Chippel, D., Scrimgeour, K. G., *Can. J. Biochem.* **48**, 999 (1970).
Cosulich, D. B., Smith, J. M., *J. Am. Chem. Soc.* **71**, 3574 (1949).
Daiber, D., Preussmann, R., *Z. Anal. Chem.* **206**, 344 (1964).
Edwards, J. O., Abbot, J. R., Ellison, H. R., Nyberg, J., *J. Phys. Chem.* **63**, 359 (1959).
Fan, T. Y., Tannenbaum, S. R., *J. Food Sci.* **37**, 274 (1972).
Fan, T. Y., Tannenbaum, S. R., *J. Agric. Food Chem.* **21**, 237 (1973).
Gough, T. A., McPhail, M. F., Webb, K. S., Wood, B. J., Coleman, R. F., *J. Sci. Food Agric.* **28**, 345 (1977).
Masui, M., Nakahara, H., Ohmori, H., Sayo, H., *Chem. Pharm. Bull.*, **22**(8), 1846 (1974).
Mirvish, S. S., *Toxicol. Appl. Pharmacol.* **31**, 325–351 (1975).
Perrin, D. D., "Dissociation Constants of Organic Bases in Aqueous Solution", Butterworth, London, 1965.
Poe, M., *J. Biol. Chem.* **252**, 3724–3728 (1977).
Purchase, I. F. H., Longstaff, E., Ashby, J., Styles, J. A., Anderson, D., Leferre, P. A., Westwood, F. R., *Br. J. Cancer* **37**, 873 (1978).
Ramasastri, B. V., Blakley, R. L., *J. Biol. Chem.* **239**, 106 (1964).
Reed, L. S., Archer, M. C., *J. Chromatogr.* **121**, 100 (1976).
Roth, B., Hultquist, M. E., Fahrenbach, M. J., Cosulich, D. B., Broquist, H. P., Brockman, J. A., Jr., Smith, J. M., Jr., Parker, R. P., Stokstad, E. L. R., Jukes, T. H., *J. Am. Chem. Soc.* **74**, 3247 (1952).
Scanlan, R. A., *Crit. Rev. Food. Technol.* **5**, 357 (1975).
Stedman, G., *J. Chem. Soc.*, 1702 (1960).
Stokstad, E. L. R., Shin, Y. S., Tamura, T., in "Folic Acid Biochemistry and Physiology in Relation to the Human Nutrition Requirement", National Academy of Sciences, Washington, DC, 1977.
Vonderschmitt, D. J., Vitols, K. S., Huennekens, F. M., Scrimgeour, K. G., *Arch. Biochem. Biophys.* **122**, 488–493 (1967).
Waller, C. N., Goldman, A. A., Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., *J. Am. Chem. Soc.* **72**, 4630 (1950).
Wogan, G. N., Paglialunga, S., Archer, M. C., Tannenbaum, S. R., *Cancer Res.* **35**, 1981 (1975).

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A New Isochroman Mycotoxin Isolated from *Penicillium steckii*

Richard H. Cox,* Oscar Hernandez, Joe W. Dorner, Richard J. Cole, and Dorothy I. Fennell[1]

Penicillium steckii (NRRL 6336) was isolated from moldy millet hay suspected of causing deaths in cattle. The mold produced a toxic metabolite identified by spectroscopic means as 3,7-dimethyl-8-hydroxy-6-methoxyisochroman. The median lethal dose of the toxin in 1-day-old chickens was 800 mg/kg.

We have isolated a toxigenic strain of *Penicillium steckii* Zalecki (NRRL 6336) as a result of our continuing efforts to identify the etiological agents responsible for naturally occurring toxicoses associated with ingestion of molded feed. The fungus was one of several toxigenic fungi isolated from moldy millet hay suspected of causing deaths in cattle. Saito et al. (1971) have reported the isolation of a strain of *P. steckii* that was toxic to cultured cells and animals. Davis et al. (1975) have also reported the isolation of a strain of *P. steckii* from chocolate syrup that was toxic in the brine shrimp and chicken embryo bioassays. In neither of these cases were the toxin(s) identified. Some strains of *P. steckii* are capable of producing the nephrotoxin citrinin (Krogh, 1974).

