JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Label Compd Radiopharm 2004; **47**: 807–815.
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jlcr.867

Note

Synthesis of 5,5,6,6-D4-L-lysine-aflatoxin B_1 for use as a mass spectrometric internal standard

Peter F. Scholl* and John D. Groopman
Department of Environmental Health Sciences, Bloomberg School of Public Health, Johns Hopkins University, 615 N. Wolfe St., Baltimore, MD 21205, USA

Summary

Human exposure to the hepatocarcinogenic mycotoxin aflatoxin B_1 results in modification of serum albumin lysine ε-amino residues to form lysine-aflatoxin adducts. A perdeuterated reference standard is now required to quantitatively measure this adduct in epidemiologic studies of liver cancer using isotopic dilution mass spectrometry. A convenient method for the preparation of D4-L-lysine-AFB_1 using commercially available 5,5,6,6-D4-L-lysine is demonstrated for the first time. The application of two standard α-amino protection methods is also reported that simplifies the production of natural isotopic abundance lysine-AFB_1 over the currently used method employing N_α-acetyl-L-lysine. *t*-Boc-N_α-lysine was used to prepare lysine-AFB_1; however, a preferred method for directing reaction of AFB_1-dialdehyde to the ε-amino group of 5,5,6,6-D4-L-lysine utilized cupric ions that were spontaneously removed during the reverse phase HPLC purification of D4-lysine-AFB_1 using 1% HOAc. This strategy eliminates the need to otherwise synthesize and purify *t*-Boc-N_α- or N_α-acetyl-5,5,6,6-D4-lysine and then TFA or enzymatically deprotect overnight to obtain the target compound. Copyright © 2004 John Wiley & Sons, Ltd.

Key Words: D4-lysine; lysine-aflatoxin adduct; isotope dilution mass spectrometry; α-amino group protection; copper

Introduction

Human exposure to the mycotoxin AFB_1 is implicated in the etiology of liver cancer in Asia and Sub-Saharan Africa by molecular epidemiologic studies.[1]

*Correspondence to: P. F. Scholl, Department of Environmental Health Sciences, Bloomberg School of Public Health, Johns Hopkins University, 615 N. Wolfe St., Baltimore, MD 21205, USA. E-mail: pscholl@jhsph.edu

Contract/grant sponsor: US National Institutes of Health; contract/grant number: P01 ES06052
Contract/grant sponsor: US National Institutes of Health; contract/grant number: P30 ES 03819

Received 14 June 2004
Accepted 29 June 2004

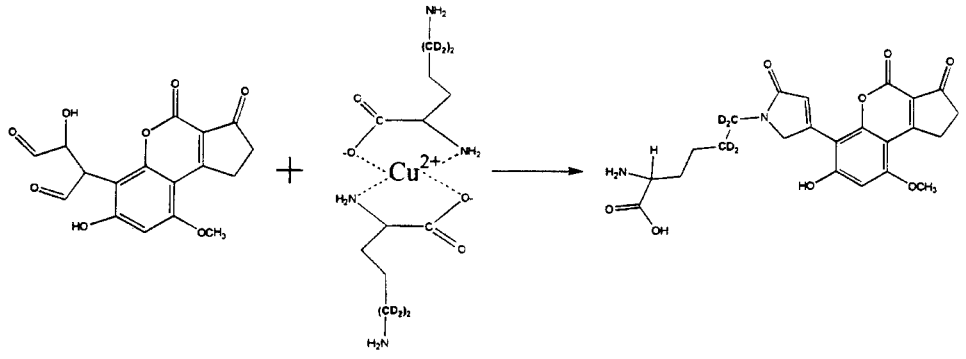
AFB_1 present in contaminated foodstuffs is consumed and subsequently metabolized to *exo*-AFB_1 epoxide that, if not detoxified by enzymatic conjugation with glutathione, reacts with proteins and macromolecules such as albumin and DNA.[2–4] DNA is a critical target as AFB_1-N^7-guanine adducts are mutagenic. Interest in measuring aflatoxin albumin adducts is due to the surrogacy of these adducts as a marker of DNA damage.[5,6] Because the $t_{1/2}$ of AFB_1-albumin adducts in blood is $\sim$20-fold longer than that of urinary metabolites ($\sim$24 h) such as the AFB_1-N^7-guanine adduct or AFB_1-mercapturic acid, albumin adducts have been more often used as a biomarker of AFB_1 exposure and disease risk.[7–13]

