The Mycotoxin Verruculogen: a 6-*O*-Methylindole

Data are presented to support a 6-*O*-methylindole-type structure for verruculogen, a tremorgenic metabolite produced by *Penicillium verruculosum* isolated from moldy peanuts. Conclusions are based on ultraviolet, nuclear magnetic resonance, and mass spectral analyses of verruculogen and two products from reductive cleavage of the tremorgen.

Fungal metabolites that elicit a severe tremorgenic response in animals have been reported to be involved in the deaths of livestock (Ciegler, 1969; Wilson *et al.*, 1968) and appear to be complex indole-type compounds that fall into two major groups based on their nitrogen content. These are the tremortins, which contain only one nitrogen atom per molecule, or the fumitremorgins and verruculogen, which contain three nitrogen atoms per molecule (Cole *et al.*, 1972; Hou *et al.*, 1971; Yamazaki *et al.*, 1971).

We have previously reported the isolation of the new mycotoxin verruculogen (TR-1) from a strain of *Penicillium verruculosum* isolated from moldy peanuts (Cole *et al.*, 1972). The toxin, when administered orally or IP, caused acute toxicity and severe tremors to mice and day-old cockerels. We now wish to report the partial chemical structure for TR-1.

EXPERIMENTAL SECTION

TR-1 was isolated by silica gel and Florisil column chromatography and subsequently crystallized from benzene–ethyl alcohol solution to yield colorless crystals (Cole *et al.*, 1972).

Reduction of TR-1 with 5% palladium-on-carbon in ethanol solution at room temperature and atmospheric pressure using an Ogg–Cooper microhydrogenator apparatus afforded two products, TR-2 and a volatile compound. The volatile compound was concentrated by fractional distillation and isolated as its 2,4-dinitrophenylhydrazone (DNP) derivative. 2,4-DNP derivatives were prepared by adding 2,4-dinitrophenylhydrazine to 4.0 ml of methanol containing one drop of glacial acetic acid and 100 mg of aldehyde. The reaction mixture was kept at room temperature overnight. 2,4-DNP derivatives were subsequently purified by silica gel column chromatography using a gradient elution from toluene to ethyl acetate.

TR-2 was isolated from the distillation residue by crystallization from benzene–ethyl acetate solution (1:1, v/v) to yield colorless crystals.

Nmr spectra were performed with a Jeolco Minimar-60 spectrometer at a frequency of 60 MHz. Spectra of TR-1 were taken in chloroform-*d* and dimethylsulfoxide-d_6; TR-2 spectra were taken in dimethylsulfoxide-d_6. Mass spectra were taken with an AEI MS-9 mass spectrometer. The samples were introduced into the instrument by direct probe and ionization was effected by electron impact at 70 eV or by chemical ionization using isobutane as the ionizing reagent.

Uv spectra were performed with a Beckman DB-G spectrophotometer in ethanol solution. ϵ_{max} were calculated with an assumed mass of 551 for TR-1 and 429 for TR-2.

Thin-layer chromatography (tlc) was done with silica gel GH-R coated onto glass plates (20 × 20 cm, 0.25 μ thickness). Plates spotted with TR-1 or TR-2 were developed using toluene–ethyl acetate–formic acid (5:4:1, v/v/v); plates spotted with the 2,4-DNP derivatives were developed in chloroform–toluene (1:1, v/v). TR-1 and TR-2 were visualized on tlc plates by spraying with 50% ethanolic sulfuric acid and heating at 100° for 5 min.

RESULTS AND DISCUSSION

TR-1 had a melting point of 233–235° (dec). The uv spectrum of TR-1 showed λ_{max}^{EtOH} 226 (47,500), 277 (11,000) and 295 nm (9750), which is typical of certain indole alkaloids (Agurell *et al.*, 1969). One product from reductive cleavage of TR-1 was an extremely volatile compound which gave a positive Schiff's test for aldehyde. The 2,4-DNP derivative of this compound showed *m/e* 266 ($C_{11}H_{14}N_4O_4$), mp 120–123°, and R_f 0.78. These data were essentially identical to those for the 2,4-DNP derivative of isovaleraldehyde (*m/e* 266 ($C_{11}H_{14}N_4O_4$), mp 120–121°, R_f 0.78).

Uv analyses of TR-2, the second product from TR-1 reduction, showed λ_{max}^{EtOH} 224 (37,400), 268 (6830), and 294 nm (7540), indicating that the indole nucleus remained unaltered. Thin-layer chromatography of TR-2 showed a single spot at R_f 0.46 compared to 0.67 for TR-1. TR-1 and TR-2 were visualized on tlc plates under long-wave uv light as mustard-colored and light brown fluorescent spots, respectively.

