Toxicity of Metabolites Produced by the "Alternaria"

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Introduction

The presence of toxin-producing fungi in foodstuffs and other agricultural commodities is well established (*1-3*). The *Alternaria*, *Aspergilli*, *Fusaria* and *Penicillia* have been repeatedly implicated as the principal co-inhabitants of products in which toxicity has been demonstrated (*4,5*). Numerous compounds have been isolated that can explain the toxicity of the *Aspergilli*, *Fusaria*, and *Penicillia*. Among the more important of these are the aflatoxins, patulin, penicillic acid, and sterigmatocystin, because of their carcinogenic potential; the ochratoxins, citrinin, cyclopiazonic acid, and the estrogenic zearalenone because of a variety of high toxicities and their frequent appearance in moldy foodstuffs.

By comparison, toxic components of the *Alternaria* have been studied to only a small extent. The *Alternaria* are found on wheat, barley, oats, sorghum, corn, and peanuts (*4,6,7*). Animal feeds and silage that contain these crops, as well as alfalfa and grass hay are also good sources (*4*). The *Alternaria* are plant pathogens and thus can contaminate food through field infection as well as through storage. Black spot of Japanese pear, brown spot of tobacco, early blight of tomato and potato, and seedling chlorosis of citrus are caused by this genus (*8*).

Christensen et al. (*4*) found *Alternaria* to be one of the genera most consistently isolated in 943 fungal isolates. Of these *Alternaria* from foods and animal feeds, 90% were lethal to rats when fed in a sterilized corn-rice mixture. The percentage was greater than that obtained with isolates of *Aspergilli*, *Fusaria*, and *Penicillia* from the same sources. Doupnik and Sobers (*9*) reported that 31 of 96 *Alternaria* isolates from tobacco were lethal to chicks. Toxic effects in geese (*10*) and a hemorrhagic syndrome in poultry (*11*) have been attributed to *Alternaria*.

Several compounds have been isolated and identified. Most interest has concerned the role of these compounds in plant pathogenesis and their potential use as antibiotics (*8,12*). An exception is tenuazonic acid, a compound of the tetramic acid class. Interest in its potential for toxicity to animals developed when the compound was shown to have antitumor activity (*13,14*). When tested, the LD_{50} doses for sodium tenuazonate administered to mice orally were 81 mg/kg for females and 186 mg/kg for males; in rats the corresponding values were, respectively, 168 and 180 mg/kg (*15*). Meronuck et al. (*16*) found that 23 of 34 *Alternaria* isolates were lethal to rats. Salts of tenuazonic acid were identified in 20 of the 23 lethal isolates, and the compounds were considered to be the

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major toxins. However, since some isolates were toxic but did not contain tenuazonic acid, other toxins appear to be present. Tenuazonic acid has been found in field-infected tobacco (*17*). Quantitative data on the amounts of tenuazonic acid in toxic extracts are not available, and this information is necessary to better correlate the degree of observed toxicity with the presence of this metabolite.

The *Alternaria* are known to produce compounds of at least two other structural classes. The first are dibenzo-α-pyrones (*12,18,19*). These are often major components of the crude fungal extracts. The structures of two, alternariol (AOH) and alternariol monomethyl ether (AME), were established in 1953 (*12*), yet almost no toxicity data are available. Recently, our laboratory isolated and elucidated the structure of two more dibenzo-α-pyrones, altenuene and altenuisol (*20,21*). We isolated two compounds, which we call altertoxin I and altertoxin II, which now represent a third structural class (unpublished data).

Initial investigation of the cytotoxicity of the dibenzo-α-pyrones has indicated a high toxicity (*22*). For HeLa and lymphoma L5178Y cells, the ID_{50} values for AOH and AME were 6 and 8 μg/ml, respectively. We now present further data on the toxicity of these compounds to bacterial and tissue cell cultures, to female mice, and to fetal mice *in utero*. The toxicities of crude extracts, pure components, and a combination of two of the components, AOH and AME, are compared.

Methods

Cytotoxicity

HeLa S3 cells were grown in monolayer culture, and the toxicity of purified mycotoxins or crude fungal extracts was evaluated as previously described (*23*). Bacterial toxicity toward *Bacillus mycoides* ATCC 6462 was carried out by using the paper-disc agar plate method (*24*).

