APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1985, p. 1225–1228
0099-2240/85/111225-04$02.00/0

Vol. 50, No. 5

7α-Hydroxytrichodermol, a New Trichothecene from *Myrothecium roridum*

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Received 11 June 1985/Accepted 15 August 1985

Five plant-pathogenic isolates of *Myrothecium roridum* from Florida produced only simple trichothecenes rather than the usual macrocyclic trichothecenes. The major metabolite was 7α-hydroxytrichodermol.

Myrothecium roridum and *Myrothecium verrucaria* are two species of the Fungi Imperfecti which are the principal sources of the macrocyclic trichothecenes (3, 14). During the past 5 years, we have examined many isolates of these *Myrothecium* spp. for the production of macrocyclic and trichoverroid trichothecenes (2). It has been our observation that these fungi produce these trichothecenes in submerged cultures, although some isolates under these culture conditions produce these antibiotics either in very low yields or in a very erratic manner. It has been our experience that the only trichothecenes produced by our *Myrothecium* isolates are the macrocyclic trichothecenes and the biosynthetically related trichoverroids (4). There are two reports of the isolation of the simple trichothecenes, trichodermol (1) and diacetylverrucarol (9), from *Myrothecium* cultures. Trichodermol was isolated as a minor metabolite accompanied by much larger amounts of the macrocyclic roridin and verrucarin trichothecenes (1). There is reason to believe that trichodermol is likely to lie along the biosynthetic trail to the macrocyclic trichothecenes, and so its isolation from *Myrothecium* cultures is not unexpected (4).

We examined several plant-pathogenic isolates of *M. roridum* from Florida and found that these isolates produce only the simple trichothecenes, both in submerged liquid cultures and on solid rice media.

MATERIALS AND METHODS

General physical and chemical methods. Melting points were determined on a Fisher-Johns hot-stage melting point apparatus and were uncorrected. Nuclear magnetic resonance (NMR) spectra were determined in deuteriochloroform on an IBM SY-200 MHz spectrometer with tetramethylsilane as an internal standard. The ^{13}C NMR signals were assigned by using ^{1}H single-frequency off-resonance decoupling techniques, chemical shift relations, and comparison with previous data. Mass spectra were determined on a VG 7070 E mass spectrometer. Optical rotations were determined on a Perkin-Elmer 241 automatic polarimeter. Filtration and flash chromatography (12) were carried out on Silica Gel 60 (230/400 mesh; E. Merck AG, Darmstadt, Federal Republic of Germany). Thin-layer chromatography (TLC) was carried out on silica gel plates (0.25 mm thick; E. Merck). The Chromatotron (model 7924; Harrison Research, Palo Alto, Calif.), which is centrifugally accelerated, was used for the preparative TLC. Visualization of trichothecenes was effected with 4-(*p*-nitrobenzyl)-pyridine and tetraethylenepentamine spray (13).

Source and maintenance of cultures. Five isolates of *M. roridum* were obtained from A. W. Engelhard (10). No attempt was made to maintain these isolates as single-spore clones; the cultures were used as received. They were stored on sterile soil particles at 4°C. To produce inoculum, infested soil particles were seeded onto Sabouraud glucose agar (containing 40 g of glucose, 10 g of peptone, and 15 g of agar per liter) 7 days before use.

Fermentation of liquid culture. The ability to produce trichothecenes was evaluated in a small-scale fermentation which involved a two-stage process, as a seed medium and as a production medium. Agar blocks bearing spores of *M. roridum* isolates grown on Sabouraud glucose agar were inoculated with 25 ml (in a 125-ml Erlenmeyer flask) of seed medium (15.6 g of glucose and 10 ml of corn steep liquor per liter) which had been sterilized at 121°C for 15 min. The incubation was conducted at 25°C for 48 h, and the agitation speed was 100 rpm. After 2 days, 5 ml of the seed medium was transferred to 50 ml (in a 250-ml Erlenmeyer flask) of production medium (1.0 g of $NH_4H_2PO_4$, 3.0 g of K_2HPO_4, 5.0 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 40 g of sucrose, and 8 ml of glycerol per liter) which had been sterilized at 121°C for 15 min. The production media were incubated at 25°C for 7 days at an agitation speed of 150 rpm. During the course of 7 days of incubation, 1 ml of liquid was taken from the production media, and trichothecene production of each isolate was monitored by TLC analysis. This experiment was repeated twice.

