Articles

Aflatoxin M_1 8,9-Epoxide: Preparation and Mutagenic Activity

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Treatment of aflatoxin M_1 (AFM_1) with dimethyldioxirane in an anhydrous mixture of CH_2Cl_2 and acetone afforded the corresponding aflatoxin M_1 8,9-epoxide (AFM_1-E) in practically quantitative yield. This highly reactive intermediate was identified by ^1H NMR and characterized by its neat conversion into the corresponding *trans*-methoxyhydrin derivative **1**. The analysis of the ^1H NMR spectrum of the above epoxide revealed that one stereoisomer, which should be that with the *exo* configuration, was present as major component. The mutagenicities of AFM_1-E, the parent mycotoxin (AFM_1), aflatoxin B_1 (AFB_1), and its epoxide (AFB_1-E) were assessed by using a sensitive improved Ames test with the *Salmonella typhimurium* strain TA-100. AFM_1 and AFB_1 had specific mutagenic activities (SMA) of 13 and 121 revertants/ng, respectively, with S9 metabolic activation. AFM_1-E was mutagenic with and without metabolic activation showing SMA of 13 and 12 revertants/ng, respectively. AFB_1-E had a SMA of 42 and 29 revertants/ng, with and without S9 metabolic enzymes, respectively. These results suggest that the epoxidation of AFM_1 can constitute a major route accounting for the cytotoxic effects elicited by this mycotoxin and that AFM_1-E is not as active as AFB_1-E in reacting with the constituents of the mutagenicity assay.

Introduction

Aflatoxin M_1 (AFM_1)[1] is a hydroxylated metabolite of aflatoxin B_1 (AFB_1), and it constitutes a widely occurring contaminant of milk and dairy products. Although AFM_1 does not seem to reach the high cytotoxicity levels exhibited by AFB_1, it is a mutagenic substance (*1, 2*) and carcinogenic effects due to its administration have been reported (*3–5*). However, important questions concerning the cytotoxicity of AFM_1 remain unsolved because of the low availability of this mycotoxin for study (*6, 7*). Thus, although data obtained from mutagenicity assays indicate that bioactivation of AFM_1 is a necessary step for eliciting its cytotoxic activity, the nature of the bioactivated species responsible for this activity has not been heretofore identified. Nevertheless, in analogy with the case of AFB_1, it can be anticipated that epoxidation of the double bond present between C-8 and C-9, which would lead to a highly reactive intermediate, could be a major pathway accounting for the cytotoxic effects induced by AFM_1.

In the case of AFB_1, the above hypothesis found a strong support when the 8,9-epoxy derivative of this mycotoxin (AFB_1-E) could be synthesized by the group of T. M. Harris (*8*). Use of dimethyldioxirane (DMD) as oxidation reagent led to the isolation of this highly reactive epoxide in pure form, which enabled the study of its reactivity (*9, 10*) and cytotoxic activity on different biological matrices (*11–13*). The results obtained from these studies converged into considering AFB_1-E as a very potent mutagen and the main intermediate responsible for the cytotoxic effects elicited by the parent mycotoxin. Concerning the stereochemical features of AFB_1-E, it was demonstrated that the *exo* stereoisomer is the major product resulting from the epoxidation of AFB_1, either by chemical or by biological means (*8*). However, more recently Raney et al. reported the isolation of the AFB_1-E stereoisomer with *endo* configuration (*14*). This compound is formed in a 1:10 molecular ratio in respect to the *exo* isomer, and it appears that it is considerably less cytotoxic than the latter epoxide (*15*).

With these antecedents, the present contribution reports the preparation and identification of the 8,9-epoxy derivative of AFM_1 (AFM_1-E). The identification of this compound was carried out by spectral (NMR) means and by its conversion to the corresponding methoxyhydrin. In addition, the mutagenic activity of AFM_1-E was investigated using the tester strain TA-100 of *Salmonella typhimurium*.

