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Mutagenic Action of Phaseolinone, a Mycotoxin Isolated from *Macrophomina phaseolina*

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Abstract

Phaseolinone was mutagenic to excision-repair-deficient strains of *Escherichia coli* WP2 and also to *Salmonella typhimurium* TA100. The repair test was indicative of covalent binding of the toxin to DNA. The side-chain epoxide and the hydroxy groups of the molecule were found to be essential for mutagenic activity.

Introduction

Fungi produce a variety of metabolites which are responsible for many toxic syndromes in both man and animals. The probability of contamination of human foodstuffs and animal feed by these naturally occurring toxic metabolites is high because of the ubiquitous distribution of fungi; the harmful effects of these metabolites are of great concern because of their carcinogenic potential.

Phaseolinone is a newly described diepoxy metabolite of the plant-pathogenic fungus *Macrophomina phaseolina* Goid. (Siddiqui *et al.* 1979; Dhar *et al.* 1982). Many expoxides are known to be mutagenic and carcinogenic (Miller 1970). Therefore, the possible role of phaseolinone as a human and animal health hazard needs to be investigated. In this paper it is established that phaseolinone is a base-pair substitution mutagen and, by using different derivatives, evidence for the functional groups involved in mutagenesis is provided.

Materials and Methods

Culture

M. phaseolina was isolated from black gram (*Phaseolus mungo* L.) seeds (Siddiqui *et al.* 1979) and maintained in Czapek–Dox agar slants at 10°C. The *Escherichia coli* and *Salmonella typhimurium* strains were obtained from Professor B. A. Bridges and Professor B. N. Ames respectively. *E. coli* strains were maintained at room temperature (30°C) in nutrient agar (Oxoid) slants and *S. typhimurium* strains were maintained for short period at 4°C in nutrient agar slants and plates. Stock cultures were maintained at −30°C.

Phaseolinone and Its Derivatives

Phaseolinone was isolated from 7-day unshaken cultures of *M. phaseolina* in Czapek–Dox broth, purified and its derivatives prepared as described by Dhar *et al.* (1982).

Reversion Test

The ability of phaseolinone to cause reversion of auxotrophic mutants of *E. coli* and *S. typhimurium* was assessed by standard methods as reported by Bridges (1972) and Maron and Ames (1983) respectively.

To determine whether mutagenesis was concentration-dependent the soft agar overlay method of Maron and Ames (1983) was used. For the *S. typhimurium* test the top agar contained 0·1 mg/ml of

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biotin and 0·1 mg/ml of histidine, 0·5% (w/v) NaCl, 0·8% (w/v) agar No. 1 (Oxoid) and the bottom agar contained 1·2% (w/v) agar in minimal medium (Maron and Ames 1983) and 12·5 μg biotin per plate. For the *E. coli* test M9 bottom agar (Miller 1972) was used and top agar contained 0·15% (w/v) nutrient broth (Oxoid), 0·5% (w/v) NaCl and 0·8% (w/v) agar No. 1 (Oxoid).

The test compounds in 0·1 ml water and 0·1 ml overnight (16 h) nutrient broth (Oxoid) culture of the test strain were mixed in 2 ml top agar (42°C) before pouring. Average counts from three plates were taken after incubation at 37°C for 48 h; revertants which appeared were again transferred to minimal media for further confirmation. Experiments were performed at least three times.

Growth Inhibition

Growth inhibition by phaseolinone was assayed in minimal medium containing histidine (2 mg/plate) for *S. typhimurium* and M9 agar containing tryptophan (2 mg/plate) for *E. coli*. Lawns of bacteria were prepared from 4 h nutrient broth cultures, and the aqueous solution of phaseolinone was placed in a central well. The diameter of the inhibition zone was measured after 48 h at 37°C.

Seed Germination Inhibition Test

Surface-sterilized black gram seeds were placed in sterile Petri dishes containing filter paper soaked with different concentrations of phaseolinone or its derivatives. The number of seeds which had germinated after 36 h was recorded. Averages of triplicate sets were scored.

