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# Macrocyclic Trichothecene Toxins Produced by a Strain of *Stachybotrys atra* from Hungary†

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A strain of *Stachybotrys atra* isolated from a field case of stachybotryotoxicosis in Hungary was cultured in Hungary. All of the compounds toxic to brine shrimp were separated from the culture extract by solvent partition, column chromatography, and preparative thin-layer chromatography. Two of the toxic compounds were identified as verrucarin J and satratoxin H by comparison with pure standards resolved by high-pressure liquid chromatography and characterized by mass spectrometry. Two other toxic components were identified as roriden E and satratoxin G on the basis of their mass spectra. The fifth toxic compound was identified as a macrocyclic trichothecene based on the following findings: a positive 4-(*p*-nitrobenzyl)pyridine color reaction, hydrolysis resulting in verrucarol verified by combined gas chromatography-mass spectrometry, and a characteristic trichothecene proton-nuclear magnetic resonance spectrum. This macrocyclic trichothecene has a molecular ion (528) identical to satratoxin H, and its mass spectrum is similar; however, its $R_f$ value on Silica Gel G differs.

Stachybotryotoxicosis is caused by the toxins of some strains of *Stachybotrys atra* (*S. alternans*), a saprophytic fungus frequently found on high-cellulose materials, e.g. straw and hay. The disease was recognized in the USSR as early as the 1930s, when it killed thousands of horses (9). This disease occurs annually in Hungary (4), where it is an important mycotoxicosis. This disease has been diagnosed in France (14) and in South Africa (20); however, there is no generally accepted method of toxin analysis, which makes diagnosis difficult.

The first researchers (8) described these toxins as steroids that give a positive reaction in the resorcinol test. More recent investigations have indicated that the toxins are not steroids but are resorcinol negative (13, 16).

Bamburg and Strong (1) suggested that the *Stachybotrys* toxins are trichothecenes because of the similarity in their action to that of trichothecenes. Eppley et al. (5, 6) isolated five macrocyclic trichothecenes, two of which were verrucarin J (7) and roridin E (24), isolated earlier from *Myrothecium* (2). The other three toxins proved to be new, macrocyclic trichothecenes (Fig. 1), named accordingly satratoxins F, G, and H.

Recently, a Hungarian strain of *S. atra* was investigated for the presence of mycotoxins (21); a macrocyclic trichothecene that yielded verrucarol upon hydrolysis was found, but its identity was not determined.

Our objective in this investigation was to isolate and identify all of the major toxic components produced by *S. atra* (ATCC 34915) as determined by the brine shrimp test. The highly toxic *S. atra* strain used in this study was chosen from among 13 isolates obtained from serious cases of stachybotryotoxicosis in Hungary. The isolate was selected by the severity of lesions obtained in the rabbit skin test.

## MATERIALS AND METHODS

**Culture of the fungus.** The strain *S. atra* Jaszapati (ATCC 34915) was isolated from straw obtained in the Hungarian village of Jaszapati from a field case of mycotoxicosis in horses. The fungus was seeded on autoclaved rice and incubated for 5 weeks at room temperature (21 to 25°C); it was shaken daily for the first 2 weeks.

**Isolation of the toxins.** The rice cultures were dried and finely ground. They were batch extracted twice with ethyl acetate overnight. The extracts were combined, concentrated, taken up in acetonitrile, and partitioned with petroleum ether (bp, 60 to 70°C) twice (Fig. 2). The concentrated material of the acetonitrile phase was chromatographed on a Silica Gel 60 (mesh size, 100 to 200; Davison Chemical, Baltimore, Md.) column, using chloroform with 0 to 5% concentrations of isopropanol. The individual toxins

