Synthesis and Mutagenicity of the Aflatoxin B_1 Model 3a,8a-Dihydro-4,6-dimethoxyfuro[2,3-b]benzofuran and Its 2,3-Epoxy Derivative

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The synthesis and mutagenic activity in the *Salmonella*/Ames test (TA-98 and TA-100 strains) of an aflatoxin model, i.e., the title furobenzofuran **6** and its 2,3-epoxy derivative **7**, are reported. In addition, the mutagenicity exhibited by compounds **6** and **7** has been compared with that obtained from aflatoxin B_1 (**8**) and its 8,9-epoxide **9**. Compound **6** was synthesized in a seven-step sequence (15% overall yield from 3,5-dimethoxyphenol), and epoxides **7** and **9** were prepared by using dimethyldioxirane in acetonitrile. Compound **6** was inactive in TA-98 strain even in the presence of S9 fraction. Conversely, it was a moderate mutagen in TA-100 strain, and the presence of S9 fraction doubled the mutagenic potency. Epoxy derivative **7** induced mutagenic effects in TA-100 strain, and this activity was enhanced in the presence of S9 fraction. The fact that in this strain indirect mutagenicity induced by compound **6** was greater than direct mutagenicity exhibited by its epoxy derivative **7** suggests that bioactivations other than epoxide formation could account for the overall activity observed for the parent furobenzofuran molecule. In this sense, compound **6** could be an attractive aflatoxin model to study these putative bioactivation mechanisms. On the other hand, epoxide derived from aflatoxin B_1 (**9**) appeared to be a very potent mutagen in both strains, and in TA-98 strain its mutagenicity was considerably higher than that exhibited by aflatoxin B_1 in the presence of S9 fraction. These results could be taken as further evidence to support the hypothesis about the crucial role that epoxide **9** plays in the cytotoxic effects elicited by aflatoxin B_1.

INTRODUCTION

The establishment of structure–activity relationships between aflatoxin-related molecules and the mutagenic and carcinogenic effects elicited by this class of compounds has been a subject of active research (Wong and Hsieh, 1976; Shahin, 1989). However, for molecules bearing the furobenzofuran moiety, all of these studies had so far to be restricted to the parent compounds since the corresponding epoxy derivatives, postulated as the ultimate species responsible for the mutagenicity exhibited by these mycotoxins, were still unknown. In this context, the recent paper by Baertschi et al. (1988) on the synthesis of aflatoxin B_1 8,9-epoxide (cf. **9** in Figure 2), constitutes a major contribution to this field. Among other important applications (Baertschi et al., 1989), this availability has brought about the possibility to evaluate the significance of these activated epoxides in the different cytotoxic effects observed for the parent aflatoxins.

The above considerations could also be extended to aflatoxin models. In this context, we deemed that aflatoxin analogue **6** (Figure 1) could be an attractive target molecule to work on. In contrast with a related analogue described previously by Coles et al. (1977), compound **6** contains a furobenzofuran moiety activated by the presence of two methoxy groups in the aromatic ring located in the same relative positions as the oxy substituents present in the more potent aflatoxins (cf. **9** in Figure 2). Therefore, compound **6**, besides its potential role as intermediate for the synthesis of natural aflatoxins, could provide information about the relative contribution of an activated furobenzofuran moiety to the overall cytotoxicity elicited by aflatoxins. With this aim, we report on the synthesis and mutagenic activity in the *Salmonella*/Ames test (TA-98 and TA-100 strains) of an aflatoxin model, i.e., the furobenzofuran **6** and its 2,3-epoxy derivative **7**. In addition, the mutagenicity exhibited by compounds **6** and **7** has been compared with that obtained from aflatoxin B_1 (**8**) and, for the first time to our knowledge, from its 8,9-epoxide **9**.

EXPERIMENTAL PROCEDURES

Synthesis of Compounds. Melting points were determined with a Koffler apparatus and are uncorrected. Elemental analyses were obtained with a Carlo Erba Model 1106 instrument. Pyrolysis and microdistillations were performed with a Büchi Model GKR-50 apparatus. The IR spectra were recorded with a Perkin-Elmer 399 B spectrometer. The ^1H and ^{13}C NMR spectra were recorded with a Varian Unity 300 spectrometer. Unless otherwise stated, all NMR spectra were performed in neutralized $CDCl_3$ solutions and chemical shifts are given in parts per million downfield from tetramethylsilane. The gas chromatographic and mass spectrometric (GC–MS) determinations were carried out with a Hewlett-Packard 5985 A spectrometer coupled to a Hewlett-Packard 5890 chromatograph (25-m OV-101 capillary column), using methane as ionization gas.

