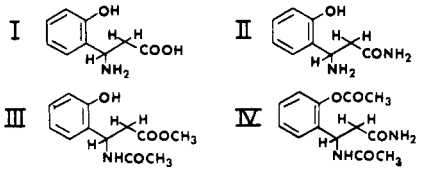
Reaction of Coumarin with Aqua Ammonia. Implications in Detoxification of Aflatoxin

Ammonia and coumarin were found to undergo a typical Michael addition reaction to give β-aminohydrocoumaric acid, which is a water soluble β-amino acid. It is suggested that a similar addition may be responsible for the detoxification of aflatoxin-containing feeds by ammonia instead of a simple opening of the lactone ring by hydrolysis.

It has been demonstrated that treatment of aflatoxin-contaminated meals with aqueous ammonia resulted in detoxification (Masri et al., 1969), as determined by chemical analysis for aflatoxin as well as by bioassay procedures with ducklings. Consequently, research was initiated to determine the products of reaction in order to understand the mechanism. Assuming that the difuro portion of aflatoxin, being an intramolecular acetal, is probably unreactive in base, we made a preliminary investigation with unsubstituted coumarin as a model system. The aflatoxins and coumarin resemble ethyl cinnamate in having a reactive position β to the carboxyl carbon atom (C_4) for Michael addition (Bergmann et al., 1959). Mild conditions were selected for the investigation to simulate one of the systems which proved successful in detoxifying aflatoxin-contaminated oil seed meals (Masri, et al., 1969).

The reaction of coumarin with aqua ammonia was carried out at room temperature with occasional stirring for 3 weeks, yielding products which varied depending on the recovery procedure. If initial concentration of the reaction mixture was on the steam bath, the main product was β-aminohydrocoumaric acid (I); concentration in vacuo yielded its amide II. The overall conversion by either method was about 30%. The amide was hydrolyzed to the acid by heating in water on the steam bath.



Proof of the structure of β-aminohydrocoumaric acid was based on infrared and ultraviolet spectra and comparison with authentic material prepared by the method of Posner (1909) and on a comparison of their acetates.

By structural analogy we may expect that the aflatoxins might undergo similar reactions with ammonia to yield relatively water-soluble β-amino acids or, possibly, further degradation products. If such a Michael addition reaction occurs with the aflatoxin, it would not be surprising if the formed β-aminohydrocoumaric acid derivatives were devoid of toxicity or were of a much lower order of toxicity. It is known that slight structural changes in the aflatoxins result in profound concomitant changes in toxicity. For example, aflatoxin B_1 is more toxic than G_1 which in turn is more toxic than B_2, B_{2a}, G_2, M_1, and Q_1. Also, the carcinogenicity of the aflatoxins appears related to epoxidation of the double bond of the terminal furan ring through microsomal action in mammals. The metabolites formed may interact with cellular DNA by intercalation and "stacking". It would appear that the flat shape of the metabolites is conducive to this interaction. Steric changes such as may result from introduction of hydroxyl groups in M_1 and Q_1 and likewise in the formation of β-amino derivatives proposed in this paper may limit the intercalation and stacking processes (Masri et al., 1967, 1974a,b). Carcinogenicity tests of Q_1 with trout as well as mutagenicity tests in bacterial systems indicate that aflatoxin Q_1 is essentially noncarcinogenic (Masri and Sinnhuber, 1976). Chloroform extract of reaction products of ammonia and aflatoxin under high-temperature conditions have been partially identified by Lee et al. (1974). Under these conditions they recovered different types of reaction products from those that could be expected from the present report. They have not reported compounds resembling the β-amino acid obtained from coumarin; but, it is possible that the compounds were missed as a result of their isolation procedures. Whether aflatoxin B_1 undergoes Michael addition with ammonia similar to coumarin is not known. However, the reaction of coumarin described here is of interest, in itself, in view of the ubiquitous distribution of coumarin derivatives in plants.

EXPERIMENTAL SECTION

Melting points were uncorrected and taken on a Kofler block viewed under a low-power microscope. The infrared spectrum of β-aminohydrocoumaric acid was taken in Nujol mull, all others in chloroform, and ultraviolet spectra in absolute ethanol or water.

β-Aminohydrocoumaric Acid (I). Finely ground coumarin (10 g) was suspended in 28% aqueous ammonia (250 mL) in a round-bottom flask and allowed to stand with occasional stirring for 20 days at room temperature in subdued light. The resulting deep-green solution was transferred to a 500-mL beaker and evaporated to small volume by immersion in live steam. Methanol (100 mL) was added yielding an off-white microcrystalline precipitate. The mixture was refrigerated and 3.1 g of product collected by filtration (mp 204–208 °C). Recrystallization from a water–methanol mixture (1:4 v/v) gave colorless microprisms of pure β-aminohydrocoumaric acid melting at 213–216 °C.

Anal. Calcd for $C_9H_{11}O_3N$: C, 59.68; H, 6.12; N, 7.73. Found: C, 59.5; H, 6.05; N, 7.78.

