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Ammoniation Products of an Aflatoxin Model Coumarin

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Treatment of 5,7-dimethoxy-9-oxocyclopenteno[*c*]coumarin (**2**) with aqueous ammonia at 37 °C produces three major products identified as 3-(2-hydroxy-4,6-dimethoxyphenyl)-2-cyclopentenone (**3**), 3,5-dimethoxyphenol (**4**), and 3-(2-hydroxy-4,6-dimethoxyphenyl)-2-hydroxy-2-cyclopentenone (**5**). Compound **5**, which is not derived from **3**, further decomposes in aqueous ammonia to **4**. The keto group on the cyclopentene ring of **2** is required for ammonia-induced decomposition because 5,7-dimethoxycyclopenteno[*c*]coumarin (**1**) fails to react under conditions that degrade **2**.

Aflatoxin levels in highly contaminated corn can be effectively reduced by treatment with low concentrations of ammonia at atmospheric pressure (Brekke et al., 1977a, 1979). In this process, contaminated corn at 17.5% moisture content is treated with 1.5% gaseous ammonia at ambient temperatures ranging from 32 to 43 °C for 13 days. The corn is then dried at 40 °C to 10% moisture. The process reduces aflatoxin to below the FDA action level of 20 ng/g. Although biological data indicate that the process is feasible for detoxification (Brekke et al., 1977b; Norred, 1979; Southern and Clawson, 1980), the chemical fate of aflatoxin during the ammoniation process is not known.

In studies of the ammoniation of aflatoxin B_1 (Figure 1) at elevated temperature and pressure, the major products were identified as aflatoxin D_1, which results from lactone ring opening followed by decarboxylation, and a molecular weight 206 phenol that lacks the cyclopentenone ring of D_1 (Lee et al., 1974; Cucullu et al., 1976). Subsequent work using aflatoxin B_1 spiked peanut and cottonseed meals showed this process to be additionally complicated by the meals, because D_1 was found in only trace amounts (Lee and Cucullu, 1978). Use of radiolabeled aflatoxin B_1 allowed approximately 50% of the added toxin to be accounted for by several products, but the remainder appeared to be lost through volatilization (Lee et al., 1979). The present study was undertaken to further explore the sequence of reactions that occurs during ammoniation of aflatoxin as they relate to the corn decontamination process, which is carried out under considerably milder conditions than the peanut–cottonseed meal process. Model coumarins **1** and **2** (Figure 2), rather than aflatoxin, were used which allowed the reactions to be carried out on a scale that facilitated isolation and characterization of the products without the concomitant hazard in using carcinogenic aflatoxin. Also, structural requirements for reactivity as influenced by other functional groups could be easily studied by using model compounds. These compounds have been shown to be nonlethal and noncarcinogenic to rats at doses greatly exceeding the effective levels of aflatoxin B_1 (Wogan et al., 1971).

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EXPERIMENTAL SECTION

Analytical Procedures. Infrared spectra (IR) were recorded in chloroform with a Beckman IR8 spectrophotometer. Ultraviolet spectra (UV) were recorded in ethanol with a Unicam SP 800 spectrophotometer. Mass spectra (MS) were obtained by electron impact at 70-eV ionizing electron energy and a source temperature of 200 °C with a Kratos MS 30 spectrometer. Samples were introduced by a direct insertion probe or a gas chromatograph (GC) inlet. For GC–MS, a 3-ft 3% OV-1 column was temperature programmed from 70 to 250 °C at 4 °C/min. The helium flow rate was 40 mL/min, and the GC was coupled to the MS by using a single-stage glass jet separator.

Proton nuclear magnetic resonance (NMR) spectra were obtained with a Varian HA-100 or Bruker WH-90 Fourier transform spectrometer. Carbon-13 NMR spectra were obtained with the Bruker instrument. Chemical shifts are reported as δ values in ppm downfield from internal tetramethylsilane. Column chromatographic separations were done on silica gel 60 (70–230 mesh; EM Laboratories). Thin-layer chromatography (TLC) was carried out on precoated 0.25-mm layers of silica gel 60, F-254 (EM Laboratories), with dichloromethane–acetone (4:1) as the developing solvent. Components were visualized under long- and short-wave UV light and with iodine vapor.

