http://informahealthcare.com/txr
ISSN: 1556-9543 (print), 1556-9551 (electronic)

Toxin Reviews

Toxin Rev, Early Online: 1–7
 DOI: 10.3109/15569543.2014.994132

informa
healthcare

REVIEW ARTICLE

Synthesis and purification of the aflatoxin B_1-lysine adduct

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Abstract

This work reports the chemical synthesis of aflatoxin B_1 (AFB_1)-lysine based on procedures available in the literature, but using lysine without a protection group in the α-amine group. AFB_1-exo-8,9-epoxide was obtained by epoxidation of AFB_1 with chloroperoxybenzoic acid in dichloromethane and phosphate buffer. Purification and identification of the AFB_1-lysine were conducted by liquid chromatography (LC), and its structure was confirmed by LC with mass spectrometer and diode-array detection. The preparation of AFB_1-lysine using lysine without a protection group in the α-amine group was completed in 24 h, being a practical modification of available methods that can be reproduced in analytical laboratories.

Keywords

AFB_1-lysine, aflatoxins, biomarkers, synthesis

History

Received 16 September 2014
Revised 17 November 2014
Accepted 28 November 2014
Published online 26 December 2014

Introduction

Aflatoxin B_1 (AFB_1)-lysine is a metabolite produced by the liver of animals and humans that have consumed foodstuffs contaminated with AFB_1, a hepatocarcinogen produced by fungus of the genus Aspergillus, mainly *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Magan & Olsen, 2004). AFB_1 is a common contaminant of agricultural commodities such as maize and oil seeds and is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC, 1993).

After oral ingestion, AFB_1 is absorbed and primary biotransformed by enzymes of the cytochrome P450 family, which generate aflatoxins M_1, Q_1, B_{2a}, P_1 and aflatoxicol (Hsieh & Atkinson, 1991). However, the enzymes also convert AFB_1 into its carcinogenic form, AFB_1-8,9-epoxide, which bonds covalently to DNA at guanine residues and serum albumin at lysine residues, producing AFB_1-N^7-guanine and AFB_1-lysine adducts, respectively (Garner & Martin, 1979; Guengerich & Johnson, 1999; Martin & Garner, 1977; Wild et al., 1986). AFB_1-lysine is a digest product of AFB_1-albumin, thus the concentration of AFB_1-lysine in serum (often expressed as AFB_1-lysine/mg albumin) indicates that an individual has been exposed to AFB_1 for a period of 2–3 months (Wild et al., 1992). Therefore, the measurement of AFB_1-lysine in the human or animal serum is a valuable biomarker of long-term exposure to AFB_1 through the diet, as demonstrated in several epidemiological studies on the toxic effects of aflatoxins in human populations (Gan et al., 1988; Sabbioni, 1990; Shuaib et al., 2010). It is estimated that 1.4 and 2.3% of AFB_1 ingested is covalently bound to albumin (Gan et al., 1988).

The determination of serum AFB_1-lysine has been used as a biomarker to indicate the involvement of aflatoxins as a causative agent for human diseases, especially hepatocellular carcinoma (HCC), and the main outputs of those studies were reviewed by Jager et al. (2011). Higher AFB_1-lysine (or AFB_1-albumin) levels were found in serum samples from China (Gan et al., 1988), Guinea (Sylla et al., 1999), Gambia (Turner et al., 2000), Benim (Gong et al., 2002) and Ghana (Tang et al., 2008), which concentrations (up to 1064 pg/mg albumin) presented significant correlations with the exposure to AFB_1 through the diet in those countries. Surveys conducted in the United States (Johnson et al., 2010) and Brazil (Scussel et al., 2004) showed percentages of positive samples of 20.6% and 62%, respectively, with levels ranging from 1.01 to 16.57 pg/mg albumin and 0 to 57.3 pg/mg albumin, respectively, which are lower than the values reported in African or Asian countries. Moreover, the measurement of AFB_1-lysine in serum samples was used to evaluate the effectiveness of intervention methods for reducing the dietary intake of aflatoxin. In a recent study conducted in China, Chen et al. (2013) observed that agricultural practices implemented in China in the 1980s dramatically reduced the aflatoxin contamination of foods thereafter, thus resulting in a remarkable decrease in the human exposure to aflatoxin. This is consistent with the lack of correlation between the levels of AFB_1-lysine in serum and the incidence of HCC as observed by Xu et al. (2010) and Wu & Santella (2012).

