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Tenuazonic Acid, a Toxin Produced by *Alternaria alternata*[1]

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Fifty-seven of 87 isolates of *Alternaria alternata* (Fr) Keissler grown on autoclaved, moist corn-rice substrate and fed to rats were lethal. The major toxin produced was isolated and characterized as tenuazonic acid. Twenty of 23 toxigenic *Alternaria* isolates examined produced tenuazonic acid. No tenuazonic acid could be detected in either of the field samples of sorghum or blackeyed peas, which were heavily invaded by *Alternaria*.

Mycelia of *Alternaria* are common and sometimes abundant under the pericarps of wheat (1, 5), and the outer portions of weathered kernels of wheat, barley, oats, and sorghum contain relatively massive amounts of it. The fungus is also prevalent in peanut fruits during their development (4). Palyusik (8) found an isolate of *Alternaria tenuis* that when grown in pure culture and fed to geese was lethal to them, and Doupuik and Sobers (2) reported that 31 of 96 isolates of *Alternaria longipes* from tobacco when grown in pure culture and fed to chicks were lethal to them. According to Lucas (7) both of these species should now be designated *Alternaria alternata* (Fries) Keissler. Forgacs and Carll (3) fed *Alternaria*-infested grain to chicks and produced diarrhea and various other symptoms in the young chicks and hemorrhaging and gizzard lesions in older ones. The work here reported was undertaken to develop further information concerning the possible toxicity of this almost ubiquitous fungus.

MATERIALS AND METHODS

Isolates of *Alternaria* from grains, seeds, and other plant materials in which it is common were grown on a 1:1 mixture of autoclaved moist corn-rice substrate for 14 to 20 days at 23 to 25 C, after which the fungus-infested grain was dried, ground, and fed either as the sole ration or as 50% of a balanced toxicological diet (12) to 50-g weanling female white rats (*Rattus norvegicus*).

The toxin was originally extracted from *Alternaria*-infested corn-rice and purified partially by thin-layer chromatography. The material to be used for characterization was obtained by twice extracting, with 5 liters of water, 2 kg of corn-rice infested by an isolate of *A. alternata* from peanuts. The aqueous extracts were reduced to 100 ml in a flash evaporator and then extracted three times with 100 ml of butanol saturated with water. The butanol extracts were dried under vacuum, dissolved in 10 ml of water, and loaded onto a 5 by 60 cm column of Sephadex G-10. The material was eluted with distilled water at a rate of 5 ml/min. The eluant was monitored with an LKB Uvicord column monitor, and 10-ml fractions were collected. The first ultraviolet absorbing peak was found in fractions collected between 450 and 600 ml and had the ultraviolet spectrum characteristic of the toxin. These fractions were pooled, dried under vacuum, dissolved in 5 ml of ethanol, and streaked silica gel PF 254 preparative plates. The plates were developed in chloroform-methanol (90:10), the top 2 cm of the ultraviolet absorbing band was scraped off, and the substances present were eluted with ethanol. After being chromatographed three times in this manner, the ethanol solution of the ultraviolet absorbing material was concentrated under a stream of nitrogen and dried under vacuum to give 75 mg of a white powder.

A standard solution was made by dissolving 5.0 mg of the isolated material in 1.0 ml of absolute ethanol. Ultraviolet spectra were taken in water, absolute ethanol, 0.09 N NaOH, and 0.09 N HCl at a concentration of 10 μg/ml.

For infrared spectroscopy, 0.6 mg of the substance was thoroughly ground with 50 mg of spectral-grade KBr and converted to a pellet in a micropress. The infrared spectrum was obtained on a Perkin Elmer model 257 grating infrared spectrometer. In addition, a portion of the purified material was chromatographed on silica gel PF 254 plates using the acid system toluene-ethyl acetate-formic acid (5:4:1), to yield the free acid. The free acid was eluted with ethanol, dried under vacuum, and dissolved in chloroform to give a 10% solution. An infrared spectrum of this solution as well as a chloroform blank was taken on the same spectrometer using a 0.1-mm salt cell.

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A 10-mg sample was dried at 40 C under vacuum for 48 hr and submitted to Clark microanalytical laboratory (Urbana, Ill.) for combustion analysis. A 10-mg sample was also submitted to emission spectroscopy.

