the 8-keto-12,13-trichothecene molecule. Selective hydrolysis of the compound afforded trichothecolone and cinnamic acid. Thus, structure 2 was assigned to this compound. The compound was not encountered before in nature nor was it prepared before synthetically.

The total chloroform extractives of the moldy anise fruits, collected from the field, produced pronounced toxic symptoms in the skin test of albino rats performed according to a published procedure (Wei et al., 1972). Likewise, the total chloroform extractives of the intracellular (mycelial) and extracellular (culture filtrate) of the fungus produced significant toxic skin reactions. A mixture of 1–4, in almost equal proportions, also produced toxic symptoms in albino rats although the manifestations of the skin reaction were much less pronounced (Table I).

With a view of determining if the toxicity of the moldy anise fruits was altered on storage, we mixed *T. roseum* infected anise fruits (1 g) with healthy fruits (99 g) and preserved the mixture in an Erlenmeyer flask, plugged with

108 J. Agric. Food Chem., Vol. 30, No. 1, 1982

Ghosal et al.

sterilized cotton, at ordinary temperature, for about 2 years. The fungus appeared on all fruits within a few weeks although the intensity of growth of the mycelium was not significant. Subsequently, the infected fruits were examined for 12,13-epoxytrichothecenes. Analytical and preparative layer chromatography of the chloroform extract of these moldy fruits revealed the presence of three more 12,13-epoxytrichothecenes in addition to the above-mentioned four compounds (1–4). A chloroform solution of TLC scrapings of the extractives of the preserved fruits, at several R_f values, when put to skin test of albino rats, produced symptoms characteristic of 12,13-epoxytrichothecenes. The total chloroform extractives also produced toxic skin reactions (Table I).

Albino rats fed with the chloroform extractives (1 mg rat^{-1} day^{-1}, for 3 weeks) of the stored moldy anise fruits in their diet were severely stunted in growth, listless, and developed diarrhea and inflammation of the skin around their noses and mouths. Those receiving a diet containing the chloroform extractives (1 mg rat^{-1} day^{-1}, for 3 weeks) of the moldy anise fruits collected from the field were also listless and suffered from diarrhea and anorexia but did not develop inflammation of the skin.

The above observations would seem to suggest that proliferation of *T. roseum* (CMI-IMI 225229) and the concomitant increase in the toxin production were facilitated on storage of anise fruits having prior infection. Baking of fruits could not reduce the content of the toxins. This was expected since 12,13-epoxytrichothecenes, as a class, are quite stable, being unchanged on storage and not destroyed by normal cooking procedures (Bamburg and Strong, 1971). Spices are normally stored for indefinite period of time before they are sold and anise is no exception. Ingestion of moldy anise therefore poses toxin risk in humans. It has not been established that the 12,13-epoxytrichothecenes present in the moldy anise has caused toxicosis in humans or animals. Notwithstanding the lack of any such clear-cut proof, circumstantial evidence provided in the present investigation and elsewhere that various trichothecenes have been responsible for poisoning, e.g., degeneration of cells in the central nervous system and exhaustion of the bone marrow (Tatsuno, 1968) of both animals and human beings, is impressive. Thus, prolonged ingestion even of a small amount of moldy anise fruits, which contain 12,13-epoxytrichothecenes, may cause neurological disorders and disfunction of the central nervous system in humans and may also predispose man to other ailments. From a public health viewpoint, therefore, further work in this direction is warranted.

EXPERIMENTAL SECTION

All melting points were taken on a Kofler block in open capillaries and were uncorrected. UV spectra were taken in ethyl alcohol in a Beckman 24 spectrophotometer. IR spectra were taken in KBr and only the major bands are quoted. ^{1}H NMR spectra, in CDCl$_3$, were recorded at 90 MHz by using Me$_4$Si as an internal standard. Mass spectra were determined in an MS-50 instrument at an ionizing potential of 70 eV. Column chromatography was done on silica gel (British Drug Houses, Poole, England; 60–120 mesh) as the adsorbent. Thin-layer chromatography (TLC) was conducted with silica gel G (E. Merck, Darmstadt) using three solvent systems, viz., C$_6$H$_6$–HOAc (98:2, solvent 1), CHCl$_3$–MeOH (98:2, solvent 2), and C$_6$H$_6$–MeOH–HOAc (22:2:1, solvent 3), as developers. For preparative layer chromatography, a 2-mm plate thickness was used. Iodine vapor, 2,4-dinitrophenylhydrazine reagent, and Ehrlich reagent were used for the staining

purposes, and a short-wave UV lamp (250 nm) was used for the detection purposes.