The measurement of serum AFB_1-lysine adduct may also be useful to assess the exposure to dietary aflatoxin among

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different population groups. In Malaysia, Leong et al. (2012) found 97% of human serum samples with detectable levels of AFB_1-lysine, which concentrations in the age group of 31–50 years were 3.08 times higher than the 18–30 years age group. In another more recent study conducted in Malaysia, the AFB_1-lysine levels in adults were higher in men than in women, and a significant positive correlation was observed between the levels of AFB_1-lysine and total bilirubin and creatinine, hence indicating that exposure to AFB_1 can affect the liver and kidney functions (Mohd Redzwan et al., 2014).

Studies involving the exposure of pregnant women and the fetus to AFB_1 have been conducted by using serum AFB_1-lysine as a biomarker. Groopman et al. (2014) determined AFB1-lysine in the serum of women from rural areas of South Asia, during the first and third trimester of pregnancy, and their children at birth and at two years of age. The authors found 94% of the serum samples of pregnant women with detectable levels of AFB_1-lysine, as well as in samples from children at the birth and at two years of age, hence indicating a long exposure period of children to aflatoxins, since the early stages of life. In Ghana, Shuaib et al. (2010) found levels of 0.44–268.73 pg/mg albumin in the serum of pregnant women and observed that participants with higher levels of AFB1-lysine were more likely to have babies with lower birth weight (Shuaib et al., 2010).

The routine analysis of AFB_1-lysine adduct requires useful quantities of reference standards, which are not commercially available (Jager et al., 2011). Consequently, several studies have been developed for the preparation of this compound by using different starting materials such as AFB_1-dibromide (Sabbioni et al., 1987), AFB_1-8,9-exo-epoxide (Sujatha et al., 2001) and AFB_1-dialdehyde (Scholl & Groopman, 2004). Sass et al. (2013) reviewed the available procedures for chemical synthesis of AFB_1-adducts and observed that the reaction for the formation of AFB_1-lysine always occurs between derivatives of AFB_1 with the ε-amine group of lysine. The AFB_1-lysine was first chemically synthesized by Sabbioni et al. (1987), from the intermediate 8,9-dihydro-8,9-dibromo-AFB_1 and N_α-acetyl-L-lysine (Sabbioni, 1990; Sabbioni et al., 1987), and the product formed was de-acetylated to form the AFB_1-lysine. Guengerich et al. (2002) performed the preparation AFB_1-lysine by using the AFB_1-diol/dialdehyde and N_α-acetyl-L-lysine in buffer, being the reaction kept overnight at 37 °C under constant stirring, and the resulting compound de-acetylated by an acylase enzyme. Scholl & Groopman (2004) also prepared the AFB_1-lysine adduct from AFB_1-diol/dialdehyde in phosphate buffer solution (pH: 7.4), but using a blue solution containing the complex $(Lys)_2Cu^{+2}$ recently prepared (Bodanszky & Bodanszky, 1994). According to the authors, the reaction was conducted overnight at 20 °C under constant stirring, and the purification of the final product and release of Cu^{+2} were carried out simultaneously by high-performance liquid chromatography (HPLC).

Therefore, the methods previously described for the preparation of AFB1-lysine used several lysine molecules derivatives, which contained a protecting group in the α-amine group, such as N_α-acetyl-lysine (Sujatha et al., 2001), t-Boc-N_α-lysine and $(lysine)_2Cu^{2+}$ (Scholl & Groopman, 2004), to avoid undesirable reactions with the α-amino group-lysine. However, the use of these derivatives of lysine, besides adding some more steps in the method, may increase the amount of interfering products in the reaction medium. However, no previous studies have been addressed in order to verify the need for a lysine protection to form the AFB_1-lysine adduct with adequate yield and purity. In this article, we describe the chemical synthesis and purification of AFB_1-lysine based on procedures available in the literature, but using lysine without the presence of a protection group in the molecule.

Materials and methods

Chemicals

All reagents were of analytical grade and water was purified by deionization (Milli-Q system, Millipore, Bedford, MA). HPLC-grade acetonitrile and methanol (JT Baker, Xalostoc, Mexico) were used for chromatographic analyses. AFB_1, m-chloroperbenzoic acid (MCPBA), L-lysine and HPLC-grade dichloromethane were purchased from Sigma (St Louis, MO).

