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Trichothecenes Produced by *Stachybotrys atra* from Eastern Europe

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A total of 17 isolates of *Stachybotrys atra* isolated from various parts of Hungary and Czechoslovakia were grown on rice, and the toxin production of each isolate was analyzed by high-performance liquid chromatography. Of the 17 isolates, 14 produced macrocyclic trichothecenes (satratoxins F, G, and H, roridin E, and verrucarin J) as well as trichoverrols A and B. Most isolates produced satratoxins G and H in higher quantities than the other trichothecenes. The yield (in milligrams) of trichothecenes produced by one isolate grown on 800 g of rice was as follows: roridin E, 12; satratoxin F, 10; satratoxin G, 75; satratoxin H, 100; trichoverrol A, 15; and trichoverrol B, 30.

Stachybotryotoxicosis is caused by contact with *Stachybotrys atra* (also known as *S. chartarum*), a saprophytic fungus frequently found on high-cellulose materials, e.g., straw and hay (12). The toxic principles believed responsible for stachybotryotoxicosis are macrocyclic trichothecene mycotoxins (satratoxins G and H) which have been isolated from straw associated with sheep and cattle toxicoses (10, 11). Stachybotryotoxicosis was recognized as a serious problem in the Soviet Union (9) and Eastern Europe (4), but scattered outbreaks have been reported in other parts of the world (17, 18). The first case of stachybotryotoxicosis reported in the United States involved the occupants of a house in suburban Chicago (3a).

Five macrocyclic trichothecenes have been identified from a culture of *S. atra* (6–8), two of which, verrucarin J and roridin E, were isolated earlier from *Myrothecium* sp. (14). The other three toxins were characterized and named satratoxins F, G, and H. Harrach et al. (12, 13) and Bata et al. (2) found satratoxins G and H, verrucarin J, roridin E, and a new macrocyclic trichothecene (molecular weight, 528) from cultures of *S. atra* obtained from Hungary and Czechoslovakia.

Since earlier studies of toxigenic isolates of *S. atra* from Eastern Europe indicated that there were as yet unidentified trichothecenes produced in these cultures (6, 12, 13), we undertook a study of trichothecene production by 17 toxigenic isolates of *S. atra* from Eastern Europe. Also, because previous studies report low toxin production (<10 mg/kg) by *S. atra*, we hoped to establish culture conditions which would lead to increased toxin production.

MATERIALS AND METHODS

Source and culture of fungi. A total of 17 isolates of *S. atra* were obtained from B. Harrach, Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary. The isolates had been cultured from incidents of stachybotryotoxicosis in various parts of Hungary and Czechoslovakia (2).

Each isolate was grown in a 1-liter Erlenmeyer flask on 200 g of moist, autoclaved Uncle Ben's Converted Rice (Uncle Ben's, Inc., Houston, Tex.). Rice cultures were incubated at 22 to 26°C for 2 weeks and then were incubated at 10°C for 2 weeks.

Based on this preliminary survey, one isolate (Debrecen 1132) was selected and grown on 800 g of rice as described above to obtain substantial quantities of trichothecenes.

Extraction and cleanup procedures for analysis of trichothecenes. The following procedure was conducted in triplicate for each culture. Rice culture (5 g) was weighed and transferred to a 125-ml Erlenmeyer flask. Then, 50 ml of 90% methanol was added to the flask, and the flask was agitated in a sonicator (Branson Cleaning Equipment Co., Shelton, Conn.) for 30 min. The flask was removed from the sonicator, and the solvent was decanted. The extraction was repeated with 50 ml of 90% methanol. The aqueous methanolic extracts were combined and filtered by gravity through Whatman no. 1 filter paper, and the solid material on the filter paper was washed with 10 ml of methanol. The filtrate was wahed two times with hexane (30 ml each time) in a 250-ml separtory funnel. The hexane layer was discarded. The aqueous methanol solution was slowly added to a solution of 100 ml of ferric gel solution. Ferric gel solution was prepared by adding 100 ml of distilled water to 10 ml of 15% ferric chloride ($FeCl_3 \cdot 6H_2O$), and the pH of the ferric chloride solution was adjusted to circa 4 to 4.5 by adding 1 N NaOH solution. The solution of ferric gel-methanolic extract was allowed to stand for 5 min and was then filtered through Whatman no. 1 filter paper. The precipitate was washed with 50 ml of 50% methanol. The filtrate and wash were combined and concentrated in vacuo to remove methanol. The resulting water solution (about 40 ml) was extracted three times with dichloromethane (25 ml each time). The organic extracts were combined and dried over sodium sulfate. After removal of the sodium sulfate, the dichloromethane extract was concentrated in vacuo, and the resulting material was transferred to a 1-dram vial with a small amount of dichloromethane. The solution in the vial was dried with a stream of dry nitrogen.

