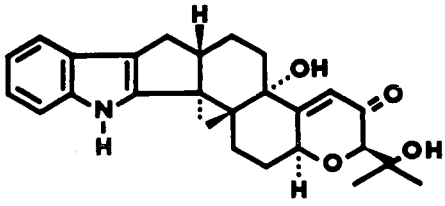
CHEMICALLY DIFFERENT TREMORGENIC MYCOTOXINS IN ISOLATES OF *PENICILLIUM PAXILLI* FROM AUSTRALIA AND NORTH AMERICA

PETER A. COCKRUM, CLAUDE C. J. CULVENOR, JOHN A. EDGAR and ALAN L. PAYNE

CSIRO Division of Animal Health, Animal Health Research Laboratory, Private Bag No.1 , Parkville, Victoria. 3052, Australia

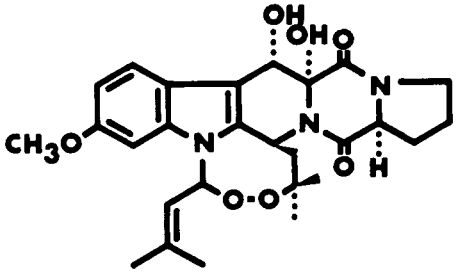
Fifteen years ago Wilson and Wilson (1) reported isolation of a fungal metabolite from *Aspergillis flavus* Link ex Fr. with the unusual property of causing sustained tremor in mice, rats, and guinea pigs. Since then, 18 tremorgenic mycotoxins have been found, some of which have yet to yield to structural elucidation. An early review by Ciegler *et al.* (2) grouped the tremorgens on the basis of the number of nitrogen atoms per molecule viz: one (the penitrem type), three (the verruculogen (**1**)—fumitremorgin type), and four (the tryptoquivaline type). This classification reflects their biosynthetic origins. A second type containing one nitrogen atom per molecule (the paspaline type) was subsequently reported, of which paxilline (**2**), isolated from *Penicillium paxilli* Bain. (3), is a representative (4).



1. VERRUCULOGEN

In the course of studies carried out in this laboratory into the neurological disorder of sheep and cattle known as ryegrass staggers, *P. paxilli* has been isolated from the feces of cattle in Victoria and from soil from pastures on which an outbreak of ryegrass staggers occurred in South Australia. Both of these isolates of *P. paxilli* produced substantial quantities of verruculogen (**1**) as the only detected tremorgen.

This result is at variance with the published isolation of the much less potent (3) and structurally dissimilar (5) tremorgen paxilline (**2**) from *P. paxilli* and prompted a direct comparison with the North American isolate (3). When cultured in quantity under conditions identical with those used to culture our isolates of *P.*



2. PAXILLINE

paxilli, this isolate produced paxilline (**2**), as reported (3), and no detectable verruculogen (**1**), thus confirming a difference in secondary chemistry of the North American and Australian isolates.

Although the biosynthesis of verruculogen has not been closely studied, it may be speculated that it is formed from the amino acids proline, tryptophan, and two separate isoprene units (2). The biosynthesis of paxilline (6) and related paspaline-type compounds (7) has been studied, however, and there is experimental evidence that paxilline is derived from tryptophan and the diterpene geranylgeraniol.

The production of verruculogen in our isolates of *P. paxilli* therefore reflects a significant difference in their biosynthetic capability when compared with the North American isolate of this species.

EXPERIMENTAL[1]

CULTIVATION OF FUNGI.—Isolates of *Penicillium paxilli* Bain. were obtained from surface soil (FRR 2196-Henninghausen, Sth. Australia) and fresh cattle feces (FRR 1973-Mickelham, Victoria) by the methods described previously (8). FRR denotes the culture collection of CSIRO Division of Food Research, Nth. Ryde, N.S.W., Australia. A culture of the North American isolate of *P. paxilli* was obtained from the American Type Culture Collection (ATCC 26601). The isolates were grown for tremorgen identification and bioassayed as described elsewhere (8).

[1]Melting (decomposition) points are corrected and were performed in sealed, evacuated tubes. Elemental analyses were performed by the Australian Microanalytical Service (CSIRO Division of Applied Organic Chemistry, Melbourne). ^1H (60 MH_z) nmr spectra were recorded on a Varian T60 spectrometer and ^{13}C nmr spectra were recorded on a Varian CFT-20 spectrometer with the sample dissolved in $CDCl_3$ and TMS as internal standard. The 100MHz ^1H nmr spectrum was run on a JEOL FX100 spectrometer with the sample dissolved in acetone-d_6 with TMS as internal standard. Low resolution mass spectra were recorded on a Varian Mat 111 GC/MS, samples being introduced by means of a heated direct insertion probe. Optical rotations were measured on a Perkin Elmer model 241 polarimeter. Column chromatography eluant was monitored with a Pye Unicam LCM 2 wire transport flame ionization detector. Tlc was performed on glass plates precoated with Silica Gel $60F_{254}$ and a concentration zone (E. Merck, Darmstadt), the developing solvent was toluene/ethylacetate/formic acid (50/40/10) and tremorgens were visualized as dark regions against a glowing green background under short wavelength (254 nm) uv irradiation or as spots showing a characteristic sequence of colors when sprayed with a 10% (w/v) solution of Cerium IV sulfate (E. Merck, Darmstadt) in concentrated phosphoric acid, diluted immediately before use with acetone (1:4). Column chromatography was performed with a glass column packed with Florisil (60–100 mesh, E. Merck, Darmstadt), which formed a bed 2.5 cm x 95 cm. Flow rates were maintained at 5 ml/minute; the pumping system was a Hewlett Packard 1010B HPLC system. All soverts were analytical grade and redistilled in the laboratory before use.

