When the reaction of an equivalent amount of nitrosyl chloride with a 5% solution of amine I in dimethylformamide was carried out in a 0.1-mm. sodium chloride cell of a Perkin-Elmer Model 21 spectrophotometer at $-46°$, a new absorption at 2090 cm.$^{-1}$ developed quickly and its decay at $-10 \pm 1°$ was followed over a period of 2–6 hr. A plot of log A_0/A *vs.* time gave a reasonably straight line with k_1 (average of 7 points) = 6.25×10^{-4} sec.$^{-1}$ and in another reaction 5.35×10^{-4} (average of 5 points). In dimethyl sulfoxide at $-11 \pm 1°$, k_1 (average of 8 points) was 5.00×10^{-4}. Details of the calculations are presented in the thesis of M. L. F.[1]

Dehydrohalogenation of the Dichloride XV. Reaction of 20 mg. of dichloride XV with 5 ml. of alcoholic potassium hydroxide on a steam bath gave an immediate orange color and after 10 min. the solution was neutralized and the products were partitioned between ether and water. Chromatography of the ether layer on neutralized alumina (elution with cyclohexane) gave 6 mg. of chlorindenone XIV, m.p. 93–94° (lit.[30] m.p. 93–94°). An authentic sample, m.p. 92–93°, was prepared from 2-phenyl-1,3-indandione and phosphorus pentachloride in carbon tetrachloride.[30]

α-Cyanobibenzyl (XX), b.p. 138–145° at 0.4–0.5 mm., m.p. 50–51° (lit.[31] m.p. 57–58°), had absorption at 2250 cm.$^{-1}$ in the infrared (chloroform) and a triplet centered at τ 6.02 (J = 14.5 c.p.s.) and a doublet centered at τ 6.87 (relative areas 1.0 and 2.1) in the n.m.r. in deuteriochloroform.

(31) C. R. Hauser and W. R. Basen, *J. Am. Chem. Soc.*, **78**, 494 (1956).

α,α-Dibenzylphenylacetonitrile (XXIII), m.p. 83–84° (lit.[31] 92–92.5°), showed weak absorption at 2250 cm.$^{-1}$ in the infrared (chloroform) and a singlet at τ 6.72 in the n.m.r. (deuteriochloroform).

α-Carboxybibenzyl (XXI), m.p. 94–94.5° (lit.[31] m.p. 88–89°), prepared from the nitrile XX by hydrolysis with sodium hydroxide in aqueous trimethylene glycol, showed in the n.m.r. in deuteriochloroform an ABC pattern[24a] with chemical shifts of H_A, H_B, and H_C at τ 6.00, 6.57, and 6.97; $J_{AB} = J_{AC} = 7.8$, $J_{BC} = 15.7$ c.p.s.

α-Cyano-o-chlorobibenzyl (XXII) was prepared from *o*-chlorobenzyl chloride by the same procedure employed with the parent nitrile XX. After distillation and crystallization there was obtained in 68% yield the chloronitrile XXII, m.p. 62–63°. The infrared absorption in chloroform showed absorption typical of the nitrile group at 2240 cm.$^{-1}$. The n.m.r. in deuteriochloroform showed a quartet centered at about τ 5.9 and another absorption at τ 7.8. Each of these showed evidence of further coupling between H_A and the *gem*-protons H_B and H_C.

Anal. Calcd. for $C_{15}H_{12}NCl$: C, 74.5; H, 5.8; N, 5.8; Cl, 14.7. Found: C, 74.8; H, 5.2; N, 5.7; Cl, 14.7.

α,α-Di(o-chlorobenzyl)phenylacetonitrile (XXIV) was obtained from the distillation residue in the preparation of XXII and recrystallized from ethanol, m.p. 92–93°. The infrared spectrum in chloroform showed absorption at 2240 cm.$^{-1}$. The n.m.r. in deuteriochloroform showed an AB quartet with centers of the two pairs of peaks at τ 6.37 and 6.55 (J = 14 c.p.s.).

Anal. Calcd. for $C_{22}H_{17}NCl_2$: C, 72.1; H, 4.7; N, 3.8. Found: C, 71.8; H, 4.6; N, 3.5.

The Structures of Aflatoxins B and G_1

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Contribution from the Department of Chemistry and the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts. *Received November 16, 1964*

The structures of the two mold metabolites aflatoxin B_1 and aflatoxin G_1 were elucidated. Acute toxicities in White Pekin ducklings are presented.

