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DNA Adduction by the Potent Carcinogen Aflatoxin B_1: Mechanistic Studies

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Abstract: Aflatoxin B_1, a potently carcinogenic fungal metabolite, is converted to the biologically active form by chemical oxidation using dimethyldioxirane and enzymatically by cytochrome P450 mixed-function oxidases. Both processes give rise to mixtures of the *exo*- and *endo*-8,9-epoxides. Methanolysis studies reveal exclusive *trans* opening of both epoxides under neutral conditions in CH_3OH and CH_3OH/H_2O mixtures; an S_N2 mechanism is postulated. Under acidic conditions, the *exo* isomer gives mixtures of *trans* and *cis* solvolysis products, suggesting that the reaction is, at least in part, S_N1; the *endo* isomer gives only the *trans* product. The *exo* isomer reacts with DNA by attack of the nitrogen atom at the 7 position of guanine on C8 of the epoxide to give the *trans* adduct; the *endo* epoxide fails to form an adduct at this or any other site in DNA. The *exo* isomer is strongly mutagenic in a base-pair reversion assay employing *Salmonella typhimurium*; the *endo* isomer is essentially nonmutagenic. Aflatoxin B_1 and its derivatives intercalate in DNA. These results are consistent with a mechanism in which intercalation of the *exo* epoxide optimally orients the epoxide for an S_N2 reaction with guanine but intercalation of the *endo* isomer places the epoxide in an orientation which precludes reaction. Thus, while the *exo* epoxide is a potent mutagen, the *endo* epoxide fails to react with DNA.

Introduction

The fungi *Aspergillus flavus* and *A. parasiticus* frequently infest peanuts, corn, and other agricultural commodities to produce aflatoxin B_1 (AFB_1, **1**) and related furofuran metabolites.[1] AFB_1 is a potent carcinogen which, after metabolic activation to an electrophilic species, reacts efficiently with DNA.[2] Reaction occurs with high regiospecificity at the N7 position of guanine residues in DNA.[3-5] Structural studies of the guanine adduct point to the *exo*-8,9-epoxide (**2**) being the genotoxic agent, although the epoxide has never been isolated from or directly detected in biological systems.[3,4,6-9] For many years, **2** eluded chemical synthesis; attempts to prepare it were frustrated by the lability of the epoxide, which invariably reacted with constituents of the epoxidation reaction system.[10-13] For example, use of

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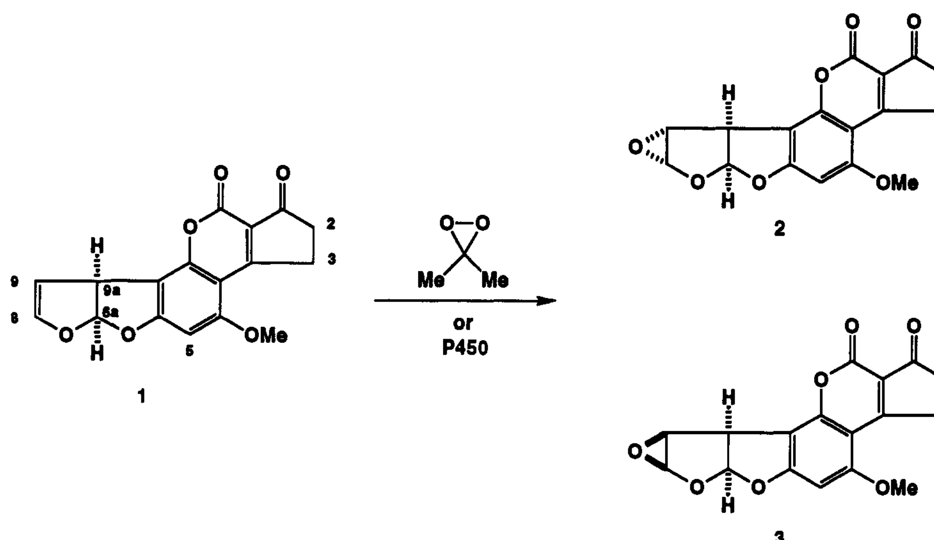
Scheme 1



m-chloroperbenzoic acid led to hydroxyesters derived from cleavage of the epoxide by *m*-chlorobenzoic acid, a coproduct of the reaction.[6,14] The epoxides of 2,3-dihydro-4*H*-pyran and other vinyl ethers can be prepared if precautions were taken to avoid solvolytic cleavage of the epoxide ring,[16,17] but the epoxides of 2,3-dihydrofurans are less accessible and through 1987 only a single, rather special case had been reported.[18] It was speculated that the epoxide of AFB_1 might not exist *per se*, undergoing dissociation of the C8–O bond to give an unisolable zwitterion.[12] In 1988, the question was laid to rest when this laboratory reported a synthesis of **2** (Scheme 1).[19] The procedure used dimethyldioxirane as the oxidant; acetone served as the solvent and was a coproduct of the reaction. High yields were obtained. Epoxide **2**, although highly reactive, is inert to acetone and other aprotic, nonnucleophilic solvents. The epoxide is stable as a crystalline solid, having spectroscopic properties consistent with formulation **2**. It reacts rapidly with H_2O, i.e., with a half-life of only a few seconds, to give 8,9-dihydrodiols and even faster with DNA to give an adduct at the N7 position of guanine.[20,21] The chemical properties of **2** are fully in accord with it being the fugitive carcinogenic electrophile.[19]

Subsequently, a close examination of the reaction of AFB_1 with dimethyldioxirane revealed that the reaction is not completely stereospecific and that small amounts of the *endo* isomer, i.e., **3**, are also formed.[22] The reaction of *m*-chloroperbenzoic acid with AFB_1 similarly gives predominantly the *exo* epoxide, but small amounts of the *endo* epoxide are also formed.[15] Even microsomal epoxidation of AFB_1 yields both epoxides. Trapping experiments using glutathione transferases to catalyze glutathione conjugation produced adducts of both epoxides.[23] The product ratio in enzymatic oxidations depends upon the species from which the microsomes are derived. Human microsomes give larger proportions of *endo* epoxide than rat or mouse microsomes. In all cases the *exo* product predominated. However, the discovery that the *endo* epoxide is also formed raised questions about the possible contribution of the *endo* epoxide to the overall carcinogenicity of AFB_1. From the chemical point of view, the availability of both stereoisomers provided an opportunity to evaluate the extent and mechanism(s) of their reactions with DNA. The study described herein shows that the *exo* epoxide reacts efficiently with DNA via an S_N2 mechanism, whereas the *endo* epoxide is incapable of reaction. The consequence is that the *exo* epoxide is a potent mutagen consistent with it being the carcinogenic form of AFB_1, whereas the *endo* epoxide is inactive.