Experimental Procedures

Caution: *Solid aflatoxins are hazardous due to their electrostatic nature and should be handled using appropriate containment procedures and respiratory mask to prevent inhalation. Aflatoxin epoxides are potent mutagenic agents. Therefore, the use of gloves, well ventilated fume cupboards, and careful destruction of residues with NaOCl is recommended for the manipulation of these compounds.*

The ^1H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded with a Varian Unity 300 spectrometer. Spectra were

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[1] Abbreviations: AFM_1, aflatoxin M_1; AFB_1, aflatoxin B_1; AFB_1-E, aflatoxin B_1 8,9-epoxide; AFM_1-E, aflatoxin M_1 8,9-epoxide; DMD, dimethyldioxirane. SMA, specific mutagenic activity.

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taken in the solvents indicated for each case, which were previously neutralized. Chemical shifts are given in ppm related to tetramethylsilane for ^1H and deuteriochloroform for ^{13}C as internal standards. The HPLC analyses were performed with a modular system formed by two Waters 510 pumps, a Merck column (LiChrospher 100 RP-18, 5 μm, 12.5 × 0.4 cm), and an Applied Biosystems detector-gradient controller (1000S diode array detector). Mass spectra and high resolution mass spectra (HRMS) were obtained with a VG Autospec Q instrument. Solutions of dimethyldioxirane were prepared as described by Adam et al. (*16*) and thoroughly dried over molecular sieves (3 Å). AFB_1 was from Aldrich Chemical Co. (Milwaukee, WI), and its 8,9-epoxide was prepared with dimethyldioxirane as previously described (*13*).

Isolation of AFM_1. The isolation and purification of this aflatoxin was carried out from the organic extracts obtained from *Aspergillus flavus* NRRL 3251 cultures following the procedure of Hsieh et al. (*7*). Briefly, the $CHCl_3$ extract obtained from cultures of the above strain on rice (1 kg, 8 days incubation at 25 °C) was filtered through anhydrous Na_2SO_4. The filtrate was subjected to two successive chromatographic separations on silica gel (first on an open column and then on a medium pressure system), to obtain a fraction which contained a mixture of AFM_1 and AFM_2 in a 8:1 molecular ratio (HPLC monitoring). Separation of this mixture by reversed-phase medium pressure liquid chromatography led to the collection of eluates containing AFM_1 free from AFM_2. Final purification of these eluates by flash chromatography on silica gel afforded AFM_1 as a white solid with a purity over 95% (HPLC monitoring: 30:70 CH_3CN/H_2O at 1 mL/min). A yield of 6–8 mg of AFM_1 was obtained by this procedure. The identification of this mycotoxin was carried out by comparing its analytical data with those of an authentic standard and its spectroscopic features with those reported in the literature (*7*). ^1H NMR (5:1 $CDCl_3/CD_3SOCD_3$): δ 6.55 (d, 1 H, J = 3 Hz, H-8), 6.50 (s, 1 H, H-6a), 6.37 (s, 1 H, H-5), 5.69 (d, 1 H, J = 3 Hz, H-9), 3.98 (s, 3 H, 4-OCH_3), 3.46–3.40 (2 H, 2 H-3), 3.20 (br, OH), 2.63–2.56 (2 H-2 masked by solvent signal). MS: 328 (M^+, 100), 299 (59), 271 (40), 149 (41). HRMS for $C_{17}H_{12}O_7$: calcd, 328.05830; found, 328.05597.

Epoxidation of AFM_1. A solution of AFM_1 (1.5 mg, 5 μmol) in anhydrous CH_2Cl_2 (5 mL), maintained at 20 °C under argon atmosphere, was treated with a 10-fold molecular excess of dry DMD (50 mM in acetone), and the mixture was stirred under the above conditions until reaction was complete (2 h, HPLC monitoring). The crude reaction mixture was diluted with hexane (3 mL) and cooled at 0 °C, and solvents and excess of reagent were eliminated under vacuum to give a white solid in high conversion yield and purity (^1H NMR, Figure 1), which was identified as the expected AFM_1 8,9-epoxide (AFM_1-E). ^1H NMR (CD_2Cl_2): δ 6.44 (s, 1 H, H-5), 5.70 (d, 1 H, J = 1 Hz, H-6a), 5.42 (dd, 1 H, J_1 = 1.5 Hz, J_2 = 1 Hz, H-8), 4.19 (d, 1 H, J = 1.5 Hz, H-9), 3.97 (s, 3 H, 4-OCH_3), 3.43–3.38 (2 H, 2 H-3), 3.29 (s, 1 H, OH), 2.62–2.58 (2 H, 2 H-2).

