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The *endo*-8,9-Epoxide of Aflatoxin B_1: A New Metabolite

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AFB_1[1] (1) is a highly toxic fungal metabolite which has been shown to be a carcinogen in animals and implicated by epidemiological studies as a carcinogen in man (*1*). Genotoxicity has been ascribed to an unstable metabolite formed by oxidative metabolism (*2*). The intermediate has never been isolated from biological systems, but interception by reaction with DNA and with glutathione points to the biologically relevant species being the *exo*-8,9-epoxide (**3**) (*3–5*). The epoxide eluded synthesis for many years due to its high reactivity; however, we recently reported a procedure for its preparation (*6*). The method uses dimethyldioxirane (**2**) as the oxidant and gives high yields of crystalline epoxide. Reactivity of the *exo*-epoxide appears to be generally concordant with the properties of the fugitive, bioactivated AFB_1.

In the earlier report we stated that the dimethyldioxirane epoxidation procedure was stereospecific for formation of the *exo*-epoxide. On closer examination of the reaction we now find that the *endo*-epoxide (**4**) is also formed, although to a much smaller extent. Herein, we report the synthesis and characterization of AFB_1 *endo*-8,9-epoxide and its formation by rat and human liver microsomes.

Experimental Procedures

Chemicals. AFB_1 was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dimethyldioxirane was synthesized as described by Murray and Jeyaraman (*7*) and Adam et al. (*8*). Solutions of dioxirane were stored over anhydrous $MgSO_4$ at −20 °C and were used within 1 month of preparation. ^1H NMR spectra were obtained on a Bruker (Billerica, MA) AM-400 instrument.

Synthesis of AFB_1 *exo*- and *endo*-8,9-Epoxides. AFB_1 (3 mg) was treated with 1.5 equiv of dimethyldioxirane (*6*). After 20 min, the solvent and excess dioxirane were evaporated under a stream of N_2. The resulting solid was dissolved in [$^2H_6$]acetone, and the ^1H NMR spectrum was obtained. The principal product was the *exo*-8,9-epoxide (**3**). Close examination of the spectrum revealed a second species, *endo*-AFB_1 8,9-epoxide (**4**), which was produced in ~10% yield and was the only other product detected.

Recrystallization. The mixture of *exo*- and *endo*-AFB_1 epoxides was dissolved in 300 μL of CH_2Cl_2. Anhydrous acetone (300 μL) was added, and the solution was stored at −20 °C for 24 h to produce crystals. The supernatant was removed from the crystals with a syringe. The crystals were washed with a small amount of anhydrous acetone and dried with N_2. The supernatant was evaporated under a stream of N_2. ^1H NMR spectra were obtained on the crystals and the residue from the supernatant. The crystals were found to be pure *exo*-AFB_1 epoxide. Integration of the signals in the spectrum of material obtained from the supernatant revealed a 4:1 mixture of *exo*- and *endo*-epoxides. Additional *exo*-epoxide was removed from the supernatant by crystallization from a smaller volume of a CH_2Cl_2/acetone mixture; the ^1H NMR spectrum of the resulting supernatant indicated a 2:1 mixture of *exo*- and *endo*-epoxides. Decoupling experiments were performed on this sample to assist in assigning the ^1H NMR spectrum of **3** (Table I).

Microsomal Incubations. Incubation mixtures (250-μL total volume) contained human or rat liver microsomes (3 mg/mL), 100 mM sodium phosphate buffer (pH 7.4), an NADPH generating system (containing final concentrations of 5 mM glucose 6-phosphate, 1 mM $NADP^+$, and 1 IU/mL yeast glucose-6-phosphate dehydrogenase), 5 mM GSH, mouse or rat liver cytosol (3 mg/mL), and 50 μM AFB_1. Incubations were carried out for 40 min at 37 °C, and reactions were quenched by addition of 50 μL of 2 M CH_3CO_2H. Product mixtures were stored at −20 °C. Samples were thawed, centrifuged, and then analyzed by reverse-phase HPLC using an Econosphere octadecylsilane (5 μm, 4.6-mm × 250-mm) column (Alltech Associates, Deerfield, IL). Elution solvents consisted of (A) 20 mM aqueous $CH_3CO_2NH_4$ (pH 4.0) and (B) a mixture of CH_3OH/CH_3CN (1:1 v/v). A multistep gradient of 90% A and 10% B (v/v) to 80% A and 20% B (v/v) over 20 min increasing to 50% A and 50% B (v/v) at 35 min was used to elute AFB_1 metabolites. Retention times of the GSH conjugate of AFB_1 *exo*-epoxide, the GSH conjugate of AFB_1 *endo*-epoxide, AFQ_1, and AFB_1 were 21.0, 21.8, 30.7, and 36.0 min, respectively. UV absorbance of metabolites was monitored at 360 nm with a single-wavelength detector. Peak areas were estimated using a Hitachi D-2500 Chromato-Integrator with AFB_1 8,9-dihydrodiol serving as an external standard (*9*, *10*).

