Production and Characterization of Aflatoxin B_2 Oximinoacetate

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Aflatoxin B_2 oximinoacetate (AFB_2-OA) was prepared by reacting aflatoxin B_2 (AFB_2) with *O*-carboxymethoxyamine. AFB_2-OA was analyzed by thin-layer chromatography, reversed-phase solvent gradient high-performance liquid chromatography, ultraviolet spectroscopy, infrared spectroscopy, thermospray and fast atom bombardment mass spectrometry, proton nuclear magnetic resonance spectroscopy, and correlated spectroscopic two-dimensional nuclear magnetic resonance spectroscopy in order to validate the proposed molecular structure. The results of spectral analyses demonstrated that the only difference between AFB_2 and AFB_2-OA was the substitution of an oximinoacetate group in AFB_2-OA for the cyclopentenone carbonyl group in AFB_2.

The aflatoxins are a group of toxic fungal metabolites produced by the species *Aspergillus flavus* and *Aspergillus parasiticus* (Wei et al., 1984). These molds occur in a variety of foods, especially peanuts and cereals, and can produce enough aflatoxins to render these foods hazardous (Linsell, 1979). Exposure to aflatoxins has been linked to a variety of acute and chronic diseases of man and animals (Campbell and Stoloff, 1974; Linsell and Peers, 1977).

Among the many methods described for detecting aflatoxins, potentially the most useful are the various enzyme-linked immunoassays (Chu, 1984). Development of enzyme-linked immunoassays for aflatoxins depends on the ability to produce aflatoxin derivatives (haptens) that can be bound to proteins while retaining enough of the original aflatoxin structure so that antibodies produced will recognize the native toxins. Six methods for producing aflatoxin haptens have been described:

(1) Any form of aflatoxin with a 2,3 double bond can be converted to either the 2-hydroxy or the 2,3-dihydro diol derivative that can form a Schiff base with amino functions in carrier proteins (Lawellin et al., 1977).

(2) The 2-hydroxy form of aflatoxin can also be reacted with glutaric anhydride to form a hemiglutarate derivative that will form a covalent bond with carrier protein (Lau et al., 1981b).

(3) Any aflatoxin with a 2,3 double bond can be metabolically activated with liver microsome preparations and reacted with nucleic acids to form aflatoxin–purine adducts that can then be complexed with protein (Haugen et al., 1981).

(4) Any form of aflatoxin with a cyclopentenone moiety can be derivatized with *O*-carboxymethoxyamine to produce the protein-reactive oximinoacetate form (Langone and Van Vunakis, 1976).

(5) Aflatoxin with a 2,3 double bond can be reacted with either chlorine or bromine to form the corresponding 2,3-dichloride or 2,3-dibromide derivatives that form covalent bonds with proteins (Sizaret et al., 1982).

(6) Aflatoxin with a 22-hydroxylated cyclopentenone moiety can be reacted with succinic anhydride to form the hemisuccinate derivative that will form a covalent bond with carrier protein (Lau et al., 1981a).

The various aflatoxin haptens are shown in Figure 1.

The structure presented by the hapten–carrier conjugate is an important factor in determining the specificity of the immune response to the hapten (Landsteiner, 1945). Antibodies generally recognize that part of the hapten furthest from the point of attachment with the carrier (Hammock and Mumma, 1980). A useful method of producing aflatoxin hapten was reported by Chu et al. (1977): aflatoxin B_1 was refluxed under mild conditions in a 1:4:1 mixture of pyridine–methanol–water in the presence of *O*-carboxymethoxyamine hemihydrochloride to yield the protein-reactive product aflatoxin B_1 1-(*O*-carboxymethyl)oxime. This product can then be coupled to proteins either in the presence of water-soluble carbodiimide (Chu and Ueno, 1977) or by mixed-anhydride reaction (Bierman and Terplan, 1980), to yield an excellent immunogen. As opposed to some other methods for producing aflatoxin hapten, the structure of the molecule is preserved with the exception of the double-bonded oxygen on the cyclopentenone moiety. In addition, this derivative, upon conjugation, forms a fairly large bridge between the hapten and the carrier protein, which improves the specificity of resulting antibodies for the native toxin (Chu, 1984).

Extensive spectral analysis is necessary to confirm the predicted structure of any aflatoxin derivative. In this report, we describe the conversion of aflatoxin B_2 to aflatoxin B_2 oximinoacetate, purification of the product, and spectral characterization of the purified product.

MATERIALS AND METHODS

Aflatoxin B_2 oximinoacetate (AFB_2-OA) was prepared by adaptation of the method of Chu et al. (1977). *O*-Carboxymethoxyamine hemihydrochloride and pyridine were purchased from Aldrich Chemical Co. (St. Louis, MO), and solvents were purchased from Fisher Scientific Co. (Springfield, NJ). Aflatoxin B_2 (50 mg) and *O*-carboxymethoxyamine hydrochloride (76 mg) were added to 40 mL of a 1:4:1 mixture of pyridine–methanol–water, and this mixture was refluxed for 2 h. After the mixture was allowed to stand overnight at room temperature, the solvent was removed by rotary evaporation to yield a yellow-orange product.

The reaction product and aflatoxin standards were dissolved in chloroform and analyzed by both normal-

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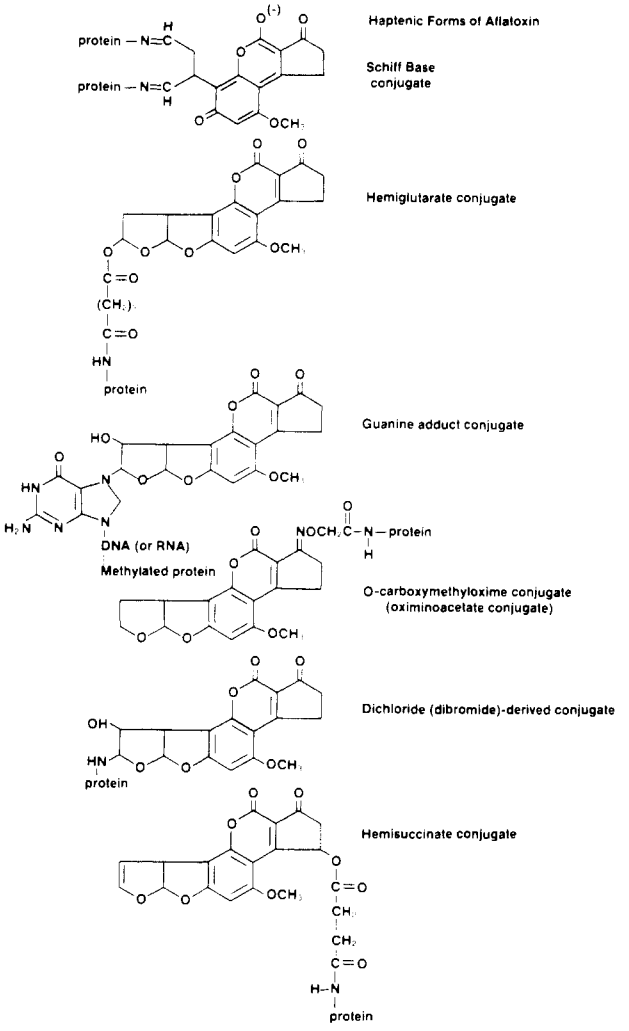