5,7-Dimethoxy-4-methylcoumarin (**1**). A mixture of 3,5-dimethoxyphenol (12.0 g, 78 mmol) and methyl acetoacetate (11.4 mL, 94 mmol) in methanesulfonic acid (40 mL) was vigorously stirred for 2 h at 70 °C. Then the crude reaction mixture was poured into ice–water (600 mL) and extracted with dichloromethane (3 × 200 mL). The joined organic fractions were washed with Na_2CO_3 saturated solution (300 mL) and water (2 × 300 mL) and dried over $MgSO_4$. The solid residue obtained after solvent removal was crystallized from a hexane–ethyl acetate mixture to give pure compound **1** (14.6 g, 86% yield): mp 173 °C; IR ($CHCl_3$) 1720, 1610 cm^{-1}; ^1H NMR δ 6.38 (d, 1 H, J = 2.4 Hz, H-8), 6.26 (d, 1 H, J = 2.4 Hz, H-6), 5.90 (q, 1 H, J = 1.2 Hz, H-3), 3.84 (s, 3 H, CH_3O), 3.83 (s, 3 H, CH_3O), 2.49 (d, 3 H, J =

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0021-8561/91/1439-1723$02.50/0

1.2 Hz, CH_3); ^{13}C NMR δ 162.8 (Ar CO), 161.0 (C=O), 159.1 (Ar CO), 156.9 (Ar CO), 154.5 (C-4), 111.2 (C-3), 104.8 (Ar C), 95.3 (Ar C), 93.4 (Ar C), 55.7 (CH_3O), 55.6 (CH_3O), 24.1 (CH_3); MS, *m/z* (%) 220 (M^+, 100), 192 (86), 177 (78). Anal. Calcd for $C_{12}H_{12}O_4$: C, 65.45; H, 5.45. Found: C, 65.62; H, 5.33.

4-Formyl-5,7-dimethoxycoumarin (**2**). A mixture of coumarin **1** (7.2 g, 32.5 mmol) and selenium dioxide (5 g, 45.5 mmol) in xylene (300 mL) was stirred for 5 h under reflux in a reaction flask equipped with a Dean-Stark trap. Then the crude reaction mixture was allowed to cool, filtered through Celite, and washed thoroughly with *tert*-butyl methyl ether. The residue obtained after solvent removal was purified by flash chromatography over silica gel eluting with *tert*-butyl methyl ether–chloroform mixtures to give pure coumarin **2** (6.8 g) in 84% yield: mp 190–91 °C; IR ($CHCl_3$) 1730, 1700, 1620 cm^{-1}; ^1H NMR δ 10.49 (s, 1 H, CHO), 6.5 (d, 1 H, *J* = 2.2 Hz, H-8), 6.35 (d, 1 H, *J* = 2.2 Hz, H-6), 6.30 (s, 1 H, H-3), 3.93 (s, 3 H, CH_3O), 3.89 (s, 3 H, CH_3O); ^{13}C NMR δ 191.3 (CHO), 164.3 (C=O), 161.2 (Ar CO), 157.6 (Ar CO), 157.2 (Ar CO), 148.9 (C-4), 110.6 (C-3), 101.3 (Ar C), 95.7 (Ar C), 94 (Ar C), 56.3 (CH_3O), 56.0 (CH_3O); MS, *m/z* (%) 234 (M^+, 100), 178 (61), 135 (16). Anal. Calcd for $C_{12}H_{10}O_5$: C, 61.54; H, 4.27. Found: C, 61.36; H, 4.31.

2,3,3a,8a-Tetrahydro-4,6-dimethoxy-2-oxofuro[2,3-b]benzofuran (**3**). Zinc dust (4.0 g, 0.06 mmol) was added portionwise to a solution of aldehyde **2** (2.8 g, 12 mmol) in acetic acid (120 mL) maintained at 100 °C, and the mixture was stirred for 1.5 h at 120 °C. Then the crude reaction mixture was allowed to cool, diluted with chloroform (100 mL), and filtered. The precipitate was washed with chloroform (100 mL), and the joined organic fractions were washed with water (2 × 100 mL) and $NaHCO_3$ saturated solution (2 × 100 mL) and dried over $MgSO_4$. The residue obtained after solvent removal was purified by flash chromatography over silica gel eluting with hexane–ethyl acetate mixtures to give pure benzofuran **3** (1.8 g) in 60% yield: mp 152–53 °C; IR (KBr) 1780, 1635, 1610 cm^{-1}; ^1H NMR δ 6.45 (d, 1 H, *J* = 7 Hz, H-8a), 6.12 (d, 1 H, *J* = 2 Hz, Ar H), 6.08 (d, 1 H, *J* = 2 Hz, Ar H), 4.15 (q, 1 H, *J* = 7 Hz, H-3a), 3.83 (s, 3 H, CH_3O), 3.80 (s, 3 H, CH_3O), 2.94 (m, 2 H, H-3, H-3′); ^{13}C NMR δ 174.4 (C=O), 163.0 (Ar CO), 159.2 (Ar CO), 157.0 (Ar CO), 108.5 (C-8a), 105.6 (Ar C), 92.9 (Ar C), 89.1 (Ar C), 55.7 (CH_3O), 55.4 (CH_3O), 40.7 (C-3a), 33.1 (C-3); MS, *m/z* (%) 236 (M^+, 18), 207 (100), 164 (8). Anal. Calcd for $C_{12}H_{12}O_5$: C, 61.06; H, 5.08. Found: C, 60.98; H, 5.03.