Toxicity to the Young Adult Mouse

Female mice weighing 18-21 g were studied. These included the 4-5 week-old CD-1 mouse (Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts) and 8-10 week-old DBA/2 mouse (Jackson Laboratories, Bar Harbor, Maine). Purified mycotoxins were administered as a single dose intraperitoneally (IP) in 0.1 ml DMSO. Crude fungal extracts were injected IP at a dose of 100 mg/kg/day for 3 consecutive days. The number of deaths that occurred within 14 days was recorded. External signs of toxicity were sought in the animals that survived. After AOH and AME administration, an animal from each test group that survived was sacrificed each month for 10 months. The liver, kidneys, heart, lungs, spleen, and thymus were fixed in 10% neutral buffered formalin for histologic examination. Liver/body and spleen/body weight ratios were calculated.

Fetotoxicity and Teratogenicity

DBA/2 mice were used. The day that a vaginal plug was found was considered day 1 of gestation. The test compounds were administered subcutaneously in DMSO or orally in honey:water (1:1) from days 9 to 12 or 13 to 16 of gestation. Controls were untreated or they received the appropriate solvent. The mice were then sacrificed on day 20 of gestation for examination of the young. The number of live, dead, and resorbed fetuses was counted, and the live fetuses were fixed in Bouin's solution for examination of the internal organs by the method of Wilson (*25*). Some were fixed in alcohol for clearing and staining of the skeleton with alizarin red S (*26*).

Isolation, identification and quantification of the compounds from *Alternaria isolates*

Ten toxic isolates of *Alternaria* were kindly supplied by Dr. Chester Mirocha, University of Minnesota, St. Paul, Minnesota. A toxic isolate of *Alternaria tenuis* (M-1) was supplied by Dr. Charles Main, North Carolina State University, Raleigh, North Carolina. The isolates were grown in pure culture for 14 days on autoclaved rice fortified with yeast extract and Czapek's Dox broth (*27*). The cultures were extracted with acetone–water

(7:3, v/v) and separated into tetrahydrofuran (THF)-soluble and insoluble fractions.

The dibenzo-α-pyrone metabolites AOH, AME, altenuene, and altenuisol were isolated from *Alternaria tenuis* by silica gel G column chromatography of the THF-soluble extract, an increasing THF-in-benzene elution series being used as reported (*20,21*).

AOH, AME, and altenuene were determined quantitatively as trimethylsilyl derivatives in crude extracts by using the gas-chromatographic method of Pero et al. (*28*). Concentrations of altenuisol in crude extracts were not determined because an analytical method was not available. However, its presence was determined by thin-layer chromatography (TLC) on 250 μ silica gel G after development in 30% THF in benzene (*21*).

Altertoxins I and II were isolated in a similar manner to that above but from *Alternaria mali* and with an increasing ethyl acetate-in-benzene elution series. The altertoxins elute in 30% ethyl acetate in benzene. Altertoxin I has not been crystallized but has the empirical formula $C_{20}H_{16}O_6$. Altertoxin II was crystallized from DMSO–water mixtures and has the empirical formula $C_{20}H_{14}O_6$.

Altertoxin I was determined quantitatively by a fluorodensimetric assay. A Zeiss thin-layer chromatogram spectrophotometer with a mercury lamp was used. Altertoxin I was excited by light passing through an M-365 filter and assayed by light emitted at 534 mμ. The concentration of altertoxin I was proportional with transmission from 0.1 to 5.0 μg. Altertoxin II gives a black quenching spot under ultraviolet light on the TLC plates. A method for analysis has not been worked out for this compound.

Results

Table 1 indicates that the *Alternaria* metabolites have considerable cytotoxic activity, especially against HeLa cells. Altertoxin II was the most toxic and had an ID_{50} value of 0.5

Table 1. Cytotoxicity of *Alternaria* metabolites.