TLC. Thin-layer chromatography (TLC) was carried out on precoated silica gel 60 TLC plates (0.25 mm thick, E. Merck, Darmstadt, Federal Republic of Germany). The developing solvent system was ethyl acetate-hexane (vol/vol, 70:30); the following R_f values were noted: verrucarin J, 0.80; satratoxin H, 0.66; satratoxin H, 0.34; satratoxin G, 0.33; trichoverrol A, 0.17; and trichoverrol B, 0.16. Visualization of trichothecenes was effected with 4-(*p*-nitrobenzyl)pyridine and tetraethylene pentamine spray (21).

The Chromatotron (Harrison Research, Palo Alto, Calif.), a centrifugally accelerated thin-layer chromatograph, was used for the preparative TLC. The Chromatotron plates were either silica gel (2 or 4 mm thick) or alumina (2 mm

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TABLE 1. Production of trichothecenes from *S. atra* isolates

S. atra isolate	Concentration (μg/g) on rice culture[a]					
	VJ	RE	SF	SG	SH	TVL
Belvardgyula	11	24	18	81	68	28
Budapest 1		23	12	46	52	18
Budapest 2	20	39	15	83	80	24
Budapest 3	13	47	16	92	100	28
Debrecen 23		35	19	13	67	18
Debrecen 1132	9	36	34	108	160	48
Debrecen 5142						
Karcag 1, S-7	9	29	29	84	104	40
Karcag 2	13	35	22	109	88	26
Miskolc 2						
Miskolc 3	8	18	17	135	111	14
Nagytarcsa		50		19	128	49
Petrivente		55	15	16	116	33
Perkata						
Zsarolyan	16	53	18	160	192	18
My 93	17	69	68	106	75	27
My 276	37	85	36	72	159	33

[a] Average of three determinations where the individual analyses agreed to within ±10%. VJ, Verrucarin J; RE, roridin E; SF, SG, and SH, satratoxins F, G, and H, respectively; TVL, trichoverrols A and B mixture (ratio of A to B, 1:2).

thick). The eluting solvent system was either methanol in dichloromethane or 2-propanol in hexane.

HPLC. The samples were dissolved in 3 ml of dichloromethane. Analysis by high-performance liquid chromatography (HPLC) was carried out with a gradient of 2-propanol in a mixture of dichloromethane and hexane at a flow rate of 1.2 ml/min. A Gilson model 302 gradient liquid chromatograph equipped with a Gilson Holochrome variable wavelength detector set at 260 nm was employed, with the following gradient system: solvent A, 30% dichloromethane in hexane; solvent B, 50% 2-propanol in dichloromethane with an increasing gradient of 5 to 50% solvent B over a period of 20 min. A 5-μm amino Spherisorb column (250 by 4.6 mm) was used. The retention times (in minutes) observed were as follows: 3.7 min verrucarin J, 3.7; roridin E, 5.7; satratoxin F, 8.3; satratoxin G, 10.1; satratoxin H, 10.8; and trichoverrols A and B, 13.4 (see Fig. 3). The trichoverrols can be separated on the amino column with 20% 2-propanol in hexane.

Each extract was analyzed three times by injecting 4.0, 7.0, and 10.0 μl of the diluted extract into HPLC apparatus. The amounts of toxins were calculated based on calibration curves for the standard toxins. The results are shown in Table 1.

A semipreparative 5-μm silica gel column (250 by 10 mm; Supelco, Inc., Bellefonte, Pa.) was used for purification of the trichothecenes. The eluting solvent was 2.5% methanol in dichloromethane at a flow rate of 1.5 ml/min.

NMR spectroscopy. Nuclear magnetic resonance (NMR) spectra were determined in deuteriochloroform on a spectrometer (IBM SY-200 MHz) with tetramethylsilane as an internal standard.

Source of standards. Roridin E, verrucarin J, and trichoverrols A and B are available in our laboratory from a liquid culture of *Myrothecium verrucaria* (ATCC 24571) (16). Satratoxins F, G, and H were isolated from the culture of *S. atra* by us, and their chemical structures were determined by NMR spectroscopy.