Preparation of AFB_1-lysine adduct

The synthesis of the AFB_1-lysine was started by the preparation of AFB_1-8,9-epoxide as summarized in Figure 1. The MCPBA (16 mg, 51 μmols) was dissolved in 1 ml of dichloromethane and washed four times with phosphate buffered saline pH 7.4 (4 × 1 ml). The aqueous phase was removed and 0.8 ml of phosphate buffer (0.1 M, pH 7.6) was added to the organic phase. The mixture was cooled to 0 °C, and 2 mg of AFB_1 (6 μmols) dissolved in 1 ml of dichloromethane was added. The reaction remained under agitation for 6 h at 0 °C. The aqueous phase was pipetted off, and the organic phase was dissolved in 1 ml of dichloromethane and washed with 0.5 M sodium thiosulfate (3 × 1 ml). Dichloromethane was removed by evaporation in a stream of nitrogen (Baertschi et al., 1988; Raney et al., 1992). The reaction mixture was submitted to ^1H NMR analysis (Figure 6) and from the data obtained in the spectrum, it was possible to verify that the product AFB_1-8,9-*exo*-epoxide, AFB_1-8,9-*endo*-epoxide and the unreacted AFB_1 were present in ratio 1:0.2:0.5, respectively.

Due to the instability of AFB_1-epoxide, the reaction mixture containing the AFB_1-8,9-*exo/endo*-epoxide was submitted to reaction with 0.8 ml of phosphate buffer (0.1 M, pH 7.6) to form the AFB_1-dialdehyde in equilibrium with AFB_1-diol, which are more stable than the corresponding AFB_1-epoxide (Scholl & Groopman, 2008). After 20 min of stirring, the solution was washed with dichloromethane (3 × 0.8 ml) to remove the unreacted AFB_1. Afterwards, L-lysine (5 mg) previously dissolved in 0.4 ml phosphate buffer (0.1 M, pH 7.6) was added in the aqueous phase containing the products AFB_1-dialdehyde and AFB_1-diol (approximately 1 mg in total). The reaction remained under stirring for 24 h to form the product AFB_1-lysine. After this period, the reaction mixture was filtered through a 0.45 μm PTFE membrane, and the product was purified by HPLC. The final product was determined by spectrophotometric analysis at pH 7, $\varepsilon_{399} = 25\,400/M\,cm$ (Guengerich et al., 2002; Scholl & Groopman, 2004).