Figure 1. Haptenic form of aflatoxins.

phase and reversed-phase thin-layer chromatography. Normal-phase TLC was performed by spotting reaction product and aflatoxin standards on Kieselgel 60 plates (American Scientific Products, Charlotte, NC) and developing the plates in one of three different solvent systems: 1:9 methanol–chloroform, 1:9 acetone–chloroform, or 16:3:1 chloroform–ethyl ether–glacial acetic acid. Aflatoxin standards and reaction product were analyzed by reversed-phase TLC on a Whatman $KC_{18}/KC_{18}F$ plate (American Scientific Products) with a 1:3 ethanol–0.5 M sodium chloride developing system. The plates were dried, and fluorescent spots were detected with a long-wavelength ultraviolet light source.

The reaction product was further analyzed by reversed-phase solvent gradient high-performance liquid chromatography using two Waters Model 6000A solvent delivery systems, a Waters RCM-100 separatory module with a Radial-Pak A column (octadecylsilane, 5-μm particle size, 8-mm internal diameter), a Waters Model 440 absorbance detector operated at 365 nm, a Waters WISP 710B automatic injector, a Waters data module dual-pen recorder–integrator, and a Waters system controller (Millipore-Waters, Milford, MA). Two mobile phases (A and B) were used: A = 5% acetonitrile in water and B = 5% glacial acetic acid in acetonitrile. The flow rate was 1 mL/min. A Waters curve 5 was employed, the injection volume was 25 μL, and the program time was 30 min. The reaction product was prepared for injection by dissolving in acetonitrile and filtering through an Acrodisc-CR disposable filter assembly with a 0.45-μm pore size (Gelman Filtration Products, Ann Arbor, MI).

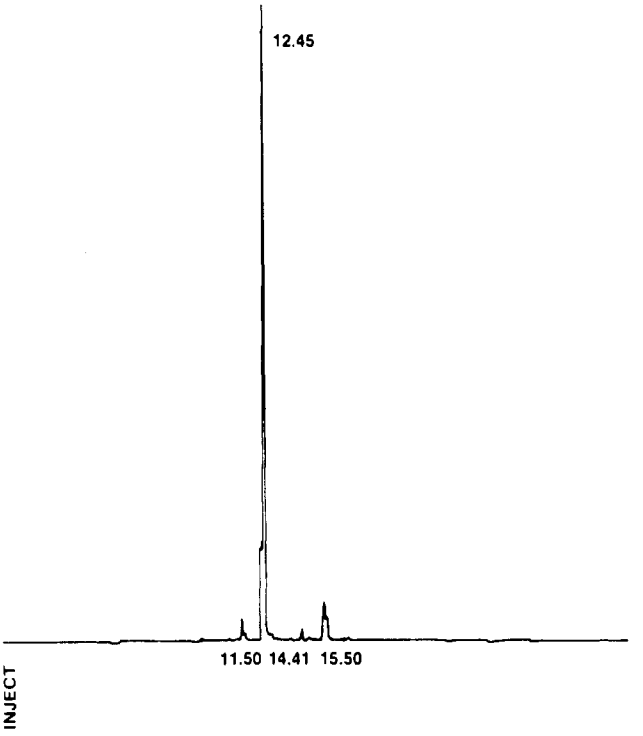

Figure 2. Reversed-phase solvent gradient high-pressure liquid chromatogram of AFB_2 at 365 nm.

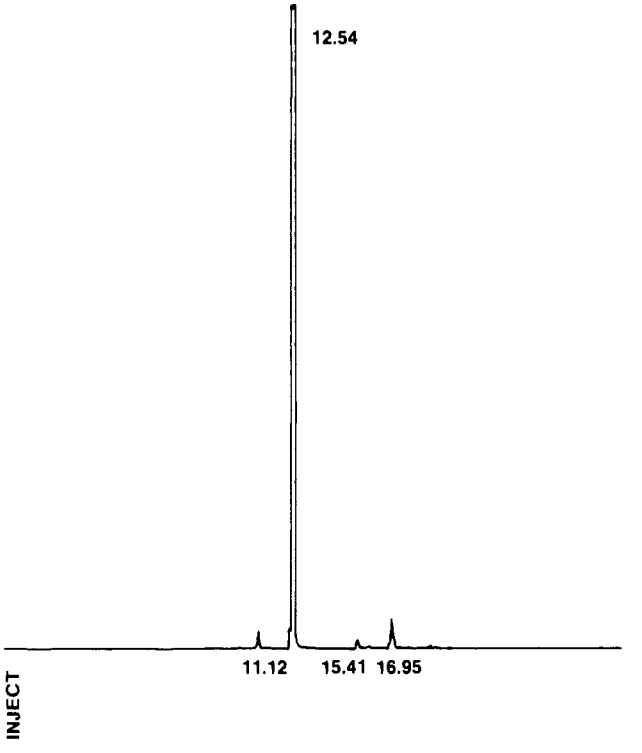

Figure 3. Reversed-phase solvent gradient high-pressure liquid chromatogram of AFB_2-OA at 254 nm.

A modification of the method of Thouvenot and Morfin (1983) was used to crystallize AFB_2-OA. The reaction product was dissolved in 25 mL of distilled water and adjusted to pH 8.0 with KOH. This solution was washed three times with benzene and acidified by addition of 0.25 M HCl. AFB_2-OA precipitated, and the aqueous layer was extracted four times with ethyl acetate. The extract was filtered over anhydrous sodium sulfate and dried under vacuum. The resulting product was crystallized from methanol and water, yielding pale yellow crystals. The

