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Mutagenicity of the *Alternaria* Metabolites Altertoxins I, II, and III

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The Ames *Salmonella typhimurium* assay was used to demonstrate that an extract of the mold *Alternaria alternata* was mutagenic. The mutagenic extract was fractionated, and the Ames test was used to determine which fractions were mutagenic. Subsequently, altertoxins I and II and a new compound referred to as altertoxin III were isolated by liquid chromatography and shown to be hydroxyperylenequinone compounds by mass spectrometry and infrared, ultraviolet, and proton magnetic resonance spectroscopy. Altertoxins I, II, and III were mutagenic to *S. typhimurium* TA98, TA100, and TA1537 with and without metabolic activation.

Molds of the genus *Alternaria* are common in soil; they are important plant pathogens that cause spoilage of commercially important commodities. Diseases caused by *Alternaria* species include black spot of Japanese pear, tobacco brown spot, early blight of potato, and tomato and citrus chlorosis (7). Fruits and vegetables kept under refrigeration can be spoiled by *Alternaria* because the mold grows at low temperatures (16). Products of *Alternaria* cultured on grain are toxic to chicks and rats. Of 87 isolates grown on corn-rice substrate and fed to chicks, 57 were lethal (4). In another study (6), of 96 strains of *Alternaria* isolated from tobacco and grown on corn, 43 were toxic to chicks. In a third study (3), 90% of the cultures isolated from food and grown on corn were lethal to rats.

The *Alternaria* produce numerous secondary metabolites, including tenuazonic acid (TA), alternariol (AOH), alternariol methyl ether (AME), altenuene, and altertoxin I (7). What little is known about the occurrence in the food supply and toxicity of these metabolites has been reviewed recently (9, 12, 17). In general, these metabolites do not have high acute toxicity, and the chronic effects have not been investigated extensively.

The Ames *Salmonella typhimurium* mutagenicity assay has been used to test *Alternaria* culture extracts. In one study (8) it was reported that extracts of all five isolates of *Alternaria* were mutagenic, with and without liver microsomal activation. A strain of *Alternaria tenuis* produced metabolites that were mutagenic without microsomal activation (2). In another study (14), AME was weakly mutagenic without metabolic activation, AOH showed a marginal response with activation, and TA was nonmutagenic. An *Alternaria* extract was fractionated, and mutagenicity was found in the fractions containing a compound tentatively identified as altertoxin I and in another fraction that may have contained altertoxin II (14). Although other fractions were also mutagenic, the compounds responsible for the mutagenicity were not identified.

In view of the mutagenicity of *Alternaria* extracts, a project was undertaken to obtain *Alternaria* isolates, determine which metabolites they produce, test the extracts for mutagenicity by using *S. typhimurium*, and isolate and characterize the mutagenic metabolites.

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MATERIALS AND METHODS

Isolation of cultures. Because *Alternaria* species are suspected of losing toxin-producing capacity during subculturing and storage in culture collections, fresh cultures of *Alternaria* were obtained. *Alternaria* organisms were isolated from cherries collected from processing lines of canners.

Culture methods. The cherries were sliced and plated on potato glucose agar containing 40 ppm (μg/ml) chlortetracycline hydrochloride and incubated at 22°C for 5 days. Suspected *Alternaria* colonies growing out of the cherries were subcultured onto diagnostic agar media and identified by the method of Ellis (5).

To test for the production of metabolites, each isolate was cultured on rice. Flasks containing 50 g of rice and 50 ml of water were autoclaved at 121°C for 30 min at 15 lb/in^2. The sterilized substrate was inoculated with spores of the mold and incubated without shaking for 20 days at 22°C. To produce larger amounts of metabolites, 10 2,800-ml Fernbach flasks containing 250 g of rice and 250 ml of water were autoclaved, inoculated with the selected isolate (isolate 14, as described below), and incubated in the same way as the 50-g cultures described above.

Extraction. Chloroform was chosen as the extraction solvent because it gave good recovery of the various metabolites in preliminary experiments. Small 50-g cultures were extracted by blending with 150 ml of chloroform. They were then filtered through fluted S&S 588 filter paper, and the filtrate was saved for chemical analysis.

The crude extract filtrate from a 50-g *Alternaria* culture (isolate 14) was dried under vacuum and found to weigh 393 mg. A 100-mg portion was dissolved in 1 ml of dimethyl sulfoxide (DMSO) and serially diluted. The extract was tested for mutagenicity with and without metabolic activation at 0.05 to 500 μg per plate at logarithmic intervals.

