of incubation. Viable embryos were characterized by reduced blastodermal area, 'relaxed' neural folds, and poorly defined somite pairs (fig. 3). Furthermore, TFP (9 µg/ml)-treated embryos showed the first signs of neural tube closure defects within 3 h of incubation compared to 4.5 h of incubation in CPZ (20 µg/ml)-treated embryos, a finding consistent with that of Levin and Weiss[10] who reported that TFP was approximately four times more effective than CPZ in inhibiting calmodulin binding activity in vitro. When embryos showing 'relaxed' neural folds (cf. fig. 2) were subcultured for 6–8 h on plain thin albumen, they underwent morpohogenesis comparable to corresponding (untreated) controls, indicating that 1) the inhibitory action of CPZ and TFP was readily reversible and 2) the observed relaxation of neural folds was not a consequence of general cytotoxicity[11].

SEM revealed that apical (luminal) surfaces of treated neuroepithelial cells were often smoother and broader than those of controls (figs 4 and 5), suggesting that the contractile activity of apical microfilament bundles responsible for apical constriction of neuroepithelial cells during elevation of neural folds had been impaired[3]. This view is supported by the observation that apical microfilament bundles in cells showing poor apical constriction were thinner and much less conspicuous than those in controls where they are known to progressively increase in thickness during apical constriction[16].

While we have shown that calmodulin antagonists reversibly inhibit elevation of neural folds through their disruptive action on the contractile activity of apical microfilament bundles in neuroepithelial cells, further work is needed to 1) elucidate the molecular basis of this inhibition and 2) determine if calmodulin indeed participates in closure of the neural tube. Experiments along these lines are presently underway in our laboratory.

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Alternariol, a dibenzopyrone mycotoxin of *Alternaria* spp., is a new photosensitizing and DNA cross-linking agent

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Summary. The mycotoxin alternariol (3,4',5-trihydroxy-6'-methyldibenzo [a] pyrone) but not alternariol monomethyl ether (3,4'-dihydroxy-5-methoxy-6'-methyldibenzo [a] pyrone) is phototoxic to *Escherichia coli* in the presence of near UV light (320–400 nm). The phototoxicity bioassays with a DNA repair-deficient mutant of *E. coli* suggested that DNA may be the molecular target for photo-induced toxicity of alternariol. Interactions between alternariol and double-stranded, supercoiled DNA suggest that alternariol interacts with DNA by intercalation. No DNA breakage was detected in this system; however, alternariol forms a complex and cross-links double-stranded DNA in near UV light. These results suggest that alternariol is a new phototoxic, DNA-intercalating agent and is a DNA cross-linking mycotoxin in near UV light.

Key words. Alternaria spp.; mycotoxin; alternariol; alternariol methylether; photosensitizer; DNA cross-linking.

Light modifies the biological activity of naturally occurring photosensitizers[1–10]. The cellular targets of these molecules have been identified as membrane components and the nucleus[3]. Indeed, DNA is the molecular target in the presence of near UV light (n-UV, 320–380 nm) for some of these photosensitizers[1–6]. Compounds that react with DNA in the presence of n-UV can cause chromosomal aberrations and are powerful mutagens[4, 11]. The possibility that the photochemical properties of such natural products can be exploited in the treatment of neoplastic disorders has been considered (see Elespuru and Gonda[1]). However, the photosensitizing nature of mycotoxins is relatively unexplored.

Mycotoxins are toxic metabolites produced by various microfungi[12]. The compounds are commonly encountered in mold-contaminated food-stuffs and pose serious threats to the health of humans and livestock. At least three classes of mycotoxins are known to be phototoxic. These are the aflatoxins[6], the extended quinone cercosporin (see Towers[3]) and the dimeric anthraquinone rugulosin[12]. Sporodesmin, an amino acid-derived fungal metabolite is a known photosensitizer[12]. Alternariol, however, a well-known myco- and phytotoxin produced by *Alternaria* spp., has not been previously examined for any phototoxic effects, although the compound is known to be cytotoxic[12]. It is therefore relevant that alternariol, as well as some other *Alternaria* spp. metabolites, were recently found to be mutagenic[13].

