

# Macrocyclic Trichothecene Toxins Produced by *Stachybotrys atra* Strains Isolated in Middle Europe

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A total of 17 strains of *Stachybotrys atra* isolated in Hungary and Czechoslovakia were cultured on Sabouraud agar, and the toxins produced by them were chemically analyzed by gas-liquid chromatography, high-pressure liquid chromatography, and mass spectroscopy. Furthermore, brine shrimp (*Artemia salina*) bioassay was used for the determination of toxicity of the compounds examined. Macrocyclic trichothecenes (satratoxins H and G, roridin E, and verrucarín J as well as two other unidentified macrocyclic trichothecenes) were found in all of the cultures tested. The identities of satratoxins H and G, roridin E, and verrucarín J were qualitatively determined by high-pressure liquid chromatography and gas-liquid chromatography. The ratio of satratoxins H and G and roridin E was found to be similar in each of the strains tested, but the amount of verrucarín J found was different in each of them. One of the unidentified macrocyclic trichothecenes was equivalent to the compound isolated by Harrach et al. (Harrach et al., Appl. Environ. Microbiol. 41:1428-1433, 1981). The other one proved to be a newly isolated macrocyclic trichothecene toxin. Stachybotryotoxicosis, one of the oldest mycotoxicoses known, and a serious problem in Middle Europe (Gy. Danko, Magy. Állatorv. Lapja 31:226-232, 1976), is believed to be caused by macrocyclic trichothecene toxins produced by *Stachybotrys atra* (R. M. Eppley, in Rodricks et al., ed., *Mycotoxins in Human and Animal Health*, p. 285-293, 1977). Forty years ago, the death of animals in the Soviet Union was associated with this fungus (C. U. Ruhliada, in Proceedings of the All-Union Sci. and Tech. Conf., p. 47-51, 1980). Today, these strains cause considerable damage in Hungary and other European countries (Gy. Danko, Magy. Állatorv. Lapja 27:241-249, 1972; Gy. Danko, Magy. Állatorv. Lapja 31:226-232, 1976; E. L. Hintikka, in Rodricks et al., ed., *Mycotoxins in Human and Animal Health*, p. 277-284, 1977; LeBars et al. Ann. Nutr. Aliment. 31:509-517, 1977; Palyusik et al., Magy. Állatorv. Lapja 26:304-306, 1971; Szabó et al., Magy. Állatorv. Lapja 25:21-27, 1970) and in the Republic of South Africa (Schneider et al., J. S. Afr. Vet. Assoc. 50:73-81, 1979). Stachybotryotoxicosis has been diagnosed in horses (Ruhliada, 1980), calves (Danko, 1972), sheep (Danko, 1976; Schneide et al., 1979), swine (Szabo et al., 1970), ruminants in zoological gardens (LeBars et al., 1977), and in humans (Andrássy et al., Mycosen 23:130-133, 1980; Szabo et al., 1970). *Stachybotrys* toxins were thought initially to be steroids (V. G. Drobotko, in *Fungus Disease of Horses and People*, 1949), and accordingly, the resorcinol test was used for their detection (R. U. Yuskiv, Mikrobiol. J. [Kiev] 30:68-71, 1968). However, this assumption was not correct, and resorcinol-negative *Stachybotrys* toxins were described (Danko, 1976; M. Nummi and M. L. Niku-Paavola, Ann. Nutr. Aliment. 31:761-770, 1977; S. V. Pathre, C. J. Mirocha, and M. Palyusik, Abstr. 2nd Int. Congr. Plant Pathol., Am. Phytopathol. Soc., St. Paul, Minn., abstr. no. 978, 1973). On the basis of clinical signs and autopsy findings of stachybotryotoxicosis, it was then presumed that *Stachybotrys* toxins, like the *Fusarium* toxins possessing similar cytotoxic effects, were trichothecene mycotoxins (J. R. Bamburg and F. M. Strong, in Kadis et al., ed., *Microbial Toxins*, p. 209-292, 1972).

Eppley et al. isolated several macrocyclic trichothecenes (satratoxins F, G, and H, verrucarín J, and roridin E; Fig. 1) from *Stachybotrys atra* (7-9). Harrach et al. confirmed these findings by isolating satratoxins G and H, verrucarín J, and roridin E from *Stachybotrys* strains of Hungarian and Finnish origin (13, 14). Direct proof of the involvement of macrocyclic trichothecenes in stachybotryotoxicosis were predicted by isolation of satratoxins G and H from straw associated with sheep and cattle stachybotryotoxicosis (11, 12).