Methanolysis of AFM_1-E. A solution of epoxide AFM_1-E obtained as described above was treated with MeOH (1 mL), and the mixture was stirred for 30 min at 20 °C. The elimination of solvents and excess of reagent under vacuum afforded a white solid in quantitative yield and high purity (^1H NMR, Figure 2, HPLC), which was characterized as the *trans*-methoxyhydrin **1**. ^1H NMR ($CDCl_3$): δ 6.33 (s, 1 H, H-5), 6.13 (s, 1 H, H-6a), 5.16 (s, 1 H, H-8), 4.49 (s, 1 H, H-9), 3.97 (s, 3 H, 4-OCH_3) 3.43–3.39 (2 H, 2 H-3) 3.21 (s, 3 H, 8-OCH_3), 2.66–2.62 (2 H, 2 H-2), 1.70 (br, OH); ^{13}C NMR ($CDCl_3$): δ 201.7 (C-1), 177.5 (C-3a), 167.6 (C-5a), 163.1 (C-4), 155.3 (C-11), 153.6 (C-9c), 117.6 (C-6a), 117.1 (C-11a), 112.1 (C-8), 107.2 (C-9b), 104.1 (C-3b), 90.8 (C-5), 85.8 (C-9a), 76.3 (C-9), 56.5 (OCH_3), 55.3 (OCH_3), 35.0 (C-2), 29.7 (C-3); MS: 376 (M^+, 30), 303 (100), 273 (29). HRMS for $C_{18}H_{16}O_9$: calcd, 376.07943; found, 376.07801.

Microsuspension Mutagenicity Assay. A microsuspension procedure previously reported was used (*17–19*). This assay, which is a modification of the *Salmonella*/microsome test (*20*), is approximately 10 times more sensitive than the standard Ames *Salmonella* procedure based on absolute amounts of material added per determination. Tester strain TA-100 was kindly provided by Dr. B. N. Ames (Berkeley, CA). Bacteria were grown overnight in Oxoid Nutrient Broth No. 2 (Oxoid Ltd., Hants, England) to approximately (1–2) × 10^9 cells/mL and harvested by centrifugation (5 000*g*, 4 °C, 10 min). Cells were resuspended in ice-cold phosphate-buffered saline (PBS, 0.15 M, pH 7.4) to a concentration of approximately 1 × 10^{10} cell/mL (determined spectrophotometrically at 550 nm). The S9 and S9 mix were prepared according to the procedure of Ames et al. (*20*). The S9 from livers of Aroclor 1254 pretreated male Sprague-Dawley rats containing 52.4 mg of protein/mL, as determined using the modified biuret method of Ohnishi and Bar (*21*), was used throughout. For the microsuspension assay, the following were added, in order, to 12 × 75 mm sterile glass culture tubes on ice: 0.1 mL of S9 mix, 0.005 mL of aflatoxin solution in DMSO, and 0.1 mL of concentrated bacteria in PBS (1 × 10^{10} cells/mL of PBS). The aflatoxin solutions were maintained in ice and tested immediately after synthesis. The mixture was incubated in the dark at 37 °C with rapid shaking. After 90 min the tubes were placed in an ice bath and taken out one at a time, immediately before adding 2 mL of molten agar (*21*) containing 90 mmol of histidine and biotin. The combined solutions were vortex-mixed and poured onto minimal glucose plates. Plates were incubated at 37 °C in the dark for 48 h. Strain markers were routinely determined for each experiment. The spontaneous number of revertants were based on plates which had only DMSO added. All procedures were carried out in a room fitted with yellow fluorescent lights (GE F40Go) to minimize potential photo-oxidation.