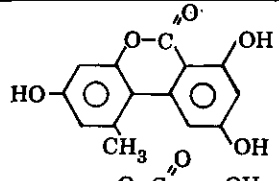
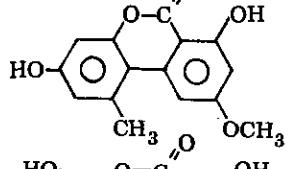
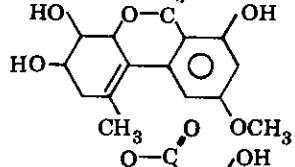
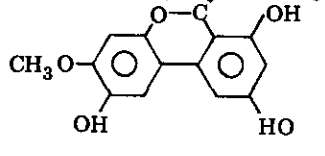
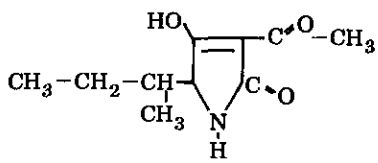
Alternaria metabolite	Structure	Toxicity to *Bacillus mycoides*, μg/disc[a]	Toxicity to HeLa cells (ID_{50}), μg/ml
Alternariol (AOH)		60	6
Alternariol monomethyl either (AME)		500	8–14
Altenuene		125	28[b]
Altenuisol		5	8
AOH + AME (1:1)		0.25	7–10
Altertoxin I	$C_{20}H_{16}O_6$	250	20

Table 1. Cytotoxicity of *Alternaria* metabolites. (Continued)

Alternaria metabolite	Structure	Toxicity to *Bacillus mycoides*, μg/disc[a]	Toxicity to HeLa cells (ID_{50}), μg/ml
Altertoxin II	$C_{20}H_{14}O_6$	250	0.5
Tenuazonic acid		50[c]	40–90, 1300, 300[d]

[a]The lowest concentration of toxin at which there is a measurable zone of inhibition around the assay disc.
[b]ID_{25}.
[c]Toxicity to *Bacillus megaterium* [from Gitterman (*13*)].
[d]Toxicity to Eagle's KB carcinoma for the *L*, *D*, and iso forms, respectively [from Gitterman (*13*)].

μg/ml. Of the dibenzo-α-pyrones, AOH, AME, and altenuisol were the most toxic. When *Bacillus mycoides* was the test organism, AOH and altenuisol were the most toxic, but the combination of AOH and AME showed a striking synergistic effect. With a 1:1 mixture of AOH and AME, only 0.25 μg of each was necessary to elicit a zone of inhibition. Similar synergism was not evident with the HeLa cells.

Table 2. Cytotoxicity of the extracts from *Alternaria* isolates to Bacillus mycoides.

Alternaria isolate		Bacterial toxicity, μg/disc[a]	
Number	Source	THF-soluble fraction	THF-insoluble fraction
M-1	Tobacco	2	5
C-2	Peanuts	5	125
C-3	Whole wheat flour	2	60
C-4	Whole wheat flour	1	30
C-5	Spring wheat	10	30
C-6	Peanuts	1	30
C-7	Sorghum	10	30
C-8	Peanuts	1	10
C-9	Potato	2	30
C-10	Wheat	10	30
C-12	Sunflower	5	30

[a]The lowest concentration of toxin at which there is a measurable zone of inhibition around the assay disc.

The crude extracts from *Alternaria* isolates that had been reported to be lethal to rats showed that most of the cytotoxicity against the bacteria was in the THF-soluble fraction (Table 2). All of the *Alternaria* compounds in Table 1 are freely soluble in THF, with the possible exception of the salts of tenuazonic acid. The activities of the crude extracts were much greater than that of the compounds taken singly. Synergism is thus, again suggested. It is notable that both AOH and AME were present in all of the extracts (Table 3). Altertoxin I was also present in most extracts but at much lower concentrations.

When administration was to mice, the toxic principles were again found to be in the THF-soluble fraction of the isolates (Table 4). AME produced the least lethality (Table 5). There was no evidence of synergism between AOH and AME as there was in the case of *Bacillus mycoides* (Table 5). The mice receiving AOH and AME often were sedated within a few minutes. When recovery occurred it was within 24 hr. The eyes of the mice were dull, and there was occasional stomach spasm and periodic panting. The altertoxins were lethal to the mice at 200 mg/kg. Toxicity with this compound was characterized by inactivity, subendocardial and subarachnoid hemorrhage and blood in the cerebral ventricles. It is seen in Table 3 that altertoxin I occurred in all of the extracts except that from isolate C-2.

Table 3. Concentration of Alternaria toxins in the THF extract of the *Alternaria* isolates.