2,3,3a,8a-Tetrahydro-4,6-dimethoxy-2-hydroxyfuro[2,3-b]benzofuran (**4**). A solution of diisobutylaluminum hydride in toluene (4 mL, 1.2 M) was added dropwise to a solution of compound **3** (0.96 g, 4 mmol) in toluene (100 mL) maintained at −20 °C in an inert atmosphere. After stirring for 75 min at that temperature, the reaction was quenched by careful addition of 2 N HCl (20 mL). Then the crude reaction mixture was allowed to reach room temperature and was extracted with *tert*-butyl methyl ether (3 × 100 mL). The joined organic fractions were washed with water (3 × 150 mL) and dried over $MgSO_4$. The residue obtained after solvent removal was purified by flash chromatography over silica gel eluting with dichloromethane–diethyl ether mixtures to give alcohol **4** as an epimeric mixture which could not be separated (0.70 g, 70% yield): IR (CCl_4) 3610, 3500–3400, 1625, cm^{-1}; ^1H NMR δ 6.40 (dd, 1 H, J_1 = 6 Hz, J_2 = 2 Hz, H-8a), 6.05 (m, 2 H, Ar H), 5.60 (br, 1 H, H-2), 3.90 (m, 1 H, H-3a), 3.85 (s, 3 H, CH_3O), 3.75 (s, 3 H, CH_3O), 3.50–2.90 (3 H, OH, H-3, H-3′); ^{13}C NMR (CD_3COCD_3) δ 162.3 (Ar CO), 159.7 (Ar CO), 156.5 (Ar CO), 106.5 (Ar C), 103.4 (C-8a), 100.6 (C-2), 92.5 (Ar C), 88.9 (Ar C), 55.9 (CH_3O), 55.6 (CH_3O), 43.8 (C-3a), 38.9 (C-3); MS *m/z* (%) 238 (M^+, 40%), 209 (100), 181 (45). Anal. Calcd for $C_{12}H_{14}O_5$: C, 60.50; H, 5.88. Found: C, 60.40; H, 5.87.

2,3,3a,8a-Tetrahydro-4,6-dimethoxy-2-[(methoxycarbonyl)oxy]furo[2,3-b]benzofuran (**5**). Methyl chloroformate (4 mL, 49 mmol) was added dropwise to a solution of alcohol **4** (0.50 g, 2.1 mmol) in pyridine (15 mL) maintained at 0 °C. When addition was completed, the reaction mixture was allowed to reach room temperature and was stirred for 24 h. Then the crude reaction mixture was poured into water–ice (250 g) and extracted with *tert*-butyl methyl ether (3 × 150 mL). The joined organic fractions were washed with water (2 × 300 mL), NH_4Cl saturated solution and dried over $MgSO_4$. The residue obtained after solvent removal was purified by flash chromatography eluting with a 4:1 hexane–ethyl acetate mixture to give pure carbonates exo (0.190 g) and endo (0.300 g) in 83% overall yield: IR (CCl_4) 2840, 1765, 1630, 1600, 1220 cm^{-1}. Anal. Calcd for $C_{14}H_{16}O_7$: C, 56.76; H, 5.40. Found: C, 56.82; H, 5.46. Exo isomer: mp 95 °C; ^1H NMR δ 6.40 (d, *J* = 6 Hz, 1 H, H-8a), 6.30–5.90 (m, 3 H, 2 Ar, H, H-2), 4.10 (m, 1 H, H-3a), 3.80 (s, 6 H, 2 CH_3O), 3.75 (s, 3 H, CH_3O), 2.60–2.20 (m, 2 H, H-3, H-3′); ^{13}C NMR δ 162.4 (Ar CO), 159.7 (Ar CO), 156.6 (Ar CO), 154.3 (C=O), 111.8 (C-8a), 107.5 (Ar C), 92.2 (Ar C), 88.8 (Ar C), 55.6 (CH_3O), 55.3 (CH_3O), 54.8 (CH_3O), 42.5 (C-3a), 37.0 (C-3). Endo isomer: mp 123 °C; ^1H NMR δ 6.40 (d, *J* = 6 Hz, 1 H, H-8a), 6.30 (m, 1 H, H-2), 6.05 (m, 2 H, Ar H), 4.00 (m, 1 H, H-3a), 3.80 (s, 3 H, CH_3O), 3.75 (s, 3 H, CH_3O), 3.60 (s, 3 H, CH_3O), 2.50–2.00 (2 H, H-3, H-3′); ^{13}C NMR δ 162.1 (Ar CO), 159.6 (Ar CO), 156.1 (Ar CO), 154.2 (C=O), 112.9 (C-8a), 107.1 (Ar C), 101.7 (C-2), 91.8 (Ar C), 88.5 (Ar C), 55.6 (CH_3O), 55.3 (CH_3O), 54.6 (CH_3O), 42.5 (C-3a), 36.8 (C-3).

Pyrolysis of Carbonates **5**: *3a,8a-Dihydro-4,6-dimethoxyfuro[2,3-b]benzofuran* (**6**). **Caution**: *All assays were carried out with glassware carefully washed with ammonia, water, and acetone. In addition, safety precautions usually taken for the manipulation of aflatoxin derivatives were also adopted in the obtention of olefin* **6** *and its further epoxidation.* A sample of one carbonate (exo epimer, 90 mg) was heated for 2 h at 225 °C under vacuum (1 Torr) in a bulb-to-bulb distillation apparatus to give olefin **6** as a white crystalline solid (40 mg, 60% yield). When the same procedure was carried out with the endo epimer, yield obtained in **6** was lower (50%). **6**: mp 87 °C; IR (CCl_4) 2840, 1625, 1500, 1470 cm^{-1}; ^1H NMR δ 6.65 (d, *J* = 7.2 Hz, 1 H, H-8a), 6.40 (dd, *J* = 2 Hz, *J* = 2.9 Hz, 1 H, H-2), 6.13 (d, *J* = 2 Hz, 1 H, Ar H), 6.03 (d, *J* = 2 Hz, 1 H, Ar H), 5.31 (t, *J* = 2.6 Hz, 1 H, H-3), 4.52 (dt, *J* = 2.4 Hz, *J* = 7.2 Hz, 1 H, H-3a), 3.80 (s, 3 H, CH_3O), 3.75 (s, 3 H, CH_3O); ^{13}C NMR δ 161.9 (Ar CO), 159.6 (Ar CO), 156.2 (Ar CO), 144.5 (C-2), 112.3 (C-8a), 107.4 (Ar C), 103.2 (C-3), 92.0 (Ar C), 88.8 (Ar C), 55.6 (CH_3O), 55.4 (CH_3O), 48.3 (C-3); MS *m/z* (%) 220 (M^+, 85), 191 (100), 174 (18). Anal. Calcd for $C_{12}H_{12}O_4$: C, 65.45; H, 5.45. Found: C, 65.38; H, 5.65.