Synthesis of Coumarins 1 and 2. The procedures described by Asao et al. (1965) were employed for the synthesis of 5,7-dimethoxycyclopenteno[*c*]coumarin (**1**) and 5,7-dimethoxy-9-oxocyclopenteno[*c*]coumarin (**2**).

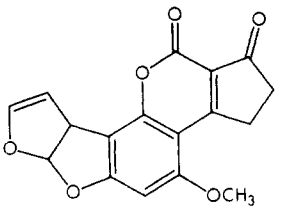

Figure 1. Structure of aflatoxin B_1.

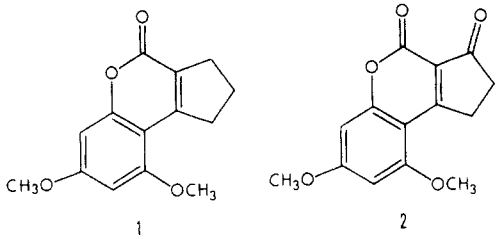

Figure 2. Structures of model compounds examined.

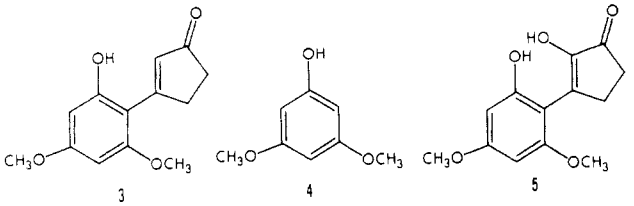

Figure 3. Structures for ammoniation products of **2**.

Reaction of Coumarin 2 with Ammonia at 50 °C. A mixture of **2** (130 mg, 0.5 mmol) and concentrated ammonium hydroxide (50 mL) was sealed, protected from light, and kept at 50 °C for 14 days with occasional swirling. The reaction mixture was concentrated to a small volume, acidified to pH 1 with 4 N HCl, and extracted with dichloromethane–methanol (9:1; 4 × 50 mL). The extract was dried (Na_2SO_4), concentrated, and chromatographed on a column of silica gel (15 g). Elution with dichloromethane–acetone (97:3) gave 3,5-dimethoxyphenol (**4**) (Figure 3) (6% yield) identified by TLC and MS comparison with an authentic sample. Elution with dichloromethane–acetone (95:5) gave 28% recovered **2**. Elution with dichloromethane–methanol (99:1) gave 3-(2-hydroxy-4,6-dimethoxyphenyl)-2-cyclopentenone (**3**) in 53% yield: mp (chloroform–hexane) 182–184 °C; IR 3600, 3550, 1705, 1690, 1675, 1610, 1150, 1100 cm^{-1}; UV λ_{max} 228 nm (ϵ 9720), 329 (ϵ 15 650); MS *m/e* (rel intensity) 234 (M^+, 100), 217 (5), 206 (19), 205 (19), 191 (64), 177 (13), 154 (7); ^1H NMR (100 MHz, $CDCl_3$–CD_3OD) δ 2.40 (2 H, m, CH_2), 3.20 (2 H, m, CH_2), 3.74 (6 H, s, 2 OCH_3), 3.86 (1 H, s, OH), 6.00 (1 H, d, J = 2.5 Hz, aromatic H), 6.08 (1 H, d, J = 2.5 Hz, aromatic H), 6.53 (1 H, t, J = 1.5 Hz, vinyl H). Irradiation of the vinyl proton signal at δ 6.53 sharpened the δ 3.20 methylene multiplet. Upon treatment of the sample with NaOD–CD_3OD to exchange active hydrogens, the resultant spectrum exhibited a diminished signal at δ 2.40. Therefore, the δ 2.40 signal is due to the methylene adjacent to the ketone and the δ 3.20 signal represents the allylic methylene. ^{13}C NMR ($CDCl_3$ – CD_3OD) δ 33.1, 35.0 (CH_2), 55.4, 55.6 (OCH_3), 91.0, 94.2 (aromatic CH), 105.9 (aromatic C), 131.5 (vinyl CH), 158.3, 160.4, 162.8 (aromatic C—O), 173.8 (vinyl C), 213.2 (C=O).