For chemical degradation studies, 20 mg of the substance was placed in a tube containing 0.5 ml of 2 N HCl. The tube was sealed and heated for 24 hr at 100 C. The tube was cooled and opened, and the hydrolysate was diluted to 2 ml with water. One milliliter of this was made to 10 N with NaOH and cooled to 0 C, and 20% I_2 in KI was added in 10% excess. After 1 hr the solution was filtered to remove the iodoform and brought to *p*H 7 with HCl, and the excess iodine was removed with 10 N sodium thiosulfate. This solution was then extracted three times with one volume of butanol, and the butanol extracts were concentrated to 1 ml under nitrogen. One microgram of each of 22 amino acids was co-spotted with 5 μliters of the butanol solution on silica gel G (0.5 mm layer) and developed to 15 cm with butanol-acetic acid-water (75:15:10). Ninhydrin in butanol was used to make the amino acids visible. One microgram of each of the amino acids which co-chromatographed with the unknown in the above system, 5 μliters of the unknown, and 5 μliters of the unknown plus 1 μg of known amino acid were spotted onto Silica Gel G plates and developed for 6 hr with methyl ethyl ketone-pyridine-water-acetic acid (70:15:15:2) in an S chamber with the top closure removed.

Thirty-four *Alternaria* isolates from various sources were tested for their ability to produce the toxin isolated from the peanut isolate. The isolates were grown on corn-rice substrate (1:1) for 2 weeks at room temperature, and then dried, ground and extracted with water (5:1) overnight. The water extracts were analyzed by ultraviolet spectroscopy for the presence of the toxin. If absorption maxima at 239 and 278 nm were obtained, the toxin was reported as present.

In addition, samples were extracted with diethyl ether (5:1). The ether extracts were concentrated and streaked on Silica Gel G plates (0.5 mm thick). After development in chloroform-methanol (90:10), the plates were examined under long-wave ultraviolet light (340 nm). The bright blue fluorescent band near the solvent front was marked; the silica was scraped from the plate, and the substance was eluted with ethanol. The eluant was examined by ultraviolet spectroscopy for the presence of alternariol monomethyl ether maxima at 335 to 342 (wide band), 301, 290, 257, and 220 nm; these absorption maxima match those reported earlier by Rosett et al. (9).

A sample of sorghum and a sample of soybeans, both of which were naturally colonized by *Alternaria*, were assayed for the toxin. A 100-g sample of each was ground in a Stein mill, 50 ml of water was added, and concentrated hydrochloric acid was used to adjust the *p*H to 2.0 or less. The sample was then extracted twice with 500 ml of ethyl acetate. The extracts were collected by filtration and concentrated first in a flash evaporator then under a stream of nitrogen to a volume of about 2 ml. This was then streaked onto preparative silica plates and developed with toluene-ethyl acetate-formic acid (5:4:1). The plate was then examined under short-wave ultraviolet light (254 nm), and the materials running at the same R_F as the toxin were obtained by scraping the silica from the plate and eluting the substances with ethanol. The ethanol solution was examined by ultraviolet spectroscopy for the toxin. The eluted materials were then chromatographed again in the same manner, and the spectroscopic procedures were repeated.

RESULTS

Fifty-seven of the 85 isolates of *Alternaria* grown on a corn-rice substrate and fed to rats were lethal to one or both rats within 10 days, and 54 or 64% were lethal to both rats to which they were fed (Table 1). All of the rats that succumbed to the *Alternaria*-infested rations lost weight, and those that survived gained weight and consumed approximately as much feed as did the controls (Table 2). Postmortem lesions in the rats included hemorrhaging in the gastrointestinal lumen and anorexia.

The partially purified material originally found in ethanol extracts of *Alternaria*-infested corn-rice proved fatal to 50-g rats when administered at a dosage of 100 to 200 mg/kg. This material showed ultraviolet absorbance maxima at 238 and 279 nm in ethanol, and reacted with ethanolic ferric chloride to give a deep red color.

The material isolated for use in the characterization study had the same above characteristics and consisted of 75 mg of a white powder having an extinction coefficient of 598 for a 1% ethanolic solution (at 278 nm).

