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Preparation of Aflatoxin B_1 8,9-Epoxide Using *m*-Chloroperbenzoic Acid

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Aflatoxin B_1 is a potent carcinogen which requires activation in order to react with DNA. The *exo*-8,9-epoxide is putatively the active form although it has thus far eluded direct detection in biological systems. This laboratory has reported a synthesis of the epoxide using dimethyldioxirane as the oxidant [(1988) *J. Am. Chem. Soc.* **110**, 7929-7931]. A new, very convenient synthesis is described herein in which *m*-chloroperbenzoic acid is used as the oxidant in a two-phase system. The reaction is carried out in CH_2Cl_2 in contact with an aqueous phosphate buffer (pH 7.2) to remove the byproduct *m*-chlorobenzoic acid, which would otherwise react with the epoxide. Excess *m*-chloroperbenzoic acid is removed with aqueous sodium thiosulfate.

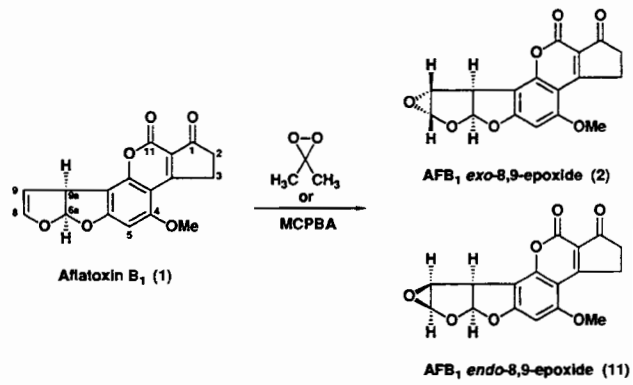
Introduction

The fungi *Aspergillus flavus* and *Aspergillus parasiticus* frequently infest peanuts, corn, and other agricultural commodities to produce aflatoxin B_1 (AFB_1, **1**)[1] and related substances (*1*). AFB_1 is a potent carcinogen and, after metabolic activation to an electrophilic species, reacts with DNA at the N7 position of guanine (*2*). The carcinogenic electrophile is putatively epoxide **2** (Scheme I). The epoxide has never been detected in biological systems, but its role as a reactive intermediate has been inferred from the structures of adducts with DNA and other bioreceptors (*3-7*).

For many years the epoxide also eluded synthesis (*8-11*). In the absence of a viable synthetic procedure, in situ methods were developed for generation of **2**, the most generally useful one, that of Martin and Garner, being a two-phase reaction (*3*). In their procedure, *m*-chloroperbenzoic acid (MCPBA) is added to a CH_2Cl_2 solution of AFB_1 which is in contact with an aqueous solution of DNA. Although epoxide has never been detected in the process, significant quantities of the DNA adduct are formed.

Early attempts to prepare **2** were frustrated by the fact that epoxidizing agents which were then available invariably destroyed the epoxide, but in 1988 we reported a synthesis of **2** using dimethyldioxirane as the oxidant (*12*). The success of this procedure is due to the epoxide being completely stable to the oxidant and to the byproduct acetone. Epoxidation with dimethyldioxirane is efficient and rapid; the pure *exo*-epoxide can be isolated in crystalline form (*12, 13*). The epoxide is a stable compound but very reactive. In aqueous solution it has a half-life substantially less than 10 s, but reaction with aqueous solutions of duplexed DNA affords high yields of the guanine N7 adduct. A shortcoming of the epoxidation procedure is that dimethyldioxirane is unstable; i.e., it must be prepared as a dilute solution in acetone and has limited storage life unless stored at low temperature. We now wish to report a simpler epoxidation procedure, one based on the two-phase in situ epoxidation procedure of Martin and Garner.

Scheme I



Materials and Methods

Caution. *AFB_1 is an extremely potent mutagen and carcinogen, the biological activity of which is ascribed to epoxide* **2**. *Prudent laboratory practices should be exercised to avoid exposure of personnel to AFB_1 and* **2**. *Hydroxy ester* **3** *and any other potentially electrophilic derivatives of AFB_1 should be handled with equal caution.*

Materials. AFB_1, MCPBA, and *m*-chlorobenzoic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). Anhydrous sodium sulfate and sodium thiosulfate were purchased from Fisher Scientific (Fairlawn, NJ). Dimethyldioxirane was synthesized as described by Murray and Jeyaraman (*14*) and Adam et al. (*15*). 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-300 instrument.