Based on the apparent ability to produce the desired compounds, one isolate (M4582) was selected to produce trichodermol and 7α-hydroxytrichodermol in a large-scale fermentation. The fungus was grown in 300 ml (in a 2-liter Erlenmeyer flask) of seed medium for 2 days at 25°C, transferred to production media (3 liters [1 liter of medium per 4-liter Erlenmeyer flask]), and incubated for 7 days at 25°C. The agitation speeds in both media were the same as those described above.

Rice culture. Production of trichothecenes on rice was done by the method of Lee and Mirocha (6). Each isolate was grown in 1-liter Erlenmeyer flasks on 200 g of moist autoclaved Uncle Ben's converted parboiled rice (Uncle Ben's Inc., Houston, Tex.). Deionized water (120 ml) was added to the rice in each flask, and the flasks were autoclaved at 121°C for 1 h twice on two consecutive days. The rice was inoculated with agar blocks bearing spores of *M. roridum*, and the flasks were shaken by hand daily for 1

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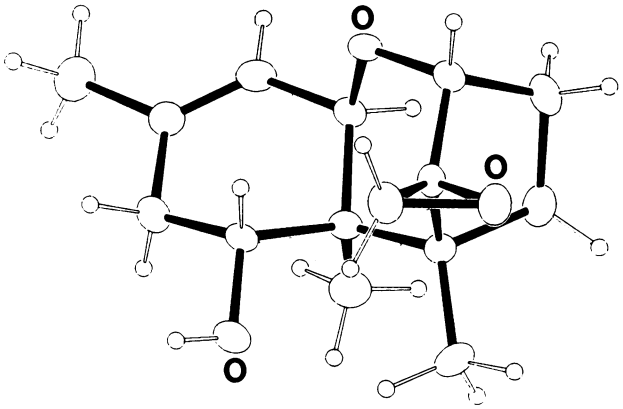

FIG. 1. Diagram of 7α-hydroxyscirpene drawn by using experimentally determined coordinates. The oxygen atoms have been labeled.

week. Rice cultures were incubated at 22 to 26°C on a laboratory bench for 2 weeks and then incubated at 10°C for 2 weeks.

Later, one isolate (M4582) was selected and grown on 4 kg of rice as described above to obtain substantial quantities of 7α-hydroxytrichodermol.

Isolation of trichodermol and 7α-hydroxytrichodermol. (i) Liquid culture. The liquid culture (3 liters) was filtered through Whatman no. 1 filter paper, and the filtrate was extracted three times with ethyl acetate (3 liters each time). The mycelia remaining from filtration were soaked in methanol overnight and filtered. The methanol filtrate was concentrated in vacuo until only an aqueous phase remained which was extracted three times with ethyl acetate (500 ml each time). The ethyl acetate extracts were pooled and concentrated in vacuo to yield 4 g of gum. The gum was subjected to filtration chromatography (50 g of silica gel) with increasing amounts of ethyl acetate in hexane to yield five fractions: 1 (800 mg, eluted with hexane), 2 (200 mg, eluted with 10% ethyl acetate in hexane), 3 (400 mg, eluted with 30% ethyl acetate in hexane), 4 (500 mg, eluted with 50 to 70% ethyl acetate in hexane), and 5 (1.5 g, eluted with ethyl acetate). Fractions 3 and 4 were subjected to purification on a Chromatotron (2-mm-thick silica gel plates, ethyl acetate-hexane) to yield, after recrystallization (ethyl acetate-hexane), 25 mg of trichodermol and 50 mg of 7α-hydroxytrichodermol. The physical and spectral properties of trichodermol matched those reported previously (1).