Experimental Section

Biological Hazards. *Aflatoxin B_1 and many of its derivatives are potently carcinogenic. Great care should be exercised to avoid personnel exposure. Crystalline material presents an inhalation hazard because the crystals develop electrostatic charge and cling to dust particles. For this reason the dust produced by the scraping of preparative TLC plates should be regarded as particularly hazardous.*

Chemicals. AFB_1 was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dimethyldioxirane was synthesized as described by Murray and Jeyaraman and by Adam *et al.*[24,25] Solutions of dimethyldioxirane were stored over anhydrous $MgSO_4$ at -20 °C and were used within one month of preparation. AFB_1 *exo*- and *endo*-8,9-epoxides were prepared according to previously published procedures; the *exo* epoxide was recrystallized as described.[19,22] Standards of AFB_1-N7-guanyl adduct and AFB_1 dihydrodiol were prepared according to published methods.[26] Reagents for oligodeoxynucleotide synthesis were purchased from Pharmacia-P.L. Biochemicals, Inc. (Piscataway, NJ) and Fisher Scientific (Pittsburgh, PA). Columns for HPLC were obtained from Alltech Associates, Inc. (Deerfield, IL). The oligonucleotide d(ATGCAT) was synthesized by standard solid-phase phosphoramidite chemistry with an automated synthesizer and quantitated spectrophotometrically by the method of Borer assuming $\epsilon_{260} = 41500\ M^{-1}\ cm^{-1}$ for single-stranded material.[27] ^1H NMR spectra were recorded on a Bruker AC-300 spectrometer.

Hydrolysis of AFB_1 Epoxides. Hydrolyses were conducted in 1:1 mixtures of acetone/10 mM aqueous sodium phosphate at pH 7.0 at 23

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(14) The failure of synthetic approaches to epoxide **2** led to development of methods for *in situ* generation of epoxide in the presence of DNA.[7,8] The most generally useful method was the two-phase procedure of Martin and Garner, which employed *m*-chloroperbenzoic acid in a mixture of CH_2Cl_2 and H_2O.[6] The effective electrophile in the reaction was generally believed to be the epoxide. We recently discovered that their procedure actually yields significant concentrations of the epoxide in the organic phase.[15]

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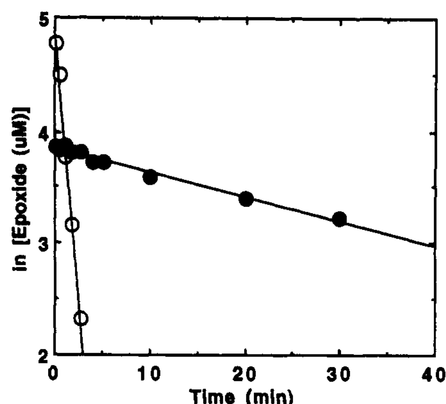

Figure 1. Hydrolysis of AFB_1 *exo* and *endo* epoxides **2** (○) and **3** (●) in 1:1 acetone/10 mM aqueous sodium phosphate, pH 7.0, at 23 °C. Aliquots were withdrawn at timed intervals and quenched in methanol. The methanol adducts were quantitated by HPLC as a measure of remaining epoxide. See Experimental Section for details. The slope of the line for epoxide **2** was $-0.95\ s^{-1}$, while that for epoxide **3** was $-0.022\ s^{-1}$.

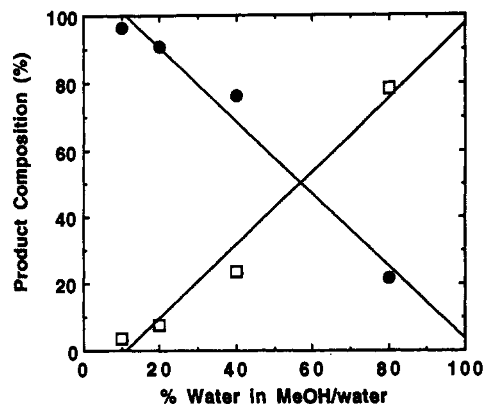

Figure 2. Methanolysis of AFB_1 *exo* epoxide **2** in buffered aqueous MeOH, pH 7.4. See Experimental Section for details. The products are dihydrodiols (□) and the *trans* methanol adduct **4** (●). *Cis* adduct **6** was not observed.

°C. A mixture of AFB_1 epoxides in acetone (2.7 mM, *exo*:*endo* 3.9:1) was added to buffer (0.5 mL) with vigorous vortex mixing to give a final concentration of approximately 200 μM. Samples (20 μL) were withdrawn for analysis and quenched with CH_3OH (0.4 mL) at time points during 5 min for the *exo* epoxide and 1 h for the *endo* epoxide. These samples were stored for 12 h at 4 °C and then diluted with 0.2 mL of 20 mM ammonium acetate. Analysis was by reverse-phase HPLC (Econosphere, 4.6 × 30 cm column eluted with 37.5% CH_3CN/CH_3OH (1:1) in 20 mM ammonium acetate buffer, pH 3.9, 1.5 mL/min). The formation of methanolysis products **4** and **6** reflects the presence of unhydrolyzed epoxides. Compounds **4** and **6** eluted at 9.5 and 10.5 min, respectively. They were quantitated by peak height using standards of known concentration as reference. Data are presented in Figure 1.