Epoxidation of AFB_1 in a Two-Phase System. MCPBA (50-60%, 20 mg, 64 μmol, 4 equiv) was dissolved in CH_2Cl_2 (1.5 mL) and purified in situ by washing with phosphate buffer (pH 7.2) (0.1 M, 4 × 2 mL). Fresh buffer (2 mL) was added to the CH_2Cl_2 solution. A solution of AFB_1 (5 mg, 16 μmol) in CH_2Cl_2 (500 μL) was then added, and reaction was allowed to proceed at room temperature for 100 min with vigorous stirring. The aqueous layer was pipetted off. The organic layer was diluted with CH_2Cl_2 (500 μL) and washed with 0.5 M sodium thiosulfate solution (3 × 2 mL). The CH_2Cl_2 solution was diluted (1-2 mL of CH_2Cl_2), dried briefly over anhydrous sodium sulfate, and

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[1] Abbreviations: AFB_1, aflatoxin B_1; MCPBA, *m*-chloroperbenzoic acid; NOE, nuclear Overhauser effect; FAB, fast atom bombardment.

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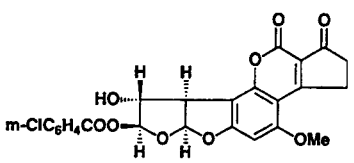
filtered through a plug of sodium sulfate. Solvent was removed by evaporation in a stream of nitrogen to afford a 3.6:1 mixture of *exo*-epoxide **2** and *endo*-epoxide **11** in a combined yield of >70%. Small amounts of unreacted AFB_1 (8%) and *trans*-hydroxy ester **3** (6%) are seen in the ^1H NMR spectrum of crude product. A larger amount (15%) of hydroxy ester **3** was formed when 5 equiv of MCPBA and longer reaction times (120 min) were used to force the epoxidation reaction to completion. The *exo*-epoxide can be crystallized from product mixtures using CH_2Cl_2/acetone (1:1 v/v) (*13*).

Stability of Epoxide 2 in Two-Phase Systems. The hydrolysis of epoxide **2** in CD_2Cl_2 in contact with 0.1 M sodium phosphate buffer (pD 7.4) in D_2O (1:1 v/v) at room temperature was monitored by ^1H NMR. Under these conditions, 9–16% of the epoxide hydrolyzed within 8 h and 22–33% hydrolyzed over 22 h, whereas the hydrolysis reaction would have been complete in less than 1 min in aqueous solution.

Results and Discussion

Among the earlier attempts to prepare the epoxide, aflatoxin B_1 had been treated with MCPBA, but only hydroxy esters were detected; these are derived from attack of *m*-chlorobenzoic acid on the epoxide (*8*, *9*). During our search for procedures for preparation of aflatoxin epoxide, an NMR study of the epoxidation of AFB_1 by MCPBA was carried out to see whether the epoxide could be detected as a transient intermediate. AFB_1 was treated with 3 equiv of MCPBA in CD_2Cl_2, and the spectrum of the mixture was monitored by NMR. At ambient temperature the epoxidation reaction was complete within 140 min as judged by disappearance of AFB_1 vinylic resonances. During the initial phase of the reaction, a small buildup of epoxide **2** (<5%) was seen, as indicated by the characteristic doublets at δ 6.13 and 4.50 corresponding to H-6a and H-9a. These signals rapidly disappeared as the epoxide reacted with the *m*-chlorobenzoic acid byproduct. After 20 min, no resonances corresponding to **2** could be seen. Even when the *m*-chloroperbenzoic acid was carefully freed of *m*-chlorobenzoic acid, it was not possible to stop the reaction at the epoxide stage. By the end of the reaction period the NMR spectrum of the solution indicated that three products had been formed. However, only one of them, *trans*-hydroxy ester **3**, could be isolated by chromatography; the others apparently decomposed.