The large 250-g cultures of isolate 14 were extracted twice. The moldy rice was broken up with a spatula, blended with 1,000 ml of chloroform, and vacuum filtered through Whatman no. 1 filter paper in a Büchner funnel. The filter cake was then reblended with an additional 750 ml of chloroform and filtered; the filtrate was added to the first filtrate. Filtrates from all 10 large cultures of isolate 14 were combined and concentrated to 400 ml on a rotary evaporator and saved for isolation of the altertoxins.

Fractionation of the extract. After mutagenicity was shown for the crude extract of the culture of isolate 14, 100 mg of

TABLE 1. Average background and average positive control revertant counts in the *S. typhimurium* mutagenicity test

Strain	Background count	Positive controls		
		Chemical	μg/plate	Count
TA98	20	4-Nitro-*o*-phenylenediamine	10	1,245
TA98 + S9	35	2-Fluorenylacetamide	20	1,163
TA100	158	Nitrofurantoin	0.5	874
TA100 + S9	135	2-Aminoanthracene	1.25	1,023
TA1537	6	4-Nitro-*o*-phenylenediamine	10	98
TA1537 + S9	10	2-Aminoanthracene	1.25	57

extract was dissolved in chloroform, added to a 25-g silica gel column (0.05 to 0.2 mm, silica gel 60; E. Merck AG, Darmstadt, Federal Republic of Germany) and eluted with 500 ml of benzene-methanol-acetic acid (94:3:3). Some altertoxins II and III were lost during the fractionation because these metabolites are unstable on silica gel and in acidic solution. Five fractions were collected from the column and analyzed by thin-layer chromatography (TLC) as described below. Each fraction was dried in vacuum before being dissolved in DMSO for the mutagenicity test. Each fraction, representing 100 mg of original extract, was tested for mutagenicity without S9 activation at 0.05 to 5,000 μg equivalents per plate at logarithmic intervals.

Analysis of extracts for metabolites. Extracts were analyzed qualitatively by TLC for AOH, AME, TA, altertoxins I and II, and an unknown compound designated altertoxin III. A 10-μl portion of each extract filtrate was spotted on a silica gel TLC plate (7GF; Mallinkrodt, Inc., St. Louis, Mo.), along with standards of each compound to be analyzed. AOH, AME, and TA standards were obtained from F. Chu, University of Wisconsin. Standards of altertoxins I, II, and III were isolated by the method described below. All standards were dissolved in chloroform at a concentration of 25 μg/ml. After the plate was developed with benzene-methanol-acetic acid (90:5:5) and dried, AOH (R_f 0.16) and AME (R_f 0.69) were detected as blue fluorescent spots under longwave UV light. TA (R_f 0.88) was detected as a dark spot under shortwave UV light as it absorbed the light and quenched the background fluorescence of the 7GF plate. Altertoxin I (R_f 0.38) and altertoxin II (R_f 0.51) were detected by exposing the plate to I_2 fumes in a glass tank. The altertoxin I and II spots changed from colorless to violet. Altertoxin III (R_f 0.94) was detected by exposing the plate to NH_3 fumes, which caused altertoxin III to change to a violet color.

TABLE 2. Production of metabolites by *Alternaria* isolated from cherries and grown on rice

isolate no. and species	AOH[a]	AME[a]	μg of toxin/g of rice			
			TA	Altertoxins		
				I	II	III
1. *A. alternata*	−	−	956	28	14	110
2. *A. alternata*	+	−	920	4	28	114
3. *A. alternata*	−	+	28	1	1	6
4. *A. tenuissima*	+	+	506	9	10	194
5. *A. tenuissima*	+	+	248	6	9	93
6. *A. tenuissima*	+	+	280	13	15	110
7. *A. alternata*	+	−	1,700	4	225	36
8. *A. alternata*	+	+	1,906	21	205	190
9. *A. alternata*	+ +	+ +	330	6	100	109
10. *A. alternata*	+ +	+ +	680	3	20	28
11. *A. alternata*	+	+	856	8	8	39
12. *A. alternata*	−	+	398	18	74	223
13. *A. alternata*	−	+	850	29	68	239
14. *A. alternata*	−	−	96	60	228	380

[a] Symbols: −, negative; +, positive (10 to 100 μg of toxin per g of rice); + +, strongly positive (100 to 1,000 μg of toxin per g of rice).