The structure of an aflatoxin adduct formed with lysine in albumin was first elucidated via a proof by synthesis using AFB_1 dibromide as the starting material.[14–16] Condensation of the dialdehyde tautomer of 8,9-dihydro-8,9-dihydroxy-AFB_1 with the ε-amino group of N_α-acetyl-lysine to form a Schiff base, followed by an Amadori rearrangement to an α-amino ketone was proposed to lead to the formation of the N_α-acetyl-lysine-AFB_1 adduct over a 24 h period. Overnight enzymatic *N*-deacetylation provided a lysine-AFB_1 congener in 14% yield. More recently, the structure and mechanism of N_α-acetyl-lysine-AFB_1 formation have been clarified.[17,18] Utilizing *exo*-AFB_1-epoxide, Guengerich *et al*. proposed adduct formation proceeds through a carbinolamine, instead of a Schiff base. Although these methods enable the preparation of lysine-AFB_1 standards for use in antibody based and fluorometric assays, N_α-acetyl-D4-lysine is not commercially available for the more direct synthesis of a perdeuterated standard now required to perform isotopic dilution mass spectrometric measurements and quantitatively assess AFB_1 exposure in epidemiologic studies.[19] Moreover, alternative protecting group strategies are available that enable the more efficient preparation of lysine-AFB_1.

As alternatives to N_α-acetylation, we demonstrate the application of two commonly used α-amino protection methods to direct reaction of AFB_1-dialdehyde to the ε-amino group of L-lysine. One method uses *t*-Boc-N_α-lysine and the preferred method (Scheme 1) uses cupric ions that are spontaneously removed during reverse phase HPLC purification of the product, lysine-AFB_1, using 1% HOAc. This technique eliminates the need to synthesize and purify N_α-*t*-Boc- or N_α-acetyl-5,5,6,6-D4-lysine and the subsequent TFA or enzymatic deprotection step otherwise required to obtain the target compound.

Results and discussion

The reaction of Cu^{2+} or *t*-Boc α-amino protected lysine with AFB_1 dialdehyde (Scheme 1) proceeds to completion in 6–7 h with the formation of uncharacterized brown precipitates and the corresponding lysine-AFB_1



Scheme 1. Reaction of AFB_1 dialdehyde is directed to the ε-amino group of lysine via protection of the N_α group in a Cu^{2+} complex that spontaneously dissociates during the reverse phase HPLC isolation of lysine-AFB_1 using 1% HOAc

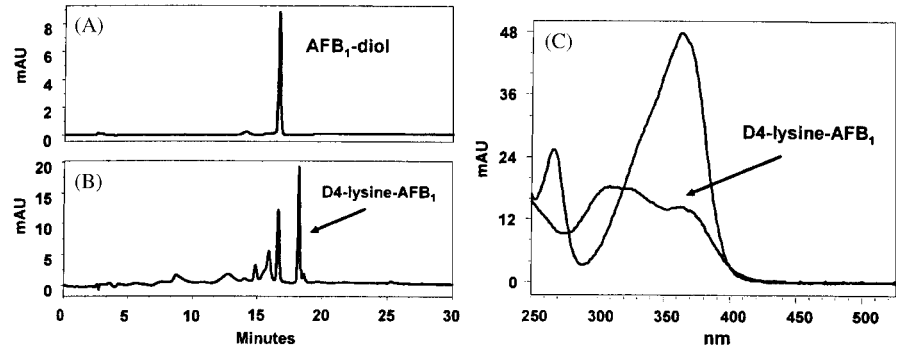


Figure 1. Chromatographic profile (310 nm) of the reaction of AFB_1 dialdehyde with the $(D4\text{-lysine})_2Cu^{2+}$ complex. (A) AFB_1 dialdehyde starting material as AFB_1 diol (17 min). (B) Reaction profile at $t = 30$ min. D4-lysine-AFB_1 $t_r = 18$ min. (C) UV absorbance spectrum of AFB_1 diol and D4-lysine-AFB_1. Chromatographic conditions. Column: Phenomenex, Luna 250 × 4.6 mm, 100 Å, 5 µm, C18(2). Flow rate = 1 ml/min. $T = 35°C$. Mobile phase A = 95% (1% HOAc/H_2O); 5% MeOH. Mobile phase B = 95% MeOH; 5% (0.1% HOAc/H_2O). Linear gradient: 20–80% B over 30 min