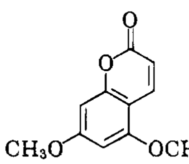
Aflatoxins are metabolites of *Aspergillus flavus* Link *ex* Fries, a fungus which under certain conditions grows prolifically on peanuts and on cereals.[1,2] The two compounds are of considerable interest because of their toxicity and carcinogenic potency in many animal species. Preliminary reports from other laboratories dealt with the isolation and characterization of two toxins,[3-5] and the present paper is concerned with the chemical structures of the two major metabolites.

Early attempts to produce the toxins in these laboratories led to concentrates with low activities, and isolation of the toxins became possible only when we received 200 mg. of a crude extract prepared by investigators of the U. S. Food and Drug Administration utilizing a different mold variant.[6] Cultures of *Aspergillus flavus* Link *ex* Fries were grown on sterilized crushed wheat and extracted with chloroform and the toxins precipitated by adding petroleum ether. Individual components were isolated from this concentrate by preparative thin layer chromatography, and the two

(1) R. Allcroft and R. B. A. Carnaghan, *Chem. Ind.* (London), 50 (1963).
(2) K. Sargeant, R. B. A. Carnaghan, and R. Allcroft, *ibid.*, 53 (1963).
(3) B. F. Nesbitt, J. O'Kelly, K. Sargeant, and A. Sheridan, *Nature*, **195**, 1062 (1962).
(4) A. S. M. van der Zijden, W. A. A. B. Koelensmid, J. Boldingh, C. B. Barrett, W. O. Ord, and J. Philp, *ibid.*, **195**, 1060 (1962).
(5) H. DeIongh, R. K. Beerthuis, R. O. Vles, C. B. Barrett, and W. O. Ord, *Biochim. Biophys. Acta*, **65**, 548 (1962).
(6) We are much indebted to Drs. H. R. Smith, F. A. Hodges, B. H. Armbrecht, and W. Horwitz for their contribution.

major components were easily located by inspection of the chromatoplates under ultraviolet light.

The substance exhibiting blue fluorescence had m.p. 268–269° dec.; $[\alpha]^{CHCl_3}D$ −558°; λ_{max}^{EtOH} 223, 265, and 362 mμ (ε 25,600, 13,400, and 21,800); $\nu_{max}^{CHCl_3}$ 1760 (very intense), 1684 (weak), 1632, 1598, and 1562 cm.$^{-1}$. These physical constants demonstrated identity with the previously described aflatoxin B_1[3–5] and the molecular weight found to be 312 by mass spectrometry agreed with the composition $C_{17}H_{12}O_6$.[7] The ultraviolet and infrared spectra of the metabolite were not particularly revealing at the outset of this study except that the absence of bands in the infrared above 3500 cm.$^{-1}$ excluded the presence of hydroxyl functions. Aflatoxin B_1 represents a highly unsaturated molecule, and it was hoped that reduction might lead to a product with more readily interpretable spectral properties. Catalytic reduction in ethanol solution over a palladized charcoal catalyst was complete after 3 equiv. of hydrogen was absorbed, and tetrahydrodesoxoaflatoxin B_1 was formed in essentially quantitative yield. It had infrared absorptions ($CHCl_3$) at 1705, 1625, and 1610 cm.$^{-1}$, and its ultraviolet spectrum [λ_{max}^{EtOH} 255, 264, and 332 mμ (ε 8500, 9200, and 13,900)] was strikingly similar in shape to that of 5,7-dimethoxycoumarin (**1**) [λ_{max}^{EtOH} 247, 250, and 324 mμ (ε 6920, 6920, 15,150)][8] but sufficiently different from other dialkoxycoumarins to allow the conclusion that tetrahydrodesoxoaflatoxin B_1 is a 5,7-



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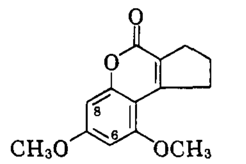