Preparation of Authentic Samples of the GSH Conjugates of AFB_1 *exo*- and *endo*-Epoxides. GSH (200 mg, 0.065 mmol) in 2 mL of CH_3OH was treated with 50 mg of Na metal (2.2 mmol), causing the GSH to dissolved immediately. AFB_1 epoxide (1.5 mg, 2:1 exo:endo in 0.75 mL of CH_2Cl_2) was added. The solution immediately became yellow and after 1 min was neutralized with ~1.5 mL of 1 M aqueous CH_3CO_2H. After evaporation in vacuo with a Speed Vac concentrator (Savant Instruments, Inc., Hicksville, NY), the residue was dissolved in 4 mL of H_2O and clarified using a 0.4-μm filter. Purification by HPLC (vide supra) gave 70% and 50% yields, respectively, of the conjugates of the *exo*- and *endo*-epoxides as determined by HPLC peak areas in the chromatogram. The products were distinguished by comparison with the adduct derived from pure *exo*-epoxide. The structure of the adduct derived from *endo*-epoxide has been established by NMR spectroscopy and by comparison with adducts of ethanethiol (K. D. Raney, unpublished results).

Results and Discussion

Synthesis of *exo*- and *endo*-AFB_1 Epoxides. Epoxidation of **1** using freshly prepared dimethyldioxirane (**2**) in anhydrous acetone gives a ~10:1 mixture of the

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[1] Abbreviations: AFB_1, aflatoxin B_1; AFQ_1, aflatoxin Q_1; GSH, reduced L-glutathione.

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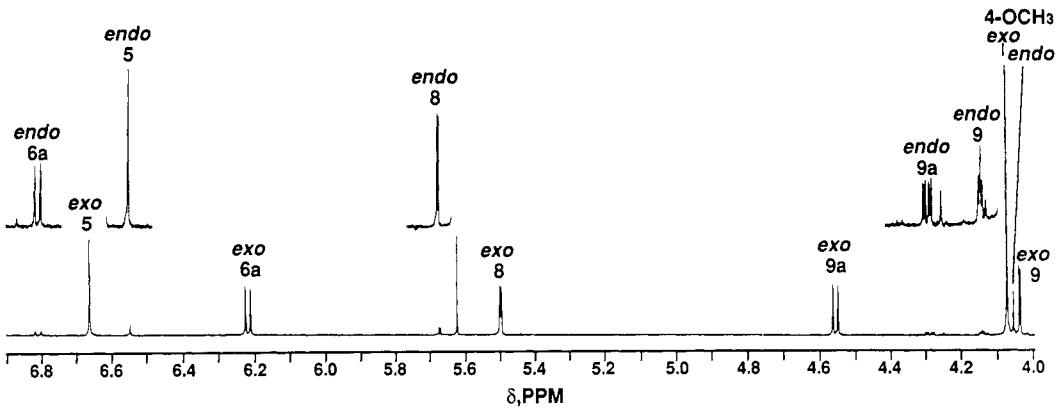
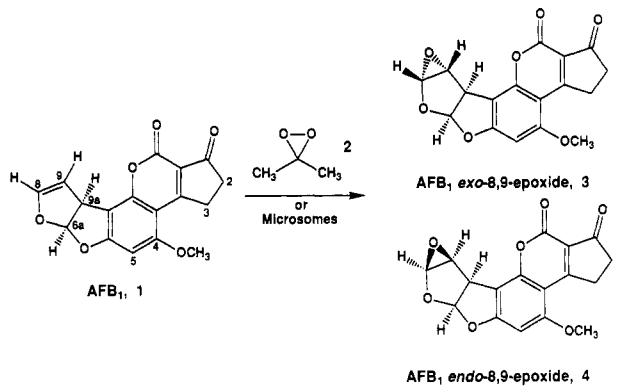


Figure 1. ^{1}H NMR spectrum of a ~10:1 mixture of AFB_1 *exo*- and *endo*-epoxides arising from epoxidation of AFB_1 with dimethyldioxirane.