TABLE 3. Mutagenicity of *A. alternata* isolate 14 extract in *S. typhimurium* TA98, TA100, and TA1537 with (+S9) and without (−S9) metabolic activation

Alternaria extract (μg/plate)	No. of revertants/plate[a]					
	TA98		TA100		TA1537	
	−S9	+S9	−S9	+S9	−S9	+S9
Negative control with DMSO	33	43	142	143	8	13
0.05	30	39	156	131	10	10
0.5	30	47	176	138	12	12
5	60	56	231	155	18	14
50	203	96	377	250	66	32
500	255	243	277	407	70	71
5,000	205	204	107	254	8	56

[a] Average values from two plates.

TA was quantitatively determined by high-performance liquid chromatography (HPLC) with a pump (6000A; Waters Associates, Inc., Milford, Mass.), an injector (U6K; Waters), a radial compression system with a C_{18} column (Waters), an absorbance detector set at 280 nm (model 440; Waters), and a data module (model M730; Waters). The mobile phase used was methanol-water (85:15) containing 300 mg of $ZnSO_4 \cdot 7H_2O$ per liter. TA forms a complex with Zn^{2+}, which elutes as a sharp peak. The flow rate was 1 ml/min. A standard of TA, 10 μg/ml in methanol, was prepared. Extract filtrate (10 μl) was injected directly into the chromatograph without further purification or concentration. Under these conditions TA eluted in 6.3 min. The amount of TA in extracts was obtained by comparing peak areas of standard and sample.

Altertoxins I, II, and III were quantitatively determined with a pump (model 6000A; Waters), an injector (U6K; Waters), a 5-μm-pore-size silica gel column (Waters), an absorbance detector set at 355 nm (model 450; Waters), and a recorder (model 7130 A; Hewlett-Packard Co., Palo Alto, Calif.). The mobile phase used was chloroform-acetic acid-methanol (98:1:1). The flow rate was 1 ml/min. Under these conditions altertoxin III eluted in 1.4 min, altertoxin II in 3.0 min, and altertoxin I in 8.5 min. Altertoxins in extracts were quantitated by injecting 10 μl of the extract filtrate directly into the chromatograph, without purification or concentration, and comparing the peak heights from known concentrations of standards, which were isolated by the method given below. The isolate that produced the most altertoxins I and II was chosen to be tested in the mutagenicity assay because preliminary work in this laboratory and the work of Scott and Stolz (14) indicated that altertoxins I and II were mutagenic.

Preparation of pure compounds. The concentrated extract from the 10 large cultures of *Alternaria* isolate 14 dissolved in 400 ml of chloroform was added to a 150-g silica gel column (0.05 to 0.2 mm; silica gel 60; E. Merck) and eluted with 3 liters of methylene chloride, then with 3 liters of chloroform, and finally with 3 liters of chloroform-methanol (98:2). Fractions (400 ml) were collected and analyzed for altertoxins I and II and for the compound designated altertoxin III by HPLC. Altertoxin III eluted with the methylene chloride, altertoxin II with the chloroform, and

TABLE 4. Mutagenicity of *A. alternata* isolate 14 extract fractions in *S. typhimurium* TA98, TA100, and TA1537 without metabolic activation

Extract fraction (μg equivalents/plate)[a]	No. of revertants/plate[b] for the indicated fraction no.														
	TA98					TA100					TA1537				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
DMSO	24	24	23	23	24	119	119	121	121	119	6	6	10	10	6
0.05	16	22	21	30	23	118	128	128	116	112	5	3	6	10	5
0.5	21	24	32	18	24	110	119	132	136	117	4	4	10	6	4
5	17	22	35	29	23	97	130	188	162	118	6	6	20	13	8
50	16	40	59	63	48	132	272	231	243	190	4	32	52	27	10
500	19	55	128	112	74	130	225	310	327	311	4	30	72	75	20
5,000	21	47	193	173	60	109	128	102	172	201	8	18	14	66	32

[a] The amount shown represents the quantity of original extract from which the fraction plated was derived.
[b] Average values from two plates.

altertoxin I with the chloroform-methanol. Fractions containing mixtures were rechromatographed on smaller columns. Repeated chromatography and crystallization from chloroform-hexane yielded 150 mg of altertoxin I, 317 mg of altertoxin II, and 451 mg of altertoxin III. The purity of the compounds was checked by HPLC. No other peaks could be seen when an amount of toxin giving a full-scale peak was chromatographed. Similarly, only a single spot for each toxin could be seen on TLC. These compounds were tested for mutagenicity with and without metabolic activation.