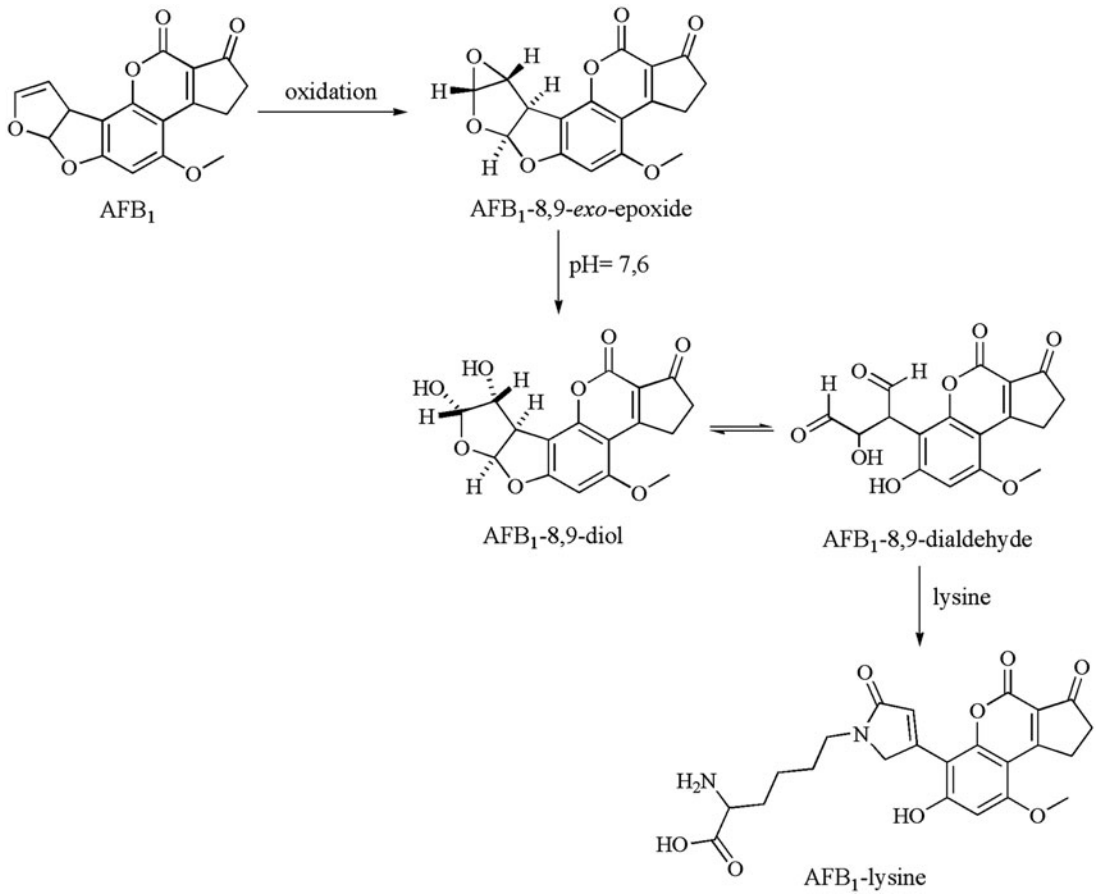

Figure 1. Pathways used for synthesis of AFB_1-lysine from AFB_1-8,9-*exo*-epoxide.

Purification of the reaction mixture containing AFB_1-lysine adduct

Purification of the AFB_1-lysine was conducted in a HPLC system (Shimadzu 10VP, Kyoto, Japan) equipped with a 10A XL fluorescence detector (excitation at 370 nm and emission above 470 nm). The separation was carried out using a reverse phase Kinetex C_{18} column (Phenomenex, Torrance, CA), 4.6×150 mm, 2.6 µm particle size. Samples and column were kept at room temperature. Mobile phases consisted of eluent A containing 1% acetic acid in water:methanol (95:5), eluent B prepared with methanol: 0.1% of acetic acid in water (95:5) and eluent C composed of 100% acetonitrile. Gradient elution started with 10% of eluent B with linear increase to 30% over 75 min followed by isocratic elution until 100 min. Throughout the chromatographic run, the percentage of eluent C (acetonitrile) remained constant at 1%. Flow rate was maintained at 0.9 ml min^{-1}.

Liquid chromatography analysis of purified AFB_1-lysine adduct

Analysis of AFB_1-lysine was conducted in the same HPLC system and chromatography column as previously described above using a 10A UV detector set at 399 nm and a 10A XL fluorescence detector (excitation at 405 nm and emission above 470 nm). Samples and column were kept at room temperature. Mobile phases consisted of eluent A prepared with 20 mM ammonium phosphate (pH 7.4):methanol (95:5) and eluent B prepared with methanol:20 mM ammonium phosphate (pH 7.4) (95:5). Gradient elution started at 5% eluent B for 2 min followed by a linear increase to 60% B over 20 min and kept constant for 15 min. Total chromatographic run time was 35 min, and the mobile phase flow rate was maintained at 1.0 ml min^{-1}.

LC-MS/MS analysis of purified AFB_1-lysine adduct

The characterization of the synthesized standard was performed on an Acquity I-Class ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA) equipped with a BEH C_{18} column (2.1×50 mm, 1.7 µm) and coupled to a Xevo TQ-S mass spectrometer (Waters). The column was kept at 40 °C during analyses. Ten microliters of samples kept at 10 °C were injected. Gradient elution was employed with mobile phase composed of water (eluent A) and acetonitrile (eluent B), both containing 0.06% of formic acid. Starting from 95% eluent A, the percentage of eluent B was linearly raised to 26% over 3.0 min and kept constant for 4 min (7.0 min). Then, eluent B was increased to 90% over 1.0 min, followed by a reduction to 5% over 0.2 min, and the column re-equilibrated to initial conditions for 0.3 min. Total chromatographic run time was 8.5 min, and the mobile phase flow rate was maintained at 0.6 ml min^{-1}.

The mass spectrometer was operated in the scan mode from *m/z* 280–650 and in the product ion scan mode from *m/z* 300–465 for the target ion of *m/z* 457.10. All experiments

were carried out with electrospray ionization in positive ion mode, with a capillary voltage of 0.75 kV, a source temperature of 150 °C and a desolvation temperature of 650 °C. Desolvation gas flow and cone gas flow were maintained at 500 L h^{-1} and 150 L h^{-1}, respectively. Product ion scan was performed with collision energy 37.5 V and cone voltage of 30 kV.

NMR spectrometry analysis of AFB_1-8,9-exo-epoxide

The NMR spectrum of AFB_1-8,9-*exo*-epoxide was accomplished with the reaction mixture containing AFB_1, AFB_1-8,9-*exo*-epoxide and AFB_1-8,9-*endo*-epoxide in acetone-d_6. The solutions were then transferred to 5-mm NMR tubes. NMR data were collected on an Avance DRX500 spectrometer system (Bruker, Billerica, MA) (500.13 MHz for ^{1}H) at 300 K.