3a,8a-Dihydro-2,3-epoxy-4,6-dimethoxyfuro[2,3-b]benzofuran (**7**). A solution of 65 mM dimethyldioxirane (1.2 molecular equiv; Murray and Jeyaraman, 1985) in acetone was added to a solution of olefin **6** (0.030 g, mmol) in dry acetonitrile (5 mL) maintained at −40 °C. When reaction was completed (HPLC monitoring), reagent excess and solvents were removed under vacuum at 0 °C and the residue was identified as the expected epoxide in high purity: ^1H NMR (CD_3CN) δ 6.16 (d, *J* = 2.1 Hz, 1 H, Ar H), 6.12 (d, *J* = 2.1 Hz, 1 H, Ar H), 5.92 (dd, J_1 = 5.7 Hz, J_2 = 0.6 Hz, 1 H, H-8a), 5.27 (dd, J_1 = 1.8 Hz, J_2 = 0.6 Hz, 1 H, H-2), 4.21 (d, J_1 = 5.7 Hz, 1 H, H-3a), 3.84 (d, J_1 = 1.8 Hz, 1 H, H-3), 3.83 (s, 3 H, CH_3O), 3.75 (s, 3 H, CH_3O); ^{13}C NMR δ 160.7 (Ar CO), 156.9 (Ar CO), 110.3 (C-8a), 101.5 (Ar C), 91.8 (Ar C), 88.8 (Ar C), 81.5 (C-2), 55.3 (C-3), 55.1 (2 CH_3O), 46.5 (C-3a); MS *m/z* (%) 236 (M^+, 27), 178 (100), 163 (36), 135 (12).

Aflatoxin B_1 *2,3-Epoxide* (**9**). This compound was prepared from aflatoxin B_1 (**8**) and dimethyldioxirane as described by Baertschi et al. (1988), by using acetonitrile as solvent, with conversion yields higher than 90% according to HPLC analysis. Compound **9** was identified by comparison of its ^1H and ^{13}C NMR data with those reported in the literature.

Mutagenicity Tests. Mutagenicity was assayed by using the standard plate incorporation *Salmonella* mutagenicity test. This test was performed as described by Maron and Ames (1983). Two tester strains, TA-98 (frameshift mutagenesis detection) and TA-100 (base substitution mutagenesis), kindly supplied by Dr. B. N. Ames (University of California at Berkeley), were used. Exogenous metabolic activation was provided by liver postmitochondrial fractions (S9) obtained from Aroclor 1254 (Monsanto) induced male Wistar rats. Protein concentration (36 mg/mL) was determined according to the Lowry method (Lowry et al., 1951) using bovine serum albumin as standard; 20 μL of S9 fraction was used per plate.

Compounds were dissolved in dimethyl sulfoxide and tested for mutagenicity by using the preincubation procedure (20 min at 37 °C) (Maron and Ames, 1983). Each compound, including controls, was assayed at four doses; two plates per dose were used. Spontaneous reversion controls were performed in triplicate. 2-Nitrofluorene (0.5 μg/plate, TA-98 without S9), 2-aminofluorene (0.5 μg/plate, TA-98 with S9), and sodium azide (0.1

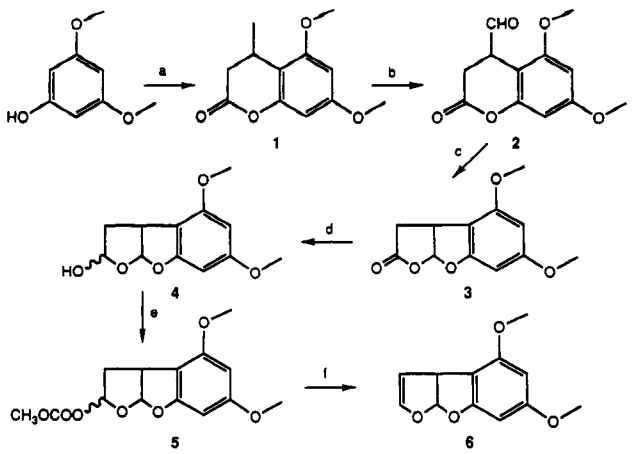

Figure 1. Synthetic procedure for the preparation of aflatoxin model 6. a, methyl acetoacetate/CH_3SO_3H/70 °C; b, SeO_2/xylene/reflux; c, Zn/CH_3COOH/120 °C; d, DIBAL/toluene/−20 °C; e, $ClCOOCH_3$/pyridine; f, pyrolysis under vacuum.

μg/plate, TA-100 without S9) were used as positive controls to check strain sensitivity and enzymatic activities, and routine controls of samples and S9 sterility were systematically carried out. Toxicity effects were detected by examination of background lawn.