The structure of **3** was established by ^1H NMR spectra, including decoupling and NOE difference experiments, and by the FAB mass spectrum. Confirmation of the



3

structure of **3** was obtained by independent synthesis involving reaction of *m*-chlorobenzoic acid with epoxide **2** in CD_2Cl_2. It is noteworthy that the NMR spectrum of the crude reaction mixture showed the three products seen in the reaction of MCPBA with AFB_1. Hydroxy ester **3** obtained from **2** was spectroscopically and chromatographically identical to that obtained from AFB_1. Coles et al. have reported that treatment of structurally similar 3a,8a-dihydrofuro[2,3-*b*]benzofuran with MCPBA leads to three isomeric hydroxy esters, resulting from trans and cis opening of the epoxide and intramolecular transfer of the acyl group between positions 8 and 9 of the cis adduct (*9*).

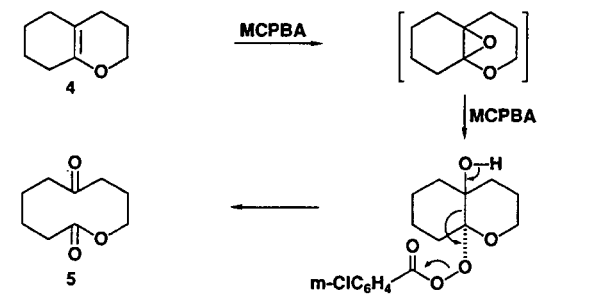
During the course of our studies using the two-phase procedure for preparation of aflatoxin adducts on DNA, we discovered that disappearance of AFB_1 is much more rapid than appearance of the DNA adduct, pointing to the presence of a long-lived intermediate. One such possibility is hydroxy ester **3**; Coles et al. have shown that this and other esters of AFB_1 8,9-dihydrodiol will react with DNA to give the guanine adduct (*5*). However, NMR monitoring of an experiment in which aflatoxin B_1 was added to a solution of MCPBA in CD_2Cl_2 in contact with a phosphate buffer (pD 7.2) in D_2O revealed that the organic phase contained substantial quantities of the epoxide and only traces of the hydroxy ester. The epoxidation was essentially complete in less than 2 h. In spite of the high reactivity of the epoxide and water being in contact with the CH_2Cl_2 solution, most of the epoxide still remained even after allowing the mixture to stand for 16 h. In an independent check of the remarkable stability of the epoxide under these conditions, a CD_2Cl_2 solution of purified epoxide was equilibrated with buffer (pD 7.2) at ambient temperature. After 8 h, ~12% of the epoxide had hydrolyzed; after 22 h, ~27%. The stability of epoxide under these conditions is undoubtedly due to the low solubility of water in the organic phase and of the epoxide in the aqueous phase. The success of the two-phase epoxidation stems in part from the fact that the *m*-chlorobenzoic acid is extracted into the aqueous phase before it has an opportunity to react with the epoxide; MCPBA is only weakly acidic and remains in the organic phase.

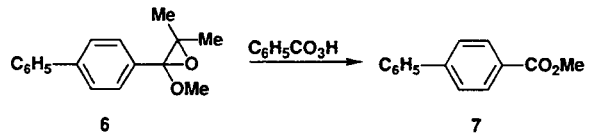
The two-phase reaction would potentially be a useful procedure for preparation of epoxide, but a method is needed for removal of excess MCPBA from the organic phase. MCPBA contamination will confound mutagenesis experiments since the peroxy acid has been shown to react with DNA (*16*). Furthermore, we find that MCPBA slowly reacts with the epoxide. Mixtures of **2** and MCPBA on standing form hydroxy ester **3** and other products. Even when the MCPBA was carefully freed of *m*-chlorobenzoic acid contamination by extraction with aqueous sodium phosphate buffer (pH 7.5), **3** was formed as a major product.

It is possible that spontaneous decomposition of MCPBA forms *m*-chlorobenzoic acid which reacts with epoxide to form hydroxy ester **3**, but it seems likely that some other mechanism is operative. Unusual side reactions have been reported previously in the epoxidation of enol ethers. The oxidation of substituted and unsubstituted tetrahydrochromans **4** with excess peroxy acid gives 6-ketononanolides **5** (*17*). Formation of **5** involves reaction of the initially formed epoxy ethers with excess peroxy acid to give hydroxy peroxy esters, which then fragment to 6-ketononanolides (Scheme II). It has been reported that reaction of excess peroxybenzoic acid with Δ^{17}- and Δ^{20}-steroidal enol ethers yields ketosteroids and esters by cleavage of the intermediate epoxide (*18*). Similarly, reaction of epoxide **6** with peroxybenzoic acid forms ester **7** as shown in Scheme II (*19*).