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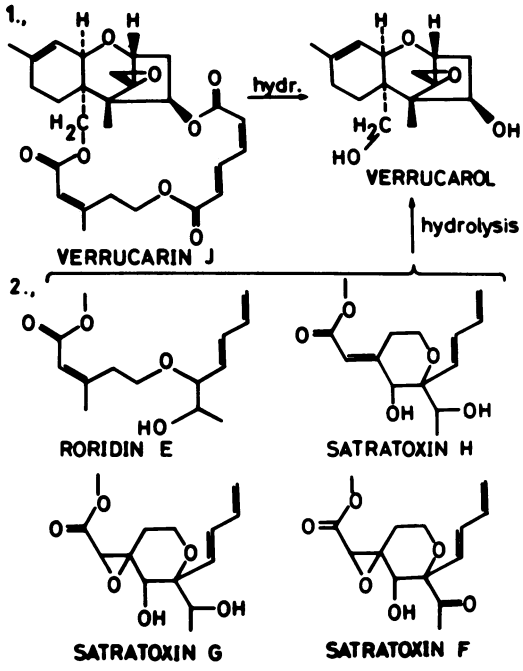


FIG. 1. *Chemical structures of verrucarol, roridin E, verrucarin J, and satratoxins F, G, and H. The macrocyclic trichothecenes yield the alcohol verrucarol upon hydrolysis.*

were prepared by intensive preparative thin-layer chromatography (TLC) (Fig. 2).

**TLC.** Separation and determination of purity were done with Merck Silica Gel 60 $F_{254}$, 0.25-mm-thick precoated plates and Merck HPTLC (high-performance TLC) Silica Gel $F_{254}$. The solvent systems used were as follows: methylene chloride-ethyl acetate (9:1) to remove the less polar compounds (verrucarin J, ergosterol, and other impurities), chloroform-isopropanol (98:2) to remove the medium-polarity materials (roridin E and component $C_{27}H_{34}O_8$), methylene chloride-methanol (95:5) to remove the most polar fractions (an unknown macrocyclic trichothecene and satratoxins G and H), and chloroform-isopropanol (94:6) to remove a material with a molecular ion of 428.

The components on the TLC plates were scraped off and eluted from the silica gel with acetone, using elution tubes.

**Brine shrimp test.** Dried *Artemia salina* eggs bought from an aquarium supply house were hatched in salt water (15 g of NaCl, 1 g of borax, 1 liter of tap water) at room temperature. The toxins in solution were placed in 1-ml wells in the glass plate and dried. The freshly hatched brine shrimps were placed in the wells of the glass plate in 0.5 ml of salt water (about 20 shrimps per partition). The toxic effects (death and immobility) were determined at 24, 36, and 48 h.

**Making the trichothecenes visible on TLC plates.** The plates were dipped in 1% 4-(*p*-nitrobenzyl)pyridine in chloroform-carbon tetrachloride (2:3). After the plates were heated at 150°C for 30 min, they were dipped in 10% tetraethylenepentamine in $CHCl_3$-$CCl_4$. The trichothecenes turned blue immediately when reacted with the latter reagent (22). The plates were then sprayed with 50% methanolic $H_2SO_4$ and heated.

**MS.** Low-resolution mass spectrometry (MS) was carried out with an LKB-9000 combined gas chromatograph-mass spectrometer at 20 and 70 eV over a mass range of 0 to 750. Appropriate background spectra were taken to correct for background contribution. Direct injections of acetone solution were used to resolve the hydrolysis products of the macrocyclic trichothecenes by combined gas chromatography (GC)-MS. The hydrolysis products were resolved as their trimethylsilyl ether derivatives; multiple scans were taken of each peak.

To obtain a high-resolution mass spectrum, a small crystal of each component was placed in a capillary tube and inserted into the direct probe inlet of an AEI-MS double-focusing mass spectrometer. Masses were calculated with an on-line computer.

**Hydrolysis and analysis by GC-MS.** A 20-μl amount from a 1-μg/μl solution of each compound was evaporated under $N_2$ in a 0.5-dram (ca. 0.5885-g) vial; then 30 μl of a 0.3 N NaOH solution in ethanol-water (9:1) was added, and the mixture was held at room temperature (21 to 25°C) overnight. After evaporation of the solvent under $N_2$, 20 μl of a silylating reagent (Tri-Sil BT; Pierce Chemicals Co., Rockford, Ill.) was reacted with the samples for at least 15 min at room temperature. The resulting trimethylsilyl ether derivatives were analyzed by GC-MS.