Alternaria isolate	Solids in the THF extract, %				
	Alternariol	Alternariol monomethyl ether	Altenuene	Altenuisol	Altertoxin I
M-1	5.50	14.00	2.30	+[a]	0.04
C-2	0.25	0.25	0	0	0
C-3	1.00	2.50	0	0	0.10
C-4	1.80	4.40	0	0	0.22
C-5	0.50	0.25	0	0	0.02
C-6	1.10	1.60	1.30	0	0.10
C-7	11.60	3.80	0	0	0.13
C-8	2.20	15.00	3.40	+[a]	0.04
C-9	10.00	6.40	0	0	0.02
C-10	0.50	0.50	0	0	0.01
C-12	2.70	1.00	0	0	0.02

[a]Detected on thin-layer chromatograms.

The effects of AOH and AME on body and organ weight are presented in Table 6. Mice dosed with either AOH or AME weighed slightly less than the controls. Liver/body weight and spleen/body weight ratios were slightly less than in controls, but this was due to reduction of body weight. Histologic examination of the tissues did not reveal abnormalities.

Table 7 shows that the combination of AOH and AME at 25 mg/kg each, administered on days 9-12 of gestation, resulted in an increased percentage of dead and resorbed fetuses/litter and runts/litter. The number of

Table 4. Toxicity of the THF-soluble and THF-insoluble extract of *Alternaria* isolates in two strains of mice[a]

Alternaria isolate	Lethality[b]			
	THF-soluble fraction		THF-insoluble fraction	
	CD-1	DBA/2	CD-1	DBA/2
M-1	2/3	2/3	0/3	1/3
C-2	1/3	3/3	0/3	1/3
C-3	2/3	2/3	0/3	1/3
C-4	1/3	3/3	0/3	0/3
C-5	3/3	3/3	0/3	1/3
C-6	1/3	3/3	0/3	0/3
C-7	1/3	2/3	0/3	0/3
C-8	1/3	3/3	0/3	0/3
C-9	1/3	3/3	0/3	1/3
C-10	1/3	3/3	0/3	0/3
C-12	3/3	3/3	1/3	0/3
Control (0.1 ml DMSO)	0/3	0/3	0/3	0/3

[a]The dose was equivalent to 300 mg/kg of the initial acetone–water (7:3) extract.

[b]Expressed as number of mice that died in 14 days/number of mice tested.

Table 5. Toxicity of *Alternaria* metabolites in three strains of mice.

Alternaria metabolite	Dose, mg/kg	Lethality[a]	Strain
Alternariol (AOH)	100	2/10	DBA/2
	200	3/10	DBA/2
	400	3/10	DBA/2
Alternariol monomethyl ether (AME)	100	0/10	DBA/2
	200	0/10	DBA/2
	400	1/10	DBA/2
AOH + AME	100 + 100	3/10	DBA/2
Altenuene	50	1/3	DBA/2
Altertoxin I	100	0/8	CD-1
	200	8/8	CD-1
Altertoxin II	100	0/2	CD-1
	200	2/2	CD-1
Tenuazonic acid	81[b]	50%[b]	Albino[b]

[a]Expressed as number of mice that died in 14 days/number of mice tested. One mouse receiving AOH at 100 mg/kg and one receiving 400 mg/kg died after 37 and 45 days, respectively.

[b]This is the LD_{50} value [from Gitterman *(13)*].

Table 6. Effects of AOH and AME on the organ weights and organ weight/body weight ratios of DBA/2 Mice.

Treatment	Final body weight, g	Weight change final 5 months, g	Liver weight, g[a]	Liver/body ratio x 100	Spleen weight, g[a]	Spleen/body ratio x100
Controls	30.56±1.51	+3.32±0.80	1.61±0.11	5.28±0.27	0.089±0.003	0.30±0.01
AOH, 100 mg/kg	29.18±1.36	+0.05±0.81	1.70±0.13	5.91±0.47	0.157±0.040[b]	0.54±0.14[b]
AOH, 200 mg/kg	25.71±1.14	+0.08±1.84	1.58±0.11	6.14±0.38	0.098±0.011	0.38±0.03
AOH, 400 mg/kg	25.92±1.36	+0.33±0.73	1.63±0.14	6.24±0.33	0.091±0.013	0.35±0.04
AME, 100 mg/kg	28.93±1.33	+0.94±1.29	1.65±0.08	5.74±0.24	0.093±0.005	0.33±0.02
AME, 200 mg/kg	27.61±1.32	−0.20±1.37	1.63±0.10	5.89±0.28	0.092±0.005	0.33±0.01
AME, 400 mg/kg	25.51±0.93	−0.82±0.85	1.57±0.10	6.15±0.28	0.081±0.004	0.32±0.01

[a]Average for the 10 month period.