Methanolysis of AFB_1 Epoxides. AFB_1 *exo* epoxide (200 μg, 0.61 μmol) was dissolved in anhydrous CH_3OH (0.5 mL). The solution was allowed to stand at room temperature for 1 h and then evaporated to dryness under a stream of nitrogen. The ^1H NMR spectrum showed the *trans* methoxy alcohol **4** to be the sole product (>95% pure). Similar results were obtained when the methanolysis was carried out using CD_2Cl_2 or THF as the solvent.

Methoxy alcohol **4**: ^1H NMR (300 MHz, $CDCl_3$) δ 6.64 (1*H*, d, H6a), 6.30 (1*H*, s, H5), 5.08 (1*H*, s, H8) 4.54 (1*H*, s, H9), 3.95 (1*H*, d, H9a), 3.93 (3*H*, s, 4-OMe), 3.38 (2*H*, m, H3,3″), 3.20 (3*H*, s, 8-OMe), 2.62 (2*H*, m, H2,2′); MS (FAB) *m/z* 361 (MH^+).

To a stirred solution of AFB_1 epoxide (*exo*:*endo* ~10:1, 2 mg, 6.1 μmol) in dry CH_2Cl_2 (0.5 mL) was added anhydrous HCl (25 μL of a saturated solution in dry benzene), followed immediately by dry CH_3OH (0.5 mL). The resulting solution was stirred overnight at room temperature. Solvents were removed under a stream of N_2, and the residue was dissolved in $CDCl_3$. The ^1H NMR of the crude product mixture indicated the formation of methoxy alcohols **4**, **5**, and **6**. The *exo* epoxide (**2**) had partitioned 84:16 between *trans* and *cis* methanolysis products **4** and **6**, as judged by integration of the 8-OMe signals. *Endo* epoxide **3** gave exclusively *trans* methanolysis product **5**; *cis* adduct **7** could not be detected. Compounds **4**–**6** were purified by HPLC on a silica gel column (Econosil, 10 μm, 250 mm × 10 mm, 3 mL/min) using ethanol/CH_2Cl_2 (3:97 v/v) as the solvent. Methoxy alcohol **4** eluted at 24 min; methoxy alcohols **5** and **6** eluted at 19 min, unresolved from each other; they were, however, separated by reverse-phase HPLC using an Econosphere ODS (5 μm, 250 mm × 4.6 mm) column, eluted isocratically with 35% CH_3CN/CH_3OH (1:1 v/v) in H_2O at a flow rate of 1.5 mL/min. The *cis* methoxy alcohol **6** derived from *exo* epoxide eluted at 6.5 min, and *trans* methoxy alcohol **5** derived from *endo* epoxide eluted at 7.6 min. Methoxy alcohol **7** was not detected.

Methoxy alcohol **6**: ^1H NMR (300 MHz, $CDCl_3$) δ 6.53 (1*H*, d, H6a), 6.36 (1*H*, s, H5), 4.97 (1*H*, d, H8), 4.53 (1*H*, m, H9), 4.09 (1*H*, dd, H9a), 3.94 (3*H*, s, 4-OMe), 3.58 (3*H*, s, 8-OMe), 3.38 (2*H*, m, H3,3′), 2.62 (2*H*, m, H2,2′); MS (FAB) *m/z* 361 (MH^+).

Methoxy alcohol **5**: ^1H NMR (300 MHz, $CDCl_3$) δ 6.42 (1*H*, d, H6a), 6.37 (1*H*, s, H5), 4.97 (1*H*, d, H8), 4.53 (1*H*, dd, H9), 4.28 (1*H*, m, H9a), 3.94 (3*H*, s, 4-OMe), 3.47 (3*H*, s, 8-OMe), 3.39 (2*H*, m, H3,3′), 2.63 (2*H*, m, H2,2′).

Methanolysis of AFB_1 Epoxides in H_2O. Recrystallized AFB_1 *exo* epoxide (~100 μg in 10 μL DMSO) was added to a series of microfuge tubes containing 0, 10, 20, 40, and 80% volume fraction of 0.1 M aqueous sodium phosphate buffer, pH 7.4, in CH_3OH (total volume 1 mL). The reaction mixtures were allowed to stand at room temperature for 2 h with occasional stirring. Aliquots (10 μL) were analyzed by reverse-phase HPLC as described above, eluting isocratically with 30% CH_3CN/CH_3OH (1:1 v/v) in 20 mM ammonium acetate buffer, pH 3.9, and a flow rate of 1.5 mL/min. Elution times of AFB_1 dihydrodiols, the *cis* methoxy alcohol **6**, and the *trans* methoxy alcohol **4** were 4.1–5.0, 9.1, and 9.9 min, respectively. UV absorbance of products was monitored at 360 nm. Peak areas were estimated using a Hitachi D-2500 Chromato-Integrator. Results are shown in Figure 2. The above experiment was repeated using H_2O instead of buffer; similar results were obtained. The experiment was repeated with a ~4:1 mixture of *exo* and *endo* epoxides. Only dihydrodiols and *trans* methanolysis products **4** and **5** were observed; compound **5** eluted at 11.0 min.