Our objective was to evaluate the toxigenic potential of *S. atra* by identifying the various mycotoxins produced by *Stachybotrys* strains isolated from straw associated with field outbreaks of apparent stachybotryotoxicosis.

## MATERIALS AND METHODS

**Culturing of fungi.** A total of 17 *S. atra* strains, isolated in various parts of Hungary and Czechoslovakia in connection with animal death, were propagated. Each strain was isolated from a different outbreak and from straw in every case. The isolation of strains was done with wet filter paper. The isolates were inoculated with a loop onto 200-ml portions of sterile Sabouraud agar, distributed in 22 2-liter Roux bottles plugged with cotton wool, and allowed to harden in horizontal positions. Culturing at room temperature lasted for 20 days.

**Toxin isolation.** Fungi grown in the bottles were extracted with 300 ml of ethyl acetate for 4 h, and then with fresh solvent overnight. The combined ethyl acetate extracts were evaporated, and the oily residue was dissolved in 60 ml of acetonitrile that was washed with petroleum ether (3 times,

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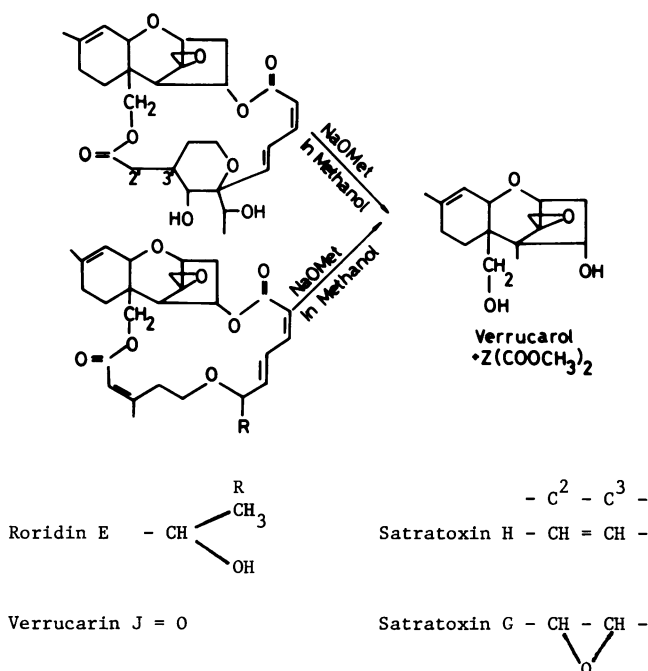


FIG. 1. The chemical structure of macrocyclic trichothecenes and their reaction with sodium methoxide reagent. Z is equivalent to the esterificated macrocyclic rings.

20 ml) and then evaporated. The residue was dissolved in aqueous methanol (1:1) and passed through a Sep-Pak C<sub>18</sub> cartridge (Waters Associates, Inc., Milford, Mass.) that was washed with 1 ml of aqueous methanol (1:1) before elution of the toxins with 4 ml of methanol-water (7:3). The cartridge could be regenerated with ethanol. The eluent containing the toxins was divided into two equal parts and evaporated to dryness under N<sub>2</sub>.

**HPLC.** The evaporated extracts were dissolved in 0.5 ml of methanol-water (7:3), and 0.1 ml was injected into a reserved-phase Polygosyl 60-D 5 C<sub>18</sub> column (25 cm by 4.6 mm; Macherey-Nagel, Dusen, Federal Republic of Germany) on a high-performance liquid chromatography (HPLC) apparatus (Waters Associates, Inc.). Isochratic elution with methanol-water (7:3) was used. Peaks which eluted at retention times identical to the standards were collected and evaporated to dryness under N<sub>2</sub> before mass spectral analysis (MS). The amounts of the individual toxins were estimated by HPLC (Fig. 2). MS (AGI MS 92 [Micromass Mass Spectrometric Co.] mass spectrometer; direct probe) was used to prove the identities of the materials separated by HPLC with the standards having the same retention time.

