Isolation, Identification, and Mutagenicity of Alternariol Monomethyl Ether

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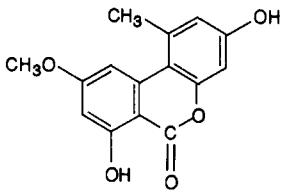
Alternariol monomethyl ether (AME), an *Alternaria* toxin, was isolated from the media of *Alternaria alternata* strain 261, a fungus from grains of Linxian County, China, an area noted for a high incidence of esophageal cancer. AME was purified by silica gel chromatography and recrystallized from acetone. The chemical structure was identified by systematic spectral analysis. The 60-MHz proton magnetic resonance spectrum exhibited long-distance couplings between 6′-CH_3 and 5′,6-hydrogens, and the assignments to two pairs of meta-coupled aromatic hydrogens were revised. Decoupling experiments supported the new assignments. Irradiation at 2.77 ppm peak (6′-CH_3) collapsed the 7.28 ppm dull doublet into sharp peaks and changed the 6.77 ppm diquadruplet into a sharp doublet, while peaks at 6.55 and 6.69 ppm remained unchanged. Therefore, the peaks at 6.55, 7.28, 6.69, and 6.77 ppm should be 3′-, 5′-, 4-, and 6-hydrogens, respectively. AME was a strong mutagen in *Escherichia coli* strain ND-160. Compared to spontaneous revertants, the number of revertants caused by 50 and 100 μg of AME/plate was 6- and 10-fold greater, respectively. Since Scott reported in 1980 that the mutagenicity of AME to *Salmonella typhimurium* TA98 was weak or marginal, it appears that the mutagenic action of AME exhibits some selectivity toward specific genomic regions or DNA sequences.

The genus *Alternaria* can cause a variety of plant diseases including tobacco brown spot (Lucas, 1959), tomato and potato blight, citrus seedling chlorosis (Templeton, 1972), and others. The reason is that they can produce toxic metabolites having diverse chemical structures (Harvan and Pero, 1976). Alternariol monomethyl ether (AME), 3,7-dihydroxy-9-methoxy-1-methyl-6*H*-dibenzo-



[*b*,*d*]pyran-6-one (Raistrick et al., 1953), is one of the members in this family, and its occurrence in discolored pecans has been reported (Schroeder and Cole, 1977). Mammalian toxicity of AME (Pero et al., 1971) toward Lymphoma L5178Y and Hela cells in tissue culture systems has been demonstrated. AME is chiefly responsible for tobacco chlorosis, while it is weakly toxic to mice (Pero et al., 1973) and to the gestation of Syrian golden hamsters (Pollock et al., 1982). It has been shown that AME has a mutagenic activity in *Salmonella typhimurium* strain TA98 while the antibiotic effects of alternariol can be synergized by AME (Pero and Owens, 1971). However, the mutagenic activity is reportedly weak (Scott and Stoltz, 1980). It has been observed that *Alternaria alternata* contamination of grains in areas with a high incidence of esophageal cancer is heavier than in areas with a low incidence and that *A. alternata* can induce esophagus and forestomach carcinoma in rats (Zhen, 1984; Liu et al., 1982). Therefore, it appeared to be important to study the mutagenic action of AME in more detail. In view of the advantages of bacterial assays for the identification of potential mutagens (Ames et al., 1975; Green and Muriel, 1976), we have employed a related assay in the present study.