Ultraviolet absorption maxima of the isolated substance and tenuazonic acid described by Stickings (11) are presented in Table 3. The most pertinent features here are the shift from 238 to 220 nm in acid, indicating an enolate to enol conversion and the nearly identical absorptions of the isolated compound and tenuazonic acid. The lack of an absorption maximum at 217 nm in the spectrum of the isolated toxin when measured in ethanol can be accounted for if the isolated material was in the form of a salt. The ratios between the extinctions at 278, 238, and 220 nm are the same for both tenuazonic acid and the isolated toxin.

The infrared spectrum of the isolated substance is presented in Fig. 1. A comparison of the infrared spectra of tenuazonic acid in carbon tetrachloride as described by Kaczka et al. (6) and the isolated substance both in a

TABLE 1. *Mortality of rats fed corn-rice substrate inoculated with Alternaria isolates from nine different sources*

Source of isolates	No. of isolates	No. of isolates lethal to:			Days to death
		0 Rats	1 Rat	2 Rats	
Peanuts	27	7	2	18	3-9
Wheat	17	2	1	14	3-8
Sorghum	9	2	0	7	4-8
Rye	9	5	0	4	5-7
Sunflower	8	2	0	6	5-10
Whole wheat flour	7	5	0	2	3-5
Soybeans	5	5	0	0	0
Cattle feed (corn)	3	0	0	3	4-10
Total	85	28	3	54	3-10

TABLE 2. *Weight change and feed consumption of rats placed on a diet of corn-rice substrate colonized by Alternaria sp.*

No. of samples	Avg wt change in rats (g)		Avg feed consumption of rats (g)	
	Succumbing to ration	Surviving ration	Succumbing to ration	Surviving ration
Fed as sole ration				
68	-13	+10	14	93
Control corn-rice				
17		+2		76
Fed mixed in a balanced ration				
17	-14	+52	10	117
Control balanced ration				
6		+65		123

KBr pellet and as the free acid in chloroform is presented in Table 4. The infrared spectrum of the toxin in either form is similar to that of tenuazonic acid. Particularly the amide absorption at 1,617 cm^{-1}, the NH absorption at about 3,045, and the absorptions in the region below 1,600 cm^{-1} are similar in both materials. No published spectra of tenuazonic acid were available for direct comparison.

The combined results of emission spectroscopy and combustion analysis are presented in Table 5. The combustion analysis data and emission spectra data are consistent with the material being a mixed K, Ca, and Mg salt of tenuazonic acid.

In the chemical degradation study, only isoleucine co-chromatographed with the unknown amino acid obtained. This result is again consistent with results with tenuazonic acid by Stickings (11).

Water and ether extracts of corn-rice infested with selected isolates from the sources listed in Table 6 were analyzed for the presence of salts of tenuazonic acid and for alternariol monomethyl ether. Of 34 isolates of *Alternaria* tested, 23 were toxic (Table 6). Twenty of these 23 isolates produced tenuazonic acid, and 14 produced alternariol monomethyl ether. Of the 11 nontoxic isolates, 2 produced alternariol monomethyl ether, and none produced tenuazonic acid. Three toxic isolates did not produce tenuazonic acid, and one of these produced alternariol monomethyl ether. Presumably, toxins other than tenuazonic acid were involved in the toxicity of these isolates.

Tenuazonic acid was not found in the two naturally infested samples examined.

DISCUSSION

Alternaria sp. have been widely reported as toxigenic, but this toxicity has not previously been attributed to any particular metabolite. In these studies a toxic substance was isolated and data were obtained to indicate that it was a mixed salt of tenuazonic acid. The lethal dose of 100 to 200 mg/kg was close to the 137 to 205 mg/kg lethal dose in female rats re-

TABLE 3. *Comparison of the ultraviolet spectra of an Alternaria toxin and tenuazonic acid*

Absorption	Solvent	*Alternaria* toxin[b]		Tenuazonic acid[a]	
		max (nm)	ϵ rel.[b] (nm)	max (nm)	ϵ rel.[b] (nm)
210-220 nm	Water	None		None	
	0.09 N NaOH	None		None	
	0.09 N HCl	220	0.41	220	0.41
	Ethanol	None		217	0.39
230-240 nm	Water	239	0.79	239	0.79
	0.09 N NaOH	240	0.78	240	0.78
	0.09 N HCl	None		None	
	Ethanol	238	0.64	None	
260-280 nm	Water	279	1.0	279	1.0
	0.09 N NaOH	279	1.0	279	1.0
	0.09 N HCl	277	1.0	277	1.0
	Ethanol	278	1.0	278	1.0

[a] Data from Stickings (11).