We have employed the relatively simple and sensitive bioassay promulgated by Ashwood-Smith et al.[9] in our study of alternariol and alternariol monomethyl ether (fig. 1). For this study we developed a rapid method for detecting DNA-cross-linkage using agarose gel electrophoresis. Alternariol was found to be a new photosensitizer, and the results suggest that DNA is the

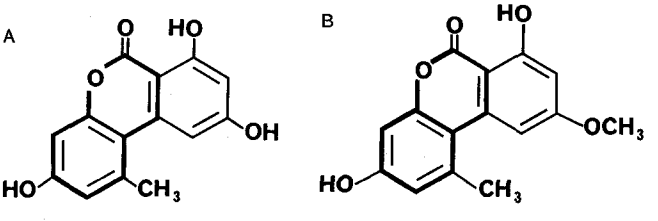

Figure 1. The chemical structures of alternariol (A), and alternariol monomethylether (B). The coumarin moiety is outlined.

molecular target for alternariol. The basis of the bioassay lies in the use of two strains of *Escherichia coli;* one strain (B/r) is able to repair damaged DNA; the other strain (B_{s-1}) is a DNA repair-deficient mutant[9]. The bioassay involves incubating the bacterial strains in the presence of the photosensitizer and irradiating with light of appropriate wavelength and irradiance.

A mixture of alternariol and its methyl ether was available as a gift from Professor R. Thomas (University of Surrey) to Dr A. Stoessl. Alternariol and its methyl ether ($R_f = 0.21$ and 0.65 respectively) were separated from this mixture by repeated TLC on silica gel 60 F254, 0.2 mm (Kieselgel, DC-Alufolien, Merck) using chloroform-methanol (95:5, v/v) as the solvent, and was chromatographically homogeneous. UV spectra for both compounds were referable to those presented in Cole and Cox[12].

Both strains of *E. coli* were grown overnight in nutrient broth at 37°C. Two petri plates containing 25 ml of nutrient agar were covered with a lawn of each strain of bacteria. In an unilluminated vertical flow sterile cabinet four replicates of 6 mm diameter, Whatman no. 1 filter paper discs were loaded with 5 µl of one of the following (0.0, 0.1, 1.0, 10.0, 50.0, 100.0 or 1000 µg/ml) concentrations of alternariol or alternariol monomethyl ether (fig. 1) in 95% redistilled ethanol. The filter paper discs were allowed to dry in dark. Subsequently, one set of the seven discs was applied in an unilluminated cabinet to the surface of each of the four bacterial plates. One set of plates containing *E. coli* B/r and *E. coli* B_{s-1} was immediately irradiated with n-UV (320–400 nm) from a bank of two Sylvania F20T12BLB fluorescent tubes at a distance of 15 cm at 35°C for 1 h. The duplicate set of plates wrapped with aluminium foil to exclude all light, was maintained under identical conditions. Subsequently, all plates were incubated at 37°C. After 24 h the organisms were scored for any phototoxic response. A clear zone of growth inhibition surrounding a disc was considered a positive (i.e. phototoxic) response. The results indicated that *E. coli* B_{s-1} is much more sensitive to the photosensitizer alternariol than is *E. coli* B/r. With 50, 100 and 1000 µg/ml alternariol zones of inhibition corresponded to 2, 8 and 10 mm diameter respectively when *E. coli* B_{s-1} was used. No zones of inhibition were detected in dark when either strain was used in the bioassay. Phototoxicity with *E. coli* B/r was only observed when a concentration of 1000 µg/ml alternariol was administered and showed an inhibition zone of 7.5 mm diameter. However, with *E. coli* B_{s-1} phototoxicity was always observed when 50–100 µg/ml compound was used. This suggests that the bacterial DNA may be the molecular target of the photosensitizer. Alternariol monomethyl ether was not phototoxic at the concentrations used.

We suspect that alternariol may intercalate with DNA. To test our hypothesis we used supercoiled, double-stranded plasmid DNA designated pCB4[14]. If alternariol intercalates with closed circular plasmid DNA (Form-I DNA) it will retard its mobility by introducing positive turns in the negatively supercoiled plasmid and thus reduce the electrophoretic mobility[15]. To test this we developed a 4-channeled agarose gel holder with two lanes per channel (fig. 2). Agarose gel (0.7% in TANAE buffer, pH 7) was prepared and channel 1 was poured with no alternariol; the gel was kept hot and the alternariol concentration was adjusted to 1 µg/ml in the remaining fraction, and channel 2 was poured; the remaining agarose was kept hot and the concentration of alternariol was raised to 5 µl/ml, and channel 3 was poured. The procedure was repeated with channel 4 containing 10 µg/ml alternariol. The first lane in each channel shows marker DNA. The other lanes contain pCB4 DNA. We consistently observed reduced mobility of supercoiled pCB4 DNA when 10 µg/ml was used (fig. 2; channel 4, lane 2, b). With 10 µg/ml alternariol in the gel the supercoiled DNA was dramatically retarded (fig. 2, arrowhead) and smeared over a relatively wide range. No DNA breakage was evident in this system since the amount of DNA migrating with the mobility of linear or open circle DNA did not increase with the treatment (fig. 2)[16].