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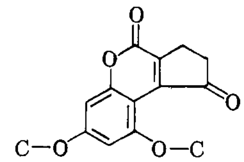
dialkoxycoumarin. For reasons which will become clear in the sequel, 5,7-dimethoxycyclopenten[*c*]-coumarin (**2**) was prepared and found to have λ_{max}^{EtOH} 248, 257, and 325 mμ (ε 7700, 7000, and 16,100). Thus, the ultraviolet spectrum of this model compound corresponded even more closely to that of tetrahydrodesoxaflatoxin B_1, and the remaining lateral displacement could be attributed to the presence of an additional carbon substituent in the reduction product. The empirical change accompanying the catalytic reduction of aflatoxin B_1 demanded the presence of an olefinic double bond and a carbonyl group in the molecule, and to rationalize the hydrogenolysis it was necessary to place the latter functionality in conjugation with either the double bond or with the coumarin ring. As already mentioned briefly, the infrared spectrum of aflatoxin B_1 possesses a high intensity absorption band at 1760 cm.$^{-1}$ and a low intensity band at 1684 cm,$^{-1}$, while coumarin **1** and tetrahydrodesoxoaflatoxin display the anticipated absorptions at 1705 and 1706 cm.$^{-1}$, respectively. The two absorption bands in the spectrum of the natural product are attributable to coumarin and ketone carbonyl groups only if these functionalities are present in a unique structural relationship. It was assumed that the ketone function was attached to the C-4 or preferably to the C-3 position of the coumarin nucleus and located on a five-membered carbocyclic ring. Furthermore, an n.m.r. spectrum[9] of aflatoxin B_1 with A_2B_2 absorption at δ 3.42 and 2.61 indicated that the remaining two carbon atoms of the cyclopentane ring are substituted by four hydrogen atoms. Of the two part structures **3** and **4**, the former was unequivocally excluded as follows. Condensation of phloroglucinol dimethyl



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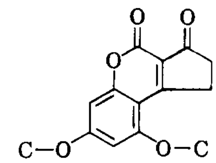
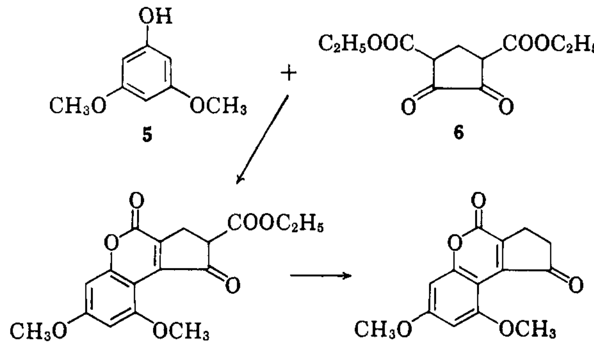


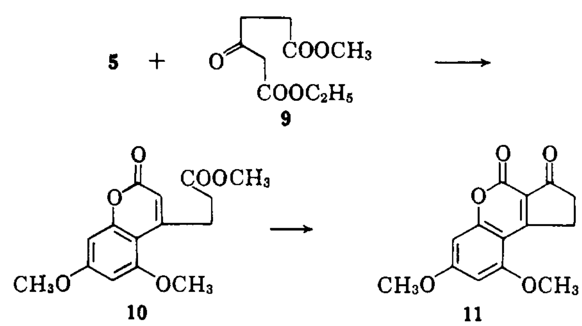
4

ether (**5**) with diethyl cyclopentane-4,5-dione-1,3-dicarboxylate (**6**) yielded the coumarin **7** (or its tautomer) which by exposure to hot hydrochloric acid was transformed to 5,7-dimethoxycyclopentenone[3,2-*c*]coumarin (**8**): $\nu_{max}^{CHCl_3}$ 1726 cm.$^{-1}$ (broad); λ_{max}^{EtOH} 245, 268, and 356 mμ (ε 13,200, 8700, and 9000). These spectral



5 **6** **7** **8**

data differ markedly from those of aflatoxin B_1 and to secure positive evidence in favor of part structure **4**, 5,7-dimethoxycyclopentenone[2,3-*c*]coumarin (**11**) was synthesized. Von Pechmann condensation of phloroglucinol dimethyl ether with the β-ketoester **9** furnished a coumarin **10** which on cyclization with polyphosphoric acid yielded the tricyclic ketone **11** whose spectral characteristics [$\nu_{max}^{CHCl_3}$ 1759 (intense), 1685 (weak), 1614, 1594, and 1550 cm.$^{-1}$; λ_{max}^{EtOH} 215, 257, and 355 mμ (ε 22,200, 9650, and 26,800)] demonstrated beyond question that aflatoxin B_1 contains part structure **4**.