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EXPERIMENTAL METHODS

Isolation. *A. alternata* 261 was cultured in corn flour for 15 days. The growth medium (50 kg) was extracted for 3 days with 10 L of ethanol, and the solution was concentrated to a volume of 200 mL by evaporation. Methanol (200 mL) was poured into the ethanol extract, resulting in the formation of a precipitate. After filtration and drying, the precipitate weighed 30 g. The methanol filtrate was evaporated to 100 mL, and an additional 400 mL of acetone was added. The resulting precipitate weighed 10 g after filtration and drying. The acetone solution was concentrated to 50 mL, and 200 mL of ethyl ether was added to yield 5 g of a precipitate. Because the methanol and ethyl ether precipitates were positive in *Escherichia coli* reversion mutation and sister-chromatid exchange assays, they were chromatographed on a scaled-up (4 × 50 cm) silica gel (160-mesh) column. A solvent system of dichloromethane, methanol, and water (55:2:2) was used to elute material from the column. The mutagenic fractions were collected with a fraction collector, and the solution was evaporated to dryness. The dry material was repurified on a 2 × 50 cm silica gel column and eluted with dichloromethane, methanol, and water (55:1:1). Crude AME crystallized from the combined fractions, and AME was recrystallized from acetone. Crystals were collected by centrifugation.

Identification. *Thin-Layer Chromatography.* Silica gel plates were prepared as follows: Water (8 mL) was poured into a beaker with 5 g of silica gel G (Quindao Ocean Chemical Industry), and the mixed paste was coated on a 5 × 20 cm glass plate. The plates were activated by heating at 120 °C for 2 h. After the samples were spotted, the plates were developed with dichloromethane, methanol, and water (55:3:3) and visualized by illumination at 254 nm with a portable UV lamp or by exposure to saturated iodine vapors. With AME, fluorescent and colored spots can be observed.

Determination of Physicochemical Properties. Organic analytic methods (Chen Yao-Zu, 1983) were used for detecting the organic functional groups and solubility properties of AME. The melting point (uncorrected) was measured with use of a microscope and heated-plate apparatus (Franz Kusiner Wacht, KG Dresden A21); the elemental composition was determined on a Carlo Erba Model 1106 elemental analyzer.

Spectroscopic Studies. Infrared (KBr pellet) and ultraviolet (absolute ethanol, 1-cm cells) spectra were recorded with Perkin-Elmer infrared spectrophotometer, Model 580V, and Specord UV Model V13, respectively. A JMS-D100 mass spectrometer

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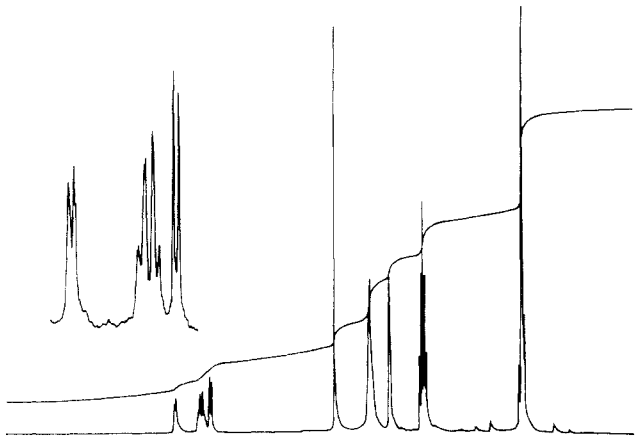

Figure 1. Proton magnetic resonance spectrum of recrystallized AME.

was used to record mass spectra at 75 eV. Proton magnetic resonance spectra of AME in acetone-d_6 (Beijing Chemical Industry) were obtained with a FX-60Q spectrometer equipped with a Fourier transform computer. Tetramethylsilane (TMS) was used as an internal standard.

Mutagenicity Assay. A reversion mutation assay was used for the mutagenicity assay (Clarke and Wade, 1975). *E. coli* strain ND160 was a gift from B. A. Bridges. The material to be tested was dissolved in dimethyl sulfoxide (Beijing Chemical Industry), and aliquots (0.1 mL or less) were screened for mutagenic activity in a 63-h incubation. The material (without metabolic activation by liver homogenate S9) was tested in the plate incorporation with assay *E. coli* strain ND-160. A 3-fold increase in spontaneous revertants was considered to be a minimum positive mutagenic response.