High-resolution mass spectral analyses (hrp) of TR-1 showed the largest detectable mass at *m/e* 511.2360 ($C_{27}H_{33}N_3O_7$) when ionization was effected *via* electron impact and at *m/e* 551 when analyzed *via* low-resolution (lrp) and chemical ionization. Preliminary X-ray crystallography of TR-1 suggested a mass larger than 551 and a value of 584 was obtained from vapor pressure osmometry analyses. Elemental analyses showed: C, 64.85%; H, 6.47%; N, 7.27%; and O, 20.68% ($C_{32}H_{38}N_3O_7$). High-resolution mass spectral analyses of TR-2, using electron impact and chemical ionization, showed a molecular ion peak (M*) at *m/e* 429.1898 ($C_{22}H_{27}N_3O_6$) and a protonated molecular ion peak (M* + 1) at *m/e* 430, respectively.

The nmr spectra of TR-1 and TR-2 were identical in the region of δ 6–9, except for the absence of a 1-proton signal in the spectrum of TR-2 (Figure 1A and 1B). This suggests that the proton was attached to the low molecular weight compound that was cleaved during reduction.

The typical 2,3,5 or 2,3,6 substitution pattern on the indole ring suggested in the nmr spectrum of TR-1 was obvious in the spectrum of TR-2 (ortho doublet at δ 7.71, J = 9.0 Hz; ortho–meta doublet of doublets at δ 6.60, J = 3.0 and 9.0 Hz; and a meta doublet at δ 6.87, J = 3.0 Hz) (Figure 1B). The chemical shift of the proton absent in the nmr spectrum of TR-2 was overlapped with that of the meta doublet in the TR-1 spectrum (Figure 1A). However, the ortho–meta doublet of doublets at δ 6.69 (J = 3.0 and 9.0 Hz) was only partially obstructed and the ortho doublet (δ 7.86, J = 9.0 Hz) was clearly visible.

At 3-proton singlet at δ 3.74 due to OCH_3 was presumed to be substituted on the indole ring (Figure 1A). On the basis of the nmr data, indole ring substitution of the OCH_3 could be located at either the 5 or 6 position. However, the 6-*O*-methyl substitution pattern was favored over the 5-*O*-methyl substitution on the basis of comparisons of the uv spectra of TR-1 and TR-2 with 5-methoxy-*N*,*N*-dimethyltryptamine (λ_{max} 274, 293, and 306 nm) (Agurell *et al.*, 1969) and with several 6-*O*-methyl-substituted indole alkaloids, *i.e.*, Vincine (λ_{max}^{EtOH} 229, 273, and 296 nm), Herbaine (λ_{max}^{EtOH} 227, 271, and 295 nm),