A number of statistical approaches for the analysis of test data have appeared in the literature (Myers et al., 1981; Berstein et al., 1982; Barale et al., 1989). In the present study *Salmonella* Assay Software, version 2.3 (Integrated Laboratory Systems and Research Triangle Institute Research Triangle Park, NC), was used. This approach integrates aspects of several previously published (Myers et al., 1981; Stead et al., 1981; Berstein et al., 1982) methods. The analysis begins by testing the fitness of the data into a linear model. If this model does not fit adequately, then two models (Lintox 1 and Lintox 2), which incorporate toxicity, are estimated. Finally, if none of these models are appropriate, a Berstein-like truncation process is used. The statistics supplied includes a p value for Anova test of dose–reponse as indication of whether each dose is significantly different from the background (t-test), the initial slope of the dose–response curve (potency estimation), and a determination as to whether the computed slope is statistically different from zero (model-dependent mutagenicity determination).

The mutagenicity ratio (MR), i.e., the ratio of the numbers of revertants induced by test compound to the numbers of spontaneous revertants for the control (Commoner, 1976; Yourtee et al., 1987), was also used as mutagenicity criteria.

RESULTS AND DISCUSSION

Synthesis of Compounds. Preparation of the aflatoxin model 6 (Figure 1) was based on the approach developed by the group of Büchi for the construction of the furobenzofuran moiety of aflatoxins B_1 and M_1 (Büchi et al., 1967; Büchi and Weinreb, 1971). However, some modifications were introduced. Thus, use of methanesulfonic acid as solvent and catalyst (Camps et al., 1980) for the condensation of 3,5-dimethoxyphenol with methyl acetoacetate afforded coumarin 1 in good yields. Subsequent allylic oxidation and further treatment of aldehyde 2 with zinc–acetic acid followed by acid-catalyzed cyclization in the same reaction medium led to the formation of the 2-oxofurobenzofuran 3, which already contained the carbon framework of the target molecule 6. Reduction of compound 3 afforded a 1:1 exo:endo epimeric mixture of the alcohols 4, which could not be separated. Conversely, the corresponding mixture of exo and endo carbonates 5, the synthetic precursors of compound 6, could be resolved by chromatographic means and their relative configuration assigned by ^1H NMR. Pyrolysis of these intermediates under controlled vacuum to avoid their concomitant distillation afforded the expected furobenzofuran 6, which was characterized by spectroscopic means and by comparison with data reported for molecules structurally related with aflatoxins. The overall yield for the seven-step sequence starting from 3,5-dimethoxyphenol was 15%.

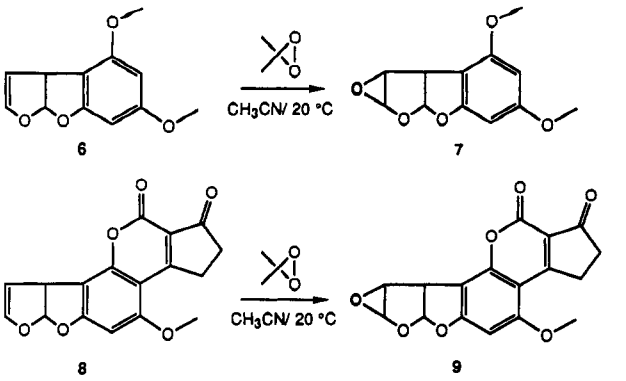

Figure 2. Preparation of the corresponding epoxides of aflatoxin model 6 and aflatoxin B_1 (8).

Epoxidation of 6 was carried out according to the dimethyldioxirane procedure described by Baertschi et al. (1988) for the synthesis of the corresponding aflatoxin B_1 epoxide 9 (see Figure 2) using acetonitrile as cosolvent. The same procedure was applied for a parallel preparation of epoxide 9. In our experience with the synthesis of highly reactive epoxides using dimethyldioxirane (Bujons et al., 1990), the use of this solvent gives good reproducible yields and confers greater stability to the reaction products. This was particularly important in the case of compound 7, which appeared to be more unstable than epoxide 9.

Mutagenicity Assays. Results from the *Salmonella*/Ames test in TA-98 and TA-100 strains for the furobenzofuran 6 are shown in Table I. Mutagenicity data obtained from parallel tests performed with aflatoxin B_1 (8) have been included for comparison purposes. As shown, compound 6 was inactive in TA-98 strain even in the presence of enzyme metabolic activation. In comparison, good dose–response curves were obtained in TA-100 strain assays with and without S9 bioactivation, and estimation of the respective initial slope values revealed that incubation with S9 fraction doubled the mutagenic potency of 6. This result indicates that the model furobenzofuran can undergo, like aflatoxins, microsomal bioactivation processes that enhance its mutagenicity. However, according to the doses used and the values of the initial slopes, the mutagenic potency of 6 was rather low in comparison with that exhibited by aflatoxin B_1 in TA-100 strain. On the other hand, a comparison of these results with those reported some years ago by Coles et al. (1977) on a related aflatoxin model (i.e., demethoxylated furobenzofuran 6) using the mutagenicity obtained for aflatoxin B_1 as reference indicates that the electronic activation derived from the presence of two methoxy groups is important to elicit mutagenicity in TA-100 strains.

