Table II. Concentration (ppm) of Cyanazine, Metabolites, and Degradation Products in the Organisms

		Concentration, equivalent ppm						
		Algae	Crab	Daphnia	Elodea	Fish	Mosquito	Snail
Cyanazine	0.55[a]	–	0.0	–	0.621	–	–	–
N-Deethylcyanazine	0.47	–	0.172	–	0.0	–	–	–
Cyanazine amide	0.37	–	0.0	–	0.0	–	–	–
N-Deethylcyanazine amide	0.26	–	0.0	–	0.0	–	–	–
Unknown A	0.16	–	0.0	–	0.0	–	–	–
Unknown B	0.07	–	0.058	–	0.0	–	–	–
Unknown C	0.00	–	0.081	–	0.008	–	–	–
Unextractable		0.127	0.209	0.020	0.025	0.016	0.075	0.062
Total ^{14}C		1.256	0.521	0.040	0.654	0.051	0.103	0.108

[a] R_f value, silica gel F-254, methanol–acetone–chloroform (5:45:50). –, extract not chromatographed because less than 500 dpm.

the radioactivity in water could not be extracted even after acid hydrolysis.

Concentration of cyanazine and degradation products in the organisms was low (Table II). The total equivalent of radioactivity in the aquatic organisms ranged from 1.3 ppm in algae [*Oedogonium cardiacum* (Huss.)] to 0.05 ppm in fish (*Gambusia affinis* Baird and Girard) and 0.04 ppm in *Daphnia magna* Strauss. The extracts of crab (*Uca minax*) and *Elodea canadensis* which contained radioactivity greater than 500 dpm were analyzed by TLC. The results showed that *Elodea* contained 0.6 ppm of cyanazine (Table II). No other degradation products were detected in *Elodea* except for a small amount of very polar metabolites. On the other hand, no cyanazine was found in the crab extracts. About 0.2 ppm of *N*-deethylcyanazine and a small amount of polar metabolites were detected in the crabs. There was no evidence to indicate that cyanazine and its degradation products concentrated through the food chain. For example, in a food chain of algae → mosquitoes (*Culex pipiens quinquefasciatus* Say) → fish, total radioactivity decreased from 1.3 ppm in algae to 0.05 ppm in fish.

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Structure of the Tremor-Producing Indole, TR-2

The structure of a tremorgenic indole, TR-2 ($C_{22}H_{27}N_3O_6$), has been established by spectroscopic studies as II. The structure consists of 6-*O*-methyltryptophan, proline, and isoprene moieties. The two amino acids are combined to form a diketopiperazine compound. TR-2 was chemically derived from verruculogen (TR-1), shown to be I. TR-1 and TR-2 are structurally similar to fumitremorgens A and B from *Aspergillus fumigatus*.

The biological, physical, and chemical properties of the tremorgenic mycotoxin, verruculogen (TR-1), isolated from *Penicillum verruculosum* (ATCC no. 24640; NRRL no. 5881), have been reported (Cole et al., 1972; Cole and Kirksey, 1973). A subsequent X-ray study of TR-1 showed the structure to be I (Fayos et al., 1974). When TR-1 in ethanol solution was hydrogenated in the presence of palladium on carbon (5%) at room temperature, two products, isovaleral-