**Gas-liquid chromatography.** Sodium methoxide reagent (1.15 g of Na in 100 ml of methanol, 100 μl) was added to the dry residue from a Sep-Pak cartridge and heated for 15 min at 60°C. After the reaction (Fig. 1), methanol (120 μl containing 0.5 N HCl to neutralize the base) was added, and the mixture was evaporated. Then, 50 μl of *N,O*-bis-trimethylsilyl-trifluoroacetamide (Pierce Chemical Co., Rockford, Ill.) was added and heated at 60°C for 15 min. The bis-trimethylsilyl ether derivate of verrucarol was separated on a glass capillary tube (10 m by 0.3 mm), wetted with SE 52 stationary phase (Supelco Inc., Supelco Park, Bellefonte, Pa.), using a heating program of 160 to 220°C (4°C/min), and the total verrucarol content of the residue was determined (10).

**Bioassay.** The brine shrimp (*Artemia salina*) bioassay was done by the method of Eppley (6).

**Sources of standards.** The roridin E sample was a gift from B. B. Jarvis, Department of Chemistry, University of Maryland, College Park, Md. Verrucarin J was a gift from C. Tamm, Institut für Organische Chemie der Universität Basel, Basel, Switzerland. Satratoxins H and G were isolated by us, and their chemical structures were determined by MS and nuclear magnetic resonance spectroscopy.

## RESULTS AND DISCUSSION

All of the 17 *S. atra* strains examined produced each of the four macrocyclic trichothecenes studied, satratoxins H and G, verrucarin J, and roridin E. The qualitative and quantitative analyses were made based on HPLC. The HPLC results were confirmed by MS or gas-liquid chromatography in the following manner. Peaks that eluted with the same retention time as the standards were injected into MS equipment, or after transesterification to verrucarol, they were injected into a gas-liquid chromatography column.

The amounts of compounds examined in the culture were different (Table 1); however, the ratios of the individual toxins to each other were similar. All of the strains investigated produced satratoxin H in the highest quantity. Almost all of them produced satratoxins H and G and roridin E in decreasing order. Only one strain produced more roridin E than satratoxin G, and one produced them in equal amounts. The production of verrucarin J was much more independent;

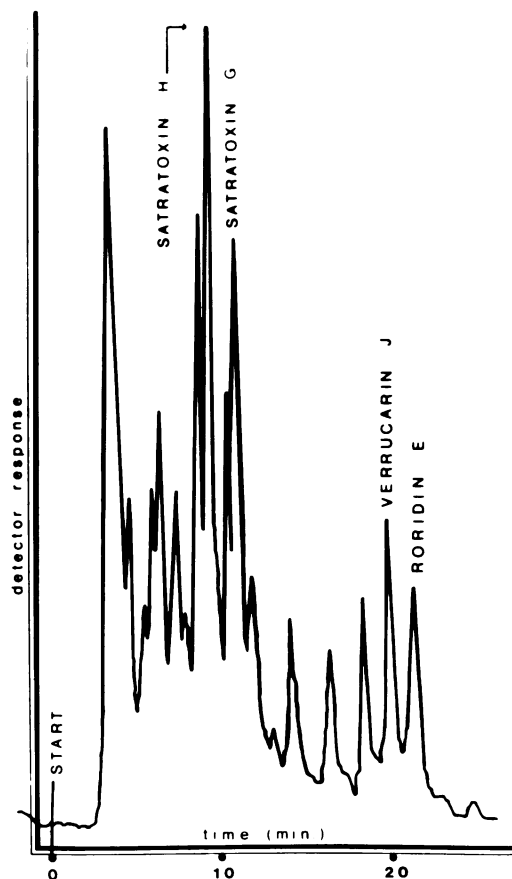


FIG. 2. The HPLC chromatogram of an *S. atra* isolate. See the text for experimental conditions.

the amount of this toxin produced was more than the amount of satratoxin G in 7 of 17 strains and less than that in 10 strains (in three strains it was even less than the amount of roridin E).