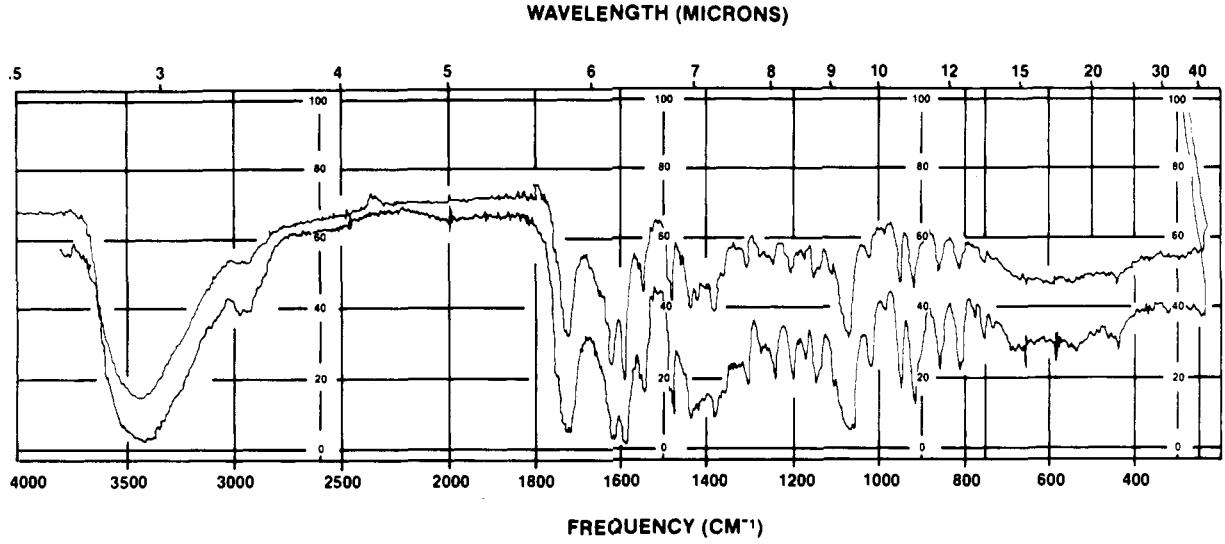

Figure 4. Infrared spectrum of AFB_2-OA.

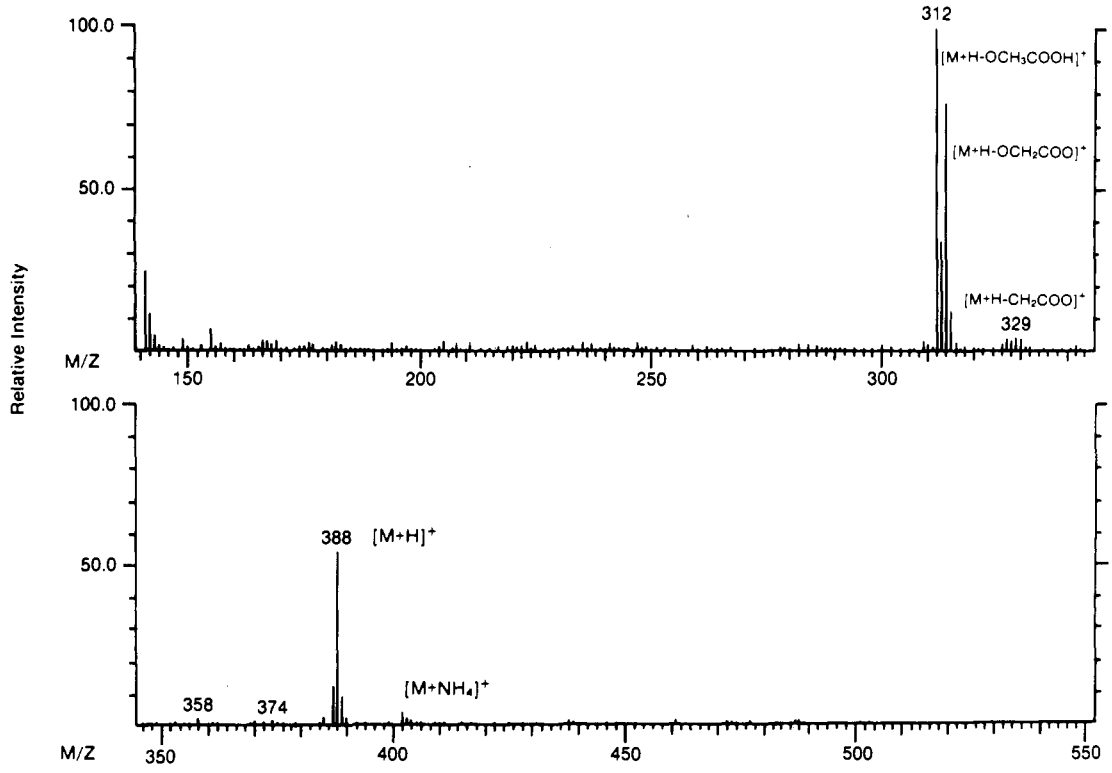

Figure 5. Thermospray mass spectrum of AFB_2-OA.

Table I. Relative Amounts of Products Formed by Reacting Aflatoxin B_2 with *O*-Carboxymethoxyamine As Measured by Reversed-Phase Solvent Gradient High-Pressure Liquid Chromatography

365 nm	peak A (RT 11.50) = 2.7%
	peak B (RT 12.45) = 87.6%
	peak C (RT 14.41) = 1.2%
	peak D (RT 15.50) = 3.7%
	resid absorbance = 3.7%
254 nm	peak A (RT 11.12) = 1.9%
	peak B (RT 12.54) = 92.3%
	peak C (RT 15.41) = 1.0%
	peak D (RT 16.95) = 3.4%
	resid absorbance = 1.4%

crystallized product was then analyzed by reversed-phase solvent gradient HPLC as described above.

AFB_2-OA crystals were used to obtain ultraviolet, infrared, and mass spectra. An ultraviolet spectrum was obtained by dissolving AFB_2-OA in acetonitrile and scanning a 1-cm light path from 200 to 400 nm with a Shimadzu UV-160 recording spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). An infrared spectrum was obtained in a KBr pellet (IR grade; Mallinckrodt Chemical Co., St. Louis, MO) on a Perkin-Elmer 521 IR spectrometer (Perkin-Elmer Corp., Norwalk, CT).

Mass spectra were obtained by electron ionization (EI), thermospray, and fast atom bombardment (FAB) ionization techniques. The EI probe spectrum was obtained on a MS-902 (AEI, Manchester, U.K.) at 70-eV electron energy with the probe cup heated to 200 °C. The instrument was scanned from m/z 35 to 450. Thermospray spectrum of the fraction AFB_2-OA was obtained on a Finnigan MAT 4500 quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) with a Vestec thermospray interface (VESTEC, Inc., Houston, TX). One microgram of the sample (dissolved in acetonitrile) was injected directly into the in-