EXTRACTION OF TREMORGENS FROM LIQUID CULTURE.—Washed mycelia were covered with acetone and allowed to stand overnight to reduce the viability of the conidia, then macerated in acetone and allowed to stand several hours with occasional stirring. After filtration, bioassay indicated that negligible tremorgenic activity remained in the mycelial material. The acetone extract was evaporated *in vacuo* below 40°, and the residue was redissolved in dry acetone and reduced to a small volume. At this stage the Australian isolates deposited crystalline tremorgen. Further quantities of relatively pure tremorgens were prepared by column chromatography on a bed of Florisil. The tremorgens were eluted with diethyl ether, after preliminary elution with hexane, and crystallized when the ether eluate was evaporated.

IDENTIFICATION OF VERRUCULOGEN (**1**).—The compound obtained from Australian isolates of *P. paxilli* (FRR 1973 and FRR 2196) was recrystallized to constant mp (236–8° decomp.) (lit. 233–5° (9)) from ethanol-acetone (4:1), and the mmp with authentic verruculogen was not depressed. Thin layer chromatography and cochromatography with authentic verruculogen produced a single spot (R_f 0.48) of characteristic coloration (pinkish blue fading to yellow green)after spraying. Elemental analysis (after drying 2 hr at 60° *in vacuo*) found: C, 63.5; H, 6.6; N, 7.9; O, 21.5 Calc. for $C_{27}H_{33}N_3O_7$: C, 63.4; H, 6.5; N, 8.2; O, 21.9) and $[\alpha]^{25}D$ (−29.9°) are in agreement with data reported by Cole *et al.* (9) and Fayos *et al.* (10). ^1H (60 MH_z) and ^{13}C nmr spectra of the isolated compound were identical with reference spectra of verruculogen, and low resolution ms showed M^- *m/e* 511 and the reported losses of 15, 18 and 84 (11) and was identical to that of authentic verruculogen run under the same conditions. The isolated compound is therefore concluded to be verruculogen.

IDENTIFICATION OF PAXILLINE (**2**).—The tremorgenic fraction from a culture of the North American isolate of *P. paxilli* (ATCC 26601) crystallized from acetone-hexane as colorless prisms, decomp. 257–264° (after drying 2 hr at 60° *in vacuo* found: C, 73.0; H, 7.7; N, 3.2; O, 16.2. Calc. for $C_{27}H_{33}NO_4$, 0.5 H_2O: C, 73.0; H, 7.7; N, 3.2; O, 16.2). Paxilline is reported to have a mp of 252° on a Kofler micro melting point apparatus (3). Under our conditions (evacuated capilliary), a sample from Dr. Cole decomposed at 242–252°, unchanged by recrystallization or by admixture with the crystals isolated here. The isolated compound and the authentic sample had the same R_f on tlc (0.52), co-chromatographed as a single spot, and gave the same characteristic color with ceric sulfate-H_3PO_4 (purple blue fading through yellow with a blue border to salmon pink). A ^1H nmr spectrum (100MHz) was

in full accord with published data for paxilline (5), and a low resolution mass spectrum showed M^+ 435 and was identical with that of paxilline run under the same conditions. Despite the small difference in decomposition temperature, due possibly to a difference in crystal form, the tremorgen isolated here is clearly identical with paxilline.

ACKNOWLEDGMENTS

The authors wish to thank Dr. J. I. Pitt, CSIRO Division of Food Research, N.S.W., 2113 for identification of the Australian *Penicillium* isolates, Dr. R. Giesecke, Institute of Medical and Veterinary Science, Adelaide, Sth. Australia for soil samples, Dr. S. R. Johns, CSIRO Division of Applied Organic Chemistry, Fishermens Bend, Victoria, 3027 for ^{13}C nmr spectral analysis, and Dr. D. Kelly, Chemistry Department, University of Melbourne for the ^1H ($100MH_z$) nmr spectrum. We are very grateful to Dr. R. J. Cole, National Peanut Research Laboratory, U.S. Department of Agriculture, Dawson, Ga., for samples of verruculogen and paxilline and reference nmr spectra.

Received 16 April 1979.

LITERATURE CITED

1. B. J. Wilson and C. H. Wilson, *Science*, **144**, 177 (1964).
2. A. Ciegler, R. F. Vesonder and R. J. Cole, *Adv. Chem. Ser.*, 149, p. 163 (1976).
3. R. J. Cole, J. W. Kirksey and J. M. Wells, *Can. J. Microbiol.*, **20**, 1159 (1974).
4. R. J. Cole, J. W. Dorner, J. A. Lansden, R. H. Cox, C. Pape, B. Cunfer, S. S. Nicholson and D. M. Bedell, *J. Agric. Food Chem.*, **25**, 1197 (1977).
5. J. P. Springer, J. Clardy, J. M. Wells, R. J. Cole and J. W. Kirksey, *Tetrahedron Lett.*, 2531 (1975).
6. M. Tanabe [as quoted by Cole (4)].
7. W. Acklin, F. Weibel and D. Arigoni, *Chimia*, **31**, 63 (1977).
8. G. W. Lanigan, A. L. Payne and P. A. Cockrum, *Aust. J. Exp. Med. Biol. Sci.* **57**, 31 (1979).
9. R. J. Cole, J. W. Kirksey, J. H. Moore, B. R. Blankenship, U. L. Diener and N. D. Davis, *Appl. Microbiol.*, **24**, 248 (1972).
10. J. Fayos, D. Lokensgard, J. Clardy, R. J. Cole and J. W. Kirksey, *J. Am. Chem. Soc.*, **96**, 6785 (1974).
11. R. J. Cole and J. W. Kirksey, *J. Agric. Food Chem.*, **21**, 927 (1973).