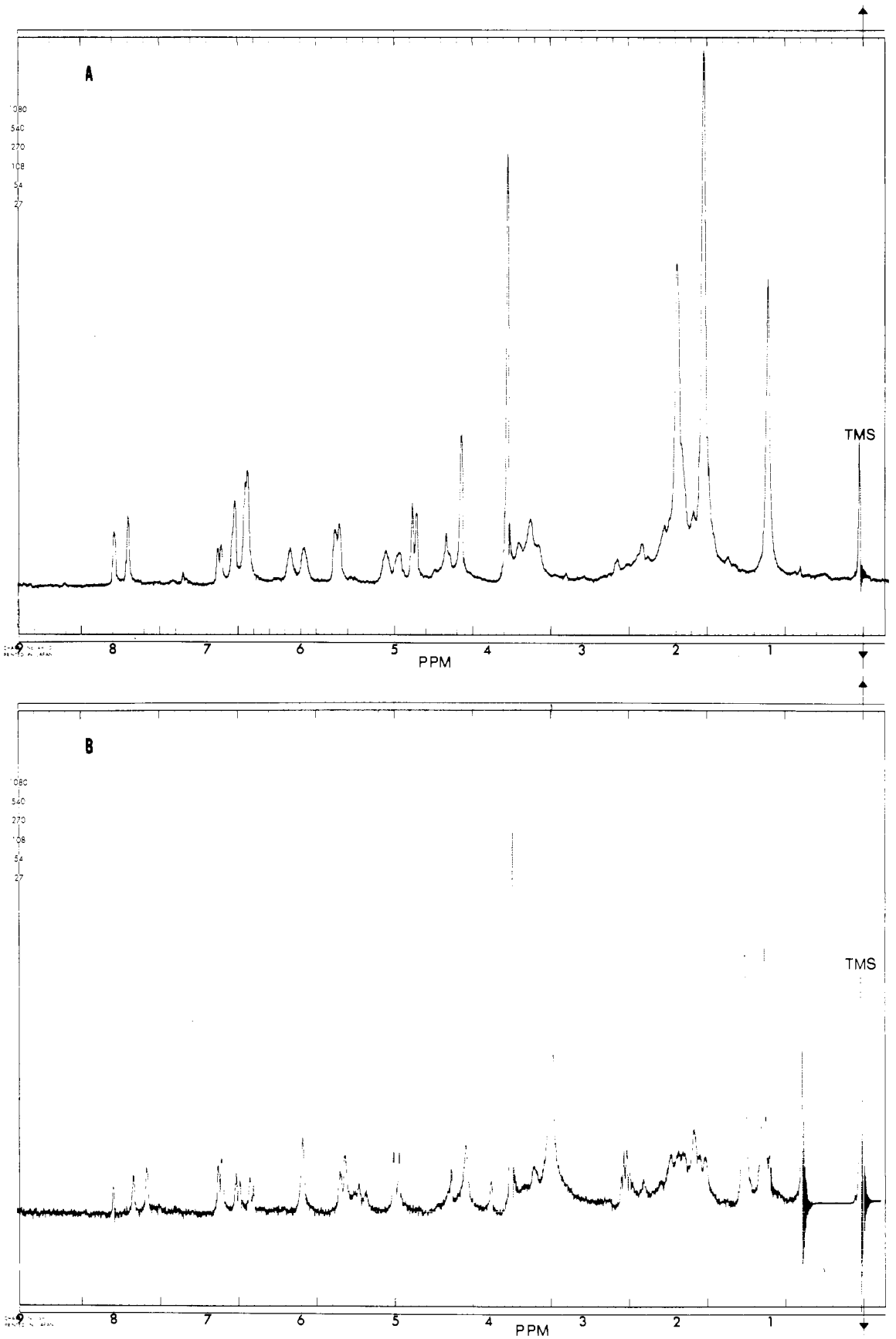

Figure 1. (A) Nuclear magnetic resonance spectrum of TR-1. (B) Nuclear magnetic resonance spectrum of TR-2.

and Vincinine (λ_{max}^{EtOH} 226, 272, and 295 nm) (Neuss, 1964).

The addition of D_2O to the nmr sample of TR-1 resulted in the disappearance of a 1-proton singlet at δ 4.10 (D_2O exchangeable) and collapse of the spin–spin coupling of two coupled 1-proton doublets at δ 5.53 (J = 2.5 Hz) and δ 4.69 (J = 2.5 Hz). TR-2 contained two D_2O exchangeable protons; these were singlets at δ 4.20 and 5.94.

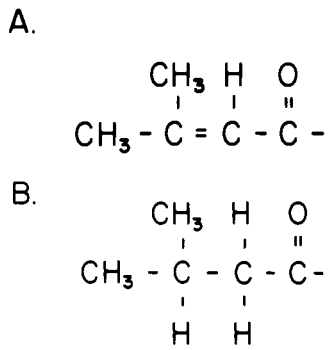

Figure 2. (A) β-Methylcrotonyl moiety. (B) Isovaleryl moiety.

The nmr spectrum of TR-1 also showed that there were two methyl groups present in addition to the two contained on the β-methylcrotonyl moiety (Figure 1B). These had chemical shifts of δ 1.96 and 0.98 (chloroform-*d*) and were not coupled to any other protons.

The methyl group at δ 1.96 experienced an upfield shift to δ 1.24 in the spectrum of TR-2, while the other methyl group remained essentially unchanged (δ 1.03) (Figure 1B).

Two 1-proton doublets in the TR-1 spectrum at δ 4.97 (J = 8 Hz) and 6.00 (J = 8 Hz) (Figure 1A) were absent in the TR-2 spectrum (Figure 1B). This presumably was caused by the loss of a double bond during reductive cleavage of TR-1.

The mass spectrum of TR-1 showed prominent losses of 15 (CH_3), 18 (H_2O), and 84 (C_5H_8O). The latter demonstrated that the same cleavage that occurred *via* chemical reduction also occurred in the mass spectrometer, with the exception that two hydrogen atoms were added to each fragment during reductive cleavage (*m/e* 86 ($C_5H_{10}O$) and *m/e* 429 ($C_{22}H_{27}N_3O_6$) compared to *m/e* 84 (C_5H_8O) and *m/e* 427 ($C_{22}H_{25}N_3O_6$)) *via* mass spectral fragmentation of TR-1.