9 **10** **11**

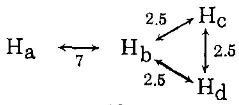
It remained to locate five carbon atoms, and one of these was part of a methoxy group because the n.m.r. spectrum of aflatoxin B_1 had a three-proton singlet at δ 4.02. The spectrum exhibits additional signals at

(7) Mass spectra were kindly measured by Professor K. Biemann and Mr. H. Schnoes, M.I.T.

(8) A. G. Caldwell and E. R. H. Jones, *J. Chem. Soc.*, 540 (1945).

(9) N.m.r. spectra were measured on a Varian Associates A-60 instrument in $CDCl_3$ solutions. Chemical shifts are given in p.p.m. from an internal tetramethylsilane standard.

δ 6.89 (doublet, J = 7 c.p.s., H_a); 6.52 (triplet, J = 2.5 c.p.s., H_c); 5.53 (triplet, J = 2.5 c.p.s., H_d); 4.81 (triplets of doublet, J = 2.5 and 7 c.p.s., H_b). This strikingly simple four-proton pattern can be explained if the three coupling constants J_{bc}, J_{bd}, and J_{cd} are identical, and the relationship of the protons is illustrated in **12**.



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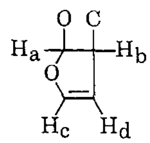
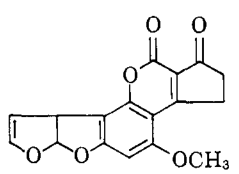


13

Such a situation has previously been encountered with 2,3-dihydrofuran,[10] and to account for its large chemical shift the H_a proton must be attached to an acetal carbon atom. When translated into structural terms, expression **13** resulted. The missing hydrogen atom in aflatoxin-B_1 appears as a one-proton singlet (δ 6.51) superimposed on the H_c triplet, and at this stage all atoms present in aflatoxin B_1 were accounted for. The remaining task was to ascertain the orientation of the dihydrofuran ring.

No signals due to vinylic protons appear in the n.m.r. spectrum of tetrahydrodesoxoaflatoxin B_1, but peaks due to the acetal (δ 6.42, doublet, J = 5.5 c.p.s.) and methoxy protons (δ 3.82, singlet) are easily discernible. Furthermore, the portion of the spectrum representing the six cyclopentane protons was identical in detail with the corresponding region in the spectrum of the model compound **2**. The peaks due to the aromatic protons of the synthetic coumarin **2** appeared at δ 6.35 (doublet, J = 2.5 c.p.s.) and 6.55 (doublet, J = 2.5 c.p.s.). Theoretical considerations[11] demand that the high-field signals are due to the C-6 proton, and because the aromatic proton in tetrahydrodesoxoaflatoxin B_1 appears at δ 6.30 it should be located on a carbon atom situated between two alkoxy substituents. Further support for the placement of the aromatic hydrogen atom in aflatoxin B_1 was provided by comparison of its resonance (δ 6.51) with that of the corresponding proton in sterigmatocystin (**18**) (δ 6.45).[12,13] These findings seem to exclude structures **16** and **17**. Aflatoxin B_1



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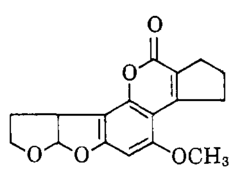

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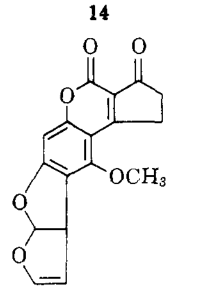

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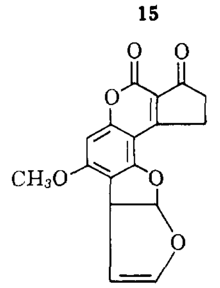


17

(10) L. M. Jackman, "Applications of Nuclear Magnetic Resonance Spectroscopy," Pergamon Press, New York, N. Y., 1959, p. 88.

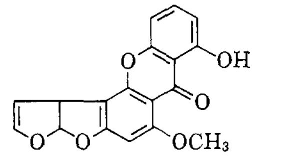
(11) P. Diehl, *Helv. Chim. Acta*, **44**, 829 (1961), and earlier references cited.

(12) E. Bullock, J. C. Roberts, and J. G. Underwood, *J. Chem. Soc.*, 4179 (1962).