EXPERIMENTAL SECTION

The toxigenic fungus was isolated from the suspect hay on potato dextrose agar (PDA) plates and was maintained at 5 °C after 7–10 days growth at 27 °C. The fungus was mass cultured in fifty 2.8-L Fernbach flasks containing 100 g of shredded wheat supplemented with 200 mL of mycological broth (pH 4.8), 15% sucrose, and 2% yeast extract. The cultures were incubated at 28 °C for 3 weeks prior to extraction. Extraction of the fungal cultures with hot chloroform yielded an oily extract that was highly toxic to day-old chickens.

The crude extract was systematically fractionated, and toxicity of the fractions was monitored with day-old chickens. The fractions were administered via crop intubation using 1 cm^3 of corn oil as the inert carrier at a rate of 1 cm^3 or less per chicken (Kirksey and Cole, 1974; Cole, 1978).

The crude chloroform extract was fractionated on a silica gel 60 column (9 × 18 cm) eluted sequentially with three column volumes each of benzene, diethyl ether, ethyl acetate, and acetone. The diethyl ether eluate was the most toxic. This fraction was evaporated to dryness and applied to a second silica gel 60 column (3.5 × 40 cm). The column was packed as a benzene slurry and the sample was applied to the column in benzene solution. The residual oil was eluted from the column with 3 L of benzene. The column was then eluted with a linear gradient from benzene to diethyl ether (240/17 mL fractions). Bioassay of every tenth tube revealed that toxicity was between the 40th and 60th tubes. These fractions were combined and thin-layer chromatography (silica gel 60F-254, 5 × 10 cm; solvent systems, chloroform/acetone, 93:7, v/v and toluene/ethyl acetate/formic acid, 5:4:1, v/v/v) showed the combined fractions to contain predominantly one metabolite. The toxic fractions were reduced in volume and placed at 5 °C overnight. Approximately 3 g of very fine microcrystals were collected. TLC showed there to be only one metabolite present. The crystals were recrystallized from acetone solution.

Infrared spectra (IR) of the metabolite were taken with a Perkin-Elmer 257 IR spectrometer equipped with a 3X condenser. Samples were coated on KBr blocks as a thin film. Ultraviolet spectra (UV) were taken with a Beckman

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Model DB-G recording spectrophotometer in methanol solution. High-resolution (HRP) and low-resolution (LRP) mass spectra were obtained with an A.E.I. MS-902 double-focus mass spectrometer. Samples were introduced into the ion source by direct probe and ionization was effected by electron impact at 70 eV. Melting points were taken with a Kofler micro-melting point apparatus.

Proton NMR spectra were obtained on a Varian Associates XL-100-12 spectrometer equipped with the 620 L data system and Gyro Code accessory. Spectra were run in $CDCl_3$ solution using the continuous wave (CW) mode with Me_4Si serving as the internal reference and the deuterium signal from the solvent serving as the internal lock signal. Natural abundance, proton-decoupled ^{13}C NMR spectra were obtained using the pulse Fourier transform (FT) mode. The following parameters were used to acquire the FT data: pulse angle, 30°; spectral width, 5000 Hz; data acquisition time, 0.8 s; pulse repetition time, 2.0 s; data points, 8K; and exponential weighting function, 1.0. Carbon-proton, one-bond coupling constants were obtained from gated decoupled spectra. Single-frequency, off-resonance decoupled (sford) spectra were obtained to aid in the assignment of the spectrum.

8-Acetoxy-3,7-dimethyl-6-methoxyisochroman. The acetate derivative (III) of the toxin was prepared by dissolving 50 mg of the toxin in a mixture of 1 mL of acetic anhydride and 1 mL of pyridine and stirring at room temperature overnight. Removal of the solvent on a vacuum line yielded a white powder (mp 96 °C) whose mass spectrum and NMR spectra were consistent with the addition of one acetate group to the toxin.