Results

Reversion

Phaseolinone was found to be mutagenic only for *S. typhimurium* strain TA100, although eight different strains were used (Table 1). Reversion occurred at a concentration of 500 μg/plate. TA1535 showed no reversion at concentrations up to 1 mg/plate either in spot tests or in overlay assays. *E. coli* strain WP2*uvrA* was found to be more sensitive

Table 1. Reversion and growth inhibition of *S. typhimurium* and *E. coli* test strains by phaseolinone

Strain[A]	Relevant character			Revertants per plate[C]	Inhibition zone[D] (mm)
	rfa[B]	*uvr*	pKM101		
S. typhimurium					
hisD3052	+	+	−	28	0
TA1534	+	−	−	23	0
TA1978	−	+	−	11	4
TA1938	−	−	−	18	15
TA98	−	−	+	40	15
hisG46	+	+	−	4	0
TA1535	−	−	−	18	8
TA100	−	−	+	568 (400)	15
E. coli					
WP2*trpE65*				6	10
WP2*uvrA*				100 (92)	15
CM5??1*recA*				7	15

[A] Strains with *uvr* mutations are deficient of excision repair.
[B] The rough mutation (*rfa*) causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increases permeability to large molecules.
[C] Solution containing 750 μg of phaseolinone was placed in the central well.
[D] Data presented in parentheses are the number of revertants excluding the number of spontaneous revertants; toxin concentration was 1 mg/plate.

to reversion by phaseolinone, reversion being detected at a concentration of 125 μg/plate (Table 2). The other two *E. coli* strains used showed no reversion by phaseolinone either in spot tests or in overlay assays (Table 1).

Growth Inhibition and Repair Test

The roughest *S. typhimurium* strains and all *E. coli* strains were sensitive to phaseolinone. A zone of inhibition of 15 mm was observed with repair-deficient strain TA1938 compared with the zone of 4 mm observed in strain TA1978 (Table 1). Similar results were also obtained in repair-deficient and repair-proficient *E. coli* strains.

Mutagenicity and Toxicity of Derivatives

Six derivatives of phaseolinone (Fig. 1) were tested for mutagenic action on *E. coli* strain WP2*uvrA* and also for inhibition of seed germination. The ketone (II) was less mutagenic

Table 2. Reversion of *E. coli* WP2*uvrA* and *S. typhimurium* TA100 strains to prototrophy induced by phaseolinone

Culture (10^8 cells) and phaseolinone (toxin) were mixed in 2 ml top agar and plated on minimal media to score trp^+ and his^+ revertants of *E. coli* WP2*uvrA* and *S. typhimurium* TA100 respectively

Concn of toxin (μg/plate)	No. of revertants per plate	
	S. typhimurium TA100	*E. coli* WP2*uvrA*
0	168	8
62	216	14
125	208	36
250	230	30
500	413	70
1000	657	102

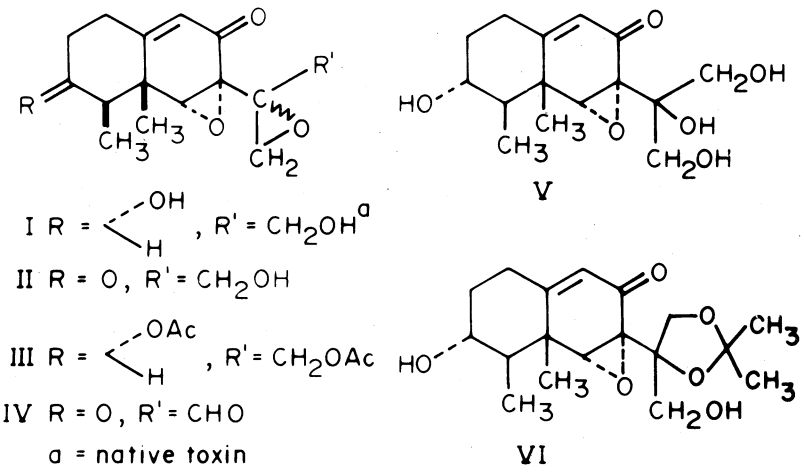

Fig. 1. Structure of phaseolinone and its derivatives.

and less toxic than phaseolinone itself (Table 3). The diacetate (III) was, however, as toxic as the native toxin (I) but was not mutagenic. The ketoaldehyde (IV), and tetrol (V) and the acetonide (VI) also showed reduced toxicity and loss of mutagenicity (Table 3).