adducts in 30% yield. The chromatographic profile and time course of the AFB_1-dialdehyde reaction with $(D4\text{-lysine})_2Cu^{2+}$ are presented in Figures 1 and 2. *t*-Boc-N_α-lysine-AFB_1 reactions exhibit the same reaction time course and a similar, but delayed, chromatographic profile in which *t*-Boc-lysine-AFB_1 elutes at 29 min. The full scan mass spectrum of *t*-Boc-lysine-AFB_1 exhibits the expected parent molecular ion at m/z 557. Additional ions were produced by in-source fragmentation corresponding to loss of the *t*-Boc protective group, $N_\alpha H_3$. CO and water from the *t*-Boc-lysine parent molecular

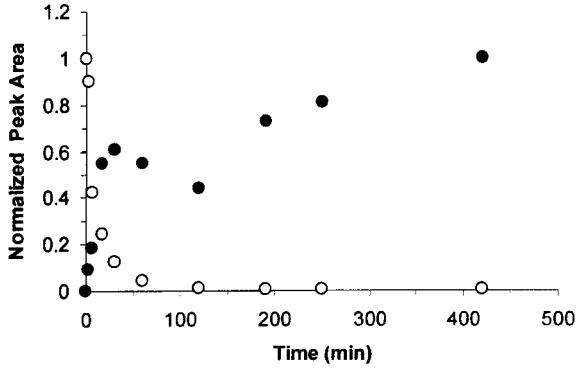


Figure 2. Time course of the reaction of AFB_1 dialdehyde with the $(D4\text{-lysine})_2Cu^{2+}$ complex. AFB_1 diol (○) and D4-lysine-AFB_1 (●) chromatographic peak areas (see Figure 1) were normalized by their individual maxima. A 42% loss of product, relative to the amount at 400 min, was observed in reactions run overnight