Mutagenicity tests. *S. typhimurium* TA98, TA100, and TA1537 were used to detect reverse mutations from histidine dependence to histidine independence by the plate incorporation method of Ames et al. (1). Bacteria from an overnight culture (0.1 ml), the test substance in DMSO (0.05 ml), and S9 mix, when used (0.5 ml), were added to 2 ml of molten top agar. A total of 50 μl of S9 per plate derived from the livers of Aroclor 1254-induced male Sprague-Dawley rats was used. Because altertoxin III was unstable in DMSO solution and formed a purple product, the DMSO was purged with a stream of nitrogen before and after solutions were prepared. This reduced the level of oxygen, which may have been responsible for the decomposition. Altertoxin III was insufficiently soluble in any other Ames test-compatible solvent. All tests were run in duplicate. The variation between duplicate samples was always less than 20%. Plates were incubated for 2 days at 37°C, and all colonies were counted manually. A revertant count twice that of the background was considered positive. Bacterial cultures were routinely tested for viable count and sensitivity to ampicillin and crystal violet. Positive controls were used as shown in Table 1.

RESULTS

HPLC and TLC analysis of extracts. The results of analyses of 14 *Alternaria* extracts are shown in Table 2. All the cultures produced TA and altertoxins I, II, and III, although sometimes in only trace amounts. Not all isolates produced detectable levels of AOH and AME. Because it was the best producer of all three altertoxins, extract from *Alternaria* isolate 14 was chosen for mutagenicity testing and as the source of the altertoxins. In addition, *Alternaria* isolate 14 did not produce AOH or AME, which, if present, are difficult to separate from the altertoxins.

Mutagenicity assay of *Alternaria* extract. The crude *Alternaria* extract was mutagenic to all three bacterial tester strains, with and without metabolic activation (Table 3).

Mutagenicity assay of *Alternaria* extract fractions. Four of the five fractions from the silica gel column fractionation of the *Alternaria* isolate 14 extract were mutagenic (Table 4). Each fraction represents portions of original extract, and the data are presented as microgram equivalents of crude extract and not actual micrograms of material. Fractions 3 and 4 were the most mutagenic. Fraction 3 contained altertoxin II and a small amount of altertoxin I; fraction 4 contained altertoxin I and a small amount of altertoxin II. Fractions 2 and 5 were less mutagenic. Fraction 2 contained altertoxin III and some TA; fraction 5 contained a small amount of altertoxin I and low-R_f material. Fraction 1, which contained TA, was not mutagenic.

TABLE 5. Mutagenicity of altertoxins, I, II, and III in *S. typhimurium* TA98, TA100, and TA1537 with (+S9) and without (−S9) metabolic activation

Altertoxin (μg/plate)	No. of revertants/plate[a]																	
	TA98						TA100						TA1537					
	−S9			+S9			−S9			+S9			−S9			+S9		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
DMSO	20	20	22	35	30	34	153	126	148	135	116	143	6	3	6	10	3	7
0.018	22	27	51	34	24	32	186	127	179	138	136	128	6	4	10	6	6	8
0.06	28	18	50	24	33	40	140	186	250	132	133	146	5	8	17	6	3	6
0.18	24	40	106	40	36	74	166	360	532	130	146	204	5	16	26	13	6	12
0.6	43	114	170	32	34	131	183	375	558	122	178	256	13	27	50	6	36	24
1.8	40	136	260	42	48	130	296	440	524	152	216	490	18	36	54	8	20	52
6	88	130	150	46	109	222	329	452	621	186	450	668	28	57	86	13	43	64
18	100	146	156	58	157	412	525	406	646	295	255	966	52	47	76	29	76	98
60	97	34	171	84	408	416	531	168	646	318	240	836	40	33	76	60	72	160

[a] Average values from two plates.