6,8-Dimethoxy-3,7-dimethylisochroman. To a suspension of oil-free sodium hydride (30 mg of 50% oil suspension) in DMF (2 mL), a solution of the toxin (30 mg in 1 mL of DMF) was added dropwise followed by the addition of iodomethane (0.25 mL). After stirring overnight at room temperature under nitrogen, the reaction mixture was partitioned between ether and water. The organic extract was washed successively with 5% HCl, water, 5% sodium thiosulfate, and then dried with sodium bisulfate. Evaporation of the solvent gave a solid (III) (28 mg) which was recrystallized from aqueous methanol, mp 93–94 °C. The NMR spectra were consistent with the methoxy derivative of the toxin.

The median lethal dose (LD_{50}) of the toxin was determined by administering to 1-day-old chickens a single 1 cm^3 dose of corn oil carrier containing the toxin at concentrations of 500, 750, and 1000 mg/kg (Kirksey and Cole, 1974). Five replicates of each concentration were tested, and animals were observed for 1 week after dosing.

RESULTS AND DISCUSSION

The purified metabolite had a melting point of 115–117 °C and exhibited UV absorption at λ_{max}^{MEOH} 285 nm and 223 (sh) nm (ϵ max 7700 and 27 000). It appeared on the TLC plates as a bright yellow spot in visible light and a bright yellow-fluorescent spot under UV light at R_f 0.46 (chloroform/acetone, 93:7, v/v) and R_f 0.65 (toluene/ethyl acetate/formic acid, 5:4:1, v/v/v). It had an elemental composition of C, 70.67%; H, 8.08%; N, none; and O, 21.24 (by difference); required for $C_{12}H_{16}O_3$: C, 69.2%; H, 7.7%; and O 23.1%). Functional groups evident from the IR spectrum were OH (3320 cm^{-1}), aromatic (3010, 1600, 1490 cm^{-1}), ether (2820 cm^{-1}), methyl (1390 cm^{-1}), and isolated H on aromatic ring (820 cm^{-1}).

Low-resolution mass spectral analysis showed a molecular ion peak (M^+) at nominal mass m/e 208 (base peak). High-resolution mass spectral analysis showed the M^+ peak to have a mass of 208.1053 with a computer

Table I. ^1H NMR Data for the Toxin and Its Derivatives

proton	I, δ[a]	III,[c] δ[a]	IV,[d] δ[a]
1	4.38, 4.94 (J = 15.5)[b]	4.55, 4.88 (J = 15.6)	4.56, 4.92 (J = 15.6)
3	3.78	3.68	3.72
4	2.55	2.52	2.54
5	6.24	6.38	6.34
11	1.38 (J = 6.0)	1.35 (J = 6.1)	1.37 (J = 6.1)
12	3.66	3.73	3.72
13	2.04	1.94	2.04

[a] In ppm downfield from internal Me_4Si. [b] Coupling constants in Hertz. [c] $CH_3C{=}O$, 2.30 ppm. [d] CH_3O on C-8, 3.83 ppm.

Table II. ^{13}C Chemical Shifts for the Toxin and Its Derivatives[a]

carbon	I, δ	III, δ	IV, δ
1	64.59 (145.3)[b]	64.53	64.69
3	70.69 (142.8)	70.04	70.50
4	34.02 (120.9)	33.93	34.17
5	96.08 (156.2)	101.59	92.89
6	153.69	153.58	153.82
7	112.91	119.30	115.53
8	152.58	147.57	156.46
9	133.87	134.28	134.06
10	114.98	121.29	115.91
11	10.03 (126.9)	10.29	10.20
12	55.95 (144.1)	55.16	57.20
13	21.63	21.67	21.90
		20.73 $CH_3C{=}O$	57.90 (OCH_3)
		169.20 $CH_3C{=}O$	