Reaction of Coumarin 2 with Ammonia at 37 °C. The same quantities and procedure were employed as for the 50 °C reaction except the mixture was kept at 37 °C for 20 days. Elution of the silica gel column (10 g) with dichloromethane–acetone (97:3) gave **4** (8–10% yield), dichloromethane–acetone (95:5) gave **2** (13–23% recovery), dichloromethane–methanol (98:2) gave **3** (4–7% yield), and dichloromethane–methanol (96:4) gave crude **5**. Preparative TLC of crude **5** was carried out, followed by visualization under long UV light and extraction of the low R_f greenish band with dichloromethane–methanol (9:1). The resulting compound was identified as 3-(2-hydroxy-4,6-dimethoxyphenyl)-2-hydroxy-2-cyclopentenone (**5**) and was obtained in 2–8% yield: mp (dichloromethane–hexane) 183–187 °C; IR 3470–3360 (OH), 1685 (C=O), 1605 cm^{-1} (C=C); MS *m/e* (rel intensity) 250 (M^+, 100), 232 (9), 194 (9), 179 (11), 167 (11), 154 (62); ^1H NMR (90 MHz, $CDCl_3$) δ 2.53 (2 H, m, CH_2), 3.02 (2 H, m, CH_2), 3.79 (6 H, s, 2 OCH_3), 5.71 (1 H, br s, OH), 6.12 (1 H, d, J = 2.2 Hz, aromatic H), 6.21 (1 H, d, J = 2.2 Hz, aromatic H); ^{13}C NMR ($CDCl_3$) δ 26.0, 32.6 (CH_2), 55.4 (2 OCH_3), 92.7, 95.5 (aromatic CH), 104.8 (aromatic C), 141.6, 147.0 (vinyl C), 157.1, 159.7, 162.9 (aromatic C—O), 203.4 (C=O). Treatment of **5** with acetic anhydride–pyridine afforded the diacetate: GC–MS *m/e* (rel intensity) 334 (M^+, 4), 292 (32), 250 (100), 154 (31); ^1H NMR ($CDCl_3$) δ 2.15 (3 H, s), 2.20 (3 H, s).

For ammoniations on solid supports, a solution of **2** (130 mg) in dichloromethane was intimately mixed with 100 g of Celite or oven-dried corn germ, and the solvent was removed at reduced pressure. A solution of concentrated ammonium hydroxide (5.5 mL) and H_2O (18.5 mL) was added to bring the NH_3 concentration to 1.5% and the moisture to 18%. The mixture was sealed, protected from light, and kept at 37 °C for 14 days. The NH_3 was then partially removed at reduced pressure below 40 °C. The mixture was acidified and extracted repeatedly with dichloromethane–methanol (9:1). The concentrated extract from corn germ was defatted by partitioning between hexane/methanol–water (9:1). The methanolic extract was concentrated, diluted with H_2O, and extracted with dichloromethane–methanol (9:1).

Stability of Ketophenol 3 toward Ammonia. A solution of **3** (42.7 mg, 0.18 mmol) in concentrated ammonium hydroxide (15 mL) was sealed, protected from light, and kept at 37 °C for 21 days. The solution was concentrated to a small volume, acidified with 4 N HCl, and extracted with dichloromethane–methanol (9:1; 3 × 10 mL). TLC examination of the extract failed to indicate **5**. The extract was chromatographed on a column of silica gel (4 g). Elution with dichloromethane–methanol (99:1) gave an 86% recovery of **3**, identified by TLC and melting point.