UV spectrum in water: λ_{max} 272 nm (ϵ 2030); 212 nm (ϵ 5770). UV spectrum in base: λ_{max} 290 nm (ϵ 3380); 238 nm (ϵ 6980).

These absorption maxima are characteristic of alkylated phenols resulting from the loss of the conjugated chromophore of coumaric acid.

The infrared spectrum had a multiple band of absorption at 3100–2600 and 1650–1500 cm^{-1}, characteristic of an amino acid zwitterion (Bellamy, 1954).

The methanolic filtrate was evaporated to a brown oil and acetone (50 mL) was added, leaving a residue of beige amorphous powder (1.36 g) which was insoluble in acetone and water but soluble in methanol. Chromatography of this material on thin layers of silica gel–starch containing fluorescent mineral phosphor (Miller and Kirchner, 1954) gave four spots when viewed under ultraviolet light. No attempt was made to identify these compounds.

The filtrate from acetone treatments was evaporated to a dark-brown oil which was triturated with water (50 mL) to yield a tan residue which was filtered off (5.4 g) and found by mixed melting point to be identical with coumarin starting material.

The monoacetate III of the methyl ester of the acid I was prepared by heating the acid I with acetic anhydride and quenching the reaction with excess methanol to yield a precipitate which melted at 146–148 °C after recrystallization from hot water.

Anal. Calcd for $C_{12}H_{15}O_4N$: C, 60.75; H, 6.37; N, 5.90. Found: C, 60.9; H, 6.36; N, 5.88.

The dibenzoate of the methyl ester of β-aminohydrocoumaric acid was prepared from the acid I by the Schotten-Baumann reaction with benzoyl chloride. The oily product mixture was taken up in methanol, and water was added to induce crystallization. The product, on recrystallization from aqueous methanol, melted at 146–147 °C.

Anal. Calcd for $C_{24}H_{21}O_5N$: C, 71.45; H, 5.25; N, 3.47. Found: C, 71.5; H, 5.18; N, 3.47.

The monoacetate and dibenzoate of methyl β-aminohydrocoumarate were prepared from β-aminohydrocoumaric acid which in turn was prepared from coumarin with hydroxylamine by the method of Posner (1909) and found to be identical with those from the reaction with ammonia.

β-Aminohydrocoumaramide (II). Finely ground coumarin (10 g) was suspended in 28% aqueous ammonia (250 mL) and allowed to stand for 20 days at room temperature in subdued light with occasional stirring. The deep-green reaction mixture was evaporated down to small volume with a rotary vacuum evaporator under water aspirator vacuum and at a bath temperature of 40 °C. The brown syrupy residue was extracted with four 250-mL portions of chloroform, and the combined extracts were evaporated on the steam bath to 100 mL at which point crystals began to appear. The mixture was cooled and 3.3 g of white crystalline material was recovered. Recrystallization from a large volume of chloroform yielded clusters of blades of β-aminohydrocoumaramide, mp 125–126 °C.

Anal. Calcd for $C_9H_{12}O_2N_2$: C, 59.98; H, 6.71; N, 15.55. Found: C, 59.7; H, 6.58; N, 15.3.

The diacetate IV of the amide II was prepared by heating on the steam bath for 3 min 0.2 g of II in 5 mL of acetic anhydride containing 0.2 g of freshly fused sodium acetate. The reaction mixture was poured into water and a white solid was collected, yielding felted needles from acetone–methanol, mp 228–230 °C.

Anal. Calcd for $C_{13}H_{16}N_2O_4$: C, 59.08; H, 6.10; N, 10.60. Found: C, 59.2; H, 6.14; N, 10.6.

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Microbial Transformation of *S*-Methyl *N*-[(Methylcarbamoyl)oxy]thioacetimidate (Methomyl) in Soils

Microbial transformations of methomyl in two tobacco-growing soils were determined in a perfusion study where the soils (10 g) were perfused with aqueous solutions (230 cm^3) containing 6 ppm of methomyl with and without sodium azide. Adsorption of methomyl was indirectly assessed. The contribution of adsorption to the loss of methomyl from solutions was small (approximately 5%) when compared with that of microbial transformation. Microbial transformation of methomyl in both soils occurred after a lag phase of about 7 to 14 days. However, in enriched soils, transformation occurred with virtually no lag phase.

The application of methomyl to soil in transplant water to control tobacco yellow dwarf in Australia necessitates the investigation of the behavior of methomyl in soil. It has been shown that methomyl decomposes in soil (Harvey and Pease, 1973); however, individual processes influencing the behavior of methomyl in soil have not been investigated. Microbial transformation and adsorption would appear to be the most important processes affecting the behavior of methomyl in soil. The combined effects of microbial transformation and adsorption of methomyl in two soils were determined (experiment 1) by perfusing the soils with solutions of methomyl and by measuring the changes in concentration of methomyl in the perfusing solutions at regular intervals. To assess indirectly the effect of adsorption (experiment 2) sodium azide was added to the solutions to prevent any microbial trans-