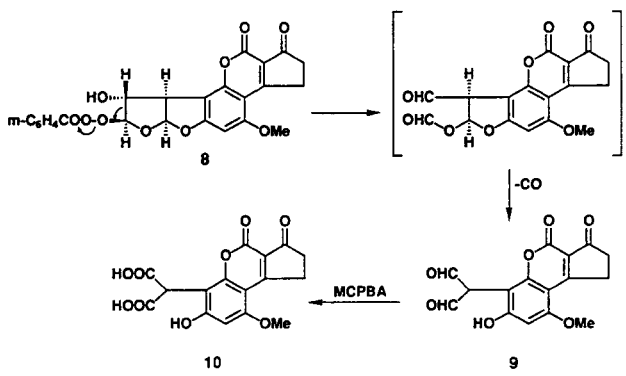
Formation of *trans*-hydroxy ester **3** in the reaction of epoxide **2** with MCPBA can be understood in the light of these results. Initial formation of *trans*-hydroxy peroxy ester **8** is followed by fragmentation of the C8–C9 bond to form bis-aldehyde **9** which can oxidize to malonate **10**

Scheme II





Scheme III



(Scheme III). The process generates 3 equiv of *m*-chlorobenzoic acid which can react with the remaining epoxide **2** to give hydroxy ester **3**. The overall process may require acid catalysis. No detectable reaction occurs when epoxide **2** is treated with MCPBA in a two-phase system. However, the epoxide decomposes if the CH_2Cl_2 solution of **2** and MCPBA is separated from the aqueous phase. Consequently, the preparation of stable solutions of the epoxide requires removal of excess MCPBA.

It is important that excess reducing agent and its oxidation product remain in the aqueous phase. Various reducing agents were examined. The method of choice involved aqueous sodium thiosulfate. One important advantage of thiosulfate is that, if attack on the epoxide occurs, the adduct will be water soluble.

The oxidation of AFB_1 has been found to give a mixture of the *exo*- and *endo*-epoxides **2** and **11**. Epoxidation with dimethyldioxirane also gives a stereoisomeric mixture of epoxides (*13*). The MCPBA procedure yields a larger fraction of the *endo*-epoxide than was observed with dimethyldioxirane (~3.6:1 of exo and endo with MCPBA as compared with ~9:1 with dimethyldioxirane), in keeping with its higher reactivity and decreased steric bulk of MCPBA. It is noteworthy that microsomes also give a mixture of the two epoxides (*13*). At this point it appears that the *endo*-epoxide does not react with DNA and is not a mutagen; however, it does undergo enzyme-mediated conjugation with glutathione. The epoxide obtained from the two-phase procedure is not as pure as that obtained with dimethyldioxirane; small amounts of AFB_1 may still be present, and some hydroxy ester inevitably arises from reaction of the epoxide with MCPBA or *m*-chlorobenzoic acid. The overall epoxidation yield is approximately 70%; when correction is made for the fraction that has the endo configuration, the yield of the exo form is still in excess of 50%. The dimethyldioxirane procedure remains the method of choice for preparation of exo-epoxide in crystalline form (fewer impurities need to be removed during the crystallization process). However, for many purposes the simpler procedure described herein will prove to be adequate for preparation of AFB_1 epoxide.

Our observation that epoxide **2**, when dissolved in a hydrophobic solvent, is stable for long periods in contact with water indicates that it could have a substantial half-life in vivo if sequestered in lipid membranes or other relatively anhydrous environments. The stability of **2** in two-phase systems must be taken into account in detoxification procedures. Although **2** undergoes rapid hydrolysis when dissolved in water, a water-miscible solvent needs to be added to solutions of **2** in CH_2Cl_2, $CHCl_3$, or other water-immiscible organic solvents to be assured of rapid hydrolysis of the epoxide. It is noteworthy that Categnaro et al. (*20*) report unexpected stability of AFB_1 dichloride (which arises during treatment of AFB_1 with NaOCl); they recommend addition of a water-miscible organic solvent to facilitate hydrolysis of the dichloride.

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