GC was done on a Hewlett-Packard 5710A equipped with a flame ionization detector. GC conditions were: a stainless steel column (1 m by 3 mm) packed with 3% OV-17 on 100- to 120-mesh Gas-Chrom Q; a column temperature programmed from 150 to 280°C at 8°C/min; and carrier gas ($N_2$) and hydrogen flow rates of 30 ml/min.

Analysis by GC-MS was done with computer-controlled selective ion monitoring. Verrucarol was determined by continuous scanning for its molecular ion and for 15 characteristic mass fragments.

**High-pressure liquid chromatography.** The samples were brought into solution in methanol-water (60:40) and filtered. Analysis by high-pressure liquid chromatography was carried out with methanol-water (65:35) at a flow rate of 0.5 ml/min, using a Waters apparatus equipped with a fluorescence and absorbance detector (254 nm) and with a μBondapack C-18 reverse-phase column.

**Determination of nuclear magnetic resonance and ultraviolet absorption spectra.** The proton-nuclear magnetic resonance spectrum was measured on a 270-MHz Brucker instrument. The proton signal from residual $[^2H]CHCl_3$ was used as an internal standard. Ultraviolet spectra were determined in methanol with a Beckman DB-GT recording spectrophotometer.

**Sources of standards.** The samples of verrucarol, verrucarin J, and verrucarin K were gifts from Ch. Tamm, Institut für Organische Chemie der Universität Basel, Basel, Switzerland. Mass spectra of satratoxins H, F, and G and roridin E were obtained from R. M. Eppley, Division of Chemistry and Physics, Food and

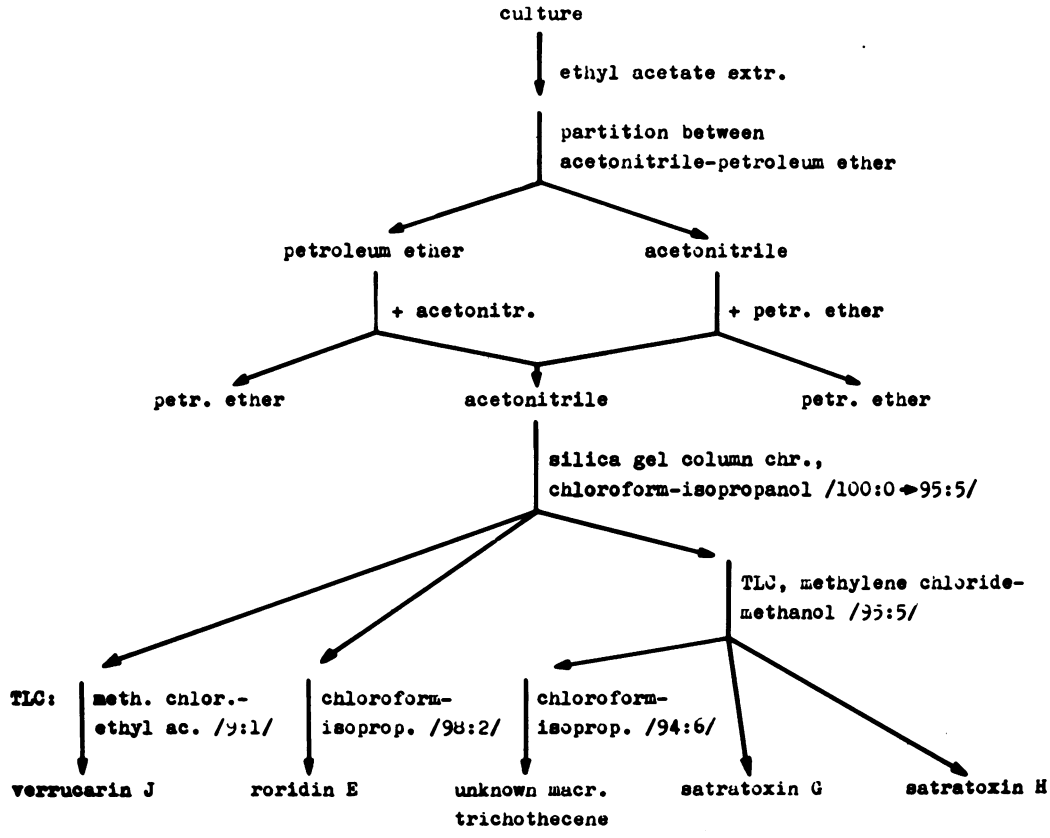


FIG. 2. *Procedure used to isolate the Stachybotrys toxins. Abbreviations: extr., extract; acetonitr., acetonitrile; petr., petroleum; chr., chromatography; meth. chlor., methylene chloride; ac., acetate; isoprop., isopropanol; macro., macrocyclic.*