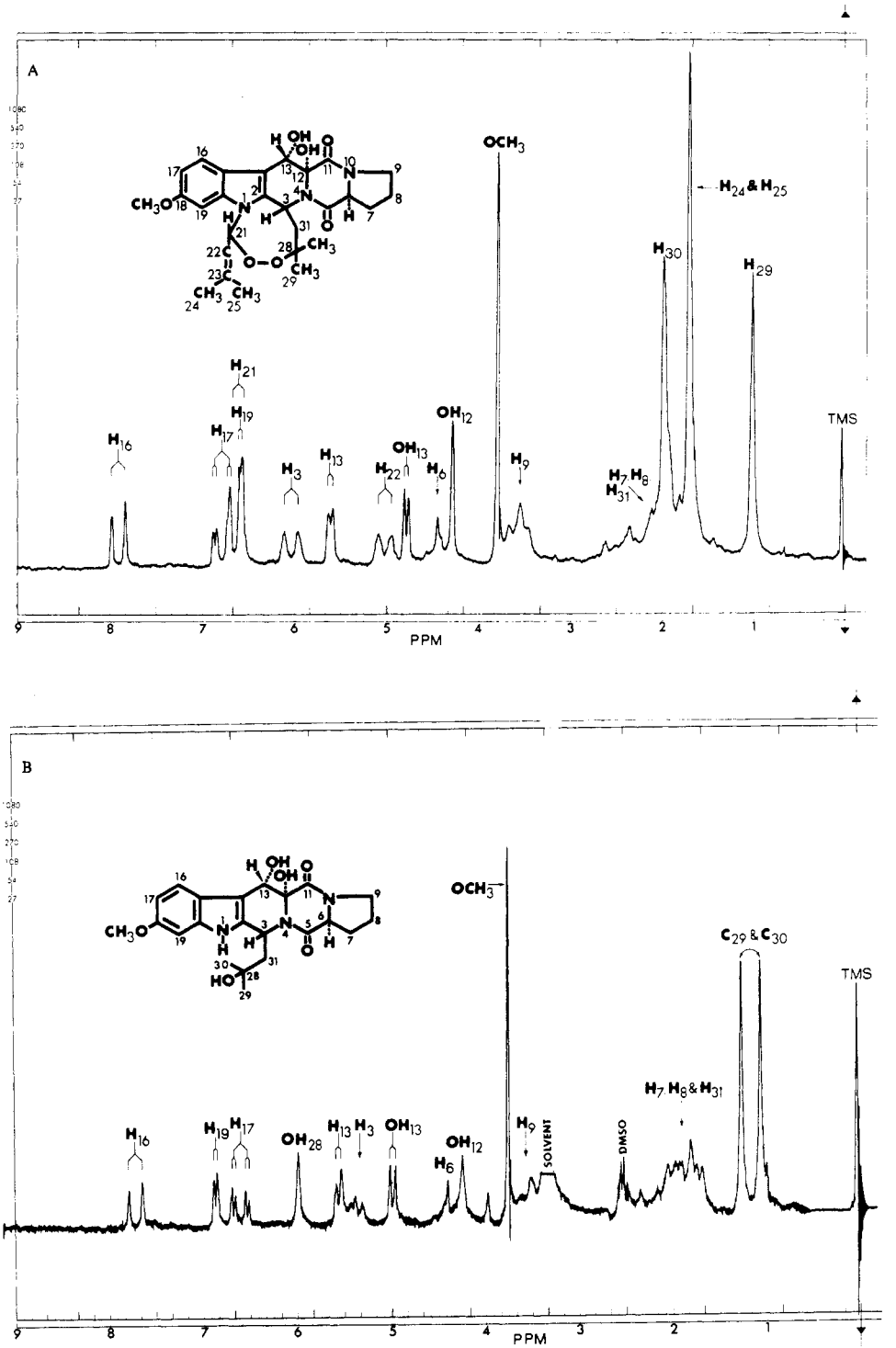
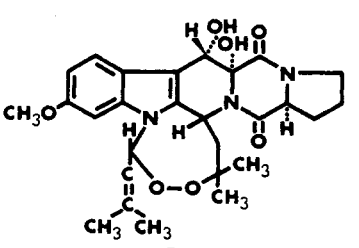
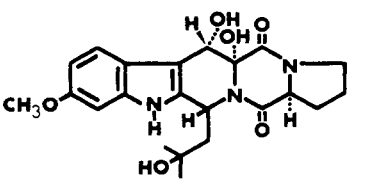


Figure 1. The proton NMR spectra of TR-1 (A) and TR-2 (B) in chloroform-*d* and dimethyl-d_6 sulfoxide solution, respectively.

dehyde and TR-2, were formed (Cole and Kirksey, 1973). Isovaleraldehyde was concentrated by fractional distillation and isolated and identified as its 2,4-dinitrophenylhydrazone (DNP) derivative. Isovaleraldehyde apparently originated via cleavage of the bonds between the indole nitrogen and C(21) and between the peroxide oxygens and reduction of the double bond in the β-methylcrotonyl moiety (Cole and Kirksey, 1973). The tremorgenic indole product (TR-2) is now assigned structure II based on deductions from comparisons of its spectral data with that of TR-1.