Alternariol could react with DNA in a manner similar to that of psoralens by forming covalent, interstand cross-links[17]. The method we used to test for crosslinkage in doubled-stranded DNA was similar to that of Cole (18) who initially found 4,5',8-methoxypsoralen to bind to DNA. The method we designed utilized the zero order reassociation kinetics of denatured cross-linked DNA. The differences between the zero order reassociation kinetics of cross-linked DNA and the 2nd order reassociation kinetics of untreated DNA has been used effectively with hydroxylapatite chromatography to show that 4,5',8-trimethylpsoralen introduces cross-links into DNA[19]. Our method utilizes a homogeneous fragment of DNA created by digesting a plasmid pECAN 1 with Eco R_1[19]. The homogeneity of this fragment permits the analysis of single and double stranded DNA by gel electrophoresis. This means that a large number of small samples of DNA can be tested simultaneously and rapidly on a single agarose electrophoretic gel.

Our method involves irradiation of 2 µl (0.25 µg DNA/µl) DNA in the presence of the photosensitizer in 16 µl TRIS-EDTA, pH 8.0 buffer for 2 h in plastic microcentrifuge tubes. The final concentration of photosensitizer was 100 µg/ml. After irradiation, the reaction mixtures were immersed in a boiling water bath for 2 min and subsequently placed in an ice bath. 5 µl of bromophenol blue in sucrose was subsequently added as a dye

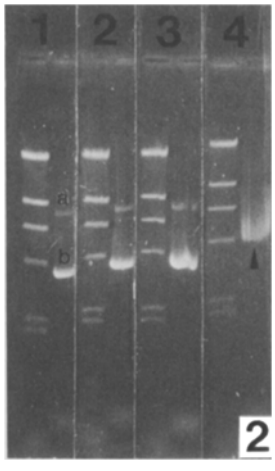

Figure 2. Agarose gel with four channels (1, 2, 3, 4) containing 0, 1, 5, and 10 µg/ml alternariol respectively. The first lane in each channel contains Hind III digested DNA as mol.wt marker; the second lane contains pCB4 DNA. In channel 1, lane 2 linear DNA and supercoiled DNA are designated a and b respectively. In channel 4 lane 2 (arrowhead) pCB4 DNA is shown to have reduced mobility in the presence of 10 µg/ml alternariol.

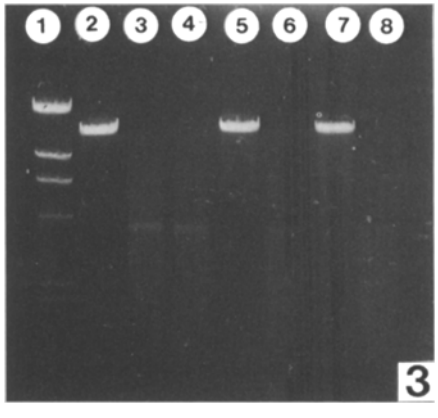

Figure 3. Cross-linking of pECAN 1 DNA as detected by agarose gel electrophoresis (lanes 5–8). Lane 1 contains Hind III digested DNA as a mol.wt marker; lane 2, untreated linearized pECAN 1 DNA; lane 3, denatured pECAN 1 DNA; lane 4, n-UV treated and subsequently denatured pECAN 1 DNA ; Lane 5, pECAN 1 DNA treated with n-UV in the presence of psoralen and subsequently denatured as described in text. Note that the DNA migrates the same distance as untreated DNA; lane 6, as above but with no n-UV irrdiation. Note that no DNA cross-linking is evident; lane 7, same treatment as lane 5 but 8-methoxypsoralen was used instead of psoralen. Note that cross-linking of pECAN 1 DNA is apparent; lane 8, as above, but with no n-UV irradiation.

marker to the solution. Aliquots (25 µl) of the DNA reaction mixtures were subjected to electrophoresis in 0.7% agarose. The procedure denatures native double-stranded DNA or n-UV-treated DNA; however cross-linked DNA, because of its snap-back property, is rapidly renatured. DNA without interstrand cross-links that is denatured to the single-stranded form migrates much faster during electrophoresis (fig. 3, lanes 3, 4, 6, 8) than boiled, cross-linked DNA (fig. 3, lanes 5, 7) which migrates the same distance as the untreated undenatured DNA (fig. 3, lane 2). DNA was visualized by staining with ethidium bromide and transillumination. Any double-stranded DNA detected after electrophoresis was evidence of DNA cross-links. Figure 3 shows this effect with psoralen or 8-methoxypsoralen, which are known DNA cross-linkers. Figure 4 shows that alternariol (lane 4), but not alternariol monomethyl ether (lane 6), reacts with DNA in n-UV to produce cross-links. Ethanol alone did not photoreact with DNA to produce cross-links. Furthermore, figure 5 shows that precipitation of the DNA photoproduct (lane 4) with 80 µl 95% cold ethanol in the presence of 40 µl 100 mM NaCl and washing the DNA with cold ethanol prior to denaturation did not remove the cross-links. This is evidence for covalent bonding of alternariol to DNA.