[a] In ppm downfield from internal Me_4Si. [b] One-bond carbon-hydrogen coupling constants.

calculated molecular formula of $C_{12}H_{16}O_3$ (calculated mass 208.1099). Fragment ions appeared at m/e 193.0866 (RI 39%) for loss of CH_3, m/e 177.0898 (RI 9.2%) for loss of OCH_3, and m/e 164.0814 (RI 77.7%) for loss of CH_3CHO. This latter fragmentation was similar to that observed for the $M^+ - 44$ ion due to loss of CH_3CHO in mellein (3-methyl-4-hydroxy-3,4-dihydroisocoumarin) and 4-hydroxymellein (3-methyl-4,8-dihydroxy-3,4-dihydroisocoumarin) (Cole et al., 1971) and represents a similar structural feature, i.e., a secondary methyl group attached to a carbon atom that is in turn bonded directly to an oxygen atom. The electron impact elimination of acetaldehyde in these cases is the result of a retro Diels-Alder reaction.

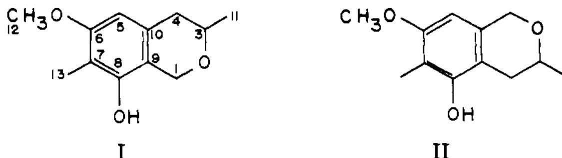
The ^1H chemical shifts and coupling constants of the toxin (I) are given in Table I. Homonuclear spin decoupling experiments established that the multiplet at 3.79 ppm is spin-coupled to the doublet at 1.40 ppm and the multiplet at 2.56 ppm. On the basis of the chemical shifts and coupling constants, the following fragments are suggested: $-CH_2(CH_3)CH-$, CH_3-, $-OCH_3$, and $-CH_2-$.

The ^{13}C chemical shifts, multiplicities, and one-bond carbon hydrogen coupling constants (Table II) confirm the presence of the above fragments and further suggest the presence of a penta-substituted benzene ring. The ^1H and ^{13}C spectra, together with the one-bond carbon–hydrogen coupling constants further suggest the presence of the fragment $-CH_2O(CH_3)CHCH_2-$. The two low-field chemical shifts in the ^{13}C spectrum suggest that the aromatic ring has two oxygen atoms attached. This, along with the IR spectrum, suggests a hydroxy group in addition to the methoxy group attached to the aromatic ring.

To confirm the presence of a hydroxy group, the acetate derivative (III) of I was prepared. Comparison of the ^{13}C chemical shifts of the acetate (III) (Table II) with those

of I, along with the shift difference between phenol and phenyl acetate (Wehrli and Wirthlin, 1976), suggests that the ^{13}C peaks at 134.0 and 153.8 ppm are meta to the OH group in I, the peaks at 112.8 and 115.4 ppm are ortho to the OH group, and the unsubstituted carbon at 96.2 ppm is para to the OH group. Differences between the proton shifts of I and the acetate (III) (Table I) are consistent with these conclusions.

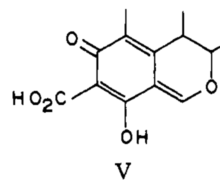
Thus, the data suggest the possibility of two structures for the toxin, I and II. Attempts to distinguish between



I and II using long-range, proton-carbon coupling constants obtained from the gated decoupled ^{13}C spectra of the toxin and its acetate were not successful. Only broad lines were observed for the substituted carbons of the aromatic ring. Furthermore, chemical shift differences for the aliphatic carbons between the toxin and its acetate (III) are not large enough to permit one to distinguish between I and II.