Extraction of Toxins from Moldy Anise Fruits. In a typical experiment, toxins from the fruit–mycelial mixture of moldy anise (about 200 g) was isolated as follows. The material was macerated in a high-speed blender with chloroform (1 L). The chloroform extract was processed according to a previously described procedure (Ghosal et al., 1978) to give a brown semisolid (1.4 g) which was biologically active (Table I). Partitioning of this substance between *n*-hexane and chloroform–ethyl acetate, as before (Ghosal et al., 1978), provided a yellow semisolid (0.23 g) which showed several UV fluorescent and DNP-positive spots on analytical TLC (solvents 1 and 2). It was dissolved in benzene–chloroform (1:1, 10 mL) and chromatographed over a column of silica gel (24 × 1.8 cm). Elution was carried out with benzene–ethyl acetate (90:10, 300 mL; 80:20, 200 mL). Fractions (10 mL) were collected and monitored by analytical TLC.

Trichothecin (4). Fractions 4–14 were combined and the solvent was evaporated under reduced pressure when an amorphous solid was obtained (31 mg). It showed a major DNP-positive spot at R_f 0.7 (solvent 2) together with a minor DNP-positive spot at R_f 0.4, and three I$_2$-positive spots (in traces). The major component was isolated by preparative layer chromatography (solvent 2). The band around R_f 0.7 was scraped and eluted with chloroform. The chloroform solution showed the presence of a single entity on analytical TLC. The solvent was removed and the residue crystallized from *n*-hexane–chloroform as tiny crystals (12.5 mg): mp 115–116 °C; $[\alpha]^{28}_D$ +42.8° (*c* 0.44, CHCl$_3$); C$_{19}$H$_{24}$O$_5$ (by combustion analysis and M$^+$); IR ν_{max} 1738, 1710, 1672 cm^{-1}; MS *m/e* (rel intensity) 332 (M$^{+\cdot}$, 5), 247 (14), 246 (100), 218 (4). The physical and spectral properties of the compound were indistinguishable from those reported for trichothecin (Freeman et al., 1959; Fishman et al., 1960). Direct comparison (mp, mixed co-TLC, and IR) of the compound with a reference sample of trichothecin established that they were identical.

4-O-Acetyltrichothecolone (1). Fractions 17–20 were combined and evaporated to give a glassy solid (3.8 mg), mp 103–105 °C. It showed a single spot on analytical TLC, R_f 0.45 (solvent 2), which was DNP positive: $[\alpha]^{28}_D$ +51.3° (*c* 0.28, CHCl$_3$); C$_{17}$H$_{22}$O$_5$ (by high resolution MS); IR ν_{max} 1745, 1670, 1205 cm^{-1}; MS *m/e* (rel intensity) 306 (M$^{+\cdot}$, 5), 264 (1), 247 (5), 246 (72), 218 (12), 122 (100); ^{1}H NMR (CHCl$_3$) δ 4.48 (1 H, m, H-4α), 3.85 (1 H, d, J = 6.5 Hz, H-2), 2.92 (2 H, q, J = 3.6 Hz, C$_{13}$-methylene protons), 2.44 (1 H, m, H-3α), 2.02 (3 H, s, OAc-4β), 1.90 (1 H, m, H-3β), 1.78 (3 H, m, Me-16), 0.98 (3 H, Me-15), 0.81 (3 H, Me-14). On treatment with 0.1 N NaOH at ordinary temperature, the compound gave trichothecolone (co-TLC and IR) and sodium acetate, crystallized from moist diethyl ether.

Trichothecolone (3). Fractions 21–38 were combined and concentrated. The concentrate on analytical TLC showed several blue fluorescent spots under UV light and one intense DNP-positive spot, R_f 0.3 (solvent 2). The mixture was subjected to preparative layer chromatography using the same solvent system. The scraping of the band around 0.3 was eluted with chloroform. The solvent was evaporated from the chloroform solution when an amorphous solid (2.7 mg) was obtained. Attempts to crystallize the compound from common organic solvents were unsuccessful. The homogeneity of the compound was tested by TLC in three different solvent systems (solvents 1–3) and by high-resolution mass spectrometry: C$_{15}$H$_{12}$O$_4$; IR ν_{max} 3450 (OH), 1672, 1612 cm^{-1}; MS *m/e* (rel intensity) 264 (M$^{+\cdot}$, 2), 247 (6), 246 (28), 233 (20), 221 (90), 218 (11),