(13) E. Bullock, D. Kirkaldy, J. C. Roberts, and J. G. Underwood, *ibid.*, 829 (1963).

and tetrahydrodesoxoaflatoxin B_1 are represented by **14** and **15**, respectively.

The second metabolite with yellow-green fluorescence [m.p. 244–246° dec.; $[\alpha]^{CHCl_3}{}_D$ −556°; λ_{max}^{EtOH} 243, 257, 264, and 362 mμ (ε 11,500, 9900, 10,000, and 16,100); $\nu_{max}^{CHCl_3}$ 1760, 1695, 1630, and 1595 cm.$^{-1}$] was identical with aflatoxin G_1.[3-5] Its molecular weight, found to be 328 by mass spectrometry,[7] agreed with that calculated for the previously proposed composition $C_{17}H_{12}O_7$. An n.m.r. spectrum of the metabolite exhibited an A_2X_2 pattern (δ 4.47, triplet, J = 6 c.p.s., and δ 3.48, triplet, J = 6 c.p.s.), and the chemical shifts and multiplicities of all other protons were identical with those of aflatoxin B_1 (**14**). We concluded that aflatoxin G_1 has structure **19**.



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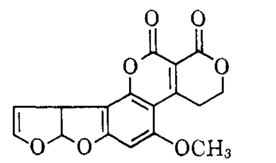
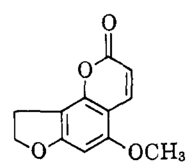


19

After publication of our preliminary communication on the structures of aflatoxins B_1 and G_1,[14] several papers appeared describing results obtained in other laboratories. The chemistry of aflatoxin B_1 was studied by Dutch investigators[15] who provided independent evidence for the presence of a methoxydihydrofuranocoumarin moiety. Furthermore, in a personal letter dated July 10, 1963, Dr. D. A. van Dorp revealed additional evidence in favor of structure **14**. The ultraviolet absorption spectra of 4′,5′-dihydroisobergaptene (**20**) and 4′,5′-dihydrobergaptene (**21**) differ in the relative intensities of the two maxima at 255 and 265 mμ, respectively, and comparison with the spectrum of tetrahydrodesoxoaflatoxin B_1 (**15**) indicated strongly that the latter compound belongs to the isobergaptene series. The benzenoid protons in the two model compounds appeared at δ 6.27 and 6.41,



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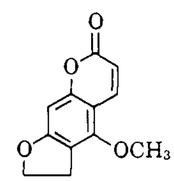

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respectively, and comparison with **15** (δ 6.30) provided a further argument in favor of structure **14** for aflatoxin B_1.

A communication from the Tropical Products Institute, London,[16] described the spectral properties of the aflatoxins in detail, and, although the presence of a dihydrofuran ring was recognized, no structures were proposed. Structures tentatively suggested by South African workers[17] are excluded by evidence presented in this paper. Finally, an X-ray analysis of aflatoxin

(14) T. Asao, G. Büchi, M. M. Abdel-Kader, S. B. Chang, E. L. Wick, and G. N. Wogan, *J. Am. Chem. Soc.*, **85**, 1705 (1963).

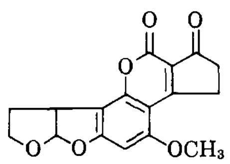
(15) D. A. van Dorp, A. S. M. van der Zijden, R. K. Beerthuis, S. Sparreboom, W. O. Ord, H. DeIongh, and R. Keuning, *Rec. trav. chim.*, **82**, 587 (1963).

(16) R. D. Hartley, B. F. Nesbitt, and J. O'Kelly, *Nature*, **198**, 1056 (1963).

(17) K. J. vander Merwe, L. Fourie, and de B. Scott, *Chem. Ind.* (London), 1660 (1963).

G_1 is in complete accord with structure **19** and revealed a *cis* fusion of the two dihydrofuran rings.[18]

Aspergillus flavus produces two additional hepatotoxic metabolites which were called aflatoxin B_2 and aflatoxin G_2. The two compounds are identical with dihydroaflatoxins B_1[15–17,19] and G_1[16,17], respectively, and are consequently represented by structures **22** and **23**.