Therefore, the small fragment from TR-1 probably existed as a β-methylcrotonyl moiety instead of an isovaleryl moiety (Figure 2, A and B). Further evidence for this was provided in the nmr spectra of TR-1 and TR-2. The chemical shifts of the two CH_3 groups lost from TR-1 during reductive cleavage were positioned at δ 1.68 as a 6-proton singlet in chloroform-*d* and at δ 1.63 and 1.74 as a 2-3 proton singlet in dimethylsulfoxide-d_6, (Figure 1, A and B), while the two methyl groups of isovaleraldehyde 2,4-DNP were positioned further upfield at δ 1.04 and 0.94 (in acetone-d_6) as a 6-proton doublet (J = 7.0 Hz). The chemical shifts of the two methyl groups of TR-1 are consistent with a β-methylcrotonyl rather than with an isovaleryl moiety. The nature of the linkage of this moiety in TR-1 is not known.

Tremorgenic compounds previously have been reported to be produced by *Aspergillus flavus* (Wilson and Wilson, 1964), *Penicillium spp* (Ciegler, 1969; Hou *et al.*, 1971; Wilson *et al.*, 1968) and *Aspergillus fumigatus* (Yamazaki *et al.*, 1971). Although all reported tremorgens appear to be indole alkaloids, TR-1 bears a closer chemical similarity to the fumitremorgins reported by Yamazaki *et al.* (1971). TR-1 and the fumitremorgins contain three nitrogen atoms (compared to one nitrogen for tremorgens from *Penicillium spp*) and an apparent 6-*O*-methoxy substitution of the indole ring, seemingly absent in the Penicillium tremorgens. However, comparisons of other physical and chemical data demonstrated that TR-1 was not identical to the fumitremorgins.

X-Ray crystallography studies are underway to determine the absolute chemical structure of TR-1.

LITERATURE CITED

Agurell, S., Holmstedt, B., Lindgren, J., *Acta Chem. Scand.* **23**, 903 (1969).
Ciegler, A., *Appl. Microbiol.* **18**, 128 (1969).
Cole, R. J., Kirksey, J. W., Moore, J. H., Blankenship, B. R., Diener, U. L., Davis, N. D., *Appl. Microbiol.* **24**, 248 (1972).
Hou, C. T., Ciegler, A., Hesseltine, C. W., *Appl. Microbiol.* **21**, 1101 (1971).
Neuss, N., "Physical Data of Indole and Dehydroindole Alkaloids," Vol. I and II, Lilly Research Laboratories, 1964.
Wilson, B. J., Wilson, C. H., *Science* **144**, 177 (1964).
Wilson, B. J., Wilson, C. H., Hayes, A. W., *Nature (London)* **220**, 77 (1968).
Yamazaki, M., Suzuki, S., Miyaki, K., *Pharm. Bull. Jap.* **19**, 1739 (1971).

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Photolysis of Parathion (*O,O*-Diethyl-*O*-(4-nitrophenyl)thiophosphate). New Products

O,O,S-Triethylthiophosphate was identified as the major product of the photolysis of parathion in aqueous THF or ethanol. Minor products were *O,O,O*-triethylthiophosphate, paraoxon, and triethylphosphate, which was formed by secondary photolysis of paraoxon.

Cook and Pugh (1957) and later Koivistoinen and Merilainen (1962) identified paraoxon (diethyl-4-nitrophenylphosphate, **2**) as one of the products from the photolysis of parathion (*O,O*-diethyl-*O*-(4-nitrophenyl)thiophosphate, **1**). Frawley (1957) and later Arterberry and Durham (1961) and Kimura (1963) have shown that 1 and 2 are inhibitors of cholinesterase. Frawley and Cook (1958) found the anticholinesterase activity of aqueous emulsions of 1 increased upon irradiation with light.

Quinby and Lemmon (1958) reported cases of parathion poisoning among farm workers, sometimes at relatively long intervals after spraying, when the parathion had reached "safe" levels. Milby *et al.* (1964) studied similar anomalous poisoning and found paraoxon on the foliage of trees which were sprayed with **1**. These authors also observed a higher incidence of poisoning among people who worked in fields repeatedly sprayed with **1**. The implication of this work is that **2** produced by the interaction of sunlight with **1** causes the poisoning of farm workers.

With these results in mind we initiated a study of the