Attempted Reaction of *Endo* Epoxide with an Oligonucleotide Duplex. A solution of AFB_1 epoxides (*exo*:*endo* ~4:1, 0.5 mg, 1.6 μmol) in anhydrous acetone (100 μL) was added to d(ATGCAT) (40 A_{260} units, 1.1 μmol) dissolved in 0.5 mL of 0.1 M sodium phosphate buffer, pH 7.4, at 0–5 °C with vigorous stirring. After reaction at 5 °C for 45 min, 50 μL aliquots were analyzed by reverse-phase HPLC (C-18 analytical column, 5–25% linear gradient of CH_3CN in 0.01 M sodium phosphate buffer, pH 7.0, over 25 min; 1.0 mL/min; UV detection at 260 nm). Retention times of unmodified d(ATGCAT) and modified d(ATGCAT)-AFB_1 were 2.5 and 8.5 min, respectively. Analyses were repeated after acid hydrolysis of purine–deoxyribose linkages. Aliquots (100 μL) of the reaction mixture were treated with 0.1 M HCl (100 μL) at 60 °C for 30 min and then analyzed for the presence of AFB_1-N7-guanyl adducts by reverse-phase HPLC (Econosphere ODS, 5 μm, 4.6 × 250 mm), eluted with 32.5% CH_3CN/CH_3OH (1:1 v/v) in 20 mM ammonium acetate buffer, pH 3.9, at a flow rate of 1.2 mL/min). UV absorbance of products was monitored at 360 nm. Retention time of the AFB_1-N7-guanyl adduct was 10.0 min.

We failed to find a chromatographic procedure for separation of the epoxides due to their lability. As an alternative, the *exo* epoxide was removed by hydrolysis, taking advantage of its higher reactivity. A partial hydrolysis of the epoxide mixture was performed by addition of an equal volume (100 μL) of 0.1 M sodium phosphate buffer, pH 7.0, to the acetone solution of epoxides 10 min before reaction with oligonucleotide. This yielded the *endo* epoxide essentially free of the *exo*. Substantial quantities of dihydrodiols were formed; however, dihydrodiols do not react with DNA. No adducts derived from the *endo* epoxide were detected either at the oligonucleotide stage or after depurination.

Reaction of AFB_1 Epoxides with DNA. A solution of AFB_1 epoxides (*exo*:*endo* ~4:1, 0.2 mg) in anhydrous acetone (100 μL) was added with stirring to ~2 mg of calf thymus DNA in 1.0 mL of 0.1 M sodium phosphate buffer, pH 7.2, at 10 °C. After 30 min, the reaction mixture was acidified to pH 2.0 with 0.1 M HCl and heated at 80 °C for 30 min. The solution was filtered through a 0.45-μm filter. Formation of guanyl-N7 adduct(s) was assayed by reverse-phase HPLC (30% CH_3CN/CH_3OH (1:1 v/v) in 20 mM ammonium acetate buffer, pH 3.9, 1.5 mL/min). Retention times of the N7-guanyl adduct and the AFB_1 dihydrodiols were 7.1 and 4.3–5.3 min, respectively. A similar experiment was

performed after partial methanolysis of the epoxide mixture by the addition of an equal volume (100 μL) of CH_3OH (room temperature, 2–3 min) prior to reaction with DNA. No adducts derived from the *endo* epoxide were observed.

Bacterial Mutagenesis Assays. For the assay of *exo* AFB_1 epoxide, crystalline *exo* AFB_1 epoxide dissolved in acetone was added to the cell suspension. For the assay of the *endo* AFB_1 epoxide, a mixture of *exo* and *endo* AFB_1 epoxides (4:1; obtained from the mother liquor after crystallization of the *exo* AFB_1 epoxide) was allowed to hydrolyze for 15 min in acetone/H_2O (pH 7.0, 1:1 v/v) at 23 °C and then used as the source of the *endo* AFB_1 epoxide. During this period the amount of *exo* epoxide decreased ~10^4-fold, while the amount of *endo* epoxide decreased only ~25%. The mutation assay was carried out by the procedure of Maron and Ames (1983) in the preincubation mode.[28] The AFB_1 epoxides (*exo* in acetone and *endo* in acetone/H_2O (1:1 v/v) with the total volume of acetone <25 μL) were added to *Salmonella typhimurium* TA100 cell suspension in 0.2 M sodium phosphate buffer, pH 7.4, and diluted to give a final volume of 700 μL. After 5 min at 23 °C, the preincubation mixture was added to molten top agar enriched with trace histidine and biotin, which was then poured on minimal glucose agar plates. The revertant colonies on each plate were scored after incubation for 48 h at 37 °C. Cytotoxicity appeared at the highest dose of each AFB_1 epoxide. AFB_1 dihydrodiol did not elicit a mutagenic response. The results for the *exo* and *endo* epoxides are presented in parts A and B of Figure 3, respectively (note differences in scales of the two plots). The data are replotted in Figure 3C to emphasize the difference in mutagenicity.

Results and Discussion

A study of the hydrolysis of the two epoxides was carried out in aqueous acetone at pH 7.4. Reaction rates were determined by removing samples from the reaction mixture and quenching with excess methanol. The study revealed that the *exo* epoxide is ~40-fold more reactive than the *endo* (see Figure 1). Presumably, a similar relationship exists in water alone; however, the reactions are too fast in pure water to measure by this technique. The substantial difference in reactivities of the epoxides is noteworthy. Steric considerations might suggest that the reactivities would be reversed. It seems likely the reactivity difference is due to an anomeric effect. Studies of *cis*- and *trans*-2,5-dimethoxytetrahydrofurans have shown them to have similar reactivity (within a factor of 2).[29] A study of tetrahydropyranyl diethers by Kirby and Martin showed the *trans*-linked species to undergo spontaneous hydrolysis 200-fold slower than the *cis*.[30] They accounted for this difference in terms of participation of the antiperiplanar lone pair of the distal oxygen of the *cis* isomer in stabilization of the transition state, whereas the distal oxygen of the *trans* isomer is sterically precluded from offering this assistance. Detailed conformational data for the aflatoxin epoxides will be required to analyze the reactivity differences. Crystal structures have been reported for aflatoxins B_1 and B_2,[31,32] however, we have not been able to prepare crystals of the *exo* epoxide suitable for single-crystal crystallography. Attempts to crystallize the *endo* epoxide must await isolation of the compound in pure form.