[b] Calculated by dividing the extinction at the given wavelength by the extinction at 260-280 nm in the same solvent.

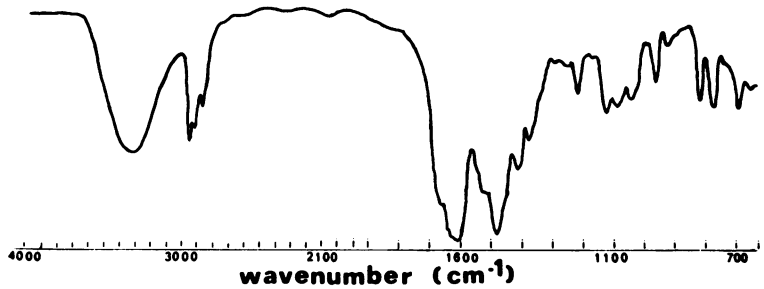

FIG. 1. *Infrared spectrum of toxin isolated from Alternaria-infested corn-rice.*

TABLE 4. *Comparison of the infrared absorption maxima of an Alternaria toxin and tenuazonic acid*

Alternaria toxin in KBr (cm^{-1})	*Alternaria* toxin free acid in $CHCl_3$ (cm^{-1})	Tenuazonic acid in CCl_4[a] (cm^{-1})
—	3,440	—
3,220	3,280	3,174
—	3,048	3,049
2,870	2,870	—
2,920	2,020	—
2,957	2,957	—
—	—	1,721
1,670	1,692	1,692
1,610	1,617	1,630
—	1,595	—
1,480	—	1,458
1,375	1,380	1,383
—	—	1,330
1,290	1,294	1,294
1,250	—	—
1,215	—	1,232
1,120	1,123	1,110
1,085	1,100	1,098
1,040	1,030	1,034
—	—	1,018
—	974	974
960	960	658
915	—	921
—	—	905

[a] Data from Kaczka et al. (6).

TABLE 5. *Combustion analysis and emission spectroscopy of an Alternaria toxin and tenuazonic acid*

Component	Per cent by weight	
	Alternaria[a] toxin	Tenuazonic[b] acid
Combustion analysis		
C	60.40	60.90
H	7.77	7.70
O	25.40	24.30
N	6.40	7.10
Emission spectroscopy		
K_2O	5.30	0.00
CaO	3.36	0.00
MgO	5.15	0.00

[a] Corrected for ash as oxides.
[b] Calculated from $C_{10}H_{15}O_3N$.

TABLE 6. *Toxicity of Alternaria isolates versus toxin production*

Isolate source	No. of isolates			
	Tested	Toxic[a]	Producing toxin	Toxic but not producing toxin
Peanuts	19	13	11	2
Wheat	3	3	2	1
Whole wheat flour	3	2	2	0
Sorghum	3	2	2	0
Sunflower	2	1	1	0
Rye	2	1	1	0
Potato	1	1	1	0
Soybeans	1	0	0	0
Total	34	23	20	3

[a] Toxicity established by feeding 20-day-old, weanling rats for 2 weeks.

ported by Smith et al. (10). In addition, a survey of isolates from various sources showed that the tenuazonic acid was produced by 20 of 23 toxic *Alternaria* isolates. It thus appears that tenuazonic acid and its salts are major toxic metabolites produced by isolates of *Alternaria* from agricultural products. The evidence for the natural occurrence of this metabolite is negative but inconclusive. In view of the relatively large doses required to produce ill effects, it seems unlikely that tenuazonic acid represents a serious mycotoxin problem, but this possibility should not be discounted

until many more naturally infested samples of feeds and foods are examined.

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