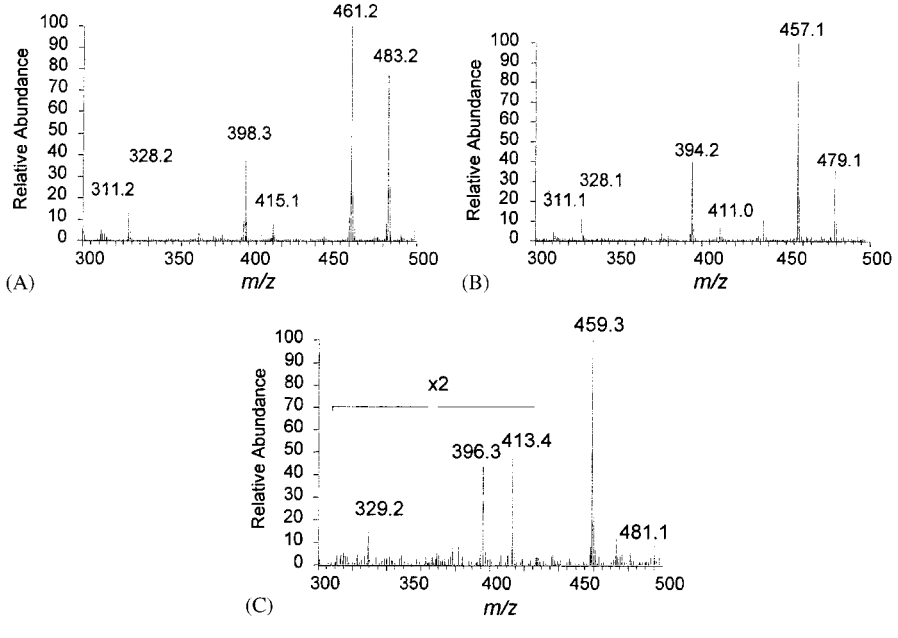


Figure 3. Full scan positive ionization ESI mass spectra of aflatoxin standards. D4-lysine-AFB_1, lysine-AFB_1 and $^{13}C_{\varepsilon}{}^{15}N_{\varepsilon}$-lysine-$AFB_1$ exhibit parent molecular ions $(M+H)^+$ at m/z 461, 457 and 459, respectively, and the corresponding $(M+22)^+$ sodium adducts. In source fragmentation produced by using a relatively high cone voltage (40 V) yields ions corresponding to $(M+H\text{-}H_2O\text{-}CO)^+$ and $(M+H\text{-}H_2O\text{-}CO\text{-}NH_3)^+$. C_{ε}–N_{ε} bond cleavage in lysine-AFB_1 and $^{13}C_{\varepsilon}{}^{15}N_{\varepsilon}$-lysine-$AFB_1$, respectively, yields m/z 328 and 329. (A) D4-lysine-AFB_1. (B) Lysine-AFB_1 (C) $^{13}C_{\varepsilon}{}^{15}N_{\varepsilon}$-lysine-$AFB_1$. Infusion 20 µl/min. Solvent: 50% MeOH/0.1% HOAc. Capillary potential = 40 V

ion. Consistent with the published spectra of lysine-AFB_1 and N_α-acetyl-lysine-AFB_1, the UV absorbance maximum of D4-lysine-AFB_1 and *t*-Boc-lysine-AFB_1 exhibits a bathochromic shift to 399 nm at pH 7 due to formation of aflatoxin phenolate.[15,17]

The full scan and CID mass spectra of D4-lysine-AFB_1 and lysine-AFB_1 are presented in Figures 3 and 4. A representation of the fragmentation pathway of lysine-AFB_1 is presented in Scheme 2. The parent molecular ion of lysine-AFB_1 undergoes dehydration and the loss of $N_\alpha H_3$ and CO. Cleavage of the C_ε–N_ε bond further yields *m/z* 328. Formation of the parent lysine-AFB_1 molecular ion via the thermodynamically unfavorable protonation of the carboxylic acid has been previously observed with other N_ε-alkylated lysine derivatives.[20–22] Fragment ions produced by the loss of water, $N_\alpha H_3$ and CO from lysine-AFB_1 are analogous to those arising from N_α-acetyl-lysine derivates mono- and di-alkylated at N_ε with hexanal.[20–22] By analogy with hexanal N_ε di-alkylated N_α-acetyl-lysine, di-alkylation of the ε-amino position in lysine-AFB_1 is indicated by the fragment ion at *m/z* 328 in Figures 3(A) and 3(B).[20] The CID spectra of the parent molecular ions of D4-lysine-AFB_1 and lysine-AFB_1 in Figure 4 exhibit $(M+H\text{-}H_2O)^+$ supporting the assignments made from the full scan mass spectra. Fragmentation of the parent molecular ion of D4-lysine-AFB_1 (*m/z* 461) to 415 yielded *m/z* 328 in MS^3 studies.

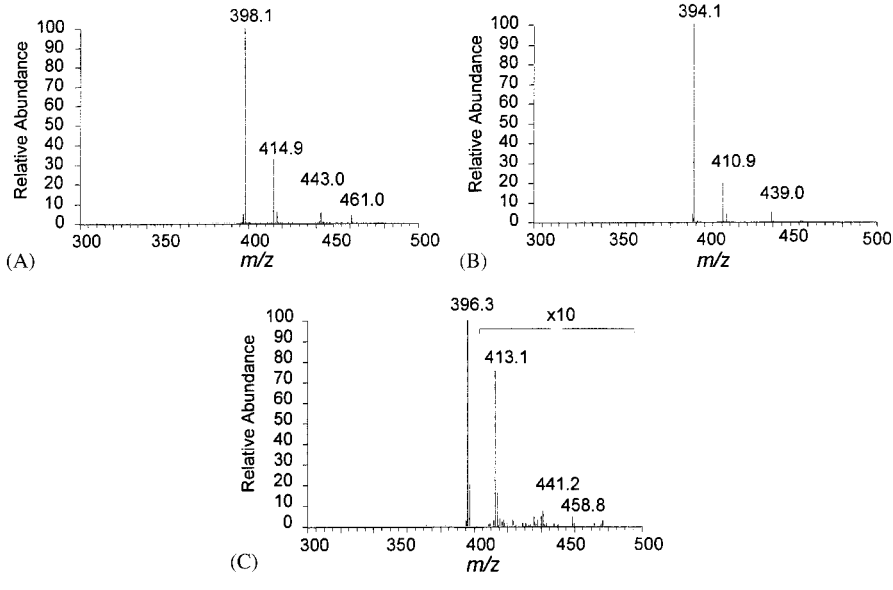
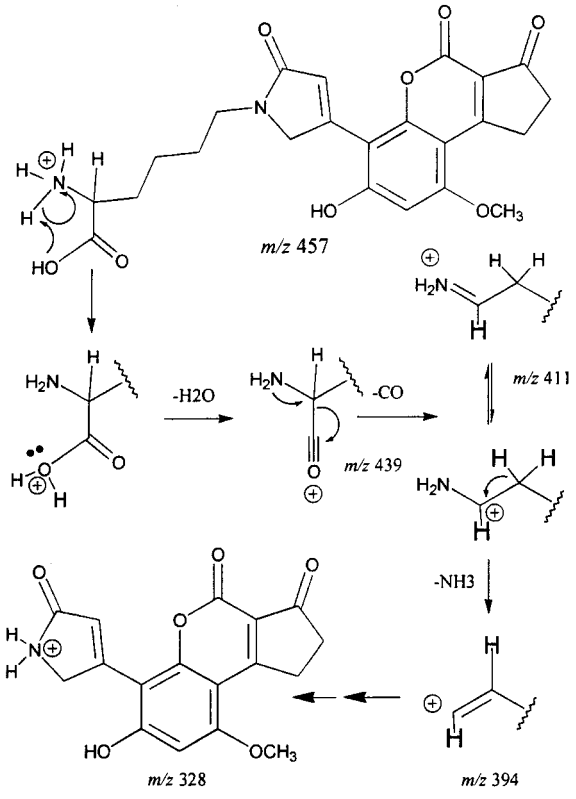