Results and discussion

The preparation of AFB_1-lysine from AFB_1-dialdehyde and free lysine as described in the experimental section was performed in a reaction time of 24 h and 25% yield (0.34 mg, 0.74 µmol AFB_1-lysine/3.0 µmol AFB_1-8,9-*exo*-epoxide).

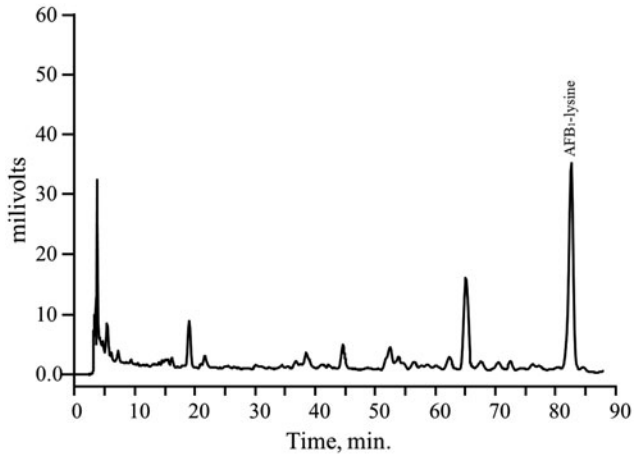

Figure 2. Chromatogram of the reaction mixture containing AFB_1-lysine.

In this reaction, unlike other procedures reported earlier (Sujatha et al., 2001; Scholl & Groopman, 2004), free lysine without the presence of a protective group on the α-amino group of lysine was used. The advantage of this procedure is the elimination of steps for protection/deprotection, although the two free amine groups in the lysine molecule allow the formation of other undesirable products leading to a decrease in the reaction yield. However, the yield obtained in this study (25%) using free lysine was similar to the yield previously reported (30%) by Sujatha et al. (2001) and Scholl & Groopman (2004) using $(lysine)_2Cu^{2+}$. The AFB_1-lysine adduct in the reaction mixture had a retention time of approximately 83 min, as shown in the chromatogram presented in Figure 2.

Purified AFB_1-lysine determined by liquid chromatography with fluorescence and ultra-violet detection presented a retention time of approximately 23 min, as shown in Figures 3(A and B). In both chromatograms, the product showed a retention time equal to that found in literature under the same chromatography conditions (Scholl et al., 2006).

Analysis of purified AFB_1-lysine by UPLC coupled to a mass spectrometry with ion scan was performed for ions with *m/z* ranging from 280 to 650. A molecular ion of *m/z* 457.10 was detected at 1.93 min as presented in Figure 4(A and B). The chromatography run was also simultaneously monitored by an in-line diode-array detector, and the UV-Vis spectrum for the chromatographic peak at 1.93 min is shown in Figure 4(C). The spectrum obtained is similar with that reported by Sabbioni et al. (1987). The product ion scan for the precursor ion *m/z* 457.10 showed a major fragment at *m/z* 394, and the fragment ions with *m/z* 311, 328, 348, 366 and 376 as also reported by Scholl et al. (2006), Scholl & Groopman (2008) and shown in Figure 5. According to Scholl et al. (2006), the ion at *m/z* 328, which is produced by the cleavage of C_6-N bond, distinguishes AFB_1-lysine from C_8-aflatoxin adduct molecules, like AFB_1-N^7-guanine and AFB_1-mercapturate.

The ^{1}H NMR spectrum of AFB_1-8,9-*exo*-epoxide was performed as described at experimental section and the product exhibited the ^{1}H NMR spectrum shown in Figure 6. The signals relating to AFB_1-8,9-*exo*-epoxide were compared