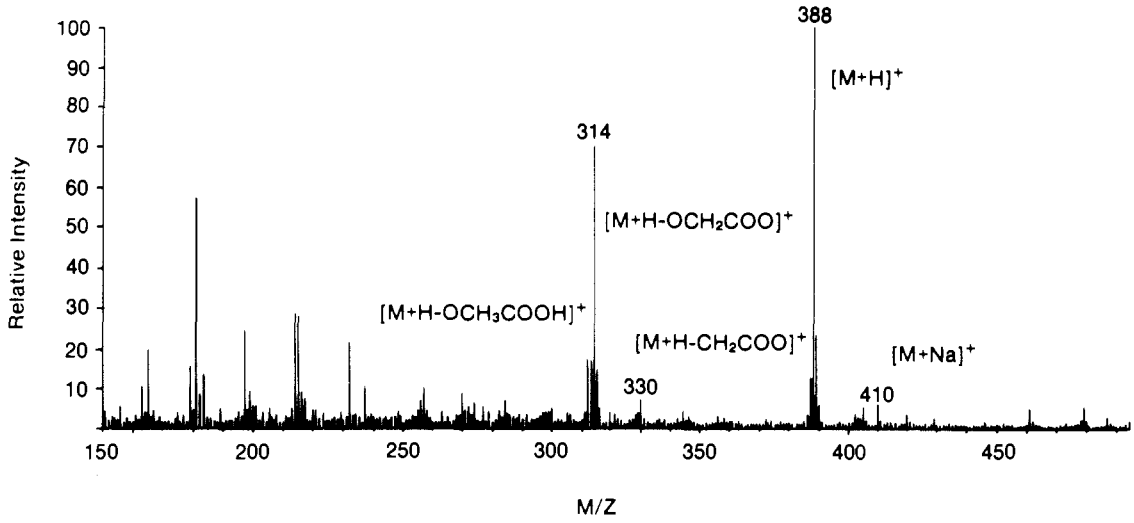

Figure 6. Fast atom bombardment mass spectrum of AFB_2-OA.

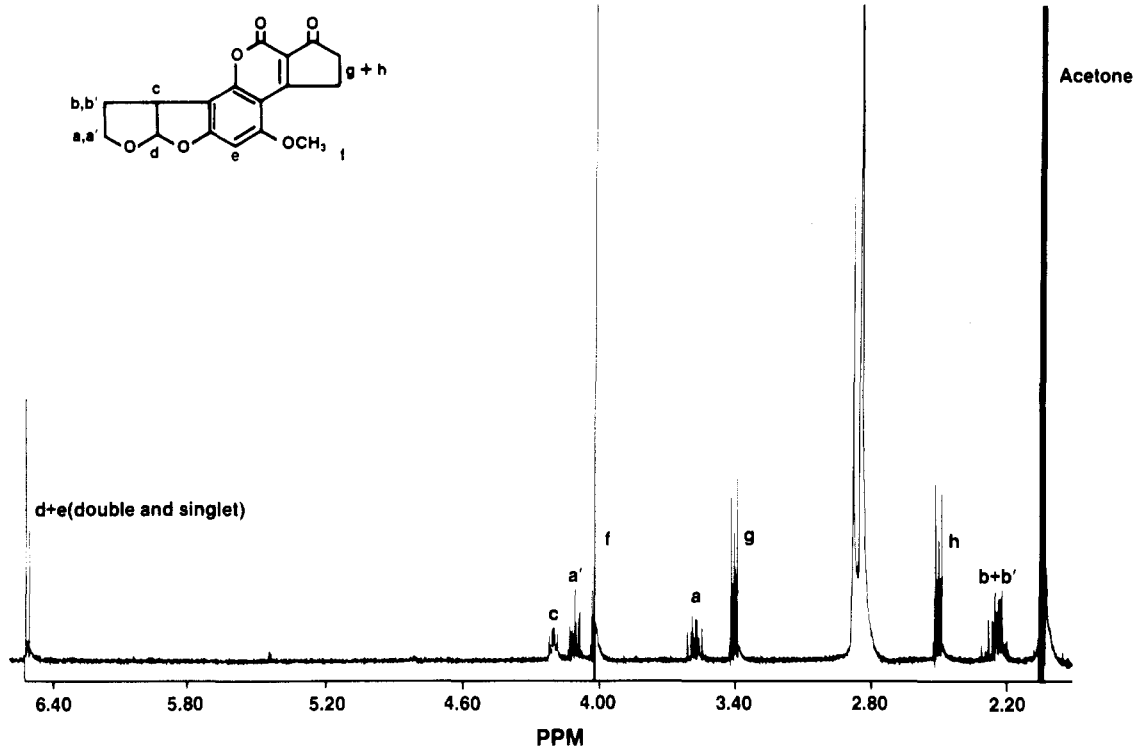

Figure 7. Proton nuclear magnetic resonance spectrum of AFB_2.

Table II. Elemental Compositions, Mass Error, and Exact Mass for Each Composition within a 20 ppm Window of the FAB High-Resolution Mass Spectrometric Measured Value of 387.0969

element				ppm	
C	H	O	N	error	accurate mass
17	15	7	4	7.3	387.094 074 1
19	17	8	1	3.8	387.095 416 8[a]
13	17	9	5	-14.8	387.102 627 4
12	15	9	6	17.7	387.090 051 4
14	17	10	3	14.2	387.091 394 1

[a] Corresponds to elemental composition for AFB_2-OA.

terface with a mobile phase of 60% acetonitrile in water with 0.1 M ammonium acetate at a flow rate of 1.2 mL/min. A thermospray vaporizer temperature of 220 °C and a source temperature of 275 °C were used for the analysis. The mass spectrometer was scanned from 140 to 700 in 2 s.

The FAB mass spectrum was obtained from a ZAB-E (VG Analytical Inc., Manchester, U.K.) mass spectrometer equipped with an Ion Tech ion gun. The FAB spectrum of AFB_2-OA was acquired of a thioglycerol matrix and 1 μL of a 1 $\mu g/\mu L$ solution (in acetonitrile) of AFB_2-OA placed in the probe tip. The ion gun was operated at 8 kV (1 mA) with xenon as the bombarding gas. The mass spectrometer was scanned from m/z 140 to 900 in 4 s. Positive-ion detection proved most sensitive for the analysis. The FAB accurate mass data were obtained at 8000 resolution (10% valley) with reference ions at m/z 344.721 69 and m/z 392.715 33 from CsI and RbI (50:50) mixture in water.