Reaction of Diketone 5 with Ammonia. A solution of **5** (1.5 mg) in concentrated ammonium hydroxide (0.5 mL) was sealed, protected from light, and kept at 37 °C for 3 days. The reaction mixture was concentrated, acidified, and extracted with dichloromethane. TLC examination of the extract showed **5** plus 3,5-dimethoxyphenol (**4**). The identity of **4** was confirmed by GC–MS: *m/e* (rel intensity) 154 (100, M^+), 125 (63), 111 (15), 94 (25), 69 (29).

Reaction of 3,5-Dimethoxyphenol (4) on Corn with Ammonia. A solution of **4** (80 mg) in dichloromethane was intimately mixed with 100 g of oven-dried corn germ, and the solvent was removed at reduced pressure. The mixture was ammoniated and processed as for the treatment of **2** on corn germ. The methanolic extract was concentrated, diluted with H_2O, and extracted with dichloromethane. Chromatography of the extract on a 2.3-cm column of silica gel (35 g) and elution with dichloromethane–acetone gave the fraction that would contain compound **4**. GC–MS analysis of this fraction as the trimethylsilyl (Me_3Si) derivative failed to indicate the presence of **4**. The Me_3Si derivative of **4** prepared from a stock solution was easily detected at the 50-ng level.

RESULTS AND DISCUSSION

Model compound 1 has the cyclopenteno[c]coumarin ring system of aflatoxin B_1 but lacks the ketone group on the cyclopentene ring. Attempts to degrade 1 under a variety of ammoniation conditions were unsuccessful and resulted in recovery of starting compound. These treatments were 5% NH_3 in aqueous ethanol at 50 °C for 14 days, concentrated ammonium hydroxide at 50 °C for 7 days, and adsorption of 1 onto Celite or corn germ and 1.5% NH_3 at 37 °C for 13 days and 50 °C for 12 days, respectively. The unreactivity of 1 is in contrast to the results of the study of Bergot et al. (1977) with an unsubstituted coumarin. They found that treatment of coumarin with concentrated ammonium hydroxide at room temperature for 20 days resulted in both Michael addition of ammonia to the α,β-unsaturated double bond and ammonolysis of the coumarin lactone to an amide.

Compound 2 is a better model than 1 because it possesses a tricyclic ring system with substitution identical with that of aflatoxin B_1. It lacks only the three carbon and one oxygen atoms that comprise the bis(dihydrofuran) moiety, which has not been shown to react with ammonia. Treatment of 2 with concentrated ammonium hydroxide at 50 °C for 14 days results in lactone ring opening and decarboxylation to give a 53% yield of ketophenol 3. Also isolated were 3,5-dimethoxyphenol (4) and unreacted 2 in yields of 6 and 28%, respectively. Compound 3 was characterized on the basis of its spectroscopic properties (see Experimental Section), and compound 4 was identified by TLC and MS comparisons with an authentic sample. Compounds 3 and 4 are analogous to aflatoxin D_1 and the molecular weight 206 compound, respectively, formed by ammoniation of aflatoxin B_1 under conditions of high temperature and pressure (Cucullu et al., 1976). Formation of compounds 3 and 4, therefore, establishes the validity of coumarin 2 as an aflatoxin model for ammoniation studies. The relative reactivities of ketocoumarin 2 and coumarin 1 toward aqueous ammonia are consistent with the proposal of Vesonder et al. (1975) that the ketone group may be involved in the initial stages of aflatoxin B_1 decomposition by ammonia.