RESULTS AND DISCUSSION

Following the isolation and purification procedure described here, colorless needles were obtained (R_f 0.41 in dichloromethane, methanol, and water, 55:3:3). The melting point is 276–277 °C, which is close to that reported by Scott and Stoltz (1980) but differs from other reports (Raistrick et al., 1953; Rosett et al., 1957). The crystals were not soluble in aqueous sodium bicarbonate, distilled water, or tetrachloromethane but were soluble in acetone or ethyl ether. They dissolved easily in cold dilute aqueous sodium hydroxide, giving a yellow-green solution that changed overnight to a cherry red color. The crystals were also moderately soluble in methanol, ethanol, chloroform, dichloromethane, and ethyl acetate. An ethanol solution gave an intense purple color with ethanolic ferric chloride. It slowly reduced Fehling's reagent while boiling.

Anal. Found (calcd) for $C_{15}H_{12}O_5$: C, 66.54 (66.18); H, 4.43 (4.41). This is in agreement with other data reported for AME (Pero and Owens, 1971).

The UV spectrum of the crystal exhibited λ_{max} (EtOH) 335–342, 301, 290, 257, and 220 nm. These values and their relative intensities appeared to be identical with previous reports for AME (Rosett et al., 1957; Thomas, 1961; Seitz et al., 1957). The infrared spectrum exactly matched the spectra reported for AME (Pero et al., 1971). Low-resolution mass spectra recorded at 75 eV exhibited a parent ion peak at m/z 272.

The 60-MHz porton magnetic resonance spectrum of the crystalline material (Figure 1) indicated proton chemical shifts as follows: 2.79 (3 H, s, CH_3), 3.21 (s, OH, H_2O), 3.97 (3 H, s, OCH_3), 6.55 (1 H, d, 3′-H, J = 2.3 Hz), 6.69 (1 H, d, 4-H, J = 2.3 Hz), 6.77 (1 H, dq, 6-H, J = 2.3 Hz), 7.28 ppm (1 H, d, dull, 5′-H, J = 2.3 Hz). The assignments to two pairs of meta-coupled aromatic hydrogens differ from ones where the 3′- and 4-hydrogens are assigned to low field (7.20, 6.70 ppm) and the 5′- and 6-hydrogens to high field (6.59, 6.62 ppm; Seitz et al., 1975) because there are the long-distance couplings between 6′-CH_3 and 5′,6-hydrogens. Decoupling experiments supported these assignments. Irradiation at 2.79 ppm (6′-CH_3) collapsed the 7.28 ppm dull doublet into sharp peaks and changed the 6.77 ppm diquadruplet into a sharp doublet, while peaks at 6.55 and 6.69 ppm remained unchanged.

Table I. Mutagenicity of AME toward *E. coli* Strain ND-160

compd added, μg/plate	revertants/plate	inc in revertants
none, 0[a]	7, 11	(1)
AME, 50	48, 57	6
AME, 100	91, 100	11
benzidine, 50	138, 130	15
atebrine hydrochloride, 250	250, 283	30

[a] Solvent (DMSO) control.

Mutagenicity of AME (without S9 activation) was readily observed in *E. coli* strain ND-160 (Table I). The levels of revertants caused by 50 and 100 μg/plate were 6- and 10-fold greater than spontaneous revertants, respectively.

Although AME was only weakly mutagenic to *Salmonella typhimurium* strain TA98 (Scott and Stoltz, 1980), the present results show that it is significantly mutagenic to *E. coli* ND-160. Thus, it is possible that this mutagenic compound may be selective for different genomic regions or sequences of the DNA. It is interesting to speculate on possible explanations for the differing mutagenicity of AME in the two strains. *S. typhimurium* strain TA98 is a strain that is defective in histidine synthetase, while the *E. coli* strain is one defective in lactase. The genes for carbohydrate-hydrolyzing enzymes would be expected to be key genes involved with the upper digestive tract, for example in the uptake of nutrition and in cellular proliferation of esophageal epithelial tissue. In any event, it seems clear that the molecular details of the relationship between AME and esophageal cancer deserve further study.