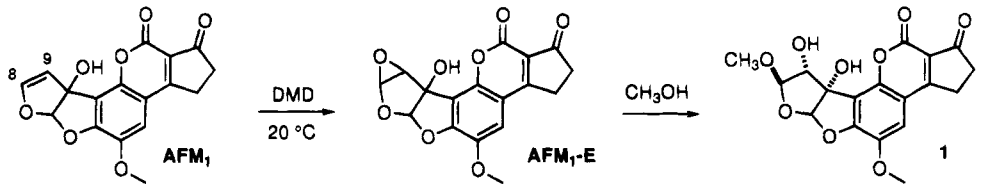
Results and Discussion

Preparation of AFM_1-E. The advent of dioxiranes, and particularly of DMD, has constituted a valuable tool for the preparation and isolation of highly reactive epoxides, as was shown for the case of AFB_1-E (*8*). The ease of manipulation, the possibility of working under neutral conditions in aprotic solvents, and simple workup of the crude reaction mixtures (*22*), also made DMD the reagent of choice for assaying the epoxidation of AFM_1. In this context, AFM_1 presents some differences in comparison with AFB_1. It is rather unavailable in the amounts needed for performing chemical reactions, and its solubility in organic solvents is considerably lower in comparison with that of AFB_1. In our case, the first drawback was partially circumvented through the production and further isolation of AFM_1 from cultures of *Aspergillus flavus* NRRL 3251. By this procedure 6–8 mg of pure AFM_1 (according to HPLC and spectroscopic criteria) were obtained from 1 kg of rice incubates after four different chromatographic processes.

Concerning the solubility problems, it was necessary to work at 1 mM concentration in CH_2Cl_2 to obtain a homogeneous solution of this mycotoxin for the epoxidation reaction. Under these conditions, treatment of AFM_1 with an excess of DMD led to the formation of the expected epoxide AFM_1-E (Scheme 1). This epoxidation reaction was slower compared to that of AFB_1, which could be mainly due to the different concentration at which the oxidations took place. In addition, special care was taken to ensure the absence of water in the reaction mixture, owing to the sensitivity of AFM_1-E toward hydrolytic conditions. Thus, precipitation in hexane permitted the isolation of the epoxy derivative in pure form, whereas the procedures assayed to recover this compound from the direct evaporation of solvents and excess of reagent from the crude reaction mixture led to extended decomposition of the epoxide.

The identification of AFM_1-E was carried out by ^1H NMR. Figure 1 shows the 300 MHz NMR spectra