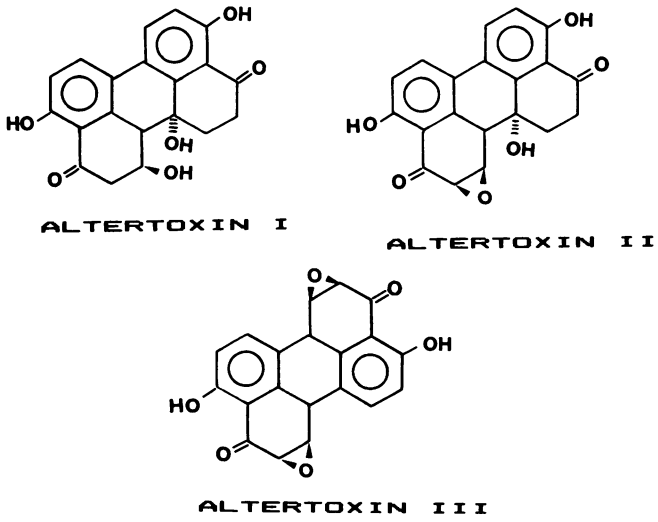

FIG. 1. Structures of altertoxins I, II, and III.

Physical properties of altertoxins I, II, and III. Altertoxins I, II, and III were isolated from *Alternaria* isolate 14 as described above. By high-resolution mass spectrometry the molecular formula of altertoxin I is $C_{20}H_{16}O_6$, that of altertoxin II is $C_{20}H_{14}O_6$, and that of altertoxin III is $C_{20}H_{12}O_6$. Infrared, UV, and ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained for altertoxins I, II, and III and were consistent with hydroxyperylenequinone-type structures.

Mutagenicity testing of altertoxins I, II, and III. The results of the mutagenicity assays of altertoxins I, II, and III isolated from the *Alternaria* extract are given in Table 5. All three compounds were mutagenic to all three strains with and without metabolic activation. In general, altertoxin III was the most mutagenic of the three compounds at virtually all doses in which a mutagenic response occurred.

DISCUSSION

Two structures have been proposed for altertoxin I, also called dihydroalterperylenol (11, 15). The mass, UV, infrared, and ^{13}C and ^1H NMR spectra of altertoxin I, dihydroalterperylenol, and the compound used in this study are identical. No structures have been proposed for altertoxin II. An isomer of altertoxin II is known in the literature as alteichin (12) and alterperylenol (11). The compound used in this study has numerous differences between its spectra and the spectra reported for alteichin and alterperylenol. To our knowledge, this is the first report of altertoxin III. The mass, UV, infrared, and ^{13}C and ^1H NMR spectra of altertoxins I, II, and III have been reported previously (M. Stack, M.S. thesis, The American University, Washington, D.C., 1984). We have recently elucidated the structures of altertoxins I, II, and III (Fig. 1), using ^1H and ^{13}C NMR spectra (manuscript in preparation).

The data show that altertoxins I, II, and III are mutagenic to *S. typhimurium* TA98, TA100, and TA1537, even in the absence of metabolic activation. Of the three compounds, altertoxin III appears to be the most mutagenic to TA98, followed by altertoxin II and altertoxin I. Aflatoxin B_1, which is one of the most potent biologically produced genotoxic substances known, has been reported to have a mutagenic potency of approximately 7 revertants per pmol with TA100 in the presence of S9 (10). By comparison, altertoxin III gave a maximum response of 0.7 revertant per pmol with TA100, whereas the maximum response of altertoxin II was about 0.5 revertant per pmol, and that of altertoxin I was <0.03 revertant per pmol. The comparison between the mutagenic potency of the altertoxins and that of aflatoxin B_1 cannot be strictly made because the mutagenicity of aflatoxin B_1 depends on the efficiency of the in vitro metabolic activation system. However, these data show that altertoxins II and III are quite potent mutagens in the *S. typhimurium* plate incorporation assay. Altertoxin I is considerably less mutagenic than the other altertoxins studied.

Some difficulties were encountered in testing altertoxin III, which was unstable in DMSO and formed an insoluble purple product. Because oxygen in the DMSO may have been responsible for the decomposition, the mutagenicity assay was performed as rapidly as possible with DMSO solutions that were purged with nitrogen. In spite of these precautions, the altertoxin III solutions were quite purple by the time all test plates were poured. It is possible that the decomposition products are mutagenic, but that determination requires further work. Altertoxin II is also unstable in DMSO but to a lesser extent than altertoxin III.

From the results of this study it was concluded that the *S. typhimurium* assay can be used to detect mutagens in crude fungal extracts and to follow the mutagens through fractionation and isolation of pure compounds. The compounds responsible for the mutagenicity of *Alternaria* extracts studied are altertoxins I, II, and III, which have hydroxyperylenequinone-type structures. Methods of analysis for the altertoxins in food should be devised if the presence of these mycotoxins is to be determined in the food supply.

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