175 (44), 161 (28), 149 (47), 122 (100); ^{1}H NMR (CDCl$_3$) δ 2.90 (2 H, dd, J = 3.8 Hz), 1.81 (3 H, s, Me-16), 0.99 (3 H, s, Me-15), 0.82 (3 H, s, Me-14). The physical and spectral properties of the compound were consistent with those reported for trichothecolone in the literature (Freeman et al., 1959; Fishman et al., 1960). Direct comparison (mp, mixed mp with an authentic sample remained undepressed, co-TLC, and IR) with an authentic sample of trichothecolone established that they were identical.

4-O-Cinnamoyltrichothecolone (2). Fractions 42–48 were combined and concentrated. The concentrate showed one major and three minor spots on analytical TLC (solvent 3). The major component was separated by preparative layer chromatography. The layer scraping of the major band, around R_f 0.5, was eluted with chloroform. The solvent was evaporated from the chloroform solution and the residue crystallized from n-hexane–chloroform as light straw crystals (7 mg): mp 107–109 °C; C$_{24}$H$_{26}$O$_5$ (by combustion analysis and M$^+$); [α]$^{28}_D$ +47.7° (c 0.23, CHCl$_3$); UV λ_{max} (ethyl alcohol) 220 nm (log ϵ 4.24), 265 (4.30), 300 sh (3.08); IR ν_{max} 1680, 1672, 1612, 1595 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 7.81 (1 H, d, J = 16 Hz, cinnamoyl-β-H), 7.5 (5 H, m, aromatic H), 6.50 (1 H, m, H-10), 6.41 (1 H, d, J = 16 Hz, cinnamoyl-α-H), 4.33 (1 H, m, H-4α), 3.90 (1 H, m, H-11), 3.88 (1 H, d, J = 5.5 Hz, H-2), 3.1–2.8 (2 H, C$_{13}$-methylene protons), 2.90 (1 H, m, H-7β), 2.43 (1 H, m, H-3α), 2.25 (1 H, m, H-7α), 1.94 (1 H, m, H-3β), 1.85 (3 H, Me-16), 1.0 (3 H, Me-15), 0.82 (3 H, Me-14); MS m/e (rel intensity) 394 (M$^+\cdot$, 1), significant fragment ion peaks at m/e (rel intensity) 247 (12), 246 (100), 218 (4), 131 (11), 122 (14), 103 (7), 77 (5). Saponification of the compound, at ordinary temperature, and the usual workup of the product gave trichothecolone (co-TLC and IR) and cinnamic acid (mp, mixed mp, co-TLC, and UV).

Isolation of T. roseum from Moldy Anise. The pink-colored mycelia of the fungus present on the surface of the anise fruits, collected from the field, were isolated on potato dextrose agar medium (PDA) according to the "single-spore" isolation method (Funder, 1961). An axenic culture of the fungus was maintained on artificially inoculated anise fruits. The fruits were stored at 0 ± 2 °C, in an aseptic culture tube, as reference.

Feeding of Chloroform Extractives of Moldy Anise to Albino Rats. Charles Foster rats (110–120 g), bred and maintained in the departmental animal house, were acclimated in the controlled conditions (28 ± 2 °C) in the experimental animal laboratory. A pilot experiment was conducted to determine the approximate food intake of each animal which was found to be 10 ± 2 g day^{-1}. An ethyl alcoholic solution of the test compound was mixed with a ground corn supplement ration and fed to the animals ad libitum. The amount of the test compound was so adjusted that its intake was about 1 mg rat^{-1} day^{-1} for 3 weeks. In the control experiment, chloroform extractives of healthy anise fruits were mixed with the ground corn supplement ration. A record was kept on the quantity of diet left unconsumed by the animals.

Extraction of Intra- and Extracellular Toxins of T. roseum. The fungus was grown in Richard's medium (200 mL) in a still culture flask (1 L) at 21 °C for 21 days. Workup of the intracellular (mycelial) and extracellular (culture filtrate) extractives, according to a previously described procedure (Ghosal et al., 1976), gave a brown oily substance (0.55 g). It was partitioned, as described before (Ghosal et al., 1978, when a light brown semisolid (0.18 g) was obtained. The semisolid substance, when subjected to preparative layer chromatography, using solvent 2 as the developer, afforded compounds 1 (1.8 mg), 3 (0.5 mg), and 4 (2.2 mg).

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