In contrast to the results at 50 °C, no one product predominated when the ammoniation of 2 was carried out at 37 °C for 20 days. Products 3 and 4 and starting 2 were isolated in respective yield ranges of 4–7%, 8–10%, and 13–23%. The same products were formed after only 13 days but in diminished yields; in this case 2 was recovered in 52% yield. Most notable, however, was the formation of a new component in 2–8% yield that migrated on TLC just below ketophenol 3. The MS of this compound exhibits a molecular ion at m/e 250, 16 mass units greater than that of 3. An intense ion at m/e 154 attributed to facile production of the 3,5-dimethoxyphenol ion was weak in the MS of 3. The ^1H NMR spectrum closely resembled that of 3 except for the absence of a vinyl proton at δ 6.53. Major differences in the ^{13}C NMR spectra were in chemical shifts of the α- and β-vinyl carbons of the cyclopentene ring, which were at δ 131.5 and 173.8 in 3 and at δ 141.6 and 147.0 in the new compound. The signal at δ 147.0 is assignable to the enol carbon of an α-diketone because the corresponding carbon in cucurbitacin diosphenols is found at δ 147.2 (Yamada et al., 1978). Acetylation gave a diacetate as shown by MS and ^1H NMR evidence. These data establish the new product as an enolized α-diketone having the structure depicted by 5.

When ketocoumarin 2 was adsorbed onto Celite and ammoniated with 1.5% NH_3 at 37 °C for 14 days, 4 and 5 were obtained in respective yields of 7 and 3% plus 5% of unchanged 2 was recovered. So that this study could be extended to a system more closely resembling the process for ammoniation of aflatoxin-contaminated corn, 2 was adsorbed onto oven-dried corn germ and ammoniated with 1.5% NH_3 at 18% H_2O and 37 °C for 14 days. Extraction of the ammoniated corn followed by column chromatographic separation failed to isolate any of the expected ammoniation products. Crude extracts and appropriate chromatographic fractions were examined by GC–MS for 4, which was thought to be an end product of the degradation; however, none was detected. In order to explain the absence of recognizable products from the ammoniation of 2 on corn, we investigated stabilities of compounds 3–5 toward NH_3.

Treatment of ketophenol 3 with ammonium hydroxide at 37 °C for 21 days resulted in an 86% recovery of 3. Traces of 4 were detected by TLC. This lack of reactivity was anticipated, because 3 is the major product of the 50 °C ammoniation and the analogous aflatoxin D_1 is the major product from high temperature and pressure ammoniation of aflatoxin B_1. Because no diketone 5 was detected, it did not appear to be formed by oxidation of 3. Treatment of 5 with concentrated ammonium hydroxide at 37 °C for 3 days and examination of the organic extract by TLC and GC–MS established the presence of 3,5-dimethoxyphenol (4) plus unreacted 5. Compound 4 was found to be stable toward concentrated ammonium hydroxide at 37 °C for 13 days. However, when 4 was adsorbed onto corn germ and ammoniated and the appropriate chromatographic fraction was examined by GC–MS for the Me_3Si derivative of 4, none could be detected. In a control experiment using one-tenth the amount of 4 but no NH_3, the Me_3Si derivative of 4 was easily detected by GC–MS [m/e 226 (M^+), 211 ($M^+ - CH_3$)]. The contribution of corn germ to the reactivity of 4 during ammoniation appears to be a general oxidative surface effect, because replacement of germ by Celite still results in disappearance of 4 to give a multitude of products.

Ammonia-induced decomposition of the aflatoxin model ketocoumarin 2 proceeds by two paths. Formation of the decarboxylated ketophenol 3 is favored at the higher temperature (50 °C), whereas at 37 °C, compounds 3–5 are formed in approximately equal amounts accompanied by considerable brown uncharacterized material. Diketone 5 readily decomposes in aqueous ammonia to 4, which in turn is unstable to ammonia when distributed over a large surface area. The mechanism for formation of 5, presumably by oxidative decarboxylation of the ammonium salt of 2, is not known. In a similar reaction, cohulupone has been reported to undergo oxidative deacylation in which an isobutyryl moiety is replaced by hydroxyl to give hulupinic acid (Burton and Stevens, 1964). The simplest analogue of 5, cyclopentanedione, readily decomposes in air to a brown material (Koetz and Neukom, 1975). Although the fate of the cyclopentanedione ring of 5 is not known, the initial cleavage product from 5 would not be expected to be particularly stable. In their study of the ammoniation of [^{14}C]aflatoxin B_1 spiked peanut meal, Lee et al. (1979) could account for only 50% of the radioactivity, with the remainder presumed to be lost as volatiles. Loss of the cyclopentenone ring and lactone carbonyl of 1-[^{14}C]acetate-labeled aflatoxin B_1 as volatiles would represent 44% of the total radioactivity.