Figure 4. CID mass spectra of lysine-aflatoxin standards. The respective parent molecular ions fragment with the loss of H_2O, CO and $N_\alpha H_3$. (A) D4-Lysine-AFB_1 (*m/z* 461). (B) Lysine-AFB_1 (*m/z* 457). (C) $^{13}C_\varepsilon^{15}N_\varepsilon$-lysine-$AFB_1$ (*m/z* 459). Infusion 20 µl/min. Solvent: 50% MeOH/0.1% HOAc



Scheme 2. Fragmentation pathway of lysine-AFB$_1$

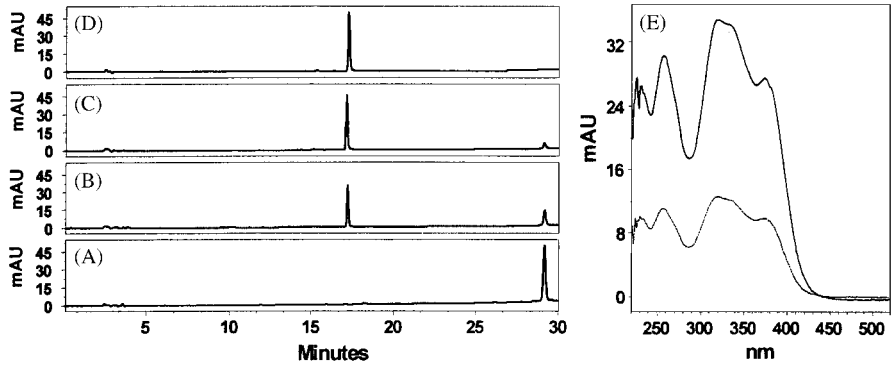


Figure 5. Time course of *t*-Boc-N$_\alpha$-lysine-AFB$_1$ deprotection using 40% TFA. (A) $t = 0$ min. (B) $t = 30$ min. (C) $t = 1$ h. (D) $t = 2$ h. (E) UV absorbance spectra of lysine-AFB$_1$ (17 min) and *t*-Boc-lysine-AFB1 (29 min) from panel B. See Figure 1 for HPLC conditions

To corroborate the structural assignment made to *m*/*z* 328 in Scheme 2, $^{13}C_\varepsilon$, $^{15}N_\varepsilon$-lysine-AFB$_1$ was prepared. The expected +2 AMU shifts in lysine-

AFB_1 ions to m/z 459, 413 and 396 were observed (Figures 3(C) and 4(C)). Because the ε-amino group of lysine is retained, the $^{13}C_{\varepsilon}$, $^{15}N_{\varepsilon}$-lysine-AFB_1 spectrum (Figure 3(C)) exhibits the fragment at m/z 329, instead of m/z 328 as in lysine-AFB_1 and D4-lysine-AFB_1.