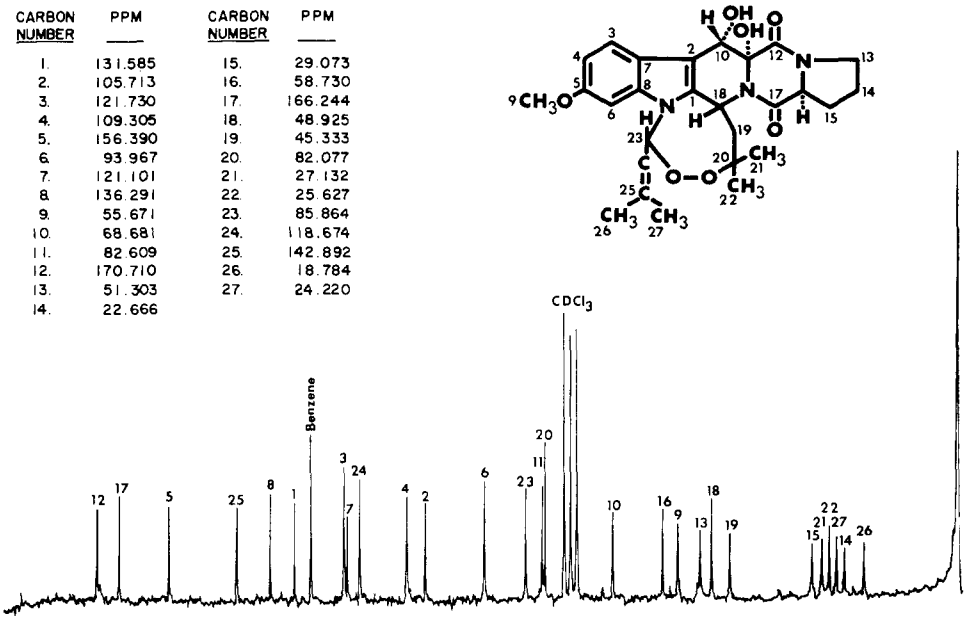


I



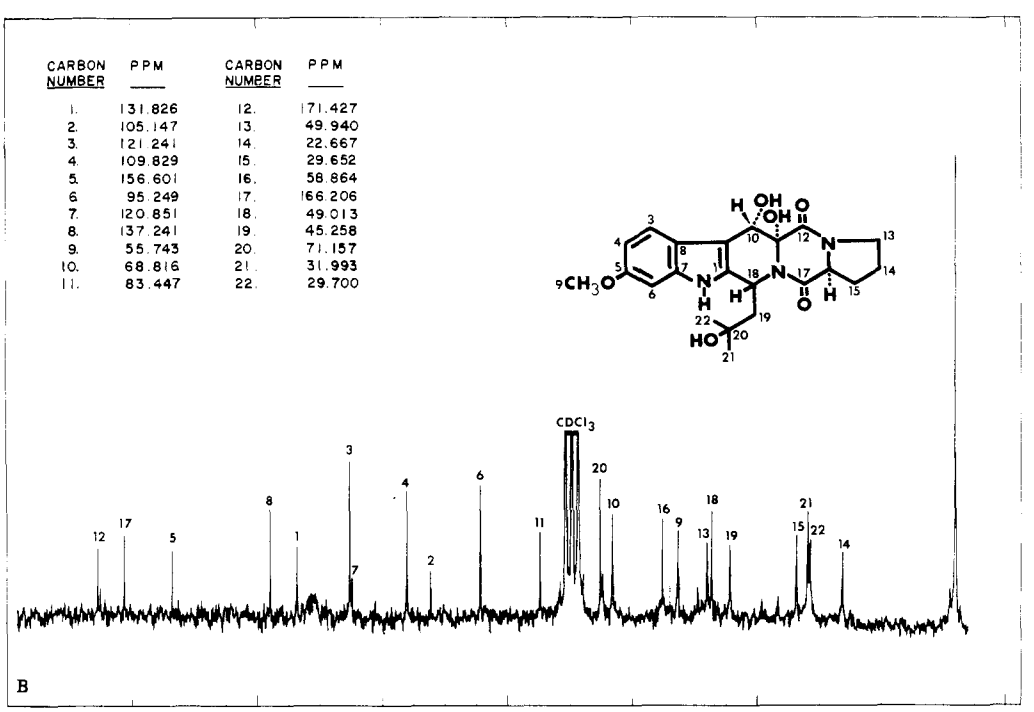
II

CARBON NUMBER	PPM	CARBON NUMBER	PPM
1.	131.585	15.	29.073
2.	105.713	16.	58.730
3.	121.730	17.	166.244
4.	109.305	18.	48.925
5.	156.390	19.	45.333
6.	93.967	20.	82.077
7.	121.101	21.	27.132
8.	136.291	22.	25.627
9.	55.671	23.	85.864
10.	68.681	24.	118.674
11.	82.609	25.	142.892
12.	170.710	26.	18.784
13.	51.303	27.	24.220
14.	22.666		



A

CARBON NUMBER	PPM	CARBON NUMBER	PPM
1.	131.826	12.	171.427
2.	105.147	13.	49.940
3.	121.241	14.	22.667
4.	109.829	15.	29.652
5.	156.601	16.	58.864
6.	95.249	17.	166.206
7.	120.851	18.	49.013
8.	137.241	19.	45.258
9.	55.743	20.	71.157
10.	68.816	21.	31.993
11.	83.447	22.	29.700



B

Figure 2. The ^{13}C NMR spectra of TR-1 (A) and TR-2 (B) in chloroform-*d* solution. All values in parts per million downfield from Me_4Si; parentheses mean assignments may be reversed; asterisk indicates assignments are uncertain.

EXPERIMENTAL SECTION

High-resolution mass spectral analysis of TR-2 showed the largest detectable mass at *m/e* 429.1898, with a computer-calculated formula of $C_{22}H_{27}N_3O_6$. Its ultraviolet (uv) absorption maxima were λ_{max} (EtOH) 224 (37,400), 268 (6830), and 294 nm (7540) which typified a 2,3,6-substituted indole compound; infrared (ir) absorptions were at 3450 (OH and/or indole), 1660 (diketopiperazine), and 1380 cm^{-1} (CH_3).

Major arguments for the assignment of structure II to TR-2 came from comparisons of the proton and ^{13}C nuclear magnetic resonance (NMR) spectra of TR-1 and TR-2 (Figures 1 and 2).