To obtain a nuclear magnetic resonance spectrum, AFB_2-OA was purified by reversed-phase isocratic HPLC using a 6000A solvent delivery system, an RCM-100 separatory module with a Radial-Pak A column, a Model 440 absorbance detector with a 365-nm filter (Millipore-Waters), and a Fisher Recordall Series 5000 strip-chart

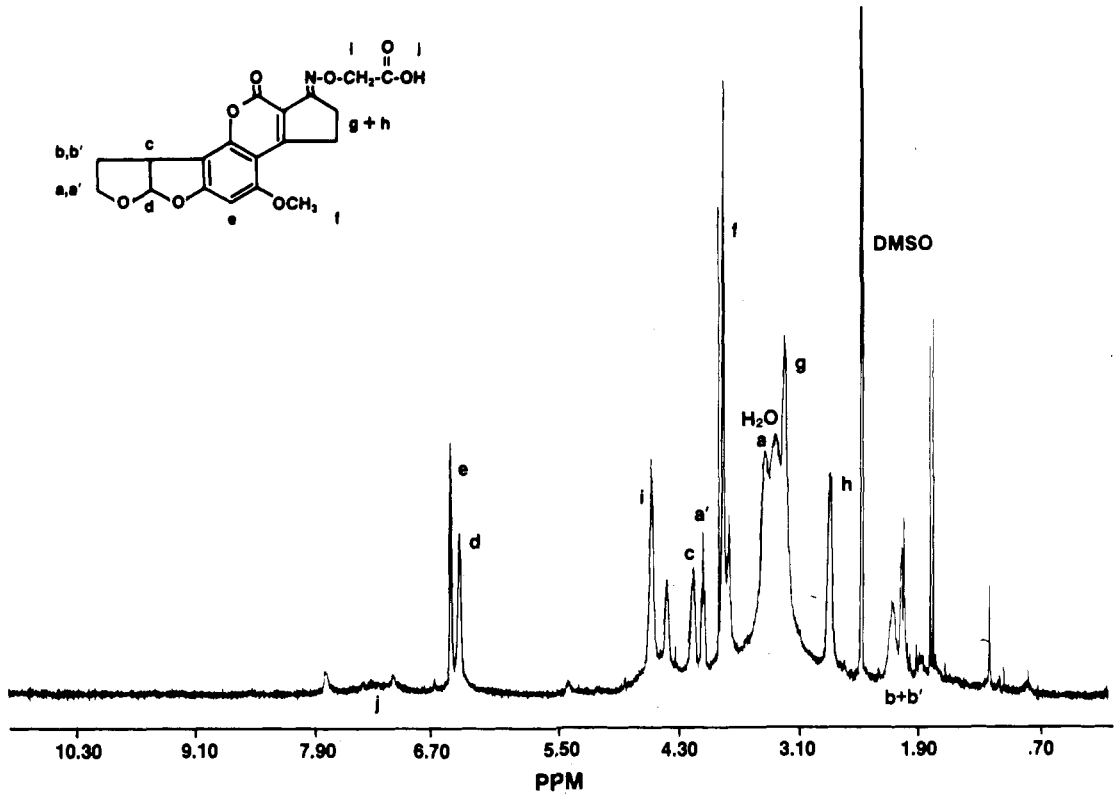

Figure 8. Proton nuclear magnetic resonance spectrum of AFB_2-OA.

Table III. Comparison of the Proton Nuclear Magnetic Resonance Spectra of AFB_2 and AFB_2-OA

proton	AFB_2	AFB_2-OA
a	3.58 (m)	3.56 (m)
a′	4.11 (m)	4.09 (m)
b + b′	2.22 (m)	2.20 (m)[a]
c	4.21 (m)	4.20 (m)
d	6.52 (d)	6.49 (d)
e	6.52 (s)	6.53 (s)
f	4.03 (s)	3.95 (s)
g	3.40 (m)	3.35 (m)
h	2.50 (m)	2.89 (m)
i		4.62 (s)
j		7.34 (s)

[a] It is possible that b′ is slightly upfield of b and is obscured by the acetone-d_6 signal. Abbreviations: s, singlet; d, doublet; m, multiplet.

recorder (Fisher Scientific). The reaction product was prepared for injection by dissolving in acetonitrile and filtering through an Acrodisc-CR disposable filter assembly, 0.45-μm pore size (Gelman Filtration Products). The solvent system was a 56:40:4 acetonitrile–water–glacial acetic acid solution. Collection conditions were 1 mL/min flow rate and an injection volume of 100 μL. Four fractions were collected, with fraction 2 being AFB_2-OA as determined by TLC. Purity was determined by reversed-phase solvent gradient HPLC as described above.

Proton and COSY-type two-dimensional nuclear magnetic resonance spectra were obtained of HPLC-purified AFB_2-OA on a Bruker AM-400 NMR spectrometer (Bruker Instruments, Billerica, MA) operating at 400 mHZ. The spectrum of AFB_2 was obtained in acetone-d_6 (Aldrich). Spectra of AFB_2-OA were obtained in DMSO-d_6 and in a 1:5 DMSO-d_6–acetone-d_6 solution.

RESULTS

Analysis of the crude reaction product by normal-phase TLC developed in a 1:9 methanol–chloroform solution yielded three fluorescent spots: one that cochromatographed with AFB_2 standard (R_f 0.44); a spot that did not migrate (R_f 0); and one very nonpolar spot (R_f 0.64). Normal-phase TLC in 1:9 acetone–chloroform and in 16:3:1 chloroform–ethyl ether–glacial acetic acid solutions yielded similar results: an intense yellow fluorescent spot that did not migrate, a spot that cochromatographed with AFB_2 standard, and a very nonpolar spot that was not identified. Reversed-phase TLC yielded a very polar spot with tailing and a small spot that was more polar, as well as a small fluorescent spot that did not migrate. The AFB_2 standard cochromatographed with the most intense polar spot. This system did not separate the presumptive AFB_2-OA spot from AFB_2 standard, but separated a minor, more polar spot that was not resolved by normal-phase TLC. AFB_2 derivatized in a 1:1:8 trifluoroacetic acid–glacial acetic acid–water solution cochromatographed with the minor, more polar spot.

Analysis of crude reaction product by reversed-phase solvent gradient HPLC (RP-SG-HPLC) demonstrated a major peak (peak B) with an absolute retention time of 12.45 at 365 nm and an absolute retention time of 12.54 at 254 nm (Figures 2 and 3). Three minor peaks were observed at both wavelengths (peak A = 11.50 at 365 nm, 11.12 at 254 nm; peak C = 14.41 at 365 nm, 15.41 at 254 nm; peak D = 15.50 at 365 nm, 16.95 at 254 nm). By RP-SG-HPLC, the relative amounts of the four products are presented in Table I.

By comparison of standards, peak A was determined to be AFB_2. Peak B was the presumptive AFB_2-OA. *O*-Carboxymethoxyamine hydrochloride was found to produce negligible absorbance at either 365 or 254 nm. The identities of peaks C and D were not established. Analysis of crystallized product demonstrated that the absorbance of peak B was increased to 93.9% at 365 nm and 96.2% at 254 nm; traces of the other peaks were still present in the crystals.