The use of *t*-Boc-N_{α}-lysine to prepare lysine-AFB_1 has been discouraged due to reports indicating lysine-AFB_1 is unstable at pH 4.[14–16] However, as shown in Figure 5, *t*-Boc-lysine-AFB_1 can be completely deprotected within 2 h without degradation of lysine-AFB_1. The overnight exposure of lysine-AFB_1 to 40% TFA results in complete conversion to three more polar aflatoxin congeners. Thus, *t*-Boc-N_{α}-lysine is a convenient reagent for the more rapid preparation of lysine-AFB_1 than N_{α}-acetyl-lysine-AFB_1, which requires an overnight enzymatic *N*-deacetylation step to obtain the target compound.

Experimental

CAUTION. AFB_1 is a potent human hepatocarcinogen. AFB_1 and its derivatives should be handled with due consideration for safety.

Preparation of AFB_1 epoxide

AFB_1 epoxide was prepared by oxidizing AFB_1 in anhydrous methylene chloride with dimethyldioxirane.[23] Solvent was evaporated under a stream of N_2 gas and AFB_1 dialdehyde was obtained by the addition of phosphate buffer (0.1 M, pH 7.4). AFB_1 diol exists in equilibrium with AFB_1 dialdehyde ($pK_a = 8.2$).[24] AFB_1 diol, pH 4, $\varepsilon_{360} = 21\,800/\text{M cm}$.[17]

Preparation of Cu^{2+}-lys_2

The α-amino groups of L-lysine and 5,5,6,6-D4-L-lysine hydrochlorides (96–98% isotopic purity, Cambridge Isotope Labs, Andover, MA) were protected by complexing with Cu^{2+}.[25] These blue solutions were directly used in subsequent reactions with AFB_1 dialdehyde.

Preparation of D4-lysine-AFB_1, lysine-AFB_1 and t-Boc-N_{α}-lysine-AFB_1

Lysine-AFB_1 adducts were synthesized via the overnight reaction of AFB_1 dialdehyde (2.88 mM) at 20°C in potassium phosphate buffer (0.1 M, pH 7.6) with an equimolar amount of the corresponding α-amino protected lysine derivative. The D4-lysine and *t*-Boc-lysine time courses were monitored by removing reaction mixture aliquots and quenching with 1% HOAc/water at 0°C for analysis by HPLC with UV or mass spectrometric detection. Lysine-AFB_1 exists in a pH-dependent equilibrium with its corresponding phenolate anion. Lysine-AFB_1, pH 7, $\varepsilon_{399} = 25\,400/\text{M cm}$.[14,15,17]

Effect of TFA concentration t-Boc-N_α-lysine-AFB_1 on deprotection

HPLC-purified *t*-Boc-N_α-lysine-AFB_1 was dissolved in 50% acetonitrile/water containing a range of TFA concentrations (0–80% TFA, v/v) at 20°C. The amounts of *t*-Boc-N_α-lysine-AFB_1 and lysine-AFB_1 present after 3 h were measured via HPLC with UV diode array detection.

Effect of exposure time on TFA (40%) deprotection of t-Boc-N_α-lys-AFB_1

HPLC-purified *t*-Boc-lysine-AFB_1 was dissolved in 50% acetonitrile/water containing a final 40% TFA (v/v) at 20°C. The deprotection reaction time course was monitored by HPLC with UV diode array detection.

Mass spectrometric instrumentation

Samples were analyzed using a Thermoquest Finnigan LCQ Classic ion trap mass spectrometer operated in the positive ionization ESI mode. Reaction products were analyzed in full scan and collision induced dissociation (CID) experiments, using He as the collision gas. Spray voltage = 4.6 kV. Capillary temp. = 200°C. Capillary potential = 8 V. Auxillary gas was not used. A mass window of $\pm$ 1.5 m/z was applied for selection of $(M+H)^+$ in CID studies.

Conclusion

Independent of the protection strategy used (Cu^{2+} or *t*-Boc), reactions of N_α-protected lysine with AFB_1 dialdehyde proceeded to completion in 5–7 h with 30% reaction yields. Lysine-AFB_1 thus prepared was indistinguishable by UV absorbance, chromatographic properties or mass spectrometry from lysine-AFB_1 prepared using N_α–acetyl–lysine. The use of cupric ions to protect the α-amino group of lysine enabled the more rapid preparation of lysine-AFB_1 and D4-lysine-AFB_1 by obviating the need for removal of N_α-*t*-Boc or enzymatic N_α-deacetylation. This method will facilitate the preparation of natural isotopic abundance and D4-lysine-AFB_1 standards for the measurement of human exposure to aflatoxin B_1 in future molecular epidemiologic studies of liver cancer.