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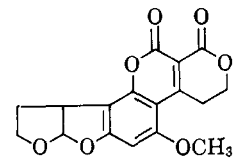

23

Acute toxicities were determined by biological assay in day-old White Pekin ducklings. Groups of ten animals weighing 51 ± 4 g. received various dose levels of the appropriate compound dissolved in propylene glycol, each animal receiving 0.1 ml. by stomach tube. Mortality and body weights were recorded 48 hr. after administration. Under these conditions the LD_{50} for aflatoxin B_1 (**14**) was calculated as 28.2 μg. with 95% confidence limits of 24.7 and 32.2 μg. The LD_{50} for aflatoxin G_1 (**19**) was estimated to be 90 μg. Administration of 50 μg. of the reduction product **15** resulted in no mortality compared to 100% mortality with the same dose of the parent compound **14**.[20]

Subacute toxicity of the compounds was determined in ducklings by their potency in the induction of hyperplasia of the bile duct epithelium. Animals received five daily 0.05-ml. administrations (by stomach tube) of dimethyl sulfoxide solutions of the compounds and were killed on the seventh day. Severity of bile duct hyperplasia was evaluated histologically. Daily doses of 0.4 μg. of aflatoxin B_1 caused detectable lesions. Comparable effects were caused by 1.56 μg./day of aflatoxin G_1, indicating the lower potency of the latter compound.[20]

Experimental

Melting points determined on a Kofler hot-stage microscope are corrected. Infrared spectra were measured in chloroform solutions on a Perkin-Elmer Infracord Model 237. Ultraviolet spectra were measured in ethanol solutions using a Cary recording spectrophotometer, Model 11. Optical rotations were determined in chloroform on a Zeiss polarimeter using a 1-dm. tube. Microanalyses were performed by the Midwest Microanalytical Laboratory, Indianapolis, Ind.

Isolation of Aflatoxins B_1 and G_1. The crude mixture of toxins (210 mg.) supplied by the U. S. Food and Drug Administration was applied to chromatoplates coated with silica gel G (according to Stahl) purchased from E. Merck AG, Darmstadt, Germany. Development with a mixture of chloroform–methanol (97:3) produced horizontal bands which showed fluorescence under ultraviolet light. The blue fluorescent band with an R_f value of 0.56 was scraped off the plates, and the silica gel was subsequently extracted with chloroform–methanol (1:3 by volume). After the solvent had been removed by evaporation *in vacuo*, the residue was dissolved in chloroform and passed through a column of Merck acid-washed alumina (5 g.). Evaporation gave colorless crystals (68 mg.) and six recrystallizations from chloroform–methanol yielded pure aflatoxin B_1 (**14**) (24 mg.), colorless prisms: m.p. 268–269° dec.; mol. wt. 312 (mass spec.); $[\alpha]^{CHCl_3}D$ −558° (*c* 1.50); λ_{max}^{EtOH} 223, 265, and 362 mμ (ϵ 25,600, 13,400, and 21,800); $\nu_{max}^{CHCl_3}$ 1760, 1684, 1632, 1598, and 1562 cm.$^{-1}$. A second crop of aflatoxin B_1 (25 mg.) with the same melting point was obtained from the mother liquors.

A band with yellow-green fluorescence and an R_f value of 0.48 was also removed from the chromatoplates, and extraction of the silica gel with chloroform (100 ml.) and with methanol (80 ml.) yielded two extracts which were combined. After the solvent was removed, the residue was dissolved in chloroform and the solution passed through a column of Merck acid-washed alumina (5 g.). Removal of the solvent from the filtrate yielded pale yellow crystals (57 mg.). Five recrystallizations from chloroform–methanol furnished pure aflatoxin G_1 (**19**) (30 mg.), colorless needles: m.p. 244–246° dec.; mol. wt. 328 (mass spec.); $[\alpha]^{CHCl_3}D$ −556° (*c* 0.455); λ_{max}^{EtOH} 243, 257, 264, and 362 mμ (ϵ 11,500, 9900, 10,000, and 16,100); $\nu_{max}^{CHCl_3}$ 1760, 1695, 1630, 1595, and 1545 cm.$^{-1}$.