To further distinguish between I and II, the methoxy derivative (IV) of the toxin was prepared. Proton and ^{13}C NMR data for IV are given in Tables I and II, respectively. Differences between the ^{13}C chemical shifts of the toxin and IV (Table II) are consistent with the conclusions revealed previously on the basis of the ^{13}C chemical shift differences between the toxin and its acetate (III). When NMR spectra of IV (^1H and ^{13}C) were obtained upon successive additions of the lanthanide shift reagent Eu(fod)$_3$, the downfield induced shifts clearly showed that europium was complexing to any appreciable extent only with the aliphatic ether oxygen. Furthermore, the induced shift data are only consistent with structure I for the toxin and not with II. Thus, the structure of the toxin is established as 3,7-dimethyl-8-hydroxy-6-methoxyisochroman (I) (Weast, 1968).

The structure of the toxin (I) is similar to that of another toxin, citrinin (V), isolated from *P. steckii* (Krogh, 1974).



A possible biosynthetic route to citrinin has been proposed (Curtis et al., 1968). However, without additional experiments, one can only speculate that I and V may be derived biosynthetically from a common pathway.

The toxin (I) had an LD_{50} of 800 mg/kg in day-old chickens. This demonstrates a case where a fungal metabolite is acutely toxic at relatively high levels and observed toxicity of the fungal extract was due to production of relatively large amounts of the metabolite. Also, a dramatic change in solubility as a result of purification may have contributed to reduced toxicity of the purified metabolite. No other toxins were detected during purification.

LITERATURE CITED

Cole, R. J., *J. Food Protect.* **41**, 138 (1978).
Cole, R. J., Moore, J. H., Davis, N. D., Kirksey, J. W., Diener, U. L., *J. Agric. Food Chem.* **19**, 909 (1971).
Curtis, R. F., Hassall, C. H., Nazar, M., *J. Chem. Soc. C*, 85 (1968).
Davis, N. D., Wagener, R. E., Dalby, D. K., Morgan-Jones, G., Biener, U. L., *Appl. Microbiol.* **30**, 159 (1975).
Kirksey, J. W., Cole, R. J., *Mycopath. Mycol. Appl.* **54**, 291 (1974).
Krogh, P., in "Mycotoxins", Purchase, I. F. H., Ed., Elsevier, New York, 1974, p 425.
Saito, M., Enomoto, M., Umeda, M., Ohtsubo, K., Ishiko, T., Yamamoto, S., Toyokawa, H., in "Mycotoxins in Human Health", Purchase, I. F. H., Ed., MacMillan Press, Ltd., London, 1971, p 181.
Weast, R. C., Ed., "Handbook of Chemistry and Physics", 49th ed, The Chemical Rubber Co., Cleveland, OH, 1968, p C-37.
Wehrli, F. W., Wirthlin, T., "Interpretation of Carbon-13 NMR Spectra", Heyden, New York, 1976, p 47.

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Metabolism of [14C]Mibolerone in the Chicken

George H. Dunn, Leo F. Krzeminski,* Ronald E. Gosline, and Charles J. Subacz

One-day-old chicks were primed on nonradioactive mibolerone for 21 days. [14C]Mibolerone was then given orally for 28 days. Mibolerone was rapidly metabolized and eliminated. Highest concentrations, determined 1 h after the last ^{14}C dose, were found in bursa, followed by liver. Intact mibolerone was found in adipose tissues. Metabolites of mibolerone were detected in adipose tissues and liver, but were present in amounts too small for identification. There were no detectable residues in eggs collected 72 days after the last dose of [14C]mibolerone. The method would have detected residues above 0.5 ppb.

Avian lymphoid leukosis (LL) is a bursa dependent lymphoid neoplasm (Kakuk et al., 1977) induced by the lymphoid leukosis viruses, a form of C-type RNA tumor virus (Romero et al., 1978a). The neoplasm occurs primarily in the bursa of fabricius. The disease progresses by metastasization of the malignant cells to visceral organs with eventual death (Burmester, 1969). Treatment has involved surgical removal of the bursa, treatment with cyclophosphamide or with androgens (Romero et al., 1978b). Associated drawbacks have included economics, survival, and loss of immunocompetance (Romero and Frank, 1977). The androgen analogue, Mibolerone (17β-hydroxy-7α,17-dimethylestr-4-en-3-one) administered

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