Scheme 1. Formation of AFM_1-8,9-epoxide and Reaction with Methanol



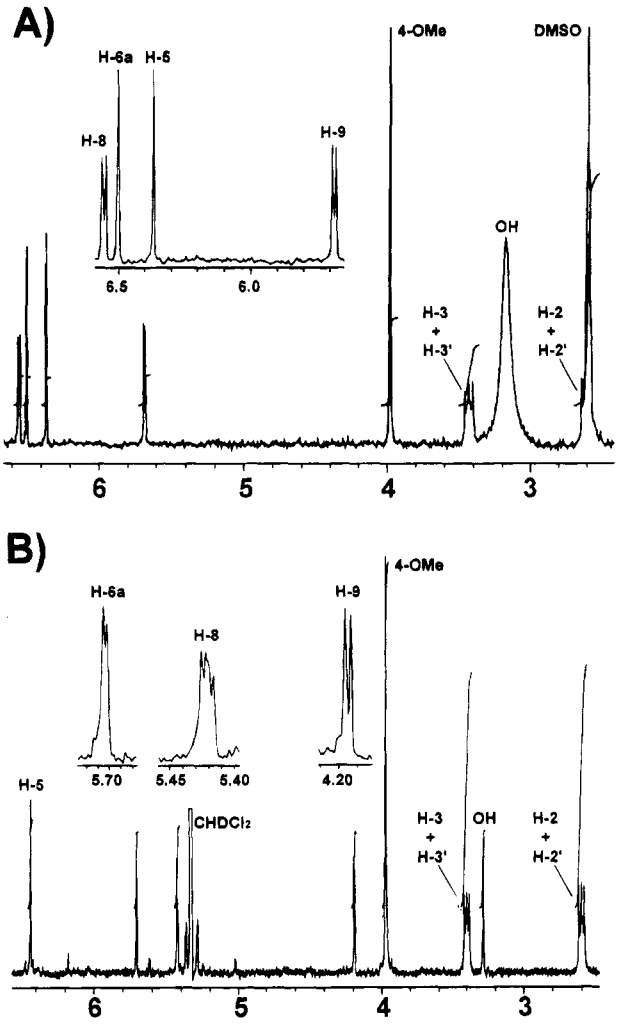


Figure 1. ^1H NMR spectra (300 MHz) of (A) AFM_1 ($CDCl_3$/CD_3SOCD_3) and (B) AFM_1 8,9-epoxide (AFM_1-E) (CD_2Cl_2).

registered for AFM_1 and AFM_1-E. As depicted, the pair of doublets corresponding to H-8 and H-9 in AFM_1 (at 6.55 and 5.69 ppm, respectively) are missing in the spectrum of the epoxy derivative. Instead, a doublet of doublets centered at 5.42 ppm and a doublet at 4.19 ppm are present in this spectrum, which could be assigned to H-8 and H-9, respectively. As expected, H-8 showed an additional coupling with H-6a, which supported its identification (*13*). In addition, the acetal proton H-6a, which appeared as a singlet at 6.55 ppm in the parent mycotoxin, was split into a doublet centered at 5.70 ppm in the epoxide. All these features are consistent with the formation of an oxirane ring at the expected position for AFM_1-E. In this sense, the shifts observed for H-8 and H-9 are similar to those described by Baertschi et al. for the case of AFB_1-E (*8*). Unfortunately, the low amount of AFM_1-E obtained did not permit the acquisition of an interpretable ^{13}C NMR spectrum.

With respect to the stereochemical features of AFM_1-E, the above data indicate that one stereoisomer, which

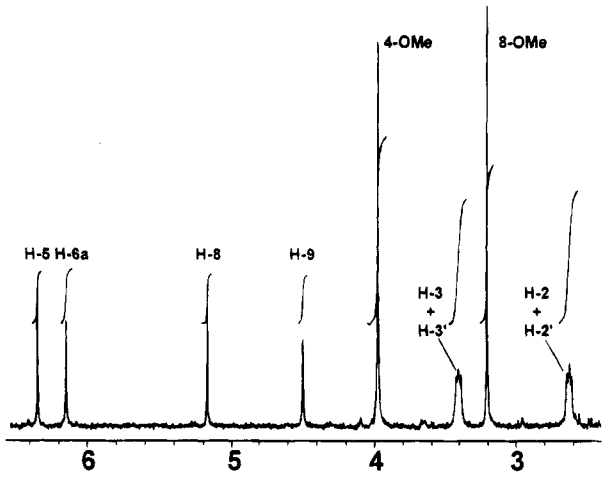


Figure 2. ^1H NMR spectrum (300 MHz, $CDCl_3$) of the *trans*-methoxyhydrin **1** derived from the reaction of AFM_1-E with methanol.

should be that with the epoxide ring in the *exo* configuration, was formed almost exclusively. In fact, a closer inspection of the ^1H NMR spectrum of this sample revealed the presence of three minor absorptions centered at 6.17, 5.62, and 5.02 ppm in a 1:1:1 ratio, which could be attributed to H-6a, H-8, and H-9 of the putative *endo* stereoisomer (cf. ref 14). Nevertheless, even assuming these peaks were due to this stereoisomer, which could not be confirmed because of the low amount of sample available, the *endo*/*exo* ratio obtained (approximately 1:9) would be not higher than that reported for the case of AFB_1-E (*14, 15*). These results suggest that under the conditions of kinetic control derived from the epoxidation with DMD, the *exo* stereoisomer of AFM_1 is formed with high preference.