For 7α-hydroxytrichodermol, high-resolution mass spectrum (EI, 70 eV), *m/e* = 266.1526 (M^+ calculated as 266.1512); $[\alpha]_D$ −7.5 (C 0.15, methyl alcohol); melting point, 214 to 215°C; ^{1}NMR δ 0.86 (3H, s, 14-H), 1.09 (3H, s, 15-H), 1.69 (3H, s, 16-H), 1.8 to 2.1 (2H, m, 3β- and 8β-H), 2.24 (1 H, dd, *J* = 12.6 and 6.0 Hz, 8α-H), 3.06 and 3.10 (1H each, AB, *J* = 4.3 Hz, 13-H), 3.61 (1H, d, *J* = 5.5 Hz, 11-H), 3.82 (1H, d, *J* = 5.0 Hz, 2-H), 4.26 (1H, dd, *J* = 7.6 and 4.0 Hz, 4-H), 4.46 (1H, dd, *J* = 12.0 and 6.0 Hz, 7-H), and 5.36 (1H, brd, *J* = 5 Hz, 10-H); ^{13}C NMR δ 79.5 (C-2), 40.4 (C-3), 74.6 (C-4), 49.5 (C-5), 44.3 (C-6), 72.7 (C-7), 39.6 (C-8), 138.4 (C-9), 118.9 (C-10), 68.5 (C-11), 65.4 (C-12), 47.0 (C-13), 8.3 (C-14), 10.4 (C-15), and 22.5 (C-16).

(ii) Rice culture. Rice cultures (4 kg) were extracted by soaking the cultures three times with 500 ml of methanol per flask each time. The methanol extracts were combined and concentrated in vacuo to give an aqueous phase (2 liters) which was defatted three times with hexane (2 liters each time) and then extracted three times with ethyl acetate (2 liters each time). The ethyl acetate extract was concentrated in vacuo to give 72 g of a gum. The gum was subjected to filtration chromatography (700 g of silica gel) with increasing amounts of methanol in methylene chloride to yield four fractions: 1 (2.5 g, eluted with methylene chloride), 2 (28.0 g, eluted with 1.2% methanol in methylene chloride), 3 (25.0 g, eluted with 3 to 5% methanol in methylene chloride), and 4 (7.5 g, eluted with 50% methanol in methylene chloride). Fraction 2, which contained both trichodermol and 7α-hydroxytrichodermol, was subjected to flash chromatography with 10 to 70% ethyl acetate in hexane. The fractions containing trichodermol were combined and subjected to additional flash chromatography on silica and preparative TLC (Chromatotron) on alumina to yield 20 mg of trichodermol. The fractions containing 7α-hydroxytrichodermol were combined, the solvent was evaporated, and the residue was triturated with a small amount of methylene chloride and filtered. The solid was recrystallized from ethyl acetate in hexane to yield 615 mg of 7α-hydroxytrichodermol. The filtrate, which still contained some 7α-hydroxytrichodermol, was saved and combined at a later step with fraction 3. Fraction 3 was first subjected to a second filtration chromatography, then the fractions containing 7α-hydroxytrichodermol were combined with the filtrate from fraction 2. This was passed through a flash chromatography column (eluted with 10 to 50% ethyl acetate-hexane). Fractions containing the desired compound were recrystallized from ethyl acetate-hexane to yield an additional 165 mg for a total yield of 780 mg of 7α-hydroxytrichodermol.