These results seem to underline the predictions of Tamm (22) that these 29-carbon-containing macrocyclic trichothecenes, satratoxins H and G and roridin E, belong to the same biosynthetic sequence, whereas 27-carbon-containing verrucarol J is the first toxic macrocyclic trichothecene member of another biogenetic sequence more independent from the first one, as reflected by the variations in toxin contents of the different strains. In addition to the Hungarian and Czechoslovakian strains described here, an American *S. atra* isolate described by Eppley produced a similar toxin ratio, i.e., 12 mg of satratoxin H, 8 mg of satratoxin G, 2 mg of verrucarol J, and 3 mg of roridin E (and 2 mg of satratoxin F not found by us) from 1 kg of oats infected by *S. atra* (7). A similar result was obtained in a study of the most polar toxins of a Finnish isolate (13); satratoxin H was found in the largest amount followed by satratoxin G (in addition to a smaller amount of a new compound with a molecular weight of 528). The origins of the isolates do not seem to result in any difference in toxin ratios. Satratoxin H comprised about half (40 to 70%) of the macrocyclic trichothecene compounds synthesized by the *S. atra* strains investigated. The total amount of satratoxin G, verrucarol J, and roridin E was 30 to 40%.

We examined the verrucarol content of the samples. Without treatment with sodium methoxide the samples did not contain verrucarol. After the treatment with sodium methoxide, the verrucarol content of the whole hydrolyzed sample of the fungus culture was always higher than the amount calculated as the sum of the theoretical verrucarol content of the four macrocyclic trichothecenes measured by HPLC. The four compounds examined gave 80 to 90% of the value that was determined from the hydrolyzed sample.

TABLE 1. Chemical analysis data of the *S. atra* strains tested

Strain	Satratoin H <sup>a</sup>	Satratoin G <sup>a</sup>	Roridin E <sup>a</sup>	Verrucarol J <sup>a</sup>	Verrucarol <sup>b</sup>	Verrucarol <sup>c</sup>
Belvárdgyula	111	20	16	24	86	97
Budapest 1	149	39	4	50	123	175
Budapest 2	90	27.5	3	4	63	77
Budapest 3	245	36	25	13	159	227
Debrecen 23	105	28	14	25	87	98
Debrecen 1132	153	41	21	85	155	202
Debrecen 5142	178	31	4	51	133	170
Karcag 1	134	41	40	65	143	171
Karcag 2	185	48	24	8	95	132
Miskolc 2	16	3	3	4	13	21
Miskolc 3	65	17	3	13	49	58
Nagytarcsa	160	21	0 <sup>d</sup>	10	95	128
Petrivente	24	6	0 <sup>d</sup>	1	16	25
Perkáta	32	8	4	6	27	28
Zsarolyán	85	16	8	15	62	78
My 93	98	17	28	45	97	116
My 276	191	28.5	2	1	109	127

<sup>a</sup> Measured by HPLC (micrograms per culture flask).

<sup>b</sup> Calculated as total verrucarol in the four macrocyclic trichothecene toxins (micrograms per culture flask; e.g., 1 µg of satratoxin H = 0.48 µg of verrucarol, etc.)

<sup>c</sup> Total verrucarol found in transesterified sample by gas-liquid chromatography (micrograms per culture flask).

<sup>d</sup> Less than 0.5 µg per Roux bottle.

One possible explanation could be the presence of other macrocyclic trichothecenes. To check this possibility, peaks eluted from the HPLC column were individually investigated. Two of them could be transmethylated into verrucarol. For the peak that eluted at a retention time of 10.4 min, it was established on the basis of MS and HPLC, using different solvent systems (13) for comparison with the standard, that this material is identical to the 528-molecular-weight, new macrocyclic trichothecene described by Harrach et al. (13) but not yet identified. The molecular weight of the compound from the other peak, eluted at a retention time of 8.8 min, also appeared to be 528. We did not find any data in the literature on the isolation of this compound. Both new compounds isolated were found to be different from the compound isolated by J. French and named i-satratoxin H (personal communication). The toxicity of the two newly isolated compounds was revealed by brine shrimp bioassay.

All of the *S. atra* strains investigated were isolated in connection with animal diseases that were diagnosed on the basis of symptoms indicating stachybotryotoxicosis. The finding that all of the strains produced the four highly toxic macrocyclic trichothecene *Stachybotrys* toxins seems to support the theories about their role in stachybotryotoxicosis, as presumed earlier (8, 11). According to our comparative and quantitative results, satratoxin H seems to be the most dangerous of the *Stachybotrys* toxins, being produced in the highest amount by all of the strains. In addition, it is the most polar of the four main *Stachybotrys* toxins, being detected most often under physiological circumstances (14) and having the ability to cause the characteristic cytotoxic effects.

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