The proton NMR spectrum of TR-2 (Figure 1B) showed the typical pattern expected for the indole ring protons as shown with doublets at 7.71 ppm (J = 9.0 Hz) and 6.87 ppm (J = 3.0 Hz) assigned to the protons on C(16) and C(19), respectively, and the doublet of doublets at 6.60 ppm (J = 3.0 and 9.0 Hz) assigned to the proton on C(17). The multiplet appearing at 5.37 ppm is assigned to the proton on C(3). The peak for the hydroxyl proton on C(28) appears at 5.94 ppm and disappears upon the addition of D_2O. Comparisons of the spectrum of TR-1 (Figure 1A) with that of TR-2 show that the peaks due to the *gem*-dimethyl group of TR-1 at 1.66 ppm are absent in the spectrum of TR-2. Furthermore, the two methine protons attached to C(21) and C(22) in TR-1 are also absent in the spectrum of TR-2.

Further evidence supporting structure II for TR-2 was provided when the ^{13}C NMR spectra of TR-1 and TR-2 were compared (Figure 2) and showed that the signals for the five carbons, C(21)–C(25), present in the spectrum of TR-1 were absent in TR-2. (The ^{13}C spectra were assigned on the basis of known chemical-shift correlations (Stothers, 1972) and off-resonance decoupling experiments.)

These data were consistent with those expected for the structure II assigned to TR-2. Both TR-1 and TR-2 have structural, spectral, and biological similarities to fumitremorgens A and B (Yamazaki et al., 1974; Eichman et al., 1975). TR-2 produced perceptible tremors in day-old cockerels dosed orally via crop intubation at dosage levels down to 12.5 mg/kg.

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