Deoxygenation of 7α-hydroxytrichodermol to give 7α-hydroxyscirpene. A solution of 200 mg (0.83 mmol) of 7α-hydroxytrichodermol and 90 μl (1.12 mmol) of pyridine in 5 ml of dry methylene chloride was treated with 120 μl (0.86 mmol) of phenylchlorothionocarbonate (Aldrich Chemical Co., Inc., Milwaukee, Wis.). The mixture was stirred at room temperature under nitrogen for 48 h. The reaction mixture was filtered, the residue was washed with methylene chloride, and the combined organic compounds were condensed in vacuo to approximately 1 ml. The solution was separated by preparative TLC (Chromatotron, silica gel, 2% methyl alcohol in methylene chloride) to yield 180 mg (64%) of an oil which slowly solidified. This was used in the next step without further purification.

The thionocarbonate (180 mg, 0.45 mmol) and 3.5 mg of 2,2′-azobis(2-methylpropanenitrile) in 25 ml of dry benzene was heated to 75°C under nitrogen. *n*-Tributyltin hydride (0.180 ml, 0.194 g, 0.66 mmol) (Aldrich) was added all at once by syringe through a septum, and heating was continued. After 24 h, the reaction was shown by TLC to be incomplete, and an additional 0.180 ml of *n*-tributyltin hydride and 1.0 mg of 2,2′-azobis(2-methylpropanenitrile) were added. Heating was continued for 6 h, at which time the reaction appeared complete. Work-up of the reaction mixture was performed as described by Schuda et al. (11), with the exception that the resulting oil was chromatographed by preparative TLC (Chromatotron, silica gel, 2% methyl alcohol in methylene chloride) to yield 75 mg of 7α-hydroxyscirpene (Fig. 1). Recrystallization from ethyl acetate-hexane gave white crystals (47 mg, 42%) with a melting point of 150 to 151°C.

For 7α-hydroxyscirpene, ^{1}H NMR δ 0.81 (3H, s, 14-H), 1.05 (3H, s, 15-H), 1.69 (3H, s, 16-H), 1.5–2.2 (6H, m, 3-H, 4-H, 8-H), 3.15 (2H, s, 13-H), 3.73 (1H, d, *J* = 5 Hz, 2-H), 3.83 (1H, d, *J* = 5 Hz, 11-H), 4.47 (1H, dd [after D_2O exchange], *J* = 12 and 6 Hz, 7-H), and 5.35 (1H, brd, J = 5

Hz, 10-H); ^{13}C NMR δ 81.0 (C-2), 34.4 (C-3), 26.6 (C-4), 46.1 (C-5), 44.5 (C-6), 73.4 (C-7), 39.7 (C-8), 138.1 (C-9), 119.7 (C-10), 69.1 (C-11), 66.2 (C-12), 48.6 (C-13), 10.3 (C-14), 14.9 (C-15), and 22.5 (C-16).

X-ray data for 7α-hydroxyscirpene. M_r = 250.34, orthorhombic space group $P2_12_12_1$ with a = 6.522(2), b = 12.187(2), and c = 16.647(2), V = 1.323 ± 0.005 nm^3, Z = 4 (one molecule per asymmetric unit), d_{calc} = 1.26 g cm^{-3}, and μ = 6.51 cm^{-1}. A total of 1,189 independent reflections were collected on a Nicolet R3M automatic diffractometer by using CuKα radiation with a graphite monochromator on the incident beam. The structure was solved by direct methods and refined by full-matrix least-squares analysis to a final R-factor (agreement between observed and calculated structure factors) of 0.034. Complete crystallographic details for this molecule will be published by the Naval Research Laboratory authors.

RESULTS AND DISCUSSION

M. roridum 4582 was obtained as a plant pathogen on *Peperomia obtusifolia* A. Dietr. and subsequently shown to be a pathogen on *Gloxinia* sp. (10). The pattern of trichothecene production by this isolate, as well as the four other Florida isolates, is unusual in that these isolates appear incapable of producing the normal array of trichoverroid and macrocyclic trichothecenes (4), which suggests that these Florida isolates lack the enzyme systems necessary to construct the macrocyclic ring system.

The rice cultures appear to be superior for toxin production by M4582, although the isolation of the trichothecenes is hampered by the presence of many interfering substances formed in the rice cultures. The liquid culture is better for the production of trichodermol since this compound was produced in only small amounts in the rice culture.