Studies of the methanolysis of epoxides **2** and **3** were carried out. The *exo* epoxide can be obtained in pure form by recrystallization. The supernatant solutions from recrystallizations are enriched in *endo* isomer, but no method has been found by which the *endo* epoxide can be fully purified. Chromatographic purifications were frustrated by the sensitivity of both epoxides to adventitious moisture. Consequently, kinetic studies of the *endo* epoxide were carried out using mixtures with the *exo* isomer.

Treatment of the *exo* epoxide with anhydrous CH_3OH gave a single product (**4**, Scheme 2). The ^1H NMR spectrum of **4** was similar to one reported by Coles *et al.*[11] for the ethanolysis product which they had assigned as the *trans* adduct. The NMR spectrum of methanolysis product **4** confirmed the *trans* assignment, i.e., negligible vicinal coupling constants between H8 and H9 and between H9 and H9a ($^3J_{8,9}$ and $^3J_{9,9a}$ = ~0 Hz), lack of NOE between H6a and the 8-methoxy group, and the presence of NOE from H6a to H8 (see Figure 4).

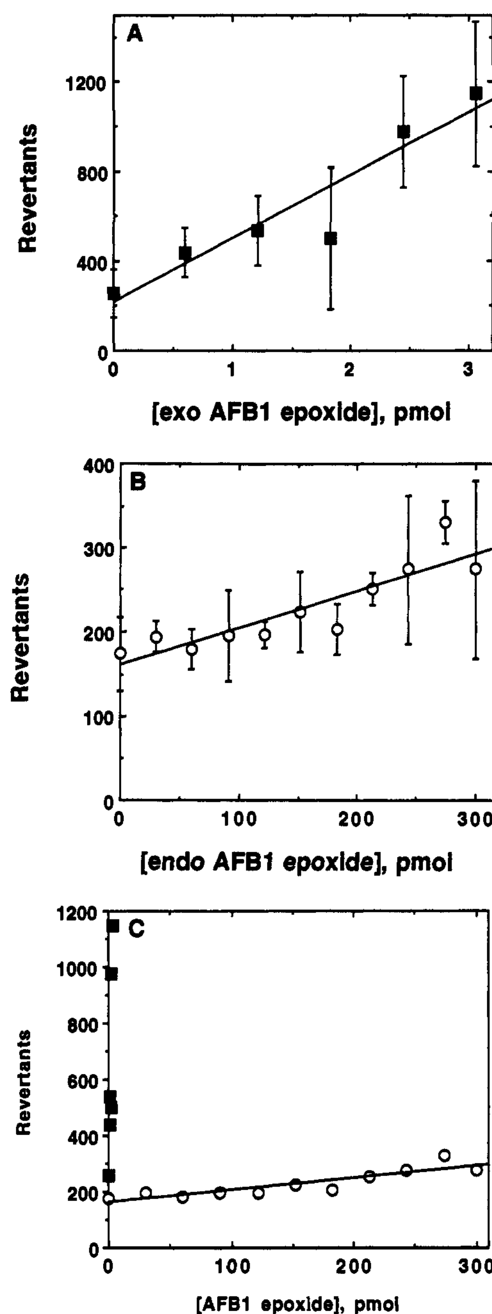

Figure 3. Revertants produced in *S. typhimurium* TA100 by AFB_1 epoxides: (A) *exo* AFB_1 epoxide (■); (B) *endo* AFB_1 epoxide (○); (C) comparison of *exo* AFB_1 epoxide (■) and *endo* AFB_1 epoxide (○) mutagenicity. Each point in plots A and B represents the mean ± SD (n = 3, duplicate assay for each of the three data).

The *endo* isomer also yielded only a single product which was isolated by HPLC. Using NMR, its stucture was established as *trans* adduct **5**. As shown in Figure 4, the H8–H9 and H9–H9a vicinal coupling constants were 2.8 and 7.5 Hz. The large H9–H9a coupling constant precluded the compound being **6**, the *cis* product of the *exo* epoxide. Nuclear Overhauser effects were examined. An NOE was observed from the 8-methoxy group to H6a, placing the methoxy group on the α face.

The methanolysis reactions were repeated under acidic conditions using HCl as the catalyst. The *exo* isomer gave a 5:1

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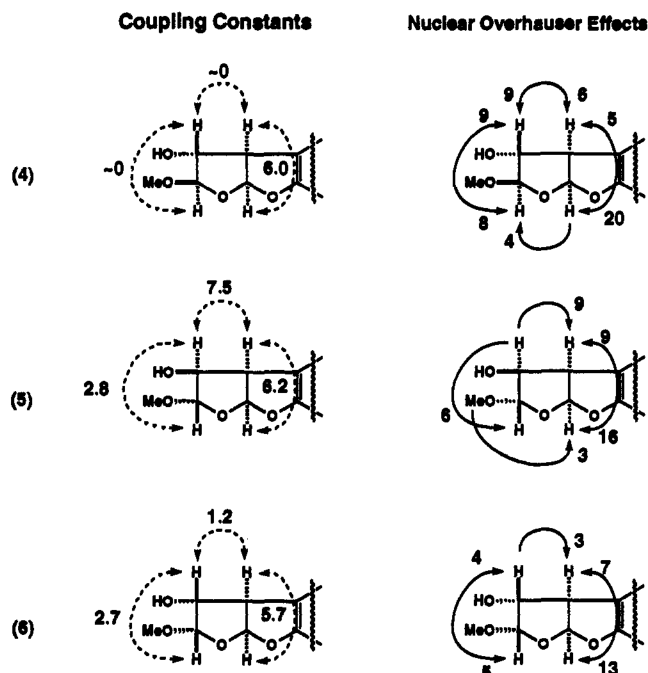


Figure 4. Coupling constants (Hz) and NOEs (%) observed for compounds 4–6 obtained from methanolysis of AFB_1 epoxides 2 and 3. Only data for the tetrahydrofurofuran portions of the molecules is reported. The arrows in the NOE data indicate the direction of the NOE effect; that is, a double-headed arrow means the effect was seen in both directions.