Drug Administration, Washington, D.C. The samples of isororidin E and 7β,8β-epoxyisororidin E and the roridin E mass spectra were provided by M. Matsumoto; the vertisporin mass spectrum was provided by S. Hayakawa, Shionogi Research Laboratory, Shionogi and Co., Ltd., Fukushima-ku, Osaka, Japan.

## RESULTS AND DISCUSSION

The most important signs and symptoms of stachybotryotoxicoses are damage of the epithelium, the endothelium, blood vessel wall, and blood cells (4, 9, 14, 20). Different bioassays used to measure these effects were developed before the actual analyses were started. Accordingly, the rabbit skin (17), chicken skin (4), and chicken embryo (17) tests were compared with tissue cultures of *Paramecium caudatum* (17), *Daphnia magna*, and *A. salina*. The brine shrimp test proved to be most practical because of its simplicity, cheapness, and sensitivity.

Five major brine shrimp-toxic metabolites were isolated. Their purity was checked by resolution on TLC plates with different solvent systems. The plates were sprayed with sulfuric acid and charred.

None of the toxic components fluoresced under 254- or 366-nm-wavelength light, but they quenched the fluorescence of the Silica Gel 60 $F_{254}$ plates under short-wavelength ultraviolet light. However, if sprayed with sulfuric acid and then heated, the toxic components fluoresced light blue under 366-nm-wavelength light. This is similar to the fluorescence of type T-2 trichothecenes. The toxic components were also positive in the 4-(*p*-nitrobenzyl)pyridine color reaction (22). Some nontoxic materials gave a positive reaction as well. Since the 4-(*p*-nitrobenzyl)pyridine reaction is not totally specific for epoxy groups, but also reacts with other alkylating compounds, we studied only the brine shrimp test-positive fractions.

Upon hydrolysis with base, each toxic fraction yielded verrucarol, the common neutral hydrolysis product of most of the macrocyclic trichothecenes.

Verrucarol was detected by GC and GC-MS by full scans and by selected ion monitoring. The latter method was the most sensitive, allow-

ing the detection of nanogram quantities.

All five toxins had an ultraviolet absorption maximum between 255 and 262 nm, characteristic of a conjugated butadiene-type system, which is a typical structure of a macrocyclic trichothecene.

The least polar of the five toxins isolated was identified as verrucarin J (7), and the most polar one was identified as satratoxin H (5). Their identity was confirmed by a mass spectroscopy comparison of authentic samples. All of the isolated toxins could be resolved by high-pressure liquid chromatography as described. Coinjection of authentic samples with verrucarin J or satratoxin H yielded only one peak.

One toxic compound was identified as roridin E (24), and another was identified as satratoxin G (6) according to their mass spectra. A comparison was also made with isororidin E (15) and vertisporin (10), but none was identical.

A fifth brine shrimp-toxic component, which had an $R_f$ value similar to that of satratoxin G and to that of another nontoxic component with a molecular ion of 428, was separated. The fifth toxin was free from all other compounds after repeated resolution by TLC and high-pressure liquid chromatography.

This component absorbed in the 255 to 262-nm range and yielded verrucarol upon hydrolysis. Its proton-nuclear magnetic resonance spectra suggested a macrocyclic trichothecene, and it had a molecular ion in its mass spectrum of 528 (Fig. 3). It differed from satratoxin H in its $R_f$ value on TLC and high-pressure liquid chromatography, but its mass spectrum was very similar to satratoxin H except for a difference in intensities at $m/e^+$ 466, 484, 110, and 231.