An additional confirmation of the structure of AFM_1-E was obtained by the characterization of the methoxyhydrin **1** isolated from the treatment of the crude reaction mixture containing this epoxide with methanol (Scheme 1). Thus, the mass spectral analysis of **1** supported the methoxyhydrin formation. The low resolution spectrum exhibited the molecular ion (m/z 376) and the base peak at m/z 303. Mass spectral studies carried out with isotopically labeled models related to AFB_1 and AFM_1 structures lacking the D and E rings (results not shown) indicate that the loss of 73 amu, which gives rise to the observed base peak, involves a $C_3H_5O_2$ fragment comprising C-6a, O-7, and C-8 linked to a methoxy group. In addition, the empirical formula of **1** was confirmed by exact mass measurement. On the other hand, the ^1H NMR spectrum of this compound (Figure 2) showed two methoxy groups (3.97 and 3.21 ppm) and three singlets corresponding to H-6a, H-8, and H-9 at 6.13, 5.16, and 4.49 ppm, respectively. The absence of multiplicity between H-8 and H-9 supports that the OCH_3 at C-8 and the OH at C-9 should be in a *trans* relative configuration. Finally, the ^{13}C NMR spectrum showed two peaks attributed to methoxy groups (56.5 and 55.3 ppm) and

Table 1. Mutagenicity of Aflatoxin B_1 (AFB_1), Aflatoxin B_1 8,9-Epoxide (AFB_1-E), Aflatoxin M_1 (AFM_1), and Aflatoxin M_1 8,9-Epoxide (AFM_1-E) toward *S. typhimurium* TA-100 Strain, Expressed as Revertants per Plate, in the Absence (−S9) or Presence (+S9) of Metabolic Activation[a]

compd	concn (ng/plage)	TA-100 (rev/plate) −S9	TA-100 (rev/plate) +S9	compd	concn (ng/plate)	TA-100 (rev/plate) −S9	TA-100 (rev/plate) +S9
AFB_1	0	87 ± 3	107 ± 13	AFM_1	0	87 ± 3	107 ± 13
	2.5	87 ± 5	436 ± 31		62.5	104 ± 5	1450 ± 16
	5	92 ± 10	835 ± 24		125	110 ± 19	1703 ± 64
	10	100 ± 14	1285 ± 12		250	127 ± 2	1703 ± 21
	20	105 ± 2	1508 ± 46		500	162 ± 13	1568 ± 25
	SMA[b]	0.9 ± 0.2	121 ± 11		SMA	0.1 ± 0.05	13 ± 5
AFB_1-E	0	87 ± 3	107 ± 13	AFM_1-E	0	87 ± 3	107 ± 13
	2.5	248 ± 10	221 ± 27		62.5	1240 ± 14	1369 ± 34
	5	330 ± 16	411 ± 36		125	1500 ± 8	1688 ± 14
	10	376 ± 31	500 ± 24		250	1736 ± 31	1840 ± 57
	20	540 ± 22	960 ± 59		500	1679 ± 17	1638 ± 19
	SMA	29 ± 9	42 ± 4		SMA	12 ± 4	13 ± 4

[a] Values represent means of duplicate plates at the indicated dose ± SD. For experimental details, see Experimental Procedures. [b] The specific mutagenic activity (SMA), expressed as rev/ng, has been estimated from the slope of the linear zone of the dose−response curve.

peaks corresponding to C-6a, C-8, C-9, and C-9a at 117.6, 112.1, 76.3, and 85.8 ppm, respectively. All these assignments are consistent with the structure for methoxyhydrin **1**.