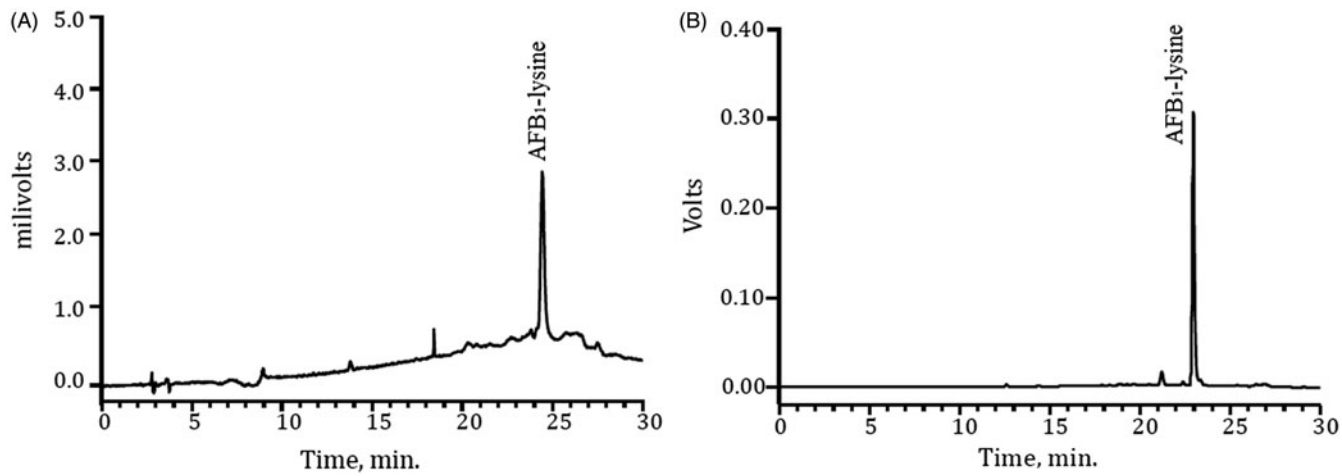

Figure 3. Chromatograms of the purified AFB_1-lysine solution ($t_R = 23$ min): (A) UV detection (399 nm) and (B) fuorescence detector in (405 nm/470 nm).

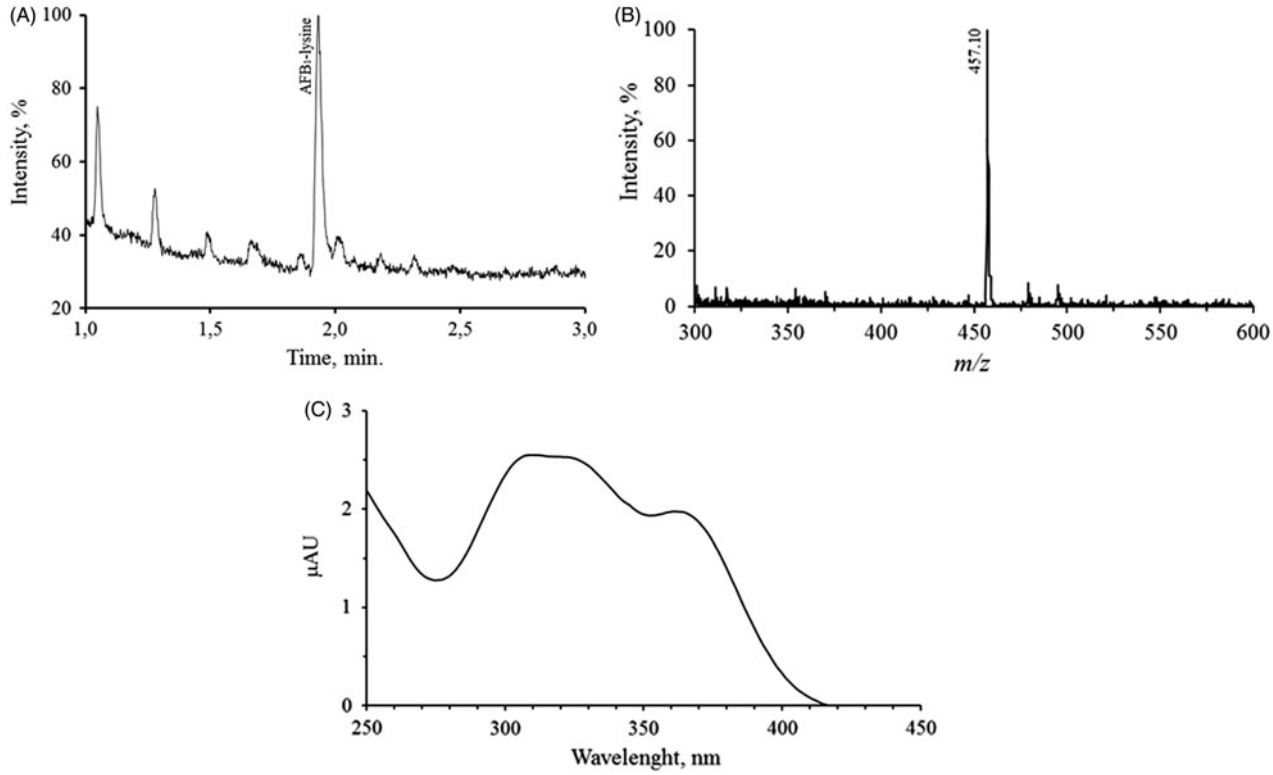

Figure 4. (A) Total ion current chromatogram of purified AFB_1-lysine adduct for a continuous scan from *m/z* 280 to 650; (B) mass spectrum for peak at 1.93 min; and (C) UV-Vis spectrum for peak at 1.93 min.