The results obtained from a parallel set of assays carried out with epoxides 7 and 9 are shown in Table II. Although satisfactory dose–response curves were obtained for epoxy derivative 7 in both strains, the initial slope values showed that mutagenicity induced in TA-98 strain could be considered negligible. In the case of TA-100 strain, it is worth noting that incubation with S9 fraction enhanced its mutagenic potency and that the compound became toxic at the highest dose tested. This result suggests that the epoxide, an already bioactivated species, could undergo additional microsomal bioactivation and that this effect

Table I. Mutagenicity of Model Furofuran 6 and Aflatoxin B_1 (8) toward *S. typhimurium* TA-98 and TA-100 Strains[a]

compd	concn, μg/plate	TA-98,[b,d] rev/plate, ±SD		TA-100,[b,d] rev/plate, ±SD	
		−S9	+S9	−S9	+S9
6	0	27.0 ± 1	39.3 ± 5	155 ± 19.1	143.3 ± 10.2
	20	39.0 ± 15.6	43.5 ± 2.1	347 ± 2.8**	464 ± 11.3**
	50	37.5 ± 9.2	46.0 ± 7.1	513 ± 4.2**	894 ± 11.3**
	100	39.5 ± 0.7**	64.0 ± 5.7*	856.5 ± 23.3**	1768 ± 14.1**
	200	50.0 ± 1.4**	82.5 ± 9.2*	1385.5 ± 17.7**	2950.5 ± 95.5**
	dose–response (Anova)	NS	$p \leq 0.005$	$p \leq 0.001$	$p \leq 0.001$
	acceptable model (F-t)	linear	linear	Lintox 1	Bernstein
	slope ± SE[c]	0.1 ± 0.03	0.2 ± 0.03	8.4 ± 0.5	15.8 ± 0.3
	mutagenicity (F-t)	$p \leq 0.01$	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$
8	0	27.0 ± 1	39.3 ± 5	155 ± 19.1	143.3 ± 10.2
	0.05	32.0 ± 1.4*	80.5 ± 9.2*	155 ± 2.8	608.5 ± 101.1**
	0.1	29.0 ± 4.2	114 ± 13.4**	180 ± 24	844.5 ± 7.8**
	0.5	33.0 ± 4.2	372 ± 55.1**	187 ± 11.3	1152 ± 36.8**
	1	44.0 ± 4.2*	774 ± 28.3**	196 ± 3.5	800.0 ± 36.8**
	dose–response (Anova)	$p \leq 0.05$	$p \leq 0.001$	NS	$p \leq 0.001$
	acceptable model (F-t)	linear	linear	linear	Bernstein
	slope ± SE[c]	14.6 ± 2.75	710.3 ± 30.3	41.4 ± 13.5	7637.1 ± 388.9
	mutagenicity (F-t)	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.05$	$p \leq 0.001$

[a] For procedures, see Experimental Procedures. [b] Mean values of duplicate plates. [c] Estimation of the initial slope. [d] *, Significant at 5%; **, significant at 1% (Student's *t*-test). [e] NS, nonsignificant ($p > 0.05$).

Table II. Mutagenicity of Model Furofuran Epoxide 7 and Aflatoxin B_1 Epoxide 9 toward *S. typhimurium* TA-98 and TA-100 Strains[a]

compd	concn, μg/plate	TA-98,[b,d] rev/plate, ±SD		TA-100,[b,d] rev/plate, ±SD	
		−S9	+S9	−S9	+S9
7	0	45.0 ± 1	53.0 ± 7.1	197.3 ± 14.6	166.7 ± 9.3
	20	56.5 ± 0.7**	83.0 ± 8.5	312.5 ± 43.1*	317 ± 8.5**
	50	90.0 ± 7.1**	102 ± 4.2*	446 ± 36.8**	690 ± 188.1*
	100	136 ± 17.0**	186 ± 5.7**	721 ± 42.4**	879.5 ± 2.1**
	200	220.5 ± 17.7**	349.5 ± 4.9**	971.5 ± 12**	toxic
	dose–response (Anova)	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$
	acceptable model (F-t)	linear	Bernstein	Lintox 1	Lintox 2
	slope ± SE[c]	0.9 ± 0.04	1.2 ± 0.1	6.4 ± 0.7	9.9 ± 1.3
	mutagenicity (F-t)	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$
9	0	45.0 ± 1	53.0 ± 7.1	197.3 ± 14.6	166.7 ± 9.3
	0.05	114 ± 24*	71.5 ± 3.5	272 ± 31.1	200 ± 50.9
	0.1	330.5 ± 6.4**	64.0 ± 5.7	544.5 ± 146.4*	230.5 ± 7.8**
	0.5	1005 ± 248.9*	164.5 ± 27.6*	1074.5 ± 20.5**	574.5 ± 13.4**
	1	2043 ± 73.5**	283 ± 29.7**	407.5 ± 258.1	649 ± 154.1*
	dose–response (Anova)	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.05$	$p \leq 0.001$
	acceptable model (F-t)	linear	linear	Lintox 2	Lintox 1
	slope ± SE[c]	1984.7 ± 98.9	225.8 ± 16.6	2743.1 ± 662.1	1145.2 ± 288.7
	mutagenicity (F-t)	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.005$	$p \leq 0.005$

[a] For procedures, see Experimental Procedures. [b] Mean values of duplicate plates. [c] Estimation of the initial slope. [d] *, Significant at 5%; **, significant at 1% (Student's *t*-test).

would counterbalance the expected deactivation of 7, a rather unstable molecule, through second-phase metabolism reactions induced by the S9 fraction.