Table 3. Mutagenic and toxic activity of phaseolinone and its derivatives

Concentration of compounds used was 1 mg/plate for reversion test and 100 μg/ml (3 ml/plate) for seed germination inhibition test

Agent (see Fig. 1)	No. of revertants of *E. coli* WP2*uvrA* per plate	Seed germination inhibition (%)
Phaseolinone (I)	180	100[A]
Ketone (II)	45	50
Diacetate (III)	26	100[B]
Ketoaldehyde (IV)	24	30
Tetrol (V)	24	46
Acetonide (VI)	22	0
None	25	0

[A] 12 μg/ml native toxin inhibited seed germination by 70%.
[B] 12 μg/ml of diacetate inhibited seed germination by 80%.

Discussion

Phaseolinone has been found to be mutagenic. It caused reversion to prototrophy in *S. typhimurium* strain TA100 but not in strain TA1535 (Tables 1 and 2). Since the difference between TA100 and TA1535 is that the former harbours plasmid pKM101, it would appear that the presence of pKM101 is necessary for phaseolinone-induced reversion of the *hisG46* marker to be detected.

All Ames' frame-shift indicator strains were insensitive to reversion by phaseolinone which suggests that the toxin is not capable of acting as a frame-shift mutagen. Growth-inhibition studies indicated that insensitivity to the mutagenic effect of phaseolinone was not simply due to killing of tester strains (Table 1). The two strains which showed reversion in this test were also among the most sensitive to growth inhibition. Thus it is unlikely that there was any masking of mutagenicity by toxicity. Moreover, the results indicated that most excision-repair-deficient strains were sensitive to killing by phaseolinone.

All smooth *S. typhimurium* strains (*hisD3052*, TA1534 and *hisG46*) were resistant to the toxic effects of phaseolinone; it is likely, therefore, that rough strains were more permeable to phaseolinone.

The repair test indicated the possibility of killing by covalent binding of phaseolinone to TA1938 DNA, and that the damage produced was excisible in strain TA1978. Repair-deficient *E. coli* strains were also more sensitive than the repair-proficient strain. These results suggest that phaseolinone-induced DNA damage can be efficiently repaired by excision-repair mechanisms.

E. coli WP2*trpE65* strain is known to be sensitive to reversion by mutagens, causing base-pair substitution at both A : T and G : C sites (Bridges 1976). No reversion was induced in *E. coli* WP2*trpE65* and its *recA* derivative (Table 2), and only the WP2*uvrA* strain was sensitive to reversion. These results suggest that phaseolinone is a base-pair substitution mutagen which produces excisible chemical damage to DNA and that reversion to prototrophy resulted from the operation of a *recA*-dependent, error-prone repair system. Phaseolinone-induced reversion in *S. typhimurium* strain TA100 may also be due to a plasmid (pKM101)-determined, error-prone repair function which is normally present in *E. coli* WP2 strains but not in *S. typhimurium* LT2 strains (Sedgwick and Goodwin 1985).

Because the ketone (II) was less mutagenic and less toxic than phaseolinone (I) (Table 3), and phaseolinone diacetate was just as toxic as the native toxin, but was not mutagenic, it may be suggested that both the primary and secondary alcoholic groups of phaseolinone are essential for mutagenic action. Modification of only one hydroxy group (ketone) resulted

in reduction of mutagenic activity, and it was lost completely when both the hydroxy groups were modified (ketoaldehyde IV and diacetate III). On the other hand, when phaseolinone was converted to tetrol (V) or acetonide (VI), a reduction in toxicity and a loss of mutagenicity was observed. This implies that a side-chain epoxide and the alcoholic groups are essential for mutagenic action. It is still not clear which functional groups are responsible for toxicity.

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