Scheme I. Formation of AFB_1 *exo*- and *endo*-Epoxides



epoxides **3** and **4** (Scheme I). No other products are observed if the reaction is carried out under anhydrous conditions. The downfield region of the ^{1}H NMR spectrum of this mixture of epoxides is shown in Figure 1 with the resonances of the endo isomer enlarged for clarity. The *exo*-epoxide can be crystallized from the mixture using CH_2Cl_2/acetone (1:1 v/v). Final traces of *endo*-epoxide could be removed from the crystals by washing with dry acetone. ^{1}H NMR analysis of the crystallization supernatant showed a 2:1 mixture of *exo*- and *endo*-epoxides. Procedures are presently being developed for complete purification of the *endo*-epoxide to facilitate studies of its chemistry and biology.

^{1}H NMR Studies. The doublet at δ 6.81 (Figure 1) was assigned to H-6a on the basis of chemical shift and by comparison with the corresponding signal in the *exo*-epoxide (*6*). Irradiation of the δ 6.81 signal led to collapse of the doublet of doublets at δ 4.29 to a doublet permitting assignment of the latter signal as H-9a. The doublet at δ 5.67 (J = 1.7 Hz) was assigned at H-8; H-8 of the *exo*-epoxide also appears as a doublet and has a similar coupling constant (δ 5.50, J = 1.8 Hz). Irradiation at δ 5.67 led to collapse of the incompletely resolved doublet of doublets at δ 4.14 into a doublet establishing the peak at δ 4.14 as arising from H-9. The vicinal relationship of H-9 and H-9a was confirmed by irradiating H-9 (δ 4.14) and observing collapse of the H-9a doublet of doublets (δ 4.29) to a doublet. Other assignments of the *endo*-epoxide were made by comparison with the *exo*-epoxide isomer (Table I). The conclusion that the major isomer is the *exo*-epoxide and the minor one the endo is consistent with the greater steric hindrance of the endo face of the 8,9 double bond. A key difference in the spectra of the two stereoisomers is the coupling between H-9 and H-9a. The trans protons in the exo isomer have a torsional angle near 90° and are not coupled to each other, whereas the angle between the cis protons in the endo isomer is small, leading to a coupling constant of 2.6 Hz.

Table I. ^{1}H NMR Assignments for AFB_1 *exo*- and *endo*-Epoxides[a]

proton	AFB_1 *exo*-epoxide, δ	AFB_1 *endo*-epoxide, δ
2,2′	2.53, 2 H, m	2.53 (overlapped with exo 2,2′)
3,3′	3.44, 2 H, m	3.44 (overlapped with exo 3,3′)
4-OCH_3	4.07, 3 H, s	4.05, 3 H, s
5	6.66, 1 H, s	6.54, 1 H, s
6a	6.22, 1 H, d	6.81, 1 H, d
8	5.50, 1 H, d	5.67, 1 H, d
9	4.03, 1 H, d	4.14, 1 H, dd[b]
9a	4.55, 1 H, d	4.29, 1 H, dd
coupling constants	AFB_1 *exo*-epoxide, Hz	AFB_1 *endo*-epoxide, Hz
$J_{8,9}$	1.8	1.7
$J_{9,9a}$	~0	2.6
$J_{9a,6a}$	5.8	6.6

[a] Spectra were obtained in CD_2Cl_2. [b] Incompletely resolved.

Microsomal Incubations. It is noteworthy that metabolic activation by hepatic microsomes also yields a mixture of the *exo*- and *endo*-epoxides. Although the epoxides cannot be detected directly in the microsomal oxidation, the GSH adducts can be trapped by carrying out oxidations in the presence of GSH transferases. The GSH conjugates were identified by comparison with authentic samples prepared by reaction of the epoxide mixture with the anion of GST.