Commoner (1976) proposed that, in addition to good dose–response relationship, a mutation ratio (see Experimental Procedures for definition) value above 2.0 should be required as a complementary criteria for establishing that a given compound is mutagenic in the *Salmonella*/Ames test. In our case, Figure 3 depicts the mutation ratios obtained for compound 6 and its epoxy derivative 7 in TA-98 and TA-100 strains in the absence (A) or presence (B) of S9 bioactivation. As shown, it could be confirmed that compound 6 is a direct mutagen in TA-100 strain at all doses assayed. On the other hand, epoxide 7 appeared to be a direct mutagen in both strains, although the activity exhibited in TA-100 strain was lower than that elicited by its parent derivative 6 and it could only be rigorously considered mutagenic at the highest doses assayed (100 and 200 μg/plate).

Mutation ratios derived for compounds 6 and 7 from assays with S9 incubation in TA-98 strain (Figure 3B) followed similar patterns to those obtained in Figure 3A. However, for the case of TA-100 strains it can be observed that microsomal bioactivation enhances mutagenicity in both compounds. Remarkably, the indirect mutagenicity exhibited by the model furobenzofuran 6 was greater than that elicited by its epoxy derivative 7 as direct mutagen, and S9 bioactivation on this latter intermediate led to toxic effects at the highest dose tested (200 μg/plate). These observations support the hypothesis that bioactivations other than epoxide formation could account for the overall mutagenic effects elicited by the model furobenzofuran 6.

The fact that aflatoxin model compounds 6 and 7 exhibited higher activities in TA-100 strains indicates that they predominantly induce base substitution mutagenicity. An examination of the mutagens which act on these strains (McCann et al., 1975; McCann and Ames, 1976)

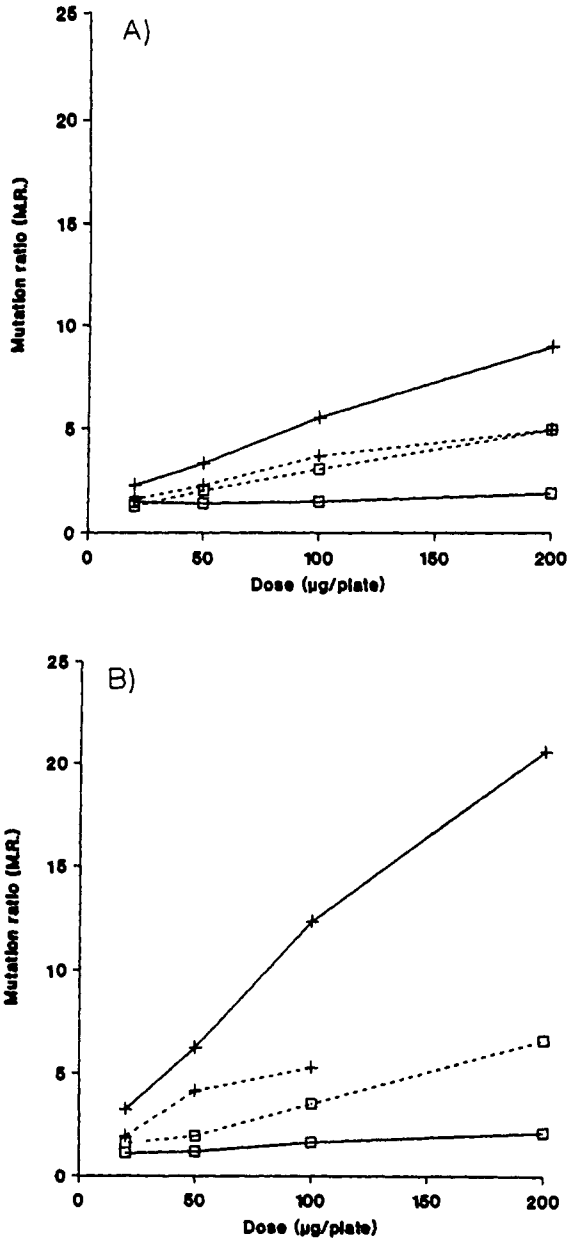

Figure 3. Mutation ratio (MR) plotted against the dose of aflatoxin model **6** (—) and of its 2,3-epoxy derivative **7** (- - -) used in the *Salmonella*/Ames test with TA-98 (□) and TA-100 (+) strains, in the absence (A) or presence (B) of microsomal enzyme preparation (S9). The parameter MR is defined under Experimental Procedures.