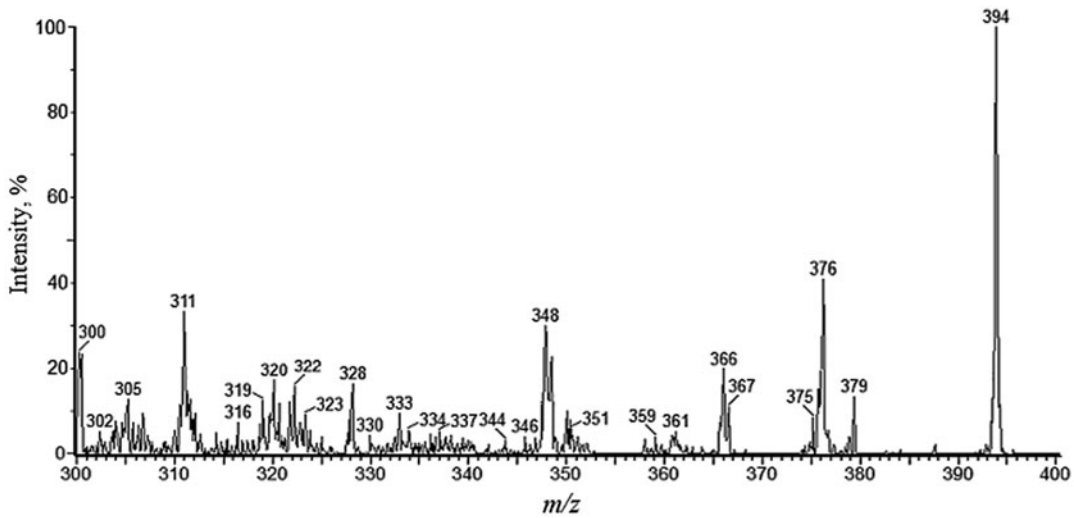

Figure 5. Product ion scan for the parent molecular ion $[(M+H)]^+ = m/z$ 457.1].

with data reported by Raney et al. (1992), for confirmation of this compound in acetone-d_6, as listed in Table 1.

According to data reported in Table 1, the chemical shifts for the product obtained is identical to data reported for AFB_1-8,9-*exo*-epoxide in the literature. It was also possible to measure the coupling constants between the hydrogens and H_{6a} H_{9a}, and between H_9 and H_{9a}, which values were also identical to those described in a previous work (Raney et al., 1992). The main coupling which differentiates AFB_1-8,9-*exo*-epoxide of AFB_1-8,9-*endo*-epoxide is the coupling between the hydrogens H_6 and H_{9a}, because in the case of the *exo* isomer, the trans protons have a torsional angle near 90° and are not coupled to each other (Raney et al., 1992). Coupling between these hydrogens is zero for the product obtained, as listed in Table 1. Moreover, it was possible through the reaction mixture spectrum to obtain the approximate yield of AFB_1-8,9-*exo*-epoxide, from the integration of the signals relating to hydrogen H_{9a}, of the following compounds: *endo* isomer (4.28 ppm), *exo* isomer (4.54 ppm) and unreacted AFB_1 (4.80 ppm); the ratio between them is present in the spectrum of 0.2:1.0:0.5, as shown in Figure 6.

The overall time for preparation and purification of AFB_1-lysine in this study (24 h) was much lower than previously reported methods that used lysine molecules derivatives

Table 1. Comparison of the ^{1}H NMR data of AFB_1-8,9-*exo*-epoxide synthesized with data reported in the literature (Raney et al., 1992).

C	δ_H (ppm) AFB_1-*exo*-epoxide	δ_H (ppm) AFB_1-*exo*-epoxide (literature)[a]
2	2.53	2.51
3	3.44	3.40
4-OCH3	4.07	4.05
5	6.66	6.64
6a	6.22	6.20
8	5.50	5.49
9	4.03	4.03
9a	4.55	4.55
Coupling constants	AFB_1-*exo*-epoxide (Hz)	AFB_1-*exo*-epoxide (Hz)
$J_{9a/6a}$	5.8	5.8
$J_{9/9a}$	0	0

[a]Raney et al. (1992).