Isolation of trichothecenes from the rice culture of *S. atra*. The rice culture (800 g) was extracted five times with 50% aqueous methanol (1-liter each time) by agitating in a sonicator. The extracts were combined and filtered through Whatman no. 1 filter papers and then defatted two times with hexane (1 liter each time). The defatted extract was concentrated in vacuo to remove the methanol, and the resulting aqueous phase was extracted five times with ethyl acetate (500 ml each time). The ethyl acetate layer was concentrated to obtain an oily gum (5 g) which was subjected to filtration chromatography on 250 g of TLC grade silica gel (placed in a Büchner funnel; chromatography conducted under reduced [aspirator] pressure) with increasing amounts of methanol in dichloromethane to yield the following fractions: I (0.5 g, eluted with dichloromethane), II (3.0 g, eluted with 5% methanol in dichloromethane), and III (0.7 g, eluted with 10% methanol in dichloromethane). TLC analysis of the three fractions showed that only fraction II contained trichothecenes.

Fraction II was subjected to flash chromatography (20) with 0 to 10% methanol in dichloromethane as eluting solvents. The following fractions were obtained: 1 (300 mg, eluted with dichloromethane), 2 (250 mg, eluted with 1% methanol in dichloromethane), 3 (750 mg, eluted with 3% methanol in dichloromethane), and 4 (250 mg, eluted with 5% methanol in dichloromethane). TLC analysis of the four fractions revealed that fractions 2, 3, and 4 contained trichothecenes.

Fraction 2 was further purified on the Chromatotron (2-mm thick silica gel plate; solvent system, 15% 2-propanol in hexane). Two trichothecenes were obtained upon recrystallization from dichloromethane-hexane and were identified as roridin E (12 mg) and satratoxin F (10 mg). Characterization of trichothecenes was done by comparing the NMR spectra of the compounds with those of authentic toxins (3, 8, 16).

Fraction 3 was purified on the Chromatotron (4-mm thick silica gel plate; solvent system, 3 to 5% methanol in dichloromethane). The fractions containing trichothecenes were combined and further purified on the Chromatotron (2-mm thick alumina plate; solvent system, 20% 2-propanol in hexane). TLC analysis of fractions from the Chromatotron showed a mixture of satratoxins G and H. The fractions containing satratoxins G and H were purified by preparative HPLC. The 75 mg of satratoxin G and 80 mg of satratoxin H were obtained by recrystallization from ethyl acetate-hexane.

Fraction 4 was purified as described for fraction 3. After HPLC purification, 20 mg of satratoxin H, 15 mg of trichoverrol A, and 30 mg of trichoverrol B were obtained.

RESULTS AND DISCUSSION

Of 17 isolates of *S. atra*, 14 produced trichothecenes based on HPLC analysis with satratoxins G and H (Fig. 1), which were usually the major toxins observed. Lesser amounts of verrucarin J, roridin E, satratoxin F (Fig. 1), and trichoverrols A and B (Fig. 2) were also detected (Table 1). The chromatogram of a culture extract (Karcag 1, S-7) is shown in Fig. 3. HPLC results were confirmed by comparing the NMR spectra of purified toxins from the rice culture with spectra of authentic standards. Trichoverrols A and B (Fig. 2) were not resolved by 2-propanol–dichloromethane–hexane eluting solvent system in HPLC, but could be resolved with 20% 2-propanol in hexane. The typical ratio of trichoverrol A to trichoverrol B in these cultures was about 1:2 which is the same ratio of trichoverrols A to B observed in cultures of *Myrothecium* sp. (14, 16). Several cultures

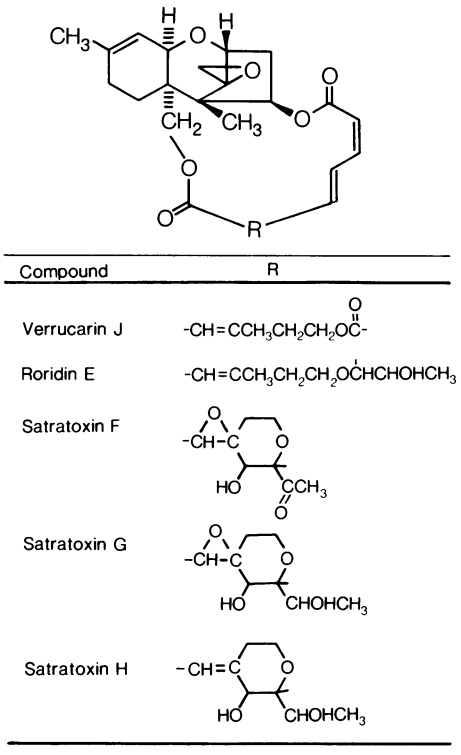

FIG. 1. Structure of macrocyclic trichothecenes.

contained small quantities (<10 μg/g) of trichoverrins A and B, which are trichothecenes that are intermediate in structure between the trichoverrols (Fig. 2) and roridin E (Fig 1) and are believed to lie along the biosynthetic pathway from the trichoverrols to the macrocyclic trichothecenes in *Myrothecium* sp. (16).