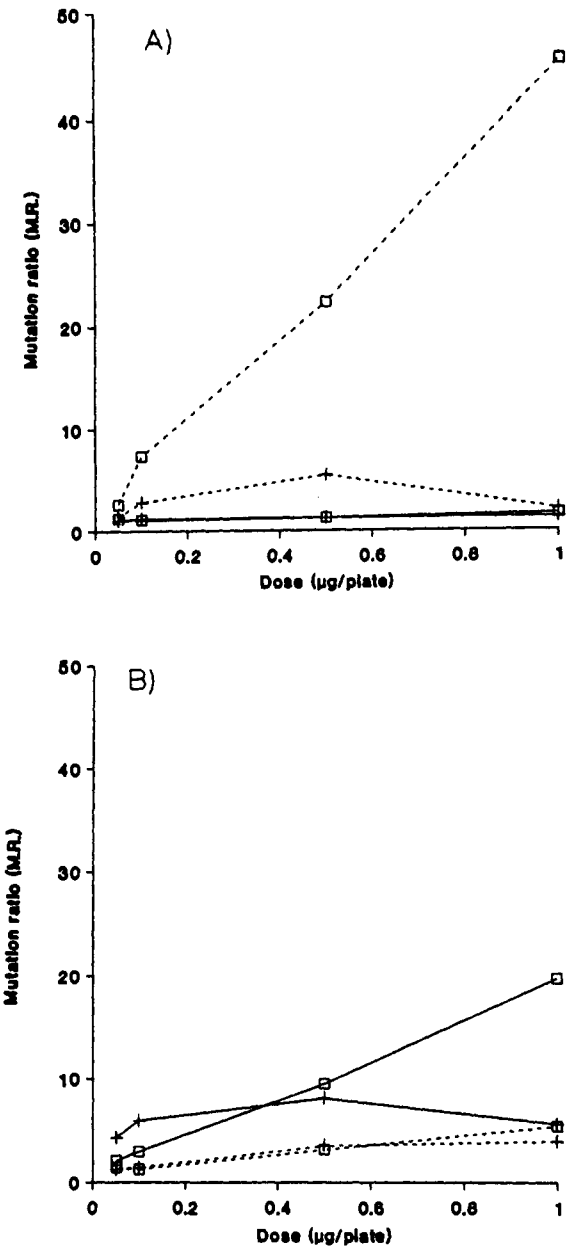

Figure 4. Mutation ratio (MR) plotted against the dose of aflatoxin B_1 (**8**) (—) and of its 8,9-epoxy derivative **9** (- - -) used in the *Salmonella*/Ames test with TA-98 (□) and TA-100 (+) strains, in the absence (A) or presence (B) of microsomal enzyme preparation (S9). The parameter MR is defined under Experimental Procedures.

shows a wide variety of strong alkylating agents which lack appropriate steric and functional group requirements for achieving an efficient intercalation into the DNA framework. Therefore, a plausible explanation for our results could be that furobenzofuran **6** would be activated enough to exert by itself an alkylating activity, probably in the fragment comprising the condensed furan rings, and from this point of view it could be considered an interesting aflatoxin model. In this respect, it seems reasonable that the incubation with S9 fraction, which usually leads to the formation of strong alkylating intermediates, would enhance the mutagenicity of **6**, as it was the case (cf. parts A and B of Figure 3). In addition, evidence found in the sense that furobenzofuran **6** is susceptible to bioactivation processes alternate to epoxidation capable of inducing mutagenicity could be taken as a model to study the extent at which similar mechanisms could be operating in related aflatoxins. Conversely, it is evident that compound **6** cannot be taken as a satisfactory aflatoxin model from those structural features that induce mutagenicity in TA-98 strain.

Finally, results obtained with the aflatoxin B_1 epoxy derivative **9** (Table II and Figure 4) deserve some comment, since to the best of our knowledge they constitute the first report on mutagenicity data derived from the *Salmonella*/Ames test related to these activated intermediates, postulated as the ultimate mutagenic species in the more cytotoxic aflatoxins (Betina, 1989). As expected, epoxide **9** is a very strong direct mutagen in TA-98 strain, and its activity is higher than the indirect mutagenicity elicited by aflatoxin B_1 itself. Concerning the effects of epoxide **9** in TA-100, strain, this compound appeared to be also a direct mutagen and initial slope values were also high in the absence of microsomal bioactivation. However, the mutagenicity elicited seems to be lower than that exhibited in TA-98 strain, although this consideration should be

taken with caution due to the inspecific toxicity obtained at the highest dose used (1 μg/plate), which could mask the overall effect. On the other hand, the presence of S9 fraction reduces the mutagenicity exhibited by epoxide **9** in both strains. In fact, according to the mutation ratio criteria, compound **9** would only be mutagenic at the highest dose assayed in TA-98 strain (MR 5.34). In contrast with the behavior of the epoxide model **7**, it seems that the aflatoxin B_1 epoxide is more sensitive to microsomal-mediated deactivation. In any case, our results could be taken as direct evidence, in agreement with those previously reported by Baertschi et al. (1989), on the crucial role that epoxide **9** plays in the cytotoxicity elicited by aflatoxin B_1.

In conclusion, from the results herein presented, we believe that compound **6** could constitute an interesting aflatoxin model for obtaining more detailed information on the chemical and xenobiochemical events involved with furobenzofuran moieties. In particular, the study of bioactivation processes leading to the formation of intermediates alternate to the 2,3-epoxy derivative could be contemplated. Work along this line is now in progress in our laboratory.

ACKNOWLEDGMENT

We thank Dr. P. Andrews for providing us the *Salmonella* assay software and P. Doménech for performing the elemental analyses. We acknowledge financial support from CICYT (Grant AGR-89-532) and CICYT-Generalitat de Catalunya (Grant QFN-89-4010).

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Received for review February 25, 1991. Accepted June 14, 1991.

Registry No. 1, 6093-80-7; **2**, 135105-85-0; **3**, 135105-86-1; **4**, 135105-87-2; **5** (isomer 1), 135105-88-3; **5** (isomer 2), 135214-35-6; **6**, 135105-89-4; **7**, 135105-90-7; **8**, 1162-65-8; **9**, 42583-46-0; 3,5-dimethoxyphenol, 500-99-2; methyl acetoacetate, 105-45-3.