[b]Two large spleens were recorded (0.410 g and 0.225 g).

malformed fetuses approached but did not reach significance at the two higher doses. AOH alone, at 100 mg/kg, also increased the percentage of dead + resorbed fetuses/litter and runts/litter. This dosage, however, was shown to have a low degree of toxicity in young adult female mice, as already described. AME, administered on the same days, did not yield effects that reached significance. When AOH was administered at 100 mg/kg on days 13-16 of gestation, the percentage of malformed fetuses increased; AME did not yield effects at the 50 mg/kg dose. Malformations, when seen, were of a variety of types. Most occurred in the controls at lower incidences. The data indicate a fetotoxic effect of AOH at 100 mg/kg and suggest synergism between AOH and AME, since the combination of each at 25 mg/kg yielded significant responses.

Conclusion

Exposure of humans to acutely toxic levels of *Alternaria* components through the food supply is unlikely if fungicidal applications and sanitary storage methods are practiced. However, low-level, long-term exposure is an additional hazard. In order to assess the importance of the components of *Alternaria* as toxic contaminants of our food supply, we must know what toxins are produced in the different species of the genus and how these vary with alterations in their environments. The present study represents a beginning for the determination of the relationship of *Alternaria* to environmental health problems.

We know of at least three classes of toxic metabolites produced by the *Alternaria*: the dibenzopyrones, the tetramic acids, and the group represented by altertoxins I and II. All have been shown in this work to have some degree of toxicity. A synergistic effect between AOH and AME has also been shown in two of the tests, and both AOH and AME are present in all of the species. Whether or not the compounds discussed represent a hazard to humans remains to be determined. It is the opinion of the authors that further research should be encouraged to establish or negate the importance of these compounds as environmental toxins.

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Table 7. Fetotoxic and teratogenic effects of alternariol (AOH) and alternariol monomethyl ether (AME) on DBA/2 mice.

Time of treatment[a]	Treatment	Dose, mg/kg	No. of live fetuses (no. of litters)	Dead + resorbed fetuses/litter (avg), %	Runts/ litter (avg), %	Malformed fetuses/ litter (avg), %
9–12	AOH + AME (1:1)	0.5–4.0[b]	133(15)	9.7	2.2	4.4
		10[b]	24(3)	3.3	0	16.2
		25[b]	35(7)	25.2[c]	22.0[d]	20.2
9–12	AOH	50[b]	51(8)	7.3	3.0	10.6
		100[b]	21(5)	41.7[c]	11.9[d]	7.8
9–12	AME	25[b]	35(6)	3.5	0	0
		50[e]	77(14)	25.0	13.1	23.6
		100[e]	32(6)	15.1	13.3	10.3
		50[f]	32(8)	37.1	6.7	22.8
9–12	DMSO (controls)	0.05 ml	101(15)	13.4	0	11.7
		0.10 ml	42(10)	23.2	13.2	6.0
9–12	Honey–water (1:1) (controls)	0.10 ml	33(7)	19.7	2.4	14.3
9–12	No treatment		77(12)	2:8	0	1.9
13–16	AOH	25[b]	32(5)	10.0	6.7	2.2
		50[b]	22(4)	14.3	0	0
		100[b]	12(4)	37.6	6.3	27.1[c]
13–16	AME	50[e]	47(7)	4.6	3.6	1.8
		50[f]	27(4)	4.2	10.0	0
13–16	DMSO (controls)	0.10 ml	36(5)	10.2	15.3	0
13–16	Honey–water (1:1) (controls)	0.10 ml	29(4)	3.1	0	3.1

[a]Days of gestation.
[b]Administered in 0.05 ml DMSO, subcutaneously.
[c]$p \leqslant 0.05$.
[d]$p \leqslant 0.01$.
[e]Administered in 0.10 ml DMSO, subcutaneously.
[f]Administered in 0.10 ml honey–water, 1:1.

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