Tetrahydrodesoxoaflatoxin B_1 (**15**). Aflatoxin B_1 (**14**) (15.363 mg.) in ethanol (15 ml.) was hydrogenated in a Hösli microhydrogenator using a 10% Pd–C catalyst (35 mg.) at 21° and 717 mm. Hydrogen absorption was complete after an uptake of 3.82 ml. of hydrogen (145 min.). The catalyst was collected on a filter and washed with chloroform. The combined filtrates were evaporated to dryness giving colorless crystals (15.3 mg.). Recrystallization from methanol yielded pure tetrahydrodesoxoaflatoxin B_1 (**15**), 13 mg., colorless plates: m.p. 249–250°; blue fluorescence in ultraviolet light; mol. wt. 300 (mass spec.); $[\alpha]^{CHCl_3}D$ −398° (*c* 0.78)[21]; λ_{max}^{EtOH} 255, 264, and 332 mμ (ϵ 8500, 9200, and 13,900); $\nu_{max}^{CHCl_3}$ 1705, 1625, 1610, and 1580 cm.$^{-1}$.

5,7-Dimethoxycyclopenteno[c]coumarin (**2**). A solution of phloroglucinol dimethyl ether (**5**) (1.54 g., 0.01 mole) and ethyl cyclopentanone-2-carboxylate (1.56 g., 0.01 mole) in glacial acetic acid (10 ml.) was saturated with hydrogen chloride gas while the mixture was cooled externally with ice. After storage at room temperature overnight, the mixture was poured into ice–water and the precipitate collected by filtration. Recrystallization from ethanol gave colorless plates (2.0 g.): m.p. 182–184°; λ_{max}^{EtOH} 248, 257, and 325 mμ (ϵ 7700, 7000, and 16,100); $\nu_{max}^{CHCl_3}$ 1706, 1608, and 1567 cm.$^{-1}$.

Anal. Calcd. for $C_{14}H_{14}O_4$: C, 68.28; H, 5.73. Found: C, 67.90; H, 5.78.

Ketoester **7**. A solution of phloroglucinol dimethyl ether (**5**) (770 mg., 0.005 mole) and diethyl cyclo-

(18) K. K. Cheung and G. A. Sim, *Nature*, **201**, 1185 (1964).

(19) S. B. Chang, M. M. Abdel-Kader, E. L. Wick, and G. N. Wogan, *Science*, **142**, 1191 (1963).

(20) P. M. Newberne, G. N. Wogan, W. W. Carlton, and M. M. Abdel-Kader, *Toxicol. Appl. Pharmacol.*, **6**, 542 (1964).

(21) The melting point and optical rotation of tetrahydrodesoxoaflatoxin B_1 given in our preliminary communication (ref. 14) are incorrect. We wish to thank Dr. D. A. van Dorp for calling our attention to these errors.

pentane-4,5-dione-1,3-dicarboxylate (**6**) (1.21 g., 0.005 mole) in glacial acetic acid (15 ml.) was saturated with dry hydrogen chloride gas. After a reaction time of 9 hr. at room temperature, the mixture was poured into water (100 ml.), and the yellow precipitate (1.5 g.) was collected on a filter and washed with water. Recrystallization from ethanol afforded yellow needles, m.p. 203–205°.

Anal. Calcd. for $C_{17}H_{16}O_7$: C, 61.44; H, 4.85. Found: 61.21; H, 5.17.

5,7-Dimethoxycyclopentenon[3,2-c]coumarin (**8**). The ketoester **7** (400 mg.) was dissolved in a mixture of 5% hydrochloric acid (15 ml.) and dioxane (20 ml.), and the resulting solution was heated at 105° for 3 hr. The dark yellow solution was then diluted with water (500 ml.) and the mixture was extracted five times with chloroform (20 ml. each). All extracts were combined and washed with 5% aqueous sodium bicarbonate and water; the solution was dried with magnesium sulfate. After concentration to 5 ml. the material was poured onto a column of Merck acid-washed alumina (70 g.) and the material eluted with chloroform. The early eluates on evaporation produced yellow crystals (164 mg.). One recrystallization from ethanol–chloroform furnished yellow needles: m.p. 178–179°; λ_{max}^{EtOH} 245, 268, and 356 mμ (ϵ 13,200, 8700, and 9000); $\nu_{max}^{CHCl_3}$ 1726 (broad), 1614, and 1565 cm.$^{-1}$.

Anal. Calcd. for $C_{14}H_{12}O_5$: C, 64.61; H, 4.65. Found: C, 64.55; H, 4.85.