Analysis of the ultraviolet spectrum of AFB_2-OA crystals

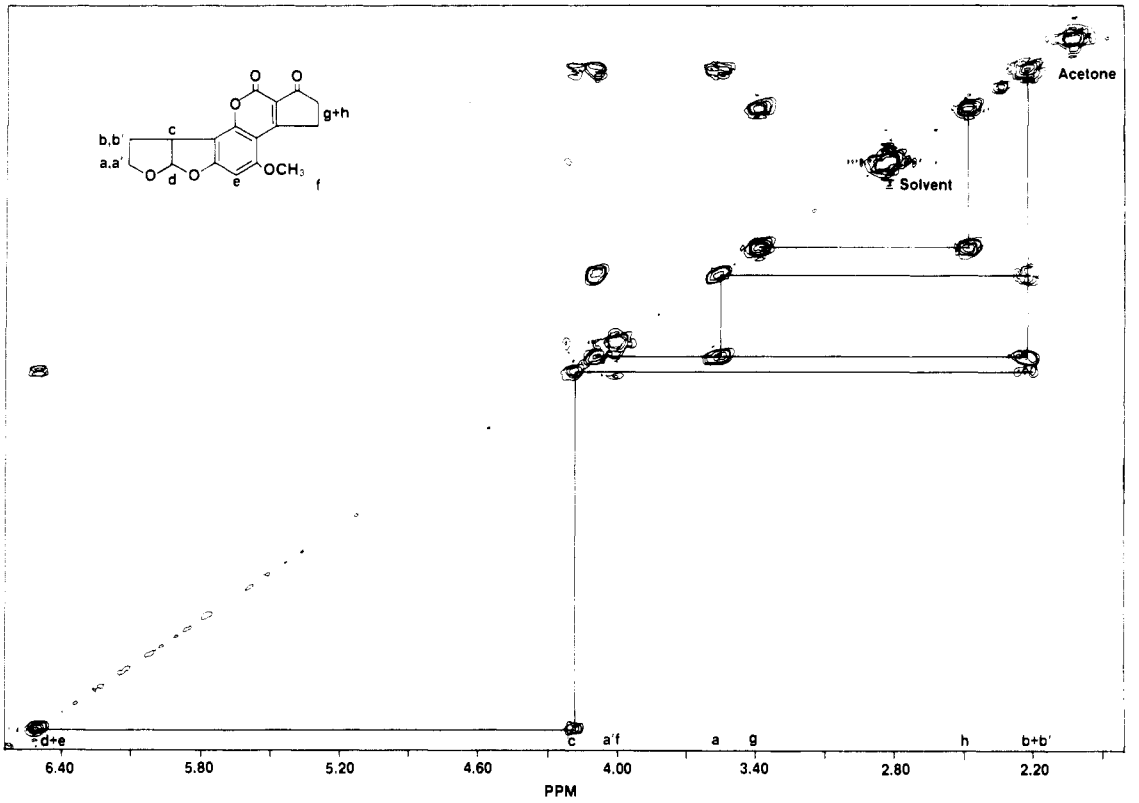

Figure 9. COSY-type two-dimensional nuclear magnetic resonance spectrum of AFB_2.

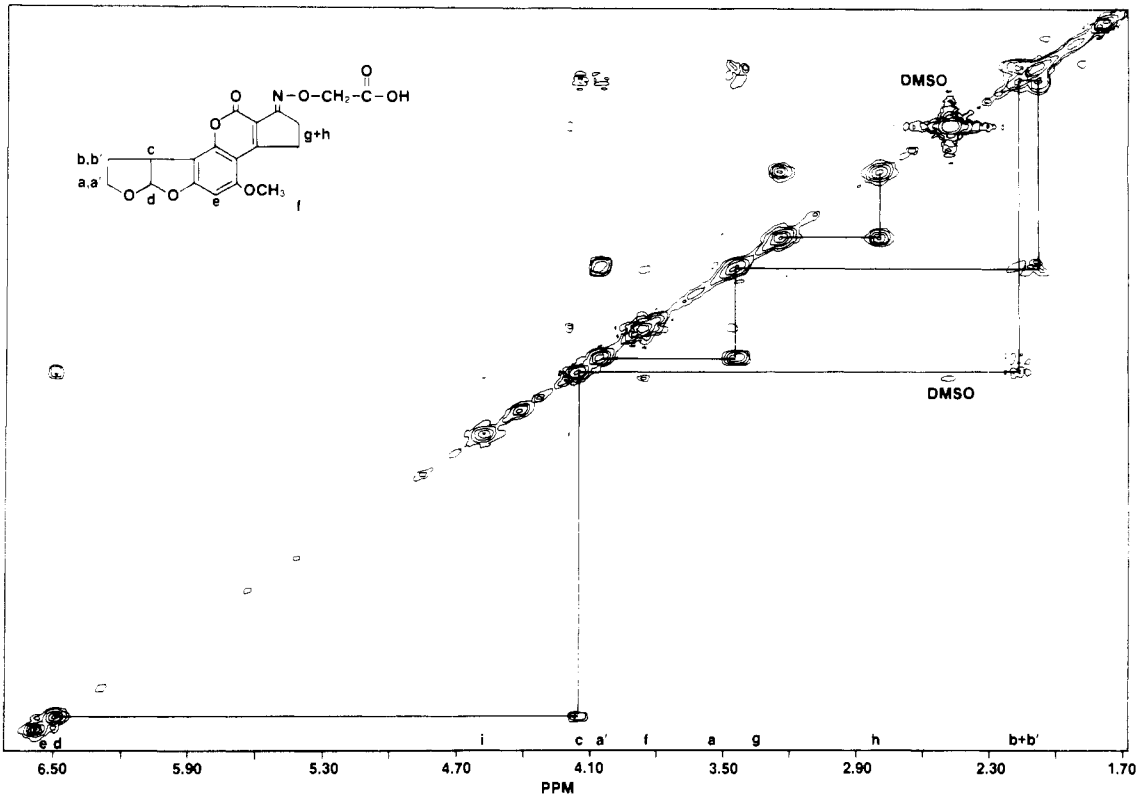

Figure 10. COSY-type two-dimensional nuclear magnetic resonance spectrum of AFB_2-OA.

in acetonitrile demonstrated a major peak at 363 nm and a minor peak at 265 nm. The molar absorption of AFB_2-OA at 363 nm was calculated to be 22 059.

The infrared spectrum (Figure 4) demonstrated an O–H stretching band partially overlapping the C–H stretching band in the 2500–3700-cm^{-1} region, indicating a carboxylic group. Taken with the presence of a C=N stretching band at 1630 cm^{-1} and the absence of absorptions at 1760 and 1685 cm^{-1}, characteristic of a coumarin nucleus with a cyclopentenone moiety, this indicates the substitution of oximinoacetate for a ketone group (Chu et al., 1977).