The *exo*-epoxide is efficiently trapped as the GSH conjugates by mouse α class GSH *S*-transferase enzymes (*11*) and can therefore be analyzed by HPLC as the GSH conjugate (*5*, *12*). On the other hand, constitutive rat GSH *S*-transferase enzymes conjugate the *exo*-epoxide inefficiently. We find the reverse to be true for the *endo*-epoxide; it is readily trapped as its GSH conjugate by rat GSH *S*-tranferase enzymes but not by mouse; thus with the rat transferase the enzymatic formation of AFB_1 *endo*-epoxide can be assayed by monitoring formation of the GSH adduct.

AFB_1 was incubated with human or rat liver microsomes using mouse cytosol or rat cytosol to trap *exo*- and *endo*-epoxides, respectively. Estimates of rates of production of AFB_1 *endo*-epoxide by three samples of human microsomes and two of rat are presented in Table II. HPLC chromatograms resulting from incubation of human liver microsomes in the presence of mouse and rat cytosol are shown in panels A and B, respectively, of Figure 2. With the human microsomes, the GSH conjugates of both epoxides are observed. The previously described adduct of the *exo*-epoxide is the predominant form produced (*5*). With rat microsomes only low levels of the *endo*-epoxide are trapped as the GSH adduct. We are presently inves-

Table II. Production of AFB_1 *exo*- and *endo*-Epoxides by Rat and Human Liver Microsomes

microsomal sample	pmol of AFB_1 epoxide–GSH conjugate formed min^{-1} (mg of protein)$^{-1}$	
	exo-epoxide–GSH[a]	*endo*-epoxide–GSH[b]
human 107	34	12.3
human 110	41	4.6
human 115	40	2.0
rat (untreated)	32	0.9
rat (phenobarbital-induced)	68	0.8

[a] 8,9-Dihydro-8-(*S*-glutathionyl)-9-hydroxy-AFB_1 arising from AFB_1 *exo*-epoxide (*5*). [b] Stereoisomer of 8,9-dihydro-8-(*S*-glutathionyl)-9-hydroxy-AFB_1 arising from AFB_1 *endo*-epoxide.

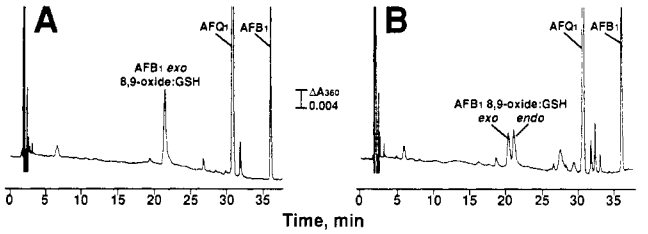


Figure 2. HPLC chromatograms resulting from incubation of AFB_1 with human microsomes in the presence of GSH and GSH *S*-transferase enzymes. All labeled peaks were identified by cochromatography with standards. (A) Human liver 107 microsomes with mouse liver cytosol as the source of GSH *S*-transferases (50-μL injection). (B) Human liver 107 microsomes with rat liver cytosol as the source of GSH *S*-transferases (80-μL injection).

tigating the abilities of individual P-450s and other enzymes to form these stereoisomeric epoxides and the abilities of various GSH *S*-transferase enzymes to conjugate them.

Incomplete stereospecificity of epoxidation of AFB_1 by dimethyldioxirane and by microsomes is certainly not unique. We find that *m*-chloroperbenzoic acid also gives stereoisomeric mixtures.[2]

Conclusions

Although the *endo*-epoxide appears to be only a minor product in chemical and enzymatic epoxidations of AFB_1, its genotoxicity and other biological activity may have disproportionate significance. Our earlier studies with epoxides of AFB_1 and related species have shown that the ratio of mutations to adducts produced is relatively constant (*10*); however, there is substantial precedent with the polycyclic aromatic hydrocarbons for a dramatic influence of epoxide stereochemistry on the types of adducts formed and on the subsequent biological activity (*13*, *14*).

Preliminary evidence suggests that the *endo*-epoxide is less susceptible than the exo to hydrolytic conversion to 8,9-dihydrodiol. Consequently, the *endo*-epoxide may have a longer lifetime in biological milieu and increased opportunity to react with critical targets. We have not yet examined the reactivity of the *endo*-epoxide with DNA, but the formation and biological disposition of adduct(s) of the *endo*-epoxide may be different.

[2] R. Iyer and T. M. Harris, unpublished observations.

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