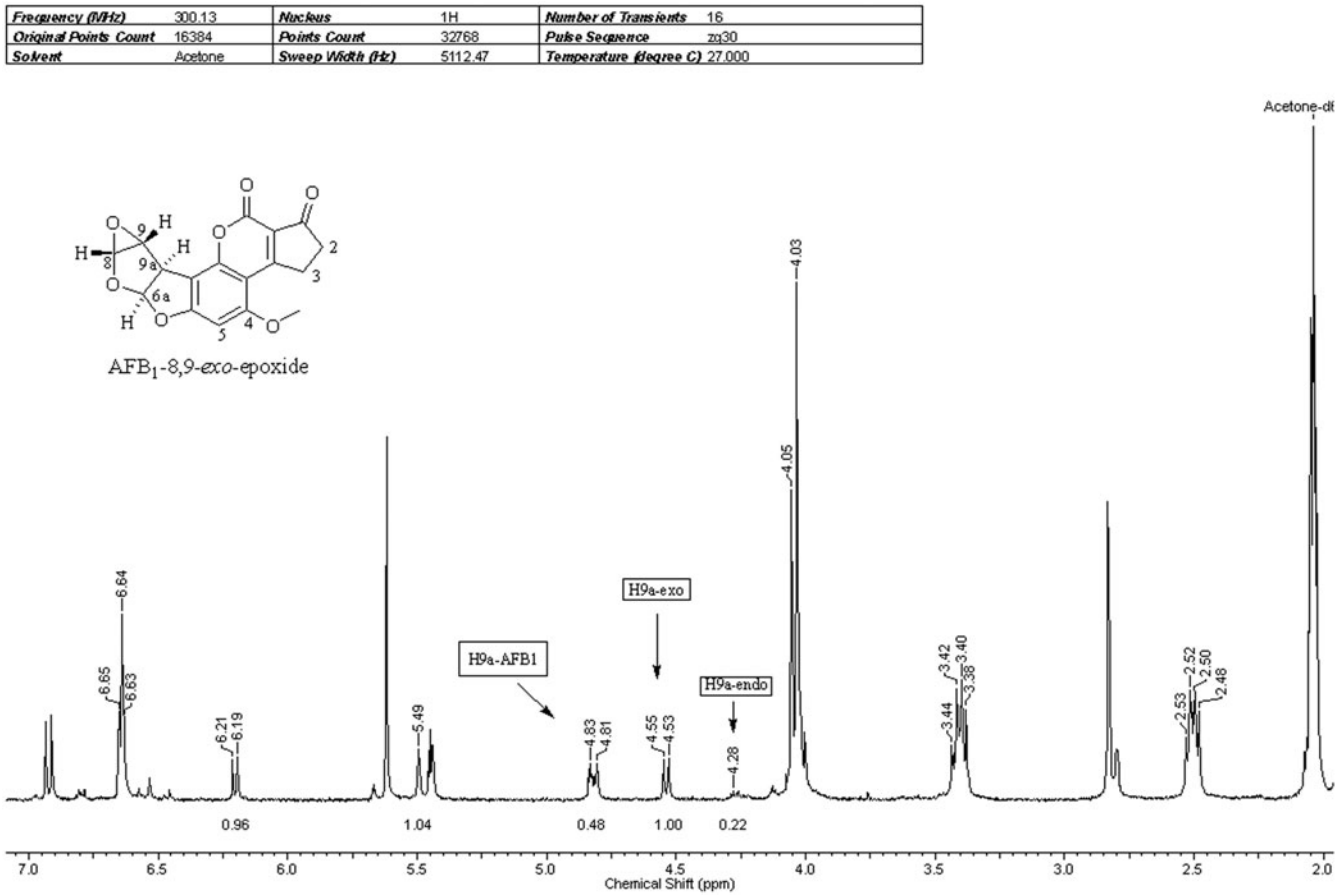

Figure 6. ^{1}H NMR spectrum of reaction mixture containing AFB_1-8,9-*exo*-epoxide, AFB_1-8,9-*endo*-epoxide in acetone-d_6.

containing a protecting group in the α-amine group, such as N_α-acetyl-lysine (Sujatha et al., 2001), t-Boc-N_α-lysine and $(lysine)_2Cu^{2+}$ (Scholl & Groopman, 2004). The procedure described had a yield of 25%, which was similar to previous methods, and had the additional advantage to avoid the formation of interfering substances and wastes containing copper.

Conclusion

The procedures, as described in this present study, require a lower time than methods previously described for preparation and purification of AFB_1-lysine, as lysine was used without the presence of a protection group in the molecule, leading to the formation of less-undesirable reaction products. The preparation and purification of AFB_1-lysine was completed in 24 h with 25% yield, being a simple and practical method that can be reproduced in analytical laboratories.

Declaration of interest

The authors report no conflicts of interest.

The authors thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support and fellowships.

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