The EI mass spectrum did not yield a molecular ion for AFB_2-OA. The highest peak detected was at m/z 313 due to the loss of OCH_2COO from the molecular ion. The probe cup was heated up to 200 °C to vaporize the sample for ionization. However, at this temperature the sample

apparently decomposed as noted by the dark residue remaining in the probe cup and the numerous low-mass ions. For this reason, soft ionization techniques such as thermospray and FAB, which did not depend on sample volatility, were evaluated. The analysis of AFB_2-OA by thermospray/MS (Figure 5) and FAB/MS (Figure 6) resulted in spectra exhibiting an $[M + H]^+$ ion at m/z 388. The thermospray and FAB spectra were similar in that they both showed losses of CH_2COO, OCH_2COO, and OCH_3 + COOH from the $[M + H]^+$ ion. The thermospray spectrum exhibited an adduct ion at m/z 405 due to $[M + NH_4]^+$. Ammonium addition is common for compounds with low proton affinity (such as AFB_2-OA) and comes from the ammonium acetate in solution necessary for thermospray ionization (Blakely and Vestal, 1983). The FAB spectrum also exhibited an adduct ion $[M + Na]^+$ due to cationization of the sample. Sodium is present in residual amounts on the steel probe tip or in solvents and glassware used in sample preparation. AFB_2-OA was also analyzed by negative-ion detection using thermospray and FAB. However, the negative-ion sensitivity for the sample was poor, and no molecular anion was detected.

Accurate mass measurement on the $[M + H]^+$ ion at m/z 388 using FAB ionization verified the empirical formula for AFB_2-OA. Table II lists all empirical formulas of compounds within 20 ppm of the measured mass minus hydrogen [measured value (388.1047–hydrogen (1.0078) = molecular weight for AFB_2-OA (387.0969)]. The expected empirical formula for AFB_2-OA ($C_{19}H_{17}O_8N_1$) was among the closest matches to the measured mass value and the only formula possible. The first formula on Table II does not follow valence laws for an electrically neutral compound. The last three formulas are outside the window of error for the accurate mass measurement. The mass spectral data acquired by thermospray and FAB are consistent with the proposed structure of AFB_2-OA.

Consistent with the results of RP-SG-HPLC, reversed-phase isocratic HPLC (RPI-HPLC) demonstrated four fractions. Fraction 2, determined to be AFB_2-OA by TLC, was found to be 94.0% pure at 365 nm by RP-SG-HPLC. Fractions 1 and 2 were not well resolved by RPI-HPLC, accounting for the lack of complete purity of fraction 2.

Comparison of the proton nuclear magnetic resonance spectrum of AFB_2 (Figure 7) with the spectrum of AFB_2-OA (Figure 8) demonstrated that the only difference was the presence of proton singlets at 4.62 and 7.34 ppm in the AFB_2-OA spectrum, consistent with the substitution of the oximinoacetate group for the ketone group in the cyclopentenone ring. AFB_2-OA was very soluble in DMSO, but the NMR spectra were so broad that coupling could not be detected. Well-resolved spectra were obtained in acetone, but the solubility of AFB_2-OA was so low that acetone, water, and impurity signals dominated the spectra. The mixed solvent, which was used at near-saturation levels of AFB_2-OA, gave well-resolved lines although various solvent signals still obscured some of the signals from the compound. Assignments, shown in Table III, were made on the basis of chemical shift, integration, comparisons of the chemical shifts, and slopes of the multiplets for AFB_2 and AFB_2-OA and from COSY-type two-dimensional proton NMR of both compounds (Figures 9 and 10). On the basis of the spectral data, the structure of AFB_2-OA was unequivocally established.

DISCUSSION

Extensive spectral analysis was necessary to confirm the predicted structure of aflatoxin B_2 oximinoacetate. Both the ultraviolet and infrared spectra were very similar to the spectra for aflatoxin B_1 1-(*O*-carboxymethoxy)oxime (Chu et al., 1977). The infrared spectrum confirmed the predicted substitution of a double-bonded nitrogen for a ketone function in the cyclopentenone moiety.

Mass spectral analysis was complicated by two factors: The crystallized AFB_2-OA was not completely pure, and AFB_2-OA proved to be thermally unstable. Thermospray has been reported to be useful for analysis of thermally unstable compounds (Covey et al., 1986), and the use of a liquid chromatography interface separated AFB_2-OA from contaminants in the crystallized product. Fast atom bombardment mass spectrometry made possible an accurate mass determination and the assignment of an empirical formula to AFB_2-OA.

Two problems were encountered in determining NMR spectra: the lack of a good solvent for AFB_2-OA that was also compatible with NMR spectroscopy and the presence of a minor contaminant that was not completely resolved by HPLC purification. COSY-type two-dimensional NMR made possible the determination of structural aspects of AFB_2-OA that were obscured in the NMR spectrum by the presence of contaminants. Combining the results of the various spectra, the absolute identity of AFB_2-OA was confirmed.

Registry No. AFB_2, 7220-81-7; AFB_2-OA, 118920-31-3; *O*-carboxymethoxyamine, 645-88-5.

LITERATURE CITED

Bierman, V. A.; Terplan, G. Nachweis von aflatoxin B_1 mittels ELISA. *Arch. Lebensmittelhyg.* **1980**, *31*, 51–57.

Blakely, C. R.; Vestal, M. L. Thermospray interface for liquid chromatography/mass spectrometry. *Anal. Chem.* **1983**, *55*, 750–754.

Campbell, A. D.; Stoloff, L. Implications of mycotoxins for human health. *J. Agric. Food Chem.* **1974**, *22*, 1006–1014.

Chu, F. S. Immunoassays for analysis of mycotoxins. *J. Food Protect.* **1984**, *47*, 562–569.

Chu, F. S.; Ueno, I. Production of antibody against aflatoxin B_1. *Appl. Environ. Microbiol.* **1977**, *33*, 1125–1128.

Chu, F. S.; Hsia, M. T. S.; Sun, P. S. Preparation and characterization of aflatoxin B_1-1-(O-carboxymethoxy)-oxime. *J. Assoc. Off. Anal. Chem.* **1977**, *60*, 791–794.

Covey, T. R.; Lee, E. D.; Bruins, A. P.; Henion, J. D. Liquid chromatography/mass spectrometry. *Anal. Chem.* **1986**, *58*, 1451A–1461A.

Hammock, B. D.; Mumma, R. O. In *Pesticide Analytical Methodology*; Harvey, J., Jr., Zweig, G., Eds.; ACS Symposium Series 136; American Chemical Society: Washington, DC, 1980; pp 321–352.

Haugen, A.; Groopman, J. D.; Hsu, I. C.; Goodrich, G. R.; Wogan, G. N.; Harris, C. C. Monoclonal antibody to aflatoxin B_1-modified DNA detected by enzyme immunoassay. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4124–4127.

Lagone, J. J.; Van Vunakis, H. Aflatoxin B_1: Specific antibodies and their use in radioimmunoassay. *J. Natl. Cancer Inst.* **1976**, *56*, 591–595.

Landsteiner, K. *The Specificity of Seriological Reactions*; Harvard University Press: Cambridge, MA, 1945; 310 pp.