Mutagenicity Assays. The mutagenic activities of AFM_1 and AFM_1-E in tester strain TA-100 are summarized in Table 1. For comparison, the mutagenic activities of AFB_1 and its corresponding 8,9-epoxy derivative AFB_1-E, are also presented. Tester strain TA-100 was chosen because it has been described as the most sensitive toward AFM_1 (*4*), and the microsuspension assay was used to enhance the sensitivity for the detection of the mutagenic activities. As shown in Table 1, AFM_1-E is a potent mutagen in the absence of S9 metabolic enzymes (−S9). An approximate SMA was calculated for each compound based on the slope of the linear portion of each dose−response curve. For AFM_1-E the SMA was 12 ± 4 revertants/ng. This value is very close to the SMA of AFM_1 with metabolic activation (+S9), which is approximately 13 ± 5 revertants/ng. The mutagenic activity of AFB_1 with metabolic activation is 121 ± 11 revertants/ng. This value is approximately 10 times higher than the activity of AFM_1. In contrast to the similar activities observed for AFM_1-E and for AFM_1 with metabolic activation, AFB_1-E has a lower activity [29 ± 9 revertants/ng (−S9)] than AFB_1 with metabolic activation. However, the activity of AFB_1-E increased with the addition of metabolic enzymes (42 ± 4 revertants/ng). It is important to remark that this increase cannot be attributed to the presence of unreacted AFB_1 as a contaminant of the epoxide sample, since the epoxidation took place quantitatively (HPLC monitoring).

The fact that no mutagenicity was obtained from tests performed with crude reaction mixtures containing AFM_1-E which had been treated with water [SMA of 0.1 (−S9) and 0.0 (+S9) revertants/ng] or methanol [SMA of 0.9 (−S9) and 0.7 (+S9) revertants/ng] supports the hypothesis that the mutagenic effects shown in Table 1 were due to the presence of the epoxy intermediate. In fact, the HPLC controls carried out in the epoxidation assays showed that AFM_1-E did not survive in the presence of water/methanol mixtures, which confirms that the above treatments led to the complete conversion of the epoxide into the corresponding diol or methoxyhydrin derivatives. As expected from the data found in the literature for related compounds, the formation of these derivatives results in an important drop of the mutagenic effects elicited in the *S. typhimurium* assay (*23*).

Several preliminary conclusions follow the results summarized in Table 1. First, although AFM_1-E is a potent direct mutagen for TA-100, it is less active than AFB_1-E. This relationship is consistent with the difference in mutagenicities reported for the parent mycotoxins (*1*). The higher mutagenicity observed for AFB_1-E with S9 (42 revertants/ng) compared to the activity observed without S9 (29 revertants/ng) may be explained assuming that the constituents in S9 may stabilize the highly reactive metabolite, AFB_1-E. Another possibility is that, due to the high reactivity of AFB_1-E, a dose−response relationship between the compound and its mutagenicity is difficult to determine. Since AFB_1 with S9 had a much higher mutagenic activity (121 revertants/ng) compared to the direct activity of AFB_1-E, which is thought to be the putative reactive metabolite for AFB_1, the lower activity obtained for the exogenous AFB_1-E could be due to its high reactivity with constituents in the assay. The reactivity between AFM_1-E and the assay constituents seems much less significant. On the other hand, the similar mutagenic activities of AFM_1 (with S9) and AFM_1-E (with and without S9) suggest that the epoxidation of the enol ether moiety should be the major route accounting for the mutagenic activity of AFM_1 and that AFM_1-E is considerably more stable than AFB_1-E.

In summary, AFM_1-E has been prepared and identified for the first time. The chemical epoxidation of AFM_1 leads to the preferential formation of the isomer with the *exo* configuration, although the presence of the *endo* isomer in minor amounts could not be discarded. AFM_1-E has shown to be a potent direct mutagen on *S. typhimurium* TA-100 strain, and metabolic activation does not modify the elicited cytotoxicity. Finally, the availability of this epoxide would facilitate its putative detection in biological matrices and will permit direct investigation of its reactivity and metabolism in order to clarify the role that this intermediate plays in the carcinogenicity of AFM_1.

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