5,7-Dimethoxy-4-(2′-methoxycarbonylethyl)coumarin (**10**). A solution of phloroglucinol dimethyl ether (**5**) (4.6 g., 0.03 mole) and the ketoester **9**[22] (6.2 g., 0.03 mole) in glacial acetic acid (80 ml.) was saturated with dry hydrogen chloride gas. After 8 hr. at room temperature, the product (8.5 g.) was isolated in the manner described above. Recrystallization from a mixture of chloroform and cyclohexane gave silky needles: m.p. 124–124.5°; $\nu_{max}^{CHCl_3}$ 1725, 1620, 1608, and 1560 cm.$^{-1}$.

(22) D. K. Banjeree and K. M. Sivanandaian, *J. Org. Chem.*, **26**, 1634 (1961).

Anal. Calcd. for $C_{15}H_{16}O_6$: C, 61.64; H, 5.52. Found: C, 61.64; H, 5.67.

5,7-Dimethoxycyclopentenon[2,3-c]coumarin (**11**). The ester **10** (8.5 g.) was heated with polyphosphoric acid (250 g.) at 105–110° with occasional stirring. After 2 hr. the brown reaction mixture was poured into ice–water (2 l.) and the crystalline precipitate (7.9 g.) collected by filtration. Chromatography over Merck acid-washed alumina (150 g.) using chloroform as eluent gave starting ester **10** (400 mg.) and colorless crystals (7.0 g.). Recrystallization from chloroform–methanol yielded pure tricyclic ketone **11**: m.p. 248–249°; λ_{max}^{EtOH} 215, 237 (shoulder), 257, 345 (shoulder), and 355 mμ (ϵ 22,200, 14,600, 9650, 25,800, and 26,800); $\nu_{max}^{CHCl_3}$ 1759 (very intense), 1685 (weak), 1614, 1594, and 1550 cm.$^{-1}$.

Anal. Calcd. for $C_{14}H_{12}O_5$: C, 64.61; H, 4.65. Found: C, 64.46; H, 4.83.

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(23) NOTE ADDED IN PROOF. Professor A. F. Peerdeman and Mr. B. van Soest (Utrecht) have confirmed the structure of aflatoxin B_2 using X-ray analysis (private communication from Dr. D. A. van Dorp).

Spectrophotometric Determination of the Kinetics of the Pepsin-Catalyzed Hydrolysis of Certain Dipeptide Substrates

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The kinetics of the pepsin-catalyzed hydrolysis of N-carbobenzoxy-L-phenylalanyl-L-tyrosine and N-acetyl-L-phenylalanyl-L-tyrosine at pH 2 in 3.4% methanol at 35° have been determined by a spectrophotometric technique. The kinetic parameters for the former compound are $K_0 = 2.1 \pm 0.3 \times 10^{-4}$ *M,* $k_0 = 1.24 \pm 0.08 \times 10^{-2}$ *sec.*$^{-1}$*, and for the latter,* $K_0 = 1.95 \pm 0.18 \times 10^{-3}$ *M,* $k_0 = 4.66 \pm 0.44 \times 10^{-2}$ *sec.*$^{-1}$*. The results with the acetyl compound are in agreement with earlier data obtained by a different procedure.*

Only two quantitative experiments have been performed on the kinetics of the hydrolysis of simple synthetic substrates by pepsin.[2] Casey and Laidler[3] utilized a potentiometric formol titration to investigate the hydrolysis of N-carbobenzoxy-L-glutamyl-L-tyrosine and N-carbobenzoxy-L-glutamyl-L-tyrosine ethyl ester. Baker[4] employed primarily a ninhydrin analysis in her studies on the hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine (Ac-PheTyr) and N-acetyl-L-tyrosyl-L-tyrosine. In this paper we report a spectrophotometric technique which promises to be useful in pursuing detailed kinetic investigations on pepsin and in quantitatively evaluating pepsin activity. The pro-

(1) (a) This investigation was supported in part by Grant AM 08005-01 of the U. S. Public Health Service; (b) taken in part from the B. A. Thesis of J. J. S.

(2) (a) F. A. Bovey and S. S. Yanari in "The Enzymes," Vol. 4, P. D. Boyer, H. Lardy, and K. Myrback, Ed., 2nd Ed., Academic Press Inc., New York, N. Y., 1960, Chapter 4; (b) R. M. Herriott, *J. Gen. Physiol.*, **45**, part 2, 57 (1962).

(3) E. J. Casey and K. J. Laidler, *J. Am. Chem. Soc.*, **72**, 2159 (1950).

(4) L. E. Baker, *J. Biol. Chem.*, **211**, 701 (1954).