Lau, H. P.; Fan, S. L.; Chu, F. S. Preparation and characterization of aflatoxin Q_1-hemisuccinate. *J. Assoc. Off. Anal. Chem.* **1981a**, *64*, 681–683.

Lau, H. P.; Gaur, P. K.; Chu, F. S. Preparation and characterization of aflatoxin B_{2a}-hemiglutarate and its use for the production of antibody against aflatoxin B_1. *J. Food Safety* **1981b**, *3*, 1–13.

Lawellin, D. W.; Grant, D. W.; Joyce, B. K. Enzyme-linked immunosorbent analysis for aflatoxin B_1. *Appl. Environ. Microbiol.* **1977**, *34*, 94–96.

Linsell, C. A. In *Carcinogenic Risk Strategies for Intervention*; IARC Scientific Publication No. 23; International Agency for Research on Cancer; Lyon, 1979; p 111.

Linsell, C. A.; Peers, F. G. Aflatoxin and liver cell cancer. *Trans.*

R. Soc. Trop. Med. Hyg. **1977**, *71*, 471–473.
Sizaret, P.; Malaveille, C.; Montesano, R.; Frayssinet, C. Detection of aflatoxins and related metabolites by radioimmunoassay. *J. Natl. Cancer Inst.* **1982**, *69*, 1375–1381.
Thouvenot, D.; Morphin, R. F. Radioimmunoassay for zearalenone and zearalenol in human serum: production, properties and use of porcine antibodies. *Appl. Environ. Microbiol.* **1983**, *45*, 16–23.
Wei, D. L.; Chen, W. L.; Wei, R. D.; Jong, S. C. In *Toxigenic Fungi—Their Toxins and Health Hazard*; Kurata, H., Ueno, Y., Eds.; Developments in Food Science 7; Kodansha: Tokyo, 1984; p 87.

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Formation of 7*H*-Cyclopenta[*b*]pyridin-7-ones as Proline-Specific Maillard Products

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In a series of proline/monosaccharide model experiments 7*H*-cyclopenta[*b*]pyridin-7-ones were formed as Maillard reaction products. Their structures were elucidated by means of MS, IR, and ^{1}H NMR spectroscopy and verified by alternative synthetic routes. 1,2,3,4,5,6-Hexahydro-7*H*-cyclopenta[*b*]-pyridin-7-one (**1**) also was found to be a main constituent in wort and beer.

As demonstrated in previous publications model reactions of L-proline and monosaccharides result in complex mixtures of proline-specific compounds (Tressl et al., 1985a–c). Recently we described the formation of 8*H*-cyclopent[*b*]azepin-8-ones by a Strecker-type reaction of proline and cyclic enolones (Tressl et al., 1985d). In this paper we demonstrate the formation of homologous 7*H*-cyclopenta[*b*]pyridin-7-ones by an analogous ring-enlargement reaction of L-azetidinic acid and cyclic enolones. These compounds are also identified in proline model systems with reducing sugars under Maillard reaction conditions. Further investigations predicted 2-acyltetrahydropyridines as possible precursors to form 7*H*-cyclopenta[*b*]pyridin-7-ones in the proline system.

EXPERIMENTAL SECTION

Sample Preparation. *Reaction of L-Proline and Monosaccharides.* Equimolar amounts of L-proline and monosaccharides (0.03 mol of glyceraldehyde, erythrose, arabinose, glucose, and rhamnose, respectively) dissolved in 50 mL of water were autoclaved for 1.5 h at 150–160 °C in a stainless steel laboratory autoclave equipped with a 100-mL duran glass tube and heated by an electric heater with magnetic stirrer. After the mixtures had cooled to room temperature, the pH (5–6) was adjusted to 10–11 by addition of 0.1 N NaOH and the reaction products were extracted three times with freshly distilled diethyl ether. The combined ether extracts were dried over anhydrous sodium sulfate and concentrated to 1 mL. Aliquot amounts of the extracts were investigated by capillary GC/MS and nitrogen-selective detector.

Reaction of 2-Acetyl-3,4,5,6-tetrahydropyridine and Aldehydes. Equimolar amounts of 2-acetyl-3,4,5,6-tetrahydropyridine, prepared according to Büchi and Wüest (1971), and aldehydes (acet-, propion-, and pyruvaldehyde, and furfural) were stirred for 2–4 h at room temperature in water at pH 11 adjusted by addition of 0.1 N NaOH. From the acetaldehyde reaction mixture was separated compound **8** by diethyl ether extraction and isolated by liquid–solid chromatography on Al_2O_3 with pentane/ether (9:1). Additionally the acet-, propion-, and pyruvaldehyde and the furfural reaction mixtures were autoclaved without further purification for 15 min at 150–160 °C. From these experiments compounds **2**, **4**, **6**, and **7** were isolated by ether extraction and liquid–solid chromatography on Al_2O_3 with pentane/ether (9:1) and preparative gas chromatography. The purified compounds were investigated by MS IR, and ^{1}H NMR spectroscopy.

Reaction of L-Azetidinic Acid and Cyclic Enolones. Equimolar amounts of L-azetidinic acid and cyclic enolones (0.01 mol of 2-hydroxy-2-cyclopententen-1-one, 0.03 mol of cyclotene = 2-hydroxy-3-methyl-2-cyclopenten-1-one, 0.01 mol of ECP = 3-ethyl-2-hydroxy-2-cyclopenten-1-one) dissolved in water were autoclaved for 1.5 h at 160–180 °C and the reaction products extracted as described for the proline/monosaccharide model experiments. From the proline/cyclotene system compounds **3**, **9**, and **11** and from the proline/ECP system compounds **5**, **10**, and **12** were separated by liquid–solid chromatography on Al_2O_3 with pentane/ether (9:1). The isolated compounds were investigated by MS, IR, and ^{1}H NMR spectroscopy.

Capillary Gas Chromatography (GC)/Mass Spectrometry (MS). Capillary gas chromatography/mass spectrometry was carried out by using a 50-m fused silica column (0.32-mm i.d.) coated with Carbowax 20 M + KOH coupled with a Finnigan MAT 4500 quadrupole instrument. Conditions were as follows: temperature program 70–180 °C at 2 °C/min; ionization voltage 70 eV; resolution 1000.

Preparative Gas Chromatography. Purification by preparative GC was carried out with a Varian Aerograph equipped with a 3-m glass column (2-mm i.d.), coated with 5% SP 2401 DB on 100–200-mesh Supelcoport, temperature program 100–250 °C at 4 °C/min.

^{1}H NMR and IR Spectroscopy. ^{1}H NMR spectra were recorded at 270 MHz on a Bruker WH 270 NMR spectrometer in $CDCl_3$ solution. Chemical shifts are referenced to tetramethylsilane (Me_4Si) as internal standard; coupling constants (J) are given in hertz. Infrared spectra were obtained from $CDCl_3$ or CCl_4 solutions with a Perkin-

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