Acknowledgements

Financial support for this work was provided by the US National Institutes of Health through Grants P01 ES06052 and P30 ES 03819.

References

1. Kensler TK, Qian G, Chen J, Groopman JD. *Nature Cancer Reviews* 2003; **3**: 321–329.
2. Eaton DL, Groopman JD (eds). *The Toxicology of Aflatoxins*. Academic Press, Inc., Harcourt Brace and Co.: New York, 1994.

3. Wild CP, Garner RC, Montesano R, Tursi F. *Carcinogenesis* 1986; **7**: 853–858.
4. Essigmann JM, Croy RG, Nadzan AM, Busby WF, Reinhold VN, Büchi G, Wogan G. *Proc Natl Acad Sci* 1977; **74**: 1870–1874.
5. Cupid BC, Lightfoot TJ, Russell D, Gant SJ, Turner PC, Dingley KH, Curtis KD, Leveson SH, Turtletaub KW, Carner RC. *Food Chem Toxicol* 2004; **42**: 559–569.
6. Groopman JD, DeMatos P, Egner PA, Love-Hunt A, Kensler TK. *Carcinogenesis* 1992; **13**: 101–116.
7. Wild CP, Jiang Y, Allen SJ, Jansen LAM, Hall AJ, Montesano R. *Carcinogenesis* 1990; **11**: 2271–2274.
8. Wild CP, Hudson GJ, Sabbioni G, Chapot B, Hall AJ, Wogan GN, Whittle H, Montesano R, Groopman JD. *Cancer Epidemiol Biomarkes Prev* 1992; **1**: 229–234.
9. Wang J, Qian G, Zarba A, He X, Zhu Y, Zhang B, Jacobsen L, Gange S, Muñoz A, Kensler T, Groopman JD. *Cancer Epidemiol Biomarkers Prev* 1996; **5**: 253–261.
10. Kensler TK, Gange SJ, Egner PA, Dolan PM, Munoz A, Groopman JD, Rogers AE, Roebuck BD. *Cancer Epidemiol Biomarkers Prev* 1997; **6**: 603–610.
11. Kensler TK, He X, Otieno M, Egner P, Jacobsen L, Chen B *et al*. *Cancer Epidemiol Biomarkers Prev* 1998; **7**: 127–134.
12. Sun C, Wu D, Wang L, Chen C, You S, Santella R. *Br J Cancer* 2002; **87**: 966–970.
13. Skipper PL, Tannenbaum SR. *Carcinogenesis* 1990; **11**: 507–518.
14. Sabbioni G, Skipper PL, Büchi G, Tannenbaum SR. *Carcinogenesis* 1987; **8**: 819–824.
15. Sabbioni G. *Chem–Biol Interact* 1990; **75**: 1–15.
16. Sabbioni G, Ambs S, Wogan GN, Groopman JD. *Carcinogenesis* 1990; **11**: 2063–2066.
17. Guengerich FP, Arneson KO, Williams KM, Deng Z, Harris TM. *Chem Res Toxicol* 2002; **15**: 780–792.
18. Guengerich FP, Voehler M, Williams KM, Deng Z, Harris TM. *Chem Res Toxicol* 2002; **15**: 793–798.
19. Wild CP, Jiang Y, Sabbioni G, Chapot B, Montesano R. *Cancer Res* 1990; **50**: 245–251.
20. Fenaille F, Tabet J, Guy P. *Rapid Commun Mass Spectrom* 2004; **18**: 67–76.
21. Dookeran NN, Yalcin T, Harrison AG. *J Mass Spectrom* 1996; **31**: 500–508.
22. Yalcin T, Harrison AG. *J Mass Spectrom* 1998; **11**: 1237–1243.
23. Baertschi SW, Raney KD, Stone MP, Harris TM. *J Am Chem Soc* 1988; **110**: 7929–7931.
24. Johnson WW, Harris TM, Guengerich FP. *J Am Chem Soc* 1996; **118**: 8213–8220.
25. Bodanszky M, Bodanszky A. *The Practice of Peptide Synthesis* (2nd edn). Springer: Berlin, Germany, 1994; 52–53.