Satratoxin F was not present in the *Stachybotrys* culture, although ergosterol and a material called SB-4 ($C_{27}H_{34}O_8$) were found (S. V. Pathre, C. J. Mirocha, and M. Palyusik, 2nd Int. Congr. Plant Pathol., Am. Phytopathol. Soc., abstr. no. 978, 1973. Ergosterol and SB-4 were verified by TLC and by high- and low-resolution mass spectroscopy. The latter material was also compared with verrucarin K (3) by TLC; how-

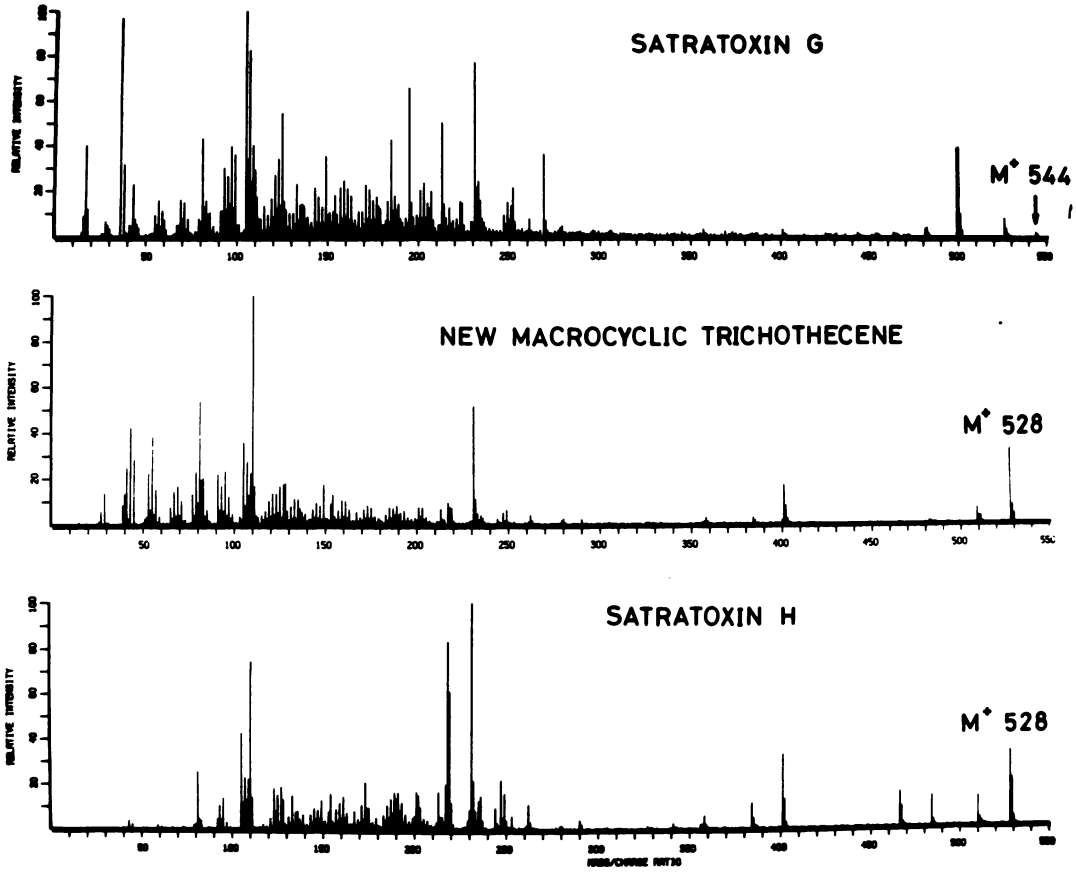


FIG. 3. *Mass spectra of satratoxins G and H and the newly reported derivative of satratoxin H.*

ever, the $R_f$ values were different, although their elemental composition is the same. SB-4 was not toxic in the brine shrimp test.

Our study suggests that the macrocyclic trichothecenes do indeed play a causal role in the disease called stachybotryotoxicosis, as each toxic component found in this study was a trichothecene. We confirmed the results of Eppley et al. (5, 6) in that we isolated satratoxins G and H, verrucarin J, and roridin E from *S. atra*. In addition to these toxins, we report a new macrocyclic trichothecene.

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