The proof of the structure for 7α-hyroxytrichodermol is based on its molecular formula, as derived by high-resolution mass spectrometry, the close resemblance of its ^{1}H- and ^{13}C-NMR spectra to those of trichodermol, and its formation of a diacetate, indicating the presence of an additional hydroxyl group relative to trichodermol. This additional hydroxyl group must be in ring A and on either position 7 or 8. In addition, from the coupling pattern of the proton (doublet of doublets with J = 12.0 and 6.0 Hz) attached to the carbon bearing this hydroxyl group, the A-ring hydroxyl group must be either 8β or 7α (7). Were the hydroxyl group either 8α or 7β, the proton on the carbon bearing the hydroxyl group would be a doublet with $J \sim 5$ Hz (7).

Two pieces of ^{1}H-NMR spectral data initially suggested to us that this new compound was 8β-hydroxytrichodermol: the chemical shift of H-10 is nearly the same (δ 5.36) as the chemical shift for H-10 (δ 5.32) in 8β-hydroxyverrucarol (5), and the ^{1}H-NMR chemical shift of the C-14 methyl group is at 0.86 ppm. When this methyl resonance is >1 ppm, it has been taken as evidence that the hydroxyl group is located at 7α rather than at 8β, since for trichothecenes bearing a 7α-hydroxyl group, δ H-14 resonates at ca. 1.1 ppm (7). However, two other pieces of NMR data suggest that the hydroxyl group is 7α. In the ^{1}H-NMR spectra of trichothecenes, both H-2 and H-11 appear as doublets between 3.5 and 4.0 ppm. H-2 is a sharp doublet, whereas H-11 is considerably shorter and somewhat broadened owing to long-range coupling (W-coupling) with H-7α. For our compound, both H-2 and H-11 are sharp doublets, suggesting the loss of long-range coupling between H-11 and H-7α. The

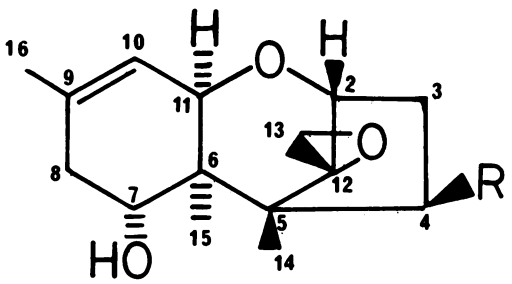

FIG. 2. Structures of 7α-hydroxyscirpene (R = H) and 7α-hydroxytrichodermol (R = OH).

second piece of evidence comes from ^{13}C-NMR data, where in going from trichodermol to our hydroxytrichodermol, the chemical shift of C-11 decreases by ca. 2 ppm; whereas, in going from verrucarol or its diacetate to the corresponding 8β-hydroxyverrucarol (or 8β-hydroxyverrucarol diacetate) the chemical shift of C-11 does not change. These data suggest that the hydroxyl group in the A-ring may be at the 7α position rather than the 8β position.

Because of the ambiguity, we sought a more secure proof of the structure. To this end, we deoxygenated C-4 by conversion to a C-4 thionocarbonate, followed by treatment with tributyltin hydride (11) to give 7α-hydroxydeoxytrichodermol (7α-hydroxyscirpene [Fig. 2]), whose structure was proved by single crystal X-ray diffraction analysis (see Materials and Methods). As in the starting 7α-hydroxytrichodermol, H-11 appears as a sharp doublet and the H-14 methyl protons resonate at 0.81 ppm. Clearly, in these 7α-hydroxylated compounds, the chemical shifts of the H-14 methyl protons are unaffected by the presence of the 7α-hydroxyl group and they resonate in the normal region (~0.8 to 0.9 ppm).