mixture of the *trans* adduct 4 and a new species identified as *cis* adduct 6. The NMR spectrum of 6 showed $^3J_{8,9}$ and $^3J_{9,9a}$ vicinal coupling constants of 2.7 and 1.2 Hz (Figure 4). The *cis* ethanolysis product derived from the *exo* epoxide of an aflatoxin model has been described;[11] it shows values of 3.3 and 1.5 Hz, respectively, for vicinal coupling constants corresponding to $^3J_{8,9}$ and $^3J_{9,9a}$ in 6. No isomerization occurred when *trans* isomer 4 was treated with acidic CH_3OH under the conditions that had been used to prepare 6, indicating that 6 had arisen directly from the epoxide, not by an acid-catalyzed isomerization of *trans* adduct 4. Methanolysis of the *endo* epoxide under acidic conditions gave *trans* product 5 as the only detectable product. This assertion must be made with caution due to the fact that *endo* epoxide was not available in pure form and small quantities of the *cis* methanolysis product might have escaped detection in the product mixture.

Reactions of the epoxides in H_2O may have more S_N1 character than in CH_3OH due to the higher dielectric constant. While it would be desirable to observe directly the stereochemistry of hydrolysis of the epoxides in H_2O, interpretation of the stereochemical outcome of such experiments is compromised by the fact that the hydrolysis products, the 8,9-dihydrodiols, are hemiacetals and equilibration of the *cis* and *trans* diols can occur via ring cleavage to the aldehyde (Scheme 3). As a probe for possible changes in mechanism of solvolysis in H_2O, a series of methanolysis reactions were carried out on the *exo* epoxide in CH_3OH/H_2O mixtures containing increasing mole fractions of H_2O. The yield of methanolysis products drops as the mole fraction of CH_3OH is decreased, but the stereochemistry of the products provides a sensitive probe of mechanism. Reactions of the epoxides carried out with pure *exo* epoxide and mixtures of *exo* and *endo* in CH_3OH/H_2O mixtures containing 20, 40, 60, and 80% H_2O were found to give only the *trans* products. Furthermore, the yield of *trans* product 4 dropped in a linear fashion, providing further evidence that no change in mechanism was occurring (see Figure 2). Quantitation of the endo product was less precise due to it being the minor constituent of a mixture, but no obvious change of mechanism was observed during the transition from pure methanol to 20:80 CH_3OH/H_2O.

The aflatoxin epoxides can be regarded as highly strained acetals. The solvolytic reactions of acetals, hemiacetals, and the hydration–dehydration of simple aldehydes and ketones have long been the subject of mechanistic investigation.[33,34] The central issue has been to identify the structures of transition states. Positive charge in the transition state will be stabilized by electron donation from oxygen. However, S_N2 processes as well as S_N1 will generally be facilitated by this process. The transition states for S_N2 processes in hydroxylic solvents are likely to involve hydrogen bonding or outright protonation of the epoxide oxygen contributing positive charge to the transition state.

Jencks has concluded that S_N1 processes are unlikely in solvolytic cleavage of acetals.[34,35] He has reasoned that oxocarbonium ions resulting from cleavage of acetals are too unstable to exist as free intermediates and predicts that these reactions should proceed through a preassociation or concerted mechanism. Oxocarbonium ions are frequently invoked in the hydrolysis of glycosides, but Sinnott and Jencks have shown that the rate of solvolysis is dependent on the leaving group.[36] Discrimination between competing entering nucleophiles is dependent upon the nature of the leaving groups. Thus, the leaving group is involved in the transition state. Jencks points out that a cationic intermediate can be stabilized by simultaneous close association with both the entering and leaving groups. Furthermore, this stabilization can occur not only in *trans* geometry but also in *cis*.

The alcoholysis reactions of the aflatoxin epoxides provide a unique opportunity for probing the structure of the transition state; the cleavage products provide direct evidence for the stereochemistry of reaction. Cleavage of the acetal linkage occurs exclusively by rupture of the bond between C8 and the epoxide oxygen. Alcoholysis reactions, such as the methanolysis reactions studied herein, are sufficiently facile that cleavage can be observed under conditions where the product methyl acetal is stable. The rigorous *trans* geometry of products derived from the *exo* epoxides argues strongly for an S_N2 process. It is likely that hydrogen bonding of solvent with the epoxide assists the displacement reaction in which the nucleophile must approach from the more hindered *endo* face.