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Procedure for Minimizing Losses in Sample Processing and Assay of Rubratoxin B from Mixed Feed

George W. Engstrom* and John L. Richard

A study was conducted on some factors that affect recovery of rubratoxin B from mixed feed. Results are presented that show that storage temperature, feed moisture content, and atmosphere have a marked affect on the deterioration rate of rubratoxin B as measured by its recovery from mixed feed. The method of sample processing and extraction that evolved from this study included brief storage at freezer temperature (−20 °C) and a 5-min Waring blender extraction with 5% acetic acid in ethyl acetate at 6 °C. The high-pressure liquid chromatography assay system included a regular-phase column and the ethyl acetate–chloroform–acetic acid (80:20:1 v/v/v) solvent system. Retention time of rubratoxin B was 7.9 min, k' was 1.53, and percent recovery was 79% for the first extraction and an additional 5–10% for the second extraction. This system provided excellent resolution and detected as little as 5 ng of rubratoxin B.

Rubratoxin B is produced by *Penicillium rubrum* Stoll and *Penicillium purpurogenum* Stoll when grown on various agricultural commodities and laboratory media (Hayes et al., 1970; Emeh and Marth, 1976). *P. rubrum* Stoll was initially described as a toxigenic organism following isolation from moldy corn (Burnside et al., 1957). *P. rubrum* and *Aspergillus flavus* Link were the only fungi of 13 isolated by Burnside et al. (1957) from that moldy corn that caused illness and death when fed to swine, horses, and mice. Oral administration of artificially contaminated feedstuffs resulted in congestion and hemorrhages in several organs, especially the liver and kidney (Forgacs et al., 1958).

Rubratoxin B was extracted and partially purified by Wilson and Wilson (1962) and purified and characterized by Townsend et al. (1966), and the chemical structure was determined by Moss et al. (1967, 1968, 1969) and Büchi et al. (1970). Rubratoxin B decomposed on melting and pyrolytic decomposition resulted in the loss of a molecule of carbon dioxide. When a UV spectrum was done in a hydroxylic solvent, the strong absorption maximum at 250 nm produced by the disubstituted maleic anhydride function decreased in intensity. This decrease demonstrated the ease with which the anhydride rings equilibrated with the open form (acid) (Moss, 1971). Moss et al. (1971) have reported that rubratoxin B acts as an acid in aqueous solutions.

Methods for extraction and purification of rubratoxin B have been reported for cultures grown on liquid media, corn, rice, and soybeans (Wilson and Wilson, 1962; Hayes and Wilson, 1968; Natori et al., 1970; Hayes and McCain, 1975; Emeh and Marth, 1977). Recently, Whidden et al. (1980) published a method for detection of rubratoxin B and seven other mycotoxins from corn. Although 67% of the rubratoxin B was extracted, only 31% was recovered at the end of the assay. They suggested that the difference was due to the length and complexity of the multimycotoxin analysis and the instability of rubratoxin B.

This paper defines some factors responsible for the deterioration of rubratoxin B and describes an improved method of sample handling, extraction, and assay of rubratoxin B from mixed feed.

EXPERIMENTAL SECTION

Materials. Crude rubratoxin B was extracted from the liquid of surface cultures of *P. rubrum* Stoll and purified by the method of Hayes and Wilson (1968). This preparation was further purified by chromatography on organic Sephadex LH-20 (100 × 2.4 cm) in acetonitrile. Purity of the rubratoxin B standard was determined by high-pressure liquid chromatography (HPLC) in three nonaqueous solvent systems: (a) ethyl acetate–chloroform–butanol–acetic acid, 40:55:4:1 v/v/v/v; (b) ethyl acetate–chloro-

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