Bata et al. (2) examined the toxin production of the same isolates used in this study by growing the fungi on Sabouraud agar; however, the yields obtained were less than $\frac{1}{100}$ of those we observed from the cultures grown on rice substrate. Four macrocyclic trichothecenes (satratoxins G and H, verrucarin J, and roridin E) were identified in their studies. They reported that the verrucarol content of the whole hydrolyzate of the fungal culture was always higher than the amount calculated as the sum of the theoretical verrucarol content of the four macrocyclic trichothecenes, suggesting that other trichothecenes were present in the extracts of the cultures. Our finding of satratoxin F and trichoverrols A and B in addition to these four macrocyclic trichothecenes supports their results. Furthermore, this is the first report of finding trichoverrols A and B in cultures of *Stachybotrys* sp. although trichoverrols are frequently found in cultures of *Myrothecium* sp. (14, 16). Trichoverroids such as trichoverrols and trichoverrins are believed to be the intermediate products in biosynthetic trail to the macrocyclic trichothecenes (16), and the isolation of trichoverrols A and B from the culture of *Stachybotrys* sp. suggests that *Myrothecium* sp. and *Stachybotrys* sp. employ a similar set of enzymes for the biosynthesis of these toxins. In addition, Smitka et al. (19) found that *M. roridum* produced isosatratoxin H from liquid culture fermentation, which demonstrated that *Myrothecium* sp. could produce satratoxin-type macrocyclic trichothecenes.

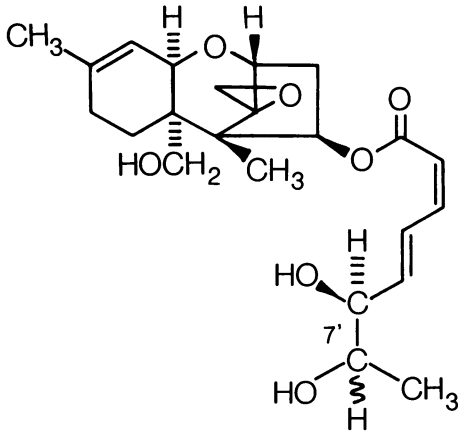

FIG. 2. Structure of trichoverrols A (7-S) and B (7-R).

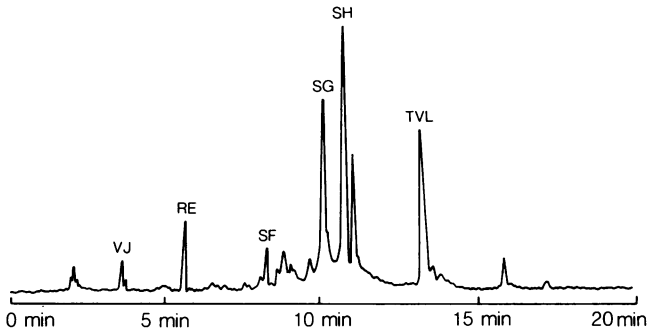

FIG. 3. Chromatogram (amino column, isopropanol–dichloromethane–hexane) of *S. atra* S-7 extract. Abbreviations: VJ, verrucarin J; RE, roridin E; SF, SG, and SH, satratoxins F, G, and H, respectively; TVL, trichoverrols A and B.

For the past few years, we have been interested in production of satratoxins because we needed substantial amounts of these toxins to pursue chemical studies. We looked briefly into toxin production in liquid culture and found no production of trichothecenes under conditions reported in the literature for any of the 17 Eastern European isolates or the two Egyptian isolates of *S. atra* (ATCC 46994 and 46996) cited in the literature (5). The isolates of *S. atra* used in this study grew poorly in submerged shake cultures and produced no detectable quantities of trichothecenes either under the conditions reported (5) or our standard conditions (15). Still cultures do produce trichothecenes, but the yields are so low (<1 μg/liter) as to be useless for toxin production. The use of Uncle Ben's rice as the production medium combined with growth of the cultures at two different temperatures resulted in a remarkable improvement of toxin production (especially satratoxins) compared with the amounts of toxin production observed by other scientists (2, 6, 12).

All of the *S. atra* isolates examined in this study were involved in stachybotryotoxicosis in Europe (2). Our finding that most isolates of *S. atra* produced highly toxic macrocyclic trichothecenes and trichoverroids supports the conclusion that the toxic principles are trichothecenes as was presumed earlier (1, 6, 10). The natural occurrence of *Stachybotrys* toxins might be more common than expected as evidenced by a recent case study showing that occupants

of a house in Chicago suffered from stachybotryotoxicosis (3a).

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