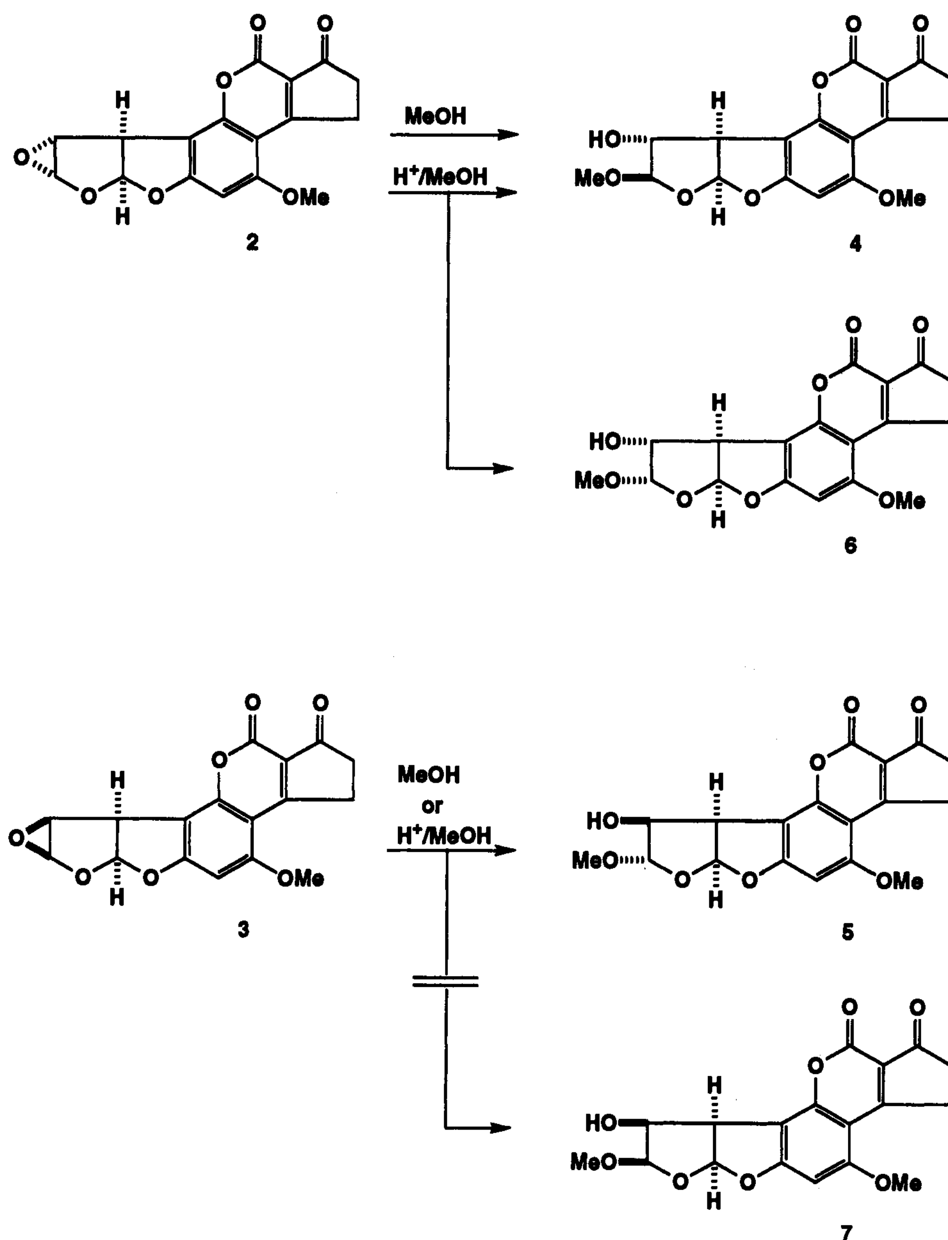
The reaction appears to undergo a change of mechanism under acidic conditions with the rigorous *trans* geometry being lost. We attribute the difference in stereochemical outcome to the acid-catalyzed process involving cleavage of the protonated epoxide, whereas the neutral condition involves, at most, an epoxide activated by hydrogen bonding. Although formation of *cis* product would appear to be *prima facie* evidence for an S_N1 process, Jencks has argued that *cis* products, as well as trans, can arise from concerted mechanisms.

Methanolysis of the *endo* epoxide is stereospecific under both neutral and acidic conditions. This result provides only limited insight on the mechanism of reaction since steric factors combine with electronic to favor *trans* entry of the incoming nucleophile. This contrasts with the *exo* situation where *trans* attack by the nucleophile requires the attack to be from the less desirable *endo* face. We conclude that near neutrality the solvolytic pathway in both CH_3OH and H_2O is S_N2 not S_N1.

Reaction of the epoxides with DNA were examined, first with the self-complementary oligonucleotide d(ATGCAT) and then with calf thymus DNA. Previous studies in this laboratory had shown that $d(ATGCAT)_2$, at temperatures where it existed as a duplex, reacted readily with the *exo* epoxide of aflatoxin to give a guanyl adduct in each chain.[20] High yields of adduct were also observed with high molecular weight DNA.[26] The slower reaction of the *endo* isomer with solvent would in itself not deter reaction with DNA and could conceivably give higher yields of the guanyl

(33) Cordes, E. H.; Hull, H. G. *Chem. Rev.* **1974**, *74*, 581–603.
(34) Jencks, W. P. *Acc. Chem. Res.* **1980**, *13*, 161–169.
(35) Young, P. R.; Jencks, W. P. *J. Am. Chem. Soc.* **1977**, *99*, 8238–8248.
(36) Sinnott, M. L.; Jencks, W. P. *J. Am. Chem. Soc.* **1980**, *102*, 2026–2032.

Scheme 2



adduct. However, in attempted reactions of the *endo* epoxide with this oligonucleotide and with calf thymus DNA using conditions with which the *exo* isomer gives high yields of the guanine N7 adduct, the *endo* isomer gave no isolable or detectable adduct(s). The potential formation of guanine N7 and other thermally labile adducts was probed without success by chromatographic examination of extractable materials after thermal depurination. In addition, the possibility of thermally stable adducts in the DNA recovered after depurination was assessed by observing the UV spectrum of DNA in the region of 350 nm. In that these experiments were conducted using a mixture of the *exo* and *endo* epoxide, we cannot rigorously exclude the possibility that a labile *endo* adduct could have co-eluted with a known product from the *exo* epoxide. However, within the limits of detection, we conclude that the *endo* epoxide did not react with DNA.

The remarkable difference in reactivity of the epoxide stereoisomers with DNA can readily be rationalized. We have previously proposed that the reaction of the *exo* epoxide of aflatoxin B_1 with DNA involves an intercalated transition state.[37] A number of lines of evidence have been obtained which indicate that aflatoxin B_1 and many of its derivatives associate with DNA and that this association involves intercalation of the planar portion of the aflatoxin moiety into the DNA.[38] This intercalation occurs with little regard for sequence; A:T regions are actually slightly better intercalation sites than G:C regions. Adduction occurs when intercalation of aflatoxin *exo* epoxide occurs on the 5′ side of guanine with the molecule oriented to place the epoxide moiety proximal to the nonbonding orbital of guanine N7.