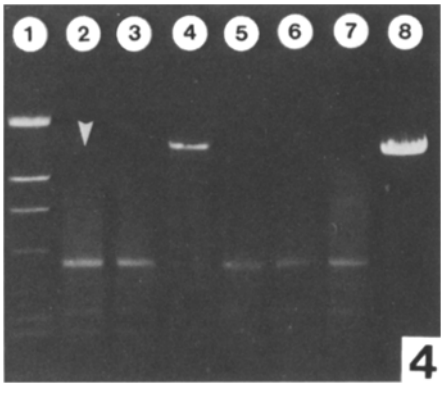

Figure 4. Cross-linking of pECAN 1 DNA by alternariol in the presence of n-UV as detected by electrophoresis (lanes 4–7). Lanes 1–3 and 8 represent controls; lane 1, marker DNA; lane 2, denatured pECAN 1 DNA, some undenatured DNA was detected when boiling was less than 2 min (arrowhead); lane 3, n-UV treated and denatured pECAN 1 DNA; lane 8, untreated pECAN 1 DNA. Lanes 4–7; lane 4, pECAN 1 DNA treated with n-UV in the presence of alternariol and subsequently denatured. Note that most of the DNA comigrates with the untreated control (lane 8); lane 5, as above but with no n-UV irradiation; lane 6, treatment of pECAN 1 DNA with alternariol monomethylether; no cross-linkage is evident; lane 7, as above but no n-UV irradiation.

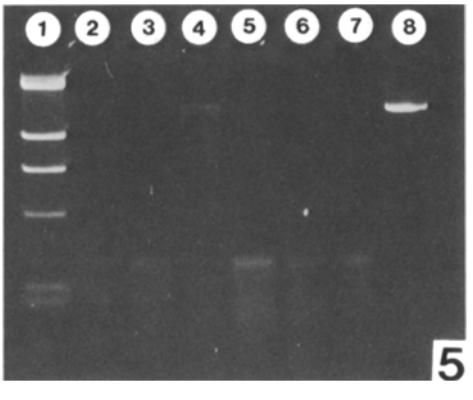

Figure 5. Lanes 1–8. All lanes represent the identical treatments of figure 4, but all the DNA was precipitated and washed as described in the text. Note that in lane 4 DNA cross-linking is evident by comparison with untreated control (lane 8).

Our study has revealed that alternariol, a planar, tricyclic, aromatic isocoumarin-containing compound is phototoxic to *E. coli;* it represents a new phototsensitizing mycotoxin, complexes with double-stranded DNA in dark and cross-links double-stranded DNA in n-UV. These are important similarities shared with DNA-crosslinking furanocoumarins[1,2,4–6,9,11]. Substituted isocoumarins are common fungal metabolites[12,20], and many other compounds containing a coumarin moiety are known to be biologically active and highly toxic, mutagenic photosensitizers[6]. The toxicity, mutagenicity and carcinogenicity of compounds of this type, which include alternariol, should be reconsidered in the presence of light because light dramatically increases activity.

The presence of phototoxic mycotoxins is a hazard to health, and a problem to the agricultural and food industries[13]. There is a need for a rapid and inexpensive method to detect compounds that react with DNA. Techniques now in use to detect DNA cross-linked include hydroxylapatite column chromatography[5,6,18] which is tedious and time-consuming when many samples are to be analyzed. The hydroxylapatite procedure also requires mg quantities of DNA, and an ample supply of purified photosensitizer. In contrast, our assay provides a rapid method to simultaneously test many such compounds for their ability to break or cross-link DNA. By using agarose gel electrophoresis and defined plasmids, the technique is considerably streamlined, is less time-consuming and the DNA phototoxicity reaction can be conveniently done in a 20 µl reaction mixture using µg quantities of photosensitizing compound and DNA. More significantly, many samples may be assayed in concert on one gel. This technique should find broad application especially when minute amounts of purified mycotoxins are available.

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