Although there are a number of trichothecene metabolites bearing 7α-hydroxyl groups (8), these metabolites invariably are formed in minor amounts unless the C-8 position is also oxygenated (as in nivalenol and deoxynivalenol). This particular fungal isolate is unusual in that it produces an A-ring oxygenated major metabolite oxygenated only at the C-7α position.

ACKNOWLEDGMENTS

We thank A. W. Engelhard for furnishing the isolates of *M. roridum*.

This investigation was supported by Public Health Service grant CA 25967 from the National Cancer Institute and by U.S. Army contracts DMAD 17-82-C-2240 and USAMRIID 85MM5511.

LITERATURE CITED

1. **Harri, E., W. Loeffler, H. P. Sigg, H. Stahelin, C. Stoll, C. Tamm, and D. Wiesinger.** 1967. Uber die Verrucarine und Roridine, eine Gruppe von Cytostatisch Hochwirksamer Antibiotica aus Myrothecium Arter. Helv. Chim. Acta **45**:839–853.
2. **Jarvis, B. B., R. M. Eppley, and E. P. Mazzola.** 1983. Chemistry and bioproduction of macrocyclic trichothecenes, p. 20–38. *In* Y. Ueno (ed.), Trichothecenes: chemical, biological, and toxicological aspects. Elsevier Science Publishing Co., Inc., New York.
3. **Jarvis, B. B., and E. P. Mazzola.** 1982. Macrocyclic and other novel trichothecenes: their structure, synthesis, and biological significance. Acc. Chem. Res. **15**:388–395.
4. **Jarvis, B. B., G. P. Stahly, G. Pavanasasivam, J. O. Midiwo, T. DeSilva, C. E. Holmlund, E. P. Mazzola, and R. F. Geoghegan,**

Jr. 1982. Isolation and characterization of the trichoverroids and new roridins and verrucarins. J. Org. Chem. **47**:1117–1124.
5. **Kupchan, S. M., D. R. Streelman, B. B. Jarvis, R. G. Dailey, Jr., and A. T. Sneden.** 1977. Isolation of potent new antileukemic trichothecenes from *Baccharis megapotamica*. J. Org. Chem. **42**:4221–4225.
6. **Lee, Y.-W., and C. J. Mirocha.** 1984. Production of nivalenol and fusarenone-X by *Fusarium tricinctum* Fn-2B on a rice substrate. Appl. Environ. Microbiol. **48**:857–858.
7. **Machida, Y., and S. Nozoe.** 1972. Biosynthesis of trichothecin and related compounds. Tetrahedron **28**:5113–5117.
8. **McDougal, P. G., and N. R. Schmuff.** 1985. Chemical synthesis of the trichothecenes. Fortschr. Chem. Org. Naturst. **47**: 153–219.
9. **Okuchi, M., M. Itoh, Y. Kaneko, and S. Dio.** 1968. A new antifungal substance produced by *Myrothecium*. Agric. Biol. Chem. **32**:394–396.
10. **Ploetz, R. C., and A. W. Englehard.** 1980. Chemical control of *Myrothecium* disease of *Gloxinia*. Proc. Fla. State Hortic. Soc. **93**:181–183.
11. **Schuda, P. F., S. J. Potlock, and R. W. Wannemacher, Jr.** 1984. Trichothecenes. I. The synthesis of 4-deoxyverrucarol from verrucarol and diacetoxyscirpenol. J. Nat. Prod. **47**:514–519.
12. **Still, W. C., M. Kahn, and A. Mitra.** 1978. Rapid chromatographic technique for preparative separations with moderate resolution. J. Org. Chem. **43**:2923–2924.
13. **Takitani, S., and Y. Asabe.** 1983. Thin-layer chromatographic analysis of trichothecene mycotoxins, p. 113–120. *In* Y. Ueno (ed.), Trichothecenes: chemical, biological, and toxicological aspects. Elsevier Science Publishing Co., Inc., New York.
14. **Tamm, C.** 1974. The antibiotic complex of the verrucarins and roridins. Fortschr. Chem. Org. Naturst. **31**:61–117.