The solvolysis studies indicate that near neutrality the stereoisomeric epoxides both react by S_N2 mechanisms. In spite of the fact that both epoxides are very labile and can form oxygen-stabilized carbocations, the stereochemistry of methanolysis reactions is consistent with bimolecular reactions exclusively involving inversion of configuration. The *exo* epoxide reacts efficiently with DNA, whereas the *endo* epoxide does not react at all. This observation is also consistent with the proposed bimolecular reaction mechanisms. Whereas intercalation of the planar portion of *exo* aflatoxin epoxide optimally orients the oxirane moiety for S_N2 attack, similar intercalation of the *endo*

(37) Gopalakrishnan, S.; Byrd, S.; Stone, M. P.; Harris, T. M. *Biochemistry* **1989**, *28*, 726–734.

(38) Raney, K. D.; Gopalakrishnan, S.; Byrd, S.; Stone, M. P.; Harris, T. M. *Chem. Res. Toxicol.* **1990**, *3*, 254–261.

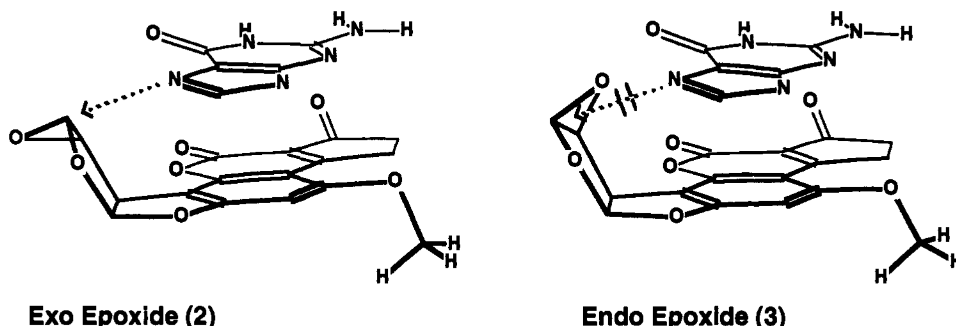
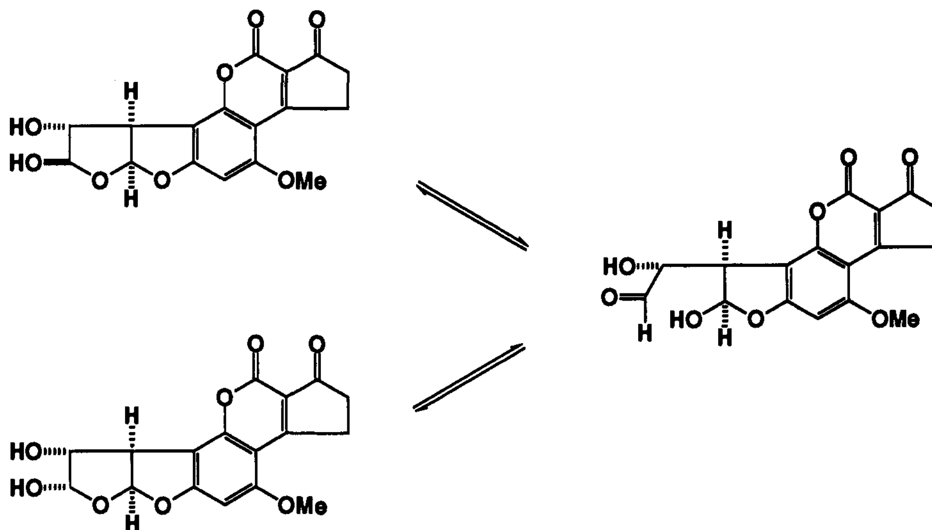


Figure 5. Trajectory of S_N2 attack of the N7 position of guanine on epoxides **2** and **3** when intercalated in DNA. Note that the orientation of **2** is optimal for backside attack on the epoxide but the orientation of **3** precludes backside attack.

Scheme 3



epoxide places the C–O bond of the oxirane ring aiming toward guanine N7 rather than away from it; these relationships are depicted in Figure 5. Consequently, DNA adduction by an S_N2 mechanism is precluded for the *endo* epoxide, and hydrolysis becomes the default process. It should be noted that, if the two epoxides had been able to undergo S_N1 cleavage processes under neutral conditions, both of them would have been able to form guanine adducts via intercalated transition states, i.e., the *exo* and *endo* epoxide would have formed *trans* and *cis* adducts, respectively.

The mutagenicity of the AFB_1 epoxides was assessed using the *S. typhimurium* TA100 reversion system of Maron and Ames.[28] This strain is highly sensitive to AFB_1 when activated by microsomal preparations. The mixture of AFB_1 epoxides produces a high revertant rate in this strain. To evaluate the mutagenicity of the two epoxides, the *exo* AFB_1 epoxide could be tested in pure form but *endo* AFB_1 epoxide had to be used in mixtures with the *exo* epoxide. However, the effect of the *exo* epoxide was minimized by hydrolysis of the AFB_1 epoxide mixture for $\sim$15 half-lives of the more reactive *exo* epoxide. Substantial amounts of the *endo* epoxide were lost ($\sim$25%) by this process, but the final solution was essentially free of *exo* epoxide. The experiments showed that, whereas *exo* AFB_1 epoxide is a potent mutagen, the *endo* AFB_1 epoxide is at least 500-fold less active. Even that level of activity for the *endo* epoxide may reflect contamination by small quantities of the *exo* epoxide rather than a low-yield reaction with DNA. The data are shown in Figure 3A,B. The *exo* and *endo* data are overlaid in Figure 3C to assist the reader in visualizing the large difference between these two plots. It should be noted that *endo* epoxide prepared by this procedure is severely contaminated with AFB_1 dihydrodiols. However, the dihydrodiols derived from both epoxides are not mutagenic in this assay. These studies are fully consistent with the *exo* epoxide being the metabolite which causes AFB_1 to be potently carcinogenic. The *endo* isomer is apparently an innocuous detoxification product.

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