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Binding of Dietary Anions to Vegetable Fiber

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Binding of chenodeoxycholate and decanoate to alcohol-insoluble residue (AIR) of carrot, cabbage, broccoli, and onion was demonstrated. Binding of chenodeoxycholate to freeze-dried calcium pectate gel under conditions used for vegetable AIR was observed and supports the idea that binding of bile acids and fatty acids to vegetable fiber occurs through salt linkages to calcium pectate of the plant cell wall. Such binding may possibly be beneficial to human health by lowering blood cholesterol levels and by reducing the risk of colon cancer.

Vegetable fiber in the human diet has been implicated in lowering serum levels of cholesterol (Selvendran, 1978, 1985; Jenkins et al., 1979; Robertson et al., 1980; Chen et al., 1981; Judd and Truswell, 1982; Nakamura et al., 1982). Several studies have shown that vegetable fiber can bind bile acids and thereby prevent their reabsorption in the small intestine (Birkner and Kern, 1974; Kern et al., 1978; Selvendran, 1978; Robertson et al., 1980; Hoagland and Pfeffer, 1986, 1987). To replace lost bile acids, the human body draws upon its cholesterol reserves in the low-density lipids of blood serum. Selvendran (1978) obtained evidence that pectin in the cell wall material of parenchymatous tissue of mature runner bean pods was involved in binding of cholate. Further investigations have suggested that binding of bile acids to carrot fiber largely occurs through calcium salt linkages to the calcium pectate of residual cell wall (Hoagland and Pfeffer, 1986, 1987). Since calcium pectate is widely distributed in plants (Jarvis, 1982), the binding of bile acids to alcohol-insoluble residue (AIR) of several vegetables was determined. In addition, since calcium pectate is implicated in binding of bile acids, binding of other dietary anions that have a great affinity for calcium ions (fatty acids and phytate) may also be possible. This investigation was undertaken to extend the range of interactions of dietary fiber with anionic components of the human diet and to detail some of the chemistry that underlies the nutritional benefits of dietary fiber. The interaction between vegetable fiber and oxalate was also investigated to clarify the involvement of calcium pectate in binding.

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EXPERIMENTAL SECTION

Materials. Chenodeoxycholic acid and sodium phytate were obtained from Sigma Chemical Co. Redistilled decanoic acid was a gift from Raymond Bistline. Citrus pectin was obtained from U.S. Biochemical Corp. Oxalic acid and KCl were Baker analyzed reagent grade. 4-(2-Hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) was obtained from Aldrich Chemical Co. Acetonitrile was the HPLC-grade product of Burdick & Jackson. Other chemicals were reagent grade.

Preparation of Vegetable Alcohol-Insoluble Residue (AIR). Washed vegetables, purchased from a local supermarket, were cut into small pieces and minced in a blender with water (300 mL/200 g of vegetable). The mixture was frozen for at least 24 h to disrupt the plant cells and then thawed. Water-soluble material was removed by vacuum filtration of several washings with water on a medium sintered-glass filter. The wet residue was then extracted with absolute ethanol (1 L/200 g of original vegetable) by refluxing for 5 h. The material was filtered and washed several times with water. The AIR was rapidly washed with dilute (10:1) water–ammonium hydroxide and water to neutralize the fiber with negligible deesterification. A slurry of the AIR was then freeze-dried to give yields ca. 3% of the starting weight of material.

Reversed-Phase HPLC. A Du Pont Zorbex ODS, 4.6-mm i.d. × 15-cm length column was used with a mobile phase of pH 7.2, 0.02 M phosphate–acetonitrile (67:35 (v/v)) as previously described (Hoagland and Pfeffer, 1987; Parris, 1977). Peak detection was recorded with an Apple IIe computer running CHROMATOCHART software by Interactive Microware.

Gel Permeation Chromatography. Chromatography was performed with a Synchrom Synchropak GPC 100, 4.6-mm i.d. × 25-cm length column and a mobile phase of pH 7.20, 0.01 M